DETERMINATION OF AFLATOXIGENIC FUNGI IN POULTRY FEEDS AND AFLATOXIN B_1 IN FRESH AND BOILED BROILER LIVERS IN LIVE BIRD MARKETS IN ZARIA, NIGERIA

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SEPTEMBER, 2017

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(P13VTPH8023)

A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE IN VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICINE

DEPARTMENT OF VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICINE
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SEPTEMBER, 2017

DECLARATION

I declare that the work in this dissertation entitled **" Determination of aflatoxigenic fungi in poultry feed and aflatoxin B_1 in fresh and boiled broiler livers in live-bird markets in Zaria Nigeria."** was carried out by me in the Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University, Zaria, Nigeria under the supervision of Professor Junaidu Kabir and Professor (Mrs). C.N. Kwanashie. The information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this Dissertation was previously presented for another degree or diploma at this or any other institution.

Muhammed Jimoh, IBRAHIM	_____	_____
Name of Student	Signature	Date

CERTIFICATION

This dissertation entitled **" Determination of aflatoxigenic fungi in poultry feed and aflatoxin B_1 in fresh and boiled broiler liver in live-bird markets in Zaria, Nigeria."** by Muhammed Jimoh IBRAHIM, meets the regulations governing the award of the degree of Master of Science in Veterinary Public Health and Preventive Medicine in the Department of Veterinary Public Health and Preventive Medicine of the Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to Almighty Allah

ACKNOWLEDGEMENTS

Firstly, my acknowledgement and appreciation go to Almighty Allah, my Supervisors, Profs. Junaidu Kabir and C.N. Kwanashie whose support, guidance and word of encouragement kept me focused on completing this thesis, thank you so much.

Next, I would like to give thanks to my parents, family, relatives, friends, colleagues and well wishers, for their prayers and support, who fully supported me during this course. They gave me wise advice and encouraged me. I am eternally indebted to Dr. karimat Hussain, Nwanga Ephraim, Hassana Tahir (cherry), and Halima Abdulmalik. I acknowledge with gratitude the kind and caring assistance I got from Dr. Friday Zakari, Abudulrazaq Bello, and Ibrahim B. Mohammed. I cannot forget the kindness of Prof. J.O. Ayo, Dr. G.S.N Kia and extreme support of Dr. M.T. Salawudeen. I appreciate the encouragement I received from Dr. Rasheedat Mustapha and Prof. Abdulkarim Augunua. This dissertation would not have been possible without their guidance in one way or another. I also want to give my gratitude to the academic and non-academic staff of the Department of Veterinary Public Health and Preventive Medicine, ABU, Zaria. Staff of the laboratory of Department of Crop Protection, Faculty of Agriculture, Department of Veterinary Microbiology, Faculty of Veterinary Medicine, ABU, Zaria, Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Science, all of ABU, Zaria and DNALABs limited, Kaduna for support and for providing all consumables, items and facilities to finish my project. Words alone can't express how indebted I am to the live bird marketers that I interacted with in the course of this study for their patience and cooperation. I am forever indebted to you all, may Almighty Allah pay and reward you all with good in this world and paradise in the hereafter. Ultimate gratitude is to Allah, He

grants us success and lightens our burden, not because we are strong, smart or formidable, but out of his mercy and grace, He made it possible, even when it seemed impossible, may He entrench humility, gratitude and sacrifice in our lives, Ameen

ABSTRACT

There is increasing concern for the contamination of poultry products with *Aspergillus* strains responsible for aflatoxin production. The occurrence of aflatoxigenic fungi in poultry feeds and aflatoxin B_1 in fresh and boiled broiler liver samples in live bird markets in Zaria, Nigeria were examined. Feed samples were cultured for fungi on Sabouraud dextrose chloramphenicol agar to detect the presence of *Aspergillus* species. Aflatoxin production potential was evaluated using Czypeck dox agar. Desiccated coconut agar was used to detect fluorescence of *Aspergillus* spp and indirect competitive enzyme linked immunosorbent assay (cELISA) was used to determine aflatoxin residue concentrations (µg/kg) in both fresh and boiled (100°C for 90 mins) liver samples. Out of the 300 poultry feed samples tested, 234(77.9%) were contaminated with *Aspergillus* spp of which 126(53.8%), 48(20.5%), 27(11.5%), 15(6.4%), 9(3.9%), 5(2.1%), 3(1.3%), and 1(0.4%) were identified as *A. flavus*, *A. fumigatus*, *A. parasiticus*, *A. niger*, *A. nidulans*, *A. terreus*, *A. nomius*, and *A. caelatus* respectively. On desiccated coconut agar, *Aspergillus* had isolation frequency of 98(51.9%) with ($P < 0.05$). Samples from Dan Magaji, had isolation frequency of 28(28.6%), Zaria City 21(21.4%), Samaru 17(17.4%), Sabon Gari, 7(7.1%), Kwangila 14(14.3%) and Tudun wada 11(11.2%). The mean and standard deviation of aflatoxins in freshly tested liver concentration were 41.45 ± 29.55, 66.40 ± 22.28, 9.90 ± 6.50, 10.00 ± 1.74, 29.80 ± 17.16 and 35.25 ± 8.14 µg/kg for Zaria city, Sabon Gari, Kwangila, Samaru, Tudun wada and Dan Magaji respectively. All the liver sample of birds in live bird markets had aflatoxin B_1 concentration higher than the maximum limit (4 µg/kg) set by World Health Organization and Standard Organization of Nigeria. Also, there was statistically significant ($P < 0.05$) difference in sample locations. Furthermore, the mean value and standard deviation of boiled liver contents were 8.2 ± 5.0, 1.8 ± 0.7, 0.6 ±

0.3, 29.0 ± 9.5, 5.2 ± 5.1, 4.5 ± 3.3 µg/kg for Samaru, Kwangila, Dan Magaji, Sabon Gari, Zaria City and Tudun wada respectively. The mean value and standard deviation of liver content before and after boiling were 15.98±15.00 and 8.22±10.52 µg/kg respectively. There was no statistical difference in the processed form of liver ($P < 0.05$). Aflatoxin content, were found in edible tissues both before and after heat treatment. Strict measures should be taken to monitor toxins in feed and chicken meat products.

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LIST OF ABBREVIATIONS AND SYMBOLS

CPA	Cyclopiazonic Acid
iELISA	Indirect Competitive Enzyme linked immunosorbent Assay
DON	Deoxynivalenol
DNA	Deoxyribonucleic Acid
DCA	Dessicated Coconut Agar
SDA	Sabouroud Dextrose Agar
PDB	Potato Dextrose Broth
CAST	Council for Agricultural Science and Technology
ASM	American Society for Microbiology
AFM	Aflatoxin M
AFB	Aflatoxin B
AFG	Aflatoxin G
HCV	Hepatitis C virus
HBV	Hepatitis B virus
UV	Ultraviolent
ZEN	Zearalenone
WHO	World Health Organization
CDC	Centres for Disease Control and Prevention
SSA	Sub Sahara Africa
OTA	Ochratoxin
LBM	Live bird market
AFs	Aflatoxin
LSU	Large subunit
SSU	Small subunit

ITS	Internal transcribed spacer
RAPD	Random amplified polymorphic DNA
ISSR	Inter simple sequence repeat
NIV	Nivanenol
DAS	Diacetoxyscrirpenol
FAO	Food and Agricultural Organization
EFSA	European Food Safety Agency
HIV/AIDS	Human immunodeficiency virus
HCC	Hepato-cellular carcinoma
LD50	Lethal dose 50
RNA	Ribosomal RNA
P450	Cytochrome 450
G	Guanine
T	Thymine
XPD	Xeroderma pigmentosum complementation gene group D
AFT	Total Aflatoxin
%	Percentage
Kg	Kilogram
µg	Microgram
ng	Nanogram
FEHD	Food and Environmental Hygiene Department

TH	Trichothecences
FAOSTAT	Food and Agriculture Organization Statistics
USA	United States of America
EU	European Union
UAE	United Arab Emirate
HPLC	High performance liquid chromatography
JEFCA	Joint Expert Committee on Food Additive
rpm	Revolution per minute
W/V	Weight by volume
Mm	Millimeter
mL	Milliliter
L	Liter
ML	Maximum limits
BSA	Bovine Serum Albumin
PBS-T	Physiological buffer saline Tween
IgG-ALP	Goat anti-rabbit
KCl	Potassium chloride
PACA	Partnership for Aflatoxin Control in Africa
FAOUN	Food and Agricultural Organization of the United Nation
SCL	Sahel Capital Limited

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

The study of mycotoxins really began in 1960 with the outbreak of Turkey X disease in the U.K, linked to peanut meal imported from Brazil (Sargeant *et al*., 1961). Mycotoxins, the secondary metabolites from toxigenic fungi, are contaminants in foods and feeds, exerting harmful effects upon animal and human health (Zahoor-ul-Hassan *et al.,* 2010). Mycotoxins are low-molecular weight secondary metabolites, produced by certain strains of filamentous fungi, such as *Aspergillus, Penicillium and Fusarium,* which invade crops in the field. Mycotoxins are structurally-diverse simple C4 compounds, for example, moniliformin, to complex substances such as the phomopsins which are filamentous fungi that vary in their chemistry and biological effects (Sudakin, 2003; Dinis *et al.,* 2007). The most important mycotoxins in naturally contaminated food and feeds are aflatoxins (AFs), ochratoxins (O), zearalenone (ZEN), T-2 toxin, deoxynivalenol and fumonisins (Sultana and Hanif, 2009). No region of the world escapes the problem of mycotoxins and according to Lawlor and Lynch (2005) and Okoli *et al*. (2006b), mycotoxins are estimated to cause loss of as much as 25 % of the world's crops each year. The mycotoxins accumulate in foods during storage under favourable conditions of ambient temperature and relative humidity for fungal growth. Mycotoxins are produced only under aerobic conditions Ratcliff (2002), where they often attack ground nut, maize, cotton seed cake, wheat, and soyabean. They are regularly implicated in toxic syndromes in animals and humans (Charoenpornsook and Kavisarasai, 2006). However, animals may have varying susceptibilities to mycotoxins, depending on physiological, genetic and **environmental factors.** Most mycotoxins such as aflatoxin B_1, T-2 toxin and ochratoxin A inhibit protein synthesis

(Charoenpornsook and Kavisarasai, 2006). The consumption of multiple doses of mycotoxin-contaminated diet may induce haematological, biochemical, physiological changes in the liver and growth depression in animals (Awad *et al.,* 2006; Shi *et al.,* 2006; Rezar *et al.,* 2007; Gowda *et al.,* 2008). Thus, the presence of mycotoxins in poultry feeds can result in significant economic losses to the poultry industries (Awad *et al.,* 2006). Mycotoxins have been detected in various food commodities from many parts of the world and are presently considered as some of the most dangerous contaminants of food and animal feeds (Okoli, 2005; Okoli *et al*., 2006a; 2006b; 2007a; 2007b). Adverse effects of mycotoxins on animal health and production have been recognized in animals kept under intensive management such as poultry, swine and cattle as a consequence of the consumption of high levels of contaminated cereals and oilseeds in the diet (Charoenpornsook and Kavisarasai, 2006).

Aflatoxins produced by the toxigenic fungi, mainly *Aspergillus flavus* and *Aspergillus parasiticus*, constitute one of the major health hazard groups of naturally-occurring toxicants, both for man and animals. There are six forms of aflatoxins: B_1, B_2, G_1, and G_2 are found in plant-based food, while M_1 (metabolite of B_1) and M_2 are found in foods of animal origin. Aflatoxin B1 is the most harmful form due to its direct link to human liver cancer (Leslie *et al*., 2008; USAID, 2012). The order of potency for both acute and chronic toxicity of aflatoxins is $AfB_1 > AfG_1 > AfB_2 > AfG_2$ (Santacroce *et al*., 2008). Among the four major groups of aflatoxins; namely B_1, B_2, G_1 and G_2; aflatoxin B_1 (AFB_1) is the most toxic and it is a known carcinogen. Acute or chronic aflatoxicosis in domestic birds results in decreased meat/egg production, immunosuppression and hepatotoxicosis (Verma *et al.,* 2004; Khan *et al.,* 2010). Human exposure to aflatoxins may result from consumption of plant-derived foods that are

contaminated with the toxins, and the carry-over of aflatoxins and their metabolites in animal products such as meat and eggs (CAST, 2003).

Aflatoxin is one of the numerous naturally-occurring mycotoxins that are found in soils and foods. Aflatoxins have been found in soil as well as in grains, nuts, dairy products, tea, spices and cocoa, as well as animal and fish feeds (Waliyar *et al.*, 2008).

1.2 Statement of the Research Problem

Mycotoxins that occur in food and/or feedstuffs have great significance in the health of humans and livestock (Tola and Kebede, 2016). The growth of this heterologous group of fungi in feed and the generation of secondary metabolites of mycotoxins have adverse effects on the poultry industry and human health as well (Monson *et al.,* 2015; Oliveira *et al.,* 2015).

Exposure of Pregnant women to aflatoxin may cause increased maternal mortality and low birth weight. Infants exposed to aflatoxin-contaminated foods may be more susceptible to stunting growth and malnutrition (Shuaibu *et al*., 2010). Aflatoxins impair livestock growth, reproduction, immune functioning and ability to metabolize vaccines (Makun *et al.,* 2012).

The economic impact of reduced animal productivity, increased incidence of disease due to immunosuppression, damage to vital organs and interference with reproductive capacity is many times greater than the impact caused by death due to mycotoxin poisoning (Akande *et al*., 2006). It has been pointed out that aflatoxin contamination of feeds of food-producing animals can result in residues of ingested aflatoxins or its metabolites in meat, milk and egg (Gizachew *et al*., 2016).

It has been estimated that more than 5 million people in developing countries world-wide are at risk of chronic exposure to aflatoxins through contaminated food (Shepard, 2005; Strosnider *et*

al., 2006). Animals exposed to aflatoxins show a variety of symptoms, depending on the animal species. However, in all animals, aflatoxins may cause liver damage, decreased reproductive performance, reduced milk or egg production, embryonic death, teratogenicity, tumors and suppressed immune function, even when low levels are consumed (Akande *et al*., 2006). Aflatoxins are both acutely and chronically toxic in animals and humans (Pimpukdee *et al*., 2004).

Aflatoxin B_1 is known to be the most toxic metabolite, especially in sensitive species such as poultry (Hussein and Brasel, 2001). Beside direct losses related to mortality, feed conversion and growth rate, recovering birds remain poor. Indeed, air sacculitis is a major reason for carcass condemnation at slaughter inspection (Kunkle, 2003; Lupo *et al.,* 2010). It is proven that aflatoxin leads to reduced egg production as well as their quality in laying hens (Rizzi *et al*., 2003).

Poultry feeds in live bird markets is at risk of unsafe aflatoxin accumulation which can negatively affect human health, food security and economic trade (Williams *et al*., 2004). Their toxicity depends on different factors including its concentration, the duration of exposure, the species, sex, age, and health status of animals (Jewers, 1990).

1.3 Justification of the Study

Ingestion of aflatoxin concentration in broiler tissues or its metabolites may be dangerous to humans who consume these products. The Nigerian poultry industry is an estimated at $ 600 million business with approximately 165 million birds. The estimated poultry meat consumption in Nigeria is approximately 1.2 metric tons as at 2013 (SCL, 2015) which contribute about 9-10% of agricultural GDP, with the provision of about 36.5% of the total protein intake of

Nigeria. Thus, livestock sector is vital to the socio-economic development of Nigeria (SCL, 2015). Zaria being in Sub Saharan Africa with high temperature and relative humidity is at risk of unsafe aflatoxin exposure in poultry which can negatively affect human health, food security and economic trade.

Live bird markets are located in a specific areas of the general market, facilities are limited with poor hygienic conditions especially stores where feeds are kept, points of sales and slaughter with no stringent biosecurity measures (FAOUN, 2008). Most birds receive feed from containers which are poorly kept leading to contamination of feed. Thus, it is therefore important to understand the level of exposure to mycotoxin contaminated feed. Most mycotoxin control efforts are targeted on feed millers and poultry farms.

There is also a paucity of information regarding aflatoxin contamination of poultry feed and carcass of chickens in the area of study. This necessitates the need to investigate the potential public health risks, associated with the consumption of food of animal origin, especially slaughtered birds in Zaria, Nigeria.

Constant monitoring of poultry feed and meat will contribute to the improvement of livestock and human health, which will also enhance the export potential leading to increased income for farmers. The findings of the study may help in bridging the information gap and help to drive a search for solutions.

1.4 Aim and Objectives of the Study

1.4.1 Aim of the study

To determine contamination of feeds in poultry cages with aflatoxigenic *Aspergillus* spp and occurrence of aflatoxin B_1 in fresh and boiled broiler liver samples from live bird markets in Zaria, Kaduna State, Nigeria.

1.4.2 Objectives of the study

1. To isolate and characterize *Aspergillus* in poultry feed fed to birds in live bird markets in Zaria.

2. To determine the occurrence of aflatoxigenic strains of fungi in feeds, fed to birds in live bird markets in Zaria.

3. To determine the level of aflatoxins B_1 in liver of slaughtered broiler chickens in live bird markets in Zaria.

4. To determine the effect of heat treatment (by boiling) on the persistence of detected aflatoxins in contaminated liver tissues.

1.5 Research Questions

1. Are there aflatoxin-producing and non-producing strains of *Aspergillus* in feeds in live bird markets in Zaria?
2. Are there detectable aflatoxins B_1 in the liver of slaughtered broilers in live bird markets in Zaria?

3 Can heat treatment by boiling of aflatoxin-containing livers of chicken affect the concentration of the aflatoxins?

CHAPTER TWO

LITERATURE REVIEW

2.1 Taxonomic Classification of Fungi

Fungi are eukaryotic organisms, that is they contain a membrane bound nucleus in their cells. Biologically they are the members of an independent taxonomic rank known as kingdom fungi (Kirk *et al.,* 1988). Fungi are mainly classified depending on the type of spore and nature of specialized structures produced for reproduction. Multiple regions of the fungal rRNA genes have been used to study fungal taxonomy and diversity; these include the small-subunit (SSU) and large-subunit (LSU) rRNA genes and the internal transcribed spacer (*ITS*) region that separates the two rRNA genes (O'Brien *et al.,* 2005; Öpik *et al*., 2006; Jumpponen and Jones, 2009). The internal transcribed spacer (ITS) regions of the ribosomal RNA have also become a useful tool in fungal taxonomy, as these regions evolved rapidly and help distinguish different species within a genus (White *et al.,* 1990).

Ascomycota is a Division/Class of the kingdom fungi that together with the *Basiodiomycota*, form the sub-kingdom *Dikarya*. Its members are commonly known as the Sac Fungi. They are the largest class of fungi, with over 64,000 species. The defining feature of this fungal group is the "ascus" meaning sac. The Eurotiales are an Order of sac fungi, also known as the green and blue moulds. The Order contains 3 families, 49 genera, and 928 species. The Order was circumscribed in 1980 (Benny and Kimbrough, 1980).

The fungi from these 5 genera: *Aspergillus*, *Fusarium*, *Penicillium, Alternaria*, and *Calviceps* are responsible for majority of mycotoxins of agricultural relevance (Jones and Toal, 2003; Cannon and Kirk, 2007).

2.2 Molecular Tools

Molecular genetic analysis using DNA marker provides valuable tools for detecting genetic relationship among *Aspergillus* (Bardakci, 2001). The random amplified polymorphic DNA (RAPD) technique has also been used to characterize and detect genetic variability between isolates of *A. flavus* and related species (Batista *et al.,* 2008; Gehlot *et al.,* 2011; Irshad and Nawab, 2012) The inter-spaced sequence repeats (ISSR) technique has been employed to investigate the diversity and population structure of *A. flavus* (Tran-Dinh *et al.,* 2009; Hadrich *et al.,* 2010; Wang *et al.,* 2012).

2.3 *Aspergillus* species

Aspergillus was first identified in 1729 and catalogued by an Italian biologist P Micheli who named it as holy water sprinkler" (Bennett, 2010) Laboratory identification of Fungi is based on their appearance and structure. The genus *Aspergillus* is one of the most important filamentous fungal genera. *Aspergillus* is a filamentous fungi as opposed to yeast which is single celled. *Aspergillus* appears as round single cell or chains of cells called hyphae. *Aspergillus* is a filamentous, cosmopolitan and ubiquitous fungus found in nature. It is commonly isolated from soil, plant, debris, and indoor air environment. While a telemorphic state has been described only for some of the *Aspergillus* others are accepted to be mitosporic, without any known sexual spore production (Bennelt, 2010). There are over 185 species of the genus *Aspergillus,* with 20 incriminated as a causative agent of opportunistic infection in man. Among these is *A. fumigatus*, which is the most frequently isolated species (Bennelt, 2010).

A. flavus, *A. niger*, *A. clavatus*, *A. glaucus group*, *A. nidulans*, *A. oryzae*, *A. terreus*, *A. ustus*, *A. fumigatus* and *A. versicolor* are among the other species less frequently isolated as opportunistic pathogens (Bennelt, 2010). *Aspergillus* species are used in the fermentation industry, but they are also responsible for various plant and food secondary spoilage with result of accumulation of mycotoxins (Perone *et al.,* 2007).

2.3.1 Growth of *Aspergillus*

Fungi reproduce by forming tiny spores which grow under a variety of conditions and many are known to be plant pathogens. *Aspergillus* can produce mycotoxins which are often found in contaminated foodstuff and hazardous to the consumer (Makun *et al.,* 2011). Many toxins from this type of mould have been identified and they depend greatly on genus and species (Fratamico, 2005).

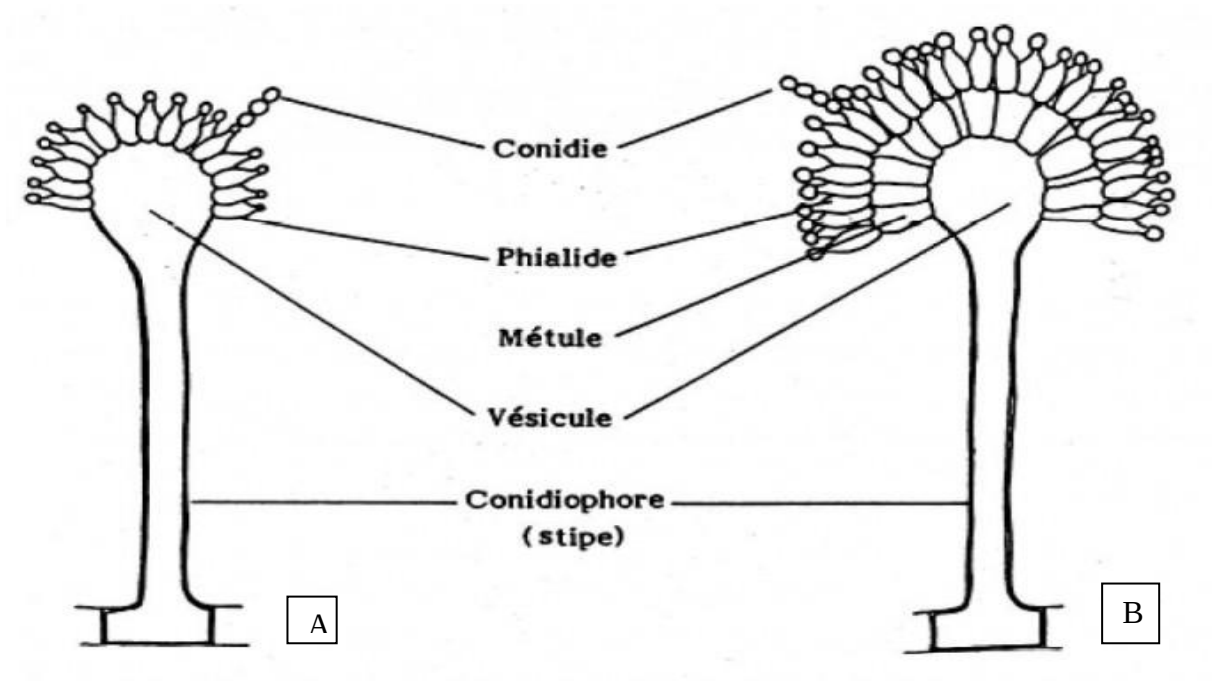

Figure 2.1: Conidial head morphology in *Aspergillus* (A) uniseriate, (B) biseriate.

Adopted from Ellis *et al* (2007).

2.3.2 Identification of *Aspergillus* species

Aspergillus species identification is based on microscopic examination and morphological characteristic of colony (MacClenny, 2005). For identification, isolates are usually inoculated at three points on Czapek Dox agar and 2% malt extract agar and incubated at 25 °C. Most species sporulate within 7 days (Ellis *et al.,* 2007). Molecular methods continue to improve and become more rapidly available. However, microscopy and culture remain commonly used for identification of *Aspergillus* (Diba *et al.,* 2007).

The 2003 American Society for Microbiology (ASM) survey documented that 89 % of laboratories performing mycological examination (morphology based), 16 % of them use serologic test and fewer than 5 % use molecular test for the identification of microbial pathogens

(Warris *et al.,* 2001). Isolation in culture and phenotypic identification of common clinical isolates of *Aspergillus* is usually quick and easy. However, culture is often described as slow and sometimes creating misconception about its value for the detection of Aspergilli (Klich, 2002). Given the continued reliance on microscopy and culture, the diagnostic value of this method must be improved by procedural changes and adequate training of laboratory personnel (Diba *et al.,* 2007).

The use of Sabouraud dextrose agar, malt extract inhibitory mould agar, or similar sporulation agar as primary isolation media for *Aspergillus* may speed growth rate and the production of conidia (Diba *et al*., 2007). The addition of antibacterial agents to isolation media help to prevent bacterial growth and reducing the need for subculture (Diba *et al.,* 2007). The initial incubation of fungal media is 35-37 °C (Tarrand *et al.,* 2002). *Aspergillus* can be detected and measured due to strong fluorescence and ultra-violet (UV) light absorbing properties (Bhatnagar *et al.,* 2002).

2.3.3 Biological properties of *Aspergillus*

Aspergillus species are capable of growing on a variety of substrates and under a variety of environmental conditions (Bradburn *et al.,* 1993). *Aspergillus* grows rapidly in tropical countries, with high temperature, extreme ranges of rainfall and humidity, which favour a large number of heterogeneous filamentous fungi that exist heterotrophically (Reddy *et al.,* 2007; Bandh *et al.,* 2012). The colonies are usually fast growing, white, yellow, yellow-brown, brown to black or shades of green, mostly consisting of a dense felt of erect conidiophores (De Hoog *et al.* 2000; Klich, 2002; Ellis *et al.,* 2007). Conidiophores terminate in a vesicle covered with either a single palisade-like layer of phialides (uniseriate) or a layer of subtending cells (metulae) which bear small whorls of phialides (biseriate) (Ellis *et al.,* 2007).

The vesicle, phialides, metulae and conidia form the conidial head. Conidia are one-celled, smooth or rough walled, hyaline are produced in long dry chains which may be divergent (radiate) or aggregated in compact columns (columnar). Some species may produce Hülle cells or sclerotia (De Hoog *et al.*, 2000; Klich, 2002; Ellis *et al.*, 2007).

2.3.4 Features unique to certain species of *Aspergillus*

Sclerotia, Cleistothecia, Aleuriconidia, and Hülle cells are the microscopic structures of *Aspergillus*. These structures are of key importance in identification of *Aspergillus species* like *A. nidulans* and *A. vesicolor*. Cleistothecium is a round, closed structure enclosing the asci which carry the ascospores (Doctor Fungus, 2007).

When the cleistothecium bursts, the asci are spread around the surroundings, cleistothecium is produced during the sexual stage of reproduction of *Aspergillus* (Doctor Fungus, 2007). During lysis of conidium it produces aleuriconodium, the base are usually truncated and carries remnant of the lysed supporting cells. These remnants form annular frills at its base. Hulle cell is a large sterile cell bearing a small lumen, similar to cleistothecium. It is associated with the sexual stage of some *Aspergillus* species (Doctor Fungus, 2007).

2.4 Common Microscopic Features of all Species of *Aspergillus*

The genus *Aspergillus* has a typical vesicle (Doctor Fungus, 2007). Conidiophores originate from the basal foot cell located on the supporting hyphae and terminate in a vesicle at the apex. Hyphae are septate and hyaline (Doctor Fungus, 2007). The morphology and colour of the conidiophores vary from one species to another (Table 2.1).

Conidiophores covers the surface of the vesicle entirely (radiate head) or partially only at the upper surface (columnar head). The flask-shaped phialides which are either uniserate or biserate are attached to the vesicle directly or via a supporting cell, metula over the phialidesare the round conidia (2-5 μm diameter), forming radial chains (Doctor Fungus, 2007). In the presence of Hülle cell which form the morphology of cleistothecia and asco spore (Okuda *et al.*, 2000)

2.4.1 Microscopic features of *Aspergillus* species

2.4.1.1 *Aspergillus flavus*

Conidia are finely roughened, variable in size and oval to spherical in shape, conidial heads are typically radiate, later splitting to form loose columns (mostly 300-400 μm in diameter), biseriate, but having some heads with phialides borne directly on the vesicle (uniseriate). Conidiophore stipe is hyaline and coarsely roughened. Conidia are globose to subglobose (3-6 μm in diameter), pale green and conspicuously echinulate (Sabatelli *et al.*, 2006; Doctor Fungus, 2007). Yellow-green colonies, rough-walled stipes, mature vesicles bearing phialides over their entire surface and conspicuously echinulate with uniseriate and biseriate conidial head (Doctor Fungus, 2007).

2.4.1.2 *Aspergillus parasiticus*

Sterigmata are uniserate, sclerotia are usually absent, conidia are coarsely echinulate, uniform in shape, size and echinulation (Reddy *et al.*, 2007).

2.4.1.3 *Aspergillus fumigatus*

Sterignate are uniseriate and columnar conidial heads with the phialides limited to the upper two thirds of the vesicle and curving to be roughly parallel to each other (Cuenca-Estrella *et al.,* 2006; Sabatelli *et al.,* 2006). Conidia are produced in basipetal succession forming long chains and are globose to subglobose (2.5-3.0 μm in diameter), green and rough-walled to echinulate (Sabatelli *et al.,* 2006).

2.4.1.4 *Aspergillus nidulans*

Conidia are globose and rough-walled, conidial heads are short columnar and biseriate. Stipes are usually short, brownish and smooth-walled. conidial heads are short columnar (up to 70 x 30 μm in diameter) and biseriate. Conidiophore stipes are usually short, brownish and smooth walled. (Espinel-Ingroff *et al.,* 2001; Espinel-Ingroff, 2003; Cuenca-Estrella *et al.,* 2006).

2.4.1.5 *Aspergillus niger*

Conidial heads are large (up to 3 mm by 15 to 20 μm in diameter), globose, dark brown, becoming radiate and tending to split into several loose columns with age. Conidiophore stipes are smooth-walled, hyaline or turning dark towards the vesicle. Conidial heads are biseriate with the phialides borne on brown, often septate metulae. Conidia are globose to subglobose (3.5-5 μm in diameter), dark brown to black and rough-walled. Conidial heads are dark brown to black rough-walled, radiate and biseriate with metulae twice as long as the phialides (Espinel-Ingroff *et al.,* 2001; Pfaller *et al.,* 2002; Diekema *et al.,* 2003; Espinel- Ingroff, 2003; Serrano *et al.,* 2003; Cuenca-Estrella *et al.,* 2006).

2.4.1.6 *Aspergillus terreus*

Conidial heads are compact, columnar (up to 500 x 30-50 μm in diameter) and biseriate. Conidiophore stipes are hyaline and smooth-walled. Conidia are globose to ellipsoidal (1.5-2.5 μm in diameter), hyaline to slightly yellow and smooth-walled. cinnamon-brown cultures, conidial heads biseriate with metulae as long as the phialides (Samson *et al.*,1995; de Hoog *et al.*, 2000; Klich, 2002).

Table 2.1: Microscopic properties of *Aspergillus* Species

Species	Conidiophore	Phialides	Vesicles
A. clavatus	long, smooth	Uniserate	Huge, clavate-shaped
A.flavus	Colourless, rough	Uni/Biserate	Round, radiate head
A. fumigatus	Short (<300µm), smooth, colourless or greenish	Uniserate	Round, columnar head
A. glaucus gr	Variable length, smooth, colourless	Uniserate	Round, radiate to very loosely columnar head
A. nidulans	Short (< 250µm), smooth, brown	Biserate short	Round, columnar head
A. niger	long, smooth, colourless or brown	Biserate	Round, radiate head
A. parasiticus	Long, smooth, colourless or greenish	Uni/biserate	Round, columnar head
A. terreus	short (<250µm), smooth, colourless	Biserate	Round, compactly columnar head
A. versicolor	Long, smooth, colourless or brown	Biserate	Round, loosely radiate head

Source:http://www3.abe.lastate.edu/AE469_569/cornmoods/Aspergillus_summary.htm,2015

2.5 Macroscopic Features of *Aspergillus* species

Variation in growth rate help in species identification (Samson and Pitt, 2000; McClenny, 2005; Diba *et al*., 2007). Surface colour variation is based on the species. The reverse is uncoloured to pale yellow in most of the isolate (Table 2.2). *A. fumigatus* is a thermolerant fungus and can grow at high temperature (Cuenca-Estrella *et al.,* 2006; Sabatelli *et al.,* 2006).

Table 2.2: Colonial properties of *Aspergillus* species

Species	Surface	Reverse
A. flavus	Yellow-green	Golden to Red brown
A. clavatus	Blue-green	White, brownish with age
A. fumigatus	Blue-green to gray	White to tan
A. parasiticus	Blue-green	White to tan
A. niger	Black	White to yellow
A. terreus	Cinnamon to brown	White to brown
A. nidulans	Green, buff to yellow	Purplish red to olive
A. versicolor	White at the beginning, turns to yellow	White to yellow or purplish red
A. glaucus	Tan, pale green or pink green with yellow areas	Yellow to brown

Source: http://www3.abe.lastate.edu/AE469_569/cornmolds/Aspergillus_summary.htm, 2015

2.5.1 *Aspergillus flavus*

Colonies are granular, flat, most at times with radial grooves, yellow at first, but quickly becoming bright to dark yellow-green with age (Sabatelli *et al.,* 2006; Doctor Fungus, 2007)

2.5.2 *Aspergillus parasiticus*

The colony is dark green on czapak's agar, remain green with age (Reddy *et al.,* 2007).

2.5.3 *Aspergillus fumigatus*

This species is thermotolerant and grows at very high temperatures, Colonies have blue-green surface pigmentation with a suede-like surface, consisting of a dense felt of conidiophores (Cuenca-Estrella *et al.,* 2006; Sabatelli *et al.,* 2006).

2.5.4 *Aspergillus nidulans*

They are plain green in colour with dark red-brown cleistothecia, developing within and upon the conidial layer. Reverse may be olive to drab-grey or purple-brown (Espinel-Ingroff *et al.,* 2001; Espinel-Ingroff, 2003; Cuenca-Estrella *et al.,* 2006).

2.5.5 *Aspergillus niger*

The colonies consist of a compact white or yellow basal felt covered by a dense layer of dark-brown to black conidial heads (Espinel-Ingroff *et al.,* 2001; Pfaller *et al.,* 2002; Diekema *et al.,* 2003; Espinel-Ingroff, 2003; Serrano *et al.,* 2003; Cuenca-Estrella *et al.,* 2006).

2.5.6 *Aspergillus terreus*

Colonies are suede-like and cinnamon-buff to sand brown in colour with a yellow to deep dirty brown reverse (Samson *et al.,* 1995; de Hoog *et al.,* 2000; Klich, 2002).

2.6 Mycotoxin Production by Moulds

Mycotoxin-producing fungi are ubiquitous (Murphy *et al*., 2006). Grains can be contaminated with mycotoxin producing fungi at all levels of production, processing, and supply chains (Enyiukwu *et al.,* 2014). Mycotoxins are secondary toxic metabolites with low molecular weight produced by members of the various mould genera (*Aspergillus*, *Fusarium*, *Penicillium*, *Calviceps*) (Valchev *et al.,* 2014). They possess a broad spectrum of biological activity comprising carcinogenic, tetratogenic, mutagenic, embryotoxic, nephrotoxic, oestrogenic and immunosuppressive effects (Valchev *et al.,* 2014). Mycotoxins are secondary metabolites produced by filamentous fungi on food crops that are capable of being potentially harmful to animals and human population such that they cause diseases or death in humans and other animals when ingested depending on the level and duration of exposure (Bennett and Klich, 2003; Viljoen, 2003; Bandyopadhyay *et al.,* 2007; Fakhruddin *et al.,* 2015). Mycotoxins are commonly associated with oil seeds, groundnut, spices, cotton seeds, soybean, cowpea as well as several cereals like maize, wheat, barley, sorghum, oats and rye which serve as main staple for humans and raw materials for livestock (Farag, 2008; Tiffany, 2013). They are secreted in the blood, urine, feaces, milk, meat, muscles, and eggs (Eaton *et al.,* 2001; Wild and Turner, 2002; Mykkänen *et al.,* 2005; Johnson *et al.,* 2008; Okello *et al.,* 2010).

In poultry production, feed is the key vehicle for introducing mycotoxin into flocks (Habib *et al.,* 2015a). Some of these fungi species especially those that produce mycotoxins can contaminate the feed of the birds which they may subsequently consume resulting in mycotoxicosis (Kwanashie *et al.,* 2013). The occurrence and incidence of mycotoxin in chicken meat and eggs are alarming (Iqbal *et al.,* 2014b). Dietary exposure to aflatoxin B1 (AFB1) is detrimental to avian health and leads to major economic losses for the poultry industry (Monson

et al., 2014). Consumption of feed contaminated with mycotoxins can adversely affect poultry performance and health (Monson *et al.,* 2014).

Over 400 different types of mycotoxins have been identified so far; however, the significant agriculturally important mycotoxins in terms of their toxicity and occurrence are aflatoxin, ochratoxins, patulin, ergot alkaloids, fumonisins, zearalenone and trichothecenes–nivanenol (NIV), deoxynivanenol (DON), diacetoxyscirpenol (DAS) and T-2 toxins (Bankole and Adebanjo, 2003; Binder *et al.,* 2007; Spiteller, 2009; Iqbal *et al.,* 2013; Iqbal *et al.,* 2014a). They are odourless and tasteless (Fapohunda, 2013, 2014). Environmental factors favouring mould growth and aflatoxin production are hot and humid conditions (Enyiukwu *et al.,* 2014). The optimum temperature and moisture content for growth and toxin production for various aflatoxigenic fungi varies between 24°C and 28°C with moisture content of at least 17.5% (Ominski *et al.,* 1994). The ambient climatic condition in most Africa countries fall within these range, which encourages high prevalence of these toxins in grains (Makun *et al.,* 2011).

These poisonous chemical compounds are capable of causing acute or chronic effects on humans including the induction of cancer, birth defects, digestive systems disorders, reproductive dysfunction, immune suppression, impediment of liver metabolism, liver cirrhosis and premature puberty in girls (FAO, 2013; Viljoen, 2003). Mycotoxins damage gastrointestinal tracts, respiratory systems, DNA and sulphyhydryl bonds in many enzymes in humans (Reddy *et al.,* 2010). It was suggested that mycotoxins exacerbates some disease conditions such as kwashiorkor, malaria, HIV/AIDS and hepatocellular carcinoma (Wagacha and Muthomi, 2008).

Mycotoxin concentrations are usually sporadic and their distribution in location varies annually. However, local weather patterns, crop damage and agronomic practices affect their production

(ISU, 2014). According to statistics, up to 25% of the world's crops are mycotoxin contaminated and over 4.5-5.0 billion people around the world especially in less developed nations are at the risk of chronic exposure to mycotoxins (Bryden, 2007; Bhat, 2008; Tiffany, 2013; Whitlow and Hagler, 2013). A significant level of grain-mould contamiantion occur in the field, is encouraged by damp conditions at time of harvest, insect infestations, delayed harvesting as well as improper post-harvest handling, transportation, poor drying and poor storage (Reddy *et al.,* 2013; Tiffany, 2013).

2.7 Aflatoxins

Aflatoxins are polyketide secondary metabolites produced by toxigenic strains of *Aspergillus* (Da Costa *et al.,* 2010; Valchev *et al.,* 2014). Chemically they are difuro-coumorins which are freely soluble in chloroform and methanol (Enyiukwu *et al.,* 2014). They grow on a variety of nutritional substrates like cereals which is the main active ingredient of poultry and human food (Banerjee, 2010). They are extremely harmful to the health of humans and animals, showing carcinogenic, mutagenic, teratogenic and immunosuppressive actions (Zinedine and mańes, 2009; Agriopoulou *et al.,* 2016). Among the 18 different types of aflatoxins identified, the major members are aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), G_2 (AFG_2). M_1 (AFM_1) and M_2 (AFM_2) are metabolites of B_1 and B_2 which are produced by *Aspergillus flavus*, *Aspergillus parasiticus*, and *A. nomius* (Kurtzman *et al.,* 1987). Additionally other species which produce aflatoxin are *A. pseudotamarii, A. ochraceoroseus, A. rambellii, A. toxicarius* (Reiter *et al.,* 2009; Sarigiannis *et al.,* 2014; Agriopoulou *et al.,* 2016). *A. flavus* can vary from non-toxic to highly toxigenic and are more likely to produce AFB_1 than AFG_1. Strains of *A. parasiticus* generally have less variation in toxigenicity and produce AFB_1 and varying amounts of AFB_2, AFG_1 and AFG_2 (Coppock and Christian, 2007). Aflatoxins B occur more frequently as

contaminants, and are also believed to be more potent, than Aflatoxins G (Bennett and Klich, 2003). Aflatoxin B_1 (AFB_1) is most relevant mycotoxins due to it toxic effects as demonstrated in human exposure as carcinogenic, mutagenic and teratogenic effect (EFSA, 2006; EFSA, 2007). AFB_1 causes acute hepatoxicity in humans and animals, and in severe intoxications may cause death (Corcuera *et al.,* 2012).

The toxicity of aflatoxins in poultry has been widely investigated by determining their teretogenic (Sur and Celik, 2003), carcinogenic, mutagenic and growth inhibitory potentials (Oguz and Kurtoglu, 2000). Aflatoxin has caused serious destructions in Africa, which has caused significant financial losses in agricultural commodities contaminated with toxins and consequently having effects on animal and human health point of view (Wu and Munkvold, 2008: Zhag and Caupert, 2012). Although most countries of the world have been affected by aflatoxin, it is sub-saharan Africa (SSA) that has suffered most (Makun *et al.,* 2012). Most of SSA agriculture occurs in impoverished rural areas and a lack of technical infrastructure in many African countries does not allow for routine quality control of even commercially produced commodities, never mind those produced by rural population for their own consumption (Makun *et al*., 2012).

2.7.1 Physical and chemical properties of aflatoxin B and G

Structurally they are dihydrofuran-coumorins moiety containing double bond which are freely soluble in chloroform and methanol. They are unstable to UV light or polar solvents (Reddy and Waliyar, 2007; Farag, 2008). Aflatoxins are secondary metabolites that have two possible designations, B or G, which relate to fluorescence under exposure to long-wave ultra violet (UV)

light. Aflatoxin B appears blue in colour, while aflatoxin G appears green in colour due to differences in their chemical structures (Verma, 2004; Reddy and Waliyar, 2007).

The aflatoxin B occur as difuro macro-cyclopentenone series and difurocoumaro lactone series (Reddy *et al.*, 2007). The order of acute and chronic toxicity is $AFB_1 > AFG_1 > AFB_2 > AFG_2$, reflecting the role played by epoxidation of the 8, 9-double bond and also the greater potency associated with the cyclopentenone ring of the B series, when compared with the six-membered lactone ring of the G series as illustrated by their LD50 values for day-old duckling (Reddy and Waliyar, 2007). Table 2.3 and Figure 2.2 show the physical and chemical properties of aflatoxins.

Table 2.3: Physical properties of aflatoxins

Aflatoxin	Molecular formula	Molecular weight	Melting point °C
B1	$C_{17}H_{12}O_6$	312	268-269
B2	$C_{17}H_{14}O_6$	314	286-289
G1	$C_{17}H_{12}O_7$	328	244-246
G2	$C_{17}H_{14}O_7$	330	237-240
M1	$C_{17}H_{12}O_7$	328	299
M2	$C_{17}H_{14}O_7$	330	293
B2A	$C_{17}H_{14}O_7$	330	240
G2A	$C_{17}H_{14}O_8$	346	190

Source: International Crop Research Institute for Semi Arid Tropics,

Adapted from: Reddy *et al* (2007).

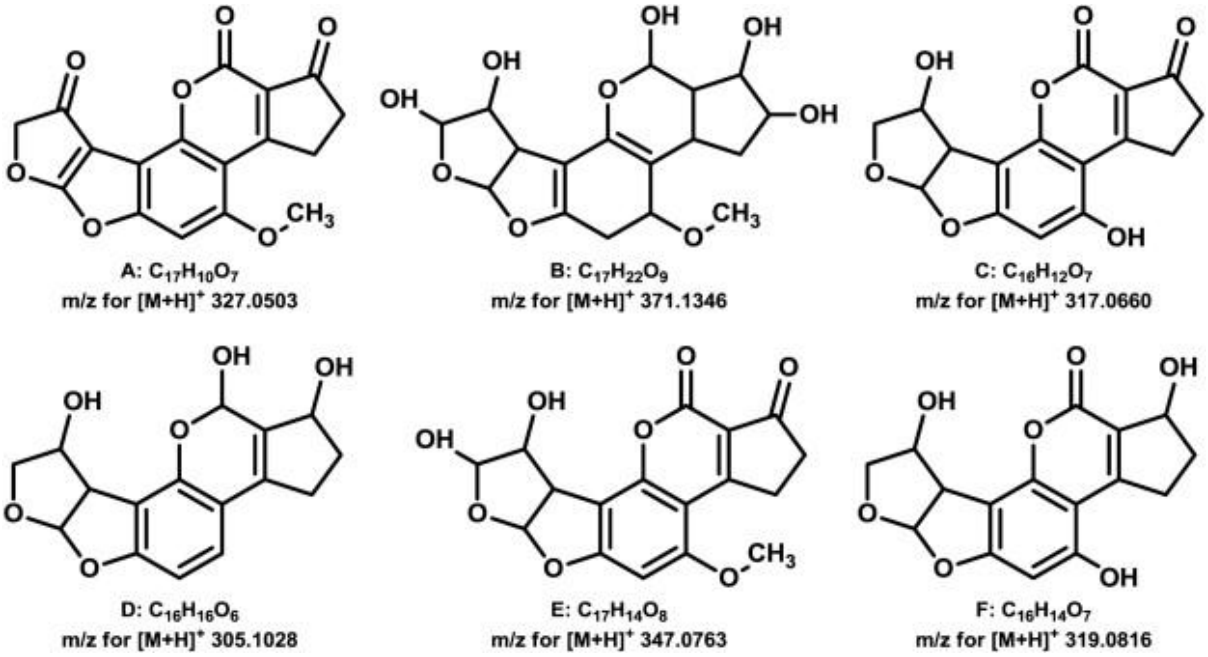

Figure 2.2: Chemical properties of aflatoxin B and G (A-F)

Source: Adapted from Agriopoulou *et al* (2016).

2.8 Toxicology of Aflatoxin

Aflatoxins are not eliminated completely by normal cooking procedure (Kishore *et al.*, 2002). They are efficiently absorbed in the duodenum and the liver is main target organ but may affect other organs based on their reactivity with DNA, RNA, enzymes and proteins. Mycotoxins undergo metabolism via cytochrome P450s specifically CYP3A4, CYP3A5, and/or CYP1A2 enzymes in the liver to exo-8, 9-epoxide (Murphy *et al*., 2006; Allameh *et al.*, 2011).

Aflatoxin epoxide (8, 9 epoxide) is the major toxic metabolite which binds to DNA or RNA to form adducts. Adducts especially at N 7-guanine implicates the compound in carcinogenesis and inducement of hepato-cellular carcinoma (HCC), leading to the conversion of guanine (G) to thymine (T) at the third nucleotide of the codon (Murphy *et al*., 2006).

It is also important to note that cytochrome P450 types also convert AFB_1 to other derivatives e.g AFM_1, AFP_1 and Q_1 (Campbell and Hayes, 1976). In the presence of water, AFB_1-8, 9-dihydrodiol is formed which reacts with serum proteins (leusine or albumin) thus informing the toxicity of aflatoxins (Allameh *et al.*, 2011; Tiffany, 2013). Hepatitis B Virus (HBV) infection aggravates with chronic ingestion of aflatoxins. Aflatoxin B1 and HBV cause human hepato-cellular carcinoma (Bhat, 2008). AFB_1 can form AFB1-DNA adducts, DNA strand breaks, DNA base damage and oxidative damage that can lead to cancer (Wang and Groopman, 1999). This damage can be repaired by various mechanisms e.g base excision repair (Wood, 1999).

Recent findings suggest that two loci in this xeroderma pigementosum complementation gene group D (XPD) are of particular importance in modulating the AFB_1 related development of liver cancer (Long *et al*., 2009). Probably of more direct importance is the action of AFB_1 on the P53 gene where it causes AGG to AGT transverse mutation at codon 249 (Bressac *et al.,* 1991). Gene P53 is responsible for producing the P53 protein which has an important role in the regulation of cell cycle and in the suppressing of genome mutation (May *et al.,* 1999).

2.9 Sensitivity of Poultry to Aflatoxin

Poultry meat, egg, and other products derived from them are very crucial in healthy food chain (Habib *et al.,* 2015a). Most mycotoxins of concern in poultry feed are aflatoxins (Monbaliu *et al.,* 2010). This feed stuff constitutes an important source of energy in poultry production (Yegani *et al.,* 2006). Poultry are highly susceptible to the immunotoxic effect of food-borne aflatoxin B1 (AFB_1). Exposure to aflatoxin may impair cell-mediated and humoral immunity, limit vaccine efficacy and increases the incidence of costly secondary infection (Monson *et al.,* 2015). Dietary exposure to aflatoxin B_1 (AFB_1) is detrimental to avian health and leads to major economic losses for the poultry industry (Melissa *et al*., 2014). Aflatoxin B_1 (AFB_1) and total Aflatoxin (AFT) contaminate feed which is deposited as residues in eggs, muscles (breast, leg) organ (liver, kidney, gizzard) Herzallah, (2013). Small quantity of aflatoxin in feed can cause reduced feed quality and animal efficiency either through poor conversion of nutrient or reproductive abnormalities (Oguz and Kurtoglu, 2000; Ortatatli *et al.,* 2002). Listlessness, anorexia, lowered growth rate, poor feed utilization, decreased egg production, anaemia, reduced immune function, hepatotoxicosis, haemorrhage and increased mortality have been observed in affected birds (Miazzo *et al.,* 2000; Oguz *et al.,* 2000; Ortatatli and Oguz, 2001).

Aflatoxicosis has the same toxic effects in poultry as it does in mammals. A dose of 0.25 μg/kg in turkey poults and duckling impairs growth, and a dose of 1.5 μg/kg in broilers and 4 μg/kg in Japanese quails has negative effects on growth rate (Ananth and Anwer, 2006). An increase in blood clotting time increases the susceptibility of the carcass to bruising even at doses below that to have an effect on growth (Ananth and Anwer, 2006). In poultry, aflatoxins impair the availability of bile salts, which decreases vitamin D3 production. It causes a decrease in the absorption of fat soluble vitamins (Ananth and Anwer, 2006). Aflatoxins also decrease the production of vitamin A in the liver, and it has secondary effect such as decrease blood calcium levels, decreased bone strength, and a decreased tissue and serum tocopherol level (Ananth and Anwer, 2006). However, decrease in tocopherol level can lead to vitamin A and E deficiencies.

2.9.1 Pathogenicity and clinical significance of aflatoxin in man and other animals

2.9.1.1 *Acute aflatoxicosis in animals*

Acute aflatoxicosis in animals was first documented in 1960, after more than 100,000 turkeys died following an outbreak in the United Kingdom (Wu *et al.*, 2011b). Aflatoxin causes a variety of symptoms depending on the animal, dose, length of exposure, species, breed and diet or nutritional status. However, in all animals, aflatoxin can cause liver damage, gastro-intestinal dysfunction, reduced productivity, decreased **feed utilization** and efficiency, decreased reproductive performance, reduced milk or egg production, embryonic death, teratogenicity (birth defects), tumours and suppressed immune system function, even when low levels are consumed (Jones *et al*., 1994; Cortyl, 2008).

Fatty liver, kidney disorders, leg and bone problems, pigmentation problems (carcasses, egg yolk), reduced hatchability, smaller eggs and reduced egg shell quality, coccidiosis, vaccine failure, reduced immunity, lower resistance to diseases, bacteria, viruses and reduced performance have been reported in exposed birds (Cortyl, 2008; Wu *et al.*, 2011a).

2.9.1.2 *Acute aflatoxicosis in human*

Acute aflatoxicosis is associated with extremely high doses of aflatoxin, and is characterized by hemorrhages, acute liver damage, oedema, and high mortality rates in humans. Acute aflatoxicosis is associated with sporadic outbreaks of the consumption of highly contaminated foods. Early symptoms of acute high level exposure to aflatoxin include diminished appetite, malaise, and low fever; later symptoms, which include vomiting, abdominal pain, and hepatitis, can signal potentially fatal liver failure (Barret, 2005). Kenya has experienced several recurrences of acute aflatoxicosis in humans and has recorded hundreds of deaths in the last 4 decades (Shephard, 2008).

2.9.1.3 *Chronic aflatoxicosis in humans and animals*

Chronic aflatoxicosis is associated with long-term exposure to low to moderate levels of aflatoxin in the food supply. It is estimated that more than 5 billion people in developing countries worldwide are at risk of chronic aflatoxin exposure through contaminated foods (Strosnider *et al.*, 2006; Shephard 2008). Chronic low-level exposure to aflatoxin, particularly aflatoxin B1, is associated with an increased risk of developing hepatocellular carcinoma, or liver cancer, as well as impaired immune function and malnutrition and stunted growth in children. Aflatoxin B1 is the most potent liver carcinogen and is found in greater concentrations than any other naturally occurring aflatoxin (Bhat and Vasanthi, 2003; Wu *et al*., 2011a)

According to the World Health Organization (WHO), hepatocellular carcinoma is the third leading cause of cancer deaths globally (WHO, 2008).

Aflatoxin exposure affects early development, as well as some aspects of human immunity and nutritional processes (Williams *et al.,* 2004). Aflatoxin B_1 is the most toxic of the aflatoxins and the strongest naturally occurring chemical liver carcinogen known. Aflatoxin, metabolized by enzymes in the liver, binds to proteins and causes acute toxicity (aflatoxicosis). Aflatoxin exposure causes acute liver damage and liver cirrhosis, as well as development of tumors or other genetic effects (FEHD, 2001; Bhat and Vasanthi, 2003; WHO and CDC, 2005)

2.9.2 Impact of aflatoxins on the liver

Liver is the main organ of detoxification and the first major organ to be exposed to dietary intake of xenobiotics. Several liver conditions have been associated with aflatoxin, particularly in under developed countries especially Africa (Makun *et al.,* 2012). However, staple food are routinely contaminated with aflatoxin Makun *et al.* (2012), and are efficiently absorbed in the small intestine especially by the duodenum. The liver is main target organ but may affect other organs based on their reactivity with DNA, RNA, enzymes and proteins (Enyiukwu *et al.,* 2014). Mycotoxins undergo metabolism via cytochrome p450 enzymes in the liver. Aflatoxin epoxide (8, 9 epoxide) is their major toxic metabolite which binds to DNA or RNA to form adducts.

There is morphological change in kidneys and some blood parameters of the renal function in broiler chickens experimentally fed with aflatoxin (Valchev *et al.,* 2014). However, AFM_1 is less toxic and possess the same carcinogenic, mutagenic and toxic activities as its parent compound

AFB1 and it is also highly stable during heat treatments such as pasteurization and are not eliminated completely by normal cooking procedure (Kishore *et al.,* 2002; Peng *et al.,* 2016)

2.9.3 Regulation for aflatoxin world-wide

The presence of aflatoxins in food caused difficulties in trading, and thus economic loss, due to difference in acceptable limits for the aflatoxins ranging from 0 to 50 ng of food by local authorities (Bekele *et al.,* 2011; Joerg and Elke, 2002; Simone *et al,* 2008). Because of the deleterious health effects of aflatoxins, their doses have been strictly regulated. For instance, by the end of 2003, approximately 100 countries (covering approximately 85% of the world's inhabitants) had specific regulations or detailed guidelines for mycotoxins in food or feed (Hans *et al.,* 2007). Twenty-six countries in Asia/Oceania which represent 54.5% of the world's inhabitants enact regulations and monitoring law on all aflatoxins contamination in food and feed (FAOU, 2004).

Regulation of mycotoxins is established in many countries, specifying the maximum limit of aflatoxin, ochratoxin (OTA), patulin, fumonisins, zearalenone, and deoxynivalenol in feed and foodstuffs (Mariko, 2012). Many factors are always considered in setting a limit for mycotoxins. These are food consumption data, scientific factors to assess risk, awareness about the level and distribution of mycotoxins in commodities, analytical methodology, level of food security, trade and economic factors in the determination of mycotoxin tolerance level (van Egmond, 2013).

The legislation of many countries states that any food containing a contaminant in an amount that is unacceptable for the public, and with high toxicological level, cannot be marketed in that

country (Mariko, 2012). In most countries, regulations are established to control the contaminants in food and feed stuffs to protect human health, as indicated in Table 2.4 and Figure 2.3. Twenty parts per billion is the allowed maximum limit of total aflatoxins in food meant for human consumption in most countries including the USA, Nigeria and UAE and as low as 4 ppb in the EU nations (Tiffany, 2013). In Africa, grains contribute about 46% of the total energy intake (FAOSTAT, 2010). Although report of aflatoxin in both meat and meat products, and egg from Africa seemed insignificant, there is however chronic intake of such amount simultaneously occurring with other food borne toxicants, which is bound to have deleterious health impact (Speijer and Speijer, 2004).

Table 2.4: Total Aflatoxin Limits in Nigeria, Canada, Codex, Gulf Cooperation Council, New Zealand, Australia, India, South Africa and United States of America

Country	Foodstuffs	Total permissible aflatoxin (µg/kg)
Nigeria	Almonds, hazelnut, pistachois, shelled brazil nut, ready to eat	10
CODEX	Peanuts, Almonds, Shelled brazil nuts, hazelnuts	15
GCC(A)	Pistachios, intended for further processing	15
Canada	Tree nuts, nut and nut products	15
South Africa	Peanuuts	15
United State of America	Brazil nuts, Peanuts and peanut products, pistachios products	20
Australia/New Zealand	Peanuts,	15
India	wheat, maize, jawar, bajra, rice, groundnut kernel, whole and split pulse masur, whole and split pulse moong, whole and split pulse chana, food grains, split pulse arhar	30

(a) Members of Gulf Cooperation Council are Saudi Arabia, United Arab Emirates (UAE), Kuwait, Bahrain, Oman, Yemen, Qatar

(Source: food and nutrition paper 64 on the situation of world wide mycotoxin regulations. FAO, 2003)

Figure 2.3: Countries with and without regulations for aflatoxins

(Source: food and nutrition paper 64 on the situation of world wide mycotoxin regulations. FAO, 2003)

2.9.4 Regulation for mycotoxins world wide

Food safety is an imperative in food production world wide and poultry meat, eggs, and poultry products derived from them are crucial in safe food chain (Habib *et al.,* 2015b). As far as safety is concerned, special attention is directed towards possible contamination of food and poultry feed with fungi and the risk of mycotoxin contamination (Radmila *et al.,* 2007). Over time families bring out their grains to clean. However, if the grain change colour or smell due to poor storage, they are sold to especially toll poultry feed miller either as whole grain or ground fine particle at a very cheap rate (Habib *et al.,* 2015b). However, regulations of mycotoxins have been established in many countries (Table 2.5) to protect consumers from the residual effects of mycotoxins that may contaminate foodstuffs (van Egmond, 2013).

Table 2.5: Geographical distribution of mycotoxins

Locations	Mycotoxins
Western Europe	Ochratoxin, Vomitoxin, Zearalenone
Eastern Europe	Zearalenone, Vomitoxin
North America	Ochratoxin, Vomitoxin, Zearalenone
South America	Ochratoxin, Vomitoxin, Fumonisins, aflatoxin
Africa	Aflatoxin, Fumonisins, Zearalenone
Asia	Aflatoxin, Fumonisins, Zearalenone
Australia	Aflatoxin, Fumonisins,

Source: adapted from Zaki *et al* (2012).

2.9.5 Economic impact of mycotoxin

Feed is the major cause of exposure to mycotoxins by animals and therefore ultimately humans (Bryden, 2012). Animals are considered the most exposed group to high concentration of aflatoxins through feed stuffs that develop several health problems which lead to large economic losses (Herzallah *et al.,* 2013). The presence of mycotoxins in food and feed is potentially hazardous to the health of humans as well as animals because of their carcinogenic, toxic and mutagenic effects (Royer *et al.,* 2004). Aflatoxin causes substantial economic losses to poultry industry impeding the growth of birds, decreasing feed conversion ratio, egg production and mortality (Miazzo *et al.,* 2000). The occurrence and incidence of AFs, OTA, and ZEN in chicken meat and eggs are alarming and it may cause health hazard on humans (Iqbal *et al.,* 2014a). These losses are pronounced in meat, and eggs in terms of quality and quantity as a result of contamination with aflatoxin residues (Bintvihok *et al.,* 2002a; Hall and wild, 2003; Farombi *et al.,* 2006).

Aflatoxins especially Aflatoxin B_1 (AFB_1) are potent hepatocarcinogens and immunosuppressant and pose greatest threat to poultry industry through low productivity and death (Chukwuka *et al.,* 2010; Pedrosa and Borutova, 2011; Ezekiel *et al*., 2014). Globally, it is estimated that aflatoxin contributes to between 4.6 and 28.2 percent of liver cancer cases. Each year, 550,000–600,000 new liver cancer cases are recorded worldwide, and of these, approximately 25,200–155,000 are attributable to aflatoxin exposure. In 2008, liver cancer was the third leading cause of cancer-related deaths world-wide (WHO World Health Statistics, 2008).

2.9.6 Analytical methods of mycotoxins for agricultural products

Mycotoxins are toxic secondary metabolites produced by fungi on agro-food products and feed stuff (Selvaraj *et al.,* 2015). Over the past few decades, many analytical methods including thin layer chromatography, high performance liquid chromatography MS/MS, High performance liquid chromatography coupled with fluorescent detectors are widely used for the detection of aflatoxin (Kamkar, 2006; Zheng *et al.,* 2006; Herzallah, 2009; Chen *et al.,* 2012; Soleiymany *et al.,* 2012; Hepsag *et al.,* 2014; Mao *et al.,* 2015; Ossa *et al.,* 2015) followed by ELISA (Thirumala-Devis *et al*., 2002; Bakirdere *et al.,* 2012). Although these methods are sensitive, but they require well equipped laboratories, trained personnel and time consuming sample preparation methods (Peng *et al.,* 2016).

However, several rapid screening of a large numbers of samples have been used widely in food safety monitoring as they are reliable, robust and rapid (Peng *et al*., 2016). These include immunoassay, particularly indirect competitive enzyme linked immunosorbent assay (ic-ELISA), nucleic acid hybridization, polymerase chain reaction, mycotoxin biosynthetic gene and combination assays for detection of mycotoxin to ensure safety and quality food (Mostafa *et al*., 2012; Peng *et al*., 2016).

2.9.7 Mycotoxin exposure assessment

Exposure assessment is a factor of risk assessment in addition to toxicity, occurrence of mycotoxin on commodities as well as data on food intake are needed to conduct exposure assessment (van Egmond and Jonker, 2007). Identifying the active ingredient of mycotoxin is very difficult. However, Joint Expert Committee on Food Additive (JECFA) emphasis the use of a current analytical methods and to ensure that the result of the survey, provide a reliable

assessment of intake (WHO, 2002b). Many countries have set maximum limits (MLs) for AFM_1 which range from 0.01-0.5µg/L in various foods (ECR, 2001; 2004). Over the past few decades, many analytical methods, including thin layer chromatography (Kamkar, 2006), high performance liquid chromatography (Herzallah, 2009; Rahmani *et al.,* 2009) and HPLC-Ms/Ms (Chen *et al.,* 2012, Soleimany *et al.,* 2012: Ossa *et al.,* 2015). Although these methods are sensitive, they require well-equiped laboratories, trained personnel, and time consuming sample preparation methods (ECR, 2001; 2004). In virtually all JECFA reviews of mycotoxins, analytical data on amounts of contamination were often inadequate for developed countries and non-existent for developing countries (van Egmond and Jonker, 2007)

2.9.8 Sampling procedure for agricultural products

Effective monitoring is required to ensure that the AFM_1 residue do not exceed the MLs in the human food chain (Peng *et al.,* 2016). About 5 billion humans in different countries are at risk of AFB_1 through different contaminated food and livestocks products (Liu and Wu, 2010). Moreover, the carry-over of AF through animal-derived products, such as meat and eggs, into the human food chain is a potential threaten to human health (Aly and Anwer, 2009; Christofidou *et al.,* 2015; Herzallah, 2013 and Iqbal *et al.,* 2014b). However significant economic losses may occur due to the presence of natural feed contaminants, such as mycotoxins which are the most widely distributed and toxic to poultry (Jia *et al.,* 2016).

Aflatoxins are sparsely distributed in different concentrations on agricultural products which is an important factor to be considered in establishing regulatory sampling criteria (van Egmond and Jonker, 2007). The distribution of mycotoxins especially aflatoxins in peanut is heterogenous (van Egmond and Jonker, 2007). The consumption of peanut could lead to an accidental high

single dose of aflatoxins, rather than the chronic intake at a relatively low level dose (van Egmond and Jonker, 2007; European commission, 2002b; FDA, 2002).

2.9.9 The problem of aflatoxin contamination of foods and feeds in Nigeria

Aflatoxins are found in "alarming concentrations in Nigeria, between 3,000 to 138,000 ug/kg in pre-harvest maize samples" (Maxwell *et al.,* 2000). Animal feeds both commercially and locally prepared is an important source of energy for livestocks industry. Standard Organization of Nigeria set limit for aflatoxin in poultry feed at 20 ppb (Tiffany, 2013). European Union, has reportedly rejected animal feed from some exporters in Nigeria (PACA, 2012). It is not clear, however, that Nigeria has been affected by trade losses in these EU rejections of livestock feed. A recent study of poultry feeds found aflatoxin levels above 20 ppb in 62% of samples among commercial producers (PACA, 2012). Investigation of 13 samples of poultry feed in Nigeria found a mean concentration of 15.5 ppb with a range of 0 to 67.9 ppb (Adebayo and Etta, 2010). About 94% food samples are contaminated with aflatoxin in Nigeria (USAID and DANYA International, 2012).

2.10 Prevention and Control of Aflatoxicosis

Most aflatoxicosis results from eating contaminated foods and there are almost no treatments for aflatoxin exposure (Lizárraga-Paulín *et al.,* 2011). There is evidence that some strains of *Lactobacillus* effectively bind dietary aflatoxins. Similarly, clay-based enterosorbents have been used to bind aflatoxins in the gastrointestinal tract (Lizárraga-Paulín *et al.,* 2011). It has been demonstrated that selenium supplementation modifies the negative effects of aflatoxin B1 in Japanese quail, while butylated hydroxytoluene gives some protection in turkeys. Oltipraz, a drug originally used to treat schistosomiasis, has been tested in human populations in China with some apparent success (Bennett *et al,* 2007). Methods for controlling aflatoxin exposure are

largely prophylactic. In a primary prevention trial, the goal is to reduce exposure to aflatoxins in the diet. A range of interventions include planting pest-resistant varieties of staple crops, attempting to lower mold growth in harvested crops, improving storage methods following harvest, and using trapping agents that block the uptake of unavoidably ingested aflatoxins. In secondary prevention trials, one goal is to modulate the metabolism of ingested aflatoxin to enhance detoxification processes, thereby reducing internal dose and subsequent risk (Groopman *et al.*, 2008).

CHAPTER THREE

MATERIAL AND METHODS

3.1 Study Area

The study was carried out in Zaria, a major city in Kaduna State in North West Nigeria. There are two Local Government Areas in Zaria town namely, Sabon Gari and Zaria Local Government Area. Zaria is known as an educational center and another major occupation of the people is poultry farming and livestock rearing (Ajala, 2004). Zaria has 6 major live bird markets with a total of poultry slaughter of 7,250 daily. The population of Zaria is estimated as 547,000 from the 2006 Nigerian census. It is situated on latitude 11°7□ , 11°12□ N and longitude 7°41□ E (Mamman *et al*., 2000). Relative humidity in Zaria is between (63.2- 68.8 %) with an average rainfall of (155.9-182.1mm), temperature range of (25-30.2°C), and with a low evaporation rate (154.2-163.91mm) (Igono and Aliu, 1982; Edem, 2009). The vegetation of Zaria is within the guinea savannah (Kadunastate.gov, 2006).

3.2 Study Design

A cross-sectional study was used to determining the occurrence of aflatoxigenic fungi, isolated from feed and aflatoxin concentration in slaughtered broilers in the study area. Samples were collected from six major live bird markets within Zaria metropolis from August, 2015 to January, 2016. Most of the feeds and chickens at these live bird markets were sourced from feed stores and poultry farms, respectively across Kaduna State by the sellers. Chickens are slaughtered in the same live-bird markets where they are sold.

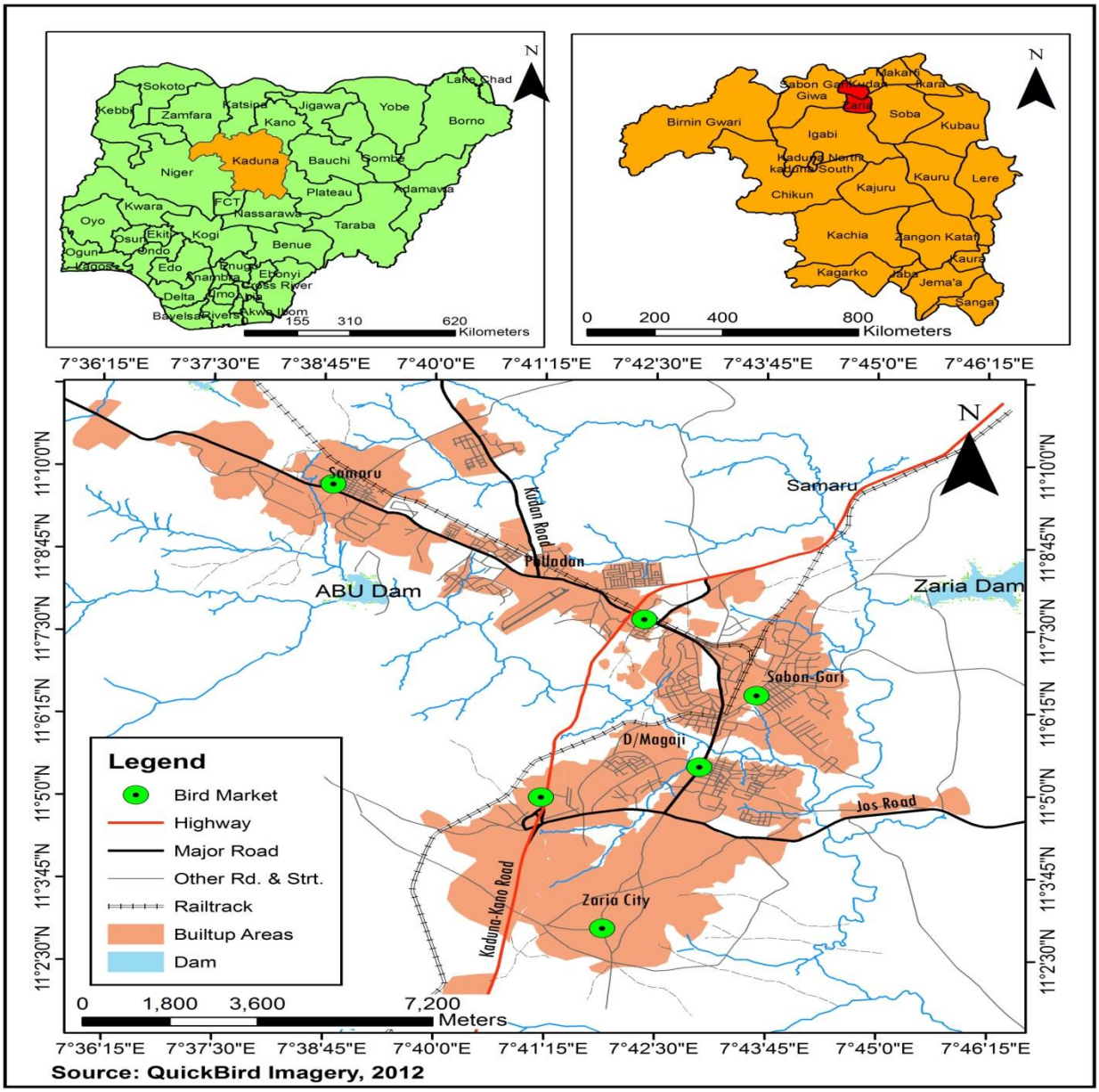

Figure 3.1: Map of live bird markets in Zaria.
Source: Quick Bird Imagery, 2013.

3.3 Sample Size

The sample size was calculated using the formula, described by Thrusfield (1997).

$$n = \frac{Z^2 Pq}{d^2}$$

n = Sample size

p= Prevalence of *Aspergillus* in feed 78% (Habib *et al.*, 2015)

d= Desired absolute precision 0.05

Z= Standard normal deviation for 95 %

q= 1-p

Feed sample size = 263

Tissues sample size = 60

The sample size for feed was increased to 300 to increase precision and minimise sampling error. Therefore, 50 feed samples were collected at each of the live bird markets in Zaria namely: Dan Magaji, Kwangila, Samaru, Sabon-Gari, Tudun wada, and Zaria city. The feeds and slaughtered broilers were selected based on availability.

A total of 60 liver samples from broilers were collected from six live bird markets. Ten broiler liver samples, where from dressed birds at the six live bird markets.

3.4 Sample and Sampling

All the six live bird markets in Zaria namely: Dan Magaji, Kwangila, Samaru, Sabon-Gari, Tudun wada, and Zaria city were enrolled in the study.

3.4.1 Preparation of culture media

Sabouraud dextrose agar

Exactly 65 g of Sabouraud dextrose agar (SDA) (CM41-Oxoid, U.K.), was added to 1000 mL distilled water and brought to boil in order to dissolve completely and sterilised by autoclaving at 121°C for 15 minutes. The mixture was allowed to cool to about 50 °C, after which chloramphenicol and cyclohexamide were added, mixed thoroughly by swirling and left to cool and solidify (According to manufacturer instruction).

Czypeck yeast agar

Exactly 65 g of Czypeck dox agar (CYA), (CM0097-Oxoid, U.K.), was added to 1000 mL distilled water and brought to boil so as to dissolve completely, and sterilised by autoclaving at 121°C for 15 minutes. The mixture was allowed to cool to about 50 °C to solidify (According to manufacturer instruction).

Dessicated Coconut Agar

Dessicated coconut impregnanted neutral red agar (NRDCA) was prepared as described by Atanda *et al.* (2011). Fresh whole coconut fruit (*Cocos nucifera*) was purchased. The coconut was cleaned with alcohol and opened with a sterilised knife. The white part (mesoderm of *Cocos*

nucifera) was removed with sterilised knife, placed on foil paper and immediately placed in an oven at 80 °C for 6 h to prevent enzymatic reaction. While in, the oven, the coconut was spread and mixed using a wooden spoon after every 30 minutes to allow even drying of the coconut.

Exactly 40 g of the white part of coconut was weighed on a foil paper and soaked in 400 mL of hot distilled water for 30 minutes. It was blended, sieved with Whatman paper No. 1 and 2 % agar weight (Bacto-agar Difco Laboratory, Australia) was added as a binder. Thereafter, 3 mL of concentrated neutral red stain (Stigma-Aldrich, USA) was added to the filtrate and brought to boil and cooled to about 50 °C. The filtrate was divided into two parts. In one part, 0.1% neutral red (sigma-Aldrich Co, LLC, USA) stain was added and to the other part 100 µg/mL phenol red stain (Abbey Colour, Philadelphia) was added. The two portions were sterilised in an autoclave at 121 °C for 15 minute and allowed to cool to 50 °C and 100 µg/mL of chloramphenicol and streptomycin were added to each. The contents (15 mL) was poured into a sterile Petri dishe (8.5 cm) while shaking.

Sterile distilled water

Exactly 10 mL of distilled water was dispensed into sterile sample bottles which were later autoclave at 121 °C for 15 minute. The mixture was allowed to cool to about 50°C and labelled according to the method described by Larone (1995).

3.4.2 Collection of poultry feed samples

A total of 300 samples of different types of poultry feeds which are fed to the birds before slaughter (concentrate, commercially prepared broiler finishers, compounded feeds, and maize offals) were collected and analyzed from live bird markets. The feed samples were collected

from feeding troughs and feeders during the time of sample collection per stand in live bird markets with a sterile spoon and polythene bag.

3.4.3 Processing of feed samples

Feed samples were prepared using the method, described by Makun *et al*, (2010) and Udom *et al,* (2012). Exactly 1 g of feed was added into 9 mL of sterile distilled water and homogenized with stomacher (Stomacher® Bag, Seward, USA).

Inoculation of feed on the media

One loopful of each mixture was inoculated into labelled Sabouraud dextrose Chloramphenicol agar plates and subculture on Czypeck dox agar, incubated at room temperature (25-26 °C) for 3-5 days. The colonies that appeared greenish-yellow colour with powdery texture and having the reverse side of pale to yellow colour were considered for microscopic characterisation of *Aspergillus* species (Mycology-Critique, 2004; Giorni *et al.,* 2007).

Spore grown on sterile distilled water was used to inoculate coconut agar plates, using sterilised tooth pick (Ezekiel *et al.,* 2011) and incubated at room temperature for 72 h. The tentative identification of *Aspergillus* section *flavi* was carried out in the Department of Pharmagnosis and Drug Development, Ahmadu Bello University, Zaria. The plates were observed under long wavelength (365 nm), ultraviolet for fluorescence or blue/green illumination (indicating ability to produce aflatoxin) between 5-7 days of incubation as reported (Ezekiel *et al*., 2014). The identification was based on the strength of fluorescence.

3.4.3.1 *Storage of fungi in sterile distilled water*

Suspected fungal colonies from the labelled plate were gently inoculated into a sterile distilled water, covered loosely and maintained as stock cultures, kept at room temperature as described by Larone (1995), in slate bottles.

3.4.3.2 *Macroscopic examination of culture*

Plates were examined grossly for morphological characterisatics of *Aspergillus* species such as the reverse and colonial morphology and colour as described (James and Natalie, 2001; Giorni *et al.*, 2007; Bandh *et al.*, 2012).

3.4.3.3 *Microscopic examination of culture*

One to two drops of lactophenol cotton blue dye was added on a labelled grease free clean glass slide using a sterilised platinum inoculating needle, a suspected fungal growth was picked from the medium and placed on the slide. This was then teased out and covered with glass cover slip. This was pressed down slightly with the tip of finger to expel any air bubble and further disintegrate the hypal growth to enhance observation. The slides were observed under x 10 and 40 magnification of a light microscope. Microscopic characterisation of fungi such as hyphae, conidial heads and arrangements vesicle, mutalae were observed according to the method described by James and Natalie, (2001) and Bandh *et al*, (2012).

3.5 Collection of Liver Samples

A total of 60 liver samples from broilers were collected from six live bird markets. Ten each of the liver samples were collected from the six live bird markets, based on convience.

3.5.1 Analyses of the liver samples

Whole liver was placed into a ziploc polythene bag aseptically and transported to the Mycotoxin Laboratory, Department of Crop Protection Laboratory, Ahmadu Bello University, Zaria. Samples were weighed and 1 g of samples was homogenized (HGBTWT, USA), with 100 mL of 70% Methanol (v/v 70 mL methanol and 30 mL distilled water) and 0.5% of KCl blended to mixed thoroughly, in a conical flask and shaken in an orbit shaker (Benchmark®) for 30 minutes at 150 rpm, the extract was filtered using Whatman paper. Exactly 150 µL of Bovine Serum Albumin conjugate (AFB1-BSA) and carbonate buffer were filled into lane B-G border wells (lane A and H, well B-G, 1and 2) were filled with distilled water and plate were incubated in a shaker incubator (Technel and Technel, USA) at 37 °C for 1 h. The plate were removed and the toxin washed with Physiological Saline Buffer Tween (PSB-T) 3 times, and allow 3 minutes for each wash. A total of 150 µL of 0.2% BSA was added to each well, while the border well was again filled with distilled water and incubated at 150 rpm, 37 °C for 30 min. The plate was then removed and the solution was discarded, then was washed with PBS-T, 3 times, allowing 3 minute for each wash. Exactly 150 µL of goat anti-rabbit IgG-ALP was added to all wells, with the exception of border wells which were filled with distilled water, and incubated for 1 h. The plate was washed as removed and the solution was discarded, then was washed with PBS-T, 3 times, allowing 3 minute for each wash. A total of 150 µL of P-nitrophenyl-phosphate in 10%

diethanol amine buffer was dispensed into each of the wells, with the exception of border wells which was filled with distilled water and incubated for 30 min. The plate was removed and read in ELISA reader using the microplate reader of 405 nm (Sarimehmetoghlu *et al*., 2004; Rosi *et al.,* 2007)

The concentration of aflatoxin B1 (µg/kg) was calculated as follows;

$$AFB1(\mu g/kg) = \frac{AxDxE}{G}$$

Where A = AFB1 Concentration from curve (ng/mL)

D = Time dilution with buffer (o)

E = Extraction solvent volume used (100 mL)

G = Sample weight used (20 g)

3.5.2 Processing of boiled liver samples

One gram (1 g) of fresh liver samples with detected aflatoxin contents were heated at boiling temperature for 90 min, the same procedure was done as in section 3.5.1 as according to the methods described (Sarimehmetoghlu *et al*., 2004; Rosi *et al.,* 2007)

3.6 Data Analyses

Data obtained from this study were expressed using tables, bar charts, mean and standard deviation (Mean ± SD). They were analysed using Graphpad prism version 5.0 and subjected to repeated-measure of analysis of variance (ANOVA), followed by Tukey's post hoc test to compare mean values among different locations. Paired t-test was used to determine the effect of heat treatment on aflatoxin residues. Chi square was used to determine the possible association

between locations and feed samples with *Aspergillus* spp which was used to determine the strength of the association amongst the mentioned variable in relation to aflatoxin. Values of $P < 0.05$ were considered to be significant (Snedecor and Cochran, 1989).

$$P\ (\%) = \frac{\text{Number of samples with a species or genus}}{\text{Total number of samples}} \times 100$$

CHAPTER FOUR

RESULTS

4.1 Prevalence of *Aspergillus* and Other Fungi in Poultry Feeds in Live Bird Markets

Out of 300 feed samples collected from cages in 6 live bird markets, 283 (94.3%) feed samples yielded growth of at least one fungal organism. *Aspergillus* species was found in 234 (78%) of the samples other fungal genera had frequencies of isolation as follows: *Rhizopus* 18(6%), Mucor 17(5.6%), Yeast 6(2%), Dermatophytes 6(2%), *Fusarium* 1(0.3%) and *Penicillium* 1(0.3%). (Table 4.1)

4.2 Distribution of *Aspergillus* Based on the Live Bird Markets

Aspergillus were the most dominant species in all samples accross the six markets; Dan Magaji, Kwangila, Sabon Gari, Samaru, Tudun wada and Zaria City with frequencies of 41(13.66%), 36(12%), 40(13.33%), 41(13.66%), 35(11.67%) and 41(13.66%) respectively. There was no statistically significant difference in the isolations rate based on location where the samples were collected ($P > 0.05$) (Table 4.2).

Table 4.1: Prevalence of fungi in poultry feed from live bird markets in Zaria

Frequency	***Aspergillus***	**Dermatophyte**	***Mucor***	***Rhizopus***	***Penicillium***	**Yeast**	***Fusarium***
No. of positive feed samples	234.0	6.0	17.0	18.0	1.0	6.0	1.0
Prevalence (%) (n=300)	78.0	2.0	5.6	6.0	0.3	2.0	0.3

%= Percentages, n=number of samples

Table 4.2: frequency of isolation of *Aspergillus* spp from feed samples collected from six live bird markets in Zaria.

Location of LBM	No of samples	Number positive for *Aspergillus* spp.	Isolation Frequency (%)
Sabon Gari	50	40	13.3
Zaria city	50	41	13.7
Samaru	50	41	13.7
Dan magaji	50	41	13.7
Tudun wada	50	35	11.7
Kwangila	50	36	12.0
Total	**300**	**234**	**78.0**

Chi square = 0.5588; df=5; P-value = 0.9898

4.3 Isolation Frequencies of Various species of *Aspergillus*

The order of feed samples contaminated with *Aspergillus* spp., were *A. flavus, A. fumigatus, A. parasiticus, A. niger, A. nidulans, A. terreus, A. nomius* and *A. caelatus* were found with the following frequencies; 126 (53.8%), 48 (20.5%), 27 (11.5%), 15 (6.4%), 9 (3.9%), 5 (2.1%), 3(1.3%) and 1 (0.4%) respectively Table 4.3. There was no stastically significant difference in the isolation rates of different species of *Aspergillus*.

4.4 Isolation Rates of Aflatoxigenic species of *Aspergillus* from Six Live Bird Markets in Zaria.

On desiccated coconut agar, 98 of the 234 (51.9%) of the *Aspergillus* spp cultured, grew with a blueish fluorescence indicative of aflatoxin production. The isolation frequencies of the aflatoxigenic *Aspergillus* spp based on location were 28(28.6%), 15(15.3%), 7(7.1%), 17(17.3%), 11(11.2%) and 20(20.4%) from Dan Magaji, Kwangila, Sabon Gari, Samaru, Tudun wada and Zaria city LBMs respectively. However, there was statistical difference in the distribution of aflatoxigenic *Aspergillus* spp on desiccated coconut agar under ultra violet light ($P > 0.05$) as seen in Table 4.4.

Table 4.3: Distribution of species of *Aspergillus* from poultry feed samples in live bird markets Zaria (n=234)

Aspergillus Isolated	No. of isolates	Isolation Frequency (%)
A. caelatus	1	0.4
A. flavus	126	53.8
A. fumigatus	48	20.5
A. nidulans	9	3.9
A. niger	15	6.4
A. nomius	3	1.3
A. parasiticus	27	11.5
A. terreus	5	2.1
Total	**234**	**100.0**

Prevalence = 77.98%, P value = 0.9945 and $\chi^2 = 0.7002$.

Table 4.4: Isolation rate of aflatoxigenic *Aspergillus* species based on live bird markets in Zaria.

Location (LBM)	No of *Aspergillus* spp. tested	Intensities of fluorescence by *Aspergillus* spp			No. -ve	*Total No. aflatoxigenic	Isolation frequency (%) of aflatoxigenic *Aspergillus* spp
		+++	++	+			
Dan magaji	41	0	9	19	13	28	28.6
Kwangila	36	7	2	5	21	15	15.3
Sabon Gari	40	3	2	2	33	7	7.1
Samaru	41	1	6	10	24	17	17.3
Tudun wada	35	4	4	4	23	11	11.2
Zaria City	41	2	3	15	22	20	20.4
Total	**234**	**17**	**26**	**55**	**136**	**98**	**100.0**

P=0.0002, df=5, χ=24.8

* isolation frequencies include all those with very strong, strong and weak fluorescence

+++: Very strong

++: Strong

+: Weak

-: Negative

4.5: Isolation Frequencies of *Aspergillus* spp from Live Bird Markets in Zaria

There are some species that were specific to particular location in this study i.e *A. caelatus* was specific to Tudun wada, *A. nomius* to Dan Magaji, *A. terreus* are specific to Sabon Gari, Tudun wada, Kwangila and Dan Magaji. However, some species are adapted to different locations within the study area such as *A. flavus, A. fumigatus, A. niger, A. parasiticus,* and *A. nidulans*. The differences in fungal profile of various locations could be due to fluctuation in weather condition as seen in Table 4.5

Table 4.5: Isolation frequencies of *Aspergillus* spp isolated from all the live bird market Zaria (n=234).

Aspergillus isolates	No. isolate from LBM (%)					
	ZR CITY	S/G	SAM	TDW	KGL	DMG
A. caelatus	0(0)	0(0)	0(0)	1(2.9)	0(0)	0(0)
A. flavus	20(48.8)	24(60.0)	17(41.5)	25(71.4)	21(58.3)	19(46.3)
A. fumigatus	10(24.4)	8(20.0)	14(34.1)	4(11.4)	6(16.7)	6(14.6)
A. niger	4(9.8)	3(7.5)	4(9.8)	0(0)	3(8.3)	1(2.4)
A. nidulans	1(2.4)	2(5.0)	2(4.9)	0(0)	2(5.5)	2(4.8)
A. nomius	0(0)	0(0)	0(0)	0(0)	0(0)	3(7.3)
A. parasiticus	6(14.6)	2(5.0)	4(9.8)	4(11.4)	2(5.5)	9(21.9)
A. terreus	0(0)	1(2.5)	0(0)	1(2.9)	2(5.5)	1(2.4)
Total	41(100)	40(100)	41(100)	35(100)	36(100)	41(100)

ZR CITY= Zaria city, S/G= Sabon Gari, SAM= Samaru, TDW = Tudun wada, KGL = kwangila and DMG = Dan Magaji

4.6 Macroscopic and Microscopic View of *Aspergillus flavus* on SDA

Plates I show the macroscopic and microscopic appearances of *A. flavus* isolated from feeds in the LBMs, which was inoculated and incubated at room temperature for 5 day. The macroscopic appearance as a yellow to green colonies, while the microscopic appearance has a colourless and rough conidiophores, phialides which are biserate, covers two-third of the vesicle with a radiate head. Other macroscopic and microscopic features of *Aspergillus* spp found in this study are shown in Appendix IV.

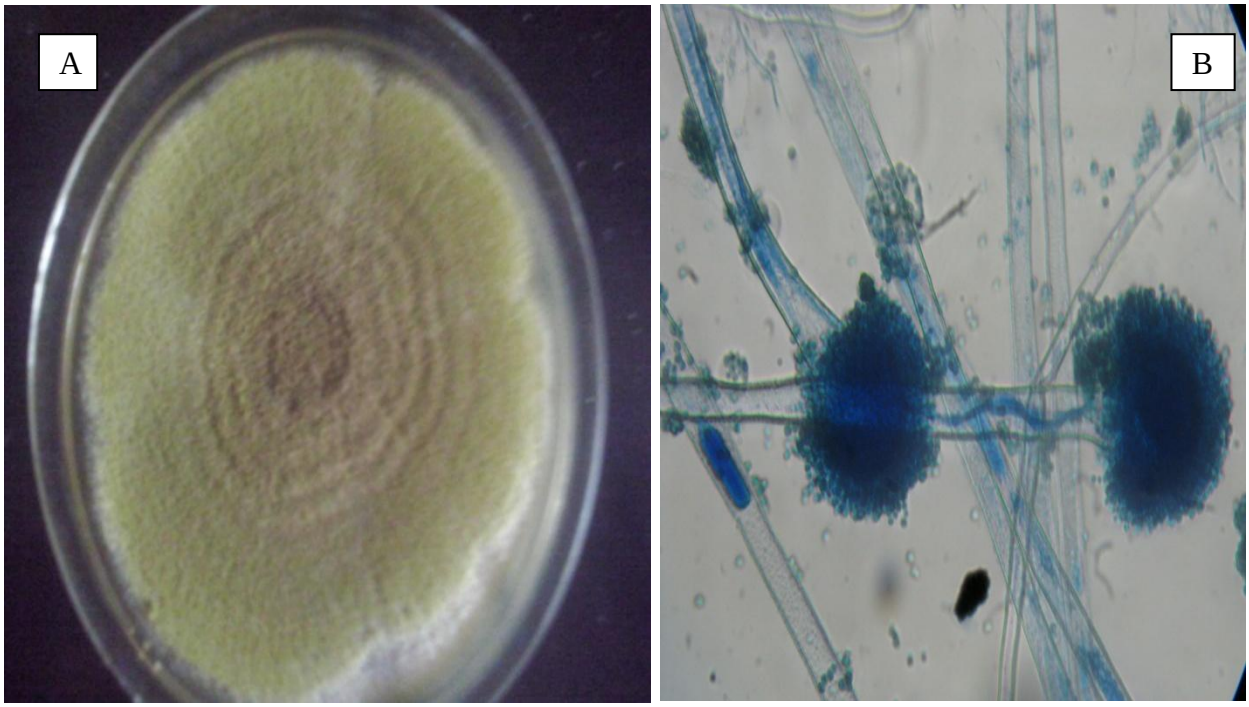

Plate I: A. Macroscopic appearance of *A. flavus* with Yellow to green colony
B: Microscopic staining of *A. flavus* using Lactophenol cotton blue dye, showing biseriate head and globose vesicle (Mag x40) B.

4.7 Macroscopic and Ultra-violent View of *Aspergillus flavus* on Dessicated Coconut Agar

Aspergillus flavus growth on dessicated coconut agar incubated for 7 days at room temperature shows a yellow to green colony appearance. Blue colour fluorescence was observed under ultra violet light as seen in Plate II.

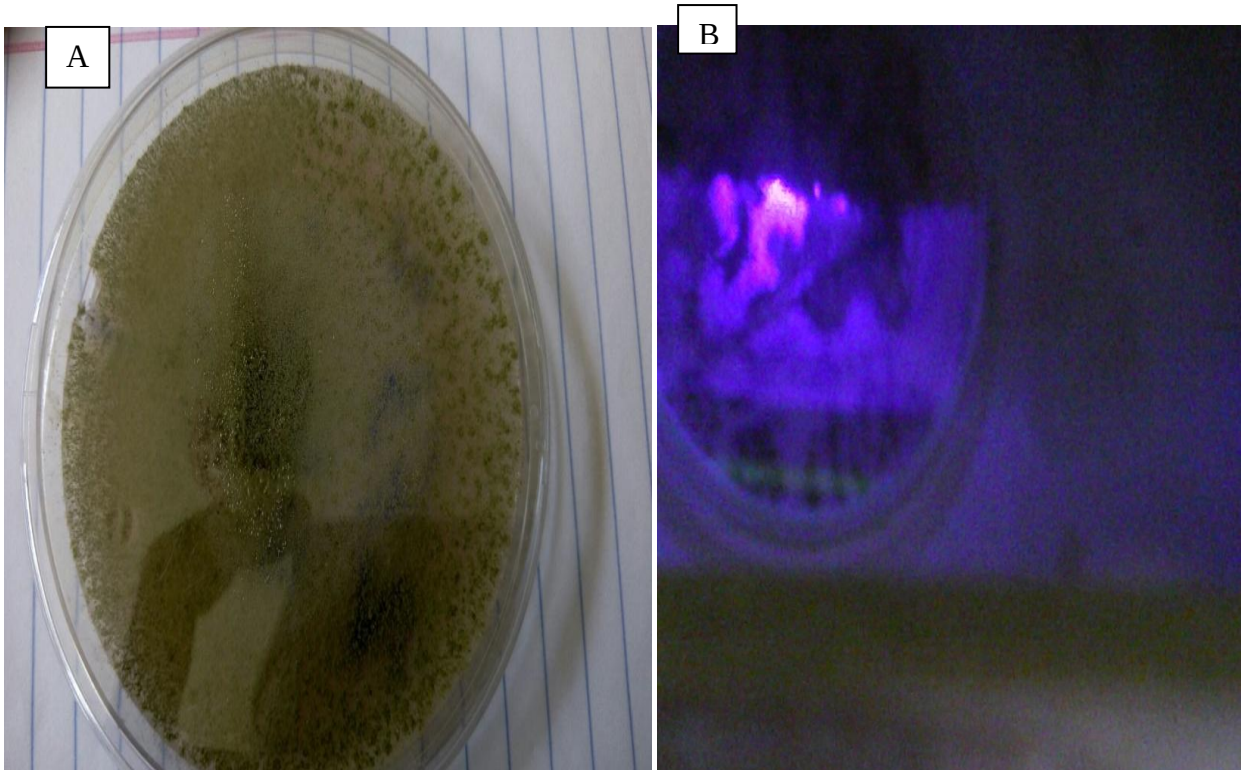

Plate II (A and B); A: 7 day growth colony of *A. flavus* on DCA at 27 °C and Blue colour fluorescence under ultra violet light (B).

4.8 Determination of Aflatoxin Contents in Fresh Broiler Liver Samples

In total 60 liver samples were tested for aflatoxin residues using ELISA; fifty nine (98.3%) out of 60 liver samples had detectable aflatoxin residues. Only 1(1.6%) did not have detectable residues. The mean concentration of aflatoxin B1 in the liver samples from all the 6 live bird market was 32.2 ug/Kg. The mean concentrations for samples from Zaria city, Sabon Gari, Samaru, Tudun wada, Dan magaji and Kwangila were 41.9, 66.4, 10.0, 29.8, 35.2 and 9.9 ug/Kg respectively. The overall mean concentration and standard deviation of aflatoxin B1 in the 59 liver samples was 32.8 µg/kg with a range of 1.9-96.2 µg/kg. The mean value of aflatoxin residue in the liver samples (41.5 ± 29.6 µg/Kg) from Zaria city was statistically significantly ($P < 0.0001$) lower than that obtained in the liver samples from Sabon Gari (66.4 ± 22.3 µg/Kg). The mean value of aflatoxin B1 residues found in liver samples collected from Zaria city LBM, was significantly ($P > 0.0001$) higher than that obtained from Kwangila (9.9 ± 6.8 µg/Kg) and Samaru (10.0 ± 10.8 µg/Kg). There was also a significant difference between the mean concentrations obtained from Zaria city, Sabon Gari, Samaru and Kwangila. There was statistical significance ($P < 0.0001$) between the mean value of liver samples obtained from Sabon Gari (66.4 ± 22.3 µg/Kg) when compared to those samples from Samaru (10.0 ± 1.8 µg/Kg), Tudun wada, Dan magaji and Kwangila (Appendix II). The mean concentration of aflatoxin B1 residues in the liver sample (35.3 ± 8.1 µg/Kg) at Dan Magaji was significantly ($P < 0.0001$) higher than that obtained in the liver sample (9.9 ± 6.7 µg/Kg) at Kwangila. The mean value of aflatoxin residue (10.0 ± 1.8 µg/Kg) from samaru was significantly ($P < 0.0001$) lower than that obtained in the liver sample (35.3 ± 8.1 µg/Kg) in Dan Magaji live bird market. The mean concentration of aflatoxin residue (35.3 ± 8.1 µg/Kg) at Dan Magaji was significantly (P <

0.0001) lower as compared to Kwangila (9.9 ± 6.7 µg/Kg) using ANOVA. Distribution of various concentrations of aflatoxin B1 contents in broiler livers in the live-bird markets were 23(1-20 µg/kg), 17(21-40 µg/kg), 9(41-60 µg/kg), 5(61-80 µg/kg) and 5(81-100 µg/kg) as shown Figure 4.1 and Appendix.

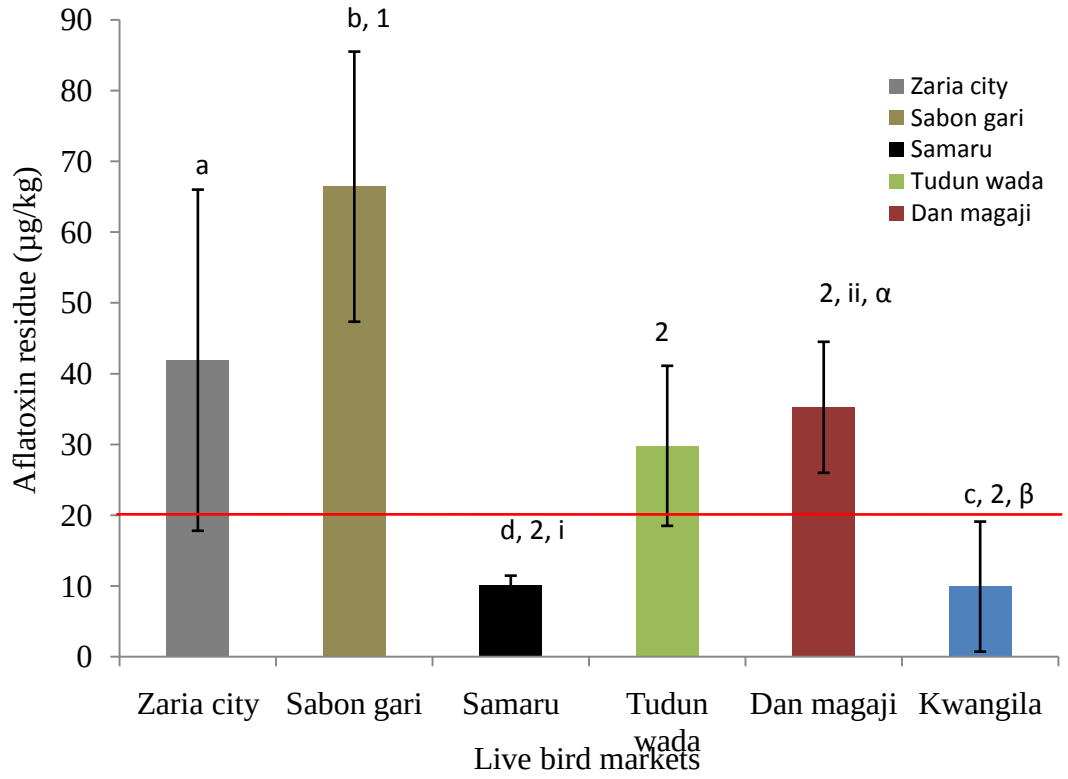

Figure 4.1: Aflatoxin contents in fresh liver tissues sampled at various locations in Zaria.

Value with different letters, numbers, roman numerals and symbols are significantly ($P < 0.05$)

4.9: Aflatoxin B1 Content in Boiled Broiler Liver Tissues from Various Live Bird Markets in Zaria.

All 59 liver samples that had detectable aflatoxin B1, were boiled at boiling temperature for 90 minutes for the effect of heat treatment on aflatoxin concentrations. The total mean value of B1 in liver samples from the 6 live bird markets was 8.2 µg/Kg. The mean value for samples from Zaria city, Sabon Gari, Samaru, Tudun wada, Dan magaji and Kwangila were 8.2, 29.0, 5.2, 4.2, 0.5 and 1.8 µg/Kg respectively against 41.9, 66.7, 10.0, 29.8, 35.2, and 9.9 µg/Kg respectively before boiling. The maximum aflatoxin B1 concentration was 46.1 µg/Kg and minimum concentration of 0.2 µg/Kg (Table 4.8). The mean concentration value and SD of aflatoxin B_1 in liver (8.2 ± 5.5 µg/Kg) in liver samples from Zaria city was significantly ($P < 0.0001$) lower than that obtained in Sabon Gari (29.0 ± 9.5 µg/Kg). The mean value of aflatoxin residue (8.2 ± 5.1 µg/Kg) Zaria city was significantly ($P > 0.0001$) higher than that obtained in Dan Magaji (0.6 ± 0.3). There was significant difference ($P > 0.0001$) between Sabon Gari (29.0 ± 9.5 µg/Kg) as compared to Samaru (5.2 ± 5.1 µg/Kg), Tudun wada (4.5 ± 3.4 µg/Kg) and Dan Magaji (0.6 ± 0.3 µg/Kg) ANOVA using a tukeys multiple comparism test (Figure 4.2) and (Appendix III).

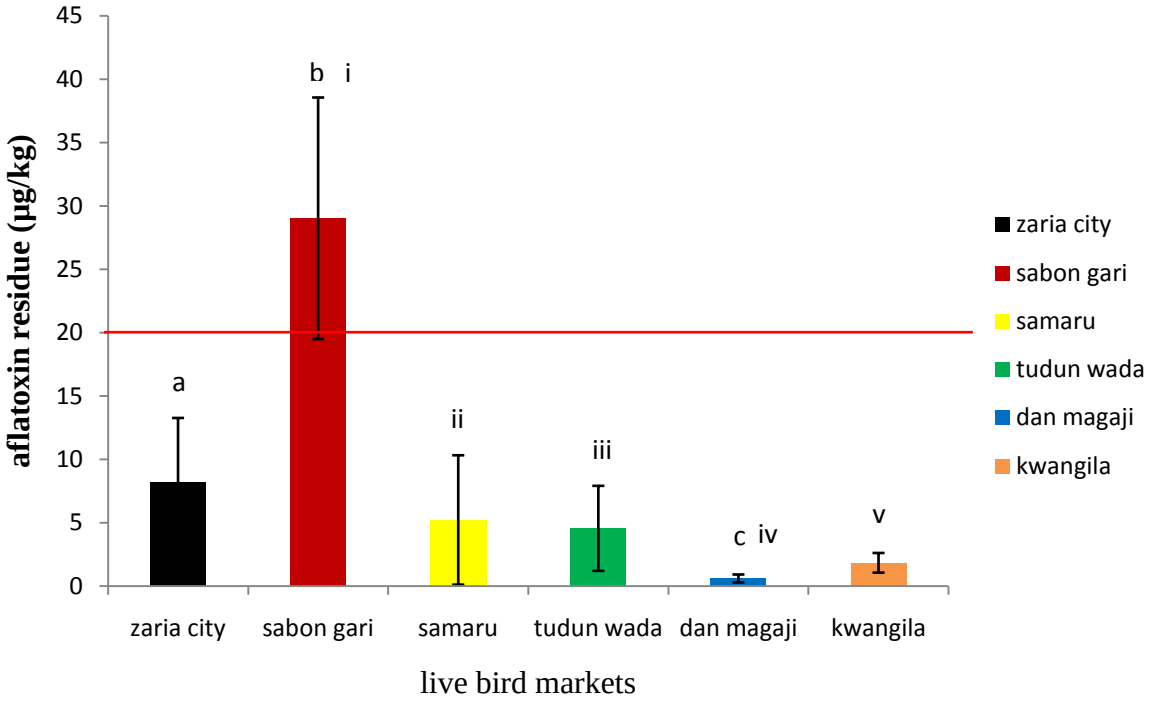

Figure 4.2: Aflatoxin content in boiled liver tissue sampled at various live bird markets in Zaria.

Value with different letters, and roman numerals are significantly ($P < 0.05$)

4.10: Aflatoxin Contents in Broiler Livers Sampled from Various Locations in Zaria Both Before and After Boiling

The aflatoxins residue concentrations in liver tissues sampled before and after boiling from various locations in Zaria was determined. The mean percentage reduction were 79.7%, 53.6%, 49.9%, 83.3%, 98.4% and 76.1% respectively. In Zaria City, Sabon Gari, Samaru, Tudun Wada, Dan Magaji and Kwangila respectively. There are significant ($P< 0.05$) different in all content levels in liver sample from broiler except Samaru using student *t-test* (Table: 4.6) and (Figure 4.3).

Table 4.6: Aflatoxin concentrations in liver samples of broiler chicken in live bird markets before and after boiling.

	fresh liver samples (µg/kg)	boiled liver samples (µg/kg)	mean reduction percentage (%)
Zaria City	41.4 ± 29.5□	8.2 ± 5.0□	79.7
Sabon Gari	66.4 ± 22.2□	29.0 ± 9.5□	53.6
Samaru	10.0 ± 1.7	5.2 ±5 .1	45.9
Tudun wada	29.8 ± 17.1□	4.5 ± 3.3□	83.3
Dan Magaji	35.2 ± 8.1□	0.6 ± 0.3□	98.4
Kwangila	9.9 ± 6.5□	1.8 ± 0.7□	76.1

* Values with different superscript letters are significantly ($P < 0.05$) different

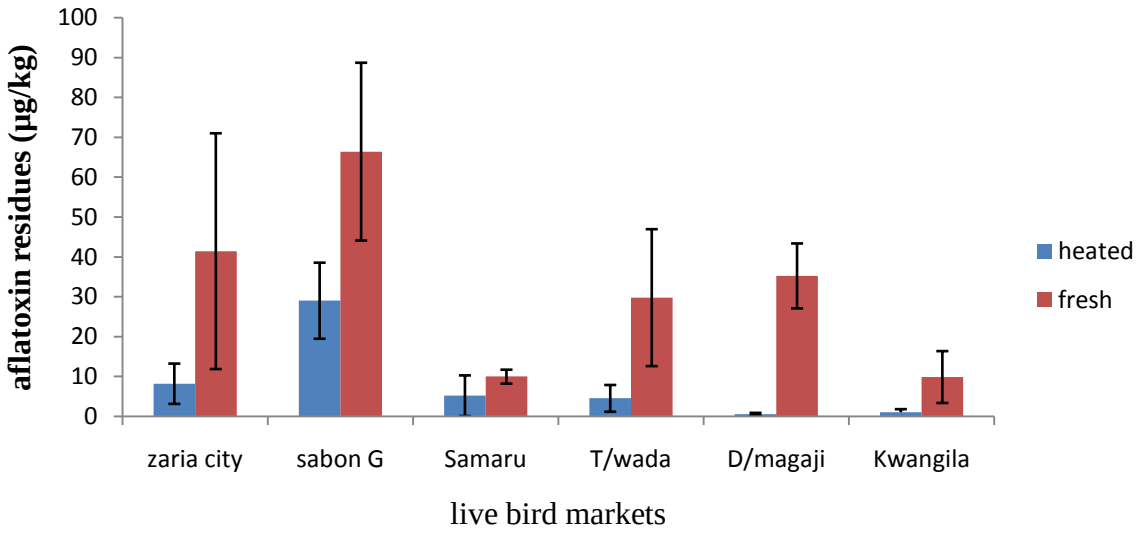

Figure 4.3: Aflatoxin content concentrations of broilers liver tissue samples in Zaria before and after boiling.

CHAPTER FIVE

5.0 DISCUSSION

The results of this study showed that there was wide spread fungal contamination (94.67%) of feeds fed to poultry in live bird markets in Zaria, which is consistent with previous reports of high fungal fungal contamination of poultry feeds in Nigeria, (Obi and Ozugbo 2007; Osho *et al*., 2007; Uwazuoke and Ogbulie 2008; Ezekiel *et al*., 2014; Habib *et al.*, 2015; Aliyu *et al.*, 2016) and other parts of the world like in Argentina (Dalcero *et al*., 1997), Brazil (Oliviera *et al*., 2006; Rossa *et al*., 2006), Serbia (Krnjaja *et al.*, 2007) and Pakistan (Saleemi *et al.*, 2010). In this study, isolation frequency of different genera of contaminating fungi ranked in this order: *Aspergillus, Rhizopus*, *Mucor,* Yeast, Dermatophytes, and *Penicillium,* which is in concordance with Saleemi *et al.* (2010), Sivakumar *et al.* (2014) and Bhuyan *et al.* (2015) who found similar fungal genera in feeds. The source of the predominance of *Aspergillus* contaminants of feeds in this study is not obvious, however it could be related to storage in poorly ventilated areas, weather condition and season of the year the research was conducted. Live-bird market facilities in Nigeria are very limited, generally hygienic condition are very poor at sales points, stores and particularly at slaughter points (FAOUN, 2008). Birds receive water and feed from containers giving rise to considerable spillage of water and contamination of feed (FAOUN, 2008). The cages may be metallic but are often in wood or other porous materials which are difficult to clean and disinfect (FAOUN, 2008). This high fungal prevalence may be due to the fact that weather conditions such as high temperature and relative humidity favour fungal growth. During the research many of the bird sellers were observed to add water to their feeds to increase the volume of feed which encourages mould growth and subsequent aflatoxin production (Habib *et al.*, 2015a)

In this study, the prevalence of *Aspergillus* spp was 234(77.9%) with *A. flavus* prevalence of 126(42%) in feed samples. The prevalence of *A. flavus* reported in this study is less than those reported by Accensi *et al.* (2004), 55% by Diba *et al.* (2007), 91.8% by Ezekiel *et al.* (2014), 83.3% by Sivakumar *et al.* (2014), 50% by Fakruddin *et al.* (2015), 51.6 % by Gherbawy *et al.*(2016) and 64.3% by Ghaemmaghami *et al.* (2016). However, the prevalence was higher than others such as 3% by Anissie *et al.* (2003), 35% by Fapohunda *et al.* (2012), 22.64% by Greco *et al.* (2014), 17.27% by Davari *et al.* (2015), 20% by Habib *et al.* (2015a) and 41.2% by Aliyu *et al.* (2016). The variation in isolation rates of *Aspergillus* species in these studies could be the varying weather conditions of various study locations and handling methods. However, since Nigeria is in tropical region with significant period of the year being humid. Therefore the environment is very conducive for the growth of fungi in feeds. This showed that fungi can easily adapt to various geographical regions, high temperature tolerance character and low (11-14%) humidity levels (Battilani *et al*., 2003; Macioro *et al*. 2007). It was also demonstrated that the high prevalence of *A. flavus* may be largely dependent on long term storage in inappropriate weather conditions. *A. flavus* possess a higher adaptability to grow in a wide range of environmental conditions and produce spores that remain viable even under extremely unfavorable conditions (Saleemullah *et al.,* 2006). Other researchers have also shown the predominance of *A. flavus* in feeds (Accensi *et al*., 2004; Reddy *et al.*, 2004; Somashekar *et al*., 2004; Fapohunda *et al.*, 2012; Davari *et al.*, 2015; Fakruddin *et al.*, 2015; Ghaemmaghami *et al.*, 2016).

The order of predominance of *Aspergillus* species isolated from feedstuffs in this study were; *A. flavus, A. fumigatus, A. parasiticus, A. niger, A. nidulans, A. terreus, A. nomius* and *A. caelatus*. This is not in agreement with Aliyu *et al*. (2016), who had the following order of predominance; *A. fumigatus, A. flavus,* Habib *et al.* (2015a), who had *Apergillus* species isolated in the order; *A. fumigatus, A. parasiticus, A. flavus, A. niger and A. terreus.* Ghaemmaghami *et al.* (2016), had the order as; *A. flavus, A. niger,* and *A. fumigatus*. Davari *et al.* (2015), had; *A. fumigatus, A. flavus, A. niger* and *A. parasiticus* as the predominant species. Sivakumar *et al.* (2014), order where; *A. flavus, A. niger* and *A. fumigatus.* Greco *et al.* (2014), reported; *A. niger, A. flavus, A. parasiticus* and *A. fumigatus* as order of predominance Saleemi *et al.* (2010), had the following order; *A. niger* followed by *A. flavus.* Diba *et al.* (2007) reported the following order; *A. flavus, A. niger* and *A. fumigatus.* Anissie *et al.* (2003), reported as*; A. niger, A. fumigatus, A. nidulans,* and *A. flavus*. The variation in the order of predominance could be due to adaptation to various geographical regions, season of the year when the research was carried out and the fact that some of the species can grow at low relative humidity.

The predominance of *A. flavus* in feed was noted in all the six live bird markets studied However, Dan magaji, Zaria city, Samaru and Sabon Gari had the highest prevalence of *Aspergillus* species with Kwangila and Tudun wada live bird markets recording the lowest. The high prevalence of *Aspergillus* in these four markets may be based on their stocking density and type of feed the birds are fed with. *Aspergillus flavus* is one of the most important producers of aflatoxin. Agricultural commodities can be contaminated with *A. flavus* at any stage of production (in field/during harvest/ transport and storage) (Berthier and Valla, 1998).

Out of the 60 liver samples tested for aflatoxin B_1, 66.7% had aflatoxin levels above maximum limit set by Standard Organization of Nigeria and European Union, 2003. The result obtained in this study is higher than the maximum tolerated levels of aflatoxins in foodstuffs, dairy products and animal feedstuff (NAFDAC, 2003). The aflatoxin contents of the liver samples relate to fungal profile in most of the live bird markets. The aflatoxin B1 contents of the liver samples could be as a result of direct ingestion of feed contaminated with *Aspergillus* species. Invariably the secondary exposure to aflatoxin contents through consumption of chicken liver is derived from the poultry fed aflatoxin contaminated feed which may pose a risk to human health and result in conditions such as acute aflatoxicosis, hepatocellular carcinoma, hepatitis B virus infection, and growth impairment (wild and gong, 2010). Considerable importance is given to the presence of aflatoxins in foods and feeds because of their carcinogenic, mutagenic and teratogenic effect (Begum and Samajpati, 2000).

In this study, 83.3%(60) of boiled liver samples had AFB_1 content levels lower than the maximum limit set by the Standard Organization of Nigeria. 16.3% of the liver samples had higher than the maximum tolerated levels of aflatoxins in foodstuffs (NAFDAC, 2003). The reduction in concentration of aflatoxin B_1 may be due to conversion into different hydroxylated or other metabolite forms of the aflatoxin, in agreement with Tripathi and Mishra (2010), that aflatoxins are thermostable, so the physical treatment by heat result in only small changes in their levels. When animals consume AFB_1, their livers tend to detoxify it, forming the less toxic hydroxylated metabolites AFM_1, AFP_1, and AFL, although there is still a health risk for humans that ingest them; therefore, it is meaningful to measure all AF metabolites. Aflatoxin M1 was the most abundant AF hydroxylated metabolite; it is frequently found in human urine and has a

direct association with increased incidence of hepatitis, cirrhosis, and hepatocellular carcinoma (Sun *et al.,* 1999). Epidemiological studies have correlated the incidence of liver cancer in animals and humans to the consumption of AF in contaminated cereal (maize or sorghum) from cereal to hens and from there to humans, and the effects of AF were increased by hepatitis B viral infection (Henry *et al.,* 1999). The traces of AFB_1 have been found in animal liver and eggs used for human consumption (Park and Pohland, 1986). Tedesco *et al.* (2004) indicated that AF contamination in fowl represents a risk for public health due to the high consumption of contaminated poultry products. Bintvihok and Kositcharoenkul (2006) and Giacomini *et al.* (2006), found higher levels of AFB_1 and AFM_1 in hen liver than in muscle, The European community and many other countries have imposed 2 ng/g AFB1 maximum tolerance level in human food products (Anonymous, 2004). The differences in AF susceptibility make the extrapolation of data from animals to humans difficult because human fatalitie due to AF acute toxicity are less common. Some adult humans can consume anywhere from 2 to 6 mg of AF per day (Krishnamachari *et al.,* 1975), and lethal dosages for adult humans have been calculated to be in the range of 10 to 20 mg of AF (Pitt, 2000).

The aflatoxin content levels obtained in the present study were higher than those reported by Bintvihok *et al.* (2002), who reported 0.1 ± 0.02 µg/kg residues of aflatoxins in liver. Zaghini *et al*. (2005) Herzallah (2009), reported 0.2 to 6.4 µg/kg in fresh meat samples. Hussain *et al.* (2010) reported a high residue level in liver of 6.9 ± 0.1 µg/kg. In addition, Iqbal *et al.* (2014), reported 2.6 ± 0.6 µg/kg of aflatoxin residue with a range LOD-6.31 in broiler chickens. The high aflatoxin content levels in this study agree with previous work that feed contamination ultimately contaminate meat (Perši *et al.,* 2014). The high concentration of AFB_1 contents

observed in this study, may be as a result of immediate ingestion of aflatoxins, concentration of aflatoxin ingested in feed (Hussain *et al.*, 2010).

The results of the present study agree with those of Oyero and Oyefolu, (2010), that aflatoxin B_1 in tissue sample cannot be destroyed totally. However in that study it was sun-dried compared to the boiling in this study. The report of heat treatment in the present study agree with report of Binder (2007), Iqbal *et al*. (2014a) and Tripathi and Mishra (2010) who reported that aflatoxins are thermostable, so the physical treatment by heat result in only minimal changes in their levels.

This findings is line with Iqbal *et al.* (2014a), who described that the presence of aflatoxin in meat pose a potential health hazard to comsumers. The high aflatoxin content levels in liver samples could be as a result of consumption of aflatoxin-contaminated poultry feeds which varied with their concentration, and duration of ingestion. The transfer of aflatoxins from poultry feed to by-products could result in health and economic implications on both poultry and human health.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusions

Eight different species of *Aspergillus* were isolated and identified, with 78% prevalence rate from poultry feeds in live bird markets in Zaria. Aflatoxin producing *Aspregillus* species were harboured in 51.9 % of the feed samples analysed using neutral red dessicated coconut agar and observed under ultraviolent light. Of the liver samples tested for aflatoxin residues, 66.7 % had varying levels of aflatoxin (29.80 - 66.40 µg/kg) above maximum limit set by Standard Organization of Nigeria, and European Union. The percentage reduction of aflatoxin B_1 level in boiled liver samples were; 79.7%, 53.6%, 45.9%, 83.3%, 98.3%, and 76.1% respectively from Zaria city, Sabon Gari, Samaru, Tudun wada, Dan Magaji, and Kwangila repectively. A total of 16.3% of the boiled liver samples had higher than the maximum tolerated levels of aflatoxins in foodstuffs, dairy products and animal feedstuff stipulated by the Standard Organization of Nigeria. The level of aflatoxin residues in liver of boilers may pose hazard to human health.

6.2 Recommendations

Based on the findings reported, the following recommendations are made:

1. Strict measures should be taken to monitor and control aflatoxins in feed and broiler chicken liver.

2. Good hygienic practices should be encouraged in live bird markets such as improved ventilation of stores and washing of feed trough to reduce the multiplication of mycotoxin producing *Aspergillus* species.

3. All live bird sellers in live bird markets should be encouraged on good agricultural practices such as addition of absorbents, binders, zeolite, wheat bran, alfalfa, and ammonia in their poultry feed.

4. Containers in which feeds are kept should be rountinely washed before introducing new feed.

5. NAFDAC should enforce maximum permissible limits in agricultural produce through the Federal Ministry of Agriculture.

6. The use of high performance liquid chromatography, immunological detection, nucleic acid hybridization, and combination assays for detection of fungi and mycotoxins should be used for further studies.

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APPENDICES

Appendix I

Table 4.1: Aflatoxin content of fresh broiler liver based on location in live bird markets.

S/no.	Zaria city	Sabon Gari	Samaru	Tudun wada	Dan Magaji	Kwangila
1	24.4	52.9	9.0	37.8	41.8	16.4
2	58.5	79.9	11.0	21.8	28.7	3.4
3	43.2	22.7	27.4	17.3	47.4	20.1
4	20.1	96.2	31.6	10.1	32.8	2.7
5	3.3	84.6	3.1	6.3	24.3	20.6
6	67.6	64.1	0	38.2	36.2	5.8
7	9.0	72.8	4.7	53.3	27.4	3.8
8	27.3	40.6	6.2	19.2	40.1	9.3
9	80.6	68.0	5.5	40.0	28.3	9.5
10	85.0	82.5	1.9	54.0	45.4	7.4

$P = < 0.0001$, $R^2 = 0.5712$, $F = 14.38$, $Df = 5$. One way ANOVA (tukeys multiple comparism test)

Appendix II

S/no.	Zaria city	Sabon Gari	Samaru	Tudun wada	Dan Magaji	Kwangila
1	5.2	23.9	6.0	6.6	0.9	2.0
2	13.0	46.1	9.3	5.0	0.4	1.6
3	12.3	19.3	11.1	4.3	1.2	3.0
4	6.7	39.8	15.4	2.0	0.4	1.1
5	0.2	36.0	2.0	1.2	0.3	3.0
6	11.7	23.5	0	4.3	0.4	2.0
7	2.1	29.7	2.3	12.6	0.4	0.7
8	5.4	17.2	2.9	5.6	0.5	2.0
9	15.5	21.7	2.4	1.9	0.4	2.0
10	10.1	33.0	0.8	2.1	0.9	1.0

Df = 5, F = 43.07, R^2 = 0.7995, P = < 0.0001 One way ANOVA (tukeys multiple comparism test)

Appendix III

Distribution of various concentrations ranges of Aflatoxin B1 residues in fresh broiler livers in 6 live bird markets in Zaria.

	1-20 (µg/kg)	21-40 (µg/kg)	41-60 (µg/kg)	61-80 (µg/kg)	81-100 (µg/kg)
All	23	17	9	5	5
Zaria	3	2	2	1	2
S/Gari	0	1	2	4	3
Samaru	7	2	0	0	0
T/Wada	4	4	2	0	0
Dan Magaji	0	7	3	0	0
Kwangila	9	1	0	0	0
Total	23	17	9	5	5

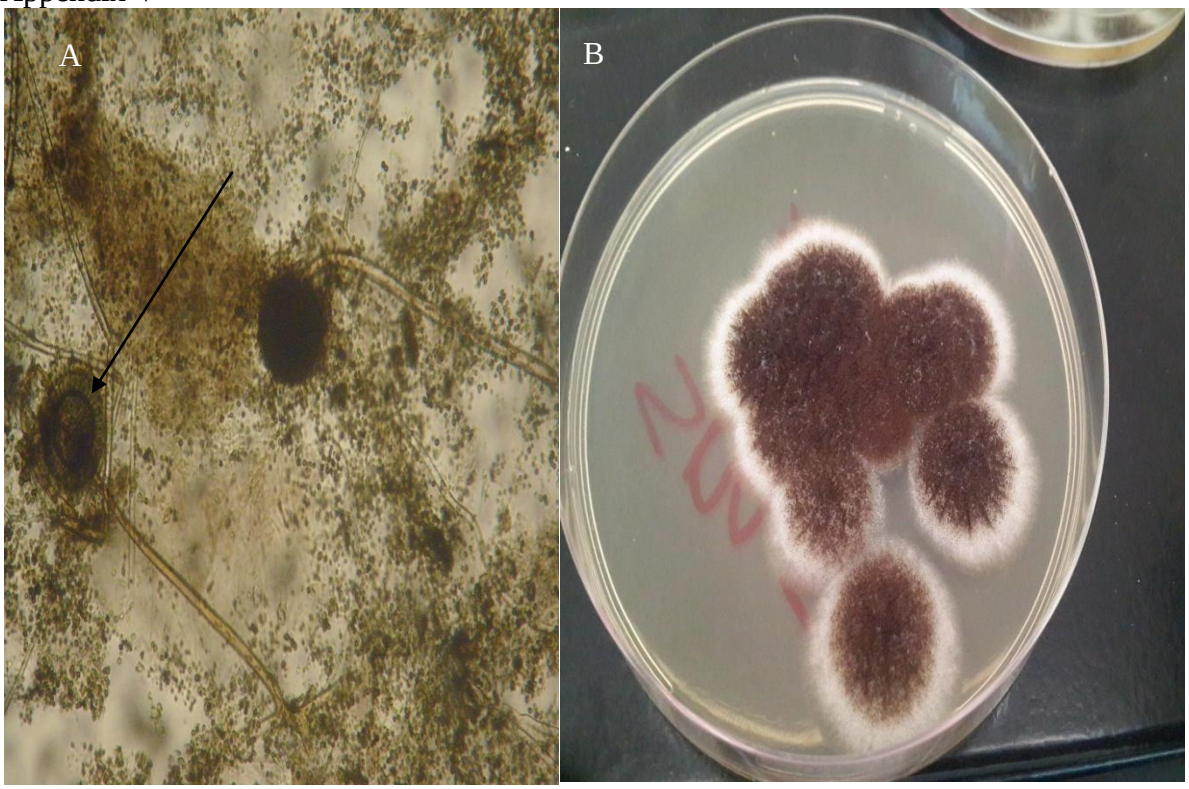
*S/Gari = Sabon Gari, T/wada = Tudun wada

Appendix IV

Aflatoxin contents in liver samples of broiler chicken from live bird markets Zaria after boiling. (Mean Ug/kg)

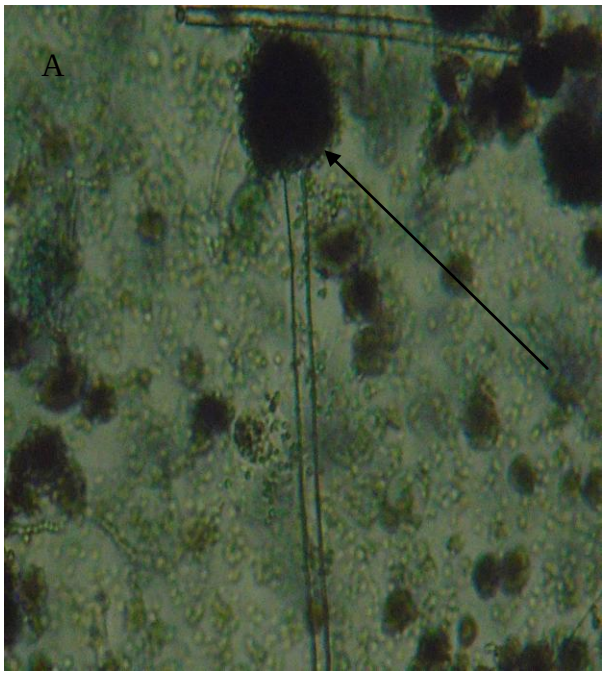
Location	Mean	Minimum	Maximum
Zaria	8.2	0.2	15.5
Sabon Gari	29	17.2	46.1
Samaru	5.2	0	15.4
T/wada	4.5	1.2	12.6
D/magaji	0.5	0.3	1.2
Kwangila	1.8	0.7	3

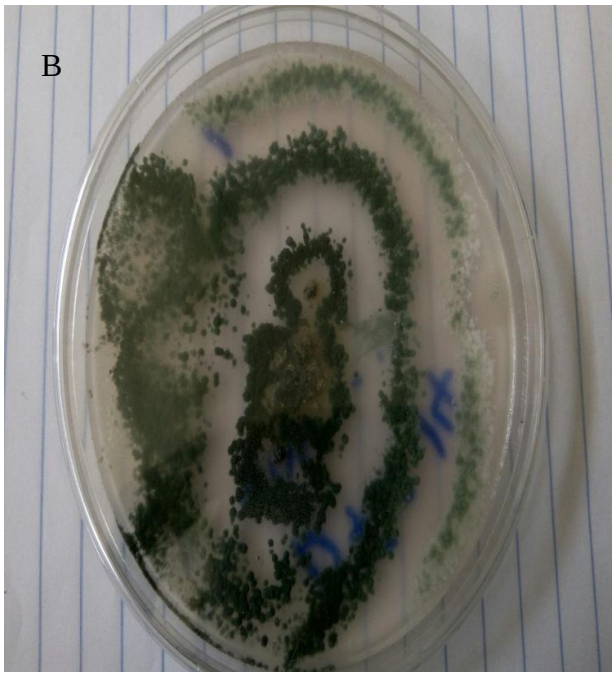
Appendix V



(*Aspergillus niger*): A: biseriate head with globose vesicle (Mag x40). B: Brown colony at 27 °C after 7 days on SDA.

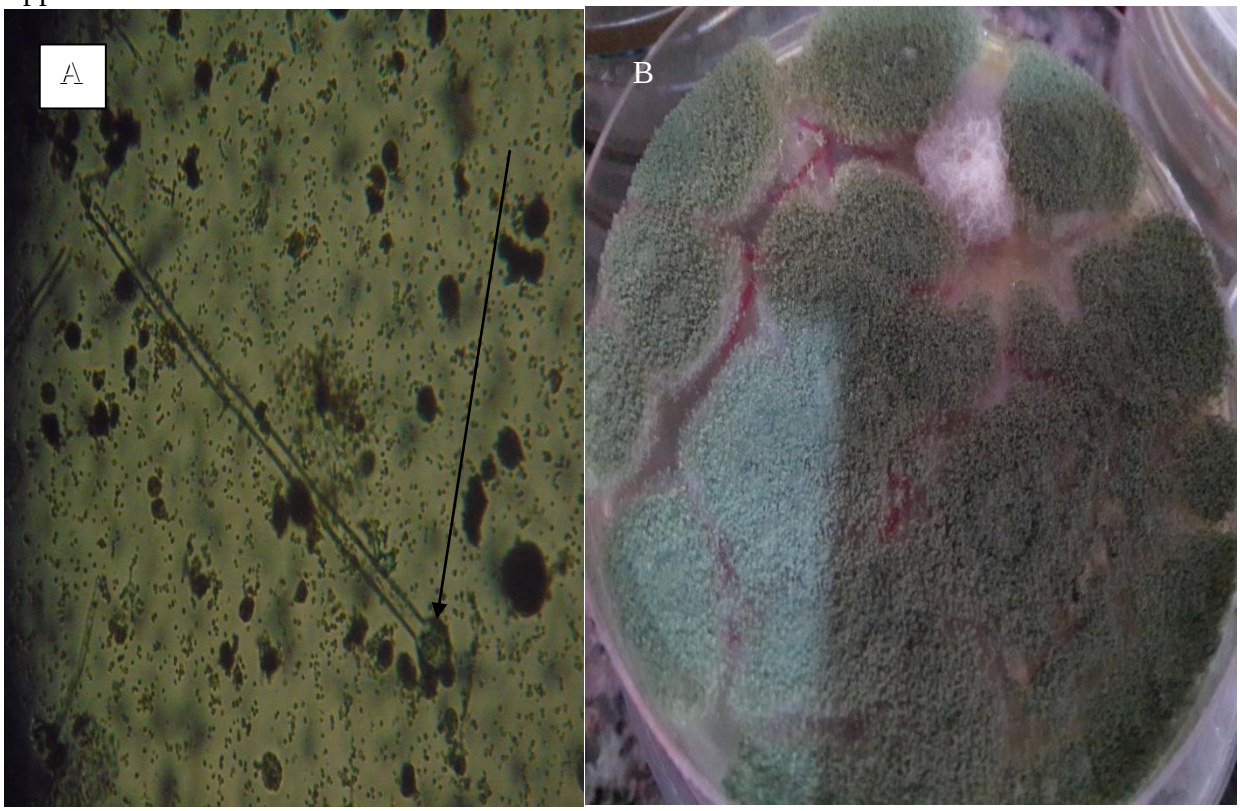
Appendix VI





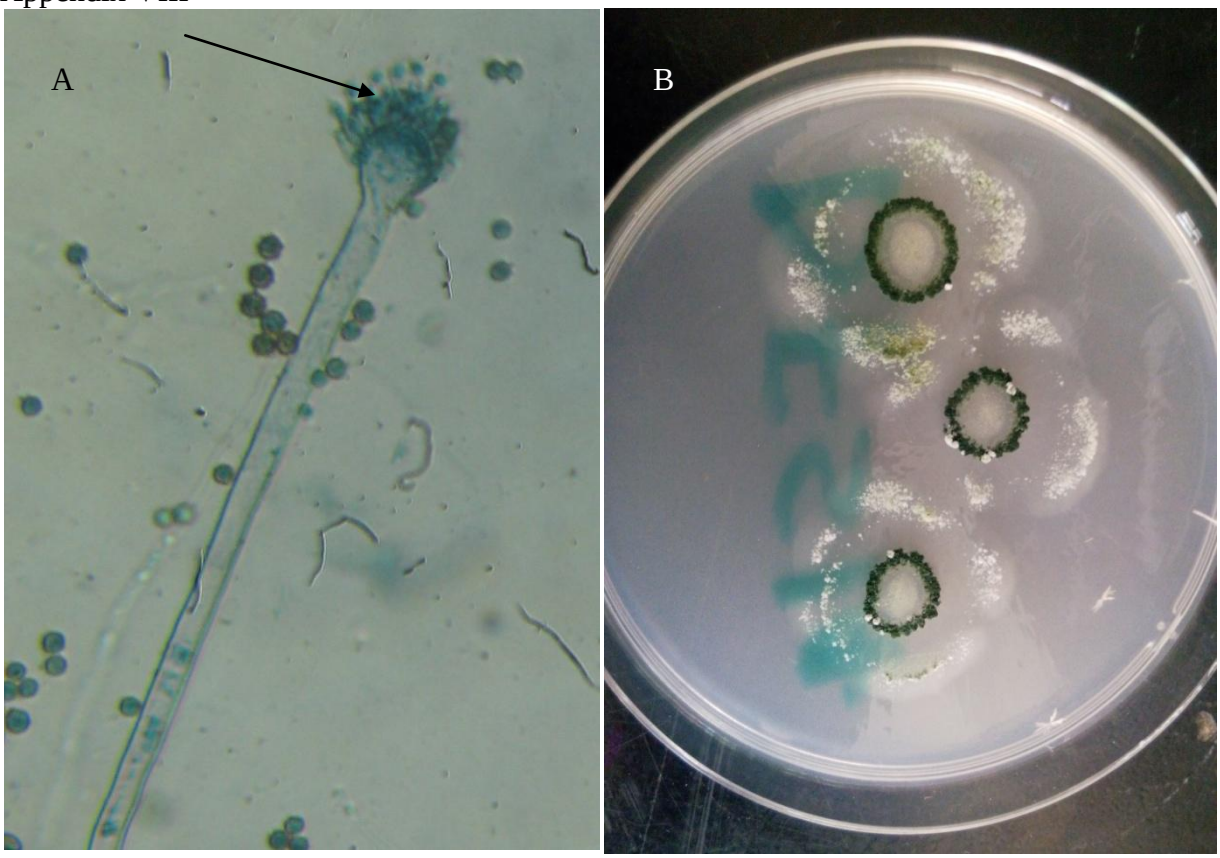
(*Aspergillus parasiticus*); A: Biseriate head with subglose and globose vesicle (Mag×40)
B: Dark green colony at 27 °C after 7 days on SDA

Appendix VII



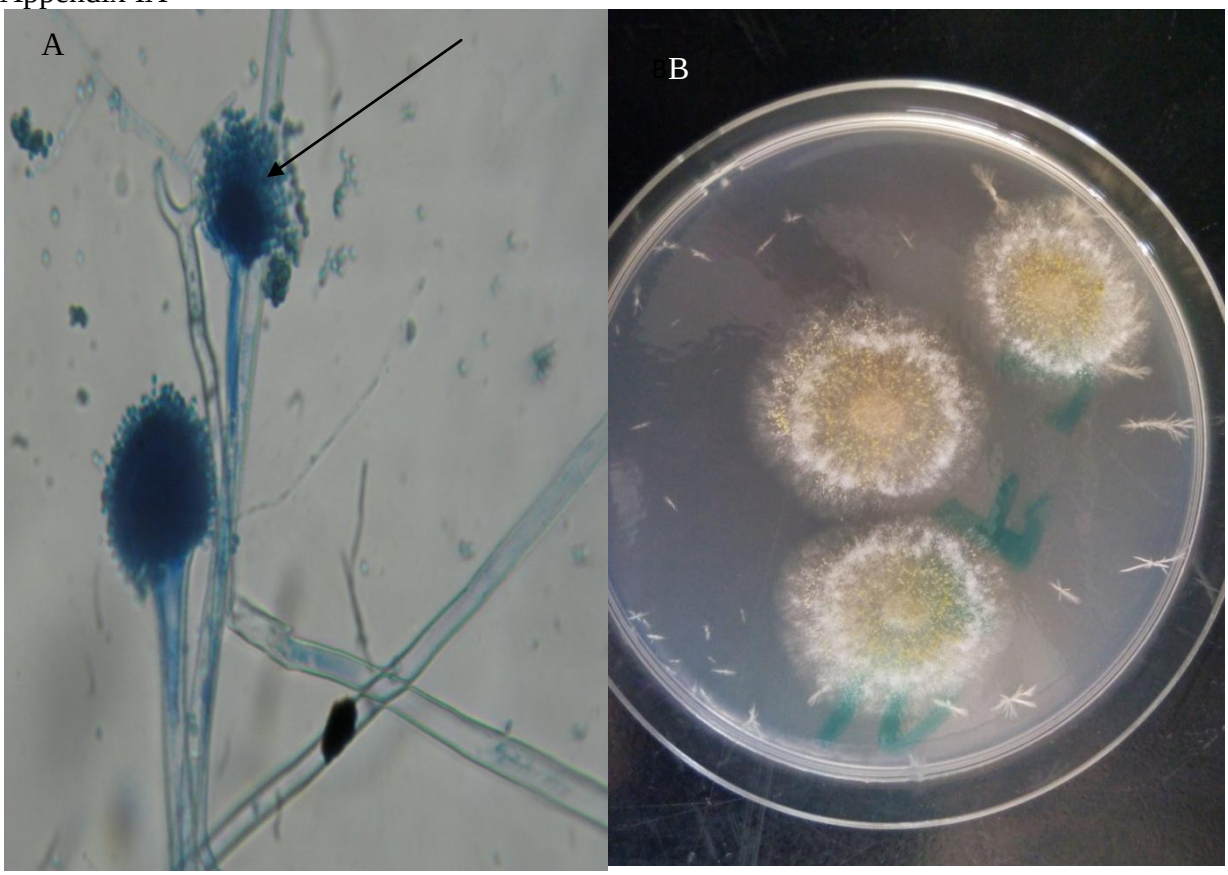
(*Aspergillus caelatus*); A: uni and biseriate head with subglose vesicle (Mag×40)
B: green colony at 27 °C after 7 days on SDA

Appendix VIII



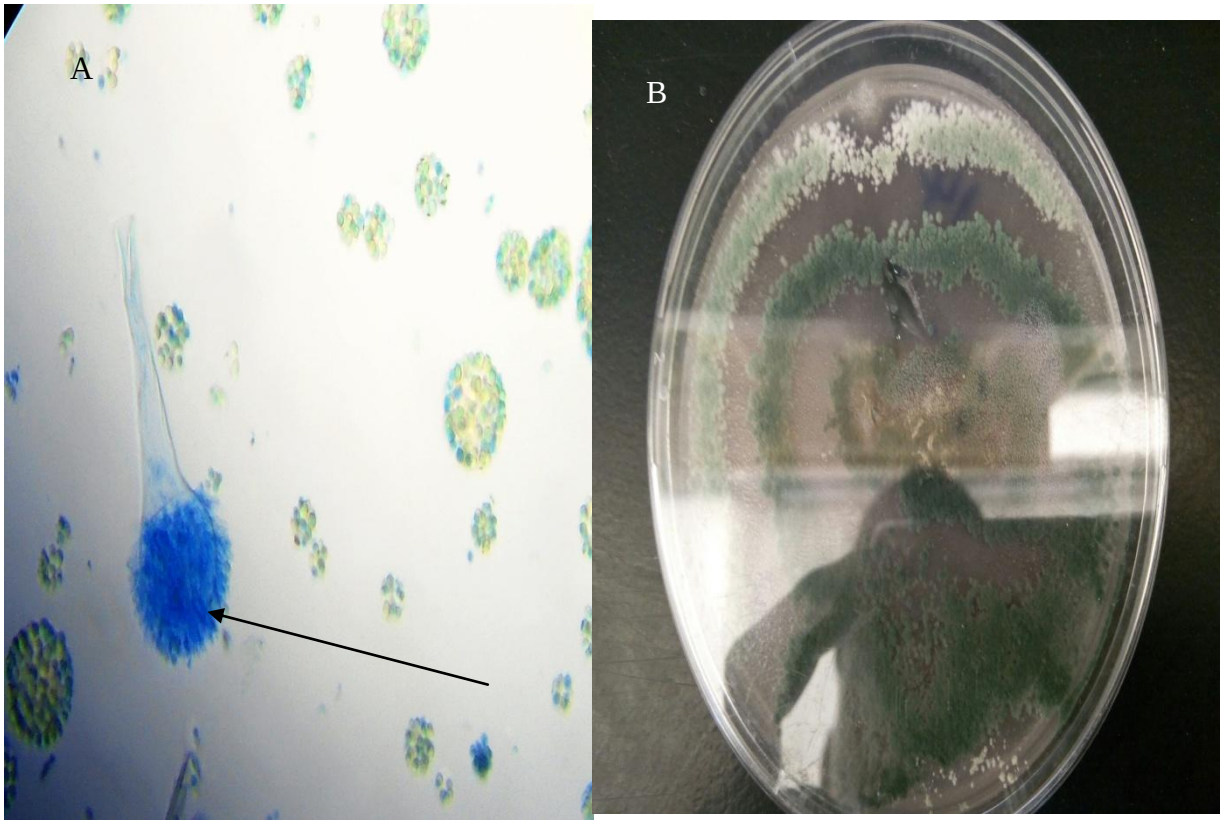
(*Aspergillus nidulans*); A: Short columnar and Biseriate head (Mag×40). B: plain green colony at 27 °C after 7 days on CYA

Appendix IX



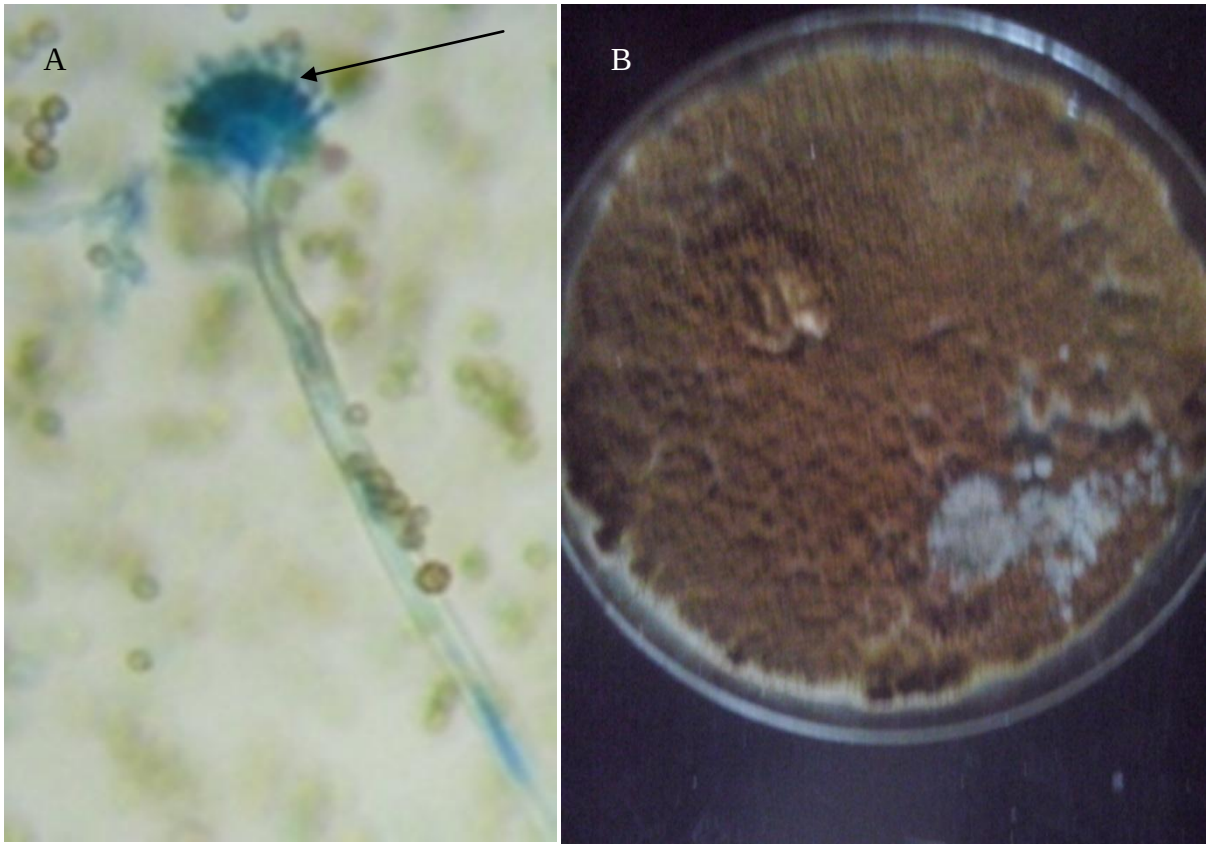
(*Aspergillus terreus*); A: Biseriate head with globose vesicle (Mag×40). B: sand brown colony at 27 °C after 7 days on CYA

Appendix X



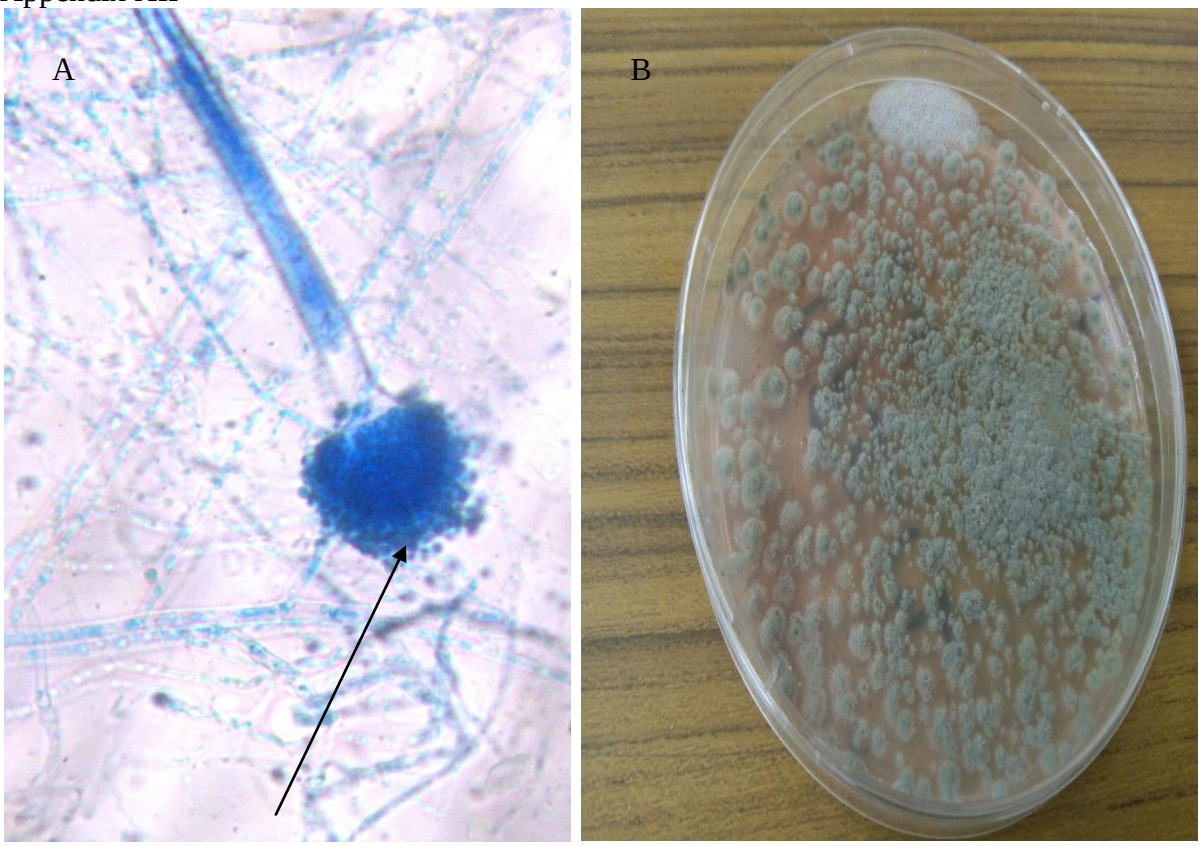
(*Aspergillus fumigatus*); A: Uniseriate with ovate to ask shape vesicle which covers upper two third (Mag×40). B: blue green colony at 27 °C after 7 days on CYA

Appendix XI



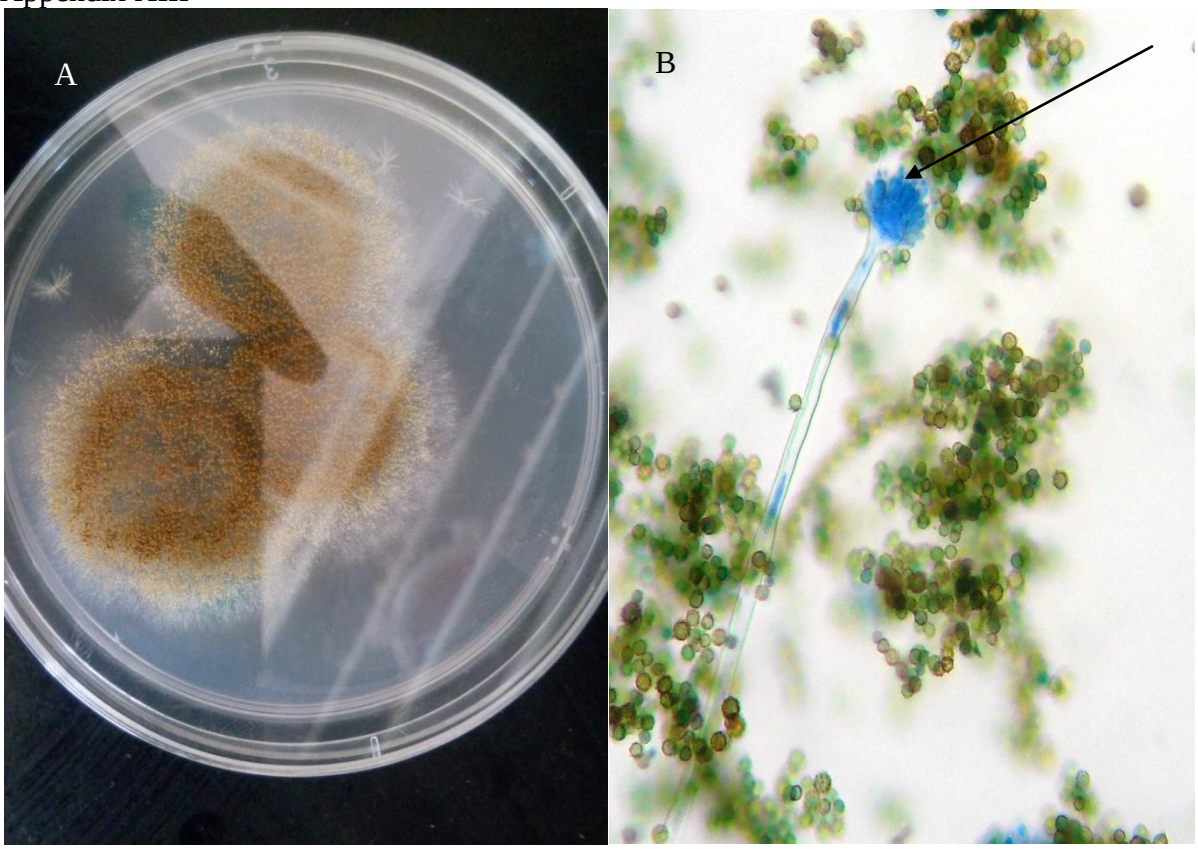
(*Aspergillus tamarii*); A: uniseriate and biseriate head with globose and subglobose vesicle (Mag×40). B: Sand brown colony of 27 °C after 5 days on CYA

Appendix XII



(*Aspergillus pseudotamarii*); A: Biseriate head with globose and subglobose vesicle (Mag×40)
B: green colony at room temperature after 7 days on DCA

Appendix XIII



(*Aspergillus nomius*); A: Golden yellow colony at 27 °C after 7 days on CYA. B: biseriate head with globose vesicle (Mag×40)