# Detection of Aflatoxins and Ochratoxins in Broiler and their feed in Khartoum State

**By**

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# DEDICATION

To my parent

To my brothers and sisters

To my uncle

and

To all people I love...

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## ABBREVIATIONS

| | |
|---|---|
| AFB1 | Aflatoxin B1 |
| AFB2 | Aflatoxin B2 |
| OTA | Ochratoxin A |
| OTB | Ochratoxin B |
| OTC | Ochratoxin C |
| ppb | Part per billion |
| DON | Deoxynivalenol |
| TDIS | Tolerable Daily Intakes |
| bw | Body weight |
| EC | European commission |
| EU | European Union |
| HPLC | High Performance Liquid Chromatography |
| AFDA | American Food and Drug Administration |
| APF | Agricultural Policy Framework |
| AOAC | Association of Official Analytical chemist |
| FSA | Food Standards Agency |
| FFSA | French Food Safety Agency |
| IFST | Institute of Food Science and Technology |
| IARC | International Agency of Research on Cancer |
| USDA | United State Department of Agriculture |
| SCF | Scientific committee for food |
| CYP 45O | Cytochrome Peroxidase 450 |

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## Abstract

This study was conducted in Khartoum state to investigate the occurrence of aflatoxin and ochratoxin in broiler feed stuff (finished ration, groundnut seed and sorghum or maize) and detected their residues in target organs (liver for aflatoxin and kidney for ochratoxin). Thirty- six samples of feed stuff, 90 livers samples and 90 kidneys samples were randomly collected from 9 farms. The feed stuff was extracted and the mycotoxins were detected using HPLC techniques and ELISA test.Aflatoxin was detected in all feed stuff sample examined (36). The mean concentration of which in total fresh prepared finished ration samples (9) was 38ppb when detected by HPLC and 35 ppb using ELISA test. In contrast the mean concentration in stored finished ration samples (9) the mean concentration was 71ppb when using HPLC and 55ppb when ELISA was used. In ground nut seed samples (9) a mean concentration was 38ppb when detected by HPLC and a mean of 93.13ppb when ELISA was used. In sorghum the mean concentration was 14 ppb and 0.37ppb when detected by HPLC and ELISA respectively. In maize the mean concentration was 92ppb and 37ppb when detected by HPLC and ELISA respectively. The mean concentration of ochratoxin in total fresh prepared finished ration samples (9) was 0.78ppb when detected by HPLC and 0.71 ppb using ELISA test. In stored finished ration samples (9) the mean concentration was 0.24ppb when using HPLC and 0.77ppb when ELISA was used. In ground nut seed samples (9), the mean concentration was 0.48ppb when detected by HPLC 1.3ppb when ELISA was used. In sorghum the mean concentration was 3ppb and 1.5ppb when detected by HPLC and ELISA, respectively. In maize the mean concentration was 2.5ppb and 2.12ppb when detected by HPLC and ELISA respectively. The toxin in liver and kidney were extracted and detected by ELISA test. Sixty –four livers out of ninety examined were positive for aflatoxins residues, with a mean concentration between 0.14ppb and 1.73ppb .

Eighty – six kidneys out of ninety kidney samples examined were positive for ochratoxin residues with a mean concentration between o.49ppb and 3.03ppb. The macroscopic change revealed wide areas of congestion, paleness, and necrosis and grease ness in affected livers. The affected kidney showed area of congestion, haemorrhage, paleness, enlargement and necrosis.

**المستخلص**

أجريت هذه الدراسة بولاية الخرطوم للكشف عن الافلاتوكسين والاوكراتوكسين وتحديد تركيزهما في علائق الدجاج اللاحم (العليقة الكاملة النهائيه وكسب الفول السوداني والذرة الرفيعة والذرة الشامية) ، و تحديد الأثر المتبقي للافلاتوكسين والاوكراتوكسين في الأعضاء المستهدفة (أكباد الدجاج اللاحم للافلاتوكسين والكلى للاوكراتوكسين). جمعت عشوائياً 36 عينة عليقه و90 عينة كبد و90 كلى من 9 مزارع . أستخلص الافلاتوكسين والاوكراتوكسين من العلائق والاكباد والكلى تم الكشف عنها بواسطة جهاز الكرموتغرافيا السائله عالية الفعالية (HPLC )واختبار الإليزا (ELISA) وجد ان جميع عينات العلائق(36) تحتوى على الافلاتوكسين. كان متوسط تركيز الافلاتوكسن في عينات العلائق النهائية (9) عينات 38 جزء من البليون عند قياسها بواسطة HPLC و متوسط تركيز 35 جزء من البليون عند قياسها بواسطة اختبار الإليزا. فى المقابل كان متوسط التركيز فى عينات العلف النهائية المخزنة (9) عينات 71جزء من البليون عند قياسها بواسطة HPLC و متوسط تركيز 55 جزء من البليون عند قياسها بواسطة اختبار الإليزا. بينما كان متوسط تركيز علائق كسب الفول السوداني 38 جزء من البليون عند قياسها بواسطة جهاز HPLC و 93 جزء من البليون عند قياسها بواسطة اختبار الإليزا. في الذرة الشامية كان متوسط التركيز 92 جزء من البليون 37 جزء من البليون عند قياسها بواسطة HPLC والإليزا على التوالى. في الذرة الرفيعة كان متوسط التركيز 14 جزء من البليون 0.37 جزء من البليون عند قياسها بواسطة HPLC والإليزا على التوالي.

أوضحت النتائج أن متوسط تركيز الأوكراتوكسين في العلائق النهائية الكاملة (9) عينات 0.78 جزء من البليون عند قياسها بواسطة HPLC و0.71 عند قياسها بالأليزا. فى المقابل كان متوسط التركيز فى عينات العلف النهائية المخزنة (9) عينات 0.24جزء من البليون عند قياسها بواسطة HPLC و متوسط تركيز 0.77 جزء من البليون عند قياسها بواسطة اختبار الإليزا. بينما كان متوسط تركيز علائق كسب الفول السوداني 0.48 جزء من البليون عند قياسها بواسطة HPLC و3.03 جزء من البليون عند قياسها بالإليزا. في الذرة الشامية كان متوسط التركيز 2.5 جزء من البليون 2.12 جزء من البليون عند قياسها بواسطة HPLC والإليزا على التوالي. في الذرة الرفيعة كان متوسط التركيز 3 جزء من البليون 1.5 جزء من البليون عند قياسها بواسطة HPLC والإليزا على التوالي. وجد إن 64 من 90 من الكباد تحتوى على بقايا الأفلاتوكسين، متوسط تركيز قدرة 0.14 و1.73 جزء من البليون.كما أن 86 من 90 من الكلى موجبة لبقايا الأوكراتوكسين متوسط تركيزها ما بين 0.49 و 3.03 جزء من البليون. اشتملت التغيرات المرضية العيانية في الاكباد المصابة على احتقان و شحوب ونخر وتدهن الكبد ، كما شملت التغييرات المرضية في الكلى على احتقان ، نزيف ، شحوب ، تضخم ونخر.

# INTRODUCTION

Mycotoxins are fungal toxins produced by mycelia structure of filamentous fungi commonly called moulds (Hussein and; Brasel, 2001). Mycotoxin produced when moulds grow in agricultural products, these toxins are a diverse group of toxic secondary metabolites, they do not belong to single class of chemical compound and they differ in their toxicological effects, (Coker,1979). The toxins produced by fungi belonging to Aspergillus, Pencillinum and Fusarium genera (Wagacha and Muthomi, 2008).

Mycotoxin contamination of agricultural commodities has attracted worldwide attention because of the significant losses associated with its effect on human health, poultry and live stock. They are formed by different kinds of fungi species and each fungi species is capable to produce more than one toxin (Chelkowski, 1991; Hussien, and Brasel, 2001).

It is estimated that 25% of the worlds food crops are contaminated annually by variable level of mycotoxins during growth and storage, and there are currently more than 400 mycotoxins known (IFST, 2006; USDA, 2006). Mycotoxins as secondary metabolites of toxigenic mould represent a great risk to human and animal health. Consumption of mycotoxins contaminated food or feed may cause acute and long term chronic effects (Kabak *et al*, 2006; Binder 2007). In addition to general toxicity, their biological effect includes immune suppressive, estrogenic and genotoxic effect, the effect depend upon mycotoxin, level in feed, period of exposure and animal species. (Sargeant *et al*., 1961). Mycotoxins with the greatest public health and agroeconomic significance include aflatoxins, ochratoxins, trichothecenes, zearalenone, and fumonisins (Hussein and Brasel, 2001).

Aflatoxin B1 (AFB1) and ochratoxin A(OA) belong to the most frequently occurring mycotoxins (Sargeant *et al*., 1961).Aflatoxin is a mycotoxin produced

by *Aspergillus flavus* and *Aspergillus parasiticus* ,commonly found to grow in animal feeds. Aflatoxins were first discovered in the early 1960s resulting from a large disease outbreak in turkeys and other poultry species. The toxicosis included marked biliary hyperplasia, acute hepatic necrosis, loss of appetite, lethargy, wing weakness and death (Blount, 1961; Lancaster *et al*, 1961; Asao *et al*, 1963). Ochratoxins are produced by several species of Aspergillus and Penicillium, predominantly several members of the *Aspergillus ochraceus* group and *Penicillium verrucosum* types I and II. The most important of these toxins is ochratoxin A (OA), which is more toxic and more frequently found than ochratoxin B (OB) or ochratoxin C. OA is a common contaminant of cereals (corn, wheat, barley, oats, rice, and sorghum), peanuts, as well as soya, coffee and cocoa beans(Krogh, 1976).

Aflatoxin were classified as carcinogenic to human (group1), ochratoxin classified as possible human carcinogenic (group 2B) by the International Agency Research on Cancer (WHO-IARC, 2002). OTA has been implicated in a diverse range of toxicological effects, including renal toxicity, mutagenicity, teratogenicity, neurotoxicity and immunotoxicity in both animals and man (O'Brien and Dietrich, 2005).OTA causes significant losses to the poultry industry due to its effects on performance and health.

## Objectives:

1- To detect the occurrence of aflatoxins and ochratoxin in broilers feed at the farm level in Khartoum State and determine their concentration

2- To determine the occurrence of aflatoxicosis and ochratoxicosis in broilers chickens in Khartoum State.

3- To describe the gross lesion of naturally occurring aflatoxicosis and ochratoxicosis.

# CHAPTER ONE

# LITERATURE REVIEW

## 1.1. Mycotoxins

Mycotoxins are diverse range of molecules that are harmful to animal and human. They are secondary metabolise secreted by mould, mostly Pencillium and Fusarium genera. They are produced in cereal, grains well as forages before, during and after harvest, in various environmental conditions. Due to the diversity of their toxic effect and their synergestic propertises, mycotoxin are considered as risky to the consumers of contaminated food and feed (Yiannikouris and Jonany, 2002).

Mycotoxins are metabolized in the liver and the kidneys and also by microorganisms in the digestive tract. Therefore, often the chemical structure and associated toxicity of mycotoxins residues excreted by animals or found in their tissues are different from the parent molecule .In farm animals, mycotoxins have negative effects on feed intake, animal performance, reproductive rate, growth efficiency, immunological defense as well as been carcinogenic, mutagenic, teratogenic, tremorgenic(cause tremor) or damage the central nervous system, haemorrhagic, as well as causing damage to the liver and kidneys. Ruminant animals are generally more tolerant to feed contaminated by mycotoxins than non-ruminant species due to the detoxifying capabilities of rumen micro organisms. Swine are generally the most sensitive with poultry intermediate (Ratcliff, 2002).

## 1.2 Mould growth and Mycotoxins production

Many species of fungi produce mycotoxin in feedstuffs .Moulds can grow and mycotoxin can be produced pre harvest or during storage, transport, processing or feeding. Mould growth and mycotoxin production are related to plant stress caused by weather extremes, to insect damage, to inadequate storage practices

and faulty feeding condition. In general, environmental condition –heat, water and insect damage cause plant stress and predispose plants to mycotoxin contamination .Mould grow over temperature range of( 10-104C),PH range of 4-8 and high relative humidity range of (70 to 90%). Moulds can grow on a dry surface (Lacey, 1991). Mould can grow on feeds containing more than 12-13% moisture. In wet feeds such as silage, higher moisture levels allow mould growth if oxygen is available. Because most moulds are aerobic, high moisture concentrations that exclude adequate oxygen can prevent mould growth. The conditions most suitable for mould growth may not be the optimum conditions for mycotoxin formation in the laboratory (Boyacioglu *et al*., 1992).

For example, the *Fusarium* moulds have been reported to grow prolifically at 25-30°C without producing much mycotoxins, but at near-freezing temperatures, large quantities of mycotoxins were produced with minimal mould growth (Joffe, 1986). Field applications of fungicides may reduce mould growth, thus reducing production of mycotoxins. However, the stress or shock of the fungicide to the mould organism may cause increased mycotoxin production (Gareis and Ceynowa, 1994).

*Aspergillus* species normally grow at lower moisture content and at higher temperatures than the *Fusarium* species. Therefore, *Aspergillus flavus* and aflatoxins in corn are favored by the heat and drought stress associated with warmer climates. Aflatoxins contamination is enhanced by insect damage before and after harvest. Similarly *Penicillium* species grow at relatively low moisture content and low temperatures and are widespread in occurrence. Because both *Aspergillus* and *Penicillium* can grow at low water activities, they are considered as storage fungi. The *Fusarium* species are generally considered to be field fungi and are thought to proliferate before harvest (Christensen *et al*., 1977). However, *Fusarium* species may also grow and produce mycotoxins

under certain storage conditions. In corn, *Fusarium* moulds are associated with ear rot and stalk rot, and in small grains, they are associated with diseases such as head blight (scab). In wheat, excessive moisture at flowering and afterward is associated with increased incidence of mycotoxin formation. In corn, *Fusarium* infections are more commonly associated with insect damage, warm conditions at wet conditions late in the growing season (Trenholm *et al*., 1988).

## 1.3 Stability of Mycotoxins

Mycotoxins are very stable, they can resist high temperature and several manufacturing processes .Most of the important mycotoxin are, in general quite resistant to most forms of food and feed processing (IARC, 2002; IFST, 2006)

For example, zearalenone is stable during storage, milling and cooking .DON and T-2toxin are stable at 120C and relatively stable at180C.Although, cooking can reduce the level of certain mycotoxin in food; it does not achieve complete detoxification. Aflatoxins decompose at their melting point, which are between 23C for aflatoxin (G1) and 299Cfor aflatoxin (M1), but are not destroyed under normal cooking condition. They can be completely destroyed by autoclaving in the presence of ammonia or by treatment with a bleach (IARC, 2002).

It is not surprising to detect low content of mycotoxin in processed foods. This is why it is important to prevent the formation of toxin in the raw material (IARC, 2002).

## 1.4 Occurrence of Mycotoxins in Developing Countries

The Food and Agricultural Organization of the United Nations (FAO) has estimated that up to 25% of the world's food crops are significantly contaminated with mycotoxins (WHO, 1999). However, the presence of mycotoxins in food is often over looked in Africa due to public ignorance about their existence, lack of regulatory mechanisms, dumping of food products, and the introduction of contaminated commodities into the human food chain during

chronic food shortage due to drought, wars, political and economic instability. Ethical considerations also play a role during the manufacturing process of food products using heavily contaminated commodities and sometimes "diluting" contaminated agricultural products such as peanuts with good quality products to an "acceptable" level below the regulatory level (MERCK, 2006; FDA, 1995).

## 1.5 Legal Limits of Mycotoxins

In order to assess the risk to public health from consumption of mycotoxins, the exposure of consumers to these toxins can be compared to safety guidelines such as Tolerable Daily Intakes (TDIs) (SCF, 1996). TDIs have been set by scientific committee for food (SCF) and are based on threshold level, identified during toxicological studies, below which the toxin are considered not to cause an adverse effect. TDI represent an estimate of the amount of a contaminant, expressed on a body weight basis, which can be ingested daily over a lifetime without appreciable health risk TDIs have been set by the SCF for mycotoxin as follow: 1µg/kg body weight /day for deoxynivalenol, 0.2µ/kg bw/day for zearalenone, and 2µ/kg bw/day for fuonisins. In addition, in 1998 the SCF expressed the opinion that exposure to ochratoxin A should be kept to the lower end of a range of TDIs of 1.2-14ng/kg bw /day below 5ng/kg bw/day because of uncertainties about the way ochratoxin A cause toxicological effect. Ochratoxin A has been shown to damage, and cause cancer of kidneys in laboratory animals .Although a define causative link has not been established, the consumption of foodstuffs highly contaminated with ochratoxin A has been associated with development of Balkan Endemic Nephropathy (a specific type of kidney disease) in certain human population (USDA, 2006).In contrast to the other mycotoxin, no TDI has been set for aflatoxins. This is because aflatoxins have been shown to cause cancer in the liver of laboratory animals by damaging DNA. They have also been linked to liver cancer in humans in a number of

developing countries, it has therefore been recommended that aflatoxin concentration in food should be reduced to the lowest levels achievable. The legal limits for aflatoxin in these food commodities were set in order to provide consumers with an increased measure of protection and prevent grossly contaminated product from entering the market (FSA, 2005).

The American Food and Drug Administration (AFDA) has established action level for aflatoxin present in food or feed. These limits are established by the Agency to provide an adequate margin of safety to protect human and animal health (USDA, 2006).

European community (EC) Regulation 2174/2003 sets limits of 2 microgram of aflatoxin B1and 4 microgram of total aflatoxins per kilogram of cereal, including maize and processed cereal product intended for direct human consumption or use as an ingredient .A maximum limit of 3 micrograms of ochratoxin A per kilogram of cereal products was set by EC Regulation 472/2002.

Recent legislation (EC Regulation No.683/2004) introduced legal limits for certain mycotoxin in baby food.

## 1.6 Health Implication

Mycotoxins are very resistant to heat treatments and to the action of chemical agents (Jouany 2001). That is why mycotoxins remain long time active in the media they were released, being a serious danger to animal health. On the other hand, some of these mycotoxins can easily pass from the forages to meat, milk or eggs posing thus serious health hazards to the people consuming products from contaminated animals *(*Conkova *et al*., 2003, Di Mello *et al*,. 1999, Hussein 2001).

The most commonly encountered mycotoxins in feedstuffs and foods are: Aflatoxins produced by *Aspegillus flavus* and *Aspergillus parasiticus*, Zearalenone produced by *Fusarium graminearum* and *Fusarium roseum*,

Ochratoxins produced by *Pencillium viridicatum* and *Aspergillus ochraceus*, Trichothecenes produced by *Fusarium tricinctum*, and Deoxynivalenol (Vomitoxin) produced by *Fusarium graminearum* (table 1)

**Table 1 Some mycotoxins and mould species which produce it**

| Mycotoxins | Mould genera | Species that produce Mycotoxin |
|---|---|---|
| Aflatoxins | *Aspergillus* | *A.flavus, A.parasiticus, A.nomius* |
| Ochratoxin | *Aspergillus* and *Penicillinum* | *P. viridicatum and A. ochraceus, A. sulphureus* |
| Cyclopiazonic acid | *Aspergillus* and *Penicillinum* | *A.parasiticus, A.versicolor, a.oryzre, A.tamari, P.verrucosum, P.patulum, P.cyclopim, P.camembertii.* |
| Deoxynivalenol (DON) | *Fusarium.* | *F.graminesrum, F. subglutinans* |
| Fumonisin | *Fusarium* | *F.verticillioides, F. moniliforme* |
| Patulin | *Penicillinum* | *P.expansum* |
| Sterigmatocystin | *Aspergillus* and *Penicillinum* | *A.flavus A.parasiticus, A.versicolor,A.rugulosus,A.nidulans, P.camembertii, P.griseofulvum* |
| T-2 toxin | *Fusarium* | *F. sportrichioide* |
| Zearalenone | *Fusarium* | *F. graminearum, F. subglutinans* |

Source :( USDA, 2006)

Mycotoxins exert their effects through four primary mechanisms:

1. Intake reduction or feed refusal.
2. Alteration in nutrient content of feed and hence absorption and metabolism.
3. Pathological effects on different organs and tissues.
4. Suppression of the immune system (Schiefer, 1990).

Mycotoxins can increase incidence of disease and reduce production efficiency. In the field, animals experiencing mycotoxicosis may exhibit few or many of a variety of symptoms, including: digestive disorders, reduced feed consumption, unthrift ness, rough hair coat or abnormal feathering, undernourished appearance, subnormal production and impaired reproduction. (Whitlow and Hagler, 2002)

Some of the symptoms observed with mycotoxicosis may be secondary, resulting from an opportunistic disease that is fliers up because of immune suppression due to exposure to mycotoxins. Therefore, the progression and diversity of symptoms are confusing and makes diagnosis difficult (Hesseltine, 1986). (Table 1.2)

Table.2 The effect of major mycotoxin and cellular molecular mechanism of action

| Toxin | Effect | Cellular and molecular mechanism of action |
|---|---|---|
| Aflatoxin B1+M1 | Hepatotoxicity .<br>Gentoxicity.<br>Oncogenicity.<br>Immunomodulation . | Formation of DNA adduct.<br>Lipid peroxidation.<br>Bioactivation by cytochroes P450.<br>Conjugation to GS transferase. |
| Ochratoxin A | Nephrotoxicity .<br>Gentoxicity.<br>Immunomodulation. | Effect protein synthesis<br>Inhibition of ATP production<br>Detoxification by peptidases |
| Trichothecenes (Toxin T-2,DON. ...) | Hematotoxicity .<br>Immunomodulation .<br>Skin toxicity . | Induction of apoptosis in haemopoietic progenitor.<br>cells and immune cell<br>Effect protein synthesis.<br>Abnormal changes in immunoglobulin. |
| Zearalenone | Fertility and Reproduction. | Binding to oestrogen receptors.<br>Bioactivation by reductase<br>Conjugate to glucuronyl transferase. |
| Fumonisin | Neurotoxicity.<br>Hepatoxicity .<br>Immunomodulation .<br>Gentoxicity . | Inhibition of ceramide synthesis<br>Adverse effect on the sphinganin/sphingosin ratio. |

Source :( FFSA, 2006).

## 1.7 Economic Impact

Mycotoxins contamination of the food chain has a major economic impact. However, the insidious nature of many mycotoxicoses makes it difficult to estimate incidence and cost (Miller,1998) .In addition to crop losses and reduced animal productivity, costs are derived from the efforts made by producers and distributors to counteract their initial loss, the cost of improved technologies for production, storage and transport, the cost of analytical testing, (Whitaker,2006). There is also a considerable cost to society as a whole, in terms of monitoring extra handling and distribution costs, increased processing costs and loss of consumer confidence in the safety of food products, the greatest economic impact is associated with human health (Miller, 1998).

## 1. 8 Mycotoxins Determination

Testing for mycotoxins is a complicated process that generally consists of three steps: (1) Sampling means to select a sample of a given size from a bulk lot. (2) Sample preparation comprises the grinding of the sample and taking a representative sub-sample of the ground material. (3) The analytical step consists of several processes where the mycotoxin is solvent extracted from the sub-sample, the solvent is purified and the mycotoxin in the solvent is quantified. The mycotoxin value, measured in the analytical step is then used to estimate the lot concentration or is compared to a maximum limit in order to classify the lot as acceptable or unacceptable. This means that a very small quantity of the lot is finally used in the quantification step to estimate the mycotoxin concentration of the whole lot. Analytical procedures for the determination of mycotoxins have improved continuously over the past years. Chromatographic methods like high performance liquid chromatography (HPLC) or gas chromatography (GC) have been used widely and can be considered as the most accurate quantification systems, but also a variety of immunological methods, in particular immuno sorbent assays (ELISAs) are

used frequently, as they require usually no further sample purification. ELISA test kits are well favored as high through put assays with low sample volume requirements and proceeding times of less than an hour, some even in less than 15 minutes. However, although the antibodies have the advantage of high specifity and sensitivity to their mycotoxin target molecule, compounds with similar chemical groups would also interact with the antibodies. This so-called matrix effect is especially evident in case of high complexity of the test material, which is in particular the case with finished feed, and can lead to overestimates, underestimates, or even false negative or false positive results, so that in such cases chromatographic detection remains the method of choice**.** **(**CAST, 2003).

## 1.9 Diagnosis of Mycotoxicosis

Diagnosis is further complicated by a lack of research and feed analysis, nonspecific symptoms and interactions with other stress factors.

A definitive diagnosis of a mycotoxicosis cannot be made directly from symptoms, specific tissue damage or even feed analysis. However, experience with mycotoxin-affected flocks increases the probability of recognizing mycotoxicosis. A process of elimination of other factors, coupled with feed analysis and responses to treatments can help identify a mycotoxicosis. Regardless of the difficulty of diagnosis, mycotoxins should be considered as a possible cause of production and health problems when such symptoms exist and problems are not attributable to other typical causes (Schiefer, 1990).

Analytical techniques for mycotoxins are improving. Cost of analysis has been a constraint, but can be insignificant compared with the economic consequences of production and health losses related to mycotoxin contamination. Newer immunoassays have reduced the cost of analysis. Collection of representative feed samples is a problem, primarily because fungi can produce very large

amounts of mycotoxins in small areas making the mycotoxin level highly variable within the lot of feed (Whittaker *et al*., 1991).

## 1.10 Ochratoxins

Ochratoxins (A, B, C) are isocoumarin and L-b-phenylalanine derivatives, and are classified as pentapeptides. *Aspergillus* and *Penicillium* species have been reported to produce one or more of the ochratoxins. Ochratoxin A (OTA) is the most common and most studied. It is produced by *Aspergillus ocraceus*, the first fungi from which it has been isolated and after which it is named. It is also produced by many other *Aspergillus* and *Penicillium* species (Studer-Rohr, I. *et al.* 1995). Recent studies showed that OTA is produced by *A. niger* (Stander, *et al.* 2000). Although all *Aspergillus* species produce OTA, the highest quantities produced by *A. carbonarius*. In temperate climates OTA is produced by *Penicillium verrucosum*, while a number of *Aspergillus* spp. (*A. ochraceous*, *A. niger*, *A. sulphureus*, *A. sclerotiorum*, and *A. melleus*) are known to be responsible for its production in tropical and pan-tropical regions of the world. (Pitt, 2000). The nephrotoxic effect of OTA has been shown in many animal species, although sensitivity is variable among them (Abarca *et al.,* 2001). It acts essentially in the proximal renal tubules, inhibiting the enzyme phosphoenolpyruvate carboxylase, which is a lipid peroxidant, and it alters the structural and functional renal ability to metabolize calcium (Betina, 1989).

## 1.10 .1 Natural Occurrence of Ochratoxin A in Animal Feed

OTA is a secondary toxic metabolite produced mainly by some strains of *Aspergillus ochraceus* and *Penicillium verrucosum* species. These species can grow in different climates. *Aspergillus* are found in tropical regions, whereas *Penicillia* are common in temperate regions; and can grow when the temperature is as low as 5 ºC (WHO 2002). In general, OTA formation occurs mainly after harvesting on insufficiently dried cereal and cereal products.

Factors influencing OTA production include environmental conditions, such as temperature and water activity, but also the type and integrity of the seeds. While *A. ochraceus* grows better in oilseeds (peanuts and soybeans) than in grain crops, such as wheat and corn, *P. verrucosum* may grow better in wheat and corn (Madhyastha *et al.*, 1990). A wide variety of nutritional based biotic factors may affect the production of OTA biosynthesis. While, different carbon sources, including glucose, sucrose, galactose or xylose, appear to repress OTA production in *A. ochraceus*; other compounds, such as lactose, and organic nitrogen, such as urea and amino acids, induces its production (Abdelhamid *et al.*, 2009). OTA has been found in cereal grains (maize, barley, wheat, oats, rye), hay and mixed feed (. Battaglia *et al.*,1996; EFSA 2006). OTA amount in animal feed varies from country to country. The highest amounts have been reported in Northern Europe and North America (WHO 2002). Specifically, the highest frequencies were reported in Denmark (57.6%), Canada (56.3%) and Yugoslavia (25.7%) ( Speijers *et al.*,1993).

## 1.10.2 Effects of Ochratoxin A on Animal Production and Health

OTA-contaminated feed has its major economic impact on monogastric animals and the poultry and pig industry. Ruminant animals are more resistant than monogastrics to OTA toxicity. In general, exposure to OTA contaminated feed reduces animal growth rates and affects animal production. Pigs are generally considered as the animal species most sensitive to the nephrotoxicity of OTA. Nephropathy, but without renal failure, was observed in female pigs fed on diets containing 1 mg OTA/kg feed, but not on diets containing 0.2 mg OTA/kg feed for two years(EFSA 2006). Degenerative changes affecting epithelial cells in some proximal tubules were observed in pigs given a diet containing OTA at 0.8 mg/kg for six months, as well as proliferative changes in the interstitium, which predominated after one year (Stoev *et al., 2002)*. The phagocytic activity and the production of Interleukin 2 (IL 2) were decreased when pigs consumed

feed contaminated by OTA (2.5 mg/kg). In another study, ingestion of feed containing 25 μg/kg decreased feed efficiency, daily gain weight and final body weight in pigs (Malagutti *et al., 2005)*. The poultry industry is also affected by OTA contamination. Turkeys, chickens and ducklings are susceptible to this toxin. Typical signs of poultry ochratoxicosis are reduction in weight gain, poor feed conversion, reduced egg production, poor egg shell quality and nephrotoxicity. OTA fed at various doses (1–5 mg/kg), to animals of various ages, altered their serum biochemistry, including decreases in cholesterol, total protein, albumin, globulin, potassium, and triglyceride levels, and increases in uric acid and creatine levels and in the activities of serum alkaline phosphatase (ALP) and gamma glutamine transpeptidase (GGT) (Denli *et al., 2008;Gentle et al., 2008)*. The effects depend on the level of the toxin and time exposure. However, numerous studies showed that even exposure to low levels of OTA (0.5 mg/kg feed) altered performance, including decreased feed consumption and growth rate and poor feed conversion efficiency (Prior *et al., 1980;Wang et al., 2009)*. Reduction in egg production and egg weight were recorded in laying hens when animals were fed a diet contaminated with OTA at 2 and 3 mg/kg levels. It was also reported that laying hens fed on a diet contaminated with 2 mg OTA/kg,, significantly, reduced daily feed consumption, egg mass production, and serum triglyceride concentrations, and increased the relative liver weight as compared with a control diet (Denli *et al., 2008)*. Weight losses, diarrhea, excessive urine excretion and renal lesions have been noted in chickens fed a diet contaminated with 2 mg OTA/kg. Exposure to OTA-contaminated feed (2.5 mg OTA/kg) also decreased the concentration of α-tocopherol in the chicken liver (Hoehler1996). Impaired chick immune function even at concentrations as low as 0.25 mg/kg of OTA was also reported (Wang2009).

### 1.10.3 Human Exposure to Ochratoxin A Contaminated Food

OTA occurrence in human food commodities of vegetable and animal origin has been recognized as a potentially global human health hazard. Several detailed risk assessments have linked kidney damage incidence to estimated OTA consumption in the diet. OTA is associated with the Balkan Endemic Nephropathy and was also linked to human renal disease. Moreover, it has been described that OTA is genotoxic and associations have been found between OTA exposure and tumor incidences in long-term animal bioassays. A general maximum OTA limit of 5 μg/kg in cereals and 3 μg/kg in cereal products was proposed by the World Health Organization (WHO 2002). OTA has been detected in human blood and human milk samples. The increase of OTA in several human fluids in the various populations of endemic regions may describe the human exposure to OTA contaminated food.

### 1.10.5 Poultry Ochratoxicosis

The consumption of poultry feed contaminated with ochratoxin, causes a reduction in growth rate and feed consumption, poorer feed conversion and increased mortality (Peckham et al ., 1971; Huff et al ., 1974; Verma et al ., 2004). Furthermore it produces a reduction in total blood proteins (Huff et al., 1988; Stoev et al., 2000), suppression of immune function (Chang et al., 1979; Dwivedi and Burns, 1984 a and b; Stoev et al., 2000, 2002; Santin et al., 2002; Politis et al., 2005) and impairment of blood coagulation (Raju and Devegowda, 2000). OTA induces degenerative changes and an increase in the weight of the kidney and liver, aswell as a decrease in the weights of the lymphoid organs (Stoev *et al.*, 2000, 2002).

## 1.11. Aflatoxins

Aflatoxins are family of mycotoxins that contaminate peanuts, cereal, cottonseed, corn, rice and other commodities with widespread contamination in hot and humid regions of the world and is a continuing worldwide problem

(LFRA, 2003; Murphy, 2006). It's extremely toxic, mutagenic and carcinogenic compounds produced by certain strain of *Aspergillus flavus* and *Aspergillus parasiticus* .The name of aflatoxin comes from A(Aspergillus) +FLA(flavus)+toxin (Whitlow *et al,* 2002). They are four major aflatoxins are called B1, B2, G1 and G2 based on their fluorescence under UV light (blue or green) and relative chromatographic mobility during thin-layer chromatography. Aflatoxin B1 is the most potent natural carcinogen known(Squire,1981**),** and is usually the major aflatoxin produced by toxigenic strains.

### 1.11.1 Poultry Aflatoxicosis

Aflatoxins were first discovered in the early 1960s resulting from a large disease outbreak in turkeys and other poultry species. The toxicosis included marked biliary hyperplasia, acute hepatic necrosis, loss of appetite, lethargy, wing weakness and death (Blount, 1961; Lancaster *et al*, 1961; Asao *et al*, 1963). Of the aflatoxins, aflatoxin B1 (AFB1) is the most potent, and is nearly universally found in many foods and feeds. Avoidance of contaminated feeds is often not possible, and feed that contains relatively low amounts of AFB1 may still have deleterious effects on sensitive species such as poultry (Carnaghan, 1965; Doerr *et al*, 1983; Giambrone *et al*, 1985). In fact, poultry is one of the most sensitive food-producing animals (Newberne, 1967; Cavalheiro, 1981; Malkinson *et al*, 1982; Dalvi, 1986; Council for Agricultural Science and Technology, 1989). A requisite to AFB1 toxicity is the metabolic activation of AFB1 to the proximal, electrophilic species, the AFB1- 8,9-epoxide (AFBO) (Hayes *et al*,1991). In most species, oxidation occurs via cytochrome P450s (CYPs), although prostaglandin synthase and select lipoxygenases can also activate AFB1 (Battista and Marnett, 1985; Liu and Massey, 1992; Raney *et al*, 1992b).

## 1.11.2 Epidemiology of Aflatoxicosis

Food systems and economics render developed-country approaches to the management of aflatoxins impractical in developing-country settings, but the strategy of using food additives to protect farm animals from the toxin may also provide effective and economical new approaches to protecting human populations (Williams *et al*, 2004).

Earlier recognized disease outbreaks called [mouldy corn toxicosis], [poultry haemorrhagic syndrome], and [Aspergillus toxicosis] may have been caused by aflatoxins (Aiello *et al*, 1998). Aflatoxins were first isolated some 40 years ago after outbreaks of disease and death in turkeys (Blount, 1961) and of cancer in rainbow trout (Halver, 1965, Rucker *et al,* 2002) fed on rations formulated from peanut and cottonseed meals. The fungi responsible are ubiquitous and can affect many of the developing-country dietary staples of rice, corn, cassava, nuts, peanuts, chilies, and spices. The result is that, at latitudes between 40°N and 40°S of the equator, contamination of stored, inadequately dried produce is possible. Fungal invasion and contamination often begin before harvest and can be promoted by production and harvest conditions (Williams *et al,* 2004). Genotypes (Mehan *et al*, 1986). Drought (Sanders *et al,* 1993), soil types (Mehan *et al,* 1991), and insect activity (Lynch and Wilson, 1991) are important in determining the likelihood of preharvest contamination (Cole *et al*, 1995). Timely harvest and rapid and adequate drying before storage are also important (Mehan *et al,* 1986). Economic pressures have created a double standard for allowable contamination of commodities destined for human and animal consumption (Williams *et al*, 2004). Human foods are allowed 4–30 ppb aflatoxin, depending on the country involved (Food and Drug Administration. 1995, Henry *et al*, 1999). Grains for animal feed in the United States are allowed 300 ppb aflatoxin (Food and Drug Administration. USA, 1994) because

this concentration not only provides protection against acute aflatoxicosis but also is low enough to allow most of the grain produced to be traded. In these animal feeding situations, the long-term risk of cancer is not a concern, except for the most susceptible species. Consequently, veterinary research has examined higher levels of exposure but for shorter time periods. This provided most of the information on the toxicities of aflatoxin at intermediate rates of exposure (100–500 ppb) and is the most potentially relevant information that is appropriate for the human situation in developing countries where no control of aflatoxin is exercised (Williams *et al,* 2004).

## 1.12 Ochratoxin A and Aflatoxin Combination

The combined effect of aflatoxin and ochratoxin in broiler chicken is synergistic. Action between them can enhance their toxic effects and alter the target organs affected (Farfan, 2000;Zinedine *et al*., 2006).

How ever the antagonistic effect of AFB1 and OTA was only manifested by the fact that the number of liver lipids was not increased in the presence of OTA, although in other cases this is a diagnostic symptom of aflatoxicosis (Huff and Doerr, 1981; Huff *et al*., 1983). The toxicity of some mycotoxin combinations was also tested in poultry and pigs, where AFB1 together with OTA were found to be most toxic (Huff *et al*., 1988).

# CHAPTER TWO

# MATERIALS AND METHODS

## 2.1 Study Area

The study was conducted in Khartoum State which lies in semi desert zone between latitude 16.45- 15.8 north and longitude 25.3- 31.45east.It is composed of seven localities which includes ( Khartoum ,Bahary , Gabelawlia, Sherganil , Omdurman , Karari and Ombeda) .According to Khartoum state census 2008 the poultry population was estimated as 34.636.000 birds .

## 2.2 Samples Collection:

The samples were collected randomly between June and December 2009 from (9) poultry (broiler) farms in Khartoum State of different capacities i.e. big intensive farms (above 25.000 chicks), medium farms (5.000 - 15.000 chicks) and small extensive farms (less than 5.000 chicks). These include three farms in each locality of Khartoum, Bahary and Omdurman (farms A, B, C) represent big extensive farm, medium farm and small farm in Khartoum locality, (farm D, E and F) in Bahary, and (farm G, Hand I) in Omdurman respectively.

### 2.2.1 Feed stuff Samples:

Form each farm four feed samples were taken, one sample from ground nut cake and another one from sorghum or maize plus two samples from prepared ration one of them was taken from fresh prepared ration the other from the stored ration.

Samples for groundnut, sorghum, maize and stored finished ration were prepared by taking about one kg from 5 sacs selected randomly, thoroughly mixed and one kg was taken. One kg was collected from different feeder which represents the feed ration .Each sample of these was divided into two parts one

for determination of aflatoxin and ochratoxin concentration using High Performance Liquid Chromatography (HPLC) technique and the other using Enzyme Linkage Immunosorbent Assay (ELISA) test.

### 2.2.2 Liver and Kidney Samples:

90 livers and 90 kidneys samples were collected from the 9 farms (10 samples from each farm). The organs were taken immediately after slaughtering. About half of each organ was blended and stored at -20˚C for ELISA test for aflatoxin and ochratoxin determination.

## 2.3 Samples Extraction:

### 2.3.1 Feedstuff Samples:

#### 2.3.1.1Feedstuff Samples Extraction for HPLC

##### 2.3.1.1.1 Extraction of Ochratoxin A:

Extraction, clean up, and determination of ochratoxin A was done using Association of Official Analytical Chemist (AOAC) official method 991.44. In brief; feed powder (50g) of mixed feed was transfered into 500 mL conical flask. 250 mL chloroform and 25 mL 0.1 M methyl phosphoric acid were then added. The flask was securely stoppered and shaken on a wrist action shaker for 30 minutes and filtered through filter paper. 50 mL of the filtrate was then transferred into a separation funnel and extracted with 10 mL 3% sodium bicarbonate and the upper (bicarbonate) phase was collected. 5 mL bicarbonate extract were then loaded into C18 cartridge that has been previously washed two times with 2 mL methanol, 2 mL water, and 2 mL 3% sodium bicarbonate. C18 column was then washed with 2 mL phosphoric acid and 2 mL water, and ochratoxin A was eluted with 8 mL ethyl acetate: methanol: acetic acid (95: 5: 0.5) into screw capped borosilicate vial containing 2 mL water. The vial was

shaken until the two phases were completely mixed and left for approximately 2 minutes. The upper phase was then collected into a new screw capped borosilicate vial. The lower phase was washed twice with 1 mL ethyl acetate to extract ochratoxin A left in a fraction of upper phase remaining in the lower phase, both fractions were combined to upper phase and then evaporated to dryness. The dry film was dissolved with 500 µL mobile phase and injected into HPLC. The chromatographic conditions were as follows:

- Column type & size: C18; 250 X 4.6 mm I.D.; 5 micron particle size.
- Temperature: ambient temperature 25 $^{o}$C.
- Fluorescence detector: 333 and 460 nm as wavelengths for excitation and emission, respectively
- Mobile phase: acetonitrile: water: acetic acid (99: 99: 2)
- Flow rate: 1 ml/min.
- Injection Volume: 20 µL.
- Calibration curve was determined, using series of dilutions containing 2 ng 20 $\mu L^{-1}$, 4 ng 20 $\mu L^{-1}$ and 8 ng 20 $\mu L^{-1}$ of ochratoxin A standard. The correlation factor was 0.999.

2.3.1.1.2 **Extraction of Aflatoxin:**

50g of mixed feed was transfered into 500 mL conical flask 250 mL methanol: water (55:45), 100 mL hexane and sodium chloride (2g) were added. The flask was securely stoppered and shaken on a wrist action shaker for 30 minutes and filtered through filter paper. 25 mL of the aqueous methanol lower phase was transferred into a separation funnel. It was then extracted three times with 25 mL chloroform. The combined chloroform extracts were concentrated to 2 mL. The concentrated extract was then carefully transferred into screw capped borosilicate vial and evaporated to dryness. The dry film was dissolved with

400 µL mobile phase (water: methanol: acetonitrile 60:20:20) and separated by HPLC (Altenkirk *et al*., 1974; Beg *et al*., 2006). A standard was used for qualitative and quantitative analysis.

**HPLC – Operation Conditions**

The HPLC-operating conditions for aflatoxin detection were as follows:

- Column Type & Size: C18; 250 X 4.6 mm I.D.; 5 micron particle size.
- Temperature: Room temperature 25 $^{o}$C.
- Detector: Photodiode Array λ 365 nm.
- Mobile Phase: Deionized Water: methanol: Acetonitrile (60:20:20)
- Flow Rate: 1 ml/min.
- Injection Volume: 20 µL.
- Microsoft Excel was used to calculate averages and coefficient of variations

**2.3.1.2 Feedstuff Sample Extraction for ELISA**

Samples were grounded using high speed blender for 5 min, 6gm of the grounded sample was weighted and 24 ml of 70% methanol was added for aflatoxin and 40ml of 50% methanol was added for ochratoxin , shacked vigorously for 3 min and 5min respectively. The extract was filtered by pouring at least 5 ml through (Neogen filter syringe) then the filtrate was collected as a sample ready for analysis.

**2.3.1.3 Liver and Kidney Samples:**

One gram of kidney and Liver were taken from each were put in a beaker then 5 ml of 70 %( v/v) methanol was added, homogenized in magnetic stirrer for 2min, transferred to 10ml test tube and centrifuged at 2,700 for 15 min, the

supernatant was collected and a portion was subjected to aflatoxin and ochratoxin analysis by ELISA (Vilar, et al 2008).

## 2. 4 Enzyme linked Immuno-sorbent Assay (ELISA):

### 2. 4. 1 Assay Principles:

The aflatoxins and ochratoxin concentration were measured by using Veratox® aflatoxins and ochratoxin quantitative kits (GIPSA FGIS 2008-111, and V – Ochra-0808 Neogen Corporation USA/Canada).

Veratox for aflatoxins and ochratoxin are a direct competitive ELISA in microwell format which allows the user to obtain the exact concentrations in parts per billion (ppb). Free aflatoxins and ochratoxin in the samples and controls are allowed to compete with enzyme-labeled aflatoxins or ochratoxin (conjugate) for the antibody binding sites. After a wash step, substrate is added, which react with the bound conjugate to produce blue color that indicates less aflatoxins or ochratoxin. The test is read in microtiter plate to yield optical densities. The optical densities of the controls form the standard curve and the samples optical densities are plotted against the curve to calculate the exact concentration of aflatoxins or ochratoxin.

### 2. 4. 2 Test Procedures:

All reagents were allowed to warm at room temperature before use.

1. A red-marked mixing well was removed for each sample to be tested plus 4 red-marked wells for controls in aflatoxin test and 5 red – marked well for control in ochratoxin test, and placed in the well holder.
2. Equal number of antibody-coated wells was removed. Antibody well which will not be used immediately was returned to the foil pack

with desiccant. The foil pack was resealed to protect antibody. One end of strip was marked with a "1", and strip was placed in the well holder with the marked end on the left.

3. Each reagent was mixed by swirling the reagent bottle prior to use.
4. 100 µl of conjugate was placed from the blue-labelled bottle in each red-marked mixing well.
5. 100 µl of controls and samples were transferred (using a new pipette tip for each) to the red-marked mixing wells as described blow:-

   1- Aflatoxin

   0 5 15 50 S1 S2 S3 S4 S5 S6 S7 S8 strip1

   S9 S10 S11 S12 S13 S14 S15 S16 S17 S18 S19 S20 strip2

   2- Ochratoxin

   0 2 5 10 25 S1 S2 S3 S4 S5 S6 S7 strip1

   S8 S9 S10 S11 S12 S13 S14 S15 S16 S17 S18 S19 strip2

6. Using a 12 channel pipettor, the liquid in the wells was mixed by pipetting it up and down three times. 100 µl was transferred to the antibody-coated wells.

7. Timer was set for 2 min in aflatoxin test and 10 min in ochratoxin test and the wells were mixed for the first 10-20 sec at room temperature by sliding the microwell holder back and forth on a flat surface without splashing reagents from wells.

8. The contents of the antibody-coated wells were shacked out, then the wells were filled with distilled water and dumped out, this step was repeated five times, then the wells were turned upside-down and taped out on a paper towel till the remaining had been removed.

9. The needed volume of substrate was poured into the substrate reagent boat.

10. Using new tips on 12-channel pipettor, 100 µl of substrate was primed and pipetted into the wells.

11. Timer was set for 3 min in aflatoxin test and 10 min in ochratoxin test and the wells were mixed for the first 10-20 sec at room temperature by sliding the microwell holder back and forth on a flat surface without splashing reagents from wells.

12. The needed volume of the red stop solution was poured into the stop solution reagent boat.

13. Using new tips on 12-channel pipettor, 100 µl of the red stop solution was primed and pipetted into the wells, and then the plate was shacked by sliding back and forth on a flat surface

14. The bottom of the microwells were wiped with a dry towel.

15. Finally the optical densities were measured by using ELISA reader (BIO-RAD 680 (USA)) using 655 nm filter.

**2. 4. 3 Calculations:**

The optical densities were determined using Neogen veratox soft ware and the exact concentrations of the aflatoxin and ochratoxin in ppb in samples were calculated.

**2.5 Data analysis:**

Data was analyzed using STATA (Version 7) 2007. Tables and histograms were used for presentation of the results. The mean and

Standard deviation were used to measure the presence of aflatoxin and ochratoxin in feed and tissues

# CHAPTER THREE

# RESULTS

## 3. 1 Feedstuff:

### 3. 1. 1.1 Samples taken from feeders

All samples collected from farms (one sample from each farm) were found positive for aflatoxin using HPLC technique with a concentration that varied between 2.5 ppb and 105.2 ppb and a mean of (37.88 ±32.36ppb). When using ELISA test the concentration varied between 2.93ppb and 115.5ppb with a mean of (35.24±29.07ppb). (Table 3).

Using HPLC technique 4 samples out of 9 were found positive for ochratoxin A. with a concentration that varied between 0.4ppb and 2.6 ppb and a mean of (0.78 ±1.12ppb). Using ELISA 8 sample out of 9 were found positive for ochratoxin A with a range of 0.4ppb and 1.2ppb and a mean 0f (0.71±0.38ppb). (Table 4).

### 3. 1. 1. 2 Stored finished ration samples

Using HPLC technique and ELISA test all 9 samples were found positive for aflatoxin with a range between 8.9 ppb and 165.33ppb and a mean concentration of (70.54±56.08ppb) and concentration varied between 8.29ppb and 173.33ppb and a mean of (55.32±50.32ppb) respectively .( Table 5).

Regarding the ochratoxin 3 samples out of 9 were found positive with a range between 0.4ppb and 1.31ppb and a mean of (0.24±0.43ppb). Using ELISA test 8 sample out of 9 were found positive with a concentration that varied between 0.4ppb and 1.5ppb and a mean concentration of (0.77±0.43ppb). (Table 6).

## 3. 1. 2 Ground nut samples

Using HPLC technique and ELISA test all 9 samples were found positive for aflatoxin with a range between5.08ppb and 123.78ppb and a mean concentration of (60.57±31.36ppb) and concentration varied between 5ppb and 146.5ppb with a mean of (93.13±51.26ppb) respectively(Table 7).

On the other hand three samples out of 9 were found positive for ochratoxin with a concentration that varied between 0.84ppb and 2.6 ppb and a mean of ( 0. 48±0.88 ppb) when HPLC was used .When ELISA test was used 8 samples out of 9 were found positive with a concentration varied between 0.4ppb and 1.5ppb and a mean of (1.3±0.9ppb).) (Table 8).

## 3.1.3 Sorghum and maize samples

Four sorghum samples and five maize samples were examined as a source of raw material in the farms. All were found positive to aflatoxin with a concentration that varied between 2.2ppb and 19.8 ppb for sorghum and 55.21ppb and 133ppb for maize and a mean concentration of (14.18±12.8ppb) and (52.29±52.8ppb) respectively when detected by HPLC technique, and a concentration between 0.3ppb and 4.15 ppb for sorghum and 2.8ppb to 86.02 ppb for maize with mean concentration of (0.37±0.75ppb) and (23.27±20.86ppb) respectively when detected by ELISA test (Table 9and 10). When using HPLC technique for determination of ochratoxin three samples out of four were found positive with a concentration varied between 1.37ppb and 2.81ppb and a mean of (1.62±1.2ppb) and two samples of maize out of five were found positive for ochratoxin with a mean concentration of( 0.98±1.7ppb). Using ELISA test three sample of sorghum were found positive with concentration that varied between 0.3ppb and 1.9ppb and a mean concentration of (0.83±0.85ppb). All maize samples were found positive for ochratoxin with

concentration varied between 0.7ppb and 2.5ppb and a mean concentration of (2.21±1.19ppb)( Table 11 and 12) .

## 3.2 Tissue samples:

### 3.2.1 Liver samples:

Sixty-four samples out of ninety samples examined were found positive for aflatoxins residues, with a mean concentration that varied between 0.14ppb and 1.73ppb. The positive liver samples were found in all farms examined. (Table 13) (Fig 1).

### 3.2.2 Kidney samples

Eighty- six samples out of ninety kidney samples examined were found positive for ochratoxin residues with a mean concentration varying between o.49 and 3.03ppb.The positive samples found in all farm examined. (Table 14) (Fig 2).

## 3.3 Clinical symptoms

Chickens showed diversity of symptoms including decreased weight gain (Fig 3) the comb and wattles were absent or not well developed dullness, poor appearance, ataxia, lameness, paralysis of the legs and wings gasping ( Fig 4).

## 3. 4 Gross lesions

The liver showed wide areas of congestion and paleness they were greasy with some necrotic foci. The kidney showed area of congestion, haemorrhage paleness and necrosis. All lesion, were clearly evident in farm (I) in which the sample collected during poultry mycotoxicosis outbreak, it showed be stressed that all bird in this farm were found positive for ochratoxin and aflatoxin residues (Fig 5, 6 and 7).

Table 3 **:** The concentration range and mean concentration of aflatoxins in fresh prepared ration in (ppb) using HPLC and ELISA test.

| Methods used | No. of samples | No. of positive samples | concentration range in ppb | Mean concentration ± SD |
|---|---|---|---|---|
| HPLC techniques | 9 | 9 | 2.5 — 105.2 | 37.88±32.36 |
| ELISA test | 9 | 9 | 2.9 —178.33 | 35.24±29.07 |

Table 4: The concentration range and mean concentration of Ochratoxin in fresh prepared ration in (ppb) using HPLC and ELISA test.

| Methods used | No. of samples | No. of positive samples | concentration range in ppb | Mean concentration ± SD |
|---|---|---|---|---|
| HPLC techniques | 9 | 4 | 0.4 —— 2.6 | 0.78±1.12 |
| ELISA test | 9 | 8 | 0.4 —1.2 | 0.71±0.38 |

Table 5: The concentration range and mean concentration of aflatoxins in stored finished ration in (ppb) using HPLC and ELISA test.

| Methods used | No. of samples | No. of positive samples | concentration range in ppb | Mean concentration ± SD |
|---|---|---|---|---|
| HPLC techniques | 9 | 9 | 8.29—165.2 | 70.54±56.08 |
| ELISA test | 9 | 9 | 8.3 —178.33 | 55.32±50.32 |

Table 6: The concentration range and mean concentration of Ochratoxin in stored finished ration in (ppb) using HPLC and ELISA test.

| Methods used | No. of samples | No. of positive samples | concentration range in ppb | Mean concentration ± SD |
|---|---|---|---|---|
| HPLC techniques | 9 | 3 | 0.4—1.31 | 0.24±0.43 |
| ELISA test | 9 | 8 | 0.4 —1.5 | 0.77±0.43 |

Table 7: The concentration range and mean concentration of aflatoxins in ground nut cake in (ppb) using HPLC and ELISA test.

| Methods used | No. of samples | No. of positive samples | concentration range in ppb | Mean concentration ± SD |
|---|---|---|---|---|
| HPLC techniques | 9 | 9 | 5.08 —123.78 | 60.57±31.36 |
| ELISA test | 9 | 9 | 5 ——146.5 | 93.13±51. 26 |

Table 8: The concentration range and mean concentration of Ochratoxins in ground nut cake in (ppb) using HPLC and ELISA test.

| Methods used | No. of samples | No. of positive samples | concentration range in ppb | Mean concentration ± SD |
|---|---|---|---|---|
| HPLC techniques | 9 | 9 | 0.84 —2.6 | 0.48±0.88 |
| ELISA test | 9 | 8 | 0.4 ——1.5 | 1.3±0. 9 |

Table 9 : The concentration range and mean concentration of aflatoxins in sorghum samples (ppb) using HPLC and ELISA test.

| Methods used | No. of samples | No. of positive samples | concentration range in ppb | Mean concentration ± SD |
|---|---|---|---|---|
| HPLC techniques | 4 | 4 | 2.2—19.8 | 14.18±12.8 |
| ELISA test | 4 | 4 | 0.3—4.15 | 0.37±0.75 |

Table 10: The concentration range and mean concentration of Ochratoxin in sorghum samples (ppb) using HPLC and ELISA test

| Methods used | No. of samples | No. of positive samples | concentration range in ppb | Mean concentration ± SD |
|---|---|---|---|---|
| HPLC techniques | 4 | 3 | 1.3—2.81 | 1.62±1.2 |
| ELISA test | 4 | 3 | 0.3—1.9 | 0.83±0.85 |

Table 11: The concentration range and mean concentration of aflatoxins in maize samples (ppb) using HPLC and ELISA test.

| Methods used | No. of samples | No. of positive samples | concentration range in ppb | Mean concentration ± SD |
|---|---|---|---|---|
| HPLC techniques | 5 | 5 | 55.21—133 | 52.29±52.8 |
| ELISA test | 5 | 5 | 2.8 —86.02 | 23.27±20.8 |

Table 12: The concentration range and mean concentration of Ochratoxins in maize samples (ppb) using HPLC and ELISA test

| Methods used | No. of samples | No. of positive samples | concentration range in ppb | Mean concentration ± SD |
|---|---|---|---|---|
| HPLC techniques | 5 | 2 | 0.93— 4 | 2.4±2.1 |
| ELISA test | 5 | 5 | 0.7—3.9 | 2.12±1.19 |

Table 13: Concentration and mean concentrations of aflatoxins residues in liver (ppb) (µg/kg)

| Farm | NO. of positive samples | Concentration of Aflatoxin in ppb (µg/kg livers) | Mean concentration in ppb(µg/kg liver ) ±SD |
|---|---|---|---|
| A | 5 | 0.1,04,0.3,03,0.3, | 0.14±0.1 |
| B | 10 | 0.3,0.8,1.1, 1,1.1,0.7,1,0.5,0.8,0.4 | 0.74±0.2 |
| C | 10 | 1.7,0.5,0.4,1.8,0.7,1.9,1,0.5,0.8,1.4 | 0.97±0.5 |
| D | 10 | 1,1.7,1.8,1.7,2.2,2.8,0.8,2.4,1,1.4 | 1.68±0.6 |
| E | 2 | 0.4,0.1 | 0.05±0.2 |
| F | 7 | 0.9,1,1.6,0.5,0.8,1,0.9 | 0.67±0.3 |
| G | 4 | 0.6,0.7,0.2,0.8 | 0.23±0.2 |
| H | 6 | 0.7,1,1.5,1.1,0.9,1.4 | 0.76±0.3 |
| I | 10 | 1,1,1.6,1.5,2.1,2.5,2.2,1.5,2,1.9 | 1.73±0.4 |

**Fig 1: The mean concentration of Aflatoxin residues in liver in (ppb)in different farms using ELISA test**

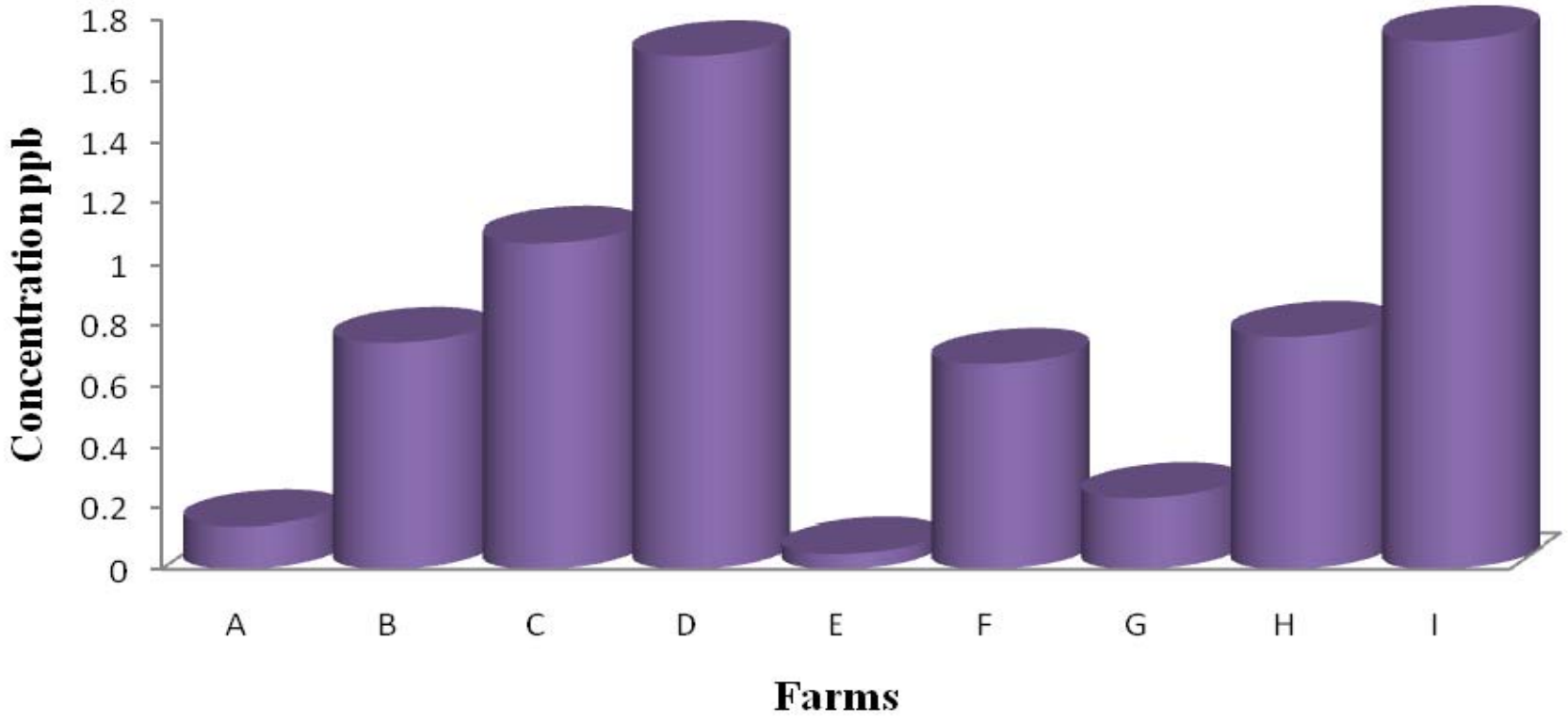

Table 14: Concentration and mean concentration of ochratoxin residues in kidney (ppb) (µg/kg)

| Farm | NO. of positive samples | Concentration of Ochratoxin ppb (µg/kg kidney) | Mean concentration ppb (µg/kg kidney) ±SD |
|---|---|---|---|
| A | 9 | 3.1,1.3,2.9,2.5,0.4,0.1,3.2,2.3,1.5 | 1.73±1.2 |
| B | 9 | 3.1,3.6,2, 2.3,3.3,4.5,4.6,4.6,2.7, | 3.03±1.4 |
| C | 10 | 2.1,3.6,2.3,1.1,1.9,1.5,2.8,0.1,1.8,2.3 | 1.78±0.75 |
| D | 10 | 2.6,2.2,3.7,0.9,1.3,2.1,2.1,2.9,4.2,0.7 | 2.27±1.1 |
| E | 10 | 0.6,0.1,0.1,0.8,0.8,0.4 ,0.3,0.1,0,7,1 | 0.49±0.3 |
| F | 9 | 2,1.5,1.2,0.6,1.9,1.1,2.2,1.8,0.9 | 1.32±0.67 |
| G | 10 | 2.6,2.1,2.7,2.2,3.7,1.2,1.6,0.7,1.7,0.1 | 1.86±1 |
| H | 9 | 0.9,0.3,1.7,0.7,1.8,0.6,4.2,1.1,0.4 | 1.17±1.1 |
| I | 10 | 0.4,3.3,1.1,1.9,3.9,2.2,1.2,3,3.6,0.2 | 2.08±1.3 |

**Fig 2 : The mean concentration of ochratoxin residues in kidneys in (ppb) in different farms using ELISA test**

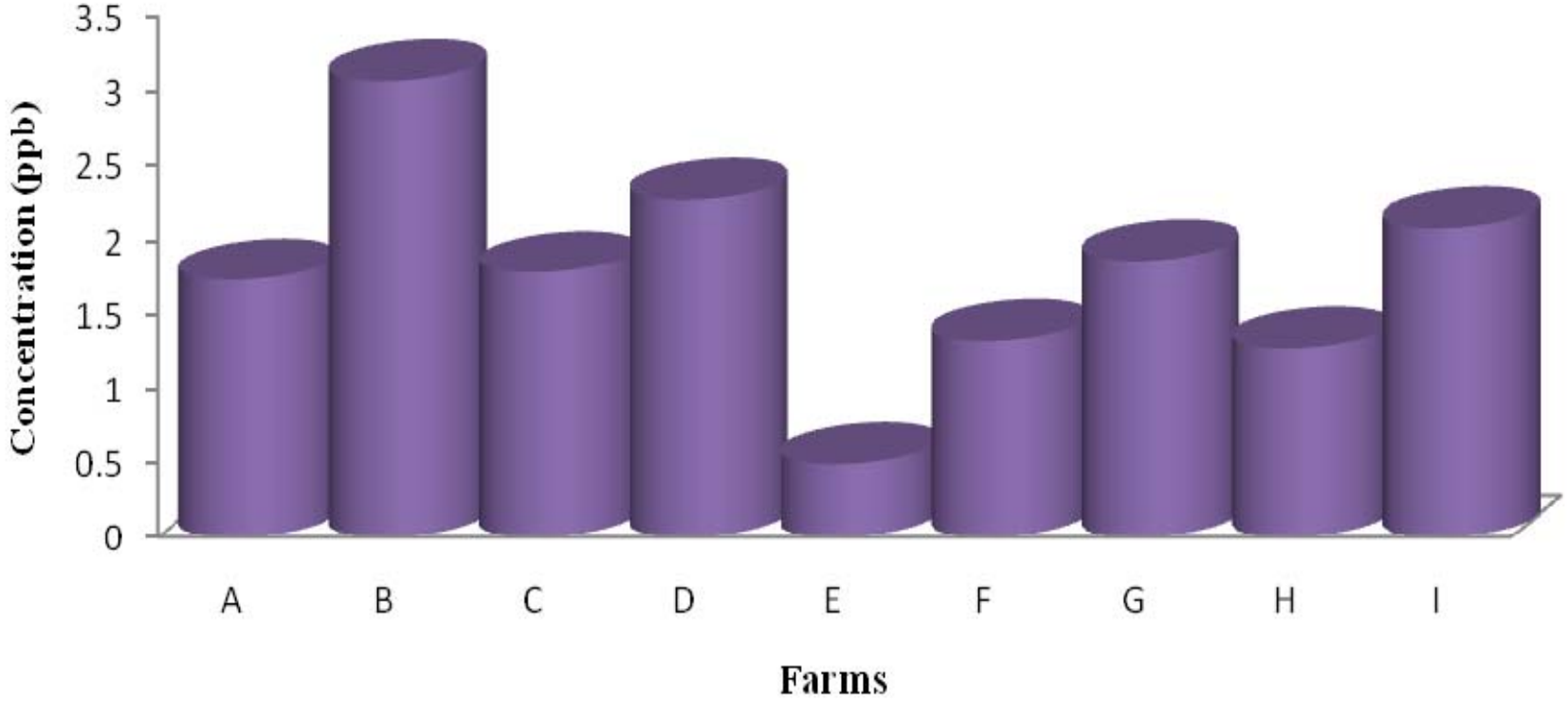

**Fig 3: Un developed comb and wattles and paralysis (45 days)**

**From farm (I), aflatoxin residue in liver was 1.73 ppb, ochratoxin residue in kidney was 2.8**

**Fig 4: Dullness of birds and reduction in body weight (45 days )**

**From farm (I), aflatoxin residue in liver was 1.73 ppb, ochratoxin residue in kidney was 2.8ppb**

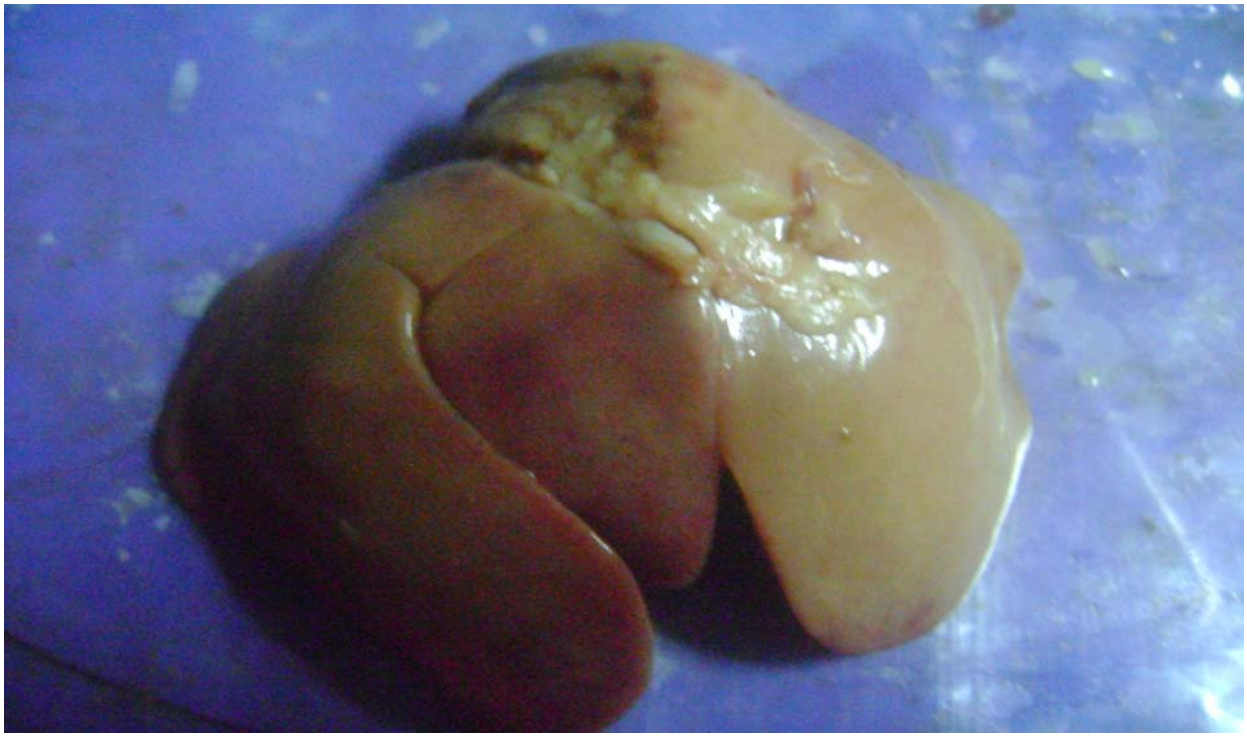

Fig: 5. Liver showing fatty change (note paleness of the right part)

(From farm (I), aflatoxin residue in liver was 1.73 ppb.

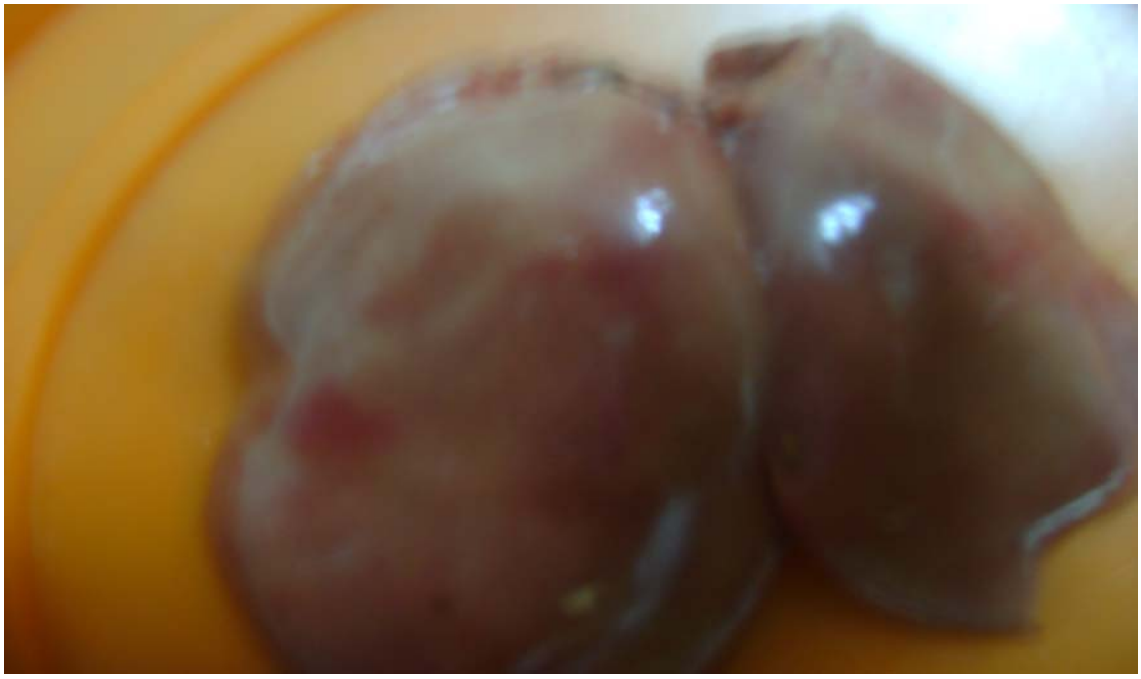

Fig: 6 .Liver showing wide areas of congestion and necrotic foci (From farm **(I), aflatoxin residue in liver was 1.73 ppb.**

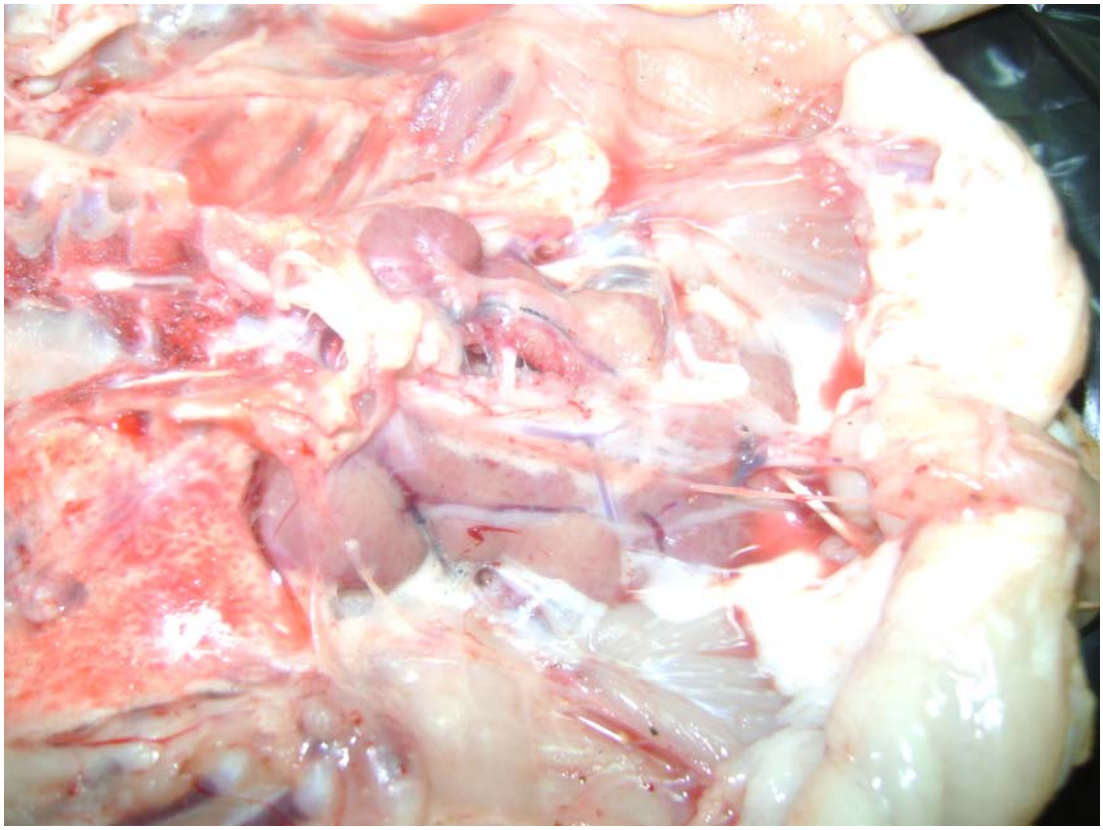

Fig 7: Kidney showing paleness and enlargement of the kidney **from farm (B) ochratoxin residue in kidney was 3.03 ppb**

# CHAPTER FOUR

# DISCUSSION

Aflatoxin and ochratoxin are two mycotoxins of current concern to animal and public health, and both produce severe economic and physiological effects in broiler chickens. Surveys of foods and feeds around the world have revealed that the problem of mycotoxicosis is not limited to any one geographic area but is a real or potential problem in all areas where moulds grow. In fact, virtually all staple food products consumed throughout the world are subject to contamination by mould toxins (Purchase, 1971). Some of the greatest difficulties in detecting mycotoxins in animal feed include their heterogeneous distribution in the raw and finished feed and inavailabilty of sensitive methods to detect low levels of these contaminant. In contrast, their direct detection and quantification in the birds themselves (in the plasma, liver, kidneys and muscles) confirms the existence of the problem and minimizes the errors involved in feed sampling and analysis (Furlan *et al.*, 2001).

Recent studies conducted in Khartoum state showed that mycotoxins contamination of poultry feed becomes a huge problem facing poultry production development in Khartoum state and Sudan at large (Elzupir, 2008, Mursal, 2009 and Babiker, 2009).

All feed samples examined from poultry farms in different localities in Khartoum state were found positive for aflatoxin, with concentration varying between (10- 97 ppb) and that 17 % of the livers examined were found to contain aflatoxin residues, the concentration of which varied between 2-12 ppb (Mursal, 2009). On the other hand Babiker, 2009 examined poultry and dairy feed samples and found that all samples were positive for one, two or three genera of *Aspergillus, Penicillium* and *Fusarium* which are the most important

fungal genera that contaminate the feed stuff with different potent mycotoxins e.g: Aflatoxin, Ochratoxin and trichothecene.

The aim of this study is to determine the occurrence of aflatoxin and ochratoxin in broilers feed and tissues. All feed samples examined in this study which included finished ration and raw material were found positive for aflatoxin. In fresh prepared ration the mean contamination concentration was 37.88ppb when measured by HPLC and mean concentration of 35.24ppb when determined by ELISA test, in contrast the stored finished ration showed mean concentration 70.54ppb measured by HPLC and mean concentration of 55.32ppb when ELISA test was used . The highest mean concentration was detected in stored finished ration and the lowest mean concentration was detected in sorghum samples 14.18 ppb when tested by HPLC and 0.37 ppb when tested by ELISA. These results were similar to those obtained by Mursal 2009. In this study the ground nut samples showed high mean concentration of contamination by the two methods of detection 60.57ppb and 93.13ppb and contamination level between 5.08ppb and 123.78 when detected by HPLC and contamination level between 5ppb and 146.5ppb when ELISA was used. In a surveys done in Malaysia and Philippines on poultry feed revealed that the concentration of aflatoxins was found to lie in the range of 0.8 – 762 ppb (Sulaiman et al. 2007) and 1-244 ppb (Ali, Hashim, and Yoshizawa, 1999) respectively. The range of the concentrations of aflatoxins in groundnut in this study was relatively narrow compared to the wide ranges obtained in the previous two studies. These differences could be attributed to the wide variation in climatic, environmental conditions and in differences in managerial levels in these countries.

This is the first study in Khartoum state to examine the occurrence of ochratoxin in poultry feed and tissues. Sixteen samples out of thirty six (44%) were found contaminated with ochratoxin A and concentration varied between 0.4-2.81ppb when tested by HPLC. On the other hand thirty two samples were

found positive when ELISA test was used, which constituted (88%) of the samples examined with concentration that varied between (0.3- 3.9ppb). Gumus *et al., (*2004) in Turkey found that 89 % of the feed samples examined were found positive for ochratoxin with a concentration varied between 0.53-12 ppb; these results were similar to our finding.

Dawlatana *et al., (*2008) in Bangladesh found the concentration of ochratoxin A in feed varied between 1-117ppb, this high concentration could be mainly due to tropical weather and poor management methods.

In these study the mean concentration of ochratoxin in sorghum samples 1.62ppb when detected by HPLC and 0.83ppb when detected by ELISA test in contrast the maize samples showed high mean contamination 2.4ppb and 2.1ppb. The high cost of sorghum in Sudan lead the producer to use the low cost material (maize) in animal feed. It was observed that the positive samples with high concentration were found in feed stuff containing maize as a main source of the feed stuff as compared to low concentration in feed containing sorghum. This is basically due to moisture content of maize which favour mould growth. The maize examined in this study was imported from South America and European countries exposed to bad storage condition over sea which favour mould growth. Sorghum is produced in Sudan and is used as fresh as possible. In a study conducted by Moretti *et al*., (1995), there was a little or no aflatoxins in sorghum and this agrees with our results that showed low levels of aflatoxins in sorghum (0.3 – 4.15 ppb) and (2.2 -19.8ppb) by the two method of detection

The two methods of detection showed different reading and it could be due to the heterogeneous distribution of the toxins in feed. The tissue samples showed aflatoxin residues in livers varying between 0.05ppb in farm E which is medium farm and 1.73ppb in farm I which is small farm and that may be due to poor storage practise in small farm regarding to small and medium farms and this

result is similar to that obtained by Mursal 2009. The mean concentration of ochratoxin residues in kidney varied between 0.46ppb in farm E and 3.03ppb in farm B which is medium farm that could be due to the samples collected during summer and autumn 2009 in which the temperature and humidity create suitable situations for mycotoxin production. There result agree with (Adler 2003) in his study showed that high moisture content, high relative humidity and warm temperatures enhance mould growth and toxin production. And also agree with (Elzupir 2008) who showed that level of aflatoxin increased in summer and autumn in Khartoum state compared to winter.

## Conclusion and Recommendation

In conclusion the amount of aflatoxin and ochratoxin in feed stuff especially finished ration, ground nut cake and maize are exceeded the maximum permitted level according to the FDA standards, which result in accumulation of considerable amount of aflatoxins in liver and ochratoxin in kidney. This evident posing dangerous problem to the poultry and human health.

- The (SSMO) Sudanese Standardization and Metrology Organization together with the Federal Veterinary Authorities should adopt regulations that limiting the amount of mycotoxin and help control it in animal feed
- Introduce analytical methods for monitoring imported mycotoxins that enter the food chain.
- Increase aware ness of producers and handlers and the public to hazards of mycotoxins

Further studies are needed to:

1. Examine the concentration of aflatoxin and ochratoxin in laying hens and their residues in broiler meat.
2. Investigate the occurrence of other mycotoxin in poultry feed stuff, tissues and their effect.

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