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ORIGINAL RESEARCH

Cytotoxic effects of aflatoxin B_1 standard in relation to aflatoxin extracts from South African compound feeds on human lymphocytes

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ABSTRACT

Cytotoxicity testing of aflatoxin (AF) on the viability of cells grown in cultures can be widely used to predict the potential toxic effects of AF in animals. To this end, an *in vitro* experimental study was conducted to ascertain the toxic effects of AF extracts obtained from compound feeds in South Africa on human lymphocytes in comparison to that of an AFB_1 standard. The approach adopted was on the basis of viable cells reducing methyl tetrazolium bromide (MTT) from blue to a purple formazan dye, which was then spectrophotometrically quantified to provide the rate of cytotoxicity. Data obtained indicated no cytotoxic response in control cells, as the viability of cells without treatment with AF standard or methanolic extracts of AF extracts (negative control) using methanol as the reconstituting solvent, was 99.9% after 24 hrs of incubation. However, cell viability significantly ($p<0.001$) decreased upon exposure to AF extracts especially for poultry feed. This was influenced by both the dose and duration of exposure, which was much more pronounced when the cells were exposed to AFB_1 standard than for all the AF extracts tested. This implies that these feeds on exposure to AF can greatly influence animal health with respect to both the contamination dose and exposure time.

Keywords: Aflatoxins, Cytotoxicity, Lymphocyte, Methyl tetrazolium bromide

1.0 Introduction

Aflatoxins (AFs) are a group of mycotoxins produced as secondary metabolites notably by *Aspergillius flavus* and *A. parasiticus*. Other species such as *A. nomius, A. bombycis, A. ochraceoroseus, A. pseudotamarii, A. tamarii,* and *A. australis* (Ito *et al*., 2001; Varga *et al.*, 2009) are also recognized producers. There are four main types of aflatoxins; aflatoxin B_1 (AFB_1), AFB_2, AFG_1 and AFG_2, owing to their colour (blue and green, denoted by the letters) and retardation factor (R_F) when viewed under UV light. The most important of these four toxins in terms of prevalence and abundance is AFB_1. Aflatoxin B_1 is also the most potent of all AFs and has been classified as a Class 1 carcinogen by the International Agency for Research on Cancer, IARC. This natural toxin is commonly reported to induce various health effects in humans and animal species; which could be hepatotoxic, mutagenic, genotoxic or carcinogenic (Wu, 2010). Chronic exposure to low levels of AF may well be a risk factor involved in the etiology of hepatocellular carcinoma and immune system suppression/dysfunction (Jiang *et al*., 2008; Wu *et al.,* 2011).

Typically, AFs as metabolites are not chemically similar to those essential metabolites (e.g. polyamine) found in cells. A peculiar problem with respect to their occurrence in compound feeds is the ability of the animal to assimilate high amounts into their system. This may result in decreased animal performance and even death. The presence of the toxin in the animal's system can be further complicated when its metabolized into animal products such as eggs, meat and milk for human consumption (Veldman *et al*., 1992).

Therefore, it is of principal importance that compound feeds (feedstuffs or animal feeds that are formulated from various raw materials and fortified with additives) are routinely screened to check for the presence and degree of AFs contamination.

There are several approaches to this, which include direct measurements of the toxins and screening of AFs-producing fungi that gives an indication of the likely AFs presence in a commodity. However, the later approach may only give an indication of the presence of the fungal strain, but not the presence of the toxin. The fact is, some potential AF producers may not produce the toxin under certain conditions (Frisvad *et al*., 2006). It might be that some strains may have died before they are screened, leaving the already produced toxins in the compound feed matrix (Vosough *et al*., 2010). Biologically, cytotoxicity testing of AFs on human lymphocytes against extracts of compound feeds is another approach to check for the presence of toxin contamination. This approach that has been used to detect toxicity in environmental systems, involves the use of target cells, rather than using animal models (Braydich-Stolle *et al.,* 2005). The test is rapid and cytotoxicity of the test material can be quantitatively expressed against that of an untreated control. Nevertheless, limitations are experienced in terms of false positive results. Although there is substantial financial investment in growing cell lines that have to be maintained under special aseptic working conditions (Pence, 2011). Apparently, due to the high expense, cytotoxicity-testing has been modified making use of cells available to laboratory workers i.e. blood cells, and in particular, lymphocytes. These can be used on a routine basis with ethical considerations and handled under sterile conditions. An advantage of this technique is that they do not require long maintenance periods under sterile conditions, unlike the case for cell cultures, due to shorter maturation times (Harry *et al*., 1998).

In this study, isolated and purified human lymphocyte cells were exposed to AFs extracts from compound feeds for investigation, as a biological means for testing the safety of these feeds.

2.0 Materials and Methods

2.1 Equipment and reagents

Unless otherwise stated, all chemicals used were of analytical grade. Tetrazolium salt [3-(4, 5-dimethylthiazol -2-yl) -2,5-diphenyltetrazolium bromide (MTT)], MTT assay kit, histopaque 1077, solvents and dimethylsulphoxide (DMSO) were purchased from Sigma Aldrich (St Louis, USA). Complete tissue culture medium (RPMI-1640 supplemented with 10% foetal calf serum (FCS) and L-glutamine were purchased from Promega Corporation (Madison, USA). Penicillin (100 U/ml) and streptomycin (100 µg/ml) were also Sigma, Aldrich. A humidified incubator (Incotherm – Labotec) was used and the temperature set at 37°C. The 96-well microtitre plates were obtained from Corning Cell Wells™ (Corning, USA). Additional materials and equipment included: Hank's Balanced Salt Solution (Adcock Ingram), AFB_1 standard (ARC, South Africa), ELISA microplate Reader (Modello: A2; Rome, Italy), centrifuge (Shalom S. Africa), haemocytometer (Shalom S. Africa) a phase contrast light microscope (Olympus B061, Wirsam Scientific, S. Africa), phytohaemagglutinin-p (PHA-p) (Sigma, Aldrich), penstrep-fungizone (Adcock Ingram), phosphate buffer saline (pH 7.4), trypan blue solution and fluorometer (Modello:A2, Rome, Italy). The PO7/V15/26.01.05 aflaprep kit with immunoaffinity column (Afla prep) was obtained from R-Biopharm Rhones Ltd (AG; Darmstadt, Germany).

2.2 Selection of aflatoxin extracts from South African compound feeds

The protocols of Candlish *et al*. (1998), for extracting and quantification of AFs in compound feeds, were adopted with some modifications in obtaining extracts for the present study. The milled sample (12.5 g) and 1 gram NaCl were weighed into a solvent resistant blender jar into which 62.5 ml methanol and distilled water (60:40, v/v) were added and blended for 60 s. The extract was filtered and diluted with distilled H_2O (62.5 ml), which was mixed thoroughly by swirling. Sample extract (25 ml) was passed through a filter paper (Whatman No. 4) and 10 ml of the filtrate obtained (equivalent to 1 g of sample) was passed through an immuno-affinity column at a flow rate of 2–3 ml/min, after which, the immuno-affinity

column was washed using 10 ml of phosphate buffered saline (PBS) at a flow rate of 5 ml/min. The analytes were then eluted (1 drop/s) using 1 ml of methanol and collected in an amber vial. Back flushing was employed thrice with the eluent to ensure complete release of AFs into the solution. The extract was dried in a fume cupboard using N_2 gas and stored at 0 °C until use for further analysis. Ten AF extracts from compound feeds, mainly for poultry and cattle, were used to perform the cytotoxicity study. Selection of the feeds was based upon most significant levels of AFs analysed in the study.

2.3 Cytotoxicity testing

Aflatoxin B_1 standard at concentration levels of 20, 40 and 80 µl/ml as well as AFs containing feed extracts at different concentration levels were tested *in vitro* to evaluate their effects on the viability of human lymphocytes after 24, 48 and 72 h of exposure. A one-way analysis of variance (ANOVA) was performed on the cytotoxicity data using a pairwise multiple comparison procedures (Holm-Sidak method). Cytotoxicity testing was done using venous blood from a healthy male volunteer after the University of Johannesburg's Clinic Ethics Committee granted ethical clearance. Blood was collected by venous puncture, using a 2 × 15 ml sterile syringe and immediately transferred into a 10 ml heparin tube. The blood was diluted with an equal volume of RPMI-1640. Ten millilitre of the mixture was layered onto 5 ml of histopaque in a 15 ml polypropylene conical tube and then centrifuged at 1,500 rpm for 30 mins at ambient temperature. The interface layer consisting of mononuclear cells was carefully removed using a sterile pipette and washed twice with Hank's Balanced Salt Solution (Adcock Ingram) by centrifugation at 4°C. About 20 µl of cell suspension, 80 µl of 0.2 % trypan blue solution were mixed in an Eppendorf tube and incubated for 2 mins at room temperature. Using a sterile Pasteur pipette, 10 µl of the trypan blue cell suspension mixture was then transferred to a haemocytometer chamber and covered with a cover glass slip. Viable and non-viable cells were counted: coloured (blue) cells were considered dead while uncoloured cells excluding the dye were considered viable. Viability was determined as:

$$\% \text{ Cell viability} = (\text{viable cell counted} / \text{total number of cells}) \times 100$$

The concentration and number of cells were calculated using the formula:

$$\text{Cell/ml} = (n / v) \times (5) \times 10^4$$

Where: n = number of cells counted

v = area of big squares counted x depth (0.1)

DF = dilution factor (10 µl of blood: 40 µl of Trypan blue) = 5

Mononuclear cells were transferred into a complete culture medium containing 1.5% L-glutamine, 10% foetal calf serum (FCS) and 1% penstrep (penicillin and streptomycin). Cells were seeded into 96 well plate containing methanol (used as a negative control), pure AFB_1 standard (used as a positive control) or AF extract reconstituted in methanol and incubated in a 5% buffered and humidified incubator for 48 hrs at 65 ^{0}C.

The cytotoxicity assay performed herein is a biological method to assess the quality of compound feeds as well as a confirmatory test for the AF contents in similar feeds obtained via HPLC as reported elsewhere (Iheanacho *et al*., 2012). In this regard, the potential cytotoxic effect of sample extracts on lymphocytes was assessed *in vitro* by 3, 4, 5-dimethylthiazol-2, 5-diphenyl tetrazolium bromide (MTT) assay as described by Meky *et al*. (2001). This method assesses the ability of cells to convert MTT to formazan crystals. 3-(4, 5-dimethylthiazol-2, 5-diphenyl tetrazolium bromide (50 mg) was dissolved in 10 ml of 0.14 M phosphate buffered saline (PBS) (pH of 7.4) and filtered through a Whatman No.1 filter paper. About 25 µl of 5 mg/ml MTT solution was added into each cell contained in the 96 well plate and gently shaken using a vibrating shaker (Wirsam Scientific, S. Africa). The contents were incubated for 2 hrs at 37°C in a 5% buffered and humidified incubator. Thereafter, 50 µl DMSO was added to each reaction and further incubated for 4 hrs to solubilize the formazan crystals formed. A microplate reader set at a wavelength of 620 nm was used to measure optical density (OD) values. The percentage of viable cells obtained after the assay was calculated as follows:

% Cell viability = [Mean OD values of treated cells */* Mean OD values of Control] × 100%.

2.4 Statistical analysis of data

A linear regression analysis was done on SigmaPlot 10.0 for Windows (Systat Inc., 2006). Data were then graphically represented. Among treatment groups, mean values were estimated to be different if the level of probability (p) was <0.05.

3.0. Results

It was observed that the viability of cells without any treatment with AFB_1 standard or extracts was 99.9%, 99.7% and 99.7% after 24, 48 and 72 h, respectively, of incubation (Figures 1, 2 and 3). The viability of cells after 24 hrs exposure was strongly influenced by the concentration of AFB_1 standard and sample extracts. This showed a higher reduction in cell viability when compared to the control or those of extracts with lower AFs contents as seen in Figure 1.

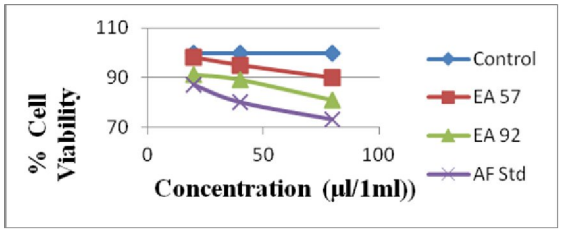

Fig 1. Toxic effects of aflatoxin extracts from compound feed samples and aflatoxin standard on human lymphocytes at 24 hours of exposure

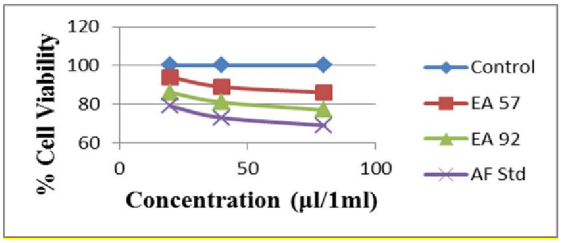

Fig 2. Toxic effects of aflatoxin extracts from compound feed samples and aflatoxin standard on human lymphocytes at 48 hours of exposure

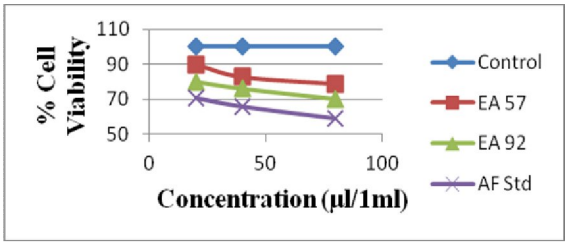

Fig 3. Toxic effects of aflatoxin extracts from compound feed samples and aflatoxin standard on human lymphocytes at 72 hours of exposure

Cell viability decreased significantly ($p<0.001$) over time due to continued exposure and increased AF dosage under the same conditions. The AFB_1 standard (80 µl/ml) used as a point of reference exhibited the greatest cytotoxic effect in causing cell mortality (73% cell viability recorded after 24 hrs of exposure), which increased over time (59% cell viability recorded after 72 hrs of exposure). The AFs extract concentrations from compound feeds, as determined by HPLC results which have already been submitted in another study (Iheanacho *et al.,* 2014) shows a relative correlation in respect to the determined cytotoxicity seen in this study, hence the relative cell percentage viabilities seen in Figure 1 and Tables 1, 2, 3.

4.0. Discussion

In this study, the cytotoxic effect of feed extracts containing AFs on cell viability of human lymphocytes cells in comparison to that of standard AFB_1 was performed *in vitro*. *In vitro* cytotoxicity testing initially described by Jelinek (1977) on chick embryos toxicity screening test was against toxic potentials of different chemicals but not on lymphocytes against AFs. Reports of Henry and Wyatt (2001) and Sehata *et al*. (2004) also established the toxicity of different chemicals which include anti-tumour drugs, antipyretics, antibiotics and ergot alkaloids on chick embryo and rat brain but not on lymphocytes against AFs. The toxico-pathological potential of AFs, both for the AFB_1 standard and AFs extracts from different compound feeds, to the lymphocytes was relatively high. This was observed as increased cytotoxicity (measured as decreased viability) as compared to the very low cytotoxicity posed to cells that received no treatment with AF standard

Table 1. Toxicity on Human Lymphocytes after 24 hrs

% Cell Viability ±SD				
Sample codes	**Compound feed types**	**20 µl/ml**	**40 µl/ml**	**80 µl/ml**
EA 92	Poultry	91±2.1	89±0.9	81±5.0
EA 57	Cattle	98±0.7	95±1.3	90±2.7
EA 83	Cattle	96±1.1	90±3.3	87±1.5
EA 26	Poultry	97±1.1	91±4.0	89±2.0
EA 91	Cattle	98±3.4	90±2.8	86±2.9
EA 70	Poultry	98±0.3	93±2.2	91±0.5
EA 88	Poultry	94±2.1	91±3.0	89±3.9
EA 78	Cattle	99±5.3	96±2.7	90±1.1
EA 21	Poultry	95±2.1	91±4.2	89±0.9
EA 34	Poultry	97±4.4	92±3.0	85±2.7
AF B_1		87±2.1	80±0.9	73±1.3

EA = Sample Code, AF Std = Aflatoxin standard, SD= Standard Deviation

Table 2. % Cell viability of human lymphocytes induced by AF extracts in feeds after 48 hrs of exposure

% Cell Viability ±SD				
Sample codes	**Compound feed types**	**20 µl/ml**	**40 µl/ml**	**80 µl/ml**
EA 92	Poultry	86±0.7	81±1.1	77±2.0
EA 57	Cattle	94±2.1	89±2.8	86±2.8
EA 83	Cattle	94±1.3	89±0.9	82±1.2
EA 26	Poultry	89±1.1	83±2.0	79±1.9
EA 91	Cattle	92±0.9	87±2.3	81±3.5
EA 70	Poultry	95±3.3	88±4.8	83±3.0
EA 88	Poultry	90±5.3	84±1.1	80±1.1
EA 78	Cattle	87±0.9	82±2.5	79±2.7
EA 21	Poultry	91±2.7	87±3.1	81±3.2
EA 34	Poultry	90±1.5	85±2.5	79±2.9
AF Std		79±1.3	73±2.0	69±2.2

EA = Sample Code, AF Std = Aflatoxin standard, SD= Standard Deviation

Table 3. Toxicity on Human lymphocytes after 72 hrs

% Cell Viability ±SD				
Sample codes	**Compound feed types**	**20 µl/ml**	**40 µl/ml**	**80 µl/ml**
EA 92	Poultry	80±3.7	76±0.7	70±2.5
EA 57	Cattle	90±2.2	83±1.9	79±4.1
EA 83	Cattle	89±4.1	81±2.0	77±2.8
EA 26	Poultry	82±2.5	79±2.0	72±3.3
EA 91	Cattle	88±2.1	81±2.5	75±2.1
EA 70	Poultry	87±3.0	83±4.1	78±3.5
EA 88	Poultry	87±2.8	80±3.6	73±0.8
EA 78	Cattle	84±2.2	79±0.9	76±2.4
EA 21	Poultry	89±3.0	84±2.1	77±1.2
EA 34	Poultry	85±2.7	79±2.0	71±3.7
AF B_1		71±0.9	66±0.6	59±0.5

EA = Sample Code, AF Std = Aflatoxin standard, SD= Standard deviation

or extract. A further decrease was observed over time as exposure dose increased. In overall, AFB_1 standard revealed the highest toxicity as expressed by the lowest percentage lymphocyte viability when compared to that for feed extracts which also exhibited some but lower cytotoxicity tendencies. This agrees with the reports of Bünger *et al*. (2004) and Meky *et al.* (2001) in which human cell mortality increased with increasing AFs levels. Our study further reveals the implication of duration in exposure. Given the high dietary intake of AFs which led to many outbreaks of aflatoxicosis in animals reported in South Africa (Arnot *et al*., 2012; Otto, 2011; Van Halderen *et al*., 2000) and other parts of the world (Akinrinmade and Akinrinde, 2012; Thomas *et al.*, 2010). This possible cause might be linked to the ability of this toxin causing immunosuppression as an initial step of immunomodulation due to loss of cells (Forsell *et al.*, 1985). This immunosuppressive effect can render the animals susceptible to secondary infections; pre-disposing chickens to candidiasis, Marek's disease, coccidiosis and salmonellosis (Robens and Richard, 1992), pigs to erysipelas and salmonellosis and cattle to fascioliasis, intramammary and clostridial infections (Smith and Moss, 1985).

Generally, aflatoxins, mainly AFB_1, are metabolized by the enzyme cytochrome P-450 enzyme (CYP450) to a toxic AFB_1-epoxide that binds to cellular DNA, RNA and protein forming an adduct that possibly results in the carcinogenic effect of AFB_1 in both animals and humans (Ayub and Sachan, 1997). Another mechanism of action of AFs involves the inhibition of DNA synthesis (El Khoury *et al*., 2011), which may result in aflatoxicosis in animals with vague initial presentations (Dixon *et al*., 2008). It is known, that T lymphocytes play a pivotal role in the immune system, being responsible for immune responses, acting as a natural defence mechanism against host invasion of diseases (Størmer and Lea, 1995). This may lead to a gradual but harmful onset of a disease condition. Some signs and symptoms due to such secondary infections may be lethargy and anorexia. Sometimes expressions like haematochezia, melena and haematemesis (Tedesco *et al.*, 2004) are present as disease condition, and widespread petechiae and ecchymoses (Galvano *et al*., 2001) may occur. Some animals may present with peripheral oedema or ascites, polyuria and polydipsia (Wilson, 2010) in the progressive stages of the disease. Though fatality rate due to aflatoxicosis is high, its signs are also consistent with liver failures that are specific for AFs aetiology. However, the toxicity of the AFs extracts is lower than that of AF standard. One of the reasons may be due to the chemical compositions of the feed samples. Another reason could also be possibility of breakdown into residues. Animal feed consists of complex adjuncts and matrice which interfere with extraction and purification of the toxins to obtain up to 99.5% purity during laboratory analysis. It was therefore not unexpected that the pure aflatoxin standard would exert more damage to the lymphocytes. This does not in any sense downplay the cytotoxic potential of the crude hepatotoxin *in vivo*, especially over a period of time. Therefore, continued ingestion of the contaminated feeds may be risky to the animals.

5.0. Conclusion

The analysis of compound feeds from South Africa for AFs presence and contamination, using the MTT assay pre-analysis screening, showed feed samples with residual toxicity. The toxicity of AFB_1 standard on human lymphocytes was compared to that of compound feed fraction extracts and adequate comparability of the results were observed. It was not, however, possible to deduce the biochemical mode of action of the AFB_1 standard and AFs extract based on the observed reduction in cell viability potentiated by either the standard or extracts. Nevertheless, cyctotoxicity results obtained showed a high through-put, which confirms the sensitivity of the test performed to an extent in the detection of AFs contained in compound feeds. This makes it a useful biological screening tool to ascertain the quality of compound feeds.

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ORIGINAL RESEARCH

Haematological Response of Growing West African Dwarf Goats to Micro Doses of Dietary Aflatoxins

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ABSTRACT

An experiment was conducted with 20 West African Dwarf male goats (4-5 months old) averaging 8.4±1.04kg to assess haematological response of the animals to varied levels of dietary aflatoxin of 0, 50, 100 and 150 µg aflatoxin/kg in diets 1 (control), 2, 3 and 4 respectively. The animals were randomly divided into four treatments with each treatment having five goats housed individually in a completely randomised design for a period of 4 weeks. Blood samples were collected from the animals through the jugular vein, at day 0, 14 and 28 during the feeding trial, for haematological studies. Results obtained showed that, there were no significant differences among the haematological indices of all animals examined at day 0. At day 14, Packed Cell Volume (PCV), Red Blood Cell (RBC), White Blood Cell (WBC), Haemoglobin (Hb), leukocyte differential counts and platelets of goats fed treatments 2, 3 and 4 were not significantly different from goats fed the control diet. However, Mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) of goats fed diets 3 and 4 were significantly ($P<0.05$) higher than the control. At day 28, RBC, Hb, MCV and MCH significantly ($P<0.05$) increased as the level of the toxin increases in the diets. There were apparent decrease in WBC, its differential counts and platelets in goats on treatments 2, 3 and 4 compared to the control at days 14 and 28. These results suggest that aflatoxin contaminated diets fed to bucks at 100µg/kg altered haematological variables at day 28 of dietary exposure of the animal to the diets. The effects elicited on the animals are dose-dependent and was also influenced by the duration of exposure.

Keywords: Feed contaminants, Dietary aflatoxins, Haematology, West African Dwarf goats,

1.0 Introduction

Mycotoxins are toxic, chemically diverse secondary metabolites produced by a wide range of fungi. According to Pitt (1996), mycotoxins are defined as 'fungal metabolites which when ingested, inhaled or absorbed through the skin cause lowered performance, sickness or death in man or animals, including birds. Infections by mould and subsequent mycotoxin production can develop at various stages of crop production: in the field, during harvesting and transportation or storage (Sultana and Hanif, 2009). Of all mycotoxins, aflatoxins probably cause the most concern (CAST, 2003). This is due to their carcinogenic and immune suppressing effects in both humans and domestic animals (Turner *et al*., 2003) and economic losses due to significant reductions in export value (Wu, 2006). Aflatoxins are one of the most potent toxic substances that occur naturally. These are a group of closely related mycotoxins produced by fungi *Aspergillus flavus* and *A. Parasiticus*. Aflatoxin contamination can occur in a wide variety of feedstuffs including corn, sorghum, barley, rye, wheat, peanuts, soya, rice, cottonseed and various derivative products made from these primary feedstuffs (Busby and Wogan, 1979). In Nigeria, comparism of aflatoxin production among agro-ecological zones showed that aflatoxin B_1 was significantly higher in Southern Guinea Savannah than in Northern Guinea Savannah, and intermediate in maize samples from the Derived Savannah agro-ecological zone (Atehnkeng *et al*., 2008). Ruminants such as cattle, sheep, and goats are less known for their sensitivity to the negative effects of mycotoxin than non ruminants. Ruminants are exposed to mycotoxin in growing pasture (fungal contamination of grasses), silage,

hay and straw (including bedding), concentrate feed formulated with contaminated grains. Mycotoxins affect ruminants by reducing feed consumption, reducing nutrient utilization, altering rumen fermentation, suppressing immunity, altering reproduction, irritating tissues, and causing cellular death. However, production (milk, beef, or wool), reproduction and growth can be altered when ruminants consume aflatoxin-contaminated feed for extended periods of time (Hussein and Brasel, 2001). Confusion results when consultants attempt to discuss the impact of aflatoxins on the ruminant animal by relying upon the research findings of feeding infected grains to monogastrics. As a result of toxic effect of aflatoxin, biochemical and haematological parameters have been reported to be affected importantly. Concentrate supplementation in ruminant diet is essential to improve performance of the animals. However, aflatoxin contamination can jeopardise the objective. Thus, understanding West African Dwarf goat response to aflatoxin intoxication prompted the design of this experiment.

2.0 Materials and Methods

2.1 Experimental materials and feeding trial

Aspergillus flavus cultured maize grains containing aflatoxin was generated at the Plant Pathology Laboratory, International Institute of Tropical Agriculture (IITA), Ibadan. Aflatoxins were quantified using scanning densitometer, CAMAG TLC Scanner 3 with win- CATS 1.4.2 software (Camag AG, Muttenz, Switzerland) as described in Suhagia *et al*. (2006). The aflatoxin contaminated maize was substituted for non-cultured maize in various proportions to formulate four treatment diets containing approximately 0, 50, 100, and 150 µg Kg^{-1} aflatoxin, constituting treatments 1 (control), 2, 3 and 4, respectively. Twenty 4-5 months old West African Dwarf male Goats weighing averagely 8.4±1.04 kg were allotted randomly into the four treatments. There were 5 animals per treatment, housed individually across the treatments in a 4-week feeding trial. The animals were fed forage and concentrate (60:40 respectively).The gross composition of the concentrate diets is as shown in Table 1. The animals were fed their respective diets *ad libitum* daily. Fresh potable water was made available throughout the experimental period.

2.2 Blood collection and evaluation

At the beginning of the feeding trial, blood samples were collected from the animals, and at 14 days interval throughout the experimental duration. The bucks were bled through the jugular vein using a sterilized disposable syringe and needle for each animal into, vaccutainer tubes, containing a calculated amount of ethylene diamine tetraacetic acid (EDTA) as anticoagulant for haematological study. The Red Blood Cell (RBC) counts, total White Blood Cell (WBC) counts, leukocyte differential counts, platelets, haemoglobin (Hb) concentration and Packed Cell Volume (PCV) were determined as described in Ewuola and Egbunike (2008). Blood constants (MCH, MCV, MCHC) were determined using appropriate formulae as described by Jain (1986).

Table 1: Gross composition (%) of concentrate diet for the experimental animals

Ingredients	T1 (0 µg/kg)	T2 (50 µg/kg)	T3 (100 µg/kg)	T4(150 µg/kg)
Uncontaminated maize	55.00	51.67	48.34	45.01
Contaminated maize	0.00	3.33	6.66	9.99
Wheat offal	28.00	28.00	28.00	28.00
Brewer dried grain	15.00	15.00	15.00	15.00
Di-calcium phosphate	1.00	1.00	1.00	1.00
Grower premix	0.50	0.50	0.50	0.50
Salt	0.50	0.50	0.50	0.50
Total	100.00	100.00	100 .00	100 .00
Calculated Nutrient (%DM)				
Crude protein (%)	12.87	12.87	12.87	12.87
DE (kcal/kg)	3572.69	3572.69	3572.69	3572.69
NDF	25.43	25.43	25.43	25.43
ADF	9.01	9.01	9.01	9.01

DM- dry matter DE- Digestible Energy; NDF- Neutral Detergent Fibre; ADF- Acid Detergent Fibre

Table 2: Haematological parameters of WAD goats at the commencement of the experiment (day 0)

PARAMETERS	T1 (0µg/kg)	T2 (50µg/kg)	T3 (100µg/kg)	T4 (150 µg/kg)	SEM	NORMAL RANGE*
Packed Cell Volume (l)	0.25	0.24	0.27	0.27	0.01	0.22-0.38
Red Blood Cells ($x10^{12}$/l)	11.18	10.52	10.57	10.86	0.25	8.0-18.0
White Blood Cells ($x10^{9}$/l)	12.41	12.44	10.76	12.46	0.91	4.0-13.0
Haemoglobin (mmol/l)	5.09	5.01	5.58	5.67	0.23	4.96-7.45
Mean Cell Volume (fl)	21.93	23.36	26.25	25.22	1.27	16.0-25.0
Mean Cell Haemoblobin (fmol)	0.45	0.48	0.54	0.52	0.03	0.32-0.50
Mean Cell Haemoglobin Concentration (x10 g/l)	33.32	33.22	33.29	33.36	0.02	30.0-36.0
Lymphocyte ($x10^{9}$/l)	7.60	5.43	7.20	6.04	0.53	2.0-9.0
Monocyte ($x10^{9}$/l)	0.15	0.29	0.32	0.277	0.04	0.0-0.55
Eosinophils ($x10^{9}$/l)	0.21	0.25	0.27	0.24	0.04	0.05-0.65
Neutrophils ($x10^{9}$/l)	4.45	6.47	2.97	5.90	0.92	1.2-7.2

SEM: Standard error mean. *Duncan and Prasse (1986)

2.3 Data Analysis

The data obtained from the haematological response of the experimental animals were subjected to Analysis of Variance of statistical analytical system (SAS, 2003) at p = 0.05

3.0 Results

The haematological parameters of WAD bucks fed dietary aflatoxin at day 0, 14 and 28, are shown in Tables 2, 3 and 4. There were no significant differences among the haematological indices of all animals examined at day 0 and all parameters examined were within the normal range reported for goats by Duncan and Prasse (1986). This ascertained that all the animals were healthy at the commencement of the feeding trial. At day 14, PCV, RBC, WBC, Hb, leukocyte differential counts and platelets of goats fed treatments 2, 3 and 4 were not significantly (*P>0.05*) different from goats fed the control diet. However, MCV and MCH of goats fed diets 3 and 4 were significantly ($p<0.05$) higher than those fed diet 3 and the control diet with the least value recorded in goats fed control diet. There were apparent increase in PCV and Hb of goats on treatments 2, 3, and 4 compared to the controls.

Table 3: Haematological response of WAD goats to varied levels of dietary aflatoxin at day 14

PARAMETERS	T1 (0µg/kg)	T2 (50µg/kg)	T3 (100µg/kg)	T4 (150 µg/kg)	SEM	NORMAL RANGE*
Packed Cell Volume (l)	0.19	0.22	0.28	0.27	0.16	0.22-0.38
Red Blood Cells ($x10^{12}$/l)	11.95	11.14	11.26	11.24	0.47	8.0-18.0
White Blood Cells ($x10^{9}$/l)	13.55	10.77	10.05	10.95	0.96	4.0-13.0
Haemoglobin (mmol/l)	3.88	4.48	5.74	5.64	0.33	4.96-7.45
Mean Cell Volume (fl)	15.73^{b}	19.52^{ab}	24.98^{a}	24.29^{ab}	1.58	16-25
Mean Cell Haemoblobin (fmol)	3.25^{b}	4.04^{ab}	5.17^{a}	5.02^{ab}	0.33	5.2-8.0
Mean Cell Haemoglobin Concentration (x10 g/l)	33.25	33.34	33.33	33.32	0.14	30-36
Lymphocyte ($x10^{9}$/l)	7.58	4.31	5.78	6.10	1.01	2.0-9.0
Monocyte ($x10^{9}$/l)	0.32	0.32	0.23	0.21	0.03	0.0-0.55
Eosinophils ($x10^{9}$/l)	0.20	0.17	0.16	0.17	0.04	0.05-0.65
Neutrophils ($x10^{9}$/l)	5.46	5.96	3.88	4.47	0.45	1.2-7.2
Platelets ($x10^{9}$/l)	250.75	199.33	164.50	199.00	19.45	300-600

ab: means in the same row with different superscript are significantly *(P<0.05)* different. SEM: Standard error mean, *Duncan and Prasse (1986)

There were apparent decrease in WBC, Its differential counts and platelets in goats on treatments 2, 3 and 4 compared to the control. At day 28, RBC, Hb, MCV and MCH of goats across the treatments were significantly ($p<0.05$) influenced by the toxin. The Hb, MCV and MCH values obtained for goats on treatment 4 were significantly (*P<0.05*) higher than those on control diet, but were not significantly different from goats fed treatments 2 and 3. Goats on control diet had significantly (*P<0.05*) highest RBC values, and was significantly (*P<0.05*) different from goats on treatment 2 which had the least values.

4.0 Discussion

Haematological indices are a reflex of the effect of diets on the physiological status of animals. Report by Aletor and Egberongbe (1992) and Aletor (1989) indicated that the blood variables most consistently affected by dietary influences include RBC counts and PCV, plasma protein and glucose. In this study, aflatoxin contaminated diets fed to goat bucks at varied concentrations influenced RBC, Hb, MCV and MCH significantly. The values obtained from these parameters increased with increase in aflatoxin concentration and also with the duration of exposure to the diets. The response by the animals may probably be an indication of macrocytic anemia, which could be due to responsive simulations of the bone marrow, or impaired synthesis of vitamin B_{12}. The result obtained in this study corroborates Clark *et al*. (1984) that observed increases in total RBC counts, PCV, haemoglobin concentration in goats fed 0.1ppm to 0.4 ppm for 34 days, and suggested that onset and magnitude of increases in PCV, haemoglobin concentration were dose-related. The result was at variance with the report of 27 kg lambs fed 2ppm aflatoxin for 37 days, in which no significant effect was observed in haematological variables (Fernaindez *et al*., 2000), suggesting that species of animal differ in response to aflatoxin (Howard and David, 1990). Thrombocyte, monocyte, lymphocyte and neutrophil counts in WAD bucks tended to decline apparently with increase in the aflatoxin level in the diets. The finding of this study corroborates previous findings (Agag, 2004; Mohiuddin *et al*., 1986), that aflatoxin affected thrombocyte and phagocyte cells in chickens. Aflatoxin has been reported to alter extrinsic and common clotting pathway in animals by causing biochemical changes in thromblastin clotting factors and reduced plasma prothrombin and fibrinogen (Doerr *et al*., 1976), due to impaired hepatic synthesis of clotting factors caused by toxicity of aflatoxin on the liver cells (Huff *et al*., 1983). Results obtained for WBC and its differential counts suggest degenerative left shift in which there is falling total leukocyte and differential counts. This may probably be as a result of alteration in bone marrow due to degeneration, depression, depletion or destruction of bone marrow induced by the toxin.

Table 4: Haematological response of WAD goats to varied levels of dietary aflatoxin at day 28

PARAMETERS	T1 (0µg/kg)	T2 (50µg/kg)	T3 (100µg/kg)	T4 (150µg/kg)	SEM	NORMAL RANGE*
Packed Cell Volume (l)	0.25	0.33	0.30	0.26	0.02	0.22-0.38
Red Blood Cells ($x10^{12}$/l)	13.22^{a}	10.55^{b}	12.44^{a}	11.88^{ab}	0.34	8.0-18.0
White Blood Cells ($x10^{9}$/l)	12.57	13.60	10.73	10.46	0.98	4.0-13.0
Haemoglobin (mmol/l)	3.86^{b}	4.27^{ab}	5.02^{ab}	5.89^{a}	0.33	4.96-7.45
Mean Cell Volume (fl)	15.92^{b}	17.55^{ab}	21.39^{ab}	25.74^{a}	1.57	16.0-25.0
Mean Cell Haemoblobin (fmol)	3.30^{b}	3.63^{ab}	4.42^{ab}	5.32^{a}	0.32	5.2-8.0
MCHC (x10 g/l)	33.32	33.34	33.33	33.34	0.003	30.0-36.0
Lymphocytes ($x10^{9}$/l)	5.73	5.95	3.99	4.12	0.48	1.2-7.2
Monocytes ($x10^{9}$/l)	6.37	7.01	6.29	6.00	1.02	2.0-9.0
Eosinophils ($x10^{9}$/l)	0.31	0.35	0.29	0.18	0.03	0.0-0.55
Neutrophils ($x10^{9}$/l)	0.15	0.28	0.16	0.17	0.04	0.05-0.65
Platelets ($x10^{9}$/l)	236.67	162.67	263.50	170.25	18.76	300-600

ab: means in the same row with different superscript are significantly *(P<0.05)* different. SEM: Standard error mean; MCHC: Mean Cell Haemoglobin Concentration

*Duncan and Prasse (1986)

5.0 Conclusion

Based on the results of this study, It can be concluded that micro doses of dietary aflatoxin in goats will have mild effect on the animal's immune response and health status within 28 days of dietary exposure.

Conflict of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Mycotoxin awareness amongst traders and farmers in Ekiti State, Nigeria

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ABSTRACT

Mycotoxins are secondary metabolites produced by microfungi that are capable of causing disease and death in humans and other animals. The present work is a preliminary study focused on mycotoxin awareness amongst traders and farmers in some local government areas of Ekiti State. A questionnaire on mycotoxin awareness was distributed to two hundred respondents in each of the four local government areas visited. Simple percentages were used to analyse data collected. The data obtained showed that in Ekiti State Nigeria, the people are ignorant of mycotoxins produced by moulds but are aware of the existence of fungi. The general finding was that the people consumed some foods with mould growth so long as the taste did not change. In view of the data obtained, it is hereby suggested that studies should be carried out to ascertain the mycotoxin content of food and feedstuff in these areas. It was concluded that an intensive effort towards awareness is necessary at the grass root level in order to inform the public and as such reduce the incidence of mycotoxins in food and feed stuff and subsequent transfer to man.

Keywords: Mycotoxins, Traders, Farmers, Ekiti

1.0 Introduction

Mycotoxins are toxic compounds produced by different types of fungus, belonging mainly to the *Aspergillus*, *Penicillium* and *Fusarium* genera. Under favourable environmental conditions, when temperature and moisture are conducive, these fungi proliferate and may produce mycotoxins. They commonly enter the food chain through contaminated food and feed crops, mainly cereals. The presence of mycotoxins in food and feed may affect human and animal health as they may cause many different adverse health effects such as induction of cancer and mutagenicity, as well as estrogenic, gastrointestinal and kidney disorders. Some mycotoxins are also immunosuppressive reducing resistance to infectious disease (EFSA, 2014).

Toxins are naturally produced by all types of moulds and fungi. Hundreds of these mycotoxins exist, and, contamination of natural materials with multiple toxins, either from one or several fungi, is common. Examples of mycotoxins of imporatnce are: Aflatoxins, ochratoxins A, Patulin, Zearalenoene, Fumonisins, T-2 toxins, Deoxynivalenol, etc.

Toxins are especially produced under certain conditions including the following:

i. Host plant stress in the field: poor soil fertility, insect damage, high/low temperatures or moisture
ii. Harvesting: late harvesting, dry crops, slow storage filling e.g. silage clamp, soil contamination
iii. Storage: wet grain, poor silage packing, incorrect fermentation of ensiled products
iv. Finished feeds and forages: poor hygiene, exposure to air/ moisture, incorrect storage (temperature/ moisture) (knowmycotoxins.com 2014)

Mycotoxins appear in food as a result of fungal infection of crops in store and in the field. Infection of humans occurs either by eating the infected crops directly or by using the crops as livestock feed. Detection of mycotoxins in food have been reported by many Researchers. For instance Corn cakes were recalled in

France following detection of Mycotoxins (Haccpeuropa 2014). Using data supplied by France, EFSA estimates that a temporary increase in the levels of three mycotoxins – deoxynivalenol, fumonisins and zearalenone – in maize and maize products is unlikely to impact significantly on public health. However, for some consumers currently estimated total exposure from all sources (including other crops) is already close to what is considered a safe level (EFSA, 2014). Atanda *et al .,* 2013 have reported high incidence of in Nigeria. Makun *et al* (2010) reported Fungal and aflatoxin contamination of some human food commodities in Nigeria. All these reports meet with no reaction from the populace because they are not aware of mycotoxins and as reported by Atanda *et al* (2013) lack of awareness of the dangers posed by mycotoxin contamination of produce is a major factor responsible for its high incidence in Nigeria. Majority of farmers produce and food handlers and/or processors are illiterate with virtually no knowledge of the implications of toxigenic mould growth.

2.0 Materials and Methods

2.1 Description of the Study Area

Ekiti state is located on 7°N 40¹N 5°15¹E. It is in South West Nigeria. The state enjoys tropical climate with two distinct seasons, these are the rainy season (April-October) and the dry season (November-March). Temperature ranges between 21°and 28°C with high humidity. Tropical forest exists in the south while savanna occupies the northern peripheries. Ekiti is an Agrarian state and the major crops produced are maize, rice, yam, cassava, cash crops like cocoa and kola nuts are also produced. The major occupation in the area is trading and farming. The farming is majorly for subsistence except in cases where the individual has inherited cocoa and kola nut trees from the family. There are 18 local Government areas out of which four were considered in this preliminary study. These four were chosen because they are cosmopolitan. There are Tertiary institutions in these study areas.

2.2. Sample and sampling methods

The sampling method for this study was purposive in which case a questionnaire meant to elicit responses on the level of awareness on mycotoxins was distributed randomly. A total of 800 persons irrespective of sex were used of which 200 respondents were drawn from each of Ado-Ekiti, Ikere, Ido-Osi and Ekiti West local government areas of Ekiti state. Where necessary the questionnaires were interpreted in the local language for easy understanding. The responses were calculated using simple percentages.

3.0. Results

The result obtained revealed that in Ekiti State Nigeria, people of age group 18-39 were more available in the market places and farm settlements visited followed by the age group 40-60(Table 1). More females were seen in the course of the survey especially in the market places since the trading activities are left mostly for women (Table 2).

Table 1: Ages of Respondents and their frequencies of occurrence in the Local Government areas

	Local governments and occurrence(%) of the age groups			
Age	**Ado-Ekiti**	**Ekiti-West**	**Ikere**	**Ido-Osi**
18-40	52	54	54	54
40-60	32	28	32	28
60 and above	16	18	14	18

Table 2: Sex of respondents

	Local governments and occurrence(%) of the sexes			
Gender	**Ado-Ekiti**	**Ekiti-West**	**Ikere**	**Ido-Osi**
Male	20	42	30	44
Female	80	58	70	56

Table 3: Respondents with knowledge of Fungi

	Occurrence(%) of the sexes in the local government areas			
Heard of Fungi	**Ado-Ekiti**	**Ekiti-West**	**Ikere**	**Ido-Osi**
Yes	90	70	82	33
No	10	30	18	67

Table 4: Respondents that eat food covered with Fungi

	Occurrence(%) of respondents in the local government areas			
Can eat food with Fungi?	**Ado-Ekiti**	**Ekiti-West**	**Ikere**	**Ido-Osi**
Yes	14	30	38	26
No	86	70	62	74

In Ado-Ekiti 90% of the respondents had heard of fungi while 20% had not. In Ekiti west 70% had heard of fungi while 30% had not, in Ikere – Ekiti 82% had heard of fungi while 18% had not while in Ido-Osi 33% had heard of fungi while 67% had not (Table 3). Many of the respondents will eat food covered with moulds so long as the taste does not change. The result obtained therefore revealed that in Ado-Ekiti 14% of the respondents said they will eat food covered with fungi while 86% will not while in Ekiti West 30% will eat food covered fungi while 70% will not. 38% of respondent in Ikere-Ekiti will eat food covered with fungi while 62% will not while in Ido-Osi 26% of respondents will eat food covered with fungi while 74% will not(Table 4)

Many people are still ignorant of mycotoxins and even the fungi that produce them.. The result of the survey showed that In Ado-Ekiti 54% of respondents had heard of mycotoxins while 46% respondents had not, in Ekiti West 70% of the respondents had heard of mycotoxins while 30% had not. In Ikere- Ekiti 36% had heard of mycotoxins but 64% had not while in Ido-Osi 66% had heard of mycotoxins while 44% had not. (Table 5).

4.0. Discussion

The eating of mouldy food by some of the respondents could be because some foods like Eko(maize meal) are eaten even when mouldy. The mould mycelia are only washed off before consumption. Some people even eat bread with signs of mouldiness like tiny black pin heads. Another reason could be because of the poor economic situation in the study area since it is seen as being wasteful when mouldy grains, cereals or seeds are thrown away.

The results obtained reflected the high level of ignorance amongst the respondents. Many researchers who have worked on mycotoxin occurrences in foods and feeds(Makun *et al*., 2009, Ilesanmi and, Ilesanmi 2011, Ezekiel *et al*., 2012, Atanda *et al*., 2013.) have suggested that educating the people can result in lower exposure to mycotoxins . Bankole and Adebanjo (2003) suggested that private nongovernmental organizations should also join in the spread of information especially to the most remote villages.

Table 5: Respondents with knowledge of Mycotoxin

	Occurrence(%) of respondents in the local government areas			
Know Mycotoxins?	**Ado-Ekiti**	**Ekiti-West**	**Ikere**	**Ido-Osi**
Yes	54	70	36	66
No	46	30	64	34

They also suggested that there should be regular programs on radio and televisions on mycotoxin hazards and discussion on the issue should also feature regularly on daily newspapers and magazines

5.0. **Conclusion and Recommendations**

There is need to intensify efforts on mycotoxin awareness at the grassroots as this will go a long way in reducing the occurrence in food and feed stuff. If the farmers and traders are not aware of these agents which need to be controlled then effecting control will be difficult.

There is therefore need for Mycotoxicology Society of Nigeria and other bodies world over to engage in more aggressive campaign aimed at farmers and traders who handle these products before they get to the consumers. A lot of seminars and workshops has been organised in and outside Nigeria but most decisions stop at the venues. The people in the local communities need to be reached and educated on good agricultural practices. This therefore calls for increased funding by foreign bodies to enable this grass root campaigns to be carried out effectively

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ORIGINAL RESEARCH

The distribution and mycotoxigenicity of fungal isolates of stored maize grains from five agro-ecological zones of Nigeria

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ABSTRACT

Seventy composite samples of stored maize grains collected from farmers' storage structures in five agro-ecological zones of Nigeria (AEZs): Sudan Savanna (SS), Northern Guinea Savanna (NGS), Southern Guinea Savanna (SGS), Derived Savanna (DS) and Humid Forest (HF) were examined for fungal contamination. The isolates were further assessed for their abilities to produce mycotoxins in culture medium. The *Aspergillus* isolates found in the grains were *A. niger*-clade, *A. fumigatus* and *A. tamari*. The *Aspergillus* population (63.7–76.8%) was significantly higher than the population of other fungal genera in the grains excluding grains from the HF zone where the *Penicillium* species (51.4%) occurred in higher abundance of 51.4% to 35.03% of *Aspergillus*. In addition the *Fusarium* isolates were more abundant (19.4%; $p<0.05$) in the NGS zone than the other zones. Of the five *Fusarium* species (*F. semitectum*, *F. verticillioides*, *F. graminearum*, *F. proliferatum* and *F. nygamai)* isolated in the grains, *F. verticillioides* recorded a high occurrence of 100% in both the NGS and SS zones respectively. The isolates were found to produce aflatoxins, fumonisins, zearalenone and ochratoxin A in culture medium and *A. flavus* was the only aflatoxin-producing species among the *Aspergilli*. Stored maize grains in Nigeria thus contain an array of mycotoxigenic moulds which may increase the risk of mycotoxin exposure since environmental and storage conditions in sub-Saharan Africa favour mycotoxin production.

Keywords: Aflatoxin, Fumonisin, Maize, Fungi, Ochratoxin A, Zearalenone**.**

1.0 Introduction

Fungal and mycotoxin contamination of foods is an increasing issue of concern in sub-Saharan Africa (SSA). Several studies have shown that *Aspergillus, Fusarium* and *Penicillium* species and their toxic secondary metabolites are the primary contaminants of stored foods in SSA (Bankole and Adebanjo, 2003; Chilaka *et al*., 2012; Atanda *et al*., 2013). These moulds invade crops on the fields, colonise them, and are often transmitted from field to store where they proliferate under favourable environmental and poor storage (post-harvest) conditions which are characteristic of SSA countries (Cotty and Jaime-Garcia, 2007). Mycotoxins may be produced by moulds on foods and when ingested by humans or animals, produce diverse health effects ranging from carcinogenicity to immune suppression and deaths (CAST, 2003). The presence of toxigenic moulds and their mycotoxins in foods usually lead to direct loss of agricultural products and/or reduced income for farmers due to rejection of such commodities in international markets even though the foods pass through sales points in the local markets where little or no regulation/monitoring are enforced.

Maize, a staple crop widely cultivated in Nigeria and other parts of the world, remains a major focal crop for mycotoxigenic fungal attack (Aja-Nwachukwu and Emejuaiwe, 1994; Kpodo *et al*., 2000; Bankole and Mabekoje 2003; Hell *et al*.,

2003; Ezekiel *et al*., 2008; Atehnkeng *et al*., 2008; Kankolongo *et al*., 2009; Egbuta, 2011; Chilaka *et al*., 2012; Atanda *et al*., 2013; Mohale *et al*., 2013). Despite this, literature is deplete of information on the distribution of these fungal isolates in farmers' storage structures across the agro-ecological zones (AEZs) of the country. Since storage and handling practices vary from one AEZ to another there is a probability that variation in the fungal population of the stored grains can occur within the AEZs. The present work therefore aims at investigating the variations in the distribution of isolates of stored maize grains sampled from five Agro ecological zones of Nigeria

2.0 Materials and methods

2.1 Survey sites

Surveys were conducted between August 2011 and February 2012 in five out of the seven AEZs of Nigeria where maize is predominantly produced (Adetunji *et al*., 2014a): SS: Kano and Sokoto states, NGS: Kaduna state, SGS: DS: Ondo, Ekiti, Osun, Oyo and Nasarawa states and HF: Lagos and Ogun states. The geographical location, temperature and rainfall patterns of the zones had been documented by previous workers (Udoh *et al*., 2000; Atehnkeng *et al*., 2008).

2.2. Sampling and sample preparation

Sampling was carried out as described by Adetunji *et al.* (2014a). Seventy composite samples (3 kg each) were collected across the five AEZs: HF (*n* = 4), SGS (*n* = 11), NGS (*n* = 11), SS (*n* = 11) and DS (*n* = 33). The samples were kept in well labelled sterile polyethylene bags and transported to the laboratory for analysis. Each sample was hand-mixed, coarse-ground and allowed to pass through a No.14-mesh screen. Sub-samples of 500 g were taken from each lot, ground with a milling machine (Greiffenberger Antriebstenchnic, Germany) and further sieved with a 1-mm mesh. Sub-samples of 50 g were taken from the lots and kept in zip lock envelopes for fungal isolation.

2.3 Mycological analysis of maize grains

2.3.1 Isolation of fungi

Fungi were isolated from the 50 g sub-samples by the dilution plating technique (Samson *et al*., 1995). Ten grams of each sub-sample was diluted in 90 ml of 0.1 % sterile peptone water and the mixture vortexed for 2 min. Aliquots (0.1 ml) were inoculated by surface plating on ½ strength Potato Dextrose Agar (PDA) plates supplemented with 0.01% chloramphenicol. Isolations were made twice from each sub-sample in triplicate PDA plates. The first set was incubated at 30 ^{o}C for 3 days for enumeration of *Aspergillus* species while the second set was incubated at 25 ^{o}C for 7 days for enumeration of *Fusarium* and *Penicillium* species. Yeasts were enumerated from PDA plates of both sets.

2.3.2 Identification of isolated fungi

Colonies of the isolates that bore resemblance to *Aspergillus*, *Fusarium* and *Penicillium* species were transferred to full strength PDA, peptone-pentachloronitrobenzene agar (PPA) and water agar (WA; 20 g agar/l of distilled water) respectively for further characterization. The cultures on PDA were incubated at 30 ^{o}C for 7 days while those on PPA and WA were incubated at 25 ^{o}C for 7 days. Cultures of *Fusarium* were incubated under fluorescent light on a 12 h day/night schedule to initiate conidia which were subsequently used for single sporing. Single spores of the isolates were manipulated and incubated overnight on a WA plate at 25 °C. Germinated spores were then maintained on a modified Czapek Dox complete medium (CM) and stored at 4^{o}C until identification.

All isolates were identified on the basis of morphological characteristics and comparison with appropriate keys in literature. Isolates belonging to the *Aspergillus* section *Flavi* group and other sections such as *Fumigati* were identified to species level according to Klich (2002), Ehrlich *et al*. (2007) and Pitt and Hocking (2009) while all black isolates of *Aspergillus* were regarded as belonging to the section *A. niger*-clade (Pitt and Hocking, 2009). For the *Aspergillus* section *Flavi* group, data

obtained from morphological character assessment (macro: colony colour, morphology and size; micro: conidia morphology and size) of each isolate was matched with the aflatoxin production profile of the isolates on yeast extract (2%) sucrose (20%) agar as illustrated below. All identified *Aspergillus* isolates were further maintained on PDA slants by the single colony transfer technique at 4 ^{o}C.

The *Fusarium* isolates stored on CM were inoculated on Carnation Leaf Agar (CLA) for 14 days after which they were identified based on morphological characteristics exhibited on CLA (sporodochia and uniform macro conidia) under the Olympus BX51 Digital Microscopy, Olympus Optical Co., LTD, Japan and for pigmentation and colony morphology on PDA. Species identification was according to the taxonomic criterion of Leslie and Summerell (2006). Macro- and micro- characters of *F. verticillioides* and *F. semitectum* isolates were compared to those of *F. verticillioides* ATCC MYA 836 reference strain (Afolabi *et al*., 2007) and *F. semitectum* BUFC 059 (Ezekiel *et al*., 2013). The identified *Fusarium* isolates were then cultured on CM slants in a 4 ml vial, and stored at 4^{o}C. All *Penicillium* isolates were identified to the genus level (Samson *et al*., 2010) while the yeast isolates were stained with lactophenol cotton blue and observed under Olympus BX51 Digital Microscope (Olympus Optical Co., LTD, Japan) for cell shape, sporulation and characteristic vegetative reproduction.

2.3.3 Assessment of mycotoxigenic potential of fungal isolates

All isolates of *Aspergillus*, *Fusarium* and *Penicillium* were assessed for their ability to produce aflatoxins (AF), fumonisins (FB), zearalenone (ZEA) and ochratoxin A (OTA) in culture. Each isolate was sub-cultured on Yeast Extract Sucrose (YES) agar and incubated for 14 days at 28 ºC (Singh *et al*., 1991). At the end of the incubation period, 10 g of the culture medium was gently removed from the Petri dish and added to a vial containing 20 ml of dichloromethane for mycotoxin extraction. The mixture was homogenised, filtered through Whatman No. 1 filter paper and spotted on a two-dimensional Thin Layer Chromatographic (TLC) plate (Chilaka *et al*., 2012). A 20 µl aliquot of the filtrate from each sample was spotted alongside the mycotoxin standards: aflatoxin B_1 (AFB_1), AFB_2, fumonisin B_1 (FB_1), ZEA and OTA. After the double development of spotted plates, the plates were dried; plates for AF and OTA determination were viewed under UV light at wavelengths of 365 and 360 nm respectively. The fluorescing colour and retardation factor (Rf) value of the spots of the extracts were compared with those of the mycotoxin standards. In addition, the dried chromatographic plates for FB_1 and ZEA were sprayed with anisaldehyde reagent and diazotiseddianisidine respectively. Plates for FB_1 determination were further heated for 1 min at 120 ºC and then viewed under UV light at 313 nm while plates for ZEA were viewed at short wavelength of 260 nm. Plates that showed the desired colouration under UV light at the various wavelengths were qualitatively assessed and considered as positive (+) for the specific mycotoxin

2.4 **Statistical analysis**

All data were analyzed by SPSS® 16.0 (Windows version, SPSS, IL, USA). One way ANOVA was performed for the distribution of fungal species across the agro-ecological zones (AEZs). All means were tested for significance by the Duncan's Multiple Range Test at 95% confidence level.

3.0 Results

3.1 Fungal profile of maize grains

A total of 566 fungal isolates belonging to four genera: *Aspergillus* (*n* = 350), *Penicillium* (*n* = 151), *Fusarium* (*n* = 45) and *Saccharomyces* (*n* = 20) were isolated from the grains (Table 1). The population of *Aspergillus* (63.7–76.8%) was higher than the population of the other fungal genera of the grains in the AEZs except the HF zone where the *Penicillium* species (51.4%) were more abundant. Among the *Aspergillus* species isolated, *A. flavus* was the only aflatoxin-producing species and was the most abundant species in all the AEZs. The incidence

of *A. flavus* in the grains from the NGS (71.5%) and the SGS (75.4%) zones was significantly (p<0.05) higher than the grains from other zones. Following *A. flavus* in hierarchical succession was the *A. niger*-clade whose incidence in the DS, HF and SS zones was significantly (p<0.05) higher than the SGS and NGS zones while it was not isolated from the NGS zone. The incidences of *A. fumigatus*, *A. tamarii* and *S. cerevisae* in the grains were very low (≤5.0%) in the AEZs (Table 1).

The highest incidence (19.4%; p<0.05) *of Fusarium* isolates was found in maize grains from the NGS zone (Table 1) and the isolated *Fusarium* species were: *F. semitectum, F. verticillioides, F. graminearum, F. proliferatum* and *F. nygamai* (Table 2). *Fusarium verticillioides* was the most dominant *Fusarium* species in the AEZs and had an occurrence of 100% in the SS and NGS zones. In addition, *F. graminearum* was isolated only from maize grains from SGS zone (Table 2). Table 3 shows the diversity of mycotoxigenic moulds (*Aspergillus*, *Fusarium* and *Penicillium*) within the agro-ecological zones and the toxins (aflatoxins, fumonisins and zearalenone, ochratoxin A) produced by the isolates respectively. The aflatoxins were the most the most commonly contaminating mycotoxins in the AEZs

4.0 Discussion

Four fungal genera: *Aspergillus, Fusarium, Penicillium and Saccharomyces*; were identified in this study as contaminants of stored maize grains across the AEZs. These fungal genera with the exception of the yeast are known to widely contaminate stored maize (Aja-Nwachukwu and Emejuaiwe, 1994; Kpodo *et al*., 2000; Hell *et al.,* 2003; Kankolongo *et al.,* 2009; Chilaka *et al*., 2012; Mohale *et al*., 2013). The high incidence of *A. flavus* in the grains across the AEZs and its significantly higher occurrence in the grains than all other *Aspergillus* species including those belonging to the section *Flavi* agrees with previous reports from Nigeria (Bankole and Mabekoje 2003; Atehnkeng *et al*., 2008; Egbuta, 2011).

The common occurrence of *A. flavus* in Nigeria's AEZs is consistent with findings of Kankolongo *et al. (*2009) that *A.flavus* and *A.niger* clade were the most prevalent fungal isolates in Zambian maize. This suggests similarity of prevalent conditions such as the occurrence of *A. flavus* in the soil and plant debris (Horn and Dorner, 1999; Nesci and Etcheverry, 2002; Jaime-Garcia and Cotty, 2004). The plant debris usually acts as reservoirs of inoculums for infection of kernels in the field. This could be due to the occurrence of *A. flavus* in the soil and plant debris (Horn and Dorner, 1999; Nesci and Etcheverry, 2002; Jaime-Garcia and Cotty, 2004) which act as the reservoir of inoculums for infection of kernels in the field.

Table 1. Incidence of fungal species in stored maize grains from five agro-ecological zones of Nigeria.

	% Occurrence of fungal species in AEZ*				
Fungal species	NGS	SGS	DS	HF	SS
Aspergillus flavus	71.52^{a}	75.42^{a}	50.91^{b}	35.03^{c}	51.41^{b}
A. niger-clade	0.00^{b}	0.89^{b}	11.16^{a}	8.97^{a}	14.19^{a}
A. fumigatus	0.00^{a}	0.00^{a}	0.05^{a}	0.34^{a}	0.06^{a}
A. tamari	3.80^{a}	0.46^{b}	1.53^{a}	0.00^{b}	5.01^{a}
Fusarium spp.	19.41^{a}	6.82^{b}	9.32^{b}	4.23^{b}	0.56^{c}
Penicillium spp.	5.27^{c}	16.22^{b}	26.95^{b}	51.44^{a}	28.21^{b}
Saccharomyces cerevisae	0.00^{a}	0.18^{a}	0.08^{a}	0.00^{a}	0.56^{a}

*****AEZ**-** Agro-ecological zones: Northern Guinea Savanna (NGS), Southern Guinea Savanna (SGS), Derived Savanna (DS), Humid Forest (HF) and Sudan Savanna (SS).

Percentage occurrence values with different superscript alphabets in a row are significantly different (α = 0.05).

Table 2. Incidence of *Fusarium* species in stored maize grains from five agro- ecological zones of Nigeria

[a]AEZ	Incidence of *Fusarium spp.*	[b]*F. verticilliodes*	*F. semitectum*	*F. proliferatum*	*F. nygamai*	*F. graminearum*
SS	1/11	100[a]	0.00[c]	0.00[c]	0.00[b]	0.00[b]
NGS	9/11	100[a]	0.00[c]	0.00[c]	0.00[b]	0.00[b]
SGS	15/33	25[c]	25.00[a]	0.00[c]	0.00[b]	50.00[a]
DS	4/11	44.45[b]	11.11[b]	11.11[b]	33.33[a]	0.00[b]
HF	3/4	50[b]	0.00[c]	50.00[a]	0.00[b]	0.00[b]

[a]Agro-ecological zones: Sudan Savanna (SS), Northern Guinea Savanna (NGS), Derived Savanna (DS), Southern Guinea Savanna (SGS) and Humid Forest (HF).
[b]Percentage occurrence of *Fusarium spp*
Percentage occurrence values with different superscript alphabets in a columm are significantly different (α = 0.05).

The non-isolation of other aflatoxigenic members of *Aspergillus* section *Flavi* (unnamed taxon SBG, *A. parasiticus and A. parvisclerotigenus*) from the grains in this study contrasts previous reports by Atehnkeng *et al.* (2008) and Perrone *et al.* (2014) who found these three species in Nigerian maize in addition to the widely distributed *A. flavus* at very low frequencies.This may be attributed mainly to (1) the choice of isolation and characterization medium- PDA, used in our study instead of modified Dichloran Rose Bengal Agar (DRBA), a selective medium for isolation of *Aspergillus* and *Penicillium* species and 5/2 agar for differentiation of *Aspergillus* section *Flavi* species (Cotty, 1989; Diedhiou et al., 2011) and (2) the relatively scarce distribution of the species in maize.

The occurrence of *A. fumigatus* in the grains is in line with previous reports on maize in Nigeria (Makun *et al.,* 2010; Egbuta *et al.,* 2011). However, Makun *et al,.* (2010) found low occurrence of *A. fumigatus* (2.3%) in maize grain from markets and farmers' storage structures in Nigeria, Egbuta *et al.* (2011) and Chilaka *et al.* (2012) found high incidence of *A. fumigatus* (>40%) than *A. flavus* in commercial maize from South Africa and Nigeria. The high incidence of some fungal species across the AEZs and low incidence of others confirm that fungi that thrive or found to be scarce in a particular area are strongly determined by the prevailing climatic conditions (Wayne, 2007).

The incidence of *Penicillium* species was higher than that of *Fusarium* species across the AEZs except for the NGS zone. The high occurrence of *Penicillium* species in the AEZs contrasts the findings of Atehnkeng *et al*. (2008) and Chilaka *et al.,* (2012) who reported low incidences of *Penicillium* species in maize grains while it corroborated the reports of Egbuta (2011) who found more *Penicillium* species (57.9%) in stored maize grains than *Fusarium* species (47.4%). In spite of the relatively low occurrence of *Fusarium* species than *Penicillium* and *Aspergillus* species in the grains in this study, its wide distribution across the AEZs of Nigeria shows that *Fusarium* species is a regular contaminant of maize grains (Fandohan *et al*., 2005; Adejumo *et al*., 2007; Chilaka *et al*., 2012; Mohale *et* al., 2013). The occurrence of *F. verticillioides* as the most common *Fusarium* species isolated from the grains is in agreement with the work of Kankolongo *et al.,* 2009 and Chilaka *et al*., 2012. In addition, *F. nygamai* is reported for the first time in Nigerian maize grain as it was found in grains from the DS zone in contrast to previous studies by Adejumo *et al.*, (2007) and Ezekiel *et al.*, (2008) that reported only *F. graminearum*, *F. verticillioides*, *and F. proliferatum* in maize from South-Western Nigeria.

The significance of this study lies in the diversity of mycotoxigenic moulds found in Nigerian

maize grains. It is known that when moulds invade and colonise a suitable agricultural commodity such as maize, they utilize the available nutrients thus deteriorating the grains. The moulds may further liberate mycotoxins in the commodity depending on the mycotoxigenic potential of the moulds, condition and duration of storage of the commodity as well as the prevailing environmental factors. The incidence of mycotoxigenic *A*. *flavus* and *Fusarium* species in this study and the toxins (aflatoxins, fumonisins, zearalenone and ochratoxin A) produced by the isolates in the conditions of storage is a potential risk to the health of consumers of the grains. A major concern is the attendant health effects that could arise from consumption of these grains by Nigerians as mycotoxins are potent carcinogens, nephrotoxins and immune system toxicants (Bondy and Pestka, 2000; CAST, 2003). Besides, multi-mycotoxin exposure has recently been reported in Nigerian population (Ezekiel *et al.,* 2014 and Adetunji *et al.,* 2014b).

Stored maize grains in Nigeria are contaminated with propagules of mycotoxigenic moulds and possible additional risk may be cross-contamination of maize and groundnuts since both are mainly co-stored in rural areas of West Africa. Intervention strategies such as: (1) development of maize varieties that are resistant to a range of fungal infections and subsequent mycotoxin formation (2) reduction of wounds on the crop during cropping and harvesting or by insects (use of insecticides) and (3) harvesting of grains prior to onset of hot, dry climate conditions that may significantly increase infections by these fungi and (4) drying of maize cobs to a moisture content of about 13% after harvest prior to storage

Conclusion

The isolates were found to produce aflatoxins, fumonisins, zearalenone and ochratoxin A in culture medium and *A. flavus* was the only aflatoxin-producing species among the *Aspergilli*. Stored maize grains in Nigeria thus contain an array of mycotoxigenic moulds which may increase the risk of mycotoxin exposure since environmental and storage conditions in sub-Saharan Africa favour mycotoxin production.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Table 3: Mycotoxin Profile of Fungal Isolates from Five agro-ecological zones of Nigeria

AEZ	Toxin	Fungal Specie								
	Parameter	*A. flavus*	*A. niger*	*A. fumigatus*	*F. verticilloides*	*F. semitectum*	*F. proliferatum*	*F. graminearum*	*F. nygamai*	*Penicillium spp*
SS	Toxin tested	AFB_1,B_2,G_1,G_2	OTA	AFB_1,B_2,G_1,G_2	FB_1, ZEA	ND	ND	ND	ND	OTA
	Toxin produced	AFB_1,B_2,G_1,G_2	ND	ND	FB_1	ND	ND	ND	ND	ND
	Intensity	+, +, +, +	ND	ND	+	ND	ND	ND	ND	ND
NGS	Toxin tested	AFB_1,B_2,G_1,G_2	OTA	AFB_1,B_2,G_1,G_2,	FB_1, ZEA	ND	ND	ND	ND	OTA
	Toxin produced	AFB_1,B_2,G_1	ND	ND	FB_1, ZEA	ND	ND	ND	ND	ND
	Intensity	+, +, +	ND	ND	++, ±	ND	ND	ND	ND	ND
DS	Toxin tested	AFB_1,B_2,G_1,G_2	OTA	AFB_1,B_2,G_1,G_2,	FB1, ZEA	FB1, ZEA	FB1, ZEA	ND	FB1, ZEA	OTA
	Toxin produced	AFB_1,B_2,G_1,G_2	OTA	ND	FB1, ZEA	FB1, ZEA	FB1, ZEA	ND	FB1, ZEA	OTA
	Intensity	++, +, +, +	+	ND	+, ++	+, +	+, +	ND	+, +	++
SGS	Toxin tested	AFB_1,B_2,G_1,G_2	OTA	AFB_1,B_2,G_1,G_2,	FB1, ZEA	FB1, ZEA	ND	FB1, ZEA	ND	OTA
	Toxin produced	AFB_1,B_2,G_1,G_2	ND	ND	FB1, ZEA	ND	ND	ZEA	ND	OTA
	Intensity	++, +, +, +	ND	ND	+, ±	ND	ND	+	ND	+
HF	Toxin tested	AFB_1,B_2,G_1,G_2	OTA	AFB_1,B_2,G_1,G_2,	FB_1, ZEA	ND	FB_1, ZEA	ND	ND	OTA
	Toxin produced	AFB_1,B_2	ND	ND	FB_1	ND	FB_1	ND	ND	ND
	Intensity	+, +	ND	ND	++	ND	+	ND	ND	ND

++ Mycotoxigenic positive isolates with high fluorescence intensity under UV light

+ Mycotoxigenic positive isolates with moderate fluorescence intensity under UV light

± Mycotoxigenic positive isolates with weak fluorescence intensity under UV light

ND- Not Detected

HF- Humid Forest; DS- Derived Savanna; SS- Sudan Savanna SGS-Southern Guinea Savanna, NGS- Northern Guinea Savanna

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ORIGINAL RESEARCH

Growth and Haematological Responses of Wistar Rats Administered Deoxynivalenol Extract

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ABSTRACT

Response of wistar rats to orally administered extract of Deoxynivalenol (DON) was assessed in this study. One hundred wistar rats with average weight of 166.85g were randomly allotted to 5 treatment groups and each group was replicated 4 times with 5 rats per replicate. Each group was administered varied DON concentration of 0, 250, 500, 750 and 1000µg/kg for T1 (control), T2, T3, T4 and T5 respectively. The DON extract was orally administered to each animal using canula at 0.1ml/100g body weight at 48 hours interval for a period of 28 days in a completely randomized design. Feed intake, body weight changes and feed conversion ratio were monitored. Blood was sampled at day 21 from the animals through ocular vein for haematology. Result showed that daily feed intake and feed conversion ratio were not significantly different among the treatments. The final live weight and average cumulative weight gain were significantly ($p<0.05$) reduced with increase in the DON concentration in the rats. Weight gain in rats administered 250 µg/kg (11.0±3.1g) and 500 µg/kg (11.5±6.3g) were significantly ($p<0.05$) higher than those rats treated with 750µg/kg (5.7±12.5g) and 1000µg/kg (5.7±6.2g) but lower than the control rats (19.3±10.4g). The packed cell volume and haemoglobin concentration in treated rats significantly ($p<0.05$) reduced with increase in the DON concentration than the control suggesting anaemic condition in the animals. However, erythrocytes, leukocytes, platelets, neutrophils, lymphocytes, eosinophils and monocytes were not significantly influenced by the treatments. This study suggests that exposure of rats to 250µg/kg and above depressed growth rate, reduced blood volume and its oxygen carrying capacity.

Keywords: Growth, Haematology, Deoxynivalenol extract, Wistar rats.

Introduction

The poor performance of livestock in developing countries has been attributed to the seasonal inadequacy of feed, both in quantity and quality (Shephard, 2003). Many feed resources could have a major impact on livestock production which ultimately reduces the growth and reproductive potential of animals. Animals consume feeds which may contain toxic substances thereby exposing the entire population of people in developing countries worldwide to risk of chronic exposure to naturally occurring mycotoxin through contaminated food (Shephard, 2003). Most of these mycotoxins such as aflatoxin, fumonisin, ochratoxin and deoxynivalenol are known to be potent carcinogens and hepatotoxic agents that pose serious hazards to human and animal health (Sidhu *et al.,* 2009).

These mycotoxins also have an impact on agricultural economy through the loss of crop production (Wu, 2004). Food and Agricultural

Organization of United nations (CAST, 2003) reported that 25% feedstuff is polluted by mycotoxin in the world and it results in over 1 billion dollars loss for poultry industry annually. However, emphasis on mycotoxin has always been on aflatoxin produces by Aspergillus species while there is little emphasis on a lot of other mycotoxins such as Zearalenone and deoxynivalenol produce by Fusarium species with respect to their toxicity in the tropics.

Deoxynivalenol (DON), also known as vomitoxin, is a trichothecene mycotoxin produced by the *Fusarium* genus which is commonly found in our environment. Deoxynivalenol and zearalenone belong to the most prevalent mycotoxins produced by the *Fusarium* species (Bucheli *et al*., 2008). The DON has been reported to occur in cereal grains worldwide (Wegulo, 2012) and can increase in stored grain with kernel moisture content of 22-25% (Vesonder *et al.,* 1978, Sanden *et al*., 2012). One ppm or more of DON has been reported to result in reduced feed intake and weight gain in swine (Côté *et al.,* 1984). Don has been shown to be the primary mycotoxin associated with swine disorders including feed refusal, diarrhea, emesis, reproductive failure and death (Vesonder *et al.,* 1978 Côté *et al.,* 1984 and Pestka, 2007). However, response of rat to DON extract in the tropics has not been adequately documented. Therefore, growth and haematological response of wistar rats to DON extract were investigated.

2.0 Materials and Methods

2.1 Experimental site

The experiment was carried out at the Animal House of the Faculty of Veterinary Medicine, University of Ibadan, Ibadan, Nigeria (7^0 20'N, 3^0 50'E; 200m above sea level).

2.2 Experimental materials and layout

One hundred wistar rats with average weight of 166.85g were collected from the rat colony, Department of Veterinary Anatomy, University of Ibadan and randomly allotted to 5 treatment groups and each group was replicated 4 times with 5 rats per replicate. Deoxynivalenol (DON) extract was obtained from SIGMA Company Ltd. Each group was administered varied Deoxynivalenol concentration of 0, 250, 500, 750 and 1000 µg/kg for T1 (control), T2, T3, T4 and T5 respectively. The DON extract was orally administered to each animal using canula at 0.1ml/100g body weight at 48 hours interval for a period of 28 days in a completely randomized design. The animals were fed commercial diets with 16% CP and 2400kcal/kg digestible energy. The animals were fed the same diet *ad libitum* daily. Portable water was made available throughout the experimental periods. Feed intake, body weight changes, mortality and feed conversion ratio were monitored.

2.2 Blood collection and evaluation

Blood samples were collected at day 21 from the rats through the ocular vein into a set of vaccutainer tubes containing a calculated amount of ethylene diamine tetraacetic acid (EDTA) for haematological study. Packed cell volume (PCV), Haemoglobin, red blood cell and leukocyte counts were determined from the blood sample in EDTA bottles as outlined in Ewuola and Egbunike (2008). Other blood indices and leukocyte differentials were determined as described by Jain (1986).

2.3 Data Analysis

Data obtained were tested using one-way analysis of variance of statistical analysis software (SAS, 2003) at $p = 0.05$. Treatment means were separated using Duncan Multiple Range Test option of the same software.

3.0 Results

3.1 Growth indices of the wistar rats

The growth indices of rats administered DON extract is as shown in Table 1. The daily feed intake and feed conversion ratio of rats

administered varied doses of DON were not significantly different among the treatments. However, final live weight and cumulative weight gain of rats administered 250 and 500µg/Kg DON were significantly ($p<0.05$) lower than the control rats but significantly ($p<0.05$) higher than those treated with 750 and 1000µg/Kg. Rats treated with 750 and 1000µg/Kg recorded least final live weight (172.5g and 172.6g respectively) and body weight gain (5.7g), while the highest value of live weight (186.05g) and cumulative body weight gain (19.25g) were recorded in the control rats.

3.2 Haematological response of the wistar rats

The haematological parameters of wistar rats administered DON extract is as shown in Table 2. The packed cell volume of rats treated with DON extract was significantly ($p<0.05$) lower than the non treated rats (control). The packed cell volume of rats administered 250, 500, 750 and 1000µg/Kg were not significantly different from one another. Haemoglobin concentration follows the same trend with packed cell volume. The haemoglobin concentration of treated rats was not significantly different from one another, however, haemoglobin of treated rats was significantly ($p<0.05$) lower than the non-treated rats. The erythrocytes, leukocytes and thrombocytes count were not significantly different among the treatments. The leukocytes differentials: Neutrophils, Lymphocytes, Eosinophils and Monocytes were not significantly influenced by the treatments.

4.0. Discussion

Deoxynivalenol (DON) has been adjudged to be one of the mycotoxins produces by Fusarium species and related generals that is commonly found in agricultural commodities (Desjardins *et al.,* 1993). In this study, feed consumption pattern of rats administered varied concentration of DON extract was not significantly influenced by toxin. This could be attributed to short period of exposure and probably the highest dose of DON (1mg/kg) was not sufficient enough to induce feed refusal effect. Besides, since the DON extract was not administered through feed, immediate effect on the feed intake within a space of 28 days has not been probably significant. This result was at variance to the report of Côté et al. (1984) who observed that DON was the primary mycotoxin associated with swine disorder including feed refusal and diarrhea. Marasas (1984) also reported that diet containing DON decreased feed consumption on a dose related basis. However, McMillan and Moran (1985) observed that poults fed 75ppm DON revealed no effect on feed consumed or growth.

The final live weight and cumulative weight gain were significantly depressed by the DON concentration administered to rats. The body weight gain in rats administered 250, 500, 750 and 1000µg DON/Kg was reduced by 42.9%, 40.3%, 70.4% and 70.4% respectively relative to mean weight gain of 19.25±10.40g in the control rats. This result corroborates the finding of Bergsjo et al. (1993) who reported a decrease body weight gain and slaughter weight in pigs fed 3.5ppm of DON in 8 weeks trial. Pigs fed diets containing 2 and 4ppm of DON exhibited a dose-related decrease in weight gain (Bergsjo *et al*., 1992). Rotter *et al*. (1995) also reported a lower feed intake and reduced weight gain in swine exposed to 4mg DON/kg diet within 42 days. Clinical data has shown an association between DON contamination of diets and poor performance in dairy herds (Whitlow *et al*., 1994). However, the observed result on the weight gain in this study was at variance with the reports of McMillan and Moran (1985) and Kubena *et al*. (1987) that DON has no effect on weight, feed consumption and growth of the animals.

Table 1: Growth indices of Wister rats administered DON extract

Parameters	0µg/Kg	250µg/Kg	500µg/Kg	750µg/Kg	1000µg/Kg
Initial weight (g)	166.80±9.24	166.95±8.08	166.85±7.19	166.80±5.87	166.85±11.61
Final weight (g)	186.05±14.53[a]	177.95±8.55[b]	178.35±4.51[b]	172.50±15.68[c]	172.55±8.45
Cumulative body weight gain (g)	19.25±10.40[a]	11.00±3.14[b]	11.50±6.33[b]	5.70±12.45[c]	5.70±619[c]
Daily feed Intake (g)	37.79±0.55	36.56±1.04	35.08±0.71	36.21±1.38	37.85±0.08
Feed Conversion Ratio	10.82±3.4	16.83±8.45	15.84±7.49	10.64±8.83	9.56±9.04

abc: Means along the same row with different superscript are significantly (P<0.05) different

Table 2: Haematological response of the wister rats administered DON extract

Parameters	0µg/Kg	250µg/Kg	500µg/Kg	750µg/Kg	1000µg/Kg
Packed cell volume (%)	49.75±0.86[a]	45.75±0.94[b]	46.63±0.63[b]	46.13±1.27[b]	44.63±1.36[b]
Haemoglobin (g/100ml)	16.92±0.29[a]	15.56±0.32[b]	15.85±0.21[b]	15.68±0.43[b]	15.17±0.46[b]
Erythrocyte ($x10^6$/L)	2.16±0.06	1.88±0.38	2.89±0.81	2.04±0.55	2.80±0.61
Leukocytes ($x10^3$/L)	2.10±0.29	2.49±0.45	2.54±0.46	2.96±0.44	2.40±0.45
Thrombocytes ($x10^3$/L)	125.50±17.04	121.63±17.50	141.13±12.64	129.38±13.04	133.50±10.57
Neutrophils (%)	26.00±1.57	29.38±3.67	27.38±1.05	30.38±1.83	29.13±1.68
Lymphocytes (%)	71.13±1.39	67.38±4.14	69.63±1.08	67.63±1.90	66.50±2.06
Eosinophils (%)	1.38±0.42	1.63±0.65	0.75±0.25	1.00±0.42	1.38±0.42
Monocytes (%)	1.50±0.42	1.75±0.37	2.25±0.31	1.00±0.42	1.75±0.31

ab: Means along the same row with different superscript are significantly (P<0.05) different

The haematological response of rats revealed a dose-dependent effect of DON on the animal health status with depression on oxygen carrying capacity and blood volume in animals administered DON extract as evidence in significantly reduced haemoglobin concentration and packed cell volume in treated rats compared to the control. This effect has been implicated in anaemic disease condition induced by the toxin (Ewuola and Egbunike, 2008; Pereira *et al*., 2013). This result was in agreement with the finding of Bergsjo *et al*. (1993) who reported temporary fall in packed cell volume in pigs fed 3.5ppm DON within 8 weeks trial. Matejova *et al*. (2014) reported decrease in mean corpuscular haemoglobin of Rainbow trouts while other haematological parameters were not statistically different between the experimental and control groups. Other haematological parameters that were not significantly influenced by the DON extract in this study corroborated Modra *et al* (2013) observation that DON has no effect on haematological variables in weaned pigs fed diet contaminated with 0.6 and 2 mg DON/kg except mean corpuscular volume that decrease in animals exposed to 2 mg DON/kg for 4 weeks. Pinton *et al*. (2008) also reported that consumption of DON-contaminated diet does not have a major effect on the haematological and biochemical blood parameters.

5.0 Conclusion

Based on the findings of this study, the deoxylnivalenol extract in treated rats reduced

growth rate, blood volume and oxygen carrying capability of red blood cells without adverse effect on other haematological variables.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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ORIGINAL RESEARCH

Natural occurrence of aflatoxins and ochratoxin A in raw and roasted groundnut from Niger State, Nigeria

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ABSTRACT

Aflatoxins (AFs) and ochratoxin A (OTA) contamination of raw and roasted groundnut from Niger State, Nigeria was assessed. Eighty-one samples were randomly collected during the rainy season (May–October) from different locations in four microclimatic zones of Niger State and analyzed for aflatoxin B_1 (AFB_1), AFB_2 and OTA using high performance liquid chromatography. The three mycotoxins (AFB_1, AFB_2 and OTA) were found in 88.9, 75.3 and 90.1% of the samples respectively and the concentrations ranged 4.0–188 µg/kg for AFB_1, 0.4–38.4 µg/kg for AFB_2 and 0.8–45.6 µg/kg for OTA. All aflatoxin positive groundnut samples contained levels above the Nigerian and European Union (2 µg/kg) action limits for AFB_1 while 55% of the samples had OTA concentrations exceeding the 5 µg/kg regulatory limit of Nigeria and EU. Since groundnut is a staple food in Nigeria, consumption of contaminated kernels may contribute to an increased dietary exposure to AF and OTA in the studied population with possible health risks associated with such exposures.

Keywords: Aflatoxins, Groundnuts, Ochratoxin A

1.0 Introduction

Aflatoxins (AFs) are poisonous secondary metabolites produced mainly by *Aspergillus flavus*, *A. parasiticus* and *A. nomius*. The four major AFs: aflatoxin B_1 (AFB_1), AFB_2, AFG_1 and AFG_2; are the most agriculturally important mycotoxins in food and feed because of their abundant presence in nature and high toxicity (Makun et al., 2012). Aflatoxins especially AFB_1 are powerful human carcinogens that also elicit mutagenic and teratogenic effects (Raisuddin et al., 1993). These toxins play causative roles in the development of hepatocellular carcinoma (Liu and Wu, 2010) and have been the cause of deaths among humans in Eastern Province of KenyaAzziz-Baumgartner et al., 2006; Probst et al., 2007), India (Krishnamachari et al., 1975) and Malaysia (Lye et al., 1995). On the other hand, ochratoxin A (OTA) elicits kidney and liver impairment in animals, especially in pigs (Battacone *et al*., 2010), and man (Reddy and Bhoola, 2010). It is also associated with Bulgarian porcine and chicken nephropathy (Stoev et al., 2009) as well as human kidney disorder commonly referred to as Balkan Endemic Nephropathy (BEN) (Peraica *et al*. 1999). Aflatoxins and OTA are known to contaminate nuts and oilseeds, cereals, roots and tubers, fruits, vegetables and animal feed (Makun et al., 2012) particularly in warm, humid regions of the world. In fact groundnut is one of the most susceptible crops to aflatoxins (Makun et al., 2012).

Groundnut (*Arachis hypogaea* L.) is one of the major sources of protein for many West African countries. Apart from its use as food or snacks and oil, groundnut is also an important source of cash and a component of compound feed in Nigeria. It generates about 60, 42 and 21% of rural cash earnings for groundnut producers in Senegal, Niger and Nigeria, respectively and accounts for about 70% of rural employment in Senegal (Ntare, *et al*. 2005). During the last four decades, West Africa lost its position in world groundnut production and export shares as its production share declined from 23 to 15% whereas export share declined from 55 to 20% (ITC, 2001). Senegal and Nigeria are among the world's largest groundnut producers (Ntare *et al.,*

2005). Groundnut is produced mainly in Northern and Middle-belt regions of Nigeria including Mokwa Local Government of Niger State (Adoga and Obatomi, 1992). The competitiveness of Nigeria with regards to international trade in groundnut is significantly reduced because the crop is primarily susceptible to contamination by mycotoxins especially AFs and OTA and its contamination by AFs is severe (Makun *et al*., 2012).

There are only three reports (Darling, 1963, Okonkwo and Nwokolo, 1978; Opadokun, 1992) on the incidence of AFs in groundnut in Niger State which is one of the major groundnut producing states in the country. More so, surveys on OTA content in nuts are very scarce in Nigeria. For Nigeria to improve its competitiveness in groundnut trade, the nation must generate data on mycotoxins in the crop so as to establish whether the levels are safe for human and animal consumption and further adopt measures to control their contamination levels. The current trend in climate change with its attendant influence on fungal contamination, mycotoxin production and distribution (Paterson and Lima, 2010) makes constant monitoring of food and feed an imperative in order to forestall outbreak of animal and human mycotoxicoses. It is for these reasons that this study was conducted to determine the incidence of AFB_1, AFB_2 and OTA in groundnut from Niger State in Nigeria.

2.0 Materials and Methods

2.1 Sampling

A total of 81 groundnut samples were randomly collected during the rainy season (August-September) from representative towns of twenty-five local government areas of Niger State. Based on the annual rainfall pattern, Niger State, a middle belt state of Nigeria has four microclimatic zones (Figure 2). Zones 1, 2, 3 and 4 are the wettest, wet, dry and driest zones with annual rainfall ranges (mm) of >1400, 1200-1400, 1000-1200 and <1000 respectively. Twenty of the samples were marketed roasted samples while 28 and 32 were collected from the store and farm respectively. With regards to sampling according to microclimatic zones, 22, 19, 19 and 21 were sampled from zones respectively. The stored samples were collected from locally built mud barns called "rumbu" in Hausa. About 0.5 kg of each sample were collected, labeled, packaged in small container and taken to the laboratory where they were ground to powder, put in sealed plastic bags and stored at -4^{o}C until analysis.

2.2 Analysis of mycotoxins

Samples were subjected to extraction of toxins, clean up and analyzed for AFB_1, AFB_2 and OTA according to the method described by Ehrlich and Lee (1984) without modification. Methylene chloride and phosphoric acid were used for the simultaneous extraction of AFB_1, AFB_2 and OTA. A separate portion of the initial methylene chloride/phosphoric acid extract was subjected to a specific clean-up procedure for each mycotoxin.

2.2.1 *Extraction of mycotoxins*

Approximately 50 g portion of pulverized groundnut sample was weighed into a 500 ml Erlenmeyer flask and 25 ml 1M orthophosphoric acid and 250 ml of methylene chloride were added. The flask was placed on a mechanical shaker for 30 minutes and the content filtered under pressure on Buchner funnel fitted through an 18 cm circle rapid filter paper. Two hundred milliliter of the filtrate was collected and 50 ml aliquot was taken from the filtrate and placed in separate 100 ml Erlenmeyer flasks with glass stoppers, for AF and OTA assay.

The fraction for AFs analysis was subjected to a specific column chromatographic clean-up method. A column was set up with a glass wool and 150 ml of dichloromethane (DCM) was poured into the column and emptied half way. Anhydrous sodium sulphate (Na_2SO_4) was added and the sides of the column washed with DCM. Silica gel was added to the green line of column together with 80 ml of DCM and this was allowed to settle half way. Three scoops of anhydrous sodium sulphate (Na_2SO_4) were added and the DCM drained off to top of packed section of the column.

About 50 ml of the filtrate was added and drained off to top of the packed part of the column. The filtrate was defatted with 130 ml of hexane and 130 ml of ether sequentially, and each fraction drained out completely. Aflatoxins were extracted into 130 ml of ether/methanol/water (96:3:1, v/v/v) that was collected off column in a new beaker. The extract was evaporated to near dryness, put into sealed amber glass vials and stored at 0 ^{o}C for a week until further analysis.

A different clean-up method to that of aflatoxin was used for OTA. The toxin was extracted into aqueous solution of $NaHCO_3$ (4 g $NaHCO_3$/100 ml distilled water) and acidified to a pH of 2 with H_2SO_4 to obtain an acid fraction in a separatory funnel. Ochratoxin A was repeatedly extracted thrice from the acid fraction with 25 ml of DCM. The pooled DCM fraction was passed through anhydrous Na_2SO_4, evaporated to dryness and stored in sealed amber glass vials at 0 ^{o}C for a week until further analysis.

2.2.2 *High Pressure Liquid Chromatography*

Aflatoxins were analyzed on a Cecil 1100 series HPLC system equipped with a UV detector set at a wavelength of 365 nm as described by Cora, Angre and Ronald (2005). The Altraspher ODS column, 4.6 mm x 250 mm was used at ambient temperature of 25 ^{o}C. Acetonitrile/water/acetic acid (10:50:40, v/v/v) was used as the mobile phase pumped at a flow rate of 0.8 ml/min. The injection volume of both samples and standards used was 20 µl. The analysis was carried out with aflatoxins standards (Sigma Chemical Company, St. Louis, MO, USA) of known concentrations. Aflatoxin B_1 and AFB_2 eluted at distinct retention times of 1.673 min and 1.524 min, respectively. Calibration curves with correlation coefficient (R^2) of 0.91 and 0.99 were established for AFB_1 and AFB_2, using a series of dilutions containing (0.004, 0.008, 0.012 and 0.016 µg/ml) and (0.01, 0.02, 0.03 and 0.04 µg/ml) respectively for each standard. The limits of detection (LOD) were estimated as follows: known concentrations of aflatoxin standards were prepared, successively diluted and subjected to HPLC until the minimum concentration at which the analyte could be detected was established. The LOD of the HPLC instrument with regards to both toxins was determined to be 0.21 and 0.18 µg/kg while the limits of quantification (LOQ) were estimated based on the standard deviations of response and slope; this gave 0.42 and 0.33 µg/kg respectively.

OTA was quantified on same HPLC machine with a UV detector set at wavelength of 254 nm as described by Engstrom, Richard and Cysewski, (1977). The operating conditions were set at ambient temperature of 25 ^{o}C. Acetonitrile/water/acetic acid (50:48:2, v/v/v) was used as a mobile phase pumped at a flow rate of 1 ml/min. Injection volume of OTA analytes and standard was 60 µl. The retention time for OTA was 1.11 min. Calibration curve with an R^2 of 0.93 (Figure 10) was generated using series of dilutions containing 0.015, 0.025, 0.035 and 0.045 µg/ml. The LOD and LOQ for OTA were estimated to be 0.15 and 0.45 µg/kg respectively.

To determine the recoveries for the tested mycotoxins, 3 samples of each food commodity that were confirmed by same HPLC methods described above not to contain any of the studied mycotoxins were each spiked with 100 µg/kg of mycotoxin standard. For AFB_1 and AFB_2, the apparent recoveries (mean ± standard deviation) obtained were 93.6% ± 8.6 and 90.3% ± 8.5, respectively, while that of OTA was 97.5% ± 2.7.

2.3 Statistical analysis

Mean $\pm$ standard deviation and analysis of variance (students' t-test) of data generated were calculated using SPSS 18 software. The statistical level of significance was fixed at $P<0.05$ (95%).

Table 1: Incidence and concentration (µg/kg) of aflatoxin B_1 (AFB1), AFB_2, and ochratoxin A in roasted, stored and farm groundnuts from Niger State

Mycotoxin	Incidence & concentration (µg/kg)	Roasted	Stored	Farm	Total
Aflatoxin B_1	Incidence	19/21	25/28	28/32	72/81
	Mean ± SE	64.78^{a}±5.11	42.78^{b} ±4.27	54.32^{c}±4.36	53.06±2.57
	Range	27.00 – 188.00	4.80 – 165.60	4.00 – 181.60	4.00 –188.00
Aflatoxin B_2	Incidence	16/21	19/28	26/32	61/81
	Mean ± SE	11.60^{a}±1.46	5.08^{b}±0.81	8.10^{ac}±0.99	8.08.±0.63
	Range	0.40 – 36.40	0.80 – 32.80	0.40 – 38.40	0.40 – 38.40
Ochratoxin A	Incidence	19/21	24/28	30/32	73/81
	Mean ± SE	13.30^{a}±0.81	13.60^{ab} ±1.17	14.46^{abc}±1.08	13.86±0.62
	Range	5.20 –32.80	0.80 – 40.00	1.60 – 45.60	0.80 – 45.60

abc: Mean with different superscripts in a row are significantly different from each other ($p<0.05$)

3.0 Results

The data (adjusted based on recovery) obtained from HPLC analysis as summarized in Table 1 shows the natural occurrence of AFB_1, AFB_2 and OTA in raw and roasted groundnut produced and marketed in Niger State, Nigeria. Generally, 88.9% of the samples were contaminated with aflatoxins (range = 0.4–188.0 µg/kg) with AFB_1 being more prevalent (incidence = 72/81 (88.9%)) and more concentrated (mean level: 53.1 µg/kg) in samples than AFB_2 (incidence = 61/81 (75.3%); mean level: 8.1 µg/kg). With respect to the type of samples collected (e.g. raw kernels from field or store, and roasted kernels from markets), AFB_1 increased in incidence from the farm (87.5%), to the store (89.3%) and market with roasting (90.5%) while the mean concentration decreased significantly ($p<0.05$) from farm (54.3 µg/kg) to store (42.8 µg/kg) with a concomitant increase from store to market (64.8 µg/kg). However, the concentrations between the farm and market were significantly different at ($p<0.05$) It was found that the highest incidence of AFB_2 was observed in farm samples (81,3%) followed by roasted marketed (76.2%) and stored samples (67.8%). However, based on the toxin content, AFB_2 occurred at the highest mean concentration of 11.6 µg/kg in roasted groundnut samples followed by farm (8.1 µg/kg) and stored (5.1 µg/kg) samples and the differences were significant ($p<0.05$).

Amongst the three mycotoxins analyzed, OTA occurred most frequently (73/81, 91%) in the samples and at moderate levels of 0.8–45.6 µg/kg (Table 1). The incidence and levels of OTA were highest in samples from the farm (30/32 (93.8%); 14.46 µg/kg) followed by those from the store (24/28 (85.7 %); 13.60 µg/kg) and market (19/21 90.5 %); 13.30 µg/kg) although there were no significant differences in the levels of the toxin between sample categories. On a general note, higher mycotoxin (AFB_1, AFB_2 and OTA) incidences and concentrations were observed in the samples from open air sources (farm and market) than those from farmers' store. The only exception to this trend is the observed higher incidence of AFB_1 in stored samples than that found in farm samples.

The effects of annual rainfall intensity on mycotoxin contamination are reported in Table 2. Aflatoxin B_1 occurred most frequently (19/19, 100%) in samples from the dry zone, followed by those from the wet (17/19, 89.5%), wettest (19/22, 86.4%) and driest (17/22, 77.3%) zones. Similarly, samples from the dry zone had the highest AFB_1 mean level (74.0 µg/kg) followed by those from the driest (56.1 µg/kg), the wettest (45.1 µg/kg) and the wet (35.4%) zones. The AFB_1 concentrations of samples from the wet zone were significantly ($p<0.05$) lower than levels of the toxin in the other zones. The frequency of occurrence of AFB_2 was also highest in samples from the dry zone (16/19, 84.2 %) while the lowest frequency was observed in samples from the

driest zone (14/21, 66.7%). The wet (15/19, 78.9%) had a higher frequency than the wettest zone (16/22, 72.7%). The mean AFB_2 content was higher in samples originating from the wettest zone (10.54 µg/kg) and dry (9.06 µg/kg) than those from driest (6.62 µg/kg) and wet (5.76 µg/kg) zones as seen in Table 2. However, the levels in the wet zone were significantly lower ($p<0.05$) than those from the other zones. Ochratoxin A was recovered from 100% of samples from the dry and wet zones. Meanwhile 17/21 (81%) and 18/22 (81.8%) of samples from the driest and wettest zones were respectively found to be contaminated with OTA. However, with regards to contamination levels of the toxin, OTA was highest in samples from the wet (mean: 15.80 µg/kg) followed by the levels recovered from samples analyzed from the wettest (mean: 13.6 µg/kg), driest (mean: 13.5 µg/kg) and dry (mean: 12.6 µg/kg) zones. Based on the above observations, the general trend was that the incidence and levels of AFs and OTA contamination were lowest in extreme dry (driest zone) and wet (wettest zones) conditions. Accordingly, the highest concentrations of AFB_1 and OTA were observed in locations (Bida (range: 6.8-188.µg/kg and Borgu range 4.0-45.6µg/kg respectively) in the dry zone. while the lowest OTA concentration was detected in locations (Wushishi, range of 0.8 and 38.0µg/kg) in the driest part of the State

In addition, the study revealed co-occurrence of aflatoxins and ochratoxin A in 37/81 (45.7%) of the groundnut samples. Co-occurrence was detected in samples across all the zones evaluated in this study. Furthermore, 72/81 (88.9%) and 45/81 (55.6%) samples had AFB_1 and OTA concentrations above the Nigerian and European Union action limits of 2 µg/kg and 5 µg/kg, respectively.

4.0 Discussion

There are quite a few studies on aflatoxin contamination of groundnut and groundnut products from Niger State (Darling, 1963; Peers, 1965; Okonkwo and Nwokolo, 1978; Opadokun, 1992) in particular and Nigeria in general (Abalaka and Elegbede, 1982; Gbodi, 1986; Akano and Atanda, 1990; Bankole *et al*., 2005; Odoemelam and Osu, 2009; Ezekiel *et al*., 2012). However, except for Ezekiel *et al*. (2012) that reported the presence of 20 fungal metabolites including AFB, AFB_2, AFG_1, AFG_2 and OTA in 29 peanut cake samples analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS), all other investigations in the country were performed using the less sensitive and subjective thin layer chromatographic (TLC) technique. The detection limit of the LC/MS/MS used by Ezekiel *et al*. (2012) for ochratoxin A was 4.0 ug/kg making it less sensitive to the toxin than our method which had an LOD of 0.1 ug/kg.

Table 2: Incidence and concentration (µg/kg) of aflatoxin B_1 (AFB1), AFB_2, and ochratoxin A in groundnuts in Niger State in accordance with rainfall pattern

Mycotoxin	Incidence & concentration (µg/kg)	Zone4 (Driest)	Zone 3 (Dry)	Zone 2 (Wet)	Zone 1 (Wettest)	Total
Aflatoxin B_1	Incidence	17/21	19/19	17/19	19/22	72/81
	Mean ± SE	56.10^{a} ±5.11	74.02^{b} ±5.94	35.44^{c} ±4.25	45.10^{cd} ±4.13	53.04 ±2.57
	Range	8.00 – 165.60	5.60 – 188.00	4.00 – 13.60	6.40 – 141.60	4.00 – 188.00
Aflatoxin B_2	Incidence	14/21	16/19	15/19	16/22	61/81
	Mean ± SE	6.62^{a} ±1.10	9.06^{ab} ±1.23	5.76^{abcd} ±1.23	10.54^{abc} ±1.47	8.08 ±0.63
	Range	0.80 – 32.40	0.80 – 32.80	0.40 – 38.40	0.40 – 36.40	0.40 – 38.40
Ochratoxin A	Incidence	17/21	19/19	19/19	18/22	73/81
	Mean ± SE	13.50^{a} ±1.4	12.64^{ab} ±0.94	15.80^{abcd} ±1.43	13.56^{abc} ±1.23	13.88 ±0.62
	Range	0.80 –38.00	1.20 – 30.40	4.00 – 45.60	1.60 – 40.00	0.80 – 45.60

abc: Means with different superscripts in a row are significantly different from each other ($p<0.05$)

The current study therefore presents for the first time the concentrations of aflatoxins and ochratoxin A in Nigerian groundnut using HPLC. The observed recoveries (above 90% for all studied toxins), LOD and LOQ indicate that the sensitivity and reliability of the methods employed were sufficient for evaluation of aflatoxins and OTA in groundnut. There is no report on OTA contamination of unprocessed groundnut in Nigeria; therefore, the present work to the best of our knowledge is the first survey on OTA in Nigerian groundnut. This survey has revealed that AFB_1, AFB_2 and OTA are common contaminants of raw and roasted groundnut. The observed higher incidence and levels of the studied mycotoxins in samples from open air sources (farm and market) than those from stores could be due to exposure of the nuts to favourable environmental conditions for fungal growth and mycotoxin synthesis in the field and at market than in the stores. High temperature and moisture conditions as well as floods experienced during the sampling period (rainy season of 2010) and the unwholesome transportation means coupled with adverse marketing practices in Africa can contribute to increased fungal proliferation and mycotoxin production (Wagacha and Muthomi, 2008), which might have resulted in increased levels of these toxins in field and marketed samples.

Optimal production of aflatoxins by *Aspergillus flavus* and *A. parasiticus* occurs at temperatures between 25 and 30 ^{o}C and kernel moisture content of about 18%, while optimal OTA production by *A. ochraceus* is attained at temperatures varying between 31 and 37 ^{o}C in grains with moisture content of 22**%** (Ominski *et al*. 1994). The above-mentioned conditions approximate to the ambient conditions in the dry and wet microclimatic zones under study (Umoh, 1997). The zones with extreme humid (wettest zone) and hot (driest zone) conditions are outside the optimal condition range hence may not be suitable for optimal mycotoxin production. It is thus not surprising that aflatoxins and OTA contamination of groundnut as reported in this work is more severe in the dry and wet zones than what was observed in the wettest and the driest zone. This is in agreement with previous data reported by Atehnkeng *et al*. (2008) who found the mean aflatoxin concentration in maize from the humid Southern Guinea Savannah (mainly from Bida) to be higher than those from drier, hotter North Guinea Savannah and the very humid, cooler Derived Savannah of Nigeria.

The absolute levels of aflatoxins reported in the present study are comparable to those observed by Bankole *et al*. (2005) in roasted groundnut (range: 5–165 µg/kg for AFB_1) and Odoemelam and Osu (2009) in raw groundnut (range: 74.03–82.12 µg/kg for AFB_1) but much lower than those reported by Darling (1963) (range: 100–2000 µg/kg), Peers (1965) (range: 100–2000 µg/kg), Okonkwo and Nwokolo (1978) (max: 900 µg/kg), Abalaka and Elegbede (1982) (max: 600 µg/kg), and Opadokun (1992) (max: 8000 µg/kg). Additionally, aflatoxin concentrations reported herein were much lower than the levels found in Nigerian groundnut cake: 20–455 µg/kg (Akano and Atanda, 1990) and 13–2824 µg/kg (Ezekiel *et al.,* 2012).

The extremely high levels of aflatoxins found in the raw and roasted forms of Nigerian groundnut that were above the Nigerian and European Union (EC, 2002) maximum acceptable limits for AFB_1 is of serious public health concern considering that the nut is regularly consumed by the population and the cake used as an inevitable source of protein in animal feed formulations. Of grievous concern is the fact that these highly aflatoxin contaminated products are consumed mostly by school aged children and young adults (Ezekiel *et al*. 2013) who are still in active reproductive and labour ages. Exposure therefore to aflatoxins at such unsafe levels by these vulnerable groups could synergically act with other carcinogens, especially hepatitis B virus, to elicit the high incidence of primary liver cancer observed among the Nigerian population (Olubuyide and Solanke, 1990).

Reports on OTA contamination of foods from Nigeria are only on cocoa and cocoa products (Bankole and Adebanjo, 2003) maize (Gbodi, *et al*. 1986; Adebajo *et al*. 1994), maize-based weaning food (Oyelami *et al*. 1996), sorghum

(Elegbede *et al*. 1982; Makun *et al*. 2009), rice (Makun *et al*. 2007; Ayejuyo *et al*. 2008; Makun *et al*. 2011), kolanut and cocoa bean (Bankole and Adebanjo, 2003) and tiger nut (Adebajo, 1993) but none exists for groundnut in the country. The high presence of OTA in over 90% of the samples with more than one half of the samples having unsafe levels of the nephrotoxin could impact negatively on human (Reddy and Bhoola, 2010) and animal health (Battacone *et al.* 2010). The nephrotoxin has long retention time in serum of pigs and other animals and therefore is persistent carryover of the toxin into edible animal tissues and products (Duarte, Lino and Pena. 2011). This associated public health impact might be aggravated in Nigeria with such high OTA prevalence.

The implications of the simultaneous occurrence of mycotoxins within the same food matrix as shown in this work to human and animal health is complex. However, it has been established that the interactive effects of mycotoxins in combinations could be synergistic, additive or antagonistic in the host organism (Miller, 1995; Speijer and Speijer, 2004). Co-occurrence of AFB_1 and OTA could be synergistic in causing nephropathy and increasing the mutagenic effect of the latter (Sedmikova *et al*. 2001). The anticipated adverse public health impact that could result from exposure to aflatoxins and OTA, singly or in combination, should necessitate the regulation of mycotoxins by the relevant agencies in Nigeria. More so, such enforcement of legislation against mycotoxins will improve Nigeria's access to high-value international trade markets. There is also the need for intensive public enlightenment on the hazards of mycotoxins to farmers and traders of agricultural products in the country.

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ORIGINAL RESEARCH

Simultaneous Occurrence of Aflatoxin and Ochratoxin A In Rice From Kaduna State, Nigeria

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ABSTRACT

Eighty six samples of field, stored and marketed rice (*Oryza sativa*) collected from the traditional rice growing areas of Kaduna state, were analyzed for aflatoxins (AFs) and ochratoxin A (OTA) by high-performance liquid chromatography (HPLC). Aflatoxins were detected in 74.4% of the samples, AFB_1 was found at concentrations between 4-292µg/kg and AFB_2 between 0.4-27.2µg/kg. OTA was found at concentrations between 0.2 µg/kg and 35.6µg/kg but at higher prevalence than aflatoxins. Co-contamination with AF and OTA was common; thirty seven (37) of the rice samples contained both aflatoxins and ochratoxin A. The AFB_1 and OTA levels in 100% and 58% of the rice samples were regarded as unsafe based on Nigerian and European Union maximum permissible levels of 2µg/kg and 5µg/kg respectively. The presence of these toxins at unacceptable concentrations and their multi-occurrences in the rice samples which might exert either additive or synergistic toxic effects in human beings raise concern with respect to public health.

Keywords: Mycotoxins, Aflatoxin, Ochratoxin A, Rice, Nigeria.

1.0 Introduction

Mycotoxins are toxic secondary metabolites produced by fungi and they contaminate different agricultural commodities before or under post-harvest conditions. They are mainly produced by fungi in the *Aspergillus, Penicillium* and *Fusarium* genera (CAST, 2003). When mycotoxins are ingested, inhaled or absorbed through the skin, they cause lowered performance, sickness or death on humans and animals (CAST, 2003). Exposure to mycotoxins can produce both acute and chronic toxicities ranging from deleterious effects upon the central nervous to death, cardiovascular and pulmonary systems, and upon the alimentary tract (CAST, 2003). Other mycotoxins occurring in food have longer term chronic or cumulative effects on health, including the induction of cancers and immune deficiency (CAST, 2003). Mycotoxins may also be carcinogenic, mutagenic, teratogenic and immunosuppressive. The ability of some mycotoxins to compromise the immune response and, consequently, to reduce resistance to infectious disease is now widely considered to be the most important effect of mycotoxins, particularly in developing countries (Coker, 1997). Mycotoxins attract worldwide attention because of the significant economic losses associated with their impact on human health, animal productivity and both domestic and international trade. It has been estimated by Miller (1993), for example, that annual losses in the USA and Canada, arising from the impact of mycotoxins on the feed and livestock industries, are of the order of $5 billion. In developing countries, where the food staples (e.g. rice, maize and groundnuts) are susceptible to contamination, it is likely that significant additional losses will occur among the human population because of morbidity and premature death associated with the consumption of mycotoxins (Miller, 2003).

Rice is one of the most important staple foods in the world. The world has increased its rice production by 27%, or 155 million tonnes, at the current estimate of 482 million tonnes (723 million tonnes of paddy), world rice production would be 3.4 percent larger than in 2010, reflecting a combination of good weather and attractive prices, which encouraged producers to expand the area under rice by an estimated 2.4 percent to 165 million hectares (FAO, 2011). The increase in world production is anticipated to be concentrated in Asia, where the five top rice producing countries: Bangladesh, China, India, Indonesia and Viet Nam, are all heading towards record output (FAO, 2011). The FAO forecast for production in Africa has changed little since September 2011, remaining in the order of 17.0 million tonnes, which is 2.6 percent more than in 2010. Many western countries are implementing expansionary rice production policies.

In particular, output is set to rise vigorously in Benin, Ghana, Mali, Nigeria and Sierra Leone, amid attractive market prices. Nigeria is the largest rice producing country in the West African region and also the largest importer of rice in the world. Rice production rose gradually over the years with area expansion to surpass major rice producing countries like Cote d Ivoire and Sierra Leone (WARDA, 1996). The principal factors driving increased rice production in Nigeria is population growth and urbanization. (WARDA, 1996). The annual demand for rice in the country is estimated at 5 million tonnes. In Nigeria, The North Central zone is the largest producer of rice accounting for about 47% of the total rice output in 2000. This was followed by Northwest (29%) Northeast (14%) southeast (9%) and the least (the southwest (4%) (Nweke *et al,.*1999). Kaduna state is the largest rice producing state in the country accounting for about 22% of the country's rice output, followed by Niger State (16%), Benue State (10%) and Taraba State (7%). (Nweke *et al,.*1999).

Kaduna State is the main traditional rice growing area in Nigeria with the highest yield (Erenston and Lacon, 2003). Rice is commonly eaten as boiled rice and in the northern parts of the country it is taken as paste "tuwo", fermented breads (‘masa') and as unleavened bread (‘Waina'). The Hausa also use it in preparation of a local snack called "nakiya". Due to the fact that rice is a highly consumed cereal and little has been done on the fungi and mycotoxin contaminating it in Kaduna state, this study was undertaken to determine the level and extent of contamination by ochratoxin A, Aflatoxin B_1 and Aflatoxin B_2 under natural conditions in Kaduna state, a leading rice producer. Many scientists in Nigeria (Okoye, 1992) and from other parts of the world (Taligoola *et al*., 2004) have studied and reported the fungi and mycotoxins contaminating rice but there seem to be little or no reports on the fungal and mycotoxin profile of rice in Kaduna State.

Table 1: Kaduna State Local Government Areas (LGAs) according to microclimatic zones

	Zone	Annual Rainfall Range (mm)	LGAs
1	(wettest)	> 1600	Kachia, Sanga, Kaura, Jama’a, Zango/Jaba
2	(Wet)	1200 – 1600	Kagarko, Birni Gwari, Kaduna North, Kaduna south, Chikun, Saabon- Gari
3	(Dry)	1000 – 1200	Zaria, Ikara, Kudan, Makarfi, Soba, Igabi, Kubaun, Lerea, Giwa
4	(Driest)	<1000	Kauru

2.0 MATERIALS AND METHODS

All chemicals used were of Analar grade and manufactured by May and Baker LTD (Dagenham England unless otherwise stated.). Silica gel 60-120 mesh, petroleum spirit (60-80^{oc}), n -Hexane, Orthophosphoric acid, methanol, sodium sulphate anhydrous, sulphuric acid, sodium hydrogen carbonate, methylene chloride. Mycotoxin standards of aflatoxins (B_1

and B_2) and OTA standards were obtained from Sigma, St. Louis, Mo., USA. HPLC was fitted with ZORBAX Eclipse XDB-C18, 4.6mm X 150mm, 3.5µm column.

2.1 Sampling

Dry sample of rice were randomly collected during the rainy season (April to October) from the twenty two local government areas of Kaduna state (Table1). Stored, marketed and field samples were collected. The field samples were collected shortly before the harvest period, the stored samples were collected from traditional storage facility called rumbu (a locally built mud barns) and the marketed samples were collected from the rice sellers in the various markets. About 1.0 kilograms of each sample were collected, labeled, packaged in a plastic bottle which were properly sealed and taken to the laboratory. In the laboratory, the samples were ground into fine powder with the aid of an electric blender, the powder were stored in the cupboard for mycotoxin analysis.

2.2 Analysis of mycotoxins.

The samples were screened and analyzed for aflatoxin B_1, B_2 and ochratoxin A using a multi-mycotoxin assay method (Ehrlich and Lee, 1984) without modification. In the method, methylene chloride and phosphoric acid were used for the simultaneous extraction of AFB_1, B_2 and OTA. A separate portion of the initial methylene chloride/phosphoric acid extract was subjected to a specific clean-up procedure for each mycotoxin.

2.2.1 Extraction of Mycotoxins

About 50g portion of pulverized rice samples was weighed into 500ml Erlenmeyer flask and 25ml 1M-phosphoric acid and 250ml of methylene chloride were added. The flask was shaken for 30 minutes using a shaker and the content filtered under pressure on Buchner funnel fitted with 18 cm circle rapid filter paper. About 200ml of the filtrate was collected and from this, 50ml aliquot each was placed in separate 100ml Erlenmeyer flasks with glass stoppers, for AF and OTA assay.

The fractions for aflatoxin analysis were subjected to a specific column chromatographic clean up method. To this end, a column was set up with glass wool, 150ml dichloromethane (DCM) poured into the column and emptied half way. Anhydrous sodium sulphate (Na_2SO_4) was added, the sides of the column were washed with DCM. Silica gel was added to green line of column and 80ml DCM added again, and this was allowed to settle half way. Three scoops of sodium sulphate (Na_2SO_4) was added and drained off to top of column. About 50ml of the filtrate was added and drained off to top of column. The filtrate was defatted with 130ml hexane and 130ml ether sequentially and each fraction drained to dump. Aflatoxins were extracted into 130ml ether: methanol: water (96:3:1) that was collected off column in a new beaker. The extract was evaporated to near dryness, put into vials and stored at 0^oC until used for analysis.

A different clean up method to that of aflatoxin was used for ochratoxin A. Using separatory funnel the toxin was extracted into aqueous sodium bicarbonate solution (4gm $NaHCO_3$/100ml distilled water) which was acidified to pH 2 with H_2SO_4 to obtain an acid fraction. OTA was further extracted from the acid fraction into dichloromethane (by rinsing with 25ml of DCM thrice). The pooled DCM fractions was drained through Na_2SO_4, evaporated and stored in amber glass vials at 0^oC until used for analysis.

2.2.2 High Pressure Liquid Chromatographic Technique

Aflatoxins were analyzed on on Cecil 1100 series HPLC with UV detection as described by Cora *et al*. (2005) at wavelength of 365nm. The altraspher ODS column, 4.6mm x 25cm was used at ambient temperature of 25^oC. Acetonitrile : water and acetic acid in ratio 10:50:40 v/v/v respectively was used as mobile

phase at flow rate of 0.8ml/min. The injection volume was 20 µl.

The analyses were carried out with aflatoxins standards (Sigma Chemical Company, St. Louis, MO, USA) of known concentrations with $AFB_1$and AFB_2 eluting at distinct retention time of 1.673 and 1.524 respectively. Calibration curves with correlation factors of 0.91 and 0.99 were obtained for $AFB_1$and AFB_2, respectively using series of dilutions containing 0.005µg/ml, 0.01µg/ml, 0.015µg/ml, 0.02µg/ml and 0.025µg/ml of each of the standard. The detection limit of the machine with regards to the toxins was 0.21µg/kg. About 10 µg/ml of AFB_1 and AFB_2 were spiked in 3 samples of rice in order to determine the recovery rates. The mean ± standard deviation obtained for the two toxins were 98.5±3.2% and 89.3±2.5% respectively.

OTA was quantified on same HPLC machine with UV detection as described by Engstrom, Richard and Cysewski (1977) at wavelength of 254nm. The operating temperature was ambient temperature of 25^{o}C. Acetonitrile : water and acetic acid in ratio 50:48:2 respectively was used as mobile phase at flow rate 1ml/min. The injection volume was 60 µl. Calibration curve with a correlation factor of 0.925 was determined using series of dilutions containing 0.023 µg/ml, 0.018 µg/ml, 0.014 µg/ml, 0.009 µg/ml and 0.004 µg/ml. The retention time for OTA was 1.11 minutes while the detection limit of the machine with regards to the toxin was 0.1 µg/kg. 10µg/ml of OTA was spiked in 3 samples of each food commodity and recovery rates determined. The Mean ± standard deviation recovery rates obtained for OTA was 99.1±6.1 The observed recoveries indicate that the sensitivity and reliability of the methods employed were sufficient for evaluation of aflatoxins and OTA in rice. The concentrations reported were adjusted based on recovery rates obtained.

3.0 Results

3.1 Mycotoxin Contamination of Rice

Aflatoxin B_1 and B_2 were detected in the samples from all the twenty two local government areas. Eighty six rice samples were analyzed for aflatoxin B_1 and 64 were contaminated with the toxin at concentrations between 4-292µg/kg with a mean value of 66.028µg1kg, Kauru had the highest occurrence of aflatoxin B_1 (157.34µg/kg) and lowest incidence was observed in Giwa. (14.66µg/kg). Similarly, of the 86 rice samples analyzed for Aflatoxin B_2, 41 were contaminated with the toxin at concentrations of between 0.4-27.2µg/kg with a mean value of 5.168µg/kg. The occurrence was highest in Makarfi (27.2µg/kg) with lowest incidences in Giwa and Birnin Gwari..

The results of mycotoxin determination according to the four microclimatic zones of Kaduna State as well as the sample sources are presented on Tables 3 and 4. Aflatoxin B_1 was a common contaminant of rice from all the four microclimatic zones of the state. The mycotoxin contents were significantly higher in samples from the driest part of the State (zone 4) than those from the other zones. There were no significant ($p<0.05$) differences between aflatoxin B_1concentrations in samples from zone I, II and III. Similarly, higher incidence of the toxin was recorded in zone IV (100%) than zones I (75%), II (66.66%) and III (78.13%). The incidence of the mycotoxin was lowest in the field samples (45.45%) when compared with the marketed (70.83%) and stored (69.44%) samples. However, there were no significant differences between the aflatoxin B_1 concentrations of the marketed, stored and field samples.

The rice samples collected were also analyzed for aflatoxin B_2, 41 of the 86 samples analyzed contained the toxin (0.4-27.2 µg/kg). There were no significant differences ($p<0.05$) in both the concentrations and incidence of aflatoxin B_2 present in the 41 positive sample analyzed from the marketed stored and field and from the four zones. The incidence and concentrations of the mycotoxins determined are presented on Table 2. Ochratoxin A was detected in samples from twenty two local government areas of Kaduna state.

Table 2: Summary of the Incidence of Mycotoxins Contamination of Rice in Kaduna State According to the 22 Local Govt. Areas

		OCTRATOXIN A				AFLATOXIN B_1				AFLATOXIN B_2			
S/N	**LGA**	**NSA**	**NPS**	**RANGE**	**MEAN± S.D**	**NSA**	**NPS**	RANGE	MEAN± S.D	NSA	NPS	RANGE	MEAN± S.D
1.	Birni Gwari	3	3	2.4-19.2	11.47±2.45	3	3	8-108	49.34±15.06	3	1	0.8	0.8±0.003
2.	Chikun	6	5	0.4-11.2	4.00± 0.95	6	3	40-80	64.0± 6.11	6	2	1.6-4.8	3.2±0.800
3.	Igabi	7	6	1.6- 4.8	5.80 ±0.59	7	6	20-140	66.66±10.49	7	3	1.6-7.2	374±0.880
4.	Ikara	2	2	1.2- 2.0	2.60 ± 0.70	2	2	20-204	112±46.00	2	2	0.4-2.4	1.4±0.500
5.	Jamaa	6	4	1.6 – 24.4	11.00 ±2.83	6	5	32-116	68.0±7.01	6	3	2.4-22.4	13.6±2.9
6.	Kaduna South	5	4	4.0 – 20.0	11.50 ±1.89	5	4	4-140	80.0±14.17	5	3	4.8-12.0	7.74±1.09
7.	Kaduna North	2	1	0. 2	0.20 ± 0.00	2	1	68	68.0±0.00	2	0	-	-
8.	Kachia	6	6	0.4 – 15.2	7.86 ±1.14	6	4	4-76	49±7.80	6	2	2	0.8-9.6	5.2±2.20
9.	Kagarko	6	5	0.2.- 22.4	6.04 ± 2.09	6	5	4-120	68.0±10.83	6	4	0.8-9.6	3.48±1.02
10.	Kaura	6	4	4.4-16.8	9.20±1.43	6	5	8-76	48.0±6.16	6	4	1.6-4.8	2.8±0.383
11.	Kauru	3	3	10.4-18.0	14.54±1.11	3	3	56-292	157.34±35.07	3	2	4.8-12.8	8.8±2.00
12.	Zangol Jaba	4	4	6.4-35.6	17.10±3.20	4	2	76-160	118±21.00	4	1	3.6	3.6±0.00
13.	Kubaun	2	2	6.4-10.4	8.40 ±1.00	2	2	40-72	56.0±8.00	2	2	0.8-4.4	2.6±0.90
14.	Kudan	2	2	1.6-4.0	2.80±0.60	2	1	48	48.0±0.00	2	1	1.6	1.6±0.00
15.	Lerea	4	4	0.4-14.0	6.10±1.44	4	2	40-76	58.0±9.00	4	2	0.8-2.4	1.6±0.40
16.	Sanga	2	2	0.2-4.8	2.50±1.15	2	2	60-108	84.0±12.00	2	1	14.4	14.4±0.00
17.	Sabon Gari.	5	4	0.4-18.8	7.80±2.22	5	2	60-108	84.0±12.00	5	1	2.4	2.4±0.00
18.	Zaria	7	6	0.2-16.24	11.20±1.16	7	6	4-144	68.0±11.88	7	3	3.6-15.2	7.76±1.86
19.	Makarfi	3	2	7.2-18.8	13.00±2.90	3	3	24-56	44.0±5.03	3	1	27.2	27.2±0.00
20.	Soba	2	2	7.2-11.2	9.20±1.00	2	0	-	-	2	0	-	-
21.	Giwa	3	1	3.2	3.20±0.00	3	3	8-56	14.66±8.62	3	3	0.4-1.6	0.8±0.01
	Total	**86**	**72**	**0.2-35.6**	**8.83±0.43**	**86**	**64**	**4-292**	**66.03±3.08**	**86**	**41**	**0.4-27.2**	**5.17±0.43**

Key: NSA- Number of samples analyzed, NPS- Number of positive samples

Of the 86 rice samples analyzed for OTA, 72 were contaminated with the toxin at concentrations of between 0.2-35.6µg/kg with a mean value of 8.832µg/kg. The occurrence of ochratoxin A. in rice was highest in Zangon Jaba (17.1µg/kg) with lowest incidences observed in Kaduna North (0.2µg/kg). The ochratoxin A concentrations in samples from the four microclimatic zones were not significantly ($p<0.05$) different, however, higher incidence of the toxin was recorded in zone IV (100%) than zones I (83.33), II (81.48) and III (84.38). There were no significant differences between the concentrations of ochratoxin A from the marketed, stored and field samples but the incidence of the mycotoxin was lowest in stored samples (66.67%) when compared with the marketed (81.25%) and field (81.8) samples.

Out of the 86 rice samples analyzed, aflatoxin B_1 and B_2 occurred together in forty five (45) samples, thirty seven (37) samples contained both the aflatoxins and ochratoxin A and 43 samples were contaminated with ochratoxin A alone.

4.0 Discussion

Rice (*oryza sativa*), is highly cultivated and consumed worldwide and this makes it one of the most important principal sources of mycotoxins to human beings and animals in the world. According to data tracked by the Food and Agricultural organization (FAO, 2011), world rice production is expected to increase to 456.2 million tonnes while consumption is expected to rise to 455.2 million tonnes. Aflatoxins and ochratoxin A are among the five most significant and abundant mycotoxins contaminating foods and feed stuffs in the world (Bhat and Vasanthi, 2003), and the results obtained in this study indicate that they are also major contaminants (Ochratoxin A, Aflatoxin B_1 and Aflatoxin B_2 in decreasing order of prevalence) of rice in Kaduna State, Nigeria.

The insignificant differences in incidence and concentrations of toxins observed due to geographical locations and types of samples i.e. field store and market samples might be as results of the seeds' microclimatic and physiological conditions. Aflatoxin B_1,and B_2 incidences and concentrations were higher in stored and marketed samples than field samples because crops are mostly infected with fungi from field due to environmental factors, farming system or insect infestation; these field fungi persist and proliferate with consequence increase in mycotoxin formation during storage when favourable conditions persist (Miller, 1995). This might have led to high incidence of aflatoxins recorded in this work in stored rice samples (store and market) than in field samples.

Although ochratoxin A incidence and contents exhibited a decreasing order from field to market and then store, the decreasing trend could be attributed to increased effectiveness of the traditional storage facilities "rumbu" (in Hausa) against ochratoxin producing fungi (Udoh *et al*., 2000), that are built on raised platforms that prevent rodent and insect attack, moisture from getting to grains and also provide anaerobic conditions within it. Such conditions can reduce fungal growth and consequently mycotoxin production (Javis, 1971). It could also be as a consequence of the pre-storage sun drying of newly harvested grains on dry surfaces rocks (Awuah and Ellis, 2002) by farmers as observed by the researchers during sampling. These processes significantly reduce the fungal and mycotoxin contamination (Hell *et al*., 2000) and might therefore, account for the consistently lower incidence and mycotoxin contents in stored samples compared to those from the field and market. Of the four microclimatic zones, a general higher incidence of toxins was observed in the driest zone (Zone IV). It could be that the stress on the crop due to excessive heat, agricultural management practices such as: irrigation, crop rotation, methods of harvesting in the zone have created unique ecological niches that promote the toxigenic potential of strains of the species of fungi isolated (Bilgrami *et al*. 1981).

Table 3: Summary of the Incidence of Mycotoxins Contamination of Rice in Kaduna State According to Microclimatic Zones

Microclimatic Zone		aflatoxin B_1	Aflatoxin B_2	Ocharatoxin A
Zone I	Mean±S.D	65.56± 4.38 [a]	7.32±1.10 [a]	10.08± 0.99 [a]
		4- 160	0.8-22.4	0.2-35.6
	Range	18/24	11/24	20/27
	nc/ ns			
Zone II	Mean±S.D	68.66±4.38 [ab]	4.26±0.57 [ab]	7.28±0.80 [ab]
		4-140	0.8-12.0	0.2-22.4
	Range	18/27	11/27	22/27
	nc/ns			
Zone III	Mean±S.D	60.96±5.01 [abc]	4.52±0.8 [abc]	7.46±0.49 [abc]
		4-204	0.4-27.2	0.4-18.8
	Range	25/32	17/32	27/32
	nc/ns			
Zone IV	Mean±S.D	157.34±35.07 [d]	8.80±1.16 [abcd]	14.54±1.11 [abcd]
		56-292	4.8-12.8	10.4-18
	Range	3/3	3/3	3/3
	nc/ns			

abcd: Means with different letters (superscripts) along column were significantly different from each other ($P<0.05$)

Aflatoxin data found here when compared with those of other studies are in conformity with the findings of Tanaka *et al*. (2007) that mycotoxin contamination is less commonly reported for rice than other crops. Such postulation is based on data reported here and others (Opadokun and Ikeorah, 1979; Ibeh *et al.,* 1991; Obidoa and Gungani, 1992, Ikeorah and Okoye, 2005; Atehnkeng *et al*., 2008), which reveal much lower levels in Nigerian rice (maximum of 174 µg/kg) than other crops especially maize, groundnuts (range: 2.2 to 2000 µg/kg). The higher seed coat integrity of rice seed acts as a barrier against fungal invasion (Stossel, 1986), thus limiting fungal growth and consequent mycotoxin production in rice relative to that of maize, groundnut and others that have less formidable coat and hence are excellent substrates for mycotoxin production.

Aflatoxin B_1 is the most toxic amongst the two aflatoxins studied ($B_1$and B_2), it is an important contaminant of food and feed crops before, during and after harvest (Shananah *et al*., 2003) and it is well establish that it is both carcinogenic and cytotoxic. The findings in this study showed that the rice samples had aflatoxins B_1: 100%, and B_2 : 22.09% and with levels exceeding acceptable limits (2 and 4µg/kg respectively) set by the Nigeria and European Union. The unwholesome quantities of aflatoxins found in the rice samples (Aflatoxin B_1 = 4 - 292µg/kg and B_2 = 0.4 - 27.2µg/kg) which though are all lower than levels (1600 – 12,000 µg/kg) that caused deaths in the two fatal outbreaks of AF poisoning in Kenya (Afla-guard, 2005), could when ingested chronically, synergistically interact with other cancer promoters especially hepatitis B virus to elicit high primary liver cancer incidence observed in Nigeria (Fakunle *et al.* 1977), which has previously been identified as the most common malignant tumour seen in medical wards (Olubuyide *et al*., 1986). It is reported to be the commonest cause of death from cancer in the middle aged (Junid, 1979) and elderly populations (Olubuyide and Solanke, 1990) in the country. Apart from causing liver cancer, continuous intake of AF at low doses could increase still-births and neonatal mortality (Maxwell *et al.*, 1998), immunosuppression with increased susceptibly to infectious diseases such as pneumonia (Oyelami *et al*.1997) and HIV/AIDS (Lane, 2005). Intake of AF has also been associated with stunted growth (Gong *et*

al., 2002, 2003, 2004) and aggravation of protein malnutrition in children (Adhikari *et al*, 1994).

Ochratoxin A contamination of cocoa and cocoa products in Nigeria has been well documented (Bankole and Adebanjo, 2003) but very few reports of its incidence in other crops from the country are available. High level of up to 150 µg/kg of the toxin was detected in maize (Sibanda *et al*., 1997) and mouldy rice (Makun *et al*., 2007 and Makun *et al.*, 2011)) from Northern Nigeria. Ayejuyo et al. (2008) found very low levels of OTA (maximum: 2.18 µg/kg) in samples of imported rice marketed in Lagos metropolis but the range observed in this study was (0.2 - 35.6 µg/kg).

The OTA contents in imported rice were all below the international regulatory limit of 5 µg/kg than those found in the present study (58.02% unsafe), which could be because there must have been compliance to the international regulatory limits at the point of processing, packaging and import. This evaluation of mycotoxins in Nigerian rice gives the quality of the cereal with regards to its acceptability for human and animal consumption. Ochratoxin A is nephrotoxic, teratogenic, carcinogenic and immuno-suppressive in many animal species (Stoev, 1998 and International Agency for Research on Cancer (IARC),1993). The international Agency for Research on Cancer has classified OTA as possibly carcinogenic in humans (group 2B carcinogen) (IARC, 1993). The demonstrated presence of AFs and OTA at concentrations above the limits acceptable to world mycotoxin regulatory agencies and the co-occurrences of toxins with possible toxic synergistic effects make these studied rice samples of low quality for human and animal consumption and in fact raises preliminarily national public health concerns.

With such simultaneous occurrences of unrelated mycotoxins (Rizzo *et al*. 2004) in similar samples, this will certainly increase the severity of health-related problems generated from consumption of such contaminated food products as consumption of multiple mycotoxin in foods may exert both synergistic and additive effects (Placinta *et al*. 1999; Casado *et al*. 2001; Creppy *et al*. 2004; Speijer and Speijer 2004; Luongo *et al*. 2008) in both animal and man.

Sedmikova *et al.*, (2001) reported the possibility of OTA increasing the mutagenic ability of aflatoxin B_1 in cases of simultaneous occurrence of the two mycotoxins in the same crop.

Table: 4. Summary of the Incidence of Mycotoxin Contamination of Rice in Kaduna State According Sample Sources

Sample source		Aflatoxin B_1	Aflatoxin B_2	Ocharatoxin A
Market	Mean±S.D	75.18± 4.39 [a]	5.34±0.72 [a]	8.72± 0.65 [a]
		4-292	0.4-27.2	0.2-35.6
	Range	34/48	20/48	39/48
	nc/ ns			
Store	Mean±S.D	69.92±5.20 [ab]	6.08±0.86 [ab]	7.14±0.56 [ab]
		4-204	0.4-22.4	0.2-15.2
	Range	25/36	16/36	24/36
	nc/ns			
Field	Mean±S.D	42.40±12.48 [abc]	3.54±0.46 [abc]	10.54±1.08 [abc]
		4-140	1.6-6.4	0.4-20.0
	Range	5/11	5/11	9/11
	nc/ns			

abc: Means with different letters (superscripts) along columns were significantly different from each other (P<0.05)

5.0 Conclusion

The demonstrated presence of aflatoxins and ochratoxin A in this highly consumed foodstuff at unsafe levels renders them the problematic mycotoxins in Nigerian rice. Therefore, rice can be regarded as a major source of mycotoxin exposure in Nigeria. In view of the forgoing, it is recommended that studies to elucidate the possible aetiologic roles of AFs and OTA in the increased incidences of liver cancer and nephropathy should be conducted in Nigeria. Regulating these toxins in foods in Nigeria is therefore also an imperative.

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ORIGINAL RESEARCH

Aflatoxigenic moulds and aflatoxin contamination of retailed fishery products in Lagos markets

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ABSTRACT

Forty fishery products (27 fin fish, 4 shell fish and 9 fish feed samples) were obtained from markets in Lagos and analyzed for the presence of aflatoxigenic moulds and aflatoxins with the view of assessing the quality of these products. Mycological analysis was performed by the dilution plating technique while aflatoxin analysis was by an Enzyme-Linked Immunosorbent Assay method. *Aspergillus flavus* and *A. tamarii* were the only *Aspergillus* section *Flavi* species recovered from all fish feed, 50% of shell fish and 37% of fin fish samples. *Aspergillus flavus* occurred more frequently ($p<0.05$) than *A. tamarii* in each category of fishery product. The incidence of non aflatoxigenic *A. flavus* isolates was higher than that of aflatoxigenic *A. flavus* isolates in all categories of fishery products. All fish and fish feed samples contained aflatoxins at concentration ranges of 1.05–25.00 µg/kg. Fish feed samples contained significantly ($p<0.05$) more aflatoxins than the fish samples. Total aflatoxin levels exceeded 10 µg/kg and 20 µg/kg in 66.67% and 22.22% of fish feed respectively. Smoked-dried fin and shell fishes may therefore represent a safe food for human consumption in view of mycotoxin contamination. Furthermore, efforts should be intensified to lower aflatoxin levels in feed formula fed to fishes.

Key words: Aflatoxin, *Aspergillus* species, Fish feed, Food safety.

1.0 Introduction

Fish is an important source of dietary protein and minerals, and from the economic standpoint, it is a source of income to many people in developing countries. Fish is highly perishable due to its high moisture and fat content. According to Akande and Tobor (1992), freshly caught fishes in artisanal fishery are usually covered with damp sack and sometimes wet grass in order to maintain a conducive temperature and to delay spoilage prior to further processing. However, this practice can serve as a source of microbial contamination to the harvested fish if processing is delayed. Additional sources of contamination include poor sanitary condition of handlers and market environs, and improper storage conditions of the fishes. In developing regions such as in sub-Saharan Africa where electricity is unstable in rural areas, fish is preserved more often through a traditional process involving salting, sun drying, smoking and a further air drying during sale. The step-wise process reduces the moisture content, limits the range and quantities of microbial flora that invade and colonize the smoke-dried fishes, and imparts flavour on the fishes. This in turn contributes to the extension of the shelf-life of the fishes (Park, 2002). However, in humid conditions, smoke-dried fish can absorb moisture from the atmosphere and this could lead to the fungal contamination and subsequent mycotoxin formation, thereby posing a challenge to the consumers of the product (Park, 2002).

Few studies have reported the presence of aflatoxins; highly toxic compounds naturally produced by *A. flavus*, *A. parasiticus* and some microsclerotial species of *Aspergillus* section *Flavi* (Pildain *et al*., 2008), in fish and fish feed (Adebayo-Tayo *et al*., 2008; Adejola, 2011; Almeida *et al*., 2011; Arowora *et al*.,

2012; Barbosa *et al*., 2013). In addition, the hazardous effects of aflatoxin contaminated feeds on fish health and production have been documented (Jantrarotai and Lovell, 1990; Hussein *et al*., 2000; Shehata, 2003; Abdelhamid *et al*., 2007; Zaki *et al*., 2008; Ruby *et al*., 2013). In spite of the available data on aflatoxin contamination of various foodstuffs and other livestock feeds, and the associated health implications, little or no data exists on the occurrence of *Aspergillus* species and aflatoxins in fin and shell fishes in Nigeria despite the fact that both fishes are widely consumed in Nigeria due to their high nutrient profiles. Adebayo-Tayo *et al*. (2008) had reported that fishes have the best sources of protein that is better digested than that of beef.

This survey work was therefore embarked upon to assess the presence and quantities of aflatoxigenic fungi and aflatoxins in some common smoked-dried fish and fish feeds sold in Lagos markets. This study will be useful for fish farmers and consumers as well as public health specialists.

2. 0. Materials and methods

2.1 *Sample collection and preparation*

Forty fishery products (27 fin fish, 4 shell fish and 9 fish feed samples) were obtained from fish retailers in Lagos markets. The fish samples had been smoked-dried and only fish and feed samples that showed no trace of visible mouldiness were purchased. The fish samples were randomly purchased from three markets (Oto, Mile 12 and Lagos Island) while the feed samples were obtained from Oko-oba market, Lagos. Each sample was collected as a bulk 1.5 kg representative sample obtained from several parts of the retailers' stock, placed in sterile polythene bags, labelled appropriately and taken to the laboratory for storage at 4^{o}C.

Fungal and aflatoxin analyses were performed on all samples within 48h of collection. Prior to analyses, each sample was ground into fine powder using a high speed blender and batched into two parts: part A for fungal analysis and part B for aflatoxin analysis. Each batch weighed 25 g as a representative sub-sample; this was taken for mycological and aflatoxin examination.

2.2 *Isolation and characterization of Aspergillus section Flavi from fishery products*

Mycological analysis was carried out on all part A sub-samples to assess the incidence of species belonging to *Aspergillus* section *Flavi*. Moulds were isolated from the samples by the dilution plating technique (Samson *et al*., 1995) and characterized by the phenotyping method for *Aspergillus* section *Flavi* described by Ezekiel *et al*. (2014a). Ten grams of each ground sub-sample was diluted with 90 ml of 0.1 % sterile peptone water, vortexed for 2 min and 1 ml aliquot of the sample was inoculated on triplicate plates of acidified ¼ strength Potato Dextrose Agar (PDA). Acidification of PDA was by addition of 0.01% lactic acid per litre of PDA. The inoculated plates were incubated at 30 ^{o}C for 3–5 days.

All isolates that bore a resemblance to *Aspergillus* section *Flavi* species were transferred from the PDA plates to neutral red desiccated coconut agar (NRDCA) plates and incubated at 30^{o}C for 5 days. The phenotyping of all isolates was performed by making (1) three-point inoculation/plate of each isolate in order to examine consistency in morphological (macroscopic and microscopic) characters, and (2) single-point inoculation/plate of each isolate for qualitative assessment of aflatoxigenicity (Ezekiel *et al*., 2014a).

2.3 *Qualitative determination of aflatoxigenic potential of isolates*

The isolates were also qualitatively screened for their ability to produce aflatoxins by observing the obverse and reverse sides of each single-point inoculated NRDCA plate at 24-hour interval under long wave UV light (365 nm) until the third day for maximum fluorescence (Ezekiel *et al*., 2014a). Pigmentation, fluorescence and characteristic colour of fluorescence were used as indices to evaluate the ability of each isolate to produce aflatoxins (Ezekiel *et al*., 2014a). The identity of each isolate was confirmed to belong to one of the following species of *Aspergillus* section

Flavi: *A. flavus* L-strain, *A. minisclerotigenes*, *A. parvisclerotigenus* and *A. parasiticus*. For confirmation of *A. tamarii* isolates, suspected isolates on NRDCA were transferred to 5/2 agar (5% V-8 juice and 2% agar, pH 5.2) and incubated at 30 ^{o}C for 5 days (Diedhiou *et al*., 2011).

2.4 *Analysis of fishery products for aflatoxins*

Assessment of fish and fish feed samples for levels of aflatoxin contamination was carried out by an Enzyme-Linked Immunosorbent Assay (ELISA) method in which free aflatoxin in the samples and control compete with enzyme-labelled aflatoxin conjugate for the antibody-binding site. This was performed as follows using the R-BIOPHARM (Darmstadt, Germany), –Ridascreen Aflatoxin Total Test kit (Art. No. R4701).

Five grams of each part B sub-sample was weighed into an extraction bottle and 25 ml of 70 % methanol added. The mixture was shaken vigorously for about 10 min and allowed to settle. The supernatant was filtered through Whatman No.1 filter paper and the filtrate was collected into a collecting tube. About 100 µL of the filtrate was then diluted with 600 µL distilled water and 50 µL of this mixture was taken for the assay. About 50 µL each of standard solution, enzyme conjugate and antibody solution were successively added to 50 µL of the samples in duplicate microtiter wells. The mixture was gently shaken and incubated in the dark at ambient temperature for 30 min. After incubation, the microtiter wells were emptied of the mixture and washed three times with washing buffer. About 100 µL of the substrate/ chromogen was then added to each well, mixed gently and incubated in the dark at ambient temperature (30^{o}C) for 15mins. This was followed by the addition of 100 µL of the stop solution to each well and subsequent measurement of the absorbance at 450 nm using Convergys ELR 96X ELISA Reader (Convergent Technologies GmbH & Co. KG, Germany).

The results were then extrapolated from a standard curve using RIDA SOFT Win (Art. No. Z999) software and reported as µg/kg total aflatoxin of the samples.

2.5 *Data analysis*

All the data obtained were analyzed by the SPSS® 14.0 Windows version (SPSS, IL, ISA). Means were calculated, tested for significant difference at α = 0.05 using the one-way ANOVA and separated by the Duncan's Multiple Range Test.

3.0 Results

3.1. *Incidence of Aspergillus section Flavi isolates*

The incidence of *Aspergillus* section *Flavi* isolates in the fishery products from Lagos markets are shown in Table 2. All fish feed, 37% of fin fish and 50% of shell fish samples were contaminated with *Aspergillus* section *Flavi* isolates. A total of 109 isolates (fish feed-59, fin fish-41 and shell fish-9) were isolated. There was no significant difference in the microbial load (cfu/g) of the *Aspergillus* section *Flavi* isolates in the fishery products although the load was highest in the fish feed samples. In particular, only *A. flavus* and *A. tamarii* species were found in the samples.

Table 1: Incidence of *Aspergillus* section *Flavi* isolates in fishery products from Lagos state, Nigeria

Fishery product	*N*[a]	Mean CFU/g[b]	*N*[c]	Incidence[d] (%) of species	
				A. flavus	*A. tamarii*
Fish feed	9/9	590a	59	49 (83.05)a	10 (16.95)b
Fin fish	10/27	380a	41	36 (87.80)a	5 (12.20)b
Shell fish	2/4	450a	9	9 (100.00)a	0 (0.00)b

[a]Number of samples contaminated with propagules of *Aspergillus* section *Flavi* isolates.
[b]Means of colony forming unit per gram of fishery products analyzed.
[c]Number of *Aspergillus* section *Flavi* isolates obtained from each fishery product.
[d]Incidence of species with different alphabets in a row are significantly different (p<0.05).

Table 2: Aflatoxin contamination of fishery products from Lagos state, Nigeria

Fishery product	N^a	Min	Max	Mean[b] ± SD	N^c (%) >10	N^c (%) >20
Fish feed	9/9	4.97	25.00	13.69a ± 7.18	6 (66.67)	2 (22.22)
Fin fish	27/27	1.05	10.00	5.40b ± 2.40	0 (0.00)	0 (0.00)
Shell fish	4/4	4.23	5.90	5.23b ± 0.77	0 (0.00)	0 (0.00)

[a]Number of samples contaminated with aflatoxins.
[b]Mean values with different alphabets are significantly different ($p<0.05$).
[c]Number of samples positive for aflatoxins above the specified limit of 10 µg/kg and 20 µg/kg.

The incidence of *A. flavus* (83.05–100.00%) was significantly ($p<0.05$) higher than that of *A. tamarii* (12.20–16.95%) in the fishery product. Only 20.40% , 11.11% and 22.22% of *A. flavus* isolates in the fish feed, fin fish and shell fish samples, respectively, produced aflatoxins on NRDCA during phenotyping while all *A. tamarii* isolates did not produce any aflatoxin (data not shown).

3.2. Occurrence of aflatoxins in processed fish and fish feed samples

Table 1 shows the total aflatoxin contamination of the fishery products. All fish and fish feed samples contained aflatoxins and the concentrations of total aflatoxins ranged between 1.05–25.00 µg/kg. The concentrations of aflatoxins in the fish feed samples (range = 4.97–25.00 µg/kg; mean = 13.69 µg/kg) were significantly ($p<0.05$) higher than the concentration in the fish samples [fin fish (range = 1.05–10.00 µg/kg; mean = 5.40 µg/kg); shell fish (range = 4.23–5.90 µg/kg; mean = 5.23 µg/kg)]. Total aflatoxin levels exceeded 10 µg/kg and 20 µg/kg in 66.67% and 22.22% of fish feed respectively.

4. Discussion

Consumption of aflatoxin contaminated feed has been a major challenge in fish farming as it causes reduced growth and several pathologies in different fish and shrimp species (Ruby *et al*., 2013; www.biomin.net). This study has shown that fishery products retailed in Lagos markets are contaminated with aflatoxigenic fungi and aflatoxin, albeit at low-to-moderate levels. Fungal propagules may have been introduced into the fish samples during handling or through improper storage of the fish or as a result of poor sanitary conditions of the markets. The recorded dominance of *A. flavus* over *A. tamarii* in the fish and fish feed samples conforms with previous studies on fishery products (smoked-dried fish and fish feed) by Wheeler *et al*. (1986), Atapattu and Samarajeewa (1990), Fafioye *et al*. (2002), Essien *et al*. (2005), Adebayo-Tayo *et al*. (2008), Almeida *et al*. (2011) and Barbosa *et al*. (2013) that isolated *A. flavus* as the predominant member of the *Aspergillus* section *Flavi* group. This possibly indicates that the nutrient profiles of fish, water activity levels, competition from related fungal species as well as other unknown factors may be responsible for the absence or non-recovery of other members of the section *Flavi* especially the highly toxigenic species (e.g. *A. minisclerotigenes* and *A. parvisclerotigenus*). However, this is the first report of *A. tamarii* occurrence in smoked fish in Nigeria.

The levels of aflatoxins in the present study is similar to that obtained from smoked-dried fish from Uyo (South-South Nigeria) (range = 1.5–8.1 µg/kg (Adebayo-Tayo *et al*., 2008) but higher than the levels in smoked-dried fishes from Abeokuta (South-Western Nigeria) (range = 0.03–1.15 µg/kg) (Adejola, 2011). Furthermore, aflatoxin levels in the smoked-dried fish were within the acceptable limit of 10 ppb. However, this does not guarantee the safety of the fishes for human consumption since (1) they are used on a daily basis for soups and stews in almost every home in Nigeria, and (2) continuous consumption of foods contaminated with low-to-moderate aflatoxin levels over a period of time can increase population exposure (Ezekiel *et al*., 2014b). This may further contribute to chronic aflatoxicosis which is characterized by hepatocellular carcinoma, severe immune

suppression and growth retardation especially in children (Eaton and Groopman, 1994; Turner *et al*., 2002, 2003). Therefore there is the need to implement good post-harvest practices for fish processing (e.g. application of longer heat- and air-drying) and to continuously monitor smoked-dried fishes and other fishery products retailed in local markets in Nigeria for aflatoxin levels so as to ensure their safety for consumption.

The higher level of aflatoxins in the fish feed samples as compared to the fish samples is supported by the fact that contamination of feed is more complex in nature (e.g. contamination from cereal adjuncts which are usually diverse in nature). Specifically, the feeds might have been contaminated with aflatoxin and aflatoxigenic moulds through the raw materials like maize, groundnut cake, soybean, and cassava used for feed formulation, during feed processing, handling and/or through inadequate storage conditions. Considering the complexity of fish feeds, likely higher contamination rates of feeds compared to contamination of fish products and possibility of horizontal transfer of aflatoxins from ingested feeds to fish, fish farmers who compound fish feeds are encouraged to source for healthy grain components as basal ingredients during feed formulation.

Conclusion

This study has shown that smoked-dried fin and shell fishes in Lagos markets are contaminated by low-to-moderate levels of aflatoxin although fish feeds contained higher aflatoxin levels. Since aflatoxin-contaminated foods are consumed daily by the populace in developing countries as the food are being transported to local markets without proper checks by regulatory agencies, the findings of the present study therefore raise serious threats to the health of consumers. Hence, it is imperative that policies and enforcement of measures for the control and management of aflatoxins in fishery products, be set in place. In addition, more awareness is needed on the dangers and effects of feeding fishes with aflatoxin-contaminated feeds.

Conflict of interest

Authors declare no conflict of interest.

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