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MYCOLOGICAL ANALYSIS AND AFLATOXINS DETECTION IN DRIED FISH SOLD IN OJA-OBA MARKET, ILORIN, NIGERIA.

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ABSTARCT

This study was carried out to determine the fungal load and aflatoxins in dried fish samples sold in Ilorin. The fish examined were *Protepterus annectens* (Eja Opolo), *Siluriformis* (Catfish), *Distichodus brevipinnis* (Eja Omu), *Oreochromus niloticus* (Tilapia fish), *Gymnarchus niloticus* (Eja Osan). The quantitative enumeration of fungi as colony-forming units per gram (CFU/g) was between 12.0±1.0 – 1.5±0.5 CFU/gx10^{-3}. Moisture content of the dried fish samples ranged between 41.0±1.0 to 55.0±5.0. Five different fungal species were found. The associated fungi were *Aspergillus flavus, Rhizopus* sp, *A. niger*, *Mucor* sp, and *Penicillium chrysogenum.* The *Aspergilus flavus* and *A. niger* had the highest rate of occurrence among the isolated fungi. Quantification of aflatoxin from the dried fish revealed samples were contaminated with aflatoxins but they did not exceed the legal limits and confirms potential exposure of this toxin from dried fish infected with fungi. Prolonged intake of dried fish with these metabolites may constitute potential public health hazard.

Keywords: Aflatoxins, Dried fish, Oja-Oba, Mycotoxins, HPLC, Chromatogram

INTRODUCTION

Fish is a major source of animal protein in the tropics, and it is particularly high in calcium (Ca), phosphate (P), and vitamins (Oluwaniyi and Dosunmu, 2009). Fish and fish products are popular, particularly in coastal areas. Fresh fish, on the other hand, is perishable and must be processed to extend its shelf life. Chilling, freeing, salting, canning, drying, and smoking are currently the most widely used preservation techniques (Kumolu-Johnson *et al.,* 2010). The most popular method is drying (Deng *et al.,* 2021).

In Africa, fungal contamination is a serious problem for both fish growers and consumers. Fungi may induce this infection as the primary deterioration agents as a secondary contaminants as a result of mechanical damage (Eyo, 2012). Several molds have been linked to fish spoilage and their ability to produce mycotoxins is harmful to consumers' health. Food instability, a lack of critical nutrients, and low income generation have all been linked to fungal fish spoilage in developing countries (Wu, 2014).

Mycotoxins are metabolites released by fungi growing on a variety of organic substrates that can cause death or serious side effects when ingested or consumed by humans or animals. Vomiting, weight loss, tumor growth, and death could all be fatal side effects. Several fungi-secreted toxins are well-known, and the bulk of them can be found in grain crops. While certain mycotoxins are

known to be carcinogens, most damaged organs in the body and can be lethal to the blood, kidneys, skin, or central nervous system (Ashiq, 2015; Yin *et al.*, 2008; Martins *et al.*, 2001).

Mycotoxins are mostly produced by fungi in foods. When it comes to food poisoning caused by mycotoxins, the fungus Aspergillus, Penicillium, and Fusarium are the most important. Aflatoxins are the most common fungus metabolites that pose a direct health risk to humans. Aspergillus flavus can be found in soil, air, and rotten plant residues. Aflatoxin contamination and manufacturing can occur in the field, during transportation, or in storage. Most infections, on the other hand, occurs in the field, but aflatoxin formation can occur at any time under favourable conditions (Ashiq, 2015; Yin *et al.*, 2008; Martins *et al.*, 2001).

Lack of adequate standardization of smoked dried fish has resulted in unhygienic practices, exposing fish and fish products to numerous forms of contamination (Wogu, 2011). In Nigeria, smoked dried fish is so scarce that it is transported and preserved in substandard conditions. This triggered the need for a mycological analysis and aflatoxins detection in dried fish sold in Ilorin. Walter *et al.* (2020) found aflatoxins produced by *Aspergillus* species, as well as other fungal species, in smoked dried fish from Bida. However, little information is known on mycotoxin generation in dried fish from Ilorin main market, Kwara State; so, the purpose of this study is to determine the mycological analysis and aflatoxins detection in dried fish sold in Ilorin main market.

MATERIALS AND METHODS

Sample Collection

Five (5) different types of dried fishes samples such as *Protepterus annectens* (Eja Opolo), Siluriformis (Catfish), *Distichodus brevipinnis* (Eja Omu), *Oreochromus niloticus* (Tilapia fish), and *Gymnarchus niloticus* (Eja Osan) were randomly sampled and purchase from different marketing sites located at Oja-Oba main market in Ilorin, kwara state, Nigeria. Twenty five (25) samples, five (5) from each related species was kept in sterile polyethylene bags and transported to the laboratory for analysis. The fishes were identified in the Department of zoology, university of Ilorin.

Sterilization of Materials

Glassware such as conical flask and test tubes were washed thoroughly with detergent and rinsed. They were wrapped in aluminium foil and sterilized in an oven at 160℃ for 1 hour. All media and diluents were sterilized by autoclaving at 121℃ for 15 minutes.

Moisture Content Determination

Sample was pulverized. A dry clean crucible was weighed (W_1) and 2g of each of the pulverized samples were placed in the crucible. The weight of the sample and crucible were recorded as W_2. The crucible with sample were placed in an oven at 105℃ for 3 hours. It was allowed to cool and weighed. Further drying, cooling and weighing were carried out until a constant weight W_3 was achieved (Nester *et al*., 2006). The percentage moisture content were given as;

% Moisture content = Loss in weight due to drying ×100/ Weight of sample.

% Moisture content = $W_1 - W_3 \times 100 / W_2 - W_1$

Where: W_1 = Weight of crucible

W_2 = Weight of crucible + sample before drying

W_3 = Weight of crucible + sample after drying.

Media Preparation

Potato dextrose agar (PDA) is the specific culture media used. The culture media was prepared according to manufacturer's instructions and sterilized in an autoclave for 15 minutes at 121℃.

Fungi Isolation and Identification

Stock solution of related species of sample were prepared by homogenizing pulverized dried fish from equal gram of the five sample of each species (which will be done by using a blender) in 20ml of sterile distilled water into conical flasks. A ten-fold serial dilution were done and 1mililitre of different dilution (10^{-2}) was dispensed in duplicates into sterile Petri dishes and molten potato dextrose agar (PDA) at about 44-45℃ containing 1 mililitre of streptomycin were poured asceptically into the Petri dishes containing the inoculum and it was swirled gently to allow even distribution, allowed to solidify and incubated at 27±2℃ for 5-7 days. Morphologically distinct colonies was subcultured on fresh media to obtain pure isolates. The isolates were identified based on their colonial morphology as well as cellular morphology.

Cellular Morphology

In describing the cellular morphology mounts was made and was stained with Lactophenol in Cotton blue. The characteristics used in describing the cellular morphology are grouped into two; Vegetative characteristics and reproductive characteristics. Vegetative characteristics to be used are nature of hyphae, whether septate or non septate, colour of hyphae, nature of branching presence of specialized vegetative structures like rhizoids and secondly reproductive structures such as types of sporophore, whether it is composed of sporangiophore or conidiophores, attachment of sporophore to the hypha. The type of spores whether conidia or sporangiospores, and shape and size of spores.

Extraction of Aflatoxin

Twenty (20) g of sample were weighed using a weighing balance into a conical flask. 80ml of 70% methanol were added to the sample and shaked for 45 minutes on orbital shaker. The solution were later filtered using sterile filter paper.

Quantitative Estimation of Aflatoxin

Total aflatoxin present in each fish sample were determined using column chromatography (AOAC, 2000). The aflatoxin present were extracted using this method, Fluroscene dye were added which served as a binding agent to the metabolite (aflatoxin). Fluorescent spectrophotometer were used to check the absorbance and used to calculate the total aflatoxin present in the fish samples.

Procedure for Aflatoxin Detection in Fish Samples

Ball of glass wool were loosely placed in the bottom of 22×300mm chromatographic column and 5gm of anhydrous sodium sulphate was added to give base for silica gel followed by 0.01g of fluorescene. Chloroform was added until tube was about half full.10gm of silica gel in slurry with chloroform was poured. Sides of the tube was washed with chloroform and stirred to disperse silica gel. Some chloroform was drained to aid settling leaving a few centimetres above silica gel. 15gm of anhydrous sodium sulphate were added slowly. Chloroform was drained to the top of sodium sulphate. About 50ml of sample extract was added to column, eluted to maximum flow rate. Aflatoxin was eluted with 150ml methanol. Fraction was collected from time of addition till flow stopped. The elute was evaporated on water bath. The residue was quantitatively transferred to a vial, solvent evaporated and re-dissolved in a known volume of

chloroform and kept in vial for quantification. The aflatoxin was quantified using fluorescent spectrophotometer at 750 and 820nm respectively.

RESULTS

Moisture Content (%) of the Dried Fishes

The moisture contents (%) of the dried fishes were presented in Table 1. The moisture content (%) ranged from 41.0±1.0 (S5=*Gymnarchus niloticus*) to 55.0±1.0 (S3=*Distichodus brevipinnis*).

Total Fungi Counts (TFC) (cfu/g) of the Dried Fishes

The total fungi counts of the dried fishes were presented in Table 2. The total fungi count ranged from 1.5±0.5 (S3=*Distichodus brevipinnis*) to 12.0±1.0 (S2= *Siluriformis*). *Siluriformis* had the highest fungal while *Distichodus brevipinnis* had the least fungal counts.

Colonial and Microscopic Characteristics of the Fungi Isolates

The colonial and microscopic characteristics of the fungi isolates which include colour, surface, shape and conidia surface were presented in Table 3. The colonial features of Iso1 were green mould, white conidiophores, long smooth and hyaline aseptate. The microscopic features of Iso1 were pale brown, spherical, globose, smoothy fine and *Aspergillus flavus* was identified. The colonial and microscopic features of Iso2 were black, conidiophores were long, smooth and hyaline aseptate. They are slightly brown, smooth, rough and irregular and *Aspergillus* niger was identified based on this features. Iso3, Iso4 and Iso5 were identified as *Penicillium chrysogenum, Mucor* sp., *Rhizopus* sp. based on colonial and microscopic features.

Aflatoxin Concentration of Fish Samples

The results for the aflatoxin concentration of *Protepterus annectens* **(**Eja Opolo), *Siluriformis* (Catfish), *Distichodus brevipinnis* (Eja Omu), *Oreochromus niloticus* (Tilapia fish) and *Gymnarchus niloticus* (Eja Osan) were presented in Table 4.

Chemical Analysis of Mycotoxin

Aflatoxin detection of *Protepterus annectens* (Eja Opolo), *Siluriformis* (Catfish), *Distichodus brevipinnis* (Eja Omu), *Oreochromus niloticus* (Tilapia fish) and *Gymnarchus niloticus* (Eja Osan) using HPLC Chromatogram were presented in Figure 1, 2, 3, 4 and 5 respectively.

Table 1: Moisture Content (%) of each Fish Sample

Fish Samples	Moisture content (%)
Protepterus annectens (Eja Opolo)	42.5±2.5
Siluriformis (Catfish)	47.5±2.5
Distichodus brevipinnis (Eja Omu)	55.0±5.0
Oreochromus niloticus (Tilapia fish)	52.5±2.5
Gymnarchus niloticus (Eja Osan)	41.0±1.0

Note: Data are means of two replicates ±SD

Table 2:Total Fungi Counts (cfu/g) of Fish Samples

Fish Samples	Total fungi counts (cfu/ml)x10^{-3}
Protepterus annectens (Eja Opolo)	2.5±0.5
Siluriformis (Catfish)	12.0±1.0
Distichodus brevipinnis (Eja Omu)	1.5±0.5
Oreochromus niloticus (Tilapia fish)	8.0±1.0
Gymnarchus niloticus (Eja Osan).	9.0±1.0

Note: Data are means of two replicates ±SD

Table 3:Colonial and Microscopic Characteristics of the Fungi Isolates

Isolate	Colonial Features	Microscopic Features	Fungi Identified
Iso1	Green mould, white conidiophores, long smooth and hyaline aseptate.	Pale brown, spherical, globose, smoothy fine.	*Aspergillus flavus*
Iso2	Black, conidiophores are long, smooth and hyaline aseptate.	Slightly brown, smooth, biseriate, globose, rough and irregular.	*Aspergillus niger*
Iso3	Green, branched, septate hyphae and open spores.	Grey in colour and watery surface.	*Penicillium chrysogenum*
Iso4	Colonies were notreally fast growing, black colour of the sporangiospore gave the sporangium a characteristics black colour.	Stolons, rhizoids and sporangioles are abent. The mycelium is coenocytic and much branched, spores were enclosed. Hyphae penetrates into the substratum.	*Mucor* sp.
Iso5	Colonies were growing rapidly, and darkens with age, reverse is white.	Unbranched sporangiophore, white spores, numerous stolons, globose.	*Rhizopus* sp.

Key: Iso=Isolates

Table 4:Aflatoxin Concentration of Fish Samples

Fish Samples	Aflatoxin level (Conc. Ppb)				
	B1	**B2**	**G1**	**G2**	**Total**
Protepterus annectens (Eja Opolo)	8.0969	11.0872	0.2773	0.2118	19.6732
Siluriformis (Catfish)	4.4258	7.5189	0.0151	BDL	11.9598
Distichodus brevipinnis (Eja Omu)	0.0145	BDL	15.6692	4.1248	19.8085
Oreochromus niloticus (Tilapia fish)	6.7012	5.9910	0.0160	0.0318	12.7400
Gymnarchus niloticus (Eja Osan)	6.8593	11.3293	1.7819	BDL	19.9705

Key: B1=AflatoxinB1, B2=Aflatoxin B2, G1=Aflatoxin G1, G2=Aflatoxin G2

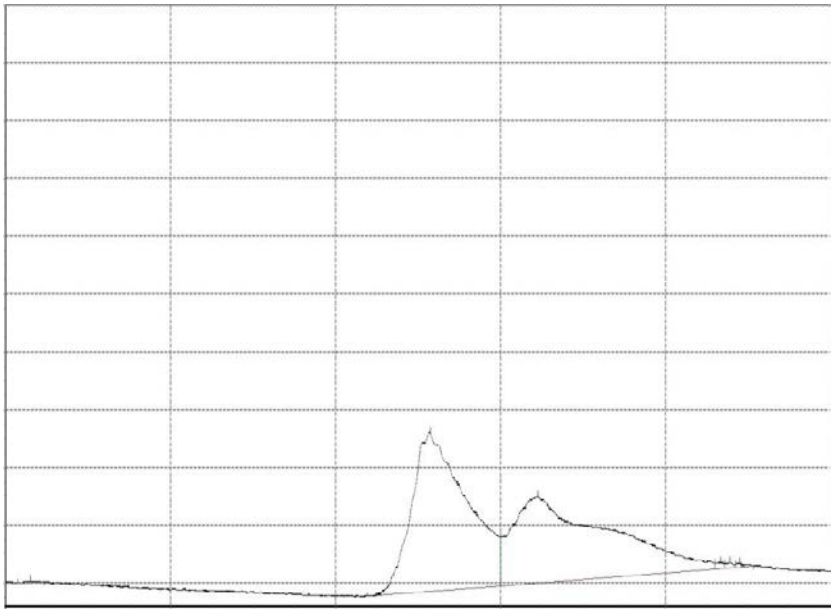

Figure 1: HPLC Chromatogram of Aflatoxin produced by *Protepterus annectens* (Eja Opolo)

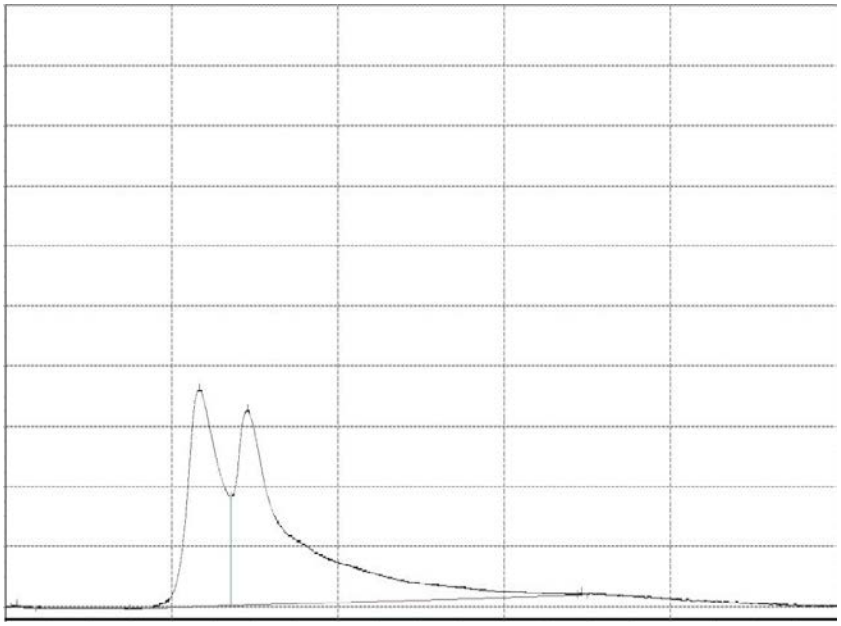

Figure 2: HPLC Chromatogram of Aflatoxin produced by *Siluriformis* (Catfish)

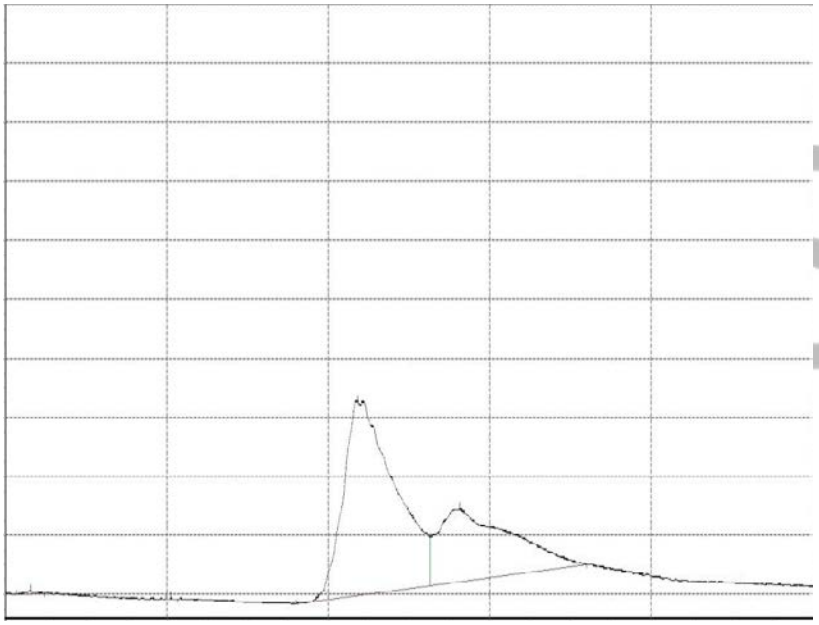

Figure 3: HPLC Chromatogram of Aflatoxin produced by *Distichodus brevipinnis* (Eja Omu)

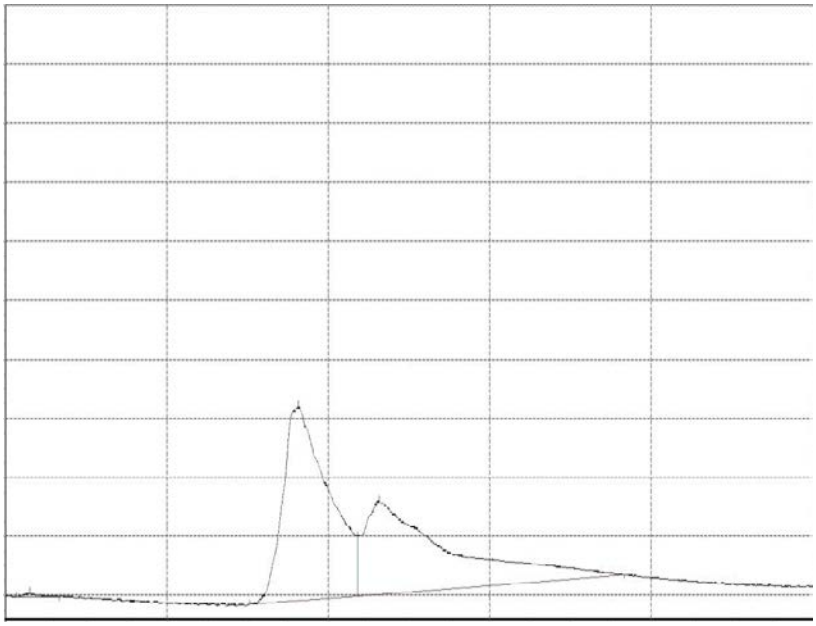

Figure 4: HPLC Chromatogram of Aflatoxin produced by *Oreochromus niloticus* (Tilapia Fish)

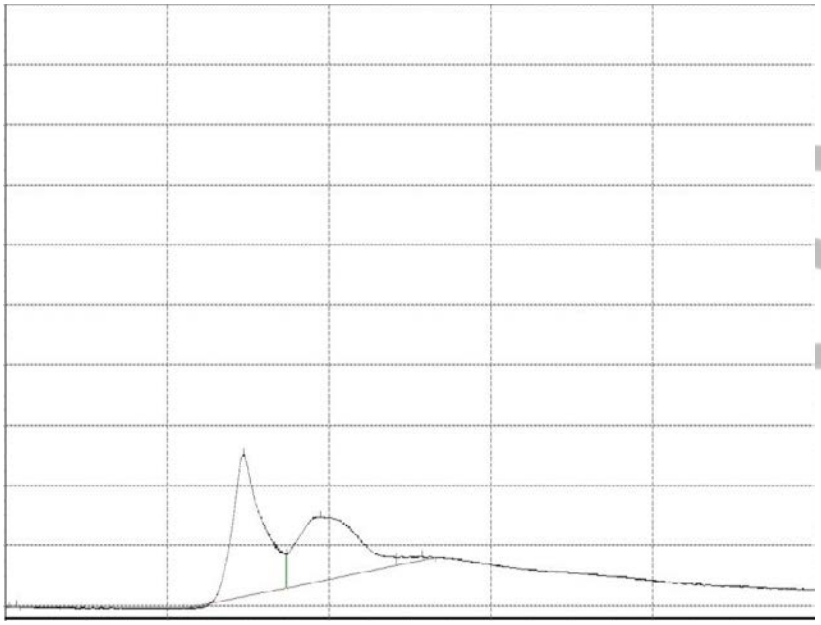

Figure 5: HPLC Chromatogram of Aflatoxin produced by *Gymnarchus niloticus* (Eja Osan)

DISCUSSION

The percentage moisture content of each of the dried fish sample was depicted in Table 1. The fish *Distichodus brevipinnis* (Eja Omu) has the highest percentage moisture content which was 55.0±5.0 and the fish *Gymnarchus niloticus* (Eja Osan) had the least moisture content. High moisture content has been reported to accelerate food spoilage. The moisture content were high which can accelerate the spoilage of the fish.

The fungal species isolated from the smoked fish sample were *Aspergillus flavus*, *Aspergillus niger*, *Penicillium chrysogenum*, *Mucor* sp. and *Rhizopus sp*. are the most dominant. These fungi isolated constitutes most of the fungi isolated from dried fish. Edema and Agbon (2010) isolated *A. flavus*, *A. niger*, *Penicillium sp*, and *Rhizzopus sp* from smoked cured fish from open markets in south western Nigeria. The growth of these fungi species in the fish is as a result of favourable condition that supports their growth. Adebayo-Tayo *et al.* (2008) reported the isolation of

Aspergillus flavus, A. terreus, A. fumigatus, Absidia sp, Rhizopous sp, A. niger, Mucor sp and some others from selected smoked fish from different market sites in Uyo, Akwa Ibom state. The presence of *Aspergillus* in the sample could be because *Apsergillus* is a common soil fungus and it could get into the fish during the handling and storage of these fishes in dirty environment. *Penicillium* is also soil a fungus that can get into fish as a result of poor hygiene practice thus leading to spoilage of fish. *Rhizopus* and *Mucor* sp present in the fish can also cause spoilage of the fish and have negative impact on consumers. The presence of all these fungi in the fishes is an indication that the fish was a good substrate for them to act upon and grow well.

The presence of these fungi in the fishes could be as a result of poor sanitation during the processing of these fishes and it could also be as a result of poor hygienic display of these fishes in the market or as a result of poor storage in unventilated environment where pest act as a carrier of inoculum or spores of fungi are dropped from air. The amounts of the fungi present were not in high amount which could be less detrimental but could be harmful in immuno-compromised individuals where the fungi could act as opportunistic pathogens. These fungi are not just detrimental in immuno-compromised individuals but they are capable of producing toxins that are highly dangerous when accumulated in the body. Since it is not possible to live in a sterile environment, what is important is to minimize high level of aflatoxin contamination. According to the United States Food and Drug Administration (FDA) action levels for aflatoxin present in food of feed is 20 to 300 ppb.

Each of the fish had low level of aflatoxin (Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, Aflatoxin G2). Aflatoxin which causes aflatoxicosis is characterized by vomiting, abdominal pain, pulmonary oedema, convulsion, coma and death (ICRI, 2000). *Gymnarchus niloticus* (Eja Osan) had the highest amount of aflatoxin, 19.9705 ppb. This study is in contradiction with study conducted by Nkwolo and Okonkwo, (1978) which stated that the highest exposure was recorded from dried fish contaminated with exceptionally high toxin level of 400-800ppb. Most countries have applied a regulatory limit of 20ppb for total aflatoxin in a wide range of food (FAO, 2004). *Siluriformis* (Catfish) had the aflatoxin level at 11.9598 ppb which was the least highest of the fishes. The presence of various fungi does not necessarily mean that aflatoxin is present in the food but the presence of toxin producing fungi such as *Aspergillus flavus* could be an indication of the presence of aflatoxin. Various factors have contributed to the presence of high amount of aflatoxin present in the fish sample such as the susceptibility of the fish to the invasion of fungi during storage or packaging. These fungi find the environment of the fish favourable for their growth and so grow abundantly and produce these metabolites.

CONCLUSION

Aflatoxin is a carcinogenic toxin that has a lot of negative effect on health of human. Various fungi produce these toxins and these toxin producing fungi are contaminants of fishes. Proper processing and storage of these fishes lead to less contamination and less amount of toxin in the fishes.

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Competing Interests

Authors have declared that no competing interests exist.

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