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Phenotypic Characterization of Toxigenic Fungi, Evaluation and Effect of Post Treatment on Aflatoxin and Ochratoxin A Content in Smoked Fish and Smoked Bush Meat

Fowoyo Patience Temitope[1*] and Bodunde Rachael Oluwaseun[1]

[1]Department of Biosciences, Salem University, P.M.B. 1060, Lokoja, Kogi State, Nigeria.

Authors' contributions

This work was carried out in collaboration between both authors. Author FPT designed the study, performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript and managed literature searches. Author BRO managed the analyses of the study and literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Smoked fish and smoked bush meat constitute a major food for humans and are sometimes consumed without further processing leading to contamination with toxins such as aflatoxin and ochratoxin A in which consumption may have serious impact on public health. This study is aimed at detecting the incidence of aflatoxin and ochratoxin A in smoked fish and smoked bush meat samples in Lokoja, Kogi state. A total of thirty-six (36) samples of smoked fish and smoked bush meat were examined; Eight (8) of which belonged to different species of smoked fish (catfish and tilapia fish) and four (4) different species of smoked bush meat samples purchased in triplicates from Old market, Lokoja. All the fish samples contained ochratoxin A with a content of between 2.3-11 µg/kg while the smoked bush meat samples had ochratoxin A content of 3.8-11.5 µg/kg. Aflatoxin content in the fish samples ranged between 4.8-12.5 µg/kg while in smoked bush meat it had 4.5-13.8 µg/kg. *Aspergillus flavus*, *Penicillium* sp., *Mucor* sp. and *Aspergillus niger* were the fungi isolated from the samples. *Aspergillus niger*, *Penicillium* sp. and *Aspergillus flavus* produced

**Corresponding author: E-mail: Patbello83@gmail.com;*

ochratoxin A while only *Aspergillus flavus* produced aflatoxin. The smoked fish and smoked bush meat samples were subjected to heat and brine treatments to determine their ability to reduce the concentration of the toxins. After heat treatment of the samples, aflatoxin concentration ranged between 0.019-0.067 µg/kg and for ochratoxin A, the range was between 0.011-0.06 µg/kg. Aflatoxin concentration ranged between 0.017-0.065 µg/kg and ochratoxin A concentration ranged between 0.057 - 0.108 µg/kg after brine treatment and this indicated that both treatments drastically reduced the aflatoxin and ochratoxin A content of the samples though heat treatment was more effective. It is therefore recommended that consumption of smoked fish and smoked bush meat as ready-to-eat without undergoing post treatment be discouraged so as to decrease the likelihood of the occurrence of food intoxication. Also the use of high temperatures of about 80℃ or soaking in brine solution (7.5%) should be encouraged for post treatment of these foods before consumption.

Keywords: Aflatoxin; ochratoxin A; smoked fish; smoked bush meat; toxigenic fungi.

1. INTRODUCTION

Mycotoxins are low molecular weight compounds that are synthesized during secondary metabolism by filamentous fungi [1]. Mycotoxins are natural contaminants in raw materials, food and feeds. Mould species that produce mycotoxins are extremely common, and they can grow on a wide range of substrates under different environmental conditions; they occur in agricultural products and other food commodity all around the world [2]. Five classes of mycotoxins are considered the most significant in agriculture and in the food industry namely aflatoxins, ochratoxin, fumonisin, citrinin and patulin. Many mycotoxins may be toxic to vertebrates and other animal groups even in low concentrations, some of them can cause autoimmune illnesses, and have allergenic properties, while others are teratogenic, carcinogenic, and mutagenic [2]. *Aspergillus, Alternaria, Claviceps, Fusarium, Penicillium and Stachybotrys* are the recognized genera of mycotoxigenic fungi [3,4]. The natural fungal flora associated with food is dominated by the *Aspergillus, Fusarium* and *Penicillium* genera [5]. A significant proportion of the Nigerian populace consume smoked fish and smoked bush meat and this serves as a major part of their meal. The aim of this study is to detect the presence of aflatoxin and ochratoxin A in smoked fish and smoked bush meat, identify the organisms responsible for their production whilst finding methods to eliminate or reduce their incidence in food.

2. MATERIALS AND METHODS

2.1 Collection of Samples

A total of 36 samples comprising of different species of smoked fish (n= 8) and smoked bush meat (n= 4) were purchased in triplicate from Old market, Lokoja, Kogi State. The species used in the study were smoked tilapia fish (*Oreochromis niloticus*) -nile tilapia, smoked tilapia fish (*Oreochromis aureus*) -blue tilapia, smoked tilapia fish (*Tilapia zilli*) -red belly tilapia, smoked tilapia fish (*Oreochromis urolepsis urolepsis*) -wami tilapia, smoked cat fish (*Pylodictis olivaris*) -flat head catfish, smoked cat fish (*Clarias gariepinus*) -sharp tooth catfish, smoked cat fish (Clarias anguillaris) -mud fish, smoked cat fish (*Arius heudeloti*) -sea cat fish, smoked bush meat (*Rattus fuscipes*) -bush rat, smoked bush meat (*Rattus norvegicus*) -brown rat, smoked bush meat (*Thryonomys swinderianus*) -cane rat and smoked bush meat (*Critecosmys gambianus*) -African giant rat.

Each of the samples were placed in sterile polyethylene bags separately and transported immediately to the laboratory for analysis.

2.2 Isolation of Fungi in Smoked Fish and Smoked Bush Meat Samples

The samples were each covered separately with sterile net and sun dried for 2 days. The dried samples were grinded separately using a high speed blender into a powdery form. Ten grams (10 g) of each of the samples was homogenized in 90 ml of distilled water and 1 ml of the homogenate was serially diluted. 1 ml of the aliquot of the selected dilutions was plated in duplicate on sterile Potato Dextrose Agar containing 1% streptomycin using standard pour plate technique. Inoculated plates were incubated at 28±2℃ for 5 days. The fungal colonies that developed were sub-cultured thrice to obtain pure cultures.

2.3 Identification of Fungal Isolates

Distinct colonies were identified on the basis of their morphological and cellular characteristics.

The isolates were examined morphologically for pigmentation, texture, spore formation, production of fruiting bodies, shape and biochemical reactions according to the identification keys used by [6-8] and were compared with already identified cultures obtained from the microbiology laboratory, Salem University, Lokoja.

2.4 Detection and Quantification of Aflatoxin and Ochratoxin A in Smoked Fish and Smoked Bush Meat

The aflatoxin and ochratoxin A detection and quantification was carried out using Agra Quant® mycotoxin ELISA test kit (Romer Lab, Singapore Pte Ltd) according to the manufacturer's instruction. The kit code for aflatoxin and ochratoxin A are COKAQ1000 and COKAQ2000 respectively. The aflatoxin and ochratoxin A was extracted by dissolving 5 g of each of the ground sample in 25 ml of 70% methanol and was filtered using the Whatman no.1 filter paper. 50 ml of the filtrate was added to 100 ml of the enzyme conjugated aflatoxin and ochratoxin A respectively from the Agra Quant kit and mixed together in the dilution well (12 blue/green-bordered dilution well placed microtitre holder). 100 ml of the mixture was transferred into the antibody coated microtitre well and incubated at 37℃ for 15 min. The mixture was discarded and washed with distilled water repeatedly for 5 times. After washing, 100 ml of the enzyme substrate was added to each antibody coated wells and incubated at 37℃ for 5 min. The development of a blue colour indicates the presence of aflatoxin and ochratoxin A using their respective kits. 100 ml of a stop solution was then added in order to stop the reaction and this changed the colour from blue to yellow immediately. The microtitre plates were measured optically using an ELISA plate reader with an absorbance filter of 450 nm (OD_{450}) and a differential filter of 630 nm. Aflatoxin and ochratoxin A concentration was expressed in µg/kg.

2.5 Identification of Toxigenic Fungi in Smoked Fish and Smoked Bush Meat Samples

Fungal isolates were qualitatively screened for toxin production by a TLC agar plug method for aflatoxin and ochratoxin A using the method by [9]. Matured spores of the isolates were single point inoculated on YES agar. The plates were then incubated at 25℃ for 7 days. The agar plug (5 mm in diameter) was removed from the centre of the colony and transferred agar side down on to the designated track on a 20 × 20 silica gel 60 TLC plate. The plug was held in position until the appearance of the application spot under it, the agar plug was discarded and the spot allowed left to dry. A second plug was removed from the same colony and superimposed on the previous spot and were developed in a tank containing toluene: ethyl acetate: formic acid 90%: chloroform (7:5:2:5, v/v/v/v). The plates were run until the eluent front was approximately 10 cm above the application line. They were then dried (in a fume hood for 20 min) and examined under the long wavelength UV light (366 mm) for aflatoxin and ochratoxin A. The aflatoxin was detected as intense blue bands whereas ochratoxin A was seen as fluorescent blue band.

2.6 Post Treatment of Smoked Fish and Smoked Bush Meat Samples

The smoked fish and smoked bush meat samples were subjected to both heat and brine treatments. The samples were heat treated at 80℃ for 30 min; and brine treatment was carried out by soaking the samples in 7.5% of sodium chloride (NaCl) solution for 5 min, covered with sterile net and air dried after which it was blended to powder.

2.7 Effect of Heat and Brine Treatment on Aflatoxin and Ochratoxin A Concentration of Smoked Fish and Smoked Bush Meat

The Agra Quant kit was used to determine the concentration of aflatoxin and ochratoxin A in treated smoked fish and smoked bush meat samples. The kit code for aflatoxin and ochratoxin A are COKAQ1000 and COKAQ2000 respectively. The aflatoxin and ochratoxin A was extracted by dissolving 5 g of each of the ground sample in 25 ml of 70% methanol and was filtered using the Whatman no.1 filter paper. 50 ml of the filtrate was added to 100 ml of the enzyme conjugated aflatoxin and ochratoxin A respectively from the Agra Quant kit and mixed together in the dilution well (12 blue/green-bordered dilution well placed microtitre holder). 100 ml of the mixture was transferred into the antibody coated microtitre well and incubated at 37℃ for 15 min. The mixture was discarded and washed with distilled water repeatedly for 5 times. After washing, 100 ml of the enzyme

substrate was added to each antibody coated wells and incubated at 37℃ for 5 min. The development of a blue colour indicates the presence of aflatoxin and ochratoxin A using their respective kits. 100 ml of a stop solution was then added in order to stop the reaction and this changed the colour from blue to yellow immediately. The microtitre plates were measured optically using an ELISA plate reader with an absorbance filter of 450 nm (OD_{450}) and a differential filter of 630 nm. Aflatoxin and ochratoxin A concentration was expressed in µg/kg.

2.8 Analysis of Data

The data generated from these investigations were subjected to analysis of variance (ANOVA). The test of significance was carried out using Duncan's multiple range tests (DMRT).

3. RESULTS AND DISCUSSION

The fungal isolates were identified as *Aspergillus flavus*, *A. niger*, *Penicillum* sp. and *Mucor* sp. from the samples. Aflatoxin concentration in the samples ranged from 4.5 µg/kg-13.8 µg/kg and the ochratoxin A concentration in the samples was between 2.3 µg/kg -11.5 µg/kg using the standard curve for calibration and determination of the toxin content (Figs. 1 and 2). The toxin concentrations of the samples examined in this study were not above the recommended limits (Food and Drug Administration (FDA) of the United States indicates that levels of aflatoxin intake for humans as a maximum of 20 ppb or µg/kg which corresponds to the maximum residue limit in Nigerian foods [10] and according to [11], the maximum daily limit varies between 5 and 20 µg/kg). However, if the smoked fish and bush meat samples are consumed extensively over a long period, it may become hazardous to human health and becomes a food safety concern. Aflatoxin and ochratoxin A content was highest in smoked bush meat as shown in Table 1. *Aspergillus flavus* was capable of producing both aflatoxin and ochratoxin A. *Aspergillus niger and Penicillum* sp. produced ochratoxin A while *Mucor* sp. did not produce any of the two toxins (Table 2). The presence of these fungi and mycotoxins in the food samples may cause serious health problems for smoked fish and smoked bush meat consumers, which

Table 1. Aflatoxin and ochratoxin A concentration in smoked fish and smoked bush meat

Samples	Concentration of aflatoxin (ug/kg)	Concentration of ochratoxin A (ug/kg)
AO	11.0 ± 0.10^{c}	2.3 ± 0.43^{j}
BP	10.8 ± 0.26^{d}	3.5 ± 0.55^{i}
CN	4.5 ± 0.42^{j}	3.8 ± 0.00^{i}
DO	4.8 ± 0.10^{j}	4.9 ± 0.20^{h}
EA	5.8 ± 0.17^{i}	5.5 ± 0.50^{g}
FA	6.2 ± 0.20^{h}	6.4 ± 0.21^{f}
GN	7.0 ± 0.20^{g}	7.5 ± 0.50^{e}
HP	8.9 ± 0.13^{f}	8.3 ± 0.38^{d}
IO	9.8 ± 1.00^{e}	9.9 ± 0.26^{c}
JN	11.0 ± 0.00^{c}	10.4 ± 0.30^{b}
KN	12.5 ± 0.50^{b}	11.0 ± 0.12^{a}
LO	13.8 ± 0.15^{a}	11.5 ± 0.57^{a}

Key:
AO: Smoked tilapia fish (Oreochromis niloticus) -nile tilapia
BP: Smoked tilapia fish (Oreochromis aureus) -blue tilapia
EA: Smoked tilapia fish (Tilapia zilli) -red belly tilapia
JN: Smoked tilapia fish (Oreochromis urolepsis urolepsis) -wami tilapia
FA: Smoked cat fish (Pylodictis olivaris) -flat head catfish
KN: Smoked cat fish (Clarias gariepinus) -sharp tooth catfish
DO: Smoked cat fish (Clarias anguillaris) -mud fish
HP: Smoked cat fish (Arius heudeloti) -sea cat fish
CN: Smoked bush meat (Rattus fuscipes) -bush rat
GN: Smoked bush meat (Rattus norvegicus) -brown rat
IO: Smoked bush meat (Thryonomys swinderianus) -cane rat
LO: Smoked bush meat (Critecosmys gambianus) -African giant rat

Note: *In Tables 2, 3 and 4, each value represents the mean and estimated standard deviation of three replicates. Means followed by the same superscript along the same column are not significantly different using Duncan's multiple range tests ($P < 0.05$)*

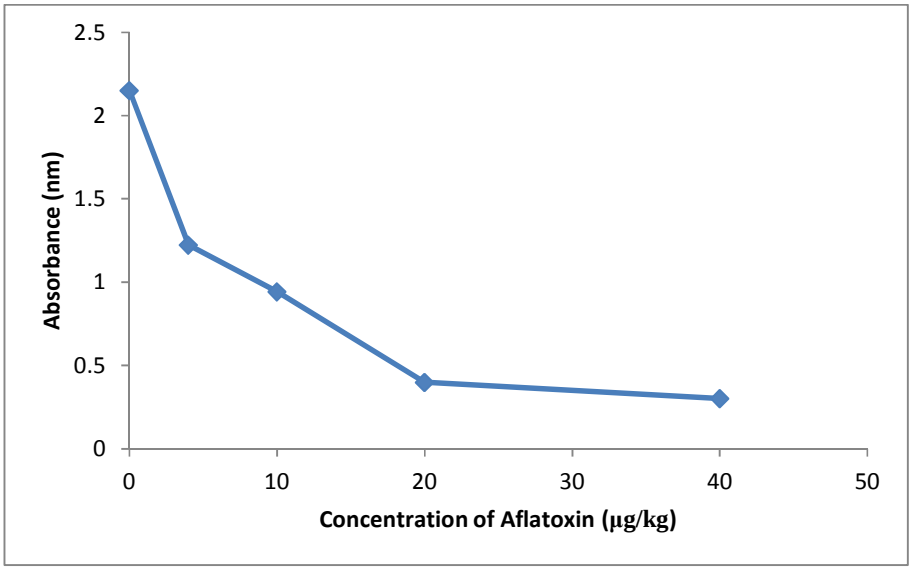

Fig. 1. Standard curve of different concentration of total aflatoxin

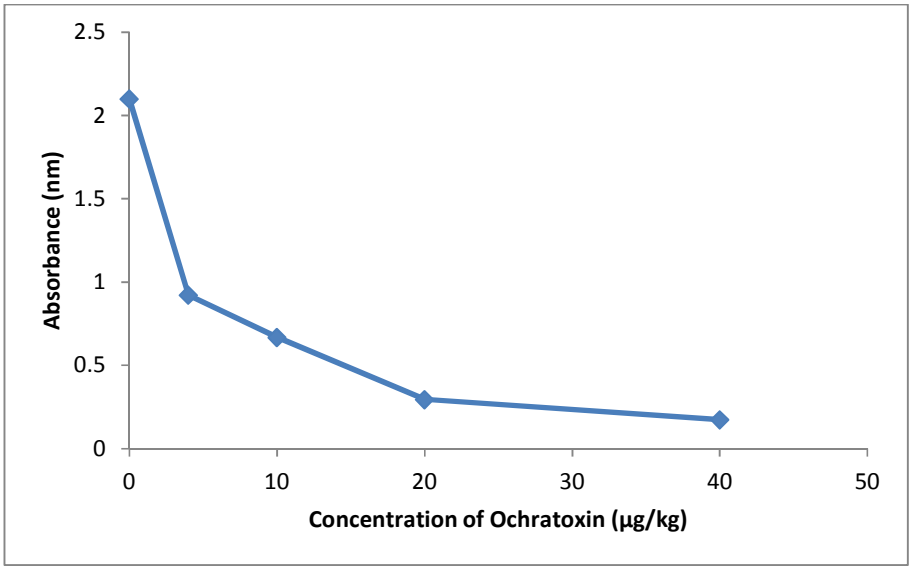

Fig. 2. Standard curve of different concentration of total ochratoxin

may result in a suppressed immune system and cancer of human organs like the liver.

The use of high temperature or heat was more effective in the reduction of the aflatoxin and ochratoxin A concentration than brine treatment in all the samples as shown in Figs. 3 and 4. Mycotoxin contamination of food can be destroyed or reduced using heat and chemical treatments [12]. Heating and cooking under pressure can destroy nearly 70% of aflatoxin [12]. Chemical treatment has been used as the most effective means for the removal of mycotoxins from contaminated commodities [12]. Chemicals used include acetic acid, ammonia gas or ammonium salts, calcium hydroxide, formaldehyde, hydrogen peroxide, methylamine, ozone gas, phosphoric acid, sodium hypochlorite. Sodium hypochlorite is also similar to sodium chloride and thus could be one of the reasons why salt may be used to reduce mycotoxin concentration in food. The aflatoxin and ochratoxin A concentration of the treated (heat and brine treatment) samples were

compared with the initial toxin concentration of the samples and it was observed that there was a drastic reduction in toxin concentration after treatment as illustrated in Tables 3 and 4. The aflatoxin and ochratoxin A level was significantly reduced when subjected to heat treatment and brine treatment. The result showed that the treatments employed may be able to reduce the mycotoxin levels to a non-significant level that may not be toxic to human health according to regulatory bodies.

Table 2. Fungal isolates capable of producing aflatoxin and ochratoxin A in smoked fish and smoked bush meat

Isolates	Organisms	Aflatoxin	Ochratoxin A
AO1	*Mucor* sp.	Negative	Negative
AO2	*Penicillum* sp.	Negative	Positive
BP1	*Aspergillus flavus*	Positive	Positive
BP2	*Mucor* sp.	Negative	Negative
CN1	*Aspergillus flavus*	Positive	Positive
CN2	*Penicillum* sp.	Negative	Positive
DO1	*Aspergillus niger*	Negative	Positive
DO2	*Aspergillus flavus*	Positive	Positive
EA	*Aspergillus niger*	Negative	Positive
FA1	*Aspergillus flavus*	Positive	Positive
FA2	*Mucor* sp.	Negative	Negative
GN1	*Penicillum* sp.	Negative	Positive
GN2	*Aspergillus flavus*	Positive	Positive
HP	*Aspergillus flavus*	Positive	Positive
IO1	*Mucor* sp.	Negative	Negative
IO2	*Aspergillus niger*	Negative	Positive
JN	*Aspergillus flavus*	Positive	Positive
KN	*Aspergillus flavus*	Positive	Positive
LO	*Penicillum* sp.	Negative	Positive

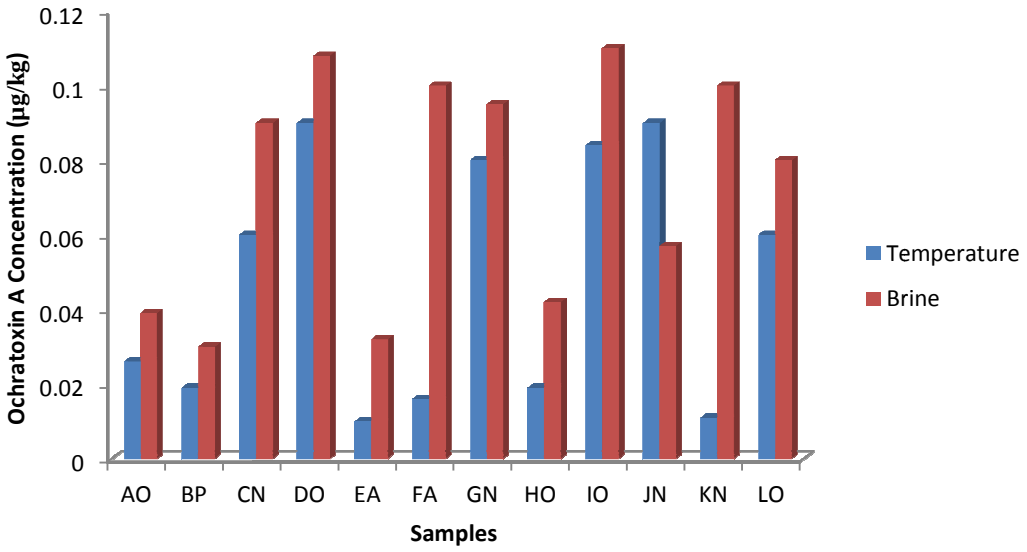

Fig. 3. Effect of temperature (80°C) and brine solution (7.5%) on ochratoxin A concentration in smoked fish and bush meat samples

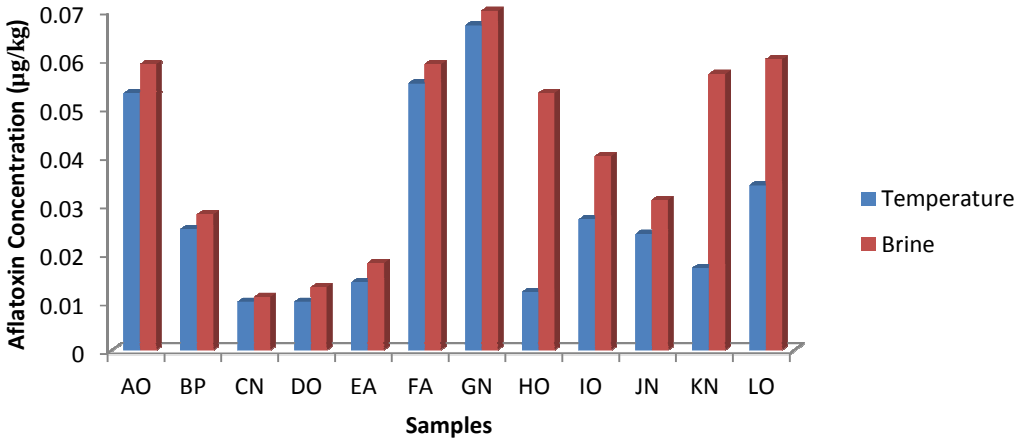

Fig. 4. Effect of temperature (80℃) and brine solution (7.5%) on aflatoxin concentration in smoked fish and smoked bush meat samples

Table 3. Comparison of the aflatoxin concentration of the treated samples and initial untreated samples in determining the effect of post treatment

Samples	Aflatoxin concentration before treatment (µg/kg)	Aflatoxin concentration after heat treatment (µg/kg)	Aflatoxin concentration after brine treatment (µg/kg)
AO	11.0 ± 0.10^{c}	0.037 ± 0.001^{d}	0.033 ± 0.00^{d}
BP	10.8 ± 0.26^{d}	0.021 ± 0.001^{e}	0.017 ± 0.00^{e}
CN	4.5 ± 0.42^{j}	0.053 ± 0.002^{b}	0.050 ± 0.002^{b}
DO	4.8 ± 0.10^{j}	0.060 ± 0.002^{a}	0.057 ± 0.002^{b}
EA	5.8 ± 0.17^{i}	0.021 ± 0.001^{e}	0.019 ± 0.013^{e}
FA	6.2 ± 0.20^{h}	0.067 ± 0.001^{a}	0.065 ± 0.001^{a}
GN	7.0 ± 0.20^{g}	0.037 ± 0.002^{d}	0.033 ± 0.003^{d}
HP	8.9 ± 0.13^{f}	0.053 ± 0.020^{b}	0.050 ± 0.002^{b}
IO	9.8 ± 1.00^{e}	0.047 ± 0.030^{c}	0.043 ± 0.002^{c}
JN	11.0 ± 0.00^{c}	0.019 ± 0.00^{f}	0.016 ± 0.030^{e}
KN	12.5 ± 0.50^{b}	0.033 ± 0.002^{d}	0.030 ± 0.002^{d}
LO	13.8 ± 0.15^{a}	0.043 ± 0.002^{c}	0.040 ± 0.001^{c}

Table 4. Comparison of the ochratoxin A concentration of the treated samples and initial untreated samples in determining the effect of post treatment

Samples	Ochratoxin A concentration before treatment (µg/kg)	Ochratoxin A concentration after heat treatment (µg/kg)	Ochratoxin A concentration after brine treatment (µg/kg)
AO	2.3 ± 0.43^{j}	0.06 ± 0.01^{b}	0.057 ± 0.002^{e}
BP	3.5 ± 0.55^{i}	0.03 ± 0.02^{b}	0.028 ± 0.02^{g}
CN	3.8 ± 0.00^{i}	0.11 ± 0.01^{a}	0.108 ± 0.013^{c}
DO	4.9 ± 0.20^{h}	0.13 ± 0.03^{a}	0.127 ± 0.001^{b}
EA	5.5 ± 0.50^{g}	0.02 ± 0.01^{b}	0.018 ± 0.014^{h}
FA	6.4 ± 0.21^{f}	0.15 ± 0.10^{a}	0.146 ± 0.003^{a}
GN	7.5 ± 0.50^{e}	0.07 ± 0.00^{b}	0.066 ± 0.003^{b}
HP	8.3 ± 0.38^{d}	0.13 ± 0.00^{a}	0.127 ± 0.001^{b}
IO	9.9 ± 0.26^{c}	0.09 ± 0.01^{b}	0.086 ± 0.001^{d}
JN	10.4 ± 0.30^{b}	0.03 ± 0.02^{b}	0.028 ± 0.001^{g}
KN	11.0 ± 0.12^{a}	0.05 ± 0.01^{b}	0.044 ± 0.002^{f}
LO	11.5 ± 0.57^{a}	0.06 ± 0.00^{b}	0.057 ± 0.002^{e}

4. CONCLUSION

The presence of fungi such as *Aspergillus, Penicillum* and *Mucor* indicates contamination in smoked fish and smoked bush meat. *Aspergillus* and *Penicillum* sp. aflatoxin and ochratoxin A. The foods may have been exposed to fungal infestation due to lack of good storage practices, inadequate ventilation of storage rooms and the unhygienic environment in which the smoked fish and smoked bush meat are displayed in the market likely responsible for toxin production. These findings indicate that there may be risk of human exposure to mycotoxins through the consumption of smoked fish and smoked bush meat and thus consumers should pay attention to properly cooking these foods, and also washing with salted water if they are to be consumed as ready-to-eat as this study has shown that heat treatment employing temperature of 80℃ and brine solution of 7.5% concentration may be used to reduce the level of toxicity.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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