UJMR, Volume 2 Number 1 June, 2017 *ISSN: 2616 - 0668*

https://doi.org/10.47430/ujmr.1721.008

Received: 28th Feb, 2017 **Accepted:** 6th April, 2017

Incidence of Fungal Flora and Aflatoxin in Some Spices Sold in Central Market, Funtua, Nigeria

Haruna, M.[a,*], Dangora D.B.[b], Khan A.U.[b], Batagarawa U.S.[b], Ibrahim H.[c], Adamu, S. U.[a]

[a]Department of Biology, Umaru Musa Yar'adua University Katsina, Katsina State, Nigeria

[b]Department of Biological Sciences, Ahmadu Bello University Zaria, Kaduna State, Nigeria

[c]Department of Biological Sciences, Kaduna State University, Kaduna State, Nigeria

*Correspondence author Email: mustaphaharuna65@yahoo.com, 08069587120

Abstract

Spices get contaminated with fungi and aflatoxin due to poor agricultural and storage practices. A total of 42 dried, raw, powdered and processed samples representing fourteen different types of spices were randomly collected in new polythene bags from Funtua Central Market, Nigeria and were screened for fungi and aflatoxin contaminations. The spices were Clove, African nutmeg, Ashanti pepper, Candlewood, Ethiopian pepper, Pineapple of the bush, Ginger, Garlic,Chillies,Kajiji,Thyme, Chilli powder (yaji), Curry and Locust bean (Dadawa). Fungi were isolated on Potato Dextrose Agar by Agar plate method for dried and raw samples and Standard Dilution Plate method for powdered samples. Seven fungal species were isolated and identified as *Aspergillus flavus, A. parasiticus, A. fumigatus, Rhizoctonia* sp*, Mucor hiemalis, Rhizopus stolonifer* and *Nigrospora sphaerica.* Percentage occurrence of fungal species ranged from 0.8% to 50.4%. Enzyme-linked Immunosorbent Assay (ELISA) was used for aflatoxin determination. Aflatoxin was only detected in 4 out of 14 spices (28.6%) namely garlic (3.3µg/kg), Ethiopian pepper (5.7µg/kg), chillies (16.6µg/kg) and African nutmeg (17.2µg/kg). African nutmeg and chillies had aflatoxin levels above maximum acceptable limit of 10µg/kg set by EU and NAFDAC are therefore, not safe for human consumption.

Key words: ELISA, Fungi, Spices, Total Aflatoxin

INTRODUCTION

Mould growth decreases the quality of food, and also creates a potential risk for human health because of the production of toxic metabolites known as mycotoxins. The notable moulds that have been reported to contaminate spices include *Aspergillus*, *Penicillium*, *Rhizopus*, *Eurotium*, *Cladosporium*, *Trichoderma*, *Mucor* and *Stachybotrys* (Freire *et al*., 2000; Elshafie *et al*., 2002; Bokhari, 2007; Hashem and Alamri, 2010). Among these fungi, *Aspergillus* remain the most prevalent contaminant of spices.

Aflatoxins are a group of mycotoxins produced by some *Aspergillus* species, notably *Aspergillus flavus* and *Aspergillus parasiticus* (Creppy, 2002). Although the presence of *Aspergillus* mould does not necessarily indicate aflatoxin contamination, there is certainly an increased risk (Robertson, 2005). Aflatoxins are the most dangerous to human health because of their highly toxic, carcinogenic, teratogenic, hepatotoxic and mutagenic characteristics (Chu, 1997; Pariza, 1996). Among the known aflatoxins, aflatoxin B1 (AFB1) remains the most prevalent in foods (Lee *et al*., 2004). AFB1 is also the most potent toxic metabolite capable of inducing hepato-carcinogenicity (Sweeney and Dobson, 1998), genotoxicity in reproductive and blood cells (Fapohunda *et al*., 2008; Ezekiel *et al*., 2011), as well as some other toxic conditions. They are also considered to cause liver cancer in humans, particularly in a number of developing countries, where high levels of aflatoxins are found in some staple foods (Mathew, 2005). Epidemiological evidence collected from East Africa, the Philippines and Thailand showed a correlation between the incidence of liver cancer and exposure to aflatoxins (Smith and Moss, 1985). Aflatoxins have also been identified as a potential biological weapon for food and water contamination (Smith, 2004).This toxin has been reported to contaminate a wide variety of spices globally (Freire *et al*., 2000; Bokhari, 2007; Ardic *et al*., 2008; Cho *et al*., 2008; Iqbal *et al*., 2011). Spices are usually produced in countries with tropical climates that have high temperature, humidity and rainfall (Martins *et al*, 2001).

Spices are aromatic vegetable substances used in minute quantities to enrich or alter the flavour of food (Bokhari, 2007). Several spices are routinely used in Nigerian homes for cooking. Regardless of the daily use of these spices there are very few documented reports on mycobiota and aflatoxin contaminations in these spices. The aim of this study was to determine the fungi and aflatoxin contaminations in different spices sold in Funtua central market, Nigeria.

MATERIALS AND METHODS

Sample Collection

Fourteen different types of spices were randomly collected in triplicates in new polythene bags from Funtua central market during the wet season of 2014. The three (3) replicates of each spice were mixed to prepare one composite sample (Farid *et al.*, 2013). This was done to prevent bias while collecting the samples because some samples were newly brought to the market while some were in the market for a long period of time. Table 1 shows list of spices with their Scientific, Common and Local names as well as parts of plant used as spices.

Isolation of Fungal Species

Agar plate method was used for isolation of fungi in dried and raw samples (Jha, 1995).Each sample was surface sterilized by putting it into 1% Sodium hypochloride for two minutes then rinsed three times with sterile distilled water. The disinfected samples were transferred with sterile forceps into petri dishes containing sterilized 15ml PDA (supplemented with chloranphenicol) at the rate of four pieces per plate, larger samples were cut into smaller pieces. These were prepared in triplicates and plates were incubated at room temperature for 7 days.

Table 1: Spices obtained from Funtua Central Market with their Scientific, Common and Local Names as well as Parts of Plant Used.

Scientific Name	Common Name	Local Name	Part of Plant Used
Eugenia aromatica(L.)	Clove	Karamfani	Bud
Monodora myristica(Gaertan)	African nutmeg	Gyadarmiya	Seeds
Piper guineense(Schumach)	Ashanti pepper	Masoro	Seeds
Fagara zanthoxyloides(Lam.)	Candlewood	Fasakwari	Stem
Xylopia aethiopica(Dunal)	Ethiopian pepper	Kimba	Fruit
Thonningia sanguinea(Vahl.)	Pineapple of the bush	Kulla	Root
Zingiber officinale(Roscoe)	Ginger	Citta maiyatsu	Rhizome
Allium sativum(L.)	Garlic	Tafarnuwa	Bulb
Capsicum frutescence (L.)	Chillies	Barkono	Fruit
Cyperus tonkinensis(Hooper)		Kajiji	Fruit
Thyme vulgaris (L.)	Thyme	Thyme	Leaves
Capsicum frutescence (L.)	Chilli powder	Yaji	Fruit
-	Curry	Kori	-
Parkia biglobosa (Jacq.)	Locust bean	Dadawa	Seed

Standard Dilution Plate method was used for isolation of fungi in powdered samples (Aziz *et al.*, 1998). One gram of each composite samples was transferred into 250 ml screw-capped Mccartney bottles containing 9ml of sterile distilled water. The solution was mechanically homogenized using a mechanical shaker at constant speed for 15 minutes. The suspension was allowed to stand for 10 minutes. Five fold serial dilution (1:5) was prepared and 1ml portions of suitable dilution (10^{-2} and 10^{-4}) was used to inoculate PDA plates. Plates were incubated at room temperature for 7 days. Pure cultures of isolates were obtained by repeated subculture on PDA.

Percentage of fungal occurrence were calculated us ing the formula:

$$\text{Fungal occurrence (\%)} = \frac{\text{Total number of individual fungal occurrence}}{\text{Total number of fungal occurrence}} \times 100$$

Fungal colonies and isolates were identified according to their morphological and microscopic characteristics using identification keys (Robert *et al.*, 2004; Davise, 2002; Klich, 2002).

Aflatoxin Assay

Spices were individually and finely ground using laboratory mill (Romer). Enzyme linked immunosorbent assay ELISA (AqraQuant Total Aflatoxin Assay 1/20) test kit was used for aflatoxin determination in the samples. This process was carried out in three stages name

Sample Extraction

Sample extraction was performed according to the manufacturer's instruction (Aqra Quant Total Aflatoxin Assay 1/20 test kit). Twenty five mililitre of aceto nitrile /water (84/16) was added to 5g of each ground spice sample and the solution was extracted by shaking for 30 minutes using orbital shaker. The sample was allowed to settle and the top layer of extract was filtered through a Whatman No. 1 filter paper and the filtrate was collected for clean up.

Sample Clean-up

Sample clean-up was performed with MycoSep 226 aflazon according to the manufacturer's instruction, so as to remove interfering substances such as colour and oil. Four mililitre of extract was transferred into a glass tube, MycoSep column was placed firmly into the top portion of the glass tube and pushed through until 0.5ml of purified extract was removed and then 0.5ml of the purified extract was transfered into 2 ml vials and evaporated to dryness. The residues in the vials were reconstituted with 0.5ml of 70/30 methanol/water, which were used for ELISA testing.

ELISA Test

This was done according to AqraQuant Total aflatoxin Assay 1/20 test kit manual. Two hundred microliter of conjugate was dispensed into each green-bordered dilution well. One hundred microlitre of each standard and sample was added into the appropriate dilution well containing 200µL of conjugate. Each well was carefully mixed by pipetting it up and down three times and 100µL of the contents from each dilution well was immediately transferred into a corresponding antibody coated microwell. It was then incubated at room temperature for 15 minutes. The contents of the microwell strips were discarded followed by washing each microwell by filling it with distilled water, and then dumping the water from the microwell strips. This was repeated for a total of five washes. Microwell strips were tapped using absorbent paper towels to expel as much residual water as possible after the fifth wash. The bottom of the microwells were dried with a dry towel. One hundred microlitre of the substrate was added into each microwell and incubated at room temperature for five minutes and a blue colour developed. One hundred microlitre of stop solution was added into each microwell strip, the color changed from blue to yellow. The strips were read with microwell reader using an absorbance filter of 450 nm.

RESULTS

Fungal Contamination of the Spices

In this study, 250 isolates representing 7 species of 5 genera were isolated from the 14 spices collected. *Aspergillus* species (42.9 %) were the most dominant fungi isolated. Among the *Aspergillus* spp., *A. flavus* and *A. parasiticus* had the highest occurrence (50.4% and 44.8% respectively) and were found to contaminate all the samples except candlewood by *A. flavus*. *A. fumigatus*, *Rhizopus stolonifer* and *Nigrospora sphaerica* had the same frequency of occurrence (1.2%). These were followed by *Mucor hiemalis* (0.4) and *Rhizoctonia* sp (0.8%) (Table 2).

Aflatoxin Contamination of the Spices

Aflatoxin was detected in only 4(28.6%) out of the 14 spices samples the concentrations of aflatoxin in the contaminated samples ranged from 3.3-17.2µg/kg. The contaminated samples were African nutmeg with the highest level (17.2µg/kg) followed by chillies (16.6µg/kg). The lowest aflatoxin concentration was recorded in Ethiopian pepper (5.7 µg/kg) and garlic (3.3µg/kg) (Table 3).

Discussion

Among the genera of fungi isolated in this study, *Aspergillus* had the highest frequency of occurrence in all spices. The result agreed largely with the result obtained by many investigators working on spices mycobiota. For example Bokhari (2007) in Saudi Arabia and Sumanth *et al*. (2010) in India isolated fungal genera from tested spices also revealed that *Aspergillus* was the most common genus isolated. Among the *Aspergillus* species encountered, *A. flavus* and *A. parasiticus* were most prevalent. This result is in agreement with the result obtained by Hashem and Alamri (2010) who reported that *A. flavus*, *A. parasiticus* and *A. tamarii* were dominant

Table 2 : Percentage Occurrence of Fungal Isolates in Spices Obtained from Funtua Central Market

Spices	*A. flavus*	*A. parasiticus*	*A. fumigatus*	*Mucor hiemalis*	*Rhizoctonia* sp	*Rhizopus stolonifer*	*Nigrospora sphaerica*	**Total**
Chillies	6	16	-	-	2	1	-	
Garlic	20	3	-	-	-		-	
Ginger	2	2	-	1	-	-	-	
Dadawa	3	2	-	-	-	1	-	
Curry	15	3	-	-	-	-	-	
Thyme	12	3	-	-	-	-	-	
African nutmeg	18	2	-	-	-	-	-	
Ashanti pepper	5	1	-	-	-	-	-	
Clove	14	5	-	-	-	-	-	
Kajiji	6	15	-	-	-	-	-	
Candlewood	-	2	-	-	-	-	3	
Yaji	3	10	-	-	-	1	-	
Pineapple of the bush	2	45	-	-	-	-	-	
Ethopian pepper	20	3	3	-	-	-	-	
Total	126	112	3	1	2	3	3	250
% occurrence	50.4	44.8	1.2	0.4	0.8	1.2	1.2	100

Table 3:Spices from Funtua Central Market with Associated Fungi and Aflatoxin Content

Spices	Fungi Isolated	Total aflatoxin conc. (µg/kg)
Chillies	*A. flavus, A. parasiticus, Rhizoctonia* sp., *Rhizopus stolonifer*	16.6
Garlic	*A. flavus, A. parasiticus,*	3.3
Ginger	*A. flavus, A. parasiticus, Mucor hiemalis*	N.D
Dadawa	*A. flavus, A. parasiticus, Rhizopus stolonifer*	N.D
Curry	*A. flavus, A. parasiticus,*	N.D
Thyme	*A. flavus, A. parasiticus,*	N.D
African nutmeg	*A. flavus, A. parasiticus*	17.2
Ashanti pepper	*A. flavus, A. parasiticus*	N.D
Clove	*A. flavus, A. parasiticus*	N.D
Kajiji	*A. flavus, A. parasiticus*	N.D
Candlewood	*A. parasiticus, Nigrospora sphaerica*	N.D
Yaji	*A. flavus, A. parasiticus, Rhizopus stolonifer*	N.D
Pineapple of the bush	*A. flavus, A. parasiticus*	N.D
Ethiopian pepper	*A. flavus, A. parasiticus, A. fumigatus*	5.7

N.D = Not Detected, Maximum limit set by EU/NAFDAC= 10µg/kg

species of *Aspergillus* that were previously isolated from spices globally with *A. flavus* occurring more frequently. The other five fungi species namely *A. fumigatus*, *Rhizopus stolonifer, Nigrospora sphaerica, Mucor hiemalis* and *Rhizoctonia sp,* had least level of occurrence. Moderate or low frequency of occurrence of *A. fumigatus* in different spices were reported by several researchers such as El-Kady *et al*. (1992), Abdulkadir *et al*. (2003). Most of these fungi were previously isolated from various kinds of spices by El-kady *et al*. (1995), Freire *et al*. (2000), Ekhuemelo and Ekefan (2013), Farid *et al*. (2013), Gnonlonfin *et al*. (2013).

In this study aflatoxin assay of spices revealed that 4 (28.6%) out of 14 spices were contaminated with total aflatoxins in the range of 3.3-17.2µg/kg. Two of these (14.3%) namely, African nutmeg and chillies had aflatoxin levels above maximum acceptable limit of 10µg/kg set by EU and NAFDAC. Contamination of African nutmeg might have occurred during extensive transportation from the southern to the northern part of the country. It is also highly rich in nutrient contents that can support fungal growth. Ezekiel *et al*. (2013) in Lagos Nigeria, reported aflatoxin B1 concentration of African nutmeg at 20µg/kg. Contamination of Chillies with aflatoxin might have occurred during drying. This is because farmers mostly spread chillies by the roadside on bare ground for drying. This provides an avenue for fungi contamination and subsequent aflatoxin contamination. Ethiopian pepper had aflatoxin concentration of 5.7µg/kg which is within aflatoxin acceptable limit. Contamination of Ethiopian pepper with aflatoxin might have occurred during transportation (from the producing area) and poor handling (unhygienic) practice in the markets. Low aflatoxin contamination in garlic might be as a result of essential oil (allicin) present in garlic which has antimicrobial activity (Haruna *et al*., 2016). Although aflatoxin producing fungi were isolated from all the samples yet only four spices contained aflatoxin. This might be due to the inability of the aflatoxigenic fungi to produce aflatoxins in the samples. Elshafie *et al*. (2002) in the Sultanate of Oman did not detect any aflatoxins in 15 selected samples out of 105 spices samples including cloves and ginger, but found 45% of their 20 *Aspergillus flavus* isolates to be aflatoxigenic. It has been established that spices may support fungal growth (e.g *A. flavus*) but inhibit the production of aflatoxins than in cereals (MacDonald and Castle, 1996). Therefore it is suggested that not all *A. flavus* strains are aflatoxigenic on this kind of matrix (Elshafie *et al*., 2002). Bokhari (2007) also reported that some spices inhibit aflatoxin biosynthesis but not the growth of the toxigenic fungi.

Conclusion

A total of 7 different fungi species were isolated with *A. flavus* (50.4 %) and *A. parasiticus* (44.8 %) more prevalent than any other species isolated. Aflatoxin assay revealed that 4 spices were contaminated in the range of 3.3-17.2µg/kg. African nutmeg and chillies had aflatoxin concentrations above maximum acceptable limit (10µg/kg) set by EU and NAFDAC.

Recommendations

Spices should be properly dried in a hygienic place so as to prevent mould contamination during drying. Market hygienic conditions need to be improved.

Acknowledgements

We acknowledge Mr. Emmanuel Ndem of National Agency for Food and Drug Administration and Control (NAFDAC), ShituAdamu and MalamTanimu of Ahmadu Bello University Zaria, Kaduna State, Nigeria for technical assistance offered

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