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Assessment of Aflatoxins and Aflatoxigenic Fungi Associated with Dried Vegetables from Selected Markets with in Kaduna Metropolis

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Abstract: Vegetable are considered as the leafy outgrowth of plants shoot used as food. These include those plants or plant part used in making soup or served as an integral part of main meal they are sources of nutrients, vitamins and minerals. Drying is a cheap means of preserving vegetables because they are prone to fungi contamination. The assessment of aflatoxigenic fungi associated with dried vegetables (Baobab, Red chilli pepper, Okro and Tomatoes) from selected markets within Kaduna metropolis were investigated. A total of forty (40) samples (ten samples each) of the dried vegetables were analyzed for fungi and total aflatoxin. Fungi were identified and characterized using the conventional and Molecular technique and Total Aflatoxin were identified using Enzyme linked immune-sorbent assay (ELISA). The fungi identified were *Aspergillus flavus and Aspergillus niger.* The result of the total aflatoxin revealed that Baobab from Sabo (SB 001) had the highest aflatoxin of 31.6µg/kg while Baobab from Barnawa (BRW 001), with aflatoxin content of 18.80µg/kg. Baobab from Tudun wada (TW 001) with aflatoxin content of 15.00µg/kg, Baobab from Malali (MLL 001) had aflatoxin content of 12.10µg/kg is higher while Baobab from Central Market had aflatoxin of 1.60µg/kg. The vegetable with the highest aflatoxin content is Baobab from Sabo (SB 001) had aflatoxin content of 31.6µg/kg, while Okra from kamazou (KMZ 003) with aflatoxin content of 27.00µg/kg and Red Chilli Pepper from Malali (MLL004) had aflatoxin content of 26.40µg/kg, Tomatoes from Malali (MLL 002) had the lowest aflatoxin content of 6.50µg /kg. These result can serve as baseline for enacting laws and observing the critical control point as the ingestion of such mycotoxins contaminated vegetables have enormous health significance. Because these toxins are capable of causing diseases in man and animals.

Keywords: Baobab, *Aspergillus flavus,* Aflatoxin, Polymerase Chain Reaction (PCR), ELISA

1. Introduction

Vegetable consumption provides a valuable contribution to the nutrition of rural and urban populations in West Africa. Numerous studies have recorded uses and consumption patterns of vegetables [22], but few studies have reported on dried vegetables. Drying of foods is practiced in Africa to make the products more durable and preserve them for food insecure periods. Drying is mainly done on an artisanal scale or through small-scale industrial units. Dried products can be infected with fungi and other contaminants either already present on the primary product, or during the drying process that takes place under unhygienic conditions; further spoilage can occur during storage, handling and transport till sale [20]. Crops or products that are susceptible to fungal growth can also be contaminated with mycotoxins [6]. Mycotoxins are hazardous to consumers' health and affect food quality leading to economic losses including loss of commercial value [25]. Fungi are generally aerobic organisms therefore storage atmosphere deficient in oxygen would lead to reduced metabolism and consequently mycotoxin production [19] reported that a reduction in oxygen content of storage environment from 5% to 1% and increase in carbon dioxide content to above 20% dramatically reduced the growth of *Aspergillus flavus* and AF production. Commodities are better stored anaerobically with the addition of organic acids such as propionic acid as preservatives in storage systems which do not absorb moisture or enhance moisture migration

[19]. Unfortunately traditional storage facilities in Africa are devoid of such standard storage Aflatoxins are poisonous secondary metabolites produced mainly by *Aspergillus flavus*, *Aspergillus parasiticus* and, *Aspergillus nomius*, which according to [10] contaminate plants and plant products like peanut, corn, cottonseed and tree nuts [14]. The four major aflatoxins, B1, B2, G1 and G2 are the most important mycotoxins in foods and feeds because of their high prevalence in nature and toxicity [19]. This group of deadly mycotoxins contaminates nuts and oilseeds, cereals, roots and tubers, fruits, vegetables and animal feeds [19] particularly in warm, humid regions of the world. Natural occurrence of mycotoxins in vegetables has been demonstrated in many countries [29]. However, reports on mycotoxin contamination of African traditional vegetables are exceptionally rare. There are reports of the association of aflatoxin contamination of plants foods particularly cereals with liver cancer in Africa and china [18]. Health hazard posed by mycotoxins to animals and humans is well recognized and over 100 countries have established regulations for major mycotoxins in food. Rapid detection and quantification of moulds and mycotoxins in food commodities and processed food products is necessary for assuring safety and quality of food and implementation of hazard analysis and critical control points (HACCP). The food stuffs which are highly susceptible for the mycotoxin contamination are corn [9], peanuts [26], spices [12] and herbs [17]. Cereal grain is usually infected or colonized by different pathogenic or saprophytic fungi during the vegetative period. Numerous fungal species are reported to occur in barley grains. [21] Mycotoxins have been detected in human foods and livestock feeds in Nigeria. On a world wide scale, relationships between mycotoxins and human illness have been clearly established. Aflatoxins have been shown to be involved with and to aggravate hepatitis B infection [16]. In Nigeria, documentation of Mycotoxin related human health problems is not extensive. Despite the awareness of the health hazard posed by mycotoxin and established regulations, there is little information about the occurrence of mycotoxin in Northern Nigeria, (*Adansonia digitata,)* okro (*Abelmoschus esculentus)* tomatoes *(Lycopersicum esculentum)* and red chili pepper (C*apsicum annuum*) is an important vegetable that is consumed in almost every home in Northern Nigeria.

2. Materials and Method

2.1. Collection of Sample

Samples of dried leaves of *Adansonia digitata*, *Abelmoschus esculentum, Lycopersicum esculentum*), and (*capsicum annum*) were collected from ten locations, and fourty samples from ten major sellers within kaduna metropolis were collected into fourty clean polythene bags and immediately transported to food and industrial microbiology research laboratory for analysis.

2.2. Preparation of Sample for Isolation of Fungi

The powdered leaves of the grinded samples were inoculated on Sabouraud Dextrose Agar plates to which streptomycin was incorporated. The inoculated plates was incubated upright at 25°C for 5 days. All observed colonies were subculture to obtain pure cultures. The growth rate and diffusible pigmentation of each sample was examined macroscopically. Tease mount using lactophenol cotton blue was adopted and microscopic features such as spore and hyphae morphology were observed and compared with the standard colour atlas [3].

2.3. Identification of Fungi

2.3.1. Molecular Identification of Fungi

The extraction of the DNA was carried out using the method of [8]. Where the fungi obtained was subjected to PCR assay. The fungal isolate were subjected to molecular technique using the method of [8]. The PCR method were used to confirm the genetic variation among the *Aspergillus* groups using ITS set of primer 4 and 5 and gel electrophoresis of PCR products confirmed the presence of *Aspergillus* species at an amplification range of 500-600 base pairs in all the sample tested.

In gel electrophoresis, the amplified products are separated by electrophoresis on a 1.5% agarose gel with 0.5% ethidium bromide in 1x TAE buffer and 100bp DNA ladder was used as molecular weight marker. After staining with gel red, the gels were visualized under ultraviolet light (UV) photographed by Gel Doc 2000. PCR clean up and DNA sequencing was carried out to obtain a pure DNA, a PCR clean-up using Nucleospin gel and PCR Clean up kit to purify the DNA 700 microlitre of wash buffer NT3 was added and centrifuged at 12000 rpm for 30 seconds and repeated twice. The gel and PCR clean –up nucleospin column was placed in a new centrifuge tube with 30 microlitre of elution buffer and incubated at room temperature (18- 25°C) and centrifuged at 12000 rpm for 1 minute the DNA is recovered in a pellet. The identity of the strain was verified by morphological characteristics and by comparing the ribosomal DNA sequences with those already deposited in the data at Genbank using blast search tool [8].

2.3.2. Sample Preparation and Extraction of Mycotoxins

Two hundred grams (200g) each of powdered dried vegetables (Tomatoes, Red chili pepper, Okro and Baobab) were weighed and pulverized into powder, twenty grams of the grinded samples were weighed into a clean bottle and 80 ethanol were added, it was shaken vigorously for 3minutes and allowed to settle. The top layer of extract was filtered using whatman No. 1 filter paper and the filtrate collected for total aflatoxin analysis [3].

2.3.3. Analysis of Total Aflatoxin

The beacon Total Aflatoxin kits were used for total aflatoxin assay. Beacon Total Aflatoxin assay is a direct competitive enzyme-linked immunosorbent assay (ELISA). The assays were performed according to the procedure described in the Beacon Assay kit manual. According to the procedure there is no need for dilution of samples for

aflatoxins determination. One hundred microlitre extracts without dilution were further used in the procedure. Absorption in microwells was measured with an ELISA microwell reader using a 450 nm absorbance filter.

3. Result and Discussion

3.1. Results

This study was conducted to determine the aflatoxigenic fungi flora of dried vegetables marketed in kaduna metropolis and the result presented in table 1 The result shows that the mycotoxigenic fungi identified are *Aspergillus flavus*, *Aspergillus niger*. The presence of this organism is not surprising as their source could be from soil, dust or the vegetable themselves at the point of harvest. It also indicates that the dried vegetable and in particular the baobabs leaves powder (*Adansonia digitata*) that are used in kaduna metropolis are highly contaminated.

Table 1. *Colonial and Microscopic Identification of Fungi Associated with Dried Vegetables Sold within Kaduna Metropolis.*

Sample	colony description	Microscopy	probable identity
Baobab		Has aerial hyphae bearing	*Aspergillus flavus*
	Light green and powdery	conidiosphores intermixed	
	Colonies		
	Brown to black colonies	The conidospore terminate in vessels, and the conidia are in chains	*Aspergillus niger*
Tomatoes			*Aspergillus flavus*
	Light green and powdery	Has ariel hyphae bearing	
		Colonies conidiospore	
Okra	Brown to black colonies	The conidiospore	*Aspergillus niger*
	terminate in vessels and Light green and powdery	the conidia are in chains.	
	Colonies		
Red chilli pepper	Light green and powdery	has ariel hypae bearing	*Aspergillus flavus*
	Colonies	Conidiospore	

Table 2. *Frequency of Occurrence of Fungal Species.*

Identified organisms	Number of organisms	Frequency (%)
Aspergillus niger	22	55
Aspergillus flavus	18	45

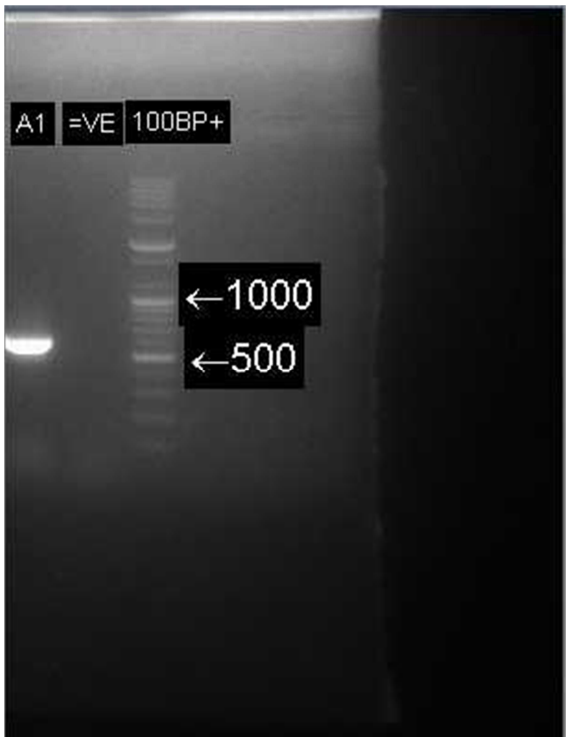

Figure 1. *Gel electrophoresis of PCR product amplified by using ITS4/ITS5.*

Table 3. *Sequencing of Fungi DNA.*

ACCTGGAAGAATGGTTGGAAAACGTCGGCAGGCGCCGGCCAATC
CTACAGAGCATGTGACAAAGCCCCATACGCTCGAGGATCGGACGC
GGNGCCGCCGCTGCCTTTCGGGCCCGTCCCCCTGTCTTGGCATCCC
CCTCCTGCCTGCAATCCTTCTCCGCTTGAAACATCATGTGATATTAA
TTCTTCTTGTGATCACTGCATCTTCTTCTCCGCCTT

Key: A: Adenine, C: Cytosine, T: Thymine, G: Guanine.

The result of the product of PCR product amplified were shown in figure 1 and the seguencing of the fungi DNA in table 3. The result of the BLAST showed the result as 99% *Aspergillus flavus*. Table 5 showed the fungi isolated from the *Adansonia digitata* are of two types depending on the location two different types were identified microscopically as *Aspergillus niger, Aspergillus flavus*. Table 6 showed the fungi obtained from *Lycopersicum esculentum* which were identified microscopically as *Aspergillus niger* and *Aspergillus flavus*. Table 7 showed the fungi obtained from *Abelmoschus esculentum* which were observed to be *Aspergillus niger* and *Aspergillus flavus* from different locations. Table 8 showed fungi obtained from *Capsicum annum* from different location were observed to be *Aspergillus flavus* and *Aspergillus niger*. Table 9 showed result of aflatoxin analysis of dried vegetables consumed in kaduna metropolis is presented from the result dried *Adansonia digitata* leaves had the highest total aflatoxin content of 31.6 ppb, followed by dried *Abelmoschus esculentum,* with 27.0 ppb and *Capsicum annum* 26.4 ppb dried *Lycopercium esculentum* had the lowest value 6.5 ppb. this variation may be due to the fact that some of the

plant may not have provided the necessary nutrient and environment necessary for fungi proliferation and subsequent aflatoxin production.

Table 4. *Basic Local Alignment Search Tool (BLAST) Query of Fungal DNA.*

Score	Expert	Identities	Gaps	Strand	Frame
220BITS	(119)	9E-54 ()	120/121	(99%)	0/12 (0%)

Features
Query1
ACCTGGAAGAATGGTTGGAAAACGTCGGCAGGCGC
CGGCCAATCCTACAGAGCATGTGAC 60
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 398
ACCTGGAAGAATGGTTGGAAAACGTCGGCAGGCGC
CGGCCAATCCTACAGAGCATGTGAC S339
Query 61
AAAGCCCCATACGCTCGAGGATCGGACGCGGNGCCG
CCGCTGCCTTTCGGGCCCGTCCCC 120
|||||||||||||||||||||||||| ||||||||||||||||||||||||
SBJCT 338 AAAGCCCCATACGCTCGAGGATCGGA
CGCGGTGCCGCCGCTGCCTTTCGGGCCCGTCCCC 279
QUERY 121 C 121
|
SBJCT 278 C 278
KEY: %: Percentage, A: Adenine, C: Cytosine, T: Thymine, G: Guanine.

Table 5. *Fungi Isolated from Dried Adansonia digitata Sold within Kaduna Metropolis.*

Isolate codes	Microscopic observation	Probable isolates
BRW 001, UGR 001, KMZ 001, BDR 001, MLL 001, TW 001 SB 001, CMK 001 KW 001, MDY 001	The conidiospores terminate in vessels, and the conidia are in chains	*Aspergillus niger*
BRW 001, ML 001, TW 001, SB 001, CMK 001	They have aerial hyphae bearing conidiospores	*Aspergillus flavus*

Key: BRW: Barnawa, UGR: Unguwan Rimi, KMZ: Kamazou, BDR: Badarawa, MLL: Malali, TW: Tudun wada SB: Sabo, CMK: Central Market, KW: Kawo, MDY: Monday, 001: *Adansonia digitata*

Table 6. *Fungi Isolated from Dried Lycopersicum esculentum Sold within Kaduna Metropolis.*

Sample codes	Microscopic observation	Probable isolates
BRW002, UGR002, KMZ002, BDR002, TW002, SB002, CMK002, KW 002, MDY002	The conidiospores terminate in vessels, and the conidia are in chains.	*Aspergillus niger*
BRW002, ML002, TW002, SB002, CMK002, MDY002	They have aerial hyphae bearing conidiospores.	*Aspergillus flavus*

Key: - BRW: Barnawa, UGR: Unguwan Rimi, KMZ: Kamazou, BDR: Badarawa, TW: Tudun wada, SB: Sabo, CMK: Central market, KW: Kawo, MDY: Monday MLL: Malali), 002 (*Lycopersicum esculentum*)

Table 7. *Fungi Isolated from Dried Abelmoschus esculentun Sold within Kaduna Metropolis.*

Sample codes	Microscopic observation	Probable isolates
UGR003, BDR003, TW003 SB003, CMK003, KW003, MDY003	The conidiospores terminate in vessels, and the conidia are in chains.	*Aspergillus niger*
BRW003, KMZ003, MLL003, TW003, SB003, CMK003, MDY003, BDR003	They have aerial hyphae bearing conidiospores.	*Aspergillus flavus*

Key:-UGR: Unguwan Rimi, BDR: Badarawa, TW: Tudun wada, SB: Sabo, CMK: Central market, MDY: Monday, BRW: Barnawa, KMZ: Kamazou, MLL: MLL, 003: Abelmuschus esculentum

Table 8. *Fungi Isolated from Dried Capsicum annum Sold within Kaduna Metropolis.*

Sample codes	Microscopic observation	Probable isolates
SB004, BDR004, TW004, CMK004, KW004, MDY004	The conidiospores terminate in vessels, and the conidia are in chains.	*Aspergillus niger*
SB004, BRW004, KMZ004 MLL004, TW004, CMK004, MDY004, BDR004	They have aerial hyphae bearing conidiospores.	*Aspergillus flavus*

Key: - SB: Sabo, BDR: Badarawa, TW: Tudun Wada, CMK: Central Market, KW: Kawo MDY: Monday, BRW: Barnawa, KMZ: Kamazou, MLL: Malali, 004: *Capsicum annum*

Table 9. *Total aflatoxin content in dried vegetables sold in kaduna metropolis.*

Sample	Sample code	Aflatoxin content (µg/kg)	Aflatoxin EU limit (µg/kg)
Baobab	BRW001	18.80 ± 0.48^{e}	2-4
	MLL 001	12.10 ± 0.40^{c}	2-4
	TW 001	15.00 ± 0.87^{d}	2-4
	SB 001	31.60 ± 1.40^{g}	2-4
	CMK 001	1.60 ± 0.24^{a}	2-4
Tomatoes	MLL 002	6.50 ± 0.85^{b}	2-4
Okro	KMZ 003	27.00 ± 0.86^{f}	2-4
Red chilli pepper	MLL 004	26.40 ± 1.20^{f}	2-4

Values are Mean ± SEM, value with different superscript within the rows are statistical significant at ($p<0.05$)
KEY- BRW 001 (Barnawa Baobab), MLL 001 (Malali Baobab), TW 001 (Tudun Wada Baobab), SB 001 (Sabo Baobab), CMK 001 (Central Market Baobab), MLL 001 (Malali Tomatoes), KMZ 003 (Kamazoub Okra), MLL 004 (Red Chilli Pepper)

3.2. Discussion

These result shows that the mycotoxigenic fungi identified are *Aspergillus flavus* and *Aspergillus niger.* A total number of two isolates were identified from 40 samples from different locations in kaduna, Nigeria. These isolates were subjected to microscopic, macroscopic and molecular identification and the result shown in these findings correlates with the findings of [2] who provided data on fungi contaminating Okra and Red chilli pepper, [3] also detected same fungal spectrum in baobab and tomatoes. [13] Findings

in Baobab, Tomatoes, Okra and Red Chilli Pepper. The occurrence of aflatoxin is highly pivoted on favourable temperature of between 24 and 28°C and substrate moisture content of at least (17.5%) [19], this might be the reason for the observed moisture dependent aflatoxin contamination trend. The identification of these toxin from various samples using ELISA have these results presented in the table 2 above and it agrees with the findings of [3] who also found aflatoxin in these vegetables using same method [24] also found the toxin in Okra and Red chilli pepper, [1] detected aflatoxin also in dried okra and red chilli pepper [23] found the toxins present in fresh tomatoes marketed in Sokoto, Nigeria. Similarly, [27] also showed the presence of aflatoxin in fresh tomatoes, powder red chilli pepper grown in Pakistan. In slight variance to these work [19] and [13] did not find aflatoxin in both fresh and dried baobab, and okra sample but found aflatoxin in red chilli pepper. The presence of aflatoxins in very low frequency of occurrence and concentration in okra in these three surveys and the detection of the mycotoxins in same crop from one location out of ten in this work imply that okra is an unsuitable substrate for aflatoxin contamination. The phytochemical composition of okra [19] which includes essential oils especially the fungicidal principles; phenols and tannins that confer inherent resistance to mould infestation and aflatoxin production [19] could be the reason for the absence of the toxins in the crop. The results obtained for RCP are similar to values obtained by [31] in red paprika at AFB1 and total AF maximum level of 5.40µg/kg and 9.68µg/kg in Morocco but higher than the estimated values observed by [13] in 1 out of 30 samples contaminated with AFB1 at concentration of 3.2µg/kg. The herein reported concentrations were lower than those determined by (35.9, 52.6 and 69.2µg/kg in three red chili powder), [15] (total AF concentration of 0.2 – 79.7µg/kg in 52 of 80 commercially dried chili) in Bahrain and Malaysia respectively. In their review on mycotoxins in botanicals and dried fruits, [29] reported alarming aflatoxin levels in *Capsicum* spp from Ethiopia and India. Eight of the 60 red pepper samples from Ethiopia contained AFB1 at concentration ranging from 100µg/kg to 500µg/kg. The toxin (AFB1) was also found in 107 out of the 182 marketed Indian chili pod samples at concentration of up to 969µg/kg. Also recorded the occurrence of AFB1 in 18 of 70 ground red pepper samples from Hungary at concentrations of between 6.1µg/kg and 15.7µg/kg. Our results therefore lay credence to the findings that *Capsicum* specie are good substrate for aflatoxigenic fungi growth and aflatoxin production and are therefore susceptible to aflatoxin contamination worldwide.

The aflatoxin content range from 1.6ppb - 31.6ppb was observed which is higher than the maximum acceptable limits in foods. The contamination of all these vegetables accessed and in particular baobab is an indication of improper drying, storage and handling of these products and may have exposed a lot of its consumers to the adverse effect of aflatoxin unknowingly. Although, it is difficult to prevent aflatoxin formation in food prior to harvesting due to high heat and moisture, it is possible to attain favorable result by correct storage [31]. Decreasing fungal growth and mycotoxins formation in food and feed are essential since it is consumed by both human and animals. *Aspergillus* is found in food storage places which produce mycotoxin at suitable moisture and temperature [28].

4. Conclusion

The result of vegetables assessed showed that most of the vegetables contain high amount of toxigenic fungi the fungi isolated were A*spergillus niger* with the highest frequency of occurrence (55%) followed by *Aspergillus flavus* (45%) with the total aflatoxin ranging from 1.6 ppb to 31.6 ppb which is above the maximum tolerable limit. The maximum tolerable limits for Aflatoxin in human foods is between 5-20 ppb, while for animal feeds is from 5 to 300 ppb with infant foods having the least regulated levels (0.05-10 ppb). These result can serve as baseline for enacting laws and observing the critical control point, Good agricultural practices (GAP) represent a primary line of defense against contamination of vegetables with mycotoxins, followed by the implementation of Good manufacturing practices (GMP) during the handling, storage, processing, and distribution of vegetables for human consumption as the ingestion of such mycotoxins contaminated vegetables have enormous health significance. Because these toxins are capable of causing diseases in man and animals.

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