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AFLATOXIGENIC FUNGI AND AFLATOXINS IN LOCALLY PROCESSED PEANUT BUTTER IN LAGOS, NIGERIA

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ABSTRACT

Aflatoxin and the producing fungi are known contaminants of farm produce at pre-harvest, post-harvest storage and post-processing. This research was carried out to detect aflatoxigenic fungi and quantify aflatoxins in locally processed peanut butter. Forty-seven samples of peanut butter were purchased from vendors in different markets in Lagos, Nigeria. Fungal species were isolated by pour plate method and identified using cultural and microscopic characteristics. Aflatoxigenic fungi (*Aspergillus flavus* and *A. parasiticus*) were isolated from 14/47 (29.79%) samples and they were screened for four aflatoxin genes (*afl*R, *nor*-1, *ver*-1, *omt*-1). Aflatoxin was quantified in samples with aflatoxigenic fungi using High Performance Liquid Chromatography with Ultra violet detection (HPLC-UV). Only seven (five *A. flavus* and two *A. parasiticus*) of the fourteen isolates screened had one or more aflatoxin genes with most isolates having *nor*-1 gene. Aflatoxin B_1 was present in all the peanut butter while aflatoxins B_2, G_2 and G_1 were present in 71.43%, 85.71%, and 57.14% of the samples respectively. The peanut butter samples had total aflatoxin content ranging between 373.6µg/kg – 6741.6µg/kg, which is above the 20µg/kg maximum permissible limit recommended by US FDA and ≤ 4µg/kg by EU. Aflatoxin B_1 content was 54.3µg/kg – 805.8µg/kg, and also far above the EU limit of 2µg/kg. The high concentration of aflatoxins and occurrence of aflatoxin B_1, the most toxic aflatoxin, in all the peanut butter is of great concern to the health of consumers. Adequate sensitization on preventive measures especially avoidance of mouldy peanut kernels by sorting before use in the production of peanut butter should be encouraged.

Keywords: Aflatoxin; peanut butter; *Aspergillus flavus*; *Aspergillus parasiticus*

INTRODUCTION

Aflatoxins are mycotoxins known to be toxic, mutagenic, carcinogenic and immunosuppressant. They are secondary metabolites produced majorly by *Aspergillus flavus* and *Aspergillus parasiticus.* These fungi are common contaminants of cereals (maize, wheat, rice and oats), **(Krnjaja *et al.*, 2019)** and nuts such as peanuts. Peanuts are rich in nutrients especially proteins, fats, carbohydrates, and minerals **(Settaluri *et al.*, 2012)** and so will support fungal growth and aflatoxin production. Infection of peanut by aflatoxigenic fungi starts right from the farm before harvest through the storage period under conducive intrinsic and extrinsic factors. In the Tropics, humid storage conditions with high temperature support fungal growth. Contamination of peanut by *Aspergillus* section *Flavi* and aflatoxins could occur at any point along the supply chain **(Norlia *et al.*, 2019)**. Peanuts are the major raw materials used for production of peanut butter. Peanut and peanut products are among foods that have been found to be incriminated in aflatoxin contamination.

Siwela *et al.* (2011) investigated levels of aflatoxin in peanuts that can be passed over to peanut butter. They however discovered reduction in the level of aflatoxin at each processing stage bringing it to total reduction of 89% and with the highest reduction recorded during roasting and blanching/skinning of the peanut. **Ndung'u *et al.* (2013)** isolated *Aspergillus niger*, *A. caeletus*, *A. alliaceus*, *A. tamari*, *A. parasiticus*, *A. flavus* (L and S strain) and *Penicillium* spp. from raw, roasted groundnut and peanut butter in Kenya. They also reported aflatoxin contamination in the nuts and butter which they attributed to the source of groundnuts and occurrence of defective nuts. High occurrence of aflatoxins was discovered in peanut and peanut butter in Zimbabwe **(Mupunga *et al.*, 2014). Yentur *et al* (2006)** obtained aflatoxin level higher than that of the recommended Turkish Food Codex in their peanut butter samples.

Foods and feeds contaminated by aflatoxins affect human and animal health and also result in economic loss **(Nazhand *et al.*, 2020).** Dietary exposure of humans to aflatoxins cause serious health problems worldwide and in some cases, it may lead to death, though the effect is usually cumulative. It has been known to cause acute hepatocellular carcinoma, liver cancer. Globally, aflatoxin has been reported to be responsible for the cause of 4.6% to 28.2% hepatocellular carcinoma yearly and aflatoxins have been found to be more prevalent in less developed countries in tropical and sub-tropical regions of the world **(Liu and Wu (2010).** Impaired digestion, slow rate of growth and teratogenic effect associated with congenital malformation have been reported as symptoms of aflatoxicosis **(Sarma *et al*, 2017).** Reye-like Syndrome was reported in Thailand, New Zealand, Czechoslovakia, the United States, Malaysia, -Venezuela, and Europe to be associated with aflatoxin **(Bbosa *et al.*, 2013).**

In Nigeria, some researches have been carried out on mycotoxin contamination of peanut and peanut products. **Oyedele *et al.* (2017)** isolated moulds from 97.6% of the 84 groundnut samples from four agro-ecological zones in Nigeria. They isolated *Penicillium*, *Fusarium* and more of *Aspergillus*. They detected both fungal and bacterial metabolites and aflatoxins were the most abundant. Peanut cake commonly called 'kulikuli' was reported to contain 20 fungal metabolites with aflatoxins above the US FDA limit of 20 µg/kg in 90% of the samples **(Ezekiel *et al.*, 2012).** Peanut butter in Nsukka, South East Nigeria was reported by **Onyeke *et al.* (2017)** to contain total aflatoxin above the permissible level of 10 µg/kg set by FAO/WHO for food meant for human consumption in developing countries and far exceeded the 4 µg/kg standard of the European Union but with aflatoxin B_1 and B_2 within permissible limits.

Lagos is a densely populated urban centre located in South West Nigeria where the populace depends largely on fast foods because of their metropolitan life style. Locally processed peanut butter bought both from road side and traditional markets is usually eaten with garden eggs. It is a delicacy which also serves as spread on bread. Peanut butter imported from other countries to Nigeria are retailed in super markets or super stores. The locally processed peanut butter is cheaper and easily accessible to consumers on the streets. The production process in local cottage industries do not adhere strictly to safety guidelines. Considering the association of aflatoxins with peanut, the raw material for production of peanut butter and the hazardous effect of aflatoxins this research work was carried out to determine the occurrence of aflatoxin producing fungi in locally produced peanut butter in Lagos, Nigeria for contamination with aflatoxins and presence of aflatoxin genes in the isolates.

MATERIALS AND METHODS

Sample collection

A total of 47 samples of locally processed peanut butter (packaged in small plastic containers) were bought from different markets in Lagos. The samples obtained were immediately labelled and transported to the laboratory for analysis. Samples were obtained from seven markets under five local government areas as shown in Table 1.

Table 1 Collection of samples

S/N	Local government	Sample source	Number of samples obtained
1	Lagos mainland	Oyingbo	8
2	Alimosho	Iyana-ipaja	5
3	Oshodi-isolo	Oshodi	8
4	Shomolu	Bariga	5
5	Eti-osa	Lekki phase one, Agungi, Ajah	21

Isolation and identification of fungi

Ten grams of peanut butter was weighed into 90ml of sterile distilled water and homogenized. Using pour plate method, 1ml of resulting mixture was plated in duplicate on Potato Dextrose Agar (PDA) and incubated at 25ºC for 5 days. Colonies obtained were sub cultured on PDA to obtain pure cultures. Sterilized inoculating needle was used to cut small portion of fungal growth, placed on the middle of clean slide, few drops of lacto phenol blue were added and teased with the inoculating needle. It was carefully covered with a cover slip avoiding air bubbles production. It was then observed under ×40 magnification of a light microscope.
Identification was based on microscopic examination, colony characteristics (colour, appearance, reverse colony colour appearance), using Pictorial atlas of soil and seed fungi **(Watanabe, 2002)** and Atlas of clinically important fungi **(Sciortino, 2017).**

Detection of aflatoxin genes

DNA extraction

The five *A. flavus* and nine *A. parasiticus* (14 in total) isolated from locally processed peanut butter were inoculated on fresh PDA and incubated at 25ºC for 72 hrs. A small amount of each fungus was transferred into sterile mortar and crushed with pestle in Phosphate buffer solution (this preliminary step was to lyse the cell wall). A solution made up of 95µl of water, 95µl of solid tissue buffer and 10µl of proteinase K was added and mixed thoroughly using a vortex mixer. It was then incubated at 55ºC for 2 hrs and centrifuged to remove insoluble debris. Then 200µl of supernatant was transferred to a tube and 400µl of genomic binding buffer was added to it. The mixture was transferred to a Zymo-spin™ IIC-XL column in a collection tube and centrifuged (≥12000×g) for 1 minute, the collection tube was discarded with the flow through. To the column in a new collection tube, 400µl DNA Pre-wash buffer was added and centrifuged and the collection tube was emptied. Seven hundred micro litres (700µl) of g-DNA wash buffer was added and centrifuged for 1 minute and the collection tube was emptied. Two hundred micro litres (200µl) of g-DNA wash buffer was added and centrifuged for 1 minute and the collection tube was discarded with the flow through. The Zymo-spin™ IIC-XL column was transferred to a 1.5ml Eppendorf tube and 50µl of elution buffer was used to elute the genomic DNA and was stored at -20ºC (Zymo Research Group, USA).

Polymerase chain reaction (PCR)

The Solis Biodyne 5X HOT FIREPol Blend Master mix was used. Primers used are as according to **Scherm *et al*. (2005)** (Table 2). The 20µl reaction mixture used consisted of 1x Blend Master mix buffer (Solis Biodyne, Estonia), 2.0mM $MgCl_2$, 200µm of each deoxynucleoside triphosphates (dNTP) (Solis Biodyne, Estonia), 20pMol of each primer (BIOMERS, Germany), 2 units of hot FIREPol DNA polymerase (Solis Biodyne, Estonia), proofreading enzyme, 5µl of the extracted DNA, and was made up with sterile distilled water.
Peltier thermal cycler 100 (MJ Research) was used for thermal cycling. The initial denaturation was at 95°C for 15 minutes, followed by 35 cycles at 95°C for 30s, 62°C for 1min. and 72°C for 1min. 30s. There was a final extension step at 72°C for 10mins. The amplification product was separated on a 1.5% agarose gel and electrophoresis was carried out for 1hr 30mins. at 80V. The DNA bands were visualized by staining with ethidium bromide. The 100bp DNA ladder was used as DNA molecular weight standard. The positive and negative controls used in this analysis were *Aspergillus flavus* ATCC 22546 and *Penicillium fellutanum* ATCC 48694 respectively.

Table 2 Aflatoxin primers used for PCR Amplification **(Scherm *et al*., 2005)**

S/N	Primer	Sequence	bp
1	*Nor*-1F	5′ -ACC GCT ACG CCG GCA CTC TCG GCAC-3′	400
2	*Nor*-1R	5′-GTT GGC CGC CAG CTT CGA CAC TCC G-3′	400
3	*Ver*-1F	5′-ATG TCG GAT AAT CAC CGT TTA GAT GGC-3′	895
4	*Ver*-1R	5′-CGA AAA GCG CCA CCA TCC ACC CCA ATG-3′	895
5	*Omt*-1F	5′-GGC CCG GTT CCT TGG CTC CTA AGC-3′	1232
6	*Omt*-1R	5′-CGC CCC AGT GAG ACC CTT CCT CG-3′	1232
7	*afl*RF	5′-TAT CTC CCC CCG GGC ATC TCC CGG-3′	1032
8	*afl*RR	5′-CCG TCA GAC AGC CAC TGG ACA CGG-3′	1032

Extraction of aflatoxin from peanut butter samples

Aflatoxin was extracted from the seven peanut butter samples which had isolates that possessed one or more aflatoxin genes. Twelve and half grams (12.5g) of the sample in addition with 2.5g NaCl and 62.5ml of 70% methanol were homogenized in a blender for 2 minutes at high speed. The mixture was then filtered through No. 1 Whatman filter paper. Thirty millilitres (30ml) of water was added to 15 ml of the filtrate and it was filtered again through a Millipore filter paper. Fifteen millilitres (15ml) of the second filtrate was passed through solid phase extraction (SPE) cartridge and then washed with 10ml of water. Aflatoxins were eluted with 1ml of ethanol and then 1ml of water **(Stojanovska *et al*., 2013).**

Determination of aflatoxin concentration in peanut butter

Aflatoxin concentration in locally made peanut butter samples and standards (Sigma-Aldrich) were determined using HPLC with UV detection. The seven samples with isolates that had one or more aflatoxin genes were those investigated for aflatoxin concentrations. The standards for aflatoxins G_2, G_1, B_2, and B_1 had concentrations 1µg/ml, 4µg/ml, 1µg/ml, and 4µg/ml respectively. The HPLC system consisted of a quad pump, degasser and a variable wavelength detector (Agilent Technologies, Germany). Aflatoxins were separated in HPLC column with mobile phase of methanol: water: acetonitrile (40:50:10% respectively). The UV detection was at a wavelength of 365nm and the flow rate was 0.7ml/min. The run time for each sample was about 10 minutes.

RESULTS

Fungal contamination of peanut butter

Out of the 47 samples of peanut butter investigated for fungal contamination, 33 (70.21%) were contaminated with fungi from two main genera; *Aspergillus* and *Rhizopus*. A total of 42 fungal isolates was obtained. The fungal isolates identified include *Rhizopus stolonifer*, *Aspergillus niger*, *Aspergillus parasiticus* and *Aspergillus flavus*. *Rhizopus stolonifer* was the most prevalent at 50% (21/42), *Aspergillus parasiticus* 21% (9/42), *Aspergillus niger* 17% (7/42) and *Aspergillus flavus* 12% (5/42) (Figure 1).

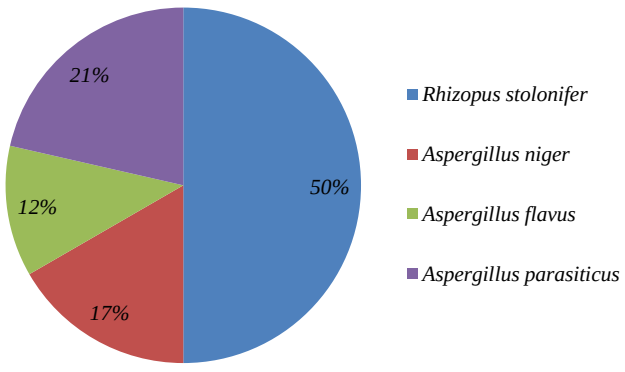

Figure 1 Prevalence of fungal isolates in locally processed peanut butter

Of greater importance in this study is that 14 (29.79%) samples were contaminated by aflatoxigenic fungi (*A. flavus* and *A. parasiticus*) (Table 3).

Table 3 Occurrence of fungal isolates in locally processed peanut butter

S/N	Sample source	Number of samples with mould growth	*Rhizopus stolonifera*	*Aspergillus niger* (Occurrence)	*Aspergillus flavus*	*Aspergillus parasiticus*
1	Lekki phase one	2	1	2	ND	ND
2	Agungi	3	1	1	1	1
3	Oyingbo	5	2	1	1	1
4	Ajah	10	8	1	3	5
5	Bariga	4	3	1	ND	ND
6	Iyana-Ipaja	4	3	ND	ND	1
7	Oshodi	5	3	1	ND	1

Legend: ND – not detected

Detection of aflatoxin genes

Of the five *Aspergillus flavus* isolates identified in this study, two had the full complement of all four aflatoxin genes present, namely nor-1, ver-1, omt-1 and aflR. One had three with aflR absent, while the last two had only nor-1 present. Of the nine *Aspergillus parasiticus* isolates only two had nor-1 present while no genes were detected in the other seven isolates (Figures 2-5)

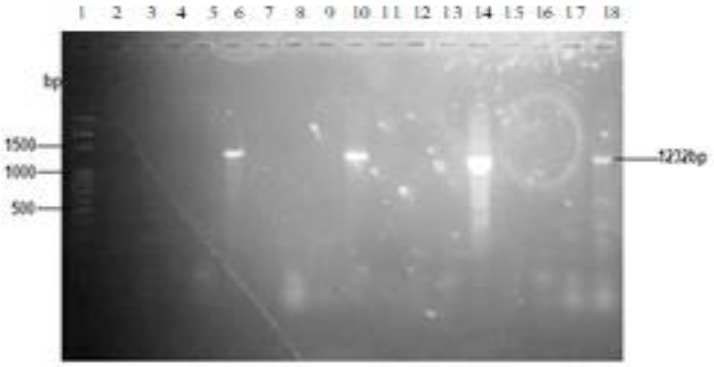

Figure 2 Detection of *omt*-1 gene; Lane 1, 100bp DNA ladder; Lane 2, negative control 1 (without genomic DNA); Lanes 6, 10, 14, isolates that were positive for *omt*-1 gene; Lane 17, negative control 2 (*Penicillium fellutanum* ATCC 48694); Lane 18 positive control (*Aspergillus flavus* ATCC 22546).

Figure 3 Detection of *ver*-1 gene; Lane 1, 100bp DNA ladder; Lane 2, negative control 1(without genomic DNA); Lanes 6, 10, 14, isolates that were positive for *ver*-1 gene; Lane 17, Negative control 2 (*Penicillium fellutanum* ATCC 48694); Lane 18 positive control (*Aspergillus flavus* ATCC 22546).

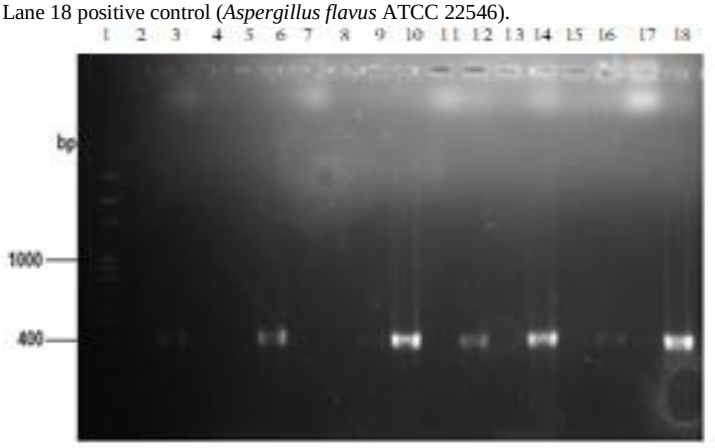

Figure 4 Detection of *nor*-1 gene; Lane 1, 100bp DNA ladder; Lane 2, negative control 1(without genomic DNA); Lanes 3, 6, 9, 10, 12, 14, 16 isolates that were positive for *nor*-1 gene; Lane 17, Negative control 2 (*Penicillium fellutanum* ATCC 48694); Lane 18 positive control (*Aspergillus flavus* ATCC 22546).

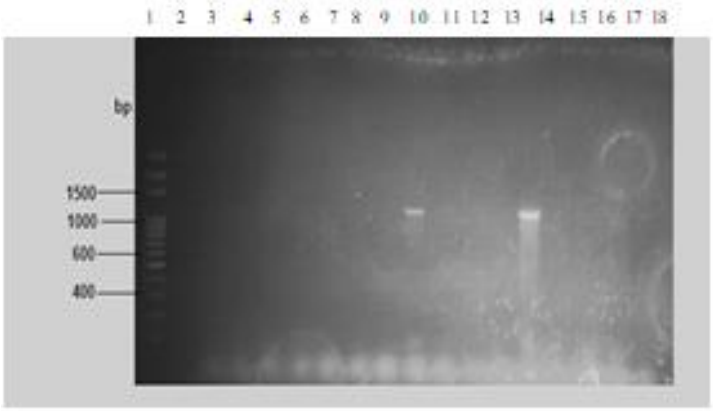

Figure 5 Detection of *afl*R gene; Lane 1, 100bp DNA ladder; Lane 2, negative control 1(without genomic DNA); Lanes 10, 14 isolates that were positive for *afl*R gene; Lane 17 negative control 2 (*Penicillium fellutanum* ATCC 48694); Lane 18 positive control (*Aspergillus flavus* ATCC 22546).

Concentration of aflatoxins in peanut butter

Aflatoxin was detected in the seven tested peanut butter samples. The concentration of aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2) and their prevalence in the tested seven peanut butter samples varied between 3.526µg/kg for aflatoxin B_2 to 4284.6µg/kg for aflatoxin G_1 and 57.14% for aflatoxin G_1 to 100% for aflatoxin B_1 respectively (Table 4). The range of total aflatoxin concentration of the tested peanut butter was 373.6µg/kg to 6741.6µg/kg (Table 4).

Table 4 Concentration and prevalence of aflatoxins in locally processed peanut butter

Sample number	Sample ID	AFG_2	AFG_1 (µg/kg)	AFB_2	AFB_1	Total aflatoxins
7	a	1006.8	2656.6	41.20	805.8	4510.4
18	b	0.00	0.00	22.2	351.4	373.6
22	c	3546	0.00	6.266	54.3	3606.57
24	d	1366	1310.4	92.2	397.8	3166.4
25	e	2001	0.00	3.526	374.0	2378.53
27	f	2110	667.0	0.00	167.6	2944
44	g	2257	4284.6	0.00	200	6741.6
	Prevalence (%)	85.71	57.14	71.43	100	100

DISCUSSION

In this study, 14 (29.79%) of the 47 samples of locally processed peanut butter from different zones in Lagos, Nigeria were contaminated with aflatoxigenic fungi (*A. parasiticus* and *A. flavus*). This is similar to the result reported by **Mupunga *et al*. (2014)** who isolated aflatoxigenic fungi from 3 (27%) of their 11 peanut butter samples in Zimbabwe. The peanuts which served as raw material for production of the peanut butter could have been contaminated with aflatoxigenic fungi and carried over to the peanut butter. In Nigeria the local production of peanut butter is done in cottage industries. Hazard Analysis and Critical Control Points (HACCP) have not been established in the production process. Therefore, fungal contaminants can set in along the processing line. Roasting of peanut is one of the steps involved in peanut butter production. However, there is no evidence of adequate monitoring of roasting temperature which may have helped to eliminate at least the vegetative forms of fungal contaminants although fungal spores could germinate later. Fungal contaminants may also have been introduced from the spices. There may have been post

processing contamination of the peanut butter by fungi during packaging or hawking from the environment.

Two of the *A. flavus* strains had the four aflatoxin genes (nor-1, ver-1, omt-1 and aflR), 1 strain had 3 genes except aflR while 2 strains each of *A. flavus* and *A. parasiticus* had only nor-1 gene. This was as opposed to the work carried out by **Davari *et al*. (2015)** on animal feedstuffs in Iran from which all aflatoxigenic isolates had the four genes. Different strains may have been isolated from both studies. In our study, two strains of *Aspergillus flavus* produced AFB_1, AFB_2, AFG_1, and AFG_2, one produced AFG_2, AFB_2, AFB_1, another one produced AFG_2, AFG_1, AFB_1 and yet another produced AFB_1, AFB_2. Each of the two *Aspergillus parasiticus* produced AFG_2, AFB_2, AFB_1 and AFG_2, AFG_1, AFB_1 respectively. This can be compared with the work of **Gherbawy *et al*. (2016)** where their aflatoxigenic *Aspergillus flavus* and *Aspergillus parasiticus* isolates produced the four aflatoxins (AFB_1, AFB_2, AFG_1, and AFG_2) in a similar pattern. The total aflatoxin concentration of all tested samples ranged from 373.6µg/kg – 6741.6µg/kg which is above the 20µg/kg maximum permissible limit by US/FDA and the ≤ 4µg/kg limit of the European Union (EU). This result differs from the 6.1 to 247ng/g obtained from peanut butter in Zimbabwe by **Mupunga *et al* (2014).** Of all the aflatoxins analysed, AFB_1 was the most prevalent (100%) which is similar to the 90% contamination of AFB_1 in peanut butter samples in South Africa reported by **Kamika *et al*., (2014).** In our study, AFB_1 concentration ranged from 54.3µg/kg – 805.8µg/kg which is above the EU maximum limit of ≤ 2µg/kg. This is in contrast with the 20µg/kg to 10,740µg/kg level of AFB_1 observed by **Njoroge *et al*. (2016)** over a three-year period in Zambia. We however obtained prevalence of 87.71%, 71.43% and 57.14% for AFG_2, AFB_2 and AFG_1 respectively. The high level of aflatoxins in the peanut butter may be possibly due to such level in peanuts which served as the major raw material. Local producers of peanut butter in Nigeria may not be aware of the dangers of aflatoxin contamination both in peanut and peanut butter. Aflatoxin B_1 is the most carcinogenic of all mycotoxins. It's association with hepatocellular carcinoma in liver and the likely synergy between the effect of hepatitis B virus and aflatoxins was reported by **Mably *et al*. (2005).**

CONCLUSION

This study has revealed contamination of locally processed peanut butter in Lagos, Nigeria with presence of aflatoxigenic fungi and aflatoxins above recommended limits. Adequate sensitization of the local producers against the use of mouldy peanuts is recommended. Our results from Lagos will help to broaden existing information on aflatoxins in Nigeria and the African continent at large.

Competing interest: None to declare

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