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Determination and Comparison of Total Aflatoxin Levels in Groundnuts from Four Major Markets in Lagos State, Nigeria

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ABSTRACT: *Aflatoxins (Aspergillus flavus toxins) are poisonous substances produced by some fungi on crops, foods, and feeds. The study was carried out to determine and to compare total aflatoxin levels in groundnuts sold in Lagos markets, Nigeria. Microbiological and toxicology tests were carried out on raw, boiled, and roasted groundnuts from the same source. The microbiological test was carried out to detect the fungi that produce aflatoxins as their secondary metabolite. The groundnuts samples were cultured on Potato Dextrose Agar (PDA). The isolates were identified using standard microbiological methods, characteristics such as presence of spores, shape, colour of spores and presence of septa were used in identification. Aspergillus flavus (75%; percentage of occurrence) was the most frequently isolated fungus from the groundnut samples. The other contaminant isolated from the samples were Aspergillus niger (25%). The toxicology analysis was carried out to determine the production of aflatoxins and the level present in the groundnut. This was carried out using a Romer Mycosep Column kit for aflatoxin to clean-up, and High-Performance Liquid Chromatography (HPLC) to detect the aflatoxins and determine the levels. Total aflatoxins were detected in the groundnuts, and they ranged between 0.36 ppb and 0.42 ppb. The levels of total aflatoxins were not beyond the regulatory standard of National Agency for Food and Drugs Administration and Control- NAFDAC (for ready to eat food, 4µg/kg while food that will still undergo processing 20µg/kg). Thus, the groundnut samples from the four markets are fit for consumption.*

KEYWORDS:*Aspergillus flavus*, Aflatoxins, toxicology test, microbiological test, mycotoxins, groundnut, food contaminants

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INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is an annual legume which is also referred to as peanut, earthnut, monkey nut and goobers and it plays a very important role in human diets as one of the world's most valued oil seed crops (Kankam *et al*., 2021). They are used extensively for innumerable industrial processes such as abstraction of oils for domestic, industrial uses, soap production, cooking, and the manufacturing of cosmetics (Abdulla, 2013). Furthermore, they are used in complementary food formulations for weaning babies and likewise, used in many animal feed formulations to achieve specific desires (Kortei *et al.,* 2021).

Groundnut occupies a very strategic position on the list of the world's cultivated crops; placing 13th in the world as well as being the 4th most important oil seed crop (Bediako *et al*., 2018). Groundnut is grown in more than 100 countries in the world particularly arid and semi-arid tropics (Ncube and Maphosa, 2020). Groundnut is cultivated in 28 million hectares of land in over 100 countries with 65% produced in Asia and 26% in Africa (FAOSTAT, 2019). The major groundnut producing countries in Africa are Nigeria (2,420,000 t), Sudan (1,641,000 t), Senegal (915, 000 t), Congo (27,315 t), Ghana (420,000 t), Chad (870,094 t) and Niger (461,842 t) (FAOSTAT, 2019). The average groundnut yield in Africa is, however, very low (964 kg/ha) compared to US (3500 kg/ha) and other developed countries (AICC, 2016).

Reddy *et al.,* 2009, Adetunji *et al.,* 2018 and Kortei *et al.,* 2021 reported that different species of fungi (mold) colonize groundnut and specialize in the different phases of activities; cropping, harvesting, handling and transportation, producing an array of mycotoxins that contaminate our foodstuffs. The physical presence of these fungi causes contaminations likewise their mycotoxins (Kankam *et al*., 2021). Mycotoxins are poisonous substances produced by certain fungi found primarily in grains and nut crops, spices, traditional herbal products, alcoholic drinks, and other produce (Odamtten, 2018). Adetunji *et al*., 2018 and IARC, 2002 reported that it poses a potential menace to both human and animal health.

Groundnut seeds are also affected by molds of the *Aspergillus* family with the common in nature been *Aspergillus flavus*, it is responsible for the production of aflatoxins contamination (structurally related toxic polyketide-derived secondary metabolites) (Kankam, 2021). The major producers of aflatoxins are *Aspergillus flavus* and *Aspergillus parasiticus* (Richard and Payne, 2003; Vargra *et al*., 2011). *Aspergillus* spp. infection and aflatoxin contamination are more pronounced where there are high temperatures, high moisture, moisture stress, poor harvest and post-harvest management and inadequate storage structures (Okello *et al.,* 2010). Contamination of groundnuts with aflatoxins can occur at every stage of the cropping season and afterwards. Contamination can occur in the field before harvest, during harvest or post-harvest (Kankam *et al*., 2021).

According to WHO in 2018, aflatoxin has been confirmed to have a great impact on a significant fraction of the most prioritized health menaces in developing countries and is probably the most well-researched mycotoxin in the world. Aflatoxin contamination results in

downgrading of grains and oilseeds, and depletion of their nutritional value (Jolly *et al.,* 2009). Prolonged consumption of aflatoxins has been reported to cause impaired immune function, malnutrition and stunted growth in children, disabilities, and death (Bbosa *et al.,* 2013; Gong *et al.,* 2004). Aflatoxin is known to have adverse effect on reproductive health and associated with liver cirrhosis, hepatitis B and C infections, and liver cancer (Bbosa *et al.,* 2013). The objective of this study is to determine the presence and level of aflatoxins in some selected peanuts sold in four major markets in Lagos State, Nigeria, to compare the effect of boiling and roasting on aflatoxins in peanuts, to isolate the fungi producing the aflatoxins. Figure 1 shows the pictures of *Aspergillus flavus and Aspergillus niger* isolated from the groundnut samples in the study.

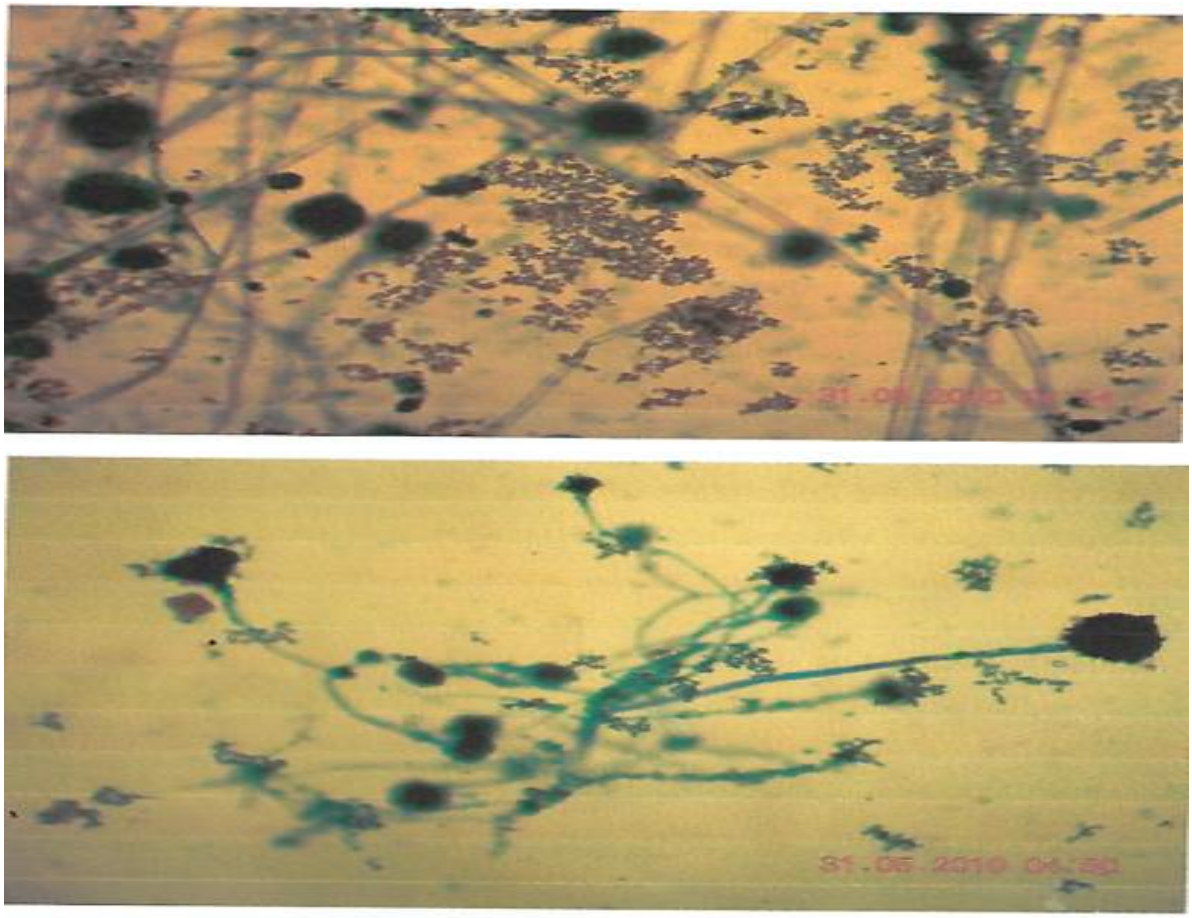

Figure 1: *Aspergillus flavus* stained with lactophenol cotton blue dye under microscopic view.

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MATERIALS AND METHODS

Sample collection

Raw peanut (groundnuts) samples were randomly purchased from four major markets located at Oyingbo, Mushin, Ile-epo and Mile 12 in Lagos State, Nigeria. They were at random collected from different stocks and properly labeled in this order sample A (Mile 12 market), sample B (Oyingbo Market), sample C (Mushin Market), Sample D (Ile-epo Market) respectively and taken to the laboratory for further analysis.

Sample preparation

The raw peanut samples were divided into three parts, the first part was left in its raw form, the second was boiled while the third was roasted using sand which is a common method of roasting groundnut in Nigeria. The peanut preparation was carried out in the laboratory the way it is prepared locally. The peanut used for the toxicology analysis were grinded.

Media preparation

To isolate the fungi (*Aspergillus flavus* and *Aspergillus parsiticus*) responsible in the production of these toxins, potato dextrose agar (PDA) was used (Chieng, 2022). The PDA used was amended with 100ppm (part per million) of the antibiotic, Chloramphenicol to inhibit bacteria growth. Chloramphenicol (0.5g) was diluted in a known volume of ethanol and sterilized using ultraviolet light overnight. 34.5g of the media was measured using a weighing balance into a conical flask with the aid of a spatula and weighing boat, it was then diluted in 500ml of distilled water. The media was uniformly dispersed in the solution by mixing thoroughly with a magnetic stirrer. The conical flask was covered with an aluminum foil, taped round, and sterilized alongside with the distilled water, using an autoclave at 121^{o}C for 15 minutes. The media was allowed to cool to a temperature of 45^{o}C after which, the chloramphenicol was added into it and mixed. The media was then poured into sterile petri-plates in the sterilization cabinet and allowed to solidify.

Microbiological sample analysis

The isolation procedure was carried out under aseptic condition using the method described by Sandes (2012) with some modifications. The work surface in the sterilization cabinet was swabbed with methylated spirit. Sodium hypochlorite was used for the surface sterilization of the peanuts (groundnut). Ten nuts were picked from each sample and suspended in a volume (10ml) of sodium hypochlorite differently for 10 minutes, after which, the nuts were rinsed thrice in sterile distilled water. With the use of sterile forceps, 5 nuts were picked from each

sample and inoculated into different petri-plates with solidified media. The plates were properly labeled and incubated at 27^{o}C for 5 days. The plates were fully observed daily.

After the incubation period, there was evidence of fungi growth on all plates, the colony characteristic of the organism was looked out for and this entails spore production, colony elevation, presence of hyphae, colour of spores, presence of cross wall or septate and the shape of growth from the reverse and top of plate. Staining techniques were also used to view the organisms present and for proper identification. The staining was carried out using lactophenol cotton blue dye. The procedure entailed making a wet mount slide and this was done by putting a drop of lactophenol cotton blue dye on a slide and picking a small amount of the isolate with an inoculating needle unto the drop of the dye. The isolate was gently teased apart with the inoculating needle. The slide was covered using a cover slip and viewed under the microscope at magnification 40.

Toxicology sample analysis

There are three basic processes involved in the toxicology analysis of aflatoxin and these include: Extraction, Clean-up, and Detection.

Extraction

Twenty-five grams of each grounded sample was weighed out using a weighing balance into a 250ml beaker. Then, 100ml of 90:10 acetonitrile/water was added to the weighed samples to extract toxins from the nuts, aluminum foil was used to seal the beaker to avoid spillage. Using an incubator shaker, the mixture was mixed for 1 hour to allow uniformity at a speed of 250 rpm at temperature favorable for fungi which is 27^{o}C (Sheibani, 2008). Using a funnel, the extract from each sample was filtered through qualitative filter paper into the brown syrup bottles. A brown bottle was used to prevent the penetration of light into the extract as aflatoxins are sensitive to light rays.

Clean up

This was done to separate the aflatoxins from the extract, and it was carried out using a Romer MycoSep column (a kit) for high performance liquid chromatography (HPLC). From the extract, 9ml was pipetted into a 15x85mm culture tube which was provided in the kit, The extracts (500µl) were poured into different MycoSep column, and the extracts were spiked through a MycoSepTM 224 clean-up column, the aflatoxins were believed to have been trapped in the column. Acetonitrile(200µl) was measured and poured into each of the columns to extract the toxins into amber vials (a small brown bottle with cover).

Detection

For detecting the aflatoxins, a standard for aflatoxin was prepared. The standard was prepared in acetonitrile (lichrosolv). An appropriate volume of working standard solution (aflatoxin) was taken to prepare 5 calibrator levels which ranged from 0.1 - 1.6ng. Derivation solution (10ml aqueous iodine + 5ml glacial acetic acid + 35ml water), 700µl was added to the above and to the extracted toxins from the samples, the amber vials were covered, allowed to heat up for 8.5 minutes at 65^{o}C. The vials were cooled at room temperature and the covers were removed. A mobile phase for the HPLC was prepared by mixing water + acetonitrile + methanol (400 + 100 +100) mL. From the content in the vials, 50µl was injected into the HPLC immediately after cooling. Calibrators were injected thrice for each level of standard to obtain the calibration curve, in the same manner, the samples were injected twice.

The HPLC conditions were kept appropriate:

Flow rate: 1ml/min.

Detector: Fluorescence, wavelength Excitation: 360nm, Emission: 440nm

Mobile phase: water + acetonitrile + methanol (400 + 100 + 100) mL

Column: C18, 150x4.6mm

Typical retention time (mins): Aflatoxin G1 = 4.0, Aflatoxin G2 = 9.8, Aflatoxin B1 = 5.6, Aflatoxin B2 = 14.8

The results were calculated as follows:

$$\text{Concentration of standard} = \frac{\text{concentration of standard stock solution}}{\text{Total volume derivatized}} \times \text{volume injected}$$

$$\text{Amount of sample injected, W} = \frac{\text{weight of sample} \times \text{volume of purified extract}}{\text{Volume of extraction solution} \times \text{total volume derivatized}} \times \text{volume injected}$$

Concentration of each aflatoxin from standard calibration curve = C (ng)

Aflatoxin ng/g (ppb) = C/W

In all, 24 samples were analyzed. Four markets were visited, raw groundnuts were bought and groundnuts from each market were divided into three parts, one part was roasted, the other was boiled while the third was left raw. The samples were in duplicates to make up the total of 24 that was analyzed.

Statistical analysis

The total aflatoxin levels for the different groundnut preparations across all four markets were measured in duplicates for each sample. These data were analyzed using a combination of Excel and SAS ANOVA tests.

RESULTS AND DISCUSSIONS

As shown in table 1, *Aspergillus flavus* and *Aspergillus niger* were positive in raw groundnuts from Oyingbo, Ile-epo, Mile 12 and Mushin. Boiled groundnut from Mushin and Ile-epo market had no growth of *A. flavus* only *Aspergillus niger* was isolated, it was observed that both *Aspergillus flavus* and *Aspergillus niger* were present in boiled groundnut from Mile 12 and Oyingbo market. *Aspergillus flavus* and *Aspergillus niger* were present in roasted groundnut from the four market except for Mushin market where *Aspergillus flavus* was absent. It is not uncommon to isolate *A. niger* from peanuts (Dania *et al*., 2021), and this was the case in this study, while *A. parasitcus* which was suspected because of its role in aflatoxin production was not isolated.

From the study carried out as shown in table 1, *Aspergillus flavus* and *Aspergillus niger* were isolated from the groundnut samples from the four markets. Both *A. flavus* and *A. niger* were isolated from Mile 12 and Oyingbo markets. There was no *A. flavus* growth in samples from Mushin and Ile-epo market. The occurrence of *Aspergillus* was more on the raw samples and roasted samples. The boiled samples had the least occurrence of *Aspergillus*. Surprisingly, no *Aspergillus spp.* was detected in the boiled and roasted preparation method of samples from Mushin market. The change in temperature is a probable reason for decrease in occurrence of *Aspergillus* because some fungi can be killed at high heat (Adejuyo *et al*., 2008; Liu *et al*., 2017; Tejero *et al*., 2021). The result as compared to that carried out by Bankole and Eseigbe in 2004 on fungi isolated from dry roasted groundnut in Southwestern Nigeria is synonymous. From Bankole and Eseigbo's work, *A. flavus* had the highest number of cases of the fourteen fungi species that were isolated. *A. niger* according to Person *et al*., (2010) has been linked to invasive pulmonary aspergillosis. Thus, they are of public health importance.

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Table 1: Groundnut samples positive to *Aspergillus species*

Markets	Raw	Boiled	Roasted	*Aspergillus Spp.* present
Mile 12	+	+	+	*A. flavus* and *A. niger*
Oyingbo	+	+	+	*A. flavus* and *A. niger*
Mushin	+	-	-	*A. niger*
Ile-epo	+	-	+	*A. niger*

Key: (+) presence of *Aspergillus species* in the groundnut sample, (-) absence of *Aspergillus species* in groundnut samples

As shown in tables 2 to 4, there was production of total aflatoxins (B_1, B_2, G_1, G_2, M_1 and M_2) in the groundnut samples. The level of the total aflatoxins varied from one market to the other and from one sample to another. Mile 12 Market generally had higher mean aflatoxin levels compared to Oyingbo, Mushin, and Ile-Epo Markets, across all preparation methods, at 0.504, 0.531, and 0.496 ppb aflatoxin levels respectively. This can be attributed to the location of Mile 12 market, also, being a major wholesale market and all food products brought into Lagos are first dropped there before they are distributed to other markets across the metropolis. Therefore, this could be the reason for the contaminations (Kumar, 2021). However, it is surprising because the food products are distributed to the other retail markets where factors that could initiate aflatoxins production such as long storage, moisture, direct sunlight, and stocking of excessive and different food products are favored. Oyingbo Market and Mushin Market show relatively lower mean aflatoxin levels compared to Mile 12 Market, while Ile-Epo Market has varying mean aflatoxin levels but tends to be lower than Mile 12 Market.

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Table 2: Average Aflatoxin Level in RAW Groundnuts

Aflatoxin Type (ppb)	Mile 12 Market	Oyingbo Market	Mushin Market	Ile-epo Market
AF B1	0.209	0.112	0.0775	0.143
AF B2	0.0105	0.01	0.007	0.006
AF G1	0.2225	0.2125	0.2115	0.2685
AF G2	0.0295	0.028	0.0185	0.0275
AF M1	0.0195	0.014	0.0055	0.02
AF M2	0.0125	0.0105	0.002	0.01
Total Aflatoxins	**0.504**	**0.387**	**0.322**	**0.475**

Table 3: Average Aflatoxin Level in BOILED Groundnuts

Aflatoxin Type (ppb)	Mile 12 Market	Oyingbo Market	Mushin Market	Ile-epo Market
AF B1	0.2225	0.1055	0.086	0.1385
AF B2	0.008	0.009	0.0095	0.007
AF G1	0.2205	0.1485	0.1145	0.2105
AF G2	0.032	0.0255	0.0185	0.041
AF M1	0.0305	0.0095	0.008	0.025
AF M2	0.017	0.0165	0.0025	0.0115
Total Aflatoxins	**0.531**	**0.315**	**0.239**	**0.434**

Table 4: Average Aflatoxin Level in ROASTED Groundnuts

Aflatoxin Type (ppb)	Mile 12 Market	Oyingbo Market	Mushin Market	Ile-epo Market
AF B1	0.198	0.11	0.0915	0.142
AF B2	0.0075	0.009	0.0075	0.0075
AF G1	0.2155	0.2135	0.1965	0.1955
AF G2	0.0295	0.0315	0.0235	0.0235
AF M1	0.0275	0.015	0.0075	0.021
AF M2	0.018	0.014	0.003	0.0105
Total Aflatoxins	**0.496**	**0.393**	**0.330**	**0.4**

Keys: AFDB1/AFDB2 – Aflatoxins B1/B2
AFG1/AFG2 - Aflatoxins G1/G2

AFM1/AFM2 - Aflatoxins M1/M2
Ppb - Part per billion
Note: 1 µg/kg = 1 ppb

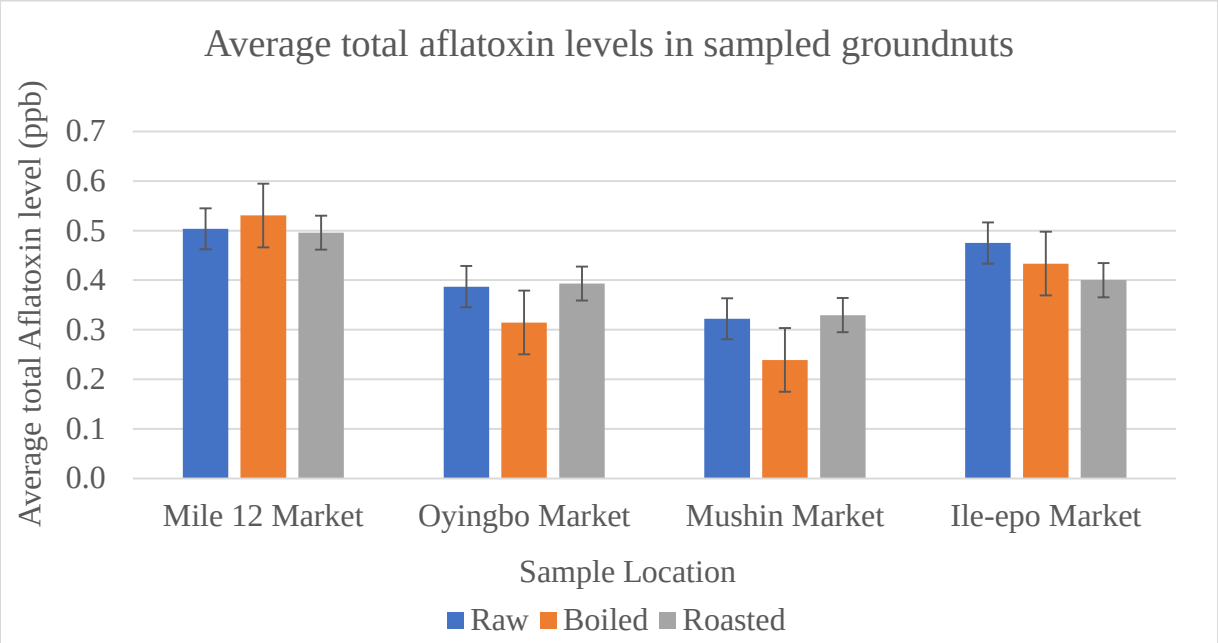

Figure 2: Average total aflatoxin levels

Figure 2 shows the total aflatoxin levels in all the samples from all the four markets. The boiled groundnuts generally have lower mean aflatoxin levels compared to raw and roasted groundnuts across all markets. The raw and roasted groundnuts show varying levels, with roasted groundnuts sometimes having lower mean aflatoxin levels. This can be attributed to the preparation method with raw sample not undergoing any form of heating and directly from the soil, while the roasted had aflatoxin level higher than the boiled because sand was used to roast the groundnut and there is probability that the sand might have been contaminated with aflatoxins before it was used. This could apply to all groundnuts roasted with sand, an alternative to the use of sand could be put in place to limit or prevent aflatoxin levels while roasting. For the boiled sample, the heat could have limited the growth of *Aspergillus flavus* thereby reducing the total aflatoxin levels in it. It can be suggestive to state that the boiling and roasting processes may influence aflatoxin content with the boiling method having a positive effect in reducing aflatoxin levels compared to raw and roasted preparation methods, although there was no significant difference in the aflatoxin levels. Overall, the p-value obtained from the analysis of variance (ANOVA) test is greater than 0.05, it suggests that there is no statistically significant difference in aflatoxin levels among the four major markets for the specific type of groundnut preparation methods being analyzed (raw, boiled, or roasted).

The total aflatoxins levels derived were not beyond the specified levels (4µg/kg for ready-to-eat food and 10µg/kg for processed foods) given by NAFDAC for Nigeria. Therefore, from a regulatory standpoint, the values are not only insignificant but also fall within acceptable limits for human consumption. This does not dismiss the aflatoxin contamination detected in the groundnut samples from the four markets. Since the fungi producing the aflatoxins were present in the samples, there are possibilities of existing lag or stationary microbiological growth phase which might suggest aflatoxin production stage. The isolation of *A. flavus* indicates that the total aflatoxin levels detected were solely produced by *A. flavus*. The presence of *A. niger* in this study was not surprising, however, there was no account for the toxins, Ochratoxins (Abarca *et al*., 1994) typically produced by this specie.

CONCLUSION

The results of our investigation demonstrate that the aflatoxin and ochratoxin producing fungus (*Aspergillus flavus* and *A. niger*), aided by moisture and other factors are common agents of contamination of groundnuts marketed in Mile 12 market, Mushin market, Oyingbo market and Ile-epo market. However, the levels of total aflatoxin in most of the sampled food commodities are within acceptable limits. The total aflatoxin levels in the raw samples were high compared with the other preparation methods because it was not subjected to temperature change, which is a major influence in aflatoxin production. The observed variations in aflatoxin levels across the different markets and preparation methods may be influenced by factors such as storage conditions, handling practices, or variations in peanut sources. These factors could be explored in future research to better understand the drivers of aflatoxin contamination. Reduction of aflatoxin levels and prevention of fungal growth in groundnut in the four market and indeed Nigeria via continuous monitoring and potential interventions should be a public health priority to ensure food safety and protection of human health.

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