Comparative Evaluation of Unshelled Groundnut Sold in Makurdi Metropolis for Aflatoxin B_1 Secondary Metabolites Using Enzyme Linked Immunosorbent Assay

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ABSTRACT (ARIAL, BOLD, 11 FONT, LEFT ALIGNED, CAPS)

Aims: The contamination of peanuts by aflatoxins (AF) results in financial losses to farmers' as well as severe food safety and public health challenges globally. This study was carried out to; (i) assess the levels of AFB_1 in stored groundnuts (ii) determine the relationship between moisture content and AF levels, and (iii) explore vendors' knowledge, attitudes and practices (KAP) of AF and their approach towards groundnut storage.
Study design: Quantitative research method was employed in this study.
Place and Duration of Study: This study was conducted at the Department of Microbiology, Benue State University, Makurdi from May – June 2019.
Methodology: Duplicate groundnut samples were collected from ten market locations in Makurdi and analyzed using Enzyme Linked Immunosorbent Assay (ELISA) quantification method.
Results: The moisture content of the groundnuts was determined, and data on Knowledge Attitude and Practice (KAP) relating to groundnut storage were obtained using questionnaires. The results obtained showed that all the sampled groundnuts were contaminated with AFB_1 levels ranging from 17.3 - 35.9 parts per billion (ppb). Furthermore, we found a correlation between high moisture content and high AFB_1 levels and vice-versa. The knowledge of AF among the groundnut retailers was low (<40%), and 40.91 % of the sellers confirmed that groundnuts were stored for ≤ one month before sale.
Conclusion: The levels of AFB_1 levels in stored groundnuts are above the Standard Organization of Nigeria (SON) permissible limit of 20 ppb for stored groundnut in Nigeria. The data obtained raises concerns for food safety considering that groundnuts are widely consumed in Makurdi. Regular evaluation of AFB_1 levels in stored grains should be conducted.

Keywords: Mycotoxins, Aflatoxin B_1, ELISA, Groundnuts, Nigeria

1. INTRODUCTION

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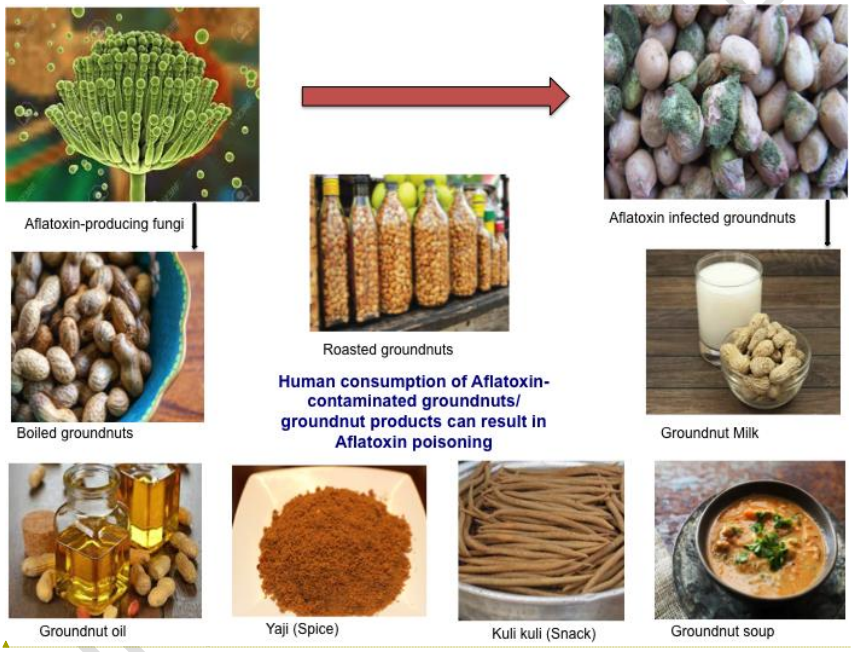
Aflatoxins (AFs) are notorious, toxic secondary metabolites produced by the black molds predominantly *Aspergillus flavus*, *A. parasiticus*, and other fungi like *Emericella* sp. (Plascencia-Jatomea et al., 2014; Varga et al., 2009). Currently, there are approximately 20 known AFs, however, the key ones are - AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, and AFM_2 (Luo et al., 2018). *Aspergillus* species are commonly soil inhabiting, and widely distributed in organic debris and decomposing flora (Sellon and Kohn, 2014). In humid, tropical and sub-tropical climates of Africa, Asia, and Latin American (between latitude 40 °N and 40 °S of the equator); the lack of adequate drying and poor storage facilities promotes the growth of, and toxin

production of these AF-producing fungi (Abbas et al., 2009; Kew, 2013; Klich, 2007; Sarma et al., 2017; Wild and Gong, 2010; Williams et al., 2004). Agricultural products are contaminated at any phase either during pre-harvest or post-harvest, including in storage and/or processing (Kader and Hussein, 2009; Kew, 2013; Winter and Pereg, 2019). Thus, any toxins present in these food and feed products are likely to pose food safety risks in large populations, contribute to increasing poverty, and threaten food security globally (Vasan and Bedard, 2019). Furthermore, AFs, if ingested can cause severe health challenges like hepatocellular carcinoma (HCC), stunted growth, malnutrition, immunotoxicity (Rushing and Selim, 2019). AF-contaminated foods also results in diminish marketability, lost profit, and product rejection during trading (Fashube, 2017; Vasan and Bedard, 2019), amongst others. AFs contaminate stored agricultural crops that make-up the staple diets of Nigerians and many other low- and middle-income countries (LMICs), for instance, groundnuts. Besides China and India, Nigeria is a main groundnut producer globally and is cultivated in many states in Nigeria with commercial quantities produced in Benue State. In Nigeria, groundnuts are very popular, are consumed by the young and old alike in various forms (raw, boiled, or roasted) as a snack, milk, oil for food, and industrial uses. Groundnuts are also incorporated in spices, used in porridge, and soup making (Figure 1). Among some ethnic groups, the consumption of raw groundnuts during breastfeeding is encouraged as it has been purported to naturally improve breast milk production. This study quantified only AFB_1 because it has been reported to be the commonest and most carcinogenic member in the AF family, and according to the International Agency for Research on Cancer (IARC), AFB_1 are Group I carcinogens. The objectives of this study therefore are to (i) assess the levels of AFB_1 in stored groundnuts (ii) determine the relationship between moisture content and AF levels and (iii) explore vendors' Knowledge, Attitude, and Practice (KAP) of AF and their approach towards groundnut storage.



~~Figure 1: Some likely sources of Aflatoxin poisoning from the consumption of common groundnut and groundnut products available in Nigeria~~

2. MATERIAL AND METHODS

2.1 Sample collection

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Raw husked groundnut seeds were randomly purchased from ten market locations in Makurdi metropolis namely: High-Level, Wurukum market, North-Bank market, Modern market, Railway market, Police check-point (Makurdi Airport), Judges quarters, International market road, Kanshio Mechanic village, and Wadata New garage. They were multiple groundnut sellers in each of the sampled markets, and for each seller that we sampled their groundnuts; a questionnaire was administered to gather KAP data. The collection was carried out in the rainy season months of May – June 2019. They were then transported to the laboratory in clean polythene bags, dehusked by hand under aseptic conditions before further experimentation.

2.1 Sample Preparation and Extraction

Two samples from each lot were analyzed using the method of Morgan et al., 1986. Fifty grams (50 g) of the groundnut samples were weighed and pulverised with the aid of a food blender. A twenty-gram (20 g) portion of the powdered sample was then weighed into a 250 ml conical flask containing 100 ml portion of 70 % methanol. This was placed on an orbital shaker set at a speed of 150 resolutions per minute (rpm) for 30 minutes. The recovered mixture was then filtered with a Whatman filter paper, and the filtrate was used as the extract for AF level determination.

2.1.1 Quantification of Aflatoxin B1 levels using Enzyme Linked Immunosorbent Assay (ELISA) Kit

The levels of AFB_1 were analyzed from the extracted groundnut samples following the procedure as described by Kadir et al., 2013. The ELISA kit (k4208, Bio visionInc.) was used following the manufacture instructions. AFB_1-Bovine serum albumin (BSA) conjugate was prepared in carbonate coating buffer at 100 ng/mL (Nanograms Per Millilitre) concentration. A 150 µl (micro-litre) aliquot of the diluted toxin-BSA was then dispensed into each wells of the ELISA plate. The plate was then incubated at 37 ^{o}C for 1 hour in an incubator (Stat fax-2200, Awareness tech Inc.). Following incubation, the toxins in the wells were discarded and the plates were washed three times at an interval of 3 minutes using 150 µl phosphate buffered saline (PBS) Tween 20. A 150 µl aliquot of 0.2 % BSA was measured into each of the wells of the ELISA plate and incubated at 37 ^{o}C for 30 min. The plate was then removed and the contents discarded, following which the plate was washed three times at an interval of three minutes. A 1.67 µl aliquot of antiserum was diluted in 10 ml of PBS-Tween 20 and mixed properly. A 150 µl aliquot of the prepared antiserum was dispensed in each of the well of the plate while filling the border wells with distilled water. The ELISA plate was then incubated again for 30 min at 37 ^{o}C. Following incubation, the contents were discarded and the plates were washed three times using the PBS-Tween 20 at 3 minutes interval after each wash. An AFB_1 standard was prepared (using 1:10 diluted groundnut extract) at concentrations ranging from 25 ng to 10 picogram/ml in 100 µl volume. The standards were added serially in well. Subsequently the samples were added to the other wells and added 50 µl of antiserum to all the wells and incubated for 1 hour at 37 ^{o}C to facilitate reaction between the toxin present in the sample with antibody. Following 1 hour of incubation, the ELISA plate was removed, the contents were discarded and plates were washed three times using PBS- Tween 20 at 3 minutes interval of wash. Goat anti-rabbit (enzyme conjugate) was prepared at a ratio of 1:4000 dilution of goat anti-rabbit IgG, labelled with alkaline phosphatase, in PBS Tween containing 0.2 % BSA. A 150µl aliquot was added to each well and incubated for 1 hour at 37 ^{o}C. The plate was removed, discarded the content and washed the plates. A 50 µl substrate solution of p-nitrophenyl phosphate was prepared in 10% diethanolamine buffer at pH 9.8, introduced into each well and incubated for 30 minutes at 37 ^{o}C. The AFB_1 level in parts per billion (ppb) was measured by taking the 405 nanometre (nm) absorbance in an ELISA reader (AC3000, Azure Biosystems ,Inc.).

2.1.1.1 Moisture Content Analysis

The balance and drying Oven method as described by Siddique and Wright, 2003 was used for the analysis of moisture content. A petri dish was weighed using a weighing balance (FX-500I, A&D) and the weight of container was measured and denoted as W1. Thereafter, five (5) g of the groundnut sample was weighed before drying. This was designated as W2. The groundnut sample was then weighed and measured for 3 hours in a 105 – 110 °C oven (DOL-24A, Gilson Company Inc.). The heated sample was then cooled in a desiccator for approximately 10 minutes. The groundnut samples were re-weighed to obtain the weight of the groundnuts after drying. Finally, the moisture content of the groundnut samples was determined by calculating the percentage of the moisture content on a wet-weight basis using the following formula:

Equation 1: Moisture content (%) = (W2-W3/W2-W1) ×100

Where: W1 = weight of the petri dish

W2 = weight of the petri dish with groundnuts sample before drying

W3 = weight of petri dish with groundnut sample after drying.

3. RESULTS AND DISCUSSION

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3.1 Levels of Aflatoxin B_1 of Stored Groundnuts in Makurdi

All the groundnuts sampled from the various locations were positive for AFB_1 indicating a 100% contamination rate. The levels of AFB_1 in stored groundnut as examined from the sampled markets ranged from 17.3 - 35.9 ppb (Figure 2).

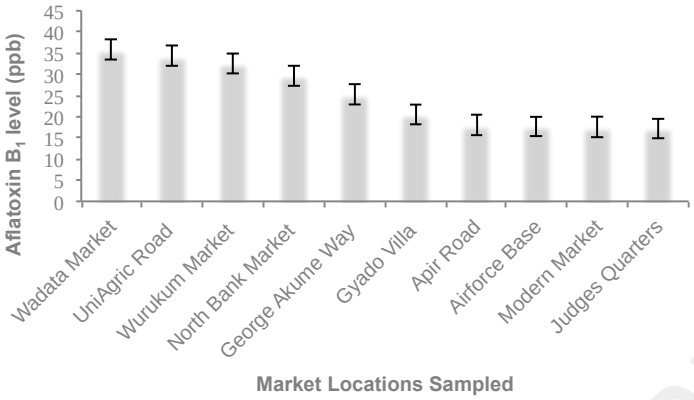

Figure 2: Plot showing AFB_1 levels versus locations sampled. The markets sampled are plotted on the x-axis, whereas the AF levels (ppb) are plotted on the y-axis (Error bars indicate range). Duplicate samples were collected and assayed from each market.

At 17.3 ppb, groundnuts collected from Judges Quarters showed the lowest AFB_1 level; modern-market (17.5 ppb); Air-force base (17.8 ppb); and Apir road (17.9 ppb). These AFB_1 levels were below the Standard Organization of Nigeria and also the Codex Alimentarius Commission (CAC), Joint FAO/WHO Food Standards Program adopted permissible limit of 20 ppb in unprocessed peanuts. Conversely, Wadata market showed the highest AFB_1 level (35.9 ppb); University of Agriculture road market (34.3 ppb); Wurukum market (32.6 ppb); North Bank Market (29.7 ppb); George Akume way (25.3 ppb) and Gyado villa (20.6 ppb), all above the 20 ppb limit. Current research on the levels of AF in Nigeria also found similar high results. For instance, in a study by Magembe et al., (2016), all the sampled groundnuts were contaminated with AFB_1 with levels ranging from 72.97- 195.17 ppb. Similarly, studies by Salau et al., (2016) on AF contamination of stored groundnut in Sokoto State Nigeria reported a contamination rate of 82.5% and AFB_1 levels between 0.9 - 646.0 ppb. Variation in levels of exposure between locations as observed in this study is similar to the report of Wartu et al., (2015) where levels of 6.0 - 28.75 ppb were reported in groundnuts sampled in Kaduna, Nigeria. Though studies performed by Hoeltz, et al., (2012) in Southern Brazil found a lower contamination rate of AFB_1 (14.0%), yet the AFB_1 concentrations were high (24.0 to 87.5 µg/kg) and exceeded the 20.0 µg/Kg limit for B1, G1, B2 and G2 in Brazil.

3.1.1 Level of Aflatoxin B1 in stored Groundnuts in Relation to Moisture Content

The relationship between the moisture content in the groundnuts sampled from various markets in Makurdi and AFB_1 levels was also investigated (Figure 3).

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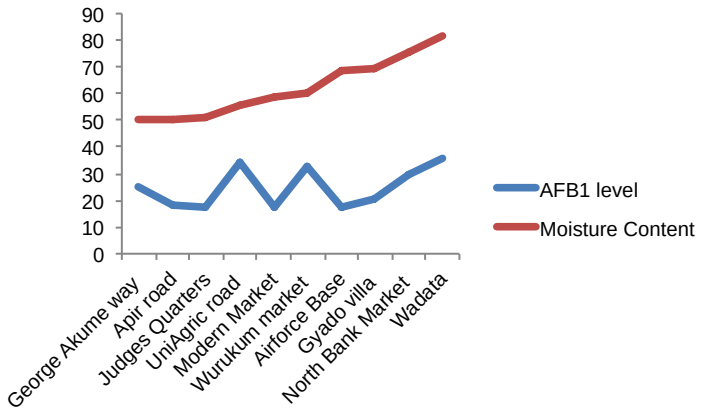

Figure 3: Level of Aflatoxin B_1 of stored Groundnuts in in Relation to Moisture Content

The results showed that the groundnuts sampled had high moisture content (>50 %) and that high moisture content tends to be associated with high AFB_1 levels and vice versa. According to Benbrook 2005, fungi can thrive under different environmental conditions; infest crops with subsequent AF production. These factors temperature, moisture content, relative humidity, and amount of rainfall promote fungal growth, infestation, and mycotoxin production in the field and during storage (Darko et al., 2018; Darwish et al., 2014; Farombi, 2003). This statement has been validated by the findings of this study, which shows a gradual rise in AF levels with increasing moisture content (Figure 3). Hence implying that contamination in stored groundnut products is dependent on the moisture content of the harvested groundnut before storage. These changes in the moisture content and its impact on AF levels have previously been reported by Salau et al., (2016) and agree with the findings of this study. Comparably, the high temperatures that characterize Northern Nigerian and Southern Brazilian states provide favourable environmental conditions for AF production, and may explain the high levels reported.

3.1.1.1 Awareness of Aflatoxins by vendors and their approach towards groundnut storage

This study also investigated the awareness of AFs by groundnut sellers in Makurdi. Using questionnaires, vendors were asked about their levels of awareness, sufficient understanding of AF risk to human health and approaches towards groundnut storage. The results are presented in Table 1.

Table 1: Awareness and Knowledge of Aflatoxins by Groundnut Sellers in Makurdi

Awareness	Number examined	Frequency (%)
Yes	8	36.36
No	14	63.64
Knowledge of Aflatoxins		
Yes	6	27.27
No	16	72.27
Total	22	100.00

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From the results obtained (Table 1), knowledge of AF was low, with only 36.36 % of the groundnut sellers admitting that they were aware of AFs, while 63.64 % were not aware of it. Similarly, 27.27 % of the traders acknowledged that they had a sufficient understanding of AFs and their risks to human health, while 72.27% did not. This low level of knowledge on AFs could be a possible reason for the high incidence rate (100 %) reported in this study since lack of knowledge about AF will result in no deliberate measure to prevent its occurrence to be put in place. The low level of AF knowledge as observed in this study is similar to that of Ilesanmi et al. (2011) where an awareness rate of 32.0 % was reported in Ibadan, Nigeria. Conversely, in a study conducted in Benin to evaluate groundnut farmers on perception, awareness, and action on AF, 77% of farmers agreed that they were aware of the negative outcomes of AF on human health (Jolly et al., 2016).

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3.1.1.2 Awareness of Aflatoxins by vendors and their approach towards groundnut storage

Data on storage time and packaging used was also collected using questionnaires as shown in Table 2.

Table 2: Storage time and packaging used by vendors

Storage Time	Number examined	Frequency (%)
≤1week	10	3 (13.63)
≤1 month	9	40.91%
≤3Months	8	36.36%
Up to 1year	2	9.09%
Packaging		
Sacks	19	86.36%
Baskets	2	9.09%
Polythene Bags	1	4.54%

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Hygienic practices, as well as the type of packaging employed in peanut storage, and the method of storage, could also be a possible reason for variations in AFB_1 levels. Reports from respondents showed that the majority of the vendors store groundnut for up to a month (40.91%) and some up to a year (9.09%). An extended storage period predisposes groundnut to attack by AF producing fungi and is a possible reason for the high rate of contamination. Similarly, concerning packaging used to store the groundnuts, 86.36 % of the respondents stored groundnuts in polypropylene grain bags, 9.09 % stored groundnuts in baskets, and 4.54 % stored in polythene bags. These are often not waterproof, and as such lead to increased moisture content and better susceptibility of the groundnuts to fungi attack. Few of the respondents spray their products with fungicides and 68.0% of them buy the groundnuts and as such are not aware of the post-harvest damage that the groundnuts might have faced before buying them for storage. All these factors are possible links to the high contamination rate recorded in this study. Countries in LMIC for instance Brazil and Nigeria suffer from poor agricultural practices that favour AF-production. In a study by Darko et al. (2018), the use of zero oxygen hermetic packaging, rather than polypropylene woven sacks had positive effects in the control of fungal growth and AF and in preserving quality. However, in this study, 86.36% of vendors reportedly stored groundnuts in woven polypropylene sacks, while 9.09% and 4.54% stored groundnuts in rattan baskets and polythene bags respectively (Table 2). Packaging materials of this type allow air, and there are indications that this may accelerate fungal contamination with subsequent aflatoxin production (Hell et al., 2000).

4. CONCLUSION

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SON has established maximum permissible levels for AFs in food and feed in Nigeria to ensure food safety, still, this is not strictly adhered to. As shown by the results obtained in this study, groundnuts sampled from all the locations revealed a 100% AFB_1 contamination rate. Over 50% of the groundnut sampled had AFB_1 levels that were above the safety limit allowed by the SON/ CAC, regulatory agency standards of 20 ppb in unprocessed peanuts. Thus, rendering them unsafe for consumption. Furthermore, data obtained from this study also indicates that the level of AFB_1 tends to increase with increased moisture content and vice-versa. Finally, the level of knowledge and awareness of AF amongst the groundnut sellers sampled in the various markets was very low. It is recommended that regular monitoring of AFB_1 in stored grains and cereals are conducted, and also public health campaigns to educate the farmers/vendors on aflatoxins in Makurdi.

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AUTHORS HAVE DECLARED THAT NO COMPETING INTERESTS EXIST. THE PRODUCTS USED FOR THIS RESEARCH ARE COMMONLY AND PREDOMINANTLY USE PRODUCTS IN OUR AREA OF RESEARCH AND COUNTRY. THERE IS ABSOLUTELY NO CONFLICT OF INTEREST BETWEEN THE AUTHORS AND PRODUCERS OF THE PRODUCTS BECAUSE WE DO NOT INTEND TO USE THESE PRODUCTS AS AN AVENUE FOR ANY LITIGATION BUT FOR THE ADVANCEMENT OF KNOWLEDGE. ALSO, THE RESEARCH WAS NOT FUNDED BY THE PRODUCING COMPANY RATHER IT WAS FUNDED BY PERSONAL EFFORTS OF THE AUTHORS.

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