OCCURRENCE OF AFLATOXIN IN READY-TO-EAT ROASTED SNACKS OBTAINED FROM PORT HARCOURT AND OWERRI, NIGERIA

ABSTRACT

Aims: Microbial infestation of nuts and dried foods with the resultant production of aflatoxin is a global challenge to human health. This study aims to ascertain the safety of vended ready-to-eat cashew, groundnut and breadfruit.
Study design: This work is based on completely randomized design with two replications and the average values calculated for mean comparison.
Place and Duration of Study: Imadavistic laboratory, Port Harcourt and the Department of Biotechnology, Federal Institute of industrial Research, Oshodi, Nigeria, between January and November, 2022.
Methodology: Forty samples purchased randomly were examined for proximate composition, microbial safety and presence of aflatoxins using standard conventional and molecular methods.
Results: The respective mean percentage proximate composition for groundnut, cashew and breadfruit were: protein (33.1±0.1, 14.9±0.3, 19.7±2.0), Carbohydrate (10.7±0.8, 16.5±2.2, 38.2±0.6), lipid (40.1±2.5, 34.3±1.3, 12.3±0.6), ash (1.8±0.1, 26.9±1.3,1.8±0.1), fiber (7.2±1.5, 20.3±0.6, 26.9±1.3) and moisture (7.0±0.1, 4.0±0.5, 7.8±1.6). Mycological count for groundnut, cashew and breadfruit ranged from 17 to 33; 5 to 17 and 2 to 17 log10 cfu/g, respectively. The mycological studies revealed that there were *Aspergillus* spp., *Candida albican*, *Mucor* sp., *Rhizopus* sp., *Fusarium* sp., *Penicillium* sp. and *Saccharomyces cerevisae*. Aflatoxigenicity results reveals that 31.3% (10 of 32) of the fungi identified were able to produce aflatoxin and they were principally Aspergillus flavus. The range of aflatoxin in the samples were 6.96362 µg/kg of aflatoxin B1 in breadfruit obtained from Oji and Ekeonuwa to 1.07668 µg/kg of aflatoxin B2 obtained in cashew nuts from Rumokoro and Choba.
Conclusion: The presence of aflatoxins though within acceptable doses underscores the need for proper processing of these roasted snacks.

Keywords: Aflatoxin, Aspergillus flavus, breadfruit, cashew, groundnut, metabolites

1. INTRODUCTION

The current life style and rising health concerns have led to an increase in the demand for food and food products, which provide health benefits beyond the basic nutrition. In third world nations like Nigeria, the intake of roasted snacks can cover a great percentage of the daily recommended intake of the basic nutrients like protein which will reduce the food insecurity. These low moisture foods are not a very good medium for microbial growth but could harbor both bacteria and fungi, with the potentials of producing aflatoxins.

African breadfruit (*Treculiaafricana*Decne), seed from a tropical tree of the family Moraceae has been reported to be rich in carbohydrate, fat, certain minerals, vitamins, fibre and high-quality essential amino acids [1, 2]. The seeds have been reported to produce aflatoxins as high as 48.59 µg kg [3, 4].

Groundnut (*Arachis hypogaea* L), a species of the legume family is a good source of edible oil, fat, protein, crude fibre and vitamin E and consumed by people of all ages because of their good flavor and are considered as a very popular snack food worldwide [5, 6, 7]. Aflatoxins ranging from 0.0 to 50.0 µg/kg have been reported in ready-to-eat roasted groundnut [8, 9, 10].

The cashew tree (*Anacardium occidentale*) is a tropical evergreen tree of the genus Anacardium believed to be native Northeastern Brazil that produces the cashew seed. It is often eaten on its own as a snack food or used as recipe because of its pleasant taste. Cashew contains fat, protein (lysine, cysteine, arginine tyrosine, valine) vitamins (C, E, D) [11]. Adetunji et al. [10] and Ubwa et al. [9] have reported the detection of aflatoxins in cashew nuts in Lagos and Makurdi, respectively in Nigeria.

Aflatoxins (AFB1, AFB2, AFG1 and AFG2) are mostly formed by aflatoxigenic strains of the fungus: *Aspergillus flavus* and *Aspergillus parasiticus*, that grow in many cereals and oilseeds, and are known to be hepatotoxic, carcinogenic and teratogenic [8, 12, 13]. *Aspergillus flavus* mostly produces AFB1 and AFB2, whereas *Aspergillus parasiticus* produces AFB1, AFB2, AFG1, and AFG2. *Aspergillus parasiticus* is more adapted to a soil environment and has a more constrained spread than *Aspergillus flavus*, preferring the aerial portions of plants, such as leaves and flowers [14]. A number of additional species that are closely related to *A. flavus* or *A. parasiticus*, such as *A. minisclerotigenes*, *A. korhogoensis*, *A. aflatoxiformans*, and *A. texensis* also produce aflatoxins B and G. [15, 16, 17, 18, 19, 20] These fungi also produce other compounds including aflatoxicol, versicolorin, and sterigmatocystin in addition to the previously described four aflatoxins [21]. In concentrations or maximum limits, the word "total" frequently refers to the sum of AFB1, AFB2, AFG1, and AFG2. In food samples that have been poisoned, AFB1 is the aflatoxin that is most frequently found; the other three are typically not reported when AFB1 is present [22]. Heat and humidity encourage the growth of fungi that produce aflatoxins, and both pre- and post-harvest fungal contamination can result in aflatoxins showing up in food.

Mycotoxins are nephrotoxic, immunotoxic, teratogenic, and mutagenic, hence their presence in our food systems and tissues has significant negative effects on public health. Consumption of an aflatoxin, also known as hepatocellular carcinoma, results in liver cancer (HCC), the third-leading cause of cancer-related deaths globally and accounting for between 550,000 and 600,000 new cases each year [23]. Among other consequences of aflatoxins on the human digestive system are toxic encephalopathy, intestinal hemorrhage, liver necrosis, and fibrosis (loss of balance, recent memory decline, headaches, insomnia, and loss of coordination). It has also been connected to the factors that have increased cases of human gastrointestinal and hepatic neoplasms in China, the Philippines, and Africa [24].

Additionally, they have the potential to cause both immediate and long-term effects on both human and animals, including fatalities and conditions affecting the central nervous system, cardiovascular, pulmonary, and digestive systems [25].

Nigeria reported significant levels of aflatoxin exposure in individuals that have been scientifically verified, as well as the largest estimated number of cases of hepatocellular carcinoma (HCC-liver cancer) related to aflatoxins worldwide [26]. Due to the sneaky nature of mycotoxin synthesis and the ensuing sickness states that make mycotoxicosis

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identification problematic, many cases of both human and animal mycotoxicosis have routinely gone unreported in Nigeria. This suggests that there has been little effort made to address mycotoxicosis in Nigeria and that mycotoxins are not well-understood. The study therefore seeks to investigate the presence of aflatoxin in breadfruit, cashew and groundnuts in south-south and south-east regions.

2. MATERIAL AND METHODS

2.1 Sampling and Study Area

Forty (40) samples of groundnut, cashew nut and breadfruit were purchased from super markets and street vendors in Port Harcourt, Rivers State and Owerri city, Imo State, and taken to the Laboratory for analysis.

2.2 Sample Preparation

The sample were separately pulverized/blended with a sterile blender, packaged and stored at 25oC.

2.3 Proximate Analysis

Proximate analysis was done with composite samples of cashew, groundnut and breadfruit using the methods previously described by AOAC [27]. The parameters determined were: protein, carbohydrate, lipid, ash, crude fiber and moisture.

2.4 Mycological Analysis

2.4.1Isolation and identification of Fungi Isolates

The dilution plating technique as described by Samson et al. [28] was applied for the isolation and enumeration of fungi present in the food samples. Each comminuted sample (10 g) was diluted in sterile distilled water (90 ml) and homogenized for 2 min. Exactly 100 µl of the homogenized mixture was surface-plated out in duplicate on potato dextrose agar (PDA). Incubation of all inoculated plates was performed at 29±2˚C for 3 to 5 days. Thereafter, fungal colonies were counted and reported as colony forming units per gram (CFU/g) of analyzed food sample. All distinct colonies on the PDA agar plates were transferred to freshly prepared plates of PDA. The purified cultures were retained at 4˚C PDA slants.

2.4.2 Aflatoxingenicity Test

Fungal isolates were examined for aflatoxingenicity on neutral red Desiccated Coconut Agar (NRDCA), after 3 days of incubation by checking for fluorescence of agar under long wavelength UV light (365 nm). The ratio of aflatoxigenic to non-aflatoxigenic isolates in the nut samples was calculated using the information acquired from toxigenic screening of isolates on NRDCA [29].

2.4.3 Aflatoxin Analysis

Standard Stock solutions of AFB1, AFB2, AFG1 and AFG2 (~10µg/ml) were prepared by dissolving the solid standard (Sigma, Sigma Chemical Co - St. Louis, MO) in benzene: acetonitrile (98:2, v/v) and the exact concentration were measured by spectrophotometer

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(Shimadzu UV-1601PC, Shimadzu Scientific Instruments, Japan). A working standard solution (µg/ml) of AFB1 (0.12), AFB2 (0.04), AFG1 (0.07), and AFG2 (0.07l) in benzene: acetonitrile (98:2, v/v), for spiking and quantification purposes, was prepared by diluting appropriate aliquots of stock solutions of each toxin [30].

2.4.4 Extraction from Groundnuts, Breadfruit and Cashew nuts

At room temperature (29±2°C), 5g of finely ground (20 mesh) samples were weighed into an appropriate flask (Mason Jar) and extracted for 5 min at a medium speed in an Omni mixer (Omni 17105, Omni International, USA), using 10 ml distilled water and 300 ml chloroform [30]. The extract was filtered through Whatman 4 paper filter under vacuum, and 20g of anhydrous sodium sulphate was added to the filtrates. The filtrate was then passed through a Whatman fiber glass membrane (GF/B 1mm) while being vacuum-filtered. The filtrate (100ml) was evaporated (40-500C) in rotary evaporator (Buchi B-481, Labortechnik AG, Switzerland).

Clean-up: The residue was moved using 6 ml of chloroform to a florisil cartridge (1g, 6 ml, vac, Supelcleanä, Supelco, USA), which had been previously conditioned using 6 ml of chloroform, fitted to a plastic stopcock, and linked to a vacuum system (Varian®, VacElut, SPS 24, Varian Associates Inc, USA). Without allowing air to travel through the cartridge, 6 ml of chloroform: hexane (1:1, v/v) and 6 ml of chloroform:methanol (9:1, v/v) were used to wash the cartridge. Then 30 ml of acetone:water (97:3, v/v) were used to elute the aflatoxins [31]. Air was allowed to circulate through the cartridge in between each 5ml aliquot. In a water bath at 40°C with nitrogen flow (Yamato Water Incubator BT 25, Yamato, Japan), the eluate was evaporated until dry. In dim light, cleanup and TLC analyses were performed.

2.4.5 High Performance Liquid Chromatography

The method described by Herzallah et al. [32] was adopted for HPLC analysis. Water, acetonitrileand methanol (60:20:20 v/v) were used as the mobile phase in a column chromatography on an Inertsil ODS C-18 (250 4.6 mm; 5 id) column with a flow rate of 1.0 ml/min and a detection time of 1 min. Ultra-violet (UV) detection was done at a wavelength of 365 nm. Sample volume of 20 µl was injected into the HPLC column and maintained at 40°C.

2.5 Molecular Analysis

2.5.1 Deoxyribonucleic Acid (DNA) Extraction Protocol

Zymo Quick DNA Fungal/Bacterial Kit was used for the DNA extraction. Fungal mycelium/bacterial culture was scrap off and transfer into a sterilized mortar, followed by the addition of 750 µl of bashing bead buffer and homogenize. Then 200 µl of nuclease-free water was added to the homogenized sample. Vortexing was done for 20 min followed by centrifuging the ZR Bashing Bead TM Lysis Tube (0.1 and 0.5mm) in a microcentrifuge at 10,000 x g for 1 min. Then about 400 µl supernatant was transferred to a Zymo-spin TM Iii-F filter in a collection tube and centrifuge at 8,000xg for 1 min. Thereafter, 1,200□ l of Genomic Lysis Buffer was added to the filtrate in the collection tube from Step 4. A Zymo-Spin TM IIC Column3 in a collection tube was then filled with 800 µl of the mixture from Step 5 and centrifuged at 10,000 x g for 1 min. After discarding the flow through from the collection tube, step 6 was performed again. Following the addition of 500□ l of DNA Wash Buffer to the Zymo—Spin TM IIC Column and centrifugation at 10,000 x g for 1 min. Deoxyribonucleic acid Pre-Wash Buffer (200□ l is added to the Zymo—Spin TM IIC Column in a fresh collection tube. The Zymo--Spin TM IIC Column is then transferred to a clean 1.5 ml

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microcentrifuge tube and 100 µl (35 µl minimum) DNA Elution Buffer added directly to the column matrix before centrifugation at 10,000 x g for 30 s to elute the DNA. The Ultra-pure DNA is now ready for use.

2.5.2 Determination of DNA Quality Using Nanodrop

DNA quality and purity were checked using NanoDrop 2000c spectrophotometer (Thermo fisher Scientific Inc. Wilmington, Delaware, USA). Purity is measured as a ratio of Ultraviolet (UV) light absorbance at 260 nm to that of 280 nm. The NanoDrop was connected to a computer system and the sensor was cleaned using a cotton wool and 70% ethanol. 1µl of elution buffer (the solution used to re-suspend the DNA) was dispensed directly on top of the NanoDrop sensor. The Nanodrop determined the blank and the DNA samples (1µl) were separately loaded onto the sensor. The sensor was usually wiped when a new sample is to be loaded to avoid contamination. Nanodrop measurement was taken in triplicates for each sample.

2.5.3 Fungi polymerase chain reaction (PCR) Protocol

Primer sequence employed for the fungi identification is ITS4: TCCTCCGCTTATTGATATGS ITS5: GGAAGTAAAAGTCGTAACAAGG

2.5.4 Polymerase chain reaction cocktail mix

This consist of 2.5 µl of 10x PCR buffer,1 µl of 25mM $MgCl_2$, 1 µl each of forward primer and reverse primer, 1 µl of DMSO, 2ul of 2.5mMDNTPs, 0.1 µl of 5u/ µl Taq DNA polymerase, and 3 µl of 10ng/ µl DNA. The total reaction volume was made up to 25 µl using 13.4 µl Nuclease free water.

Polymerase chain reaction cycling parameters for ITS (Internal Transcribed Spacer Region for identification purpose)

Initial denaturation at 94˚C for 5 min, followed by 36 cycles of denaturation at 94˚C for 30 s, annealing at 54˚C for 30 s and elongation at 72˚C for 45 s. Followed by a final elongation step at 72˚C for 7 min and hold temperature of 10 ˚C forever. Amplified fragments were visualized on Safe view-stained 1.5% agarose electrophoresis gels. The DNA ladder employed is Bioline's Hyper ladder, and the amplicon diameter is around 650 base pair (Bp). [33].

2.6 Statistical analysis

Statistical analysis was performed on the data obtained using SPSS Version 21-Statistical package for social and biological sciences.

3. RESULTS AND DISCUSSION

3.1 Proximate composition

The results of the percentage mean proximate composition of the three roasted snacks examined is presented in Table 1. Groundnut had the highest percentage protein (33.1±0.1) and lipid (40.1±2.5) contents while the least were in cashew (14.9±0.3) and breadfruits (12.3±0.6), respectively. Breadfruit had the highest carbohydrate (38.2±0.6) and moisture

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(7.8±1.6) contents while the least were observed in groundnut (10.7±0.8) and cashew (4.0±0.5), respectively. Cashew nut had the highest ash (3.6±0.2) and fibre content (26.9±1.3) while the least were observed in breadfruit (1.8±0.1), and groundnut (7.2±1.5), respectively.

Table 1. Comparison of Proximate Composition

Nuts	Protein (%)	CHO (%)	Lipid (%)	ASH (%)	Moisture (%)	Fibre (%)
Groundnut	33.1±0.1c	10.7±0.8a	40.1±2.5c	1.8±0.1a	7.0±0.1ab	7.2±1.5a
Breadfruit	19.7±2.0b	38.2±0.6c	12.3±0.6a	1.8±0.1a	7.8±1.6b	20.3±0.6b
Cashew-nut	14.9±0.3a	16.5±2.2b	34.3±1.3b	3.6±0.2b	4.0±0.5a	26.9±1.3c
ANOVA	63.207	109.006	75.328	74.126	4.362	71.877
p-value	0.000	0.000	0.000	0.000	0.047	0.000
Decision	Significant	Significant	Significant	Significant	Significant	Significant

Row mean ± standard error with different alphabet is significant

3.2 Aflatoxigenicity of the fungal isolates

The result of the screening for aflatoxin production by the fungal isolates is presented in Table 2. It revealed that only 10 (31.25%) of the 32 isolates had the ability to produce aflatoxin.

Table 2. Aflatoxigenicity of the mycological isolates

Isolates	Sample	Morphology	Aflatoxigenicity
C1	Cashew nut	Green-*Aspergillus*	+
C2	,,	Grey-*Aspergillus*	+
C3	,,	Green-*Aspergillus*	+

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C4	,,	Green-*Aspergillus*	+
C5	,,	Black	+
C6	,,	Black with Filamentous white	+
C7	,,	Dark green	+
C8	,,	Green-*Aspergillus*	+
Ba	Breadfruit	Fluffy Grey *Aspergillus flavus*	+
Bb	,,	Fluffy Grey *Aspergillus flavus*	+

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3.3 Aflatoxin profile of the examined samples.

The results of the aflatoxin profile of the examined samples are presented in Figure 1. Cashew nuts had aflatoxin B2 and G2. Breadfruits has aflatoxin B1 and G1, while groundnut had B1 and G1. The highest occurrence of aflatoxin was observed in breadfruit which had 6.96362 µg/kg (B1) followed by groundnut which had 5.68522 µg/kg (G1) while the least was observed in cashew nut which had 1.07668 µg/kg (B2).

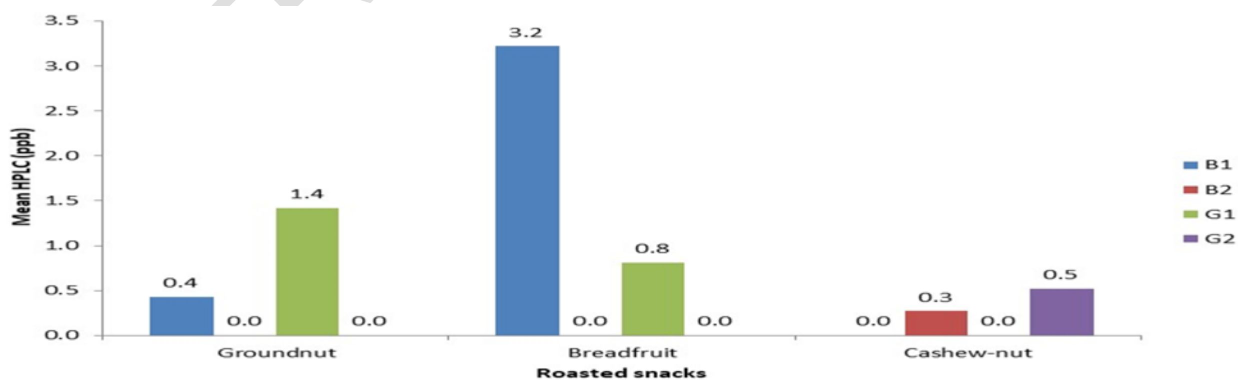

Figure 1. Mean aflatoxin of the roasted snacks

B1- Aflatoxin B1, B2- Aflatoxin B2, G1- Aflatoxin G1, G2- Aflatoxin G2.

3.4 Mycological Count and Molecular identification of the aflatoxin-producing fungal

The results of the average counts from the examined roasted snacks are presented in Table 3. The counts ranged from 1.6×10^2 ±6.8 in groundnut to 9.8×10^3±2.8 cfu/g in cashew. Representative phylogenic trees of the aflatoxin-producing fungal isolates are presented in Figure 2. They were principally *Aspergillus flavus*.

Table 3. Mycological count of the roasted snacks

Snacks	Mycological count
Groundnut	$1.6x10^2 \pm 6.8$
Breadfruit	$5.8x10^1 \pm 3.8$
Cashew-nut	$9.8x10^3 \pm 2.8$
ANOVA	12.286
p-value	0.003
Decision	Significant

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Fig. 2: Phylogenic tree of A. flavus.

NAME: *Aspergillus flavus*. Strain: RCBBR_CH3. Assertion NO: ON584559

The examined roasted snacks beside their pleasant flavor that that attracts consumers, they are also very rich in basic nutrients, hence usually eaten as healthy snack by all ages without restriction, though more by adults as snack in Nigeria.

The percentage proximate composition of groundnut revealed the following: Protein (33.1%±.1), carbohydrate (10.7±0.8), lipid (40.1±2.5), ash (1.8±0.1), moisture (7.0 ±0.1) and fibre (7.2±1.5). Breadnut had: Protein (19.7±2.0), carbohydrate (38.2±0.6), lipid (12.3±0.6), ash (1.8±0.1), moisture (7.8 ± 1.6) and fibre (20.3±0.6) while cashew nut had protein (14.9±0.3), carbohydrate (16.5±2.2), lipid (34.3±1.3), ash (3.6±0.2), moisture (4.0 ±0.5) and fibre (26.9±1.3). Olatunya et al. [7], reported similar trend with groundnut where lipid (38.2) was highest, followed by protein (26.3) and the least was ash (3.5). The fibre contents are comparable (9.63/7.20) whereas, there were variations in the moisture and carbohydrate

contents. Their finding with respect to breadnut showed a wide margin in the moisture (45.0/7.8) and fibre (1.38/20.3) contents but had comparable values for protein (12.6/19.7), lipid (16.1/12.3) and ash (2.0/1.8). Akinhamni et al. [11] reported the percentage proximate composition for cashew which had as highest lipid (49.1) and the least being ash (2.8) as reported in the present study. The other parameters were varied. The moisture content in this study is slightly above the range reported by Ubwa et al. [9] for roasted cashew (2.19 to 2.76%) and groundnut (1.80 to 4.20%) in samples examined in Makurdi, Nigeria.

Low moisture foods like these snacks are not a good medium for microbial growth and survival but they were not sterile either. The microbial contamination of the roasted snacks examined could have occurred during the growth, harvesting, processing and packaging of these snacks. Moreso, fungi can produce aflatoxin in "postharvest" conditions like: storage, transportation, and food processing. The main predisposing factor in postharvest aflatoxin accumulation in food is poor storage conditions, namely: excessive heat and moisture, pest-related crop damage, and extensive periods of time spent in storage (exceeding several months) [12].

The mycological results revealed the presence of predominantly Aspergillus flavus. Others were *Aspergillus spp.*, *Candida albican*, *Mucor* sp., *Rhizopus* sp., *Fusarium* sp., *Penicillium* sp. and *Saccharomyces cerevisiae*. Authors have reported the isolation of similar fungi for peanuts, roasted groundnuts and cashew nuts [10, 35].

A total of 31.5% of the *Aspergillus flavus* isolates were positive for aflatoxin production. This finding is comparable to the report of 35.6% of aflatoxin production by *Aspergillus flavus* isolated from ready-to-eat corn and groundnut-based snacks in Lagos, Nigeria by Ezekiel et al. [8], in the compounded Neutral Red Desiccated Coconut Agar. On their part, Adetunji et al. [10] reported 55.0% of *Aspergillus flavus* producing aflatoxin. The detected aflatoxin in the examined rosids were: AFB1 and AFG1 (Groundnut), AFB1 and AFG1 (Breadfruit) and AFB2 and AFG2 (Cashew nuts). Ezekiel et al. [8] have also reported AFB1, AFB2 and AFG1 in examined snacks which included groundnut and other nut-based foods.The aflatoxin concentration of the breadfruit obtained from Oji and Ekeonuwa, Owerri in Imo State had the range of 6.96362 µg/kg and 1.07668 µg/kg in samples from Rumuokoro and Choba, in Rivers State. These values are within the permissible limit of 20 and 10 µg/kg recommended by the Standard Organization of Nigeria (SON) and National Agency for Food Drug Administration and Control (NAFDAC) and Codex Alimentarius Commission for ready-to-eat food substances, respectively [36]. Adetunji et al. [37] reported a range of 0.01 -0.28 µg/kg and 0.03-0.77 µg/kg of aflatoxin in cashew from Nigeria and South Africa, respectively. Ubwa et al. [9] reported a 0.10 -0.40 µg/kg and 12.00 ->20 µg/kg in roasted cashew and groundnut, respectively in parts of Makurdi, Benue State, Nigeria. On their part, Tor et al. [39] reported values of 0.30 and >20.00 µg/kg of aflatoxins in cashew samples collected from separate sites, while also reporting a range of 0.50 to 12.20 µg/kg of aflatoxin detection in roasted groundnut from several sites in Gboko, Benue State, Nigeria. Gachomo et al. [35] reported that there has been a higher concentration of aflatoxin in Nigeria of 216 µg/kg. Daily consumption of low concentration (below regulatory limits) of aflatoxin especially AFB1 for a considerable length of time may culminate in the development of hepatocellular carcinoma [40]. Wu et al. [13] estimated that daily intake of at most 9µg/g aflatoxin in foodstuff would cause an increase of 1 (one) hepatocellular cancer case per 10,000 population.

Aspergillus is a common contaminant of plant based dry foods and aflatoxigenic type were present on/in the nut. The aflatoxigenic type were Aspergillus flavus. Aflatoxin was found on the nuts but in low amount lesser than the set limit but its presence even at little pose a health concern.

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4. CONCLUSION

The examined ready-to-eat roasted snacks are rich in desirable nutrients, but were however found to be contaminated with Aspergillus flavus which secretes metabolites like aflatoxin which has dire public health significance, notwithstanding that the produced aflatoxins were within the acceptable limits in Nigeria. Adequate sanitary procedure like the Hazard Analysis and Critical Control Point (HACCP) should be employed in preparation, packaging and storage of these snacks.

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