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An Assessment of Aflatoxin Contamination in Tiger Nut using HPLC and Aflatoxin Reduction with Citrus Juices

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Abstract

Aflatoxins are metabolites that have been implicated to be directly responsible for several diseases. This present study assesses the aflatoxin contamination of tiger nut (*Cyperus esculentus*) collected randomly from the markets in the capital cities of Kano (Ka), Kaduna (Kd) and Gombe (Gm) States in Nigeria between December 2012 and March 2013. High performance liquid chromatography (HPLC) was used to determine the concentrations of aflatoxins (B1, B2, G1 and G2) in the samples (n = 90). The results indicated that all the tiger nut samples collected were contaminated. The concentration of the four aflatoxins in the samples collected from the three locations was in the order: AFB1 > AFB2 > AFG1> AFG2. The highest concentrations for the individual aflatoxins in the tiger nut samples were AFB1 (21.100 ± 0.100 µg/kg), AFB2 (0.910 ± 0.000 µg/kg), AFG1 (1.510 ± 0.150 µg/kg) and AFG2 (0.905 ± 0.000 µg/kg) for Ka; AFB1 (21.050 ± 0.050 µg/kg), AFB2 (2.105 ± 0.000 µg/kg), AFG1 (3.115 ± 0.000 µg/kg) and AFG2 (0.105 ± 0.000 µg/kg) for Kd; and AFB1 (11.110 ± 0.010 µg/kg), AFB2 (1.300 ± 0.010 µg/kg), AFG1 (1.685 ± 0.000 µg/kg) and AFG2 (0.600 ± 0.000) µg/kg for Gm. The result shows that 90% of the tiger nut was above the 10 µg/kg limit, while about 17% was beyond the alarm limit of 20 µg/kg aflatoxins set by United State Food Safety Regulations and the Codex Alimentarius. The treatment of the samples with varied concentration of orange extract showed significant reduction in the concentration of aflatoxin. Aflatoxin B1 in the tiger nut samples was reduced by 75% and 67% respectively when treated with 50% and 100% (v/v) orange juice extract; the orange juice (100% (v/v)) also reduced aflatoxin G2 by 75%. Rinsing with water serve better in the remediation of aflatoxins B2 and G1 than does the orange juice extract. The study infers that consumption of the nut with citrus juice or rinsing the nut with highly diluted citrus juice would significantly reduce aflatoxin contamination in tiger nut, since the nut is normally consumed raw.

Keywords: Tiger nut, Aflatoxins, Remediation, Citrus juice, HPLC

1. Introduction

Cyperus esculentus (Tiger nut) is a plant of the family *Cyperaceae*, which produces rhizomes from the base and tubers that are somewhat spherical (Cortes *et al.*, 2005). The plant is not really a nut but a tuber first discovered in Egypt some 4000 years ago (Daniel and Maria, 2000; Moshe, 1992).

It has other names like yellow nutsedge, chufa, flatsedge, rush nut, water grass, earth almond,

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northern nut grass and nut grass (Shilenko et al., 1979). *Cyperus esculentus* is known in Nigeria as *aya* in Hausa, *ofio* by Yoruba and *akihausa* by Igbo.

Extensive surveys have been undertaken with regard to aflatoxin contamination in cereals and oilseeds and a number of reviews have also appeared on these aspects (Cismileanu *et al*., 2008; Rajarajan *et al.*, 2013). Comparatively, there is dearth of information on aflatoxin problem in nut and spices which constitute an important fraction of human diet; coupled with the fact that, these are usually consumed without severe heating, a means of drastically reducing contaminations.

Aflatoxins are metabolites that are produced by toxigenic species of Aspergillus of which *Aspergillus flavus* and *Aspergillus parasiticus* are by far the most important (Pittet, 1998). Attention is increasingly being given to these mycotoxins for several reasons, as they have been shown to be directly responsible for several diseases. Aflatoxins are found to be hepatotoxic and potent hepatocarcinogens in animals (Wogan, 1992). They are known to be hazardous to the health of humans, and in some cases directly causing illness and even death. Aflatoxins are also implicated in liver cancer in human and animal population (Moss, 2002).

Efforts to determine the specific role of insects in the *A. flavus* infection process increased dramatically when aflatoxins were recognized as a health concern, leading to the recognition that ear feeding insects (e.g. corn earworm (*Helicoverpa zea*); European corn borer (*Ostrinia nubilalis*); fall armyworm (*Spodoptera frugiperda*); western bean cutworm (*Striacosta albicosta)*; and southwestern corn borer (*Diatraea grandiosella*) can increase aflatoxin levels in pre-harvest and post-harvest corn (Otsuki *et al*., 2001).
Tiger nuts are usually stored improperly for long time and sometimes consumed unprocessed - this poses higher risk to aflatoxin exposure. Since aflatoxin contamination is unavoidable, numerous strategies for its detoxification been proposed are by heat, chemical or biological methods. Detoxification procedures should not only reduce the concentration of toxins to safe levels, but should also prevent production of toxic degradation products as well as any reduction in the nutritional value of the treated commodity (Sánchez *et al*., 2005).

Use of natural plant extracts and biological methods constitute efficient and low-hazardous method for removing these toxins (Reddy *et al*., 2009). Natural products of plants, such as the extracts of *Agave* species (Sánchez *et al*., 2005), *Garcinia indica* (Tamil *et al*., 2003) and

Satureja hortensis L. essential oil (Razzaghi-Abyaneh *et al*., 2008) can inhibit the growth of *Aspergillus flavus* and aflatoxin biosynthesis.

AflaTest™ from VICAM is an advanced biotechnology used for the quantitative measurement of all the major aflatoxins (including AFB1, AFB2, AFG1, AFG2 and AFM1) without the use of toxic solvents like chloroform or methylene chloride and with precise numerical results. It uses a monoclonal antibody-based affinity chromatography. Chemical inactivation of aflatoxin B1 and aflatoxin B2 in maize grain was carried out using 1N aqueous citric acid, the AflaTest™ assay showed that aflatoxins in the maize grain, with an initial concentration of 29 ng g^{-1} was completely degraded, while 96.7% degradation occurred in the maize contaminated with 93 ng g^{-1} aflatoxin. Aflatoxin fluorescence strength of the acidified samples was much weaker than the untreated samples, when observed in HPLC chromatograms (Méndez-Albores *et al*., 2005).

Nigeria has a climatic condition that is very favourable for fungal growth and mycotoxin contamination. In 1996, Bankole and Eseigbe (1996) recognized tiger nut as one of the commodities susceptible to aflatoxin contamination and detected aflatoxins in 35% of tiger nut samples collected from Nigeria, with concentrations ranging from 10 to 120 μg/kg. Adebajo (1993) also reported the presence of aflatoxin in tiger nut at toxicological unsafe levels.

Nigeria has experienced high aflatoxin exposure levels in humans, and is also reported to have the highest estimated cases of hepatocellular carcinoma (HCC - liver cancer-) attributable to aflatoxins (Liu and Wu, 2010). Therefore this study is aimed at providing scientific data on the level and the extent of aflatoxin remediation of tiger nut by its treatment with various concentrations of citric acid obtained from locally available limes and oranges.

2. Materials and Methods

Reagents

All reagents used in this study were of Analytical grade, and all solvents used to quantify aflatoxins in the samples were of HPLC grade. Individual aflatoxin (B1, B2, G1 and G2) standards were obtained from Sigma-Aldrich Co., USA.

Sample Collection

A total of 90 tiger nuts made up of 30 samples randomly collected from the capital cities of Gombe (Gm), Kano (Ka) and Kaduna (Kd) in the dry season between December 2012 and March 2013 were used for the study. The samples were purchased from street hawkers, local markets and retail shops and were estimated to have been imported at least five months from the date of purchase. The samples were collected in nylon bags and then conveyed to the laboratory and stored at 4°C prior to analyses.

Sampling Locations

The three selected sites for this study are: Kano (latitude 12º 25'N to 12º 40'N, longitude 8º 35'E to 8º 45'E) has for centuries been the most important commercial and industrial nerve centre of Northern Nigeria. Kaduna metropolis (latitude 10°28'N, longitude 7°25'E) is located in the central area of what used to be called the Northern Region of Nigeria. Gombe State is situated in the north-eastern part of Nigeria (latitude 10º19'N and 11º02'E. It is characterized by tropical climate (FRN, 2007; Lazarus, 2008). These states are hot and humid, providing a conducive environment for mould growth for most part of the year.

Sample Preparation

All the samples collected were divided into two parts, labeled Sample A and Sample B.

Sample (A) are those meant for the determination of aflatoxin, these were preserved at 2 - 4ºC, prior to analysis (n = 15 for each state).

Sample (B) were the samples meant for the physical and proximate analyses, these were properly cleaned and then analyzed immediately. The samples belonging to this group were further sub-divided into portion B1 (Those meant for moisture content, pH and sugar content determinations). These were finely powdered and stored in an airtight container in a freezer until analyses was carried out. Portion B2 were analyzed for physical parameters such as weight, length and diameter and colour, insect infestation and mechanical damage.

Determination of Physical Parameters

The shape, colour and appearance, weight, length and diameter of each of the ninety samples were determined. The length and diameter were measured using a micrometer screw gauge, while the weights were determined using a Mettler- analytical weighing balance.

Determination of Aflatoxins using the HPLC system

Aflatoxins concentrations were determined using a High Performance Liquid Chromatography (HPLC) (Waters 616/626 HPLC Systems) located at the International Institute for Tropical Agriculture (IITA) Ibadan, Nigeria.

Preparation of Standard Aflatoxin Solutions

Total Aflatoxins Standard (R-Biopharm Rhone) of quantified methanol solution of 250 ng/ml AFB_1, 250 ng/ml AFB_2, 250 ng/ml AFG_1 and 250 ng/ml AFG_2 was used as the stock for the calibration curve. From this working standards of 2.50, 2.00, 1.25, 1.00, 0.50, and 0.25 ng/ml were prepared.

Aflatoxin standard solutions were prepared for the purpose of quantitative analysis by HPLC. From aflatoxins (AFB1, AFB2, AFG1 and AFG2) samples purchased from Sigma-Aldrich Co., USA, appropriate amounts were weighed out to prepare a stock solution in toluene-acetonitrile (9/1). The absorbance (A) read at 350 nm as well as the molar absorbance coefficient (ε) and the molecular weight (MW) for each aflatoxin were used to determine the concentration of each standard. Using the equation below

$$Aflatoxin - \frac{\mu g}{Kg} = A \times MW \times 1000/\varepsilon,$$

where (MW of AFB1 = 312; AFB2 = 314; AFG1 = 328; AFG2 = 330) .

While the molar absorbance (ε) was calculated with the following equation:

$$\varepsilon = Abs \times 100/mM,$$

where Abs is the absorbance, mM the millimolar concentration.

Extraction for HPLC analysis

Exactly 5.00 g of ground sample of tiger nut was weighed into a set of centrifuge tubes and 10.00 mL of petroleum ether added and then thoroughly mixed. 5.00 mL of chloroform was then added and the tubes covered properly, after which it was shaken for 30 min and centrifuged for 20 min at 5000 rpm (Zheng *et al*. 2006).

Clean-up by immunoaffinity chromatograph

This step involves removal of the substances, which may interfere with the detection of the analyte. A number of alternate clean-up techniques for mycotoxins have been described in the literature (Cismileanu *et al*., 2008). 2.00 mL of the final tiger nut extract was diluted with 48 mL of phosphate buffered saline (PBS, pH 7.4) -in order to protect the antibodies in immunoaffinity columns-. The column (EASI-EXTRACT) was obtained from R-Biopharm Rhone, Scotland.The mixture was then allowed to pass through the column by gravity at a flow rate of 5 mL/min. The column contained monoclonal antibodies for aflatoxins bound to a solid support. The diluted extract was then allowed to pass through the column, following which any aflatoxins present in the sample was bound to the antibody within the column. The column was then washed with 20 mL PBS. The elution of aflatoxins was carried out with 1.5 mL methanol and 1.5 mL deionized water; so as to allow the complete denaturing of the monoclonal antibodies, with the subsequent release of the toxin into the solution (Pena, 2010; Pena *et al*., 2002).

Analytical determination of aflatoxin using HPLC

The aflatoxin analysis was carried out according to the method reported by Gnonlonfin et al. (2010) with slight modification, 10.00 g of ground tiger nut samples was vortexed for 20 min. 1.00 g of sodium chloride was then added followed by 25 mL of 80% methanol; the mixture was then shaken at 250 rpm for 30 min using an orbital shaker. After which the mixture was centrifuged at 4000 rpm for 5 min at 5°C, the filtrate was then obtained using a Whatman No.1 filter paper, in order to avoid particles in the supernatant that was obtained.

A 10.00 mL portion of the filtrate was then diluted with 40 mL of deionized water, after which, 10.00 mL of the diluted filtrate was passed through the AflaTest® immunoaffinity column at a flow rate of 1 to 2 drops per second. The column was first washed with 15 mL of distilled water and then the aflatoxin was eluted with 3 mL of methanol. The eluent was dried in a water bath and then reconstituted using 200 µL methanol and stored at 4°C until use.

Aflatoxins (B1, B2, G1 and G2) were then analyzed in HPLC system (Waters 616/626 HPLC Systems) equipped with a quaternary pump set at a flow rate 1.5 mL/min and connected to a fluorescence detector (FLD) set at 365 nm as an excitation wavelength and 435 nm as emission wavelength. A post-derivatization was performed using a photochemical reactor for enhanced detection (PHRED). The mobile phase used was 0.1M

KH_2PO_4: acetonitrile: methanol: acetic acid (690/150/75/20, v/v/v/v) and the recovery of aflatoxins from the spiked tiger nut samples determined. The tiger nut samples were spiked with 5 µg/kg, 10 µg/kg and 20 µg/kg AFB1, AFB2, AFG1 and AFG2 respectively.

Reduction in the Level of Aflatoxins in Tiger Nut using Orange Extracts

Extract of ripe oranges was obtained from citrus fruits purchased from Samaru market, Zaria, Nigeria. A 15 mL portion of undiluted orange juice was added to 5.0 g of pulverized tiger nut in a beaker and shaken for 5 min, this set was labeled $OR_{100\%}$; to another experimental group 15 mL made up of 50% orange juice and 50% distilled water was added to 5.0 g of pulverized tiger nut in a beaker and shaken for 5 min, this set was labeled $OW_{50\%}$; the third group consist of 15 mL distilled water only being added to 5.0 g pulverized tiger nut in a beaker and shaken for 5 min, this was labeled $WT_{100\%}$; all procedure was carried out at room temperature.

Following which the tiger nut was analyzed for aflatoxins. The reduction in the level of aflatoxin was then calculated. Triplicate measurements were taken for each experimental set-up. This was carried out like the remediation in date fruit samples conducted by Me´ndez-Albores *et al*., 2004.

Statistical Analysis

Data presented are mean of replicate observations (n =30) ± standard deviation. The data obtained was statistically analyzed using SPSS and Graph Pad Prism Statistical Software. The test for relationships was carried out using the Pearson Correlation Index and Student's t-test, at 95% confidence limit.

3. Results and Discussion

Study of the Physical Properties of Tiger Nut

All the tiger nut samples were brown in colour, oval shaped and dried. The highest mean length of 1.393 ± 0.689 cm was obtained from samples collected at Kano, while the least was 1.236 ± 0.648 cm for the samples collected from Gombe. As depicted in Figure 1, the least weight (0.672 ± 0.117 g) and the least diameter (0.584 ± 0.043 cm) were recorded for the tiger nuts obtained from Gombe. There was no significant difference in the weight, length and diameter of the nuts obtained from the three cities (Student's t-test, $P < 0.05$).

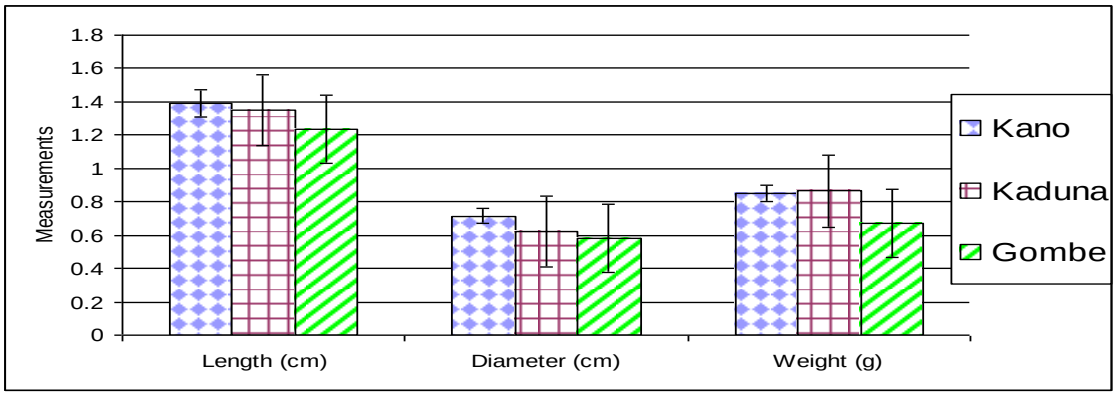

Figure 1: Weight, length and diameter of tiger nut from the three states

Examination of the physical state of the tiger nut samples with respect to insect and mechanical damage revealed that the incidence of insect infestation was highest in samples obtained from Kano, with 30% of the nuts found to be insect infested, compared to 20% in the nuts from Kaduna and 10% in the ones from Gombe. Mechanical damage of the nut occurred in 15% and 10% of the nuts from Kano and Kaduna respectively, while those obtained from Gombe had only 3% being mechanically damaged.

From Table 1, there is incidence of aflatoxin (B1, B2, G1 and G2) contamination in the entire tiger nut samples analyzed. The HPLC result indicated that the level of AFB1 ranges from 8.050 to 21.100 µg/kg, AFB2 ranges from 0.115 to 0.910 µg/g, AFG1 ranges from 0.500 to 1.510 µg/kg and AFG2 ranges from 0.075 to 0.905 µg/kg for Kano tiger nuts. For the tiger nuts obtained from Kaduna, the level of AFB1 ranges from 8.005 to 21.050 µg/kg, AFB2 ranges from 0.900 to 2.105 µg/kg, AFG1 ranges from 1.195 to 3.115 µg/kg and AFG2 ranges from 0.080 to 0.105 µg/kg. On the other hand, the samples from Gombe had AFB1 at levels ranging from 7.005 to 11.110 µg/kg, AFB2 ranges from 0.920 to 1.320 µg/kg, while AFG1 ranges from 0.195 to 1.825 µg/kg and AFG2 ranges from 0.090 to 0.600 µg/kg.

The concentration of the four aflatoxins in the samples collected from the three locations follows the ranking: AFB1 > AFB2 > AFG1> AFG2. Generally, the concentration of AFB1 was about ten-fold that of AFB2 and AFG1, while the concentration of AFB2 and that of AFG1 was about ten-fold that of AFG2.

Determination of aflatoxins in Tiger nut samples using the HPLC system

Table 1: Concentration of Aflatoxin in Tiger nut samples using HPLC method

Sampling location	AFB1	AFB2	AFG1	AFG2 (µg/kg)
Ka	9.005±0.000[a]	0.115±0.000[a]	0.505±0.000[a]	0.075±0.00[a]
	12.050±0.005[b]	0.170±0.000[a]	0.985±0.000[b]	0.110±0.001[b]
	8.050±0.005[a]	0.605±0.000[b]	0.500±0.100[a]	0.900±0.000[c]
	21.100±0.100[c]	0.910±0.001[c]	1.510±0.100[c]	0.905±0.000[c]
Kd	8.005±0.000[a]	0.905±0.000[a]	1.195±0.000[a]	0.080±0.000[a]
	21.050±0.050[c]	2.105±0.000[c]	3.115±0.000[b]	0.105±0.000[a]
	13.000±0.000[b]	0.935±0.000[b]	1.200±0.010[a]	0.090±0.000[a]
	15.050±0.050[b]	0.900±0.000[a]	1.310±0.000[a]	0.090±0.010[a]
Gm	11.050±0.050[b]	1.205±0.000[b]	1.725±0.000[b]	0.125±0.000[a]
	7.005±0.000[a]	0.920±0.010[a]	1.195±0.000[a]	0.090±0.000[a]
	11.110±0.010[b]	1.300±0.010[c]	1.685±0.000[b]	0.600±0.000[b]
	9.005±0.000[a]	1.320±0.000[c]	1.825±0.000[b]	0.505±0.000[b]

Values are mean of triplicate determinations ± SD, n = 4 for each city, $P < 0.05$, aflatoxin values with different superscripts indicated as a, b, c down the column of each sampling location are statistically different

AFB1: Aflatoxin B1 Ka: Kano

AFB2: Aflatoxin B2 Kd: Kaduna

AFG1: Aflatoxin G1 Gm: Gombe

AFG2: Aflatoxin G2

Although all the samples analyzed were contaminated, tiger nut samples collected from Kano and Kaduna appeared to be more contaminated than those collected from Gombe which had the highest AFB1 levels of 11.110 µg/kg, compared to 21.100 µg/kg and 21.050 µg/kg recorded for Kano and Kaduna respectively. Generally, AFB1 was the most predominant of all the aflatoxins; this is in agreement with the findings of Alam *et al.* (2010) who reported that amongst all aflatoxin species, AFB1 is normally the most predominant in food and feed products.

Pearson Correlation Index ($r = 0.543$) for the data, using the sampling locations of the tiger nut as discriminating variable indicates that there is slight statistical difference in the mean level of AFB1 in the tiger nut from Kano compared to those from Gombe. Also, the correlation indices of 0.689 and 0.662 indicate that the concentrations of AFG1 and AFG2 respectively were significantly elevated in the tiger nut from Kano compared to the ones from Kaduna and Gombe.

However, the level of AFG2 in all the nut samples does not differ significantly ($P < 0.05$).
The present results clearly indicate that AFB1 and total aflatoxin levels exceeded the NAFDAC permissible limits of 4 µg/kg and 10 µg/kg, European Union 2 µg/kg and 4 µg/kg and the World Health Organization permissible limits of 5 µg/kg and 10 µg/kg respectively (Moss, 2002; Papp *et al*., 2002).

The result of spiking the tiger nut extracts indicates that the recoveries of AFB1 and AFB2 were found to be generally higher than those of AFG1 and AFG2, this conforms to previous report by Gnonlonfin *et al*. (2010). The study also indicates that there is no significant relationship between aflatoxin contamination of the nut and the level of insect or/and mechanical damage on the samples.

Reduction in the Level of Aflatoxins in Tiger Nut using Orange Juice

By using the orange juice extract of 100% and 50% concentration to remediate the aflatoxins in the tiger nut, the result shows that total aflatoxins in the tiger nut samples were reduced drastically while maintaining the room temperature (28^{o}C). For the remediation of total aflatoxins in tiger nut, it is evident that all treatments were more effective than plain water in reducing the level of aflatoxins. Increase in the concentration of orange juice from 50 to 100% significant reduced the levels of AFG1 and AFG2 by 30% and 75% respectively

compared to 7% and 24% reduction recorded in the 50% extract; but increased concentration of the extract did not lead to increased reduction of AFB1 and AFB2 (Table 2).

Aflatoxin B1 in the tiger nut samples was reduced by 75% and 67% respectively when treated with 50% and 100% (v/v) orange juice extract; while the tiger nut sample treated with distilled water recorded a 38% reduction. The 100% orange juice extract also led to a 75% reduction in the level of aflatoxin G2.

The orange juice extract had less effect in the reduction of aflatoxin B2 and G1, compared to water only which led to a 50% reduction in aflatoxin B2 and a 45% reduction in AFG1 in the samples. This implies that rinsing of tiger nut with water alone serve better in the remediation of aflatoxins B2 and G1 than does the orange juice extract.

Table 2: Aflatoxin remediation of tiger nut with varying concentration of extract

	AFB1 (µg/kg)	**AFB2** (µg/kg)	**AFG1** (µg/kg)	**AFG2** (µg/kg)
$IOW_{50\%}$	3.450	1.105	0.985	0.285
$FOW_{50\%}$	0.850	0.985	0.915	0.215
$IOR_{100\%}$	7.150	1.025	1.895	0.895
F $OR_{100\%}$	2.350	1.008	1.320	0.220
$IWT_{100\%}$	8.850	2.130	2.165	0.465
F $WT_{100\%}$	5.450	1.065	1.205	0.205

$IOW_{50\%}$ Initial concentration of aflatoxin with orange/water 50%
$FOW_{50\%}$ Final concentration of aflatoxin with orange/water 50%
$IOR_{100\%}$ Initial concentration of aflatoxin with orange 100%
$FOR_{100\%}$ Final concentration of aflatoxin with orange 100%
$IWT_{100\%}$ Initial concentration of aflatoxin with water 100%
F $WT_{100\%}$ Final concentration of aflatoxin with water 100%

4. Conclusion

The tiger nut samples from Kano were found to be more contaminated with aflatoxins compared to those from Kaduna and Gombe; this would not be unconnected to high humidity, high temperature, poor crop handling and storage in the location, since these create a conducive condition for the growth of mould and subsequently aflatoxin production.

All of the tiger nut samples collected in this study were contaminated with aflatoxins; with about 17% of these recording levels beyond the maximum limit of 20 µg/kg aflatoxins set by United State Food Safety Regulations, National Agency for Food, Drug Administration and Control (NAFDAC) and the Codex Alimentarius. Aflatoxin B1 in the tiger nut samples was reduced by 75% and 67% respectively when treated with 50% and 100% (v/v) orange juice extract; the orange juice (100% (v/v)) also reduced aflatoxin G2 by 75%. Distilled water serve better in the remediation of aflatoxins B2 and G1 than does the orange juice extract. The use of orange juice extract and water for the reduction of aflatoxins in tiger nut serve as a relief to consumers of the nut, as it is consumed raw, unlike in cooked foods that will have these toxins being reduced by the processing.

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