A SURVEY OF FUNGAL CONTAMINATION AND THEIR METABOLITES IN STREET-VENDED TIGER NUTS AND DATES IN LAGOS STATE, NIGERIA USING LCMS/MS ANALYSIS

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Abstract:
Tiger nuts (Cyperus esculentus) and dates (Phoenix dactylifera) are commonly consumed raw, posing potential health risks. This study investigated fungal contamination and mycotoxin presence in 54 composite samples from 18 Lagos State markets. Moisture content, frequency of occurrence of fungi, aflatoxigenic potential and presence of fungal metabolites were determined using standard methods and Liquid Chromatography with tandem Mass spectrophotometry (LC-MS/MS) analysis. Moisture content and CFU/mL of nuts ranged between 8.00 - 38.83%, and 1.60 x 10^4 - 3.10 x 10^5cfu/mL. Fungal genera isolated include *Aspergillus* (11.80%), *Fusarium* (3.89%), *Penicillium*, *Saccharomyces* (38.25%), and Candida (41.2%). An aggregate of 26 fungal metabolites (15 regulated and 11 unregulated) were detected in the nuts and dates. Fusarium-producing toxins; FB1, FB2 and FB3 (regulated mycotoxin) and Fusaric acid metabolite (emerging mycotoxins) produced the highest concentration (20.00 ±0.0 and 81.5µg/kg ± 2.4 respectively) of toxins among the metabolites, while Aflatoxin B1 and B2 concentrations ranked the least (0.2-2.05µg/kg ±0.1). This is the first report to document the occurrence of fusaric acid and emerging mycotoxins (Enniatin A, A1, B, and B1) in tiger nuts and dates consumed in Lagos, Nigeria. Notably, the unusually high levels of fusaric acid detected in these nuts raise concerns due to its potential health impacts. These findings will inform policymakers and relevant institutions in ensuring public safety.

Keywords: Cyperus esculentus, Phoenix dactylifera, LC-MS/MS, mycotoxins, fungal metabolites, ready-to-eat snacks, Lagos.

1. INTRODUCTION

Cyperus esculentus, also known as tiger nut, is a small root tuber crop that flourishes in tropical and Mediterranean regions. It is mostly found in Southern Asia, Middle East, Southern Europe, Africa and Madagascar (Ntukidem et al., 2020). In Nigeria, it is frequently consumed in its raw form, either as a fresh fruit or in its dried alternative, and in other cases, it is crushed and juiced to produce tiger nut milk. Consumption of raw tiger nuts and tiger nut milk has numerous health benefits; it is a valuable source of vegetable oils (which reduces the risk of heart disease), research has reported the versatility of tiger-nut in Lowering colon cancer risk, Stimulating heart function, Soothing diarrhea symptoms and Reducing inflammation (Bazine & Arslanoglu, 2020).

Phoenix dactylifera (Dates) are tropical fruits that grow on their parent tree in small clusters. Dates are cultivated in the Middle East and North African countries, with a global annual production records of about 6 million tons (FAOSTAT, 2021). Egypt produces about 1.7 million tons a year and rated as the largest producer of dates globally (FAOSTAT, 2021). There are different subtypes of dates which vary geographically. The most common subtype available in Nigeria is the 'deglet nour' type which originated from Algeria (Abdulaziz *et al.*, 2021). The chemical profile of date fruit reveals a diverse array of nutrients, including carbohydrates, dietary fiber, proteins, lipids, minerals, vitamins, enzymes, phenolic acids, and carotenoids, contributing to its exceptional nutritional and health value (Ibrahim *et al.*, 2021).

Varieties of tiger nut— Yellow, brown and black are grown in Nigeria and they are referred to as "Ofio" in Yoruba, "Akiausa" in Igbo and "Aya" in Hausa. The yellow type is preferred due to its intrinsic qualities, which include its size, beautiful hue, and fleshier structure (Figure 1b and 1c shows the fresh and dried yellow tiger nut). Moreover, it produces more milk after extraction, which contain fewer fats, more proteins, and lower amounts of anti-nutritional compounds, such as polyphenols (Ihenetu *et al.*, 2021). Due to its ability to prevent thrombosis, heart issues, and promote blood circulation, tiger nut milk has been reported to be beneficial for preventing arteriosclerosis (Edo et.al., 2023). As a result of the high arginine content, which releases the hormone insulin,

and its optimal combination of sucrose and starch without glucose, tiger nuts without sugar can be used to treat diabetes (Mohammed *et al.*, 2022). The milk is also a suitable beverage for celiac patients who cannot tolerate gluten, and also an deal option for people with lactose intolerance who require dairy-free substitutes.

Date fruits contain 70% carbohydrate (mostly invert sugars such as glucose and fructose), which is beneficial for people who cannot tolerate sucrose and is easily absorbed by the human body without the digestion that ordinary sugar needs to perform. Dates consumers in Saharan areas have the lowest cancer rate, which may be attributed to the magnesium found in dates, which is considered a mineral-rich "mine" (AbdulQadir *et al.*, 2011).

Since both dates and tiger nuts are sold in open market spaces where they are usually left uncovered for long periods of time, they are both prone to microbial contamination by both bacteria and fungi via deposition through air. They can also be contaminated by the water that is used to prevent dehydration of the fruit's skin and during handling of the fruits. This contamination can have adverse effects on the consumer of these foods, such as gastrointestinal infections, organ damage, food poisoning, and, in some severe cases, death (Oyedele et.al., 2020, Obande et.al.,2017, Tshipamba et.al., 2018) These fruits can also be affected by mycotoxigenic fungi which has been shown to cause organ failure and cancer. (Adewunmi *et al.*, 2021, Adetunji *et al.*, 2017). In addition, information on the microbiological quality and incidence of fungal metabolites in tiger nuts and dates sold in Lagos Markets is scarce. Therefore, this research work aims at assessing the microbiological quality and the possible fungal metabolites present in tiger nuts and dates retailed in various markets within Lagos state. The result of this research will inform policies makers and concerned regulatory agencies on appropriate measures needed to ensure the safety of the consumers of these nuts.

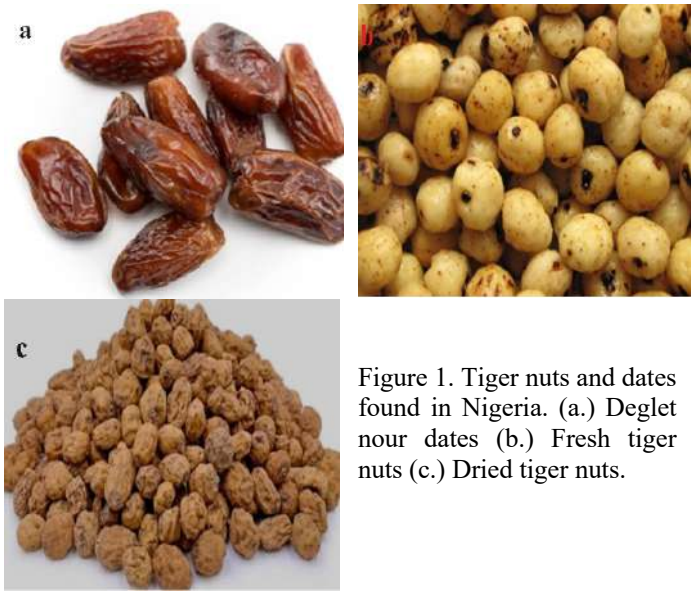

Figure 1. Tiger nuts and dates found in Nigeria. (a.) Deglet nour dates (b.) Fresh tiger nuts (c.) Dried tiger nuts.

2.0 Methods

Study Sites And Sample Collection

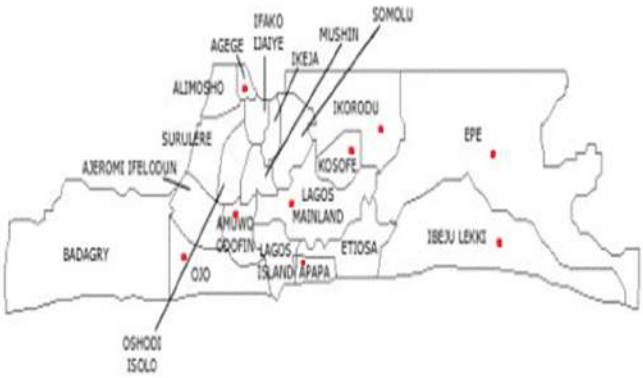

Figure 2: Map of Lagos showing the sample collection sites

The selection of the study area of this research was based on the divisions of Lagos State as detailed by the government of Lagos State. These divisions include; Ikeja, Badagry, Ikorodu, Lagos (Eko), and Epe (Figure 2). An approximate weight of 100 g each of fresh Tiger nut (TGW), dried Tiger nut (TGD) and dates (DT) were purchased randomly from 90 vendors, in a situation where the vendor does not have the three samples, the purchase was made based on availability. A total number of 90 TGW, 90 TGD and 90 DT were purchased from 18 major markets within 9 Local Council Development Areas (LCDAs). Similar samples from the same market were composited to form the sample for each market, and the samples were kept in the refrigerator at 4°C for microbial analysis.

Moisture analysis

As described by AOAC, 2000, an empty glass petri dish was dried in the oven at 105°C for 1 h and transferred to a desiccator to cool before weighing. A 5g of individual milled samples of tiger nuts and dates were weighed and spread uniformly in the dish in triplicates. The dishes containing the sample were placed in the oven and dried for 4 h at 105°C. After drying, the dishes were put into the desiccator to cool, reweighed, and the procedure was repeated until a constant weight was achieved.

The moisture content was calculated using the formula:

$$\frac{W_2 - W_3}{W_2 - W_1} \times 100$$

W1- weight of empty Petri dish.
W2- weight of sample and Petri dish before drying.
W3 - weight of sample and Petri dish after drying.

Isolation of Fungi

A gram of each milled representative samples from each market were weighed into test tubes with 9 ml of sterile distilled water and decimally diluted until 10^{-2} dilutions (for tiger nut samples) and 10^{-1} dilutions (for date samples) were

achieved (Adetunji *et al.*, 2018). A 0.1 ml of 10^{-2} dilution for both dry and wet tiger nuts and 10^{-1} dilution for dates was transferred into already prepared Sabouraud Dextrose Agar (SDA) containing chloramphenicol (100 mg/litre). Using a spreading glass rod, the inoculum was cultured using the spread plating technique, allowed to dry and incubated at 25°C for 5 days. The fungal count was done after 72 h and further incubated until the fifth day. The resultant colonies on the SDA plates were counted after incubation and subcultured on SDA and Potato Dextrose Agar (PDA) for proper purification and identification of isolates using standard methods. The plates with mold isolates were incubated at 25°C for 5 days.

Morphological identification of fungi Isolates

Isolates were observed both microscopically and macroscopically to determine the genus and species (where applicable) of each organism. The isolates were identified according to previously published methods as Aspergillus (Nyongesa *et al.*, 2015); Penicillium (Kim *et al.*, 2007), Fusarium (Rivas-García *et al.*, 2018; Parikh *et al.*, 2018). The fungal morphology was studied macroscopically by observing the colony feature: Colony diameter, Right side shape, colour on media, Reverse shape and color on media.

They were observed microscopically with a digital camera compound microscope using a lactophenol cotton blue-stained slide mounted with a small portion of the mycelium (Gaddeyya et al., 2012). The result of the morphological and microscopy examination were compared with various reference manuals, taxonomic books and monographs available on various groups of fungi (Aneja, 2003) for fungal identification.

Toxigenicity of Mycotoxigenic Fungi

Suspected isolates of Aspergilli section Flavi were examined for aflatoxigenicity using Neutral Red Desiccated Coconut Agar (NRDCA) as described previously by Atanda *et al.*, 2011. The data obtained from toxigenic screening of *A. flavus* isolates on NRDCA was used to determine the ratio of aflatoxigenic isolates to non-aflatoxigenic isolates in the tiger nut and date samples (Adetunji *et al.*, 2018).

Liquid Chromatography tandem mass spectrometry (LC-MS/MS) analysis for fungal metabolite quantification

Thirty six composite samples (18 dry tiger nut, 9 fresh tiger nut and 9 dates) were analysed for the presence of fungi metabolites using the direct injection LC-MS/MS technique (Varga et al., 2012). The procedure describes a quantitative method where all mycotoxins present in the samples were identified and quantified simultaneously.

Extraction of Metabolites and LCMS/MS analysis for nut sample

Ground and homogenized tiger nuts and dates samples (5.00 ± 0.01 g) were weighed into 50-mL polypropylene tubes (VWR International) using a mill and mixer before a test portion is removed for analysis. The first extraction step was performed by adding 20ml of the solvent 1 (acetonitrile/water/formic acid, 80:19.9:0.1, *v*/*v*/*v*) to the weighed sample and the mixture was shaked in an orbital shaker (Hechingen, Germany) at 250 rpm for 90min at room temperature. After extraction, the tubes were centrifuged for 5 min (4200 rpm) with an Eppendorf AG Centrifuge 5804 R (Hamburg, Germany), then 450µl of the supernatant was removed to a glass vial. Then 750 µl (V2) ultrapure water was added to the supernatant and Vortexed. The vortexed 1200µL aliquot was filtered across membrane syringe filter into a glass vial and vortexed again. The content of the vial was mixed and 5 µL thereof was injected directly into the LC-MS/MS system (Tripple Quad LC-MS Agilent 6460 C, Singapore).

LC-MS/MS parameters and methods Validation for LCMS/MS analysis

For the analysis, a Tripple Quad LC-MS Agilent 6460 C,(Singapore) LCMS/MS machine was used. The accuracy of the method was evaluated through recovery studies by spiking blank samples with multi-mycotoxin standards in triplicates, recoveries were obtained using the identical extractions methods as for samples and the same analysis techniques and mean % recovery was recorded for each of the toxins. For the spiking procedures, three different blank nut samples were spiked with a mix standard solution at one concentration. A 5g each of the homogenized and finely ground sample were weighed into a 50ml conical tube and 200µL multi-mycotoxin spiking solution was added to each nut type and homogenized with 1ml of the extraction solvent (acetonitrile/water/formic acid, 80:19.9:0.1, *v*/*v*/*v*), the homogenous mixture was extracted, diluted and 5µL injected into the LCMS/MS machine at the temperate of 50°C (Varga et al., 2012) . The sensitivity of the methodology or system used was examined by limit of detection (LOD) and limit of quantification (LOQ), which were estimated for a signal-to-noise ratio (S/N) x 3 and x 10, respectively, from chromatograms of samples spiked at the lowest level validated

Determination of mycotoxin mass fraction

$$C_{\text{Mycotoxin}}\ [\mu g/kg] = \frac{Cx(VxR)}{m}$$

Where:

C –concentration of mycotoxin [ng/ml]

V – extraction volume (= 20ml) [ml]

R – dilution = 2.66

m – amount of sample [g]

Statistical Analysis of Data

The results were expressed as mean values of triplicate measurements. One-way analysis of variance (ANOVA) was performed at a 95% confidence interval ($p < 0.05$) to determine significant differences. Statistical analysis was conducted using IBM SPSS Statistics version 16.0 for Windows.

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3.0 Results

Moisture analysis and total fungal counts

The moisture content of fresh and dry tiger nut samples ranged from 24.2 - 44.3% and 5.6 - 13.8% respectively, while the moisture content of dates samples ranged from 6.0 – 14.2% (Table 1). In addition, the total plate counts of molds in fresh and dry tiger nut ranged from 1.62×10^5 - 3.75×10^5 CFU/mL, and 1.39×10^5 - 3.75×10^5 CFU/mL respectively and 1.41×10^4 – 2.61×10^4 CFU/mL for dates. The mean moisture contents

and fungi colony counts of the three different samples across the five divisions are not significant at a 95% confidence interval ($p < 0.05$).

Identification of fungal isolates

A sum of 44 isolates were obtained from all samples. The distribution of the isolates is as presented in Table 2. Based on morphology and microscopic view of the colonies as interpreted by the taxonomic reference manual, 17 were identified as *Aspergillus spp*., while 6 were identified as *Penicillium* species and 5 were identified as *Fusarium species* (Table 3a-3c). On the contrary, sixteen (16) yeasts colonies were isolated where morphological identification showed that they belonged to the genera Candida, Saccharomyces, Pichia and Rhodotorula (Table 3c). The isolated fungi comprised are;

Table 1, Mean moisture content and Mean Colony count in tiger nuts and dates across the divisions.

	FRESH TIGER NUT		DRY TIGER NUT		DATES	
Division	Mean Moisture Content (%)	Mean Colony Count (CFU/mL) x 10^5	Mean Moisture Content (%)	Mean Colony Count (CFU/mL) x 10^5	Mean Moisture Content (%)	Mean Colony Count (CFU/mL) x 10^4
Ikeja	38.83 ±4.68*	2.40±0.17	10.25±2.66	2.35±0.98	9.75±2.63	1.80±0.15
Badagry	33.45±4.23	2.50±0.86	9.30±2.54	2.65± 0.69	8.58±1.68	1.83±0.30
Ikorodu	30.50±7.78	3.14±0.62	8.70±1.56	2.43± 0.51	8.00±1.41	2.34±0.38
Eko	34.13±7.81	2.74±0.87	8.05±1.93	2.40±0.91	11.75±2.66	1.60±0.16
Epe	33.33±5.66	2.81±0.27	11.8±1.68	2.42±0.42	11.30±2.78	1.94± 0.42

*±S.D

Table 2, Distribution of Fungi Species in Tiger nuts and Dates

FUNGI	FRESH TIGERNUT	DRY TIGERNUT	DRY DATES	TOTAL ISOLATES ACROSS
Aspergillus spp.	5	7	5	17
Penicillium spp.	1	2	3	6
Fusarium spp.	2	3	0	5
Candida spp.	2	2	2	6
Saccharomyces spp.	2	2	2	6
Rhodotorula spp.	1	1	-	2
Pichia spp.	1	1	-	2
Total	14	18	12	44

Table 3a, Microscopic and Macroscopic characteristics of *Aspergillus* isolates

***ASPERGILLUS* SPECIE**	**MACROSCOPIC CHARACTERISTIC**			**MICROSCOPIC CHARACTERISTIC**			
	Colony Diameter (mm)	Conidia Colour	Conidia Reverse Colour	Colour of Conidial Head	Shape	Vesicle Seration	Conidial Surface
A. flavus	56	Yellow-green	Pale yellow	Yellow-green	Globose ellipsoid	Biserate	Smooth, finely roughned
A. niger	50	White to Carbon black	Pale yellow	Black	Globose	Biserate, large size	Very rough, irregular
A. fumigatus	50	Blue-green	Pale yellow	Bluish-green	Globose	Uniserate	Smooth or Spinose
A. terreus	35	White to Brown	Light yellow	Light Brown	Spherical	Biserate spatulate	Smooth
Aspergillus spp.	37	Black	Cream	Brown	Globose	Biserate	Rough
A. novofumigatus	41	Olive green	Brown	Green	Globose	Radiate	Smooth
Aspergillus Spp	39	Cream To Dark Brown	Cream	Dark Brown	Globose	Uniseriate	Rough

Table 3b, Microscopic and Macroscopic characteristics of *other fungal isolates*

***Fusarium* Species**	**Macroscopic Characteristic**		**Microscopic Characteristic**
	Surface Colour	Reverse Colour	
F. oxysporum	White and fluffy	Orange	Fusiform microconidia within hyaline hyphae
Fusarium sp.	White and grey; fluffy	Grey	Oval shaped microconidia lacking septa Macroconidia were present with a curved shape
F. avenaceum	Cotton white, fluffy	Deep red	Septate spindle shaped macroconidia, septate microconidia present in small numbers.
Penicillium sp.	Blue-grey	Pale yellow	aerial hyphae that were smooth-walled, rami cylindrical, phialids ampliform, flask-shaped with short necks, smooth walled conidia
Penicillium solitum.	Blue-green	Pale cream	subsurface hypha which are rough-walled, smooth-walled conidia

Table 3c, Microscopic and Macroscopic characteristics of Yeast isolate

YEAST SPECIES	***MACROSCOPIC CHARACTERISTIC**					***MICROSCOPIC CHARACTERISTIC**
	Colony Diameter (mm)	Colony Colour	Texture	Elevation	Margin	
Candida spp.	45	Creamy white	Pasty	Slightly Domed	Entire	Small, oval cells that occurred mostly in clusters
Candida spp.	38	Shiny, Creamy white	Creamy	Domed	Entire	Small, oval cells that occurred mostly in clusters
Saccharomyces cerevisiae	41	Dull, Cream with a 'bread-like'smell	Creamy	Flat	Entire	oval, slightly cylindrical, and short cells
Saccharomyces spp.	39	Slightly shiny cream coloured with a 'bread-like'smell	Creamy	Slightly raised	Entire	oval, slightly cylindrical, and short cells
Rhodotorula spp.	25	Pink-Orange	Creamy	Raised	Entire	Round cells with pseudo hyphae present
Pichia spp.	35	White	Dry	Flat	Entire	elongated tubular and spheroidal shaped blastoconidia

Morphological Identification of Isolates

The suspected isolates were compared with results from previous research based on fungal macroscopy. The features examined for all isolates include the colony diameter, the conidia colour, and the conidial reverse colour. For Aspergillus, the Conidia colour ranged from dark brown to black for the Nigri section (Figure 3A), from light brown to medium brown for the terrei section (Figure 3B), yellow-green to blue-green for the Flavi section (Figure 3C), and from blue-green to dark green for the Fumigati section. The reverse colour for all Aspergillus ranged from pale yellow to medium brown. Microscopically, the colour of the conidial head ranged from yellow green to dark brown across all isolates, and the shape of the conidial head ranged from globose to globose-ellipsoid and spherical. The conidia surface also varied between smooth and rough.

For yeast, all isolates had entire margins, although margin shape may be entire, wavy, or irregular. The colony diameter measured 30-40mm for Candida spp., 35-41mm for Saccharomyces spp., 20-25mm for Rhodotorula sp. and 30-35mm for Pichia sp. when subcultured. Due to Rhodotorula sp.'s ability to produce carotenoid pigments, it is easily differentiated by the colour (pinkish orange) on SDA medium. Pichia appeared as pure white, Candida spp. appeared creamy-white and Saccharomyces spp. appeared creamy. Microscopically, the isolates were described and differentiated based on the shape of their cells which were oval for Candida spp., slightly cylindrical oval for Saccharomyces spp., and elongated spherical for Pichia.

The conidia colours of the Penicillium spp. ranged between blue-green and blue grey; the reverse colour was pale cream and pale yellow (see Figure 3D). The isolates were confirmed as *Penicillium spp*. by microscopy, in which the distinct elongated flask-shaped structures (phialides) were present. The species were then differentiated based on hyphal and phialidal morphology (Kim *et al.*, 2007).

The macroscopic and microscopic profiling for the Fusarium spp. was compared to the study conducted by Rivas-García *et al.*, (2018) and Parikh *et al.*, (2018). Macroscopically, the colours of the Fusarium spp. ranged between cotton white to grey-white and fluffy. The reverse ranged from orange to yellow with dark red to deep red while microscopically, the isolates were confirmed as Fusarium because of their distinct curved macroconidia and aseptate microconidia (Rivas-García *et al.*, 2018 and Parikh *et al.*, 2018.).

Figure 3; Some Fungi isolated from the samples on SDA (A) *Aspergillus niger* (B) *Aspergillus terreus* (C) *Aspergillus flavus* (D) *Penicillium solitum*

Toxigenicity of Mycotoxigenic Fungi

The intensity of the fluorescence produced by the isolates (section Flavi and section Fumigati) on Neutral Red Desiccated Coconut Agar (NRDCA) was reported as either absent (-), weak (+), moderate (++) or high (+++), and no fluorescence was produced by the two negative controls (Aspergillus sp. and Penicillium sp.) as expected. The *A.flavus* isolated from dried tiger nut showed strong fluoresence (+++) while that of fresh tiger nut showed moderate intensity while the *A.flavus* from dates produced no fluorescence (-). Other aspergillus species tested were *A.fumigatus* and *A.novofumigatus* both of them and the control isolates produced no fluorescence.

LC-MS/MS analysis

Settings and Performance Parameters of LCMS/MS Machine

The recoveries rate for all the metabolites ranged from 81% to 103.0% with ENNIATIN A & B and Fumonisins and Deoxynivalenol having the lowest and maximum recovery respectively. The values for the limit of Quantification for the metabolites ranged between (0.4-25 µg/kg with aflatoxins group having the least LOQ while the Fumonisins (FB1-FB3) recorded the highest LOQ. The regression coefficients (r^2) for the calibration curves were between 0.998 and 0.999, which demonstrated a good linearity.

Incidence and Quantification of Mycotoxins and Metabolites in Nuts and Dates Samples

A total of 26 fungal metabolites belonging to 7 major categories of mycotoxins family were detected in 36 composited samples of dried and fresh tiger nuts and dates using the LC-MS/MS methods. An aggregate of 15 regulated mycotoxins were detected and quantified. The nuts and dates samples recorded 100% incidence for all the 26 fungal metabolites detected though larger percentage were lower than the limit of Quantification (LOQ).

The metabolites concentrations ranged between LOQ-2.05 µg/kg for Aflatoxin B1 and LOQ-0.81 µg/kg for Aflatoxin B2 in dry tiger nuts. All other regulated mycotoxin levels were below the levels of Quantification (LOQ). In fresh tiger nuts only 11.11% (1 of 9) of the samples recorded values above the LOQ while all other regulated mycotoxins levels were below the LOQ value (Table 5). In dates, all regulated mycotoxins were below the LOQ values. The concentration of AFB_1 and AFB_2 present in the samples fell within the acceptable EU standard (<4.0µg/kg) and within the standard of the National Agency for Food and Drug Administration and Control (NAFDAC) for ready-to-eat foods (<10 µg/kg).

Eleven non-regulated metabolites were detected and quantified in the nuts and dates samples. The non-regulated metabolite concentrations ranged between LOQ-81.5µg/kg for Fusaric acid in dry tiger nuts. Eight samples out of 18 (44.44%) were within the levels of detection for Fusaric acid. All other non-regulated mycotoxin levels were below the LOQ values. In dates, the Fusaric acid levels ranged from LOQ-12.1µg/kg, in which five out of nine samples (55.55%) had values above the LOQ. All other non-regulated mycotoxin levels were below the LOQ (Table 6). In fresh tiger nuts, all non-regulated mycotoxins were below the LOQ values.

Table 4, LCMS/MS Performance Parameters

Mycotoxin family	Mycotoxin	Abbreviation	% Recoveries	LOQ; (µg/kg)	LOD (µg/kg)
aflatoxins	AFLATOXIN B1	AfB1	96	0.4	0.1
	AFLATOXIN B2	AfB2	93	0.4	0.1
	AFLATOXIN G1	AfG1	98	0.4	0.1
	AFLATOXIN G2	AfG2	99	0.4	0.1
Zearalenone + Metabolite	α – ZEARALENOL	α -ZEL	87	10	3.0
	β – ZEARALENOL	β -ZEL	101	10	3.0
	ZEARALANONE	ZAN	103	10	3.0
	ZEARALENONE	ZEN	102	5	1.5
A-Trichothecenes	DIACETOXYSCIRPENOL	DAS	96	3	0.9
	HT-2	HT-2	92	9.6	2.9
	T-2	T-2	98	9.6	2.9
B-Trichothecenes	3-ACETYL DEOXYNIVALENOL	3-ADON	80	20	6.1
	15-ACETYL DEOXYNIVALENOL	15-ADON	83	20	6.1
	DEOXYNIVALENOL	DON	103	20	6.1
	NIVALENOL	NIV	85	20	6.1
Fumonisins + Metabolite	FUMONISIN B1	FB1	103	25	7.6
	FUMONISIN B2	FB2	82	25	7.6
	FUMONISIN B3	FB3	86	25	7.6
	FUSARIC ACID	FA	102	3	0.9
	MONILIFORMIN	MON	82	3	0.9
Ochratoxin	OCHRATOXIN A	OTA	94	1.6	0.5
Emerging Mycotoxins	BEAUVERICIN	BEA	85	1	0.3
	ENNIATIN A	ENNI A	81	1	0.3
	ENNIATIN A1	ENNI A1	84	1	0.3
	ENNIATIN B	ENNI B	86	1	0.3
	ENNIATIN B1	ENNI B1	81	1	0.3

4.0 Discussion

Fungal contamination of foods is a significant factor contributing to global food loss (Simon et al., 2019). The infection of crops by field fungi during cultivation, followed by the proliferation and growth of fungi during storage, can lead to foodborne fungal infections. This, in turn, exacerbates poverty and hunger, counteracting the Sustainable Development Goals (SDG) 2030 agenda (Pederson, 2018). Consequently, the high fungal loads found in nuts present a cause for concern and necessitate immediate intervention.

Fungi are ubiquitous, spreading through spores, and their proliferation in plants often begins from the soil, which serves as their natural habitat. Fungi also enter plants through bruises and damage on crops, enabling microbial growth. Post-harvest practices, storage conditions, and handling techniques by vendors are further factors that contribute to fungal contamination (Adejumo and Okeleye 2022). Additionally, extrinsic factors such as temperature, relative humidity, and environmental gaseous composition are key determinants in promoting fungal growth (Orole et al., 2017). The copious fungal load recorded in this study could be attributed to one or more of these extrinsic factors. The total fungal counts of the tiger nut tubers reported (2.35×10^5 - 3.10×10^5) align with those found by Sa'Id, Abubakar, and Bello (2017), which exceeded the recommended limit of $<10^5$ as specified by the International Commission on Microbiological Specifications for Food (ICMSF, 1986). Consumption of foods contaminated with high levels of fungi, particularly toxigenic species, poses serious risks to human health. Mycotoxicosis and other health complications have been documented in individuals consuming fungi-contaminated food (Pitt and Milller et al., 2017).

Table 5, Regulated mycotoxin levels in all dry and fresh tiger nut samples

Regulated Mycotoxins	Concentration Range (µg/kg)		Mean Conc. (µg/kg)		Standard Deviation	
	Dry TN (n=18)	Fresh TN (n=9)	Dry TN (n=18)	Fresh TN (n=9)	Dry TN (n=18)	Fresh TN (n=9)
Aflatoxin B1	0.2-2.05	0.2-0.61	0.55	0.25	0.68	0.14
Aflatoxin B2	0.2-0.81	0.2-0.4	0.28	0.2	0.18	0.00
Aflatoxin G1	0.2-0.4	0.2-0.4	0.2	0.2	0.00	0.00
Aflatoxin G2	0.2-0.4	0.2-0.4	0.2	0.2	0.00	0.00
Beauvericin	0.5-1.0	0.5-1.0	0.5	0.5	0.00	0.00
Fumonisin B1	20-40	20-40	20.0	20.0	0.00	0.00
Fumonisin B2	20-40	20-40	20.0	20.0	0.00	0.00
Fumonisin B3	20-40	20-40	20.0	20.0	0.00	0.00
Deoxynivalenol	10-20	10-20	10.0	10.0	0.00	0.00
Moniliformin	1.5-3.0	1.5-3.0	1.5	1.5	0.00	0.00
Nivalenol	10-20	10-20	10.0	10.0	0.00	0.00
Ochratoxin A	0.8-1.6	0.8-1.6	0.8	0.8	0.00	0.00
α-Zearalenol	5-10	5-10	5.0	5.0	0.00	0.00
β- Zearalenol	5-10	5-10	5.0	5.0	0.00	0.00
Zearalenone	5-10	5-10	5.0	5.0	0.00	0.00

***** samples recorded as LOQ are considered positive for the metabolite tested and half of the LOQ value for each analyte was recorded as the concentration for the metabolite

This study investigated the fungal contamination of tiger nuts and dates sold by street vendors. Four fungal genera were identified: *Aspergillus, Penicillium, Fusarium*, and yeast. *Aspergillus* species dominated the microbial population, followed by yeast*, Penicillium*, and *Fusarium* (with the lowest incidence). Among *Aspergillus* spp., *A. niger* was the most prevalent in both fresh (7%) and dry (4%) tiger nuts, while *A. novofumigatus* dominated the dates. Notably, *A. fumigatus* was absent in dates and minimally present in tiger nuts (0.3-0.6%). These findings align with previous reports by Al-Sheikh (2009), Chukwu et al. (2013), Orole et al. (2017) and Cohen *et al.* (2021), who identified *A. niger, A. flavus,* and *A. terreus* in both fresh and dry tiger nuts.

Fusarium and *Penicillium* species are common storage fungi that proliferate on agricultural products due to inadequate post-harvest practices. These molds were detected in tiger nuts and dates likely due to poor handling and harvesting methods. The findings align with previous studies by Piombo et al. and Orole et al., who identified these fungi and yeasts as prevalent microorganisms in these products. Fumonisins, tricothecenes, Zearalenone, Ochratoxin A, among others are common mycotoxins produced by fusarium and penicillium spps. These mycotoxins have been attributed as the causative agents of human and animal diseases viz; cancer: Linked to esophageal and liver cancer, Neurological disorders, Liver damage, reproductive disorders, haematological disorders etc (IARC 1993, 2002, Bennett & Klich 2003). Hence, the incidence of these toxin-producing mycotoxin could pose a huge danger on the health of the populace. This report documents the isolation of six yeast types from tiger nuts and dates. Notably, the yeast-to-mold ratio varied across samples, with fresh tiger nut, dry tiger nut, and dates exhibiting ratios of 5:1, 4:1, and 6:1, respectively. The pH levels of tiger nut beverages ranged from 6.3 to 6.8, while dates had a pH of 6.5, characterized as slightly acidic (Rosello-Soto et al., 2018). This acidic environment favors the growth of most yeasts, particularly *Saccharomyces cerevisiae.* Tiger nuts provide a nutrient-rich environment, comprising carbohydrates, proteins, and fiber, which supports yeast growth. Additionally, the high water activity of tiger nuts fosters yeast proliferation. Dates, with their sugary substrate (70-80% sugar content), also support yeast fermentation (Boulenouar et al., 2017). The high moisture content (20-30%) and slightly alkaline pH (6.5-7.5) of dates create suitable conditions for yeast growth (Makhlouf et al., 2017). Contamination during ripening, particularly if dates are not properly stored, presents another possible route for yeast proliferation (Essali et al., 2017).

The results of the toxigenicity test confirmed that the aspergilli section flavi group are the main producers of aflatoxins; however, not all the flavi section groups are aflatoxins producers. In this report, only 33% (2 out of 6) isolates tested positive for aflatoxin production . The result was further corroborated by the LCMS/MS analysis where AFB1 and AFB2 were detected and quantified in two of the tiger nuts samples. This further confirms the suitability of Neutral Red Desiccated Coconut Agar (NRDCA) for screening of aflatoxins and aflatoxigenic fungi in foods and agricultural produce in underdeveloped and developing nations where resources for testing for aflatoxins are scarce (Atanda *et al.*, 2011; Adetunji *et al.*, 2017).

Table 6, Unregulated mycotoxin levels in all dry tiger nut and date samples

Unregulated Mycotoxins	Concentration range (µg/kg)		**Mean concentration (µg/kg)		Standard Deviation	
	*TN (n=27)	Dates (n=9)	TN (n=27)	Dates (n=9)	*TN (n=27)	Dates (n=9)
Zearalanone	5.0-10.0	5.0-10.0	5.0	5.0	0.00	0.00
Diacetoxyscirpenol	1.5 -3.0	1.5-3.0	1.5	1.5	0.00	0.00
H-T2	4.8 -9.6	4.8-9.6	4.8	4.8	0.00	0.00
T2	4.8 -9.6	4.8-9.6	4.8	4.8	0.00	0.00
3-Acetyl Deoxynivalenol	10.0-20.0	10-20.0	10.0	10.0	0.00	0.00
15-Acetyl Deoxynivalenol	10-20.0	10-20.0	10.0	10.0	0.00	0.00
Fusaric Acid	1.5-81.5	1.5-12.1	6.32	4.38	16.0	3.47
Enniatin A	0.5-1.0	0.5-1.0	0.5	0.5	0.00	0.00
Enniatin A1	0.5-1.0	0.5-1.0	0.5	0.5	0.00	0.00
Enniatin B	0.5-1.0	0.5-1.0	0.5	0.5	0.00	0.00
Enniatin B1	0.5-1.0	0.5-1.0	0.5	0.5	0.00	0.00

*TN-both fresh and dried Tiger nut

** samples recorded as LOQ are considered positive for the metabolite tested and half of the LOQ value for each analyte was recorded as the concentration for the metabolite

Previous studies have documented instances where samples exhibited discordant results, testing positive for aflatoxigenicity but negative for aflatoxin production (Atanda et al., 2011; Adetunji et al., 2019). This discrepancy may be attributed to various factors such as; Analytical method limitations. The quantity of aflatoxins produced may fall below the detection limit, contingent upon the analytical method and stock standard concentration (Atanda et al., 2011). Also, slow growth dynamics and extrinsic factors such as relative humidity, incubation temperature, and pH of the growth medium can influence aflatoxin production (Adetunji et al., 2019). It has been reported that the method of sample preparation, variation in analytical methods, matrix interference effects, and sensitivity of analytical methods can impact toxin detection and quantification (Krska et al., 2009). The possibilities of toxin degradation during storage, handling, or analysis can also contribute to discordant results (Karlovsky, 2015). These factors underscore the complexity of mycotoxins detection and highlight the potential for conflicting results between aflatoxigenicity tests and quantitative analyses. For instance, in this research work, Penicillium toxins were not detected in the nuts samples, despite that penicillium species were isolated in the nuts during mycological analysis. One or more of the above reasons could be responsible for this.

The presence of aflatoxins and aflatoxin-producing fungi in nuts has been previously established (Adetunji *et al.*, 2019; 2020). However, there is a scarcity of recent reports on aflatoxin contamination in tiger nuts and dates sold in Nigeria, particularly in Lagos State. Most of the available reports on contamination of tiger nuts with aflatoxins in Nigeria were done on samples from the northern part of the country (Omoniyi *et al.*, 2014; Robert *et al.*, 2012; Umar *et al.*, 2016). This can be associated with the fact that the nut is native to the northern part of Nigeria and a generally acceptable snack to all and sundry, unlike in Lagos State, where the consumption rate is lower. Omoniyi *et al.* (2014) reported a high concentration of aflatoxins (8–20 µg/kg) in raw tiger nuts meant for direct consumption in the northern part of the country. To our knowledge, this report is the first on aflatoxin and other fungi metabolites contamination of tiger nuts and dates in Lagos State using LCMS/MS analysis. The only report available online as at the time of this report on aflatoxin contamination of tiger nuts is that of Robert *et al.* (2012), and this was not done solely for tiger nuts but on the evaluation of aflatoxins in snacks consumed in Lagos State. No information regarding aflatoxin contamination of dates sold in Lagos State, Nigeria was found when writing this report. This research provides novel evidence of aflatoxin and fungal metabolite contamination in dates sold in Lagos State, Nigeria.. Nonetheless, there are global reports of aflatoxin production in tiger nuts and dates. Gil-Serna, Vázquez, and Patiño (2020) reported that AFB1 concentration ranged from 0.7–4.5 µg/kg and AFB2 concentration ranged from 2.2–3.5 µg/kg in tiger nut beverages in Spain. The maximum permitted limits for total aflatoxins and AFB1 in processed foods and products meant for human consumption or as food ingredients by the European Union are 4 and 2 µg/kg, respectively (EU, 2023). Therefore, the tiger nut sample in this report, which was above 2 µg/kg, was above the EU limit; however, it was still within the NAFDAC limit of 5 µg/kg. Shamsuddeen and Aminu (2016) also tested for aflatoxins in tiger nuts, with concentrations ranging from 0.00 - 23.3µg/kg, only 2 of the 20 samples tested had an aflatoxin concentration of 23.2µg/kg and 23.3µg/kg, respectively.

This report, along with other scholarly studies, confirms that tiger nuts and dates exhibit relatively low susceptibility to mycotoxin contamination. Analysis revealed the presence of

regulated mycotoxins (Aflatoxins, Ochratoxin A, Deoxynivalenol, Zearalenone, and Fumonisins) at concentrations below permissible limits in ready-to-eat foods. Additionally, emerging mycotoxins (Enniatin A, A1, B, B1, Fusaric acid, DAS, and HT-2 toxins) and masked mycotoxins (3-Acetyl Deoxynivalenol and 15-Acetyl Deoxynivalenol) were also detected.

Regulated mycotoxins, are toxins that are subject to strict monitoring and control, well-studied, widely occurring, and highly toxic compounds (EU, 2020; EFSA, 2019). In contrast, unregulated toxins, such as Enniatin and Fusaric acid, lack established maximum residue limits (MRLs) or tolerable intake levels. Enniatin has been linked to cytotoxicity, genotoxicity, immunotoxicity, neurotoxicity, and reproductive issues (Dall'Asta et al., 2013; Prosperini et al., 2016). Fusaric acid, detected in all nut samples (up to 81.5 µg/kg), poses risks of neurological disorders, liver damage, kidney damage and cancer (Jestoi et al., 2008; Li et al., 2018; Song et al., 2014). Fusaric acid affect tubers and roots by stunting their growth; therefore, it is entirely plausible that it would be present in tiger nut tubers (López-Díaz *et al.*, 2017). Oubraim *et al.* (2018) inferred that the high fusaric acid content in the fruit of date palm plants is a side effect of the growth of *Fusarium spp*. on these plants. The presence of the fusaric acid metabolite in tiger nuts and dates is reported for the first time in this study.

Notably, this study reports the contamination of tiger nuts and dates with Fusaric acid for the first time. Previous research suggests Fusaric acid's presence in plant-based foods may result from *Fusarium spp.* growth (López-Díaz et al., 2017; Oubraim et al., 2018). The absence of regulatory limits for unregulated toxins underscores the need for regulatory agencies to expedite action in setting standards for the toxins in foods.

5.0 Conclusion

In conclusion, the detection of AFB1, AFB2, and aflatoxigenic fungi in tiger nuts and dates from Lagos State markets suggests potential human exposure to aflatoxins through the consumption of these products. While most samples complied with EU and NAFDAC regulatory limits, indicating relatively safe consumption, the presence of fusaric acid in dry tiger nuts and dates raises concerns due to its potential for acute toxicity with continuous consumption. Given the lack of established maximum permissible limits for fusaric acid in food, moderate consumption of these nuts is advised to minimize toxicity risks. This study's findings provide valuable insights for policymakers, food industries, and regulatory agencies to inform decisions on health issues related to date and tiger nut consumption, processing, and utilization. The key implications of this research includes; Enhanced awareness of aflatoxin and fusaric acid contamination in tiger nuts and dates, the need for continuous monitoring and control measures to assure food safety and it also emphasized the importance of moderation in consuming these nuts to mitigate toxicity risks. This study contributes to the development of evidence-based guidelines for ensuring the safe consumption of tiger nuts and dates, ultimately protecting public health.

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