Mycotoxigenic Fungi Associated with *Cyperus esculentus* L. on retail in Lagos Metropolis and its Public Health Implications

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Abstract

The purpose of this study was to isolate and identify mycotoxigenic fungi associated with C. esculentus L. on retail in Lagos, Nigeria, in an attempt to protect the populace from mycotoxicosis and cancers that could emanate from its consumption. Fifty (50g) of both dry and fresh samples of tiger nuts were purchased by random sampling from three different markets in Lagos. These samples were ground into powder, and fungal isolation was carried out by spread plating of 100µl of serially diluted samples on Potato Dextrose Agar (PDA) containing 500mg/litre streptomycin. Molds and yeasts identified using molecular characterization with internally transcribed spacer regions ITS3F and ITS4R were Aspergillus niger, A. terreus, Penicillium guaibinense, P. citrinum, Candida sorboxylosa and Pichia kudraivzevii. These molds have been known to be mycotoxigenic and the yeasts pathogenic on mammals including humans. Hence, consumption of tiger nuts could be of public health concerns.

Key words: Mycotoxicosis, Public health, Tiger nuts, mycotoxigenic fungi, Lagos, Nigeria.

1.0 Introduction

Cyperus esculentus L. is a tuber that grows freely and is consumed widely in Nigeria and in various part of West and East Africa (Abaejoh et al, 2006; Ike et al, 2017). It is a perennial plant which has scaly rhizomes at its base that gives rise to hard spherical tuber, and consumed widely in the fresh uncooked state (Nyarko et al, 2011). *Cyperus esculentus* is commonly called tiger nuts (though not actually nuts), with its other names as Rush nuts, yellow nutsedge, Zulu nuts, chufa, water grass, earth almond and edible rush (Ire et al, 2020). In Nigeria, it is called ofio, aya, aki-hausa or imumu depending on the ethnic group. They are the size of chickpea but wrinkling with a chewy texture and sweet mitty flavour almost similar to coconut. *C. esculentus* was first cultivated in Egypt and traditionally used as both food and medicine (Alina Petre, 2022).

Cyperus esculentus has been recognized as one of the best nutritional crops used to augment diets because of its high calcium and iron content, necessary for body growth and development. As food, they are a good source of plant-based protein, carbohydrates, fats, fibre, and an array of minerals including phosphorus, magnesium, zinc, calcium and also vitamins C and E. It is a rich source of antioxidants which protects against aging and diseases such as cancer and heart diseases (Jing et al, 2013). Hence, they improve cardiovascular health by lowering Low-Density Lipoprotein (LDL) or bad cholesterol levels. The high antioxidant content help fight free radicals, consequently lowering the risk of chronic inflammation and certain cancers. *C. esculentus* also promotes digestion and gut health. Research has shown that germinating *C. esculentus* prior to eating them increases their antioxidants content (Badejo et al, 2014). It has also been reported that substantial consumption of *C. esculentus* reduced various cases of cardiovascular diseases, diabetes, cancers, and obesity (Ekeanyanwu & Ononogbu, 2010), and it is ideal for children, older persons and sportsmen (Martnez, 2003). Ade-Omowaye et al (2008) in their study suggested that tiger nuts could serve as alternative to cassava in the bread industry due to its inherent nutritional and therapeutic advantages. Tiger nut tubers have been used as raw material for the production of tiger nut milk drink which is rich in energy, fat, starch, glucose, fibre and protein (Maxwell et al, 2019) and is a preferred drink to sodas which are highly caffeinated and sugary. The milk is packaged in PET bottles and sold in shops and super markets, and is a great substitute for those who are lactose intolerant (Ike et al, 2017).

In Nigeria, tiger nuts are hawked on the streets usually in a wide open wheelbarrows and exposed to unhygienic environmental conditions. It is highly patronized due to its health benefits, especially by the male gender due to its perceived aphrodisiac property (Nyarko et al, 2011). The sellers (mostly of Hausa descent), use their bare hands to dispense the tiger nut into tiny polythene packaging bags, and these are bought ready-to-eat. According to Ibenyassine et al (2007), *C. esculentus* from farm to the point of sale are likely to be exposed to all forms of microbial contamination: contamination during storage, handling by street marketers, packaging and transport, and hence food safety is compromised. In Nigeria, these tubers are mostly eaten in a raw, fresh and uncooked state, there is no processing step to destroy any microorganism, which could result in public health concerns.

The objective of this study was to isolate, identify and characterize the types of fungi including mycotoxigenic fungi present in *C. esculentus*, on retail in Lagos Metropolis, Nigeria, in an attempt to protect the populace from mycotoxicosis and cancers that could emanate from its consumption.

2.0 Materials and methods

2.1 Study sites and sample collection

The study area of this research was limited to markets in Lagos Metropolis namely: Mushin market, Mile 12 market, and Berger markets in Lagos mainland. This is because tiger nuts tubers are mostly sold in these areas of Lagos, Nigeria. Lagos generally has a warm and humid weather throughout the year which presents favourable climatic conditions conducive for fungal growth and mycotoxin production on marketed and stored farm commodities.

Purposive sampling method was used for this study. It was purposive because the researcher purchased samples (both wet and dry tubers) directly from retail outlet (markets) where these tubers are dispensed with bare hands into polythene bags and sold to consumers directly. Random sampling followed within each market. Samples were bought from five traders randomly selected within a market and the samples were pooled per market. The samples were placed in sterile plastic bags and transported to Microbiology laboratory of McPherson University, Seriki – Sotayo, Ogun State for analysis.

2.2 Moisture analysis

The classical oven-dry method was used to determine the moisture content of the tiger nuts. Five (5) grams of ground samples of wet and dry *C. esculentus* tubers (in triplicates) were weighed to determine the initial weight and then dried in an oven at a temperature of 100^{o}C and weighed hourly until a constant weight was achieved (the final weight). Moisture content was determined by subtracting the final weight (oven dry weight) from the initial weight / initial weight x 100%.

2.3 Isolation of Fungi

Samples of dry and fresh *C. esculentus* were ground into powder using a grinder and 1 gram each was weighed into 10ml of sterile distilled water and mixed. Using a micropipette, 50ul and 100ul were transferred on to separate plates of already set Potato Dextrose Agar (PDA) containing Streptomycin (500mg/litre) in duplicates. Using bent glass rod, the inoculum was cultured using spread plating technique, allowed to dry and incubated at 25^{o}C for 5 days. Fungal colonies from plates were counted, sub-cultured using the 3-point culture technique on fresh PDA plates for purification of the isolates. The plates were incubated at 25^{o}C for 7 days for good sporulation to take place and then used for morphological identification. Pure cultures of the isolate were sub-cultured on cryovials containing PDA and incubated at 25^{o}C for 7 days, after which the pure cultures were covered with sterile distilled water and kept at 25^{o}C for molecular characterization.

The morphological identification of the isolate was based on the method described by Jedidi et al (2018). Molecular characterization and identification of the isolates was carried out using internally transcribed spacer regions (ITS3 and ITS4) of the ribosomal RNA gene, and the process included DNA extraction, Polymerase Chain Reaction (PCR) and DNA sequencing. Genomic DNA was extracted from the pure fungal isolates by using PrestoTM Soil DNA extraction kit (Geneaid, Taiwan) according to the manufacturer's protocol. The quantity and quality of DNA extracted was assessed on Nano drop spectrophotometer (ND-1000, Thermofisher Scientific) and 1.5% agarose gel electrophoresis. PCR was carried out using the general-purpose primers ITS3F (5'-GCATCGATGAAGAACGCAGC-3' and ITS4R (5'-TCCTCCGCTTATTGATATGC-3' (White et al, 1990). Analysis of the DNA sequencing for the identification of moulds and yeasts species using forward and reverse sequences combined to form a single contiguous sequence was done using BioEdit. Sequence identity was verified by blasting against NCBI database using Blastin (Zhang et al, 2000), as deployed on NCBI. Sequence analysis and blast tree was generated on NCBI, trimming of aligned sequences carried out on BioEdit and phylogenetic tree viewed on MEGA X.

2.4 Statistical Analysis of Data

All data were analyzed by general statistics and one-way ANOVA using the Statistical Product and Service Solutions (SPSS) software package 21.0.

3.0 Results and discussion

3.1 Moisture analysis and total fungal counts:

The results of moisture analysis of dry tiger nuts ranged from 12.5% - 13% while the moisture contents of fresh tiger nuts were from 45-46 % (Tables 1 & 2). There were predominantly moulds on the dry tiger nuts plates and a few suppressed yeast colonies from Berger, Ojodu samples while only yeasts were present in the fresh tiger nuts plates.

The total mould counts for the dry tubers ranged from 1.0 $x10^3$ cfu/ml – 1.4 x 10^4 cfu/ml while the total yeast counts for fresh, wet tubers were quite high and ranged from 1.9 $x10^6$ cfu/ml – 2.1 $x10^6$ cfu/ml (Tables 1 & 2).

Studies show that high moisture levels can increase fungal growth and deterioration in food (Barbosa-Canovas & Juliano, 2007). The moisture content of dry tiger nuts samples was between 12.5 - 13%. According to Likhayo et al (2018) food commodities are usually dried to lengthen shelf-life and to maintain good quality during storage. However, in spite of the low moisture content, fungal growth still occurred probably due to the high nutrients content which play important role in metabolism, in contrast to wet tiger nuts where high yeasts counts were observed, thus indicating the role of moisture in improving microbial metabolism, amongst other factors. In fresh tiger nuts with moisture content of 45-46 %, yeasts colonies prevailed. Yeasts grow best in foods with high moisture and high sugar contents which serve as source of carbon and energy for its growth. Yeast is usually commercially cultured on aerated suspension of molasses (sugar), in which the yeast uses oxygen to release the energy from the sugar in the process of respiration. The more sugar there is, the more active the yeasts will be and the faster they will grow. Therefore, the presence of high moisture content of fresh tubers, in addition to the sugar content inherent in the tubers served as good sources of metabolism for yeasts.

3.2 Morphological identification of fungal isolates

A total of 19 moulds were isolated from dry tiger nuts samples. Based on morphology of colonies, 10 of these were identified as *Aspergillus* spp., while 9 were identified as *Penicillium* species. On the contrary, twenty-two (22) yeasts colonies were isolated from the fresh tiger nuts samples. There were no moulds colonies. The morphological identification and numbers of isolates from wet and dry tiger nuts is shown in table 3.

3.3 Molecular identification of fungal isolates

Fungal species identified by molecular characterization in *C. esculensis* were *Aspergillus niger, Aspergillus terreus, Penicillium guaibinense, Penicillium citrinum, Candida sorboxylosa and Pichia kudriavzevii*. The phylogenetic tree for the isolates is shown in figure 1.

Aspergillus terreus is a fungus known for many applications in the biotechnology industry as producers of itaconic acid, and also a producer of a number of secondary metabolites such as the drugs lovastatin, terrain, asperfuranone and cyclosporine A used as immunosuppressant, anti-cholesterol, anti-cancer, and other bioactive compounds (Okabe et al 2009, Nadumane et al, 2016). *A. terreus* is also known to produce tremorganic mycotoxins which elicit either intermittent or sustained tremors in mammals (Evans and Gupta, 2018), hence of public health concerns.

Aspergillus niger is more prevalent in warmer climates both in the field and in stored foods. It possesses a bulk warehouse of prolific genes which are involve in regulation of primary and secondary metabolism (Pel et al, 2007). It produces ochratoxin A, fumonisin B2 and have been known to produce aflatoxins (Adewunmi et al, 2021; Noonimabc, et al, 2009; Al-Abdalall, 2009; Schuster et al, 2002; Abarca, et al, 1994) in stored grains. OTA causes nephrotoxicity and renal tumors in a variety of animals and is hazardous to human health through consumption. It is an opportunistic pathogen causing lung aspergillosis, liver and kidney disease and genital tract infections (Muntanola, 1987). It is a storage fungus posing serious threat to stored grains in the tropical warm weather. *A. niger* produces secondary metabolites such as oxalic acids, kojic acids, and cyclic pentapeptide with moderate to high acute toxicity. Oxalate crystals of oxalic acid causes pulmonary oxalosis (Nakagawa et al, 1999). Therefore, the consumption of tiger nuts containing *A. niger* could have public health implications.

Penicillium species are widely distributed in the environment and easily isolated from air and soil. Therefore, its presence in tiger nuts could be from soil during harvest or from handling by sellers. *Penicillium* are very diverse and cosmopolite fungi, about 350 species are recognized within the genus, and they produce various range of mycotoxins including Ochratoxin A (OTA) and Patulin for which regulation are imposed in a number of countries (Perrone & Susca, 2017). *Penicillium citrinum* produces citrinin, also a regulated mycotoxin. Other secondary metabolites produced to a lesser extent include cyclopiazonic acid, oxaline, griseofulvin, citreoviridin, pestalotin, quinolactacin A, curvularin, Agroclavine, Flavoclaucin, etc. The main risk of *Penicillium* is related to ingestion of food contaminated by mycotoxins produced by several species including *P. guaibenense* (*P. austrosinense*) and *P. citrinum* isolated from the tiger nuts samples. One of the regulated mycotoxin, OTA is very significant in food safety. The target organs for OTA toxicity in mammals are the kidneys and liver.

OTA has been reported as a potent renal carcinogen in rodents (Mally, 2012) and in poultry (Bondy et al, 2015), however, the epidemiological evidence in humans is very scarce according to Bui-Klimeke and Wu (2015). Lee & Ryu (2017) reported the maximum concentration and incidence of OTA in raw cereal grains to be 1,164ug/kg and 29% respectively. Therefore, the risk of OTA exposure cannot be completely avoided. The consequence of exposure to OTA include disruption of the gut microbiota homeostasis, teratogenicity, carcinogenicity, mutagenicity, hepatotoxicity (Liew & Mohd-Redzwan, 2018; Pfoh-Leszkowicz & Manderville, 2012; Zheng et al, 2013; Qi et al, 2014). Others are genotoxicity (Pfohl-Leszkowicz & Manderville, 2007), immunotoxicity (Marin & Taramu, 2015), embryotoxicity (Hong et al, 2000), developmental toxicity, neurotoxicity (Bhat et al, 2016),

testicular toxicity (Schwart, 2002), blood-brain barrier damage (Jackson & Ryu, 2017) and nephrotoxicity (Zhao et al, 2017).

Pichia kudriavzevii is a yeast fungus involved in beverage and food fermentations. It is a teleomorph of Candida krusei. It is considered an opportunistic pathogen which infects those with immunodeficiency and can lead to mastitis in mammals such as dogs and cattle (Hurst, 2016). The anamorph of *P. kudriavzevii* (*Candida* species) can cause sepsis with symptoms including fever, hypothermia, fast heart rate, swelling and high blood glucose levels, high white blood cell counts, low oxygen level, low urine output, high lactate in blood and a decrease in capillary filling (Nagarathnamma et al, 2017; Sepsis.org).

Conclusion:

The results in this study revealed high contamination of the *C. esculentus* samples with mycotoxigenic fungi capable of causing serious public health risks to consumers. Therefore, major stakeholders (the hawkers and the consumers) need to be educated on the inherent risks associated with the crude handling practices, and the best practices necessary such as thorough cleaning of tubers before consumption, washing and disinfection of hands before handling the tubers, in order to avert food safety issues that could lead to health risks.

Conflict of Interest

The authors declare no conflict of interest.

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Table 1: Total moulds counts and moisture content of dry *C. esculentus* L. tubers.

Sample code	Sample location	% Moisture	Total Moulds Counts	Total Yeast Counts
Dry *C. esculentus* tubers				
AD1	Mile 12, Lagos	13%	1.0×10^3	Nil
AD2	Berger, Ojodu, Lagos	13%	2.0×10^3	2.0×10^1
AD3	Mushin, Lagos	12.5%	1.4×10^4	Nil

Table 2: Total yeasts counts and moisture content of wet *C. esculentus* L. tubers.

Sample Code	Sample location	% Moisture	Total Yeasts counts	Total moulds count
WET *C. esculentus* tubers				
AW1	Mile 12, Lagos	46%	2.1×10^6	Nil
AW2	Berger, Ojodu, Lagos	45%	1.9×10^6	Nil
AW3	Mushin, Lagos	46%	2.0×10^6	Nil

Table 3: Morphological identification and numbers of fungal isolated from dry and fresh *C. esculentus*

Fungi	**No. of isolates from Fresh/wet *C. esculentus***	**No. of isolates from Dry *C. esculentus***	**Colony morphology on Potato Dextrose Agar**
Aspergillus spp.	-	10	Black fluffy colony
Penicillium spp.	-	9	Bluish-green with white margin
Yeasts	22	2	Small pale whitish colonies with characteristic fruity smell.
Total	22	21	

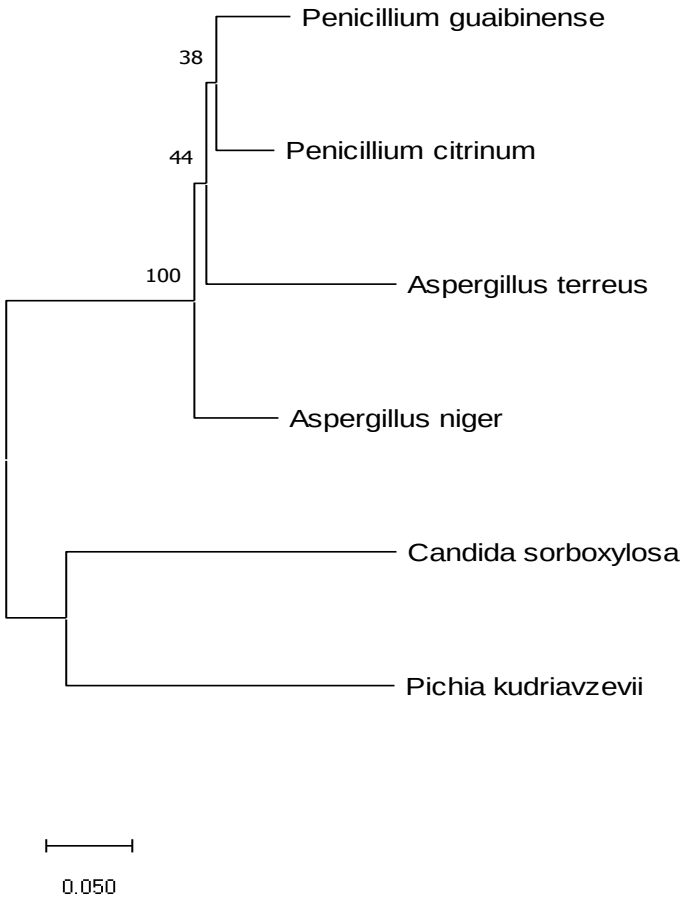

Fig.1: Phylogenetic tree for fungal species isolated from *C. esculensis L.*