EVALUATION OF TOXIGENIC FUNGI FOUND IN TIGER-NUT DRINKS SOLD IN LAGOS STATE

BY

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18/4883

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ABSTRACT

Tiger-nut drink is a refreshing drink prepared mainly with tiger-nuts (*Cyperus esculentus*), sugar and water. It is locally produced and consumed in Lagos State for its health benefits which could be compromised due to contamination, hence the need for evaluation. This study is aimed at the evaluation of toxigenic fungi in tiger-nut drinks sold in Lagos State. 10 (ten) branded and unbranded samples (six branded samples and four unbranded samples) of tiger-nut drinks were purchased in duplicates from different vendors in Lagos State (Oshodi and Ikorodu). Standard analytical methods were employed. Isolation of toxigenic fungi was carried out using spread plate agar method. The toxigenic fungi isolated were identified using morphological analysis and toxin extraction. The pH of the tiger-nut drink samples ranged from 4.60 to 5.14. On potato dextrose agar, the branded tiger-nut drinks stored at fridge temperature have the lowest colony counting while the highest colony counting was recorded in the branded tiger-nut drinks stored at room temperature. The isolates produced aflatoxin varying from 0.00000 to 22.48874 e^{-5}. The microorganisms identified were M*ucor spp.*, *Acremonium spp.*, *Aspergillus flavus* and *Aspergillus niger*. A high level of toxigenic fungi has been observed from this study and they are capable of posing health threats of food borne infections to the society. This truncates the nutritional value of the tiger-nut drinks, and points to either a production flaw during the processing stage, lack of good manufacturing practices or an exposure to contamination of the raw materials. The environments where tiger-nut drinks are being produced should be monitored, good manufacturing practices should be adhered to and storage conditions should be monitored. This will contribute to the reduction and eventual elimination of the high microbial contamination of tiger-nut drinks sold in Lagos State.

DECLARATION

I, UZAMA OSARUGUEMWEN PEACE, hereby declare that the project work titled **EVALUATION OF TOXIGENIC FUNGI FOUND IN TIGERNUT DRINKS SOLD IN LAGOS STATE** is a record of an original work done by me, as a result of my research effort carried out in the Department of Biological Sciences and Biotechnology, Caleb University, Imota, Lagos, Nigeria.

_____

Student's Signature & Date

CERTIFICATION

This is to certify that this research work was carried out by Uzama Osaruguemwen Peace with matric number 18/4883 in the Department of Biological sciences and Biotechnology, College of Pure and applied sciences, Caleb University Imota, Lagos, Nigeria.

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EXTERNAL EXAMINER **DATE**

DEDICATION

I dedicate this project to God, for never leaving me, just as He promised. I dedicate this project also to my parents, Mr Henry Uzama and Mrs Aituari Uzama, and to my siblings Esosa, Osaretin, Osaivbie and Osariemen for their endless support.

Lastly, I dedicate this project to my course-mates, HOD and lecturers, and the microbiology field at large.

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CHAPTER ONE

1.0 BACKGROUND STUDY

Tiger-nut (*Cyperus esculentus*) is an underutilized crop which belongs to the which belongs to the division *Magnoliophyta*, class *Liliopsida*, order *Cyperales* and family *Cyperaceae* (Uva *et al*., 1997). It grows as a tuber in Nigeria and in various other parts of West and East Africa. It possesses a rhizomatic radicular system. Small roots shoot out of this, forming tiger-nuts. Tiger-nuts are nutty, flavored and edible tubers which contain carbohydrates, sugars, protein, oil and fiber (FAO, 1988).

Tiger-nut is a grass-like perennial plant with spherical tubers and a pale-yellow cream kernel encased in a fibrous sheath. It is commonly known as chufa, Zulu nuts and chew-fa. It is also referred to as yellow nut grass and earth or ground almonds (Odoemelan, 2003; Belewu and Belewu, 2007).

Tiger-nuts grow wild along rivers and are grown mostly by rural farmers in the Northern part of Nigeria. It is locally known as Aya in Hausa, Ofio in Yoruba and Akiausa in Igbo, where three categories are cultivated- yellow, black and brown, although only yellow and brown tiger-nuts are readily available in the market. Because of its intrinsic features, such as its larger size, appealing color, and fleshier body, the yellow type is preferred to all others. The yellow type yields more milk when extracted, has less fat and more protein, and has fewer anti-nutritional elements, including polyphenols (Okafor *et al*., 2003).

As a result of its nutritional benefits, it has been discovered that tiger-nuts can be used as a substitute for cassava in the baking industry (Ade-Omowaye *et al*., 2008). It is often collected

and eaten raw as a snack, roasted, dried or ground to flour. Porridge, ice-cream, sherbet, and milky drinks can be made from ground flour mixed with sorghum (Ndubuisi L.C, 2009). Tiger-nuts can also be used to produce Tiger-nut oil; or be processed into a common beverage known as Tiger-nut Drink, or Horchata De Chufas.

Tiger-nut drink is a refreshing drink prepared mainly with tiger-nuts, sugar and water. It is rich in oleic acid, protein, fat, starch, glucose and energy unlike industrially-produced 'soft drinks' which could lead to type 2 diabetes and teeth enamel erosion because of toxic substances including benzene and phosphoric acid contained in most soft drinks. The tiger-nut milk is rich in nutrients but the high nutritive value of this milk is compromised due to the effects of some microorganisms on the milk (Abaejoh *et al*., 2006). Interestingly, tiger-nut drink could be beneficial in managing and controlling type 2 diabetes since it contains natural sugar. It protects the internal mechanisms and helps to prevent constipation and diarrhea.

Tiger-nut drink has a short shelf life which could be as a result of poor hygienic practices from the preparation to the storage which exposes the product to microbial contamination. Microbes are frequently introduced during the harvesting, processing, and storage processes (Kotun *et al*., 2017; Kotun and Odebode, 2019). It could also be linked to poor handling and dispensing conditions by the hawkers. The use of bare hands, dirty cups and contaminated water during washing of nuts and regular sprinkling to wet the nuts surfaces for freshness could also be responsible for the microbial contamination. Some of the different microbial species identified with tiger-nuts include *Aspergillus flavus*, *Aspergilius niger*, *Sacchromyces cerevisiae*., *Saccharomyes fubiligera Fusarium solani*, *Candida pseudotropicalis Bacillus subtilis*, and *Staphylococcus aureus*. Because a variety of species produce mycotoxins, it is critical to determine the mycotoxigenic status of these fungi species isolated from tiger-nuts. Examples that

produce poisonous secondary metabolite include *Aspergillus niveus, Aspergillus ochrceus, Aspergillus purpureus, Aspergillus terreus, Aspergillus ruber, Aspergillus oryzae, Penicillium camemberi.* (Kotun *et al*., 2017; Kotun and Odebode, 2019).

Based on a study carried out by Hiko A., [Hiko A, Muktar Y. 2020], no sample of commercially prepared tiger-nut beverage contained viable microorganisms but Escherichia coli, Bacillus spp., Shigella sp., yeasts and molds were isolated from home-made tiger-nut drinks.

1.1 Statement of the Problem

Hundreds of residents of Lagos State consume tiger-nut drinks from different locations where it is highly produced without efficient monitoring. The unmonitored production of tiger-nut drink brings about an uncertainty regarding the safeness of the drink for consumption and allegiance to food safety protocols during the production process which, if not followed, could lead to diseases or illnesses. As the people of Lagos State develop and show more interest in Tiger-nut drinks and its production for sale, the need to evaluate, identify and isolate toxigenic fungi found in tiger-nut drink is arising. It is now more necessary to examine the microbial safety of the drink to ensure the safety and health of the consumers. Therefore, this study was carried out for the evaluation and identification of toxigenic fungi found in tiger-nut drinks sold in Lagos state.

1.2 Research questions

This study will be guided by the following questions:

- What are the toxigenic fungi found in tiger-nut drinks sold in Lagos State?

- What are the frequency and occurrence of these microorganisms found in tiger-nut Samples sold in Lagos State?

1.3 Significance of the study

The result of this study still will be significant to other students and researchers as a reference material for further research on the subject matter.

1.4 Aim of the study

The aim of this study is to evaluate, isolate and identify toxigenic fungi found in Tiger-nut drinks sold in Lagos State.

1.5 Objectives of the study

The broad objective of this study is to measure the toxigenicity of tiger-nut drinks sold in Lagos. Specific objectives are as follows:

- To isolate toxigenic fungi found in tiger-nut drinks.
- To extract toxins from the pure fungi isolates and identify them.
- To characterize and identify isolates using morphological techniques.
- To determine how often these microorganisms are present in tiger-nut drinks.

CHAPTER TWO

2.0 History of Tiger-nuts

Tiger-nut (*Cyperus esculentus*) was first discovered 4000 years ago. The tubers were first cultivated in the Nile valley by ancient Egyptians. Their cultivation was then spread to other parts of the world with a temperate climate and fertile soil. According to reports, tiger-nuts arrived in Spain from Africa (IHS, 2005; HBR, 2005; CVNews, 2006; Deatra, 1999). Common names given to tiger-nut are yellow nut-grass, chufa, water grass, zulu nut and rush nut. plant was first initiated by the Arabs in the Valencia region.

Tiger-nut is actually a tuber and not a nut. The tiger-nuts acquire two forms: largueta (prolonged) and armela (rounded). At first, the root crop produces leaves like plants but as the days become shorter and cooler, leaf production will cease and tubers will be formed. High temperatures and low nitrogen levels increase tuber production. Tiger-nut tubers contain myristic acid as the main saturated acid and oleic acid as the predominant unsaturate present in the sample to the extent of 8.8-27.4% (Eteshola and Oraedu, 1996).

Tiger-nut is widely consumed in various forms including dried, baked, fried and roasted. As a food, tiger-nut can be soaked in water. It is used to make Kunu Aya, which is consumed in Northern Nigeria to quench thirst and as a source of energy and protein which is of high value, considering the amino acids present.

2.1 Botany of Tiger-nuts

Cyperus esculentus (tiger-nut / chufa sedge / yellow nut sedge / earth almond) is a highly adaptable crop and grows well under a wide range of climatic and soil conditions. It can be found

in the tropics, subtropics, and warm climates all over the world. It is grown in Western Africa, but it is a severe weed of cotton, grains, potatoes, and sisal in Eastern African countries. It is also grown in south America, Europe and Asia. The tuber grows 50- 250 tubers per plant and weigh 2 – 26 g per tuber (FAO, 1988). Tiger-nut is a perennial plant, which grows up to 90 cm tall.

The flowers of tiger-nut are distinct, having a cluster of flat oval seeds, surrounded by four degrees that are 90 degrees apart. The leaves are stiff and fibrous, giving it the appearance of a grass (Deatra, 1999).

2.2 Classification of Tiger-nuts

Tiger-nut (*Cyperus esculentus*) belongs to the Division–*Magnoliophyta*, Class–*Liliopsida*, Order– *Cyperales* and Family–*Cyperaceae*, Species- *Cyperus esculentus.*

Tiger-nut is classified in the division: *Magnoliophyta;* class: *Liliopsida*; order: *Cyperales*; family: *Cyperaceae*. in the order *Cyperales*, and the species *Cyperus esculentus*. The word esculentus means "edible" in Latin (Negbi, 1992). Although there are different tiger-nut varieties, the black and yellow varieties are the most common (Barminas *et al*., 2001). They are most common in their long and round sizes.

The varieties are:

- *Cyperus esculentus* var. *esculentus*.
- *Cyperus esculentus* var. *hermannii.*
- *Cyperus esculentus* var. *leptostachyus*.
- *Cyperus esculentus* var. *macrostachyus.*
- *Cyperus esculentus* var. *sativus*
- *Cyperus* esculentus var. *rotundus*

2.3 Nutritional composition of Tiger-nuts

Tiger-nuts are valued for their highly nutritive starch content (20-30% of DW), fibre, carbohydrate and are rich in sucrose (17.4-20.0%), fat (25.5%), protein (8.0%). They are also rich in sodium, calcium, phosphorus, potassium, magnesium, zinc, iron and traces of copper, energy content (starch, fat, sugar and protein), and vitamins E and C (Belewu *et al.,* 2007; Oladele *et al.,* 2007). Tiger-nut is high in phosphorus, potassium, and iron. Magnesium, calcium, zinc, copper, sodium, and manganese are also present (TTSL, 2005).

Because phosphorus in plants is usually bound to a compound called phytate, it is poorly absorbed from the gut into the body. The mineral substance of the bones and teeth is primarily composed of phosphorus (P) and calcium. It aids in the formation of ATP (an energy compound required for "activating" glucose, fatty acids, and other molecules) and the enhancement of cognitive performance. Phosphate is essential in the body. It acts as a buffer to help regulate acidity and alkalinity (Moore, 2004).

2.4 Nutrients per Serving

One ounce of raw tiger-nuts contains:

- Calories: 120
- Protein: 2 grams
- Fat: 7 grams
- Carbohydrates: 19 grams
- Fiber: 10 grams

- Sugar: 9 grams

When raw and when grounded, tiger-nuts contain vitamin C, B6, iron, calcium, phosphorus, potassium, magnesium and zinc.

A 200 ml glass of 'horchata' contains about 1.12% starch, 1.30% fat, 12.60%, protein; 0.35% carbohydrate, 0.38% fibre and 132 Cal energy value (TTSL, 2005).

2.5 Utilization of Tiger-nuts

Tiger-nut can be used in making tiger-nut drink, which is rich in vitamins, minerals, digestive enzymes (amylase, catalase, and lipase) as well as in phytochemicals and antinutritional factors (tannins, phytic acids, saponins and glycosides). Tiger-nut drink has been observed to be good in preventing arteriosclerosis, and tiger-nut drink could be beneficial in managing type 2 diabetes since it contains natural sugar. It can help prevent heart problems, and it can be consumed by lactose-intolerant individuals. The excesses after processing tiger-nuts can be used as in producing bio-ethanol. It is also reported to be aphrodisiac and carminative, promoting urine production and menstruation (Wills 1962). Tiger-nut is high in dietary fiber content, and could be effective in the treatment of colon cancer. According to Abodunrin and Belewu (2008), tiger-nuts are a great substitute for cereal grains. They are valued for their rich starch and mineral content, such as sodium, magnesium and traces copper (Abodunrin and Belewu, 2008).

Tiger-nut is an important food crop for some African tribes. Children frequently collect and consume it. Since ancient times, it has been cultivated and eaten raw or roasted, used as hog feed, or pressed for juice to make a beverage. Tiger-nuts are frequently used as an almond substitute or as a coffee and cocoa additive (FAO, 1988; NUTRA, 2005). Fresh tiger-nuts have been fermented to make a local alcoholic beverage (Barminas *et al*, 2001).

Tiger-nut oil is used as an alternative to olive oil. Tiger-nut oil is golden brown, possessing a rich nutty taste (TTSL, 2005). Tiger-nut oil is also an excellent ingredient in cosmetics. It has a high oleic acid content and is low in acidity, making it ideal for the skin. The industrial applications of tiger-nut oil include high-value cosmetics and instrument lubricants. Because it was extracted without the use of any external heat, the oil is tasty, stable, and of high quality (cold pressed oil). It is preferred over other cooking oils because it is more resistant to chemical decomposition at high temperatures.

Tiger-nuts make tasty farm family snacks and can be processed into fine, powdery flour and substituted for wheat flour in bread and other baked goods. Tiger-nut flour has a distinct sweet flavour that makes it ideal for use in the baking industry. It can be used to make cakes and cookies, as well as to complement fruit flavours. The flour is combined with sorghum to make porridge (TTSL, 2005). Tiger-nuts could be used in bread, cereals, and puddings. It could be added to rice, cassava, custard, pap, and couscous to make them have more flavour. Tiger-nuts and their extract could be combined with wheat flour and other local flours to make baked goods.

Yogurt can be made from coconut and tiger-nut milk, in combination with fresh cow milk, through fermentation with *Lactobacillus bulgaricus* and *Streptococcus thermophilus* starter cultures (Akoma *et al.*, 2000). Many researchers have suggested that tiger-nut could be used as a livestock feed (Wikipedia, 2005; Bamgbose *et al.*, 2003; ONRG, 2005).

Raw tiger-nuts can also be eaten as a snack, and can be processed into fine flour which could be used at an alternative to wheat flour in bread.

2.6 Health Benefits of Tiger-nuts

1. Tiger-nut is a good source of antioxidants, known to prevent heart attacks and improve blood circulation.
2. Tiger-nut is a rich source of dietary fibre. Tiger-nuts are made up of 30% of fibre, which aids in digestion, prevents constipation and helps constant bloating.
3. Tiger-nuts are one of the best non-artificial source of protein.
4. Tiger-nuts are a natural source of magnesium. Magnesium helps the immune system function optimally, and fights infections. Researchers found in a cell study that tiger-nut extract was effective against *salmonella* bacteria and *staphylococcus* in a petri dish environment. Tiger-nut extracts might also be effective against antibiotic-resistant bacterial infections. (Seukoup JA *et al*, 2013)
5. The insoluble fibre in tiger-nut drinks helps to regulate blood sugar levels by slowing down the digestion of sugar in the gut.
6. Tiger-nut drinks contain healthy fat (monounsaturated fats) and oil, which reduces the risks of heart diseases.
7. According to research, tiger-nut helps to fight prostrate cancer in men.
8. Tiger-nut drinks can be used as an alternative to food, considering the numerous health benefits and nutritional composition.
9. Tiger-nut drinks can be used as a worthy alternative to cow milk, suitable for lactose intolerant individuals.
10. Tiger-nut is highly composed of vitamins and minerals, especially vitamin E, C, potassium and phosphorus.

2.7 Processing Techniques

To modify its appearance, develop its natural flavor, destroy harmful microorganisms, improve nutritional quality, and prevent decomposition, various food processing techniques can be applied to tiger-nut processing. Tiger-nuts can be processed in a variety of ways including selection, washing, boiling and roasting, milling, blending, sieving, drying, soaking, fermentation, malting, packaging, storing, and preserving.

To achieve maximum quality, mature, healthy tubers should be selected. Tiger-nuts must be washed in clean water to remove sand and dirt, reducing microbial load. Heating causes protein coagulation and gelatinization of starch granules. When tiger-nuts are milled or blended, a large fraction is broken down into tiny particles (powder, flour, or fine paste). Drying is done primarily to reduce moisture content to the point where insufficient water remains to support the growth of microorganisms.

Tiger-nuts could be packaged, stored, and preserved at room temperature in airtight containers. To avoid rapid color changes, microbial growth, or spoilage, tiger-nuts should be frozen at constant and mild temperatures. Microorganisms found in food are classified into groups based on their preferred growth temperature, each group having an ideal temperature and a temperature range (Kotun, 2017; Kotun *et al*., 2017; Kotun and Odebode, 2019). They could be mesophilic (they grow at room temperature), thermophilic (they grow at high temperatures), psychrophilic (they grow at low temperatures), or psychotrophic (they thrive at low temperatures).

2.8 Microorganisms that can be found in Tiger-nut drinks

Due to the factors such as poor handling and unhygienic preparation area, some microorganisms have been commonly observed in tiger-nut drinks:

2.8.1 *Aspergillus*

Aspergillus is a large genus of molds that is found all over the world, with species involved in food spoilage, mycotoxin production, and fermentation (Encyclopedia of Food Sciences and Nutrition, Second Edition, 2003). *Aspergillus* belongs to the phylum *Ascomycota*, containing about 200 species. *Aspergillus* species are abundant in nature and have long been recognized as common contaminants in human food. The presence of *Aspergillus* in food is determined by the substrate and environmental factors including temperature, preservative presence, water activity and microbial competition. The most frequently occurring *Aspergillus* species are *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus flavus* and *Aspergillus fumigatus*.

Aspergillus flavus is a common soil fungus which grows on dead plant tissues in the soil. It is a facultative parasite, colonizing majorly corn, barley, wheat, rice, and peanuts. Aspergillosis infections have been found to be caused more often by *Aspergillus flavus*, and by *Aspergillus fumigatus*. *A. flavus* has been isolated from a vast range of food items including cocoa, dried meat products and sour lime (Hom B.W, 2007).

A. flavus produces secondary metabolites such as cyclopiazonic acid, aflatrem, aflavinin, aspergillic acid, neoaspergillic acid, paspalinine and Aflatoxins. Aflatoxins are extremely dangerous to both human and animal health.

2.8.2 *Saccharomyces*

Saccharomyces is a yeast genus in the *Saccharomycetaceae* family, phylum *Ascomycota*, and kingdom Fungi. The most common species is *Saccharomyces cerevisiae* and it is used in the food industry to make a variety of foods, wines, and beers. *Saccharomyces cerevisiae* is a yeast that is genetically tractable and is closely related to Candida albicans. As a result, Saccharomyces cerevisiae is a common model yeast in fungal molecular research, such as DNA sequence analysis (Cardinali G. *et al*, 2002), mechanism of action, antifungal drugs, and investigation of pathogenicity factors such as adhesion (Kanbe T. *et al,* 1998).

2.8.3 *Fusarium*

Fusarium is one of the most significant mycotoxigenic fungal genera in food and feed. Almost all species are capable of producing mycotoxins, many of which are subject to international regulation. *Fusarium* mycotoxins that are well-known include *fumonisins*, *zearalenone*, *deoxynivalenol*, and other *trichothecenes* (U. Thrane, 2014). *Fusarium* is one major cause of chickpea disease, and like *ascochyta*, it causes economic damage in many producing countries. *Fusarium* species infect crop plants all over the world in temperate climate zones. *Fusarium* head blight, a well-known crop disease caused by various *Fusarium* species, can result in yield loss and grain quality degradation. Infection of cereals such as barley, wheat, and maize is frequently accompanied by mycotoxin contamination and as a result, has a significant impact on human and animal diets. *Trichothecenes*, *zearalenone*, *fumonisins*, and *enniatins* are the four major groups of *fusarium* mycotoxins.

2.8.4 *Candida*

Candida normally lives on the skin and inside the body, in places like the mouth, throat, intestine, and vagina, without causing any problems. Some species of *Candida* causes infections in humans, but the most common and prevalent species is *Candida albicans*. *Candida albicans* is an opportunistic human fungal pathogen that causes candidiasis (M.A Kabir *et al*, 2012). It is found in the human digestive tract as part of the normal microflora. It is only one of about 200 species in the genus *Candida*, but it accounts for up to 75% of all candidal infections. These infections can be superficial, affecting only the skin or mucous membranes, or they can invade the bloodstream and spread to internal organs.

Surgery (especially abdominal surgery), burns, long-term stay in an intensive care unit, and previous administration of broad-spectrum antibiotics and immunosuppressive agents are all risk factors for invasive candidiasis (Kontoyiannis DP, 2003).

Commercial antifungal agents used to treat candidiasis include polyenes, fluoropyrimidines, echinocandins, and azoles. However, the presence of both intrinsic and acquired resistance to azole antifungals has been well observed in certain *Candida* species. *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, and *Candida krusei* are also found in healthy people (MacCallum DM., 2012). These five species are responsible for more than 90% of invasive infections, though the relative prevalence of the species varies depending on geographical location, patient population, and clinical settings (Miceli MH *et al*, 2011).

2.9 Traditional Preparation of Tiger-nut drink

Commonly, tiger-nut drink is prepared by soaking washed tiger-nuts in water for 2-8 hrs. After soaking, it is ground for about 10 minutes into a fine paste and mixed with water in the ratio of 3litres of water for 1 kg of tiger-nuts. The mixture is allowed to sit for some time. Afterwards, sugar is added and it is sieved for the pure drink. Larger nuts are sweeter, while the smaller nuts produce more milk. Tiger-nut drink is nutritious and can be consumed by both old and young people. However, the quality can be compromised due to poor production or inappropriate handling of materials used to prepare the drink. It can also be compromised by the hygienic state of the environment in which it is prepared.

2.10 Factors affecting nutritional quality of tiger-nut drinks

1. Hygienic handling is important when processing tiger-nuts to avoid tissue invasion which may contribute to microbial fungal contamination.

2. Preservation of tiger-nuts can affect the quality of tiger-nut drinks. Fresh tiger-nuts can be kept in water changed daily for up to 10 days.

3. Temperature: Under warm conditions, tiger-nuts ferment rapidly. This is because of naturally present microorganisms growing on the tubers (FAO, 1988).

4. Water activity

5. Shelf life of tiger-nuts

CHAPTER 3

3.0 Materials

The materials that were used include 10 samples of tiger-nut drinks, Potato dextrose agar, Malt Extract Agar, streptomycin, Incubator, lactophenol blue, Filter paper, Electronic weighing balance, Test tubes, Petri dishes, Autoclave, Water bath, Tiger-nuts, Sugar, Sterile syringes, Glass slides, Coverslips, Microscope, Paper tape, Ethanol, Inoculating loop, Bunsen burner, Measuring cylinder, pH meter, refrigerator, forceps, foil paper, universal bottle, cotton wool, nose mask, conical flask, cryovial tube, beaker, spatula, durham tubes,marker, masking tape, MacCartney bottle, marker and Distilled water.

Major Equipment and Apparatus

- Incubator: It is used to create artificial growth environment for microbes.
- Weighing balance: A weighing balance is used to determine the weight or mass of agar, to ensure accurate measurement.
- Test tube: It is used for the transfer of small volumes (5–10 cm3) of liquid media/agar for inoculation (held in test tube rack; dry non-absorbent cotton wool plug or plastic cap prevents contamination).
- Autoclave: This is used in the Sterilization of media, solutions and equipment before use and contaminated items afterwards. It is also used for melting solidified agar media for use.
- Glass slides: A microscope slide is a thin piece of glass, typically 75 by 26 mm (3 by 1 inches) and about 1 mm thick, used to hold objects for examination under a microscope.

- Cover slip: It protects the microscope and prevents the slide from drying out when it's being examined.
- Ethanol: It is used for killing harmful organisms that could be on surfaces.
- Microscope: It is used to examine microorganisms to identify them and their morphological characteristics.
- Inoculating loop: It is a simple tool used to pick up and transfer a small sample (inoculum) from a culture of microorganisms and used to streak on plates.

3.1 Methods

3.1.1 Study area

This study was carried out in 7 (seven) locations in Lagos State namely Igbogbo, Kairo market, Oshodi, Mile 12, Bolade market, Obafemi Awolowo Road and Iyano Brown.

3.1.2 Sample Collection and Treatment

Ten (10) freshly prepared/packaged (branded and unbranded) tiger-nut drinks were randomly purchased in duplicates from vendors in seven (7) locations within two local governments in Lagos State. Each set of samples collected from the vendors was kept in a sterile container, labeled respectively and taken to the laboratory for analysis. Ten samples were stored at room temperature and the other 10 were stored in a refrigerator for 3 days. The seven locations were assigned different codes TA and TD, TB and TE, TC and TF, T1, T2, T3 and T4. Samples TA and TD were different branded tiger-nut drinks gotten from Igbogbo market, Samples TB and TE were different branded tiger-nut drinks gotten from Obafemi Awolowo Road, and Samples TC and TF were different branded tiger-nut drinks gotten from Kairo market. Sample T1 was an

unbranded tiger-nut drink gotten from Bolade market, Sample T2 was an unbranded tiger-nut drink gotten from Oshodi Market, Sample T3 was an unbranded tiger-nut drink gotten from Iyano Brown and Sample T4 was an unbranded tiger-nut drink gotten from Mile 12 market, making 20 (twenty) samples in total. A control sample was prepared in the laboratory using dry tiger-nuts, which were bought from the market and transported to the laboratory in a clean polythene bag for analysis.

3.1.3 Preparation of the laboratory Tiger-nut drink control sample

About 2kg of dried tiger-nuts was weighed and sieved to remove stones and other foreign materials which may alter the taste and quality of the extracted drink before being rinsed in water to remove sand. Afterwards, it was washed in sterile distilled water to remove sand. It was then boiled for 15 minutes and sieved. It was grinded with 4 litres of potable water for 10 minutes into a fine paste using a clean grinding machine. 2 litres of water was then added to the milled tiger-nut. After that, the slurry sample was filtered using a clean muslin cloth until no extract was recovered. 120g of Sugar was added to the 6 litres of water. 2.4g of potassium sorbate (preservative), 3.0g of ascorbic acid and 2 drops of milk was added (due to the fact that the samples obtained were sweetened and treated with preservative). After that, it was boiled again while stirring for about 15 minutes. The filtrate was divided into two portions: one stored at room temperature for three days and one stored at refrigerating temperature, making 2 different treatments.

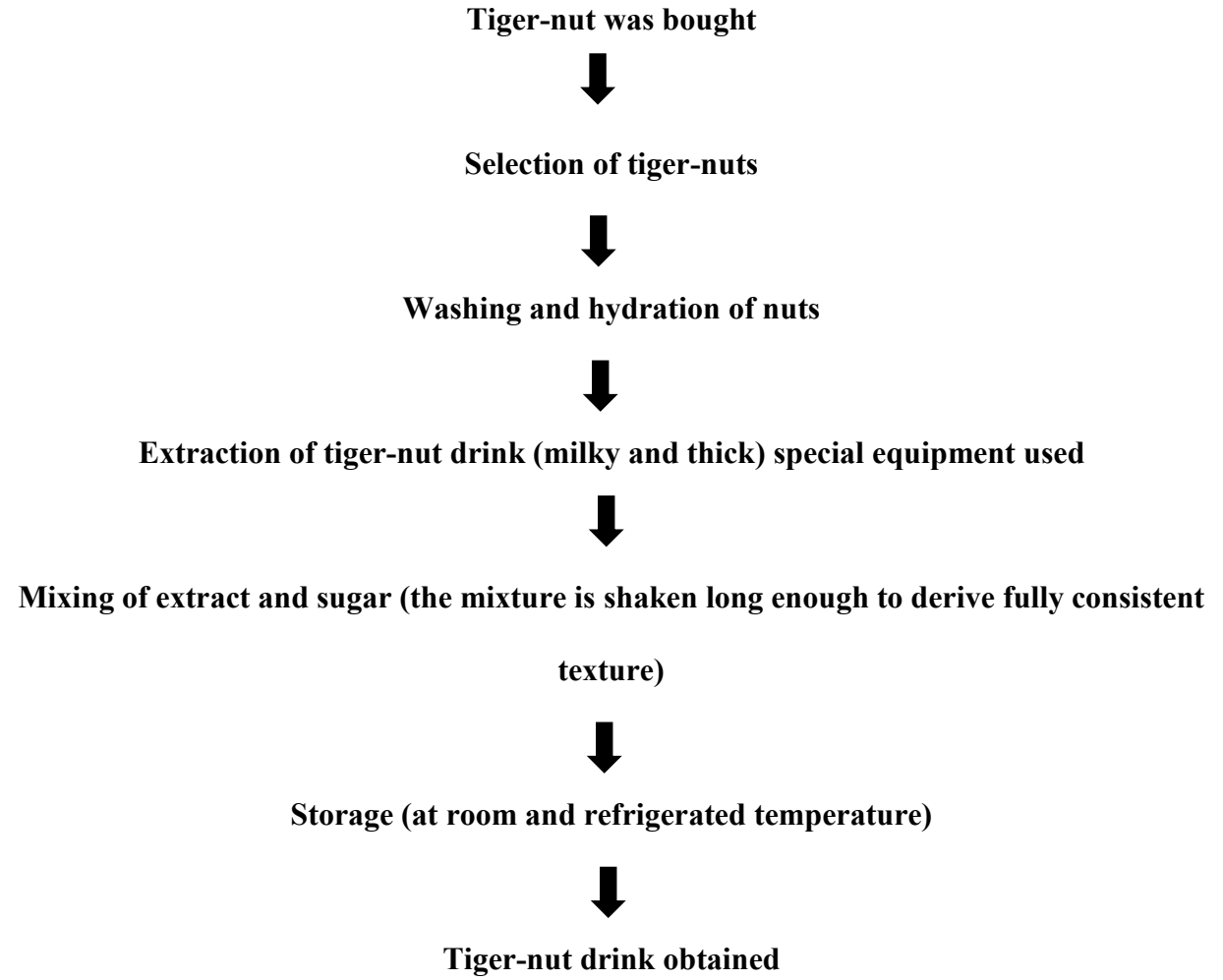

Figure 3.1: Flow chart of local preparation of tiger-nut drink

3.1.4 Determination of PH of the Tiger-nut drinks

The PH of each tiger-nut drink was determined directly using a PH meter at room temperature. About 10ml of each sample was placed in the measuring cup of the pH meter and then the measuring arm lowered into the cup to determine the pH. The measurements were taken and recorded.

3.1.5 Serial Dilution

Three-fold serial dilution was carried out by transferring 1 ml of each sample of tiger-nut drink into a test tube containing 9 ml of sterile distilled water using sterile syringes and the tube was mixed and labeled. 1 ml of dilution 10^{-1} was then transferred into another test tube (10^{-2}) containing 9 ml of distilled water. Using separate 1 ml pipette, the transfers were repeated until dilution 10^{-3} was achieved. The dilution was carried out on the samples of tiger-nut drink stored in the refrigerator and under room temperature.

3.1.6 Preparation of Culture Media

The fungal growth media was prepared according to the manufacturer's instructions and heated for sterilization. Streptomycin was be added to inhibit the growth of bacteria.

3.1.7 Culture Media for Growing Fungi

An aliquot (0.1 ml) of the dilutions of the tiger-nut drink samples was transferred aseptically into freshly prepared potato dextrose agar in triplicates in well labeled Petri dishes and incubated at room temperature (28 ±2°C) for 5 days.

3.1.8 Sub-Culturing

All plates that visible mold growth was observed were sub cultured on Malt Extract Agar to obtain pure isolates. They were incubated at 30°C for 3-5 days, after which the cultured plates were identified morphologically and microscopically using wet mount.

3.1.9 Identification of Fungal Isolates from Tiger-nut drinks

On a clean microscope slide, a drop of ethanol (70%) was dropped, and material from cultures of filamentous fungi was picked using a rigid inoculating wire and a small amount of the culture was removed. The wire was flamed by holding it upright in the hottest part of the Bunsen flame. The fungal material was immersed in the ethanol and the material was released very gently. About two drops of lacto-phenol in cotton blue was added, and the cover-slip was gently placed on the slide, avoiding bubbles.

3.1.10 Enumeration of moulds and yeast on the Tiger-nut samples

The yeasts and molds count on the tiger-nut drink samples was determined using pour plate method. 0.1 ml of each dilution was ascetically plated into potato Dextrose agar and Malt extract agar. The Agar plates were incubated invertedly at 28°C for 3-5 days. Colonies that grew on the medium were counted and expressed as colony forming units (cfu) per millimeter of tiger-nut drink sample. Numbers of colony forming units were calculated as follows:

$$\text{cfu g-1} = \frac{\text{count x 1/ dilution}}{\text{inoculum}}$$

3.1.11 Aflatoxin Determination by GC-MS

Equipment and materials: Volumetric flasks (100, 250ml) and beenLom, graduated pipette (1, 5, 10 ml), analytical balance, dark brown sample bottles 10ml, disposable nose mask, hand gloves and pasteur pipette, 5ml plain sample bottles, 5ml needles and syringe, vortex mixer,

centurim centrifuge and HPLC1100 Agilent series manual injection with quaternary pump and thermostatic column compact.

Chromatographic condition: The mobile plane comprises of a Methanol:Water:Acetonitrite (40:50:10 %) composition. The column used was Zorbay SB-C8 Extended 4.6 X 150mm summed at a flow rate of 0.0500ml/mms^{-}. The ultraviolent-visible detector was set at 365nm.

Sample preparation: 1ml of the sample was taken with the aid of a disposable Pasteur pipette and 3ml of Acetonitrite was added. The solution shaken and vortexed for 2 minutes to deprotemize the sample. It was centrifuged for 5minutes at 5,000rpm. The supernatant was filtered using micro Millipore filter of 0.45µm particle seize. After equilibrating the column for about 50minutes, 20ml of each sample was individually injected manually and the peak areas recorded and integrated by enhanced integrator. The amount of aflatoxin present in the sample was calculated on the printout.

CHAPTER 4

4.0 Results

The pH values of tiger-nut samples ranged from 4.66 – 5.14. The results are expressed in Table 4.1.

The result of Colony counting of fungi in tiger-nut samples at Room temperature and fridge temperature in each PDA. On PDA, the branded tiger-nut drinks stored at fridge temperature have the lowest colony counting while the highest colony counting was recorded in the branded tiger-nut drinks stored at room temperature. The results are recorded in Table 4.2 and 4.3.

The unbranded tiger-nut drinks stored at fridge temperature have the lowest colony counting while the highest colony counting was recorded in the branded tiger-nut drinks stored at room temperature. The results are recorded in Table 4.4 and 4.5.

Four species of fungi were isolated from both branded and unbranded tiger-nut drinks. The isolates were identified and characterized based on morphology and microscopy. The fungi species are identified as *Aspergillus flavus, Acremonium sp, Mucor spp.,* and *Aspergillus niger* and are characterized in Table 4.6.

The isolates produced different volumes of aflatoxin which varies from 0.00000 to 22.48874 e^{-5} . The results are expressed in the Table 4.7.

Acremonium spp. has the highest frequency, followed by *Aspergillus niger. Mucor spp. and Aspergillus flavus* have the lowest occurrence as expressed in Figure 4.1.

Table 4.1: pH values

S/N	Sample Code	Area obtained	pH value
1	TA	Igbogbo	4.96
2	TB	Obafemi Awolowo Road	4.90
3	TC	Kairo market	4.98
4	TD	Igbogbo	5.14
5	TE	Obafemi Awolowo Road	5.04
6	TF	Kairo market	4.73
7	T1	Bolade market	4.66
8	T2	Iyano Brown market	4.81
9	T3	Oshodi	4.92
10	T4	Mile 12	5.02
11	PD	Control sample	4.78

*Key: Sample code 1-4 represent unbranded tiger-nut drink samples, and sample code A-F represent branded samples. Sample PD represents control samples.

Table 4.2: Frequency of colonies on the Potato Dextrose Agar plates (fridge samples)

Sample Code (fridge samples)	Molds count	Yeast count	Number of colonies obtained. CFU/100µL
PDA	12	60	72
TF1	2	5	7
TF2	4	6	10
TF3	5	31	36
TF4	8	20	28
TFA	4	25	29
TFB	4	7	11
TFC	2	4	6
TFD	1	2	3
TFE	1	1	2
TFF	4	4	8

*Key: Sample code 1-4 represent unbranded tiger-nut drink samples, and sample code A-F represent branded samples. Sample PD represents control samples.

Table 4.3: Frequency of colonies on the Potato Dextrose Agar plates (room samples)

Sample Code (Room Samples)	Molds Count	Yeast Count	Number of colonies obtained. CFU/100µL
PD2	28	120	128
TR1	30	178	208
TR2	14	44	58
TR3	10	30	40
TR4	9	31	40
TRA	29	111	140
TRB	38	102	140
TRC	7	25	32
TRD	29	85	114
TRE	8	40	48
TRF	9	40	49

*Key: Sample code 1-4 represent unbranded tiger-nut drink samples, and sample code A-F represent branded samples. Sample PD represents control samples.

Table 4.4: Frequency of colonies on the Malt Extract Agar plates (fridge samples)

Sample Code (fridge Samples)	Molds Count	Yeast Count	Number of colonies obtained. CFU/100µL
PDA	13	117	130
TF1	6	20	26
TF2	2	4	6
TF3	5	31	36
TF4	8	19	27
TFA	9	33	42
TFB	6	20	20
TFC	10	19	29
TFD	14	30	44
TFE	1	2	3
TFF	10	20	30

*Key: Sample code 1-4 represent unbranded tiger-nut drink samples, and sample code A-F represent branded samples. Sample PD represents control samples.

Table 4.5: Frequency of colonies on the Malt Extract Agar plates (room samples)

Sample Code (room Samples)	Molds Count	Yeast Count	Number of colonies obtained. CFU/100µL
PD2	33	159	192
TR1	52	100	152
TR2	6	50	56
TR3	8	32	40
TR4	14	33	47
TRA	11	49	60
TRB	13	123	136
TRC	9	55	64
TRD	52	100	152
TRE	20	40	80
TRF	6	50	56

*Key: Sample code 1-4 represent unbranded tiger-nut drink samples, and sample code A-F represent branded samples. Sample PD represents control samples.

Table 4.6: Identification and Characterization of Fungal Isolates

Isolate code	Color on Potato Dextrose Agar	Shape	PRESUMPTIVE IDENTITY
TFF	**White, reverse orange**	**Flat**	***Acremonium spp.***
TF1	**White, reverse orange**	**Flat, round**	***Acremonium spp***
TF4	**Yellow at first, becoming greenish-yellow afterwards**	**Flat**	***Aspergillus flavus***
TR1	**White at first, then frequently developing from dark brown to black**	**Flat at border and raised in the middle**	***Aspergillus niger***
TFA	**White at first, then frequently developing from dark brown to black**	**Flat at border and raised in the middle**	***Aspergillus niger***
TFC	**White, reverse orange**	**Flat, Radially spherical**	***Acremonium spp***
PD2	**White at first, then dull brown and finally brownish-black**	**Round**	***Mucor spp.***

*Key: Sample code 1-4 represent unbranded tiger-nut drink samples, and sample code A-F represent branded samples. Sample PD represents control samples.

Table 4.7: Aflatoxin Production from Fungal Isolates

Sample code	Aflatoxin AfB1	Aflatoxin AfB2	Aflatoxin AfG1	Aflatoxin AfG2	Total produced
Mould TFF	—	—	$0.00827e^{-3}$	___	$0.00827e^{-3}$
Mould TF1	—	—	$0.00136e^{-3}$		$0.00136\ e^{-3}$
Mould TF4	$22.48874\ e^{-5}$	—	___	___	$22.48874\ e^{-5}$
Mould TFA	___	—	—	$0.00086\ e^{-5}$	$0.00086\ e^{-5}$
Mould TR1	—	—	___	$0.00094\ e^{-4}$	$0.00094\ e^{-4}$
Mould TFC	___	___	$0.02934\ e^{-4}$	—	$0.02934\ e^{5}$
Mould PD2	—	—	___	—	0.00000

*Key: Sample code 1-4 represent unbranded tiger-nut drink samples, and sample code A-F represent branded samples. Sample PD represents control samples.

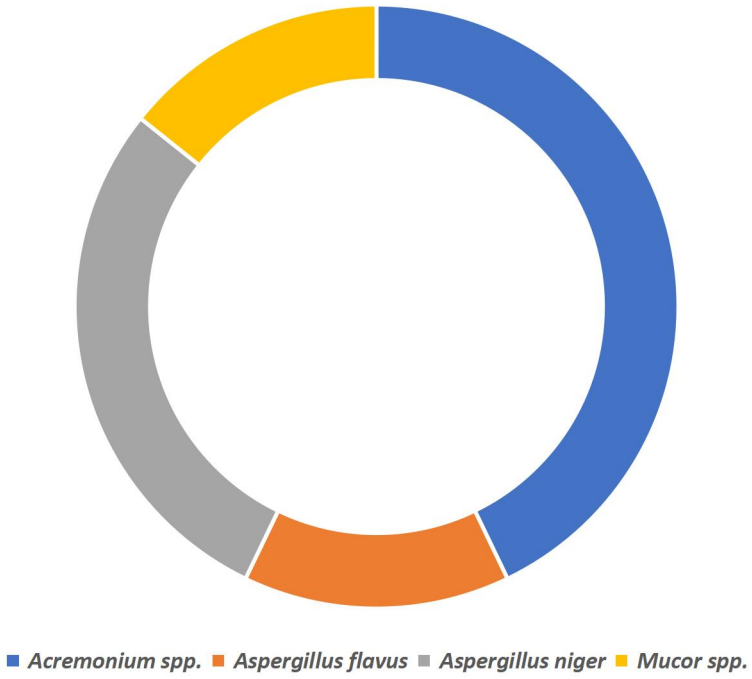

Figure 4.1: Frequency of occurrence of isolates

CHAPTER FIVE

5.0 Discussion and Conclusion

This study on Evaluation of toxigenic Fungi found in Tiger-Nut Drinks sold in Lagos State provides information on the nutritional value, factors affecting quality of tiger-nut drinks, utilization of tiger-nuts and health benefits of tiger-nuts. Tiger-nuts are rich in energy giving nutrients and preventive or protective nutrients (fiber, iron, copper, zinc, vitamins C and E).

The results obtained from this study shows that the samples were heavily contaminated with mycotoxigenic fungi, posing a threat to public health, according to a study by Kotun and Odebode, 2019. This could be as a result of poor hygienic practices from the preparation to the storage, exposing the tiger-nut drinks to contamination. The detection of fungi species such as *Aspergillus flavus, Acremonium spp.* and *Aspergillus niger* in the isolated tiger-nut samples indicates contamination that could be due to poor handling and packaging, use of contaminated water, dirty cups and bare hands during processing stages. According to a study by Kotun *et al (*2017), microbes are frequently introduced during the harvesting, processing, and storage processes.

The pH observed for both brand and unbranded tiger-nut drink samples were within the range of data previously reported (Ndubuisi, 2009). The results obtained from this study of the evaluation of toxigenic fungi found in tiger-nut drinks sold in Lagos, showed unacceptable levels of fungi, as observed in the volume of aflatoxin produced by the tiger-nut samples.

Tiger-nuts and its products could bring many benefits to people (young and old) in developing countries by playing important roles in providing food security, enhancing livelihoods, improving nutritional status and social well being of vulnerable groups. However, a high level of

toxigenic fungi has been observed from this study, and they are capable of posing health threats of food borne infections to the society. This truncates the nutritional value of the tiger-nut drinks, and points to either a production flaw during the processing stage, lack of good manufacturing practices or an exposure to contamination of the raw materials, according to Kotun *et al* (2017).

As a result, the environments where tiger-nut drinks are being produced should be monitored, good manufacturing practices should be adhered to and storage conditions should be monitored. This will contribute to the reduction and eventual elimination of the high microbial contamination of tiger-nut drinks sold in Lagos State.

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LIST OF TABLES AND FIGURES