EVALUATION OF FUNGI ASSOCIATED WITH GROUNDNUT CAKE (*KULIKULI*) SAMPLES FROM SELECTED NORTHERN NIGERIAN STATES AND THEIR CONTROL WITH SOME BOTANICALS

BY

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CERTIFICATION

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DEDICATION

I dedicate this research work to my able God for sustaining me to complete this project despite all odds. Also, to the memory of my late father, Prof. Badamasi Mustapha who gave me educational foundation to be who I am today. Your memories still linger in my heart, but there is a time to live and leave. I miss you, Dad! Rest on until that day when we shall all rise again, when death's prison bars are broken.

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ABSTRACT

Groundnut cake (*Kulikuli*) is a common snack in Nigeria prepared from groundnut (*Arachis hypogaea* L.). Food contamination by aflatoxin-producing fungi remains a major health challenge in Nigeria. Fungal contamination in *Kulikuli* occurs during production, handling, storage and transportation. However, research on *Kulikuli* has focused on microbial characterisation and nutritional value. There is dearth of information on fungal infestation, mycotoxin production in *Kulikuli* and their control with botanicals having anti-fungal properties. Therefore, this study was aimed at evaluating fungi associated with *Kulikuli* production, aflatoxin secretion and their control using selected botanicals.

Twenty five *Kulikuli* samples each were purchased from selected markets with *Kulikuli*production centres in Kano (Rimin Gado, Janguza, Kurmi, Sabongari, Rimi); Kaduna (Bacci, Kaduna Central, Kawo, Sabuwar Gari, Kakuri); Sokoto (Kanawa, Ilaela, Dange Shuni, Jabo, Yan tumatir); Zamfara (Bungudu, Tsohuwar Kasuwa, Dampa Sabuwar Kasuwa, Tudun Wada) and Abuja (Utako, Wuse, Madalla, Dutse, Bwari). Fungi associated with the *Kulikuli* samples were isolated and identified using morphological and molecular characterisations. *Kulikuli* with botanicals (MLB) and without botanicals (ML) were also prepared at the Mycology Laboratory Department of Botany as controls. The botanicals used were garlic, ginger and turmeric.Proximate analysis and aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) contents of the samples were determined using standard procedures. Ten Simple Sequence Repeat primers were used for aflatoxin genes (*AT, AN, AP, AFUM* and *FUS*) in each isolated fungus. Data obtained were subjected to descriptive statistics and Analysis of Variance (ANOVA) at $\alpha_{0.05}$.

One hundred and six fungi were isolated and identified as *Aspergillusflavus* (n=28), *A. parasiticus* (n=26), *A. niger* (n=7), *A. tamarii* (n=9), *A. fumigatus* (n=3), *Penicillium oxalicum* (n=11), *P. chrysogenum* (n=6), *Fusarium oxysporum* (n=10)and *F.compaticum* (n=6). All fungi isolated from market samples, except *A. fumigatus* and *P. oxalicum* were also isolated from *Kulikuli* samples prepared in ML without botanicals. *Kulikuli* samples with botanicals had no fungal incidence. *Kulikuli* samples from the markets compared with MLB and ML samples were significantly different in moisture content (11.91, 6.64, and 12.64%), ash (3.91, 4.84, and 4.84%), crude protein (49.23, 59.23, and 50.25%), fibre (3.20, 4.55, and 4.05%), fat (4.83, 3.26, and 3.83%) and pH (6.30, 6.88, and 6.84), respectively. Market samples had the highest AFB_1, AFB_2, AFG_1 and AFG_2 of 1.64, 2.10, 0.10 and 0.13 µg/kg, respectively, while samples treated with garlic had the least AFB_1 (0.34µg/kg), AFB_2 (0.37µg/kg), AFG_1 (0.01µg/kg) and AFG_2 (0.02µg/kg). Aflatoxin concentrations in MLB and ML samples were within tolerance limits of NAFDAC and European Union 4 µg/kg. The *aflR, nor*, *ver* and *omt* biosynthetic pathway genes found in aflatoxin producing fungi were detected in all fungi isolated from the purchased *Kulikuli* except strains from Kaduna (Kaduna Central) samples. However, the MLB samples yielded no fungal growth.

Kulikuli samples from the studied areas were infested with nine fungi species. *Aspergillus spp* were the dominant fungi found to be most prevalent in the market samples. The addition of garlic during production of *Kulikuli*best suppressed fungal infestation and reduced aflatoxin levels.

Keywords: *Kulikuli*, *Arachis hypogaea*, Aflatoxins, Botanicals.

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CHAPTER 1
INTRODUCTION

1. 1. The study background

Groundnut is a leguminous species with seeds that grow underground. Taxonomically, groundnut is classified as *Arachishypogaea* Linn.It is widely cultivated in Asia, Africa and United States. It is known to be a crop rich in numerous macro and micro nutrients with its seeds containing oil, protein, magnesium, phosphorus, manganese as well as vital vitamins such as niacin, lysin, B-complex (Ntare *et al*., 2008). Groundnut according to Ntare *et al*. (2008) generates about 21 % rural cash earnings in Nigeria. This oil seed is a cheap protein source, which contains oil perfect for animal feed formulation and cash making in West Africa countries including Nigeria.

Groundnut is very important in Nigeria and it is often processed into many local foods among the African populace.Itcan be eaten raw, cookedreferred to “Epa”, “Daffafiyarar”, gyadar” or processed into cake groundnut cake (*Kulikuli*), roasted cookies (Epa yiyan, marao marao, or tupus) or groundnut ball. These are often seen hawked across the streets or roadsides both in rural or urban areas of Nigeria. One of the most prominent streets vended foods in Nigeria is Groundnut cake commonly called ‘*Kulikuli*’. *Kulikuli* is mainly produced in Northern States such as Kano, Sokoto, Kaduna, Abuja, and Katsina.

The production of *Kulikuli*is through milling, moulding and frying of groundnut with residual crude oil extracted from them (Adebesin *et al*., 2001). Just like the parent substrate material, *Kulikuli* as a groundnut product has been analysed and reportedly rich in crude fat and protein (Adebesin *et al*., 2001). It is widely consumed raw as a snack, with mixture of garri (cassava flour) and water or as a supplement with other local foods such as Koko, Fura, or Kamu. Among the local Hausa tribe in Nigeria, *Kulikuli* is also further processed

by grinding it as ingredient for local cuisines such as Gurasa, Suya, Dambu, Kwaddo and Kilishi.

Kulikuli is a local convenience consumed across Sub-Saharan Africa. However, there is dearth of data and information on the microbial interference and mycotoxin contamination in the locally hawked *Kulikuli* in Nigeria as well as other countries in which *Kulikuli* are popularly consumed. It may also be due to the fact that it is extensively consumed in rural areas and among the low income or people below middle class, therefore, *Kulikuli* is not regarded as a major food that is worthy of scholarly attention. However, because*Kulikuli* is hawked and sold massively in different markets across Nigeria,there is an urgent needto evaluate its quality and microbial value continually as failure to do this may result in mass food poisoning injurious to the human health.

However, groundnut as well as their product foods are commonly prone to fungal deterioration and mycotoxin (fungal toxin) contamination. This may be as a result of the numerous nutrients it contains or as a result of unhygienic handlings and prolong storage time. Mycotoxins as fungal toxins are of different types such as Aflatoxins, Fumonisin, Deoxynivalenol (DON), Ochratoxins, Patulins, Coumarins, Zearalenols, Trichothecenes, Fusaric Acids, Fumagillin, and several others. However, Aflatoxins (AFs) are the most popular and common ones in Nigeria. AFs involve toxins, which are produced as extracellular metabolised by some certain fungi such as *Aspergillus*, especially *Aspergillus flavus* and*Aspergillus parasiticus.*

Aflatoxins (AFs) was the major mycotoxin threat on the food products in many developing countries especially in West Africa sub region (Bankole and Adebanjo, 2003). AFs are of different types; major examples include the aflatoxins B (AFB_1 and AFB_2), G (AFG_1 and AFG_2) and M (AFM_1 and AFM_2). Other types are AFD_1, AFRM, AFRo, and so on. AFs have been widely reported on many foodstuffs in Nigeria, many of which are of vast importance like rice, nuts, maize, beans, guinea corn, millet, cacao, sorghum, chilli pepper, yam, potatoes, tomatoes, peanuts and so on according to Makun *et al*. (2010). AFs have also been linked with environmental pollution, ecosystem imbalance and some

critical health hazards such as cancer, mutations as well as cardiac, skin, and respiratory disorders (Yassin *et al*., 2011).

Garlic commonly known as *Allium sativum* has been recognised as a medicinal plant at the time of early Olympic Greece when it was popularly used to feed the athletes in order to boost their strength and stamina (Petrovska and Cekovska 2010).Over many years, has been widely publicised for its richness in notable phytochemicals against many microorganisms including aflatoxigenic fungi. It has been used in many herbal preparations for the treatments of several plant and animal diseases and in controlling microbes in foods (Onyeagba *et al*., 2004). Dietary intake of garlic in many epidemiological studies have been reportedly applied for reduction of several diseases in humans such as the colon cancer, stomach ulcers, and cancers of the breast and prostate (Melino*et al.* 2011).Garlic contains a phytochemical compound called allicin witha potent antimicrobial and antifungal agent.

Ginger (*Zingiber officinale*) is another plant that has been documented with several phytochemical and antifungal properties (Tagoe *et al.,* 2009). Ginger has been used widely as spice in several cuisines and folk medicines; its most common part 'rhizome'is a popular root system, which is commonly refers to as ginger. It helps in alleviating nausea and reduce vomiting during pregnancy, others includes anti-inflammatory, anti-allergic and anti-osteoarthritis.Ginger extract has potent antifungal properties against a wide range of fungal species such as *Aspergillusniger* and *A. flavus* (Onyeagba*et al*., 2004)

Turmeric (*Curcuma longa* L.) is another plant of wide medicinal importance, it is a flowering angiosperm of the family Zingiberaceae (same as that of ginger), also producing root that is widely employed for cooking. The phytochemicals in this plant have made it popular medicinal plant worldwide. The diarlheptanoids content of turmeric for example is a class of various curcuminoids like curcumin, bisdemethoxycurcumin as well as demethoxycurcumin. These phytochemical components of turmeric made up of 3.14 % of turmeric powder. It also contains essential oils such as turmerone, atlantone, zingiberene and germacrone. This plant has been employed in several clinical trials for the diagnosis and treatments of different human ailments such as inflammations of several organs (Sutarno *et al.* 1999).

The importance of garlic, ginger and turmeric also inspired interest of this research is to investigate some importance of ginger additive on the incidence of toxigenic fungi and aflatoxin level control as well as their effects on proximate compositions of Groundnut cake (*Kulikuli*) collected from five selected states in Northern Nigeria.

1. 2. Statement of research problem

Many groundnut-producing countries are combating the problems of fungal spoilage and toxins during cultivation and storage.Fungal spoilage is causing massive losses of groundnuts in Nigeria due to prevailing environmental and storage conditions. Unhygienic handlings and poor storage facilities in Nigeria often expose groundnuts and their products to fungal and aflatoxin contaminations, and these often result in *Aspergillus, Fusarium and Penicillium* contaminations that are groups of mycotoxigenic fungi (fungi that produce toxic metabolites). The incidence and consumption of such toxins in human foods and animal feeds has been associated withvarious diseases like cancer, respiratory problems, skin and cardiac disorders and so on.

Many studies have reported aflatoxins poison in African countries and in many indigenous Nigerian local foods with some levels higher than the tolerance limit for humans (Shephard 2008a; Makun *et al.,* 2010). These researches also suggests many control measures and advocated the importance of biological control measures which have not been widely implemented in Nigeria.Despite increasing reports on high levels of aflatoxins in many indigenous Nigerian foods, there is still limited information and data on the analysis and assessment of *Kulikuli.* In addition, fungal contamination and molecular implications of such fungi and mycotoxinshave not been well researched on *Kulikuli*. The presenceof these microorganisms on *Kulikuli* in Nigeria may pose critical health threats to the populace if not curbed.

1. 3. Justification of this research study

*Kulikuli*is widely produced in thenorthern part of Nigeria, however, its consumption has been adopted massively in many other regions of the country especially among low-income earners. This food is nutritionally rich due to its raw material (groundnut).It has been established that many moulds are also responsible for its bio deterioration or post-harvest decay especially during storage. However, the public health concerns become

imperative if mycotoxin contents of this stored food become elevated above the tolerance level of 2ppb given by European commission, whichis injurious to human health. This poses serious threat not only to the food security and quality of stored *Kulikuli* but also to the human consuming it.

Considering the importance of stored *Kulikuli* to the Nigerian populace, there is need for adequate control of these toxin-producing fungi from growing on our food. The results of this research will necessitate strict control of quality of *Kulikuli* and can then be utilized in both industries and other developing countries to avoid such outbreaks.

It is clear that *Kulikuli*handlers need to take safety sanitary and hygienic precautions. However, the doctrine of poor packaging and keeping it for longer period due to poor transportation may be signalling otherwise measures needed for controlling the incidence of moulds and their toxins in this food product. In this study, the effects of selected botanicals such as ginger, turmeric and garlic were investigated on the nutrient composition, fungal incidence and aflatoxin concentration of *Kulikuli*.In addition, this study also aimed at evaluating the molecular characteristics of aflatoxigenic fungi associated with*Kulikuli*from some selected states in Northern Nigeria as a case study.

1.4. Aim and objectives

1.4.1. Aim of study

The aim of this study was to evaluate the fungi associated with groundnut cake (*Kulikuli*) samplesfrom five selected Northern Nigerian states, aflatoxin secretionand proximatecompositions with their control using selected botanicals.

1.4.2. Objectives of the study

Specific objectives of the study

The specific objectives of this study are to:

- **i.** Find out the prevalence of aflatoxigenic moulds on *Kulikuli* based on percentage incidence across the study states.
- **ii.** Establish the relationship of the percentage prevalence of mould, nutrient composition and aflatoxins in studied *Kulikuli* samples.
- **iii.** Isolate and characterize aflatoxigenic fungi associated with bio-deterioration of *Kulikuli.*

iv. Assess the biocontrol potential of garlic, ginger and turmeric on aflatoxigenic fungi and aflatoxin contents of *Kulikuli.*

v. Find the presence aflatoxin biosynthetic genes in the isolated strains using Simple Sequence Repeat (SSR) molecular amplifications.

CHAPTER 2
LITERATURE REVIEW

2.1.Groundnut biology, origin and geographical distribution

Groundnutis botanically called *Arachishypogaea* Linn (Plate 2.1) andsometimes known as 'goober' or 'monkey nut'. Groundnut is a leguminous crop grown mainly for its edible seeds. It a member of the family Fabaceae and it is botanically grouped as follows:

Kingdom: Plantae
Phylum: Angiosperms
Sub-Phylum: Eudicots
Sub-Phylum: Rosids
Order: Fabales
Family: Fabaceae
Sub-Family: Faboideae
Tribe: Dalbergieae
Genus: *Arachis*
Species: *Arachis hypogaea*

The two subspecies in the genus *Arachis* are *A. hypogea* spp. *Hypogea* and *A. hypogea* spp. *Fastigiata*. Each of these subspecies has two varieties: var *hypogea* and var *hirusta* for *A. hypogea* spp. *hypogea,* and var. *fastigiata* and var. *vulgaris* for *A. hypogea* spp. *fastigiata* (Gibbons *et al.*, 1972). Groundnut is popularly and widely grown in the tropical areas of the world and is imported in small or large quantities commercially. This plant is classified as grain legume and it is as well known as oil crop because it contains high level of vegetable oil. Shelled kernels of groundnuts production in 2016 was above 44 million tonnes with china having 38% of the total world production. The pods develop underground typically

different from other leguminous crops, this condition is termed 'Geocarpy' and as Linnaeus as named it*hypogaea* (meaning under earth) based on this nature.

Groundnut plant can harbour some nitrogen-fixing bacteria symbiotically, and forms root nodules.These bacteria, including *Enterobacter arachis* and *Bradyrhizobium subterraneum*are able to fix atmospheric nitrogen to the soil fertility (Madhaiyan *et al*., 2010; Grönemeyer *et al*., 2015). This makes groundnut a natural bio fertilizer and an important component used for crop rotation. This was made evident in the study of Inal *et al*., (2007) who showed that the concentration of phosphorus in shoots of maize and groundnut was higher when they were intercropped.

Groundnut is one of the major crop in the world. It is the sixth out of the most important oil seed crops in the world which originates from Brazil, and commonly grown in the tropics as well as many of the temperate areas in the world. It is a self-pollinating legume and rarely undergoes cross-pollination. The groundnut variety *hypogaea* is the most popularly grown commercially (Krapovickas and Gregory, 2004).

2. 2. Nutritional and mineral composition of groundnut seeds

Groundnut as a leguminous plant is well fit for its nutrient values; it has also been processed into different products. Groundnut is commonly eaten in different styles; roasted, grinded, powdered, and milled paste, boiled or eaten raw. In many developing countries like India, groundnut as oil seeds is commonly used for making oils through different extraction methods. Their product is usually employed for use as supplements for feeding animals. Groundnut seed is often regarded as one of major protein supplements in many of West African countries. It is also a very important cash seed in Nigeria that could be processed into snacks, cookies, fried or roasted seeds and extracted oil.

In a study by Kumar *et al.* (2013), they investigated comparative physicochemical, proximate, and mineral compositions of raw and roasted groundnut seeds and reported that roasting had effect in reduction of the mineral and physicochemical contents of groundnut. They determined 4.6 % total ash, 26.1 % protein, 39.1 % fat contents of raw groundnut while the roasted seeds had 4.1 % crude protein, 26.5 % ash contents, and 38.1 % fat. Other proximate and minerals were analysed higher in the raw shelled groundnut

seeds as compared with those of roasted ones, for examples magnesium, potassium, calcium and zinc) properties were higher in the raw shelled groundnut seeds.

Plate 2.1. Groundnut plant and seeds (Source:https://en.wikipedia.org/wiki/Peanut).

In another study, Eshun *et al*. (2013) assessed the nutrient, mineral and lipid contents as well as the calorific values of four seed pastes made from groundnut varieties in Ghana. The varieties studied included *Arachis hypogaea* variety called sinkarzie, the f-mix, jl 24 variety and manipintar variety. They reported that groundnut contain protein range of 23.63 % in cinkazie to 28.95 % in variety jl 24, the crude fat ranges from 38.10 % in variety jl 24 to 48.80 % in sinkazie, they also reported that carbohydrate ranges from 19.65 % in jl 24 to 11.54 % in sinkazie. They also reported that the groundnuts had energy content ranged from 537.06 kcal/100 g in jl 24 to about 24.00 kcal/100g measured in manipintar as well as 128.30 kcal/100g in f-mix. They observed that these groundnuts have seeds that at room temperature contain pale yellow oil in all the four varieties with nutty odour.

Saponification values weredetermined by Eshun *et al*. (2013) for the four selected groundnut varieties and reported that they contain a ranged between 144.70 mgKOH/ g in jl 24 to 208.97 mgKOH/ g in sinkazie, iodine value ranged between 79.20 mg/ 100 g in sinkazie up till 87.10 mg/100 g measured f-mix. They also reported that the fatty acids in the groundnuts ranged from 8.10 % FFA analysed in manipintar to 14.30 % FFA analysed in F-mix. They concluded according to the report of this study that groundnut can be used to fight malnutrition and improve food security if adequately produced in large quantities. They recommended that the oils extracted from groundnuts could be explored as raw materials especially for domestic and for industrial productions.

Mustapha *et al*. (2013) also investigated nutrient and functional value of unshelled groundnut seeds from Nigeria. Their study focused on major groundnut markets; Bosso market in Minna, Niger State, Nigeria. The groundnut seeds were study for proximate/nutrient contents and some key minerals and functional properties. They reported that the studied groundnut seed samples contained a ranged between 40.19 to 42.23 % in crude fat, and also 23.50 to 27.40 % in crude protein, as well as 4.30 to 6.35 % of carbohydrate content. They also observed that the seeds contained 3.03 to 5.10 % range moisture, 16.33 to 23.69 % crude fibre and 1.55 to 2.36 ash. They reported that the groundnut seed samples milled into flours ranged in mineral compositions like sodium, calcium, potassium, zinc, iron, phosphorus, copper, and manganese but the dominant

macro mineral were potassium, manganese and calcium which are good elements for dietary supplements.

In this research, the functional properties of the groundnut samples ranged from 198.44 to 202.10 % for water absorption capacity, they also contained a ranged of 170.29 to 191.00 % for foaming absorption capacity, 25.30 to 32.00 % emulsion capacity, as well as 9.40 % to 14.00 % emulsion stability. They also reported that the groundnuts contain 4.00 to 5.00 % foaming absorption capacity, and 0.60 to 0.79 g/cm^3 of bulk density. They reported that the groundnut samples flours contain higher functional properties as compared to the roasted and sundried groundnut samples flours but all these characteristics approved the fact that their application in the food systems.

In a different study, Alenyorege *et al*. (2015) studied and reported the influence of different fertilisers on the proximate and mineral contents of fresh and stored groundnut seeds; they focused on two major fertilizers; the yaralegume and the humate green ok fertilisers.They were the commonly used fertilisers across different communities in upper-west region of Ghana. They analysed twenty-four (24) fresh and stored samples and reported that the fresh groundnut sample contain mean values of 3.65 %, 24.78, 44.79 %, 5.65 %, 2.18 % and 19.16 % of moisture, protein fibre, crude fat, ash and carbohydrate respectively while the dried samples contained 3.88 %, 27.08, 47.62 %, 6.75 %, 2.28 % and 12.28 % of moisture, protein, fibre, crude fat, ash and carbohydrate respectively. However, the values all reduced after a storage time. They also observed that fertilisers do not significantly affect the nutrient compositions of the groundnut seed therefore the choice on fertilizer should be on the quantity (yield and growth influence) while less attention should be given to their impact on the quality of the seeds.

2.3. Economic value of groundnuts

Groundnut is well cultivated acrossover 100 countries in the world both in the tropical and in the temperate regions. Groundnut production worldwide has been estimated toabout 24.00 million hectareswith a total mean annual production of about 37.00 million tons with an average yield of about 1530 kg per hectares in year 2009. Its production was estimated to total mean of about 48.7 million tonnes per annum in 2019(FAOSTAT, 2019).

Groundnut is widely cultivated in over 100 countries around the world while China, India, USA, Indonesia, Senegal, Mayanmer, and so on as the major producers. Groundnut is very important cash crop andoil seed, and it is mostly consumed in the US and Africa (Sahayaraj and Martin, 2003).

In Senegal, Niger Republic and Nigeria, groundnut generates about 21 to 60 % of total rural cash earnings. It accounts for over 75 % of rural populace employment among the Senegalese and its local populace (Ntare *et al*., 2008). Also, in the Sub Sahara African countries accounts for about 8.2 million tonnes of groundnut production per year with 9.5 million hectares of groundnut cultivation (USDA, 2012). This oil seed crop is commonly served as good source of protein for inclusion in many animal feeds, groundnut after corn is the most cultivated crop in West African countries and used as snacks (Ezekiel *et al*., 2013). There are many food derivatives made from groundnut, which are often seen hawked along roadside or in the rural markets of Nigeria.It is also commonly seen on the streets and along the roadside in the urban areas or in the cities andurban streets.

Groundnut seeds has been used as table kernel nankeens such as fried, soaked, boiled, and roasted groundnuts. Groundnut kernels as well are commonly used as spices for vegetable mix and sprouts mix in salad making. Groundnut due to its nutritional value and palatability is good for making animal and poultry feeds. The oil extracted from groundnut have been used in many preparations; mainly as cooking oil but also in other uses such as soap making, cosmetics, shaven cream, leather dressing materials, furniture lubricants, and so on. The groundnut seeds also arebeing widely exploited as raw material in industrial production of fatty acids and vanaspati ghee. It is used to preserve butters, milk, candy, chocolates, laddu, and barfi commonly known as chukii and even to preserve groundnut packs.

The groundnut oil commonly used in making preservatives, like its use as medium for the preservation of pickles, chutneys and so on. It has been widely used in the production of different types of herbal and medication ointments, plasters, syrups and emulsions for medication applications. The groundnut shells also have great commercial importance, they are used for filter in cattle feed, hard particleboard, activated carbon, cork substitute, and they are commonly used as fuel among the local populace. Groundnut green shoots

and roots are used as animal feed while the shells of the pods used as cattle feeds. They also have importance in crop cultivations as they are used for making feed and compost.

2.4.*Kulikuli* as a product made from groundnuts

Kulikuli is a groundnut based snack popularly eaten among the Nigerian populace, it is an indigenous local food to the North Western Nigeria but has gained popularity all over the country. It is produced from fried residue after groundnuts seeds are milled and their oil extracted (Adebesin *et al*., 2001). *Kulikuli* as a groundnut product is very rich in nutrients and minerals. Studies have established that it is rich in protein and fat andpreferably eaten dried. As a snack, *Kulikuli*arecommonly eaten among young and adults,as it is a common convenience among school age children due to its affordability. It is used as ingredients in poultry, and fish formulated feeds (Akano and Atanda, 2010). *Kulikuli* is now popularly eaten across the West African coasts as snack.

It is however, unfortunate that this popularly consumed food is still locally made with poor packing system. Nzeako *et al*. (2010) observed that some bacteria such as *Escerichial coli*, *Salmonella* species and some other enteric bacteria are commonly associated with this food as they studied *Kulikuli* samples from different markets in Nigeria and raised concerns on poor handling of this local food. They also recommended an urgent need for the assessments of this food product in correction to enteric diarrhoea cases in schoolchildren and adolescent in Nigeria. In another study, some cases of enteric diarrhoea were reported to be as results of consumption of some *Kulikuli* samples as they isolated bacteria species like *Klebsiella sp*, *Shigella sp* and *Proteus* (Nzeako *et al*., 2010).

2. 5. Fungi bio deterioration and mycotoxins implications on Nigeria local foods

2.5.1. Fungi and mycotoxins

Fungi are unique wide group of spore bearing organisms that lack photosynthetic pigments living parasitically or saprophitically on organic substratum. They are mostly microorganisms except those that regarded as macro fungi due to their macroscopic reproductory structures called basidiocarp and commonly involved in decomposition of different wastes (dirty work).They have shown to be capable of decomposing different substrates such as woods, plant polymers, lignin, cellulose and huge arrays of some other organic molecules such as waxes, feathers, cuticle of insects, rubber, and animal flesh

(Feng *et al.,* 2011). Fungi have been known to be among the major organisms involved in the conversion of living and dead tissues through decomposition because of their characteristic saprophytic mode of living.

Fungi are unique in structure and as a result they are found in different habitats of the world, hence they are regarded as cosmopolitan or ubiquitous organisms meaning that they can grow or found anywhere in soils, water, air, in extreme environments and in living organisms. Some are known to be tolerant to high concentration of chemicals, heat or cold resistant while some prefer normal temperate environment (Gock *et al*., 2003).They are robust group of organisms, which have been widely studied since the invention of the microscope in the 17th century for biodegradation or bioremediation potentials. They are also being employed for many industrial and scholarly applications such as the brewer's yeasts for brewing, bakers' yeasts for baking, antibiotic production, commercial enzymes, many commodity chemicals, even as food such as the mushrooms (Hyde *et al.,* 2019).

As bio degraders, fungi have found their fame in many environmental issues; they are well associated with the decomposition of wide arrays of wastes including agricultural wastes, chemical effluents as well as dead plant and animals. They have been well reported to be responsible for several food spoilage including plant diseases, crop yield loss and post-harvest food spoilage. The areas of food spoilage potentials of fungi have over the years gained public attention and raised concerns on the possible health hazards. This is because of the discoveries of fungal toxins (mycotoxins) which are significantly hazardous to the well-beings of man and animals (Shephard, 2008b).Mycotoxins arefungal toxins;they aresecondary metabolites that arecommonly produced by certain class of fungi called moulds. They belong to the groups of *Aspergillus, Fusarium*and *Penicillium* species. Major groups of mycotoxins are Aflatoxins, Ochratoxins, Critinins, Patulins, and Fusarium toxins (Makun *et al*., 2010).

(a) Aflatoxins

Aflatoxins(AFs) are acategory of mycotoxins produced by fungi in the *Aspergillus*genus.These species include *A. flavus* and *A. parasiticus*(Martins *et al.,* 2001). However, the general terminology "aflatoxin" refers to four distinctclasses of mycotoxins,

namelyAFB_1, AFB_2, AFG_1 and AFG_2 (Yin *et al.,* 2008).Aflatoxin B_1(AFB_1), the most toxic of them, is a powerfulcarcinogen and has been directly associated with several health issues, especiallyliver cancer in animals(Martin *et al.*, 2001). These toxins are mainly associated with food commodities and agricultural products originating fromtropical and subtropical areas, including maize, spices, cotton, peanuts andpistachio (Martin *et al.,* 2001; Yin *et al.,* 2008).

Aflatoxins are the most popular and common in Nigeria, AFs are toxins produced as secondary metabolites by some special groups of fungi called *Aspergillus*, especially *Aspergillus flavus* and*Aspergillus parasiticus.* AFs have been widely reported on many food stuffs in many states of Nigeria especially on maize, guinea corn, rice, beans, nuts sorghum, cocoa, chilli pepper, dried vegetables, beverages, peanuts and so on (Makun *et al*., 2010). Unfortunately, the mechanism of action of many mycotoxins are still unknown but it is reported to be more potent in immunosuppress individuals.

(b) Ochratoxins

Ochratoxin is a group of mycotoxins that are present in three forms, namelyA, B and C. Ochratoxinsare secondary metabolites produced byAspergillus andPenicilliumspecies. The differences in the three forms of Ochratoxins are as follows: Ochratoxin B (OTB) is a nonchlorinated form of Ochratoxin A (OTA), in other words, OTB does not possess the Chlorine atom present in OTA;on the other hand, Ochratoxin C (OTC) is an ethyl ester form Ochratoxin A, that is, an ethyl ester group differentiates these two form of ochratoxins (Bayman and Baker 2006).*Aspergillus ochraceus* is a major contaminantof a wide range of commodities including beer and wine, while the closely related species*Aspergillus carbonarius* is mainly present in vine fruit, and releases its toxins during the process of juice-making (Mateo *et al.* 2007).Ochratoxin A has been labelled as a potential carcinogen and an important nephrotoxin;it has been associated with the formation of tumours in the urinary tract, though research in human subjects is limited due toconfounding factors (Bayman and Baker 2006; Mateo *et al.,* 2007).

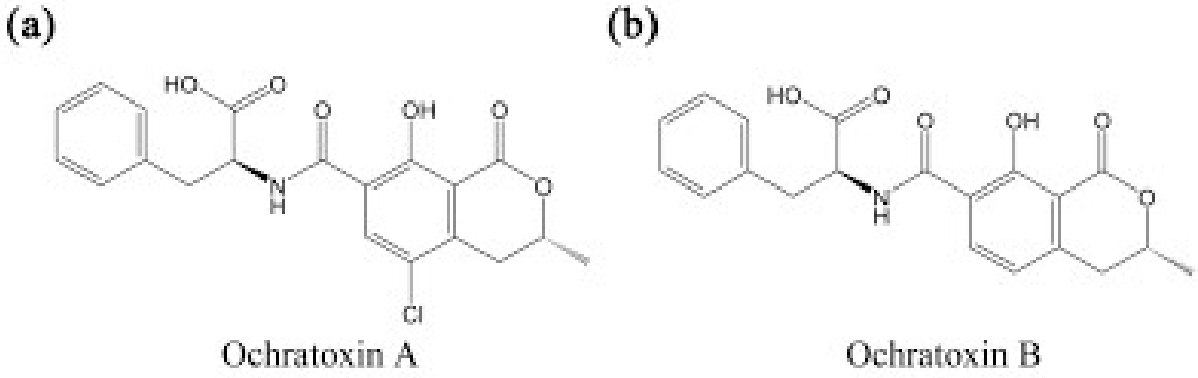

Figure 2.1. Chemical structures of ochratoxin.

Source: Ha (2015).

(c) Citrinin

Citrinin is a fungal toxin that was isolated for the first time from *Penicillium citrinum*, but over a dozen of *Penicillium* species and several *Aspergillus* species also produce this toxin. Some of these species, especially *Penicillium camemberti* and *Aspergillus oryzae* have been used industrially in theproduction of human foodstuffs, including cheese, sake, soya sauce and miso. In Japan, citrinin was associated with the yellowed rice disease, ita majornephrotoxin animals(Bennett and Klich 2003a).However, its effects on human health still remain poorly understood, although it has been associated with a wide range of human foods, including rice, wheat, barley, corn, rye and oats. Citrinin can in synergists with Ochratoxin A to slow down RNA synthesis in murine kidneys (Bennett and Klich 2003b).

(d) Patulins

The fungal toxin patulinisproduced by *Penicillium expansum*, *P. aecilomyces* andsome Aspergillus species. *Penicillium expansum* particularly common on mouldy vegetables, fruits, and rotting figs and apples (Moss 2008; Trucksess and Scott 2008). However, it's absent in fermented beverages such as beverages, such as cider because it cannot survive the fermentation process. Although there is no evidence on the carcinogenic potential of patulin so far, this toxin, has been shown to impair the immune system in animals (Moss 2008). The European Community in 2004 established the standard limit of patulin concentrations in food products as follows: 50 µg/kg for fruit juice, 25 µg/kg for all solid apple products and 10 µg/kg for children's apple derivative products (Trucksess and Scott 2008).

(e) Fusarium toxins

Fusarium toxins are another major class of fungal toxins produced by more than 50 species of *Fusarium* and with a history of infecting grain in developing cereals, especially maize and wheat (Schaafsma and Hooker, 2007). Fusarium toxins include fumonisins, which impairs the nervous systems in horses and cause cancer in rodents. Other toxins in this group, such as trichothecenes are widely associated with chronic and fatal

toxicologies in animals and humans. According to Desjardins and Proctor (2007) some other major groups of *Fusarium* toxins include fusarins, beauvercin, enniatins, equisetin and butenolide.

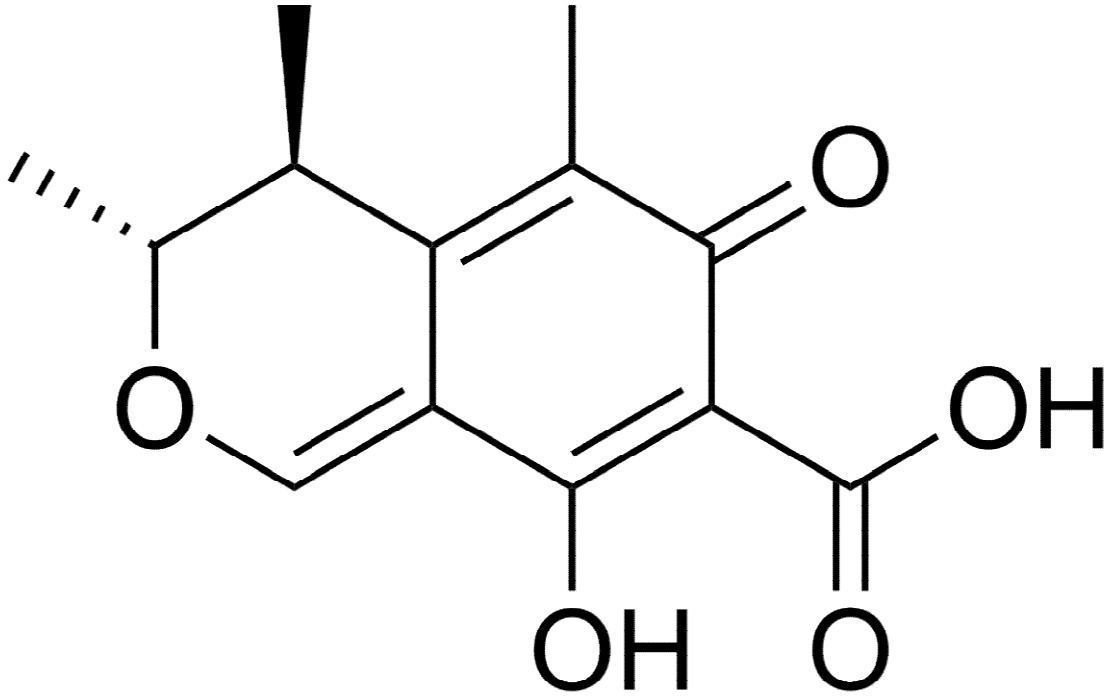

Figure 2.2. Chemical structure of Citrinin

Source: https://en.wikipedia.org/wiki/Citrinin

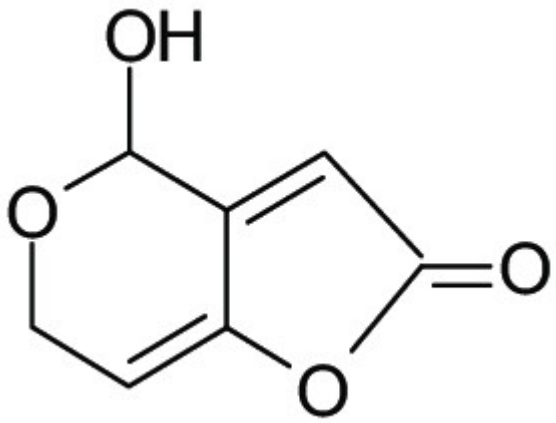

Figure 2.3. Chemical structure of patulin

Source: Fathi-Achachloue *et al.* (2007)

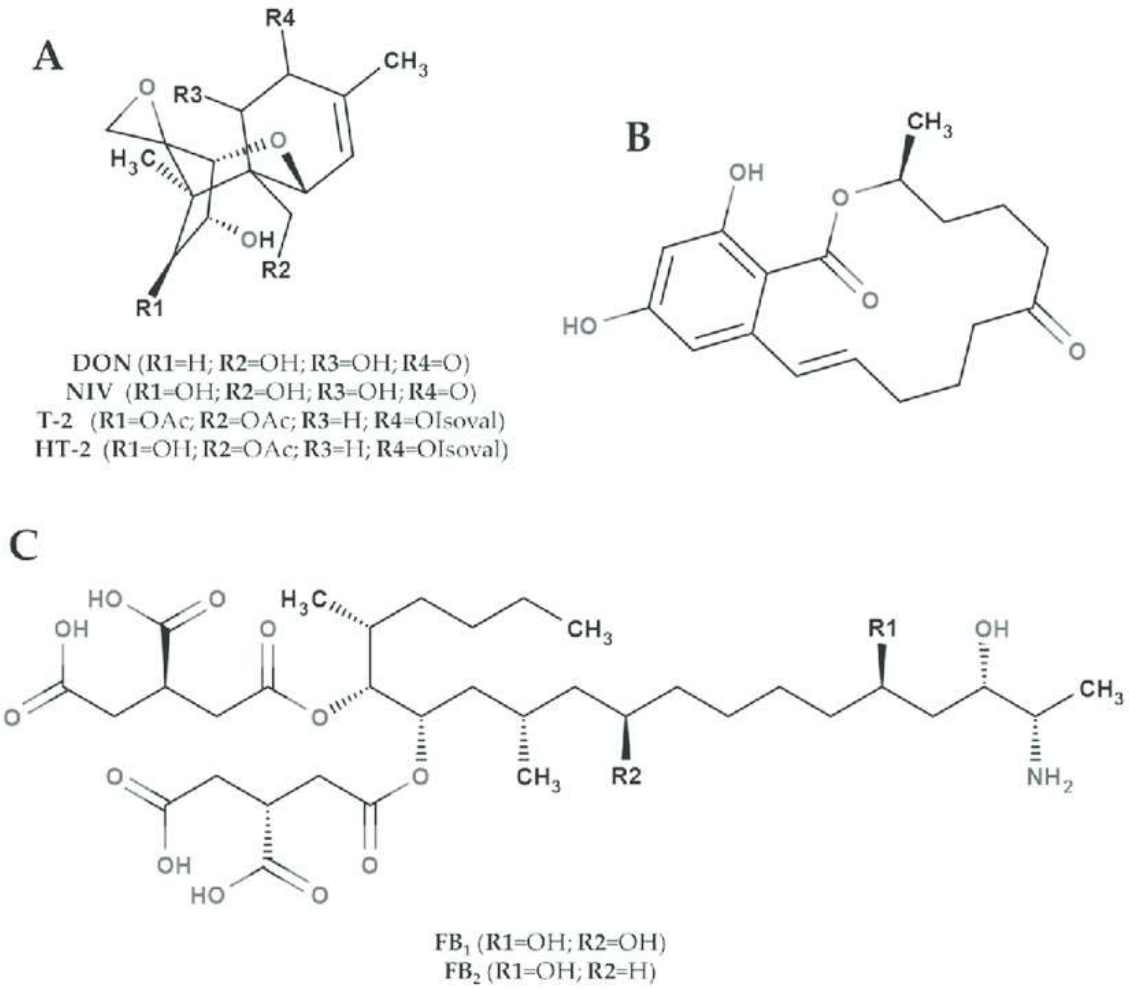

Figure 2.4. Chemical structure of the main *Fusarium* mycotoxins.

(A) Trichothecenes; (B) Zearalenone; (C) Fumonisins.Source: Ferrigo et al. (2016).

2. 5. 2. Morphology of some important aflatoxigenic fungi

Although molecular methods have improved and become widespread in the identification of aflatoxigenic fungi, microscopic technique have also been used to morphologically characterize these organisms. Morphological identification of aflatoxigenic fungi has often been the first step in ascertain fungal identity, especially in resource-limited laboratories. The microscopic morphological characters that have been useful in fungi identification include size, stipe length, shape, vesicle serration and conidia surface as well as colony colour and shape. The study of Gautam and Bhadauria (2012) highlighted the similarities and differences between the morphological and microscopic characteristics of major Aspergillus species. Only those of *A. niger, A. flavus* and *A. fumigatus* are presented below.

In terms of colony surface colour, *A. niger* varies between dark brown to black, while *A. flavus* and *A. fumigatus* are yellow/greyish green and green to dark green, respectively. Colonies of these three species present similar margins, often entire, while the reverse side colourless (*A. niger*) and colourless to pale yellow (*A. flavus* and *A. fumigatus*). *Aspergillus niger* and *A, flavus* often show rapid growth on potato dextrose agar medium as opposed to *A. flavus,* which has a moderate growth.

With respect to microscopic characters, *A. flavus, A. fumigatus* and *A. niger* all possess branched and septate hyphae. Conidiophore morphology varies as follows: *A. niger* (200–400µm in length and 7 – 10 µm in diametre), *A. flavus* (600– 800µm in length and 15 – 20 µm in diametre) and *A. fumigatus*(250– 500µm in length and 2 –8 µm in diametre). This implies that *A. flavus* is microscopically larger than the other two species. Furthermore, the vesicle of their conidiophore is globose, subglobose and dome-shaped, respectively. Conidia are usually larger in *A. niger,* measuring 30 to 75 µm with a brackish brown head and an exine spiny ornamentation. On the other hand, conidia are almost smooth, greyish green to yellow and 20–40 µm in length in*A. flavus* and 20–30 µm , with a roughened blue green head in *A. fumigatus.*In terms of philiades, *A. niger* and *A. flavus* possess biseriate (two series) of philiades that entirely cover the vesicle, while they are uniseriate and cover only the upper regions of the vesicle in *A. fumigatus* (Gautam and Bhadauria 2012).

Fusarium moniliforme, F. equiseti, F. solani and *F. oxysporum* are among the most prevalent Fusarium species in Africa (Onyike and Nelson, 1992). The differentiating morphological characters presented below were described Rodrigues and Menezes(2005) on Fusarium species associated with cowpea seeds. These traits were in line with those described by Hafizi *et al.* (2013) for *F. solani* and *F. oxysporum* that cause crown disease in oil palm. For brevity, only *F. oxysporum* and *F. solani* were considered.

In *Fusarium solani*microconidia formfrom lateral long monophialides and narrow at the apex. They are unicellular andin shape. Chlamydospores are either single or in pairs, globular, with a wrinkled or smooth wall. Colonies of *F. solani* on PDA havean abundant aerial mycelium and are pale to brown or in some cases white greyish to white creamy. Colony size varies between 3.1 to 3.7 cm (Hafizi *et al.* (2013). On the other hand, the identification of *F. oxysporum* can be made based on microconidia which, produce short monophialides that are mostly unicellular, and vary from oval to ellipsoid and even cylindrical. These macroconidia areabundant and possess anattenuated apical cell. Colony colour is pale, white or white-violet and growth varies between 3.4 to 4 cm.

Species in the genus Penicillium are known globally for their importance in food and drug industry, and for their mycotoxic activities. Colonies of Penicillium are fast-growing. They consist of a dense felt of conidiophore, and are often greenish and occasionally white. Under the microscope, phialides produce chains of one-celled conidia in a basipetal succession.

Phialides can be produced either individually or in groups, thereby showing brush-like appearance referred to as penicillus. Sometimes the penicillus is made up of branches and metulae, and the branching type may be simple, that is monoverticillate or one-stage, referred to as biverticillate-symmetrical, and two-stage, biverticillate-asymmetrical. There are also case of more than two branches. The macroscopic and microscopic characters of some important Penicillium species on PDA as described in the study of Tiwari *et al.* (2011) listed as follows: Colonies of *Penicillium citrinum* are often granular or powdery, with a dark green colouration, and the underside of the colony has a pale yellow appearance. On PDA media colonies of *P. oxalicum* are compact and cream yellow on the underside. This species has a very rapid growth compared to *P. citrinum. Penicillium*

notatum is green, radially plicate. The colony margins and underside are off white in colour (Tiwari *et al.,* 2011)

2.5.3. Types and incidences of mycotoxin in agricultural products

The incidence of mycotoxins on foodstuffs and feeds have risen public health concerns. Mycotoxin producing fungi are known to be moulds, especially those that contaminate and involve in the deterioration of foods. They contaminate and destroy many agricultural products, seeds, fruits, tubers, grains, kernels and so on especially during storage and cause great economic loss and reduction in their nutrient values (Jimoh and Kolapo, 2008).

Mycotoxin contaminants generally posehealth threats to animal and humans (Iheshiulor *et al*., 2011). They have great economic impact due to their producers that deteriorate agricultural products and cause serious economic losses. It is however difficult to determine the total financial loss caused by mycotoxin producing fungi due to different factors, but direct annual costs can be estimated. The annual loss of agricultural produce due to mycotoxin infestation in the United States for instancewas estimated to about 1.66 billion dollars and mitigation cost for fungi infestation estimated to annual cost of 466 million dollars.The main factor for mycotoxin incidence in foods, feeds or agricultural products is the suitability of the environmental conditions for the growth of toxigenic fungi. This have been well reviewed and studied by many researchers according to Bankole and Adebanjo (2003), Bankole *et al*. (2006), Wild and Gong (2010).

Fungi are highly diverse organisms and with verse biochemical and molecular characteristics, they possess diverse metabolic pathways and plate test techniques have been used to construct their morphological characteristics (Bakri, 2010). Morphological, biochemical as well as the molecular characteristics of toxigenic fungi can beadapted for identification of toxigenic fungi. Other plate tests techniques for the characterisation of toxigenic fungi include the polymeric dye compounds, guaiacol media, and tannic media (Kiiskinen *et al*., 2004). Detection and quantification of mycotoxins on food, crops, and feeds today has improved due to availability of advanced in technology, chemistry and biochemistry methods. Different analytical methods such as the use of Tin Layer

Chromatograph (TLC), High Performance Liquid Chromatograph (HPLC), Polymerace Chain Reaction (PCR), Gass Chromatogragh and Mass Spectrophotometer (GC/MS). Several molecular methods have also been widely employed for fungal identification. These methods include the use of universal primers to target conserved regions within rRNA complex and the Internal Transcribed Spacer (ITS) techniques. Molecular sequence technology has facilitated this purpose in identifying broad range of toxigenic fungi to species level (Gong *et al*., 2002).

Mycotoxin have over the years, attracted public attention worldwide due to their impacts on the human and animal health, crops loss and economy (Fandohan *et al*., 2005; Hell *et al*., 2008). Mycotoxin affects the human health directly or indirectly and different mycotoxins have been reported in different African countries (Shephard, 2008a). Their occurrence on maize in many parts of the world has made it the ideal cereal prone to mycotoxin contamination. However, they have been embedded in sorghum, millet and some kernels, seeds as well as some fruits.Mycotoxins were detected in Benin Republic and Togo, causing impaired growth in young children (Gong *et al*., 2002; Bandyopadhyay *et al*., 2007). The incidence of mycotoxins on groundnuts have been reported in Benin Republic and Nigeria (Ezekiel *et al*., 2012). Aflatoxins were detected on kernels that had no visible symptoms of fungal infestation (Gong *et al*., 2002). They have also been reported in groundnut due to combine situations of prolonged high temperature and injuries from insect attacks (Craufurd *et al*., 2006).

Nuts are also commonly contaminable crops by different fungi and mycotoxins. The most researched are the groundnuts and pistachio nuts, which are the major important crops imported throughout the world. Aflatoxins have been reported as major mycotoxin affecting these nuts especially in the United States (Clevelan*et al*., 2003), in Asia (Bonjar, 2004) and in African continents (Bankole *et al*., 2006) while there are less or no report of this toxin on these nuts from Europe. Ochratoxin-A has been detected on pistachio nuts in a survey done by the British Food Standard Agency in 2002 this toxin was detected at a lower level and it is often not considered as toxin of concern (Herpoel *et al*., 2002).

2.5.4. Aflatoxins as an important type of mycotoxin of concern

Aflatoxins (AFs) are the group of mycotoxin produced as secondary metabolite only by certain *Aspergillus* fungi (Kurtzman *et al*., 1987); *Aspergillus flavus* and *Aspergillus parasiticus.* The name Aflatoxin is a compound name derived from the word 'A' *Aspergillus*, 'fla' *flavus* and 'toxins' poisons. Therefore, aflatoxin can as well be defined as *Aspergillus flavus* poisons. AFs are of different types such as AFB_1 (Aflatoxin B_1), the AFB_2(Aflatoxin B_2), the AFG_1 (Aflatoxin G_1), AFG_2 (Aflatoxin G_2), AFM1 (Aflatoxin M_1), AFM_2 (Aflatoxin M_2), as well as others such as AFD1, AFRM, AFRo, and so on (Fig. 2.1).Aflatoxins (AFs) have been widely reported in many foods, feeds, crops (such as nuts, kernels, grains, seeds, fruits, and so on), animal products such as extracted cattle milk and processed local foods (Galvano *et al*., 1996). Generally, it is understood that the first biosynthetic products of *Aspergillus flavus* is the Aflatoxin B_1 (AFB_1) while it metabolise into AFB_2 (D'mello *et al.,* 1998). It is also believed that *A. flavus* only produces aflatoxin B_1 and B_2 while fungus *A. parasiticus* is believed to produce aflatoxin G_1 and G_2.

The strains of aflatoxigenic fungi vary in different geographical areas and environmental conditions as well as the kind of food substrate available for the fungal growth. Some Aspergillus strains are pathogens of plants while some are saprophytes on decaying organic matters. Some are found growing on food while some cause deterioration of fruits, seeds, nuts and different stored crops. Aspergillus species are unique organisms; they grow at temperatures of 12 to 48 $^{\circ}$C, they can proliferate under water condition as low as -35 MPa, they are semitermophiles and semixerophytes fungi with well-adapted growth characteristics fit for tropical and sub-tropical areas. They are commonly seen as secondary opportunistic pathogens in many diseased host plants (Payne, 1998). Many Aspergillus fungi are insect transmitted as they usually take opportunities of insect infestation to gain attachment with several crop plants. They are usually not aggressive fungi but they tend to contaminate and spoil foods, feeds and crops due to their fast growth (Payne, 1998).

Aflatoxin is regarded as the most potent mycotoxin. It is a natural carcinogenic compound that have been linked with many foods and feed poisoning in Africa with resulting hepatocellular cancer (Williams *et al*., 2004). The aflatoxin interference has also been

linked with viral attack Hepatitis B and Hepatitis C to effect the cancer of the liver (Williams *et al*., 2004). The outbreak of aflatoxins has been reported in many parts of Africa and this has been termed Aflatoxicosis a disease caused by Aspergillus flavus such as the one reported in Kenya by CDC (2004) and Williams *et al*. (2004).

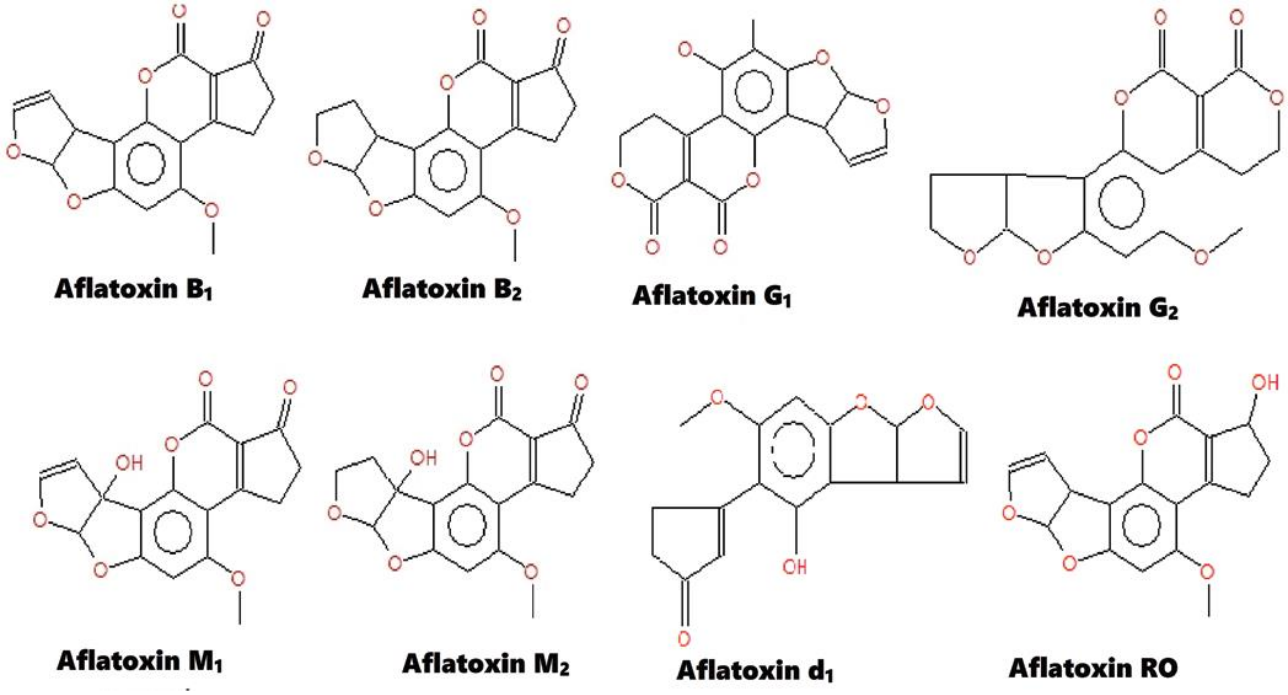

Figure 2.5. Chemical structure of common aflatoxins

(Source: Asemoloye *et al*. 2017)

According to the report from a research carried out by Turner *et al*. (2005a), aflatoxin incidence was linked to the suppression of immune system. Exposed infants/children to aflatoxin contamination through infested foods, crops and feeds were observed to develop stunted growth with poorly developed hair (Gong*et al*., 2002). They reported that these toxins also cause malnutrition in animals.

Furthermore, AFB_1 is refer to as most toxic aflatoxins and this is responsible for the reason why it is the mostly researched aflatoxin. The International Agency for Research on Cancer (IARC) reportedly classify this toxin as class 1 natural carcinogenic toxins (Castegnaro and Wild, 1995). They are hepatotoxic, carcinogenic and mutagenic in nature which governments in many countries have enforced regulatory agencies to monitor the aflatoxin levels in market crops, feeds and foods (van Egmond, 2002). According to IARC (1993), above 4.6 billion peoplewere exposed to aflatoxins in many developing countries (Williams *et al*., 2004) and this has been reported to increase due to different factors.Poor harvest tools/practices, storage facilities, transportation, packaging, policies have been found to be responsible for increased aflatoxin incidences in many developing countries including Nigeria (Bankole *et al*., 2005; Zain *et al.,* 2011).

2. 5. 5. Molecular diagnosis techniques for mycotoxins

(a) Polymerase chain reaction (PCR)

Polymerase Chain Reaction (PCR) was invented by Kary Mullis (Mullis and Faloona 1987; Mullis 1990). PCR is one of the most widely used and highly developed method for nucleic acid analysis. This procedure is based on the capacity of the enzyme DNA polymerase to make copies of a strand of DNA by elongatingthe complementary strands using on a pair of closely spaced primers.PCR a repetition of cycles of amplification of the selected nucleic acid sequences (Mullis, 1990). This procedure runs in three step in each

cycle, namely, DNA denaturation during which the DNA double strands separate. This is followed by primer annealing, which is done at a lower temperature. At this stage,primers attach themselves to their complementary target sequences. The last step is known as extension reaction step. Here,the enzyme DNA polymerase elongates the DNA sequences between primers on each strand. The quantity of PCR products are doubled at the end of each cycle.The PCR procedure is executed in a programmable thermal cycler. In general, after performing 30 to 50 thermal cycles, there would be an exponential increase in the amount synthesized DNA molecules(White *et al*., 1992).

(b) Reverse Transcriptase (RT)-PCR

The standard PCR protocol has been severally modified since its inception (Erlich *et al*., 1991). Some modifications have brought about effectively expandeddiagnostic capabilities, especially in clinical settings.Reverse Transcriptase PCR (RT-PCR) was established to amplify RNA fragments. In this procedure, RNA fragments are converted to a complementary DNA fragment (cDNA) by means of a reverse transcriptase (RT) enzyme, then cDNA isamplified by standard PCR. The RT-PCR protocol has played amajor role in the diagnosis ofviral infections, detection of *Mycobacteria* species and observations ofantimicrobial therapiesas described by Jou *et al.*(1997). Unlike PCR, the standardRT-PCR reactions are demanding.This is mainly because the enzymes are unable to withstand higher temperatures therebyhindering wide application in clinical diagnosis. The temperature-resistant DNA polymerase,*Tth pol* and its closely related thermostable variantsobtained from other living things possess aneffectual reverse transcription potential and as such can useful in the detection of RNA targets without needinga distinct reverse transcription step (Myers and Gelfand1991).

(c) Nested PCR

This is a highly sensitive technique, developed to detect very small quantities of DNA fragments based on two sets of primers (Erlich *et al.,* 1991). One set of amplification primers are used for the first stage ofamplification, which is 15 to 30 cycles. The resulting products are made to go through another amplification round using a different set of primers, which possess an internal sequence that was amplified by the first primer pair

(Roberts and Storch 1997). This double amplification enhances the sensitivity of nested PCR.However, this technique has a major disadvantage, as it is highly prone to contamination when the amplification products are transferred from the first to the second stage. One way to avoid contamination during nested PCR consists inthe physical separation of amplification mixtures using a layer of waxor by developing primers based on distinct annealing temperatures (Erlich *et al.,* 1991).

(d) Multiplex PCR

In multiplex PCR, as the name implies, more than two primerpairs with a specificity for distinct targets are added in the same reaction tube. This means that at least one unique target DNA sequence will be amplified (Roberts and Storch 1997). A major disadvantage of this technique is that the primers sets must be prudently designed to have the same annealing temperatures, and this requires extensive tests. The concomitant amplification of multiple targets in multiplex PCR can be serve ina variety of purposes. For example, in diagnostic analyses,this technique can serve in the detection of multiple pathogens in a one specimen (Jou *et al.* 1997; Roberts and Storch 1997).

2. 5. 6. Analysis of amplification products

(a) Hybridization Protection Assay (HPA)

In HPA Hybridization Protection Assay, a probe is labelled with an acridinium ester and added to a sample containing PCR products for identification. Both the probe and the products are incubated in one test tube. Then the binding of probe to the target DNAcan be measured without further manipulation (Arnold *et al.,* 1989).In a positive sample, the bound probe is protected from alkaline hydrolysis and, when peroxides are added, the probe emits detectable light. As an advantage, the binding of the amplified product to a solid support by DNA is not required in HPA. Thus, this technique can be executed in a short time, typically a few hours. In addition, there is no need for excess DNA probe to beremoved (Nelson and Kacian 1990).

(b) DNA Enzyme Immunoassay (DEIA)

DNA Enzyme Immunoassay (DEIA) is one of the recently developed methods for the analysis of nucleic acids previously amplified by PCR.In this method, an anti-dsDNA antibody entirely recognizes the hybridization productsthat result from the reaction

between a DNA probe and a target DNA. The final product is detected using a colorimetric reactionas described by Mantero *et al.*(1991). This approachaugments the sensitivity of PCR products by including enzymatic reactions. The specificity of DEIA is also enhanced by hybridization between the probe and PCR-amplified DNA, and the formation of target DNA/probe hybrids and anti-dsDNA antibody complex.

(c) Single-Strand Conformational Polymorphisms (SSCP)

This method was first described for the first time byOrita *et al*. (1989).In this approach, PCR is performed on DNA with a potential region polymorphism. The PCR products, usually incorporating a detector marker are visualised by means of gel electrophoresis. The changes in the physical structure of DNA is mirrored in differential gel migration. SSCP is very sensitive as it can detect single nucleotide substitutions. It can be useful in the evaluation of mutations arising from resistance mechanisms. Modifications of SSCP have been used to investigate the genes that contribute to the multidrug resistance of *M. tuberculosis* (Felmlee *et al.,* 1995).

(d) Random Fragment Length Polymorphism Analysis (RAFLP)

This is post amplification process, whereby amplified DNA fragments are sliced by an enzyme, referred to as a restriction endonuclease, then separated by gel electrophoresis and transported to a nitrocellulose membrane. Fragment with specific sequences can then be detected using a labelled homologous oligonucleotide, which acts as a probe. The variations in the size and number of the detected fragments is defined as RFLPs. These reflect variations in both the location of the restriction sites near those loci and the number of loci that correspond to the probe (Van Embden *et al.,* 1993).

(e) Simple Sequence Repeat/Microsatellite Markers (SSR)

Microsatellites markers/simple sequence Repeat (SSRs), also called simple tandem Repeat (STRs) (Tautz, 1989), consist of tandem repeat of short DNA sequences (1–20 base long) that are widely distributedin the genome of eukaryotic cells and account for up to 10 to 80 % of genomic content. SSR markers can more easily generate information compared with previous markers, namely RFLP and RAPD because of its increased discriminatory power due to high allelic diversity(Diouf and Hilu, 2005). Valuable

attributes SSR markers include co-dominance, sensitivity, technical and analytical simplicity, that is, data can be unambiguously scoredin a highly reproducible way. Microsatellite are becoming popular tools in molecular genetic analysis, it's application in the analysis of genetic structure of genes, percentage gene flow of plant and animal populations (Kent*et al.,* 2002).

Olufowote *et al.,* (1997) used microsatellite markers/simple sequence repeats to detect variation within rice varieties. They concluded that well-chosen microsatellites are an efficient method for assessingheterogeneity of rice accessions. Ahmad (2002) also assessed the genomic diversity among Wheat (*Triticum aestivum L*) based on simple sequence repeat and detected a high level of polymorphism among the cultivars used. (Yu *et al.*2002) studied allelic diversity among the elite inbred lines of cultivated sunflower (*Helianthus cannuus L.)* using SSR markers (Chen-Dao *et al*., 2001) determined the genetic similarities and relationship among cowpea breeding lines and cultivars using simple sequence repeat markers.

2. 6. Factors affecting fungal and aflatoxin incidence inagricultural products

2.6.1 General factors

There are various factors affecting the fungi and aflatoxin incidence in food, feeds and agricultural product. These factors can be characterised as intrinsic or extrinsic factors, they may also be categorised as reported by Zain*et al.* (2011) based on ecology, environmental and storage factors. Zain *et al.* (2011) stressed that factors such as fungi, substrate and environmental conditions are the key factor that determine the incidence of fungi in food, feeds and agricultural product irrespective of any grouping we wish to place them.

Aflatoxins are groupedinto pre-havest and post harvest aflatoxins. Pre-harvest aflatoxins are those that attack crop plants (shoot, root, seeds, fruits or tubers) during the plant cultivation young or matured but it occurred on the field without harvest while the post harvest aflatoxins are those caused by the *Aspergillus* infestation of crop plant after they have been harvested from the farm (postharvest crops). The incidence and levels of aflatoxin contamination can also be due to the geographical location and seasonal factors. This can also be determined by cultivation, harvesting methods, stocking, transport

conditions and so on. In the postharvest storage, moisture and temperature control is of paramount importance as these are the main factors that determine the fungal growths and their capability of their biosynthesis of different mycotoxins (Bryden, 2007).

2.6.2. Climatic conditions

The climatic condition such as the temperature and moisture are the two most important factors that determine the incidence of *Aspergillus* fungi or their production of aflatoxins. These fungi are common in areas that have prolonged hot and humid conditions; these encouraged the growth of toxigenic fungi and concurrent aflatoxin production. They also occurred in temperate and tropical zones such as Nigeria where this kind of climate occurs (Mclean and Berjak, 1987). The ability of some fungi to produce aflatoxin (Aflatoxin biosynthesis) is highly dependent on the moisture, water activity, temperature, pH, and oxygen status of the substrate on which the toxins will be secreted. Aflatoxins are produced as secondary metabolite and secreted extracellularly on their growth substrates. Therefore, these conditions are very crucial for this to happen (Bryden, 2007).

The optimum temperature of 24 ^{o}C and 28 ^{o}C as well as moisture of 17.5 % humidity have been reported to be the best condition for mould growth and their production (biosynthesis) of toxins (Ominski *et al*., 1994). Also, the conditions which favours mould growths are often the ones observed in most West African countries and this could be the reason why many aflatoxin outbreaks have been reported in Africa. Sharp drought condition after a prolonged wet condition often creates a stress factor to the fungi rendering them to produce mycotoxins, this condition also stress plant and make them more susceptible to the fungi pests. These conditions were affirmed to aid the aflatoxin outbreak that was reported from Kenya in 2004 according to CDC (2004). In another report, Edema and Adebanjo (2000) as well as Makun *et al*. (2009 a and b) reported a higher aflatoxicosis symptoms during the rainy season as compared to the dry season in some harvested agricultural produced in Nigeria.

2.6.3. Nutrients availability and the proliferation of aflatoxigenic fungi

The aflatoxigenic fungi are mainly moulds of the fungal genus *Aspergillus,* they grow saprophytically on foods, feeds, wood, dead and living cells/tissues of animals, plants and other organic materials. They have genetic capabilities to secrete aflatoxins extracellularly

on their substratum. They mechanism behind their nutrient uptake and why they secret aflatoxins is not fully understood but it is established that the availability of nutrients on the substrate on which they grow enhances their ability to produce the toxin as it enhances their general metabolic rates.

Moulds generally require energy for growth, which they derived from organic substrates such as carbohydrates, vegetable oils, carbon/nitrogen sources and so on. They also obtain nutrients by predigestion of organic substrates and extracts nutrients from them a process termed '*Mineralization*'. In a report by rachaputi *et al*. (2002), varying levels of aflatoxins were detected in maize, sorghum and rice that were harvested, stored and analysed at the same time. They associated this variation based on the diverse nutrients contained in these crops as they were exposed to the same fungus (*Aspergillus flavus*). They also observed that the same fungus produces varying aflatoxin B1 concentrations when cultured on groundnut and cottonseeds. Rachaputi et al. (2002) carried out further investigation on the influence of mineral compositions on mycotoxin production on many other toxigenic fungi.

Rachaputi et al. (2002) observed that some strains of *Penecillium viridicatum* isolated from grain were more virulent and produced ochratoxins and citrinin than those strains isolated from meat, some had even lost their toxin producing capabilities. Field strains of *Fusarium* and *Alternaria*species, which are isolated from grains during or before harvesting, weremore capable of producing mycotoxins than others, which were isolated from soils. Temperature at different degrees has also been shown to directly influence the aflatoxin production, prolong end season drought with elevated temperatures may increase the mycotoxins contaminations. Moulds generally favour temperatures between 20 to 35 ^{o}C for growth and hence for toxin production, they however can produce toxins not in correlation to growth needs. *Fusarium trincinctum* for example can grow well under the temperature of 25 ^{o}C but produce T-toxins best at very low temperatures. *Pecinillium martensii* can best produce penicillic acid at 20 to 30 ^{o}C but more of their toxins accumulate better around 4 to 10 ^{o}C.

2.6.4.Agricultural systems and farming techniques

There are many agricultural systems if poorly handled can increase the aflatoxin incidence while some farming techniques may support the fungal growth.

(a) Poorly handled crop rotation systems

Some cultivated produce harvested with infested produce and replanted in another soils. Some unsorted infected samples may infect other crops if stored together without a good control measure. Soils, which were cultivated previously with rye, oats potatoes or melon with poor agricultural managements may also increase mycotoxins if used for the cultivation of groundnut seeds. Crop rotation in other words may promote *Aspergillus* infestation if not wisely done.

(b) Type and condition of cultivated soils

The soil is a natural home for many microorganisms, many pathogens also passes off-season periods by hiding their spores in the soil. The soil structure and type may also support the growth of some fungi than others depending on the available nutrients and physicochemical compositions. Groundnuts grown on sandy soils may better support mould proliferation than the clay soils. Moulds may also be less survived in soil with prolong dried conditions while soil with higher water holding capacity may better support the growth of moulds during the rainy season (Codex Alimentarius Commission, 2004).

(c) Poor pre-harvest management

This include genotypes, plant diversity, drought, soil type, fertilization, insect control and so on and all these linked with likelihood of increased aflatoxin levels if not carefully managed (Cole *et al*., 1988). Corresponding infection and fungal growth due to these factors may also enhance the aflatoxin incidence in the cultivated crops. Stressed plants may loss their defence against toxigenic fungi and become more susceptible and these factors are more significant as aflatoxigenic fungi are opportunistic pathogens of different plants (Payne, 1992).

(d) Time of harvest

Prolong time before harvest can increase fungal infestation of many crop plants, harvest as the first stage of production need to be done at time when the crops are still freshly matured and free of pathogens. This should be done carefully to avoid storage loss due to

carry over pathogens; some seeds need to be dried before harvest, while some have to be harvest freshly. Plants are less defensive after fruiting; disease plants can be destroyed while infected seeds should be sorted out. Matured crops attracts pests especially insects and some other higher pests that cause mechanical damage on the crop. This often create opportunities for many aflatoxigenic fungi to colonise the crop and possibly produce aflatoxin on them.

(e) Unhygienic harvesting handlings

This involves the use of unhygienic tools for harvesting matured crops, many tools are carriers of fungal spores (Mazzoni *et al*., 2011), and using the same tools for harvesting different crops without proper hygienic treatment may spread the toxigenic fungi into healthy crop and hence contaminate them with the toxins. Avantaggio *et al*. (2002) reported that insect and damages caused by harvesting tools increase *Fusarium* mycotoxins contaminations. Some fungal spores inoculated on plant surfaces during harvesting get entrance in the crop. Rachaputi *et al*. (2002) reported that early harvesting of groundnuts saved 27 % returns or less aflatoxins.

(f) Poor postharvest handlings

The postharvest stage is a very crucial stage in crop production especially in local areas or areas where the crops are to be transported to distant location. This leads to primary processing stages such as milling, and usually involves cleaning, drying (for some crops), storage and transportation. At all these stages, fungal contamination need to be strictly controlled. Unfortunately, lack of facilities, storage space, unpredicted weather conditions, labour constraints, cash availability and insect's invasionare factors sabotaging such necessities in Africa (Bankole and Adebanjo, 2003). In a study carried out by Kaaya and Kyamuhangire (2006), they observed that aflatoxin levels increased four folds after the third week of harvest in some harvested crops. They also observed that this aflatoxin level increased 7 folds after 4 weeks of harvest.

(g) Illiteracy and lack of awareness

The aflatoxin incidence are mostly detected in rural or local area than in the industrialized urban areas. This may be because of illiteracy or non-awareness of fungal contamination

or food poisoning. Majority of farmers in many developing countries including Nigeria are illiterates and they are completely unaware of aflatoxins.

2. 7. Implications of aflatoxin/mycotoxin contaminations in Nigeria

Fungal toxins areoften reported from many crops and animal feeds in Nigeria, some of which were detected with aflatoxin levels above the tolerance limit for humans. Ingestion of these affect the physiological body conditions, metabolizing fungi toxins affects the gastrointestinal tracts, liver, kidney and skin (Bankole *et al*., 2003).Many mycotoxins have been listed as class 1 natural carcinogens and mutagens, which have been well linked, with many human health issues. According JECFA (2001), aflatoxins have shown to aggravate hepatitis B and C infections, while Fumonisin in South Africa have reported to cause oesophageal cancer (Makaula *et al*., 1996).

Aflatoxins in Nigeria have been documented in relation to many human's health hazards. Mycotoxicosis and aflatoxicosis issues have not been well documented in Nigeria but may have link with many deaths in immunosuppress individuals. Ikeorah and Okoye (2005) linked the deaths of some children in Ibadan metropolis to aflatoxicosis contracted because of consumption of mould infested *Kulikuli*. In several studies, Aflatoxins were detected at different concentrations in urine and blood of diseased liver patients (Adegoke *et al*., 2004). In Zaria (a city in Nigeria), aflatoxins were detected in some organs of some dead children, Aflatoxin b_1 (AFB_1) was detected in some children who died shortly after tested positive to have kwashiorkor in Western part of Nigeria and in the semens of some male adults from Benin city Nigeria.

In similar studies carried out in Nigeria, Aflatoxin M_1 (AFM_1) was discovered in breast milk of some nursing mothers and in the blood collected from some babies' umbilical cords (Adejumo and Awosanya, 2012). Doctors have also detected aflatoxins causes deaths in some liver cancer patients at the National hospital Abuja (FCT) Nigeria and many other hospitals have given reports of aflatoxicosis, ochratoxicosis and zearalenotoxicosis symptoms in some patients.

However, many Nigerians are yet to be educated on fungal toxins in foods, as this area has not been popularised. Unfortunately, there are less or no policies on the aflatoxin

quarantine in street hawked or road vended local foods in Nigeria (Idahor *et al*., 2010; Makaula *et al*., 1996). Aflatoxin have contaminated livestock feeds in Nigeria, young and pregnant animals are usually more susceptible to aflatoxicosis.It has been causing losses of ruminants, some aves, horses, donkeys and ponies with a syndrome called quine leuko-encephalomalacia associated with fatal mycotoxic diseases in relation to *Penicillium purpurogenum*detected in Vom Township in Plateau State of Nigeria (Ocholi *et al*., 1992).

Idahor*et al*. (2008) reported mycotoxin infections in some rabbits leading to reduce sperm production, weight loss, and less ability to consume foods. They also linked abnormalities in placenta due to high levels of fumonisin B_1 with associated difficult pregnancy during the first trimesters (Idaho *et al*., 2008). Ogunlade *et al*. (2004) investigated fumonisin B_1 and reported that at concentration of 1650-1990 µg in diet can cause cancer in rabbits. Ewuola *et al*. (2003) on the other hands reported the effects of fumonisin B1 on weight loss in New Zealand white rabbits.

Mycotoxicosis symptoms commonly observed on the fieldlivestock include digestive disorders, reduced eating rate/ malnutrition, thriftiness, rough air coat/abnormal feathers, less production, impaired reproduction, and mixed disease profile (Kao and Robinson, 1972; Sharma *et al.,*1993). Mycotoxicosis is not easily diagnosed as they sometimes increase the intensity of different disease in immunosuppressed individuals (Hesseltine, 1986).

2. 8. Control of aflatoxin in foods, feeds and agricultural products

2.8.1. Sanitation

Basic sanitary measures are crucial across all the stages of crop and food productions, sorting and debris removal from previously harvested or stored products could minimize fungal spores and infestation. Sorting out infected or physically damaged crops from healthy ones can reduce fungal attack by 40 to 80 %.

2.8.2. Good agricultural practices

Agricultural practices involve all the processes that were applied to cultivation and storage process of farm production. This should be done to ensure a safe healthy foods and agricultural products. The bottom line of this is to handle every steps in such a way that

fungal spores or inoculums are avoided to tackle the incidence of mycotoxins. Land or soil management must be practice such that it would not promote the growth of toxigenic fungi and as well the crop cultivation, planting, harvesting processes, life stock care, crop rotation, irrigation and so on must be done hygienically to avoid fungal contaminations (CAC RCP 51, 2003; Negedu *et al*., 2011).

2.8.3. Drying

Drying of some crops have been reported to aid mycotoxin reduction, rapid drying reduces the moisture content and creates harsh condition for fungal growth. By this, it rather preserved the crop, feeds or foods for longer period. The overall recommendation is to quickly dry healthy agricultural products before fungi infestation. The safe moisture level is set at 10 to 13 %. This is recommended not only during storage but also during transportation in to long distances and marketing.

In a study carried out by Hell *et al*. (2008), it was observed that there was an increasing aflatoxin levels as harvested maize were stored at humid environment for 3-day period after harvest but recorded less aflatoxin contents in those stored in dried environment. In another study by Ayodele *et al.* (2010), the analysed Critical Control Point (CCP) of yam tubers by making them into chips and recorded less aflatoxin contents as compared to the main tubers.

2.8.4. Storage conditions

The storage condition is a key factor that determines the incidence of aflatoxins in stored foods, feeds and crops. Moisture should be avoided from leaking roofs or condensation because of poor ventilation. Aflatoxin contamination of stored materials depends on the inadequate handling and storage conditions that favour the mould growths. Biological activities during storage should be reduced by maintaining drying condition of less than 10 % moisture during storage. Insect interference should be removed as they can increase the moisture content of the storehouse from respiration (Negedu *et al*., 2011; Turner *et al*., 2005b).

Care must be taken that stored materials are free from mould contaminats, daily temperature, moisture should be noted and adjusted in the storehouse.Wetgrains and

kernels preferably should be dried before storage to avoid fungal deterioration. Bagged materials can be stacked on pallets. The storehouse should be well ventilated and well protected from rain, drainage of underground water and insects, rodents, reptiles, birds or any other pests. All these should also be maintained for transport containers. In addition, the storehouses need to be well washed, fumigated and drained with registered pesticides before they are reuse.

2.8.5. Traditional or cultural methods

Some cultural methods can reduce aflatoxin contamination. Fandohan *et al*. (2005) reported that aflatoxins and fumonisins could be reduced in maize and some other agricultural crops through some hygienic handlings in Benin Republic. They observed that sorting, winnowing, crushing, washing, dehulling of maize grains significantly reduce the toxin levels in the studied samples and this is similar to the observations made Lopez-Garcia and Park (1998). It is important to note that heating, roasting, milling, baking, frying and drying can reduce aflatoxin concentrations in foods and feeds.

2.8.6. Food management system

Food management system for many local or streets vended foods in developing countries will help to reduce the aflatoxin contaminations in many ways. The Hazard Analysis Criritical Point (HACCP) is designed to prevent problems of food poisoning by food industries.It involves conducting a detail analysis at all steps of food process using different principles that involves test for mycotoxins at pre-harvest, postharvest, until production, packaging and marketing stages and implement measures at all these levels to avoid mycotoxin contamination. It has been used for aflatoxin control of corn and coconuts in Southeast Asia, in Africa for groundnuts and some groundnut-processed products and for some nuts in West Africa. In South Africa, HACCP has been used to control patulin a mycotoxin commonly associated with pistachio nut (FAO/IAEA, 2001). Several HACCP schemes have been developed for wheat and wheat-based and for the control of mycotoxins in the atmosphere (Lopez-Garcia *et al*., 1999).

2.8.7. Mycotoxin combating policies and regulations

Strong legislation against mycotoxins in foods will reduce the incidence of food poisoning due to aflatoxins. Foods, crops or feeds containing higher levels of aflatoxins above the

tolerance limit can be ban seize and diverted for other use before they get to the consumers. This requires analysis of foods, feeds and crops at every levels of marketing to ensure their quality controls (Mariko-kubo, 2012). It was also reported in 2003 by FAO after a worldwide study on mycotoxin regulations among different countries.They reported that there are some permissive levels at which humans and animals could tolerate mycotoxins. This study increased mycotoxin regulation in these countries by 30 % increase as compared to 1995 (FAO, 2003). In countries where strict regulations are enforced against mycotoxins, infected products are diverted for some other use instead of consumption, for example, infected corn can be used for bio-diesel production (Desjardins *et al*., 1993).

Unfortunately, there are no specific government regulations on mycotoxins in most African countries, although there are about 15 countries with known regulation against mycotoxins in Africa and many of these countries do not have standard advance tools to adequately monitoring the regulations especially in the rural areas. In Africa, Morocco has the most extensive mycotoxin regulations, while Nigeria follows the European commission on mycotoxin regulations and these are primarily applied to the exported crops and food products but not on the local foods.

2.8.8. Breeding of resistant varieties

Breeding of varieties that are resistant to fungi attacks have been shown to adequately reduce aflatoxin incidence. A key example of this is the production of '*Bt corn*' a corn breed that is resistant to *Aspergillus flavus.* This breed was developed due to increased aflatoxin incidence in cereals like maize and legumes like groundnut, beans and so on. Genetic approach has yielded some breeder crops that are resistant to aflatoxigenic fungi. Bt corn is an example, it possess a bacterium *Bacillusthuringiensis* gene called cyst a protein that codes for the production of some insecticides (Huang and Kang, 2007). This gene has also been activated in other crops such as rice, cotton, tomato, peanuts and so on (Williams *et al*., 2004; Desjardins *et al*., 1993).

2.8.9. Application of supplements against aflatoxins

The Novasil clay approach involve the use of highly purified clay commonly used as supplement for foods to absorb any aflatoxin present in the gastrointestinal tract. In

Ghana, novasil was used in clinical trials as dietary supplement, and proven to be good absorbant of mycotoxins. It was observed that novasil significantly reduced AFB_1 in albumin adducts as tested from the blood serum. It also tested to reduce AFB_1, other mycotoxins and Polycyclic Aromatic Hydrocarbons (PAHs) in urine by 60 % after a three-month treatment. This material has been well used with contaminated feeds for rats, poultry, swine and cattles to reduce mycotoxin contamination (Diaz *et al*., 1997).

Activated carbon has also been used as absorbent for mycotoxins, it has been used to reduce aflatoxin in milk according to Galvano *et al*. (1996), 0.1 % activated carbon in cattle feeds was reported to reduce the aflatoxin content by 20 % (Diaz *et al*., 1999). Also addition of glucomannan at 0.05 % in dietary feeds and bentonites at 1 % were found to reduce aflatoxins significantly in the milk of cattles (Diaz *et al*., 1999). In most cases, clay was used at 1% of novasil clay and recommended in animal feeds.

2.8. 10. Education and awareness

Majority of farmers in many developing countries including Nigeria are illiterates and they are completely unaware of aflatoxins. The mycotoxin Mycotoxicology Society of Nigeria has tried to educate people but the supports are needed from Government at all levels for this. Many local farmers believed that powdery substances on some crops can simply be dusted off to clean it; some believe that washing can remove and purify crops from fungi without further treatment.

2.8.11. Chemical control measures

Many fungicides have been successfully used to control aflatoxigenic fungi, sodium bisulphite, ozone, ammonic acids and so on have been commonly used to fumigate many crop plants and to control mycotoxin incidences (Magan and Alfred, 2007). Ammonification of grains have been shown to reduce some mycotoxin levels (Diaz *et al*., 1999).Many synthetic chemicals have been discourage for used in recent years due to their residual effects. They are very potent against the fungal pests but may also have some negative effects on the environment. Some other factors such as the cost, miss-application,

bioaccumulation and so on account for the dissatisfaction on the use of chemicals. These have resulted in some strict regulations on the use of chemicals; some chemicals have been completely ban in some countries (Paland and Hofte, 2006).

2.8.12. The use of biological entities

Biological controls involve the use of living things, their parts or products to fight or remove aflatoxigenic fungi or aflatoxins in crops, animals, feeds, or foods. Many countries such as the US and Mozambique has developed agencies that involves the use of biological measures for different fungal diseases especially those of *Aspergillus flavus*. This is the most promising system as compared to the chemical and mechanical control of mycotoxins (Murphy *et al*., 2006) and efforts have been made to create improved varieties of crops that are resistant to *Aspergillus* attack.

In Nigeria, the international Institute for Agricultural Research (IITA) is pioneering many research on the biological control of *Aspergillus fungi* some other toxigenic fungi. They developed a technique called 'Aflasave' which focuses mainly on aflatoxin controls and this has been successful as tested positive in many case studies (Bandyopadhay *et al.* 2003). The physical removal of aflatoxin infested produce from the healthy ones was advocated by Lopez-Garcia (1999) but it is advisable that handlers try to avoid this contamination before hands. Some organisms that can inhibit aflatoxigenic fungi has been used to reduce aflatoxins in many crop plants. Some virulent species of *A. flavus* for example have been tested in the control of the virulent ones through a mechanism of competition on the fields.

2. 9. Importance of botanicals in the control of aflatoxins

2.9.1. Botanical pesticides

Many African countries including Nigeria are blessed with several plants that have fungicidal potentials; they can be exploited in preparations and applied for the removal or reduction of aflatoxins in food and feeds (Anjorin and Salako, 2009). Recently, many plant derived products like some plant extracts and essential oils from local plants have been proven in different researches to be environmental friendly and applicable as effective antifungal biocides due to their phytochemical composition (Anjorin and Salako, 2009).

The use of Botanicals is a form of biological control of aflatoxins and this present a better opportunity due to their availability, cost effectiveness and environmental friendly. The use of botanicals cannot be easily abused and effective (Bianchini and Bullerman, 2010). Botanicals includes phytochemicals that are fungicidal in nature and can be used instead of synthetic chemicals because they are locally renewable (Anjorin and Salako, 2009)

2.9.2. Plant volatiles

Natural plant volatiles include ethylene, carbon dioxide, crotyl alcohol and so on.Many of the plant volatiles could be used to activate plant response against toxigenic fungi. Some plant has been genetically modified to produce some volatile compounds, phytohormones, and phytochemicals against aflatoxigenic fungi. These plants can be planted without the fear of aflatoxins or fungal attacks. Many studies have established that volatile compounds can modulate plants response against toxigenic fungi examples of such volatiles include CO_2 as proven by Sharma (1985) and as supported reported by Roze *et al*. (2004a). Ethylene was also reported by (Roze *et al*. (2004b), as well as Gunterus *et al*. (2007) while crotyl alcohol was reported in the studies of Roze *et al*. (2004b).

2.10. Potentials of garlic as biocontrol agent against toxigenic fungi

2.10.1. Biology of garlic

Garlic as plant is placed in the onion species under the genus *Allium*. It is called *Allium sativum* in Botany but regarded as garlic as early as the times of William Woodville book on medieval Botany published in 1793. Garlic is very related to onions, shallot, chive, leek and the likes and classified scientifically as follows:

Kingdom Plantae: Plantae
Phylum: Angiosperm
Sub Phulum: Monocots
Order: Asparagales
Family: Amaryllideceae
Sub Family: Allioideae
Genus: Allium

Species: *Allium Sativum*

Binomial name: *Allium sativum* Lin.

It is synonymous with Allium arenarium, Allium contr*versum*, *Allium longicuspis*, *Allium pekinense*, *Porrum ophioscorodon*, and *Porrum sativum*. The word 'Garlic' is derived from old English 'garleac', which means 'peer shaped leek' from the words 'gar' spear and 'leek'.

Garlic is bulbous in nature, growing up to 1 metre height, it has hermaphrodite flowers and pollinated commonly by bees, butterflies, moths and other insects. It is a native of central Asia and northern Iran. It has been long used as seasoning plant all over the world for thousands years, it is known in ancient Egypt, Greece, Rome, China, and India traditional medicine. China is the country that produces the highest quantity of garlic in the world with over 80 % of the world's supply.

2. 10. 2. Nutritional and medicinal properties of garlic

Garlic is very nutritive in nature and has been used for thousands of years as spice. The garlic bulb according to Blumenthal *et al*. (2000) is rich with about 65 % of water, about 29 % carbohydrates, 2.40 % organic Sulphur compounds 2.5 % protein, 1.20 % amino acids and 1.49 % fibre. It contains more than two hundred of chemically important compounds including volatile oil, ajoene, allin, allicin, peroxidase, allinase, myrosinase, phellandrene, linalool, citral, geraniol and many vitamins such as A, B1, and C as well as mineral elements to mention a few. Therefore, garlic is biochemically dynamic in nature, it contains 17 amino acids, more than 20 kinds of sulfide compounds and several chemicals of therapeutic significance (Ariga and Seki, 2006). It has novel aroma and flavour and commonly used as spice in foods in many civilizations and cultures.

Garlic has long been referenced as medicinal plant and several data are emanating on its use as in the prevention of heart diseases, cancer and many other ailments (Milner, 1996). It has been shown that garlic act as protective against cancer (Fleischauer and Arab, 2001), several data have been generated like those of Ip *et al*. (1992) have established in garlic in suppression of breast, skin, colon, uterine, oesophagus, and lung cancers due to its sulphur components. Allicin a bioactive compound found in garlic is a sulphur

compound that gives garlic its special odour and flavour. This compound can reduce the risks of colon, pancreas, and breast cancer.

Although the mechanism at which garlic used in fighting cancers is not well understood but research has suggested that garlic can block the formation of N-nitroso compound $(NOC)_2$ and suppress its bio activation which leads to carcinogenesis (Lin *et al*., 1994; Fleischauer and Arab, 2001). In another study, it was reported that garlic can enhance DNA repair, reduces the cell growth and enhances the apoptosis induction to create protections against cancer cells. It was suggested that the allylsulphur compound found in garlic have been the active compound that fight the cancer cells through the influence of some materials in the diet.

Plate 2.2. Garlic bulbs and plant (Source: Bayan *et al.* 2014)

Thus, garlic is popular for its anticancer, antitumor, anti-bacterial and antifungal properties. Mei *et al*. (1989) earlier reported that 5 grams of garlic per day can reduce the formation of nitrosamine in humans; it can block the urinary excretion of nitroproline that arises from nitrate and proline in food supplements (Yang, 2001). In another study conducted by Sigounas *et al*. (1997), they demonstrated that garlic has the ability to suppress tumor cells of different origins; it enhances the inhibition of allyl sulfides. Researchers have compared the effects of feeding on mixture of fabricated allitridum extracted from garlic and the selenium intake to intake of foods with no additives in China for five years and results obtained confirmed high significant reduction of tumors by 30 % and stomach cancer by 52 % (Li *et al*., 2004).They also observed that this bacterium in laboratory model organisms fed with garlic additives also showed protective capabilities against skin, liver, and breast cancers.

Many researchers have also claimed that garlic can control high blood pressure according to Fleischauer and Arab, 2001). In some other studies, the anti-inflamatory and pro-inflamatory activities of garlic have been established. In some the immune modulatory effect of garlic was established while some also affirmed its anti-oxidant and neuroprotective properties (Blumenthal *et al*., 2000). Two compounds called S allylmercapto L custeine and S allylcysteine had been extracted from garlic and tested positive for their utmost radical scavenging activities (Thomson and Ali, 2003).

2.11. Potentials of ginger as biocontrol agent against toxigenic fungi

2.11.1. Biology of ginger

Ginger is taxonomically placed in the phylum Angiosperm, a monocot plant under Family *Zingiberaceae* and Genus *Zingiber*. It is classified in botany as follows:

Kingdom:	Plantae
Clade:	Angiosperms
Clade:	Monocots
Clade:	Commelinids
Order:	Zingiberales
Family:	Zingiberaceae
Genus:	*Zingiber*

Species: *Zingiber officinale* Roscoe

It is a flowering plant that produces rhizome in the root popularly known as ginger. The rhizome is the most popularly explored parts of the plant. Ginger is widely consumed as spice and commonly explored in traditional medicine. An herbaceous plant exhibits perennial life cycle but grows annually as pseudo stems during which it produces a false stem at the base of its leaves. It can grow about 1 meter with long narrowed leaf blades(Singh and Singh, 2019). This plant produces its inflorescences in pale yellow often with purple flowers and produces rhizome on separate shoot. Ginger is in the same family as turmeric, cardamom and galanqal, it originates from Island Southeast of Asia and then first domesticated among the Austronesians (Singh and Singh, 2019). It is one of the first spice transported from Asia to Europe and used by ancient Greeks and Romans. It produces white and pink clusters of flower buds which blooms into yellow flowers and this explains why it was commonly adopted for landscaping in sub-tropical houses (Ravindran and Nirmal-Babu, 2016).Ginger rhizome often gathered whenever the stalk withers and it has fragrance used as spice, sialagogue and condiment.Today, ginger is majorly produced in India (1, 109, 000 tonnes), Nigeria (522, 964 tonnes), China (463, 707 tonnes), Indonesia (340, 341 tonnes), Nepal (271, 863 tonnes) and so on with average annual world total of 3, 270, 762 tonnes.

2. 11. 2. Nutritional and medicinal properties of ginger

It is a key natural ingredient in Indian and Nigeria cuisine commonly eaten among the vegetarians and meat consumers. Ginger has been employed as substitute in many drinks and local beers, yoghurts, beverages, teas, and coffee. It is also consumes in salads, candies, cookies, in many dishes and snacks. It has crucial roles in traditional avuivedic medicine with popular usage for making pulse and lentil curie (Ravindran and Nirmal-Babu, 2016).

Plate 2.3. Ginger plant and rhizome (Source: Sutarno *et al.* 1999)

According to USDA (2012), 100 grams of ginger was analysed to contain; 79 g of water, 333 kJ of energy (80 kcal), 17.77 g of carbohydrates, 1.7 g of sugars, 2 g of dietary fiber, 0.75 gfat, 1.82 g protein with several vitamins such as thiamin (2 %, 0.025 mg), riboflavin (3 %, 0.034 mg), niacin (5 %, 0.075 mg), pantothenic acid (4 %, 0.203 mg), Vitamin B6 (12 %, 0.16 mg), folate (3 %), vitamins C (6 %, 0.026 mg), vitamin E (2 %, 0.026 mg) as well as many minerals such as Ca (2 %, 16 mg), Fe (5 %, 0.6 mg), Mg (12 %, 43 mg), Mn (11 %, 0.23 mg), P (5 %, 34 mg), K (9 %, 415 mg), Na (1 %, 13 mg), Zn (4 %, 0.34 mg). Ginger is used fresh or dried for pregnant women or nursing mothers for strength. It contains essential phytochemicals such as zingerone such as gingerols that gives it its pungent smells, shogaolsand gingerols, and enzymes such as zingibain a cysteine protease (Ravindran and Nirmal-Babu, 2016). It posses several medicinal properties such as alleviation of nausea, vomiting (especially during pregnancy), dysmenorrhea, osteoarthritis, heartburns, allergic reactions like rashes and so on. It has been characterised as powerful antioxidant, anticoagulant, antimicrobial and so on (Ravindran and Nirmal-Babu, 2016).

2.12. Potentials of turmeric as biocontrol agent against toxigenic fungi

2.12.1. Biology of turmeric

Turmericis taxonomically placed in the phylum Angiosperm, a flowering plant under Family *Zingiberaceae* and Genus *Zingiber*. It is classified in botany as follows:

Kingdom:	Plantae
Clade:	Angiosperms
Clade:	Monocots
Clade:	Commelinids
Order:	Zingiberales
Family:	Zingiberaceae
Genus:	*Curcuma*
Species:	*Curcuma longer* L.

It is flowering plant that produces root, which is commonly used for cooking in different dishes. It is a perennial plant, produces rhizome and grows as an herbaceous plant. It is native to India and generally to Asia continent, requires 20 to 30 ^{0}C temperature for

growth and a handful amount of rainfall. It is propagated through its rhizome and this is the most explored part of turmeric (Priyadarsini, 2014).

2. 12. 2. Nutritional and medicinal properties of tumeric

The rhizome of turmeric is the most popularly eaten part, often consumed fresh, dried or boiled in water. It is also commonly processed into powder for use as coloring for several meals and cuisines. Turmeric powder is characteristically warm, bitter with peperlike flavor with mustard like aroma (Priyadarsini, 2014).

It is commonly used in Avurvedic medicine due to its phytochemical constituent called curcumin. Analysis of 100 g of turmeric powder showed that it contains about 60 to 70 % carbohydrates, 6 to 30 % water, 3 to 7 % minerals, 6 to 8 % protein, 3 to 7 % essential oils, 5 to 10 % fat, 2 to 7 % fibre, and 1 to 6 % curcuminoids. The phytochemical components of turmeric were analysed to include key chemicals such as curcumin, demethoxycurcumin and biseemethoxycurcumin (Priyadarsini, 2014).

Turmeric was reported to contain about 34 different essential oils such as turmerone, atlantone, germacrone, zingiberene and so on (Priyadarsini, 2014). This plant has also been used fresh or dried for several medicinal properties such as alleviation of oxidants, inflammation, heartburns, allergic reactions like rashes and so on. It has also been characterized as powerful antimicrobial material (Ravindran and Nirmal-Babu, 2016).

Plate 2.4. Turmeric plant and rhizome (Source: Kew England, 2018)

2.11. Status of mycotoxin control and research in Nigeria

The hazardous influence of mycotoxins against the human and animal health cannot be overemphasised. Aflatoxin research started in Nigeria as far as since 1961 when a team from United Kingdom arrived Nigeria on sole objective of alerting Nigerian populace on the incidence of fungal toxin called aflatoxin under the Tropical Products Research Institute, UK. The then Government of the Northern Nigeria according to Blount (1961) enacted a government committee, departments and research institutes. Key examples are the Institute of Agricultural Research (IAR) located at Samaru community in Zaria Nigeria and the Nigerian Stored Products Research Institute (NSPRI) located at Ibadan Oyo State Nigeria.

The NSPRI relies on the use of an analytical protocol of Thin Layer Chromatography (TLC) for quantification of aflatoxins in Nigerian foods and feeds.The IAR between 1961 to 1966 through Mac-Donald and colleagues run mycotoxins analysis through the use of Laboratories of the Tropical Products Research Institute London according to Mac-Donald *et al.* (1965). This IAR is a Unit under the Department of Crop Protection although this unit later changed their mycotoxin analytical methods after the establishment of their mycotoxin research laboratory in 1980 (although no more function today). Nigeria is unfortunately face with so many challenges on the control of mycotoxins despite the fact that the researches on mycotoxins have started in Nigeria as far as early 1960s sequel their realization worldwide. Several problems such as limited government and interagency interests, inadequate fund, unavailability of modern machine/techniques, inadequate skills and expertise among several others.

The TLC is an easy protocol for mycotoxin analysis but still face with challenges of scarcity and expensive mycotoxin standards to run the process, the standards are not available in the country and hence have to be imported from abroad at a price which many Nigerian institutions could not continually afford nor either sending the samples abroad. The Universities in Nigeria could not afford necessities for mycotoxin researches due to fund unavailability and poor structural adjustment program in 1986. Many foreign

companies are also sceptical of selling out their mycotoxin standards as it can be used as bioweapons.

Up till date, the IITA remained the only institute that continue carrying out reliable researches and awareness programmes such as workshops on mycotoxins in Nigeria (Bankole and Adebanjo, 2003). Presently, there have been increase interests for researches on mycotoxins in Nigeria which require the use of of more advanced methodologies such as the use of Enzyme Linked Imunosorbent Assay (ELIZA), the use of High Performance Liquid Chromatography (HPLC), the use of Gass Chromatograph linked with Mass Spectophotometry (GCMS), and so on. These methods can provide better and faster quantification and support multi analysis of mycotoxins. Fortunately, there are now kits with which some companies and organisations can afford, but there is need to use some advance equipment and personnel for interpreting them. Many institutions in West Africa including Nigeria do not have necessary funds to maintain the use of these machines, presently no LC-MS/MS in any University at all and there is no or less cooperation between research institutions and the government in formulating policies or regulations on mycotoxins especially on many streets vended foods and feeds.

The Mycological Society of Nigeria (MSN) has been involved in many sensitization program with the government at all levels for creation of mycotoxin control unit in Nigeria. This was to establish extension services on the risks of mycotoxin incidence in Nigeria but it is yet to get full mandates from some concerned governmental agencies such as the Ministries of Environments and Agricultures, National Agency for Food and Drug Administration and Control (NAFDAC), the Standard Organisation of Nigeria (SON) and so on. Many of these government agencies are also not interested in deep mycotoxin research on local foods as their attentions focused on the imported and exported goods. Neither training nor funds are made available for researchers to carry out mycotoxin assessment works across Nigeria.

The NAFDAC and IITA were formally involved in training under certain arrangements on mycotoxin training (MYCORED) but funds are no more available to intensify such training.Unfortunately, more capacity building and concerted efforts are needed from

government and stakeholder organization to work intensively on mycotoxin incidence in Nigeria especially on locally/streets vended foods.

2.12. Molecular studies on aflatoxins

Aflatoxins (AFs) is one of the major mycotoxins of significant impact in many areas of the human life. AFs is the most popular mycotoxin and one of the most hazardous carcinogens. Aflatoxin was firstly discovered in relation to the Turkey X disease (a disease with a symptom of hepatotoxicity) in poultry at Great Britain in the early 1960s. The AFs were isolated from this poultry field and identified to cause the death of several animals (Nesbitt *et al*., 1962). The aflatoxin was then discovered to be produced by a fungus Aspergillus flavus from which it was named aflatoxin (Williams *et al*., 2004) and since then has sparkled several researches on AFs in crops, foods and feeds across many countries as reported by Turner *et al*. (2007).

The aflatoxin synthesis mechanism by *A*. *flavus* and *A*. *parasiticus* has receive research interests over the years. The first gene that was revealed to be involved in aflatoxin biosynthesis were identified as *nor* and *ver* genes which include clan *nor1* and *ver1* that were cloned, expressed and sequenced in 1992 (Chang *et al*., 1992). As more research unfolds in this subject area it was later observed that full aflatoxin cluster was sequenced and characterized in 1995 as reported by Trail *et al*. (1995) Many Aflatoxin biosynthesis genes and control enzymes for biosynthesis of AFs were reported and documented today through the advancement in molecular biology and biotechnology.

This has helped much understanding on aflatoxin biosynthesis. Thirteen (13) *Aspergillus* species were studied and observed that they were able to secret certain AFs and sterigmatocystin (ST), they observed that these fungi were able to make these secretions through an intermediate biosynthesis aided by some enzymes (Varga *et al*., 2011), they identified *A. flavus, A. parasiticus*, and two other new aflatoxigenic species *A. pseudocaelatus* and *A. pseudonomius* to be the major aflatoxin producing fungi.

Some other fungi which are not *Aspergillus* species were also being reported to be able to produce some structural analogous aflatoxin compounds. *Fusarium kyushuense* according to Schmidt-Heydt *et al*. (2009) was reported to be capable of producing AFB_1 and AFG_1.

Another filamentous ascomycete fungus called *Podospora anserina* was recently reported to process ST biosynthesis gene which enables its ability to produce ST toxins. In another study by Slot and Rokas (2011), it was reported that the ST gene found in *Podospora anserina* must have been horizontally acquired from *Aspergillus nidulans*.Incomplete aflatoxin gene clusters are found in many fungal species that are pathogens of some conifer plants across the world.Many of these fungi were found in the class of *Dothideomycetes* such as fungi *Dothistroma septosporium* and *Dothistroma pini*. These fungi were reported to be able to produce red compound called dothistromin that is structurally similar to an intermediate aflatoxin biosynthetic product called versicolorin B (Zhang *et al*., 2010).

It has been established that the toxicity of aflatoxin B1 in in sighting cancer, mutations as well as cytotoxicity in human and in some animal tissues is commonly controlled by their reaction with some short-lived electrophilic aflatoxin epoxides, which in turn affect the DNA amino acids. Examples of these epoxides are the AFB_1 exo epoxide and the AFB_1 endo-8,9-epoxide. In the liver, biosynthesis of aflatoxin is carried out by principal enzyme called cytochrome (Cyt) P-450 1A2 and 3A4 the protective structural enzyme in the liver against aflatoxin epoxides is the main aflatoxin-detoxifying enzyme known as glutathione. However, any attacks or defect in GST controlling detoxification of aflatoxin can completely shut down the process (Klein *et al*., 2000).

It has also been reported that high resistance of mice to aflatoxin contamination is as a result of their high level of GST isozyme which is active against Aflatoxin epoxide in the liver according to Chanda *et al*. (2009a) and Roze *et al*. (2007a). It was also revealed in a study by Chanda *et al*. (2010), who with the use of fluorescence microscopy and some specific antibody to suppress aflatoxin toxicity in cell patches using a wild type *Aspergillus parasiticus* SU-1. They also observed that the aflatoxin inducing condition for the fungus correlates with onset of transcriptional enzymes which are involved in biosynthesis of aflatoxins and also observed that some polyclonal antibodies can specially recognize some early or middle or late enzymes which are involved in the biosynthesis of aflatoxins.

The use of western blot and semi quantitative transcriptional analysis of aflatoxin expression established the transcripts of *nor, ver, omtA* and *vbs* genes expressed within 24 h and 50 hr in many aflatoxigenic fungi in the presence of carbon sources such as glucose and sucrose (Roze *et al*., 2007b). In another study, on intracellular localization, fluorescence and some researches on scanning microscopy and on transmission electron microscopy were used to study the nor, ver, omt-A and vbs aflatoxin biosynthetic enzymes in fungi localized to the cytoplasm shortly after these genes were synthesised and moved into the vesicles and vacuoles some differential agar media that can induce aflatoxin (Hong *et al.*, 2008).

They also observed that *old* and *vbs* were expressed through the fungal cytoplasmic reticulum with observable ring and patches characteristics around the nucleus after 24 to 48 hrs.The researchers suggested that the early, middle and late nor, ver, omtA and vbs fungal aflatoxin biosynthetic enzymes move to the vacuole through fungal cytoplasm-vacuole pathway.

Chanda *et al* (2009b) gave direct experimental evidence showing some membrane bound organelles taking part as sites of aflatoxin biosynthesis. In their research, they purified the V-fraction which is a subcellular fraction that houses some vesicles, endosomes and small vacuoles, this fraction was purified and aflatoxin biosynthesis enzymes such as the nor, ver, omtA and vbs were detected in it. It was later confirmed that this fraction could convert ST and VA intermediate pathways to aflatoxin formation. This was verified in the middle and late aflatoxin enzymatic activities in the study of Linz *et al*. (2012) in the V fraction.

The aflatoxin biosynthesis needs to be linked distinctively with vesicle and this was explained by chanda *et al*. (2009a; 2010), the transfer of aflatoxins via the inner membrane of vesicles was destroyed biochemically using some bioactive compounds (sortins) and genetically by distruption of avaA gene tethering complex. AvaA is a common homotypic fusion protein protein sortin (HOPS) which controls the fusion of perivascular chambers into the vacuole. They observed that sorting application and avaA destruction in small vesicles resulted in the production of more aflatoxin biosynthesis

enzymes and hence the quantity of aflatoxin that was produced extracellularlly into the growth medium.

This is an indication that the aflatoxin production and storage in the perivascular chambers or in the early or late endosomes are factors to be considered in the extracellular production of aflatoxins by toxigenic fungi. Popular novel functional structures and chemical characteristics of proteins in the vesicle fraction was revealed by Linz *et al*. (2012) which working on fungi that are capable of producing aflatoxins using high throughput machines like LC-MS MS and NMR. They revealed that the vesicle fraction apart from being capable of acting as houses for biosynthetic enzymes, they also take crucial parts in aflatoxin biosynthesis. This v fraction also houses several other enzymes that are involved in biosynthesis of other secondary metabolites like patulin, polyamides, amines, isoprene and shikimate.

Some proteins, which are concerns with the cell response and management of heat and other external stresses like osmotic and oxidative stresses, have been detected in the V fraction. The involvements of these proteins in the vesicle chambers have raise intriguing hypothesis that localisation and migration of secondary metabolite biosynthetic enzymes are produced in the v fraction. This then anoted the whole functional and regulatory interactions in relation to stress responses and secondary metabolism to the v fraction. It was also concluded that the secondary metabolites have some protective roles in the response, survival and adaptation of different biotic organisms.

CHAPTER 3
MATERIALS AND METHODS

3. 1. Sites and sampling procedures

Surveyand collection of *Kulikuli* samples were carried out across five (5) different states in Northern Nigeria, sampled groundnut cake (*Kulikuli*) were located using the Geographical Information System (GIS) where 2 kg each of the sample were collected at five (5) markets in each state. The locations covered as described in figure 3.1 were within two (2) geo-political zones namely Northwest (NW) and North Central (NC). The Northwesternstate occupies 80% of the sampled state with North central occupying only 20% of the sampled state.

The samples were collected with both random and purposive techniques. The purposive technique of sampling was employed to select five (5) states in Northern Nigeria while the method of Simple Random Technique was use to locate twenty-five (25) markets from each of the selected state were the sampling would be done. The simple random technique of sampling followed the procedures of writing the names of all market from each state on pieces of paper, folded and packed in a container, they were mixed thoroughly and then five papers were selected randomly from the container without replacement. The market picked were the ones selected for sample collections and this was done separately for each state.

The North-western states include Kano, Kaduna, Zamfara and Sokoto while the North central state sampled was only Abuja-FCT. The Kano state is located besides the Katsina state in the North/West part, borders with Jigawa state in the North/East side, Bauchi state in the South/East while it borders with Kaduna state in the South/West part. Kano state is made up of about 20, 131 km^2 land mass and located around the latitude of 11^o 30 ‘ N and at the longitude of 8^o 30 ‘ E. Kano state is one of the most populous states in Nigeria.. It is

dominated by the Hausa people, and has 44 local government area, including the capital city, Kano

Kaduna state is also found in the Northern part of the country's high plains. It has vegetation of Sudan savannah characterised by many scattered grasses, shrubs and short trees. This state is of a total land mass of 46,053 km^2 which is about 17,781 sq mi, it lies at the latitude of 10^o 20 ' N and located around the longitude of 7^o 45' E. Kaduna state comprises of 23 local government areas including about 63 different ethnic groups.

Zamfara state on the other hand comprised of 14 local government areas with a land mass of 39,762 km^2 which is 15,352 sq. mi, it lies on the latitude of 12^o 10 ' N and located around the longitude of 6^o 15 ' E. This state is located besides Niger Republic in the North side and Kaduna State in the South side. It borders with Katsina state in the East side and borders with Sokoto state as well as the Niger state in the West side.

Sokoto State is located towards the extreme North/Western part of the country around the confluence of Sokoto river as well as the Rima river. Sokoto state lies around the latitude of 13^o 05 ' Nand the longitude of 28^o 03 ' E, it has a land mass of 25,973 km^2 which is about 10,028 sq mi, and average annual temperature of 28 ^{0}C equivalent to 82.9 ^{o}F. It is a relatively hot state, the rainy season in Sokoto usually start in the month of June and often runs till October each year most of which includes showers on daily basis.

The Federal Capital Territory(FCT) Abuja is located at the central part of Nigeria, it has a total land mass of 7,315 km^2 that is 2,824 sq. mi,. It is located toward the North of the confluence of River Niger and River Benue and it is bordered in the West/Northern side with Niger state. It also shares borders with Nasarawa State at the East/Southern part as well as Kogi State in the South/Western part. It lies between the latitude of 8.25^o 9.20 'N and longitude of 6.45^o 7.39'E. Geographicaly, Abuja at the centre of Nigeria with six local councils including the Abuja city and five (5) local government areas.

Geographically, the North-western states of Nigeria include; Kano (total landmass of 20,131 km^2 and lies within latitude 11°30′N and longitudes 8°30′E), Kaduna (total landmass of 46,053 km^2 or 17,781 sq mi and located at the latitude of 10^o 20 'N and at the longitude of 7^o 45 'E). The Zamfara state (total landmass 39,762 km^2 or15,352 sq mi and located around the latitude of 12^o 10 ' N and at the longitude of 6^o 15 ' E) and Sokoto (total

landmass of 25,973 km^2 or 10,028 sq mi and lies within latitude 13°05′N and longitude 05°15′E) while the North central state sampled was only Abuja-FCT (total landmass of 7,315 km^2 or 2,824 sq mi).Notably, the samples were collected between February and March 2016, the identity of vendors from which the samples were sourced are not mentioned for confidential reason.

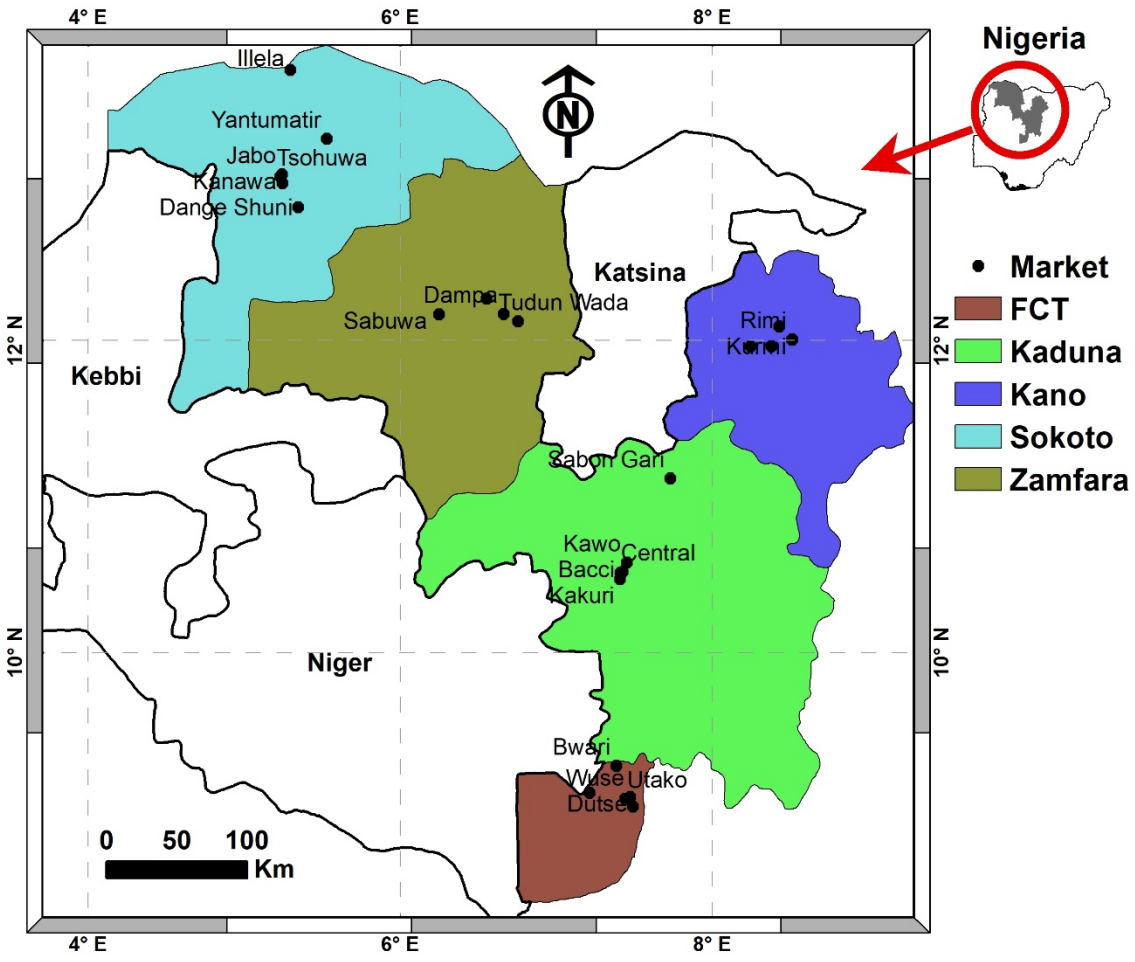

Figure 3.1. Map showing the locations of collected *Kulikuli* samples

Table 3.1. Details of sampling locations and number of *Kulikuli* samples

GPZ	State	Market	Sample	Code
NW	Kano	Dawanau	1	Kano 1
		Janguza	1	Kano 2
		Kurmi	1	Kano 3
		Rimi	1	Kano 4
		Rimingado	1	Kano 5
		Total	**5**	
NW	Zamfara	Bungudu	1	Zamfara 1
		Dampa	1	Zamfara 2
		Sabuwar Kasuwa	1	Zamfara 3
		TsohuwarKasuwa	1	Zamfara 4
		Tudunwada	1	Zamfara 5
		Total	**5**	
NW	Kaduna	Bacci	1	Kaduna 1
		Central	1	Kaduna 2
		Sabuwar Gari	1	Kaduna 3
		Kakuri	1	Kaduna 4
		Kawo	1	Kaduna 5
		Total	**5**	
NW	Sokoto	Dange Shuni	1	Sokoto 1
		Ilaela	1	Sokoto 2
		Jabo	1	Sokoto 3
		Kanawa	1	Sokoto 4
		Yan tumatir	1	Sokoto 5
		Total	**5**	
NC	Abuja-FCT	Bwari	1	Abuja 1
		Dutse	1	Abuja 2
		Madalla	1	Abuja 3
		Utako	1	Abuja 4
		Wuse	1	Abuja 5
		Total	**5**	
Control 1	ML prepared UI *Kulikuli* without botanical: garlic additive	**Total**	**5**	ML
Control 2	ML prepared UI *Kulikuli* with botanical: garlic additive	**Total**	**5**	$MLBG_1$

GPZ=Geopolitical Zone; NW = North West; NC = North Central.

3.2.Production of *Kulikuli* and garlic additive

Modified methods of Jimoh and Kolapo (2008) as well as that of Ezekiel *et al*. (2013) were adopted to bake *Kulikuli* in this study. Groundnuts were roasted and grounded to form a paste normally refers to as Labu. Labu is further process by mixing it with salt and powdered pepper and depending on the desires outcome spices was added to give it desired smell. The mixture of Labu and other added ingredients are mixed into paste and moulded into desired shapes round, cylindrical and spiral.

Kulikuli were prepared under aseptic laboratory condition at the Mycology Laboratory (ML) Department of Botany, UI using fresh healthy dried groundnuts, this preparation was done and treated as control. The laboratory samples were prepared in different groups as coded below:

- **i.** Mycology Laboratory, Department of Botany, UI *Kulikuli* without botanicals (ML).
- **ii.** Mycology Laboratory, Department of Botany, UI *Kulikuli* with botanical: garlic additive ($MLBG_1$).
- **iii.** Mycology Laboratory, Department of Botany, UI *Kulikuli* with botanical: ginger additive ($MLBG_2$).
- **iv.** Mycology Laboratory, Department of Botany, UI *Kulikuli* with botanical: turmeric additive(MLBT).
- **v.** Mycology Laboratory, Department of Botany, UI *Kulikuli* with botanicals: garlic and ginger additives ($MLBG_1G_2$).
- **vi.** Mycology Laboratory, Department of Botany, UI *Kulikuli* with botanicals: garlic and turmeric additives ($MLBG_1T$).
- **vii.** Mycology Laboratory, Department of Botany, UI *Kulikuli* with botanicals: ginger and turmeric additives ($MLBG_2T$) and
- **viii.** Mycology Laboratory, Department of Botany, UI *Kulikuli* with botanicals: garlic, ginger and turmeric additives ($MLBG_1G_2T$).

Plate 3.1. Different shapes and sizes of *Kulikuli* samples

Garlic, ginger and turmeric were supplemented at 0.05 mg/100g of milled groundnut in the MLB following the recommendation of the European Union on the addition of spices in foods.

Thesamples wereprepared in three replicates and arranged in different groups specifically as follows:

ML = No Garlic additive (control).

MS = Market samples: Kano, Kaduna, Sokoto, Zamfara, and Abuja FCT with no additives.

$MLBG_1$ = Garlic additive (0.05 mg) + 100 g total groundnut.

$MLBG_2$ = Ginger (0.05 mg) + 100 g total groundnut.

MLBT = Turmeric (0.05 mg) + 100 g total groundnut.

$MLBG_1G_2$ = Garlic (0.025 mg) + Ginger (0.025 mg) + 100 g total groundnut.

$MLBG_1T$ = Garlic (0.025 mg) + Turmeric (0.025 mg) + 100 g total groundnut.

$MLBG_2T$ = Ginger (0.025 mg) + Turmeric (0.025 mg) + 100 g total groundnut.

$MLBG_1G_2T$ = Garlic (0.013 mg) + Ginger (0.013 mg) + Turmeric (0.013 mg) + 100 g total groundnut.

The oil removed was thereafter heated and employed for frying themoulded labu pastes to form *Kulikuli*. The procedure employed for production of *Kulikuli* in this study is presented in Figure 3.2 below.

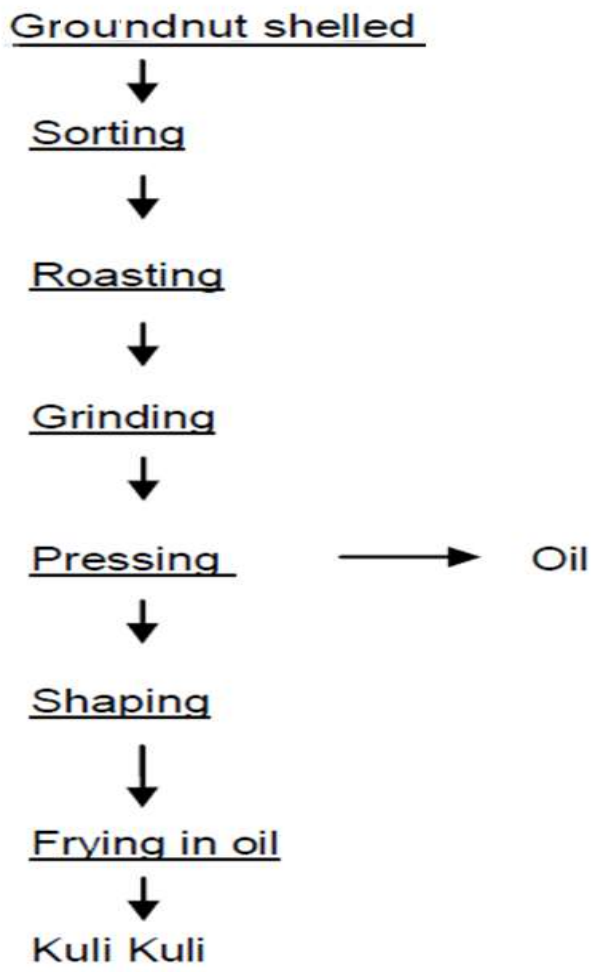

Figure 3.2. Flowchart of laboratory preparation of *Kulikuli*

Source: Jimoh and Kolapo(2008)

3.3.Proximate and chemical analysis of *Kulikuli*

The collected *Kulikuli* from markets across all the selected states were analysed to detect their nutrient composition as compared to the laboratory prepared ones with or without garlic additive. The samples were analysed for nutrient compositions by following the methods of the Association of Official Analytical Chemistry AOAC. (2008). The samples (market and laboratory samples) were predigested each based on the type of analysis to be carried as follows:

3.3.1. Analysis of crude protein content

The crude protein content of each of the sample was analysed with semi-micro kjeldahl method. This method consists of three phases namely; the Digestion, Distillation and Titration. The Digestion method involves the addition of 0.5 g of fine grinded *Kulikuli* sample into the kjedahl digestion chamber followed by the addition of concentrated N_2SO_4, the mixture was briefly shaken and then transferred in to the hole of the digestion block heater in the fume cupboard. Here, the mixture was left to digest for four(4) hrs until a clear colorless solution is formed in the tube.Then the chamber was cooled and the mixture therein (100 mL) was transferred into a clean volumetric flask and made up to 150 mL by adding distilled water.

Distillation procedures followed the digestion process;the process was carried out using the Markham Distillation Apparatus (MDA) through which volatile substances like ammonia were steam distilled with several other collections of distillates. The MDA was streamed for some minutes and the steam generator was removed thereafter from heat source in order to let the developing vacuum remove the condensed water after which the steam generator was replaced back on the heat mantle which is the heat source and other component of the kjeldahl apparatus was properly fixed.

After this, 5 mL of the digest was pipetted into the apparatus body through the aperture of its small funnel and then 5 mL of 40 % NAOH (W/V) was added and stired up. The mixture was further steamed and distilled for 2 min and pipetted 5 mL into another conical flask, which is already containing 10 mL of 2 % Bone Acid solution of mixed indicator.

The Bone Acid solution change the color of the solution from red to greenish coloration and this indicated that the trapping of the ammonia liberated.

The Titration procedures follow after obtaining the green colored solution. The solution was thereafter titrated against 50 mL of OO1N111.C in burette and the green color turned to wine at the stage of end point also known as equivalent point, this was an indication that all the Nitrogen trapped as Ammonium borated NH_4BO_3 had been removed as Amonium chloride solution NH_4CL. The percentage of this removed Nitrogen in the analysis was calculated using the formula below:

% Nitrogen = Titer value X Atomic mass of N X Normality of the used HCL X 4% Nitrogen = Titer value X Normality or Mortality of the used HCL X Atomic mass of N X volume of the flask containing the digest.

Weight of the digested sample in milligram X volume of the digested steam distillation. The crude protein (CP) content in the samples is therefore determined by multiplying the percentage Nitrogen by a constant factor of 6.25, i.e. the CP (%) = % N X 6.25.

3.3.2. Analysis of crude fat content

The crude fat content in each of the *Kulikuli* samples was analysed by using soxhlet extraction method. 1 gram of dried sample powder in Fat Free Extraction Thimble (FFET) and put inside a lightly cotton wool. The FFET was placed inside an extractor and fitted with a reflux condenser, 250 mL of soxlet flask that have been previously oven dried, cooled in dissector and weighed. The soxlet flask was then filled up to a three quarter of its volume by pouring petroleum ether of 40-60 ^{0}C bpt. The extraction plus condenser was set and placed on the heater for six hours with constantly running water from the tap in other to condense the vapour.

This was closely monitored for ether leaks and in other to adjust the heat source for the ether to brit gently. Thereafter the ether is left to siphon over sometimes up to15 times until siphoning is exhausted and it was later realised that all the ether contents extracted was completely drained into the ether stock bottle. The thimble that is containing the sample was then remove after this observation and dried on a clock glass placed on the bench.

The procedures followed by replacing the extractor, flask and thecondenser to continue distillation until the flask is completely dried. The flask containing the fat was detached and its exterior cleaned and weighed constantly after placing in the oven. The fat content of the sample is calculated as follows:

$W_1 - W_0$ X 100/1 W_t

Where W_0 is the initial weight of the dry soxlet flash, W_1 is the final weight of the oven dried flash and oil/fat content.

3.3.3. Analysis of moisture content

The moisture content of each of the *Kulikuli* samples was analysed using 2 g of each sample. Each sample was placed in a crucible of known weight. The crucible and the sample then placed inside an oven programed at 100 ^{0}C for 12 hrs after which the crucible and the sample was placed on desiccator, allowed to cool and weighed. Thereafter, it was returned back in the oven, and repeatedly weighed after 12 hrs until a constant weight is achieved. The moisture content of the sample was calculated as:

$$\% \text{ MC} = \frac{W_1 - W_0}{W_3 - W_0} \; x \; \frac{100}{1}$$

Where MC is the moisture content, W_0 is the initial weight of the crucible, W_1 is the weight of sample together with the crucible, and W_3 is the weight of crucible plus the oven dried sample.

3.3.4. Analysis of ash content

The ash content was analysed by placing 2 g of each of the *Kulikuli* sample in a porcelain crucible and together placed on muffle furnace of 55 ^{0}C setting and this was heated for 4 hours, the sample was thereafter turned into white ash. The crucible and content were allowed to cool in air to about 100 ^{0}C, and further allowed to cool up till room temperature in a dessicator. The ash content was weighed in three replicates and the percent ash was calculated as follows:

$$\% \text{ Ash content} = \frac{wt\ of\ ash}{Original\ weig \quad of\ sample} \; x \; \frac{100}{1}$$

3.3.5. Analysis of crude fibre content

The crude fibre of the *Kulikuli* samples was analysed by using 2 g of the sample placed in flask and added with 100 mL of 0.255 molar NH_23O_1 solution. The mixture was briefly mixed and heated in the heating mantle of a reflux machine for 1 hr. The hot mixture was filtered through a fibre sieve cloth. The result fluid filterate was discarded and the residue was returned back to the fibre flask. The residue was thereafter added with 100 mL OF 0.313 NNaOH solution and again heated under heating mantle of a reflux machine for another I hr. The resulting mixture was again filtered through a fibre sieve cloth.

The result fluid filtrate was discarded and the residue was returned back to the fibre flask. 10 mL of acetone was added to the residue to dissolve the organic constituents and the mixture was washed twice with 50 mL of hot water on the sieve cloth and thereafter returned back into the crucible. The crucible with its new content was oven dried at 100 ^{0}C for 12 hrs to remove moisture content and weighed to obtain W_1, the crucible together with the $W_1$was transferred into the muffle furnace and washed at 55 ^{0}C up till 4 hrs. The crucible and the white ash content was thereafter cooled in dessicator and reweighed again to have $W_{2..}$ The percent fibre content was calculated as follows:

$$\% \text{ fibre} = \frac{W_1 - W_2}{wt\ of\ sample}\ x\ \frac{100}{1}$$

3.3.6 Analysis of pH

The pH of the *Kulikuli* sample was calculated using a pH meter. 10 g of the *Kulikuli* sample was powdered and dissolved in 50 mL of distilled water, the resulting solution was then filtered in a conical flask and the pH was taken using the calibrated meter by standardising it in buffer of 4.0 and neutralise in 7.0 pH.

3.4.Fungal analysis of *Kulikuli*samples

The collected *Kulikuli* samples were briefly rinsed with 99 % ethanol to surface sterilize it. Each sample was point inoculated into a prepared Potato Dextrose Agar (PDA) plate for

fungal isolation and incubated at 35±2 ^{o}C for 4-7 days in the incubator as described by Jonathan, *et al*. (2008).

There was inclusion of streptomycin sulphate in the agar during preparation for bacterial growth prevention (Jonathan and Fasidi, 2001). Inoculation was done in three replicates, the mixed fungal cultures were separated on different PDA plates and fungal growth characteristics were recorded within 4-7 days of incubation. Each isolate was mounted on microscope slide using 0.1% lactophenol cotton blue for microscopic study (Jonathan and Fasidi, 2001).Incidence of each fungus was calculated on percentages across each state to determine the dominant fungus associated with *Kulikuli* in each state (equation 1).

$$\text{Percentage incidence} = \frac{Number\ of\ a\ particular\ fungal\ spcie}{Total\ Number\ of\ fungal\ species} \text{ X } 100 \qquad (1)$$

3.5. Characterization of fungal isolates

This was done using the growth and morphological characteristics of the isolated fungal strains. The fungi were checked for their features such as colony size and shape, surface feature on plate and underside such as Colonial appearance color, texture, growth pattern, microscopic features such as conidia, sporangium and hyphae arrangements and these were compared with those of the already identified fungal strains. The strains were mounted on slides according to Pubert and Winton (2013) and Asemoloye *et al*. (2017).

The colonies of the fungal strains were studied on plates and observed for peculiar characteristic as follows;

i. Colonial morphology.
ii. Growth rate.
iii. Colony colour.
iv. Texture.
v. Upper and underside appearances.

The young (4-day-old) fungal mycelium was mounted using Lactophenol cotton blue stain on the microscope examined using X10 and X40 objectives, studied for fungal microscopic characteristics based on the size and shape, as well as the surface features of conidia and the arrangement pattern of hyphae. This preparation was done following the methods in

accordance with Andrew and Okorokov (1994) and Asemoloye *et al*. (2017). Each and the total isolated fungal strains were compared (equation 1) to know the most abundance fungal specie that is associated with the sample

3.6. Chemical analysis for detection of aflatoxin in *Kulikuli*

The chemical analysis used for the detection and quantification of aflatoxins in the *Kulikuli* samples involved the use of High Performance Liquid Chromatograph (HPLC) machine model S433 (Sykam). The process adopted for the purpose was the protocols of Oluwafemi and Ibeh (2011) and that of Jonathan *et al*. (2016a). The HPLC used contained column LDC, made of pump called Milton Riy Constametric 1 pump and a column type Lichrosorb which is a RP 18 column (Merk Hiber).

The machine has a particle size of 5.0 μm with a length of 125 mm and inside diameter of 4.0 mm. The machine is also made up of 60 MPa pump pressure packaged with an automatic type injector made of version of Rheotype Gilson Abimed 231. The machine is also made up of 535 fluorenscence spectrophotometer detector coupled with 365 gamma excitation and 444 nm gamma emission with a flow rate of 1 mL per one minute while the injection volume was 50 μL with a mobile phase of which contains water and acetonitrite solutions at 75 ratio 25 respectively. It has a flow rate of 1.2 mL per minute for 20 min.

The analysis started through the digestion of samples (40 g per sample), this was done using soxlet type extractor. The solvent used for the extraction was N-hexane. The defatted *Kulikuli* (40 g each) was placed in the soxlet extraction thimble and 50 mL of solution containing ethyl acetate was added and extracted three times. The extracts were poured together and dried up by adding anhydrous sodium sulphate; the remaining mixture was further concentrated near dryness under the vacuum pump and transferred into a brown glass vial to be evaporated under nitrogen stream.

The remaining mixture was cleaned up and the extract was resuspended in 1 mL chloroform and applied to 14 x o.8 cm column containing 2.5 Kiesel type of 60 gel and silica gel of 70/230. The aflatoxin analysis was carried out by loading 1.5 μL on the HPLC column and compared with aflatoxin standards. The aflatoxin analysed included the following:

i. AFB_1

ii. AFB_2

iii. AFG_1

iv. AFG_2

3.7.Molecular studies

The fungal strains isolated from *Kulikuli* samples were characterized molecularly based on the use of Simple Sequence Repeats (SSRs) primers, which is known as microsatellites. This method has been proven to be the best in the last decade for plant research because of the hyper-variability of markers and ease of gene detection. SSR markers were developed through the SSR enriched libraries to be used for amplification of aflatoxin biosynthesis genes in each fungus (Rahimi *et al*., 2007). Ten (10) SSR primers were designed (Table 3.2); the *Aflr* gene Encoding the Pathway-Specific Transcription Factor, which is specific for all aflatoxigenic fungi.The*Nor*, *Ver* and *Omt* genes are the three structural cluster genes in biosynthesis aflatoxin pathway essential for aflatoxin production. These cluster genes contain structural, regular and unassigned genes, which plays a key role in the production of aflatoxins. Six (6) other primers are to be used for amplification of targeted regions of a particular fungal strain for mycotoxin biosynthesis pathway (Table 3.2).

3.7.1. Fungal DNA extraction

The DNA of each selected strains of fungi was extracted with a DNA extraction kit called ZR Fungal DNA Miniprep Kit following the manufacturer procedures. The Kit was prepared at 30^0C. 100 mg of fungal mycelium was first briefly marcerated in isotonic buffer (PBS) of 200 µL volume in the PBS bottle and then 200 µL of ZR Bashing Bead lysis buffer was added with the mixture in the beading tube. A bead beater was thereafter fitted with 2 ml tube for about 5 minutes to holder and assembled to secure a maximum speed. This mixture was then centrifuged at spinning rate of 10,000 x g for 1 min and 400 µL of the supernatant was decanted into another Spin filter tube of the Zymo Spin orange top collecting tube. The tube was again spinned at the rate of 7, 000 x g for 1 min and the supernatant was decanted in another tube and 1,200 µL of DNA binding buffer was added to the mixture, briefly vortexed and then spinned again at 10, 000 x g for 1 min. Thisspinning step was repeated again and 800 µL of supernatant was then decanted into a new collection Zymo Spin tube.

Table 3.2.SSR primers used for identification of aflatoxigenic moulds

S/N	TARGET REGIONS		PRIMER SEQUENCE(F)	100µm		PRIMER SEQUENCE ® R	100µm	Expected Amplicon (bp)
1	Nor	NOF	5'-ACC GCT ACG CCG GCA CTC TCG GCAC-3'	487.06µl	NOR	5'-GTT GGC CGC CAG CTT CGA CAC TCC G-3	550.85µl	400bp
2	Ver	VEF	5'-ATG TCG GAT AAT CAC CGT TTA GAT GGC-3	426.38µl	VER	5'-CGA AAA GCG CCA CCA TCC ACC CCA ATG-3	440.28µl	895bp
3	Omt	OMF	5'-GGC CCG GTT CCT TGG CTC CTA AGC-3	414.43µl	OMR	5'-CGC CCC AGT GAG ACC CTT CCT CG-3'	541.35µl	1232bp
4	aflR	AFLF	5'-TAT CTC CCC CCG GGC ATC TCC CGG-3'	480.98µl	AFLR	5'-CCG TCA GAC AGC CAC TGG ACA CGG-3'	497.64µl	1032bp
5	*A. Flavus*	AFF	5'GTAGGGTTCCTAGCGAGCC-3'	393.79µl	AFR	5'-GGAAAAAGATTGATTTGCGTTC-3'	447.05µl	796bp
6	*A. Niger*	ANF	5'-ATTCGCCGGAGACCCCAACA-3'	559.23µl	ANR	5'-TGTTGAAAGTTTTAACTGATTGCATT-3'	427.55µl	1232bp
7	*A. Tamari*	ATF	5'-CCAAGCTTTGCAACCAAGCCTGTCG-3'	457.21µl	ATR	5'-CTGCAGGTGTAGTGTGCTTGGCCGA-3'	532.77µl	1024bp
8	*A. Parasiticus*	APF	5'-AGTCAAAGGTTGAATACC-3'	386.1µl	APR	5'-GCTCAGCCATGACCTTGACTG-3'	519.85µl	840bp
9	*A. Fumigatus*	AFUF	5'-TATGTCTTCCCCTGCTCC-3'	550.02µl	AFUR	5'-CTATGCCTGAGGGGCGAA-3'	504.44µl	250bp
10	Fusarium	FUSF	5'-AACTCCCAAACC CCTGTGAACATA-3'	407.25µl	FUSR	5'-TTTAACGG CGTGGCCGC-3'	420.82µl	431bp

SSR = Simple Sequence Repeats. Source:Inqaba Biotechnical Industry, South Africa

The newly prepared mixture was then washed with Prewash Buffer by adding 200 µL of this buffer to the mixture and again spinned at 10, 000 x g for 1 min. 500 µL of the wash buffer mixture was then again added to the supernatant and re-spinned at 10, 000 x g for 1 min. The Zymo Spin tube containing the supernatant of this mixture was then transferred into a clean micro centrifuge tube of 1.5 µL capacity and then 100 µL of DNA elution buffer was added directly into the tube from the Zymo Spin tube matrix.

The tubes were together spinned at 10, 000 x g for 30 sec to complete the process of DNA elution and then the Zymo Spin tube was discarded. The new solution in the micro centrifuge tube contains the desired fungal DNA.

3.7.2.DNA quantification

The quality of DNA of each sample was determined using NANO-drop Spectrophotometer in the Bioscience laboratory of International Institute of Tropical Agriculture (IITA), Ibadan. 1µl of extracted DNA sample was measured at ultraviolet region of the spectrum at wavelength of 260nm and 280nm. The concentration was calculated by the Nanodrop spectrophotometer and the result was recorded for molecular analysis.

3.7.3. Determination of the quality of the isolated fungal DNA

The quality of isolated fungal DNA was checked by running the DNA samples with the aid of gel electrophoresis on 1% agarose gel in 0.5M TAE buffer at 100 volt for 1hr. Visualization of the DNA band was done by staining in ethidium bromide and distaining in distilled water and the gel images were taken and documented.

3.7.4. Amplification of aflatoxigenic gene from the isolated fungi

Most frequent fungal strains were selected among the isolates; the genes were amplified on their extracted gDNA. The concentration of DNA was first determined using Nano-drop Spectrophotometer. The same quality of DNA concentration (5 ng) was taken from each fungi and amplified using the gene primers. Total volume of 25 µl (Table 3.3) was prepared and this was subjected to PCR Amplification using Themoscientific Thermal cycler set at initial denaturation temperature of 94 ^{0}C/3 min followed by 30 cycles of 94^{0}C

per 1 min, annealing at 55^0C, 72^0C per 45 second for extension and final temperatures and final state of 4^0C till ∞.

Procedure	Thermocycling conditions		
Denaturation	94^0C	3 mins	30 Cycles
Denaturation	94^0C	1 min 45 secs	30 Cycles
Annealing Temperature	55^0C	1 mins	30 Cycles
Extension	72^0C	45 secs	30 Cycles
Final extension	72^0C	5 mins	30 Cycles
Final state	4^0C	∞	

Table 3.3. Procedures for amplification of aflatoxigenic genes

Table 3.4. PCR amplification reaction volumes

Mix	1x	50x
DNA	2.5µl	-
Primer Forward (1µm/µl)	1.0µl	50µl
Primer Reverse (1µm/µl)	1.0µl	50µl
Master mix	12.5 µl	-
Sterile distilled water	8.0 µl	-
Total	25 µl	1

3.7.5. Agarose Gel Electrophoresis

About 1.5% (w/v) Agarose Gel was prepared by stirring 1.5g of agarose in 100ml 1 x TAE buffer and heated in microwave oven.The solution was allowed to cool and a drop of 2mg/ml of ethidium bromide was added. The solution was poured in a tray fixed with comb to create wells upon solidification. After cooling, the comb was carefully removed. Loading dye was added to the amplified PCR product and carefully loaded into each well and applied voltage of 120V was used to run the loaded gel for 1h 30mins.

3.8. Statistical data analysis

The quantitative data obtained were analysed using statistical Package for Social Sciences (SPSS Version 22) and Microsoft Excel 2016. Means, standard deviation, variance and range were separated using mean comparison technique and tested for significance by Analysis of Variance (ANOVA) at $p = 0.05$.

CHAPTER 4
RESULTS

It was observed in this study that the addition of ginger and turmeric altered the aesthetic value and the choice of *Kulikuli* production. Turmeric for example changed the color and the aroma of the *Kulikuli* and this would in no way allow consumers to purchase or cunsume it while ginger on the other side changed the appearance, smell and taste of the produced *Kulikuli*. Therefore, the samples with ginger and turmeric additives were excluded from the analysed components. Garlic seems to be the most promising when compared with the other two spices for overall acceptability. Hence, the choice of garlic as control.

4.1. Sampled areas and mould incidence in*Kulikuli*

4.1.1. Fungal occurrence in *Kulikuli* samples

The studied *Kulikuli*samples were collected from five (5) states in Northwestern and North Central Nigeria. These states include Kano, Zamfara, Kaduna as well as Sokoto and Abuja-FCT. In addition, the studied *Kulikuli* were randomly collected from five major markets in each state. Therefore, 25 samples of *Kulikuli* were collected from 25 markets in five Northern States-Nigeria. These 25 samples of Groundnut cake (*Kulikuli*) were found valid for the study and hence all samples were used in conducting the molecular studies.

There are 48 fungal strains which are majorly nine fungal species are isolated from the *Kulikuli* samples.These fungal strains are *Aspergillus flavus, Aspergillus parasiticus, Aspergillus tamarii, Aspergillus niger, Aspergillus fumigatus, Penicillium oxalicum, Penicillium chrysogenum, Fusarium oxysporum and Fusarium compaticum.* In Figures 4.1–4.5 below, nine (9) varieties of mould (*Aspergillus flavus, Aspergillus parasiticus, Aspergillus tamarii, P. oxalicum, F. oxysporum, P. chrysogenum, A. niger, F. compaticum anid A. fumigatus)* occurred 94 timesin the sampled states (Sokoto, Abuja, Kano, Kaduna

and Zamfara). Specifically, Kaduna (Fig. 4.4) appeared to have the highest occurrence of moulds while Sokoto has the least occurrence of moulds among the sample states

Table 4.1. Descriptive statistics for mould prevalence

Provenance	N = 94	Mean	Std. Deviation	Variance	Range
Sokoto	17	2.647	1.730	2.993	6
Abuja	18	3.167	2.036	4.147	6
Kano	20	3.600	2.644	6.989	8
Kaduna	21	3.952	2.801	7.848	8
Zamfara	18	3.000	2.142	4.588	8
ML	5	2.333	1.332	2.753	4
$MLBG_1$	5	2.221	1.123	2.122	4

ML = Mycology Laboratory Department of Botany UI *Kulikuli* without botanicals;$MLBG_1$= Mycology Laboratory Department of Botany UI *Kulikuli* with garlic additive

It was however observed that the laboratory prepared samples with and without garlic additive $MLBG_1$ and ML had the least fungal incidence. The $MLBG_1$ (Fig 4.8) had the least fungal incidence with all the fungi occurring below 0.5 followed by ML (Fig. 4.7) in which *A. flavus* had the highest incidence.

From Figure 4.1, it can be observed that 6 varieties of moulds were identified in the sampled Groundnut cake (*Kulikuli*) collected from markets in Sokoto States. The frequency occurrence of this moulds are *Aspergillus flavus* (5)*, Aspergillus parasiticus* (5)*, Aspergillus tamarii* (3)*, P. oxalicum* (1)*, F. oxysporum* (2)*,* and *A. niger* (1). This implies that the most frequently observed mould are *Aspergillus* flavu*s and Aspergillus parasiticus* while the least frequently observed are *P. oxalicum,* and *A. niger.*

In Figure 4.2 above, 7 varieties of moulds were identified in the sampled Groundnut cake (*Kulikuli*) collected from markets in Abuja-FCT. The frequency occurrence of this moulds are *Aspergillus flavus* (5)*, Aspergillus parasiticus* (4)*, Aspergillus tamarii* (2)*, P. oxalicum* (1)*, F. oxysporum* (3)*, P. chrysogenum* (2) and *A. niger* (1). This implies that the most frequently observed mould is *Aspergillus flavus* while the least frequently observed are *P. oxalicum,* and *A. niger.*

From Figure 4.3, it can be observed that 7 varieties of moulds were identified in the sampled *Kulikuli* collected from markets in Kano States. The frequency occurrence of this moulds are *Aspergillus flavus* (5)*, Aspergillus parasiticus* (5)*, Aspergillus tamarii* (1)*, P. oxalicum* (3)*, F. oxysporum* (2)*,* and *A. niger* (2), *F. compaticum* (2). This implies that the most frequently observed mould are *Aspergillus flavus and Aspergillus parasiticus*while the least frequently observed is *A. tamarii.*

From Figure 4.4, it can be observed that 9 varieties of moulds were identified in the sampled Groundnut cake (*Kulikuli*) collected from markets in Kaduna States. The frequency occurrence of this moulds are *Aspergillus flavus* (5)*, Aspergillus parasiticus* (5)*, Aspergillus tamarii* (1)*, P. oxalicum* (2)*, F. oxysporum* (1), *P. chrysogenum* (2)*, A. niger* (1), *A. fumigatus* (3) and *f. compaticum* (1). This implies that the most frequently observed mould are *Aspergillus flavus and Aspergillus parasiticus*while the least frequently observed are *A. tamarii, F. oxysporum, A. niger and F. compaticum.*

From Figure 4.5, it can be observed that 7 varieties of moulds were identified in the sampled *Kulikuli* collected from markets in Zamfara States. The frequency occurrence of this moulds are *Aspergillus flavus* (5)*, Aspergillus parasiticus* (5)*, Aspergillus tamari* (1)*, P. oxalicum* (4)*, F. oxysporum* (1)*, P. chrysogenum* (1)*,* and *F. compaticum* (1). This implies that the most frequently observed mould are *Aspergillus flavus and Aspergillus parasiticus* while the least frequently observed are *A. tamarii, F. oxysporum, P. chrysogenum*and *F. compaticum.*

From Figure 4.6, it can be observed that 7 varieties of moulds were identified in the sampled *Kulikuli* isolated from the laboratory prepared *Kulikuli* without garlic additive (ML). It was observed that these samples contained fewer fungi as compared with the market samples. The frequency occurrence of this moulds are *Aspergillus flavus* (2)*, Aspergillus parasiticus* (1)*, Aspergillus tamarii* (1)*, P. oxalicum* (1)*, F. oxysporum* (1)*,* and *A. niger* (1), *F. compaticum* (1). This implies that the most frequently observed mould are *Aspergillus flavus.*

From Figure 4.7 however, the fungi that were isolated from the laboratory prepared Kulikuli with garlic additive were significantly lowered due to the garlic added as the fungal prevalence were generally lower than 1 for all the identified fungi (Fig 4.7). It can be observed that 7 varieties of moulds were identified from the laboratory prepared *Kulikuli* with garlic additive (ML). It was observed that these samples contained less fungus as compared with the market samples. The frequency occurrence of this moulds are *Aspergillus flavus* (0.30)*, Aspergillus parasiticus* (0.30)*, Aspergillus tamarii* (0.30)*, P. oxalicum* (0.30)*, F. oxysporum* (0.30)*,* and *A. niger* (0.30), *F. compaticum* (0.30). This implies that the most frequently observed mould are *Aspergillus flavus.*

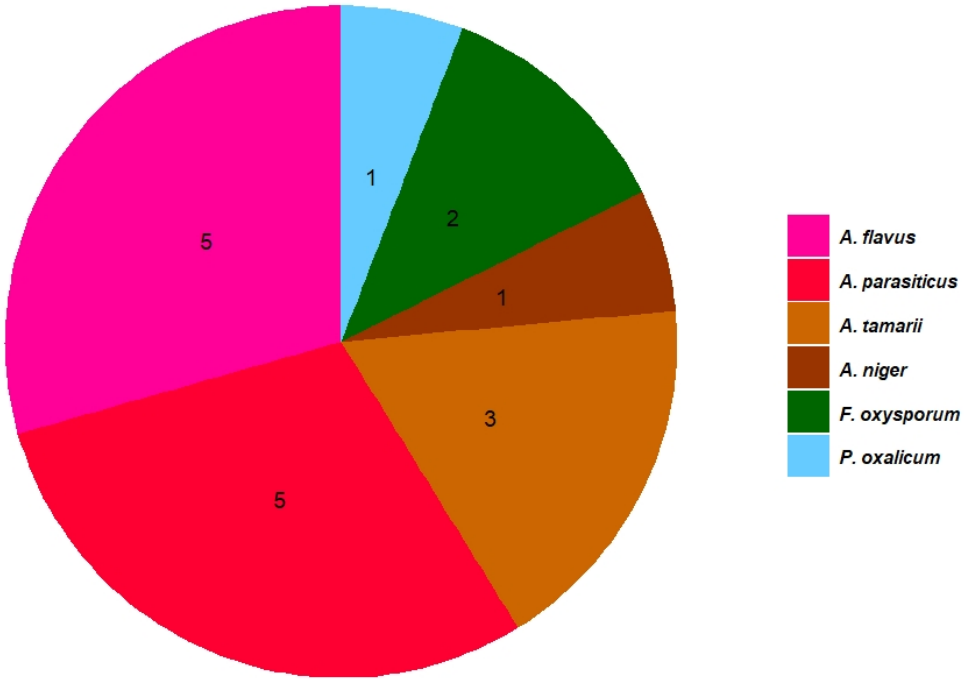

Figure 4.1. Mould prevalence in*Kulikuli* samples from Sokoto State.

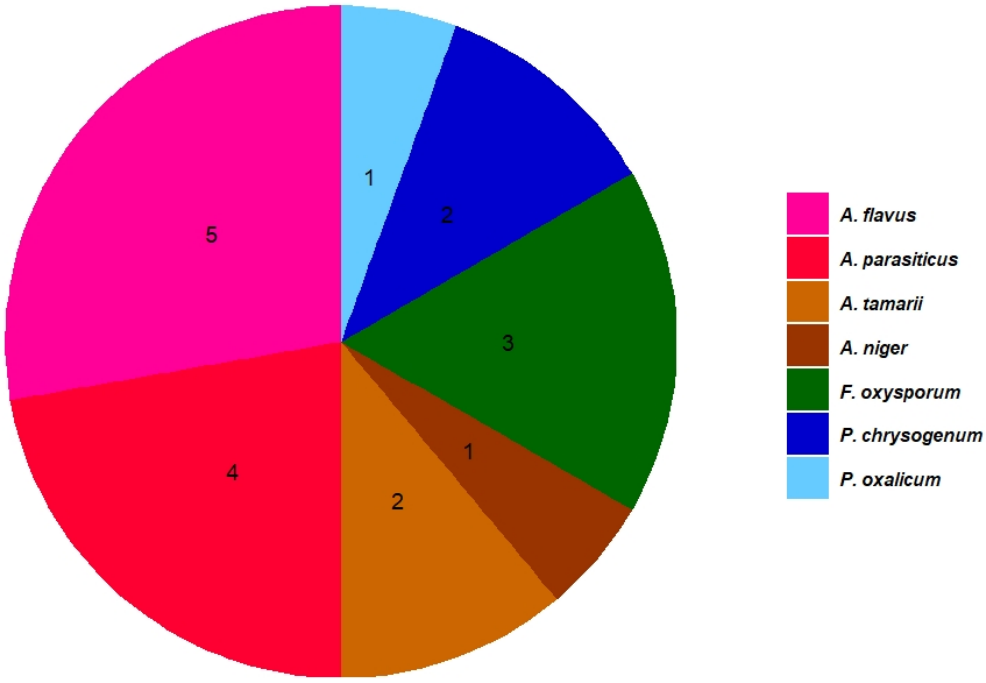

Figure 4.2. Mould prevalence in *Kulikuli* samples from Abuja

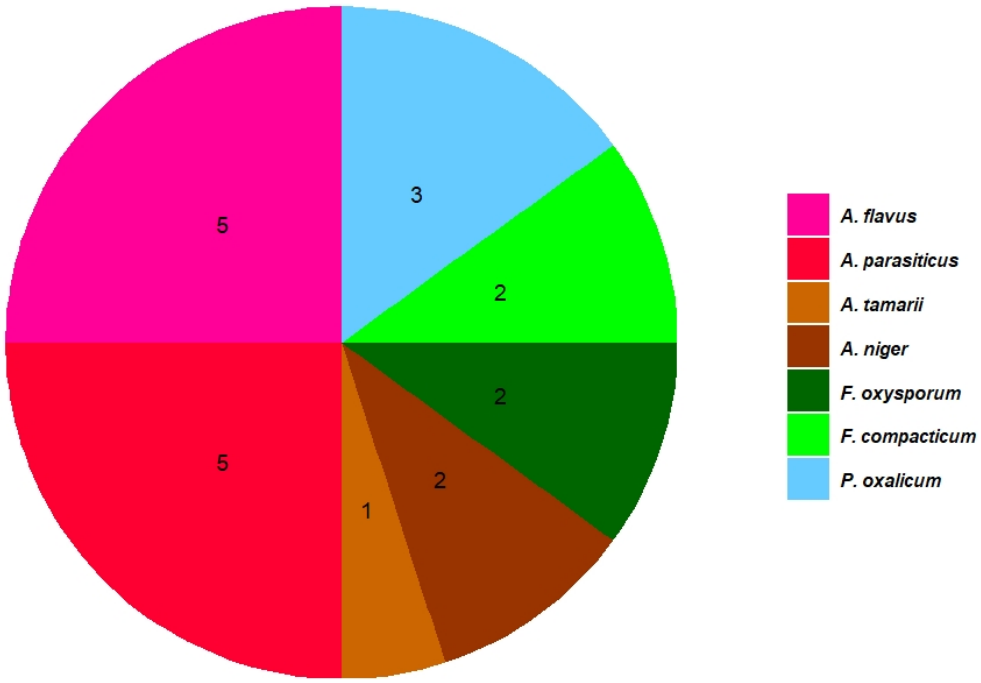

Figure 4.3. Mould Prevalence in *Kulikuli* samples from Kano State

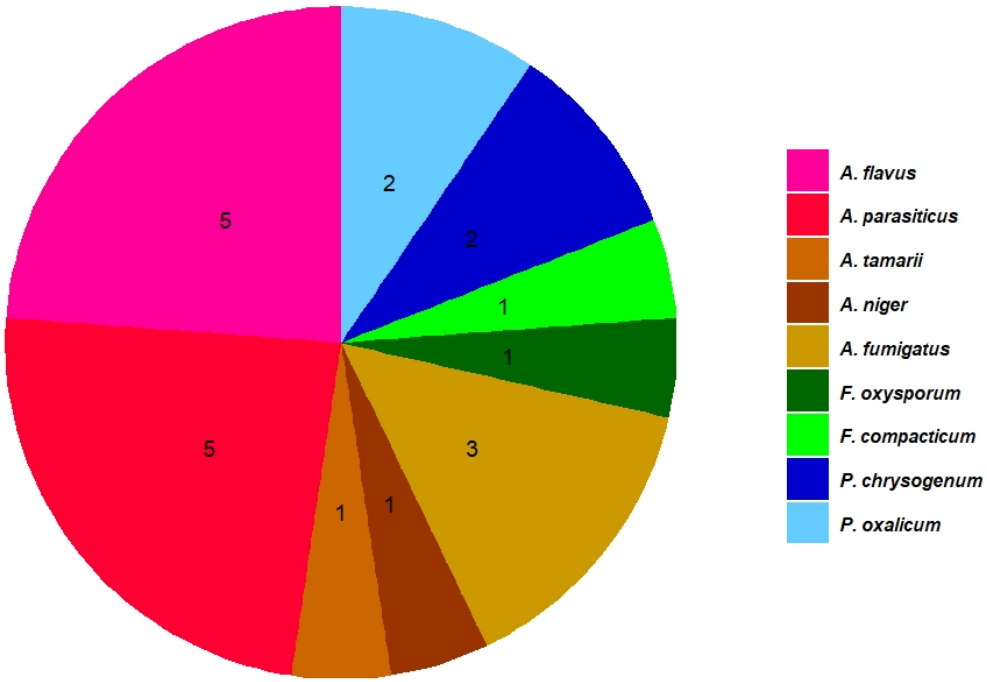

Figure 4.4. Mould prevalence in *Kulikuli* samples from Kaduna State

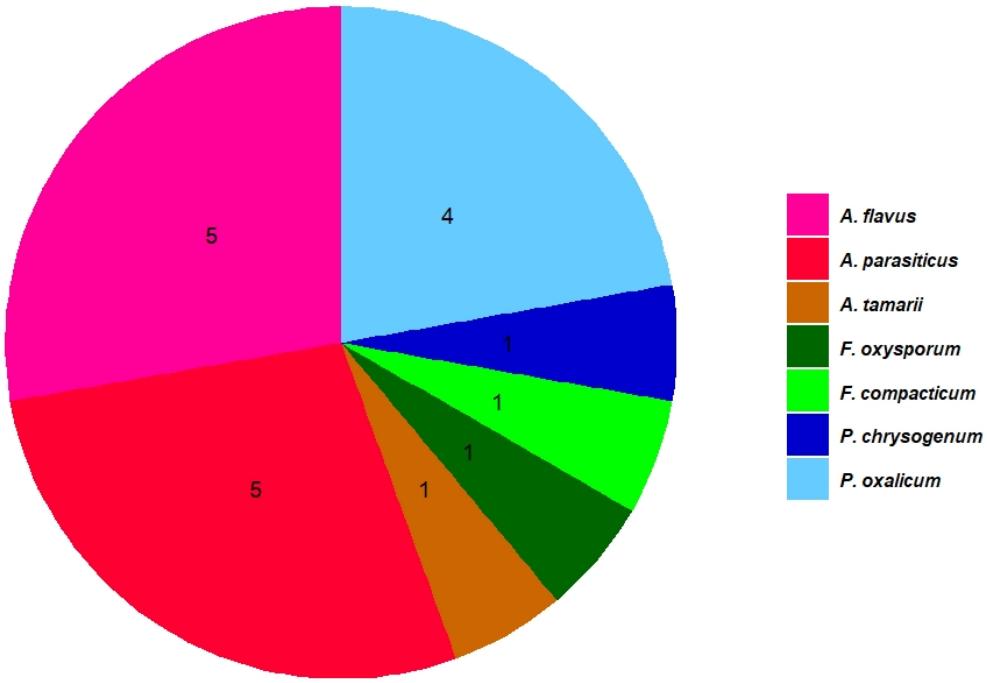

Figure 4.5. Mould prevalence in *Kulikuli* samples from Zamfara State

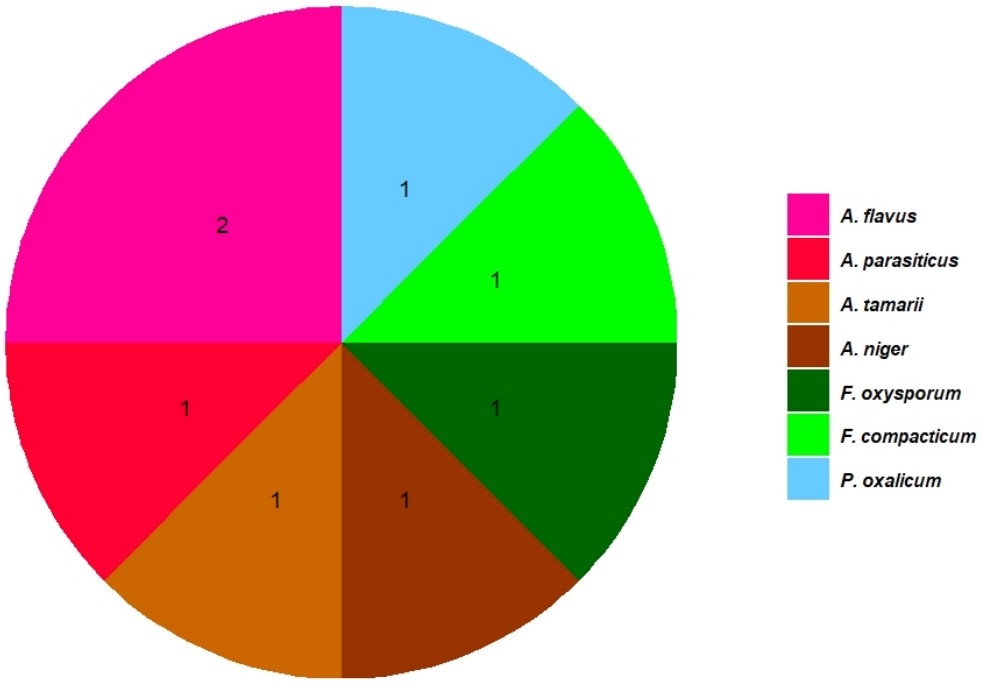

Figure 4.6. Mould prevalence in prepared *Kulikuli* without garlic additive (ML)

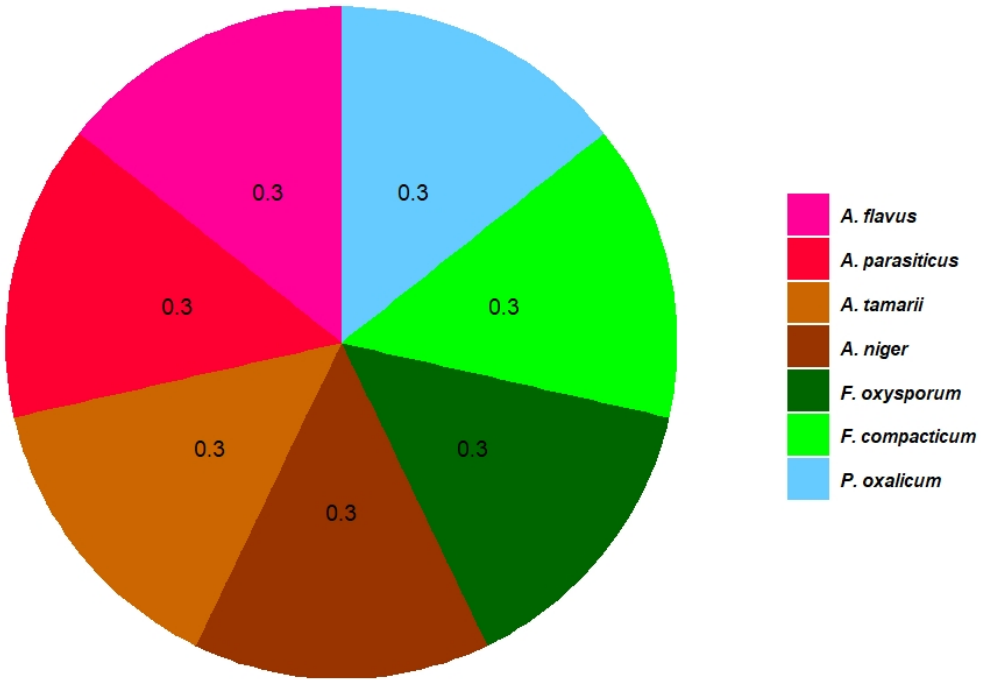

Figure 4.7. Mould prevalence in prepared *Kulikuli* with garlic additive (MLBG1)

4.1.2.Dominant fungal strain based on percentage incidence

The effect of garlic additive reflected on the total number of fungi associated with the samples. TheMycology Laboratory, Department of Botany UI *Kulikuli* with Garlic additive ($MLBG_1$) generally had the least total fungal incidence (2 ± 0) followed by the Mycology Laboratory, Department of Botany UI *Kulikuli* without Garlic (ML) which had 2 ± 0 incidence. Kaduna appeared to have the highest occurrence of moulds while Sokoto had the least occurrence among the market samples (Table 4.2). The most frequently observed mould are *Aspergillus* flavus and *Aspergillus parasiticus* which are well associated with aflatoxin productions while the least frequently observed are *A. fumigatus* and *F. compaticum* (Table 4.2).

Also, mean count showed that Kaduna has the highest mean count of four. Likewise, Kano has a mean of four; Abuja 3; Zamfara 3; and Sokoto 3. The standard deviation of three was observed in Kaduna; three in Kano; 2 in Zamfara; 2 in Abuja and 2 in Sokoto. The range of mould in the states ranged between six in Sokoto and Abuja toeight in Kano, Kaduna and Zamfara.

Based on the percentage incidence, *A. flavus* and *A. parasiticus* which are the most often reported aflatoxigenic fungi were the most dominant fungi asthey gave the % incidences of 26.42 and 24.58 respectively (Table 4.2). The next dominant fungi were *Penicillium oxalicum* and *F. oxysporum*, which gave the percentage incidences of 10.38 and 09.43 followed by *A. tamarii* and *A. niger* with the percentage incidences of 08.49 and 06.60 while the least dominant fungi were *A. fumigatus*, *P chrysogenum* and *Fusarium oxysporum* which had the % incidences of 02.83, 05.66 and 05.66 respectively.

Table 4.2. Descriptive statistics for mould prevalence in *Kulikuli* samples

Fungal strain	Sokoto	Abuja	Kano	Kaduna	Zamfara	ML	$MLBG_1$	Total Incidence	% Incidence
Aspergillus flavus	5	5	5	5	5	2	1	28	26.42
A. parasiticus	5	4	5	5	5	1	1	26	24.58
A. tamarii	3	2	1	1	1	1	-	9	08.49
A. niger	1	1	2	1	-	1	1	7	06.60
A. fumigatus	-	-	-	3	-	-	-	3	02.83
Penicillium oxalicum	1	1	3	2	4	-	-	11	10.38
P. chrysogenum	-	2	-	2	1	1	-	6	05.66
Fusarium. Compaticum	-	-	2	1	1	1	1	6	05.66
F. oxysporium	2	3	2	1	1	1	-	10	09.43
Total	17	18	20	21	18	8		106	100.00
Mean±SD	3±2	3±2	4±3	4±3	3±2	2±0	2±0		

Values are means of three replicates ± Standard Deviation
ML = Mycology Laboratory, Department of Botany UI *Kulikuli* without garlic;
$MLBG_1$= Mycology Laboratory, Department of Botany UI *Kulikuli* with garlic
Key: Higher = ; Lower =

4.2. Characterisation of the isolated fungal strains

The isolated fungi with highest percentage incidence from the studied five (5) states includes nine (9) different fungi and they were identified based on their morphological and molecular characteristics as *Aspergillus flavus, Aspergillus parasiticus, Aspergillus niger, Aspergillus tamarii, Aspergillus fumigatus. Penicillium oxalicum, Penicillium chrysogenum, Fusarium oxysporum* and *Fusarium compaticum* (Table 4.3).

Aspergillus niger is characterized withblack-brown mycelium and greenish-yellow to yellow orange coloration at the plate reverse side, a floccose head splitting with age.It has long, closely packed and brownish metulae. *A. tamarii* showed a rusty-brown colony on plate and creamish-brown at reverse with long and rough stipe, its head is partly globular with thick orange yellow conidia, which is strongly roughened. *A. flavus* is distinguished with yellowish-green becoming green with age coloration on plate, creamish-yellow on reverse side, it has radiating head which becomes loosely columnar with age and a long, verrucose and hyaline stipe with small metulae. Similarly, *A. fumigatus*had 3.0 to 3.2 cm growth diameter on plate with bluish-green on plate and creamish-yellow in its reverse, its head is columnar in shape, short, smooth and greenish-grey stipe, metulae is absent and its phialides are ampuliform, closely packed, roughly parallel to the axis of the stipe. It also has globose, echinate and greenish conidia.

A. parasiticus was also characterised with a light sparse of grey greenish color to pale bluish green or parrot green colouration.It has mycelium typical of fluffy cream white color that appears as dull white at maturity. It has exudates present at the surfaces and reverse underside of the plate appears yellowish or orange colour with observable wrinkled mycelium. It produces few sclerotia, which appears wheat brown in colour. The conidia appear very sparse dull green reverse yellowish to orange light peach.

Penicillium chrysogenum is characterised by its 2.7 to 4.5cm colony diameter on plate; the texture is sulcate and velutinous.It is bluish-green to (dark) green in surface and yellow (occasionally creamish) in reverse. It has a short, smooth stipe. The penicillin is terverticillate, phialidesampuliform, collula very short, both divergent and appressed branches. The conidia are ellipsoidal to spherical, smooth and greenish.

Penicillium oxalicum is characterised by velutinous texture and heavy sporulation with greyish-green upper and pale yellow reverse plate colour, it has long and smooth long stipe, and with asymmetrically biverticillate metulae that is closely oppressed, phialides are across with a very short collula. The conidia are ellipsoidal, large, smooth and pale green.

Fusarium oxysporum has floccose texture mycelium, which is whitish-cream with pale to bluish-violet reverse plate colour; chlamydospores are abundant and usually single while *Fusarium compaticum*also has floccose texture with whitish-cream and deep rose red in plate reverse side. Chlamydospores are abundant in a clusters rough golden yellow.

Moreover, Kaduna appeared to have the highest occurrence of moulds while Sokoto has the least occurrence (Table 4.3). In addition, mean count showed that Kaduna has the highest mean count of four followed by Kano (4), Abuja (3), Zamfara (3) and Sokoto (2).

The range of mould in the states ranged between six in Sokoto and Abuja to eight in Kano, Kaduna and Zamfara. The most frequently observed mould are *Aspergillus* flavu*s* and *Aspergillus parasiticus* which are well associated with aflatoxin productions while the least frequently observed are *A. fumigatus* and *F. compaticum* (Table 4.3).

Table 4.3. Morphological and microscopic characteristics of the fungal strains

Fungal species	Morphological/Microscopic features on PDA
Aspergillus flavus	It appears as yellowish green but becomes green at maturity; the plate underside appears creamish yellow. It has columnar head that becomes loose at maturity. It is characterised microscopically by a short broomish appearance, and its possession of long verrucose and a hyaline stipe, which has small metulae.
Aspergillus parasiticus	It appears on plate as light sparse grey green colouration, which becomes pale blue green to parrot green at maturity. The mycelium appears fluffy with creamy to dull white colour, and this appears with exudates. The underside plate colour appears yellowish with wrinkled mycelium. It produces few sclerotia, which appears wheat brown, and conidia are very sparse in dull bluish green to reverse yellowish orange to light peach colour.
Aspergillus niger	It appears black or blackish brown on plates often with yellowish mycelium. It appears on the plate underside with greenish yellow-to-yellow orange colour. It is characterised microscopically with a short broomish appearance, it has a globose head metulae, which split at maturity while their metulae is long, closely packed and brownish.
Aspergillus tamarii	It has colony that appears rusty brown on plate, and appears as creamish brown on the plate underside. It is characterised microscopically by a long and rough stipe with a thick, strongly roughed and globular conidia, which appear with orange yellow colouration.
A. fumigatus	It is 3.0 to 3.2 cm in diameter, bluish-green at upper and creamish-yellow in its reverse. Its head is columnar in shape. Short, smooth and greenish-grey stipe. Metulae is absent andphialides are ampuliform, closely packed, roughly parallel to the axis of the stipe. The conidia are globose, echinate and greenish.

Penicillium oxalicum	It appears on plates with gelatinous texture, it is often heavily sporulated which appears greyish green and pale yellow at the plate underside. It is characterised microscopically by a long broom appearance. It has a long and smooth stipe.It is asymmetrically biverticillate and its metulae are closely oppressed, with an accrose phialides, and short collula. It produces smooth, ellipsoidal conidia in large quantities that appear with pale green coloration.
Penicillium chrysogenum	It produces colonies with a 2.7 to 4.5 cm diameter. It has mycelium with sulcatetextures and velutinous. It appears bluish green to dark green on plates and yellowish or occasionally creamish on the plate underside. It is characterised microscopically by possession of terverticillate, with phialides appearing ampuliform. It has a very short collula both appear divergent and appressely branched. It produces ellipsoidal to spherical smooth and greenish conidia
Fusarium oxysporum	This fungus has a floccose texture and whitish cream coloration on plates, with pale to bluish violet plate underside colour. It is characterised microscopically by abundant and single chlamydospores.
Fusarium compaticum	It has a floccose texture, appears whitish cream on plates with deep rose red appearance on the plate underside. It produces abundant, clustered, rough golden yellow chlamydospores.

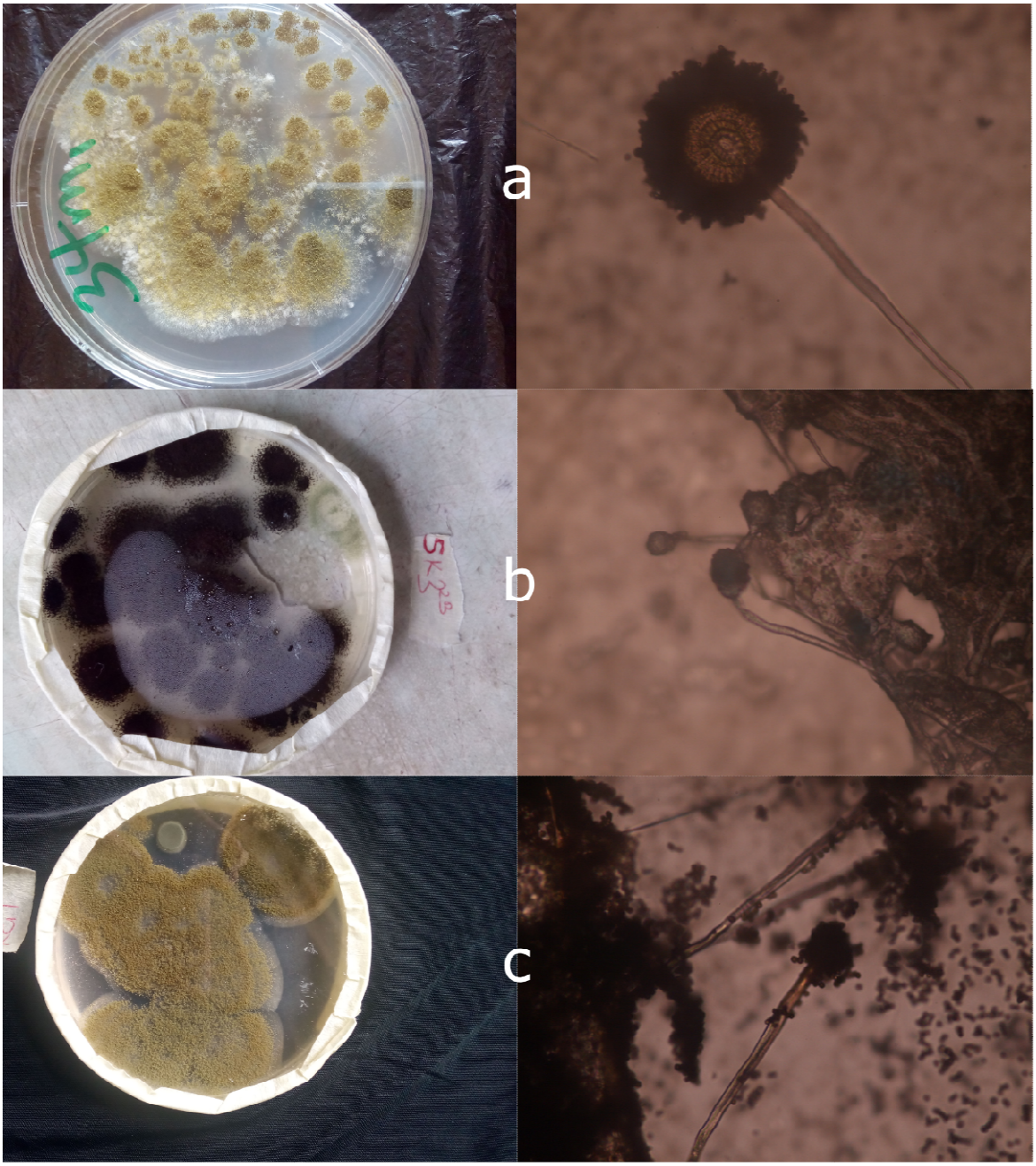

Plate 4.1. Deteriorating fungi isolated from *Kulikuli* samples

a = *Aspergillus flavus*;**b** = *A. niger*;**c** = *A. fumigatus*

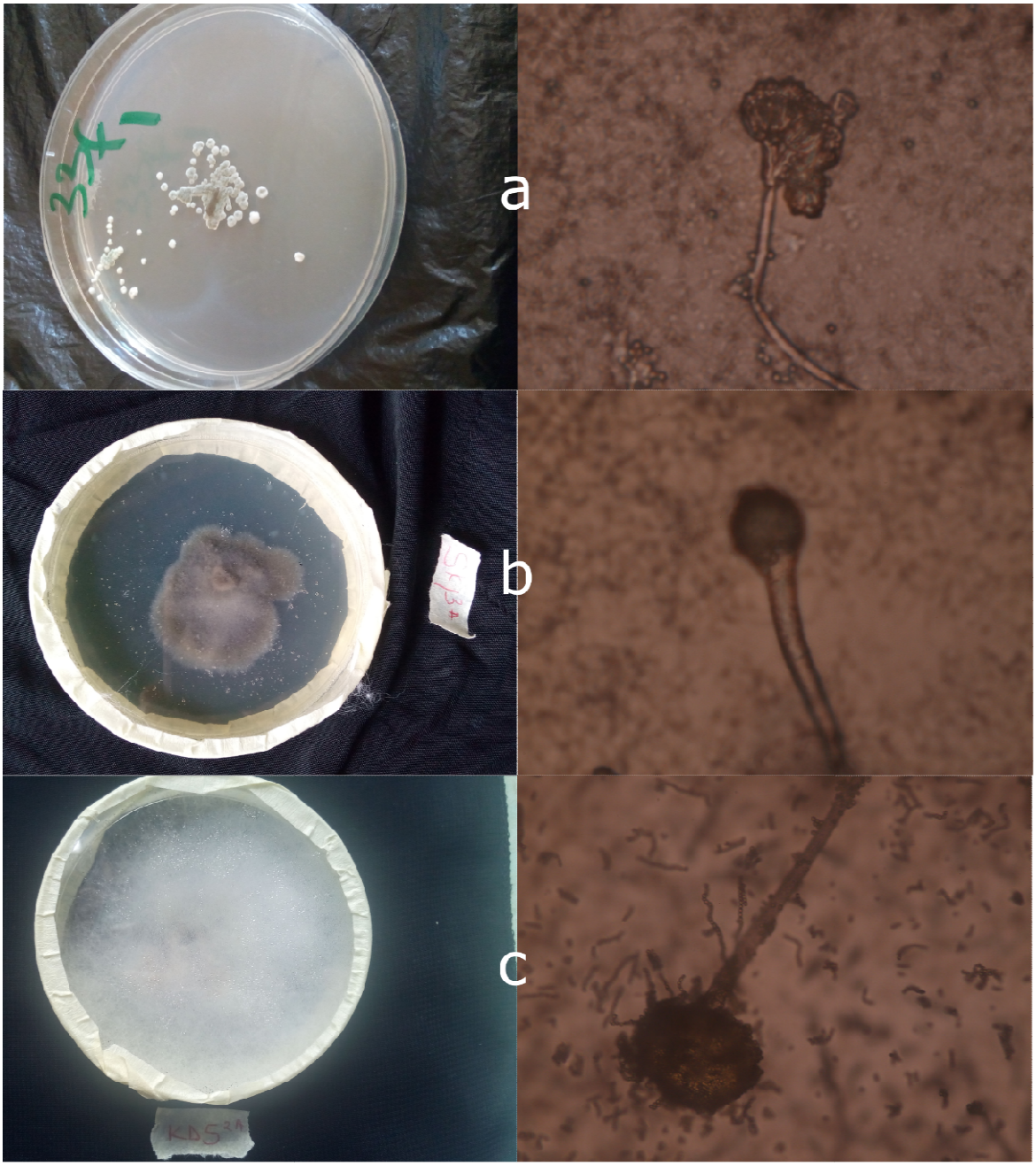

Plate 4.2. Deteriorating fungi isolated from *Kulikuli* samples

a= *Penicilliumsp;* **b**= *A. tamarii;* **c**= *Fusarium compaticum*

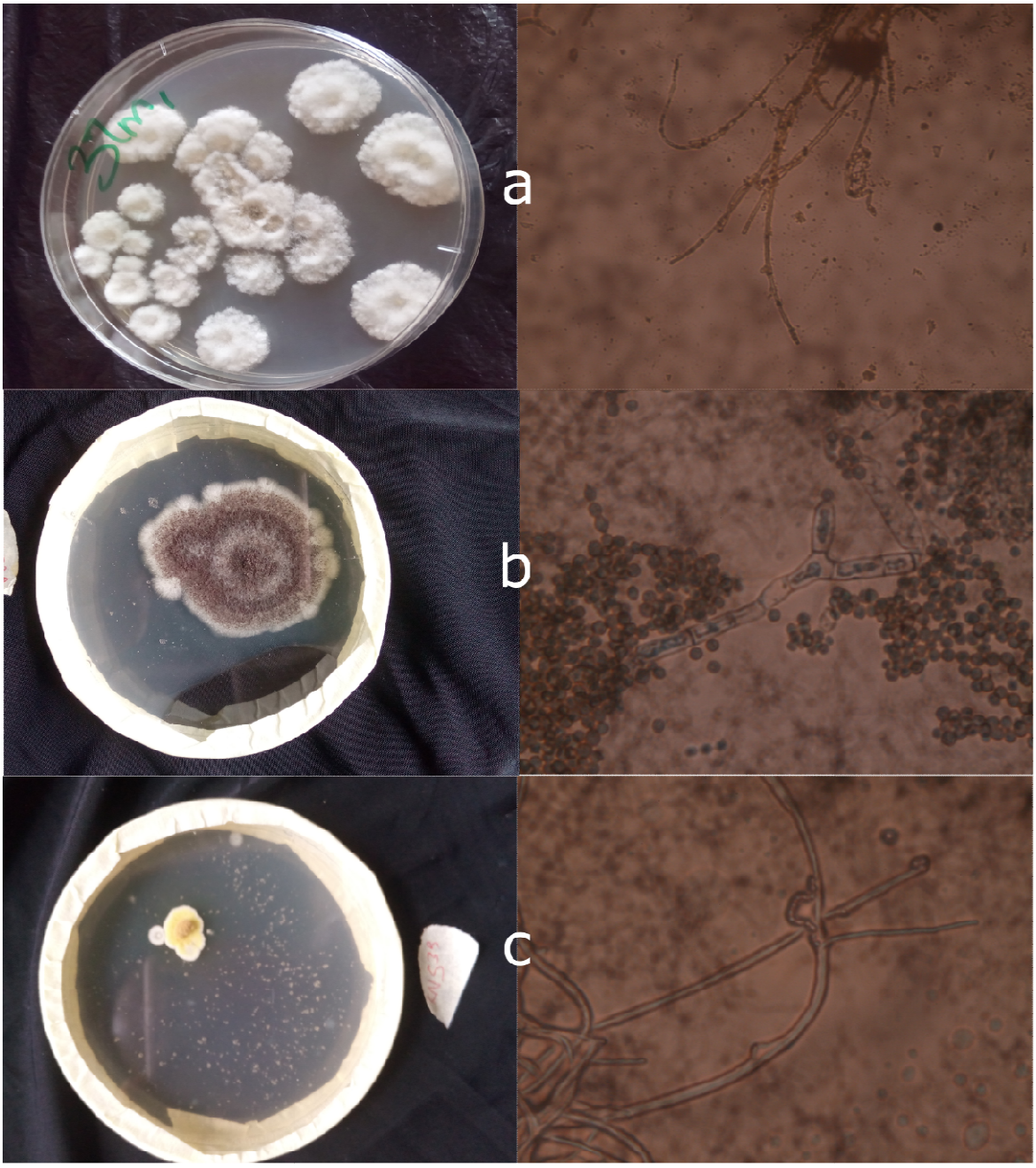

Plate 4.3. Deteriorating fungi isolated from *Kulikuli* samples

a= *F. oxysporum;* ***b*** = *A. parasiticus;* **c** = *P. chrysogenum*

Generally, *Aspergillus flavus* and *Aspergillus parasiticus* had the highest incidences in all the states. It was observed that six (6) most abundant fungal strains were identified in the sampled (*Kulikuli*) collected from markets in Sokoto States. The frequency occurrence of this moulds are *Aspergillus flavus*(50 %)*, Aspergillus parasiticus* (50 %)*, Aspergillus tamari* (30 %)*, P. oxalicum* (10 %)*, F. oxysporum* (20 %)*,* and *A. niger* (10%).

This implies that the most frequently observed mould are *Aspergillus flavus and Aspergillus parasiticus* while the least frequently observed are *P. oxalicum,* and *A. niger*. Seven varieties of moulds were however identified in the sampled *Kulikuli* collected from markets in Abuja-FCT and Kano respectively.The most frequently observed mould in Abuja FCT was*Aspergillus flavus,* the least frequently observed were*P. oxalicum* (10 %)and *A. niger* (10 %).However, the highest in Kano were*Aspergillus flavus* (50 %) and *Aspergillus parasiticus* (50 %) and the least frequently observed was*A. tamari, F. oxysporum, F. chrysogenum and A. niger* with all having 10% incidences.

Kano and Zamfara had the highest numbers of fungal strains with 9 and 7 most frequent fungal strains respectively. The most frequently observed moulds in Kano are *Aspergillus flavus* (50 %) and *Aspergillus parasiticus* (50 %) while the least frequently observed are *A. tamarii, f. oxysporum, A. niger* and *F. compaticum.* The frequent mould in Zamfara are *Aspergillus flavus* (50 %)*, Aspergillus parasiticus* (50 %)*, Aspergillus* while *P. oxalicum* (40) *A. tamari* (10 %)*, F. oxysporum* (10 %)*, P. chrysogenum* (10 %) and *F. compaticum* (10 %) are the least.

4.3. Effect of location on the proximate composition of *Kulikuli* samples

This section details the result of the proximate analysis done between the sampled states. The nutrient values between the states analysis was done to ascertain the level of pH, crude protein, crude fat, ash and moisture content among the states. These results are presented using charts to obtain a better visualization of the phenomena under discourse.

From Figure 4.8, it is evident that all the sampled *Kulikuli*were very edible because their pH level is slightly lower than 7 which implies a slight acidic level. State-wisely, *Kulikuli* collected from Kaduna state appears to be the most edible with pH value of 6.24, while *Kulikuli* from Abuja had the least pH value of 5.96. In addition, Sokoto, Zamfara and

Kano had pH values of 6.14, 6.03 and 5.97 respectively.However, the laboratory prepared samples with and without garlic additive ($MLBG_1$ and ML, respectively) had the highest overall pH values (Fig 4.8). The $MLBG_1$samples had a pH value closest to neutral (6.88) while ML hada pH of 6.84.

From Figure 4.9, the sampled *Kulikuli* were very rich in crude protein. Among the sampled states, Sokoto appeared to have the most protein-rich *Kulikuli* with percentage level of 49.23%. In addition, the least-protein-rich *Kulikuli* was observed from Abuja with a percentage crude protein of 43.18%. This shows a percentage difference of 6.05% between the crude protein content of Sokoto and Abuja *Kulikuli*. Accordingly, a percentage crude protein of 48.371%, 46.407% and 46.31% was observed in Kaduna, Kano and Zamfara respectively. However, the laboratory prepared samples with ($MLBG_1$) and without (ML) garlic additive has the highest overall crude protein values (Fig 4.9). The $MLBG_1$ samples had the best overall crude protein of 59.23 % crude protein while ML had the best overall pH of 50.25% crude protein respectively.

The graphical summary in Figure 4.10 also shows that, Kaduna state has the *Kulikuli* with the highest fat content of 4.83%. In addition, an insignificant percentage difference in fat content was observed between Abuja (4.64 %) and Kano (4.55%). Furthermore, the percentage fat content of *Kulikuli* from Sokoto and Zamfara are 4.45 % and 3.888 %. This implies that Zamfara state had *Kulikuli* with the least fat content. However, the laboratory prepared samples with ($MLBG_1$) and without (ML) garlic additive had the least overall fat content (Fig 4.10). The $MLBG_1$ samples had the least overall fat content of of 3.26 % while samples from ML had the least fat of 3.83 % respectively.

The crude fibre summary presented in Figure 4.11 shows that *Kulikuli* from Kano state had the highest crude fibre (3.20 %) while Sokoto has the least crude fibre rich *Kulikuli*. The percentage crude fibre between the two states were 3.20 % and 2.99 % respectively, which implies a percentage difference of 0.3 %. Accordingly, a percentage crude fibre of 3.14 %, 3.05 % and 3.02 % was obtained in Abuja, Zamfara and Kaduna respectively.

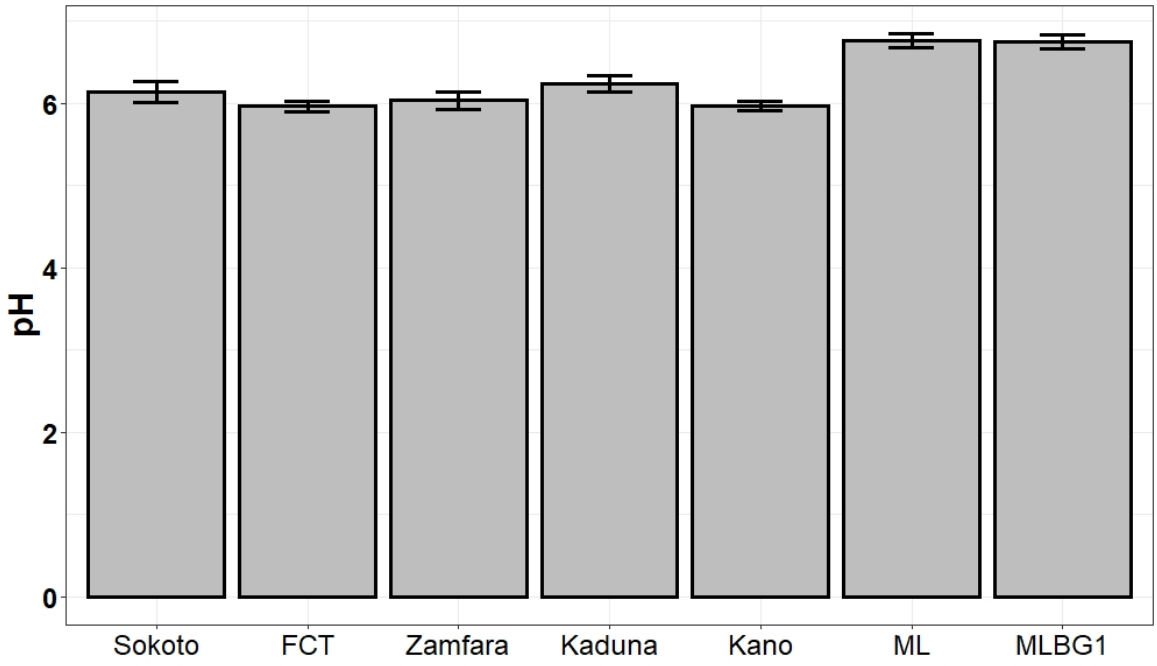

Figure 4.8. pH of markets and laboratory-prepared *Kulikuli* samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

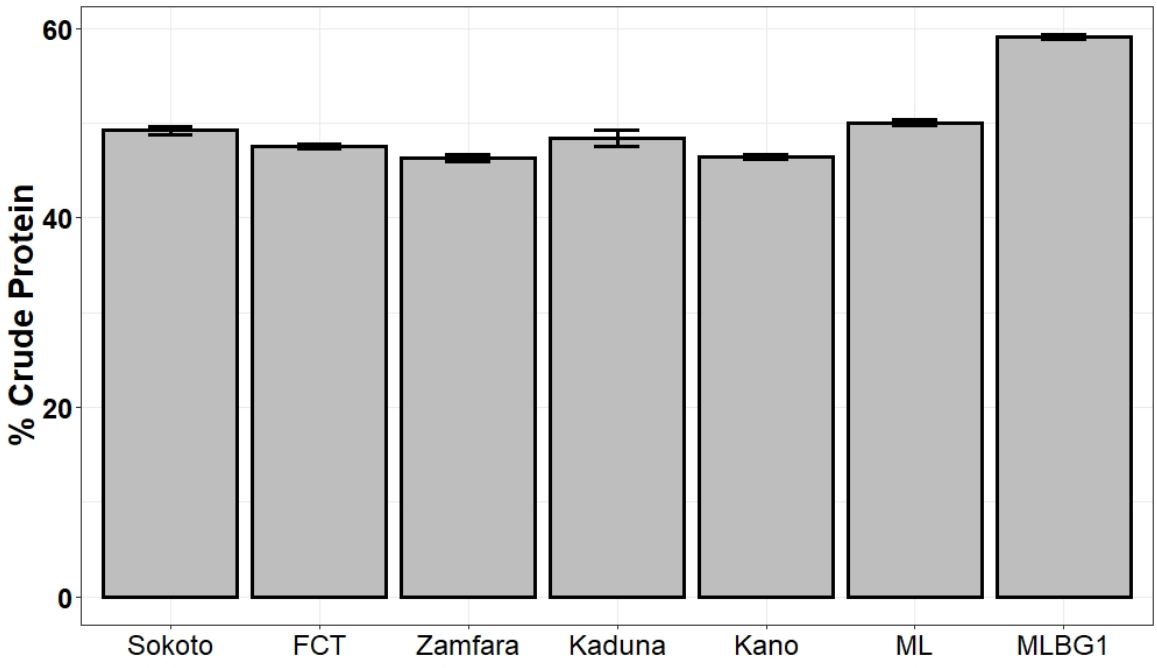

Figure 4.9. Crude protein of markets and laboratory-prepared *Kulikuli* samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

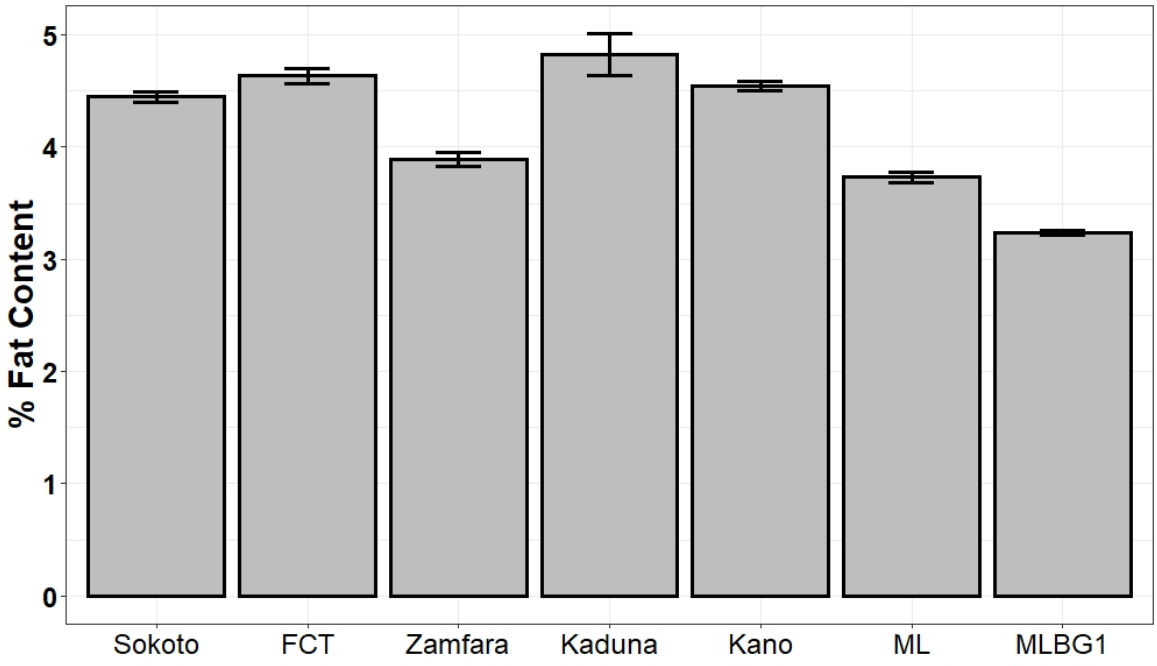

Figure 4.10. Percentage fat content of markets and laboratory-prepared *Kulikuli* samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

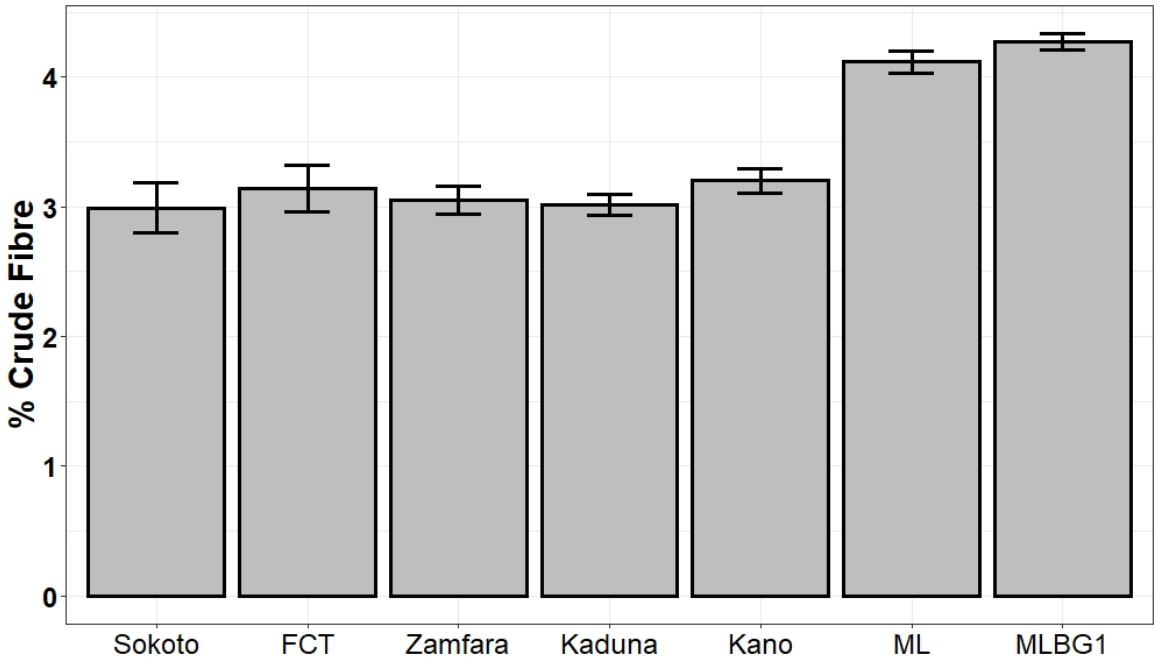

Figure 4.11. Crude fibre of markets and laboratory-prepared *Kulikuli* samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

From Figure 4.12, *Kulikuli* collected from Kano State appears to be the richest in percentage ash content with Abuja being the least rich in ash content. Item-wisely, Kano has a percentage ash content of 3.91 %; Sokoto (3.84 %); Kaduna (3.07 %); Zamfara (2.95 %) and Abuja (2.89 %). Difference-wisely, there exist a slight percentage difference of 0.069 % between the ash content of Sokoto and Kano State, while a percentage difference of 0.126 % and 0.063 % were observed between Kaduna and Zamfara as well as Zamfara and Abuja respectively. However, the laboratory prepared samples with ($MLBG_1$) and without (ML) garlic additive also had the highest overall crude ash content (Fig 4.11). The best overall ash content of 4.55 % was obtained from laboratory prepared samples with garlic additive ($MLBG_1$) followed by ML, with 4.05 %, respectively.

Figure 4.13 shows the percentage prevalence of moisture content between the states. From the results, samples of *Kulikuli* collected from Zamfara state appear to have the highest moisture content of 11.91 % compared to other states like Kaduna (11.37 %), Abuja (10.64 %), Sokoto (10.64 %) and Kano (8.88 %). However, the laboratory prepared samples with garlic ($MLBG_1$) and without garlic (ML) garlic additive also had the highest and lowest overall moisture contents (Fig 4.13). The $MLBG_1$ samples had the least overall moisture content of 6.64 % while ML had the best highestmoisture content(12.64 %).

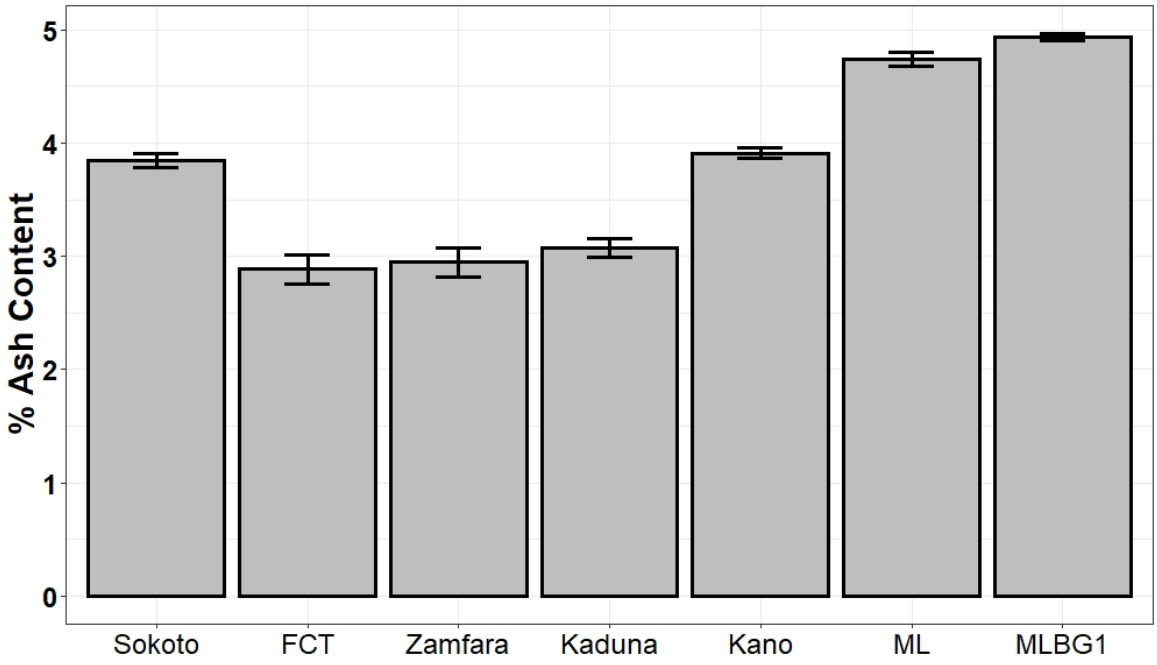

Figure 4.12. Percentage ash of markets and laboratory-prepared *Kulikuli* samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

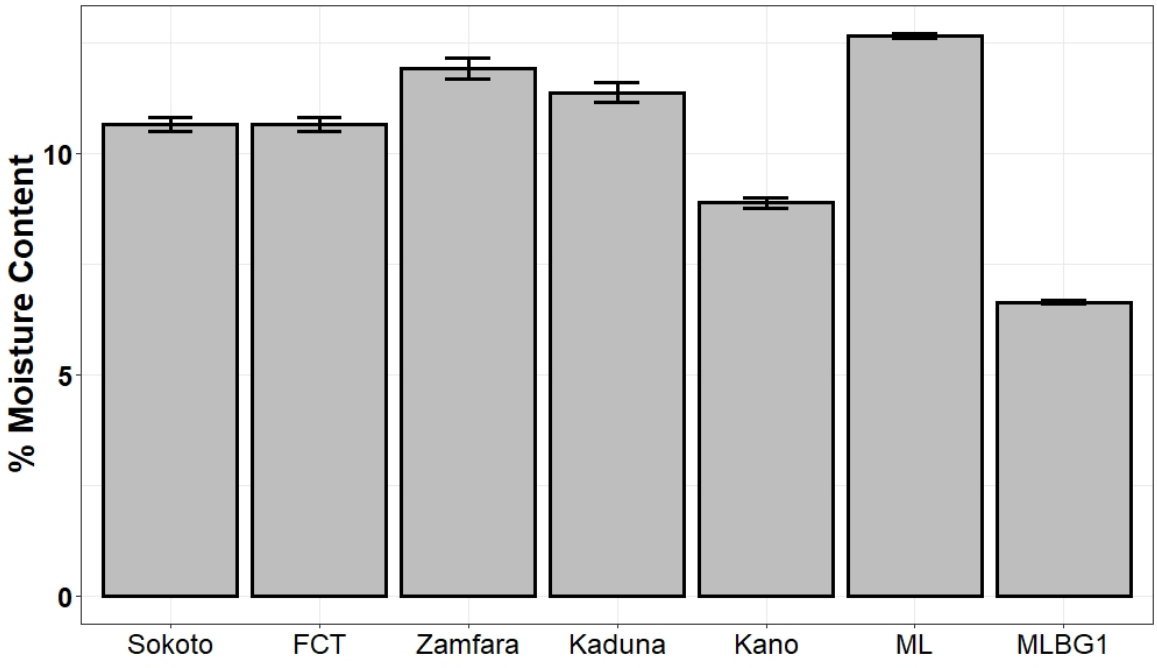

Figure 4.13. Moisture content of markets and laboratory-prepared *Kulikuli* samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

The Figure 4.14 to Figure 4.19 show the comparative proximate compositions of the *Kulikuli* from the studied States and control. From the results obtained, pH and crude protein for *Kulikuli* collected from Zamfara state as presented in Figure 4.14 showed that there was no significant differences in pH. However, the highest crude protein, which was within the range of 58.9% and 59.3 % was obtained in samples prepared in the laboratory with garlic additive. This was significantly different from crude protein content across all market samples, which ranges from 44.8 % and 47.8 %. However, laboratory-prepared samples with garlic had the lowest fat content (3.13 %) and the highest crude fibre (4.65 %), which were significant different from market samples (Figure 4.15). Ash and moisture content for *Kulikuli* samples as compared with controls are presented in Figure 4.16. It was observed thatboth controls, that is, *Kulikuli* samples prepared in the laboratory had the highest ash content 4.84 % and 4.74 %, respectively for samples with and without garlic respectively. These values were significantly different from that of market samples, which ranged between 2.48 % and 3.55 % (Figure 4.16). On the contrary, moisture content was relatively high in all market samples (Figure 4. 16) and lowest in control samples with garlic additive (6.64 %).

Likewise, the comparative proximate compositions of *Kulikuli*samples collected from Sokoto State is presented in Figure 4.17 to Figure 4.19. From the results obtained, the proximate compositions of *Kulikuli* collected from Sokoto state as presented in Figure 4.17 appear to have the highest crude protein, which was within the range of 47.8 to 51.4 % compared to other nutrients such as fat content (4.3 to 4.7 %), crude fibre (2.3 to 4.4 %) and ash (3.6 to 4.2 %). In addition, the laboratory prepared samples in comparison with market samples from this state also had the highest crude protein, ash and pH Figure 4.17 and Figure 4.19).

The comparative proximate compositions of *Kulikuli* samples collected from Abuja is presented in Figure 4.20, Figure 4.21 and Figure 4.22. From the results obtained, the proximate compositions of *Kulikuli* collected from FCT as presented in Figure 4.20had the highest crude protein which was within the range of 46.4 to 48.8 % compared to other nutrients such as fat content (4.3 to 4.6 %) as shown in Figure 4.21, crude fibre (2.5 to 3.9 %) and ash (2.6 to 3.7 %) (Figure 4.22). Also, the laboratory prepared samples in

comparison with samples from this state also had the highest Crude Protein, Ash and pH. This implies that samples of *Kulikuli* collected from Sokoto state and the laboratory prepared ones has more protein value as compared to ash and fibre. Justifiably, many research evidences have also shown that protein content is significantly necessary for the human's health.

Moreover, the comparative proximate compositions of *Kulikuli* samples collected from Kano State is presented in Figure 4.23, Figure 4.24 and Figure 4.25. From the results obtained, the proximate compositions of *Kulikuli* collected from Kano State as presented in Figure 4.23 appear to have the highest crude protein which was within the range of 45.4 to 47.8% compared to other nutrients such as fat content (4.4 to 4.8 %) (Figure 4.24), crude fibre (2.8 to 3.6 %) and ash (3.7 to 4.1 %) (Figure 4.25). In addition, the laboratory prepared samples in comparison with samples from this state also had the highest crude protein, ash and pH (Figure 4.23).

The comparative proximate compositions of *Kulikuli* samples collected from Kaduna State is presented in Figure 4.26, Figure 4.27 and figure 4.28. From the results obtained, the proximate compositions of *Kulikuli* collected from Kaduna State as presented in Figure 4.26 had the highest crude protein which was within the range of 44.8to 51.8 % compared to other nutrients such as fat content (3.8 to 5.5 %)(Figure 4.27), crude fibre (2.8 to 3.4 %) and ash (2.8 to 3.4 %) (Figure 4.28).In addition, the laboratory prepared samples in comparison with samples from this state also had the highest crude protein, ash and pH (Figure 4.28).

4.4. Nutrient composition *Kulikuli* as affected by location and garlic

It was observed that there were significant differences ($p \leq 0.05$) in the nutrient content between all the market samples across different states and the laboratory prepared samples with and without garlic additive. This may be a reflective of the fact that there were differences in the associated fungi incidence.However, the $MLBG_1$and ML generally had the nutrient contents as compared to the market samples across the states (Table 4.5). The highest crude protein content was detected in samples from Sokoto State (49.23 ± 0.63 %).However, protein content for $MLBG_1$(59.23±1.43%) and ML (50.23±1.40%) was significantly higher than all market samples.

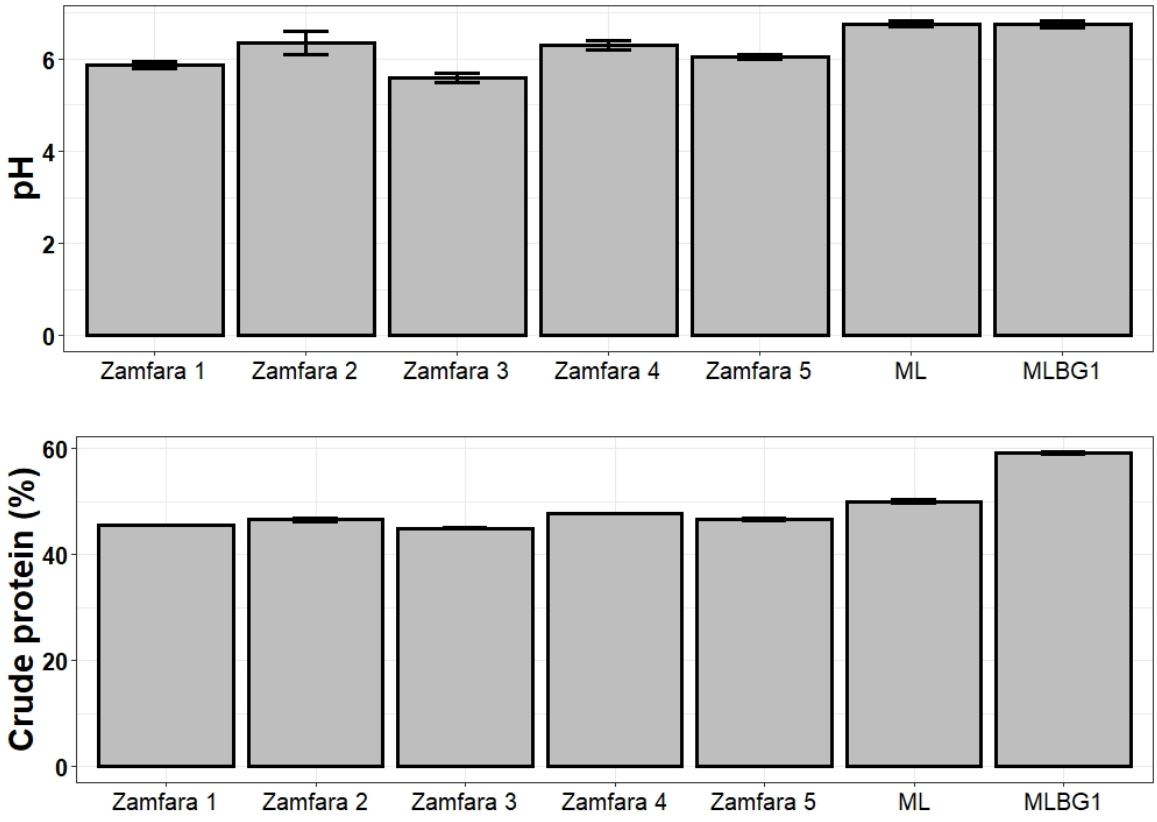

Figure 4.14.pH and crude protein of Zamfara*Kulikuli*andcontrol samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

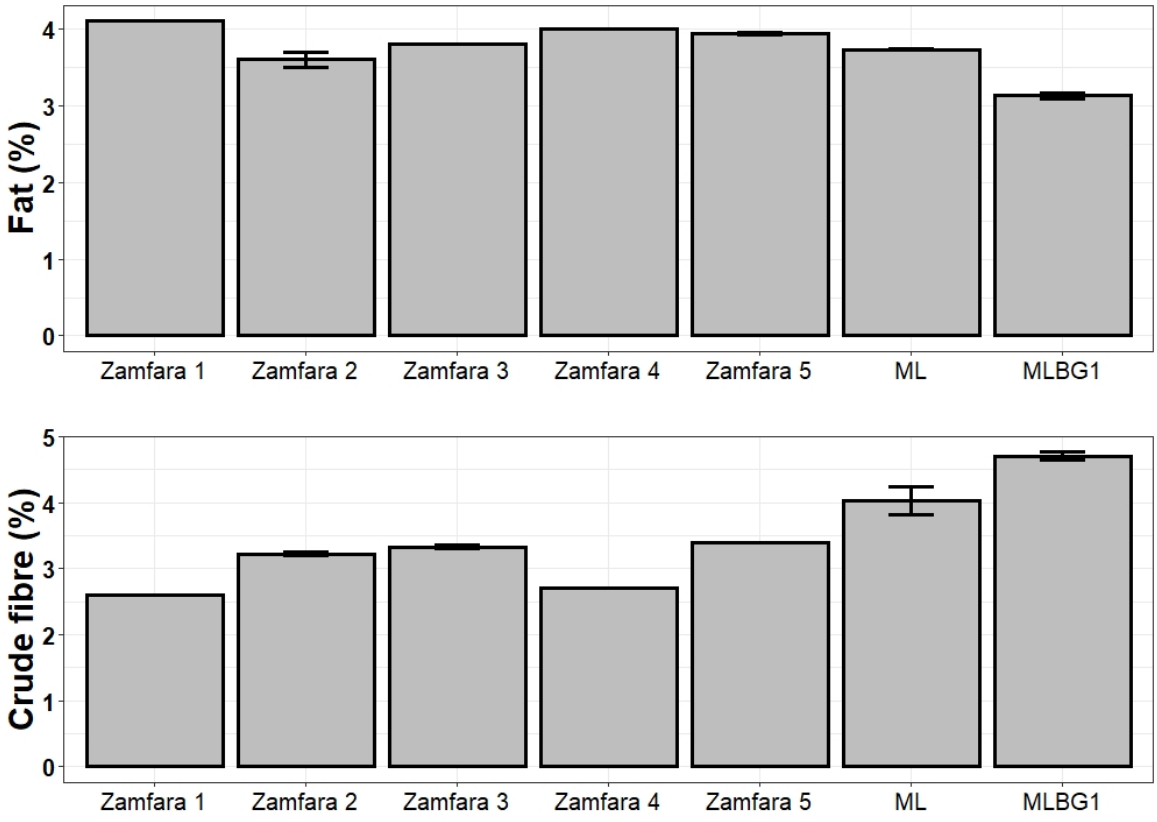

Figure 4.15.pH and crude protein of Zamfara *Kulikuli* and control samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

Figure 4.16. Ash and moisture content of Zamfara *Kulikuli* and control samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

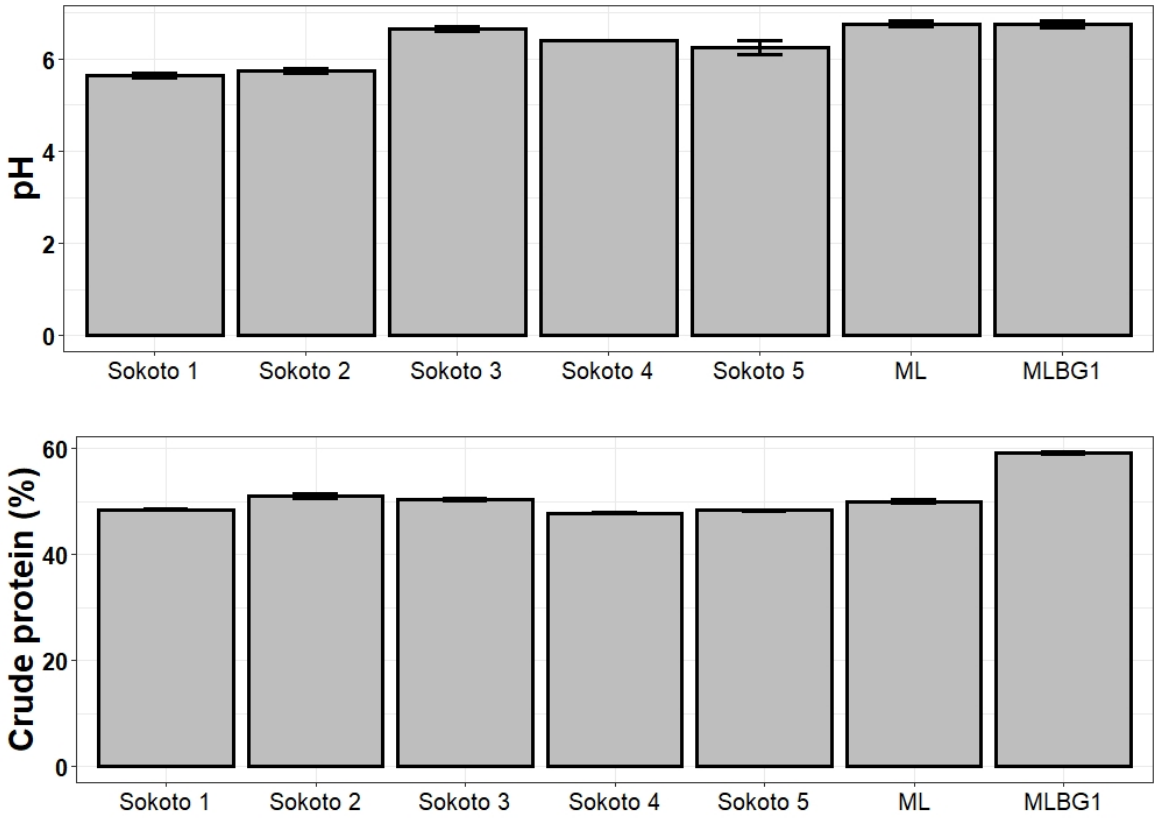

Figure 4.17. pH and crude protein of Sokoto*Kulikuli*and control samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

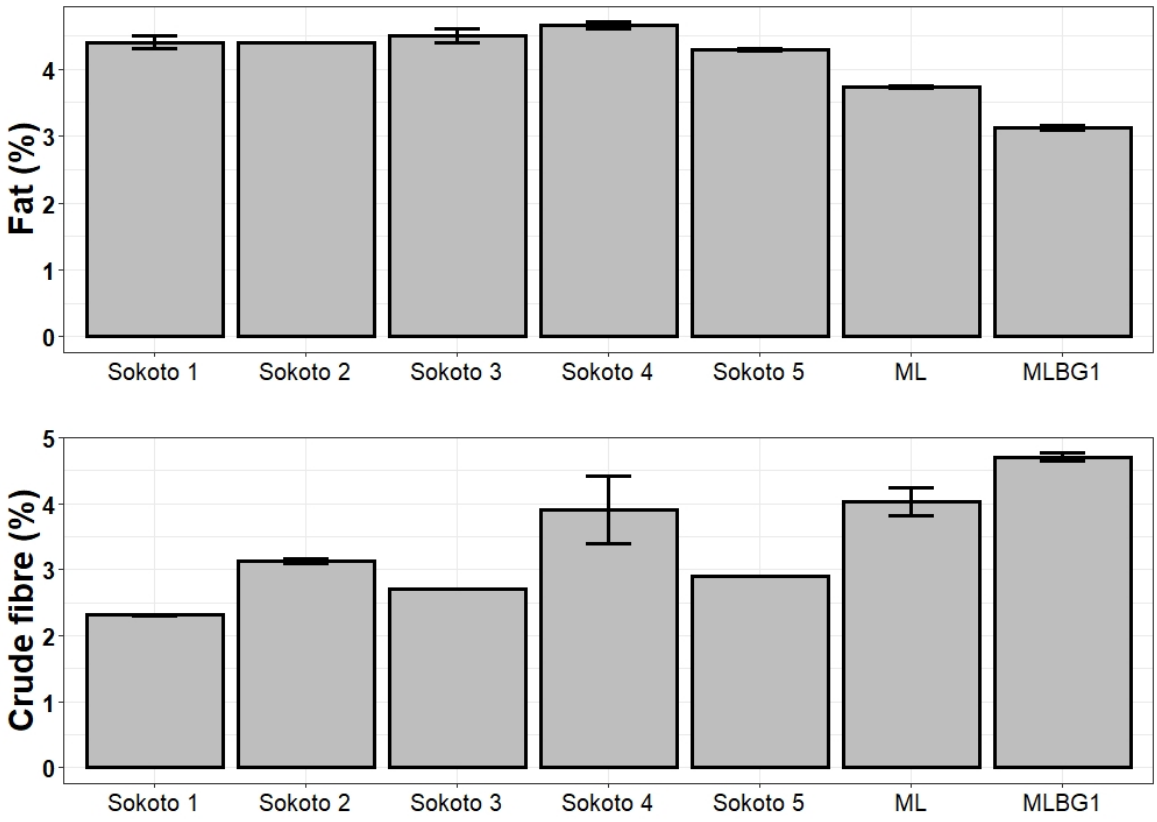

Figure 4.18. Fat and crude fibre Sokoto *Kulikuli* and control samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

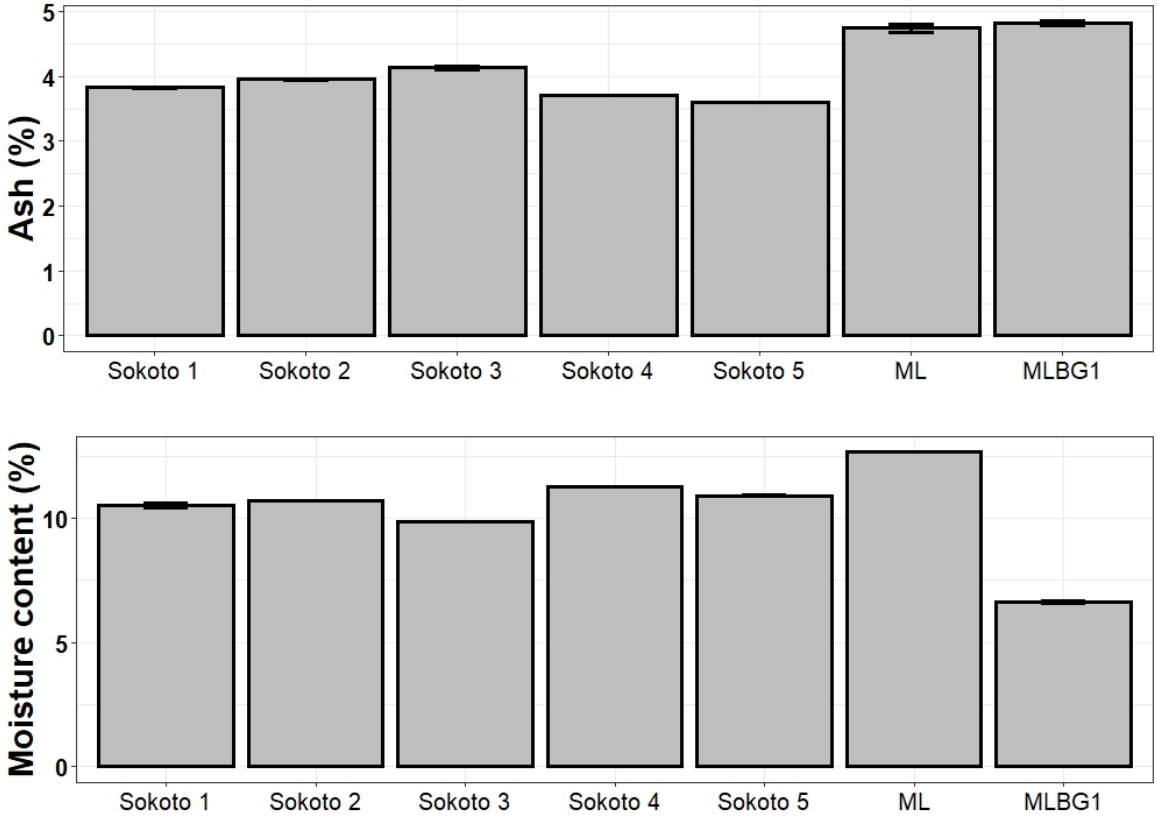

Figure 4.19. Ash and moisture content of Sokoto *Kulikuli* and control samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

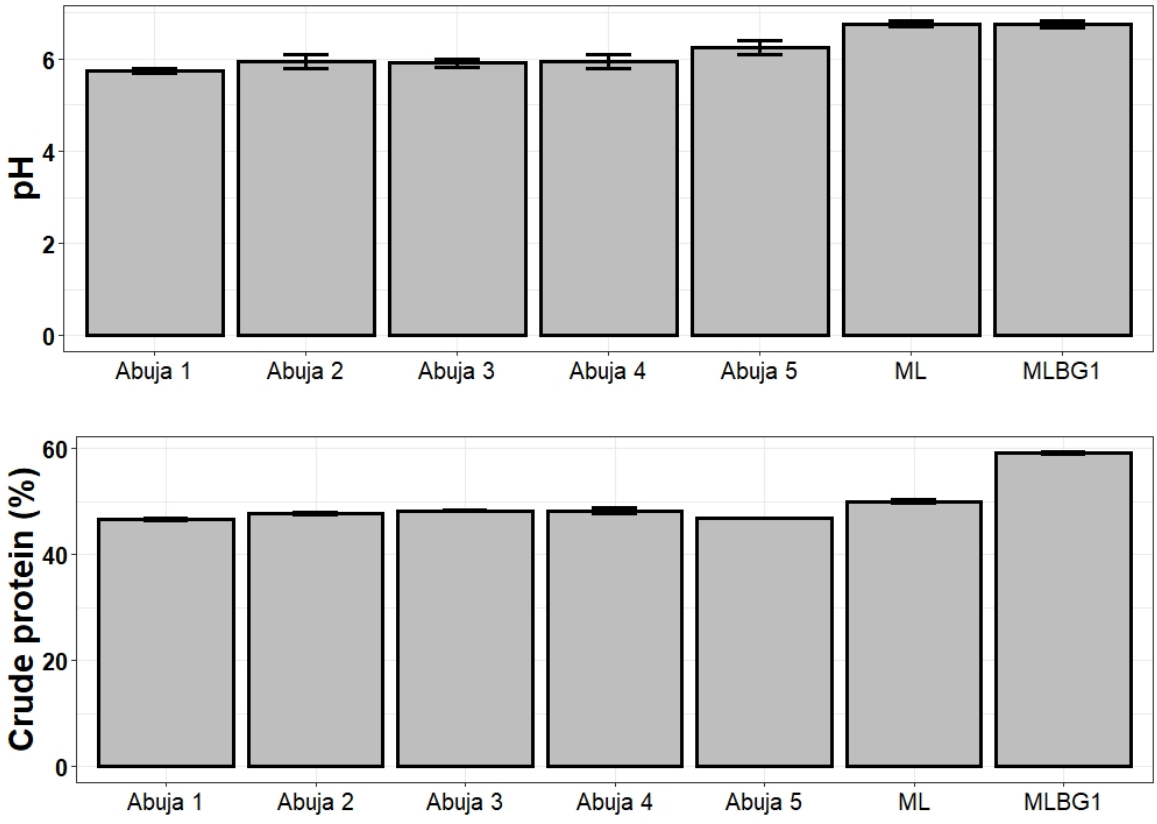

Figure 4.20.pH and crude protein of Abuja*Kulikuli* and prepared samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

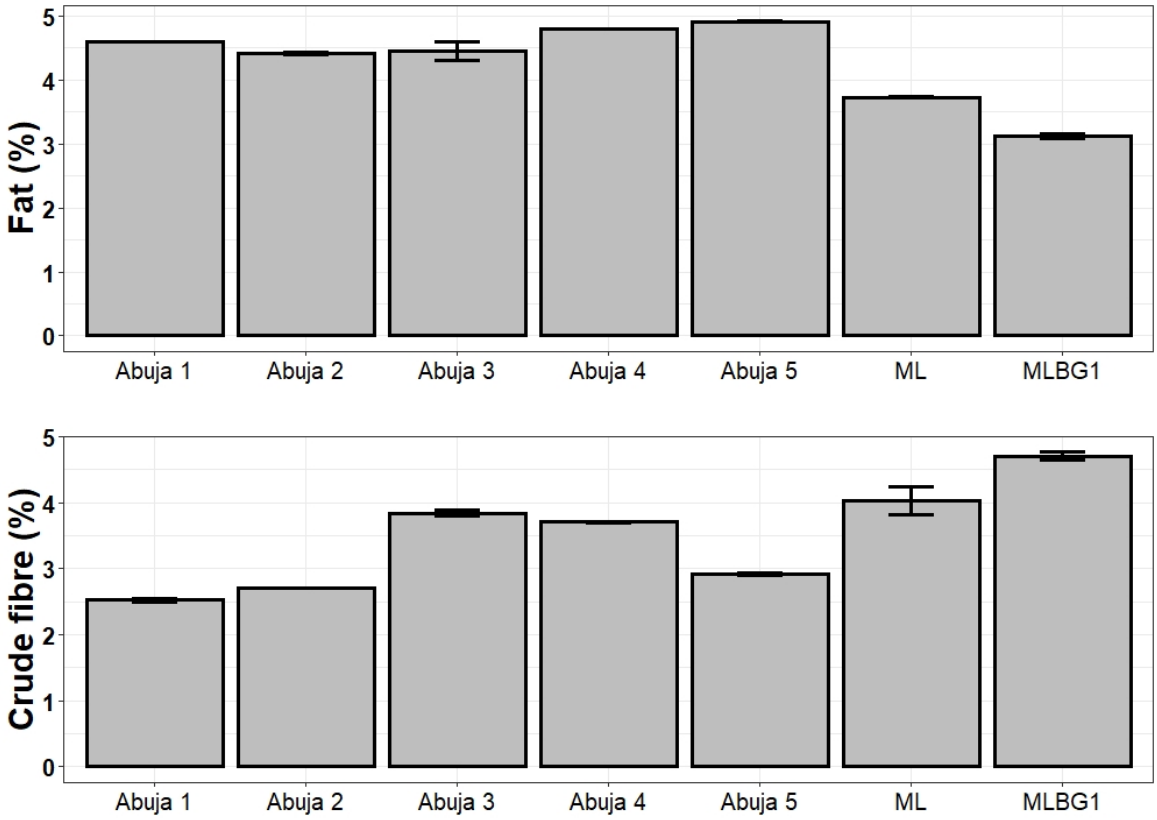

Figure 4.21 Fat and crude fibre of Abuja *Kulikuli* and prepared samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

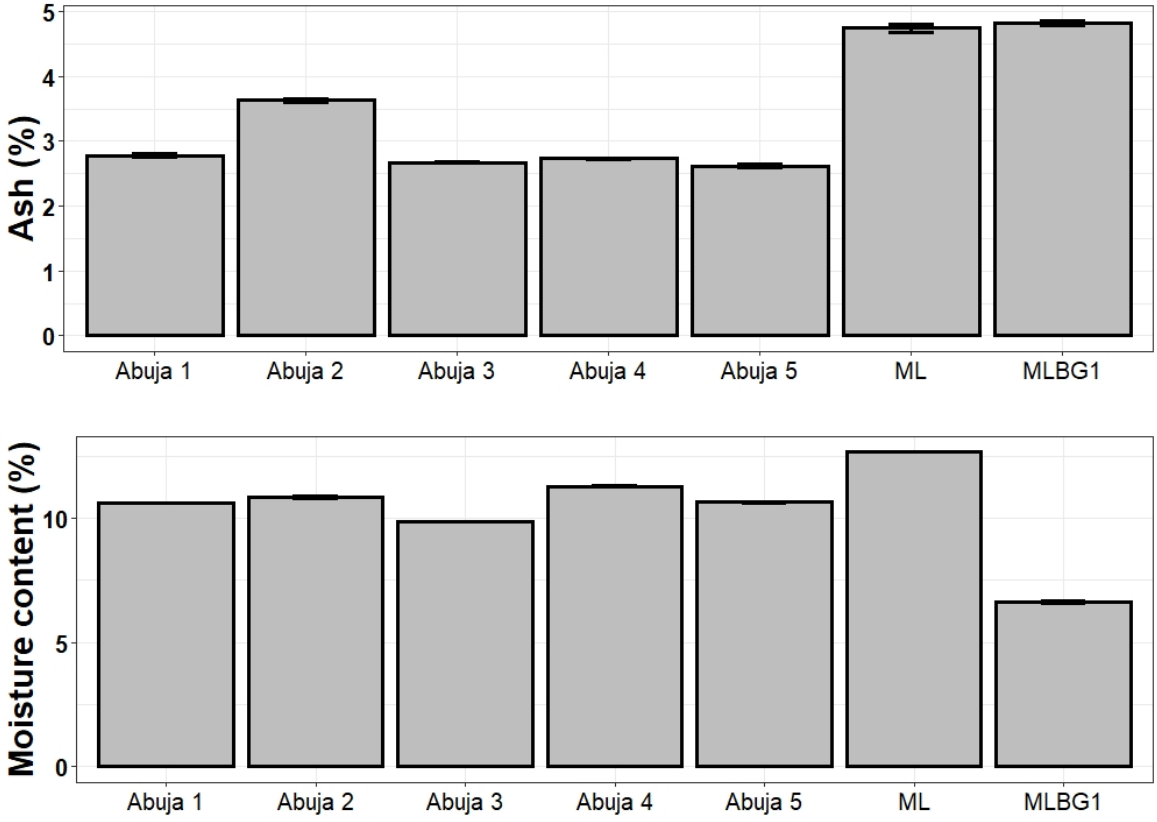

Figure 4.22. Ash and moisture content of Abuja *Kulikuli* and prepared samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

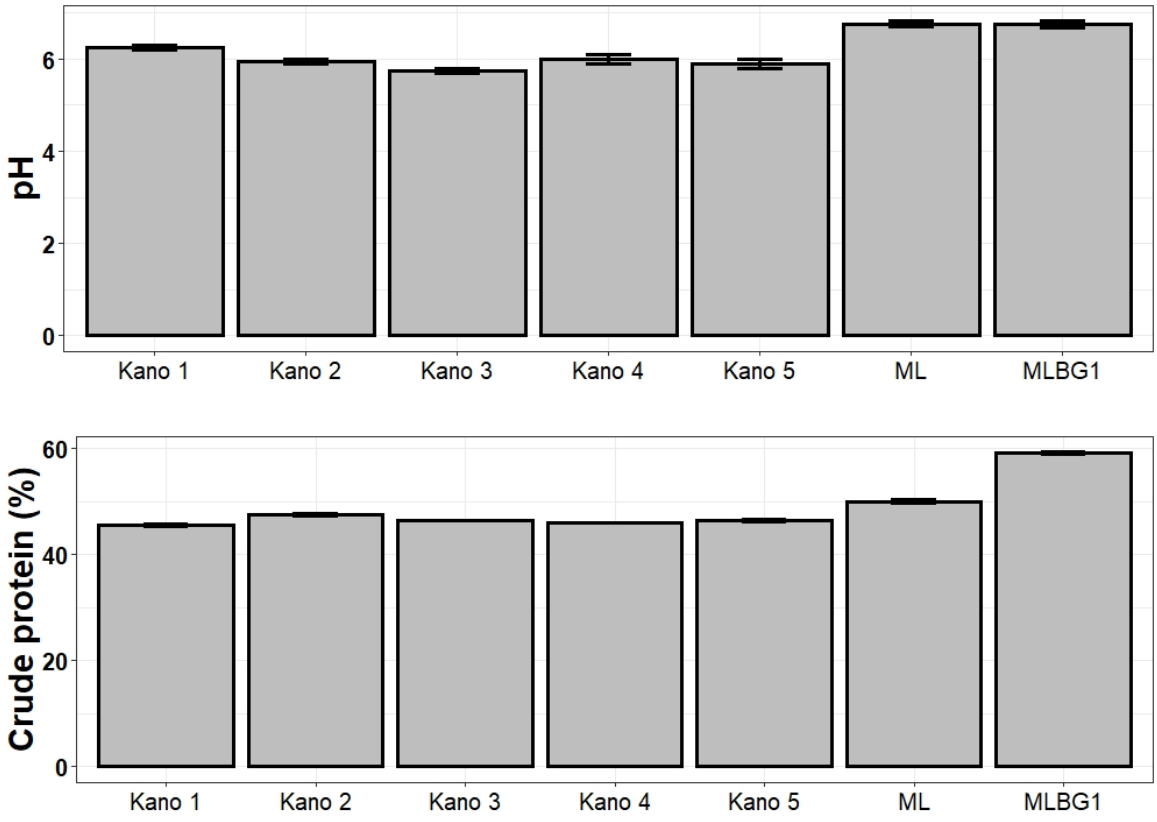

Figure 4.23.pH and crude protein of Kano*Kulikuli* and prepared samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

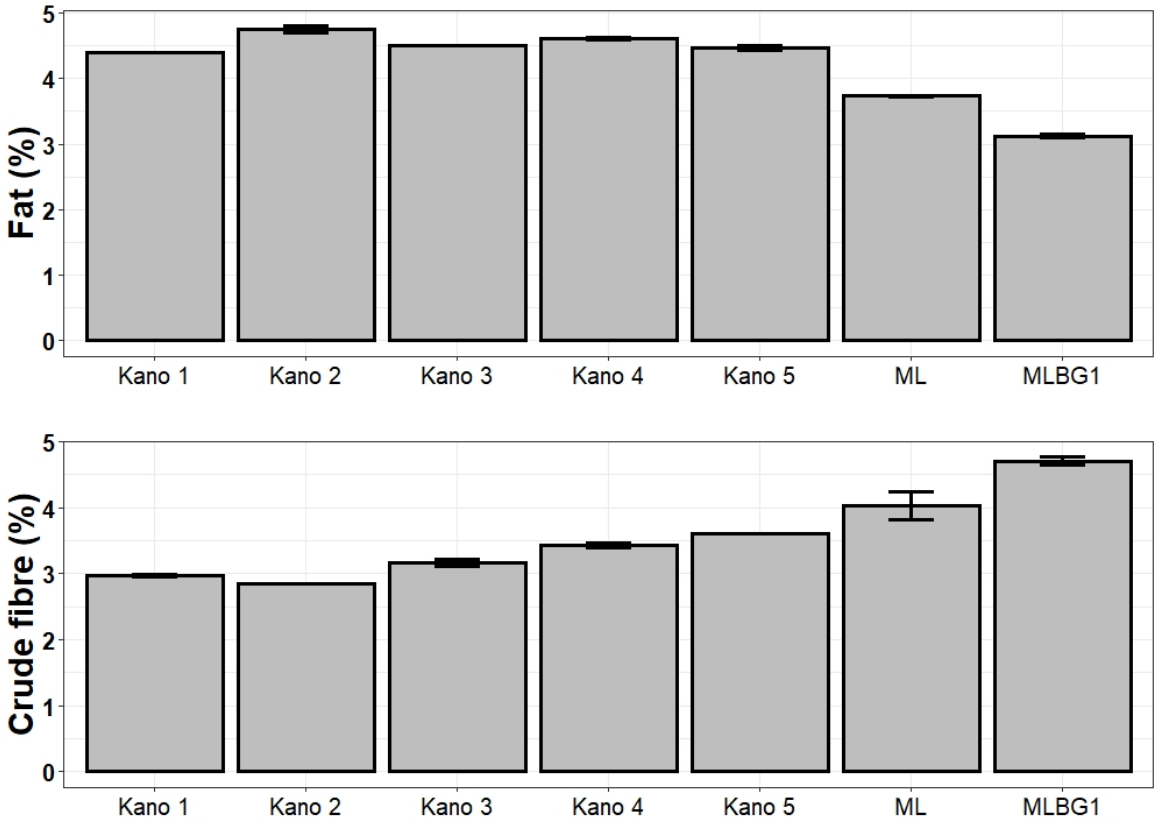

Figure 4.24. Fat and crude fibre of Kano *Kulikuli* and prepared samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

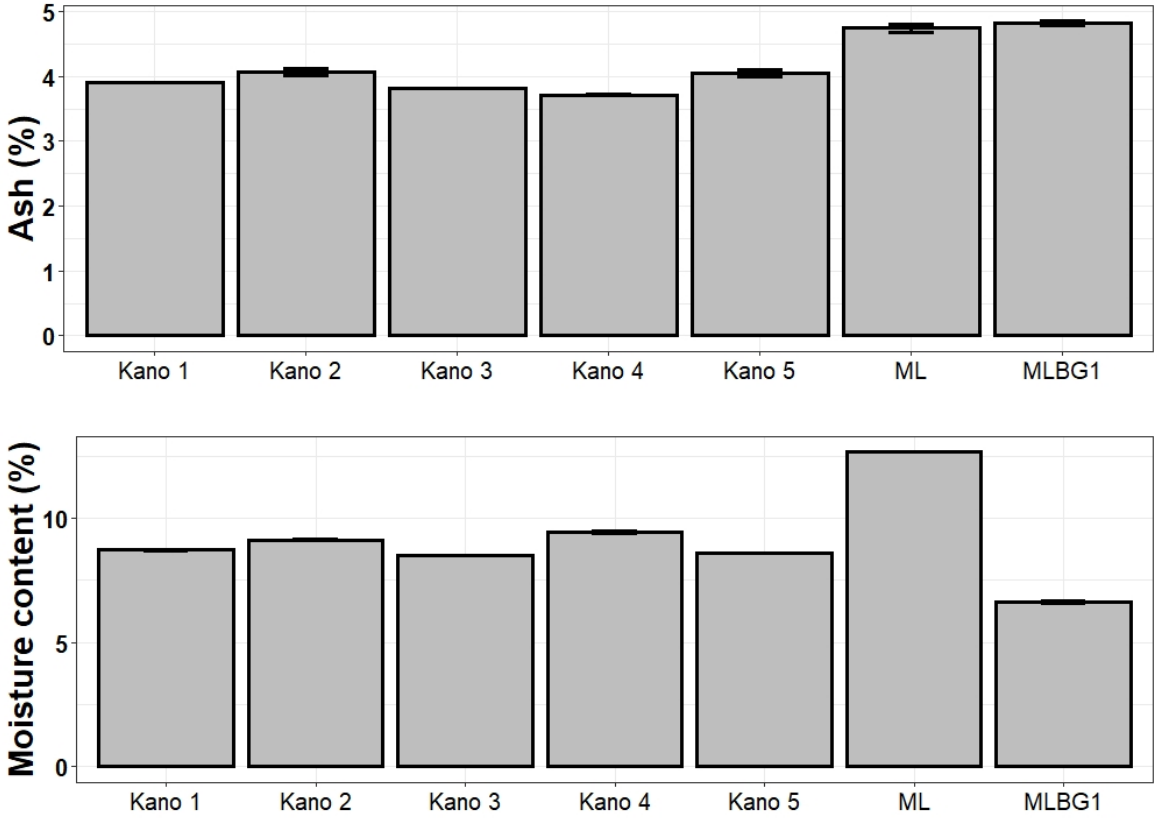

Figure 4.25. Ash and moisture content of Kano *Kulikuli* and prepared samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

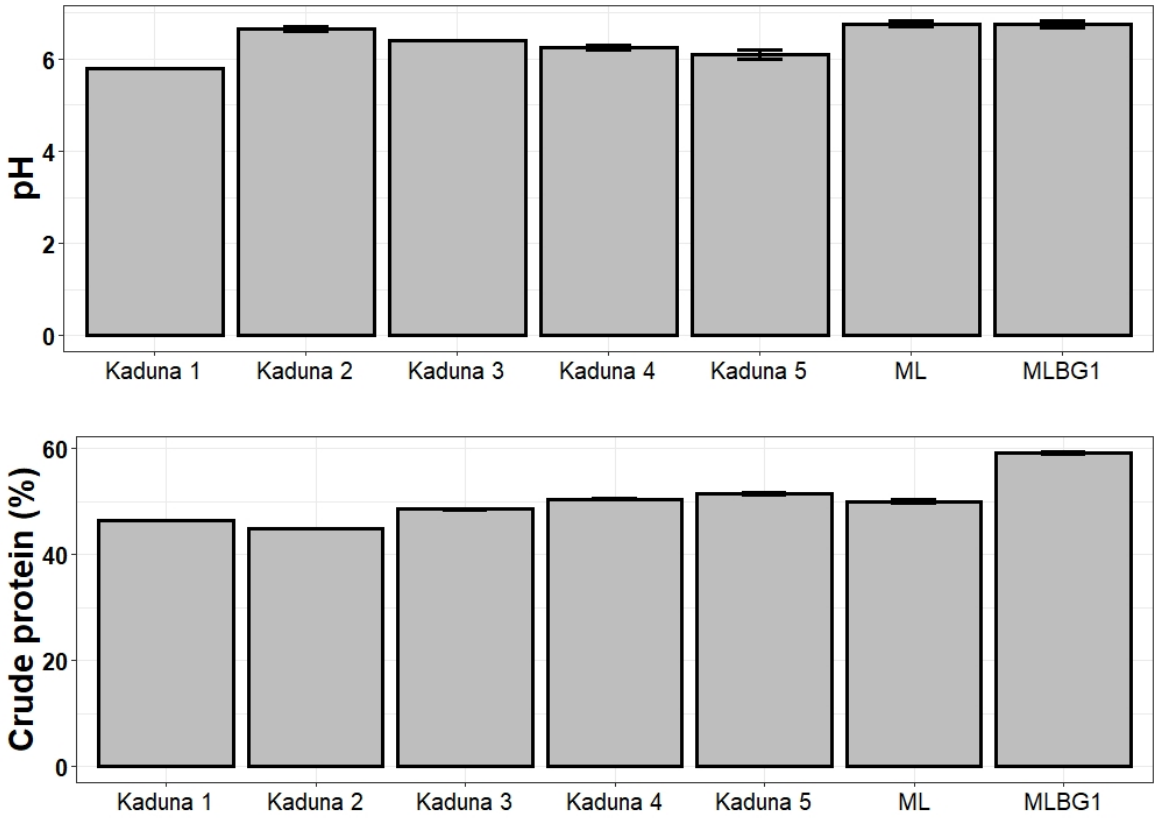

Figure 4.26.pH and crude protein of Kaduna*Kulikuli* and prepared samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

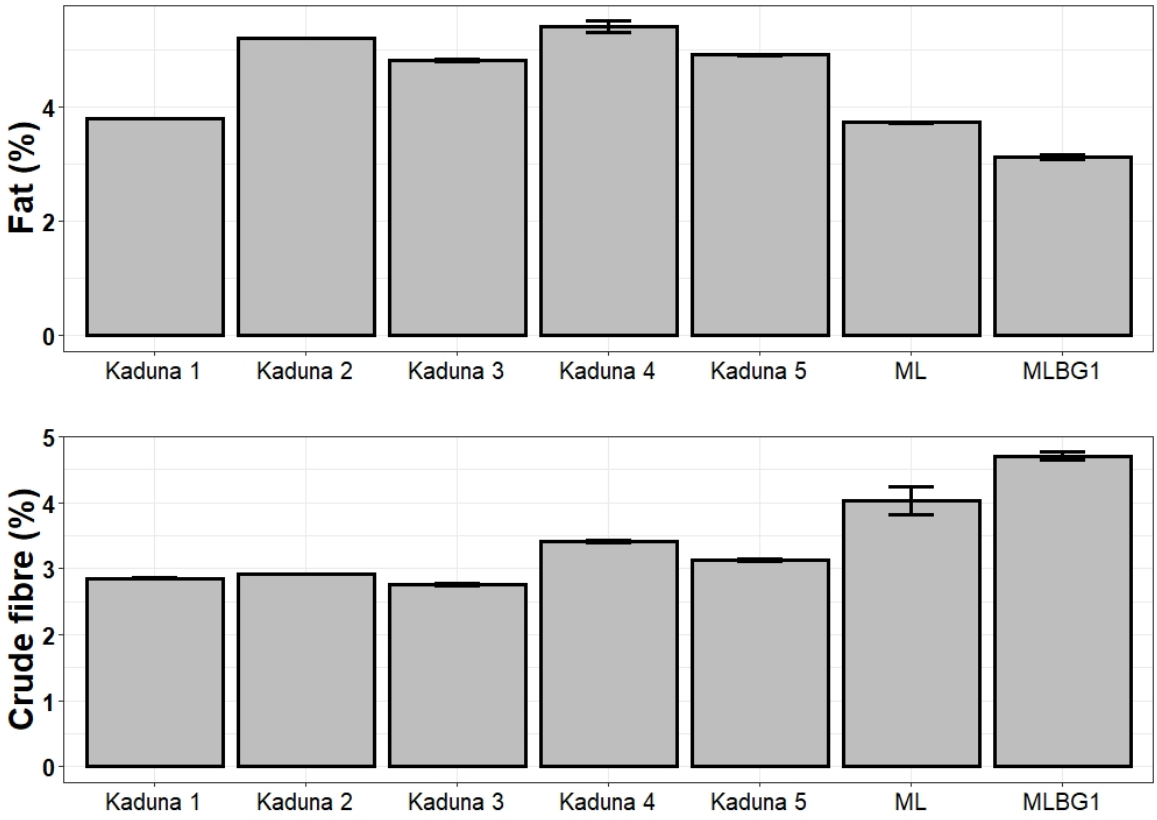

Figure 4.27. Fat and crude fibre of Kaduna *Kulikuli* and prepared samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

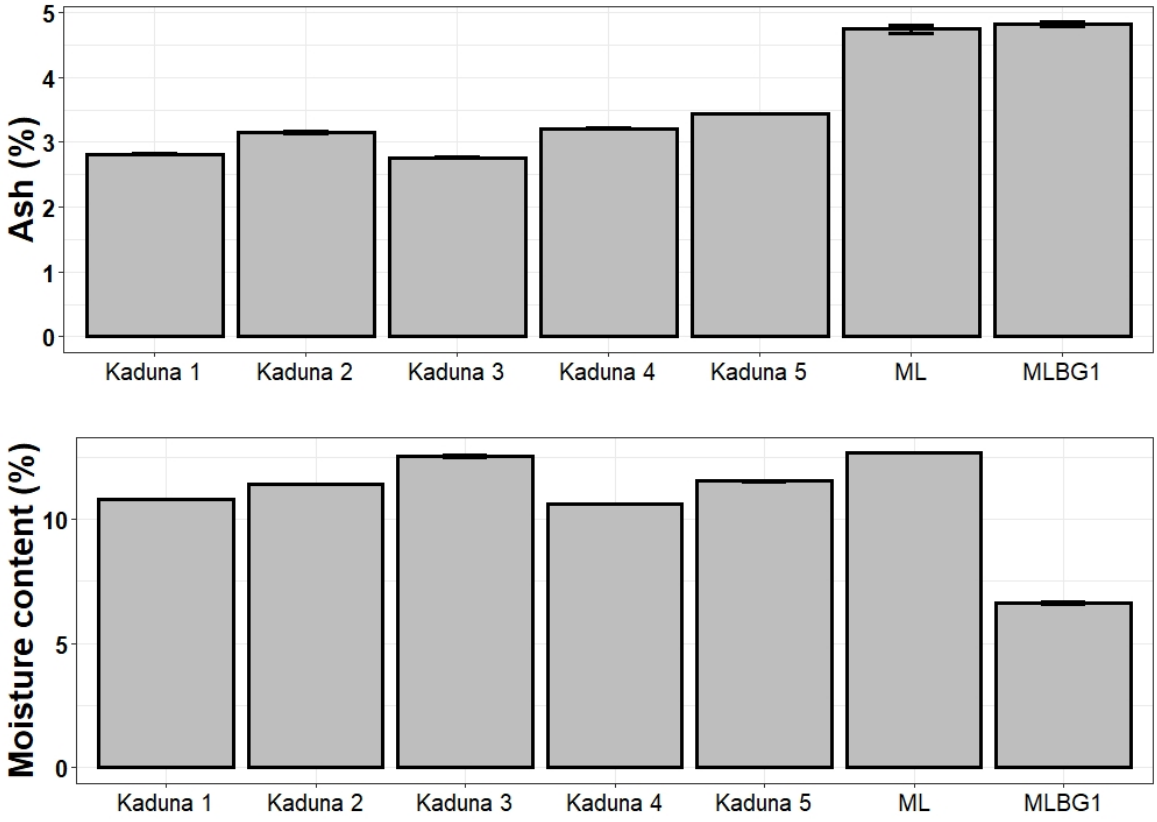

Figure 4.28. Ash and moisture content of Kaduna *Kulikuli* and prepared samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

The percentage moisture content in the sampled *Kulikuli* ranged between 8.88 % (Kano) and 11.91 % (Zamfara).However, laboratory samples without garlic additive, ML had the highest moisture content (12.64±0.51%) as opposed to those with garlic, $MLBG_1$(6.64±0.66%). The range of fat content from market samples was narrow, varying from 3.89 % (Zamfara) to 4.83 % (Kaduna). In contrast, fat content was least for both types of laboratory prepared samples, $MLBG_1$ (2.6 %) and ML (3.4 %). In addition, ash content for market samples was between 2.89 % (Abuja) and 3.91 % (Kano). This was significantly lower than ML (4.84 ± 0.27 %) and $MLBG_1$ (4.84±0.21 %). Regarding pH, both market and laboratory prepared *Kulikuli* had an acidic value that ranged from 6.35 (ML) to 5.6 ($MLBG_1$).

There were no significant differences in the nutrient content of market sampleacross the five studied states, despite differences in the associated fungal incidence in the samples. However, high crude protein content was detected in the samples, 43.18 % (Abuja) to 49.23 % (Sokoto) (Figure 4.18).All *Kulikuli* samples from the five selected states had a crude fibre content of about 3.0 % (Table 4.5). This value was higher for both laboratory prepared samples with 4.05 % (ML) and 4.55 % ($MLBG_1$) as shown in Table 4.4.

Table 4.4. Proximate analysis of market and laboratory prepared *Kulikuli* samples

	Zamfara	Kaduna	Sokoto	Abuja	Kano	ML	$MLBG_1$
pH	6.034±0.31b	6.24±0.32b	6.140±0.42b	5.962±0.18b	5.970±0.18b	6.84±0.34a	6.88±0.13a
% Crude protein	46.31±0.19b	48.37±0.62b	49.23±1.40b	43.18±9.44c	46.41±0.78b	50.23±1.40a	59.23±1.43a
% Fat	3.888±0.19b	4.826±0.19a	4.448±0.14a	4.636±0.21a	4.546±0.14a	3.83±0.21b	3.26±0.37b
% Crude Fibre	3.051±0.37b	3.018±0.26b	2.990±0.60c	3.139±0.60b	3.202±0.31b	4.05±0.47a	4.55±0.30a
% Ash	2.948±0.43c	3.074±0.29b	3.842±0.21b	2.885±0.42c	3.911±0.15b	4.84±0.27a	4.84±0.21a
% Moisture content	11.91±0.78a	11.37±0.76a	10.64±0.52b	10.64±0.51b	8.876±0.39c	12.64±0.51a	6.64±0.66d

Values are Mean of three replicates ± Standard Deviation; Means ± Standard Deviation have the same alphabet letter in the same row are not significantly different according to Duncan Multiple Range Test (DMRT) at differences ($P \leq 0.05$).
ML = Mycology Laboratory, Department of Botany UI *Kulikuli* without garlic;
$MLBG_1$= Mycology Laboratory, Department of Botany UI *Kulikuli* with garlic

Key: = Higher; = Lower

The descriptive statistics shown in Table 4.5 revealeda percentage difference of 6.05 % between the crude protein content of *Kulikuli* from Sokoto and Abuja. Accordingly, a percentage crude protein of 48.37 %, 46.41 % and 46.31% was observed in Kaduna, Kano and Zamfara,respectively. Furthermore, the highest fat content of 4.83 ± 0.61 % was recorded in Kaduna samples as opposed to Zamfara, which had the least (3.89±0.19%). Similarly, ash content was highest in Kano samples (3.91±0.15%) and lowest in Abuja (2.89±0.42%). Moisture content was highest in Zamfara (11.9±0.78%) and lowest in Kano (8.87±0.39%).

Table 4.5. Descriptive statistics of proximate analysis between the States

	State	N	Mean	Std. Deviation	Std. Error	CI Lower Bound	CI Upper Bound
pH	Zamfara	5	6.034	0.3107	0.139	5.648	6.420
	Kaduna	5	6.240	0.319	0.143	5.844	6.636
	Sokoto	5	6.140	0.428	0.191	5.609	6.671
	Abuja	5	5.962	0.181	0.081	5.737	6.187
	Kano	5	5.970	0.182	0.082	5.744	6.196
	Total	25	6.069	0.294	0.059	5.948	6.191
CP	Zamfara	5	46.310	1.076	0.481	44.975	47.65
	Kaduna	5	48.372	2.7715	1.239	44.931	51.813
	Sokoto	5	49.234	1.400	0.626	47.496	50.972
	Abuja	5	43.184	9.440	4.222	31.463	54.905
	Kano	5	46.408	0.778	0.348	45.442	47.374
	Total	25	46.702	4.614	0.923	44.797	48.607
FAT	Zamfara	5	3.888	0.194	0.087	3.647	4.129
	Kaduna	5	4.826	0.618	0.277	4.058	5.594
	Sokoto	5	4.448	0.135	0.060	4.280	4.616
	Abuja	5	4.636	0.215	0.096	4.369	4.903
	Kano	5	4.546	0.137	0.061	4.376	4.716
	Total	25	4.469	0.433	0.087	4.290	4.648
CF	Zamfara	5	3.052	0.374	0.167	2.588	3.516
	Kaduna	5	3.018	0.262	0.117	2.692	3.344
	Sokoto	5	2.990	0.596	0.267	2.250	3.730
	Abuja	5	3.140	0.598	0.267	2.398	3.882
	Kano	5	3.202	0.315	0.141	2.811	3.593
	Total	25	3.080	0.420	0.084	2.907	3.254
ASH	Zamfara	5	2.948	0.429	0.192	2.415	3.481
	Kaduna	5	3.074	0.286	0.128	2.719	3.429
	Sokoto	5	3.842	0.208	0.093	3.583	4.101
	Abuja	5	2.886	0.420	0.188	2.364	3.408
	Kano	5	3.912	0.153	0.068	3.722	4.102
	Total	25	3.332	0.543	0.109	3.108	3.557
MC	Zamfara	5	11.912	0.784	0.351	10.938	12.886
	Kaduna	5	11.372	0.757	0.338	10.433	12.312
	Sokoto	5	10.642	0.523	0.234	9.993	11.291
	Abuja	5	10.638	0.515	0.230	9.999	11.277
	Kano	5	8.876	0.390	0.175	8.392	9.361
	Total	25	10.688	1.186	0.237	10.198	11.178

CP= crude protein; CF, crude fat; MC, moisture content. CI= 95% Confidence Interval for Mean

From the descriptive summary in Table 4.5, pH level washighest in Kaduna (6.24±0.32) and lowest in Kano (5.97±0.18) and Abuja (5.96±0.18) respectively. In addition, the crude protein level washighest in Sokoto (49.2±1.40 %) and lowest in Abuja (43.2±9.44 %). Furthermore, the fat content washighest in Kaduna (4.83±0.61 %) and lowest in Zamfara (3.89±0.19 %). Ashcontent in Kano was highest (3.20±0.31 %) and lowest in Sokoto (2.99±0.60 %), while ash content washighest in Kano (3.91±0.15 %) and lowest in Abuja (2.89±0.42 %). The highest value of moisture content was obtained from Zamfara State (11.9±0.78 %) and the lowest in Kano State (8.87±0.39 %).

Table 4.6. Anova results of proximate analysis of *Kulikuli* samples

		Sum of Squares	Df	Mean Square	F	p value
	BG	0.284	4	0.071	0.793	0.543
pH	WG	1.789	20	0.089		
	Total	2.073	24			
	BG	109.082	4	27.270	1.357	0.034
CP	WG	402.054	20	20.103		
	Total	511.136	24			
	BG	2.496	4	0.624	6.202	0.002
FAT	WG	2.013	20	0.101		
	Total	4.509	24			
	BG	0.156	4	0.039	0.191	0.940
CF	WG	4.079	20	0.204		
	Total	4.235	24			
	BG	5.047	4	1.262	12.391	0.000
ASH	WG	2.037	20	0.102		
	Total	7.084	24			
	BG	26.270	4	6.567	17.484	0.000
MC	WG	7.512	20	0.376		
	Total	33.782	24			

Key: BG = Between Groups; WG = Within Groups

The analysis of variance (Table 4.6) shows that moisture contentF (4,20) = 17.484, ash content F(4,20) = 12.391, crude protein F(4,20) =1.357as well as fat content F(4,20) = 6.202 has significance values of 0.000, 0.000, 0.034 and 0.002 respectively which are below *p*= 0.05. Therefore, there is a statistically significant difference in the mean moisture, ash as well as crude protein and fat content between the studied *Kulikuli* in the sampled states. On the contrary, the variance of pH F(4,0) = 0.793 and crude fat F(4,20) = 0.940 has significance value of 0.543 and 0.940 respectively which are above *p* = 0.05. Therefore, there is a statistically significant difference in the mean pH and crude fat between the studied *Kulikuli* in the sampled states.

4.5.Aflatoxins contents of*Kulikuli*

There were varying proportions of Aflatoxins B_1, B_2, G_1 and G_2 in the*Kulikuli* samples that were collected from Sokoto State (Figure 4.29 and Figure 4.30).However, no sample from this state contained aflatoxin concentration that is higher than the tolerance limit of 2 µg/kg. Generally, aflatoxins B_1ranging from 0.84–1.27 µg/kg,B_2ranging from 0.93–1.32 µg/kg(Figure 4.29) werethe most prevalent while aflatoxins G_1(0.01–0.03 µg/kg) and G_2(0.03–0.05 µg/kg) (Figure 4.30) were the least prevalentwithin the sampled *Kulikuli*.This corroborated the highest incidence of *A. flavus* and *A. parasiticus*fungi thatwere the major aflatoxigenic fungal strains.

Generally, there was lower aflatoxin content in the laboratory prepared samples with garlic additive and without garlic ($MLBG_1$ and ML, respectively) as compared with all market samples from Sokoto State, and this was concurrent with lessercount of aflatoxigenic fungi in $MLBG_1$and ML as compared with the market samples.The laboratory prepared samples ($MLBG_1$ and ML) had the least overall aflatoxin contents (Figure4.29 and Figure 4.30). Aflatoxin concentrations for $MLBG_1$were as follows: AFB_1(0.34 ± 0.01 µgkg^{-1}), AFB_2(0.37 ± 0.00 µgkg^{-1}), AFG_1(0.01 ±0.00 µgkg^{-1}) as well as AFG_2(0.02 ± 0.00µgkg^{-1}).Similarly, the MLsamples had the second least overall aflatoxin B_1, B_2, G_1 and G_2concentration of 0.70± 0.01 µgkg^{-1}, 0.74 ± 0.00µgkg^{-1}, 0.02 ± 0.01µgkg^{-1} and 0.034 ± 0.00µgkg^{-1}, respectively.

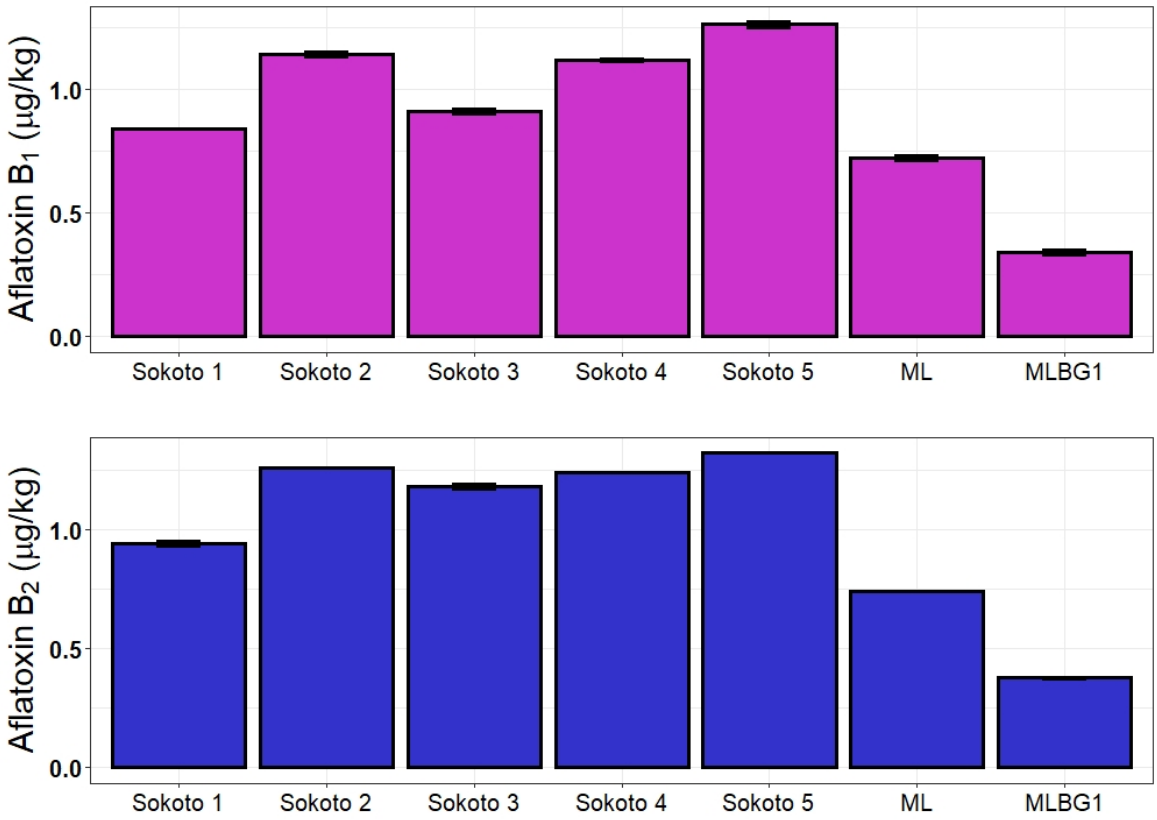

Figure 4.29. Aflatoxins B_1 and B_2 levels of *Kulikuli* from Sokoto and control samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

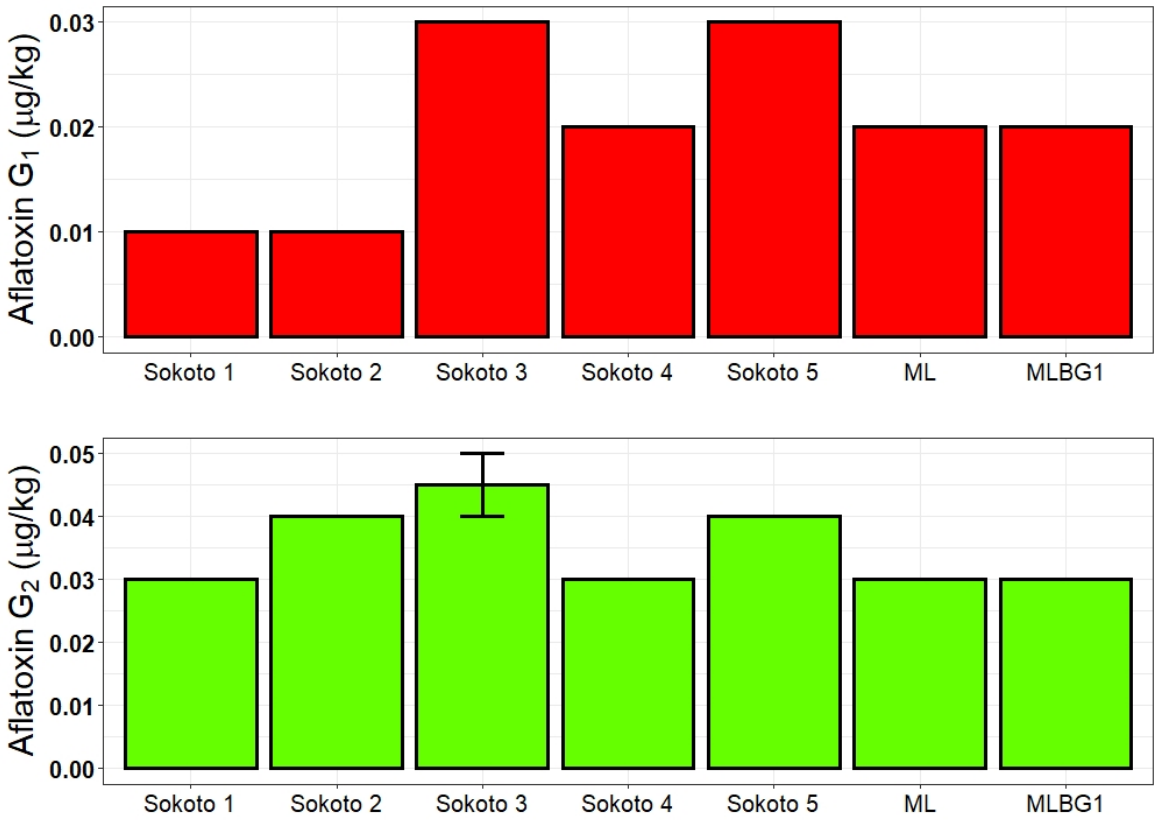

Figure 4.30. Aflatoxins G_1 and G_2 levels of *Kulikuli* from Sokoto and control samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

In addition, there were varying concentrations of Aflatoxins B_1, B_2, G_1 and G_2 in the *Kulikuli* samples that were collected from Abuja (Figure 4.31 and Figure 4.32), as well no sample from this state contains aflatoxin concentration that is higher than the tolerance limit of 2 µg/kg. Generally, aflatoxins B_1 ranging from 1.12–1.32 µg/kg, B_2 ranging from 1.18–1.62 µg/kg (Figure 4.31) were the most prevalent while aflatoxins G_1 (0.06–0.12 µg/kg) and G_2 (0.08–0.15 µg/kg) (Figure 4.32) were the least prevalent within the sampled *Kulikuli*.This corroborated the highest incidence of *A. flavus* and *A. parasiticus*fungi, which are the major aflatoxigenic fungal strains.

Generally, there was lower aflatoxin content in the laboratory prepared ($MLBG_1$ and ML) as compared with all the market samples across all the states, and this was concurrent with the presence of less aflatoxigenic fungi in $MLBG_1$ and ML as compared with the market samples. In all, the garlic treated laboratory prepared *Kulikuli*($MLBG_1$) had the least aflatoxin B_1, B_2, G_1 and G_2 content of 0.34 ± 0.01 μgkg^{-1}, 0.37 ± 0.00μgkg^{-1}, 0.01 ± 0.00μgkg^{-1} and 0.02 ± 0.00μgkg^{-1}, respectively as shown in Figure 4.31 and Figure 4.32.Aflatoxin contents in theMLsamples were 0.70± 0.01 μgkg^{-1} (AFB_1), 0.74 ± 0.00μgkg^{-1} (AFB_1), 0.02± 0.01 μgkg^{-1}, (AFG_1) and 0.03 ± 0.00μgkg^{-1} (AFG_2).

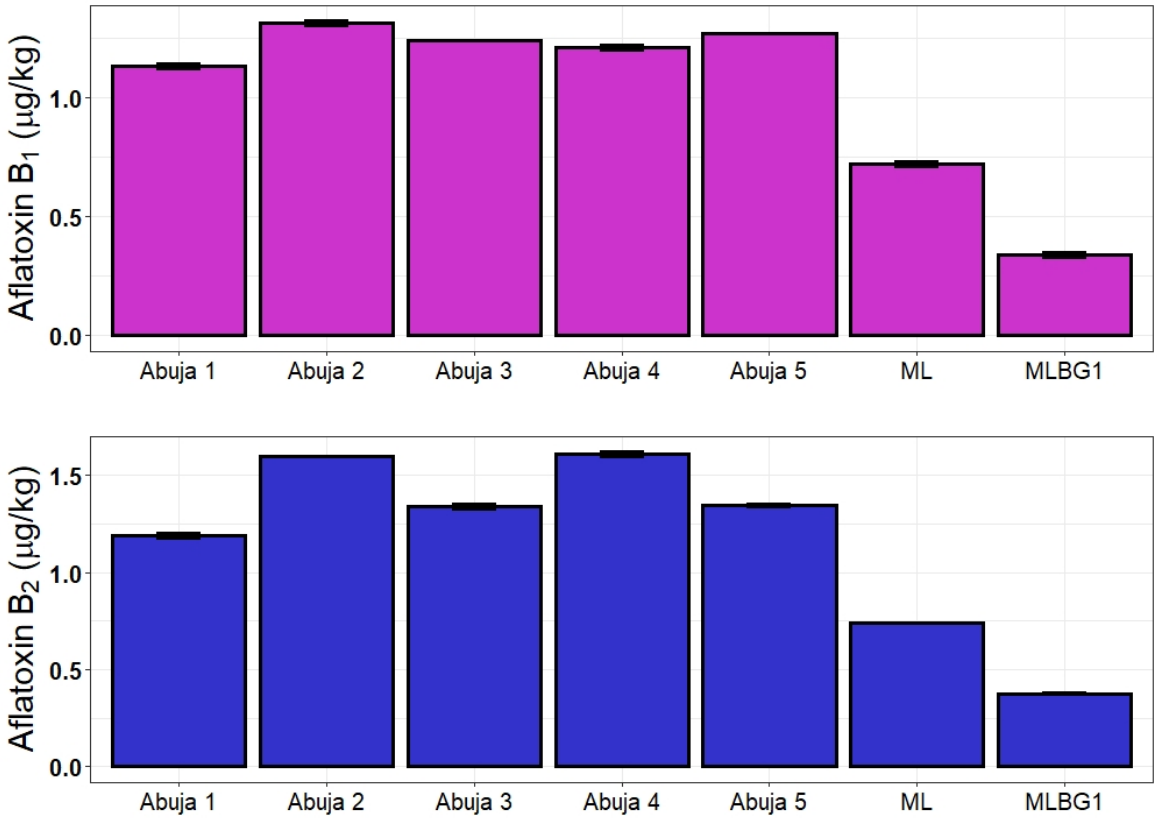

Figure 4.31. AflatoxinsB_1 and B_2levels of *Kulikuli* from Abuja and control samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

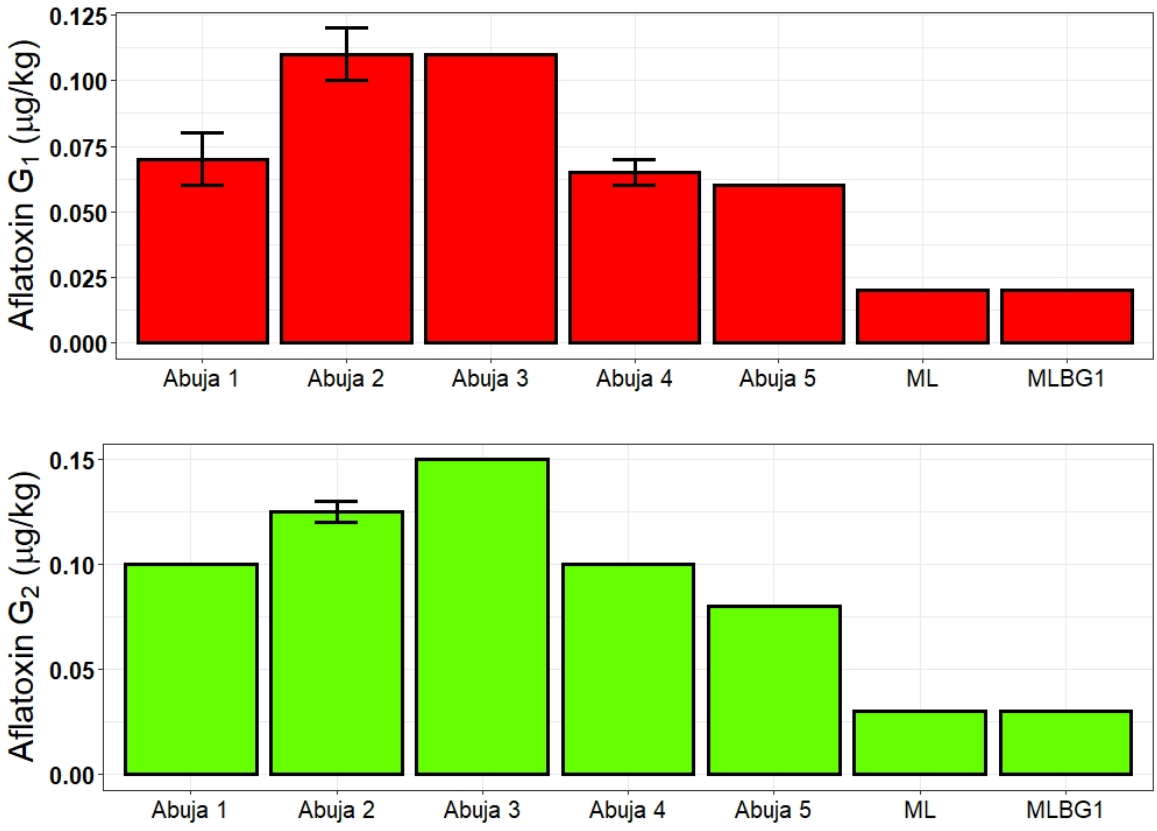

Figure 4.32. AflatoxinsG_1 and G_2levels of *Kulikuli* from Abuja and control samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

Furthermore, there were varying concentrations of aflatoxins B_1, B_2, G_1 and G_2 in the *Kulikuli* samples that were collected from Zamfara State (Figure 4.33 and 4.34) but there was a sample from Dampa Market(Zamfara 2) that contained aflatoxin concentration higher than the tolerance limit of 2 µg/kg according to the standard of the European Union.

Generally, aflatoxins B_1which ranged from 1.20 to 1.96µg/kg and B_2 (1.44–2.16 µg/kg) were the most prevalent, while aflatoxinsG_1 (0.03–0.05 µg/kg) and G_2 (0.02–0.07 µg/kg) were the least prevalentwithin the sampled *Kulikuli*.This corroborated the highest incidence of *A. flavus* and *A. parasiticus*, whichwere the major aflatoxigenic fungal strains. There was however lower aflatoxin content in the laboratory prepared samples ($MLBG_1$ and ML) as compared with all the market samples across all the states, and this was concurrent with the presence of less aflatoxigenic fungi in $MLBG_1$ and ML as compared with the market samples (Figure 4.33 and Figure 4.34).

In all, the garlic treated laboratory prepared *Kulikuli*samples ($MLBG_1$ and ML)had the least aflatoxin contents as presented in Figure 4.21. The $MLBG_1$ had the least overall aflatoxin B_1 content of 0.34 ± 0.01 μgkg^{-1}, B_2(0.37 ± 0.00μgkg^{-1}), G_1(0.01 ± 0.00μgkg^{-1}) and G_2(0.02 ± 0.00 μgkg^{-1}),while ML had the least overall aflatoxin B_1, B_2, G_1 and G_2content of 0.70± 0.01 μgkg^{-1}, 0.74 ± 0.00μgkg^{-1}, 0.02± 0.01 μgkg^{-1} and 0.03 ± 0.00μgkg^{-1}, respectively.

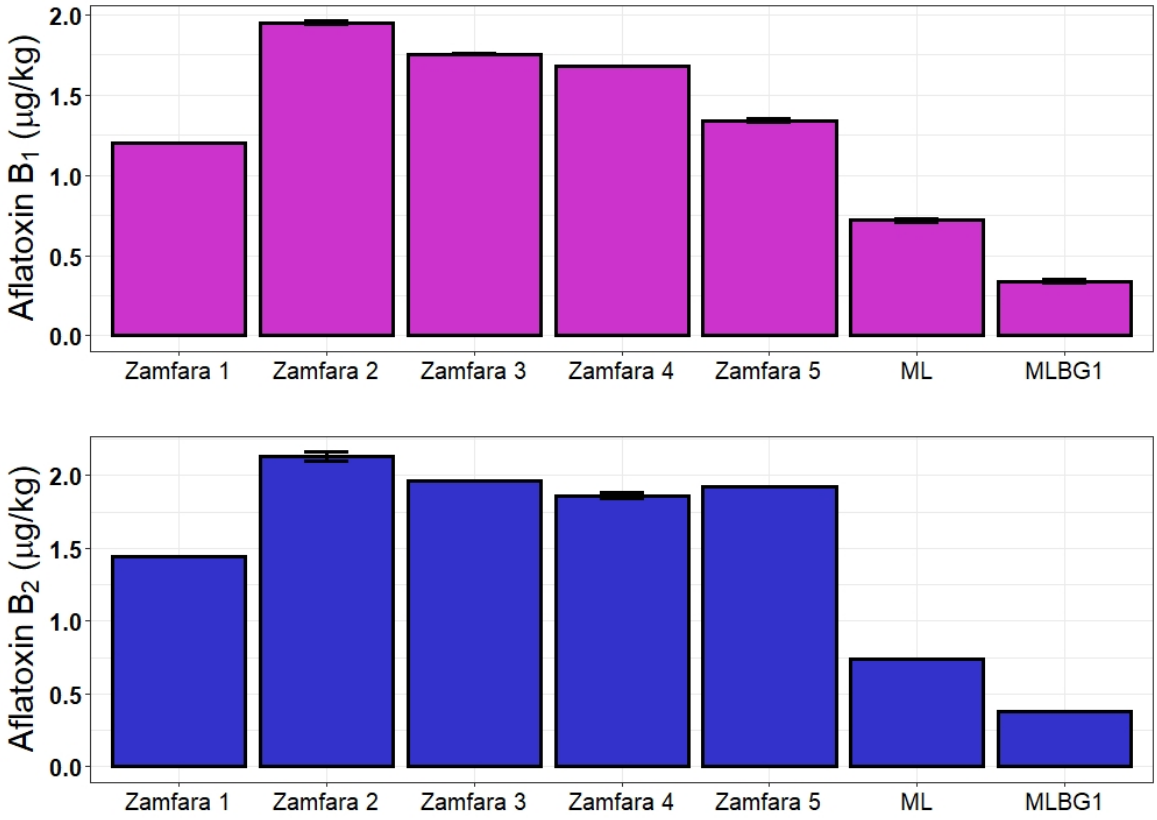

Figure 4.33. Aflatoxins B_1 and B_2 levels of *Kulikuli* from Zamfara and control samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

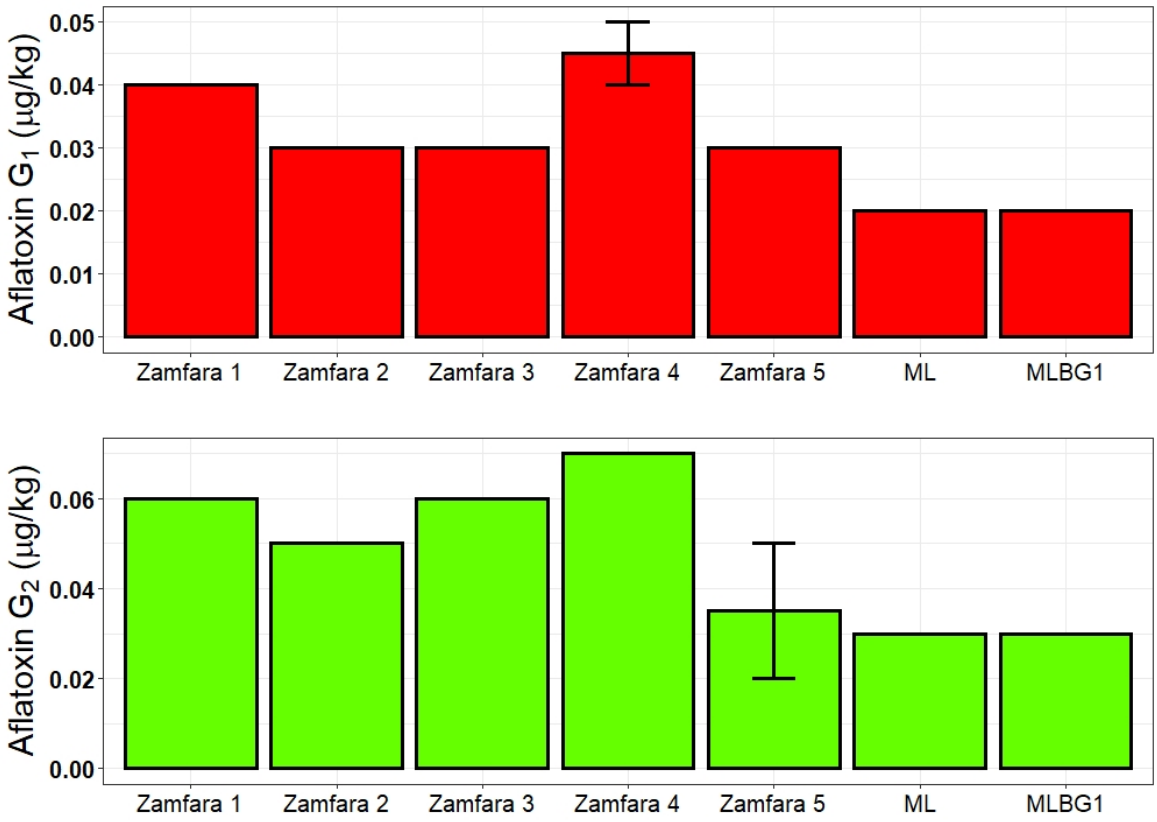

Figure 4.34. Aflatoxins G_1 and G_2 levels of *Kulikuli* from Zamfara and control samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

Moreover, varying concentrations of aflatoxins B_1, B_2, G_1 and G_2were detected in the *Kulikuli* samples that were collected from Kano State (Figure 4.35 and Figure 4.36) but there was no sample from this state that contained aflatoxin concentration higher than the tolerance limit of 2 µg/kg according to the standard of the European Union.

Generally, aflatoxins B_1 (0.88–1.13 µg/kg) and B_2 (1.27–1.60 µg/kg) (Figure 4.35) werethe most prevalent, while aflatoxins G_1 (0.01–0.03 µg/kg) and G_2 (0.02–0.36 µg/kg) (Figure 4.36)were the least prevalent within the sampled *Kulikuli*.Hence, this corroborates the highest incidence of *A. flavus* and *A. parasiticus,* which were the major aflatoxigenic fungal strains in the market samples. There was however lower aflatoxin content in the laboratory prepared samples ($MLBG_1$ and ML) as compared with all the market samples across all the states.This was concurrent with the prevalence aflatoxigenic fungi in $MLBG_1$ and ML as compared with the market samples.

The laboratory prepared samples with and without garlic ($MLBG_1$ and ML)had the least overall aflatoxin concentrationacross all the samples (Figure 4.35 and Figure 4.36). The $MLBG_1$ had the least overall aflatoxin B_1, B_2, G_1, G_2 concentrations of $0.34 \pm 0.01 \mu gkg^{-1}$, $0.37 \pm 0.00\ \mu gkg^{-1}$, $0.01 \pm 0.00\ \mu gkg^{-1}$and $0.02 \pm 0.00 \mu gkg^{-1}$, respectively. On the other hand, aflatoxin levels in ML sampleswere $0.70 \pm 0.01\ \mu gkg^{-1}$ (B_1), $0.74 \pm 0.00 \mu gkg^{-1}$ (B_2), $0.02 \pm 0.01 \mu gkg^{-1}$ (G_1) and $0.03 \pm 0.00 \mu gkg^{-1}$ (G_2).

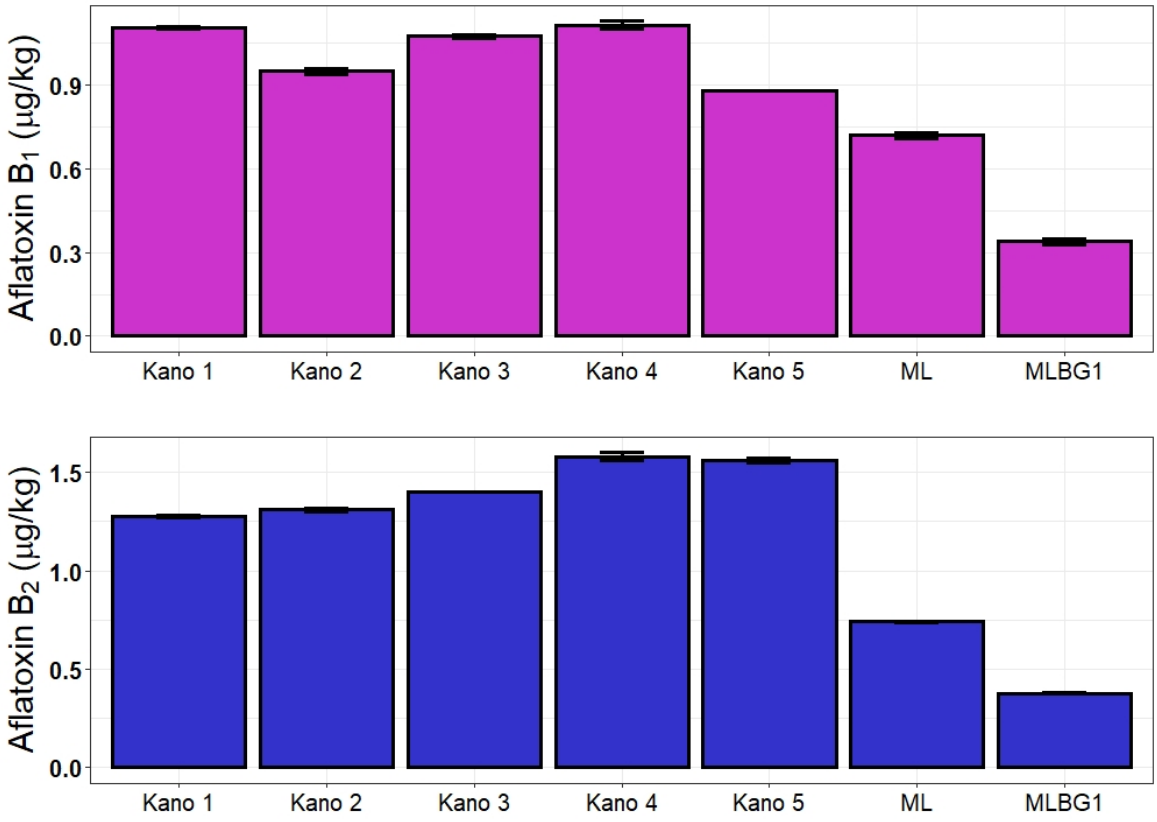

Figure 4.35. Aflatoxins B_1 and B_2 levels of *Kulikuli* from Kano and control samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

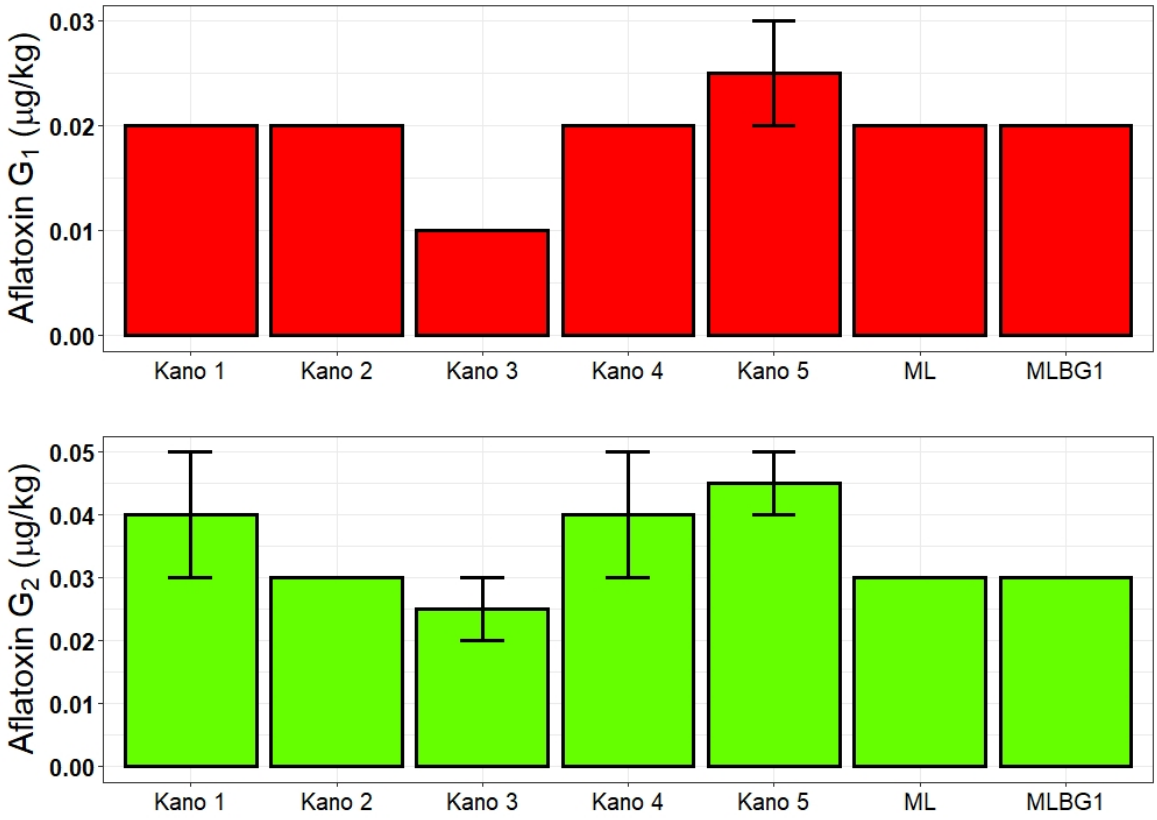

Figure 4.36. Aflatoxins G_1 and G_2 levels of *Kulikuli* from Kano and control samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

In addition, varying concentration of aflatoxins B_1, B_2, G_1 and G_2were detected in the *Kulikuli* samples collected from Kaduna State (Figure 4.37 and Figure 4.38).There were samples from this state (Kaduna 3 (Sabuwar Gari) and Kaduna 4 (Kakuri)) that contained aflatoxin concentrations higher than the tolerance limit of 2 µg/kg according to the standard of the European Union.

Generally, aflatoxins B_1 (1.47–1.80 µg/kg) and B_2 (1.76–2.73 µg/kg) (Figure 4.37) were the most prevalent, while aflatoxins G_1 (0.06–0.15 µg/kg) and G_2 (0.07–0.21 µg/kg) (Figure 4.38) were the least prevalent within the sampled *Kulikuli*.This corroborated the highest incidence of *A. flavus* and *A. parasiticus*fungi, whichwere the major aflatoxigenic fungal strains in market samples. There was however lower aflatoxin content in the laboratory prepared samples with garlic ($MLBG_1$) and without garlic (ML) additive as compared with all the market samples across all the states.This result was concurrent with the presence of less aflatoxigenic fungi in $MLBG_1$ and ML as compared with the market samples.

In all, laboratory prepared *Kulikuli*with garlic ($MLBG_1$) had the least aflatoxin contents as presented in Figure 4.37 and Figure 4.38. These samples and the ones prepared in the laboratory without garlic (ML) had the least overall aflatoxin contents across all the samples (Fig 4.23). The Aflatoxin B_1, B_2, G_1 and G_2concentrations for $MLBG_1$were 0.34 ± 0.01 μgkg^{-1}, 0.37 ± 0.00μgkg^{-1}, 0.01 ± 0.00μgkg^{-1} and 0.02 ± 0.00 μgkg^{-1}, respectively. Similarly, aflatoxin concentration for ML sampleswere 0.70 ± 0.01μgkg^{-1} (AFB_1), 0.74 ± 0.00 μgkg^{-1} (AFB_2), 0.02 ± 0.00μgkg^{-1} (AFG_1)and 0.03 ± 0.00μgkg^{-1} (AFG_2).

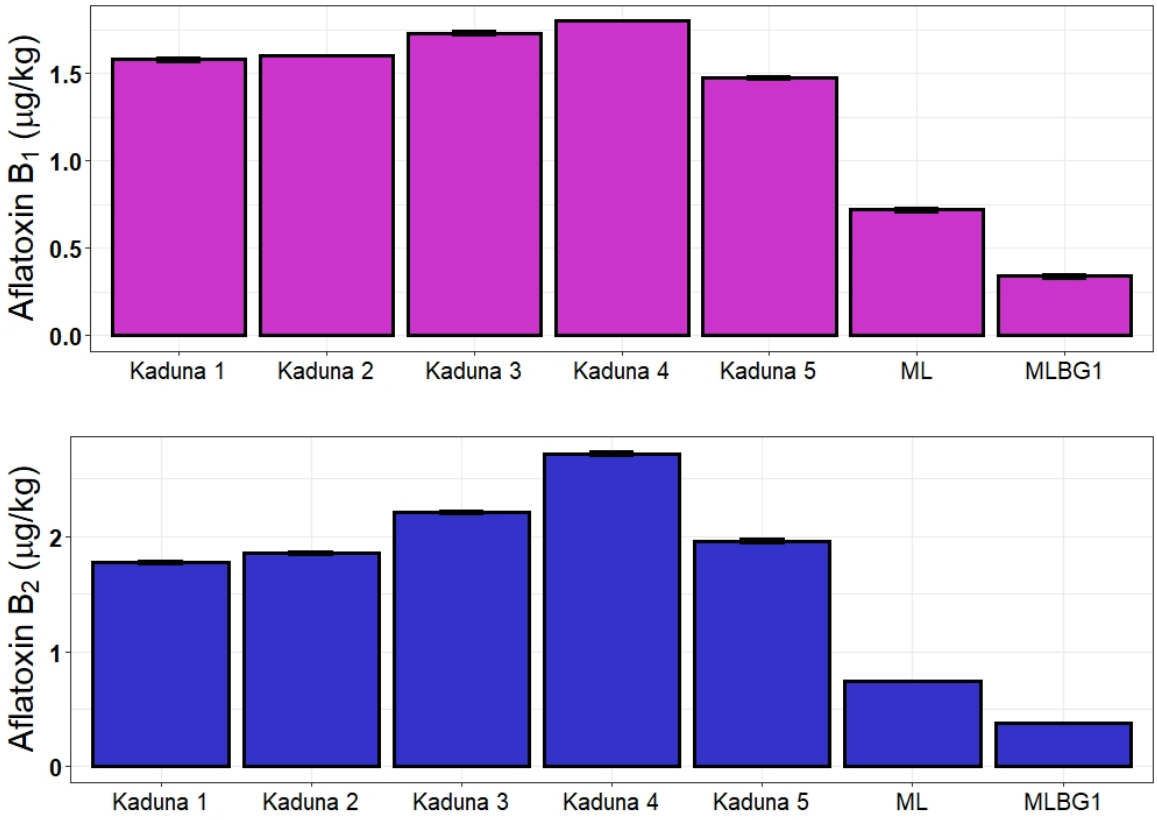

Figure 4.37. Aflatoxins B_1 and B_2 levels of *Kulikuli* from Kaduna and control samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

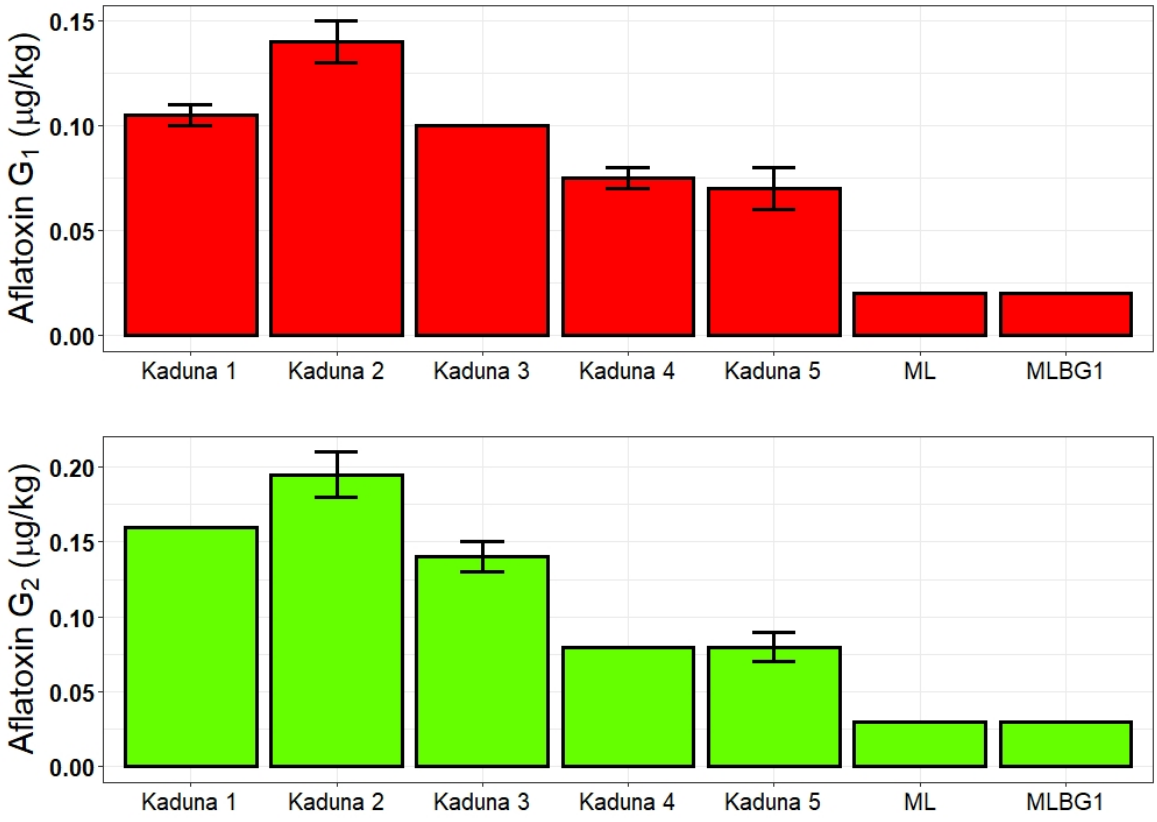

Figure 4.38. Aflatoxins G_1 and G_2 levels of *Kulikuli* from Kaduna and control samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

The comparative aflatoxin concentrations in all the sampledStates and the laboratory prepared *Kulikuli* with and without garlic additive are presented in Figure 4.39 and Figure 4.40 below. Among the sampled states, *Kulikuli*from Kaduna and Zamfara had the highest Aflatoxin B_1concentrations of 1.64±0.13 and 1.59±0.31µgkg^{-1}, respectively (Figure 4.39). In the same note, aflatoxin B_2concentrations were also highest in these two States with2.10 ± 0.38 and 1.86± 0.26 µgkg^{-1}, respectively (Figure 4.39).Kano and Sokoto had comparable concentrations of AFB_1 (1.03±0.11 and 1.05 ±0.17µgkg^{-1}), which were the lowest across the sampled States. Sokoto recorded least aflatoxin B_2 of 1.19 ± 0.15 µgkg^{-1}.Aflatoxin $G_1$was recorded highest in Kaduna (0.10 ± 0.03 µgkg^{-1}) and lowest in Kano (0.02 ± 0.01 µgkg^{-1}) and Sokoto (0.02 ± 0.01 µgkg^{-1}). Similarly, aflatoxin G_2 was at its peak in Kaduna (0.13 ± 0.05 µgkg^{-1}) and lowest in Sokoto (0.04 ± 0.01 µgkg^{-1}) and Kano (0.04 ± 0.01 µgkg^{-1}) (Figure 4.40).

In all, laboratory prepared*Kulikuli*samples with garlic ($MLBG_1$) had the least aflatoxin concentration as presented in Figure 4.39 and Figure 4.40. The ML samples had the second least overall aflatoxin levels across all the samples. Aflatoxin B_1, B_2, G_1 and G_2contentsin the $MLBG_1$ samples were lowest as follows: 0.34 ± 0.01µgkg^{-1},0.37 ± 0.00µgkg^{-1}, 0.01 ± 0.00 µgkg^{-1} and 0.02 ± 0.00µgkg^{-1}while those without garlic (ML)had the second least overall aflatoxin B_1 content (0.704 ± 0.01 µgkg^{-1}), B_2(0.74 ± 0.00µgkg^{-1}), G_1(0.02 ± 0.01µgkg^{-1})and G_2(0.03 ± 0.00µgkg^{-1}).

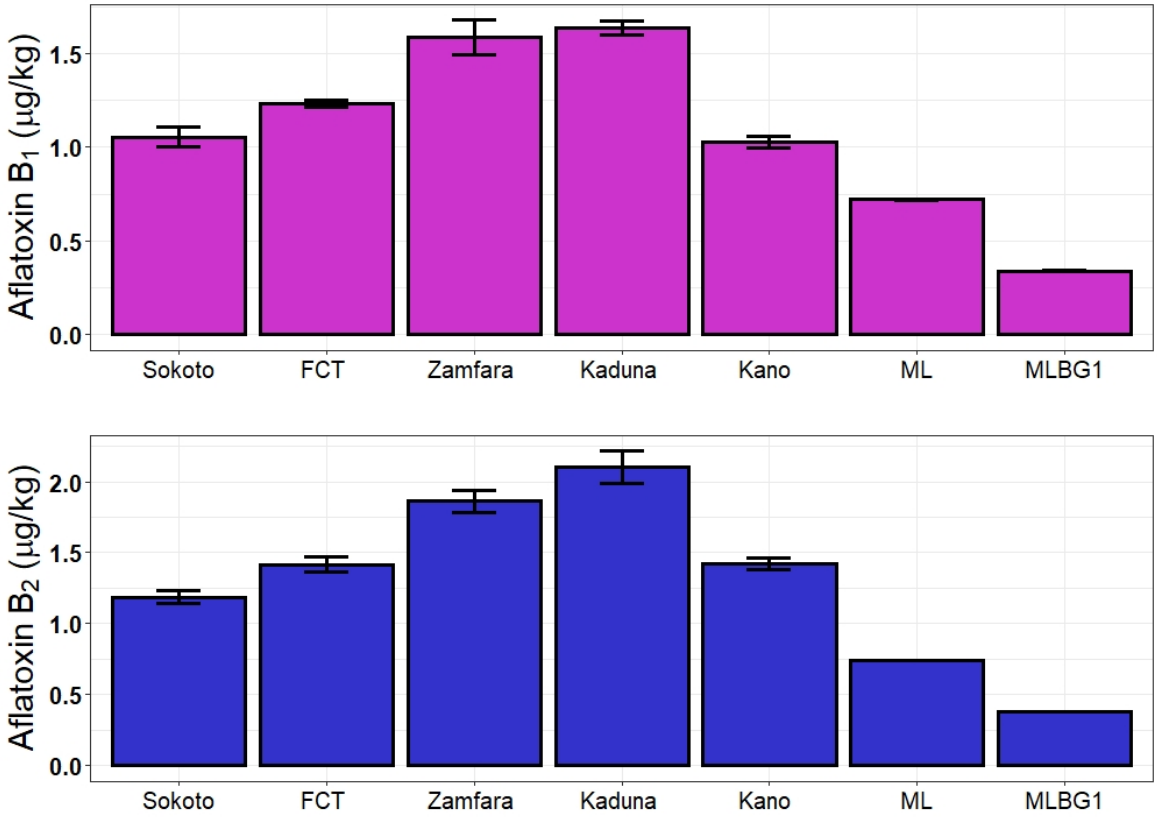

Figure 4.39. Aflatoxins B_1 and B_2 levels of all studied *Kulikuli* samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

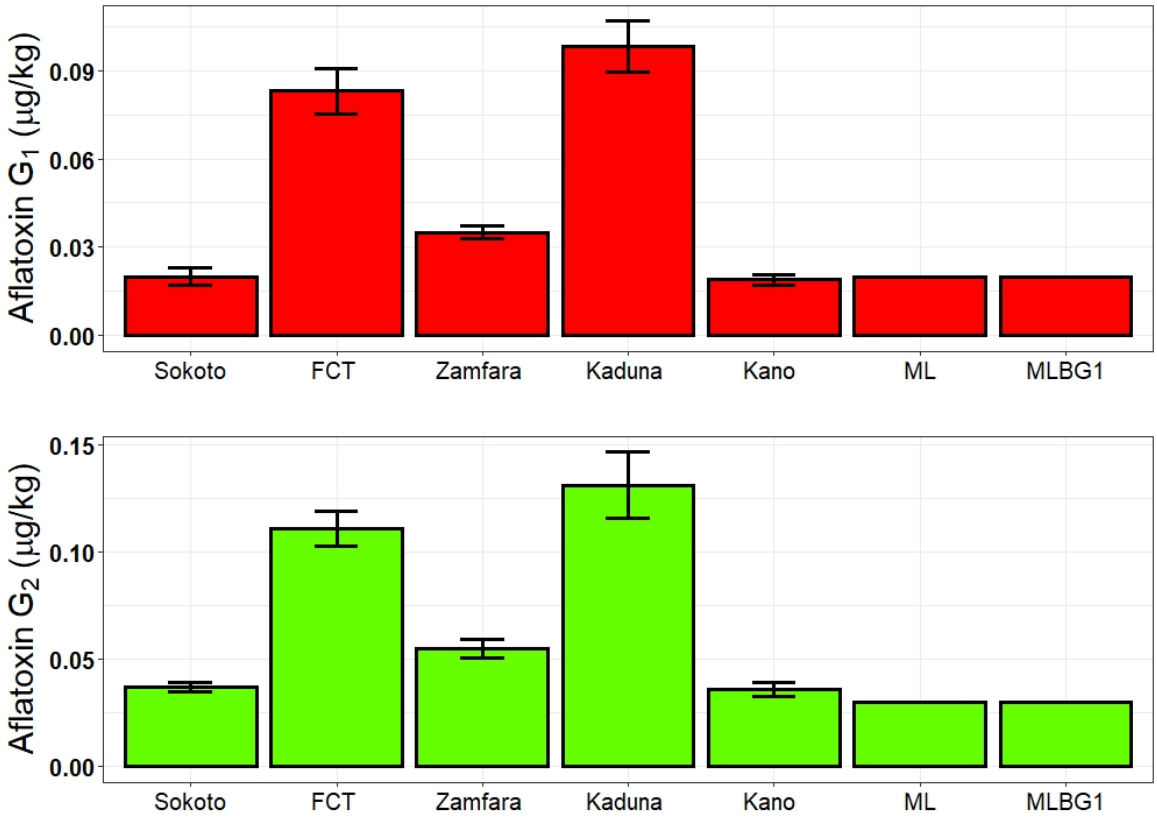

Figure 4.40. Aflatoxins G_1 and G_2 levels of all studied *Kulikuli* samples

$MLBG_1$ = Laboratory prepared *Kulikuli* with garlic additive.

ML = Laboratory prepared *Kulikuli* without garlic additive.

The sampled*Kulikuli*from markets had the highest concentration of Aflatoxin B_1 and B_2 across all states considered (Table 4.7). Among the sampled States, Kaduna had the highest Aflatoxin B_1 with a mean concentration of 1.64 ±0.13 μgkg^{-1}, while Kano recorded least with 1.03 ±0.11 μgkg^{-1}. Kaduna state had the highest aflatoxin B_2 content of 2.10 ± 0.38 μgkg^{-1}. Zamfara had 1.86 ± 0.26 μgkg^{-1} while Kano, Abuja and Sokoto had 1.43 ± 0.14, 1.42 ± 0.18 and 1.19 ± 0.15 μgkg^{-1} respectively. Aflatoxin G_1highest concentration was recorded in Kaduna (0.10 ± 0.03) and lowest in Kano (0.02 ± 0.01).Similarly, aflatoxin $G_2$was at its peak in Kaduna (0.13± 0.05 μgkg^{-1}) and lowest in Sokoto (0.04 ± 0.01 μgkg^{-1}) and Kano (0.04 ± 0.01 μgkg^{-1}).

Table 4.7. Aflatoxins contents of the *Kulikuli* samples

	State					Laboratory	
	Zamfara	Kaduna	Sokoto	Abuja	Kano	ML	$MLBG_1$
AFB_1	1.59±0.31	1.64±0.13	1.05±0.17	1.23±0.07	1.03±0.11	0.70±0.01	0.34±0.01
AFB_2	1.86±0.26	2.10±0.38	1.19±0.15	1.42±0.18	1.43±0.14	0.74±0.00	0.37±0.00
AFG_1	0.04±0.01	0.10±0.03	0.02±0.01	0.08±0.02	0.02±0.01	0.02±0.00	0.01±0.00
AFG_2	0.06±0.01	0.13±0.05	0.04±0.01	0.11±0.03	0.04±0.01	0.03±0.00	0.02±0.00

Values are means of three replicated samples

The analysis of variance below (Table 4.8) shows that aflatoxin B_1F(4, 20) = 13.27, aflatoxin B_2F(4, 20) = 12.07, aflatoxin G_1 F(4, 20) = 22.56 and aflatoxin G_2 F(4, 20) = 12.83had significance values of 0.093, 0.074, 0.006and 0.020, respectively. Therefore, there was no significant difference in aflatoxin B_1 and B_2in the *Kulikuli* samples across the studied states. However, significant differences were found among the amount of aflatoxin G_1 and G_2 across the States.

Table 4.8. ANOVA results for Aflatoxins contents

		Sum of Squares	Df	Mean Square	F	p value
	BG	1.674	4	0.419	13.274	0.093
AFB_1	WG	0.631	20	0.032		
	Total	2.305	24			
	BG	2.769	4	0.692	12.070	0.074
AFB_2	WG	1.147	20	0.057		
	Total	3.916	24			
	BG	0.028	4	0.007	22.564	0.006
AFG_1	WG	0.006	20	0.000		
	Total	0.034	24			
	BG	0.039	4	0.010	12.827	0.020
AFG_2	WG	0.015	20	0.001		
	Total	0.054	24			

Key: BG = Between Groups; WG = Within Groups; Df=Degree of freedom

5. 5. Molecular analyses

The amplification of the aflatoxigenic genes *aflR*, *Nor*, *Ver* and *omt* in all the isolated *Aspergillus* strains is shown in Plate 4.2. Generally, *aflR* gene was amplified (1032 bp) in all the strains except in strain 17 (Plate 4.2), this was the aflatoxin biosynthesis that is mostly present in all the strain. Moreso, *Nor* gene (400 bp) was amplified in 40 out of 48 strains, *Ver* and *omt* genes were both amplified (1,232 bp) in 36 strains respectively.

A. flavus toxin biosynthesis gene was amplified (796 bp) in 37 while 29 strains showed *A. tamari* toxin biosynthesisgenes (1024 bp). Also, *A. niger* and *A. fumigatus* genes were amplified in 11 strains with a band of 800 bp and 400 bp respectively. However, none of the isolates showed the presence of both the *A. parasiticus* and *Fusarium* toxin biosynthesis gene despite the presence of some *A. parasiticus* and *Fusarium* species among the strains (Plate 4.3).

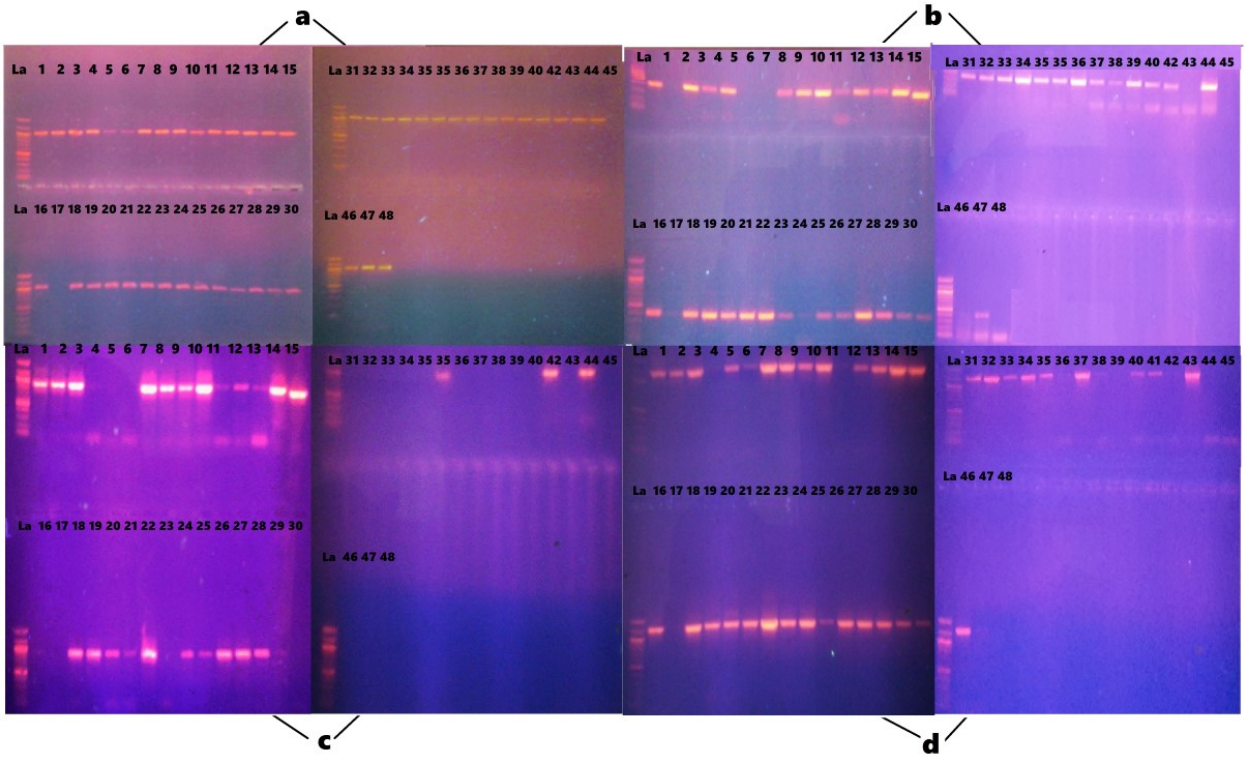

Plate 4.4. Agarose gel electrophoresis of the fungal strains isolated from *Kulikuli*

(a) *aflR* gene; (b) *Nor* gene; (c) *Ver* gene and (d) *Omt* gene

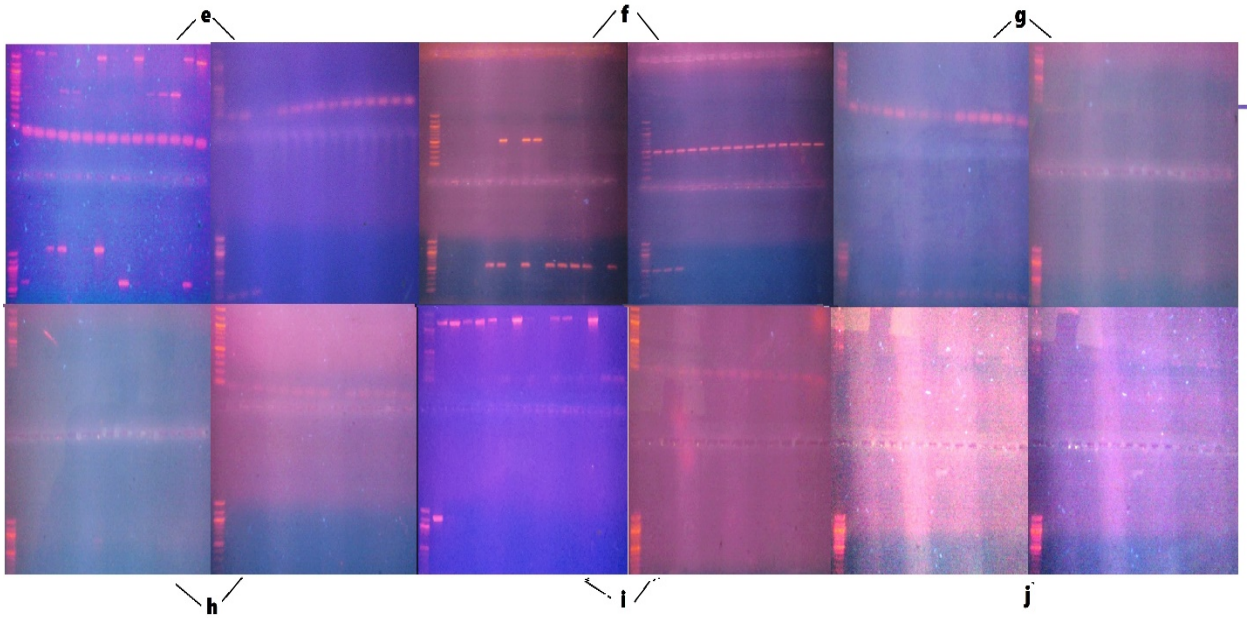

Plate 4.5. Agarose gel electrophoresis of the fungal strains isolated from *Kulikuli*

(e) *A. flavus* (f) *A. Tamari* (g) *A. niger* (h) *A. parasiticus* (i) *A. fumigatus* and (j) *Fusariumspp*

Table 4.9. Molecular amplification of aflatoxigenic genes using SSR technique

Strain Codes	*Aflr*	*Nor*	*Ver*	*Omt*	*AF*	*AP*	*AN*	*AT*	*AFUM*	*FUS*
SK2^{2b}	+	+	+	+	+	-	+	-	+	-
SK3^{3c}	+	-	+	+	+	-	+	-	+	-
KN5^{1a}	+	+	+	+	+	-	+	-	+	-
ZM3^{2b}	+	+	-	-	+	-	+	-	+	-
KN2^{1b}	+	+	-	+	+	-	+	-	+	-
FG3^{2c}	+	-	-	-	+	+	-	-	-	-
KD3^{2b}	+	-	+	+	+	-	-	-	+	-
KD2^{3b}	+	+	+	+	+	+	-	-	-	-
ZM5^{3c}	+	+	+	+	+	+	-	-	-	-
ZM3^{1b}	+	+	+	+	+	-	+	-	+	-
ZM5^{1b}	+	+	+	-	+	-	+	-	+	-
ZM1^{2a}	+	+	+	+	+	-	+	-	-	-
KM5^{3b}	+	+	+	+	+	-	+	-	+	-
SK1^{3a}	+	+	+	+	+	-	+	-	-	-
SK3^{2a}	+	+	+	+	+	-	+	-	-	-
KD2^{2b}	+	+	-	+	+	-	-	-	+	-
KD2^{1c}	-	-	-	-	-	-	-	-	-	-
FG1^{1a}	+	+	+	+	+	-	-	-	-	-
SK1^{1a}	+	+	+	+	+	-	-	-	-	-
KN1^{2a}	+	+	+	+	-	+	-	-	-	-
KD5^{2a}	+	+	+	+	-	+	-	-	-	-
KN5^{1c}	+	+	+	+	+	-	-	-	-	-
KD5^{2d}	+	+	-	+	-	+	-	-	-	-
KN3^{2a}	+	-	+	+	+	-	-	-	-	-
KN5^{3c}	+	+	+	+	-	+	-	-	-	-
KD2^{1a}	+	+	+	+	-	+	-	-	-	-
SK2^{1a}	+	+	+	+	-	+	-	-	-	-
SK2^{2b}	+	+	+	+	-	+	-	-	-	-
SK3^{2b}	+	+	-	+	+	-	-	-	-	-
KD4^{1a}	+	+	-	+	-	+	-	-	-	-
FCT2^{2a}	+	+	-	+	+	+	-	-	-	-
FCT1^{1a}	+	+	-	+	+	+	-	-	-	-
FCT3^{1a}	+	+	-	+	-	+	-	-	-	-
FCT2^{1b}	+	+	-	+	-	+	-	-	-	-

FCT2^{2b}	+	+	-	+	+	+	-	-	-	-
FCT3^{1c}	+	+	+	-	+	+	-	-	-	-
KN2^{2a}	+	+	-	+	+	+	-	-	-	-
FCT3^{3b}	+	+	-	-	+	+	-	-	-	-
KN3^{3a}	+	+	-	-	+	+	-	-	-	-
KN4^{1a}	+	+	-	+	+	+	-	-	-	-
KN3^{1c}	+	+	-	+	+	+	-	-	-	-
ZM2^{3a}	+	+	+	-	+	+	-	-	-	-
ZM4^{2a}	+	+	-	+	+	+	-	-	-	-
KN4^{2a}	+	+	+	-	+	+	-	-	-	-
ZM4^{1a}	+	-	-	-	+	+	-	-	-	-
KD1^{3b}	+	+	-	+	+	+	-	-	-	-
ZM2^{3C}	+	-	-	-	+	+	-	-	-	-
SK3^{2a}	+	+	-	-	+	+	-	-	-	-

Key: + = presence; - = absence

CHAPTER 5

DISCUSSION

5.1.Fungal incidence

This study revealed that ginger and turmeric werenot appropriate botanicals for the control of fungi associated with *Kulikuli* production in Nigeria.Ginger, having a unique smell and flavourbecause of its component of volatile oils, and some phytochemical components such as zingerone, shogaols and gingerols (An *et al*., 2016) altered the smell of the *Kulikuli* oil during heating therebymaking it not palatable for consumption.In addition, turmeric has some phytochemicals such as diarylheptanoids and several kinds of curcuminoids like demethoxycurcumin and curcumin (Siewek, 2013; Hu *et al*., 2014) which changed the colour of the *Kulikuli* samples thereby making them not appealing to consumers.

Kulikuli is preferably eaten solid with a characteristic smell and colour from the original raw material (groundnut) which change on the addition of turmeric and garlic. It was however established in this study that the garlic met all the aforementioned necessities in *Kulikuli* preparation. It was also established in this study that the garlic additive in *Kulikuli* production enhanced the nutrient composition, and as well reduced the fungal and aflatoxin composition. Garlic also served as a natural preservative by enhancing the shelf life of *Kulikuli.*

Specifically, 48 fungal strains isolated from *Kulikuli* (groundnut cake) that were collected from different markets across five states in Northern Nigeria. On the basis of percentage (%) incidence 9 strains had highest incidence and they are mostly *Apergillus* fungi. *Aspergillus* fungi most especially *A. flavus* have been known to be capable of producing aflatoxins (Van Hove *et al*., 2011; Susca *et al*., 2010).

Specifically, this study identified nine (9) variants of moulds in groundnut cakes (*Kulikuli*) collected from five (5) states of Nigeria, the moulds include *Aspergillus flavus, Aspergillus parasiticus, Aspergillustamarii, A. niger, A. fumigatus, P. oxalicum, P. chrysogenum, F. oxysporum,* and *F. compaticum.* Based on the percentage incidence, *Aspergillus flavus* and *A. parasiticus*were most dominant fungal species associated with the studied *Kulikuli* samples, similar fungal strains have been earlier reportedly associated with many groundnut products (Jimoh and Kolapo 2008; Varga *et al.,* 2012).

It was revealed in this study that *Aspergillus flavus* and *A. parasiticus* were the most frequently associated fungi with the studied *Kulikuli* samples in all the states based on percentage incidence. These two fungal strains are well documented for aflatoxin biosynthesis (Cotty and Cardwell 1999; Barros *et al*. 2006).

These *Aspergillus* strains are characterized with unique conidial head, for example the observed *A. flavus* had conidial head with shades of yellow-green to brown and somehow dark sclerotia as also observed by Varga *et al*. (2012). Most of the earlier works to distinguish these *Aspergillus* strains made use of growth attribute, type of metabolite produced by each strain, and their spore or mycelial morphology (Klich, 1998; Jonathan*et al.*, 2015). It is therefore important to screen their ability to produce aflatoxin to be able to establish the safety of this food product.

In addition, it was also established in the study that the laboratory prepared *Kulikuli* with and without garlic additive had the least fungal incidences as compared to the market samples and this maybe a reflective of the fact that garlic had different phytochemical components, which are active against the growths of many fungal and bacterial pathogens (Lin *et al.*, 2017). This observation is also in accordance with the claims of Onyeagba *et al*. (2004) who reported that addition of garlic in some traditional antifungal decoction increased their potencies.

5. 2. Proximate analysis of *kulikuli*

Investigation was also carried out on the nutrient contents of the *Kulikuli*market samples available for sale in North-western (Kano, Zamfara, Kaduna and Sokoto) and North central (Abuja-FCT) Nigeria. It was observed that the laboratory prepared samples with garlic additive had better key nutrient contents as compared to the market samples; this might bebecause of the less fungal incidence in the garlic added which tend to preserved them from fungal deterioration.

The percentage of fat between the states corroborates with the recommended health beneficial fat (38 Kj). Justifiably, the percentage prevalence of fat indicates the presence of acidity. This corresponds to the pH level of the sampled *Kulikuli* which was identified to be slightly acidic in nutrient. However, the laboratory prepared samples with garlic ($MLBG_1$) and without garlic (ML) additive had the least overall fat values (Fig 4.10). The $MLBG_1$ had the least overall crude protein of 3.26 % fat while ML had the least overall pH of 3.83 % fat respectively. It was evident that all the sampled *Kulikuli* were very edible because their pH level was slightly lower than 7, which implies slight acidity. *Kulikuli* collected from Kaduna state had pH value of 6.24, while *Kulikuli* from Abuja has the least pH value of 5.96. This could be as a result the difference in the acidity of the water used to prepare *Kulikuli* in these different locations.

The richest *Kulikuli* in terms of protein content was obtained from Sokoto (49.23%) as opposed to Abuja (43.18 %). This may be explained by several factors, including the difference in groundnut varieties cultivated in these States, the cultural practices and the *Kulikuli* processing. For example, a protein content of 13.40–30.88 %, which is lower than the values obtained in the present study was reported by Emelike and Akusu (2018) from Southern Nigeria. Our results imply that samples of *Kulikuli* collected from Sokoto state and the laboratory prepared ones had more ash and fibre. Justifiably, many research evidences have shown that protein content is significantly necessary for human's health.

Despite the fact that protein content was relatively high in market samples, this study revealed that there is a potential for even higher protein in *Kulikuli* when better hygienic procedures are followed. This is indicated by an increase in protein content of 50 to 59 % in *Kulikuli* samples prepared in the laboratory under strict hygienic conditions.

Furthermore, the increased protein content due to the addition of garlic can be explained by its antimicrobial and antifungal properties. This is in line with the study of Ayoade and Adegbite (2016).

In all, protein appears to be the most prevalent nutrient in the tested samples and this correlates the report of Ntare *et al*. (2008) who identified groundnuts to be rich in protein, Njintang and Sarma (2001) also reported that peanut also contains some nutritional factors such as phytic acid, condensed tannins, and trypsin. In addition, the pH levels of all the tested samples are slightly lower than seven, whichimply a slight acidic level, hence very suitable for human consumption.

This study reported nutrient composition of *Kulikuli* from Nigeria, this is very important as Nigeria is being listed as one of the major producer of groundnuts after China and India (Okaka, 2005). Ezekiel *et al*. (2013) had earlier carried out studies on groundnut products and reported that these products importantly serve as various sources of protein in animal feeds/rations. In many Sub Saharan African countries, groundnut is cultivated widely and used to produce snacks popularly eaten as local convenience.

In many of these countries, groundnut is very popular just like maize, it is often boiled, roasted, or processed into candies, cakes or ball form snacks and these are often seen hawked along the local streets or on the roadside of the urban cities in Nigeria. Groundnut, be it raw or processed however may be prone to fungal deterioration or mycotoxin contamination if not properly handled. This is due to its numerous nutrients which attract the fungi growth and hence their toxic metabolites called mycotoxins.

5. 3. Aflatoxin concentrations in *Kulikuli*

Nigeria has been documented with several cases of mycotoxin contaminations, which resulted from unhygienic handling and poor road or storage facilities. Many groundnut products have been detected to contain variouslevels.Unfortunately, the attentions have been mainly focused on the microbiological assessment of the safety of food products in Nigeria other than mycotoxin contaminations (Akano and Atanda, 1990; Jimoh and Kolapo, 2008). There is also dearth of knowledge with little or no data on the aflatoxin

assessment of *Kulikuli* samples in the major areas where *Kulikuli*is mainly consumed in Nigeria.

Incidence of many aflatoxigenic fungi and their contamination of foods and feeds has been linked to the poor processing and storage methods. In this study, samples of groundnut cake (*Kulikuli*) from Kaduna State appeared to have the highest occurrence of moulds while Sokoto had the least occurrence of moulds. This may be because of handling as reported by Fernandez-Cruz *et al*. (2010) that ranging aflatoxigenic fungi may occur in food products in different locations depending on sanitary measures adopted in processing or storing them. Many other factors may as well affect the incidence of aflatoxigenic fungi in a food product, pH, nutrient, water activity (aw), redox potential of the substrate, temperature, oxygen availability (Nagwa *et al*., 2013).

Kulikuli is one of the most common snacks in Nigeria, it was reported by Ezekiel *et al.* (2012), Fakoor *et al*. (2012), Fapohunda *et al*. (2012) and Jonathan *et al*. (2016b) that *Aspergillus* are the major cause of aflatoxigenic fungal contamination of snacks in Nigeria and other Sub-Sahara Africa. This was also established in this study as we recorded highest incidence in *A. flavus* and *A. parasiticus* in all the collected *Kulikuli* samples across all the studied states. This in addition, corresponded with the detected aflatoxin levels in the samples are below the tolerance limit, there are however still no adequate multi-aflatoxin analyses of this snack (groundnut cake) in Nigeria and based on the economic challenge/literacy level of many producers, this situation of aflatoxin incidence could be alarming.

5. 4. Molecular analyses

It is imperative today to combine morphological and molecular (genetic) characteristics of these *Aspergillus* fungal for their identification due to their complex and diverse characteristics. SSR was used to characterize the fungal strains based on aflatoxin biosynthesis genes on peanuts. We report similar results with Montiel *et al*. (2003), who characterized 24 fungal strains and clearly distinguished *A. flavus, A. parasiticus, A. oryzae* and *A. sojae* using the AFLP technique.Similar results have also been previously reported by many other researcher using other molecular techniques (Bennet and Klich 2003a).

To distinguish between aflatoxigenic and nontoxigenic fungi, 10 aflatoxin biosynthesis genes were tested on all the isolated fungal strains in this study using SSR method. Conventional methods for this purpose were through culturing methods in suitable media, and extracting aflatoxin from then using organic solvents (Lin and Zayas, 1998). Recently, several molecular methods such as SSR, AFLP, RAPD, ITS sequence comparison, DNA amplification fingerprinting techniques, quadruplex PCR and other molecular methods have been reported (Montiel *et al*. 2003; Baird *et al*. 2006; Perrone *et al*. 2006).

Extensive biochemical and genetic studies have been employed to give better understanding of the molecular regulation of aflatoxin biosynthesis. This studies employed DNA based detection system as a tool for detecting and identifying aflatoxin-producing fungi. *Aspergillusflavus* and *A. parasiticus* have been previously isolated from peanut as the dominant fungal species in Argentina by Barros and Feltham (2003), and characterized based on their sclerotia characteristics, inoculum distribution, aflatoxin production and genetic diversity using VCG analysis (Barros *et al*. 2006). This study provides similar characteristics of this two dominant fungal species as associated to *Kulikuli* which is a groundnut product.

The isolated strains of *Aspergillus* species in this study from *Kulikuli* showed possession of *aflR* gene, this gene has been well linked with aflatoxin biosynthesis and had been employed for characterizing the aflatoxigenic fungi (Cotty, 2006). In many other molecular PCR based studies, non aflatoxigenic fungal strains was studied to show absence of the *aflR* gene and hence this gene could not be amplified in them (Criseo *et al.*, 2008; Scherm *et al*., 2005). Gallo (2012) examined molecular differences between aflatoxigenic fungi and non aflatoxigenic ones and reported that *A. flavus* possess seven different variants of *afl* genes such as *aflD, aflM, aflO, aflP,* and *aflQ.* In that study, they grouped the *Aspergillus*strains into four types based on their amplification results. All the aflatoxigenic strains gave complete set of a particular amplification patterns while the other three groups that were non-aflatoxin producing fungi gave no amplification product for all the tested seven genes.

Gallo (2012) further stated that *A. flavus* and *A. parasiticus* have genetic complexities of these genes which has led to the provision of different markers that can be used to track

and monitor variations in these fungal species, hence they can be evaluated for risk they can cause humans or animals when they are present in the food. Also, this has led to more understanding of the genetic variability in the *afl* gene clusters a key to the selectively safe and effective aflatoxigenic fungi for biocontrol measures for reduction of losses in crop yields.

Also, *Nor, Ver* and *Omt*genes are three clusters which are structurally similar as aflatoxin biosynthetic genes, they take crucial part in the pathways of aflatoxin biosynthesis essential for aflatoxin production. These cluster genes contain structural, regular and unassigned gene which plays a key role in the production of aflatoxins. Their result indicates that *Nor* was able to amplify 40, *Ver* and *Omt* were each able to amplify 36 out of 48 DNA samples. Notably, no DNA amplification observed with *A. niger*, *A. parasiticus*, *A. fumigatus*and *Fusarium* even at the highest level indicating high specificity of SSR.

However, none of the strain showed the presence of *Fusarium* and *A. parasiticus* toxin biosynthesis despite the fact that Fusarium species are among the most economically significant mycotoxins producing fungi globallygiven their potency and widespread geographical distributionin economically important grain crops such as maize,barley and wheat (Maier *et al*., 2006).

More so, this study reported presence of aflatoxin biosynthesis genes in 48 fungal strains isolated from *Kulikuli*from five states in Nigeria. Four aflatoxigenic genes *aflR*, *Nor*, *Ver* and *Omt* were tested present in most of the strains.The *aflR* is a gene which encodes aflatoxin biosynthesis specific transcription factor useful for all aflatoxigenic fungi, while the *Nor*, *Ver* and *Omt* genes are structural cluster genes in aflatoxin biosynthesis pathway, they are cluster genes which contain structural, regular and unassigned genes which plays a key role in the production of aflatoxins. Our results imply that samples of *Kulikuli* collected from Zamfara state may have a more negative impact on human health compared to other states. Justifiably, molecular evidences have shown that moisture content is a significant determinant of Aflatoxins in food.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6. 1. Summary

Kulikuli is one of the most common local snacks in Nigeria and mainly produced in Northern Nigeria. So far, manual traditional methods have prevailed in the production and storage of *Kulikuli* in Nigeria. Strict hygene rules are not often observed during the production of *Kulikuli,* thereby introducing the risk of contamination by fungi and other food-spoiling microorganisms. There is a paucity research on the fungal communities associated with *Kulikuli* and the mycotoxins with serious health implications resulting from fungal biological activities.

This study revealed the occurrence of 106 fungi on *Kulikuli* samples from the selected markets. The identified fungi were counted as follows: *Aspergillusflavus* (n=28), *A. parasiticus* (n=26), *A. niger* (n=7), *A. tamarii* (n=9), *A. fumigatus* (n=3), *Penicillium oxalicum* (n=11), *P. chrysogenum* (n=6), *Fusarium oxysporum* (n=10)and *F.compaticum* (n=6). *Kulikuli* samples prepared in the laboratory to serve as standard also contained all fungi isolated from market samples, except *A. fumigatus* and *P. oxalicum*.

Apart from garlic,the other botanicals introduced altered the taste, aroma, colour and palatability of the standard *Kulikuli* samples, and were excluded from further analyses.There was no fungal incidence in laboratory-prepared *Kulikuli*with 0.5 g of freshgarlic.*Kulikuli* samples from the markets compared with the standard with and without garlic (MLB and ML, respectively) were significantly different in moisture content (11.91, 6.64, and 12.64%), ash (3.91, 4.84, and 4.84%), crude protein (49.23, 59.23, and 50.25%), fibre (3.20, 4.55, and 4.05%), fat (4.83, 3.26, and 3.83%) and pH (6.30, 6.88, and 6.84), respectively.

The highest aflatoxin (AFB_1, AFB_2, AFG_1 and AFG_2) levels were recorded in market samples as follows: 1.64, 2.10, 0.10 and 0.13 µg/kg, respectively. Laboratory-prepared samples with garlic had the least AFB_1 (0.34µg/kg), AFB_2 (0.37µg/kg), AFG_1 (0.01µg/kg) and AFG_2 (0.02µg/kg) which were all within tolerance limits of NAFDAC and European Union 4 µg/kg. The *aflR, nor*, *ver* and *omt* biosynthetic pathway genes found in aflatoxin producing fungi were detected in all fungi isolated from the purchased *Kulikuli* except strains from Kaduna (Kaduna Central) samples. However, the MLB samples yielded no fungal growth.

6.2.Conclusions

It was observed that the *A. flavus* and *A. parasiticus* were the major aflatoxigenic fungal strains associated with the sampled *Kulikuli* across all the states. Several health benefits were associated with the introduction of garlic in the production of *Kulikuli*, including better nutritional value with low moisture content, high protein andlow fat. Garlic effectively suppressed fungi species present in *Kulikuli* and reduced their aflatoxin levels.

6.3. Recommendations

1. There is need for adequate quality control for *Kulikuli* and other similar groundnut-based products in Northern Nigeria.
2. Strict hygiene rules such as sorting to remove dead groundnut seeds andother particles, and sterilization of materials should be observed in the production of *Kulikuli.*
3. No more than 0.5 g of fresh garlic should be introduced into 100 g of groundnut pasteto control fungi associated with *Kulikuli.*
4. Government at all levels should take continuous education and campaign on aflatoxin seriously.

6.4. Contributions to knowledge

1. This study showed that *Aspergillusflavus* and *A. parasiticus* are the major fungi infesting *Kulikuli* in the study areas.
2. The introduction of garlic during *Kulikuli* production suppresses fungal growth and reduces aflatoxin levels.
3. Garlic improves the shelf life of *Kulikuli* by reducing its moisture content.

4. This study showed that garlic introduction during *Kulikuli* production enhanced its nutritional values by increasing protein content and loweringfats.

6.5. Suggestions for further studies

1. There is a need to carry out further studies to cover other major *Kulikuli* production centres in Nigeria such as Nupe in Niger State.

2.Further investigations are needed to explore the efficiency turmeric and ginger as antifungal agents in *Kulikuli* production.

3. There is need for more in depth molecular studies on different aflatoxin biosynthesis genes and their expression in these fungi with detailed phylogenetic analysis of the strains.

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Appendices

Appendix 1. Tukey HSD Post Hoc Proximate Analysis

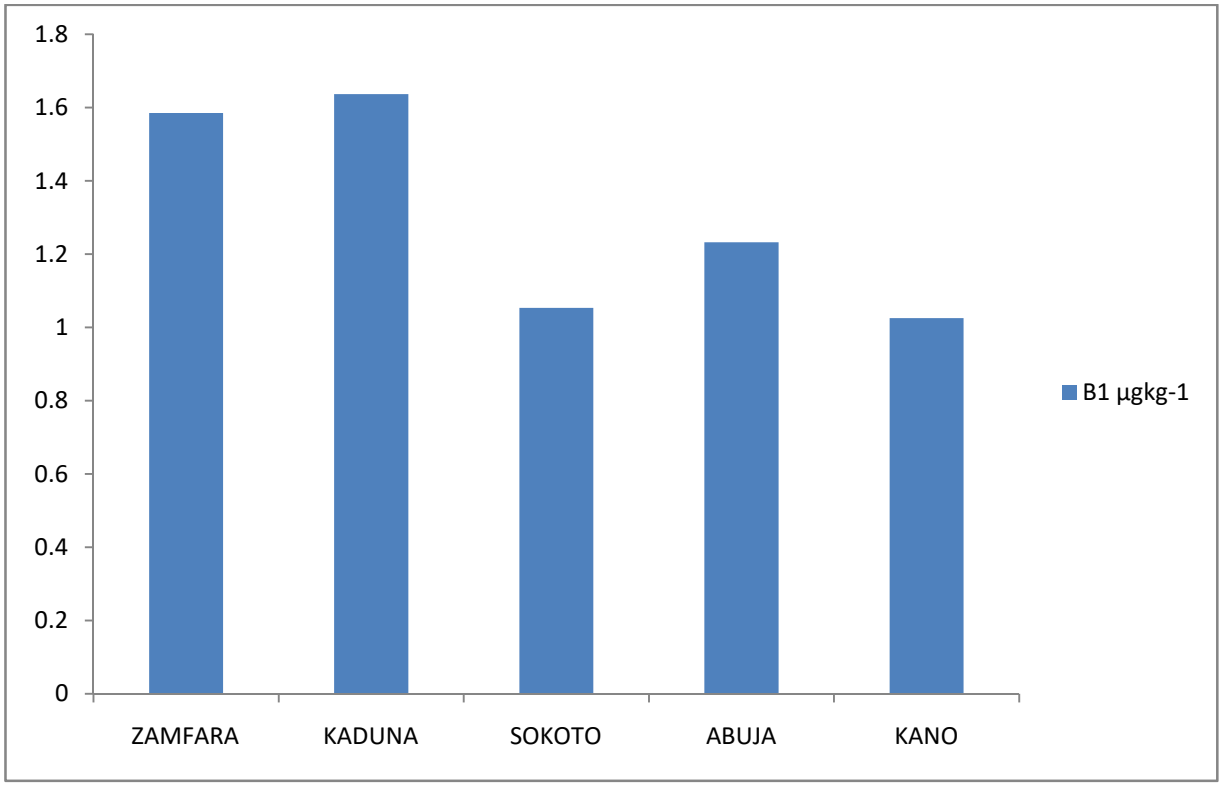
Multiple Comparisons							
Dependent Variable	**(I) proximate**	**(J) proximate**	**Mean Difference (I-J)**	**Std. Error**	**Sig.**	**95% Confidence Interval**	
						Lower Bound	**Upper Bound**
pH	Zamfara	Kaduna	-.20600	.18916	.810	-.7720	.3600
		Sokoto	-.10600	.18916	.979	-.6720	.4600
		Abuja	.07200	.18916	.995	-.4940	.6380
		Kano	.06400	.18916	.997	-.5020	.6300
	Kaduna	Zamfara	**.20600**	.18916	.810	-.3600	.7720
		Sokoto	**.10000**	.18916	.983	-.4660	.6660
		Abuja	**.27800**	.18916	.593	-.2880	.8440
		Kano	**.27000**	.18916	.618	-.2960	.8360
	Sokoto	Zamfara	.10600	.18916	.979	-.4600	.6720
		Kaduna	-.10000	.18916	.983	-.6660	.4660
		Abuja	.17800	.18916	.877	-.3880	.7440
		Kano	.17000	.18916	.894	-.3960	.7360
	Abuja	Zamfara	-.07200	.18916	.995	-.6380	.4940
		Kaduna	-.27800	.18916	.593	-.8440	.2880
		Sokoto	-.17800	.18916	.877	-.7440	.3880
		Kano	-.00800	.18916	1.000	-.5740	.5580
	Kano	Zamfara	-.06400	.18916	.997	-.6300	.5020
		Kaduna	-.27000	.18916	.618	-.8360	.2960
		Sokoto	-.17000	.18916	.894	-.7360	.3960
		Abuja	.00800	.18916	1.000	-.5580	.5740
CP	Zamfara	Kaduna	-2.06200	2.83568	.948	-10.5474	6.4234
		Sokoto	-2.92400	2.83568	.838	-11.4094	5.5614
		Abuja	3.12600	2.83568	.803	-5.3594	11.6114
		Kano	-.09800	2.83568	1.000	-8.5834	8.3874
	Kaduna	Zamfara	2.06200	2.83568	.948	-6.4234	10.5474
		Sokoto	-.86200	2.83568	.998	-9.3474	7.6234
		Abuja	5.18800	2.83568	.385	-3.2974	13.6734
		Kano	1.96400	2.83568	.956	-6.5214	10.4494
	Sokoto	Zamfara	**2.92400**	2.83568	.838	-5.5614	11.4094
		Kaduna	**.86200**	2.83568	.998	-7.6234	9.3474
		Abuja	**6.05000**	2.83568	.245	-2.4354	14.5354
		Kano	**2.82600**	2.83568	.854	-5.6594	11.3114
	Abuja	Zamfara	-3.12600	2.83568	.803	-11.6114	5.3594
		Kaduna	-5.18800	2.83568	.385	-13.6734	3.2974
		Sokoto	-6.05000	2.83568	.245	-14.5354	2.4354
		Kano	-3.22400	2.83568	.785	-11.7094	5.2614
	Kano	Zamfara	.09800	2.83568	1.000	-8.3874	8.5834

		Kaduna	-1.96400	2.83568	.956	-10.4494	6.5214
		Sokoto	-2.82600	2.83568	.854	-11.3114	5.6594
		Abuja	3.22400	2.83568	.785	-5.2614	11.7094
FAT	Zamfara	Kaduna	-.93800*	.20063	.001	-1.5383	-.3377
		Sokoto	-.56000	.20063	.075	-1.1603	.0403
		Abuja	-.74800*	.20063	.010	-1.3483	-.1477
		Kano	-.65800*	.20063	.027	-1.2583	-.0577
	Kaduna	Zamfara	**.93800***	.20063	.001	.3377	1.5383
		Sokoto	**.37800**	.20063	.357	-.2223	.9783
		Abuja	**.19000**	.20063	.875	-.4103	.7903
		Kano	**.28000**	.20063	.637	-.3203	.8803
	Sokoto	Zamfara	.56000	.20063	.075	-.0403	1.1603
		Kaduna	-.37800	.20063	.357	-.9783	.2223
		Abuja	-.18800	.20063	.879	-.7883	.4123
		Kano	-.09800	.20063	.988	-.6983	.5023
	Abuja	Zamfara	.74800*	.20063	.010	.1477	1.3483
		Kaduna	-.19000	.20063	.875	-.7903	.4103
		Sokoto	.18800	.20063	.879	-.4123	.7883
		Kano	.09000	.20063	.991	-.5103	.6903
	Kano	Zamfara	.65800*	.20063	.027	.0577	1.2583
		Kaduna	-.28000	.20063	.637	-.8803	.3203
		Sokoto	.09800	.20063	.988	-.5023	.6983
		Abuja	-.09000	.20063	.991	-.6903	.5103
CF	Zamfara	Kaduna	.03400	.28564	1.000	-.8207	.8887
		Sokoto	.06200	.28564	.999	-.7927	.9167
		Abuja	-.08800	.28564	.998	-.9427	.7667
		Kano	-.15000	.28564	.984	-1.0047	.7047
	Kaduna	Zamfara	-.03400	.28564	1.000	-.8887	.8207
		Sokoto	.02800	.28564	1.000	-.8267	.8827
		Abuja	-.12200	.28564	.993	-.9767	.7327
		Kano	-.18400	.28564	.966	-1.0387	.6707
	Sokoto	Zamfara	-.06200	.28564	.999	-.9167	.7927
		Kaduna	-.02800	.28564	1.000	-.8827	.8267
		Abuja	-.15000	.28564	.984	-1.0047	.7047
		Kano	-.21200	.28564	.944	-1.0667	.6427
	Abuja	Zamfara	.08800	.28564	.998	-.7667	.9427
		Kaduna	.12200	.28564	.993	-.7327	.9767
		Sokoto	.15000	.28564	.984	-.7047	1.0047
		Kano	-.06200	.28564	.999	-.9167	.7927
	Kano	Zamfara	**.15000**	.28564	.984	-.7047	1.0047
		Kaduna	**.18400**	.28564	.966	-.6707	1.0387
		Sokoto	**.21200**	.28564	.944	-.6427	1.0667
		Abuja	**.06200**	.28564	.999	-.7927	.9167
ASH	Zamfara	Kaduna	-.12600	.20183	.969	-.7299	.4779
		Sokoto	-.89400*	.20183	.002	-1.4979	-.2901

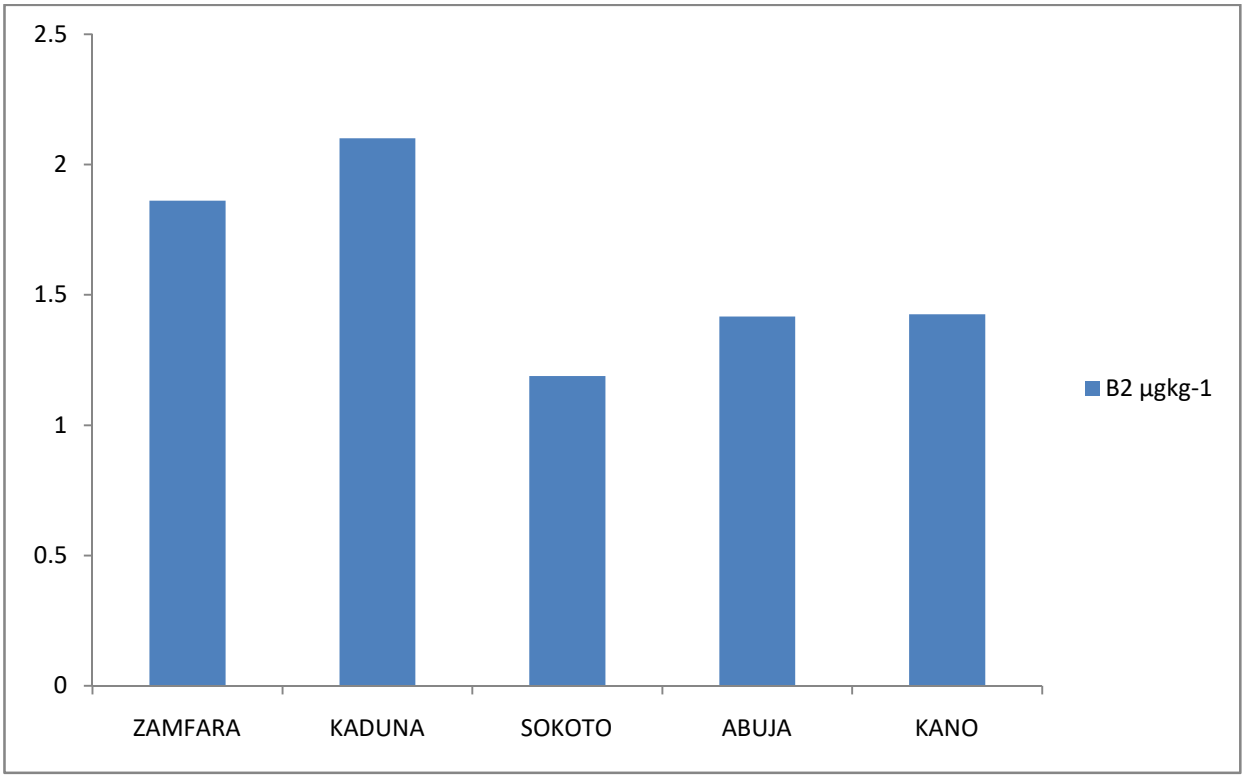
		Abuja	.06200	.20183	.998	-.5419	.6659
		Kano	-.96400*	.20183	.001	-1.5679	-.3601
	Kaduna	Zamfara	.12600	.20183	.969	-.4779	.7299
		Sokoto	-.76800*	.20183	.009	-1.3719	-.1641
		Abuja	.18800	.20183	.881	-.4159	.7919
		Kano	-.83800*	.20183	.004	-1.4419	-.2341
	Sokoto	Zamfara	.89400*	.20183	.002	.2901	1.4979
		Kaduna	.76800*	.20183	.009	.1641	1.3719
		Abuja	.95600*	.20183	.001	.3521	1.5599
		Kano	-.07000	.20183	.997	-.6739	.5339
	Abuja	Zamfara	-.06200	.20183	.998	-.6659	.5419
		Kaduna	-.18800	.20183	.881	-.7919	.4159
		Sokoto	-.95600*	.20183	.001	-1.5599	-.3521
		Kano	-1.02600*	.20183	.000	-1.6299	-.4221
	Kano	Zamfara	**.96400***	.20183	.001	.3601	1.5679
		Kaduna	**.83800***	.20183	.004	.2341	1.4419
		Sokoto	**.07000**	.20183	.997	-.5339	.6739
		Abuja	**1.02600***	.20183	.000	.4221	1.6299
MC	Zamfara	Kaduna	**.54000**	.38762	.639	-.6199	1.6999
		Sokoto	**1.27000***	.38762	.028	.1101	2.4299
		Abuja	**1.27400***	.38762	.027	.1141	2.4339
		Kano	**3.03600***	.38762	.000	1.8761	4.1959
	Kaduna	Zamfara	-.54000	.38762	.639	-1.6999	.6199
		Sokoto	.73000	.38762	.358	-.4299	1.8899
		Abuja	.73400	.38762	.352	-.4259	1.8939
		Kano	2.49600*	.38762	.000	1.3361	3.6559
	Sokoto	Zamfara	-1.27000*	.38762	.028	-2.4299	-.1101
		Kaduna	-.73000	.38762	.358	-1.8899	.4299
		Abuja	.00400	.38762	1.000	-1.1559	1.1639
		Kano	1.76600*	.38762	.002	.6061	2.9259
	Abuja	Zamfara	-1.27400*	.38762	.027	-2.4339	-.1141
		Kaduna	-.73400	.38762	.352	-1.8939	.4259
		Sokoto	-.00400	.38762	1.000	-1.1639	1.1559
		Kano	1.76200*	.38762	.002	.6021	2.9219
	Kano	Zamfara	-3.03600*	.38762	.000	-4.1959	-1.8761
		Kaduna	-2.49600*	.38762	.000	-3.6559	-1.3361
		Sokoto	-1.76600*	.38762	.002	-2.9259	-.6061
		Abuja	-1.76200*	.38762	.002	-2.9219	-.6021

*. The mean difference is significant at p< 0.05 level.

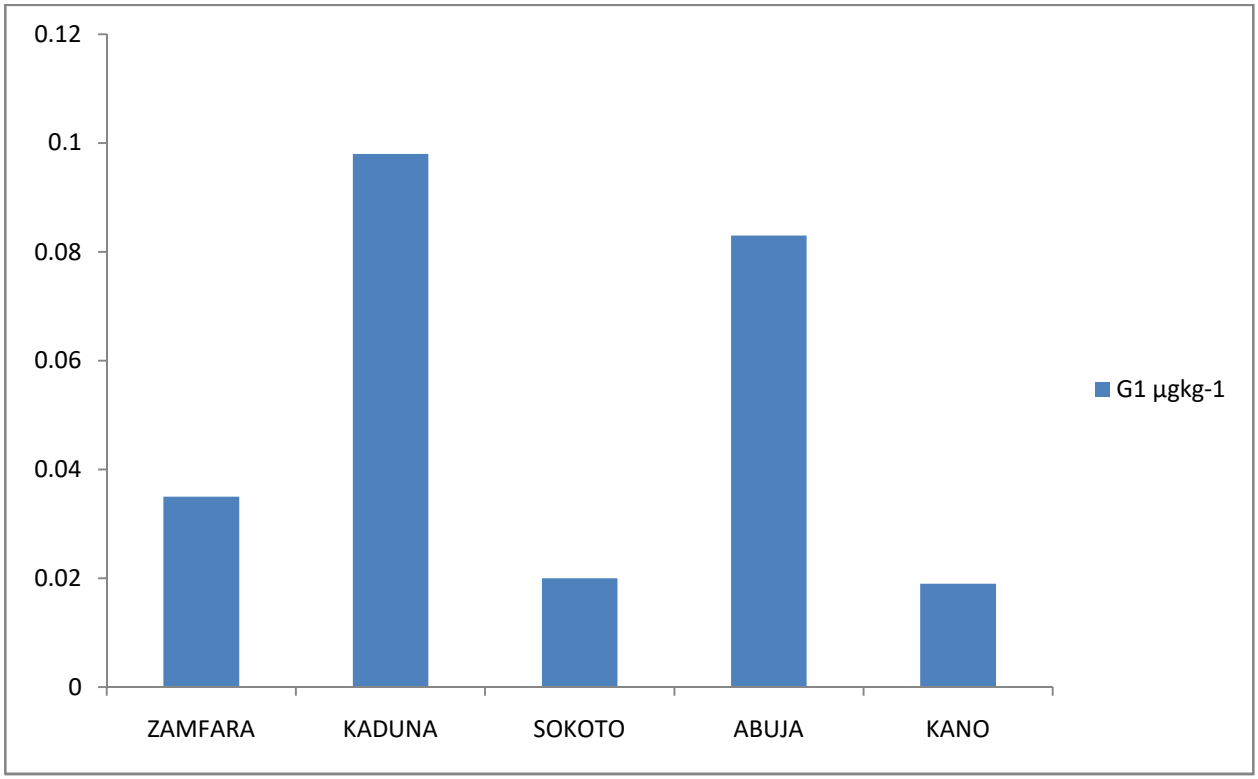
Appendix 2. Aflatoxin B1 between the States



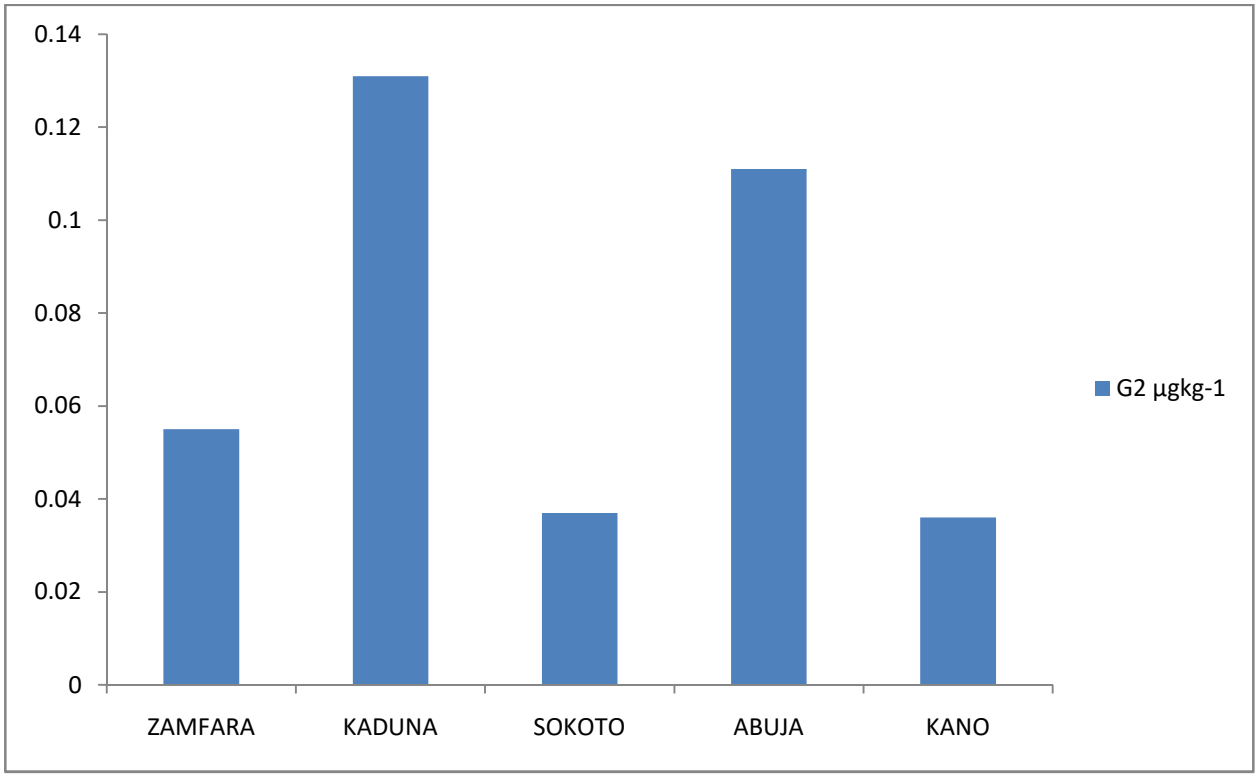
Appendix 3. Aflatoxin B2 between the States



Appendix 4. Aflatoxin G1 between the States



Appendix 5. Aflatoxin G2 between the States



Appendix 6. Descriptive summary of aflatoxigenic analysis between the States

Aflatoxins/States		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
						Lower Bound	Upper Bound
B_1	Zamfara	5	1.5860	.30867	.13804	1.2027	1.9693
	Kaduna	5	1.6380	.12696	.05678	1.4804	1.7956
	Sokoto	5	1.0540	.17372	.07769	.8383	1.2697
	Abuja	5	1.2320	.06797	.03040	1.1476	1.3164
	Kano	5	1.0280	.10710	.04790	.8950	1.1610
	Total	25	1.3076	.30991	.06198	1.1797	1.4355
B_2	Zamfara	5	1.8620	.25636	.11465	1.5437	2.1803
	Kaduna	5	2.1020	.38324	.17139	1.6261	2.5779
	Sokoto	5	1.1880	.14738	.06591	1.0050	1.3710
	Abuja	5	1.4180	.18213	.08145	1.1919	1.6441
	Kano	5	1.4260	.13885	.06210	1.2536	1.5984
	Total	25	1.5992	.40394	.08079	1.4325	1.7659
G_1	Zamfara	5	.0360	.00894	.00400	.0249	.0471
	Kaduna	5	.1000	.02739	.01225	.0660	.1340
	Sokoto	5	.0200	.01000	.00447	.0076	.0324
	Abuja	5	.0840	.02408	.01077	.0541	.1139
	Kano	5	.0200	.00707	.00316	.0112	.0288
	Total	25	.0520	.03786	.00757	.0364	.0676
G_2	Zamfara	5	.0560	.01140	.00510	.0418	.0702
	Kaduna	5	.1320	.05215	.02332	.0672	.1968
	Sokoto	5	.0380	.00837	.00374	.0276	.0484
	Abuja	5	.1120	.02775	.01241	.0775	.1465
	Kano	5	.0380	.00837	.00374	.0276	.0484
	Total	25	.0752	.04727	.00945	.0557	.0947

Appendix 7. Tukey HSD Post Hoc aflatoxins analysis

Multiple Comparisons							
Dependent Variable (Aflatoxin)	**(I) aflatoxins**	**(J) aflatoxins**	**Mean Difference (I-J)**	**Std. Error**	**Sig.**	**95% Confidence Interval**	
						Lower Bound	**Upper Bound**
B_1	Zamfara	Kaduna	-.05200	.11231	.990	-.3881	.2841
		Sokoto	.53200*	.11231	.001	.1959	.8681
		Abuja	.35400*	.11231	.036	.0179	.6901
		Kano	.55800*	.11231	.001	.2219	.8941
	Kaduna	Zamfara	**.05200**	.11231	.990	-.2841	.3881
		Sokoto	**.58400***	.11231	.000	.2479	.9201
		Abuja	**.40600***	.11231	.013	.0699	.7421
		Kano	**.61000***	.11231	.000	.2739	.9461
	Sokoto	Zamfara	-.53200*	.11231	.001	-.8681	-.1959
		Kaduna	-.58400*	.11231	.000	-.9201	-.2479
		Abuja	-.17800	.11231	.523	-.5141	.1581
		Kano	.02600	.11231	.999	-.3101	.3621
	Abuja	Zamfara	-.35400*	.11231	.036	-.6901	-.0179
		Kaduna	-.40600*	.11231	.013	-.7421	-.0699
		Sokoto	.17800	.11231	.523	-.1581	.5141
		Kano	.20400	.11231	.392	-.1321	.5401
	Kano	Zamfara	-.55800*	.11231	.001	-.8941	-.2219
		Kaduna	-.61000*	.11231	.000	-.9461	-.2739
		Sokoto	-.02600	.11231	.999	-.3621	.3101
		Abuja	-.20400	.11231	.392	-.5401	.1321
B_2	Zamfara	Kaduna	-.24000	.15146	.523	-.6932	.2132
		Sokoto	.67400*	.15146	.002	.2208	1.1272
		Abuja	.44400	.15146	.057	-.0092	.8972
		Kano	.43600	.15146	.063	-.0172	.8892
	Kaduna	Zamfara	**.24000**	.15146	.523	-.2132	.6932
		Sokoto	**.91400***	.15146	.000	.4608	1.3672
		Abuja	**.68400***	.15146	.002	.2308	1.1372
		Kano	**.67600***	.15146	.002	.2228	1.1292
	Sokoto	Zamfara	-.67400*	.15146	.002	-1.1272	-.2208
		Kaduna	-.91400*	.15146	.000	-1.3672	-.4608
		Abuja	-.23000	.15146	.563	-.6832	.2232
		Kano	-.23800	.15146	.531	-.6912	.2152
	Abuja	Zamfara	-.44400	.15146	.057	-.8972	.0092
		Kaduna	-.68400*	.15146	.002	-1.1372	-.2308
		Sokoto	.23000	.15146	.563	-.2232	.6832
		Kano	-.00800	.15146	1.000	-.4612	.4452
	Kano	Zamfara	-.43600	.15146	.063	-.8892	.0172
		Kaduna	-.67600*	.15146	.002	-1.1292	-.2228

		Sokoto	.23800	.15146	.531	-.2152	.6912
		Abuja	.00800	.15146	1.000	-.4452	.4612
G_1	Zamfara	Kaduna	-.06400*	.01117	.000	-.0974	-.0306
		Sokoto	.01600	.01117	.615	-.0174	.0494
		Abuja	-.04800*	.01117	.003	-.0814	-.0146
		Kano	.01600	.01117	.615	-.0174	.0494
	Kaduna	Zamfara	**.06400***	.01117	.000	.0306	.0974
		Sokoto	**.08000***	.01117	.000	.0466	.1134
		Abuja	**.01600**	.01117	.615	-.0174	.0494
		Kano	**.08000***	.01117	.000	.0466	.1134
	Sokoto	Zamfara	-.01600	.01117	.615	-.0494	.0174
		Kaduna	-.08000*	.01117	.000	-.1134	-.0466
		Abuja	-.06400*	.01117	.000	-.0974	-.0306
		Kano	.00000	.01117	1.000	-.0334	.0334
	Abuja	Zamfara	.04800*	.01117	.003	.0146	.0814
		Kaduna	-.01600	.01117	.615	-.0494	.0174
		Sokoto	.06400*	.01117	.000	.0306	.0974
		Kano	.06400*	.01117	.000	.0306	.0974
	Kano	Zamfara	-.01600	.01117	.615	-.0494	.0174
		Kaduna	-.08000*	.01117	.000	-.1134	-.0466
		Sokoto	.00000	.01117	1.000	-.0334	.0334
		Abuja	-.06400*	.01117	.000	-.0974	-.0306
G_2	Zamfara	Kaduna	-.07600*	.01734	.002	-.1279	-.0241
		Sokoto	.01800	.01734	.835	-.0339	.0699
		Abuja	-.05600*	.01734	.031	-.1079	-.0041
		Kano	.01800	.01734	.835	-.0339	.0699
	Kaduna	Zamfara	**.07600***	.01734	.002	.0241	.1279
		Sokoto	**.09400***	.01734	.000	.0421	.1459
		Abuja	**.02000**	.01734	.777	-.0319	.0719
		Kano	**.09400***	.01734	.000	.0421	.1459
	Sokoto	Zamfara	-.01800	.01734	.835	-.0699	.0339
		Kaduna	-.09400*	.01734	.000	-.1459	-.0421
		Abuja	-.07400*	.01734	.003	-.1259	-.0221
		Kano	.00000	.01734	1.000	-.0519	.0519
	Abuja	Zamfara	.05600*	.01734	.031	.0041	.1079
		Kaduna	-.02000	.01734	.777	-.0719	.0319
		Sokoto	.07400*	.01734	.003	.0221	.1259
		Kano	.07400*	.01734	.003	.0221	.1259
	Kano	Zamfara	-.01800	.01734	.835	-.0699	.0339
		Kaduna	-.09400*	.01734	.000	-.1459	-.0421
		Sokoto	.00000	.01734	1.000	-.0519	.0519
		Abuja	-.07400*	.01734	.003	-.1259	-.0221

*. The mean difference is significant at $p<0.05$ level.

Appendix 8. Molecular detection of aflatoxin geness using SSR

Fungal strain	Genes									
	Aflr	*Nor*	*Ver*	*Omt*	Tamari	*Flavus*	*A.niger*	*A.parasiticus*	*A.fumigatus*	*Fusarium*
1	+	+	+	+	+	-	-	-	-	-
2	+	-	+	+	+	-	-	-	-	-
3	+	+	+	+	+	-	-	-	-	-
4	+	+	-	-	+	-	-	-	-	-
5	+	+	-	+	+	-	-	-	-	-
6	+	-	-	-	-	+	-	-	-	-
7	+	-	+	+	-	-	-	-	-	-
8	+	+	+	+	-	+	-	-	-	-
9	+	+	+	+	-	+	-	-	-	-
10	+	+	+	+	+	-	-	-	-	-
11	+	+	+	-	+	-	-	-	-	-
12	+	+	+	+	+	-	-	-	-	-
13	+	+	+	+	+	-	-	-	-	-
14	+	+	+	+	+	-	-	-	-	-
15	+	+	+	+	+	-	-	-	-	-
16	+	+	-	+	+	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-
18	+	+	+	+	+	-	-	-	-	-
19	+	+	+	+	+	-	-	-	-	-
20	+	+	+	+	-	+	-	-	-	-
21	+	+	+	+	-	+	-	-	-	-
22	+	+	+	+	+	-	-	-	-	-
23	+	+	-	+	-	+	-	-	-	-
24	+	-	+	+	+	-	-	-	-	-
25	+	+	+	+	-	+	-	-	-	-
26	+	+	+	+	-	+	-	-	-	-
27	+	+	+	+	-	+	-	-	-	-
28	+	+	+	+	-	+	-	-	-	-
29	+	+	-	+	+	-	-	-	-	-
30	+	+	-	+	-	+	-	-	-	-
31	+	+	-	+	+	+	-	-	-	-
32	+	+	-	+	+	+	-	-	-	-
33	+	+	-	+	-	+	-	-	-	-
34	+	+	-	+	-	+	-	-	-	-
35	+	+	-	+	+	+	-	-	-	-

36	+	+	+	-	+	+	-	-	-	-
37	+	+	-	+	+	+	-	-	-	-
38	+	+	-	-	+	+	-	-	-	-
39	+	+	-	-	+	+	-	-	-	-
40	+	+	-	+	+	+	-	-	-	-
41	+	+	-	+	+	+	-	-	-	-
42	+	+	+	-	+	+	-	-	-	-
43	+	+	-	+	+	+	-	-	-	-
44	+	+	+	-	+	+	-	-	-	-
45	+	-	-	-	+	+	-	-	-	-
46	+	+	-	+	+	+	-	-	-	-
47	+	-	-	-	+	+	-	-	-	-
48	+	+	-	-	+	+	-	-	-	-