Aflatoxin Contamination in Spices Sold in Ilorin, North-Central, Nigeria

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Abstract: Spices are commonly contaminated with aflatoxins, a group of potent mycotoxins produced by moulds. This study investigated the incidence of aflatoxigenic fungi and aflatoxin contents in spices commonly used in Ilorin, North Central Nigeria. Sixty samples of thirteen different spices were purchased from three markets. Fungal isolates were identified using phenotypic characteristics. Aflatoxin contamination was investigated qualitatively and quantitatively using thin layer chromatography and ELISA techniques respectively. Six species belonging to the fungal genera *Aspergillus, Rhizopus* and *Penicillium* were recovered. *A. flavus* and *A. niger* were the most dominant species. Thirty-one (47.1 %) out of sixty (60) samples analyzed were contaminated by total aflatoxin while 29 (52.9 %) were not. Three samples had aflatoxin contents above the 20 ppb (µg/Kg) limit value by the EU while the others did not exceed the 20 ppb (µg/Kg) limit. These results provide baseline information on fungal contamination in spices in Ilorin. Proper spices handling that will limit risks of fungal contamination and their metabolites accumulation at all production and storage stages is recommended while monitoring of aflatoxin contamination and creation of local benchmarks by regulatory agencies is recommended.
Key words: mycotoxin, *Aspergillus,* contamination, dried spices, Ilorin.

INTRODUCTION

Mycotoxins are pharmacological, active secondary metabolites produced by toxigenic strains of moulds in a strain-specific way that evokes some complex toxicological activities (Bennet and Klich, 2013). Mycotoxins are produced by fungal genera notably *Aspergillus*, *Penicillium* and *Fusarium,* and they contaminate various food substances, with toxic side effect on man or animals (Ghali *et al*., 2008). Over 400 types of mycotoxins have been determined and are generally divided into groups based on their structural similarities (Bennet and Klich, 2013) and their major toxic effects. The most toxic mycotoxins produced include aflatoxins, ochratoxins, deoxynivalenol, zearalenone, fumonisins and trichothecenes (Adegoke, 2004).

Aflatoxins are the most harmful mycotoxins and are of major concern and importance in food chains due to their potent effects to human and animal health. They are associated with neurodegenerative diseases (Smith and Groopman, 2018; Kara and Oztas, 2020) and are some of the most potent carcinogens known. They are produced in stored products when environmental conditions are ideal. Specifically, high temperatures of up to 45°C, high humidity of between 65 % - 90 %, moisture contents of above 9%, and physical damage caused by insects and rodents favour aflatoxin production (Okello *et al*., 2010 cited in Ncube *et al.,* 2021).

Spices are a group of plant substances with a strong taste and aroma which are used in small amounts as flavoring agents in various foods (Kulshrestha *et al*., 2014). They may be consumed raw or half cooked e.g. black pepper, ginger, red chilli powder and sesame. Spices are valued for their colors, taste and aroma, which makes them versatile and widely-used ingredients in food preparations globally. Many spices are also consumed due to their associated health benefits such as antioxidants, anti-allergens and antimicrobial effects (Toma and Abdulla, 2013; Yilmaz and Garipoğlu, 2014).

Microbial contaminants such as filamentous fungi can grow or contaminate spices at various stages during harvest, processing, transportation, storage and handling. This contamination compromises spices quality and safety, mainly via mycotoxin production. Spices can be contaminated with mycotoxins at various stages of processing, and when consumed by humans or animals can cause mycotoxicosis which are potentially fatal.

Studies have shown that spices are contaminated with mycotoxigenic moulds especially *Aspergillus* sp which produce aflaxoxins (Shapira and Paster, 2004; Fazekas *et al*., 2005; Barac, 2019).

In Nigeria most reports on spices' contamination with aflatoxins were mostly from the far North and the Southern parts of the country, which have a different climate than the Northern part. Ezekiel *et al*. (2013) studied aflatoxin contamination in three spices (black pepper, ashanti pepper and African nutmeg) in Lagos; Oloyede *et al*., (2016) studied aflatoxins contamination of some edible grains in Lagos and Ota while Odu *et al.* assessed suya spice mixes in Port Harcourt.

This study thus investigates the fungal contaminants associated with commonly-consumed spices in Ilorin, North-Central Nigeria and the associated contamination of the spices with aflatoxin. The findings will provide baseline information about the incidence of aflatoxin contamination in spices in the city, and contribute to the growing body of knowledge regarding aflatoxin contamination of various foods in Nigeria.

MATERIALS AND METHODS

Sampling, moisture content determination, and storage

The 13 spices investigated were chosen based on their availability and popularity and they include cinnamon *(Cinnamon verum),* clove (*Syzygium aromaticum*), ginger (*Zingiber officinale*), fenugreek (*Trigonella foenum-graecum*) seeds, ground red chilli peppers (*Capsicum annuum*), turmeric (*Curcuma longa*), garlic (*Allium sativum*), thyme (*Thymus vulgaris*), nutmeg (*Myristica fragrans*), grains of Selim (*Xylopia aethiopica*), alligator pepper (*Aframomum melegueta*), black pepper (*Piper nigrum*) and African nutmeg (*Monodora myristica*). The samples were verified and deposited in the University of Ilorin Herbarium.

A total of 60 fresh, dried or powdered samples (depending on availability) of thirteen different spices were purchased from three markets between March and July 2019. The Yoruba road market primarily serves the upper income residential areas; Ipata market is a traditional and buslting food market while Oja tuntun is the largest market in Ilorin City and is also known as Central Market.. Spice samples were collected in paper bags and the moisture content immediately determined by the oven drying method 100°C in a hot oven till constant weight. They were then either immediately analyzed or stored at 4°C pending analysis.

Culturing, isolation and identification of fungi

Fungi were isolated following standard dilution and spread plate methods.

Non-powder spice samples were first pounded in a sterilized mortar. One gram of each sample was dissolved in 20 ml of Maximum Recovery Diluent (HiMedia®). Then 200 µl of the mixture was inoculated into sterile plates of Malt extract agar (MEA) (Difco™) and Potato Dextrose Agar (HiMedia®) (PDA) amended with chloramphenicol (25 mg/l). The plates were incubated at 25°C for up to 7 days. Single spores of recovered fungal isolates were inoculated into MEA and incubated again for 5-7 days. The mycelia were then stained with lactophenol cotton blue dye, observed microscopically, and identified using identification manuals (Davise, 2002; Klich, 2002; Robert *et al*., 2004).

PCR Amplification of 5.8S–ITS rRNA gene and DNA nucleotide sequencing

Three fungal isolates that were not conclusively identified by phenotypic means were subjected to molecular identification. The 5.8S–ITS rRNA gene was amplified with the universal ITS1 and ITS4 primers TCCGTAGGTGAACCTTGCGG and TCCTCCGCTTATTGATATGC respectively (White, *et al*., 1990) followed by DNA nucleotide sequencing at International Institute of Tropical Agriculture (IITA), Ibadan. Nucleotide sequence cleaning and analysis was done as previously described (Temu, 2016).

Comparison of nucleotide sequences to those available in the National Center for Biotechnological Information (NCBI) using nucleotide basic alignment search tool (nBLAST) identified the fungi to the nearest similar accession. The two most similar accessions to each fungal isolate were taken to infer the phylogenetic relationship using Unweighted Pair Group Method with Arithmetic Averages (UPGMA) method with Kimura 80 parameters and 1000 bootstrap values (Alam, *et al*., 2015).

Aflatoxin analysis

Spice preparations were ground and mixed to uniform consistency using a laboratory mill. The samples were analyzed for total aflatoxins using a commercial direct competitive ELISA (AqraQuant Total Aflatoxin Assay 1/20, Singapore) kit. Briefly, the samples were extracted in a methanol-water mix (80:20). Sample defatting was performed in n-hexane chloroform (60:40). In the test, free aflatoxins in the sample compete with added enzyme-labeled aflatoxin (conjugate) for antibody binding site. The intensity of the colour produced by reaction of the substrate with the enzyme attached to the toxin, in both standards and sample extract wells, was determined by reading the absorbance at 450 nm using an ELISA microtitre plate reader within 10-15 minutes after addition of the stop solution. The detection limit was 60 µg/kg.

STATISTICAL ANALYSIS

Data collected were subjected to ANOVA analysis using Statistical Package for Social Sciences (SPSS) version 20.0. Means were separated using Tukey's-b Test at 5 % level of significance ($P< 0.05$).

Total Aflatoxin analysis

Aflatoxin extraction

Dried spices were ground using a laboratory mill. Extraction was performed according to the manufacturer's recommendation using the Aqra Quant Total Aflatoxin Assay 1/20 test kit (Romer Labs Singapore Pte Ltd). To defat, 2 g of each spice was mixed with 10 ml of 60:40 n-hexane-chloroform and shaken on an orbital shaker for 1hr. The mixture was allowed to settle, then the liquid filtered through a Whatman No 1 filter paper and the filtrate collected for clean-up.

Detection of aflatoxin contamination

Aflatoxin contamination of the spices was determined using Thin-Layer Chromatography (TLC). Sample clean-up was performed using MycoSep 226 AflaZon according to the manufacturer's instructions. Briefly, each spice was suspended in 80 % methanol (1:4), shaken on an orbital shaker for one hour, then the supernatant was pipetted into test tubes. Next it was incubated at 55^{o}C for two to three minutes in a water bath. Filter paper was placed in the TLC chamber and the 80% methanol was added into the chamber, the lid was closed and the chamber was tilted to wet the filter paper. The TLC plate coated with silica gel was labelled with pencil and then spotted with the sample extracts and aflatoxin buffer as standard, then placed into the chamber. The TLC was allowed to develop for 10-15 minutes, and then the plate was removed from the chamber. The process was completed when there was presence or absence of colour to show if total aflatoxin was present or absent in the samples. Then 8 % of Ninhydrinin acetone was used to stain the TLC plate and it was viewed under UV light at wavelength 254 nm (Lin, *et al.*, 1998).

Quantitative analysis of aflatoxins

Spice samples that tested positive with the TLC were further tested to quantitatively estimate aflatoxins. Aflatoxin analysis of the spices was carried out according to AqraQuant total aflatoxin Assay 1/20 test kit manual. Exactly 200 µL of conjugate was dispensed into each green-bordered dilution well and 100 µL of each of aflatoxin standard (0, 1.0, 2.0, 4.0, 10.0 and 20.0 µg/kg) and the samples was added into the appropriate dilution well. Each well was carefully mixed by pipetting it up and down three times and 100 µL of the contents from each dilution well was immediately transferred into a corresponding antibody coated microwell.

Next, 100 µL of HRP-conjugate reagent was dispensed into each microtitre plate well, covered with adhesive strip and incubated for 60 minutes at 37^{o}C. The wells were then aspirated and washed with distilled water five times. Each well was washed by filling with 400 µL Wash solution with a manifold dispenser, the plate was inverted and blotted against clean paper to remove any remaining wash solution. Next, 50 µL of chromogen solutions A and B were added to each well, and gently mixed and incubated for 15 minutes at 37^{o}C. Once a blue colour developed, then 50 µL of stop solution was added into each well which changed the blue colour to yellow. The wells, containing both the aflatoxin standards and the samples were read with a microwell reader (0, 1.0, 2.0, 4.0, 10.0, 20.0 40.0, 60.0, 80.0 and 100.0 µg/kg) using an absorbance filter of 450 nm. The aflatoxin content of samples was interpolated from the standards' values.

RESULTS AND DISCUSSION

Moisture content

Most of the spices had a moisture content ranging from 6.8 -14.7 % (Table 1). These values meant the spices were held at moisture levels that complied with reputable standards like those of the European Spice Association. The only exceptions were clove and cinnamon which exceeded the limits of 12 % and 14 % respectively (European Spice Association, 2015). Spice contamination may arise from poor hygienic conditions during harvesting, processing, transportation and storage. Fungal contamination is further intensified by warm, humid tropical conditions and partial drying which provide optimal conditions for fungal growth and consequently production of mycotoxins (Ahmed El-Imam *et al.,* 2012; Mwangi *et al*., 2014). Thorough sun-drying or dry-heat sterilization to reduce the moisture content of spices, proper packaging and storage can minimize growth of microorganisms such as fungi, bacteria, and yeasts.

Table 1: Moisture content of the spice samples obtained from open-air markets in Ilorin, North-Central Nigeria

Sample	Moisture content (%)
Cinnamon	14.7
Clove	14.5
Ginger	11.7
Fenugreek seeds	11.3
Turmeric	9.6
Garlic	9.4
Thyme	8.7
Nutmeg	8.0
Ground chilli pepper	7.5
Grains of Selim	6.9
Alligator pepper	6.8
Black pepper	6.8
African nutmeg	6.8

Fungal incidence and identification

Fifty-seven (57) fungal isolates comprising six (6) fungal species were isolated from the sixty spice samples. Their distribution among the markets was variable (Table 2).

Table 2: Frequency of occurrence of fungal isolates (%) recovered from spices sold in three markets in Ilorin, Nigeria

Fungal isolates	Market			
	Oja tuntun (%)	Ipata market (%)	Yoruba road market (%)	Total (%)
Aspergillus flavus	14.04	8.77	12.28	35.09
A. niger	8.77	10.53	14.04	33.34
A. fumigatus	1.75	-	3.51	5.26
A. parasiticus	3.51	3.51	5.26	12.28
Penicillium citrium	-	-	5.26	5.26
Rhizopus stolonifer	1.75	3.51	3.51	8.77

It was observed that the samples procured from the Yoruba road market had the highest fungal load and this may be due to the lower patronage of this market as compared to the larger markets. Relatively lower patronage results in longer storage times of the spices in the trucks and stores which could encourage fungal proliferaton.

The fungal isolates recovered from the various samples are presented in Table 3.

Table 3: Fungal isolates recovered from spice samples

S/No	Spices	Fungal isolates*
1	Turmeric	*Aspergillus niger*, *A. parasiticus* (AY373859)
2	Alligator pepper	*A. flavus* (MG554231), *A. niger*
3	Ground chilli pepper	*Rhizopus stolonifer, A. flavus*
4	Garlic	*A. niger*, *Penicillium citrinum*
5	Grains of Selim	*A. niger*, *A. flavus, A. fumigatus*
6	African nutmeg	*R. stolonifer*, *P. citrinum*
7	Cinnamon	*P. citrinum*, *A. flavus*, *A. niger*
8	Black pepper	*A. parasiticus*, *A. flavus* JNYG35

*Fungal species identified by molecular means have accession information in brackets

Despite having the second-highest moisture content, no fungi were recovered from cloves. This may be attributed to the antimicrobial properties of its essential oils which have been shown to be effective against microbes including fungi (Munoz Castellanos *et al.*, 2020). Similarly, no fungal genera were recovered from fenugreek seeds, ginger, nutmeg and thyme and this disagrees with reports (Dixit and Singh, 2011; Jeswal and Kumar, 2015) who isolated aspergilli and other fungi. These results are however in agreement with Hashem and Alamri (2010) who also observed low levels of fungal contamination from cloves. This phenomenon could be attributed to the spices being freshly-harvested or well-preserved, or possessing potent anti-fungal abilities.

Nutmeg contains terpinen-4-ol, terpinene, limonene, α-pinene, β-pinene, p-cymene, β-caryophyllene, and carvacrol which have been reported to have antimicrobial activity and supposed to involve in membrane disruption by the lipophilic compounds that can inhibit fungal growth as reported by (Gupta *et al*., 2013; Carradori *et al*., 2016). Grains of Selim and cinnamon had the largest species diversity.

This can be remedied by further sun-drying the cinnamon to lower the moisture content, while the grains of Selim (which had low moisture content) should be harvested and stored for shorter periods to prevent the growth of *Aspergillus* sp and other storage fungi.

The most frequently-occuring fungal genus was *Aspergillus*. Thirty-two samples were contaminated with at least one *Aspergillus* spp. *A. flavus* and *A. niger* were the dominant species recovered from the spices, and this is in agreement with the findings of Jeswal and Kumar (2015) who analysed nine Indian spices. The high incidence of *Aspergillus* spp on spices calls for attention since among others, *A. flavus* and A. *parasiticus* are known aflatoxin producers (Noreddine, 2020). A. *fumigatus* was also isolated, and it is a known human pathogen. Some *Penicillium* spp are known to produce toxic metabolites like ochratoxin and citrinin (Jeswal and Kumar, 2015; Geisen *et al.,* 2018). Darwish *et al*. (2014) also isolated *Penicillium* and its metabolite ochratoxin A in ground chilli and other various spices. Although not all strains in these two genera are toxin producers, their presence in food stuffs is still of major concern.

Aflatoxin content

The aflatoxin-contaminated spice samples and the levels of contamination are presented in Table 3.

Table 3: Level of total aflatoxin in spices

Sample	**Aflatoxin levels**	
	< 20 (µg/kg)	**>20 µg/kg**
Turmeric	-	+
Alligator pepper	+	-
Garlic	+	-
Ginger	-	+
Ground red chilli pepper	+	-
African nutmeg	+	-
Grains of Selim	+	-
Cinnamon	-	+
Cloves	+	-
Thyme	+	-
Black pepper	+	-

Aflatoxins were found in all spices except fenugreek and nutmeg, and this was not surprising as aflatoxigenic fungi were not recovered from them. It was intriguing to find aflatoxin contamination in cloves, despite the aflatoxigenic fungi were not recovered. This may indicate an earlier colonization by the fungi. Using simple qualitative detection of total aflatoxin, (Temu, 2016) also reported total aflatoxin amounting to or higher than 4 ppb (µg/kg) in red chilli powder. Mwangi *et al.* (2014) similarly observed lower levels of aflatoxin in nutmeg. The draft maximum content of total aflatoxins in spices proposed by the electronic working group of the Food and Agricuture Organisation (FAO) and World Health Organisation (WHO) is 20 -30 µg/kg of these spices (Codex Alimentarius, 2018). Most of the tested spices contained less than 20 µg/kg of aflatoxins which makes them within the limits acceptable in the African Union and India among other countries. They are however above the 10 µg/kg limit set by the European Union since 2002 (Codex Alimentarius, 2018). To determine if ginger and cinnamon which had over 20 µg/kg exceeded the 30 µg/kg upper limit proposed, more accurate empirical methods like GC-MS may need to be applied.

CONCLUSION

This study reports the contamination of several commonly-used spices in Ilorin with mycotoxigenic fungi. *Aspergillus* sp. was the most frequently encountered fungus, with both pathogenic and aflatoxigenic species recovered. Aflatoxins were detected in all but two of the tested spices namely fenugreek and nutmeg. While the levels were lower than domestic standards, improved handling would further lower aflatoxin contamination and allow for potential value-addition by export to the European Union among others.

Further work to determine the exact contamination levels of these spices with aflatoxins and other mycotoxins is recommended.

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