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AFLATOXIGENIC FUNGI AND AFLATOXIN CONTAMINATION OF *COCHLOSPERMUM TINCTORIUM* ROOT POWDER USED AS SPICE IN SOUP PREPARATION

[*1]Aliyu, A., [1]Ibrahim, A. D., [1]Jodi, A. M., [3]Muhammad, A. S., [4]Ahmadu, U., [2]Dandare, S. U, [5]Yusuf, A. B., and [6]Jodi, S. M.

[1]Department of Microbiology, Usmanu Danfodiyo University Sokoto
[2]Department of Biochemistry, Usmanu Danfodiyo University Sokoto
[3]Department of Biology, Al-Qalam University, Katsin,
[4]Department of Science Laboratory Technology, Ramat Polytechnic Maiduguri
[5]Department of Microbiology, Federal University Birnin Kebbi
[6]Department of Pharmaceutics and Pharmaceutical Microbiology, Faculty of pharmaceutical Sciences, Usmanu Danfodiyo University Sokoto, Nigeria

*Corresponding authors' email: ibrahim.aliyu@udusok.edu.ng

ABSTRACT

Cochlospermum tinctorium root powder (Kwata) is commonly used as spice in foods and for medicinal purposes in West African sub-region. This research evaluated the rate of contamination of C. tinctorium root powder with aflatoxigenic fungi and aflatoxins. Kwata samples obtained from different sellers in Sokoto market were analyzed for presence of fungi using standard mycological methods. Aflatoxins and their concentrations in the kwata were also evaluated using EnzymeLinked Immunosorbent Assay (ELISA). The total aflatoxin concentrations of the different Kwata samples ranged from 125 to 580 ppb while aflatoxin B1 concentrations in the samples were found to be 35.5 ppb, 24.5 ppb, 32 ppb, 23.8 ppb and 45 ppb all of which are above the limits (20 ppb for adult food and 0 ppb for infant food) stipulated by regulatory bodies in Nigeria. The Aspergillus species isolated and their percentage occurrences in Kwata samples were Aspergillus niger (50% in sample A and above 25% in other samples), Aspergillus flavus (50% in sample A and 25% in other samples), Aspergillus fumigatus (33% in samples B and E and 20% in other samples). In conclusion, this study revealed high contamination of Kwata sold within Sokoto metropolis with Aspergilus species and aflatoxins, the cummulative effect of which may cause serious public health problems in populations that use this preparation in soup.

Keywords: Aspergillus flavus, AgraQuant, Aflatoxin, Kwata, ELISA

INTRODUCTION

Kwata is the name given to the root powder of *Cochlospermum tinctorium* commonly used as spice in soup preparation. *Cochlospermum tinctorium* is a bushy plant that is about 50 cm in height. The plant has a widespread occurrence in Savannah shrubs land and throughout the drier areas of the West African region. It has common names in Nigeria, which are Rawaya orkyamba (Hausa), obazi orobanzi (Igbo) and sewutu (Yoruba).

The plant is commonly used for medicinal purposes in West African sub-region. It is used in management of various conditions such as pain and inflammation. The root parts of the plant are used traditionally to cure fever, hepatitis, and abdominal pain. They are also used traditionally for the treatment of gonorrhoea, jaundice and gastrointestinal diseases (Ahmad *et al.,* 2011). An extract of the root of kwata (*Cochlospermum tinctorium*) is used to treat malaria in Burkina Faso; in Nigeria, a concoction of the root with tamarind fruits is used to cure snake bites. A decoction is used in a bath to treat urogenital disorders, kidney pain and pain between the ribs. The body is washed with water extract of the root to cure skin diseases. In Cote d'voire, powder of the root is applied topically to treat skin diseases; also the root is chewed as a tonic (Burkill, 2000).

During their metabolic processes, fungi often produce secondary metabolites called mycotoxins, which are poisonous chemical compounds capable of causing disease and death in humans and livestock (Bennett and Klich, 2003; Marta et al., 2016). Mycotoxins are not necessary for the growth and development of fungi; they are thought to be used by the fungus to weaken its host as a strategy to make the environment conducive for fungal proliferation (Hussein and Brasel, 2001). The presence of mycotoxins in agricultural products pose serious threats to human health as they could cause significant economic losses in several countries (Horn 2003; Wu *et al.,* 2014).

Among the several types of mycotoxins, aflatoxins are of major concern, with aflatoxin B1 being the most toxic to humans and animals (Olivier *et al.,* 2017). Generally, aflatoxins are genotoxic, carcinogenic, immunosuppressive substances and can cause both acute and chronic toxicity. Worldwide, aflatoxins are estimated to cause 28% of the total cases of the most common liver cancer, hepatocellular carcinoma (HCC) (Wu, 2014). Wu (2014) suggested that approximately 172,000 cases of HCC per year are caused by consumption of aflatoxin-contaminated diets and the majority of cases occurs in sub-Saharan Africa. In addition to HCC, consumption of aflatoxincontaminated foods can cause stunted growth in children, acute poisoning and immune-system dysfunction (Groopman *et al.,* 2008). Related health problems are difficult to diagnose, mainly due to cryptic, long-term and chronic exposures. However, as previously shown and recognised by the Kenyan government in 2004 and 2005, hundreds of human death cases can be ascribed to the consumption of aflatoxin-contaminated products (Lewis *et al.,* 2005).

Plants and herbs are used in most households for culinary purposes; they are often used as additives to enhance flavour

and aroma. The consumption of plants or herbs contaminated with mycotoxins may cause ill effects in an individual. This study was carried out to determine the occurrence of aflatoxigenic fungi as well as the levels of aflatoxin B1 in Kwata (*Cochlospermum tinctorium*), a popular root powder used commonly by some Nigerian communities in preparing soups. In addition, a key aflatoxin biosynthesis gene was amplified from some of the isolated aflatoxigenic organisms.

MATERIALS AND METHODS

Sample collection

Samples of Kwata (*Cochlospermum tinctorium*) root powder, designated Samples A, B, C, D and E, were collected at Sokoto central market and then transported to Microbiology laboratory in Usmanu Danfodiyo University, Sokoto and used for this study.

Isolation and culture condition

The fungi associated with the 'Kwata' was isolated following serial dilution and inoculation on Sabouraud Dextrose Agar (SDA) at 25 ^{o}C for seven days and stored as spore's suspension on 20% glycerol for further analysis (Olivier *et al.,* 2017). Morphological and growth characterizations of the fungi on the Sabouraud Dextrose Agar (SDA) were carried out. Physiological analysis was carried out on Desiccated Coconut Agar (DCA) (Frisvad and Samson 2004), and yeast extract agar (YES) (Pitt *et al.,* 1983).

Identification of isolates

Isolates were identified using cultural and morphological features such as growth pattern, conidial morphology and pigmentation described by Tafinta *et al.* (2013). Microscopic observations were then carried out by placing a drop of lactophenol cotton blue stain on a glass slide. To this, a portion of the fungal mycelia from a pure culture was added and covered with a coverslip, while avoiding air bubbles in the process. The preparation was then viewed using the x10 and x40 objective lens. The organisms were identified using a comprehensive fungi atlas by Samson and van ReenenHoekstra-1988 (Oyeleke and Manga, 2008).

DNA extraction from Isolates

The DNA isolation was carried out using the zymoBIOMICs kit (Zymo research). The cultured spores were added into zymoBIOMICs lysis tube and 750 µl of zymoBIOMICs lysis solution was then added. It was secured in a bead beater fitted with a 2 ml tube holder assembly and processed at maximum speed for 5 mins. The lysis tube content was then centrifuged using a microcentrifuge at 10000 x g for 1 min. Exactly 400 µl of the supernatant was transferred to a Zymo-spin and centrifuged at 8000 x g for 1 min. Then, 1200 µl of zymoBIOMICs DNA binding buffer was added to the filtrate. Exactly 800 µl of the mixture (DNA binding buffer + centrifuged supernatant) was transferred to a Zymo-spin in a collection tube and centrifuged at 10000 x g for 1 min. The flow from the collection tube was then discarded and the step was repeated. ZymoBIOMICs DNA wash buffer 1 (400 µl) was then added to the Zymo-spin in a new collection tube and was centrifuged at 10000 x g for 1 min; the flow was discarded. Seven hundred microlitres (700 µl) of zymoBIOMICs DNA wash buffer 2 was added to the Zymo-spin in a collection tube and centrifuged at 10000 x g for 1 min and the flow through was discarded. Two hundred microlitres (200 µl) of DNA wash buffer 2 was added into Zymo-spin and centrifuged at 10000 x g for 1 min. The Zymo-spin was transferred to a clean 1.5 ml microcentrifuge tube and 100 µl (50 µl minimum) zymoBIOMICs DNase/RNase free water was added directly to the column matrix. It was incubated for 1 min, then centrifuged at 10000 x g for 1 min to elute the DNA.

The Zymo-spin IV-HRC spin filter was prepared by removing the base of the Zymo-spin IV-HRC spin filter and placed into a clean collection tube centrifuged at 8000 x g for 3 min and the flow through was discarded. The cap was removed and 400 µl zymoBIOMICs DNase/RNase free water was added to the Zymo-spin IV-HRC spin filter. The Zymo-spin IV-HRC spin filter was loosely capped and centrifuged at 8000 x g for 2 minutes. The eluted DNA was transferred to a prepared Zymo-spin IV-HRC spin filter in a clean 1.5 ml microcentrifuge tube. The Zymo-spin IV-HRC spin filter was loosely capped and centrifuged at precisely 8000 x g for 1 min.

PCR confirmation of aflatoxigenic fungi

The aflatoxigenic fungi were confirmed using the method of Sadhasivam *et al.* (2017) to optimise the PCR amplification assay for direct detection of mycotoxigenic fungal species by targeting the aflatoxin biosynthesis gene, the primers used for species-specific detection and gene amplification were tested and confirmed. The PCR amplification was standardised by empirically varying critical factors that affect amplification such as primer concentration, amount of template and annealing temperature. The set of primers used for amplification are avf723F (5'-ATGGTCACATACGCCCTCCTCGGG-3') and avf1675R (5'-GCCTCGCATTCTCTCGGCGACCGAA -3'), with annealing temperature 58^{o}C. These primers amplify the *avfA* gene that is involved in the conversion of averufin (AVF) to versiconal hemiacetal acetate (VHA). The expected amplicon size is 950 bp (Yu *et al.,* 2000).

The PCR reaction was performed in 0.2 ml thin-wall PCR tubes with flat frosted caps in 17.5 µl PCR reaction volume containing 3 µl of template DNA, 1µl each of primer avf723F and avf1675R, 12.5 µl Taq master mix - containing 20 µM of deoxynucleoside triphosphates (dNTP), Taq DNA polymerase and Tag buffer with $MgCl_2$.

The PCR thermocycling conditions include initial heat activation of DNA polymerase at 95 ^{o}C for 15 min, followed by 35 cycles of denaturation at 94 ^{o}C for 30 secs, annealing at 58 ^{o}C for 1 min, extension at 72 ^{o}C for 90 secs and a final extension at 72 ^{o}C for 10 min. The PCR products were electrophoresed on a 1% agarose gel with a 100bp DNA size marker at 96V for 1 hour (Sadhasivam *et al.,* 2017). The PCR products were then purified using PCR product purification kit and the purified PCR products were sent to Inqaba Biotech South Africa for sequencing service. The quality of the sequences from Inqaba was analysed using finch TV and sequence analysis were performed by comparing the sequences against the known sequences in the NCBI database.

Quantification of Aflatoxin in Kwata

The total aflatoxin and aflatoxin B1 concentrations were determined in the laboratory of Animal care services Konsult (Nig) Ltd. The analysis of total aflatoxin and aflatoxin B1 (AFB1) content of the 'Kwata' samples were performed using ELISA kit AgraQuant® ELISA total and Aflatoxin B1 (Romer Labs, Singapore). Five grams (5 g) of Kwata was weighed and added to 25 ml of methanol (70%). It was allowed to stand for 10 mins in order to aid aflatoxin extraction, then filtered using a No. 1 Whatman filter paper. Fifty microlitres (50 µl), each, of sample filtrate and aflatoxin standard were dispensed in separate dilution wells and each was with 100 µl of the conjugate. One hundred microlitres (100 µl) from the filtrate/standard-conjugate mixture was taken and dispensed in the antibody-coated

wells. It was then incubated at room temperature for 15 mins. The content of the wells was discarded and the wells were washed 3 – 4 times with distilled water. Exactly 100 µl of substrate was added to each well and incubated for 5 mins to allow for colour change (different shades of blue to colourless). A volume of 100 µl of stop solution was added which converted the blue end-point to yellow. The mixture was read with an ELISA plate reader at 450 nm. The optical densities of standards and those of samples were recorded. A standard curve was generated which was used to extrapolate the concentrations of total aflatoxin and aflatoxin B1 of the samples. Samples with high levels of aflatoxin were diluted further with 70% methanol to either 1/10th or 1/20th (or more) of the original concentration in order to obtain readings within the range of the standard curve.

RESULTS AND DISCUSSION

Aflatoxin B1 concentrations in the samples of Kwata (*Cochlospermum tinctorium* root powder) from old market Sokoto and its limits for food in Nigeria showed that Sample E had the highest concentration of aflatoxin B1 (45 ppb). Samples A, B, C and D had aflatoxin B1 concentrations of 35.5 ppb, 24.5 ppb, 32.0 ppb and 23.8 ppb, respectively, with the aflatoxin B1 concentration in Sample D the lowest (Figure 1).

The fungi associated with the contamination of Kwata (*Cochlospermum tinctorium* root powder) were identified in this study on the basis of morphological and microscopic features as *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus fumigatus*. The morphological and microscopic features of the fourth organism did not match any of the ones described on the chart for identification of fungi (Table 1).

The frequency of occurrence of fungi associated with the *Cochlospermum tinctorium* root powder indicated that *A. niger* and *A. flavus* occurred at a frequency of 50% each in Sample A and both occurred at >25% frequency in all the other Kwata samples (Figure 2). *A. fumigatus* occurred at a frequency of 33.3%, each, in Samples B and E, and 20%, each in Samples C, and sample D. The uncharacterised organism appeared in Samples B and D with occurrence frequencies of 16.6% and 20%, respectively.

PCR analysis detected the presence of the aflatoxin biosynthesis gene (*avfA*) in *A. flavus* and in the uncharacterised organism from the Kwata. Presence of the *avfA* gene in the *A. flavus* and the uncharacterised organism with 99 and 100 % identity to *A. flavus* NRRL 3357 and an *avf A*gene from *A. flavus* isolate AF70, respectively, was confirmed (Table 2).

Phylogenetic analysis, based on neighbour-joining tree, showed that the aflatoxin biosynthesis genes from the isolates in this study had some degree of difference based on their nucleotide sequences with the divergent from the root at 0.2 confidence limit based on the bootstrap analysis (Figure 3).

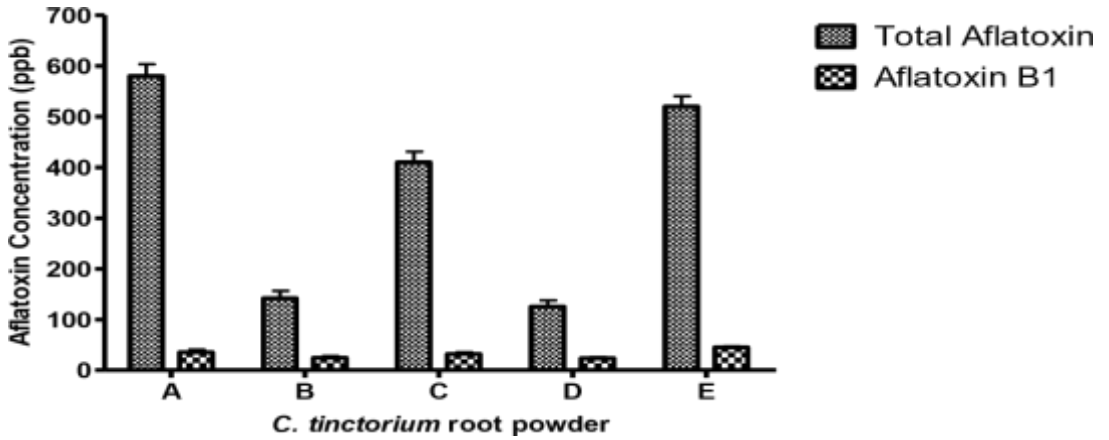

Figure 1: Total Aflatoxin and aflatoxin B1 concentrations in different *C. tinctorium* root powder samples obtained within Sokoto Market, Nigeria. Bars represent means of triplicate measurements and error bars are standard deviations (SD) from the means.

Table 1: The Phenotypic identity of fungal species isolated from Kwata (*Cochlospermumtinctorium* root powder) obtained within Sokoto market.

Identified organism *Aspergillus*	**Colony description**	**Microscopy**
niger	It is black having a round shape,and it is powdery. The reverse is yellow.	The conidiophore terminates in vessels, and the conidia are in chains.
Aspergillus flavus	It is green in colour and powdery.	They have hyphae bearing conidiophores
Aspergillus fumigatus	It is blue and smooth	They have hyphae with conidiophores
Uncharacterized	Black in colour with crystals	They have hyphae conidiophores

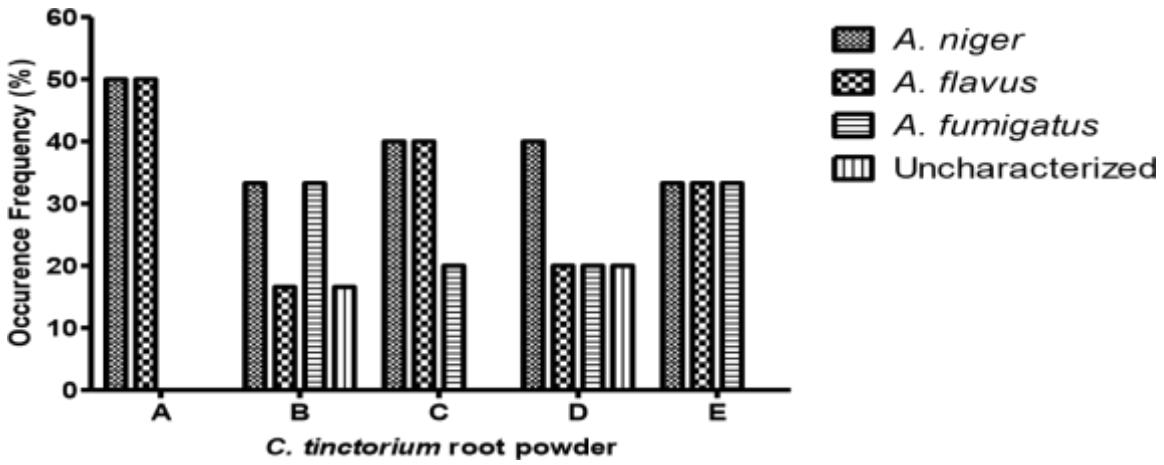

Figure 2: Frequency of occurrence of fungi in the root powder of *C. tinctorium* (kwata) obtained from Sokoto market, Nigeria.

Table 2: Molecular confirmation of aflatoxigenic fungi based on PCR amplification of aflatoxin biosynthetic gene (*avfA*)

Seq ID	Best Hit	% Query coverage	% Identity	Accession
AF1	*Aspergillus flavus* NRRL 69 3357 SAGA Complex component (sgfl3)		99	XM002382146. 1
SB	*Aspergillus flavus* isolate 100 AF70 aflatoxin biosynthesis gene cluster complete sequence		100	XM002382146. 1

Figure 3: Neighbour-joining tree showing the relationship of the aflatoxin biosynthesis genes of identified isolates to their closest relatives available on the NCBI database.

In this study, presence of aflatoxins and aflatoxigenic fungi in *Cochlospermum tinctorium* root powder samples (Kwata), obtained from Sokoto market, northwestern Nigeria was established. The concentrations of aflatoxin B1 (AFB_1) in all the *Cochlospermum tinctorium* root powder samples, as recorded in this study were higher than the maximum acceptable limits of aflatoxins in foods (Badmus et al., 2021). The Kwata obtained from Sokoto market and used widely for its culinary importance is therefore unsafe for human or livestock consumption. This is because aflatoxins were correlated to adverse health effects, such as cancer, with aflatoxin B1 (AFB_1) being the most toxic amongst them (Dohnal and Kuča, 2014, Yun *et al.,* 2016). Aflatoxin B1 (AFB_1 is a potent carcinogen and was directly associated with development of liver cancer in several animals **(**Hamid *et al.,* 2013**)**. AFB_1 is carcinogenic because it is metabolized by the liver to the highly reactive and electrophilic epoxide intermediate which causes hepatotoxicity (Dohnal and Kuča, 2014). The Nigerian limits for AFB_1 concentrations in adults and infants' foods are 20 and 0 ppb (Tiffany, 2013; PACA, 2018), respectively.

The presence of mycotoxin-producing fungi, namely *Aspergillus niger*,*Aspergillus flavus*, *Aspergillus fumigatus*, as established in this study, is not surprising as they could come from soilor field (preharvest) or during storage (postharvest) as previously reported (Marta *et al.,* 2016). *Aspergillus spp*. are widely distributed, especially in food storage places. At suitable moisture and temperature

conditions, *Aspergillus* produces mycotoxins (Surekha *et al*., 2011). Amongst all the *Aspergillus spp*., *A. flavus* is the major producer of AFB_1 andAFB_2. Other species such as *A. parasiticus* also synthesizes AFB_1 and AFB_2 alongside other aflatoxins such as AFG_1 and AFG_2 (Bennett and Klich, 2003). *A. niger* and *A. fumigatus* were not reported to produce aflatoxin; however, some strains were found to produce ochratoxin A. and gliotoxin (Schuster *et al.*, 2002; Nieminen *et al.*, 2002). Both biological, chemical and physical conditions of *Aspergillus* were reported to influence its production of aflatoxins (Kumar *et al.*, 2017).

Amongst the four organisms isolated in this study, *A. niger* has the highest frequency of occurrence in all the samples. Interestingly, *A. niger* has been shown to inhibit the biosynthesis of AFB_1 in *A.flavus* through down-regulation of the expression of major biosynthetic genes **(**Xing *et al.*, 2017**)**. Remarkably, 19 out of 20 aflatoxin biosynthetic genes were reported to be down-regulated by *A. niger* (Xing *et al.*, 2017). Despite this known crucial biological role of *A. niger* in inhibiting AFB_1 biosynthesis, the concentration of AFB_1 recorded in the present study, which was at least 4-fold higher than the acceptable limit is alarming.

The presence of a critical aflatoxin biosynthesis gene (*avfA*) in the isolated *A. flavus* and in the uncharacterized organism from the Kwata and confirmation of the presence of the gene in the *A. flavus* isolate with 99% identity to that from *A.flavus* NRRL 3357 and100% identity to aflatoxin biosynthesis gene from *A. flavus* isolate AF70 in the uncharacterised organism showed that the isolates obtained in the present study are indeed aflatoxigenic.

Decreasing or controlling fungal growth and eliminating aflatoxins formation in foods for human consumption and animal feed is essential for food security and health. Although it is difficult to prevent aflatoxin formation in food before harvesting due to heavy rainfall, temperature and moisture content, it is possible to reduce their level by establishing good hygienic practices during transport and storage (Marta *et al.*, 2016; Zinedine and Maes, 2009).

CONCLUSION

In conclusion, the findings in this study show that kwata (*C. tinctorium* roots powder) obtained within Sokoto metropolis contained fungal species that are capable of producing aflatoxins. Also, four different fungal species were isolated, which included *A. niger, A. flavus* and *A. fumigatus*) and one uncharacterized organism. Furthermore, PCR and molecular analysis of the key aflatoxin biosynthetic gene (*avfA*) confirmed that two of our isolates (*A. flavus* and uncharacterised) are indeed aflatoxigenic organisms. The 100% identity of the *avfA*gene from the uncharacterised isolate to *A. flavus* isolate AF70 suggests that the uncharacterized organism is highly likely to be an *A. flavus* strain. The concentrations of total aflatoxins and aflatoxin B1 determined in all the samples exceeded the acceptable limits. Therefore, these results show that *C. tinctorium* root powder is not safe for consumption and it can be used in establishing public health awareness on the consumption of contaminated foods.

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