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Identification of Fusarium Species Contaminating *Hibiscus sabdariffa* in Jigawa state and Quantitative Assessment of Fumonosin (FB1) Produced

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ABSTRACT

Fungal species are known to be notorious fungal contaminants causing damage to food and feed samples and at the same time producing mycotoxins that compromise the health status of both human and animals. Twenty seven (27) samples of dried Hibiscus sabdarifa were sourced from nine (9) local government areas (L.G.As). Three (3) L.G.As were randomly selected from each of the three senatorial districts of Jigawa State (11º 47' 00''N, 4º 44' 00''E) viz: Babura, Roni, Maigatari, Hadejia, Kafin Hausa, Auyo, Buji, Gwaram and Miga L.G.As. Fumonisin producing fungal species were isolated from the collected samples by the mycological analytical procedures under aseptic condition. The macro and microscopic identifications of Fusarium species was done following the standard identification keys while other identification keys were employed for other species. Their mycotoxigenicity was also determined using the appropriate methods. Extraction and quantitative determination of the fumonisin was performed using high performance liquid chromatography tandem mass spectroscopy (HPLC/MS). Auyo being a wetland area and more humid, has the heighest concentration of the mycotoxin (FB1) at 2052.93±24.34 µg/kg while sample extract from Roni was observed to have a mean value of 1712.9±18.4 µg/kg and Maigatari has the lowest concentration of the toxin at 1630.27±21.76 µg/kg. Values obtained from the study areas are quite higher than the daily tolerable intake limits set by regulatory bodies such as WHO, Codex alimentarius and European food Safety Agency (EFSA) and therefore, call for immediate and coordinated mobilization of mitigation strategies to safeguard the health of the consumers and sustain the relevance of the commodity in the international markets.

Keywords: *Fungi, Fusarium species, FumonisinB1, Mycotoxigenicity, Hibiscus sabdariffa*

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INTRODUCTION

Detrimental health hazards on humans as well as animals are known to be posed by groups of some ubiquitous fumonisin producing fungal species called *Fusarium verticillioides*. These species are known to contaminate several food matrices (Anumudu *et al.*, 2024). More so, *Fusarium verticillioides* aside of being a notorious fumonisin producer is also one of the most prevalent fungi associated with contamination of corn throughout the world (Anumudu *et al.*, 2024; Edeghor *et al.*, 2023). In 1988, the mycotoxin named fumonisins believed to be the causative agent of a field outbreak of leukoencephalomalacia in horses in South Africa were first isolated from cultures of *F. verticillioides* strain MRC 826.2 (Bacelar *et al.*, 2016; Abeywickrama, 1993). In addition to

F. verticillioides, which is the major fumonisin-producing strain, fumonisins may also be produced by *F. proliferatum, F. anthophilum, F. dlamini, F. napiforme, and Alternaria alternate.*

Fumonisin contamination of food, particularly corn and corn-based products, is a widespread global issue. While the exact prevalence varies by region and crop, it's a significant concern for food safety and human health. Fumonisins, produced by certain Fusarium fungi, can cause various health problems, including cancer, and have been linked to diseases in animals (Gomes *et al*., 2024). The fifteen (15) different fumonisins that have so far reported, have been grouped into four main categories (A, B, C, and P). Of these groups, the most abundant is fumonisin B1 (FB1). However, the co-occurrence of Small amounts of other mycotoxins, mainly FB2 and FB3 were however, found to co-occur in FB1-containing samples. For some decades, FB1 has already been known as a harmful mycotoxin. (Mohammed *et al*., 2022) It can cause fatal diseases in animals and is classified as a potential human carcinogen (Awuchi *et al*., 2022; EFSA, 2018). As hypothesized by Wang *et al*. (2023), in their de novo sphingolipid biosynthesis in primary rat liver hepatocytes, the main mechanism behind the effects is the inhibition of the enzyme ceramide synthase, because of its clear structural similarity to the long-chain base backbones of sphingolipids. More so, an outbreak of neural tube defects (NTD (embryonic defects of the brain and spinal cord resulting from failure of the neural tube to close) in 1090 to 1091 along the Mexican-American border and certain regions in China and South Africa has also been hypothesized by epidemiologists to be due to the high levels of FB1 observed in corn consumed in those areas (Copp *et al*., 2013). Number of research groups have also reported histopathologic effects in rat livers associated with apoptosis, characterized by small, rounded eosinophilic hepatocytes and necrotic hepatocytes after both short-term and long-term treatments with FB1 (Kumari *et al*., 2024; Szabó *et al*., 2018).

FB1 has been detected in corn in all parts of the world. The concentrations recorded vary enormously, but levels up to 300 mg/kg have been observed in corn feed in Hungary and United States. It has been estimated that the mean daily intake of FB1 may range between 12 and 140 µg per person, depending on geographical factors. However, there are individuals who may consume doses as high as 2500 µg/day. In addition to corn, FB1 has also been detected in rice, sorghum, wheat bran, soybean meal, and poultry feed (Chen *et al*., 2021). Therefore, it will be of paramount importance to screen and identify fungal contaminants with fumonisin producing ability and further determine in quantitative terms, the amount of mycotoxin produced by the isolated species in a controlled environment. This is with a view to mount surveillance and device mitigation strategies.

Hibiscus sabdariffa, commonly known as Roselle, is an important plant cultivated for its economic and nutritional value in many tropical and subtropical regions, including Nigeria. The calyces of *Hibiscus sabdariffa* are widely used in the production of beverages, herbal teas, jellies, jams, and medicinal products due to their rich content of anthocyanins, antioxidants, vitamins, and minerals (Onyeukwu *et al*., 2023). Jigawa State, located in northern Nigeria, is a significant producer of *Hibiscus sabdariffa*, contributing to the livelihood of many farmers and serving as a valuable export commodity (Jekayinfa *et al*., 2020).

The contamination of food products by fungi and mycotoxins is a major global food safety issue, particularly in developing countries where environmental conditions and poor post-harvest management favor fungal growth. Previous studies have reported that *Hibiscus sabdariffa* and other agricultural products are susceptible to contamination by fungi such as Aspergillus, Penicillium, and Fusarium species (Adeyeye *et al*., 2022). These fungi are capable of producing dangerous mycotoxins, including aflatoxins, ochratoxins, and fumonisins, which pose serious health risks to consumers.

Due to the aforementioned scenario reported by Bhandare and Malode (2024), the safety and quality of *Hibiscus sabdariffa* are often compromised by fungal contamination. Fungi such as *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp are known to infect agricultural products, particularly under humid and poorly managed storage conditions (Enyiukwu *et al*., 2020). These fungi produce secondary metabolites called mycotoxins, which are toxic to humans and animals when consumed, even at low concentrations. Mycotoxins such as fumonisins, aflatoxins, and ochratoxins have been associated with various adverse health effects, including liver damage, kidney dysfunction, immunosuppression, and carcinogenicity (Awuchi et *al*., 2022).

Particularly in Jigawa State, despite the economic and nutritional importance of *Hibiscus sabdariffa* in Nigeria, there is limited research on the specific fungi and mycotoxins associated with its production. Given the climatic conditions and post-harvest handling challenges in Jigawa State, the risk of fungal contamination and mycotoxin production in *Hibiscus sabdariffa* is significantly high. The scanty studies and available records makes it challenging to assess the extent of contamination, enforce safety standards, and develop effective mitigation strategies. Given the reliance of local communities on *Hibiscus sabdariffa* for income and nutrition, the presence of mycotoxins if not studied and mitigation strategies devised, could have serious public health and economic consequences. Therefore, this study aims to identify the fusarium fungal species associated with *Hibiscus sabdariffa* cultivated in Jigawa State and determine the levels of mycotoxins (fumonisin) contaminating the product. The findings will provide critical insights into food safety concerns and help in formulating strategies to mitigate contamination risks. The study will also go a step further to fill the knowledge gap by identifying the fusarium fungal contaminants capable of fumonisin production and quantitatively determine mycotoxin (Fumonisin B1) thus produced in *Hibiscus sabdariffa* from Jigawa State which

will contribute to safeguarding public health and enhancing the quality of *Hibiscus sabdariffa* for local and international markets.

METHODOLOGY

Sampling of hibiscus sabdariffa

Samples of dried *Hibiscus sabdarifa* were sourced from nine (9) local government areas. Three (3) Local government areas (L.G.A.) were randomly selected from each of the three senatorial districts of Jigawa State (11º 47' 00''N, 4º 44' 00''E), The centre for the collection of the samples were: Gumel local government area (12° 37' 51" N, 9° 23' 36" E) from jigawa North-West senatorial district (sourced from:Babura, Roni and Maigatari L.G.As). Hadejia local government area (12° 26' 53" N, 10° 2' 37" E) from jigawa North-East senatorial district (sourced from: Hadejia, Kafin Hausa and Auyo L.G.As) and Birnin Kudu local government area (11° 27' 0" N, 9° 30' 0" E) from Jigawa South-West senatorial district (sourced from: Buji, Gwaram and Miga L.G.As).

Simple conventional identification of fungi species

The mycological analytical procedures (Kaufman *et al.,* 1963) was performed under aseptic condition. One gram of milled sample was weighed into a test tube and diluted in 9ml of sterile Ringer's solution, vortexed/shaken and serially diluted further to $10^{-6.}$ One ml from each test tube was cultured by pour plate technique on potato dextrose agar (PDA) and incubated for 4-5 days at 25ºC. Plates were counted for fungal colonies using a colony counter and the number of Fungal colonies per gram was calculated and expressed in colony forming units per gram of sample (CFU/g) was calculated in percentages viz:

$$\frac{CFU}{g} = \frac{\text{Number of colonies}}{\text{Plating volume (1ml)}} \times \text{Reciprocal of the dilution factor}$$

Morphological identification of fungal species

Isolated fungal colonies were further sub-cultured on PDA, Czapek yeast agar (CYA) and malt extract agar (MEA) according to Kaufman *et al.* (1963) under aseptic conditions and incubated at 25ºC for 7 days. Pure fungal colonies were harvested and stained with lactophenol in cotton blue and mounted on microscope slides for identification. The macro and microscopic identifications of *Fusarium* species was done following the identification keys of Pitt and Hocking, (1985) and Nelson *et al.* (1983) while Pitt and Hockings, (2022) keys was employed for other species (Table 1).

Screening of fusarium fungal isolates with fumonisin producing potentials

For the fusarium fungal isolates, test for their toxigenicity was carried out as described by Makun *et al.* (2011), without modification. Briefly, these isolates were cultured individually on solid yeast extract sucrose agar (YES) agar in a 90-mm Petri dish and incubated at 25ºC for 28 days according to the method of Singh *et al.* (2000). Mycotoxins (fumonisin) synthesized by each fungus were extracted by dissolving 5 g of isolate including the medium in 10 mL of dichloromethane (DCM). The crude extract obtained was filtered through a Whatman no. 2V filter paper and the filtrate was put in a screw cap vial and dried on a steam bath and stored at 4ºC until analyzed.

Fumonisin extraction and clean-up procedures for quantitative HPLC

Because fumonisins are only soluble in lower alcohols especially methanol and water, a different extraction method to that for the other mycotoxins was employed. This extraction of FBs and clean-up was done according to the method of Sydenham *et al.* (1992) without modification. Sub sample 25g of the pulverized *Hibiscus sabdariffa* (from which *F. Verticelloides* and *F. graminaerum* was isolated) was mixed with 50ml of methanol/water (3:1) in a volumetric flask and shaken for one hour and then filtered through Whatman No1 filter paper. The pH of the extract was adjusted to 6-6.5 using acetic acid (to enable the binding of the fumonisin on the SAX column). The SAX cartridge was first conditioned by washing with 5ml methanol (MeOH) and 5ml MeOH/H_2O (3:1v/v). The flow rate was maintained at 2ml/min as described by Sydenham *et al.* (1992). The column was then washed with 5ml of MeOH/H_2O (at 3:1v/v) and subsequently with 3ml MeOH. The fumonisin (FB_1) was finally eluted at the flow rate of 1ml/min with 10ml of 1% acetic acid in MeOH. The eluate was dried under the stream of nitrogen gas at 60ºC and stored at 4 – 8ºC until further analysis.

Fumonisin (mycotoxin) analysis

Residues for FB analysis were reconstituted in methanol and aliquots derivatized with o-phthaldialdehyde (OPA) prior to separation on a reversed-phase HPLC system using fluorescence detection at excitation and emission wavelengths of 335 and 440 nm respectively (Shephard *et al.*, 2000). The isocratic mobile phase made up of 0.1mol/L sodium dihydrogen phosphate/methanol (80:20) that had its pH adjusted to 3.5 using Acetic acid, was pumped at a rate of 1 ml/min.

RESULTS AND DISCUSSION

Triplicate readings of Fumonisin B1 concentration indicated that the three isolated *Fusarium verticelloides*

Table 1: Morphlogical Identification of fungal fusarium species based on the Macroscopic and Microscopic Features.

	MORPHOLOGICAL FEATURES					
Fungal species	**Macroscopic features**			**Microscopic features**		
	Colony diameters	**Colony colours**	**Colony Textures**	**Conidial heads**	**Stipes**	**Phialledes**
Fungal species	**FUSARIUM SPECIES**					
Fusarium graminearum	Colonies on petridish on PDA covers the whole dish	Rose to golden brown to yellowish brown colour.	Flocose mycelium. The reverse is ruby to dark ruby colour	Macronidia has about 5 septa, straight to moderately curved		Micronidia are absent
Fusarium verticelloides	Colonies on petridish on PDA covers the whole dish	Mycellium appeared felty and coloured to pale salmon	Reverse is deep plurry coloured	Macronidia (often rare) appeared relatively short and stout		Micronidia shows poorly defined annulations
Fusarium culmorum	Colonies covers the whole petridish	Mycelium was observed to be Pale yellow brown.	Mycelium was dense and floccose and the reverse was observed to be red to deep red	Macronidia were found to be relatively short and stout.		Micronidia not produced.

Table 2: Colony-forming unit (CFU/g) and fungal load from the sampled areas.

	RONI	**BABURA**	**MAIGATARI**	**K/HAUSA**	**HADEJIA**	**AUYO**	**BUJI**	**MIGA**	**GWARAM**
CFU/g ($x10^6$)	2.249	1.249	2.333	1.916	2.917	1.833	2.166	2.082	2.25
Fungal Load($x10^6$)	4.083	3	5.75	5.25	11.499	1.833	6.999	4.916	3.665

Table 3: Concentration of Fumonisin B1 (FB1) (µg/kg) extracted from *Fusarium verticelloides* Isolated from *Hibiscus sabdariffa* sampled in Jigawa State determined by HPLC/MS method.

Fumonisin B1 producing fungi	**Sampling area**	**Concentration of the mycotoxin (FumonisinB1) (µg/kg) 1st reading**	**Concentration of the mycotoxin (FumonisinB1) (µg/kg) 2nd reading**	**Concentration of the mycotoxin (FumonisinB1) (µg/kg) 3rd reading**	**Mean Conc. Of FB1 ± SEM (µg/kg)**
Fusarium verticelloides	Maigatari	1910.0	1450.8	1530.0	1630.27±21.76
Fusarium verticelloides	Auyo	2034.8	2924.0	1200.0	2052.93±24.34
Fusarium verticelloides	Roni	1760.6	1758.1	1620.0	1712.9±18.4

species from three local government areas of Auyo, Roni and Maigatari in Jigawa State are mycotoxigenic. As shown in (Tables 2-3) and chromatograms in (Figures 1A –C), Auyo being a wetland area and more humid, has the heighest concentration of the mycotoxin (FB1) at 2052.93±24.34 µg/kg while Maigatari has the lowest concentration of the toxin at 1630.27±21.76 µg/kg, reason being it is located in a more drier location and very much less humid.

Assessment of fusarium fungal contamination and by extension the mycotoxin (fumonisin B1) concentration in commodities such as *Hibiscus sabdariffa* widely consumed across all tribe and religious lines and an emerging cash crop of high economic value in Nigeria becomes imperative. The truth of this issue becomes even glaringly clear due the fact that number of research groups have reported histopathologic effects in rat livers after both short-term and long-term treatments with FB1. These effects include: Apoptosis, characterized by small, rounded eosinophilic hepatocytes, and also necrotic hepatocytes (Cao *et al*., 2022). Reduced liver weight, elevated serum alanine aminotransferase (Tomaszewska *et al*., 2022) and cytoplasmic vacuolation of adrenal cortex (Szabó *et al*., 2018) are typical signs of hepatotoxicity also observed in experimental rats administered different doses of fumonisin. In general, several reports and observations has it that: Female rats seem to be more sensitive/susceptible to the fumonisin toxicity than males and if this observation is extrapolated to humans, it is then an alarming reason for a serious concern. The colony forming unit (CFU/g) was determined and observation thus made (Table 1) clearly indicated high level of contamination by various species of fungi. This observation correlates with the previous findings by Garba *et al*. (2023) that reported high level of fungal contamination of agricultural products in the Sudan and Sahel savannah regions of Nigeria. Based on the studies reported by Oyedele *et al*. (2024); Mabekoje *et al*. (2023);Waller and Brayford (1990), fusarium species are field fungi that are mostly prevalent in the humid southern part of Nigeria while the tropical hinterland of the country is dominated by the Aspergillus species (Nji *et al*., 2023; Uzo and Currah, 20180). Therefore, identification of *Fusarium verticilloides* in samples from Maigatari and Roni

local government areas located in semi-arid zone of northen Jigawa State, is a testimony of climate change and the associated changes that comes along with it. Auyo local government area situated along the course of Hadejia-Jama'are River present a climatic condition that will support the prevalence of *Fusarium verticilloides* and so, identification of this fungal specie conform to the expectation. However, due to the constant movement/translocation of products through commercial activities, the problem of product contamination by the fungal metabolite (fumonisin) will unfortunately not be restricted to the area (Auyo) alone.

As could be observed from the result obtained in this study, triplicate readings of Fumonisin B1 (FB1) concentration indicated that the three isolated *Fusarium verticelloides* species from three local government areas of Maigatari, Auyo and Roni in Jigawa State are mycotoxigenic (Tables 2-3) and chromatograms 1A –C), the highest concentration of the mycotoxin (FB1) at 2052.93±24.34 µg/kg was observed in a sample from Auyo local government area, probably being a wetland area with high humidity, while FB1 at concentrations of 1712.9±18.4 and 1630.27±21.76 µg/kg were detected in Roni and Maigatari respectively. This observation (result) corroborates the findings of Garba *et al*. (2022), Vismer *et al*. (2015) that reported high concentrations of this mycotoxin from the Derived and Southern guinea savannah regions of Nigeria. However, findings from some African countries such as Ghana, Malawi, Zambia, and Kenya reported quite lesser concentrations of 11–1655, 20–115, 20–1420, and 110–120 µg/kg (Kpodo *et al*., 2000; Doko *et al*., 1996; Doko *et al*., 1995; Kedera *et al*., 1999). While South Africa and Zimbabwe reported higher ranges of 1000–30,000 and 2500–6000 µg/kg (Gamanya, and Sibanda, 2001; Mashinini and Dutton, 2006) respectively compared to the report obtained in this our study. This high concentration of FB1 in the hibiscus sample from Nigeria is quite a bad omen for the country as far as the public health and competitiveness of the product in the international market space is concern. Reason for the aforementioned assertion being that this toxins (FBs) are linked with several health issues like cancer of the esophagus as evident from different regions of the world. (Xue *et al*., 2019). The molecular basis of the toxicities attributed to FB1 is due to the fact that it consists of a diester with propane-1,2,3-tricarboxylic acid (TCA) and 2-amino-12,16-dimethyl-3,5,10,14,15
pentahydroxyleicosane where hydroxyl (OH-) groups at the C-14 and C-15 positions involved with the carboxyl groups (-COOH) of TCA to form an ester that ultimately inhibits ceramide synthesis (El-Sayed *et al*., 2022; Shephard, 1998). More so, FB1 is implicated with the incidences of hepatocarcinoma, stimulation and suppression of the immune system, defects in the neural-tube, nephrotoxicity, as well as other ailments (Madhu *et al*., 2019). It is prominent as a promoter of hepatocarcinoma (Gelderblom *et al*., 1988) where its synergistic interactions with aflatoxin B1 (AFB1) has been exhibited in animal models for two stages, i.e., initiation and promotion of cancer (Xue *et al*., 2018). FB1 toxicity has also been associated with marked immunological effects. The effects of FB1 on the cytokine profile of different organs and different cell types have been described in findings reported by (Chen *et al*., 2021). An increased expression of tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) has been detected in mouse liver and kidney and also in primary cultures of hepatic cells after exposure to FB1 (Gamiel, 2024). In addition, report by (Sharma *et al*., 2003) indicated that FB1 can increase the expression of interferon-γ (IFN-γ), IL-1α, IL-6, IL-10, IL-12, and IL-18 in mouse liver,and in human dendritic cells, FB1 has also been shown to increase the expression of IFN-γ and the related chemokine CXCL9 (Pourmasumi *et al*., 2024). Both international and regional regulatory bodies such as the United Nations' Codex alimentarius, world health organization (WHO) and the European food safety agency (EFSA) has set regulatory limits for mycotoxins in agricultural commodities/products traded at the international market or destined for export into their countries (EU). For instance, while the codex alimentarius recommended 4000 µg/kg and 1000 µg/kg of FB1 for both unprocessed and processed agricultural products, the WHO has in its regulation approved 1000 µg/kg and 2 µg/kg for the same commodities. The most stringent regulation comes from the EFSA with *tolerable* daily intake (TDI) for *fumonisin*B1 (FB1) at 1.0 µg/kg body weight (Gilbert *et al*., 2020). The member countries of EFSA are always the preferred destination for the Nigerian hibiscus because of its high market value. If at this point the concentration of the FB1 obtained in this study is compared to the maximum tolerable limits set by these regulatory bodies, it becomes a real cause for concern for Nigeria as far as international trade is concern. This becomes apparent as this commodities that will be presented by Nigeria at the foreign markets might face outright rejection that will result in the fall of foreign earnings and poor balance of trade and hence low GDP for the country. Moreover, such rejection may certainly results in glut of the product in the domestic market and subsequent fall in the income of farmers that have been hitherto leveraging on the appreciable market value of the commodity (hibiscus). This unfortunate scenario if not mitigated might lead to increased poverty among the farmers and other players along the hibiscus value chain in Nigeria.

Conflict of interest

The authors declared that there is no conflict of interest.

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