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Investigation of Fungal Contamination of Different Cigarette Tobacco Brands Consumed In Lapai and Minna Metropolis of Niger State Nigeria

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ABSTRACT
Tobacco is among the world's crop with annual worrisome mycotoxins burden. This research aim at determining the presence and load of associated fungi with their mycotoxins in brands of cigarette tobacco commonly consumed in Lapai and Minna metropolis of Niger State Nigeria. Thirty-six (36) brands of cigarette tobacco were purchased in open markets in Minna and Lapai towns of Niger state, Nigeria. Using standard procedures, they were cultured on potato dextrose agar (PDA) plates and observed for 72 hours for fungal growth. Pure fungal colonies were morphologically identified, fermented at 35°C for 21 days to extract their secondary metabolites. The acute toxicity profile of the secondary metabolites elicited by the isolated fungi were determined using the Lorke's procedure at a dose of 3000 mg/kg body weight. Results showed a total of one hundred and fifty-eight (158) fungal contamination of the cigarette brands comprising Apergillus niger, Aspergillus fumigatus, Aspergillus nidulans, Chrysonillia sitophilia and Aspergillus flavus with a percentage contamination of 77.8, 97.2, 8.3, 16.7 and 44.4 respectively. All isolated fungi elicited Aflatoxin B_1 and Ochratoxin A mycotoxins production except for A. fumigatus and A. nidulans that produced only the Ochratoxin A mycotoxins with a contamination load less than equal to 25 µg/kg. No mortality was reported for the isolated fungi except for A. nidulans and A. fumigatus were extreme and moderate toxicity was reported at 3 g/kg p.o after 24 hours and no sign of delayed toxicity or mortality after 14 days of observation. The study concludes that cigarettes sold and consumed in Lapai and Minna towns in Niger State have high incidence of fungal contamination with some eliciting AFB_1 and OTA under favorable environmental conditions.

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INTRODUCTION

Tobacco is one among the 25% of world crops affected by mycotoxins every year (FAO, 1997). Diverse microorganisms community ranging from bacteria (both Gram positive and negative), bacterial

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spores and toxins (endotoxins and exotoxins), molds, yeasts, as well as fungal spores and their secondary metabolites such as aflatoxin B_1 are involved in the production process of cigarette from tobacco, with the spores of such fungi capable of surviving the entire manufacturing process and the level of these toxins production influenced by the substrate on which the fungi grow as well as the moisture level, temperature, and presence of competitive micro-flora (Musa and Gbodi, 2020; Verweij *et al.,* 2012).

Mycotoxins are naturally occurring toxins produced by certain fungi found on different agricultural crops like cereals, coffee, nuts, dried fruits, and spices among others capable of extensive proliferation under favourable environmental conditions. They are low molecular weight toxic metabolites produced by species of molds belonging to various genera ranging from *Aspergillus, Penicillium, Fusarium* and *Byssochlamys* capable of causing serious health risks to animals and human upon acute and chronic exposures (Azizi *et al*., 2012; Makun *et al*., 2009). Among the mycotoxins, aflatoxins a group of highly toxic secondary metabolic products namely aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2) and recently aflatoxin M_1 (AFM_1) and aflatoxin M_2 (AFM_2) are being elicited by these fungal mycotoxins. While these aflatoxins are produced by certain *Aspergillus spp* namely *A. flavus* (producing AFB_1 and AFB_2) and *Aspergillus parasiticus* (producing AFB_1, AFB_2, AFG_1 and AFG_2), *Aspergillus alliaceus*, *Aspergillus niger, Aspergillus carbonarius*, *Aspergillus auricomus*, *Aspergillus glaucus*, and *Aspergillus melleus* have been reported to be Ochratoxin producers (Abarca *et al.*, 1994; Bayman *et al.,* 2002). Ochratoxin A, a nephrotoxin to virtually all animal species studied to date have a longer elimination half-life than any of the most other species. It is an immune suppressant, a toxin to the liver, a carcinogen, as well as a potent teratogen (Beardall *et al*., 1996). Judging from the carcinogenic, mutagenic, teratogenic and immunosuppressive effects of aflatoxins coupled with the speculative nature of Ochratoxin A as an immune suppressant, hepatic and nephrotic poison, carcinogen, a potent teratogen, this research was thus conducted to determine the extent of fungal contamination of cigarette tobacco largely consumed in Minna and Lapai towns of Niger State, Nigeria.

MATERIALS AND METHODS

Sample Collection

Thirty six (36) different cigarette brands were procured from randomized cigarette wholesale and retail outlets in Lapai and Minna towns of Niger State

Experimental Animals

Matured Wistar rats of either sex weighing (100 - 200 g) were obtained. They were housed in ventilated cages and maintained under standard nutritional and environmental conditions of normal relative humidity, room temperature, and 12 hour light and 12 hour dark cycle. The animals were fed with standard food pellets and clean water *ad libitum* for 14 days. All experimental protocol is in accordance with the standard ethical guidelines for laboratory animal use and care.

Preparation of Potatoes Dextrose Agar (PDA) Nutrient

Five hundred grams of thinly sliced peeled white potatoes were heated with 1000 mL of distilled water at about 60^{o}C for 1 hr before been filtered hot through muslin cloth. 0.50 g of chloramphenicol, 20 g of glucose and 15 g of agar-agar were accurately weighed and added to the filtered potato broth before been made up to 1000 mL with distilled water. The mixture was shaken to homogeneity and then

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sterilized in an autoclave using standard procedure. Sterile pipette was used to transfer 20 mL cooled PDA broth into sterile Petri dishes before being allowed to cool and gel (Musa and Gbodi, 2020).

Inoculation of Sample

Three cigarette sticks of each cigarette brands were emptied into a well-labelled sterile PDA plates. The plates were well sealed and left to stand at room temperature. The plates were observed for 48 hours for fungal growth. Non-inoculated PDA plates serves as the negative control. The procedure were prepared in triplicate for all brands (Musa and Gbodi, 2020).

Isolation and Identification of Pure Fungal Cultures

Distinct fungal colonies were subcultured into freshly prepared PDA plates. The procedure were repeated until pure colonies were obtained. Standard microscopic and morphological identification were performed (Musa and Gbodi, 2020).

Fermentation of Pure Fungal Cultures

Distilled water (100 mL) was added to 250 g of sterilized local rice in a 1000ml conical flask before been allowed to stand for 24 hours at room temperature for moisture equilibration. Pure fungal isolates were inoculated onto the appropriately labelled sterile rice medium. The inoculated rice medium were then maintained in a Grieve laboratory oven LW 201C at about 35^{o}C for 21 days (Musa and Gbodi, 2020).

Extraction of Secondary Metabolites

After 21 days of fermentation, 750 mL of methylene chloride was added to the conical flask and allowed to stand for 1 hour before been pulverized using a Christison commercial blender. The pulverized rice culture was then poured into an Erlenmeyer flask fitted to another Erlenmyer flask and then speedevac 2 suction pump through a Buchner funnel fitted with Whatman paper No 4. The resulting filtrate was subjected to Soxhlet extraction for the complete evaporation of the methylene chloride. The resulting residue was mixed with petroleum ether in the ratio 1:15, refrigerated for 24 hours, with the resulting petroleum ether filtered off using Whatman No 4 filter paper. The filter paper containing its precipitate was maintained at 40^{o}C until complete dryness in a Grieve laboratory oven LW 201C. The dried extract was used for toxicity screening (Musa and Gbodi, 2020).

Mycotoxins Extraction

The crude extracts elaborated by the fungi cultured in rice media were screened and analyzed for aflatoxin B_1 (AFB_1) and Ochratoxin A (OTA). Fifty gram (50 g) of the pulverized metabolites were weighed into separate 500 mL Erlenmeyer flask followed by the addition of 25 mL of 1M phosphoric acid and 250 mL of dichloromethane. The flasks were shaken for 30 minutes using a mechanical shaker with its content filtered under pressure using a Buchner funnel fitted with an 18 cm circle rapid filter paper. 200 mL of filtrate was collected with 100 mL aliquot each placed in a separate 250 mL Erlenmeyer flask with glass stoppers, for AFB_1 and OTA assay (Makun *et al*., 2009).

Mycotoxins Identification and Analysis Using Thin Layer Chromatography (TLC)

The standards for AFB_1 and OTA identification were obtained from Food Toxicology and Contaminants Laboratory, Centre for Applied Science and Technology Research (CASTER) IBBU Lapai. The TLC plates were developed in petroleum ether-methanol-water (96:3:1) and were estimated by visual comparison of fluorescence intensity of samples with that of standards. AFB_1 was confirmed by

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spraying the thin layer chromatographic plates with aqueous sulphuric acid (50:50, v/v), dried and viewed under long wave and the spots fluoresced blue (AOAC, 1980). For OTA identification, 1M phosphoric acid and dichloromethane were used in place of 4M phosphoric acid and chloroform respectively. The intensities of the standards and test samples were compared visually. To confirm the presence of OTA, the TLC plates were sprayed with alcoholic aluminum chloride (20 g in 100 mL alcohol), exposed to ammonia vapor before being viewed under long wave. The change in fluorescence from blue-green to bright blue confirms OTA spots (Makun *et al*., 2007).

Animal Grouping and Toxicity Testing

Eighteen animals in regardless of sex were randomly divided into 5 groups with 3 animals per group. While group 1 (the control) received 1 mL cold press olive oil, groups 2, 3, 4, 5 and 6 intra-peritoneally received 3000 mg/kg body weight extract produced from *Aspergillus flavus*, *Aspergillus nidulans*, *Chrysonillia sitophilia*, *Aspergillus niger* and *Aspergillus fumigatus* respectively. General symptoms of toxicity and mortality in each group were observed within 24 h. Animals that survived after 24 hours were observed for another 14 days (no extract administration) for any sign of delayed toxicity (Lorke, 1983).

RESULTS AND DISCUSSSION

The frequency and distribution of the fungi contamination from the analyzed cigarette brands are presented in Figure 1 and Table 1 respectively.

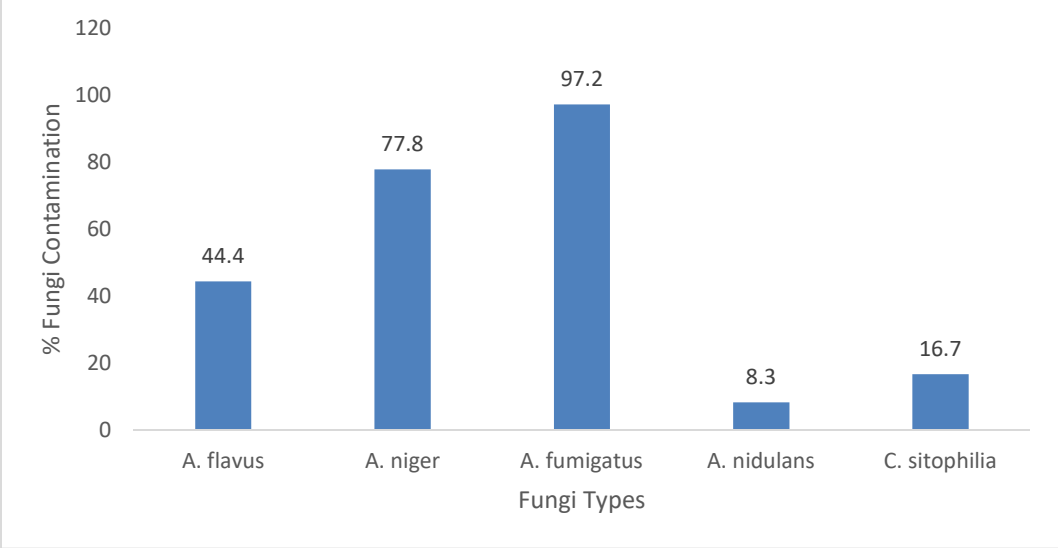

Fig.1: Frequency of fungi contamination of all cigarette samples.

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Table 1: Fungi types in all cigarette samples.

Sample	Plate I Isolate I	Plate I Isolate II	Plate I Isolate III	Plate II Isolate I	Plate II Isolate II	Plate II Isolate III	Plate III Isolate I	Plate III Isolate II	Plate III Isolate III
CG1	*A. niger*	-	-	*A. niger*	*A. fumigatus*	-	*A. fumigatus*	*A. flavus*	-
CG2	*A. niger*	*A. fumigatus*	-	*A. niger*	-	-	*A. fumigatus*	*A. flavus*	-
CG3	*A. fumigatus*	-	-	*A. niger*	*A. flavus*	-	*A. niger*	-	-
CG4	-	-	-	*A. niger*	*A. fumigatus*	*A. flavus*	*A. niger*	-	-
CG5	*A. niger*	-	-	*A. fumigatus*	*A. flavus*	-	*A. niger*	*A. fumigatus*	-
CG6	*A. niger*	*A. fumigatus*	-	*A. niger*	*A. fumigatus*	*A. flavus*	*A. niger*	*A. fumigatus*	*A. flavus*
CG7	*A. fumigatus*	-	-	*A. niger*	*A. fumigatus*	-	*A. niger*	*A. fumigatus*	-
CG8	*A. niger*	*A. fumigatus*	-	*A. niger*	*A. fumigatus*	*A. flavus*	*A. niger*	*A. fumigatus*	*A. flavus*
CG9	*A. niger*	-	-	*A. fumigatus*	-	-	*A. niger*	-	-
CG10	*A. niger*	*A. fumigatus*	*A. flavus*	*A. niger*	*A. fumigatus*	*A. flavus*	*A. fumigatus*	*A. flavus*	-
CG11	*A. niger*	*A. fumigatus*	*A. flavus*	*A. fumigatus*	-	-	*A. niger*	*A. fumigatus*	*A. flavus*
CG12	*A. niger*	*A. fumigatus*	*A. flavus*	*A. niger*	*A. fumigatus*	*A. flavus*	*A. fumigatus*	-	-
CG13	*A. fumigatus*	-	-	*A. fumigatus*	-	-	*A. fumigatus*	-	-
CG14	*A. fumigatus*	-	-	*A. fumigatus*	-	-	*A. niger*	-	-
CG15	*A. fumigatus*	*A. flavus*	-	*A. niger*	-	-	*A. fumigatus*	*A. flavus*	--
CG16	*A.fumigatus*	-	-	*A. niger*	-	-	*A. fumigatus*	-	-
CG17	*A. niger*	*A. flavus*	*A. nidulans*	*C. sitophilia*	-	-	*A. niger*	-	-
CG18	*A. fumigatus*	*A. nidulans*	-	*A. niger*	*A. fumigatus*	*A. flavus*	*A. fumigatus*	-	-

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CG19	-	-	-	*A fumigatus*	-	-	*A. fumigatus*	-	-
CG20	*A. niger*	*A. fumigatus*	-	*A. fumigatus*	-	-	*A. niger*	*A. fumigatus*	-
CG21	*A. niger*	-	-	*A. fumigatus*	-	-	-	-	-
CG22	-	-	-	*A. niger*	*A. fumigatus*	-	*C. sitophilia*	-	-
CG23	*A. niger*	*A. fumigatus*	-	*C. sitophilia*	-	-	-	-	-
CG24	*A. fumigatus*	-	-	*A. fumigatus*	-	-	*C. sitophilia*	-	-
CG25	*A. fumigatus*	-	-	-	-	-	-	-	-
CG26	*A. fumigatus*	-	-	*A. niger*	*A. fumigatus*	-	*A. fumigatus*	-	-
CG27	*A. niger*	-	-	*A. fumigatus*	-	-	*A. fumigaus*	-	-
CG28	*A. fumigatus*	-	-	*A. niger*	-	-	*A. niger*	*A. fumigatus*	-
CG29	*A. niger*	*A. fumigatuus*	-	*A. niger*	*A. fumigatus*	*A. flavus*	*A. niger*	-	-
CG30	*A. niger*	*A. fumigatus*	*A.flavus*	*A.niger*	*A. fumigatus*	*A. flavus*	*A. fumigatus*	-	-
CG31	-	-	-	*A. fumigatus*	-	-	-	-	-
CG32	*A. niger*	*A. fumigatus*	-	*A. niger*	*A. fumigatus*	-	*A. niger*	-	-
CG33	*C. sitophilia*	-	-	*A. fumigatus*	-	-	*C. sitophilia*	-	-
CG34	*A. fumigatus*	*A. flavus*	-	*A. niger*	-	-	*A. niger*	*A. nidulans*	-
CG35	*A. niger*	-	-	*A. niger*	*A. flavus*	-	*A. niger*	*A. fumigatus*	*A. flavus*
CG36	*C. sitophilia*	-	-	*A. fumigatus*	-	-	*A. fumigatus*	-	-

Key: CG = Cigarette; (-) = no fungi detected

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This finding is in agreement with the report of Papavassiliou *et al*., 1971 who reports hundreds of fungi strains prominent among them were *Aspergillus* strains. *Aspergillus fumigatus* has the highest incidence of contamination similar to the findings of Moularat *et al*., 2008 and Krause *et al*., 2003 who reported the fungal as the prominent microorganism from 14 brands of cigarettes analyzed. Table 2 showed the aflatoxin B_1 and Ochratoxin A concentrations produced by the fermented fungi.

Table 2: TLC analysis of AFB_1 and OTA

Fungi	Concentration (µg/kg)	
	Aflatoxin B_1	**Ochratoxin A**
A. niger	10	25
A. fumigatus	-	25
A. flavus	25	< 25
A. nidulans	-	< 25
C. sitophilia	< 10	< 25

Key: (-) = Not detected; < = less than

The fungi were all able to produce both AFB_1 and OTA mycotoxins except *A. fumigatus* and *A. nidulans* who were not able to elicit AFB_1 mycotoxin production. This is the first time the fungal *Aspergillus niger*, *Aspergillus flavus,* and *Crysonillia sitophilia* has been reported to elicit mycotoxins production. It is known that not all strains of fungus produces toxins. It is reported that not all fungal strains produce mycotoxins as they may be subdued by nature since fungi have been known to mutate and thus, lose the ability to elicit toxins (Kendrick, 1992).

Toxicity Screening

The toxicity profile of the secondary metabolites of the fungi is presented in Table 3.

Table 3: Toxicity profile of fungal secondary metabolite

Group/Extract Administration	Mortality Ratio	% Mortality	Remark
1 (Olive oil control)	0	0	Not lethal
2 (*A. flavus*)	0	0	Not lethal
3 (*A. nidulans*)	2/3	66.67	Extremely Toxic
4 (*C. sitophilia*)	0	0	Not lethal
5 (*A. niger*)	0	0	Not lethal
6 (*A. fumigatus*)	1/3	33.33	Moderately toxic

The administered extract of the secondary metabolite of the fungal did not result in mortality over the 24-hour period as well as no delayed toxicity was observed in the animals after 14 days at the administered dose of 3000 mg/kg body weight except for *A. nidulans* and *A. fumigatus* were extreme and moderate toxicity was reported. These findings agree with the report of *Gbodi et al*., 1991 who reported that *A. quadrilineatus* and *A. nidulans* at 2560 mg/kg body weight exhibited a 100% mortality.

CONCLUSION

The presence of the isolated fungi and its associated mycotoxins confirms that cigarettes poses a significant health risk to

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its consumers although no definite inference from this study could be drawn of their role as etiological agents in human health problem.

REFERENCES

Abarca, M. L., Bragulat, M. R., Sastella, G. and Cabanes, F. J. (1994). Ochratoxin A production by strains of *Aspergillus niger* var. niger. *Appl. Environ. Microbiol*. 60**:** 2650-2652.

Association of Official Analytical Chemists AOAC (1980). Method of analysis of Aflatoxins, Ochratoxin A, Zearalenone, Vomitoxin and Secalonic acid.

Azizi, I. G., Ghadi, H. and Azarmi, M. (2012). Determination of Aflatoxin B_1 levels of feedstuffs in traditional and semi-industrial cattle farms in Amol, Northern Iran. *Asian Journal of Animal and Veterinary Advances*. 7: 528 – 534.

Bayman, P.J. Baker, L. Doster, M. A. Michailides, T. J. and Mahoney. N. E. (2002). Ochratoxin production by the *Aspergillus ochraceus* group and *Aspergillus alliaceus*. *Appl. Environ. Microbiol*. 68**:** 2326 – 2329.

Beardall, J. M. and Miller, J. D. (1996). Disease in humans with mycotoxins as possible causes. pp. 487-539.

Food and Agricultural Organization. (1997). Food and Agricultural Organization of the United Nations food and nutrition paper 64. Worldwide regulations for mycotoxins. A compendium. Food and Agricultural Organization, Rome, Italy.

Gbodi, T. A., Nwude, N., Aliu, Y. O., Ikediobi, C. O. and Cheneme, C. O. (1991). Acute toxicity of crude extracts of *Aspergillus guadrilineatus* isolated from acha (*Digitaria exilisstapf*). *Vet. Hum. Toxicol*. 33: 27 - 31.

Kendrick, B. (1992). Mycotoxin in food and feed in the fifth kingdom. 5th Edn. Mycologue Publications Waterloo Ontario. pp. 316 - 333.

Krause, J. D., Hammad, Y. Y. and Ball L.B. (2003). Application of a fluorometric method for the detection of mold in indoor environments. *Applied Occupational and Environmental Hygiene*, 18: 499 - 503.

Lorke D. (1983). A new approach to practical acute toxicity testing. Archive of Toxicol. 4: 275 - 287.

Makun, H.A., Gbodi, T.A., Akanya, H.O., Salako, E.A. and Ogbadu G.A. (2007). Fungi and some mycotoxins contaminating rice (*Oryza sativa*) in Niger State, Nigeria. *African Journal of Biotechnology*. 6: 211 – 217.

Makun, H.A., Gbodi, T.A., Akanya, O.H., Salako, E.A. and Ogbadu G. A. (2009). Fungi and some mycotoxins found in mouldy sorghum in Niger State, Nigeria. *World Journal of Agricultural Sciences*. 5 (1): 05-17.

Moularat, S., Robine, E., Ramalho, O. and Oturan M.A. (2008). Detection of fungal development in closed spaces through the determination of specific chemical targets. *Chemosphere*. 72: 224-234.

Musa, D.A. and Gbodi, T.A. (2020). Mycotoxin associated risks of cigarette tobacco sold in Nigeria. Lambart Academic Publishing, Beau Bassin 71504, Muritius. pp. 31 – 35.

Papavassiliou, G., Piperakis, J. and Marcelou-Kinti, U. (1971). "Mycological flora of cigarettes" *Mycopathology Mycology Applied*. 44(2): 117–120.

Verweij, J. J., Kerremans, A., Voss, P. and Meis, J. F. G. M. (2012). "Fungal contamination of tobacco and marijuana," *Journal of the American Medical Association*. 284(22): 2875 – 2881.

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