IDENTIFICATION AND CHARACTERIZATION OF MULTI-MYCOTOXINS RECOVERED FROM DRY CORN IN SELECTED MARKETS IN ENUGU METROPOLIS, ENUGU STATE, NIGERIA.

Abstract

Corn is an important food crop that forms a major meal plan in Nigeria. Its high nutritious value also makes it perfect for fungi to thrive and produce its metabolites. Mycotoxin is a fungi metabolite that is toxic to both humans and animals. This study aimed at isolation of fungi from corn vended in selected markets in Enugu metropolis, identification and characterization of mycotoxin produced from the isolates. A total of 9 corn samples were collected from 3 different markets in Enugu metropolis comprising of Emene ,Ogbete and New markets respectively . The samples were cultured on potatoes dextrose agar for isolation, isolates were identified and characterized by cultural, biochemical and microscopic analysis of the organisms; selected isolates were further characterized by polymerase chain reaction (PCR). The isolates were subjected for mycotoxin production by culturing in potatoe dextrose broth for 14 days at 35°C after which the filtrates were assayed for the presence of mycotoxin using gas chromatography-mass spectroscopy (GC-MS) and individual aflatoxins were further characterized using high performance liquid chromatography (HPLC). From the study, *Fusarium* and *Aspergillus flavus* occurred at 66% respectively, *Aspergillus niger* at 55%, *Mucorspp* at 77%, *Penicilliumspp* at 33%, and *Aspergillus fumigates* at 44%. PCR amplified products of the selected isolates showed positive amplification at 650bp for internal transcribed spacer gene. Mycotoxins identified from isolates were aflatotoxin, ochratoxin A (OTA), citrinin, patulin, citreoviridin, T-2 toxin, deoxynivalenol (DON), diacetoxyscirpenol, zearalenone (ZEN), nivalenol, Ht-2 toxin, cyclopiazonic acid with ochratoxin A, Diacetoxyscirpenol and Ht-2 toxin being above consumption limit at 2.64ug/kg,1.27ug/kg and 424.48ug/kg respectively. The isolates showed varied concentration of individual aflatoxins comprising of B1, B2, G1 and G2. These aflatoxins were produced by *Aspergillus flavus* strain ZMXL11, *Aspergillus flavus* strain AF-13, and *Penicillium citrinin* PEN-65 among, B1 was found to be above the maximum limit at 99% in all the isolates.Aflatoxin has been found as to be a group 1 carcinogen implicated in liver cancer. Constant consumption of mycotoxin contaminated corn could lead to varying health risk that includes liver cancer, kidney and gastrointestinal disturbances. This study showed that corn sold in selected markets in Enugu metropolis were contaminated with mycotoxigenic fungi that produced high levels of mycotoxin concentrations which is of public health concern.

Key words. Mycotoxin, Aflatoxins, Gas chromatography-mass spectroscopy, High performance liquid chromatography

1. INTRODUCTION

"Corn is an important food crop in Nigeria. Its high nutritious value also makes it perfect for fungi to thrive and produce mycotoxin. Mycotoxin is a secondary metabolite produced by some filamentous fungi including *Aspergillus spp., Penicilliumspp, Fusariumspp,*and*Alternariaspp* among others. These fungi are ubiquitous in nature and capable of attacking corn and other crops both on the field, during harvest and in storage". (68). "They thrive within a range of environmental factors that include a temperature range between 25 and 30°C, relative humidity between 88% and 95%, a water activity higher than 0.78, and an acidic pH range. The World Health Organization estimates that over 500 million of the poorest are exposed to unsafe mycotoxin levels, most of who live in sub-Saharan Africa. Starting in childhood, chronic exposure leads to increased mortality and morbidity. Currently, more than 300 mycotoxins are known and possess wide variations in fungal origin, structure, function, and biological effect, but only a few of them appear to have a significant effect on health and agriculture" (1). . "Mycotoxins greatly resist decomposition or being broken down in digestion, so they remain in the food chain even after heat treatment, such as cooking and freezing" (62). "The most common mycotoxins are aflatoxins, ochratoxin A, fumonisins, deoxynivalenol, T-2 toxin, and zearalenone . Of these aflatoxin poses more toxicological importance. Aflatoxins frequently contaminate corn and sorghum. Aflatoxin exposure results in acute toxicosis mainly in children, a substantial cancer burden and is associated with child stunting. Aflatoxin is potently immunosuppressive" (70,71,72). "The fatalities from consuming highly contaminated corn have been recorded since the late 1960s until recently in Eastern Africa" (73,72). "To put this into perspective, globally, acute aflatoxin exposure may cause hundreds of deaths while chronic exposure causes nearly a-third of all of the liver cancer cases in Africa" (69). "In some parts of Africa, zearalenone contamination may be a concern" (74). "The feeding of highly contaminated corn affects animal health and productivity, thus lowering the supply of protein" (72). The health impact of exposure is grossly underreported due to lack of coordinated monitoring and medical surveillance. Mycotoxins are neglected as major public health problems and their control is inadequately funded and not prioritized by many African governments. This work aims to isolate mycotoxin-producing fungi from corn sold in selected markets in Enugu Metropolis, identify and characterize the mycotoxin present. This study will help determine the safety of corn grains sold in the markets.

2. MATERIALS AND METHODS

2.1 Materials/Equipment:

They include , Autoclave, petri dishes, microscope, bijou bottles, dried corn grains, Electric hot plate, UV-Vis spectrophotometer, electrothermal drying oven, Weighting balance, plastic bucket/Crucible/Measuring cylinder, sieve cloth/Beakers/Conical flask, Whatman's filter paper/Spatula, distilled water/Cotton wool.

2.1.1 Chemicals/Reagents:

They includes , Lactophenol cotton blue, , Ammonium Hydroxide Solution, analytical ethanol, analytical diethyl ether, analytical N-Butanol, sodium chloride 5% solution, phenol reagent, 3,5-dinitrosalicylic acid, potassium acetate, aluminum chloride.

2.1.2 Media

Media used includes PDA (potato dextrose agar), SDA (sabroaud dextrose agar), and PDB (potato dextrose broth)

2.2. Sample Collection: Dried corn grains were randomly purchased from three (3) different markets located in Enugu metropolis, Nigeria comprising of Emene, Ogboete and New Market respectively. Three samples were purchased from each of the markets. The samples were kept in a sterile polyethylene bags and transported to the Applied Microbiology and Brewery laboratory, Enugu State Science and Technology for analyses.

2.3. Determination of Physicochemical Properties of Corn Samples:

2.3.1. Temperature: The temperature of the maize where it was stored was determined at the study site *(in situ)* by inserting the thermometer into the silos bag; this was left for 15seconds before reading was taken.

2.3.2. Maize pH: The pH of the maize sample was determined using pH meter in accordance with method of Cheesbrough, (2012). A total of 20g of the maize sample was added into a 100ml beaker and mixed with 20ml of distilled water. The solution was mixed uniformly by stirring for 30 minutes using a stirrer. The mixture was allowed to stand for 1hr and the pH meter was placed in maize samples for readings to be taken.

2.3.3. Moisture content: A total of 1g of the sample was weighed and placed into a crucible. Similarly, the crucible and the sample were weighed before being put into the oven for drying. After that, the sample was placed in the oven at 105°C for 1hr. Once removed, it was cooled in a desiccator, and the weight was noted. The sample was further dried for 30 minutes until a constant weight was achieved. The percentage of moisture content loss was calculated using the formula: % moisture content loss = (W1 – W2) / WT × 100/1, where W1 represents the initial weight, W2 represents the weight after drying, and WT represents the total weight.

2.4. Isolation of fungal species: A total of 10g of the corn samples obtained from each of the markets were weighed aseptically and macerated in 90ml distilled water and agitated for 20mins. From this, subsequent tenfold dilution was made up to 10-4. One milliliter (1ml) of each dilution was dispensed in sterile potato dextrose agar plates by pour plate method. They were gently rotated to ensure even dispersion, allowed to solidify and were incubated at 37ºC for 3days.

2.5. Identification of Fungal Species: Identification of fungal isolates was done according to the manuals of Oyeleke and Manga (2012). The fungal isolates were identified using cultural and morphological features such as colony growth pattern, conidial morphology, and pigmentation. After isolation, fungal isolates were sub-cultured on the SDA slants. Isolates obtained were viewed with microscope using x40 lens to observe morphology of mycelium and spore structures (57)

2.5.1. Molecular Identification: Fungal isolates were further characterized by PCR

2.6. Mycotoxin Production: Potato dextrose broth (PDB) was used for production of mycotoxin. PDB was prepared routinely and sterilized. A total of (25 ml) of broth was dispensed into sterile conical flasks and autoclaved for 1 hr, allowed to cool o 40^0C. The flasks were inoculated respectively with a loopful of the test fungi. The inoculated flasks were incubated on a shaker at 30^0C for 12 days. The isolates were filtered with a sterile whatman filter paper and filtrates obtained for furtheranalysis.. (Sign *et al, 2022*).

3. RESULT

Physioco-Chemical Properties of Corn Samples from the Study Areas. :
The physicochemical properties of the corn samples varied with moisture content all within the normal range at 14.0-15.4, 55.5% of samples have higher temperature of 34-36.2^{o}C; while 44.4% were within normal temperature range at 30.2-32^{o}C. pH values were above normal range with 6.89-7.2 pH values (Table 1).

Table 1: Physioco-Chemical Properties of Corn Samples from the Study Areas.

Sample	MC (%) NR- 14-16	inference	Temperature (°C) NR-24 -32°C	Inference	pH NR-(5.8-6.2)	Inference
Emene market						
Sample 1	15.4	Within	36.2	above	7.2	slightly alkaline
Sample 2	16.5	within	36	above	7.2	slightly alkaline
Sample 3	15.4	within	34	above	7.2	slightly alkaline
Ogboete market						
Sample 4	14.9	within	34	above	6.87	Slightly neutral
Sample 5	14.3	within	32	within	6.87	Slightly neutral
Sample 6	14.0	within	35.1	**above**	**6.87**	Slightly neutral
New market						
Sample 7	15.45	within	30	within	6.89	Slightly neutral
Sample 8	14.25	within	32	within	6.89	Slightly neutral
Sample 9	14.0	within	30.2	within	6.89	Slightly neutral

Key:

NR- Normal Range , MC- Moisture content

Cultural and Microscopic Characteristics of Isolates :

All the isolates were septate hyphea but differed in their cultural characteristics and microscopy (Table 2)

Table 2: Cultural and Microscopic Characteristics of Isolates

Sample id	Appearance	Microscopic appearance	Probable Organism
1	Fluffy white hyphe	Septate hyphae	*Fusarium verticillioides*
2	Dry blue surface with a red pigment	Septate hyphae	*Aspergillus flavus*
3	Dry dark blue surface with a white pigment	Septate hyphae	*Aspergillus niger*
4	Black cottony with a pale yellow pigment	Septate hyphae	*Mucor spp*.
5	Dusty Green appearance	Septate hyphae	*Penicillum*spp
6	Grayish surface with white background	Septate hyphae	*Aspergillus fumigatus*

Occurrence and Distribution of the Isolates from the Study Areas:

Mucor spp. was higher in occurrence at 77%. Other isolates showed varied occurrence rates.(Table 3)

Table 3: Occurrence and Distribution of the Isolates from the Study Areas

Isolates	**Sample (Total number of sample)** (%)			
	Emene market (n=3)	**Ogbete market (n=3)**	**New market (n=3)**	**Total no n=9**
Fusarium verticillioides	3 (100)	2 (67)	1 (33)	6 (66)
Aspergillus flavus	3 (100)	2 (67)	1 (33)	6(66)

Aspergillus niger	1(33)	2 (67)	2 (67)	5 (55)
Mucor spp.	2 (67)	3 (100)	2 (67)	7 (77)
*Penicillum*spp	1(11)	2 (67)	0 (0)	3(33)
Aspergillus fumigates	2 (67)	1 (33)	1 (33)	4(44)

Key:

n- Number of sample

Molecular identification of Isolates

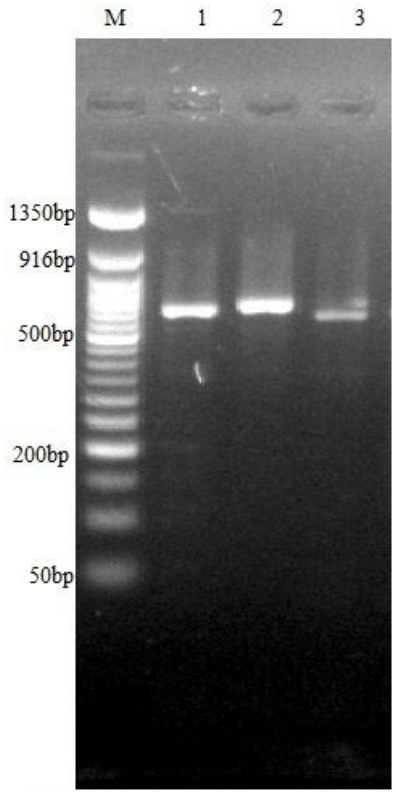

Fig. 1: Agarose gel electrophoresis of PCR amplified products of *Aspergillus flavus* strain ZMXL11, *Aspergillus flavus* strain Af-13 and *Penicilliumcitrinum* strain PEN 65. Lane M shows 50bp DNA ladder; lanes 1, 2 and 3 showed positive amplification for 650bp for ITS gene

Table 4: Molecular Characterization of Isolates

Source	**Isolates**	**Strain**	**% Identity**	**Accension Number**
New Market	*Aspergillus flavus*	**ZMXL11**	**96.7**	**MT446180.1**
Ogboete Market	*Aspergillus flavus*	**AF13**	**94.27**	**CPO59864.1**
Emene Market	*Penicilliumcitrinum*	**PEN-65**	**83.69**	**MK089541.1**

Identification and Characterization of mycotoxins using GC-MS

A total of 12 different mycotoxins were extracted from *Aspergillus flavus*zmxl, *Aspergillus flavus* AF13, and *Penicilliumcitrinum*Pen-13 respectively at different peak levels.(table 5)

Table 5:Characterization of Mycotoxin Using GC-MS

Mycotoxins	M.W (ug/kg)	Peak Area *Aspergillus flavus* strain ZMXL1	RT (min)	Peak Area *Aspergillus flavus* strain AF13	RT (min)	Peak Area ***Penicillium citrinin* PEN-65**	RT (min)	ML set by EC	Inference
Aflatoxin	312.279	1.47	8.752	0.19	8.875	0.01	15.691	20	within
Cyclopiazonic acid	336.384	1.21	10.384	0.02	10.352	0.02	10.218	NA	Na
Ochratoxin A	403.81	2.64 +	10.384	0.08	10.518	0.03	10.619	0.2-5	above
Citrinin	250.25	1.39	10.891	0.13	10.852	BDL	10975	19-1000	within
Patulin	154.12	2.42	12.250	0.11	12.309	BDL	12.308	10-50	within
Citreoviridin	402.484	0.13	12.749	0.02	12.648	BDL	12.829	NA	NA
T-2 Toxin	466.527	1.46	13.462	BDL	13.363	BDL	13.382	50	within
Deoxynivalenol	296.315	0.31	13.913	BDL	13.890	BDL	13.764	1750.0	within

Diacetoxyscirpenol	366.410	1.27 +	14.382	0.01	14.417	0.01	14.219	1	above
Zearalenone	318.364	1.18	14.675	0.03	14.849	BDL	14.638	350	Within
Nivalemol	15.392	312.318	15.392	0.02	15.691	BDL	15.947	NA	NA
HT-2 Toxin	15.859	424.48	15.859	0.11	15.691	0.07	15.813	50	above

Keys

NA: Not available

ML: maximum level

M.W: molecular weight

BDL – Below detectable level

EC- European commision

Types of Aflatoxin produced from the isolates using HPLC:

From the study**,**G2, G1, B1 and B2 were the different types of aflatoxins identified from the isolates . The aflatoxins were all above acceptable limit. (Table 6).

Table 6: Different Aflatoxins produced Isolates Using HPLC.

Types of Aflatoxin	Amount Ug/kg *Aspergillus flavus* strain ZMXL11	Peak Area *Aspergillus flavus* strain ZMXL11	RT (min)	Amount Ug/kg *Aspergillus flavus* strain AF13	Peak Area *Aspergillus flavus* strain AF13	RT (min)	Amount Ug/kg ***Penicillium citrinin* PEN-65**	Peak Area ***Penicillium citrinin* PEN-65**	RT (min)	ML set by EC	Inference
G2	9.44867	7.40769	10.856	8.39882	32.11761	10.846	1.20733	38.55759	10.855	10-20	within limit
G1	3.13936	13.44311	12.997	2.633992	72.57869	12.992	3.62588	57.65141	12.998	10-20	within limit
B2	12.3687	108.41510	16.049	11.33630	121.8996	16.039	16.71232	146.40175	16.047	2-20	within limit
B1	1416.65520	257.79791	18.066	1357.00155	246.94095	18.055	1684.49624	306.54498	18.064	2-20	Above limit

total	1429.33882	387.06381	1368.60208	473.53620	1701.57236	549.57236

ML-maximum level

EC – European commission

RT- retention time

Common mycotoxins Identified, their classifications and Potential Health Risks.

Aflatoxin identified are in group 1 of IRAC classification with ochratoxin A in 2 B .

Table 7. Groups of Mycotoxin According to IARC (International Agency for Research on Cancer) Identified and Characterized and Potential Health Risks.

Types of Aflatoxin	**IARC Grouping**	**Potential Health Risk**	**Target Organ**
Aflatoxin G2	Group 1	Hepatotoxic and immune-suppressive	Liver
Aflatoxin G1	Group 1	Liver and Kidney disorders	liver
Aflatoxin B2	Group 1	Hepatocellular carcinoma	liver
Aflatoxin B1	Group 1	Hepatotoxic and immune-suppressive	liver
HT-2 Toxin	Not classified		
Cyclopiazonic acid	Not classified	Immunotoxic and hepatotoxic	Muscle, hepatic tissue and spleen
Ochratoxin A	Group 2B	Carcinogenic, teratogenic, Immuno-suppressive, nephrotoxic and causing upper urinary tract disease	Kidney, liver
Citrinin	Group 3	Nephrotoxic	Kidney
Patulin	Group 3	lesions in the duodenum,	GIT

		hemorrhages in the intestines, and gastric ulcers, and also alters intestinal function	
Citreoviridin	Not classied	Teratogenic and immunotoxic	Not specific
T-2 Toxin	Not classified	Hepatotoxic, genotoxic and immune-suppressive	GIT, Immune system
Deoxynivalenol	Group 3	Nausea, vomiting, diarrhea, reproductive effects and toxicosis	Reproductive organs, GI
Diacetoxyscirpenol	Not classiffied		
Zearalenone	Group 3	Carcinogenic, hormonal imbalance and reproductive effects	Reproductive organs, GI
Nivalemol	Group 3	Anorexic, immunotoxic, haematotoxic and genotoxic	GIT, immune system

4. Percentage Production of Aflatoxin from Isolates

From the study ,Aspergillus flavus strain ZMXL11, produced the highest aflatoxin B1 and B2 at 67% and 28% respectively (fig. 2,3,4).

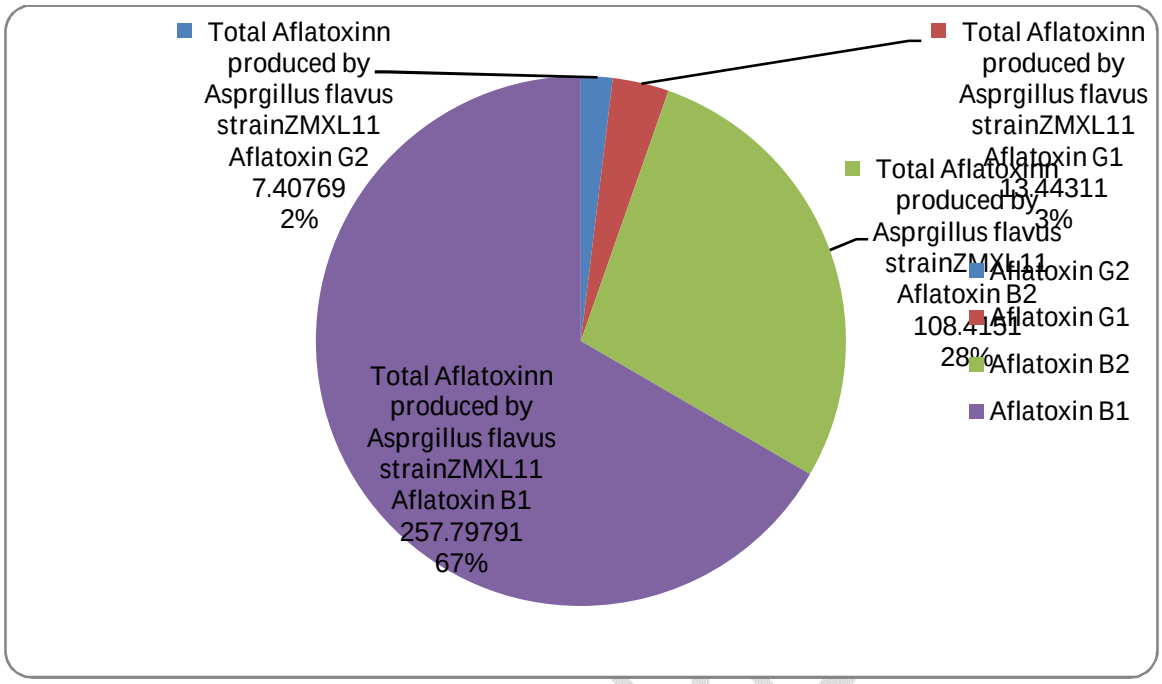

Fig. 2 Total Aflatoxin Produced by *Aspergilusflavus* strain ZMXL11

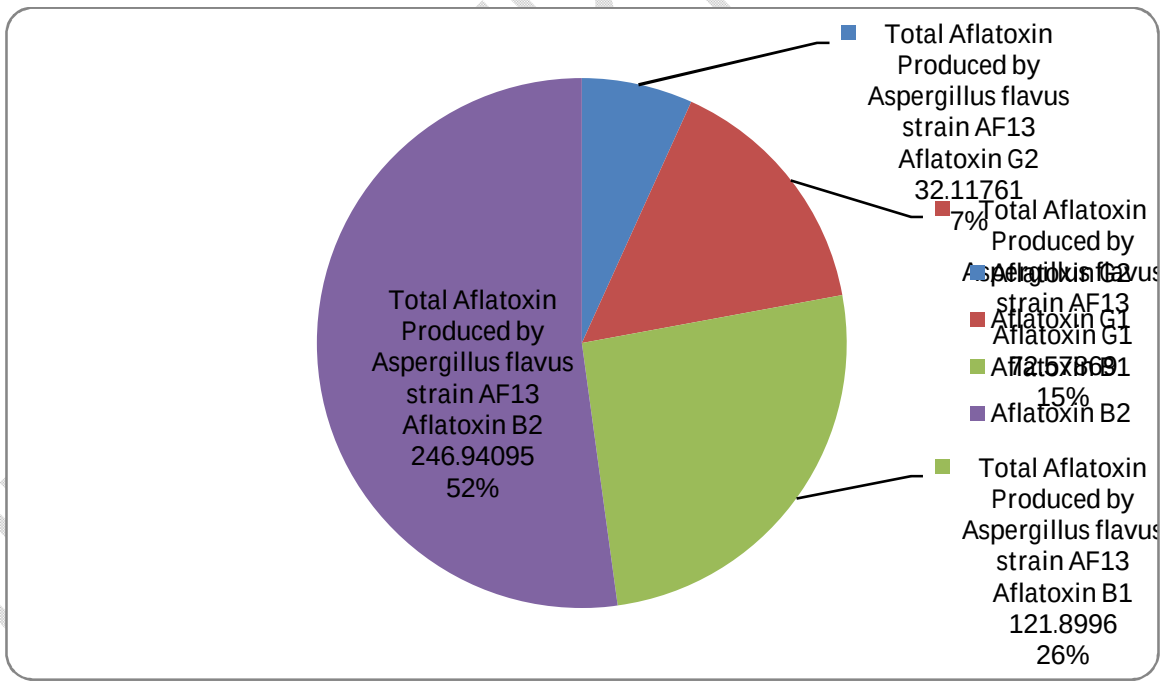

Fig. 3 Total Aflatoxin Produced by *Aspergilusflavus* strain AF 13

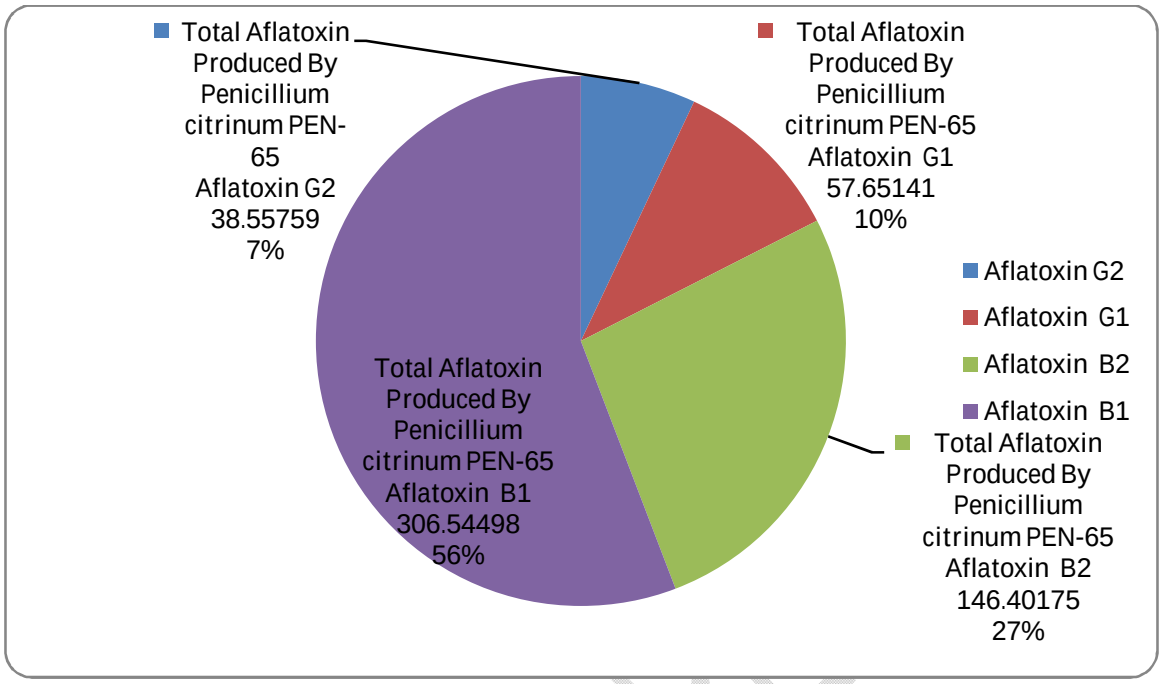

Fig. 4 Total Aflatoxin Produced by *Penicilliumcitrinum* strain PEN-65

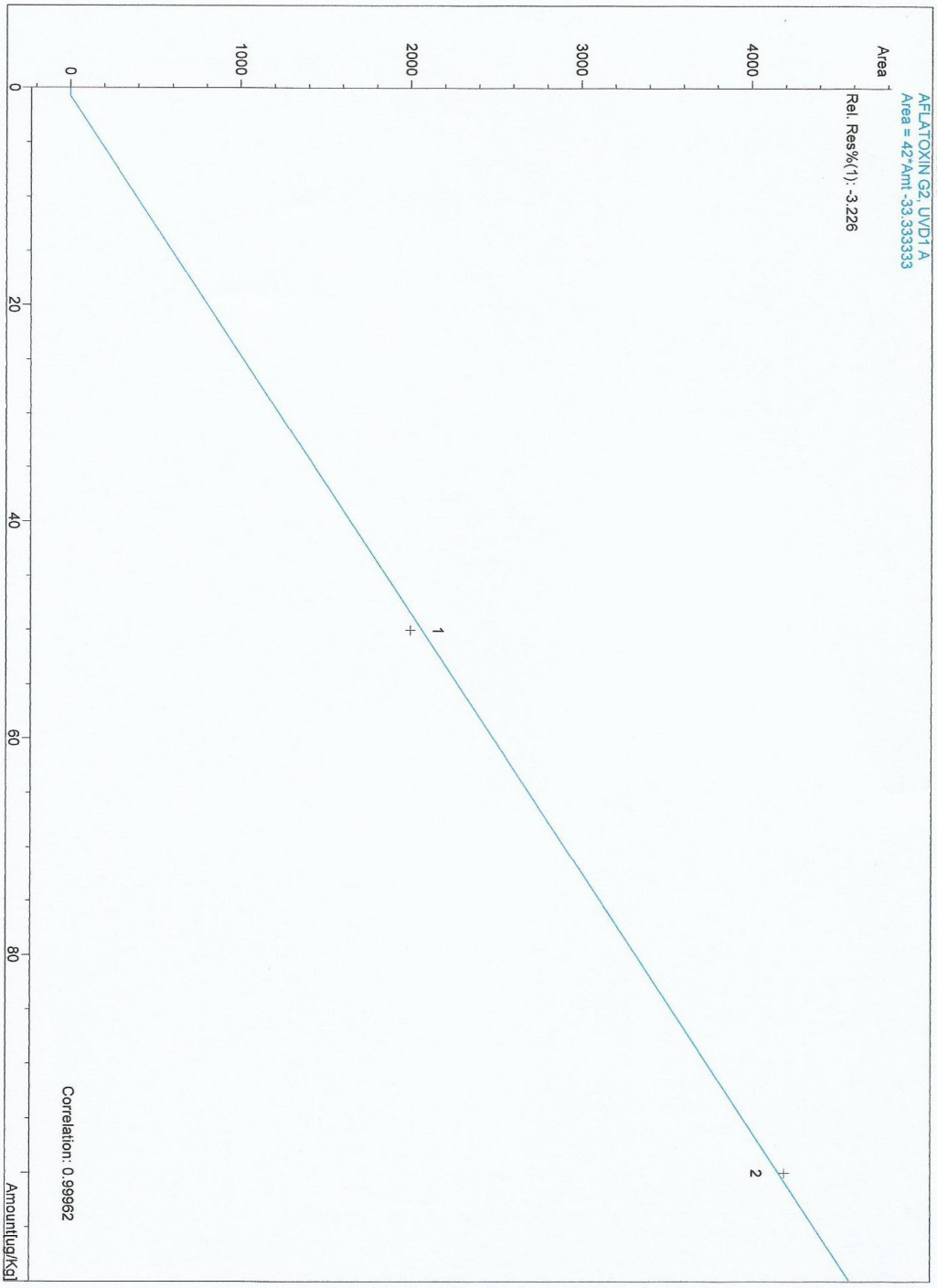

Fig 5: Calibration curve for Aflatoxin G2

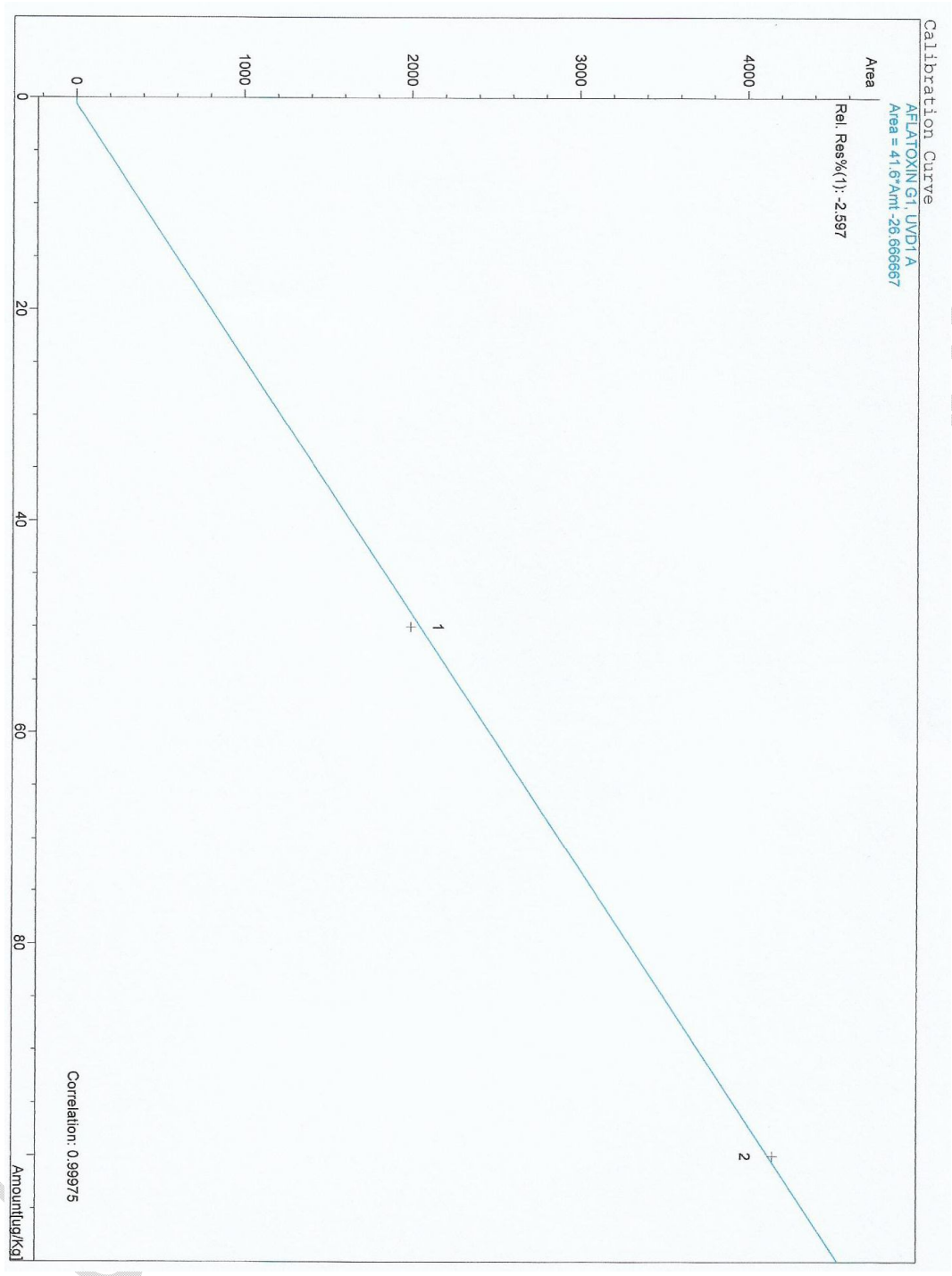

Fig 6" Calibration curve for G1

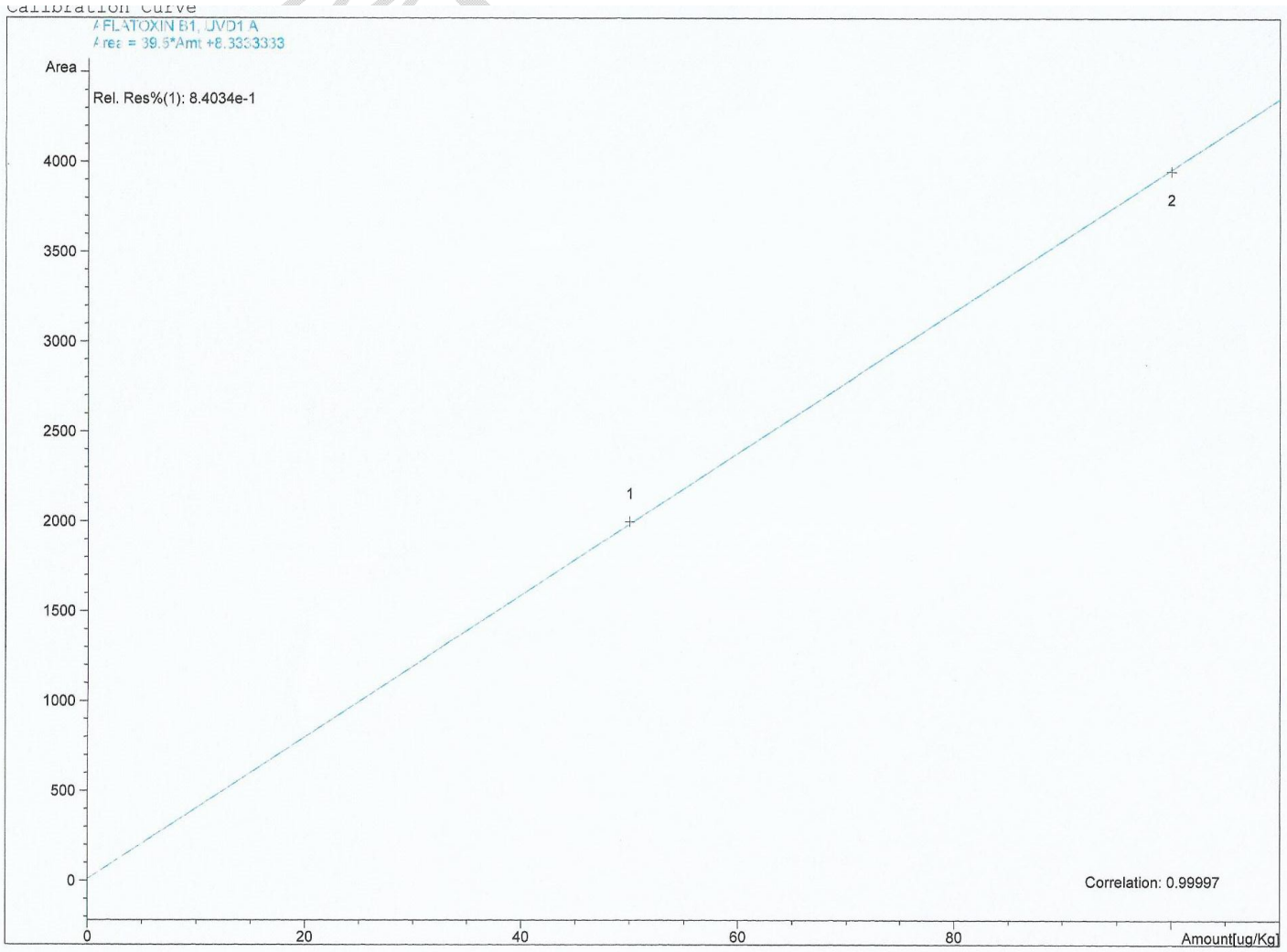

Fig 7 : Calibration Curve for Aflatoxin B1

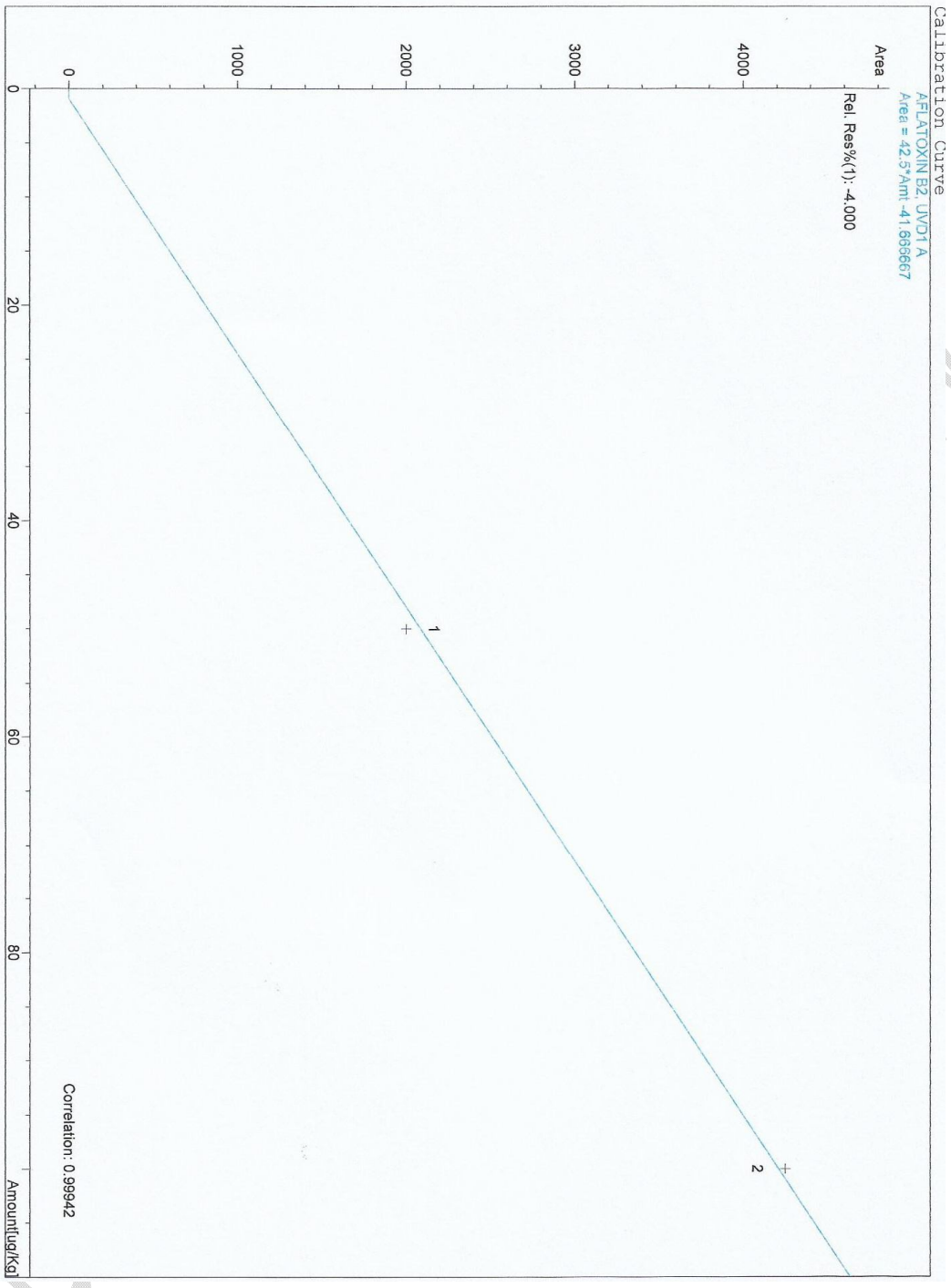

Fig 8. Calibration Curve for Aflatoxin B2

5. DISCUSSION

Mycotoxins are toxic secondary metabolite produced by fungi and are capable of causing disease and death in both humans and other animals. Mycotoxins are naturally occurring toxins produced by certain moulds (fungi) and can be found in food. The mould grows on a variety of different crops and foodstuffs including cereals, nuts, spices, dried fruits, apples and coffee beans, often under warm and humid conditions. Corn is a staple cereal which forms a major part of the Nigerian menu. It is cultivated by local farmers within the country but the larger quantities are gotten from the northern part of Nigeria, from where they are transported to various markets for sale. We investigated mycotoxin contamination from corn sold in selected markets in Enugu metropolis.Environmental factors play a key role in determining fungal occurrence (57) so the activity of the fungi and their level of colonization are much determined by predominant environmental conditions most importantly humidity and temperature specially on the field (41) . "Those factors, according to (15) influence the development, survival, distribution, and frequency of mycotoxigenic fungi and their subsequent toxin accumulation. Temperature and humidity also affect plant growth, strength, and health and influence the competitiveness of mycotoxigenic fungi" (51). Each fungi have an optimal temperature and moisture content, water activity range for growth, germination, and mycotoxin production. Therefore, no single range of temperature and water activity can be defined as inducing to fungal activity.

From the study, the physicochemical parameters for moisture content, temperature, pH values varied. The moisture content of the samples were within the acceptable limit at 14-16.5% while some of the samples showed higher results of temperature and pH (Table 1). Moisture content of corn ranged from 13% to 16% with production of high level of aflatoxin .(5) on his study on effect of Moisture Contents on mycotoxin level got similar result with high level of aflatoxin produced at high moisture content.

Temperature is another environmental factor that affects fungi growth and mycotoxin contamination. From the study, samples showed varied temperature of 32°C to 35°C. This is in line with the works of (53) who found similar results in his study, where he observed fungi growth at 30°C to 35°C. Duarte *et al.*, 2012 found optimal growth for fungi at 25-30°C. Our studies noted that the samples had a temperature range that supports fungi growth and especially mycotoxigenic fungi with optimal growth range of 30°C to 35°C. Mobley (1980) in his study found out that *Aspergillus* produced maximum amounts of aflatoxin Bi at 32°C and 20% moisture content (MC) and no aflatoxin Bi at 20°C and 16% MC and 20°C and 20% MC. Other researchers have reported maximum production at temperatures of 30°C (11), 35°C (11), 28-30°C and 30-35C (12). Optimum toxin production occurred in the range of 24-32C and 20-28% MC. (12) also agree that the optimum temperature range for maximum aflatoxin production is 30°C -35°

Fungi generally grows well in acidic conditions (13). But some species favor neutral to slightly alkaline conditions. From the study, the pH values ranged from 6.87-7.2 (Table 1). The obtained results were not in line tothe study carried out by (65), who observed that the optimum pH of fungi spp. growth was 5-6. According to Dix and Webster 1995, the pH values for mycotoxin production were indicated at 4-5.0, and does not agree with the present study where pH was indicated at 6.87 – 7.2.
"From the current study, the fungal isolates were firstly identified to a genus level using a morphological examination depending on the colors of colony formed at sides, the top and reverse of the fungal cultures. The microscopic examination of the shape of the spore-producing structures was used for further identification. Isolates were septate with varied morphological characteristics. Mixed genera was observed in the study, they included*mucorspp.*,*Fusarium spp.* ,*Penicilliumspp* and *Aspergillus spp.* The morphological examination and identification of fungi are useful for the identification of isolates up to the family or genus level" (64). However, this identification is not enough to identify the isolated fungi up to the species level (Lutzoni et al., 2004).thus selected isolates were subjected to further characterization with PCR.
Percentage occurrence showed that *mucor spp*. was higher in occurrence at 77%. Other isolates showed varied occurrence rates. According to the work of Chandrashekar*et al*. 2014, ten species belonging to three genera (*Aspergillus*, *Penicillium* and *Mucor*) were found, which is similar with the present study with mixed genera ocuuring simultaneously in one sample.

From the study, the molecular identification with PCR showed three species of selected organisms that includes , *Aspergillus flavus*strain ZMXL11, *Aspergillus flavus AF-13* and *Penicilliumcitrinum* strain PEN 65.Agarose gel electrophoresis of PCR amplified products of *Aspergillus flavus* strain ZMXL11, *Aspergillus flavus* strain Af-13 and *Penicilliumcitrinum* strain PEN 65. Lane M shows 50bp DNA ladder; lanes 1, 2 and 3 showed positive amplification for 650bp for ITS gene.(fig.1). according to Anderson and Parkin, 2007 , "ITS rDNA region sequence is one of the most important tools for the identification of the fungal species isolated from environmental sources hence, it has been widely used to detect the fungal community, and as an improvement of the classical identifications".

From the study, the biodiversity of the isolated fungal species is clearly observed among the different locations. The distribution and abundance of the fungi differ from one isolation location to another. Some species such as the *A. flavus* were isolated and identified from three locations, whereas *P. spp.* was isolated only from two locations. The biodiversity of fungi refers to the physiochemical properties of the environment such as; PH of the sample, temperature, and humidity while taking into consideration that all locations belong to the tropical environment.

From the study, a varied number of mycotoxin were detected from the isolates using Gas chromatography-mass spectrometry (GC-MS). They comprise of aflatoxin, cyclopiazonic acid, ochratoxin A, citrinin, patulin citreoviridin, T-2 Toxin, deoxynivalenol, diacetoxyscirpenol, zearalenone, nivalemol and HT-2 Toxin

Aspergillus flavus ZMXL1, ochratoxin A was highest in concentration at (2.64) , patulin (2.42mm),aflatoxin (1.47) T-2 toxin (1.46), citrinin(1.39), diacetoxyscirpenol(1.27), cyclopiazonic acid (1.21), zearalenone(1.18mm), HT-2 toxin (0.54mm), nivalenol (0.42), deoxynivalenol (0.31) and citreoviridin (0.13). With a molecular weight range of 154 ug/kg to 466ug/kg for the lowest and highest levels.

Aspergillus flavus AF-13 produced fewer mycotoxins ,comprising of aflatoxin, cyclopiazonic acid, ochratoxin A, citrinin, patulincitreoviridin,diacetoxyscirpenol, zearalenone, nivalemol and HT-2 Toxinwithaflatoxins having the highest peak at 0.19

Penicillium citrinin PEN-65 recorded the lowest number of mycotoxin comprising of aflatoxin, cyclopiazonic acid, ochratoxin A, diacetoxyscirpenol and HT-2 Toxin with HT-2 Toxinfound to be responsible for the peak area at (0.07) , followed by ochratoxin A (0.03) ,cyclopiazonic acid (0.02), aflatoxin and diacetoxyscirpenol(0.01). citrinin, patulin, zearalenone, nivalenol, citreoviridin, T-2 toxin and deoxynivalenol concentration were below detection limit.(table 5)

From the study, aflatoxin analysed comprised of B1, B2, G1 and G2. All individual aflatoxins recorded unacceptable consumption limits for AFB1 at 257ppb, 246ppb and 306ppb from*A.flavus* strain ZMXL11, *A flavus* strain AF13 and *P. citinum* respectively. (Table 6). All the sample had individual aflatoxin above consumption limit.The current result agrees with the study done by Victor et al, 2022, who found aflatoxins to be above regulatory limit in his study . Victor et al in their study also noted the presence of multiple mycotoxin which agrees with the current study where 12 different mycotoxin where identified from a single organism.

It is very worrisome that all the corn samples were contaminated with varying levels of different mycotoxins including the group 1 and group 2 mycotoxins which are proven carcinogens (Table 7). With Nigeria not having regulations for most of the toxins detected , this is a very serious public health concern as this suggest that even though most of the mycotoxins are within international acceptable limits , yet continous exposure could lead to potential toxicity, since accordingKemboi et al., 2020; Mwihia et al., 2020; Njobeh et al., 2010 ,when food is contaminated with multiple mycotoxins, even in relatively low concentrations, their potential additive effects becomes a great public health concern. From the study AFB1 is present in very high concentration in the all the samples tested. It is important to note that all doses of aflatoxin have cumulative effect on the risk of cancer Williams (2004) Aflatoxins have been reported to be responsible for 4.6% to 28.2% of cases of hepatocellular carcinoma globally (Liu and Felicia,(2010) Therefore, the presence of aflatoxins in corn has the potential to cause toxicity particularly in chronic consumers. Also, since susceptibility to aflatoxin toxicity is highest in the young, children must not be fed with corn or its derivatives that is contaminated with aflatoxin.

"From the study, ochratoxin A is also found to be above consumption limit. Ochratoxins A role and risk assessment in animal and human disease has been reviewed. The estimated tolerable dosage in humans was estimated at 0.2 to 4.2 ng/kg body weight based upon national toxicology program (NTP) study in rats. Ochratoxins A is mutagenic, immunosuppressive and teratogenic in several species of animals. Its target organs are the kidneys (nephropathy) and the developing nervous system" (38,37). "Recently, it has been suggested that Ochratoxins A can cause testicular cancer in humans" (54). "The hypothesis that consumption of foods contaminated with OTA causes testicular cancer was tested" by Schwartz (2002). Schwartz concluded: "Thus, Ochratoxins A is a biologically plausible cause of testicular cancer. Future epidemiologic studies of testicular cancer should focus on the consumption of OTA-containing foods such as cereals, pork products, milk and coffee by mothers and their male children." Comparing this with the present study, Ochratoxin A was present at 2.64ug/kg from *Aspergillus flavus* ZMXL1 , this is already a potential danger as it is above the acceptable consumption level, it is true that we are yet to confirm Ochratoxin A effects ,but above literature suggest that OTA has potential harmful effects on human health and its consumption should be controlled.

Patulin is a secondary metabolite of certain *Penicillium, Aspergillus*, and *Byssochlamys* species and a common contaminant of apples and apple products but commodities such as grains and other fruits and vegetables are also susceptible although to a lower extent. Even though the contamination incidence is high, it is fortunate that the levels are usually low. Patulin has been a reason for concern due to its carcinogenic properties; however, the International Agency for Research on Cancer (IARC) was unable to establish the severity of its effects on humans and as a consequence no classification exists at present. Regulation levels set in several countries vary from 30 to 50 µg/kg. The EC proposed limits of 50 p.p.b. in unprocessed grain, in the present study wedetected traces of patulin contamination but they were insignificance to cause any problem to human within the enugu metropolis.

"Zearalenone (ZEN) represents xenoestrogens having a chemical structure analogous to natural estrogens that permits its binding with estrogenic receptor sites leading to amplified estrogenicity. Exposure to this contaminant is accompanied by reduced levels of progesterone and serum testosterone in the bloodstream resulting in infertility and reduced incidences of pregnancy in animals like cows, pigs and rats"Yang*et al,* (2007). "It has also been shown to exert immunotoxic effects at low concentration levels. ZEN toxicity brings about numerous changes in the target cells by altering various metabolic events such as cell proliferation and apoptosis" Taranu *et al* ,(2015). "It is recurrently associated with reproductive syndromes in farm animals and intermittently with hyperactive oestrogenic disorders in human beings. ZEN has been categorized as a Group 3 carcinogen by the International Agency for Research on Cancer (IARC) due to its unclassifiable carcinogenicity to humans with inadequate evidence" (Rai *et al*,2019) . "However, owing to its continual incidence and extensive damage to both human and animal health, there is a need to

adopt effective management strategies to control ZEN toxicity" (Fruhauf*et al*., 2019). "ZEN is found to cause contamination at different stages of food chain leading to adverse health effects in both human and animal" Romero *et al*.,(2016). ZEN is commonly found in animal feeds and grains stored improperly Li N*et al*.,(2020) which makes them undesirable for consumption Eskola *et al* (2018) . "Moreover, as processing methods are unable to completely degrade the toxin, therefore, a tolerable daily intake for human adults of 0.25 µg/kg by weight has been recommended by the European Food Safety Authority for ZEN. Further to minimize the health risk, the European Union has specified limits for ZEN in food products. For instance, the maximum permissible limit of ZEN in unprocessed grains is 100–200 µg/kg while for processed grains; the limit has been reduced to 75 µg/kg , EFSA Panel on Contaminants in the Food Chain" (2011). from the current study , ZEN was found to have a concentration of 1.18 which does not pose much concern to public health in Enugu metropolis.
"Nivalenol / Deoxynivalenol are often found to co-contaminate foods, such as cereals, legumes, and their processed products Ok H.E" et al (2011). "At a cellular level, DON inhibits ribosome binding and protein synthesis, which disrupts intracellular signaling and, ultimately, leads to cell death McCormick"., (2003). The acute toxic effects of DON include vomiting, diarrhea, and loss of appetite. Chronic exposure to DON may cause anorexia, delayed growth, immunotoxicity, and reproductive toxicityPestka (2010) NIV is reported to be more toxic than DON, although it differs from DON only by an additional hydroxyl group Cheat., (2015) . To avoid the potential health risks after consuming DON, NIV, and their glucoside conjugates, legislation concerning tolerable daily intake (TDI) amounts of these toxins has been published. The Joint FAO/WHO Expert Committee in Food Additives (JECFA) established a maximum TDI of 1 µg kg^{-1} b.w. day^{-1} for the sum of DON, 3-ADON, and 15-ADON .For NIV, a TDI of 0.4 µg kg^{-1} b.w. day^{-1} was established by the Food Safety Commission of Japan (FSCJ).DON/NIV are frequent contaminats in maize , comparing our rwsults to the one from above, we realized that this mycotoxin is often accompanied by other contaminants like zearalone, T2 Toxin and Ht2-Toxin. DON was found to be slightly above limit at 1.27ug/kg . this is worrisome as Nigeria is yet to establish a consumption limit for both DON and NIV. People could possibly be exposed to the potential danger of toxicity of DON/NIV without proper guidelines.
"Mycotoxins are a public health concern, mainly due to their multiple types and prevalence that can lead to adverse effects due to chronic exposure even when contaminating food at low levels. If ingested, mycotoxins can cause episodes of acute or chronic diseases, such as various types of cancer, food poisoning, liver disease, various hemorrhagic syndromes, immune and neurological disorders in humans"Capriotti*et al*., (2012). In addition, mycotoxin contamination of food has been linked to cytotoxicity or even genotoxicityAlshannaq A, Yu J-H (2017) which can also induce toxic effects on the liver and kidneys, immune reproduction and fetal toxicity, and teratogenicity and carcinogenicity Chen and Wu (2017).. "Moreover, exposure to a mycotoxin diet has been associated with an increased incidence of esophageal and gastric carcinomas in certain regions" of China Hsia et al., (2004)

"In recent years, large-scale poisoning incidents and international trade disputes caused by fungal contamination are extremely common. For example, severe outbreaks of aflatoxinosis have been reported in Kenya, India, and Malaysia, killing hundreds of people. In the United States, mycotoxin corn infection is a chronic economic and health problem. The European Union's food and feed rapid alert system has placed mycotoxins in second place based on the total number of hazard notifications"Yang et al (2020). This suggest that Nigeria especially Enugu metropolis is already in danger bracket of mycotoxin toxicology especially with Aflatoxin which has a far above acceptable limit of consumption.

Samples for this study were collected around July which is a rainy season in Enugu with Temperature range of between 26.7 to 37.8°C and moisture content of about 18% which are optimum for the growth of *Aspergillus flavus*, this explained the level of contamination of the corn samples. One limitation with this work is that samples were not collected during dry season to ascertain if there will be significant different in the levels of contamination, thus more work needs to be done in this regard

Farmers and sellers must be periodically educated on good storage methods which they should adopt as preventive and control measures, to reduce exposure the food products to fungi infestation and mycotoxin contamination. This will ensure the safety of corn and its derivatives for human consumption, especially in Enugu metropolis. All doses of aflatoxins have progressive effect on the risk of cancer, therefore, monitoring of corn similar foods sold in the market is important for protection of the public from aflatoxin and mycotoxin toxicity.

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6. CONCLUSION

This study unraveled 2 species of *Aspergillus flavus* and*Penicillium Citrinin* which all produced 12 different mycotoxins present in corn sold in markets from Enugu Metropolis. This should be of public health concern factoring in the potential synergistic effects that these mycotoxins could have. Further, studies on the toxicological effects of emerging mycotoxins – especially the ones without proper regulation should be done. This will aid in filling of gaps currently existing in toxicity and exposure data that is required to support legislative decisions for mycotoxins control.

Disclaimer (Artificial intelligence)

Option 1:

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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Details of the AI usage are given below:

1.

2.

3.

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