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Detection and analysis of mycoflora associated corn and wheat flours and their toxins

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Abstract

Corn and wheat flours are a significant part of the daily diet for millions of people. This work aimed to detect all the fungal flora associations and determine the mycotoxin production in the local corn and wheat flours. All corn and wheat flours were packed in 50kg bags as commercial presentations of each type. Samples were collected from three localities area. These samples were analyzed for mycoflora contaminant using the Potato dextrose agar (PDA) method and tested for mycotoxins production by using HPLC technique for the separation process. The results indicated that, total fungal counts yielded 138 isolates belonging to 40 isolates from corn flour samples, equal to 29%, and 98 isolates from wheat flour samples, equaling 71%. Location (Z) gave a higher total fungal count with corn flour samples which recorded 34 isolates, equal to 85%, followed by location (A), which gave six isolates, equal to 15%. Corn flour samples collected from location (B) were better than others which recorded zero fungal flora percent. On the other hand, location (B) was the most total fungal count isolated from wheat flour samples which record 60 isolates equal 61.2% followed by location (A) which gave 32 isolates equal 32.7% and location (Z) which record 6 isolate equal 6.1%. Five fungal species belonging to three fungal genera were identified from different corn flour samples i. e. *Aspergillus niger, A. flavus, A. parasiticus, Fusarium moniliform* and *Penicillium* spp. *Aspergillus flavus* was the most fungal frequency occurred. Whereas seven fungal species belonging to five fungal genera were identified from different wheat flour samples. These are *Alternaria alternate, Aspergillus niger, A. flavus, A. parasiticus, Fusarium moniliform, Penicillium* sp., and *Rhizopus stolonifer*. Detection of mycotoxins presented that, only aflatoxins were produced by some isolates of *A. flavus* and *A. parasiticus*. From wheat flour, *A. flavus* isolate No. A/N/ 5 gave 0.59 ng/g of total aflatoxins, *A. flavus* isolate No. A/N/ 6 gave 0.71 ng/g of total aflatoxins and *A. parasiticus* (isolate No. A/N/ 94) gave 0.66 ng/g of total aflatoxins respectively. From corn flour, only *Aspergillus flavus* No. Z/N/ 22 which isolated gave 0.81 ng/g of total aflatoxins. Fumonisin B_1 was not detected.

Key words: Corn flour, wheat flour, fungi, mycotoxin, HPLC.

1. Introduction

Cereals represent the most critical food source in many countries; mycotoxins molecules and toxic secondary metabolites can contaminate them, as they play no prominent role in the essential metabolic pathways used for growth and energy production. Some of these compounds, such as Penicillin, have antibiotic properties, whereas others are potential toxins[11]. The Food and Agricultural Organization (FAO) states that mycotoxins infect 25% and 50% of crops worldwide. According to reports, a variety of fungal species found in corn belongs to the genera *Aspergillus*, *Fusarium*, and *Penicillium*, all of which have been linked to the production of mycotoxins that cause mycotoxicosis in humans and animals [21,19]. The primary degradation agents of foods and feedstuffs are fungi, ubiquitous plant pathogens. Fungal infection of plants results in poor crop yield and quality, which translates to economic losses; toxicity impacts mycotoxins[16]. Corn (*Zea mays* L. belongs to the family Gramineae) is one of the most popular crucial economic cereal crops for human consumption and animal feed worldwide[19]. It is one of the largest food grain crops after wheat in Egypt. Wheat flour (*Triticum aestivum* L. belongs to the family Gramineae) is mainly utilized for making flatbreads. Other uses of wheat flour are in bakery product manufacturing. It is an essential constituent of people's daily diet [28].

Flour is a fine powder produced by grinding grains used for human consumption, and molds can contaminate all its products at all phases of the production chain. As well as corn, wheat flour is a powder produced from the grinding of wheat used for human utilization, and all its items can be contaminated by molds at all phases of the production chain[2]. When the storage conditions in grains store-houses are not standard, this toxin will accumulate on them [7]. Nowadays, over 400 mycotoxins are known. However, the most investigated include aflatoxins, trichothecenes, fumonisins, earalenone, and ochratoxins[6]. However, scientists pay more attention to those proven to be carcinogenic and toxic to humans and animals. The mycotoxins that are the most significant medically and in the food, industry include aflatoxins (aflatoxin B_1), ochratoxins (ochratoxin A), fumonisins (fumonisin B_1), zearalenone, patulin and trichothecenes (deoxynivalenol)[12,23]. Mycotoxin-producing fungi, which are associated with groundnuts, peanuts, cereals such as maize, rice, sorghum, wheat, barley, and oats, and spices such as black

pepper, ginger, nutmeg, chilly, etc., are of greater significance for all over world[15,3]. Among mycotoxins, aflatoxin is a potent mycotoxin that is naturally produced from several fungi species e. g., *Aspergillus* species. The four significant aflatoxins (AFs) i. e., aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), and G_2 (AFG_2), are of great importance owing to the pose a considerable risk to human health and widespread occurrence [25].

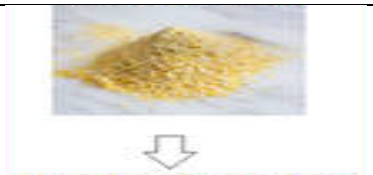
Aflatoxins are produced by *Aspergillus flavus*, *A. parasiticus*, and *A. nominus*. They contaminate a wide range of agricultural food commodities, including beans, sorghum, groundnuts, millet, peas, cassava, rice, and maize, with the latter being the most significantly contaminated by aflatoxins[15,18]. Microbiological analysis should be used to examine the presence of active micro-organisms, such as saprophytic and toxin-producing fungi. More information is needed regarding the prevalence and concentration of mycotoxins in flour foods. One of the best ways to control food contamination and mycotoxin problem is to investigate potentially toxigenic fungal populations in the flour used. This study aimed to understand the nature of associated fungi, monitor the distribution of fungi in different types of corn and wheat flour samples that are commonly consumed in Egypt, isolate and identify the fungal flora that commonly occurred, to calculate the percentage of total fungal count and fungal frequencies that contaminated corn and wheat flour samples. In addition, this work was focused on the toxigenic fungi association that may be harmful.

2. Materials and Methods

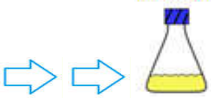
1-Samples collection: Randomized corn and wheat flour samples were collected from three different localities. The list of collected flour samples, package types, weight / (kg) which analyzed were tabulated in **Table (1).** One kilogram was taken from each corn and wheat flour in each location market.

Table (1) List of collected flour samples package type and analyzed

Type of flour	Corn	Wheat
Package weight / (kg)	50	50
Samples weight / (g)	1000	1000
Each Subsamples weight / (g)	10	10



Flour sample



Stock suspension each contain 10g / 90 ml of sterile water

2-Mycoflora analysis:

2. a- Isolation and purification of the fungal flora association, ten grams of each sub-sample (corn and or wheat flours) was transferred to 90 ml of sterile water in a sterilized bottle to give a dilution of 1:10. The suspension obtained was considered as the stock suspension. Subsequently, successive dilutions were made from that sample; 1 ml of suspension was transferred to a sterilized tube containing 9 ml of sterile water to dilute 1:100 (10^{-2}) and mixed well. This step was repeated to give 10^{-3}, 10^{-4}, and 10^{5} dilutions. To estimate the mold population, for this purpose, 0.1 mL of each dilution was transferred to sterilized Petri dishes, and for each dilution, 3 Petri dishes were inoculated as replicates. The optimal dilutions (10^{-4} to 10^{-5}) were used. The Petri plates were then incubated at 26±2°C for five days. After incubated dishes, all devolving fungi were purified on plates of PDA (with traces of Streptomycin sulfate) using the hyphal tip or single spore techniques. The colonies were counted then the results were expressed in colony-forming units (CFU). Pure cultures of growing fungi were maintained in test tube slants containing PDA medium [23].

2. b- Fungal identification: Pure cultures seven days old were identified at the genus or species level was carried out according to the cultural and morphological characters with the help of available literature found principally in publications by [27] for the genus *Aspergillus*,[5]for the genera of imperfect [29] for *Aspergillus* and *Penicillium*). The frequency of identified fungal species was calculated according to the number of isolates of a genus or species/total number of fungal isolates x 100.

3-Testing of mycotoxin production: All isolates of toxigenic fungi were propagated as a pure culture in 100 ml yeast extract sucrose (YES) to be tested for mycoxin production. Each flask was inoculated with 0.1 ml spore suspension containing approximately 10^5 spores/ml. Cultures were incubated at 26±2°C for 14 days. Then tested for mycoxin production by using High-Performance Liquid Chromatography (HPLC)

according to the methods of [4]. Also, mycotoxins contents were determined by using HPLC according to the methods of [9].

3. Results and Discussion

1-Total fungal count: Data presented that the total fungal count isolated from different flour samples resulted in 138 isolates belonging to 40 isolates from corn flour samples, equal to 29%, and 98 isolates from wheat flour samples, equivalent to 71%, as shown in **Table (2).** On the other hand, data indicated that location (Z) gave a higher total fungal count with corn flour samples which recorded 34 isolates, equal to 85%, followed by location (A), which gave six isolates, equal to 15%. Corn flour samples collected from location (B) were better than others which recorded zero percent. On the other hand, location (B) was the total fungal count isolated from wheat flour samples, recording 60 isolates equal to 61.2%, followed by location (A), which gave 32 isolates equal to 32.7%, and location (Z), which recorded six isolates equal to 6.1%. Similar results were obtained by [6], who reported those total fungal counts of maize flours ranged from <10 to $8.4 \times 10^{(4)}$ CFU/g and predominant mycobiota belonged to *Aspergillus* spp. and *Penicillium* spp. Also,[11] reported that Fungi of corn flour counts varied between 1.79 and 4.7 x 10 CFU of fungi/g of the sample, indicating that they were under the level established by the CONVENIENT Guideline 1337-90 of 10^4 CFU/g. The viable count of mycoflora in the maize and poultry feed samples is 1.0 x 10^2—3.6 x 10^6 cfu/gm.[11] stated that from the 33 cereals samples evaluated (biscuits, wheat flours, semolinas, and corn products),13 samples (39,4%) were contaminated by fungal flora, of which eight samples (24,2%) were contaminated by mold, yeasts contaminated three samples (9,1%), and both molds and yeasts contaminated two samples (6,1%). [23] reported that the highest mean microbial count (9.30 x 10^{13} cfu/g) was observed in a plantain wheat flour bought from the Sango market, while the lowest (1.16 x 10^{12} cfu/g) was observed in wheat flour from Oja-Ota market. [23] show that the sample with the highest microbial load is plantain flour from Sango at 9.30 x 10^{13} cfu/g, while the example with the lowest microbial load is wheat flour from Oja-Ota at 1.16 x 10^{12} cfu/g. According to [28] who found that all the raw wheat flour samples were found positive for fungus; however, the colonies were found in the acceptable range. The fungal load in raw flour was found in the field of $1.0x10^2$ to $4.5x10^3$ cfu/g.

2-Fungal frequency occurred:

2. a-Percentage of fungal frequency associated with tested corn flour was recorded in Table (3). Data show that five fungal species belonging to three fungal genera were identified from different flour samples. *Aspergillus niger*, *A. flavus*, *A. parasiticus*, *Fusarium moniliform*, and *Penicillium* sp. *Aspergillus flavus* was the most fungal frequency occurred which record in 19 isolates, equal to 47.5%, followed by *A. parasiticus* 13 isolates, equal to 32.5%, *Fusarium moniliform* five isolates (12.5%) and *Aspergillus niger* two isolates (5%). *Penicillium* sp., was less fungal frequency occurred, which recorded only one isolate equal to 2.5%. According to [1] who found that, in maize flours, predominant mycobiota belonged to *Aspergillus* spp., and *Penicillium* spp., and the most frequent species were *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp. Also,[11] **isolated** and identified several fungal species associated with corn flour as *Aspergillus* spp., A. *flavus*, *A. niger*, A. *terreus*, and *Penicillium* spp.[29]tested some food grains and grain flour market samples. They found that contamination of aflatoxin B_1 was 68.18% in food grains, whereas 100% in grain flour, possibly due to improper post-harvest technology and storage conditions.[35] isolated five fungal genera, namely: *Aspergillus*, *Penicillium*, *Rhizopus*, *Fusarium*, and *Botrytis*, from maize and maize products. *Aspergillus* was the most predominant genera isolated (62%). Among the *Aspergillus*, three species were identified: *A. niger*, *A. tamari* and *A. flavus*.[16] reported that the identification of molds showed that 57.1% of tested cereals were contaminated by *Aspergillus niger*, 28.6% of molds *Penicillium* species, including *Penicillium notatum* and *Penicillium* spp., and 14.3% of samples contaminated by ***Fusarium* sp.**[9] reported that, from one hundred samples of maize grains and 100 samples of maize flour, four fungal genera, namely: *Aspergillus*, *Penicillium*, *Fusarium* and *Rhizopus*, were isolated. *Aspergillus* was the most isolated genus in the samples.

Table (2) Percentage of total fungal count isolated from different flour samples

Localities	Type of flour				Total	
	Corn		Wheat			
	T. c.	%	T. c.	%	T. c.	%
Location (A)	6	15.0	32	32.7	38	27.5
Location (B)	0	0.0	60	61.2	60	43.5
Location (Z)	34	85.0	6	6.1	40	29.0
Total	40	29.0	98	71.0	138	100.0

T.c.=Total colonies

Table (3) Percentage of fungal frequency associated of tested corn flour.

Fungal isolates	Total	
	T. c	%
Aspergillus niger	2	5.0
A. flavus	19	47.5
A. parasiticus	13	32.5
Fusarium moniliform	5	12.5
***Penicillium* sp.**	1	2.5
Total	40	100.0

T. c = Total colonies

2. b-Percentage of fungal frequency associated with tested wheat flour presented that seven fungal species belonging to five fungal genera were identified from different flour samples. These are *Alternaria alternate*, *Aspergillus niger*, *A. flavus*, *A. parasiticus*, *Fusarium moniliform*, *Penicillium* sp., *and Rhizopus stolonifer*, as shown in **Table (4).** Higher fungal frequency was recorded with *A. niger* which gave 50.0%, followed by *A. flavus* 22.5, *A. parasiticus* 9.2, *Penicillium* sp., *Rhizopus* stolonifer 6.2, and *Alternaria alternate* 4.1%. Less fungal frequency was recorded with *Fusarium moniliform* at 2.0%.[2] reported that the most frequently isolated molds from wheat flour samples were *Aspergillus*, *Penicillium*, and *Fusarium*, with frequency percentages of 21.3%, 15.84%, and 12.23%, respectively.[17] stated that species of *Aspergillus*, *Penicillium*, and *Rhizopus* contaminated wheat flour. These filamentous fungi, belonging to Ascomycotina and Zygomycotina groups, have been categorized as common contaminants of wheat flour. *A. flavus* and *A. terreus* occurred most frequently, with a percentage frequency of 95.65% and 67.33%. The percentage of occurrence of *Rhizopus* spp., and A. *niger* was found to be 70% and 56.66%, respectively, and was recorded in both samples.[23] reported that seven fungi were isolated from Plantain/Yam and Wheat Flour purchased from four markets. The isolated fungi include *Aspergillus flavus*, *Aspergillus niger*, *Rhizopus* spp., *Geotrichum* spp., *Yeast, Penicillium* spp., *and Paecilomyces* spp.

3- Reaction of mycotoxins production: All toxigenic fungi isolated from corn and wheat flour samples were tested for mycotoxins production. Data in **Table (5)** indicated that, only four fungal isolates were found to gave positive reaction to produce mycotoxins. Out of them one isolate of *A. parasiticus* (isolate No. A/N/ 94) and two isolates of *A. flavus* (as isolate No. A/N/ 5 and isolate No. A/N/ 6) which were isolated from wheat flour were found to produce one or more aflatoxins. Only one isolate of *A. flavus* No. Z/N/ 22 which isolated from corn flour was found to produce aflatoxins. Fumonisin B_1 was not detected. These results fully supported the results obtained by[1], who found that Aflatoxins were caught in 14 maize flours and two popcorn kernels samples. *Aspergillus flavus* and *Aspergillus parasiticus* produced aflatoxins. It was discovered that aflatoxins and ochratoxin A co-occur in four ochratoxin-positive maize flour samples.

Table (4) Percentage of fungal frequency associated of tested wheat flour.

Fungal isolates	Total	
	T. c	%
Alternaria alternata	4	4.1
Aspergillus niger	49	50.0
A. flavus	22	22.5
A. parasiticus	9	9.2
Fusarium moniliform	2	2.0
***Penicillium* sp.**	6	6.1
Rhizopus stolonifer	6	6.1
% Total	98	100.0

Table (5) Reaction of mycotoxins production.

Source of flour	Isolate No.	Tested fungi	Type of tested mycotoxins					
			AFB_1	AFB_2	AFG_1	AFG_2	FB_1	OTA
	A/N/ 5	***Aspergillus flavus***	+	+	ND	+	ND	ND
	A/N/ 6	***Aspergillus flavus***	+	+	+	+	ND	ND
Wheat		***A. niger***	ND	ND	ND	ND	ND	ND
	A/N/94	***A. parasiticus***	+	+	+	+	ND	ND
		Fusarium moniliform	ND	ND	ND	ND	ND	ND
	Z/N/ 22	***Aspergillus flavus***	+	+	+	+	ND	ND
		A. niger	ND	ND	ND	ND	ND	ND
Corn		***A. parasiticus***	ND	ND	ND	ND	ND	ND
		Fusarium	ND	ND	ND	ND	ND	ND

ND =Not detected + = Positive producer

4- Quantities of Aflatoxin (Afs) produced:

Determination of Aflatoxin (Afs) produced were tabulated in Table (6) and Figs. (1 – 5). Data indicated that, *A. flavus* isolate No. A/N/ 5 from wheat flour, gave 0.59 ng/g of total aflatoxins belonging to 0.05, 0.31, and 0.23 ng/g of B_1, B_2, and G_2, respectively. Aflatoxin G_1 was not detected with the same isolate (No. A/N/ 5) as showing in **Fig. (2)**. *A. flavus* isolate No. A/N/ 6 from wheat flour, gave 0.71 ng/g of total aflatoxins belonging to 0.05, 0.34, 0.17, and 0.15 ng/g of B_1, B_2, G_1, and G_2, respectively **(Fig. 3)**. Finally, *A. parasiticus* No. A/N/ 94 from wheat flour, gave 0.66 ng/g of total aflatoxins belonging to 0.05, 0.10, 0.18, and 0.33 ng/g of B_1, B_2, G_1, and G_2, respectively **(Fig. 4).** On the other hand, data show that only one isolate of *Aspergillus flavus* No. Z/N/ 22, which was isolated from corn flour, gave 0.81 ng/g of total aflatoxins belonging to 0.17, 0.33, 0.16, and 0.15 ng/g of B_1, B_2, G_1, and G_2 respectively **(Fig. 5).** These results agreed with those of Chavarri et al., 2012 who stated that the aflatoxin content complied with the tolerance level allowed for corn flour (20 ng/g). **[15]** foundthat the mean levels of total aflatoxin and aflatoxin B_1 in wheat flour were 1.99 and 0.53 ng g^{-1}, respectively. The levels of total AF ($p = 0.03$), AFG_2 ($p = 0.02$), and AFB_1 ($p = 0.003$) were significantly higher in samples obtained from high-risk areas. Also, **[29]** reported that the contamination of aflatoxin B1 was found to be 63.16% in food grains, whereas 100% in grain flour and 68.18% in total food grains and grain flour with a mean concentration (ppb) of 75.18, 60.4 and 72.23 respectively.**[19]** found 23 samples (57.5%) out of 40 samples of corn flour have shown positive for aflatoxin (35% of samples have shown positive for G_1, 5% of samples have shown positive for G2, 50% of samples have shown positive for B_1, 32.5% samples have shown positives for B_2).**[16]** reported that AF contamination was mainly observed in 88.8% of samples analyzed (N=32) with a mean level of contamination of 42.0µg/kg. The levels of cereals samples, biscuits, wheat flour, semolinas, and corn products were higher than 100µg/kg.**[31]** determined aflatoxin levels in maize flour samples by high-performance liquid chromatography. The results indicated that, in 11 of the 69 (16%) analyzed samples, aflatoxin was detected in the range of 0.379-24.54 µg/kg total aflatoxin. In two examples, the total aflatoxin level in one sample and the aflatoxin B1 level was above the permissible toxin limits of Turkey and the European Union.**[30]** found that the mean aflatoxin level in maize flour was 174ppb, while the maize bran had a mean aflatoxin level of 213ppb. Significant differences exist between the mean aflatoxin contaminations of old maize, fresh maize, maize flour, and maize bran.**[4]** determined the levels of mycotoxins, quantifying different including zearalenone (ZEA), T_2-toxin, aflatoxin B_1 (AFB_1), deoxynivalenol (DON), and ochratoxin A (OTA) in 30 and 10 corn flour samples. Spiked calibration curves based on external and internal standards were used to overcome matrix effects and were reported as linear between 2 and 50 ng g−1 for aflatoxin B1, T-2 toxin, ochratoxin A; 50 and 1250 ng g−1 for zearalenone; and 75 and 1800 ng g−1 for deoxynivalenol. AFB_1, OTA, and ZEA were detected and quantified in 23 (76.6%), 6 (20%), and 14 (46%) of 30 samples, with an average contamination of 154.1 ng g−1, 25 ng g−1, and 358.7 ng g−1, respectively.**[25]** analyzed seventy-five maize samples and 27 samples of maize flour from three regions. Examples from Eastern Kenya had the highest contamination at 22.54±4.94 ppb, while those from Nairobi had the lowest (7.92±1.57 ppb). Aflatoxin in maize flours was slightly above the international upper limit of 5ppb, but all the results were lower than the Kenya standard, whose upper limit is 10ppb. Samples of maize flour from Eastern Kenya had the highest aflatoxins concentrations at 6.98± 0.53 ppb due to higher levels of aflatoxin contamination in maize grains about maize flours.**[28]**reported that, from Plantain/Yam and Wheat Flours, *Aspergillus flavus* was the predominant (31%) aflatoxigenic fungi isolated compared to *A. niger* (21%). The other fungi isolated include *Rhizopus* spp., Geotrichium spp., Yeast, *Penicillium* spp., and *Paecilomyces* spp. Aflatoxin was detected in all the food samples tested in this study at concentrations ranging from 0.2 ppb to 5.9 ppb, all within the CODEX Alimentarius Commission (CAC) aflatoxin acceptable limit of 15 ppb.

Table (6) Quantities of Aflatoxin (Afs) (ng/g) production

Source of flour	Type of fungi	Isolate No.	Aflatoxin (Afs) conc. (ng/g)				
			AFB_1	AFB_2	AFG_1	AFG_2	Total of (Afs)
Wheat	***A. flavus***	**A/N/ 5**	0.05	0.31	ND	0.23	0.59
Wheat	***A. flavus***	**A/N/ 6**	0.05	0.34	0.17	0.15	0.71
Wheat	***A. parasiticus***	**A/N/ 94**	0.05	0.10	0.18	0.33	0.66
Corn	***A. flavus***	**Z/N/ 22**	0.17	0.33	0.16	0.15	0.81

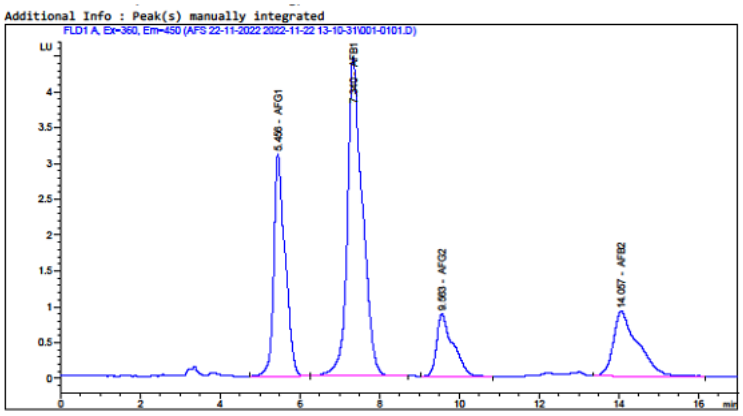


Fig. (1): Standard (STD) spiked in the HPLC chromatogram of aflatoxins AFG_1, B_1, G_2& B_2

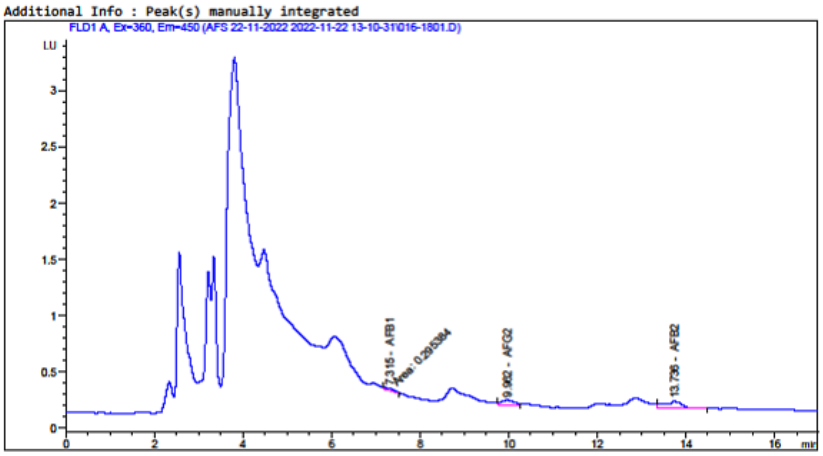


Fig. (2) HPLC chromatogram of aflatoxin produced by *A. flavus* isolated from wheat flour samples (No.A/N/5).

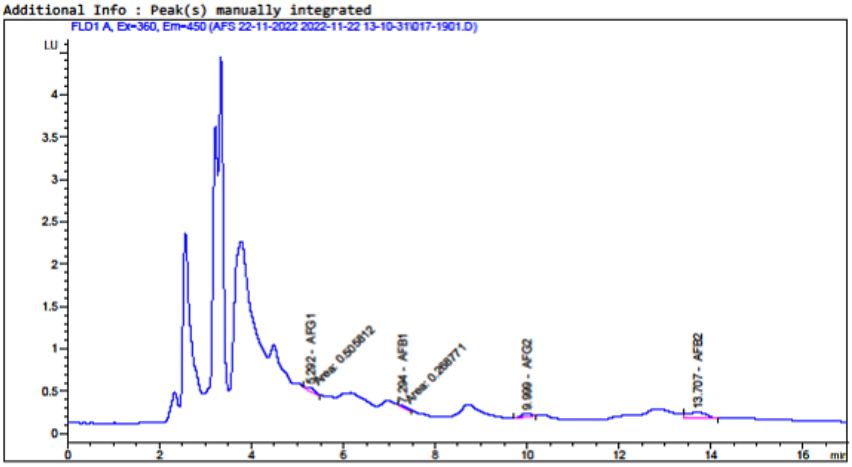


Fig. (3) HPLC chromatogram of aflatoxin produced by*A. flavus*isolated from wheat flour samples(No.A/N/6).

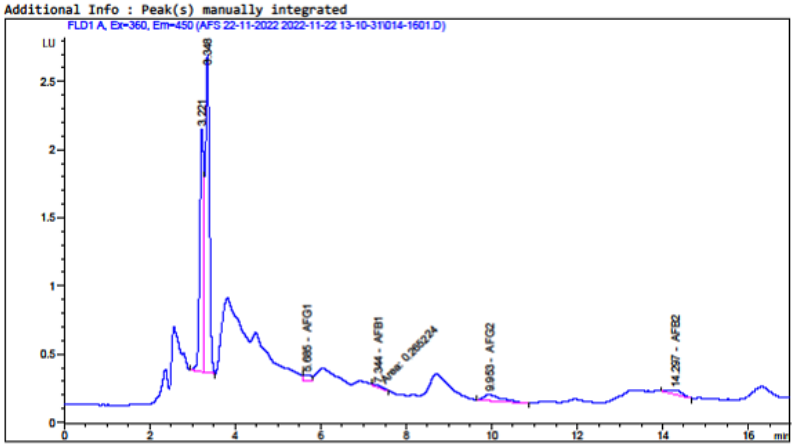


Fig. (4) HPLC chromatogram of aflatoxin produced by *A. parasiticus* isolated from wheat flour samples (No.A/N/ 94).

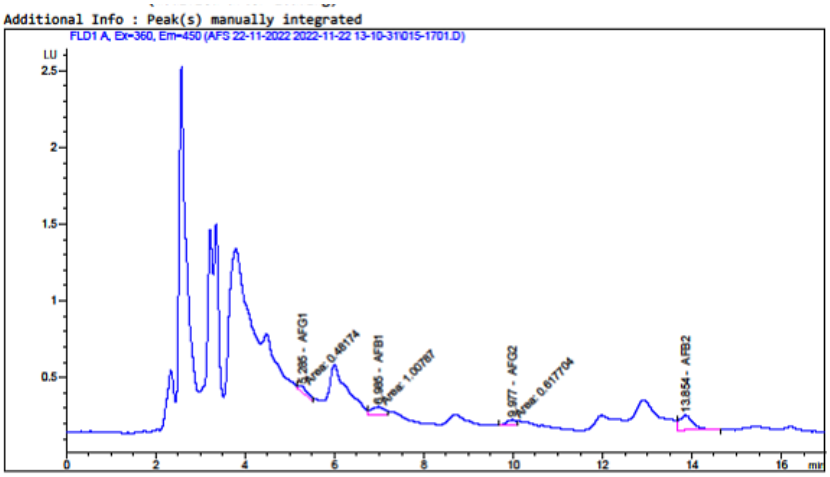


Fig. (5) HPLC chromatogram of aflatoxin produced by *A. flavus* isolated from corn flour samples(No.Z/N/22).

4. Conclusion

The tested corn and wheat flour samples were positive for fungal infection and were within the recommended range. *Aspergillus spp*. are the most dominant fungi which were isolated in this study. The fungal species and mycotoxins identified help develop effective control strategies.

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