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Original Article

Isolation and Identification of Aflatoxin and Ochratoxin Producing Fungi from Some Local Food Items in the Markets of Karbala Governorate

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Abstract:- This study showed the isolation and identification of fungi contaminating some foodstuffs and their ability to produce Aflatoxin and Ochratoxin toxins. The results showed the isolation of 17 isolates of fungi isolated from different food sources, which are 10 isolates of Aspergillus niger in the samples red pistachio, basmati rice, amber rice, Nestle and corn and 4 Aspergillus flavus isolates in noodles, soybean, white pistachio and corn samples. One isolate of Aspergillus fumigates in a sunflower seed sample and one isolate of Rhizopus sp. in the biscuit sample and one isolate of Penicillium sp in the red pistachio sample.

Thin plate chromatography test TLC showed that Aspergillus niger isolated from red pistachios produced Ochratoxin and Aflatoxin together, while samples of soybean rice and amber rice produced only Ochratoxin, and Aspergillus flavus isolated from soybean produced Aflatoxin and Ochratoxin from maize. It was an Aflatoxin producer. Penicillium sp isolates isolated from red pistachios were Ochratoxin-producing.

Aim of the study:

A survey study of the fungi in the most traded foodstuffs in the community of Karbala governorate and confirming the most prevalent and polluting fungi in food samples that produce the toxins Aflatoxin and Ochratoxin

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Introduction:

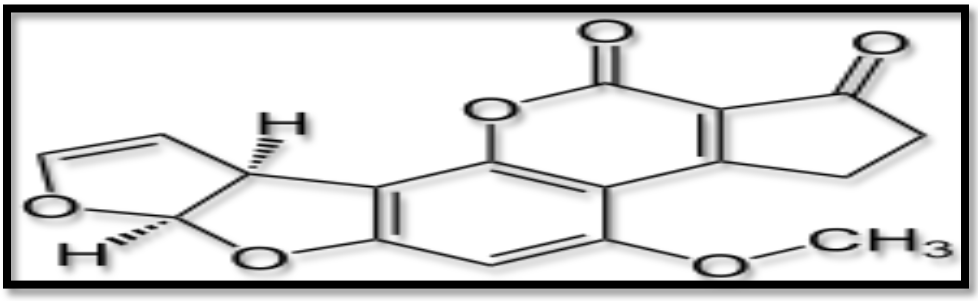
Mycotoxins are found in a wide range of Environments due to their ability to utilize A variety of substrates and to their relative Tolerance to low pH, low water activity, and Low temperature (Huis in't Veld, 1996). Generally, foods have essential nutrients for Fungal growth, thus fungi can appear and Spoil different foods. Fungal spoilage of food Causes economic losses worldwide (Dantigny Et al., 2005). In addition, some fungi Synthesize mycotoxins, which can be a Hazard for human health. (Pitt, 1996) defined Mycotoxins as fungal metabolites that whenIngested, inhaled or absorbed through the Skin cause illness on human and animalDeath. Mycotoxin is produced during the growthOf fungi and can be found out or in the Hyphae and spores of these organisms(Zucchi and Melo, 2009; Köppen et al., 2010). If ingested, mycotoxins may causeAcute or chronic disease episodes, termed Mycotoxicosis (Köppen et al., 2010;Medeiros et al., 2012). Mycotoxin long-term Exposure has also been related to several Mycotoxicosis, such as carcinogenic,Mutagenic, teratogenic, estrogenic, Hemorrhagic Immunotoxic, nephrotoxic, hepatotoxic, Dermotoxic neurotoxic and Immunosuppressive (Richard, 2007; Medeiros et al., 2012). Currently, more than 500 differentMycotoxins have been discovered and this Number do not stop increasing. Among the Most economically and toxicological Important mycotoxins that pose greatestPotential risk to human and animal health asFood and feed contaminants are: aflatoxins,Strigmatocystin, trichothecenes, fumonisins, Zearalenone, ochratoxin, alternariol, patulin, And certain ergot alkaloids (CAST, 2003; Bennett and Klich, 2003; Richard, 2007; Köppen et al., 2010).

2-1.Aflatoxins

Aflatoxins (AFs) are some of the most important and harmful mycotoxins. As of 2020, 60 years have already passed since their discovery. AFs are one of the five agriculturally most important mycotoxins(Res,1995) .Chemically, the AFs are difuranocoumarin derivatives with a bifuran group attached to the coumarin nucleus and a pentanone ring (in the case of aflatoxin AFBs) or a lactone ring (in case of aflatoxin AFGs)(Schuda,1980). There are more than 20 known AFs, but the most common are aflatoxin B1 (AFB1) (PubChem CID: 186907), aflatoxin B2 (AFB2) (PubChem CID: 2724360), aflatoxin G1 (AFG1) (PubChem CID: 14421), and aflatoxin G2 (AFG2) (PubChem CID: 2724362) (PubChem, 2020), from which AFB1 is the major representative in food crops(European Food Safety Authority,2018) .Aflatoxin M1 (AFM1) (PubChem CID: 15558498) and M2 (AFM2) (PubChem CID: 10903619) are the hydroxylated metabolites of AFB1 and AFB2 (PubChem ,2020 and Hussain,2008)

Structure of aflatoxin

Are a group of mycotoxins produced by the fungus Aspergillus and are potent hepatotoxins and carcinogens in the liver. Structurally all **aflatoxins** contain a coumarin ring and an unsaturated lactone moiety**.**(Williams,H.Jaesschke,2011)



Most human exposure comes from nuts and grains

Two closely related species of fungi are mainly responsible for producing the Aflatoxins of public health significance: Aspergillus flavus and A. parasiticus. Under favorable conditions typically found in tropical and subtropical regions, including high temperatures and high humidity, these moulds, normally found on dead and decaying vegetation, can invade food crops. Drought stress, insect damage and poor storage can also contribute to higher occurrence of the moulds including in more temperate regions. Several types of Aflatoxin (14 or more) occur in nature, but four – Aflatoxins B1 , B2 , G1 and G2 are particularly dangerous to humans and animals as they have been found in all major food crops; but most human exposure comes from contaminated nuts, grains and their derived products. Additionally, Aflatoxin M1 (AFM1), a product of Aflatoxin B1 (AFB1) metabolism, can be found in milk in areas of

high Aflatoxin exposure.(/WHO- 2018)

Long-term exposure can have serious health consequences

Long-term or chronic exposure to aflatoxins has several health consequences including: ‹

1- Aflatoxins are potent carcinogens and may affect all organ systems, especially the liver and kidneys; they cause liver cancer, and have been linked to other types of cancer – AFB1 is known to be carcinogenic in humans; the potency of Aflatoxin to cause liver cancer is significantly enhanced in the presence of infection with hepatitis B virus (HBV) (WHO ,2017)‹

2- Aflatoxins are mutagenic in bacteria (affect the DNA), genotoxic, and have the potential to cause birth defects in children(WHO ,2007)

3- children may become stunted, although these data have yet to be confirmed because other factors also contribute to growth faltering e.g. low socioeconomic status, chronic diarrhoea, infectious diseases, malnutrition(WHO ,2002)

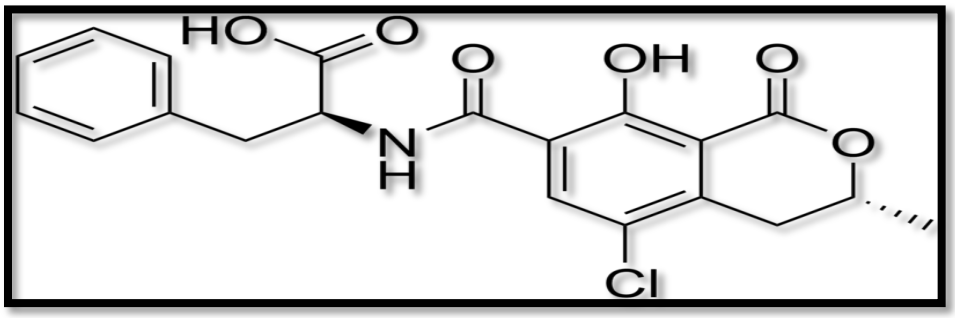
4- Aflatoxins cause immune suppression, therefore may decrease resistance to infectious agents (e.g. HIV, tuberculosis) (WHO,1999)

2-2 Ochratoxin

Ochratoxin A (OTA) is a naturally occurring food borne mycotoxin found in a wide variety of agricultural commodities worldwide, ranging from cereal grains to dried fruits to wine and coffee. It is produced by several different fungi including *Aspergillus ochraceus*, *A. carbonarius*, *A. niger* and *Penicillium verrucosum*. These fungi vary in their optimal growing temperatures and water activity, and contaminate various commodities. Contamination generally occurs as a result of poor storage of commodities and suboptimal agricultural practices during the drying of foods (Moss, 1996). Ingestion is the main source of exposure to OTA.

Structure of Ocratoxin

Ochratoxin A (OTA) is a mycotoxin produced by secondary metabolism of many filamentous species belonging to the genera Aspergillus and Penicillium , Biosynthetically, it is a pentaketide derived from the dihydrocoumarins family coupled to β-phenylalanine. Its chemical name is: L-phenylalanine-N-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyrane-7-yl)carbonyl]-(R)-isocoumarin and its chemical structure is presented in Figure (Keeper-Goodman;1989,Breenkamp;1994).



Health effect of ocratoxin

OTA is a chemically stable compound; hence, ordinary food processing measures fail to substantially reduce its presence in foods and beverages. OTA has been shown to be toxic and carcinogenic in animals. The kidney is the main target organ for OTA.

1-OTA is a potent renal carcinogen in several animal species (Duarte et al., 2011; Hagelberg et al., 1989; IARC, 1993; Krogh, 1974; Kuiper-Goodman and Scott, 1989; World Health Organization (WHO), 2002).

2-Other adverse effects of OTA include immunotoxicity (Bondy and Pestka, 2000; Pestka and Bondy, 1994).

3- inhibition of macromolecular synthesis, increased lipid peroxidation, and inhibition of mitochondrial respiration (Kuiper-Goodman and Scott, 1989; Marquardt and Frohlich, 1992).

4-OTA has been suspected as a cause of various human nephropathies since the 1970s including Balkan Endemic Nephropathy (BEN) (Barnes et al., 1977; Castegnaro et al., 2006; Elling and Krogh, 1977; Pfohl-Leszkowicz et al., 2002; Sattler et al., 1977) .

5-chronic interstitial nephropathy (CIN) (Abid et al., 2003).

6- The International Agency for Research on Cancer (IARC) has classified OTA as a Group 2B possible human carcinogen, based on demonstrated carcinogenicity in animal studies (Fazekas et al., 2005; IARC, 1993)

Materials and Methods

2-1. Devices and materials used in the implementation of the study.

No	Devices	Company manufactured	Origin
1	Incubator	Lab-Teach	Korea
2	Blender	National	Japan
3	Electrical oven	Memmert	Germany
4	Autoclave	Sermite	Germany
5	Sensitive balance	Sartorius	Germany
6	Petri dish	Unisonics LTD	England
7	Flask	Unisonics LTD	England
8	Refrigerator	ISHTAR	Iraq

2-2: chemical substances

No	Substance	Company manufactured	Origin
1	Cholorophorm	Fluka	Switzerland
2	Methanol	BDH	England
3	Hypochlorite sodium		
4	TLC	Fluka	Switzerland
5	Chloramphenicol	Oxoid	England

2-3: culture media

No	Media	Company manufactured	Orgin
1	PDA	Hi-media	India

2-4: Sample collection

15 items were collected from different food sources, which are nuts (white pistachios, red pistachios and red grains), biscuit, Nestle, Lattice , macaroni, lentils, indomie, gypsum with pistachios, corn, bazmaki rice, amber rice, soybeans, White grain. . At random from the local market Karbala Governorate, with two samples for each food item and a weight of 250 gm for each sample for the purpose of obtaining isolates from Fungi producing toxins

2-5: Cultures

Prepare this media according to instructed by the manufacturer, taking 39 grams of powder media and dissolving in a liter of distilled water and sterilise in autoclave in temperature121C° and under the pressure of 1.5 atm for 15 minutesand then cooled to the point of 45C° is added to the antibiotic chloramphenicol concentration of 50 mg/l,Chloramphenicol acts as a selective agent to inhibit bacterial overgrowth of competing microorganisms from mixed specimens, while permitting the selective isolation of fungi.

2-6: Isolation and diagnosis of fungi associated with food item

The food samples were transferred to the toxicology laboratory in the Environmental Health Department – Faculty Medical Sciences – University of Karbala and cut into small pieces 5 mm and surface sterilized with a solution of

sodium hypochlorite at a concentration 2% for two minutes, after which it was washed with sterile distilled water and then placed on filter papers to get rid of the water and then They were implanted in plastic dishes (8 cm in diameter) containing PDA medium by placing four pieces of the food item, At a distance of 3 cm from the edge of the plate and a fifth piece in the center of the plate and the process was repeated twice (iterations) for each material All dishes were incubated at 30 ± temperature for a period of seven days (Mikhail and Peder, 1982) after which the isolates were purified.by incubating the dishes at 30 ±2 °C for 10-14 days Fungi and isolates were diagnosed according to their phenotypic and microscopic characteristics (2009, and Pitt Hocking and Moubasher (1993).

2-7: Testing the fungus susceptibility to the production of aflatoxin and Ochratoxin by using Thin Layer Chromatography (T.L.C)

Is the development of isolation fungi on central PDA and by puting the tablets of the fungi studied and a diameter of 5 mm old a week in the center of each dish then incubated at 25 C°±2 c for one week after this cutting the middle and transported the pieces by a sterile needle to a blender container then 20ml of choloform and blending the mixture for 10 minutes and then is filtrerd mix by filter paper then taking the filtrate and placed in a beaker is clean sterilized then move to electric oven at a temperature of 40C° where quantitative focus to approximately 1ml only .Not detect the presence of toxin , sheets chromatographic technique using thin TLC with dimensions (20*20)cm where platelets are active in the electric furnace degree (120)C° for one hour befor use . the system uses Chapter cholorform : methanol 95:5.

Is the work of a straight line on the plate TLC is a distance of 1.5 cm from the base plate , and takes 15 mycroliter of standard toxin and placed on line a distance of 2cm from the left the plate and at a distance of 2cm from the spot of fungal extract at the same distance and a quantity equal to the amount of standard toxin, then leave spots to dry and the placed in basin chapter containing a mixture of choloform : methanol and by 95:5 and are monitored until the arrival of the solution to a distance of approximately 2 cm from the upper end of the plate , extracted it from the chromatographic process then dried under situation labratory for a period of 5 minutes, then examined under a UV and long wavelength 360 nm and is detected the presence of toxins matching coefficient deportation (Rf) Relay factor color and sparkle to the posion index with color to migrate fungi extract samples (Sobolev and Dorner ,2002)

Result and Discussion

3-1: Isolation and diagnosis of fungi associated with food item

Isolation and diagnosis of fungi isolated from different food materials the results of the isolation of fungi15 food samples were obtained from 17 isolates of fungi (five species)were obtained the number of isolates of the fungus *A. niger , A. flavus, Penicillium sp. , A. Fumigatus* and *Rhizopus sp*. was 10, 4, 1, 1, and 1 consecutive. table (1) .

Where the appearance of the A. niger , A. flavus is the most isolated species compared to the other fungal species, and the reason is that the species of this genus has the ability to secrete a large number of enzymes that decompose nutrients that are used in nutrition and growth, in addition to its wide spread and that some of its types can grow in content Low humidity, in addition to the relative density of the spoors it produces. (Eaton & Groopman , 1994) .

Table(1): showing Fungi isolated from different food materials:

No	Fungi species	Number of isolates
1	*Aspergillus niger*	10
2	*Aspergillus flavus*	4
3	*Aspergillus Fumigatus*	1
4	*Rhizopus sp.*	1
5	*Penicillium sp.*	1
6	Total	17

Table(2): show fungi species in food materials

Fungi species	Macaroni	Lattice	Indomie	Amber rice	Soybean	White pistachio	Red pistachio	Corn	Basmati rice	Red sunflower seeds	Nestle	Biscuits	Adas	Pistachio Gypsum	Whitesunflower seeds
Aspergillus niger	+	-	+	++	+	-	+	+	+	-	++	-	-	-	-
Aspergillus flavus	-	+	-	-	+	+	-	+	-	-	-	-	-	-	-
Aspergillus Fumigatus	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Rhizopus sp.	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Penicillium sp.	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-

The results of the frequency of the fungi in Table (2) showed that the fungus *A.niger* recorded the highest frequency in (Macaroni, Indomie, Amber, soybeans, red pistachios, Bismaki honey) and *Aspergillus flavus* had the highest frequency in (noodle, soybean, pistachio, corn) and *Penicillium spp* recorded the highest frequency in red pistachio, while *A. fumigatus* had the highest frequency in red sunflower seeds, while *Rhizopus sp.* had the highest frequency in red pistachio. Frequency in the biscuit As for the rest of the samples, no percentages of his presence were recorded. Has Several studies have shown that toxin-producing fungi, especially *Aspergillus*, are among the main pollutants in Dried fruits and that the fungi *A. niger* and *A. flavus* are the most isolated species in dried apricots(Zohri and Abdel-Gawad, 1993; Aziz and Moussa, other dried fruits and the rest, prunes and dates) 2008., *al et* Benlioglu; 2006, Heperkan; 2002. (The current study agrees with the results of other studies in The dominance of the genus Aspergillus in samples of maize and potato gypsum (Shakroakhron, 2012) and confirmed by the same study. One of the most common species found in food sources is *A. niger, A. Ochraceus , A. flavus., A. terreus*.. The cause of the appearance of the fungus *Aspergillus* in foodstuffs is *A. fumigatus., .A. versicolor, A. parasiticus*. It is the species of this genus having the ability to secrete a large number of enzymes that decompose the nutrients that are used In nutrition and growth, as well as increasing the capacity of its spread, especially that some types can grow in a low content ofHumidity as well as the relative density of the boards produced by them (1994, Groopman & Eaton.**)**

3-2: The results of detection using thin layer chromatography showed

that five fungal isolates out of 17 isolates that were isolated from different food items , were producing mycotoxins,

Aflatoxin and Ochratoxin. These results showed that the isolate of the fungus *Aspergillus niger* isolated from soybean was producing Ochratoxin, the isolate of the fungus *Aspergillus flavus* isolated from soybean produced Aflatoxin, and the isolate of the fungus *Penicillium sp*. Isolated from red pistachios also produced Ochratoxin , by comparing the color of the brilliance of the extract of each isolate and relay factor with the standard toxin of Aflatoxin and Ochratoxin, image (1). Image (2) showed that the isolate of the fungus *Aspergillus niger* isolated from red pistachio was producing Aflatoxin and Ochratoxin. In addition to the ability of the isolate of the *Aspergillus flavus* isolated from corn to produce Aflatoxin compared to the standard toxin represented in the image (3).

The results proved the ability of the fungus Aspergillus niger isolated from the amber rice to produce Ochratoxin, by comparing with the standard toxin , image (4) The results were similar to the study (Al-Aboudi *et al*., 2015), where the results showed that 71 isolates of *Aspergillus flavus* produced Aflatoxin B1 out of 149 isolates that were isolated from different foodstuffs collected randomly from local markets in Babylon and in a study conducted by Al-Khalaf in 2011, it was shown that 40 isolates out of a total of 49 isolates of the fungus *Aspergillus flavus* produce Aflatoxin B1. In another study conducted by (Deabes, and Al-Habib. 2011) the fungi producing mycotoxins were included in 30 samples of nuts collected in the Kingdom of Saudi Arabia. The study revealed that the percentage of samples producing Aflatoxin was 80, 80, 60, 40, 40. 20% for pistachios, peanuts, walnuts, almonds, hazelnuts and cashews, and also showed that the concentration of Aflatoxin B1 ranged from 0. 3 mcg/kg in cashews to 140 mcg/kg in peanuts. Honk *et al*., 2011 indicated that five of 11 samples of maize-processed food products were contaminated with high levels of Aflatoxin and with concentrations of (18.2 , 12.2 , 17.2 , 10.8 , 21,0 ppm. The current study supported many

studies, such as the study (Al-Janabi, 2009), which showed that 36% of the isolates of *Aspergillus flavus* that infects maize produces Aflatoxins, Another study showed that 29 isolates from a total of 49 isolates of *Aspergillus flavus* isolated from nuts: field pistachio, pistachio nuts, walnuts and white seeds, Red sunflower seeds, cashews and chickpeas produce Aflatoxin B1(Al-Khalaf, 2011).In a study conducted by Fernane *et al*. (2010) in Algeria, showed that 5.56% of isolates of *Aspergillus flavus* isolated from pistachio samples produced Aflatoxins B1 and B2. Al-Fatlawi's study (2014) showed that 6.56 percent of the total isolates *Aspergillus flavus* isolate from nuts producing Aflatoxin toxins.

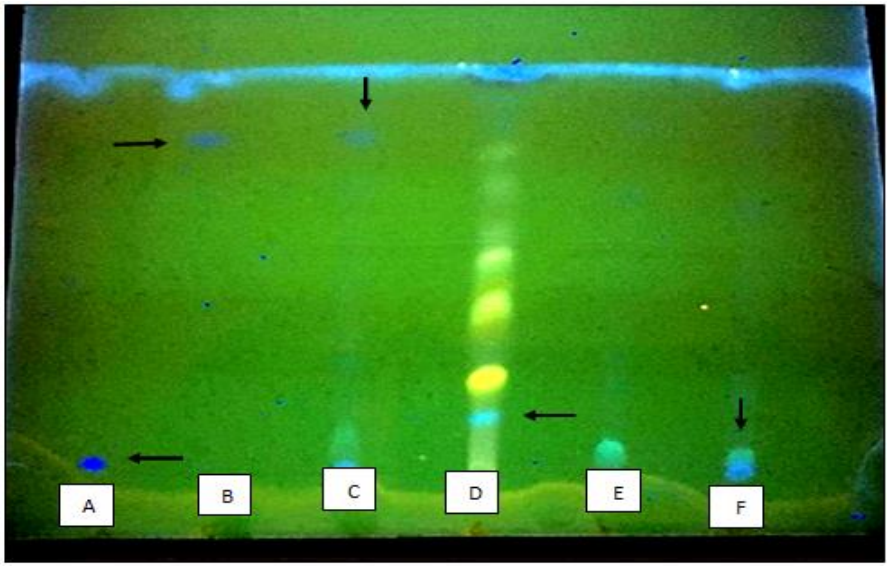


Image (1) The TLC test method demonstrates the ability of the fungal isolates Aspergillus flavus, Aspergillus niger, and Penicillium ssp. to produce Ochratoxin and Aflatoxin , in two food item. A- Ochratoxin control B- Aflatoxin control C- Aspergillus flavus (soybean) D- Aspergillus niger (soybean). F- Penicillium ssp (pistachio)

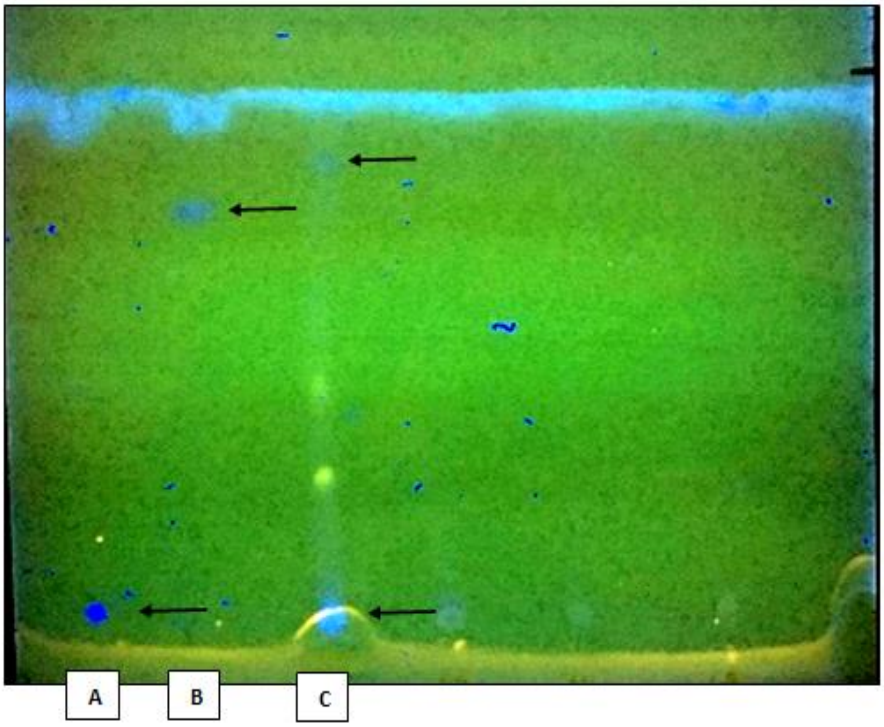


Image (2) The TLC test method demonstrates the ability of the fungal isolate, Aspergillus niger, to produce Ochratoxin and Aflatoxin in one food item

A- Ochratoxin control B- Aflatoxin control C- Aspergillus niger (pistachio)

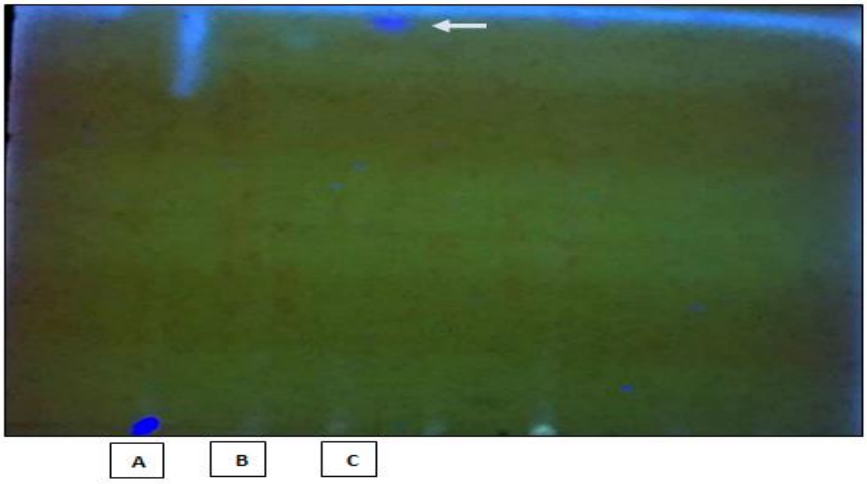


Image (3) The TLC test method demonstrates the ability of the fungal isolate, Aspergillus flavus, to produce Aflatoxin in one food item. A-Ochratoxin control B- Aflatoxin control C- Aspergillus flavus (corn).

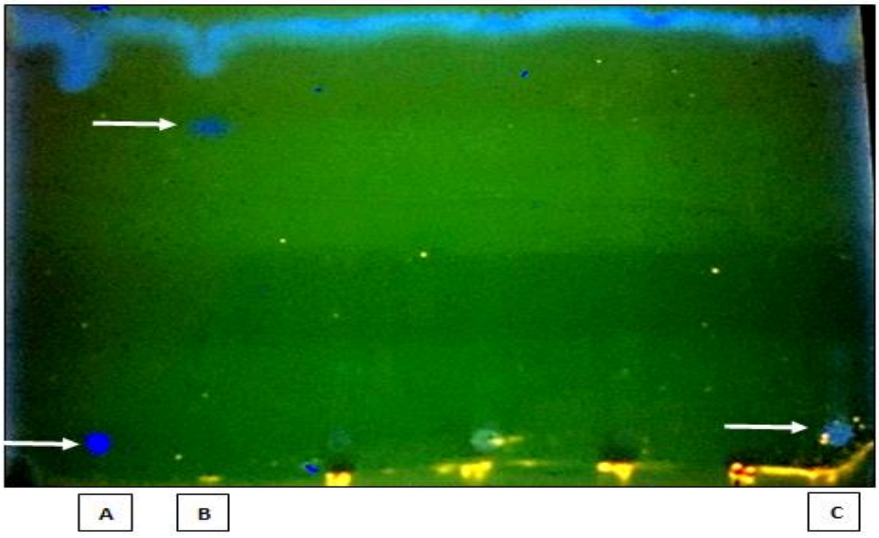


Image (4) The TLC test method demonstrates the ability of the fungal isolate, *Aspergillus niger* to produce Ochratoxin in one food item . A- Ochratoxin control B- Aflatoxin control C- *Aspergillus niger (Anbar rice)*

CONCLUSION

The results of this study showed the of five types of fungi contaminating the food items under study, and that three of them Aspergillus flavus , Aspergillus niger , Penicillium ssp were producing the most dangerous types of mycotoxins, Aflatoxin and Ochratoxin .

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