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Occurrence, Characterizations of Ochratoxin a and Molecular Identification of *Aspergillus Ochraceous* Contaminating Dried Fruits

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ABSTRACT

Background: Dried fruits (raisins, sultanas, and currants) are regarded as ‘‘healthy foods’’ and are also ingredients in muesli, biscuits, cakes, and other foods. **Aim:** The aim of this study was characterize the ochratoxinogenic fungus *Aspergillus ochraceus* in dried fruit. **Methods:** The results indicated the dominant *of A. ochraceus* among the isolated fungi, Morphological characterizations of each isolate, their occurrence and frequency were determined. Also, the possibilities control ochratoxigenic fungi using beneficial fungi in addition to the molecular identification,the capabilities of *Aspergillus ocraceius* to produce ochratoxin A, through Thin Layer Chromatography and HPLC techniques. **Results:** The results revealed that seven fungal species contaminated dried fruits were traditional identified. As, *A. niger* (80), A. *terrus (50), A. flavus (55), Fusarium (66) Nigrosora (35), Penicillium (54) and A. ochraceus (40)*. PCR product analysis of 18S rRNA gene ITS2 gene from extracted DNA of A. ochraceus isolates at (420bp) PCR product. The results of detection using TlC showed that 6 isolates of *ochraceus* were mostly produced *ochratoxi*n in different types of dried fruits of figs, raisins, apricots, Iraqi raisins and apricot. 90% were produced OTA by comparing the color of shine with the extract of each isolate. First sample there is no quantity for ochratoxin, sample carryover factor is 32.35 in sample 3, 4 there is another toxin in sample 5 and carry over factor is 31.76. In Sample No. 6, the carry-over factor was 32.94.
Keywords: *A. ochraceus,* Mycotoxin,PCR, Dried fruits.

Introduction

Fungi are the primary spoiling and toxin-contaminating agents in food and feed during storage and processing, rendering them unfit for human and animal use and resulting in significant financial losses. Improper storage conditions provide a favorable environment for the growth of *Aspergillus* spp. and the development of mycotoxin(s). Mycotoxin contamination of crops and other agricultural products is a major food safety issue [1]. Following many toxicological studies, *Ochratoxin* (OTA) has been shown to have a variety of health consequences for humans and animals, making it one of the most significant mycotoxins of global concern. Renal toxicity, mutagenicity, genotoxicity, teratogenicity, immunotoxicity, and probably neurotoxicity has all been linked to OTA [2]. The International Agency for Research on Cancer has listed OTA as a potential human carcinogen (group 2B) [3].*Ochratoxin* was discovered in 1965 in South Africa, represent a group of related compounds produced by *Aspergillus* ochraceus, *Penicilliumverrucosum, and other Penicillium* species [4]. *Ochratoxin* A (OTA)is the most important fungal toxin of this group than Ochratoxins B and C. OTAexhibits potent nephrotoxicity, hepatotoxicity, cytotoxicity, and immunotoxicity. Also, it is a potential teratogenicandimmunosuppressive. It is possibly a human carcinogen and is of special

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interest as it can be accumulated in the meat of animals. [4,5] Ochratoxin is a group of related compounds formed by *Aspergillus ochraceus, Penicillium verrucosum,* and other *Penicillium* species. It was first discovered in South Africa in 1965 (5). The most important fungal toxin in this community is ochratoxin A (OTA), which is followed by ochratoxin B and C. OTA causes nephrotoxicity, hepatotoxicity, cytotoxicity, and immunotoxicity in humans. It may also be teratogenic and immunosuppressive. It has the potential to be a human carcinogen and is of particular concern because it can accumulate in animal meat [4,5]. Ochratoxin A (OTA), along with aflatoxins, fumonisins, trichothecenes, and zearalenone, is one of the most common mycotoxins with global occurrence and toxicity (2). OTA is produced by toxigenic organisms such as *Aspergillus and Penicillium*. In the case of grapes, Aspergillus niger, A. carbonarius are considered to be potential OTA producers[6]. OTA has been found in a wide range of foods, including cereals, green coffee, spices, nuts, dried fruits, beer, wine, oranges, and grape juice[7]. The kidney organ, is a OTA's target, but it has a variety of other toxic effects, including hepatotoxicity, immunotoxicity, teratogenicity, and neurotoxicity[8].

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Materials and Methods

The Fungi was completely collected in Al hashmiaa in Babylon The fungi from associated with dried fruit used in this research were isolated by surface sterilization of the seeds using a solution of sodium hypochlorate at a concentration of 1 % for a minute and then washed with sterile distilled water three times. The seeds was planted in Petri dishes contained PDA medium with 10 seeds in each dish and in three Repeats and dishes were placed in the incubator at 25˚temperture for a period of 7 days. Fungi isolates were purified and diagnosed according to taxonomic and microbiological characteristics depending on [9], the occurrence and frequency were calculated according to the following equations

Fungal DNA extraction

Fungal genomic DNA was extracted from *A. Ochraceus* isolates by using (**G-Spin DNA extraction kit with modification**) as and done according to company instructions as following steps:

a- 1 ml cultured fungal cells was transferred to 1.5 ml microcentrifuge tube then centrifuged at 10000 rpm for 1 minute then the supernatant was discarded.

a- 180µl GT buffer and 250mg glass bead were added to the tube and the cell pellet suspended by vortex, then 20 µl of Proteinase K was added and the mixtures were incubated at 60ºC for 30 minutes. During incubation periods the mixtures tubes were inverted every 3 minutes.

b- 200µl GB buffer were added to each tube and mixed by vortex for 10 seconds. Then the tubes incubated at 60ºC for 10 minutes with inverted the tubes every 3 minutes through incubation periods.

a- 200µl absolute ethanol were added and immediately mixed by vortex, then precipitates if happen was broken by pipetting.

b- A GD column was placed in a 2 ml collection tube and all mixtures were transferred (including any precipitate) to the GD column. Then centrifuged at 10000 rpm for 1

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minute. The 2 ml collection tubes that contains the flow-through were discarded and placed the GD column in a new 2 ml collection tube.

a- 400µl W1 buffer were added to the GD column, then centrifuged at 10000rpm for 1 minute. The flow-through was discarded and placed the GD column back in the 2 ml collection tube.

b-600µl Wash Buffer were added to the GD column. Then centrifuged at 10000 rpm for 1 minute. The flow-through was discarded and placed the GD column back in the 2 ml collection tube. and the tubes were centrifuged again for 2 minutes at 12000 rpm to dry the column matrix.

a- The dried GD column was transferred to a clean 1.5ml microcentrifuge tube and 100µl of pre-heated elution buffer were added to the center of the column matrix.

b- The tubes were let stand for at least 3 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 10000 rpm for 1 minutes to eluted the purified DNA.

3-2-4-2-2: Estimation of extracted total DNA:

The extracted total DNA was checked by using Nanodrop (**Thermo Scientific NanoDrop Lite UV Visible Spectrophotometer**. **USA**) that measured DNA concentration (ng/µL) and checked the RNA purity at absorbance (260 /280 nm) as following steps:

1. After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, DNA).
2. A dry wipe was taken and cleaned the measurement pedestals several times. Then carefully pipetted 2µl of free nuclease water and placed onto the surface of the lower measurement pedestals for blank the system.
3. The Nanodrop sampling arm was lowered and 1µl DNA sample measured.

PCR master mix preparation

PCR master mixreactions for all genes was prepared by using (Maxime PCR PreMix kit) and this master mix done according to company instructions as following table (2)

Table 2. Standard PCR master mix protocol

PCR Master mix	Volume
DNA template 5-50ng	5µL
Forward primer (10pmol)	2µL
Reveres primer (10pmol)	2µL

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PCR water	13µL
Total volume	20 µL

After that, these PCR master mix components that mentioned in table above placed in standard Maxime PCR PreMix kit tubes that containing all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$,and loading dye). Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes, and then placed in T100 PCR Thermocycler (BioRad-USA).

PCR product analysis

The PCR products were analyzed by agarose gel electrophoresis method as following steps:1.5% Agarose gel was prepared in using 0.5X TBE and dissolving in microwave for 5 minutes, and left to cool for 50°C. Then 3µl ethidium bromide stain were added into agarose gel solution. Agarose gel solution was poured in tray after fixed the comb in proper position and left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray. The gel tray was fixed in electrophoresis chamber and filled by 0.5X TBE buffer. 10µl PCR product were loaded in to each well with added 5µl (DNA marker Ladder) in first well. Then electric current was performed at 100 volt and 80 AM for 1hour.PCR products were visualized by using UV Transilluminator.

Test The Ability of *A.Niger* and *A. Ochraceus* Strains to Produce *Ochratoxin A*

Development of *A.Niger* and *A. Ochraceus* Strains

The Fungus Strains were purified on a sterile PDA media, the discs from the strains were planted with a diameter of 5mm and with 3 replicates per strain, and these discs were placed in the center of the dishes, after which they were incubated at 30˚c for 10 days.

Ochratoxin A Extraction

The Ochratoxin A was extracted from 10 days old cultures of *A. nige*r and *A. ochraceus* isolates grown on PDA at 30 ^{o}C by taking agar plugs according to Macdonald et al., (1999). Small plugs were cut out of the agar near the middle of the mycelium from each plate and then transferred to an electric blender. 100 ml of a mixture consisting of water and chloroform in a ratio of 1:1, after which the mixture was filtered by a 4 layers muslin cloth and then transfer the filtrate to a separating funnel and shake well, then take a chloroform layer and concentrate it by placing it in the electric oven at 45˚C.

High Performance Liquid Chromatography

Ocratoxin A was extracted from samples (5ml or 5gm) by homogenization with 20 mL acetonitrile: H2O (6:4, v=v) for 2 minutes. The extract was filtered and 4 mL of filtrate was diluted with 25mL phosphate buffer saline pH 7.4 (PBS). The samples were degassed in a sonic bath for 30 minutes, then the pH was adjusted to 7.2 using 2 M sodium hydroxide5 mL of acetonitrile is added to the sample and then stored until the analysis is performed

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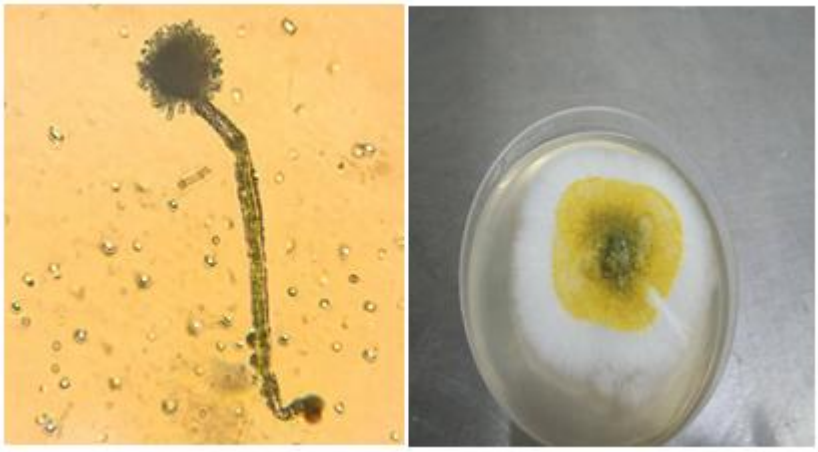
Statistical Analysis

Statistical analysis of results was performed with SPSS (version 16) software (SPSS Chicago, IL, USA). The mean OTA concentration in dried fruit was com- pared by one-way analysis of variance (ANOVA) test.

Result andDiscussion

Isolation and morphological identification of fungi

Analysis of different fungal species isolated from dried fruit samples for morphological and cultural characteristics showed that there was substantial variation in the colony colour, margins, and texture and colony reverse colours. Based on morphological features, 7 species were identified as specified in Figure 1, four of them are belong to Aspergillus: *A. niger*, *A. terrus, A. flavus* and *A. ochraceus*. The fungal analysis of the dried fruits samples showed that 380 of the fungal isolates were counted on PDA in apricot, raisin, Iraqi raisins, and fig samples distribution of fungal contaminated dried fruits. The isolated species belonged to four different genera; namely, Aspergillus spp., Fusarium, Nigrospora and Penicillium. However, the most common genus was Aspergillus spp. as shown in Figure (1).



Ochraceus in micrscop In plate colony A.ochraceus

Figure 1. morphological identification of fungi

Table 1. Number of fungi species isolated from dried fruits

Fungal species	Number of isolates	Proportion of each isolate %
A. Niger	**80**	**21**
A. Terrus	**50**	**13.1**
A. Flavus	**55**	**14.47**
A. Ochraceus	**40**	**10.5**

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Fusarium	**66**	**17.4**
Nigrospora	**35**	**9.2**
Penicllium spp.	**54**	**14.2**
Total	**380**	**99.87**

the percentage of *A. niger* appearing in the Iraqi raisin and raisins is 100%, and in the apricot is 83.3% and 15% in the Fig plant, and it did not appear in the Nut plant. And *A. Terrus* fungus in Iraqi raisins at a rate of 50% and in apricot and raisins at a rate of 33.3%, and it did not appear in the rest of the plants. The fungus *A. flavus* had the highest percentage in the Iraqi raisin, at a rate of 83.3, and the apricot 66.6, and its appearance in the Iraqi raisin 5 and Nut 15 plant did not appear in the rest of the girls. As for the emergence *of fusarium* in Iraqi raisins, its occurrence rate is 100%, and in apricots 50%, raisins and Nut 33.3, and it did not appear in the rest, the fungus *Nigrospora* its occurrence in apricots is the highest 66.6% and in raisins 33.3% and Iraqi raisins 15% did not appear in the rest of the dried fruits, the fungus *penicllium* Its appearance in apricots is 100%, in Iraqi raisins it is 83.3, while raisins are 66.6% and in Fig 15%. It did not appear in the rest of the dried fruits, as for the *Ochraceus* mushroom, its occurrence in Iraqi raisins, raisins, and figs was 50%, and it did not appear in the rest of the dried fruits. studies have shown that the toxin-generating fungi, especially A*spergillus*, are among the main pollutants in dried fruits, and that the fungi *A .niger* and *A.flavus* are the most frequently isolated species in dried apricots, dates, plums and other dried fruits[10,11,12,13]. The present study agrees with the results of other studies in the dominance of the genus *Aspergillus* in samples of corn gypsum, potatoes, dried fruits, and nuts[14]. The same study also confirmed that it is one of the most common species Found in food sources are *A.terreus, A.flavus, A.ochraceus, A.niger .A.parasiticus, A.versicolor A. fumigatus*

Table 2. The percentage of the appearance of fungi isolated from dried fruits

dried fruits	**A.niger**	**A.terrus**	**A.flavus**	**Fusarium spp**	**Nigrospora spp**	**Penicliumspp**	**Ochraceusspp**
Apricot	83.3	33.3	66.6	50	66.6	100	33.3
Raisins	100	33.3	83.3	33.3	33.3	66.6	50
Raisins Iraqi	100	50	50	100	15	83.3	50

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Nut	0	0	15	33.3	0	0	0
Figs	15	0	0	0	0	33.3	50
	15	0	0	15	0	0	33.3

Polymerase Chain Reaction (PCR)

species belonging to the genus *Aspergillus* are potential producers of OTA, a mycotoxin with nephrotoxic, immunosuppressive, teratogenic, and carcinogenic effects. The aim of the present study was to identify the contaminate species of *Aspergillus* found inside of dried fruit. The collected samples were from AL hashimiaa located in the Babylon city and differed in maturity stage and drying status .Concerning the ability of *A. ochraceus* species to produce OTA, all the isolates were able to pro-duce this mycotoxin. These data are similar to those [15] After phenotypic and microscopic diagnosis, molecular diagnosis is made using pcr technique As it is a modern and developed technology and one of the most sensitive and accurate techniques that depends on DNA as amplification by means of it facilitates the identification of the diameter [16,17] These results are similar to what they reached[18] *he initiator pER correlated appropriately with the target genotype and gave positive result Pe-1,Pe-2,Pe-3,Pe-4 ,Pe-5,Pe-6,Pe-7 Pe-8. In 420pb.The PCR assay developed for A. ochraceus identication* in pure culture was also *suc-cationinpureculturewasalsosuc-cation in pure culture was also suc*-cessfully applied for detecting an amplicon of 400 bp. The specicity and sensitivity of the as-say reported by[16].

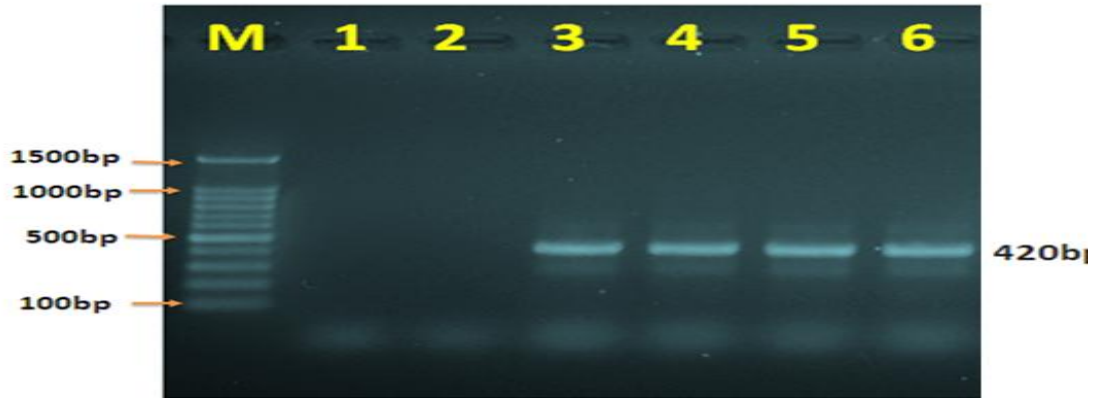

Figure 2. Agarose gel electrophoresis image that showed the PCR product analysis of 18S rRNA gene ITS2 gene from extracted DNA of *Aspergillus ochraceus* isolates. Where M: marker (1500-100bp) and the positive *Aspergillus ochraceus* isolates were showed at (420bp) PCR product

Thin layer chromatographic(TLC)

The results of detection using TlC showed that 6 isolates of *ochraceus* were mostly produced ochratoxin in different types of dried fruits of figs, raisins, apricots, Iraqi raisins and dried apricots, and 90% produced the toxin by comparing the color of shine with the extract of each isolate. First sample there is no quantity for ochratoxin, sample carryover factor is 32.35 in sample 3, 4 there is another toxin in sample 5 and carryover factor is

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31.76. In Sample No. 6, the carry-over factor was 32.94. Similar to our findings, [19]recorded the presence of *ochratoxin A* in dried figs in Turkey. In USA, [20] examined 50,000 figs for fungal infections and measured ochratoxin content in figs with visible fungal colonies. Pooled figs infected with *Aspergillus alliaceus* contained ochratoxin A, figs infected with the *A. ochraceus* group had little or none, and figs infected with *Penicillium* had none. [12]reported that all the samples tested of dried fruits were contaminated by ochratoxin A and the concentrations ranged between 50-110 µg/kg of apricots, 60-120 µg/kg of They also found that all samples of raisins were naturally free from mycotoxins.(18) recorded a detectable amount of ochratoxin A in two samples of dates (360-450 µg/kg). *Ochratoxin* A is a secondary metabolite produced by several species of *Aspergillus and Penicillium* that has been found in a wide variety of cereal grains, coffee beans, cocoa, beer, red wine and recently found in raisins produced in several countries[19]. The difference in toxin concentration may be attributed to Genetic factors, as it was found that the toxin producing isolates had four genes Responsible for the B1 biosynthesis pathway as there Another gene responsible for regulating the activity of these genes is called Structural gene is[21].

Figure3. Thin layer chromatographic(TLC) of ochratoxin in *A. ochraceus*

HPLC Analysis

*H*PLC was performed in a stationary phase and a step gradient polarity system of mobile phase which appropriate *Ochratoxin* A in *ochraceus* compound in the raisin dry fruit sample was identified under the chromatographic conditions Figure(3),appearance of retention time min (4.81) *Ochratoxin A in ochracuse* compound in the fig dry fruit sample was identified under the chromatographic conditions Figure(3),appearance of retention time min (4.86) Other studies confirm the presence *Ochratoxi*n A *in raisin* f. plant like the result [22] The *Ochratoxin* A HPLC chromatogram of the standard appearance of retention time min (0.236) Other studies confirm the presence *Ochratoxin* A in raisin. *Ochratoxin* A in *A.niger* compound in theApricat.raisin iniraq ,nut a dry fruit sample was identified under the chromatographic conditions Figure(4-4),appearancethe maximum of retention time min (4.86) Ochratoxin A in ochracuse compound in the fig dry fruit sample was identified under the chromatographic conditions f. plant like the result[23] The Ochratoxin A HPLC chromatogram of the standard (Fig4) and the positive sample of the maximum concentration(2.887))

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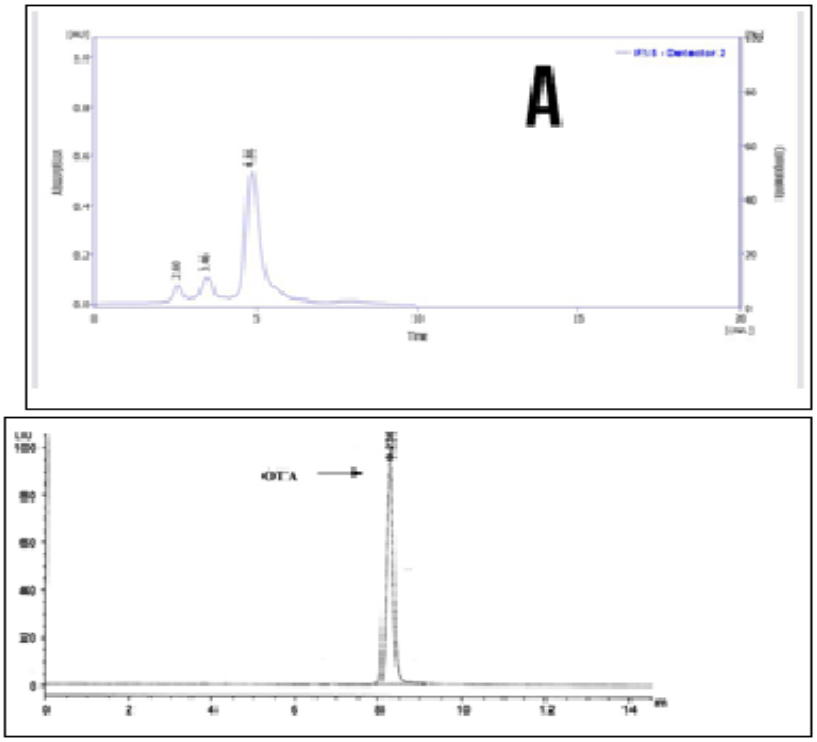

Figure X.:xxxxxxxxxxxxxxxxxxxxxxxxxxxxxx

Table X. xxxxxxxxxxxxxxxxxxxxxx

	Reten. Time	**Area**	**Height**	**Area**	**Height**	**W05**
	[min]	[mAU.s]	[mAU]	[%]	[%]	[min]
1	2.597	305.873	32.834	5.7	9.3	0.16
2	3.460	395.666	32.456	7.3	9.2	0.22
3	4.863	4691.075	285.938	87.0	81.4	0.29
	Total	5392.614	351.229	100.0	100.0	

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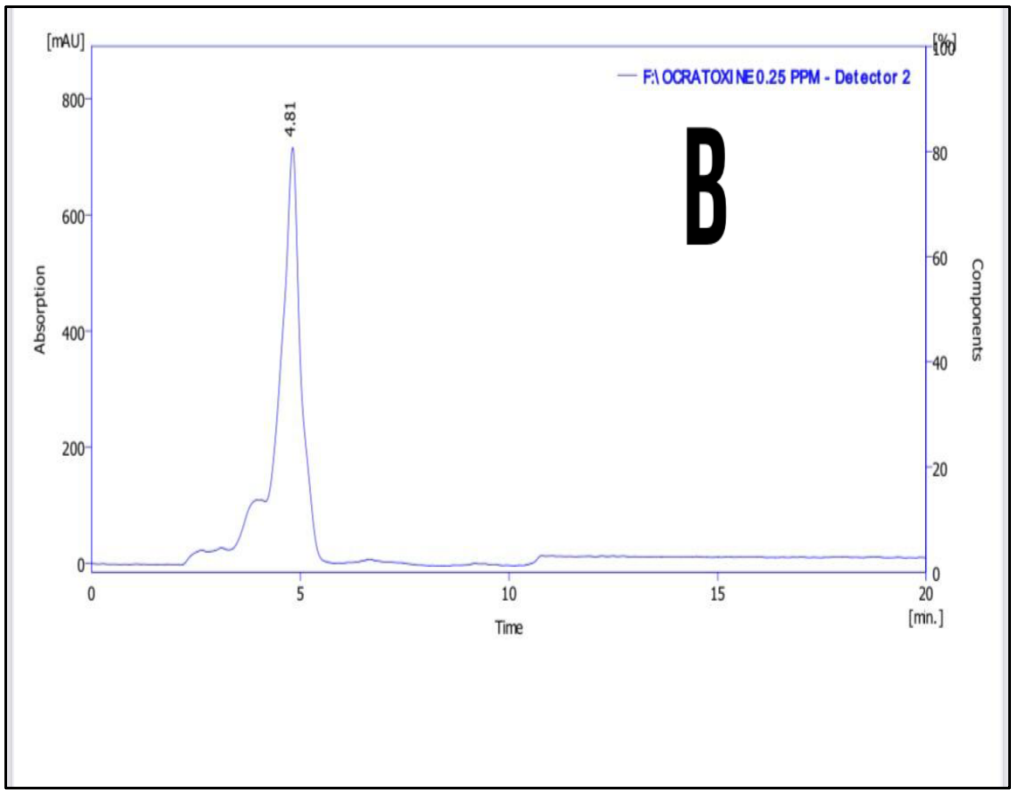

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	[min]	[mAU.s]	[mAU]	[%]	[%]	[min]
1	2.583	93.029	12.798	10.4	16.2	0.13
2	3.443	182.933	18.049	20.5	22.9	0.18
3	4.860	617.765	48.139	69.1	60.9	0.22
	Total	893.727	78.985	100.0	100.0	

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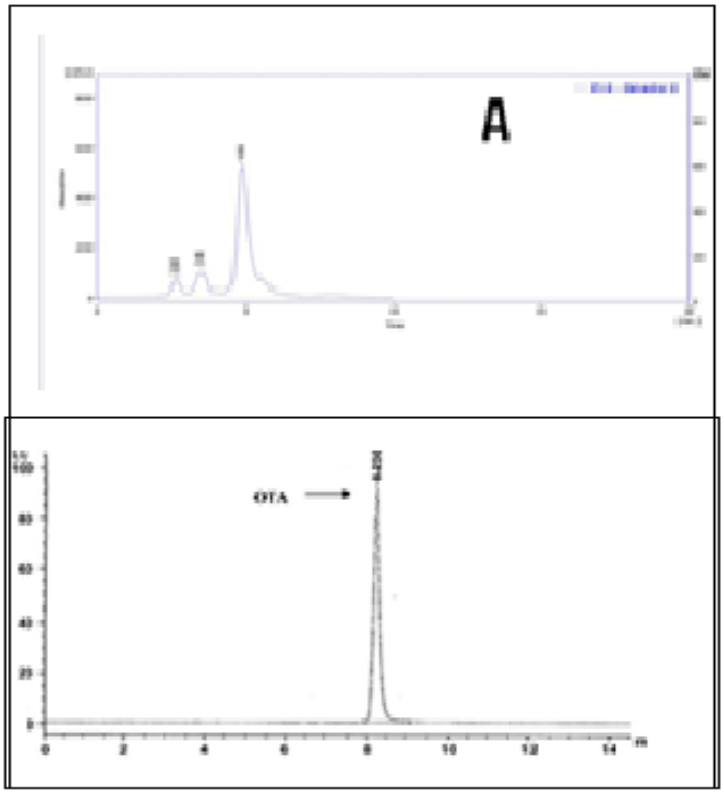

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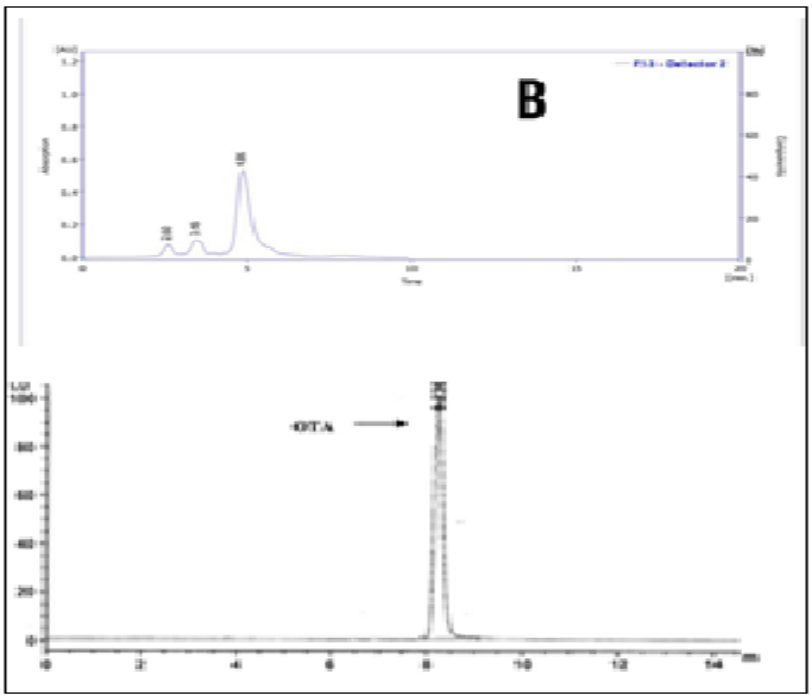

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2	3.460	407.810	32.055	7.2	9.1	0.22
3	4.863	4966.510	286.382	87.4	81.5	0.29
	Total	5680.194	351.271	100.0	100.0	

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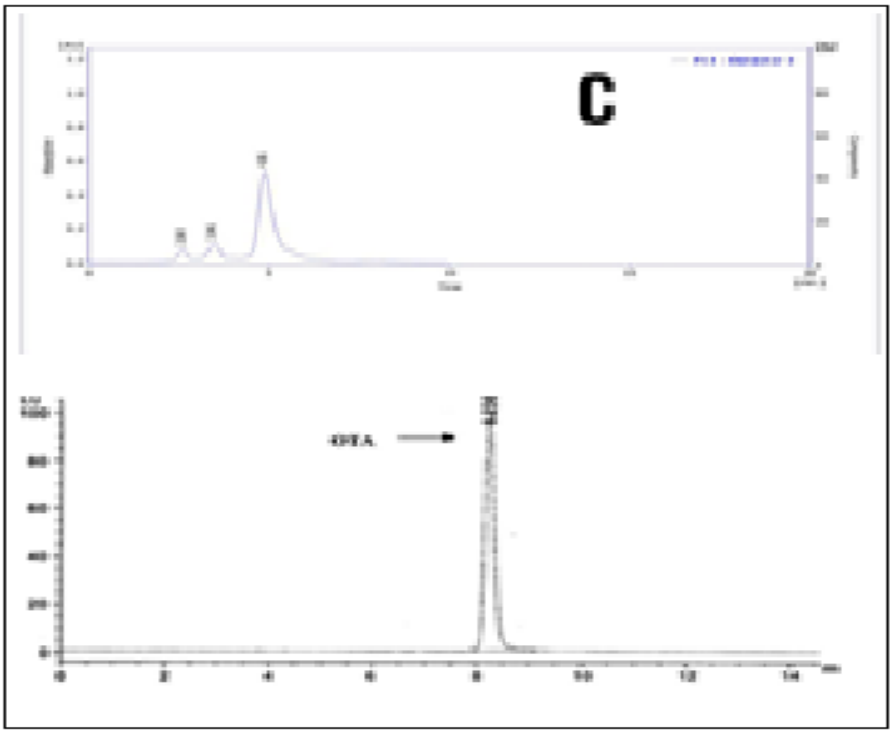

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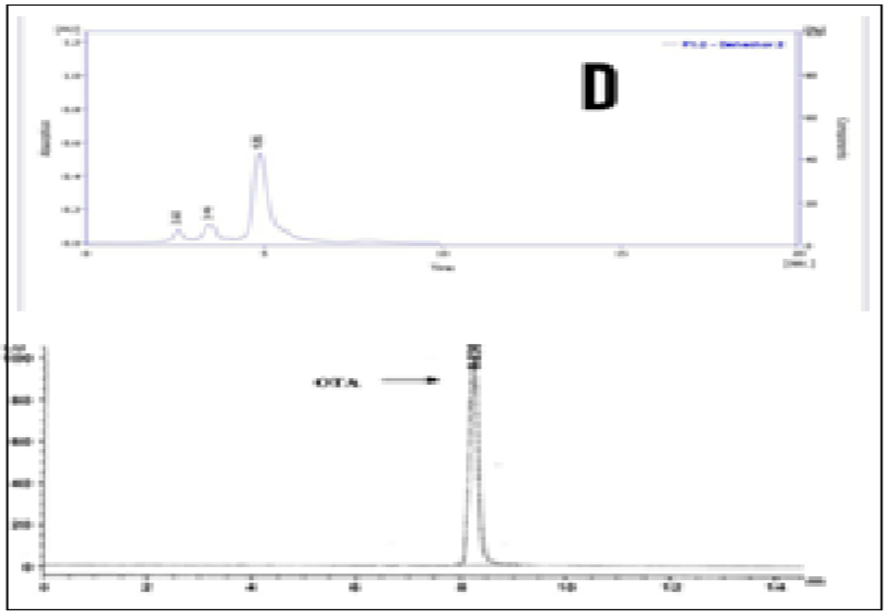

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2	3.460	95.845	12.182	6.4	7.9	0.14
3	4.863	1242.453	123.202	83.6	79.4	0.18
	Total	1486.264	155.164	100.0	100.0	

Conclusions

Based on the data provided in this paper, the analyzed samples focused on the contribution of toxicological risk of *ochratoxins.* However, it is essential to inspect and track dried fruits in the presence of OTA on a regular basis in order to assess hygienic managements. This is the first research to demonstrate that beneficial fungi can reduce OTA levels in dried fruits. During storage periods, a survey of a large number of dried

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fruits is needed at regular intervals. Furthermore, this study is the first step toward conducting a large-scale OTA survey in Iraqi dried fruits.

Reference

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