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FUNGI ASSOCIATED WITH *Sesamum indicum* L. SEEDS AND OCHRATOXIGENIC POTENTIAL OF SOME *Aspergillus* AND *Penicillium* ISOLATES IN DUHOK

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ABSTRACT

Sesame the oil-based seed crop *Sesamum indicum* L. has been widely planted and consume in Iraq. Unfortunately, sesame seeds are particularly susceptible to different infectious agents especially fungi in the field specially if stored improperly. Using culture and immunological approaches, the current study tried to analyze the ochratoxigenic potential of several *Aspergillus* and *Penicillium* isolates from sesame samples in Duhok city.

The results showed the isolation of 24 fungal species belong to 14 genera with number of sterile mycelia and yeasts, also showed the potency of 17 isolates of *Aspergillus* and *Penicillium* to produce OTA. Ochratoxin A (OTA) were detected in cultures of six species of section nigri (*A.carbonarius* and *A.niger* aggregate), one of *Aspergillus* section circumdati species (*A .ochraceus*) and one species of *Penicillium* (*P.verrucosum*). OTA was found at level from 0.63 to 0.70 ng\ml in *A.carbonarius* isolates; from 0.60 to 0.64 ng\ml in *A.niger* aggreg. isolates; in *P.verrucosum* from 0.52 to 0.61 ng\ml and in *A.ochraceus* isolates 0.23 -0.62 ng\ml while none of both *A.sclerotiorum*, *A.japonicus* isolate produce. According to our findings, the occurrence of ochratoxigenic *Aspergillus* and *Penicillium* isolates in sesame seed samples poses a specific risk to consumer health.

INTRODUCTION

The sesame (*Sesamum indicum* L.) belongs to the family Pedaliaceae and one of the world's most and oldest important oil-seeds. It contains 58 to 44 percent oil, 25 percent protein. 13.5 percent carbohydrates and 5 percent Ash. It also contains lot of dietary fibers and micronutrient including iron, phosphate, calcium and potassium, as well as vitamins E, and niacin, thiamine, tocopherols, lignin, and phyto-sterols (8). Sesame is significant in Iraq due of its several applications.

Fungal genera associated with sesame seeds such as *Alternaria, Curvularia, Fusarium, Helminthosporium, Memnoniella, Penicillium,* and *Rhizophus* sp. have been observed associated with sesame, causing seed degeneration in soil before

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germination, seedling mortality, and foliage infection at the adult stage Altaf ets. (8) *Althernaria* sesame is the most damaging disease of sesame, causing little brown patches on leaves ranging from 1 to 8 mm in diameter and reducing seed viability. *Fusarium* sp. causes dark necrotic patches on sesame leaves and yellowing of the foliage is the initial indication of *Fusarium* sp. infection Abarca et. al. (2) Seed rot caused by *Aspergillus* and *Fusarium* caused seed rot, which inhibited seed germination. *Fusarium moniliforme* causes root rot and seedling blight by causing brown necrotic lesions on roots and later becoming a seedling invader.

Contamination from fungal agricultural pests, on the other hand, is a big worry. Nowadays, people pay a lot of knowledge to food -safety and pollutants (35, 55). Fungi-created mycotoxin are among the greatest common food pollutants, posing a threat food safety, to public health, and the economies of many countries, particularly developed ones. Moisture, inherent characteristics and long shelf life, nutrients, high water activity and pH are the most important elements in mycotoxin contamination of Food (53). *Aspergillus* fungus, particularly *A. ochraceus* and *A . carbonarius* and occasionally *A. niger* produce ochratoxins OTA.

Sesame (*Sesamum indicum* L.) is a popular tropical oil seed crop sub-tropical and temperate locations around the world, covering approximately 9,398,770 hectares (ha). Sesame is regarded as a nutrient-dense food as a result of its high oil content, which ranges from 44 to 58% proteins and its nutritious, cosmetics qualities and medicinal (29). Sesame seeds have been proven to have anti-oxidant, anticancer, wound-healing, antihypertensive, neuro-protective, anti-inflammatory properties due to the presence of different bioactive chemicals (23, 39).

The majority of Ochratoxin A (OTA) was first discovered in *Aspergillus* section *Circumdati* (*A .ochraceus*) and *Penicillium* species (*P.nordicum* and *P.verrucosum*) Other species such as *P. crustosum*, *P. chrysogenum*, *P. brevicompactum*, *P. oxalicum* and *P. olsonii* have been claimed as ochratoxin A developers (35). However, multiple studies in the recent decade have found that few species of the *Aspergillus* section *nigri* (*A.niger aggr.* and *A.carbonarius*), which are frequent contaminants of fruits like grapes and their derivatives, are ochratoxigenic (3, 22). In *Aspergillus* section *Circumdati* (previously the *Aspergillus ochraceus* group), the follows species can produce OTA: *Aspergillus. flocculosus, A. cretensis, A. roseoglobulosus A. pseudoelegans, A. sclerotiorum, A. sulphureus* and *Neopitromyces muricatus*. They are the primary cause of OTA contamination in grapes and grapevine products (1, 25). In the Province of Duhok, no investigations on mycotoxins contamination in sesame seeds have been undertaken. As a result, the current investigation attempted to assess the ochratoxigenic ability of various *Aspergillus* and *Penicillium* isolate from sesame samples using cultural and Immunological methods (ELISA) Figure1.

MATERIALS AND METHODS

1 SAMPLES COLLECTION

Figure1: some of the sesame seed samples used incurrent study.

During August 2021 to January 2022, 100 samples (250 g) of sesame seed were collected in sterile polyethylene bags from different places in Duhok province, north of Iraq. Sesame seeds were transformed to the University of Duhok Mycology Research Laboratory to be tested for fungal contamination, ochratoxin testing, and fungal identification. The seeds were confined to 4°C until they were needed.

2 Isolation and Identification of Fungi

The direct plating method as used to analyze sesame seed samples (034). One hundred seeds were surface sterilized with 2% sodium hypochlorite solution for 2 min. at room temperature, followed by two washes with sterilized distilled water and placement on three culture media using international seed testing association ISTA techniques (10-20 seeds per plate): and used different agar culture (Dichloran rose bengal chloramphenicol Agar medium (DRBC) (Germany,Fluka), malt extract Agar (MEA) medium (France, Biokar Diagnostics), and Potato dextrose agar (PDA) (LAB-M, UK). Plates were incubated for 5-7 days at 28°C. The fungus that grew on the seeds were identified either directly or by sub culturing them on another plates of (PDA) and Czapeck's agar media. The identification of fungal species was done using descriptions from earlier investigations (2, 15, 18, 24, 34, 54).

3 Percentage of Frequency

The following formula was used to calculate the percentage frequency of species isolated.

$$\text{Isolation Frequency \%} = \frac{\text{Number of samples on which a fungus appeared}}{\text{Total number of samples}} \times 100$$

4 Identification of *Aspergillus* and *Penicillium* species

Daily, samples of sesame seed were investigated for sporulation fungi using a stereomicroscope (AmScope, China). For identification, pure colonies were produced on appropriate media. Based on physical and cultural features, the majority of observed taxa were identified to species level. Pure colonies of *Penicillium* and *Aspergillus* were grown on four media for identification (24). The following media were used: Czapeck yeast extract agar (CYA25)(K_2HPO

41 gr, Czapek concentrates 10gr, Powdered Yeast Extract 5 gr, sucrose 30gr,agar 15gr and distilled water 1 liter , incubated for seven days at 25°C, czapeck yeast extract agar (CYA37) incubated for seven days at 37°C, czapeck yeast extract agar with 20% Sucrose incubated for seven days at 25°C (CY20S) K_2HPO_4 1 gr, Czapek concentrates 10gr, Powdered Yeast Extract 5 gr, sucrose 200 gr ,agar 15gr and distilled water 1 liter incubated for seven days at 25°C (24, 34). For each culture, two plates of CYA and one plate each of CY20S and MEA were used. Each plate is inoculated in the center and incubated for seven days in the light at 37°C, one CYA is incubated. The others are being incubated at 25 degrees Celsius.

5 Confirmation test for *Aspergillus* and *Penicillium* species identification

5a Ehrlich test

The Ehrlich test was developed by Frisvad et. al (17) to distinguish species of the *Penicillium* subgenus *Penicillium* by detecting alkaloids interacting with Ehrlich reagent (18) using a filter paper method. The Ehrlich reagent is 2 g 4-dimethylamino benzaldehyde in 96 percent ethanol (85 ml) combined with 15 ml 10 N HCl. On the mycelia side of an established colony on CYA (incubated for 5–9 days at 25°C), a four-mm agar plug is cut out, and a piece of wetted filter paper (Whatman No. 1) is placed on the mycelia side. In section Nigri Samson et. al (44) employs this method to classify numerous *Aspergillus* species.

5b Growth on Creatine Sacrose Agar (CREA)

On Creatine Sucrose Agar medium (creatine(1H2O),3g; sucrose,30g; KCl,0.5g; MgSo4.7H2O,0.5g; FeSO4.7H2O,0.1g; K2HPO4.3H2O,1.3g; bromocresol purple,0.05g; agar,15.0g and distilled water,1L), the growth capacities of all *Aspergillus* species in section Nigri isolates were examined CREA is a semi-selective medium that can be used to classify diverse fungal cultures, particularly *Penicillium* species. Formation of acid (changing the medium from purple to yellow) and production of base can be employed as diagnostic features on CREA colonial growth characteristics. CREA can be used to divide all black aspergilli into groups in a semi-selective manner (44). All species were identified using the keys and descriptions provided by the author (2, 18, 24, 43, 45).

6 Determination

The toxigenic potential of fungus in culture media Cabanes et. al (13) and Saito and Machida (43) Saito and Cabanes rapid methods for detecting ochratoxigenic isolates section Nigri based on color change following exposure to ammonia vapor, were used to study the ochratoxigenic potential of all reported *Aspergillus* isolates from section Nigri. Coconut cream agar Petri plates were used to grow the isolates. The medium was prepared according to the instructions of Dyer and McCammon (14), Lin and Dianese (26) Each strain was inoculated in the center of solidified coconut cream agar medium in 9-cm Petri dishes and incubated at 27°C in the dark. After 4 days of incubation, the dishes were flipped upside down and a drop of ammonia solution was placed into the lid of the dish to see the color shift of the colony reversal.

7 Extraction of Ochratoxin A from Fungal culture

The ability of isolates from the *Aspergillus* and *Penicillium* genera to produce OA was tested. The extraction of ochratoxin A was done using the Bragulat et. al. (12) technique. The isolates were cultivated for 7 days on Czapek

yeast autolysate agar (CYA) medium at 25°C. Each colony had three plugs (7 mm diameter) removed from the inner, middle, and outside portions. Plugs were placed in 3-ml vials, 1 ml methanol was added, the vials were shaken, and the vials were incubated at 25oC for 60 minutes. The extracts were centrifuged three times at 4000 rpm for five minutes each time. The supernatant was filtered via a Millipore filter (0.22)m filtration membrane (Millex GP Filter unit Coringhwohill Co Ireland). The extracts were then analyzed using ELISAs (Enzyme linked Immunosorbent Assays) Figure 2.

a b

Figure 2: Green fluorescent a on the back of a colony developed on coconut cream agar (an Ochratoxin A (OTA)-producing strain) (365 nm). Under the same circumstances, a non-OTA producing strain showed no fluorescence (b).

8 Ochratoxin Analysis

The enzyme linked immunosorbent assay was used to produce a quantitative study of OA (ELISA). This was done in accordance with the manufacturer's instructions (Veratox quantitative ochratoxin test-Neogen Corporation-USA). The OTA concentration was estimated using a standard curve derived from OTA standards and expressed in ng/ml.

9 Preservation of the Isolated isolates

Preservation of all fungi isolates was done using culture of PDA in sterilized vial glass and preservation with glycerol and stored at 5˚C in a refrigerator.

RESULTS AND DISCUSSION

Fungal species, frequency percentages of genera, and species of fungus isolated during the investigation are shown in Table 1 and figure 3 there were 15 genera and 24 species identified from 100 samples, as well as unidentified yeasts and sterile mycelium. Seven species of *Aspergillus* were found, with the most diversity of all of the retrieved taxa. Three species of black aspergilli were found (*A. carbonarius* 1.3%, *A. japonicas* 0.44% and *A. niger* 3.0%). Other aspergillus species include *A.ochraceous* 1.5%, *A.flavus* 1.0% ,*A.fumigatus* 0.33% and *A.sclerotiorum* 1.0%.In the Kurdistan region, several species were detected in soil and other agricultural items (7, 40) The possibility for contamination of commodities by *Aspergillus* and their toxins is increased by climatic circumstances characterized by humidity and temperature, as well as poor storage techniques 0(36) and Several investigators have noted the abundance of *Aspergillus* in preserved nuts bean (7, 39) figure 4.

Table 1: The genera and species of fungal isolates and the frequency percentages

S.N	Frequency (%) of fungal genera on 7PDA MEA and DRBC 1	
	Fungal genera	(%) Frequency
1	*Absidia* sp.	2.0
2	*Alternaria alternata*	0.5
3	*Aspergillus carbonarius*	1.3
4	*A.flavus*	1.0
5	*A.fumigatus*	0.33
6	*A.niger*	3.0
7	*A.ochraceous*	1.5
8	*A.japonicus*	0.44
9	*A.sclerotiourum*	1.0
10	*Penicillium citrinum*	1.5
11	*P.expansum*	1.0
12	*P.verroucosum*	0.5
13	*Curvularia* sp.	1.0
14	*Emericella nidulans*	0.77
15	*Eurotium amstelodami*	0.66
16	*Fusarium oxysforum*	0.33
17	*Geotrichum candidum*	0.3
18	*Monilia* sp.	0.28
19	*Mucor circinelloides*	0.25
20	*Rhizopus stolonifera*	0.22
21	*Sterile mycelium*	0.17
22	*Trichoderma* sp.	0.15
23	*Cephalosporium* sp.	0.1
24	Yeast non identified	0.1

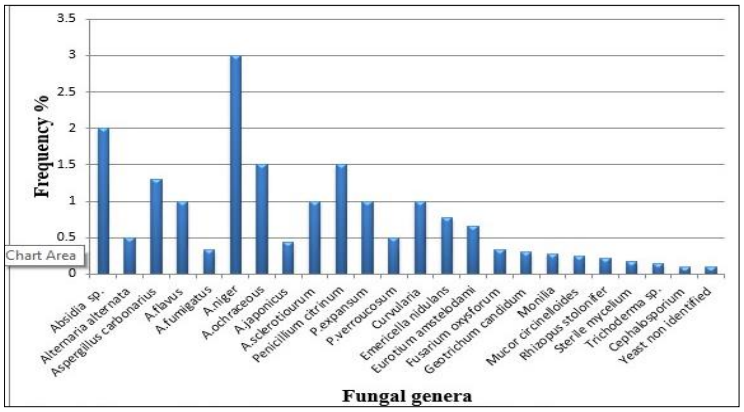

Figure 3: Frequency (%) of fungal genera on DRBC.PDA and MEA medium.

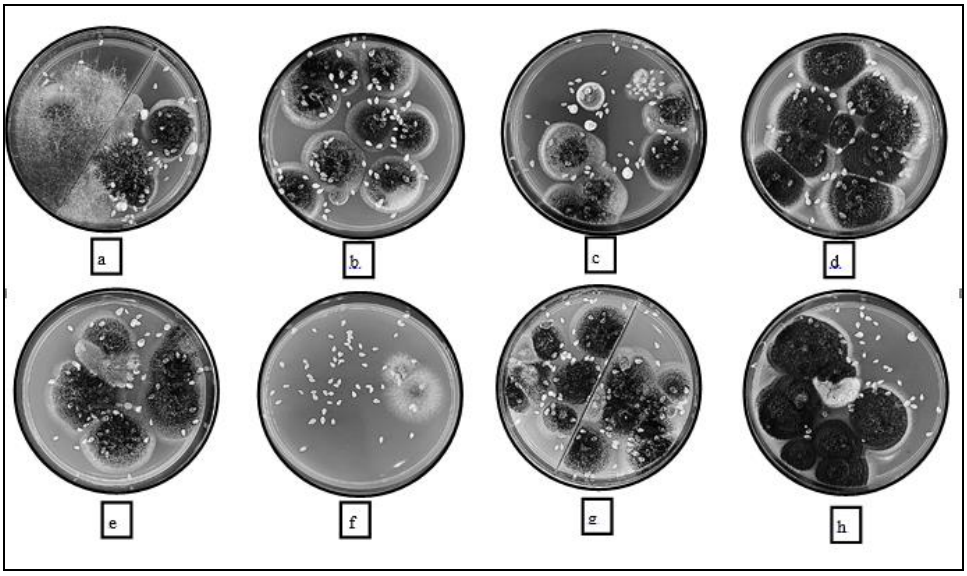

Figure 4a: Growth of *A.niger* and *A.carbonarius* , *Rhizopus stolonifer* and *M.circinelloides* on sesame seed bean on DRBC, b. Growth of *A.niger* and *A.japonicus* and *P.expansum* on sesame seed bean on DRBC medium, c. Growth of *A.niger* and *A.japonicus* and *P.verrocusum* on sesame seed bean on DRBC medium, d. Growth of *A.niger* and *A.carbonarius* and on sesame seed bean on MEA medium, e. Growth of *A.niger* and *A.flavus* and *A.carbonarius* on sesame seed bean on MEA medium, f. Growth of *A.fumigatus* and *E.amstelodami* on sesame seed bean on PDA medium, g Growth of *A.niger* and *P.citrinum* ,*A.flavus* and *A.carbonarius* on sesame seed bean on PDA medium h. Growth of *A.niger* and *A.japonicus,* and *A.ochraceous* on sesame seed bean on MEA.

***Penicillium* was second in the number of isolated species from seed and was represented by three species. *P.citrinum* was the most common species (1.5 %), followed by *P. expansum* (1%) and *P. verrocusum (*0.5%). *Aspergillus* and *Penicillium* species mainly grow during storage. Two teleomorphic ascomycetes, namely, *Emericella nidulans and Eurotium amstelodami*_were detected with percentage frequencies 0.77% and 0.66% respectively. Isolates of *E. amstelodami* from turkish dried figs produced Aflatoxin B1 and ochratoxin A when grown on potato- dextrose -broth (PDB) medium0 (47).**

Other genera and species include *Absidia* sp., *Alternaria alternate*, *Curvularia* sp., *Fusarium oxysoprum*, *Geotrichum candidum*, *Monilia, Mucor circinelloides* ,*Rhizopus stolonifer* ,*Trichoderma sp Cephalosporium* sp. with frequency percentages of (2.0, 0.5, 1.0, 0.33, 0.3, 0.28, 0. 25, 0.22, 0.15)% and 0.1% and yeast 1%.

After 7 days at 25°C, the capacity of the fungal isolates to produce OA was tested using solid laboratory medications (CYA). As indicated in 2.6, a fast method of extraction of OA by Bragulat et. al. [0](12) was used. Table 3.

Eleven isolates belonged to four species wrer showed ochratoxigenic potential,two pecies from section Nigri (*A.carbonarius, A.niger aggr*.), one *Aspergillus* species from section circumdati (*A.ochraceus,*), and one species of *Penicillium* were found to contain ochratoxin A. *(P.verrucosum*). For *A.carbonarius, A.ochraceus* the proportion of OTA generating isolates examined by ELISA technique was 100 percent, while the percentages of ochratoxigenic potential in *A.niger* aggr. and *P.verrucosum* were 60 percent and 66.6 percent,

respectively (Table 3). Isolates from *A. carbonarius a*nd, to a lesser extent, *A. niger*, demonstrated the ability to produce ochratoxin A and were isolated often from dried vines naturally contaminated with orchratoxin A in the Kurdistan region (41).

The proportion of ochratoxigenic isolates in the genera *Aspergillus* and *Penicillium* reported from various parts of the world varies considerably based on the number of isolates studied and geographical regions. Several publications have investigated the ochratoxigenic potential of *A.carbonarius* strains (3, 24,29). All of these research revealed that this species has a constant ability to create OA, with ochratoxigenic potential of *A.carbonarius* isolates ranging from 41.7 to 100%.

Several publications have reported a percentage of ochratoxigenic isolates in *A.niger* ranging from 0.8 to 18.5 percent (3, 09, 037, 50).

Magnoli revealed that 30% of *A.niger* from Brazilian grapes was an OA producer and (28, 51) discovered an abnormally high percentage (43.1%) of ochratoxigenic strains in *A.niger* from Italian grapes.

The uniseriate black *Aspergillus japonicas* showed no ochratoxigenic producing ability (Table 2). This is in line with what is mentioned by literature 0(11, 24, 28,) All the three isolates of *A.ochraceus* were positive for ochratoxin A producing ability. However Serra et. al. (50) and according to the study, 50 percent of *A.ochraceus* strains isolated from Portuguese wine grapes were able to produce OA. Some authors in Argentina, Brazil, and Spain have reported a greater percentage of OA positive isolates among *A.ochraceus*. (20, 28).

Out of 3 strains of *P.verrucosum*, two were positive for OA production. *P.verrucosum* is the major species producing OA in cereals such as wheat and barley in temperate and cold climate Cabafies et. al. (13) however, the fungus was isolated from samples of dried vine grapes.

Table 2: Percentage (%) of ochratoxin A producing strains and range of OTA detected

Fungus (from culture)	Ochratoxin A ng/ml
A.japonicus	0.00
A.carbonarius	0.65
A.carbonarius	0.63
A.carbonarius	0.65
A.ochraceus	0.62
P.verrucosum	0.52
A.niger aggr.	0.64
A. niger aggr.	0.00
A.carbonarius	0.70
P.verrucosum	0.00
A.niger aggr.	0.62
P.verrucosum	0.00
A. sclerotiorum	0.00
A.ochraceus	0.23
A.niger	0.00
A.ochraceus	0.21
A.niger aggr.	0.60

Table 3: List of fungal isolate examined for ochratoxin A by ELISA technique

Species	No. of isolates tested	OA producing isolate (%)	Rangeng/ml.
A.carbonarius	4	100	0.63-0.70
A.japonicus	1	0	N.D
A.niger aggr.	4	75	0.60-0.64
A.ochraceus	2	100	0.23-0.62
A.sclerotiorum	1	0	N.D
P.verrucosum	3	66.6	0.52-0.61

N.D =Not detected.

The possibility for contamination of commodities by *Aspergillus* and their toxins is increased by climatic circumstances characterized by humidity and temperature, as well as poor storage techniques Riba et. al. (36) and several investigators have noted the abundance of *Aspergillus* in preserved nuts bean (10, 39).

CONCLUSION

According to our findings, the occurrence of ochratoxigenic *Aspergillus* and *Penicillium* isolates in sesame seed samples poses a specific risk to consumer health. As a result, such contaminated products should be examined for the presence of fungi. It's crucial to identify the fungi in order to determine out which mycotoxins are present inexpensive culture approaches for detecting ochratoxigenic contamination when restricted resources prevent the use of analytical procedures. To limit the risk of fungal infection, seeds should be treated with a suitable chemical before sowing.

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الفطريات المرافقة لبذور السمسم وقابلية بعض عزلات من الفطرين اسبرجلس وبنسيليوم على انتاج الاوكراتوكسين في محافظة دهوك

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الملخص

محصول السمسم من المحاصيل الزيتية المهمة في العراق، يتعرض المحصول للإصابة بالعديد من المسببات المرضية خاصة الفطريات المرافقه للبذور. تضمنت الدراسة عزل وتشخيص الفطريات المرافقة لبذور السمسم في محافظه دهوك والتحري عن قابلية بعض عزلات الفطرين اسبرجلس وبنسليوم على إنتاج السم الفطري اوكراتوكسين باستخدام الطرق الزرعية والمناعية. أظهرت النتائج عزل 24 نوعاً تعود الى 14 جنساً فضلاً عن عدد من الفطريات العقيمة والخمائر، أظهرت نتائج قابلية 17 عزلة من الفطرين اسبرجلص و بنسليونم لانتاج السم الفطري اوكراتوكسين ستة انواع من مجموعة nigri (*A. carbonarius* and *A. niger* aggreg.) ونوع واحد من مجموعة circumdati (*A. ochraceus*) ونوع واحد من جنس بنسليوم (*P. verrucosum*). اوكراتوكسين A وجدت بمعدل (ng/m 0.63-0.70) في *A. carbonarius* و بمعدل (0.60-0.64 ng\ml) في *A.niger* aggreg وجدت بمعدل (0.52-0.61 ng/ml) في*P.verrucosum* ; وبمعدل (0.23-0.62 ng\ml) في*A.ochraceus* بينما لم يفرز كل من *A.japonicus* و *A.sclerotiorum*. بخصوص النتائج المكتشفة فأن وجود الفطريات اسبرجلس والبنسليوم المنتجة للسم الفطري اوكراتوكسين في بذور السمسم يمثل خطراً مهدداً لصحة المستهلك.

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