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Isolation, Diagnosis and Characterization of *Aspergillus sp* from Tea Bags Samples and Its Ability to Produce *Ochratoxin A*

ABSTRACT

This study was carry out to investigate from the contamination of packed tea samples collected from different markets with Aspergillus species. The extent of contamination of these samples with *Ochratoxin* A (OTA) was examined by using ELISA and the ability of *Aspergillus niger* to produce this toxin was determined by using the (HPLC) technique. The results showed that Aspergillus species contaminated 66 packed tea samples. *Aspergillus niger* was isolated from 34 black tea samples while *Aspergillus ochraceus* isolated from 20 samples and Aspergillus *fumigatus* from 23 samples. On the other hand, the green tea showed 22 samples contaminated with *A.niger*, 21 *A. ochraceus* and 15 A. *fumigatus*. The rate of fungi isolation from the different tea samples were *Aspergillus niger* (41.48 %), *A. ochraceus*)30.37%(and *Aspergillus fumigatus* (28.1%).The quantitative detection of *Ochratoxin A* by using ELISA technique showed the presence of OTA in tea samples as it reached in the samples of Ahmad black tea (2.27 µg/kg), which is the least concentrated than the rest of the studied teas and the highest concentration rate (6.46 µg /kg) it appeared in samples of Ahmed green tea. The results of quantitative estimation using (HPLC) also showed the ability of *A. niger* to produce OTA as it produced this toxin at a concentration of 12.36 ppb / 100 ml.

INTRODUCTION

Tea (Camellia sinensis) is a famous aromatic beverage all over the world. After water, it's a popular and enjoyable drink (Gebrewold, 2018). Black, Green, Oolong, Pu-erh, and White teas are the five fundamental types of tea, each having its own distinct flavor. Teas have a strong fragrant flavor and contain theine and caffeine, but in less amounts than coffee. (Eshiet and Smith, 2018). Also, tea has been used as a traditional treatment for different conditions, such as blood pressure reduction, decreasing LDL cholesterol, being anti-diabetic, recovering gut health, improving heart health, and reducing the risk of stroke and cancer, Black tea has numerous antioxidant chemicals that are helpful to one's health and can also aid in the reduction of inflammation. Green tea is also one of the healthiest beverages on the globe since it is high in polyphenol antioxidants, such as atechin, as well as minerals. (Sentkowska and Pyrzyńska, 2019). These combinations provide a range of health benefits, including improved brain function and anti-neurodegenerative (anti-Parkinson and anti-Alzheimer), as well as antidepressant effects. These compounds can also prevent the generation of free radicals in the body, which play a key role in aging and the development of a variety of diseases. Tea also contains a variety of nutrients, such as vitamin E, vitamin C, fluoride, and potassium (Natarajan et al., 2019; Akbarialiabad et al., 2021).

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Mycotoxins are secondary fungal metabolites produced by specific mold (fungi) strains that are well-known for posing a serious health risk to humans. Skin necrosis, leukopenia, immunodeficiency, and possibly liver cancer are all possible side effects of mycotoxins (Perdoncini et al., 2019). Due to the non-protein structure of these toxins, they are often resistant to heat and might compromise the health of those consuming such contaminated foods, despite the cooking process (Peromingo et al., 2019).

Mycotoxins that have been shown to be carcinogenic, genotoxic, teratogenic, and renal and hepatotoxic. Fungi from the genera *Aspergillus*, *Fusarium*, *Claviceps*, *Penicillium*, *Stachybotrys*, and *Altenaria*, among others, create mycotoxins (Makhuvele et al., 2020). Even at low doses, they can injure humans and animals (Adekoya et al., 2019). During manufacturing, pre-harvest, and post-harvest, mycotoxins contaminate mostly cereals, grains, nuts, and their by-products (Gbashi et al., 2018). Toxins enter the body mostly through ingesting, but also through inhalation, parental, and dermal exposure. Toxins can also enter the food chain through contaminated crops, which are consumed directly or indirectly as feed sources by humans or animals. As a result, they can be found in meat, milk, and eggs (Hojnik et al., 2017). *Ochratoxin* A (OTA), the most common and significant fungal toxin generated by Aspergillus species and Penicillium species (Liuzzi et al., 2017) is found in cereals, coffee, wine, dried fruits and nuts, and meat products. (Fink-Gremmels, 2005). OTA has a wide range of harmful effects on the host, including carcinogenic consequences (Polovic et al., 2018), nephrotoxic (Vettorazzi et al., 2019), hepatotoxic (Sobral et al., 2018). Because OTA is processed and stored mostly in the liver and kidney, these organs are the primary targets for OTA's harmful effects OTA has been linked to liver inflammation and potentially cancer in previous studies (Wang et al., 2019).

The most prominent global fungi linked with the post-harvest degradation of various materials is *Aspergillus niger*, which is a filament of low efficiency and has the capacity for rapid growth and pH tolerance, It is globally distributed, as it can be isolated from all continents, and it is not very picky about environmental conditions, as it can grow at temperatures ranging from 6 to 47 Cº, pH ranging from 1.5 to 9.8, and aqueous activity ≥ 0.77..(Pitt and Hocking, 2009). A. *niger* is one of the fungus that has been classed as GRAS (Generally Recognized as Safe) by the FDA (Tsang et al., 2009), A. *niger* was discovered to produce high amounts of citric acid in a medium containing sugar (Currie, 1917).

MATERIAL AND METHODS

A total of 70 teabags sample, commercially and packed were randomly purchased. The samples include 40 black tea, 20 green tea and 10 herbal tea from different brands. Ten grams of each sample added to a 90 mL portion of sterile saline (85%) in a 500 mL Erlenmeyer beaker and homogenized completely by an electric shaker at a constant speed for 15 minute (Aziz et al., 1998). Then a series of dilution were performed. One ml of each dilution was added to sterile petri dishes and then sterilized Potato dextrose agar medium poured. Plates were incubated at 28 C° for 7 days and then examined for fungal growth. The isolates identified by using the main keys for mold diagnosis (Mohammed, 2011).

Ochratoxin A Determination:

the toxin was extracted from Tea bags samples by Enzyme linked immune sorbent assay technique (ELISA). The procedure of extracting and determination of the toxin concentration was carry out according to the instruction of the manufacturing company (Shenzhen- China).

Determining the most productive isolate of *ochratoxin* A from A. *niger*:

Extract the OTA according to the method described by Tan and Richard (2012) with some modifications as follows:

In a 500 ml beaker, mix 50 grams of milled wheat with 50 ml of distilled water, then sterilize at 121°C for 15 minutes. The sterilized wheat medium was then injected with A. niger using a 1 cm diameter disc and incubated for 21 days at 28 ± 2cº with manual shaking more than 10 times daily After the incubation period is through, sterilize the flask with an autoclave, then put with 150 ml of a 30: 105: 15 mixture of water, acetonitrile, and acetic acid, in that sequence, close it, and shake it slowly by electric vibrator and suspension was filtered using filter paper (What man No 2), then

was transferred 100 ml of the filtrate to the separating funnel, 40 g of anhydrous magnesium sulfate and 10 g of NaCl were added. The funnel was shaken well for one minute, the lid was opened to get rid of gases, and then left on an iron holder until the separation is completed, the lower layer is pulled out, and then the filtration process is carried out again. The filtrate is concentrated using a rotary evaporator, then stored in an opaque glass bottle at 5 C^{o} until quantification is performed by HPLC.

Quantitative determination using HPLC technology:

The examination was carried out were compared with standard toxin supplied by Shenzhen company in the laboratories of the Ministry of Science and Technology - Department of Environment and Water, using a high-performance liquid chromatography device, model (SYKAMN) of German origin, where the mobile phase was acetonitrile: distilled water: (70:30) and separation column: (C18 -ODS). (25 cm * 4.6 mm) and a fluorescence detector (ex=365nm, em=445nm) was used, where the flow velocity of the transporting phase was: 0.7 ml/minute (Liu et al., 2012).

RESULTS AND DISCUSSION

The result of the study showed close proportions for the fungal contamination in the different types of tea bag that collected randomly from different market in Ramadi – Iraq. The fungi that contaminated tea samples belong to the genus Aspergillus, represented by A. *niger*, *A. ochraceus* and A. *fumigatus*. The identification of Aspergillus species based on the morphological characteristics of colony on PDA medium and microscopic examination of fungal structure (hyphae and spore). A. *niger* showed black powdery colony on PDA medium while upon the microscopic examination showed septate and branched hyphae with thick – walled conidiophore that carry on their end a large spherical vesicle (Zulkifli and Zakaria, 2017) as shown in (Figure 1).

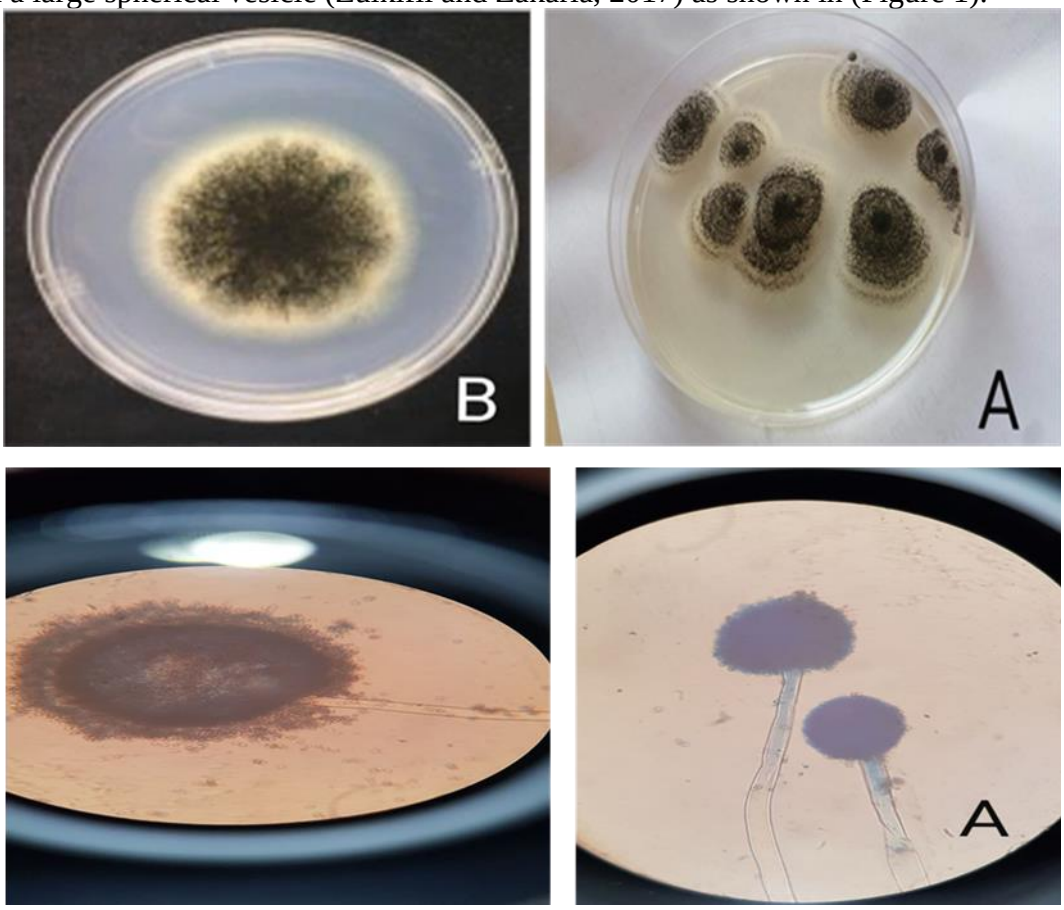

Figure (1) Morphological and microscopic characteristics of *A. niger*

A. *fumigatus* growing on the PDA showed white fungal hyphae on the edge of circular growth while the spores found in the center of circular growth with blue to green color. For a microscopic examination, the *sporangiophore* appeared above its vesicles, which

appear in a semi-circular shape carrying chains of small conidia (O, Gorman et al., 2009) as shown in (Figure 2).

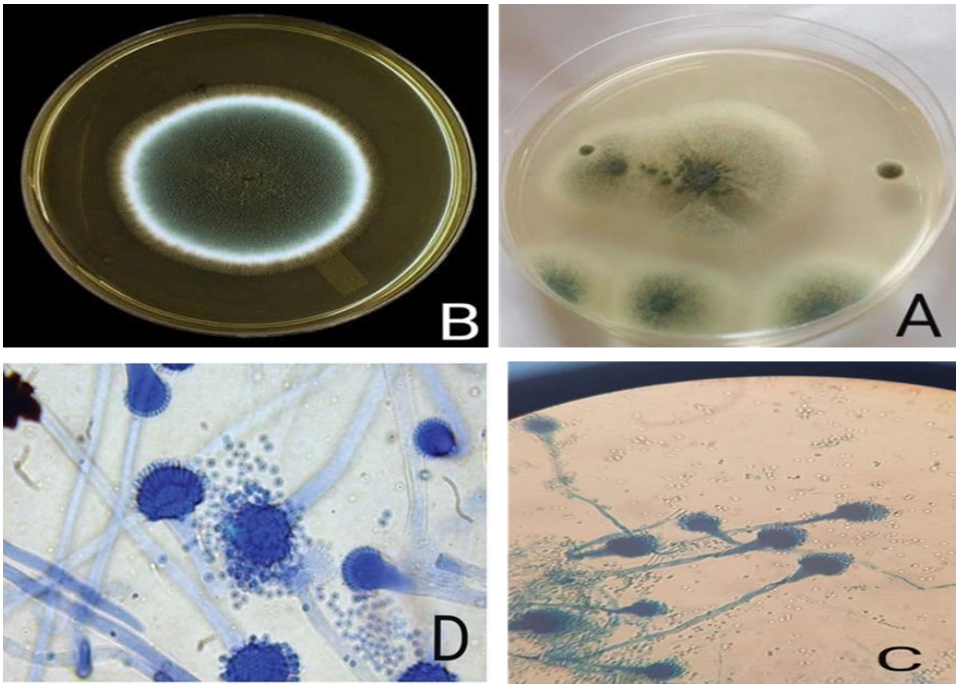

Figure (2) Morphological and microscopic characteristics of A. *fumigatus*

A. *ochraceus* colonies grew on PDA appeared in yellow color and some colonies may be colored pink to purple irregular pebble-like. Upon microscopic examination, the carriers of A. *ochraceus* appeared densely and the phialides were arranged finely or coarsely on the heads of the conidia in a bilateral way (the phialides are attached to intermediate cells called metulae, which in turn are attached to the vesicle) (Refai and Hassan, 2013) as shown in (Figure 3).

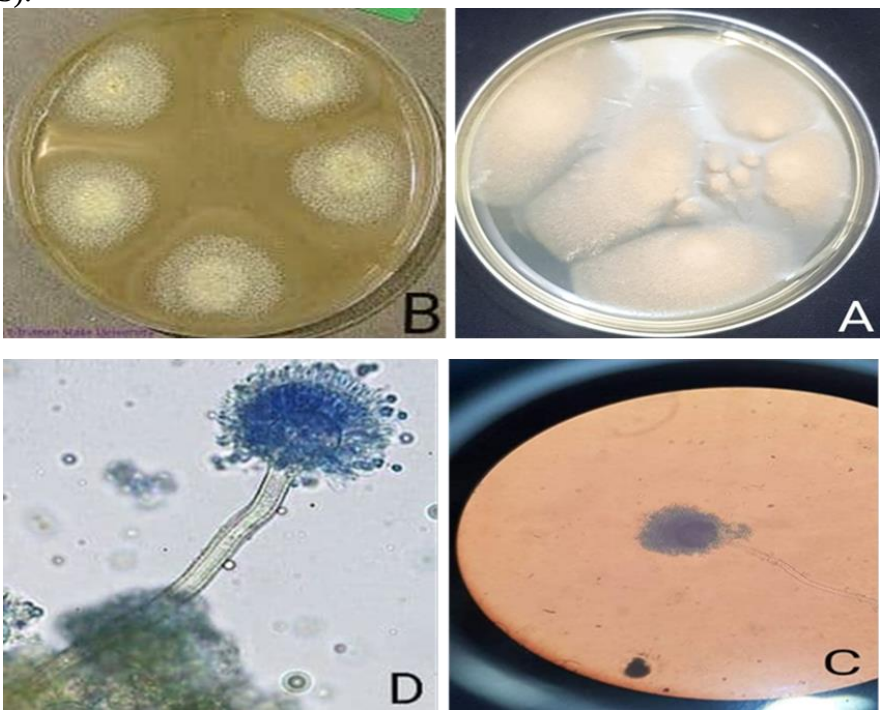

Figure (3) Morphological and microscopic characteristics of A. *ochraceus*

The results of isolation and diagnosis showed that the frequency of A. *niger* in Ahmad black tea samples was (9) and the type A. *fumigatus* was (7) then A. *ochraceus* was followed by (3) samples. As for Ahmad black tea infused with cardamom, the frequency of Aspergillus species was

as follows A. *niger* (8), A. *ochraceus* (5) and A. *fumigatus* (3), While the frequency of A. *niger* in Mahmood black tea samples was (8) and A. *ochraceus* (6), and A. *fumigatus* was (5, and in Mahmood black tea infused with cardamom, A. *niger* appeared at a frequency (9) followed by A. *fumigatus* at frequency (8), then A. *ochraceus* at frequency (6).

As for green tea, the results of isolation and diagnosis showed that A. niger fungus in Ahmad tea samples had a frequency in (8) samples, while the frequency of A. ochraceus was in (7) and *A. fumigatus* (5), as well as in Mahmood tea the frequency was *A. niger* (8) and *A. ochraceus* (6) and *A. fumigatus* had a frequency (7), while A. niger appeared in green tea with mint samples at a frequency (7) and *A. ochraceus* with a frequency (8), while *A. fumigatus* appeared in samples its frequency was (3) as shown in Table -1.

Table (1) The total counts of *Aspergillus* species contaminated the tea samples

Samples	*A. niger*	*A. fumigatus*	*A. ochraceus*	Total
Ahmed Black Tea	9	7	3	19
Ahmed Black tea with cardamom	8	3	5	16
Mahmood Black Tea	8	5	6	19
Mahmood Black tea with cardamom	9	8	6	23
Ahmed Green Tea	7	5	7	19
Mahmood Green Tea	8	7	6	21
Green tea with mint	7	3	8	18
Total	56	38	41	135

The percentage of fungi frequency in the total samples of Tea bags (black + green):

The results of the study shown in Table (2) that the percentage of contamination with *A. niger* fungus amounting to 41.48% of the total tea samples (black, green) agreed with the results of previous studies, which showed that A. *niger* fungus is the most common in tea samples contamination, reaching up to the percentage of contamination with this fungus is 80% of the total samples used in the study (Zhou et al., 2019), as indicated by Ye et al. (2020) to the contamination of tea samples with *A. ochraceus*, which causes the production of a large part of the OTA present in tea, which is what it agrees with the results of the current study, as the contamination rate with this fungus was 30.37%, and it was shown by Xu et al. (2019) that the contamination of tea with *A. fumigatus i*s at a lower level and this agreed with the findings of the current study, as the percentage of this fungus reached 28.15% of the total fungi developing the results also showed that the pollution rate in green tea is less than that in black tea .

Table (2) The rate of Aspergillus species in different tea samples

Tea sample brand	*A. niger*	*A. ochracues*	*A. fumigatus*	Total %
Black tea	34	20	23	(57%) 77
Green tea	22	21	15	(43 %) 58
Total %	(41.48 %) 56	(30.37%) 41	(28.15%) 38	(100 %)135

Determination of *Ochratoxin- A* concentrations in tea bags by ELISA technique:

The results of the ELISA-based analysis showed the presence of OTA in all tested samples and these results were divided according to the types of tea used in the research as shown in Table – 3

Table (3) *Ochratoxin-A* average concentration in tea samples

Samples	OTA concentration (µg/kg)
Ahmed Black Tea	2.27
Ahmed Black tea with cardamom	3.42
Mahmood Black Tea	3.79
Mahmood Black tea with cardamom	3.33
Ahmed Green Tea	6.46
Mahmood Green Tea	6.44
Green tea with mint	5.17

The results showed a variation in the average OTA concentration in the packed tea samples, as it reached in the samples of Ahmad black tea (2.27) µg/kg, which is the least concentrated than the rest of the studied teas, followed by Mahmood black tea infused with cardamom with a

concentration rate of (3.33) µg/kg and Ahmad tea Black grafted with cardamom at a rate of (3.79) µg/kg, then the results began to gradually rise, as the average concentration of OTA toxin A in green tea with mint reached (5.17) µg/kg and Mahmood green tea (6.44) µg/kg, and the highest concentration rate was (6.46) µg/kg in Ahmad green tea, and this is consistent with the range indicated by Ye, et al.,(2020) which mentioned that the OTA ratio ranged between (2-10) µg/kg, and the concentration rate also agreed with what was reached by Pakshir et al. (2020) , which showed that the percentage of OTA in tea samples ranged between (2.81-6.9) µg/kg for both green and black tea samples, while the concentration rate (2.27) µg/kg for Ahmed black tea was less than the range reached by the latter.

The *Ochratoxin-A* concentration rate of Mahmood green tea and Ahmad green tea samples was higher than the limit established in the regulations of the Joint Committee between Food and Agriculture Organization and the World Health Organization on Food Additives (JECFA) of (5) µg/kg, while the rest of the tea types were within the permissible limits. Results for all types of tea with EU regulations that indicated that the maximum permissible limits (OTA) ranged between (2-10) µg/kg for all foods (Pakshir et al., 2020).

Determination of *Ochratoxin-A* in vitro *by A. niger* isolates using HPLC technique:

A.niger isolate producing the highest concentration of *ochratoxin* A were cultured for 21 days at 28 °C, then the toxin was extracted and measured by HPLC, where the result showed that A. *niger* produced an OTA at a concentration of 12.36 ppb / 100 ml.

The results agreed with what was indicated by Passamani et al. (2014) which showed that isolates of A. *niger* have the ability to produce OTA with concentrations ranging between 1.47 - 9.96 ppb, and also Freire et al. (2018) showed that isolates of A. *niger* produce OTA with different concentrations according to The growth period, as the lowest rate of toxin production was between 2.86-11.14 µg/kg during the growth period 3-6 days. The highest rate of toxin production occurred during 15 days of growth as it reached 17.58 µg/kg, then the percentage decreased after that to reach the toxin concentration to 13.45 µg/kg after 21 days of development.

This type of fungi has strains that have the ability to produce more than one type of toxins depending on the basic components and environmental conditions surrounding the fungus (Frisvad et al., 2011).

CONCLUSIONS

From the results, it was conclude that the packed tea samples were contaminated with *Aspergillus spp* (*A. niger, A. ochraceus and A. fumigatus*) and A.*niger* was the most common fungi isolated from tea packed samples . The results of ELISA technique showed that all studied samples were contaminated with OTA. The quantitative estimation by using (HPLC) technique showed that A. *niger* has the ability to secrete OTA in the suitable conditions for growth.

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عزل وتشخيص وتوصيف فطر *Aspergillus niger* من عينات الشاي المعلب ((Tea bags)) ودراسة قابليته على إنتاج سم الاوكرا-A

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الخلاصة

أجريت هذه الدراسة بهدف فحص أنواع من جنس *Aspergillus sp* الملوثة لعينات مختلفة من الشاي المعلب المباع في الأسواق المحلية ثم فحص مدى تلوث هذه العينات بسم الاوكرا A باستخدام تقنية ELIZA وتحديد مدى قدرة *Aspergillus niger* على إنتاج هذا السم باستخدام تقنية السائل عالي الأداء (HPLC) .

أظهرت نتائج العزل والتشخيص أن أنواع *Aspergillus sp* التي لوثت 66 عينة من الشاي المعلب هي *A. niger* و *A. ochraceus* و *A. fumigatus* حيث بلغ تردد *A. niger* في عينات الشاي الأسود بواقع 34 عينة ، بينما تردد *A. ochraceus* في 20 عينة ، اما النوع *A. fumigatus* فقد تردد في 23 عينة. أما بالنسبة لعينات الشاي الأخضر، فقد تردد فطر *A. niger* في 22 عينة و *A. ochraceus*تردد بواقع 21 عينة بينما تردد *A. fumigatus* في 15 عينة، بينما بلغت النسبة المئوية لهذه الفطريات في مجموع عينات الشاي (41.48٪) ، (30.37٪) ، (28.15٪) على

الكلمات المفتاحية: *A.niger* , الشاي المعلب , الاوكرا A , HPLC , ELISA

التوالي ، ومن خلال مقدار التردد والنسبة المئوية تبين أن فطر *A. niger* هو أكثر أنواع الفطريات شيوعاً في عينات الشاي المدروسة.

بينت نتائج الكشف الكمي باستخدام تقنية ELIZA إن معدل التركيز في شاي احمد الأسود (2.27 µg/kg) والذي يعد الأقل من باقي أنواع الشاي المدروسة أما أعلى معدل للتركيز (6.46 µg/kg) فقد ظهر في عينات شاي احمد الأخضر.

كما أظهرت نتائج التقدير الكمي باستخدام تقنية السائل عالي الأداء (HPLC) قدرة فطر *A. niger* على إنتاج الاوكرا A حيث أنتج هذا السم بتركيز 12.36 جزء في البليون / 100 مللتر.