

## Identification of Fungi and Detection of Mycotoxins Associated with Infected Fig Fruits

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**Abstract:** The aim of this research is to isolate and identify the fungi associated with infected fig fruits in different Governorates in Egypt (Alexandria, Qalubia, Ismailia, Giza and Sinai), as well as study their ability to produce aflatoxins. Samples of fresh fig fruits were collected, small slices of peel were disinfected and transferred to Petri-dishes containing PDA medium and incubated at 25°C for 5 days. The higher total fungal counts as 201, 111 and 102 isolates were recorded on the samples from Qalubia, Sinai and Ismailia, respectively and the least as 48 isolates was found on samples from Giza. Seven fungal genera were identified (*Alternaria tenuis*, *Aspergillus flavus*, *A. niger*, *A. parasiticus*, *A. terreus*, *Botryodiplodia theobromae*, *Cladosporium* sp., *Fusarium solani*, *Penicillium* sp. and *Rhizopus* sp.). All *Aspergillus* genera produced mycotoxins except *Aspergillus terreus* which was negative producer of mycotoxins (AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub>).

**Keywords:** Aflatoxins, *Aspergillus*, figs, fungi, mycotoxins

### INTRODUCTION

Edible figs (*Ficus carica* L.) is a deciduous tree or shrub belonging to the Moraceae family and native to Asia Minor, from where it spread into the Mediterranean regions.

The fig is a nutritious fruit, rich in fibers, potassium, calcium, iron and is free of sodium, fat and cholesterol (Stover *et al.*, 2007). Additionally, figs are an important source of vitamins, amino acids and antioxidants (Solomon *et al.*, 2006).

In Egypt, figs are widely grown in North West Coast and North Sinai and the total cultivated area is about 69865 Acer. Egypt is the second largest producer of fig worldwide after Turkey with annual production of 176.105 tons per year according to FAOSTAT (2014).

Fungal infection of the fig fruits may occur during the growing season, harvesting, handling, transport and post-harvest storage and marketing conditions. The fig fruits contain high levels of sugars, nutrients, and with low pH the fruits are vulnerable to fungal decaying (Singh and Sharma, 2007). About 70% of crop diseases were due to fungi causing high economic losses, major crop disease and postharvest losses of agriculture products (Agrios, 2005).

The problem of fig fruits is the developing of fungal decay inside the fig fruits; furthermore ostiole size seems to be correlated with the incidence of fungal decay and with poor quality of fruit (Ferguson *et al.*, 1990).

Doster and Michailides (2007) and Coviello *et al.* (2009) reported that the major fruit disease affecting figs are smut in dried fruits caused by *Aspergillus niger*, Alternaria rot caused by (*Alternaria alternata* or other *Alternaria* spp. and often associated with other fungi such as *Cladosporium herbarum* or *Ulocladium atrum*),

gray mold or Botrytis rot (caused by *Botrytis cinerea*), fig endosepsis (caused by *Fusarium moniliforme* and other *Fusarium* spp.) and sour rot or souring (caused by various yeasts and bacteria including species in the genus *Hanseniaspora*, *Saccharomyces*, *Pichia* or *Bacilli*). They also found that *Aspergillus flavus* and *A. parasiticus* can produce the mycotoxins known as aflatoxin.

Sekar *et al.* (2008) identified *Aspergillus flavus*, *A. niger*, *Rhizopus* sp., *Mucor* sp. and *Penicillium* sp. from different samples of figs, maize, dates and grapes which collected from storage shops.

Bhale (2011) stated that the common postharvest and storage fungi of fig fruits are *Alternaria* spp., *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp.

Embaby *et al.* (2012) isolated 140 isolates from fresh figs and 80 isolates from dried figs. Five fungal genera were identified *i.e.* *Alternaria*, *Aspergillus*, *Fusarium*, *Mucor* and *Rhizopus* from fresh and dried figs. Some isolates of *Aspergillus* were positive producer of aflatoxins while the others were negative producer.

Saadullah and Abdullah (2014) collected samples from four types of dried fruits (apricot, fig, grapes and plum). A total of 20 species of *Aspergillus* were isolated on Dichloran Rose Bengal Chloramphenicol Agar medium (DRBC medium). *Aspergillus awamori*, *A. carbonarius*, *A. flavus*, *A. fumigatus*, *A. japonicas*, *A. niger*, *A. ochraceus*, *A. parasiticus* and *A. tubingensis* were detected from all types of dried fruits.

Farjood and Banhashemi (2013) isolated 24 *Aspergillus* species from soil, leaf and fig fruit samples. The *Aspergillus* species percentages were *A. alliaceus* (3.13), *A. carbonarius* (7.44), *A.*

*flavus* (35.1), *A. fumigatus* (6.38), *A. japonicus* (2.13) and *A. terreus* (2.13).

The present research aims to isolate and identify the fungi associated with fig fruits in different regions in Egypt, as well as study their ability to produce aflatoxins.

## MATERIALS AND METHODS

This work was carried out during the seasons 2015-2017 at the Laboratory of Plant Pathology, Department of Agricultural Botany, Faculty of Agriculture, Suez Canal University.

Samples of fresh fig fruits (*Ficus carica* L.) were collected from Alexandria, Giza, Ismailia, Qalubia, and Sinai Governorates in Egypt, put into sterile polyethylene bags. Date and place of samples collections were recorded, and then the bags were sealed properly and brought into the laboratory of plant pathology for further studies.

Fig fruits were washed with tap water followed by sterile distilled water several times. Then they were surface sterilized by dipping in 1% sodium hypochloride (NaOCl) for 2 min., after that, the fruits were washed twice with sterile distilled water. Small slices of peel from sterilized fruits were again disinfected with 0.5% NaOCl for 1 min, followed by rinsing with sterile distilled water and dried on sterilized filter papers. The slices were transferred to Petri-dishes containing Potato Dextrose Agar medium (PDA) and incubated at 25°C for 5 days. Fungal growth on fruits were isolated and purified on PDA slant.

All fungal isolates (7-14 days old) were identified in Plant Pathology Dept., Faculty of Agriculture, Suez Canal University, based on cultural and morphological characteristics on specific PDA medium according to Maren and Johan (1988) for genus *Aspergillus*, Nelson *et al.* (1983) for *Fusarium*, and Barent and Hunter (1977) for the genera of imperfect fungi, Singh *et al.* (1991) for either *Aspergilli*, *Fusaria*, and *Penicillia*, Samson and Varga (2007), Watanabe (2010), Oranusi and Wesley (2012), Agwa *et al.* (2014) and El-Fadaly *et al.* (2015).

All identified fungi were cultured on PDA slants (Tournas and Katsoudas, 2005) and stored at 4°C until used. Total fungal counted and frequency percent of naturally occurred fungi in fruit samples were calculated.

### Mycotoxin analysis:

Samples of fresh fig fruits were prepared according to the method described by (AOAC, 2007) in Food Toxicology and Contamination Dept. National Research Center (NRC) for detection of aflatoxins (AFs).

To prepare spore suspension, spores from colonies (6-10 days old) of *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus parasiticus*, and *Aspergillus terreus* were collected by adding 5 ml of sterile distilled water to each Petri dish

containing spores and rubbing the surface with a sterile L-shaped spreader (3 times). The suspension was collected and then centrifuged at room temperature (Tzortzakis, 2009). The suspension was diluted with the sterile water to obtain the spore suspension ( $10^5$  spores/ml).

Mycotoxins were detected using 100g of fig fruits which were surface sterilized with 70% ethanol and then wounded. Five microliters of the spore suspension at  $10^5$  CFU/ml were injected into each wound then incubated at 26°C for 15 days.

Aflatoxins AFB1, AFB2, AFG1 and AFG2 were determined in artificially inoculated fig fruits using High Performance Liquid Chromatography (HPLC) according to (AOAC, 2007).

The HPLC instrument used for aflatoxins determination was waters (474) system, equipped with the quaternary pump fluorescence detector set system at 360 nm excitation and 440 nm emission wave lengths. The chromatography column was phenomenex c18 (250 x 4.6 mm), 5  $\mu$ m. The mobile phase was water: methanol: acetonitrile (H<sub>2</sub>O: MeOH: CH<sub>3</sub>CN) 3:1:1v/v/v) was isocratically at a flow rate of 1 ml/min (Han *et al.*, 2004).

Data were collected and integrated using Total Chrome Navigator Chromatography Manager Software.

## RESULTS AND DISCUSSION

### Isolation and Identification of Fungi:

Data in Figure (1) showed that, fig fruit samples from Qalubia area gave the highest total fungal count as 201 isolates of fungi equal 37.43% comparing with other governorates. Sinai samples produced 111 isolates equal 20.67% then, Ismailia samples which recorded 102 isolates of fungi equal 18.99%. The least total fungal isolates with 48 isolates equal 8.94% was found on the fig fruit samples from Giza.

### Fungal frequency:

The fungal frequency detected with rotted fig fruits samples from the different governorates in Egypt is presented in Table (1). The fungal species which belong to seven genera were identified. These were *Alternaria tenuis*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus parasiticus*, *Aspergillus terreus*, *Botryodiplodia theobromae*, *Cladosporium* sp., *Fusarium solani*, *Penicillium* sp. and *Rhizopus* sp. (Figures 2-8).

Also, data showed that, the genus *Aspergillus* was the most frequently isolated fungus with 303 isolates. Eighteen of these isolates (3.35%) for *Aspergillus flavus*, 186 isolates (34.64%) for *Aspergillus niger*, 81 isolates (15.08%) for *Aspergillus parasiticus* and 18 isolates (3.35%) for *Aspergillus terreus*.

The detected fungi which followed genus *Aspergillus* were *Rhizopus* sp. which recorded 63 isolates (11.73%), *Penicillium* sp. 45 isolates

(8.38%), *Cladosporium* sp. 42 isolates (7.82%), *Alternaria tenuis* 36 isolates (6.70%), *Fusarium solani* 36 isolates (6.70%) and *Botryodiplodia theobromae* was the fungal species occurred with 12 isolates (2.23%).

Briefly, data showed that *Aspergillus niger* was the higher fungal frequency (34.64%) associated with fig fruits samples. Whereas, *Botryodiplodia theobromae* was the lowest fungal frequency (2.23%) followed by *Aspergillus flavus* and *Aspergillus terreus* (3.35%).

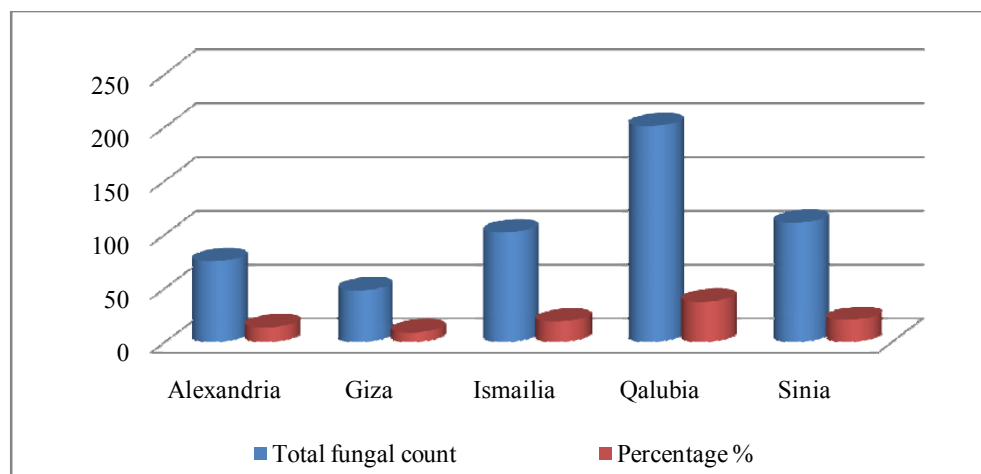


Fig. (1): Total count and percentages of fungi associated with rotted fig fruits from different governorates in Egypt

Table (1): Fungal frequency associated with rotted fig fruits from different governorates in Egypt

Fungal isolates	Governorates										Total	
	Alexandria		Giza		Ismailia		Qalubia		Sinai			
	T.C.F	%	T.C.F	%	T.C.F	%	T.C.F	%	T.C.F	%	T.C.F	%
<i>Alternaria tenuis</i>	ND	0.00	9.00	1.68	9.00	1.68	ND	0.00	18.00	3.35	36.00	6.70
<i>Aspergillus flavus</i>	3.00	0.56	ND	0.00	9.00	1.68	3.00	0.56	3.00	0.56	18.00	3.35
<i>Aspergillus niger</i>	18.00	3.35	18.00	3.35	30.00	5.59	75.00	13.97	45.00	8.38	186.00	34.64
<i>Aspergillus parasiticus</i>	9.00	1.68	9.00	1.68	15.00	2.79	30.00	5.59	18.00	3.35	81.00	15.08
<i>Aspergillus terreus</i>	ND	0.00	ND	0.00	3.00	0.56	15.00	2.79	ND	0.00	18.00	3.35
<i>Botryodiplodia theobromae</i>	3.00	0.56	ND	0.00	ND	0.00	9.00	1.68	ND	0.00	12.00	2.23
<i>Cladosporium</i> sp.	30.00	5.59	ND	0.00	ND	0.00	12.00	2.23	ND	0.00	42.00	7.82
<i>Fusarium Solani</i>	3.00	0.56	3.00	0.56	3.00	0.56	18.00	3.35	9.00	1.68	36.00	6.70
<i>Penicillium</i> sp.	ND	0.00	ND	0.00	18.00	3.35	18.00	3.35	9.00	1.68	45.00	8.38
<i>Rhizopus</i> sp.	9.00	1.68	9.00	1.68	15.00	2.79	21.00	3.91	9.00	1.68	63.00	11.73
<b>Total</b>	75.00	13.97	48.00	8.94	102.0	18.99	201.00	37.43	111.0	20.67	537.00	100.0

ND= Not detected T.C.F= Total count of fungi %= Fungal frequency occurred

These results were in agreement with Embaby *et al.* (2012) who isolated 140 isolates from fresh figs and 80 isolates from dried figs. Five fungal genera were identified as *Alternaria*, *Aspergillus*, *Fusarium*, *Mucor* and *Rhizopus* from fresh and dried figs. Some isolates of *Aspergillus* were positive producer of aflatoxins, while the others were negative producer. Embaby *et al.* (2014) isolated *Alternaria citri*, *Botryodiplodia theobromae*, *Fusarium* sp., *Penicillium digitatum* and *Penicillium italicum* from citrus fruits and reported that the genus *Penicillium* had the most

isolation frequency from both Behira and Qalyoubia governorates in Egypt.

In the present study, results revealed that *Aspergillus niger* was the most frequently isolated fungal species (34.64%) followed by *A. parasiticus* by (15.08%), whereas *A. flavus* and *A. terreus* by (3.35%) for each. This is in line with data presented by Saadullah and Abdullah (2014) who found that the most fungal species isolated from figs were *A. niger* followed by *A. flavus*, *A. carbonarius* and *A. parasiticus* with a percentage

frequencies of 76.65%, 66.6%, 33.1% and 33.1%, respectively.

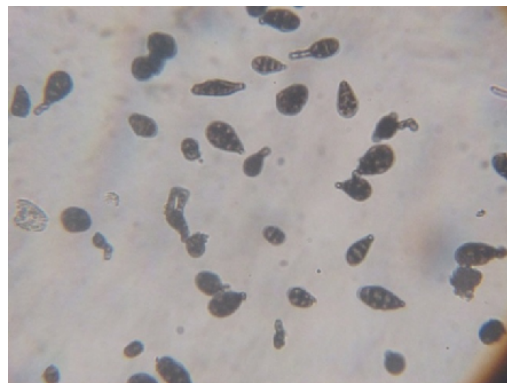
*Aspergillus flavus* and *A. niger* were reported as being the most common species on dried figs, Pitt and Hocking (2009). Also Embaby *et al.* (2012) noted that *A. niger*, *A. flavus* and *A. parasiticus* were the most frequent species on dried figs in Egypt. Similar results were reported by Doster *et al.* (1996) from figs in California.

Javanmard (2010) found that the most frequently isolated species from Iranian figs was *A. niger* aggregate (90%) followed by *A. flavus* (63.76%).

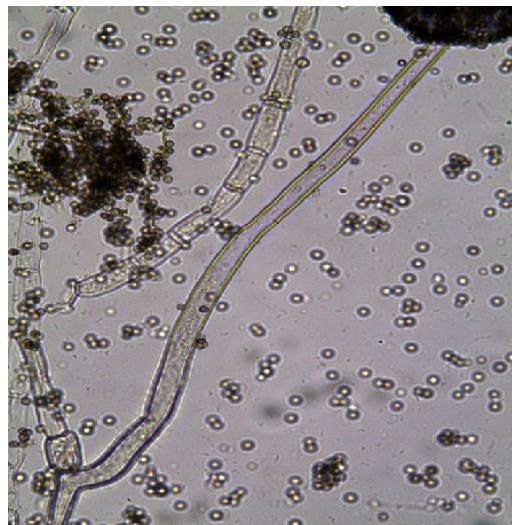
To explain these results, high incidence of black *Aspergillus* can be referred to their black spores that can provide protection from sunlight and ultraviolet light, giving them competitive advantages, these fungi are xerophilic, thus can tolerate high sugar concentrations and low water activity (Iamanaka *et al.*, 2005).



**Fig. (2):** Natural infected fig fruits in the orchard (left); healthy fig fruits (right)



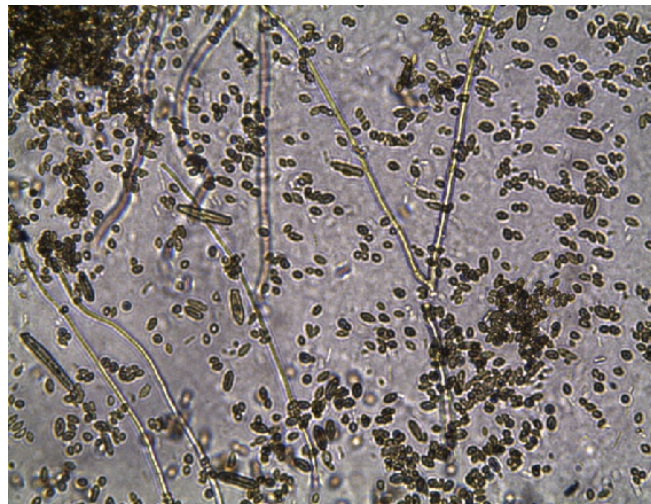
**Fig. (3):** *Alternaria* spores under light microscope



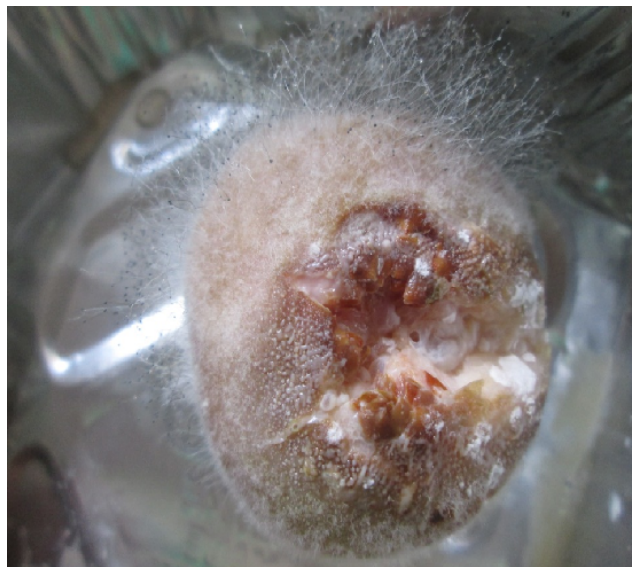
**Fig. (4):** *Aspergillus sp.* under light microscope



**Fig.(5):** Fig fruits infected with *Aspergillus niger*      **Fig.(6):** *Aspergillus niger* under light microscope



**Fig. (7):** *Botryodiplodia* spores under light microscope



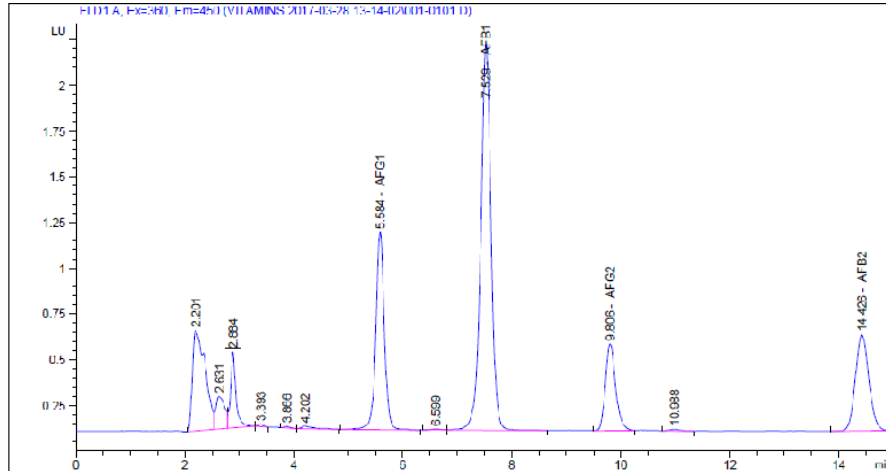
**Fig.(8):** Fig fruits infected with *Rhizopus* sp.

### Mycotoxin production by *Aspergillus* genera

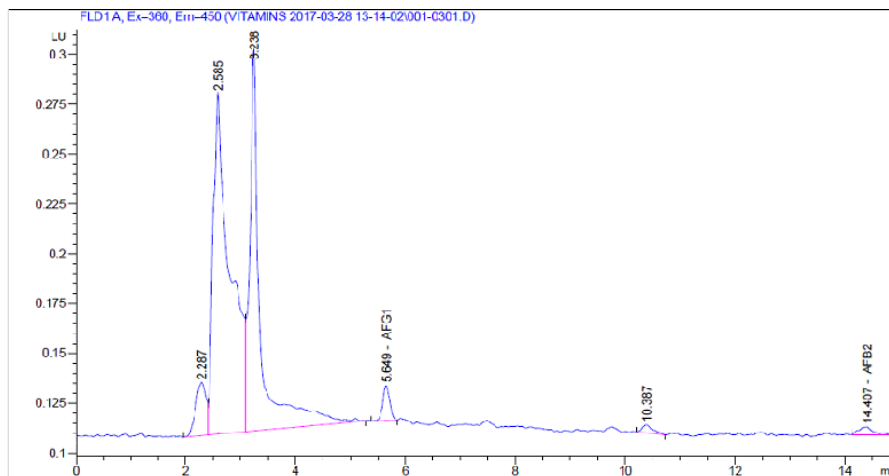
*Aspergillus flavus*, *Aspergillus niger*, *Aspergillus parasiticus* and *Aspergillus terreus* were tested using thin layer chromatography (TLC) for production of mycotoxins.

Identification of aflatoxins could easily be deduced from the constant retention time

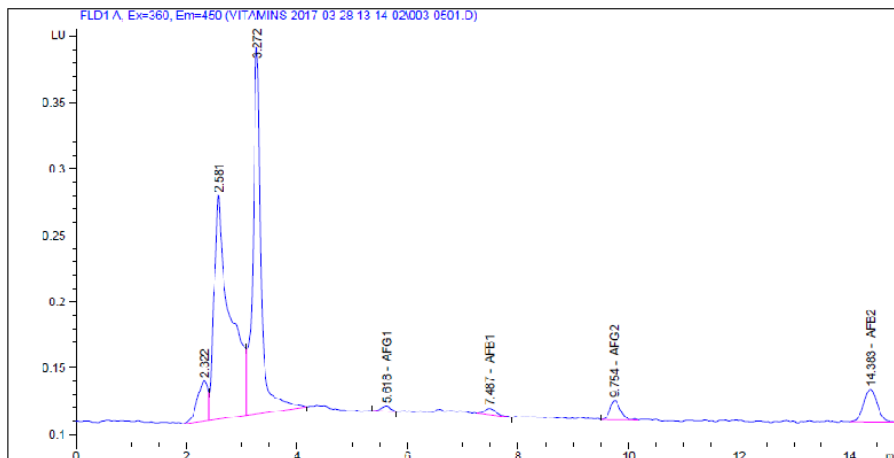
compared with the standard spiked in the HPLC chromatogram. In this regard, Figure (9) showed the standard curve of Mycotoxin production of Aflatoxin (AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub>), while Figures (10, 11, 12 and 13) presented the Aflatoxins produced by *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus parasiticus* and *Aspergillus terreus*, respectively.



**Fig. (9):** Standard curve of Mycotoxin production of Aflatoxins (AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub>)



**Fig. (10):** Aflatoxins produced by *Aspergillus flavus*



**Fig. (11):** Aflatoxins produced by *Aspergillus niger*

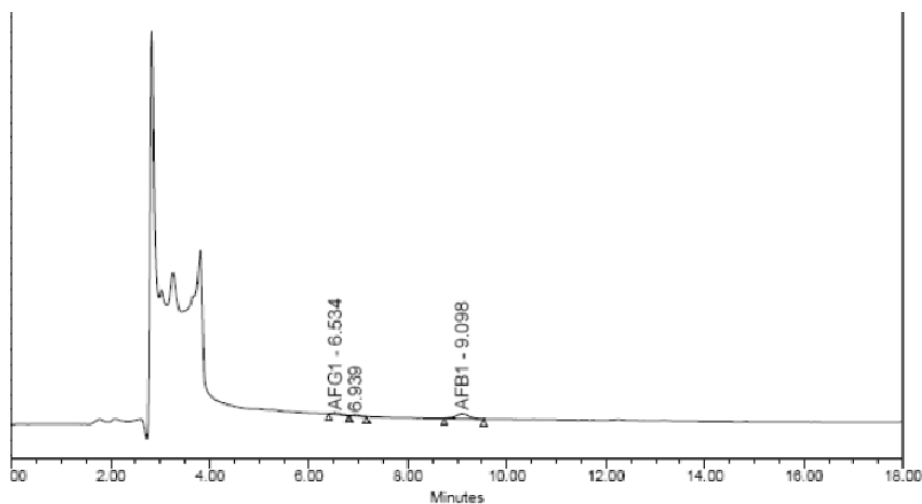


Fig. (12): Aflatoxins produced by *Aspergillus parasiticus*

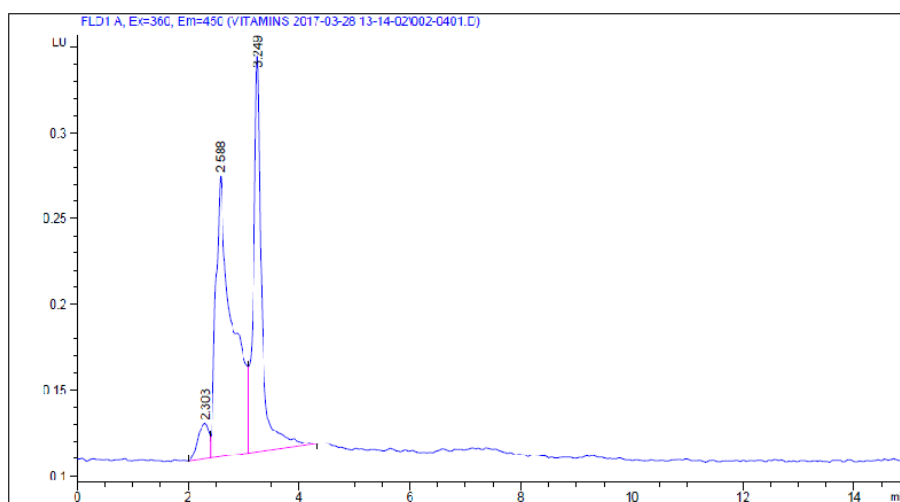


Fig. (13): Aflatoxins produced by *Aspergillus terreus*

Data in Table (2) revealed that all *Aspergillus* genera produced mycotoxin except *Aspergillus terreus* which was negative producer of mycotoxins (AFB1, B2, G1 and G2).

On the other hand, *A. flavus* produced AFB1, AFB2 and AFG1 with concentrations 0.01 ng/ml, 0.05 ng/ml, 0.01 ng/ml, respectively and

AFG2 not detected. *A. niger* produced AFG1 and AFB2 with concentration 1.00 and 0.01 ng/ml, respectively, while, AFB1 and AFG2 were not detected. *A. parasiticus* produced AFB1 and AFG1 with concentrations 0.36 and 0.12 ng/ml, respectively while; AFB2 and AFG2 were not detected.

Table (2): Production of mycotoxin from different species of *Aspergillus* genera

Fungal isolates	Mycotoxin (ng/ml)				Total
	AFB1	AFG1	AFB2	AFG2	
<i>Aspergillus flavus</i>	0.01	0.01	0.05	ND	0.02
<i>Aspergillus niger</i>	ND	1.00	0.01	ND	1.01
<i>Aspergillus parasiticus</i>	0.36	0.12	ND	ND	0.48
<i>Aspergillus terreus</i>	ND	ND	ND	ND	ND

ND= not detected

Aflatoxins are secondary toxic metabolites produced by certain isolates of *Aspergillus flavus*, *A. nomius* and *A. parasiticus* however not all isolates are toxigenic.

The ability to synthesize aflatoxins is strain dependent and occurs in the fungal hyphae, conidia and sclerotia. Maximum mycotoxin level are encountered when fungal mycelium reach an optimum level (Varma and Verma, 1987; Baird *et al.*, 2006).

These findings are similar to these of Abramson and Clear (1996) who found that four strains of *Aspergillus parasiticus* were producing of B1, B2, G1 and G2 aflatoxins, Aspergillic acid and Kajic acid, detection of agar plates of *A. flavus* and *A. parasiticus* showed aflatoxins after 21 days and it was confirmed by TLC.

Our results are in agreement with Kheiralla (1994) who found that three isolates of *Aspergillus flavus* produced aflatoxins B1 and B2. One isolate of *A. parasiticus* produced B1, B2, G1 and G2. Previous studies of Doster *et al.* (1996) found that the high level of aflatoxins (>100 ng/g) were detected in 83% of figs infected by *A. parasiticus* and 38% of the figs infected by *A. flavus*.

Embaby *et al.* (2007) reported that *Aspergillus niger* and *A. parasiticus* were isolated from rotted apricot fruits and found that two isolates of *A. parasiticus* produced one or more of aflatoxins B1, B2, G1 and G2.

Al-Abdalall (2009) found that *Aspergillus niger* produced aflatoxins B1, B2 and G2 in field beans.

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## عزل وتعريف الفطريات المسببة لأعفان ثمار التين وتحديد السموم الفطرية الناتجة

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أجرى هذا البحث بقسم النبات الزراعي بكلية الزراعة جامعة قناة السويس خلال الفترة من ٢٠١٥ حتى ٢٠١٧م، بهدف عزل الفطريات المسببة لأعفان ثمار التين المأخوذة من ٥ محافظات (الإسكندرية، الجيزة، الإسماعيلية، القليوبية، سيناء). تم تعقيم الثمار وأخذت منها شرائح وأعيد تعقيمها مرة أخرى ثم وضعت هذه الشرائح في أطباق بتري تحتوى على بيئة PAD وتم تحضينها على درجة ٢٥ درجة مئوية لمدة ٥ أيام. وفي هذه التجربة تم التعرف على العديد من الفطريات على ثمار التين منها *Alternaria tenuis*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus parasiticus*, *Aspergillus terreus*, *Botryodiplodia theobromae*, *Cladosporium* sp., *Fusarium solani*, *Penicillium* sp. and *Rhizopus* sp. وأثبتت النتائج أن أعلى معدل لوجود الفطريات تم تسجيله في العينات الواردة من محافظة القليوبية (٢٠١ عزل). ثم عينات سيناء (١١١ عزل) وعينات الإسماعيلية (١٠٢ عزل). أقل وجود للفطريات (٤٨ عزل) تم تسجيله على الثمار الواردة من الجيزة. سجل فطر *Aspergillus niger* أعلى معدل للتواجد بنسبة ٣٤.٦٤٪ على ثمار التين بينما سجل *Botryodiplodia theobromae* أقل معدل بنسبة ٢.٢٣٪ في حين سجل فطر *Aspergillus terreus* ٣.٣٥٪. وقد وجد أن جميع أنواع الاسبرجلس لها القدرة على إفراز الأفلاتوكسين عدا فطر *A. terreus* والذي لم يفرز أي من الإفلاتوكسينات (AFB1, B2, G1, G2). من جانب آخر وجد أن *A. flavus* أفرز كلاً من AFB1, AFB2, AFG1 بتركيز ٠.٠١ و ٠.٠١ و ٠.٠٥ نانوجرام/مل على التوالي. كما أفرز فطر *A. parasiticus* افلاتوكسين من نوعي AFB1 و AFG1 بتركيز ٠.٣٦ و ٠.١٢ نانوجرام/مل لكل منهما. كما أفرز فطر *A. niger* افلاتوكسين من نوعي AFB2 و AFG1 بتركيز ١.٠٠ نانوجرام/مل و ٠.٠١ نانوجرام/مل لكل منهما.