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Evaluation and Detection of Ochratoxins and Aflatoxins of *Aspergillus piperis* by Fluorometric Spectroscopy, Gel Electrophoresis and Western Blotting Techniques

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TOTAL ochratoxins (OTs) and aflatoxins (AFs) of *Aspergillus piperis* were quantitively detected by fluorometric spectroscopy in both fungal biomass (FB) and culture filtrate (CF). OTs were detected with high concentrations; 38×10^{-3} and 32×10^{-3} µg/g in fungal biomass and culture filtrate of *A. piperis* respectively, while, AFs recorded lower concentrations; 3.3×10^{-3} and 2.3×10^{-3} µg/g. Consequently, different types of OTs produced in FB and CF of *A. piperis* were identified using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) method. The molecular weights (MWs) of protein bands from SDS-PAGE step revealed the presence of ochratoxin B, ochratoxin α and ochratoxin C (OTB, OTα and OTC) in FB of *A. piperis* but, the results revealed that, only OTB and OTα were found in the CF. The MWs of OTB, OTα and OTC were found to be 363, 260,430 KDa respectively. Also, these findings were confirmed by western blotting technique using the antibodies of OTB, OTα and OTC. Toxicity of different concentrations of CF of *A. piperis* was tested in vitro on seed germination of broad bean (*Vicia faba*).

Keywords: Aflatoxins, *Aspergillus piperis*, Chromatography, Gel electrophoresis, Ochratoxins, Western.

Introduction

Aspergillus piperis is a member from black aspergilli which, in previous studies of the author, it exhibited an important antagonistic activities against some phytopathogenic fungi (El-Debaiky, 2017, 2018). Moreover, Aspergilli among other genera of fungi such as fusaria and penicillia are considered important producers of mycotoxins which are toxic secondary metabolites causing health hazards to animals and human beings (Reddy et al., 2010). Several types of mycotoxins attracted attention of many mycologists such as aflatoxins (AFs), ochratoxins (OTs), trichothecenes, zearalenone, fumonisins, ergot alkaloids and deoxynivalenol where AFs and OTs are the most important (Reddy et al., 2010; Cendoya et al., 2014; Covarelli et al., 2015; Frisvad et al., 2019; Singh & Cotty, 2019). The main mycotoxins found produced by black aspergilli were OTs especially ochratoxin A (OTA) which produced in large amounts especially by *A. carbonarius* (Abarca et al., 1994; Samson et al., 2004; Esteban et al., 2006).

Several studies reported that, some species of the genus *Aspergillus* were found to be good antagonists against some plant pathogenic fungi (Bandyopadhyay & Cardwell, 2003; Adebola & Amadi, 2010; Bosah et al., 2010). But more attention should be taken while using them in the field of biological control of plant diseases due to their mycotoxins production which may be accumulated in plant parts. In addition, where plants are considered the base of human food and animal feed so, accumulation of mycotoxins in them will transfer to food and feed stuffs causing lot of hazardous effects on human and animal health (Magan & Olsen, 2004; Fink-Gremmels, 2008; Nácher-Mestre et al., 2015).

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There has been an international research efforts, aimed to identify and quantify mycotoxins and evaluation of their biological effects on humans and animals (Zain, 2011). For this purpose, some conventional accurate analytical methods were used such as thin-layer chromatography, high-performance liquid chromatography, diode array, fluorescence or mass spectrometry detectors, gas chromatography coupled with electron capture, flame ionization or MS detectors (Dowell et al., 2002; Visconti et al., 2005; Cucci et al., 2007; Lippolis et al., 2008; Longobardi et al., 2012; Bueno et al., 2015) and enzyme-linked immunoassay (Zheng et al., 2005; Wang et al., 2011). Though all these techniques are accurate and precisely detect the mycotoxins in food or feed samples, they have some disadvantages where they require skilled operators, extensive sample pretreatment, equipment and may not accurate at low concentrations (Goryacheva et al., 2007; Kralj Cigić & Prosen, 2009). Therefore, rapid, sensitive and specific assay techniques are required. The recent advanced methods developed for mycotoxins detection are depend on using biomolecules such as antibodies, DNA and enzymes (Chauhan et al., 2016).

Fungal organisms and their mycotoxins cause serious economic losses in several crops where they play significant role in causing pre- and post-infections viz., seed rot, reduction or elimination of germination capacity, seedling damage during growth, harvest and storage (Mohana et al., 2011; Labbé & García, 2013; Aiyaz et al., 2015; Divakara et al., 2015).

Accordingly, the present study was designated to examine and determine AFs and OTs in the FB and CF of the used strain of *A. piperis* using fluorometric spectroscopy, SDS-PAGE and western blotting techniques. In addition, toxicity of different concentrations of CF of *A. piperis* was tested on seed gemination of *V. faba in vitro*.

Materials and Methods

Source of A. piperis

Culture of *A. piperis* (AUMMC No.9043) was purchased from Assiut University Moubasher Mycological Centre (AUMMC), Assuite University, Assuite, Egypt. The culture was maintained on potato dextrose agar (PDA) plates and slants at 4°C ± 2.

Used medium and preparation of fungal biomass

Sabouraud's glucose broth (SGB) medium was used for sub-culturing process of *A. piperis*. The medium has the following constituents (g/L), peptone, 10 and D-Glucose, 20 (Moubasher, 1993). Fungal fragments were transferred to 500mL of SGB and agitated in shaking incubator (3x g) at 25°C for 5-7 days. Thereafter, the FB was separated from the medium by using Whatman No. 1 filter paper and Buchner funnel then washed three times with sterile phosphate buffered saline (PBS) (Rath, 2001). Then, the total amount of AFs and OTs in both FB and CF were detected using immunoaffinity column chromatography by fluorometric spectroscopy (ICCFS).

Detection of total AFs and OTs using ICCFS

Extraction of AFs and OTs

For extraction process, 50g of FB or 50mL of CF were mixed separately with 5g of analytical, United States Pharmacopeia (USP) grade sodium chloride and 100mL of 80% HPLC grade methanol into Eberbach glass blender jars. After covering each jar, the blending process was done at high speed for 1min. Then, the extracts were filtered by pouring through clean funnels lined with fluted filter papers into clean VICAM disposable beakers. Extracts must be used within 30min. Each extract was diluted by adding 40mL dist. water (for AFs test) or PBS (for OTs test) to 10mL extract and filtered again using VICAM MicroFiber Filter paper 11cm.

Separation and quantification of AFs

The VICAM AflaTest column was used for separation of total AFs from the prepared extracts. The column coupling was used to attach the AflaTest column to the bottom of the glass syringe barrel on the pump stand. The waste collection vessel was placed under the column outlet. Each filtered extract (10mL) was added to the syringe barrel then, the coupling was inserted at the end of the pump tubing into the top of the glass syringe barrel. After that, the pump was activated, and a steady flow rate was maintained of approximately 1:2 drops of extract/s until all the extract has passed through the column. Washing process was performed by adding 10 ml of deionized distilled water to the syringe barrel and activating the pump. Maintain a steady flow rate of 1 to 2 drops of water/s until all the water has passed through the column. The last step was repeated.

A clean VICAM borosilicate glass cuvette was place under the outlet of the AflaTest column. The column was detached and 1mL of HPLC grade methanol directly pipetted into the column headspace. Then, the column was reattached, and the pump activated. Enough air pressure applied to pass the methanol through the column at a rate of approximately 1 drop/s. Collect all the methanol (1mL) in the cuvette. One ml of freshly made developer solution (VICAM AflaTest Developer) was added directly to the sample eluate in the cuvette and mixed well for 3 to 5s. The cuvette was cleaned quickly from outside with a Kimwipe. Immediately, the cuvette was placed in a calibrated fluorometer (VICAM Series 4EX) and the sample concentration (µg/g) recorded after 60s.

Separation and quantification of OTs

Ten mL of the extract was added to a syringe barrel with an OchraTest column attached. The bottom cap from the OchraTest column was removed and the diluted extract completely passed through the OchraTest column at a rate of about 1:2 drops/s until air comes through the column. After that, washing process was performed by filling the syringe barrel with 10mL of 0.1% Tween-PBS solution which pass through the OchraTest column at a rate of 1:2 drops/s. The washing process was repeated using 10mL of deionized distilled water.

Add/Elute Ochra Test column with 1.5mL of Ochra Test eluting solution into the syringe barrel. The solution passed through the OchraTest column at a rate of 1 drop/s or slower and collect all the sample eluent (1.5mL) in a clean glass put under the OchraTest column. The cuvette was cleaned quickly from outside with a Kimwipe. Immediately, the cuvette was placed in a calibrated fluorometer (VICAM Series 4EX) and the sample concentration (µg/g) recorded after 60 s.

Identification of different OTs of A. piperis using gel electrophoresis

The previous experiment indicated that *A. piperis* produced high concentrations of total OTs either in FB or CF. SDS-PAGE method was carried out to evaluate different types of OTs in total soluble protein of *A.piperis* according to the method of (Laemmli, 1970a).

Extraction of protein

Where OTs are protein in nature, so all the coming procedures concerning protein were performed either using FB or the FC of *A. piperis* to determine the presence or absence of different OTs. Consequently, for obtaining total protein, small portion of FB (50-100mg) or CF (50-100mL) were added separately to 1 ml of TriFast™ (PeQlab a VWR company) solution in glass -Teflon or power homogenizer. The sample volume should not exceed 10% of the volume of TriFast. After complete homogenization, 0.2mL chloroform per each 1mL of TriFast was added, and the mixture shake vigorously by hand for 15s and kept to phase separate for 3-10min at room temperature. Then, the mixture was centrifuged for 5min at 12000x g (max) where it separated into 3 phases; the lower red phenol-chloroform phase, containing the protein, the interphase and the colorless upper aqueous phase.

Precipitation and preparation of protein for gel electrophoresis

The protein from FB and CF samples was precipitated and prepared for gel electrophoresis according the method mentioned in (Habiba et al., 2018). Isopropanol (1.5mL) was added to the phenol-chloroform phase for precipitation of protein at room temperature for 10min. Then, the mixture was centrifuged at 12000x g for 10min at 4°C. The supernatant was discarded, and the protein pellet washed triplicate using 2mL 0.3mol guanidine hydrochloride in 95 % ethanol for 20 min at room temperature before centrifuging at 7500x g for 5min at 4°C. After that, the protein pellet was mixed with 2mL of 100% ethanol and settle for 20min at room temperature, then centrifuged at 7500x g for 5min at 4°C. Next the ethanol was removed, and the protein pellet let dry for 5-10min under vacuum and dissolved in 1% SDS by pipetting it up and down. The protein samples may be incubated at higher temperatures (50-100°C) for complete solubilization of protein. Also, the un-soluble impurities were removed by centrifugation at 10000x g for 10min at 4°C. The soluble protein samples should be transferred to fresh tubes for immediate usage or kept frozen at -20°C for future use.

The concentration of protein fraction was determined using the method of (Bradford, 1976) which based on the interaction between protein and Coomassie Brilliant Blue G250 (CBBG-250) in acidic conditions. An amount of 50µL of protein sample was mixed with 50µl of distilled

water and 200µL CBBG-250 for 5min till color stabilization. Consequently, the absorbance was recorded at 595nm and the concentrations of protein samples are determined based on previously prepared standard of Bovin Serum Albumin (BSA) at concentration range from 0 to 150µg.

Gel electrophoresis process

The stock solutions used for this step were prepared according to (Laemmli, 1970b) and summarized in Table 1.

Gel preparation: The slab gel (13%) was prepared according to the method illustrated in (Laemmli, 1970b) by mixing the previous stock solutions in the following quantities: acrylamide methylene bis-acrylamide, 10mL; resolving gel buffer, 3.7mL; SDS , 0.3mL; ammonium persulphate, 1.5mL; distilled water, 14.45mL and TEMED, 0.015mL. While, the stacking gel (4%) was prepared using acrylamide- methylene bis-acrylamide, 2.5mL; stacking gel buffer stock, 5.0mL; SDS, 0.2mL; ammonium persulphate, 1.0mL; distilled water 11.3mL and TEMED, 0.015mL.

Loading of samples and electrophoresis: After gel polymerization, 30µg from each protein sample was loaded and electrophoresis performed at 75V through stacking gel followed by 125V during approximately 2hrs. After complete electrophoresis, gels were stained by 0.1% Coomassie Brilliant Blue R- 250 for 2hrs. then distained with a solution (1:3:6) of glacial acetic acid: methanol: water, respectively. Then, the obtained data were analyzed by gel documentation system (Geldoc-it, UVP, England) and Totallab analysis software, ww.totallab.com, (Ver.1.0.1).

Western Blotting Technique

Anti-Ochratoxin mixed antibodies HRB (abcam) was detected via western blotting technique as manufacturer protocol. Tris-buffered saline (25m mol Tris, pH 7.4, 0.15M NaCl) containing 0.1% Tween 20 (TBST buffer) was used for preparing different dilutions of the antibodies. Electrophoresed proteins on SDS-PAGE were transferred to a Hybond™ nylon membrane (GE Healthcare) and incubate for 1hr at room temperature in blocking buffer (2-5% nonfat dry milk in TBST buffer, pH 7.4). Then, it incubated overnight at 4°C in antibody solution containing appropriate dilutions of primary antibody prepared in 1-5% nonfat dry milk in TBST buffer, pH 7.4. Membrane was washed three times at room temperature in TBST Buffer, 5 min for each wash. Then, membrane was incubated for 1hr at room temperature in antibody solution containing 0.1-0.5µ ml^{-1} of HRP- conjugated secondary antibody. Membrane was washed three times at room temperature in TBST Buffer, 5min for each wash.

TABLE 1. Stock solutions used in gel electrophoresis process.

Solution	Preparation
Acrylamide methylene bis- acrylamide (30: 0.8 % w/v)	30g of acrylamide plus 0.8 g methylene bis- acrylamide in 100mL distilled water then filtered through Whatman filter paper No.1. Stored at 4°C in a dark bottle.
Tetramethylethylenediamine (TEMED)	Used concentrated and stored at 4°C in a dark bottle.
Ammonium Persulphate (1.5 % w/v)	Freshly prepare by adding 0.15g to 10mL water.
SDS (10 % w/v)	Dissolve 10g of SDS in 100mL distilled water.
2- mercaptoethanol	Used concentrated.
Staking gel buffer (0.5 M Tris-HCl pH 6.8)	6g Tris in 40mL distilled water. Using 1mol HCl to adjust pH at 6.8 and complete to 100mL with distilled water. Stored at 4°C after filtration through Whatman filter paper No. 1.
10x Resolving Gel buffer (3.0 M Tris- HCl, pH 8.6)	30.3g Tris (0.25 M); 144.0g glycine (1.92mol) and 10.0g SDS (1%) in 1L distilled water. The solution was stored at 4°C until used.

Toxicity test

Potato dextrose broth medium prepared according to (Moubasher, 1993) was used for growing of *A. piperis* culture at 27°C for 10 days. After incubation period, CF was achieved by filtration process to remove FB through filter paper. The crude CF was assumed to be 100% concentration, then, different concentrations (10, 30, 50, 60%) were prepared from it using dist. H_2O. Effect of different concentrations of CF on seed germination were performed using seeds of *V. faba*. Intact seeds were selected and sterilized using sodium hypochlorite 5% for 5min, then washed by dist. H_2O for another 5min. After that, seed germination was determined by placing 10 seeds on cotton piece in small plastic box and wetted separately by about 10mL from each previously prepared concentration then kept for incubation in dark at 26±1°C. The incubated seeds should be wetted daily by the used solutions. Control was prepared by wetting seeds with dist. H_2O. Germination percentage and germination index of control and treatments were determined after 5 days according to (Gupta, 1993).

Results and Discussion

Detection of total AFs and OTs using ICCFS

Determination of total quantities of AFs and OTs found in both FB and CF of *A. piperis* was performed using the ICCFS analysis. The results revealed occurrence of both mycotoxins in different concentrations. But the obtained data strongly indicated that, the major distinguished mycotoxins were OTs which represented higher concentrations than AFs either in FB or CF (Table 2). As reported in the table, the quantity of AFs and OTs was highly concentrated in FB where AFs were 3.3×10^{-3}µg/g and OTs were 38×10^{-3}µg/g, respectively, while they were lower concentrated in CF where the values were 2.3×10^{-3}µg/g for AFs and 32×10^{-3}µg/g for OTs. Accordingly, special attention was directed to accurate identification of the possible different types of OTs using gel electrophoresis and Western blotting techniques. My results about production OTs by *A. piperis* which is a member of black Aspergilli, were in accordance with many other researchers who demonstrated that, several species of black-spored *Aspergillus* section Nigri had the capability to produce different types OTs (Samson et al., 2004; Frisvad et al., 2007; Palumbo & O'Keeffe, 2013; Perrone & Gallo, 2017). While my results about the production of AFs by the black fungus; *A. piperis* disagreed some other literatures, which restricted the production of AFs to certain groups of Aspergilli rather than black Aspergilli such as *A. flavus* group (Bayman & Cotty, 1993; Geiser et al., 1998, 2000; Criseo et al., 2001). However, some other studies stated the production of AFs by some isolates of *A. flavus* and *A. niger* (Al-Abdalall, 2009; Soares et al., 2013).

TABLE 2. Total AFs and OTs in FB and CF of *A. piperis.*

Mycotoxins	Concentration (µg/g)	
	FB	CF
AFs	3.3×10^{-3}	2.3×10^{-3}
OTs	38×10^{-3}	32×10^{-3}

Identification of different OTs by SDS-PAGE fingerprinting technique

Analysis of data retrieved from SDS-PAGE fingerprinting technique revealed the presence of different OTs according to their molecular weights (MW), compared to the used marker, in both FB and CF of *A. piperis*. Regarding to the analyzed data; ochratoxin B (OTB) and ochratoxin α (OTα) were expressed with different expression levels for Fb and CF, while, ochratoxin C (OTC) was only expressed for FB (Photos 1, 2 and Tables 3-5). Also, the MW of the detected bands of OTB, OTα and OTC were found to be 363, 260,430KDa respectively, this is in accordance with the results reported in other literatures (Diniz & Paulo, 2016; Freire et al., 2018). Moreover, Table 6 showed that FB of *A. piperis* was superior for OTB expression level (12.9%) comparing with CF (11.1%), but, in contrary, CF was over expressed OTα (11.5%) than FB (8.9%). In the present study, results exhibited the presence of OTB, OTα and OTC in the both FB and CF of *A. piperis* and absence of ochratoxin A (OTA) which agreed (Samson et al., 2004).

Western Blotting Technique

The SDS-PAGE fingerprinting indicated presence of OTB, OTα and OTC in both FB and CF of *A. piperis*. Also, these findings were confirmed by western blotting technique using the specific antibody of each detected ochratoxin. Photo 3A illustrated the positive reactions among the used antibodies with their specific OTs. In addition, the detected bands were computerized to detect the MWs of the OTs (Photo 3 B, C, 4 and Table 7). The obtained data indicated that the MW of OTC and OTB are 454.5 and 360KDa, respectively while, MW of OTα is ranged from 361.79 to 266.23KDa.

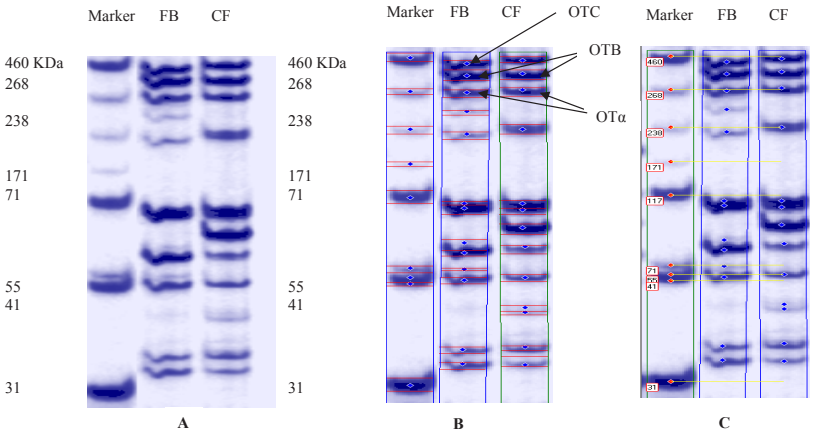

Photo 1. Protein fingerprinting patterns for FB and CF of *A. piperis* (A: Gel bands as observed & B and C: computerized for molecular weight detection). Arrows in B referred to the detected OTs bands.

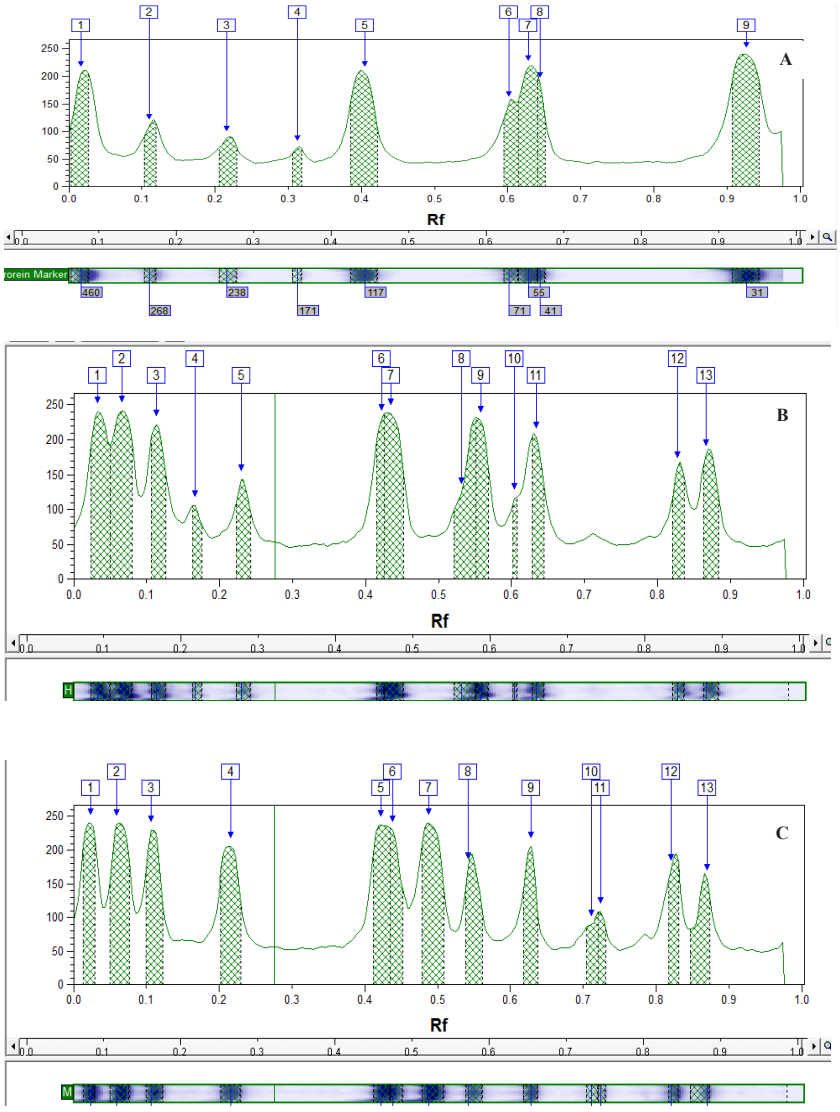

Photo 2. Data analysis of protein patterns parameters for protein marker (A), FB (B) and CF (C).

TABLE 3. Detected MW from bands analysis for protein marker in photo.2.

Band No	Peak Height	Area	Band %	MW (KDa)
1	205.83	168.00	12.04	460
2	109.50	120.00	5.31	268
3	88.63	168.00	5.70	238
4	70.71	96.00	2.65	171
5	205.46	264.00	20.05	117
6	161.25	144.00	8.62	71
7	216.08	192.00	15.67	55
8	186.13	72.00	5.39	41
9	239.67	264.00	24.56	31

TABLE 4. Detected MW from bands analysis for FB in photo.2.

Band No	Peak Height	Area	Band %	MW (KDa)
1	239	192	12.73	430 (Ochratoxin C)
2	230	192	12.96	363 (Ochratoxin B)
3	220	144	8.94	260 (Ochratoxin α)
4	105	96	2.86	239
5	142	144	5.53	111
6	230	72	4.68	108
7	236	192	13.02	108
8	124	216	10.00	92
9	227	120	7.94	86
10	115	48	1.61	69
11	204	120	7.00	49
12	161	120	5.39	33
13	179	144	7.37	32

TABLE 5. Detected MW from bands analysis for CF in photo.2.

Band No	Peak Height	Area	Band %	MW (KDa)
1	240	120	7.4	460
2	238	192	11.1	363 (Ochratoxin B)
3	202	240	11.5	260 (Ochratoxin α)
4	205	192	9.8	238
5	237	168	10.3	111
6	229	120	6.6	107
7	239	216	12.9	99
8	174	168	7.5	90
9	205	144	6.8	55
10	89	120	2.9	39
11	108	72	2.0	38
12	171	96	4.5	33
13	157	192	6.1	32

TABLE 6. OTs expression levels for FB and CF of *A. piperis*.

OTs	OTs expression levels for *A. piperis*	
	FB	CF
B	12.9 %	11.1%
C	12.7 %	-
α	8.9%	11.5%

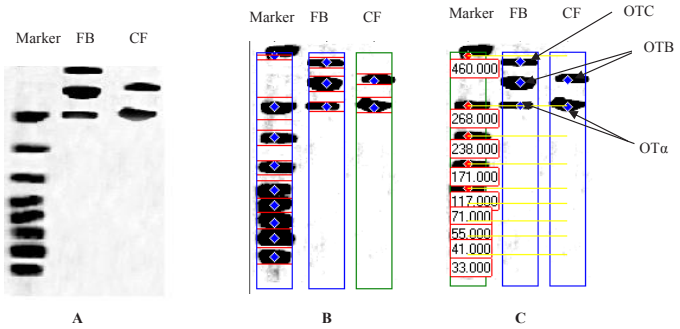

Photo 3. Bands appeared with OTs-antibodies positive reactions in western blot analysis (A: Bands as observed & B and C: Computerized for molecular weight detection) [FB: Fungal biomass and CF: Cultural filtrate of *A.piperis*].

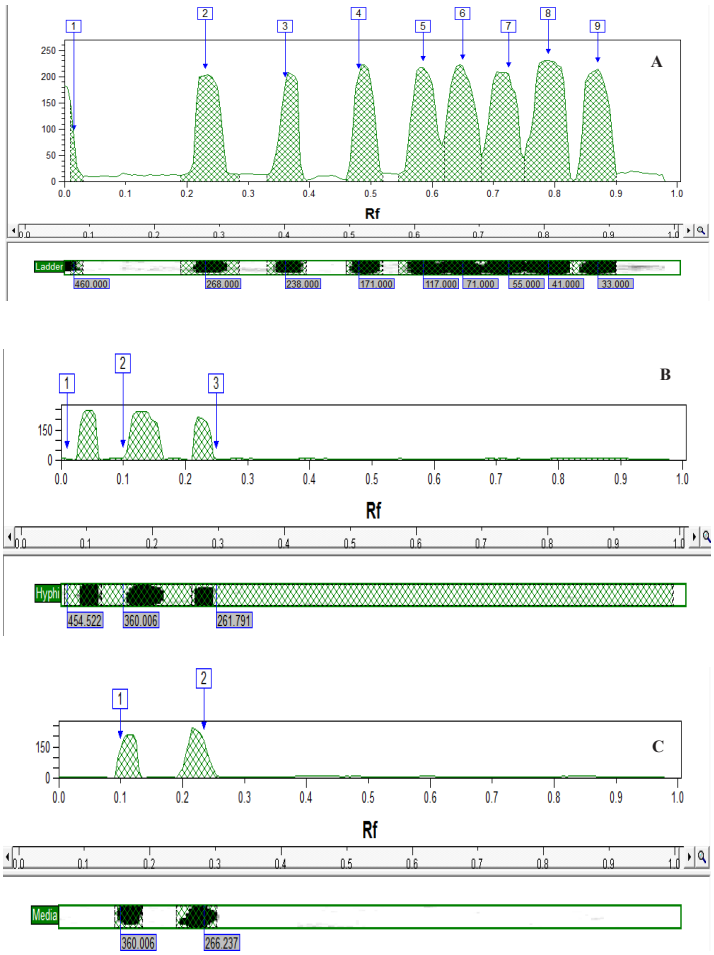

Photo 4. Data analysis of protein patterns parameters for protein marker (A), FB (B) and CF (C) for bands appeared in western blot analysis.

TABLE 7. MW of detected OTS from western blotting analysis.

OTs	MW of OTs (KDa)	
	FB	CF
C	Band 1: 454.5	-
B	Band 2: 360	Band 1: 360
α	Band 3: 261.79	Band 2: 266.23

Toxicity test

The results of the toxic effect of CF of *A. piperis* on seed germination percentage after 5 days and germination index are shown in Table 8. Germination percentage and germination index were decreased linearly with increased concentration of CF over untreated control. Maximum inhibition in seed germination was observed by 100% concentration. Value of germination index was 5.23 and 3.56 at 10% and 50% concentration respectively despite the germination percentage was the same value (70%). This means that seeds at 10% were more vigorous than at 50%, on other words the adverse effects of mycotoxins in CF on seeds increased by increasing concentrations. This is in agreement with the findings of other researchers (Crisan, 1973; Jones et al., 1980; Bokhari, 2002; Janardhan et al., 2011).

Conclusion

From the present study, it was concluded that, AFs and OTS were found in both FB and CF of the black fungus *A. piperis* using fluorometric spectroscopy method. However, percentage of AFs was very small compared to OTs, so the attention was directed to identify the types of OTs found in FB and CF of *A. piperis* using SDS-PAGE fingerprinting and western blotting techniques. In details, OTB and OTα were detected in FB and CF of *A. piperis* while OTC was found only in the FB. Interestingly, from the results of this study, it was noticed that; the carcinogenic OTA was absent in both FB and CF of *A. piperis*, and this is very important to whom interested in the field of biological control of plant diseases where, in a previous study of the author (El-Debaiky, 2017), this fungus exhibited an antagonistic activity against some phytopathogenic fungi. Additional studies still needed before recommendation of *A. piperis* for field using as bio-controlling agent against plant diseases. Toxicity of different concentrations of CF of *A. piperis* was tested on the germination of broad bean (*Vicia faba*) seeds. The results showed that with increasing the concentration, the germination percentage decreased.

TABLE 8. Toxicity effect of CF of *A. piperis* on germination percentage and germination index of *V.faba* seeds.

Concentration (%)	Germination Percentage after 5 days (%)	Germination Index
0 (Control)	100	7.5
10	70	5.23
30	Not determined	-
50	70	3.56
60	40	2.13
100	0	0

Ethical approval: Not applicable.

References

Abarca, M., Bragulat, M., Castella, G., Cabanes, F. (1994) Ochratoxin A production by strains of Aspergillus niger var. niger. *Applied and Environmental Microbiology*, **60**(7), 2650-2652.

Adebola, M., Amadi, J. (2010) Screening three Aspergillus species for antagonistic activities against the cocoa black pod organism (*Phytophthora palmivora*). *Agriculture and Biology Journal of North America*, **1**(3), 362-365.

Aiyaz, M., Divakara, S.T., Nayaka, S.C., Hariprasad, P., Niranjana, S.R. (2015) Application of beneficial rhizospheric microbes for the mitigation of seed-borne mycotoxigenic fungal infection and mycotoxins in maize. Biocontrol Science and Technology, **25**(10), 1105-1119.

Al-Abdalall, A.H.A. (2009) Production of aflatoxins by *Aspergillus flavus* and *Aspergillus niger* strains isolated from seeds of pulses. *Journal of Food, Agriculture and Environment*, **7**(2), 33-39.

Bandyopadhyay, R., Cardwell, K.F. (2003) Species of Trichoderma and *Aspergillus* as biological

control agents against plant diseases in Africa. In: "*Biological Control in Integrated Pest Management Systems in Africa*". Wallingford: CABI publishing pp. 193-206.

Bayman, P., Cotty, P.J. (1993) Genetic diversity in *Aspergillus flavus*: Association with aflatoxin production and morphology. *Canadian Journal of Botany*, **71**(1), 23-31.

Bokhari, F. (2002) Aflatoxins production by A*spergillus flavus*, isolated from different food stuffs commonly used in Jeddah region, Saudi Arabia. *Pakistan Journal of Biological Sciences*, **5**(1), 69-74.

Bosah, O., Igeleke, C., Omorusi, V. (2010) *In vitro* microbial control of pathogenic *Sclerotium rolfsii*. *International Journal of Agriculture and Biology*, **12**, 474-476.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**(1-2), 248-254.

Bueno, D., Istamboulie, G., Muñoz, R., Marty, J.L. (2015) Determination of mycotoxins in food: A review of bioanalytical to analytical methods. *Applied Spectroscopy Reviews*, **50**(9), 728-774.

Cendoya, E., Monge, M.P., Palacios, S.A., Chiacchiera, S.M., Torres, A.M., Farnochi, M.C., Ramirez, M.L. (2014) Fumonisin occurrence in naturally contaminated wheat grain harvested in Argentina. *Food Control*, **37**, 56-61.

Chauhan, R., Singh, J., Sachdev, T., Basu, T., Malhotra, B. (2016) Recent advances in mycotoxins detection. *Biosensors and Bioelectronics*, **81**, 532-545.

Covarelli, L., Beccari, G., Prodi, A., Generotti, S., Etruschi, F., Juan, C., Ferrer, E., Mañes, J. (2015) *Fusarium* species, chemotype characterisation and trichothecene contamination of durum and soft wheat in an area of central Italy. *Journal of the Science of Food and Agriculture*, **95**(3), 540-551.

Crisan, E.V. (1973) Effects of aflatoxin on germination and growth of lettuce. *Applied and Environmental Microbiology*, **25**(3), 342-345.

Criseo, G., Bagnara, A., Bisignano, G. (2001) Differentiation of aflatoxin-producing and non-producing strains of *Aspergillus flavus* group. *Letters in Applied Microbiology*, **33**(4), 291-295.

Cucci, C., Mignani, A., Dall'Asta, C., Pela, R., Dossena, A. (2007) A portable fluorometer for the rapid screening of M1 aflatoxin. *Sensors and Actuators B: Chemical*, **126**(2), 467-472.

Diniz, S.D.S., Paulo, S. (2016) "*Mycotoxins: Biochemical Approach*". Editora Albatroz.

Divakara, S., Aiyaz, M., Moore, G., Venkataramana, M., Hariprasad, P., Nayaka, S.C., Niranjana, S. (2015) Analysis of genetic and aflatoxin diversity among *Aspergillus flavus* isolates collected from sorghum seeds. *Journal of Basic Microbiology*, **55**(11), 1255-1264.

Dowell, F.E., Pearson, T.C., Maghirang, E.B., Xie, F., Wicklow, D.T. (2002) Reflectance and transmittance spectroscopy applied to detecting fumonisin in single corn kernels infected with *Fusarium verticillioides*. Cereal Chemistry, **79**(2), 222-226.

El-Debaiky, S.A. (2017) Antagonistic studies and hyphal interactions of the new antagonist Aspergillus piperis against some phytopathogenic fungi in vitro in comparison with T*richoderma harzianum*. *Microbial Pathogenesis*, **113**, 135-143.

El-Debaiky, S.A. (2018) Effect of the new antagonist; *Aspergillus piperis* on germination and growth of tomato plant and Early Blight incidence caused by Alternaria solani. *Merit Research Journal of Agricultural Science and Soil Science*, **6**(4), 41-49.

Esteban, A., Abarca, M., Bragulat, M., Cabanes, F. (2006) Study of the effect of water activity and temperature on ochratoxin A production by *Aspergillus carbonarius*. *Food Microbiology*, **23**(7), 634-640.

Fink-Gremmels, J. (2008) Mycotoxins in cattle feeds and carry-over to dairy milk: A review. *Food Additives and Contaminants*, **25**(2), 172-180.

Freire, L., Guerreiro, T.M., Pia, A.K., Lima, E.O., Oliveira, D.N., Melo, C.F., Catharino, R.R., Sant'Ana, A.S. (2018) A quantitative study on growth variability and production of ochratoxin A and its derivatives by *A. carbonarius* and *A. niger* in grape-based medium. *Scientific Reports*, **8**(1), 145-73.

Frisvad, J.C., Larsen, T.O., Vries, R.De, Meijer, M., J. Houbraken, Cabañes, F., Ehrlich, K., Samson, R. (2007) Secondary metabolite profiling, growth profiles and other tools for species recognition and important Aspergillus mycotoxins. *Studies in Mycology*, **59**, 31-37.

Frisvad, J.C., Hubka, V., Ezekiel, C., Hong, S.-B., Nováková, A., Chen, A., Arzanlou, M., Larsen, T., Sklenář, F., Mahakarnchanakul, W. (2019) Taxonomy of *Aspergillus* section Flavi and their production of aflatoxins, ochratoxins and other mycotoxins. *Studies in Mycology*, **93**, 1-63.

Geiser, D.M., Pitt, J.I., Taylor, J.W. (1998) Cryptic speciation and recombination in the aflatoxin-producing fungus Aspergillus flavus. *Proceedings of the National Academy of Sciences*, **95**(1), 388-393.

Geiser, D.M., Dorner, J.W., Horn, B.W., Taylor, J.W. (2000) The phylogenetics of mycotoxin and sclerotium production in *Aspergillus flavus* and *Aspergillus oryzae*. *Fungal Genetics and Biology*, **31**(3), 169-179.

Goryacheva, I.Y., De Saeger, S., Nesterenko, I.S., Eremin, S.A., Van Peteghem, C. (2007) Rapid all-in-one three-step immunoassay for non-instrumental detection of ochratoxin A in high-coloured herbs and spices. *Talanta*, **72**(3), 1230-1234.

Gupta, P. (1993) Seed vigour testing. "*Handbook of Seed Testing*". New Delhi, pp. 242-249.

Habiba, H.E., Bondok, A.T., Abdel-Aal, M.S. (2018) Evaluation of some promising teosinte genotypes for morphological and genetic parameters under Egyptian Conditions. *Alexandria Science Exchange Journal*, **39**(2), 307-332.

Janardhan, A., Subramanyam, D., Praveen, A., Reddi, M., Narasimha, G. (2011) Aflatoxin impacts on germinating seeds. *Annals of Biological Research*, **2**(2), 180-188.

Jones, H., Chancey, J., Morton, W., Dashek, W., Llewellyn, G. (1980) Toxic responses of germinating pollen and soybeans to aflatoxins. *Mycopathologia*, **72**(2), 67-73.

Kralj Cigić, I., Prosen, H. (2009) An overview of conventional and emerging analytical methods for the determination of mycotoxins. *International Journal of Molecular Sciences*, **10**(1), 62-115.

Labbé, R.G., García, S. (2013) "*Guide to Foodborne Pathogens*". Wiley Online Library.

Laemmli, U.K. (1970a) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**(5259), 680.

Laemmli, U.K. (1970b) Protein electrophoresis in slab gels. *Nature*, **227**, 680-683.

Lippolis, V., Pascale, M., Maragos, C.M., Visconti, A. (2008) Improvement of detection sensitivity of T-2 and HT-2 toxins using different fluorescent labeling reagents by high-performance liquid chromatography. *Talanta*, **74**(5), 1476-1483.

Longobardi, F., Iacovelli, V., Catucci, L., Panzarini, G., Pascale, M., Visconti, A., Agostiano, A. (2012) Determination of ochratoxin A in wine by means of immunoaffinity and aminopropyl solid-phase column cleanup and fluorometric detection. *Journal of Agricultural and Food Chemistry*, **61**(8), 1604-1608.

Magan, N., Olsen, M. (2004) "*Mycotoxins in Food: Detection and Control*". Woodhead Publishing.

Mohana, D., Prasad, P., Vijaykumar, V., Raveesha, K. (2011) Plant extract effect on seed-borne pathogenic fungi from seeds of paddy grown in southern India. *Journal of Plant Protection Research*, **51**(2), 101-106.

Moubasher, A. (1993) "S*oil Fungi in Qatar and Other Arab Countries*". The Centre for Scientific and Applied Research, University of Qatar.

Nácher-Mestre, J., Serrano, R., Beltrán, E., Pérez-Sánchez, J., Silva, J., Karalazos, V., Hernández, F., Berntssen, M.H. (2015) Occurrence and potential transfer of mycotoxins in gilthead sea bream and Atlantic salmon by use of novel alternative feed ingredients. *Chemosphere*, **128**, 314-320.

Palumbo, J.D., O'Keeffe, T.L. (2013) Distribution and mycotoxigenic potential of *Aspergillus* section Nigri species in naturally contaminated almonds. *Journal of Food Protection*, **76**(4), 702-706.

Perrone, G., Gallo, A. (2017) *Aspergillus* species and their associated mycotoxins. In: "*Mycotoxigenic Fungi*", pp. 33-49, Springer.

Rath, P.M. (2001) Phenotypic and genotypic characterization of reference strains of the genus *Aspergillus*. *Mycoses*, **44**(3-4), 65-72.

Reddy, K., Salleh, B., Saad, B., Abbas, H., Abel, C., Shier, W. (2010) An overview of mycotoxin contamination in foods and its implications for human health. *Toxin Reviews*, **29**(1), 3-26.

Samson, R.A., Houbraken, J., Kuijpers, A.F., Frank, J.M., Frisvad, J.C. (2004) New ochratoxin A or sclerotium producing species in *Aspergillus* section Nigri. *Studies in Mycology*, **50**(1), 45-56.

Singh, P., Cotty, P.J. (2019) Characterization of Aspergilli from dried red chilies (*Capsicum* spp.): Insights into the etiology of aflatoxin contamination. *International Journal of Food Microbiology*, **289**, 145-153.

Soares, C., Calado, T., Venâncio, A. (2013) Mycotoxin production by *Aspergillus niger* aggregate strains isolated from harvested maize in three Portuguese regions. *Revista Iberoamericana de Micología*, **30**(1), 9-13.

Visconti, A., Lattanzio, V.M.T., Pascale, M., Haidukowski, M. (2005) Analysis of T-2 and HT-2 toxins in cereal grains by immunoaffinity clean-up and liquid chromatography with fluorescence detection. *Journal of Chromatography A*, **1075**(1-2), 151-158.

Wang, X., Zhang, H., Liu, H., He, C., Zhang, A., Ma, J., Ma, Y., Wu, W., Zheng, H. (2011) An immunoarray for the simultaneous detection of two mycotoxins, Ochratoxin A and Fumonisin B1. *Journal of Food Safety*, **31**(3), 408-416.

Zain, M.E. (2011) Impact of mycotoxins on humans and animals. *Journal of Saudi Chemical Society*, **15**(2), 129-144.

Zheng, Z., Humphrey, C.W., King, R.S., Richard, J.L. (2005) Validation of an ELISA test kit for the detection of total aflatoxins in grain and grain products by comparison with HPLC. *Mycopathologia*, **159**(2), 255-263.

تقييم وتعيين سموم الأوكراتوكسينات والأفلاتوكسينات فى أسبرجيللس بيبيرس بواسطة التحليل الطيفي الفلوري، الفصل الكهربى وتقنية استخدام الأجسام المضادة

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تم الكشف الكمى عن السموم الفطرية (أوكراتوكسينات OTs والأفلاتوكسينات AFs) فى كلا من الكتلة الحية للخيوط الفطرية والجراثيم FB والرشيح CF الخاصين بفطر أسبرجيللس بيبيرس *Aspergillus piperis* بواسطة التحليل الطيفي الفلوري. وقد بينت النتائج أن الأوكراتوكسينات تتواجد بتركيزات عالية ؛ 38×10^{-3} ، 32×10^{-3} ميكروجرام/جرام في كلا من الكتلة الحية والرشيح على التوالي. بينما سجلت الأفلاتوكسينات تركيزات أقل ؛ 3.3×10^{-3} ، 2.3×10^{-3} ميكروجرام/جرام. بناءا على ما تقدم تم تعريف أنواع مختلفة من الأوكراتوكسينات المنتجة في الكتلة الحية والرشيح الخاصين بفطر *A. piperis* باستخدام طريقة الفصل الكهربائي (SDS-PAGE) حيث دلت الأوزان الجزيئية للبروتينات المنفصلة عن وجود الأوكراتوكسين B و α و C (OTB و OTα و OTC) في الكتلة الحية *A. piperis* بينما تم تسجيل وجود OTB و OTα فقط في الرشيح. كانت الأوزان الجزيئية لكل من OTB و OTα و OTC هى 363 ، 430،260 KDA على التوالي. تم تأكيد هذه النتائج من خلال تقنية استخدام الأجسام المضادة لكل من OTB و OTα و OTC. وترجع أهمية هذا البحث إلى التعرف على السموم الفطرية الخاصة بعزلة الفطر محل الدراسة نظرا لأنه تم الكشف مؤخرا عن صفات تضاد حيوى لهذا الفطر ضد الفطريات الممرضة للنبات مما يؤهله للاستخدام فى حماية النباتات ضد الأمراض الفطرية المختلفة وبالتالى وجب التعرف على سمومه جيدا قبل ترشيحه للاستخدام على المحاصيل المستخدمه من قبل الانسان. تم اختبار السمية لتركيزات مختلفة من رشيح المستعمرة الفطرية للأسبرجيلس بيبيرس على نسبة ومؤشر الانبات لبذور نبات الفول وقد بينت النتائج أن بزيادة التركيز تقل نسبة الإنبات للبذور.