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MYCOFLORA AND MYCOTOXINS OF SUNFLOWER (*HELIANTHUS ANNUUS L.*) SEEDS IN EGYPT 1. SUGAR FUNGI AND NATURAL OCCURRENCE OF MYCOTOXINS

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ABSTRACT

Eighteen genera and sixty-three species, in addition to three varieties were isolated from thirty six samples of sunflower seeds collected from different places in Egypt. *Aspergillus* and *Penicillium* followed by *Rhizopus* and *Fusarium* were the most frequent genera. *A. niger, A. flavus, A. fumigatus, A. terreus* and *P. chrysogenum* were the most common species. Samples 11, 4 and 8 of 36 sunflower samples were of high, moderate and low toxicity, respectively, to brine shrimp larvae and were naturally contaminated with aflatoxins (B_1, B_2, G_1 and G_2), sterigmatocystin, ochratoxin A, zearalenone, T-2 toxin and diacetoxyscirpenol.

INTRODUCTION

In the years since 1960, it became clear that aflatoxins were a problem not just in groundnuts but in a wide range of materials used for human food and animal feed, including other oilseeds such as cotton seed, sunflower and soybean. Surveys for the mycoflora and mycotoxins in cotton seed and peanut were conducted in this laboratory (Sabah Saber, 1982 and El-Maghraby and El-Maraghy, 1986).

The present work was designed for studying the mycoflora and mycotoxins in sunflower samples collected from different places in Egypt.

World production of sunflower, in 1975 was 9.6 million tons (FAO, 1979). The largest producer countries are U.S.S.R, Argentina, United States and Turkey. No reports are available from the producing countries on the incidence of aflatoxins in

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domestic sunflower seed or cake. However, three countries have reported results of surveillance of imported sunflower cake. These are : Hungary, Germany and Italy (FAO, 1979).

MATERIALS AND METHODS

Thirty-six samples of sunflower seeds of 1985 crop, half kg each, were collected from the markets in Egypt.

Determination of seed-borne fungi

This was made by using the dilution-plate method as described by Christensen (1963). Modified Czapek's agar medium was used in which glucose replaced sucrose (10 g/1) and to which rose bengal (1/15000) combined with streptomycin (1/30000) were used as bacteriostatic agents (Smith & Dawson, 1944, and Martin, 1950). Five plates were used for each sample. The plates were incubated at 28°C for seven days and the developing fungi were identified and counted. The colonies of slow growing fungi were transferred to slants to ensure precise counting and then to plates for identification. Other agar media were also used (Czapek's + 0.05 yeast extract, malt extract, potato-dextrose and corn meal agar).

Toxins extraction from sunflower seeds

Twenty-five g of sunflower sample were defatted by extraction with n-hexane for 10 h using a Soxhlet-type extractor. The defatted residue was extracted for another 10 h with chloroform. The chloroform extract was dried over anhydrous sodium sulphate, then filtered and distilled to dryness under vacuum. The residue was diluted with chloroform to 1 ml. The chloroform extracts were analyzed for the presence of known mycotoxins, aflatoxins (B_1, B_2, G_1 & G_2, citrinin, ochratoxin A, patulin, sterigmatocystin, zearalenone, T-2 toxin and diacetoxyscirpenol using thin-layer chromatographic plates and tested for toxicity using brine shrimp larvae.

Detection and assay of toxins

Brine shrimp *(Artemia salina)* larvae were used for mycotoxins bioassay according to the method described by Korpinen (1974).

a) 15 – 20 drops of brine shrimp eggs (HADLOW, KENT, ENGLAND) were hatched in artificial sea water (NaCl, 30 g; $CaSO_4$, 2 g; $MgSO_4 . 7 H_2O$, 3 g; MgCl, 8.5 g; KCl, 0.8 g; and MgBr, 0.1 g; per liter distilled water and adjusted to pH 10 with NaOH) and kept at room temperature (22 – 24°C). Air is usually conducted into the water in small bubbles through a tube. Three days after the emergence, the hatched larvae were used as test animals. In order to obtain the desired concentration of the larvae, they were filtered through ordinary filter paper and resuspended in a known volume of sea water.

b) 0.05 ml of chloroform extract was placed in each test tube, the chloroform was evaporated and about 20 – 40 shrimp larvae in 1 ml sea water were transferred into the tubes. The tubes were kept at room temperature (22 – 24°C). Control tubes with 0.05 ml of chloroform were also made.

c) After 24 hours, the mortality was determined with a stereoscopic microscope.

Thin – layer chromatographic determination of toxins

For qualitative determination, thin – layer chromatographic technique adopted in this laboratory (El-Kady and Moubasher, 1982 and El-Kady, 1986) was employed using precoated silica gel plates type 60 F_{254} (MERCK).

RESULTS AND DISCUSSION

1. Mycoflora of sunflower seed

Sixty three species and 3 varieties belonging to 18 genera were isolated in the present investigation on glucose – Czapek's medium (Table 1). All of these species were isolated before from Egyptian seeds and grains (Moubasher *et al.*, 1972 and 1979; Abdel-Kader *et al.*, 1979; El-Kady *et al.*, 1982; Mazen *et al.*, 1984 and El-Maghraby, 1984).

Aspergillus and *Penicillium* were extremely dominant followed by *Rhizopus* and *Fusarium*. The previous genera were also dominant in Egyptian cereal grains (Assawah and Elarosi, 1960; Moubasher *et al.*, 1972; Abdel-Kader *et al.*, 1979; El-Kady *et al.*, 1982; Mazen *et al.*, 1984; and El-Maghraby, 1984) and oil seeds (Moubasher *et al.*, 1979; Sabah Saber, 1982).

Aspergillus was the most common genus and occurred in 100 % of the samples representing 85.7 % of the total fungi. Twenty-three species in addition to 3 varieties of this genus were obtained. *A. niger, A. flavus, A. fumigatus* and *A.*

terreus were of high occurrence. They were isolated from 91.7 %, 83.3 %, 77.8 % and 55.6 % of the samples, thus constituting 29.4 %, 39.3 %, 15.8 % and 2.9 % of total Aspergilli and 29.4 %, 33.7 %, 13.5 % and 2.5 % of total fungi, respectively. The previous four species were also the most common Aspergilli in peanut shells, covered and uncovered seeds (Moubasher *et al.,* 1979) and in cotton seeds, crushed cotton seeds, cooked meal and cotton seed cake (Sabah Saber, 1982). Four species and one variety were isolated in moderate frequency and these were *Aspergillus flavus* var. *columnarise, A. nidulans, A. sydowi ,A. tamarii* and *A. ochraceus* occurring in 41.7 %, 41.7 %, 36.1 %, 30.6 % and 25 % of the samples comprising 1.1 %, 3 %, 1.5 % 0.6 % and 1 % of total Aspergilli and 0.9 %, 2.6 %, 1.3 %, 0.5 % and 0.9 % of total fungi, respectively.

Penicillium was second in frequency of occurrence and was recovered from 88.9 % of the samples, yielding 6 % of the total fungi. It was represented by 14 species of which *P. chrysogenum* was the most prevalent followed by *P. corylophilum* and emerged from 63.9 % and 36.1 % of the samples comprising 41.1 % and 7.3 % of total penicilli and 2.5 % and 0.4 % of total fungi, respectively. *P. chrysogenum (P. notatum)* and *P. corylophilum* were the most prevalent *Penicillium* species in cotton seeds, crushed cotton seeds, cooked meal and cotton seed cake (Sabah Saber, 1982). Moubasher *et al.* (1979) isolated *P. chrysogenum* at low to rare frequency of occurrence from peanut shells, covered and uncovered peanut seeds.

Rhizopus and *Fusarium* were of moderate occurrence. They emerged in 38.9 % and 36.1 % of the samples accounting 0.4 % and 4.3 % of total fungi, respectively. *Rhizopus* was represented by *R. stolonifer* only. Four species of *Fusarium* were identified, namely *F. oxysporum, F. moniliforme, F. equiseti and F. semitectum. F. oxysporum* was the most frequent species occurring in 25 % of the samples and *constituting* 15.4 % of total *Fusarium* and 0.7 % of the total fungi. *F. moniliforme* was isolated in low occurrence, but its count comprised 81.3 % of total *Fusarium* and 3.5 % of total fungi. *Fusarium oxysporum* and *F. moniliforme* were isolated from Egyptian cereal grains and oil seeds as reported by Moubasher and his *collaborators* (1972).

Table 1

Total counts of fungal propagules per sunflower seed and number of samples from which each fungus was isolated

Genera and Species	T. C. (propagules)	N. C. I.
Total count	4498.9	
A spergillus	3853.6	36
A. niger Van Tieghem	1132.9	33
A. flavus Link	1514.8	30
A. fumigatus Fresenius	608.0	28
A. terreus Thom	110.9	20
A. flavus var. columnaris Raper & Fennell	42.0	15
A. nidulans (Eidam) Wint	117.4	15
A. sydowi (Bain. & Sart.) Thom & Church	57.7	13
A. tamarii Kita	23.4	11
A. othraceus Wilhelm	39.4	9
A. nidulans var. *latus* Thom & Raper	41.8	7
A. parasiticus Speare	81.0	5
A. melleus Yukawa	3.8	4
A. candidus Link	2.5	3
A. quadilineatus Thom & Raper	52.0	3
A. terreus var. *aureus* Thom & Raper	5.2	3
A. versicolor (Vuill.) Tiraboschi	1.8	3
A. carneus (v. Tiegh.) Blochwitz	3.2	2
A. chevalieri (Mang.) Thom & Church	4.4	2
A. terricola Marchal	1.2	2
A. amstelodami (Mang.) Thom & Church	0.6	1
A. clavatus Desmazieres	0.6	1
A. egyptiacus Moubasher & Moustafa	5.2	1
A. flavipes (Bain. & Sart.) Thom & Church	1.3	1
A. nidulans species (Hulle cells)	0.6	1
A. sulphureus (Fres.) Thom & Church	0.6	1
A. ustus (Bain.) Thom & Church	1.3	1
Penicillium	271.3	32
P. chrysogenum Thom	112.3	23
P. corylophilum Dierckx	19.9	13
P. nigricans (Bain.) Thom	39.1	8
P. viridicatum Westling	39.0	7
P. citrinum Thom	16.8	5
P. camemberti Thom	9.8	4
P. jenseni Zaleski	3.1	4

Table 1 : Contd.

Genera and Species	T. C. (propagules)	N. C. I.
P. cyclopium Westling	13.2	2
P. roqueforti Thom	6.5	2
P. funiculosum Thom	0.6	1
P. kapuscinskii Zaleski	3.9	1
P. piscarium Westling	2.6	1
P. purpurogenum Stoll	0.6	1
P. simplicissimun (Oud.) Thom	3.9	1
Rhizopus stolonifer Ehrenb ex Fr. Lindt	16.3	14
Fusarium	194.3	13
F. oxysporum Schlecht ex Fr.	29.9	9
F. moniliforme Sheldon	157.9	6
F. equiseti (Corda) Sacc	1.3	1
F. semitectum Berk & Ray.	5.2	1
Acremonium	9.8	7
A. implicatum Gilman & Abbott	8.5	6
A. strictum W. Gams	1.3	1
Cladosporium	85.3	7
C. herbarum (Pers.) Link ex Fr.	43.1	4
C. sphaerospermun Penzig	22.1	4
C. cladosporioides (Fres.) de Vries	20.1	3
Alternaria alternata (Fr.) Keissler)	19.3	4
Mucor	5.1	4
M. racemosus Fresenius	4.5	3
M. hiemalis Wehmer	0.6	1
Stachybotrys	8.7	4
S. albipes Berk. & Br Jong & Davis	2.6	2
S. chartarum (Ehrenb. ex Link) Hughes	4.8	2
S. microspora (Mathur & Sankhla) Jong & Davis	1.3	1
Syncephalastrum racemosum (Cohn Schroeter)	4.5	3
Gliocladium	11.0	2
G. catenulatum Gilman & Abbott	0.6	1
G. roseum (Link) Thom	10.4	1
Humicola grisea Traaen	3.5	2
Trichothecium roseum (Pers.) Link ex Fr.	2.6	2
Botryotrichum atrogriseum Van Beyma	0.6	1
Epicoccum purpurascens Ehrenb. ex. Schlecht	2.6	1
Paecilomyces terricola Bainier	1.3	1
Sporotrichum olivaceum Fries	2.6	1
Trichoderma viridi Pers. ex. S. F. Gray	1.3	1
Sterile mycelium	5.2	2

T.C. = Total count per mg seeds in every sample.
N.C.I. = Number of cases of isolation (out of 36).

High occurrence = more than 18 cases.
Moderate occurrence = between 9 – 18 cases.
Low occurrence = between 4 – 8 cases.
Rare occurrence = Less than 4 cases

2 . Mycotoxins of sunflower seed

Eleven, 4 and 8 crude extracts of sunflower seeds out of 36 samples tested (63.9 % of the samples) were of high, moderate and low toxicity, respectively to brine shrimp larvae (Table 2). The crude extracts were found by TLC analysis to contain aflatoxins (B_1, B_2, G_1 and G_2), 2 trichothecene derivatives (T-2 toxin and dia-cetoxyscirpenol) , ochratoxin A, sterigmatocystin and zearralenone.

Aflatoxin derivatives (B_1, B_2, G_1 and / or G_2) were detected in 41.7 % of the samples, most of these samples were highly infected with *Aspergillus flavus* and / or *A. parasiticus.* From the view point of direct hazard to health, aflatoxins are the most important among the known mycotoxins (Scott, 1973). Aflatoxins are mutagenic, carcinogenic and teratogenic and actually toxic to most experimental and domesticated animals and man (El-Zawahri *et al.,* 1977 and Davis and Diener, 1978).

Sterigmatocystin was reported in 8.5 % of the crude extracts of samples in which *A. nidulans, A. nidulans* var. *latus* and *A. quadrilineatus (A. nidulans* group) were predominant. The previous species were reported as sterigmatocystin - producing fungi in this laboratory by El-Kady and Abdel-Hafez (1981) .

Ochratoxin A was identified in the crude extract of one sample only. This sample was naturally contaminated by 6 species of Aspergilli *(A. flavus, A. melleus, A. niger, A. ochraceus, A. sydowi* and *A. terreus),* two species of penicilli *(P. chrysogenum* and *P. corylophilum) .* Ochratoxin A was produced by *A. ochraceus* and *A. melleus* as previously reported by Ciegler (1972), Ciegler *et al.* (1972), Hesseltine *et al.* (1972) and Krogh (1978).

Zearalenone was detected in the crude extracts of only two samples in which *F. oxysporum* was found in the first and *F. moniliforme* in the other. Zearalenone was previously found in maize, wheat, oats, sorghum, sesame seed, potato, hay, silage cassava and commercially formulated processed feed (Mirocha *et al.,* 1974, 1976 and 1977, Mirocha, 1983, and El-Maraghy, 1984).

T-2 toxin and diacetoxyscirpenol were detected in 11 % and 13.8 % of the samples, respectively. These samples were molded with species of *Stachybotrys, Fusarium, Acremonium* and *Trichthecium.* In this respect, more than 62 different trichothecenes have been produced in laboatory cultures by species of *Fusarium, Cephalosporium, Myrothecium, Trichothecium, Trichoderma* and *Stachybotrys.* T-2 toxin and diacetoxyscirpenol were naturally detected in Egyptian paddy and wheat grains (El-Maghraby, 1984 and El-Maraghy, 1984) .

Table 2

Moisture content, propagule counts of fungi in each sample per mg sunflower seed, dominant species and their counts, toxicity test and toxins identified in 36 sunflower seed samples

Sample No.	% Moisture content	Total (Propagules / mg dry seeds)	Dominant species	Count (propagules / mg dry seeds)	Sample extracts	
					Toxicity test	Toxins identified
1	3.4	50.6	*Aspergillus flavus*	6.6	A	aflatoxins B_1, B_2 G_1 and G_2
			A. fumigatus	8.8		
			A. niger	6.6		
			Penicillium citrinum	11.0		
2	4.9	35.2	*A. fumigatus*	4.4	A	diacetoxyscirpenol and T – 2 toxin
			Cladosporum cladosporioides	6.6		
			C. herbarum	6.6		
			Stachybotrys chartarum	2.2		
3	5.2	39.6	*A. fumigatus*	11.0	D	
			C. herbarum	6.6		
			P. cyclopium	11.0		
4	4.7	521.3	*A. fumigatus*	198.9	A	aflatoxins B_1, and B_2
			A. quadrilineatus	23.4		
			A. nidulans	14.3		
			A. niger	244.4		
			Fusarium oxysporum	5.2		
			P. nigricans	16.9		
5	4.0	174.0	*A. flavus*	63.3	C	aflatoxins B_1, B_2, G_1 and G_2.
			A. niger	100.3		
6	4.4	23.4	*A. flavus*	4.0	C	aflatoxins B_1 , B_2 G_1 and G_2.
			A. niger	6.5		
			A. fumigatus	5.2		
7	4.5	41.6	*A. niger*	7.8	C	T – 2 toxin
			C. cladosporioides	9.1		
			C. sphaerospermum	5.2		
			F. oxysporum	1.3		
			S. albipes	1.3		
8	4.5	35.1	*A. flavus*	4.0	C	aflatoxins B_1 and B_2
			A. niger	6.5		
			C. herbarum	5.2		
9	4.9	42.9	*A. flavus*	7.8	B	diacetoxyscirpenol
			C. sphaerospermum	5.2		
			Gliocladium roseum	10.4		
			S. albipes	1.3		
			S. microspora	1.3		
10	4.5	224.9	*A. flavus*	149.5	A	diacetoxyscirpenol
			A. nidulans	15.6		
			A. niger	19.5		
			F. moniliforme	29.9		
11	5.9	336.5	*A. flavus*	247.5	D	
			A. niger	83.2		

Table 2: Contd.

Sample No.	% Moisture content	Total (Propagules / mg dry seeds)	Dominant species	Count (propagules / mg dry seeds)	Sample extracts	
					Toxicity test	Toxins identified
12	4.4	205.4	*A. flavus*	143.0	D	
			A. niger	20.8		
			A. quadrilineatus	16.9		
13	7.3	232.7	*A. flavus*	111.8	A	aflatoxins B_1 and B_2
			A. nidulans	13.0		
			A. niger	68.9		
			A. quadrilineatus	11.7		
14	5.3	7.1	*A. nidulans var. latus*	3.9	D	Sterigmatocystin
			A. parasiticus	1.3		
			A. terreus	1.3		
15	5.3	150.2	*A. niger*	26.4	D	aflatoxins B_1 and B_2
			A. parasiticus	73.2		
16	5.3	113.3	*A. flavus*	44.2	B	afltoxins B_1, B_2 G_1 and G_2
			A. niger	46.9		
17	5.1	101.2	*A. flavus*	31.7	A	diacetoxyscirpenol and aflatoxins B_1, B_2, G_1 and G_2
			A. niger	31.0		
			A. ochraceus	5.2		
			A. sydowi	11.9		
			F. oxysporum	1.3		
18	4.4	151.7	*A. flavus*	87.8	D	sterigmatocystin
			A. fumigatus	14.5		
			A. niger	23.4		
			A. quadrilineatus	22.4		
19	5.5	33.2	*A. flavus*	5.3	A	aflatoxing B_1, B_2, G_1 and G_2
			A. fumigatus	7.9		
			A. niger	11.2		
			A. parasiticus	2.2		
20	5.6	40.0	*A. flavus*	9.9	D	
			A. niger	10.6		
			A. sydowi	5.9		
21	4.7	40.1	*A. flavus*	3.9	A	aflatoxins B_1 B_2, G_1 and G_2
			A. fumigatus	3.2		
			A. niger	7.8		
22	5.3	191.2	*A. flavus*	34.2	D	ochratoxin A
			A. niger	71.9		
			A. ochraceus	18.5		
			A. sydowi	20.5		
			A. terreus	17.2		
			P. chrysogenum	21.1		
23	5.1	95.5	*A. flavus*	29.1	A	aflatoxins B_1 and B_2
			A. niger	44.2		
			terreus	10.6		
			P. chrysogenum	3.9		

Table 2 : Contd.

Sample No.	% Moisture content	Total (Propagules / mg dry seeds)	Dominant species	Count (propagules / mg dry seeds)	Sample extracts	
					Toxicity test	Toxins identified
24	5.5	105.1	*A. flavus*	3.1	D	
			A. niger	52.8		
			P. nigricans	5.9		
25	5.0	94.1	*A. flavus*	7.9	B	aflatoxins B_1, B_2, G_1 and G_2
			A. niger	40.3		
			A. terreus	33.0		
			P. chrysogenum	3.3		
26	4.8	78.0	*A. flavus*	58.7	D	
			A. niger	11.2		
27	4.3	24.6	*Acremonium implicatum*	2.0	C	T – 2 toxin
			Alternaia alternata	7.9		
			Trichothecium roseum	2.0		
28	4.1	19.0	*Al. alternata*	7.5	C	T – 2 toxin
			A. fumigatus	2.0		
			T. roseum	0.6		
29	5.3	164.3	*A. fumigatus*	85.8	D	
			A. niger	31.2		
			F. oxysporum	10.8		
			P. viridicatum	20.8		
30	5.7	78.0	*A. fumigatus*	20.8	D	
			C. herbarum	23.4		
			C. sphaerospermum	10.4		
			P. chrysogenum	7.8		
31	5.8	418.6	*A. flavus*	267.2	B	sterigmatocystin
			A. nidulans	46.8		
			A. niger	75.4		
			F. semitictum	5.2		
32	5.2	52.0	*A. egyptiacus*	5.2	D	zearalenone
			A. fumigatus	7.8		
			A. niger	5.2		
			F. oxysporum	5.2		
			P. chrysogenum	10.4		
33	6.2	124.8	*A. fumigatus*	93.6	C	
			A. niger	5.2		
			P. corylophilum	5.2		
34	6.1	70.2	*A. fumigatus*	31.2	C	
			A. niger	20.8		
			Epicoccum purpurascens	2.6		
			P. chrysogenum	7.8		
35	5.5	358.8	*A. flavus*	137.8	A	aflatoxins B_1, B_2 G_1 & G_2; & diacatoxyscirpenol and zearalenone
			A. fumigatus	28.6		
			A. niger	36.4		
			F. moniliforme	122.2		

Table 2 : Contd.

Sample No.	% Moisture content	Total (Propagules / mg dry seeds)	Dominant species	Count (propagules / mg dry seeds)	Sample extracts	
					Toxicity test	Toxins identified
36	4.4	68.9	*A. flavus* *A. fumigatus* *A. niger* *A. terreus* *P. chrysogenum*	5.2 24.7 5.2 13.0 5.2	A	aflatoxins B_1, B_2 G_1 and G_2

Toxicity test

A = High, 75–100 % of larvae tested died.
B = Moderate, 50–74 % of larvae tested died.
C = Low, 25–49 % of larvae tested died.
D = None, 0–24 % of larvae tested died.

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الفلـورا الفطـرية وسـموم الفطـريات لبـذور عبـاد الشـمس في مصـر
(١) فطريات السكر والتلوث الطبيعي لسموم الفطريات

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تم في هذا البحث عزل وتعريف ٦٣ نوعاً تنتمي إلى ١٨ جنساً من الفطريات من ٣٦ عينة من بذور عباد الشمس جمعت من المناطق المختلفة بجمهورية مصر العربية ، وذلك باستخدام طريقة التخفيف والوسط الغذائي شابكس المحتوي على ١ ٪ جلوكوز. كانت أكثر الأجناس سيادة هي **اسبرجيلس ، بنسيليوم ، ريزوبس وفيوزاريم** ، كما كانت أكثر الأنواع إنتشاراً **اسبرجيلس نيجر ، اسبرجيلس فلافس ، اسبرجيلس فيوميجاتس ، اسبرجيلس تيريس وبنسيليوم كريزوجينم .**

درست سمية مستخلص العينات المختبرة بالإستعانة ببرقات قشريات الملاحات رخوية الهيكل (ارتيميا ساليني) وهي شديدة الحساسية للتركيزات الضئيلة من سموم الفطريات ، وقد تم التعرف على السموم المتواجدة بطرق التحليل الكروماتوجرافي باستخدام رقائق السيلكا وعديد من المذيبات والكواشف اللونية وقد ثبت أن مستخلص ١١ عينة عالية السمية ، ٤ متوسطة السمية و ٨ قليلة السمية ، كما أظهر التحليل تلوث العينات ببعض السموم الفطرية منها **الافلاتوكسينات B_1, B_2, G_1 & G_2 الاسترجماتوسيستين ، اوكراتوكسين A ، الزيرالينون ، سم T-2 ثنائي استيكوسي سكرابينول .**