Role of Dinaferm in Improvement of Aflatoxicosis in Cultured Sea bass

Abou El-Gheit, E. N.[1] , Saad, T.T.[2] and El-Hammady, A. K. I.[1]

[1]Aquatic pathology Lab, National Institute of Oceanography and Fisheries, Egypt.
[2]Poultry and Fish Diseases Department, Faculty of Veterinary Medicine Alex University, Egypt.
abouelgheit5374@yahoo.com

Abstract: This study was carried-out on Sea bass collected from different private fish farms at Alexandria governorate. Role of dinaferm in improvement of aflatoxicosis was investigated. Fish treated with AFTB1 revealed erratic swimming with respiratory and nervous manifestations, haemorrage and congested lesions on the skin, increasing the amount of slime mucous on the skin and gills, severe bilateral exophthalmia, blindness in addition to fin and tail rot. Postmortem lesions showed that, the liver was friable with pale brownish color, congested kidney and congested gall bladder. The posterior portion of the kidney was tinged with blood. The economic losses resulted from AFTB1 appeared in the form of increasing the mortality rates by about 70 % in the group treated with AFTB1, while the group fed Dinaferm, the mortality rates reached 43.75 % and there was a clear decrease in the body weight, weight gain and economic returns. The addition of Dinaferm improve the weight gain in the group treated with Dinaferm by about 0.35, 0.63, 0.46, 0.16, 0.02, 0.05, 0.29 and 0.6 Kg, and the economic returns improved by about 2.45, 4.41, 3.22, 1.12, 1.14, 0.35, 2.03 and 4.20 LE than the group treated with AFTB1 for the period from 1 to 8 weeks, respectively.This study concluded that, the toxicity of fish with Aflatoxin B1 causes a high economic losses to cultured fish via its increasing mortality, decreasing body weight, body weight gain and the economic returns and the addition of Dinaferm to the fish diet can improve body weight, body weight gain and the economic and productive efficiency of fish production farms .
[Abou El-Gheit, E. N. , Saad, T.T. and El-Hammady, A. K. I. **Role of Dinaferm in Improvement of Aflatoxicosis in Cultured Seabass.** *Life Sci J* 2013; 10 (2): 1285-1291] (ISSN:1097-8135). http://www.lifesciencesite.com. 178

Keywords: Dinaferm; Aflatoxicosis; Cultured Sea bass.

1. Introduction

Fish and fish products play an important and increasing role in solving the human nutritional problems in Egypt.

One of the main factors affecting fish production and efficiency were fish diseases and especially that resulted from mycotoxins and mainly Aflatoxins especially Aflatoxin B1 (AFTB1) which commonly contaminated the human, animal and fish feed (Scudmore et al., 1999; Creepy et al., 2004; Bayder et al., 2005; Bejaoui et al. 2006 and Timperio et al., 2006).

In human the mycotoxins Aflatoxins constitute a major hazards on newly weaned children (Jonsyn et al. 1995 ; Oylami et al., 1996; Scudmore et al., 1999 and Bejaoui et al., 2006). Also there is a common relationship with Balkan Endemic Nephropathy and AFTB1 (Stefanovic et al., 2006), also between AFTB1 and Procine Nephropathy (Radic et al., 1997 and Sauvant et al., 2005) and there is a report called the Aflatoxins is a mycotoxins endemic in Egypt (El-Kady et al., 1995; El-Shaboury 1998 and Wafa et al., 1998).

Aflatoxins causes severe economic losses to fish industry (Jonsyn and Lahai, 1992), through decreasing fish weight, feed conversion ratio and causes severe mortality among fish farms (Samira-Rezeaka, 1991 and El-Shaboury, 1998).

The Aflatoxins causes severe losses among different fish species especially Tilapia Nilotica, Mugil and Common Carp and these species considered the most distributed fish species among Egyptian fish farms and lakes (El-Zarka et al., 1993 and El-Shaboury , 1998) . Also, these species accepted by Egyptian consumers and play an important economic role in fish industry in Egypt.

Aflatoxins are a group of structurally related, toxic metabolites produced by seven species of Aspergillus and six species of Penicillium. *Aspergillus flavus* from which the toxins acquired their name appears to be the predominant Aflatoxin producer . Furthermore, the toxin production by these fungi is influenced by species and even strain of fungi, time and temperature of incubation, moisture content of substrate and type of substrate. (Scudmore et al., 1999; Bayder et al., 2005 and Tangani and Pussemier, 2006).

The most important clinical signs that appeared on the fish suffered from Aflatoxicosis include , nervous and respiratory manifestations as well as skin erosion, congestion of the skin, fin and tail rot, exophthalmia and congestion. While, the most important postmortem changes were enlargement of all internal organs especially liver, kidney, spleen and gall bladder. (Easa, 1997 and Saad , 2002).The main economic losses resulted from affection of cultured

fish with Aflatoxins resulted from decrease of fish body weight, weight gain and hindrance of the food conversion efficiency. (Easa, 1997 and Saad, 2002).

The addition of some immunostimulants as the Dinaferm can inhibit the effect of Aflatoxins on the cultured fish and increase the economic and productive efficiency of fish production farms via increasing the body weight and weight gain (Easa, 1997 and Saad, 2002).

The aim of this study, is the determination of the effect of Aflatoxins on the efficiency of fish farms and determination of the lesions and clinical signs of the infected fish, in addition to determination of the role of the Dinaferm as feed additives in improving the losses resulted from Aflatoxins.

2. Materials and Methods

Fish:

A total number of 320 Sea bass random fish samples were collected from different private marine fish farms. The fish were transported alive to the laboratory in plastic bags containing water enriched by air (2/3). The average body weight of the fish about 30 ± 5 gm.

Aquaria:

Fish were kept in prepared glass aquaria (90 x 50 x 35 Cm). These aquaria were used for holding the experimental fish throughout the period of the present study, supplied with saline water. The continuous aeration was maintained in each aquarium using an electric air compressors. Water temperature was kept at 22 ± 1 ^{o}C . All fish were acclimatized for at least 2 weeks prior to the experiment. (El-gamal, 2005).

Fish diets:

Fish were fed on commercial fish food containing 43-45% crude protein (obtained from Barseek fish culture factory) the diet was daily provided at 3% of body weight and the daily amount of food was offered on two occasions over the day (Regular diet), in addition to acute and chronic experiment as described by (El-gamal, 2005).

Aflatoxin B1:

The mycotoxin Aflatoxin B1[(C_{20} H_{18} $CINO_6$) (7-Carboxy-5-Choloro-8-Hydroxy-3, 4-dihydro-3-R-methyl isocoumarin)] was kindly provided by Sigma Chemical Co. U. S. A. also by Sigma-Aldrich Chemie Gmbh, Germany. The AFTB1 that used its number 0-1877 (1 mg) Lot. No. 76 H 4084.

Dinaferm:

Is an immunostimulant containing the extract of Ginseng plant and sucroses that increase the vitality and activity of the fish. Also, it increase and activate the antibodies and immunity of the fish against any stress that the fish exposed to it also contain some useful bacteria that help in destruction of the AFTB1 and hindered its effects, also the Dinaferm activate the liver to get ride of the AFTB1 and also, it considered as a stomachic to the fish that improve the fish appetite. Moreover, the Dinaferm improve the water quality and decrease the ammonia level with improvement the excretion of heavy metal from the fish body.

Toxicity of Aflatoxin- B1.:

The fish (30±5 gm each) in this experiment divided into 4 groups, two groups of them was injected with 1/10 LD50 (10000 ng) (Shehata et al., 1985), the first group take toxin with Dinaferm ® and the second group take toxin with regular diet.

The other two groups act as a control and one of them take regular diet only and the other take regular diet with Dinaferm. All the experimental groups were kept under daily observation for 8 weeks.

The clinical signs, mortality and postmortem lesions were recorded throughout the 8-week experimental period .

Fish weight:

During the experiment the fish was weighted weekly and the body weight gain was calculated for every week according to the following equation (Shewita, 2004):

Weight gain = Body weight 2 – Body weight 1.

Economic returns:

The return from fish sale per LE according to the following equation (Shewita,

2004 El-Tahawy, 2004):

Returns = Wight of fish X price of fish (LE).

Statistical analysis:

The data of body weight, body weight gain and return losses of exposed fish to AFTB1 were statistically analyzed using ANOVA test according to (SAS, 1987 and Macarthur et al., 2006) to examine the significant effect of the main variables on the studied parameters.

3. Results and Discussion

The mortalities due to exposure to AFTB1 in (Table, 1). Showed that the groups which fed AFTB1 with regular diet was recorded high mortality rate (70 %) than the group fed AFTB1 with diet contain Dinaferm (43.75 %). While the control groups not show any mortalities.

The economic losses for the groups fed AFTB1 with regular diet about 15.8 LE and for the groups fed Dinaferm with AFTB1 was about 9.8 LE.

Table (2) explain the significant differences ($P < 0.01$) due to the effect of different treatments on body weight of the fish, as the groups fed diet contain AFTB1 show severe decrease in body weight along the period of the experiment , as the body weight of this groups were 2.34, 2.27, 2.04, 2.24, 2.16, 1.74, 1.50 and 1.18 Kg, while the group fed Dinaferm with AFTB1 showed improvement in its body weight as

its body weight were 2.69, 2.90 , 2.50, 2.40, 2.18, 1.79, 1.79 and 1.78 Kg.

The return results in (Table, 3) indicated that ,the AFTB1 causes severe economic losses but by the addition of Dinaferm there is improvement in the economic returns of this groups. The economic returns of the groups fed AFTB1 were 16.38, 15.89, 14.28, 15.68, 15.12, 12.18, 10.50 and 8.26 LE, but, by the addition of the Dinaferm the economic returns by reached to 18.3, 20.30, 7.50, 16.8, 5.26, 12.53, 12.53 and 12.46 LE, for the weeks 1, 2, 3, 4, 5, 6, 7 and 8, respectively.

Also, the results in table (4) indicated that, the addition of Dinaferm to fish diet improves he weight gain and its returns, than the groups without Dinaferm treatment or AFTB1 feeding diet. The weight gain of the fish due to addition of Dinaferm were 0.35, 0.63, 0.46, 0.16, 0.02, 0.05, 0.29 and 0.6 Kg and the returns improved by about 2.45, 4.41, 3.22, 1.12, 0.14, 0.35, 2.03 and 4.20 LE for the weeks from 1 to 8, respectively.

This results agreed with those of (Saad, 2002) who reported that the AFTB1 causes decrease of body weight, body weight gain and so reduction of economic and productive efficiency of fish production farms and the addition of Dinaferm to the fish diet will improve body weight and weight gain with increasing of the fish production farms profits. Saad (2002) and El-gamal (2005) attributed this results to the Dinaferm act as immunostimulant as it contain the extract of Ginseng plant which increase the vitality and activity of the fish. Also, the Dinaferm increase and activate the antibodies and immunity of the fish against any stress that the fish exposed to it, also it contain some useful bacteria that help in destruction of the AFTB1 and hindered its effects, moreover the Dinaferm activate the liver to get ride of the AFTB1, in addition the Dinaferm considered as a stomachic to the fish that improve the fish appetite, the Dinaferm improve the water quality and decrease ammonia level and improve the excretion of heavy metal from the fish body.

The most important behavioural changes appeared on the infected fish with AFTB1 were - erratic swimming with respiratory and nervous manifestations, which appears in the form of swimming vertically and on the lateral sides, swimming in the circle movement, with signs of asphyxia, decreasing of feeding reflex, decrease vitality, losing of body reflex and don't stimulate to external stimuli, at the end of injection the fish appear off-food and death occur suddenly without any clinical appearance.

Table (1): Mortality number and rates, as well as the economic losses resulted from Aflatoxin-B1administration to Sea bass fish.

Treatment condition	Week number	Dinaferm treated diet		Regular diet	
		Mortality number	Return losses (LE)	Mortality number	Return losses (LE)
Control fish	1	-	-	-	-
	2	-	-	-	-
	3	-	-	-	-
	4	-	-	-	-
	5	-	-	-	-
	6	-	-	-	-
	7	-	-	-	-
	8	-	-	-	-
	Total mortalities	0/80	-	0/80	-
	Mortality % after 8-weeks	0%	-	0%	-
Treated fish with AFTB1	1	-	-	-	-
	2	5	1.40	16	4.48
	3	10	2.80	-	-
	4	5	1.40	-	-
	5	5	1.40	6	1.68
	6	10	2.80	12	3.36
	7	-	-	10	2.80
	8	-	-	12	3.36
	Total mortalities	35/80	9.8	56/80	15.68
	Mortality % after 8-weeks	43.75%	-	70%	-

The most important clinical signs of Aflatoxicosis appeared in Fig. (1 and 2) and it started in the 1st week of administration and continued until the end of the experiment (8th week) and it include, abnormal skin pigmentation, increase the amount of mucous (Slimness) in skin and gills and then decreased gradually, leading to appearance of rusty spots in the external surface of the fish, the eye affected as follow, in the beginning, appearance of eye cataract, which increased gradually leading to severe exophthalmia (bilateral) after that there is destruction of the eye and the fish become blind especially at the end of the experiment (10-20 %), there is fin and tail rot, which leads to appearance of vertebral column in severe cases (40%), mild ascitis changed to severe ascitis and severe abdominal distension, congested gills with mild signs of gill rot and stunted growth

At the post-mortem findings (Fig. 3 and 4) we noticed that, the liver is friable with a pale brownish colour, congested kidney especially at the later stages of injection, gas bladder is congested and covered with inflammatory exudates, the abdominal cavity is filled with viscous bloody fluid and sanguineous fluid, at the later stages appearing of gastro-enteritis, blotched area in the kidney especially in the posterior part, while the anterior part was slightly pale in colour, the liver at the beginning of injection was edematous and slightly pale with a focal necrotic area, the spleen is congested.

These results agreed with those of Safinaze (2000) and Saad (2002) where they reported that Aflatoxicosis causes severe behavioral, clinical and post-mortem changes similar to our results in toxicated fish.

This study concluded that, the toxicity of fish with Aflatoxins causes a high economic losses to cultured fish via its increasing mortality, decreasing body weight, body weight gain and the economic returns and the addition of Dinaferm to the fish diet can improve body weight, body weight gain and the economic and productive efficiency of fish production farms.

Table (2): Explain the effect of Aflatoxin-B1treatment on Sea bass weight (Kg) with and without Dinaferm treatment .

Group	Parameter	Fish number	Dinaferm® treated diet	Fish number	Regular diet
Control fish	Week1	80	3.16±0.03A	80	2.71±0.0B
	Week2	80	3.17±0.03A	80	2.82±0.03AB
	Week3	80	3.19±0.02A	80	2.89±0.02AB
	Week4	80	3.23±0.02A	80	2.99±0.01AB
	Week5	80	3.25±0.02A	80	3.03±0.02A
	Week6	80	3.26±0.02A	80	3.14±0.02A
	Week7	80	3.33±0.03A	80	3.17±0.03A
	Week8	80	3.45±0.04A	80	3.20±0.03A
Treated fish with Aflatoxin- B1	Week1	80	2.69±0.02B	80	2.34±0.03B
	Week2	75	2.90±0.02AB	64	2.27±0.02B
	Week3	65	2.50±0.02B	64	2.04±0.02B
	Week4	60	2.40±0.04B	64	2.24±0.01B
	Week5	55	2.18±0.01B	58	2.16±0.03B
	Week6	43	1.79±0.01C	48	1.74±0.01C
	Week7	43	1.79±0.02C	38	1.50±0.01C
	Week8	43	1.78±0.02C	26	1.18±0.02C

Means within the same column carrying similar superscripts are significantly different at (P< 0.01)

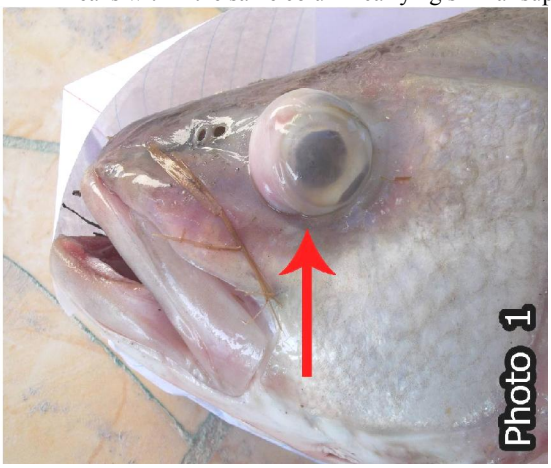

Photo (1): Sea bass fish suffering from Aflatoxicosis . showing erythema in the mouth and severe exophthalmia.

Table (3): Explain the effect of Aflatoxin-B1treatment on Sea bass returns (LE) with and without Dinaferm treatment .

Group	Parameter	Fish number	Dinaferm® treated diet	Fish number	Regular diet
Control fish	Week1	80	22.12±2.20B	80	18.97±1.98C
	Week2	80	22.19±2.23B	80	19.74±1.74BC
	Week3	80	22.33±3.22B	80	20.23±2.23AB
	Week4	80	22.61±2.66B	80	20.93±2.99AB
	Week5	80	22.75±2.75B	80	21.21±3.22A
	Week6	80	22.82±3.27B	80	21.98±3.21A
	Week7	80	23.31±2.27AB	80	22.19±2.18A
	Week8	80	24.15±2.15A	80	22.40±2.49A
Treated fish with Aflatoxin- B1	Week1	80	18.83±2.87C	80	16.38±1.37D
	Week2	75	20.30±2.35B	64	15.89±1.55E
	Week3	65	17.50±2.36CD	64	14.28±1.14F
	Week4	60	16.80±2.37DE	64	15.86±1.61E
	Week5	55	15.26±2.26E	58	15.12±1.12E
	Week6	43	12.53±2.23F	48	12.18±1.13G
	Week7	43	12.53±2.55F	38	10.50±1.14H
	Week8	43	12.46±2.47F	26	8.26±1.12I

Means within the same column carrying similar superscripts are significantly different at (P< 0.01)

Table (4): Explain the effect of Aflatoxin-B1treatment on Sea bass (body weight gain and its returns / LE) with and without Dinaferm treatment.

Group	Parameter	Gain due to addition of Dinaferm	Returns due to addition of Dinaferm
		Kg	LE
Control fish	Week1	0.45±0.02B	3.15±0.01A
	Week2	0.35±0.01C	2.45±0.02C
	Week3	0.38±0.02BC	2.66±0.02BC
	Week4	0.24±0.03D	1.68±0.01D
	Week5	0.22±0.02D	1.54±0.01D
	Week6	0.12±0.01F	0.84±0.03F
	Week7	0.16±0.01F	1.12±0.02F
	Week8	0.25±0.02E	1.75±0.01E
Treated fish with Aflatoxin- B1	Week1	0.35±0.03BC	2.45±0.02C
	Week2	0.63±0.06A	4.41±0.03A
	Week3	0.46±0.02B	3.22±0.02A
	Week4	0.16±0.01F	1.12±0.01F
	Week5	0.02±0.01H	0.14±0.002H
	Week6	0.05±0.02H	0.35±0.001H
	Week7	0.29±0.01E	2.03±0.02E
	Week8	0.6±0.05G	4.20±0.02G

Means within the same column carrying similar superscripts are significantly different at (P< 0.01)

Photo (2): Seabassfish suffering from Aflatoxicosis showing severe hemorrhagic patches on the body and severe hemorrhages at base of pectoral fins.

Photo (7): Seabass fish suffering from Aflatoxicosis showing serous ascetic fluid tinged with blood dropping on behind paper upon opening the fish.

Photo (8): Seabass fish suffering from Aflatoxicosis showing serous ascetic fluid with different degrees congestion on the liver of infected fish.

References

Baydar, T.; Engin, A.; Girgin, G.; Aydin, S. and Sahin, G. (2005): Aflatoxin and Ochratoxin in various types of commonly consumed retail ground samples in Ankara, Turkey. Ann. Agric. Environ. Med. 12 (2): 193 – 7.

Bejaoui, H.; Mathieu, F.; Taillandier, P. and Lebrihi, A. (2006): Biodegradation of Aflatoxin B1by Aspergillus section Nigri species isolated from French grapes: a potential means of Aflatoxin-B1decontamination in grape juices and musts. FEMS Microbiol. Lett. 255 (2): 203 – 8.

Creepy, E. E.; Chiavappa, P.; Baudrimont, I.; Borracci, P.; Moukha, and Carratu, M. R. (2004): Synergistic effects of fumonisin B1 and Aflatoxin B1: are in vitro cytotoxicity data predictive of in vivo acute toxicity?. Toxicology 201 (1 – 3): 115 – 23.

Easa, A. A. M. (1997): Effect of Aspergillus ochraceus mould and its metabolites on some cutlutred freshwater fishes in Egypt. MVSc. Thesis Dept. of Int. Medicine. Infectious diseases and fish. Fac. of Vet. Med. Cairo. Univ.

El-gamal, M. H. (2005): Some studies on the effect of Yersinia species in cultured freshwater fish Ph. D. Thesis Fac. Of Vet. Med. Alex. Univ.

El-Kady, I. A; S. S. M. El-Maraghy and M. E. Mostafa. (1995): Natural occurrence of mycotoxins in different species in Egypt. Folia Microbiol (Praha) 40 (3): 297-300.

El-Shaboury, F. A. (1998): Fungal flora of Brolus lake fish at Kafr El-Sheikh Province. Alex. J. Vet. Science, Vol. 14. No. 3: 117-128.

El-Tahawy, A. S. (2004): Factors affecting the profitability of fish farms and their relation to veterinary management. M. V. Sc. Thesis Fac. of Vet. Med. Alex. Univ.

El-Zarka, S; Shaheen, A. H.; Aleem, A. A and Abdel Hady, A.M. (1993): Tilapia fisheries in lake Mearіut age and growth of Tilapia Nilotica in the lake. Bull. Inst. Ocean. Fish. Cairo, 1, 149-182.

Jonsyn, F. E. and G. P. Lahai. (1992): Mycotoxic flora and mycotoxins in smoke-dried fish from Sierraleone. Nahrung; 36 (5): 485-9.

Jonsyn, F. E.; S. M. Maxwell and R. G. Hendrickse. (1995): Aflatoxin B1and aflatoxins in breast milk samples from SierraLeone. Mycopathologia Aug; 131 (2): 121-6.

Macarthur, R.; Macdonald, S.; Brereton, P. and Murray, A. (2006): Statistical modeling as an aid to the design of retail sampling plans for mycotoxins in food. Food Addit. Contam. 23 (1): 84 – 92.

Oylami, O. A.; S. M. Maxwell and E. Adeoba. (1996): Aflatoxins and Aflatoxin B1in the weaning food of Nigerian children. Ann. Trop. Paediatr Jun; 16 (2): 137-40.

Radic, B.; R. Fuchs; M. Peraica and A. Lucic. (1997): Aflatoxin-B1in human sera in the area with endemic nephropathy in Croatia. Toxicol. Lett. Apr. 28; 91 (2): 105-9.

Saad, T. T. (2002): Some studies on the effects of Ochratoxin on cultured Seabass and Carp species. M. V. Sc. Thesis, Avian and Aquatic Animal Med. Fac. of Vet. Med. Alex. Univ.

Samira-Rezeaka. (1991): Integumentery mycosis in cultured freshwater fish and shrimps. M. V. Sc. Thesis Fac. Of Vet. Med. Alexandria University.

SAS (1987): Statistical analysis system. User's Guide Statistics. SAS Institute Cary, North Carolina.

Safinaz, G. M. I. (2000): Effect of Phenol on the immune-response of Tilapia fish and susceptibility to diseases. Ph. D. Thesis Fac. Of Vet. Med. Suize Canal Univ.

Sauvant, C.; Holzinger, H.; Mildenberger, S. and Gekle, M. (2005): Exposure to nephrotoxic Aflatoxin B1enhances collagen secretion in humoral renal proximal tubular cells. Mol. Nutr. Food Res. 49 (1): 31 – 7.

Scudmore, K. A.; S. Patel and V. Breeze. (1999): Surveillance of stored grain from the 1997 harvest in the United Kingdom for Aflatoxin B1. Food Addit. Contam. Jul; 16 (7): 281-90.

Shehata, A.; T. A. Ibrahim and A. A. Shaaban. (1985): Acute and subchronic toxicity studies of Bayluscide in Tilapia nilotica fish. Assiut Vet. Med. J., 17 (34): 214-221.

Shewita, R. S. M. (2004): Effect of dietary supplementation of ascorbic acid and/or vitamin E on productive performance, immune response and body composition of tilapia nilotica (O. Niloticus). M. V. Sc. Thesis Fac. of Vet. Med. Alex. Univ.

Stefanovic, V.; Toncheva, D.; Atanasova, S. and Polenakovic, M. (2006): Etiology of Balkan Endemic Nephropathy and associated urothelial cancer. Am. J. Nephrol. 26 (1): 1 – 11.

Tangani, E. K. and Pussemier, L. (2006): Aflatoxin B1and citrinine loads in stored wheat grains: dust and possible prediction using ergosterol measurement. Food Additive Contam. 23 (2): 181 – 9.

Timperio, A. M.; Magro, P.; Chilosi, G. and Zolla, L. (2006): Assay of Aflatoxin B1in grape by high-pressure liquid chromatography coupled on line with an ESI-mass spectrometry. J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 2006 Jan. 24.

Wafa, E. W.; R. S. Yahya; M. A. Sobh; I. Eraky; M. El-Baz; H. A. El-Gayar; A. Betbeder and E. E. Creppy. (1998): Human aflatoxicosis and nephropathy in Egypt: a preliminary study. Hum. Exp. Toxicol. Feb; 17 (2): 124-9.