

## NEW TRIALS OF USE OF MOLASSES AND GARLIC EXTRACTS FOR COMPATING MYCOTOXICOSIS

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### ABSTRACT

*Two hundreds and fifty samples of feeds (125 of each of ingredients of plant origin "yellow corn, white corn, soya bean, soya bean meal, wheat and beans" and compound manufactured feed and animal protein concentrates (meat-bone meal, fish meal, poultry offal and mixed feed). Samples were collected from various poultry farms at Giza and Cairo Governorates. Fungal isolation from ingredients of plant origin revealed lower rate of mould contamination in comparison to compound manufactured feed and animal protein concentrates. For instance, Aspergillus species was isolated from 48-68% and 80-100% of feed ingredients and animal concentrates respectively. This findings was correlated with higher levels of mycotoxins in manufactured feed and animal protein concentrates than feed ingredients of plant origin. For aflatoxins and ochratoxin A the mean levels in feed ingredients were ranged from (18-31 ppb and 15-28 ppb, respectively), while in manufactured feed and animal protein concentrates they ranged from (23-42 ppb and 32-47 ppb) respectively. The isolated Aspergillus flavus and A. ochraceus produced significant levels of respective mycotoxins. The induction of aflatoxicosis and ochratoxicosis in quails with particular reference to their partial elimination by garlic and molasses were carried out. The changes in biochemical parameters (AST, ALT, ALP, LDH, total lipid, triglyceride, cholesterol, LDL, HDL and VLDL), also in serum total protein and protein electrophoresis due to aflatoxicosis and ochratoxicosis were significantly improved under supplementation of garlic extract and molasses in diet.*

### INTRODUCTION

Up to date the high productivity of animal and poultry gain essential goal in the improvement of the human life. Hence all relating elements including health of feed and feeding become interesting factors to be study. Recently in developing countries the offal of abattoir, fish byproduct and unsuitable meat for human consumption are used for feed manufacture for animal and poultry. These products carry a dangerous toxic factors which affect the animal health (**Bendell et al., 1985**). Fungi and mycotoxins pollution increased in manufactured feed and animal protein concentrates than ingredients of plant origin (**Mahmoud, 1993; Adebajo et al., 1994; Skrinigar et al.,1995** and **Casstell'a et al.,1999**). It causes many disease problems in human and animal particularly respiratory affections, Alimentary Toxic Alukia, Teratogenic, Dermatogenic and Carcinogenic diseases which reflected directly on the performance and health of man and animal (**Viscoli et al., 1990; Li et al., 1999; Kubena et al., 1999** and **Wang et al., 2000**).

The aflatoxin (AF) and ochratoxin A (OA) were considered to be most important dangerous mycotoxins, possess acute and chronic toxicity depending on dosage and on species of animals (**Dihter, 1984 and Jones, 1993**). Several workers had documented the toxicity of ochratoxin and the relative lack of toxicity of aflatoxin in Japanese quails (**Doster et al., 1973** and **Chang and Hamilton, 1982**). Ochratoxin A is a mycotoxin that has been demonstrated to be approximately three more time toxic to game birds (Ringnck phesant) than aflatoxin (**Ruff et al., 1992**). Aflatoxin are secondary fungal metabolites that can be produced by *Aspergillus flavus* and *A. parasiticus*. Whereas ochratoxin A is the most prevalent one produced by *Aspergillus ochraceus*. The main toxic manifestations of these toxins are hepatotoxicosis, nephrotoxicosis, immunosuppressive, oxidative damage, alterations in serum constituent, enzyme activity and carcinogenesis that can link to aflatoxicosis and ochratoxicosis in various species of animals (**Ruff et al.,1992; Edrington et al., 1995; Hochler and Marquardt, 1996; VinitketKumnuen et al., 1999** and **Rastogi et al., 2001**). Hence, the use of safe methods to the animal health to eliminate the toxic effects of aflatoxin and ochratoxin become critical demand. The most recent method is the use of natural

substance as garlic and molasses (*Dowd and Shen, 1991* and *Kirubaharan et al., 1999*).

Therefore, the present study, aimed to investigate the prevalence of moulds and their toxins in single natural feed and those manufactured from waste product. The most predominant mycotoxins were employed for the purpose of their elimination by garlic and molasses. The biochemical parameters of laboratory animals were taken as indicator for detection the efficiency of all treatments.

## MATERIALS & METHODS

### 1.Feed samples:-

Two hundred and fifty feed samples were collected from poultry farms at Cairo and Giza Governorates. They include two main types:-

**A.**Ingredients of plant origin (yellow corn, white corn, soya bean, wheat and beans), 25 samples of each.

**B.**Animal protein concentrates and compound manufactured feed (meat-bone meal, fish meal, poultry offals, mixed feed and Soya beans meal) (25 samples of each).

### 2.Quail chicks:-

Seventy healthy Japanese quail one day old were kindly obtained from private farms at Alexandria Governorate. They were kept in wire floored brooder batteries with electric heat and constant lighting. Feed and water provided *ad libitum*.

### Preparation of garlic extract and molasses:-

The garlic pulps were broken into cloves. These cloves were washed in sterile distilled water and dried on a filter paper. They were crushed in juice crusher and the juice collected. To the garlic, tryptic soya broth was added to a ratio 1:4 and mixed will using a magnetic stirrer for 30 minutes and centrifugated at 8000 rpm for 60 minutes. The supernatant was filtered through a membrane filter. The extract prepared was used on the same day (*Kirubaharan et al., 1999*).

For molasses the obtained crude extract from sugar cane manufactories was used as it is (*Churchil et al., 2001*).

**Screening of samples for fungal contamination (ICMSF, 1978 and Conner et al., 1992):-**

At least 1 kg of each feed was finally ground in electric grinder and a desired amount taken for mycological and mycotoxicological examination.

**Isolation and identification of fungi (ICMSF, 1978):-**

Each sample was moistened and held at room temperature until heavy growth was observed. Suspension of each of these commodities were made in 1/4 strength Ringer's solutions by blending and then 10 fold serial dilutions were prepared. One ml portions of each dilutions of each sample inoculated in sterile petri dishes (each containing 15 ml malt extract agar containing oxytetracycline).

**Incubation and reporting the result:-**

The plates were then incubated 7-10 days at 25-28°C and examined visually. Individual colonies were selected, purified and briefly identified according to macroscopic and microscopic characters as reported by *Al-doory (1980); Refai (1988); Raper and Fennel (1965); Smith and Yarrow (1988) and Thomas and Carter (1990)*.

**Production of aflatoxin by *A. flavus* and ochratoxin A by *A. ochraceus* in yellow corn (Trenk et al., 1971 and Benett, 1979):-**

250 gm of yellow corn was dispensed in 2 liter flasks and autoclaved for 15 min. at 121°C. The flasks were inoculated with conidial suspension of *A. flavus* (in case of aflatoxin) and *A. ochraceus* (in case of ochratoxin). The moisture content of flask was raised by addition of distilled water to 30-40%. The flasks were incubated at 30°C for 15-30 days before extraction of toxin.

**Extraction of aflatoxin and ochratoxin:-**

Aflatoxin and ochratoxin from 50 gm portions of feed were extracted as mentioned by (*Basil et al., 1981*). Methanol: water (55:45) was used as primary extraction solvent before purification and clean up

by chloroform. The immunoaffinity column loaded and developed according to the recommended procedures by *Hansen (1993)*. After development of all columns, they were observed under U.V. light and photographed or its content is dissolved in methanol to be measured by fluorometer.

**Experimental elimination of Aflatoxicosis and ochratoxicosis by garlic extract and molasses (*Doster et al., 1973; Ruff et al., 1992; Kirubaharan et al., 1999; Parlet et al., 1999 and Curchil et al., 2001*):-**

The quail chicks were divided into seven groups (ten of each) and maintained for 3 weeks period for experimental work. The design of treatment and doses of all materials were shown in table (1)

**Table (1):** Experimental design of the elimination of ochratoxicosis and Aflatoxicosis by garlic and molasses.

Group Treatment	G1	G2	G3	G4	G5	G6	G7
Aflatoxins (total) 2 ppm	-	+	+	+	-	-	-
Ochratoxin 2 ppm	-	-	-	-	+	+	+
Garlic 5% of total diet	-	-	+	-	-	+	-
Molasses 5% of total diet	-	-	-	+	-	-	+
Healthy diet	+	-	-	-	-	-	-

### Blood sampling:-

Blood samples were collected at end of experiment and centrifuged at 3000 rpm for 5 minutes and then the sera were kept in deep freeze at  $-20^{\circ}\text{C}$ .

### Biochemical analysis:-

Sera were used for measuring the concentration of total proteins (*Sonnen-wirth and Jareet, 1980*), protein electrophoresis pattern (*Davis,*

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1964 and *Ornstein, 1964*). Total lipids, cholesterol, triglyceride, HDL, LDL described according to *Knight et al. (1972)*, *Watson (1960)*; *Gordon and Amer (1977)* and *Pesce and Kaplan (1987)*, respectively.

Activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined according to *Reitman and Frankel (1957)*; alkaline phosphatase (ALP) were determined according to *Refield and Goldbery (1971)*. The determination of urea were conducted according to *Wybenga et al. (1971)* and creatinine as described by *Gaoria (1974)*. All kits from BioMerieux including lipids and liver enzymes.

The obtained data were statistically analysed using t-test after *Petrie and Watson (1999)*.

## RESULTS & DISCUSSION

The mycoflora of feed ingredients of plant origin (white, yellow corn, Soya bean, Soya bean meal, wheat and beans) revealed isolation of 5 genera of moulds. Genus *Aspergillus* was the predominant isolates (60, 52, 52, 48, 68%) followed by *Penicillium* (20, 20, 12, 12, 8%). *A. flavus* and *A. ochraceus* were the most frequent species persisted in samples. Whereas other genera including (*Fusarium*, *Rhizopus* and *Mucor*) rarely isolated. The same findings were observed in many previous studies (*Ahmed, 1993*; *Abd El-Fattah, 1994* and *Jand and Singh, 1995* and *Desjardins et al., 2000*).

These cereals which were used as a source of diet for human and animal contained few mould contaminants (Table, 2) in comparison to the animal protein concentrates which were used recently in animal feeding (meat-bone meal, fish meal, poultry offal and mixed feed) (Table, 3) which when subjected to isolation of fungi a relatively large number of mould were isolated. Nine genera of fungi were isolated from these feed samples. *Aspergillus* and *Mucor* isolated from all samples of compound manufactured feed and animal concentrates with particular reference to *A. flavus*, *A. ochraceus* and *A. candidus* which predominantly species isolated. For instance, in meat and bone meal *A. flavus* was present in 100% of samples, *A. ochraceus* present in 60% of samples and *A. candidus* was

isolated from 32% of samples. Whereas *A.niger*, *A. glaucas*, *A. terrus* and *A. fumigatus* were rarely isolated species (24,12,4 and 0%) respectively. Other genera of fungi were frequently present *Penicillium* (68%), *Rhizopus* (16%), *Fusarium* (8%), *Rhodotorula* (8%), *Scopulariopsis* (4%), *Cladosporium* (4%) and *Alternaria* (0%). Regarding genera of *Penicillium* and *Fusarium*, *P. citreanum*; *P. implicatum* and *P. digitatum*, *F. moniliforme* and *F. poea* were isolated from manufactured and animal concentrates samples. Generally, these levels of fungal contamination in meat and bone meal was nearly observed in other manufactured feed (fish meal, poultry offal and mixed feed) (Table, 3). These findings were detected previously by *Mahmoud (1993)* in mixed feed (*Saber, 1993*) in Marketed manufactured feed (*Skrinijar et al., 1995*). *El-Far et al. (1993)* who detected the over incidence of fungal contamination near 83% in bone meal, fish meal, blood meal and concentrates.

Comparison between mycoflora of feed ingredients in table (2) and compound manufactured and animal protein concentrates feed (Table, 3) revealed that the manufactured compound feed were relatively higher in contamination this may be due to many reasons related to the source of feed, changes of environmental factors, procedures of production and transportation to the place of animal rearing. All these factors play an important role in increasing the fungal contamination of manufactured waste products (Fish meat, meat bone meal, mixed feed and poultry offal. These products may have a useful compound for animal health but the presence of microbial contamination which give a chance for toxin production and decaying of protein and vitamins contents affect the animal health (*Deiner et al., 1976; Abramson et al., 1983* and *Park and Bullermon, 1983*).

Table (4) and (5) reported mycotoxins contamination of ingredients and compound manufactured animal protein concentrates feeds. All samples of both types of feeds had a various levels of aflatoxins and ochratoxins. Otherwise, the rate of aflatoxin contamination in feed ingredient varies from 8-20% of samples with the mean levels of (18-31 ppb), whereas the same toxin in compound manufactured animal protein concentrates had the range of (48-60 %) with mean levels of (23-42 ppb).

These observations also seen in ochratoxin contamination which ranged from (4-12%) of feed ingredient at mean levels of (15-28 ppb). However in manufactured compound and animal protein concentrates feed ranged from (32-48%) at mean levels of (42-67 ppb). Other mycotoxins as zearalenon and T2 had also the same pattern of contamination. These results come in parallel with (*El-Far et al., 1993; Abdebajo et al., 1994; Jimenez et al., 1996* and *Nikulin, 1996*).

The higher toxins contamination of compound manufactured waste product increased the health hazard and danger of their consumption by animals (*Hsu et al., 1972*). This also may be due to the unknown sources of single materials which used in manufacture of compound feed.

The waste product used as meat, bone, fish and offal suggested to be from diseased animals which appeared as clear high contamination by mould and mycotoxins and reflected as disease outbreak or bad productivity of animal. Whereas the ingredients of plant origin from natural cereals which not exposed to various unhygienic handling and reflected as high quality of feed which had low relative contamination of cereals in contrary of manufactured waste product (Tables, 2, 3, 4 and 5). To fulfill our above suggestion the predominant isolates in this study *A. flavus* and *A. ochraceus* screened for mycotoxins production to support the results in Table (2-5). While, the *A. flavus* which isolated from ingredients of plant origin produced aflatoxin at rate ranged from 20-50% at mean levels of (20-31 ppb) (Table, 6). The isolates from compound feed and animal protein concentrates product produced aflatoxin at rate ranged from (60-80 %) at mean levels of (27-52) (Table, 7).

The isolates of *A. ochraceus* from cereals yielded ochratoxin at rate ranged from (12.5-40%) with mean levels of (22-41 ppb). In contrary, the isolates from compound manufactured animal protein concentrates feed from waste product had levels of ochratoxin of(40-70%)(40-61 ppb) (Tables,8 and 9). These results supported by (*Bendel et al.,1985;Li, 1999* and *Wang et al., 2000*).

The damage of liver and kidney are the main dangerous effects of aflatoxins and ochratoxin A in animals and poultry (*Dihter,1984; Bendel*



*et al., 1985* and *Jones, 1993*). These effects are due to selective suppression of the activity of natural killer cells (*Pegram and Waytt, 1986*). Other problems due to the moulds and their toxins would be recorded including, teratogenic, dermatogenic and carcinogenic effects to human and animal (*Viscoli et al., 1990; Lie et al., 1999 and Wang et al., 2000*).

For preservation of all types of feeds from these contaminants trials of using natural materials of no side effect on hygienic quality of animal product was become of critical recommendation. Garlic (*Allium sativum*) is one of the common additive in food and the molasses which used as a source of trace elements and vitamins for human (*Kirubaharan et al., 1999 and Churchil et al., 2001*). The trial of elimination of aflatoxin and ochratoxin by molasses and garlic was conducted in Japanese quails.

The biochemical changes in quails due to aflatoxicosis and ochratoxicosis shown in table (10) which shows significant increase in serum enzyme activities (AST, ALT, ALP and LDH). Similar findings were obtained by *Badawy et al. (1996)* and *Mobarak et al. (1996)* in Japanese quails and *Sahar and El-Meadawy (2001)* in broiler chickens.

This may be due to necrosis of hepatic cells and release of these enzyme into circulation (*Lyuch et al., 1971*). Such hepatic toxic effect of aflatoxin attributed to its active metabolite in liver as expoxide, *Netke et al. (1997)* which covalently bind to DNA and may affect structural and enzymatic protein function (*Culler and Newbern, 1994*). Ochratoxin and aflatoxin are a protein synthesis inhibitor that have an affect on mitochondrial oxidative enzyme activity (*Marquardt and Frohlich, 1992*). The aflatoxin had adverse effect on liver cells more than ochratoxin. Inversely ochratoxin found to be has greater toxic effect on the kidney than aflatoxin. This was expressed as a significant increase in serum urea and creatinine (Table, 10). These results were attributed to a significant alteration in tubular function caused by aflatoxin or ochratoxin (*Ruff et al., 1992; Badawy et al., 1996 and Mobarak et al., 1996*).

Treatment with garlic and molasses (groups 3, 4, 6 and 7) led to reduced enzyme activities. This protective action of garlic may be due to the presence of diallyl sulfide which conjugated with epoxide and decrease the degenerative effects induced by aflatoxin or ochratoxin (**Jeong and Lee, 1998 and Helen et al., 1999**). Yeast and vitamins are considered as molasses constituent which counteracted the toxic effect of aflatoxins (**Churchil et al., 2001**) and other mycotoxins (**Dowd and Chen, 1991**). Aflatoxin and ochratoxin induced a severe effect on serum biochemistry of quails which were represented by significant decrease of total lipids, triglyceride, cholesterol, HDL-C and VLDL-C, where a significant increase will be noticed in LDL-c (Table, 11). These abnormalities may be attributed to hepatocellular injury induced by aflatoxin or ochratoxin where the liver is the major source for plasma Total lipid, lipid profiles and lipoproteins (**Tietz, 1996**). As well as, mycotoxins binding to DNA leading to inhibition of RNA polymerase allowing inhibition of lipid synthesis and transport, **Donaldson et al. (1992)** and **Weibking et al. (1993)**. Following garlic or molasses supplementation, partially ameliorated the toxic effect of aflatoxin or ochratoxin (groups 3, 4, 6 and 7). Garlic extract decreased total cholesterol, LDL-cholesterol, increase HDL-C, reduction of triglyceride and VLDL (**Siegel et al., 1999** and **Zhang et al., 2001**).

Molasses contain a large amount of trace element such Cu, Zn, Mn, e.t.c. and some vitamin like B-complex such these constituents are catalysts for oxidative enzyme of liver and decrease the adverse effects of aflatoxin or ochratoxin (**Awadallah et al., 1984** and **Webster et al., 1996**).

In regards to serum total protein and electrophoretic pattern (Table, 12). Our observation demonstrated an apparent significant decrease in the total protein, prealbumin, albumin, total alpha, total beta and total globulin as well as A/G ratio among quails fed on diets supplemented with aflatoxin or ochratoxin (groups 2,5). Similar results were also recorded by **Badawy et al. (1996)** and **Sahar and El-Meadawy (2001)**. Aflatoxin or ochratoxin causes inhibition of DNA and protein synthesis as well as immunosuppressive due to the inflammation, cirrhosis of liver and nephrotoxic effect (**Harvey et al., 1990 and Tietz, 1996**).

The globulin component (Table, 13) showed significant drop in  $\alpha 1$ ,  $\beta 1$  and  $\beta 2$  globulin with significant increase in  $\alpha 2$ ,  $\gamma 1$  and  $\gamma 2$  globulins. This result correlated with *Burguera (1983) Tietz, 1996*.

Supplementation of garlic and molasses induced good effect that minimize the alterations of serum protein and its fraction due to aflatoxin or ochratoxin (Groups G3, G4 and G7) Table (13).

Dially sulfid of garlic inhibit the activity of cytochrom P-450 (Oxidative metabolism) which activated by aflatoxin or ochratoxin. So its inhibition produced immunospressive effects on humarol and cellular response (*Jeong and Lee, 1998*). Yeast as a competent of molasses has the same effects on the cytochrome P-450 (*Sengslag and Wurgler, 1994*). Also molasses contain vitamin B-complex in high concentrations this well restored the activity of B-globulin which decreased by mycotoxins (*Harvey et al., 1990*).

It is become apparent that the levels of mould and their toxin contamination observed significantly in compound manufactured animal protein concentrates from waste products reflected the bad methods of manufacturing of feed which used a waste unhygienic materials in industry of animal feeds. In addition, contamination of diet with aflatoxin or ochratoxin caused alteration of serum Japanese quails as enzyme activities, kidney function, T. lipids, Lipid profiles, T. protein and its fractions. The supplementation of Garlic extract or molasses improve these biochemical changes. This attributed to the presence of Dially sulfide of garlic and trace elements and vitamins of molasses.







**Table(6):**Screening of *A. flavus* isolated from single natural feed for production of aflatoxins.

A. flavus isolated from:-	Aflatoxins (ppb)				
	No. of +ve	%	Max. ppb	Mean ppb	Min.ppb
White corn	5/10	50	33	22	18
Yellow corn	3/10	30	36	31	23
Soya bean	3/10	30	24	20	15
Wheat	2/10	20	25	23	20
Beans	2/10	20	44	22	16
Soya bean meal	5/10	50	42	30	20

**Table(7):**Screening of *A. flavus* isolated from manufactured poultry and animal waste products for production of aflatoxins.

A. falvus isolated from:-	Aflatoxins (ppb)				
	No. of +ve	%	Max.ppb	Mean ppb	Min ppb
Fish meal	7/10	70	56	32	45
Meat and bone meal	8/10	80	65	41	52
Poultry of glows	6/10	60	48	32	43
Mixed feed	6/10	60	30	21	27

**Table (8):** Screening of *A. ochraceus* isolated from ingredients of plant origin for production of ochratoxins.

A. ochraceus isolated from:-	Ochratoxins (ppb)				
	No. of +ve	%	Max. ppb	Mean ppb	Min. ppb
White corn	0/5	-	-	-	-
Yellow corn	1/5	20	30	30	30
Soya bean	1/5	20	41	41	41
Wheat	2/5	40	45	36	40.5
Beans	1/8	12.5	22	22	22
Soya bean meal	6/10	60	30	28	23

**Table(9):**Screening of *A. ochraceus* isolated from manufactured feed from waste product for production of ochratoxin.

A. ochraceus isolated from:-	Ochratoxins (ppb)				
	No. of +ve	%	Max.ppb	Mean ppb	Min. ppb
Meat and bone meal	7/10	70	65	46	50
Fish meal	5/10	50	47	32	40
Poultry offal	6/10	60	42	33	44
Mixed feed	4/10	40	53	43	47











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محاولات حديثة لاستخدام العسل الأسود ومستخلص الثوم فى مقاومة التسمم الفطرى.  
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قد تم فحص عدد 250 عينة من العلائق (125 من كلا من:- مكونات العلائق ذات الأصل النباتى "ذرة صفراء وبيضاء وفول صويا والقمح والفول البلدى" والعلائق المركبة ومركبات البروتين الحيوانى "مسحوق لحم وعظم ومسحوق أسماك ومخلفات الدواجن وعلائق مخلوطة). وقد تم جمع هذه العينات من مزارع الدواجن بمحافظة الجيزة والقاهرة. وقد أفاد العزل للفطريات لمكونات العلائق ذات الأصل النباتى بتواجد التلوث بالفطريات بمعدل منخفض بالمقارنة بالعلائق المصنعة ومركبات البروتين الحيوانى فعلى سبيل المثال تم عزل جنس الاسبرجيلس من 48-68% و80-100% من مكونات العلائق ومركبات الحيوان على التوالى وقد كانت هذه النتائج مرتبطة بتواجد نسب عالية من السموم الفطرية فى الأعلاف المصنعة ومركبات البروتين الحيوانى بالمقارنة بمكونات الاعلاف ذات الأصل النباتى فبالنسبة لسموم الافلاتوكسين والاوكراتوكسين أ كان متوسط معدل تواجدها فى مكونات الأعلاف يتراوح بين 18-31 جزء من البليون و15-28 جزء من البليون على التوالى فى حين أن معدل هذه السموم فى العلائق المصنعة ومركبات البروتين الحيوانى كانت تتراوح بين 23-42 جزء من البليون و32-47 جزء من البليون على التوالى.

وقد أعطت العترات المعزولة (اسبرجيلس فلافس والاسبرجيلس اوكرائشيس) كميات ملحوظة من السموم الفطرية.

وقد تم استحداث التسمم بسموم الافلاتوكسين والاوكراتوكسين فى طيور السمان واستخدام العسل الأسود وثمار الثوم فى التخلص من التسمم الفطرى. وقد تبين أن التغيرات فى كيمياء الدم الناتجة عن التسمم الفطرى شاملة (وظائف الكبد والكلى ونسبة الدهون) قد تحسنت بوضوح عند استخدام مستخلص الثوم والعسل الأسود فى الأغذية.