**Egyptian Journal of Veterinary Sciences**
**https://ejvs.journals.ekb.eg/**

# The synergistic Effect of Both Mannan and Chitosan Oligosaccharides on Broilers Intoxicated with Aflatoxicosis

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NUMEROUS techniques were used for detoxification of aflatoxin. The present study was conducted to evaluate the addition of mannan oligosaccharides (MOS) and/or Chitosan (Chit) to lessen the toxicity of AFs in broiler chickens (cobb500), improving the performance, biochemical, hematological, antioxidant, and histopathological parameters and gene expression analysis of caspase3 and interleukin 1-β.Five equal groups of 50 broilers each were formed (n=10); negative control (G1) given the basal diet, positive control (G2) basal diet+ 100 ppb AFs kg-1diet; MOS received group (G3) given as basal diet+ 100 ppb AFs kg-1diet + MOS (1 g kg-1 diet); Chit received group (G4) given basal diet+ 100 ppb AFs kg-1diet +Chit (0.5 g $kg^{-1}$ diet) ; and MOS/Chit received group (G5) given basal diet+ 100 ppb AFs kg-1diet + MOS (1 g $kg^{-1}$ diet)+ Chit (0.5 g $kg^{-1}$ diet). All treatments were given to broilers between 6 and 25 days of age. The combination of both MOS and Chit improved hematological parameters in aflatoxicosis-intoxicated groups. Both renal and hepatic markers were significantly improved in chicks intoxicated with aflatoxins and treated with MOS and/or Chit. A significant improvement of antioxidant markers in both MOS and Chit groups exposed to aflatoxicosis. Gene expression of Caspase-3 and IL-1β were significantly improved in chicks intoxicated with aflatoxins and treated with MOS and/or Chit. MOS and/or Chit reduced the levels of accumulation of aflatoxin in muscles, liver, and kidney tissues. The liver sections of the groups (G3, G4, and G5) exhibited significant enhancement in the hepatic parenchymal architecture.

**Keywords:** aflatoxins, Chit, MOS, antioxidants, Caspase-3, IL-1β

## Introduction

There are various factors that can affect the quality and safety of the feed, including its origin, processing, handling, and storage[1]. The chicken business in Egypt depends heavily on imported feed components; hence, the risk of fungus contamination is very significant, either during production stages, during transit, or possibly even during market storage stages.[2].

Aflatoxins (AFs) are naturally occurring, secondary metabolites of mycotoxins generated from *Aspergillus flavus* and *Aspergillus parasiticus*. Its high poisonousness and carcinogenicity are widely known. Aflatoxin B1, B2, G1, G2, M1, M2, and other dangerous aflatoxins. Due to contaminated food being consumed by humans and animals, AFs injection has negative consequences on humans and many species of animals, including being hepatotoxic [3], teratogenic, mutagenic, and carcinogenic effects [4].

The tendency of aflatoxins to contaminate numerous types of poultry and poultry products, including chicken liver, wings, nuggets, and thighs, has drawn particular attention. Different species of the genus Aspergillus fundamentally had been identified to be capable of producing these toxins[5].

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(Received 25/09/2023, accepted 27/11/2023)
DOI: 10.21608/EJVS.2023.238221.1627

Due to the production of free radicals and reactive oxygen species (ROS), which are thought to be involved in the primary mechanism of aflatoxin toxicity, it has been claimed that aflatoxins cause oxidative stress. By lowering the level of free radicals, antioxidants are thought to reduce oxidative stress during mycotoxicosis [6] to prevent mycotoxins' negative effects in chicken feeds, mannan-oligosaccharides (MOS) and ß-glucans had been suggested [7]. Similarly, several studies have assessed the binding activity of Chit against a number of mycotoxins [8].

According to Van den Abbeele, [9], MOS is a polysaccharides that are commonly found in nature, including plants, bacteria, yeasts, and other species. Due to their physicochemical characteristics (water solubility, viscosity, and stability), they have a variety of commercial uses, the most common forms of MOS are as an emulsion stabilizer and an ingredient in hardeners. MOS also demonstrates important health advantages, such as reducing pathogen adhesion, controlling bacterial growth, and enhancing immune function. In general, immunostimulant polysaccharides interact directly with numerous immune system components, boosting various immunological pathways in the process[10].

Chitin, a polysaccharide made up of N-acetyl-D-glucosamine units found in insects, marine diatoms, algae, fungi, and crustacea, is the source of Chit. Chit is produced through a process that involves deacetylation, demineralization, deproteinization, and decolorization. The performance of animal growth was said to be enhanced by dietary supplementation with Chit [11-13]. Chit has been recognized as an additive having multiple uses, such as its ability to fight off bacteria that cause food poisoning[14]. Therefore, the effects of dietary MOS and/or Chit supplements in the growth performance, hematological parameters, blood biochemical changes, gene expressions, and organ histopathology of broiler chicks given an aflatoxin-containing diet were examined.

## Methods

**Chemicals: mannan oligosaccharide (**MOS) and Beta glucan powder from ABChem (pharmaceutical Row Materials, Egypt). Yeast cell wall powder food grade, chitosan oligosaccharide (Chit.) from *Tokyo Chemical Industry Co., Ltd.(TCI).*

**Production of Aflatoxin in Peanut Seeds:**

Five provinces in Egypt were used to gather 500 g samples of peanuts in shells: Alexandria, El-Beheira, El-Daqahliya, El-Sharqiya, and Asyut. Forty-five peanut seeds (5 ×3× 3 duplicates) from each location were immediately plated on Petri dishes with malt extract agar (MEA; Oxoid, Basingstoke, Hampshire, UK), dichloran 18% glycerol agar (DG18; Oxoid), as well as MEA + 10% NaCl (0.95 aw, MS; Oxoid).The frequency of fungi was measured 7 days after incubation at 25°C. The cultures had a large crop of green spores, which were destroyed by applying 3 ml of 0.005% Triton X-100 /dish. The spores were dislodged using a loop, and the dish was agitated to achieve a homogeneous spore suspension. The strains of *Aspergillus flavi, Aspergillus nigri, Asperagillus parasitics,* and *Aspergillus circumdati* were isolated via peanut seeds and subsequently cultured at malt extract agar (MEA) slants in a temperature of 4°C. These cultures were then preserved until further analysis to determine their capacity for producing toxins[15]. The analysis of aflatoxins (Afs) was conducted by using high-performance liquid chromatography (HPLC). In this study, three replicates from peanut samples of each location were subjected to analysis to aflatoxins (AFs) over two different seasons. The extracting process involved the use of afla-prep columns, namely the Neogene Europe variant with a wide bore. Following a 10-day incubation period at a temperature of 25°C at yeast extract sucrose (YES) agar obtained from Fisher (Loughborough, Leicestershire, UK), three to five plugs were taken from each colony originating from strains isolated from peanuts. These plugs were subsequently transferred to individual 2 ml Eppendorf tubes, and their weights were recorded. To extract AF, 800 l of chloroform had put in each Eppendorf tube and agitated by 1 hour. The chloroform extraction subsequently transferred into fresh vial and subjected to gentle air drying in preparation for derivatization, following the established technique [16], and afterwards, quantitative HPLC analysis. The 200-l stock solution of the AFs mix standard at methanol (Supelco, Bellefonte, Pa., USA), was dried under nitrogen gas and divided as samples. It contained 200 ng B1, 60 ng B2, 200 ng G1, and 60 ng G1. For injection into the HPLC, four concentrations were made. The HPLC detection limit for AFB1 was 0.012 ng g-1 for peanut seeds and 0.8 ng g-1 for YES medium, respectively.

**Experimental Chickens:**

Five groups of ten healthy one-day-old broiler chickens (Cobb500 from El-Daqahliya poultry) each were produced from a total of fifty healthy chickens.

Chicks were grown in litter under typical environmental and hygienic circumstances and fed a balanced basal meal prepared in accordance with the NRC [17] (Table 1). Stainless steel enclosures 1.75 x 1.55 $m^2$ were used to house broiler chickens. For the first three days, the temperature was kept at 33 °C. After that, it decreased gradually by 3 °C each week until it reached 24 °C, where it remained for the duration of the experiment. The relative humidity was maintained at 60%. The experimental groups involved: control negative (G1) obtained the normal diet; control positive (G2) normal diet+ 100 ppb AFs kg-1 diet [18]; MOS group (G3) obtained as control positive + MOS (1 g kg-1 diet)[18]; Chitosan group (G4) obtained as control positive Chitosan (0.5 g kg-1 diet) [19]; and MOS/Chitosan group (G5) obtained as control positive + MOS (1 g kg-1 diet)+ Chitosan (0.5 g kg-1 diet).The desired amount of aflatoxins was mixed gradually with the given diet to ensure complete distribution of aflatoxins with the diet. All of the treatments were given for 20 days.

**TABLE 1. Nutrient requirements of Cobb500Broiler performance & Nutrition Supplement according to the National Research Council [20].**

**Broiler Nutrition**

| Recommended minimum specifications | | | |
|---|---|---|---|
| | **Starter** | **Grower** | **Finisher** |
| Feeding Amount/bird | 250g | 1000g | |
| | 0.55 | 2.20 Ib | |
| Feeding period days | 0-10 | 11-20 | 21-30 |
| Feed Structure | Crumb | Pellet | Pellet |
| Crud protein % | 21-22 | 19-20 | 18-19 |
| Metabolizable energy MJ/kg | 12.70 | 13.00 | 13.30 |
| (AMEn*) Kcal/kg | 3035 | 3108 | 3180 |
| Kcal/Ib | 1380 | 1410 | 1422 |
| Lysine % | 1.32 | 1.19 | 1.05 |
| Digestible Lysine % | 1.18 | 1.05 | 0.95 |
| Methionine % | 0.50 | 0.48 | 0.43 |
| Digestible Methionine % | 0.54 | 0.42 | 0.43 |
| Met+Cys % | 0.98 | 0.89 | 0.82 |
| Digestible Met+Cys % | 0.88 | 0.80 | 0.74 |
| Tryptophan % | 0.20 | 0.19 | 0.19 |
| Digestible Tryptophan % | 0.18 | 0.17 | 0.17 |
| Threonine % | 0.86 | 0.78 | 0.71 |
| Digestible Threonine % | 0.77 | 0.69 | 0.65 |
| Arginine % | 1.38 | 1.25 | 1.13 |
| Digestible Arginine % | 1.24 | 1.10 | 1.03 |
| Valine % | 1.00 | 0.91 | 0.81 |
| Digestible Valine % | 0.89 | 0.81 | 0.73 |
| Calcium % | 0.90 | 0.84 | 0.76 |
| Available Phosphorus % | 0.45 | 0.42 | 0.38 |
| Sodium % | 0.16-0.23 | 0.16-0.23 | 0.15-23 |
| Chloride % | 0.17-0.35 | 0.16-0.35 | 0.15-0.35 |
| Potassium % | 0.60-0.95 | 0.60-0.85 | 0.60-0.80 |
| Linoleic Acid % | 1.00 | 1.00 | 1.00 |

**Sample collection:**

The broilers were weighed on a digital scale and were recorded on day 1, 7, 10, and 20. .At the end of trail, blood samples were collected from the wing vein into a sterile syringe. Samples for serum analysis were after that collected in non-heparinized tubes. Sera were separated after centrifugation at 3,000 × g for 15 min and kept at -18°C till usage for kidney and liver functions. Heparinized blood samples were withdrawn to evaluate complete blood count (CBC). Following blood collection, the broilers were individually weighed and euthanized via cervical dislocation.

**Determination of complete blood picture:**

Total Erythrocyte Count (TEC) counts were done using Hayem's fluid. The concentration of haemoglobin was measured using the van Slyke apparatus and the PCV - Hacksley haematocrit centrifuge (UK) [21] and [22].

**Biochemical analysis:**

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) serum levels were measured using commercial kits from Spectrum in Cairo, Egypt [23]. Gamma-glutamyl transpeptidase enzyme (GGT) was measured by spectrophotometry via commercial kit from Spectrum in Cairo, Egypt, and as previously scribed [24]. Total protein, creatinine

and urea were measured calorimetrically based on the method reported by [25] . Cholesterol was determined calorimetry as described by [26, 27] utilizing commercial kit from Spectrum, Cairo, Egypt.

**Determination of antioxidant status and lipid peroxidation**

To eliminate any red blood and clots, 1g of hepatic tissue was perfused with a pH 7.4 phosphate buffer saline (PBS) solution containing 0.16 mg/ml heparine. A 5–10 ml cold buffer containing 50 mM potassium phosphate, pH7.4, 1 mM EDTA, and 1 mM Triton X100 was used to homogenize the tissue. After 15 minutes of centrifuging at 4000 rpm, gather the supernatant for analysis. Superoxide dismutase activity (SOD) was estimated by determination of the ability of the enzyme to reduce nitroblue tetrazolium [28], catalase activity (CAT) was determined by inhibition of catalase activity by hydoxy benzene-sulphonic acid and amino phenazone [29], malondialdehyde (MDA) levels were determined by estimating the products of reacting MDA with thiobarbituric acid in presence ofn-butanol [30, 31], reduced glutathione (GSH) levels was determined by reacting GSH with di-thio-dinitro-benzene compounds [32]**.**

**Gene expression by RT-PCR**

A quantitative real-time PCR (qRT-PCR) assay was performed using the RNeasy Mini Kit's instructions [33]. Total RNA was extracted from the samples. Using the recombinant Ribolock RNase inhibitor and the RevertAid Reverse Transcriptase for First Strand cDNA Synthesis Kit, the mRNA was next reverse transcribed into cDNA. For qRT-PCR reactions, 25 µL mixtures were made by the use of Quantitect SYBR green qPCR Master Mix (2X) kit, containing 12.5 µL QuantiTect SYBR Green qPCR Master Mix (2X) Plus, 0.25 µL Reverse transcriptase, 0.5 µL of forward and 0.5 µL of reverse primer (Table1), 8.25 µL RNAase-free water and 3 µL cDNA. The Qiagen- GmbH Company in Germany sold us all the kits. Real-time PCR was carried out using the Stratagene MX3005P thermal cycler for qPCR (according to $2^{-\Delta\Delta ct}$ [34] Where: $\Delta\Delta Ct = \Delta Ct$ treference – $\Delta Ct$ target; $\Delta Ct$ target = Ct control – Ct treatment and $\Delta Ct$ reference = Ct control- Ct treatment).

**TABLE 2. List of primer sequence used in gene expression analysis**

| Gene | Forward | Reverse | accession number |
|---|---|---|---|
| **IL-1β** | 5′-TCCTGGAGGAGGTTTTTGAG-3′ | 5′-CACGAAGCACTTCTGGTTGA-3′ | NM_204524.1 |
| **Caspase3** | 5′-TGGCGATGAAGGACTCTTCT-3′ | 5′-AGAGTTTGGGTTTCCCTGCT-3′ | NM_204725.1 |
| **GAPDH** | 5’-AAGGCTGAGAACGGGAAACT-3’ | 5’-TCTCCATGGTGGTGAAGACA-3’ | NM_204305.1 |

**Quantification of aflatoxins by HPLC**

Tissue from liver, kidney and muscles were analyzed by HPLC for quantitative detection of aflatoxins according to [35].

**Sample preparation for analysis**

The samples of chicken were homogenized according to the method described by [36]. 25 g of each defrosted sample were mixed with five grams of diatomaceous earth and 2.5 mL of a 20% aqueous citric acid solution after being homogenized. 50 mL of dichloromethane was used to extract the mixture, which was shaken for 30 minutes. An aliquot (10 mL) of the filtered extraction was evaporated to dryness, dried by adding $Na_2SO_4$, and then filterated once more. The residue was extracted with 5 mL of hexane to extract the fat and reconstituted with 10 mL of acetonitrile: $H_2O$ (75:25, V/V). The aqueous phase (bottom) was then taken in 5 mL amounts, evaporated, and dried. Reconstituted the concentration in 50 mL of an methanol: $H_2O$ (80:20, v/v). Without preconditioning, a 40 mL aliquot was poured onto the immunoaffinity column (AFLAPREP® R-Biopharm). Phosphate buffered saline (PBS, pH 7.6) (10 mL) was used to wash the column. Aflatoxin removal at the column required 2 mL of methanol, which was then evaporated to dryness.

**Analytical procedures of HPLC**

After being resuspended in 200 L of mobile phase (AOAC 2000), the residue was derivatized using 700 L of 20:10:70 trifluoroacetic acid, acetic acid, and water. A 50 L aliquot of the derivatized extract was injected into a Waters 2475 Multi-Wavelength Fluorescence Detector equipped with a guard column and a reverse phase column (Luna 5 mm C18 (2) 100A, 150 x 4,6mm, Phenomenex Inc.) in excitation of 360 nm and emission in 440 nm. Methanol, acetonitrile, and water were mixed to form the mobile phase for the chromatographic process. The pump program began with acetonitrile-methanol-

water (1:1:4) over the course of 7 minutes, washed for 5 minutes (2:2:1), and then stabilized for 6 minutes before the next injection. 1.5 mL/min of fluid were flown.

**Histopathological examination:**

For histochemical study the hepatic tissue specimens were collected from the five groups after euthanasia. All of the hepatic tissues were fixed for 120 minutes use 70% alcohol, then for 90 minutes using 90% alcohol, and finally for two hours each using 100% alcohol. Then stained with H&E stain according to [37].

**Statistical Analysis**

Utilizing the statistical software program SPSS for Windows (version 17; SPSS Inc., Chicago, IL, USA), statistical analysis was conducted. $P \leq 0.05$ was used to determine whether two mean values were statistically significant. One-Way ANOVA and Duncan's multiple comparison Post Hoc tests were used to analyze group differences. Data was represented by mean± standard error of means.

## Results

As showed in table (3), broilers fed on diet intoxicated with AFs had significantly decreases at body weight (BW) starting from week 1 as compared with the negative control. The addition of MOS and/or Chit to an AFs containing diet increased the BW significantly in the $7^{th}$ , $10^{th}$ and $20^{th}$ day of experiment as compared to positive control group.

**TABLE 3.Effect of dietary chitosan and *mannan* on broiler chicks body weight fed on diet containing 100 ppb AFs / kg diet after 20 days of dietary supplementation**

| Body weight/g broiler | -ve control | +ve control | AF+mannan | AF+ chitosan | AF+ mannan+ chitosan | p |
|---|---|---|---|---|---|---|
| **Day0** | 46.7±0.5$^{a}$ | 46.1±0.3$^{a}$ | 47.2±0.6$^{a}$ | 46.3±0.6$^{a}$ | 46.3±0.4$^{a}$ | 0.871 |
| **Day 7** | 147.2±0.4$^{a}$ | 125.1±1.1$^{c}$ | 135.2±1.0$^{b}$ | 132.3±0.9$^{b}$ | 136.3±1.3$^{b}$ | 0.001 |
| **Day 10** | 355.4±2.5$^{a}$ | 230±3.3$^{d}$ | 329±2.5$^{c}$ | 325±3.0$^{c}$ | 337±4.1$^{b}$ | 0.001 |
| **Day 20** | 650.9±6.1$^{a}$ | 550±4.7$^{c}$ | 610.7±5.22$^{b}$ | 605.4±6.3$^{b}$ | 645.3±7.22$^{a}$ | 0.001 |

$^{a, b, c,d}$ Means in the same row with different superscripts differ significantly ($p < 0.05$).G: group. AF: aflatoxin (100 ppb/kg diet). Values expressed as means ± SE. Significance difference was conducted on each time point for all groups (Row )

As described in table (4), the effects of AFs also had a considerable impact on hematological parameters. Hemoglobin, PCV%, and TEC were all lower than the control. When added to a diet containing AFs, MOS and/or Chit significantly lessened the detrimental effects of AFs on the hematological parameters.

Table (4) lists the effects of MOS, chit, and both MOS+chit on renal function. At day 20, there was a statistically significant increase (p 0.05) in blood creatinine, urea, and uric acid when G2 was compared to the G1. When G5 was compared with the G1, no significant difference at the concentration of creatinine, urea, and uric acid was found, but there was a substantial drop in these levels when the treated groups for MOS and Chit were compared to the positive control group.

In Table (4), the influences of aflatoxin, MOS, Chit, and both MOS+Chit on cholesterol have been analyzed and summarized. As comparing to the control negative group, the blood cholesterol of control positive group (G2) at day 20 drastically reduced ($p \leq 0.05$). Additionally, as compared to the control positive group, the cholesterol levels in the MOS, Chit, and both MOS+Chit treated groups were considerably higher (p 0.05).

The impacts of aflatoxin, MOS, Chit and both MOS+Chit at liver function were shown in table (4). In day 20, AST, ALT and GGT was significantly elevated ($p \leq 0.05$) (41.50±0.76, 250.2±1.6and 17.5±0.44), respectively, in G2 group as compared with G1 group (18.90±.0.60, 211.9±.1.4and 7.25±0.45). In addition, AST, ALT and GGT were significantly lowered ($p \leq 0.05$) at MOS, Chit and both MOS+Chit received groups as compared with the control positive group.

On the 20th day of the experiment, as shown in table 4, intoxicated chicks at the G2 group exhibited a substantial reduction ($p \leq 0.05$) in total protein concentrations in comparison to G1group. The inclusion of MOS, Chit, and both MOS+Chit as feed additives resulted in a statistically significant ($p \leq 0.05$) elevation on total protein concentration compared with the control positive group G2.

**TABLE 4. Effect of dietary chitosan and *mannan* on some hematological and biochemical parameters for broiler chicks fed on diet containing 100 ppb AFs / kg diet after 20 days of dietary supplementation.**

| | -ve control | +ve control | AF+mannan | AF+ chitosan | AF+ mannan+ chitosan | p |
|---|---|---|---|---|---|---|
| **Hemoglobin mg/dl** | 8.9±0.003$^{a}$ | 4.8 ±0.011$^{c}$ | 7.5±0.004$^{b}$ | 7.3 ±0.003$^{b}$ | 8.3±0.001$^{a}$ | 0.001 |
| **PCV%** | 34.2±1.62$^{a}$ | 20.6 ±1.011$^{b}$ | 28.2±1.04$^{c}$ | 28.3±1.03$^{c}$ | 33.3±1.14$^{a}$ | 0.001 |
| **TEC10$^{6}$/µL** | 2.78±0.62$^{a}$ | 1.81±0.011$^{c}$ | 2.29±0.04$^{b}$ | 2.31±0.03$^{b}$ | 2.63±0.14$^{a}$ | 0.001 |
| **Creatinine mg/dl** | 030±0.003$^{c}$ | 0.52 ±0.011$^{a}$ | 0.38±0.004$^{b}$ | 0.39±0.003$^{b}$ | 0.31±0.004$^{c}$ | 0.001 |
| **Urea mg/dl** | 7.86±0.19$^{d}$ | 17.53±0.13$^{a}$ | 12.4±0.12$^{b}$ | 12.07±0.0$^{b}$ | 10.38±0.12$^{c}$ | 0.001 |
| **Uric acid mg/dl** | 2.87±0.045$^{d}$ | 7.05 ±0.086$^{a}$ | 5.87±0.13$^{b}$ | 6.13±0.12$^{b}$ | 4.19±0.12$^{c}$ | 0.001 |
| **cholesterol mg/dl** | 194.0±2.8$^{a}$ | 96.80±2.3$^{d}$ | 123.50±0.74$^{c}$ | 125.40±1.1$^{c}$ | 144.30±2.1$^{b}$ | 0.001 |
| **ALTIU/L** | 18.90±0.60$^{d}$ | 41.50±0.76$^{a}$ | 29.20±0.47$^{b}$ | 30.10±0.57$^{b}$ | 24.1±0.56$^{c}$ | 0.001 |
| **AST IU/L** | 211.9±1.4$^{d}$ | 250.2±1.6$^{a}$ | 224.1±1.5$^{c}$ | 225.0±1.4$^{c}$ | 217.2±2.0$^{d}$ | 0.001 |
| **GGTIU/L** | 7.25±0.45$^{d}$ | 17.5±0.44$^{a}$ | 12.51±0.34$^{b}$ | 12.95±0.26$^{b}$ | 9.3±0.29$^{c}$ | 0.001 |

$^{a, b, c,d}$ Means in the same row with different superscripts differ significantly ($p < 0.05$). G: group. AF: aflatoxin (100 ppb/kg diet). Values expressed as means ± SE.

The effects of aflatoxin, MOS, Chit and both MOS+Chit on homogenized liver tissue antioxidant and oxidative stress were evaluated and presented in Figures(1:A-D). Aflatoxin exposure had a substantial impact on the liver as compared to the control group (G1); it raised (P=0.001) the amount of MDA in the liver and lowered (P=0.001) the activity of the liver's CAT, SOD, and GSH. Dietary MOS and/or COS supplementation lowered (P=0.001) liver MDA content and improved (P=0.001) liver CAT, SOD, and GSH activity when compared to the AFs group (Figure.1 A-D)

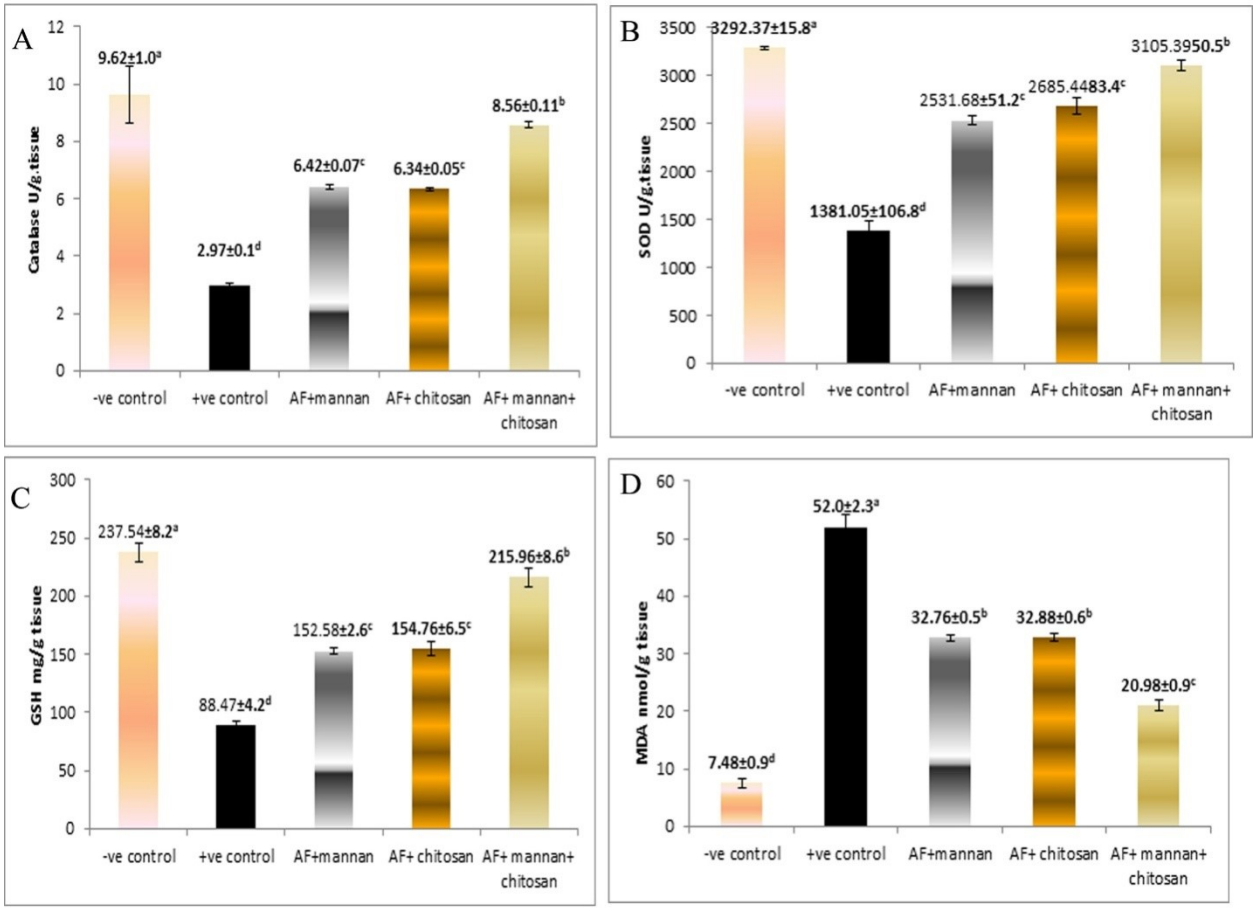


**Fig. 1. Effect of chitosan and/or mannan on antioxidants and lipid peroxidation on aflatoxicosis in broilers (a) catalase, (b) Superoxide dismutase (SOD),(c) Glutathione reduced (GSH), (d) malondialdehyde (MDA).**

Aflatoxin-free, aflatoxin, and aflatoxin diets with Chit/MOS alone or in combination were fed to the various treated groups, and the histopathology of their liver tissues was shown in Figure. 2. The hepatic parenchyma of the control negative group (G1) was seen in the liver sections. The experimental group labeled as the control positive group (G2) exhibited significant alterations characterized by substantial vascular congestion, bleeding, diffuse infiltration of mononuclear cells, and widespread necrosis of hepatocytes. The liver sections of the groups (G3, G4, and G5) exhibited significant enhancement in the hepatic parenchymal architecture, accompanied to modest vascular congestion, as depicted in Figure 2.

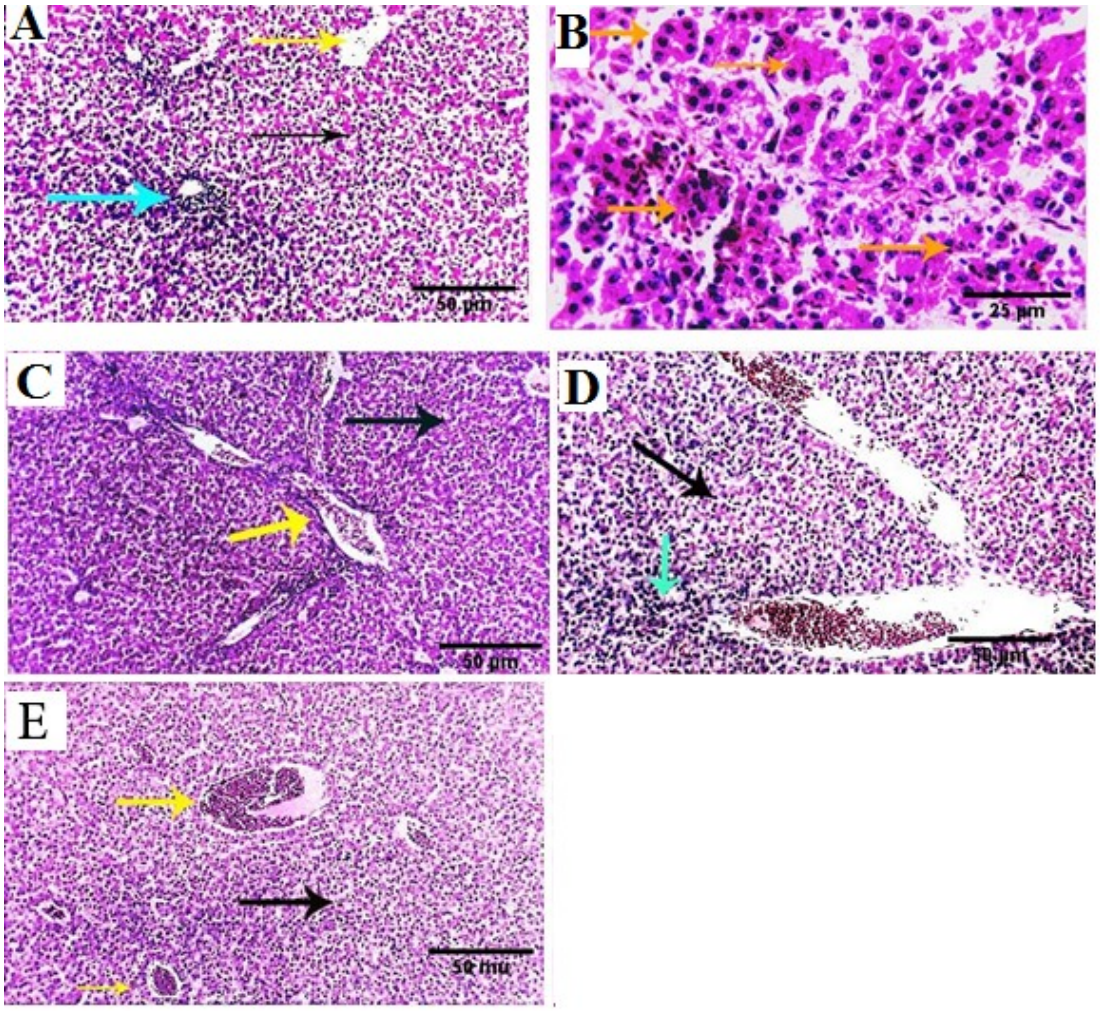


**Fig. 2. H&E. Scale bars are 25um and 50um for hepatic tissue (A)the liver of the control negative group (G1); (B)aflatoxicoses group (G2 ); (C)mannan-treated group (G3); (D)Chitosan-treatedgroup (G4 )and (E)mannan-chitosan-treated group (G5)**

The data presented in Table 5 and Figures 3-5 indicate that the mean total aflatoxin residues in chicken samples varied across different groups. The aflatoxin group (G2) exhibited a notable rise in hepatic aflatoxin residues, kidney, and muscle tissues towards the conclusion of the experiment, in comparison with the other groups ($p < 0.05$). The mean values of the chicken muscle, liver, and kidney samples exhibited significant variability, while the liver exhibiting higher residue levels compared to the muscles and kidneys. With the addition of MOS and Chit that were pertinent to the aflatoxin group, this residue was significantly reduced ($p \leq 0.005$). When compared to the results of the other groups at the end of the experiment, tissue from the aflatoxin at G5 indicated a significant decrease in aflatoxin residue ($p \leq 0.05$).

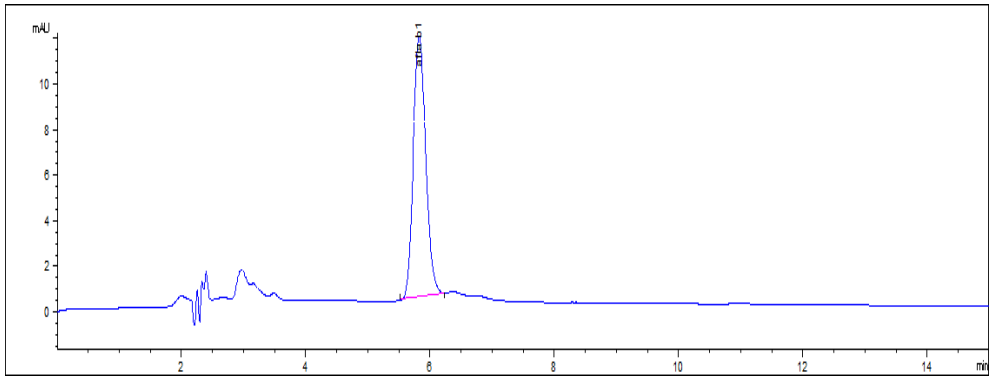


**Fig. 3. chromatograms of total aflatoxins residues in chicken muscles**

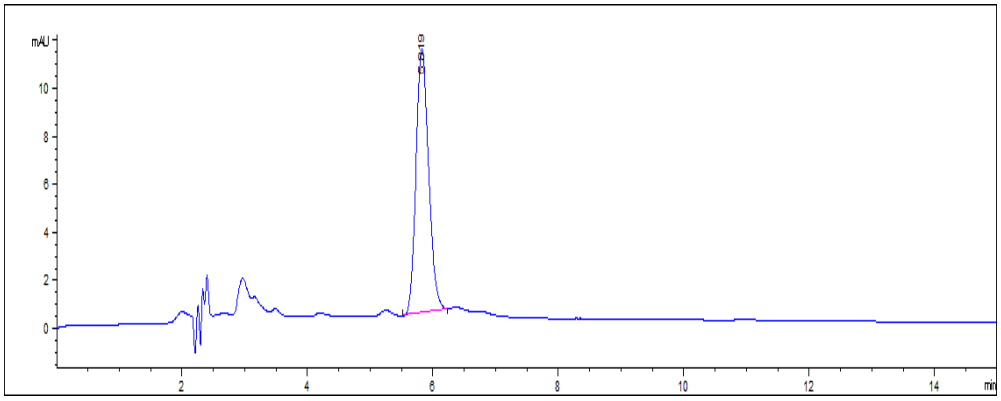


**Fig. 4. chromatograms of total aflatoxins residues in chicken liver**

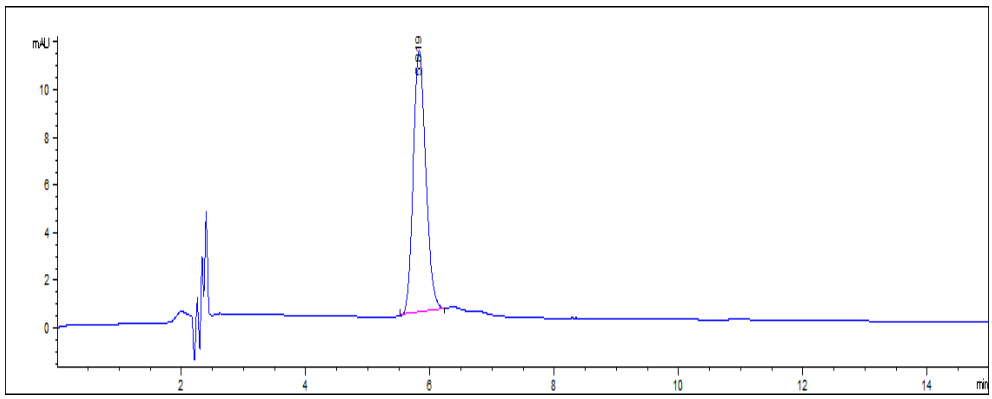


**Fig. 5. chromatograms of total aflatoxins residues in chicken kidney**

**TABLE 5. The concentrations of Aflatoxins residues in chicken muscles, liver and kidney tissues:**

| | +ve control | AF+mannan | AF+ chitosan | AF+ mannan+ chitosan | p |
|---|---|---|---|---|---|
| Muscles (ppt) | 86.50±1.6[a] | 40.71±1.2[b] | 65.28±2.1[c] | 38.73±1.1[b] | 0.001 |
| Liver (ppt) | 183.00±18.27[a] | 87.26±8.89[b] | 127.96±11.85[c] | 81.83±8.92[b] | 0.001 |
| Kidney (ppt) | 163.00±3.88[a] | 77.72±2.33[b] | 131.51±2.93[c] | 72.31±1.76[b] | 0.001 |

[a, b, c] Means in the same row with different superscripts differ significantly ($p < 0.05$). Values expressed as means ± SE.

Broilers Liver Caspase-3 gene expression levels was significantly increased in aflatoxin group (G2) when compared to negative control group (G1). With the addition of MOS and Chit the Caspase-3 was significantly reduced (P≤0.001) (Figure 6A). Aflatoxin-fed broilers showed elevated IL-1β mRNA abundances in the liver. However, dietary MOS and/or Chit addition decreased the levels of IL-1β gene expression in the liver broilers (P ≤0.001) (Figure 6B) .

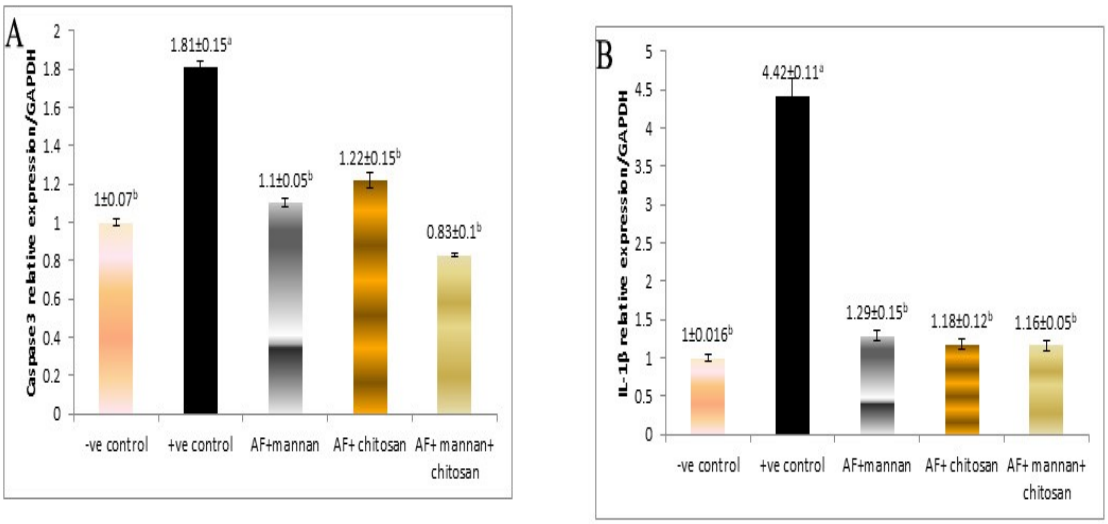


**Fig. 6. Gene expression analysis of caspase 3 and IL-1β in chicks exposed to aflatoxicosis and supplementedwith mannan oligosaccharides and chitosan oilgosaccharides**.

## Discussion

Mycotoxins are widely accepted as dietary pollutants that cannot be avoided. The most prevalent mycotoxin found in chicken feed is aflatoxins [38].The reduction of performance parameters is one of the most economical consequences of AFBs in broilers [39]. The use of 100 ppb in the (G2) control positive group demonstrated a substantial reduction in body weight as compared to the control negative group from the first week to the end of the study (20 days).

The amount of (100 ppb) AFB1 declined daily weight gain and average daily feed intake in broilers, which slowed their growth. This harmful impact on growth condition of broiler chickens may be owing to the fact that aflatoxins slow down protein synthesis[40]-[41].

When MOS, or Chit was added to a diet containing 100 ppb of aflatoxin, its bad effects on these performance measures were greatly reduced. [42] observed that adding 2 g/kg of MOS oligosaccharides to the diet helped groups that had been exposed for 21 days to 200 ppb AF recover their growth performance; [43] looked at the effect of glucoMOS on broilers that had been exposed to Aflatoxin 2 mg/kg during 21 days and found that weight gain and feed effectiveness were a partial restoration; [44] who discovered that adding Chit to chickens has an effective impact on growth parameters,[45] observed that duck fed diets with

Chit had significant (P≤0.05) enhancements in body weight. Additionally, fish fed a diet enriched with chitosan and folic acid nanoparticles at a dose of 1 mg/kg will develop faster and have a higher growth index [46], and ; Quils fed diets with a medium MOS content (3 g/kg feed) experienced significant (P≤0.05) increases in body weight, weight gain, and effectiveness of feed conversion, according to research by Abdel-raheem and Esmail [47].

Before significant symptoms appear, alterations in haematological parameters can be used to diagnosis chronic and sub-clinical aflatoxicosis cases [48]. These variables serve as sensitive markers of the harmful impacts of AF in the intended organs. The hazardous haematological effects of AF have been extensively researched and are well known. In the current investigation, these harmful consequences were also clearly seen. TEC, PCV%, and hemoglobin counts in G1 significantly decreased in the current study (P ≤0.05). [49];[50] found that AFs had a negative impact on hematological parameters in boilers. An AF-containing diet that included MOS and/or Chit greatly lessened the negative impacts of AF on haematology. The negative impacts of AF on haematology were greatly reduced when glucoMOS (1 g per kg) was added to a diet containing AF[51].

Serum ALT, AST, and GGT values that are raised are signs of liver damage[52], [53] and [54]. When comparing G2 group with the G1 group, there was a notable rise in ALT, AST, and GGT at 20 days (p ≤0.05) in this study.

According to the findings of[55] and [56], the serum levels of ALT, AST, and GGT were considerably lower in the MOS supplemented group at 20 days (p≤0.05). As opposed to the positive control group, liver enzymes significantly decreased when Chit was fed a food contaminated with aflatoxin. Findings from this study indicate that inclusion of Chit to diets containing aflatoxins appears to be helpful at lowering toxicity. [57] Study who stated that Chit and Chit oligosaccharides can offer defense against the deadly combination of ZEN and aflatoxins (AFs) in duck.

In our study, total protein (TP) was considerably lower at AFs group compared with negative control and other examined groups after 20 days of AFs consumption. [58-61] studies indicated that broiler chickens TP was significantly lowering by AFs contaminated food. It was suggested by Quezada et al. ,2000 study that a serum protein is a sensitive early sign of this toxicity, which was worse in chickens that were still growing[55].

Compared to the negative control group, broilers in the control positive group (G2) indicated a significantly elevated level of blood creatinine, urea, and uric acid at day 20 of the trial, according to our results for renal function tests. Elevated serum creatinine levels serve as a sign of impaired renal function and kidney injury, maybe attributable to the deleterious impact of aflatoxin-induced toxicity. Furthermore, it has been established that the elevation of plasma urea concentrations can be attributed to the nephrotoxic effects generated by AFB1[62] and [63]. Additionally, it was reported that elevated levels of urea and creatinine were observed in broilers aged 2 and 6 weeks who were fed feed contaminated with 3 mg/kg of AFB1. These findings were found to be being connected with the renal tubules' inflammatory and dystrophic processes [64]**.** The findings of this study imply that exposure to AFB1 may potentially result in degenerative alterations in the kidney, thus leading to impair renal function.

When the amount of reactive oxygen species (ROS) produced surpasses what the body's antioxidant defense system can tolerate, oxidative stress is the result. Lipid peroxidation (LPO) refers to an autocatalytic process in which polyunsaturated fatty acids present in cell membranes undergo breakdown, resulting in the formation of lipid hydroperoxides. This process is mediated by free radicals and is known for its destructive nature [60], illustrates the extent of overall lipid oxidation of the body. MDA formed at the end of lipid peroxidation. However, an antioxidant defense mechanism that includes enzymatic components like SOD and CAT as well as non-enzymatic components like GSH and vitamin E is able to limit these quantities of free radicals and lipid peroxidation[65].

Natural antioxidants are less likely to have negative side effects and are healthier and more beneficial. Medicinal herbs, chitin, and other substances are suggested to be abundant natural antioxidant sources. The use of compounds and natural plant materials and chitin, etc., have significant improvement in both signs of oxidative stress and free radicals, this antioxidant effects comes when the compounds are used[66-68].

The results of the present study suggest that the amount of AF (100 ppb/kg) could raise the liver's oxidative state of broilers by increasing ROS as a result of the oxidative stress condition in the presence of AF intoxication. This was indicated by the results of increased MDA in the liver of the birds of G2. The livers of broilers given a feed contaminated with AFs contained much more MDA and had significantly lower antioxidant enzyme activity and GSH levels than those of the control group [69].

MOS and Chit supplementation improved the catalase, SOD enzyme's decreased activity and the GSH level caused by AFs to be nearly at control levels. These results imply that MOS and Chit may mitigate the harms brought on by AFs.

In the study of [70] found that, Dietary intake of *Saccharomyces Cerevisiae* improved the SOD enzyme's lowered activity and the GSH level caused by AF to be nearly at control levels. These results imply that SC may mitigate the harms brought on by AFB1. Our finding was compatible with [71]and [72] where MDA was decreased in broiler liver after MOS addition GSH levels was improved.

According to [73] in broilers under heat stress, dietary Chit supplementation improved the antioxidant status of the muscles (elevated muscle CAT, SOD, and GPx activity) and reduced lipid peroxidation (less MDA content), indicating that COS could enhance the meat quality of broilers under heat stress through direct antioxidant capacity. In addition, the [74]study's findings suggest that dietary Chit supplementation at a dose of 500 mg/kg may enhance the antioxidant function.

Additionally, the phenomenon of apoptosis, also known as programmed cell death, is a crucial sign of toxicity and is characterized by a number of typical morphological characteristics, including cell shrinkage, fragmentation into membrane-bound apoptotic bodies, and rapid phagocytosis by nearby cells[75]. One of the cysteine proteases that is essential for the execution of apoptosis is caspase-3 (cysteinyl aspartate proteinase). Caspase activation appears to be necessary for the development of the apoptotic phenotype of cell death, according to a number of genetic and biochemical investigations [76]. Caspase-3 expression levels as an apoptotic regulator mRNA gene were found to be considerably ($P\leq 0.05$) higher in broiler livers during the G2, indicating severe apoptosis in the tissue. Biochemical abnormalities in the system that controls the balance of oxidants and antioxidants were linked to increased caspase-3 activation. All of this might be linked on the pathogenic network of AFs toxicity, as an imbalance between ROS and antioxidant enzymes can change the structure and function of proteins, lipids, and DNA, damage lipid membranes, start cellular catalytic processes, and cause cell death [77].

Additionally, in the current investigation, Caspase-3 gene expression levels in MOS and/or Chit were considerably reduced ($P \leq 0.05$) in comparison to with AF alone. The food additive had a diminishing impact on the expression of Caspase-3, a protein involved in the dysregulation of cell death, in the liver. This suggests that the additive promotes the regeneration of hepatic cells and prevents liver cell death by restoring the balance between oxidants and antioxidants. Consequently, it facilitates a prompt and thorough recovery from Aflatoxicosis [78].

The findings of the aflatoxins residue in this study were in agreement with those of [79] and [80], who stated that despite aflatoxins residues can be discovered in the liver, muscles, stomach, kidneys, adipose tissue, and meat, the liver is where they are most concentrated. [81] found that the liver had the highest concentration of AFB1 and total aflatoxins, followed by the kidneys and the gizzard, while the thigh and breast had the lowest concentrations.

The inflammatory response brought on by various stresses has also been linked to animal liver damage. An excess of free radicals that can attack hepatocytes and harm liver structure and function can bring on inflammation in the liver. IL-1β are among the inflammatory cytokines that are crucial early mediators of liver injury[82]In the current study aflatoxins increased production of IL-1β in the liver, suggesting that aflatoxins can result in hepatic inflammatory response. The current research work was limited to evaluate the toxicity of aflatoxin in hepatic tissues, where investigation of toxicity markers in different organs are also required.

## Conclusion

The current study provided evidence in that supports the hypothesis that natural additives such mannan and chitosan could enhance the vital signs and antioxidant capacity of broilers exposed to 100 ppb aflatoxins.

**List of abbreviation:**

| | |
|---|---|
| AFs | Aflatoxins |
| ALT and AST | Alanine and aspartate transaminase |
| CAT | Catalase |
| CBC | Complete blood count |
| Chitosan | Chit |
| GGT | Gamma glutamyl transpeptidase |
| GPx | Glutathione peroxidase |
| GSH | Reduced glutathione |
| HPLC | High performance liquid chromatography |
| MDA | Malondialdehyde |
| MEA | Malt extract agar |
| MOS | Mannan oligosaccharides |
| $Na_2SO_4$, | Sodium sulphate |
| NRC | National Research Council |
| PCV | Packed cell volume |
| qRT-PCR | Quantitative real-time PCR |
| SOD | Superoxide dismutase |
| TEC | Total erythrocytic count |
| YES | Yeast extract sucrose |

*Acknowledgement*

The authors would like to acknowledge the efforts excreted by Department of pathology, Faculty of Veterinary Medicine, Mansoura University for their efforts for establishing histopathological examination.

*Conflict of Interest*

All the authors declared that there was no conflict of interest.

*Funding statement*

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

*Author`s contributions*

Al-Zahraa Al-Habashi and Mohamd El-Adl: Conduction of experiment design, statistical analysis, biochemical analuysis and gene expression analysis, Ghada Abd El Dayem: Formulate the dietary requirement of studied groups and EL-Said EL-Sherbini: Supervise the conduction of the experiment.

*Ethical approval*

The current study complies with national and international guidelines. The current study was approved by the research ethics committee at Mansoura University. The current study was conducted based on the guidelines of ARRIVE guidelines 2.0.

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## التأثير التآزري لكل من مانان وشيتوسان أوليغوساكشاريديسون على برويلات المسكرات المسكرات المُتَمَرَّجَة بسموم افلا الفطرية

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استُخدمت تقنيات عديدة لإزالة متبقيات السموم الفطرية. أُجريت هذه الدراسة لتقييم إضافة مادة المنّان (MOS oligosaccharides) و/أو (Chitosan (Chit للتخفيف من سمية الـ AFs في دجاج التسمين (cobb500)، وتحسين الأداء، والكيماويات الحيوية، وصورة الدم، والمضاد للأكسدة، والبارامترات الوبائية المرضية، وتحليل التعبير الجيني الكاسباس 3 والإنترلوكين 1-ب .تم تشكيل خمس مجموعات متساوية من 50 فرخ لكل منها (عشر افراخ/مجموعة) ؛ المجموعة الضابطة (G1) و تتغذى على عليقة بادئة و نامية و ناهية دون اى اضافات، المجموعة الإيجابية (G2) نفس عليقة المجموعة الضابطة + 100 جزء من كل منها /Afs كيلوغرام ؛ تلقى مجموعة MOS (G3) مقدمة باعتبارها حمية بالوزن + 100 جزء من كل منها /AFs كيلوغرام MOS + غرام واحد كيلوغرام - 1 غذاء)؛ تلقى تشيت مجموعة (G4) مع الأخذ بعين الاعتبار حمية البزال + 100 جزء من الوجبة + 100 جزء من الوجبة + 100 جزء من الوجبة (0.5 Cchit + ccg-1det AFs غرام كيلوغرام - 1 غذاء)؛ واستلمت MOS/Cit مجموعة (G5) مجموعة (G5) معطاة حمية بالجازال + 100 جزء من الغرامات AFs كيلوغرام-1) MoS + غرام) + شيت (0.5 diet 1-kg g). وأُعطيت جميع العلاجات للمعالجات التي تتراوح أعمارها بين 6 أيام و25 يوماً. وأدى الجمع بين إلى تحسين البارامترات الوراثية في مجموعات التسمم - التسمم - التسمم. وتحسنت علامات الكلى والهباء تحسناً كبيراً في الفتيات اللاتي يُسمرن بالسموم المتفائلة ويُعالجن بموجات مائية و/أو تشيت. حدوث تحسن كبير في العلامات المضادة للأكسدة في كل من مجموعتي وزارة الصحة وتشيت المعرّضتين لسمّيّة الغدة الدرقية. وقد تحسن كثيراً التعبير عن جينات الكازباز-3 وLL-1B بالتسمم بالفلاتوكسينات والمعالجات بMOS و/أو Chit. و/أو قلل MOS و/أو Chit من مستويات تراكم الأفلاتوكسين في العضلات والكبد وأنسجة الكلى. وأظهرت أجزاء كبد المجموعات (G3, G4, G5) تحسناً كبيراً في الانسجة الكبدية.

**الكلمات الرئيسية**: الأفلاتوكسينات، تشيت، MOS، مضادات الأكسدة، كاساسيل-3، IL-1.