JJBS

Volume 11, Number 5,December 2018
ISSN 1995-6673
Pages 549 - 555

Jordan Journal of Biological Sciences

# Immunological Influences of Experimental Aflatoxicosis in Broilers and the Protective Role of Mannan Oligosaccharides

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*Received April 19, 2018; Revised June 4, 2018; Accepted June 7, 2018*

**Abstract**

The present study is carried out to highlight the toxic effects of Aflatoxin B1 on humoral and cell-mediated immune responses of broilers, and to evaluate the efficacy of Mannan oligosaccharides (MOS) to ameliorate these toxic effects. In this study, experimental diets were designed, and the broiler chicks were divided into four groups. The first group was fed a normal diet. The second group was fed normal diet with MOS. The third group was fed aflatoxin B1 at the dose of (250 parts per billion"ppb"), while the last group was fed diet containing the aflatoxin in addition to MOS. The birds were checked for humoral and cell-mediated assays. The results revealed that there was a significant decreasing effect of 250 ppb AFB1 on both the antibody titer against the Newcastle disease virus (NDV) and lymphocyte blastogenesis and transformation. The investigation of differential leukocytic counts revealed lymphocytopenia and heterophilia. Dietary supplementation of a 1g MOS/Kg diet during aflatoxicosis could ameliorate some of the toxic effects of AFB1 in broilers, and improve the immune response. These outcomes confirm the immunosuppressive impact of aflatoxin and the role of mannan oligosaccharides to overcome its adverse effects.

**Keywords:** Aflatoxicosis, Lymphocyte, HPLC, TLC, Broilers, Mannan

## 1. Introduction

Mycotoxins are chemically stable, secondary toxic metabolites which are produced by certain fungi. They constitute serious and ever-present environmental health hazards as may maintain their toxic effects over an extended period of time. In addition to their economic impact as they cause several hundred million dollar losses annually in the poultry industries, mycotoxins residues in poultry meat and eggs represent a concern for human health since they are known to have strong hepatotoxic and carcinogenic effects (Zain, 2011).

Aflatoxins are one of the most widespread mycotoxins in poultry and human food that contribute to significant health disorders and decrease in the production performance. Moreover, they have been proven to be carcinogenic and/or toxic to humans and animals as well (Mazzoni *et al*., 2011)**.**

The name ''aflatoxin" is derived from the first letter of the word *Aspergillus* and the first three letters of *flavus*, because it is mostly produced from two species, *Aspergillus flavus* and *Aspergillus parasiticus*, which thrive under hot and humid conditions in the fields and stores where contamination is usually a consequence of interactions among the causative fungi, the host animal and the environment. The response to mycotoxin contamination also depends on a number of factors including the level of exposure, environmental conditions, the type and susceptibility of the invader species (Richard, 2007).

In poultry, aflatoxicosis causes listlessness, anorexia with a low growth rate; poor feed utilization, decreased egg production and increased mortality. In addition to anemia, reduction in the immune function, hepato-toxicosis, hemorrhage, teratogenesis, carcinogenesis and mutagenesis (Oguz, 2012). Among the known aflatoxins, AFB1 is the most commonly encountered and the most potent among the naturally occurring carcinogens, and is classified as a group I carcinogen by the International Agency for Research on Cancer (Yunus *et al*., 2011).

In general, mycotoxins cannot be immediately detected by visual examination; nonetheless, they are revealed when poultry are, for instance, faced with infectious agents, and this might be contributing to their immunosuppression effect (Girish and Smith, 2008).

Accordingly, due to their health problems in poultry flocks and the potential economic losses, poultry producers tried several strategies in order to avoid mycotoxicosis, aiming to protect their flocks against the adverse action of the toxins. In this context, mannan oligosaccharides (MOS) has been proposed as a mycotoxin binder for preventing the adverse effects of mycotoxins in poultry feeds (Pazhanivel and Balachandran 2014)**.**

Mannan oligosaccharides (MOS) is polysaccharide-protein complex derived from the cell wall of

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*Saccharomyces cerevisiae*. The MOS product is reported to have at least three probable modes of action by which the broiler performance is improved by the adsorption of pathogenic bacteria containing type 1 fimbriae with mannose-sensitive lectins, and by improving the intestinal function or "gut health" in addition to the immune modulation and simulating gut associated and systemic immunity by acting as a non-pathogenic microbial antigen, giving an adjuvant-like effect (Spring *et al*., 2000).

Considering the above-mentioned facts, the present study was planned with the aim of studying the impact of aflatoxin (AFB1) on avian immunity and to assess the efficacy of MOS as a mycotoxin binder in counteracting the adverse effects of aflatoxicosis in broilers.

## 2. Materials and Methods

### *2.1. Extraction of aflatoxin from Aspergillus flavus (Bauer et al., 1983)*

*Aspergillus flavus* NRRL-500 purchased from the Microbiological Resources Centre (Cairo MIRCEN) was grown on potato dextrose agar slants for approximately fourteen days at room temperature (20-22 $^{o}$C) until well sporulated. Spores were harvested, adjusted to approximately 5 X $10^{6}$ spores/ mL. Spore suspension was cultured and incubated at room temperature (20-22 $^{o}$C) in dark conditions for twenty days.

At the end of the incubation period, mycelia of cultures were carefully overlaid with 25 mL chloroform and kept for twenty-four hours in a dark room. Chloroform layers were combined and concentrated in rotary flash evaporator.

### *2.2. Chromatographic Estimation of Extracted Aflatoxin:*

#### *2.2.1. Qualitative Determination of Aflatoxin by Thin Layer Chromatography (TLC) (Gimeno, 1979)*

Activated coated silica gel chromatographic plates were spotted parallelly from the chloroform extract together with the standard aflatoxin B1, and were left to dry in the air. The prepared TLC plates were transferred into the developing tank containing the developing solvent system [toluene- ethyl acetate – 90 % formic acid (60:30:10)]. The plates were inspected under UV light (365 nm), and the rate of flow (RF) values, colors, and intensities of the unknown spots were compared with those of the standard ones.

#### *2.2.2. Quantitative Determination of Aflatoxins by High Performance Liquid Chromatography with Postcolumn Fluorescence Derivatization (HPLC-FLD)*

Aflatoxin B1, B2, G1 and G2 standards, and HPLC grade solvents were purchased from Sigma, Chemical Co (St. Louis, MO, U.S.A.).

Derivatization of aflatoxins was performed as described in (Manetta *et al*., 2005). The stock solution of aflatoxin standards was dissolved in acetonitrile: methanol (9:1) (AOAC, 2000). HPLC analysis was performed using Agilent Technologies 1100 series liquid chromatograph equipped with an auto sampler and fluorescence detector. The analytical column was Eclipse XDB-C18 (150 X 4.6 µm; 5 µm) with a C18 guard column (Agilent, USA). The mobile phase consists of Acetonitrile / Water / Methanol (1:6:3), then detected at 360 nm fluorescence wave length for excision and 440 nm for emission. The peaks were identified by congruent retention times and were compared with those of the standards.

### *2.3. Experimental Diet*

Known amounts of AFB1 was added and thoroughly mixed with the basal ration (tested negative for the presence of any possible residual mycotoxins) in proportion so as to arrive at the respective dose levels. The amount of crude AFB1 was added at a dose of 250 ppb/kg (parts per billion). Samples were taken from the mixed diet and further quantified for the concentration of the mycotoxin by HPLC to ensure proper mixing.

The experiment was carried on 121-day-old Cobb broiler chicks obtained from Misr Arab Poultry Company. The chicks were acclimatized for a period of one week before the commencement of the trial and were divided into four equal groups, n=30 each, the experimental design was as follows:-

**Group 1 (control negative)** healthy chicks were given normal ration from one day of age till the end of the experiment.

**Group 2 (control positive)** healthy chicks were given normal ration containing 1g MOS/kg feed from the second week of age till the end of the experiment.

**Group 3** was given a ration containing 250 ppb AFB1/kg feed from the second week of age till the end of the experiment.

**Group 4** was given a ration containing 250 ppb AFB1 plus 1g MOS/ kg feed from the second week of age till the end of the experiment.

*Immunization:* All chicks were vaccinated against Newcastle disease on day seven (Anil *et al*., 2003).

### *2.4. Assessment of Immunological Parameters*

#### *2.4.1. Differential Leukocyte Count*

Moderately thin blood films were fixed with methyl alcohol, stained with diluted Giemsa stain for thirty minutes and examined microscopically. The percentage values of different leukocytes were calculated according to this principle (Feldman *et al*. 2000).

#### *2.4.2. Serological Examinations and Assessment of Humoral Immune Response*

The serum samples from each group were obtained on day twenty-eight and the vaccine immune response was evaluated by hemagglutination inhibition test according to this principle (Beard, 1980).

#### *2.4.3. Assessment of Cell-mediated Immune Response*

#### *2.4.4. Lymphocyte Proliferation Assay*

##### *2.4.4.1. Separation of Lymphocytes (Burrells and Well, 1977)*

The obtained heparinized blood samples were layered carefully on the surface of lymphocyte separation medium ficoll hypaque (1:1), centrifuged, and washed then re-suspended in 1 ml RPMI 1640 containing 10 % fetal calf serum.

##### *2.4.4.2. Viable Lymphocyte Count (Hudson and Hay, 1980)*

A hundred µL of 0.4 % trypan blue were added to the lymphocyte suspension, then mixed well and immediately

transferred to the haemocytometer. At least one-hundred viable lymphocytes were counted. The number of viable lymphocyte per ml was calculated according to the following equation:

Viable cell yield = (Viable cell count / Quadrants counted) X Dilution factor X

Hemocytometer factor X Current volume (mL)

*2.4.4.3. Standardization of Lymphocyte Concentration for Blastogenesis :*

According to the viable cell count, the viable lymphocytes were adjusted at a final concentration of 2 $x10^6$ cells/mL and suspended in RPMI medium containing 10 % fetal calf serum (FCS).

*2.4.4.4. Preparation of Mitogen (non-specific mitogen): Phytohemagglutinin (Rai-el-Balhaa et al., 1985)*

Phytohemagglutinin (PHA) was obtained as a powder and reconstituted in 5 mL RPMI medium. The required concentration could be made to 15 µL $mL^{-1}$.

*2.4.4.5. Setting up of Lymphocyte Culture (Meky et al., 2001).*

Sterile microtiter tissue culture flat bottom 96 well plates were used for cultivation of lymphocyte. The wells were used in triplicate manner; one contained 100 µL of suspended lymphocytes ($2 \times 10^6$ cells) in 50 µL growth medium (RPMI + 10% FCS) and served as cell control. Another well contained 100 µL of suspended lymphocytes + 50 µL PHA (non-specific mitogen) as (15 µg / mL), and a third well group contained 150 µL of RPMI-1640 medium only. The total volume per well was adjusted to 150 µL. The plates were incubated at 37°C in a $CO_2$ incubator, lymphocyte transformation and blastogenesis were assayed after 48-72 hours using methyl tetrazolium bromide dye (MTT) assay.

Methyl tetrazolium dye (MTT) was added at $1/10^{th}$ of the total sample volume, and incubated at 37 $^oC$ in a 5 % $CO_2$ incubator for four hours. After incubation, the lysing buffer was added in 50 µL/ well. The plate was incubated overnight for complete cell transformation, then applied on a microplate reader ELx 800 UV (Bio-Tek) at 570 nm wave length.

*2.5. Statistical Analysis*

The obtained data were analyzed using SPSS software (SPSS, 2006) via applying analysis of one way ANOVA together with Duncan's Multiple Rang Test for testing the significant differences among the treated groups. Also, Student's T test for independent samples was used to detect the difference between the second and the third week results within the same group. The data were significant at ($P$<0.05).

## 3. Results

*3.1. Culture of Aspergillus flavus*

The culture of toxigenic *Aspergillus flavus* strain was maintained on potato dextrose agar plate; macroscopy revealed velvety, yellow to green or brown reverse goldish to red-brown figures (1 and 2). Microscopic morphology showed unbranched conidiophores, which were enlarged at the tip forming a swollen vesicle. Vesicles are completely covered with flask-shaped phialides, the phialides produce chains of mostly round conidia (figure 3).

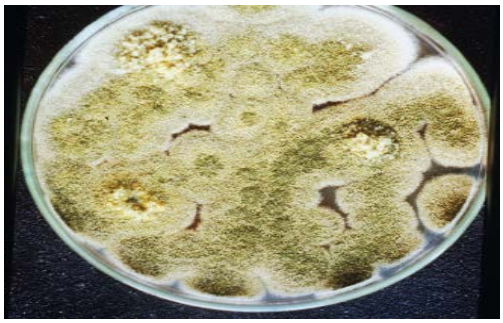

**Figure 1**. Potato dextrose agar plate showing growth of *A. flavus.*

**Figure 2**. Macroscopical morphology of *A. flavus* on potato derxtrose agar**.**

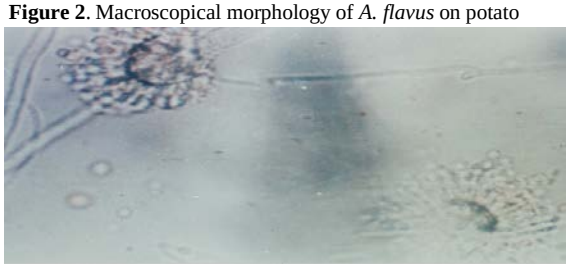

**Figure 3**. Microscopical morphology of *A. flavus* staining by lactophenol cotton blue (X 100) .

*3.2. Qualitative Determination of Aflatoxin by (TLC)*

The fungal extract was observed under UV light at 365 nm, and was matched with standard aflatoxin B1 as shown in figure 4.

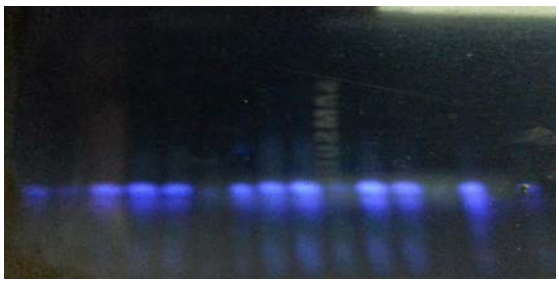

**Figure 4.** TLC for determining the toxigenicity of *Aspergillus flavus* strain.

*3.3. Quantitative Determination of Aflatoxin B1 Using HPLC*

Figure 5 shows two graphs (a, b). The first graph was for standards and the second one was for the samples. The quantification of aflatoxin B1 was determined from the area of the peak.

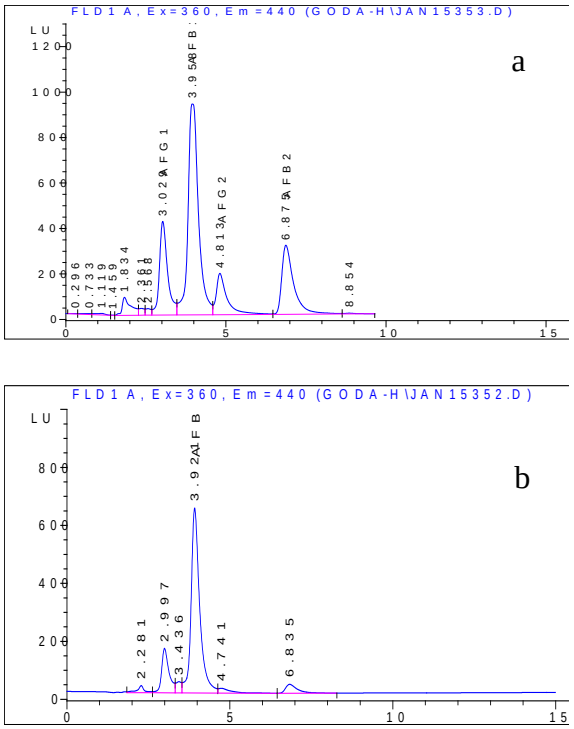


**Figure 5**. Typical high performance Liquid Chromatogram of aflatoxins (a) standards (AFG1,AFB1, AFG2 and AFB2); (b) Chromatogram of the chloroform extract of *Aspergillus flavus* strain.

### *3.4. Assessment of Immunological Parameters*

#### *3.4.1. Differential Leukocyte Counts*

Differential leukocyte counts in different experimental groups of broilers after three weeks of feeding were represented in table 1. The lymphocyte percentage was significantly decreased in group three (lymphocytopenia) compared with the control negative group, while the heterophils percentage was in higher value, and these percentages were restored in group four. Regarding the monocytes percentage, all groups were significantly increased compared with the control negative group. The eosinophils in all groups were within the normal range.

**Table 1**. Differential leukocyte counts in different experimental groups of broilers after three weeks of exposure to the experimental diet.

| Leukocytes % | | | | | |
|---|---|---|---|---|---|
| Groups | Heterophils | Eosinophils | Lymphocytes | Monocytes | H/L Ratio |
| 1 | 30.20±0.75c | 3.13±0.15a | 62.50±0.40a | 4.17±0.29c | 0.48±0.02c |
| 2 | 37.33±0.43b | 3.13±0.15a | 54.07±0.18b | 5.47±0.12ab | 0.69±0.01b |
| 3 | 45.67±0.35a | 2.80±0.06a | 45.90±0.25c | 5.63±0.20ab | 1.00±0.01a |
| 4 | 37.90±0.51b | 3.13±0.09a | 52.90±0.30b | 6.07±0.15a | 0.72±0.01b |

The relationship between heterophils and lymphocytes was represented as the H/L ratio as shown in figure 6. There was a significant increase in H/L ratio in the aflatoxin feed group compared with other experimental groups after two and three weeks of exposure to the experimental diet.

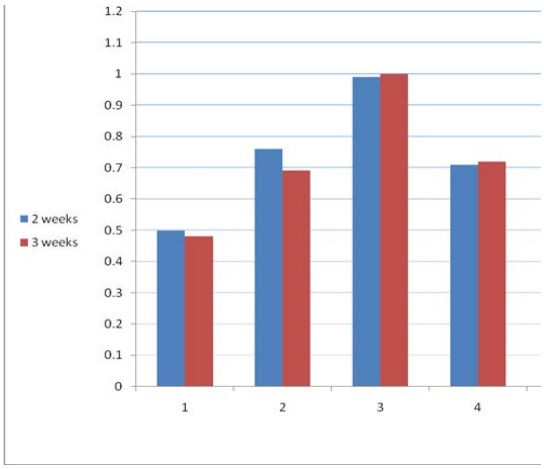


**Figure 6**. The relationship between heterophils and lymphocytes (H/L) ratio. Means within the same column carrying different superscripts are significantly differed at ($P \leq 0.05$).

#### *3.4.2. Assessment of Humoral Immune Response*

Haemagglutination inhibition test (HI) showed that there were significant differences of anti- NDV antibody titer among the treated groups after three weeks of exposure. The aflatoxin treated groups revealed a significant decrease compared with the negative control group. The supplementation of MOS to the aflatoxin-treated diet revealed a non-significant increase as shown in figure 7.

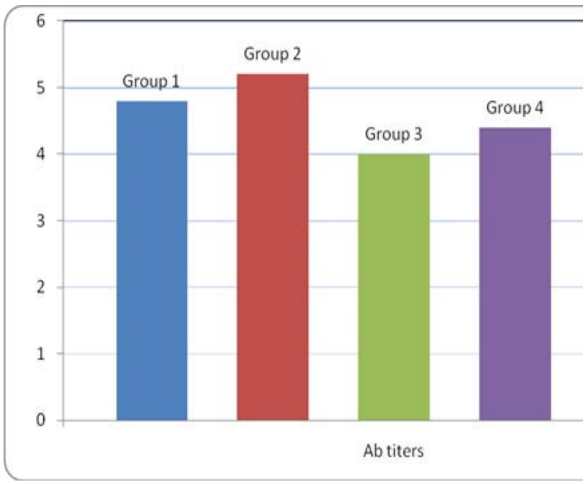


**Figure 7.** Antibody titers of the HI test against NDV vaccine. Means within the same column carrying different superscripts are significantly differed at ($P \leq 0.05$).

*3.4.3. Assessment of Cell-mediated Immune Response "Lymphocyte Transformation Test"*

Figure 8 represented the lymphocyte transformation after two weeks of feeding. The results showed that group three was decreased significantly compared with the two control groups. The control positive group was higher than the negative, while group four was similar to the control negative one.

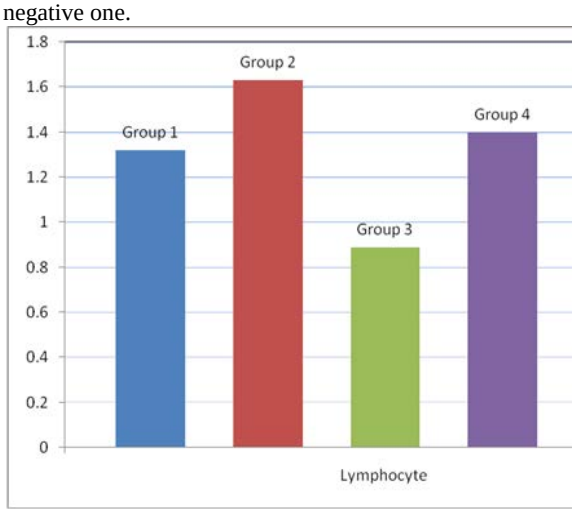


**Figure 8.** Lymphocyte transformation after two weeks of exposure to experimental diet. Means within the same column carrying different superscripts are significantly differed at ($P \leq 0.05$).

Figure 9 represented the Lymphocyte transformation after three weeks of feeding. The results still showed that group three was decreased significantly compared with the two control groups. The control positive group was higher than the negative one.

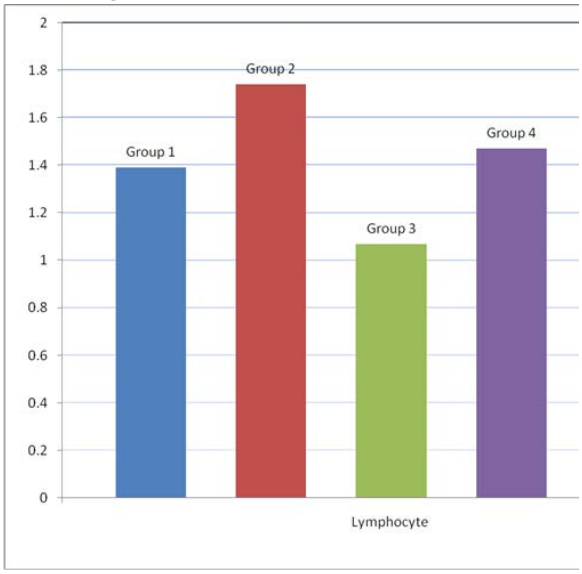


**Figure 9.** Lymphocyte transformation after three weeks of exposure to experimental diet. Means within the same column carrying different superscripts are significantly differed at ($P \leq 0.05$).

## 4. Discussion

Mycotoxins are one of the major factors affecting broiler's performance and immunity (Yunus *et al*., 2011). Therefore, the present study was carried out to investigate the effect of the aflatoxin (AFB1), and the efficacy of MOS on broiler's immune response parameters.

The results of differential leukocyte counts in table 1 demonstrated that the addition of AFB1 induced significant leukocytopenia, lymphocytopenia and heterophilia with a significant increase of monocytes. The increase of heterophil counts suggested that adding mycotoxins may elicit an inflammatory response in the chicks as was mentioned by (Keçeci *et al*., 1998). The decrease in the total leukocyte count, lymphocytes and the increase of heterophils can be attributed to the toxic effects of aflatoxins on circulating cells, sequestration of cells in tissues and / or the effects of aflatoxins on bone marrow and lymphoid tissues. The heterophil: lymphocyte ratio (H/L ratio) is a better indicator of stress in poultry representing changes in the number of circulating leukocytes, in particular, a pronounced heterophilia and lymphocytopenia (Maxwell, 1993). There was a significant increase in the H/L ratio represented in figure 6 in the aflatoxicated group compared with other experimental groups after three weeks of exposure to the experimental diet. The pervious findings are in accordance with the results obtained by (Andretta *et al*., 2012; Mohaghegh *et al*., 2017).

Mycotoxin-induced immunosuppression may be manifested as depressed T- or B-lymphocyte activity, suppressed antibody production and impaired macrophage/neutrophil-effector functions. As the immune system is primarily responsible for defense against invading organisms, so the suppressed immune function by aflatoxins may eventually decrease resistance to infectious diseases, reactivate chronic infections, and/or decrease vaccine and drug efficacy (Oswald *et al*., 2005).

Humoral immunity is mediated by antibodies released by B-cells into the bloodstream and effectively against extracellular antigens. Cell-mediated immunity (CMI) is based on specific antigen recognition by thymus derived T-lymphocytes, and specializes in the elimination of intracellular antigens. In birds, precursors of T-cell and B-cell originate in bone marrow, while the actual development of T-cells take place in the thymus and B-cells in the bursa of Fabricius (Surai and Dvorska, 2005). Concerning the assessment of humoral immune response through the estimation of antibody titers against NDV vaccine using HI test, the results in figure 7 revealed that there was a significant decrease in the titer. These results agreed with those obtained by (Otim *et al*., 2005) who reported that there was a significant reduction in the haemagglutination inhibition of ND antibody titer of 0.250 mg aflatoxin B1 per bird.

In this regard, the humoral immune response from broilers may decrease depending on the level and duration of the exposure to the toxins. These results were confirmed also by (Manafi *et al*., 2012) who reported that the biphasic nature of AFB1 on humoral immunity, humoral immune response might decline depending upon the dose and duration of exposure. Also, the results in figure 7 showed that supplementation of MOS could improve the immune response of broilers which indicates that MOS has immunomodulatory properties. These results agree with (Khalaji *et al*., 2011; Yalcin *et al*., 2013; and Mohaghegh *et al*., 2017).

Concerning the lymphocyte blastogenesis assay for the assessment of cellular immunity, the figures 8 and 9 revealed a significant reduction of the cell-mediated immune response in te aflatoxin treated group compared with the other control groups. These results exhibited the toxic and harmful effects of these mycotoxins on the cellular immunity. Similar results were obtained by (Girish and Smith, 2008, Jayaramu *et al*., 2012; and Anjorin and Cyriacus 2014).

This study revealed that MOS has the ability to detoxify aflatoxicosis in broilers through improving both humoral and cell-mediated immunity profile of broiler. The current results are in agreement with (Santin *et al*., 2003; Oguz and Parlat, 2004; and Pizzolitto *et al*., 2013) who confirmed the ability of MOS to reduce the deleterious effects of aflatoxin.

## 5. Conclusion

The present study concludes that the aflatoxin B1 caused toxic effects on humoral and cell-mediated immune responses of broilers with adverse lymphocytopenia and heterophilia. On the other hand, adding MOS to aflatoxin-treated ration led to the improvement of the immune health and the reduction of the toxic effects of AFB1on immune profile. Attention must be taken to avoid mycotoxicosis as it doesn't only cause harm to poultry, but also it has dangerous effects on human health because of its residual and cumulative nature.

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