



**Immunomodulatory Effect of Dietary Turmeric against Aflatoxins in Mice:  
Histological and Immunohistochemical Study**

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**ABSTRACT**

Mycotoxins contribute to serious wide range of adverse health concerns, especially on the immune system. The current research aimed to investigate the possible ameliorative effect of dietary turmeric on aflatoxicosis induced immune impairments in Albino mice. Twenty-eight female albino mice 25-27 g were divided into 4 groups. Group I, is the control group received a basal diet. Group II, received a basal diet with 5% turmeric. Group III, were given basal diet with added aflatoxins (5  $\mu\text{g}/\text{kg}$ ). Group IV, co-administered aflatoxins and turmeric at the same previous doses. All treatments continued for 1 month. Feed efficiency ratio (FER), weight gain, food intake, thymus weights as well as spleen relative weights were recorded. The serum levels of super oxide dismutase (SOD), Malondialdehyde (MDA) and Interleukin-6 (IL-6) were estimated. Histopathological examination and immunohistochemistry of TNF- $\alpha$  of thymus and spleen were performed. Aflatoxins significantly ( $P<0.05$ ) reduced weight gain, FER and spleen weight than control while food intake and thymus relative weight were non-significantly altered. Serum MDA and IL-6 were significantly ( $P<0.05$ ) increased in afla-intoxicated group than control. Meanwhile, SOD was significantly ( $P<0.0$ ) depleted than control. Aflatoxins deteriorated splenic architecture, as presented by depleted lymphoid follicles, lymphocytopenia as well as edema. The co-administration of turmeric with aflatoxins significantly ameliorated the aflatoxins induced pro-inflammation. Moreover, turmeric possesses antioxidant effect that ameliorated aflatoxins induced immune impairments.

## INTRODUCTION

The second most common toxic metabolites are mycotoxins. Mycotoxins are identified by more than one name like, "Silent killers", "natural toxicants" and "unavoidable contaminants" (Surai and Mezes, 2005). World Health Organization (WHO) (1999) expected that about 25% of the world's harvest manufacture be polluted with mycotoxins. The mycotoxins show at any stages of crop production. The contamination by mycotoxins depends on the humidity of the crop (Surai and Mezes, 2005). *Aspergillus*, *Fusarium* and *Penicillium* are the main precursors of mycotoxins in our food (Abdallah *et al.*, 2015). There are 300 types of mycotoxins or more, which have the ability to induce symptoms of toxicity in human and animal species (Fink-Grenmels, 1999). The most important mycotoxins that contaminate the food are aflatoxins, ochratoxins, zearalenone, deoxynivalenol, fumonisins, and T-2 toxin (Dvegowda *et al.*, 1998). The pathological lesion induced after eating mycotoxins will differ according to the dose of the toxin. It varies from primary biochemical disorder to loss of cellular functions or even toxic injury of the cells (Bryden, 2012). Digestive, urinary, nervous, reproductive and immune systems of the body are affected even by a low dose of mycotoxins but generalized cytotoxicity can be caused by a high dose of mycotoxins (Maresca and Fantini, 2010).

Mycotoxins are considered as one of the most important immunosuppressive toxin which increase the rate of susceptibility to diseases and reduce the productivity of animals (Corrier, 1991; Surai and Mezes, 2005). This immunosuppressive effect is due to depression of the activity of T or B lymphocyte and inhibition in the activity of natural killer cells (Berek *et al.*, 2001).

*Aspergillus flavus* and *Aspergillus parasiticus* produce aflatoxins (Klich *et al.*, 2009). There are several types of aflatoxins, the most effective one is aflatoxin B<sub>1</sub> which cause hepatic toxicity, hepatic carcinoma (Magnussen and Parsi, 2013), suppression in the immune system (Surai and Mezes, 2005), teratogenic and mutagenic effects (Smith *et al.*, 2017). Aflatoxins, at first, affect the cell-mediated immunity and the function of phagocytic cell, with reduction in the functions of lymphocyte and macrophages that aid in the functions of lymphocyte (Surai and Mezes, 2005). Moreover, Hinton *et al.* (2003) documented that aflatoxin B<sub>1</sub> causes toxicity of the lymphoid cells with disturbance in the non-specific defense mechanism, the function of macrophage and number of the cells in the bone marrow, spleen and thymus of rats.

Turmeric is one of the important food additives, the powder of its dehydrated roots used as spices in many countries. The yellowish pigments in its powder are curcuminoids, which have a protective effect against aflatoxicosis specially aflatoxin B<sub>1</sub> toxicity (Soni *et al.*, 1997). Also, it has an anti-inflammatory, hepatoprotective and anticarcinogenic effect (Pal *et al.*, 2001). Furthermore, administration of curcumin to diabetic rats regulate the gene expression of antioxidant enzymes activity (El-Bahr, 2013).

The aim of this study is to investigate the protective effect of turmeric on aflatoxicosis mediated immune-impairments.

## MATERIALS AND METHODS

### Tested Animals and Experiment Design:

Twenty-eight female albino mice weighing 25-27 g were obtained from the Organization for Biological Products and Vaccines, Helwan, Egypt. They were kept for 2 weeks for accommodation prior to the onset of the experiment. Mice were kept in a ventilated room under

room temperature  $24 \pm 2^\circ\text{C}$ , natural light/dark rhythm and humidity  $49\% \pm 1$ . Mice received *ad libitum* drinking water and diet. Rats were randomly distributed to four groups; seven mice each.

**Group I**, is the control group received basal diet.

**Group II**, is the control turmeric group and were given a basal diet with 5% turmeric that was purchased from the local market.

**Group III**, is aflatoxins treated group, they were given a basal diet with added aflatoxins in a rate of  $5\mu\text{g}/\text{kg}$ .

**Group IV**, is combination of turmeric and aflatoxins group and were given both aflatoxins and turmeric at the same doses of groups II and III, All treatments continued for 1 month.

#### **Aflatoxins:**

The Aflatoxins used in the present research was a product of Animal Health Research Institute, El-Mansura, Egypt. They were obtained by inoculation toxigenic fungus strain of *Aspergillus parasiticus* NRRL 2999 to parboiled rice to be fermented. The previously mentioned procedures were done according to (Shotwell et al., 1966). Fermented moldy rice was dried and ground into a fine powder. The level of Aflatoxins mounted in rice powder were estimated by HPLC at Mycotoxins Central Laboratory and Food Safety of the National Research Centre, El Dokki, Giza, Egypt according to the method of Nabney and Nesbitt (1965). Aflatoxins (B1 and B2) standards were purchased from (Biopure Referenzsubstanzen GmbH Co., Austria). The level of Aflatoxins B1 in rice was  $6.8\text{ mg}/\text{Kg}$ , B2 was 0.09, G1 was  $0.15\text{ mg}/\text{kg}$  and G2 was  $0.16\text{ mg}/\text{kg}$  with total aflatoxins contents  $7.2\text{ mg}/\text{kg}$ .

#### **Food Intake, Weight Gain and Feed Efficiency Ratio:**

Experimental mice were weighed at day 1 of the experiment then weighed at the end of the experimental period. The weight gain was obtained by

subtracting final weight from the initial one. Food intake was recorded all over the experimental time. Feed efficiency ratio (FER) was calculated as follow:

$\text{FER} = \text{body weight gain (g) after 4 weeks} / \text{food intake (g) for 4 weeks}$

#### **Blood and Tissue Sampling:**

At the end of the experiment, the mice at diestrus phase of the estrous cycle were anesthetized. Blood samples were collected from retro-orbital venous plexus of the eye into plain tubes. The later tubes left for clotting kept in the refrigerator then sera were separated, collected and stored at  $-30^\circ\text{C}$ . Thereafter, animals were euthanized by cervical dislocation. Spleen and thymus were excised then weighed.

#### **The Relative Lymphoid Organs Weights:**

Thymus and spleen weights were calculated as follow: absolute thymus or spleen weight at the end of the experiment / final body weight X 100.

#### **Superoxide Dismutase (SOD) and Malondialdehyde (MDA):**

The levels of MDA and SOD were determined in sera according to Mihara and Uchiyama (1978) and Nishikimi *et al.* (1972). Both were assayed using commercial kits of OxisResearch Co., USA.

#### **Interleukin-6 Assay:**

Interleukin -6 (IL-6) was assayed using ELISA commercial kits (IBL Co., Japan). The procedures were followed according to the manufacturer's protocol.

#### **Histopathological Examination and Immunohistochemistry (IHC):**

Thymus and spleen were immersed in 10% Formalin buffer, paraffinized in blocks and were cut into  $5\text{-}\mu\text{m}$ -thickness sections. These sections were stained with hematoxylin and eosin (H & E) according to Drury and Wallington (1980) and examined under microscope. The IHC was performed on  $5\text{-}\mu\text{m}$  sections on positively charged slides using a primary antibody for TNF- $\alpha$  (Thermo Co., USA). Sections were

dewaxed and subjected for antigen retrieval in autoclave at 120°C for 10 minutes using 10 Mm citrate buffer (PH 6). Then the slides were washed and subjected for blocking of endogenous peroxidase via the addition of 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for fifteen minutes. Slides were rewashed again with PBS and blocking buffer was added. The slides were incubated with the blocking buffer for 30 minutes at room temperature. Primary antibody was put at concentration 5 µg/mL and incubated for 1 hour. Biotinylated polyvalent secondary antibody (Thermo Scientific Co., UK) was put on tissue sections and -incubated for one hour.

Immunoreactive parts were examined under light microscope and subjected to image analysis to calculate IHC-stained area % which is expressed as integrated density % (Integ Den %). This analysis was made by taking photos for nine random fields / slide for each experimental mouse then using ImageJ software according to the method of Helmy *et al.* (2014).

#### Statistical Analyses:

Statistical analyses were performed by One-way analysis of variance (ANOVA) followed by Duncan's multiple comparison tests (SPSS v. 16.0, SPSS Inc., IL, USA). All values were represented as mean ± standard errors. A

probability level of P<0.05 denoted significance.

## RESULTS

Table 1, demonstrated the effect of turmeric on food intake, weight gain and FER. The administration of aflatoxins showed non-significant alteration in food intake than control. The control turmeric group showed significant (P<0.05) reduction in food intake than aflatoxins group. The weight gain exhibited significant (P<0.05) reduction in turmeric control and aflatoxins groups than control however, combination of turmeric and aflatoxins exhibited non-significant variation than control and other groups. The FER exhibited significant (P<0.05) reduction in aflatoxins group than control. The administration of turmeric to aflatoxins group demonstrated significant (P<0.05) improvement in FER than aflatoxins group. Both control turmeric and turmeric +aflatoxins groups exhibited significant (P<0.05) reduction in FER than control. However, the two groups were non-significantly differed from each other. The relative spleen weight showed a significant reduction in aflatoxins group than the control. Co-administration of turmeric with aflatoxins significantly ameliorated (P<0.05) the reduction in splenic weight than aflatoxins group. Thymus relative weight demonstrated non-significant alterations among all tested groups (Table 1).

**Table (1):** Effect of turmeric on food intake, body weight gain, FER, relative spleen weight and relative thymus weight in aflatoxins-intoxicated mice.

	Control	Turmeric Control	Aflatoxins	Turmeric +aflatoxins
Food intake (g/ 1 month)	143.70±3.71 <sup>ab</sup>	121.70±2.19 <sup>a</sup>	155.30±8.67 <sup>b</sup>	140.00±2.31 <sup>ab</sup>
Weight gain (g/1 month)	31.67±1.66 <sup>a</sup>	22.67±1.20 <sup>b</sup>	20.33±1.45 <sup>b</sup>	25.67±1.76 <sup>ab</sup>
FER	0.22±0.02 <sup>a</sup>	0.19±0.00 <sup>b</sup>	0.13±0.01 <sup>c</sup>	0.19±0.01 <sup>b</sup>
Spleen relative weight (%)	0.58±0.04 <sup>a</sup>	0.61±0.04 <sup>a</sup>	0.34±0.06 <sup>b</sup>	0.49±0.02 <sup>c</sup>
Thymus relative weight (%)	0.13±0.03 <sup>a</sup>	0.14±0.02 <sup>a</sup>	0.07±0.01 <sup>a</sup>	0.10±0.01 <sup>a</sup>

Different superscripts between columns are considered significant at P<0.05.

**Superoxide Dismutase (SOD) and Malondialdehyde (MDA):**

The activity of SOD was significantly (P<0.05) depleted in aflatoxins group than control and other groups. The co-administration of turmeric with aflatoxins significantly improved the antioxidant level of SOD than that of aflatoxins group. On the

other hand, the level of MDA as an indicator for lipid peroxidation was significantly (P<0.05) increased in aflatoxins group than control and other groups. Adding turmeric to aflatoxins group significantly (P<0.05) reduced the MDA level than that of aflatoxins group.

**Table (2):** Effect of turmeric on SOD, MDA, IL-6, spleen TNF-  $\alpha$  Integ Den %, and thymus TNF-  $\alpha$ , Integ Den % in aflatoxins mice.

	Control	Turmeric Control	Aflatoxins	Turmeric +aflatoxins
<b>SOD (U/mL)</b>	6.54± 0.05 <sup>a</sup>	6.69± 0.05 <sup>a</sup>	4.13±0.04 <sup>b</sup>	5.67 ±0.08 <sup>c</sup>
<b>MDA (nmol/ mL)</b>	0.57±0.01 <sup>a</sup>	0.54±0.01 <sup>a</sup>	1.14±0.03 <sup>b</sup>	0.78±0.01 <sup>c</sup>
<b>IL-6 (pg/mL)</b>	9.89±0.04 <sup>a</sup>	9.37±0.04 <sup>a</sup>	33.16±0.86 <sup>b</sup>	23.35±0.37 <sup>c</sup>
<b>TNF-<math>\alpha</math> spleen (Integ Den %)</b>	18.20±1.16 <sup>a</sup>	11.50±0.64 <sup>a</sup>	56.25±4.27 <sup>b</sup>	29.25±1.65 <sup>c</sup>
<b>TNF- <math>\alpha</math> thymus (Integ Den %)</b>	25.33±2.5 <sup>a</sup>	23.33±1.95 <sup>a</sup>	45.33±4.23 <sup>b</sup>	30.67±3.58 <sup>c</sup>

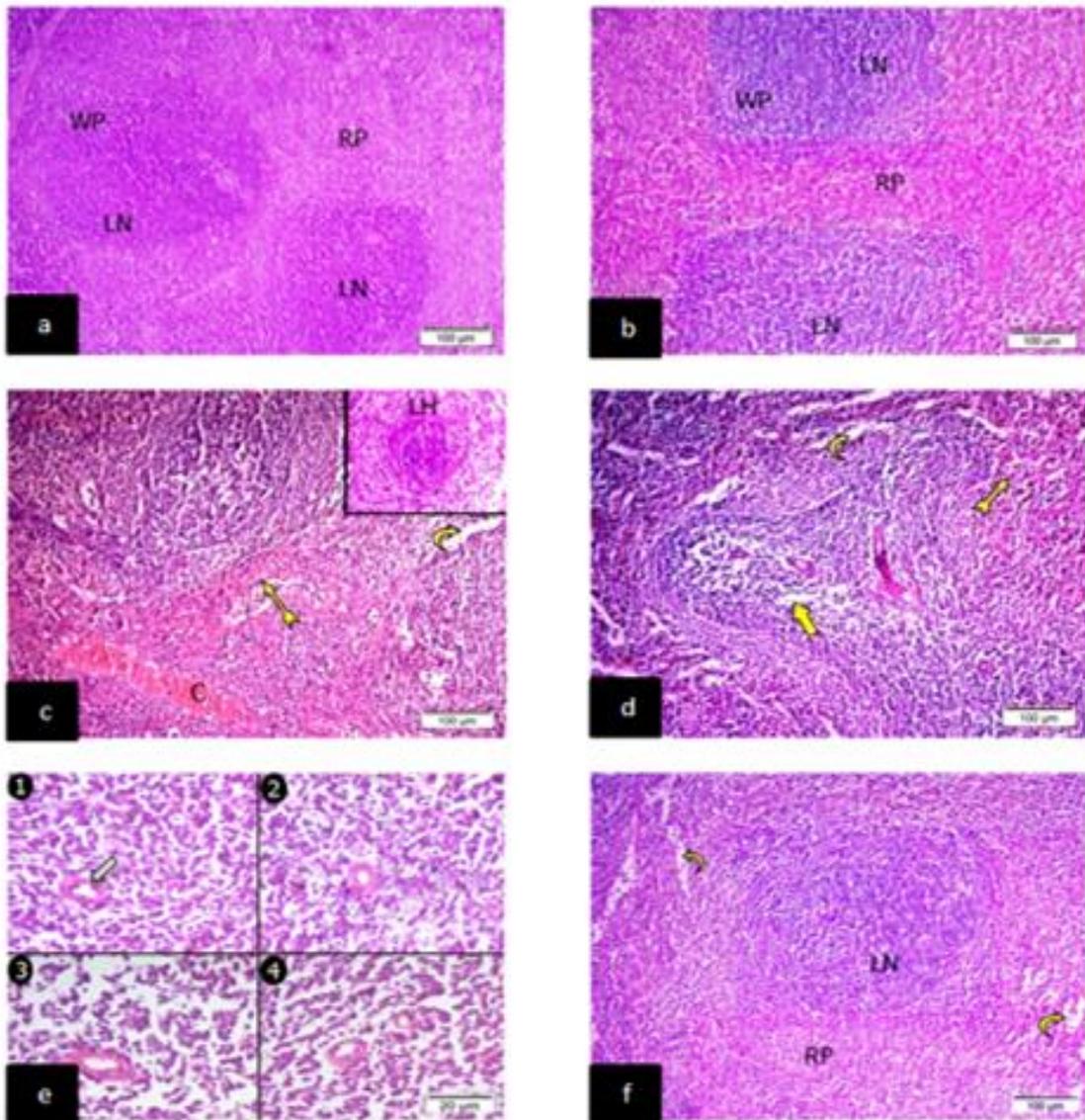
Different superscripts between columns are considered significant at P<0.05.

**Histopathological Examination and IHC:**

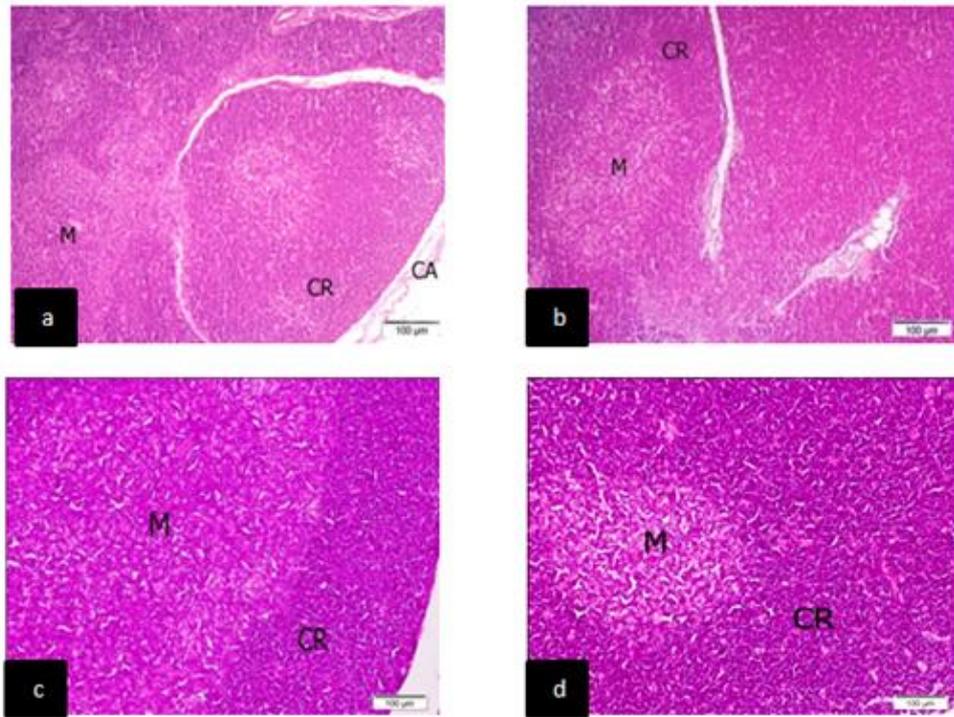
The histological structure of the spleen in control and turmeric groups showed the normal structure of the spleen displayed as white pulp contained (lymphatic nodules and periarterial sheaths), the red pulp (splenic cord and splenic sinus) and marginal zone (Fig. 1a,b). However, aflatoxins group showed loss of the normal architecture of the spleen, hypoplasia in some lymphatic nodules and depletion, represented by lymphocytopenia, in others. The red pulp showed mild congestion and edema with enlarged and congested blood vessels (fig. 1c,d&e). On the other hand, aflatoxins exposed turmeric treated group showed amelioration in the aflatoxins-induced pathological lesions apart from edema (Fig. 1f).

Non-significant histopathological changes were observed in the thymus. The changes represented by mild depletion of the lymphocyte in the cortical region of aflatoxins group as compared by others (Fig.2)

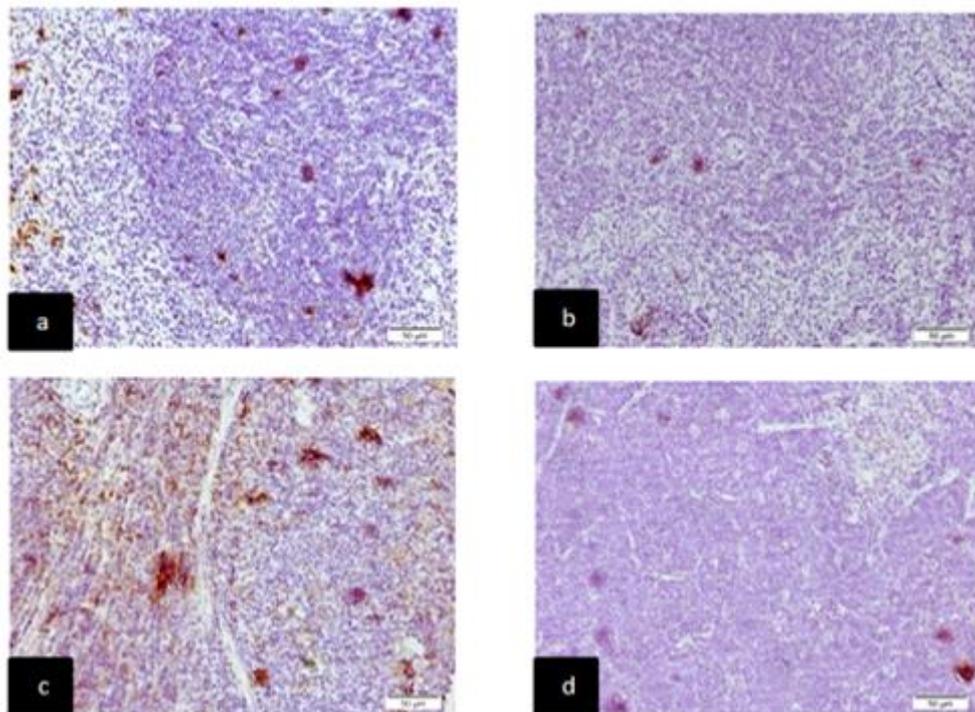
The Integ Den % of TNF- $\alpha$  in aflatoxins treated spleens was significantly (P<0.05) higher than control and other groups. However, treatment of aflatoxins mice with turmeric significantly (P<0.05) decreased Integ Den % of TNF-  $\alpha$  than that of aflatoxins group (Table 2, Fig 3). On the same line, The Integ Den % of TNF- $\alpha$  in aflatoxins treated thymus was significantly (P<0.05) higher than control and other groups. Meanwhile, treatment of aflatoxins mice with turmeric significantly (P<0.05) decreased thymus Integ Den % of TNF- $\alpha$  than aflatoxins group (Table 2, Fig 4).



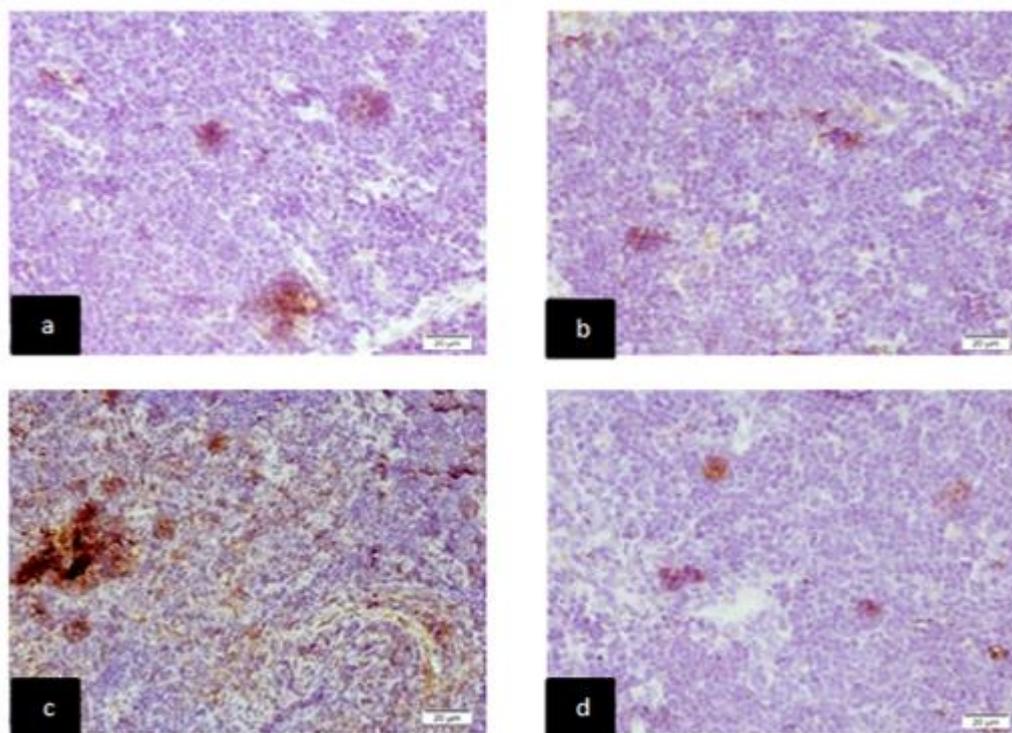
**Fig. (1):** Photomicrographs of mice spleen stained with H&E. Control (a) and turmeric (b) groups showed normal structure of the spleen displayed as white pulp (WP), lymphatic nodules (LN) and red pulp (RP). Aflatoxins group (c&d) showed loss of the normal architecture of the spleen, depletion (thick arrow), hypoplasia (LH) of lymphatic nodules, edema (curved arrow), enlarged and congested blood vessels (C) and mild congestion of the red pulp (tailed arrow). (e): showed lymphocytopenia around the nodular arteries (thick arrow) in the lymphatic nodules in aflatoxins (e3) group as compared with control (e1) turmeric (e2) and turmeric aflatoxins co-treated (e4) groups. Turmeric aflatoxins co-treated group (f): showed lymphatic nodules (LN) and red pulp (RP) with little edema (curved arrow).



**Fig (2):** Photomicrographs of mice thymus stained with H&E. Control (a), turmeric (b), Aflatoxins (c) and turmeric aflatoxins co-treated (d) groups showed: connective tissue capsule (CA), cortex (CR) and medulla (M).



**Fig (3):** Photomicrographs of mice spleen representing the intensity of TNF- $\alpha$  in control (a), turmeric (b), aflatoxins (c) and turmeric aflatoxins co-treated (d) groups with scale bar=50  $\mu$ m. The aflatoxins group showed hyper immunostaining of TNF- $\alpha$  than control and turmeric groups. The co-administration of turmeric ameliorated the hyper immunostaining of splenic TNF-  $\alpha$  than aflatoxins group.



**Fig (4):** Photomicrographs of mice thymus represented the intensity of TNF- $\alpha$  in control (a), turmeric (b), aflatoxins (c) and turmeric aflatoxins co-treated (d) groups, with scale bar=20  $\mu$  m. The aflatoxins group showed hyper immunostaining of TNF- $\alpha$  than control and turmeric groups. The co-administration of turmeric ameliorated the hyper immunostaining of splenic TNF- $\alpha$  than aflatoxins group.

### DISCUSSION

There are growing interests about the effect of Aflatoxins, that are widely spread, on both human and animal health (Wild *et al.*, 2015). The use of a dietary supplement to counteract the adverse effects of such toxins is mandatory. Therefore the usage of experimental animals for detection of adverse health consequences of such toxins as well as possible protecting effects of some substances is an applicable solution to control such problem (Udomkun *et al.*, 2017). The current study investigated the effect of dietary turmeric 5% to counteract Aflatoxins immune impairments in albino female mice model. Our results showed that control turmeric group was significantly reduced in food intake than aflatoxins group. The weight gain exhibited

significant ( $P < 0.05$ ) reduction in control turmeric and aflatoxins groups than the control. These results were in the same line of Han *et al.* (2016) who demonstrated the reduction of weight gain as well as food intake in turmeric fed group. Kocabas *et al.* (2003) found that aflatoxins significantly reduced weight gain. The FER exhibited a significant decline in aflatoxins group than control. This reduction could be attributed to the decreased of weight gain in spite of non-affected food intake. The administration of turmeric to aflatoxins group demonstrated significant improvement in FER than aflatoxins group. This data suggested the ameliorative effect of turmeric on aflatoxins induced weight loss and reduced FER.

Aflatoxins significantly depleted the antioxidant SOD while increased

lipid peroxidation (MDA) than control our data was in harmony with Farag *et al.* (2018). The aflatoxins are able to produce oxidative stress that depletes SOD and leads to the promotion of membrane lipid peroxidation these changes could promote the production of IL-6 that noted in the present study. The IL-6 is involved in several immune responses and inflammatory reactions cascades (Debruyne and Delanghe, 2008). The dysregulated IL-6 in afla-intoxicated group is indicative of tissue damage and inflammatory reactions by aflatoxins inside the body as well as predictive for auto immunity (Tanaka *et al.*, 2014). Current results of IL-6 were in accordance with those of Hinton *et al.* (2003). On the parallel line, aflatoxins significantly increased TNF- $\alpha$  immunoreactivity than control. Our results concord with Qian *et al.* (2014). The elevation of IL-6 and TNF- $\alpha$  may be attributed to the increased oxidative stress that depleted SOD and increased MDA. The oxidative stress or reactive oxygen species production are potent stimulant for nuclear factor kappa that further promote level TNF- $\alpha$  that provoke IL-6 and pro-inflammatory response (Lawrence, 2009). The co-administration of turmeric with aflatoxins significantly ameliorated the elevated lipid peroxidation and SOD levels that reduced IL-6 production and nuclear factor kappa induction of TNF- $\alpha$ . These effects ensured the anti-inflammatory effect of turmeric (Lawrence, 2009).

Histopathological changes in spleen demonstrated hypoplasia in some lymphatic nodules and depletion represented by lymphocytopenia. These changes may be attributed to the oxidative stress and lipid peroxidation induced by aflatoxins, that promoted DNA damage to splenic cellular components (Abdel-Wahhab and Aly, 2003). This splenic depletion led to

decrease in its relative weight as shown in the present study. The thymus histopathological changes were non-significant that harmonize with the non-significant alteration in its weight. This may be due to the older age of the experimental mice that regressed thymus and made it less sensitive to aflatoxins induced changes (Aspinall and Andrew, 2000; Gui *et al.*, 2012). However, the expression of thymus TNF- $\alpha$  protein was significantly altered than control. Administration of turmeric ameliorated the histopathological alterations in the spleen due to its reducing effect of oxidative stress and lipid peroxidation that protected cells from DNA damage.

In conclusion, the Aflatoxins have a serious effect on the immune system in albino female mice as it promoted pro-inflammatory cytokines; IL-6 and TNF- $\alpha$  through oxidative stress mediated mechanism. Moreover, Aflatoxins later immune alterations were reflected adversely on FER and body weight gain. The co-administration of turmeric is beneficial as it alleviated the oxidative stress induced pro-inflammatory changes.

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## ARABIC SUMMARY

## التأثير المناعي للكرم الغذائي ضد السمية بالأفلاتوكسين في الفئران: دراسة نسيجية و نسيجومناعية

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تشكل السموم الفطرية العديد من المخاوف الصحية الضارة وخاصة على الجهاز المناعي. تهدف الدراسة الحالية إلى اختبار التأثير المحسّن المحتمل للكرم الغذائي على الإصابة بالأمراض المناعية التي تحدثها السموم الفطرية (أفلاتوكسين) في الفئران البيضاء. تم تقسيم ثمانية وعشرون أنثى من الفئران البيضاء يتراوح وزنها 25-27 غرام إلى 4 مجموعات. المجموعة الأولى ، هي المجموعة الضابطة و تلقت النظام الغذائي الاساسى. المجموعة الثانية ، تلقت النظام الغذائي الاساسى مع الكركم 5 ٪. المجموعة الثالثة ، تلقت النظام الغذائي الاساسى مضافا اليه (5 ميكروغرام / كيلوغرام) من الأفلاتوكسين. المجموعة الرابعة ، تلقت الأفلاتوكسين مع الكركم بنفس الجرعات السابقة الجرعات السابقة. استمرت جميع العلاجات لمدة شهر واحد. تم تسجيل نسبة كفاءة التغذية (FER) ، معدل زيادة الوزن ، معدل تناول الطعام ، أوزان الغدة التيموسية و الطحال.النسبية. تم تقدير مستويات فوق اكسيد الديسميوتاز و و ثنائى الدهيد المألون و الانترلوكين- 6 فى مصل الدم.تم إجراء الفحص النسيجي مرضى و النسيجو مناعى لمعامل نخر الورم الفا على الغدة التيموسية و الطحال. يقلل الأفلاتوكسين بشكل معنوى معدل زيادة الوزن و الكفاءة الغذائية و وزن الطحال النسبى عن المجموعة الضابطة بينما تم تغيير معدل استهلاك الغذاء و الوزن النسبى للغدة التيموسية. ازداد كل من ثنائى الدهيد المألون و الانترلوكين- 6 فى مجموعة الافلاتوكسين عن المجموعة الضابطة فى الوقت نفسه استنفذ فوق اكسيد الديسميوتاز معنوياً عن المجموعة الضابطة. تسببت الأفلاتوكسين فى تدهور ملحوظ لبنية الطحال لأنها استنفدت البصيلات الليمفاوية و ادت الى قلة الكريات اللمفاوية وكذلك وجود ورم بالنسيج. استخدام الكركم مع الأفلاتوكسين حسن بشكل ملحوظ آثار الالتهاب الناجمة عن الأفلاتوكسين. يمتلك الكركم تأثيراً مضاداً للأكسدة يحسن من الخلل المناعى الناجم عن الأفلاتوكسين.