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Evaluation of Effects of Spirulina Extracts on Immunologic Dysfunction and Inflammation Associated with Aflatoxin B1 Induced Toxicity in Mice

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Abstract

The contamination of foods by various mycotoxins has been reported as a major public health concern across the world. The most predominant type of fungal toxins are aflatoxins which are synthesized by certain fungi that contaminate agricultural crops or produce. The main aflatoxin producing fungi are Aspergillus flavus and Aspergillus parasiticus which contaminate food crops in the farm and after harvesting. Out of all the types of aflatoxins, the most potent type is aflatoxin B1. The mechanism of toxicity and health effects of aflatoxins have been studied widely and it has been shown that aflatoxin B1 leads to liver necrosis, inflammation and liver cancer. The use of natural products as a remedy to health consequences of aflatoxins in humans and animals is gaining popularity. Owing to its anti-inflammatory effects, Spirulina plantesis has been studied for its immunomodulatory effects. This study evaluated the effects of spirulina extract on aflatoxin B1 (AFB1) induced immune dysfunction and inflammation. Male BALB/c mice weighing 28-34g were randomly placed into 6 groups and orally treated as follows: Group 1 was not treated but received food and water for entire experimental period. Group 2 received 200 µg/kg b.w of aflatoxin B1 orally. Group 3 received 1g/kg b.w of activated charcoal and an hour later 200 µg/kg b.w of aflatoxin B1 orally. Group 4, 5 and 6 received 50mg/kg, 100mg/kg and 150mg/kg b.w of Spirulina plantesis respectively then an hour later each group received 200 µg/kg b.w of aflatoxin B1 orally. Treatments were done on a daily basis for 14 days. At the last day of the experiment, all the mice were denied food and water for 12 hours, thereafter sacrificed and samples processed for immunological studies. The results indicated that body weight significantly increased when treated with 100mg/kg spirulina+AFB1 and 150mg/kg spirulina+AFB1 groups in compared to AFB1 treated group ($p < 0.05$). AFB1 was shown to increase serum level of IFN- γ and IL 2 and decrease levels of IL 4. Treatment with spirulina extract had no significant effect on the serum concentrations of IL-4 and IL-2 ($p > 0.05$) in comparison with aflatoxin B1 treated group, the serum levels of IFN- γ and IL-2 reduced significantly ($p<0.05$). Treatment with spirulina extract at different doses had no significant effect on serum levels of immunoglobulins A, G and M ($p < 0.05$). The mRNA expression of IL-4 was downregulated while that of TNFα, and IFNγ were upregulated. The results showed that increasing mRNA expressions of TNFα, and IFNγ as a result of AFB1was prevented ($p\leq0.01$) by administration of spirulina extract. These findings suggests that spirulina extract could be used as a remedy to AFB1 induced immune dysfunction and inflammation.

Keywords: *Spirulina plantesis*, Aflatoxin B1, Immune dysfunction, Inflammation, Liver necrosis.

INTRODUCTION

Aflatoxins are group of biological toxins synthesized by fungi that infect agricultural crops or contaminate produce. The main strains that produce aflatoxins are *Aspergillus flavus* and *Aspergillus parasiticus* [1, 2]. The proliferation of these strains are enhanced under warm and humid conditions [3]. Studies have documented more than 20 variant types of aflatoxins and the predominant variants contaminating food are AFB1, AFB2, AFG1 and AFG2. The hydroxylation of aflatoxin AFB1 and AFB2 yields AFM1 and AFM2. Out of all these types, the most potent type is aflatoxin B1 [1]. Aflatoxins pose a significant impact to human health [4]. Severe contamination of food crops and their consumption results in sickness or even death when consumed in high doses. The most common sources of aflatoxin include food crop and animal products. Some of the susceptible food crops include wheat, corn and nuts. Contamination of animal feeds with aflatoxins is a serious public health issue that not only affects livestock production but also human health [5]. Animals that have consumed aflatoxin contaminated feeds end up contaminating the produce, for example meat, milk and eggs [6].

The mechanism of toxicity and health effects of aflatoxins have been studied widely. According to a study by [7], aflatoxins mainly target liver. Their findings showed that aflatoxin B1 treatment results in liver necrosis, inflammation and increased concentrations of Alanine Aminotransferase and Aspartate Aminotransferase [7, 8]. It is well established that aflatoxins have a great potential to cause cancer. Aflatoxin metabolism occur in the liver and this result in generation of reactive metabolites which cause liver cancer [9, 10, 11]. Aflatoxins have also been demonstrated to cause immunotoxic effects. The breakdown of AFB1 in the liver produces Aflatoxin B1 – exo – 8, 9 epoxide (AFBO) which is immunotoxic. AFBO compound interact with immune cells in the body thus affecting their role in mediating immune responses [12, 13, 14].

The use of natural products as a remedy to aflatoxins complication in humans and animals is gaining popularity. Owing to its anti-inflammatory effects, *Spirulina plantesis* has been studied for protective effects [15]. *Spirulina plantesis* is a blue – green alga that grow in basic lakes. It is multicellular and filamentous cyanobacteria that is commercially grown for nutritional benefits. *Spirulina plantesis* has been shown to provide remedy to inflammatory diseases, for example, arthritis, colitis and allergic reactions, though how it achieves this function has not been understood [16, 17, 18]. Based on similar findings, this study aims at evaluating the effects of spirulina extract against aflatoxin B1 (AFB1) induced immune dysfunction and inflammation.

MATERIALS AND METHODS

Animal model

The study was approved by KEMRI Animal Care and Use Committee (ACUC) (Approval number KEMRI/ACUC/02.06.19). Male BALB/c mice weighing 28-34g and 8 weeks old were sourced from KEMRI's Animal Unit. The mice were raised in clean rooms with restricted access and controlled environmental factors (temperature of 23 – 25ºC and humidity of 50 to 60%). Commercially produced chow diet for mice and water were provided as often as necessary or required, and all potential stress factors kept minimum. The handling of mice was carried out following good laboratory animal care standards provided by Scientific Ethics Review Unit (SERU) – KEMRI.

Chemicals and Reagents

Aflatoxin B1 was sourced from FERMENTEK Ltd., Jerusalem, Israel (purity by HPLC and TLC ≥98%). *Spirulina plantesis* was sourced from Masinde Muliro University of Science and Technology where it is commercially produced, dried and packaged in plastic containers. ELISA kits for quantification of serum levels of cytokines (IL-2, TNF, IF- γ) and immunoglobulins (IgM, IgA, IgG) were sourced from Solarbio Science and Technology Co., Ltd., China. RNA extraction kit for tissue samples was purchased from QIAGEN Co., Germany. FIREScript RT cDNA Synthesis Kit and HOT FIREPol® EvaGreen® qPCR Supermix were purchased from Solis BioDyne Company, Estonia.

Study Design

Thirty male BALB/c mice weighing 28-34g were randomly selected and put into cages. Before the study began, mice were allowed to acclimatize with the environment for one week while monitoring their health status. Mice were given food and water *ad libitum*. The mice were placed into six groups, 5 mice in every group and treated on a daily basis for 14 days: Group 1 was not treated but received food and water for entire experimental period. Group 2 received 200µg/kg body weight of aflatoxin B1 orally. AFB1 200µg/kg b.w has been reported to be effective in eliciting toxic effects [19, 20]. Group 3 received 1g/kg b.w of activated charcoal and an hour later 200µg/kg body weight of aflatoxin B1 orally. Activated charcoal has been reported to be potent in ameliorating the effects of AFB1 [21], and thus was included for comparison with *Spirulina plantesis* treatment. Group 4 mice were administered 50mg/kg b.w of *Spirulina plantesis* and an hour later 200 µg/kg b.w of aflatoxin B1 orally. Group 5 and 6 received 100 and 150 mg/kg b.w of *Spirulina plantesis* respectively then an hour later each group received 200 µg/kg b.w of aflatoxin B1 orally.

During the experiment period, the weight of the mice were recorded every 4 days. During the last day of the experiment, all the mice were denied food and water for 12 hours, thereafter sacrificed. The abdominal cavity of mice was opened and cardiac puncture was performed. The blood was collected using a sterile needle and syringe and transferred into blood collection tubes without additives. Serum was prepared and stored at -20ºC for subsequent quantification of IL-2, TNF, IF- γ, IgM, IgA and IgG. The spleen was isolated, washed and stored at -80ºC for gene expression analysis later.

Measurement of serum levels of cytokines and antibodies

The levels of cytokines (IL-2, TNF, IF- γ) and immunoglobulins (IgM, IgA and IgG) in serum were measured using ELISA technique as outlined by the manufacturer. The procedure for quantification of serum levels of cytokines and immunoglobulins was provided by the manufacturer (Solarbio Science and Technology Co., Ltd., China).

Gene Expression Analysis

Thirty milligrams of spleen tissue was used during the extraction of total RNA. Manufacturer's protocol was followed in the process. After extraction, quantification of RNA extracted and its purity was assessed. The purity of RNA was evaluated using ratio of absorbance at 260nm/280nm. The ratio for all the samples were between 1.9 – 2.0, implying that the RNA isolated was pure. cDNA was synthesized using total RNA isolated. The mRNA expression in the spleen for mice IL-2, TNF, and IF- γ were determined using qRT-PCR. Hprt was used as a normalizer gene to standardize gene expression for cytokines of interest [22, 45]. The primers for the genes of interest were obtained from PubMed database (Table 1). qRT-PCR was done using HOT FIREPol® EvaGreen® qPCR Supermix and QuantStudio™ 5 RT PCR machine. The data obtained was analysed using the $2^{-\Delta\Delta}$ Ct method.

Table 1: Primer sequence for RT-PCR [22]

Gene	Primer Sequence
Hprt	Fwd: 5′-CTGGTGAAAAGGACCTCTCG-3′ Rev: 5′-TGAAGTACTCATTATAGTCAAGGGCA-3′
IL-4	Fwd: 5′-AGATCATCGGCATTTTGAACG-3′ Rev: 5′-TTTGGCACATCCATCTCCG-3′
TNF	Fwd: 5′-CTCCAGGCGGTGCCTATGT-3′ Rev: 5′-GAAGAGCGTGGTGGCCC-3′
IFN-γ	Fwd: 5′-GGATGCATTCATGAGTATTGC-3′ Rev: 5′-CCTTTTCCGCTTCCTGAGG-3′

Data Analysis

The level of expression of cytokines mRNA was determined using $2^{-\Delta\Delta}$ Ct method after performing qPCR. The concentrations of unknown samples after performing ELISA was calculated based on the standard using Microsoft Excel. Statistical calculations were done using GraphPad Software Version 8.0.2. The variation in the results were expressed as the $\bar{x}$ ± standard error of the mean. One-way ANOVA followed by Tukey's multiple comparison test was performed to determine the level of significance of difference between means. $p < 0.05$ was considered statistically significant.

RESULTS

Effect on body weight

At the end of the study, it was observed that the body weight of mice given AFB1 was significantly low compared to group not treated with AFB1 ($p < 0.05$). However, compared to AFB1 treated group and low dose (50mg/kg+AFB1) treated group, the body weight did not increase significantly ($p > 0.05$). The findings also show that there was a significant increase in body weight in group administered 100mg/kg and 150mg/kg of spirulina in comparison to the group treated with AFB1

alone ($p < 0.05$). In AFB1+Activated Charcoal group, the body weight significantly increased compared to the group given aflatoxin B1 ($p < 0.05$).

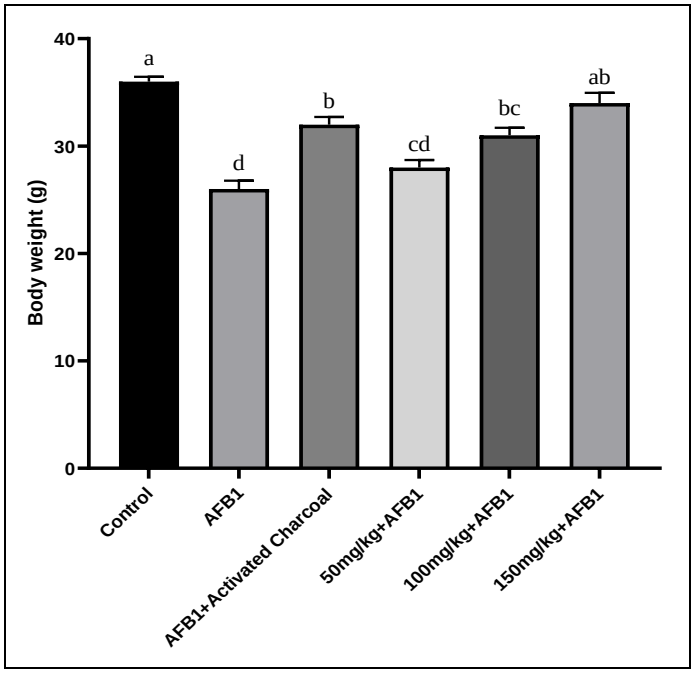
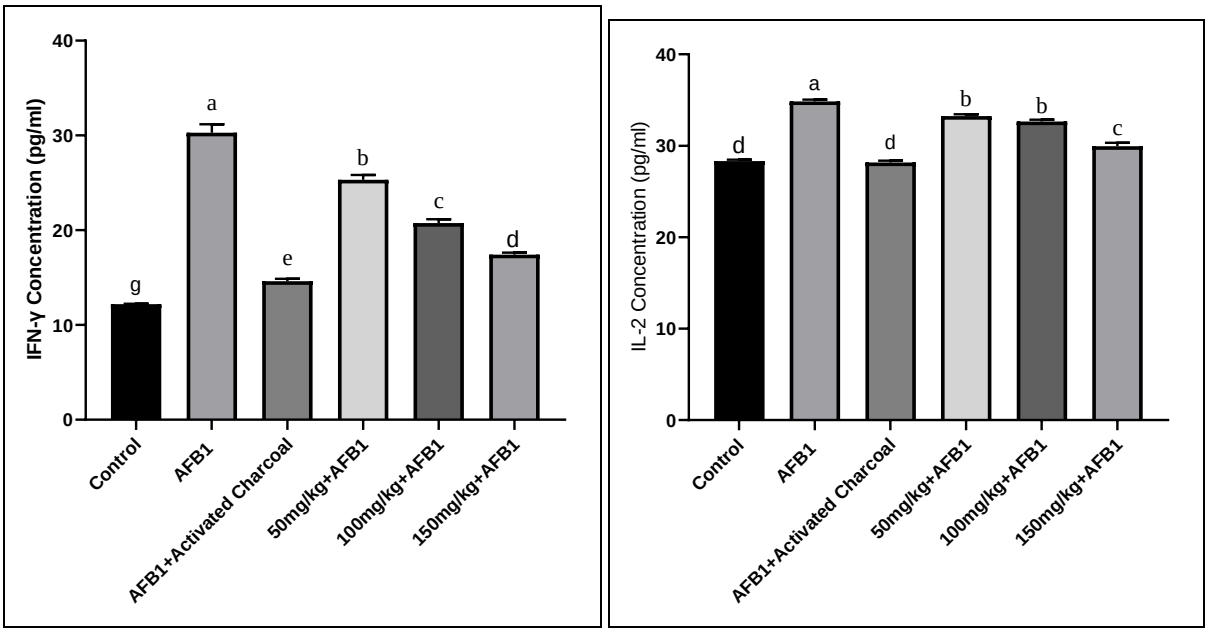


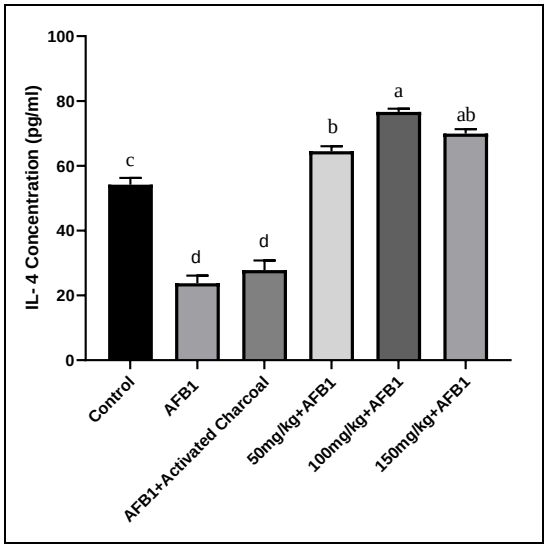
Figure 1: Graph showing the weights of the mice from the different treatment groups on the last day of the experiment. The data is presented as the $\bar{x}$ ±SEM, n=5.

Effects on serum levels of cytokines

The impact of spirulina on AFB1 treated mice on IFNγ IL 20, and IL 4 is shown in Fig. 2. In comparison with the untreated group, there is significant upregulation of IFNγ and IL 2 in group treated with aflatoxin B1 ($p < 0.05$). However, a significant reduction in the serum concentration of IL 4 in group administered AFB1 compared to untreated group was noted ($p < 0.05$). The result also indicates that doses of spirulina extract at 100mg/kg body weight and 150mg/kg body weight have no significant impact on the concentrations of IL 4 ($p > 0.05$). Similarly, doses at 50mg/kg and 100mg/kg have no significant impact on the serum levels of IL 2 ($p > 0.05$). In comparison with AFB1 treated group, a significant decrease in the serum concentrations of IFN- γ and IL 2 was reported ($p < 0.05$) in group administered 100mg/kg and the 150mg/kg of spirulina extract. This implies that spirulina extract may play a role in reducing AFB1-induced inflammation in mice.



A. **B.**



C.

Figure 2: Graphs showing the concentrations of cytokines **(A)** IFNγ, **(B)** IL 2 and **(C)** IL 4 after 14 days of treatment. The mean values are presented as the x̄ ±standard error of the mean, *n*=5. Statistical significance is denoted by superscript letters, where different letters shows statistical significance ($p < 0.05$).

Effects on serum levels of immunoglobulins A, G and M

After the experiment period, the serum concentrations of IgA and IgM in group administered with aflatoxin B1 was not significantly lowered in comparison with untreated group as shown in Table 1 ($p>0.05$). This implies that aflatoxin B1 at a dose of 200 µg/kg administered daily for 14 days does not have a significant impact on antibody-mediated immunity. However, serum levels of IgG significantly reduced in AFB1 treated group as compared to untreated group ($p < 0.05$). Further, treatment with spirulina extract at different doses had no significant effect on serum concentrations of IgA, IgG and IgM.

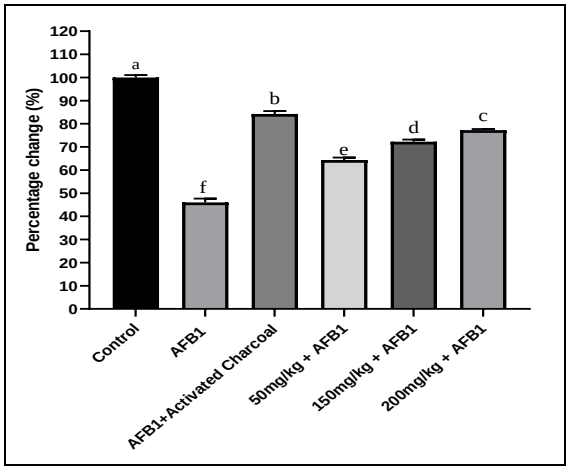
Table 2: Effect of spirulina extract on serum concentrations of immunoglobulins in mice administered aflatoxin B1.

Group	IgG (ng/ml)	IgA (ng/ml)	IgM (ng/ml)
Control	15.51±0.11	0.730±0.004	2.168±0.004
AFB1 (200 µg/kg)	14.54±0.04	0.729±0.005	2.167±0.004
Activated Charcoal 1g/kg+AFB1 200µg/kg	15.36±0.06	0.731±0.004	2.175±0.009
Sp. 50mg/kg + AFB1 200 µg/kg	15.7±0.12	0.737±0.004	2.162±0.002
Sp. 100mg/kg + AFB1 200 µg/kg	14.59±0.06	0.737±0.004	2.166±0.006
Sp. 150mg/kg + AFB1 200 µg/kg	15.58±0.17	0.735±0.004	2.169±0.006

(Sp – Spirulina; AFB1 – Aflatoxin B1)

Effect on mRNA expressions of IL-4, TNFα, and IFNγ

The *m*RNA expression of IL 4 was downregulated in all treatments across the groups as compared to untreated group ($p< 0.05$). In comparison with the untreated group, mRNA expressions of TNF-α, and IFN-γ were upregulated in activated charcoal, aflatoxin B1 and spirulina treated groups. Increasing mRNA expressions of TNFα, and IFNγ as a result of AFB1was prevented ($p\leq0.01$) by administration of spirulina extract.



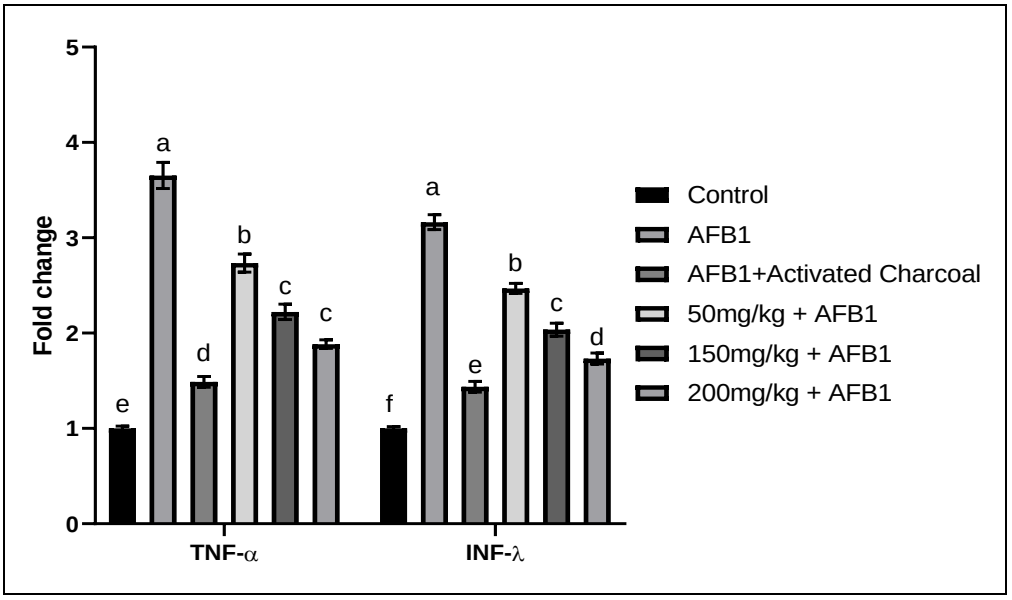


Figure 3: Graphs showing the effects of different treatments on (**A**) IL-4 (**B**) TNF and INF following AFB1-induced in mice. Bars with different lowercase letter are significantly different.

DISCUSSION

The effect of AFB1 on weight of mice has been studied extensively. Our study found that mice fed with 200µg/kg b.w of aflatoxin B1 led to significant reduction of body weight. This echoes the findings by [23] and [24] where mice significantly lost weight after 2 to 3 days after AFB1 administration. Studies have found that aflatoxin B1 damages intestinal barrier integrity. The destructive nature of AFB1 on the gut compromises absorption of essential nutrients thus leading to slow growth and body weight loss [25]. The reduction in body weight in AFB1 treated group is also attributed to decreased activity of digestive system enzymes and disruption of gluconeogenesis and fatty acid synthesis induced by AFB1 which eventually leads to decreased body weight [26, 27, 28]. The intervention with spirulina extract at low dose (50mg/kg body weight) has no significant effect in reversing body weight loss in mice induced by AFB1. However, it was noted that increasing the dose to 150mg/kg body weight restores body weight loss due to AFB1 ingestion. Intake of spirulina is associated with weight gain [29]. A study by [30], it was noted that spirulina enhances development and strengths of bones because of its role in stimulation of parathyroid hormone (PTH) and growth hormone (GH). The increase in body weight occurs because spirulina offers supplementation because it is rich in proteins [29]. The use of activated charcoal in management of aflatoxicosis is widely reported. Activated charcoal has pores that can trap chemicals and toxins. It has been reported that feeding animals with activated charcoal alongside aflatoxin B1 reduces the effects of AFB1 on weight [31]. This is because activated charcoal is capable of adsorbing AFB1 efficiently both in *in-vitro* and in *in-vivo* studies [32].

Determinations of the levels of inflammatory cytokines both in spleen tissues and serum is adequate to assess the status of the immune system [33]. During normal physiological state, the levels of inflammatory cytokines are lower but become elevated under pathological state. Our study demonstrated that exposure of mice with aflatoxin B1 resulted in elevated mRNA expressions of TNFα, and IFNγ. Treatment with spirulina extract was noted to result in a significant reduction in the mRNA expressions of TNFα, and IFN γ. This is probably because spirulina has phycocyanin which has anti-inflammatory effects [34, 35]. However, exposure of mice to AFB1 resulted in downregulation of the mRNA expression of IL-4. This finding supports the finding by [36] where IL-4 levels decreased upon treatment with aflatoxin B1.

Aflatoxin B1 has been documented to elevate the serum level of cytokines. [37] in their study found out that broilers fed with AFB1 contaminated feed led to increase in cytokine production including IFN-γ. In comparison with untreated group, there was a significant reduction in serum level of IL-4 in mice treated with AFB1. Interleukin 4 is an anti-inflammatory cytokine and it is produced during inflammatory response. It has been established by numerous studies that AFB1 inhibit mRNA and protein expression of interleukin 4 [38, 39, 40]. Treatment with spirulina extract at 100mg/kg bw and 150mg/kg bw does not result in any significant change in the levels of IL-4. This could be because spirulina extract downregulates the expression of IL-4 mRNA and protein levels [17]. In addition, it could be that the dosages used are not high enough to cause significant variations in the concentration of this cytokine. In comparison to untreated group, the findings also pointed out that serum concentrations of IFNγ and IL2 were reduced significantly in groups that received spirulina extract and activated charcoal after AFB1 administration. This suggests that spirulina extract can be used to downregulate inflammatory reaction as a result of AFB1 ingestion by mice. Similar findings were reported by [15] where spirulina extract was reported to have anti-inflammatory effect.

The humoral immune response is the branch of the immune system that involves antibodies, which are proteins produced by the body's white blood cells (B lymphocytes) that recognize and bind to specific antigens [41]. Determining the serum levels of immunoglobulins IgA, IgG and IgM is an approach used to measure the humoral immunity when triggered by AFB1 [42]. In comparison with untreated group, the levels of IgA and IgM in group given 200µg/kg body weight of AFB1 were reduced but not significantly. This suggests that aflatoxin B1 at the dose of 200µg/kg bw does not exhibit any significant immunosuppressive effect in mice. Our findings support the findings of [24] in which there were no significant interaction effects when broiler chickens are fed with AFB1. [43] also reported that feeding pigs with AFB1 contaminated feeds will result in no significant variation in concentrations of IgG, IgA, and IgM in serum. However, our study found that IgG was significantly reduced in AFB1 treated group, which confirms the findings by [44] in their study. Intervention of spirulina extract however does not result in major effect in serum levels of IgA, IgG and IgM. This could be because of dosage, duration of treatment and susceptibility of animal model.

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Conflicts of Interest: The authors of this manuscript have no conflict of interest to declare.

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