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Article

Impact of a Single Oral Acute Dose of Aflatoxin B_1 on Liver Function/Cytokines and the Lymphoproliferative Response in C57Bl/6 Mice

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Academic Editor: Chiara Dall'Asta
Received: 16 October 2017; Accepted: 15 November 2017; Published: 17 November 2017

Abstract: Aflatoxin B_1 (AFB_1), a mycotoxin found in food and feed, exerts harmful effects on humans and animals. The liver is the earliest target of AFB_1, and its effects have been evaluated in animal models exposed to acute or chronic doses. Considering the possibility of sporadic ingestion of AFB_1-contaminated food, this study investigated the impact of a single oral dose of AFB_1 on liver function/cytokines and the lymphoproliferative response in mice. C57BL/6 mice were treated with a single oral AFB_1 dose (44, 442 or 663 µg AFB_1/kg of body weight) on the first day. Liver function (ALT, γ-GT, and total protein), cytokines (IL-4, IFN-γ, and IL-17), histopathology, and the spleen lymphoproliferative response to mitogens were evaluated on the 5th day. Although AFB_1 did not produce any significant changes in the biochemical parameters, 663 µg AFB_1/kg-induced hepatic upregulation of IL-4 and IFN-γ, along with liver tissue injury and suppression of the lymphoproliferative response to ConA ($p < 0.05$). In conclusion, a single oral dose of AFB_1 exposure can induce liver tissue lesions, liver cytokine modulation, and immune suppression in C57BL/6 mice.

Keywords: cytokines; inflammatory response; immunosuppression; mycotoxin

1. Introduction

Aflatoxins (AFs) are mycotoxins produced by *Aspergillus flavus* and *A. parasiticus* that contaminate agricultural commodities under harvest and post-harvest conditions. Human and animal health issues caused by ingestion of food contaminated with AFs are considered a permanent risk and a worldwide problem [1,2]. The mammalian liver, an early target of AFs, is the major drug-detoxifying organ, responsible for the metabolic activation and elimination of toxic components [3].

Among the AFs, aflatoxin B_1 (AFB_1) is generally predominant and is considered the most toxic analog [4]. Ingestion of AFB_1-contaminated products can result in immunosuppressive, teratogenic, mutagenic, and carcinogenic effects. The International Agency for Research on Cancer classifies AFB_1 as Group 1, i.e., carcinogenic to humans [5,6]. Furthermore, AFB_1 exerts many immunotoxic effects, ranging from alterations in innate immunity or antigen-presenting cells [7–9] to changes in adaptive immunity, resulting in a reduced number of circulating lymphocytes, the inhibition of lymphocyte blastogenesis, and the alteration of cytokine expression in animals of various species [9,10].

In studies of the different effects and impact of AFB_1 exposure, animals such as mice have been exposed to acute or chronic AFB_1 doses for an extended period [11–14]. Considering the possibility of sporadic ingestion of AFB_1-contaminated food, this study investigated the impact of a single oral dose of AFB_1 (with low doses; <to ~1% of the median lethal dose (LD_{50})) on liver function/cytokines and the lymphoproliferative response in C57Bl/6 mice.

2. Results

2.1. Evaluation of the Effect of Aflatoxin B_1 on Serum ALT, γ-GT, and Total Protein Levels

Oral administration of AFB_1 did not produce any significant change in the ALT and γ-GT serum enzymes or protein levels ($p > 0.05$). Additionally, the vehicle group treated with saline:ethanol (95:5) presented similar results to those obtained for the water-treated controls ($p > 0.05$) (Table 1).

Table 1. Effects of aflatoxin B_1 on alanine aminotransferase (ALT), gamma glutamyl transpeptidase (γ-GT), and total protein levels in mice serum five days after a single oral dose of aflatoxin B_1.

Group	Parameters		
	ALT (U/L)	**γ-GT (U/L)**	**Total Protein (g/dL)**
Control	26.67 ± 9.37 [a]	6.83 ± 0.75 [a]	5.63 ± 0.49 [a]
Vehicle	43.25 ± 15.52 [a]	6.5 ± 0.71 [a]	5.46 ± 0.54 [a]
AFB_1 44 µg/kg	37.33 ± 8.64 [a]	6.3 ± 1.03 [a]	5.83 ± 0.27 [a]
AFB_1 442 µg/kg	28.33 ± 7.23 [a]	5.50 ± 0.71 [a]	5.12 ± 0.34 [a]
AFB_1 663 µg/kg	22.80 ± 3.70 [a]	7.00 ± 2.00 [a]	5.46 ± 0.30 [a]

[a] $p < 0.05$.

Means values within column with different superscript letters were statistically significant ($p < 0.05$), as determined through Tukey's test. The reference values for mice serum enzymes are 23.00 ± 4.92 units/L of ALT, 7.57 ± 4.2 units/L of γ-GT, and of 5.07 ± 0.2 g/dL of total protein [15,16].

2.2. Evaluation of Aflatoxin B_1 on Histological Lesions in Liver Tissue

Cytoplasmic hepatocyte vacuolation, megalocytosis, nuclear vacuolation, inflammatory infiltrate, and necrosis were present in the liver of treated animals; the last two lesions were more frequently observed. Lesions recorded in the liver were considered moderate to severe in mice treated with AFB_1. A significant increase in the lesional score was observed in animals exposed to 663 µg of AFB_1/kg of body weight (b.w.) compared with animals exposed to vehicle ($p = 0.001$ (Figure 1). Animals treated with 663 µg of AFB_1/kg of b.w. showed more pronounced intensity of megalocytosis, nuclear vacuolation, and necrosis than animals treated with vehicle, whereas the main lesions in the animals treated with 44 and 442 µg of AFB_1/kg of b.w. were necrosis.

2.3. Effects of Aflatoxin B_1 on Cytokine Expression in the Liver

Mice treated with 663 µg of AFB_1/kg b.w. showed significant upregulation of IL-4 and IFN-γ ($p = 0.002$) compared with the vehicle group (Figure 2). There was no difference in the IL-17 cytokine levels between animals treated with AFB_1 and untreated animals ($p > 0.05$). In contrast, there were differences between animals treated with 44 and 442 µg of AFB_1/kg of b.w. and animals treated with 663 µg of AFB_1/kg of b.w. ($p < 0.05$).

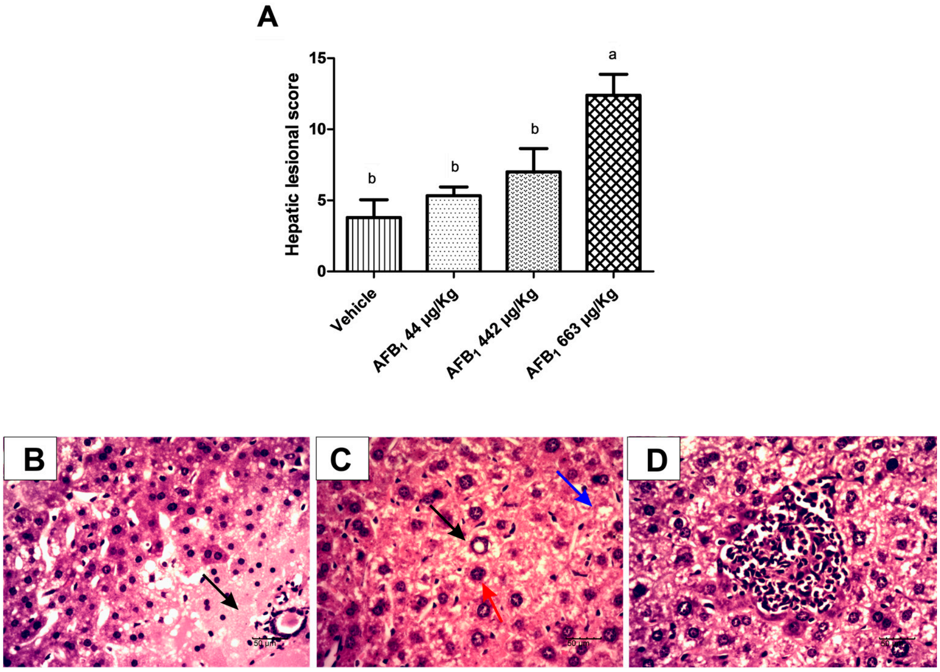

Figure 1. Effect of aflatoxin B_1 on the livers of mice exposed to 44 µg, 442 µg, and 663 µg of AFB_1/kg b.w. at 5 days after exposure. (**A**) Lesional score. The data are expressed as the mean ± SD, $n = 5$. Means without a common letter were statistically significant ($p < 0.05$), as demonstrated by Tukey's test. The liver lesions observed in animals treated with AFB_1 were (**B**) focal necrosis of hepatocytes (arrow), HE, 40×, 50 µm; (**C**) vacuolar hepatocyte degeneration (nuclear (black arrow) and cytoplasmic (blue arrow)) and megalocytosis (red arrow), HE, 40×, 50 µm; and (**D**) centrolobular inflammatory infiltrate, HE, 40×, 50 µm.

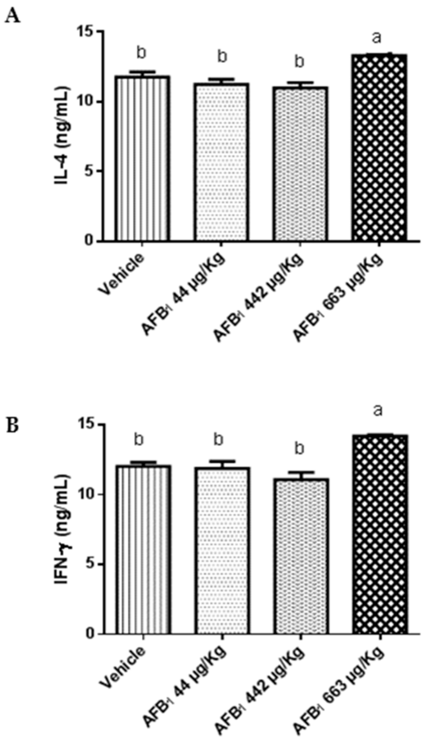

Figure 2. *Cont.*

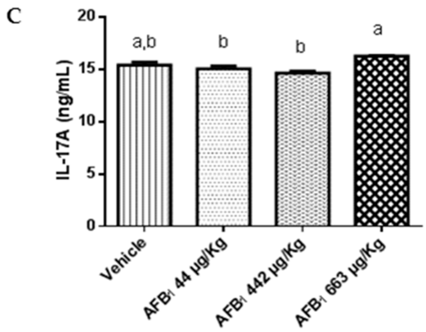

Figure 2. Effect of aflatoxin B_1 on the expression levels of (**A**) interleukin 4 (IL-4), (**B**) interferon γ (IFN-γ), and (**C**) interleukin 17 (IL-17) in the liver at 5 days post-aflatoxin exposure. Data are expressed as the mean ± SD, n = 5. ^{a,b} columns with different superscript letters were statistically significant ($p < 0.05$), as determined by Tukey's test.

2.4. Effects of Aflatoxin B_1 on Lymphoproliferation Assay

Significant suppression of the proliferative response was observed for concanavalin A (ConA)-stimulated lymphocytes under treatment with 442 and 663 µg AFB_1/kg of b.w. ($p < 0.05$). The control animals showed a lymphoproliferation index of 1.21 ± 0.14, whereas the experimental animals treated with doses of 442 µg and 663 µg AFB_1/kg of b.w. showed indexes of 0.94 ± 0.07 and 0.97 ± 0.01, respectively. However, no significant depression of the proliferative assay was observed in lipopolysaccharide (LPS)-stimulated lymphocytes when the data were compared with those of the control animals (Figure 3).

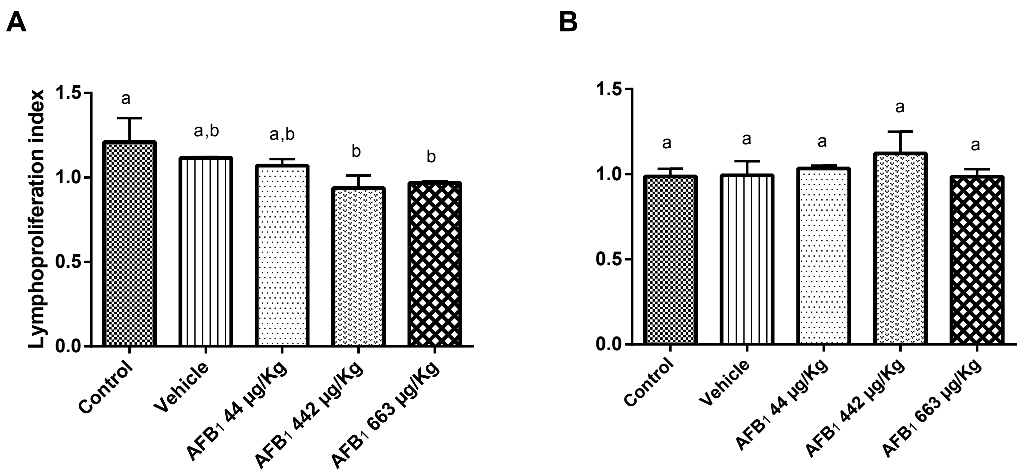

Figure 3. Effects of aflatoxin B_1 on mouse splenocyte proliferative responses in the presence of (**A**) concanavalin A or (**B**) lipopolysaccharide 5 days after aflatoxin treatment. Significance levels were determined based on the comparison of experimental animal data with control animal data. The data are expressed as the mean ± standard deviation (SD) of the proliferative index (optical density (OD) of the test well/OD of control well) for five animals. ^{a,b} columns with different superscript letters were statistically significant ($p < 0.05$), as determined by Tukey's test.

3. Discussion

Among mycotoxins, AFs are of major concern worldwide in terms of their risks to human and animal health. Their harmful effects have generally been demonstrated by administering repeated AF doses over long periods of exposure in animal models. In this study, we investigated the effects of exposure to single oral doses of AFB_1 in C57Bl/6 mice. AFB_1 did not have a major impact on the investigated hepatic biochemical parameters in the mice 5 days after administration. It is possible that the time of sampling was too long and the measurement of liver functions levels in earlier days was be different in animals treated with AFB_1 compared to the control group. However, such results are in accordance with the results of a study conducted by Almeida et al. [15], who did not detect differences in the alkaline phosphatase levels in the serum of C57Bl/6 mice after 168 h of AF treatment (60 mg/kg animal weight). Moreover, our results are consistent with those observed by Baptista et al. [17], who fed albino rats 400 µg AFB_1/kg of b.w. over 28 days and did not observe any significant differences in ALT, AST, ALP, or γ-GT enzyme activities or albumin levels.

The sensitivity degree and toxicity of AFB_1 varies between species due to differences in its biotransformation. Some animals, such as sheep, dogs, pigs, and rats, are considered extremely susceptible to $AFB_{1,}$ whereas others, such as monkeys, chickens and mice, are considered resistant species [18]. The LD_{50} described in the literature for mice is variable, with values ranging from 9 to 60 mg of AFB_1/kg of b.w. [15]. The doses of AFB_1 used in this experiment (44, 442, and 663 µg/kg of b.w.) might appear high when applied to humans; however, aflatoxicosis cases have been reported to occur at similar or higher consumption levels of AFB_1 [19,20].

Changes in the hepatic cellular architecture and organization were detected by histopathological analysis. The extent of liver damage was directly correlated with the concentration of AFB_1 and the duration of the exposure [17]. In this study, a 7.7-fold increase in the lesional score was observed in AFB_1-exposed animals. Notably, this study constitutes the first evaluation of the effects of a subclinical, single AFB_1 exposure in mice, and the hepatic lesions observed here are consistent with those observed in other studies that used different doses or frequencies of exposure [21].

In the present study, upregulation of the production of the hepatic inflammatory cytokine IFN-γ, along with increased expression of the anti-inflammatory cytokine IL-4, were observed with higher doses of AFB_1. There is no consensus regarding the cytokine responses induced by AF exposure [22–24]. Here, the difference obtained in anti- and pro-inflammatory cytokines levels might be due to time, a single and higher dose (663 µg of AFB_1/kg of b.w.), or different organs and species. Because the evaluations performed in this study were at early stages (5 days) prior to the adaptive response, increases in IL-4 and IFN-γ expression were attributed to innate immune cell activation. Additionally, many innate immune cell populations produce IL-17 in response to stress, injury, or pathogens [25]. Our results indicate that IL-17 levels were significantly different among the highest and lowest doses of AFB_1, but no differences were detected between the treated and control groups. Thus, even a single dose of AFB_1 might induce hepatic cytokine immunomodulation, but whether all of the cytokines evaluated are involved in hepatic injury remains uncertain, and further studies are required.

AFB_1 has a selective effect on cell-mediated immunity, with a relatively weak effect on the humoral immune system [21]. Consistently, in this study, we detected the inhibitory effects of AFB_1 on ConA-stimulated lymphoblastogenesis (ConA is a lectin widely used as a polyclonal T-cell activator) but not on LPS-stimulated lymphoblastogenesis. Reddy & Sharma [26] reported the inhibitory effects of AFB_1 on both LPS- and ConA-stimulated lymphoblastogenesis in animals exposed to low, repeated doses of AFB_1. The difference in the LPS response detected in this investigation could be due to the use of a single dose instead of repeated doses.

According to a review by Peraica et al. [27], the acute hepatotoxic effects of AFs recorded in humans have mostly been observed among adults in rural populations with poor nutritional levels. The same authors cited the case of a young woman who ingested a total of 5.5 mg of AFB_1 over 2 days, and whose laboratory examinations were normal and suggested that the hepatotoxicity of AFB_1 might be lower in well-nourished persons.

In this study, the biochemical parameters of hepatic functions were not altered in mice exposed to AFB_1. However, several other important parameters, such as liver tissue injury, cytokine levels, and cellular responses, were altered even with a single dose. Moreover, in a previous study, we verified that a single AFB_1 exposure induces changes in the gut microbiota in C57Bl/6 mice [28].

It is important to investigate the effects of a single dose of AFs due to the possibility of sporadic ingestion of aflatoxin-contaminated food. In this study, harmful effects were observed even with one low oral dose of AFB_1 (~1% of the median lethal dose (LD_{50}) for mice [26]). These effects might be temporary but could contribute to exacerbation in cases of patients with liver disease or other diseases associated with immunosuppression, requiring further studies.

4. Conclusions

In conclusion, even a single, oral, low dose of AFB_1 can induce liver tissue lesions, liver cytokine modulation and immune suppression in C57BL/6 mice.

5. Material and Methods

5.1. Aflatoxin B_1 Standard, Dose Criteria, and Duration of Exposure

The AFB_1 standard from *Aspergillus flavus* (A663, Sigma, St. Louis, MO, USA) was quantified according to the methods indicated by Instituto Adolfo Lutz [29]. The molar absorptivity of AFB_1 considered for calculating AFB_1 concentrations in methanol was 21,800 at 360 nm. The AFB_1 solution was dried under gaseous N_2, and the standard was dissolved in a 95:5 saline:ethanol solution for experiments.

The lowest AFB_1 dose (44 μg/kg of b.w.) was calculated based on the maximum level allowed (5 μg/kg) in both rice and beans [30,31], according to the average bean (183 g/day) and rice (160 g/day) consumption levels, which are components of the traditional diet in Brazil [32]. The highest tested AFB_1 dose (663 μg/kg of b.w.) was based on the chronic AFB_1 dose used in other studies [13,33] and represents approximately 1% of the LD_{50} for mice. The duration of exposure was based on the plasma half-life of AFB_1 in male rats, which is approximately 92 h [34].

5.2. Animals, Housing, and Experimental Design

A total of 25 male C57Bl/6 mice (10 weeks of age, average weight: 22.55 ± 0.89 g) were obtained from the University of São Paulo—Ribeirão Preto City, Brazil. C57Bl/6 mice were selected for this study because they are highly susceptible to the acute effects of aflatoxin B_1 [15]. The mice were acclimatized for 3 weeks and housed in polyethylene boxes with a bedding of wood shavings. They were maintained under standard conditions, which included a temperature of approximately 25 °C with a regular 12 h light/12 h dark cycle. All mice were given standard rodent pellet food and water *ad libitum*.

The experimental design used in this study was randomized with five repetitions (each animal represented one repetition) for each group. Group 1 consisted of untreated control mice; Group 2 received only the vehicle (saline:ethanol, 95:5) on the first day; Group 3 received a single dose of 44 μg AFB_1/kg b.w. on the first day; Group 4 received a single dose of 442 μg AFB_1/kg b.w.; and Group 5 received a single dose of 663 μg AFB_1/kg b.w. AFB_1 suspended in saline:ethanol (95:5) was administered via oral gavage (0.1 mL per 10 g of body weight). After 5 days, the animals were bled and euthanized, and their organs (liver and spleen) were then removed.

Biochemical parameters were analyzed from the serum, and a lymphoproliferation assay was performed using splenocytes. This study was approved by Committee of Animal Ethics of State University of Londrina (CEUA n° 26362.2014.65 process, 18 December 2014).

5.3. Measurements of Liver Function from Serum

ALT and γ-GT enzymes and total protein levels in serum were evaluated with a Dimension® Clinical Chemistry System (Siemens, Newark, NJ, USA).

5.4. Histopathological Analysis

Liver samples were fixed in 10% buffered formalin solution, dehydrated in increasing alcohol concentrations, and embedded in paraffin for histological analysis. The tissue samples were sectioned at 5-μm thickness, stained with hematoxylin and eosin (HE), and mounted with coverslips. Histological changes were evaluated using an adapted tissue score based on the intensity and severity of lesions as previously described by Gerez et al. [35]. Briefly, the criteria used to establish the lesional score were hepatocyte megalocytosis, inflammatory infiltrate, hepatocyte nuclear vacuolation, hepatocyte cytoplasmic vacuolation, and necrosis. The extent of each lesion was scored as follows, megalocytosis (the mean of five fields per histological section): 1–10 = 0, 1–20 = 1+, 2–30 = 2+, and 3–40 = 3+; inflammatory infiltrate and nuclear vacuolation (histological section): absent = 0, 1 = 1+, and >2 = 2+; hepatic cell vacuolation (histological section): mild = 0, and moderate = 1+; and necrosis (histological section): absent = 0, mild = 1+, moderate = 2+, and severe = 3+. For each type of lesion, the score of the extent was multiplied by the severity factor [35].

5.5. Cytokine Assays

The liver samples were macerated in an adjusted volume of 0.1 M phosphate buffered saline (PBS), pH 7.4 (1:5000 dilution). The IL-4, IFN-γ, and IL-17 levels in the liver were determined by commercial sandwich ELISA kits (BioSource International, Inc., Camarillo, CA, USA), according to the manufacturer's instructions.

5.6. Lymphoproliferation Assay

The spleens were removed from the mice aseptically, and their erythrocytes were lysed with Tris-ammonium chloride solution. In 96-well flat-bottom culture plates, 100 µL of the splenocytes (1×10^5 cells/mL) from each mouse were cultured in duplicate wells in RPMI 1640 medium (containing L-glutamine and, 10% fetal calf serum), with 0.5 µg/mL LPS (Sigma, St. Louis, MO, USA) or with 0.5 µg/mL ConA (Gibco Life Technologies, Grand Island, NY, USA). The cells were cultured for 84 h at 37 °C with 5% CO_2, and 100 µL of RPMI medium and 10 µL of 3-(4,5-dimethylthiazol-2-ly)-2,5-diphenyltetrazolium bromide (5 mg/mL MTT in PBS, Sigma, St. Louis, MO, USA) were then added to each well. The plates were then further incubated for 4 h at 37 °C, and formazan crystals were subsequently solubilized by adding 200 µL of dimethyl sulfoxide (DMSO). The optical density was subsequently measured with an ELISA microplate reader (iMarkTM, Bio-Rad, Hercules, CA, USA) at 550 nm, and the proliferation index (P. I.) of the stimulated/nonstimulated cells was calculated in duplicate [36].

5.7. Statistical Analysis

The biochemical parameter and lymphoproliferative assay data were analyzed using Statistica software (version 7.0, 2004, Stat Soft, Tulsa, OK, USA) and are presented as the mean ± standard deviation. Before the analysis, homogeneity of variance (Levene's test) and the normality of the data distribution (Shapiro-Wilk's test) were tested. One-way analysis of variance (ANOVA) followed by Tukey's test was performed, and p values < 0.05 were considered statistically significant.

Acknowledgments: This work was supported by CAPES (the Brazilian government organization for grant aid and fellowship to Brazilian researchers)—Nanobiotec Network, n°04/2008, CNPq and the Araucária Foundation (Paraná State grant).

Author Contributions: A.T.I., E.Y.H. and E.N.I. conceived and designed the experiments; A.T.I., P.L.A.S., C.Y.A. and E.N.I. performed the experiments; A.T.I. and E.I.N. analyzed the data; E.Y.H., A.P.F.R.L.B., K.K.M.d.C.F., O.K., M.C.C. and E.N.I. contributed reagents/materials/analysis tools; A.T.I., A.P.F.R.L.B. and E.I.N. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

AFB_1	aflatoxin B_1
AFs	aflatoxins
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
b.w.	body weight
ConA	concanavalin A
DMSO	dimethyl sulfoxide
ELISA	enzyme-linked immunosorbent assay
LD_{50}	median lethal dose
LPS	lipopolysaccharide
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
P. I.	proliferation index
PBS	phosphate buffered saline
RPMI	Roswell Park Memorial Institute
γ-GT	γ-glutamyl transpeptidase

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