Ameliorative effects of Sida acuta and vitamin C on serum DNA damage, pro-inflammatory and anti-inflammatory cytokines in roosters fed aflatoxin B1 contaminated diets

Olumuyiwa Joseph Olarotimi (olumuyiwa.olarotimi@aaua.edu.ng)
Adekunle Ajasin University https://orcid.org/0000-0002-5934-2529

Francis Ayodeji Gbore
Adekunle Ajasin University

Olufemi Ademola Adu
Federal University of Technology Akure

Olugbenga David Oloruntola
Adekunle Ajasin University

Olatunji Abubakar Jimoh
Federal Polytechnic Ado-Ekiti

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Abstract

The ameliorative effects of *Sida acuta* leave meal (SALM) and vitamin C on the serum pro-inflammatory and anti-inflammatory cytokines as well as DNA damage to cocks fed aflatoxin B_1 (AFB_1) contaminated diets were examined. The experiment was a completely randomized design with a total of 250 sexually mature Isa White cocks aged 24 weeks, randomly allotted into five experimental diets; each diet contained 5 replicates with 10 roosters. The diets were A (control), B (containing 1 mg/kg AFB_1), C (B + 200 mg/kg vitamin C), D (B + 2.5 g/kg SALM) and E (B + 5.0 g/kg SALM). Fresh and clean water was also provided for the whole experimental period of twelve weeks. Inclusion of 1 mg/kg AFB_1 without vitamin C or SALM increased TNF-α and IL-1β as well as 8-OHdG and NF-κB in the serum significantly ($P < 0.05$) among the cocks on diet B. However, the fortification of AFB_1 contaminated diets with vitamin C and SALM depressed serum TNF-α, IL-1β, 8-OHdG and NF-κB concentrations of the cocks significantly ($P < 0.05$). Conversely, serum IL-4 and IL-10 in birds given 1 mg/kg AFB_1 without vitamin C or SALM decreased significantly ($P < 0.05$) in comparison with the roosters on the control. However, improvements ($P < 0.05$) in IL-4 and IL-10 concentrations with corresponding reduction ($P < 0.05$) in TNF-α, IL-1β, 8-OHdG and NF-κB concentrations were recorded among cocks fed Diets C, D and E, respectively. Therefore, dietary addition of SALM at the level used in this study was beneficial and has comparable effects with inorganic antioxidant (C vitamin) by significantly reducing the inflammatory cytokines and oxidative damage biomarkers as well as enhancing the anti-inflammatory cytokines thereby promoting the health status of the cocks fed AFB_1 contaminated ration.

Introduction

The role of animal agriculture, especially the poultry sub-sector, in the developing economies of the world has been severally stressed (NEA 2020). However, the potential of this sub-sector to optimally support the fast-growing population in developing countries is limited due to the myriads of challenges facing this industry. Chief among these is the incidence of aflatoxin contamination of feeds which always has severe economic effects on the farmers' profitability and the productivity of the chickens (Nakavuma et al. 2020). The molds *Aspergillus flavus* and *A. parasiticus* are responsible for naturally producing the mycotoxins called aflatoxins. Agricultural products used in feed production such as groundnut, millet, sorghum, maize, wheat, and soybeans are easily contaminated by aflatoxin because of the favourable attendant pre- and post-harvest conditions predisposing these products to fungal infections. Aflatoxin contamination of agricultural products is so hazardous to both man and animals that only a low concentration is required to cause aflatoxicosis.

Aflatoxicosis is a disease caused by aflatoxin consumption and acute aflatoxicosis could result in death (Mahato et al. 2019). In poultry, hens fed diets containing aflatoxin had been reported having reduced performance indices such as reduced laying efficiency and feed intake as well as compromised health status as indicated by decreased vaccination efficiency, impaired immunity to diseases, and ultimately increased mortality rate (Fouad et al. 2019). Aflatoxins are equally not left out in playing significant roles

on cytokines and chemokines. Cytokines and chemokines, like hormones and neurotransmitters, are potent signaling molecules very vital in living cells to mediate intercellular communication (Arango and Descoteaux 2014). Cytokines, both the pro-inflammatory and anti-inflammatory, regulate the immune response in health and disease situations. However, the potential of cytokines to play pro-inflammatory or anti-inflammatory roles, or both, is dependent on the specific local microenvironments per time (Su et al. 2012). Therefore, it is worthy of note that aflatoxins have been reported to create an enabling environment in adversely affecting the health of animals by inhibiting certain anti-inflammatory cytokines (Marin et al. 2002; Qian et al. 2014) as well as the stimulation of some pro-inflammatory cytokines (Yarru et al. 2009; Huang et al. 2019). Furthermore, aflatoxin B_1 (AFB_1) was previously found culpable to cause oxidative damage to the DNA resulting from the generation of reactive oxygen species (ROS) in the cells. Cellular generation of ROS indirectly attacks the membrane phospholipids and gives off various aldehydes which are mutagenic in nature (Benkerroum 2020). Indicators of serum DNA damage such as 8-OHdG (8-hydroxy-2'-deoxyguanosine) (Groopman et al. 2008; Sun et al. 2018) and NF-κB (nuclear factor kappa B) have been reported to be highly favoured by AFB_1 (Huang et al. 2019). In another study, AFB_1 was found to significantly elevate IL-6 (interleukin 6), IL-1β (interleukin 1 beta) and TNF-α (tumor necrosis factor-alpha) serum concentrations (Benkerroum 2020). Similarly, Rajput et al. (2019) equally reported serum elevation of IFN-γ as well as TNF-α concentration among others in broiler chickens exposed to AFB_1. Traditionally, measurements of serum cytokines are useful biomarkers in the studies of toxicities to highlight the inflammation and immune responses in tissue damage.

Since aflatoxin contamination of agricultural products used in feed production is detrimental to animals, especially poultry birds and pigs, the use of mycotoxin binders, in recent times, is the rule of thumb for commercial feed millers to prevent unwanted contamination of animal feed by aflatoxin. A toxin binder is a substance added to poultry feed in micro amounts capable of neutralizing mycotoxins within the animal's gastrointestinal tract. A classical example of this is bentonite clay which is effective in binding aflatoxin B1 when added to poultry feed usually at a 2% inclusion rate. However, clay-based toxin binders have their negative effects such as binding nutrients even at lower inclusions, thus, reducing essential nutrient availability needed for growth and production (Kumari and Mohan 2021). For instance, Elliot et al. (2020) highlighted the European Authority for Food Safety Authority (EFSA) report indicating the possibility of binding potential of manganese when bentonite is used at a dosage higher than 0.5% in poultry feed. Away from binding essential micronutrients, the efficacy of medicinal substances, such as coccidiostats, in the feed is also altered by clays. Small protein molecules that are positively charged can also be bound into the interlayer of clay (montmorillonite) through the cation exchange (Damato et al. 2022; Kloprogge and Hartman 2022). Apart from these, many of the clay-based toxin binders are costly and increase the cost of feed production, hence, reducing farmers' profitability margin.

A novel approach to adopt in solving this problem is, therefore, looking in the direction of using phytogenic-based strategy in mitigating the incidence of aflatoxin in poultry production. Generally speaking, it has been established that phyto-additives have the potential of improving performance and maintaining the physiological status in terms of enhancing the immunity and antioxidant status in birds

(Oloruntola et al. 2018). *Sida acuta* is a common phytotherapeutic plant and rich in bioactive compounds. The leaves of *S. acuta* are a reservoir of antioxidants possessing anti-inflammatory, anti-cholinergic, anticancer and anti-cytotoxic properties (Mah et al. 2017; Rodrigues and de Oliveira 2020). It is equally a sources of antibacterial, antifungal (Hoffman et al. 2004) as well as anti-hyperglycemic (Okwuosa et al. 2011) activities, etc. This study, hence, focuses on the ameliorative potentials *of Sida acuta* leaf meal on the serum DNA damage, pro-inflammatory and anti-inflammatory cytokines of cocks fed aflatoxin B1 contaminated diets.

Materials and methods

Site, materials and diets

The research took place at the Adekunle Ajasin University's Teaching and Research Farm, Nigeria. Animal Ethics and Guidelines Unit of the institution permitted the study. The *S. acuta* leaves were sourced within the immediate environment of the institution and identified by an ethnobotanist at the University's herbarium while feed grade vitamin C was purchased from a reliable agrochemical store. The leaves were properly washed, drained and left dried in a shady screen which allows adequate air flow and away from direct sunlight for a period of 7 days. Thereafter, the leaves were pulverized to make *S. acuta* leaf meal (SALM), and then, securely stored till when needed for experimental diets composition.

For production of AFB_1, maize was grounded to grits and cultured with a toxigenic strain of *Aspergillus flavus* and quantified for AFB_1 and other Aspergillus mycotoxins as described by Mgbeahuruike et al. (2018). A total of 4 kg of the contaminated maize was combined with non-cultured maize to produce a contaminated ration containing 1mg AFB_1 (Table 1). The contaminated ration was divided into four batches. One batch was labeled diet B (positive control: containing 1mg AFB_1), other batches were labeled diets C (1mg AFB_1 + 200 mg Vit. C), D (1mg AFB_1 + 2.5 g SALM), and E (1mg AFB_1 + 5.0 g SALM)/kg diet. Vitamin C and SALM were added as supplement to diets while diet A was the negative control (0 mg AFB_1).

Table 1
Ingredient composition of the experimental Cock diets

Ingredients (kg)	A	CONTAMINATED DIETS			
		B	C	D	E
Maize	370	366	366	366	366
Cultured Maize	0	4	4	4	4
Soya Bean Meal	40	40	40	40	40
Bone Meal	15	15	15	15	15
Limestone	15	15	15	15	15
Salt	2.5	2.5	2.5	2.5	2.5
Wheat Bran	370	370	370	370	370
Palm Kernel Cake	183	183	183	183	183
Lysine	0.5	0.5	0.5	0.5	0.5
Methionine	1.5	1.5	1.5	1.5	1.5
*Vitamin-Mineral Premix	2.5	2.5	2.5	2.5	2.5
Total	1000	1000.00	1000.00	1000.00	1000.00
Analyzed Nutrients					
ME (Kcal/Kg)	**2467.77**	**2467.77**	**2467.77**	**2467.77**	**2467.77**
Crude Protein (%)	**14.79**	**14.79**	**14.79**	**14.79**	**14.79**
Calcium (%)	**1.17**	**1.17**	**1.17**	**1.17**	**1.17**
Phosphorus (%)	**0.43**	**0.43**	**0.43**	**0.43**	**0.43**
Lysine	**1.09**	**1.09**	**1.09**	**1.09**	**1.09**
Methionine	**0.45**	**0.45**	**0.45**	**0.45**	**0.45**
Crude Fibre (%)	**5.41**	**5.41**	**5.41**	**5.41**	**5.41**

*Composition of premix: 2.5 kg of premix contains: Vit. A (10000000 iu), Vit. D3 (2500000 iu), Vit. E (12000 iu), Vit. B1 (2000 mg), Niacin (15000 mg), Vit.B6 (1500 mg), Vit.B12 (10 mg), Vit. K3 (2000 mg), Biotin (20 mg), Folic Acid (600 mg), Panthothenic Acid (7000 mg), Chlorine Chloride (150000 mg), Manganese (80000 mg), Iron (40000 mg), Copper (10 mg), Zinc (60000 mg), Selenium (150 mg), Iodine (1000 mg), Magnesium (100 mg), Ethoxyquine (500 g), BHT (700 g)

Experimental animals and procedures

Sexually mature Isa White cocks, 250 in total, of 24 weeks old were sourced from a very reliable farm. The cocks were housed in two tiers battery cages and managed under strict bio-security system. Two cocks

were housed per cell. On arrival, the cocks received the basal ration for 14 days with fresh cold water *ad libitum* for stabilization. After the acclimatization period, the cocks were evenly distributed over the five experimental diets. Each group was in 5 replicates containing 10 cocks. The roosters had unrestricted access to both the experimental diets and clean water throughout the twelve weeks of the experiment. On experimental termination day, plain bottles were labeled to collect blood from 5 cocks in each replicate. The blood samples were left slanted in the rack for a period of a quarter of an hour before being spun for 5 minutes at 5000 rpm and a clean supernatant liquid (serum) was subsequently harvested and stored at -4°F. This was later used for the analyses of TNF-α, IL-10, IL-1β, 8-OHdG, IL-4, and NF-κB.

Determination of serum cytokine levels and DNA damage

The concentrations of the TNF-α, IL-1β, IL-4 and IL-10 were determined using CUSABIO commercial Chicken ELISA Kits while the concentrations of 8-OHdG and NF-κB were equally assayed using MyBioSource Inc. Chicken ELISA Kit.

Statistical analysis

Data collected from the study were statistically analyzed with one-way analysis of variance using SAS software. Duncan Multiple Range Test was used for the post hoc analysis at 5% level.

Results

Effects of AFB_1, Sida acuta and vitamin C on serum pro- and anti-inflammatory cytokines

Figure 1 is the result of serum concentration of interleukin 4 (IL-4). Among the cocks fed diet containing 1 mg/kg AFB_1, serum concentration of IL-4 was lower ($P < 0.05$) compared to the same parameter in the control cocks. However, inclusion of 200 mg/kg vitamin C (Diet C) enhanced the serum IL-4 concentrations of the birds significantly ($P < 0.05$) unlike those fed A and B diets respectively. Furthermore, Diet D, containing 2.5 g/kg SALM, significantly ($P < 0.05$) better improved the IL-4 concentrations of the roosters unlike what recorded for the cocks in groups A to C. Furthermore, inclusion of 5.0 g/kg SALM (Diet E) statistically ($P < 0.05$) increased the IL-4 concentration among the group E cocks. Figure 2 shows the serum IL-10 of cocks fed AFB1, *Sida acuta* and vitamin C. The lower significant ($P < 0.05$) value of IL-10 was recorded among the roosters on Diet B as compared with the means from cocks on all other diets. It was further observed that inclusions of C vitamin as well as varied inclusion of SALM significantly ($P < 0.05$) enhanced the IL-10 concentrations unlike in the control group. The serum concentrations of IL-10 cytokines among the cocks fed SALM were better ($P < 0.05$) than values recorded for roosters on C vitamin.

Conversely, the pro-inflammatory cytokines were significantly ($P < 0.05$) raised in group B roosters. For instance, in Fig. 3 that showed the serum concentration of interleukin 1 beta (IL-1β), the cocks fed 1 mg/kg AFB_1 without C vitamin and SALM displayed a raised ($P < 0.05$) value above the mean values

recorded for the roosters on control diet as well as other experimental diets respectively. However, the inclusion of 200 mg/kg vitamin C reduced significantly ($P < 0.05$) the IL-1β level among the roosters on Diet C unlike as observed in group B and the resultant mean value was comparably ($P > 0.05$) similar with the control group. Also, the inclusions of 2.5 and 5.0 g/kg SALM in the AFB_1 contaminated diets D and E respectively brought about statistical ($P < 0.05$) reductions in IL-1β concentrations when individually compared with those on Diet B as well as the basal diet. However, IL-1β concentrations of birds on diets D and E when compared were notably ($P > 0.05$) similar.

For serum concentration of tumor necrosis factor alpha (TNF-α) (Fig. 4), group B birds had higher noticeable ($P < 0.05$) mean value than those on all other diets. Inclusions of vitamin C and SALM notably ($P < 0.05$) reduced TNF-α level as against the value presented by roosters in group B. The inclusions of vitamin C and 5.0 g/kg SALM brought about statistical ($P < 0.05$) TNF-α value reduction unlike in the basal diet group while the TNF-α concentrations among the control birds and group D were similar ($P > 0.05$) statistically.

Effects of AFB_1, Sida acuta and vitamin C on serum DNA damage

The results of serum DNA oxidation as indicated by serum NF-κB and 8-OHdG concentrations are represented by Figs. 5 and 6 respectively. It was evidently clear that the inclusion of 1 mg/kg diet AFB_1 in the cock's diet significantly ($P < 0.05$) elevated the serum concentrations of 8-OHdG and NF-κB among the cocks in group B as against those in group A. It was further observed that serum DNA oxidation among group C birds was significantly ($P < 0.05$) lowered when compared with birds in group B. Furthermore, inclusions of phyto-antioxidant (SALM) at 2.5 and 5.0 g/kg adequately ($P < 0.05$) suppressed serum DNA oxidation among the cocks on diets D and E. It was also noted that doubling the inclusion of SALM in the diet (diet E) further reduced significantly ($P < 0.05$) the serum DNA oxidation.

Discussion

Pro-inflammatory cytokines are known for enhancing the up-regulation of inflammatory reactions. They midwife the inflammatory diseases of some infectious and noninfectious diseases. For instance, pro-inflammatory cytokines are direct mediators of insulin resistance (Chakraborty et al. 2017). It is a common knowledge that pathological pain process has been explained to be triggered by inflammatory cytokines, chiefly serum IL-1β and TNF-α, in animal physiology (Zhang and An 2007). A study earlier reported that the infusion of TNF-α has proved to initiate insulin resistance in vivo (Chakraborty et al. 2017). The significant elevations of TNF-α and IL-1β levels among the cocks on Diet B in the present study indicated that AFB_1 contamination of the diet had resulted in inflammatory damage. Conversely, the significant depression of serum concentrations of IL-10 and IL-4 respectively, proved that AFB_1 contamination in the diet could potentially militate to offset the immunity of roosters. The results further strengthened Hou et al. (2022) who documented suppression of growth performance and immunity as

well as inflammatory and oxidative stress in chicks in response to dietary AFB_1 inclusion dose-dependently. This was also agreed with the observation of Choi et al. (2010) recording suppression of serum anti-inflammatory cytokines in mice induced with AFB_1. In another experiment, pigs fed AFB1 contaminated diets were observed to have recorded an elevated serum pro-inflammatory cytokine production (Pu et al. 2021). However, our results contradicted Marin et al. (2002) which documented decreased IL-1β and TNF-α levels as well as increased IL-10 level in weanling piglets fed AFB_1 contaminated diets. Furthermore, cytokines have been highlighted to be important immunomodulatory elements within the male gonad. For instance, TNF-α has been implicated in the reduction of fertility in men (Perdichizzi et al. 2007). Another study has equally implicated TNF-α and IL-1β in adversely affecting the functions of sperm cells as a result of increased nitric oxide generation (Lampiao and du Plessis 2008). It has been suggested that leukocytospermia in animals could be linked to increasing levels of IL-1β and has been helpful in the diagnosis of the inflammation of male accessory gland (Stankiewicz et al. 2002). From our results, the elevated serum TNF-α and IL-1β concentrations among the birds on diet B are also indicators of impaired spermatogenesis and fertility occasioned by inclusion of AFB_1 in the diet.

Ascorbic acid (C vitamin) is pharmacologically used to attenuate oxidative stress and inflammation as well as to restore endothelial and organ function due to its antioxidative, anti-inflammatory and immune-boosting effects (Spoelstra-de et al. 2018). The significant reductions in serum TNF-α and IL-1β as well as enhancements of IL-4 and IL-10 concentrations observed among the cocks fed Diet C showed the potency of vitamin C in ameliorating the aflatoxigenic effects of the diet on the experimental birds. This study has given credence to several earlier studies that have documented the potency of vitamins C in reducing the oxidative and inflammatory effects induced by aflatoxins on animals (Alpsoy et al. 2009, Alpsoy and Yalvac, 2011; Dana et al. 2018). Additions of SALM in the diets further proved the inherent therapeutic potency of natural antioxidant in ameliorating the inflammatory and oxidative effects AFB_1 on animals. The fact that the reductions recorded in the serum TNF-α and IL-1β and improvements in IL-4 and IL-10 concentrations of birds on Diets D and E were comparable or better than the results on the artificial antioxidant (diet B) proved that SALM at the inclusion levels used in this study could be employed in ameliorating the negative impacts of AFB_1 in chickens' diets. The results of this study has enlisted SALM as a candidate among other phytoadditives used as therapeutic alternatives to the conventional toxin binders or artificial antioxidants in countering the negative impacts of AFB_1.

Serum 8-OHdG and NF-κB concentrations are among the biomarkers of measuring the DNA damage effect of any treatment in animals. The significant elevation of the two biomarkers in the serum of cocks fed diet B in the present study proved the serum DNA oxidative damage tendency of AFB_1 in cocks when fed up to 1 mg/kg diet. This result corroborated earlier report by Aleissa et al. (2020) who also observed an increased serum 8-OHdG in mice treated AFB_1. When AFB_1 is absorbed into the blood, it is mainly metabolized in the liver to AFB_1-exo-8, 9-epoxide (AFBO) which is a toxic metabolite of AFB_1 by the cytochrome P450 enzyme system and cytoplasmic reductase enzymes. The AFB_1-exo-8, 9-epoxide then binds to DNA to produce AFB_1-DNA adducts which leads to mutations and carcinogenesis (Deng et al.

2020). Our result was also on this same page as Sun et al. (2018) reporting a significantly elevation in serum 8-OHdG of rabbits fed AFB_1 contaminated diet. In the same vein, the significant elevation in serum NF-κB recorded among the cocks on Diet B supported Woźniakowski et al. (2021) documenting a notably high concentration in serum NF-κB of rats fed AFB_1 contaminated diet. Aflatoxin B_1 causes DNA damage by acting as an oxidant which generates reactive oxygen species (ROS) instigating NF-κB and MAPK (mitogen-activated protein kinase) pathways. The regulation of apoptosis, immunity and inflammation is hugely dependent on NFκB-mediated pathway (Li and Verma 2002). It is clear that hetero/homo-dimers are formed from p50 and p65 by NF-κB protein. It is noteworthy that p50 and p65 are in relatively inert state in the cytoplasm due to the formation of a trimer complex with the inhibitory protein IκB. After being activated by an oxidant, such as AFB_1, there is release of IκB away from the trimer. Then, NF-κB dimer is equally opened up to nuclear localization sequences. This also hastily gained access from the cytoplasm into the nucleus and attach to a particular sequence on the nuclear DNA, thereby, promoting the transcription of related genes (Zhang and Ghosh 2001).

The improvement observed in the serum concentration of NF-κB among the cocks fed AFB_1 with vitamin C (diet C) was testimonial of the potency of vitamin C as an antioxidant in ameliorating the serum DNA damage effect of AFB_1. Bowie and O'Neill (2000) had previously explained that vitamin C has the tendency to block the phosphorylation of I-kappa Balpha (inhibitory protein that is released from NF-kappaB) as a result of inhibition of I-kappa B kinase (IKK) activation. The significant reduction in the serum concentrations of AFB_1 among the cocks on diets D and E equally demonstrated the usefulness of phytoadditives in ameliorating the serum DNA destructive effects of AFB_1. Phytochemicals have been proved to be potent enough in activating NF-κB and MAPK signaling pathways and enhancing health conditions by suppressing inflammation (Huang and Lee 2018). Phytochemicals such as curcumin from turmeric, cinnamaldeyde and some others were reported in inhibiting NF-κB activation by modifying the NF-κB pathways and thereby protecting against oxidative stress and induction of inflammatory responses and consequently bringing about the improvement of animal health and growth performance (Yang et al. 2015). The reduction of the serum NF-κB observed among the birds on diets D and E, therefore, qualified SALM to be enlisted among the natural antioxidant candidates to be considered in ameliorating the serum DNA damage effects of AFB_1 in chickens.

Conclusions

In conclusion, contamination of cocks' diet with AFB_1 up to 1.00 mg/kg diet is capable of instigating pro-inflammatory cytokines and suppressing the anti-inflammatory cytokines as well as predisposing birds to DNA oxidative damage thereby compromising the health status of the animals. However, the inclusion of 200 mg vitamin C, 2.50 and 5.0 g/kg diet *S. acuta* leaf meal conferred an ameliorative and immunomodulatory effects on the cocks thereby enhancing their immunities against any external stressor.

Declarations

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Author contributions

Olumuyiwa Joseph Olarotimi: Conceptualization, Methodology, Formal analysis, Writing- Original Draft, Writing - Review and Editing; Francis Ayodeji Gbore: Supervision; Olufemi Ademola Adu: Resources; Olugbenga David Oloruntola: Project administration; Olatunji Abubakar Jimoh: Validation

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Data availability

The data collected in the course of this study are accessible through the corresponding author on reasonable request.

Ethical statement

The entire procedure complied with the guidelines for the Care and Use of Laboratory Animals, and the experimental protocol was approved by the institution's Animal Research Ethics Committee. Ethics Reference No: AAUA/FA/ANS/4750/2023.

Declaration of Competing Interest

We have no conflict of interest.

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Figures

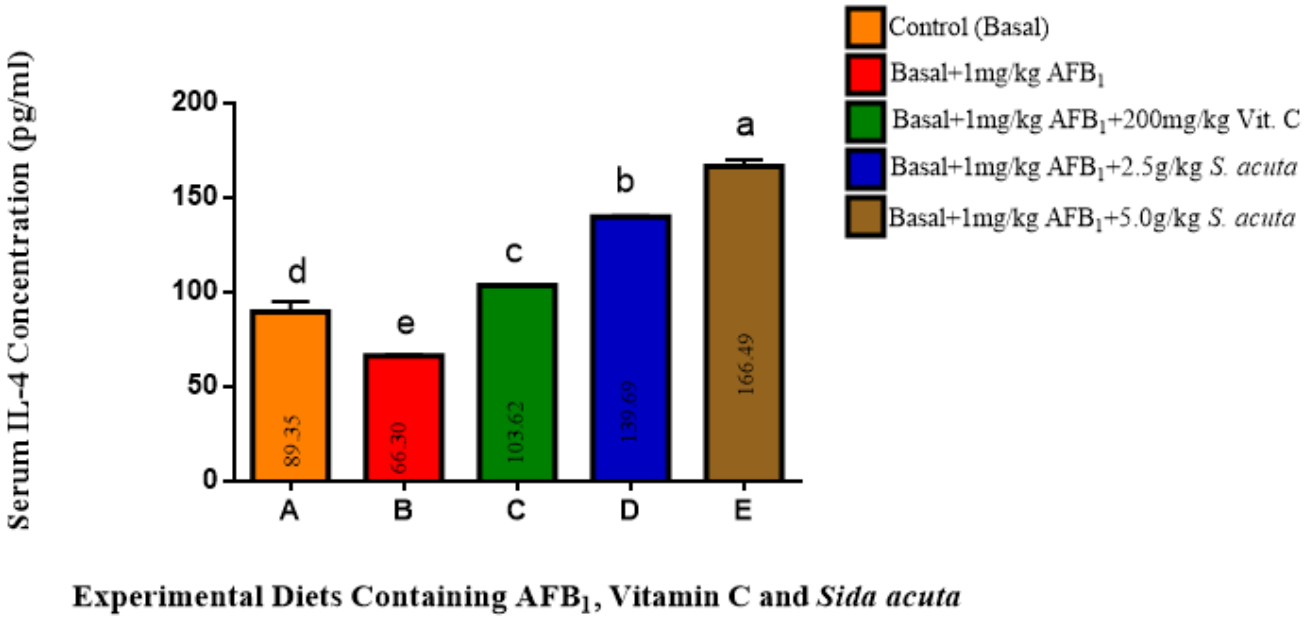

Figure 1

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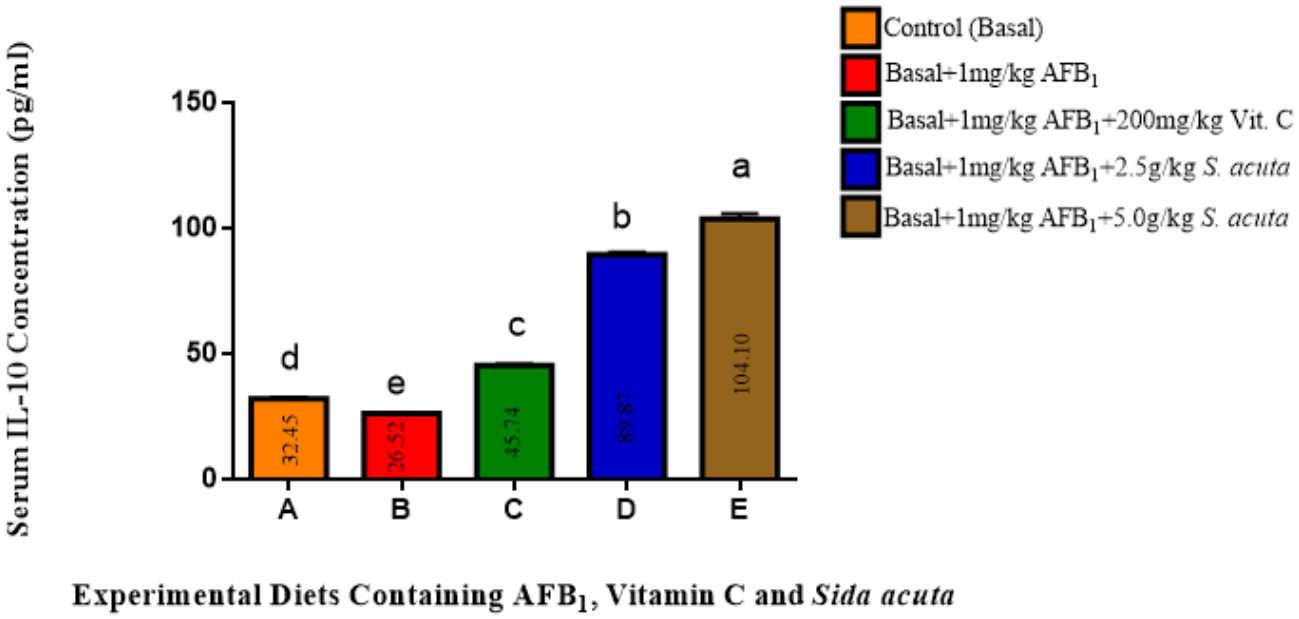

Figure 2

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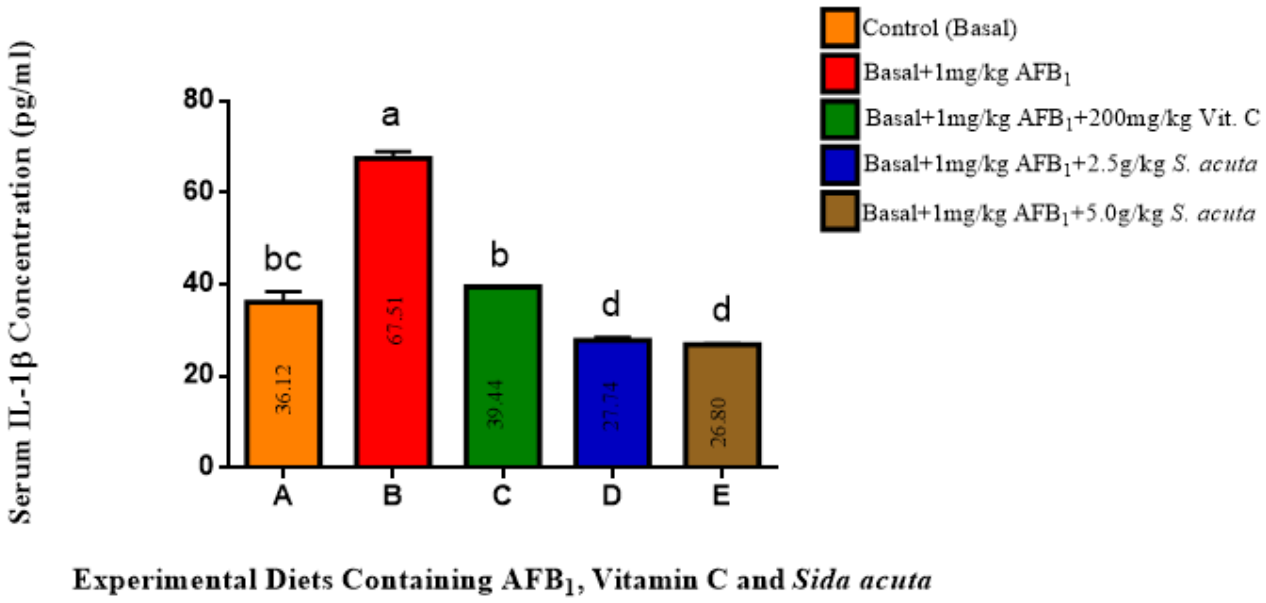

Figure 3

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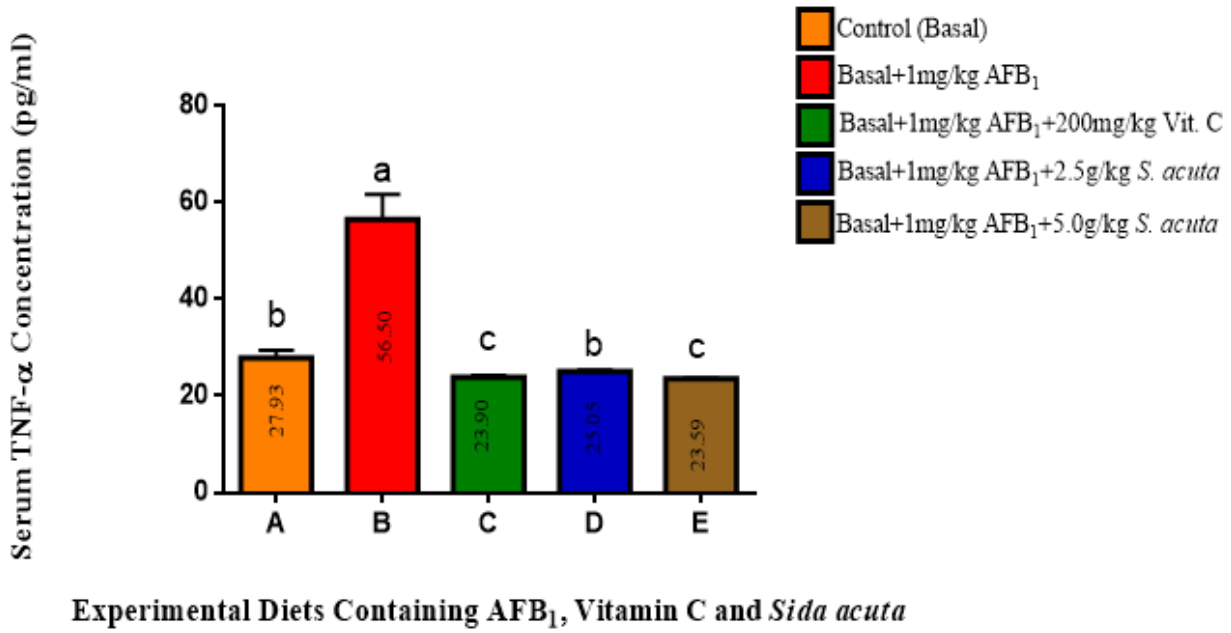

Figure 4

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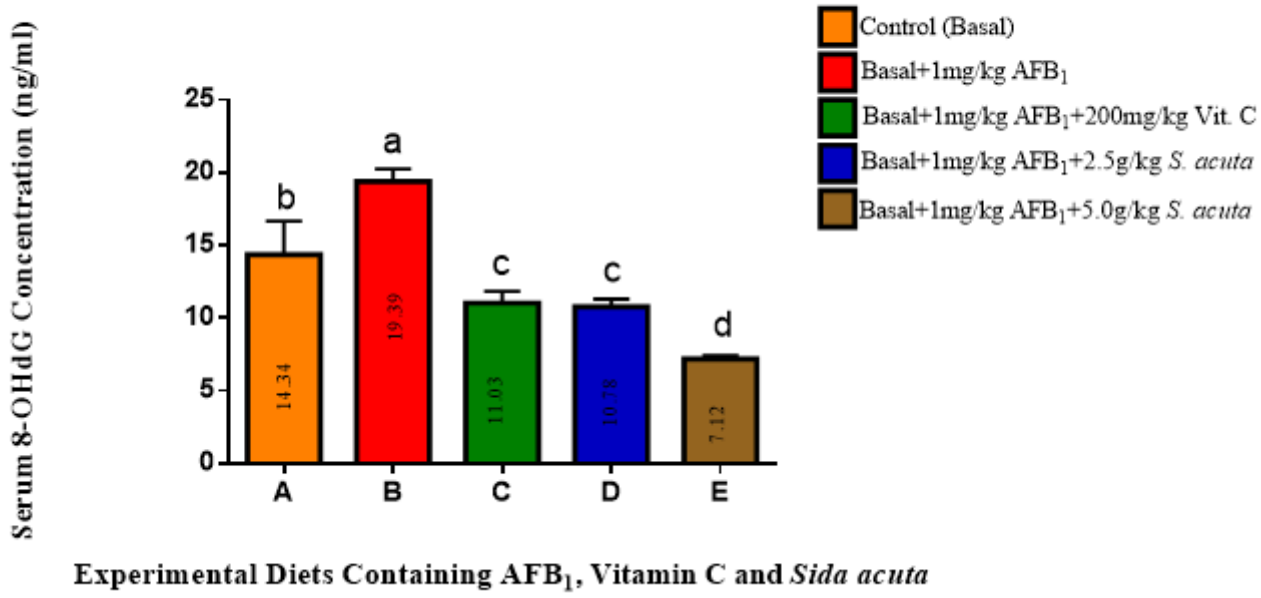

Figure 5

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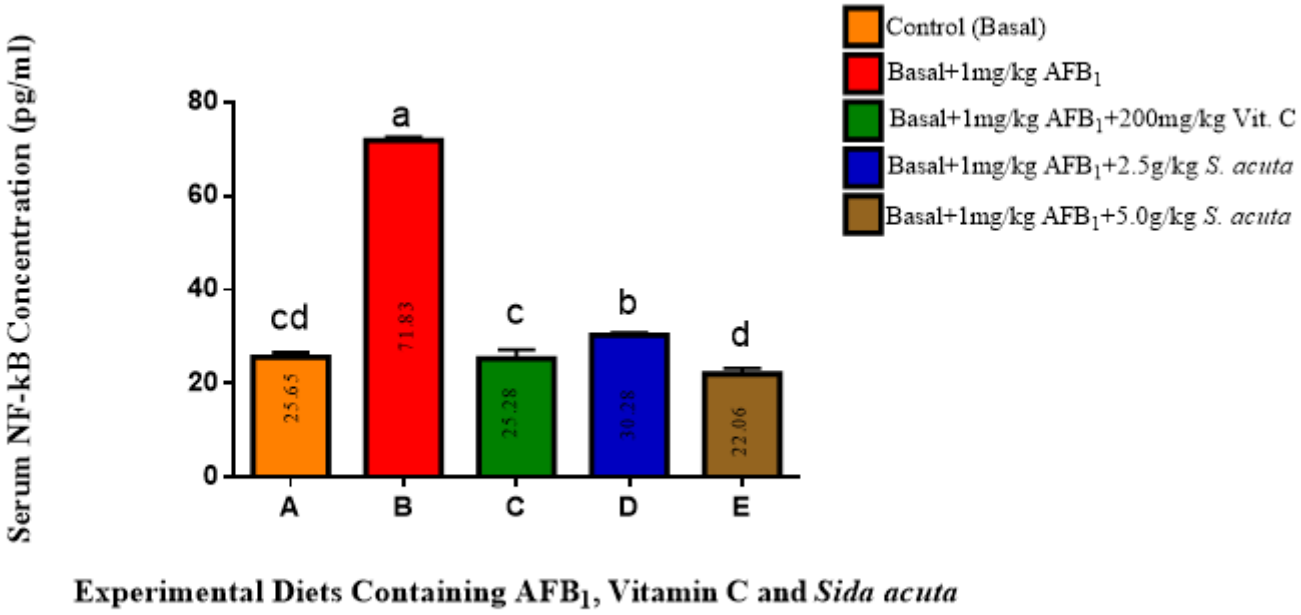

Figure 6

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