Anti-aflatoxin B1 effects of Shirazi thyme (*Zataria multiflora*) in broilers: evaluation of performance and liver histopathology

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Abstract

An experiment was conducted to study the effect of *Zataria multifora* (ZM) on the performance and liver histopathology of broiler chickens contaminated with aflatoxin B_1 (AFB1). One hundred and sixty Ross 308 male broilers (one-day-old) were divided into four treatment groups with four replicates with 10 birds in each replicate. The chickens were reared on the floor for 35 days. The groups were contaminated with AFB1 at two different concentrations, *i.e.*, 0 and 1000 ppb, and fed ZM in their feed at the concentrations of 0 and 20 gr Kg^1. The evaluated performance parameters were subjected to a completely randomized design with a 2×2 factorial arrangement of the treatments using SAS software (version 9/1). AFB1 had a statistical lowering effects on the feed intake, body weight, body weight gain and average weight of the carcass, thigh, chest, bursa of fabricius, back and neck. Also, the weights of liver, gizzard, pancreas, proventriculus, abdominal fat, full intestine, and heart were increased with AFB1 ($P<0.05$). In histopathological evaluations, the liver of chickens that received feed containing AFB1 showed multifocal and varied cytoplasmic vacuolization, severe fatty change, degenerating foci, fibrosis of the portal regions, and bile duct hyperplasia. The variables that were evaluated in this study showed that ZM had significant efficacy in diminishing the aflatoxins negative effects on the chickens.

Introduction

Aflatoxins (AFs) are a major group of mycotoxins that are produced by certain species of *Aspergillus*, *i.e.*, *A. flavus, A. parasiticus, Penicillium* and *Rhizopus spp*. Among this group, *A. parasiticus* is the most common fungus, and *A. flavus* produces the highest amount of aflatoxin B_1 (AFB1).[1,2] Common AFs in the poultry industry are B_1, B_2, G_1, and G_2, and the most prevalent aflatoxicosis is caused by AFB1.[1-3] The toxic effects of AFs arise primarily from the binding of their particular epoxide derivation to deoxyribonucleic acid (DNA).[4,5] Also, the toxic effects of AFB1 can be either acute or chronic, based on the dosage and duration of usage.[1,3] Exposure to AFs may lead to one or more biological activities, such as oxidative stress, acute toxicity, teratogenicity, mutagenicity, and carcinogenicity.[6] *Zataria multiflora Boiss* (ZM) is a thyme-like plant that belongs to the *Lamiaceae* family. This plant has the vernacular name of Avishan-e-Shirazi (Shirazi thyme), and it is a valuable medicinal plant in Iran.[7] ZM is composed of a variety of compounds, including alpha-pinene, alpha-thyjene, thymol, cis-sabinene hydrate, para cymene, 1.8-cineole, myrcene, and sabinene.[8] Probably, the presence of phenolic compounds, such as thymol and carvacrol, as the main compounds results in effective anti-*A. flavus* and anti-*A. parasiticus* properties.[9,10] A few studies have been conducted to determine the anti-fungal effects of ZM on histopathological changes in and the functioning of the liver in broilers infected with AFs. The main target organ for AFs is the liver.[5,11] AFB1 causes pathological injuries to the liver.[12] Chand and colleagues[13] reported that giving broilers feed containing 80 µg kg^{-1} of AFB1 alone, can decrease the FI and body weight.[13] This experiment was performed to assess the protective effects of ZM on the performance of the liver as well as any morphological and histopathological changes that occurred in the livers of chickens infected with AFB1.

Materials and Methods

Experimental chickens and diets

In this study, we used three different feeds for birds, depending on their age, *i.e.*, i) starter feed (1-14 days); ii) grower feed (15-28 days); and iii) finisher feed (29-35 days) (Table 1). The different feeds were set based on the broilers' requirements according to the recommendations of the Ross 308 Company, and they were adjusted by the User-Friendly Feed Formulation Program (UFFDA) software. The study included 160 broiler chickens acquired from Ross 308 and that were reared on the floor until they were 35 days old. They were divided randomly into four groups with four replicates and 10 birds in each experimental unit. This study was conducted as a 2×2 factorial plan and based on a completely randomized design. The experimental groups were as follows: i) the control group with the basal diet alone; ii) the basal diet contaminated with 1000 ppb AFB1; iii) the basal diet plus 20 g kg^{-1} ZM; and iv) the basal diet containing 20 g kg^{-1} of ZM and 1000 ppb AFB1. ZM and AFB1 were administered daily by gavage to ensure the administration of the correct dose. The experiment was approved by the animal welfare committee in the Research Center for Special Domestic Animals, Zabol University, Zabol, Iran.

Performance variables

Feed intake (FI), body weight (BW), and body weight gain (BWG) were measured every week for each replicate of groups. Feed conversion ratio (FCR) was calculated.[14] At the end of the study, after four hours without any feed, two male birds from each experimental unit were randomly selected and slaughtered by cervical dislocation. Immediately after slaughtering and defeathering, they were eviscerated, and the relative weight of the carcass,

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thighs, and breasts to the percent of carcass weight and the ratio of the weight of internal and sensitive organs to 100 g of carcass weight were determined. The relative weight of the carcass, thigh, breast, full intestine, abdominal fat, and sensitive internal organ weights were recorded.

Morphological and histopathological variables

After slaughter, chicken livers from each replicate were weighted. The liver tissues were examined morphologically and then fixed in natural formalin buffer 10% (Merck, Germany) for histopathologic studies. The tissues were immersed in paraffin, and blocks were made. The paraffin blocks were cut and colored with hematoxylin and eosin (H&E stain), and slides were prepared and studied with light microscopy.[15]

Statistical analyses

The data were analyzed in a completely randomized design with a 2×2 factorial arrangement of the treatments using statistical analysis software (SAS).[16] The data were compared with Tukey-Kramer post hoc tests. Least squares means ± standard errors were reported.

Results

Performance

The results of the chickens' performance are presented in Supplementary Tables S1-6. Feeding the chickens different levels of AFB1 had a significant effect on the amount of FI, BW, and BWG ($P<0.01$). In contrast, statistical analysis results related to interactions of FI at the ends of the first through the fourth weeks of the study demonstrated no significant changes compared to the controls (Supplementary Table S1). The results of the statistical analysis related to interaction effects of BW and BWG at the end of the first through the third weeks of the experiment showed no significant effects of the consumption of the experimental feeds compared to the weights of the chickens that received the control feed (Supplementary Tables S2 and S3, respectively). The results of this study indicated that feeding with different levels of AFB1 plus ZM had no effect on FCR in chickens during the experimental period (Supplementary Table S4) ($P>0.05$). The results of interaction effects related to carcass variables at the end of the period (35 days) indicated that there were no significant changes in the composition of the carcasses; for example, in comparison with the controls, there were no significant changes in the weights of the legs, wings, and spleens (Supplementary Tables S5 and S6) of the treated chickens. However, the different levels of AFB1 in the feeds did results in weight changes in abdominal fat, full intestine, liver, gizzard, pancreas, proventriculus, heart, and bursa of Fabricius ($P<0.05$).

Morphological and histopathological studies

At the end of the experimental period (35 days), the birds that received feed contaminated only with 1000 ppb AFB1 had larger, more delicate and tender livers with round edges (Figures 1 and 2). The histopathological find-

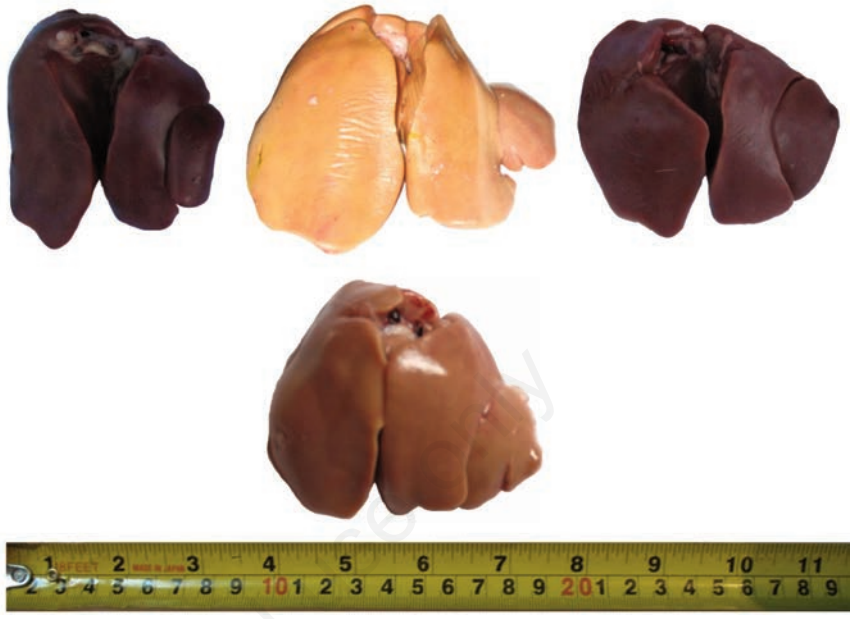

Figure 1. Liver morphology of birds intoxicated with aflatoxin B_1 (AFB1): control (upper left); AFB1 toxicosis: paleness and yellow discoloration (middle and upper); ZM: normal liver morphology (upper right); AFB1 + Shirazi thyme (*Zataria multifora*, ZM): yellow discoloration (bottom).

Table 1. Composition of the starter, grower and finisher diets fed to broilers (as fed).

Feed stuffs	Starter period (1-14 day)	Grower period (15-28 day)	Finisher period (29-35)
Corn	54.43	50.42	45.99
Wheat	-	10.00	20.00
Soybean meal (44% CP)	35.00	30.29	25.56
Fish meal (60% CP)	3.070	2.040	1.060
Soybean fat	3.290	3.570	3.760
Di-calcium phosphate	1.730	1.470	1.490
Oyster shell	1.160	1.040	1.020
Mineral premix*	0.500	0.500	0.500
Vitamin premix°	0.500	0.500	0.500
Salt	0.210	0.210	0.211
DL-methionine	0.350	0.280	0.241
L-lysine	0.240	0.190	0.211
Analyzed values			
ME (Kcal kg^{-1})	2980	3050	3100
CP (%)	22.00	20.00	18.00
Lys (%)	1.430	1.240	1.090
Met + Cys (%)	1.070	0.950	0.860
Thr (%)	0.310	0.280	0.260
Ca (%)	1.050	0.900	0.850
P (%)	0.520	0.450	0.420

*Provided at the following rates per kilogram of diet: Mn (from $MnSO_4$-H_2O), 0.63 mg; Zn (from ZnO), 0.52 mg; Fe (from $FeSO_4$-$7H_2O$), 22 mg; Cu (from $CuSO_4$-$5H_2O$), 3 mg; I (from Ca $(IO_3)2$-H_2O), 0.63 mg; Se, 0.08 mg (from sodium selenite). °Provided at the following rates per kg of diet: 3400 IU vitamin A, 800 IU vitamin D_3, 11 IU vitamin E, 0.74 mg vitamin B_1, 4.3 mg vitamin B_2, 0.4 mg vitamin B_3, 1.6 mg vitamin B_6, 0.41 mg vitamin B_{12}, 1.8 mg vitamin K_3, 0.6 mg folic acid, 1.8 mg H2, 200 mg Choline chloride.

ings in the liver sections of broilers that received feed with 1000 ppb AFB1 showed multi-nuclear cells in the portal area (Figure 3A), hemorrhage, and hyperplasia (Figure 3B), necrosis, and fat decaying in hepatocytes (Figure 3C). These pathologic findings were more obvious in chickens that received 1000 ppb of AFB1 compared to the chickens that received feed containing 1000 ppb of AFB1 plus 20g Kg^{-1} of ZM (Figure 4).

Discussion and Conclusions

In accordance with some research, no changes were observed in the appearance of the treated chickens in the first and second weeks of study.[1,5] Beginning with the third week, we observed the gradual development of low growth rate, feathers confusion, pallor crown, and aggressive and nervous symptoms in chickens that received feed containing only 1000 ppb AFB1 compared to chickens that received the control feed.[11,13] In comparison with chickens that received the control feed, no similar symptoms were observed in birds fed with ZM.[10] However, FI in chickens that received feeds with 1000 ppb AFB1 was lower than other groups in the fifth week ($P<0.01$). The use of ZM in the diets (20 g Kg^{-1} of feed) increased the FI of contaminated diets with 1000 ppb AFB1. Some authors have reported that the phenolic compounds found in ZM, such as thymol and carvacrol, can increase FI and BW in rats that received AFB1.[10,17] Also, a few studies have been performed to determine the interaction effects of AFB1 plus ZM in humans and other animal species. After the end of the third week, the results demonstrated that the average BW and BWG in the treated chickens that took diets contaminated with 1000 ppb AFB1 were significantly lower than the control ($P<0.01$). The main effects of the FI and BW of the chicks fed only with ZM were consistent with the findings of Cross and colleagues;[18] they fed chickens different levels of phenolic ZM compounds as beverages. However, the results were inconsistent with those of other authors.[19,20] Nevertheless, with increasing levels of AFB1 plus ZM at the first through the fifth week of this study (35 days old), FCR did not change ($P>0.05$). The effective impacts of plant products and derivatives on the growth and functional properties relate to several variables, including the stimulatory effects of these products on the gastrointestinal digestion process, stimulating and increasing the secretion of digestive enzymes, increasing the efficiency of nutrient feed,

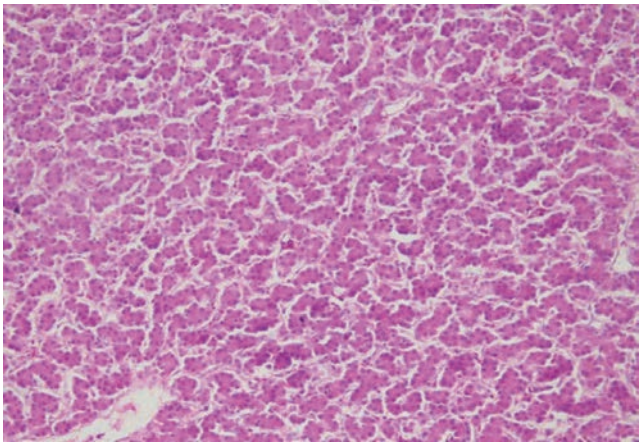

Figure 2. Normal histological appearance of the liver from a control broiler chicken (fed basal diet alone), hematoxylin and eosin 100×.

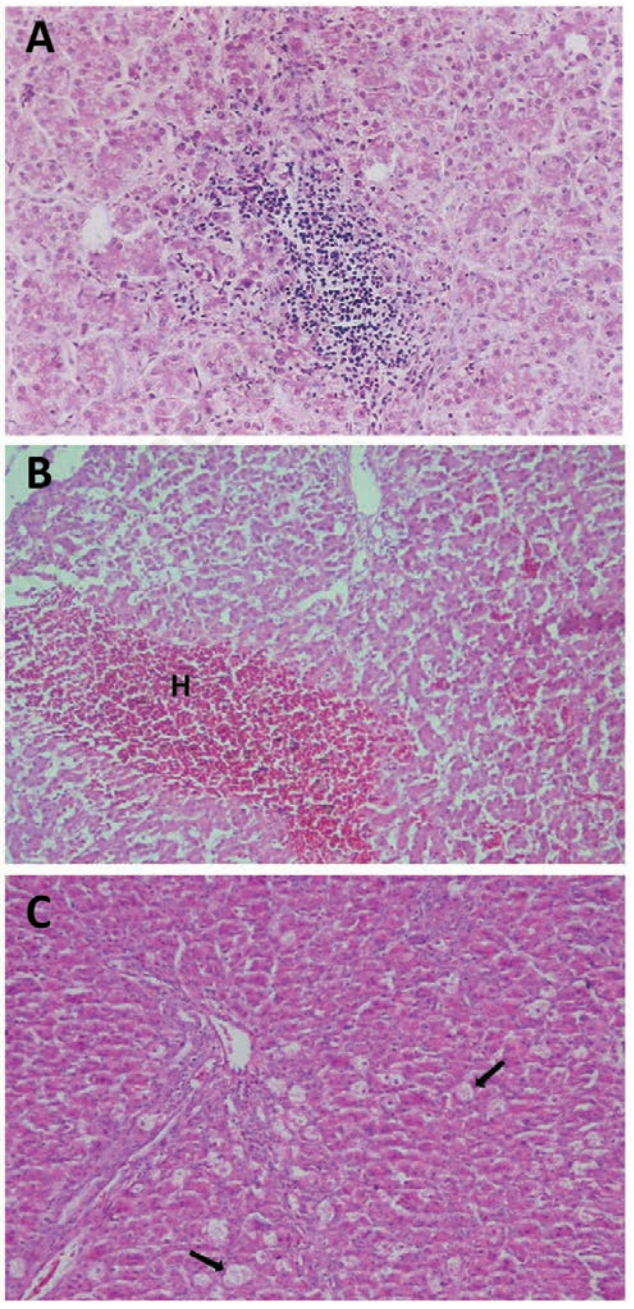

Figure 3. A) Parenchymal and mononuclear cell aggregation in liver of chicken fed 1000 ppb AFB1 contaminated diet, [hematoxylin and eosin (H&E) 100×]. B) Liver histology from a broiler chicken contaminated with 1000 ppb AFB1. Note the hemorrhage (H) in the liver tissue, (H&E ×100). C) Liver histology from a broiler chicken contaminated with 1000 ppb AFB1. A severe degeneration of the hepatocytes and loss of some nuclei (arrows) were observed, (H&E ×100).

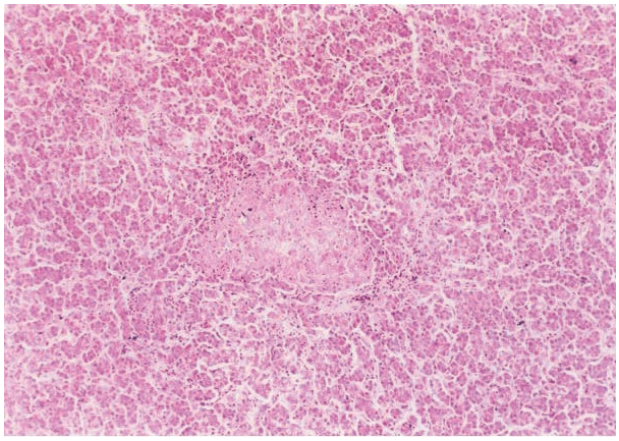

Figure 4. Restoration in focal area of hepatic necrosis in chicken fed 1000 ppb Aflatoxins + 20 gr kg^{-1} Shirazi thyme (*Zataria multifora*); hematoxylin and eosin ×100.

increasing the efficiency of the liver, and increasing the appetite due to flavors.[21]

The statistical analysis of carcass efficiency data presented as a proportion of the live weight showed a significant decrease ($P<0.01$) in the carcass ratio, thighs, breast, back, and necks of the chickens that received feeds that were contaminated with 1000 ppb of AFB1 compared to chickens that received feeds containing 20 g Kg^{-1} of ZM alone or combined with 1000 ppb AFB1. Other researchers have observed a significant decrease in the weights of the breasts and thighs of the treated chickens that received feed containing AFB1 alone compared to chickens that received the control feed.[2,21] The results showed that increasing the AFB1 concentrations in the feeds increased the variability of the weight levels (abdominal fat, full intestine, liver, gizzard, pancreas, proventriculus, and heart) and, on the contrary, significantly decreased the weight of the bursa of Fabricius compared to the controls. Miazzo and colleagues[22] reported that feeding with only 2.5 ppm of aflatoxin caused a significant increase in the weights of most of the commercial male Ross 308 broiler chickens' organs (liver, spleen, kidney, and gizzard). Also, Kermanshahi and colleagues[23] reported that feeding with 500 and 1000 µg Kg^{-1} AFB1 caused a significant increase in the relative weights of the livers and brains in male Ross 308 broiler chickens. No results have been reported concerning the effects of ZM and AFB1 on morphological and histopathological changes in the broiler chicks' liver tissues. The livers of birds that received feed contaminated with AFB1, were pale and yellowish. Pale livers were more obvious in the birds fed with AFB1 alone. Our observations of liver damage caused by AFB1 were consistent with those of some other authors.[24-26] The supplementation of feeds contaminated with 1000 ppb AFB1 plus 20 g Kg^{-1} of ZM indicated that ZM significantly decreased the effects of AFB1 on the experimental birds. These results are in accordance with some other authors' reports on Japanese quail[24] and rats.[9,10] These necrotic centers were surrounded by a delicate connective tissue and consisted of large multi-nuclear cells and hyperplasia without any fat vacuoles.[9,24,25] Some liver cells contained a lot of large nuclei and chromatin (megasitosis).[9,10,24] Fibrosis of the portal area was observed to be increasing the connective tissue with the proliferation of small bile ducts.[9,10,24-26] The histological findings confirmed the biochemical results and indicated that AFs induced severe histological changes in the hepatic tissues. Similar histological changes in the liver have been documented previously in rats.[4,6,27] The livers of the birds that received feeds containing 20 g kg^{-1} ZM were roughly normal and showed discrete diffusion cell swelling with mild effects on the hepatocytes along with mild cytoplasmic changes. However, mild positive effects on hepatocytes, infiltrating of mononuclear cells, and hyperplasia were observed in the livers of the birds that received 20 g Kg^{-1} ZM and 1000 ppb AFB1. The improved histological and histochemical appearance of the liver in broilers fed with AFB1-contaminated feed and treated with ZM revealed the protective role of the ZM against AFB1-induced liver injury.[28] These effects may be related mainly to the anti-oxidant and free radical scavenging properties of ZM.

In conclusion, the phenolic compounds that exist in the seeds of ZM can ameliorate the histopathological liver damages caused by AFB1. According to this research, 20 g Kg^{-1} of ZM is effective. Further investigations in this field in the future are recommended.

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