Efficacy of *Silybum Marianum* Seeds in Ameliorating the Toxic Effects of Aflatoxin B_1 in Broilers

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ABSTRACT

Background: This study aims to evaluate the efficacy of *silybum marianum* seeds (SMSs) on blood biochemical profile of broiler chickens contaminated with Aflatoxin B_1 (AFB1).

Methods: Combinations of three levels of AFB1 (0, 250 and 500 ppb) with three levels of SMSs (0, 0.5, and 1.0 %) were incorporated into the basal diet (corn and soybean meal). The effect of nine experimental treatments was assessed using 216 One-d-old Ross 308 male broiler chicks in a complete randomized design based on factorial design with 4 replicates of six birds. The individual effects of dietary AFB1 and SMSs on serum biochemistry factors and liver enzymes were evaluated at 35 days of age. Statistical package SAS (9.1) was used to perform the analysis.

Results: The main effects of uric acid, glucose, total bilirubin and liver enzymes (such as; aspartate amino-transferase (AST), alanine amino-transaminase (ALT) and γ-glutamyl transferase (GGT)) in groups received different levels of AFB1 significantly increased ($P<0.01$). In contrast, albumin, direct bilirubin, calcium, and phosphorus significantly decreased ($P<0.05$). However, the SMSs supplemented diets significantly decreased uric acid, glucose, AST and GGT enzymes compare to control group ($P<0.01$).

Conclusion: SMSs might prevent the adverse effects of AFB1 in contaminated food and improve safety and quality of poultry products for human use.

Keywords: Aflatoxin B_1, Broiler Chickens, Liver Enzymes, Silybum Marianum Seeds.

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INTRODUCTION

Aflatoxins (AFs) are primarily produced by *Aspergillus flavus* and *Aspergillus parasiticus*. They contaminate a wide variety of foods and seeds, including maize, oilseeds, spices, groundnuts, tree nuts, milk, and dried fruits [1]. Presence of aflatoxins in food chain is associated with a decrease in quality and quantity of food and feed materials. Consumption of aflatoxin-contaminated products poses risk of developing various diseases in human and animals. AFs are produced by toxigenic fungi after undergoing biosynthesis pathway involving several enzymes and chemical reactions. Upon consumption of aflatoxin-contaminated products by human and animals, the toxin undergoes metabolism via cytochrome P450 enzymes in the liver [1]. Different toxic metabolites are produced by aflatoxin (AF) metabolism in mammalian organs which can exert adverse effects. AF epoxide (8, 9-epoxide) is the main toxic metabolite which can bind to DNA and induce hepatocellular carcinomas [2]. For these reasons, intensive research has been pursued to develop cost-effective and safe methods to reduce the effects of AFB_1, including supplementation of foodstuffs with non-nutritive sorbents, such as bentonite or

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hydrated sodium aluminosilicate [3]. These compounds produce large molecules that cannot be carried across the intestinal wall. Moreover, the binding spectra of these substances are broad and nonspecific nutritional components, such as vitamins and minerals that may also be removed [4]. Several studies have recently focused on the inhibition of AF biotransformation into its 8, 9-epoxide constituents through interaction with cytochrome P450 enzymes [5] or natural compounds [6, 7].

The bioactive extract from *silybum marianum* (milk thistle), silymarin, contains a mixture of flavonolignans, though its residual fraction has not yet been defined chemically in detail [8]. Silymarin is used in humans for the treatment of numerous liver disorders characterized by degenerative necrosis and functional impairment [9]. Its mechanism of action still seems to be poorly understood, but data suggest that it acts as an antioxidant, cell membrane stabilizer, and permeability regulator as well as a promoter of DNA, RNA, and protein synthesis [10]. Silybin, a major constituent of silymarin [11], has been shown to preserve the functional and structural integrity of hepatocyte membranes by preventing alterations of their phospholipid structure produced by carbon tetrachloride and by restoring alkaline phosphatase and GGT activities [12, 13]. The hepatoprotective actions of silymarin and silybin have been studied in acute liver intoxication induced by toxic agents. Rastogi *et al*. (2000) reported that silymarin alleviates pathological changes in liver and serum in AFB_1 intoxicated rats, indicating its hepatoprotective action in preventing AFB_1-induced injury [14]. An antitoxic effect is also suggested which causes lower milk excretion of AFM_1, an AFB_1 metabolite observed in dairy cows receiving silymarin [15]. This suggests that silymarin may contribute to the prevention of aflatoxicosis-induced damage. The present study was conducted to evaluate the efficacy of silybum marianum seeds against aflatoxicosis in experimentally-intoxicated broiler chickens.

MATERIALS AND METHODS

Experimental Design

Three levels of AFB_1 (0, 250, and 500 ppb) with three levels of SMSs (0, 0.5 and 1.0 %) were incorporated into the basal diet (corn and soybean meal). The basal diet was formulated using commonly available food ingredients which had been screened for AFB_1 prior to the formulation of diets. The effect of nine experimental treatments (3×3 factorial design) was assessed using 216 One-d-old Ross 308 male broiler chicks in a complete randomized design with four replicates of six birds each at five weeks of age. Food and water were provided *ad libitum* and a continuous lighting schedule was used all through the experimental period.

Experimental Diets

Diets were isoenergetic and isoproteinous and they were provided in mashed form. The dietary treatment in a 3×3 factorial fashion was as follows: i.e., the first group was kept as control (T_1) and the other groups received the feed contained 250 ppb of AFB_1 (T_2), 500 ppb of AFB_1 (T_3), 0.5% of SMSs (T_4), 0.5% of SMSs Plus 250 ppb AFB_1 (T_5), 0.5% of SMSs Plus 500 ppb of AFB1 (T_6), 1.0% of SMSs (T_7), 1.0% of SMSs Plus 250 ppb AFB1(T_8), and 1.0% of SMSs Plus 500 ppb AFB1 (T_9) for 5 weeks. Basal diet was formulated and compounded to meet the nutritional requirements of broiler chicks based on Ross 308 recommendation strain during the whole period of experiment without inclusion of either AF or binder (Table 1). Treatments were administered daily to assure the correct dose administration and to ensure the safety of the operators. The objective of this study was to assess the biochemical parameters of broiler fed with AFB1 and evaluate the counteracting effects of the SMSs binding agent.

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

Finisher Period (28-35)	Grower Period (14-28 day)	Starter Period (1-14 day)	**Feed Stuffs**
45.96	50.42	54.46	Maize
25.56	30.29	35	Soybean meal (44 % CP)
20	10	-	Wheat
1.06	2.04	3.07	Fish meal (60 % CP)
3.76	3.57	3.29	Soybean Fat
1.49	1.47	1.73	Dicalcium phosphate
1.02	1.04	1.16	Oyster shell
0.25	0.25	0.25	Mineral Premix[2]
0.25	0.25	0.25	Vitamin premix[3]
0.2	0.2	0.2	Salt
0.24	0.28	0.35	DL-methionine
0.21	0.19	0.24	L-lysine
Analyzed values			
3100	3050	2980	ME (Kcal kg^{-1})
18	20	22	CP (%)
1.09	1.24	1.43	Lys (%)
0.86	0.95	1.07	Met + Cys (%)
0.26	0.28	0.31	Thr (%)
0.85	0.90	1.05	Ca (%)
0.42	0.45	0.52	P (%)

[1] For preparing Aflatoxin (AF) and *silybum marianum* seeds (SMSs) diets, AFB1 and SMSs was added to the basal diets at 0, 250 & 500 ppb and 0, 0.5 & 1% respectively.

[2] 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).

[3] Provided at the following rates per kilogram 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 H_2, 200 mg choline chloride.

Biochemical Parameters and Intestinal Length Variables

Blood samples from two chicks of each group were collected by puncturing the brachial vein. The blood sample was allowed to stand for one hour and centrifuged at a speed of 3.000 rpm for 10 minutes. The clear serum was collected in sterilized disposable plastic tubes and stored at -20 ºC. The individual serum samples were analyzed for total proteins, albumin, uric acid, glucose, total bilirubin, direct bilirubin, calcium, phosphorus, alanine amino transferase (ALT), aspartate amino transferase (AST), and gama-glutamyl transferase (γ-GT). Also, the methodology for each parameter was based on recommendations of the manufacturer of the analytical system.

Statistical Analysis

The obtained data were analyzed using the General Linear Model procedure of Statistical Analysis System (SAS) software [16]. Period-wise data were analyzed by 3×3 factorial manner. Overall period data were analyzed by repeated measurement design. Tukey-kramer test at 0.05 and 0.01 probability levels was employed for comparison of the means [17]. The experiment was approved by the Animal Welfare Committee of the Agriculture Faculty of University of Birjand with special respect to ethical issues of experimental birds.

RESULTS

Statistical analysis of the main effects of diets at the end of the experiment (day 35) indicated no significant changes in total protein level of serum on broilers fed with different levels of AFB_1 (T_2 and T_3), compared to the control (Table 2). However, addition of 500 ppb of AFB1 to the diet significantly decreased albumin (12.1 gr/L), direct bilirubin (2.22 µmole/L), calcium (7.67 mmole/L) and phosphorus (4.85 µmole/L) compared to the control ($P<0.01$). In contrast, uric acid (2.95 mmole/L), glucose (14.4 mmole/L), total bilirubin (7.37 µmole/L), ALT (19.81 U/L), AST (248.91 U/L), and γ-GT (18.58 U/L) significantly increased ($P<0.01$), (Table 2 and 3). Supplementation of diets with SMSs alone supplemented markedly decreased uric acid and glucose ($P<0.01$) as well as AST and γ-GT ($P<0.01$) (Table 3).

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Table 2. Effect of aflatoxin B_1 (AFB_1) and *Silybum marianum* seeds (SMSs) on serum biochemical values of broilers at the end of the period (35 days).

Phosphorus	Calcium	Direct bilirubin	Total bilirubin	Glucose	Uric acid	Albumin	Total protein	**Treatment**	
(µmole/L)	(mmole/L)	(µmol/L)	(µmol/L)	(mmol/L)	(mmole/L)	(g/L)	(gr/L)	SMSs (%)	AFB1 (ppb)
6.78 [a]	9.85 [a]	2.65 [a]	6.78 [b]	12.5 [b]	1.62 [b]	16.5 [a]	30.8	-	0
5.31 [b]	8.24 [b]	2.40 [b]	7.23 [a]	12.5 [b]	2.89 [a]	15.1 [ab]	31.2	-	250
4.85 [b]	7.67 [b]	2.22 [c]	7.37 [a]	14.4 [a]	2.95 [a]	12.1 [b]	30.8	-	500
5.66	8.54	2.23 [b]	7.08	14.2 [a]	2.77 [a]	13.9	27.8 [b]	0	-
5.88	8.62	2.45 [a]	7.10	12.7 [b]	2.31 [b]	14.9	32.7 [a]	0.5	-
5.40	8.61	2.58 [a]	7.20	12.6 [b]	2.38 [b]	14.7	32.1 [a]	1.0	-
								Intraction effects	
6.93	8.55 [ab]	2.39	5.21	12.91	1.46 [bc]	15.4	31.4	0	0
9.44	9.34 [ab]	2.24	6.21	13.81	2.73 [a]	13.2	28.6	0	250
6.39	11.11 [ab]	2.33	7.32	15.31	3.31 [a]	13.6	25.2	0	500
7.10	10.3 [ab]	2.64	6.91	12.21	1.61 [c]	16.2	33.6	0.5	0
5.70	8.45 [ab]	2.41	7.14	11.51	2.85 [a]	15.2	31.2	0.5	250
4.85	7.07 [b]	2.32	7.24	13.52	2.47 [ab]	13.2	34.0	0.5	500
7.20	11.45 [a]	2.82	6.81	11.53	1.30 [c]	17.5	36.2	1.0	0
4.65	7.50 [b]	2.62	7.33	10.52	2.75 [ab]	16.4	32.3	1.0	250
4.37	6.85 [b]	2.30	7.45	14.32	2.81 [ab]	10.7	28.4	1.0	500
								Probabilities	
0.05	0.05	0.05	0.05	0.01	0.01	0.05	NS		AFB1
NS	NS	0.05	NS	0.01	0.01	NS	0.01		SMSs
NS	0.05	NS	NS	NS	0.05	NS	NS		AFB1×SMSs

[a,b] Main effect means within a column lacking a common superscript differ significantly ($P<0.05$) and ($P<0.01$)

Ns: Not significant.

Table 3. Effect of aflatoxin B_1 (AFB_1) and *Silybum marianum* seeds (SMSs) on serum enzyme activities of broilers at the end of the period (35 days).

Enzyme activities (U/L)			**Treatments**	
γ-GT	ALT	AST	SMSs (%)	AFB1 (ppb)
10.41 [c]	12.83 [b]	190.16 [c]	-	0
13.33 [b]	14.66 [b]	231.44 [b]	-	250
18.58 [a]	19.81 [a]	248.91 [a]	-	500
19.67 [a]	11.51 [b]	195.17 [a]	0	-
12.75 [b]	17.25 [a]	184.13 [b]	0.5	-
6.89 [c]	18.58 [a]	188.41 [b]	1.0	-
			Interaction effect	
14.11 [b]	22.55 [a]	173.12	0	0
25.57 [a]	9.24 [b]	171.75	0	250
29.55 [a]	6.38 [b]	239.21	0	500
10.05 [b]	20.5 [a]	259.11	0.5	0
9.37 [b]	16.5 [a]	242.51	0.5	250
10.25 [b]	15.75 [a]	242.75	0.5	500
8.67 [b]	21.25 [a]	265.75	1.0	0
8.85 [b]	18.21 [a]	262.01	1.0	250
8.01 [b]	15.5 [a]	268.51	1.0	500
			Probabilities	
0.01	0.01	0.01		AFB1
0.01	0.01	0.01		SMSs
0.05	0.05	NS		AFB1×SMSs

[a,b] Main effect means within a column, lacking a common superscript differ significantly ($P<0.05$) & ($P<0.01$).

NS: Not significant.

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DISCUSSION

Intraction effects of AFB1 alone, increased the concentration of serum uric acid and γ-GT enzyme activation ($P<0.05$). In addition, main effects from liver enzymes such as; i.e., ALT, AST and γ-GT rose markedly in a dose-dependent manner in broiler chicks contaminated with AFB1 alone. SMSs significantly decreased the serum glucose, uric acid, AST, and γ-GT activities at the end of experiment (day 35) ($P<0.01$). This indicated the positive effects of SMSs on counteracting the adverse effects of aflatoxicosis on serum markers and enzyme activities [18]. Raju and Devegowda [19] reported lower total serum protein in broilers exposed to 0.3 ppm AF-contaminated diet. However, several other authors, including Tedesco *et al*. [18], did not find any effects for higher doses of AFB1 on this variable. There was no difference between serum activity and liver enzymes when different levels of SMSs and AFB1 were fed to birds compared with controls. In contrast, Fernandez *et al*. (1994) in their study on laying hens found that cholesterol, triglycerides, calcium, phosphorus, ALT, γ-GT and lactate dehydrogenase levels in serum did not vary in broilers fed AF-contaminated food [20,21]. Other authors reported a decrease in serum ALT concentration after AFB1 intoxication in chickens [22]. Solter *et al*. (2000) observed a relationship between sub-chronic exposure to a hepatotoxic agent (microcystin-LR) and decreased hepatic ALT synthesis [23]. The mechanism of silymarin action against AFB1 intoxication can only be hypothesized. Many authors have demonstrated that the activation of AFB_1 in human and rat liver is a complex process controlled by multiple cytochrome P450 enzymes [24]. As reported by Baer-Dubowska *et al*. (1998), Silymarin can inhibit the cytochrome P450 system, and, consequently, inhibit AFB1 activation [13]. Silymarin is known to be a potent antioxidant since it acts as a radical scavenger [25] and can influence enzyme systems associated with glutathione and superoxide dismutase [26, 27]. Either the action on cytochrome P450 or the inhibition of oxidative damage could be responsible for the protective effect against AFB_1-induced alteration in liver enzymes, such as that observed in the present study. Tedesco *et al*. (2004) observed no mortality due to AFB1 ingestion [18]. The toxin enters the body through the food and ultimately leads to numerous adverse effects. Silymarin, the standardized extract of SMSs, is used as a hepatoprotector in human, and it is a potent antihepatotoxic agent. The results of the present study showed that consumption of different levels of SMSs from 0.5 to 1.0% in poultry diets can reduce the toxicity of AFB_1 in broiler chickens.

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