Effect of Milk Thistle *(Silybum marianum L.)* on Biochemical Parameters and Immunity of Broiler Chicks Fed Aflatoxin B1 after Three Weeks

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Received: 20.02.2014 *Accepted: 30.03.2014*

ABSTRACT

Background: This study was conducted to determine the efficacy of milk thistle seeds (MTSs) in counteracting the toxic effects of aflatoxin B1 (AFB_1) in a contaminated diet fed to broilers.
Methods: Two dietary inclusion rates of AFB_1 (0, 0.250 and 500 ppb) and MTS (0, 0.5 and 1%) were tested in a 3×3 factorial manner. The effect of nine experimental treatments was assessed using 216 one-d-old Ross 308 male broiler chicks in a randomized complete design with 4 replicates of 6 birds each from one to 21 days of age. The effects of dietary AFB_1 and MTS on serum biochemistry factors, antibody titer against Newcastle disease (ND) and influenza disease (ID) in broilers were evaluated at the end of this period.
Results: Statistical analysis of the main effects of diets indicated no significant changes in uric acid, cholesterol, triglycerides, low density lipoprotein (LDL), ID, and phosphorus compared to the control ($P>0.01$). Also, addition of 500 ppb of dietary AFB_1 into the diet was associated with significant decreases in serum glucose, calcium, high density lipoprotein (HDL), and ND compared to the control group ($P<0.01$). The contaminated diet significantly increased the activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) ($P<0.05$).
Conclusion: Milk thistle showed protective effects and resulted in some serum enzyme activities and serum biochemical changes associated with aflatoxin toxicity.
Keywords: Aflatoxin, Antibody Titer, Broiler, Lipid Profiles, Liver Enzymes, Milk Thistle.

IJT 2014; 1098-1103

INTRODUCTION

Mycotoxins are a group of structurally diverse secondary fungal metabolites that occur worldwide as contaminants of grain [1]. Various mycotoxins, like aflatoxin (AF), ochratoxin A (OA), zearalenone, T-2 toxin, vomitoxin, and fumoninsin, naturally contaminate foods and feed of poultry [1, 2]. Aflatoxins are a group of heterocyclic toxic metabolites of toxigenic fungi *Aspergillus flavus* and *A. parasiticus* [2]. Aflatoxicosis in poultry is a chronic illness characterized by high mortality, anorexia, decreased growth rates, negative feed conversions, fatty liver, decreased egg production, poor pigmentation, and increased susceptibility to some diseases [3]. In order to eliminate or reduce mycotoxin in animal diets, different physical, chemical, and biological methods are used [4]. The mechanisms of action of AFB1 involve their metabolism to reactive intermediates, which bind to macromolecules with consequent disruption of transcriptional and translational processes [5]. Milk thistle seed (MTS), a medicinal herb, has been extensively used in folk medicine for treating liver diseases[6]. Certain active ingredients found in the seed of this plant possess numerous medicinal properties. Earlier in 1960, a German scientist isolated a flavonoid 'silymarin' from milk thistle [6]. Chemically, it is composed of four flavonoids- silybin, isosilybin, silydianin, and silychristin [7]. Silybin is the major component constituting 50 to 70% of silymarin and exhibit greater biological activities [8]. Chakarverty and Parsad reported that milk thistle has hepatoprotective and hepatorestorative functions and protects liver and kidney from both exo- and endo-toxins [7]. It reduces liver enzyme production and shows anti-inflammatory and T-cell modulating effects [9]. Birds affected by AFB_1 can effectively be recovered if treated with milk thistle [10, 11]. Increase in AST concentration after challenge with aflatoxin has been reported [12, 13]. In broilers, higher levels of silymarin afford partial protection against a

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35-day challenge with 0.8 ppm (body weight) aflatoxin [10]. Basaga *et al*. (1997) reported that milk thistle supports the immune system through its powerful antioxidant and free radical scavenging action. It has the ability to preserve the supply of glutathione and has direct effects on immune cells [14]. The main objective of this study was to investigate the effects of MTS on serum parameters, liver enzymes, lipid profiles, and immune performance of broiler chicks contaminated with AFB_1 against Newcastle and influenza diseases (ND and ID).

MATERIALS AND METHODS

Contaminating Poultry Feed

A known AFB_1 producing strain of *A. flavus* (PTCC NO: IR 111) was maintained on Potato Dexterose Agar (PDA) at optimum conditions in the Animal Science Institute, Birjand University, Iran. This culture was, then, introduced to commercial feed placed in a humid (15%) and hotter (27^oC) regions to fully propagate the toxin in feed. The AFB_1 was semi-quantified according to Tapia [15] using thin layer chromatography (TLC). Commercial feed was contaminated weekly to maintain the toxin within the required limits. The AFB_1 in the rice powder consisted of 60 ppm AFB_1.

Bird Husbandry and Experimental Protocol

A total of 216 one-d-old Ross 308 male broiler chicks were randomly allocated to nine groups in a 3×3 factorial experiment. During this study, the birds were submitted to conventional broiler chicken management and housed in floor pens in an environmentally controlled broiler house with litter floors. The chicks were divided into nine treatment groups, with four replicates per treatment and 12 chicks per replicate: T1) basal diet (BD) free of toxin, T2) BD contaminated with 250 ppb AFB_1, T3) BD contaminated with 500 ppb AFB_1, T4) BD supplemented with 0.5% MTS, T5) BD supplemented with 0.5 % MTS plus 250 ppb AFB1, T6) BD supplemented with 0.5% MTS plus 500 ppb AFB_1, T7) BD supplemented with 1% MTS, T8) BD supplemented with 1.0 % MTS plus 250 ppb AFB_1, T9) BD supplemented with 0.1 % MTS plus 500 ppb AFB_1. MTS was collected from the outskirts of Zabol district in Sistan and Blochestan province, Iran (Figure 1). Chickens consumed the diets and water *ad libitum*. They received a commercial diet formulated to meet or exceed the nutritional requirements of broilers (for 21 day of age) as recommended by the National Research Council (NRC) (Table 1).

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

Feed Stuffs	Starter Period (1-14 day)	Grower Period (15-21 day)
Maize	5443	50.42
Soybean meal(44%CP)	35	30.29
Wheat	-	10
Fish meal (60 % CP)	3.07	2.04
Soybean Fat	3.29	3.57
Dicalcium phosphate	1.73	1.47
Oyster shell	1.16	1.04
Mineral Premix	0.5	0.5
Vitamin premix	0.5	0.5
Salt	0.2	0.2
DL-methionine	0.35	0.28
L-lysine	0.24	0.19
Analyzed values		
ME (Kcal Per kg)	2980	3050
CP (Percent)	22	20
Lys (Percent)	1.43	1.24
Met + Cys (Percent)	1.07	0.95
Thr (Percent)	0.84	0.74
Ca (Percent)	1.05	0.90
P (Percent)	0.52	0.45

Figure 1. Milk thistle seed (MTS): Collected from the outskirts of Zabol district in Sistan balochestan province, Iran.

Serum Biochemical Parameters

Blood was collected in nonheparinized tubes from two broilers of each treatment by puncturing the brachial vein on the last day of

the period. Serum was separated after 8 to 10 hours through the standard procedures and was stored at -20°C for subsequent analysis. The individual and combined effects of dietary AFB1 and MTS on serum biochemistry factors, lipid profile, and liver enzymes of broilers were evaluated. The individual serum samples were analyzed for uric acid, creatinine, cholesterol, triglycerides, glucose, LDL, HDL, iron, phosphorus, and calcium, and the activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by the ultraviolet spectrometry method (Anonymous, 1984) - using an automatic analyzer (Boehringer Mannheim Hitachi 704 automatic analyzer, Japan). Then, the serum samples were assayed for Newcastle and Influenza vaccine titers using ELISA technique on the last day of the period (day 21).

Statistical Analysis

The data were subjected to ANOVA as a completely randomized design in the factorial procedure (Macros software, 2010). Differences were considered significant at $P<0.05$. Also, the Tukey-Kramer test was adapted to compare mean values. All of the procedures used in testing the chickens in this experiment were approved by the Department of Poultry Science at University of Birjand, Iran.

RESULTS

Data presented in Tables 2 and 3 demonstrate that AFB_1 and MTS had no significant effect on uric acid, iron, phosphorus, cholesterol, triglycerides, LDL, and influenza vaccine titer compared to the uncontaminated diet. In addition, MTS alone did not have any significant effects on uric acid, cholesterol, triglycerides, LDL, phosphorus, and influenza vaccine titer compared to the contaminated diet alone. As shown in Tables 2 and 3, addition of 500 ppb of AFB_1 into the diet resulted in a significant decrease in glucose (228.6 mole/L), HDL (65.1 mmole/L), calcium (7.82 mmole/L), and Newcastle vaccine titer (7.42) compared to the control ($P<0.01$). Moreover, thistle-supplemented diet showed a significant increase in glucose level compared to the control groups ($P<0.01$). Feeding diets contaminated with AF resulted in a significant increase in creatinine (1.99 µmole/L), AST (221.5 U/L), and ALT (31.49 U/L) ($P<0.05$) compared to the control group. Moreover, the mean total of AST was significantly lower in the birds fed with MTS diets compared to the values exhibited by the chicks in the control group ($P<0.05$).

Table 2. Effect of aflatoxin B1 (AFB1) and Milk thistle seeds (MTS) on serum biochemistry and some liver enzymes of broiler chickens at the end of period (21 days).

Groups									
AFB1 (ppb)	**MTS (%)**	**Glucose (mole/L)**	**Creatinine (µmole/L)**	**Uric acid (mmole/L)**	**Iron (mole/L)**	**Phosphorus (mmole/L)**	**Calcium (mmole/L)**	**AST (U/L)**	**ALT (U/L)**
0	-	242.1 [a]	0.56 [b]	5.24	396.9	7.14	9.31 [a]	198.5 [b]	21.12 [b]
250	-	245.3 [a]	1.61 [a]	6.05	359.8	6.41	8.93 [ab]	206.9 [b]	29.48 [a]
500	-	228.6 [b]	1.99 [a]	6.61	355.1	6.62	7.82 [b]	221.5 [a]	31.49 [a]
±SEM		2.89	0.13	0.27	2.99	0.15	0.19	2.7	1.64
-	0	218.8 [b]	1.54	6.49	248.1	6.31	8.22	220.8 [a]	28.91
-	0.5	246.3 [a]	1.35	5.58	254.1	6.84	9.12	204.1 [b]	27.75
-	1.0	251.1 [a]	1.27	5.84	289.4	6.92	8.91	202.1 [b]	25.41
±SEM		24.18	0.021	0.28	28.41	0.12	0.16	17.54	3.11
Probabilities									
AFB1		0.01	0.05	NS	NS	NS	0.01	0.05	0.05
MTS		0.01	NS	NS	NS	NS	NS	0.05	NS
AFB1×MTS		NS	NS	NS	NS	NS	NS	NS	NS

(a,b) Main effect means within a column lacking a common superscript differ significantly ($P<0.05$) & ($P<0.01$). [Main effect values are mean ± SEM from 6 observations each].
Ns: Not significant.

Table 3. Effect of aflatoxin B1 (AFB1) and Milk thistle seeds (SMS) on Lipid profiles and antibody titers at the end of period (21 days).

Groups		Lipid profiles				Antibody titers (Log2)	
AFB1 (ppb)	**MTS (%)**	**Cholesterol (mole/L)**	**Triglycerides (mole/L)**	**LDL (mmole/L)**	**HDL (mmole/L)**	**Newcastle Disease**	**Influenza Disease**
0	-	154.5	133.8	39.5	88.5 [a]	8.46 [a]	4.49
250	-	153.5	133.8	38.8	84.5 [a]	8.06 [ab]	4.34
500	-	156.1	144.9	29.9	65.1 [b]	7.24 [b]	4.29
±SEM		4.21	3.02	2.42	2.46		
-	0	157.2	143.7	36.9	74.6	8.36	3.66
-	0.5	152.7	135.3	41.4	81.4	8.46	3.69
-	1.0	145.1	133.5	30.3	82.1	8.49	3.89
±SEM		4.22	2.42	1.37	2.13	0.41	0.35
Probabilities							
AFB1		NS	NS	NS	0.01	0.01	NS
SMS		NS	NS	NS	NS	NS	NS
AFB1×SMS		NS	NS	NS	NS	NS	NS

¬(a,b) Main effect means within a column lacking a common superscript differ significantly (P<0.05) & (P<0.01). [Main effect values are mean ± SEM from 6 observations each].
Ns: Not significant.

DISCUSSION

AFB_1 is known to be hepatotoxin and causes genetic damage [16, 17]. In the present study, significant increases in the levels of AST and ALT were observed upon treatment with AFB_1 during 21 days. This increase in the values of serum AST and ALT might be due to hepatotoxic effects of AFB_1 which is in agreement with the findings of Fani Makki *et al*. [18]. Several studies have reported similar hepatotoxic property for AFB_1, which is in agreement with the findings of the present study [9-11]. Bares *et al*. (2008) reported the hepatoprotective property of silybin, which is the active ingredient of milk thistle [16]. In addition, the findings of the present study are in close agreement with those of Schrieber *et al*. (2008) who reported reduction of ALT and AST by milk thistle in human hepatitis C patients [17]. The findings of the present study are supported by others who reported reduction of AST by supplementation of feed with medicinal herbs as well [10, 13, 18].

Serum total glucose, HDL, and AST levels were significantly different between groups contaminated with different levels of AFB_1. Creatinine and ALT experienced significant increases and, in fact, were the values which were significantly affected in groups contaminated by AFB_1 alone, but not influenced by milk thistle consumption alone, whereas calcium and HDL decreased after aflatoxin administration. However, treatments did not influence the serum concentrations of uric acid, cholesterol, triglycerides, LDL, phosphorus, and iron. Previous studies performed with high levels of AFB_1 (0 and 1.0 ppm) showed significant decreases in serum total glucose, protein, albumin, total cholesterol, and uric acid levels [19]. However, 1.0 ppm of AFB_1 did not affect uric acid, γ-GT, and phosphorus levels [19]. Rastogi *et al*. (2000) reported that silymarin reversed biochemical changes in liver and serum in AFB_1 intoxicated rats, indicating that it has a hepatoprotective action in preventing AFB_1 induced injury [20]. Lutensko *et al*. (2008) reported that silymarin phytosome caused no difference in ALT and AST serum activity, while total protein, calcium, phosphorus, albumin, and globulin levels increased compared with the control (P<0.05) [21].

Serum antibody titer against Newcastle and influenza diseases did not significantly change in treatments containing thistle seed, whereas lowest titers were recorded in groups AFB_1. It is clear from our observations and those of other researchers that aflatoxin causes severe immunosuppression that might be due to reduction in phagocytic activity of blood monocytes and depressed complement activity, hence, depressed opsonization and phagocytic activity [22]. Moreover, there are some reports on the immune-stimulatory effects of milk thistle

[23]. The results of the present study are supported by Tedsco *et al*. (2004) who reported the negative role of contaminated feed in the presence of immunosuppressant aflatoxin in diet [10]. In conclusion, the results of the present study showed that increases in the amount of liver enzymes occurred as a result of AFB_1. The fact that milk thistle supported liver function in conjunction with acute aflatoxin challenge is encouraging and warrants further research.

ACKNOWLEDGEMENTS

This study was conducted at University of Birjand and was supported by the Office of Graduate Study as a thesis project.

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