



## Effect of milk thistle seeds (*Silybum marianum* L.) on the immune system, intestinal related variables, appearance and mortality of broilers contaminated with Aflatoxin B<sub>1</sub>

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### ABSTRACT

**Background & Aim:** Aflatoxin (AF) is the most important fungus that contaminates food and feed and can enter the food chain of humans. The aim of this study was to investigate the ability of thistle seeds (*silybum marianum* L.) to reduce the adverse effects of aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) on the immune system, variables related to the intestine, appearance and mortality of the broiler chickens.

**Experimental:** In this experiment, a total of 216 male Ross 308 broiler chickens in a completely randomized factorial design 3 × 3 with nine treatments, four replications, and six chickens unit were grown on the ground for 35 days. Treatments was included three levels of AFB<sub>1</sub> (0, 250, and 500 ppb) and three levels of milk thistle seed (0, 0.5 and 1%). Influenza and Newcastle diseases virus (IDV & NDV) titers in two steps (25 and 34 days), variables related to the intestine at the end of the experiment along with the weekly appearance and mortality of broiler chickens were evaluated.

**Results & Discussion:** Analysis of IDV and NDV results in the day 34 showed a reduction in titers of them in birds receiving diets contaminated with 500 ppb AFB<sub>1</sub> ( $p \leq 0.05$ ). Also, the length of the ileum and the total intestine at the end of the experimental period (day 35) indicated decrease in the group of birds received diets containing 500 ppb AFB<sub>1</sub> ( $p \leq 0.05$ ). Chicks receiving diet containing 500 ppb of AFB<sub>1</sub> had the most of feather abnormalities, aggressive behavior and minimal body size compared with other treatments. Mortality due to AFB<sub>1</sub> consumption in various experimental treatments was not significant.

**Recommended applications-industries:** Probably phytosomes of milk thistle seeds (MTS) reduce the mortality rate, nervous and aggressive behavior and increase the immunity of broilers against IDV & NDV in AFB<sub>1</sub> exposure time.

## 1. Introduction

Aflatoxins (AFs), potent mycotoxins produced by *Aspergillus flavus* and *A. parasiticus*, are the major concern in human and animal nutrition. Even though 18 different AFs have been identified, i.e., B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub> have been detected as natural contaminants of feed and food (Tedesco *et al.*, 2004; Fani makki *et al.*, 2013a). Several researchers have recently focused on the inhibition of AFs biotransformation into its 8, 9-epoxide constituents through interaction with cytochrome P450 enzymes using oltipraz (Kuilman *et al.*, 2000) or natural compounds (Kim *et al.*, 2000; Lee *et al.*, 2001). Milk thistle (*Silybum marianum* L.) have been used from about 2000 years ago as a natural treatment for the liver and biliary duct disorders. Silymarin presents a pharmacologically effective substance containing four main constituents, i.e., silybin (50 – 60%), isosilybin (5%), silychristin (20%) and silydianin (10%) (Zahid & Durrani, 2007). Silymarin acts in four different ways; as an antioxidant, absorber and regulator of the intracellular glutathione, as a stabilizer and regulator of cell membrane permeability that prevents the entering of hepatotoxic substances into hepatocytes, as the ribosomal ribonucleic acid (RNA) synthesis promoter simulating regeneration of the liver, and an inhibitor of the transformation of liver stellate cells into myofibroblasts (Tedesco *et al.*, 2004). These processes are responsible for the deposition of collagen fibers in the liver (Fraschini *et al.*, 2002). Furthermore, absorption of free radicals is considered to be one of the key mechanisms securing the liver Kalorey *et al.* (2005) reported that MTS improved feed intake in the presence of AFB<sub>1</sub>. The intestine length has been reported to decrease after three weeks of dietary exposure to AFB<sub>1</sub> at levels as low as 0.02 (Kana *et al.*, 2010), and 0.7 mg kg<sup>-1</sup> (Yunus *et al.*, 2011). As the width of muscularis tends to be relatively constant, the density of intestine could be a good indication of the unit absorptive area. On this variable, the effects of higher AFB<sub>1</sub> dosage in broilers are unknown. Contrary to these reports, no histo-pathological changes in duodenum, jejunum, cecum, and ileum could be mentioned by Ledoux *et al.* (1999) when male broilers were exposed to 4 mg kg<sup>-1</sup> AFB<sub>1</sub> diet for three weeks. Generally, the immunotoxic dose of AFB<sub>1</sub> is considered as less than the dose required eliciting a reduction in bird performance. Though several

contradictory reports are available, the threshold dose of AFB<sub>1</sub> may be generalized to be 0.4 and 1 mg kg<sup>-1</sup> for the negative effects on cell mediated and humoral immunity (HI), respectively. However, the question regarding the susceptibility of modern broiler regarding immunotoxicity remains yet to be answered. So far, there is evidence regarding the biphasic nature of the effects of AFB<sub>1</sub> on HI (Yunus *et al.*, 2011). In a later study, these authors reported non-significantly higher titers against Influenza and Newcastle diseases virus (IDV & NDV) in birds fed 0.1 to 0.8 mg kg<sup>-1</sup> AFB<sub>1</sub> rations as compared to the birds fed a control ration (Giambrone *et al.*, 1985a). The underlying mechanisms for this temporary increase in HI response are not known. The present study was conducted to investigate the effect of MTS on the feed intake, immune performance of broiler chicks against IDV and NDV, total length of the intestine, appearance and mortality in broiler chickens contaminated with AFB<sub>1</sub>.

## 2. Materials and Methods

### 2.1. Production of aflatoxin on Rice

*A. flavus* obtained from the Center of Scientific and Industrial Research Organization in Iran, PTCC NO: 5004 (IR111), and proliferate on potato dextrose agar (PDA) medium and used for *in vitro* studies. AFB<sub>1</sub> was produced by the fermentation process on the rice. AF analyses were performed by thin layer chromatography (TLC). The yield of AFB<sub>1</sub> produced per 25 g sample was 60 ppm.

### 2.2. Chickens and diets

The study included 216 male Ross-308 broiler chicks (One-day-old at the beginning of the study). They were divided randomly into nine treatment groups, each of which had four replications of six broiler chicks. 36 steel cages (40 × 65 × 98 cm) were used to accommodate six chicks per m<sup>2</sup>. Continuous lighting was provided during the experimental period. The room temperature was gradually decreased from 32 °C on day 0 to 25 °C on the day 14, and remained constant thereafter. The chicks were allowed *ad libitum* access to feed and water. The study lasted for 35 days and consisted of; (T<sub>1</sub>) as a control, and groups received for five weeks the feed contained, i.e., (T<sub>2</sub>) 250 ppb of AFB<sub>1</sub>, (T<sub>3</sub>) 500 ppb of AFB<sub>1</sub>, (T<sub>4</sub>) 0.5% of MTS, (T<sub>5</sub>) 0.5% of MTS plus 250 ppb AFB<sub>1</sub>, (T<sub>6</sub>) 0.5% of MTS

plus 500 ppb AFB<sub>1</sub>, (T<sub>7</sub>) 1.0% of MTS, (T<sub>8</sub>) 1.0% of MTS plus 250 ppb AFB<sub>1</sub> and (T<sub>9</sub>) 1.0% of MTS plus 500 ppb AFB<sub>1</sub>. Treatment was administered daily by gavage to assure the correct dose administration. The experiment was approved by the animal welfare committee of the Agriculture Faculty of Birjand University, Iran.

### 2.3. Data Collection

**2.3.1. Performance and Intestinal length.** Chickens in each week were examined for the aggressive behavior, disarray wings, lethargy and mortality. At the end of the experimental period (day 35) different parts of the intestine, including the ileum, duodenum plus jejunum and total of intestinal length per cm were measured and recorded.

**2.3.2. Evaluation of Humoral Immune Response.** Two broilers from each replicate of treatments were randomly selected to challenge with sheep red blood cells (sRBCs). Blood samples for antibody analysis were taken by puncture of the brachial vein. Serum antibody titers against Newcastle Disease Virus (NDV) and Influenza Disease Virus (IDV) were measured by the hemagglutination inhibition test (HI). HI antibodies were converted into log to further evaluation. In this experiment, the titer of IDV & NDV was evaluated in two stages. At the first injection on the day 20, two birds per each group were injected 0.4 ml of sRBCs (8%) in the right wing vein. Five days later, the blood samples were taken from the wing vein. On day 27, 0.8 ml of sRBCs (8%) was injected into the muscle of the breast (Fani makki *et al.*, 2013a). Blood samples were taken seven days after the injection (34 days-old of broiler chicks).

### 2.4. Statistical Analysis

The data were subjected to analysis of variance (ANOVA) as a factorial completely randomized design using the generalized linear models (GLM) procedure of statistical analysis software (SAS) (SAS Inst., 2001). The model contained the effects of mortality, intestinal length, a titer of IDV & NDV. The data were compared with Turkey - Kramer post-hoc test. Least squares means  $\pm$  standard error are reported and  $p \leq 0.05$  indicating statistical significance.

## 3. Results and Discussion

### 3.1. Appearance

In the first and second weeks of the experiment, there were not seen any changes in the appearance of the birds such as feathers, confusion, lethargy, neurological signs and mortality in the broiler chicks received different treatments.

In the third week of the experiment, the chicken consumed 500 ppb of AFB<sub>1</sub> were showing the highest level of aggressive behavior, disarray wings and lowest body size compared to other experimental treatments (Tedesco *et al.*, 2004). At the end of the period (day 35), these signs were shown in poultry received the 250 ppb of AFB<sub>1</sub>. Similar symptoms not observed in the treatments which received the different levels of (MTS) alone and plus with AFB<sub>1</sub> compared to the control group (Tedesco *et al.*, 2004).

### 3.2. Mortality and Intestinal length

In this experiment, there was no significant difference for the mortality rate. But the highest and the lowest mortality rate was related to the broiler fed 500 ppb of AFB<sub>1</sub> and the birds received 1.0% of MTS alone, respectively. Tedesco *et al.* (2004) were observed no mortality due to AFB<sub>1</sub> ingestion. A result from the present study is in agreement with (Miazzo *et al.*, 2000 and 2005) that showed that AFB<sub>1</sub> affected the mortality of chickens. The lower intestine length was seen in the broilers received 500 ppb of AFB<sub>1</sub> (135.25 cm) ( $p \leq 0.05$ ). There were no significant changes in total intestine length in broilers received different levels of AFB<sub>1</sub> (Table 1). However this difference in broilers received different levels of AFB<sub>1</sub> was significant compared with other experimental treatments ( $p \leq 0.05$ ). Other researchers reported that the whole intestine length has been decreased after three weeks of exposure to AFB<sub>1</sub> at levels of 0.02 ppm (Kana *et al.*, 2010) and 0.7 ppm (Yunus *et al.*, 2011). In the present study, the minimum and maximum length of ileum and duodenum plus jejunum was observed in the chickens received 500 ppb of AFB<sub>1</sub> and 1.0% of MTS alone, respectively (Table 1).

### 3.3. Humoral Immunity

**3.3.1. The Newcastle disease Virus (NDV) Titer.** Results of statistical analysis of NDV titer on the day 25 revealed a significant reduction ( $p \leq 0.05$ ) in birds received the contaminated diet with 500 ppb of AFB<sub>1</sub> (6.02), compared with the control group (8.86) (Table 2). The NDV titer has not significantly altered in the broiler received 250 ppb of AFB<sub>1</sub> (7.38) compared with

control group and the broilers received different levels of MTS alone. The lowest amount of HI titer for NDV was belonged to the broilers received 500 ppb of AFB<sub>1</sub> (6.02) (Yunus *et al.*, 2011). Feeding broilers with different levels of MTS did not alter NDV titer compare the control group (8.86). The results of NDV titer on day 34 of the experiment did not show any significant decrease in the group of broilers received 250 and 500 ppb of AFB<sub>1</sub> (7.45 and 6.10, respectively), compared with the control group (10.23) and the broilers received different levels of MTS (Fani makki *et al.*, 2013a). There were not significant changes in the titer of NDV in the broilers received different levels of AFB<sub>1</sub>. The lowest amount of NDV titer was seen in the birds received the diet contaminated with 500 ppb of AFB<sub>1</sub> (6.10) (Fani makki *et al.*, 2013a). However, broilers fed different levels of MTS alone, did not show significant changes in the NDV titer compared to control group (10.23). Some authors reported non-significant higher titers against IDV & NDV in the birds fed 0.1 to 0.8 mg kg<sup>-1</sup> of AFB<sub>1</sub> rations compared to the birds fed a control ration (Giambrone *et al.*, 1985a). The underlying mechanisms for this temporary increase in humoral immune (HI) responses are unknown. These results suggest that treatment with silymarin can be effective in combating with the negative effects of AFB<sub>1</sub> intoxication on NDV titer in broiler chicks.

### 3.3.2. The Titer of Influenza disease Virus (IDV) Titer.

IDV titer at the day 25 of the experiment did not show any significant changes in experimental groups,

compared with the control (4.42), (Table 2). The lowest amount of the IDV titer (2.13) was seen in the birds received diets contaminated with 500 ppb of AFB<sub>1</sub> (3.12). On the day 34, a significant reduction ( $p \leq 0.05$ ) of IDV titer was seen in the birds received the contaminated diet with 500 ppb of AFB<sub>1</sub> (2.89), compared with the control group (4.78). The titer of IDV in chickens received diets containing 250 ppb of AFB<sub>1</sub> (3.99) did not show any significant differences with the broilers received diet of control group and the broilers fed different levels of MTS.

The lowest amount of the IDV titer was seen in the birds received the diet contaminated with 500 ppb of AFB<sub>1</sub> alone. The diets containing about 0.5 and 1.0 percent of MTS, did not show a significant change in the titer of IDV compared to control group. Also, we did not see any significant increase in the titer of IDV in groups received 250 ppb of AFB<sub>1</sub> plus 0.5 percent of MTS (4.49), compared with the broiler received 250 ppb of AFB<sub>1</sub> (3.99) alone.

Giambrone *et al.* (1985a,b) in two separate experiments on Hubbard broilers, indicated a non-significant increasing trend in ND titer along with an increase in the AFB<sub>1</sub> content of ration from zero to 0.5 mg kg<sup>-1</sup>. In the other experiment, higher IDV and NDV titers were shown in the birds fed 0.1 and 0.2 mg kg<sup>-1</sup> of AFB<sub>1</sub>, respectively (Yunus *et al.*, 2011). These results suggest that treatment with silymarin may be effective on counteracting the negative effects of AFB<sub>1</sub> intoxication on IDV titer in broiler chicks.

**Table 1.** Effect of aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) and milk thistle seeds (MTS) on mortality and intestinal length

Aflatoxin B <sub>1</sub> (ppb)	Milk thistle seed (%)	Mortality	Total of Intestinal length	Duodenum plus jejunum	Ileum
0	0	0.5±0.28	151.35±2.28 <sup>a</sup>	90.25±2.04 <sup>ab</sup>	61.5±1.25 <sup>a</sup>
250	0	1±0.4	145±1.91 <sup>ab</sup>	85±1.23 <sup>ab</sup>	60.11±1.21 <sup>ab</sup>
500	0	1.5±0.64	135.25±1.46 <sup>b</sup>	80.25±1.48 <sup>b</sup>	55.25±1.42 <sup>b</sup>
0	0.5	0.25±0.25	151±4.43 <sup>a</sup>	90.75±1.52 <sup>a</sup>	60.25±1.34 <sup>ab</sup>
250	0.5	0.75±0.47	150.25±2.10 <sup>a</sup>	89.75±1.03 <sup>ab</sup>	60.5±1.52 <sup>ab</sup>
500	0.5	0.5±0.28	148.75±4.60 <sup>a</sup>	87.25±1.41 <sup>ab</sup>	61.5±1.44 <sup>a</sup>
0	1.0	0±0	157±1.40 <sup>a</sup>	93.21±2.53 <sup>a</sup>	64.11±1.92 <sup>a</sup>
250	1.0	0.5±0.28	153±1.64 <sup>a</sup>	91.5±2.01 <sup>a</sup>	62.53±1.87 <sup>a</sup>
500	1.0	0.75±0.25	148.75±2.32 <sup>a</sup>	87.24±1.09 <sup>ab</sup>	61.75±1.55 <sup>a</sup>

<sup>(a,b)</sup>Means ±standard error within a row with a common superscript differs significantly ( $p \leq 0.05$ ).

**Table 2.** Effect of experimental treatments against of influenza and newcastle disease virus titer (IDV & NDV)

Treatment		Titers of (NDV), (Log 2)		Titers of (IDV), (Log 2)	
Aflatoxin B <sub>1</sub> (ppb)	Milk thistle seed (%)	Step 1	Step 2	Step 1	Step 2
		Day25	Day34	Day25	Day34
0	0	8.86±0.35 <sup>a</sup>	10.23±0.75 <sup>a</sup>	4.42±0.87	4.78±0.43 <sup>a</sup>
250	0	7.38±0.67 <sup>ab</sup>	7.45±0.79 <sup>bc</sup>	3.43±0.41	3.99±0.08 <sup>ab</sup>
500	0	6.02±0.28 <sup>b</sup>	6.10±0.11 <sup>c</sup>	3.12±0.37	2.89±0.08 <sup>b</sup>
0	0.5	8.70±0.86 <sup>a</sup>	9.96±0.64 <sup>a</sup>	4.28±0.19	4.82±0.24 <sup>a</sup>
250	0.5	8.46±0.18 <sup>a</sup>	9.50±0.24 <sup>ab</sup>	4.43±0.19	4.49±0.39 <sup>a</sup>
500	0.5	8.23±0.15 <sup>a</sup>	9.30±0.45 <sup>ab</sup>	3.51±0.47	4.32±0.40 <sup>a</sup>
0	1.0	8.55±0.34 <sup>a</sup>	10.36±0.29 <sup>a</sup>	4.48±0.24	4.35±0.19 <sup>a</sup>
250	1.0	8.33±0.41 <sup>a</sup>	9.1±0.03 <sup>ab</sup>	3.97±0.05	4.33±0.20 <sup>a</sup>
500	1.0	7.47±0.12 <sup>ab</sup>	8.21±0.10 <sup>abc</sup>	3.24±0.11	4.07±0.38 <sup>ab</sup>

<sup>(a,b)</sup> Means ±standard error within a row with a common superscript differs significantly ( $p \leq 0.05$ ).

#### 4. Conclusions

Treatment with MTS decreases the toxic severity of AFB<sub>1</sub> in broilers. These findings suggest that silymarin might be used in chickens to prevent the effects of AFB<sub>1</sub> in contaminated feed. This information provides a basis for further studies for the establishment of the mechanisms existing between silymarin and protection against AFB<sub>1</sub> toxicity. However, more research on this topic especially on the farm and field condition needs to be done to improve the safety and quality of poultry products (Fani makki et al., 2013b).

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