




The Protective Role of Silymarin to Ameliorate the Adverse Effects of Ochratoxin-A in Laying Hens on Productive Performance, Blood Biochemistry, Hematological and Antioxidants Status

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ABSTRACT

This study was planned to examine the effect of silymarin on diminishing adverse effects of ochratoxin-A (OTA) in laying hens. A total number of 300 Inshas, local layer hens of 28 weeks of age were randomly distributed into 4 groups with 5 replicates each (15 hens). The birds were fed on the following treatments, (Control): fed a hen diet without any supplementation; (SL): fed the control diet supplemented with SL (1000 mg/kg feed); (OTA-diet): was fed the control diet contaminated with 1 ppm of OTA/kg diet, and (OTA+SL): fed the OTA-diet plus SL (1ppm OTA+1000 mg SL/kg feed). Results showed that feeding OTA at 1 ppm reduced productive performance compared with those fed the control diet. In OTA treated groups the total erythrocytes count, leukocytes count, PCV and Hb were decreased when compared to the control and SL groups. Albumin, globulin and serum total proteins in OTA treated groups were significantly lower when compared to the control and SL groups. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were significantly increased in OTA fed groups in comparison with the control and SL groups. Creatinine and uric acid were increased in OTA treated groups but were almost normal in the SL group. The results showed that OTA had a severe effect on liver and kidney, but SL treated group had normal liver and kidneys showing its hepatoprotective effects.

INTRODUCTION

Ochratoxicosis occurs less frequently in poultry than aflatoxicosis, but due to its acute toxicity it is more deadly. Ochratoxins are a family of toxic mixtures combined of three items, A, B and C, which are structurally linked and are produced by numerous species of fungus as secondary metabolites. Signs of chronic ochratoxicosis connected to renal illnesses appear in chicken after a prolonged OTA consumption of 0.5 mg OTA/kg in feed, followed by a decrease in feed intake and problems with egg production and egg quality (Vila-Donat *et al.*, 2018). It's vital to remember that in the field, non-specific indications of mycotoxicosis might emerge due to the presence of several contributing factors, making a clinical diagnosis challenging. Because OTA has the capacity to bioaccumulate in the body, ingestion of OTA by birds may be linked to the appearance of OTA residues in edible tissues and eggs (Wang *et al.*, 2018), thereby compromising the safety of egg production across the food chain. All efforts should therefore be founded to safeguard commercial poultry birds from ochratoxin's toxic effects (Attia *et al.*, 2013; 2016). There is little research on the effect of silymarin with aflatoxin and ochratoxin on broiler chickens, but there is no research on the effect of silymarin with ochratoxin A on the productive performance of laying hens. In the event of clinical ochratoxicosis, another



significant approach is to diminish damage and loss by reducing the harmful impacts of OTA by several hepato-protective and renal protective substances. A range of toxicants is presently using several chemical substances and plant extracts worldwide to treat liver, kidney damage and immunosuppression. Because of these mycotoxins, many hepatoprotective drugs are currently being used to avoid liver damage. Silymarin is an important composite of antioxidants among these natural substances. Silymarin, an extract of *Silybum marianum* plants and fruits, is one of the most significant natural hepato-renal protective substances comprising a combination of isomers of flavonoids such as silibinin, isosilibinin, silidianin and silichristin (He *et al.*, 2004). It was noted that Silymarin promotes immune status in the body (Wilasrusmee *et al.*, 2002). It stopped the tumor necrosis factor (TNF)- α release from isolated neurons of Kupffer and the perfused rat liver (Al-Anati *et al.*, 2009).

Silybum marianum, or milk thistle, is a well-known plant- mostly involved in Silymarin, its hepatotonic extract, Silymarin includes numerous flavonolignans (silibinin, isosilibinin, silychristin, isosilychristin, and silydianin) and a flavonoid (taxifolin; Federico *et al.*, 2017) that together use antioxidant, anti-inflammatory, antifibrotic, anti-lipid peroxidative, cell membrane stabilization and liver restoring effects (Attia *et al.*, 2017 and 2019).

Metabolically, Silymarin stimulates the hepatic cells and induces the synthesis of ribosomal RNA to promote protein production (Vargas-Mendoza *et al.*, 2014).

Given the foreseen importance of detoxification or neutralization of mycotoxins in poultry diets, this research aimed to further estimate the positive effects of feeding silymarin on productive performance and biochemical serum profile in laying hens object to dietary ochratoxin A.

MATERIALS AND METHODS

This research was carried out at the Poultry Research Station and at the laboratories of the Animal and Poultry Research Institute, Agricultural Research Center, Agriculture Ministry, Egypt.

Production of Ochratoxin

Fresh cultures of *Aspergillus ochraceus* (NRRL-3174) (Department of the National Institute of Animal Health, Dokki, Egypt) were used to inoculate the broken wheat grains for the production of OTA in the present experiment. Briefly, broken wheat grains (80 g) were soaked with 200 ml of distilled water for 2 h in flat-

bottom Erlenmeyer flask (1000 ml). Prior to inoculation with spore suspension (3 ml) of *A. ochraceus*, each flask contents were autoclaved at 121 °C for 20 min. These flasks were incubated for 14 days in the dark followed by OTA extraction by using acetonitrile-water (60:40). Quantification of OTA was performed using High Performance Liquid Chromatography (HPLC) equipped with florescent detector as described by Bayman *et al.* (2002).

Table 1 – Chemical composition and calculated analysis of the basal experimental diet.

Ingredients	%
Yellow corn	66.00
Soybean meal (44%)	66.00
Di-calcium phosphate	24.00
Limestone	1.71
DL. Methionine	7.59
Sodium chloride	0.10
Vit.& Min. Mixture*	0.30
Total	100
Calculated analysis**	
Crude Protein, %	16.43
Metabolizable energy (kcal/kg)	2750
Ether extract, %	2.70
Crude fiber, %	3.20
Calcium, %	3.33
Available phosphate, %	0.45
Total phosphorus, %	0.66
Methionine, %	0.39
Lysine, %	0.86
Determined analysis	
Crude Protein, %	16.45
Ether extract, %	2.68
Crude fiber, %	3.18
Calcium, %	3.50
Total phosphorus, %	0.70

*Supplied per kg of diet: vit.A, 10000 IU; D₃, 2000 IU; Vit.E, 10mg; Vit.K₃, 1mg; vit.B₁, 1mg; vit. B₂, 5mg; vit.B₆, 1.5mg; vit. B₁₂, 10mcg; Niacin, 30mg; Pantothenic acid, 10mg; Folic acid, 1mg; Biotin, 50 μ g; Choline, 260mg; Copper, 4mg; Iron, 30mg; Manganese, 60mg; Zinc, 50mg; Iodine, 1.3mg; Selenium, 0.1mg and Cobalt, 0.1mg.

** Calculated according to NRC. (1994)

A total number of 300 Inshas, (Sina X Plymoth Rock) hens of 28 weeks of age were randomly allocated into 4 groups with 5 replicates each (15 hens). The birds were fed on the following treatments, (Control): hen diet without any supplementation; (SL): control diet supplemented with SL (1000 mg/kg feed); (OTA-diet): control diet contaminated with 1 ppm of OTA/kg, and (OTA+SL): OTA-diet plus SL (1000 mg/kg feed). Dosages of OTA were based on those reported by Sawale *et al.* (2009), and SL doses were based on those reported by Attia *et al.* (2017 and 2019). The experimental duration spanned twelve weeks. The birds were provided a commercial diet with 16% crude



protein and 2750 kcal ME / Kg diets. There was no detectable ochratoxin or aflatoxin (< 1 µg / kg diet) in the basal diet. The birds were raised in an open-sided house on the ground under the same managerial circumstances. Artificially, the light schedule was kept at 16 hours a day. During the experimental period, feed and water were offered *ad libitum*.

Silymarin

Silymarin is a unique flavonoid complex – containing silybin, silydianin, and silychrisin – that is derived from the milk thistle plant. These unique phytochemicals from the milk thistle have been the subject of decades of research into their beneficial properties.

Silymarin with molecular weight 482.44 g/mol and purity (UV 60%) used in this search was manufactured by Samwon International LTD., Nanjing, China.

Parameters studied

Body weight (BW) was recorded at the beginning and end of the study to determine BW changes. Feed intake was measured weekly. The number of eggs and egg weight were recorded daily throughout the experiment.

At the end of the experimental period, five blood samples, each in heparinized tube therapy were used to evaluate subsequent hematological research. WBCs and RBC's counts using the method described by Natt and Herrick (1952) were measured with the assistance of a hemocytometer. The technique described by Sharaf *et al.* (2010) has determined hemoglobin and PCV; the levels of total protein, serum albumin, serum creatinine, AST, ALT, total cholesterol, and glucose. Globulin (determined by subtracting the albumin value from total protein). All biochemical blood constituents were commercially determined using Bio-Diagnostics Company, Egypt, commercially diagnosed kits. Glutathione activity (GSH) was assessed using whole blood heparinized spectrophotometrically and the Beutler *et al.* (1963) decrease technique of 5, 5

dithiobis (2- nitrobenzoic acid) (DTNB) glutathione (GSH). Lipid peroxide (Malondialdehyde) has been assessed spectrophotometrically using heparinized plasma, according to Ohkawa *et al.* (1979), a method based on thiobarbituric acid (TBA) reacted with malondialdehyde (MDA) in acid medium at 95 ° C for 30 minutes.

Statistical analysis

Data from all factors of reaction were subjected to one-way variance assessment (SAS, 2000). Using Duncan's Multiple Range Test (1955), variables with an important F-test ($p \leq 0.05$) were compared.

RESULTS

Productive performance

Results of body weight, feed intake (FI), egg production, and feed conversion ratio are presented in (Table 2), showing ochratoxin-A decreased productive performance parameters compared with the control birds, while supplementing hen diets with SL in absence of OTA recorded significantly a higher final body weight and change in the body weight, egg production and feed intake ($p \leq 0.05$) than all other experimental groups. Incorporating SL into the OTA-contaminated diets alleviated the adverse effects of ochratoxin on productive performance.

Serum biochemical parameters

Total protein, albumin and globulin concentrations in the group fed the contaminated diet with OTA at the end of the experimental period were considerably smaller than the control group. At the end of the experimental period, in the group treated with ochratoxin, AST, ALT, creatinine and uric acid concentration were significantly higher than in the control group, while in the group fed contaminated diets with SL, the AST, ALT, creatinine and uric acid concentration was significantly lower

Table 2 – Effect of Silymarin (SL) for detoxification of ochratoxin contaminated diets on productive performance.

Items	Dietary treatments				SEM	Sig
	Control	SL	OTA	OTA+SL		
Final body weight, (g)	1571 ^a	1590 ^a	1530 ^c	1560 ^b	8.31	*
Body weight change, (%)	9.43 ^a	9.64 ^a	7.25 ^c	8.71 ^b	0.85	*
Egg production, (%)	71.4 ^b	75.0 ^a	66.7 ^e	69.0 ^{cd}	0.11	*
Egg weight, (g)	50.1 ^b	50.8 ^a	48.9 ^c	49.5 ^b	0.23	*
Egg mass (g/hen/day)	35.8 ^b	38.1 ^a	32.6 ^d	34.2 ^{bc}	0.18	*
Feed intake (g/hen/day)	110 ^a	112 ^a	109 ^b	111 ^a	0.38	*
Feed conversion (g feed/ g egg mass)	3.08 ^c	2.95 ^c	3.34 ^a	3.23 ^b	0.04	*

^{a-d} Means in the same row with different letters, differ significantly ($p \leq 0.05$). SEM: Standard error of the means.



than in the group fed contaminated diet only. In the group fed SL (T2), the AST, ALT, creatinine, and uric acid concentration were non-significantly different from the control group (Table 3). At the end of the experimental period, the level of cholesterol in the groups fed contaminated diets was considerably smaller than in the control group, while non-significantly different from the control group among all other groups. At the end of the experimental period, glucose concentration in the groups fed contaminated diets with or without SL was significantly higher than in the control group while the T2 was non-significantly different from the control group.

Hematological parameters

At the end of the experimental period, the hemoglobin, packed cell volume and erythrocyte count of the group-fed contaminated diet with OTA was significantly lower than the control group, while the group-fed contaminated diets with SL, differed substantially from the control group. The hemoglobin, packed cell volume and count of erythrocytes in groups (SL), was not significantly distinct from the control group (Table 3), on the other hand, the count of white blood cells was.

Oxidative statuses

The present results in Table 3 showed that MDA decreased significantly ($p \leq 0.05$) while GSH increased significantly in the group that received SL compared to the group fed OTA alone.

DISCUSSION

Productive performance

Ochratoxin alone caused lower feed consumption, egg production, egg mass and average egg weight. Incorporating SL into the OTA-contaminated diets alleviated the adverse effects of ochratoxin on productive performance. The reduction in productive performance due to contaminated diets with ochratoxin in laying hens is in agreement with Stoev *et al.* (2002). Also, Elaroussi *et al.* (2006) noticed a significant decrease in body weight of chicks fed OTA- diet of 400 and 800 ppb. The diminution in productive performance during ochratoxicosis in this study may be attributed to several factors. OTA affects protein synthesis through competitive inhibition of phenylalanine-t-RNA-synthesis by phenylalanine moiety of the toxin. Moreover, ochratoxin-A interferes with DNA, RNA and protein synthesis and affects carbohydrate metabolism, particularly glucogenolysis (Kourad & Roschenthaler, 1998).

Concerning silymarin in absence of OTA recorded significantly higher production performance. In the same line, Muhammad *et al.* (2012) found that milk thistle addition (at 10 g/kg diet) significantly increased feed intake and improved feed conversion ratio. Abdalla, *et al.* (2018) found that the addition of milk thistle 25g /kg diet (equal to 1g Silymarin /kg diet) improved the performance of developed chickens under summer conditions. Moreover, birds with access to the silymarin exhibited greater productive performance and provided

Table 3 – Effect of Silymarin (SL) for detoxification of Ochratoxin-A contaminated diets on some blood constituents of laying hens.

Items	Control	SL	OTA	OTA+SL	SEM	sig
T. protein (g/dl)	5.80 ^a	5.83 ^a	5.00 ^b	5.46 ^{ab}	0.067	*
Albumin (g/dl)	2.76 ^a	2.80 ^a	2.40 ^b	2.50 ^{ab}	0.038	*
Globulin (g/dl)	3.04 ^a	3.03 ^a	2.60 ^b	2.96 ^a	0.046	*
Cholesterol (mg/dl)	138 ^a	135 ^a	120 ^b	132 ^{ab}	2.04	*
AST (U/L)	42.7 ^c	40.0 ^c	60.0 ^a	47.3 ^b	1.50	*
ALT (U/L)	10.5 ^c	11.0 ^c	22.3 ^a	14.7 ^b	0.549	*
Uric acid (mg/dl)	4.50 ^c	4.43 ^c	7.86 ^a	5.30 ^b	0.120	*
Creatinine (mg/dl)	0.453 ^c	0.450 ^c	0.956 ^a	0.686 ^b	0.010	*
Glucose (mg/dl)	225 ^c	229 ^c	296 ^a	251 ^b	3.14	*
Hematological parameters						
Hb (g/dL)	11.6 ^a	11.9 ^a	9.10 ^c	10.3 ^b	0.13	*
PCV (%)	28.0 ^a	27.6 ^a	20.5 ^c	25.2 ^b	1.35	*
RBC's (10 ⁶ /μL)	2.75 ^a	2.68 ^a	2.15 ^c	2.44 ^b	0.12	*
WBC's (10 ³ /μL)	24.3 ^b	24.6 ^b	27.3 ^a	25.2 ^b	1.24	*
Oxidative status						
MDA (n mol/ml)	15.2 ^b	10.6 ^c	25.2 ^a	17.3 ^b	3.10	*
GSH (mU/ml)	15.8 ^b	21.5 ^a	7.56 ^c	15.0 ^b	0.56	*

^{a-d} Means in the same row with different letters, differ significantly ($p \leq 0.05$). SEM: Standard error of the means, AST: Aspartate aminotransferase; ALT: Alanine aminotransferase, Hb: hemoglobin, PCV: Picked cell volume, RBC's: Red blood cells, WBC's: White blood cells, MDA: Malondialdehyde, GSH: Glutathione.



further indication for possible mycotoxin counteracting effects of silymarin. These results are in the line with the findings of Khaleghipoura *et al.* (2020). Gowda & Sastry (2000) showed the improvement of silymarin on productive performance and attributed these effects to antioxidant activity that stimulated protein synthesis by the bird's enzymatic system.

Serum biochemical parameters

The decrease in serum total protein, albumin, and globulin values in birds supplied with OTA could be due to inhibition of hepatic protein synthesis that happened at post-transcription stage by competitive inhibition of phenylalanine-t-RNA-synthesis, thus stopping amino-acylation and peptide elongation. One of the main impacts of binding albumin on OTA was to delay its elimination by restricting the transfer of OTA from the bloodstream to the hepatic and renal cells that contribute to its long half-life.

In the present investigation, the AST and ALT levels in ochratoxin treated birds increased significantly in comparison to the control birds. In the current research, the rise in AST level could be attributable to enzyme leakage owing to liver damage. Our findings agree with Wang *et al.* (2009), who discovered that OTA-contaminated diets for 21-day-old broiler chickens could considerably increase ALT and AST blood serum operations.

Regarding silymarin evidence, these findings are consistent with Shaker *et al.* (2010), who suggested that *Silybum marianum* was used to alleviate liver disease and that this may be due to a potent mixture of silymarin and its mechanism of action mainly as antiradical and anticarcinogenic functions that could be attributed to lower liver enzyme levels. Besides, Khaleghipoura *et al.* (2020) found that the inclusion of 500 mg / kg silymarin-nanohydrogels in drinking water could substantially offset the impaired growth output and alter hepatic function in Japanese quails fed on a 2 mg aflatoxin-contaminating diet.

In this research, at the end of the experimental period, the creatinine values of the groups fed contaminated diets with or without SL were considerably greater from the control group. Our findings of enhanced creatinine values are consistent with earlier reported outcomes from broilers (Sawale *et al.*, 2009). The groups fed diets containing SL, were non-significantly different from the control group. It indicates amelioration of adverse effects of OTA with these agents like SL. The finding is consistent with Bhattacharya (2011) who stated that silymarin helps preserve normal renal function and that

silibinin reduces oxidative damage to in vitro kidney cells.

During induced ochratoxicosis, reduction in serum cholesterol levels reflects impaired liver metabolism, resulting in lower cholesterol synthesis, as was also evident in this study. The substantial increase in the amount of SL augmented mycotoxic layer serum cholesterol is representative of its protective function. Significantly enhanced levels of blood glucose in layers fed on OTA contaminated diets may eventually result from liver tissue damage. OTA also influences the metabolism of carbohydrates, especially gluconeogenesis. It decreases the renal mRNA coding of carboxykinase phosphoenolpyruvate (PEPCK), which is the main enzyme in gluconeogenesis. PEPCK is the connection in glucose and glycogen between the citric acid cycle intermediates and their precursors (Leeson *et al.*, 1995). Interference in gluconeogenesis with this rate-limiting step plays the main role in the growth of renal cortex functional harm (Ueno, 1991). Verma and Shalini (1998), found that OTA was able to cause hyperglycemia in rabbits, which support our results. Experimental results exhibited that compared to the control group, blood glucose level was not substantially affected by diets supplied with silymarin. However Bhattacharya (2011), stated that silymarin can protect the pancreas against certain types of injury.

Hematological parameters

Blood white cells counts (WBCs) of the group fed contaminated diets with ochratoxin without SL, was substantially greater than the control group at the end of the experimental period, while non-significantly different from the control group and all other groups. Similar to our results, Elaroussi *et al.* (2006) exposed anemia (diminution of RBC's, PCV and Hb) in OTA poisoned broiler birds, this is associated with feed intoxication by OTA. The decrease in the amount of hemoglobin in founding during ochratoxicosis, as noted in the current research, could be due to decreased protein synthesis (Table 3). Supplementation of SL on the parameters examined in haematology was seen as resisting the change caused by OTA. Abdalla, *et al.* (2018) found that, cockerels fed diets supplied with 25g Milk thistle /kg diet (equal to 1g silymarin /kg diet) showed significantly improved red blood cells counts (RBCs), hemoglobin (Hb), blood white cells counts (WBCs) and packed cell volume (PCV) compared to the control group during summer season.



Oxidative statuses

Because several mycotoxins including aflatoxin B1, fumonisin B1, deoxynivalenol, T-2 toxin, and ochratoxin-A are known to damage lipid peroxidation cell membranes, the therapeutic characteristics of antioxidant nutrients have been explored against mycotoxins (Attia *et al.*, 2013; 2016).

The present results in Table 3 showed that MDA decreased significantly ($p \leq 0.05$) while GSH increased significantly in the group that received SL compared to the group fed OTA alone.

Only one can hypothesize the mechanism of silymarin action against OTA intoxication. Many writers have shown that OTA activation is a complicated method regulated by various cytochrome P450 enzymes in human and rat liver (Gallagher *et al.*, 1996). Silymarin can inhibit the cytochrome P450 system, as stated by Baer-Dubowska *et al.* (1998), and thus inhibit OTA activation. Silymarin increases hepatic glutathione and may boost the antioxidant defense of the liver. It has also been shown that Silymarin increases protein synthesis in hepatocytes by stimulating RNA polymerase I activity (Yu *et al.*, 2018). Abdalla, *et al.* (2018) found that the addition of 1 g silymarin / kg diet significantly improved TAC, GSH, MDA during the summer season compared with the control. Similar results were reported by Attia *et al.* (2017 and 2019).

It may be concluded from the results of this research that the birds held on silymarin, showed comparable hemato-biochemical reactions when compared to the control group reactions. Birds fed SL, with 1000 ppb/OTA showed enhancement in hemato-biochemical reactions, almost comparable to the control birds, indicating a protective impact of SL against OTA. These agents/substances may be used as a protective agent in poultry to ameliorate the adverse effects of ochratoxin A.

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