



Effects of Silymarin on Growth Performance, Internal Organs and Some Blood Parameters in Japanese Quail Subjected to Oxidative Stress Induced by Carbon Tetrachloride

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Abstract

The effects of Silymarin on growth performance, internal organs, and some blood parameters were investigated in Japanese quail that were subjected to oxidative stress induced by carbon tetrachloride (CCl₄). An experiment was conducted as a completely randomized design in a factorial arrangement (2 × 2) with four replicates of 30 birds each. Factors included two levels of Silymarin (0 and 1 mL/kg of body weight (BW)) and two levels of CCl₄ (0 and 1 mL/kg of BW). Results showed that Silymarin did not affect productive parameters, whilst CCl₄ significantly ($P < 0.05$) reduced feed intake and body weight gain. Silymarin did not affect the relative weights of breast, gizzard and heart, whereas CCl₄ reduced relative weights of breast and heart. Both Silymarin and CCl₄ administration resulted in higher pancreases relative weight. Birds treated with Silymarin had greater blood serum concentration of total protein and lower concentrations of glucose, triglyceride and total cholesterol ($P < 0.05$). In contrast, birds that received CCl₄ showed decreased total protein and increased glucose concentrations of blood serum ($P < 0.05$). The interaction effect between Silymarin and CCl₄ showed that Silymarin ameliorated the adverse effects of CCl₄ on blood albumin. Treatment of CCl₄ increased blood concentration of alkaline phosphatase compared with Silymarin ($P < 0.05$). This study showed that Silymarin may be a useful antioxidant source to ameliorate the adverse effects of oxidative stressors in Japanese quail.

Introduction

Stress results in reactive oxygen species (ROS) production. Excess generation of ROS can cause oxidative damage to macromolecules resulting in lipid peroxidation, mutagenesis, and carcinogenesis (Khan and Sultana, 2009).

Tetrachloride carbon (CCl₄) has long been known as a model toxicant and has been shown to induce oxidative stress both *in vitro* and *in vivo* (Manibusan *et al.*, 2007). ROS produced by CCl₄ cause serious damage to some tissues by

stimulating lipid peroxidation (Janbaz and Gilani, 2002).

In poultry farms, stress can reduce productive performance. Researchers have reported positive effects of medicinal plants on broiler's performance when challenged with stress (Ponte and Rosado, 2008). Medicinal plants possess beneficial properties including anticoccidial, antifungal, and antioxidant capabilities (Knekt *et al.*, 2002) and they have potential to prevent side effects of oxidative stress (Sonkusale *et al.*, 2011) by removing hydrogen peroxide (Schaffer *et al.*, 2004). Milk thistle (*Silybum marianum*) is a mixture of flavonolignans, and is known as Silymarin. The main active ingredients of its seeds are silybin, isosilybin, silycristin, and silydianin (Ding *et al.*, 2001). Some features of *S. marianum* include anti-oxidation and immune modulators (Katiyar, 2004). *S. marianum*, is also used in hepatoprotection against experimentally induced oxidative stress by various chemicals including CCl₄ (Gadgoli and Mishra, 1999).

In the present study, the effects of *in vivo* Silymarin administration was assessed on growth performance, internal organs, and some blood parameters in Japanese quail subjected to oxidative stress induced by CCl₄.

Materials and Methods

Plant preparation and extraction

S. marianum used in this experiment was collected from the heights of Ravansar (34°00' - 52" north latitude and 46°00' - 27" east longitude; altitude: 1650 m), Kermanshah province, Iran. Its total values of phenolic compounds, flavonoids, and antioxidants were measured colorimetrically, using Folin-Ciocalteu method (Guo *et al.*, 2000; Chang *et al.*, 2002). To prepare Silymarin, seeds were powdered with an electrical mill. Then powder was defatted by petroleum ether, dried, and mixed with ethanol 80% (2:10). Thereafter, it was shaken and passed through filter paper, and the remaining alcohol was removed by distillation under vacuum (Harborne, 1998; Bahraminejad *et al.*, 2008).

Birds, diets and experimental design

A total of 480 day-old Japanese quail chicks were reared for 42 days at a research farm of Gorgan University of Agricultural Sciences and Natural Resources (Gorgan, Golestan, Iran). The ambient temperature on d 1 was 38±1°C and then

decreased by 1°C every two days until a constant temperature of 24°C was reached. The lighting schedule provided 23 hrs of light per day. The experiment was performed as a completely randomized design with 4 replicates of 30 birds in each, using a 2 × 2 factorial arrangement with Silymarin and CCl₄ (olive oil solution at the volume ratio of 1:1) as main effects. Factors included 2 levels of Silymarin (0 and 1 mL/kg per body weight) and CCl₄ (0 and 1 mL/kg per body weight).

Silymarin was fed directly into crop using a syringe equipped with a plastic nozzle and a feeding tube (Nova Cath®, No. 10). Intraperitoneal injection of CCl₄ was performed on day 22 and again every three days thereafter. Chickens in the CCl₄ control group received an intraperitoneal injection of 0.9% sodium chloride solution instead (Sharma *et al.*, 2006) and those in the Silymarin control group were fed distilled water (1 mL/kg body weight) by an oral gavage. Birds were provided with free access to feed and water throughout the experimental period. The composition of the basal diet is shown in Table 1. All experimental protocols were approved by the Animal Care and Use Committee of the College of Animal Science of the Gorgan University of Agricultural Sciences and Natural Resources (Gorgan, Golestan, Iran).

Traits measured

Body weight gain (BWG) and feed intake (FI) were measured weekly and used to calculate feed conversion ratio (FCR) which is the quotient of FI divided by BWG. At the end of experiment, two birds from each replicate were selected and blood samples were collected in nonheparinized tubes from the brachial vein. Serum was obtained by centrifuging at 1500 × g for 7 min at 4°C and stored at -20°C until biochemical analysis. The serum samples were analyzed for triglyceride, total cholesterol, high density lipoprotein-cholesterol (HDL-c), total protein, albumin, glucose, and various liver enzymes including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP), using enzyme kits (Pars-Azmoon Co., Tehran, Iran). The relative weight (percentage of body weight) of carcass, trunk, and chest as well as internal organs (stomach, gizzard, pancreas, proventriculus, liver and heart) were evaluated to assess carcass performance at the end of the period.

Table 1. Composition and nutrients of basal diet¹

Ingredients	(%)
Corn	49.16
Soybean meal (44% protein)	45.05
Soybean oil	2.76
Calcium carbonate	1.30
Dicalcium phosphate	0.75
Salt	0.35
Vitamin premix ²	0.25
Mineral premix ³	0.25
DL-Methionine	0.13
<i>Calculated analysis:</i>	
ME (Kcal/kg)	2900
CP (%)	24.00
Calcium (%)	0.80
Available phosphorus (%)	0.30
Sodium (%)	0.15
Lysine (%)	1.30
Methionine (%)	0.50
Methionine + Cystine (%)	0.88

¹ Calculated composition according to NRC (1994).

² Vitamin premix (each kg contained): Vitamin A, 3600000 IU; Vitamin D3, 800000 IU; Vitamin E, 9000 IU; Vitamin K3, 1600 mg; Vitamin B1, 720 mg; Vitamin B2, 3300 mg; Vitamin B3, 4000 mg; Vitamin B5, 15000 mg; Vitamin B6, 150 mg; Vitamin B9, 500 mg; Vitamin B12, 600 mg; Biotin, 2000 mg.

³ Mineral premix (each kg contained): Mn, 50000 mg; Fe, 25000 mg; Zn, 50000 mg; Cu, 5000 mg; Iodine, 500 mg; Choline chloride 134000 mg.

Statistical analysis

This study was performed in a completely randomized design with four treatments and four replicates, each with 30 broilers, using a 2 × 2 factorial arrangements with Silymarin and CCl₄ as main effects. Data were analyzed using GLM procedure of SAS software (SAS, 2003). The main effect means and the interactions are reported and Duncan's multiple range tests were

used to compare the treatment effects. Differences were considered statistically significant at $P < 0.05$.

Results

Total phenol, flavonoids and antioxidant of Silymarin

The amounts of total phenol, flavonoids and antioxidants in Silymarin are shown in Table 2.

Table 2. Total phenol, flavonoids and antioxidants of Silymarin

Compounds	Dry weight
Total phenol	2.60 mg/g
Flavonoids	1.96 mg/g
Antioxidants	74.23%

Growth performance

The main and interaction effects of Silymarin and CCl₄ on growth performance of Japanese quails at 42 d of experiment are shown in Table 3. Silymarin did not affect productive parameters (FI, BWG and FCR) while CCl₄ significantly ($P < 0.05$) reduced BWG and FI. Experimental treatments did not affect FCR.

effects between Silymarin and CCl₄ on the relative weights of some internal organs ($P < 0.05$) including breast, gizzard, pancreas, and heart (Table 4). Silymarin did not affect the relative weights of breast, gizzard and heart, but CCl₄ reduced relative weights of breast and heart. Both Silymarin and CCl₄ resulted in higher relative pancreas weight.

The relative weight of internal organs

There were significant main and interaction

Table 3. Effect of Silymarin and carbon tetrachloride (CCl₄) on weekly growth performance of Japanese quails at 42 d (d 1 to 42)

	BWG ¹ (g)	FI ² (g)	FCR ³
Silymarin			
0 mL	36.26	143.60	3.96
1 mL	34.60	141.10	4.08
SEM	0.79	1.33	0.09
CCl ₄			
0 mL	36.71 ^a	144.80 ^a	3.94
1 mL	34.15 ^b	140.00 ^b	4.10
SEM	0.79	1.33	0.09
Interactions			
0 mL Silymarin × 0 mL CCl ₄	37.72	145.07	3.85
1 mL Silymarin × 0 mL CCl ₄	35.70	144.52	4.05
0 mL Silymarin × 1 mL CCl ₄	34.80	142.17	4.07
1 mL Silymarin × 1 mL CCl ₄	33.50	137.85	4.10
SEM	1.11	1.88	0.13
<i>Significance</i>			
Silymarin	0.16	0.22	0.41
CCl ₄	0.04	0.02	0.34
Silymarin × CCl ₄	0.75	0.33	0.52

¹ Body weight gain; ² Feed intake; ³ Feed conversion ratio.

^{a-b} Means within a column without a common superscript differ significantly ($P < 0.05$).

Table 4. Effect of Silymarin and carbon tetrachloride (CCl₄) on internal organs of Japanese quails at 42 d (% of live body weight)

	Carcass	Breast	Corpus	Gizzard	Proventriculus	Pancreas	Liver	Heart
Silymarin								
0 mL	0.38	0.26	0.12	0.02	0.004	0.004 ^a	0.03	0.01 ^a
1 mL	0.39	0.27	0.12	0.02	0.005	0.003 ^b	0.02	0.008 ^b
SEM	0.006	0.006	0.004	0.001	0.0002	0.0003	0.002	0.0003
CCl ₄								
0 mL	0.38	0.28 ^a	0.12	0.01 ^b	0.004	0.004 ^a	0.02	0.01 ^a
1 mL	0.38	0.25 ^b	0.13	0.02 ^a	0.005	0.002 ^b	0.03	0.008 ^b
SEM	0.006	0.006	0.004	0.001	0.0003	0.0002	0.002	0.0003
Interactions								
0 mL Silymarin × 0 mL CCl ₄	0.37	0.27	0.11	0.02	0.004	0.005	0.03	0.01
1 mL Silymarin × 0 mL CCl ₄	0.40	0.27	0.12	0.01	0.004	0.003	0.02	0.01
0 mL Silymarin × 1 mL CCl ₄	0.39	0.24	0.13	0.02	0.004	0.002	0.03	0.01
1 mL Silymarin × 1 mL CCl ₄	0.37	0.26	0.13	0.02	0.005	0.003	0.03	0.01
SEM	0.007	0.008	0.005	0.002	0.0002	0.0002	0.002 ₃	0.0004
<i>Significance</i>								
Silymarin	0.53	0.22	0.63	1.000	0.14	0.005	0.14	0.01
CCl ₄	0.75	0.018	0.07	0.18	0.61	0.0002	0.14	0.01
Silymarin × CCl ₄	0.014	0.22	0.17	0.18	0.14	0.033	0.61	0.07

^{a-b} Means within a column without a common superscript differ significantly ($P < 0.05$).

Blood biochemical parameters

Birds administered Silymarin showed greater blood serum concentrations of total protein and albumin but lower concentrations of glucose, triglyceride, and total cholesterol ($P < 0.05$). CCl₄ decreased total protein concentrations but

increased glucose concentrations of blood serum ($P < 0.05$). The interaction effect between Silymarin and CCl₄ showed that Silymarin ameliorated the adverse effect of CCl₄ on albumin (Table 5).

Table 5. Effect of Silymarin and carbon tetrachloride (CCl₄) on blood parameters of Japanese quails at 42 d

	Total protein (g/dL)	Albumin (g/dL)	Glucose (mg/dL)	Triglyceride (mg/dL)	Total cholesterol (mg/dL)	HDL-c ¹ (mg/dL)
Silymarin						
0 mL	2.80 ^b	1.08 ^b	306.25 ^a	192.63 ^a	170.12 ^a	39.25
1 mL	3.20 ^a	1.37 ^a	295.25 ^b	157.38 ^b	152.62 ^b	36.37
SEM	0.093	0.016	1.77	8.99	2.86	1.83
CCl₄						
0 mL	3.21 ^a	1.22	297.50 ^b	161.88	162.00	36.87
1 mL	2.75 ^b	1.23	304.00 ^a	188.13	160.75	38.75
SEM	0.093	0.016	1.77	8.99	2.86	1.83
Interactions						
0 mL Silymarin × 0 mL CCl ₄	3.07	1.15 ^c	303.25	171.25	174.25	37.50
1 mL Silymarin × 0 mL CCl ₄	3.35	1.45 ^a	291.75	152.50	149.75	36.25
0 mL Silymarin × 1 mL CCl ₄	2.52	1.02 ^d	309.25	214.00	166.00	41.00
1 mL Silymarin × 1 mL CCl ₄	3.05	1.30 ^b	298.75	162.25	155.50	36.50
SEM	0.131	0.023	2.51	12.72	4.05	2.59
Significance						
Silymarin	0.01	0.0001	0.001	0.016	0.001	0.28
CCl ₄	0.007	0.611	0.023	0.061	0.76	0.48
Silymarin × CCl ₄	0.362	0.0001	0.841	0.219	0.11	0.54

¹ High-density lipoprotein-cholesterol.

^{a-d} Means within a column without a common superscript differ significantly ($P < 0.05$).

Blood liver enzymes

Data on hepatic enzyme activities in blood serum (AST, ALT, and ALP) are presented in Table 6. Alkaline phosphatase (ALP) was the only hepatic enzyme that was influenced by

experimental treatments, as CCl₄ significantly increased its activity compared to Silymarin treatment ($P < 0.05$).

Table 6. Effect of Silymarin and carbon tetrachloride (CCl₄) on blood liver enzymes of Japanese quails at 42 d

	AST ¹ (U/L)	ALT ² (U/L)	ALP ³ (U/L)
Silymarin			
0 mL	274.50	7.12	179.50
1 mL	280.25	6.00	167.00
SEM	7.84	0.61	14.27
CCl₄			
0 mL	279.00	6.00	146.00 ^b
1 mL	275.75	7.12	200.50 ^a
SEM	7.84	0.61	14.27
Interactions			
0 mL Silymarin × 0 mL CCl ₄	271.50	6.50	154.00
1 mL Silymarin × 0 mL CCl ₄	286.50	5.50	138.00
0 mL Silymarin × 1 mL CCl ₄	277.50	7.75	205.00
1 mL Silymarin × 1 mL CCl ₄	274.00	6.50	196.00
SEM	11.09	0.86	20.18
Significance			
Silymarin	0.61	0.21	0.54
CCl ₄	0.77	0.21	0.01
Silymarin × CCl ₄	0.42	0.88	0.86

¹ Aspartate aminotransferase; ² Alanine aminotransferase; ³ Alkaline phosphatase.

^{a-c} Means within a column without a common superscript differ significantly ($P < 0.05$).

Discussion

Oxidative stress caused by excessive reactive oxygen species is one of the main factors that negatively affect organismal performance

(Dalloul et al., 2006; Lin et al., 2006) and underlies pathogenesis of several important diseases (Kris-Etherton et al., 2004). Oxidative

stress induced by carbon tetrachloride is involved with the cytochrome P₄₅₀-NADPH enzyme system through the metabolism of chloromethyl and proxy chloromethyl reactive radicals (Ha *et al.*, 2005). These radicals attack unsaturated fatty acids, alkalizing proteins, and other cellular macromolecules that lead to lipid peroxidation of the cell membrane, changes in enzyme function, and ultimately cell damage (Kodai *et al.*, 2007). In the current study, CCl₄ significantly reduced feed intake and body weight gain, which is consistent with previous studies (Khorramshahi *et al.*, 2014; Khodadust *et al.*, 2015). Reduced body weight gain could be due to reduced nutrient digestion and absorption as a result of low bile secretion (Panovska *et al.* 2007). Silymarin did not improve growth performance in birds treated with CCl₄ which contrasts the results of Ebrahimi *et al.* (2013), who used powdered Silymarin at various concentrations (0, 100, and 200 mg/kg) in broilers. This difference may be due to species differences and experimental doses. In this regard, susceptibility of broilers due to genetic selection should be taken into consideration. The relative increase in gizzard weight in birds treated with CCl₄ may be due to fat accumulation in gizzard. It was documented that CCl₄ can increase blood lipid concentrations which can lead to the fat accumulation in visceral organs (Devarshi *et al.*, 1986). However, although changes in the relative weight of pancreas and heart require further investigation, the relative weight changes may be due in part to changes in cardiac blood circulation and basal metabolism. The loss of relative carcass and breast weight in birds treated with CCl₄ can be due reduced secretion of bile due to liver oxidative damage and thereby reduced digestion and absorption of nutrients (Panovska *et al.*, 2007). In addition, it was reported that toxins produced through the destruction of intestinal epithelial cells or released during changes in the intestinal ecosystem have an adverse impact on performance parameters (Applegate *et al.*, 2009).

Hepatocytes are involved in protein metabolism; therefore, any damage to hepatocytes can lead to reduced circulation of protein concentrations (Chlopčiková *et al.*, 2004). In congruence with our results, there are some studies reporting adverse effects of CCl₄ on blood serum protein concentration (Kumar *et al.*, 2009; Sonkusale *et al.*, 2011; Jothi *et al.*, 2012).

CCl₄ reduces blood serum albumin concentration, possibly because of ribosome breakdown (Redman, 1969). In agreement with our findings, positive effects of some medical plants including Silymarin (Neshatgharamaleki and Mohajeri, 2014) and peppermint (Mehri *et al.*, 2015) on hepatocyte protein synthesis have been reported. Additionally, Huseini *et al.* (2006) stated that using medicinal plants enhances concentrations of total protein, albumin and globulin; hence albumin: globulin ratio in blood serum of broiler chickens.

The mechanism by which the *Silybum marianum* reduces blood sugar is not well understood, but it may work by inducing beta cells of the pancreas to produce insulin (Soto *et al.*, 2004). Silymarin repairs and renovates the pancreatic tissue, which plays an important role in the regulation of blood sugar (Soto *et al.*, 2004). Soto *et al.* (2003) studied the effect of Silymarin on pancreatic function in diabetic animals and noted that Silymarin plays a protective role in pancreatic tissue against damaging elements, thereby exerting its hypoglycemic effect. Indeed, we observed a reduction in blood glucose levels with Silymarin treatment. In addition, flavonoids such as Silymarin could regulate liver enzymes involved in the metabolism of carbohydrates, and therefore reduce blood sugar and restore weight. This occurs as a result of reduced liver phosphorylase enzyme activity and increased activity of glucokinase and glycogen synthase (Abascal and Yarnell, 2003a-b).

It has been reported that beta-oxidation of fatty acids and hydrolysis of triglyceride lessens under the influence of CCl₄. This leads to enhanced availability of fatty acids for esterification, and consequently, CCl₄ facilitates the synthesis of fatty acids and triglycerides through acetate. This process can be the result of acetate transfer to liver cells, followed by cholesterol upsurge (Boll *et al.*, 2001). Hasani-Ranjbar *et al.*, (2010) reported that there is a significant decrease in levels of total cholesterol and LDL-c, and improvement in total antioxidant power after treatment with *S. marianum*, garlic, and wheat germ. Banaee *et al.*, (2011) showed that oral administration of Silymarin to fish significantly reduced plasma glucose and total cholesterol levels. Tumova *et al.*, (2010) reported that administration of Silymarin resulted in lower serum cholesterol levels and mild increase in HDL-c levels. These

results may be due to fat-mediated improved bioavailability and/or by inhibition of resorption of dietary cholesterol. *S. marianum* seed extract (Silymarin) caused a significant decrease in LDL-c (Huseini et al., 2006).

Liver cells contain high concentrations of ALT, AST and ALP enzymes. ALT and AST are enzymes present in hepatocytes and liver parenchymal cells, respectively. Increased levels of these transaminases in hepatocytes are indicators of poor hepatic integrity. Destruction of liver cells leads to the leakage of these enzymes into the blood stream (Parmar et al., 2012). Therefore, an increased concentration of liver enzymes in the blood circulation is one of the main indicators of liver damage due to the toxins (Hetrog and Hollmann, 1998). CCl₄ is a toxic agent that can destroy hepatocyte membrane integrity and raise blood levels of AST, ALT, and ALP enzymes (Tsukamoto et al., 1990; Soni and Nishant, 2008). It has been reported that the most important causes of increased serum AST in birds are liver diseases (Campbell and Coles, 1986). The impact of Silymarin on liver transaminases has been reported. Silymarin can treat damaged hepatocytes and restore normal liver function (Muriel & Mourelle, 1990; Wang et al., 2013). Neshatgharamaleki and Mohajeri, (2014) showed that administration of Silymarin in rats

resulted in a significant reduction in ALP. Similar to our observations, Silymarin also lowered alanine aminotransferase levels in broiler chickens under stress induced by aflatoxin B₁ (Fani Makki et al., 2014).

In general, this study showed that Silymarin did not affect productive parameters, while CCl₄ significantly reduced feed intake and body weight gain. CCl₄ reduced relative weights of breast and heart. Birds treated with Silymarin showed greater blood serum concentration of total protein and lower concentrations of glucose, triglyceride and total cholesterol, while CCl₄ decreased total protein and glucose concentrations in blood serum. The interaction effect between Silymarin and CCl₄ showed that Silymarin ameliorated the adverse effects of CCl₄ on albumin. CCl₄ significantly increased blood levels of ALP compared to Silymarin treatment. Therefore, this study suggests that Silymarin may be a useful antioxidant source to ameliorate the adverse effects of oxidative stressors in poultry farms.

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