



The Effects of Silymarin on Oxidative Status and Bone Characteristics in Japanese Quail Subjected to Oxidative Stress Induced by Carbon Tetrachloride

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Abstract

This experiment was conducted to assess the effects of Silymarin on oxidative status, bone characteristics, and some blood parameters in Japanese quail subjected to oxidative stress induced by carbon tetrachloride (CCl₄). The experiment was performed as a completely randomized design with four replicates, each with 30 birds, using a 2 × 2 factorial arrangement with two doses of Silymarin (0 and 1 mL/kg BW) and CCl₄ (0 and 1 mL/kg BW). Results revealed that the interaction between Silymarin and CCl₄ on concentrations of total cholesterol, triglycerides, glucose, albumin, calcium, and alkaline phosphatase were significant ($P < 0.05$). In contrast, concentrations of phosphorus, total protein, and high density lipoprotein-cholesterol in blood serum did not differ between experimental treatments. Experimental treatments had a significant effect on superoxide dismutase activity in blood serum ($P < 0.05$), but not on glutathione peroxidase activity and malondialdehyde concentration. Experimental treatments significantly affected the weight, thickness, and external and internal diameters of tibia bone ($P < 0.05$), but not its length, ash, volume, and density. This study shows that Silymarin has potential to attenuate adverse effects of oxidative stress induced by CCl₄ in Japanese quail.

Introduction

Poultry experience various stressors which can result in oxidative stress. Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds their elimination by different antioxidant agents. ROS damage biomolecules by disrupting intracellular reduction-oxidation balance (Khan and Sultana, 2009).

Carbon tetrachloride (CCl₄) is a model toxicant that has been assessed in both *in vitro* and *in vivo* toxicological studies (Manibusan *et*

al., 2007). CCl₄ causes serious damage to tissues by stimulating lipid peroxidation (Janbaz *et al.*, 2002). Bone tissue is sensitive to free radical damage (Basu *et al.*, 2001) as ROS influences bone mineralization by modulating bone cell function (Wauquier *et al.*, 2009).

It was reported that medical plants can remove hydrogen peroxide and have a role in the preventing the side effects of oxidative stress (Schaffer *et al.*, 2004; Sonkusale *et al.*, 2011). Milk

thistle (*Silybum marianum*), known as Silymarin, is a herb with antioxidant properties and contains a mixture of flavonolignans with the active ingredients. The main substances in seed extract of this plant are silybin, isosilybin, silycristin, and silydianin (Ding *et al.*, 2001). *Silybum marianum* has numerous functions including antioxidant (Katiyar, 2005), anti-inflammatory (Horváth *et al.*, 2001) and immune modulation (Katiyar, 2005), and protection against non-melanoma skin cancer (Vayalil *et al.*, 2003). As an antioxidant polyphenol flavonoid, Silymarin has the natural ability to clean up free radicals (Skottova & krecman 1998). In this study, *in vivo* antioxidant function of Silymarin was assessed against oxidative stress induced by CCl₄ in Japanese quail.

Materials and Methods

Plant preparation and extraction

Silybum marianum used in this experiment was collected in the summer from the heights of Ravansar (34° 52' N 46° 27' E; altitude: 1650 m), Kermanshah, Iran. The total values of phenolic compounds, flavonoids, and antioxidants were measured calorimetrically according to the Folin-Ciocalteu method (Chang *et al.*, 2002). To prepare Silymarin, *S. marianum* seeds were powdered by a full electric mill (DM-WP120), then defatted by petroleum ether. Samples were dried and mixed (2:10 v/w) with 80% ethanol, then shacked for 24 hrs and passed through paper filter. The alcohol fraction was removed under vacuum (Harbone, 1998).

Birds, diets and experimental design

A total of 480 day-old Japanese quail chicks (BW±SE, 7±1 g) were obtained from a local commercial hatchery and raised over a 42-d experimental period. The birds were housed in an environmentally controlled poultry house with paper roll as litter at the research farm of Animal Science Faculty, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Golestan, Iran. Ambient temperature was initially set at 38±1°C and then decreased by 1°C every 2 days until a constant temperature of 24°C was reached at day 35 of experiment. The lighting schedule provided 23 hrs of light per day.

The experiment was performed as a completely randomized design with four replicates of 30 birds each, using a 2 × 2 factorial

arrangement with two doses of Silymarin (0 and 1 mL/kg BW) and CCl₄ (0 and 1 mL/kg BW). Experimental treatments were applied beginning on day 22 of the study. Silymarin was fed directly into crop *via* a syringe equipped with a plastic nozzle and feeding tube (Nova Cath®, No. 10). Intraperitoneal injection of CCl₄ was performed beginning on day 22, and again every three days (Sonkusale *et al.*, 2011). In order to homogenize the stress, the control group received 1 mL/kg BW 0.9% sodium chloride solution *via* intraperitoneal injection (Sharma *et al.*, 2006) and also 1 mL/kg BW of distilled water by oral gavage. Birds had free access to feed and water throughout the experiment. The composition of the basal diet is shown in Table 1.

Table 1. Ingredients and chemical compositions of the 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 was 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.

Traits measured

On day 42, two birds from each replicate were selected, and blood samples were collected in nonheparinized tubes from the brachial vein. Sera were obtained by centrifuging at 1500 × g for 7 min at 4°C and stored at -20°C until biochemical analysis (Khodadust *et al.*, 2015). The serum samples were analyzed for total

protein, albumin, glucose, triglyceride, total cholesterol, high-density lipoprotein cholesterol (HDL-c), calcium, phosphorous, and alkaline phosphatase (ALP) activity using enzymatic related kits (Pars-Azmoon Co., Tehran, Iran).

The activities of the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPX) were measured in red blood cells. Blood serum SOD activity was measured according to the Woolliams *et al.*, (1983). Briefly, the inhibition of nitroblu tetrazolium (NBT) reduction with xanthine-xanthine oxidase was used as a superoxide generator. One unit of SOD function was defined as the amount of protein that inhibits the rate of NBT reduction by 50%. Activity of GPX was determined according to methods described by Paglia and Valentine (1967).

Following the slaughtering on day 42, the right tibia from two birds of each replicate was collected. Tibia length was measured as distance between the two ends of the bone using a digital caliper (ACCUD SERIES 111). Tibia volume was measured under the assumption that the specific gravity of water is 1 g/cm³ at room temperature (Kim *et al.*, 2004). External and internal diameters and tibia thickness were determined using a digital caliper. To measure tibia density, bones were placed for 48 hrs in an oven at 60°C to dry. Thereafter, they were cooled in desiccators and weighed. Bone density was calculated by dividing the dry weight of each bone to its volume (Zhang & Coon, 1997). Tibia ash was

determined following its defatting by chloroform (AOAC, 1980).

Statistical analysis

The data was analyzed using GLM procedures of SAS software (SAS, 2003). Duncan's multiple tests were used to compare the effects of treatment. Differences were considered statistically significant at $P < 0.05$.

Results

Total phenol, flavonoids, and antioxidant of Silymarin

In vitro examinations showed that the prepared Silymarin contained 2.606 mg/g total phenol, 1.964 mg/g flavonoids, and 74.231% antioxidants.

Blood biochemistry

The main effects of Silymarin, CCl₄, and their interactions on blood serum biochemistry in Japanese quail chicks are depicted in Table 2. In birds treated with Silymarin, albumin concentration was greater, whereas ALP hepatic enzyme activity and cholesterol and glucose concentrations were lower ($P < 0.05$). Administration of CCl₄ resulted in lower concentrations of calcium and triglyceride and a higher concentration of glucose ($P < 0.05$). There were significant interactions between Silymarin and CCl₄ on albumin, calcium, ALP, triglycerides, cholesterol, and glucose in blood sera ($P < 0.05$).

Table 2. Effect of Silymarin and carbon tetrachloride (CCl₄) on blood biochemical parameters of Japanese quail at 42 d

	Total protein (g/dL)	Albumin (g/dL)	Calcium (mg/dL)	Phosphor (mg/dL)	ALP ¹ (U/L)	Triglyceride (mg/dL)	Cholesterol (mg/dL)	HDL ² (mg/dL)	Glucose (mg/dL)
Silymarin (mL/kg BW)									
0	2.97 ^b	1.1 ^b	9.9	8.5	218.8 ^a	156.4	165.9 ^a	76.0	304.7 ^a
1	3.25 ^a	1.3 ^a	10.1	8.5	188.7 ^b	156.9	151.4 ^b	70.6	294.3 ^b
SEM	0.05	0.01	0.3	1.5	13.48	5.10	1.88	3.32	0.73
CCl₄ (mL/kg BW)									
0	3.15	1.2	10.4 ^a	8.3	206.5	178.3 ^a	162.7	74.2	297.5 ^b
1	3.07	1.1	9.5 ^b	8.7	201.0	135.0 ^b	154.6	72.4	304.5 ^a
SEM	0.07	0.01	0.12	1.4	13.97	3.57	2.13	3.45	0.74
Silymarin × CCl₄									
0 × 0	3.0	1.1 ^{bc}	10.3 ^a	8.1	206.7 ^a	179.3 ^a	170.6 ^a	81.2	301.4 ^a
1 × 0	3.3	1.3 ^a	10.3 ^a	8.5	206.4 ^a	177.4 ^a	154.8 ^{ab}	67.2	287.6 ^b
0 × 1	2.9	1.1 ^c	9.3 ^b	8.8	231.0 ^a	133.6 ^b	161.2 ^{ab}	70.8	308.0 ^a
1 × 1	3.2	1.2 ^{ab}	9.7 ^b	8.5	171.0 ^b	136.4 ^b	148.0 ^b	74.0	301.0 ^a
SEM	0.05	0.01	0.1	1.6	13.05	3.78	1.86	82.9	53.0
P-value									
Silymarin	0.01	0.00	0.36	0.95	0.02	0.97	0.01	0.23	0.01
CCl ₄	0.50	0.41	0.00	0.50	0.70	0.00	0.19	0.69	0.02

¹Alkaline phosphatase. ²High-density lipoprotein cholesterol.

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

Antioxidant status

The main effects of Silymarin, CCl₄, and their interactions on the activities of antioxidant enzymes and MDA content in sera of Japanese quails are shown in Table 3. The interaction

between Silymarin and CCl₄ was significant on SOD activity ($P < 0.05$), but not GPX activity and MDA content. Silymarin decreased activity of SOD hepatic enzyme in blood serum, while CCl₄ increased SOD activity ($P < 0.05$).

Table 3. Effect of Silymarin and carbon tetrachloride (CCl₄) on antioxidant enzymes and MDA of Japanese quail at 42 d

Treatments	SOD ¹ (U/gHb)	GPX ² (U/gHb)	MDA ³ (nmol/L)
<u>Silymarin (mL/kg BW)</u>			
0	1271.4 ^a	50.26	2.08
1	1007.1 ^b	47.08	2.22
SEM	3.89	2.36	0.11
<u>CCl₄ (mL/kg BW)</u>			
0	1098.5	49.02	2.27
1	1180.0	48.32	2.03
SEM	4.86	2.51	0.10
<u>Silymarin × CCl₄</u>			
0 × 0	1362.0 ^a	51.84	2.34
1 × 0	835.0 ^c	46.20	2.20
0 × 1	1180.8 ^b	48.68	1.82
1 × 1	1179.2 ^b	47.96	2.24
SEM	2.38	2.4	0.08
<u>P-value</u>			
Silymarin	0.00	0.14	0.36
CCl ₄	0.42	0.75	0.11
Silymarin × CCl ₄	0.04	0.31	0.06

¹Superoxide dismutase. ² Glutathione peroxidase. ³ Malondialdehyde.

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

Table 4. Effect of Silymarin and carbon tetrachloride (CCl₄) on Index of Tibia Bone of Japanese quail at 42 d

Treatments	Weight (g)	Length (mm)	Volume (cm ³)	Density (g/ cm ³)	Thickness (mm)	External diameter (mm)	Internal diameter (mm)	Ash (%)
<u>Silymarin (mL/kg BW)</u>								
0	0.78 ^a	51.55	0.78	0.96	0.59	3.64 ^a	2.89	0.55
1	0.72 ^b	51.23	0.74	0.96	0.53	3.44 ^b	3.11	0.53
SEM	0.002	0.44	0.002	0.000	0.004	0.02	0.08	0.002
<u>CCl₄ (mL/kg BW)</u>								
0	0.76	51.60	0.78	0.97 ^a	0.60 ^a	3.63 ^a	2.81 ^b	0.56 ^a
1	0.74	51.20	0.75	0.96 ^b	0.52 ^b	3.45 ^b	3.19 ^a	0.52 ^b
SEM	0.003	0.43	0.002	0.001	0.004	0.02	0.06	0.001
<u>Silymarin × CCl₄</u>								
0 × 0	0.80 ^a	51.54	0.81	0.97	0.56 ^b	3.44 ^b	2.87 ^b	0.56
1 × 0	0.71 ^b	50.84	0.74	0.97	0.63 ^a	3.46 ^b	2.76 ^b	0.57
0 × 1	0.71 ^b	51.56	0.75	0.96	0.50 ^b	3.45 ^b	2.92 ^b	0.51
1 × 1	0.72 ^b	51.61	0.74	0.96	0.54 ^b	3.81 ^a	3.46 ^a	0.53
SEM	0.002	0.44	0.002	0.000	0.03	0.01	0.01	0.001
<u>P-value</u>								
Silymarin	0.01	0.44	0.12	0.4	0.07	0.01	0.11	0.44
CCl ₄	0.47	0.34	0.14	0.02	0.01	0.03	0.00	0.02
Silymarin × CCl ₄	0.04	0.52	0.08	0.14	0.01	0.01	0.01	0.12

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

Bone indexes

The main effect of Silymarin, CCl_4 , and their interactions on bone indexes of Japanese quail at day 42 are shown in Table 4. Silymarin and CCl_4 had significant interactions on weight, thickness, and external and internal diameters of tibia. Both Silymarin and CCl_4 decreased tibia weight ($P < 0.05$). Tibia thickness increased in quails treated with Silymarin, but CCl_4 did not have an effect ($P < 0.05$). The external and internal diameters of tibia bone increased in birds that received both treatments ($P < 0.05$). CCl_4 resulted in lower density and ash percentage of tibia bone ($P < 0.05$).

Discussion

Stressful conditions are unavoidable problems in poultry production systems. There is an increasing interest in the use of herbs and medicinal plants in poultry feeding to help overcome the stress. Medicinal plants have positive effects on performance of broilers due to their secondary metabolites, which can be ergogenic, antiphrastic, antibacterial, anti-flatus, antifungal, and antiseptic (Huang et al., 1992). It has been shown that Silymarin reduces the concentrations of glucose, cholesterol, and triglyceride in blood of broilers challenged with lead (Ebrahimi et al, 2013). Consistent with the current study, Case et al. (1995) reported that plant secondary metabolites (for example, thymol, carvacrol, and silybin) may inhibit the function of hepatic enzymes involved in the synthesis of cholesterol and fatty acids. Tollba et al. (2010) demonstrated that using various herbal ingredients can reduce blood lipids in broilers, hens and quails. Silymarin can also impact the kinetics of glucose 6-phosphatase and inhibit gluconeogenesis to decrease blood glucose (Guigas et al, 2007). Fanimakki et al., (2014) reported that Silymarin has no effects on total protein and albumin in blood serum, which contrasts our findings in albumin. It seems that this difference is due to the sensitivity of different species, as well as the form of Silymarin (powder or extract) used. It was reported that Silymarin has no effect on blood serum calcium and phosphorus (Amiridumari et al., 2013), which is consistent with the current study. Sonkusale et al (2011) reported that intraperitoneal injection of CCl_4 to broilers significantly increased blood concentrations of total cholesterol and decreased serum

concentrations of albumin, total protein and HDL-c. Samudram et al. (2008) reported a lower total protein levels in blood serum following the destructive effects of CCl_4 on the function of rat liver. The decrease in blood serum levels of total protein and albumin could be due to its reduced biosynthesis, which could occur by impairing ribosomal protein biosynthesis of endoplasmic reticulum (Clawson, 1989). Similarly, Gad et al. (2011) showed that CCl_4 decreases serum protein and albumin levels, but increases serum lipid levels, showing the adverse effect of CCl_4 on liver function. Owen (1990) noted an increase in serum cholesterol under liver diseases related to undermining role of the liver to remove cholesterol from the blood stream.

CCl_4 can lead to liver damage and necrosis by inducing production of free radicals. Hepatic necrosis elevates blood serum enzymes such as ALP and decreases activities of antioxidant enzymes such as SOD and catalase (Eidi, 2010) which is consistent with our findings of SOD. Studies have shown that the antioxidant properties of Silymarin can increase cellular glutathione, superoxide dismutase, and glutathione peroxidase function in brain tissue of rat (Nencini et al, 2007) which contrasts the current experiment. These differences could be due to differences in the duration of the experiment, the amount of Silymarin used, experimental materials, and the studied tissues (brain or blood stream). The administration of Silymarin in rats with diabetes mellitus leads to appropriate changes in MDA (an index of lipid peroxidation) and antioxidant enzymes such as SOD (Baluchnejadmojarad et al, 2010). However, in our study, CCl_4 did not increase lipid peroxidation, which could be due to the sensitivity of experimental species and usage dose.

Studies in humans and animals have shown that secondary metabolites of plants have modulatory effects on bone mineralization (Sunita & Pattanayak, 2011). The current study shows that Silymarin has more positive effects on tibia thickness than CCl_4 , perhaps due to the similarity between Silybin and estrogen in the mineralization of bone tissue (Gebhardt, 2002). Estrogen has positive effects on bone mineralization by increasing the function of the vitamin D receptor in intestinal mucosa and osteoblast cells, which leads to synthesis of bone

matrix protein and mineral storage (Liel *et al.*, 1999). Similarly, Kim *et al.*, (2012) documented osteoblastic function of Silymarin in tibia-fractured mice. The positive effects of medicinal plants in the chemical composition and physical properties of tibia in broiler chickens were reported (Pisarski & Kwiecien, 2003). Tahmasebi *et al.* (2012) showed that herbal phytoestrogens such as *Withania somnifera* enhances bone calcification. Few studies have been conducted on the effects of toxins, especially CCl₄, on tissues and bone characteristics. It has been documented that CCl₄ can disrupt the balance between oxidants and antioxidants, which can cause oxidative damage to biological molecules (Soni *et al.*, 2008), such lipid peroxidation in bone tissue. For example, teratogenic effects of the

pesticide diazinon were reported on the growth of cartilage and bone (Meneely & Wyttenbach, 1989) in chicken and quail embryos. Field studies have correlated exposure to organochlorines with loss of alveolar bone in gray seals (Bergman *et al.*, 1992), reduced bone density in deer mice (Johnson *et al.*, 2009), and altered mineralization in clapper rails (Rodriguez-Navarro *et al.*, 2006). In general, it can be concluded that Silymarin has potential to attenuate adverse effects of oxidative stressors in Japanese quail by stabilizing lipid peroxidation.

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