https://doi.org/10.52973/rcfcv-e34375 Revista Científica, FCV-LUZ / Vol. XXXIV, rcfcv-e34375

Pathological and biochemical investigation of the effects of *Silybum marianum* against Methomyl damage in broiler liver

Investigación patológica y bioquímica de los efectos de *Silybum marianum* contra el daño por metomilo en hígado de pollo

Bahadır Kilinc[1]*, Ertan Oruc[2]

[1]*Veterinary Control Central Research Institute, Pathology Laboratory. Ankara, Türkiye.*
[2]*Selcuk University, Faculty of Veterinary Medicine, Department of Pathology. Konya, Türkiye.*
**Corresponding Author: patbhdr@gmail.com*

ABSTRACT

This study was aimed to investigate the protective/preventive and/or curative effects of *Silybum marianum* seed powder against Methomyl toxication by examining some biochemical parameters and pathological changes hepatic in broiler chicks fed with feeds supplemented with Methomyl and *S. marianum* seed powder. For this purpose, 4 different groups, each containing 32 animals, were used; Control group (CONT), Methomyl group (MET), *S. marianum* seed powder group (SMT) and Methomyl+*S. marianum* seed powder group (MET+SMT). In the study, Methomyl was added to the feeds as 20 ppm and *S. marianum* seed powder as 10 g·kg^{-1}. The trial period was planned as 28 days, and necropsies of animals from each group were performed daily, and samples were taken for histopathology and biochemistry. In the study, liver enzyme activities and liver tissue oxidative stress markers GPx, MDA and SOD values were found to be similar in the CONT and SMT groups, but statistically higher in the MET group. In the MET+SMT group, the increased parameters were lower than the MET group. The histopathological examinations of liver sections, hyperemia, hemorrhage, hydropic degeneration and fatty changes of hepatocytes, focal necrosis, dissociation of remark cords, mononuclear cell infiltration in the portal area and bile ducts hyperplasia were detected. It has been observed that *S. marianum* given for preventive/healing purposes reduces histopathological damage and contributes positively in all groups. It has been concluded that *S. marianum* can be added to poultry diets against Methomyl residues or contaminations according to this study.

Key words: Histopathology; liver; methomyl; *Silybum marianum;* broiler

RESUMEN

En este estudio, el objetivo fue investigar los efectos protectores/preventivos y/o curativos del polvo de semillas de *Silybum marianum* contra la toxicidad del Metomilo examinando algunos parámetros bioquímicos y cambios Hepáticos en pollos de engorde alimentados con alimentos y cambios patológicos en pollos de engorde a los cuales se les suministró alimento mezclado con Metomilo y/o *S. marianum.* Para ello, se crearon 4 grupos diferentes, cada uno compuesto por 32 animales: grupo control (CONT), grupo Metomilo (MET), grupo de semillas de *S. marianum* en polvo (SMT) y grupo de Metomilo+Polvo de semillas *S. marianum* (MET+SMT). En la investigación, se añadió Metomilo al pienso en 20 ppm y 10 g·kg^{-1} de polvo de semilla de *S. marianum*. El período de prueba se planificó en 28 días y se realizaron necropsias semanales de los animales de cada grupo y se tomaron muestras para histopatología y bioquímica. En el estudio, se encontró que las actividades de las enzimas hepáticas y los valores de los marcadores de estrés oxidativo del tejido hepático GPx, MDA y SOD eran similares en los grupos CONT y SMT, pero estadísticamente más altos en el grupo MET. Los parámetros crecientes en el grupo MET+SMT fueron menores que en el grupo MET. El examen histopatológico de las secciones del hígado reveló hiperemia, sangrado, degeneración hidrópica y cambios grasos en los hepatocitos, necrosis focal, disociación de los cordones de estimulación, infiltración de células mononucleares en el área portal e hiperplasia en los conductos biliares. Se ha observado que *S. marianum*, administrado con fines preventivos/curativos, reduce el daño histopatológico y realiza una contribución positiva en todos los grupos. Segun los resultados de este estudio, se concluyó que *S. marianum* se puede añadir a las dietas de aves de corral para disminuir los efectos adversos de residuos de Metomilo o contaminaciones de Metomilo.

Palabras clave: Histopatología; hígado; metomilo; *Silybum marianum;* pollos

Received: 07/01/2023 Accepted: 13/02/2024 Published: 12/06/2024

INTRODUCTION

Pesticides used in agriculture not only protect crops from damage, but also improve their yield during the production process [1]. In cases where pesticides are necessary but not used, it is known that there are quality and yield losses reaching up to 60% in crops. For this reason, it is mandatory to use pesticides in plant protection in order to suppress harmful organisms that cause yield reduction [2, 3]. The most common way farm and poultry animals are exposed is through the consumption of feed contaminated with pesticides [4]. Methomyl has been used as a pesticide since 1968 [5]. Besides it was reported that it was also used maliciously in various criminal situations such as the killing of guard dogs for the purpose of theft [6]. Long-term exposure to methomyl is known to cause hepatotoxicity, cytotoxicity and neurotoxicity in animals [7]. The oral LD50 dose of Methomyl has been reported as 28 ppm in poultry [8]. There are limited studies showing the effects of pesticide residues in poultry diets on animal performance [9].

In a study conducted by Veltmann Jr and Linton [10] with thiram in broilers, it was reported that lameness, decreased weight gain and reluctance to move were observed in broilers. The severely exposed broilers were also observed to lie in the sternal position. In some studies, the toxic effects of methomyl in liver tissue were determined histopathologically. A study performed in mice showed histopathologically that hepatocellular damage was accompanied by congestion and dilation in the central and portal veins, mononuclear cell infiltration in the portal area and necrosis of hepatocytes [11].

S. marianum plant is a powerful antioxidant and shows hepatoprotective effect [12]. Seeds contain a very large amount of silymarin [13]. The extracts prepared from its seeds contain 70–80% Silymarin. *S. marianum* is a liver supporting and preservative plant [14]. This plant has been benefited from for 2000 years against liver toxicity [12, 15, 16].

This study was aimed to investigate it was aimed to investigate the toxic effects of Methomyl on liver tissue and hepatoprotective effect of *S. marianum* seed powder by histopathologic and biochemical methods in broiler chickens.

MATERIALS AND METHODS

Study design

Methomyl (®Coupon 90 SP) and *S. marianum* seed powder used in the study were commercially purchased. In the study, 20 ppm Methomyl (MET) was added to the standard broiler rations to the MET groups. SMT groups received 10 g *S. marianum* (SMT) seed powder per kg broiler ration. In order to ensure the standard of *S. marianum* seed powder, Silymarin was quantified in the commercially purchased product according to the European Pharmacopoeia [17]. The analysis by HPLC (Agilent brand 1100 model, USA) method showed 1.8% Silymarin content. In the present study, broiler rations containing Methomyl and *S. marianum* seed powder were prepared separately for each group by homogenizing with a mixer (Chrome Master brand Ribbon model Türkiye) in a special feed mill. Homogenized feeds were placed in sealed kraft bags and stored in the reinforced concrete building, at a distance from the floor and side walls.

The 128 (7-day-old) broiler chicks Ross 308 (*Gallus gallus domesticus*) used in the study were obtained from a commercial breeder. The chicks taken into the experimental environment were kept under controlled care and feeding for 7 days (d) for adaptation. Thus, the chicks were ready for the experiment at the age of 14 d. Afterwards, blood was taken from the animals at 7-day intervals and necropsies were performed. The experimental procedure was terminated at 42 d of age. The experiment period lasted 28 d. The experimental groups were formed as follows. CONT Group (n=32): Broilers in this group were given standard broiler ration and drinking water *ad libitum* for 28 d. MET Group (n=32): Broilers in this group were given standard broiler ration containing 20 ppm Methomyl and drinking water *ad libitum* every day for 28 d. SMT Group (n=32): Broilers in this group were given standard broiler ration containing 10 g·kg^{-1} *S. marianum* seed powder and drinking water *ad libitum* every day for 28 d. MET+SMT Group: (n=32) Broilers in this group were given standard broiler ration containing 20 ppm Methomyl + 10 g·kg^{-1} *S. marianum* seed powder every day for 28 d and drinking water *ad libitum.* A lighting program of one hour dark and 23 hours light was applied for all broiler groups.

A total of 122 broilers were euthanised by decapitation method, including 8 broilers randomly selected from each group (96 animals in total) on the 7th, 14th and 21st d and the remaining 26 animals (7 broilers each from CONT, SMT and MET–SMT groups and 5 broilers from MET group) on the 28th d. The study was approved by the Local Ethics Committee of the Central Research Institute of Veterinary Control Directorate (ethics committee approval dated 05.05.2021 and decision number 2021/07).

Histopathological examination

Macroscopic findings of the liver were recorded during the necropsy. For histopathological examinations, liver specimens were placed in 10% buffered formalin solution for fixation and routine tissue preparation procedure was performed. Hematoxylin Eosin (H×E) staining was performed on 5 µm thick tissue sections [18, 19, 20, 21]. In order to evaluate the histopathological findings, the entire section area of the tissue was examined under 4×, 10×, 20× and 40× objective (Leica DM2500, Germany) respectively. Scoring was achieved by modifying the method reported by Meyerholz *et al.* [22]. For this purpose, 9 areas were examined under 20× objective and scored according to TABLE I. Histopathologically, hyperaemia, haemorrhage, degeneration, fatty degeneration, necrosis, dissociation, mononuclear cell infiltration in the portal area and bile duct hyperplasia were examined. Randomly taken eight liver samples from each experimental group on the 7th, 14th, 21st d and 7 animals from the CONT, SMT and MET+SMT groups and 5 animals from the MET group left behind on the 28th d were histopathologically evaluated. The histopathological scores were compared on the basis of total scores.

Biochemical Analyzes

During the study, a total of 122 broiler chicks, including 8 broilers randomly selected from each group on d 7, 14 and 21 (96 animals in total) and the remaining 26 animals on d 28 (7 broilers each from

TABLE I
Histopathologic scoring

Score	Identification
0	No histopathologic findings were found
1	Histopathologic finding was seen in 1/3 of the section (1–3 microscopic areas).
2	Histopathologic findings were seen in 2/3 of the section (4–6 microscopic areas).
3	Histopathologic findings were seen in 3/3 of the section (7–9 microscopic areas).

the CONT, SMT and MET–SMT groups and 5 broilers from the MET group), were intracardiac blood was collected before necropsy for biochemical tests. Before the blood collection procedure, feed was removed from the feeders and a 12-hour fasting period was established from 8:00 pm to 8:00 am. Serum samples to be used in the analysis were obtained by centrifugation of blood tubes at 2683 G for 10 min at 4°C with a refrigerated centrifuge (Mipro Series MPS–1000, Türkiye). Serum samples were transferred into eppendorf tubes and stored in a deep freezer at -80°C until analysis. ALT, AST and ALP parameters of liver enzymes were measured in serum samples by colorometric method in a private laboratory (Triolab – Ankara) using Improgen liquid reagent commercial kit (TABLE II) on a autoanalyzer (Beckman Coulter brand AU640 model, USA).

TABLE II
List of commercial kits used for biochemical analyses

Reactive	Lot Number	Brand
ALT	95827	Improgen, (Türkiye)
AST	954420	Improgen, (Türkiye)
ALP	80051	Improgen, (Türkiye)

In order to determine oxidative stress parameters, liver samples were washed with saline solution to remove blood. Liver tissue samples stored at -80°C (Sanyo brand ultra-low freezer model, Japan) for analysis were washed with 1mL pH 7.4 PBS to remove blood residues. The samples were then weighed and diluted 1/10 (w/v) with PBS and transferred to eppendorf tubes for homogenization. After the samples were isolated on dry ice, they were homogenized for 30 s in a homogenizer (Thermo brand Fastprep FP120 model, Germany) and centrifuged in a refrigerated centrifuge (Hettich brand Mikro 220R model Germany) at 32689 G at +4°C for 10 min. The supernatant liquid remaining at the top of the tubes was used to determine oxidative stress parameters. These analyzes were performed at Rel Assay Diagnostics Gaziantep–Turkey laboratory. SOD and GPx parameters were determined by colorimetric method using commercial kits (TABLE III) on a (Dutch Vital Scientific brand Selectra/Flexor E model, Nedherland) autoanalyzer. The MDA parameter was determined by spectrophotometric method on a (Biotek TS800 model USA) ELISA reader set at 450 nm using a commercial kit.

Statistical analysis

In the statistical analysis of the data obtained, IBM SPSS 25.0 computer software was used. The distribution of the data was tested using Brown–Forsythe and Bartlett's test. One–Way ANOVA for measurable data was lettered by applying Post Hoc Tukey's test. Histopathological score data were analyzed by Mann–Whitney U test. Test results were considered significant according to $P<0.05$. Results are given as Mean±SE.

TABLE III
Kits used for the determination of oxidative stress parameters

Oxidative Stress Parameter	Commercial Kit Used	Brand
SOD	Ransod Test Reagent (REF:SD125, LOT:514313)	Randox, (England)
GPx	Ransel Test Reagent (REF:SD125, LOT:514313)	Randox, (England)
MDA	Chicken MDA Elisa Assay Kit Catalog number E0171Ch	Bioassay Technology Laboratory (China)

RESULTS AND DISCUSSION

Macroscopic and histopathological results

In the study, a total of 6 chicks were found dead, one on the 6th d in the CONT group, one on the 10th, 16th and 25th d in the MET group, one on the 4th d in the SMT group and one on the 5th d in the MET+SMT group. Macroscopic findings and the number of occurrences in the experimental animals are given in TABLE IV below and showed FIG 1.

TABLE IV
Macroscopic findings and number of cases in the livers of experimental animals

Day	Groups	Ascites	Liver			
			Paleness	Crunchy consistency	Haemorhagie	Swelling
7	CONT	–	–	–	–	–
	MET	1	2	–	–	–
	SMT	–	–	–	–	–
	MET+SMT	–	–	–	–	–
14	CONT	–	–	–	–	–
	MET	4	3	3	3	2
	SMT	–	–	–	–	–
	MET+SMT	1	–	–	–	–
21	CONT	–	–	–	–	–
	MET	6	6	6	5	5
	SMT	–	–	–	–	–
	MET+SMT	2	2	2	2	2
28	CONT	–	–	–	–	–
	MET	5	5	5	5	5
	SMT	–	–	–	–	–
	MET+SMT	3	3	2	3	2

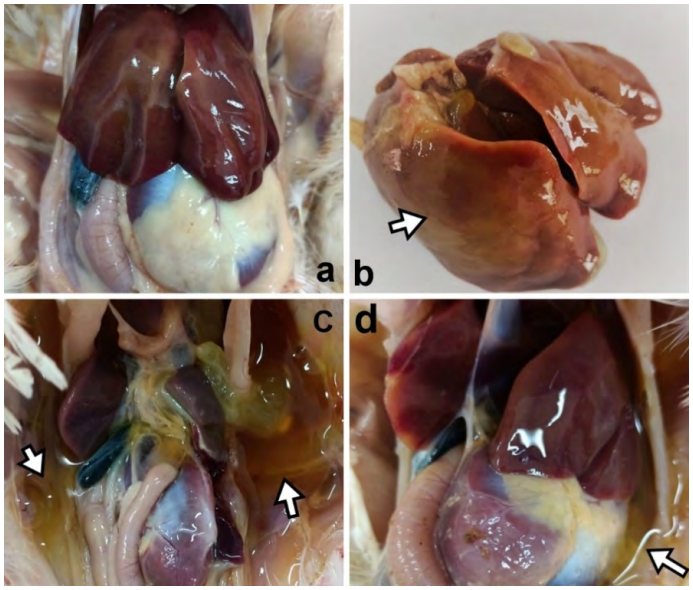

FIGURE 1. Normal appearance of the liver and abdominal cavity of the control group (a). Swollen and pale areas (arrow) and enlargement in a liver from the MET group on day 21(b). Severe proteinaceous fluid accumulation in the abdominal cavity (arrows) of a chicken from the MET group on day 28 (c). Slightly milder proteinaceous fluid accumulation in the abdominal cavity (arrow) in a chicken of the MET+SMT group on day 21 (d)

Histopathologic results

Scoring of the histopathological findings in the livers of experimental animals according to groups and days were displayed in TABLE V and Statistical scores between groups are presented in TABLE VI. Some histopathological pictures of the experimental groups are presented in FIG 2.

TABLE V
Scoring of the histopathological findings in the livers of experimental animals according to groups and days.

Liver		7th day								14th day								21th day								28th day						
		1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7
KONT	Hyperemia	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	1	-	-	2	-	-	-	-	-	-	-	-	-
	Bleeding	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Hydropic degeneration in hepatocytes	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Fattyness in hepatocytes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-
	Focal necrosis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Dissociation in remark cords	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MNH infiltration in the portal area	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-
	Bile duct hyperplasia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Total score	0	0	2	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	1	0	0	4	0	0	0	1	0	0	0	0	0
SMT	Hyperemia	-	-	-	-	2	-	-	-	-	-	-	-	-	2	-	-	-	-	-	2	-	2	-	-	-	-	-	2	-	-	-
	Bleeding	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Hydropic degeneration in hepatocytes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
	Fattyness in hepatocytes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Focal necrosis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Dissociation in remark cords	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MNH infiltration in the portal area	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
	Bile duct hyperplasia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Total score	0	0	0	0	2	0	0	0	0	0	0	0	0	2	2	0	0	0	0	2	0	2	1	0	0	0	0	2	0	0	1
MET	Hyperemia	2	1	-	3	2	-	1	-	3	3	2	3	3	1	3	2	3	3	2	3	3	3	2	3	2	2	2	3	2		
	Bleeding	-	-	-	-	-	-	-	-	2	2	-	2	2	-	-	-	1	1	-	2	3	1	-	-	2	1	2	3	2		
	Hydropic degeneration in hepatocytes	3	-	2	-	2	1	2	1	2	2	1	3	1	1	1	3	3	3	2	3	3	3	3	2	3	3	2	3	3		
	Fattyness in hepatocytes	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	1	2	1	-	3	2	-	-	-	2	2	2	3	2		
	Focal necrosis	-	-	1	-	-	1	-	-	1	-	-	1	-	-	-	1	2	1	1	2	2	2	-	-	2	2	2	2	2		
	Dissociation in remark cords	2	2	-	1	-	1	1	2	1	1	1	2	1	1	1	2	3	3	2	3	2	2	1	2	2	2	2	3	2		
	MNH infiltration in the portal area	-	-	-	-	1	-	-	-	2	1	-	2	-	-	1	-	2	1	-	2	2	-	-	-	3	1	-	2	3		
	Bile duct hyperplasia	-	-	-	-	-	-	-	-	1	1	-	1	-	-	1	1	2	1	1	2	3	1	1	1	3	2	2	3	2		
	Total score	7	3	3	4	5	3	4	3	12	10	4	16	7	3	7	10	18	14	8	20	20	12	7	8	19	15	14	22	18		
MET+SMT	Hyperemia	-	-	2	-	1	1	-	1	2	3	-	-	3	2	2	-	-	2	1	-	-	1	-	2	-	1	1	-	1	1	-
	Bleeding	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	1	-	-
	Hydropic degeneration in hepatocytes	1	1	-	2	1	-	-	-	1	2	-	-	2	2	-	-	-	1	2	-	-	1	1	-	1	-	1	-	1	2	1
	Fattyness in hepatocytes	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	1	-	1	-	-
	Focal necrosis	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	1	-	-	-	-	-	1	-	-	-	1	-	-
	Dissociation in remark cords	-	-	1	-	1	1	-	1	1	2	-	-	2	1	1	-	-	1	1	-	-	1	1	-	1	1	1	-	1	1	-
	MNH infiltration in the portal area	-	-	-	-	-	-	-	-	2	-	-	-	2	-	-	-	-	-	3	-	-	2	-	-	-	-	1	-	-	1	-
	Bile duct hyperplasia	-	-	-	-	-	-	-	-	1	1	-	-	2	-	-	-	-	1	1	-	-	1	1	-	1	-	1	-	1	1	-
	Total score	1	1	3	2	3	2	0	2	7	11	0	0	13	5	3	0	0	5	12	0	0	6	3	2	4	2	6	0	7	6	1

TABLE VI
Statistical comparison of histopathologic findings in the liver.

Days	Groups				
	CONT	SMT	MET	MET+SMT	*P* value
7	0.25 ± 0.25^{C}	0.25 ± 0.25^{C}	4.00 ± 0.5^{cA}	1.75 ± 0.36^{B}	*P*<0.001
14	0.37 ± 0.37^{B}	0.50 ± 0.32^{B}	8.62 ± 1.51^{bcA}	4.87 ± 1.80^{AB}	*P*<0.001
21	0.62 ± 0.49^{B}	0.62 ± 0.32^{B}	13.37 ± 1.93^{abA}	3.50 ± 1.46^{B}	*P*<0.001
28	0.20 ± 0.20^{C}	0.42 ± 0.29^{BC}	17.60 ± 1.43^{aA}	3.71 ± 1.04^{B}	*P*<0.001
P value	*P*>0.05	*P*>0.05	*P*<0.001	*P*>0.05	

^{a,b,c}: differences in column, ^{A,B,C}: differences in row.

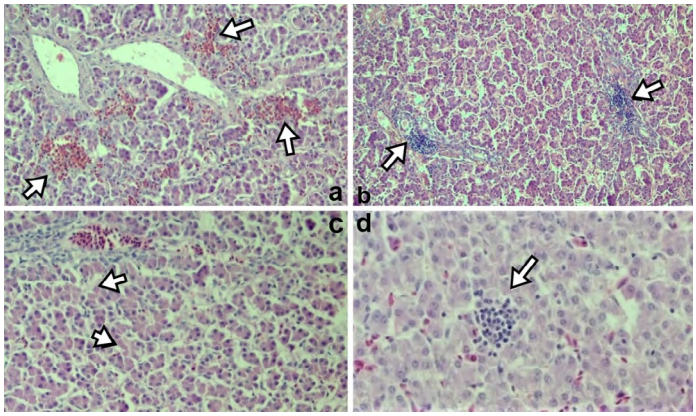

FIGURE 2. Hemorrhage foci (arrows) in a liver in the MET group. H–E. Original Magnification 200× (a). Mononuclear cell infiltration in the portal regions in a liver section in the MET Group. H–E. Original Magnification 100× (b). Degenerated and necrotic hepatocytes (arrows) in a liver section in MET Group. H–E. Original Magnification 100× (c). Focal hepatitis (arrow) in a liver section in the MET–SMT group. H–E. Original Magnification 400X× (d)

When the sum of the scores of histopathologic changes in liver tissue were analyzed, no statistically significant difference (*P*>0.05) was found between the CONT, SMT and MET+SMT groups. In the MET group, it was statistically found that it was the most severe on the twenty–eighth day, severe on the twenty–first day, moderate on the fourteenth day and mildly damaged on the seventh day (*P*<0.001). The results of the MET+SMT group on the twenty–eighth, fourteenth and seventh days were higher than the MET group, lower than the CONT and SMT groups (*P*<0.001), and the results of MET+SMT on the twenty–first day were the same as the MET group, lower than the CONT and SMT groups (*P*<0.001).

Biochemical results

There was no statistically significant difference in serum ALP enzyme activity between CONT and SMT groups in the 7th, 14th, 21th and 28th d (*P*>0.05). When CONT groups and MET groups were compared, there were significant differences in serum ALP enzyme activity in the 7th, 21th and 28th d (*P*<0.001) and significant differences in the 14th d (*P*<0.01). When MET groups and MET+SMT groups were compared with each other on the basis of days, while there was no statistically significant difference in the 7th d (*P*>0.05), significant differences were found in the 14th, and 28th d (*P*<0.01) and statistically significant differences were found in the 21th d (*P*<0.05) (FIG 3).

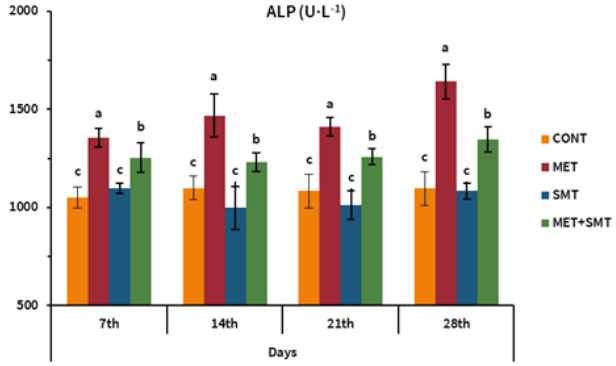

FIGURE 3. Comparison of serum ALP level according to days and groups

There was no statistical difference in serum ALT enzyme activity between the CONT groups and SMT groups at the 7th, 14th, 21th and 28th d. There was a significant (*P*<0.05) difference in serum ALT enzyme activity in the 7th d and a very significant (*P*<0.001) difference in the 14th, 21th and 28th d. When the statistical results of the MET group and MET+SMT group were compared, there was no statistical difference in the 7th and 14th d (*P*>0.05), while a significant difference was found in the 21th d (*P*<0.01) and a significant difference was found in the 28th d (*P*<0.05) (FIG 4).

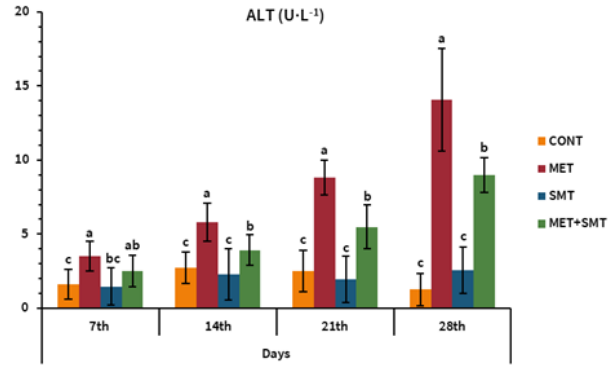

FIGURE 4. Comparison of serum ALT level according to days and groups

There was no statistically significant difference in serum AST enzyme activity between CONT, SMT and MET+SMT groups at 7th, 14th, 21th and 28th d (*P*>0.05). When CONT groups and MET groups were compared, significant (*P*<0.05), significant (*P*<0.01) and highly significant (*P*<0.001) results were found in serum AST enzyme activity in the 7th, 14th, 21th and 28th d, respectively. A significant difference (*P*<0.01) was found between MET groups and MET+SMTT groups in the 7th and 14th d and a significant difference (*P*<0.01) was found in the 21th and 28th d (FIG 5).

There was no statistical difference in GPx enzyme activity between the CONT groups and SMT groups at the 7th, 14th, 21th and 28th days (*P*>0.05). When CONT groups and MET groups were compared, there was a significant difference in the 7th d (*P*<0.05), a significant difference in the 14th d (*P*<0.01) and a very significant difference in the 21th and 28th d (*P*<0.001). The difference between MET group and

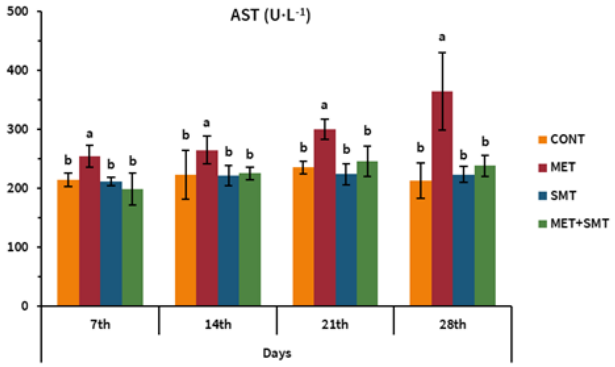

FIGURE 5. Comparison of serum AST level according to days and groups

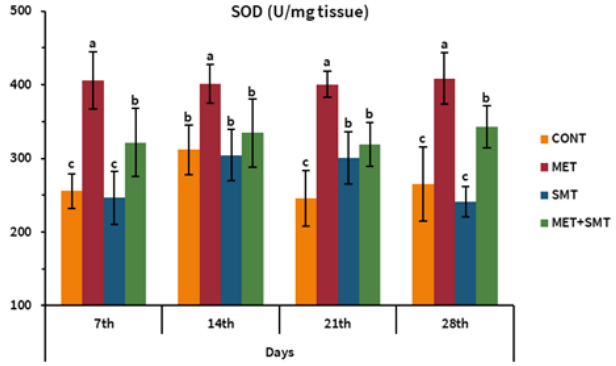

FIGURE 7. Comparison of SOD enzyme activity by days and groups

MET+SMT group was significant ($P<0.05$) in the 7th, 21th and 28th d, but significant in the 14th d ($P<0.01$) (FIG 6).

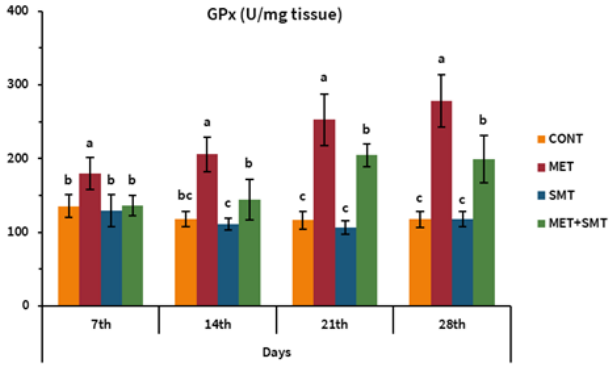

FIGURE 6. Comparison of GPx enzyme activity by days and groups

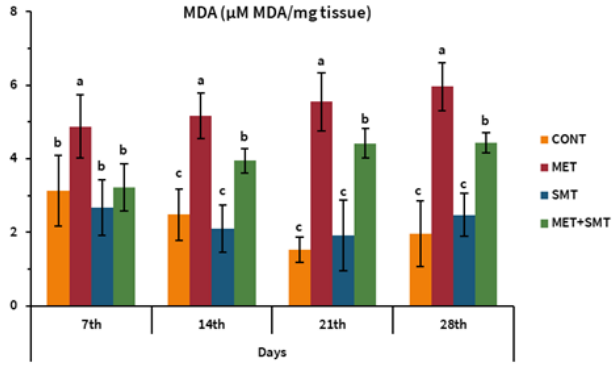

FIGURE 8. Comparison of MDA levels by days and groups

There was no statistically significant difference in SOD enzyme activity between the CONT and SMT groups in the 7th, 14th and 28th d ($P>0.05$), while a significant difference was found in the 21th d ($P<0.05$). When the CONT group was compared with the MET group, a significant difference was found in the 14th d ($P<0.01$) and a very significant difference was found in the 7th, 21th and 28th d ($P<0.001$). When the SOD enzyme activity of MET groups and MET+SMT groups were compared statistically, a significant ($P<0.05$) difference was found in the 14th and 28th d and a significant difference was found in the 7th and 21th d ($P<0.01$) (FIG 7).

There was no statistical difference ($P>0.05$) between the MDA levels in the 7th, 14th, 21th and 28th d between the CONT and SMT groups. When CONT groups and MET groups were compared, there was a statistically significant difference ($P<0.01$) in MDA levels at 7th, 14th, 21th and a very significant difference ($P<0.001$) at 21th and 28th d. There was a statistically significant ($P<0.05$) difference between the MDA levels of the MET group and MET+SMT group at the 14th and 21th d, and a significant ($P<0.01$) difference at the 7th and 28th d (FIG 8).

In methomyl hepatotoxicity, it has been reported by many researchers that the arrangement of hepatocytes is disrupted then hydropic degeneration and vacuolar degeneration are observed [7, 22, 23]. In this current study, liver histopathology of MET group civets revealed hyperemia and hemorrhage in V. centralis and sinusoids, hydropic degeneration and necrosis in hepatocytes, dissociation in Remark cords and enlargement in sinusoids. When the scores of these findings were evaluated, it was noteworthy that the finding scores increased with increasing exposure time. Aboushouk *et al.* [24] reported microscopically necrosis in hepatocytes and mononuclear cell infiltration in the portal area in chronic hepatotoxicity induced by methomyl in their study. In this study, focal necrosis in hepatocytes was found to be very high in the livers of broilers in the MET group compared to the other study groups. Periportal fibrosis and bile duct hyperplasia were observed in the liver of broilers fed chlorpyrifos, an organophosphorus compound [25]. In this study, bile duct hyperplasia was more severe in the MET group. When these findings were evaluated, it was determined that Methomyl showed hepatotoxic effects in broilers like other carbamate compounds.

Biological effects of silymarin such as antioxidant, antiviral, antiapoptotic, anti-inflammatory, anticarcinogenic, antiangiogenic and antifibrotic have been revealed by researches [26]. It was found that silymarin or silybin active substances used in toxications induced by substances such as acetaminophen (APAP), phalloidin, carbon tetrachloride and D-galactosamine, which were experimentally induced *in vivo*, alleviated liver damage [27]. In the present study, it was found that silymarin visually reduced the severity of histopathological findings such as hydropic degeneration, fatty deposits and focal necrosis in the liver caused by Methomyl. Silymarin was found to be effective in reducing and/or preventing the hepatotoxic effect of Methomyl.

Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) Liver enzymes such as ALP are widely used biomarkers to monitor hepatocellular integrity and liver injury. It has been reported that the highest ALP concentrations are recorded in periportal hepatocytes and tend to decrease gradually towards centrilobular hepatocytes [28]. In a study conducted in rabbits, El-Sheikh *et al.* [29] investigated the effects of vitamin E + Selenium injection alone or weekly at different time intervals against the adverse effects of Methomyl on growth performance, blood biochemical parameters and histopathological changes observed in the liver and as a result of Methomyl exposure; total protein, urea, AST and ALP enzyme activities and red blood cell count decreased, ALT enzyme activity and creatinine level increased. In this study, it was determined that ALT, AST and ALP enzyme activities in the blood serum of broilers in the MET group increased remarkably throughout the study compared to the CONT group. The differences between in this study and conducted by [29] in rabbits may be due to the differences in liver metabolism and anatomical structures in mammals and poultry. In addition, the duration of exposure to Methomyl in the study conducted by El-Sheikh *et al.* [29] was longer than in this study. Unlike mammals, more than 70% of the de-novo synthesis of fatty acids in poultry occurs in the liver, 5% in adipose tissue, and the rest comes from the feed consumed. The liver is the main metabolic organ involved in the synthesis, degradation and transport of lipids. In some cases, lipid metabolism in the liver is disrupted due to damage to hepatocytes and hepatosteatosis occurs [30]. In the present study, histopathologic examinations revealed findings compatible with hepatosteatosis.

In this study, macroscopic and microscopic examinations of liver tissue samples showed that tissue damage was severe in the MET group compared to the other groups. Muriel and Mourelle [31] found that silymarin, The active ingredient of *S. marianum* favored the reduction of ALP and GGT enzyme activities in a study in which rats were acutely exposed to carbon tetrachloride for 24 hours. It was reported that portal blood flow was impaired and portal blood flow rate decreased with the formation of cirrhosis Chawla *et al.* [32]. In the study conducted by Muriel and Mourelle (30), the decrease in enzyme activities with toxication may probably be due to the decrease in blood flow and enzyme synthesis. Enzymatic activities improved with liver regeneration caused by silymarin. In another study Kazemifar *et al.* [33], it was revealed that silymarin administered after paracetamol poisoning reduced the increase in AST, ALT and ALP plasma enzyme activities. Similarly, in this study, it was found that serum AST, ALP and ALT enzyme activities decreased statistically ($P<0.05$) in the MET+SMT group compared to the MET group. Against the hepatotoxic effect caused by Parasepamol and Methomyl, silymarin is thought to have hepatoprotective effect by regulating enzyme activities by protecting the structural integrity and functions of hepatocyte membranes.

Carbamate compound derivatives such as methomyl elicit oxidative stress by causing the generation of free radicals in rat tissues, and these released free radicals play an important role in the toxicity of pesticides by stimulating depletion of antioxidants or changes in the enzyme system that captures free oxygen radicals. Free Oxygen Radicals are produced by living organisms as a result of normal cellular metabolism [34]. SOD protects oxygen metabolizing cells against the damaging effects of superoxide free radicals [35, 36]. In this study, it was determined that Methomyl significantly decreased SOD activity in the liver.

Manawadi and Kaliwal [37], in their study, they investigated the effects of Methomyl on mouse liver tissue and its duration of action. Mice were administered 2, 3 and 4 $mg \cdot kg^{-1} \cdot day^{-1}$ Methomly for 30 d and the effective dose of 4 $mg \cdot kg^{-1} \cdot day^{-1}$ methomyl for 5, 10 and 20 d. As a result, they found that SOD levels decreased significantly. The lack of the expected increase in SOD enzyme activity in defense against oxidative stress in relation to toxication may have been due to the depletion of SOD activity in the samples taken before the test. In this study, it was found that Methomyl significantly increased SOD activity in the liver in all days compared to the CONT group. In addition, in this study, there was a significant decrease in SOD enzyme activity in the MET+SMT group in all days compared to the MET group. It was interpreted that the significant increase in SOD enzyme activity in the Methomyl group during the experiment was due to the reduction of oxidative damage during detoxification in the liver tissue. A situation similar to the increase in SOD enzyme activity in the liver of broilers given Methomyl was detected in the heart tissue of rats injected with CCl_4 [38]. The decrease in SOD enzyme activity in the MET+SMT group in this study suggests that the bioactive substances contained in *S. marianum* plant may be the result of interaction with free oxygen radicals.

In a study investigating the effect of silymarin and lycopene on lipid peroxidation in mice, lycopene and silymarin were given orally. *S. marianum* seed powder showed antioxidant properties in the SMT group compared to the CONT group and SOD enzyme activity was found to be statistically lower [39]. As a result, the statistically low SOD enzyme activity in this study was found to be consistent with the study of Süloğlu *et al.* [39].

Silymarin triggers the production of antioxidant enzymes such as CAT, SOD and GPx [40]. Silymarin and silibin show their effects in metabolism in different ways. They act as antioxidants by scavenging free oxygen radicals and regulating the intracellular amount of glutathione; by keeping cell membrane permeability under control and preventing the passage of substances toxic to hepatocytes by working as cell membrane stabilizers; by stimulating liver regeneration by increasing ribosomal RNA synthesis and by preventing the accumulation of collagen fibrils that cause cirrhosis by stopping the transformation of stellate cells into myofibroblasts [16]. GPx is an important intracellular enzyme that degrades H_2O_2 to water and converts lipid peroxides to the corresponding alcohols mainly in mitochondria and sometimes in the cytosol [41]. Djeffal *et al.* [42] reported that the antioxidant system was impaired in liver tissues as a result of oxidative stress and cellular damage caused by Methomyl-induced toxicity and that there was an increase in lipid peroxidation and GPx enzyme activity. Similarly, in this study, increased lipid peroxidation-related MDA level and GPx enzyme activity were found in the MET group compared to the CONT group. SMT had a positive effect on the reduction of oxidative stress by scavenging free radicals.

Silymarin is thought to act as an antioxidant agent by reducing cell death by reducing lipid peroxidation and preventing glutathione depletion [27]. Lipid peroxidation (LPO) is a chain reaction between polyunsaturated fatty acids and reactive oxygen species (ROS) that yields lipid peroxides and hydrocarbon polymers [43]. Peroxidation of polyunsaturated fatty acids and related esters produces MDA as an end product. MDA therefore functions as a biomarker of LPO [44]. Lipid peroxidation occurs in a wide range of areas and causes disorders of lipoprotein metabolism due to degeneration of cell membranes in the liver and peripheral tissues. Mansour *et al.* [45] reported that the increase in MDA level caused an increase in LPO level in rats given Methomyl. In this study, it was determined that Methomyl increased the MDA level in liver tissue. Debnath and Mandal [44] and Mansour *et al.* [45] in both studies, it was reported that there was an increase in MDA level as a result of lipid peroxidation since hepatocyte cell membranes were disrupted after Methomyl toxicity. In this study,

it was determined that *S. marianum* seed powder added to the ration in the MET+SMT group caused a decrease in MDA level by showing antioxidant properties compared to the MET group.

Long-term use of high doses of isotretinoin (vitamin A derivative) in male mice often causes disorders in liver tissue. In a study Kumaş *et al.* [46] examining the effect of silymarin application on histopathological and biochemical parameters as a therapeutic against cardiac tissue damage, serum SOD and GPx enzyme activities, plasma MDA and erythrocyte MDA levels were found to be increased in isotretinoin and isotretinoin+silymarin groups compared to the CONT group. MDA levels increased with isotretinoin administration and decreased with silymarin administration. Similar results were also found in this study. In this study, it was noticed that there was a statistically significant decrease in MDA level in the MET+SMT group compared to the MET group (P<0.05). In this study, Silymarin is thought to prevent lipid peroxidation by regulating the antioxidant system against oxidative damage caused by Methomyl in hepatocytes.

CONCLUSIONS

It was concluded that it can be added to *S. marianum* poultry diets against Methomyl residues or contaminations. For this purpose, new studies on ration dosage are needed. In addition, it was evaluated that longer-term studies will contribute to this field in order to clarify the protective/healing effect of SMT against the cumulative accumulation of MET depending on time.

Conflict of interest

The authors declare that they have no conflict of interest.

Note

This article was produced from the first author's doctoral thesis titled "PATHOLOGICAL AND BIOCHEMICAL STUDIES IN BROILER FEEDS ADDITIONED WITH METHOMYL AND SILYBUM MARIANUM".

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