The effects of herbal cream and silymarin on liver in carbon tetrachloride-treated animals

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ARTICLE INFO

Article History
Received : 04.01.2024
Accepted : 15.08.2024
DOI: 10.33988/auvfd.1414074

Keywords
Carbon tetrachloride
Herbal cream
Liver
Oxidative stress
Silymarin

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How to cite this article: Kandil A, Kılıç A, Gürel Gürevin E, Söğüt İ, Üstünova S, Eren Ş, Caner M, Demirci Tansel C (2025): The effects of herbal cream and silymarin on liver in carbon tetrachloride-treated animals. Ankara Univ Vet Fak Derg, 72 (1), 47-57. DOI: 10.33988/auvfd. 1414074.

ABSTRACT

Many medical or pharmaceutical plants have been widely used for the treatment of the liver injury. Silymarin is now used as a food-supporting material for the liver as a patented product. Herbal cream has anti-inflammatory and antioxidant effects on wound healing in a hemorrhoid model. This study aimed to evaluate the effects of herbal cream and silymarin on the liver in carbon tetrachloride (CCl_4)-treated animals. Male Wistar rats were divided into seven groups as Intact control, Control, Herbal cream (0.5 ml, intrarectal), Silymarin (70 mg/kg, intrarectal), CCl_4 (2 ml/kg, intraperitoneal), CCl_4+Herbal cream (0.5 ml herbal cream for 21 days, 3 weeks after administration of CCl_4) and CCl_4+Silymarin (70 mg/kg silymarin for 21 days, 3 weeks after administration of CCl_4). Herbal cream reduced damage and leukocyte distribution induced by CCl_4 and increased catalase. There was no significant change in superoxide dismutase (SOD) and glutathione peroxidase (GPx). The levels of SOD, catalase, and GPx in the liver increased significantly in the group treated only with herbal cream. These results point out that herbal cream may have antioxidant properties in the liver and a role in preventing liver damage. As a result, it has been detected that herbal cream is not a toxic agent and recovers liver damage with antioxidant properties.

Introduction

Liver diseases are a widespread public health problem all over the world. The use of herbal products is increasing worldwide. Many plants are used traditionally and commercially for treatment and support. *Silybum marianum* (Camel thistle) extract is used as a cytoprotectant in liver injury induced by carbon tetrachloride (CCl_4), alcohol, radiation, acetaminophen, iron overload, phenyl hydrazine, and *Amanita phalloides* (1, 27, 51, 61). *Silybum marianum* consists of flavanone derivatives such as silybin, silydianin, isosilybin, and silychristin (1, 58). *Silybum marianum* was suggested to be potentially beneficial for liver diseases because of its anti-inflammatory, antifibrotic, antioxidant, and hepatoprotective properties (2). In addition, silymarin exerts a significant anti-inflammatory effect by diminishing edema and inhibiting leukocyte migration to the inflammation region (14).

Artichoke (*Cynara scolymus* L.) leaf extracts were shown to have antimicrobial, antioxidative (64), hepatoprotective, and anti-cholestatic effects (22, 30). It was suggested to have antioxidative potential in hydroperoxide-induced oxidative stress (22). It was indicated to improve cadmium-induced hepatorenal oxidative injury in rats (19).

The benefits of walnuts (*Juglans regia*) have been known for many years. The cosmetic and pharmaceutical industries use green walnuts, leaves, shells, and seeds (56). Walnuts may be a protective agent against the toxicity of chemicals and drugs (25). It has been proposed that the bioactive compounds of walnut green husks can trigger the death of prostate carcinoma cells through apoptotic pathways (7). It has been indicated that the extract of green walnut husks had potential antioxidant activity (43).

Ficus carica is used in traditional medicine to treat cardiovascular and respiratory disorders. It has been investigated whether fig (*Ficus carica*) leaves have anti-inflammatory and antioxidant properties (23). It has been reported that the extract of *Ficus carica* has the capacity of antioxidant and anti-inflammatory properties (4). The administration of *Ficus carica* leaf extracts has been shown to affect the oxidative stress in diabetes (48). It was observed that its leaves have hypoglycaemic activity in diabetic animals (49).

Aesculus hippocastanum (Hippocastanaceae) is known as horse chestnut tree. The therapeutic benefit of horse chestnut extract is supported by *in vivo* and *in vitro* experimental researches, and it has anti-inflammatory and antioedematous properties. Escin is the major active form of *Aesculus hippocastanum* and has pharmacodynamic actions (31, 34, 50, 55). Aescin have protective effects associated with anti-inflammatory effects on endotoxin-induced liver injury (28). It has also been demonstrated that *Aesculus hippocastanum* seeds have beneficial effects on diabetic nephropathy (20). A gel formulation containing *Aesculus hippocastanum* was shown to have stability, and skin permeability ability (44).

The herbal cream used in this study contains the most active ingredients including *Cynara scolymus*, *Aesculus hippocastanum*, *Juglans regia*, and *Ficus carica* leaves. The herbal cream has been detected to be effective in wound healing and have antioxidant properties (24). The herbal cream was reported to have antioxidant, anti-inflammatory, and wound-healing effects in our experimental studies for patent research. Moreover, a patents (European Patent No: 2022504) have been taken from the European Patent Office at the end of this study. Therefore, it is considered that different studies should be performed with this cream. For this purpose, it was decided to investigate whether this cream has a role in liver toxicity.

The aim of this study was to investigate whether herbal cream has an ameliorating effect against CCl_4-induced liver injury.

Materials and Methods

Animals: All experiments in this study were approved and reviewed by the Istanbul University Local Committee on Animal Research Ethics (Decision no: 2013/92). Care and handling of the animals were in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals. Experiments were performed with 3 months old male Wistar albino rats (250-300 g). Animals were obtained from Istanbul University, Aziz Sancar Experimental Medicine Research Institute. Animals were fed a pelleted diet and tap water as free access. Animals were divided into intact control, control, silymarin (70 mg/kg, intrarectal) (n=7), herbal cream (0.5 ml, intrarectal) (n=7), CCl_4 (2 ml/kg, intraperitoneal) (n=7), CCl_4+herbal cream (0.5 ml herbal cream for 21 days 3 weeks after administration of CCl_4) (n=7) and CCl_4+Silymarin (70 mg/kg silymarin for 21 days 3 weeks after administration of CCl_4) (n=7).

CCl_4 (2 ml/kg of the 30% CCl_4 solution) was dissolved in olive oil and administrated intraperitoneally twice weekly (Monday-Tuesday) for 3 weeks. Silymarin (Solgar®) was dissolved in isotonic saline solution. The herbal cream and silymarin were administrated intrarectally twice daily (09.00 and 17.00) for 21 days. The herbal cream or silymarin was administrated intrarectally by a syringe without a needle. The tip of the insulin syringe without a needle was inserted into the rectum reaching approximately 1 cm proximal to the anus. The herbal cream in the syringe was slowly injected into the rectum, and then the tip of the syringe was kept in this position for 1 minute to remain substances into the rectum. All herbs and plant oils used in this study were obtained from certified herb sellers. The herbal cream is comprised of an aqueous-based liquid containing herbal extracts, vegetable oils, and gelling agents according to the invention application (24). A more detailed description of the herbal cream is presented in the Supplementary Material. The control group was received twice daily (09.00 and 17.00) intrarectal injection of isotonic saline solution for 21 days after olive oil was given intraperitoneally twice weekly for 3 weeks. The intact group did not receive any substance administration. Animals were anesthetized at the end of the experiment. Blood and liver were taken for analysis.

Biochemical Analyses: Blood samples were centrifuged at 4000 x *g* for 10 minutes to obtain serum. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatases (ALP), lactate dehydrogenase (LHD), albumin, total bilirubin, and total cholesterol were measured in the serum sample by enzymatic methods on AutoAnalyzer (Roche Hitachi Cobas c311).

The liver samples were homogenized and centrifuged at 6000 x *g* for 10 minutes, and then supernatants were collected from homogenates. Malondialdehyde (MDA) levels (nmol/mg protein) were

DOI: 10.33988/auvfd.1414074

measured according to the method of Ohkawa et al. (42). Total sialic acid (TSA) levels (nmol/mg protein) were determined according to the method of Katopodis et al. (29). SOD activity (U/mg protein) was calculated according to the method of Winterbourn et al. (59). SOD was defined as the amount of enzyme that causes the inhibition of nitroblue tetrazolium reduction by 50% and the enzyme activity. Catalase (CAT) activity (U/mg protein) was determined by the method of Beutler (10). The decrease in optical density per minute and enzyme activity were determined. GPx activity (U/mg protein) was measured using the methods of Paglia and Valentine (45). The protein levels of homogenates were detected using the Bradford assay (11).

Histological Analysis: The liver samples were fixed in 10% neutral buffer formalin solution and embedded in paraffin. Liver sections were stained with Hematoxylin-Eosin (HE). Liver damage was scored and evaluated in the section. The degree of damage was defined as follows: 0-normal (0-5%); 1-Mild (5-25%); 2-Moderate (25-75%); 3-Severe (>75%).

Immunohistochemical Analysis: Sections were prepared to evaluate the myeloperoxidase (MPO)-stained leukocytes as described previously (15, 32). The distribution of MPO-stained leukocytes was evaluated and scored in selected 210 areas of liver sections. The photographs of the sections were taken with Image Pro-Plus.

Statistical Analysis: The results are expressed as the mean ± SEM. The Shapiro-Wilk test was used to test the normality of the data. Significant differences were evaluated using one-way ANOVA and Kruskal Wallis test with Bonferroni and Dunn post-tests using GraphPad Prism software (San Diego, CA, USA). A value of P<0.05 was considered significant.

Results

Biochemical Results: The albumin levels enhanced in the CCl_4+S (P<0.01) and CCl_4+HC (P<0.05) groups compared to the CCl_4 groups. Total bilirubin level was enhanced in the CCl_4 group compared to the HC group (P<0.01). Cholesterol in serum was not different between groups (Figure 1). Unlike albumin, bilirubin levels that increased with CCl_4 application decreased especially after cream application and became closer to the C group (Figure 1).

In the CCl_4 group, AST levels were similar to the C groups while ALT levels increased (P<0.01) (Figure 2). ALT and AST levels in the groups applied with herbal cream application resembled the control group.

ALP levels in the S group were increased compared to the control group (P<0.01). In addition, ALP levels in the CCl_4+S (P<0.05) and S groups (P<0.05) were higher according to the CCl_4 group. LDH level decreased in the S group (P<0.05) compared to the control group (Figure 2).

MDA levels increased in the CCl_4+S group compared with IC and the HC groups (P<0.01). TSA increased in the S group (P<0.01) compared to the IC group. Moreover, TSA increased in the CCl_4+S group compared to the control groups (P<0.05) (Figure 3).

SOD level increased in the HC (P<0.001, P<0.05, P<0.05, P<0.001, and P<0.05, respectively) or S groups (P<0.001, P<0.01, P<0.05, P<0.001, and P<0.05, respectively) compared with IC, C, CCl_4, CCl_4+S, and CCl_4+HC groups. SOD levels in the CCl_4 group were similar to the control groups (Figure 4).

CAT levels increased in the HC groups compared with IC, C, CCl_4, and CCl_4+S groups (P<0.001, P<0.05, P<0.001, and P<0.001, respectively). CAT levels increased in the CCl_4+HC and S groups compared to the CCl_4 group (P<0.01 and P<0.001, respectively).

The levels of GPx increased in the HC group compared with IC, C, CCl_4, and CCl_4+HC groups (P<0.05, P<0.001, P<0.001, and P<0.05, respectively). GPx levels increased in the S group compared with the C and CCl_4 groups (P<0.01 and P<0.001, respectively) (Figure 4).

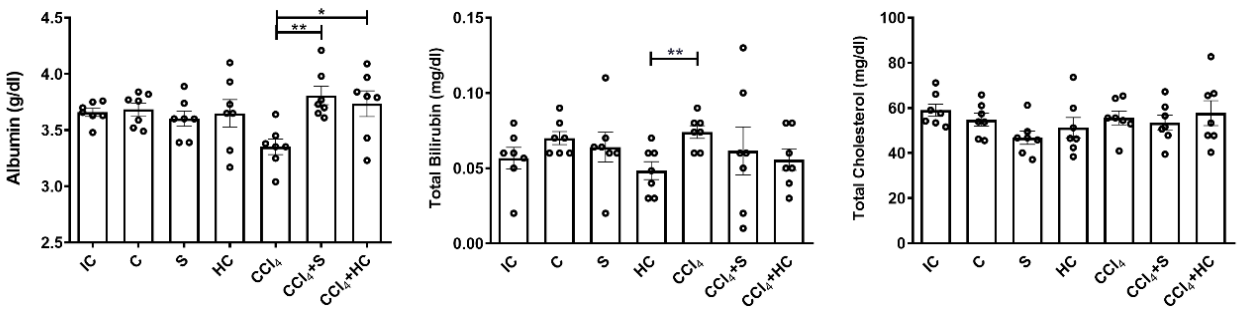

Figure 1. Albumin, total bilirubin and total cholesterol levels in the groups. *P<0.05, **P<0.01.

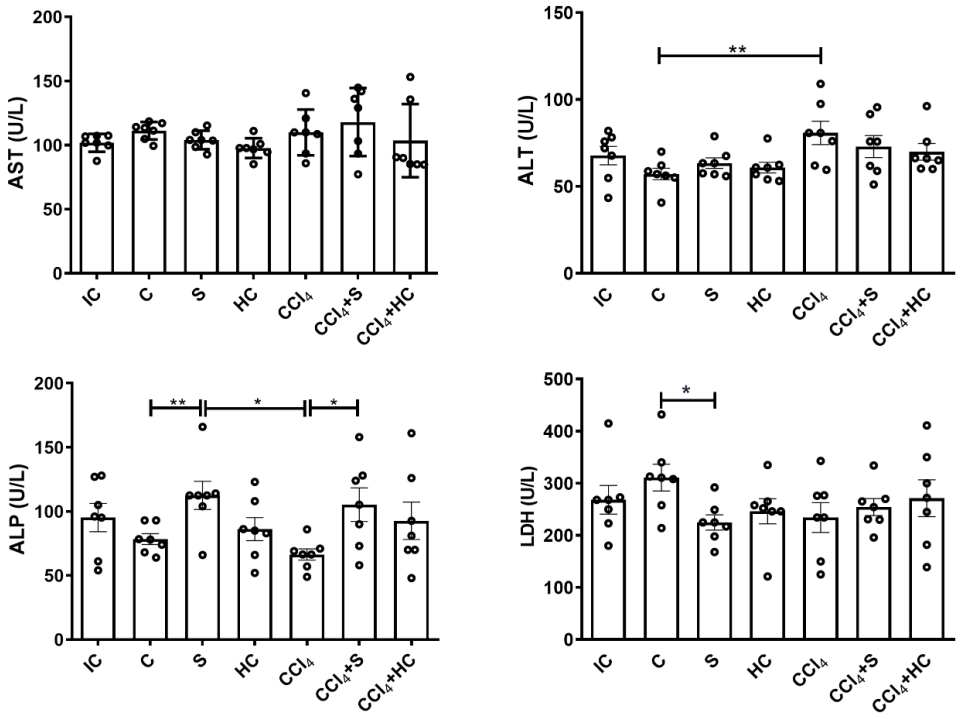

Figure 2. Aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) levels in the groups. *P<0.05, **P<0.01.

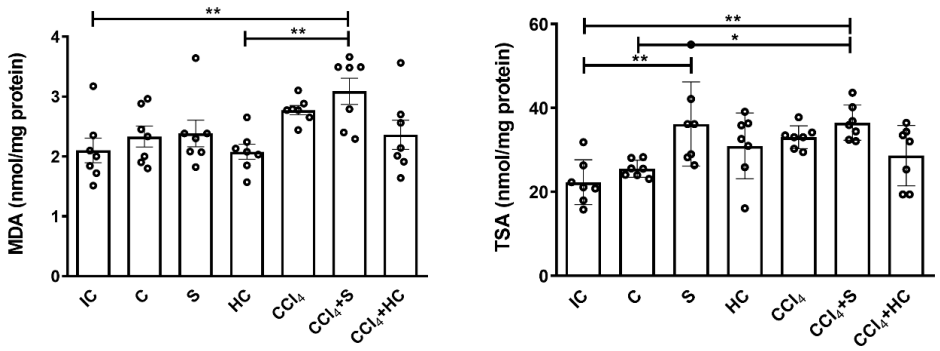

Figure 3. The malondialdehyde (MDA) and total sialic acid (TSA) levels in the groups. *P<0.05, **P<0.01.

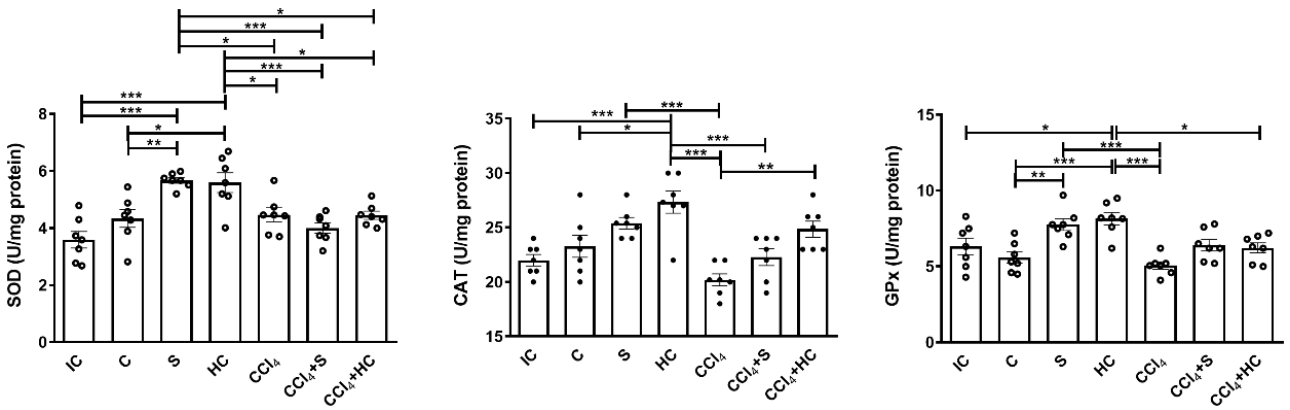

Figure 4. The superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) levels in the groups. *P<0.05, **P<0.01, ***P<0.001.

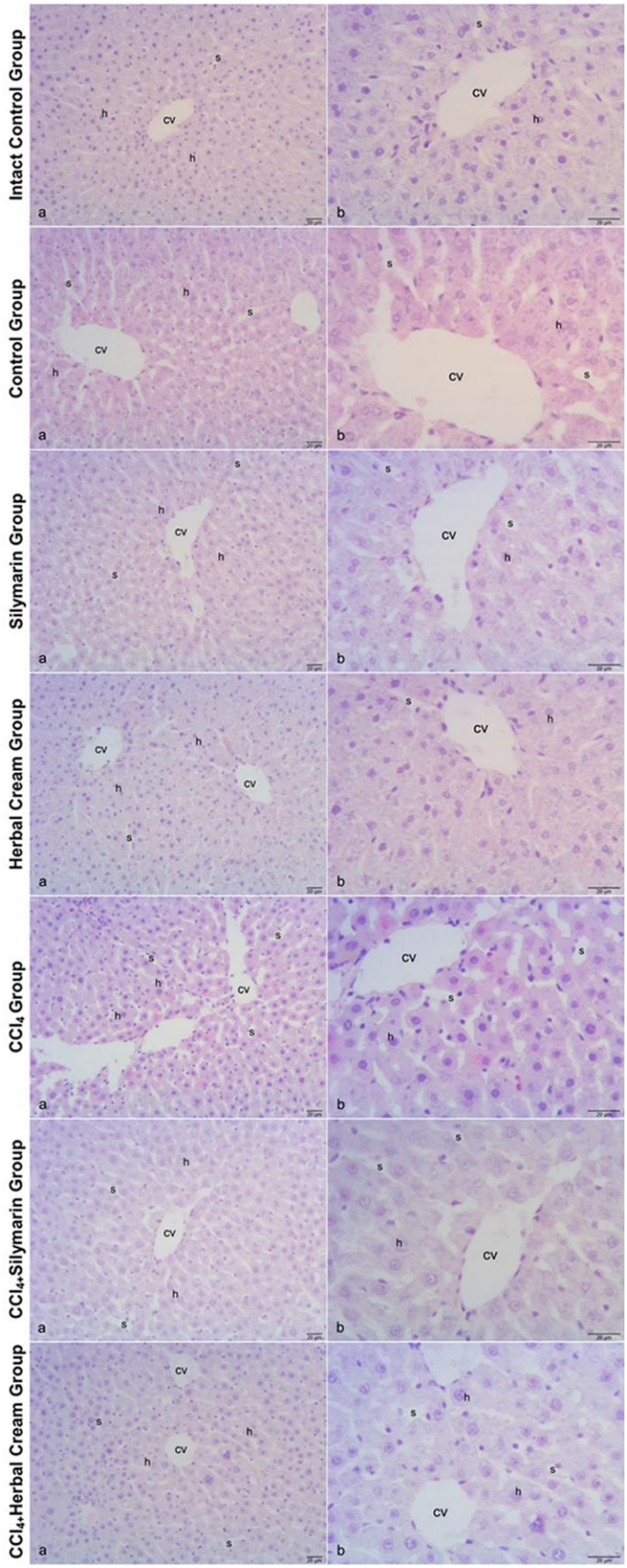

Figure 5. Central vein (cv), sinusoid (s) and hepatocyte (h) on liver tissue of the groups. Bar: 20 µm, HE.

Histological Results: The liver sections were examined under light microscopy. Histopathological changes were observed in HE-stained liver sections of CCl_4-treated animals. CCl_4 caused degeneration and expansion of sinusoids, damages in the endothelial layer, activation of Kupffer cells, and portal inflammation in liver tissue. The herbal cream or silymarin administration decreased CCl_4-induced liver damage. The liver sections in the HC or S groups were similar to the control group (Figure 5, Table 1).

Immunohistochemical Results: Leukocyte infiltration (MPO-stained leukocytes) in liver tissue increased in the CCl_4 groups compared to the IC and C groups ($P<0.001$ and $P<0.01$, respectively). The herbal cream or silymarin administration decreased leukocyte infiltration in the CCl_4 group ($P<0.001$) (Figure 6). Leukocytes were detected in portal areas, sinusoids, and vessels of liver tissue (Figure 7).

Table 1. The histological scores of groups to evaluate the damage in the liver.

Parameters	Intact Control	Control	Silymarin	Herbal Cream	CCl_4	CCl_4+ Silymarin	CCl_4+ Herbal cream
Degeneration and expansion of sinusoids	0.675	0.633	0.92	0.78	1.08	0.816	0.933
Damages in endothelial layer	0.275	0.233	0.26	0.22	0.9	0.3	0.254
Vacuolation of hepatocytes	0	0.366	0.02	0	0.2	0.016	0.133
The activation of Kupffer cells	0.675	0.366	0.38	0.44	0.86	0.65	0.683
Portal inflammation	0.475	0.666	0.36	0.62	1.18	0.516	0.181
Total score	**2.1**	**2.25**	**1.94**	**2.06**	**4.22**	**2.298**	**2.184**
Mean±SEM	**0.42±0.13**	**0.45±0.08**	**0.39±0.15**	**0.41±0.14**	**0.84±0.17**	**0.46±0.14**	**0.44±0.16**

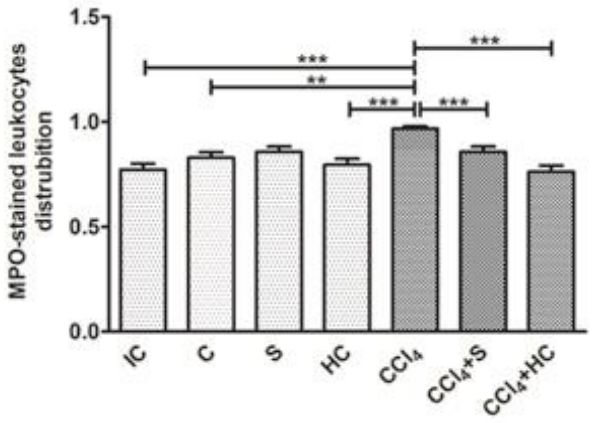

Figure 6. MPO-stained leukocyte distributions in liver tissues of groups. **P<0.01, ***P<0.001.

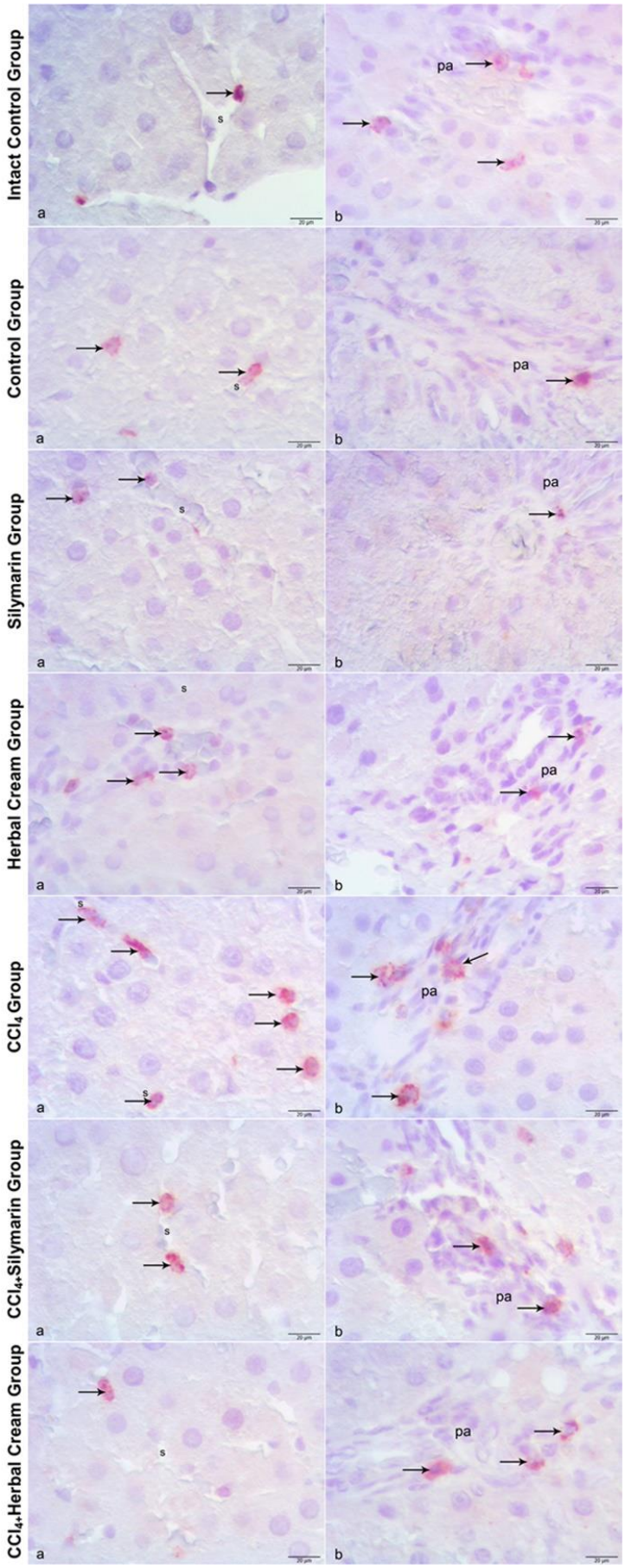

Figure 7. MPO-stained leukocytes (→) in liver tissue of the groups. Sinusoid (s) and portal area (pa). a-b) Bar: 20 µm.

Discussion and Conclusions

Liver diseases are among the most serious health problems worldwide. Liver disease is a term that indicates damage in cells, tissues, and structure, or liver dysfunction. Liver injury can occur by a variety of factors including viruses, bacteria, parasites, drugs, chemical compounds (CCl_4, dimethyl nitrosamine, thioacetamide, D-galactosamine/lipopolysaccharide), and excessive alcohol consumption (33). Inflammation and oxidative stress participate in the pathogenesis and progression of liver diseases. For this reason, many treatment procedures including various plants such as silymarin (58), *Cynara scolymus* L. (22), and *Physalis peruviana* L. (5) are developed to prevent liver disease (57). Many plants and their extracts are used to prevent liver damage (33).

In our study, it was evaluated the potential effects of herbal cream on liver toxicity. It was previously determined that intrarectal administration of this cream was effective in wound healing and reduced inflammation and oxidative stress in rats (24). It suggests that the intrarectal application of the cream can be effective by penetrating the blood more quickly. Therefore, the protective properties of herbal cream may play an effective role in different pathological conditions in addition to hemorrhoids. As a result, it was investigated whether the herbal hemorrhoid cream has beneficial effects on the liver in the CCl_4-induced hepatotoxicity model in this study. Liver toxicity was also created with CCl_4, which is widely used in the literature (6, 35, 39). CCl_4 is used as a liver injury model to investigate the mechanism of hepatotoxic effects in animals (39). CCl_4 increases free radical formation (12). The free radicals induce lipid peroxidation, tissue damage, necrosis and releasing of AST, ALT, and ALP enzymes (16, 63). In addition, toxic agents induce an inflammatory response and activate especially Kupffer cells (47). CCl_4 increased ALT and AST levels and decreased GPx, CAT, and SOD levels. Moreover, CCl_4 caused fibrosis and necrosis in the liver (60). CCl_4 increased serum ALT and induced hepatic necrosis and inflammation (62). CCl_4 increased MDA levels (8). The administration of CCl_4 increased total bilirubin, amino transaminases, ALP, glutathione, and protein levels, while decreasing the TBARS and total protein level (26, 46). In this study, CCl_4 resulted in an increase in ALT in serum. Albumin, cholesterol, and bilirubin levels did not change in the CCl_4 group. It was observed that CCl_4 administration caused histopathological changes including degeneration and enlargement of sinusoids, endothelial damage and inflammatory cell infiltration in portal areas. The leukocyte infiltration in liver tissue indicated that CCl_4 induced the inflammatory response depending on its toxicity.

The effects of these herbal products and silymarin have been investigated in CCl_4-induced oxidative stress. Milk thistle protected the liver against oxidative damage and decreased MDA in the liver in CCl4-administrated rats (8). The protective effect of *Silybum marianum* extracts was determined in CCl_4-treated animals (52). The nanoemulsion formulation of silymarin decreased alkaline phosphatase, total bilirubin, tissue lipid peroxides, pyruvate transaminase, and glutamate oxaloacetate transaminase, and increased tissue glutathione, total protein, albumin, and globulin in CCl_4-treated rats (46). *Silybum marianum* total extract, silymarin, and silibinin administrations improved the liver damage induced by diethylnitrosamine/2-acetylaminofluorene/CCl_4, decreased ALT, AST, total bilirubin, ALP levels, and increased the serum total protein levels (61). Silymarin reduced the migrating neutrophils and caused a dose-dependent inhibition of leukocyte accumulation in carrageenan-induced inflammation (14). Beta-aescin was suggested to have antioxidative and antifibrotic properties on CCl_4-induced liver injury (54). Extract of artichoke was detected to prevent hepatotoxicity on CCl_4-caused liver injury (36). Chicory and/or artichoke leaf extracts were reported to have a protective effect on CCl_4 and gamma-irradiation-induced chronic nephrotoxicity in rats (17). *Cynara scolymus* leaf extract exhibited therapeutic effects on liver injury (13, 21). A chemically-characterized extract from artichoke (*Cynara scolymus* L.) showed hepatoprotective effects against aflatoxin B1-induced toxicity in rats (40). The walnut extract exhibited protective and antioxidant effects in CCl_4-induced oxidative liver damage (18). Aydın et al. (9) have reported that *Juglans regia* L. (walnut) extract has protective effects against lipid peroxidation formation in the brain, kidney, and liver tissues in CCl_4-applied Wistar rats. The leaf extract of *Ficus carica* had a preservative effect against CCl_4-induced hepatic damage (3, 37, 38, 41, 53). In our study, herbal cream increased CAT levels and reduced CCl_4-induced liver injury and leukocytes infiltration. The antioxidant enzymes in the liver increased significantly in the HC group. These results indicate that herbal cream may have antioxidant properties on the liver tissue and a role in preventing liver damage and also does not have hepatoxicity effects.

In conclusion, it has been determined that herbal cream contains the most active ingredients including *Cynara scolymus*, *Aesculus hippocastanum*, *Juglans regia* and *Ficus carica* leaves have antioxidant and anti-inflammatory effects, and intrarectal application of natural and herbal compounds also has an important effect on pathophysiological processes such as liver damage. The researches that investigate whether there are other potential effects of the combination of fig leaves, artichoke leaves, walnut husks, and horse chestnut fruit and intrarectal therapies on different diseases may contribute to improving the life quality of patients.

DOI: 10.33988/auvfd.1414074

Acknowledgments

The authors would like to thank Feyyaz Küçükay for the drug preparation.

Financial Support

This study was supported by the Scientific Research Projects Coordination Unit of Istanbul University. Project No: 37789.

Ethical Statement

This study was approved by the Istanbul University Local Committee on Animal Research Ethics (Decision no: 2013/92).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

AK, AK, EGG, IS, SU, MC, and CDT conceived and planned the experiments. AK, AK, EGG, IS, SU, and CDT carried out the experiments. AK, AK, EGG, IS, SU, SE, and CDT contributed to sample preparation. AK, AK, EGG, IS, SU, and CDT contributed to the interpretation of the results. AK took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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