Determination of the effects of vitamin D and Nettle (*Urtica dioica* L.) extract administration on SOD-2 and TNF-α levels in liver tissue of TNBS (2,4,6 trinitrobenzene sulfonic acid)-induced rats

ABSTRACT

The aim of this study was to investigate the effects of vitamin D and nettle (*Urtica dioica* L.) extract on SOD-2 and TNF-α levels in liver tissue of rats induced with TNBS (2,4,6 Trinitrobenzene Sulfonic Acid) by immunohistochemical methods. All rats used in the study were weighed and randomly divided into four groups. Liver tissue samples were taken at the end of the experiment. They were blocked in paraffin by applying routine tissue tracking procedure. Histologic and immunohistochemical methods were applied to the sections taken from the paraffin blocks. It was determined that histopathologic changes were intense in the TNBS group and less in the TNBSD and TNBSI groups. Strong SOD-2 immunoreactivity was detected in the cytoplasm of hepatocytes in control and TNBSD groups, weak in TNBS group and moderate in TNBSI group. TNF-α immunoreactivity was weak in the cytoplasm of hepatocytes in control group, strong in TNBS group, and moderate in TNBSD and TNBSI groups. In conclusion, it is thought that vitamin D and nettle extract may have positive effects on liver tissue and both substances may be protective against liver damage due to their antioxidant and anti-inflammatory effects.

Keywords: Liver, nettle, SOD-2, TNF-α, vitamin D

Research Article

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Article info

Submission: 30-01-2024
Accepted: 06-09-2024
Online First: 30-10-2024
Publication: 27-12-2024

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
http://dergipark.org.tr/vetbio

INTRODUCTION

Inflammatory bowel diseases (IBD) affect the liver as well as the intestines, leading to the development of different diseases such as hepatobiliary disorders (HD) or nonalcoholic fatty liver disease (Gizard et al., 2014; Larsen et al., 2010). It has been reported that hepatobiliary disorders may occur in both ulcerative colitis (UC) and Crohn's disease (CD). However, hepatic symptoms occur more commonly in ulcerative colitis (Gizard et al., 2014). Although hepatic disorders are associated with IBD, the clinical picture is usually independent of IBD. It is important to perform screening for HD in IBD patients because it is predicted that approximately 5% of adults with IBD will develop liver disease. Chronic liver disease has also been reported in IBD patients with normal liver biochemical diagnostic tests. The most specific hepatobiliary complication associated with IBD is primary sclerosing cholangitis (PSC). Development of cholangiocarcinoma and colon cancer is observed in these patients (Mendes et al., 2007).

Tumor necrosis factor alpha (TNF-α) is a cytokine with effects known to be involved in the pathogenesis of some inflammatory and autoimmune diseases (Bradley, 2008). Superoxide dismutase (SOD), one of the enzymatic antioxidants, is the primary enzyme for the antioxidant,

How to cite this article

Yediel Aras Ş., (2024). Determination of the effects of vitamin D and Nettle (*Urtica dioica* L.) extract administration on SOD-2 and TNF-α levels in liver tissue of TNBS (2,4,6 trinitrobenzene sulfonic acid)-induced rats. *Journal of Advances in VetBio Science and Techniques, 9*(3), 197-205. https://doi.org/10.31797/vetbio.1428195

is the primary enzyme for the antioxidant defense system (Fridovich, 1975). SOD has been shown to have important contributions in protecting the host from intracellular pathogens (Beaman and Beaman, 1990; Özel and Birdane, 2014).

Vitamin D is an important vitamin that regulates calcium and phosphorus metabolism in the body. It also has a role in the regulation of immune system functions. It has been reported that the incidence of Crohn's disease is high in patients with vitamin D deficiency (Del Pinto et al., 2015; Suibhne et al., 2012). Vitamin D deficiency has been suggested in patients with inflammatory bowel disease. However, it is not yet certain whether vitamin D deficiency is the cause or the result of the disease (Palmer and Weaver, 2013; Sentongo et al., 2002). Nettle (*Urtica dioica* L.) is a wild plant belonging to the Urticaceae family. It has been used in traditional medicine for many years in the treatment of diseases such as rheumatism and arthritis. In addition, its hemostatic and diuretic effects have also been utilized. Nettle has the ability to increase the level of vitamin B12, folate and iron binding in the blood. Therefore, it has been suggested that brewing nettle and using it as detox may have positive effects in the treatment of many diseases (Upton, 2013).

This study aims to reveal the effects of vitamin D and nettle (*Urtica dioica* L.) extract administration on SOD-2 and TNF-α levels in liver tissue by immunohistochemical methods in rats induced with TNBS.

MATERIALS AND METHODS

Material

Thirty-two male *Sprague-Dawley* rats weighing 200-250 g were used for the study. The rats were housed in standard cages at an ambient temperature of 22 ± 2°C, 12 hours of light and 12 hours of darkness, and fed *ad-libitum* with tap water.

Method

All rats were weighed and randomly divided into four groups.

1. Control group (C, n=8): No treatment was given to the rats in this group.

2. TNBS group (TNBS, n=8): Rats in this group were administered 150 mg/kg TNBS (2,4,6 trinitrobenzene sulfonic acid) rectally in a single dose. Then, 1ml physiologic saline solution was given by oral gavage at the same time every day for 10 days (Xia et al., 2019).

3. TNBS + vitamin D group (TNBSD, n=8): In this group, 150 mg/kg TNBS (2,4,6 trinitrobenzene sulfonic acid) was administered rectally in a single dose. Then, 7,500 IU vitamin D was administered by oral gavage once a day at the same time every day for 10 days (Xia et al., 2019).

4. TNBS + Nettle group (TNBSI, n=8): In this group, 150 mg/kg TNBS (2,4,6 trinitrobenzene sulfonic acid) was administered rectally in a single dose. Then, 2.5 ml/kg nettle extract was given by oral gavage once a day at the same time every day for 10 days (Genc et al., 2011).

Histopathological Examinations

At the end of 10 days, liver tissue samples were taken. They were blocked in paraffin by applying routine tissue follow-up procedure. Hematoxylin & Eosin staining was applied to examine the general structure of the liver tissue.

Immunohistochemical Investigations

Sections of 5 µm were taken on slides coated with chromium alum gelatin and Streptavidin-biotin peroxidase method was applied. After deparaffinization and rehydration, the sections were rinsed in PBS (0.1 M, pH, 7.2). Then they were kept in 3% H_2O_2 prepared in 0.1 M PBS for 15 min. Boiled in citrate buffer solution for 10 min in a microwave oven at maximum temperature. Incubated with Large Volume Ultra V Block solution for 10 min. SOD-2 (B-1): sc-133254, 1/500 dilution) and TNFα (52B83) (sc-52746, 1/500 dilution) primary antibodies were added to the sections and kept at room temperature and humidified for 1 hour. The sections were washed with PBS and Biotinylated

Goat Anti B Polyvalent and Streptavidin Peroxidase solutions were added and incubated at room temperature for 15 min each. DAB-H_2O_2 (Diaminobenzidine hydrogen peroxide) Substrate Solution was added for chromogen application. Modified Gill III hematoxylin solution was used for counterstaining. The preparations were examined under a research microscope and photographed. To determine whether the immunoreactivity was specific or not, the sections were kept in PBS without the addition of primary antibody (negative control) and the other procedures were applied exactly the same. Immunohistochemical evaluation was performed by looking at the staining characteristics of the target cells and the staining intensity in the stained target cells. In the evaluation, two independent observers assigned values from 0 to 3 for no staining (0), weak staining (1), moderate staining (2), and strong staining (3). For each group, 20 regions were determined and immunohistochemical scoring was performed from these regions (Yediel Aras and Karadağ Sarı, 2021).

Statistical analysis

One-way analysis of variance was performed to compare SOD-2 and TNF-α immunoreactivity scores. Before analysis of variance, normality and homogeneity assumptions were examined. The kurtosis and skewness coefficients of SOD-2 (kurtosis=-0.48, skewness=-1.33) and TNF-α (kurtosis=-0.04, skewness=-0.93) scores were found to meet the normality assumption. Levene's test results of SOD-2 (Levene's statistic=1.379, $p>0.05$) and TNF- α (Levene's statistic=0.814, $p>0.05$) scores also showed that the variances were homogeneous. Therefore, Scheffe test was used for post-hoc comparisons between groups after analysis of variance. Analyses were performed in SPSS 22 package program and the significance level was set as 0.05.

RESULTS

Histopathologic results

Control group liver tissues were found to have normal histologic structure. Microvesicular fat droplets, shrinkage in hepatocyte nuclei and apopitotic areas were detected in TNBS group. Histopathologic changes were found to be less in TNBSD and TNBSI groups. In both groups, hepatocyte nuclei were similar in size to the control group and remark cords were more regular. While lymphocyte foci were observed in both TNBSD and TNBSI groups, microvesicular fat droplets were observed at a small level in TNBSI group (Figure 1).

Immunohistochemical Results

SOD-2 immunoreactivity

Strong immunoreactivity was detected in the cytoplasm of hepatocytes in control and TNBSD groups, weak in TNBS group and moderate in TNBSI group. In addition, moderate immunoreactivity was observed in lymphocyte foci in TNBSI group (Figure 2).

TNF-α immunoreactivity

Weak TNF-α immunoreactivity was detected in the cytoplasm of hepatocytes in Control group, strong in TNBS group, and moderate in TNBSD and TNBSI (Figure 3).

As a result of the analysis, it was determined that there was a significant difference between the groups in SOD-2 ($F=1043.442$, $p<0.001$) and TNF-α ($F=778.898$, $p<0.001$) immunoreactivity scores. In SOD-2 immunoreactivity scores, Control and TNBSD groups had higher mean scores than the other groups and TNBSI group had higher mean scores than TNBS group (Table 1).

In TNF-α immunoreactivity scores, TNBS group had a higher mean score than the other groups and TNBSD and TNBSI groups had a higher mean score than Control group (Table 1).

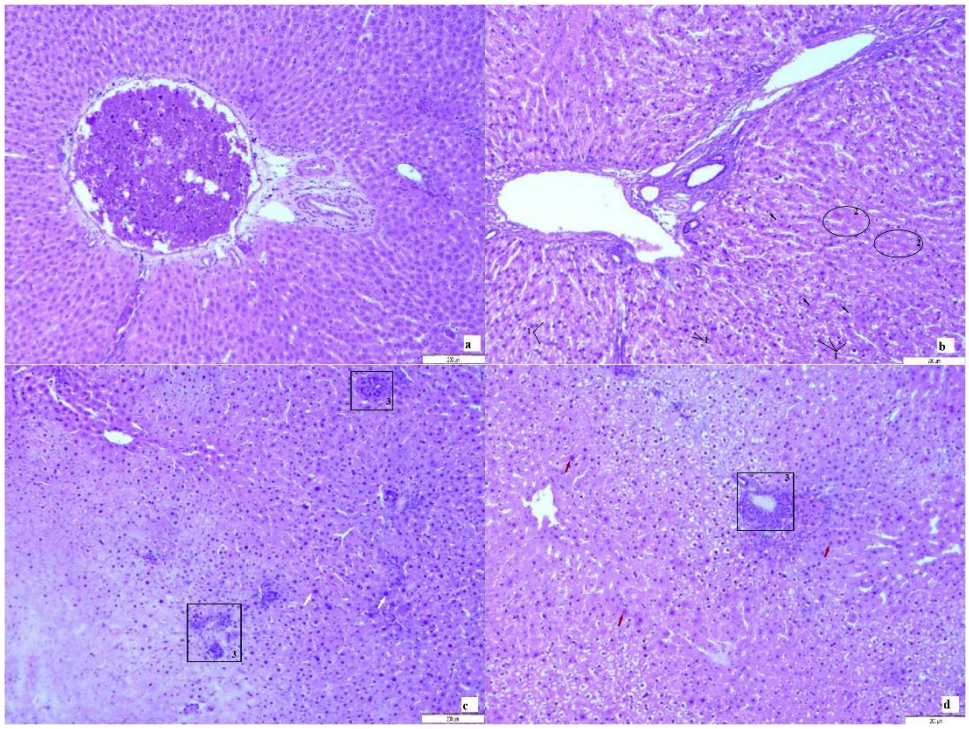

Figure 1. Rat liver tissue. a: Control group. b: TNBS group, 1: microvesicular fat droplets, 2: apopitotic areas, arrow: hepatocyte nuclei shrinkage. c: TNBSD group, white arrow: hepatocyte nuclei close to control, 3: lymphocyte follicle. d: TNBSI group, red arrow: hepatocyte nuclei close to control, 3: lymphocyte follicle. H-E staining.

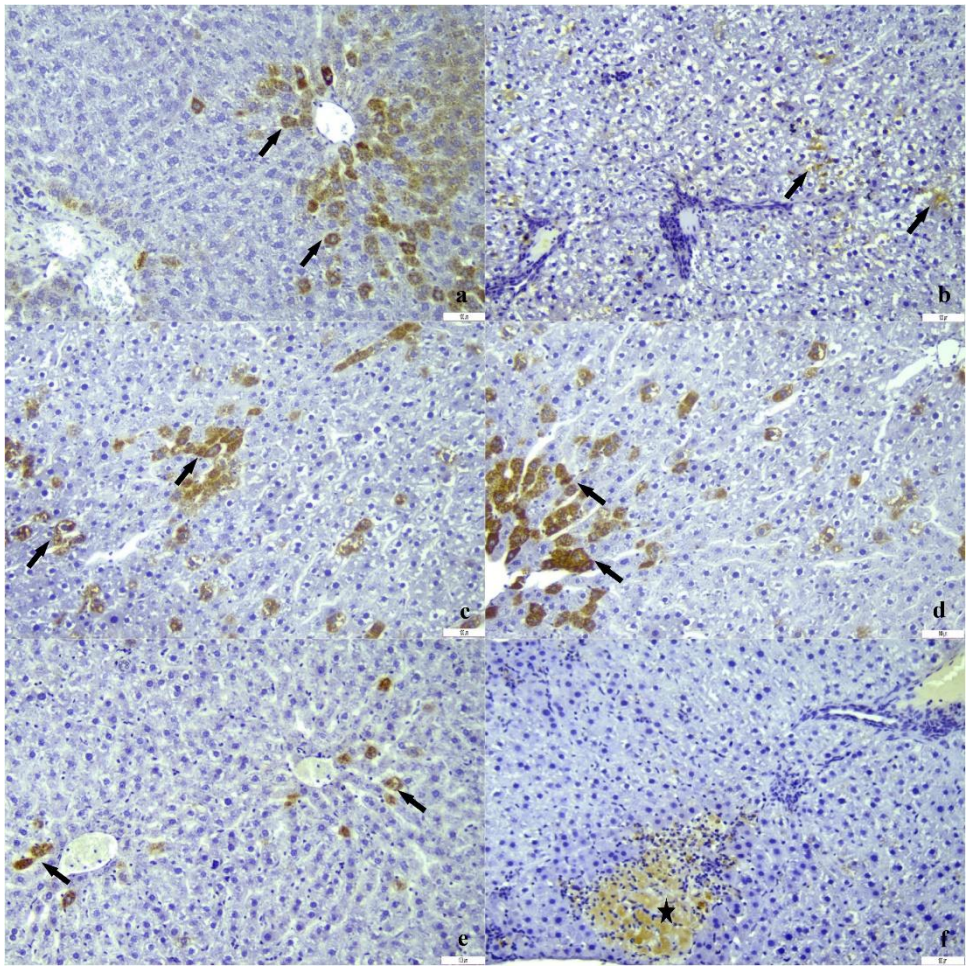

Figure 2. SOD-2 immunoreactivity in rat liver tissue. a: Control group, b: TNBS group, c, d: TNBSD group, e, f: TNBSI group. Hepatocytes (arrow), lymphocyte follicle (asterisk). a, c, d: strong immunoreactivity; weak immunoreactivity. e, f: moderate immunoreactivity.

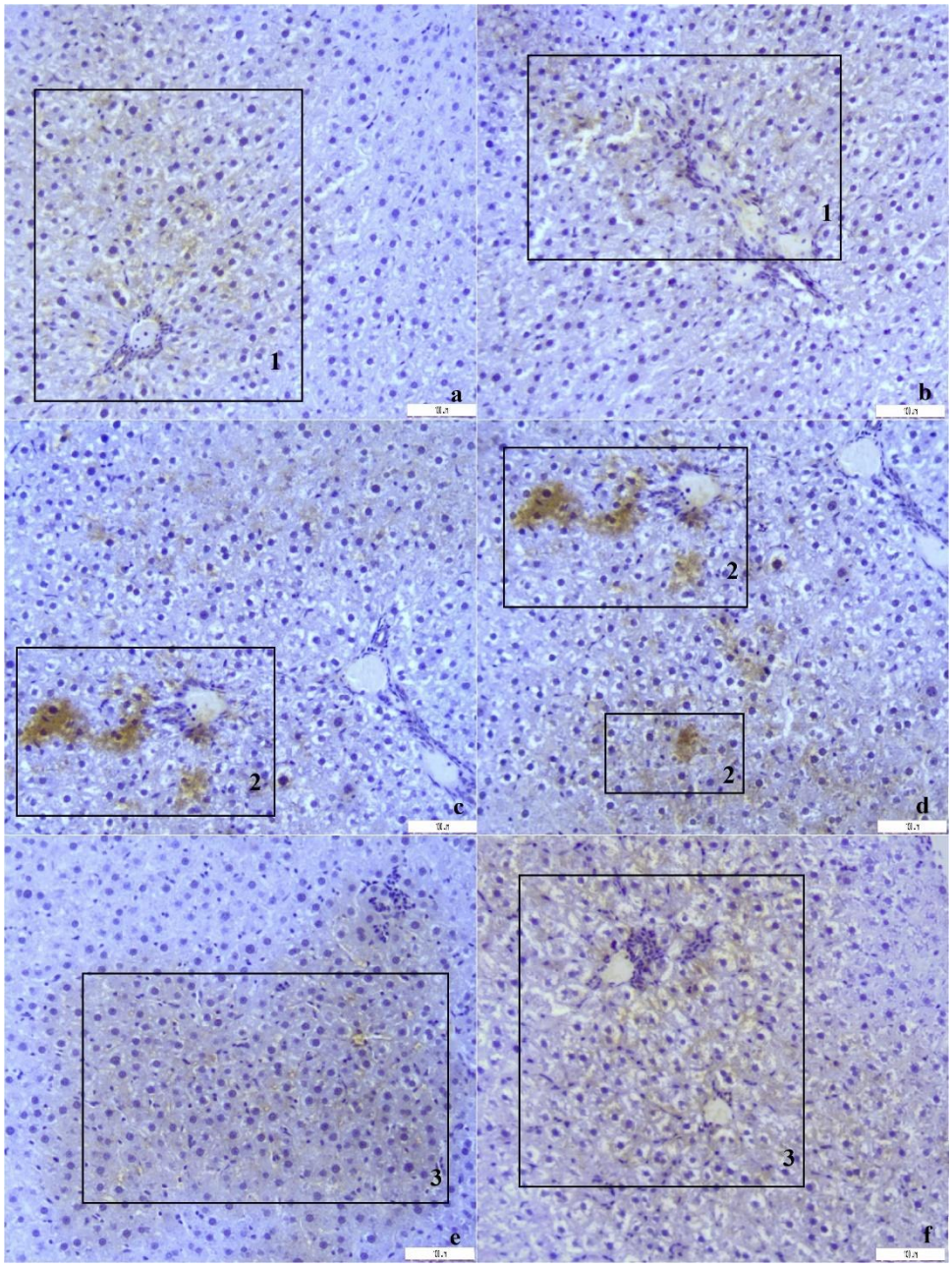

Figure 3. TNF-α immunoreactivity in rat liver tissue. a,b: Control group, c,d: TNBS group, e: TNBSI group, f: TNBSD group. 1: Weak immunoreactivity in hepatocytes. 2: Strong immunoreactivity in hepatocytes. 3: Moderate immunoreactivity in hepatocytes.

Table 1. Comparison of SOD-2 and TNF-α immunoreactivity scores between groups.

		n	Ort	SS	F	p	post-hoc
SOD-2 immunoreactivity	C (a)	20	2.95	0.13	1043.442	0.000	a, c > d > b
	TNBS (b)	20	1.05	0.10			
	TNBSD (c)	20	2.94	0.16			
	TNBSI (d)	20	2.01	0.10			
TNF-α immunoreactivity	C (a)	20	1.05	0.10	778.898	0.000	b > c, d > a
	TNBS (b)	20	2.94	0.16			
	TNBSD (c)	20	2.03	0.11			
	TNBSI (d)	20	2.03	0.11			

DISCUSSION

Inflammatory bowel diseases (IBD) such as ulcerative colitis (UC) and Crohn's disease (CD) are chronic diseases that, like cardiovascular disease, can reduce a patient's life expectancy. Its global prevalence is predicted to increase up to 1% in many regions by 2030 (Kaplan and Windsor, 2021). Non-alcoholic fatty liver disease (NAFLD), a disease characterized by fatty liver and determined by liver fat content (LFC) measurements, is among the most important causes of liver diseases even in lean patients. Its global prevalence is estimated to be over 24% (Younossi et al., 2018). It has been suggested that IBD is associated with NAFLD and these two diseases usually coexist (Lin et al., 2021). However, another study compared IBD patients with and without NAFLD and found no significant difference in the characteristics of IBD disease in both groups (Magrì et al., 2019). However, it has been suggested that a high-fat diet has an effect on the quality of the intestinal barrier and the composition of the gut microbiome, affects the pathogenesis of IBD, and LFC may also be associated with UC (Jamali et al., 2017). The development of hepatic fat accumulation (steatosis) is a common finding in CD, but the pathophysiologic processes leading to steatosis have not yet been fully elucidated. It has been suggested that insulin resistance, lipotoxicity, immune cell response and inflammation may be involved in the pathogenesis of steatosis, along with strong environmental and genetic influences. It has even been reported that steatosis may progress and lead to nonalcoholic steatohepatitis (NASH) and liver fibrosis (Bechmann et al., 2012). Studies have emphasized that changes in bile acid metabolism and intestinal microbiota dysbiosis may also be effective on the development and progression of steatosis (Sydor et al., 2020). On the other hand, it has been reported that intestinal microbiota is also impaired in CD and has a favorable course in relation with the treatment of the disease (Connors et al., 2020). Determination of microvesicular fat droplets, shrinkage in hepatocyte nuclei and apopitotic areas in the TNBS group suggested that inflammatory bowel diseases may have pathologic effects in the liver tissue and may cause dysfunction of the liver and other digestive system organs related to the liver.

In studies conducted to investigate the effectiveness of vitamin D as an immunomodulator in preventing liver tissue damage, vitamin D deficiency was found in 91% of HBV-infected patients and low vitamin D levels were found to be significantly associated with high viral replication (Ilkovska et al., 2019). It has been suggested that vitamin D deficiency is associated with disease severity in patients with chronic hepatitis (Rahman and Branch, 2013). In another study, a significant relationship was found between low vitamin D levels and increased levels of inflammation in the liver (Hoan et al., 2016). Vitamin D is an anti-inflammatory vitamin that inhibits the expression of tumor necrosis factor alpha and interleukin-1, which are key inflammatory markers of NAFLD-related liver injury. It has been suggested that vitamin D deficiency leads to severe liver inflammation and oxidative stress (Abe et al., 2021). Vitamin D treatment has been reported to be effective in improving hepatic lesions in NAFLD (Bingül et al., 2021). In addition to its direct antioxidant effect, vitamin D has also been reported to act by increasing the gene expression of proteins/enzymes in the antioxidant system (Mokhtari et al., 2017). It was reported that mRNA expressions and activities of SOD and GSH-Px increased in the liver of vitamin D-treated rats (Bingül et al., 2021). The decrease in SOD-2 immunoreactivity and increase in TNF-α immunoreactivity in the liver tissues of the TNBS group suggested that inflammation may have a negative effect on antioxidant and anti-inflammatory cytokines and cause impairment in liver function. In TNBSD group, an increase in SOD-2 immunoreactivity

and a decrease in TNF-α immunoreactivity were determined.

Nettle is a plant that should be taken into the body because of its rich nutrient content and being a good antioxidant (Hoşbaş, 2008). It was observed that nettle seed extract decreased the levels of liver enzymes and liver lipid peroxidation levels which increased due to carbon tetrachloride (CCl4). The extract shows a protective effect on the liver by reducing the hepatoxic effect of CCl4 (Şener et al., 2010). It was reported that nettle treatment effectively protected the liver against aflatoxin-induced hepatotoxicity, decreased AST, ALT and GGT levels and increased antioxidant levels (Yener et al., 2009). In another study, it was suggested that nettle significantly decreased serum lipid hydroperoxide and ceruloplasmin levels and increased catalase, paraoxonase and arylesterase levels. It has also been reported to significantly reduce liver tissue damage and to have a protective effect on the liver (Kandis et al., 2010). In the TNBSI group, an increase in SOD-2 immunoreactivity and a decrease in TNF-α immunoreactivity were determined.

CONCLUSION

Inflammatory bowel diseases negatively affect the quality of life of patients and cause many complications in the long term. The main involvement of the intestines, but it can also affect other organs, especially the liver. Inflammatory bowel diseases often cause non-alcoholic fatty liver disease in the liver. The results of the study suggested that vitamin D and nettle extract may have positive effects in alleviating the complications of the disease in the liver and that both substances may be protective against liver damage due to their antioxidant and anti-inflammatory effects. However, it is thought that many more studies are needed to elucidate the protective mechanisms of vitamin D and nettle.

ACKNOWLEDGMENT

Financial support: There is no funding source.

Conflict of interest: The authors declare no conflict of interest.

Ethical statement or informed consent: Approval for the study was obtained from Kafkas University Animal Experiments Local Ethics Committee (Decision No: KAU HADYEK/2023-160).

Author contributions: Concept, Design, Control, Data Collection, Analysis: ŞYA.

Availability of data and materials: Data and materials may be used subject to the author's permission.

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