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Research Article

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The effect of *Ulva rigida* (C. Agardh, 1823) against cadmium-induced apoptosis and oxidative stress

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Abstract

Cadmium (Cd) is known as a pollutant source in recent years with the increase in industrialization. Algae have secondary metabolites with high biological activity, used for pharmaceutical agents. The liver and kidney are the primary organs involved in the elimination of systemic cadmium and the main targets of cadmium toxicity. In the previous research, it was determined the ameliorative effects of the extract obtained from Ulva rigida in the liver tissue of rats induced by cadmium. 35 female Wistar rats between 225-240 g were used. The subjects were injected subcutaneously with 1 mg/kg cadmium chloride ($CdCl_2$) four times a week for four weeks. The study was carried out by groups as control (G1), Cadmium group (1 mg/kg $CdCl_2$-G2), Algae group (100 mg/kg-G3), Cd+algae group (1 mg/kg $CdCl_2$+50 mg/kg algae extract-G4) and Cd+algae group (1 mg/kg $CdCl_2$+100 mg/kg algae extract -G5). The subjects were sacrificed by cervical dislocation. Liver tissue and cardiac blood were collected. It was determined that oxidative stress with iNOS, inflammation and apoptosis with TNF-α increased with cadmium induction, while there was a statistically significant decrease in the groups that were given algae extract. In addition, biochemical changes in SOD, CAT and MDA values were found to be significant ($p<0.05$). As a result, it was determined that algae extract could play a protective role with its antioxidant and antiapoptotic properties in experimentally induced cadmium toxicity in rats.

***Keywords**: Cadmium (Cd), Ulva rigida, apoptosis, oxidative stress, experimental study*

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Introduction

Cd uptake by the food chain can reach about 1 mg per day (1). Many organs and systems such as the liver, kidney and testis can be damaged with cadmium poisoning (2). The distribution of cadmium in the body varies depending on the way that it is taken, the dose, and the duration. The liver and kidney are the vital organs that ensure the systemic elimination of cadmium and are the main targets of cadmium toxicity (3). Symptomatic treatment methods are applied in cadmium poisoning. However, it has been reported that selenium, vitamin E, vitamin

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C, lycopene, taurine, melatonin, acetylcysteine, progesterone, ß-carotene, chlorpromazine, and glutathione were used in studies conducted to protect or prevent from cadmium poisoning (4, 5).

Algae, one of the most important organisms of the aquatic ecosystem, are used in pharmacy and cosmetics, agriculture and industry. Antioxidant activities of algae originate primarily from the polyphenols, polysaccharides, pigments (chlorophylls, carotenoids) and vitamins they contain. Antioxidants protect the organs against damage caused by reactive oxygen species. Some of the synthetic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene, which are generally used, are limited due to their harmful effects, and thus natural antioxidants are preferred instead (6). Phenolic antioxidants, which are among the natural antioxidants, are also commonly found in algae (7-9). In another study on rats, it was determined that red and green algae had a protective effect against skin, breast, and intestinal cancer. This effect was realized by increasing the activity of antioxidant enzymes in the rat liver and reducing lipid oxidation by the phenolic components in the algae extracts (10, 11).

In this study, the protective and ameliorative effects of the extract obtained from *U.rigida* against fibrosis and inflammation caused by the activation of stellate cells in the liver tissue of cadmium-induced rats and apoptosis in the tissue were determined biochemically and immunohistochemically.

Material and Method

Preparation of the extracts

Extraction procedures were applied as described elsewhere. *U. rigida* was collected, dried, and crushed into small pieces with a grinder. The powdered samples were shaken in pure ethanol at 140 ppm at room temperature for 2 days. It was then filtered into evaporation flasks with filter paper. The filtrates were evaporated at 45°C. Finally, the weight of the residue remaining from the algae was measured, dissolved in distilled water, and the doses were determined (12).

35 adult female Wistar rats, between 225-240 g, were used. Groups were determined as follows:-

Control group (G1: 7): Group fed with standard rat chow and water, injected subcutaneously (sc) with saline for four weeks.

Cd group (G2: 7): 1mg/kg CdCl2 sc injected and fed with standard rat food and water for weeks.

Algae group (G3: 7): The group fed with standard rat food and water and given 100 mg/kg/day algae extract by gavage method.

Cd + Algae group (G4: 7): The group fed with standard rat food and water, injected sc 1 mg/kg CdCl2 and given 50 mg/kg/day algae extract by gavage method.

Cd + Algae group (G5: 7): The group fed with standard rat food and water, injected sc 1 mg/kg CdCl2 and given 100 mg/kg/day algae extract by gavage method.

U. rigida was collected from Çanakkale Strait. The extract material was given to the rats at the determined doses.

Ethical statement

Animal Experiments Local Ethics Committee (2020/06-11) by Çanakkale Onsekiz Mart University.

Collection and Tracking of Tissue Samples

The animal model of this research lasted for 21 days. Aftter sacrificing, liver tissues were placed in tissue transport cassettes after trimming and fixed in immunofix for 24 hours.

Immunohistochemistry

Tissue samples cut at a thickness of 5 microns were kept at 60 °C, de-paraffinized, and then passed through xylene twice to completely remove

the paraffin from the tissues. Afterwards, tissue samples were cleaned of xylene and their water was removed (dehydration) by passing them through graded alcohols (absolute alcohol, 96%, 80%, 70%, 50%). After this procedure, it was kept in a 0.3% TritonX100 (Santa Cruz Biotechnology) solution prepared with deionized water for 10 minutes. The solutions were passed through the pores in the cell and nuclear membranes. Afterwards, primary antibodies iNOS (ab15323) and PCNA (ab218310) were added. Then, biotinylated Seconder antibody solution (LAB-SA Detection System, Histostain-Plus Bulk Kit; A solution, Invitrogen) was applied for 30 minutes.

TUNEL Assay

Terminal Transferase dUTP Nick End Labeling technic, which allows staining apoptotic cells, was used to determine cell death.

Evaluation of Tissue Samples and Statistics

All stained tissue samples were evaluated under research microscope and photographed with a digital camera (AxioCam ICc 3). Cells showing PCNA, iNOS and TUNEL reactivity were detected using the Leica LAS V3.8 image analysis system. By counting 1000 cells from the stained sections, immunoreactive cells were determined among these cells. For evaluation this formula was used:

Immune positive cells

---------- X 100% = … %

Total number of cells (1000)

Biochemical analysis

Malondialdehyde (MDA) nmol/L

The MDA level was determined by thiobarbituric acid (TBA) at 90-1000°C. In the reaction, MDA or MDA-like substances and TBA react with the production of the pink pigment with absorption at 532 nm (Relassay, Turkey)

Super Oxide Dismutase (SOD) U/ml

Superoxide dismutase (SOD) speeds up the dismutation of the toxic radical into hydrogen peroxide and molecular oxygen during oxidative energy processes. In this method xanthine and xanthine oxidase are used to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride. SOD activity is measured by the degree of inhibition (Relassay, Turkey)

Catalase (CAT) U/L

The first sample is incubated with a known amount of hydrogen peroxide. The sample converts hydrogen peroxide into water and oxygen. The enzyme is stopped and after a fixed incubation period the remaining hydrogen peroxide is determined using a chromogen. The absorbance is at 405 nm and the results are shown as U/L (Relassay, Turkey).

Statistical analysis

SPSS, version 19.0. (SPSS, IBM Company) was used to analyze to the differences between the groups for performance, biochemical, and histological parameters. The data distribution normality was examined with the Kolmogorov-Smirnov normality test. In comparing the experimental and control groups, Mann-Whitney U and Kruskal-Wallis tests were used for continuous variables, and a chi-square test was used for categorical variables ($p<0.05$).

Results

Morphological findings

In the histological staining performed with routine Hematoxylin-Eosin, histopathological results were not found in the liver tissues of the control group (G1) (Figure 1-a). Among the Cd-induced rats, 3 subjects in the untreated group (G2) died approximately 48 hours after the application. In other subjects of the same group,

significant weight loss and slowdowns in vital activities were observed throughout the experiment. When the subjects were sacrificed 28 days later, dilatation of the vena centralis and portal veins, increased infiltration of mononuclear cells around the portal areas, and congestion in the central and portal veins were observed. In addition, histopathological tissue content characterized by an increase in the series of hepatocytes leading to necrosis, hepatocytes with pycnotic nuclei and cytoplasmic vacuolization was observed (Figure 1-b). No histopathological findings were found in the group given only algae extract. It was observed that there was occasional congestion in the sinusoids (Figure 1-c). On the other hand, it was observed that there was a regression in all these degenerative results and an increase in regenerative activity. Although the lobular integrity of the liver was impaired in the G4 group, inflammatory regression decrease in congestion and a slight decrease in the losses due to vacuolization in hepatocytes were observed (Figure 1-d, e). Regenerative changes in parallel with the increase in the dose of algae extract in the G5 group were remarkable. Especially the regression of inflammation and the decrease in hepatocyte degeneration were very important (Figure 1-e). Results obtained in routine H&E staining show that green algae extract plays a protective role against agents, such as cadmium, that cause liver toxicity. Masson trichrome triple staining also showed fibrotic changes in the liver (Figure 2).

iNOS and TNF-α Findings

It was observed that Cd-induced groups showed higher immunoreactivity compared to the control group, and the staining was mostly in the cell cytoplasm (Figure 3). It was observed that iNOS

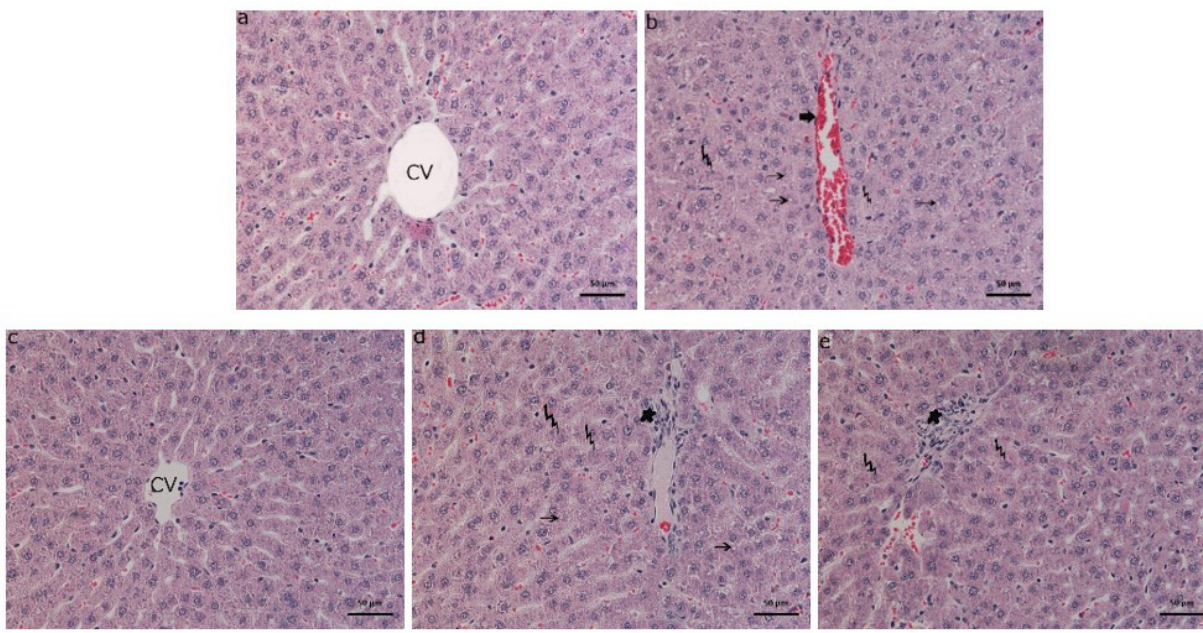

Fig. 1. a- The tissue of control group (G1), b- The tissue of only 1 mg/kg CdCl2-induced group (G2), c- The tissue of only algae extract group (100 mg/kg- G3), d- The tissue of algae (50 mg/kg) + 1 mg/kg CdCl2 induced group (G4), e- The tissue of algae (100 mg/kg) + 1 mg/kg CdCl2 induced group (G5), H&E staining, (CV: Central vein, thick arrow: congestion, thin arrow: hepatocyte vacuolization, lightning: necrotic hepatocytes, star: inflammation).

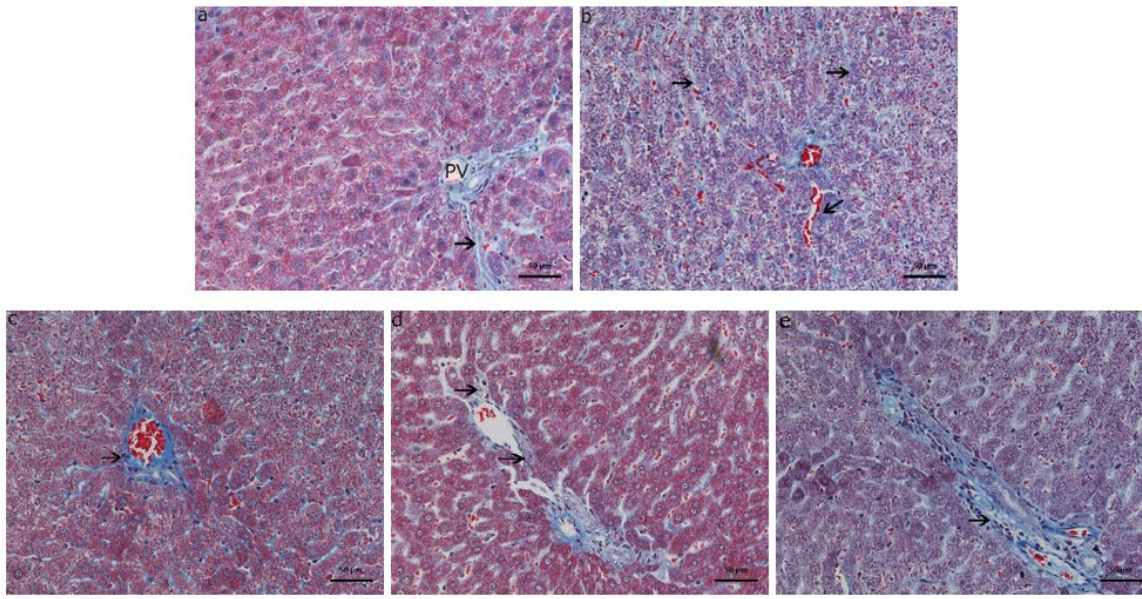

Fig. 2. a- The tissue of control group (G1), b- The tissue of only 1 mg/kg CdCl2-induced group (G2), c- The tissue of only algae extract group (100 mg/kg- G3), d- The tissue of algae (50 mg/kg) + 1 mg/kg CdCl2 induced group (G4), e- The tissue of algae (100 mg/kg) + 1 mg/kg CdCl2 induced group (G5), all subjects were treated with Masson trichrome staining (arrow: fibrosis, PV: Portal vein).

immunopositivity was more severe especially in the Cd group (G2) and in the liver tissues of the groups given 0.05 g/kg algae extract for treatment. It was determined that as the algal extract dose increased (0.1 g/kg), the immunoreactivity decreased (Figure 3d, 3e). The iNOS reactivity of only the algae treated group was similar to the control group (Figure 3c).

TNF-α expression showed lower immunoreactivity in the liver due to the increase of the algal extract dose and the staining was mostly in the cell cytoplasm (Figure 4). It was determined that apoptotic activity was at the lowest level in the control and G3 (Figure 4a, 4c). The apoptotic mechanism was very high in the tissues of the subjects in Cd group (G2) (Figure 4b). Immunoreactivity was significantly decreased in G5 (Figure 4d, e).

The control and experimental groups have significant differences for iNOS and TNF-α staining ($p<0.05$). Significance was determined as $p<0.0001$ between control and G2, $p<0.001$ between control and G4, $p<0.01$ between control and G4. In addition, $p<0.001$ significance was found between the G2 and G5. No statistically significant difference was found between the control and algae groups ($p>0.05$) (Figure 5, 6).

TUNEL Findings

In hepatocellular damage, especially oxidative stress and inflammation cause hepatocytes to be dragged into apoptosis with released cytokines. Disruption of functional processes in tissue is one of the important triggering conditions for apoptosis. In this study, in which liver damage was caused by Cd induction, it was observed that *U. rigida* extract, used as a treatment agent, slowed down the apoptosis and rapidly regenerated hepatocytes. TUNEL reactivity was observed to be severe in liver tissues of only Cd-induced groups (Figure 6b). It was determined that the apoptosis level decreased in the groups given different

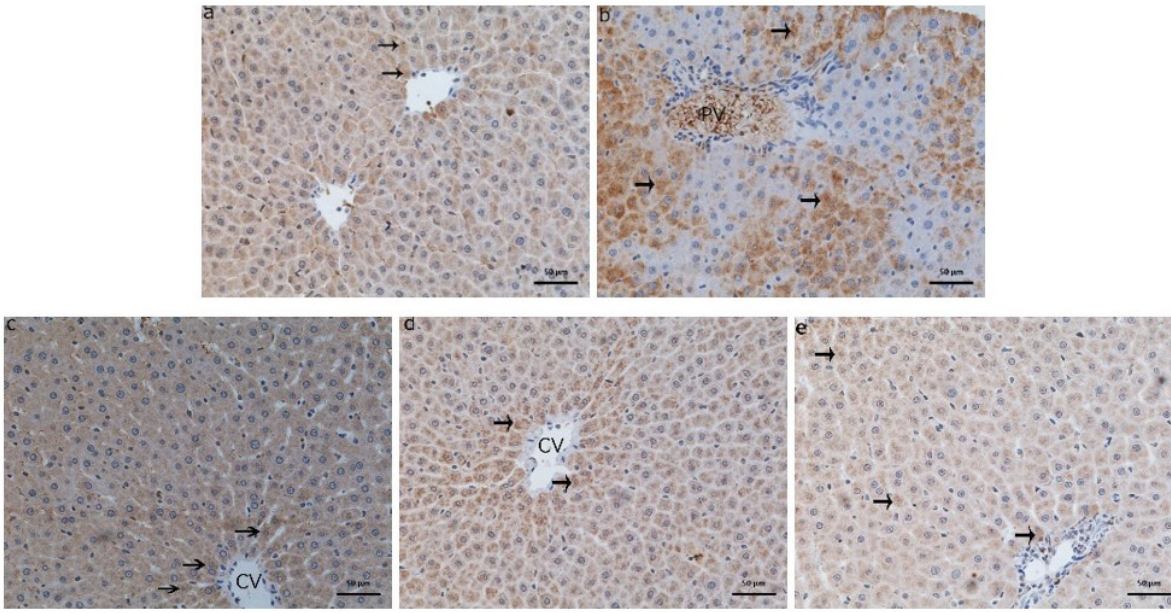

Fig. 3. a- The tissue of control group (G1), b- The tissue of only 1 mg/kg CdCl2-induced group (G2), c- The tissue of only algae extracts group (100 mg/kg-G3), d- The tissue of algae (50 mg/kg) + 1 mg/kg CdCl2 induced group (G4), e- The tissue of algae (100 mg/kg) + 1 mg/kg CdCl2 induced group (G5), iNOS staining, (Arrow: immunopositive cells).

doses of *U. rigida* extract (Figure 6d, e), and that apoptosis was similar to the control group, especially in the liver tissues of the subjects given 0.1 g/kg dose of algae extract. In statistical comparisons, this table was confirmed with significance. The highest significance in TUNEL positivity was determined between the control and Cd groups ($p<0.0001$) (Figure 7). While the least significance was observed between the control group and G4 (*$p<0.05$), the $p<0.001$ significance was observed between the control and G4 (Figure 8). The apoptotic index values increased especially in the Cd groups, so this situation was caused by the high hepatotoxicity of cadmium, while the apoptotic regression in G5 was caused by the antioxidant properties and algae extract.

Biochemical Findings

MDA level was significantly higher in the Cd-induced ($p<0.05$), but MDA levels have no significant difference between the treatment groups ($p> 0.05$). SOD and CAT levels in Cd-induced group were significantly decreased ($p<0.05$). The treatment groups had higher SOD and CAT levels ($p<0.05$) compared to the Cd-induced group (Table 1).

Discussion

Cd, which is one of the important pollutants and known for its toxic effects on living things, is a heavy metal type that cannot be produced directly from mineral ores (13). Experimental studies have shown that the effect and distribution of this metal changes in organs exposed to cadmium (14). Cadmium intake causes severe damage for organs such as liver, lungs and kidneys. However, the severity of these damages varies according to the duration and dose of cadmium exposure (15-17). It can cause some pathological ğroblems, such as liver fibrosis, renal tubular dysfunction, osteoporosis, hypertension, and cancer (18). The cytotoxic effects of cadmium

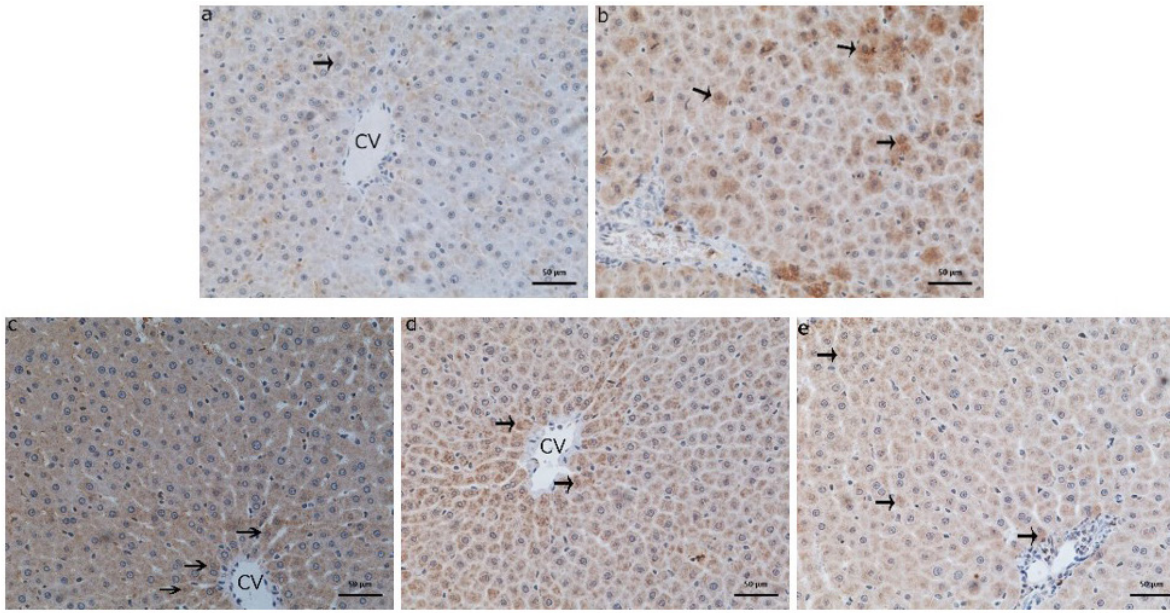

Fig. 4. a- The tissue of control group (G1), b- The tissue of only 1 mg/kg CdCl2-induced group (G2), c- The tissue of only algae extract group (100 mg/kg- G3), d- The tissue of algae (50 mg/kg) + 1 mg/kg CdCl2 induced group (G4), e- The tissue of algae (100 mg/kg) + 1 mg/kg CdCl2 induced group (G5), TNF-α staining (Arrow: immunopositive cells).

are due to the formation of free radicals and the defect in the antioxidant defense system (19).
In a study with chickens, it has been reported that cellular damage due to endoplasmic reticulum and mitochondrial stress occurs as a result of high accumulation of Cd in chicken liver (20) and another study determined that the high values of the heavy metal (Cd etc.) can be considered as a reason for histopathological results in rats (21). As a result, this picture not only affects the egg production of chickens but also reveals the possibility that heavy metals such as Cd can pass into the human body as a result of consuming their meat (20). The findings in this study also

Table 1. Spectrophotometric analysis of MDA, SOD and CAT in experimental groups

Groups	MDA (nmol/L)	SOD (U/ml)	CAT (U/L)
1 Control	6.93±0.84	227.12±32.45	98.89±20.14
2 Cd	8.64±1.01 A*G*	179.39±24.12 A*G*	84.10±12.32 A*G*
3 Algae	7.22±0.72 E*	234.48±26.56 E*	102.40±17.25 E*
4 Cd+ algae (G4)	7.79±1.15 B*F*	193.20±20.23 B*F*	81.60±22.58 B*F*
5 Cd+ algae (G5)	10.85±1.72 C*D*	145.84±18.66 C*	75.70±15.90 C*

Mean±SD (Standard deviation) data. *p< 0.05. Comparison of groups: A: 1 and 2, B: 1 and 3, C: 1 and 4, D: 1 and 5, E: 2 and 3, F: 2 and 4, G: 2 and 5. Abbreviations: MDA, Malondialdehyde; SOD, Superoxide dismutase; CAT, Catalase

showed that cadmium-induced apoptotic and necrotic damage on liver tissue increased.

Cd can induce ROS accumulation by modulating the activities of NOS (22). This oxidative damage leads to pathological disorders in functions and cellular structure (23). In this study, findings showing liver pathology such as increased Cd level and inflammatory response from serious pathological changes, periportal fibrosis and inflammatory cells infiltrating around the hepatic central vein were obtained.

In animals, stimulation of cytokine expression by chemicals in the environment is considered an inflammatory response. Therefore, in this study, the proinflammatory and anti-inflammatory cytokine TNF-α in liver tissues and iNOS immunoreactivity levels caused by oxidative stress were examined and it was observed that these parameters increased with Cd exposure and decreased with algae extract supplementation. TNF-α secreted by active macrophages is an adequate mediator of local and systemic inflammation (24). The results obtained in the study show parallelism with the literature. Liver fibrosis and degeneration due to lipid peroxidation are seen in the results, and the protective effects of *U. Rigida* extract, which has antioxidant properties and has been used in a few studies in vivo, have been investigated. It has been determined that this green algae species has protective properties against agents that cause liver damage, which will contribute to the literature.

Algae are important sources of bioactive substances used in nutrition. Phenolic antioxidants, which are among the natural antioxidants, are also commonly found in algae (25). Recent studies have revealed that *U. rigida* contains high amounts of important bioactive compounds, and is a quality protein source with its amino acid composition (26). The addition of algae in diets inhibits disaccharidase activities, and shows the algae consumption may be beneficial in chronic problems with disruptions in glucose homeostasis. In the experimental study, in which liver damage was caused as a result of Cd induction, it was observed that this situation manifested itself with the apoptotic mechanism. It was evaluated histopathologically that the degenerative results from lipid peraoxidation changed depending on the amount of algae extract.

It has been determined that some substances used in experimental model studies on algae have protective properties. Dieckol, obtained from marine algae, was given to rats induced with N-Nitrosdiethylamine for therapeutic purposes, to prevent lipid peroxidation, cell damage and scavenges free oxygen radicals in the liver (27). Both edible blue-green algae *N. commune* var *sphaeroides* (NO) and *S. platensis* (SP) supplementation reduced the development of atherosclerotic lesions in atherosclerosis mouse model induced in apolipoprotein E knockout (ApoE(-/-)) mice (28). In a different study, it was reported that brown algae polysaccharides, especially fucoidan, alginate and laminarin, have potential effects against the onset and progression of fatty liver (29, 30). Altinok-Yipel et al. investigated the protective effects of *S. platensis*, *C. vulgaris*, *L. Japonica* and *Sargassum* sp. in the acute hepatotoxicity model with carbon tetrachloride. According to the results, they determined that *S. platensis*, which is one of the algae species that can minimize the toxic effects of CCl_4, can be used for protection against chemical-induced hepatotoxicity (31). Another study showed that *Galaxaura oblongata* (Ellis and Solander) extract has protective effects against acute liver and kidney injuries by reducing apoptosis, attenuating the inflammatory response and oxidative stress load, in a lipopolysaccharide-induced liver and kidney injury model (32). It has been reported that hepatic MDA levels are reduced by 42%, vacuolar degeneration, fat infiltration and fibrosis are prevented in the liver, following the application of *S. platensis* as a result of inducing oxidative stress, fat accumulation, and fibrosis

with cadmium (33). Similar antioxidant effects of *S. platensis* supplementation on prevention of hepatotoxicity were also described, with mercury-induced liver injury (34) and CCl_4 attenuating hepatic lipid peroxidation, congestion, and hepatocyte necrosis in the livers of rats (35). In this study, hepatocyte damage accompanied by intense fibrosis around the portal area with Massson triple staining was observed. It is noteworthy that the results are compatible with the literature. However, many studies have shown that marine algae may have protective and preventive properties in liver damage models (34, 36). In previous research, the focus was on the therapeutic properties of green algae extract in the model of acute liver injury.

As a result, Cd is one of the heavy metals released into the environment by industrial activities. It can easily reach the human body in different ways. Therefore, the severity of negative consequences depends on the duration and amount of exposure. As the extent of the damage increases, the healing options decrease. Although definitive and clear, treatments for liver damage are very limited in medical terms. Alternative and more effective treatment options are always sought for heavy metal toxicity. Histopathological data show that *U. rigida* extract has a protective role against Cd-induced liver injury. Although the effects of the extract obtained from the algae species used in the study are shown texturally, it is recommended to clarify its chemical composition and to investigate the role of active substances more comprehensively. However, there are some limiting factors at this stage. The high toxic effect of the inducing agent used allows the study to be conducted only experimentally. Rat resistant to toxic effects was used in the experimental model. Subjects more resistant to heavy metal toxicity may be preferred. Dose adjustment and administration time were made based on the literature. This study is a preliminary step for studies that will allow higher dose preference.

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Authors' contribution

Author LCİ designed the study, wrote the draft of the manuscript, and managed the structure of the manuscript.

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