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Protective effect of *Allium scorodoprasum* L. ethanolic extract in cyclophosphamide-induced hepatotoxicity model in rats

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Abstract

Objectives Cyclophosphamide is a chemotherapeutic agent and immunosuppressant drug; however, it damages the liver. This study investigates the protective effect of ethanolic extract of *Allium scorodoprasum* (ASE) on cyclophosphamide-induced liver injury.

Methods Twenty-eight Wistar albino rats were randomly divided into four groups (n = 7 per group): healthy rats, cyclophosphamide (200 mg/kg), cyclophosphamide (200 mg/kg) + ASE (100 mg/kg) and cyclophosphamide (200 mg/kg) + ASE (200 mg/kg). ASE was administered for 14 days, and the rats were euthanized 24 h after cyclophosphamide administration.

Key findings Cyclophosphamide treatment leads to an increase in serum levels of alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, total cholesterol, triglycerides, low-density lipoprotein and very low-density lipoprotein, as well as an increase in the liver levels of malondialdehyde, tumour necrosis factor, interleukin (IL)-1β and IL-6, while high-density lipoprotein levels decrease. Treatment with cyclophosphamide caused liver necrosis and postnecrotic cell infiltration; however, pathological changes were prevented by ASE. 8-Hydroxy-2′-deoxyguanosine, anti-4-hydroxynenal antibody and anti-dityrosine levels increased in rats treated with cyclophosphamide and decreased in the groups treated with ASE. These changes were dose dependent in the ASE-treated groups.

Conclusions Treatment with cyclophosphamide caused liver damage due to oxidative stress and inflammation. ASE regulated the damage at high doses because it has potent antioxidant and anti-inflammatory ingredients. In future studies, it may be beneficial to administer ASE in higher doses or for longer periods of time.

Keywords: *Allium scorodoprasum* extract; cyclophosphamide; inflammation; liver toxicity; oxidative stress

Introduction

Cyclophosphamide (CP) is one of the most effective and widely used immunosuppressant and anticancer chemotherapeutic drugs.[1] It is often used in the treatment of multiple rheumatic diseases and cancers (breast, lymphoid and paediatric malignancies).[1, 2] CP is a prodrug that is converted to active metabolites by the P450 enzyme system in the liver. In addition, it can function as an autoinducer of the enzyme system. The two active metabolites of CP are phosphoramide mustard and acrolein. Phosphoramide mustard creates the antineoplastic and cytotoxic effects of CP, and acrolein creates other toxic effects. Phosphoramide mustard acts by binding to DNA and preventing cell division, while acrolein increases the formation of free radicals and induces the toxic effects of CP.[1, 3]

Acrolein directly induces cellular oxidative stress by decreasing glutathione (GSH) levels.[4] Free radicals are found within molecules associated with enzymes, receptors and ion pumps and disrupt their functions.[4–6] Acrolein also activates multiple signalling cytotoxicity pathways, including increased lipid peroxidation, nuclear transcription factor kappa-B (NF-κB) and mitogen-activated protein kinase.[7] Therefore, these toxic effects need to be eliminated using antioxidant agents.[6, 8]

CP toxicity varies in a dose-dependent manner. A dose of 120 mg/kg for more than 2–4 days has been found to cause acute toxicity. CP causes bone marrow suppression and leads to neutropenia. The administration of high doses of CP causes cardiotoxicity and can be fatal by causing haemorrhagic necrotic myopericarditis. In addition, side effects such as hepatotoxicity, hyponatremia and cardiovascular failure can be seen, depending on the dose of CP. Bladder cancer, secondary acute leukaemia and skin cancer are common in CP therapy that lasts for more than 1 year.[1, 2] The organs that are the most vulnerable to CP toxicity are the kidneys and liver, as these organs are responsible for the metabolism and excretion of CP.[9]

Many attempts have been made to use antioxidant substances to reduce the toxic effects of CP.[5, 6] The administration of CP at high doses causes hepatotoxicity[10] because acrolein triggers endoplasmic reticulum stress and mitochondrial permeability in hepatocytes.[4] The level of

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pro-inflammatory cytokines, such as tumour necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β), increase in the hepatotoxicity model induced by CP in rats. These cytokines activate the pro-apoptotic caspase cascade and lead to the apoptosis of hepatocytes.[11] It has been reported that the administration of antioxidant substances, such as vitamin E, with CP chemotherapy can reduce oxidative stress and prevent hepatotoxicity.[6]

Until the 20th century, natural products were used to treat many diseases. With the development of modern medicine and drugs, the active chemical compounds of natural products have been synthesized to treat human and animal diseases.[12] *Allium* species have been used in daily life for many years due to their aroma and taste. *Allium* metabolites have anti-inflammatory, antioxidant, antibacterial, antifungal and antiplatelets effects.[13] In addition, many species of the *Allium* genus are used for medicinal purposes as antioxidant and for the prevention of many diseases.[14–17] *Allium* plants are 25–90 cm tall, with dark red and purple flowers and strand leaves that are 2–8 mm in diameter.[13] Pharmacologically, *Allium* species are used as an antiseptic for wound healing and as a diuretic to treat hypotension; the plant also has hepatoprotective and antitumour activities.[13, 18]

Allium scorodoprasum LINNAEUS (L.) (Amaryllidaceae family) is widely grown in Northern and Eastern Anatolia and is used in the production of cheese, yoghurt and bread.[13] Different parts of *A. scorodoprasum* L. (bulb, stem, flower) have a strong antioxidant effect due to their rich phenolics content.[19, 20] *A. scorodoprasum* L. leaves contain carotenoid, flavonoid and allicin compounds.[13] The plant is characterized by superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities and contains flavonoids, phenolic acids, vitamin C and reduced glutathione.[17, 19] In addition, *A. scorodoprasum* L. has strong ferric-reducing power and radical-scavenging capacity.[19]

The antioxidant and anti-inflammatory effects of *A. scorodoprasum* L. ethanolic extract in mice have been determined.[21] Previous research on hepatotoxicity has reported that different *Allium* species and their extracts reduce lipid peroxidation and free radicals through antioxidant effects and prevent cell death. In addition, *Allium* species contribute to the recovery of thiol groups in hepatic damage, and the structure of flavonoids, phenolics and alkaloids gives them a protective effect against oxidative damage through hepatotoxicity.[22, 23] However, no previous study has been conducted to investigate the protective efficacy of *A. scorodoprasum* L. ethanolic extract against hepatotoxicity caused by cyclophosphamide.

The aim of this study was to determine the effects of *A. scorodoprasum* L. extract (ASE), which has strong antioxidant and free oxygen radical scavenging properties, on the CP-induced hepatotoxicity mouse model. In this study, it was hypothesized that the chemical contents of ASE would prevent oxidative stress, lipid peroxidation, inflammation, endoplasmic reticulum stress and mitochondrial damage induced by CP.

Materials and Methods

Animals

The 28 male Wistar albino rats (10–12 weeks age, 200–220 g) were used in the study and were housed in Sivas Cumhuriyet University Animal Experiments Center. The research protocol was approved by the Ethics Committee of Sivas Cumhuriyet University Animal Experiments (Approved number: 323, Date: 20.05.2020). The animals were adapted to the environment and kept under standard laboratory conditions (12 h dark, 12 h light at 26–28°C) during the experiment. The feed and water requirements were permitted as *ad libitum*.

Drug and plant extraction preparation

CP was purchased ready to use from Baxter Oncology GmbH, Halle, Germany (ENDOXAN 1 g solution for i.v. infusion).

The leaves of the *A. scorodoprasum* plant were collected from the Kösedağ region (an altitude of 1400–1500 m, 40° 5′ 26″" N, 37° 59′ 7″ E) of Sivas province in April–May. The taxonomic identification of plant materials (voucher specimen number: AA 4761, 2016 years) was confirmed by a senior plant taxonomist, H. Aşkın Akpulat, in the Department of Biology, Cumhuriyet University, Sivas, Turkey.

The leaves were cut into small pieces and dried in an oven at 40°C. The powdered material (10 g) was extracted with 100 ml of an extractive solution of EtOH-H2O (25:75). All extraction protocols were performed in the amber bottle at 78°C for 119 min at a shaking speed of 180 rpm. The extract was filtered and then dried with an evaporator. After evaporation, 2 g of final material was obtained with 80% loss. The phytochemical composition of the ASE was determined by HPLC (Agilent 1200 chromatograph, Agilent Technologies, Santa Clara, CA, USA). The analysis was performed by modifying the HPLC method proposed by Aloqbi *et al*.[24] ZORBAX RR, (SB-C18, 80 Å, 3.5 µm, 4.6 × 150 mm) HPLC Column was used. The mobile phase consists of 2% acetic acid in distilled H_2O (solvent A) and 0.5% acetic acid in 50% acetonitrile in water (solvent B). The flow rate was 1 ml/min with a gradual gradient of 10%, 55%, 100%, 10% and 10% of solvent B at 0, 10, 13, 15 and 20 min, respectively. Samples were injected into the HPLC column (25°C) with 10 µl after passing through 0.2 mm PTFE filters, and peak areas were calculated from the peaks observed at 280 nm. The concentrations of ASE contents were calculated using the mean peak areas by comparing the mean peak areas with the standard mix.

Experimental design

The 28 rats were randomly divided into four groups:

Group 1 [Healthy (control, n:7)]: The saline (2.5 ml/kg, p.o.) was administered for 14 days and a single dose of sterile saline (1 ml/kg, i.p.) was given 1 h after the last administration.

Group 2 [CP (200 mg/kg) (*n*:7)]: The saline (2.5 ml/kg, p.o.) was administered for 14 days, and a single dose of 200 mg/kg CP was administered (1 ml/kg, i.p. in saline) 1 h after the last administration.[25]

Group 3 [CP (200 mg/kg) + ASE (100 mg/kg) (*n*:7)]: ASE (100 mg/kg/day, p.o.) was administered by saline (2.5 ml/kg) to rats in this group for 14 days. The single dose of CP (200 mg/kg) was administered (1 ml/kg, i.p. in saline) 1 h after the last administration.

Group 4 [CP (200 mg/kg) + ASE (200 mg/kg) (*n*:7)]: ASE (200 mg/kg/day, p.o.) was administered by saline (2.5 ml/kg) to rats in this group for 14 days. The single dose of CP (200 mg/kg) was administered (1 ml/kg, i.p. in saline) 1 h after the last administration.

All rats were anaesthetized (thiopental Na anaesthesia, 40 mg/kg, i.p.) 24 h after the last CP administration. The

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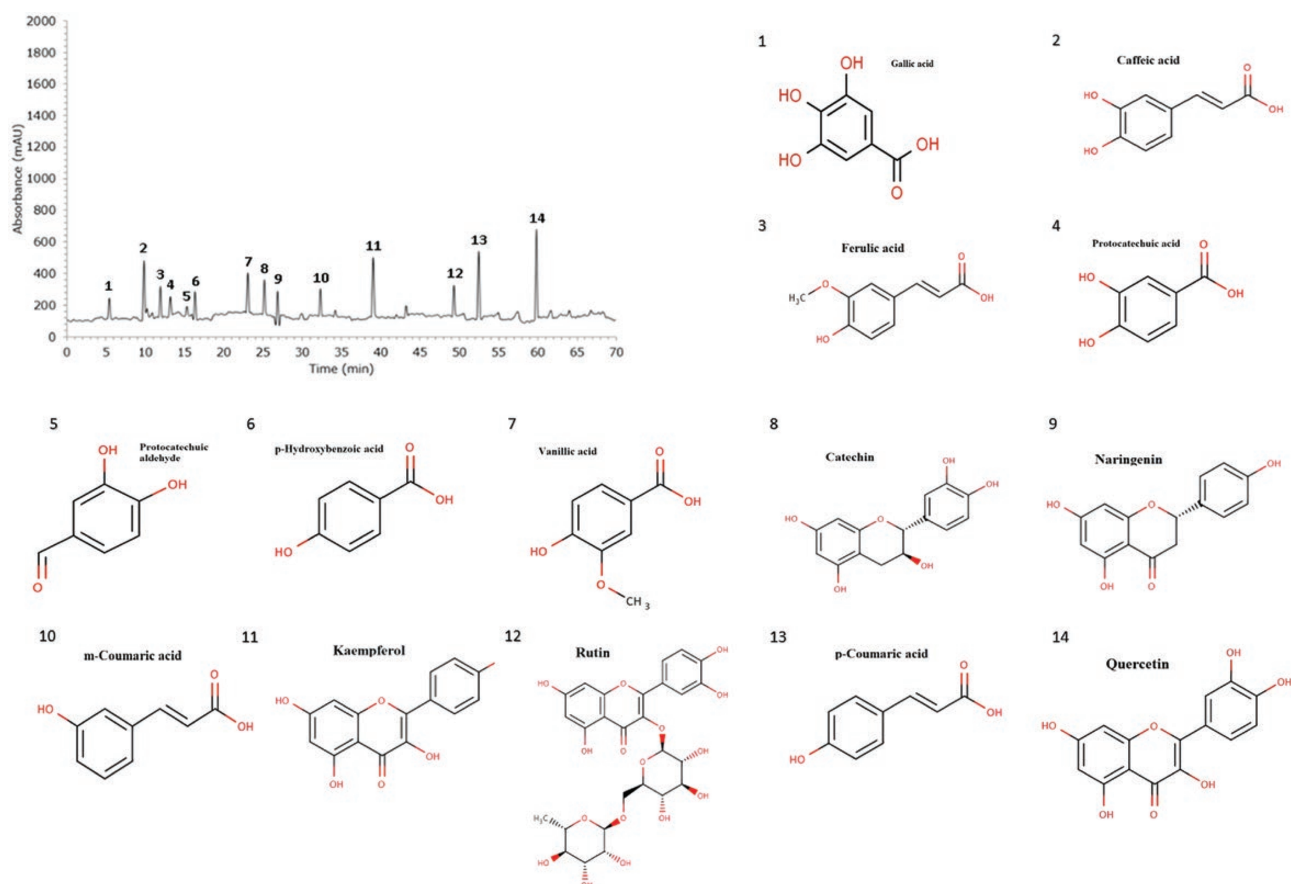

Figure 1 The phytochemical content of ASE (50 mg/ml) on HPLC analysis.

blood samples were collected from the heart. The serum and plasma were separated for analysis. Then, the animals were euthanized by decapitation and the livers were removed.

Biochemical and cytokine analyses

Serum biochemistry parameters [aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), total cholesterol, triglyceride, high-density lipoprotein (HDL), low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL)] were analysed by the autoanalyzer (Mindray BS 200, China).

The liver tissues were rinsed with phosphate-buffered saline (PBS) and homogenized in PBS (final concentration 80–100 mg protein/ml). The malondialdehyde (MDA) (Rat MDA ELISA kit, Cat no: SH0020, Bioassay Technology Laboratory, Shangai, China), TNF-α (Rat TNF-α ELISA kit, Cat no: E0764Ra, Bioassay Technology Laboratory, Shangai, China), IL-1β (Rat IL-1β ELISA kit, Cat no: E0119Ra, Bioassay Technology Laboratory, Shangai, China and IL-6 (Rat IL-6 ELISA kit, Cat no: E0135Ra, Bioassay Technology Laboratory, Shangai, China) in the liver samples were determined following to manufacturer's protocol by the ELISA reader (Bio-Tek Instruments Inc., MWGt Lambda Scan 200).

Histopathological and immunohistochemical analysis

The liver tissues were fixed in a 10% neutral formalin solution for 24–48 h. They were then washed under running water for 8 h to remove the formalin in the tissue. The tissues were placed in paraffin blocks after routine alcohol-xylol follow-up procedures. The paraffin blocks were cut to obtain 5-µm-thick sections on glass slides coated with a 10% polylysine solution and stained with hematoxylin-eosin. Histopathological changes in the tissues were evaluated under light microscopy as either 'no changes' (0), 'mild changes' (1), 'moderate changes' (2) or 'severe changes' (3).

Liver pathological status	Score	Definition
Post necrotic cell infiltration	0	No
	1	Less than half the around of the vena centralis
	2	The half around the vena centralis
	3	All around the vena centralis
Hepatocelluar necrosis in the vena centralis and around	0	No
	1	Less than half the around of the vena centralis
	2	The half around the vena centralis
	3	All around the vena centralis

The sections on slides with polylysine were deparaffinated in a xylol and alcohol series and washed with PBS. Afterwards, the tissues were kept for 10 min in 3% H_2O_2 for endogenous peroxidase inactivation. The tissues were treated with antigen retrieval solution for 2 × 5 min at 500 watts. The liver samples were washed with PBS and then incubated in

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Table 1 Effect of *Allium scorodoprasum* L. ethanolic extract (100 and 200 mg/ kg, p.o.) treatment on biochemical parameters in cyclophosphamide-induced hepatotoxicity model in rats (mean ± SD)

Serum	AST (U/L)	ALT (U/L)	LDH (U/L)	Total cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)
Control	69.14 ± 6.20^{c}	28.28 ± 4.53^{b}	32.01 ± 3.01^{c}	53.92 ± 1.65^{b}	45.28 ± 6.05^{b}	52.66 ± 10.55^{a}	21.28 ± 6.45^{b}	9.06 ± 1.21^{b}
CP	142.71 ± 9.41^{a}	69.57 ± 5.29^{a}	65.28 ± 2.14^{a}	81.10 ± 11.94^{a}	62.30 ± 3.83^{a}	33.11 ± 1.74^{b}	38.83 ± 5.40^{a}	12.70 ± 1.09^{a}
CP + ASE100	123.71 ± 12.71^{b}	54.14 ± 5.78^{b}	49.44 ± 7.72^{b}	63.36 ± 7.53^{b}	51.40 ± 3.64^{ab}	51.55 ± 11.47^{ab}	21.63 ± 5.84^{b}	10.32 ± 0.80^{ab}
CP + ASE200	114.85 ± 7.86^{b}	36.85 ± 2.11^{c}	44.43 ± 5.85^{b}	61.22 ± 4.07^{b}	49.78 ± 8.77^{b}	63.27 ± 11.14^{a}	22.61 ± 2.96^{b}	9.96 ± 1.75^{b}
df (between groups)	3	3	3	3	3	3	3	3
F	109.38	77.88	49.45	12.16	7.38	8.48	12.75	7.58
P	0.00	0.00	0.00	0.00	0.03	0.01	0.00	0.02

a, b, c: The different letters in the same column are statistically significant ($P < 0.05$). CP: cyclophosphamide, ASE: *Allium scorodoprasum* extract, df: degrees of freedom, AST: aspartate aminotransferase, ALT: alanine aminotransferase, LDH: lactate dehydrogenase, HDL: high-density cholesterol, LDL: low-density cholesterol, VLDL: very low-density lipoprotein.

8-hydroxy-2′-deoxyguanosine (8-OHdG, cat. no. Sc66036, dilution 1/200; Santa Cruz), anti-4-hydroxynenal antibody (4-HNE, cat. no. ab46545, dilution 1:200; Abcam) and anti-dityrosine (diTYR, cat. no. MDT-020P, dilution 1:200; JaICA) primary antibody at room temperature for 30 min. The samples were incubated with biotin-conjugated secondary antibodies (Large Volume Detection System: anti-polyvalent, HRP [Thermo Fisher Scientific Inc., cat. no.TP-125-HL]). The samples were incubated with the chromogenic substrate diaminobenzidine (3,3′-diaminobenzidine). The samples were counterstained with Mayer's haematoxylin and then covered with entellan. The samples were analysed under a light microscope. Immunoreactivity was graded in liver tissues as 'none' (0), 'mild' (1), 'moderate' (2) or 'severe' (3).

Statistical analysis

The data were analysed on SPSS 25.0 (SPSS, Inc., Chicago, IL, USA) software. The biochemical data were evaluated as mean ± standard deviation and histopathological and immunohistochemical data were evaluated as median [interquartile range (IQR)]. Statistical significance between groups was tested using one-way ANOVA followed by a posthoc Scheffe test for normally distributed parameters (parametrics parameters) and using the Kruskal–Wallis and post hoc Mann–Whitney U test for non-parametrics (histopathological and immunohistochemical data). The $P < 0.05$ was statistically significant.

Results

The phytochemical content of ASE (50 mg/ml) after HPLC analysis is presented in Figure 1. ASE contains phenolic acids such as gallic acid, caffeic acid, ferulic acid, protocatechuic acid, *p*-hydroxybenzoic acid, *m*-coumaric acid, vanillic acid, as well as flavonoids such as catechin, naringenin, *p*-coumaric acid, kaempferol and rutin has a high content of natural flavonoids.

The changes in serum biochemical parameters, liver MDA and cytokine parameters after ASE treatment (100 and 200 mg/kg, p.o.) in CP-induced hepatotoxicity rat models are provided in Tables 1 and 2, respectively. The pathological changes in the liver are illustrated in Figure 2, and the immunohistochemical changes in the liver are illustrated in Figures 3–5 after CP-induced hepatotoxicity.

Although ALT, AST, LDH, total cholesterol, triglyceride, LDL and VLDL levels in the CP group statistically increased, the HDL value statistically decreased compared to the control group. ALT, AST and LDH levels in the ASE (100 and 200 mg/kg) groups were statistically significantly decreased compared to the CP group, while these values were statistically higher than those of the control group ($P < 0.05$). Total cholesterol, triglyceride, HDL, LDL and VLDL levels in the CP + ASE100 and CP + ASE200 groups were statistically lower compared to the levels of the CP group ($P < 0.05$) and were similar to the levels of the control group (Table 1).

MDA, TNF, IL-1β and IL-6 levels were statistically higher in the CP group than in the control group, and the levels of these parameters were statistically lower in the CP + ASE100 and CP + ASE200 groups compared to the CP group ($P < 0.05$, Table 2).

A statistically significant difference was found between the groups in the histopathological examination of the liver

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Table 2 Effect of *Allium scorodoprasum* L. ethanolic extract (100 and 200 mg/kg, p.o.) treatment on MDA and cytokines in cyclophosphamide-induced hepatotoxicity model in rats (mean ± SD)

Liver	MDA (nmol/ mg protein)	TNF-α (ng/ mg protein)	IL-1β (ng/ mg protein)	IL-6 (pg/ mg protein)
Control	62.33 ± 5.64[d]	34.88 ± 8.54[c]	22.53 ± 4.38[d]	63.02 ± 5.89[d]
CP	120.34 ± 13.1[a]	93.99 ± 12.3[a]	46.15 ± 6.28[a]	115.19 ± 9.16[a]
CP + ASE100	104.52 ± 10.2[b]	74.07 ± 6.56[b]	38.24 ± 4.45[b]	90.89 ± 6.25[b]
CP + ASE200	89.38 ± 5.89[c]	69.76 ± 9.80[b]	30.19 ± 3.21[c]	79.42 ± 4.13[c]
df (between groups)	3	3	3	3
F	49.80	46.60	32.76	77.06
P	0.00	0.00	0.00	0.00

a, b, c, d: The different letters in the same column are statistically significant ($P < 0.05$). CP: cyclophosphamide, ASE: *Allium scorodoprasum* extract, df: degrees of freedom, MDA: malondialdehyde, TNF-α: tTumour necrosis factor-alpha, IL-1β: interleukin 1 beta, IL-6: interleukin 6.

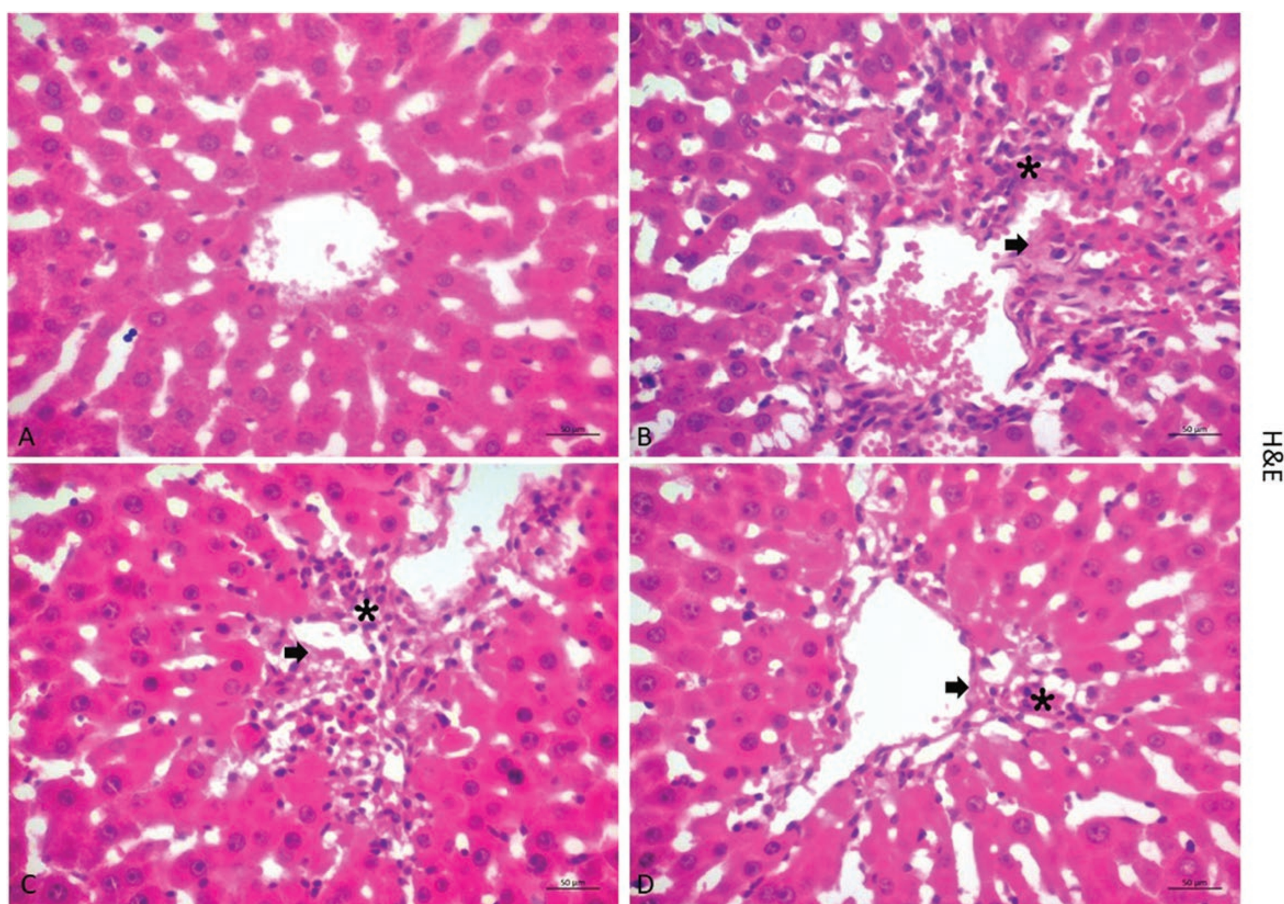

Figure 2 Liver sections stained with haematoxylin-eosin (H&E). (A) Control group, normal histological appearance, (B) CP group, severely necrotic area (*) and severe post-necrotic cell infiltrates (→), (C) CP + ASE100 group, moderately necrotic area (*) and moderate post-necrotic cell infiltrates (→), (D) CP + ASE200 group, mild necrotic area (*) and mild post-necrotic cell infiltrates (→). Liver – H&E. x40.

tissues. No pathology was detected in the control group. In the CP group, severe multifocal necrotic areas were observed. In addition, these areas were characterized by post-necrotic cell infiltrations, and disorganizations were observed in the hepatic cords. These histopathological changes were moderate in the CP + ASE100 group and mild in the CP + ASE200 group (Figure 2). The amount of liver necrosis and post-necrotic cell infiltration was statistically higher in the CP group than in the control group, while it was statistically lower in the treatment groups (ASE100 and ASE200) than in the CP group ($P < 0.05$). As a result, ASE200 treatment was observed to prevent liver necrosis quite well ($P < 0.01$, Table 3).

The immunohistochemical detection of liver 8-OHdG, 4-HNE and diTYR showed no reaction in the control group. While 8-OHdG, 4-HNE and diTYR severely increased in the CP group, moderate immunopositivity was detected in the CP + ASE100 group and mild immunopositivity was detected in the CP + ASE200 group after CP administration (Figures 3–5). The CP group was statistically different from the control group, and the treatment groups (ASE100 and ASE200)

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were statistically different from the CP group in terms of liver 8-OHdG, 4-HNE and diTYR ($P < 0.05$, Table 4).

Discussion

CP is an alkylating agent used in cancer chemotherapy, although CP's active metabolite, acrolein, has a toxic effect. Its toxic effects are based on lipid peroxidation and inflammation.[26] CP causes the development of hepatotoxicity due to excessive reactive oxygen species (ROS) production as a result of lipid peroxidation.[27] *Allium scorodoprasum* L. has strong antioxidant and anti-inflammatory effects due to its phenolic and flavonoid content, including quercetin, protocatechuic acid, coumaric acid, hydroxybenzoic acid and rutin (Table 1).[28] The contents of ASE have a hepatoprotective effect by reducing lipid peroxidation, hepatocyte swelling, leukocyte infiltration and necrosis and by renewing the activities of antioxidant enzymes and inhibiting apoptosis.[29–32] CP and acrolein increase lipid peroxidation (MDA levels) and ALT, AST and LDH levels, while decreasing the level of SOD and GSH through their toxic effects.[6, 33, 34] In this study, the lipid peroxidation, inflammation and apoptosis inhibitory effects of antioxidant and anti-inflammatory substances such as quercetin, caffeic acid, protocatechuic acid, hydroxybenzoic acid and naringenin may have prevented CP-induced liver damage dose-dependently. The protective effects of ASE may have decreased serum ALT, AST, LDH and MDA levels.

CP increases serum cholesterol, triglyceride, LDL and VLDL levels by increasing cholesterol biosynthesis and the peroxidation of unsaturated membrane lipids, causing these lipids to leak into the circulation.[35] Quercetin has a regulatory effect on lipid metabolism-related gene expression in the liver. It reduces the biosynthesis of hepatic fatty acids and triglycerides through a regulatory effect.[35] Gallic acid exerts antihyperlipidemic effects by inducing adipocyte differentiation through peroxisome proliferator-activated receptor gamma activation.[36] Protocatechuic acid has a lipid-lowering effect in liver injury because it decreases LDL, VLDL and triglyceride levels and increases HDL levels.[37] In addition, protocatechuic acid decreases ALT, AST and cholesterol levels, while it increases HDL levels in the damaged liver.[38] Naringenin reduces hyperglycaemia and hyperlipidaemia and has a liver-protective effect through the antioxidant effect.[39] In the present study, treatment with ASE may have caused reductions in the levels of LDL, VLDL, triglyceride and total cholesterol and increased HDL levels through the antioxidant and antihyperlipidaemic effects of the contents of ASE.

CP increases ROS formation and lipid peroxidation and leads to DNA damage and cellular dysfunction. Moreover, it disrupts the pro-oxidant–antioxidant balance. CP causes oxidative damage in vital organs by decreasing the levels of antioxidant enzymes such as GSH and SOD, GPx, glutathione reductase and CAT and increases MDA levels.[40, 41] In addition, CP induces liver and serum TNF-α synthesis and

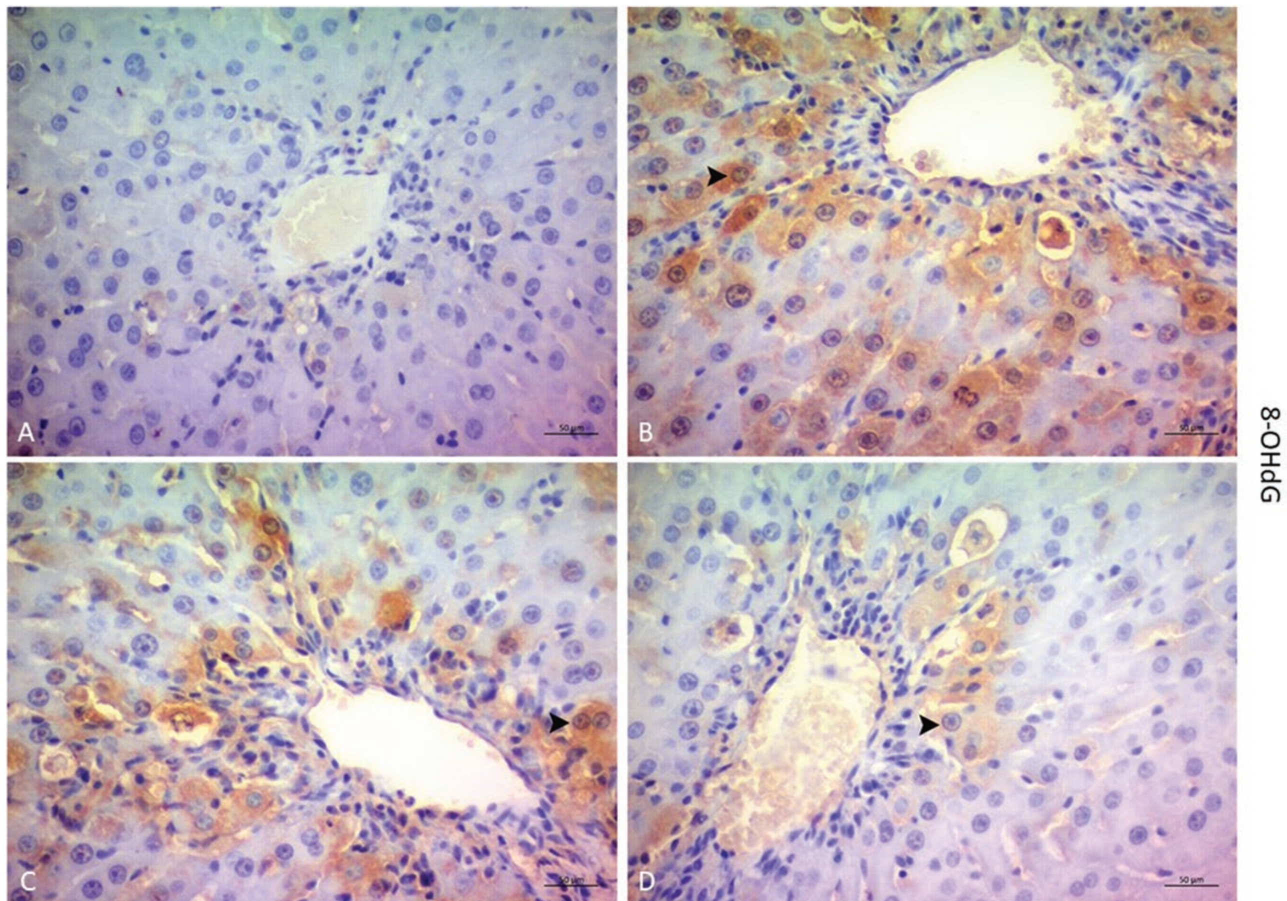

Figure 3 The liver 8-OHdG immunohistochemistry. (A) Control group, (B) severe positive reaction for 8-OhDG in CP group (►), (C) moderate positive reaction for 8-OhDG in CP + ASE100 group (►), (D) mild positive reaction for 8-OhDG in CP + ASE200 group (►). Liver – IHCx40.

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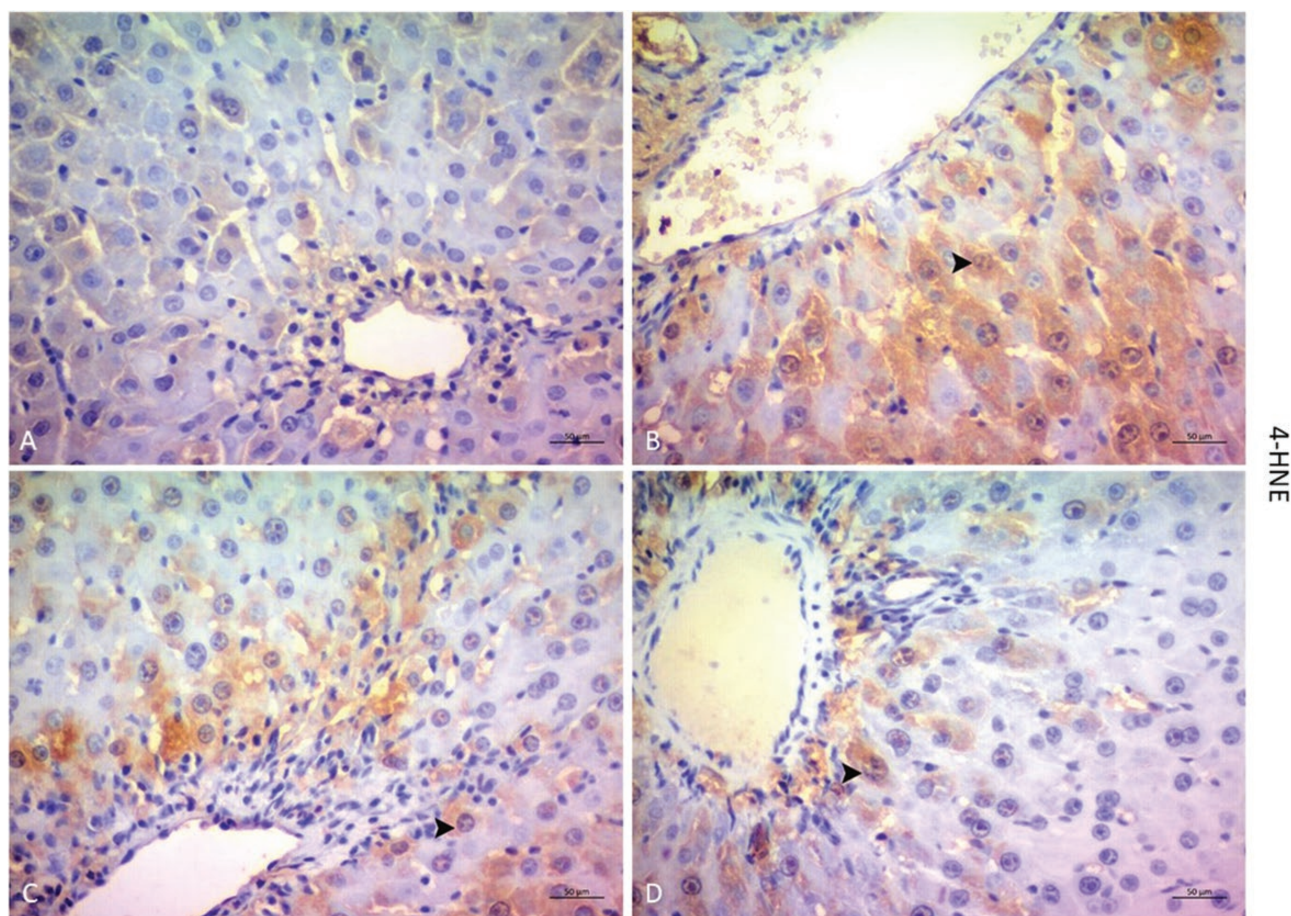

Figure 4 The liver 4-HNE immunohistochemistry. (A) Control group, (B) severe positive reaction for 4-HNE in CP group (►), (C) moderate positive reaction for 4-HNE in CP + ASE100 group (►), (D) mild positive reaction for 4-HNE in CP + ASE200 group (►). Liver – IHCx40.

related nitric oxide release. Nitric oxide triggers the release of superoxide radicals and causes an increase in MDA.[42] CP causes liver toxicity by inducing NF-κB, which results in the overproduction of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α). As a result, liver necrosis develops.[40, 41] Naringenin in ASE reduces inflammation and oxidative stress by suppressing the transcription of pro-inflammatory cytokine genes.[43, 44] In addition, naringenin is reported to suppress NO, NF-κB and pro-inflammatory cytokine production. Previous studies have found that naringenin is cytoprotective and hepatoprotective through its anti-inflammatory and antioxidant properties.[45] Protocatechuic acid in ASE plays an anti-inflammatory and antioxidant role in the liver by modulating the NF-κB/COX-2 pathway.[38] Moreover, ASE at doses of 400 and 600 mg/kg has suppressed proinflammatory cytokines (IL-6, TNF-a and IL-1B) and MDA levels and increased total antioxidant capacity in mice with paw oedema.[21] In this study, CP-induced inflammation and oxidative stress may have caused liver toxicity. The antioxidant and anti-inflammatory effects of the substances in ASE may have decreased the levels of MDA, TNF-α, IL-1β and IL-6 in a dose-dependent manner.

Histopathologically, CP causes liver tissue damage and inflammatory cell infiltration, which is also reflected in serum biochemistry. Previous studies have reported that CP causes mild fatty changes and necrosis in the liver.[46] Apoptotic and necrotic hepatocytes and neutrophil infiltration have been noted in CP-treated rats.[47] CP has led to leukocyte infiltration and adiposity through oxidative stress, inflammation and DNA damage in the liver.[48] In the previous study, it was shown that ASE reduced the MDA and total oxidant status levels and ALT, AST, alkaline phosphatase in the liver and improved liver damage in mice.[21] Therefore, ASE may have prevented liver necrosis and post-necrotic infiltration, through its anti-inflammatory, antioxidant and hepatoprotective effects in this study. It may also have prevented DNA and liver damage because it suppressed pro-inflammatory cytokines (TNF-a, IL-1β and IL-6) and DNA oxidation (8-OHdG), especially at high doses (ASE, 200 mg/kg).

CP induces ROS, and ROS induces hepatocyte apoptosis and DNA damage.[11,49] As a result, CP increases the expression of liver 8-OHdG, which is an oxidized nucleoside of DNA, and the levels of 4-HNE.[50] Increased 8-OHdG can cause base modifications and strand breaks in DNA. Naringenin reduces oxidative DNA damage by preventing apoptosis, autophagy, inflammation and oxidative stress.[11] diTYR is a protein oxidation marker that can be produced by ROS. Although its level has never been determined in CP studies, it increases in cases of liver damage.[51] Quercetin prevents the formation of diTYR by inhibiting myeloperoxidase in a dose-dependent manner.[52] Rutin is a powerful antioxidant that prevents protein oxidation, especially diTYR.[53] As a result, antioxidant substances such as quercetin, rutin and naringenin in ASE may inhibit DNA and protein oxidation

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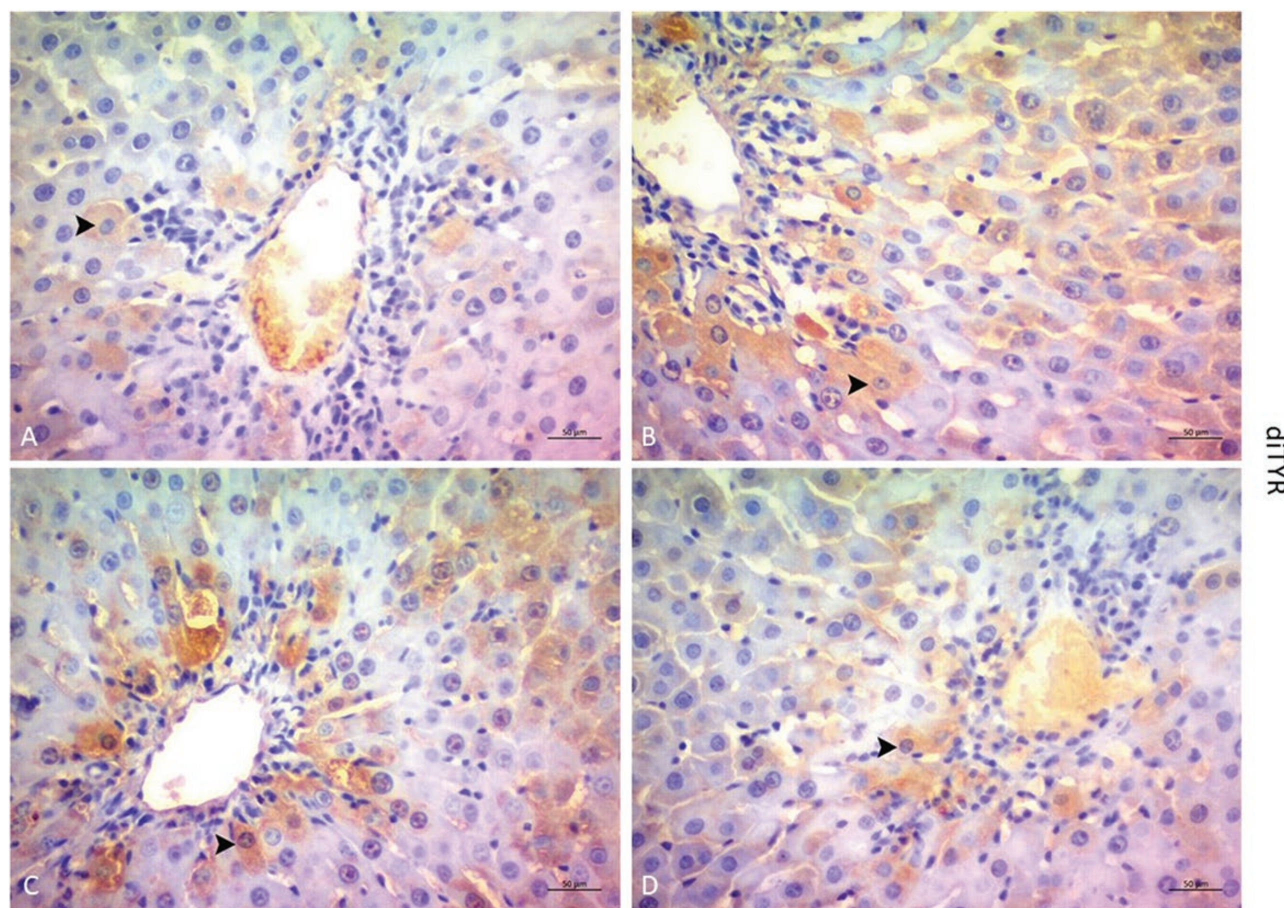

Figure 5 The liver diTYR immunohistochemistry. (A) Control group, (B) severe positive reaction for diTYR in CP group (►), (C) moderate positive reaction for diTYR in CP + ASE100 group (►), (D) mild positive reaction for diTYR in CP + ASE200 group (►). Liver – IHCx40.

Table 3 Effect of *Allium scorodoprasum* L. ethanolic extract (100 and 200 mg/kg, p.o.) treatment on necrosis and post-necrotic cell infiltration in liver tissue in cyclophosphamide-induced hepatotoxicity model in rats [median (interquartile range)]

Groups	Necrosis	Post-necrotic cell infiltration
Control	0.00(1)	0.00(1)
CP	3.00(0.25)t	3.00(0.13)t
CP + ASE100	2.00(0.25)*	2.00(0.25)#
CP + ASE200	1.00(0.25)#	1.00(0.25)#
df (between groups)	3	3
P	0.00	0.00

CP: scyclophosphamide, ASE: *Allium scorodoprasum* extract, df: degrees of freedom.
t denotes significant difference vs. healthy control group at $P < 0.05$.
* denotes significant difference vs. CP group at $P < 0.05$.
# denotes significant difference vs. CP group at $P < 0.01$.

Table 4 Effect of *Allium scorodoprasum* L. ethanolic extract (100 and 200 mg/kg, p.o.) treatment on 8-OHdG, 4-HNE and diTYR as immunohistochemical in liver tissue in cyclophosphamide-induced hepatotoxicity model in rats [median (interquartile range)]

Groups	8-OHdG	4-HNE	diTYR
Control	0.00(1)	0.50(1)	0.00(1)
CP	3.00(0.25)*t*	3.00(0.25)*t*	3.00(0.25)*t*
CP + ASE100	2.00(1) #	2.00(1)#	2.00(1)#
CP + ASE200	1.00(1) #	1.00(1)#	1.00(1)#
df (between groups)	3	3	3
P	0.00	0.00	0.00

CP: cyclophosphamide, ASE: *Allium scorodoprasum* extract, df: degrees of freedom.
t denotes significant difference vs. healthy control group at $P < 0.05$.
# denotes significant difference vs. CP group at $P < 0.05$.

by preventing ROS formation. Furthermore, ASE treatment may have reduced the levels of 8-OHdG, 4-HNE and diTYR induced by CP.

Finally, CP is converted to its active metabolites through CYP2A6, 2B6, 3A4, 3A5, 2C9, 2C18 and 2C19[54]; however, naringenin enantiomers inhibit CYP2C19 and CYP3A.[55] As a result, naringenin in ASE may have reduced the toxic effects of CP by inhibiting CP metabolism.

Conclusion

The chemical content of ASE has strong antioxidant and anti-inflammatory effects. Substances such as quercetin, naringenin and protocatechuic acid may prevent the degeneration of hepatocytes by interfering with mitochondrial stress, endoplasmic reticulum stress and lipid peroxidation. In this way, these substances can be beneficial in preventing liver toxicity. However, increasing the dose and duration of use of ASE

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may reduce oxidative stress, inflammation and liver damage more effectively.

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The draw structures of the phenolic compounds were created by using the Exposome-Explorer database (http://exposome-explorer.iarc.fr) and validated by PubChem which is an open chemistry database at the National Institutes of Health (NIH) (https://pubchem.ncbi.nlm.nih.gov).

Author Contributions

HG, ME and BD conceived and designed the research. HG and ME conducted experiments and OK conducted pathological analyse. HG and BD analysed data. BD wrote the manuscript. All authors read and approved the manuscript.

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Conflict of Interest

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report.

Data Availability Statement

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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