# Protective effect of Allium Scorodoprasum L. ethanolic extract in cyclophosphamide- induced hepatotoxicity model in rats Short Title: Allium Scorodoprasum L. extract can be protective in hepatotoxicity

**Hüseyin GUNGOR**
Sivas Cumhuriyet University
**Mehmet EKICI**
Sivas Cumhuriyet University
**Ozhan KARATAS**
Sivas Cumhuriyet University
**Burak DiK** ( burakdik@selcuk.edu.tr )
Selcuk University

**Research Article**

**Keywords:** Cyclophosphamide, Allium scorodoprasum extract, liver toxicity, oxidative stress, inflammation

**Posted Date:** May 20th, 2022

**DOI:** https://doi.org/10.21203/rs.3.rs-1664682/v1

# Abstract

**Purpose:** Cyclophosphamide(CP) is a chemotherapeutic agent and immunosuppressant drug however it damages the liver. The aim of this study is to investigate the protective effect of ethanolic extract of Allium scorodoprasum(ASE) on CP-induced liver injury.

**Methods:** A total of 28 Wistar albino rats were randomly divided into four groups (n=7/group): Healthy, CP(200 mg/kg), CP(200 mg/kg)+ASE(100 mg/kg), CP(200 mg/kg)+ASE(200mg/kg). ASE was administered for 14 days, and the rats were euthanized 24 hours after CP administration.

**Results:** CP Treatment leads to an increase in serum levels ALT, AST, LDH, total cholesterol, triglycerides, LDL, VLDL and liver levels MDA, TNF, IL-1β and IL-6, while HDL levels decrease, statistically. Treatment with CP caused liver necrosis and postnecrotic cell infiltration; however, the pathologic changes were prevented by ASE. Immunohistochemically, 8-OhDG, 4-HNE, and diTYR levels increased in the treatment with CP, whereas they decreased in the groups treated with ASE. All these changes were dose dependent in the ASE-treated groups.

**Conclusion:** The treatment with CP 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 more beneficial to use ASE in higher doses or for a longer period of time.


# Introduction

Cyclophosphamide (CP) is one of the most effective and widely used immunosuppressant and anticancer chemotherapeutic drugs (Emadi et al. 2009). It is often used in the treatment of multiple rheumatic diseases and cancers such as breast, lymphoid and pediatric malignancies (Emadi et al. 2009, Teles et al. 2017). CP is a prodrug, and it is converted to active metabolites by the P450 enzyme system in the liver. In addition, it can be autoinducer 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 its other toxic effects. Phosphoramide mustard acts by binding to DNA and preventing cell division, while acrolein increases the formation of free radicals and induces toxic effects of CP (Roy et al. 1999, Emadi et al. 2009).

Acrolein directly induces cellular oxidative stress by decreasing glutathione (GSH) levels (Mohammad et al. 2012). The free radicals are interstrand with molecules such as enzymes, receptors, and ion pumps and disrupt their functions (Senthilkumar et al. 2006, Mohammad et al. 2012, Cuce et al. 2015). It also activates multiple signaling cytotoxicity pathways including increased lipid peroxidation, nuclear transcription factor kappa-B (NF-κB), and mitogen-activated protein kinase (MAPKs) (Kim et al. 2007). Therefore, these toxic effects should be eliminated by using some antioxidant agents (Basu et al. 2015, Cuce et al. 2015).

CP toxicity varies in a dose-dependent manner. The CP treatment at a dose of 120 mg/kg for more than 2–4 days has been stated 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 hemorrhagic necrotic myopericarditis. In addition, side effects such as hepatotoxicity, hemorrhagic cystitis, hyponatremia, squamous-cell carcinoma, gonadal toxicity (amenorrhea), nausea, and vomiting can be seen depending on the dose of CP. Bladder cancer, secondary acute leukemia, and skin cancer are common in CP therapy for more than 1 year (Emadi et al. 2009, Teles et al. 2017). The most important organs for CP toxicity are the kidney and liver, as these organs are responsible for metabolism and excretion (Zhai et al. 2018).

Many antioxidant substances have tried to reduce these toxic effects of CP (Senthilkumar et al. 2006, Cuce et al. 2015). The administration of CP at high doses causes hepatotoxicity (Subramaniam et al. 2013) because acrolein triggers endoplasmic reticulum stress, and mitochondrial permeability in hepatocytes (Mohammad et al. 2012). The level of pro-inflammatory cytokines such as tumor 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 apoptosis of hepatocytes (Caglayan et al. 2018). It has been reported that the administration of antioxidant substances such as Vit E with CP chemotherapy can reduce oxidative stress and prevent hepatotoxicity (Cuce et al. 2015).

Until the 20th century, natural products have been used to treat many diseases, but the chemical compounds were synthesized to treat human and animal disease*s* with the discovery of modern medicine and drugs (Newman and Cragg 2016). *Allium* species have been used in daily life for many years due to their flavor, aroma, and taste. In addition, many species of the *Allium* family are frequently used for medicinal purposes as a high antioxidant and for the prevention of many diseases (Štajner et al. 2006, Nencini et al. 2010, Fallah et al. 2017, Kumar et al. 2017). One of the strongest antioxidant activities among Allium species is *Allium scorodoprasum* L. It has a high amount of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities, and contents of flavonoids, phenolic, vitamin C, and reduced glutathione (Štajner et al. 2006, Stojanovic 2014). Furthermore, *Allium scorodoprasum* has high ferric reducing power and radical scavenging capacity (Stojanovic 2014). *A. scorodoprosum* has hepatoprotective, diuretic and antitumor activities (Tasci and Koca 2015).

The aim of this study was to determine the effects of extract of *Allium scorodoprasum* (ASE), which has strong antioxidant and free oxygen radical scavenging properties, on the CP-induced hepatotoxicity model.

## Materials And Methods

# Animals

The 28 male Wistar albino rats (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). 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 IV infusion).

The leaves of the *Allium scorodoprasum* plant were collected from Kosedag region of Sivas province in April-May. It was cut into small pieces and dried in an oven at 40°C. The powdered material was extracted with 100 ml of extraction solution (25% EtOH: $H_2O$). All extraction protocols were performed in the amber bottle at 78°C for 119 minutes at a shaking speed of 180 rpm. The extract was filtered and then dried with an evaporator. 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. (2016). 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, po) was administered for 14 days and a single dose of sterile saline (1 ml/kg, i.p.) was given 1 hour after the last administration.

Group 2 [CP (200 mg/kg) (n:7)]: The saline (2,5 ml/kg, po) was administered for 14 days, and a single dose of 200 mg/kg CP was administered (1 ml/kg, i.p. in saline) 1 hour after the last administration (Oyagbemi et al. 2016).

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 hour after the last administration.

Group 4 [CP (200 mg/kg) + ASE (200mg/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 hour after the last administration.

All rats were anesthetized (thiopental Na anesthesia, 40 mg/kg, i.p.) 24 hours after the last CP administration. The 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 Analyzes

Serum biochemistry parameters (AST, ALT, LDH, Total Cholesterol, Triglyceride, HDL, LDL, VLDL) were analyzed 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 taken were fixed in 10% neutral formalin solution for 24–48 hours. They washed under running water for 8 hours to remove the formalin in the tissue. The tissues were taken into 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% poly-lysine solution and were stained with hematoxylin-eosin. Histopathological changes in the tissues were evaluated under light microscopy as no (0), mild (1), moderate (2) and severe (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 poly-lysine were deparaffinated in xylol and alcohol series and washed with PBS. Afterwards, the tissues were kept on hold in 10 minutes in 3% $H_2O_2$ for endogenous peroxidase inactivation. The tissues were treated with antigen retrieval solution for 2x5 minutes at 500 watts. The liver samples were washed with PBS and then they were incubated 8-Hydroxy-2'-deoxyguanosine (8-OhDG, cat. no. Sc66036, dilution 1/200; Santa Cruz), Anti-4 Hydroxynenal antibodies (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 antibody [Large Volume Detection System: anti-Polyvalent, HRP (Thermofischer, Catalog no: TP-125-HL)]. The samples were incubated with the chromogenic substrate diaminobenzidine (DAB, 3,3'-Diaminobenzidine). The samples were counterstained with Mayer's haematoxylin and they were covered

with entellan. The samples were analyzed under a light microscope. Immunoreactivity was graded in liver tissues as none (0), mild (1), moderate (2), and severe (3).

# Statistical Analysis

The data were analyzed on SPSS 25.0 (SPSS, Inc., Chicago, IL, USA) software, and hematological and biochemical values were evaluated as mean ± standard error of the mean (SEM). The values were statistically analyzed using one-way ANOVA followed by a posthoc Scheffe test. The statistical significance between the groups for histopathological and immunohistochemical data was determined by the Kruskal Wallis test and the Mann Whitney U test ($p < 0.05$). The $p < 0.05$ was statistically significant.

# Results

The phytochemical content of ASE (50 mg/mL) after HPLC analysis is presented in Fig. 1. The changes in serum biochemical and liver MDA, cytokines parameters after ASE (100 and 200 mg/kg, p.o.) treatment in CP-induced hepatotoxicity model in rats are illustrated in Table 1 and Table 2, respectively. The pathological changes in the liver are illustrated in Fig. 2 and immunohistochemical changes in the liver are illustrated in Figs. 3, 4, and 5 after the CP-induced hepatotoxicity model in rats.

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 ± SEM).

| 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 ± 2,34[c] | 28,28 ± 1,71 d | 32,01 ± 1,14 c | 53.92 ± 0.74 [b] | 45.28 ± 2.71 [b] | 52.66 ± 4.72 [a] | 21.28 ± 2.88 [b] | 9.06 ± 0.54 [b] |
| **CP** | 142,71 ± 3,55[a] | 69,57 ± 1,99 a | 65,28 ± 0,80 a | 81.10 ± 5.34 [a] | 62.30 ± 1.71 [a] | 33.11 ± 0.78 [b] | 38.83 ± 2.42 [a] | 12.70 ± 0.50 [a] |
| **CP + ASE100** | 123,71 ± 4,80 b | 54,14 ± 2,18 b | 49,44 ± 2,91 b | 63.36 ± 3.37 [b] | 51.40 ± 1.62 [ab] | 51.55 ± 5.13 ab | 21.63 ± 2.62 [b] | 10.32 ± 0.36 ab |
| **CP + ASE200** | 114,85 ± 2,97 b | 36,85 ± 0,79 c | 44,43 ± 2,21 b | 61.22 ± 1.82 [b] | 49.78 ± 3.92 [b] | 63.27 ± 4.98 [a] | 22.61 ± 1.32 [b] | 9.96 ± 0.78 [b] |

a, b, c: The different letters in the same column are statistically significant (*p < 0.05*). CP: Cyclophosphamide, ASE: *Allium scorodoprasum* extract.

AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, LDH: Lactate dehydrogenase, HDL: High density cholesterol, LDL: Low density cholesterol, VLDL: Very low density lipoprotein

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 ± SEM).

| Liver | MDA | TNF-α | IL-1β | IL-6 |
|---|---|---|---|---|
| Control | 62,33 ± 2,13 [d] | 34,88 ± 3,22 [c] | 22,53 ± 1,65 [d] | 63,02 ± 2,22 [d] |
| CP | 120,34 ± 4,96 [a] | 93,99 ± 4,64 [a] | 46,15 ± 2,37 [a] | 115,19 ± 3,46 [a] |
| CP + ASE100 | 104,52 ± 3,84 [b] | 74,07 ± 2,47 [b] | 38,24 ± 1,68 [b] | 90,89 ± 2,36 [b] |
| CP + ASE200 | 89,38 ± 2,22 [c] | 69,76 ± 3,70 [b] | 30,19 ± 1,21 [c] | 79,42 ± 1,56 [c] |

a, b, c, d: The different letters in the same column are statistically significant ($p < 0.05$). CP: Cyclophosphamide, ASE: *Allium scorodoprasum* extract.

MDA: Malondialdehyde, TNF-α: Tumor necrosis factor-alpha, IL-1β: Interleukin 1 beta, IL-6: Interleukin 6

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 they were statistically higher than the control group ($p < 0.05$). Total cholesterol, triglyceride, HDL, LDL, and VLDL levels in the CP + ASE100 and CP + ASE200 groups statistically decreased compared to the CP group ($p < 0.05$) and they were similar to 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 liver tissues. No pathological finding was found in the control group. The severe multifocal necrotic areas were observed in the CP group. In addition, these areas have post-necrotic cell infiltrations and disorganizations were observed in hepatic cords. These histopathological changes were moderate in the CP + ASE100 group and were low in the CP + ASE200 group (Fig. 2). The liver necrosis and post-necrotic cell infiltration were statistically higher in the CP group than in the control group, while it was statistically less 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).

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 [mean(median) ± SEM].

| Groups | Necrosis | Post-necrotic cell infiltration |
|---|---|---|
| **Control** | 0,33 (0,00) ± 0,21 | 0,33 (0,00) ± 0,21 |
| **CP** | 2,91 (3,00) ± 0,08 t | 2,83 (3,00) ± 0,17 t |
| **CP + ASE100** | 2,16 (2,00) ± 0,16 * | 1,83 (2,00) ± 0,17 # |
| **CP + ASE200** | 1,16 (1,00) ± 0,16 # | 0,83 (1,00) ± 0,17 # |
| CP: Cyclophosphamide, ASE: *Allium scorodoprasum* extract. | | |
| 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 $P < 0.01$. | | |

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 in the CP + ASE200 group after CP administration (Fig. 3, 4, 5). The CP group was statistically different from the control group and the treatment groups (ASE100 and ASE200) were statistically different from the CP group in the liver 8-OhDG, 4-HNE, and diTYR ($p < 0.05$, Table 4).

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 [mean(median) ± SEM].

| Groups | 8-OhDG | 4-HNE | diTYR |
|---|---|---|---|
| **Control** | 0,33 (0,00) ± 0,21 | 0,50 (0,50) ± 0,22 | 0,33 (0,00) ± 0,21 |
| **CP** | 2,83 (3,00) ± 0,17 t | 2,83 (3,00) ± 0,17 t | 2,83 (3,00) ± 0,17 t |
| **CP + ASE100** | 1,67 (2,00) ± 0,21# | 1,66 (2,00) ± 0,21# | 1,66 (2,00) ± 0,21# |
| **CP + ASE200** | 0,66 (1,00) ± 0,21# | 0,66 (1,00) ± 0,21# | 0,83 (1,00) ± 0,17# |
| CP: Cyclophosphamide, ASE: *Allium scorodoprasum* extract. | | | |
| t denotes significant difference vs. healthy control group at $P < 0.05$. | | | |
| # denotes significant difference vs. CP group at $P < 0.05$. | | | |

# Discussions

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 (Sheeja and Kuttan 2006). CP causes the development of hepatotoxicity due to the excessive ROS production as a result of lipid peroxidation (Zarei and Shivanandappa 2013). *Allium scorodoprasum* L. has strong antioxidant and anti-inflammatory effects because it has the phenolic and flavonoid content such as quercetin, protocatechuic acid, coumaric acid, hydroxybenzoic acid and rutin (Table 1) (Demir et al. 2022). The contents of ASE have a hepatoprotective effect by reducing lipid peroxidation, hepatocyte swelling, leukocyte infiltration, necrosis, renewing the activities of antioxidant enzymes, and inhibiting apoptosis (Liu et al. 2002, Liu et al. 2010, Marcarini et al. 2011, Li et al. 2014). CP and acrolein increase lipid peroxidation (MDA level) and ALT, AST, LDH levels, while they decrease the level of SOD and GSH by their toxic effects (Bhattacharya et al. 2003, Cuce et al. 2015, Habibi et al. 2015). In the current 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, decreasing cholesteryl ester hydrolysis, and peroxidation of unsaturated membrane lipids and causes these lipids to leak into the circulation (Mythili et al. 2006). Quercetin has a regulatory effect on lipid metabolism-related gene expression in the liver. It reduces the biosynthesis of hepatic fatty acids and triglycerides by the regulatory effect (Mythili et al. 2006). Gallic acid exerts antihyperlipidemic effects by inducing adipocyte differentiation through PPARγ activation (Bak et al. 2013). Protocatechuic acid has a lipid-lowering effect in liver injury because it decreases LDL, VLDL and triglyceride levels and increases HDL levels (Radhiga et al. 2016). In addition, protocatechuic acid decreases ALT, AST, and cholesterol levels while it increases HDL levels in liver damage (Adeyanju et al. 2022). Naringenin reduces hyperglycemia, and hyperlipidemia and has a liver protective effect through the antioxidant effect (Priscilla et al. 2015). In the present study, treatment of ASE may have caused to regress in the level of LDL, VLDL, triglyceride, and total cholesterol and induce HDL levels by the antioxidant and antihyperlipidemic effects of the contents.

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 level (Jnaneshwari et al. 2013, Mahmoud 2014). Besides, CP induces liver and serum TNF-α synthesis and related nitric oxide release. Nitric oxide triggers the release of superoxide radicals and causes an increase in MDA (Germoush and Mahmoud 2014). CP causes liver toxicity by inducing NF-κB and induces the release of overproduction of proinflammatory cytokines (IL-1β, IL-6, and TNF-α). As a result, liver necrosis develops (Jnaneshwari et al. 2013, Mahmoud 2014). Naringenin in ASE improves inflammation and oxidative stress by suppressing the transcription of proinflammatory cytokines genes (Bodet et al. 2008, Nguyen-Ngo et al. 2019). In addition, naringenin is reported to suppress NO, NF-κB, and proinflammatory cytokines production. It was emphasized that it is cytoprotective and hepatoprotective

by anti-inflammatory and antioxidant properties (Jayaraman et al. 2012). Protocatechuic acid in ASE has an anti-inflammatory and antioxidant role in the liver by modulating the NF-κB/COX-2 pathway (Adeyanju et al. 2022). In the current study, CP induced inflammation, and oxidative stress and 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 as dose-dependent.

CP causes liver tissue damage and inflammatory cell infiltration histopathologically as well as reflected in serum biochemistry. It has also been reported that it causes histopathologically mild fatty changes and necrosis in the liver (Abdelfattah-Hassan et al. 2019). Apoptotic and necrotic hepatocytes and neutrophil infiltration has been noted in CP-treated rats (Sun et al. 2021). CP has led to leukocyte infiltration and adiposity by oxidative stress, inflammation, and DNA damage in the liver (Aladaileh et al. 2019). Histopathologically, ASE may have prevented liver necrosis and post-necrotic infiltration by its anti-inflammatory, antioxidant, and hepatoprotective effects. It may have prevented DNA and liver damage because it suppressed proinflammatory cytokines (TNF-a, IL-1β, and IL-6) and DNA oxidation (8-OhDG), especially at high doses (ASE, 200 mg/kg).

CP induce ROS and the ROS induce hepatocyte apoptosis and DNA damage (Caglayan et al. 2018, Li et al. 2020). As a result, it increases the expression of liver 8-OHdG which is an oxidized nucleoside of DNA, and the level of 4-HNE (Stankiewicz et al. 2002). The increased 8-OHdG can cause base modifications and strand breaks in DNA. Naringenin has reduced oxidative DNA damage by preventing apoptosis, autophagy, inflammation, and oxidative stress (Caglayan et al. 2018). diTYR is a protein oxidation marker that can be produced by ROS. Although its level has never been determined in CP studies, its level increases in cases of liver damage (Sun et al. 2011). Quercetin prevents the formation of diTYR by inhibiting myeloperoxidase in a dose-dependent manner (Shiba et al. 2008). Rutin is a powerful antioxidant and prevents protein oxidation, especially diTYR (Dias et al. 2021). As a result, antioxidant substances such as quercetin, rutin, and naringenin in ASE may inhibit DNA and protein oxidation by preventing ROS formation. Further, ASE treatment may have reduced the levels of 8-OHdG, 4-HNE, and diTYR by CP-induced.

Finally, CP is converted to its active metabolites through CYP2A6, 2B6, 3A4, 3A5, 2C9, 2C18, and 2C19 (De Jonge et al. 2005) however naringenin enantiomers inhibit CYP2C19 and CYP3A (Lu et al. 2011). As a result, naringenin in ASE may have reduced the toxic effects of CP by inhibiting CP metabolism.

# Conclusion

ASE which contains strong antioxidant and anti-inflammatory contents, can be beneficial in liver toxicity. However, increasing the dose and duration of use of it can reduce oxidative stress, inflammation, and liver damage more effectively.

# Declarations

### Author Contribution Statement

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

### Funding

This study was carried out by using the facilities of Sivas Cumhuriyet University together with self-funding.

### Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

# References

1. Abdelfattah-Hassan, A., S. I. Shalaby, S. I. Khater, E. S. El-Shetry, H. Abd El Fadil and S. A. Elsayed (2019). Panax ginseng is superior to vitamin E as a hepatoprotector against cyclophosphamide-induced liver damage. Complementary Therapies in Medicine 46: 95-102.
2. Adeyanju, A. A., F. O. Asejeje, O. R. Molehin, O. Owoeye, E. O. Olatoye and E. N. Ekpo (2022). Protective role of protocatechuic acid in carbon tetrachloride-induced oxidative stress via modulation of proinflammatory cytokines levels in brain and liver of Wistar rats. Journal of Basic and Clinical Physiology and Pharmacology 33(2): 143-154.
3. Aladaileh, S. H., M. H. Abukhalil, S. A. Saghir, H. Hanieh, M. A. Alfwuaires, A. A. Almaiman, M. Bin-Jumah and A. M. Mahmoud (2019). Galangin activates Nrf2 signaling and attenuates oxidative damage, inflammation, and apoptosis in a rat model of cyclophosphamide-induced hepatotoxicity. Biomolecules 9(8): 346.
4. Aloqbi, A., U. Omar, M. Yousr, M. Grace, M. A. Lila and N. Howell (2016). Antioxidant activity of pomegranate juice and punicalagin. Natural Science 8(06): 235.
5. Bak, E.-J., J. Kim, S. Jang, G.-H. Woo, H.-G. Yoon, Y.-J. Yoo and J.-H. Cha (2013). Gallic acid improves glucose tolerance and triglyceride concentration in diet-induced obesity mice. Scandinavian journal of clinical and laboratory investigation 73(8): 607-614.
6. Basu, A., A. Bhattacharjee, A. Samanta and S. Bhattacharya (2015). Prevention of cyclophosphamide-induced hepatotoxicity and genotoxicity: Effect of an L-cysteine based oxovanadium(IV) complex on oxidative stress and DNA damage. Environ Toxicol Pharmacol 40(3): 747-757.
7. Bhattacharya, A., R. A. Lawrence, A. Krishnan, K. Zaman, D. Sun and G. Fernandes (2003). Effect of dietary n-3 and n-6 oils with and without food restriction on activity of antioxidant enzymes and lipid peroxidation in livers of cyclophosphamide treated autoimmune-prone NZB/W female mice. Journal of the American College of Nutrition 22(5): 388-399.

8. Bodet, C., V. La, F. Epifano and D. Grenier (2008). Naringenin has anti-inflammatory properties in macrophage and ex vivo human whole-blood models. Journal of periodontal research 43(4): 400-407.
9. Caglayan, C., Y. Temel, F. M. Kandemir, S. Yildirim and S. Kucukler (2018). Naringin protects against cyclophosphamide-induced hepatotoxicity and nephrotoxicity through modulation of oxidative stress, inflammation, apoptosis, autophagy, and DNA damage. Environmental Science and Pollution Research 25(21): 20968-20984.
10. Cuce, G., S. Cetinkaya, T. Koc, H. H. Esen, C. Limandal, T. Balci, S. Kalkan and M. Akoz (2015). Chemoprotective effect of vitamin E in cyclophosphamide-induced hepatotoxicity in rats. Chem Biol Interact 232: 7-11.
11. De Jonge, M. E., A. D. Huitema, S. Rodenhuis and J. H. Beijnen (2005). Clinical pharmacokinetics of cyclophosphamide. Clinical pharmacokinetics 44(11): 1135-1164.
12. Demir, T., Ö. Akpınar, H. Kara and H. Güngör (2022). Phenolic profile and investigation of biological activities of Allium scorodoprasum L. subsp. rotundum. Food Bioscience: 101548.
13. Dias, D. T. M., K. R. Palermo, B. P. Motta, A. K. Kaga, T. F. O. Lima, I. L. Brunetti and A. M. Baviera (2021). Rutin inhibits the in vitro formation of advanced glycation products and protein oxidation more efficiently than quercetin. Revista de Ciências Farmacêuticas Básica e Aplicada 42: 1-13.
14. Emadi, A., R. J. Jones and R. A. Brodsky (2009). Cyclophosphamide and cancer: golden anniversary. Nat Rev Clin Oncol 6(11): 638-647.
15. Fallah, V., J. A. Mahabadi, M. Y. Mahabadi, H. H. Kashani and H. Nikzad (2017). Protective Effect of Allium cepa (Onion) Seeds (AC) Extract on Histopathology of Testis in STZ-Induced Male Rats. International Journal of Morphology 35(4).
16. Germoush, M. O. and A. M. Mahmoud (2014). Berberine mitigates cyclophosphamide-induced hepatotoxicity by modulating antioxidant status and inflammatory cytokines. Journal of cancer research and clinical oncology 140(7): 1103-1109.
17. Habibi, E., M. Shokrzadeh, A. Chabra, F. Naghshvar, R. Keshavarz-Maleki and A. Ahmadi (2015). Protective effects of Origanum vulgare ethanol extract against cyclophosphamide-induced liver toxicity in mice. Pharmaceutical biology 53(1): 10-15.
18. Jayaraman, J., V. A. S. Jesudoss, V. P. Menon and N. Namasivayam (2012). Anti-inflammatory role of naringenin in rats with ethanol induced liver injury. Toxicology Mechanisms and Methods 22(7): 568-576.
19. Jnaneshwari, S., M. Hemshekhar, M. S. Santhosh, K. Sunitha, R. Thushara, C. Thirunavukkarasu, K. Kemparaju and K. S. Girish (2013). C rocin, a dietary colorant mitigates cyclophosphamide-induced organ toxicity by modulating antioxidant status and inflammatory cytokines. Journal of Pharmacy and Pharmacology 65(4): 604-614.
20. Kim, H. G., D. H. Yoon, W. H. Lee, S. K. Han, B. Shrestha, C. H. Kim, M. H. Lim, W. Chang, S. Lim and S. Choi (2007). Phellinus linteus inhibits inflammatory mediators by suppressing redox-based NF-κB

and MAPKs activation in lipopolysaccharide-induced RAW 264.7 macrophage. Journal of ethnopharmacology 114(3): 307-315.

21. Kumar, V. M., A. Henley, C. Nelson, O. Indumati, Y. P. Rao, S. Rajanna and B. Rajanna (2017). Protective effect of Allium sativum (garlic) aqueous extract against lead-induced oxidative stress in the rat brain, liver, and kidney. Environmental Science and Pollution Research 24(2): 1544-1552.
22. Li, B., W. Li, Y. Tian, S. Guo, L. Qian, D. Xu and N. Cao (2020). Selenium-alleviated hepatocyte necrosis and DNA damage in cyclophosphamide-treated geese by mitigating oxidative stress. Biological trace element research 193(2): 508-516.
23. Li, H., H. S. Lee, S. H. Kim, B. Moon and C. Lee (2014). Antioxidant and anti-inflammatory activities of methanol extracts of Tremella fuciformis and its major phenolic acids. Journal of food science 79(4): C460-C468.
24. Liu, C.-L., J.-M. Wang, C.-Y. Chu, M.-T. Cheng and T.-H. Tseng (2002). In vivo protective effect of protocatechuic acid on tert-butyl hydroperoxide-induced rat hepatotoxicity. Food and Chemical Toxicology 40(5): 635-641.
25. Liu, C.-M., Y.-L. Zheng, J. Lu, Z.-F. Zhang, S.-H. Fan, D.-M. Wu and J.-Q. Ma (2010). Quercetin protects rat liver against lead-induced oxidative stress and apoptosis. Environmental Toxicology and Pharmacology 29(2): 158-166.
26. Lu, W. J., V. Ferlito, C. Xu, D. A. Flockhart and S. Caccamese (2011). Enantiomers of naringenin as pleiotropic, stereoselective inhibitors of cytochrome P450 isoforms. Chirality 23(10): 891-896.
27. Mahmoud, A. M. (2014). Hesperidin protects against cyclophosphamide-induced hepatotoxicity by upregulation of PPARγ and abrogation of oxidative stress and inflammation. Canadian journal of physiology and pharmacology 92(9): 717-724.
28. Marcarini, J. C., M. S. F. Tsuboy, R. C. Luiz, L. R. Ribeiro, C. B. Hoffmann-Campo and M. S. Mantovani (2011). Investigation of cytotoxic, apoptosis-inducing, genotoxic and protective effects of the flavonoid rutin in HTC hepatic cells. Experimental and Toxicologic Pathology 63(5): 459-465.
29. Mohammad, M. K., D. Avila, J. Zhang, S. Barve, G. Arteel, C. McClain and S. Joshi-Barve (2012). Acrolein cytotoxicity in hepatocytes involves endoplasmic reticulum stress, mitochondrial dysfunction and oxidative stress. Toxicol Appl Pharmacol 265(1): 73-82.
30. Mythili, Y., P. T. Sudharsan, V. Sudhahar and P. Varalakshmi (2006). Protective effect of DL-α-lipoic acid on cyclophosphamide induced hyperlipidemic cardiomyopathy. European Journal of Pharmacology 543(1-3): 92-96.
31. Nencini, C., G. G. Franchi, F. Cavallo and L. Micheli (2010). Protective effect of Allium neapolitanum Cyr. versus Allium sativum L. on acute ethanol-induced oxidative stress in rat liver. Journal of medicinal food 13(2): 329-335.
32. Newman, D. J. and G. M. Cragg (2016). Natural products as sources of new drugs from 1981 to 2014. Journal of natural products 79(3): 629-661.
33. Nguyen-Ngo, C., J. C. Willcox and M. Lappas (2019). Anti-diabetic, anti-inflammatory, and anti-oxidant effects of naringenin in an in vitro human model and an in vivo murine model of gestational

diabetes mellitus. Molecular nutrition & food research 63(19): 1900224.

34. Oyagbemi, A. A., O. T. Omobowale, E. R. Asenuga, A. S. Akinleye, R. O. Ogunsanwo and A. B. Saba (2016). Cyclophosphamide-induced Hepatotoxicity in Wistar Rats: The Modulatory Role of Gallic Acid as a Hepatoprotective and Chemopreventive Phytochemical. Int J Prev Med 7: 51.
35. Priscilla, D. H., M. Jayakumar and K. Thirumurugan (2015). Flavanone naringenin: An effective antihyperglycemic and antihyperlipidemic nutraceutical agent on high fat diet fed streptozotocin induced type 2 diabetic rats. journal of functional foods 14: 363-373.
36. Radhiga, T., A. Sundaresan, P. Viswanathan and K. V. Pugalendi (2016). Effect of protocatechuic acid on lipid profile and DNA damage in D-galactosamine-induced hepatotoxic rats. Journal of basic and clinical physiology and pharmacology 27(5): 505-514.
37. Roy, P., J. Y. Li, C. L. Crespi and D. J. Waxman (1999). Development of a substrate-activity based approach to identify the major human liver P-450 catalysts of cyclophosphamide and ifosfamide activation based on cDNA-expressed activities and liver microsomal P-450 profiles. Drug Metabolism and Disposition 27(6): 655-666.
38. Senthilkumar, S., T. Devaki, B. Manohar and M. Babu (2006). Effect of squalene on cyclophosphamide-induced toxicity. Clin Chim Acta 364(1-2): 335-342.
39. Sheeja, K. and G. Kuttan (2006). Ameliorating effects of Andrographis paniculata extract against cyclophosphamide-induced toxicity in mice. Asian Pac J Cancer Prev 7(4): 609-614.
40. Shiba, Y., T. Kinoshita, H. Chuman, Y. Taketani, E. Takeda, Y. Kato, M. Naito, K. Kawabata, A. Ishisaka and J. Terao (2008). Flavonoids as substrates and inhibitors of myeloperoxidase: molecular actions of aglycone and metabolites. Chemical research in toxicology 21(8): 1600-1609.
41. Štajner, D., N. Milić, J. Čanadanović-Brunet, A. Kapor, M. Štajner and B. Popović (2006). Exploring Allium species as a source of potential medicinal agents. Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives 20(7): 581-584.
42. Stankiewicz, A., E. Skrzydlewska and M. Makiela (2002). Effects of amifostine on liver oxidative stress caused by cyclophosphamide administration to rats. Drug metabolism and drug interactions 19(2): 67-82.
43. Stojanovic, G. (2014). In vitro antioxidant activity of methanol extract of Allium scorodoprasum. Bulgarian journal of agricultural science 20(5): 1130-1136.
44. Subramaniam, S. R., R. A. Cader, R. Mohd, K. W. Yen and H. A. Ghafor (2013). Low-dose cyclophosphamide-induced acute hepatotoxicity. Am J Case Rep 14: 345-349.
45. Sun, D., C. Sun, G. Qiu, L. Yao, J. Yu, H. Al Sberi, M. S. Fouda, M. S. Othman, M. S. Lokman and R. B. Kassab (2021). Allicin mitigates hepatic injury following cyclophosphamide administration via activation of Nrf2/ARE pathways and through inhibition of inflammatory and apoptotic machinery. Environmental Science and Pollution Research 28(29): 39625-39636.
46. Sun, J., A. Sugiyama, A. Masuda, T. Ochi and T. Takeuchi (2011). Expressions of protein oxidation markers, dityrosine and advanced oxidation protein products in acetaminophen-induced liver injury in

rats. Journal of Veterinary Medical Science: 1104210497-1104210497.

47. Tasci, B. and I. Koca (2015). Use of Allium scorodoprasum L. subsp. rotundum as food. VII International Symposium on Edible Alliaceae 1143.
48. Teles, K. A., P. Medeiros-Souza, F. A. C. Lima, B. G. Araujo and R. A. C. Lima (2017). Cyclophosphamide administration routine in autoimmune rheumatic diseases: a review. Rev Bras Reumatol Engl Ed 57(6): 596-604.
49. Zarei, M. and T. Shivanandappa (2013). Amelioration of cyclophosphamide-induced hepatotoxicity by the root extract of Decalepis hamiltonii in mice. Food and chemical toxicology 57: 179-184.
50. Zhai, X., Z. Zhang, W. Liu, B. Liu, R. Zhang, W. Wang, W. Zheng, F. Xu, J. Wang and Y. Chen (2018). Protective effect of ALDH2 against cyclophosphamide-induced acute hepatotoxicity via attenuating oxidative stress and reactive aldehydes. Biochemical and biophysical research communications 499(1): 93-98.

# Figures

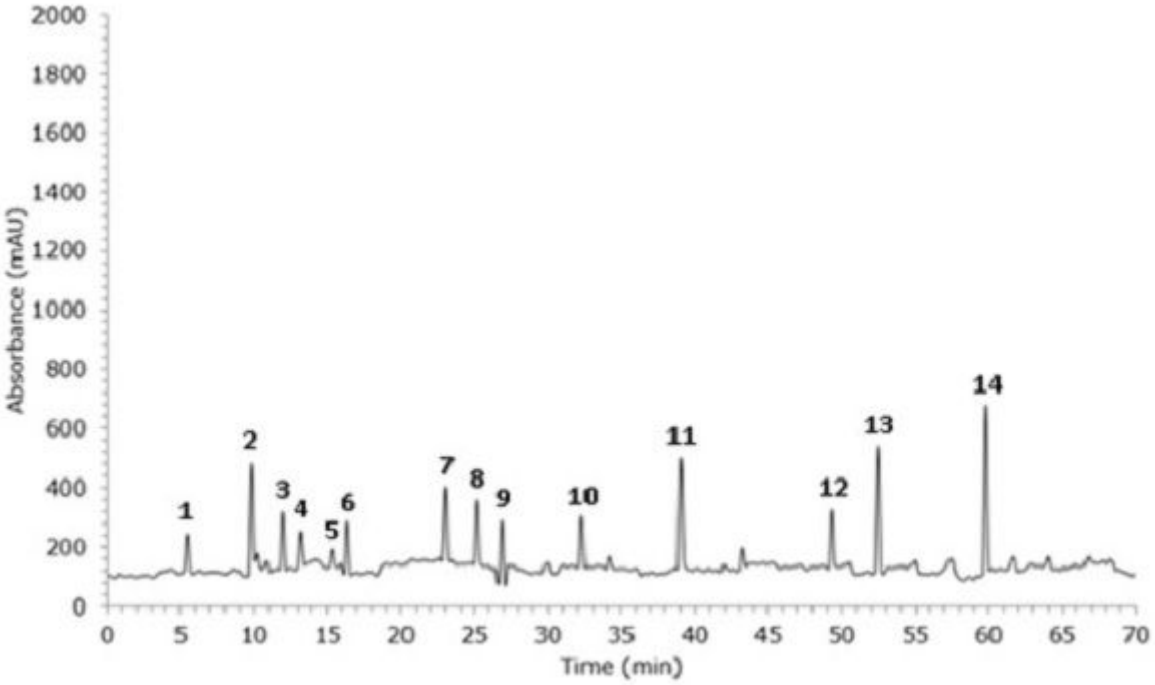


**Figure 1. The phytochemical content of ASE (50 mg/mL) on HPLC analysis**

**The phytochemical content of ASE**

1. Gallic acid
2. Caffeic acid
3. Ferulic acid
4. Protocatechuic acid
5. Protocatechuic aldehyde
6. p-Hydroxybenzoic acid
7. Vanillic acid
8. catechin
9. naringenin
10. m-Coumaric acid
11. kaempeferol
12. Rutin
13. p-Coumaric acid
14. Quercetin

## Figure 1

Please See image above for figure legend.

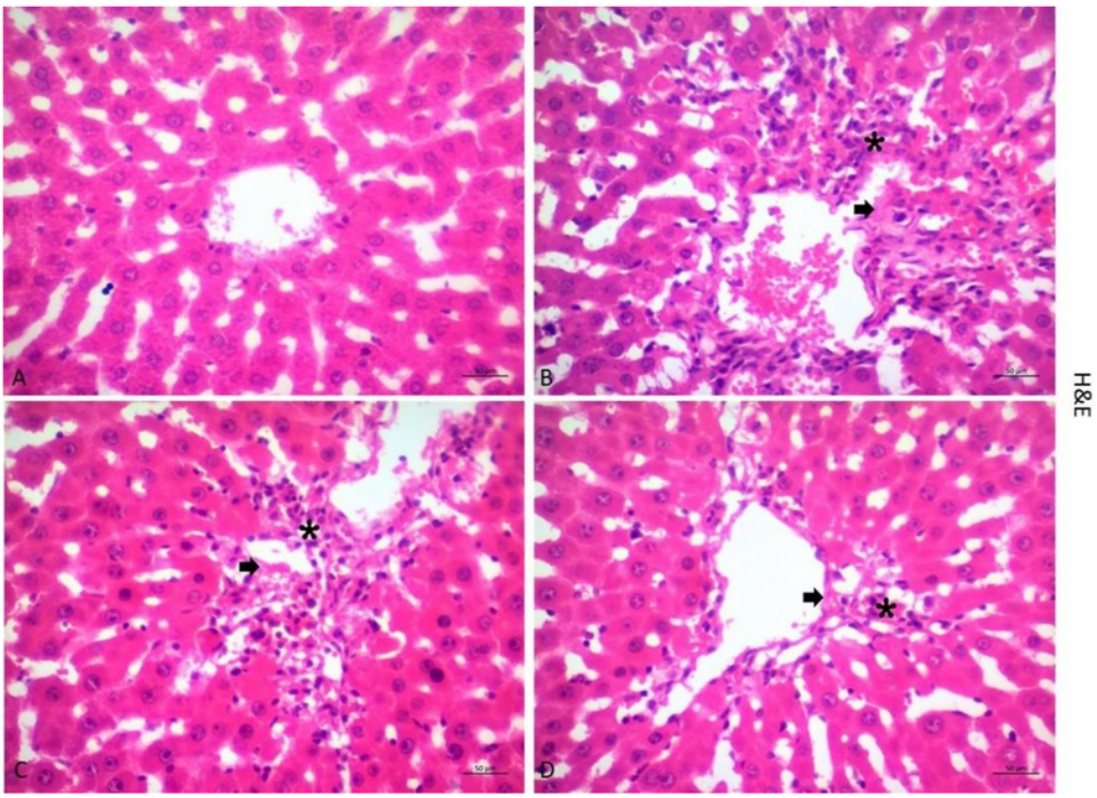


**Figure 2. Liver sections stained with hematoxylin-eosin (H&E).**

**Figure 2. 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

## Figure 2

Please See image above for figure legend.

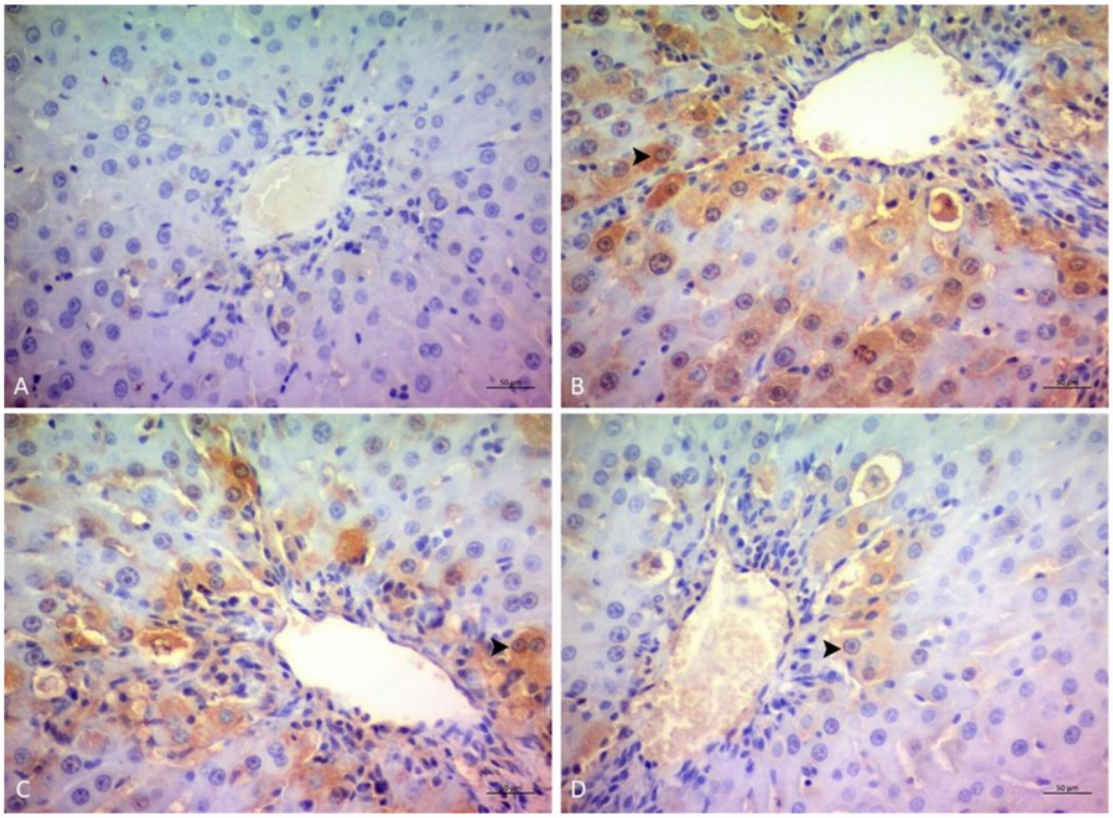


**Figure 3. The liver 8-OhDG immunohistochemistry**

**Figure 3. 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

## Figure 3

Please See image above for figure legend.

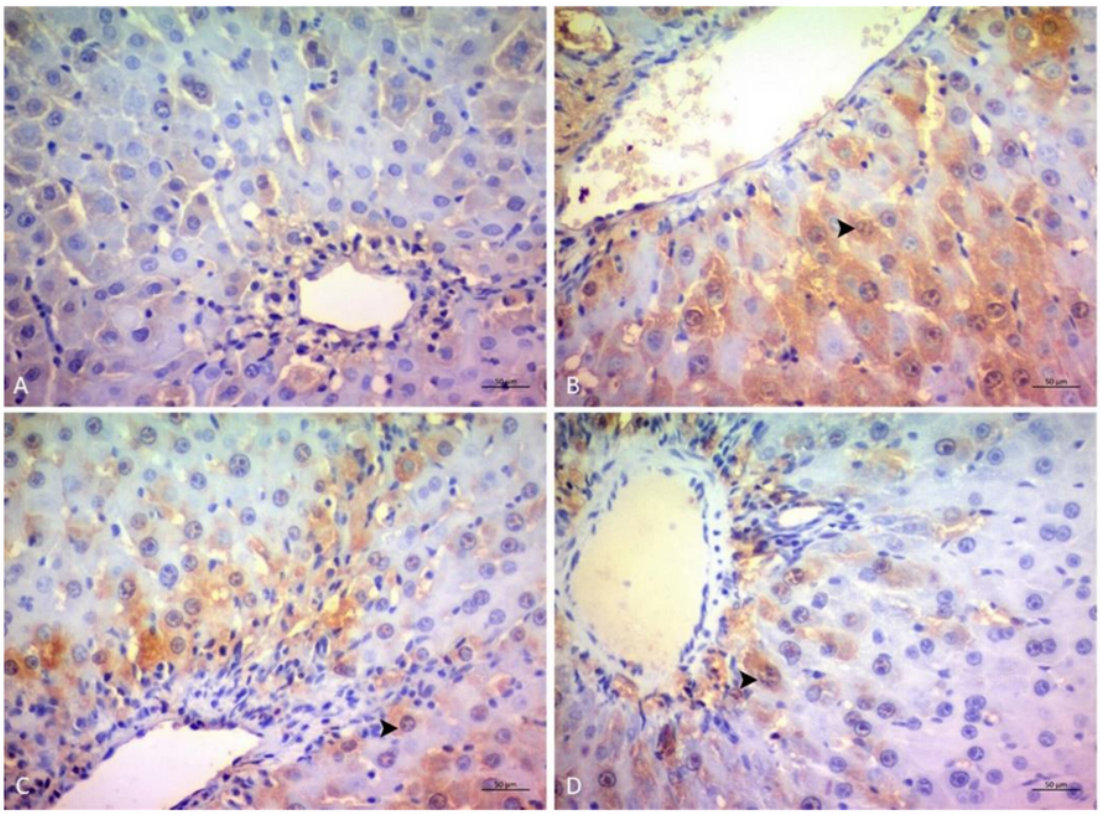


**Figure 4. The liver 4-HNE immunohistochemistry**

**Figure 4. 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

## Figure 4

Please See image above for figure legend.

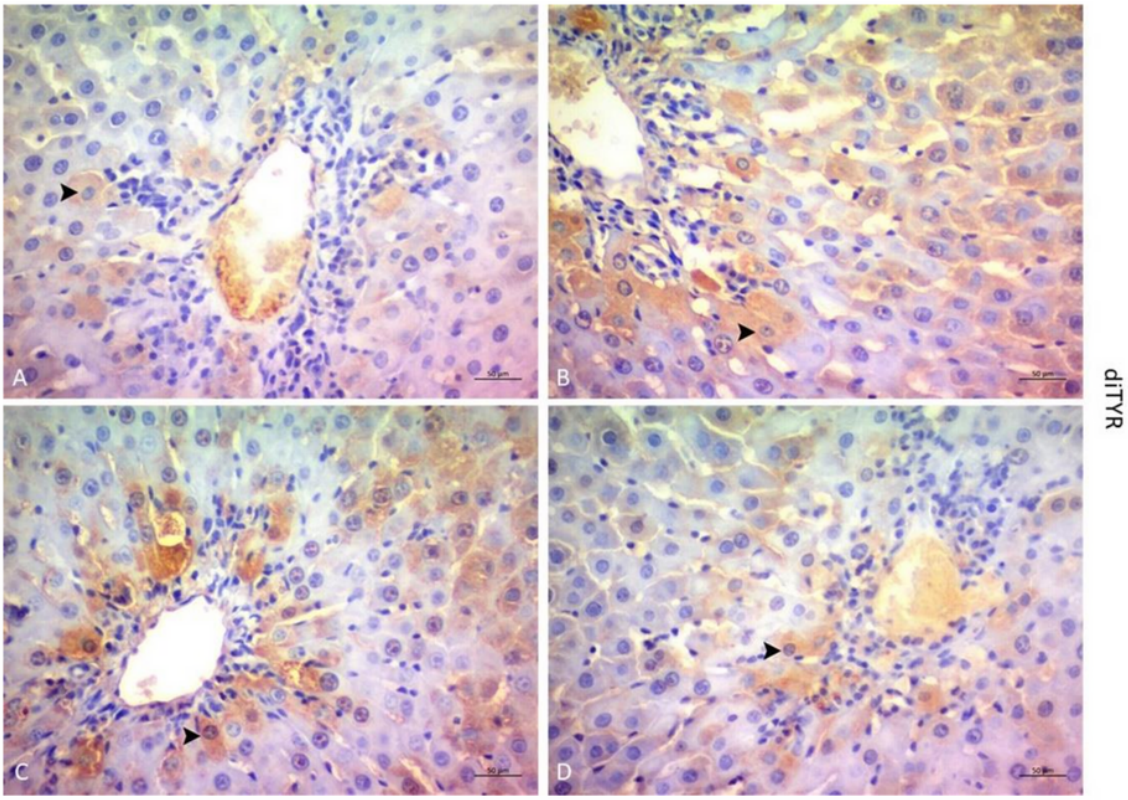


**Figure 5. The liver diTYR immunohistochemistry**

**Figure 5. 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

## Figure 5

Please See image above for figure legend.