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Research Article

Oleuropein attenuates placental growth factor expression by regulating oxidative stress and apoptosis in acrylamide hepatotoxicity

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ABSTRACT

The liver is susceptible to toxic effects, as it is the main site of acrylamide biotransformation and detoxification. Researchers have claimed that placental growth factor (PlGF) and its pathway are potentially involved in numerous diseases, including liver fibrosis and angiogenesis. Oleuropein is a natural phenolic compound with potent antioxidant effects. The purpose of this study was to examine the role of PlGF and the potential protection provided by oleuropein in acrylamide hepatotoxicity. Wistar albino rats were assigned into control, acrylamide (ACR) (5 mg/kg), oleuropein (OLE) (4.2 mg/kg), and ACR+OLE groups. Acrylamide and oleuropein were administered for 21 days. The control group received only physiological saline. Liver tissues were evaluated histologically and immunohistochemically. Histological examinations revealed significant enlargement of the sinusoidal vessels and abundant hepatocytes with pyknotic nuclei in the ACR group. Acrylamide toxicity resulted in elevated PlGF, accumulation of 8-hydroxydeoxyguanosine (8-OHdG), and increased Caspase-3 immunoreactivity in the liver. Oleuropein treatment reduced the increased expression of PlGF, 8-OHdG, and Caspase-3 against these deleterious effects observed in the ACR group. A positive correlation was observed between PlGF levels as well as oxidative stress and apoptosis markers in acrylamide toxicity. Oleuropein probably counteracted this mechanism by exhibiting antioxidant activity.

INTRODUCTION

Acrylamide was first discovered in 1893, and in 2002 researchers reported its presence in commonly consumed foodstuffs (Omar et al., 2015). Acrylamide is classified as a "possible carcinogen" and is found in heat-treated products, as well as cigarettes, soaps, plastic products, cleaning products, and baby foods (Esposito et al., 2021; Pietropaoli et al., 2022). Exposure to acrylamide causes toxic effects in the digestive tract, liver, kidney, heart, lung, and brain. A previous experimental study reported elevation in total oxidative state and malondialdehyde (MDA) levels in the rat liver following 21-day exposure to acrylamide, while the expression level of some antioxidant enzymes decreased (Rifai and Saleh, 2020). Acrylamide can also cause dysfunction and considerable reactive oxygen species (ROS) production by altering the mitochondrial membrane potential of hepatocytes (Seydi, 2015). Exposure to acrylamide in animal models results in several ultrastructural abnormalities with increases in MDA and reduced glutathione (GSH) and superoxide dismutase (SOD) levels in liver tissue (Gao et al., 2022). This means that the liver is directly affected by oxidative stress.

Although numerous studies have addressed acrylamide hepatotoxicity from different perspectives, the manner in which placental growth factor (PlGF) expression changes has not been previously examined. Acting as a pleiotropic cytokine, PIGF stimulates the growth, migration, and survival of endothelial cells and promotes pathological angiogenesis and wound healing (Li et al., 2017). Nutritional factors may cause changes in the liver, such as hepatocyte degeneration, necrosis, and replacement of the parenchyma by fibrotic tissue, resulting in loss of function. These events actually occur as a result of the activation of proinflammatory and profibrotic pathways. On the other hand, PlGF pathway blockade was found to cause no harm to healthy blood vessels, and pathological angiogenesis decreased (Li et al., 2017). Increased expression of PlGF has been shown in cirrhotic liver and hepatocellular carcinoma in both human and rodent models (Dewerchin and Carmeliet, 2012). Inhibition of PIGF has been shown to suppress liver fibrogenesis, reduce portal hypertension, and inhibit hepatocellular carcinoma (Heindryckx et al., 2013).

Polyphenols are natural antioxidants and chemoprotective agents that exhibit protective effects against diseases associated with oxidative stress and mitochondrial dysfunction. Oleuropein is the most important bioactive phenolic glycoside in olive leaves (Topuz, 2022). It exhibits powerful antioxidant and anti-apoptotic effects in several diseases (Alarcon de la Lastra, et al., 2001). On the other hand, inhibition of oxidative stress development can prevent structural and functional abnormalities in the liver. Various studies have reported strong evidence that oleuropein exerts a hepatoprotective effect (Yoon, 2018).

This study investigated the relationship between PlGF expression and oxidative stress, and the possible protective action of oleuropein, a subject which has not been investigated in previous studies of acrylamide-induced liver toxicity.

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MATERIAL AND METHODS

Experimental Design

Twenty-four Wistar albino rats were assigned into four groups - control, oleuropein-treated (OLE group - 4.2 mg/kg), acrylamide (ACR group - 5 mg/kg acrylamide BioShop, Cat No. 79.06.1), and oleuropein+acrylamide (ACR+OLE group - 5 mg/kg ACR+ 4.2 mg/kg OLE). The control group was given saline solution only. All animals received reagent for 21 days in saline solution via oral gavage. All animal procedures were approved and supervised by the Balıkesir University Ethical Committee (ethical approval number 2022/10-2 with dated 05.01.2023).

On the 21st day of the experiment, the animals were anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg) and sacrificed by cervical dislocation.

Oleuropein was obtained from Southern Marmara olive tree leaf extract. The amount of oleuropein in 100 mg/ml extract was determined as 4.2 mg using the HPLC method (Sharif et al., 2021)

Histological procedure

Ten percent neutral buffered formalin solution was used to fix the liver tissues. For histological tissue processing, tissues were washed overnight in running water and embedded in paraffin after dehydration and xylene clearing. Five-micrometer sections were taken from the tissues in the paraffin blocks by means of a microtome (Leica, Multicut, Germany) and stained with hematoxylin and eosin. Finally, histopathological changes in the liver were evaluated and photographed under light microscopy (Zeiss Axiolab 5, Jena, Germany).

Immunohistochemical procedure

Five micrometer-thick sections were taken from the paraffin blocks. After deparaffinization, these were incubated with citrate buffer for antigen retrieval. They were then treated with 3 % hydrogen peroxide for inhibition of endogenous peroxidase activity. Ten percent normal goat serum was used for non-specific binding inhibition.

The primary antibodies PlGF, 8-OHdG, and Caspase-3 (PlGF 1:250, NBP2-67067, Novus Biologicals; 8-OHdG: 1:400, BS-1278R, Bioss; Caspase-3: 1:250, BS-0081R, Bioss) were next incubated with the liver sections for one night and subsequently treated for 60 min at room temperature with the horseradish peroxidase-conjugated secondary antibody. Immunostaining was visualized with fresh DAB solution. The sections were finally counterstained with Gill's hematoxylin and mounted. The staining results were evaluated under a light microscope (Zeiss Axiolab 5, Jena, Germany).

Assessment of immunohistochemical reactivity

The histoscore (H-score) of the semi-quantitative staining intensities was obtained by evaluating the proportion of positive cells and the degree of staining (0, unstained; 1, weak; 2, median; and 3, strong), and values between 0 and 300 were found. The expression level of each antibody was subjected to statistical evaluation based on the median H-score value. Evaluations were performed by two different researchers (Numata et al., 2013).

Statistical Analysis

The study data were expressed as mean ± standard deviation and analyzed on SPSS software package (version 22; SPSS Inc., Chicago, IL, USA). Significance differences were determined by means of One-Way ANOVA and Tukey's multiple comparison post-hoc test. *$p<0.05$ and **$p<0.01$, as appropriate, were regarded as statistically significant.

RESULTS

Acrylamide causes serious histopathological changes in the liver

The histopathological findings for the study groups' H&E-stained liver tissues sections are given in Figure 1. Accordingly, while the tissues from the control group exhibited a normal histological appearance, a few cells in the OLE group exhibited acidophilic staining (thin arrows). In the ACR group, the presence of diffuse acidophilic staining cells around the portal vein was noteworthy, and these cells were thought to be necrotic. Additionally, dilated sinusoids surrounding the dilated central vein were noted (arrow heads). The cytoplasmic borders of hepatocytes were unclear, and many contained pyknotic nuclei (thick arrow).

Oleuropein lowered increased PlGF immune expression in liver tissues exposed to acrylamide

The H-scores of the angiogenic marker PlGF were examined immunohistochemically in liver sections from all the experimental group, as shown in Figure 2A. Statistical analysis of the positive PlGF- stained cells in liver sections revealed that exposure to acrylamide (291 ± 25.88) significantly increased PlGF expression in the liver compared to the control group (118 ± 18.17) ($p < 0.01$). However, significant decreases were found in the ACR+OLE (145 ± 16.75) and OLE (123 ± 13.54) groups compared to the ACR group ($p < 0.01$) (Figure 2A and Figure 3).

Oleuropein attenuates acrylamide-induced liver apoptosis in rats

This study next investigated the roles of oleuropein in acrylamide-induced apoptosis. The results showed that oleuropein significantly inhibited the activation of apoptotic markers against the promotion of Caspase-3 in acrylamide application. Statistical analysis of the Caspase-3 positive stained cells in liver tissue are shown in Figure 2B.

The OLE group exhibited mild expression (140 ± 25.64). Acrylamide treatment caused a marked elevation in H-scores (296 ± 21) compared to the control group (91.6 ± 29.43), while a significant decrease was found in the ACR+OLE group H-scores (229 ± 17.17) compared to the ACR group ($p < 0.01$) (Figure 2B and Figure 4).

Oleuropein mitigates oxidative stress-induced DNA damage

Since DNA damage has been identified in case of acrylamide exposure, we stained liver tissue sections for 8-OHdG,

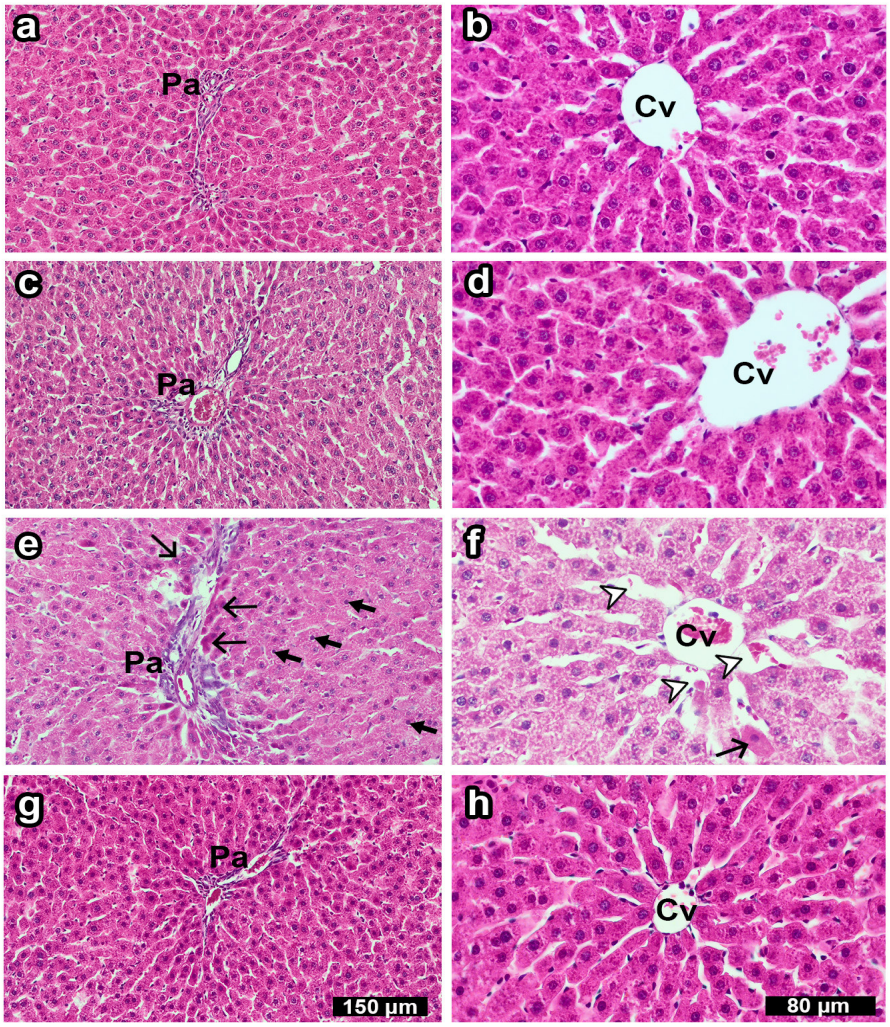

Figure 1. Photomicrographs representing H&E staining in liver sections. The control group (a, b) exhibits normal histological architecture in both the portal area (Pa) and central vein (Cv). Although acidophilic staining cells were noted in hepatocytes near the portal area in the OLE group (c), the structure of the central vein and surrounding hepatocytes were normal in appearance (d). However, in the ACR group, numerous hepatocytes with impaired integrity, unclear borders, and acidophilic staining (thin arrows) were observed near the portal area (e). Also, some hepatocyte nuclei have pyknotic nuclei (thick arrows) (e). The central vein was dilated, and dilatation was also present in the sinusoidal vessels between the hepatocyte cords (arrow heads) (e, f). However, oleuropein successfully protected the normal structure against the destructive effects of acrylamide in both the portal area and central vein structures in the ACR+OLE group (g, h). Paraffin, Scale bar: 150 µm and 80 µm for left and right panels.

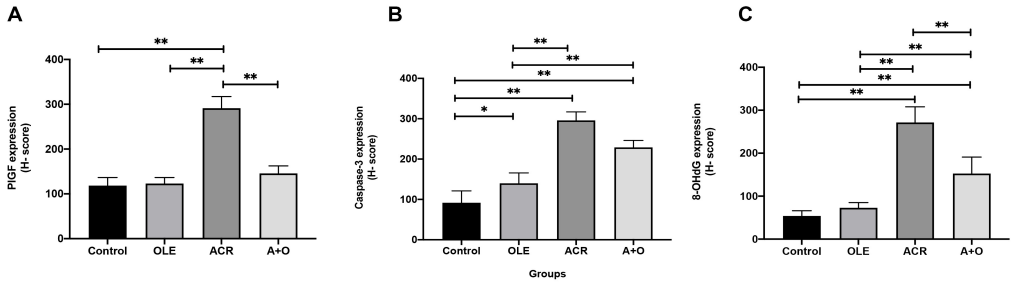

Figure 2. Statistical expression of the H-scores for PlGF, Caspase-3, and 8-OHdG expression in all the study groups. In the ACR group PlGF, Caspase-3, and 8-OHdG H-score was significantly increased compared to the other groups ($p < 0.01$). On the other hand, the A+O group H-score of PlGF and 8-OHdG was significantly decreased from the ACR group ($p < 0.01$). Besides this, the A+O group PlGF H-score did not differ from the control group ($p > 0.01$).

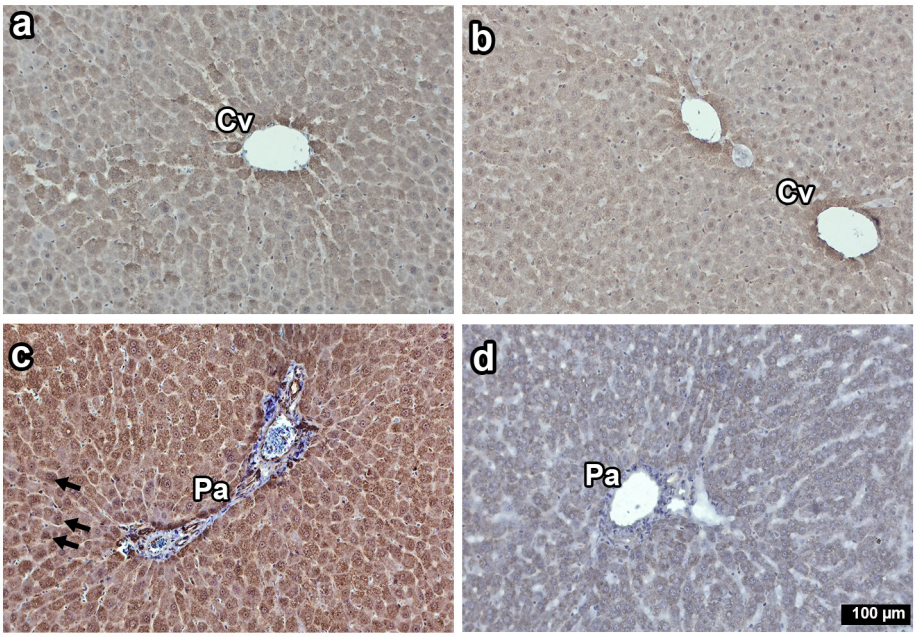

Figure 3. Immunohistochemical staining of PlGF in all four groups. Staining intensity of control, OLE and A+O groups (a, b, d) was similar. On the other hand, significantly increased cytoplasmic and nuclei staining of PlGF was observed in the ACR group (c). In addition, PlGF was widely expressed in most of the hepatic parenchyma of the ACR group and around the portal area. Arrow, positive stained cells; Pa, portal area; Cv, central vein. Strept-ABC, DAB, paraffin section. Scale bar: 100 µm for all panels.

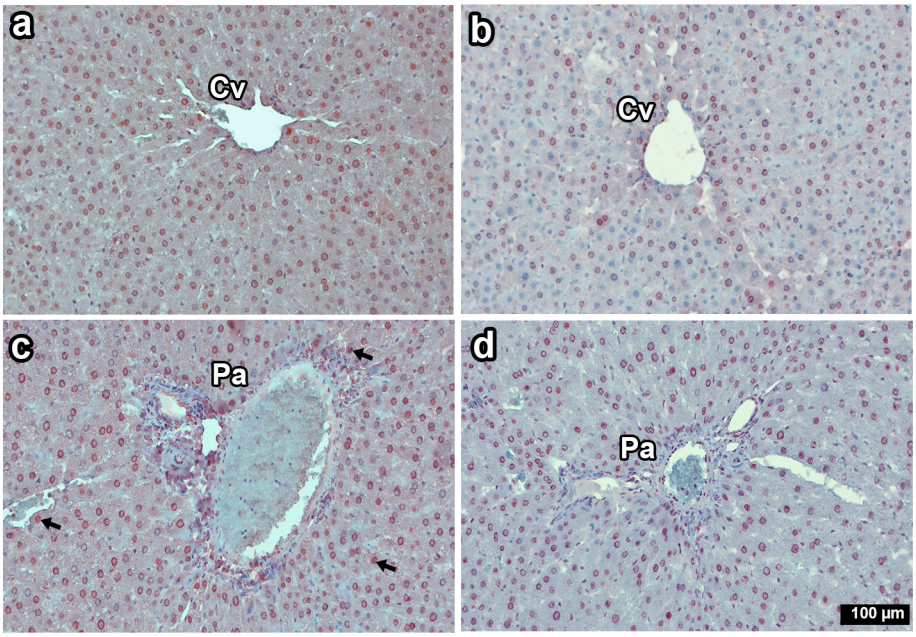

Figure 4. Immunohistochemical staining of Caspase-3 in all four groups. The control, OLE, and A+O groups (a, b, d) showed moderate immunoreactivity while increased cytoplasmic and nuclei staining of hepatocytes against the Caspase-3 antibody was observed in the ACR group (c). Arrow, positive stained cells; Pa, portal area; Cv, central vein. Strept-ABC, DAB, paraffin section. Scale bar: 100 µm for all panels.

a marker of oxidative stress-induced DNA lesions, in order to investigate the association between oleuropein and increased oxidative stress levels. Immunohistochemical analysis revealed stronger 8-OHdG staining in liver tissues (271 ± 36.53) in the ACR group compared to the control group ($p < 0.01$). In contrast, the OLE+ACR group liver tissue sections exhibited lower 8-OHdG expression (152 ± 38.35) than the ACR group ($p < 0.01$) (Figure 2C and Figure 5). It may therefore be concluded that oleuropein mitigates acrylamide-induced DNA damage.

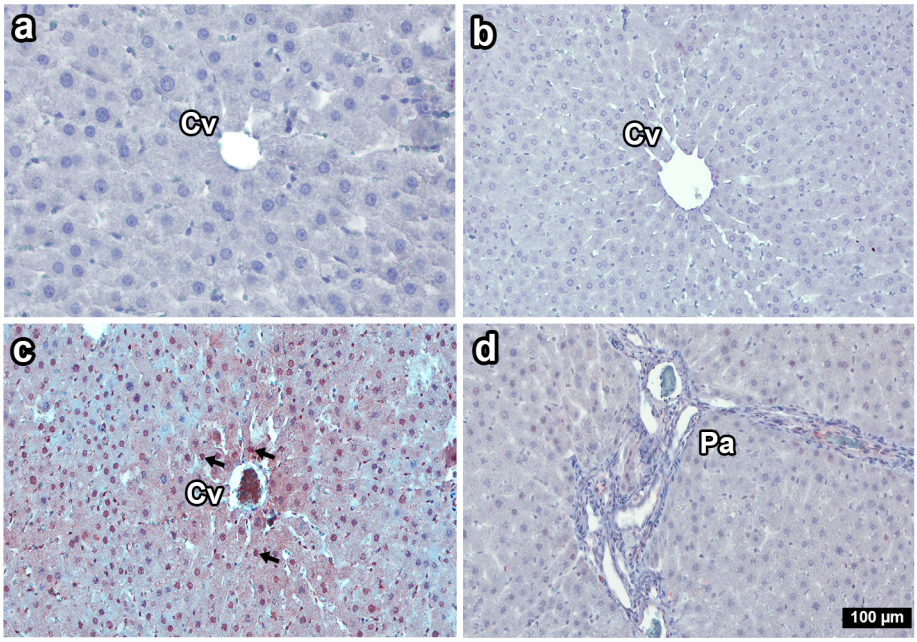

Figure 5. Immunohistochemical staining of 8-OHdG in all four groups. Staining intensity of control, OLE and A+O groups (a, b, d) was similar. On the other hand, it was determined that the expression of 8-OHdG increased significantly in the nucleus and cytoplasm of hepatocytes around the central vein. Arrow, positive stained cells; Pa, portal area; Cv, central vein. Strept-ABC, DAB, paraffin section. Scale bar: 100 µm for all panels.

DISCUSSION

The findings of this study revealed that oleuropein regulates PlGF levels by reducing Caspase-3 and 8-OHdG immunoreactivity, which increased notably with exposure to acrylamide. Our findings also support the idea that PlGF plays an important role in the pathogenesis of acrylamide hepatotoxicity and indicate that it may be a potential therapeutic target in acute acrylamide exposure. In addition, reduction of this angiogenic factor by oleuropein in acrylamide-exposed rats resulted in improvements in hepatic structures.

Increases PlGF in the liver is reported to cause liver damage, activation of monocytes and hepatic macrophages, fibrosis development, and hepatic inflammation (Li et al., 2017). An animal study in which PIGF was blocked by antibodies or genetic ablation reported decreased fibrogenesis and portal hypertension, as well as inhibition of the activation of hepatic stellate cells (Yano et al., 2008). In another study, Yano et al. reported that increased cytokine levels may contribute to an increase in PIGF levels (Yano et al., 2006). Various studies have reported that ACR supports inflammation, and that increased ROS levels promote a rise in the levels of proinflammatory cytokines (Ghorbel et al., 2015; Sengul et al., 2021). Another previous study reported significantly increased TNF-α and IL-1β levels in acrylamide-induced neurotoxicity (Santhanasabapathy et al., 2015). Researchers have also reported that, in addition to these cytokines, IL-6 also increased with acrylamide toxicity (Ghorbel et al., 2015). The increase in PlGF in the present study may have occurred through this mechanism. Although we did not measure proinflammatory cytokine levels, oxidative stress and DNA damage markers were correlated with increased PlGF in acrylamide exposure.

Oxidative stress plays a key role in the pathogenesis of acrylamide toxicity (Sengul et al., 2021; Uthra et al., 2022). The oxidative stress induced by acrylamide is related to changes in cellular antioxidant status. A previous study of acrylamide-induced nephrotoxicity observed that renal lipid peroxidation raised MDA levels and also resulted in a significant reduction of GSH, GPx, SOD, and CAT activities (Sengul et al., 2021). Significantly increased expression of Caspase-3, a key protease activated in the early period of apoptosis, is occurs in the sciatic nerves, spinal cord, and kidneys of rats treated with acrylamide (Li et al., 2006; Sengul et al., 2021). In the present investigation, and similarly to previous studies, acrylamide significantly increased the immunodensity of apoptosis protein Caspase-3 and stimulated apoptosis (Seydi, 2015). An increase was also determined in the immunoactivity of 8-OHdG, the principal indicator of DNA damage, in the ACR group. Similarly, there are studies showing marked 8-OHdG elevation in groups treated with acrylamide compared to normal healthy groups (Bin-Jumah et al., 2021; El-Beltagi, 2016).

The histopathological investigations in this study also corroborated the hepatotoxic effects of acrylamide. Previous studies have demonstrated that acrylamide causes severe histopathological changes in the liver (Gao et al., 2022; Gedik et al., 2017). Researchers reported that these changes in liver tissue are based on oxidative stress (Gedik et al., 2017). Our histopathological results also support this view corelated with literature.

In addition, in the present study, treatment with oleuropein after acrylamide intoxication reduced Caspase-3 and 8-OHdG expression levels in liver tissues. This may be attributed to the fact that oleuropein exhibits antioxidative and anti-inflammatory activities (Bakir et al., 2018). The hydroxyl groups (especially

the 1,2-dihydroxybenzene part) in the chemical structure of oleuropein can donate hydrogen to prevent oxidation, bestowing strong antioxidant activity (Hassen, 2015). In agreement with our findings, it has been proved in various experimental toxicology models that oleuropein is capable of reducing 8-OHdG formation and oxidative stress (Bakir et al., 2018; Koc et al., 2019). Numerous previous studies have also shown that oleuropein attenuates neuronal toxicity (Khalatbary and Ahmadvand, 2012; Khalatbary et al., 2015), myocardial damage and liver toxicity (Jemai et al., 2020) by mitigating several apoptotic factors (Manna et al., 2004).

Studies specifically addressing the hepatoprotective effects of oleuropein show that it attenuates 3-Nitrotyrosine formation, and NF-kB and Caspase-3 activation (Domitrovic et al., 2012). In addition, immunohistochemical analyses have confirmed that oleuropein regulates smooth muscle α-actin, toll-like receptor-4, NADPH oxidase, collagen α1 types I and III, transforming growth factor β1, and fibroblast growth factor receptor 1, finally reducing liver fibrosis and necrosis (Kim et al., 2014).

CONCLUSION

The findings of this study strongly support the idea that PlGF is a marker of acrylamide toxicity and correlates with increased apoptosis and DNA damage in liver tissue.

Taken together, our findings show that oleuropein reduces increased liver damage and PlGF expression levels induced by acrylamide treatment in rats. The mechanism underlying these effects may be at least to some degree related to the protection provided by oleuropein against acrylamide-induced apoptosis and DNA damage in the rat liver. On the other hand, it is a limitation in this study that the mRNA expression levels of PlGF, Caspase-3 and 8-OHdG have not been investigated. It is recommended to use western blot and qRT-PCR methods in studies to elucidate the underlying mechanisms.

DECLARATIONS

Ethics Approval

This study was approved by the Balıkesir University Ethical Committee (number 2022/10-2 with dated 05.01.2023).

Conflicts of Interest

The authors declare that they have no conflict of interest.

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Author Contribution

Idea, concept and design: MT

Data collection and analysis: MT, KKT

Drafting of the manuscript: KKT

Critical review: MT, KKT

Data Availability

The data used to prepare this manuscript are available from the corresponding author when requested.

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