Fish Physiol Biochem (2023) 49:441–454
https://doi.org/10.1007/s10695-023-01193-6

RESEARCH

Dietary effect of grape (*Vitis vinifera)* seed extract mitigates hepatic disorders caused by oxidized fish oil in rainbow trout (*Oncorhynchus mykiss*)

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Received: 1 December 2022 / Accepted: 6 April 2023 / Published online: 25 April 2023

Abstract The major goal of this study was to determine the effect of grape seed extract (GSE) on liver damage in rainbow trout (*Oncorhynchus mykiss*) that was caused by the consumption of dietary oxidized fish oil (OFO). Rainbow trout were fed six different experimental diets coded OX-GSE 0 (OFO diet), OX-GSE 1 (OFO and 0.1% GSE), OX-GSE 3 (OFO and 0.3% GSE), GSE 0 (fresh fish oil and 0.0% GSE), GSE 1 (fresh fish oil and 0.1% GSE), and GSE 3 (fresh fish oil and 0.3% GSE) for 30 days. The lowest % hepatosomatic index (HSI) result was calculated in fish fed with OX-GSE 0 and the highest HSI was determined in fish fed with GSE 1 diets ($p<0.05$). Histopathologically, hydropic degeneration in hepatocytes significantly increased OX-GSE 0 and GSE 3 compared to GSE 1 diets ($p<0.05$). Deposition of lipid droplets in hepatocytes was significantly increased in OX-GSE 0 and OX-GSE 3 groups than others ($p<0.05$). Liver biochemistry parameters such as superoxide dismutase (SOD), glutathione (GSH), and malondialdehyde (MDA) were significantly affected by OX and GSE treatments ($p<0.05$). There were significant differences in alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) among the liver

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enzymes analyzed in serum in OX and GSE ($p < 0.05$), meanwhile no difference was observed in lactate dehydrogenase (LDH) values between groups ($p > 0.05$). In conclusion, liver biochemistry and histopathology of rainbow trout consuming diets containing oxidized fish oil were negatively affected. However, it was determined that the supplementation of 0.1% GSE to the diet had a significant ameliorative role in these adverse effects.

Keywords Oxidized oil · Grape seed extract · Liver biochemistry · Oxidative stress · Antioxidant capacity

Introduction

Fish needs polyunsaturated fatty acids (PUFA), including docosahexaenoic acid (DHA, 22:6, n-3), and eicosapentaenoic acid (EPA, 20:5, n-3) in sufficient levels to maintain optimal growth, skeletal development, and all physiological functions (Meng et al. 2019; Sargent et al. 1995). The most effective fat source in the diet of rainbow trout, like in other farmed fish species, is fish oil, which is high in PUFA. If adequate care is not exercised in the production and storage of these high-fat diets, the fats can readily be autoxidized by exposure to ambient oxygen (Hsieh and Kinsella 1989). Furthermore, this oxidative process can be accelerated by the presence of ions and exposure to light and heat (Turner et al. 2006). Aldehydes, ketones, alcohols, hydrocarbons, volatile organic acids, and epoxy compounds, as well as additional oxidation products including hydroperoxides, are typically produced when omega-3 fatty acids are oxidized (DeLany et al. 2000; Fontagné et al. 2008; Labuza and Dugan Jr 1971), and these toxic substances may negatively affect the health and development of fish (Laohabanjong et al. 2009).

Fish fed oxidized oil diets had lower feed intake, survival, and growth performance, as well as depleted vitamin E reserves in tissues, hemolysis, liver degeneration, and skeletal deformation (Chen et al. 2013; Hamre et al. 2001; Peng et al. 2016). Moreover, oxidized fish oil not only causes oxidative stress by increasing reactive oxygen species (ROS) but also affects antioxidant enzymes (Yang et al. 2015). As a result, fish that consume oxidized oil sources employ their antioxidant systems to combat oxidative stress and the harm it causes (Chen et al. 2012). ROS is mainly composed of hydroxyl radicals (•OH), hydrogen peroxide (H_2O_2), and superoxide anions (O_2) (Misra and Niyogi 2009; Teimouri et al. 2019; Vutukuru et al. 2006). Antioxidant defense enzymes such as NADH/NADPH, reduced glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) are effective against oxidative stresses caused by oxidized oil in fish. For this reason, monitoring the differences in these enzymes is a useful tool for understanding the oxidative stress in fish and the damage caused by this stress (Kitts et al. 2012; Yuan et al. 2014; Zhang et al. 2021). In the studies about the toxicity of oxidized oil, above this ratio was used such as 100, 200, 300 and 400 meq O_2/kg levels were frequently used in that studies (Chen et al. 2019; Long et al. 2021). Malondialdehyde (MDA) is a type of aldehyde that is produced when arachidonic acid is oxidized, or when polyunsaturated fatty acids undergo non-enzymatic oxidative degradation. MDA is a well-known form of lipid peroxide (Alak et al. 2021). In addition, high levels of ROS, depletion of antioxidant defense enzymes, and hepatocellular damage cause an increase in liver enzymes such as alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) in the blood (Elbialy et al. 2021). Serum liver enzymes are frequently used to diagnose liver injury; on the other hand, histopathological examinations are the most effective method for explaining the damage. The most prevalent histological alterations of the liver, such as hepatocyte vacuolization, fatty degeneration of the liver, changes in the liver parenchyma, and necrosis, were generally categorized by semiquantitative scoring in investigations on the influence of modified fish diets on liver health (Acar et al. 2021; Demirci et al. 2021; Figueiredo-Silva et al. 2005).

Various researches have shown that diverse portions of fruits, vegetables, and grains are also rich in flavonoids, which are antioxidant phytochemicals (Rice-Evans 2001). Grape seed extract (GSE) contains polyphenolic components such as catechin, epicatechin, gallic acid, and proanthocyanidins (Alves et al. 2018; Chedea et al. 2010; Mostafavi et al. 2022; Perumalla and Hettiarachchy 2011; Shekarabi et al. 2022). In addition, GSE is known to have antioxidant as well as antimicrobial, anti-inflammatory, and antiapoptotic effects (Mehrinakhi et al. 2021; Pasqua et al. 2016). It has been suggested that the combination of grape-origin products in fish diets has remarkable benefits in aquaculture industry world-wide. It has been shown that the supplementation of GSE enhances fish growth performance and flesh quality, and also considerably improves fish antioxidant status, immunological responses, and

disease resistance (Kesbiç and Yigit 2019; Mehrinakhi et al. 2021; Mohammadi et al. 2021; Zhai et al. 2014). However, the potential of GSE for ameliorative effect on damage caused by oxidized fish oil in fish liver remains to be elucidated. As a result, the current study used morphometric, histological, and biochemical procedures to illustrate the liver curative effect of grape seed extract in rainbow trout-fed oxidized fish oil.

Materials and methods

Preparation of oxidized fish oil and experimental diets

Oxidized fish oil was produced by keeping a laboratory oven (Nüve EN 120, Turkey) at a stable temperature and continually aerating it. A flask was filled with fresh fish oil, set it in a 70 °C constant temperature laboratory oven (Nuve FN 120, Turkey), inserted the air pump pipe, and aerated it for two days (Shi et al. 2021). The peroxide value was analyzed and calculated in triplicate at the end of the oxidation process, according to Cunniff and Washington (1997) (Cd 8b-90). The peroxide value of fresh fish oil was 8.57 ± 0.92 meqO_2/kg, while the oxidized fish oil was 300.54 ± 3.35 meqO_2/kg (Chen et al. 2019; Kop et al. 2019). In this experiment, six isonitrogenous (CP: 45.51%) and isolipid (CF: 15.75%) experimental diets were designed. The formulation of experimental diets was given in Table 1. Only, industrial fish oil (SÜRSAN A.Ş. Samsun, Turkey) produced by anchovy was used in two different forms fresh and oxidized as the main fat source in the feed formulation. Grape seeds (Syrah Grape aka Shiraz (*Vitis vinifera*)) used in the extraction process were obtained from a commercial wine production company and also, the GSE used in this study was produced with the method that was previously tested in the rainbow trout study by Kesbic and Yiğit (2019).

To investigate this, six different experimental diets OX-GSE 0 (containing oxidized fish oil diet), OX-GSE 1 (containing oxidized fish oil and 0.1% grape seed extract), OX-GSE 3 (containing oxidized fish oil and 0.3% grape seed extract), GSE 0 (containing fresh fish oil and 0.0% grape seed extract), GSE 1 (containing fresh fish oil and 0.1% grape seed extract), and GSE 3 (containing fresh fish oil and 0.3% grape seed extract) were fed to rainbow trout for 30 days.

Table 1 Formulation, proximate composition and fatty acid content of the experimental diets

%	OX-GSE 0	OX-GSE 1	OX-GSE 3	GSE 0	GSE 1	GSE 3
Fish meal	45	45	45	45	45	45
Soybean meal	30	30	30	30	30	30
Corn starch	4	4	4	4	4	4
Wheat flour	6	5.90	5.70	6	5.90	5.70
Vit.-min. Mix[1]	4	4	4	4	4	4
Fresh fish oil[2]	–	–	–	11	11	11
Oxidized fish oil[3]	11	11	11	–	–	–
Grape seed extract[4]	0	0.1	0.3	0	0.1	0.3
g/100 g diet	Nutritional content					
Protein	45.87	45.41	45.77	45.51	45.47	45.57
Fat	15.72	15.66	15.35	15.75	15.60	15.72
Ash	8.18	8.31	8.30	8.25	8.23	8.27

[1]Vitamin-mineral premix: Vitamin A, 4×10^6 IU/kg; Vitamin D_3, 4×10^5 IU/kg; Vitamin E, 4×10^4 mg/kg; Vitamin K_3, 2400 mg/kg; Vitamin B_1, 4000 mg/kg; Vitamin B_2, 6000 mg/kg; Vitamin B_6, 4000 mg/kg; Vitamin B_{12}, 10 mg/kg; Vitamin C, 4000 mg/kg; Niacin, 4000 mg/kg; Calcium d–pantothenate, 4000 mg/kg; D-biotin, 100 mg/kg; Folic Acid, 1200 mg/kg; Inositol, 6×10^4 mg/kg

[2]Peroxide value (POV): 8.57 ± 0.92 meqO_2/kg

[3]300.54 ± 3.35 meqO_2/kg

[4]Catechin: 0.667 ppm, epicatechin: 0.819 ppm (Kesbiç and Yigit 2019)

OX-GSE 0 oxidized fish oil diet, *OX-GSE 1* oxidized fish oil and 0.1% grape seed extract, *OX-GSE 3* oxidized fish oil and 0.3% grape seed extract, *GSE 0* fresh fish oil and 0.0% grape seed extract, *GSE 1* fresh fish oil and 0.1% grape seed extract, *GSE 3* fresh fish oil and 0.3% grape seed extract

Experimental procedure

Rainbow trout (*Oncorhynchus mykiss*) fingerlings with a mean body weight of 11.01 ± 1.25 g were allocated randomly to 100-L aquariums with 10 fish/tank (30 fish each group) and acclimatized to the rearing conditions two weeks before the experiment started. During the acclimatization period fished was fed with basal diet. The feeding experiment was carried out in a laboratory type recirculating aquaculture system (RAS). The daily water exchange rate of the unit was limited to 10%. Water quality parameters of the system were measured and recorded throughout the feeding experiment. Dissolved O_2 7.9 ± 0.3 mg/L, pH 7.6 ± 0.3 and temperature 13.6 ± 0.2 °C values were recorded throughout the experiment. To achieve visual satiation, fish were hand-fed three times a day (09:00, 12:00, and 19:00). During a 4-week feeding experiment, each diet was supplied to three replicate groups of fish and natural photoperiod was applied throughout the feeding trial. At the end of the feeding experiment, feed was withheld for 24 h before sampling. At the end of the 4-week feeding trial, three fish from each replication were captured, anesthetized (benzocaine, 30 mg/L), and blood samples were collected from the caudal vein using heparinized syringes. Following euthanasia with over-dose anesthesia (100 mg/L) (Neiffer and Stamper 2009), the fish's liver was dissected and weighed in order to calculate hepato-somatic indices (HSI). The hepatosomatic index by HSI, % = [liver weight (g)/total body weight (g) × 100] (Htun-Han 1978). The part of liver tissue was fixed in formalin for histopathological analysis. The remaining liver tissue was frozen in liquid nitrogen and stored at − 80 °C until it was time to measure antioxidant parameters.

Morphometric measurements

In the feeding experiment, rainbow trout (six fish per group) were euthanized with a high-dose anesthetic and necropsied for liver sampling. Morphometric measurements (width and length) of the liver and gallbladder were made with a digital caliper (Insize). For histopathological analysis, the measured tissues were preserved in histological cassettes in a 10% formalin solution.

Histopathological analysis

From being sliced, the tissues were transferred to cassettes. After the cassettes were cleaned up under clean water and a paraffin block was placed, routine histopathology follow-up was done. Pieces of 5 µm thick paraffin blocks that were sliced using a microtome and put on sticky slides with coverslips underwent hematoxylin and eosin staining. Then, sections were inspected under a light microscope. Histopathological changes were scored semiquantitatively as follows: − (0): absent; + (1): mild; + + (2): moderate; + + + (3): severe (Demirci et al. 2021; Öz et al. 2020).

Biochemical analyses

Preparation of tissue homogenates

Liquid nitrogen was used to shatter plate plate-derived tissues in a porcelain mortar. They were then placed in sterile Eppendorf tubes after being weighed to 25 mg, and homogenate buffers suitable for the enzymes under investigation were then added (LPO: 10% KCl; SOD: 50 mM KH2PO4; GSH: 50 mM Tris–HCl; CAT: 50 mM KH2PO4, pH 7). Samples in the buffers were homogenized using a tissue homogenizer (TissueLyser II, QIAGEN, Germany) with a 5-mm steel ball for 1 min at a frequency of 35 Hz. After that, the homogenates were centrifuged in a chilled centrifuge (Universal 320 R, Hettich GmbH & Co. KG, Germany) at 4 °C and 4000 rpm for 30 min for LPO and GSH (Ohkawa et al. 1979; Sedlak and Lindsay 1968), and 6000 rpm for an hour for SOD (Sun et al. 1988). After that, the isolated supernatants were measured using the relevant techniques.

Determination of lipid peroxidation levels

The technique based on the reaction between thiobarbituric acid and malondialdehyde developed by Ohkawa et al. (1979) was used to determine the amounts of lipid peroxidation (LPO) in liver tissue. Utilizing a standard graph generated with 1,1,3,3-tetramethoxypropane, absorbances measured at 532 nm by a spectrophotometer (Bio-Tek EPOCH, Bio-Tek, USA) were used to compute the tissue LPO level, with the findings represented as nmol MDA/g tissue(Ohkawa et al. 1979).

Determination of superoxide dismutase enzyme activity

A technique based on the measurement of formazan dye reduction by superoxide radicals with xanthine oxidase activity was utilized to measure the superoxide dismutase (SOD) activities in liver tissue (Sun et al. 1988). The activity was estimated using the equation given in the referenced literature and represented as U/mg tissue from absorbances obtained at 560 nm.

Determination of glutathione levels

A previously published technique was used to assess the glutathione (GSH) levels in the tissue samples of the liver. (Sedlak and Lindsay 1968). At 412 nm, the samples' GSH levels were measured and represented as nmol/mg of liver tissue.

Blood sampling and biochemical analyses

A total of 9 fish (3 fish per tank) from each group were administered blood samples at the finish of the 30-day feeding trial. Benzocaine (30 mg/L) was utilized to anesthetize the fish before application (Neiffer and Stamper 2009). Using a 1-ml plastic syringe, blood was drawn from the caudal vein and placed into serum tubes (MiniCollect® Tubes) that were also centrifuged at 5000 g for 10 min (Yılmaz et al. 2015). Serum samples that had been separated were kept at − 80 °C for further biochemical evaluation. A spectrophotometer and commercial kits (Bioanalytic Diagnostic Industry, Germany) were used to assess the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) following the manufacturer guidelines.

Statistical analysis

The normality, of the data, was tested with Shapiro–Wilk and parametric results were statistically analyzed using one-way analysis of variance (ANOVA). The differences between means were tested by Tukey as a post-doc test ($p < 0.05$). Non-parametric data were analyzed by using the Kruskal–Wallis test followed by the Mann–Whitney *U* test as post-hoc. The value of $p < 0.05$ is considered statistically significant. All data are presented as mean ± standard deviation. Statistical differences of data were analyzed by using IBM SPSS Statistics 25.0 software.

Results

Morphometric and histopathological results

Table 2 shows morphometric measures of the liver and gallbladder, as well as histological results of the liver. The heptosomatic index (HSI) of rainbow trout that were fed the OX-GSE 0 group feed decreased

Table 2 Morphometric, gravimetric and histopathologic examination of the liver sampled from *O. mykiss* fed with different experimental diets

	OX-GSE 0	OX-GSE 1	OX-GSE 3	GSE 0	GSE 1	GSE 3	*P* value
Hepatosomatic index (HSI %)*	1.21 ± 0.28^{b}	1.30 ± 0.26^{ab}	1.42 ± 0.23^{ab}	1.33 ± 0.32^{ab}	1.54 ± 0.29^{a}	1.49 ± 0.14^{ab}	0.012
Liver width (mm)	9.33 ± 1.51	9.75 ± 0.76	9.24 ± .0.99	9.48 ± 1.01	8.93 ± 1.19	9.71 ± 1.38	0.699
Liver length (mm)	8.61 ± 0.97	8.39 ± 0.81	9.51 ± 1.44	8.79 ± 0.91	9.39 ± 0.73	9.26 ± 1.69	0.203
Gallbladder width (mm)	2.83 ± 0.65	3.17 ± 0.77	2.87 ± 0.37	2.99 ± 0.53	2.85 ± 0.37	3.07 ± 0.44	0.715
Gallbladder length (mm)	7.09 ± 0.93	6.41 ± 2.74	6.49 ± 0.99	7.46 ± 0.91	8.25 ± 0.97	7.38 ± 0.93	0.71
Hydropic/vacuolar degeneration	2.10 ± 0.73^{a}	1.44 ± 0.52^{a}	1.33 ± 0.50^{ab}	0.62 ± 0.74^{bc}	0.44 ± 0.52^{c}	1.55 ± 0.72^{a}	0.00
Lipidosis	1.00 ± 0.47^{a}	0.22 ± 0.44^{b}	1.22 ± 0.66^{a}	0.12 ± 0.35^{b}	0.00 ± 0.00^{b}	0.77 ± 0.97^{ab}	0.00

Data represent mean ± SD from two fish per each tank ($n = 6$)

Values with different letters in the same row indicate significant differences between their groups ($P < 0.05$)

OX-GSE 0 oxidized fish oil diet, *OX-GSE 1* oxidized fish oil and 0.1% grape seed extract, *OX-GSE 3* oxidized fish oil and 0.3% grape seed extract, *GSE 0* fresh fish oil and 0.00% grape seed extract, *GSE 1* fresh fish oil and 0.1% grape seed extract, *GSE 3* fresh fish oil and 0.3% grape seed extract

*Hepatosomatic index (HSI) (%) = 100 × (liver weight/total body weight)

significantly compared to those that were fed the GSE 1 group feed ($p < 0.05$). The morphometric assessments of liver and gallbladder size revealed that the oxidation of fat sources and/or GSE supplementation in the experimental diets supplied to the fish did not result in significant changes in gallbladder and liver size ($p > 0.05$).

Histopathologically, the minimum level of hydropic degeneration, defined as hepatocyte enlargement by water absorption, was found statistically in the GSE 1 group (Fig. 1B), whereas the highest level of hydropic degeneration was detected in the livers of trout fed OX-GSE 0 and GSE 3 group diets ($p < 0.05$, Fig. 1A–C). Lipidosis, or the deposition of lipid droplets in hepatocytes, was not detected in the livers of fish fed GSE 1 group diets; however, it increased significantly in the livers of fish fed oxidized fatty feeds ($p < 0.05$, Fig. 1 D–F).

Tissue biochemical results

Antioxidant properties of rainbow trout livers were examined for the SOD activity and GSH levels. The SOD activity was shown to be maximum in rainbow trout livers fed OX-GSE 0 group diets as compared to the other experimental groups (Fig. 2A). It was also observed that adding GSE to the diets significantly reduced the SOD activity in all other experimental groups ($p < 0.05$). There was no statistically significant difference in the level of GSH between the OX-GSE 1, OX-GSE 3, and GSE 3 groups ($p > 0.05$). However, there was a statistically significant increase in the level of GSH in both the OX-GSE 0 and GSE 1 groups ($p < 0.05$, Fig. 2B). The OX-GSE 0 group had the highest MDA level that is an indicator of lipid peroxidation in the cells (Fig. 2C). The level of MDA was significantly lower in the OX-GSE 3, GSE 0, and GSE 1 groups compared to the other study groups ($p < 0.05$).

Blood serum biochemical results

Serum ALT levels of rainbow trout fed with oxidized fatty diets were significantly increased. GSE supplementation significantly decreased serum ALT levels in the oxidized fatty diets, whereas 0.3% of GSE in fresh fish oil diets significantly increased ALT levels ($p < 0.05$, Fig. 3A). The same significant differences were observed in AST levels ($p < 0.05$, Fig. 3B). A significant increase in serum ALP value was observed with oxidized fat consumption of fish. In addition, GSE supplementation to the diets significantly decreased serum ALP levels in both oxidized and fresh oil fed fish ($p < 0.05$, Fig. 3C). LDH levels in the oxidized oil and grape seed extract groups were not significantly different ($p > 0.05$, Fig. 3D).

Discussion

It is a reality underlined by research that fish oils with a peroxide value higher than 20 meqO_2/kg are not a wise option as they are a source of peroxidants in fish feeds (Korkut et al. 2007). The fresh fish oil used in the current investigation had a peroxide value of 8.57 ± 0.92 meqO_2/kg. The oil that underwent experimental thermal oxidation increased in peroxide value to 300.54 ± 3.35 meqO_2/kg. Fish oil is still the most effective source of fat in aquafeeds for rainbow trout because it is rich in PUFA, particularly eicosapentaenoic acid (EPA, 20:5, n-3) and docosahexaenoic acid (DHA, 22:6, n-3) (Oliva-Teles et al. 2022). Because once exposed to air oxygen, these omega-3 fatty acids in feeds can rapidly oxidize (Hsieh Kinsella 1989) that can also lead to physio-metabolic disorders in fish (Yu et al. 2022). Recently, antioxidant activity has been provided using the naturally generated active component of grape seed extract (Priyadarshi et al. 2021). For that reason, the preventive effects of GSE on rainbow trout-fed oxidized oil were the main focus of the current study.

Oxidized fish oils in fish diets cause oxidative stress and affect antioxidant enzymes, mitochondrial dysfunction, and lipid peroxidation (Yang et al. 2015; Yin et al. 2019; Zhang et al. 2021). All aerobic organisms typically engage two different types of antioxidant defense mechanisms to prevent lipid peroxidation, such as low molecular weight free radical scavengers and antioxidant enzymes (Fontagné et al. 2008). The use of some synthetic antioxidants such as ethoxyquin (Ojeu 2022) in fish feed is prohibited and/or restricted by the authorities, mainly due to residual problems (OJEU 2003). SOD, CAT, and GPx are important antioxidant enzymes (Zhang et al. 2021). Recent research demonstrated that feeding oxidized lipids to rainbow trout enhanced the liver's SOD and GPx enzyme levels (Chen et al. 2012; Yin et al. 2019; Yuan et al. 2014). All cell compartments contain

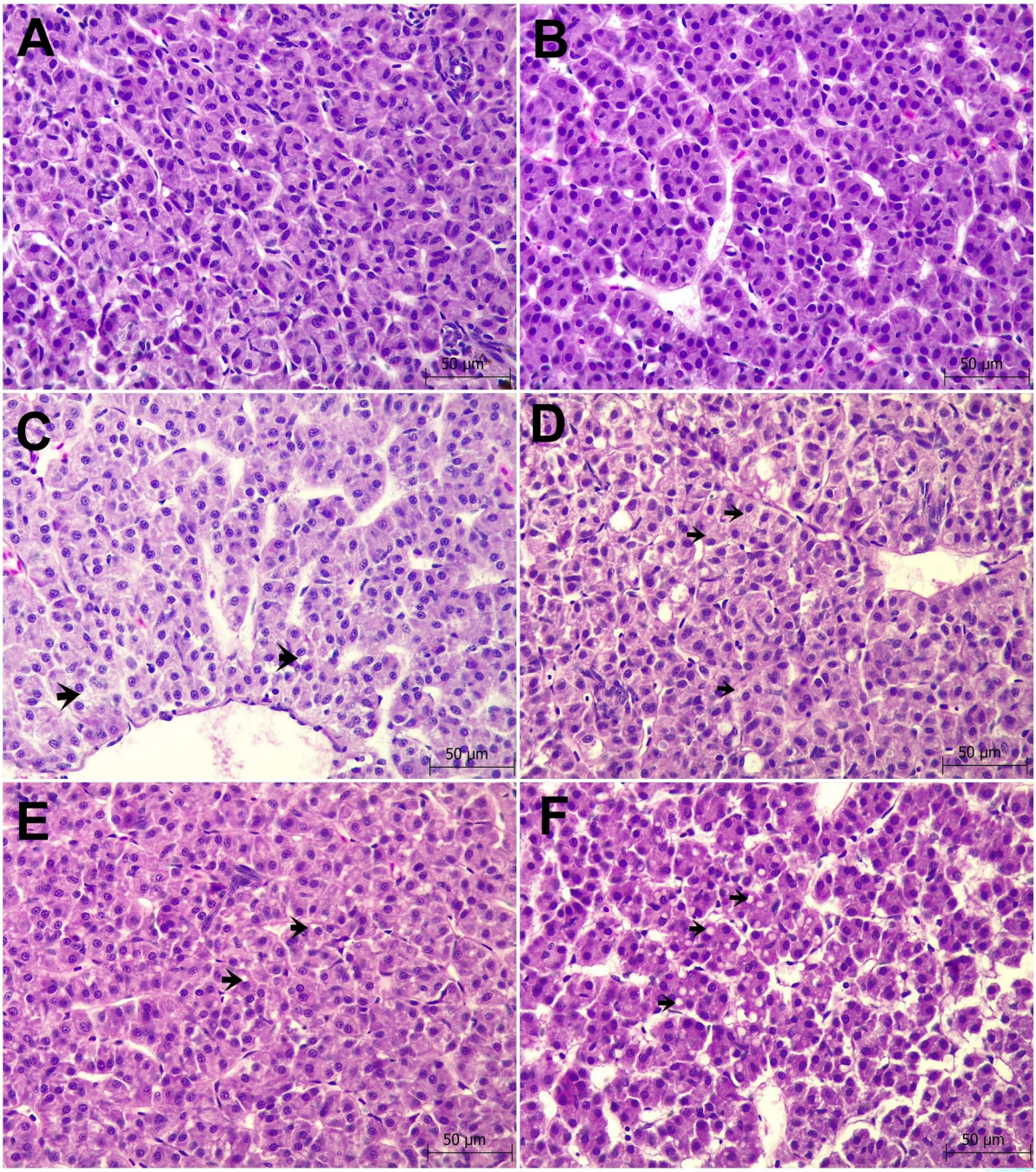

Fig. 1 Hematoxylin–eosin (H&E) staining. rainbow trout, liver. **A**, **B** Normal appearance of hepatocytes. GSE 0 and GSE 1 groups. Bar: 50 µm. **C** Moderate hydropic degeneration (black arrows) in the cytoplasm of hepatocytes.GSE 3 group. Bar: 50 µm. **D** Mild hydropic degeneration (black arrows) and lipid droplet in the cytoplasm of hepaocytes. OX-GSE 0 Bar: 50 µm. **E** Mild hydropic degeneration (black arrows) in the cytoplasm of hepatocytes. OX-GSE 1 group. Bar: 50 µm. **F** Lipid droplet in the cytoplasm of hepaocytes. OX-GSE 3 groups. Bar: 50 µm

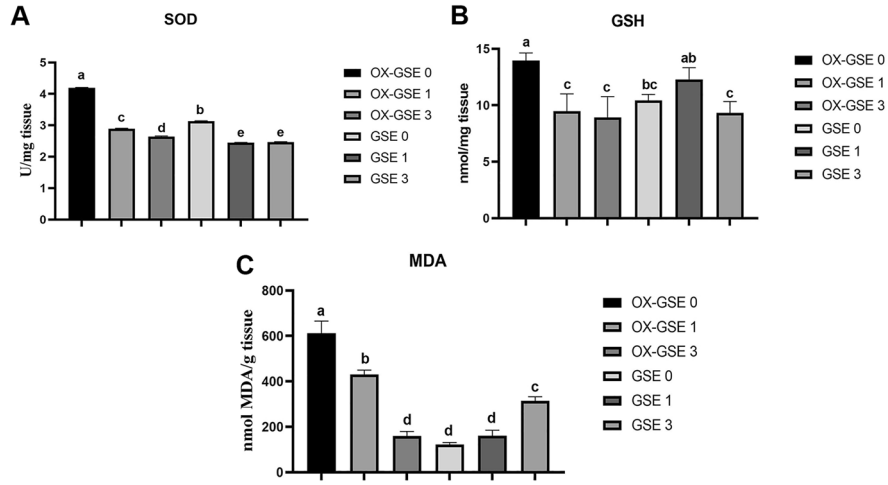

Fig. 2 The effects of 30 days OX and GSE administration on liver SOD (**A**), GSH (**B**), and MDA (**C**) activities in rainbow trout. Different letters above the bars show significant differences among the treatments ($n = 6$)

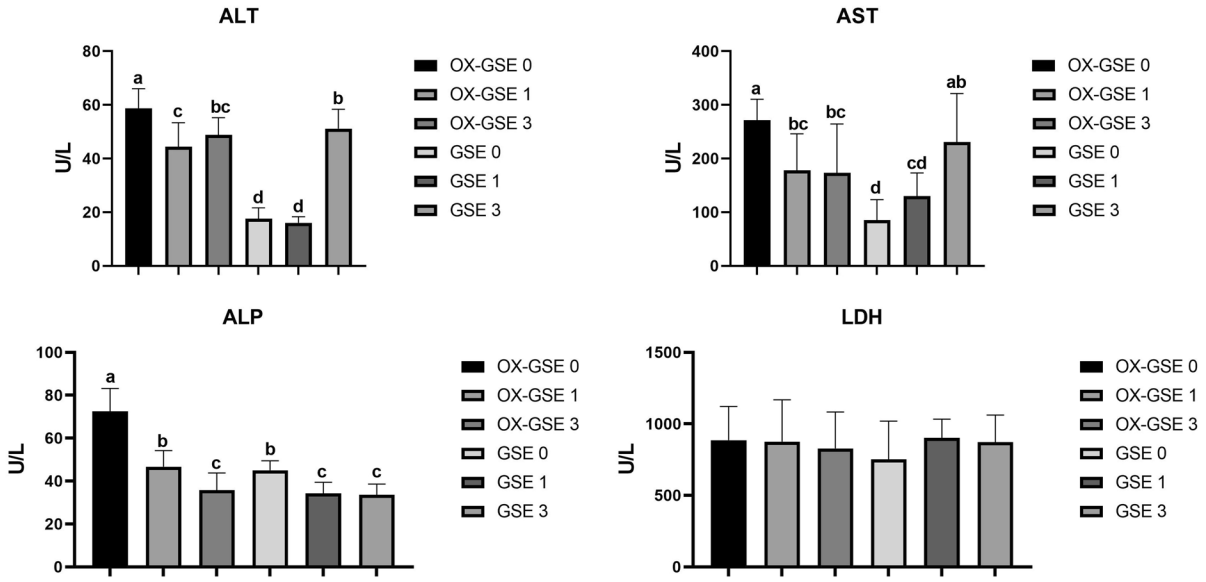

Fig. 3 The effects of 30 days OX and GSE administration on serum ALT (**A**), AST (**B**), ALP (**C**), and LDH (**D**) activities in rainbow trout. Different letters above the bars show significant differences among the treatments ($n = 18$)

large amounts of GSH that is the main soluble antioxidant. Additionally, GSH serves as a cofactor for several detoxification enzymes, including GPx and transferase (Birben et al. 2012). In the current study, antioxidant parameters such as SOD, GSH, and MDA measured in liver tissue were significantly increased in trout fed with oxidized fish oil (OX-GSE 0) compared to those fed with fresh fish oil (GSE 0). However, GSE supplementation to the diets significantly decreased the antioxidant parameter measurements in liver tissue, especially in fish consumed oxidized fish oil groups ($p < 0.05$). Grape seeds are well-known for their effective antioxidant cocktail, including includes phenols, catechins, epicatechins, procyanidins, and proanthocyanidins (Gabetta et al. 2000). Polyphenolic grape seed products improve fish growth performance, feed utilization, and antioxidant characteristics (Arslan et al. 2018; Kesbiç and Yigit 2019). In our investigation, GSE supplementation reduced liver SOD activity and GSH levels in rainbow trout-fed diets including dietary oxidized fish oil. In summary, oxidized oil triggered oxidation in contrast that, grape

seed product increased antioxidant defense. Arslan et al. (2018), has been supplemented *Oncorhynchus mykiss* juveniles' diet with 250, 500, and 1.000 mg/kg grape seed crude oil and reported that SOD activity was lower than the control group, while GPx enzyme activity increased in the 1.000 mg/kg feed group compared to the control group. In a previous study, Epigallocatechin-3-gallate (EGCG), one of the flavan-3-ol monomers with known antioxidant properties in grape seed extract, was added to rainbow trout feeds; however, no change in hepatic SOD activity was detected in fish that consumed these feeds (Thawonsuwan et al. 2010). In a previous study, dandelion flower extract (DF) reported to be rich in phenolic substances such as GSE was added to rainbow trout diets. It was reported that SOD gene expression of fish fed with DF-supplemented diets decreased in rainbow trout (Mostafavi et al. 2022). SOD activity reduced dramatically depending on the amount of GSE supplemented into the diets in our current study. Due to their high concentration of bioactive substances, previous and current studies have shown that using plant sources as a feed supplement in aquafeed can equilibrate the generation of ROS and the antioxidant system of fish. The GSH level was shown to be considerably lowered inversely proportional to the GSE ratio in the experimental diets using oxidized fish oil, however, the GSE supplemented groups were unable to show a significant difference compared to the control in the experimental diets utilizing fresh fish oil. GSE was increased in the GSE 1 group compared to GSE 0 but reduced in the GSE 3 group. As a consequence, it was revealed that adding 0.1% grape seed extract to the diet of rainbow trout greatly enhanced hepatic antioxidant enzymes.

Malondialdehyde (MDA) is a reactive end product of lipid peroxidation (Gęgotek and Skrzydlewska 2019) and is toxic. Thiobarbituric acid reactive material concentration is used to monitor lipid peroxidation in tissues and diet. Also according to research, the quantity of oxidized fish oil in the diet of many fish species. The study about hybrid grouper (♀ *Epinephelus fuscoguttatus*×♂ *Epinephelus lanceolatus*) reported that hepatic MDA levels increased by the percentage of oxidized fish oil (POV: 231 mmol/kg) in diet (Long et al. 2021). Oxidated fish oil and MDA relation result have been presented in another study about different fish species Largemouth black bass (*Micropterus salmoide*). It has been reported that liver MDA levels increased in fish fed with diets containing oxidized fish oil (128.5 meq O_2/kg) even with vitamin E supplementation (Chen et al. 2013). The same results have been reported in a different study on Atlantic halibut (*Hippoglossus hippoglossus*), in fish consuming oxidized fish oil (POV = 94.3 meq O_2/kg), liver MDA levels increased depending on the level of fat oxidation. (Lewis-McCrea and Lall 2007). To solve that problem, research has been carried out about the use of E and C vitamins as an antioxidant supplement for dietary oxidized oil and it was observed that dietary supplementation with antioxidant vitamins (E and C) can reduce hepatic MDA levels in the fish fed on oxidized oil diets (Chen et al. 2013; Gao et al. 2012, 2013; Lewis-McCrea and Lall 2007). Similar to prior research, we found that rainbow trout fed oxidized oil in its diet had higher levels of MDA in the liver. In our research, it was clearly demonstrated that liver MDA levels in rainbow trout decreased considerably once GSE was introduced to the oxidized oil diet. As a result, it was established that GSE could have an antioxidant impact after metabolic action, similar to E and C vitamins, that are known to have high antioxidant properties. The grape seed extract was demonstrated to be beneficial in reducing oxidized oil-induced hepatocellular damages. However, MDA levels in the liver of rainbow trout fed diets containing fresh fish oil climbed in proportion to the amount of GSE supplementation. This is assumed to be owing to GSE's pro-oxidant characteristics (Chedea et al. 2010). There was no significant change in the MDA level of 0.1% GSE addition to the experimental diet formulated with fresh fish oil in the current investigation, however, the liver MDA level of the fish fed 0.3% supplementation was dramatically elevated. Previous research on the pro-oxidant activity of GSE found that high dosages of GSE may have deleterious pro-oxidant effects (Shao et al. 2003). For this reason, it is predicted that the use of more than 0.1% GSE in diets containing fresh fish oil could have toxic effects on rainbow trout.

In our study, rainbow trout feeding an oxidized fat diet had liver hepatocytes that had hydropic degeneration and an increase in lipid droplets. In confirmation of the current study, it has been reported in previous studies that similar negative effects were observed in the liver of different fish species such as *Micropterus salmoides* (Chen et al. 2012), *Misgurnus anguillicaudatus* (Zhang et al. 2017), and hybrid grouper (♀

Epinephelus fuscoguttatus×♂ *Epinephelus lanceolatus*) (Long et al. 2021) that consumed oxidized oils in their feed. All these findings indicate that dietary supplementation with oxidized fat causes hepatocellular damage and fatty deposits in fish. In our study, it was found that the lipid droplets in hepatocytes caused by oxidized oil decreased with the supplementation of GSE. We also noticed that the addition of 3% GSE increased the degenerative changes in the hepatocytes of the fish. As a result, the addition of high doses of GSE to the fish diet negatively affected the health of the fish, as evidenced by histopathological examinations in the fish liver.

Fish serum AST and ALT metabolic enzyme activity analysis is a crucial diagnostic tool for identifying hepatotoxic alterations (Mousavi et al. 2020). In the present study results, rainbow trout fed oxidized oil in the diet had higher blood serum levels of ALT, AST, and ALP. Similar to the current findings, Xie et al. (2020) reported that *Micropterus salmoides* feeding a high oxidized oil (POV: 564 meq kg^{-1}) diet dramatically higher AST and ALT levels in its blood plasma than the control group (fresh oil diet). The current investigation found that adding GSE to diets prepared from oxidized oils considerably reduced the serum enzyme levels. In addition to this serum ALT and AST, values significantly increased in rainbow trout fed with fresh fish oil and with high-dose GSE extract (GSE 3). The most frequently used liver function tests to identify liver damage are ALT and AST. Increased ALT and levels suggest moderate tissue injury (Gowda et al. 2009; Hall and Cash 2012). Rainbow trout hepatocellular damage brought on by dietary oxidized oil was shown to be reduced and/or prevented by grape seed extract when both histopathological and blood serum enzyme test data were taken into consideration. However, it was observed that the supplementation of high doses of GSE in diet formulations prepared with fresh fish oil-induced liver damage in rainbow trout. In a previous study, adverse effects were observed in rainbow trout fed diets supplemented with 0.2% GSE (Kesbiç and Yigit 2019).

Previous research has suggested that the amount of time fish have been exposed to oxidized lipids in its diet could affect its liver weight and HSI (Chen et al. 2013; Yin et al. 2019). In this study, we discovered that rainbow trout given an oxidized lipid diet had a lower HSI value based on gravimetric measurement, even if we did not detect a statistically significant difference between the study groups in morphometric measures. Studies on fish fed diets containing oxidized lipids have similarly noted a decreased hepatosomatic index (Chen et al. 2013, 2012; Dong et al. 2012; Yin et al. 2019). In this study, gravimetric and morphometric measurements of the liver confirmed the histopathological scoring. As it is known, weight per unit volume decreases as lipid accumulation increases in tissues. A similar trend was observed in the liver of fish fed with OX-GSE0 group feeds. While morphometric measurements of the liver did not show any significant difference between the groups, the lowest HSI was observed in the OX-GSE0 group. Also, the highest lipid accumulation was detected in this group. The opposite of the described model was found in the GSE1 group, where the best results were obtained in almost all results.

One of the most crucial liver secretions associated with fat metabolism is bile acid, which is believed to aid in fat absorption and regulate cholesterol levels (Xie et al. 2020). Bile acids (BAs) play significant functions in lipid metabolism, are exclusively produced by the liver from cholesterol, and are kept in the gallbladder (Liao et al. 2020). The previous studies carried out about bile and gallbladder volume reported that the volume is directly related to macro nutritional elements of diets such as fat and protein (Grosell et al. 2000; Staessen et al. 2021)(Grosell et al. 2000; Staessen et al. 2021). In the present study, there was no statistically significant difference between the groups in the width and length measurements of the gallbladder in the groups supplemented with dietary oxidized oil and GSE. The fact that no significant difference in gallbladder size was observed in the experimental groups is considered to be due to the fact that all of the experimental feeds were isonitrogenous and isolipidic.

Conclusion

It was concluded as a result that the oxidized oil used in the preparation of the rainbow trout diet adversely affected serum and liver's biochemistry, liver histopathology, and that the groups over which 0.1% of grape seed extract was added to the diet formulation displayed a protective effect against the harmful effects brought on by the oxidized oil. The usage of high levels in oxidized and non-oxidized lipid groups resulted in negative effects on serum and liver biochemistry as well as fish histology, and it was also discovered that 0.3% grape seed extract

was above the tolerance limits of trout due to its potential pro-oxidant qualities. The findings of our research suggest that supplementing GSE to rainbow trout diets at a rate of 0.1% could protect the fish from any potentially harmful effects that may be caused by an oxidized oil diet. Therefore, the effects of GSE supplementation on growth in oxidized fat diets should be studied in other common aquaculture species, especially rainbow trout in future studies.

Acknowledgements The authors would like to thank to Kastamonu University Central Research Laboratuvary for supporting about sample storage.

Author contribution All authors participated in the study's design, interpretation of the findings and analysis of the data, and review of the manuscript. Osman Sabri Kesbic and Ümit Acar prepared oxidized fish oil and experimental ration. Funda Terzi, Osman Sabri Kesbiç, Huseyin Serkan Erol, Beste Demirci, and Süleyman Yıldırım carried out the animal experiments. Funda Terzi performed the histopathological analysis; Beste Demirci performed morphometric measurement; Huseyin Serkan Erol and Çağatay Salum carried out the biochemical analysis. All authors read and approved the final version of the manuscript.

Funding The histopathologic sections in this project were prepared with a microtome provided by the infrastructure project to the Kastamonu University Scientific Research Projects (Project No: KÜ-BAP05/2021–1).

Data availability Selected data will be made available on request.

Declarations

Ethical approval All animal experiments were approved by carried out by European Union Directive no: 2010/63 and the Local Ethics Committee of Kastamonu University (approval no: 2020/32(2200086733)). The animals were handled and used based on the international laboratory animal care and use guidelines. Furthermore, the study was conducted in agreement with the ARRIVE guidelines.

Consent for publication and consent to participate Not applicable.

Competing interests The authors declare no competing interests.

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