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Protective Role of Dietary Anthocyanidin Against Genotoxicity and Hepatotoxicity Influenced by Imidacloprid in the Nile Tilapia

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ABSTRACT

Neonicotinoid pesticides have been extensively used as plant protectants against weeds and insects. However, their unregulated usage has led to ecological pollution, causing severe impacts on non-target organisms, including fish and humans, who may be exposed to these chemicals through the consumption of contaminated fish meat. This research aimed to investigate the genotoxic and hepatotoxic influences of imidacloprid (IMI, a neonicotinoid pesticide) on juveniles *Oreochromis niloticus*, and assess the appropriate and affordable price treatment of grape seed extract "GRSE" (anthocyanidin) to minimize the IMI impacts. Exactly 120 fish, weighing 8.85± 0.09g, were distributed into four groups equally; group 1 was the control (fed 0% GRSE), group 2 nourished on 4% GRSE, and both groups were reared in IMI-free water. Group 3 received a 0% GRSE diet and was reared in 2.5 IMI mg/L in water, while the fourth was given a 4% GRSE diet and exposed to 2.5 IMI mg/L in water. After 30 days, blood was collected and analyzed for liver transaminases, lipid profile, leptin hormone and tumor necrosis factor-alpha (TNF-α). Liver tissue samples were assessed for total oxidant capacity (TOC), total antioxidant capacity (TAC) and DNA damage. Gills and livers were sectioned for histopathology; also, the liver was subjected to histochemical and caspase 3 immunohistochemical evaluations. IMI caused hepatotoxicity via enhancement of DNA damage and impairment of liver enzyme, lipid profile, and leptin hormone levels, as well as increased TNF-α and hepatic TOC levels. Additionally, IMI caused histopathological, histochemical and immunohistochemical alterations. These detrimental signs were fully or partially reversed by the administration of a diet enriched with 4% GRSE due to its strong antioxidant and immuno-stimulatory properties, counteracting the negative effects of IMI toxicity in *O. niloticus.*

INTRODUCTION

Neonicotinoids are pivotal organic insecticides that are commercialized worldwide. They are highly effective at low dosage, highly water-soluble, and non-volatile compounds against a broad spectrum of insects **(Vieira *et al.*, 2018)**.

Imidacloprid (IMI) was the first introduced neonicotinoid in 1991 for crop protection. It affects insects by blocking the acetylcholine receptors (nicotinic receptors) and preventing acetylcholine from conveying impulses and signals among nerves, ultimately leading to paralysis followed by insect death **(Clements *et al.*, 2016)**. Indeed, IMI is slowly degraded and absorbed in the soil, permitting it to flow into the groundwater. It can persist, with poor biodegradability with 30 days half-life **(Samojeden *et al.*, 2022)**. It has the potential to be used in aquaculture to reduce fish parasites. However, it can cause hematologic changes and disturb biological structures, leading to genotoxicity **(Iturburu *et al.*, 2018)**. Thus, the aquatic habitat is continuously polluted with chemicals, affecting the health and survival of the aquatic organisms, mainly fish **(El-Garawani *et al.*, 2021a)**.

Fish are non-target essential organisms in aquatic ecosystems since they transport energy from lesser basal trophic levels to advanced levels, which is a fundamental ecological role for food chains **(Almeida *et al.*, 2021)**. Moreover, fish are the most impacted species by pollution since they absorb contaminants through their gills and skin **(Alm-Eldeen *et al.*, 2018)**. Their tissues undergo morphological, histological, and biochemical changes due to their extended contact with aquatic pollutants, even at minimal concentrations **(Haredi *et al.*, 2020)**. The tilapia is a major human food source and is cultivated in most subtropical and tropical regions **(El-Garawani *et al.*, 2021a)**. Globally, the tilapia is the most prevalent freshwater fish aquaculture. It is considered the most commonly farmed fish species in Egypt, making up 24% of all fisheries production, and 43.5% of farmed fish **(H Omar *et al.*, 2021)**. *Oreochromis niloticus* (the Nile tilapia), is a significant commercial fish caught in Egypt, contributing significantly to the total annual capture along the River Nile **(Kadry *et al.*, 2015)**. It is especially resistant to many environmental stresses such as salt and can withstand highly polluted habitats **(El-Garawani *et al.*, 2022)**. Thus, it serves as an indicator species in ecotoxicological investigations **(Günal *et al.*, 2020)**.

Indeed, pesticides and associated substances tend to accumulate in large quantities in fish organs like gills, muscles and liver **(El-Garawani *et al.*, 2022)**. IMI leads to severe inflammation, oxidative stress, and histopathological lesions in fish brains, liver and gills. Moreover, it affects its genetic integrity **(Iturburu *et al.*, 2018)**. It causes a genotoxic effect on the Nile tilapia, causing irreversible damage to the liver due to protein oxidation and reactive oxygen species (ROS) **(Günal *et al.*, 2020)**. Furthermore, it suppresses acetylcholinesterase activity in the brain of the neotropical *Rhamdia queen* and induces an oxidative damage in muscles, brain, liver and gills **(Marins *et al.*, 2021)**. Moreover, it induces genotoxicity in the gills and muscles of the neotropical freshwater fish *Astyanax altiparanae*. In addition, it causes genotoxicity and neurotoxic effects in the muscles **(Almeida *et al.*, 2021)**. IMI was reported to cause DNA damage, triggering hepatic oxidative load, and disrupting the intestinal microbiota of fish. It enhances the

gene expression of proinflammatory TNF-α, NF-kB, IL-6 and IL-1β **(Miao *et al.*, 2022)**. It stimulates apoptosis through the release of cytochrome-c, reducing B-cell lymphoma (BCL)-2, and increasing Bcl-2-associated X protein (BAX), caspase 9, and caspase 3 **(Miao *et al.*, 2022; Subaramaniyam *et al.*, 2023)**.

Protein and lipid oxidation can cause fish meat to deteriorate and spoil by producing various undesired compounds like alcohols, ketones and aldehydes **(Yang *et al.*, 2023)**. These compounds can harm individuals' health status due to their genetic and cellular toxicity. Nowadays, various artificial and natural compounds as food additives are utilized to raise the antioxidant content of fish to improve meat quality **(Xu *et al.*, 2021)**. Anthocyanidins are polyphenolic compounds that are frequently present in vegetables and fruits that have health-promoting properties. They can reduce inflammation and act as antioxidants, as well as improve resistance to toxic substances **(Zhai *et al.*, 2014)**. Grape seeds are one of the most significant sources of anthocyanidins, with about 50mg/ kg of entire seeds **(Xu *et al.*, 2021)**. It is a naturally occurring antioxidant supplement that improves muscle protein deposition and growth **(Yang *et al.*, 2023)**.

This study aimed to explore the possible protective role of grape seed extract (GRSE) supplemented diet on the Nile tilapia to overcome the dangers of the insecticide IMI. It was crucial to assess the antioxidant biomarkers, histopathological effects, liver enzymes and inflammatory cytokines. Moreover, the genotoxic influence of the IMI was assessed via the comet assay.

MATERIALS AND METHODS

1. Insecticide and GRSE

IMI (Confidor > 70% purity IMI) was purchased from the Bayer Crop Science Company (Germany). A solution of IMI insecticide was weekly prepared using deionized water, and the stock IMI solution was stored in a brown glass with a double sealing reagent bottle to prevent photolysis. The GRSE (95% purity, red powder), which contains 95% anthocyanidin, was purchased from Hangzhou Jaymore Technology Company (China).

2. The Nile tilapia and formulated diets

Juveniles *O. niloticus* weighing 8.85± 0.09g were obtained from the Aquatic Fish Research Center, Suez Canal University (Ismailia, Egypt) and cultured in glass aquaria (60 × 30 × 40cm) in a Fish Biology and Immunology Laboratory at the Zoology Department, Faculty of Science, Suez Canal University. Four groups of 120 fish were divided at a rate of 10 fish per 60L well-aerated aquarium. Aquarium heaters were used to maintain a water temperature of 26- 28°C, a pH of 7.56, and dissolved oxygen near saturation using air pumps. Cleaning of aquaria was performed twice a week.

The obtained GRSE was added to the formulated pellets to prepare the tested diets. Two experimental diets were prepared, the control diet at a level of 0% GRSE and a supplement diet at a level of 4% GRSE (400mg GRSE/kg); this selected dosage was previously determined by **Günal *et al.* (2020)**, **Mohammadi *et al.* (2021)**, and **Jahanbakhshi *et al.* (2023)**. The dietary composition was formulated according to the method of **El-Fahla *et al.* (2022)**; the trial diets were prepared to include 4100 kcal kg^{-1} gross energy and 37.69% crude protein (CP) (NRC, 2011). Diet elements were thoroughly mixed with water (100mL for 1kg diet), made as pellets, and subjected to room temperature air dryness for 24h. Afterward, the dried pellets were kept at refrigeration temperature (4°C). The GRSE was freshly added every week by mixing the required amount of extract with 1% fish gel (100ml gel/ Kg diet) as a binder and spraying it to produce the experimental diets. Control pellets were mixed with 1% fish gel (100ml gel/ Kg diet) without GRSE.

3. Experimental designs for 30 days

The experimental Nile tilapias were allocated into four equal groups (each group contained 3 aquaria), with 10 fish/aquarium, and the experimental fish were kept for 10 days to acclimate prior the start of the trial. The first group was designated as the control group. It was fed on a control diet (0% GRSE) and reared in IMI-free water. The second group was nourished on a diet provided with 4% GRSE and was reared in IMI-free water. The third group received a 0% GRSE diet and was reared in 2.5 IMI mg/L in water. The fourth group was given a 4% GRSE diet and was exposed to 2.5 IMI mg/L in water. Only healthy fish were picked for this study, and the weight of all studied fish body weight (W initial) was recorded on day zero of the trial. The treatments remained for 30 days, and the herein research was authorized by the Animal Ethics Committee, Faculty of Science, Suez Canal University. At the termination of the experiment (30 days), the body weights of all remaining fish were recorded to estimate fish weight gain (W gain).

W gain = final weight (g) - initial weight (g).

4. Sampling

After thirty days of IMI exposure and GRSE supplementation, all fish were subjected to an overnight fasting prior sampling. Every viable fish was transferred to a 10L glass aquarium and anesthetized with clove oil dissolved in ethanol (1mL clove oil : 10mL ethanol), according to **Van Doan *et al.* (2020)**. Fish heart blood samples were drained and stored in sterilized Eppendorf tubes. The collected blood (six samples/group) was coagulated and subjected to centrifugation for 15 minutes at 1000 x g, and then the sera were kept at -80°C. The frozen sera were subjected to analysis of liver transaminases: aspartate transaminase (AST) and alanine transaminase (ALT), as well as high-density lipoproteins (HDL)-cholesterol, low-density lipoproteins (LDL)-cholesterol, leptin hormone and TNF-α. After dissection, six livers per treatment were stored at -80°C

to analyze total oxidant capacity (TOC) and total antioxidant capacity (TAC). Other samples of the liver were tested for DNA damage by comet assay. Pieces of gills and the right lobe of the tilapia livers (6 tissues per group) were removed to assess histopathology. While, liver tissues (6 tissues per group) were subjected to histochemical studies and caspase 3 immunohistochemical evaluation.

5. Serum transaminases

Following the procedures of **Reitman and Frankel (1957)**, the activities of AST and ALT activities were assayed calorimetrically using the manufacturing kit (CliniChem Ltd, Company, Budapest, Budafoki, Hungary, Catalog. NO.: 46361, Normal range: < 40 U/L for ALT & Cat. No.: 46263, Normal range: < 37 U/L for AST).

6. HDL-cholesterol and LDL-cholesterol profile

Sera (6 samples/group) were subjected to estimate HDL-cholesterol and LDL-cholesterol levels in the tilapias calorimetrically according to **Gordon *et al.* (1977)** and **Assmann *et al.* (1984)** protocols, respectively, following the HDL-cholesterol manufacturer kit protocol (CliniChem Ltd Company, Budapest, Budafoki, Hungary, Catalog. NO.: 41411,41411S, Normal range: > 1.7mmol/ L) and the LDL-cholesterol manufacturer kit protocol (CliniChem Ltd Company, Budapest, Budafoki, Hungary, Catalog. NO.: 43161,43161S, Appropriate range: < 80mg/ dL).

7. TNF-α

Serum TNF-α (6 samples/group) levels were analyzed according to **Beutler *et al.* (1985)** method following the protocol of manufacturer-specific fish TNF-α ELISA kit (CUSABIO Company, USA, Catalog No.: CSB-E13254Fh, Detection range: 125pg/ mL - 5000pg/ mL and sensitivity: less than 125pg/ mL).

8. Leptin assay

A specific fish ELISA kit (CUSABIO Company, USA, Catalog No.: CSB-EL 012870FI, Detection range: 62.5 -1000pg/m L and sensitivity: less than 31.25pg/m L) was used to determine leptin hormone levels in the collected sera from the Nile tilapia (6 samples/group).

9. TAC and TOC levels

Six liver samples (0.5g) were subjected to homogenization via Pro 200 (Pro Scientific Inc., USA) homogenizer with an ice-cold 154 mmol/L NaCl solution (1.9 w/v). Then, the homogenates were subjected to centrifugation for 15min at 1059 x g and at

4°C. TAC and TOC levels were determined spectrophotometrically in the supernatants following the guidelines of the commercial kits. TAC was measured using a specific ELISA kit (Labor Diagnostika Nord "LDN" Company, Nordhorn, Germany, Catalog No. EK750261). TOC concentrations were measured with a specific ELISA kit (LDN Company, Nordhorn, Germany, Catalog No. EK750261).

10. Single-cell gel electrophoresis (Comet assay)

Single-cell gel electrophoresis was implemented to investigate the DNA damage that occurred due to genotoxicity according to **Abdelrazek *et al.* (2015)**. Tissues were sliced into minute pieces and gently homogenized. 120µL of 0.5% low-melting agarose was put with 10µL of treated cells, and then they were put as a layer on glass slides surface that were formerly coated with normal-melting agarose (140µL of 1% mixture). Then, coverslip was put to cover the slides and left to cool for 20min at 4°C. After that, removal of coverslips was performed, and another layer of 0.5% low melting agarose was layered onto the slides and left for 20min to cool and form gel at 4°C. Slides with no coverslip's coverings were submerged overnight at 4°C in a cold lysing solution.

Equilibration of the slides, for 20min, was conducted in alkaline electrophoresis solution (1 mM disodium salt of EDTA and 300 mM sodium hydroxide, pH >13). Electrophoresis was performed using the former buffer at 25V and 300mA for 25min. After that, gentle washing for the slides was conducted three times, 5min each, with 0.4 M Tris buffer at pH 7.5 (neutral buffer). The neutral buffer was drained and the slides were washed. After air dryness, the slides were subjected to 0.2mg/ mL silver nitrate staining. A minimum of 100 cells were scanned per slide. The entire process was performed in the dark to reduce DNA damage artifacts. After coding the slides, the examination was performed at a magnification power of 100× using a Nikon (China) light microscope.

11. Histopathology

The removed gills and livers were washed in 0.6% NaCl (physiological fish saline), immersed for 48hr in 10% buffered formalin, and then preserved in 70% ethanol. Paraffin blocks were equipped, and the required sections were cut at a thickness of 5µm. The cutting sections of gills and liver were stained with hematoxylin and eosin (H&E), while collagen-specific Mallory's trichrome and glycogen-specific Periodic Acid Schiff (PAS) were the stains for liver tissues.

12. Immunohistochemistry

Immunohistochemical (IHC) detection of caspase 3, a cysteine aspartate protease, was conducted using the ABC technique on 5µm thick, formalin-fixed, paraffin-

embedded sections. The detection was performed according to **Hsu *et al.* (1981)** following the manufacturer's protocol, with an HRP/DAB detection IHC kit (Abcam Company, China, Catalog No: ab80436). Slides were subjected to active caspase 3 specific primary antibody at a rate of 1:50. Antigen retrieval was applied for all slides by boiling the slides for 25 minutes in a 10 mM sodium citrate buffer pH 6.0 (Abcam, Company, China, Catalog No: ab64236) in a microwave. The avidin-biotin complex procedure was implemented. Staining was implemented with an automated immunostainer (DAKO) after the blockage of the endogenous biotin. Subsequent detection was performed with a detection system (streptavidin-biotin, DAKO). Negative and positive control slides were implemented for each test. A positive caspase 3 reaction was seen as brown cytoplasmic staining in the hepatocytes.

13. Statistical analysis

Data analysis was carried out statistically via the analysis of variances test, one-way ANOVA, and graphing using GraphPad Prism version 8 (GraphPad Software, San Diego, CA). A *post-hoc* test "Tukey's multiple comparison tests" was conducted to recognize differences between groups. The obtained values were expressed as means and standard errors. The differences between means were considered significant at $P< 0.05$.

RESULTS

1. Body weight (W) final and gain

As depicted in Fig. (1A), no statistical variances were noted in body W initial values among all groups. The GRSE group displayed a statistical ($P< 0.05$) increase in body W final and W gain compared to the control**.** While, the IMI group demonstrated statistical ($P< 0.05$) lower W final and W gain values than those of the control. Adding anthocyanidin (4% GRSE) to the diet of the IMI group statistically ($P < 0.05$) promoted the body W final and W gain of the IMI group (Fig. 1B, C).

2. ALT and AST

The serum ALT and AST activities were significantly ($P< 0.05$) elevated in the IMI-intoxicated fish compared to those in the control group. Contrarily, nourishing the IMI group within anthocyanidin extract (4% GRSE) statistically ($P< 0.05$) lowered the AST and ALT levels. As presented in Table (1), the GRSE-supplemented group exhibited non-significant values in blood transaminases compared to control values.

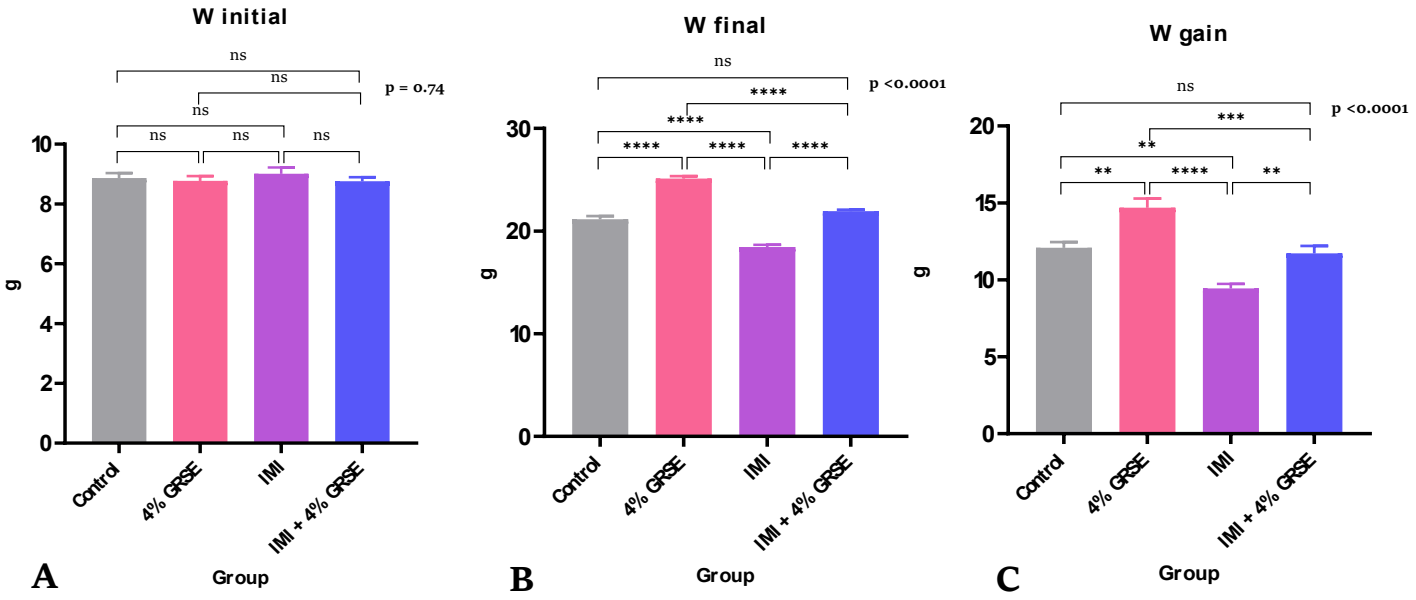

Fig. 1. Graph **(A)** represents the mean values of initial weights at zero day of treatment, while graphs **(B)** and **(C)** indicate the ameliorative effect of grape seed extract (4% GRSE) on weight final and weight gain, respectively of *O. niloticus*, after 30 days of exposure to 2.5 mg IMI/L. **, ***, and ******** denote significant difference between groups at P <0.01, P<0.001, and P<0.0001, respectively.
GRSE: grape seed extract, IMI: imidacloprid, W: weight, ns: non-significant

Table 1. Efficacy of grape seed extract (4% GRSE) as a dietary supplement on the levels of serum transaminases in *O. niloticus* after 30 days of the trial period

Group / Parameters	Control	4% GRSE	2.5 mg IMI L^{-1}	2.5 mg IMI L^{-1} + 4 % GRSE	P-value
ALT (U/L)	18.40 ± 0.36 ^{ns}	17.80 ± 0.27 ^{ns}	59.81 ± 1.25 ****	28.93 ± 1.03 ****	<0.0001
AST (U/L)	54.85 ± 1.66 ^{ns}	52.16 ± 1.51 ^{ns}	98.62 ± 8.98 ****	77.70 ± 4.37 ****	<0.0001

Values expressed as mean ± SE (***n* = 6 per group**). ******** denote that the corresponding means differ significantly at P <0.0001, according to the one-way ANOVA followed by Tukey's multiple tests.

GRSE: Grape seed extract, **IMI:** Imidacloprid, **ALT:** Alanine transaminase, **AST:** Aspartate transaminase, **ns:** Non-significant.

3. HDL-cholesterol and LDL-cholesterol

Feeding the Nile tilapia on 4% GRSE did not statistically influence the levels of HDL-cholesterol and LDL-cholesterol, in comparison with the control and supplement groups. IMI-polluted fish with 0% GRSE feeding revealed significant ($P < 0.05$) lesser HDL-cholesterol with increased LDL-cholesterol levels. Fish under IMI pollution and fed a 4% GRSE diet displayed ameliorated *(P*< 0.05) HDL-cholesterol and LDL-cholesterol levels than IMI-exposed fish-fed control diet, as presented in Table (2).

Table 2. Efficacy of grape seed extract (4% GRSE) as a dietary supplement on the levels of HDL-cholesterol and LDL-cholesterol in sera of *O. niloticus* after 30 days of the trial period

Groups / **Parameter**	**Control**	**4% GRSE**	**2.5 mg IMI L^{-1}**	**2.5 mg IMD L^{-1} + 4% GRSE**	***P*-value**
HDL-cholesterol (mg/dL)	**13.24 ± 0.30 ^{ns}**	**15.95 ± 2.02 ^{ns}**	**9.24 ± 0.22 ****	**11.62 ± 0.24 ****	**= 0.0016**
LDL-cholesterol (mg/dL)	**54.69 ± 0.72 ^{ns}**	**49.65 ± 2.80 ^{ns}**	**102.00 ± 2.06 ******	**84.30 ± 3.87 ******	**<0.0001**

Values expressed as mean ± SE (n = 6 per group). ** or ******** denote that the corresponding means in the same row differ significantly at P< 0.001 and P< 0.0001, respectively, according to the one-way ANOVA followed by Tukey's multiple tests.

GRSE: Grape seed extract, IMI: Imidacloprid, HDL: High-density lipoproteins, LDL: Low-density lipoproteins, ns: Non-significant.

4. TNF-α

The Nile tilapia reared in 2.5mg IMI/L revealed higher TNF-α concentrations than the control ones or 4% GRSE without IMI exposure. Feeding IMI-contaminated fish on an anthocyanidin extract reduced significantly the TNF-α levels ($P < 0.05$) to be lower than the values of the IMI group. It was noted that there was no statistical variation in TNF-α levels between groups fed on control and 4% GRSE diets without IMI exposure**,** as shown in Fig. (2).

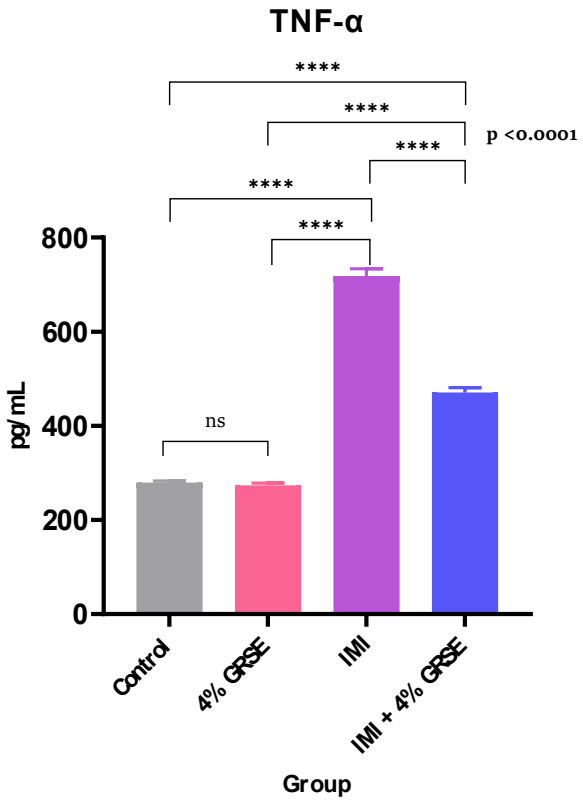

Fig. 2. Graph indicates the lowering effect of dietary grape seed extract (4% GRSE) on TNF-α levels influenced by 2.5 mg IMI/L in sera of *O. niloticus* after 30 days of treatment. **** denotes that the corresponding means differ significantly at $P< 0.05$.
GRSE: Grape seed extract, IMI: Imidacloprid, TNF-α: Tumor necrosis factor-alpha, ns: Non-significant.

5. Leptin hormone

After 30 days of the trial period, the sera of the reared tilapias in 2.5mg IMI/L exhibited a statistical ($P< 0.05$) elevation in leptin hormone levels (Fig. 3) compared to control and supplement groups. However, adding anthocyanidin extract (4% GRSE) to the IMI diet significantly ($P< 0.05$) lowered the leptin concentration than that of the IMI group. Comparable leptin hormone levels between control and 4% GRSE groups showed no significant differences.

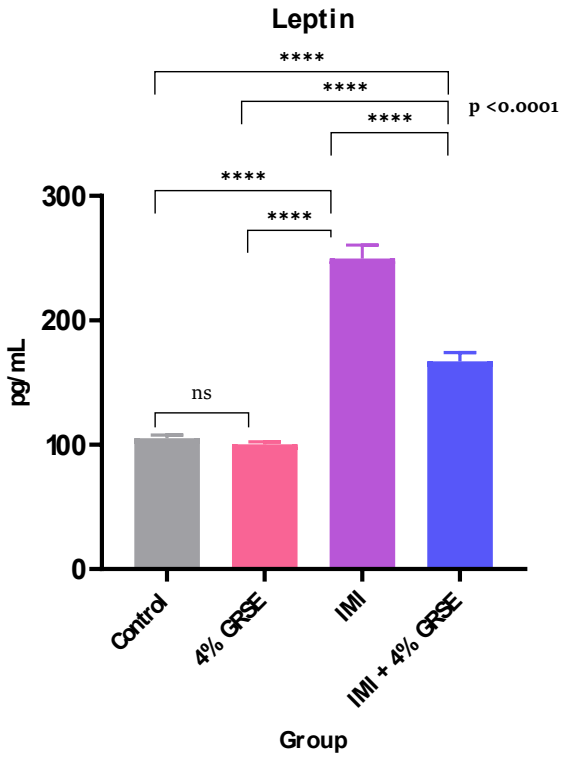

Fig. 3. Graph indicates the ameliorative role of dietary grape seed extract (4% GRSE) on leptin levels influenced by 2.5 mg IMI/L in sera of *O. niloticus* after 30 days of treatment. ******** denotes that the corresponding means differ significantly at $P< 0.05$.
GRSE: Grape seed extract, IMI: Imidacloprid, ns: Non-significant.

6. Hepatic TAC and TOC

Exposure to IMI statistically increased ($P< 0.05$) the TOC levels in the tilapia's hepatic tissues compared to the control value. However, IMI statistically decreased ($P< 0.05$) the TAC levels in fish hepatic tissues relative to the control value. Supplementing the diet of the IMI fish group with 4% GRSE resulted in a significant decrease in the TOC levels ($P< 0.05$) and a significant increase in TAC levels ($P< 0.05$) compared to the unsupplemented IMI group. Conversely, no significant differences were observed in TAC and TOC levels between the control group (0% GRSE) and the supplemented group (4% GRSE), as shown in Table (3).

Table 3. Efficacy of grape seed extract (4% GRSE) as a dietary supplement on total antioxidant and oxidant capacities in liver tissues of *O. niloticus* after a 30- day trial

Group / Parameters	Control	4% GRSE	2.5 mg IMI L^{-1}	2.5 mg IMD L^{-1} + 4% GRSE	P-value
TAC (mmol/g wet tissue)	**2.69 ± 0.07 ^{ns}**	**2.81 ± 0.06 ^{ns}**	**1.83 ± 0.07 ******	**2.26 ± 0.05 ******	**<0.0001**
TOC (mmol/g wet tissue)	**0.63 ± 0.00 ^{ns}**	**0.61 ± 0.01 ^{ns}**	**0.82 ± 0.04 ******	**0.72 ± 0.02 ******	**<0.0001**

Values expressed as mean ± SE (n = 6 per group). **** denotes that the corresponding means in the same row differ significantly at $P< 0.0001$, according to the one-way ANOVA followed by Tukey's multiple tests.

GRSE: Grape seed extract, IMI: Imidacloprid, TAC: Total antioxidant capacity, TOC: Total oxidant capacity, ns: Non-significant.

7. Comet assay

Our results demonstrated that the IMI group showed a statistical increase ($P< 0.05$) in the DNA damage of hepatic tissues in comparison with the control group (0% GRSE) (Figs. 4, 5A, C). In contrast, a statistical decrease ($P< 0.05$) in DNA damage was determined in the 4% GRSE group in comparison with the control one (0% GRSE) (Figs. 4, 5A, B). IMI exposure statistically elevated ($P< 0.05$) the DNA damage in the tilapia hepatic tissues compared to the 4% GRSE fish group (Figs. 4, 5B, C). Supplementing the diet of the IMI fish group within 4% GRSE statistically declined ($P< 0.05$) the DNA damage when matched with the IMI fish group (Figs. 4, 5C, D). No statistical alterations in the DNA damage between the control (0% GRSE) and the treated IMI group supplemented with 4% GRSE were observed (Figs. 4, 5A, D).

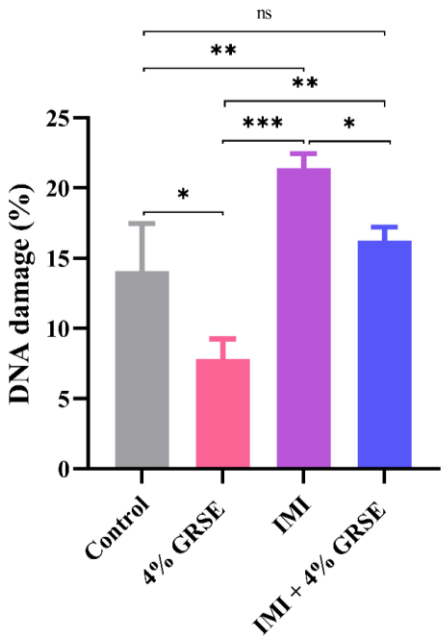

Fig. 4. Single-cell gel electrophoresis (comet assay) showing the mean values of the percentage of DNA damage in different groups. It indicates the genotoxic effect of IMI and the ameliorative effect of grape seed extract (4% GRSE) in the liver of *O. niloticus.* *, **, ***, and **** denote significant differences between groups at $P<0.05$, $P<0.01$, $P<0.001$, and $P<0.0001$, respectively.
GRSE: Grape seed extract, IMI: Imidacloprid, ns: Non-significant.

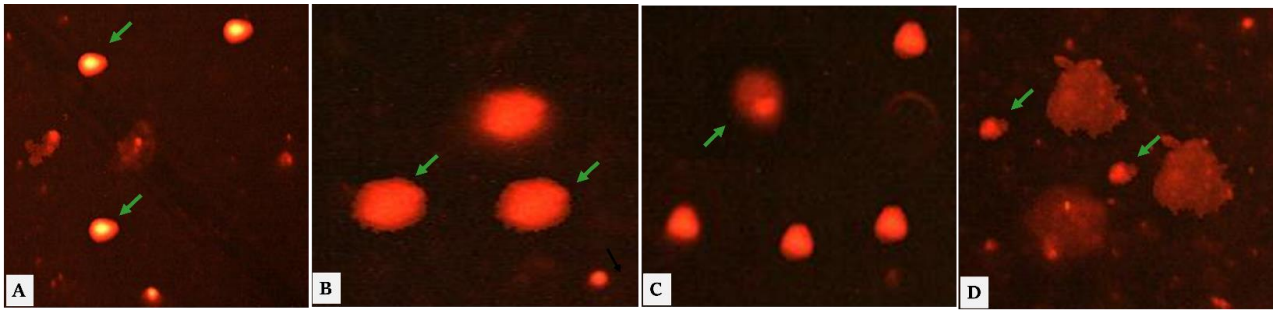

Fig. 5. Micro-photographs of single-cell gel electrophoresis (comet assay) showing DNA damage represented by the comet tails in the liver of *O. niloticus*. **(A)** Control, **(B)** 4% GRSE-supplemented group, **(C)** IMI group supplemented with 0% GRSE, and **(D)** IMI group fed on 4% GRSE

8. Gill and liver histopathology

The obtained gill tissues from both the control and the 4% GRSE groups, without IMI exposure, showed normal features of primary (PF) and secondary filaments (SF), with no detectable abnormalities in the examined gill sections **(**Fig. 6A, B, respectively). IMI toxicity resulted in significant alterations in both PF and SF. In PF, the primary blood vessel was congested with erythrocytes; in addition, PF showed complete necrosis that extended to SF, attributed to leukocytic infiltrations and hemorrhage (Fig. 6C). The SF revealed detrimental signs, including epithelial lifting in relation to edema (Fig. 6D), shortage of SF (Fig. 6E), and aneurysms (Fig. 6F). The sections exposed to IMI and nourished on 4% GRSE declared improvement in both PF and SF epithelium appearance; however, some alterations, such as congestion of blood vessels and aneurisms, are still frequently detected (Figs. 6G, H).

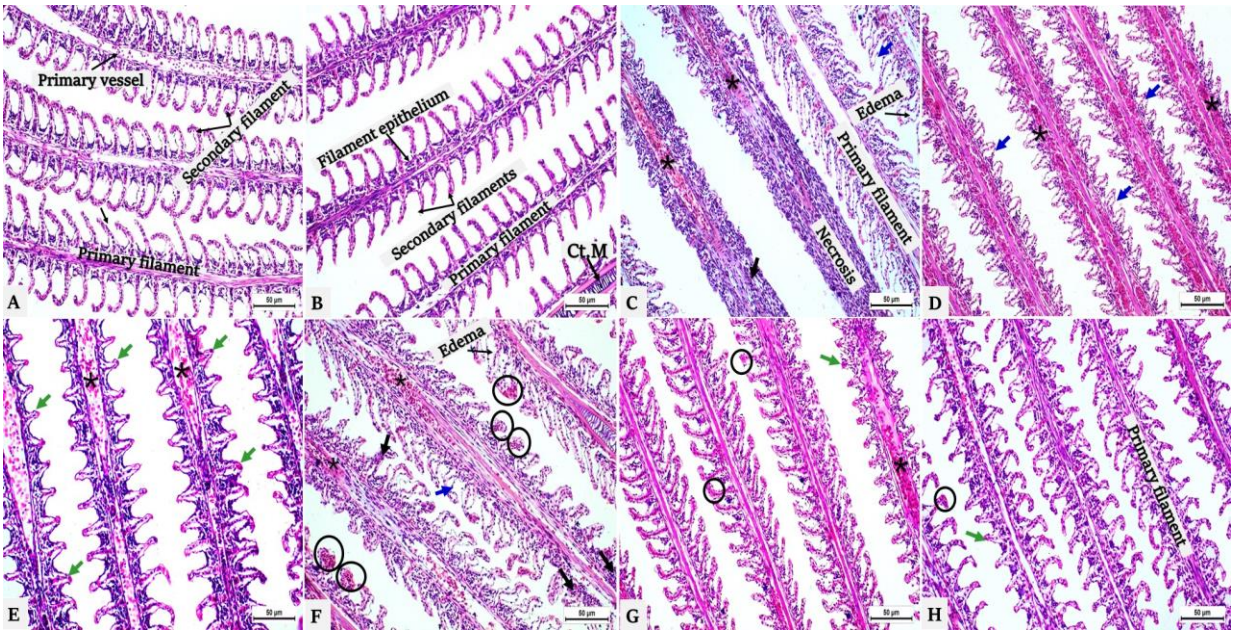

Fig. 6. Light micrographs in the gills of *O. niloticus*, following 30 days of treatment period. **(A)** Control and **(B)** 4% GRSE supplemented groups, indicated histological features in the primary and secondary filaments. **(C-F)** Micrographs of the IMI exposed group displayed congested primary blood vessels with erythrocytes (asterisks), complete necrosis of primary and secondary filaments, leukocytic infiltrations (black arrows), epithelial lifting (blue arrows) with edema, shortage of secondary filament (green arrows)**,** and aneurisms (circles)**.** **(G)** and **(F)** Micrographs of IMI group fed on 4% GRSE, showed improvements in both primary and secondary filaments with some noticeable alterations. H&E stain, X200

In the case of control and supplemented liver sections, the liver parenchyma showed typical polygonal hepatocytes with homogenous cytoplasm arranged in branched cords separated by sinusoids (Figs. 7A, B). The tilapia liver from the IMI intoxicated group exhibited severe erosion in hepatic parenchyma including hydropic (vacuolar) degeneration referring to lipidosis, necrosis, hyperplasia of hepatocytes, dilatation and congestion of central hepatic veins, hemorrhage, and mononuclear cells infiltrations (Fig. 7C-F). Co-treatment of IMI within 4% GRSE ameliorated the hepatic abnormalities induced by IMI exposure (Fig. 7G, H).

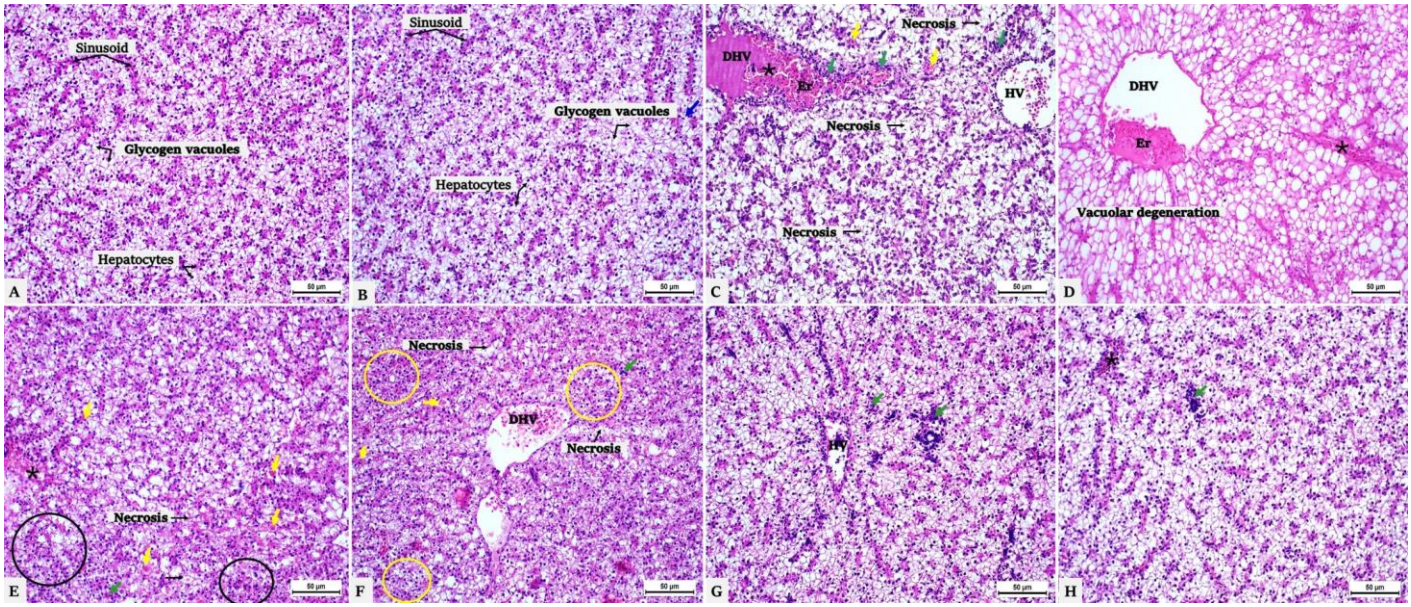

Fig. 7. Light micrographs in the liver tissues (right lobe) of *O. niloticus*, following 30 days of treatment period. **(A)** Control and **(B)** 4% GRSE supplemented groups, indicated normal hepatocellular histological features. **(C-F)** Micrographs of the IMI-intoxicated liver displayed congestion (*) and dilatation of central hepatic vein (DHV) and blood sinusoids, vacuolar degeneration, necrosis associated with lymphocytic infiltration and hemorrhage (black circles), and hyperplasia of hepatocytes (yellow circles). Yellow arrows refer to haemorrhage within the hepatic parenchyma**. (G)** and **(F)** Micrographs of the IMI group supplemented with 4% GRSE, showed marked improvement in hepatic tissue architecture, with slight leukocytic infiltrations (green arrows) and sinusoidal congestion (*). H&E stain, X200. Er**:** erythrocytes

Masson's trichrome liver-stained slices of control and GRSE-supplemented groups showed sporadically spread connective tissue fibers (collagen) around the central vein of the liver **(**Fig. 8A, B), while sections from the IMI group exhibited considerable connective tissue fiber gathering around the hepatic vein (Fig. 8C). Such fibrosis was entirely overturned by the addition of 4% GRSE within the diet of the IMI group (Fig. 8D).

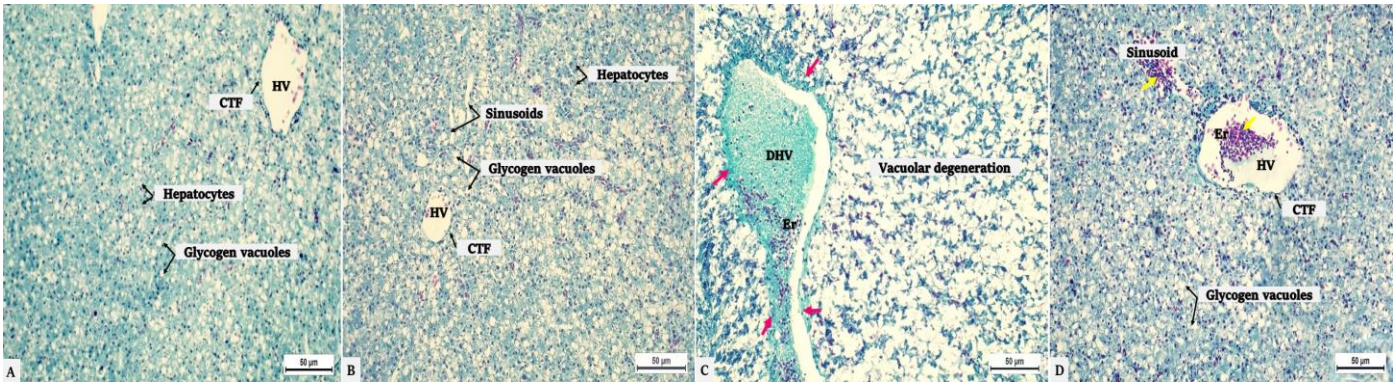

Fig. 8. Light micrographs of Masson's trichrome-stained sections in the liver (right lobe) of *O. niloticus*, following 30 days of IMI exposure. **(A)** Control and **(B)** 4% GRSE groups, indicated typical hepatic histological features, including a prominent central hepatic vein (HV), surrounded by connective tissue fibers (CTF). **(C)** Micrograph of the IMI exposed group displayed a marked increase in CTF (red arrows) around the hepatic vein (fibrosis). **(D)** A reversal of the fibrosis surrounding the hepatic vein was observed in the liver micrograph section of the IMI group fed on 4% GRSE, indicating improvement in hepatic tissues, X200.
DHV: Dilated hepatic vein, Er: Erythrocytes, yellow arrow refers to lymphocyte aggregation

As depicted in Fig. (9A, B), the PAS technique positively stains glycogen reserves that occlude the hepatocellular' cytoplasm in stained hepatic tissues of the control and 4% GRSE group. No differences was detected in glycogen store appearance between these groups. In addition, these groups had a significantly stronger PAS-positive reaction. However, the PAS technique revealed differences in glycogen distributions based on the intensity of the PAS reaction in each treatment group. In IMI hepatic sections, PAS staining showed a considerable depletion in hepatic cells glycogen contents (weak PAS-positive reaction) (Fig. 9C), which was partially reversed by adding 4% GRSE to the diet of the IMI group (Fig. 9D).

9. Caspase 3

A mild immune-histochemical reactivity of caspase 3 was detected in the hepatic cells of the control and dietary GRSE groups, in which fish were not exposed to IMI, as shown in Fig. (10A, B). IMI exposure triggered strong immune reaction of caspase 3, as seen by brown color within the hepatocyte cytoplasm and sinusoids (Fig. 10C). In contrast, the hepatic tissue of the IMI + GRSE group showed a mild caspase 3 immunohistochemistry reactivity (Fig. 10D).

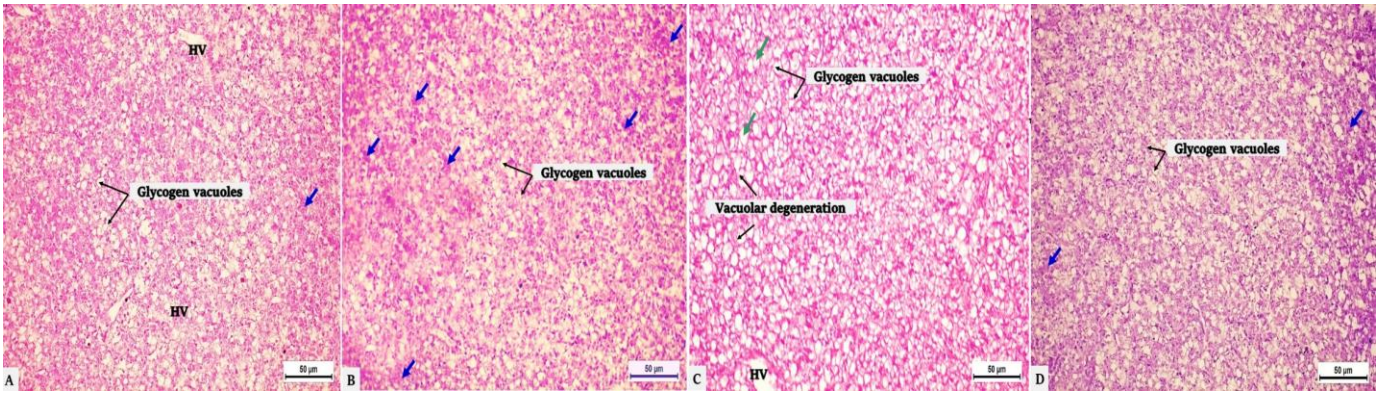

Fig. 9. Light micrographs of Periodic Acid Schiff (PAS)-stained liver (right lobe) sections in *O. niloticus*. **(A)** Control and **(B)** 4% GRSE supplemented groups, showed a positively strong PAS reaction (color intensity increased) that clarified typical aggregation of glycogen granules (blue arrows) in hepatocellular cytoplasm or glycogen vacuoles. **(C)** Micrograph of the IMI exposed group displayed a depletion in glycogen content (green arrows) with marked vacuolar degeneration within the hepatic parenchyma. **(D)** Micrograph section of the IMI group cotreatment with 4% GRSE, exhibited improvement of hepatic parenchyma with reversal of glycogen content, X200. HV: hepatic vein

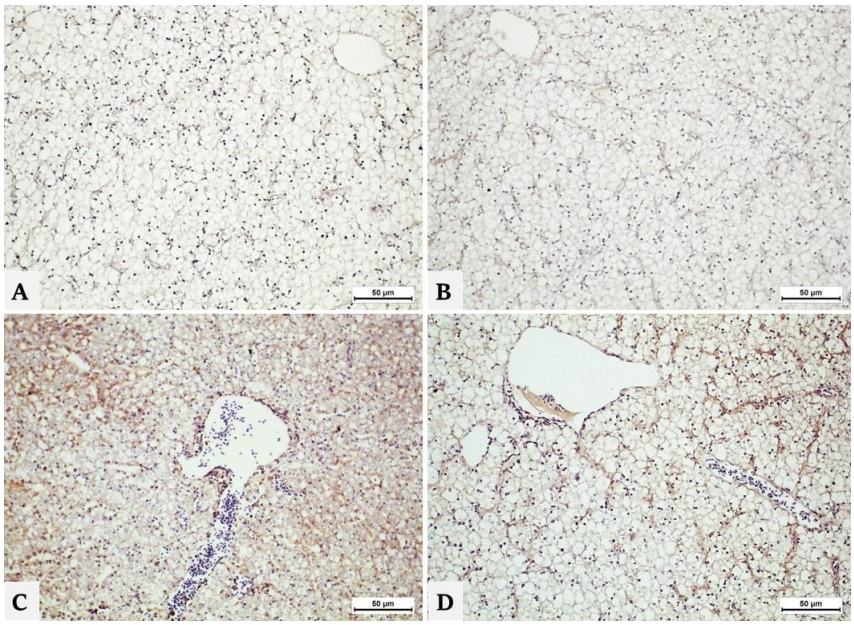

Fig. 10. Light micrographs of caspase 3 immuno-reactivity in the liver (right lobes) sections of *O. niloticus*, after 30 days of treatment. **(A)** Control and **(B)** 4% GRSE supplemented groups, showed a negative immune-histochemical expression of caspase 3. **(C)** Micrograph of the IMI group fed on the control diet displayed a strong immune-histochemical expression of caspase 3, as brown color within the hepatocyte cytoplasm and sinusoids. **(D)** Micrograph section of the IMI group nourished on 4% GRSE, exhibited a mild caspase 3 immunohistochemistry reactivity, X200

DISCUSSION

The body W final and W gain increased after treatment with GRSE. The current results are consistent with those of **Yang *et al.* (2023)**; they reported an increase in the W gain of *Oreochromis niloticus*, showing a higher growth performance than the control group. Similar results were also noted in the common carp *Cyprinus carpio* that were supplemented with GRSE in their diet **(Mohammadi *et al.*, 2021)**. Indeed, dietary supplementation with GRSE directly decreases the ROS content in the Nile tilapia, affecting the weight of fish **(Yang *et al.*, 2023)**.

As reported by **Kesbiç and Yigit (2019)**, fish diets supplemented with GRSE enhanced growth performance and antioxidant enzyme activities in the rainbow trout juveniles, which is in close agreement with our findings. This effect could be attributed to modifications in the gut microbiota brought about by the GRSE supplementation **(Kesbiç & Yigit, 2019)**. The growth-promoting impact of GRSE might be due to an increase in the activity of the gut digestive enzymes. Furthermore, the high degree of diversity of the intestinal bacteria causing modifications of the gut morphology is another important factor **(Zhai *et al.*, 2014)**. According to **Ozdal *et al.* (2016)**, supplementation with GRSE in the diet could protect the gut mucosa from oxidative damage and various pathogens. This leads to improving nutritional absorption and has a positive effect on health and growth **(Ozdal *et al.*, 2016)**.

It was noted that IMI reduced the weight of the fathead minnow *Pimephales promelas* **(Gibbons *et al.*, 2015)**. Fish are susceptible to ROS invasion due to the substantial and polyunsaturated fatty acids and amino acid contents causing protein and lipid degradation. These findings were validated as environmental stress, like exposure to IMI, resulting in oxidation and the buildup of ROS in *Tilapia nilotica* **(Collins *et al.*, 2023)**. Moreover, IMI exposure resulted in genotoxic and morphological alterations in the tadpoles of *Physalaemus cuvieri* and *Leptodactylus luctator*. They exhibited a decline in their body weight and length, indicating changes in their metabolism **(Samojeden *et al.*, 2022)**.

The elevation in the level of cholesterol may be an insignia of hepatic injury that could be due to the influence of pesticides on the hepatocytes' cell membrane permeability and or the obstruction of bile ducts, producing a decrease of its secretion **(Reda, 2018)**. Especially, fish liver is considered a biomarker of carcinogen and toxicant exposure damage and is the primary organ of multiple metabolic pathways **(Erkmen *et al.*, 2017)**. Thus, liver enzyme activities are correlated with liver function and the nutritional status of the organism, as they are deemed as critical diagnostic indices **(Ahmad *et al.*, 2021)**. Indeed, AST and ALT are considered gold standards for

monitoring hepatotoxicity and health conditions of fish **(Zhai *et al.*, 2014)**. The results of our study revealed an increase in the activities of AST and ALT in the blood of the group exposed to IMI to neutralize toxic substances. These results indicate liver injury or damage by the IMI, which agrees with the data stated by **Ramírez-Coronel *et al.* (2023)**, who showed an increase in liver enzyme activities AST and ALT in the common carp *Cyprinus carpio* treated with IMI. Moreover, **Abdel Rahman *et al.* (2022)** confirmed an increase in both enzymes upon IMI exposure in *Clarias gariepinus*. Furthermore, similar results were also reported by **Subaramaniyam *et al.* (2023)** when exposing the African catfish *Clarias gariepinus* to IMI. Similarly, high activities of AST and ALT have previously been recorded from IMI exposure, indicating damaged liver tissues resulting from oxidative stress **(Hussein *et al.*, 2019)**. This can be explained as free radicals generation interacting with the cellular membrane, triggering changes in the fluidity of the cell membrane and causing leakage in these markers in the blood **(Attia *et al.*, 2021)**.

On the other hand, supplementing the diet with GRSE lowers the activities of AST and ALT in the blood. **Zhai *et al.* (2014)** reported the same findings in the Nile tilapia. They explained their results as the GRSE exerts its protective role against adverse effects on health status and improves liver damage, which results in lowering the levels of serum biochemical markers AST and ALT. They concluded that GRSE can reduce organ injury by balancing the oxidant-antioxidant status **(Zhai *et al.*, 2014)**. Furthermore, GRSE contains several polyphenols, which exert their protective effect via the indirect stabilization property of the cell membrane, causing a decline in the AST and ALT levels in the blood **(Attia *et al.*, 2021)**.

IMI was reported to cause mitochondrial dysfunction in the grass carp *Ctenopharyngodon idellus*, which induced inflammation and boosted TNF, NF-kB, IL-6 and IL-1 expression. Moreover, it stimulated apoptosis through the cytochrome-C release, caspase 3 and 9 upregulation, and BCL-2 downregulation. IMI was confirmed to cause mitophagy, enhanced inflammation, and apoptosis mediated via mitochondria in the grass carp liver cells through the NF-kB/JNK pathway **(Miao *et al.*, 2022)**. These findings explained the higher TNF-α levels in the IMI group than the fish fed on control diet or 4% GRSE without IMI exposure.

Results showed that the leptin hormone level was elevated in the IMI group; however, adding anthocyanidin extract (4% GRSE) to the diet lowered the leptin levels more than that of the IMI group. This can be explained by the fact that fish that live in contaminated environments suffer from hypoxia, which stimulates different responses at the metabolic and behavioral levels. Indeed, exposure to hypoxia stimulates leptin signaling **(Copeland *et al.*, 2011)**. Leptin expression in teleost is extremely affected by environmental factors like water salinity and hypoxia. Indeed, changes in environmental parameters result in stressful conditions for fish, leading to a rise in leptin levels **(Blanco**

***et al.*, 2021)**. Generally, several previous studies have also reported high leptin mRNA levels as a result of hypoxic conditions in the liver of the zebrafish, their embryos **(Chu *et al.*, 2010; Yu *et al.*, 2012)** and the common carp **(Bernier *et al.*, 2012)**.

It was reported in many organisms that IMI alters the equilibrium between exogenous and endogenous ROS, decreases the antioxidant mechanism, or causes macromolecule oxidative damage **(Özkan *et al.*, 2012)**. As mentioned by **El-Garawani *et al.* (2021b)**, IMI produced alterations in the antioxidant levels of the hepatocytes of the Nile tilapia juveniles by enhancing gene expression and the activities of GPX, CAT, LPO, and SOD. These mentioned data support the current study's findings that IMI increased TOC and decreased TAC levels in the tilapia's hepatic tissues. On the contrary, enhancing the antioxidant status may be associated with antioxidant potency by lowering the number of free radicals generated during lipid peroxidation induced by IMI **(Attia *et al.*, 2021)**. This explanation supports the current study's findings that the GRSE decreased the TOC levels while increasing the TAC levels compared to the values of the IMI ones.

The comet assay was performed to detect the damage of DNA at the individual cellular level. It is recognized as one of the utmost hopeful biomarkers of genotoxicity for detecting a wide range of DNA abrasions in aquatic species. Fish exposed to IMI were shown to have DNA damage in their hepatocytes and gills **(Alvim *et al.*, 2019)**. IMI causes oxidation to different biological molecules, including proteins and lipids **(Vieira *et al.*, 2018)**. The disruption of biological functions and structures related to DNA damage is due to genotoxicity **(El-Garawani *et al.*, 2021a)**. This could be attributed to the formation of H_2O_2, which is hard to discard and causes oxidative DNA damage, especially when antioxidant enzyme activities are reduced **(Iturburu *et al.*, 2017)**. Moreover, IMI suppresses the activity of DNA repair enzymes that are responsible for the DNA repair mechanism causing nuclear abnormalities **(Odetti *et al.*, 2020)**.

Our results agree with those of **El-Garawani *et al.* (2021a)**, who reported an increase in the DNA damage in the hepatic tissues of the Nile tilapia due to genotoxicity **(El-Garawani *et al.*, 2021a)**. Similar results were observed in *Misgurnus anguillicaudatus* **(Xia *et al.*, 2016)** and *Gobiocypris rarus* **(Hong *et al.*, 2018)** after exposure to IMI. The molecular mechanism for the DNA damage can be explained as the chemical insecticide invading the nucleus through cell membranes, whereupon they interact with DNA, unfolding the DNA and damaging the genetic material **(El-Garawani *et al.*, 2021a)**. Furthermore, IMI was reported to form DNA adducts by acting as an alkylating agent **(Alvim *et al.*, 2019)**. Additionally, the DNA damage could be attributed to the interface with the produced radicals of oxygen and the establishment of DNA-DNA crosslinks or DNA-protein crosslinks **(Bolognesi & Cirillo, 2014)**. The liver was selected to measure the percentage of DNA damage because it is the principal organ that performs detoxification as it mainly collects toxic substances **(Campos *et al.*, 2017)**. Additionally,

IMI has a diverse effect on the liver, affecting its enzyme production, detoxification, and metabolism. Furthermore, chemical substances abundantly accumulate in the liver and muscles, leading to the highest injury **(Žegura *et al.*, 2004; El-Garawani *et al.*, 2022)**.

However, the protective role of GRSE can be proved by the decline in the percentage of DNA damage. Indeed, the natural food additive GRSE reduces stress levels and enhances the nutritional value and antioxidant capacity of the Nile tilapia by reducing ROS and protein and lipid oxidation products **(Yang *et al.*, 2023)**. Moreover, it showed a significant effect in protecting cells against free radicals and DNA damage **(Jahanbakhshi *et al.*, 2023)**. It reduced the stress response and enhanced the Nile tilapia growth. Additionally, the Nile tilapia exhibited higher growth performance due to the supplementation of GRSE, which likely activated the IGFs/PI3K/Akt/TOR/S6K1/4E-BP1 pathway and promoted muscle protein deposition and myofiber growth. **(Yang *et al.*, 2023)**. These mentioned data discuss the current study`s findings concerning that GRSE improved the flesh quality parameters of fish for maximum growth and muscle protein deposition.

Gills are a well-thought-out reflection of the quality of the water and robust biological indicators of toxicant contact due to their unswerving contact with the adjacent medium and susceptible structures **(Erkmen *et al.*, 2017, Günal *et al.*, 2020)**. Consistent with earlier studies, the IMI-intoxicated tilapia, herein, exhibited deformities of filaments, necrosis, epithelial lifting, and edematous forms in examined gills **(Özdemir *et al.*, 2018; Günal *et al.*, 2020; El-Garawani *et al.*, 2022)**. Epithelial lifting and edema in secondary filaments are considered nonspecific defense responses that protect the fish from the increased uptake of numerous toxicants by expanding the distance between blood capillaries and toxicants **(El-Garawani *et al.*, 2022)**.

Neonicotinoids affect the liver in a variety of ways, such as metabolism, enzyme production, and detoxification **(Sharma *et al.*, 2019)**. Serious signs of hepatic deformations in the liver exposed to IMI were detected in this study, including dilatation and congestion of the wall of the central hepatic vein, cytoplasmic vacuolation, hemorrhage, hepatocellular necrosis, and leukocyte infiltration. Hepatic deterioration may develop from aggregations of mononuclear leukocytes within the hepatic parenchyma as detected in this study **(Javed *et al.*, 2016)**. Moreover, **Ansoar-Rodríguez *et al.* (2016)** confirmed that the hydropic (vacuolar) degenerative conditions might be due to water and electrolyte accumulation inside the cell.

The accumulation of ROS has been recognized as causing hepatocellular injury through direct influence on mitochondrial DNA **(Padmini & Usha Rani, 2011)**. ROS may develop because free fatty acids remain unprocessed, as identified herein in hepatic tissues, increasing the region of vacuolization (lipidosis illness); also those radicals induce damage of mitochondrial DNA, resulting in the collapse of sinusoid structures and

hemorrhage in the hepatic tissue through the later mechanisms and ultimately severe histopathological alterations **(Özdemir *et al.*, 2018)**. These mentioned data support the current study's findings that IMI caused hepatic DNA damage and histological alterations via elevation of blood LDL levels and hepatic TOC. These influences could ultimately harm the body's physiological status including growth **(Haredi *et al.*, 2020)**. Thus, evaluating the histopathological changes in the tissues of the fish is essential for determining the health condition of fish and the level of pesticide toxicity **(Abdel Rahman *et al.*, 2022)**.

Other pathology in IMI intoxicated liver was studied such as the gathering of connective tissue fibers around the central hepatic vein (fibrosis) and the considerable decline in cytoplasmic glycogen granules; both were confirmed previously by **Priya *et al.* (2013)**. In the same line, **Lv *et al.* (2020)** investigated that prolonged exposure to IMI generated oxidative stress and inflammation that caused hepatic injury and led to hepatic fibrosis via the instigation of the TGF-β1/Smad signaling pathway. As the liver is a chief organ storing glycogen, IMI may have obvious harmful influences on systemic carbohydrate metabolism contributed to glycogen depletion, as documented by **Priya *et al.* (2013)**. Generally, numerous earlier research have also described signs of liver damage following IMI toxicity **(Priya *et al.*, 2013; Günal *et al.*, 2020; Abdel Rahman *et al.*, 2022)**.

Supplementing the IMI group with a GRSE-enriched diet partially or fully reversed the deterioration of histopathological effects caused by IMI toxicity in gills and liver tissues. This protective role may be attributable to the antioxidant activity and immune-protective roles of GRSE. These findings were also confirmed by boosted TAC levels while decreasing TOC and TNF-α levels detected in this study in the GRSE group. According to **Rodríguez-Pérez *et al.* (2019)**, grape seed proanthocyanidins are a typical antioxidant and have 50 and 20 times the antioxidant activity of vitamins E and C, respectively, making them commonly employed in nutritional supplements and food additives.

The current investigation exhibited a strong immunohistochemical reaction for hepatic caspase 3 subjected to IMI. These consequences may be linked to IMI-induced generation of ROS and oxidative destruction that surpasses the fish body's defensive machinery producing liver injury and inauguration of apoptosis **(Abdel Rahman *et al.*, 2022)**. These conclusions were validated previously by **Bal *et al.* (2012)**, who documented the existence of caspase 3 as an apoptotic marker, fragmentation of the DNA, and serious cytotoxicity outcome of IMI contact. A strong caspase 3 immunohistochemical reaction was also detected in *C. carpio* which was post-subjected for 96 h to IMI **(Özdemir *et al.*, 2018)**. On the other hand, the IMI-subjected tilapia that consumed a 4% GRSE enriched diet revealed a moderate caspase 3 immunoreactivity. This can be a result of GRSE's antioxidant-protecting efficacy that was confirmed by the

current findings of increasing hepatic TAC levels, which might induce a boost in the fighting of cells versus apoptosis, generating a strong immunoreactivity **(Yang *et al.*, 2023)**.

CONCLUSION

It is impossible to sidestep using and applying pesticides in fields of agriculture, as well as controlling aquatic pollution. As a result, safe and alternative solutions are in great demand. According to the study findings, IMI harms the health of *O. niloticus*. However, due to its strong antioxidant and immunostimulatory properties, GRSE may be able to counteract the negative effects of IMI, such as genotoxicity, hepatic toxicity, impairment of liver enzyme, lipid profile, leptin hormone, oxidative damage, immunosuppression, histological, histochemical, and immunohistochemical variations in *O. niloticus*. Therefore, the findings obtained from this study could contribute to improving the understanding of pesticide toxicity in non-target organisms, while also defending GRSE practices and ecosystem safety. Moreover, this research is significant for *O. niloticus* because of its economic reputation for the safety of human consumption.

DECLARATION OF COMPETING INTEREST

The authors claim that there is no conflict of interest.

AUTHOR'S CONTRIBUTION

Conceptualization, R.M.A, H.M.A.; Methodology, A.S.F., H.M.A., N.A.E., N.M.T., Formal analysis, H.M.A., N.A.E, N.M.T., A.S.F; Investigation, R.M.A, H.M.A.., N.A.E., N.M.T.; Resources, R.M.A, H.M.A.., N.A.E., N.M.T.; Writing- original draft preparation, A.S.F, N.A.E., N.M.T.; Writing-review and Editing, R.M.A, H.M.A.., N.A.E., N.M.T., all authors have read and agreed to the published version of the manuscript.

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