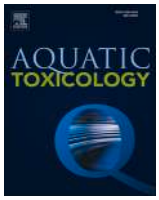
Aquatic Toxicology 265 (2023) 106738

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox



Potential role of dietary *Boswellia serrata* resin against mancozeb fungicide-induced immune-antioxidant suppression, histopathological alterations, and genotoxicity in Nile tilapia, *Oreochromis niloticus*

Afaf N. Abdel Rahman [a,*], Dalia E. Altohamy [b], Gehad E. Elshopakey [c], Abdelwahab A. Abdelwarith [d], Elsayed M. Younis [d], Nora M. Elseddawy [e], Aya Elgamal [f], Shefaa M. Bazeed [g], Tarek Khamis [h,i], Simon J. Davies [j], Rowida E. Ibrahim [a,*]

[a] *Department of Aquatic Animal Medicine, Faculty of Veterinary Medicine, Zagazig University, PO Box 44511, Zagazig, Egypt*
[b] *Department of Pharmacology, Central Laboratory, Faculty of Veterinary Medicine, Zagazig University, PO Box 44511, Zagazig, Egypt*
[c] *Department of Clinical Pathology, Faculty of Veterinary Medicine, Mansoura University, PO Box 35516, Mansoura, Dakahlia, Egypt*
[d] *Department of Zoology, College of Science, King Saud University, PO Box 2455, Riyadh 11451, Saudi Arabia*
[e] *Department of Pathology, Faculty of Veterinary Medicine, Zagazig University, PO Box 44511, Zagazig, Egypt*
[f] *Department of Animal Histology and Anatomy, Faculty of Veterinary Medicine, Badr University in Cairo (BUC), Cairo, Egypt*
[g] *Department of Biochemistry and Animal Physiology, Faculty of Veterinary Medicine, Badr University in Cairo (BUC), Cairo, Egypt*
[h] *Department of Pharmacology, Faculty of Veterinary Medicine, Zagazig University, PO Box 44511, Zagazig, Egypt*
[i] *Laboratory of Biotechnology, Faculty of Veterinary Medicine, Zagazig University, PO Box 44511, Zagazig, Egypt*
[j] *Aquaculture Nutrition Research Unit ANRU, Carna Research Station, Ryan Institute, College of Science and Engineering, University of Galway, Galway H91V8Y1, Ireland*

ARTICLE INFO

Keywords:
Nile tilapia
Mancozeb
Indian frankincense
Cytokines
Histological picture
Molecular level

ABSTRACT

This study was established to look into the toxicological consequences of chronic exposure to a fungicide (mancozeb; MAZ) on the immune-antioxidant response, gene expressions, hepato-renal functions, and histological pictures of Nile tilapia (*Oreochromis niloticus*). Additionally, the effectiveness of Indian frankincense resin extract (IFRE) to mitigate their toxicity was taken into account. Fish (n =240; average body weight: 22.45 ± 2.21 g) were randomized into four groups for eight weeks in six replicates (control, IFRE, MAZ, and IFRE + MAZ), where ten fish were kept per replicate. The control and IFRE groups received basal diets that included 0.0 and 5 g/kg of IFRE without MAZ exposure. The MAZ and IFRE+MAZ groups received the same diets and were exposed to 1/10 of the 96-h of LC_{50} of MAZ (1.15 mg/L). The outcomes displayed that MAZ exposure resulted in a lower survival rate (56.67 %) and significantly decreased levels of immune-antioxidant variables (antiprotease, complement3, phagocytic activity, lysozyme, glutathione peroxidase, superoxide dismutase, and total antioxidant capacity) compared to the control group. The MAZ-exposed fish showed the greatest levels of lipid peroxide (malondialdehyde), alkaline phosphatase, alanine amino-transferase, and stress indicators (cortisol and glucose). Additionally, histopathological alterations, including vacuolation, severe necrosis, degeneration, and mononuclear cell infiltrations in the hepatic, renal, and splenic tissues resulted, besides a reduction in the melanomacrophage center in the spleen. A down-regulation of immune-antioxidant-associated genes [toll-like receptors (*TLR-2* and *TLR-7*), nuclear factor kappa beta (*NF-κβ*), transforming growth factor-beta (*TGF-β*), phosphoinositide-3-kinase regulatory subunit 3 gamma b (*pik3r3b*), interleukins (*IL-1β* and *IL-8*), glutathione synthetase (*GSS*), glutathione peroxidase (*GPx*), and superoxide dismutase (*SOD*)] were the consequences of the MAZ exposure. Remarkably, the dietary inclusion of IFRE in MAZ-exposed fish augmented the immune-antioxidant parameters, including their associated genes, decreased stress response, and increased survival rate (85 %) compared with the MAZ-exposed fish. Moreover, dietary IFRE improved hepato-renal function indices by preserving the histological architecture of the hepatic, renal, and splenic tissues. The insights of this study advocate the use of an IFRE-dietary addition to protect Nile tilapia from MAZ toxicity, which provides perspectives for future implementations in enhancing fish health for sustainable aquaculture.

* Corresponding authors.
E-mail addresses: afne56@gmail.com (A.N.A. Rahman), rowidakamhawey@yahoo.com (R.E. Ibrahim).

https://doi.org/10.1016/j.aquatox.2023.106738
Received 8 September 2023; Received in revised form 14 October 2023; Accepted 28 October 2023
Available online 29 October 2023

1. Introduction

Pollution is a major concern that impacts everyone, and it is exacerbated by the world's industrialization and growing population (Stehle and Schulz, 2015; Ukaogo et al., 2020). Aquatic pollution is a current issue because it impedes sustainable aquaculture growth by negatively impacting fish health (Abdel-Tawwab et al., 2023; Aliko et al., 2022). A wide range of harmful contaminants have been introduced into the ecosystem as a result of agricultural operations and development processes, including pesticides (Bhat et al., 2022; Sinha et al., 2022). Pesticides are chemicals that contaminate the feed components and aquatic environment. Because they can bioaccumulate in fish tissues, they pose serious risks to fish and other creatures (Petrovici et al., 2020; Dawood et al., 2020; Abdel Rahman et al., 2022b).

Among agricultural pesticides, mancozeb (dithane or 1, 2-ethanedicarbamic acid; MAZ) is a broad-ranging fungicide used in agriculture (Banaee et al., 2023). MAZ is commonly used to treat a wide range of fungal infections of the field grains, including vegetable downy mildew and brown spots (Gullino et al., 2010). MAZ can get into water bodies after application via seepage, runoff, and spray-drift processes, contaminating aquatic ecosystems and creating possible environmental and public health hazards (De Joode et al., 2016; Liu et al., 2019). MAZ residues have been identified in many food crops (0.2–5.09 mg/kg) (Khorshed et al., 2010). In drainage water, sediment, and soil samples, the residues of MAZ were found to be 5.9–13.8 μg/L, 0.01 mg/kg, and 2 mg/L, respectively. Surface waters had measurable MAZ concentrations ranging from 0.455 to 1.279 μg/L. Also, MAZ concentrations in the aquatic systems have reached as high as 1.30 μg/L (Yıldırım and Ozcan, 2007; Flores-García et al., 2011). MAZ can endure in the sediment for one to seven days before degrading to a serious metabolite called ethylene-thiourea. This metabolite can remain for five to ten weeks, and its concentration in surface and subsurface waters was found to be 4.30 and 22.50 μg/L (Srivastava and Singh, 2013; Marques et al., 2016).

Fish is an excellent model for assessing pesticide toxicity in aquatic environments because of its high pesticide sensitivity, ability to metabolize contaminants, and bioaccumulation (Fazio et al., 2013; Abdel Rahman et al., 2022b). The Nile tilapia, *Oreochromis niloticus,* is a widely cultivated fish in the world due to its suitability for aquaculture, great palatability, and quick development (El-Sayed, 2019). A number of toxicological adverse effects on the metabolic and biological processes of the fish (non-target species) resulted from pesticide exposure. MAZ is extremely hazardous for a variety of fish species (Atamaniuk et al., 2014; Marques et al., 2016; Zizza et al., 2017; Costa-Silva et al., 2018), including Nile tilapia (Ibrahim et al., 2023), induced neurotoxicity, behavioral alterations, mutagenicity, oxidative damages, immune/antioxidant and reproductive retardation, and hormonal disturbance. In aquaculture, recent research focuses on using dietary medicinal plants, their extracts, or essential oils to alleviate pesticide toxicity and promote the growth and health of fish (Paduraru et al., 2021; Mansour et al., 2022; Abdel Rahman et al., 2022a; Ahmadniaye Motlagh et al., 2023; Rashidian et al., 2023).

One such dietary additive is frankincense, produced from the *Boswellia* genus. *Boswellia serrata* is among the numerous species of *Boswellia* that have been identified. The *B. serrata,* or Indian frankincense herb, is grown in India, northeast Africa, and Yemen (Afsharypuor and Rahmany, 2005; Hamm et al., 2005). Frankincense is a fragrant resin that turns into a granular material known as olibanum, which is yellow-brown. Granules, pellets, or powder are the common commercial forms (Al-Yasiry and Kiczorowska, 2016). Frankincense is widely recognized for its wide range of biological implications. It exhibited antibacterial, antifungal, antioxidant, hepatoprotective, neuroprotective, anti-inflammatory, and anti-cholinergic potentials associated with anti-diarrheal activities (Sadhasivam et al., 2016; Ameen et al., 2017; Ayub et al., 2018; Rajabian et al., 2020). These biological effects are connected to its active components, which include monoterpenes, diterpenes, sesquiterpenes, and polysaccharides, with the main active ingredient being boswellic acid (Niebler and Buettner, 2015; Mannino et al., 2016; Vakayil et al., 2021). The use of Indian frankincense as an additive in animal feed has been authorized and confirmed its safety (European et al., 2021). Moreover, the benefits of Indian frankincense for health have been verified in numerous researches (Schrott et al., 2014; Umar et al., 2014

Adding Indian frankincense resin extract (IFRE), a natural substance, to the Nile tilapia diet has many advantages. It boosted immune-antioxidant response and resistance to *staphylococcus aureus* and encouraged growth while lowering total cholesterol, glucose, and triglyceride levels (Montaser et al., 2021). Furthermore, including frankincense oil in the broilers' feed has a good impact on the animals' growth and health status (Kiczorowska et al., 2020; Amer et al., 2023). However, the potency of this plant in reducing fish toxicity from pesticides has not been extensively studied. Because of this, the current study evaluated the impact of dietary IFRE against oxidative damage, immunological stress, hepato-nephrotoxicity, histopathological disruptions, and gene expression alterations caused by MAZ exposure in Nile tilapia.

2. Materials and Methods

2.1. Animal ethics and mancozeb (MAZ) preparation

This work was approved (ZU-IACUC/2/F/139/2022) by the Zagazig University Authority for Animal Use in Research. From Sigma Aldrich (St. Louis, MO, USA), a technical grade MAZ (98 % purity) was purchased and employed in this study. MAZ was dissolved in distilled water to prepare a stock solution. A dilution of 1/10 of 96 h median lethal concentration (LC_{50}) of MAZ (1.15 mg/L) was prepared. The 96 -h LC_{50} of MAZ was determined in our recent report to be 11.49 mg/L (Ibrahim et al., 2023).

2.2. Preparation of Indian frankincense resin extract (IFRE) and gas chromatography/mass spectrometry (GC-MS) assay

The dry and hard resin of Indian frankincense was purchased from the local market in Zagazig City, Egypt. This resin was milled into a fine powder and then retrieved using ethyl alcohol (95 %) in a Soxhlet apparatus to obtain an extract. After the extraction was complete, the ethyl alcohol was evaporated to create a brown-colored thick syrup. This extract was divided into two portions; the first was used in the trial. The other portion was used to apply GC-MS assay following the protocol of Vakayil et al. (2021). The chromatographic analysis of this extract was performed at the Regional Centre for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt, using an Ultra Gas Chromatography system (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.3. Tested diets preparation and culturing conditions

According to NRC (2011) guidelines, two iso-energetic and iso-nitrogenous diets (Table 1) were created by adding IFRE to the basal diet at 0 (control diet) and 5 (IFRE diet) g/kg levels (Montaser et al., 2021). Pellets with a diameter of 1.5 mm were created after the ingredients were mechanically combined and pelletized. These pellets were allowed to air dry with regular rotation to promote consistent drying for 24 h and then stored at 4 °C in the refrigerator until use. The basal diet was chemically analyzed following the AOAC (2003) guidelines.

Two hundred and forty Nile tilapia (average weight, 22.45 ± 2.21 g) were collected from a private fish farm in Kafr ELSheikh Governorate, Egypt. The fish had no history of disease outbreaks and were thoroughly checked to determine their health according to CCAC (2005) guidelines. Each ten fish were stocked in well-ventilated indoor glass tanks (80 × 70 × 40 cm). Each tank had dechlorinated tap water and was supported with air stones for continuous aeration connected to a central air compressor. Fish were acclimated for two weeks before the trial, where a

Table 1
Diets formulation and proximate composition (on a dry weight basis).

Ingredients (g/kg)	Control diet	IFRE diet
Corn gluten 67 % CP	80	80
Fish meal 70.7 % CP	230	230
Soybean meal 49 % CP	230	230
Yellow corn	220	220
Wheat flour	100	100
Fish oil	30	30
Wheat bran	50	50
Soy oil	30	30
Vitamins and minerals mixture	30	30
IFRE	0	5
Proximate composition (%)		
Crude Fat	10.19	
Crude protein	37.17	
Crude fiber	3.29	
Ash	6.94	
NFE *	42.39	
GE (MJ/kg)	20.72	

IFRE, Indian frankincense resin extract; NFE, Nitrogen free extract; GE, Gross energy.
* NFE = 100 − (crude fat + crude protein + crude fiber + ash).

basal diet was given to them up to satiation three times daily (8:00, 12:00, and 16:00 h).

Water quality variables were monitored throughout the acclimation and trial periods following the APHA (2005) recommendations and maintained within the acceptable ranges. These variables included pH (6.6 ± 0.40), dissolved oxygen (6.42 ± 0.3 mg/L), temperature (22 ± 2.10 °C), nitrite (0.02 ± 0.003 mg/L), and ammonia (0.008 ± 0.005 mg/L). Daily siphoning occurred to remove any excreta from the bottom of the tank, and complete water renewal occurred twice weekly.

2.4. Experimental design

Fish were randomly allotted into four groups for eight weeks in six replicates (control, IFRE, MAZ, and IFRE + MAZ), where ten fish were kept per replicate. The control and IFRE groups received basal diets that included 0 (control diet) and 5 (IFRE diet) g/kg of IFRE without MAZ exposure. The MAZ and IFRE+MAZ groups received the same diets and were exposed to 1/10 of the 96 h of LC_{50} of MAZ (1.15 mg/L). The fish was given the tested diets up to satiation three times daily (8:00, 12:00, and 16:00 h). The tank water was entirely renewed twice a week, and the MAZ exposure dose was mixed with a small amount of water before being expanded into the tank. Throughout the exposure period (eight weeks), clinical signs, mortalities, and post-mortem lesions were all tracked.

2.5. Blood and tissue sampling

By the end of eight weeks, the fish was anesthetized with 100 mg/L benzocaine solution, and eighteen fish per group were taken for blood collection. These samples were aspirated using anticoagulant free-sterile syringes and left to clot overnight at 4 °C. The serum was obtained by centrifugation of these samples at 1750 *xg* for 10 min and used for immunological, antioxidant/oxidant, and biochemical assays. The fish were euthanized with an excess dose of benzocaine solution (300 mg/L) for 10 min, accompanied by decapitation (AVMA, 2007). Hepatic, renal, and splenic tissues (18 fish/group) were collected for histopathological investigations. Furthermore, approximately 50 mg of each head kidney tissue (18 fish/group) was collected in 1 mL Quiazol (Qiagen, Germany) and preserved at -80 °C until a gene expression assay was performed.

2.6. Serum immunological and antioxidant/oxidant assays

The level of antiprotease activity was estimated according to Bowden et al. (1997) approach by mixing the serum samples (10 μL) with 20 μL 0.1 % trypsin (HiMedia) for 5 min. Then, 470 μL of a buffer and 500 μL of sodium-benzoyl-DL-arginine-4-nitroanilide hydrochloride (BAPNA) of SRL chemicals, Chennai, India, were added. As a trypsin blank, a trypsin-BAPNA mixture was employed. The proportion of trypsin inhibition revealed serum antiprotease activity. Using Cusabio kits, complement 3 (C3; Catalog No.: CSB-E09727s) was measured by spectrophotometry following the instructions included with the kit packaging.

The serum lysozyme (LYZ) activity was evaluated following the Ellis (1990) protocol. About 0.25 mg/mL *Micrococcus lysodeikticus* (Sigma-Aldrich Chemie GmbH, Darmstadt, Germany) was suspended in 2 mL 0.05 M sodium phosphate buffer (pH 5.9) and then incubated at 30 °C for 5 min. After that, serum samples (200 μL) were added, and the absorbance at 450 nm was measured. According to the Cai et al. (2004) methodology, the phagocytic activity (PA) % of the phagocytes was assessed using heat-inactivated *Candida albicans*. PA (%) was calculated using the following formula: PA (%) = 100 × (Number of macrophages with bacteria engulfed/Total number of Macrophages).

The diagnostic test kits (Biodiagnostic Co., Egypt) were used to determine the serum levels of malondialdehyde (MDA; CAT. No. MAD 25 29), glutathione peroxidase (GPx; CAT. No. GP 2524), superoxide dismutase (SOD; CAT. No. SOD 25 21), and total antioxidant capacity (TAC; CAT. No. TA 25 13) according to the previous methods (Paglia and Valentine, 1967; Nishikimi et al., 1972; Uchiyama and Mihara, 1978; Koracevic et al., 2001).

2.7. Serum biochemical assays

Alkaline phosphatase (ALP), alanine aminotransferase (ALT), and creatinine levels in fish serum (CAT. NO. AP 10 20, AL 10 31 (45), and CR 12 50) were measured using diagnostic test kits (Biodiagnostic Co., Egypt) under previously described procedures (Reitman and Frankel, 1957; Perakis and Wolff, 1984; Wenger et al., 1984). The cortisol and glucose levels as stress-associated indicators were evaluated calorimetrically in the serum according to Saliu et al. (2017) and Trinder (1969) techniques.

2.8. Gene expression analysis

The total RNA was isolated from the head kidney tissues following the Abdel Rahman et al. (2022c) approach. Real-time quantitative PCR (RT-qPCR) evaluation of the expression of the immune-associated genes [the toll-like receptors (*TLR-2* and *TLR-7*), nuclear factor kappa beta (*NF-κβ*), transforming growth factor-beta (*TGF-β*), phosphoinositide-3-kinase regulatory subunit 3 gamma b (*pik3r3b*), interleukins (*IL-1β* and *IL-8*)] and antioxidant-associated genes [glutathione synthetase (*GSS*), *GPx*, and *SOD*] were measured.

The specified primers obtained from Sangon Biotech, Beijing, China) were used (Table 2). Following the previously reported approach (Schmittgen and Livak, 2008), gene expression was assessed as a relative fold change to a control reference gene (beta-actin, *β-actin*). The initial denaturation was performed for 10 min at 95 °C, followed by 40 cycles of denaturation, annealing, and extension at 95, 60, and 72 °C for 10, 15, and 15 sec, respectively.

2.9. Histopathological investigation

Hepatic, renal, and splenic tissues were fixed in 10 % buffered neutral formalin, dried in alcohol that was gradually increased (70–100 %), cleaned in xylene, and then embedded in paraffin. The paraffin sections of five micron-thick were produced with a microtome (Leica RM 2155, England). They were stained with H & E (hematoxylin and eosin) dyes and microscopically inspected (Suvarna et al., 2018).

Table 2
Primers of Nile tilapia's genes used for RT-PCR amplification.

Gene	Sequences	Accession number
TLR-2	TGGCACAGGACACTTAAGCA	XM_019360109.2
	GCGACGAGCACTGAGATACT	
TLR-7	CTTGGTCACGCTGTCCATCT	XM_019352834.2
	TGGCCCTGCAGAAATGGTAG	
NF-κβ	TCGGTGTAGCAGGCTTTTGT	XM_013277333.3
	GCTGCAGAGATGTGGGTGAT	
TGF-β	CCAGAGCAGAGCTACGGATG	NM_001311325.1
	CCAGGTCTGCAGAGGTTCAG	
pik3r3b	AGGCATATGACAGGAGCACAC	MH319856.1
	GCATCATCACGTCCCTCCAG	
IL-1β	CTCATGTCTGTCCGCTACCC	XM_019365842.2
	TGAAGCTTCTGTAGCGTGGG	
IL-8	CAAGATCATGTCCAGCAGATCC	NM_001279704.1
	TCGTGAAAGGAACACGGTGA	
GSS	TAGCAAGCTAAAATGCGCGG	XM_025901610.1
	AGAGCCGAGTTCATCAGCA	
GPx	GTGCCCTGCAATCAGTTTGG	NM_001279711.1
	CGAGGAGCTGGAACTTTGGT	
SOD	TCACAGCAAGCACCATGCTA	XM_003449940.5
	GCAACCTGTGTTGTCACGTC	
β-actin	CCACCCAAAGTTCAGCCATG	XM_003443127.5
	ACGATGGAGGGGAAGACAG	

TLR-2, Toll-like receptor 2; *TLR-7,* Toll-like receptor 7; *NF-κβ*, nuclear factor kappa beta; *TGF-β,* transforming growth factor-beta; *pik3r3b*, phosphoinositide-3-kinase regulatory subunit 3 gamma b; *IL-1β,* interleukin 1beta; *IL-8*, interleukin 8; *GSS*, glutathione synthetase; *GPx*, glutathione peroxidase; *SOD;* superoxide dismutase; *β-actin*, beta actin

2.10. Data analysis

Via Levene's test, all collected data were examined for homogeneity of variances. Then, the one-way ANOVA was performed on all variables using SPSS 20.0 (IBM Corp.). Duncan's post hoc test was applied to check for variations between means at $p < 0.05$. The outcomes were presented as means ± standard error (SE). Using the Kaplan-Meier approach, the survival probability for the experimental groups was calculated. To investigate any variances, the log-rank test was chosen to examine group differences in pairwise comparisons.

3. Results

3.1. GC-MS results of Indian frankincense resin extract (IFRE)

GC-MS findings of IFRE are shown in Table 3. The most prevalent compounds included α-boswellic acid (18.84 %), 3-O-acetyl-β-boswellic acid (16.69 %), oleanolic acid (12.40 %), incensole acetate (9.63 %), α-pinene (7.81 %), and camphene-6-ol (5.37 %).

3.2. Clinical observations and survival rate

The control and IFRE groups did not reveal any obvious clinical signs (Fig. 1A). However, mancozeb (MAZ) exposure induced various clinical abnormalities, including surfacing, restlessness, fin rot, skin darkening, body hemorrhages, and skin ulcerations (Fig. 1B and C). The IFRE + MAZ group displayed an improvement in the noticed symptoms, except some fish showed darkness, slight fin rot, and hemorrhages on the caudal fin (Fig. 1D).

Fig. 2 displays the data of the Kaplan-Meier curves, where the control and IFRE groups exhibited a 100 % survival rate. Meanwhile, the MAZ group had clearly the lowest survival rate (56.67 %); this rate increased in the IFRE+MAZ group (85 %). The groups showed a statistically significant differences between them ($p < 0.0001$), as well.

3.3. The Results of the Levene's test

The homogeneity of variances for all measured variables using Levene's test was presented in Supplementary Table 1. The findings revealed that all variables were homogeneous ($p > 0.05$).

Table 3
The GC-MS results of Indian frankincense resin extract (IFRE).

Peak	Compound	Retention time (min.)	Area (%)
1	α-Pinene	6.52	7.81
2	Limonene	8.08	4.66
3	α-Boswellic acid	12.69	18.84
4	1,1-Dimethyl-decyl-mercaptan	15.22	1.76
5	3,4-Dimethylbenzaldehyde	22.39	0.82
6	Stearic acid	28.01	2.36
7	3-O-Acetyl-β-boswellic acid	28.39	16.69
8	Incensole acetate	29.30	9.63
9	α-Phellandren-8-ol	30.15	2.70
10	Sabinene	32.45	3.87
11	3-Cyclohexen-1-ol	33.03	0.95
12	Dehydrosabinene ketone	36.80	2.80
13	cis-11-Eicosenoic acid	37.87	1.92
14	cis-p-Mentha-2,8-dien-1-ol	42.15	0.97
16	Terpinen-4-ol	43.82	3.57
17	13-Docosenoic acid, (E)-	48.50	0.91
18	Camphene-6-ol	50.30	5.37
19	Myristic acid	56.31	1.97
20	Oleanolic acid	59.49	12.40

3.4. Immunological and oxidant/ antioxidant responses

Table 4 demonstrates the immune-antioxidant response of the fish after MAZ exposure and/or dietary IFRE for eight weeks. Levels of the immune parameters (antiprotease, C3, PA% and LYZ) and antioxidants biomarkers (GPx, SOD, and TAC) showed a marked augmentation ($p < 0.0001$) in the IFRE group compared to the control group. The MDA did not alter in the IFRE group other than the control. The exposure to MAZ revealed a clear decrease in immune/antioxidant parameters with a rise in the MDA compared to the control. Meanwhile, feeding of MAZ-exposed fish on the IFRE diet (IFRE + MAZ) significantly ($p < 0.0001$) enhanced these parameters and declined MDA ($p = 0.0002$) compared with that of the MAZ-exposed fish fed on a basal diet Table 4.

3.5. Hepato-renal functions and stress-associated indicators

Table 5 reveals that dietary IFRE did not significantly alter the fish biochemical parameters regarding ALP, ALT, creatinine, and cortisol and reduced the glucose level compared to the basal diet. The MAZ exposure induced a noticeable increase in these parameters. On the contrary, the co-exposed group (IFRE+MAZ) exhibited a decline in these parameters compared to the MAZ group.

3.6. Genes expression

The expression of immune-antioxidant-associated genes in the head kidney after eight weeks of trial was illustrated in Figs. 3 and 4. Relative to the control fish, the highest expression ($p < 0.0001$) values of *TLR-2* (2.51-fold), *TLR-7* (2.01-fold), *NF-κβ* (3.56-fold), *TGF-β* (3.93-fold), *pik3r3b* (3.42-fold), *IL-1β* (2.91-fold), *IL-8* (2.96-fold), *GSS* (2.29-fold), *GPx* (2.15-fold), and *SOD* (2.12-fold) were obvious in the IFRE group.

The MAZ exposure induced a notable down-regulation ($p < 0.0001$) in the *TLR-2* (0.13-fold), *TLR-7* (0.09-fold), *NF-κβ* (0.18-fold), *TGF-β* (0.19-fold), *pik3r3b* (0.17-fold), *IL-1β* (0.15-fold), *IL-8* (0.16-fold), *GSS* (0.18-fold), *GPx* (0.13-fold), and *SOD* (0.11-fold) in comparison with control. In contrast, these genes were significantly up-regulated ($p < 0.0001$) in the IFRE+MAZ group, where the fold changes were 0.88-, 0.80-, 0.85-,0.91-, 0.83-, 0.90-, 0.78-, 0.84, 0.79, and 0.82-fold for the *TLR-2, TLR-7, NF-κβ, TGF-β, pik3r3b, IL-1β, IL-8, GSS, GPx*, and *SOD*, respectively.

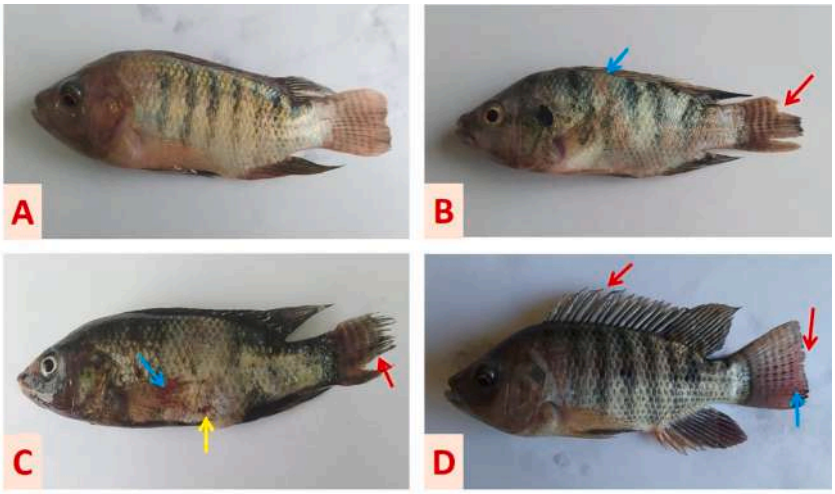


Fig. 1. Clinical signs of Nile tilapia fed dietary Indian frankincense resin extract (IFRE) and exposed to mancozeb (MAZ) for eight weeks. [A and B] Fish of the control and IFRE groups revealing normal appearance. [C and D] Fish of the MAZ group revealing skin darkening, fin rot (red arrows), body hemorrhages (light blue arrows), and skin ulcerations (yellow arrow). [E] Fish of IFRE +MAZ group revealing a slight fin rot (red arrows) and hemorrhages on the caudal fin (light blue arrow).

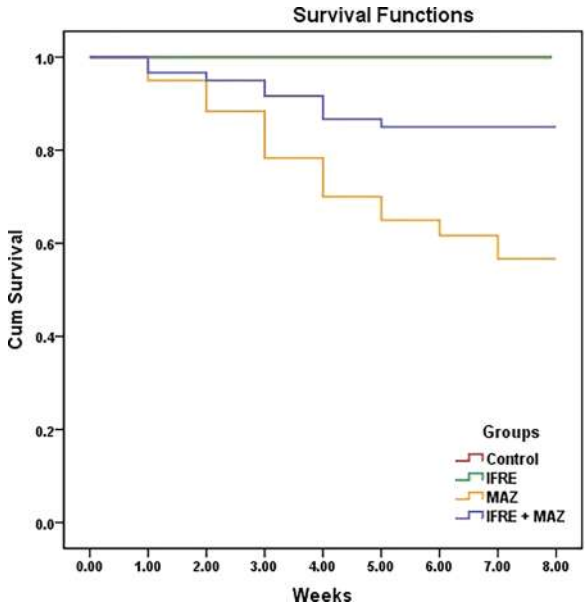


Fig. 2. Survival curves (Kaplan–Meier) of Nile tilapia fed dietary Indian frankincense resin extract (IFRE) and/or exposed to mancozeb (MAZ) for eight weeks.

Table 4
Immuno-antioxidant parameters of Nile tilapia fed dietary Indian frankincense resin extract (IFRE) and/or exposed to mancozeb (MAZ) for eight weeks.

Parameters	Control	IFRE	MAZ	IFRE+MAZ	*p*-value
Immunological parameters					
Antiprotease (ng/mL)	57.58 ± 1.49^{b}	87.69 ± 2.01^{a}	9.91 ± 2.37^{d}	32.44 ± 3.06^{c}	<0.0001
C3 (μg/mL)	157.25 ± 5.22^{b}	251.00 ± 14.43^{a}	52.78 ± 2.62^{d}	88.33 ± 1.63^{c}	<0.0001
LYZ (ng/mL)	5.02 ± 0.09^{b}	8.47 ± 0.36^{a}	0.50 ± 0.15^{d}	2.30 ± 0.26^{c}	<0.0001
PA (%)	72.50 ± 1.44^{b}	82.25 ± 1.01^{a}	37.75 ± 1.58^{d}	59.00 ± 2.31^{c}	<0.0001
Oxidant/antioxidant parameters					
MDA (nmol/mL)	2.37 ± 0.31^{c}	3.46 ± 0.76^{c}	14.91 ± 2.07^{a}	8.18 ± 0.62^{b}	0.0002
GPx (U/mL)	58.46 ± 1.65^{b}	74.76 ± 2.09^{a}	20.67 ± 2.49^{d}	38.53 ± 1.52^{c}	<0.0001
SOD (U/mL)	75.82 ± 2.42^{b}	96.60 ± 3.92^{a}	14.85 ± 3.89^{d}	43.45 ± 2.74^{c}	<0.0001
TAC (ng/mL)	9.18 ± 0.62^{b}	14.07 ± 0.82^{a}	0.77 ± 0.08^{d}	5.71 ± 0.55^{c}	<0.0001

C3, complement 3; LYZ, lysozyme; PA, phagocytic activity; MDA, malondialdehyde; GPx, glutathione peroxidase; SOD, superoxide dismutase; TAC, total antioxidant capacity. Values (mean ± *SE*) that don't share superscripts in the same row significantly differ at $p < 0.05$ (One-way ANOVA; Duncan's post hoc test).

3.7. Histopathological findings

In the control and IFRE groups, livers exhibited a normal histological structure where normal hepatocytes with a central spherical nucleus, a densely stained nucleolus, and arrangement with hepato-pancreatic tissue near the portal vein were obvious (Fig. 5A and B), respectively. However, the MAZ exposure induced vacuolation of most hepatocytes, pyknotic nucleus, and a focal aggregation of lymphocytes with the presence of brown pigment of hemosiderin and hepatolysis of fewer hepatocytes (Fig. 5C). Also, hepato-pancreatic cells showed degeneration and aggregation of melano-macrophages (Fig. 5D). These alterations were improved in the IFRE + MAZ group, where the liver restored normal histological structure with a regeneration of the hepato-pancreatic cells and aggregation of melanomacrophages in its cells and between hepatocytes (Fig. 5E).

The kidney tissues from the control and IFRE groups revealed the typical histological structure, which included multiple renal tubules with well-developed glomeruli (Fig. 6A and B). The kidneys of the MAZ-exposed group showed vacuolation in the lining epithelium of several renal tubules, pyknotic nucleus, focal aggregation of melanomacrophages, and infiltration of mononuclear cells. Some glomeruli showed shrinkage of their tufts with a wide Bowman's capsule (Fig. 6C). Necrosis which replaced by mononuclear cells infiltration and melanomacrophages were also appeared in the other renal tubules (Fig. 6D). Some renal tubules have been re-epithelized with restoring normal tufts of the glomeruli in the kidneys of IFRE + MAZ group (Fig. 6E).

The splenic tissues of the control and IFRE groups had a typical histological architecture, where it was divided into white pulp (devoid of lymphoid follicle) and red pulp and splenic capsule with melanomacrophages center found throughout the parenchyma (Fig. 7A and B). However, the MAZ exposure caused a thickening in the splenic capsule with necrosis of white pulp represented by the presence of vacuoles and a decrease of melanomacrophages center (Fig. 7C). The splenic tissues restored their normal structure with an increase of melanomacrophages by feeding the IFRE to the MAZ-exposed fish (Fig. 7D).

Table 5
Biochemical parameters of Nile tilapia fed dietary Indian frankincense resin extract (IFRE) and/or exposed to mancozeb (MAZ) for eight weeks.

Parameters	Control	IFRE	MAZ	IFRE+MAZ	*p*-value
Hepato-renal functions indicators					
ALP (IU/L)	5.86 ± 0.35^{c}	6.99.10 ± 0.64^{c}	44.00 ± 0.78^{a}	25.48 ± 1.37^{b}	<0.0001
ALT (U/mL)	6.38 ± 0.10^{c}	6.96 ± 0.03^{c}	67.48 ± 0.36^{a}	40.34 ± 0.46^{b}	<0.0001
Creatinine (mg/dL)	0.40 ± 0.03^{c}	0.41 ± 0.01^{c}	1.36 ± 0.25^{a}	0.91 ± 0.05^{b}	0.003
Stress-associated indicators					
Cortisol (ng/mL)	53.70 ± 1.23 ^{c}	51.80 ± 0.37 ^{c}	103.51 ± 2.07 ^{a}	71.40 ± 2.07 ^{b}	<0.0001
Glucose (mg/dL)	65.91 ± 0.87^{c}	50.89 ± 0.45^{d}	110.37 ± 4.72 ^{a}	86.52 ± 2.53^{b}	<0.0001

ALP, alkaline phosphatase; ALT, alanine aminotransferase. Values (mean ± *SE*) that don't share superscripts in the same row significantly differ at $p < 0.05$ (One-way ANOVA; Duncan's post hoc test).

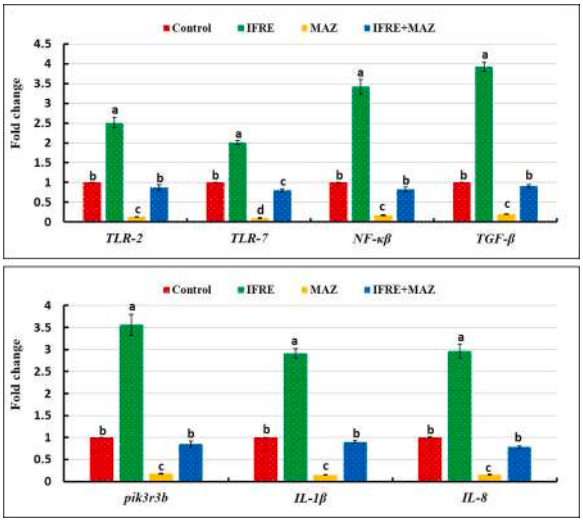


Fig. 3. The immune-associated genes expression ($p < 0.0001$) in the head kidney of Nile tilapia fed dietary Indian frankincense resin extract (IFRE) and/or exposed to mancozeb (MAZ) for eight weeks. Values (mean ± *SE*) that don't share superscripts significantly differ at $p < 0.05$ (One-way ANOVA; Duncan's post hoc test).

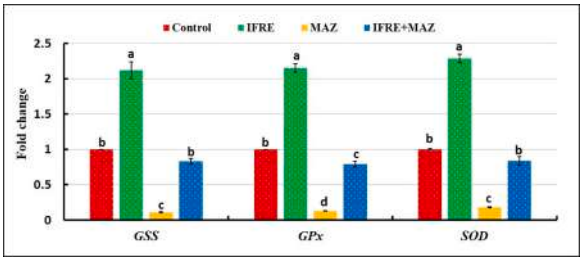


Fig. 4. The antioxidant-associated genes expression ($p < 0.0001$) in the head kidney of Nile tilapia fed dietary Indian frankincense resin extract (IFRE) and/or exposed to mancozeb (MAZ) for eight weeks. Values (mean ± *SE*) that don't share superscripts significantly differ at $p < 0.05$ (One-way ANOVA; Duncan's post hoc test).

4. Discussion

Pesticides are frequently employed in agriculture practices to safeguard crops and boost harvests (Bhat et al., 2022; Mansour et al., 2023). As a result of their dangerous impacts on aquatic life and human health, pesticide contamination of the environment is a serious issue (Dawood et al., 2020; Hamed et al., 2021; Saha et al., 2023). Mancozeb (MAZ) is one of many hazardous pesticides that pose a significant threat by impairing fish health (Liu et al., 2019; Ibrahim et al., 2023). Hence, given this framework, it is critical to better understand the adverse effects of MAZ on aquatic creatures utilizing Nile tilapia as a model to examine the toxicological impacts of MAZ. Furthermore, we investigated the potential protective role of Indian frankincense resin extract (IFRE) against MAZ-induced immunological stress, oxidative damage, histopathological disruptions, and gene expression alterations.

MAZ-exposed fish suffered various clinical symptoms, such as surfacing (gasping air), restlessness, fin rot, skin darkening, and body hemorrhages, with a lower survival rate (56.67 %). Similar manifestations induced by MAZ were found in walking catfish (*Clarius batrachus*) and Mozambique tilapia (*Oreochromis mossambicus*) were reported (Srivastava and Singh, 2013; Sinha et al., 2022). These findings could be linked to the hazards of MAZ on essential physiological systems. The respiratory impairment caused by MAZ exposure may be due to the direct action on the gills (the tissues that have direct contact with the watery contaminant), inducing oxidative damage (Kubrak et al., 2012). Also, abnormal behaviors can be linked with the effect on brain function by preventing acetylcholinesterase (neurotransmitter) from functioning, resulting in its accumulation in the synapses (Ibrahim et al., 2023). Then, the muscles are stimulated continuously until they are exhausted and tight, accompanied by limiting various physiological processes and abnormal behaviors (Sikka and Gurbuz, 2006; Saha et al., 2016).

However, when MAZ-exposed fish were given IFRE, clinical symptoms and survival rate (85 %) were improved. The strong antioxidant potential and free radical scavenging activities in IFRE against reactive oxygen species (ROS) of MAZ may be responsible for this improvement by limiting cellular oxidation. This could be accredited to the α-boswellic acid, 3-O-acetyl-β-boswellic acid, and oleanolic acid detected in our GC-MS profile, which exerted a potent scavenging and antioxidant capability. Other research corroborated this attribution (Sharma et al., 2011; Ebrahimpour et al., 2017; Gupta et al., 2022).

The immune function biomarkers (antiprotease, C3, PA%, and LYZ) in serum were evaluated. Also, gene expression was investigated to gain a better understanding of MAZ-induced toxicity at the molecular level. These parameters are crucial in fish humoral non-specific defense mechanisms and alter Nile tilapia's toxicity (Sahoo et al., 2021; Amer et al., 2022; Ibrahim et al., 2022a). In this investigation, the MAZ exposure caused immune-depression evident by decreasing the levels of antiprotease, C3, PA%, and LYZ and down-regulation of the expression of *TLR-2, TLR-7, NF-κβ, TGF-β, pik3r3b, IL-1β*, and *IL-8* genes. Also, various histopathological changes were obvious in the splenic tissues, with depletion in the melanomacrophages center (MMC) confirming the immune-suppressive effect of MAZ. MMCs have reportedly been identified as a fish immune function indicator, according to Steinel and Bolnick (2017). The immuno-suppressive impact of MAZ might be due to the lowered lymphocyte proliferation and decrease in the production of cytokines [interleukins (*IL-2* and *IL-4*), and interferon-gamma (*IFNc*)] in lymphocytes besides the induction of oxidative stress and DNA damage. This can increase the risk of infection during MAZ exposure (Medjdoub et al., 2011). Similar responses were obtained as a result of MAZ exposure in rainbow trout (*Oncorhynchus mykiss*) (Atamanalp and Yanik, 2003) and Nile tilapia (Ibrahim et al., 2023; Kanu et al., 2023).

Immune variables, including immune-associated genes, were noticeably up-regulated in the MAZ-exposed fish fed on the IFRE diet relative to the MAZ-exposed fish fed on the basal diet. No pathological alterations were noted in the splenic tissues with increased MMC. These results validated IFRE's immuno-stimulant properties, reflected in increased immuno-antioxidant response. This may be due to the boswillic acid content of IFRE detected in our GC-MS assay. According to previous researches (Chevrier et al., 2005; Beghelli et al., 2017), boswillic acids increased the expression of anti-inflammatory cytokines and secondary antibodies, macrophage phagocytosis, and stimulated lymphocyte proliferation. Consequently, they act to boost the immune

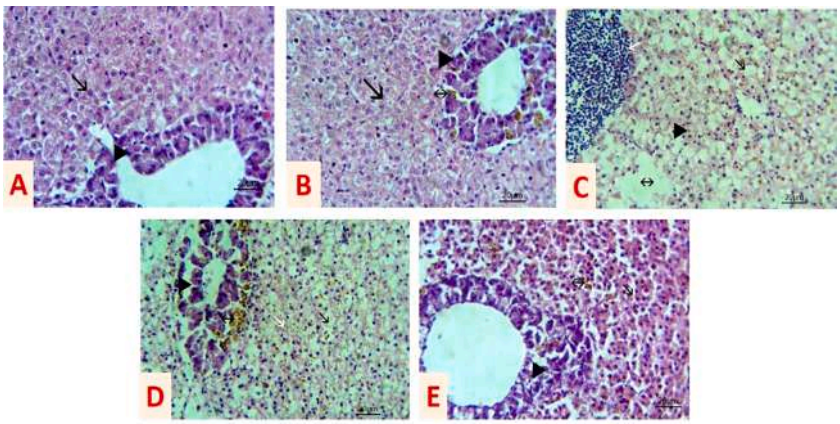


Fig. 5. Photomicrographs of liver sections of Nile tilapia fed dietary Indian frankincense resin extract (IFRE) and/or exposed to mancozeb (MAZ) for eight weeks. [A and B] Liver of the control and IFRE groups revealing a normal arrangement of the hepatocytes separated by sinusoid (arrows) and hepato-pancreatic tissue near the portal vein (arrows head) with aggregation of melanomacrophages (two heads arrow). [C and D] Liver of the MAZ group revealing vacuolation of hepatocytes with pyknotic nucleus (arrow) and focal aggregation of lymphocytes (white arrow) and hemosiderin pigment (arrowhead), and hepatolysis (two heads arrow) [C], degeneration of hepato-pancreatic cells (arrowhead) and aggregation of melano-macrophages (two heads arrow) with hemosiderin pigment (white arrow) [D]. [E] Liver of IFRE + MAZ group revealing a restoration of normal histological structure (arrow) and regeneration of hepato-pancreatic cells (arrowhead) with aggregation of melanomacrophages (two heads arrow). H&E; scale bar: 20 μm.

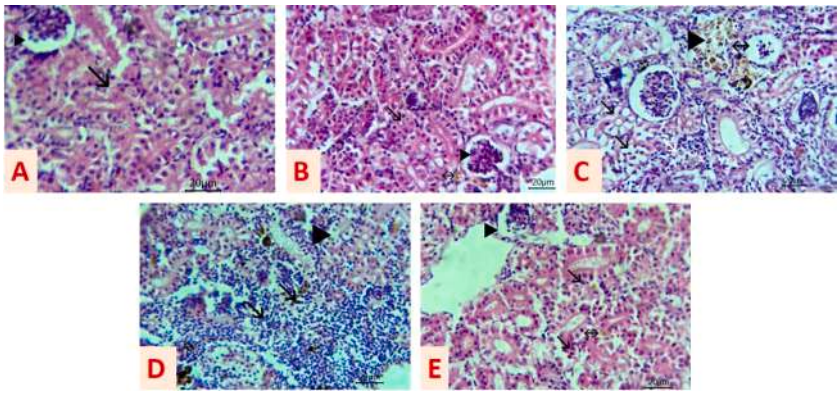


Fig. 6. Photomicrographs of kidney sections of Nile tilapia fed dietary Indian frankincense resin extract (IFRE) and/or exposed to mancozeb (MAZ) for eight weeks. [A and B] Kidneys of the control and IFRE groups revealing a normal histological structure of renal tubules (arrows) and glomeruli (arrowheads) with mononuclear cell infiltration (two heads arrow). [C and D] Kidney of the MAZ group revealing vacuolation of epithelium with pyknotic nucleus (black arrows) and melano-macrophages (arrowhead), mononuclear cells (white arrow), shrinkage of glomeruli tufts (two heads arrow) (C), necrotic epithelium replaced by mononuclear cells and melanomacrophages (black arrows) and vacuolated epithelium (arrowhead) (D). [E] Kidney of IFRE + MAZ group revealing a re-epithelization of the renal lining epithelium (arrows), glomeruli restoring normal tuft (arrowhead), and melanomacrophages (two heads arrow). H&E; scale bar: 20 μm.

system, which in turn reduces the toxicity of the MAZ. Additionally, Montaser et al. (2021) found that feeding IFRE improved non-specific immunological measures in Nile tilapia, such as LYZ and NO, and enhanced the histological architecture of the spleen.

Pesticide exposure may trigger oxidative damage and lipid peroxidation (Ndonwi et al., 2019). MDA represents the lipid peroxidation marker (Zengin, 2021). To deal with the stress and stop too much ROS formation, fish under oxidative stress generate a number of anti-oxidative protections, such as SOD and GPx (Ibrahim et al., 2019; Borges et al., 2022). Our study found that Nile tilapia exposed to MAZ had higher MDA and lower GPx, SOD, and TAC levels. Also, the antioxidant-associated genes (*GSS, GPx*, and *SOD*) were down-regulated by MAZ. It is believed that MAZ toxicity disrupted the balance of free radicals production and clearance from the body. Hence, ROS buildup then cause lipid peroxidation. Our results have been verified by Costa-Silva et al. (2018), who found that common carp (*Cyprinus capio*) underwent oxidative stress after being exposed to MAZ.

Interestingly, dietary supplementing of the MAZ-exposed fish with IFRE improved the antioxidant variables, including antioxidant-associated genes, compared to the MAZ-exposed fish, only reflecting its antioxidant potential. IFRE can lessen the generation of nitric oxide and tissue oxidative damage brought on by increased free radicals (Hartmann et al., 2014). Indeed, according to Gupta et al. (2022) and Teng et al. (2018), IFRE contains a wide variety of active ingredients, mainly phenols (boswellic acids) and glycosides (oleanolic acid) that exert positive synergy in antioxidant activity, and this was confirmed in our GC-MS profile. The *in vitro* antioxidant potential of IFRE was established by Afsar et al. (2012).

The hepatic and renal function measures are key diagnostic markers because they represent general health and fish toxicity (Ibrahim et al., 2022b; Mahboub et al., 2022). Additionally, investigating the level of pesticide toxicity requires an evaluation of the histopathological changes in fish tissues (Georgieva et al., 2021). We found an increase in hepato-renal markers (ALP, ALT, and creatinine) in MAZ-exposed fish, indicating hepato-renal dysfunctions. This result could be attributed to the histopathological alterations caused by MAZ exposure on the hepatic and renal tissues, which were obvious in this study. It is hypothesized that MAZ led to oxidative damage by ROS production, which destroys hepatic and renal cells, impairing their functioning and generating enzymatic leakage into the blood. Simakani et al. (2018) recorded

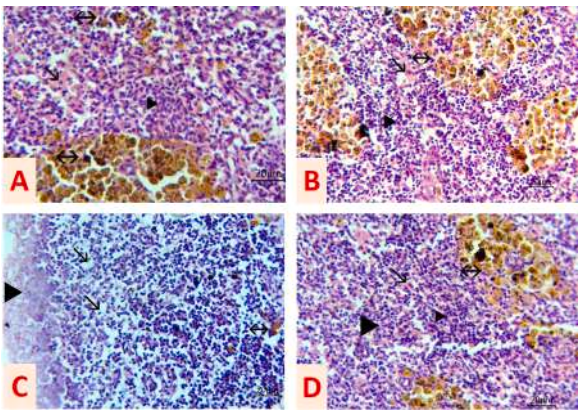


Fig. 7. Photomicrographs of splenic sections (H&E) of Nile tilapia fed dietary Indian frankincense resin extract (IFRE) and/or exposed to mancozeb (MAZ) for eight weeks. [A and B] Spleen of the control and IFRE groups revealing a typical histological structure of white pulp (arrows), red pulp (arrowheads), and melanomacrophage center (two heads arrow). [C] Spleen of the MAZ group revealing thickening of the splenic capsule (arrowhead) with necrosis of white pulp represented by the presence of vacuoles (arrow) and decrease of melanomacrophages center (two heads arrow). [D] Spleen of IFRE + MAZ group revealing a normal histological structure of white pulp (arrow), red pulp (arrowhead), and melanomacrophage center (two heads arrow). H&E; scale bar: 20 μm.

elevations in levels of renal and hepatic biomarkers of *C. carpio* following the MAZ exposure. In line with our findings, a recent investigation by Choudhury and Das (2020) found that hepatic and renal cells of spotted snakehead (*Channa punctatus*) underwent degenerative alterations after exposure to MAZ-contaminated water over an extended period.

In this regard, it was observed that MAZ-exposed fish and given IFRE as a food supplement for eight weeks caused an improvement in the hepato-renal markers and restored the tissues' histological structures. This may be explained by the strong antioxidant action exerted by the IFRE active components, restoring the hepato-renal function. Similarly, Montaser et al. (2021) found no histopathological changes in the kidney of Nile tilapia received an IFRE (5 g/kg diet) enriched diet. The nephro-protective impact of IFRE was reported in albino rats against gentamicin toxicity (Alam et al., 2011). *Boswellia sacra* resin exerted a hepato-protective impact in the rats' liver against carbon tetrachloride toxicity (Asad and Alhumoud, 2015).

Crucial physiological signals for assessing physiological status in fish are blood glucose and cortisol, which are influenced by numerous factors, including toxicity (Polakof et al., 2012; Sadoul and Geffro, 2019). Herein, MAZ exposure caused a significant stress response, as evidenced by higher cortisol levels and glucose values. Cortisol (an active glucocorticoid) regulates the metabolism of carbohydrates, protein, and lipids. During stress, blood cortisol levels rise to stimulate the creation of glucose from proteins to fulfill greater energy needs (Simonato et al., 2013; Bhanu, 2016). Also, the higher cortisol level in our study could cause the immuno-suppressive effect in this investigation. According to Dunier (1996), the immuno-suppression processes induced by pollutants might be seen as a direct negative impact on immune cells or a more indirect effect via the neuroendocrine system (corticoids). By reducing the amounts of circulating LYZ, cortisol impacts the immune system of fish (Guo and Dixon, 2021). Previously, *O. mykiss* exposed to MAZ showed similar results (Bisson and Hontela, 2002).

Conversely, a fish-fed IFRE-included diet showed marked improvement in the levels of these biomarkers after exposure to MAZ, implying that dietary IFRE could lower the stress response of Nile tilapia. This is explained by the abundance of oleanolic acid, α-boswellic acid, and 3-O-acetyl-β-boswellic acid in the IFRE, the main bioactive substances obvious in the GC-MS profile. Boswellic acids could decrease blood glucose by activating β-cells to produce excess insulin, besides increased peripheral glucose consumption by skeletal muscles (Jadhav and Puchchakayala, 2012). Oleanolic acid reduces hepatic insulin resistance by limiting mitochondrial ROS and exerting anti-inflammatory and hypo-lipidemic actions (Wang et al., 2013; Andreani et al., 2017).

As a result, it is possible to conclude that IFRE's key role is a substantial positive endpoint for its high antioxidant, immune-stimulant, and anti-inflammatory activity. Furthermore, dietary IFRE protects Nile tilapia against the immune-depression, oxidative damage, and hepato-nephrotoxicity caused by MAZ and helps to maintain tissue architecture.

5. Conclusion

It is challenging to prevent the use of pesticides in agriculture or to manage water pollution. Safe alternative protocols are, therefore, desperately needed. According to the study's findings, MAZ had a negative effect on the health of Nile tilapia, including immune-antioxidant depression and hepato-renal toxicity, including histopathological changes. Through its strong antioxidant and immuno-enhancing potentials, dietary inclusion of IFRE could counteract the MAZ-associated adverse impacts. The results of this study may help to better understand how pesticides are hazardous to creatures (fish) and safeguard the ecosystem. More research is needed to understand how chronic MAZ exposure affects the health of various fish species and the ameliorative role of IFRE.

CRediT authorship contribution statement

Afaf N. Abdel Rahman: Conceptualization, Methodology, Investigation, Resources, Software, Data curation, Writing – original draft, Writing – review & editing. **Dalia E. Altohamy:** Methodology, Investigation, Data curation, Writing – review & editing. **Gehad E. Elshopakey:** Conceptualization, Methodology, Investigation, Writing – review & editing. **Abdelwahab A. Abdelwarith:** Methodology, Investigation, Resources, Writing – review & editing. **Elsayed M. Younis:** Methodology, Investigation, Resources, Writing – review & editing. **Nora M. Elseddawy:** Methodology, Investigation, Resources, Data curation, Writing – review & editing. **Aya Elgamal:** Methodology, Investigation, Resources, Writing – review & editing. **Shefaa M. Bazeed:** Conceptualization, Methodology, Investigation, Writing – review & editing. **Tarek Khamis:** Methodology, Resources, Data curation, Writing – review & editing. **Simon J. Davies:** Investigation, Writing – review & editing. **Rowida E. Ibrahim:** Conceptualization, Methodology, Investigation, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors claim that there is no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by the Researches Supporting Project number (RSPD2023R700), King Saud University, Riyadh, Saudi Arabia. Also, the authors thank all Aquatic Animal Medicine Department staff, Faculty of Veterinary Medicine, Zagazig University, for their kind support during the study.

Supplementary materials

Supplementary material associated with this article can be found, in

the online version, at doi:10.1016/j.aquatox.2023.106738.

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