



Dietary lemon essential oil mitigates metalaxyl-induced oxidative stress, hematological disruptions, and immunotoxicity in Nile tilapia (*Oreochromis niloticus*) with associated changes in splenic MAPK-and caspase-related gene expression

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ABSTRACT

The agricultural accumulation of the phenylamide fungicide metalaxyl (MXL) poses an increasing threat to aquatic organisms. Natural compounds, such as *Citrus limon* peel essential oil (CLPO), offer antioxidant and immunostimulant properties that may counteract these toxic effects. This study evaluated the protective effects of a CLPO-enriched diet against MXL-induced toxicity in *Oreochromis niloticus*. For 12 weeks, fingerlings (33.46 ± 0.28 g; $n = 50$ per group) were assigned to four treatments: Control, CLPO (1 g/kg diet), MXL (12.34 mg/L), and CLPO+MXL. MXL exposure reduced survival to 78%, disrupted hematological parameters (decreased white and red blood cells, hemoglobin, and hematocrit), and elevated glucose and cortisol levels. Immune dysfunction was evidenced by reductions in complement 3, lysozyme, nitric oxide, and myeloperoxidase. Furthermore, MXL triggered oxidative stress, marked by diminished antioxidant enzyme activities and increased malondialdehyde levels. At the molecular level, MXL exposure upregulated the stress-signaling transcripts (*mapk1* and *mapk14*) and pro-apoptotic genes (*casp-3* and *casp-8*), while concurrently suppressing the anti-inflammatory cytokine *il-10*. These physiological changes correlated with splenic depletion and enlarged melanomacrophage centers. Conversely, dietary CLPO enhanced immune-antioxidant function, restored hematological profiles, mitigated gene expression disturbances, and preserved splenic structure. Overall, dietary CLPO supplementation significantly attenuated several adverse effects under the present experimental conditions, highlighting its potential as a protective additive in aquafeeds.

1. Introduction

The expanding contribution of aquaculture to global food security highlights the need for deeper immunological insights to protect fish health and enhance productivity (Hoppe et al., 2024; Gebauer et al., 2025). The immunity of aquatic species is an intricate system of innate and adaptive mechanisms that defends against environmental

stressors, toxins, and pathogens (Abdul Kari, 2025). As the first line of defense, the innate immune system is composed of lysozyme (LYZ), phagocytic cells, and complement proteins. The adaptive one ensures long-lasting and precise immune response through lymphocytes and antibodies (Smith et al., 2019; Makesh et al., 2022). Unfortunately, the environmental pollution by pesticides, heavy metals, and industrial effluents can profoundly impair immune function (Mustafa et al., 2024;

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Ibrahim et al., 2025). These contaminants, through the production of reactive oxygen species (ROS), may damage immune cells, suppress antibody responses, and disrupt cytokine production (Kataoka and Kashiwada, 2021). Hence, they increase fish susceptibility to pathogens and ultimately impairs sustainability of aquaculture (Satkar et al., 2024).

Agrochemical pollutants, in particular the widely applied fungicides, pose hazardous concerns for fish health (Burch et al., 2025). These chemicals are applied to treat fungal pathogens through frequent use that elevates the environmental hazards (Brauer et al., 2019). Upon escape into aquatic systems, these substances can build up in fish tissues and adversely affect physiological functions (Rohani, 2023). Among the phenylamide fungicides, metalaxyl [MXL; methyl N-(2, 6-dimethyl-phenyl)-N-(methoxyacetyl)-DL-alaninate] is a highly water-soluble compound. MXL is commonly employed to combat Oomycete-induced infections in soybeans, ornamentals, grasses, and fruits (Sukul and Spiteller, 2000; Panek et al., 2022). The half-life of MXL is 106 days (Hamed et al., 2020), where its levels in surface waters were 0.022–0.288 µg/L (Bermúdez-Couso et al., 2013).

Acute exposure tests reveal that MXL, R-MXL, and rac-MXL are lethal to *Danio rerio* (zebrafish) at 227–242 mg/L and *Oreochromis niloticus* (Nile tilapia) at 0.82 mg/L, and triggers notable negative health impacts (Yao et al., 2009; Adene et al., 2022). Additionally, MXL disturbed Na⁺/K⁺ ATPase activity, disrupted gene expression involved in physiological processes, suppressed immune response (Yao et al., 2009; Zhang et al., 2016), and induced oxidative stress in *D. rerio* (Banaee et al., 2023). Accordingly, it is essential to implement safe measures to shield fish against such contaminants.

Recently, phytochemical products have gained attention in aquaculture as safe and effective agents for promoting growth and immune responses, alongside minimizing stress resulting from pollution (Abdel-Aziz et al., 2025; Abdel Rahman et al., 2025b; Hassan et al., 2025; Pudota et al., 2025). Concisely, plant-based essential oils are being widely utilized as nutritional feed supplements in the aquaculture sector (Dawood et al., 2022). A member of the Rutaceae family, *Citrus limon* (bitter lemon) is an extensively cultivated flowering species around the world (Inglese and Sortino, 2019). Its peels represent 30–40% of the fruit's weight, and they are an abundant source of beneficial bioactives, including polyphenols, flavonoids, and essential oils; however, they are frequently discarded as waste (Saini et al., 2022; Maqbool et al., 2023). Citrus essential oils are predominantly comprised of volatile and non-volatile compounds with percentages of 85–99% and 1–15%, respectively (Bhandari et al., 2021; Sheelmarevaa et al., 2025). These molecules include monoterpenes (mainly limonene), sesquiterpenes, sitosterols, coumarins, and flavonoids (Singh et al., 2024; Xu et al., 2025), which exhibit strong antimicrobial, immunostimulant, anticancer, and antioxidant characteristics (Saleem et al., 2023; Li et al., 2024).

Regarding its application in aquafeeds, feeding *O. niloticus* with *C. limon* peel essential oil (CLPO) diet resulted in enhanced growth rate, intestinal health, and immune-antioxidant functions (Abdo et al., 2024). In addition, dietary CLPO increased resilience against *Edwardsiella tarda* in *Oreochromis mossambicus* (Mozambique tilapia) (Baba et al., 2016). Moreover, its protective role in *Oncorhynchus mykiss* (rainbow trout) against deltamethrin toxicity was also reported (Amiri Resketi et al., 2021). Despite these documented benefits of CLPO, a critical scientific gap remains concerning its potential to mitigate the immunotoxic and oxidative stress induced by phenylamide fungicides, specifically MXL. While the general toxicity of MXL in fish is recognized, its specific molecular impacts on cellular mediators of inflammatory signaling and apoptotic pathways, and systemic immune suppression in *O. niloticus* are still virtually unknown, and no dietary intervention using CLPO has been explored to counteract these specific pathways.

As *O. niloticus* is extensively farmed in many countries due to its fast growth, ease of culture, and economic value (FAO, 2025), it serves as a critical biological model, especially in ecotoxicology (Henke et al.,

2024). Thus, gaining deeper insight into the interactions between xenobiotics, particularly MXL, and fish immunity is fundamental for evaluating aquaculture safety and environmental health. To address this gap, the primary objective of the present study was to thoroughly investigate the potential ameliorative role of dietary CLPO against prolonged sub-lethal MXL exposure in *O. niloticus*. Specifically, this study aimed to evaluate the modulation of hematological profiles, systemic immunological indicators, and tissue-specific antioxidant responses. Furthermore, we sought to elucidate the underlying molecular mechanisms by profiling the gene expressions related to stress and apoptosis, thereby offering a comprehensive strategy for protecting tilapia aquaculture from fungicide contamination.

2. Materials and methods

2.1. Fish husbandry

From the Fish Research Unit at Zagazig University, Egypt, *O. niloticus* fingerlings of 33.46 ± 0.28 g (average body weight) were used in this trial. Firstly, fish were acclimated for two weeks in 160-L glass aquaria, where 10 individuals were maintained per tank. During this period, fish were closely monitored to ensure overall well-being. Dechlorinated water was provided to each tank, alongside continuous aeration using compressed air. Fish were fed a basal diet at a rate of 3% of their biomass daily (09:00, 13:30, and 16:00 h). Following the APHA (2005) directions, the physicochemical parameters of the culturing water were examined throughout the trial. The outcomes exhibited values of 6.09 ± 0.70 mg/L, 6.80 ± 0.03, 0.03 ± 0.02 mg/L, 28.10 ± 0.08 °C, 0.025 ± 0.004 mg/L, and 5.77 ± 0.80 mg/L for dissolved oxygen, pH, ammonia, temperature, nitrite, and nitrate, respectively. Additionally, a 12-h light: 12-h dark photoperiod was upheld for the duration of the experiment.

2.2. Preparation of CLPO and its analysis by gas chromatography–mass spectrometry (GC–MS)

C. limon fruits procured from a local market in Zagazig were cleansed with deionized water and peeled. Following that, the peels were finely chopped, and the hydrodistillation method was conducted using a Clevenger apparatus for 4 h to extract CLPO (Öntaş et al., 2016). Following extraction, CLPO samples were transferred into separate dark-colored glass bottles and maintained pending further use at 4 °C. At the Central Laboratories Network (National Research Centre, Egypt), CLPO was analyzed using GC–MS (Agilent Technologies, Santa Clara, CA, USA), consisting of a 7890B gas chromatograph coupled with a 5977 A mass spectrometric detector. To perform the analysis, the carrier gas (hydrogen) was applied at a flow rate of 1.1 mL/min, with a split ratio of 1:10 and an injection volume of 1.0 µL. The oven temperature was initially set to 40 °C for 1 min, then increased at a rate of 10 °C/min to 200 °C, held for 1 min. It was further ramped up at 20 °C/min to 220 °C for 1 min, followed by an increase at 30 °C/min to 300 °C for 3 min. The detector and injector temperatures were maintained at 300 °C and 250 °C. Electron ionization was used at 70 eV to obtain mass spectra with a solvent delay of 1.50 min and spectral ranges of 33–800 m/z. The constituents of CLPO were identified through comparison of their fragmentation spectra with the reference spectra from the Wiley and the National Institute of Standards and Technology library (NIST) mass spectral libraries.

2.3. Dietary formulation and toxicant handling

Two isocaloric and iso-nitrogenous diets were formulated by integrating CLPO into the basal diet at 0 g/kg (control diet) and 1 g/kg (CLPO diet) based on a prior approach (Mohamed et al., 2021). The dietary ingredients were first ground, followed by pelleting into 2-mm pellets, and subsequently air-dried for 24 h at 25 °C. The criteria of NRC (2011) were followed to assemble the tested diets based on

O. niloticus requirements for optimum nutrition. Moreover, the chemical analysis of the basal diet was conducted using AOAC (2005) techniques, and the findings are demonstrated in Table 1. Moreover, Sigma-Aldrich Co., St. Louis, USA, provided the MXL ($\geq 98\%$ purity). A stock solution of MXL was formulated by dispersing MXL in distilled water, and its level was adjusted before administration in the experimental tanks.

2.4. Experimental trial setup

The methodology was carried out based on ethical standards and was approved by the Institutional Animal Care and Use Committee of Zagazig University with approval number ZU-IACUC-2-F-266-2026. For 12 weeks, a total of 200 fingerlings were randomly allocated into four treatments, each comprising 50 fish (10 fish/replicate, 5 replicates/group). The groups were designated as control, CLPO, MXL, and CLPO+MXL. The first two groups (control and CLPO) were fed the control and CLPO diets, respectively, without exposure to MXL. The second two groups (MXL and CLPO+MXL) were fed the control and CLPO diets, respectively, and subjected to MXL at 12.34 mg/L. The exposure concentration (12.34 mg/L) was selected as 1/10th of the 96 h lethal concentration 50 (123.38 mg/L) determined in a preliminary acute toxicity study (Abdel Rahman et al., 2026). In addition, the MXL exposure and feeding were initiated on day 0 and continued for 12 weeks. Daily siphoning was conducted to remove the excretory products. The whole-tank water was renewed twice weekly with freshly dechlorinated water, and MXL was re-dosed following each water exchange to achieve the nominal exposure concentration. Daily monitoring of the fish was performed to report any mortality.

2.5. Biological sampling

Following the 12-week trial, specimens were obtained from two fish per tank ($n = 10/\text{treatment}$). Anesthesia was induced (Neiffer and Stamper, 2009) by immersing the fish in a benzocaine solution at 100 mg/L. After that, bleeding was employed by puncturing the caudal vessels, and samples were partitioned into two fractions. For hematological analysis, heparinized syringes were used to collect the first set. Meanwhile, anticoagulant-devoid syringes were applied to collect the

blood of the second fraction. After that, blood of the second fraction was maintained at 4 °C for 6 h to facilitate clot formation and then was centrifuged (1198 $\times g/10$ min) to obtain serum for stress and immune assays. Following the bleeding, fish were euthanized (AVMA, 2007) using a benzocaine solution at a dose of 300 mg/L. Spleen tissues were retrieved for antioxidant, gene expression, and histological assays, with two fish per tank assigned to each assay.

2.6. Hematological profiling assay

Hematological profile, including white blood cell counts (WBCs), differential WBC counts, hemoglobin (Hb), red blood cell counts (RBCs), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and MCH concentration (MCHC), was analyzed. For this, an automatic veterinary hematology analyzer of Hospitex Diagnostics, Italy, was utilized, which was optimized for fish blood. In addition, blood smears were prepared, Giemsa-stained, and microscopically examined to validate the hematological findings and account for nucleated fish RBCs. Manual differential WBC counts and morphological evaluations demonstrated high consistency with the automated results.

2.7. Serum stress and immune response assays

The stress (glucose and cortisol) and immune [complement 3 (C3), LYZ, nitric oxide (NO), and myeloperoxidase (MPO)] indicators were evaluated in the serum. Spectrophotometrically, glucose level was quantified by Bio-Diagnostics CO. kits (Cat No.: GL 1320, Cairo, Egypt), following the Trinder (1969) approach, depending on oxidation of gluconic acid through glucose oxidase. In addition, cortisol concentrations were assessed by employing the My-BioSource, Inc. kits (San Diego, CA, USA) with Cat No. MBS704055 at 450 nm. Moreover, C3 and MPO levels were determined using kits of My-BioSource, Inc. (Cat. Nos. MBS005953 and MBS1607797, respectively) at 450 nm in accordance with the manufacturer's protocol. LYZ activity was evaluated (Ellis, 1990), based on the lysis of *Micrococcus lysodeikticus* (Sigma-Aldrich Chemie, Germany) by LYZ, and its concentration was detected at 540 nm. Furthermore, a technique of Bai et al. (2013) was performed to estimate NO levels using the Griess reaction spectrophotometrically at 546 nm.

2.8. Splenic antioxidant/oxidant assays

The antioxidant defenses of the spleen [glutathione peroxidase (GPx), glutathione reductase (GSR), superoxide dismutase (SOD), and catalase (CAT)], simultaneously with the concentration of lipid peroxidation marker [malondialdehyde (MDA)], were assessed. To prepare the tissue homogenates before analysis, the methodology of Fernandez-Botran et al. (2002) was applied. Subsequently, Bio-Diagnostic Co. kits, Egypt, were utilized to quantify GPx at 340 nm (Paglia and Valentine, 1967), GSR at 340 nm (Goldberg and Spooner, 1983), SOD at 480 nm (Nishikimi et al., 1972), CAT at 510 nm (Aebi, 1984), and MDA at 534 nm (Ohkawa et al., 1979) with Cat. Nos of GP25–24, GR 25–23, SD25–21, CA25–17, and MD25–29, respectively. Additionally, total protein levels in the supernatants were quantified following the Lowry et al. (1951) approach.

2.9. Spleen gene expression analysis

Expression levels of mitogen-activated protein kinases (*mapk1* and *mapk14*), interleukin-10 (*il-10*), and cysteine-aspartic acid proteases (*casp-3* and *casp-8*) were studied in the splenic tissues. The reference gene was ubiquitin-conjugating enzyme E2 (*ube2*) owing to its consistent expression and minimal variation among samples. Specific primers sourced from Sangon Biotech, Beijing, China were demonstrated in Table 2. To isolate total RNA, spleen specimens were treated with QIAzol (QIAGEN, Germany) at a level of 1 mL/30 mg tissue following the manufacturer's instructions. The extracted RNA was treated with

Table 1

Basal diet ingredients and its chemical composition (dry matter basis).

Ingredients	g/kg
Fish meal 66% CP	80
Soybean meal 48% CP	300
Corn gluten meal 60% CP	120
Ground yellow corn	350
Wheat bran	80
Premix* [#]	30
Corn oil	20
Cod liver oil	20
Total	1000
Chemical analysis	
Nitrogen-free extract [#] (g/kg)	494.20
Crude protein (g/kg)	310.70
Crude fiber (g/kg)	53.60
Crude lipids (g/kg)	72.10
Gross energy (kcal/kg)	4468

* Premix composition: Each 1 kg of premix has manganese sulfate (5.30 g), calcium iodide (0.34 g), folic acid (0.70 mg), iron sulfate (20 g), cobalt sulfate (70 mg), copper sulfate (2.70 g), sodium selenite (70 mg), zinc sulfate (4 g), nicotinic acid (20 g), vitamin B₁₂ (7 mg), pantothenic acid (7 g), vitamin D₃ (2,000,000 IU), vitamin B₆ (1 g), vitamin B₁ (0.70 g), vitamin B₂ (3.50 mg), vitamin A (8,000,000 IU), biotin (50 mg), vitamin K₃ (1.50 g), vitamin E (7 g), and CaHPO₄·2H₂O up to 1 kg.

[#] Nitrogen-free extract was calculated by difference: 1000 – (crude protein+ crude fiber+ crude lipids+ ash) (in g/kg).

Table 2

Primer sequences of target genes and real-time PCR conditions.

Gene name	Primers sequences	Efficiency	Accession no.	Product size	TM
<i>mapk1</i>	CTTTGGTTTGGCTCGTGTGG	95.43	XM_003444474.5	171	59.97
	TTGGACAGCATCTCAGCCAG				60.04
<i>mapk14</i>	GTTTCCCGGCACTGACCATA	100.5	XM_003448218.5	98	60.04
	CCTCATAGCTGGGCATCCTG				59.96
<i>il-10</i>	CAGCATTTCTGTGGACCAGC	94.55	KP645180.1	200	59.48
	TGAGCTTCTTGAGCCTGACG				60.04
<i>casp-3</i>	TTCTTTGGTACGACGGCTC	99.35	NM_001282894.1	116	60.04
	CCTCTGCAAGCCTGGATGAA				60.03
<i>casp-8</i>	AGCACAATCAGCCTGGAGTC	95.50	XM_005475143.4	134	60.04
	CAGCTAGTCGAAGAGCTCGG				59.97
<i>ube2</i>	CTCTCAAATCAATGCCACTTCC	102.5	XM_003460024.5	130	57.63
	CCCTGGTGGAGGTTCTTGT				61.43

mapk1:mitogen-activated protein kinase 1; *mapk14*: mitogen-activated protein kinase 14; *il-10*: interleukin-10; *casp-3*: cysteine-aspartic acid protease 3; *casp-8*: cysteine-aspartic acid protease 8; *ube2*: ubiquitin conjugating enzyme E2; TM: melting temperature.

DNase I to remove any residual genomic DNA, subsequent to the addition of an RNase inhibitor to preserve RNA integrity. Specimens were homogenized in QIAzol (1 mL) using a tissue homogenizer to ensure complete cell lysis, following the manufacturer's protocol. After homogenization, chloroform from Sigma-Aldrich, USA (200 µL) was added to each sample. The mixture was vigorously vortexed for 30 s and then centrifuged at 12,000 rpm for 15 min to enable clear phase separation. The latter was collected and combined with isopropanol (400 µL; Sigma-Aldrich, USA) and centrifuged for 10 min. The resulting RNA pellets were washed with 75% ethanol twice, briefly air-dried for about 5 min, and finally dissolved in RNase-free water.

RNA concentration and purity were evaluated using a Quawell UV-Vis Q3000 spectrophotometer based on the 260/280 and 260/230 nm. RNA integrity was evaluated by gel electrophoresis. Samples with A260/A280 ratios of 1.8–2.0 and A260/A230 ratios of 2.0–2.2 were considered suitable for further analysis. For synthetization of cDNA, a high-capacity reverse transcription kit (Applied Biosystems, USA) was employed. The expression level of the target genes was determined by applying real-time quantitative PCR (RT-qPCR). Following an initial denaturation step (95 °C/10 min), amplification was carried out for 40 cycles consisting of denaturation (95 °C/10 s), annealing (60 °C/15 s), and extension (72 °C/15 s). Melting curve analysis was utilized to verify amplicon specificity by excluding non-specific amplification and primer-dimer artifacts. Gene expression was determined based on the $2^{-\Delta\Delta Ct}$ assay of Schmittgen and Livak (2008), based on relative fold change normalized against the *ube2* gene.

2.10. Analysis of generated data

To assess the survival probability and differences among treatments, Kaplan–Meier and log-rank tests were applied. Moreover, Levene's and Shapiro–Wilk assays were conducted to verify normality and homoscedasticity of the findings. Subsequently, GraphPad Prism software (San Diego, USA) was employed to analyze the data, particularly the one-way ANOVA test. Pairwise comparisons were also detected among treatments using Tukey's post hoc test, where the significance was $P < 0.05$.

2.11. Histological evaluation

Standard histopathological protocols (Suvana et al., 2018) were conducted to prepare spleen tissues for histopathological assessment. In this sense, approximately 0.50 cm spleen samples were fixed in buffered formalin (10%/24 h), followed by rinsing with distilled water, dehydration through graded ethanol concentrations, and xylene clearing. The tissues were embedded in paraffin, sectioned at 5 µm thickness using a microtome (Leica Biosystems, Germany). After staining with hematoxylin and eosin (H&E), the sections were tested using an AmScope microscope (Irvine, California, USA). To ensure objectivity, all histological evaluations were performed independently by two observers who were

unaware of the treatment assignments. Moreover, histological lesions were scored semiquantitatively (10 random fields/group, 400×), where 0, 1, 2, and 3 represented no, mild, moderate, and severe changes, respectively (Gibson-Corley et al., 2013).

3. Results

3.1. Findings of GC–MS analysis

Table 3 shows GC–MS characterization of CLPO, where the limonene (48.01%), α -terpineol (32.17%), and *p*-cymene (12.28%) were the predominant bioactive components. Other detected constituents included α -phellandrene (3.40%), (*E*)- β -farnesene (1.73%), terpinolene (1.07%), β -pinene (0.69%), and α -pinene (0.65%).

3.2. Fish survival

Kaplan–Meier assay (Fig. 1) revealed 100% survival in both the control and CLPO groups, whereas it decreased to 78% in the MXL group and was partially restored to 94% in the CLPO+MXL group. Additionally, the intergroup differences were highly statistically significant ($P < 0.0001$).

3.3. Blood profile and stress response

Substantial rises in WBCs ($P < 0.0001$), lymphocytes ($P < 0.001$), neutrophils ($P < 0.05$), monocytes ($P < 0.05$), Hb ($P < 0.05$), RBCs ($P < 0.01$), and Hct ($P < 0.05$) were observable in the CLPO group versus the control (Tables 4 and 5). However, the CLPO diet decreased the glucose and cortisol values ($P < 0.05$ and < 0.01) (Fig. 2). Contrarily, the MXL exposure reduced WBCs, lymphocytes, neutrophils, monocytes, Hb, RBCs, and Hct ($P < 0.0001$, < 0.0001 , < 0.0001 , < 0.0001 , < 0.001 , $<$

Table 3

Gas chromatography–mass spectrometry (GC–MS) characterization of the bioactive constituents identified in *Citrus limon* peel oil (CLPO).

Peak	Compounds	Chemical formula	Molecular weight (g/mol)	Retention time (min)	Area sum (%)
1-	α -Pinene	C ₁₀ H ₁₆	136.23	1.962	0.65
2-	β -Pinene	C ₁₀ H ₁₆	136.23	2.445	0.69
3-	α -Phellandrene	C ₁₀ H ₁₆	136.23	2.883	3.40
4-	<i>p</i> -Cymene	C ₁₀ H ₁₄	134.22	3.089	12.28
5-	Limonene	C ₁₀ H ₁₆	136.23	3.134	48.01
	Terpinolene				
6-	(Cyclohexene, 3-methyl-6-(1-methylethylidene)-)	C ₁₀ H ₁₆	136.23	3.958	1.07
7-	α -Terpineol	C ₁₀ H ₁₈ O	154.25	5.355	32.17
8-	(<i>E</i>)- β -Farnesene	C ₁₅ H ₂₄	204.35	6.133	1.73

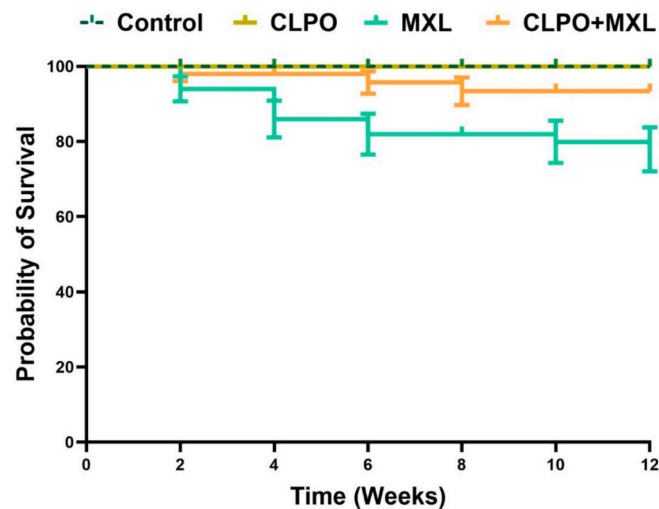


Fig. 1. Survival rate (Kaplan–Meier curves) of *O. niloticus* exposed to metalaxyl (MXL) and fed *Citrus limon* peel oil (CLPO)-supplemented diet for 12 weeks. Control and CLPO: fish fed on basal and CLPO diets, respectively, without MXL exposure. MXL and CLPO+MXL: fish were fed on basal and CLPO diets, respectively, and exposed to MXL.

Table 4
Leukogram of *O. niloticus* exposed to metalaxyl (MXL) and fed *Citrus limon* peel oil (CLPO)-supplemented diet for 12 weeks.

Parameters	Control	CLPO	MXL	CLPO+MXL
WBCs (10 ³ /mm)	23.20 ± 0.08	29.23 ± 0.87****	13.39 ± 0.34****	19.50 ± 0.66**/####
Lymphocytes (10 ³ /mm)	10.90 ± 0.09	15.18 ± 0.83***	4.94 ± 0.62****	7.81 ± 0.69*/##
Neutrophils (10 ³ /mm)	9.14 ± 0.01	10.52 ± 0.23*	6.37 ± 0.52****	8.93 ± 0.06 ^{ns} /####
Monocytes (10 ³ /mm)	1.71 ± 0.02	2.00 ± 0.14*	0.92 ± 0.02****	1.38 ± 0.03*/##
Eosinophils (10 ³ /mm)	0.90 ± 0.10	0.94 ± 0.13 ^{ns}	0.78 ± 0.17 ^{ns}	0.88 ± 0.10 ^{ns}
Basophils (10 ³ /mm)	0.54 ± 0.08	0.58 ± 0.02 ^{ns}	0.37 ± 0.10 ^{ns}	0.49 ± 0.10 ^{ns}

WBCs: white blood cell count. Control and CLPO: fish fed on basal and CLPO diets, respectively, without MXL exposure. MXL and CLPO+MXL: fish were fed on basal and CLPO diets, respectively, and exposed to MXL. Values are presented as mean ± SE (*n* = 5 / group), with each tank considered an independent experimental unit. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001 vs control and # *P* < 0.05, ## *P* < 0.01, ### *P* < 0.001, and #### *P* < 0.0001 vs MXL.

0.0001, and < 0.0001) respectively, with an increase of glucose and cortisol levels (*P* < 0.001 and 0.0001) relative to the control. Whereas, supplementing the MXL group with dietary CLPO increased WBCs, lymphocytes, neutrophils, monocytes, Hb, RBCs, and Hct (*P* < 0.0001, < 0.05, < 0.0001, < 0.01, < 0.01, < 0.001, and < 0.01) respectively, and diminished glucose and cortisol levels (*P* < 0.01) against the non-supplemented one (MXL). The values of eosinophils, basophils, MCV, MCH, and MCHC did not vary significantly across treatments.

3.4. Immune and antioxidant responses and MDA level

Fig. 3 and Table 6 declare marked increases in the C3, LYZ, NO, MPO, GPx, GSR, SOD, and CAT of the CLPO group versus the control, where *P* values were < 0.05, < 0.001, < 0.001, < 0.01, < 0.01, < 0.01, < 0.05, and < 0.001, respectively. Still, MDA levels remained unchanged following the CLPO diet. By contrast, sharp decreases of the C3, LYZ, NO, MPO, GPx, GSR, SOD, and CAT (*P* < 0.0001, < 0.0001, < 0.0001, < 0.0001, < 0.0001, < 0.0001, < 0.001, < 0.05, and < 0.01, respectively) and

Table 5
Erythrogram of *O. niloticus* exposed to metalaxyl (MXL) and fed *Citrus limon* peel oil (CLPO)-supplemented diet for 12 weeks.

Parameters	Control	CLPO	MXL	CLPO+MXL
Hb (g/dL)	8.53 ± 0.12	9.63 ± 0.27*	6.49 ± 0.25***	7.93 ± 0.32 ^{ns} /##
RBCs (10 ⁶ /mm)	3.55 ± 0.02	4.01 ± 0.10**	2.80 ± 0.02****	3.32 ± 0.11 ^{ns} /###
Hct (%)	27.48 ± 0.14	30.15 ± 0.77*	22.60 ± 0.56****	26.05 ± 0.45 ^{ns} /##
MCV (fL)	77.42 ± 0.70	75.15 ± 1.23 ^{ns}	80.61 ± 1.71 ^{ns}	78.65 ± 2.22 ^{ns}
MCH (pg)	24.03 ± 0.21	24.01 ± 0.49 ^{ns}	23.13 ± 0.76 ^{ns}	23.86 ± 0.68 ^{ns}
MCHC (g/dL)	31.06 ± 0.47	31.96 ± 0.57 ^{ns}	28.71 ± 0.81 ^{ns}	30.43 ± 1.19 ^{ns}

Hb: hemoglobin; RBCs: red blood cell count; Hct: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: MCH concentration. Control and CLPO: fish fed on basal and CLPO diets, respectively, without MXL exposure. MXL and CLPO+MXL: fish were fed on basal and CLPO diets, respectively, and exposed to MXL. Values are presented as mean ± SE (*n* = 5/group), with each tank considered an independent experimental unit. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001 vs control and # *P* < 0.05, ## *P* < 0.01, ### *P* < 0.001, and #### *P* < 0.0001 vs MXL.

elevation of MDA (*P* < 0.0001) were noted in the MXL group with respect to the control. Meanwhile, the addition of CLPO in the diet of MXL-exposed fish significantly elevated the C3, LYZ, NO, MPO, GPx, GSR, SOD, and CAT (*P* < 0.01, < 0.001, < 0.001, < 0.001, < 0.001, < 0.01, < 0.05, and < 0.05, respectively) and decreased MDA (*P* < 0.001) in relation to the MXL group.

3.5. Gene expression

Markedly increased expressions of the *mapk1*, *mapk14*, *casp-3*, and *casp-8* (*P* < 0.01, 0.0001, 0.0001, and 0.0001) and downregulation of *il-10* (*P* < 0.01) were observed upon MXL exposure against the control (Figs. 4 and 5). By contrast, fortifying the MXL group with CLPO resulted in suppression of the *mapk1*, *mapk14*, *casp-3*, and *casp-8* (*P* < 0.05, < 0.001, < 0.01, and < 0.001, respectively) and overexpression of *il-10* (*P* < 0.01) as compared to the MXL group. Moreover, the CLPO diet enhanced the *il-10* expression (*P* < 0.0001) relative to the control, with no notable changes in the other genes (*mapk1*, *mapk14*, *casp-3*, and *casp-8*).

3.6. Histological outcomes

The spleen of the control and CLPO groups exhibited normal micromorphology of white pulp lymphoid follicles around ellipsoid arterioles, with melanomacrophage centers (MMCs) beside normal red pulp, which involved splenic sinusoids, numerous erythrocytic and lymphocytic elements (Fig. 6A and B). Yet, MXL exposure induced significant (*P* < 0.0001) depletion of splenic tissues, particularly white pulp around ellipsoidal arterioles, besides a considerable increase (*P* < 0.05) in the contour of MMCs (Fig. 6C and Table 6). On the other hand, the spleen of CLPO+MXL (Fig. 6D) showed re-population of lymphocytes around ellipsoidal arterioles and marked reduction of depleted lymphoid follicles (*P* < 0.0001).

4. Discussion

The fungicide MXL is identified as a potential toxicant to aquatic species, particularly fish. Its exposure, even at low concentrations, may motivate physiological and immunological disruptions (Zeng et al., 2022; Banaee et al., 2023). Therefore, the adverse influences of MXL subjection were evaluated on splenic functional integrity by assessing hemato-immunological markers, antioxidant status, and gene

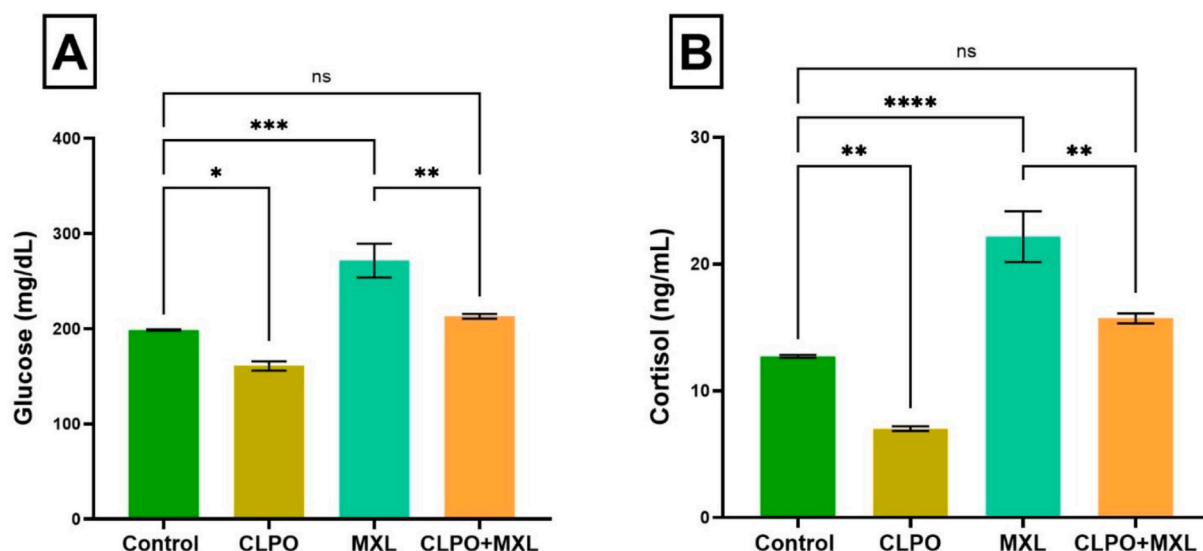


Fig. 2. Stress response of *O. niloticus* exposed to metalaxyl (MXL) and fed *Citrus limon* peel oil (CLPO)-supplemented diet for 12 weeks. [A] Glucose. [B] Cortisol. Control and CLPO: fish fed on basal and CLPO diets, respectively, without MXL exposure. MXL and CLPO+MXL: fish were fed on basal and CLPO diets, respectively, and exposed to MXL. Values are presented as mean $\pm$ SE ($n = 5/\text{group}$), with each tank considered an independent experimental unit. Statistical significance is indicated above the bars and denotes differences between groups [$*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$].

expression analysis of target genes, in addition to histological architecture. Moreover, the ameliorative effects of dietary CLPO against MXL-induced alterations in *O. niloticus* were investigated.

The GC-MS analysis of CLPO revealed a diverse profile of volatile substances, with limonene (48.01%) and α -terpineol (32.17%) being the predominant components along with lower proportions of *p*-cymene, α -phellandrene, (*E*)- β -farnesene, terpinolene, β -pinene, and α -pinene. These findings align with earlier research (Ngugi et al., 2017; Mohamed et al., 2021; Meryem et al., 2023), where these constituents exhibited antioxidant, antibacterial, immunostimulant, and growth-promoting activities, thereby providing a molecular basis for their protective role against MXL-induced toxicity.

Assessing the hematological profile provides fundamental insights into the physiological responses of fish to toxicants, and it remains a widely adopted approach in toxicology investigations (Bojarski et al., 2025). In this trial, MXL exposure for 12 weeks decreased hematological variables (Hb, RBCs, and Hct) of *O. niloticus*. Parallel outcomes were documented in albino mice (Al-Amoudi, 2012) as a result of MXL exposure. The observed decreases in Hb and RBCs in our findings suggest that MXL exposure provoked anemic conditions. This anemic effect could be a multifaceted response; while it might be partially linked to secondary oxidative damage that compromises the RBC membrane, leading to hemolysis (Al-Amoudi, 2012; Witeska et al., 2023), the primary cytotoxicity of MXL may also play a direct role. For instance, toxicant exposure could directly impair or destroy hemopoietic tissues such as the spleen, causing its dysfunction and hindering erythropoiesis (David et al., 2015; Rohani, 2023). Decreased hematopoiesis was previously reported in *D. rerio* following MXL exposure (Zeng et al., 2022), validating disturbances in blood cell formation. According to Kondera (2019) and Witeska et al. (2023), the hematological parameter shifts frequently stem from pathological changes affecting hematopoietic organs, with the spleen being especially impacted. Microscopic analysis revealed histological abnormalities in the spleen of MXL-exposed fish, which reinforced the proposed link between MXL exposure and hematological alterations. Similar hematotoxic responses were reported in *O. niloticus* by other fungicides such as copper oxychloride (Hassaan et al., 2014) and metiram (Shaalan et al., 2024).

In contrast, CLPO-fortified diet restored the disrupted hematological profile in MXL-exposed fish. The observed improvements extend beyond general antioxidant protection; while CLPO's antioxidant potential

(Zhao et al., 2022) effectively scavenged free radicals to protect RBC membranes from oxidative hemolysis, its bioactive constituents likely exerted direct cytoprotective and proliferative effects on hematopoietic tissues. This, in turn, promoted the recovery of splenic tissue structure and subsequently enhanced its primary function in hematopoiesis. The multi-targeted protective characteristics of CLPO are attributed to its abundant content of limonene (48.01%) (Bouzenna et al., 2016; Moosavy et al., 2017; Meryem et al., 2023), as identified in the present GC-MS findings. Comparable enhancement in hematological indices of ningu (*Labeo victorinus*) and *O. niloticus* receiving CLPO-enriched diets was documented (Ngugi et al., 2017; Abdo et al., 2024).

Monitoring the immune responses (cellular and humoral) is a fundamental approach for evaluating the impact of xenobiotics on fish health (Mansour et al., 2025; Moezzi et al., 2025). Reduction in these markers reflects the harmful effects of toxicant exposure and hinders the fish's protective immunity to pathogens (Mokhtar et al., 2023). In this trial, immune-suppression was observed in MXL-exposed fish as evidenced by decreased counts of WBCs, lymphocytes, neutrophils, and monocytes, alongside reduced levels of C3, LYZ, NO, and MPO. This was accompanied by a reduced fish survival (78%). Comparable effects of MXL exposure were documented in *D. rerio*, where it decreased macrophages, neutrophils, and thymus T cells (Zeng et al., 2022). While this immune-inhibition is widely associated with pesticide-induced oxidative stress and excessive ROS generation (Hussein et al., 2017), it may also involve independent chemical-induced cellular damage. Such exposure may potentially impair hematopoietic stem cell function via a suggested disruption of Wnt signaling, which might consequently lead to reduced immune cell differentiation (Zeng et al., 2022). Accordingly, the histopathological alterations observed in the spleen tissue in our study coincide with this explanation, given that the spleen is the major hematopoietic-immune organ for the synthesis of blood cells and immune defenses (Zapata, 2024). The decreased immune cells in our results are consistent with the recorded lymphoid depletion and alterations in MMCs under toxic exposure (MXL) as previously outlined by Yang et al. (2021).

Conversely, administration of a CLPO diet restored immune function in the MXL group, which reflected an enhanced survival rate (94%). Similarly, CLPO had a potent immunomodulatory effect in *O. mossambicus* and *O. niloticus* by boosting WBC count, LYZ, and MPO (Baba et al., 2016; Mohamed et al., 2021; Abdo et al., 2024). This

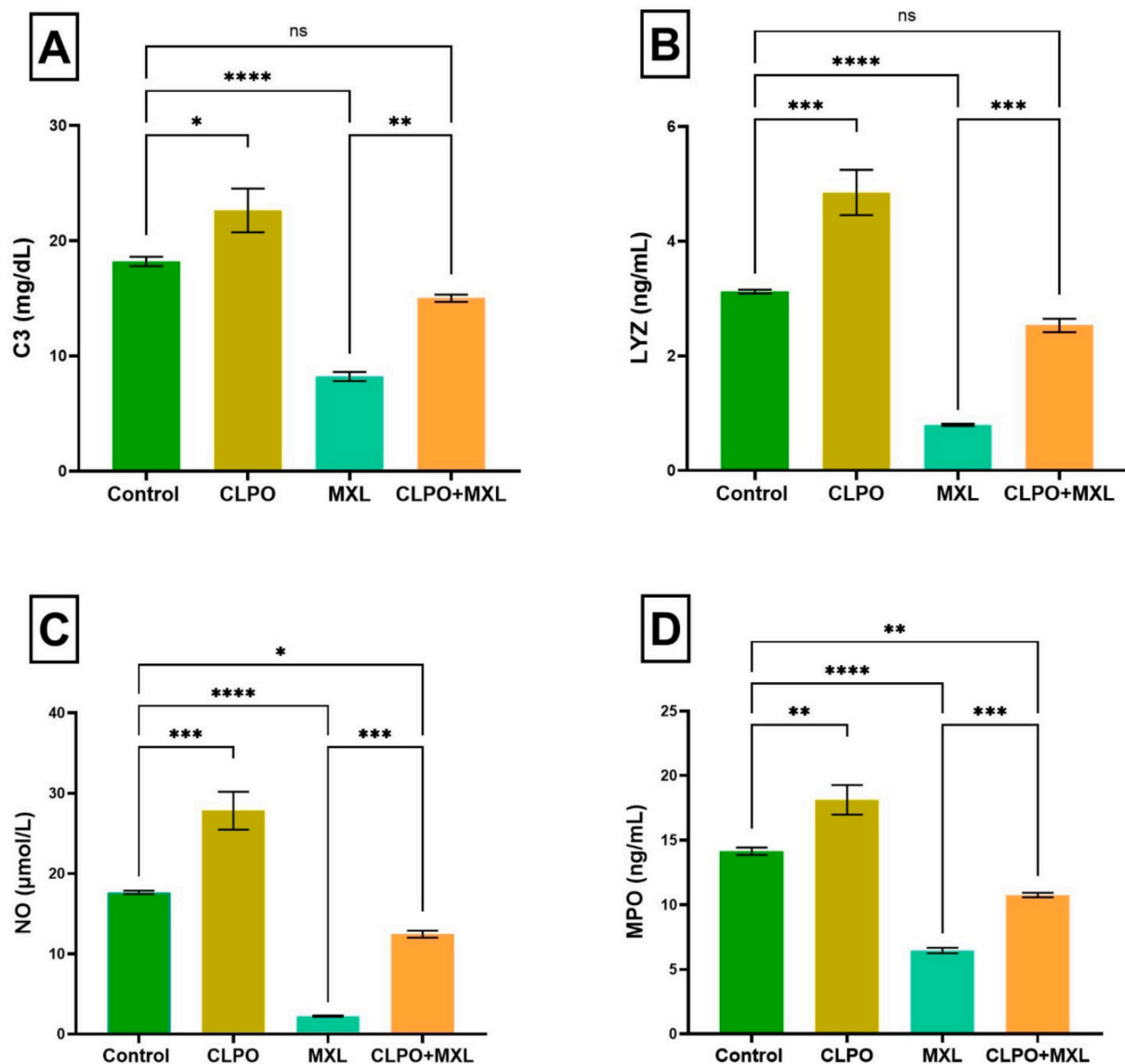


Fig. 3. Immune response of *O. niloticus* exposed to metalaxyl (MXL) and fed *Citrus limon* peel oil (CLPO)-supplemented diet for 12 weeks. [A] C3: complement3. [B] LYZ: lysozyme. [C] NO: nitric oxide. [D] MPO: myeloperoxidase. Control and CLPO: fish fed on basal and CLPO diets, respectively, without MXL exposure. MXL and CLPO+MXL: fish were fed on basal and CLPO diets, respectively, and exposed to MXL. Values are presented as mean $\pm$ SE ($n = 5$ /group), with each tank considered an independent experimental unit. Statistical significance is indicated above the bars and denotes differences between groups [$*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$].

immunostimulant influence of CLPO is strongly linked to the capacity of its major monoterpenes—particularly limonene (48.01%), α -terpineol (32.17%), and *p*-cymene (12.28%), as observed in our GC–MS analysis and supported in previous studies (Lu et al., 2019; Pucci et al., 2020). Rather than relying solely on antioxidant actions, these molecules have been documented to directly modulate specific immune-related signaling pathways, regulate immune cell networks, suppress upstream pro-inflammatory mediators, and modulate immune-related gene cascades (Amorim et al., 2016; Klimek-Szczykutowicz et al., 2020; Zhao et al., 2022). Consequently, this systemic immunomodulation effectively counteracts the chemical-induced immunosuppression caused by MXL.

In fish, cortisol is crucial for the regulation of glucose metabolism and serves as a stress-responsive marker, including pollution (Pandya et al., 2018; Yuan et al., 2025). This physiological reaction is suggested to involve the activation of the hypothalamus-pituitary-interrenal (HPI) axis (Nazar and Chatterjee, 2025), which potentially triggers increased cortisol secretion as a compensatory response to cope

with chemical stress. Cortisol, in turn, is known to promote glycogenolysis in the liver and glucose production to meet elevated energy demands (Faught and Vijayan, 2016; Lemos et al., 2023). Prior study by Banaee et al. (2023) supports this probable mechanism, where a decrease in hepatic glycogen level of *D. rerio* exposed to MXL was reported. Additionally, glucose 1-phosphate was upregulated following MXL exposure in Wistar albino rats, implying an acceleration of glycogenolysis during stress periods (Nazar and Chatterjee, 2025). Furthermore, chronically elevated cortisol levels are documented to suppress immune responses, including reduced antibody production and altered immune cell functions (Gonzalez Herrero and Kuehn, 2021; Alotiby, 2024), which represents a plausible contributing factor to the decreased immune parameters observed in our study. Comparable outcomes were documented in *O. niloticus* exposed to different pollutants (Abdel Rahman et al., 2025a; Haque et al., 2025).

On the other hand, dietary CLPO reduced stress indices in the MXL-exposed fish, verifying its anti-stress role and preserving liver glycogen. Analogously, CLPO-enriched diet decreased glucose and cortisol

Table 6

Antioxidant response and intestinal lesion scoring of *O. niloticus* exposed to metalaxyl (MXL) and fed *Citrus limon* peel oil (CLPO)-supplemented diet for 12 weeks.

Parameters	Control	CLPO	MXL	CLPO+MXL
GPx (U/mg protein)	98.25 ± 0.69	120.10 ± 6.96**	52.93 ± 2.55****	81.14 ± 0.52%/###
GSR (U/mg protein)	3.58 ± 0.32	5.58 ± 0.62**	0.95 ± 0.03***	2.94 ± 0.09ns/##
SOD (U/mg protein)	86.75 ± 0.71	97.35 ± 4.58*	73.33 ± 1.82*	85.44 ± 1.48ns/##
CAT (U/mg protein)	5.78 ± 0.04	8.42 ± 0.44***	4.09 ± 0.05**	5.51 ± 0.46ns/##
MDA (nmol/mg protein)	2.62 ± 0.16	2.22 ± 0.11 ^{ns}	8.15 ± 0.85****	3.74 ± 0.59ns/###
Depleted white pulp lymphoid follicles	0.00 ± 0.00	0.00 ± 0.00 ^{ns}	2.60 ± 0.24****	0.60 ± 0.24ns/####
Melanomacrophage centers	1.00 ± 0.00	1.00 ± 0.00 ^{ns}	1.60 ± 0.24*	1.00 ± 0.00ns/##

GPx: glutathione peroxidase; GSR: glutathione reductase; SOD: superoxide dismutase; CAT: catalase; MDA: malondialdehyde. Control and CLPO: fish fed on basal and CLPO diets, respectively, without MXL exposure. MXL and CLPO+MXL: fish were fed on basal and CLPO diets, respectively, and exposed to MXL. Values are presented as mean ± SE (n = 5/group), with each tank considered an independent experimental unit. For histopathological scoring, n = 10 random fields/group, 400×. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 vs control and #P < 0.05, ##P < 0.01, ###P < 0.001, and ####P < 0.0001 vs MXL.

concentration of *O. mossambicus* (Baba et al., 2016), *L. victorianus* (Ngugi et al., 2017), and *O. niloticus* (Mohamed et al., 2021). Mechanistically, the phytochemicals of CLPO, particularly limonene, can modulate neuroendocrine responses; by mitigating cellular stress, they potentially suppress the hyperactivation of the HPI axis, thereby limiting excessive cortisol release (Yang and Park, 2025; Yuan et al., 2025). The reduction of cortisol subsequently stabilizes downstream glucose metabolism, preventing excessive hepatic glycogenolysis (Wang et al., 2026). In addition, CLPO may directly regulate glucose homeostasis through metabolic pathway modulation, such as acting as an α -glucosidase inhibitor to reduce blood glucose availability (Tshiyoyo et al., 2025). Furthermore, the presence of bioactive substances of CLPO, particularly limonene, could exert direct neuro-protective, providing a dual-action mechanism that contributes to the decreased glucose and cortisol levels (Lopes Campêlo et al., 2011; Salehi et al., 2019; Eddin et al., 2021).

Under oxidative stress, ROS damage vital cellular macromolecules involving nucleic acids, proteins, and lipids. The elevated levels of MDA reflect ROS-induced lipid injury (Chowdhury and Saikia, 2020; Ma et al., 2025). Antioxidant defenses in fish are mediated by integral key enzymes, which neutralize excess ROS and preserve normal physiology and metabolism (Zeng et al., 2025). In this investigation, MXL triggered oxidative damage through the inactivation of antioxidants (GPx, GSR, SOD, and CAT) and the rise in MDA concentrations. In *D. rerio*, MXL exposure elicited comparable oxidative injury (Tang et al., 2025). As stated by Saquib et al. (2021), the weakened antioxidant defenses by MXL undermine the fish's ability to neutralize harmful free radicals (ROS) and thereby exacerbate tissue damage and functional impairment. In addition, MXL exposure induced oxidative injury in rats (Hussein et al., 2024b), which is an indicative of impaired tissue homeostasis. The splenic tissue lesions recorded in the MXL group further characterize the extent of this tissue injury.

Conversely, CLPO markedly mitigated MXL-caused oxidative injury by enhancing antioxidant enzymes and reducing MDA levels, thereby restoring redox balance. These advantageous effects were reflected in the improved splenic structure as seen in the histological results. CLPO's antioxidant activity is largely attributed to its bioactive compounds (Saini et al., 2022; Haokip et al., 2023). This interpretation is directly supported by our GC-MS assay, which confirmed the presence of main

antioxidant monoterpenes, including limonene (48.01%), α -terpineol (32.17%), and *p*-cymene (12.28%). These molecules can scavenge toxic free radicals, suppress lipid peroxidation, and attenuate oxidative stress (Singh et al., 2021; Agarwal et al., 2022). In fish, dietary CLPO enhanced antioxidant defenses of *O. niloticus* as described by Abdo et al. (2024). Moreover, a prior study (Bouzenna et al., 2016) reported a tissue-protective effect in rats of CLPO against aspirin toxicity.

Following MXL exposure, the transcript levels of splenic pro-inflammatory signaling mediators (*mapk1* and *mapk14*) and apoptotic mediators (*casp-3* and *casp-8*) were upregulated, together with a downregulation of *il-10*. Likewise, *D. rerio* exhibited an increased transcription of *casp-6* and *il-6* genes upon exposure to MXL, as reported by Zeng et al. (2022). In addition, Hussein et al. (2024a) documented an upregulation of hepatic *Mapk1* in rats following MXL exposure. Moreover, MXL induced upregulation of *Nf- κ B*, *Casp-3*, and *Casp-8* in rats (Hassa et al., 2018; Hussein et al., 2024b). These outcomes could be attributed to the direct xenobiotic-induced activation of inflammatory and cell death responses, which may run parallel to, or be further driven by, ROS-mediated pathways. MAPKs are key mediators of cell survival, apoptosis, stress response, and inflammatory mechanisms (Chakraborty et al., 2023). It has been suggested that the activation of these MAPKs under stress conditions might initiate cellular apoptosis via the potential recruitment of *casp-8* (Fong et al., 2017). Concurrently, the elevated expression of *casp-3* and *casp-8* points to a possible involvement of apoptosis pathways (intrinsic and extrinsic). This suggested coordination between MAPKs and caspases likely reflects a complex cellular response to MXL toxicity, involving interconnected pathways of inflammation, cytotoxicity, and oxidative stress that ultimately culminate in tissue damage (Liu et al., 2025). This hypothesis is further supported by the suppressed anti-inflammatory signaling of *il-10* highlighted in our findings.

In contrast, CLPO supplementation effectively restored *il-10*, *mapk1*, *mapk14*, *casp-3*, and *casp-8* transcript levels of the MXL group, which strongly underscores its specific anti-inflammatory and anti-apoptotic signaling modulation. This protective effect is attributed to the capacity of CLPO's bioactive molecules to cross-talk with intracellular signaling pathways. Specifically, these citrus bioactive compounds have been documented to suppress the phosphorylation and activation of the MAPK pathway and downstream caspase cascades in challenged models (Zhao et al., 2022; Li et al., 2024). This clearly supports our findings where CLPO supplementation effectively halted the transcription of splenic *mapk1*, *mapk14*, *casp-3*, and *casp-8*, thereby preventing executioner apoptotic and pro-inflammatory signaling in *O. niloticus*. Concurrently, CLPO upregulates the critical anti-inflammatory signaling network governed by *il-10*, thereby restoring cellular homeostasis at the transcription level. Comparably, *C. limon* peel extract downregulated inflammatory mediators and *Casp-3* in rats intoxicated with cyclophosphamide (Mobasher et al., 2022). In addition, CLPO reduced transcription of pro-inflammatory cytokines (*Il-6* and *Il-1 β*) following *Escherichia coli* challenge in mice (Zhao et al., 2022).

This study comprehensively evaluated numerous immunological and antioxidant parameters to assess the impact of MXL and the protective role of CLPO-fortified diet against these consequences. Nevertheless, several limitations should be considered. First, a key limitation is the absence of monitoring the actual MXL concentration between biweekly exchanges through analytical chemical approaches. While nominal concentrations were applied, future research should incorporate chemical tracking of MXL degradation kinetics under similar conditions. Additionally, although we profiled critical gene expressions related to inflammatory signaling and apoptosis, our analysis was restricted to the transcriptional level. Future studies should further validate these molecular pathways at the protein level through expression and phosphorylation analyses. Finally, while the protective effects of CLPO were evident in *O. niloticus*, further investigations across different aquatic species and under multi-stressor conditions are warranted to confirm the broader applicability and field-scale feasibility of this dietary intervention.

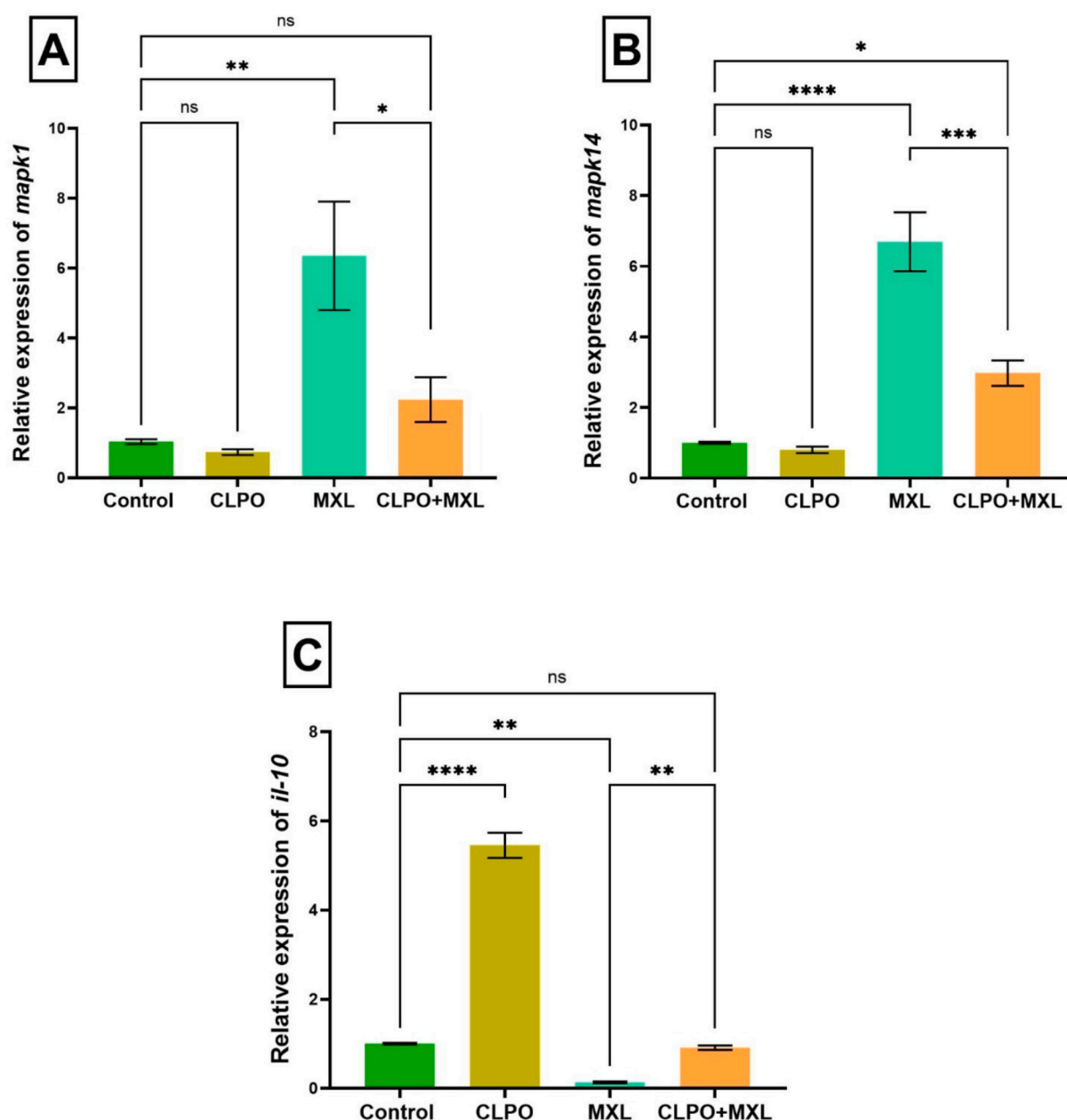


Fig. 4. Expression of pro-inflammatory signaling modulators and anti-inflammatory cytokine in *O. niloticus* exposed to metalaxyl (MXL) and fed *Citrus limon* peel oil (CLPO)-supplemented diet for 12 weeks. [A] *mapk1*: mitogen-activated protein kinase 1. [B] *mapk14*: mitogen-activated protein kinase 14. [C] *il-10*: interleukin-10. Control and CLPO: fish fed on basal and CLPO diets, respectively, without MXL exposure. MXL and CLPO+MXL: fish were fed on basal and CLPO diets, respectively, and exposed to MXL. Values are presented as mean $\pm$ SE ($n = 5$ / group), with each tank considered an independent experimental unit. Statistical significance is indicated above the bars and denotes differences between groups [$*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$].

5. Conclusion

Taken together, our findings demonstrate that long-term MXL exposure triggers systemic physiological distress in *O. niloticus*. The study highlights that this toxicity manifests through severe hematological disruptions, redox imbalance, immunological suppression, and structural damage to splenic tissues. Crucially, we identified the molecular mechanism behind these shifts as the overexpression of pro-inflammatory signaling activators and apoptotic pathways (*mapk1*, *mapk14*, *casp-3*, and *casp-8*). On the other hand, dietary fortification with CLPO (1 g/kg) proved to be an effective intervention. CLPO successfully neutralized the toxic impacts of MXL by strengthening the antioxidant-immune defenses and restoring tissue architecture. Most notably, it exerted potent anti-inflammatory and anti-apoptotic effects

by modulating the altered gene expression levels. These results position CLPO as a practical and sustainable strategy to protect fish health and ensure productivity in polluted aquatic environments.

CRediT authorship contribution statement

Elsayed M. Younis: Writing – review & editing, Methodology, Investigation, Funding acquisition. **Afaf N. Abdel Rahman:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Tarek Khamis:** Writing – review & editing, Resources, Investigation, Formal analysis, Data curation. **Abdelwahab A. Abdelwarith:** Writing – review & editing, Resources, Investigation, Funding acquisition. **Simon J. Davies:** Writing – review & editing,

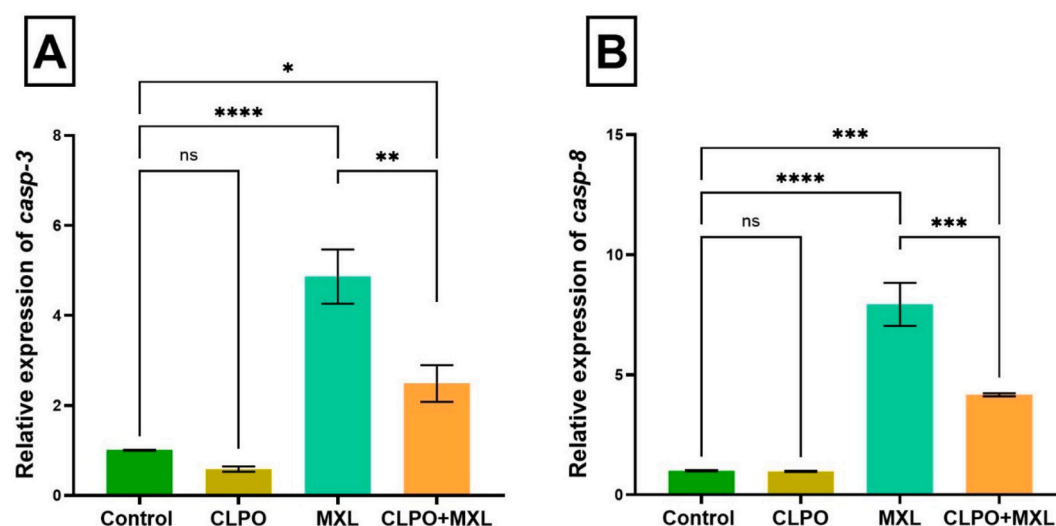


Fig. 5. Apoptosis-related pathways in *O. niloticus* exposed to metalaxyl (MXL) and fed *Citrus limon* peel oil (CLPO)-supplemented diet for 12 weeks. [A] *casp-3*: cysteine-aspartic acid protease 3. [B] *casp-8*: cysteine-aspartic acid protease 8. Control and CLPO: fish fed on basal and CLPO diets, respectively, without MXL exposure. MXL and CLPO+MXL: fish were fed on basal and CLPO diets, respectively, and exposed to MXL. Values are presented as mean $\pm$ SE ($n = 5$ / group), with each tank considered an independent experimental unit. Statistical significance is indicated above the bars and denotes differences between groups [$*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$].

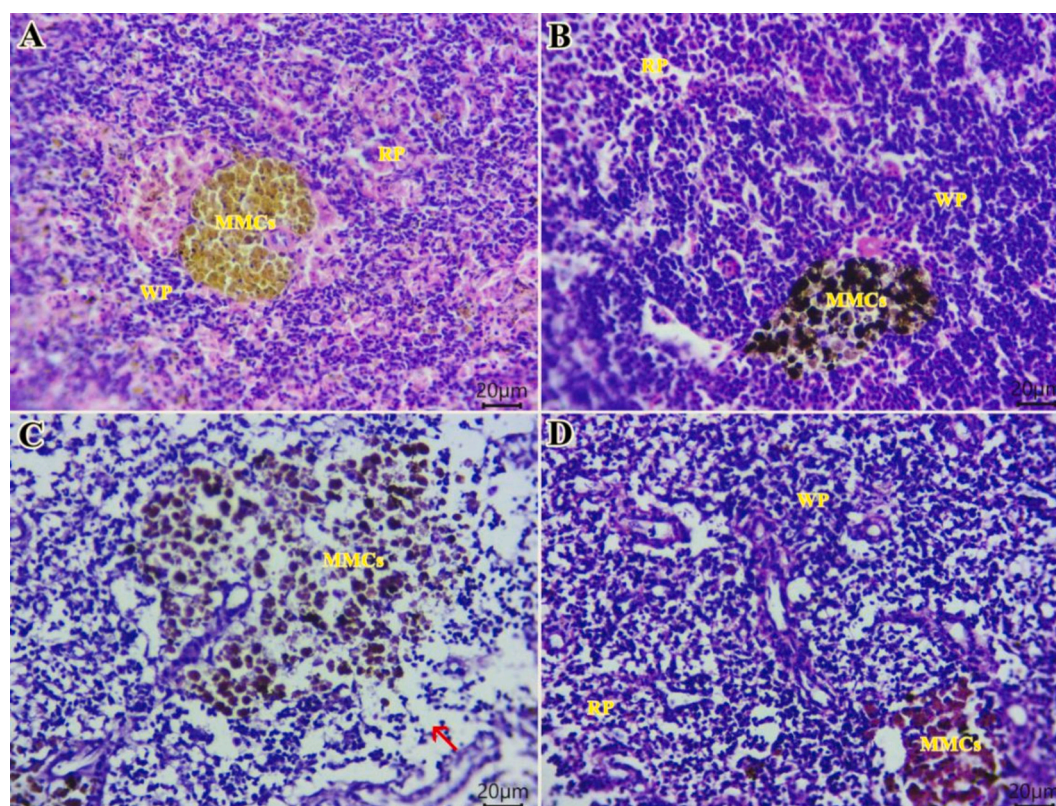


Fig. 6. Representative photomicrograph of spleen sections (H&E) of *O. niloticus* exposed to metalaxyl (MXL) and fed *Citrus limon* peel oil (CLPO)-supplemented diet for 12 weeks. [A and B] Splenic tissues of the control and CLPO groups reveal normal micromorphology of white pulp lymphoid follicles around ellipsoid arterioles, with melanomacrophage centers (MMCs) beside normal red pulp. [C] Splenic tissue of the MXL group reveals depletion of splenic tissue, especially white pulp (arrow) around ellipsoid arterioles beside increased contour of MMCs. [D] Splenic tissue of the CLPO+MXL group reveals re-population of lymphocytes around ellipsoid arterioles. RP: red pulp. WP: white pulp. MMCs: melanomacrophage centers. Control and CLPO: fish fed on basal and CLPO diets, respectively, without MXL exposure. MXL and CLPO+MXL: fish were fed on basal and CLPO diets, respectively, and exposed to MXL—Scale bar: 20 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data are available upon a reasonable request from the corresponding author.

References

- Abdel Rahman, A.N., Gharib, H.S.A.-R., Ahmed, A.I., Zagloul, A.W., Khamis, T., Sobh, M. S., Ras, R., Elhakim, Y.A., Ibrahim, R.E., 2025a. Dietary Egyptian mallow attenuates metiram-evoked neuro-ethological, endoplasmic reticulum stress genes, and biochemical/histopathological disruptions in *Oreochromis niloticus*. *Aquaculture* 599, 742193.
- Abdel Rahman, A.N., Sobh, M.S., Mansour, A.T., AlSaqufi, A.S., Alkhamis, Y., Hassanien, H.A., Abbas, A., Khamis, T., Ibrahim, R.E., Elazab, S.T., 2025b. Protective effects of dietary *Cucurbita pepo* on oxidative damage, histopathological alterations, and endoplasmic reticulum stress in the brain and gills of *Oreochromis niloticus* exposed to trace elements. *Vet. Res. Commun.* 50, 79.
- Abdel Rahman, A.N., Elshopakey, G.E., Mansour, A.T., Ashour, M., Mathew, R.T., Abdelwahid, H.H., Moafad, S.M., Khamis, T., Ibrahim, R.E., Behairy, A., 2026. Dietary *Citrus limon* Essential Oil Attenuates Metalaxyl Fungicide-Induced Toxicity in Nile tilapia Through Regulation of Growth, Digestive Efficiency, Oxidative Balance, GH/IGF Signaling, and Autophagy Pathways [Manuscript submitted for publication].
- Abdel-Aziz, M.F., Abdel Rahman, A.N., El-Dakar, A.Y., Khalil, K.M., Abbas, M.M.M., Thabet, R.Y., 2025. Dietary *Carica papaya* and alpha-lipoic acid synergistically promote resistance to *Aeromonas hydrophila*, digestive function, growth, physiological, and antioxidant responses of Nile tilapia (*Oreochromis niloticus*) raised under high densities. *Aquaculture* 604, 742478.
- Abdo, S.E., El-Nahas, A.F., Abdellatif, R.E., Mohamed, R., Helal, M.A., Azzam, M.M., Di Cerbo, A., El-Kassas, S., 2024. Combined dietary *Spirulina platensis* and *Citrus Limon* essential oil enhances the growth, immunity, antioxidant capacity and intestinal health of Nile tilapia. *Vet. Sci.* 11, 474.
- Abdul Kari, Z., 2025. Abiotic and biotic factors affecting the immune system of aquatic species: a review. *Comp. Immunol. Rep.* 9, 200230.
- Adene, C., Olutumise, A., Ayanboye, A., Oyedokun, O., Oloye, T., Akinbobola, T., 2022. Survival analysis and biochemical response of the juvenile Nile tilapia (*Oreochromis niloticus*) exposed to metalaxyl fungicides. *Egypt. J. Aquat. Biol. Fish.* 26, 295–320.
- Aebi, H., 1984. Catalase in vitro. *Methods Enzymol.* 105, 121–126.
- Agarwal, P., Sebghatollahi, Z., Kamal, M., Dhyani, A., Shrivastava, A., Singh, K.K., Sinha, M., Mahato, N., Mishra, A.K., Baek, K.-H., 2022. Citrus essential oils in aromatherapy: therapeutic effects and mechanisms. *Antioxidants* 11, 2374.
- Al-Amoudi, W.M., 2012. Haematological and biochemical effects of metalaxyl fungicide on albino mice. *Am. J. Biochem.* 2, 62–66.
- Alotiby, A., 2024. Immunology of stress: a review article. *J. Clin. Med.* 13, 6394.
- Amiri Resketti, M., Yeganeh, S., Jani Khalili, K., 2021. Dietary sour lemon (*Citrus Limon*) peel essential oil supplementation for reduction of deltamethrin-induced stress in rainbow trout (*Oncorhynchus mykiss*). *J. World Aquacult. Soc.* 52, 105–123.
- Amorim, J.L., Simas, D.L.R., Pinheiro, M.M.G., Moreno, D.S.A., Alviano, C.S., da Silva, A. J.R., Dias Fernandes, P., 2016. Anti-inflammatory properties and chemical characterization of the essential oils of four citrus species. *PLoS One* 11, e0153643.
- AOAC, 2005. Official Methods of Analysis of Association of Official Analytical Chemists, 18th Ed. Association of Official Analytical Chemists, Washington, DC.
- APHA, 2005. Standard Methods for Examination of Water and Wastewater, 21st ed. American Public Health Association, American Water Works Association, Water Pollution Control Federation, New York.
- AVMA, 2007. In: American Veterinary Medical Association (Ed.), Guidelines on Euthanasia (Formerly the Report of the AVMA Panel on Euthanasia).
- Baba, E., Acar, Ü., Öntaş, C., Kesbiç, O.S., Yılmaz, S., 2016. Evaluation of *Citrus Limon* peels essential oil on growth performance, immune response of Mozambique tilapia *Oreochromis mossambicus* challenged with *Edwardsiella tarda*. *Aquaculture* 465, 13–18.
- Bai, F., Ni, B., Liu, M., Feng, Z., Xiong, Q., Xiao, S., Shao, G., 2013. *Mycoplasma hyopneumoniae*-derived lipid-associated membrane proteins induce apoptosis in porcine alveolar macrophage via increasing nitric oxide production, oxidative stress, and caspase-3 activation. *Vet. Immunol. Immunopathol.* 155, 155–161.
- Banaee, M., Sagvand, S., Suredd, A., Amini, M., Haghi, B.N., Sopjani, M., Faggio, C., 2023. Evaluation of single and combined effects of mancozeb and metalaxyl on the transcriptional and biochemical response of zebrafish (*Danio rerio*). *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 268, 109597.
- Bermúdez-Couso, A., Fernández-Calviño, D., Álvarez-Enjo, M.A., Simal-Gándara, J., Nóvoa-Muñoz, J.C., Arias-Estévez, M., 2013. Pollution of surface waters by metalaxyl and nitrate from non-point sources. *Sci. Total Environ.* 461–462, 282–289.
- Bhandari, D.P., Poudel, D.K., Satyal, P., Khadayat, K., Dhami, S., Aryal, D., Chaudhary, P., Ghimire, A., Parajuli, N., 2021. Volatile compounds and antioxidant and antimicrobial activities of selected citrus essential oils originated from Nepal. *Molecules* 26, 6683.
- Bojarski, B., Witeska, M., Kondra, E., 2025. Blood biochemical biomarkers in fish toxicology: a review. *Animals* 15, 965.
- Bouzenna, H., Dhibi, S., Samout, N., Rjeibi, I., Talarmin, H., Elfeki, A., Hfaiedh, N., 2016. The protective effect of *Citrus Limon* essential oil on hepatotoxicity and nephrotoxicity induced by aspirin in rats. *Biomed. Pharmacother.* 83, 1327–1334.
- Brauer, V.S., Rezende, C.P., Pessoni, A.M., De Paula, R.G., Rangappa, K., Nayaka, S., Gupta, V., Almeida, F., 2019. Antifungal agents in agriculture: friends and foes of public health. *Biomolecules* 9, 521.
- Burch, E., Hussein, M.A., Zaki, M., Kamal, L.T., Zaki, G., Shoeib, T., Dawood, M., Sewilam, H., Abdelnaser, A., 2025. Assessing the effects of pesticides on aquacultured fish and ecosystems: a comprehensive environmental health review. *Fishes* 10, 223.
- Chakraborty, J., Chakraborty, S., Chakraborty, S., Narayan, M.N., 2023. Entanglement of MAPK pathways with gene expression and its omnipresence in the etiology for cancer and neurodegenerative disorders. *Biochim. Biophys. Acta* 1866, 194988.
- Chowdhury, S., Saikia, S., 2020. Oxidative stress in fish: a review. *J. Sci. Res.* 12, 145–160.
- David, M., Sangeetha, J., Shrinivas, J., Harish, E.R., Naik, V.R., 2015. Effects of deltamethrin on haematological indices of Indian major carp, *Cirrhinus mrigala* (Hamilton). *Int. J. Pure Appl. Zool.* 3, 37–43.
- Dawood, M.A.O., El Basuini, M.F., Yilmaz, S., Abdel-Latif, H.M.R., Alagawany, M., Kari, Z.A., Abdul Razab, M.K., Hamid, N.K., Moonmanee, T., Van Doan, H., 2022. Exploring the roles of dietary herbal essential oils in aquaculture: a review. *Animals* 12, 823.
- Eddin, L.B., Jha, N.K., Meeran, M.F.N., Kesari, K.K., Beiram, R., Ojha, S., 2021. Neuroprotective potential of limonene and limonene containing natural products. *Molecules* 26, 4535.
- Ellis, A.E., 1990. Lysozyme Assays. In: Stolen, J.S., Fletcher, T.C., Anderson, D.P., Roberson, B.S., Van Muiswinkel, W.B. (Eds.), *Techniques in Fish Immunology*. SOS Publications, Fair Haven, pp. 101–103.
- FAO, 2025. *Oreochromis niloticus*. In: Cultured aquatic species information Programme. Fisheries and Aquaculture Department, Rome. Text by Rakocy, J. E. In: Fought, E., Vijayan, M.M., 2016. Mechanisms of cortisol action in fish hepatocytes. *Comp. Biochem. Physiol. B* 199, 136–145.
- Fernandez-Botran, R., Gorantla, V., Sun, X., Ren, X., Perez-Abadia, G., Crespo, F.A., Oliver, R., Orhun, H.I., Quan, E.E., Maldonado, C., 2002. Targeting of glycosaminoglycan-cytokine interactions as a novel therapeutic approach in allotransplantation. *Transplantation* 74, 623–629.
- Fong, Y., Wu, C.Y., Chang, K.F., Chen, B.H., Chou, W.J., Tseng, C.H., Chen, Y.C., Wang, H.D., Chen, Y.L., Chiu, C.C., 2017. Dual roles of extracellular signal-regulated kinase (ERK) in quinoline compound BPIQ-induced apoptosis and anti-migration of human non-small cell lung cancer cells. *Cancer Cell Int.* 17, 37.
- Gebauer, T., Gebauer, R., Stejskal, V., Verleih, M., Folorunso, E.A., Korytář, T., Rebl, A., 2025. Current status and opportunities of immunological research on percid fishes. *Rev. Fish. Sci. Aquac.* 33, 286–318.
- Gibson-Corley, K.N., Olivier, A.K., Meyerholz, D.K., 2013. Principles for valid histopathologic scoring in research. *Vet. Pathol.* 50, 1007–1015.
- Goldberg, D.M., Spooner, R.J., 1983. Glutathione reductase. In: Bergmeyer, H.U., Bergmeyer, J., Graßl, M. (Eds.), *Methods of Enzymatic Analysis*, 3rd ed. Verlag Chemie, Weinheim, pp. 258–265.
- Gonzalez Herrero, M.E., Kuehn, C., 2021. A qualitative mathematical model of the immune response under the effect of stress. *Chaos* 31, 061104.
- Hamed, S.M., Hassan, S.H., Selim, S., Wadaan, M.A., Mohany, M., Hozzein, W.N., Abdelgawad, H., 2020. Differential responses of two cyanobacterial species to metalaxyl toxicity: growth, photosynthesis and antioxidant analyses. *Environ. Pollut.* 258, 113681.
- Haokip, S.W., Sheikh, K.H.A., Das, S., Devi, O.B., Singh, Y.D., Wangchu, L., Heisnam, P., 2023. Unraveling physicochemical profiles and bioactivities of citrus peel essential oils: a comprehensive review. *Eur. Food Res. Technol.* 249, 2821–2834.
- Haque, M.A., Anwar, M.B., Hossain, A.K.M.A., Ahmed, S., Zahangir, M.M., Shahjahan, M., 2025. Co-exposure toxicity of microplastic and sumithion in Nile

- tilapia – changes in growth, hematology, histopathology of internal tissues and immune-antioxidant genes expression. *J. Hazard. Mater. Adv.* 19, 100840.
- Hassa, M., Hussein, S., Senosi, Y., Mansour, M., Amin, A., 2018. Role of ginger as anti-inflammatory and anti-apoptotic in protection of liver damage induced by metalaxyl fungicide in male albino rats. *J. Clin. Exp. Pathol.* 8, 346.
- Hassaan, M., Gooda, A., Mahmoud, S., Tayel, S., 2014. Protective effect of dietary vitamin E against fungicide copperoxychloride stress on Nile tilapia, *Oreochromis niloticus* (L.) fingerlings. *Int. Aquat. Res.* 6, 58.
- Hassan, H.U., Ali, A., Al Sulivany, B.S.A., Bilal, M., Kanwal, R., Raza, M.A., Arslan, A., Ijaz, M.Z., Kabir, M., Khan, M.R., Rasdi, N.W., Arai, T., 2025. Investigation of the effects of phytogetic dietary additives on growth performance, nutrient utilization, economic efficiency and health of *Pangasius hypophthalmus*: implications for sustainable aquaculture development. *Sci. Rep.* 15, 22661.
- Henke, A.N., Chilukuri, S., Langan, L.M., Brooks, B.W., 2024. Reporting and reproducibility: proteomics of fish models in environmental toxicology and ecotoxicology. *Sci. Total Environ.* 912, 168455.
- Hopo, M.G., Mabrok, M.A., Abu-Elala, N.A., Yu, Y., 2024. Navigating fish immunity: focus on mucosal immunity and the evolving landscape of mucosal vaccines. *Biology* 13, 980.
- Hussein, S.A., El Senosi, Y.A., Mansour, M.K., Hassan, M.F., 2017. Role of antioxidant and anti-inflammatory of ginger (*Zingiber officinale roscoe*) against metalaxyl induced oxidative stress in rats. *Benha Vet. Med. J.* 33, 504–516.
- Hussein, A.S., Senosi, Y.A., Mahfouz, M.K., Arafa, M.M., Hassan, M.F., 2024a. Epigenetic impact and ameliorative potential role of quercetin or rosemary extract on metalaxyl or manganese chloride-induced toxicity via mitigation of microRNA, DNA methylation and regulation of MAPK phosphorylation in rats. *J. Adv. Vet. Res.* 14, 1160–1167.
- Hussein, A.S.A., El-Senosi, Y.A., Mahfouz, M.K., Arafa, M.M., Elmaghaby, I., 2024b. Hepatoprotective, anti-apoptotic and anti-inflammatory efficacy of quercetin or rosemary extract against metalaxyl toxicity-induced liver damage in rats: a role of Nrf2/HO-1 signaling pathways. *J. Adv. Vet. Res.* 14, 920–925.
- Ibrahim, R.E., Farag, M.F.M., Sobh, M.S., Abdelwarith, A.A., Younis, E.M., Bazeed, S.M., Elgamel, A., Khamis, T., Davies, S.J., Rahman, A.N.A., 2025. Ameliorative potential of *Populus alba* leaf powder against hexaflumuron exposure in Nile tilapia: immune-antioxidant, biochemical, histological, and transcriptomic analysis. *Fish Physiol. Biochem.* 51, 67.
- Inglese, P., Sortino, G., 2019. *Citrus History, Taxonomy, Breeding, and Fruit Quality*. Oxford University Press, Oxford.
- Kataoka, C., Kashiwada, S., 2021. Ecological risks due to immunotoxicological effects on aquatic organisms. *Int. J. Mol. Sci.* 22, 8305.
- Klimek-Szczykutowicz, M., Szopa, A., Ekiert, H., 2020. *Citrus Limon* (lemon) phenomenon - a review of the chemistry, pharmacological properties, applications in the modern pharmaceutical, food, and cosmetics industries, and biotechnological studies. *Plants* 9, 119.
- Kondera, E., 2019. Haematopoiesis and haematopoietic organs in fish. *Anim. Sci. Genet.* 15, 9–16.
- Lemos, L.S., Angarica, L.M., Hauser-Davis, R.A., 2023. Cortisol as a stress indicator in fish: sampling methods, analytical techniques, and organic pollutant exposure assessments. *Int. J. Environ. Res. Public Health* 20, 6237.
- Li, Y., Li, W., Ye, Z., Ji, C., Zhou, Z., 2024. Antioxidant, anti-inflammatory, and anticancer activities of five citrus peel essential oils. *Antioxidants* 13, 1562.
- Liu, S., Liu, J., Wang, Y., Deng, F., Deng, Z., 2025. Oxidative stress: signaling pathways, biological functions, and disease. *MedComm* 6, e70268.
- Lopes Campêlo, L.M., Moura Gonçalves, F.C., Feitosa, C.M., de Freitas, R.M., 2011. Antioxidant activity of *Citrus Limon* essential oil in mouse hippocampus. *Pharm. Biol.* 49, 709–715.
- Lowry, O., Rosebrough, N., Farr, A.L., Randall, R., 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265–275.
- Lu, Q., Huang, N., Peng, Y., Zhu, C., Pan, S., 2019. Peel oils from three *Citrus* species: volatile constituents, antioxidant activities and related contributions of individual components. *J. Food Sci. Technol.* 56, 4492–4402.
- Ma, W., Zeng, W., Zhang, D., Zhou, Y., Huang, Y., Hong, Y., 2025. Oxidative stress in aquaculture: pathogenic mechanisms and preventive strategies in farmed aquatic animals. *Curr. Issues Mol. Biol.* 47, 873.
- Makesh, M., Bedekar, M.K., Rajendran, K.V., 2022. Overview of fish immune system. In: Makesh, M., Rajendran, K.V. (Eds.), *Fish Immune System and Vaccines*. Springer Nature, Singapore, pp. 1–16.
- Mansour, A.T., Abdel Rahman, A.N., Al mutairy, M., Alkhulaifi, F.M., Khamis, T., Mahboub, H.H., Elsheshtawy, H.M., 2025. Waterborne alkylphenol xenobiotic induces alteration of innate immunity, stress response, and immune/apoptotic related genes of *Cyprinus carpio*: alleviation effect of *Salvia officinalis*. *Aquacult. Rep.* 43, 102928.
- Maqbool, Z., Khalid, W., Atiq, H., Koraqi, H., Javaid, Z., Alhag, S., Al-Shuraym, L., Bader, D., Almarzuq, M., Afifi, M., Al-Farga, A., 2023. Citrus waste as source of bioactive compounds: extraction and utilization in health and food industry. *Molecules* 28, 1636.
- Meryem, S., Mohamed, D., Nour-eddine, C., Faouzi, E., 2023. Chemical composition, antibacterial and antioxidant properties of three Moroccan citrus peel essential oils. *Sci. Afr.* 20, e01592.
- Mobasher, M., Hassen, M., Ebiya, R., Alturki, N., Alzamami, A., Mohamed, H., Awad, N., Khodeer, D., Abd El-Motelp, B., 2022. Ameliorative effect of *Citrus limon* peel extract and resveratrol on premature ovarian failure rat model: role of iNOS/caspase-3 pathway. *Molecules* 25, 122.
- Moezzi, S.A., Ramezani, S., Rezaei, K., Khoei, A.J., 2025. Mechanisms of pesticide toxicity in fish: insights into the ameliorative role of plant-derived compounds—a review. *Aquac. Nutr.* 2025, 5328773.
- Mohamed, R., Yousef, Y., El-Tras, W., Khalafalla, M., 2021. Dietary essential oil extract from sweet orange (*Citrus sinensis*) and bitter lemon (*Citrus Limon*) peels improved Nile tilapia performance and health status. *Aquac. Res.* 52, 1463–1479.
- Mokhtar, D.M., Zaccane, G., Alesci, A., Kuciel, M., Hussein, M.T., Sayed, R.K.A., 2023. Main components of fish immunity: an overview of the fish immune system. *Fishes* 8, 93.
- Moosavy, M.-H., Hassanzadeh, P., Mohammadzadeh, E., Mahmoudi, R., Khatibi, A., Mardani, K., 2017. Antioxidant and antimicrobial activities of essential oil of lemon (*Citrus Limon*) peel *in vitro* and in a food model. *J. Food Qual. Hazards Control* 4, 42–48.
- Mustafa, S.A., Al-Rudainy, A.J., Salman, N.M., 2024. Effect of environmental pollutants on fish health: an overview. *Egypt. J. Aquat. Res.* 50, 225–233.
- Nazar, N., Chatterjee, N.S., 2025. Unveiling potential biomarkers and associated health risks of chronic experimental exposure to environmentally relevant concentration of metalaxyl: an untargeted metabolomics approach. *J. Hazard. Mater. Adv.* 17, 100615.
- Neiffer, D.L., Stamper, M.A., 2009. Fish sedation, anesthesia, analgesia, and euthanasia: considerations, methods, and types of drugs. *ILAR J.* 50, 343–360.
- Ngugi, C.C., Oyoo-Okoth, E., Muchiri, M., 2017. Effects of dietary levels of essential oil (EO) extract from bitter lemon (*Citrus Limon*) fruit peels on growth, biochemical, haemato-immunological parameters and disease resistance in juvenile *Labeo victorinus* fingerlings challenged with *Aeromonas hydrophila*. *Aquac. Res.* 48, 2253–2265.
- Nishikimi, M., Rao, N.A., Yagi, K., 1972. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. *Biochem. Biophys. Res. Commun.* 46, 849–854.
- NRC, 2011. *Nutrient Requirements of Fish and Shrimp*. The National Academies Press, Washington, DC.
- Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* 95, 351–358.
- Öntaş, C., Baba, E., Kaplaner, E., Küçükaydin, S., Öztürk, M., Ercan, M.D., 2016. Antibacterial activity of *Citrus Limon* peel essential oil and *Argania spinosa* oil against fish pathogenic bacteria. *Kafkas Univ. Vet. Fak. Derg.* 22, 741–749.
- Paglia, D.E., Valentine, W.N., 1967. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J. Lab. Clin. Med.* 70, 158–169.
- Pandya, P., Upadhyay, A., Thakkar, B., Parikh, P., 2018. Evaluating the toxicological effects of agrochemicals on glucocorticoid receptor and serum cortisol level in Mozambique tilapia. *Cogent. Biol.* 4, 1480338.
- Panek, M., Ali, A., Helmer, S., 2022. Use of metalaxyl against some soil plant pathogens of the class Peronosporomycetes - a review and two case studies. *Plant Prot. Sci.* 58, 92–109.
- Pucci, M., Raimondo, S., Zichittella, C., Tinnirello, V., Corleone, V., Aiello, G., Moschetti, M., Conigliaro, A., Fontana, S., Alessandro, R., 2020. Biological properties of a citral-enriched fraction of *Citrus Limon* essential oil. *Foods* 9, 1290.
- Pudota, B.A., Tambireddy, N., Chennu, R., Chethurajupalli, L., Shaik, H., Nadella, R.K., Chatterjee, N.S., Paturi, A.P., 2025. Exploring the perspectives of phytochemicals and their role in aquaculture: present status and future trends. *Microbe* 8, 100496.
- Rohani, M.F., 2023. Pesticides toxicity in fish: histopathological and hemato-biochemical aspects – a review. *Emerg. Contam.* 9, 100234.
- Saini, R.K., Ranjit, A., Sharma, K., 2022. Bioactive compounds of citrus fruits: a review of composition and health benefits of carotenoids, flavonoids, limonoids, and terpenes. *Antioxidants* 11, 239.
- Saleem, M., Durani, A.I., Asari, A., Ahmed, M., Ahmad, M., Yousaf, N., Muddassar, M., 2023. Investigation of antioxidant and antibacterial effects of citrus fruits peels extracts using different extracting agents: phytochemical analysis with *in silico* studies. *Heliyon* 9, e15433.
- Salehi, B., Upadhyay, S., Erdogan Orhan, I., Kumar Jugran, A., Jayaweera, D., Dias, A., Sharopov, F., Taheri, Y., Martins, N., Baghalpour, N., Cho, W.C., Sharifi-Rad, J., 2019. Therapeutic potential of α - and β -pinene: a miracle gift of nature. *Biomolecules* 9, 738.
- Saqui, Q., Siddiqui, M.A., Ansari, S., Alwathnani, H., Musarrat, J., Al-Khedhairi, A., 2021. Cytotoxicity and genotoxicity of methomyl, carbaryl, metalaxyl, and pendimethalin in human umbilical vein endothelial cells. *J. Appl. Toxicol.* 41, 832–846.
- Satkar, S., Kumar, A., Bhusare, S., Sahu, A., Gautam, R., 2024. Impact of pollution and toxic stress on fish health: mechanisms, consequences, and mitigation strategies. *Uttar Pradesh J. Zool.* 45, 29–45.
- Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative CT method. *Nat. Protoc.* 3, 1101–1108.
- Shaaan, M., Elbealy, M.A., Darwish, M.I.M., Younis, E.M., Abdelwarith, A.A., Abdelaty, A.I., Davies, S.J., Ibrahim, R.E., Rahman, A.N.A., 2024. Toxicological insight of metiram: immuno-oxidative, neuro-behavioral, and hemato-biochemical changes during acute exposure of Nile tilapia (*Oreochromis niloticus*). *BMC Vet. Res.* 20, 303.
- Sheelmareva, F.A., Vasall, P.R.N., Permadi, N., Harja, A., Nurjanah, S., Al-Anshori, J., Julaha, E., 2025. Composition and medicinal applications of *Citrus* essential oils: current insights and future perspectives. *Phytomed. Plus* 5, 100836.
- Singh, B., Singh, J.P., Kaur, A., Yadav, M.P., 2021. Insights into the chemical composition and bioactivities of citrus peel essential oils. *Food Res. Int.* 143, 110231.
- Singh, N., Garg, M., Prajapati, P., Chopra, R., 2024. Chemistry of citrus fruits. In: Gupta, A.K., Kour, J., Mishra, P. (Eds.), *Citrus Fruits and Juice: Processing and Quality Profile*. Springer Nature, Singapore, pp. 45–68.
- Smith, N.C., Rise, M.L., Christian, S.L., 2019. A comparison of the innate and adaptive immune systems in cartilaginous fish, ray-finned fish, and lobe-finned fish. *Front. Immunol.* 10, 2292.

- Sukul, P., Spiteller, M., 2000. Metalaxyl: persistence, degradation, metabolism, and analytical methods. *Rev. Environ. Contam. Toxicol.* 164, 1–26.
- Suvarna, S.K., Layton, C., Bancroft, J.D., 2018. *Bancroft's Theory and Practice of Histological Techniques*, 8th ed. Churchill Livingstone, Elsevier, England.
- Tang, X., Meng, Y., Li, H., Liu, K., Wang, X., Zhong, Z., Ye, J., Chen, J., 2025. Characterization of metalaxyl-induced notochord toxicity based on biochemical and transcriptomics in zebrafish (*Danio rerio*) model. *J. Hazard. Mater.* 486, 136985.
- Trinder, P., 1969. Determination of blood glucose using 4-amino phenazone as oxygen acceptor. *J. Clin. Pathol.* 22, 246.
- Tshiyoyo, K.S., Rabbad, A., Yusuf, A.A., Malgas, S., 2025. Combination of citrus peel-derived essential oils with acarbose to inhibit amylolytic enzymes – a potential type II diabetes treatment approach. *Int. J. Biol. Macromol.* 306, 141504.
- Wang, Y., Nie, M.Z., Shi, P., Liu, Y.S., Lan, W.B., Dai, Z.R., Adeel, M., Javed, H.U., 2026. From waste to wellness: Citrus by-products as nutritional and immunological enhancers in aquaculture. *Food Chem. X* 33, 103523.
- Witeska, M., Kondera, E., Bojarski, B., 2023. Hematological and hematopoietic analysis in fish toxicology—a review. *Animals* 13, 2625.
- Xu, Y., He, P., He, B., Chen, Z., 2025. Bioactive flavonoids metabolites in citrus species: their potential health benefits and medical potentials. *Front. Pharmacol.* 16, 1552171.
- Yang, J., Park, M.J., 2025. Antioxidant effects of essential oils from the peels of citrus cultivars. *Molecules* 30, 112.
- Yang, C., Lim, W., Song, G., 2021. Immunotoxicological effects of insecticides in exposed fishes. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 247, 109064.
- Yao, K., Zhu, L., Duan, Z., Chen, Z., Li, Y., Zhu, X., 2009. Comparison of R-metalaxyl and rac-metalaxyl in acute, chronic, and sublethal effect on aquatic organisms: *Daphnia magna*, *Scenedesmus quadricauda*, and *Danio rerio*. *Environ. Toxicol.* 24, 148–156.
- Yuan, M., Fang, Q., Lu, W., Wang, X., Hao, T., Chong, C.-M., Chen, S., 2025. Stress in fish: neuroendocrine and neurotransmitter responses. *Fishes* 10, 307.
- Zapata, A.G., 2024. The fish spleen. *Fish Shellfish Immunol.* 144, 109280.
- Zeng, S., Peng, Y., Ma, J., Ge, Y., Huang, Y., Xie, S., Lu, H., 2022. Hematopoietic stem cell and immunotoxicity in zebrafish embryos induced by exposure to metalaxyl—M. Sci. Total Environ. 809, 152102.
- Zeng, Y., Song, Z., Song, G., Li, S., Sun, H., Zhang, C., Li, G., 2025. Oxidative stress and antioxidant biomarker responses in fish exposed to heavy metals: a review. *Environ. Monit. Assess.* 197, 892.
- Zhang, Y., Zhang, Y., Chen, A., Zhang, W., Chen, H., Zhang, Q., 2016. Enantioselectivity in developmental toxicity of rac-metalaxyl and R-metalaxyl in zebrafish (*Danio rerio*) embryo. *Chirality* 28, 489–494.
- Zhao, C., Zhang, Z., Nie, D., Li, Y., 2022. Protective effect of lemon essential oil and its major active component, D-limonene, on intestinal injury and inflammation of *E. Coli*-challenged mice. *Front. Nutr.* 9, 843096.