



***Nigella sativa* and carvacrol supplementation modulates physiological, immune, and disease resistance responses in *Oreochromis niloticus*: evidence of interaction in selected parameters**

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Abstract

Intensified aquaculture practices have increased the vulnerability of farmed fish to infectious pathogens, highlighting nutritional strategies to increase health and reduce antibiotic reliance. Dietary antioxidants such as *Nigella sativa* (NI) and carvacrol (CA) have shown potential as functional feed additives because of their synergistic effects under bacterial challenge. This study evaluated whether dietary supplementation with NI aqueous extract, CA, or their combination could enhance growth, antioxidant defense, immune function, and disease resistance in *Oreochromis niloticus* challenged with *Aeromonas hydrophila*. A total of 120 fish (mean weight 32.28 ± 0.32 g) were randomly assigned to four groups with three replicates per group ($n = 30$ fish/group, 10 fish/replicate): control, NI (10 g/kg diet), CA (1 g/kg diet), and NI+CA. Fish were fed for eight weeks, followed by experimental infection with *A. hydrophila*. Growth performance, hematological and biochemical indices, antioxidant enzymes (SOD, GST), lipid peroxidation (MDA), immune parameters (phagocytic activity, myeloperoxidase, total immunoglobulin), and bacterial re-isolation were assessed. Dietary inclusion of NI, CA, or NI+CA significantly improved growth parameters, with the combined group showing the greatest final body weight, weight gain, and specific growth rate, along with a reduced feed conversion ratio. Hematological and biochemical analyses revealed enhanced hemoglobin and leukocyte profiles and favorable modulation of protein, lipid, and glucose metabolism. Supplementation elevated antioxidant enzymes and reduced lipid peroxidation, indicating improved redox balance. Immune assays revealed increased phagocytic activity, myeloperoxidase response, and total immunoglobulin. Following *A. hydrophila* challenge, the NI+CA group displayed reduced clinical signs, milder lesions, a lower bacterial load, and the highest relative percent survival. Co-supplementation with NI and CA exerted synergistic protective effects across multiple physiological systems, increasing the antioxidant capacity, immune competence, and resistance to bacterial infection in *O. niloticus*. This study is among the first to demonstrate the synergistic benefits of NI and CA against bacterial challenge in *O. niloticus*, supporting their application as natural, cost-effective immunostimulants in functional aquafeeds.

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Keywords *Aeromonas hydrophila* · Antioxidants · Aquaculture industry · Medicinal plants · Nutritional immunomodulation · Oxidative stress

Introduction

The aquaculture industry is increasingly adopting natural alternatives to synthetic additives and antibiotics because of concerns over antimicrobial resistance, environmental impacts, and food safety (Qin et al. 2024). Medicinal plants and their bioactive compounds are of particular interest because of their antimicrobial, antioxidant, and immunostimulatory properties (Awad & Awaad 2017; Abu-Zahra et al. 2024, 2025a).

Nigella sativa (black seed, NI) contains bioactive compounds such as thymoquinone, thymol, and carvacrol, which contribute to its antimicrobial, antioxidant, and immunomodulatory effects (Yousefi et al. 2021; Ciesielska-Figlon et al. 2023). In aquaculture, dietary supplementation with NI has demonstrated significant benefits across various fish species. Specifically, it has been shown to increase growth, improve feed efficiency, and increase the activity of antioxidant enzymes (SOD, CAT, and GSH) (Abdel-Daim et al. 2025). Furthermore, NI supplementation helps reduce oxidative stress and hepatic damage caused by toxicants, supporting better overall fish health and resilience under stress (Andrawes et al. 2021; Yousefi et al. 2021). These findings highlight the potential of NI as a functional feed additive that can enhance fish performance and health under challenging aquaculture conditions.

Carvacrol (CA), a major phenolic constituent of oregano (*O. vulgare*) oil, has broad-spectrum antimicrobial, antioxidant, and anti-inflammatory activities (Mączka et al. 2023; Baser et al. 2025). CA has potent inhibitory effects on both gram-positive and gram-negative pathogens, including *Aeromonas hydrophila*, *Edwardsiella piscicida*, *Staphylococcus aureus*, *Vibrio* spp., and *Escherichia coli* (Hussein et al. 2021; Al-Tawalbeh et al. 2024; Qin et al. 2024).

Its antimicrobial effects are mediated through several mechanisms, including disruption of bacterial membranes, leading to cell leakage and ultimately cell death (Hussein et al. 2021). In addition, CA impairs energy metabolism by interfering with ATP synthesis in bacterial cells, disrupting their ability to perform essential cellular functions (Hussein et al. 2021). Moreover, CA suppresses the expression of virulence genes, including those involved in the inhibition of quorum sensing and biofilm formation, both of which are critical to bacterial pathogenicity and persistence in pathogens such as *Escherichia coli* and *A. hydrophila* (Lu et al. 2023; Morgaan et al. 2023). In aquaculture, dietary supplementation with CA has been shown to improve hematological profiles, enhance antioxidant defenses, and positively affect the gut microbiota composition in species such as catfish (Suarez et al. 2025). CA, classified as generally recognized as safe (GRAS), is considered safe for use in food and feed applications (Cetinkaya & Ayseli 2025). Furthermore, advances in CA encapsulation have increased its stability and bioavailability, expanding its applications across pharmaceuticals, agriculture, and aquaculture (Baser et al. 2025).

Nile tilapia (*Oreochromis niloticus*), a key aquaculture species, is highly susceptible to *A. hydrophila*, the causative agent of hemorrhagic septicemia and major production losses (Sittien et al. 2024). The emergence of multidrug-resistant *A. hydrophila* underscores the urgency of finding sustainable, antibiotic-free disease control measures (Abu-Zahra et al. 2025b).

The present study aimed to determine whether dietary supplementation with NI extract, CA, or their combination could improve growth, biochemical balance, anti-oxidant status, immune responses, and resistance to *A. hydrophila* in *O. niloticus*. By directly comparing NI, CA, and their co-supplementation under infectious challenge, this study provides novel insights into their synergistic potential. Unlike previous studies that evaluated these additives separately, our work is among the first to investigate their combined effects across integrated endpoints, linking growth, redox homeostasis, immunological markers, and survival outcomes. This approach highlights the importance of this study, offering evidence-based support for the use of NI + CA as a sustainable, antibiotic-free strategy to increase fish health and disease resistance in aquaculture.

Materials and methods

Preparation of the NI aqueous extract

Nigella sativa (NI) seeds, which are small (1–2 mm), angular, and black with a rough texture, have a distinct, pungent aroma and a bitter, peppery taste. These seeds were sourced from a certified herbal supplier in Kafrelshiekh Province, Egypt. The seeds were washed and gently rinsed with tap water to remove dust and debris and then air-dried at room temperature for 24 h. The dried seeds were ground into a fine powder via an electric grinder. To prepare the aqueous extract, 50 g of the powdered seeds were soaked in 500 mL of sterilized distilled water (1:10 w/v). The mixture was kept at room temperature overnight under continuous shaking, followed by centrifugation at 5000 rpm for 10 min. The resulting supernatant was collected, freeze-dried, and stored at -20°C until further analysis (Chauhan et al. 2018).

Carvacrol (86–90% purity) was purchased from Aromavita, a local supplier in Egypt, and stored in a tightly sealed amber bottle at 4°C in the dark until use.

Chromatographic profiling of NI via GC–MS

Gas chromatography–mass spectrometry (GC–MS) analysis of the NI was conducted at the National Research Centre, Giza, Egypt, to characterize its phytochemical constituents (Ismail et al. 2024). Before analysis, the extract was treated with BSTFA N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 1% trimethylchlorosilane (TMCS) at 70°C for 30 min to increase the volatility and stability of the polar compounds under heat. The GC–MS system comprised an Agilent 7890B gas chromatograph coupled with an Agilent 5977A mass selective detector equipped with an HP–5MS capillary column ($30\text{ m} \times 0.25\text{ mm ID} \times 0.25\text{ }\mu\text{m}$ film thickness). The oven temperature was initially set at 50°C (held for 2 min), then increased to 280°C at a rate of $10^{\circ}\text{C}/\text{min}$ and held for 10 min. Helium was used as the carrier gas at a constant flow rate of $1.0\text{ mL}/\text{min}$. The mass spectrometer was operated in electron impact (EI) mode at 70 eV with a scan range of 50–650 m/z. Compounds were identified by comparing their mass spectra with those in the NIST/EPA/NIH mass spectral library, including the Wiley spectral collection, and by matching retention indices.

In vitro antibacterial activity of the NI and CA

The antibacterial activity of NI and CA against *A. hydrophila* was evaluated via the agar disk diffusion method (Abu-Zahra et al. 2025a). A previously isolated and molecularly characterized virulent strain of *A. hydrophila* from *O. niloticus* (El-Gammal et al. 2025) was used for this assay. The isolate was cultured on Mueller–Hinton agar (MHA) and standardized to 0.5 McFarland turbidity (1.5×10^8 CFU/mL). Sterile 6 mm filter paper discs were impregnated with 20 μ L of each test substance: NI (100 mg/mL), CA (10 mg/mL in 1% DMSO), oxytetracycline (OXY; 10 μ g/disc, as a positive control), or 1% DMSO (negative control). The inoculated MHA plates were seeded evenly via sterile cotton swabs, and the prepared discs were placed aseptically on the agar surface. The plates were incubated at 37 °C for 24 h. Antibacterial activity was determined by measuring the diameters of the inhibition zones around each disc in millimeters via a digital caliper. All the assays were conducted in triplicate, and the results are expressed as the means $\pm$ standard errors (SEs), with comparisons made against the positive and negative controls.

Preparation of feed diets

Four experimental diets were prepared from commercial ingredients and formulated according to the nutritional requirements of *O. niloticus* as outlined by Jobling (2011). A basal diet (Table 1) was used as the control (CNT) and served as the base for the incorporation of the supplements. The dietary groups included:

- CNT: Basal diet without any additives.

Table 1 Physical and nutritional composition of the experimental diet of *O. niloticus*

Proximate analysis		Chemical analysis	
Ingredient	%	Nutrient	%
Yellow Corn	34.75	Moisture	8.5
Corn Gluten	10.00	Dry matter	91.5
Fish Meal	9.00	Crude Protein	30.9
Soybean Meal	39.00	Crude fiber	5
Wheat Bran	2.00	Ether Extract	5.0
Soy Oil	4.50	Ash	3.5
¹ Mineral-Vitamin Mix	0.30	Nitrogen free extract (NFE)	47.1
Salt	0.25	Calcium	0.8
Carboxy Methyl Cellulose	0.20	Total Phosphorus	0.6
		Lysine	1.7
		Methionine	0.73
		² Digestible energy	3439.08 kcal

¹Mineral–vitamin premix was obtained from El Gomhouria Company, Egypt. Each kg of premix contains the following: vitamin A (10,000,000 IU), vitamin D3 (1,000,000 IU), vitamin E (10 g), vitamin K3 (1 g), vitamin B1 (1 g), vitamin B2 (5 g), vitamin B6 (1.5 g), vitamin B12 (10 mg), niacin (30 g), pantothenic acid (10 g), folic acid (1 g), biotin (50 mg), choline chloride (250 g), iron (30 g), zinc (50 g), manganese (40 g), copper (10 g), iodine (0.3 g), cobalt (0.1 g), and selenium (0.1 g) and is carried up to 1 kg

²Based on the chemical component of the utilized feedstuffs, the digestible energy (DE) was computed

- NI: Basal diet supplemented with 1% (w/w) NI.
- CA: Basal diet supplemented with 0.1% (w/w) CA.
- NI + CA: Basal diet supplemented with 1% NI + 0.1% CA.

Both additives were thoroughly homogenized with the basal diet, formed into 2-mm pellets via a laboratory pelletizer, and air-dried at ambient temperature for 48 h. The pellets were stored at 4 °C until use. The fish were fed the experimental diets at 3% of their body weight per day and split into two equal meals (8:30 a.m. and 1:30 p.m.) over an 8-week feeding trial.

Experimental design and rearing conditions

The experimental methodology, protocols, and animal care used in the present study were performed according to the relevant guidelines and regulations of the Animal Health Research Institute, Agriculture Research Center, Giza, Egypt (Code Number: ARC/AHRI 83429/2024). Healthy juvenile *O. niloticus*, with an average initial body weight of 32.28 ± 0.32 g, were obtained from a local farm at Kafrelsheikh governorate, Egypt, and acclimated to laboratory conditions for two weeks prior to the experiment. During acclimation, the fish were fed a commercial basal diet (Aller-Aqua Co., Egypt) containing, on the basis of the manufacturer's label, 36% crude protein, 5.1% crude fat, 7.7% ash, 37.2% nitrogen-free extract, and 1% phosphorus and provided 10.2 MJ/kg digestible energy. The fish were maintained under optimal water quality conditions during this period.

A total of 120 fish were randomly distributed into four treatment groups, with three replicates per group ($n=30$ fish/group, 10 fish/replicate). The fish were reared in 12 glass aquaria (50×40×40 cm, 80 L total capacity; the usable water volume in each aquarium was approximately 50 L). The stocking density was 0.2 fish per liter, which was determined on the basis of the usable water volume via the following formula:

$$\text{Stocking Density} = \frac{\text{Usable Water Volume}}{\text{Number of Fish}}$$

The tanks were maintained under a controlled photoperiod (12 h light:12 h dark) for 8 weeks. The water parameters, including temperature, ammonia (NH₃), dissolved oxygen (DO), and pH, were checked daily throughout the trial. The temperature and pH were monitored with a digital waterproof pHep® pH/temperature tester (HI98128; Hanna Instruments Inc., RI, USA), and the NH₃ concentration was determined via a spectrophotometric method. The DO concentrations were measured via a portable dissolved oxygen meter (Standard Polarographic DO Probe -HI76407-Hanna Instruments Inc.). The recorded values were within the acceptable limits: temperature: 26 ± 1 °C; ammonia and nitrite levels: <0.02 mg/L; DO = 6.50 ± 0.25 mg/L; and pH = 7.0 ± 0.2 .

Uneaten feed and fecal matter were siphoned daily, and 50% of the water in each aquarium was exchanged with dechlorinated freshwater to maintain hygiene and water quality. The fish were weighed every two weeks to calculate growth parameters.

Growth performance

As stated by Abu-Zahra et al. (2024), the following equations were used to calculate the growth indices:

Weight Gain (WG, g) = Final Body Weight (FW) – Initial Body Weight (IW)

$$\text{Gain Percentage (G\%)} = \frac{\text{FW} - \text{IW}}{\text{IW}} \times 100$$

$$\text{Specific growth rate (SGR, \% / \text{day})} = \frac{\ln(\text{FW}) - \ln(\text{IW})}{T (\text{Days of experiment})} \times 100$$

$$\text{Feed Conversion Ratio (FCR)} = \frac{\text{Total Feed Intake (FI)}}{\text{Total Weight Gain (WG)}}$$

$$\text{Protein Intake (PI, g)} = \text{FI (g)} \times \frac{\text{Crude protein (\%)}}{100}$$

$$\text{Protein Efficiency Ratio (PER)} = \frac{\text{WG}}{\text{Protein Intake (PI)}} \times 100$$

$$\text{Survival rate (SR\%)} = \frac{\text{No of fish at the end}}{\text{No of fish at the start}} \times 100$$

Blood sampling and euthanasia

At the end of the 8-week feeding trial, the fish were fasted for 24 h before sampling. Nine fish per treatment were randomly selected and euthanized with 40 mg/L clove oil (Abu-Zahra et al. 2025c). Once opercular movement ceased and no tail-pinch response was observed, blood was aseptically collected from the caudal vein via sterile 1 mL syringes. Whole blood was placed into EDTA tubes for hematology, phagocytic activity, and serum bactericidal assays. Additional blood was collected into plain tubes, allowed to clot (15–20 min, room temperature), and centrifuged (3000 rpm, 10 min). Sera were separated and stored at -20°C for biochemical analysis.

Hematological parameters

Hematological parameters were assessed following the method described by Yılmaz et al. (2021). An automated hematology analyzer (Model: DH36, Shenzhen Dymind Biotechnology Co., Ltd., China) was used to determine the following parameters: red blood cell count (RBC, $\times 10^6/\mu\text{L}$), hemoglobin concentration (Hb, g/dL), hematocrit (Ht, %), mean corpuscular volume (MCV, fL), mean corpuscular hemoglobin (MCH, pg), mean corpuscular hemoglobin concentration (MCHC, g/dL), thrombocytes ($\times 10^3/\mu\text{L}$), total white blood cell count (WBC, $\times 10^3/\mu\text{L}$), and differential leukocyte counts (lymphocytes, granulocytes, MID, $\times 10^3/\mu\text{L}$).

Biochemical parameters

Serum biochemical parameters were analyzed via commercial diagnostic kits and measured via a semiautomated chemistry analyzer (ARX-199, Micro Lab Instrument, India). All the assays were conducted strictly according to the manufacturer's protocols as specified by the respective kit suppliers. Details of the kit brands, catalog numbers, methods, and detection wavelengths used for each parameter are provided in Table 1S (supplementary file). Globulin was calculated by subtracting the serum albumin concentration from the total protein concentration. The albumin/globulin (A/G) ratio was then computed accordingly.

Antioxidant and immune biomarkers

Superoxide dismutase (SOD) activity was determined according to Nishikimi et al. (1972) on the basis of the inhibition of nitroblue tetrazolium (NBT) reduction and expressed as U/mg protein. Lipid peroxidation (LPO) was measured as thiobarbituric acid reactive substances (TBARS) following the methods of Ohkawa et al. (1979) and expressed as μmol MDA/mL. Glutathione-S-transferase (GST) activity was assayed via the use of 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate (Habig et al. 1974) and expressed as U/mg protein. Reduced glutathione (GSH) was quantified via the DTNB reagent according to Moron et al. (1979) and expressed as μmol /mL. Nitric oxide (NO) levels were determined via the Griess reagent (Green et al. 1982) and expressed as μmol /L.

Myeloperoxidase (MP) activity was measured spectrophotometrically at 623 nm using TMB as a substrate (Quade and Roth 1997). Total immunoglobulin (TIg) was quantified by polyethylene glycol (PEG) precipitation followed by protein estimation via the Lowry method (Siwicki and Anderson 1993) and expressed as μg /mL. Phagocytic activity (PA%) was determined by incubating peripheral blood leukocytes with heat-killed *A. hydrophila* for 1 h, followed by Giemsa staining and microscopic counting of phagocytic cells (Anderson and Siwicki 1995). Serum bactericidal activity (SBA) was assessed by serial dilution of whole blood in sterile phosphate-buffered saline (PBS), plating on tryptic soy agar (TSA), and incubation at 28 °C for 24–48 h. The results are expressed as colony-forming units (CFUs) $\times 10^7$ /mL blood.

Experimental infection

An in vivo challenge trial was conducted to assess the protective effects of dietary NI and CA against *A. hydrophila*. At the end of the feeding period and after initial sampling, 21 fish remained per group. A virulent *A. hydrophila* strain (El-Gammal et al. 2025) was subcultured in tryptic soy broth (TSB; Hi-Media, India) and incubated at 37 °C for 24 h. The suspension was adjusted to 3×10^8 CFU/mL using McFarland standard No. 1. Ten fish per group were intraperitoneally injected with 0.2 mL of the bacterial suspension, while 11 fish received 0.2 mL of sterile physiological saline (negative control). The fish were observed for clinical signs and mortality over 14 days post-infection, and the survival data were analyzed via standard equations.

$$\text{Mortality\%} = \frac{\text{No fish mortalities}}{\text{Total no population}} \times 100$$

$$\text{RPS}\% = 1 - \left(\frac{\text{mortality\% in the treated group}}{\text{mortality\% in the control group}} \right) \times 100$$

Re-isolation of *A. hydrophila* from internal organs

At 14 days post-challenge, five fish per group were euthanized and aseptically dissected to collect liver, kidney, and spleen samples. Each organ was inoculated into TSB and incubated at 37 °C for 24 h, after which turbid cultures were streaked onto *Aeromonas* isolation agar (Hi-Media, India) and incubated under the same conditions. Colonies with a characteristic dark green appearance and central darkening were presumptively identified as *A. hydrophila* and confirmed by standard biochemical tests (Austin and Austin 2012). Bacterial recovery was semi-quantitatively scored as – (no growth), ± (1–3 colonies), + (few), ++ (moderate), +++ (heavy), or +++++ (very heavy). The percentage of positive fish was calculated from individuals that yielded *A. hydrophila* from any organ. All procedures were performed aseptically to avoid cross-contamination. An overview of the experimental design, including dietary treatments, feeding duration, assessed parameters, and challenge protocol, is provided in Fig. 1S.

Statistical analysis

All the data are expressed as the means ± standard errors (SEs). The assumptions of normality and homogeneity of variance were verified via the Shapiro–Wilk test and Levene’s test, respectively. Two-way analysis of variance (ANOVA) was used to evaluate significant differences among treatment groups for continuous variables. When significant differences were found ($P < 0.05$), Tukey’s post hoc test was applied for pairwise comparisons. For categorical and ordinal data, such as clinical and postmortem lesion scores, a nonparametric Kruskal–Wallis test was performed, followed by Dunn’s post hoc test (Østerlind 1998) when appropriate. All the statistical analyses were conducted via SPSS software (version 22; IBM Corp., Armonk, NY, USA).

Results

Phytochemical composition of the NI

Phytochemical analysis of the NIs revealed 13 bioactive compounds, including phenolic acids, flavonoids, esters, aldehydes, and glycosides (Fig. 1, Table 2). Chlorogenic acid was predominant (95.14 µg/mL), followed by methyl gallate (47.23 µg/mL), rutin (44.14 µg/mL), and gallic acid (38.74 µg/mL), all of which are recognized for their potent antioxidant and antimicrobial activities. Catechin (14.41 µg/mL) and caffeic acid (5.62 µg/mL) were also present, contributing to free radical scavenging and anti-inflammatory effects. Minor constituents, such as naringenin, daidzein, hesperetin, vanillin, ferulic acid, and p-coumaric acid, were detected and are likely to contribute synergistically to bioactivity. Compounds such as syringic acid, ellagic acid, rosmarinic acid, quercetin, kaempferol, and pyrocatechol were undetected, possibly because of their absence or sub-detection concentrations.

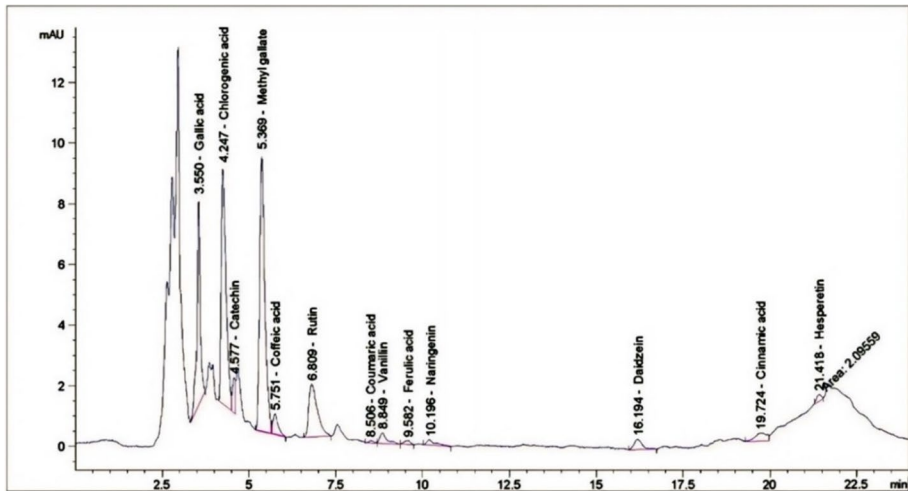


Fig. 1 Chromatographic profile of the NI as analyzed by GC–MS. The identified peaks corresponded to phenolic acids, flavonoids, and related bioactive compounds

Overall, the phytochemical profile supports the potential of NI as a multifunctional antioxidant and antimicrobial feed additive.

In vitro antibacterial activity of NI & CA

The antibacterial effects of NI and CA against *A. hydrophila* are presented in Table 2S (Supplementary file). NI resulted in the largest inhibition zone (20.67 ± 0.67 mm), followed closely by CA (20.00 ± 1.15 mm), with no significant difference between them ($P > 0.05$). The antibiotic control, oxytetracycline (OXY, 10 µg/mL), produced a smaller zone (14.60 ± 1.10 mm), indicating moderate efficacy. No inhibition zone was observed for the negative control (1% DMSO), as expected.

Growth performance

Prior to infection, all the fish displayed normal behavior, with no signs of abnormal swimming, stress, or clinical symptoms. The fish remained healthy and stable throughout the feeding trial, and no mortalities or pathological changes in the visceral organs were observed before the infection challenge. After 8 weeks, growth performance improved significantly in all supplemented groups compared with that in the CNT group (Table 3). The NI+CA group presented the greatest increases in FW, WG, and SGR (1.24-, 1.44-, and 1.27-fold greater than those of the CNT group, respectively). The FCR was reduced by nearly 19% in the NI+CA group, although not significantly ($P = 0.1316$), whereas the NI and CA groups individually presented significantly lower FCRs ($P < 0.05$). PER was significantly elevated in all the supplemented groups, with the NI+CA group achieving the greatest improvement (1.24-fold increase over the CNT group, $P = 0.0287$). Survival remained 100% across all groups, confirming the absence of adverse effects.

Table 2 Chemical composition of the NI aqueous extract identified via GC–MS analysis

Peak	Retention Time (min)	Compound Name	Peak Area (mAU·s)	Area %	Concentration (µg/mL)	Relative Abundance	Class
1	3.550	Gallic acid	43.80	15.72	38.74	Moderate	Phenolic acid
2	4.247	Chlorogenic acid	73.32	26.32	95.14	High	Phenolic acid
3	4.577	Catechin	6.68	2.40	14.41	Low	Flavanol
4	5.369	Methyl gallate	93.73	33.64	47.23	High	Phenolic ester
5	5.751	Caffeic acid	7.26	2.61	5.62	Low	Phenolic acid
6	6.350	Syringic acid	0.00	0.00	0.00	Not Detected	Phenolic acid
7	6.575	Pyro catechol	0.00	0.00	0.00	Not Detected	Phenol derivative
8	6.809	Rutin	29.92	10.74	44.14	Moderate	Flavonoid glycoside
9	7.108	Ellagic acid	0.00	0.00	0.00	Not Detected	Polyphenolic compound
10	8.506	p-Coumaric acid	8.14	0.29	0.29	Trace	Phenolic acid
11	8.849	Vanillin	4.84	1.74	1.80	Low	Phenolic aldehyde
12	9.582	Ferulic acid	1.50	0.54	0.87	Trace	Phenolic acid
13	10.196	Naringenin	3.16	1.13	2.88	Low	Flavanone
14	11.670	Rosmarinic acid	0.00	0.00	0.00	Not Detected	Phenolic acid
15	16.194	Daidzein	5.28	1.89	2.96	Low	Isoflavone
16	17.179	Quercetin	0.00	0.00	0.00	Not Detected	Flavanol
17	19.724	Cinnamic acid	6.19	2.22	1.11	Low	Phenylpropanoid
18	20.521	Kaempferol	0.00	0.00	0.00	Not Detected	Flavanol
19	21.418	Hesperetin	2.10	0.75	1.03	Trace	Flavanone

The values represent retention times (Rt) in minutes. Compound classification is based on structural and functional group identity. The compounds were identified on the basis of their retention times and UV spectral data. Relative abundance was estimated from the integrated peak area (%): high (>20%), moderate (5–20%), low (1–5%), trace (<1%), and not detected (0%)

Table 3 Effects of dietary supplementation with NI or CA on growth performance parameters in *O. niloticus*

Parameter	CNT	NI	CA	NI + CA	P value		
					NI	CA	NI + CA
IW (g)	32.15 ± 0.12 ^a	32.18 ± 0.23 ^a	32.24 ± 0.73 ^a	32.56 ± 1.00 ^a	0.321 (ns)	0.619 (ns)	0.9636 (ns)
FW (g)	68.50 ± 0.36 ^c	80.60 ± 1.00 ^b	78.00 ± 0.95 ^b	84.77 ± 0.60 ^a	< 0.0001 ***	< 0.0001 ***	0.0001 ***
WG (g)	36.36 ± 0.45 ^c	48.42 ± 1.15 ^{ab}	45.76 ± 1.17 ^b	52.21 ± 1.48 ^a	< 0.0001 ***	0.0001 ***	0.0456 *
G %	113.11 ± 1.77 ^c	150.51 ± 4.42 ^b	142.20 ± 6.07 ^b	160.90 ± 9.24 ^a	0.0001 ***	0.0005 ***	0.8193 (ns)
FI (g)	84.55 ± 0.25 ^c	94.73 ± 0.74 ^b	92.60 ± 1.04 ^b	98.56 ± 0.61 ^a	< 0.0001 ***	< 0.0001 ***	0.0005 ***
FCR	2.33 ± 0.02 ^a	1.96 ± 0.03 ^b	2.03 ± 0.05 ^b	1.89 ± 0.06 ^{ab}	0.0001 ***	0.0193 *	0.1316 (ns)
SGR (%/day)	1.35 ± 0.01 ^b	1.64 ± 0.03 ^a	1.58 ± 0.04 ^a	1.71 ± 0.06 ^a	< 0.0001 ***	0.0004 ***	0.0188 *
PI (g)	25.36 ± 0.07 ^c	28.42 ± 0.22 ^{ab}	27.78 ± 0.31 ^b	29.57 ± 0.18 ^a	0.0001 ***	0.0005 ***	0.1129 (ns)
PER	1.43 ± 0.01 ^b	1.70 ± 0.03 ^a	1.65 ± 0.04 ^a	1.77 ± 0.06 ^a	0.0012 **	0.0035 **	0.0287 *
SR%	100	100	100	100	-	-	-

The values ($n=30$ /group, 10/replicate) are the means ± SEs. Superscript letters indicate significant differences among groups ($P<0.05$). The asterisks accompanying the P values represent the degree of statistical significance in the ANOVA. ns: nonsignificant results ($P>0.05$); *: significance ($P<0.05$); **: high significance ($P\leq 0.01$); and ***: very high significance ($P\leq 0.001$)

Hematological parameters

Dietary NI, CA, and NI+CA significantly modulated the hematological profile of *O. niloticus* (Table 4). Hb levels were significantly higher in the NI and NI+CA groups ($P<0.05$), indicating improved oxygen-carrying capacity, whereas CA alone had no effect. RBC counts and Ht values increased numerically, particularly in the NI+CA group, but were not significant ($P>0.05$).

Total WBC and lymphocyte counts were significantly elevated in all supplemented groups ($P<0.001$), with the highest values in the NI+CA group, reflecting additive rather than synergistic effects. Granulocyte counts were significantly reduced across all the treatment groups ($P\leq 0.01$), suggesting a similar anti-inflammatory response. Thrombocyte counts tended to increase in the NI+CA group, but the difference was not significant. No significant changes were observed in the MCV, MCH, MCHC, or MID, indicating stable erythrocyte morphology.

Biochemical parameters

Dietary NI, CA, and NI+CA significantly affected the serum biochemical profile of *O. niloticus* (Figs. 2, and 3). Total protein (TP) was highest in the NI+CA group ($P<0.001$), with moderate increases in NI and CA. Globulin levels increased significantly in all

Table 4 Effects of dietary supplementation with NI or CA on serum hematological parameters in *O. niloticus*

Parameter	Control	NI	CA	NI+CA	P value	
					NI	NI+CA
RBCs ($\times 10^6/\mu\text{L}$)	1.67 $\pm$ 0.14	1.99 $\pm$ 0.03	1.90 $\pm$ 0.16	2.01 $\pm$ 0.04	0.076 (ns)	0.183 (ns)
Hb (g/dL)	8.13 $\pm$ 0.57 ^b	9.97 $\pm$ 0.19 ^a	8.50 $\pm$ 0.40 ^b	9.77 $\pm$ 0.38 ^a	0.013 (*)	0.542 (ns)
Ht (%)	25.27 $\pm$ 0.82	30.43 $\pm$ 1.33	25.60 $\pm$ 1.40	31.90 $\pm$ 2.65	0.556 (ns)	0.078 (ns)
MCV (fL)	165.28 $\pm$ 5.50	165.23 $\pm$ 5.58	157.71 $\pm$ 14.14	142.45 $\pm$ 8.69	0.997 (ns)	0.576 (ns)
MCH (pg)	51.19 $\pm$ 2.80	52.13 $\pm$ 1.84	52.47 $\pm$ 4.99	50.50 $\pm$ 0.41	0.830 (ns)	0.772 (ns)
MCHC (g/dL)	30.95 $\pm$ 0.97	31.65 $\pm$ 2.15	33.24 $\pm$ 0.34	33.25 $\pm$ 0.95	0.785 (ns)	0.383 (ns)
Thrombocytes ($\times 10^3/\mu\text{L}$)	6.33 $\pm$ 0.33	7.00 $\pm$ 0.58	7.00 $\pm$ 0.58	8.67 $\pm$ 0.33	0.347 (ns)	0.347 (ns)
WBCs ($\times 10^3/\mu\text{L}$)	37.75 $\pm$ 0.91 ^c	55.94 $\pm$ 0.87 ^b	57.56 $\pm$ 0.82 ^b	79.33 $\pm$ 1.62 ^a	<0.001 (***)	<0.001 (***)
Lymphocytes ($\times 10^3/\mu\text{L}$)	34.52 $\pm$ 0.64 ^c	51.66 $\pm$ 0.79 ^b	53.07 $\pm$ 1.09 ^b	72.94 $\pm$ 2.13 ^a	<0.001 (***)	<0.001 (***)
Granulocytes ($\times 10^3/\mu\text{L}$)	1.62 $\pm$ 0.21 ^a	0.57 $\pm$ 0.03 ^c	0.80 $\pm$ 0.05 ^b	0.65 $\pm$ 0.20 ^{bc}	0.001 (***)	0.004 (**)
MID ($\times 10^3/\mu\text{L}$)	4.70 $\pm$ 0.47	3.91 $\pm$ 0.40	3.53 $\pm$ 0.70	4.01 $\pm$ 1.21	0.488 (ns)	0.311 (ns)

The values are presented as the means $\pm$ SEs ($n=9/\text{group}$). Different superscripts within a row indicate significant differences ($P<0.05$) according to Tukey's post hoc test. MID: Intermediate cells (total count of white blood cells that are not categorized as granulocytes or lymphocytes). The asterisks accompanying the P values represent the degree of statistical significance in the ANOVA. ns: nonsignificant results ($P>0.05$); *, significance ($P<0.05$); **, high significance ($P\leq 0.01$); and *** very high significance ($P\leq 0.001$)

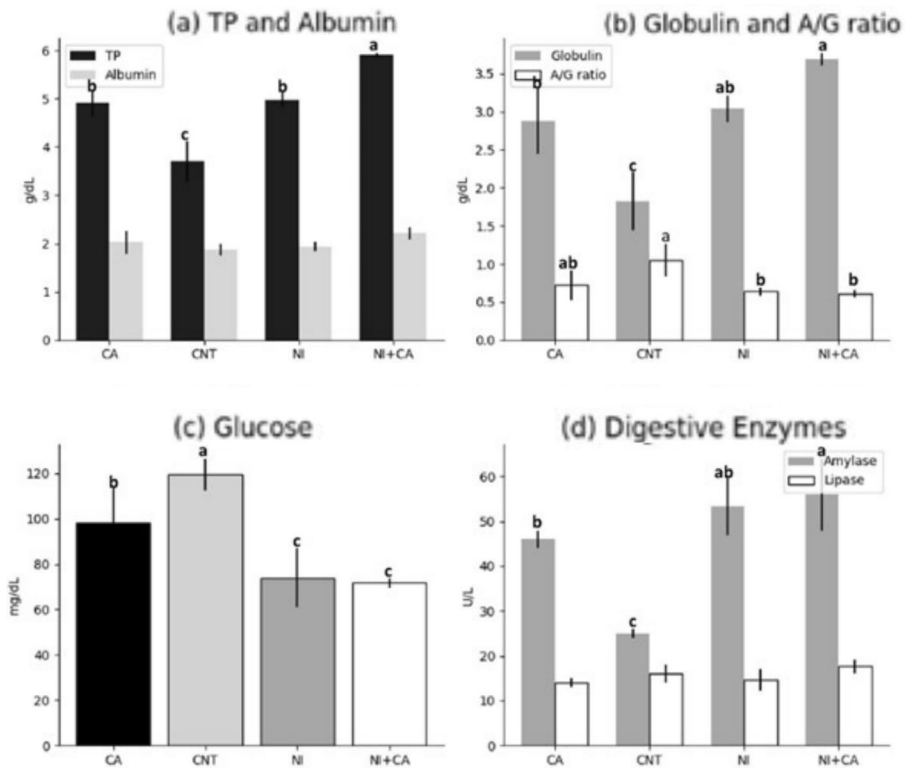


Fig. 2 Effects of dietary supplementation with NI or CA on the serum biochemical parameters of *O. niloticus*. The values are presented as the means $\pm$ SEs ($n=9$). The error bars represent SEs. Means within the same parameter with different superscript letters differ significantly ($P < 0.05$). Statistical significance (P values) for main effects and interactions: TP:NI ($P=0.0005$), CA ($P=0.0004$), interaction ($P=0.0008$); albumin:NI ($P=0.180$), CA ($P=0.056$), interaction ($P=0.483$); globulin:NI ($P=0.0004$), CA ($P=0.0003$), interaction ($P=0.0005$); A/G ratio:NI ($P=0.014$), CA ($P=0.063$), interaction ($P=0.123$); glucose:NI ($P=0.0007$), CA ($P=0.0011$), interaction ($P=0.0008$); amylase:NI ($P=0.0001$), CA ($P=0.0002$), interaction ($P=0.0003$); and lipase:NI ($P=0.306$), CA ($P=0.652$), interaction ($P=0.057$)

supplemented groups ($P < 0.001$), whereas the level of albumin was unchanged ($P > 0.05$). The A/G ratio decreased significantly only in the NI group ($P=0.014$) because of elevated globulin. Amylase activity was significantly higher in all the treatments, with the greatest increase occurring in the NI+CA treatment. Lipase activity remained unchanged ($P > 0.05$). Serum glucose was significantly lower in the NI and NI+CA groups ($P < 0.01$) than in the CA alone group.

The serum activities of GPT, GOT, LDH were significantly reduced in all the supplemented groups ($P < 0.05$) (Fig. 3). ALP activity showed a significant reduction in the NI+CA group, while NI and CA alone showed no significant differences compared to the CNT group. Urea levels decreased significantly in the NI, CA, and NI+CA groups ($P < 0.05$), with no significant change in creatinine ($P > 0.05$). The cholesterol and triglyceride concentrations were significantly lower in all the treated groups ($P < 0.001$), with the most pronounced decreases in the CA and NI+CA groups. This suggests that CA and the combination of NI+CA have a strong lipid-lowering effect, which could contribute to better metabolic regulation and reduced risk of lipid-related disorders.

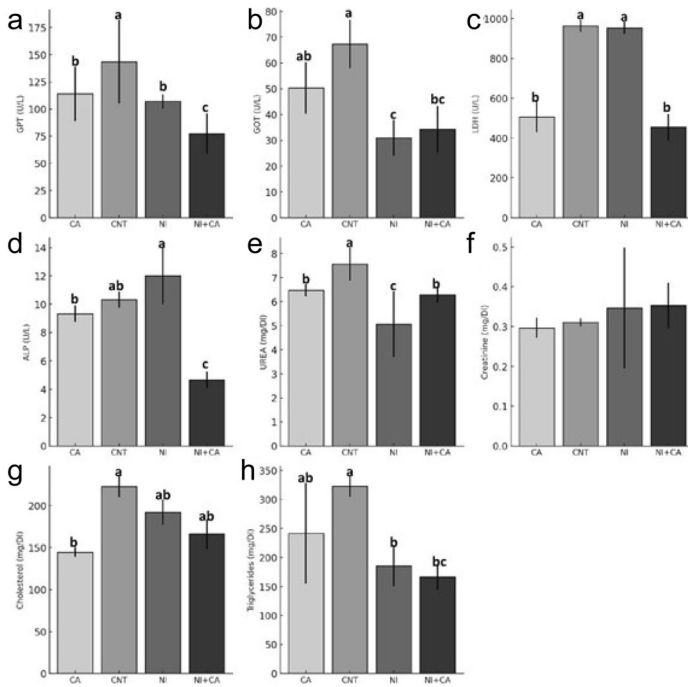


Fig. 3 Effects of dietary supplementation with NI or CA on the serum biochemical parameters of *O. niloticus*. The values are presented as the means $\pm$ SEs ($n=9$). The error bars represent SEs. Means within the same parameter with different superscript letters differ significantly ($P < 0.05$). Statistical significance (P values) for main effects and interactions: GPT: NI ($P=0.0014$), CA ($P=0.0001$), interaction ($P=0.0002$); GOT: NI ($P=0.0004$), CA ($P=0.0047$), interaction ($P=0.0010$); LDH: NI ($P=0.0007$), CA ($P=0.0017$), interaction ($P=0.0011$); ALP: NI ($P=0.0486$), CA ($P=0.0002$), interaction ($P=0.0012$); Urea: NI ($P=0.0243$), CA ($P=0.0181$), interaction ($P=0.0127$); Creatinine: NI ($P=0.3540$), CA ($P=0.9457$), interaction ($P=0.8383$); Cholesterol: NI ($P=0.0005$), CA ($P=0.0007$), interaction ($P=0.0006$); Triglycerides: NI ($P=0.0006$), CA ($P=0.0004$), interaction ($P=0.0005$)

Antioxidant and immune responses

Dietary supplementation (NI, CA, or their combination) significantly enhanced antioxidant and immune defenses in *O. niloticus* (Fig. 4). SOD and GST activities increased in all supplemented groups, peaking in the NI+CA group, indicating improved radical scavenging and detoxification capacity. GSH levels rose significantly with CA but not with NI, suggesting CA-driven enhancement of intracellular antioxidant reserves. LPO levels were markedly reduced in all the treatment groups, with the NI+CA group showing the greatest protection against lipid peroxidation.

Immunologically, the number of SBAs (CFU counts) decreased significantly across the treatment groups, with NI+CA exerting the strongest bactericidal effect. The PA and MP activities increased significantly, with the highest responses occurring in the NI+CA group, reflecting synergistic stimulation of innate immunity. TIg concentrations were also highest in the NI+CA group, indicating enhanced humoral responses. NO levels were not significantly altered, although a mild numerical increase suggested limited immunomodulation.

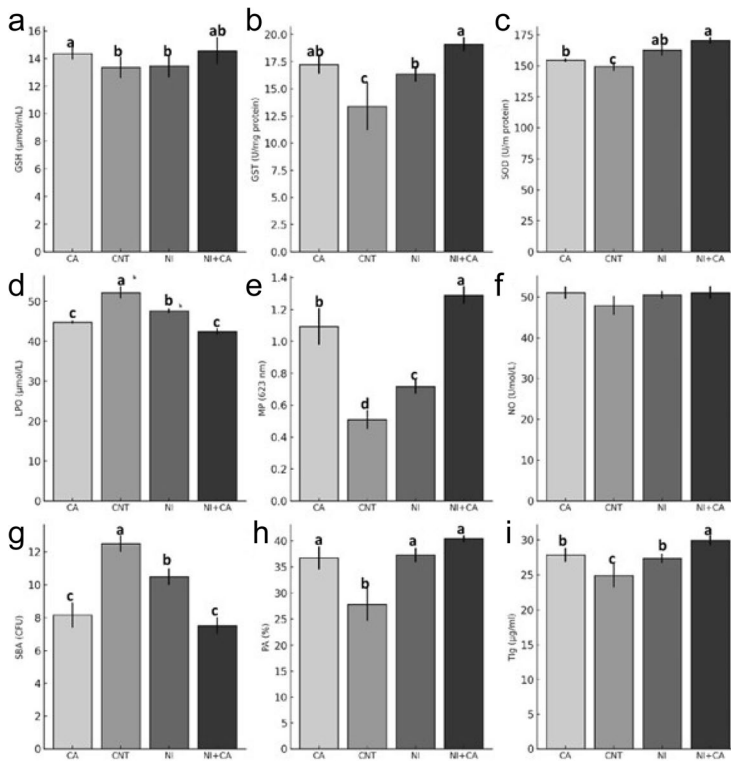


Fig. 4 Effects of dietary supplementation with NI, CA, or their combination (NI+CA) on antioxidant and immune parameters in *O. niloticus*. The values are presented as the means $\pm$ SEs ($n=9$). The error bars represent SEs. Means within the same parameter with different superscript letters differ significantly ($P<0.05$). Statistical significance (P values) for main effects and interactions: GSH: NI (0.7421), CA (0.0474), and interaction (0.9200); GST: NI (0.0113), CA (0.0021), and interaction (0.4773); SOD: NI (0.000051), CA (0.0085), and interaction (0.5320); LPO: NI (0.0002), CA (0.000003), and interaction (0.0759); MP: NI (0.0015), CA (0.0000086), and interaction (0.9100); NO: NI (0.2000), CA (0.0978), and interaction (0.2221); PA: NI (0.0006), CA (0.0011), and interaction (0.0422); TIg: NI (0.0071), CA (0.0024), and interaction (0.8084); SBA: NI (0.0043), CA (0.0000043), and interaction (0.0748)

Clinical and postmortem lesion severity following *A. hydrophila* challenge

Marked differences in clinical and postmortem lesion severity were observed among the groups (Table 5, Fig. 5). The CNT fish exhibited severe systemic pathology, including eye hemorrhage, tail erosion, ascites, and skin hemorrhage. NI supplementation markedly reduced lesion scores, with only mild fin hemorrhage and discoloration remaining. CA supplementation led to further improvement, with complete resolution of hemorrhages, ascites, and scale loss, although mild skin ulceration persisted. The NI+CA combination provided the greatest protection, with near-zero scores for most parameters and complete absence of skin ulceration and tail erosion, indicating synergistic mitigation of infection-induced pathology.

Table 5 Clinical and postmortem lesion scores (0–5 scale) in *O. niloticus* experimentally infected with *A. hydrophila* after 8 weeks of dietary supplementation with NI and/or CA

Sign	CNT	NI	CA	NI + CA
Skin hemorrhage	2.00 ± 0.01 ^a	0.83 ± 0.00 ^b	0.00 ± 0.00 ^c	1.88 ± 0.10 ^a
Fin hemorrhage	0.00 ± 0.00 ^c	1.67 ± 0.00 ^a	1.11 ± 0.40 ^a	0.62 ± 0.03 ^b
Tail hemorrhage	1.00 ± 0.00 ^a	0.42 ± 0.12 ^b	0.00 ± 0.00 ^c	0.00 ± 0.00 ^c
Eye hemorrhage	3.50 ± 0.21 ^a	0.00 ± 0.00 ^c	0.56 ± 0.11 ^b	0.00 ± 0.00 ^c
Eye protrusion	0.00 ± 0.00 ^c	0.00 ± 0.00 ^c	0.56 ± 0.13 ^b	0.62 ± 0.00 ^a
Skin discoloration	0.50 ± 0.00 ^b	0.83 ± 0.21 ^a	1.11 ± 0.00 ^a	0.00 ± 0.00 ^c
Fin erosion	1.00 ± 0.20 ^a	1.25 ± 0.00 ^a	0.56 ± 0.06 ^b	0.00 ± 0.00 ^c
Tail erosion	3.00 ± 0.21 ^a	1.25 ± 0.30 ^b	0.00 ± 0.00 ^c	0.00 ± 0.00 ^c
Skin ulcer	1.00 ± 0.09 ^b	0.42 ± 0.15 ^b	2.22 ± 0.00 ^a	0.00 ± 0.00 ^c
Scale detachment	1.50 ± 0.00 ^a	0.83 ± 0.30 ^b	0.56 ± 0.21 ^b	0.62 ± 0.14 ^b
Tail detachment	0.50 ± 0.00 ^a	0.42 ± 0.08 ^a	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b
Distended gall bladder	0.00 ± 0.00 ^c	0.42 ± 0.05 ^b	0.00 ± 0.00 ^c	1.88 ± 0.30 ^a
Ascites	3.23 ± 0.21 ^a	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	1.0 0.00 ^b

The values are the means ± SEs ($n=10$). Each value represents the average lesion severity for each sign across the four groups. Superscript letters indicate significant differences between groups ($P<0.05$). Scoring: 0 = no sign, 1–2 = mild, 3 = moderate, 4–5 = severe/generalized

Effect of dietary treatments on post-challenge survival

Following *A. hydrophila* challenge, the CNT-treated fish presented the highest mortality (50%). NI or CA supplementation significantly reduced mortality to 20%, whereas the NI + CA combination further decreased mortality to 10% (Fig. 6). The RPS values followed the same trend (60% for NI or CA, 80% for NI + CA), confirming the enhanced protective efficacy of the combined treatment. These results are consistent with the observed reductions in clinical signs and lesion severity.

Re-isolation of *A. hydrophila* and bacterial load assessment

Re-isolation of *A. hydrophila* revealed substantial differences in the bacterial load among the groups (Table 3S, Supplementary file). All CNT fish (5/5) were positive, showing very heavy kidney growth (+++), heavy liver growth (+++), and moderate spleen growth (++). NI and CA supplementation reduced the re-isolation rates to 40% and 20%, respectively, with only mild organ growth (+). The NI + CA group presented the strongest effect, with no recovery from the kidney or liver and minimal spleen growth (±; 1/5 fish).

Discussion

GC–MS analysis of the NI revealed phenolic acids and flavonoids as the predominant bioactive compounds, which likely explain the improvements in antioxidant capacity, immune function, and infection resistance observed in *O. niloticus*. The key constituents chlorogenic acid, gallic acid, and caffeic acid are potent ROS scavengers and immunomodulators

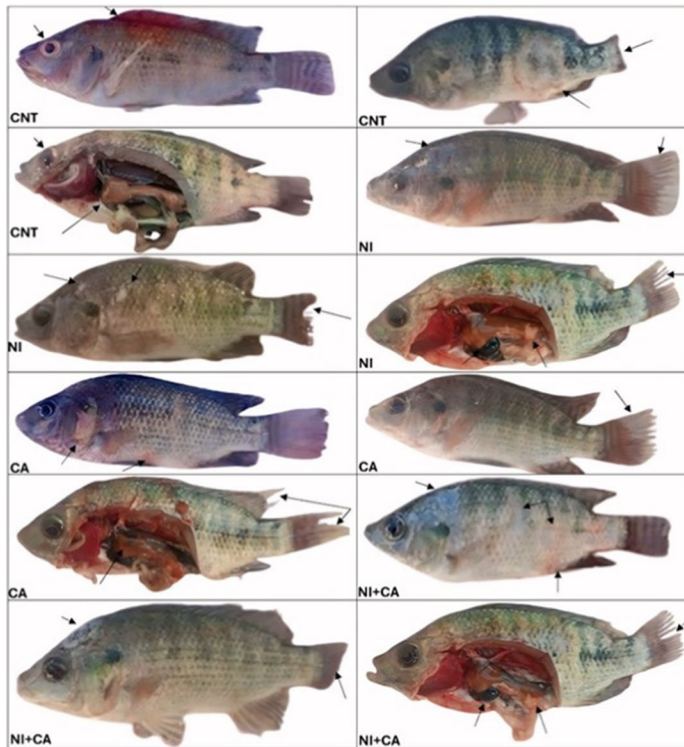


Fig. 5 External and internal pathological observations of *O. niloticus* after being fed different experimental diets and subsequently challenged with *A. hydrophila*. The CNT group presented severe external hemorrhages, scale loss, skin ulcers, eye hemorrhages, tail erosions, and darkened body color; internally, pronounced inflammation in the liver and intestines with ascites was detected. The NI group presented moderate hemorrhages, scale loss, and fin erosion; internal hemorrhaging was reduced, with a distended gall bladder. The CA group presented milder skin lesions, tail damage, skin discoloration, and moderate internal inflammation with bile retention. The NI + CA group presented minimal lesions, intact organs, and very low internal pathology

that are known to suppress proinflammatory cytokines and enhance antioxidant enzymes (Nguyen et al. 2024; Yazgan & Yazgan 2024). High levels of gallic acid correlate with reduced lipid peroxidation and lesion severity, whereas flavonoids such as rutin, naringenin, and hesperetin may strengthen vascular integrity and innate immunity, supporting the reduced hemorrhagic lesions and elevated phagocytic and MPO activity observed.

Minor compounds, including ferulic acid, vanillin, and p-coumaric acid, likely contribute additional antimicrobial effects via bacterial membrane disruption and biofilm inhibition (Daglia 2012). Methyl gallate, cinnamic acid, and low levels of daidzein may offer cytoprotective and anti-inflammatory benefits during infection stress (Borges et al. 2014). Collectively, the phytochemical richness of NI appears to act synergistically with CA, as evidenced by maximal antioxidant enzyme activity, reduced lesion severity, and minimal *A. hydrophila* re-isolation in the NI+CA group, supporting its potential as a multifunctional additive for enhancing fish health and disease resistance.

Both NI and CA exhibited strong in vitro antimicrobial activity, producing inhibition zones comparable to or slightly larger than those of oxytetracycline (OXY), confirming

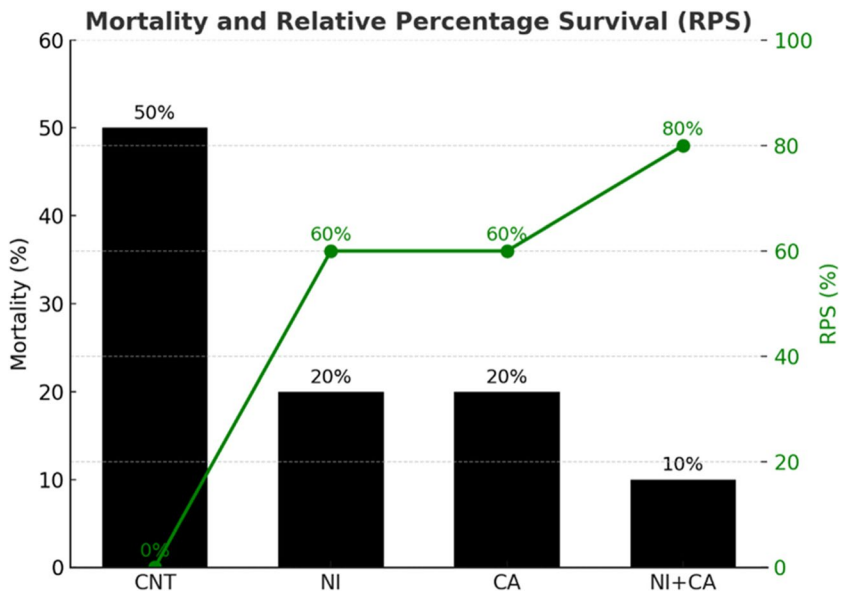


Fig. 6 Cumulative mortality and RPS in *O. niloticus* following *A. hydrophila* challenge under different dietary treatments. Mortality is represented as a bar graph (left Y-axis), whereas RPS is shown as a line graph (right Y-axis)

their broad-spectrum efficacy as medicinal plants against *A. hydrophila* (Marchese et al. 2017; Abu-Zahra et al. 2025a). The activity of NI is attributed to its high levels of phenolic acids (gallic, chlorogenic, caffeic, and ferulic acids), which disrupt bacterial membranes, inhibit key enzymes, and prevent biofilm formation (Sato et al. 2011; Daglia 2012). Flavonoids such as rutin and naringenin likely enhance this effect by destabilizing membranes and inhibiting quorum sensing. CA's well-documented membrane-disruptive action appears to act synergistically with NI phytochemicals, explaining the slightly larger inhibition zones in the combined treatments. The absence of inhibition in the DMSO control confirmed that the activity originated from the plant compounds. Compared with the moderate inhibition zone of OXY (14.60 mm), these results highlight the potential of NI and CA as natural antimicrobial alternatives or adjuncts to reduce reliance on synthetic antibiotics in aquaculture (Manoharan et al. 2021).

The dietary inclusion levels of NI and CA were selected on the basis of prior evidence of their efficacy and safety in aquaculture species. For NI, a 1% inclusion level was chosen because it consistently enhances growth, antioxidant defense, and immune function in *O. niloticus* without adverse effects on feed intake or health (Yousefi et al. 2021; Öz et al. 2023). While Abdel-Daim et al. (2025) reported that 3% NI improved lysozyme and IgM levels and resistance to *Edwardsiella tarda*, lower doses (1–1.5%) have shown comparable antioxidant and immunostimulatory benefits, with better feed efficiency and cost-effectiveness (Yousefi et al. 2021; Öz et al. 2023). CA was incorporated at 0.1% (1 g/kg feed), a dose supported by studies showing antimicrobial, anti-inflammatory, and antioxidant benefits without compromising palatability or liver health. For example, Suarez et al. (2025) reported that 0.5–1.0 g/kg CA improved red blood cell counts, antioxidant enzyme activity, and gut microbiota diversity in *Ictalurus punctatus*, whereas Mahboub and Tartor (2020) reported enhanced survival in *O. niloticus* challenged with *E. tarda* at 0.2 g/kg. However,

higher doses ($> 1.5\text{--}2\text{ g/kg}$) have been linked to hepatotoxicity and reduced feed intake in some species (Ahmed et al. 2023), suggesting that 1 g/kg is a safe and effective inclusion level.

The combination diet (NI+CA) was formulated to investigate potential additive or synergistic effects between the two bioactive compounds. Both NI and CA possess complementary antioxidant and immunomodulatory mechanisms; NI primarily enhances humoral and cellular immunity, whereas CA exerts strong antimicrobial and anti-inflammatory effects. Their combined use at the same safe and effective individual levels (1% NI and 0.1% CA) allows assessment of whether co-supplementation provides greater physiological benefits than either additive alone.

The improved growth performance, particularly in the NI+CA group, appeared to result from the synergistic effects of phenolic acids and flavonoids that enhance gut health, digestion, and nutrient utilization. Compounds such as gallic, chlorogenic, and caffeic acids promote intestinal villi development, modulate the gut microbiota, stimulate digestive enzymes, and suppress intestinal inflammation, collectively improving nutrient absorption and feed efficiency (Sharifi-Rad et al. 2018).

Cinnamic acid, ferulic acid, and naringenin support lipid metabolism by activating the AMPK and PPAR α pathways, increasing fatty acid oxidation, and reducing hepatic lipid accumulation (Zhou et al. 2016). The appetite-stimulating effect of CA, combined with the hepatoprotective and anti-inflammatory properties of NI, likely contributed to the superior SGR, FCR, and PER in the combined treatment group. These findings align with reports that phytogetic additives improve gut morphology, microbial balance, and muscle deposition in *O. niloticus* (Shourbela et al. 2021; Magouz et al. 2022). The 100% survival across all groups confirms the safety of NI and CA supplementation and supports their use as functional feed additives to increase growth without adverse effects.

Dietary supplementation with NI, CA, or NI+CA significantly improved hematological profiles, indicating enhanced physiological and immune function. Hb levels were significantly elevated in the NI and NI+CA groups, suggesting improved oxygen-carrying capacity. This enhancement is likely supported by NI polyphenols (gallic acid, caffeic acid, and rutin), which are known to protect erythrocytes and stimulate hematopoiesis (Ganeshpurkar & Saluja 2017). RBC counts and Ht values showed nonsignificant upward trends, suggesting potential stimulation of erythropoiesis without altering the MCV, MCH, or MCHC, confirming stable red cell morphology. Total WBC and lymphocyte counts were significantly increased in all treated groups, with the NI+CA group showing the highest values, reflecting additive stimulation of immune function. These findings align with previous studies demonstrating that NI can stimulate both cellular and humoral immune responses in fish species, including increased lymphocyte proliferation and protection against immune suppression and oxidative stress (Yousefi et al. 2021). These hematological improvements correspond with reduced lesion severity and mortality following *A. hydrophila* challenge, supporting enhanced disease resistance. Granulocyte counts were significantly reduced in all treated groups, which is consistent with the anti-inflammatory action of phenolics that suppress proinflammatory cytokines (Daglia 2012). Thrombocyte counts showed a nonsignificant upward trend, suggesting a potential improvement in hemostatic function.

Dietary supplementation with NI, CA, or their combination significantly improved the serum biochemical profile of *O. niloticus*. This enhancement indicated better protein metabolism, digestive efficiency, glycemic control, hepatic function, and lipid regulation. Elevated TP and globulin levels, especially in the NI+CA group ($P < 0.001$), suggest increased protein synthesis and humoral immunity, which is consistent with prior findings on the immunostimulatory effects of NI (Yousefi et al. 2021; Youssif et al.

2020). Albumin remained unchanged ($P > 0.05$), whereas the A/G ratio decreased in the NI group ($P = 0.014$), reflecting globulin-driven immune activation (Abou Khalil et al. 2017). Amylase activity increased significantly in all treated groups ($P < 0.05$), peaking in the NI + CA group, indicating improved carbohydrate digestion (Öz et al. 2023). Serum glucose levels were lower in the NI and NI + CA groups ($P < 0.01$), suggesting that NI has a hypoglycemic effect via insulinotropic and antioxidant mechanisms (Kaat-abi et al. 2015).

Liver enzymes (GPT, GOT, LDH) decreased significantly ($P < 0.05$), indicating that hepatoprotection is likely mediated by phenolic acids (Aly et al. 2023; Abd-Elkareem et al. 2023; Öz et al. 2023). Serum urea decreased in all supplemented groups ($P < 0.05$), reflecting improved nitrogen metabolism, whereas creatinine remained stable ($P > 0.05$), indicating preserved renal function (Reverter et al. 2014). The marked reductions in cholesterol and triglyceride levels ($P < 0.001$), especially in the CA and NI + CA groups, suggest hypolipidemic effects via the inhibition of cholesterol synthesis and increased lipid metabolism (Sahebkar et al. 2016; Farhangi et al. 2018). Overall, NI and CA, particularly in combination, confer metabolic and protective benefits without compromising renal health.

Dietary supplementation with NI, CA, or their combination (NI + CA) significantly enhanced the antioxidant status of *O. niloticus*, with the NI + CA group showing the strongest effects. This improvement is likely driven by phenolic compounds (e.g., gallic, caffeic, and ferulic acids; rutin), which are known to activate antioxidant enzymes and reduce oxidative stress (Parham et al. 2020). SOD and GST activities increased significantly with NI and CA ($P < 0.05$), peaking in the NI + CA group, indicating additive enhancement of endogenous defenses. LPO levels declined across all the treatments and were lowest in the NI + CA group, reflecting reduced membrane oxidative damage (El-Gammal et al. 2025; Abu-Zahra et al. 2025a). The GSH level increased only in the CA group ($P = 0.0474$), suggesting CA-specific modulation of the intracellular redox balance.

Immunologically, PA and MP activities were significantly elevated by all the treatments, with NI + CA resulting in the highest values (PA: 40.4%; MP: 1.29). A significant interaction in PA ($P = 0.0422$) indicates synergistic enhancement of phagocytic function, while MP activation appeared additive. TIg levels increased significantly in all groups ($P < 0.05$) and were highest in the NI + CA group, suggesting enhanced humoral immunity via compounds such as rutin, naringenin, and cinnamic acid (Firmino et al. 2021). SBA results revealed reduced CFUs in the CA and NI + CA groups, indicating improved antimicrobial defense, likely through combined immune activation and direct antimicrobial effects (Friedman et al. 2004). NO levels did not significantly increase ($P > 0.09$), suggesting immune modulation without excessive inflammation. Overall, NI and CA, especially in combination, enhanced the antioxidant capacity and immune competence of *O. niloticus*, with additive effects predominating and synergistic activation evident in phagocytic responses.

Dietary supplementation with NI, CA, or their combination (NI + CA) significantly improved *O. niloticus* survival following *A. hydrophila* challenge. The CNT group presented 50% mortality, confirming pathogen virulence and host susceptibility. Mortality decreased to 20% in the NI and CA groups (RPS = 60%), which is consistent with their known antimicrobial, immunostimulatory, and antioxidant properties (Mahboub et al. 2022; Ciesielska-Figlon et al. 2023). The NI + CA group exhibited the greatest protection (10% mortality; RPS = 80%), indicating synergistic effects of the combined bioactive agent. This enhanced survival likely reflects complementary mechanisms, including elevated phagocytic activity and antioxidant enzyme levels, and aligns with reduced clinical signs, lower bacterial loads, and improved immune parameters.

Overall, these findings underscore the efficacy of combined phytogetic additives as sustainable tools for disease mitigation in aquaculture.

Re-isolation of *A. hydrophila* from the liver, kidney, and spleen markedly reduced the bacterial loads in all the treated groups, especially the NI + CA group. In contrast, the CNT-treated fish exhibited heavy organ colonization, confirming pathogen virulence and host susceptibility. The antimicrobial effects of NI and CA are likely mediated by bioactive compounds such as gallic acid, cinnamic acid, and naringenin, which disrupt bacterial membranes, inhibit efflux pumps, and suppress virulence genes (Tiji et al. 2022). The broad-spectrum activity of CA, reflected by the 20% recovery rate, stems from membrane destabilization and ATP synthesis inhibition.

The NI + CA group presented the greatest degree of protection, with no bacterial growth in the liver or kidney and minimal spleen colonization. This finding aligns with prior evidence that synergistic phytogetic combinations enhance antimicrobial efficacy (Rattanachaikunsopon & Phumkhachorn 2010). Although thymoquinone was not chromatographically detected, its potential contribution to NI activity may explain the observed synergy. These results support the use of phytogetic combinations as effective, sustainable alternatives to antibiotics in aquaculture.

The bioactivity, safety, and sustainability of medicinal plant-based feed additives are increasingly valued in aquaculture. While phytoGENICS such as garlic, turmeric, ginger, and green tea offer benefits in terms of growth, immunity, or antioxidant capacity, the NI + CA combination delivers broader, multitarget effects with superior efficacy in *O. niloticus*. Unlike single-function additives, NI + CA enhances multiple physiological systems. NI contains chlorogenic acid, rutin, gallic acid, and catechin, which support antioxidant defense, erythropoiesis, and immune cell proliferation. CA, a phenolic monoterpene from oregano and thyme, has potent antimicrobial, anti-inflammatory, and hepatoprotective effects.

The key advantages of NI + CA over individual phytoGENICS include the following:

- Superior growth performance was observed, with the highest SGR and lowest FCR, indicating efficient nutrient utilization.
- Enhanced immunity is reflected by increased phagocytic activity, TIg, and lymphocyte counts.
- Robust antioxidant defenses, with elevated SOD and GST and reduced LPO, surpass typical single-additive outcomes.
- Strong resistance to *A. hydrophila*, as evidenced by reduced clinical signs, minimal organ colonization, and low bacterial recovery, is rarely associated with other phytoGENICS.

Importantly, NI and CA are effective at low inclusion levels (1% and 0.1%, respectively), unlike turmeric or ginger, which often require higher doses or solvent extraction. This enhances their practicality and cost efficiency in commercial aquafeeds.

The synergistic efficacy of NI + CA likely stems from complementary actions: NI modulates systemic antioxidant and immune responses, whereas CA delivers targeted antimicrobial and hepatoprotective effects. Together, these strains confer greater resilience and disease resistance than either strain alone.

Conclusion

Dietary supplementation with NI, CA, or their combination (NI+CA) significantly improved growth, antioxidant status, immune function, and resistance to *A. hydrophila* in *O. niloticus*. The NI+CA combination was the most effective, showing synergistic benefits in terms of digestive efficiency, metabolic balance, hepatic function, and innate immunity. These results highlight the potential of NI and CA, especially together, as phytogetic feed additives that increase disease resistance and productivity in tilapia aquaculture. Their use offers a natural alternative to synthetic growth promoters and antibiotics, supporting the move toward safer, eco-friendly aquafeeds. In summary, combining NI and CA offers a comprehensive strategy to increase fish health and performance, emphasizing the importance of selecting phytoGENICS on the basis of complementary biochemical profiles and synergistic effects.

Study limitations and future recommendations

While the present study demonstrated the promising effects of NI and CA supplementation on the health and performance of *O. niloticus*, several limitations should be acknowledged. The experiment was conducted under controlled laboratory conditions, which may not fully reflect the complexities of commercial aquaculture environments, where stressors and pathogen exposure are variable. Additionally, the present study focused on a single species, limiting the generalizability of the results. Future research should validate these findings in other fish species to assess their broader applicability.

Another limitation is the testing of only one dosage level for each additive, leaving the dose–response relationship and potential toxicity thresholds unexplored. Future studies should investigate a range of dosages to optimize the benefits of both NI and CA in fish feeds. Moreover, the long-term effects of continuous administration, potential interactions with other dietary ingredients, and the economic feasibility of large-scale application were not assessed. Long-term feeding trials under commercial aquaculture conditions would provide valuable insights into the practical effectiveness of NI+CA supplementation. While the phytochemical profile of NI has been characterized, the bioavailability and metabolism of individual compounds in fish tissues remain unknown. Future research should explore the pharmacokinetics of these compounds, including potential residual effects and their safety for human consumption.

In summary, the combined use of NI and CA offers a promising strategy for enhancing fish health and performance. To build on these findings, future studies should focus on determining optimal dosage levels, exploring the synergistic mechanisms of action, and conducting trials under real-world aquaculture conditions. Additionally, evaluating the effects on other aquaculture species and investigating their pharmacokinetics will be crucial for understanding their full potential and ensuring safe, sustainable applications in the aquaculture industry.

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Author's contribution Nagwa I.S. Abu Zahra: Methodology, Formal analysis, Writing—original draft, Formulation of overarching research goals and aims, Writing—Review & editing, Resources, Supervision, Investigation, Visualization, Shaimaa Elbaz: Ideas, Writing—original draft, Formulation of overarching research goals and aims, Writing—Review, Shireen Soliman: Resources, Investigation, Visualization, Validation, Writing – Review, Amany M. Ghoniem: Ideas, Formulation of overarching research goals and aims, Project administration, Writing – Review.

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Data availability The raw data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Animal welfare and ethics statement The experimental methodology, protocols, and animal care used in the present study all followed the relevant guidelines and regulations of the Animal Health Research Institute, Agriculture Research Center, Giza, Egypt (Code Number: ARC/AHRI 83429/2024), and all procedures were carried out in accordance with the relevant guidelines and regulations of the European Union directive 2010/63UE. The results of this study are in accordance with the ARRIVE guidelines (<https://arriveguidelines.org>). None of the authors' investigations involving human subjects are included in this paper.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest disclosure The authors declare that they have no competing financial interests or personal relationships that could influence the work reported in this paper.

Competing interests The authors declare no competing interests.

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