



Impacts of *Cinnamomum tamala* leaf extract on growth, skin mucosal and serum immunity, antioxidant activity, and expression of cytokine genes in *Cyprinus carpio* fingerlings

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Abstract This study aimed to assess the effect of *Cinnamomum tamala* leaf-extract (CTE) on the growth, immune response, antioxidant status, gene expression, and disease resistance of *Cyprinus carpio*. Over a 70-day period, fish (8.82 ± 0.04 g) were fed diets supplemented with graded levels of CTE (mg/kg): 0 (Control), 200

(CTE2), 300 (CTE3), 400 (CTE4), 500 (CTE5), and 600 (CTE6). Cytokine gene expression was studied 3 days post-challenge with *A. hydrophila*. CTE at 400–600 mg/kg maximized the growth performance. Key immune parameters, such as total protein, lysozyme, ACH_{50} , alkaline phosphatase, skin mucosal protease activity, serum phagocytosis, and peroxidase levels were enhanced ($P < 0.05$) in CTE4 and/or CTE5 than in the control. Dietary CTE did not influence serum glucose, cortisol, creatinine, urea, total immunoglobulin, or liver metabolic enzyme levels. Intestinal amylase activity, and serum antioxidant levels were higher in CTE4–CTE6; whereas, serum MDA level was decreased in CTE5 ($P < 0.05$). The 15-day relative post-challenge survival was highest in CTE5 (54.76%). The transcript levels of *sod*, *cat*, and *nrf2* were strongly upregulated in the liver tissues of CTE4 and CTE5; whereas *keap1* transcription was downregulated in CTE5. The expression of *tnf- α* , *il-1 β* , and *hsp70* was down-regulated in CTE4 and CTE5. Conversely, *il-10* expression was significantly upregulated in liver tissues of CTE4. The expression of *occludin*, *occludin 7*, and *zo-1* were significantly upregulated in the intestinal tissues of CTE4 and CTE5. Overall, dietary CTE at appropriate level can enhance growth performance, immunity, and disease resistance in *C. carpio*.

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Highlights

- *C. tamala* leaf extract (CTE) at 400–500 mg/kg improved growth performance of *Cyprinus carpio*.
- Dietary CTE at 400–500 mg/kg modulated the skin mucosal immunity of fish.

- CTE enhanced the resistance against *Aeromonas hydrophila* challenge.
- CTE at 400–500 mg/kg enhanced serum antioxidant enzymes.

Keywords *Cinnamomum tamala* leaf-extract · Common carp · *Aeromonas hydrophila* · Health · Tight-junction proteins · Oxidative stress

Introduction

Fish is a key source of protein for human nutrition. Over the past two decades, intensive and semi-intensive aquaculture systems have rapidly developed to satisfy the protein requirements of the human diet (Bilen et al. 2021). Fish health is influenced by the water quality, handling practices, stocking density, disease prevalence, and nutritional management. However, intensive aquaculture results in high mortality due to increased disease resistance (Olivia-Teles 2012). Bacterial infections are among the most prevalent diseases in aquaculture. *Aeromonas hydrophila* is a well-known opportunistic bacterium that infects almost all freshwater fish, causing substantial mortality and financial losses (Semwal et al. 2023). Antibiotics are widely used in aquaculture to prevent and cure fish illnesses. However, excessive antibiotic use can result in environmental risks and antibiotic resistance (Siddik et al. 2025). Fish and human health may be negatively impacted by therapeutic drugs (Bilen et al. 2021). Therefore, natural, inexpensive, easily accessible, and ecologically friendly additives can prevent and treat these illnesses. One way to strengthen fish immunity against diseases is to improve their general wellbeing and non-specific immune system (Yousefi et al. 2025, Lin et al. 2026, Shehata et al. 2025, Giri et al. 2019, Van Doan et al. 2020, Yilmaz et al. 2024, Gupta et al. 2023). Herbal medicines are extensively used for the prevention and control of aquatic diseases because of their bioactive compounds and reduced susceptibility to drug resistance (Lin et al. 2026). Reduced expression of pro-inflammatory cytokines, enhanced antioxidant activity and immunological responses, and improved intestinal barrier integrity are some of the ways in which herbal components protect fish from disease (Lin et al. 2026).

Commonly referred to as Indian bay leaf ('Tejpatta'), *Cinnamomum tamala* belongs to the Lauraceae family.

It is a valuable plant species because of its medicinal and aromatic properties. This tree is primarily found in subtropical and temperate forests of India, Nepal, and Bhutan (Kumar et al. 2025). *C. tamala* (CT) is a key ingredient in conventional Ayurvedic medicine and is used as a remedy for heart problems, diarrhea, vomiting, nausea, and colic. CT leaves have insecticidal and antifungal properties (Kumar et al. 2025). Several bioactive constituents have been identified in the leaves of CT, including furanogermentone, β -caryophyllene, curcumenol, curzerenone, and furanodiene. The leaves contain cinnamaldehyde, eugenol, α -linalool, carophyllene oxide, benzaldehyde, acetophenone, cinnamyl alcohol, 2-methyl benzofuran, pinocarpone, tepinen-4-ol, and niroldiol (Paul et al. 2025). CT leaves have potent therapeutic effects against cancer, inflammation, and cardioprotective and neurological illnesses (Husain et al. 2025). The presence of phenolic, hydroxyl, and carbonyl compounds has been confirmed in methanolic extracts of CT leaves (Chowdhury et al. 2026). Methanolic CT extract has strong in vitro antioxidant and antimicrobial properties (Chowdhury et al. 2026).

The aqueous extract of another kind of bay leaves (i.e. *Laurus nobilis*) was tested for its effectiveness as a feed additive in Nile tilapia culture. Dietary administration of 300–400 mg/kg *L. nobilis* aqueous extract improved intestinal and liver health and metabolic stability, reduced oxidative stress, and modulated inflammation and growth-related gene expression in Nile tilapia (Shehata et al. 2025). However, evidence of the effects of *C. tamala* extract on growth performance, antioxidant activities, skin mucosal immune responses, disease resistance, and cytokine gene expression in fish is scarce. The present study was conducted to evaluate the effects of *C. tamala* extract (CTE) on the growth performance, digestive enzymatic activities, antioxidant responses, skin mucosal immune responses, and cytokine gene expression in *Cyprinus carpio*. To our knowledge, this is the first study to investigate the effects of CTE on skin mucosal immune responses, immune function, and resistance to *A. hydrophila* challenge in *C. carpio*.

Materials and methods

Preparation of *Cinnamomum tamala* extract (CTE)

Dry *C. tamala* leaves were purchased from the market. The leaves were cleaned with tap and distilled

water to remove impurities. The leaves were dried at temperature between 25 and 40 °C and then placed in a hot-air oven for several hours. A blender was used to grind the dried leaves into a fine powder. In conical flasks, 100 g of the plant was dissolved in 300 mL of a solvent (1:1 (v/v) water: methanol). The flasks were placed in a water bath with a thermostat and shaken for 24 h after being wrapped in aluminum foil (Giri et al. 2026). A Whitman No. 1 filter paper was used to filter the mixture. The extract was lyophilized and stored at − 18 °C for further study.

Preliminary phytochemical screening of CTE

The presence of alkaloids, saponins, tannins, glycosides, steroids, phenolic compounds, terpenoids, carbohydrates, and flavonoids in CTE was screened as described previously (Bharadwaj et al. 2022; Farhana et al. 2023).

Diet preparation

The basal diet containing 30.97% protein, 9.03% lipid, and 10.22% ash was prepared as previously described (Giri et al. 2023). The composition of basal diet is shown in Table S1. The basal diet was supplemented with CTE as follows: control (0- basal diet), CTE2 (200 mg kg^{−1}), CTE3 (300 mg kg^{−1}), CTE4 (400 mg kg^{−1}), CTE5 (500 mg kg^{−1}), and CTE6 (600 mg kg^{−1}). The ingredients were mixed thoroughly, pelleted (~2 mm diameter), and air-dried in an oven (60 °C) until the moisture level was <10%. Prepared feeds were packed in

appropriate containers, labelled, and stored at − 4 °C until further use.

Experimental setup

Healthy *C. carpio* fingerlings (average weight: 8.82 ± 0.04 g) were acclimatized for 2 weeks. During the acclimatization period, the fish were fed a basal diet. Subsequently, 360 fish were randomly distributed among 18 fiberglass tanks. Each group consisted of 60 fish with three tanks × 20 fish in each tank. The tank capacity was 200 L, and the tanks were properly aerated using air stones connected to compressed air pumps. The feeding trial was continued for 10 weeks. The fish were fed to satiation twice daily with the corresponding experimental diets. Key parameters were maintained: dissolved oxygen at 6.3–7.0 mg L^{−1}, ammonia concentrations at 0.04–0.07 mg L^{−1}, and pH ranged from 7.2–7.7. All the experiments complied with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines and were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Growth performance

After the feeding trial, the fish were fasted for 24 h. Fifteen fish from each tank were counted and weighed to evaluate their growth performance. Growth metrics including percent weight gain (PWG), specific growth rate (SGR), feed conversion ratio (FCR), and survival rate (%) were measured as outlined by Giri et al (2019).

$$\text{PWG} = [(W_t - W_0)/W_0] \times 100.$$

$$\text{SGR}(\% \text{ day}^{-1}) = [(\ln W_t - \ln W_0)/t] \times 100.$$

$$\text{FCR} = [\text{total dry feed intake (g)} / \text{wet weight gain (g)}].$$

$$\text{Survival rate (\%)} = [(\text{final number of fish} / \text{initial number of fish}) \times 100].$$

Where, W_t and W_0 are the final and initial weights (g) of the fish, respectively, and 't' is the duration of feeding (in days).

Sample collection

Five fish from each tank were anesthetized with MS222 (100 mg L^{−1}) (Merck, USA) and placed in polythene

bags containing 10 mL of 50 mM NaCl. The fish were gently rubbed inside the bag for 2 min. Then the solution from each group were pooled separately, homogenized (4 °C), and centrifuged at 1500 × g for 10 min at 4 °C. The supernatants were stored at − 20 °C.

Blood was drawn from the caudal vein with three fish per tank using a 1 mL syringe. Blood was poured into a 1.5 mL Eppendorf tube and incubated

at room temperature for 1 h and at 4 °C for 4 h. It was then centrifuged at 10,000 g for 5 min at 4 °C. Serum was collected and stored at −80 °C for further analysis. After blood collection, the same fish were dissected aseptically to extract the liver and intestinal tissues. Individual tissue samples were placed in 1.5 mL tubes with TRIZOL reagent (Invitrogen, Waltham, MA, USA) and stored at −80 °C.

Skin mucosal immunity parameters

Lysozyme activity (LA) of skin mucus was determined using a turbidimetric method with a commercial assay kit (Sigma-Aldrich, USA). Mucus alkaline phosphatase (ALP) levels were determined using a commercial kit (Sigma-Aldrich, USA). The total immunoglobulin (Ig) level in skin mucus was measured using a described previously (Hoseinifar et al. 2017). Skin mucosal peroxidase and protease activities were measured as previously described (Guardiola et al. 2016). The alternative complement pathway (ACP) was determined as previously described (Van Doan et al. 2020).

Haemato-immunological parameters

Serum glucose, total protein, and albumin levels were estimated using a diagnostic kit (Merck, Rahway, NJ, USA), following the manufacturer's instructions. Serum cortisol levels were determined using a commercial kit (Zellbio Co., Germany). Serum immune parameters, including lysozyme, total Ig, and ACP were measured using commercial kits as described in the previous section. The percentage (PA; %) of head kidney macrophages was determined as described by Geng et al. (2012). The number of phagocytic cells per 100 adherent cells was determined under a microscope. The PA was calculated using the following formula: $PA = (\text{phagocytic leukocytes} / \text{total leukocytes}) \times 100$. Peroxidase and protease activities were measured according to the procedure described earlier (Guardiola et al. 2016). The activity was expressed as units mL^{-1} and unit/mg protein, respectively.

Serum antioxidant and intestinal digestive enzymes

Serum antioxidant enzyme parameters, including glutathione peroxidase (GPx), catalase (CAT), superoxide dismutase (SOD), and malondialdehyde (MDA) levels, were assessed using commercial kits (Jiancheng Bioengineering Ltd., Nanjing, China) according to the manufacturer's instructions.

After homogenizing the intestinal samples, they were centrifuged for 20 min at 4 °C (25,000×g). Following the manufacturer's instructions, lipase, amylase, and protease activities were measured using commercial kits (Sigma-Aldrich, USA).

Liver metabolic enzymes and kidney function biomarkers

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) activities were measured using commercial kits (Jiancheng Bioengineering Ltd., Nanjing, China) following the guidelines provided for commercial kits. The enzyme activity was expressed as U mL^{-1} . Serum activities of kidney function enzymes, that is, creatinine and urea, were measured using commercial kits (Sigma-Aldrich, USA).

Challenge study

Fifteen fish from each tank ($15 \times 3 = 45$ fish per group) were injected with PBS (100 μL) containing 1×10^7 live *A. hydrophila* ATCC7966 as described previously (Giri et al. 2019). Another group of fish, fed on basal diet for 10 weeks were challenged with only 100 μL PBS and denoted as the negative control (NC). The challenged fish were fed the basal diet. The cause of death and pathological changes were confirmed by re-isolating the pathogen from dead or moribund fish, and a PCR test was performed to confirm the bacterial isolates. Mortality was recorded daily in each group over 15 days.

Mortality rates (%) and relative survival percentages (RPS, %) were calculated using the following equation:

Mortality rate (%) = (no. of dead fish
/total number of fish of the challenged fish) × 100.

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

Gene expression analysis

One week after the challenge experiment, two fish from each tank were dissected aseptically to collect the liver and intestinal tissues. Total cellular RNA was extracted from the tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the standard protocol. Liver tissue was used for the expression of *sod*, *cat*, *nrf2*, *tnf-α*, *il-1β*, *il-10*, *keap1*, and *hsp70*. Meanwhile, intestinal tissue was used for the expression *occludin*, *occludin 7*, and *zo-1*. The purity of the RNA was checked, and the concentration was determined as described previously (Giri et al. 2023). RNA was reverse-transcribed to Complementary DNA (cDNA) using the cDNA synthesis kit (Life Technologies, Carlsbad, CA, USA).

The qRT-PCR was performed using a CFX96 Thermal Cycler (Bio-Rad). The primers and thermocycling conditions used are listed in Table S2. A total of 25 µL reaction mixture was prepared using 12.5 µL 2×SYBR Green RT-PCR mater Mix (TAKARA, JAPAN), 0.5 µL (10 µM) primer, 1 µL diluted cDNA template, and 10.5 µL dH₂O. By comparing the expression differences and stability analysis of several housekeeping genes (*18S rRNA*, *β-actin*, and *gapdh*), *β-actin* was selected as an internal control gene. Reactions for all genes were performed in triplicates. After amplification, a melting curve analysis was performed to verify the accuracy of each amplicon. The expression levels were analyzed using the $2^{-\Delta\Delta C_t}$

value, calculated using the comparative threshold cycle method (Livak and Schmittgen 2001).

Statistical analysis

Data were analyzed using Analysis of Variance (ANOVA), and multiple comparisons of differences between experimental groups were performed with Tukey's test using OriginPro software (version 8; OriginLab Corporation, Northampton, MA, USA). The significance level ($p < 0.05$) of the results was expressed as the mean ± standard error of the mean (SEM). Regression analysis of selected parameters was performed to find optimal CTE level.

Results

Phytochemical analysis

The phytochemical analyses indicated the presence of carbohydrates, tannins, glycosides, phenolic compounds, terpenoids, and flavonoids (Table S3).

Growth performance

The growth performance of the carp fed the CTE diet is shown in Table 1. The FBW was significantly higher in the CTE3 – CTE6 than in the control group, with the highest in CTE5 (55.54 ± 0.46 g). PWG

Table 1 Growth performance of *C. carpio* after a 10-week feeding with 0–600 mg/Kg CTE

Parameters	Control	CTE2	CTE3	CTE4	CTE5	CTE6
Initial weight (g)	8.95±0.02	8.9±0.03	8.86±0.01	9.01±0.05	8.98±0.02	8.93±0.03
FBW (g)	41.62±0.62 ^a	43.13±0.27 ^a	46.05±0.58 ^b	53.2±0.34 ^{cd}	55.54±0.46 ^c	51.16±0.37 ^d
PWG (%)	365.01±6.51 ^a	384.34±4.04 ^a	419.74±6.31 ^b	490.55±4.23 ^c	518.47±6.69 ^d	472.93±5.98 ^c
SGR (% day ⁻¹)	2.19±0.06 ^a	2.25±0.03 ^a	2.35±0.01 ^b	2.52±0.08 ^c	2.6±0.07 ^d	2.48±0.13 ^c
FCR	1.72±0.05 ^a	1.59±0.07 ^{ab}	1.47±0.09 ^{ab}	1.38±0.06 ^b	1.35±0.03 ^b	1.44±0.07 ^b
Survival (%)	100	100	100	100	100	100

Values are mean±S.E.M of three replicates (45 fish per group). Means with different superscript letters within a row indicate significant differences ($P < 0.05$)

Table 2 Skin mucosal immune activities of the *C. carpio* fed with diets enriched with different levels of CTE for a 10-week trial regime

Parameters	Control	CTE2	CTE3	CTE4	CTE5	CTE6
Lysozyme (U/mL)	12.17±0.36 ^a	13.62±0.33 ^{ac}	13.98±0.23 ^{ac}	17.1±0.69 ^b	16.84±0.54 ^{bc}	14.68±0.47 ^c
Protease activity (U/mg Protein)	6.41±0.27 ^a	7.04±0.20 ^a	7.48±0.23 ^{ab}	8.66±0.15 ^{bc}	8.92±0.21 ^c	7.34±0.40 ^a
ACH ₅₀ (U/mL)	107.82±2.51 ^a	109.03±2.96 ^{ab}	116.24±1.93 ^{ab}	119.82±3.8 ^{ab}	123.07±3.25 ^c	118.53±2.32 ^{ab}
ALP (U/mL)	43.28±1.83 ^a	44.91±1.14 ^{ad}	47.03±1.68 ^{ab}	48.72±1.37 ^{bc}	51.13±1.46 ^c	48.39±1.26 ^{bcd}
Total Ig (mg/mL)	3.97±0.17 ^a	4.38±0.12 ^a	4.51±0.26 ^a	4.63±0.13 ^a	4.42±0.22 ^a	4.13±0.09 ^a
Peroxidase activity (µg/mL)	0.63±0.04 ^a	0.68±0.03 ^{ac}	0.74±0.07 ^{ac}	0.91±0.06 ^b	0.93±0.03 ^b	0.79±0.04 ^{bc}

Values are presented as mean±S.E.M of three replicates (15 fish per group). Means with different superscript letters within a row indicate significant differences ($P < 0.05$). Means with same superscript letters within a row indicate no significant difference

values were significantly higher in CTE3–CTE6, with the highest in CTE5. The SGR value was also significantly higher in CTE3–CTE6 than in control, with highest in CTE5. FCR values were significantly lower in the CTE4–CTE6 groups than in the control group, with lowest in CTE5.

Skin mucosal immune responses

The skin mucosal immune activities are shown in Table 2. Lysozyme and ALP activities were higher ($P < 0.05$) in CTE4–CTE6, with the highest activities observed in CTE4 and CTE5, respectively. Dietary inclusion of CTE significantly enhanced skin mucosal ACH₅₀ activity only in the CTE5 compared to the control. Protease activities were higher ($P < 0.05$) in CTE4 and CTE5 than in the control. CTE administration had no significant effect on skin mucosal immunoglobulin levels. The mucus peroxidase activities

increased ($P < 0.05$) in CTE4–CTE6, with highest in CTE5 (0.93 ± 0.03 µg/mL).

Haemato-immunological responses

The effects of CTE on haemato-immunological indices are shown in Table 3. Dietary CTE had no significant influence on serum glucose, albumin, protease, cortisol, or total Ig levels. However, supplementation with 400–600 mg/kg CTE led to a significant increase in serum total protein and lysozyme levels, compared to the control. Globulin level was significantly higher in CTE4 and CTE5, compared to the control. Serum complement level (ACH₅₀) was significantly higher only in CTE5 (152.13 ± 2.44 U/mL) than in the control. Phagocytic activities were significantly higher in CTE3–CTE6, with the highest in the CTE4 ($28.91 \pm 1.16\%$). Peroxidase activity markedly augmented ($P < 0.05$) in CTE4 and CTE5, with the highest CTE5 (0.32 ± 0.02 µg/mL).

Table 3 Haemato-immunological indicators of the *C. carpio* fed with diets supplemented with different levels of CTE for 10 weeks

Parameters	Control	CTE2	CTE3	CTE4	CTE5	CTE6
Total protein (g/dL)	1.87±0.03 ^a	1.89±0.06 ^a	1.94±0.04 ^{ac}	2.23±0.05 ^{bc}	2.36±0.04 ^b	2.11±0.02 ^c
Albumin (g/dL)	0.69±0.02 ^a	0.73±0.01 ^a	0.81±0.05 ^a	0.83±0.02 ^a	0.78±0.02 ^a	0.81±0.03 ^a
Globulin (g/dL)	1.18±0.01 ^a	1.16±0.05 ^a	1.13±0.04 ^a	1.39±0.02 ^{bc}	1.58±0.01 ^c	1.3±0.02 ^{ac}
Serum glucose (mg/dL)	77.38±1.7 ^a	75.18±0.57 ^a	76.22±1.52 ^a	78.6±1.93 ^a	77.09±1.07 ^a	79.63±1.37 ^a
Cortisol (ng/mL)	64.72±3.53 ^a	65.24±2.17 ^a	63.98±1.84 ^a	65.84±2.24 ^a	66.47±3.13 ^a	67.11±2.83 ^a
Lysozyme (U/mL)	167.72±3.8 ^a	171.28±1.47 ^a	178.06±2.69 ^{ab}	191.46±3.67 ^{bc}	203.18±3.31 ^c	186.91±2.85 ^b
Protease activity (U/mg Protein)	8.49±0.61 ^a	9.13±0.37 ^a	9.28±0.42 ^a	9.71±0.26 ^a	10.59±0.63 ^a	10.34±0.54 ^a
Total Ig (mg/mL)	11.97±1.16 ^a	12.26±0.84 ^a	12.82±0.98 ^a	14.69±1.26 ^a	15.07±0.69 ^a	13.79±0.88 ^a
ACH ₅₀ (U/mL)	137.6±3.06 ^a	140.28±4.48 ^{ab}	142.04±2.33 ^{ab}	148.77±4.1 ^{ab}	152.13±2.44 ^b	144.16±1.81 ^{ab}
Phagocytic activity (%)	19.64±0.65 ^a	20.43±0.97 ^{ab}	24.13±0.7 ^{bd}	28.91±1.16 ^c	28.76±0.56 ^c	26.52±1.09 ^{cd}
Peroxidase activity (µg/mL)		0.21±0.02 ^{ab}	0.23±0.04 ^{ab}	0.28±0.01 ^{bc}	0.32±0.02 ^c	0.25±0.01 ^{ac}

Data are presented as mean±S.E.M of three replicates (9 fish per group). Different superscript letters within a row indicate significant differences ($P < 0.05$). Means with same superscript letters within a row indicate no significant difference

Table 4 Effects of dietary supplementation with CTE for 10 weeks on liver metabolic enzymes and kidney function biomarkers of carp. Data are presented as mean±S.E.M ($n = 3$). Means with same superscript letters within a column indicates no significant difference ($P < 0.05$)

Experimental groups	Liver metabolic enzymes			Kidney function biomarkers	
	ALT (U/mL)	AST (U/mL)	ALP (U/mL)	Creatinine (mg/dL)	Urea (mg/dL)
Control	5.81±0.38 ^a	43.29±0.8 ^a	14.7±0.32 ^a	0.33±0.02 ^a	7.29±0.38 ^a
CTE2	5.7±0.41 ^a	44.13±1.28 ^a	14.91±0.42 ^a	0.35±0.02 ^a	7.13±0.24 ^a
CTE3	5.62±0.3 ^a	42.79±1.73 ^a	16.18±0.5 ^a	0.32±0.03 ^a	6.88±0.48 ^a
CTE4	5.07±0.33 ^a	40.86±0.67 ^a	15.06±0.63 ^a	0.30±0.04 ^a	7.02±0.33 ^a
CTE5	4.94±0.2 ^a	41.77±0.5 ^a	13.87±0.57 ^a	0.34±0.05 ^a	6.71±0.11 ^a
CTE6	6.11±0.26 ^a	44.21±0.53 ^a	16.49±0.48 ^a	0.33±0.03 ^a	6.96±0.36 ^a

Liver metabolic enzymes and kidney function biomarkers

Dietary administration of CTE had no significant effect on the activities of liver function enzymes, that is serum ALT, AST, and ALP (Table 4). Also, dietary CTE had no significant effect on serum creatinine or urea levels.

Digestive enzyme activities

The effects of CTE on intestinal enzyme activity are shown in Fig. 1. Dietary CTE did not influence the intestinal lipase or protease activities. However, amylase activity was significantly enhanced in CTE4–, CTE5–, and CTE6 compared to that in the control or CTE2, with the highest activity was recorded in CTE5.

Serum oxidative stress

Dietary CTE affected the serum antioxidant activity of carp (Fig. 2). Serum SOD and CAT activities were significantly elevated in CTE4– CTE6, with the strongest activity observed in CTE5. However, dietary CTE had no considerable effect on serum GPx levels. The MDA levels were lower in the CTE-fed groups, but the difference was significant in the CTE5 only than in the control.

Challenge study

Mortality (%), survival (%), and RPS of fish challenged with pathogenic *A. hydrophila* and mortality was recorded for 15 days are presented in Table 5. The highest mortality (93.33%) was recorded in the control group. Infected patients show symptoms of hemorrhagic septicemia, followed by mortality. The

highest RPS (54.76) was recorded for CTE5, followed by CTE4 (42.85), and CTE6 (35.71). No mortality was observed in negative control group.

Gene expression analysis

CTE supplementation was beneficial for upregulating the transcriptional expression of antioxidant-related genes in the liver tissues of challenged fish (Fig. 3). Compared with the control group, dietary administration of 300–600 mg/kg CTE showed varying degrees of upregulation in the transcription levels of *sod*, *cat*, and *nrf2* in the liver. A significantly higher level of transcription was recorded in the CTE3 – CTE6 than in the control, except for *sod* in CTE6. The highest levels of transcriptional expression of *sod*, *cat*, and *nrf2* were recorded in the CTE5. *A. hydrophila* infection significantly upregulated the expression of *nrf2* in the control group than in the NC. However, 70-day pre-treatment with CTE at 300–600 mg/kg ameliorated the expression of *keap1*, and the expression level was significantly lower compared to control group.

Challenge with *A. hydrophila* significantly upregulated the expression of *il-1 β* , *tnf- α* , *hsp70* in the pathogen-aggravated control group than in the NC (Fig. 4). However, supplementation of CTE attenuated the expression of *il-1 β* , *tnf- α* , and *hsp70* in liver tissues of infected fish in a dose dependent manner. A considerably lower expression ($P < 0.05$) of *il-1 β* , *tnf- α* , *hsp70* was recorded in CTE3–CTE6, except *hsp70* expression in CTE3 or CTE6, compared to the control. Expression of anti-inflammatory factor *il-10* was higher in the CTE groups, but the difference was significant in CTE4 and CTE5 than in the control (Fig. 4).

The expression of tight junction (TJ)-related genes (*occludin*, *zo-1*, and *occludin 7*) is shown in Fig. 5.

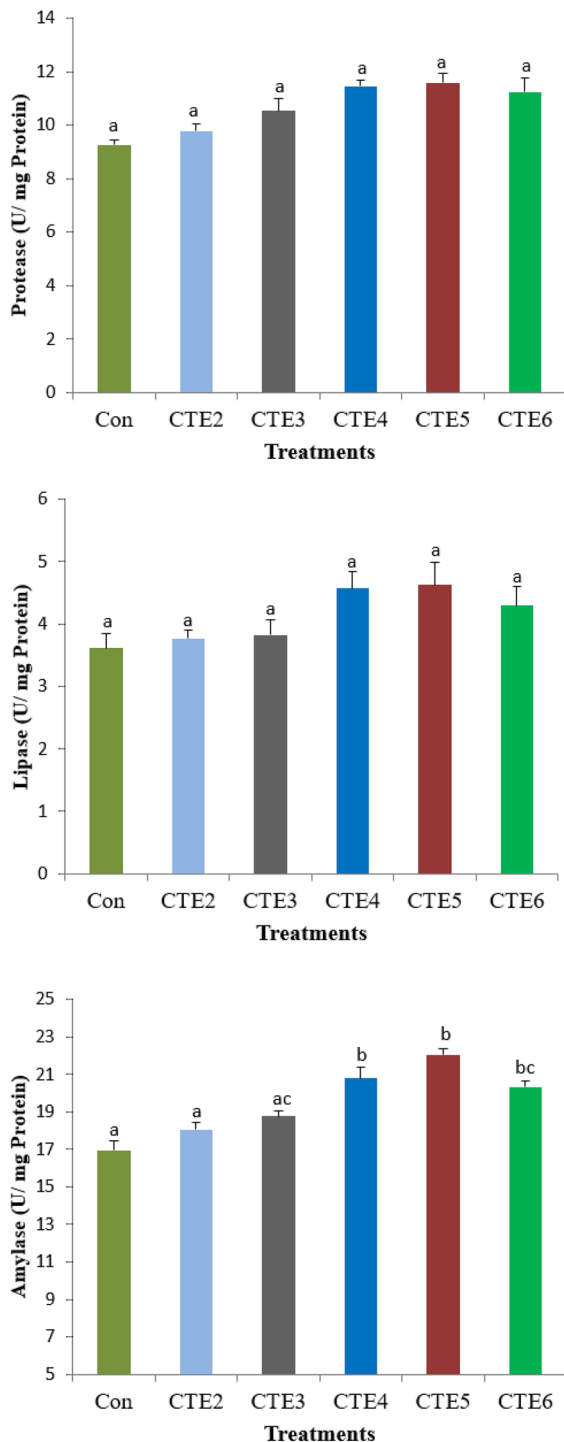


Fig. 1 Intestinal digestive enzyme activity of *Cyprinus carpio* after a 70-day of feeding with 0–600 mg/Kg CTE. Different letters above the bars indicate significant differences among treatments ($n=3$; $P<0.05$)

Compared to the NC group, significantly lower expression of *occludin*, *zo-1*, and *occludin 7* was observed in the intestines of pathogen-infected fish, that is, the control group. However, treatment with 400–600 mg/kg CTE variably enhanced ($P<0.05$) the transcription of *occludin*, and *zo-1* in the intestine of pathogen-infected fish compared to the NC or control group. Expression of *occludin7* was significantly higher in the CTE4 and CTE5 than in the control. Although, those differences were not significant compared to the NC.

Regression analysis

The results of regression analysis of selected parameters are shown in Fig. S1. Results revealed the optimal value of CTE as 538.75, 506.16, 516.16, mg/kg for FCR, SOD, and MDA, respectively. Accordingly, CTE at 506.16–538.75 mg/kg can be supplemented to carp diet.

Discussion

Phytobiotics favorably modulate immune responses in fish when added to aquaculture feeds at optimal concentrations. Phytochemicals and polyphenols in phytobiotics provide various health benefits for fish (Yilmaz et al. 2023). Dietary administration of various plant leaf extracts, for example, walnut, papaya, bay leaf, medlar, fern, and marjoram, has beneficial immunoboosting properties in cultured fish (Shehata et al. 2025; Yilmaz et al. 2023; Giri et al. 2026; Hoseinifar et al. 2017, 2020; Yousefi et al. 2021). The inclusion of CTE at 400–600 mg/kg significantly modulated the growth performance of fish in terms of FWG, PWG, SGR, and FCR. Similarly, the dietary administration of papaya leaf extract (PLE) at 2 g/kg for 60 days (Giri et al. 2026), medlar leaf extract (MLE) at 5.0 g/kg for 49 days (Hoseinifar et al. 2017), and *Cistus laurifolius* ethanolic extract (CLE) at 1.0 g/kg for 45 days (Bilen et al. 2021) to *C. carpio* resulted in better growth performance. These variations in duration may be correlated with factors, such as the herbal extract used, including the dose, type, or form, and the duration of feeding (Yilmaz et al. 2023). Methanolic CTE contains high amounts of phenolic compounds, which may explain

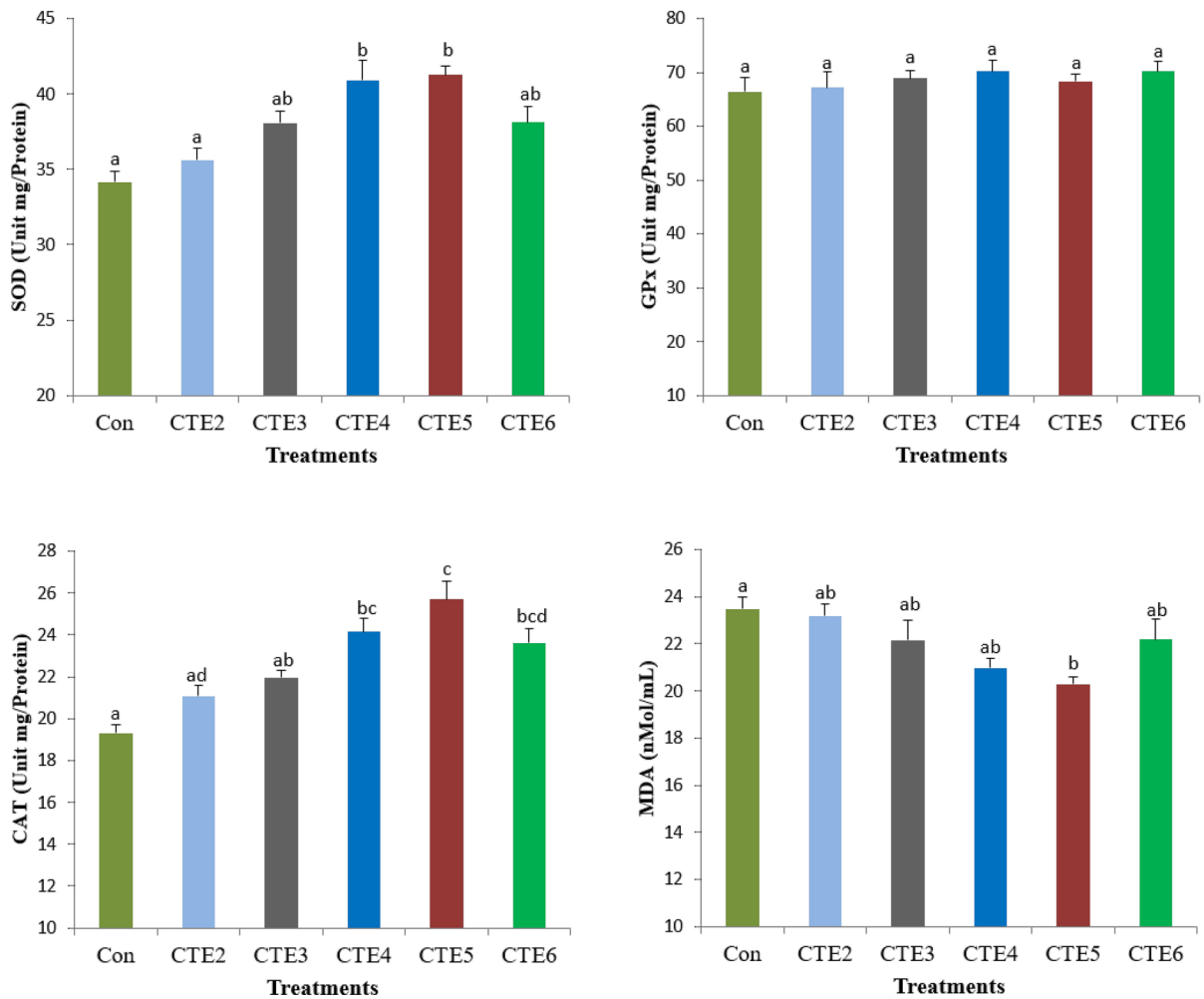


Fig. 2 Serum oxidative stress-related indices of carp fed CTE-supplemented diets for 70 days. Different letters in the bar graph represent significant differences ($P < 0.05$). Values are presented as mean $\pm$ S.E.M ($n = 3$)

Table 5 Survival percentage (%), mortality percentage (%), and relative percent survival (RPS) of fish fed diets enriched with CTE for 10 weeks and then challenged with *A. hydrophila* infection and observed for 15 days

Experimental group	Number of fish of death challenged	Number of death	Mortality Percent (%)	Survival Percent (%)	RPS
Control	45	42	93.33	6.67	–
Negative Control (NC)	45	0	0	100	–
CTE2	45	37	82.22	17.78	11.90
CTE3	45	32	71.11	28.89	23.80
CTE4	45	24	53.33	46.67	42.85
CTE5	45	19	42.22	57.78	54.76
CTE6	45	27	60.00	40.00	35.71

the observed growth performance (Chowdhury et al. 2026). The increased levels of intestinal digestive enzymes may have contributed to higher growth

performance, indicating improved nutritional digestion. Digestive enzyme activities are crucial for the hydrolysis of proteins, lipids, and carbohydrates and

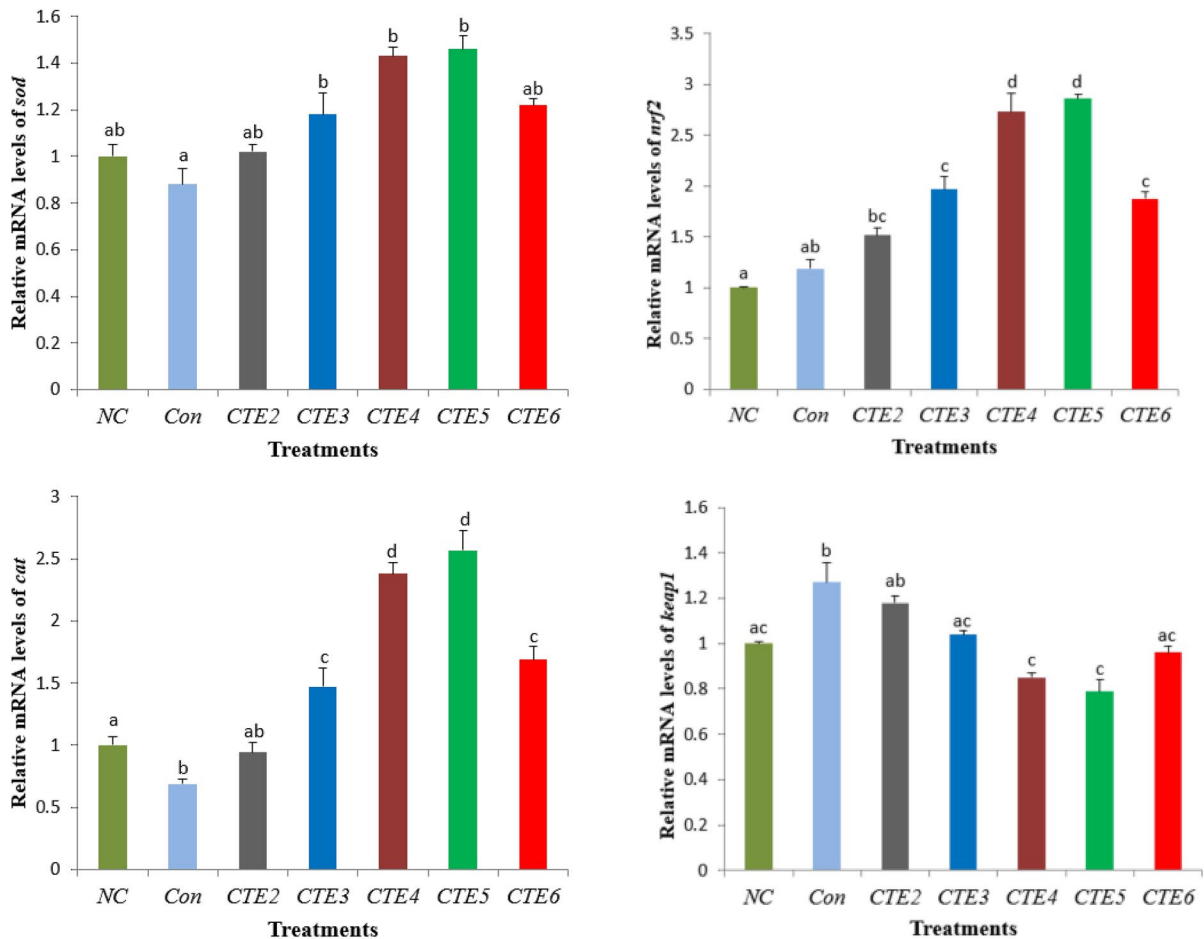


Fig. 3 Relative expression of antioxidant-related genes in the liver of *C. carpio* post-challenge with *Aeromonas hydrophila*. Data are shown as mean $\pm$ S.E.M of three replicates. Different letters in the bar graph represent significant differences ($P < 0.05$)

are linked to growth performance (Bilen et al. 2021). Amylase activity was significantly higher in the CTE4, CTE5, and CTE6 groups. Similarly, dietary herbal supplements enhance intestinal enzyme activity in various fish species (Bilen et al. 2021; Yousefi et al. 2025; Giri et al. 2023). Amylase activity may have been affected by the CTE components, allowing fish to use their diets better. A CTE dose above 500 mg/kg had a slight negative effect on growth indices. This might be because CTE contains anti-nutritional components such as tannins. Protein and fat digestion and absorption are hampered by increased concentrations of anti-nutritional substances (Gupta et al. 2023).

The initial line of defense in the fish immune system is skin mucosal immunity, which comprises

several immunocytes and immune-related agents (Hoseinifar et al. 2020). The mucus produced by goblet cells in the skin serves as an essential physical, biological, and immunological barrier against foreign particles, external stimuli, and naturally existing microbes (Van Doan et al. 2020). As an opsonin, lysozyme triggers phagocytes and the complement system sub-path to eradicate both gram-positive and gram-negative bacteria (Adineh et al. 2023). In this study, significantly higher lysozyme and ACH₅₀ activities were observed in the CTE5 group. Similarly, dietary administration of marjoram (*Origanum majorana*) extract [OME] at 200 mg/kg enhanced lysozyme and ACH₅₀ levels in the skin mucus and serum of *C. carpio* (Yousefi et al. 2021). Fish fed diets supplemented with optimum levels of plant

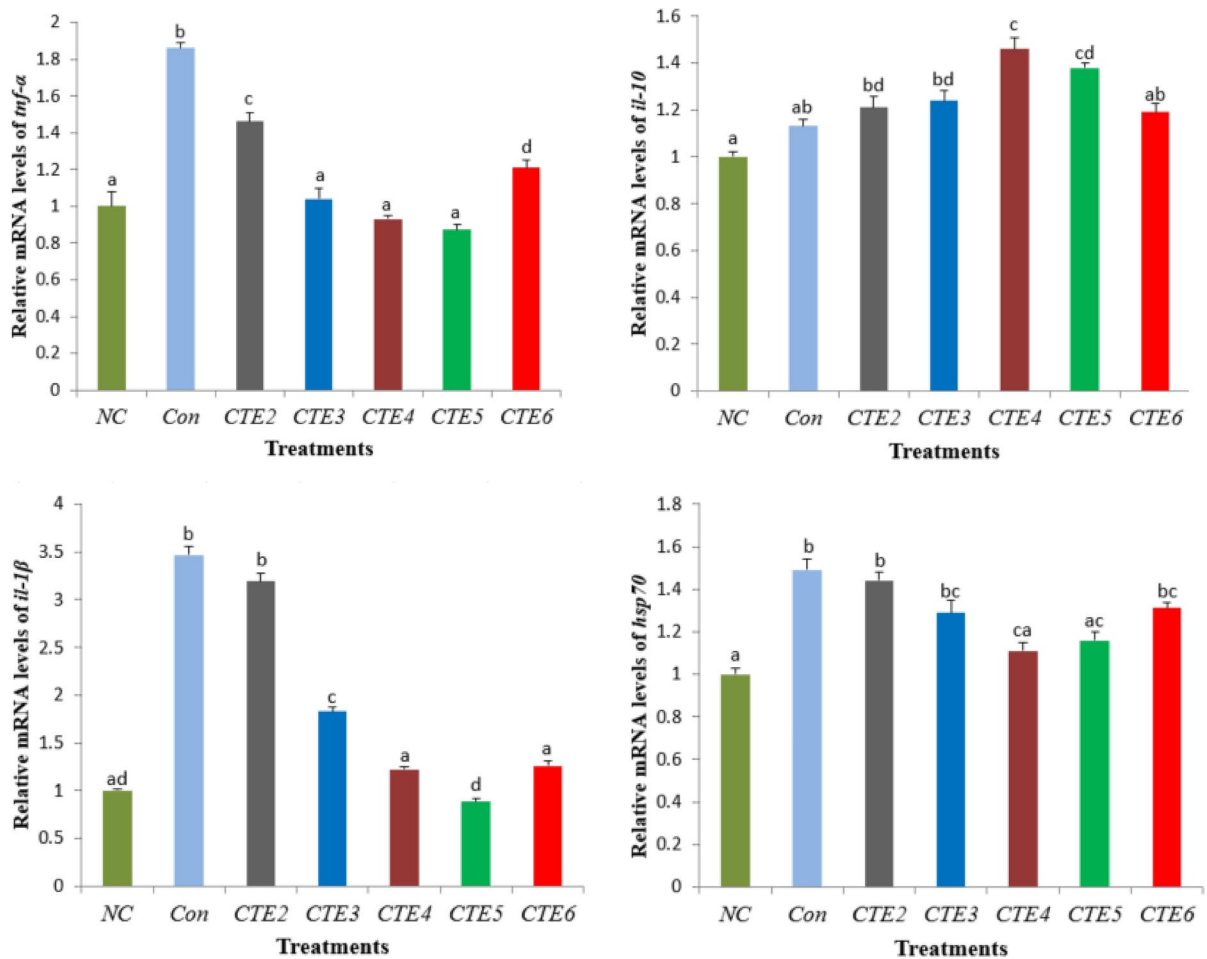


Fig. 4 Effects of dietary CTE on the relative expression of *tnfr-α*, *il-1β*, *il-10*, and *hsp70* in the liver of *C. carpio* post-challenge with *A. hydrophila*. Different letters in the bar graph represent significant differences ($P < 0.05$)

immunostimulants, for example, PLE, curcumin, savory essential oil (SEO), and fern leaf powder (FLP), had higher ($p < 0.05$) lysozyme and/or ACH_{50} levels (Giri et al. 2019; Hoseinifar et al. 2017, 2020, Ghafarifarsanti et al. 2023). Proteases may protect against pathogens in skin mucus and help in activating other innate immune components in fish mucus, such as complement and Igs. Dietary administration of 400 or 500 mg/kg CTE significantly enhanced skin mucosal protease levels. Similar to our results, dietary inclusion of MLE (Hoseinifar et al. 2017) or *B. glabra* leaf (BGL) Giri et al. 2023) significantly enhanced skin mucosal protease activity in *C. carpio*. ALP may have distinct physiological functions depending on the tissue. Owing to its hydrolytic activity, mucosal ALP is linked to fish immunity

and is essential for innate immune responses (Lallés 2019). Similarly, dietary supplementation of OME at 200 mg/kg (Yousefi et al. 2021), PLE at 1.5–2.0 g/kg (Giri et al. 2026), and curcumin at 10 g/kg for 8 weeks (Giri et al. 2019) enhanced ($P < 0.05$) the skin mucosal ALP activity in carp.

Peroxidases are antibacterial agents that successfully eliminate H_2O_2 and preserve the redox equilibrium (Van Doan et al. 2020). In the present study, significantly higher peroxidase levels were observed in CTE4– and CTE5. Similarly, dietary administration of *Bosenbergia rotunda* powder at 10 g/kg (Van Doan et al. 2019) or berberine powder at 1.0 g/Kg (Van Doan et al. 2020) significantly enhanced skin mucosal and serum peroxidase activities in Nile tilapia. Ig is released to identify antigens, such as microorganisms,

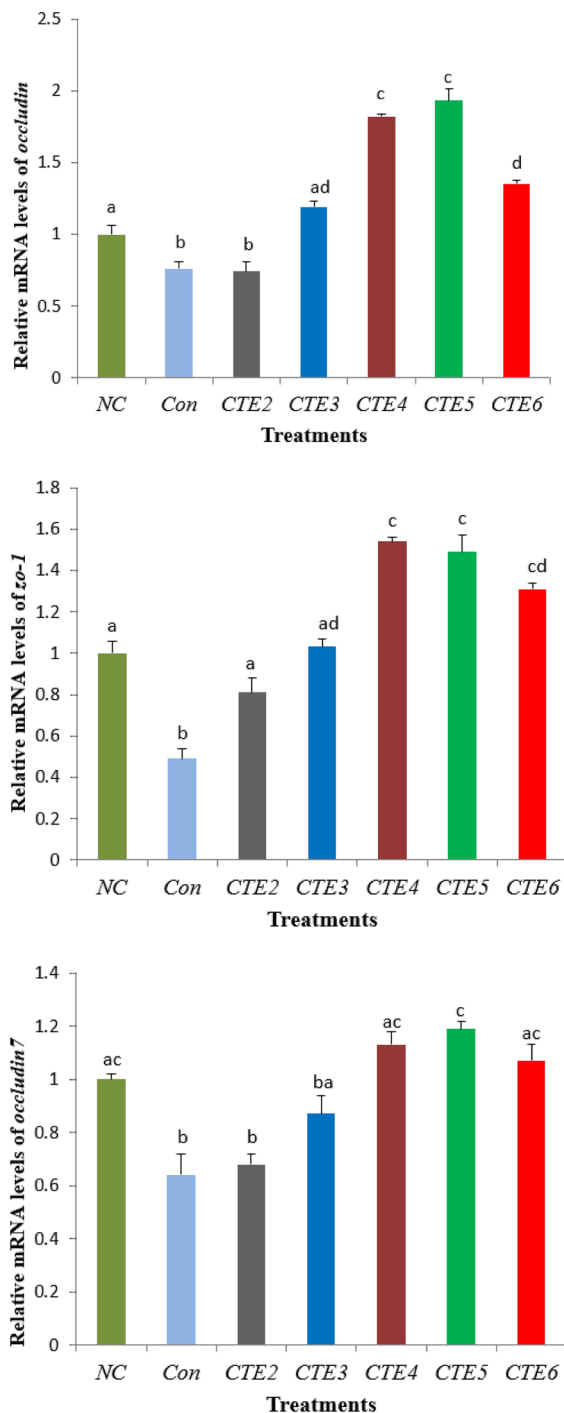


Fig. 5 Expression analysis of intestinal tight junction genes in the intestine of *C. carpio* post-challenge with *A. hydrophila*. Data are shown as mean $\pm$ S.E.M of three replicates. Different letters in the bar graph represent significant differences ($P < 0.05$)

lymphocytes, and/or epithelial cells (Yousefi et al. 2021). In the present study, dietary CTE had no significant effect on Ig levels in the serum and skin mucus of fish. Similarly, dietary PLE had no significant effect on skin mucosal IgA levels in *C. carpio* (Giri et al. 2026). Igs, which bind to and neutralize antigens, are essential components of the adaptive immune system (Parra et al. 2015). Previous studies have suggested that augmentation of immunoglobulin levels is a short-term phenomenon attributable to immunostimulants.

Hematological factors are important biomarkers for determining the health and nutritional status of fish. Serum protein levels in fish are fundamental indicators of the innate immune system (Mazandarani et al. 2017). The increased total protein in the CTE4- and CTE6 groups suggests strong innate immunity in fish. Herbal supplements enhance serum protein levels in fish (Van Doan et al. 2020; Gupta et al. 2023; Giri et al. 2023; Ghafarifarsani et al. 2023). Lower protein levels in the CTE6 group may be related to immunosuppression in fish at higher doses of CTE (Gupta et al. 2023). Because the liver produces albumin and globulin, assessing the amounts of these proteins offers important information about the health of the liver (Bojarski et al. 2025). Serum glucose and cortisol levels are bioindicators of stress in fish. Cortisol affects the growth, immunity, and reproduction (Yousefi et al. 2025). The elevated serum protein and globulin levels in CTE4 and CTE5 may be attributed to reduced stress, as shown by unaltered glucose and cortisol levels. By identifying and binding pathogens, phagocytosis limits their growth and dissemination, ensuring that no diseases infect the fish (Harikrishnan et al. 2011). The significant increase in serum PA in CTE3–CTE6 is in line with earlier studies that reported a positive influence of herbal supplements on PA in fish (Van Doan et al. 2019; 2020, Ghafarifarsani et al. 2023). The increased PA, ACH₅₀, peroxidase, and lysozyme activities in the CTE groups, particularly in the CTE5 group, indicate that dietary CTE at an appropriate dose may stimulate the immune system and increase resistance to infectious illnesses.

Serum ALT, AST, and ALP levels can be used to gauge the extent of liver damage. An elevated concentration of these enzymes is a sign of hepatic injury, which results in the leakage of these enzymes from

hepatic tissues into the bloodstream (Harikrishnan et al. 2011). Dietary CTE had no significant effect on serum AST, ALP, or ALT levels, indicating the absence of cytolytic effects on hepatocytes. Our results are in line with previously reported studies which demonstrated that dietary PLE at 2.0 g/Kg to *C. carpio* (Giri et al. 2026), and walnut leaf extract (WLE) at 750 mg/kg to *Oreochromis niloticus* (Yilmaz et al. 2023) for 60 days had no significant effect on serum ALT, AST, and ALP levels. The AST levels were higher than those of ALT, suggesting that aerobic metabolism is more prominent (Giri et al. 2026). The higher amount of flavonoids in CTE confers hepatoprotective effects (Davila et al. 1989). Dietary CTE had no significant effect on serum uric acid or creatinine levels, indicating the positive effects of CTE on renal function in fish (Nelson et al. 1999). Similarly, dietary administration of *Silybum marianum* extract (SME) had no significant influence on serum uric acid or creatinine levels in striped catfish (Abdel-Latif et al. 2023).

The higher antioxidant capacity of the CTE diets, especially CTE5, may provide greater protection to hepatocytes by exerting antioxidant effects. Important antioxidant enzymes, including SOD, CAT, and GPx, are the first line of defense against oxidative stress-induced damage in fish. These enzymes reduce the harmful effects of reactive oxygen species (ROS) and free radicals (Khalid et al. 2025). Our results demonstrated that serum SOD and CAT levels were enhanced ($p < 0.05$) in CTE4 and CTE5. Meanwhile, serum MDA levels were lower, in the CTE5 group than in the control group. By detecting MDA, a byproduct of lipid peroxidation caused by free radical assault in the host, tissue damage caused by ROS can be monitored (Giri et al. 2023). Similarly, dietary administration of SME (Abdel-Latif et al. 2023), dill (*Anethum graveolens*) essential oil (Ghafarifar-sani et al. 2023), *Echinacea purpurea* extract (EPE) (Lin et al. 2026), OME (Yousefi et al. 2021), CEO (Yousefi et al. 2023), and curcumin (Giri et al. 2019) significantly enhanced the enzymatic activities of SOD and CAT and reduced MDA levels in various fish species. The observed antioxidant activity was linked to corresponding mRNA levels. The expression of *sod* and *cat* was significantly upregulated in the liver tissues of fish fed 400 or 500 mg/kg CTE. Similarly, dietary administration of PLE (Giri et al. 2026), *Yucca schidigera* extract (YSE) (Wang et al.

2020), and cornelian cherry fruit extract (CCE) (Ahmadifar et al. 2022) in *C. carpio*, EPE in largemouth bass (Lin et al. 2026), and WLE in *O. niloticus* (Yilmaz et al. 2023) upregulated the expression of *sod* and *cat* genes. Chowdhury et al. (Chowdhury et al. 2026) revealed that the presence of high concentrations of phenolic compounds, for example, phenol, 3,5-bis(1,1-dimethylethyl), and phenol, 2-methoxy-4-(2-propenyl)-, acetate) in the methanolic fraction of CTE was responsible for its strong scavenging capacity. Through single-electron and hydrogen atom transfer processes, these compounds efficiently transfer electrons or hydrogen atoms to neutralize free radicals. These phenolic compounds can prevent ROS production by chelating metal ions, such as Cu^{2+} and Fe^{2+} (Chowdhury et al. 2026; Zeb 2020).

Numerous antioxidant and detoxifying genes in vertebrates are transcriptionally activated by the *nrf2-keap1* signaling pathway. *Keap1* is a critical post-induction repressor of *nrf2*. Nuclear translocation is crucial for *nrf2*'s activation of antioxidant enzyme gene transcription, although *keap1* can bind to *nrf2* and block its nuclear translocation (Wang et al. 2020; Sun et al. 2007). Here, expression of *nrf2* gene was significantly upregulated in the CTE4 and CTE5 groups. Meanwhile, *keap1* gene expression was downregulated in these groups compared to the control group. Similarly, dietary PLE at 1.5–3.0 g/kg upregulated the expression of *nrf2* while downregulated expression of *keap1* in carp intestinal tissues (Giri et al. 2026). Dietary administration of YSE to *C. carpio* upregulates the expression of *nrf2* while downregulating the expression of *keap1* in intestinal cells (Wang et al. 2020). Based on earlier studies, we propose that CTE downregulated *keap1* transcription in fish tissues, promoting *nrf2* translocation to the nucleus and upregulating the transcription of antioxidant genes in the hepatopancreas.

Cytokines are a diverse group of protein molecules that act as mediators of cell signaling by attracting specific immune cell types in response to pathogenic infection. Monocytes and macrophages predominantly produce the pro-inflammatory cytokines *il-1 β* and *tnf- α* , which are crucial for modulating different facets of the immune response (Gioacchini et al. 2008). In this study, dietary supplementation with CTE at 400–500 mg/kg significantly downregulated the expression of pro-inflammatory cytokines. Meanwhile, it upregulated *il-10* expression in the

liver tissues of fish post-challenge with *A. hydrophila*. Interleukin-10 (il-10) is a key anti-inflammatory cytokine that plays a crucial role in regulating the expression of pro-inflammatory cytokines. It is essential for maintaining homeostasis during host–pathogen interactions (Wang et al. 2015). Similarly, dietary administration of PLE at 2.0 g/kg (Giri et al. 2026), curcumin at 15 g/kg (Giri et al. 2019), and YSE at 400 mg/kg to *C. carpio* (Wang et al. 2020) suppressed the expression of pro-inflammatory genes (*il-1 β* and *tnf- α*) and upregulated the expression of *il-10* in tested tissues of host. Conversely, dietary PPE at 0.5% significantly upregulated the expression of *il-1 β* and *tnf- α* . Meanwhile, it downregulated the expression of *il-10* in tested tissues of *L. rohita* (Gupta et al. 2023). Herbal remedies can reduce inflammation by increasing the production of anti-inflammatory cytokines and decreasing pro-inflammatory cytokines (Lin et al. 2026). Excessive ROS generation stimulates the production of pro-inflammatory cytokines, which can lead to inflammatory illness (Mirshafiey et al. 2008). Higher mRNA levels of antioxidant-related genes, namely, *sod*, *cat*, and *mnf2*, may decrease the expression of pro-inflammatory cytokines by alleviating ROS production (Wang et al. 2020). These anti-inflammatory properties may be attributable to the presence of 9-octadecenamide (z) in CTE (Chowdhury et al. 2026).

As stress biomarkers linked to protein folding, heat shock proteins (HSPs), particularly HSP70, protect cells from the damaging effects of stress. The reactions of fish to biotic and abiotic stressors are indicated by HSPs expression (Iwama et al. 1999). In this study, dietary administration of CTE at 400 or 500 mg/kg alleviated the expression of *hsp70* in the liver tissues of challenged fish compared to the control group. Similarly, the dietary administration of a phyto-genic additive, that is, a mixture of *Valeriana officinalis* and *Passiflora incarnata* extracts, significantly reduced expression *hsp70* in the head-kidney of *Oncorhynchus mykiss* (Yilmaz et al. 2024). Adding PLE or BGL to the diet of *C. carpio* has no effect on the expression of *hsp70* (Giri et al. 2023, 2026). The results of the present study suggest that dietary CTE at 400–500 mg/kg could reduce stress due to its higher antioxidant effects.

TJs are protein complexes that form seals between adjacent epithelial cells in the intestinal lining, preventing uncontrolled passage of microbes, toxins, and

other harmful substances from the gut lumen into the underlying tissues and bloodstream (Niklasson et al. 2011). In fish, TJ genes encode claudins, occludins, and zonula occludins (ZO) proteins (Chasiotis et al. 2012). In the present study, dietary administration of CTE at 400 or 500 mg/kg reversed the expression of *zo-1*, *occludin*, and *occludin7* in pathogen aggravated fish. The enhanced expression of TJ-related genes can enhance the intestinal resistance of pathogens by increasing intestinal barrier function. Dietary administration of YSE (Wang et al. 2020), and PLE (Giri et al. 2026) to *C. carpio* significantly upregulated the mRNA levels of TJ-related genes in the tested tissues. Dietary administration of *Echinacea purpurea* extract at 1050 mg/kg to largemouth bass for 8 weeks significantly upregulated the expression of intestinal barrier markers (*zo-1*, *occludin*, and *muc2*) in intestinal tissues (Lin et al. 2026). These studies suggest that dietary administration of herbal extracts decreases the expression of pro-inflammatory cytokines, which could upregulate the expression of TJ-related genes in fish (Lin et al. 2026; Giri et al. 2026; Wang et al. 2020). We suggest that dietary CTE at 400–500 mg/kg resulted in significant downregulation of *il-1 β* and *tnf- α* , and upregulation of *zo-1* expression likely enhanced the intestinal epithelial permeability.

Herbal compound formulas, extracts, and single compounds have proven to be highly effective in increasing fish resistance to bacterial pathogens, while enhancing their immune response (Giri et al. 2026, 2023; Wang et al. 2015). Analysis of the carp survival rate showed that administration of CTE at 500 mg/kg resulted in 54.76 RPS. An RPS greater than 50% indicates a positive effect of the administered bioactive compounds or vaccines (Amend 1981). The higher survival rate may have attributed to rich phenolic and flavonoid compounds in CTE which have strong immunomodulatory, anti-inflammatory, and antioxidant properties (Semwal et al. 2023; Bharadwaj et al. 2022; Naiel et al. 2023). The phenolic compounds found in *C. tamala* extract can damage microbial cell membranes, resulting in the release of intracellular materials, inhibition of enzymatic functions, or cell destruction (Chowdhury et al. 2026). Dietary administration of curcumin (Giri et al. 2019), PLE (Giri et al. 2026), *Cistus laurifolius* ethanol extract (Bilen et al. 2021), FLP (Hoseini-far et al. 2020), SEO (Ghafarifarsani et al. 2023), OME (Yousefi et al. 2021), and BGL (Giri et al.

2023) enhanced the resistance of *C. carpio* against *A. hydrophila* challenge. The increased disease resistance of fish may be attributed to the following factors: (a) increased skin mucosal immune responses; (b) modulating haemato-immunological indicators; (c) enhanced antioxidant activities & their corresponding gene expression; (d) improved intestinal enzymatic activities; (e) improved expression of TJ genes, protecting gut permeability; and (f) downregulated pro-inflammatory genes (*tnf- α* , *il-1 β*) whereas, upregulated *il-10* transcription. However, CTE at 600 mg/kg did not result in highest post-challenge survival might be an outcome of immunosuppression.

Conclusion

The present study showed that dietary administration of 500 mg/kg CTE for 10 weeks substantially improved growth performance, skin mucosal and serum immune responses, intestinal digestive enzymatic activities, and antioxidant activities and regulated the transcript levels of important cytokine genes in carp tissues. CTE had no adverse effects on the activities of liver metabolic enzymes, which may be due to its high antioxidant activity. Regression analysis revealed that CTE at 507.16–538.75 mg/kg is required enhance growth performance and immunity of fish. However, excessive amounts of CTE could negatively affect the fish health. Furthermore, proteomic and metabolome investigations are warranted to explore the mode of action of CTE.

Author contributions Conceptualization, Methodology, Investigation; Data curation, Writing-original draft; Data curation: Sib Sankar Giri. Conceptualization, Methodology, and Resources: V. Sukumaran. Writing-review and editing, formal analysis, and Validation: Ji Hyung Kim. Supervision, Resources, Writing-review and editing, and Project administration: Se Chang Park. All authors reviewed the manuscript.

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Data availability All data generated or analyzed during this study are included in this article.

Declarations

Ethical approval All experiments complied with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines and were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Consent for publication Not applicable.

Clinical trial number Not applicable.

Competing interests The authors declare no competing interests.

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