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Curcumin as a protective agent against chromium and ammonia toxicity using molecular and biochemical approaches in fish

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In aquatic ecosystems, metal and ammonia pollution pose major concern as they contaminate the environment and induces toxicity in fish. The present study addresses the toxicity induced by chromium (Cr) and ammonia (NH_3) toxicity in fish and investigates the potential of dietary curcumin in mitigating the effects of concurrent exposure to theses stressors in *Pangasianodon hypophthalmus*. Three isonitrogenous and isocaloric diets were formulated: a control diet (0% curcumin) and two curcumin-supplemented diets containing 0.1% and 0.2% curcumin. Four experimental groups were designed in a completely randomized design: (1) control, (2) concurrent exposure to Cr and NH_3 toxicity and fed with control diet, (3) 0.1% curcumin with Cr and NH_3 exposure, and (4) 0.2% curcumin with Cr and NH_3 exposure. Fish fed with 0.2% curcumin diet, followed by the 0.1% curcumin diet under Cr and NH_3 stress, exhibited significantly reduced cortisol levels compared to the control and Cr + NH_3 groups. Similarly, the expression of *HSP70* and *iNOS* genes in liver tissue was significantly downregulated in the 0.1% and 0.2% curcumin-fed groups compared to other groups. Concurrent exposure to Cr and NH_3 led to a considerable increase in oxidative stress enzyme in liver and kidney tissues, including glutathione S-transferase (GST), catalase (CAT), and superoxide dismutase (SOD). However, dietary supplementation with 0.1% curcumin significantly reduced oxidative stress enzyme activities. The stressors markedly reduced acetylcholinesterase (AChE) activity, but supplementation with 0.1% curcumin restored AChE activity. The expression of stress-related genes such as *cytochrome P450 (CYP450)*, *caspase-3a (Cas3a)*, and *tumor necrosis factor-alpha (TNF-α)* was noticeably downregulated in the curcumin-fed groups, reducing the impact of Cr + NH_3 stress. Furthermore, total immunoglobulin (Ig) levels and growth-related gene expression, including *growth hormone (GH)* and *growth hormone receptor 1 (GHR1)*, were significantly upregulated in the 0.1% curcumin-fed group under Cr + NH_3 stress compared to all other groups. Additionally, *myostatin (MYST)* gene expression was significantly downregulated in the 0.1% curcumin-fed group. Activities of cellular metabolic and digestive enzymes were significantly improved with curcumin supplementation, mitigating the adverse effects of Cr + NH_3 stress compared to the control and other groups. Moreover, Cr bioaccumulation in different fish tissues was reduced in the 0.1% curcumin-fed group. This study highlights the potential of dietary curcumin in mitigating the adverse effects of concurrent Cr and NH_3 exposure through gene regulation, thereby improving the physiological and productive performance of *Pangasianodon hypophthalmus*.

Keywords Curcumin, Chromium, Ammonia, Gene regulation, Fish

The aquatic ecosystem is house to various pollutants, including heavy metals, pesticides, ammonia, biological waste, and nano/microplastics[1]. These pollutants pose significantly threats to the life cycle of aquatic organism, including fish. Ammonia is a major water contaminant; primarily originating from agriculture runoff and decomposition of biological waste in aquatic ecosystem. It is highly toxic to aquatic animals because elevated NH_4^+ levels depolarizes neurons, displaces K^+, activates the N-methyl-D-aspartate (NMDA) type glutamate receptor, and trigger an excessive influx of Ca^{2+}, leading to cell death in the central nervous system[2]. Furthermore,

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total ammonia exists in two forms: ionized (NH_4^+) and un-ionized (NH_3). The un-ionized form (NH_3) readily diffuses across biological membranes or induces toxicity by disrupting cellular processes[3,4]. According to Hoseini et al.[5], uneaten feed and fish excrement are the primary sources of ammonia toxicity in aquaculture, impairing essential organs and leading to mass mortality in aquatic animals[6].

Chromium (Cr) is a heavy metal found in water bodies that poses a significant harm to aquatic life, the environment, and human health. It is classified among 25 most hazardous substances due to its mutagenic, neurotoxic, and carcinogenic properties[7,8]. Cr is highly bioaccumulative, non-biodegradable, and toxic even at low concentration which can have a severe impact on aquatic life[9]. The primary source of Cr pollution in aquatic systems is textile, through industrial discharge of petroleum refining, printing, tanneries, fertilizer, mining, photography, pharmaceutical wastes, and electroplating[10–13]. The environmental waste management is often ineffective in controlling heavy metal pollution, particularly lithophilic or class B metals, which pose significant threats to ecosystems and aquatic life[10]. Chromium is especially concerning due to its long-term characteristics of biomagnification, bioaccumulation, and non-biodegradability[14]. According to Ahmed et al.[10] and Velma et al.[15], Cr exists in three oxidation states divalent (Cr^{2+}), trivalent (Cr^{3+}), and hexavalent (Cr^{6+}). Among these, hexavalent (Cr^{6+}) is the most hazardous due to its high membrane permeability, which facilitates cellular entry and toxicity. In contrast, trivalent chromium (Cr^{3+}) is the predominant form found in biological systems and plays a crucial role in mammals, contributing to protein, lipid, and carbohydrate metabolism[16]. Trivalent chromium (Cr^{3+}) has very low toxicity, limited membrane permeability, non-corrosiveness, and very little potential to biomagnify in the food chain. Due to its high membrane permeability, Cr^{6+} is significantly more toxic than other chromium forms[10]. In natural surface waters, Cr concentrations typically range from 1 to 10 ppb, while the provisional guideline limit is 50 ppb[17]. The maximum allowable intake of Cr is 0.05–0.15 mg per kg of body weight, as recommended by WHO[18] and FEPA[19].

Surprisingly, the adverse effects of pollution can be mitigated through dietary supplements such as curcumin. Curcumin (Diferuloylmethane), a polyphenol derived from *Curcuma longa* L, possess a wide range of bioactive properties, including immunostimulant, antioxidant, anti-inflammatory, anti-apoptosis, anti-stress, and antibacterial effects[20,21]. Additionally, curcumin has been shown to reduce metal and ammonia (NH_3) toxicity in fish. Its application in aquaculture enhances fish immunity without the adverse effects associated with antibiotics and other medications[22,23]. Therefore, supplementing fish diets with curcumin can help minimize the risk of microbial infections and improve residue management in aquaculture. Curcumin also activates the transcription factor nuclear factor erythroid 2–related factor, or nrf2, which plays a crucial role in regulating cellular antioxidant defenses[24]. Stress-responsive genes in fish that react to low-dose Cr and NH_3 stress include those associated with apoptosis, cytokines, nuclear factor kappa B (NF-κB), immune response, growth regulation and antioxidant defense. Nrf2 and NF-κB can actively respond to the intake of plant active ingredients, such as plant polysaccharides[25,26]. All species, including fish, rely on the crucial process known as "programmed" cell death, or apoptosis, which includes immunological and hormone-related gene development, regular cell repair, cellular function, and chemical cell death[27]. Cytokines, important signaling molecules, play a vital role in both normal and pathological conditions. Under stress, they modulate the host's inflammatory response and immune system[28].

Pangasianodon hypophthalmus is a robust, fast growing fish capable to adapting to a wide range of abiotic and biotic stressors[29–31]. According to Kumar et al.[32], this fish species is most suited for studying abiotic-related gene expression. The present study employs gene regulation analysis to elucidate the protective role of dietary curcumin against chromium and ammonia toxicity in *P. hypophthalmus*.

Materials and methods

Ethics statement

NIASM aquaculture Central Wet Laboratory facilities and experimental protocols were approved under the Committee for the Purpose of Control and Supervision of Experiments on Animals (CCSEA) as 2190/GO/RReBi/SL/2022/CCSEA. This study also strictly adhered to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. All methods were performed in accordance with the relevant guidelines and regulations.

Experimental animal and design

In this study, *P. hypophthalmus* (mean weight 47.15 ± 0.40 and length 16.30 ± 0.25 cm) was used. The fish were obtained in good health from NIASM farm pond and acclimatized for two weeks in a fiber-reinforced plastic (FRP) tank before the experiment commenced. Ten fish were kept in each replicate in 150-liter plastic rectangle tank. The experiment followed a completely randomized design (CRD) with four treatment groups, each replicated three times. The treatments were as follows:

1. Control: No exposure to chromium (Cr) or ammonia (NH_3) and fed a control diet.
2. Cr + NH_3 Exposure: Concurrent exposure to ammonia (NH_3) and chromium (Cr) and fed a control diet.
3. Curcumin 0.1% + Cr + NH_3: Concurrent exposure to Cr and NH_3 and supplemented with a 0.1% curcumin diet.
4. Curcumin 0.2% + Cr + NH_3: Concurrent exposure to Cr and NH_3, and supplemented with a 0.2% curcumin diet.

Fish were fed the experimental diets twice a day, at 8:45 AM and 5:15 PM. Continuous aeration was provided using an aerator and faeces and uneaten food were removed daily through siphoning. Water quality parameters were monitored regularly following APHA[33] guidelines and remained within the optimal range throughout the experiment. A stock solution of chromium trioxide (CrO_3, Hi Media) (100 mg L^{-1}) was prepared, and ammonia

toxicity (1/10th of the LC_{50}; ammonia sulphate, 2.0 mg L^{-1})[34] was applied along with 1/20th of the LC_{50} of Cr (2.5 mg L^{-1})[13]. The two third of the (2/3rd) water were manually replaced every alternate day. During the trial, fish were fed curcumin diets that were iso-nitrogenous (35% crude protein) and iso-caloric (352.89 kcal/100 gram) in pellet form (Table 1). Gross energy was computed using the Halver method[35].

Tissue homogenate preparation and blood collection

Under aseptic condition, fish were anaesthesia using clove oil (50 µl L^{-1}), after that, gills, muscles, brain, liver, and kidneys tissues were collected. To homogenize the tissue for enzyme analysis, a homogenizer(Omni Tissue Master Homogenize, Kennesaw, GA) was used with chilled sucrose (5% w/v, 0.25 M) and EDTA solution (1 mM). For gene expression analysis, muscle and liver tissues were processed using liquid nitrogen. The homogenized tissues were then centrifuged at 5,000 × g for 15 min at 4 °C to obtain homogenates for enzyme analysis. The collected supernatants were stored at -20 °C for further examination. Blood samples were drawn from five fish per tank during dissection and used for serum collection. Tissue protein analysis was conducted using the Lowry protein assay[36].

RNA isolation and quantification

Total RNA was isolated from *P. hypophthalmus* liver tissue using the TRIzol method. The liver tissue was homogenized using liquid nitrogen. The homogenized samples were then mixed chloroform and allowed to phase separate for five minutes. RNA was separated by centrifugation with 75% ethanol, followed by air drying of the RNA pellet. The dried pellet was dissolved in nuclease free water and stored at -80 ˚C for further use. To assess RNA integrity, a 1% agarose gel was used, and RNA bands were visualized using the Gel documentation system (ChemiDocTM MP imaging system, Bio-Rad). Thermo-scientific's nano-drop spectrophotometer was utilized to quantify RNA.

cDNA synthesis and quantitative PCR

First-strand cDNA synthesis was carried out using a cDNA synthesis kit (Thermo-scientific's). The reaction mixture contained 12 µL of RNA template (100 ng) and 15 pmol of oligo dT primers. The mixture was heated to 65 °C for five minutes and then immediately cooled on ice. Subsequently, 1 µL of RiboLock RNase Inhibitor (20 U/µL), 2 µL of dNTP Mix (10 mM), and 1 µL of reverse transcriptase enzyme were added. The reaction mixture was briefly centrifuged and incubated at 60 °C for 42 min, followed by 70 °C for 5 min. The synthesized cDNA was stored at -20°C for further analysis. The quality of synthesized cDNA was confirmed using β-actin as a housekeeping gene. Real-time PCR (qPCR) was performed using SYBR Green and gene-specific primers (Bio-Rad). The qPCR setup included an initial denaturation step at 95 °C for 10 min, followed by 39 cycles of

Ingredients	Control	Curcumin-0.1%	Curcumin-0.2%
Soybean meal[a]	35.5	35.5	35.5
Fish meal[a]	25	25	25
Groundnut meal[a]	10	10	10
Wheat flour[a]	17.2	17.1	17
Sunflower oil[a]	4.5	4.5	4.5
Cod liver oil[a]	1.5	1.5	1.5
CMC[b]	2	2	2
Vitamin and mineral mix*	2	2	2
Vitamin C[c]	0.3	0.3	0.3
Lecithin[b]	2	2	2
Curcumin	0	0.1	0.2
	100	100	100
Proximate composition of the diets			
Crude protein (CP)	34.88 ± 0.50	35.24 ± 0.09	35.36 ± 0.32
Ether extract (EE)	9.28 ± 0.09	9.44 ± 0.19	9.34 ± 0.17
Total carbohydrate (TC)	37.25 ± 0.78	36.46 ± 0.23	36.37 ± 0.56
Organic matter (OM)	90.75 ± 0.04	90.71 ± 0.03	90.51 ± 0.14
Dry matter (DM)	90.62 ± 0.16	90.47 ± 0.10	90.55 ± 0.07
Digestible energy (DE)	353.49 ± 0.56	352.92 ± 0.70	352.25 ± 0.38

Table 1. Ingredient composition and proximate analysis of experimental diets (% dry matter) of Curcumin fed to *Pangasianodon hypophthalmus* during the experimental period of 40 days. [a]Procured from local market, [b]Himedia Ltd, [c]SD Fine Chemicals Ltd., India. *Manual prepared Vitamin mineral mixture; Composition of vitamin mineral mix (quantity/250 g starch powder): vitamin A 55,00,00 IU; vitamin D3 11,00,00 IU; vitamin B1:20 mg; vitamin E 75 mg; vitamin K 1,00 mg; vitamin B12 0.6 mcg; calcium pantothenate 2,50 mg; nicotinamide 1000 mg; pyridoxine: 100 mg; Zn 500 mg; I 1,00 mg; Fe 750 mg; Cu 200 mg; Co 45 mg; Ca 50 g; P 30 g; Se: 2 ppm. Digestible energy (DE) (Kcal/100 g) = (% CP × 4) + (% EE × 9) + (TC × 4). Data expressed as mean ± SE, $n = 3$.

amplification, with denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min[37]. The details of the primers used are listed in Table 2.

Genes

The genes nitric oxide synthase (*iNOS*), heat shock protein (*HSP 70*), caspase 3a, cytochrome P450 (*CYP 450*), tumor necrosis factor (*TNFα*), growth hormone receptor (*Ghr1*), immunoglobulin (*Ig*), and growth hormone (*GH*) were examined in liver tissues and myostatin (*MYST*) in muscle tissue in this study in order to measure the data in real time.

Antioxidant enzyme activities

The Catalase (CAT) (EC 1.11.1.6) assay was carried out using the Takahara et al.[38] method. The reaction mixture consisted of 1 mL of freshly prepared hydrogen peroxide substrate solution, 50 µL of tissue homogenate, and 2.45 mL of phosphate buffer (50 mM; pH 7). After through mixing the decrease in absorbance was recorded at 240 nm over three minutes. The Superoxide dismutase (SOD) (EC 1.15.1.1) assay was carried out using Misra and Fridovich's[39] method. Briefly, the reaction mixture contained 0.5 mL of freshly made epinephrine, 1.5 mL of phosphate buffer, and 50 µL of tissue homogenate. After complete mixing, absorbance was measured at 480 nm over three minutes using a UV spectrophotometer. The Glutathione-S-Transferase (GST) (EC 2.5.1.18) assay was performed spectrophotometrically following the protocol of Habig et al.[40], using S-2,4-dinitrophenyl glutathione (CDNB) as the substrate. This method is based on the principle that glutathione (GSH), specifically S-2,4-dinitrophenyl glutathione, forms an adduct with CDNB. The formation of this adduct was monitored by measuring the increase in absorbance at 340 nm relative to a blank.

Neurotransmitter enzyme activities

The activity of acetylcholinesterase (AChE) (EC 3.1.1.7) was determined using the method of Hestrin modified from Augustinsson[41]. Briefly, the assay mixture consisted of 1 mL of phosphate buffer, 1 mL of acetylcholine buffer, and 0.2 mL of the sample. The mixture was then incubated at 37 °C for 30 min. After incubation, alkaline hydroxylamine, hydrochloric acid (HCl), and ferric chloride were added to the solution. The reaction was subsequently measured at 540 nm.

Cortisol

ELISA kit (commercially available Cortisol EIA kit, catalogue no. 500360, Cayman Chemicals, USA) was used to measure serum cortisol levels. The assay was carried out using an ELISA plate reader (Biotek India Pvt. Ltd.) in accordance with the instructions included with the kit.

Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and malate dehydrogenase (MDH)

Aspartate aminotransferase (AST) (EC 2.6.1.1) and alanine aminotransferase (ALT) (EC 2.6.1.2) activities were determined using the Wooten[42] method. Briefly, the assay involved the preparation of sodium hydroxide (1 N), potassium dihydrogen phosphate, dipotassium hydrogen phosphate, and 2,4-dinitrophenylhydrazine (DNPH). For AST, the substrates used were DL-aspartic acid and β-ketoglutarate, while for ALT, DL-alanine and α-ketoglutarate were used. The tissue homogenate was mixed with the respective substrate and incubated at

Gene	Primer sequence (5′ – 3′)	Accession number	Product length	Annealing temperature
HSP 70	F- CTCCTCCTAAACCCCGAGTC R- CCACCAGCACGTTAAACACA	XM_026934573.2	106	60
iNOS	F-ACACCACGGAGTGTGTTCGT R-GGATGCATGGGACGTTGCTG	XM_026931613.2	119	61
TNFα	F-TGGAGTTCTGCTTGCCGTGG R-GCAGCCTTTGCAGTCTCGGA	XM_026942329.2	141	62
Ghr1	F-TATTGGCTACAGCTCGCCGC R-AATCACCCCGACTGTGCTGC	XM_034306157.1	122	62
Ig	F- GGCCAGTAATCGTACCTCCA R- CTTCGTAAGGTCCCCACTGA	XM_026923540.2	139	59
MYST	F-GGGAAAGACCTGGCCGTGAC R-TCGAGGCCGGATTCTCGTCT	XM_026910492.2	113	62
GH	F-CCCAGCAAGAACCTCGGCAA R-GCGGAGCCAGAGAGTCGTTC	GQ859589.1	133	62
CYP P450	F-GATTCGGCATCCGTGCGTGC R-CGATGTGGCTGGGACGAGCA	NC_047599.1	155	60
Cas 3a	F-CCGGCATGAACCAGCGCAAC R-TCCACCGCACCATCTGTCCC	NC_047622.1	229	62
β-Actin	F-CAGCAAGCAGGAGTACGATG R-TGTGTGGTGTGTGGTTGTTTTG	XM_026929614.3	136	58

Table 2. Details of primer for relative quantitative real-time PCR. *HSP* Heat shock protein, *iNOS* Nitric oxide synthase, *TNFα* Tumor necrosis factor, *Ig* Immunoglobulin, *MYST* myostatin, *CYP P450* Cytochrome P450, *Cas 3a* caspase 3, *GH* Growth hormone.

37 °C for one hour. Following incubation, DNPH was added, and absorbance was measured at 540 nm. Lactate dehydrogenase (LDH) activity was assessed using the method of Wroblewski and Ladue[43]. In brief, 0.1 M sodium dihydrogen phosphate and disodium hydrogen phosphate were used to prepare a phosphate buffer. Freshly prepared NADH and sodium pyruvate were then added to this buffer. After introducing the sample/enzyme extract, the mixture was incubated for 20 min, and the absorbance was recorded at 320 nm. Similarly, malate dehydrogenase (MDH) activity was determined following the method of Ochoa[44]. The reaction mixture was similar to that used for LDH, except that oxaloacetate was used as the substrate instead of sodium pyruvate.

Growth performance

The following procedure was evaluated in order to determine the growth performance. The fish was sampled and weighed every 15 to 40 days on average.

Body weight gain (BWG) = Final weight (g) – Initial weight (g)

Feed conversion ratio (FCR) = Total dry feed intake (g)/Wet weight gain (g)

Specific growth rate (SGR) = 100 (ln FBW-ln IBW)/ number of days.

Weight gain (%) = Final body weight (FBW)-Initial body weight (IBW)/Initial body weight (IBW) ×100

Relative feed intake, (FI) (%/d) = 100 × (TFI/IBW)

Protein efficiency ratio (PER) = Total wet weight gain (g)/crude protein intake (g)

Thermal growth coefficient, (TGC) = $(FBW^{1/3} - IBW^{1/3}) \times (\Sigma D0)^{-1}$, where ΣD0 is the thermal sum (feeding days × average temperature, °C)

Daily growth index, DGI (%) = $(FBW^{1/3} - IBW^{1/3})$/days × 100

Chromium (Cr) analysis in fish tissues and experimental water

The Cr bioaccumulation in the different fish tissues such as brain, kidney, gills, liver, and muscles were measured. The tissues were processed according to Kumar et al.[13] method utilizing Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Agilent 7700 series, Agilent Technologies, USA). The samples were digested in a microwave digestion system (Microwave Reaction System, Multiwave PRO, Anton Paar GmbH, Austria, Europe).

Statistics

Statistical Package for the Social Sciences (SPSS) version 16 was used to analyze the data. Shapiro-Wilk and Levene's test and Shapiro-Wilk's test, respectively, were used to assess the data for normality and homogeneity of variance. The current study used a one-way ANOVA (analysis of variance) with Duncan's multiple range tests. After analysis, the data was significant at $p < 0.05$.

Results

Cortisol, heat shock protein (*HSP 70*) and inducible nitric oxide synthase (*iNOS*)

In the present study, cortisol levels were significantly elevated under concurrent exposure to low dose of Cr (1/20th of LC_{50} of Cr) and NH_3 stress compared to the control and curcumin-supplemented diets. However, cortisol levels significantly decreased ($p = 0.0051$) with dietary curcumin at 0.2%, followed by 0.1% with exposure to stressor (Fig. 1A). Similarly, concurrent exposure to Cr and NH_3 resulted in a significant upregulation of *HSP 70* ($p = 0.001$) and *iNOS* ($p = 0.0021$) expression compared to the control and curcumin supplemented groups. Conversely, dietary curcumin supplementation at 0.1% and 0.2% significantly downregulated *HSP70* and *iNOS* expression compared to the control and other groups (Fig. 1A,B).

Anti-oxidative enzymes

The liver and kidney tissues of *P. hypophthalmus* were to assess anti-oxidative enzymes, including as catalase (CAT), superoxide dismutase (SOD), and glutathione-s-transferase (GST) (Fig. 2A–C). Upon exposure to a low dose of concurrent exposure to Cr and NH_3, CAT activity significantly increased in the liver ($p = 0.0016$) and kidney ($p = 0.0013$) tissues. However, CAT activity was considerably reduced in the group provided dietary curcumin at 0.1% and exposed to Cr + NH_3 compared to the control and the group fed curcumin at 0.2%. In contrast, the group fed curcumin at 0.2% and subjected to Cr + NH_3 exhibited significantly higher CAT gene expression than the control and the 0.1% curcumin group (Fig. 2A). SOD activities in the kidney ($p = 0.022$) and liver ($p = 0.0032$) was significantly upregulated upon concurrent exposure to low dose of Cr + NH_3 compared to control and curcumin supplemented groups. Furthermore, dietary curcumin at 0.1% significantly reduced ($p < 0.05$) SOD activity in liver and kidney compared to the control and 0.2% curcumin fed group (Fig. 2B). Similarly, GST activity was significantly elevated in the liver ($p = 0.0018$) and kidney ($p = 0.0025$) under concurrent Cr and NH_3 stress compared to 0.1% curcumin group. However, dietary, curcumin at 0.1% significantly reduced GST activity in liver and kidney tissue compared to control and 0.2% curcumin fed group at 0.2%. However, dietary curcumin at 0.1% significantly reduced GST activity in both tissues compared to the control and the 0.2% curcumin-fed group. Additionally, GST activity in the kidney was significantly lower in the 0.2% curcumin group compared to the control, while GST activity in the liver was also markedly reduced in the 0.2% curcumin group (Fig. 2C).

Neurotransmitter enzymes

Figure 3A shows the neurotransmitter enzyme in the brain tissue of *P. hypophthalmus* reared under control or stressors group (Cr + NH_3). Compared to dietary curcumin fed groups, the acetylcholinesterase activity (AChE) in the brain was significantly decreased ($p = 0.0046$) in the group fed a control diet and concurrently exposed to

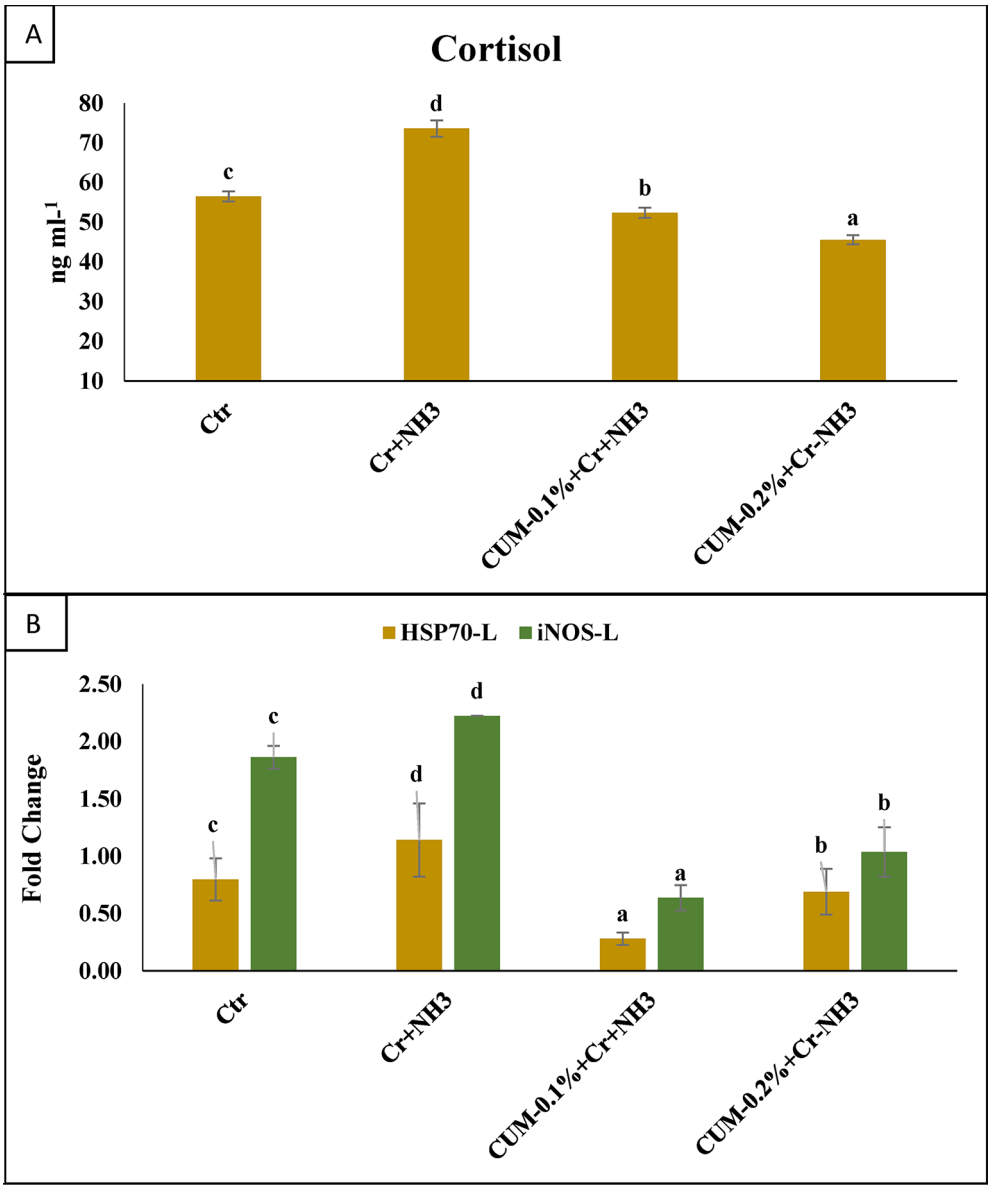

Fig. 1. (**A**, **B**) Effect of dietary curcumin on cortisol ($p = 0.0051$), and gene expression of heat shock protein (*HSP*, $P = 0.001$) and inducible nitric oxide synthase (*iNOS*, $P = 0.0021$) of *P. hypophthalmus* reread under chromium and ammonia toxicity for 40 days. Within endpoints and groups, bars with different superscripts differ significantly (**a**–**d**). Data expressed as Mean ± SE ($n = 3$).

Cr and NH_3. Furthermore, as compared to the control group that also had concomitant exposure to Cr and NH_3 stress, the AChE activity was noticeably enhanced with dietary curcumin at 0.1% followed by 0.2% fed group.

Cellular metabolic enzymes

The activities of lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) in liver and gill tissues of *P. hypophthalmus* are presented in Fig. 3B,C. Upon concurrent exposure to Cr + NH_3, LDH activity was significantly higher in the liver ($p = 0.017$) and gill ($p = 0.019$) compared to the control and curcumin-supplemented fed groups. However, in the group supplemented with dietary curcumin at 0.1% and exposed to Cr + NH_3, LDH activity in the liver and gill was significantly lower than the control and 0.2% curcumin fed group. Additionally, a substantial difference in LDH activity was observed in the liver and gill tissues (Fig. 3B), with liver LDH level being significantly higher in the 0.2% curcumin group compared to the control. Moreover, compared to the control, stressors group (Cr + NH_3) and 0.2% curcumin fed group, MDH activity was significantly lower in the liver ($p = 0.002$) and gill ($p = 0.001$) tissue of *P. hypophthalmus* in the 0.1% curcumin fed group exposed to Cr + NH_3. Conversely, the 0.2% curcumin group exhibited significantly higher MDH activity in the gill tissue compared to all other groups (Fig. 3C). Similarly, ALT (liver, $p = 0.0026$; gill, $p = 0.0018$) and AST (liver, $p = 0.0031$; gill, $p = 0.0011$) activities in both liver and gill tissues were significantly higher in the control diet group subjected to Cr + NH_3 exposure compared to the control and curcumin-supplemented diet groups.

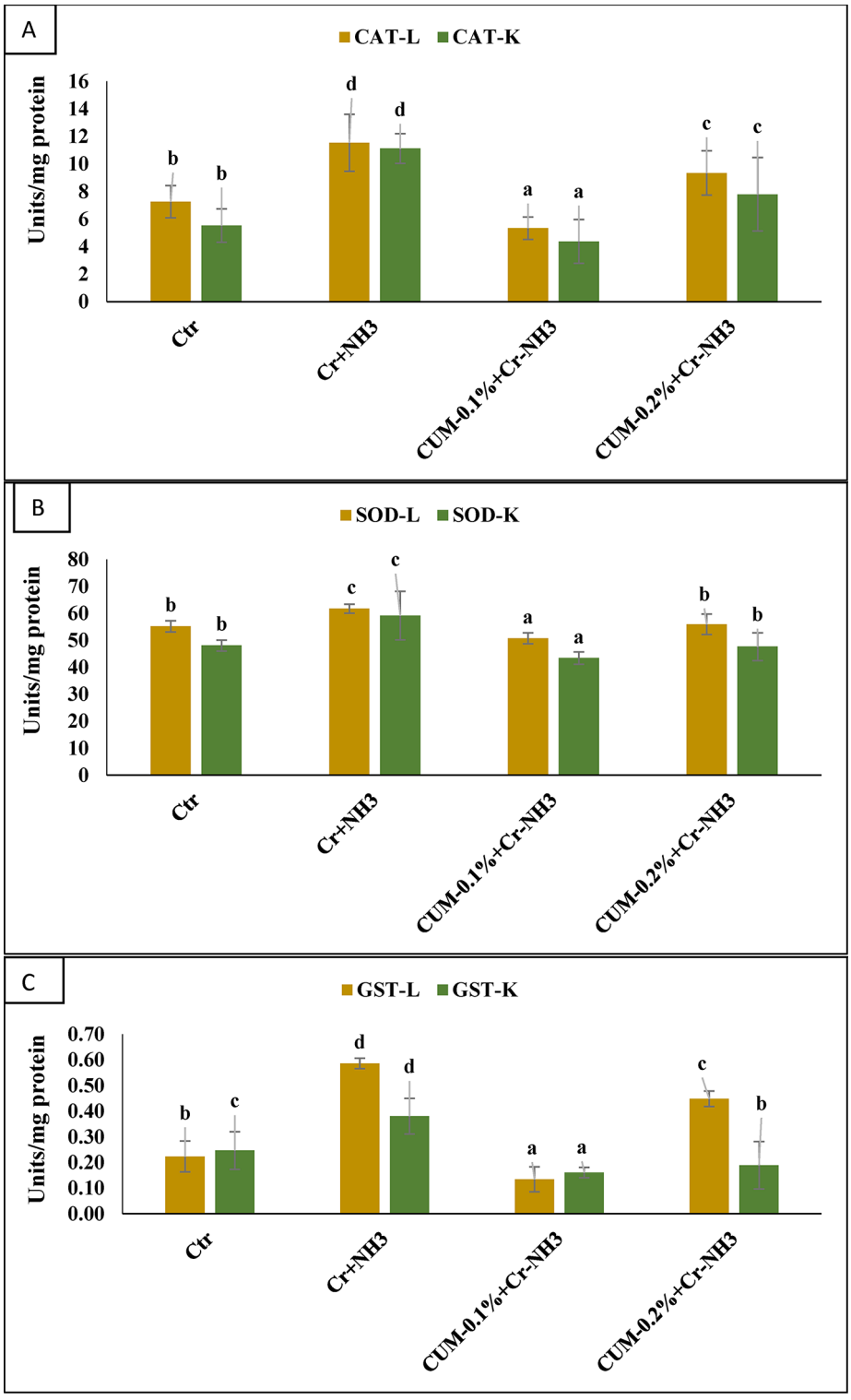

Fig. 2. (**A**–**C**) Effect of dietary curcumin on CAT (Liver, $p = 0.0016$; Kidney, $p = 0.0013$), SOD (Liver, $p = 0.0032$; Kidney, $p = 0.022$) and GST (Liver, $p = 0.0018$, kidney, $p = 0.0025$) in liver and kidney tissues of *P. hypophthalmus* reread under chromium and ammonia toxicity for 40 days. Within endpoints and groups, bars with different superscripts differ significantly (**a**–**d**). Data expressed as Mean ± SE ($n = 3$).

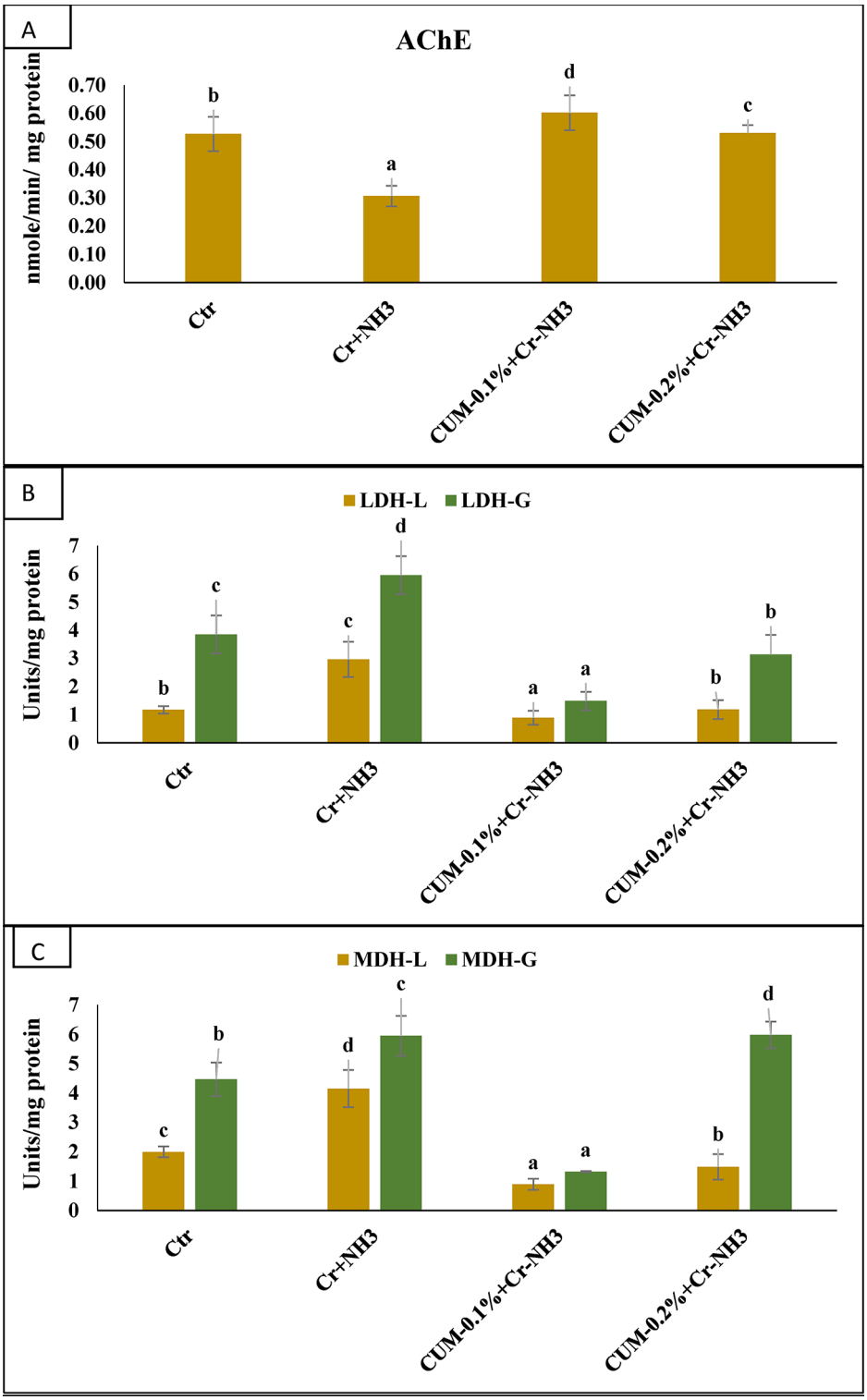

Fig. 3. (**A**–**C**) Effect of dietary curcumin on acetylcholinesterase (AChE, $p = 0.0046$) in brain, lactate dehydrogenase (LDH, liver, $p = 0.017$, gill $p = 0.019$) and malate dehydrogenase (MDH, liver, $p = 0.002$, gill, $p = 0.001$) in liver and gill tissues of *P. hypophthalmus* reread under chromium and ammonia toxicity for 40 days. Within endpoints and groups, bars with different superscripts differ significantly (**a**–**d**). Data expressed as Mean ± SE ($n = 6$).

However, supplementation with curcumin at 0.1% significantly reduced ALT activity in both tissues compared to the control, stressor-exposed ($Cr + NH_3$), and 0.2% curcumin-fed groups. While curcumin at 0.1% effectively reduced ALT activity in both tissues, curcumin at 0.2% was less efficient in modulating ALT levels (Fig. 4A). Furthermore, supplementation with curcumin at 0.1% and 0.2% significantly reduced AST activity in the liver ($p = 0.016$) and gill ($p = 0.002$) compared to the control diet group concurrently exposed to $Cr + NH_3$ (Fig. 4B).

Digestive enzymes

The intestinal tissue of *P. hypophthalmus* was analysed under controlled conditions and concurrent exposure to Cr and NH_3, to assess protease and amylase activities (Fig. 4C). Protease activity was significantly increased ($p = 0.0028$) in the groups supplemented with curcumin at 0.1% and 0.2% compared to the control group and the group exposed to $Cr + NH_3$ while receiving a control diet. Conversely, protease activity was significantly lower in the control diet group exposed to $Cr + NH_3$ compared to the control and curcumin-supplemented groups. Similarly, amylase activity was significantly enhanced ($p = 0.016$) in the group supplemented with curcumin at 0.1% and exposed to $Cr + NH_3$ compared to the control, 0.2% curcumin, and $Cr + NH_3$-exposed groups. Amylase activity was comparable between the control and 0.2% curcumin-fed groups, while the lowest amylase activity was observed in the control diet group concurrently exposed to Cr and NH_3.

Apoptotic and detoxifying gene

Compared to the control and curcumin-supplemented groups, the gene expression of cytochrome P450 (*CYP450*) in liver tissue was significantly upregulated ($p = 0.0018$) in the control diet group concurrently exposed to Cr and NH_3. Notably, *CYP450* gene expression was significantly downregulated in the group supplemented with 0.1% curcumin relative to the stressor-exposed ($Cr + NH_3$) group and in the stressor-exposed group fed a control diet (Fig. 5A). Similarly, caspase (*Cas 3a*) expression was significantly downregulated ($p = 0.0013$) in the 0.1% curcumin-supplemented group exposed to $Cr + NH_3$ compared to the control, stressor-exposed, and 0.2% curcumin-fed groups. Among all groups, the control diet group concurrently exposed to NH_3 exhibited the highest *Cas 3a* expression in liver tissues (Fig. 5A).

Total immunoglobulin and tumor necrosis factors genes

Figure 5B presents the gene expression levels of total immunoglobulin (*Ig*) and tumor necrosis factor-alpha (*TNFα*) in the liver tissue of *P. hypophthalmus* exposed to low doses of Cr and NH_3. Compared to the control and stressor-exposed ($Cr + NH_3$) groups, *Ig* gene expression was significantly upregulated ($p = 0.0011$) in the group supplemented with 0.1% curcumin, followed by the 0.2% curcumin group exposed to stressors. Conversely, *Ig* expression was markedly downregulated in the group fed a control diet and concurrently exposed to $Cr + NH_3$. Similarly, *TNFα* expression exhibited an inverse correlation with Ig expression. In the presence of stressors, dietary supplementation with 0.1% curcumin resulted in a significant downregulation of *TNFα* ($p = 0.0011$) compared to the control diet group concurrently exposed to Cr and NH_3.

Growth performance

The growth parameters of *P. hypophthalmus* reared under control conditions, with or without exposure to Cr and NH_3, were assessed, including weight gain percentage, feed conversion efficiency (FCE), specific growth rate (SGR), protein efficiency ratio (PER), daily growth index (DGI), and relative feed intake (RFI). The results are presented in Table 3. Compared to the control group and the group fed a control diet while concurrently exposed to Cr and NH_3, the weight gain percentage ($p = 0.0042$), FCE ($p = 0.0031$), SGR ($p = 0.0027$), PER ($p = 0.0023$), DGI ($p = 0.016$), and RFI ($p = 0.026$) were significantly higher in the group supplemented with 0.1% curcumin, followed by the 0.2% curcumin group. Conversely, the group exposed to Cr and NH_3 while fed a control diet exhibited a significant reduction in all growth-related parameters, including weight gain percentage, FCE, SGR, PER, DGI, and RFI, compared to the other groups. Additionally, Fig. 6A,B present the expression of growth-related genes (*GH*, *GHR1*, and *MYST*) in *P. hypophthalmus* reared under control conditions or exposed to Cr and NH_3 toxicity and fed curcumin-supplemented diets. Compared to the control and stressor-exposed ($Cr + NH_3$) groups, the genes regulating growth hormone (*GH*) ($p = 0.0034$) and growth hormone receptor 1 (*GHR1*) ($p = 0.0018$) were significantly upregulated in the groups fed 0.1% curcumin and subjected to $Cr + NH_3$. Additionally, *GH* and *GHR1* were significantly downregulated in the group fed a control diet and exposed to Cr and NH_3 compared to the control and curcumin-supplemented groups (Fig. 6A). Furthermore, dietary supplementation with 0.1% curcumin significantly downregulated *MYST* expression ($p = 0.0037$) compared to the other groups. In contrast, *MYST* expression was significantly elevated in the group exposed to Cr and NH_3 and fed a control diet compared to the control and curcumin-supplemented groups (Fig. 6B).

Chromium bioaccumulation

The chromium (Cr) concentration in water samples and its bioaccumulation in various tissues, including muscle, liver, kidney, gill, and brain, are presented in Table 4. The Cr concentrations in water varied across different groups, with levels of 0.01 μg L^{-1} in the control group, 2352 μg L^{-1} in the $Cr+NH_3$-exposed group, and 492 μg L^{-1} and 580 μg L^{-1} in the groups supplemented with 0.1% and 0.2% curcumin, respectively. Furthermore, the highest Cr bioaccumulation was observed in the liver (3.40 mg kg^{-1}), followed by the kidney (3.17 mg kg^{-1}) and gill (2.47 mg kg^{-1}). The results also indicate that dietary supplementation with 0.1% curcumin led to the lowest Cr bioaccumulation across all tissues.

Discussion

In the present investigation, concurrent exposure to chromium (Cr) and ammonia stress led to an elevated cortisol level in *P. hypophthalmus*. The Cr has strong association with cortisol secretion in stress condition,

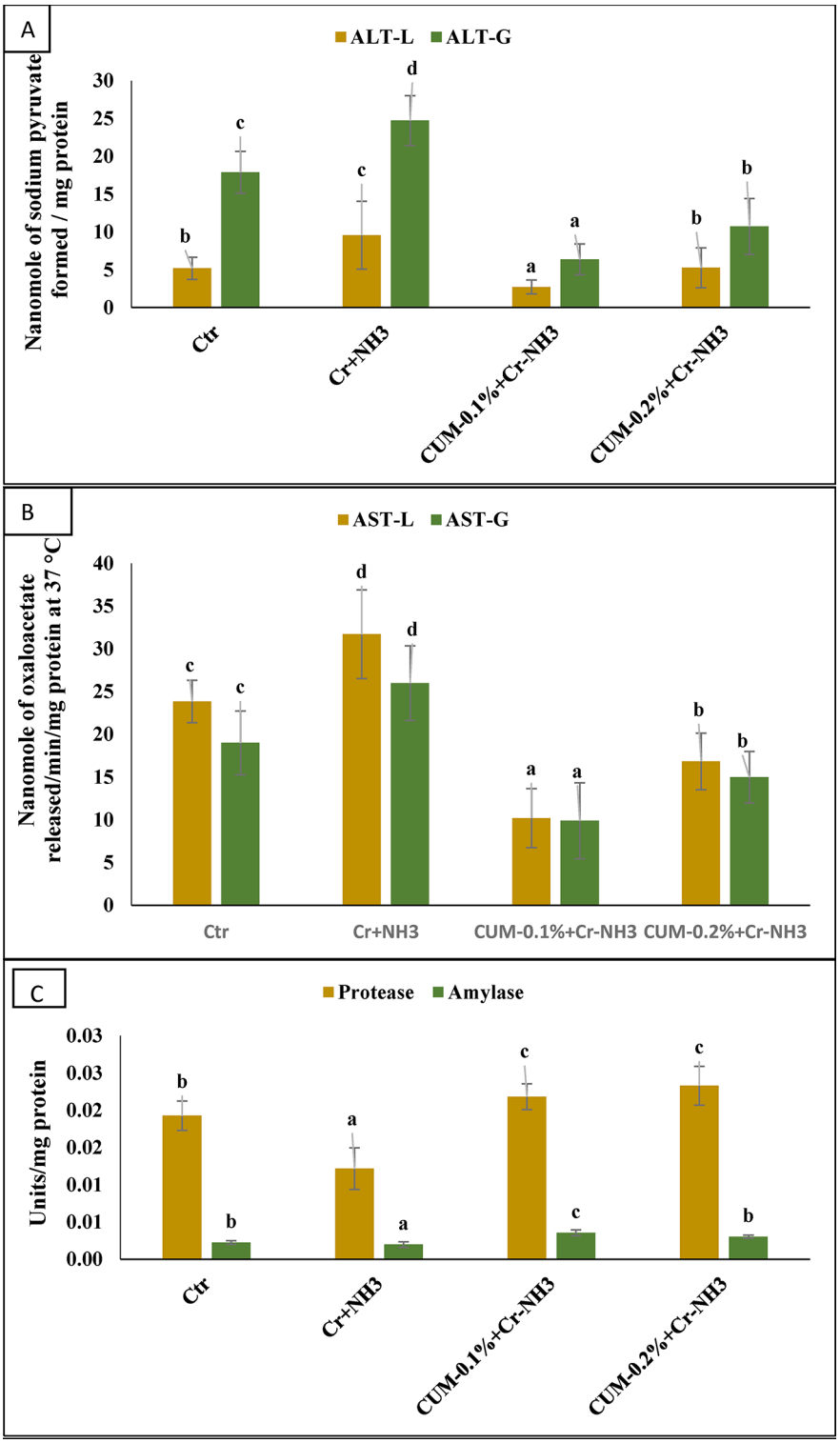

Fig. 4. (**A**–**C**) Effect of dietary curcumin on alanine aminotransferase (ALT, liver, $p = 0.0026$, gill, $p = 0.0018$), aspartate aminotransferase (AST, liver, $p = 0.0031$, gill, $p = 0.0011$) in liver and gill and protease ($p = 0.0028$) and amylase ($p = 0.016$) activity of *P. hypophthalmus* reread under chromium and ammonia toxicity for 40 days. Within endpoints and groups, bars with different superscripts differ significantly (**a**–**d**). Data expressed as Mean ± SE ($n = 6$).

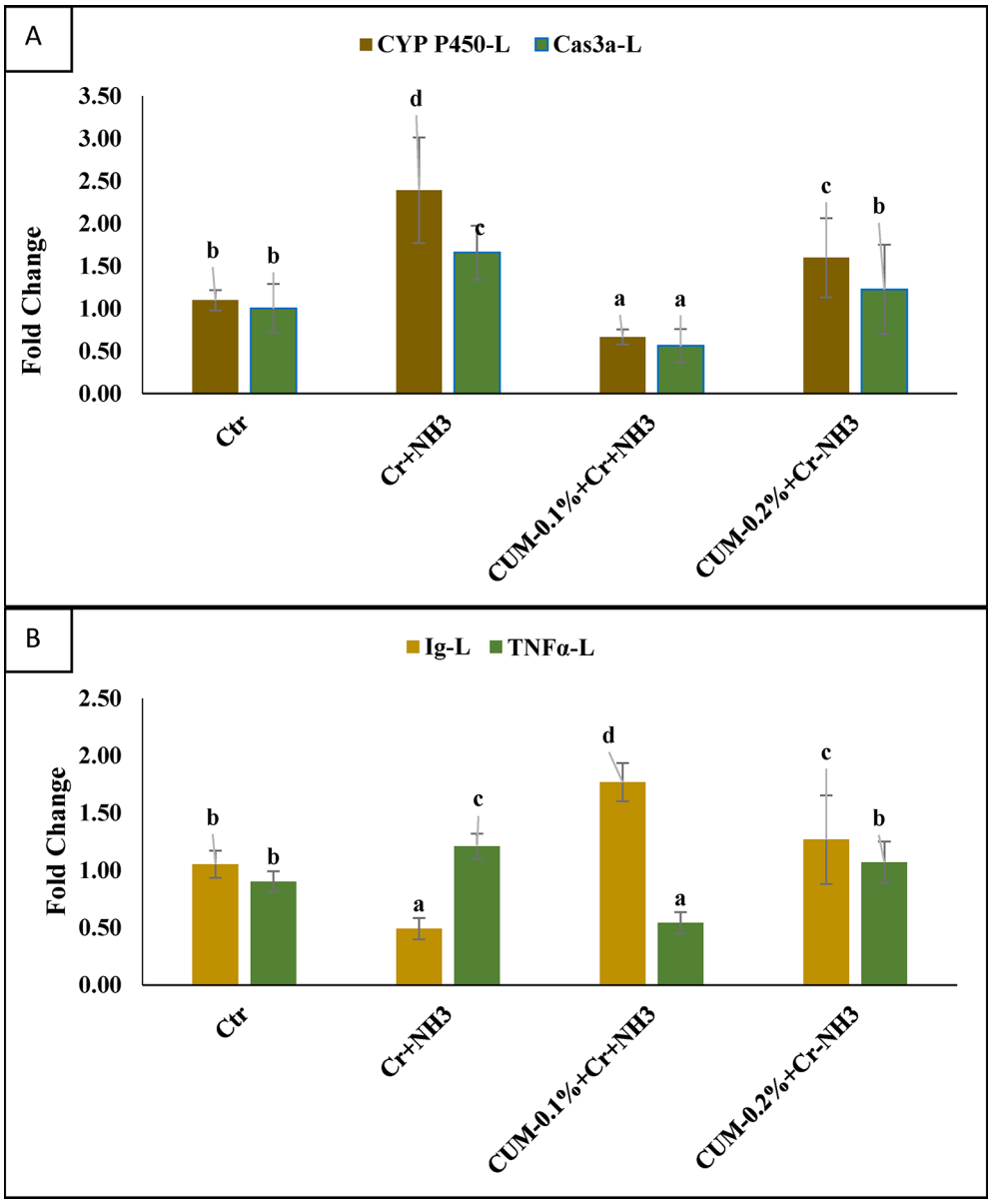

Fig. 5. (**A**, **B**) Effect of dietary curcumin on gene expression of *CYP P450* ($p = 0.0018$), *Cas 3a* (0.013), *Ig* ($p = 0.011$), and *TNFα* ($p = 0.0027$) in liver tissue of *P. hypophthalmus* reread under chromium and ammonia toxicity for 40 days. Within endpoints and groups, bars with different superscripts differ significantly (**a**–**d**). Data expressed as Mean ± SE ($n = 3$).

activating metabolic process in response to Cr toxicity[45]. It has also been demonstrated that the elevated cortisol levels may be physiological response to restore homeostasis in fish, protecting them against Cr toxicity[46]. Surprisingly, concurrent exposure to Cr and NH_3 disturbs the central nervous system, affecting glycogenolysis and gluconeogenesis[13,47]. Interestingly, dietary curcumin at 0.1% effectively reduced cortisol levels, likely due to polyphenolic nature of curcumin. Polyphenol in curcumin exhibit antioxidant properties, neutralizing lipid radicals in the cell membrane and converting them into phenoxyl radicals, ultimately reducing cortisol levels[48–50]. Abdel-Ghany et al.[51] reported that nano-curcumin supplementation at 50 and 100 mg kg^{-1} significantly reduces cortisol levels in Tilapia reared under heat stress. Furthermore, the cortisol levels observed in this study were consistent with *HSP 70* gene expression in *P. hypophthalmus* liver tissue. The current investigation found that concurrent exposure to low doses of Cr and NH_3 upregulated *HSP 70* gene expression likely due to Cr and NH_3 altering catecholamine levels, leading to excessive *HSP 70* expressions[13]. Interestingly, dietary curcumin at 0.1% protects the chaperon's family *HSP 70* by downregulating its production, thereby preventing protein degradation[23]. Curcumin also facilitates HSP *70* binding to denatured proteins, preventing their further misfolding and promoting their recovery to maintain tissue function[52] (Heredia-Middleton et al., 2008).

Surprisingly, Cr and NH_3 stress significantly upregulated *iNOS* expression, whereas dietary curcumin supplementation downregulated it. Cr and NH_3 exposure stimulate nitric oxide (NO) production, leading to

Diet	Control	Control	Curcumin diets	
Exposure		$Cr + NH_3$	$Cr + NH_3$	$Cr + NH_3$
Treatments	Control		Cum-0.1%	Cum-0.2%
Weight gain %	$74.96^{b} \pm 1.23$	$67.47a \pm 0.57$	$85.65^{d} \pm 1.59$	$77.83^{c} \pm 1.26$
FCE	$0.38^{b} \pm 0.01$	$0.27^{a} \pm 0.01$	$0.49^{d} \pm 0.02$	$0.41^{c} \pm 0.01$
SGR	$0.53^{b} \pm 0.02$	$0.37^{a} \pm 0.01$	$0.68^{d} \pm 0.03$	$0.59^{c} \pm 0.02$
PER	$1.09^{b} \pm 0.01$	$0.77^{a} \pm 0.03$	$1.39^{d} \pm 0.04$	$1.25^{c} \pm 0.08$
DGI	$4.59^{b} \pm 0.10$	$3.12^{a} \pm 0.04$	$6.09^{d} \pm 0.15$	$4.99^{c} \pm 0.07$
RFI	$160.99^{b} \pm 0.78$	$145.79^{a} \pm 0.22$	$171.41^{d} \pm 1.31$	$161.33^{c} \pm 0.93$

Table 3. Effect of dietary Curcumin on growth performance (Weight gain %, FCE, SGR, PER, DGI and RFI) of *P. hypophthalmus* reread under chromium and ammonia toxicity for 40 days. Values in the same row with different superscript (a, b, c, d) differ significantly. Data expressed as Mean ± SE (*n* = *3*). FCR feed conversion ratio, SGR specific growth rate, PER protein efficiency ratio, DGI Daily growth index, RFI relative feed intake.

oxidative damage by nitrating biomolecules such as DNA[53]. Additionally, ammonia accumulation in various tissues contributes to NO production, with ammonia being a key factor influencing *iNOS* gene expression in muscle tissue[54]. Interestingly, dietary curcumin may play a crucial role in suppressing constitutive NF-kappaB (NF-κB) and IKK activity[55]. Furthermore, curcumin inhibits *iNOS* expression by reducing NF-κB translocation[56]. The findings suggest that curcumin mitigates the harmful effects of Cr and NH_3 by modulating NF-κB signaling pathways[57].

The activities of CAT, SOD and GST in liver and kidney were significantly elevated under Cr and NH_3 toxicity. However, dietary curcumin effectively substantiated the CAT, SOD and GST in liver and kidney tissues. Concurrent exposure to Cr and NH_3 accelerates free radical production in these tissues. Cr is generally required as a catalyst for reversible oxidation using hydrogen oxide (HO)[58]. Cr (VI) is particularly responsible for production of reactive oxygen species (ROS) through a Fenton-like redox cycling mechanism[58]. It also interferes with mitochondria regulation[59]. ROS attack biomolecules such as lipids, protein, nucleic acids, disrupting cellular function and integrity[60]. Interestingly, 0.1% dietary curcumin supplementation significantly enhanced the anti-oxidative status of *P. hypophthalmus* by modulating CAT, SOD, and GST activities in liver and kidney tissues. This effect is most likely attributed to curcumin's antioxidant properties as it functions both as a direct radical scavenger and an inducer of anti-oxidant responses[61]. Additionally, curcumin contributes to reducing oxidative damage by enhancing various anti-oxidant defences, thereby protecting fish from Cr and NH_3 stress[62–64].

Acetylcholinesterase (AChE) activity in brain tissue of *P. hypophthalmus* was significantly inhibited under Cr and NH_3 stress. AChE inhibition is a key indicator of nervous systems damage, leading to behavioural, feeding, and respiration alteration in the organism[60,65]. The combined exposure also blocks the enzyme's active site, alter its structure and affects the amino acid sequence[13,66]. Surprisingly, dietary curcumin protects neurotransmitter from damage and blockage by enhancing AChE activity in brain tissue. Moreover, curcumin has neuroprotective nature due to their role in cross blood-brain barrier[67]. Many investigations have shown that curcumin lowers AChE activity following exposure to hazardous chemicals[67,68].

The supplementation of curcumin diet improved AChE activity. The current investigation found that exposure to Cr and NH_3 significantly increased LDH and MDH activities in liver and gill tissues. However, dietary curcumin at 0.1% significantly reduced LDH and MDH activity. It has been shown that under condition of oxygen and/or glucose deprivation, cells containing LDH are damaged or killed, leading to increased cell membrane permeability or rupture causing enzyme leakage[69–72].

ALT and AST activities in liver and gill tissues were significantly elevated under concurrent exposure to Cr and NH_3. However, dietary curcumin at 0.1% noticeably reduces the ALT and AST activities. This is likely due to ability of curcumin to protect the liver and gill tissue against oxidative stress caused by hydrogen peroxide[72]. Curcumin also acts as anti-inflammatory agent. Its ability to reduce ALT and AST activities suggests a hepatoprotective effect against toxic materials[73]. Additionally, curcumin may play a crucial role in stabilizing tissues cell membrane, preventing the leakage of the intracellular enzymes[74]. The present result of ALT and AST activities suggest that curcumin protects liver and gill tissues from the stress ($Cr + NH_3$) while maintaining cell membranes integrity and function[75].

The present study also revealed that concurrent exposure to Cr and NH_3 reduced the activity of digestive enzymes such as protease and amylase. However, curcumin supplementation improved digestive enzymes activity by enhancing intestinal enzymes function and increasing the number of mature intestinal cells[76]. Digestive enzyme activity directly influences nutrient absorption in fish[77]. A study on *Oreochromis niloticus* demonstrated that diets supplemented with 0.5 and 1.0% curcumin significantly improved the activities of digestive enzymes, including protease, amylase and lipase[78]. Furthermore, digestive enzymes activity is closely associated with fish growth performance. Interestingly, the present study indicates that curcumin has the potential to enhance digestive enzymes activity, intestinal absorption efficiency and overall nutrient utilization[79].

In this study, concurrent exposure to Cr and NH_3 upregulated *CYP450* gene expression. In contrast, dietary curcumin supplementation reduced *CYP450* activity in liver tissue. *CYP450* plays a key role in metabolic pathway involving lipoxygenase, arachidonic acid, and cyclooxygenase. Moreover, it contributes to the formation of carcinogenic electrophilic intermediates from naturally occurring compounds like curcumin and is involved in detoxification and xenobiotic metabolism. However, our findings indicate that Cr and NH_3 exposure

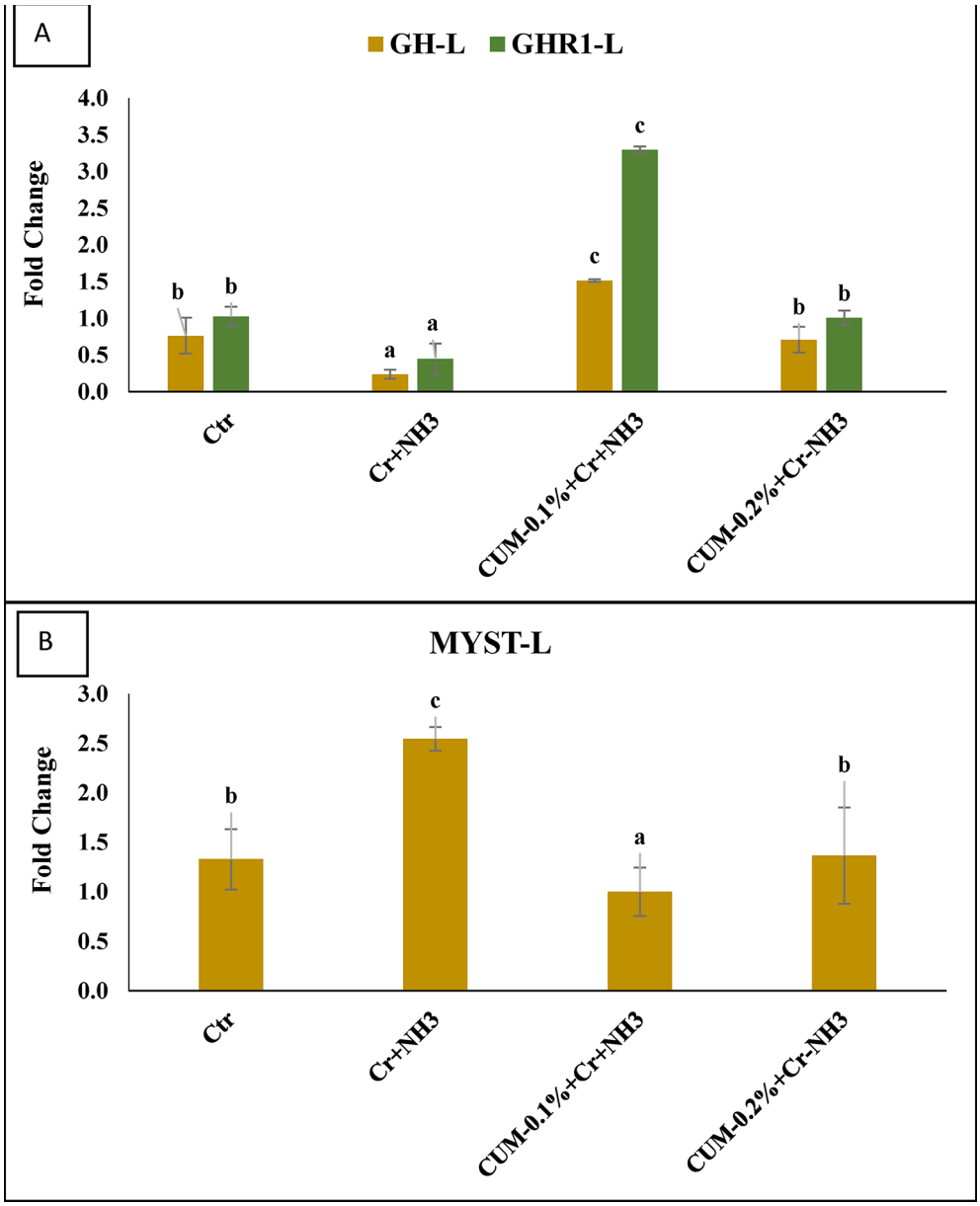

Fig. 6. (**A**, **B**) Effect of dietary curcumin on gene expression of *GH* ($p = 0.0034$), *GHR1* ($p = 0.0018$) and *MYST* ($p = 0.0037$) in liver tissue of *P. hypophthalmus* reread under chromium and ammonia toxicity for 40 days. Within endpoints and groups, bars with different superscripts differ significantly (**a**–**d**). Data expressed as Mean ± SE ($n = 3$).

Treatments	Control	Cr + NH_3	Cum-0.1%-Cr + NH_3	Cum-0.1%-Cr + NH_3
Water (μg L^{-1})	0.01 ± 0.0	2352.94 ± 11.21	492.05 ± 9.14	580.54 ± 3.23
Muscle (mg L^{-1})	ND	1.76 ± 0.07	0.17 ± 0.03	0.70 ± 0.18
Liver (mg L^{-1})	0.04 ± 0.0	3.40 ± 0.09	1.57 ± 0.04	1.86 ± 0.05
Kidney (mg L^{-1})	0.01 ± 0.0	3.17 ± 0.03	1.62 ± 0.08	2.05 ± 0.05
Gill (mg L^{-1})	0.02 ± 0.0	2.47 ± 0.21	1.41 ± 0.22	1.14 ± 0.02
Brain (mg L^{-1})	ND	0.88 ± 0.05	0.40 ± 0.09	0.27 ± 0.03

Table 4. Effect of dietary Curcumin on chromium bioaccumulation in water and different fish tissues of *P. hypophthalmus* reread under chromium and ammonia toxicity for 40 days. Data expressed as Mean ± SE ($n = 5$).

significantly upregulated *CYP450* gene expression. Previous studies have also reported that exposure to arsenic, ammonia, and high temperature, upregulates *CYP450* expression[80–82]. Notably, Giri et al.[83] found that curcumin downregulates *CYP450* gene expression in fish, suggesting its protective role against oxidative stress.

Cas 3a plays a crucial role in apoptosis, which is closely linked to oxidative stress and inflammatory responses[84]. In this study, Cr and NH_3 stress induced apoptosis via *p53*, a key regulator of apoptosis[85]. The findings were correlated with *CYP450*, which is implicated in stress-induced apoptosis and up-regulates the transcription of bcl2-associated X protein (Bax)[86,87]. Surprisingly, Bax is a major pro-apoptotic gene in fish, triggering the release of cytochrome C and caspase activation[88]. Furthermore, dietary curcumin inhibited the *Cas 3a* gene expression in liver tissue by activating caspase-3 and cleaving poly (ADP-ribose) polymerase 1 (PARP)[89].

Curcumin is effective anti-inflammatory agent, protecting tissues from damage caused by toxin substances. In this study, Cr and NH_3 exposure downregulated *TNFα* gene expression in liver tissue indicating damage. NFκB regulates genes involved in the production of proinflammatory cytokines, including TNF-α, thereby contributing to inflammation[90]. Curcumin is known to reduce inflammation and protect tissues from injury[57].

Interestingly, Cr and NH_3 stress significantly impaired fish growth performance However, dietary curcumin at 0.1 and 0.2%, dramatically improved growth performance and mitigated the toxic effect of Cr and NH_3 toxicity. Curcumin's growth-promoting effects may be attributed to its role in enhancing digestive enzyme activity[91], modulating immunity[92], and exerting anti-oxidative and anti-stress properties[93,94]. The growth promoting benefits of curcumin are primarily due to its ability to enhance digestive enzymes, particularly those involved in protein metabolism. Additionally, curcumin contributes to overall health by acting as a selective growth factor and substrate for beneficial gut microbiota while inhibiting harmful intestinal bacteria. Previous studies have also reported curcumin as a growth enhancer in fish[93,94].

The present findings are further supported by the expression of *MYST*, *GH*, and *GHR1* genes. While NH_3 exposure upregulated *MYST* gene expression in liver tissue, curcumin supplementation inhibited its expression. Furthermore, curcumin upregulated *GH* and *GHR1* gene expression, whereas NH_3 stress downregulated them. Curcumin's ability to enhance growth-related gene expression and improve growth performance may be attributed to its role in RNA, DNA, and protein synthesis, particularly the regulation of *GH* gene production. Additionally, curcumin modulates the insulin-like growth factor signaling pathway to promote growth[95]. Interestingly, curcumin has been shown to enhance Cr detoxification in muscle, liver, kidney, gill, and brain tissues. The findings related to *CYP450* and caspase-3 corroborate these results. Overall, curcumin exhibits significant potential as a detoxifying agent, effectively reducing Cr bioaccumulation in various organs.

Conclusion.

The current study revealed that incorporating a 0.1% curcumin diet significantly enhanced the antioxidative capacity, growth performance, and digestive efficiency of *P. hypophthalmus* reared under chromium and ammonia toxicity (Cr + NH_3). Moreover, curcumin supplementation at 0.1% positively influenced the regulation of key genes, including *HSP 70*, *iNOS*, *TNFα*, and *Ig*, particularly in fish exposed to when fish were subjected to simultaneous exposure to Cr and ammonia toxicity. Significant improvements were also observed in the expression of detoxification-associated genes, such as *CYP450* and *caspase*, when dietary curcumin at 0.1% was introduced, strengthening the fish's resilience against the combined effects of Cr and ammonia toxicity. Additionally, growth-related genes such as *GH* and *GHR1* exhibited upregulation, while *MYST* showed downregulation with 0.1% curcumin supplementation, contributing to an overall enhancement in the fish's growth performance. Furthermore, the supplementation of curcumin at 0.1% in the diet led to a substantial reduction in Cr accumulation. Notably, this study is the first to highlight the role of curcumin in mitigating the adverse effects of simultaneous Cr and NH_3 stress in fish.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Declarations

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Ethics approval
Institute Research Advisory Committee (RAC) has approved the experimental procedures. The present study was in complied with ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. The methodology and care and maintenance of the fish were conducted in accordance with the relevant guidelines and regulations.

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