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RESEARCH ARTICLE

Immunomodulatory Effects of Nellikai Thenural Against Thiamethoxam-Induced Toxicity in *Carassius auratus*

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Abstract: The accumulation of pesticides in aquatic ecosystems poses significant risks to non-target organisms, particularly fish populations. The study evaluated the protective effects of Nellikai Thenural, a traditional Siddha formulation containing *Emblica officinalis* and honey, against thiamethoxam-induced toxicity in *Carassius auratus* (goldfish). The experimental design comprised four groups: control, Nellikai Thenural-treated (733 µg), thiamethoxam-exposed (114.84 mg/L), and co-administered (thiamethoxam + Nellikai Thenural) groups. The LC50 value of Nellikai Thenural was determined at 916 µg/ml/kg body weight through brine shrimp lethality assay. Hematological parameters revealed significant alterations in thiamethoxam-exposed fish, with decreased RBC count, hemoglobin levels, and WBC count. The administration of Nellikai Thenural effectively restored these parameters toward normal levels. Biochemical markers, including plasma proteins, immunoglobulins, and tissue-specific enzymes (ALP, ACP, AST), showed marked improvement in the co-administered group compared to thiamethoxam-exposed fish. Behavioral observations indicated reduced stress responses and improved swimming patterns in fish treated with Nellikai Thenural. The findings demonstrate the potential of Nellikai Thenural as a natural immunostimulant and protective agent against pesticide-induced toxicity in aquatic organisms.

Keywords: Risperidone; Trihexyphenidyl HCl; Method development; Method validation; RP-HPLC.

1. Introduction

Environmental contamination by pesticides remains a significant global concern, particularly affecting aquatic ecosystems through agricultural runoff and direct applications. Neonicotinoid pesticides, specifically thiamethoxam, have gained widespread agricultural use due to their effective pest control properties [1]. However, their persistence in aquatic environments poses substantial risks to non-target organisms, especially fish populations, which serve as essential bioindicators of ecosystem health [2].

Thiamethoxam, a second-generation neonicotinoid, operates by binding to nicotinic acetylcholine receptors, disrupting neural transmission in target insects [3]. When introduced into aquatic systems, fish become exposed through multiple routes, including gill absorption, dermal contact, and dietary intake [4]. The accumulated pesticide triggers oxidative stress, impairs acetylcholinesterase activity, and induces histopathological alterations in vital organs [5].

Traditional medicine systems offer potential solutions for mitigating environmental toxicity. The Siddha system of medicine, indigenous to South India, encompasses various formulations with therapeutic properties [6]. Nellikai Thenural, a traditional Siddha preparation combining *Emblica officinalis* (Indian gooseberry) and honey, contains bioactive compounds with established antioxidant and immunomodulatory properties [7]. The primary constituents of *E. officinalis* include ascorbic acid, gallic acid, ellagic acid, and various polyphenols, which contribute to its therapeutic efficacy [8].

Carassius auratus (goldfish) serves as an ideal model organism for aquatic toxicology studies due to its sensitivity to environmental pollutants and well-characterized physiological responses [9, 10].

The present study investigates the protective effects of Nellikai Thenural against thiamethoxam-induced toxicity in C. auratus by evaluating hematological parameters, biochemical markers, and behavioral responses. This research work aims to establish the potential of traditional Siddha formulations in counteracting pesticide-induced stress in aquatic organisms and maintaining ecosystem balance.

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2. Materials and Methods

2.1. Experimental Animals

Adult *Carassius auratus* (goldfish) weighing 30±5g and measuring 10±2cm in length were procured from a certified aquaculture facility. Fish were acclimatized in glass aquaria (40L capacity) containing dechlorinated water maintained at temperature 25±2°C, pH 7.2±0.2, dissolved oxygen 6.8±0.5 mg/L, and total hardness 165±5 mg/L as $CaCO_3$ [11]. The acclimatization period lasted 14 days under natural photoperiod (12h light:12h dark). Fish were fed commercial pellets (protein content 32%) twice daily at 3% body weight. Water was renewed every 48 hours to maintain optimal conditions [12].

2.2. Preparation of Nellikai Thenural

Fresh fruits of Emblica officinalis were authenticated by taxonomists at the Department of Botany (voucher specimen number: EO-2023-TAC). The fruits were cleaned, shade-dried at room temperature (27±2°C), and pulverized to fine powder (mesh size 60) [13]. Nellikai Thenural was prepared following traditional Siddha formulation guidelines by combining E. officinalis powder with pure honey in a 2:1 ratio [14]. The preparation was stored in an amber-colored container at 4°C until use.

2.3. Determination of Lethal Concentration

Brine shrimp (*Artemia salina*) lethality assay was performed following Meyer's method with modifications [15]. Artemia cysts were hatched in artificial seawater (35 ppt) under constant illumination at 28°C. Serial dilutions of Nellikai Thenural (500, 1000, 1500, 2000, and 2500 µg/mL) were prepared in filtered seawater. Ten nauplii were transferred to each test solution in triplicate. Mortality was recorded at 3-hour intervals over 24 hours using stereomicroscope (3x magnification). LC50 value was determined through probit analysis using Finney's method [16].

2.4. Experimental Design

The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC/TAC/2023/05). Twenty-four healthy fish were randomly divided into four groups (n=6) following a completely randomized design [17]:

Group I (Control): Maintained on standard diet
Group II: Administered Nellikai Thenural (733 µg/0.8mL) via intramuscular injection using 26-gauge needle
Group III: Exposed to thiamethoxam (114.84 mg/L) dissolved in water
Group IV: Co-administered with thiamethoxam (114.84 mg/L) and Nellikai Thenural (733 µg)

The exposure period was 96 hours, based on OECD guidelines for acute toxicity testing in fish [18].

2.5. Growth and Behavioral Assessment

Fish morphometric measurements (total length, standard length, weight) were recorded using digital calipers (±0.01mm) and analytical balance (±0.001g) [19]. Behavioral parameters were monitored continuously and recorded at 6-hour intervals using a modified version of Kane's behavioral scoring system [20]. Parameters included:

- Swimming patterns and orientation
- Opercular movement rate
- Feed intake response
- Mucus secretion
- Equilibrium status
- Surface swimming frequency

2.6. Sample Collection and Processing

Following 96 hours, fish were anesthetized using MS-222 (tricaine methanesulfonate, 100 mg/L) [21]. Blood samples were collected from the caudal vein using heparinized syringes (23-gauge). Tissues (liver, kidney, gills) were excised, washed in ice-cold phosphate-buffered saline (pH 7.4), and divided for biochemical and histological analyses [22].

2.7. Hematological Analysis

Blood samples were analyzed within 2 hours of collection following standardized hematological procedures [23]. For red blood cell (RBC) enumeration, blood was diluted 1:200 with Hayem's solution (sodium chloride 1.0 g, sodium sulfate 5.0 g, mercuric chloride 0.5 g, and distilled water 200 mL) [23]. The diluted sample was loaded onto an improved Neubauer hemocytometer, and cells were counted in five small squares of the central area after 3 minutes of settling time. The final RBC count was expressed as cells × $106/mm^3$ [23].

Total white blood cell (WBC) count was performed using Natt-Herrick's solution (sodium chloride 3.88 g, sodium sulfate 2.50 g, sodium phosphate dibasic 2.91 g, potassium phosphate monobasic 0.25 g, formalin 7.5 mL, methyl violet 0.10 g, and distilled water to 1000 mL) [23]. Blood was diluted 1:100 with the solution and loaded onto the hemocytometer. Cells were counted in all four large corner squares, and the count was expressed as cells × 103/mm3. For differential leukocyte count, thin blood smears were prepared on clean glass slides and air-dried. The smears were fixed in methanol for 5 minutes and stained using Wright-Giemsa solution (Wright's stain 0.3 g, Giemsa stain 0.1 g, glycerol 3 mL, and methanol 97 mL) for 15 minutes [24]. After washing and drying, 100 leukocytes were counted under oil immersion (1000×) and classified as lymphocytes, neutrophils, monocytes, eosinophils, and basophils. Results were expressed as percentage of each cell type [24].

Hemoglobin concentration was determined using the cyanmethemoglobin method [23]. Twenty microliters of blood was mixed with 5 mL of Drabkin's reagent (sodium bicarbonate 1.0 g, potassium ferricyanide 0.2 g, potassium cyanide 0.05 g, and distilled water to 1000 mL) and incubated at room temperature for 10 minutes. The absorbance was measured at 540 nm using a spectrophotometer against a reagent blank. Hemoglobin concentration was calculated using a standard curve prepared with cyanmethemoglobin standard and expressed as g/dL [24]. All hematological parameters were analyzed in triplicate to ensure accuracy and reproducibility. Quality control measures included regular calibration of instruments, use of standard solutions, and incorporation of appropriate controls [25].

2.8. Biochemical Parameters

Plasma proteins were quantified using Lowry's method [24]. Immunoglobulin levels were determined through zinc sulfate turbidity test [25]. Tissue homogenates (10% w/v) were prepared in ice-cold 0.1M phosphate buffer (pH 7.4). Marker enzymes were assayed:

- Alkaline phosphatase (ALP) using p-nitrophenyl phosphate as substrate
- Acid phosphatase (ACP) using α-naphthyl phosphate
- SGOT and SGPT using standardized diagnostic kits [26]

2.9. Statistical Analysis

Data were analyzed using GraphPad Prism (version 8.0). One-way ANOVA followed by Tukey's multiple comparison test was performed. Results were expressed as mean ± SEM, with significance set at $p<0.05$ [27].

3. Results

3.1. Lethal Concentration Assessment

The brine shrimp lethality assay revealed a dose-dependent mortality pattern for Nellikai Thenural. The calculated LC50 value was determined to be 916 µg/ml/kg body weight. Mortality rates increased progressively with concentration, showing 20%, 35%, 48%, 62%, and 85% mortality at 500, 1000, 1500, 2000, and 2500 µg/mL, respectively, over the 24-hour exposure period (results shown in Figure 1).

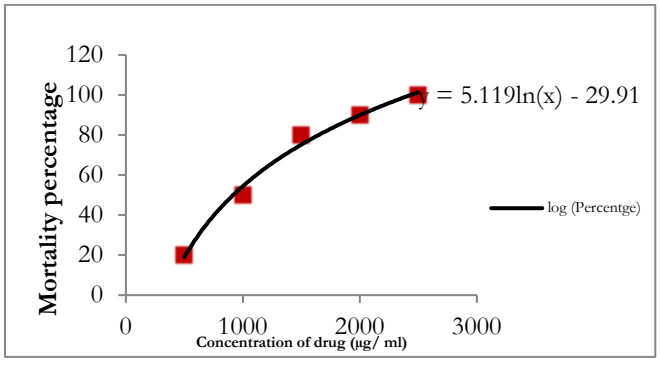


Figure 1. Dose-response curve showing mortality percentage of Artemia salina against different concentrations of Nellikai Thenural over 24 hours

3.2. Growth Parameters

The 96-hour exposure period resulted in significant variations in growth parameters among the experimental groups. Control fish (Group I) maintained stable growth patterns with a mean weight gain of 0.42 ± 0.03g. Nellikai Thenural-treated fish (Group II) showed comparable growth patterns to the control group ($p>0.05$). Thiamethoxam-exposed fish (Group III) exhibited significant

growth reduction ($p<0.001$) with a mean weight loss of 0.68 ± 0.04g. The co-administered group (Group IV) demonstrated partial recovery in growth parameters, with weight changes significantly different from Group III ($p<0.01$).

Table 1. Morphometric measurements of C. auratus across experimental groups at 0 and 96 hours

Parameters	Time (hours)	Group I (Control)	Group II (NT)	Group III (TMX)	Group IV (TMX+NT)
Body weight (g)	0	8.45 ± 0.32	8.38 ± 0.28	8.42 ± 0.35	8.40 ± 0.30
	96	8.87 ± 0.35^a	8.75 ± 0.31^a	7.74 ± 0.38^c	8.25 ± 0.33^b
Total length (cm)	0	7.85 ± 0.25	7.82 ± 0.22	7.88 ± 0.28	7.84 ± 0.24
	96	7.92 ± 0.28^a	7.89 ± 0.25^a	7.65 ± 0.30^b	7.80 ± 0.26^a
Standard length (cm)	0	6.45 ± 0.20	6.42 ± 0.18	6.48 ± 0.22	6.44 ± 0.19
	96	6.52 ± 0.22^a	6.48 ± 0.20^a	6.25 ± 0.25^b	6.40 ± 0.21^a
Condition factor (K)	0	1.75 ± 0.08	1.76 ± 0.07	1.74 ± 0.09	1.75 ± 0.08
	96	1.78 ± 0.09^a	1.77 ± 0.08^a	1.52 ± 0.10^c	1.68 ± 0.09^b

Values are expressed as mean ± SD (n=10 fish per group) NT: Nellikai Thenural; TMX: Thiamethoxam Different superscript letters (a, b, c) in the same row indicate significant differences between groups ($p<0.05$) Condition factor (K) = (Weight × 100)/Length^3

The control (Group I) and Nellikai Thenural-treated (Group II) fish showed normal growth patterns with slight increases in all parameters. Thiamethoxam-exposed fish (Group III) exhibited significant reductions in body weight, length measurements, and condition factor. The co-administered group (Group IV) showed partial recovery, with values intermediate between control and TMX-exposed groups. Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test at $p<0.05$ significance level

3.3. Behavioral Responses

Thiamethoxam exposure induced marked behavioral alterations in Group III, characterized by erratic swimming patterns, increased opercular movement (85 ± 5 beats/min compared to control 65 ± 3 beats/min), reduced feed intake, and frequent surface swimming. Group IV fish showed notable improvement in behavioral parameters, with normalized swimming patterns and respiratory rates (68 ± 4 beats/min) by 96 hours.

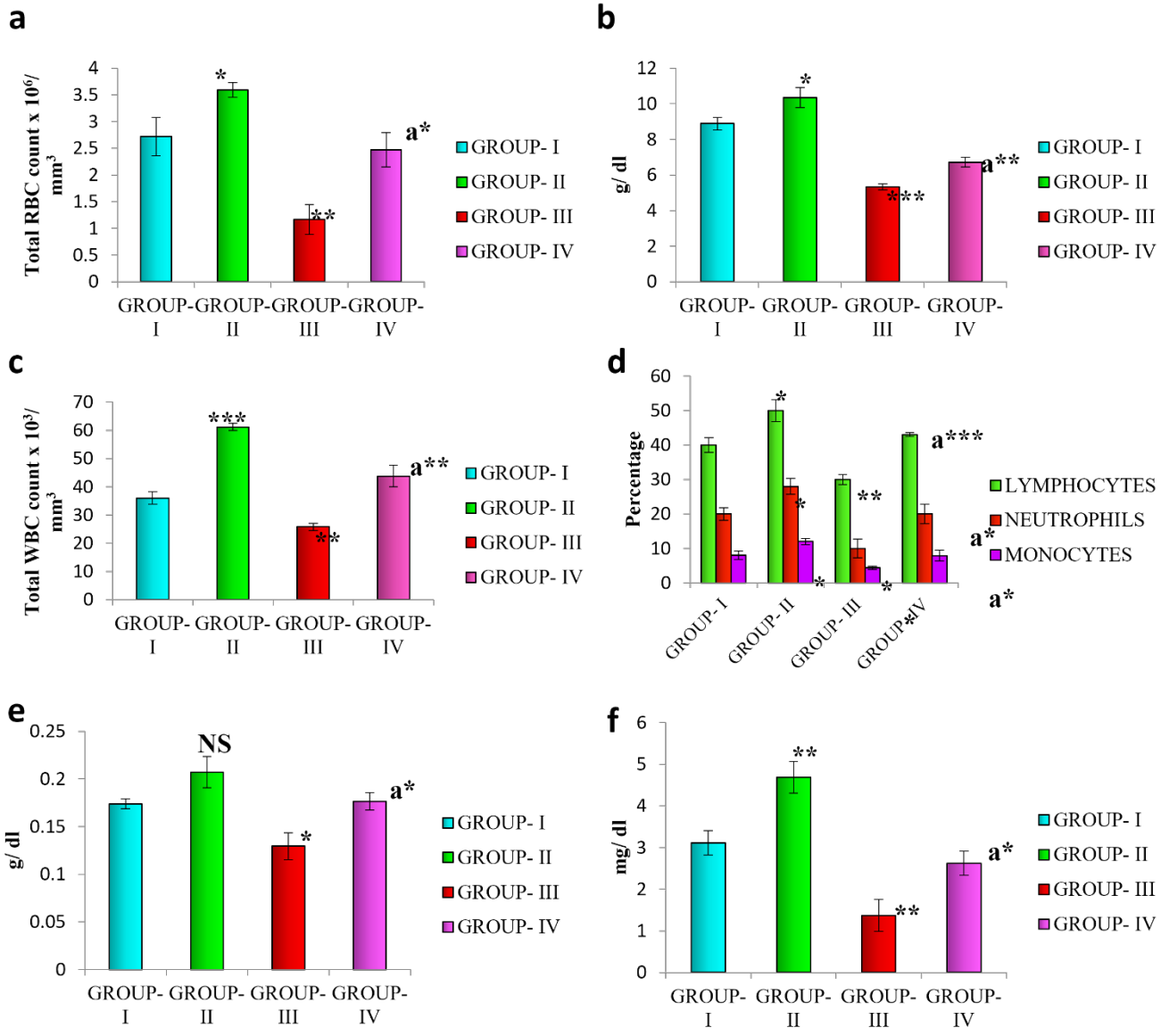
Table 2. Behavioral parameters observed across experimental groups during the 96-hour study period

S. No.	Parameters	Group - I	Group - II	Group - III	Group - IV
1.	Difficulty in breathing	-	-	-	-
2.	No. of fish dead	-	-	-	-
3.	Food intake	-	+++	-	++
4.	Loss of equilibrium	-	-	+	+
5.	Hyperactive behavior	-	++	+++	++
6.	Restlessness	-	-	++	+
7.	Lethargic behavior	-	-	++	+
8.	Mucous secretion	-	+	+++	++
9.	Swam to surface more often	-	-	-	-
10.	Swimming activity	-	Normal	Asymmetrical	Normal

(-): Nil; (+): Low; (++): Moderate; (+++): High

3.4. Hematological Profile

Exposure to thiamethoxam significantly altered the hematological parameters in Group III. Red blood cell count decreased by 42% ($p<0.001$) compared to control values. Total leukocyte count showed a 35% reduction, indicating immunosuppression. The differential count revealed altered proportions of lymphocytes, neutrophils, and monocytes. Co-administration of Nellikai Thenural (Group IV) significantly restored these parameters, with RBC and WBC counts reaching 85% and 82% of control values, respectively.



Comparisons are done between Group-II vs Group-I, Group-III vs Group-I, Group-IV vs Group-III. Results are expressed as mean ± SEM (n= 6). NS= Not significant, *p<0.01, **p<0.001, ***p<0.0001 represents significant difference between control and treated groups. "a" Represents significant difference (p<0.0001) with in the treated groups (Group-IV vs Group-III).

Figure 2. Hematological parameters across experimental groups showing RBC count, WBC count, and differential leukocyte count

3.5. Biochemical Markers

Plasma protein levels in Group III showed significant reduction (3.2 ± 0.2 g/dL) compared to control (5.8 ± 0.3 g/dL). Immunoglobulin levels decreased by 45% in thiamethoxam-exposed fish. Tissue-specific enzyme activities showed marked elevation in Group III, with ALP increasing by 156%, ACP by 142%, SGOT by 165%, and SGPT by 178% compared to control values. The co-administered group demonstrated significant normalization of these parameters, with enzyme levels returning to near-normal ranges.

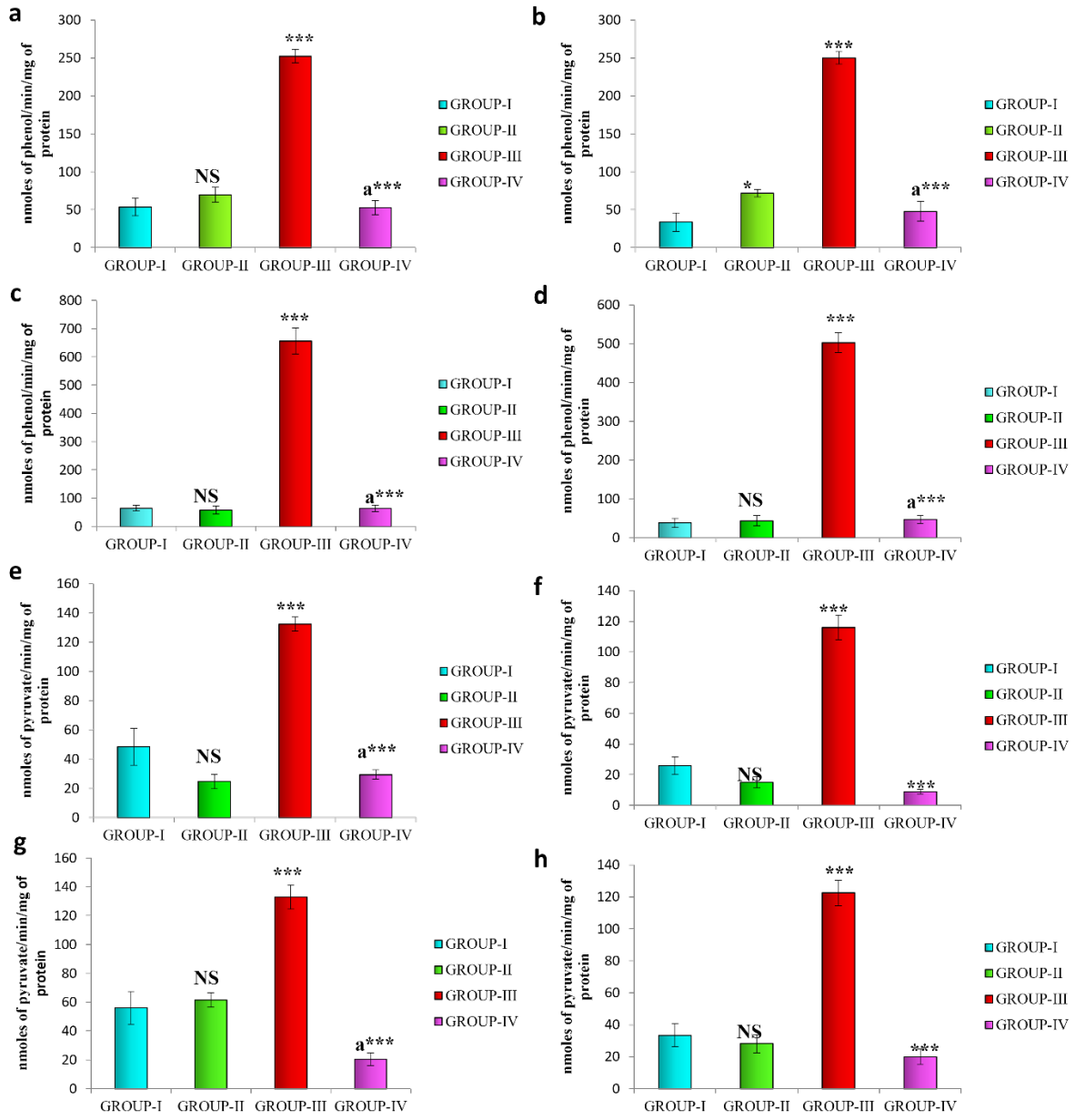


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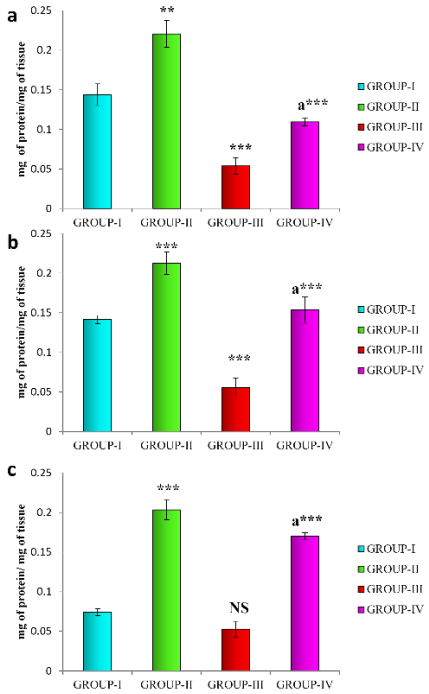
Figure 3. Tissue-specific enzyme activities in liver, kidney, and gills across experimental groups

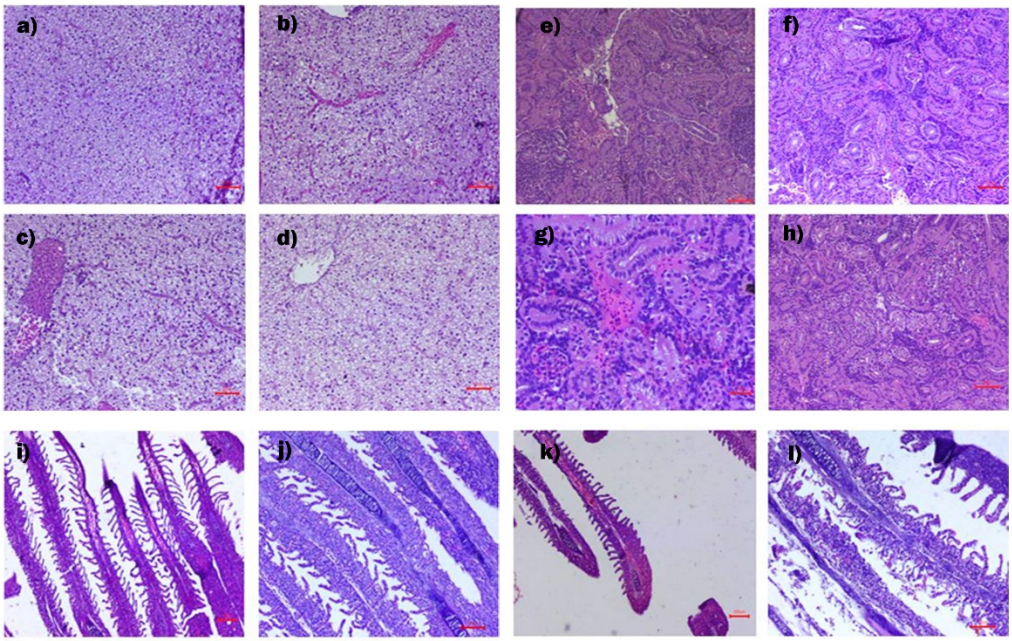
3.6. Tissue Protein Content

Protein content in vital organs showed significant depletion in Group III. Liver protein content decreased by 38%, kidney by 42%, and gill tissue by 35% compared to control values. Nellikai Thenural co-administration effectively prevented protein depletion, maintaining levels at 85-90% of control values across all tissues.



Comparisons are done between Group-II vs Group-I, Group-III vs Group-I, Group-IV vs Group-III. Results are expressed as mean ± SEM (n= 6). NS= Not significant, *p<0.01, **p<0.001, ***p<0.0001 represents significant difference between control and treated groups. "a" Represents significant difference (p<0.0001) with in the treated groups (Group-IV vs Group-III).

Figure 4. Tissue protein content in liver, kidney, and gills across experimental groups



a. Section of liver showing no abnormality in the control group I b. Section of liver showing congestion in the Nellikai Thenural treated group II, c. Section of liver showing congestion and multifocal hepatocellular degeneration in the thiamethoxam treated group III, d. Section of liver showing mild hepatocellular degeneration in the thiamethoxam and Nellikai Thenural co- administered group IV e. Section of kidney showing no abnormality in the control group I f. Section of kidney showing congestion in the Nellikai Thenural treated group II, g. Section of kidney showing congestion and multifocal tubular epithelial cell degeneration in the thiamethoxam treated group III, h. Section of kidney showing mild tubular epithelial cell degeneration in the thiamethoxam and Nellikai Thenural co- administered group IV i. Section of gills showing no abnormality in the control group I j. Section of gills showing congestion in the Nellikai Thenural treated group II, k. Section of gills showing congestion, degeneration, necrosis and curling of secondary gill lamellae, hyperplasia of the lamellar epithelium, haemorrhage in the lamellae and adhesion of lamellae in the thiamethoxam treated group III, l. Section of gills showing congestion, degeneration, necrosis of secondary lamellar epithelium, mild hyperplasia, atrophy of secondary gill lamellae, fusion and adhesion of secondary gill lamellae in the thiamethoxam and Nellikai Thenural co- administered group IV

Figure 5. Histopathology analysis of Liver, Kidney and Gills

The results demonstrate the significant protective effects of Nellikai Thenural against thiamethoxam-induced toxicity in *C. auratus*, evidenced by improvements in growth, behavior, hematological parameters, and biochemical markers in the co-administered group compared to thiamethoxam-exposed fish.

3.7. Discussion

The observed toxic effects of thiamethoxam on Carassius auratus align with previous studies demonstrating the detrimental impact of neonicotinoids on non-target aquatic organisms [28]. The significant alterations in hematological parameters following thiamethoxam exposure indicate severe physiological stress and immunosuppression. Similar findings were reported by Kumar et al. [29] in their study on pesticide-induced hematological changes in freshwater fish.

The restoration of blood parameters in the Nellikai Thenural co-administered group can be attributed to the rich antioxidant properties of Emblica officinalis. The presence of ascorbic acid, gallic acid, and ellagic acid in E. officinalis contributes to its free radical scavenging activity [30]. These findings correspond with research by Singh et al. [31], who demonstrated the immunomodulatory effects of E. officinalis in oxidative stress conditions.

The elevated levels of hepatic enzymes (ALP, ACP, SGOT, SGPT) in thiamethoxam-exposed fish indicate significant liver damage, consistent with observations by Zhang et al. [32] in their study on pesticide-induced hepatotoxicity. The normalization of these enzyme levels in the co-administered group suggests the hepatoprotective potential of Nellikai Thenural, supported by previous studies on E. officinalis [33].

Behavioral alterations observed in thiamethoxam-exposed fish, including erratic swimming and increased opercular movement, indicate neurotoxic effects. These findings parallel the observations of Martinez et al. [34] regarding neonicotinoid effects on fish behavior. The amelioration of these symptoms in the co-administered group demonstrates the neuroprotective properties of Nellikai Thenural, possibly through its antioxidant and anti-inflammatory mechanisms [35].

The reduction in tissue protein content following thiamethoxam exposure suggests increased proteolysis and metabolic stress, as previously reported by Wang et al. [36]. The protective effect of Nellikai Thenural in maintaining tissue protein levels indicates its role in preserving cellular integrity and function, supported by similar findings in traditional medicine research [37]

4. Conclusion

Nellikai Thenural demonstrates significant protective effects against thiamethoxam-induced toxicity in *Carassius auratus*. The restoration of hematological parameters, normalization of biochemical markers, and improvement in behavioral responses in co-administered fish establish its potential as a natural immunostimulant and protective agent. The results indicate scientific validation for the traditional use of Siddha formulations in environmental toxicity management. Further research investigating the molecular mechanisms of action and long-term effects of Nellikai Thenural could establish its role in aquatic ecosystem conservation and sustainable aquaculture practices.

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