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Research article

Toxicological assessment of cannabidiol (CBD) in Koi carp (*Cyprinus rubrofuscus*): Dose-dependent effects on hematological profiles, serum biochemistry, and multi-organ histopathology

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Abstract

Despite growing interest in phytogenic additives in aquaculture, the toxicological profile of cannabidiol (CBD) in teleost fish remains poorly characterized, with existing data limited to acute embryotoxicity and behavioral endpoints in zebrafish. This study aimed to conduct a systematic, dose-dependent toxicological assessment of dietary CBD in Koi carp (*Cyprinus rubrofuscus*), evaluating hematological, serum biochemical, and multi-organ histopathological responses following 28-day sub-chronic exposure. One hundred thirty-five Koi carp (24.0 ± 2.2 g) were randomly assigned to six dietary groups: feed control, vehicle control (soybean oil), and four CBD-supplemented groups (0.001%, 0.01%, 0.1%, and 1%) for 28 days. CBD isolate was incorporated via soybean oil carrier. Low-dose exposure (0.001% feed) enhanced erythropoiesis without adverse effects. Splenic melanomacrophage centre proliferation emerged at 0.01% feed, representing the most sensitive histopathological endpoint. Hepatic vacuolar degeneration and elevated serum AST occurred at 0.1% feed, progressing to multi-organ necrosis at 1% feed. Serum cholesterol was elevated across all CBD concentrations. A dietary NOAEL of 0.001% feed and LOAEL of 0.01% feed are established for ornamental cyprinid aquaculture, with the spleen and liver identified as principal target organs.

Keywords: Cannabidiol (CBD), Hematology, Histopathology, NOAEL, Ornamental fish, Toxicity

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INTRODUCTION

Cannabidiol (CBD), a major non-psychoactive phytocannabinoid derived from *Cannabis sativa* L., has attracted considerable scientific and commercial interest over the past decade. Unlike its psychoactive counterpart Δ9-tetrahydrocannabinol (THC), CBD exhibits low affinity for the cannabinoid receptors (CB1 and CB2) and does not elicit intoxicating effects, rendering it a candidate of broad therapeutic applications (Mechoulam and Parker, 2013; Blessing et al., 2015). CBD also acts as a transient receptor potential vanilloid type 1 (TRPV1) agonist, a negative allosteric modulator of GPR55, and a partial agonist at serotonin 5-HT1A receptors mechanisms with potentially distinct toxicological implications in ectothermic vertebrates (McPartland et al., 2015; Russo and Marcu, 2017). CBD is recognized for its analgesic, anti-inflammatory, anxiolytic, antioxidant, and neuroprotective properties reported in preclinical and clinical investigations without causing psychoactive effects (Cásedas et al., 2024). However, the broad pharmacological profile raises important questions regarding off-target toxicological effects, particularly in non-mammalian species whose endocannabinoid systems differ substantially from those of mammals (Elphick, 2012). Teleost fish, for instance, possess functionally conserved homologues of CB1 and CB2 receptors, as well as endocannabinoid ligands such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG), suggesting that exogenous cannabinoids, including CBD, may perturb physiological processes in fish in a species-specific manner (Cottone et al., 2013; Cristino et al., 2020, Fakhoury et al., 2022).

The use of bioactive plant compounds in aquaculture has surged as the industry seeks natural alternatives to antibiotics. Specifically, phytogenic additives such as polyphenols, essential oils, and cannabinoids are being researched for their immunomodulatory, growth-promoting, and anti-inflammatory benefits in commercial fish (Reverter et al., 2014; Bulfon et al., 2015). In this context, CBD has recently emerged as a candidate dietary supplement in ornamental and food fish aquaculture, predicated largely on its reported anti-inflammatory effects in mammals and a limited number of studies in fish (Bartončíková et al., 2023; Martini et al., 2023). The absence of standardized toxicological data for fish species represents a critical biosafety gap, as no evidence-based dietary thresholds for CBD currently exist for any aquaculture species. Establishing a safety profile inclusive of hematotoxic, hepatotoxic, nephrotoxic, and histopathological endpoints prior to broad dietary application in fish has not been systematically achieved. This is particularly concerning given that orally administered CBD at pharmacologically relevant doses may reach systemic concentrations sufficient to disturb hepatic metabolic function, erythropoietic activity, and multi-organ structural integrity.

Koi carp (*Cyprinus rubrofuscus*) hold significant economic and cultural importance as ornamental fish globally (Balon, 1995; Noga, 2010). Due to their physiological similarity to other cyprinids and sensitive hematological profiles, they serve as an ideal model for ecotoxicological studies (Fazio, 2019). Hematological and serum biochemical analyses are fundamental, integrative biomarkers for detecting systemic physiological disruption in aquatic toxicology (Hrubec et al., 2000; Fazio, 2019). Erythrocytic parameters indicate oxygen transport and hematopoietic status, while leukocyte dynamics reflect immunological stress. Organ-specific injury is further assessed through hepatic (ALT, AST, ALP) and renal (BUN, creatinine) markers and histopathological examination of the liver, kidney, gill, pancreas, and spleen provides essential morphological evidence of structural damage (Georgieva et al., 2014; Suyapoh et al., 2021). Integrating semi-quantitative lesion grading with dose-response data is crucial for determining safe dietary thresholds in aquaculture. As a highly lipophilic compound, CBD was incorporated into diets using soybean oil as a vehicle. A soybean oil-only control group was included to isolate CBD specific effects from potential vehicle-induced responses,

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as lipid supplementation can independently alter hepatic and hematological parameters in teleosts (Hemre et al., 2007).

Current CBD research in teleost fish remains limited, primarily focusing on acute embryotoxicity in zebrafish or behavioral endpoints (Wei et al., 2024). In parallel, plant-derived dietary supplements in cyprinid and catfish species have been studied predominantly for their beneficial effects on growth and health indices, such *as Tinospora cordifolia* in common carp (Ridha et al., 2025) and *Curcuma longa* in African catfish (Wei et al., 2024), rather than for toxicological characterization. A systematic evaluation of sub-chronic dietary CBD toxicity across hematological, biochemical, and histopathological parameters in cyprinid species is conspicuously absent from peer-reviewed literature, representing a substantial knowledge gap that constrains evidence-based decision-making for both practitioners and regulatory bodies. Furthermore, mammalian data reporting dose-dependent hepatic injury cannot be directly extrapolated to fish due to fundamental differences in metabolism, thermoregulation, and physiology (Ewing et al., 2019; Chen et al., 2024).

The present study therefore aimed to conduct a systematic, dose-dependent toxicological assessment of dietary CBD in koi carp (*C. rubrofuscus*) over a 28-day trial, utilizing five graded concentrations (0%, 0.001%, 0.01%, 0.1%, and 1% w/w). Our objectives were to: (1) characterize the effects of CBD on hematological and serum biochemical profiles; (2) evaluate multi-organ histopathological alterations in the gills, liver, kidney, pancreas, and spleen using a semi-quantitative scoring system; (3) correlate dietary CBD levels with total lesion burden via Spearman rank correlation; and (4) determine whether the soybean oil vehicle independently affected physiological status. Collectively, these findings establish an initial sub-chronic toxicological profile for CBD in koi carp, providing evidence-based reference values to inform safe supplementation practices and regulatory oversight in ornamental cyprinid aquaculture.

MATERIALS AND METHODS

Experimental animals and housing conditions

One hundred thirty-five clinically healthy koi carp (*Cyprinus rubrofuscus*) with an initial body weight of 24.0 ± 2.2 g were procured from a commercial aquaculture facility in Bangkok, Thailand. Upon arrival, fish were quarantined for 14 days in 650 L fiberglass tanks supplied with dechlorinated freshwater under continuous aeration. Animals were monitored daily for abnormal behavior and clinical signs of disease. A randomly selected subset (3%) underwent parasitological screening by microscopic examination of skin and gill scrapings and molecular or standard diagnostic screening for Koi herpesvirus (KHV) following established procedures (Arthur et al., 2008). After quarantine, fish were acclimated for 7 days in 60 L glass aquaria equipped with aeration and biological filtration prior to dietary intervention. Water quality parameters were monitored daily throughout the experimental period and maintained within optimal ranges for koi carp. Water temperature was maintained at 25 ± 1.1°C, pH at 7.5 ± 0.3, total ammonia nitrogen (TAN) below 0.5 mg/L, nitrite below 0.1 mg/L, total hardness at 150–200 mg/L $CaCO_3$, and total alkalinity at 100–150 mg/L $CaCO_3$. Dissolved oxygen was maintained above 6.0 mg/L throughout the study period via continuous aeration. Twenty percent of tank water was renewed daily to maintain water quality stability. Fish were fed a commercial basal diet at 2% of body weight per day, administered in two equal feedings (Arthur, 2008; Jia et al., 2014).

CBD-supplemented diet

Cannabidiol (CBD) isolate powder (>99% purity) was obtained from Eastern Spectrum Group Co.Ltd., Ratchaburi, Thailand (Lot no. L.0416102301B). A commercial koi carp diet (Optimum®, PCG, Thailand) was used as the basal diet

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and served as the feed control group. Vehicle control and CBD-supplemented diets were prepared from the same commercial pellets to ensure formulation consistency across treatments. The pellets were finely pulverized and blended with 3% (w/w) gluten powder as a binder. CBD isolate was incorporated into the diet at graded concentrations of 0% (vehicle control), 0.001%, 0.01%, 0.1%, and 1% (w/w), corresponding to 10 mg, 100 mg, 1,000 mg, and 10,000 mg CBD per kg of feed, respectively. For each formulation, the required amount of CBD was dissolved in soybean oil (2% w/w of feed) using a heat–magnetic stirrer at 70°C with continuous agitation to ensure complete solubilization. The CBD–oil solution was then uniformly incorporated into the powdered feed by mortar-assisted levigation, followed by mechanical mixing and pelletization. The pellets were dried in a forced-air oven at 70°C for 1 h and stored at 4°C for no longer than 3 days prior to use (Banerjee et al., 2021). To confirm concentration stability, 100 g of each CBD-supplemented diet was randomly sampled on day 0 and at 14-day intervals throughout the experimental period for HPLC quantification. A deviation of ≤3% from the nominal CBD concentration at the time of preparation was defined as the acceptable threshold for continued experimental use.

Sampling and experimental design

A completely randomized design was employed for the feeding trial. Fish were randomly allocated into six dietary groups: (1) feed control group (commercial basal diet), (2) vehicle control group (0% CBD with soybean oil inclusion), and (3-6) four CBD-supplemented groups prepared as ten-fold serial dilutions based on a reference concentration previously reported for use in fish (U.S. Environmental Protection Agency, 1996). Each treatment consisted of three replicate tanks with six fish per tank (n = 18 fish per group). Fish were fed their respective experimental diets at 2% of body weight per day, divided into two equal feedings, for 28 consecutive days. Throughout the experimental period, fish were monitored daily for behavioral alterations, respiratory distress, abnormal swimming activity, and mortality. At day 28, nine fish per treatment were randomly selected for terminal sampling. Fish were anesthetized with eugenol (20–100 mg/L; 3% w/v) prior to blood collection. Approximately 0.8 mL of blood was obtained from the caudal vein using a 1-mL syringe fitted with a 23-gauge needle. Following blood sampling, fish were euthanized by immersion in an overdose of eugenol (>400 mg/L) in accordance with established guidelines for fish anesthesia and euthanasia (Sneddon, 2012). A complete necropsy was subsequently performed, and tissue samples from gill, liver, kidney, pancreas, and spleen were carefully excised for further histopathological evaluation.

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Chulalongkorn University (Protocol No. 2331019) and conducted in accordance with institutional guidelines for the care and use of laboratory animals.

Hematological and serum biochemical analyses

Blood samples were collected into two separate tubes: lithium heparin-containing tubes for hematological analyses and heparinized tubes for plasma biochemical analyses. Samples were maintained at 4°C prior to processing. Hematocrit (Hct) was determined using heparinized microcapillary tubes by standard microhematocrit procedures. Hemoglobin (Hb) concentration was measured using the cyanmethemoglobin method at 540 nm (Drabkin and Austin, 1935; Fazio, 2019). Red blood cell (RBC) and white blood cell (WBC) counts were quantified using a Neubauer hemocytometer. Erythrocyte indices, including mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), were calculated according to established hematological formulas (Groff and Zinkl, 1999; Fazio, 2019). For biochemical analyses, heparinized blood was centrifuged at 3,500 ×g for 10 min to separate plasma (Potki et al., 2018). Plasma biochemical parameters, including

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alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein (TP), creatinine, blood urea nitrogen (BUN), and cholesterol were measured using a VETSCAN® VS2 Chemistry Analyzer (Abaxis, USA) in accordance with the manufacturer's instructions.

Tissue collection and histopathological examination

Following euthanasia, gill tissues were fixed in Davidson's fixative, while liver, kidney, pancreas, and spleen tissues were promptly excised and fixed in 10% neutral buffered formalin. Fixed specimens were processed using routine histological techniques, including graded dehydration, clearing, and paraffin embedding. Paraffin blocks were sectioned at 4 μm thickness using a rotary microtome. Sections were deparaffinized in xylene, rehydrated through a graded ethanol series, and stained with hematoxylin and eosin (H&E) according to standard histopathological protocols (Georgieva et al., 2014; Jantrakajorn et al., 2024). Stained sections were examined under a light microscope (Nikon ECLIPSE Ni-U, Nikon, Tokyo, Japan) equipped with a digital imaging system for documentation. Histopathological evaluation focused on identifying structural alterations consistent with toxicopathological responses. Representative lesions were recorded and documented for subsequent semi-quantitative grading analysis.

Semi-quantitative evaluation of histopathology

Semi-quantitative grading of histopathological lesions was conducted for gill, liver, kidney, pancreas, and spleen using an organ-specific scoring system adapted from established teleost toxicopathological assessment protocols (Peebua et al., 2006; McHugh et al., 2011; Georgieva et al., 2014). All tissues and corresponding histopathological parameters, as summarized in Table 1, were evaluated using a standardized seven-point distribution-based grading system. Lesion severity was scored according to the proportion of affected tissue area as follows: score 0 = absent; score 1 = <5% of tissue affected; score 2 = 5-10%; score 3 = 10-25%; score 4 = 25-50%; score 5 = 50-75%; and score 6 = >75% of the examined tissue area.

Table 1 Organ-specific histopathological parameters evaluated in gill, liver, kidney, pancreas, and spleen following dietary CBD exposure

Organ	Histopathological Parameters Evaluated
Gill	Lamellar arrangement; Gill congestion; Gill epithelial hyperplasia; Lamellar aneurysm and Eosinophilic granule cell infiltration
Liver	Melano-macrophage centers (MMC); Congestion; Hepatocyte vacuolar degeneration; Glycogen degeneration; Fatty degeneration; Hepatocyte necrosis
Kidney	Melano-macrophage centers (MMC); Glomerular alteration; Renal tubular vacuolation; Hyaline droplet degeneration; Tubular necrosis; Tubular hemorrhage
Pancreas	Melano-macrophage centers (MMC); Vacuolar degeneration; Cell swelling
Spleen	Melano-macrophage centers (MMC); Splenic congestion

Note: Severity for most parameters was graded on a six-point scale (0–6) based on the percentage of affected tissue area (<5%, 5–10%, 10–25%, 25–50%, 50–75%, >75%)

For each fish, five non-overlapping microscopic fields were systematically selected and evaluated at standardized magnifications to ensure consistency across specimens (Sornying et al., 2025) Lesion severity was determined semi-quantitatively according to the estimated percentage of tissue area affected within each field. The percentage value represents the proportion of the total examined tissue section exhibiting the specified histopathological alteration. All histopathological sections were evaluated by two independent observers in a blinded manner, and discrepancies were resolved by consensus.

Statistical analysis

Prior to inferential testing, normality of distribution was assessed using the Shapiro-Wilk test and homogeneity of variances was evaluated using Levene's test.

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All statistical analyses were performed using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA). Data are presented as mean ± standard deviation (SD) for parametric variables and as median with interquartile range (IQR) for non-parametric data.

To determine whether the feed control and vehicle control groups differed prior to dose-response evaluation, hematological and serum biochemical parameters were compared using paired t-tests. Descriptive statistics and percentage change relative to the feed control group were calculated to confirm suitability for subsequent dose-response analysis. For parametric data, including hematological and serum biochemical parameters, differences among dietary treatment groups were analyzed using one-way analysis of variance (ANOVA). When a significant main effect was detected, pairwise comparisons were performed using Dunnett's multiple comparisons test to identify differences between CBD-treated groups and the vehicle control.

Histopathological scores, expressed as semi-quantitative ordinal data (0-6), were analyzed using non-parametric methods. Differences between feed control and vehicle control groups were first evaluated using the Mann-Whitney U test to verify baseline comparability. Lesion scores for these comparisons are presented as median (IQR). Differences among multiple treatment groups were subsequently analyzed using the Kruskal–Wallis test followed by Dunn's multiple comparison test when appropriate. The association between dietary CBD concentration and total histopathological lesion scores across examined organs was assessed using Spearman's rank correlation coefficient (ρ) with a two-tailed test of significance. A p-value < 0.05 was considered statistically significant for all analyses.

RESULTS

Clinical observations and survival

No mortality occurred in any treatment group during the study (survival = 100%). Fish in the feed control, vehicle control, and 0.001-0.1% CBD groups exhibited normal swimming behavior, feeding activity, and opercular movement throughout the experiment. In the 1% CBD group, fish remained able to consume all offered feed but showed a slower feeding response from approximately Day 15 onward. In addition, mild and transient loss of equilibrium was occasionally observed 2-4 h after feeding. These signs resolved spontaneously, and affected fish returned to normal swimming behavior thereafter. No persistent abnormal swimming behavior or respiratory distress was observed in any group.

Effects of CBD on hematological and serum biochemical parameters

Comparison between feed control and vehicle control groups

To evaluate the potential impact of the delivery medium, hematological and serum biochemical profiles were compared between the feed control group (non-vehicle) and the vehicle control group (Figure 1 and Table 2). The majority of parameters including WBC, mean MCV, and key liver function markers (ALT, AST, and ALP) exhibited no significant deviations between the two groups ($p > 0.05$), indicating a high degree of systemic stability.

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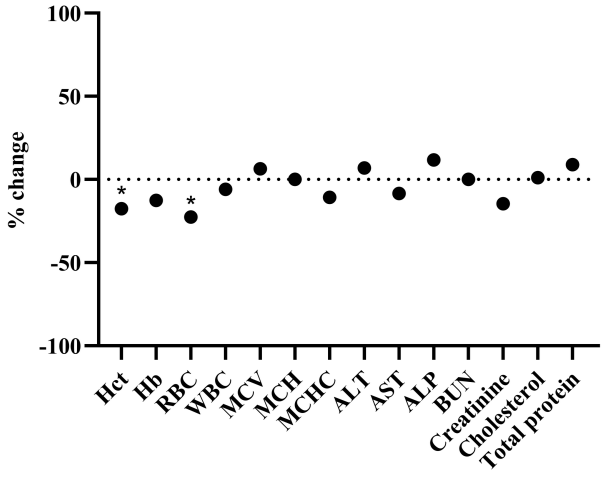


Figure 1 Summary of vehicle-related effects on hematological and serum biochemical parameters. Data are presented as percentage change of the vehicle control relative to the feed control. Each point represents an individual parameter. The dotted horizontal line indicates no change (0%). Statistical significance between control and vehicle groups was assessed using paired t-tests. Asterisks (*) indicate significant differences between groups ($p < 0.05$) as determined by paired t-tests.

Table 2 Hematological and serum biochemical parameters in untreated control and vehicle control groups. Data are expressed as mean ± standard deviation (SD). Percentage change represents the relative difference of the vehicle control compared with the untreated control. Statistical comparisons were performed using paired t-tests.

Parameters (Units)	Control (Mean ± SD)	Vehicle control (Mean ± SD)	% Change	Paired t-test (p)
Hct (%)	18.33 ± 1.23	15.11 ± 1.62	-17.57	0.00
Hb (g/dL)	3.71 ± 0.71	3.24 ± 0.72	-12.58	0.30
RBC (×10^6 cells/µL)	0.84 ± 0.09	0.65 ± 0.13	-22.58	0.00
WBC (×10^3 cells/µL)	1.57 ± 0.21	1.48 ± 0.32	-5.87	0.20
MCV (fL)	169.60 ± 11.06	180.60 ± 13.87	6.49	0.19
MCH (pg)	49.07 ± 0.75	49.10 ± 3.22	0.06	0.82
MCHC (-g/dL)	30.53 ± 1.19	27.23 ± 1.36	-10.81	0.15
ALT (U/L)	14.50 ± 2.89	15.50 ± 2.08	6.90	0.70
AST (U/L)	198.00 ± 8.48	181.30 ± 43.11	-8.43	0.56
ALP (U/L)	4.25 ± 0.50	4.75 ± 0.96	11.76	0.50
BUN (mg/dL)	2.5 ± 0.58	2.50 ± 0.03	0.00	1.00
Creatinine (mg/dL)	0.19 ± 0.02	0.17 ± 0.03	-14.51	0.13
Cholesterol (mg/dL)	112 ± 2.31	113.30 ± 1.50	1.16	0.39
Total protein (g/dL)	2.25 ± 0.28	2.45 ± 0.24	8.89	0.27

While statistical analyses identified a modest decrease in certain erythroid parameters, specifically hematocrit (Hct; -17.57%; $p = 0.0001$) and red blood cell (RBC) counts (-22.58%; $p = 0.0010$), these shifts were interpreted as a baseline physiological adjustment to the vehicle administration rather than indicative of systemic toxicity. Notably, renal function markers (BUN and creatinine) and lipid profiles remained largely stable. Creatinine showed a non-significant decrease ($p = 0.13$), staying well within biological relevance. These findings collectively demonstrate that the vehicle does not induce overt organ dysfunction or gross biochemical alterations. Consequently, the vehicle control group serves as a reliable and robust baseline for subsequent dose-dependent evaluations of CBD.

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a. Dose-dependent effects of CBD on hematological parameters

Treatment with CBD induced a significant, dose-related enhancement in erythroid-related parameters compared to the vehicle control (Figure 2). A marked elevation in Hct, Hb concentration, and RBC count was observed across the majority of CBD concentrations (0.001%–1%) (Figure 2A-2C). Specifically, Hb and RBC levels were significantly elevated beginning at 0.001% CBD ($p = 0.0048$ and $p = 0.007$), reaching a plateau at concentrations ≥ 0.01% ($p < 0.0001$). Similarly, Hct and RBC counts demonstrated significant upward shifts, particularly at 0.01% and 1% doses, suggesting a positive modulation of erythropoiesis or red cell stability by CBD. In contrast, total white blood cell (WBC) counts (Figure 2D) and red cell indices, including MCV, MCH, and MCHC (Figure 2E-2G), remained stable with no statistically significant alterations across all treatment groups ($p > 0.05$).

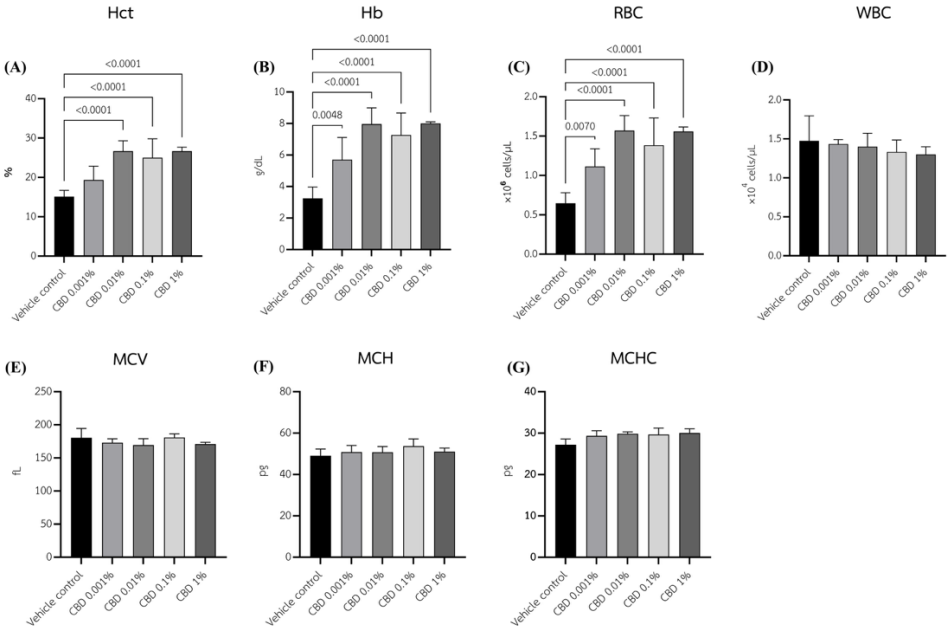


Figure 2 Effects of increasing cannabidiol (CBD) concentrations on hematological parameters. (A) Hematocrit (Hct), (B) hemoglobin (Hb), (C) red blood cell count (RBC), (D) white blood cell count (WBC), (E) mean corpuscular volume (MCV), (F) mean corpuscular hemoglobin (MCH), and (G) mean corpuscular hemoglobin concentration (MCHC) were measured following exposure to CBD at concentrations of 0.001, 0.01, 0.1, and 1% and compared with the vehicle control. Data are presented as mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparisons test versus the vehicle control. Exact p-values for statistically significant comparisons are indicated above the corresponding bars.

b. Dose-dependent effects of CBD on biochemical parameters

Serum cholesterol concentrations were significantly elevated in all CBD treated groups compared with the vehicle control, beginning at 0.001% CBD ($p = 0.004$). Cholesterol levels showed a modest incremental increase at 0.01% CBD and subsequently reached a plateau at 0.1%, with no further statistically distinguishable rise at higher concentrations (Figure 3F). Among hepatic enzyme markers, AST activity demonstrated a significant dose-dependent increase (Figure 3B). A statistically significant elevation was first detected at 0.01% CBD ($p = 0.00022$), with progressive increases at higher concentrations, reaching the greatest magnitude at 0.1% and 1% CBD ($p = 0.0008$). In contrast, ALT and ALP activities did not differ significantly between CBD treated groups and the vehicle control ($p > 0.05$) (Figure 3A, 3C). Renal function biomarkers, including BUN and creatinine (Figure 3D, 3E), remained comparable across all experimental groups (p

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> 0.05). Likewise, total protein concentrations were not significantly affected by CBD exposure (p > 0.05) (Figure 3G).

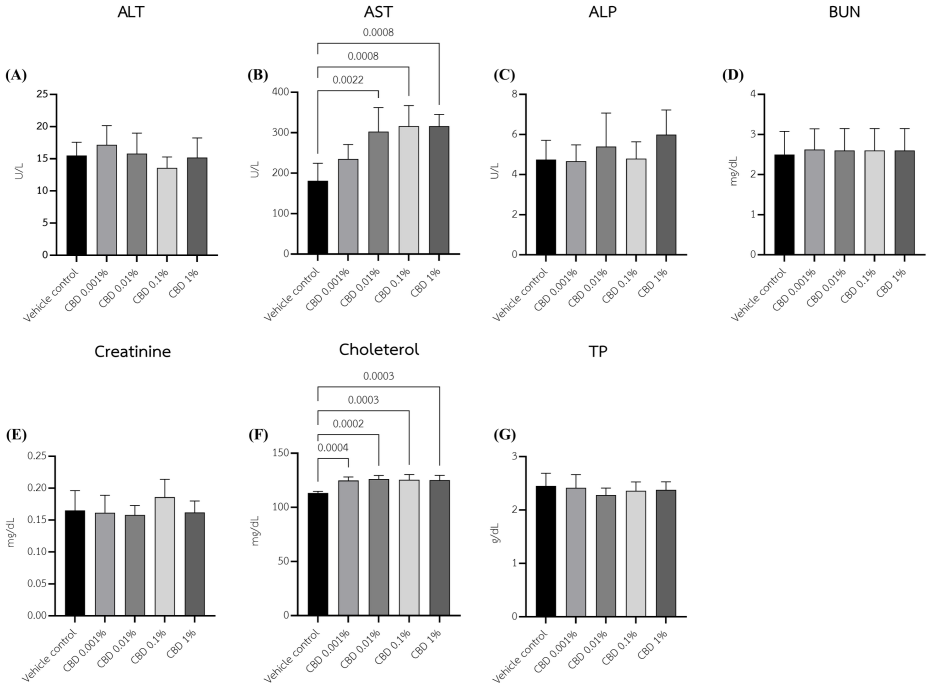


Figure 3 Effects of increasing cannabidiol (CBD) concentrations on biochemical parameters. (A) Alanine aminotransferase (ALT), (B) aspartate aminotransferase (AST), (C) alkaline phosphatase (ALP), (D) blood urea nitrogen (BUN), (E) creatinine, (F) cholesterol and (G) total protein were evaluated following exposure to CBD at concentrations ranging from 0.001 to 1% and compared with the vehicle control. Data are presented as mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparisons test versus the vehicle control. Exact p-values for statistically significant differences are indicated above the corresponding bars.

Histopathological findings and lesion severity grading

Comparison of histopathological grading between control and vehicle control groups

Histopathological grading of all examined organs including gills, liver, kidney, pancreas, and spleen revealed no biologically relevant differences between the feed control and vehicle control groups (Figure 4). Grading scores for all evaluated parameters were low and comparable between groups, with no statistically significant differences detected (Mann-Whitney U test, all p > 0.05). These findings confirm that soybean oil vehicle administration did not induce detectable histological alterations at baseline, establishing the vehicle control as an appropriate reference for subsequent dose-response comparisons.

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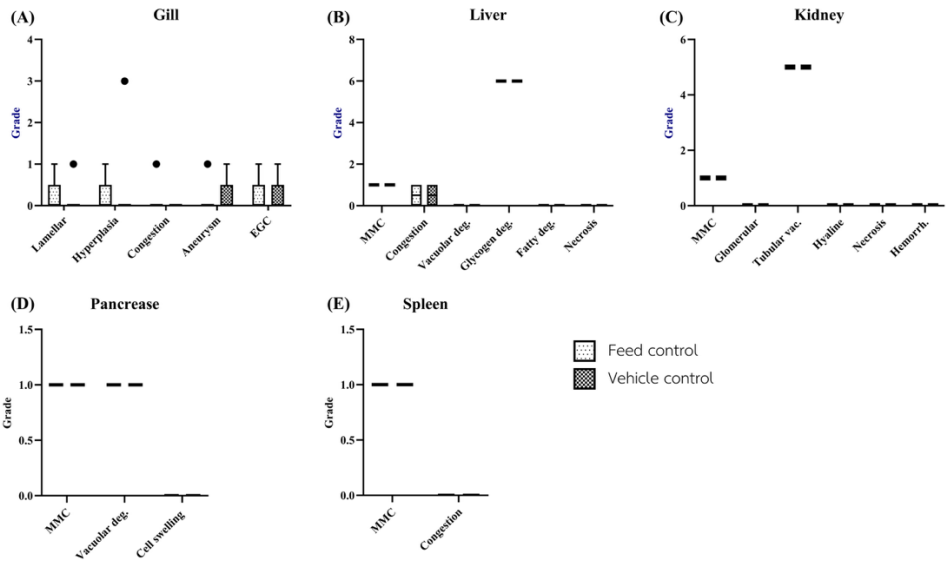


Figure 4 Histopathological grading scores for gill (A), liver (B), kidney (C), pancreas (D), and spleen (E) parameters in the feed control and vehicle control groups. Individual histopathological parameters are shown for each organ. Data are presented as Tukey boxplots with median and interquartile range. Outliers are shown as individual points. For panel (A) Gill, evaluated parameters include Lamellar, lamellar arrangement; Hyperplasia, epithelial hyperplasia; Congestion, vascular congestion; Aneurysm, lamellar aneurysm; and EGC, eosinophilic granule cell infiltration. For panel (B) Liver, parameters include MMC, melanomacrophage center; Congestion, sinusoidal congestion; Vacuolar deg., hepatocyte vacuolar degeneration; Glycogen deg., glycogen degeneration; Fatty deg., fatty degeneration; and Necrosis, hepatocyte necrosis. For panel (C) Kidney, parameters include MMC, melanomacrophage center; Glomerular, glomerular distortion/dilation; Tubular vac., renal tubule vacuolation; Hyaline, hyaline droplet degeneration; Necrosis, tubular necrosis; and Hemorrh., tubular hemorrhage. For panel (D) Pancreas, parameters include MMC, melanomacrophage center; Vacuolar deg., vacuolar degeneration; and Cell swelling, cellular swelling. For panel (E) Spleen, parameters include MMC, melanomacrophage center; and Congestion, splenic congestion.

Histopathological grading scores across treatment groups

All evaluated gill parameters, including lamellar arrangement, epithelial hyperplasia, vascular congestion, lamellar aneurysm, and EGC infiltration, were predominantly absent or minimal, with scores clustering near grade 0 across both the vehicle control and CBD treated groups (Figure 5). No dose-dependent increases were observed for any gill parameter at concentrations ranging from 0.001% to 1%.

Liver histopathology demonstrated parameter-specific, dose-dependent responses to CBD exposure (Figure 6). Glycogen degeneration was observed in all treatment groups as diffuse, pale-stained cytoplasmic clearing of hepatocytes, with no dose-related differences in severity (Figure 6D). MMC abundance and sinusoidal congestion remained low and focal across all treatment groups (Figure 6A). In contrast, hepatocellular vacuolar degeneration exhibited a clear dose-related increase, characterized by multiple small to coalescing cytoplasmic vacuoles displacing the nucleus, with predominantly diffuse distribution at the higher concentrations; severity grades were significantly elevated at CBD 0.1% ($p = 0.0038$) and 1% ($p < 0.001$) (Figure 6C, Figure 7A and 7B). Hepatocellular necrosis at the CBD 1% group appeared as multifocal areas of eosinophilic, shrunken hepatocytes with pyknotic or karyolytic nuclei and loss of normal cord architecture, and was significantly elevated compared with the vehicle control ($p < 0.0001$) (Figure 6F, Figure 7C and 7D).

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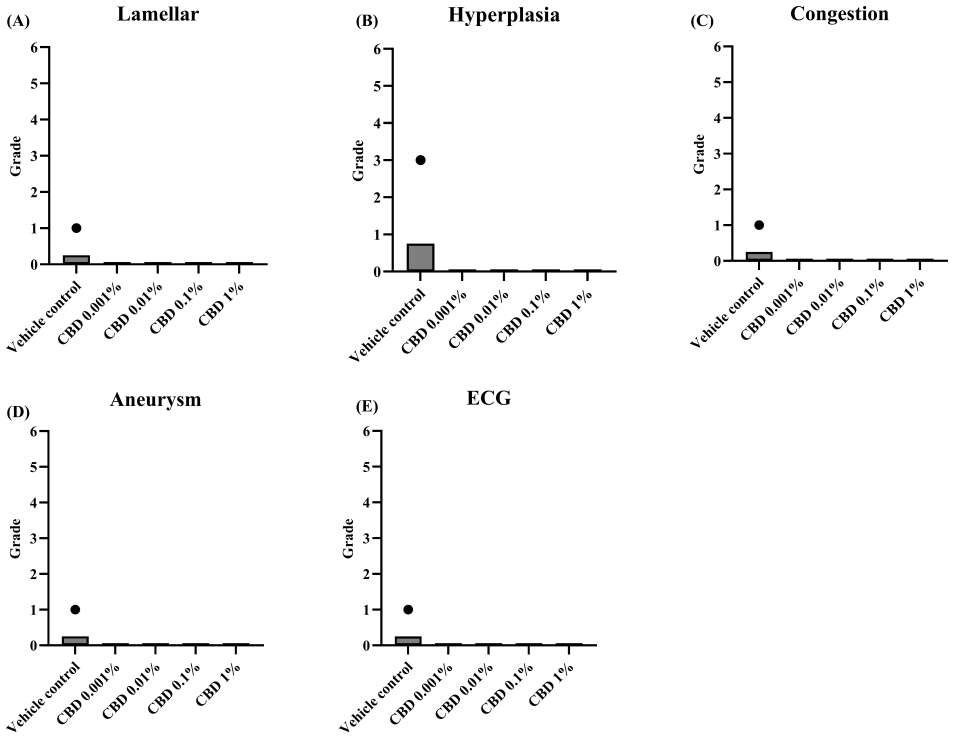


Figure 5 Histopathological grading scores of gill parameters across CBD treatment groups. Histopathological grading scores for gill parameters in the vehicle control and cannabidiol (CBD)-treated groups at increasing dose levels are shown. Evaluated parameters include (A) lamellar arrangement, (B) epithelial hyperplasia, (C) vascular congestion, (D) lamellar aneurysm, and (E) eosinophilic granule cell (EGC) infiltration. Data are presented as Tukey boxplots with median and interquartile range. Outliers are shown as individual points.

Renal histopathological evaluation revealed selective alterations at higher CBD concentrations (Figure 8). Glomerular architecture, including Bowman's capsule and capillary tufts, was preserved across all groups, and renal hemorrhage was minimal and did not differ among groups (Figure 8B, 8F). Renal tubular vacuolation was consistently present across all treatments as small, clear cytoplasmic vacuoles, reflecting baseline rather than CBD-specific changes (Figure 8C). Hyaline droplet degeneration, observed as bright eosinophilic intracytoplasmic granules within proximal tubular epithelial cells, was significantly elevated at CBD 0.1% and 1% ($p < 0.0001$) (Figure 8D). MMC scores, characterized by aggregates of pigment-laden macrophages within the interstitium, were significantly increased in the CBD 1% group ($p < 0.001$) (Figure 8A, Figure 9A and 9B). Tubular necrosis at the CBD 1% group was characterized by loss of brush border, epithelial detachment, and pyknotic nuclei, with occasional intraluminal cellular debris ($p = 0.0002$) (Figure 8E, Figure 9C and 9D). These findings indicate that high-dose CBD exposure induced mild to moderate proximal tubular injury in a dose-dependent manner.

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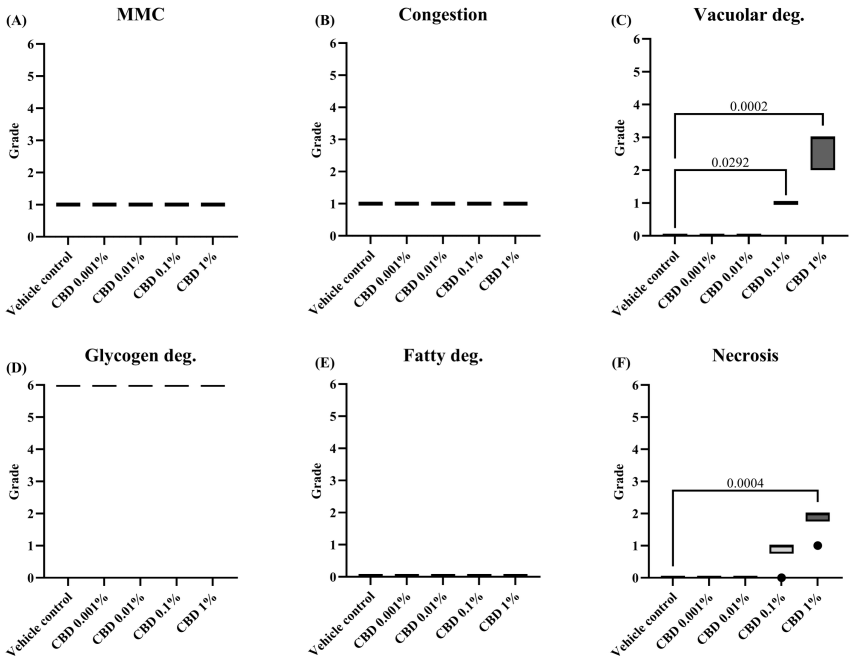


Figure 6 Histopathological grading scores of liver parameters across CBD treatment groups. Histopathological grading scores for liver parameters are presented for the vehicle control and cannabidiol (CBD)-treated groups across increasing dose levels. Assessed parameters comprised (A) melanomacrophage center (MMC), (B) sinusoidal congestion, (C) hepatocellular vacuolar degeneration, (D) glycogen degeneration, (E) fatty degeneration, and (F) hepatocellular necrosis. Data are presented as Tukey boxplots with median and interquartile range. Outliers are shown as individual points.

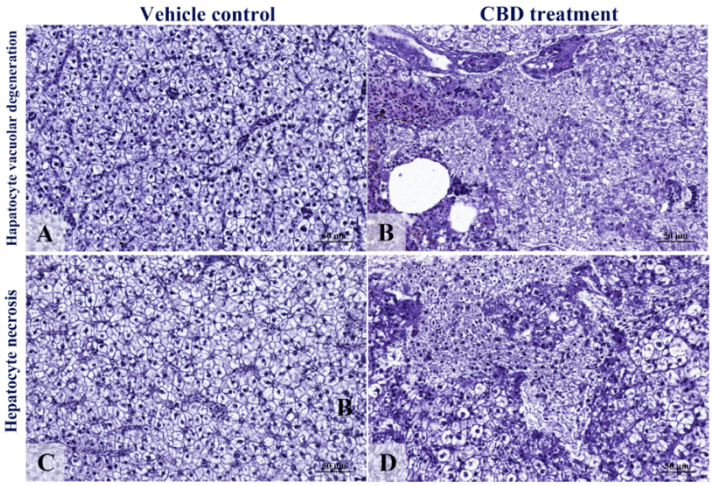


Figure 7 Representative histopathological alterations in the liver following cannabidiol (CBD) exposure. Hematoxylin and eosin (H&E) stained liver sections comparing vehicle control and cannabidiol (CBD) treated groups are shown. (A) Vehicle control sections demonstrate normal hepatic architecture without hepatocellular vacuolar degeneration. (B) Liver sections from the 1% CBD treated group exhibit marked hepatocellular vacuolar degeneration (grade 3) (arrow). (C) Vehicle control sections show no evidence of hepatocellular necrosis. (D) Liver sections from the 1% CBD treated group display increased hepatocellular necrosis (grade 2). Representative images were selected based on the predominant lesions identified through semiquantitative histopathological grading analysis. Scale bars = 50 µm (original magnification ×40).

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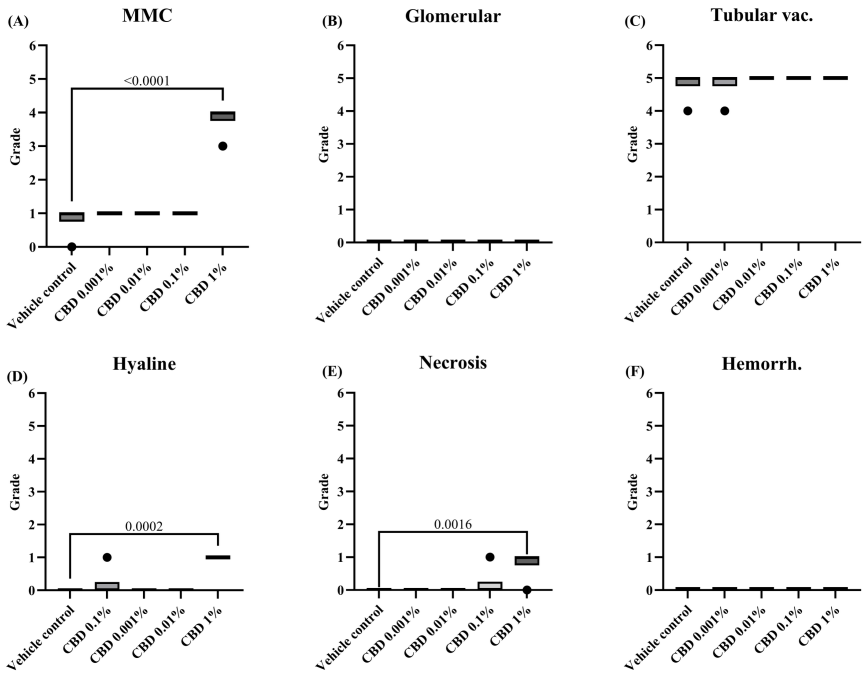


Figure 8 Histopathological grading scores of kidney parameters across CBD treatment groups. Histopathological grading scores for kidney parameters are presented for the vehicle control and cannabidiol (CBD)-treated groups across increasing dose levels. Assessed parameters comprised (A) melanomacrophage center (MMC), (B) glomerular alterations, (C) renal tubular vacuolation, (D) hyaline droplet degeneration, (E) tubular necrosis, and (F) renal hemorrhage. Data are presented as Tukey boxplots with median and interquartile range. Outliers are shown as individual points.

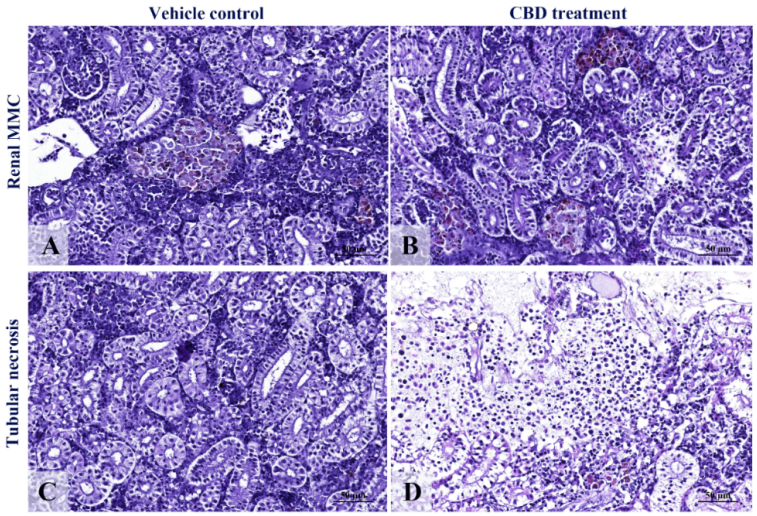


Figure 9 Representative histopathological alterations in kidney following cannabidiol (CBD) exposure. Hematoxylin and eosin (H&E) stained kidney sections comparing vehicle control and cannabidiol (CBD) treated groups are shown. (A) Vehicle control sections demonstrate normal kidney parenchyma with scattered melanomacrophage centers (MMCs) at grade 1. (B) show MMC 1% CBD treated at grade 4 (arrow). (C) Vehicle control show no tubular necrosis with normal tubular structure (arrow) while (D) 1% CBD treated fish show grade 1 marked tubular necrosis. Representative images were selected based on the predominant lesions identified in the semiquantitative histopathological grading analysis. Scale bars = 50 µm (original magnification ×40).

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Pancreatic histopathology demonstrated mild but statistically significant changes confined to the exocrine acinar tissue at higher CBD concentrations (Figure 10). Cellular swelling remained minimal across treatment groups. Vacuolar degeneration of acinar cells, characterized by clear cytoplasmic vacuoles with peripherally displaced nuclei and partial loss of zymogen granulation, was significantly elevated at the highest CBD concentration ($p < 0.0001$) (Figure 10B, Figure 11A and 11B). MMC scores, observed as discrete pigmented aggregates within the interstitial connective tissue, were similarly increased in the CBD 1% group compared with the vehicle control ($p = 0.0005$) (Figure 10A). Islet tissue architecture appeared preserved across all groups.

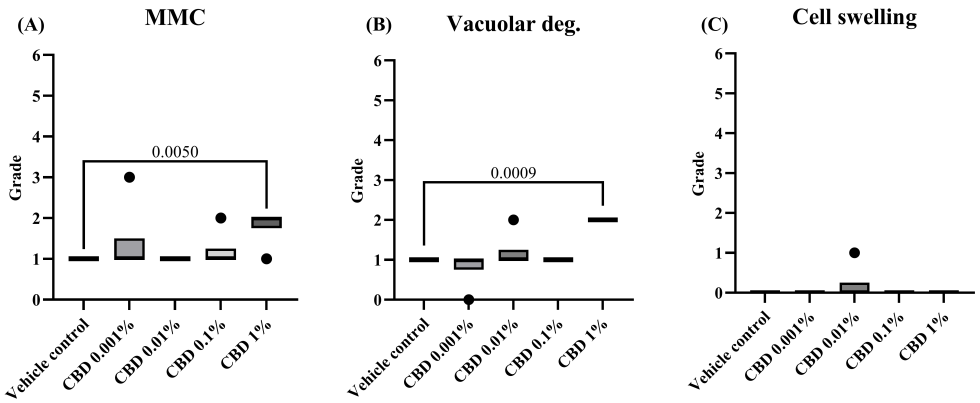


Figure 10 Histopathological grading scores of pancreatic parameters across CBD treatment groups. Histopathological grading scores for pancreatic parameters are presented for the vehicle control and cannabidiol (CBD)-treated groups across increasing dose levels. Assessed parameters comprised (A) melanomacrophage center (MMC), (B) vacuolar degeneration, and (C) cellular swelling. Data are presented as Tukey boxplots with median and interquartile range. Outliers are shown as individual points.

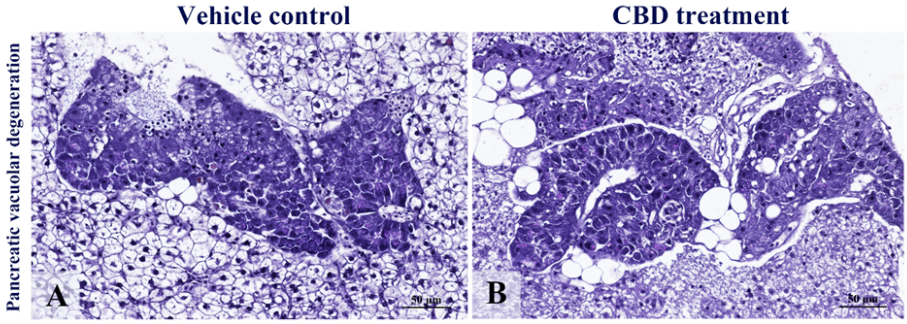


Figure 11 Representative histopathological alterations in pancreas. Hematoxylin and eosin (H&E) stained pancreas sections comparing vehicle control and CBD treated groups are shown. (A) Vehicle control sections demonstrate low grade 1 pancreatic vacuolar degeneration while (B) show pancreatic vacuolar degeneration grade 2 on 1% CBD group. Representative images were selected based on the predominant lesions identified in the semiquantitative histopathological grading analysis. Scale bars = 50 µm (original magnification ×40).

Splenic histopathological analysis revealed a dose-dependent increase in both MMC abundance and splenic congestion (Figure 12). Splenic congestion, appearing as marked dilation and engorgement of red pulp sinusoids with erythrocytes, increased progressively with CBD concentration and reached

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statistical significance in the CBD 0.1% ($p = 0.0010$) and 1% groups ($p < 0.0001$) (Figure 12B, Figure 13D and 13E). MMC scores, characterized by enlarged aggregates of golden-brown pigment-laden macrophages within the red pulp, were significantly elevated in CBD-treated groups from 0.01% onward ($p = 0.0041$), with the highest grades observed at CBD 0.1% ($p < 0.0001$) and 1% ($p = 0.0016$) (Figure 12A, Figure 13A, 13B and 13C). The progressive enlargement and increased density of MMCs is consistent with enhanced erythrocyte turnover and metabolic clearance of damaged cellular constituents.

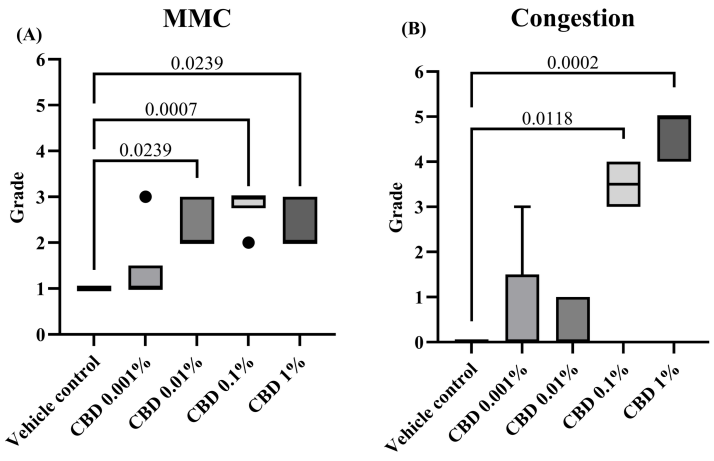


Figure 12 Histopathological grading scores of splenic parameters across CBD treatment groups. Histopathological grading scores for splenic parameters are presented for the vehicle control and cannabidiol (CBD)-treated groups across increasing dose levels. Assessed parameters comprised (A) melanomacrophage center (MMC) and (B) splenic congestion. Data are presented as Tukey boxplots with median and interquartile range. Outliers are shown as individual points.

Dose–response correlations between CBD exposure and histopathological lesions

Spearman rank correlation analysis revealed organ-specific dose-response relationships between cannabidiol (CBD) concentration and total histopathological lesion scores (Table 3). A very strong positive correlation was observed in the spleen ($\rho = 0.93$, $p < 0.0001$), indicating a marked increase in lesion severity with increasing CBD exposure. Similarly, the liver demonstrated a strong positive association ($\rho = 0.89$, $p < 0.0001$). Significant positive correlations were also identified in the kidney ($\rho = 0.77$, $p < 0.0001$) and pancreas ($\rho = 0.65$, $p < 0.0001$), reflecting strong and moderate dose-dependent increases in histopathological alterations, respectively. Correlation strength was interpreted according to Schober et al. (2018). In contrast, no significant correlation was detected in the gill ($\rho = -0.26$, $p = 0.1609$), indicating that lesion scores in this organ were not significantly associated with CBD concentration under the experimental conditions.

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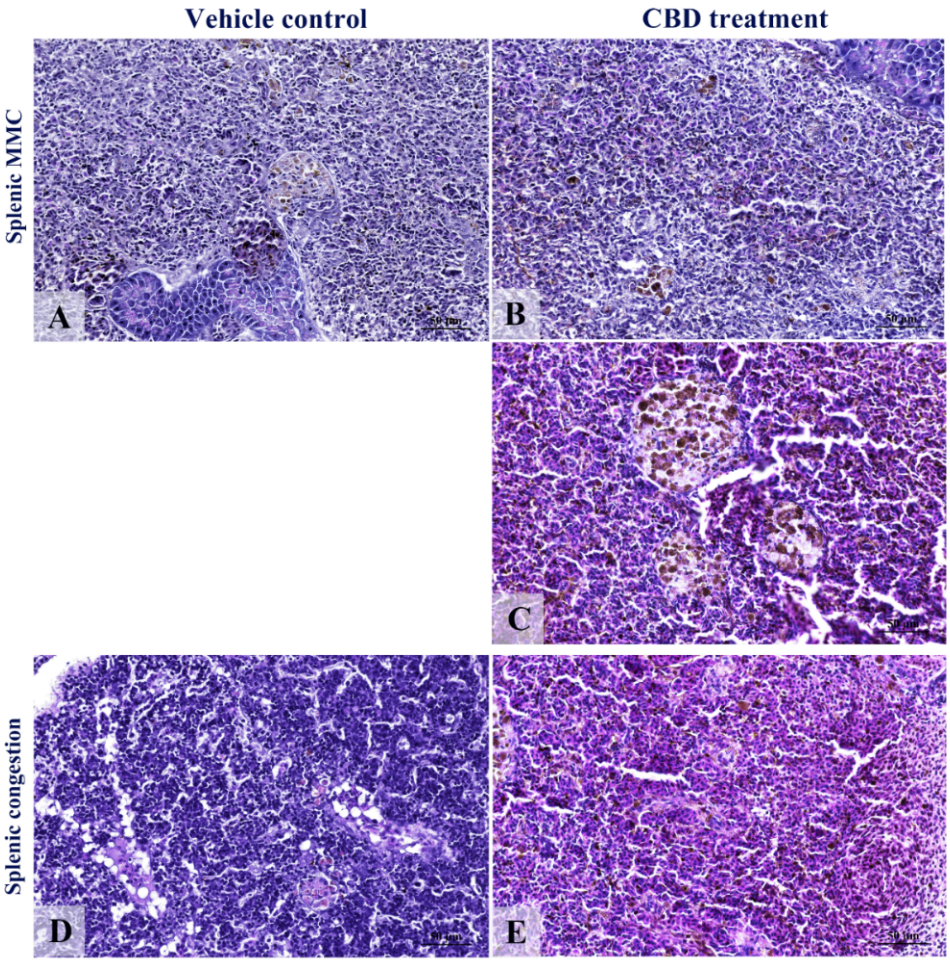


Figure 13 Representative histopathological alterations in spleen. Hematoxylin and eosin (H&E) stained splenic sections comparing vehicle control and CBD treated groups are shown. (A) Vehicle control sections demonstrate low grade scattered melanomacrophage centers (MMCs) at grade 1 (arrow). (B) show MMC 0.1% CBD treated at grade 2 and (C) 1% CBD treated fish display increased grade 3 MMC (arrow). (D) show splenic parenchyma with no congestion while (E) show splenic congestion at 1% CBD-treated at grade 5 (arrow). Representative images were selected based on the predominant lesions identified in the semiquantitative histopathological grading analysis. Scale bars = 50 µm (original magnification ×40).

Table 3 Dose–associated correlations between cannabidiol (CBD) exposure and total histopathological lesion scores across examined organs. Spearman rank correlation coefficients (ρ) and corresponding two-tailed p-values are shown.

Organ	ρ	p-value
Gill	-0.26	0.1609
Liver	0.89	<0.0001
Kidney	0.77	<0.0001
Pancreas	0.65	<0.0001
Spleen	0.93	<0.0001

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DISCUSSION

The present study provides the among first dose-dependent sub-chronic toxicological characterization of dietary cannabidiol in koi carp (*C. rubrofuscus*), integrating hematological, serum biochemical, and multi-organ histopathological endpoints across five graded concentrations over 28 days. Our findings demonstrate a dose-dependent spectrum of toxicological effects from dietary cannabidiol exposure in koi carp. Low concentrations (0.001%) preserved homeostatic balance across hematological, biochemical, and histopathological endpoints, whereas intermediate levels (0.01-0.1%) induced subtle splenic and hepatic alterations consistent with emerging physiological stress. High-dose (1%) exposure precipitated overt multi-organ pathology, thereby defining initial empirical safety thresholds for CBD in cyprinid species.

Soybean oil is widely used as a lipid carrier in aquaculture diet formulations. Notably, lipid supplementation in teleosts can alter hepatic lipid metabolism, influence erythropoiesis, and modulate inflammatory pathways depending on the fatty acid composition and inclusion level (Caballero et al., 2006). At 2% w/w inclusion, the vehicle produced no significant differences from the feed control in biochemical parameters, WBC, or erythrocyte indices, with only modest reductions in hematocrit and RBC (hemoglobin unaffected and histopathological scores indistinguishable). Although studies specifically on erythropoiesis are limited, Chen et al. 2024 reported that higher soybean oil levels elevated triglycerides and cholesterol in grass carp serum, whereas Jiang et al. 2013 demonstrated improvements in immune parameters such as lysozyme activity and complement levels. The erythrocytic depression in vehicle controls likely stems from PUFA-induced lipid peroxidation, increasing membrane fragility and hemolysis in antioxidant-deficient teleosts (Tocher, 2003). Interestingly, CBD induced dose-dependent recovery at low doses likely due to cytoprotective antioxidant stabilization of the lipid bilayer (Atalay et al., 2019), but marked erythrocytosis at 1% concurrent with multi-organ pathology, reflecting a compensatory stress response (Fazio, 2019; Witeska, 2023). These biphasic effects from oxidative buffer to systemic stressor underscore the necessity of vehicle controls in aquatic toxicology, confirming erythroid shifts as physiological baseline adjustments to dietary oil rather than hemolytic toxicity. Accordingly, all CBD treatment groups were interpreted relative to the vehicle control to partition CBD-specific effects from those attributable to the lipid carrier.

Building on this biphasic framework, the erythroid response at low CBD concentrations merits mechanistic discussion beyond antioxidant cytoprotection alone. The stability of MCV, MCH, and MCHC across all groups confirms that individual erythrocyte morphology and hemoglobin loading remained intact, excluding compensatory or pathological regenerative erythropoiesis such as that associated with hemolytic anemia or nutritional deficiency as explanatory mechanisms (Adewoyin et al., 2019). The absence of leukocyte modulation further supports this interpretation, indicating that CBD did not elicit a systemic innate immune response detectable at the blood level within the 28-day observation window. The increase in circulating RBC and Hct therefore reflects augmented erythrocyte number through extended lifespan or enhanced production, not stress-driven hemoconcentration (Mithoowani et al., 2020). A complementary mechanism involves indirect modulation of erythropoietic pathways via the teleost endocannabinoid system; CB2 receptor activation has been linked to erythroid progenitor proliferation in vertebrates, and functionally conserved CB1 and CB2 homologues are well-documented in cyprinids (Cottone et al., 2013; Cristino et al., 2020). The plateau in Hb and RBC at ≥0.01% CBD, rather than continued dose-linear escalation, is consistent with receptor saturation or physiological ceiling effects (Taborda et al., 2024), further supporting a controlled cytoprotective mechanism at sub-toxic concentrations (Stanley et al., 2013). Taken together, the erythroid data suggest a low-dose facilitative effect of CBD on red cell production

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or stability in koi carp, reflecting the compound's antioxidant or endocannabinoid-modulatory properties rather than an adverse toxicological signal.

In teleosts, AST is widely distributed across hepatic, muscular, cardiac, and renal tissues, and its elevation in serum is generally interpreted as a marker of hepatocellular membrane disruption or necrosis rather than a liver-specific indicator in isolation (Fazio, 2019; Hrubec et al., 2000). In this study, AST activity rose significantly from 0.01% CBD onward, increasing progressively to 0.1% and 1% while ALT and ALP remained unchanged across all CBD concentrations. In cyprinids, AST substantially exceeds ALT in hepatic expression, making AST the more sensitive marker of early hepatocellular injury (Groff and Zinkl, 1999; Fazio, 2019). The selective AST elevation therefore indicates hepatic membrane stress concordant with the histopathological degeneration and necrosis observed at higher doses. These concordant biochemical and histological findings are consistent with findings in rodent models (Ewing et al., 2019) and the clinical meta-analysis by Lo et al. (2023), which reported a significantly elevated risk of liver enzyme elevation with CBD use, particularly at doses exceeding 20 mg/kg/day. Serum cholesterol was elevated at all CBD concentrations, including the lowest, reaching a plateau at 0.1%. CBD modulates the endocannabinoid system via indirect mechanisms including inhibition of endocannabinoid degradation and interactions with GPR55, TRPV1, and 5-HT1A receptors (McPartland et al., 2015; Russo and Marcu, 2017), potentially perturbing CB1-regulated hepatic lipogenesis and cholesterol efflux pathways (Christopoulou and Kiortsis, 2011). That cholesterol dysregulation occurred at concentrations insufficient to cause structural liver damage suggests a functional lipid-metabolic sensitivity preceding overt hepatotoxicity. This observation is supported by evidence from human cell lines demonstrating that CBD exposure induces upregulation of cholesterol biosynthesis, transport, and storage pathways, accompanied by increased membrane incorporation (Guard et al., 2020). These findings further highlight the capacity of CBD to modulate systemic lipid homeostasis. However, species-specific differences in lipid metabolism between mammals and teleosts necessitate cautious extrapolation of these mechanisms to koi carp.

The absence of histopathological alterations in the gills across all treatment groups reflects the pharmacokinetic partitioning associated with dietary exposure. Unlike waterborne toxicants that directly insult the respiratory surface, orally administered CBD is absorbed via the gastrointestinal tract, leading to a concentrated metabolic burden in the liver and kidneys rather than the branchial tissues (Hrubec et al., 2000; Perucca and Bialer, 2020). Gill integrity thereby serves as an internal validation of the exposure route, confirming route-specific distribution.

The spleen demonstrated the strongest dose-response association across all organs, with MMC proliferation detectable from 0.01% CBD, earlier than any other histopathological endpoint. Teleost MMCs serve as integrated sites of erythrocyte catabolism, oxidized lipid clearance, and immune surveillance; their expansion under xenobiotic exposure is a well-validated sentinel of systemic oxidative stress and immune activation (Agius and Roberts, 2003; Steinel and Bolnick, 2017; Carreras-Colom et al., 2022). The dose-dependent MMC proliferation observed here likely reflects augmented phagocytic clearance of oxidatively modified erythrocytes or pro-oxidative CBD biotransformation byproducts (William and Acker, 2024). Splenic MMC expansion concurrent with circulating erythroid augmentation suggests dynamic equilibrium between low-dose erythroprotection and accelerated splenic red cell turnover, a shift from antioxidant cytoprotection at low doses to pro-oxidative metabolite burden at high doses (Atalay et al., 2019; Dawidowicz et al., 2021).

Beyond serving as a histopathological biomarker of xenobiotic exposure, increased MMC abundance may also reflect alterations in overall fish health status. MMC proliferation has been associated with enhanced phagocytic activity, erythrocyte turnover, sequestration of oxidized cellular debris, and activation of

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innate immune responses in teleosts (Agius and Roberts, 2003). Increased MMC abundance has also been reported following exposure to infectious agents, environmental pollutants, heavy metals, and other xenobiotics, reflecting physiological stress and inflammatory stimulation (Kaewamatawong et al., 2013; Sayed and Younes, 2017; Viana et al., 2021; Sayed et al., 2022; Carreras-Colom et al., 2022; Jantrakajorn et al., 2026). These responses are thought to be associated with oxidative stress and the generation of reactive oxygen species (ROS), which can induce cellular injury, apoptosis, and tissue damage (Passantino et al., 2014). As damaged cells, oxidized materials, and cellular debris accumulate, MMCs increase in abundance to facilitate their sequestration and phagocytic clearance, resulting in MMC proliferation (Passantino et al., 2014). Therefore, the dose-dependent increase in MMC scores observed in the present study suggests that higher dietary CBD concentrations may impose increasing immunological and oxidative burdens. A limitation of the present study is that MMCs were evaluated using a semi-quantitative scoring system rather than morphometric measurements. Quantitative assessment of MMC number, size, or area relative to tissue area may provide a more precise evaluation of immune activation and should be considered in future studies.

The liver demonstrated dose-dependent histopathological changes with hepatocyte vacuolar degeneration emerging as the primary lesion at intermediate concentrations and hepatocellular necrosis at the highest dose. The observed vacuolar degeneration likely reflects intracellular lipid accumulation resulting from CBD-mediated impairment of mitochondrial fatty acid β-oxidation, an early potentially reversible hepatocellular response consistent with mammalian in vitro models (Ewing et al., 2019; Drummond-Main et al., 2023). At 1% CBD, progression to necrosis indicates that lipid-induced stress exceeded adaptive cellular capacity, representing a well-recognized dose-escalation continuum in aquatic toxicology (Georgieva et al., 2014; Liu et al., 2024). The concurrent pronounced elevation of serum AST corroborates this histological transition from reversible vacuolation to irreversible membrane disruption, providing biochemical validation of the structural injury observed. High-dose CBD may further propagate hepatocellular damage through the release of proinflammatory cytokines, as reported in human clinical contexts (Larson et al., 2025). The hepatotoxic threshold identified here (≥0.1%) is directionally consistent with murine data (Ewing et al., 2019) and clinical evidence that CBD-associated liver injury follows a dose-dependent trajectory, generally well-tolerated at lower exposures (Eadie et al., 2024). However, direct mammalian-to-teleost dose equivalence cannot be assumed owing to fundamental differences in first-pass cannabinoid biotransformation and thermoregulation. Pancreatic histopathology demonstrated mild but statistically significant alterations. Given the periportal anatomical embedding of the teleost exocrine pancreas (Wallig et al., 2024), this may represent secondary injury propagated from hepatic inflammation rather than a primary pancreatic toxic mechanism as found in studies of rats and humans (Brenneman et al., 2014; Rickels et al., 2020).

Renal histopathology identified the proximal tubule as the primary target of CBD-induced nephrotoxicity, with hyaline droplet degeneration significant at 0.1% CBD and tubular necrosis emerging exclusively at 1% CBD. Proximal tubular cells are among the most metabolically active cells in the vertebrate kidney, relying almost exclusively on oxidative phosphorylation and therefore highly vulnerable to mitochondrial dysfunction and oxidative stress (Georgieva et al., 2014). Hyaline droplet accumulation reflects impaired reabsorption of filtered plasma proteins, an early marker of tubular epithelial dysfunction, while tubular necrosis at the highest dose indicates that oxidative burden exceeded the regenerative threshold of the proximal epithelium. Notably, serum total protein, BUN, and creatinine remained unaffected across all treatment groups, consistent with early-stage tubular injury of insufficient severity to reduce glomerular filtration rate or deplete circulating protein pools, a dissociation characteristic of sub-chronic toxicological exposure in teleosts (Fazio, 2019; Hrubec et al., 2000). However, while low doses of CBD have

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been studied for their potential protective effects against renal damage, mitigating oxidative stress and inflammation in various kidney injury models (Hokmabadi et al., 2023; Soliman et al., 2023), high doses may induce mitochondrial stress and lipid dysregulation, potentially contributing to renal damage, as observed in our study (Rokicki et al., 2024).

Synthesizing all endpoints along a dose continuum, the low dose (0.001%) was associated with erythroprotection without clinical signs or histological injury; intermediate doses (0.01-0.1%) elicited the earliest splenic MMC proliferation and hepatic AST/cholesterol changes in the absence of clinical signs; and the high dose (1%) produced multi-organ pathology accompanied by the clinical and behavioural signs reported above. This dose-dependent progression explicitly links behaviour, clinical signs, hematology, and histopathology to dietary CBD exposure, reinforced by the Spearman correlations presented in Table 3.

Based on this integrated dose–response pattern, a provisional no-observed-adverse-effect level (NOAEL) of 0.001% CBD (10 mg/kg feed) and a lowest-observed-adverse-effect level (LOAEL) of 0.01% CBD (100 mg/kg feed) are proposed for koi carp under sub-chronic dietary exposure. These thresholds are practically significant; for instance, a recent study in Nile tilapia demonstrated welfare improvements as reduced aggression or reduced stress biomarkers at dietary CBD of 10-20 mg/kg feed with no adverse outcomes reported (Camargo-dos-Santos et al., 2022; Mortuza et al., 2023). Concentrations at or above 1,000 mg/kg feed should be considered hepatotoxic and nephrotoxic in cyprinids under sub-chronic conditions. Notably, this welfare-effective dose range overlaps with the NOAEL established in the present study, yet falls below the LOAEL, suggesting a potentially navigable therapeutic window in cyprinids, though interspecies differences in CBD sensitivity cannot be excluded. From a practical standpoint, dietary CBD can be readily incorporated into commercial koi feeds by top-dressing with an oil-based carrier, requiring no specialised equipment. However, given the relatively high cost of purified CBD compared with conventional functional additives, continuous prophylactic use is not economically justified. Targeted, short-duration supplementation within the NOAEL range (≤10 mg/kg feed) during defined high-stress events such as transport, grading, or quarantine is therefore recommended as the most cost-effective application in sustainable ornamental aquaculture, while strictly avoiding inclusion at or above 1,000 mg/kg feed given the hepato- and nephrotoxicity demonstrated here. The limitations of this study include the use of a single species and a 28-day exposure period, which restrict extrapolation to chronic exposure scenarios, other developmental stages, or non-cyprinid taxa. In addition, sex was not systematically differentiated; therefore, potential sex-specific differences in CBD biotransformation reported in rodent models cannot be excluded (Forner-Piquer et al., 2024). Future research should incorporate toxicokinetic profiling, molecular biomarkers, and multi-generational designs across commercially relevant aquaculture species to comprehensively characterize the risk-benefit profile of dietary CBD supplementation.

CONCLUSIONS

Sub-chronic dietary CBD in koi carp follows a dose-dependent toxicological continuum, with erythroprotective effects at 10 mg/kg feed, subclinical immune activation at 100 mg/kg, hepatic and renal injury at 1,000 mg/kg, and multi-organ toxicity at 10,000 mg/kg. The spleen and liver are the principal target organs. A dietary NOAEL of 10 mg/kg feed and LOAEL of 100 mg/kg feed are proposed as evidence-based reference values to guide safe CBD supplementation and regulatory oversight in ornamental cyprinid aquaculture.

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CONFLICT OF INTEREST

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