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Dietary *Withania coagulans* fruit powder modulates immune and antioxidant responses in zebrafish

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Withania coagulans modulates immunity in zebrafish

Abstract

This study evaluated the effects of dietary supplementation with Indian rennet (*Withania coagulans*) fruit powder (WCF) on growth performance, mucosal immune responses, hepatic stress indicators, antioxidant status, and the expression of immune- and antioxidant-related genes in zebrafish (*Danio rerio*) as a model species. Fish were fed a basal diet supplemented with 0 (control), 0.5, 1, or 2% WCF for 60 days. Growth performance did not differ significantly among treatments ($P > 0.05$). Fish fed the 1% WCF diet showed significantly higher skin mucus total immunoglobulin, total protein, and lysozyme activity compared with the control and other treatment groups ($P < 0.05$). Dietary WCF also modulated immune-related gene expression, with TNF-α, IL-1β, and LYZ transcripts showing the highest expression in the 1% group ($P < 0.05$). The 1% treatment also significantly increased superoxide dismutase (SOD) and glutathione peroxidase (GPx) mRNA levels, while catalase (CAT) gene expression was significantly elevated in all WCF-fed groups ($P < 0.05$). In contrast, the 2% inclusion level was associated with elevated AST, ALT, and LDH activities and reduced immune and antioxidant responses, suggesting physiological stress at the highest dose. These findings suggest that dietary inclusion of 1% *W. coagulans* fruit powder may improve mucosal immunity and antioxidant defense in zebrafish without compromising growth, whereas higher levels may be detrimental.

Keywords: *Withania coagulans*; zebrafish; mucosal immunity; antioxidant defense; lysozyme; phytogenic feed additive.

1. Introduction

The global expansion of aquaculture has intensified the search for natural, safe, and sustainable feed additives that can enhance growth, immunity, and physiological resilience in farmed fish (Abdul Kari 2025). Traditional reliance on synthetic additives and antibiotics has raised concerns over environmental contamination, antimicrobial resistance, and residue accumulation in aquatic organisms (Singh et al., 2024; Sun et al., 2026). This has prompted a paradigm shift toward plant-derived nutraceuticals as viable alternatives (Reverter et al., 2017; Yousefi et al., 2026).

Phytogenic compounds rich in bioactive metabolites have demonstrated significant potential in improving growth performance, reducing oxidative stress, and modulating immune responses across various teleost species (Gatlin et al., 2007; Awad and Awaad, 2017). Medicinal plants have been shown to exert beneficial effects on innate immune parameters, including IgM, lysozyme activity, respiratory burst, and cytokine gene expression, thereby enhancing disease resistance (Harikrishnan et al., 2011; Abdel Mohsen Hassan et al., 2018; Yan et al., 2026). In aquaculture, herbal feed additives can also significantly enhance antioxidant enzyme activities such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx), while upregulating genes involved in oxidative defense (Gharaei et al., 2020; Asif et al., 2026). These extracts contain bioactive compounds such as saponins, terpenoids, flavonoids, and alkaloids that stimulate the immune system, increase antioxidant capacity, and may improve growth (Reverter et al., 2017; Sumana et al., 2025).

Zebrafish (*Danio rerio*) is widely recognized as a suitable model organism for studying physiology, immunity, and nutrition, owing to its short life cycle, ease of handling, genetic tractability, and physiological similarity to many farmed species (Lee-Estevez et al., 2018; Abdollahpour et al., 2025). Despite the growing body of evidence on medicinal plants in fish, studies on *Withania* species remain limited. In juvenile greasy grouper (*Epinephelus tauvina*), dietary supplementation with 200 mg/kg *Withania somnifera* extract significantly reduced mortality following *Vibrio harveyi* infection (Sivaram et al., 2004). Similarly, in Indian major carp (*Labeo rohita*), *W. somnifera* root powder stimulated immunological parameters and increased disease resistance against *Aeromonas hydrophila* (Sharma et al., 2010).

Indian rennet, *Withania coagulans* (family Solanaceae), is native to Iran, Afghanistan, Pakistan, and India (Tahir et al., 2024). *Withania* species have been used in indigenous medicine for over 3000 years to treat various diseases, including cancer, hypercholesterolemia, inflammation, diabetes, and neurological disorders (Khan et al., 2021). *W. coagulans* contains potent bioactive substances, including withanolides, alkaloids, phenolics, and flavonoids (Shahzad et al., 2021). Recently, Fazio et al. (2023) reported that dietary supplementation with *W. coagulans* fruit extract improved growth performance, haematological parameters, and lysozyme activity, while reducing mortality following bacterial challenge in Labeo rohita, aligning with the immunostimulatory potential of this plant in aquaculture. However, the biological effects of *W. coagulans* fruit powder, rather than extract, remain poorly characterized in zebrafish. Therefore, the present study aimed to investigate the effects of graded dietary levels of *W. coagulans* fruit powder on growth performance, mucosal immune responses, antioxidant defense mechanisms, hepatic health indicators, and the expression of immune- and antioxidant-related genes in zebrafish. We hypothesized that *W. coagulans* supplementation would improve immune and antioxidant responses in a dose-dependent manner, whereas excessive inclusion levels may result in diminished or adverse physiological effects.

2. Materials and methods

2.1 Ethical information

All experimental procedures were conducted in strict accordance with the ARRIVE guidelines and in full compliance with Directive 2010/63/EU and internationally accepted standards for the care and use of laboratory animals. The experimental protocols were reviewed and approved by the Research Ethics Committee of the Research Institute of Zabol (no specific approval code was issued for this study). All efforts were made to minimize animal suffering throughout the study, including the use of appropriate anesthesia (50 mg/L clove powder) during handling and sampling, careful monitoring of fish health and behavior, and humane endpoints to prevent any unnecessary pain or distress.

2.1.1 Plant material

W. coagulans plants were collected from their natural habitat in the Baluchistan region of southeastern Iran. The plant species was authenticated by Mehdi Dehghani, a faculty member of the Department of

Biology and curator of the Zabol University Herbarium (UOZH). A voucher specimen was deposited in the Zabol University Herbarium under herbarium code UOZH2750. Collection of plant material was conducted in accordance with relevant institutional, national, and international guidelines and legislation. No specific permits were required for the collection of this species in the region, as it is not listed as endangered or protected under Iranian law or the IUCN Red List. The study complies with the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

2.2 Preparation of experimental diets

W. coagulans plants were collected from the Baluchistan region of southeastern Iran and taxonomically identified prior to use. The fruits were separated, dried, ground using an electric grinder, and sieved to obtain a fine fruit powder (WCF).

The physicochemical and phytochemical characteristics of WCF were subsequently determined using standard analytical procedures. Moisture content was measured by oven drying at 105°C to constant weight, whereas total ash content was determined by incineration in a muffle furnace at 550°C according to AOAC methods. Total phenolic content (TPC) was determined using the Folin–Ciocalteu method and expressed as mg gallic acid equivalents (GAE)/g dry weight. Total flavonoid content (TFC) was measured using the aluminum chloride colorimetric assay and expressed as mg quercetin equivalents (QE)/g dry weight. Total tannin content was determined using the Folin–Denis method and expressed as mg tannic acid equivalents (TAE)/g. Mineral composition (Mg, Ca, Fe, and Zn) was quantified after acid digestion using atomic absorption spectroscopy (AAS). Fatty acid composition was analyzed by gas chromatography–mass spectrometry (GC–MS) following lipid extraction and conversion to fatty acid methyl esters (FAMEs). Total withanolides were quantified by reverse-phase high-performance liquid chromatography (RP-HPLC) using a C18 column with UV detection at 227–230 nm. Chromatographic fingerprinting of characteristic withanolides, including withacoagulin and coagulin derivatives, was performed using HPLC/UPLC to verify powder quality and authenticity (Table 1).

A commercial basal diet (Biomar, France; dry matter 93%, crude protein 39%, crude lipid 14.8%, ash 10.6%) was used as the control diet. WCF was incorporated into the basal feed at inclusion levels of 0.5%, 1%, and 2% (w/w). The experimental diets were thoroughly mixed, dried, packaged, and stored at 4°C for a maximum of 7 days before feeding. The overall experimental design and analytical workflow of the study are summarized in Figure 1.

2.3 Fish husbandry and experimental conditions

Healthy zebrafish (*Danio rerio*) with an initial body weight of 94.6 ± 5.3 mg were obtained from Gorgan Mahi Co. (Gorgan, Iran). Fish were acclimated for 2 weeks in a 600-L polyethylene tank. After acclimation, fish were randomly allocated to 12 aquaria (100 L each) at a density of 50 fish per aquarium, with triplicate aquaria per treatment. Aquaria were continuously aerated. Waste was siphoned every 2 days, and 50% of the water was replaced with dechlorinated tap water. Water quality parameters were monitored regularly: temperature 27 ± 2°C, pH 8.1 ± 0.6, dissolved oxygen 7.7 ± 0.3 mg/L, nitrate 0.06 ± 0.01 mg/L, and ammonia 0.1 ± 0.0 mg/L. The feeding trial lasted 60 days, and fish were fed their respective experimental diets three times daily (07:30, 13:00, and 19:30) to apparent satiation (Abdollahpour et al., 2024).

2.4 Growth performance assessment

At the end of the feeding trial, fish were anesthetized prior to handling and weighing to minimize stress and ensure accurate measurements. Anesthesia was induced by immersing fish in a solution of clove powder at a concentration of 50 mg/L prepared in dechlorinated tap water. Fish were placed in the anesthetic bath and monitored until they reached the appropriate stage of anesthesia, characterized by loss of equilibrium, reduced opercular movement, and absence of response to external stimuli. Once fully anesthetized, each fish was gently removed from the anesthetic bath using a soft net, blotted dry with a paper towel to remove excess water, and individually weighed using a precision balance. After weighing, fish were immediately returned to their respective aquaria containing fresh, aerated water for rapid recovery. All fish from each aquarium were weighed within a short timeframe to minimize handling stress. Following the growth performance assessment, fish were returned to their tanks and monitored until full recovery was observed before the continuation of the experiment. Growth

performance parameters were calculated using standard formulas (Abdollahpour et al., 2024). The aquarium was considered the experimental unit for statistical analysis. The aquarium was considered the experimental unit for statistical analysis.

Weight gain (WG; mg) = final body weight – initial body weight

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

Specific growth rate (SGR; %/day) = (Ln final body weight – Ln initial body weight) × 100 / days

Survival rate (SR; %) = (final number of fish / initial number of fish) × 100

2.5 Mucus and whole-body sampling

At the end of the experiment, nine fish per treatment (three from each replicate) were anesthetized with 50 mg/L clove powder after 24 h of fasting. For skin mucus collection, fish were transferred to polyethylene bags containing 5 mL of 50 mM NaCl and gently rubbed for 2 min. The collected mucus was centrifuged at 1500 × g for 10 min at 4°C, and the supernatant was stored at −80°C (Safari et al., 2019). Whole-body extracts (WBE) were prepared for biochemical assays (Holbech et al., 2001) and stored at −80°C.

2.6 Immune and antioxidant assays

Alkaline phosphatase (ALP) activity was measured using a commercial kit (Man Co., Iran) at 405 nm. Lysozyme activity was determined by the turbidimetric assay using *Micrococcus lysodeikticus* (Sigma; 0.375 mg/mL) (Ellis, 1990). Total immunoglobulin (Ig) concentration was measured by the Biuret method after 12% polyethylene glycol (PEG) precipitation (Amar et al., 2002). Total protein was measured by the Biuret method at 540 nm (Melingen and& Wergeland, 2000). Antioxidant enzyme activities (SOD, GPx, and CAT) and malondialdehyde (MDA) levels were measured using ZellBio kits (GmbH, Germany) according to the manufacturer's instructions. Hepatic enzymes AST, ALT, and LDH were measured using commercial kits (Man Co., Iran; Pars Azmoon, Iran).

2.7 Expression of antioxidant and immune-related genes

2.7.1 RNA extraction and cDNA synthesis

At the end of the experiment, nine fish per treatment (three fish per replicate tank) were collected. Total RNA was extracted from 50 mg of liver tissue using a Sambio kit (Sam Azma Gene Co., Iran). RNA concentration and purity were assessed using a Nanodrop spectrophotometer (DS-11, DeNovix Inc.,

USA), and RNA integrity was verified by 1.5% agarose gel electrophoresis. Only high-quality RNA samples were used for cDNA synthesis. Total RNA (500 ng) was reverse-transcribed to cDNA using a cDNA synthesis kit (Sam Azma Gene Co., Iran).

2.7.2 Real-time PCR

Primers for antioxidant- and immune-related genes are shown in Table 2. β-Actin was used as the housekeeping gene. Quantitative real-time PCR was performed using a Cielo 6 system (Azure Cielo, USA) with SYBR Green Master Mix (Sambio, Iran). Each 20 µL reaction contained 1 µM of each primer, 10 µL SYBR Green Master Mix, and up to 500 ng cDNA. The thermal cycling protocol consisted of 94°C for 20 s, 58°C for 30 s, and 72°C for 40 s. Relative gene expression was calculated using the 2^−ΔΔCt method (Livak & Schmittgen, 2001). Primer specificity was confirmed by melt-curve analysis, and amplification efficiency was verified for each primer pair.

2.8 Statistical and multivariate analyses

All statistical analyses were performed using R version 4.4.1 (R Core Team, 2024). Normality and homogeneity of variance were assessed using the Shapiro–Wilk and Levene's tests, respectively. Differences among dietary treatments were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's HSD post hoc test. Statistical significance was accepted at $P < 0.05$. Results are presented as mean ± SD.

To evaluate potential hormetic dose–response relationships, Brain–Cousens models were fitted to selected immune and antioxidant endpoints using the 'drc' package (Ritz et al., 2015). Effect sizes (Cohen's d) and their 95% confidence intervals were calculated using the 'effsize' package to quantify the magnitude of treatment effects relative to the control group.

Multivariate analyses were performed to visualize treatment separation and relationships among measured biomarkers. Principal Component Analysis (PCA) was conducted using the 'FactoMineR' and 'factoextra' packages. Pearson correlation coefficients among measured variables were calculated and visualized as a hierarchical clustering heatmap using the 'pheatmap' package.

Additional exploratory analyses, including Multiple Factor Analysis (MFA), Redundancy Analysis (RDA), Canonical Correlation Analysis (CCA), Partial Least Squares Regression (PLSR), benchmark dose modelling, and bootstrap validation, are provided in the Supplementary Materials and should be

interpreted with caution due to the limited sample size (n = 12 tank means). These analyses were exploratory and were used to visualize multivariate patterns rather than to test confirmatory causal hypotheses

3. Results

3.1 Survival rate and growth performance

Survival rates ranged from 90% to 93%, with no significant differences among treatments ($P > 0.05$; Table 3). Growth performance parameters are presented in Table 2. Weight gain (WG) and specific growth rate (SGR) did not differ significantly among treatment groups ($P > 0.05$). No significant differences were observed among control, 0.5%, and 1% groups ($P > 0.05$).

3.2 Immune response

ALP activity significantly increased in the 2% WCF group ($P < 0.05$). Lysozyme activity was significantly higher in the 1% WCF group compared to control and 2% groups ($P < 0.05$). Total immunoglobulin and total protein concentrations were significantly higher in the 1% WCF group compared with control and other treatments ($P < 0.05$; Fig. 2).

3.3 Antioxidant enzyme activities

The lowest SOD activity was observed in the 2% WCF group ($P < 0.05$). No significant differences in GPx activity were detected among treatments ($P > 0.05$). CAT activity was significantly higher in the 1% WCF group ($P < 0.05$). MDA levels were lowest in the 0.5% WCF group and highest in the 2% WCF group ($P < 0.05$; Figure 3).

3.4 Hepatic enzymes

All measured hepatic enzymes (AST, ALT, LDH) significantly increased in the 2% WCF group ($P < 0.05$; Figure 4). Conversely, ALT and LDH activities decreased in the 0.5% and 1% WCF groups compared to the control ($P < 0.05$).

3.5 Immune and antioxidant gene expression

Expression of *TNF-α*, IL-1β, and *LYZ* was significantly upregulated in the 1% WCF group compared to control and other treatments ($P < 0.05$; Fig. 5). *SOD* and *GPx* expression were significantly higher

in the 1% WCF group ($P < 0.05$; Fig. 6). *CAT* expression was significantly higher in the 2% WCF group compared to all other groups ($P < 0.05$; Fig. 6).

3.6 Dose-response hormesis modelling

To explore potential biphasic (hormetic) dose–response relationships, Brain–Cousens models were fitted to selected key endpoints (Fig. 7). The inverted-U shape was significant for IgM (ED_{50} = 2.32% WCF) and mucus protein (ED_{50} = 2.24% WCF), with peak responses at 1% WCF. TNF-α and GPx gene expression also exhibited strong hormetic responses (peak $\log_2$ fold changes of 4.66 and 4.30, respectively).

3.7 Effect sizes (Cohen's d)

Effect sizes for 1% WCF vs. control were very large (Cohen's d > 2.5) for all beneficial parameters: TNF-α (26.43), GPx gene (19.31), mucus protein (3.40), CAT activity (2.95) and IgM (2.52). MDA showed a large reduction (d = –5.36). In contrast, 2% WCF caused large adverse effects: MDA (+6.09), LDH (+8.96) and ALT (+2.97), while SOD activity was strongly suppressed (d = –4.42) (Fig. 8). All 95% confidence intervals excluded zero.

3.8 Multivariate discrimination of treatments

Principal Component Analysis (PCA) revealed clear separation among treatment groups, with the first two principal components explaining more than 77% of the total variance (Fig. 9). Variables contributing most strongly to treatment discrimination included IgM, CAT activity, MDA, and TNF-α expression.

3.9 Correlation network analysis

A hierarchical clustering heatmap of all measured parameters revealed strong positive correlations among immune genes (TNF-α, IL-1β, LYZ) and among antioxidant genes (SOD, GPx, CAT), while MDA and hepatic enzymes (AST, ALT, LDH) formed a separate cluster associated with the 2% treatment group (Fig. 10). Additional exploratory analyses, including benchmark dose (BMD) modelling, optimal dose calculation, bootstrap validation, canonical correlation analysis, redundancy analysis, partial least squares regression, self-organizing maps, mediation analysis, and Bayesian network analysis, are presented in the Supplementary Results.

4. Discussion

The present study investigated the effects of dietary *W. coagulans* fruit powder on growth, immunity, antioxidant status, and gene expression in zebrafish. Our findings demonstrate that WCF supplementation up to 2% did not significantly enhance growth metrics. The absence of significant improvements in weight gain and SGR indicates that *W. coagulans* may not directly promote growth under the conditions of this study. This is consistent with previous studies showing that medicinal plant extracts often produce neutral or dose-dependent growth responses in fish (Taştan and Salem, 2021). At elevated doses, saponins and other secondary metabolites may reduce feed palatability or interfere with nutrient absorption (Reverter et al., 2017).

The 1% inclusion level emerged as optimal for immune enhancement. It significantly increased total immunoglobulin, total protein, and lysozyme activity in skin mucus, suggesting that moderate doses of *W. coagulans* enhance humoral immunity and mucosal defense mechanisms. This response may be associated with bioactive constituents such as withanolides and flavonoids, which have previously been reported to possess immunomodulatory properties (Zahran et al., 2018). Conversely, the 2% inclusion level reduced lysozyme activity and total protein, which could reflect diminished immune responsiveness at excessive inclusion levels. This pattern is consistent with a biphasic (hormetic) response reported for other phytogenic feed additives (Gharaei et al., 2020). The findings of Fazio et al. (2023) in *Labeo rohita* showed that *W. coagulans* fruit extract enhanced growth and immunity, with increased lysozyme activity and plasma proteins across all treatment groups. In contrast, the present study found no significant growth enhancement and identified 1% as the optimal immunostimulatory dose in zebrafish, with 2% inducing hepatic stress. These species-specific differences underscore the importance of dose optimization for each target species.

Zebrafish fed the 1% WCF diet exhibited increased catalase (CAT) activity and decreased malondialdehyde (MDA) levels, indicating improved oxidative balance. These findings suggest that *W. coagulans* may enhance antioxidant capacity through bioactive phytochemicals and pathways associated with cellular oxidative stress responses, including Nrf2-related mechanisms (Xu et al., 2013). In contrast, the 2% WCF group showed diminished SOD activity and elevated MDA levels, implying that excessive supplementation may induce oxidative stress.

Hepatic enzyme activities (AST, ALT, LDH) are reliable indicators of liver damage (Sheikhlangi et al., 2024). Significant increases at 2% WCF were consistent with physiological stress and possible hepatic disturbance, while decreased ALT and LDH at 0.5% and 1% suggested potential hepatoprotective effects, consistent with previous reports on *Withania* species (Sharma et al., 2014; Ramadan and Ghareeb, 2023).

The transcriptional activities of immune- and antioxidant-related genes were consistent with biochemical results. Moderate WCF inclusion (1%) significantly upregulated IL-1β, *TNF-α*, and *LYZ*, indicating enhanced innate immune capacity. The heightened transcription of these cytokines is consistent with activation of innate immune pathways, influenced by withanolides and flavonoids (Kumbarwar and Dalvi, 2024). However, the 2% inclusion level suppressed immune gene expression, consistent with reduced lysozyme activity and total protein.

Similarly, 1% WCF increased mRNA levels of *SOD*, *GPx*, and *CAT*, correlating with enzymatic activity and reduced MDA. This pattern is consistent with coordinated transcriptional regulation of antioxidant defense mechanisms and may involve signaling pathways associated with cellular oxidative stress responses, including Nrf2-related pathways (Reverter et al., 2017; Qin et al., 2022). Similarly, dietary WCF influenced the expression of antioxidant-related genes. The 1% inclusion level significantly increased SOD and GPx transcript abundance, consistent with enhanced antioxidant capacity and reduced oxidative damage. Interestingly, CAT expression was highest in the 2% WCF group despite the absence of a corresponding increase in CAT enzyme activity and despite elevated MDA and hepatic enzyme activities. This pattern may represent a compensatory transcriptional response to increased oxidative stress rather than improved antioxidant protection. Together, these findings suggest that moderate WCF supplementation supports antioxidant defense, whereas excessive inclusion levels may impose physiological stress.

To integrate the physiological, biochemical, and molecular responses observed in the present study, a conceptual framework of the proposed mode of action of W. coagulans fruit powder is presented in Figure 11. The findings suggest that bioactive constituents of WCF, including withanolides, flavonoids, phenolic compounds, and saponins, may act synergistically to modulate immune and antioxidant pathways in zebrafish. At the optimal dietary inclusion level (1%), these compounds appear to enhance

mucosal immune function, stimulate the expression of immune-related genes (TNF-α, IL-1β, and LYZ), and strengthen antioxidant defenses through increased antioxidant enzyme activity and reduced lipid peroxidation. In contrast, excessive supplementation (2%) may shift the physiological response toward oxidative imbalance, as reflected by elevated MDA levels, increased hepatic enzyme activities, and reduced immune responsiveness. Collectively, these findings support a hormetic dose–response model in which moderate dietary supplementation promotes physiological resilience and homeostasis, whereas excessive exposure may impose metabolic and oxidative stress.

The present study was conducted in zebrafish under controlled laboratory conditions and did not include pathogen challenge tests or histopathological evaluation of liver tissue. Therefore, the mechanisms underlying the observed immune and hepatic responses require further investigation.

Conclusion

In conclusion, dietary supplementation with *W. coagulans* fruit powder modulated immune and antioxidant responses in zebrafish in a dose-dependent manner. The 1% inclusion level produced the most favorable biological responses, including enhanced mucosal immunity, increased antioxidant capacity, and upregulation of immune- and antioxidant-related genes without adversely affecting growth performance. In contrast, the 2% inclusion level was associated with elevated oxidative and hepatic stress indicators, suggesting that excessive supplementation may compromise physiological homeostasis. Collectively, these findings support the potential application of *W. coagulans* fruit powder as a functional phytogenic feed additive in aquaculture, while highlighting the importance of dose optimization for maximizing beneficial effects.

Authors' contribution

Abdolali Rahdari: Methodology, Investigation, Formal Analysis, Conceptualization, Project Administration, Resources, Writing Draft, Editing Final Version; Ahmad Gharaei: Conceptualization, Writing Draft; Hossein Barahoei: Methodology, Investigation, Formal Analysis, Writing Draft; Roghayeh Karami: Investigation, Writing Draft; Hamed Abdollahpour: Software, Formal Analysis, Writing Draft, Editing Final Version.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data availability statement

Data used in this study are available upon reasonable request from the corresponding authors.

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Table 1. Physicochemical composition and phytochemical characteristics of *Withania coagulans* fruit powder. Total withanolides are expressed as percentage of dry weight (% DW). Total phenolic content (TPC) is expressed as milligrams of gallic acid equivalents per gram of sample (mg GAE/g). Total flavonoid content (TFC) is expressed as milligrams of quercetin equivalents per gram of sample (mg QE/g). Total tannins are expressed as milligrams per gram of extract (mg/g extract). Ash content is expressed as percentage (%). Mineral contents (Mg, Ca, K, Fe, and Zn) are expressed as milligrams per kilogram of dry weight (mg/kg DW).

Parameter	Value
Total withanolides	0.3 (%/dry weight)
Total phenolics	40 (mg GAE/g)
Total flavonoids	30 (mg QE/g equivalent)
Total tannins	50.6 (mg/g extract)
Ash	3.2 (%)
Magnesium (Mg)	30.120 (mg/kg)
Calcium (Ca)	9.04 (mg/kg)
Potassium (K)	2.1 (mg/kg)
Iron (Fe)	95.8 (mg/kg)
Zinc (Zn)	37.1 (mg/kg)

Abbreviations: GAE, gallic acid equivalents; QE, quercetin equivalents; DW, dry weight; TPC, total phenolic content; TFC, total flavonoid content; Mg, magnesium; Ca, calcium; K, potassium; Fe, iron; Zn, zinc.

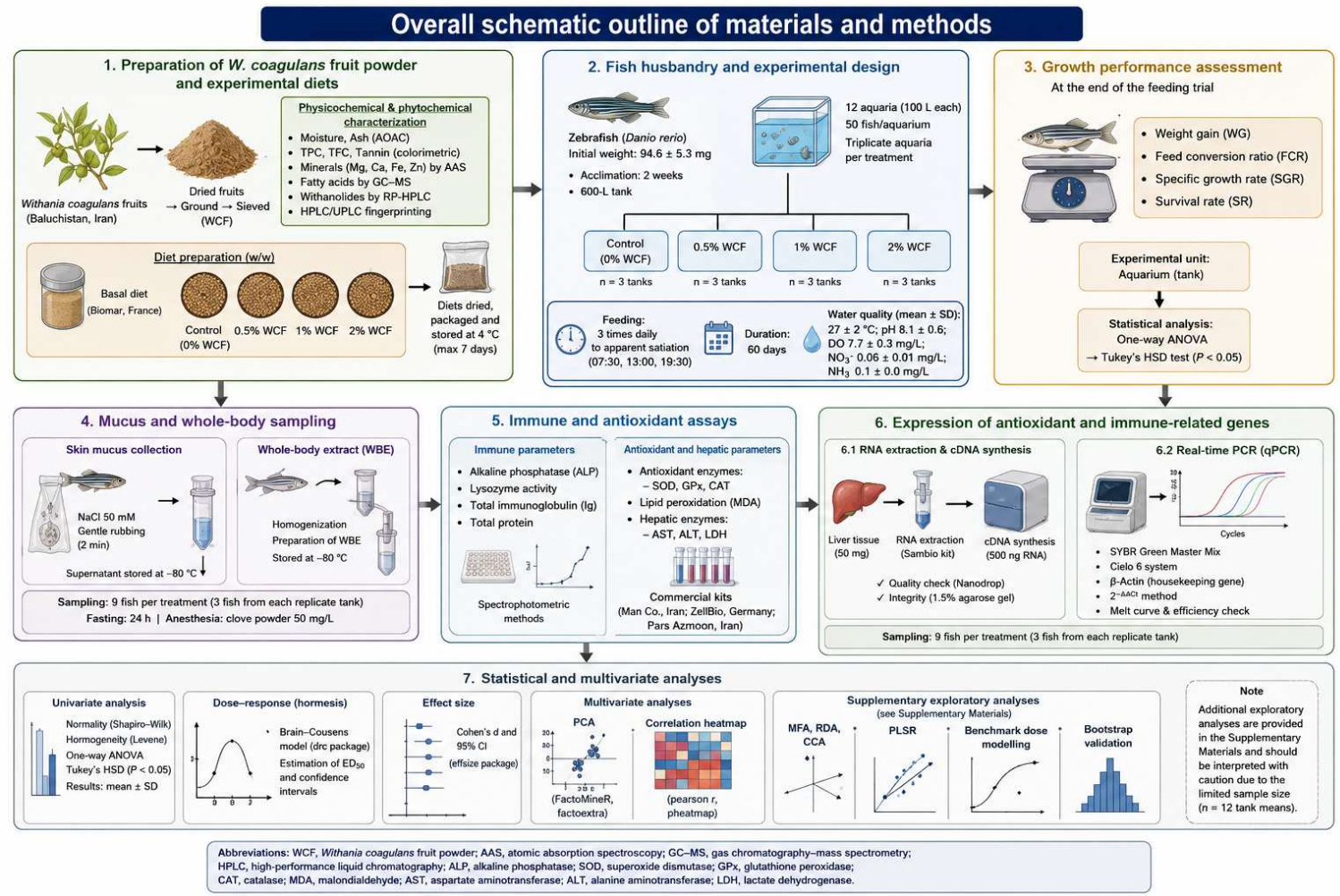
Table 2. Primer sequences and accession numbers of target genes used for quantitative real-time PCR (qRT-PCR) analysis in zebrafish (*Danio rerio*). β-Actin served as the internal reference gene.

Genes	Primer	Primer sequence (5´-3´)	Accession no.
SOD	F	GGGTGGCAATGAGGAAAG	BC055516.1
	R	GCCCACATAGAAATGCACAG	
CAT	F	GCATGTTGGAAAGACGACAC	AJ007505.1
	R	GTGGATGAAAGACGGAGACA	
GPX	F	CCAAGTAAACCAGCGGCTTCT	NM-001007281
	R	TGATGTGCAGTGGACGGTTTAT	
il-1β	F	CGTCTCCACATCTCGTACTCA	AY340959.1
	R	GTGTCTTTCCTGTCCATCTCC	
TNF-α	F	CTGCTTCACGCTCCATAAGA	AY427649.1
	R	CTGGTCCTGGTCATCTCTCC	
lyz	F	GGCAGTGGTGTTTTTGTGTC	NM-139180.1
	R	CGTAGTCCTTCCCCGTATCA	
β-Actin	F	AGCAGATGTGGATCAGCAAG	NM131031.1
	R	TACCTCCCTTTGCCAGTTTC	

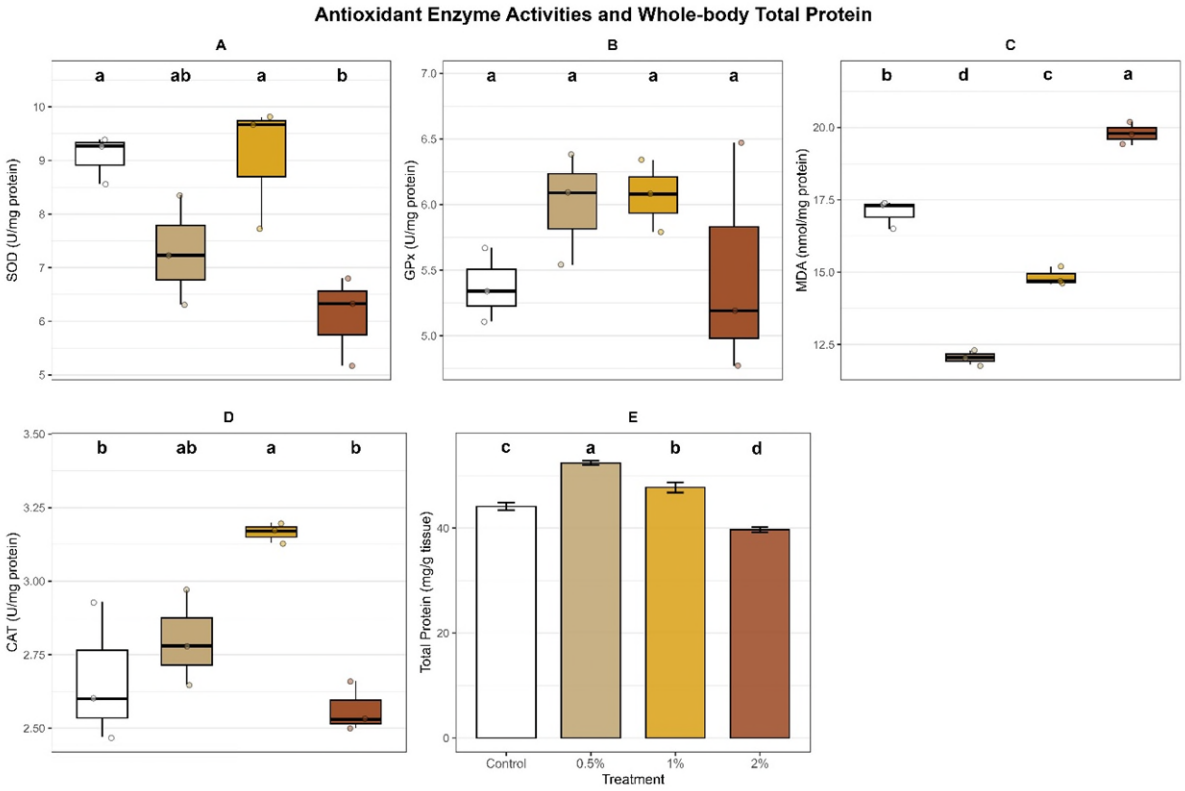
Table 3. Growth performance and survival of zebrafish (*Danio rerio*) fed diets containing different levels of *Withania coagulans* fruit powder for 60 days. Values are presented as mean ± SD. Different superscript letters within the same row indicate significant differences among treatments ($P < 0.05$).

	Treatments			
	0% (control)	0.5%	1%	2%
Initial weight (mg)	94.73 ± 1.93	95.27 ± 1.17	94.40 ± 1.55	94 ± 1.13
Final weight (mg)	222.67 ± 3.21[a]	219 ± 8.72[ab]	219.67 ± 7.57[ab]	208 ± 8.18[b]
WG (mg)	127.93 ± 2.39	123.73 ± 4.74	125.27 ± 10.4	114 ± 14.93
FCR	1.91 ± 0.15	2.2 ± 0.19	1.95 ± 0.11	1.9 ± 0.15
SGR	1.42 ± 0.06	1.39 ± 0.06	1.41 ± 0.10	1.32 ± 0.11

Figure 1. Experimental workflow of the dietary *Withania coagulans* fruit powder (WCF) trial in zebrafish. Fish were fed diets containing 0, 0.5, 1, or 2% WCF for 60 days (3 replicate tanks per treatment, 50 fish per tank). Growth performance, mucosal immune responses, antioxidant status, hepatic enzyme activities, and expression of immune- and antioxidant-related genes were evaluated. Statistical analyses included ANOVA, hormesis modelling, effect-size estimation, PCA, and correlation analyses, with additional exploratory multivariate analyses provided in the Supplementary Materials.



598 **Figure 2.** Antioxidant enzyme activities in whole-body extracts of zebrafish fed diets containing different levels
599 of *Withania coagulans* fruit powder for 60 days. Values are presented as mean ± SD. Different letters indicate
600 significant differences among treatments ($P < 0.05$).



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Figure 3. Activities of hepatic biomarkers (AST, ALT, and LDH) in whole-body extracts of zebrafish fed diets containing different levels of *Withania coagulans* fruit powder for 60 days. Values are presented as mean ± SD. Different letters indicate significant differences among treatments ($P < 0.05$).

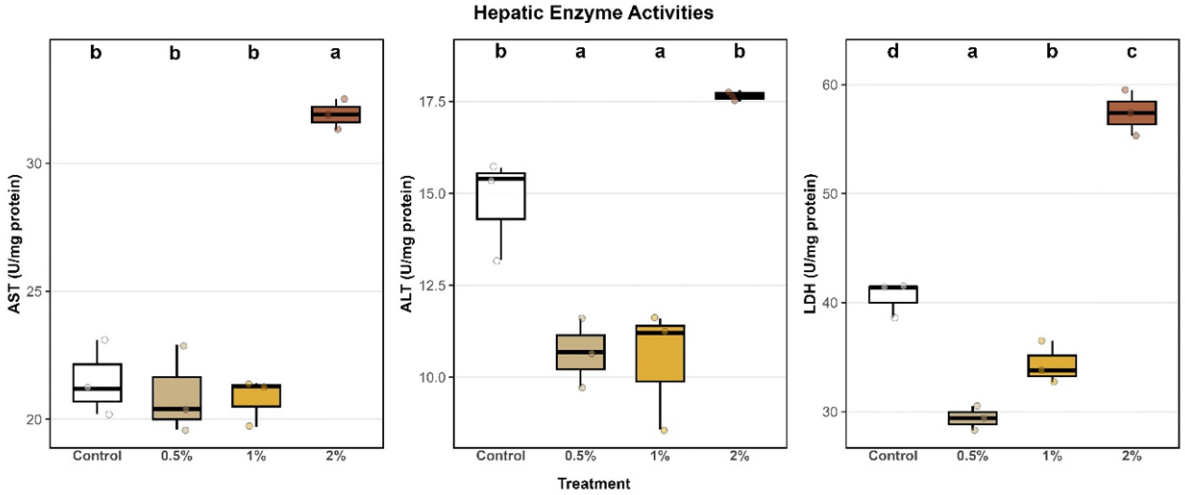


Figure 4. Alkaline phosphatase (A), lysozyme activity (B), total immunoglobulin (C), and total protein (D) in skin mucus of zebrafish fed diets containing different levels of *Withania coagulans* fruit powder (0, 0.5, 1, and 2%) for 60 days. Values are presented as mean ± SD. Different letters indicate significant differences among treatments ($P < 0.05$).

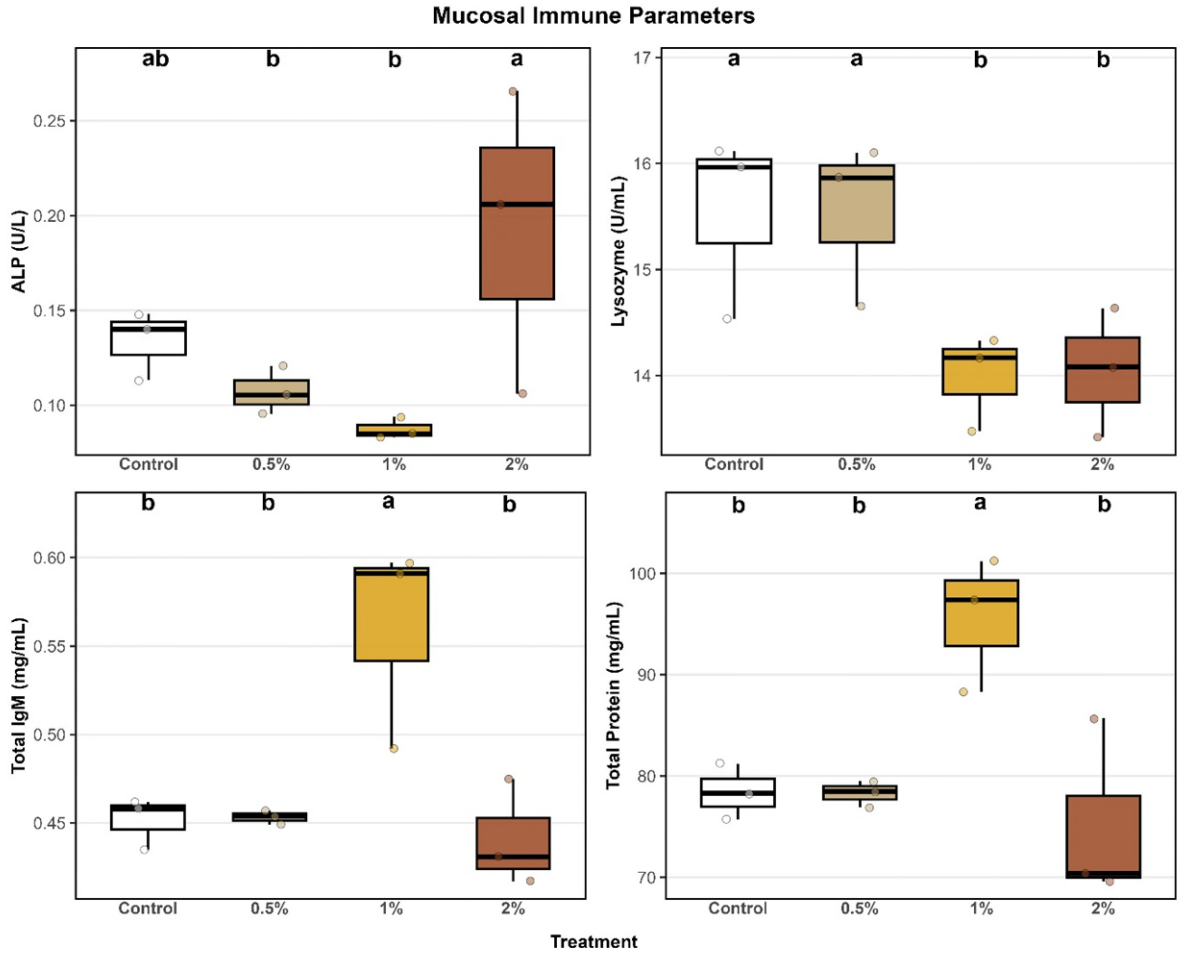


Figure 5. Expression of immune-related genes tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), and lysozyme (LYZ) in zebrafish fed diets containing different levels of *Withania coagulans* fruit powder for 60 days. Values are presented as mean ± SE. Different letters indicate significant differences among treatments ($P < 0.05$).

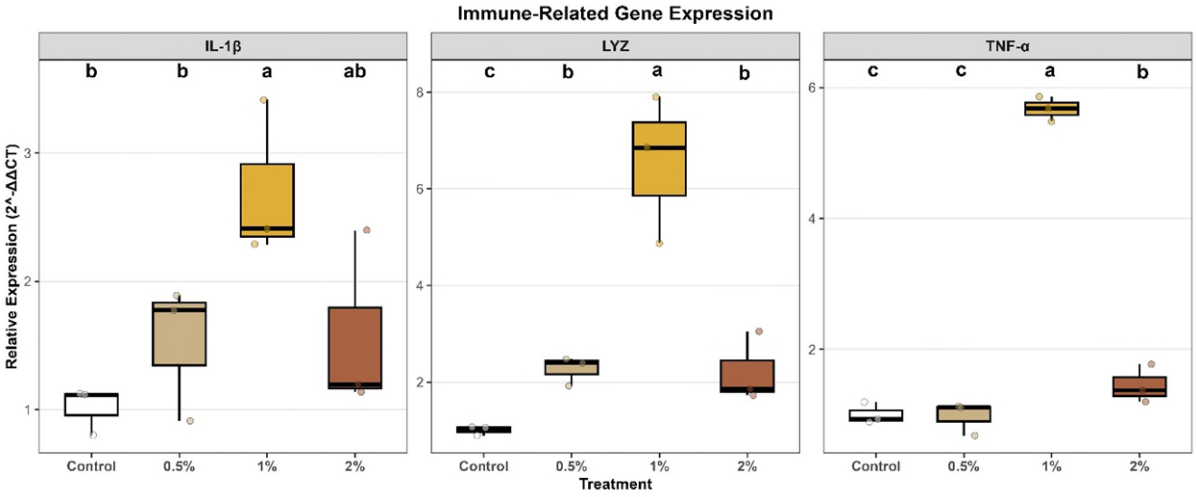


Figure 6. Expression of antioxidant-related genes superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) in zebrafish fed diets containing different levels of *Withania coagulans* fruit powder for 60 days. Values are presented as mean ± SE. Different letters indicate significant differences among treatments ($P < 0.05$).

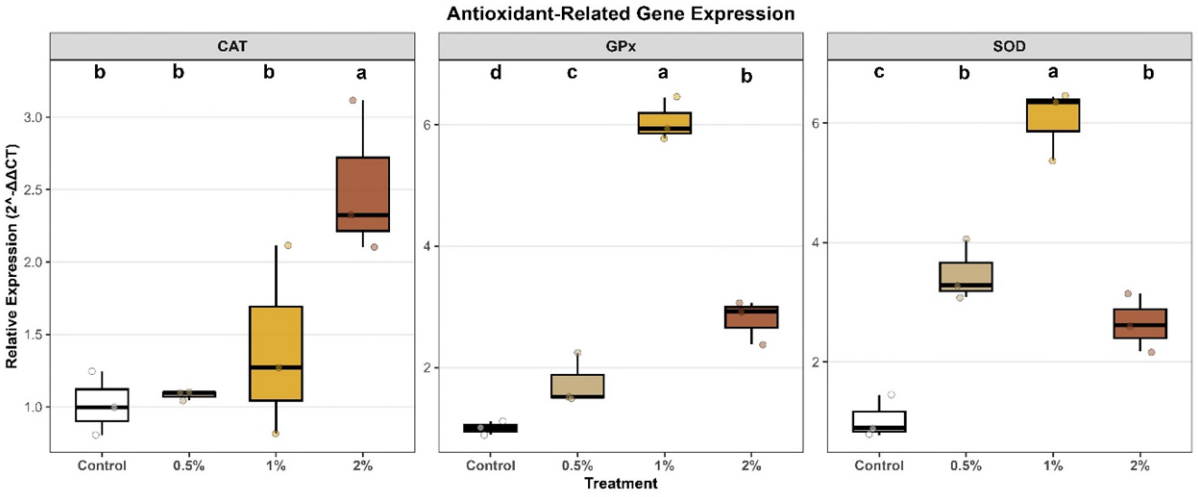


Figure 7. Hormesis dose-response curve for total immunoglobulin in zebrafish fed diets containing different levels of *Withania coagulans* fruit powder (0, 0.5, 1, and 2%) for 60 days. The inverted-U shape indicates low-dose stimulation and high-dose suppression, with the peak response at 1% WCF. Shaded area represents the 95% confidence interval (ED_{50} = 2.32% WCF).

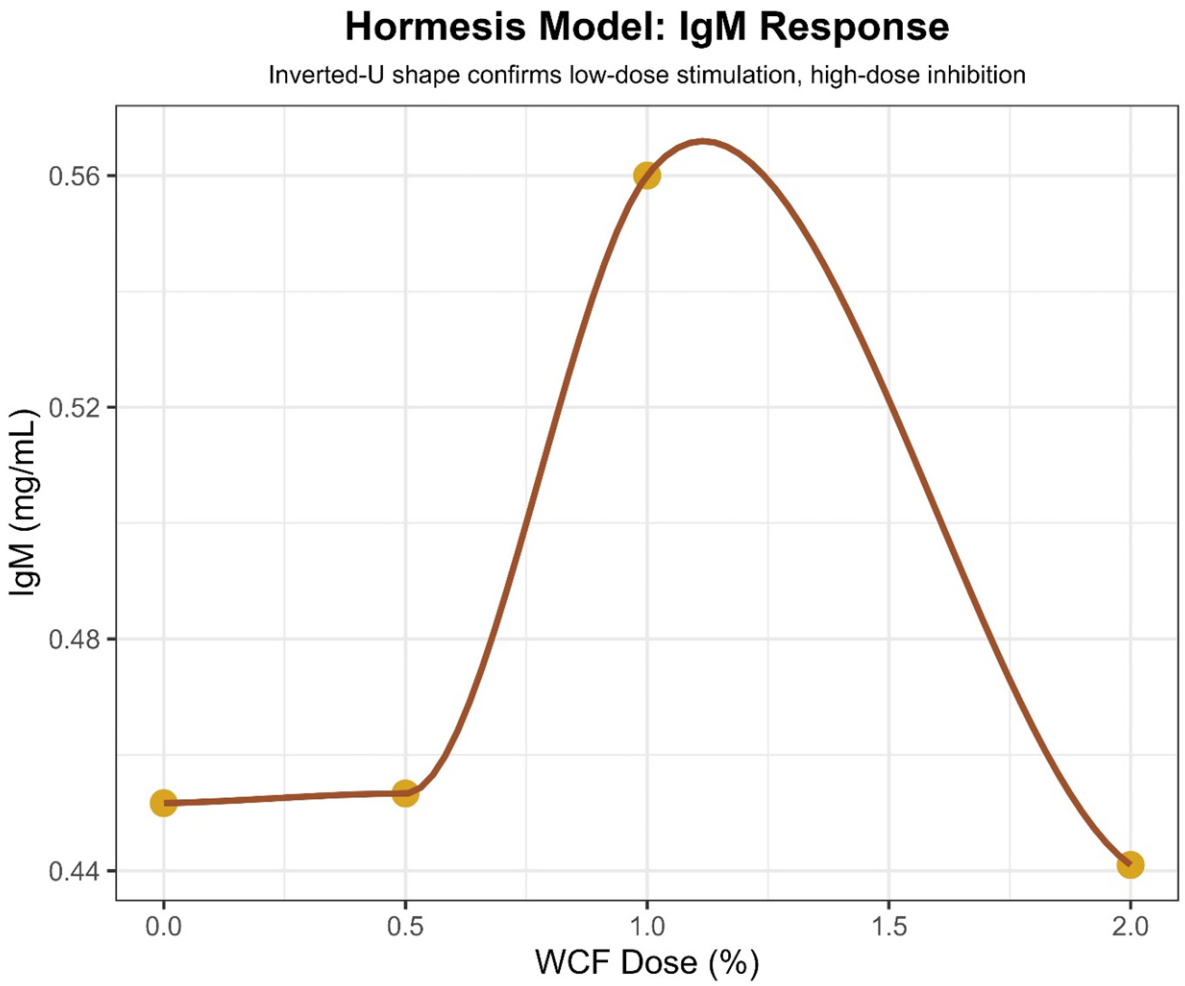


Figure 8. Forest plot showing effect sizes (Cohen's d) with 95% confidence intervals for key parameters at 1% WCF compared with the control. Positive values indicate beneficial effects on immune and antioxidant parameters, whereas negative values indicate reductions in oxidative stress (MDA). All effect sizes are large (|d| > 2.5), and all confidence intervals exclude zero.

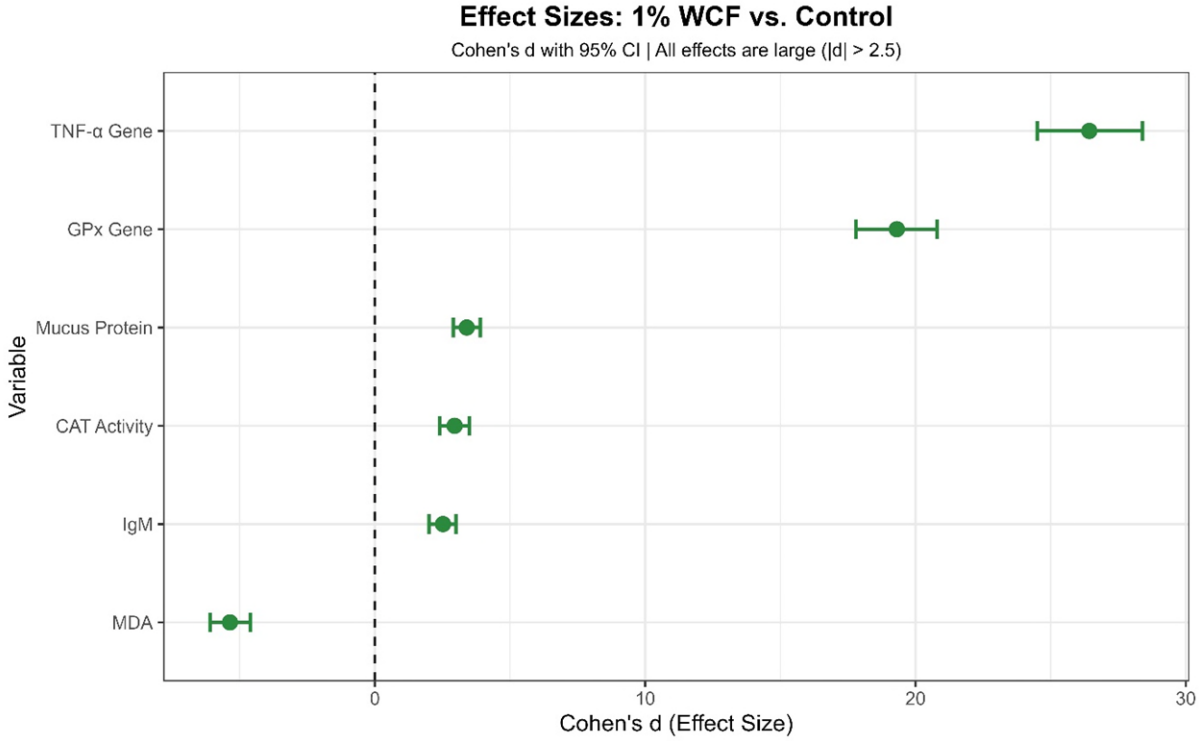


Figure 9. Principal component analysis (PCA) biplot showing multivariate separation of treatment groups based on all measured biomarkers. The first two principal components explain 77.2% of the total variance, with clear separation of the 1% and 2% treatment groups. Ellipses represent 95% confidence intervals for each treatment group.

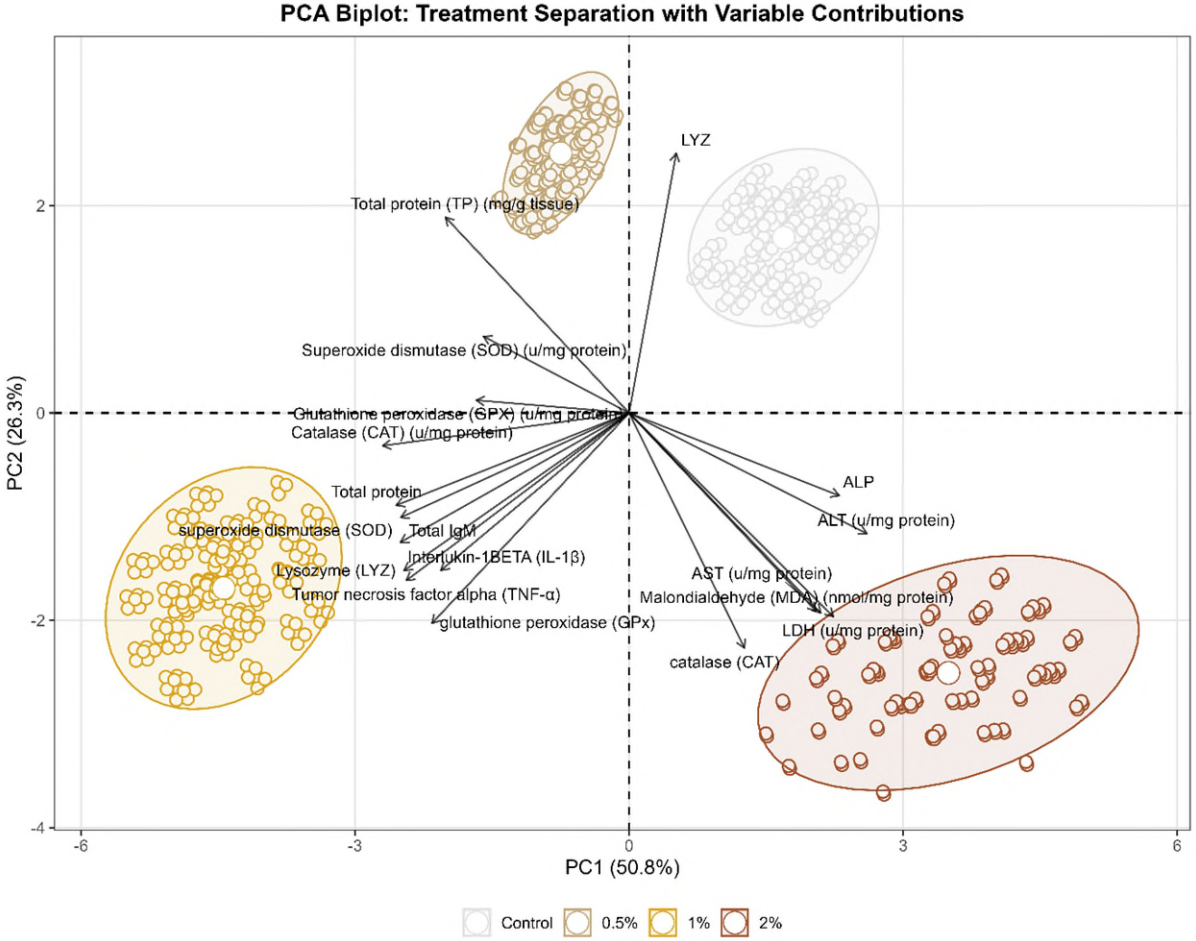


Figure 10. Hierarchical clustering heatmap shows Pearson correlation coefficients among all measured parameters. Red indicates positive correlation and blue indicates negative correlation. Dendrograms show clustering of variables based on similarity. Immune and antioxidant genes cluster together, whereas MDA and hepatic biomarkers form a separate cluster associated with stress response.

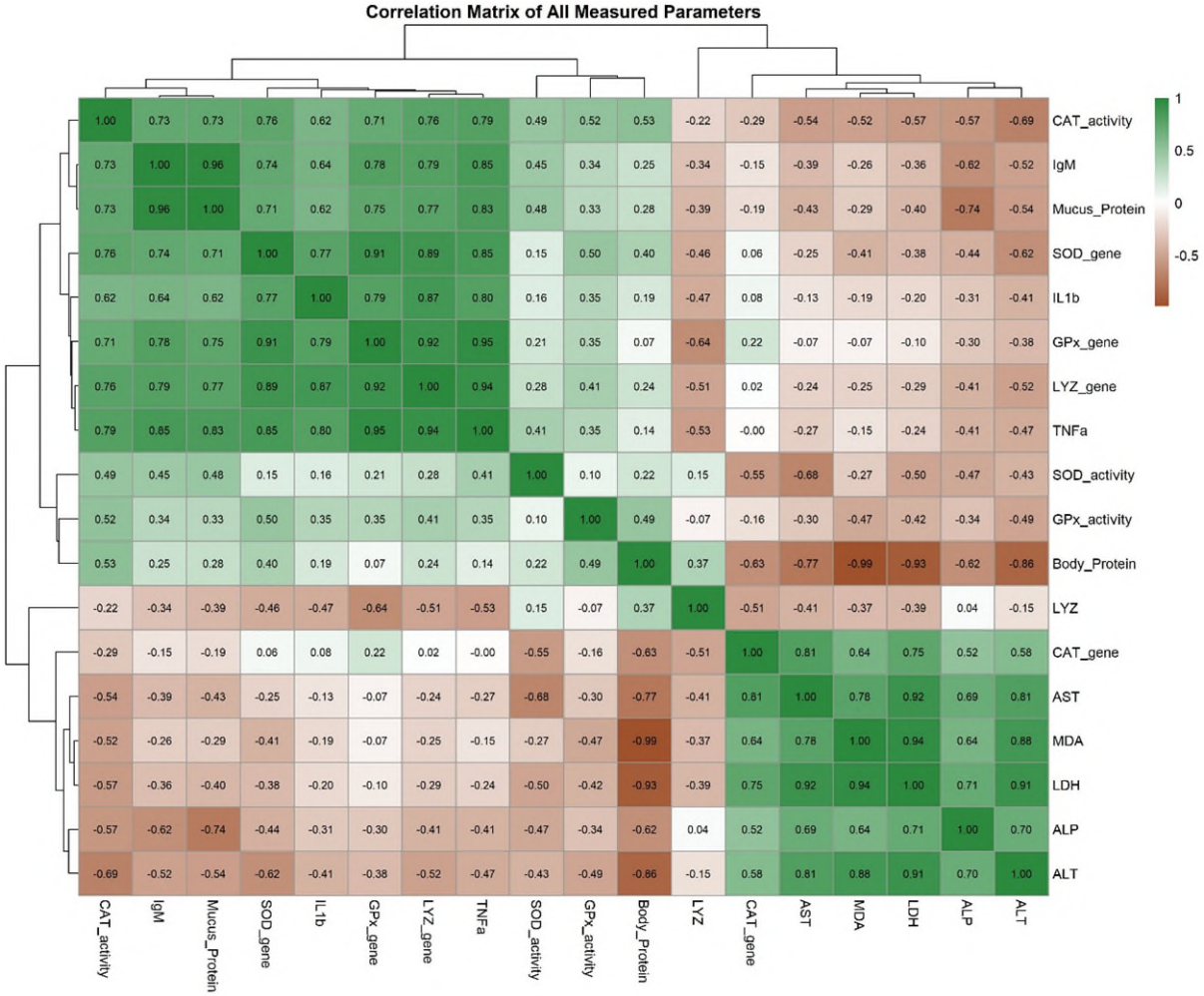
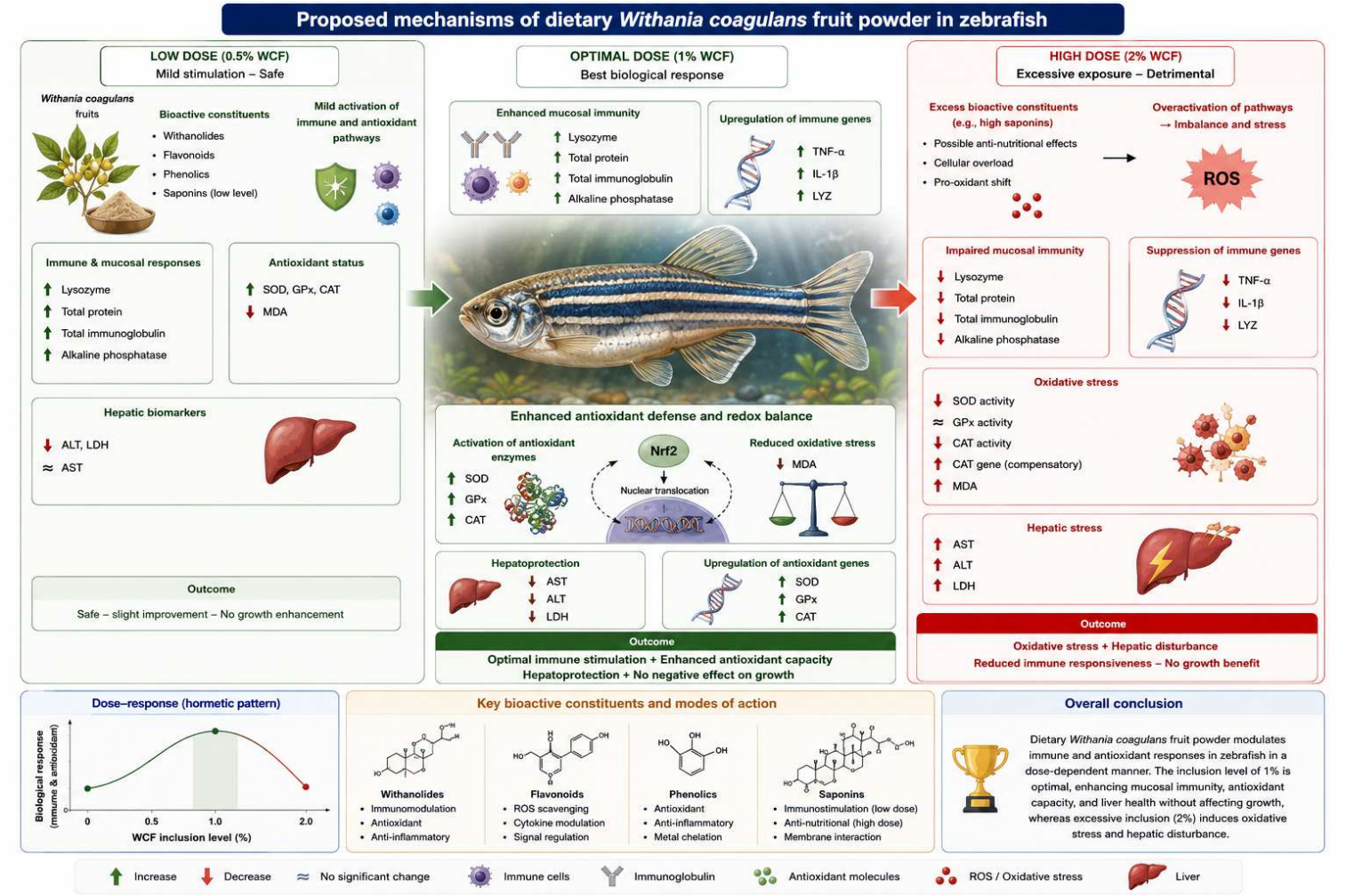


Figure 11. Proposed mechanism of action of dietary *Withania coagulans* fruit powder (WCF) in zebrafish. Bioactive compounds present in WCF, including withanolides, flavonoids, phenolic compounds, and saponins, are proposed to modulate immune and antioxidant pathways. Dietary supplementation at the optimal level (1% WCF) enhances mucosal immunity, stimulates expression of immune-related genes (TNF-α, IL-1β, and LYZ), increases antioxidant defense (SOD, GPx, and CAT), and reduces lipid peroxidation (MDA), thereby improving physiological resilience. In contrast, excessive supplementation (2% WCF) may induce oxidative imbalance and hepatic stress, resulting in elevated AST, ALT, LDH, and MDA levels together with suppression of key immune responses. The framework represents a hypothesized mechanism based on the experimental findings of the present study.



Supplementary Files

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