

RESEARCH ARTICLE OPEN ACCESS

Long-Term Administration of *Pueraria lobata* Radix Ethanol Extract Enhances Growth by Modulating Antioxidant Defense and Inflammatory Responses in Juvenile Eels (*Anguilla japonica*)

Walimuni Randika Harshan Mendis¹  | Tae-Ho Ahn² | Jin Hyeok Jang² | Byung-Cheul Jeong² | Seunghan Lee³ | So Young Kang¹  | Jun Cheul Ahn² 

¹Department of Aqualife Medicine, Chonnam National University, Yeosu 59626, Republic of Korea | ²Central R&D Center, KOFEC, Gokseong 57525, Republic of Korea | ³Department of Aquaculture and Aquatic Science, Kunsan National University, Kunsan 54150, Republic of Korea

Correspondence: So Young Kang (sykang1@chonnam.ac.kr) | Jun Cheul Ahn (andoryong@hanmail.net)

Received: 14 August 2025 | **Revised:** 4 February 2026 | **Accepted:** 4 February 2026

Keywords: *Anguilla japonica* | anti-inflammation | antioxidant | extract optimization | growth performance | *Pueraria lobata*

ABSTRACT

This study was aimed to determine the optimized extract for *Pueraria lobata* radix (PLR) and to evaluate the effects of PLR dietary supplementation in juvenile eels (*Anguilla japonica*). A 2³ full factorial design used was to determine PLR, selecting extraction's ethanol (EtOH) concentration, temperature, and time as factors. PLR's extraction conditions were established as 100% EtOH at 25°C for 10 h, maximizing the yield of key bioactive isoflavonoids—puerarin and daidzein—while minimizing cytotoxicity. In a feeding trial with juvenile eels, dietary supplementation with PLR (1%–5%) enhanced mean growth performance, yet not statistically significant due to high individual variance. Interestingly, supplemented groups exhibited higher coefficients of variation in body size, reflecting increased interindividual growth variability. Despite these growth benefits, condition factor and hepatosomatic index (HIS) remained unaffected. Hematological parameters did not differ significantly between groups, although trends toward elevated alkaline phosphatase (ALP), total cholesterol (TCHO), HDL cholesterol (CHO), and triglyceride levels—and reduced glucose (GLU) concentrations—were observed in the PLR-fed eels. Nonspecific immune responses, including lysozyme and phagocytic activity, showed no significant differences. However, gene expression of hepatic antioxidant enzymes (Cu/Zn-SOD and Mn-SOD) were significantly upregulated, while key oxidative stress-related genes (Keap1, Nrf2, and Gclc) and pro-inflammatory markers (Hsp70, IFN- α , and Myd88) were markedly downregulated. These results indicate that long-term dietary PLR supplementation promotes growth in juvenile eels, likely by enhancing hepatic antioxidant defenses and suppressing inflammation.

1 | Introduction

The aquaculture and consumption of freshwater eel (*Anguilla japonica*) are primarily concentrated in East Asia, especially in countries such as Republic of Korea, Japan, and China. The scale of eel farming is largely determined by the annual catch of elvers,

also known as glass eels or fry, which are captured in the wild. However, the availability of these wild elvers has been rapidly declining since the 1960s due to overfishing, habitat loss, and environmental changes. Despite these challenges, demand for freshwater eel continues to increase, particularly in East Asia, leading countries like Republic of Korea and China to expand

This is an open access article under the terms of the [Creative Commons Attribution](https://creativecommons.org/licenses/by/4.0/) License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

Copyright © 2026 Walimuni Randika Harshan Mendis et al. *Aquaculture Research* published by John Wiley & Sons Ltd.

their aquaculture facilities in an effort to meet consumer demand.

To achieve sustainable growth in eel aquaculture, the development and commercialization of artificially bred elvers are widely regarded as the most essential long-term solution. Although artificial breeding research has been ongoing since the 1970s, particularly in Japan, the production of artificial juveniles has remained at the experimental level. Commercial-scale success has not yet been achieved, and aquaculture operations still depend heavily on wild-caught juveniles for seed stock [1]. As a result, realistic and immediate alternatives for the continued growth of eel farming include efforts to reduce production costs by improving growth rates, feed conversion efficiency, and reducing mortality rates.

In pursuit of these goals, researchers and aquaculture producers have been investigating innovative and environmentally friendly approaches. These include the development of alternative protein sources, the use of probiotics and prebiotics, and the introduction of ICT-based smart aquaculture technologies. Among these strategies, the use of functional feed additives is receiving increasing attention due to its practicality and promising results in enhancing growth, health, and stress resilience.

Feed additives for aquaculture species, including eels, range from synthetic compounds to natural extracts and have been reported to enhance growth performance, disease resistance, hematological profiles, and nonspecific immune responses. In particular, plant-derived extracts are gaining attention because of their eco-friendly and sustainable production, as well as the rising consumer preference for natural products [2]. These characteristics make plant-based additives appealing for use in freshwater eel aquaculture.

Pueraria lobata, a plant widely distributed in East Asia, has been used in traditional medicine for centuries due to its high productivity and low cost. Its root, or radix, contains various bioactive compounds, including flavonoids, triterpenoids, and polysaccharides. Among these, the isoflavonoid puerarin is the major pharmacologically active compound, reported to have multiple health-promoting effects in both humans and animals. These effects include vasodilation, cardio protection, neuroprotection, antioxidant and anticancer properties, anti-inflammatory activity, analgesia, promotion of bone formation, inhibition of alcohol absorption, and improvement of insulin resistance. Puerarin has also been reported to exhibit antioxidant [3], antiobesity [4, 5], and hypoglycemic effects [6].

Despite these promising properties, there are relatively few studies investigating the functional roles of *Pueraria lobata* radix (PLR) extracts or puerarin in aquaculture species, particularly as antioxidants or anti-inflammatory agents [7, 8]. Moreover, studies on eel are extremely limited. Given the known bioactivity of constituents of PLR, its use in eel aquaculture could potentially improve immune function, mitigate oxidative stress, and enhance growth performance. However, to realize these benefits in practice, challenges such as variability in bioactive compound content—due to differences in harvest location, timing, and extraction methods—must be addressed. Additionally, the safety of the extract must be evaluated to prevent potential toxicity in cultured species. In addition, the conditions of the extractions could greatly affect the properties of the extracts. Unless a proper selection of extraction conditions were carried out, the bioactivities of the plant materials

could be overlooked, and the extraction conditions that yields the optimum desired bioactivities could not be identified. Instead of a one-factor-at-a-time (OFAT) approach, statistical models could be used to study the influence of each factor of the extraction process to the studied responses.

Therefore, the present study aimed to (1) optimize the extraction process for PLR to maximize the yield of key isoflavonoids, particularly puerarin, while minimizing cytotoxicity using a full factorial design and (2) evaluate the effects of dietary supplementation with the optimized PLR extract on the growth performance, physiological indices (including condition factor, hepatosomatic index (HIS), and viscerosomatic index), blood biochemistry, nonspecific immunity, and hepatic gene expression related to antioxidant capacity and inflammation in juvenile eels over a long-term feeding period.

2 | Material and Methods

2.1 | Plant Materials, Chemicals, and Reagents

Dried PLR was obtained from a commercial supplier in the Republic of Korea. Ethanol (EtOH) for extractions was purchased from Daejung Chemicals and Metals (Siheung, Republic of Korea). HPLC-grade methanol (MeOH), acetonitrile (ACN), formic acid (FA), and water were purchased from Honeywell B&J (NJ, USA) for LC-MS analyses. Dimethyl sulfoxide (DMSO; cell culture grade) was obtained from Sigma-Aldrich (USA). Puerarin and daidzein standards were sourced from Santa Cruz Biotechnology Inc. (TX, USA).

2.2 | Extraction of PLR

Three independent variables were selected for extraction optimization: EtOH concentration (% v/v; X_1), extraction temperature ($^{\circ}\text{C}$; X_2), and extraction time (h; X_3). A full factorial 2^3 design of experiments was employed to apply a polynomial linear regression model to evaluate the relationships between factors and responses. Each factor was tested at two levels (low and high; Table 1). The design matrix included 11 runs with triplicates at the center point (Supporting Information 1). The polynomial model was $Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{123} X_1 X_2 X_3$, where Y is the predicted response, β_0 is the intercept, β_1 – β_3 are the linear coefficients, and β_{12} , β_{13} , β_{23} , and β_{123} are the interaction coefficients. PLR was ground with a kitchen blender, and 2.0 g was extracted under the determined conditions in the design matrix. Extracts were filtered (Whatman Number 4), concentrated via rotary evaporation, dried under nitrogen, and freeze-dried. Dried extracts were dissolved in DMSO for cell culture and LC-MS analyses.

TABLE 1 | Coded and natural independent variables in 2^3 full factorial design.

Level	Variable		
	Percent EtOH (X_1)	Temperature ($^{\circ}\text{C}$) (X_2)	Time (h) (X_3)
High (+1)	100	75	10
Low (−1)	20	25	5

2.3 | Cytotoxicity of the Extracts

Cytotoxicity was assessed using fathead minnow (FHM) cells cultured in Dulbecco’s modified eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 IU/mL penicillin, and 50 µg/mL streptomycin (Gibco, USA) at 20°C. Cells (1.2 × 10⁵ cells/well) were seeded in 96-well plates and treated with extracts after 20–24 h, once 80%–100% confluence was achieved. The neutral red (NR) assay, with modifications, was used. Extracts dissolved in DMEM (final 2% FBS) were incubated for 96 h. Cells were stained with 50 µg/mL NR solution for 2 h at 20°C, washed twice with PBS, and treated with 1% acetic acid in 50% EtOH for 10–15 min in the dark. Absorbance was measured at 540 and 690 nm (VersaMax, Molecular Devices, USA). Cytotoxicity was expressed as growth rate (OD ratio of treated to control cells). Calculated cytotoxicity was used as a response in the full factorial design.

2.4 | Yield of Major Isoflavonoids in Extracts

Isoflavonoids were analyzed by LC-MS-Q-TOF (X500R, AB Sciex). Samples (10 µL, 500 µg/mL) were injected into a Kinetex C18 column (150 mm × 4.6 mm, 5 µm; Phenomenex) at 40°C. A mobile phase of 0.1% FA in water (A) and ACN (B) was applied using a gradient (0–25 min: 5%–100% B; 25–35 min: 100% B; 35–35.1 min: 100%–5% B; 35.1–40 min: 5% B) at 1 mL/min with a 1/5 splitter. MS conditions: positive ESI mode, ion spray voltage 5500 V, declustering potential 80 V, gas 1 and 2 at 50 psi, curtain gas 25 psi, and temperature 400°C. Major compounds were identified via precursor/fragment masses and database matching. The sum of the peak areas for puerarin and daidzein was used as a yield response in the full factorial design.

2.5 | Ethics Statement, Experimental Fish, and Culture Conditions

All animal procedures were conducted at the Central R&D Center, KOFEC, Gokseong, Republic of Korea, in accordance with the ethical guidelines and approved by the Silla University Animal Care and Use Committee (SUACUC-2023-003). Approximately

0.15 g of juvenile eels were obtained in March 2024 from a commercial supplier in Republic of Korea. The experimental elvers were randomly stocked at a density of 60 eels per 60 L tank, with triplicates per treatment group. Feed was formulated using a commercial diet (Gurogo Gold King, KOFEC, Republic of Korea) supplementing with the selected optimized extract (PLR) from the analysis of the responses in the 2³ full factorial design (extracted with 100% EtOH, 25°C, 10 h) at 1%–5% in the feed (Table 2). Fish were fed to apparent satiation once daily at 10:00 AM. Uneaten feed was collected, dried, and weighed. Conditions: 29 ± 1°C, DO 6.5 ± 0.5 mg/L, and pH 5.9 ± 0.5. Water (90%) was changed daily and tanks cleaned weekly.

2.6 | Growth Performance

Growth performance was assessed at the end of the 175-day feeding trial. Each fish was individually counted and weighed to determine final weight (FW). Weight gain (WG) was calculated as the difference between final and initial body weight. The WG rate (WGR) was expressed as (WG / initial weight) × 100. Specific growth rate (SGR) was determined using the formula [(ln FW – ln initial weight)/days] × 100. Uneaten feed was collected 20–30 min after feeding, dried, and weighed to accurately calculate feed intake and feed efficiency. The final fish number in each group were 56, 53, 56, and 41 in CTL, PLR-1, PLR-3, and PLR-5, respectively.

2.7 | Chemical Analysis

The proximate composition of the experimental diets was determined according to the Association of Official Analytical Chemists (AOAC) [9]. Moisture content was assessed by drying samples at 105°C until constant weight was achieved, typically around 6 h. Crude protein was analyzed using the Kjeldahl method, and ash content was determined by incineration in a furnace at 550°C for 6 h. Crude lipids were extracted using a modified acid–base method described by Duongbia et al. [10]. Gross energy content was calculated using conversion coefficients of 23.6 kJ/g for protein, 38.9 kJ/g for fat, and 16.7 kJ/g for carbohydrates [11]. Amino

TABLE 2 | PLR supplemented diet formulation for juvenile eel.

Component	CTL	PLR-1	PLR-3	PLR-5
Commercial base diet	90	90	90	90
Cellulose	10	9	7	5
<i>Pueraria lobata</i> radix EtOH extract (PLR)	0	1	3	5
Total	100	100	100	100
Proximate components				
Moisture ^{ns}	3.84 ± 0.42	3.92 ± 0.71	4.03 ± 0.25	4.12 ± 0.37
Protein ^{ns}	46.29 ± 1.24	45.68 ± 2.21	46.25 ± 2.36	46.68 ± 3.45
Lipid ^{ns}	6.93 ± 0.57	6.91 ± 0.24	6.95 ± 0.37	6.78 ± 0.57
Carbohydrate ^{ns}	21.5 ± 0.51	21.8 ± 0.22	21.7 ± 0.54	22.0 ± 0.72
Ash ^{ns}	22.1 ± 1.70	21.4 ± 1.81	20.2 ± 0.72	19.0 ± 0.75

Note: Values are presented as mean ± SD (n = 3). Statistical significance was determined by one-way ANOVA. Differences were considered significant at p < 0.05. Abbreviation: ns, not significant.

acid composition was analyzed by the ninhydrin postcolumn derivatization method using ion exchange chromatography, with modifications from Peace and Gilani [12]. For the analysis of 16 amino acids, samples (0.2 g) were hydrolyzed with 6 N HCl at 110°C under an aerobic condition for 24 h. The hydrolyzed samples were concentrated under reduced pressure, diluted in 50 mL of 0.2 M sodium citrate buffer, filtered, and analyzed. Methionine and cysteine were analyzed using the performic acid oxidation method, and tryptophan via alkali hydrolysis. Lipid composition was determined following the method of Folch et al. [13] using gas chromatography (Hewlett Packard 6890, USA) with an HP-88 column. The samples underwent transesterification using 14% BF₃-MeOH and were analyzed under specific temperature conditions: initial 140°C for 5 min, then ramping at 4°C/min to 240°C and holding for 20 min. Injector and detector temperatures were set at 260 and 270°C, respectively.

2.8 | Nonspecific Immunity

Nonspecific immune responses were evaluated by measuring lysozyme activity and phagocytic activity in the serum of experimental fish. Lysozyme activity was assessed using a modified turbidimetric method as described by Koskela et al. [14]. In brief, each reaction mixture (1 mL) consisted of 50 µL of serum, 0.05 M sodium phosphate buffer (pH 6.2), and *Micrococcus lysodeikticus* (0.2 mg/mL). The reduction in turbidity was monitored at 530 nm using a microplate reader (Molecular Devices, USA) at 20°C, with absorbance measured after 0.5 and 5 min. Lysozyme activity was expressed in units per mL, where one unit was defined as the amount causing a decrease in absorbance of 0.001 per minute. Phagocytic activity was determined using a modified spectrophotometric method based on Seeley et al. [15]. Equal volumes (50 µL each) of serum and nitro blue tetrazolium (NBT) solution (Sigma, USA) were mixed and incubated at 25°C for 30 min. After incubation, 1 mL of dimethylformamide was added to terminate the reaction and dissolve the formazan product. The mixture was centrifuged at 2000 × g for 5 min, and the absorbance of the supernatant was measured at 540 nm to quantify NBT reduction, using dimethylformamide as the blank control.

2.9 | Blood Biochemistry and Biological Indices

Blood samples were collected from the caudal vein of 10 anesthetized fish per group using heparinized syringes. Samples were centrifuged at 2000 rpm for 15 min at 4°C. The serum was then stored at -20°C for further biochemical analyses. Serum parameters analyzed included total protein (TP), total cholesterol (TCHO), triglycerides (TGs), glucose (GLU), glutamic oxaloacetate transaminase (GOT), and alkaline phosphatase (ALP), using commercial enzymatic kits and a blood analyzer (NX500i, FUJI FILM, Japan). The condition index (CI) was estimated using $K = W/L^3 \times 100$, where *W* is the weight of the fish (g) and *L* is the length of the fish (cm). HIS was estimated using $LW \times 100/BW$, where *LW* is the fresh weight of liver (g) and *BW* is the weight of fresh body weight (g).

2.10 | Target Gene Expression

To analyze hepatic gene expression, five individuals were randomly selected from each experimental tank. Livers were dissected and immediately at -80°C until analysis. Total RNA was extracted from individual liver samples using TRIzol reagent (Invitrogen Co., USA) according to the manufacturer’s protocol. The concentration and purity of RNA were assessed using a SpectraMax ABS Plus microplate reader (Molecular Devices, USA), and RNA integrity was verified prior to complementary DNA (cDNA) synthesis. For reverse transcription, equal amounts of total RNA were converted into cDNA using the GoScript Reverse Transcription System (Promega, USA) with oligo(dT) primers, following the manufacturer’s instructions. Gene-specific primers (Table 3) were used for quantitative real-time PCR (RT-qPCR) analysis. RT-qPCR was performed using SYBR Green Master Mix (Applied Biosystems, USA) on a QuantStudio 1 RT-qPCR system (Thermo Fisher Scientific, USA). The thermal cycling conditions were as follows: an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 30 s. Relative gene expression levels were calculated using the 2^{-ΔΔCT} method, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as the internal reference gene for normalization.

TABLE 3 | Primers for PCR analysis in this experiment.

Genes	Primers	
	Forward	Reverse
Nrf2	GGC AGT CAA GCA TCC TCT TC	CTC TGT CTT CGG GTG CTT GA
Gclc	GGT TTG GGA TGG GTA ACT GC	TAG CCA GCT GGT CAT ACA GG
Keap1	GCA GAT CTT CCA GGA CCT CA	GGT TGT AGG CCT CCA GGT AG
STAT3	GAG GTG TCC CAG GAA CTG AA	TTC TGC ACA AAC TCC TTG GC
STAT1	GAA GGA GCC ATC ACC TTC AC	GCA CTG TCT TTG GGG ATG TT
Mn-SOD	TCT GGA CAA ACC TGT CCC CT	GCC CGC TCT CCT TGT CAT AG
Cu/Zn-SOD	GGG AGA CTT GGG CAA CGT AA	TGG GAC ACC CTT ACT GGT CA
IgM	CCC TCA CAG CTA GGT TTC CA	GGC AAT ACG GTT CAC ACG AT
IFNα	GTG AGC GGC GAA TCC TTG T	TTT GTC ATC CAC CTG TGC TTT G
HSP70	CCA TCC TGA CCA TCG AAG AC	GTT CTC TTG GCC CTC TCA CA
MyD88	TGTGTGTGTTTCGACAGGGAT	CACCACTGGGATGAGACGTT

2.11 | Statistical Analysis

All data are presented as mean $\pm$ standard deviation (SD). The design and analysis of the full factorial experiment and the validation of in vivo data were performed using Minitab 17 (Minitab Inc., USA). Graphs were generated and statistical analyses were conducted using GraphPad Prism 5.0 (GraphPad Software, USA). Growth performance data were analyzed without removing outliers, because a dose-dependent increase in variance in body weight and length was observed among treatment groups. As the assumption of homogeneity of variance was violated, growth parameters were initially analyzed using Welch's one-way ANOVA, which does not assume equal variances, followed by Dunnett's T3 post hoc test. Alternatively, growth data were $\log_{10}$ -transformed to stabilize variance and improve normality. After transformation, no outliers were detected according to Grubbs' test, normality was confirmed using the Anderson–Darling test, and homogeneity of variance was verified using Levene's test. The log-transformed data were then analyzed using one-way ANOVA followed by Tukey's post hoc test. Blood biochemical parameters and target gene expression data were analyzed using one-way ANOVA followed by Tukey's post hoc test after confirming normality and homogeneity of variance using the same statistical procedures described above. All statistical analyses were two-tailed, and differences were considered statistically significant at $p < 0.05$.

3 | Results

3.1 | Cytotoxicity of the PLR Extracts (2^3 Full Factorial Design)

The cytotoxicity of each PLR extract increased in a dose-dependent manner. For the analysis of factor–response relationships and model construction, cytotoxicity at 750 $\mu\text{g/mL}$ was used as the response variable and is hereafter referred to simply as cytotoxicity. The final model for cytotoxicity included all linear and interaction terms, as removing nonsignificant terms introduced lack-of-fit. The model was statistically significant ($p < 0.05$), with an R^2 of 98.00% and an adjusted R^2 of 93.32%, indicating a strong fit between factors and response. No significant curvature effects were detected; therefore, center point data were excluded from the final model. Cytotoxicity tended to increase with all three factors (EtOH concentration, temperature, extraction time; Figure 1A). However, the main effects of EtOH concentration and extraction time were not statistically significant when compared to the interaction effects. Notably, significant two-way and three-way interactions were identified among all three factors (Figure 1A). The final linear model describing the relationship between the extraction factors and cytotoxicity was as follows: $Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{123}X_1X_2X_3$, where Y is the predicted cytotoxicity, β_0 is the intercept, β_1 – β_3 are the linear cPLRfficients, and β_{12} , β_{13} , β_{23} , β_{123} are the interaction cPLRfficients.

3.2 | Yield of Major Isoflavonoids

The LC-MS-Q-TOF information-dependent acquisition (IDA) analysis of PLR extracts identified puerarin (retention time: 5.2 min) and daidzein (9.7 min) as the major isoflavonoids. Additional isoflavonoids detected included genistin (7.6 min), ononin

(9.0 min), and calycosin (10.1 min; Supporting Information 2). The sum of the peak areas of puerarin and daidzein (hereafter referred to as peak area sum) was used as the response variable for isoflavonoid yield in the factorial analysis. The final model for peak area sum included % EtOH, temperature, and their interaction. However, only % EtOH had a statistically significant effect (Figure 1B). The model was significant ($p < 0.01$) with $R^2 = 88.21\%$ and adjusted $R^2 = 83.16\%$. Although removing terms reduced the R^2 values, no lack-of-fit was observed, confirming model adequacy. Isoflavonoid yield (peak area sum) increased with increasing % EtOH, while temperature and interaction effects were minimal (Figure 1B). The relationship was described by the following linear model: $Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_{12}X_1X_2$, where Y is the peak area sum, β_0 is the intercept, β_1 and β_2 are the linear cPLRfficients for % EtOH (X_1) and temperature (X_2), and β_{12} the interaction cPLRfficient.

3.3 | Optimization of PLR Extraction Conditions

Response optimization in Minitab 17 identified the optimal extraction conditions for maximizing peak area sum and minimizing cytotoxicity as 100% EtOH, 25°C, and 10 h. The extract produced under these conditions was designated as the optimized PLR extract (PLR Optimized Extract; Figure 2). The composite desirability for these dual objectives was 0.9006 (on a 0–1 scale), with individual desirabilities of 0.8111 for peak area sum and 1.0000 for cytotoxicity. The predicted values under optimal conditions were a peak area sum of 18,542,317 and cytotoxicity of 38.8%. Experimentally, values of 17,477,832 (94.3% of predicted) and 39.6% (102.1% of predicted) were obtained, confirming the robustness of the model (Supporting Information 1). The quantity of the major compound in PLR optimized extract, puerarin, was quantified and the method of quantification was validated to confirm the reliability of the quantification process using an LC-MS-QTRAP. Results showed that PLR–PLR contains $25.86 \pm 1.48\%$ puerarin (Supporting Information 2).

3.4 | Growth Performance and Biological Indices

Over the 175-day feeding trial, PLR supplementation led to improvements in growth performance compared to the control group, yet they were not statistically significant with both Welch's ANOVA, and one-way ANOVA with the log-transformed data. This insignificance may have resulted due to the high variance among individuals. Compared to CTL, WG was increased by 13.6%, 4.5%, and 50.3% with the supplementation with PLR-1, PLR-3, and PLR-5, respectively. Similarly, WGRs reflected these improvements, with the PLR-5 group achieving the highest WGR of 10090.99% compared to 6669.44% in the control. SGRs were slightly, but consistently elevated in PLR–fed groups compared to the control. Analysis of size distribution revealed that PLR supplementation was associated with increased variability in individual body length and weight, with the PLR-5 group showing the greatest dispersion and evidence of cannibalism. Although no statistically significant differences in mean body length or weight observed between the control and PLR-supplemented groups, these findings suggests that while PLR promotes growth, higher inclusion levels may exacerbate population heterogeneity (Figure 3). No significant differences were detected in condition factor or HIS across groups (Table 4).

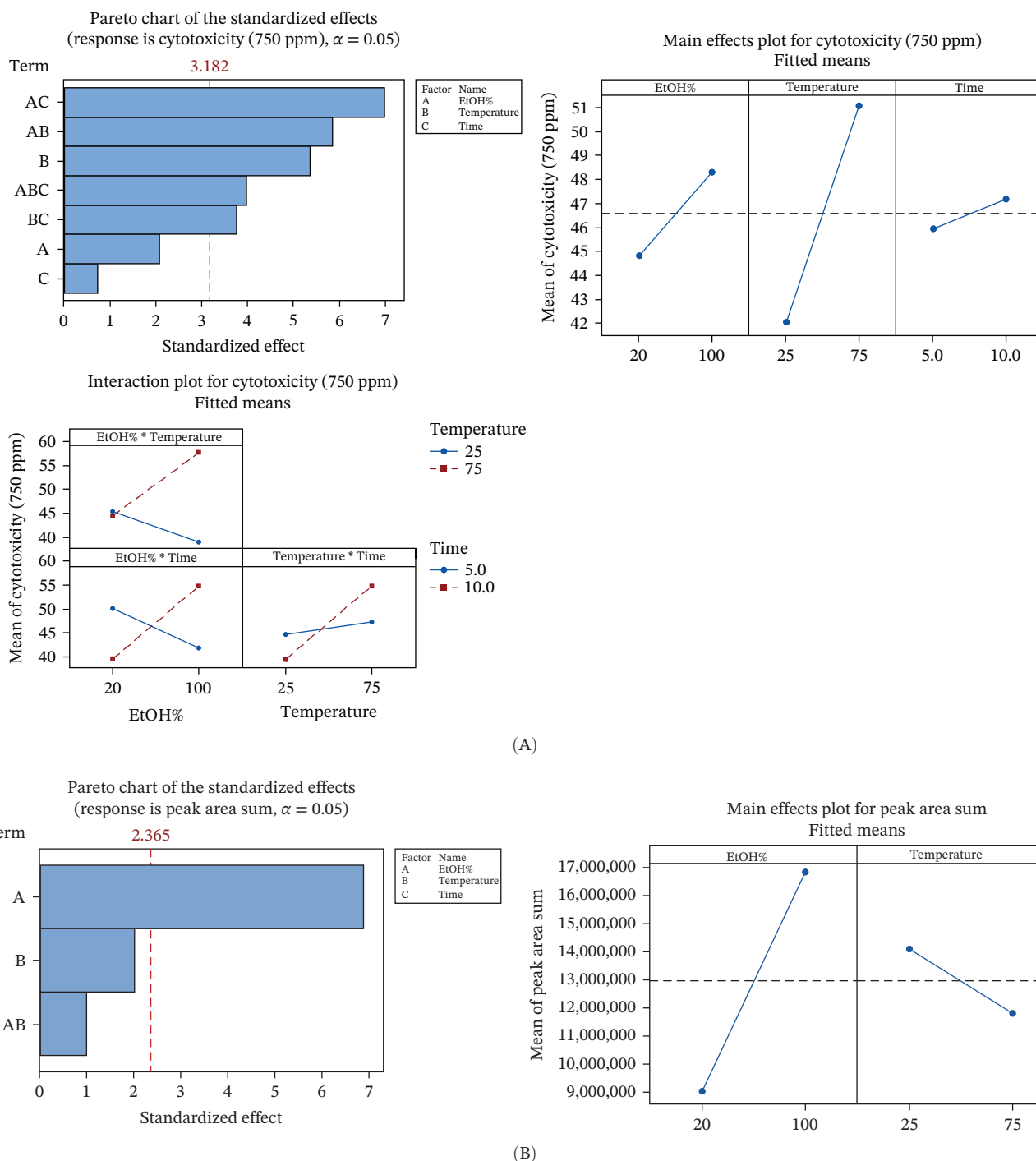


FIGURE 1 | Pareto charts of the standardized effects, main effects plots, and the interaction plots of the cytotoxicity at 750 ppm ($\mu\text{g/mL}$) in FHM cells (A), and peak area sum for puerarin and daidzein (B) for the 2^3 full factorial extractions of *Pueraria lobata* radix. Final models included all the terms because model reduction caused lack-of-fit and nonsignificance of the model in Subpart A ($p < 0.05$). Corner points were not included in both models because models did not show curvature effects ($p < 0.05$).

3.5 | Biochemical Parameters in Blood

Aspartate aminotransferase (AST, also known as GOT), a blood biomarker used to assess liver function, showed minimal changes in all experimental groups. Similarly, ALP levels were slightly higher in the PLR-supplemented groups compared to the control, but the differences were not statistically significant. There were no significant differences observed in serum TG, TCHO, TP,

or high-density lipoprotein cholesterol (HDL-C) between the control group and any of the PLR-treated groups. Although GLU levels were slightly lower in the 5% PLR group, the differences were not significant compared to the 1% and 3% PLR groups (Figure 4). In summary, dietary supplementation with PLR at concentrations of 1%, 3%, and 5% did not have any significant effects on blood health parameters, including liver function (Figure 4).

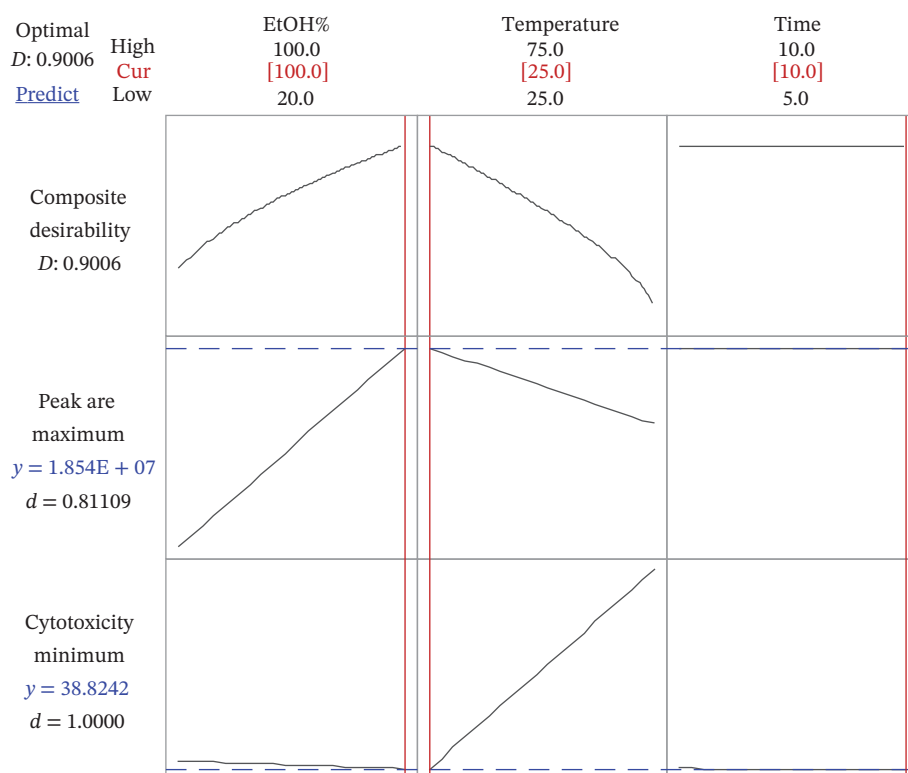


FIGURE 2 | Optimization plot for the 2^3 full factorial extractions of *Pueraria lobata* radix. Optimization was carried out to return the extraction conditions that results in maximum sum of peak area for puerarin and daidzein, and the minimum cytotoxicity percentage at 750 ppm ($\mu\text{g/mL}$).

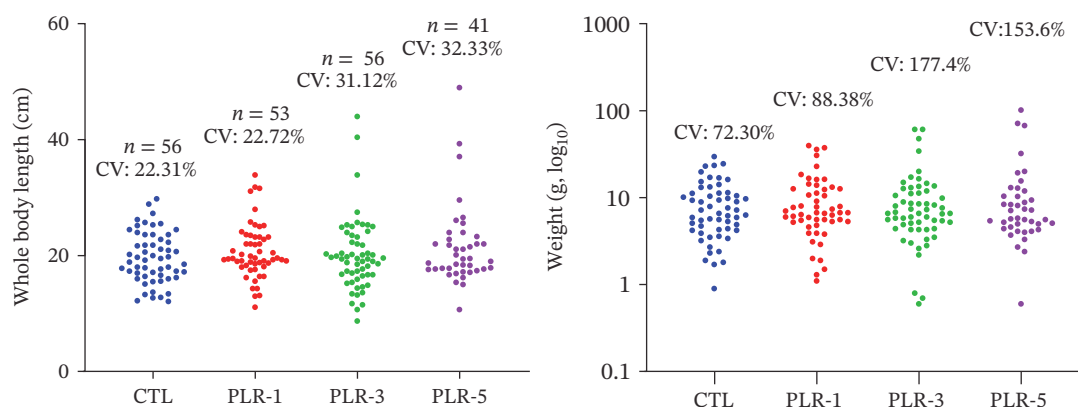


FIGURE 3 | Length and weight of all test fish of feeding with control diet (CTL) and PLR supplemented diets (PLR-1, PLR-3, and PLR-5) in juvenile eel culture for 175 days. n is the number of live eels among the 60 eels stocked in each experimental tanks. CV, coefficient of deviation. Groups were not significantly different from the one-way ANOVA ($p < 0.05$).

3.6 | Nonspecific Immunity

Nonspecific immune responses, as measured by lysozyme and phagocytic expression, showed no significant differences between PLR-supplemented groups and the control. While phagocytic activity was numerically highest in the PLR-3 group, the increase was not statistically significant (Figure 5).

3.7 | Major Liver Function Gene Activity

PLR supplementation significantly upregulated hepatic CuSOD and MnSOD gene expression at all inclusion levels, indicating an enhancement of the antioxidant defense system ($p < 0.05$;

Figure 6). Conversely, expression levels of Nrf2, Keap1, and Gclc was significantly downregulated across all PLR-treated gops ($p < 0.05$; Figure 7). RXR α expression was significantly reduced in PLR-3 and PLR-5 groups compared with the control ($p < 0.05$), STAT1 expression was significantly decreased in PLR-1 and PLR-3 groups ($p < 0.05$) but was significantly increased in PLR-5 group ($p < 0.05$). In contrast, STAT3 expression did not differ significantly between groups. For immune-related genes, MyD88 was significantly downregulated in all PLR-fed groups compared to the control ($p < 0.05$), while the expression levels of HSP70, IgM, and IFN- α showed no statistically significant changes among groups (Figure 7).

TABLE 4 | Growth performance and biological index after feeding of control diet (CTL) and PLR supplemented diets in juvenile eel (*Anguilla japonica*) culture for 175 days.

Parameter	CTL	PLR-1	PLR-3	PLR-5
<i>n</i> (end of 175 days)	56	53	56	41
Survival rate (%)	93.3	88.3	93.3	68.3
Initial weight (g)	0.13	0.13	0.13	0.13
Final weight (g) ^{ns}	8.82 ± 6.37	10.02 ± 8.85	9.21 ± 12.92	13.25 ± 20.35
Weight gain (g) ^{ns}	8.65 ± 6.37	9.89 ± 8.85	9.21 ± 12.92	13.12 ± 20.35
Weight gain rate (%) ^{ns}	6669.44 ± 4919.60	7603.92 ± 6808.36	6985.16 ± 9938.95	10090.99 ± 15656.61
Specific growth rate (%) ^{ns}	2.27 ± 0.3	2.31 ± 0.46	2.13 ± 0.57	2.33 ± 0.54
Condition factor (g/cm ³) ^{ns}	0.098 ± 0.013	0.091 ± 0.013	0.084 ± 0.017	0.091 ± 0.019
Hepatosomatic index (%) ^{ns}	1.31 ± 0.38	1.31 ± 0.28	1.20 ± 0.11	1.40 ± 0.25

Note: Data were analyzed using Welch's ANOVA followed by Dunnet's T3 post hoc test ($p < 0.05$). Data are presented mean ± SD. Abbreviation: ns, not significant.

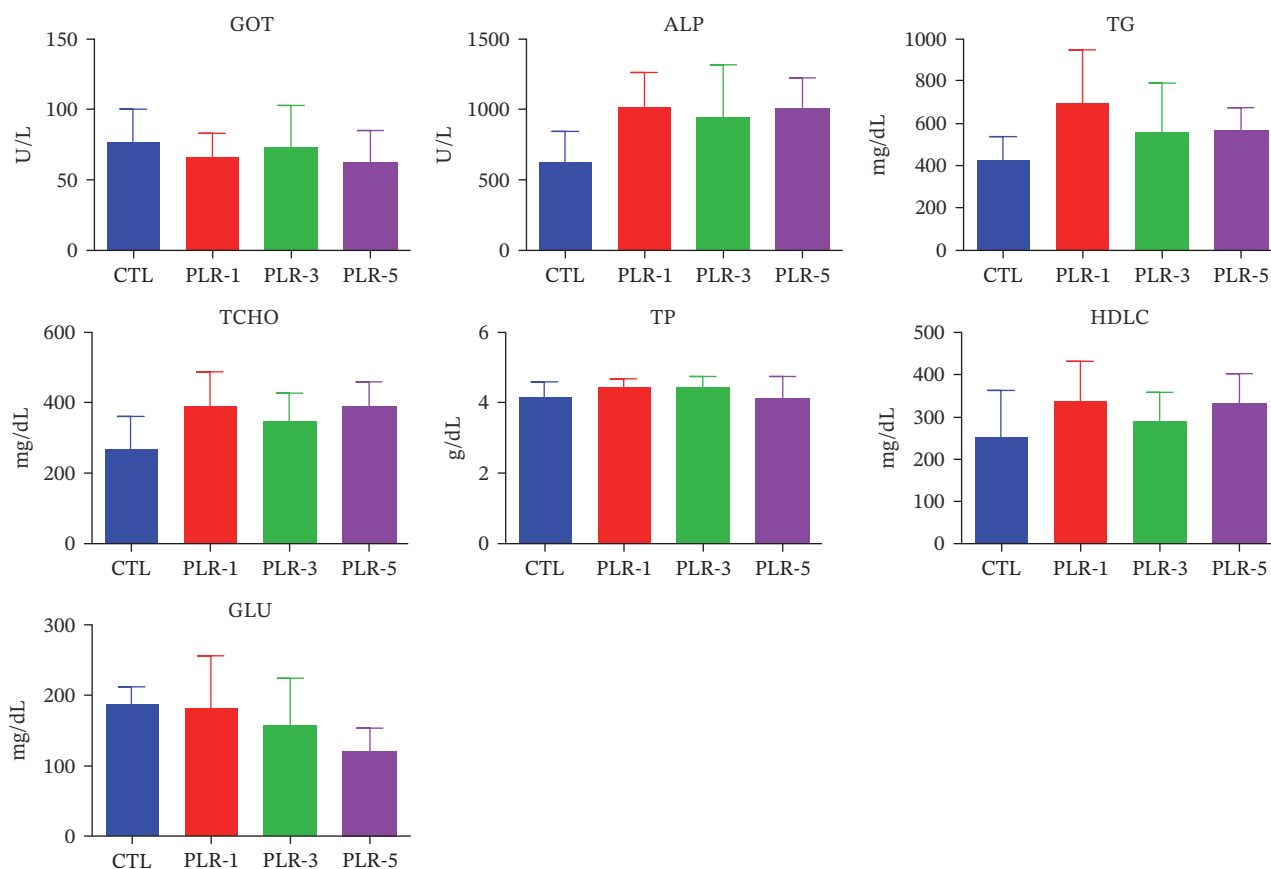


FIGURE 4 | Effects on blood health after feeding of control diet (CTL) and PLR supplemented diets (PLR-1, PLR-3, and PLR-5) in juvenile eel (*Anguilla japonica*) culture for 175 days ($n = 10$). ALP, alkaline phosphatase; GLU, glucose; GOT, aspartate aminotransferase; HDLC, high density lipoprotein; TCHO, total cholesterol; TG, triglyceride; TP, total protein. Bars represent mean ± SD. Groups were not significantly different from the one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$).

3.8 | Proximate Composition and Fatty Acid Composition

The proximate composition of whole-body eel showed no significant differences in moisture, crude protein, crude fat, or ash content between PLR-fed groups and the control after 175 days. Similarly, fatty acid composition—including total saturated fatty acids, unsaturated fatty acids, polyunsaturated fatty acids, and

omega-3:6 ratio—remained unaffected by dietary PLR supplementation (Supporting Information 3).

4 | Discussion

Eels cultured in high-density systems, such as recirculating aquaculture systems (RAS), as commonly used in Republic of Korea,

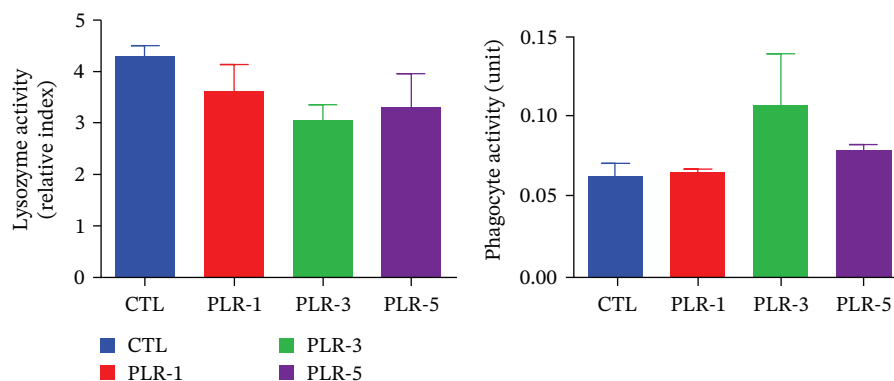


FIGURE 5 | Phagocytic activity and lysozyme activity after feeding of control diet (CTL) and PLR-supplemented diets (PLR-1, PLR-3, and PLR-5) in juvenile eel (*Anguilla japonica*) culture for 175 days ($n=10$). Bars represent mean $\pm$ SD. Groups were not significantly different from the one-way ANOVA followed by Tukey's post hoc test ($p<0.05$).

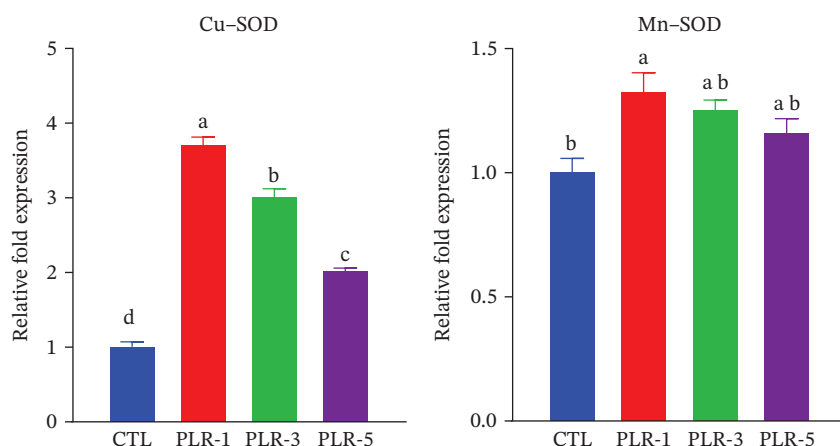


FIGURE 6 | Effects on CuSOD and MnSOD gene expression after feeding of control diet (CTL) and PLR supplemented diets (PLR-1, PLR-3, and PLR-5) in elver eel culture for 175 days ($n=5$). Cu-SOD, copper containing superoxide dismutase; Mn-SOD, manganese-dependent superoxide dismutase. Bars represent mean $\pm$ SD. Statistical differences were assessed using one-way ANOVA followed by Tukey's post hoc test ($p<0.05$). Bars that do not share the same lowercase letter are significantly different.

are exposed to a variety of environmental stressors. These include physical factors such as temperature fluctuations, light intensity, dissolved oxygen levels, and noise; chemical stressors such as ammonia, nitrite, nitrate, water hardness, pH, and alkalinity; biological stressors including handling, tank transfer, and high stocking density. These stressors activate the hypothalamus–pituitary–interrenal (HPI) axis, elevating circulating levels of cortisol and catecholamines, which in turn trigger a cascade of metabolic, immune, and physiological changes that ultimately reduce growth and productivity and cause economic losses for farmers [16]. While improving environmental management and culture systems remains a key strategy for mitigating stress, dietary interventions using functional additives have emerged as a complementary approach with considerable potential [17, 18].

In this context, plant-derived feed additives have gained attention due to their sustainability, eco-friendliness, and alignment with consumer demand for natural products. Among these, PLR extract has long been used in traditional medicine for its various pharmacological properties. Puerarin, the major isoflavonoid constituent of PLR, has been shown in mammalian models to possess antioxidant, anti-inflammatory, cardioprotective, and hypoglycemic activities [3, 4, 6]. Although some studies have investigated puerarin or PLR extracts in aquaculture species—demonstrating

growth-promoting and antioxidative benefits in largemouth bass [7] and yellowfin seabream [19]—their application in freshwater eel aquaculture has remained largely unexplored.

Our study addressed this gap by optimizing an extraction method for PLR that maximized the yield of major isoflavonoids (puerarin and daidzein) while minimizing cytotoxicity, ensuring the extract's suitability for use in aquafeeds. The optimized extraction conditions (100% EtOH, 25°C, 10 h) provided high isoflavonoid yield with low cytotoxicity, establishing a standardized method that could facilitate future applications.

Long-term feeding trials demonstrated that PLR supplementation enhanced growth performance in *A. japonica*. FW and WGR were markedly higher in PLR-fed groups compared to control, with the greatest effects observed at 5% inclusion. This aligns with findings from Huang et al. [7] and Kong et al. [19], who reported similar growth benefits from puerarin supplementation in other fish species. Notably, the coefficient of variation (CV) in size increased with higher PLR inclusion, leading to greater individual size disparity and observed cannibalism in the PLR-5 group. This suggests that the increased size variability observed at the 5% PLR inclusion may be linked to uneven feed intake and competitive interactions among faster-growing individuals, which can exacerbate size

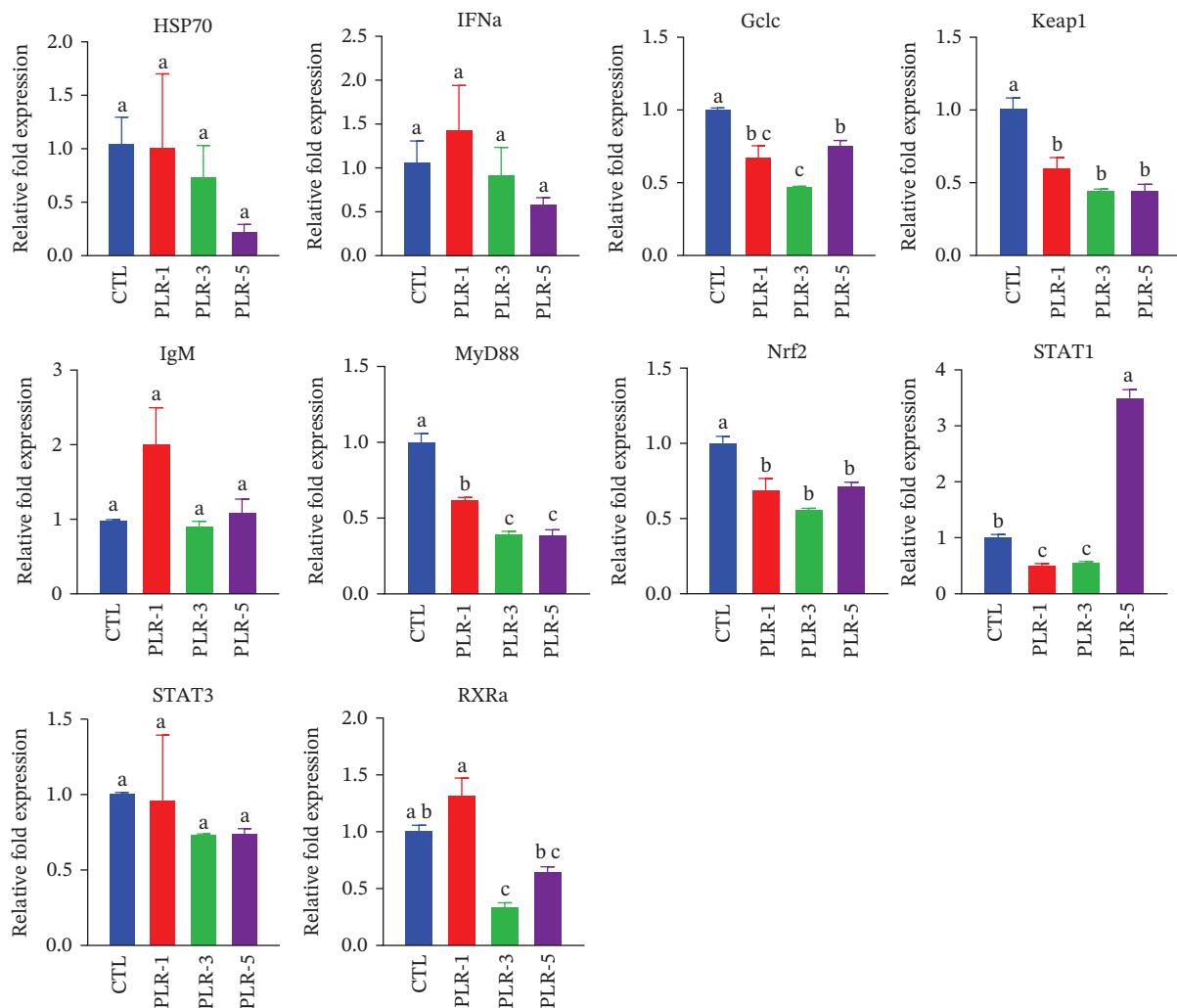


FIGURE 7 | Effects on major gene expressions after feeding control diet (CTL) and PLR-supplemented diets (PLR-1, PLR-3, and PLR-5) in juvenile eel (*Anguilla japonica*) culture for 175 days ($n = 5$). Gclc, glutamate-cysteine ligase catalytic subunit; HSP70, 70 kilodalton heat shock proteins; IFN α , interferon alpha; IgM, immunoglobulin M; Keap1, kelch-like ECH associated protein 1; MyD88, myeloid differentiation primary response 88; Nrf2, nuclear factor erythroid-2-related factor 2; RXR α , retinoid X receptor alpha; STAT1, signal transducer and activator of transcription 1; STAT3, signal transducer and activator of transcription 3. Bars represent mean $\pm$ SD. Statistical differences were assessed using one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$). Bars that do not share the same lowercase letter are significantly different.

disparities and trigger cannibalistic behavior. Such size-dependent competition is a well-recognized factor contributing to cannibalism in eel and other carnivorous fish species, highlighting the need for careful consideration of PLR dosage in practical aquaculture applications.

Blood biochemical analyses revealed no significant alterations in key markers, though trends indicated reduced GOT (AST) and GLU levels, and elevated ALP, TG, TCHO, and HDL-C levels in PLR-fed groups. These trends suggest potential effects of PLR on hepatic enzyme activity, lipid metabolism, and GLU homeostasis, consistent with studies in other species [20]. The reduction in stress markers and favorable modulation of lipid profiles could contribute to the observed improvements in growth and overall physiological status.

On the molecular level, PLR supplementation significantly upregulated hepatic CuSOD and MnSOD gene expression, indicating an enhancement of antioxidant defense capacity. In contrast, the mRNA expression levels of Nrf2, Keap1, and Gclc were consistently downregulated across PLR-treated groups. Although this pattern

may appear contradictory given the central role of Nrf2 in regulating antioxidant gene expression, it is important to recognize that Nrf2 signaling is predominantly controlled at the posttranslational level, including protein stabilization, nuclear translocation, and interactions with Keap1, rather than by transcriptional regulation alone. Therefore, changes in Nrf2-related mRNA expression do not necessarily reflect functional Nrf2 activity.

The observed downregulation of Nrf2 pathway transcripts may represent a negative feedback response following sufficient activation of antioxidant defenses, aimed at maintaining redox homeostasis [21]. Supporting this interpretation, dietary supplementation with antioxidants such as coenzyme Q10 has been reported to reduce oxidative stress and subsequently attenuate ROS sensing and NRF2 activation, leading to decreased NRF2 mRNA expression [22]. Moreover, accumulating evidence indicates that certain antioxidants can enhance the expression of antioxidant enzymes, including SOD, through signaling pathways independent of the NRF2 axis [23, 24].

In this context, the upregulation of CuSOD and MnSOD observed in the present study may reflect the involvement of alternative regulatory mechanisms rather than direct transcriptional activation by Nrf2. While pathways such as MAPK or PI3K/Akt have been proposed as potential mediators of NRF2-independent antioxidant responses [25], the present study is limited to transcript-level analysis and does not allow definitive conclusions regarding the underlying signaling mechanisms. Further investigations incorporating protein-level and posttranslational analyses would be required to clarify the precise regulatory pathways involved.

PLR supplementation also resulted in downregulation of pro-inflammatory and innate immune-related genes, including HSP70, IFN- α , and MyD88. This molecular signature suggests that PLR may exert anti-inflammatory effects in *A. japonica*, consistent with the suppression of chronic low-grade inflammation. Such effects are advantageous under high-density aquaculture conditions, where oxidative stress and inflammation can impair growth and health. The lack of significant changes in lysozyme and phagocytic activity indicates that PLR's effects were more pronounced at the transcriptional level rather than in functional immune responses within the tested time frame.

The key contributions of this study include the development of a standardized, low-toxicity extraction method for PLR suitable for aquafeeds, demonstration of long-term growth promotion in *A. japonica*, elucidation of antioxidant and immune modulation mechanisms, and identification of potential risks such as increased size variability at higher inclusion levels. Limitations include the absence of direct measurement of cortisol or oxidative stress biomarkers (e.g., MDA and GSH-Px activity) and the lack of validation under commercial farming conditions. Future research should aim to address these gaps, explore synergistic effects with other additives (e.g., probiotics), and refine dosing strategies to balance growth benefits with population uniformity. Collectively, these findings support the potential of PLR extract as a functional feed additive for freshwater eel aquaculture, capable of enhancing growth, antioxidant capacity, and immune balance.

5 | Conclusion

This study demonstrates that PLR extract is a promising functional feed additive for juvenile eel. A standardized extraction method was developed to ensure high isoflavonoid yield and low toxicity. Long-term feeding trials showed that PLR significantly improved growth, enhanced antioxidant defenses, and modulated immune-related gene expression, suggesting reduced inflammation. However, high inclusion levels increased size variability and cannibalism risk. These findings suggest that PLR extract has potential as a functional feed additive for promoting growth in eel aquaculture. Future work should focus on refining optimal inclusion levels to maximize benefits while minimizing size disparity and associated risks.

Author Contributions

Walimuni Randika Harshan Mendis: writing – original draft, data curation, formal analysis, investigation, methodology, validation, visualization. **Tae-Ho Ahn, Jin Hyeok Jang, Byung-Cheul Jeong, and Seunghan Lee:** data curation, formal analysis. **So Young Kang and**

Jun Cheul Ahn: conceptualization, funding acquisition, investigation, methodology, supervision, validation, writing – review and editing.

Funding

This study was funded by the Ministry of SMEs and Startups (Grant S3366885) and the RISE program through the Jeonbuk RISE Center, funded by the Ministry of Education and the Jeonbuk State, Republic of Korea (Grant 2025-RISE-13-KSU).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data will be made available upon request.

References

1. A. Okamura, N. Horie, Y. Yamada, N. Mikawa, and K. Tsukamoto, "Obtaining High-Quality Larvae for Mass Production of Glass Eels: Can We Refine Our Approach?" *Aquaculture* 521 (2020): 735049.
2. M. Reverter, N. Bontemps, D. Lecchini, B. Banaigs, and P. Sasal, "Use of Plant Extracts in Fish Aquaculture as an Alternative to Chemotherapy: Current Status and Future Perspectives," *Aquaculture* 433 (2014): 50–61.
3. Q. Zhou, M. Zhou, Q. Li, R. Wang, Y. Fu, and T. Jiao, "Facile Biosynthesis and Grown Mechanism of Gold Nanoparticles in *pueraria lobata* Extract," *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 567 (2019): 69–75.
4. E. Buhlmann, C. Horváth, J. Houriet, et al., "Puerariae lobatae Root Extracts and the Regulation of Brown Fat Activity," *Phytomedicine* 64 (2019): 153075.
5. X. Wang, C. Li, Z. Zhou, and Y. Zhang, "Identification of Three (Iso) flavonoid Glucosyltransferases From *Pueraria lobata*," *Frontiers in Plant Science* 10 (2019): 28.
6. X.-Z. Fan, F. Yao, C.-M. Yin, D.-F. Shi, and H. Gao, "Mulberry Twig and Pueraria Root Residue Improve the Biological Efficiency and Potential Functional Value of *Hericium erinaceus*," *Scientia Horticulturae* 286 (2021): 110214.
7. Y. Huang, W. Ma, D. Zhang, X. Chen, Z. Huang, and Y. Hong, "Dietary Puerarin Enhances Growth, Immune Function, Antioxidant Capacity, and Disease Resistance in Farmed Largemouth Bass, *Micropterus salmoides*," *Fishes* 10, no. 5 (2025): 197.
8. Y. Gao, X. Wang, and C. He, "An Isoflavonoid-Enriched Extract From *Pueraria lobata* (kudzu) Root Protects Human Umbilical Vein Endothelial Cells Against Oxidative Stress Induced Apoptosis," *Journal of Ethnopharmacology* 193 (2016): 524–530.
9. Association of Official Analytical Chemists-AOAC, *Official Methods of Analysis of AOAC International*, 17th ed. (AOAC, 2000).
10. N. Duongbia, S. Chaiwongsar, C. Chaichana, and S. Chaiklangmuang, "Acidic Hydrolysis Performance and Hydrolyzed Lipid Characterizations of Wet *Spirulina platensis*," *Biomass Conversion and Biorefinery* 9, no. 2 (2019): 305–319.
11. I. Miglavs and M. Jobling, "The Effects of Feeding Regime on Proximate Body Composition and Patterns on Energy Deposition in Juvenile Artic Charr, *Salvelinus alpinus*," *Journal of Fish Biology* 35, no. 1 (1989): 1–11.
12. R. W. Peace and G. S. Gilani, "Chromatographic Determination of Amino Acids in Foods," *Journal of AOAC International* 88, no. 3 (2005): 877–887.
13. J. Folch, M. Lees, and G. H. S. Stanley, "A Simple Method for the Isolation and Purification of Total Lipides From Animal Tissues," *Journal of Biological Chemistry* 226, no. 1 (1957): 497–509.

14. J. Koskela, R. Rahkonen, M. Pasternack, and H. Knuutinen, "Effect of Immunization With Two Commercial Vaccines on Feed Intake, Growth, and Lysozyme Activity in European Whitefish (*Coregonus lavaretus* L.)," *Aquaculture* 234, no. 1–4 (2004): 41–50.
15. K. R. Seeley, P. D. Gillespie, and B. A. Weeks, "A Simple Technique for the Rapid Spectrophotometric Determination of Phagocytosis by Fish Macrophages," *Marine Environmental Research* 30, no. 1 (1990): 37–41.
16. A. Ciji and M. S. Akhtar, "Stress Management in Aquaculture: A Review of Dietary Interventions," *Reviews in Aquaculture* 13, no. 4 (2021): 2190–2247.
17. C. I. M. Martins, E. H. Eding, M. C. J. Verdegem, et al., "New Developments in Recirculating Aquaculture Systems in Europe: A Perspective on Environmental Sustainability," *Aquacultural Engineering* 43, no. 3 (2010): 83–93.
18. M. Zhao, L. Li, and C. Ai, "Research Progress of Additives in Compound Feeds for Marine Ornamental Fish and Its Sustainable Development," in *Proceedings of 2022 7th International Conference on Environmental Engineering and Sustainable Development (CEESD 2022)*, Environmental Science and Engineering, (Springer, 2023): 29–34.
19. L. Kong, J. Ma, S. Zhou, et al., "Effects of Puerarin on Growth, Liver Immunity and Antioxidant Capacity of Yellowfin Seabream (*Acanthopagrus latus*) Under Oxidized Fish Oil Stress," *Aquaculture Reports* 37 (2024): 102212.
20. W. Hu, X. Yang, C. Zhe, Q. Zhang, L. Sun, and K. Cao, "Puerarin Inhibits iNOS, COX-2 and CRP Expression via Suppression of NF- κ B Activation in LPS-Induced RAW264.7 Macrophage Cells," *Pharmacological Reports* 63, no. 3 (2011): 781–789.
21. M. E. Giuliani and F. Regoli, "Identification of the Nrf2-Keap1 Pathway in the European Eel *Anguilla anguilla*: Role for a Transcriptional Regulation of Antioxidant Genes in Aquatic Organisms," *Aquatic Toxicology* 150 (2014): 117–123.
22. E. M. Yubero-Serrano, L. Gonzalez-Guardia, O. Rangel-Zuñiga, et al., "Postprandial Antioxidant Gene Expression Is Modified by Mediterranean Diet Supplemented With Coenzyme Q(10) in Elderly Men and Women," *AGE* 35, no. 1 (2013): 159–170.
23. X. Wang, T. Shen, J. Lian, et al., "Reveratrol Reduces ROS-Induced Ferroptosis by Activating SIRT3 and Compensating the GSH/GPX4 Pathway," *Molecular Medicine* 29, no. 1 (2023): 137.
24. M. Dell'Orco, P. Milani, L. Arrigoni, et al., "Hydrogen Peroxide-Mediated Induction of SOD1 Gene Transcription Is Independent From Nrf2 in a Cellular Model of Neurodegeneration," *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms* 1859, no. 2 (2016): 315–323.
25. A. K. Jaiswal, "Nrf2 Signaling in Coordinated Activation of Antioxidant Gene Expression," *Free Radical Biology and Medicine* 36, no. 10 (2004): 1199–1207.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.

[Supporting Information 1.](#) Supporting Information 1: Responses for the 2³ full factorial design extracts.

[Supporting Information 2.](#) Supporting Information 2: Identification and quantification of the major compounds of PLR.

[Supporting Information 3.](#) Supporting Information 3: Proximate and fatty acid composition of eel supplemented with PLR.