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Araştırma Makalesi

Cyanus depressus ve *Schizochytrium* sp. Ekstraktlarının Sazan Balığında (*Cyprinus carpio*) Büyüme Endeksleri, Antioksidan ve Bağışıklıkla İlgili Genlerin İfade Düzeyleri Üzerindeki Sinerjik Etkileri

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ÖZ

Bu çalışmada, gökbaş bitkisi (*Cyanus depressus*) ve mikroalg (*Schizochytrium* sp.) özütlerinin sinerjik etkileri sazan balığının (*Cyprinus carpio*) büyüme performansı ve gen ifade düzeyleri üzerinde incelenmiştir. Ortalama canlı ağırlığı 3,71 ± 0,16 g olan sazanlar, 60 gün boyunca 0 (kontrol), 0,05 (CS05) ve 0,1 (CS1) g/kg oranlarında bu özütlerin karışımı (CS) ilave edilmiş yemlerle beslenmiştir. Araştırma sonuçları, 0,05 ve 0,1 CS g/kg katkılı yemlerin sazanların canlı ağırlık kazancı ve spesifik büyüme oranında önemli iyileşmeler sağladığını göstermiştir ($p<0,05$). Özellikle 0,05 CS g/kg katkılı yemle beslenen balıklarda süperoksit dismutaz (SOD) ve katalaz (CAT) genlerinin ifade düzeylerinde anlamlı artışlar kaydedilmiştir ($p<0,05$). Ayrıca bağışıklık göstergesi olarak değerlendirilen interlökin-1 beta (IL-1β) gen ekspresyonunun, CS içeren yemlerle beslenen tüm gruplarda kontrol grubuna göre daha yüksek olduğu belirlenmiştir. Sonuç olarak, *Cyanus depressus* ve *Schizochytrium* sp. özütlerinin %0,05 seviyesinde yem katkısı olarak kullanılması, sazan balığı yetiştiriciliğinde büyüme ve bağışıklık parametrelerini iyileştiren, verim artırıcı bir alternatif yem katkı maddesi olarak önerilmektedir.

Anahtar kelimeler: *Cyanus depressus, Ekstrakt, Yem takviyesi, Gen ifadesi, Schizochytrium sp.*

Synergistic Effects of *Cyanus depressus* and *Schizochytrium* sp. Extracts on Growth Indices and The Expression Levels of Antioxidant and Immunity-Related Genes in Common Carp (*Cyprinus carpio*)

ABSTRACT

This study investigated the synergistic effects of cornflower (*Cyanus depressus*) and microalgae (*Schizochytrium* sp.) extracts as alternative feed additives on the growth performance and expression levels of antioxidant and immunity-related genes in common carp (Cyprinus carpio). Juvenile carp with an average weight of 3.71 ± 0.16 g were fed diets supplemented with 0 (Control), 0.5 (CS05), and 1 (CS1) g/kg of extract blend (CS) for 60 days. The results revealed significant improvements in weight gain and specific growth rate in carp fed diets containing 0.5 and 1 g/kg CS ($p<0.05$). Notably, fish fed the diet with 0.5 g/kg CS exhibited the highest expression levels of superoxide dismutase (SOD) and catalase (CAT) genes ($p<0.05$). Additionally, the interleukin-1 beta (IL-1β) gene, considered a marker of immune response, was upregulated in all experimental groups fed CS-supplemented diets compared to the control. In conclusion, the supplementation of *Cyanus depressus* and *Schizochytrium* sp. extracts at a level of 0.5 g/kg in the diet is recommended as an effective alternative feed additive to enhance growth performance and immune health in common carp aquaculture.

Key words: *Cyanus depressus, Extract, Feed supplement, Gene expression, Schizochytrium sp.*

INTRODUCTION

The growing global demand for high-quality fish products has positioned aquaculture as vital to food security. This sector faces the challenge of ensuring sustainability while meeting consumer expectations for health benefits and environmental responsibility (Boyd et al., 2020; Ababouch et al., 2023; Estim et al., 2024). To address these challenges, innovative nutritional strategies focused on promoting growth, enhancing immunity, and improving the overall health of farmed fish are essential (Dawood et al., 2018; Ahmadifar et al., 2021a; Sarker, 2023). Recent trends reveal a significant shift toward natural feed additives, particularly those derived from marine algae and terrestrial plants, as viable alternatives to synthetic growth promoters and immune stimulants (Idenyi et al., 2022; Rombenso et al., 2022; Eroldoğan et al., 2023). These natural compounds not only support fish physiology but also help reduce environmental impact, aligning with consumer preferences for cleaner, more sustainable aquaculture practices (Gephart et al., 2020; Reverter et al., 2021; Wang et al., 2024).

Among promising natural additives, *Schizochytrium* sp., a marine microalga, has garnered particular attention due to its high omega-3 PUFA content, especially docosahexaenoic acid (DHA) (Bélanger et al., 2021; Trevi et al., 2023). DHA is an integral part of fish cell membranes, contributing to membrane fluidity, immune modulation, and gene expression regulation (Hashimoto et al., 2017; Magalhães et al., 2021; Koven et al., 2024). Numerous studies have demonstrated that incorporating *Schizochytrium* sp. into fish diets enhances growth metrics, boosts antioxidant defences, and strengthens immune responses across species, including Nile tilapia (Souza et al., 2020), Atlantic salmon (Kousoulaki et al., 2022), and rainbow trout (Serrano et al., 2021). Furthermore, DHA supplementation has been associated with enhanced antioxidant defences that mitigate oxidative stress, a common concern in intensive aquaculture systems (Bi et al., 2018; Zhang et al., 2019; Ji et al., 2025).

Parallel to research on marine-derived compounds, terrastial plant extracts rich in bioactive molecules, such as phenolic compounds, have shown beneficial effects in aquaculture (Ahmadifar et al., 2021b; Tadese et al., 2022; Naiel et al., 2023; Kalaiselvan et al., 2024). *Cyanus depressus*, a terrestrial plant noted for its high phenolic and flavonoid content, exhibits strong antioxidant and immunomodulatory properties (Khammar & Djeddi, 2012; Escher et al., 2018; Fattaheian-Dehkordi et al., 2021; Gawlik-Dziki et al., 2023). According to Karataş (2024), this plant may serve as an effective alternative feed additive, with potential benefits for fish growth and overall health. While previous research has established the individual benefits of each extract, there remains a significant knowledge gap regarding their combined effects on enhancing growth and improving fish physiological status, particularly through the regulation of antioxidant and immune-related pathways. Investigating the interactions between these extracts and their effects on growth indices and key gene expression markers is crucial for optimizing feed efficiency and fish health in commercial aquaculture. Including these natural additives not only supports the physiological needs of fish but also aligns with increasing consumer demand for chemical-free aquaculture products.

This study investigates the synergistic effects of *Cyanus depressus* and *Schizochytrium* sp. extracts on growth and gene expression in common carp (*Cyprinus carpio*), a species of considerable commercial importance. Like many aquaculture species, carp face challenges related to oxidative stress and disease susceptibility under intensive farming conditions (Martos-Sitcha et al., 2020; Ciji & Akhtar, 2020; Song et al., 2023). By analyzing growth indices and the expression of key antioxidant genes (SOD and CAT) and the immune-related gene IL-1β, this research aims to provide valuable insights into how these natural additives can support fish health. In conclusion, this study evaluates the potential of *Cyanus depressus* and *Schizochytrium* sp. extracts as synergistic dietary supplements for improving growth performance and enhancing the expression of antioxidant and immune-related genes in carp. The findings aim to contribute to the development of sustainable and effective dietary strategies in aquaculture, fostering healthier fish and optimizing production outcomes.

MATERIALS AND METHODS

Ethical approval

This experiment was approved by the Van Yüzüncü Yıl University Local Ethics Committee for Animal Experiments (protocol no: 2023/13-31) and conducted in accordance with established ethical guidelines.

Preparation of extracts

The *Cyanus depressus* plants used in this study were collected from the campus of Van Yüzüncü Yıl University (Van, Turkey). For preparing the ethanol extracts, dried flower parts were ground into powder, and a total of 40 g of flower powder was combined with 1 L of 80% ethanol, shaken for 24 hours (Duman et al., 2022; Karataş 2024). *Schizochytrium* sp. was obtained in powder form from Marine Biotechnology Products (Aydın, Turkey). The microalgae powder was combined with 1 L of 80% ethanol at a ratio of 1:5 and gently shaken for 48 hours (Kiadaliri et al., 2020). Both mixtures were filtered initially through sterile cheesecloth, then centrifuged at

3500 rpm for 5 minutes. The filtrates were further clarified using Whatman filter paper (No. 1). After filtration, ethanol was evaporated at 40°C in a rotary evaporator to yield a concentrated extract. The concentrate was freeze-dried at −85°C and stored at −20°C until further experimental applications (Karataş 2024).

Incorporation of extracts into experimental diets

The extract mixture was prepared by thoroughly mixing equal volumes of each extract. To formulate experimental diets, basal diet (46% crude protein, 19% crude lipid, 2.7% crude fiber, 8.7% crude ash, 1.7% calcium, 1.23% phosphorus, and 0.3% sodium; Skretting (Milas-Muğla/Turkey)) was supplemented with extract mixtures at levels of 0 (Control), 0.5, and 1 g/kg. Briefly, the basal diet was moistened with 300 ml of water per kg until a smooth dough formed, then the extract mixture was added and thoroughly mixed. The dough was passed through a mincer to form sticks, which were dried under a fan at 22°C. These dried sticks were pelletized (3 mm) and stored at 4°C in plastic bags until use (Hoseinifar et al., 2017; Rajabiesterabadi et al., 2020; Ghafarifarsani et al., 2021).

Fish rearing and feeding protocols

The study was conducted at the Aquatic Organisms Research Unit of Van Yüzüncü Yıl University (Van, Turkey). Carp (*C. carpio*) were obtained from this facility and acclimated to experimental conditions for two weeks. Afterward, 108 healthy fish with an average weight of 3.71 ± 0.16 g were randomly distributed into nine aquaria (100 L each) at a density of 12 fish per aquarium, with three replicates. Fish were fed twice daily at 3% of their body weight for 60 days (Ghafarifarsani et al., 2021). Bi-weekly, total fish weights per aquaria were assessed, and feed amounts were adjusted based on the latest average weights. During the experiment, each aquarium was aerated by a central air pump, and 50% of the water was replaced daily with dechlorinated water. Additionally, waste materials were regularly siphoned from the aquaria bottom. Physicochemical parameters of the rearing water were maintained at 22.46 ± 0.33°C (temperature), 8.2 ± 0.1 (pH), and 6.71 ± 0.18 mg/L (dissolved oxygen). A photoperiod of 12:12 h light-dark cycle was provided using artificial lighting.

Growth indices and sampling

Fish were weighed at the start of the feeding trial and after 60 days, and growth indices were calculated using the following standard formulas:

Weight gain (WG; g/fish) = Final weight (g) − Initial weight (g),
Daily weight gain (DWG; g/fish) = (Final weight (g) − Initial weight (g)) / days,
Specific growth rate (SGR; %/day) = ((ln (final weight) – ln (initial weight)) / days) x 100,
Thermal growth coefficient (TGC) = (((final weight)1/3 – (initial weight)1/3) / temperature in °C x time in days) x 1000,
Feed conversion ratio (FCR) = Total feed given (g) / Weight gain (g),
Survival rate (SR; %) = (Final number of fish / Initial number of fish) x 100.

Prior to sampling, fish were fasted for 24 hours to allow for gut clearance. For gene expression analyses, six fish per treatment (two fish per aquarium) were randomly selected and anesthetized with clove powder (150 ppm) (Ghafarifarsani et al., 2021). Liver tissues were rapidly dissected from the sampled fish, with 25–50 mg of liver tissue collected and preserved in RNAlater solution until RNA isolation.

Gene expression analysis

Total RNA isolation was performed using the DiaRex® Total RNA Extraction Kit (TR-0877-100, Diagen, Ankara, Turkey) following the manufacturer's protocol. RNA quantity and purity were evaluated using a nanospectrophotometer (QIAxpert) at 260 and 280 nm. cDNA synthesis was subsequently performed with the Solver ArGe cDNA Synthesis Kit (SLV-M-2021-10-100, Van, Turkey)(Önalan, 2019). Real-time PCR was conducted on a RotorGene Q 9000 (Qiagen) using Solver ArGe qPCR Master Mix (SLV-M-2021-01-0.5ML Van, Turkey). A total of four genes were analyzed, including three target genes and one reference gene. The PCR reaction mixture consisted of 21 µL total volume: 12 µL SybrGreen qPCR Master Mix, 2.5 µL forward and reverse primers, 4 µL H2O, and 4 µL cDNA. The PCR protocol involved an initial incubation at 95°C for 10 minutes, followed by 45 cycles of denaturation at 94°C for 15 seconds and annealing at 63°C for 45 seconds. Beta-actin served as the reference gene to normalize Ct values from real-time PCR. Data analysis was conducted using the ΔΔCT method (Livak and Schmittgen, 2001).

Table 1. Primer sequences used to determine the expression of antioxidant and immune response genes in carp.

Gene name	Primer sequence (5´ to 3´)	Primer Efficacy	Annealing Tm (°C)	Reference
β-actin (Beta-actin)	F: CCTGTATGCCAACACCGTGCTG R: CTTCATGGTGGAGGGAGCAAGG	%98	63	Hoseini et al., 2020; Fazelan et al., 2020

CAT (Catalase)	F: AGACGACACCCATCGCTGTTCG R: AAGGTCCCAGTTGCCCTCATCG	%99	63	Hoseini et al., 2020; Fazelan et al., 2020
SOD (Superoxide dismutase)	F: TGAGCTGTCGGAAGCCATCAAG R: TTGGTTCCCACATGCAGCAATCC	%97	63	Hoseini et al., 2020; Fazelan et al., 2020
IL-1β (Interleukin-1 Beta)	F: ACCAGCTGGATTTGTCAGAAG R: ACATACTGAATTGAACTTTG	%99	55	Hoseini et al., 2020; Fazelan et al., 2020

Statistical analysis

All statistical analyses were performed with SPSS version 20. Data were checked for normality and homogeneity of variance before proceeding. Differences among groups were evaluated using one-way analysis of variance (ANOVA). When significant differences ($p < 0.05$) were found, Duncan's multiple range test was applied for post hoc comparisons. Results are reported as means ± standard error.

RESULTS

Growth indices

Growth indices for carp are presented in Table 2. During the 60-day feeding trial, no mortality was observed in the control or extract mixture groups (CS). The inclusion of a mixture of *Cyanus depressus* and *Schizochytrium* sp. extracts in the diet significantly improved FW, WG, SGR, DWG, and TGC values in fish compared to the control group ($P < 0.05$). Growth indices were highest in the CS1 group among the CS treatment groups. The most favorable (i.e., lowest) FCR was observed in the CS05 and CS1 groups ($P < 0.05$).

Table 2. Growth performance parameters of carp fed diets supplemented with different levels of extract mixture for 60 days.

Growth performance	**Experimental diets**		
	Control	**CS05**	**CS1**
Initial weight (g)	3.71 ± 0.03	3.71 ±0.01	3.71±0.02
Final weight (g)	17.67 ± 0.24 ^{b}	21.84 ± 0.78 ^{a}	22.01 ± 0.65 ^{a}
WG (g)	13.96 ± 0.25 ^{b}	18.14 ± 0.79 ^{a}	18.30 ± 0.65 ^{a}
DWG (g)	0.24 ± 0.00 ^{b}	0.32 ± 0.01 ^{a}	0.32 ± 0.01 ^{a}
SGR (%/day)	2.78 ± 0.02 ^{b}	3.16 ± 0.07 ^{a}	3.17 ± 0.05 ^{a}
TGC	0.84 ± 0.01 ^{b}	0.99 ± 0.02 ^{a}	0.99 ± 0.02 ^{a}
FCR	2.19 ± 0.01 ^{b}	1.62 ± 0.02 ^{a}	1.58 ± 0.01 ^{a}
SR (%)	100	100	100

Results are presented as mean ± S.E. (n = 3). Different superscript letters within the same row indicate significant treatment differences. Letters a and b in the same row denote statistical significance ($p<0.05$).

Abbreviations: WG, Weight Gain; SGR, Specific Growth Rate; DWG, Daily Weight Gain; TGC, Thermal Growth Coefficient; FCR, Feed Conversion Ratio; SR, Survival Rate.

Gene expression associated with antioxidant and ımmune responses

Changes in the expression levels of genes encoding the antioxidant enzymes CAT and SOD are presented in Figure 1. Results indicate that CAT gene expression was significantly upregulated in the CS05 group compared to the control and CS01 groups ($p < 0.05$). For SOD expression levels, upregulation was observed in the CS05 group compared to the control, while downregulation was noted in the CS1 group relative to the control ($p < 0.05$). The expression levels of the IL-1β gene, associated with immune response, are shown in Figure 1. According to these findings, IL-1β gene expression was significantly upregulated in the CS05 and CS1 groups compared to the control group.

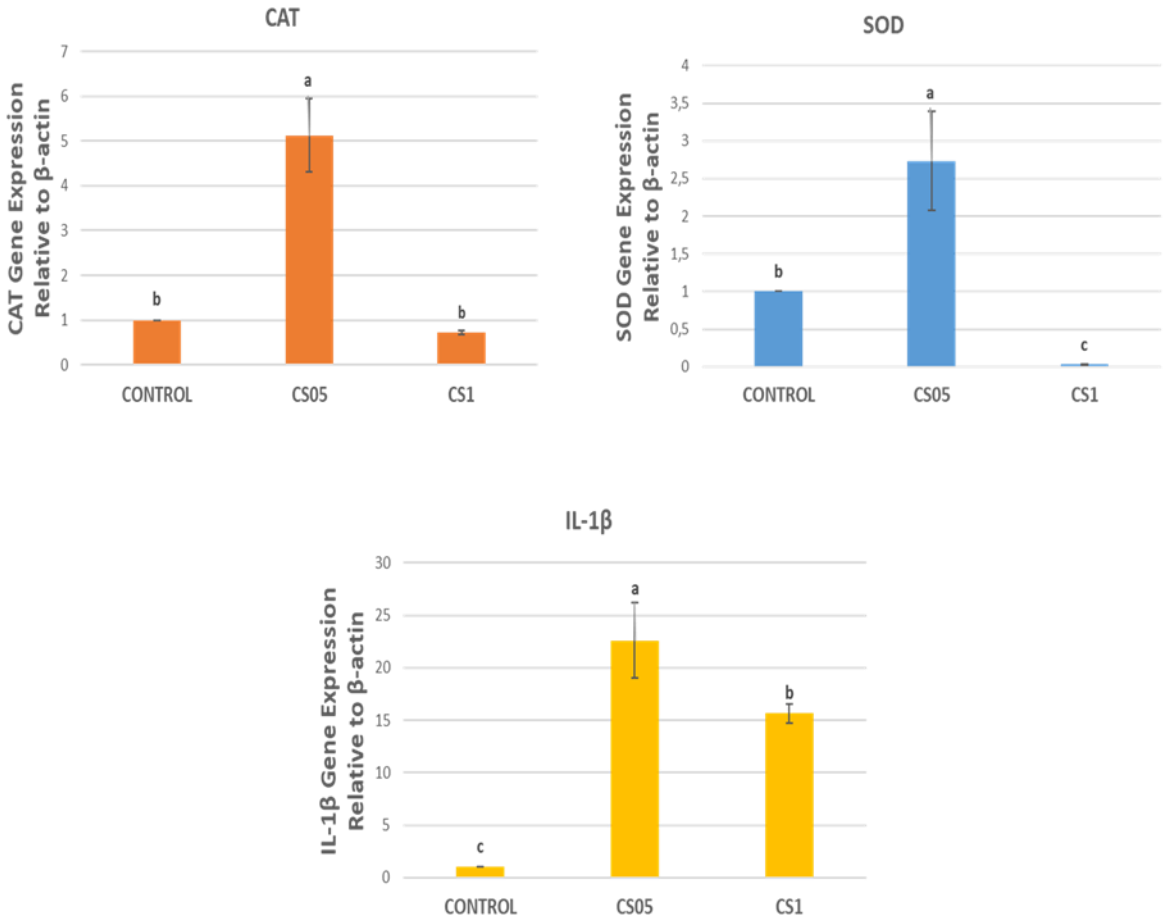

Figure 1. Synergistic effects of *Cyanus depressus* and *Schizochytrium* sp. extracts on the expression levels of antioxidant and immunity-related genes in carp after 60 days of feeding trial (n = 6).

DISCUSSION

With the increase in fish production in aquaculture, numerous microalgae and medicinal plants are now commonly used as feed additives due to their beneficial effects (Ivanova et al., 2024; Jitendrasinh et al., 2024; Onomu and Okuthe, 2024; Vijayaram et al., 2024). Studies have reported that these additives, or their extracts, improve growth indices, antioxidant status, and immune responses in aquatic animals (Vijayaram et al., 2022; Tadese et al., 2022; Bahi et al., 2023). However, these effects mostly focus on the individual use of medicinal plants and microalgae. To our knowledge, no prior research has examined the combined dietary supplementation of *Cyanus depressus* and *Schizochytrium* sp. extracts in fish. Therefore, this study was designed to evaluate the effects of combined dietary supplementation of *Cyanus depressus* and *Schizochytrium* sp. extracts on growth indices and gene activities related to antioxidant and immune responses in carp.

Findings from the present study indicate that a combination of *Cyanus depressus* and *Schizochytrium* sp. extracts added to carp diets had a beneficial effect on fish growth indices. Compared to the control (C) group, fish fed diets containing a combination of plant and microalgae extracts (CS) demonstrated significant improvements in growth indices such as WG and SGR, regardless of supplementation level. Additionally, all groups receiving diets with CS extracts showed marked improvements in FCR values. The observed improvements in growth indices may be attributed to various bioactive compounds found in plant and algae extracts that enhance feed palatability and intake (Dawood et al., 2018; Nagarajan et al., 2021; Kuebutornye et al., 2024). Previous studies have also shown that compounds like flavonoids and phenolic acids, present in *Cyanus depressus* and *Schizochytrium* sp. extracts, positively influence growth indices in various fish species (das Neves et al., 2021; Xu et al., 2022; Ghafarifarsani et al., 2023; Jin et al., 2023; Lin et al., 2024). Furthermore, as reported in prior studies (das Neves et al., 2021; Zhang et al., 2023; Shang et al., 2024), these bioactive compounds can stimulate digestive enzyme production, thereby improving feed digestibility and nutrient absorption. Improved growth indices observed here may thus be linked to increased digestive enzyme activity and enhanced nutrient absorption (Jeney et al., 2015; Yousefi et al., 2021; Karataş, 2024). Additionally, previous studies investigating

Cyanus depressus (Karataş, 2024) and *Schizochytrium* sp. (Dos Santos et al., 2019; Xie et al., 2019; Li et al., 2023) as individual feed additives reported their positive impact on fish development. To date, no data on the combined effects of *Cyanus depressus* and *Schizochytrium* sp. extracts on fish growth indices exists. However, prior studies on combined feed additives have highlighted the beneficial effects of combination applications over single additives (Abu-Elala et al., 2016; Ghafarifarsani et al., 2021; Sattanathan et al., 2022; Yousefi et al., 2022; Rashidian et al., 2023).

The efficacy of antioxidants in reducing oxidative stress is often influenced by specific aquaculture conditions, dietary applications, and environmental parameters (Zhang et al., 2020; Mugwanya et al., 2023; Karataş, 2024). In the present study, SOD and CAT gene expression levels were significantly elevated in the CS05 group compared to the control and CS1 groups. These findings suggest that the combination of *Cyanus depressus* and *Schizochytrium* sp. extracts can enhance the capacity of the antioxidant system, potentially protecting organisms from oxidative damage. However, determining the appropriate dosage is crucial in combination applications. In this study, among the concentrations evaluated, the 0.5 g/kg treatment demonstrated the most beneficial effect on antioxidant capacity, indicating that specific dosages can impact antioxidant status. The increase in CAT and SOD levels in the CS05 group may be attributed to bioactive compounds present in *Cyanus depressus* and *Schizochytrium* sp. extracts. These bioactive compounds function as potent antioxidants, neutralizing reactive oxygen species (ROS) before they cause harmful physiological effects in cells (Pradhan et al., 2021; Zhou et al., 2022). Furthermore, previous research has highlighted beneficial impacts of compounds such as fumaric acid, quinic acid, 4-hydroxybenzoic acid, chlorogenic acid, luteolin, and gallic acid components of the *Cyanus depressus* and *Schizochytrium* sp. extract blend on enhancing antioxidant capacity in aquatic species (Ghafarifarsani et al., 2023; Jin et al., 2023; Zhang et al., 2023; Lin et al., 2024; Karataş, 2024).

IL-1β is a cytokine protein secreted by various immune cells, primarily monocytes and macrophages, and plays a role in inflammatory processes (Hoseinifar et al., 2022). IL-1β is essential for regulating the innate immune response, and several studies have suggested that monitoring expression levels can provide insights into immune response changes (Ahmadifar et al., 2023; Hoseinifar et al., 2023; Shang et al., 2024). In our study, notable differences in IL-1β gene expression levels related to immunity were recorded among treatments, with the highest IL-1β expression observed in fish subjected to CS containing diets, irrespective of supplementation level. The elevated IL-1β levels compared to the control group may be attributed to compounds such as flavonoids and antioxidants in the *Cyanus depressus* and *Schizochytrium* sp. extract combination. Research has shown that specific phenolic compounds present in these materials possess immune-enhancing properties (Jin et al., 2023; Zhang et al., 2023; Lin et al., 2024). Consistent with these findings, the elevated levels of the selected immune gene in this study demonstrate the immunostimulatory effect of the dietary *Cyanus depressus* and *Schizochytrium* sp. extract combination at a molecular level in carp.

CONCLUSION

In conclusion, this study demonstrates that the combined dietary supplementation of *Cyanus depressus* and *Schizochytrium* sp. extracts significantly enhances growth performance, antioxidant capacity, and immune responses in carp (*Cyprinus carpio*). The combination effectively improved growth indices, feed conversion ratio, and upregulated the expression of key antioxidant genes (SOD, CAT) and the immune-related gene (IL-1β), suggesting enhanced nutrient utilization and immune function. Importantly, a dosage of 0.5 g/kg of the extract blend proved to be the most beneficial in this study. These findings underscore the potential of this natural extract combination as a sustainable and efficient feed additive, providing valuable insights for promoting fish health and optimizing aquaculture practices.

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