Sweet Potato Leaf Extract as a Protective Antioxidant: Improving Hematological Health in Ammonia-Exposed Mahseer Fish

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Abstract

The Mahseer fish (*Neolissochilus soroides*) is a highly valuable aquaculture species due to its economic potential. Currently, this species is being developed in intensive aquaculture systems. As a result, ammonia levels are increasing, which could potentially disrupt the growth and survival of the fish. This study aimed to investigate the effects of sweet potato (*Ipomoea batatas*) leaf extract supplementation as an antioxidant on the hematological profile of Mahseer fish exposed to NH_4Cl. Four supplementation treatments were employed: 0%, 2.5%, 5%, and 7.5%, each with five replicates. The fish were supplemented with the extract for 40 days and exposed 10 ppm NH_4Cl for 48 hours. Blood samples were collRected before rearing, after 40 days of supplementation and NH_4Cl exposure. The results demonstrated that supplementation with sweet potato leaf extract (SPLE) positively influenced the hematological profile of mahseer. Specifically, higher doses of the extract enhanced immunity across all treatments. Notably, only the highest doses of 7.5% and 5% effectively mitigated fish stress induced by ammonia exposure for 24 hours. Furthermore, there were no significant differences observed among treatments in response to NH_4Cl exposure throughout the study period. These findings underscore the potential of SPLE as an antioxidant supplement to bolster immune function and alleviate oxidative stress in mahseer under ammonia exposure conditions. Further research could focus on optimizing the dosage of SPLE to maximize its antioxidant benefits in aquaculture setting.

Keywords: aquaculture, immunity, Mahseer fish, NH_4Cl, sweet potato

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INTRODUCTION

The Mahseer (*Neolissochilus soroides*) is native to southern and Southeast Asia, including Indonesia, and has a fantastic price ranging from IDR 350,000 to IDR 1,500,000 per kilogram (Arief *et al*., 2021; Khaironizam *et al*., 2015; Putri *et al*., 2020; Subagja *et al*., 2021). In Indonesia, it inhabits rocky rivers and clear waters of Kalimantan, Sumatera, and Java (Jaafar *et al*., 2021). However, habitat degradation from human activities and overfishing has led to its endangered status (Risky *et al*., 2020). Several developments are underway on this species such as domestication and intensification of cultivation (Larashati *et al*., 2020; Muchlisin *et al*., 2021; Camargo and Alonso, 2006). However, a consequence of aquaculture intensification is increasing ammonia due to the build-up of organic matter, feed residues and fish metabolism (Chatvijitkul *et al*., 2017). Elevated levels of ammonia in the blood and tissues causes physiological disorders such as gill epithelium damage, reduced hemoglobin, inhibited ATP production, vascular disorders, and impaired osmoregulatory activity (Xu *et al*., 2021; Fikri *et al*., 2022). In addition, it increases reactive oxygen species (ROS), reduces hemoglobin-oxygen affinity, and disrupts blood electrolyte balance (Khan *et al*., 2013; Parvathy *et al*., 2023). Reduced hemoglobin-oxygen affinity leads to tissue hypoxia, affecting organs such as the kidney and disrupting hematopoiesis (Eissa *et al*., 2023). Untreated ammonia exposure can result in

mortality. Current practices to mitigate ammonia impacts include feed management, water quality maintenance, and bacterial use (Estaiano de Rezende *et al*., 2021). Internal strategies to minimize ammonia accumulation are also crucial to enhance fish resistance.

Previous studies have shown that supplementation of feed with phytobiotics can improve survival and tolerance of fish to stressors such as ammonia (Alam, 2021). Phytobiotic compounds are known to be antioxidant, anti-inflammatory, and immunomodulatory, as well as to increase fish resistance (Eissa *et al*., 2023; Salsabila *et al*., 2024). Sweet potato leaves (*Ipomoea batatas*) contain phenolic compounds that act as natural antioxidants, improving fish growth and immunity (Gulcin, 2020; Maqsood *et al*., 2013). Moreover, phenolic derivatives like flavonoids can eliminate ROS in vitro, while anthocyanins enhance immunity (Tomori *et al*., 2023). Sweet potato leaf extract (SPLE) has been shown to improve the hematological profile of tilapia exposed to ammonia, demonstrating its antioxidant effects in combating oxidative stress (Hendrik *et al*., 2021; Ulkhaq *et al*., 2024). This study aims to determine the effect of SPLE supplementation as herbal antioxidant in enhancing fish immunity and stress-reducing agents by Mahseer fish from ammonia exposure.

MATERIALS AND METHODS

Ethical Approval

The research adhered to ethical standards and received supervision and approval from the Faculty of Health, Medicine and Life Sciences, Universitas Airlangga, as specified in the Dean's letter 1627/B/UN3.FIKKIA/I/TD.06/2024.

Study Period and Location

This study was conducted from January to June 2024 at the Aquaculture Teaching Farm, Faculty of Health, Medicine, and Life Sciences, Universitas Airlangga. The study consisted of two stages: first, cultivation with feed supplementation for 40 days, followed by exposure to stressor materials for 24 hours consecutively over a period of 2 days.

Experimental Design

This study employed a completely randomized design (CRD) with four treatments and three replications. The treatments were as follows: (P1) the control group, where fish were reared and fed without supplementation of SPLE and exposed to 10 ppm NH_4Cl for 48 hours; (P2) which involved supplementation with 2.5% SPLE and exposure to 10 ppm NH_4Cl for 48 hours; (P3) where fish were supplemented with 5% SPLE and exposed to 10 ppm NH_4Cl for 48 hours; and (P4) which included supplementation with 7.5% SPLE and exposure to 10 ppm NH_4Cl for 48 hours. Each treatment was replicated five times to ensure robustness and reliability in the experimental results.

Materials Used

A total of 200 fish with each weight 28.66 ± 7.85 g and length 13.30 ± 1.34 cm, were sourced from the Fish and Environmental Health Laboratory in Pasuruan, East Java, for this study. SPL were obtained from farms in Banyuwangi and used to prepare extracts using 96% ethanol solvent, which were subsequently filtered with Whatman paper. Ammonium chloride (NH_4Cl) was used as the stressor material. The active compounds contained in SPL were phytochemically tested by spectrophotometric method to determine the amounts of active compounds that are antioxidant such as total flavonoids, total phenols and tannins (Table 1). Blood observation materials included Ethylene diamine tetra-acetic acid (EDTA), immersion oil, and Giemsa dye. Meanwhile Nessler reagent was used for measuring ammonia levels.

For fish rearing, 30 × 20 × 20 cm^3 aquariums equipped with aerators were utilized as containers. Equipment for preparing SPLE included a blender, digital scale, jar, spatula, Buchner bottle, Buchner funnel, vacuum pump, vacuum rotary evaporator, vial bottles, plastic wrap, and aluminium foil. Moreover tools for analyzing the hematological profile included 1 mL syringes, 1 mL microtubes, object glasses, cover glasses, a drop pipette, a binocular microscope (Olympus, Japan), and a hemocytometer. Water quality observation tools

consisted of sample bottles, a thermometer, a pH pen, a DO meter, and a spectrophotometer for measuring ammonia levels.

Preparation of Sweet Potato Leaf Extract

SPL extraction followed the method described by (Baldo *et al*., 2022), where 500 g of SPL powder was soaked in five liters of 70% ethanol solvent for 72 hours. The selection of 70% ethanol as the solvent aligns with findings from (Hamid *et al*., 2018; Nguyen *et al*., 2021; Purnama *et al*., 2021), which indicates that it yields the highest content of anthocyanins and flavonoids. After soaking, the mixture was filtered using Whatman paper no. 42. Subsequently, the filtrate was concentrated using a rotary evaporator at 45°C and 100 rpm. The resulting thick extract was then stored in a refrigerator at 4°C. Next, the extract was dissolved in 10 mL of ethanol and mixed with binder (Progol™, Indonesia) at a dose of 5 g/kg, then homogenized using a vortex. The dissolved extract was evenly sprayed onto the feed and air-dried before storage, following the procedure outlined by (Ariyanti *et al*., 2022).

Table 1. Qualitative phytochemical composition and levels of flavonoids, phenolic, tannins of purple sweet potato leaves

Parameters	Content	Test method
Total flavonoids	10.54 mg/g	
Total phenol	6.53 mg/g	Spectrophotometry
Total tannins	6.45 mg/g	

Ammonia Stressor Challenge Test

The ammonia stressor test followed the protocol outlined by (Li *et al*., 2019), adapting the dosage based on the Sub-lethal Concentration (LC_{50}) study previously conducted on *mahseer*. All fish were reared for 40 days, with some receiving supplementation of SPLE and others serving as controls without supplementation. After this period, all fish were exposed to 10 ppm of ammonium chloride (NH_4Cl) for 48 hours.

Blood Sampling and Analysis

Blood sampling in this study followed a methodology adapted from (Li *et al*., 2019), with specific modifications. Samples were collected at three critical time points: initially at the start of the experimental period, after administering the SPLE, and following exposure to ammonia. From each treatment group, 10% of the fish population was selected for sampling. The blood was obtained via caudal vein using a 1 mL syringe and rinsed with EDTA as an anticoagulant. Each sample was carefully transferred into microtubes for quantification, while additional samples were used to prepare blood smears for the detail examination of granulocyte types. This approach ensured comprehensive assessment of hematological profiles throughout the study, providing valuable insights into how the fish responded physiologically to extract supplementation and ammonia stress.

Observed Parameters

Parameter calculations of erythrocytes, hemoglobin, hematocrit, total leukocytes, and platelet were performed manually. The hemoglobin level was performed by Sahli's hemoglobinometer. Hematocrit level was measured using microhematocrit tubes and centrifuged at 3500 rpm for 15 minutes then read using a hematocrit reader. Erythrocytes, leukocytes, and platelet were counted using a hemocytometer accordance (Witeska *et al*., 2016).

$$\text{Erythrocytes} = \frac{\text{A}}{\text{N}} \times \frac{1}{\text{V}} \times \text{fp} \quad \text{.....(1)}$$

$$\text{Leukocytes} = \frac{\text{A}}{\text{N}} \times \frac{1}{\text{V}} \times \text{fp} \quad \text{.....(2)}$$

$$\text{Platelet} = \frac{A \times \text{P}}{\text{V}} \quad \text{.....(3)}$$

Description:
A = Counted cell number
N = Number of observed hemocytometer box
V = Volume of observed hemocytometer box
Fp = Dilution factor

Data Analysis

The erythrocytes, hemoglobin, hematocrit, and platelet data were obtained and analyzed using a one-way ANOVA test to determine the effect of SPLE supplementation as an immunostimulant on *N soroides*. Furthermore, to determine the best dose, a Duncan test ($p < 0.05$) was conducted. Both analyses were also performed on all blood parameters after exposure to NH_4CL for 24 consecutive hours over 2 days.

RESULTS AND DISCUSSION

Effects of SPLE on Erythrocytes, Leukocytes and Platelet

Sweet potato leaf extract (SPLE) supplementation had a positive effect on the hematological profile of Mahseer fish. SPLE supplementation doses of 5% (P3) and 7.5% (P4) significantly increased erythrocyte count, hematocrit, and hemoglobin levels after 40 days of rearing compared to the P1. Whereas 5% of SPLE supplementation produced the best values for erythrocytes and hematocrit, recorded at 1.77 ± 0.035 and 32.8 ± 0.764, respectively. Meanwhile, the best hemoglobin increase was found in the treatment with a dose of 7.5% at 6.50 ± 0.23 (Table 2). This shows that the content of bioactive compounds such as flavonoids can stimulate the production of erythropoietin in the kidney (Aini *et al*., 2022). Erythropoietin plays a crucial role in enhancing the production of myeloid-erythroid cells in the bone marrow, significantly boosting the production of erythrocytes (Gabriel and Idu, 2021; Syahbirin *et al*., 2024). In addition, flavonoid content functions as an anti-stress by reducing free radicals that cause oxidative stress (de Rezende *et al*., 2021). Oxidative stress in fish can reduce the self-renewal capacity of hematopoietic stem cells (HSCs) (Samimi *et al*., 2018). The enhancement of erythrocyte and hemoglobin levels are important for maintaining fish health (Nikinmaa *et al*., 2019; Rani *et al*., 2022). This finding aligns with a report from Baldo *et al*. (2022) that the administration of sweet potato leaf extract increased the erythrocyte, hematocrit, and hemoglobin values of Tilapia. Furthermore, supplementation of sweet potato was also proven to increase linear erythrocyte counts with increasing supplementation dose in healthy fish (Baleta *et al*., 2022). Besides that, SPLE supplementation for 40 days of rearing also increased the number of Mahseer fish leukocytes. Whereas a significant increase ($p < 0.05$) was obtained at the highest dose with a value of 96.53 ± 2.81. Sweet potato leaf extract contains flavonoids and is an immunostimulant (Hutagaol *et al*., 2022). These compounds can activate cellular defense cells by increasing macrophages, granulocytes, T and B cell, and lymphocytes (Rosmawaty *et al*., 2016; Safira *et al*., 2022). In addition, flavonoids and other antioxidant components in SPLE have been shown to enhance immunological responses by increasing lymphocyte proliferation and improving humoral and cellular immunity (Venkatalakshmi *et al*., 2016; Osuntokun *et al*., 2020; Ayeleso *et al*., 2017). An increase in leukocyte count is a good indicator of improved immunological function, especially in fish that are frequently exposed to various stressors, including ammonia.

The Effects of Ammonia Exposure on Erythrocytes and Leukocytes Parameters

The erythrocyte, hematocrit, and hemoglobin profiles of Mahseer fish were impacted by a 48-hour exposure to NH_4Cl. Furthermore, the fish's capacity to protect themselves from exposure depended on the application dose. In contrast to the control (P1), the highest doses of 7.5% and 5% were able to preserve distinct erythrocyte, hematocrit and hemoglobin levels. Because the SPLE's ability to lessen the harmful effects of ammonia exposure declined over time, these values ultimately did not survive more than 24 hours. After 48 hours of exposure to NH_4Cl, it was clear that none of the treatments differed significantly ($p > 0.05$). This suggests that fish can only be temporarily protected from ammonia exposure by SPLE supplementation. Phenolic and flavonoid components in SPLE are known for their antioxidant activities, which help prevent oxidative damage (Maqsood *et al*., 2013), as well as their ability to provide a balance between ROS

production and clearance under normal physiological settings. The ammonia accumulation over time can exacerbate toxicity, destroying the defense system against free radicals and increasing the quantity of hydrogen peroxide and singlet oxygen (Yan *et al*., 2021; Hardiansyah and Lamid, 2022).

Table 2. Red blood profile of Mahseer fish fed with supplemented diets containing sweet potato leaf extract and exposed to NH_4Cl

Parameters	Treatment	Time period			
		H-0	H-40	H-41	H-42
Erythrocytes ($\times 10^6$ mm^3)	P1	1.31 ± 0.04^a	1.39 ± 0.055^a	1.46 ± 0.089^a	1.59 ± 0.012^a
	P2	1.31 ± 0.04^a	1.41 ± 0.119^a	1.50 ± 0.042^a	1.56 ± 0.053^a
	P3	1.31 ± 0.04^a	1.77 ± 0.035^b	1.71 ± 0.146^b	1.75 ± 0.060^a
	P4	1.31 ± 0.04^a	1.71 ± 0.136^b	1.73 ± 0.126^b	1.69 ± 0.163^a
Hematocrit (%)	P1	25.7 ± 1.155^a	28.8 ± 0.764^a	30.0 ± 1.000^a	30.8 ± 0.289^a
	P2	25.7 ± 1.155^a	28.3 ± 0.577^a	31.0 ± 1.323^{ab}	31.0 ± 1.323^a
	P3	25.7 ± 1.155^a	32.8 ± 0.764^b	33.7 ± 1.607^b	32.8 ± 1.893^a
	P4	25.7 ± 1.155^a	32.0 ± 1.000^b	34.0 ± 1.000^b	33.3 ± 1.528^a
Hemoglobin (g/dL)	P1	4.30 ± 0.462^a	5.10 ± 0.306^a	5.40 ± 0.000^a	5.40 ± 0.200^a
	P2	4.30 ± 0.462^a	5.20 ± 0.400^a	5.90 ± 0.115^b	5.60 ± 0.400^a
	P3	4.30 ± 0.462^a	6.10 ± 0.416^b	6.50 ± 0.115^c	6.00 ± 0.400^a
	P4	4.30 ± 0.462^a	6.50 ± 0.231^b	6.30 ± 0.416^c	6.20 ± 0.529^a

Fish rearing began on H-0, followed by the administration of SPLE until H-40. Subsequently, on H-41 and H-42, the cells were exposed to NH_4Cl for 24 and 48 hours, respectively. (P1) The control group, where fish were raised and fed without SPLE supplementation and exposed to 10 ppm NH_4Cl. (P2) Supplementation with 2.5% SPLE and exposure to 10 ppm NH_4Cl for 48 hours. (P3) Supplementation with 5% SPLE and exposure to 10 ppm NH_4Cl for 48 hours. (P4) Supplementation with 7.5% SPLE and exposure to 10 ppm NH_4Cl for 48 hours. Significant differences are indicated by superscript letters following the mean values (n = 3) and standard errors in the same row ($p < 0.05$).

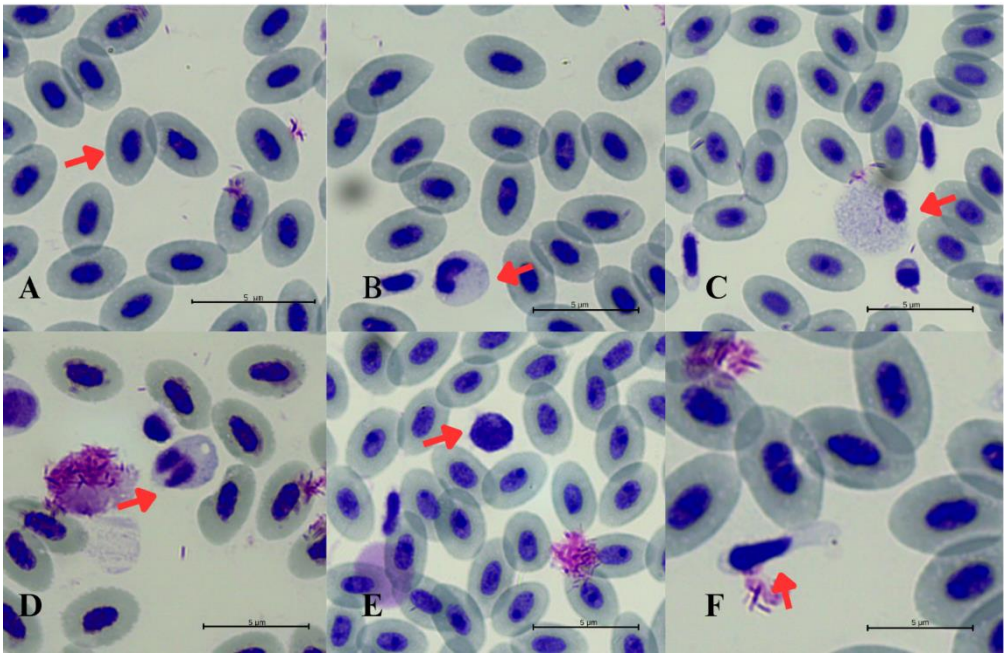

Figure 1. Blood cells of Mahseer fish. Red arrows indicate (A) Erythrocytes, (B) Monocytes, (C) Eosinophils, (D) Neutrophils, (E) Lymphocytes, (F) Platelet.

Table 3. Platelet and leukocyte component of Mahseer fish fed with supplemented diets containing sweet potato leaf extract and exposed to NH_4Cl

Parameters	Treatment	Time period			
		H-0	H-40	H-41	H-42
Platelet ($\times 10^3$ mm^3)	P1	28.67 ± 2.309^a	40.67 ± 6.429^a	36.67 ± 1.155^a	31.33 ± 4.163^a
	P2	28.67 ± 2.309^a	42.00 ± 2.000^a	37.33 ± 5.657^a	34.67 ± 3.487^a
	P3	28.67 ± 2.309^a	42.67 ± 5.774^a	42.00 ± 4.000^a	40.67 ± 2.444^b
	P4	28.67 ± 2.309^a	42.67 ± 4.619^a	44.00 ± 4.000^a	40.00 ± 2.000^b
Leukocytes ($\times 10^3$ mm^3)	P1	76.00 ± 2.650^a	85.07 ± 3.607^a	81.60 ± 5.769^a	65.87 ± 14.584^a
	P2	76.00 ± 2.650^a	84.00 ± 4.157^a	76.53 ± 9.938^a	78.40 ± 19.637^{ab}
	P3	76.00 ± 2.650^a	87.73 ± 4.406^a	84.53 ± 10.653^a	97.87 ± 25.778^{bc}
	P4	76.00 ± 2.650^a	96.53 ± 96.53^b	101.60 ± 4.000^b	111.47 ± 18.319^c
Lymphocytes (%)	P1	65.67 ± 0.707^a	66.33 ± 3.055^a	64.00 ± 2.646^a	62.00 ± 6.083^a
	P2	65.67 ± 0.707^a	66.00 ± 2.646^a	65.67 ± 0.577^a	63.00 ± 4.583^a
	P3	65.67 ± 0.707^a	67.67 ± 2.517^a	66.67 ± 1.155^a	64.00 ± 1.000^a
	P4	65.67 ± 0.707^a	68.00 ± 2.646^a	67.67 ± 2.082^a	66.67 ± 1.528^a
Monocytes (%)	P1	5.67 ± 1.155^a	6.33 ± 1.528^a	6.67 ± 2.517^a	7.00 ± 1.000^a
	P2	5.67 ± 1.155^a	5.33 ± 1.528^a	5.67 ± 2.309^a	6.67 ± 2.887^a
	P3	5.67 ± 1.155^a	6.00 ± 1.000^a	5.67 ± 0.577^a	7.00 ± 1.000^a
	P4	5.67 ± 1.155^a	5.67 ± 1.155^a	5.00 ± 1.000^a	6.00 ± 1.732^a
Granulocytes (%)	P1	28.67 ± 2.517^a	27.33 ± 2.082^a	29.33 ± 2.082^a	31.00 ± 7.000^a
	P2	28.67 ± 2.517^a	28.67 ± 3.786^a	28.67 ± 2.082^a	30.33 ± 2.082^a
	P3	28.67 ± 2.517^a	26.33 ± 1.528^a	27.67 ± 1.528^a	29.00 ± 1.000^a
	P4	28.67 ± 2.517^a	25.33 ± 2.082^a	27.33 ± 2.887^a	27.33 ± 2.517^a

Fish rearing began on H-0, followed by the administration of SPLE until H-40. Subsequently, on H-41 and H-42, the cells were exposed to NH_4Cl for 24 and 48 hours, respectively. (P1) The control group, where fish were raised and fed without SPLE supplementation and exposed to 10 ppm NH_4Cl. (P2) Supplementation with 2.5% SPLE and exposure to 10 ppm NH_4Cl for 48 hours. (P3) Supplementation with 5% SPLE and exposure to 10 ppm NH_4Cl for 48 hours. (P4) Supplementation with 7.5% SPLE and exposure to 10 ppm NH_4Cl for 48 hours. Significant differences are indicated by superscript letters following the mean values (n = 3) and standard errors in the same row ($p < 0.05$).

However, SPLE at 5% (P3) and 7.5% (P4) in Mahseer fish led to much higher levels of white blood cells after 48 hours of exposure to NH_4Cl ($p < 0.05$) (Table 3). The reduction of leukocytes by less than 5% in the supplementation group occurred because leukocyte cells migrated from the bloodstream to the injured tissue to eliminate the underlying inflammatory trigger or to aid in tissue repair (Nourshargh and Alon, 2014; Fikri *et al*., 2023). Tissues under stress or with dead cells release damage-associated molecular patterns (DAMPs), which trigger leukocyte migration (Medzhitov, 2008). Despite the considerable rise in leukocyte counts, there was no significant ($p > 0.05$) change in leukocyte differentials across treatment groups. This shows that, whereas SPLE stimulate overall immunological activity, their effects on individual immune system components may be less significant (Figure 1).

Although the values of red blood cell and white blood cell profiles increased significantly after 40 days of maintenance, there was no significant difference in platelet values across treatments. Significant alterations were only detected after 48 hours of NH_4Cl exposure. Greater doses of SPLE, 5% (P3) and 7.5% (P4), resulted in significantly greater platelet counts than the control group (P1). The decline in platelets in the control group (P1) is thought to be mediated by platelet cell migration to cells or

tissues that are bleeding (Correa *et al*., 2017; Safira *et al*., 2023). Ammonia can harm fish in a variety of ways, including hemorrhaging in the gills (Rahmati *et al*., 2022). This suggests that Mahseer fish in the P1 sustained higher tissue injury. The reduction in damage at SPLE supplementation dosages of 5% (P3) and 7.5% (P4) could be attributed to the anthocyanin content, which can improve fish lifespan and stress tolerance when exposed to ammonia (Yilmaz, 2019).

CONCLUSION

SPLE supplementation can enhance the hematological profile and immune function of mahseer under ammonia exposure. The doses of 5% and 7.5% SPLE was most effective in improving hematological parameters and mitigating stress effects. These findings suggest that SPLE supplementation could be a valuable strategy for enhancing fish health and resilience in aquaculture systems. Further research is warranted to optimize the dosage and application methods of SPLE in different aquaculture settings.

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AUTHORS' CONTRIBUTIONS

The all author had contributions of this manuscript as follows: SFA collected data, drafted the manuscript, and designed tables and graphs. SCY and DSB designed the main conceptual ideas and made critical revisions to the article. HKJ and AI proofread the manuscript. All authors discussed the results and contributed to the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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