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Research article

Effects of *Peperomia pellucida* (L.) Kunth on the growth performances, and health of red hybrid tilapia (*Oreochromis* spp.)

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Abstract

Peperomia pellucida (PP), is an herb that has been proven rich in antioxidant property. However, the beneficial effects and mode of action of PP on disease resistance in fish are still unclear. In the present study, we revealed the potential impacts of PP on the growth performance, blood parameters, antioxidant capacity, immune-related gene expression and disease resistance against *Aeromonas hydrophila* in red hybrid tilapia, *Oreochromis* spp. Experimental fish were given a diet containing different doses of PP (100, 200 and 300 mg/kg diet) in triplicates for eight weeks. After feeding trials, the experimental fish were subjected to an intraperitoneal infection of *A. hydrophila*. Findings from the present study showed a significant ($P < 0.05$) improvement in growth performances (final weight, specific growth rate, and feed conversion rate), immune-related gene expression and disease resistance against *A. hydrophila* in fish fed PP supplemented diets in comparison to the control diet. Present study also demonstrated significant ($P < 0.05$) improvement in blood parameters by dietary PP provision. Dietary PP noticeably enhanced the antioxidant status of red hybrid tilapia with the increased activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) in the liver. On top of that, upregulation of growth and immune-related genes such as Lysozyme G (LysG), nuclear factor kappa B (NF-κB), transforming growth factor-β1 (TGF-β1), heat shock protein 90 (HSP90), and beta actin (β-actin) were also observed in the dietary PP group. Overall, polynomial regression analysis revealed that dietary PP at the dosage of 108 – 151 mg/kg improved growth and health performances of red hybrid tilapia.

Keywords: Antioxidant capacity, Disease resistance, Gene expression, Growth performance, *Peperomia pellucida* (L.) Kunth, Red hybrid tilapia

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INTRODUCTION

Tilapia is the second most important farmed fish in the world, after carp. The taste and texture of this aquaculture species is well-accepted and favourable especially in Asian cuisine. Tilapia survive under tough environment, breed easily, consume a wide range of diets, and withstand stress due to high stocking density, low dissolved oxygen and other environmental factors (Abdel-Tawwab et al., 2014; Shi et al., 2020). These characteristics account for the commercial culture of tilapia over a hundred countries. The total world production of tilapia in 2018 almost doubled compared to 2010 (FAO, 2020). Fish is an affordable protein source to human which can be harvested from natural waterbodies such as lake, river and open sea. However, supplies of fish from the wild has no longer feasible to cater for the market demand as the human population grows. As an alternative, aquaculture is gearing towards intensification with high stocking density of aquaculture species in an aquaculture system. Under intensive aquaculture, water quality is readily impacted by the unconsumed fish feed and waste, as well as aquaculture species may become stressed and prone to disease infections (Van Doan et al., 2017).

Use of antibiotics could be effective to control disease infections in aquaculture species, however, over- and misuse of antibiotics can lead to adverse effects on environment and public health in long term. One of the impacts is the emergence of antibiotic resistance cases in aquaculture sites (Suyamud et al., 2024). The excessive increase of antibiotic resistance cases among pathogenic bacteria is alarming in worldwide (Church and McKillip, 2021). In addition, antibiotic residues in the aquaculture species tissue may induce allergic effects and development of antimicrobial resistance in humans, thus exert influence on the confidence of consumer to acquire aquaculture products (Hua et al., 2022). Alternatively, vaccination is adopted by fish farmers to control disease infections in aquaculture (Harikrishnan et al., 2011). By developing fish immunity against pathogens, vaccination has been widely accepted as a reliable way to prevent disease infections such as Motile Aeromonas Septicemia (MAS), vibriosis, streptococcosis, and a number of viral diseases caused by the rhabdovirus (Collins et al., 2019; Irshath et al., 2023). However, vaccination program is expensive and needs intensive labour work (Sakai, 1999). As a result, increasing attention has been paid to the application of phytobiotics as prophylactic agent in aquaculture species via dietary supplementation, which is considerably practical on a large scale. Phytobiotics are referring to any plant-based products, compounds, secondary metabolites or in any forms that can contribute to the growth and health of organisms, including the preparation using extracts from seed, fruit, root, leaf or whole plant. The preparation of phytobiotics produce bioactive compounds such as essential oils, polysaccharides, flavonoids, alkaloids and etc. (Terzi et al., 2021). According to (Skoufos et al., 2020), phytobiotics may possess properties such as immune enhancer, anti-inflammatory, antioxidant, antibacterial, and anti-parasitic. Therefore, phytobiotics have been applied in aquaculture for disease control, as well as to improve growth performance, enhance immune system, increase antioxidant capacity and stimulate disease resistance of aquaculture species (Anirudhan et al., 2021). For instance, a recent study revealed that dietary supplementation with *Curcuma longa* leaf enhanced the growth and health of African catfish (Wei et al., 2024).

Peperomia pellucida (L.) Kunth is a weed that most widely found in tropical and subtropical regions such as Africa, Australia, and Southeast Asia, including Malaysia (Raghavendra and Prashith Kekuda, 2018; Alves et al., 2019). It is known as shiny bush and pepper elder in North America (Oloyede et al., 2011). In some countries, *P. pellucida* is planted commercially for nutraceutical and cosmeceutical products (Alves et al., 2019). This weed with heart–shaped leaves were reported to possess many medicinal values such as anti-hypertension, lowering blood cholesterol, anti-inflammatory, antioxidant, anticancer, and antimicrobial (Aziba et

al., 2001; de Fátima Arrigoni-Blank et al., 2004). Bioactive compounds such as alkaloids, tannins, anthraquinones, cardiac glycosides, and flavonoids in the herbs are responsible to the medicinal values of the weed (Egwuche et al., 2011; Raghavendra and Prashith Kekuda, 2018). However, the impact of this weed to aquatic animal is still lacking. Therefore, investigation on the beneficial effects of *P. pellucida* on the growth and health of farmed red hybrid tilapia (*Oreochromis* spp.) was carried out in the present study through feeding trial, which involved growth performance analysis, digestive enzyme activity, antioxidant capacity activity, immune-related genes, and disease resistance against *A. hydrophila* infection in red hybrid tilapia.

MATERIALS AND METHODS

Ethics statements

This work was approved by the Animal Ethical Committee of Universiti Malaysia Kelantan (UMK) and experiments conducted were in compliance with UMK ethical protocol and guidelines for experimental animals. The work has been registered under research code: UMK/FIAT/ACUE/PG/2023/2.

Experimental design

A total of 500 red hybrid tilapia fingerlings was purchased from a tilapia commercial farm located at Tanah Merah, Kelantan, Malaysia. The fingerlings were kept in the Aquaculture Laboratory of UMK for 14 days before project commencement. During the acclimatization period, fingerlings were given commercial pellet (Star Feed, Malaysia) with 32% crude protein. After the acclimatization period, only healthy fingerlings with an average initial weight of 5.2 g were selected for further use. Thirty fish were kept in each 50 L aquarium equipped with aeration. Formulated feeds were given at 2% based on body weight to the experimental fish twice per day in the morning. Total feed intake was recorded daily whereas total weight gain was weighed weekly for eight consecutive weeks. Each treatment was represented by three replicates. During the experiment, water parameters were maintained at the following range: pH 6.6 to 7.5, temperature 24 to 26 °C, dissolved oxygen 6.2 to 7.3 ppm and ammonia <0.05 ppm.

Preparation of formulated feed

Whole plant of PP was air-dried and powdered using a blender (Panasonic, Malaysia). The powdered PP was then kept in -20°C for further use. Isonitrogenous and isolipidic diets were formulated with the addition of powdered PP at 100 mg/kg, 200 mg/kg and 300 mg/kg, respectively (Table 1). All ingredients were mixed homogenously and water was added gradually until a dough was formed. The dough was passed through meat grinder to produce pellets with a diameter of 1.5 mm. The produced feed pellets were oven-dried at 60 °C overnight and subjected to proximate analysis. Finally, the pellets were kept in a freezer at -20 °C. The proximate composition analysis of the experimental fish at the end of experiment was shown in Table 2.

Table 1 Diet formulation in the present study

Ingredients	Diet formulation (%)			
	Control	PP1	PP2	PP3
Soybean meal	22	22	22	22
Fish meal	50	50	50	50
Wheat bran	17	16.99	16.98	16.97
Premix*	2	2	2	2
Fish oil	3	3	3	3
Vegetable oil	3	3	3	3
Carboxymethyl cellulose (CMC) binder	3	3	3	3
Peperomia pellucida (PP) powder	0	0.01	0.02	0.03
Total	100	100	100	100
Nutritional profiles				
Carbohydrate	45.2	44.8	45.8	44.6
Protein	31.4	30.8	32.1	31.8
Ash	6.6	5.9	6.3	6.4
Lipid	6.4	6.8	6.2	7.1
Fiber	3.8	4.2	4.8	4.2
Moisture	6.6	7.5	4.8	5.9

PP1 = *P. pellucida* powder 100 mg/kg; PP2 = *P. pellucida* powder 200 mg/kg; PP3 = *P. pellucida* powder 300 mg/kg
*Premix (Aquavita, Indonesia) contains Vitamin A, D_3, E, K, B_1, B_2, B_{12}, C, calcium pantothenate, folic acid, lactose acid, biotin, amino acids, inositol, manganese sulphate, copper sulphate and cobalt chloride.

Table 2 Proximate composition analysis of experimental fish

Parameters	Proximate composition profile (%)			
	Control	PP 1	PP 2	PP 3
Crude protein	31.4 ± 0.27^{b}	30.8 ± 0.28^{c}	32.1 ± 0.76^{a}	31.8 ± 0.58^{a}
Ash	2.6 ± 0.03^{b}	2.7 ± 0.02^{a}	2.5 ± 0.02^{c}	2.7 ± 0.02^{a}
Lipid	2.6 ± 0.01^{b}	2.7 ± 0.01^{a}	2.7 ± 0.01^{a}	2.5 ± 0.01^{c}

Data expressed as mean ± SD.
Different superscripts in the same row indicates significant difference ($P < 0.05$).
PP1 = *P. pellucida* powder 100 mg/kg; PP2 = *P. pellucida* powder 200 mg/kg; PP3 = *P. pellucida* powder 300 mg/kg

Determination of growth performance

Experimental fish were sampled randomly on weekly basis. After eight weeks, growth performance parameters of the experimental fish were determined using the following formula:

Total weight gain = Final body weight - Initial body weight

Weight gain (WG, %) = (Total weight gain / Initial body weight) × 100

Specific growth rate (SGR, %) = (Total weight gain / Experimental days) × 100

Hepatosomatic index (HSI) = Total liver weight / Total body weight

Viscerosomatic index (VSI) = Total viscera weight / Total body weight

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

Proximate composition analysis

At the end of feeding experiment, fish carcasses from each treatment and the experimental diets were subjected to proximate composition analysis. Moisture content of the samples was determined by oven-drying the samples at 105 °C for two consecutive hours until constant weight was obtained. The crude protein (CP) and crude lipid (CL) values were determined by Kjeldahl and Soxhlet extraction methods, respectively (Wang et al., 2020; Li et al., 2022).

Amino acid profiling of formulated diets

Formulated diets supplemented with PP powder in the present study were analysed for amino acid profiles. The samples were hydrolysed using 6 N HCL at 110 °C for 24 h. Subsequently, the samples were subjected to High Performance Liquid Chromatography (HPLC) system for amino acid quantification in triplicates. In the quantification process, α–aminobutyric acid (AABA) was used as an internal reference. Finally, the obtained database was analysed using BreezeTM software (Kari et al., 2022). Table 3 showed the amino acid profiling of the formulated feeds. All diets were formulated to meet the nutrition requirement for Nile tilapia fish (do Nascimento et al., 2020). In this study, heuristic thresholds were developed as a practical tool to guide field-

Table 3 Amino acid profiles (%) of formulated diets is expressed in percentage of protein fed to *Oreochromis* spp. in the present study and the requirements for *O. niloticus* (do Nascimento et al., 2020). Data expressed as mean ± standard deviation

Amino acids	Mean ± Standard deviation (%)			
	Control	PP1	PP2	PP3
Arginine	7.68±0.01	7.53±0.02	7.42±0.01	7.32±0.01
Lysine	6.58±0.01	6.62±0.01	6.54±0.01	6.55±0.01
Threonine	6.43±0.01	6.52±0.01	6.08±0.01	6.32±0.01
Histidine	1.83±0.01	1.82±0.01	1.80±0.01	1.78±0.01
Isoleucine	3.43±0.01	4.52±0.01	3.88±0.01	3.51±0.01
Leucine	5.12±0.01	5.32±0.01	5.31±0.01	5.01±0.01
Methionine	2.43±0.01	2.71±0.01	2.56±0.01	2.43±0.01
Phenylalanine	4.42±0.01	5.11±0.01	4.89±0.01	4.33±0.01
Valine	4.81±0.01	4.79±0.01	3.89±0.01	4.23±0.01
Tryptophan	2.43±0.01	2.12±0.01	2.09±0.01	2.11±0.01

PP1 = *P. pellucida* powder 100 mg/kg; PP2 = *P. pellucida* powder 200 mg/kg; PP3 = *P. pellucida* powder 300 mg/kg

Fish Euthanasia Procedure

At the end of the trial, three fish (n = 3) were randomly selected from each replicate for analysis after a 24-hour fasting period. To minimize stress and ensure compliance with ethical standards, fish were first anaesthetized using clove oil at a concentration of 100 mg/L until opercular movement ceased and no response to external stimuli was observed. Euthanasia was then carried out by spinal severance, in accordance with the guidelines approved by the institutional animal ethics committee.

Hematology analysis

Fish blood was withdrawn and kept in heparin tube for blood count test and other blood parameters analysis using automated hematology analyzer (Mythic 18 Vet, USA). Blood serum samples were subjected to blood biochemical test by using VetTest analyzer (IDEXX, USA) whereas globulin levels were determined by subtraction of albumin from total protein results (Wei et al., 2024).

Antioxidant enzyme activities

At the end of feeding experiment, livers of the experiment fish (n = 3) were sampled for antioxidant enzyme activities characterization. The livers were homogenised in cold physiological saline water and centrifuged at 10000 rpm for 10 min. The supernatants were used for colorimetric detection for catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) at 280 nm using biophotometer (BioRad, USA) with commercial detection kit (Escienceslab, USA) (Wei et al., 2024).

Histological examination

For histological examination, three experimental fish were sampled and dissected to obtain their liver after the feeding experiment. Liver samples were fixed in 10% neutral-buffered formalin solution and replaced after 24 h. The liver samples were then subjected to sectioning at 1 mm thickness followed by dehydration via a series of ethanol concentrations. The samples were cleared using xylene and embedded in paraffin blocks. Transverse sections of each sample were prepared and mounted on glass slide for overnight drying at 40 °C. In the following day, hematoxylin and eosin was used to stain the samples. The samples were observed under light microscope (Olympus BX43, Malaysia) and micrographs were captured using CellSens imaging software. Finally, the liver samples were examined for any abnormality. The liver abnormality was determined if fatty liver change in the cells was observed (Abdul Kari et al., 2021).

Gene expression of growth and immune regulatory genes

RNA extraction

At the end of the feeding trial, three experimental fish were sampled for their head kidney and distal intestine after 24 h fasting. Both organs are known to be the best tissues for growth and immune response analysis. DNase/RNase wiper together with 70% ethanol were used in sterilization work. Dissection works was performed under temperature around 20 °C by using ice block. A total of 100 mg of each kidney and intestine samples was obtained and kept in NucleoProtect RNA solution (Bioanalysis, Germany). The tissues were stored at -80 °C until further analysis. RNA from kidney and intestine samples were extracted using RNA extraction kit (iNtRON Biotechnology, USA). Then, the RNA samples were subjected to purity analysis using NanoPhotometer (Implen, USA).

Quantitative real-time PCR (qPCR) analysis

Quantitative real-time PCR was used to measure growth and immune gene expressions. β-actin was used as housekeeping and reference gene. Growth and immune-related genes that were investigated including Lysozyme G (LysG), nuclear factor kappa B (NF-κB), transforming growth factor-β1 (TGF-β1), heat shock protein 90 (HSP90), and beta actin (β-actin). All the primers were designed using online Primer3 software and sequences were confirmed with GenBank and NCBI BLAST software (Table 4). qPCR reactions were carried out using LightCycler 480 (Roche) with SYBR Green (Qiagen) to quantify mRNA levels by following procedures described by (Kari et al., 2022). The gene expressions were presented as a relative expression in comparison with control group. The obtained data were measured and calculated as described in the study of (Schmittgen and Livak, 2008).

Aeromonas hydrophila challenge

At the end of the feeding trial, 10 experimental fish from each treatment were sampled randomly and subjected to *A. hydrophila* challenge trial. The experimental fish were exposed to the bacteria through intraperitoneal injection at 1×10^8 cfu/mL (Wei et al., 2024). The survival rate of bacteria infected experimental fish was monitored for four weeks and calculated as follows:

Survival rate (%) = (Total number of survived fish / Total number of infected fish) × 100

Table 4 The primers sequences used in present gene expression study

Primer pair		Gene sequence	Gene accession number
LYZg1	Forward	GAAGACTGACAGTGAGAGAATG	FR872377
	Reverse	TGGACTCTCTGGAGATGATG	OM249765
NF-κβ	Forward	CCTAAATATCGGGACCAGAAC	KY613788
	Reverse	CTGTGGATGGTAGGTGAAAG	XM_026225073
TGF-β1	Forward	TCCAGCAAGCTCAGAATAAC	NM_182873
	Reverse	GGGATTCTTGATCCGAAGAC	
HSP90a	Forward	CATCACAGGTGAGACCAAAG	DQ662233
	Reverse	CCAGGTTCTTGCCATCATAC	MN262637
β-actin	Forward	GCGTGACATCAAGGAGAAG	LC770391
	Reverse	CAAGACTCCATACCCAAGAAAG	HM768299

Lysozyme G (LyZ $g_{1)}$, nuclear factor kappa B (NF-κB), transforming growth factor-$β_1$ ($TGF-β_{1)}$, heat shock protein $_{90}$ ($HSP_{90)}$, and beta actin (β-actin)

Statistical analysis

All experimental data were subjected for normality before analysis using SPSS software 20.1. One–way analysis of variance (ANOVA) test was used to determine significant differences among the treatment and control groups in the present study ($P < 0.05$). Grouping of the differences was done by using Tukey's Post Hoc test at $P < 0.05$. All data were presented in mean ± standard deviation (SD). The best PP dose was determined through polynomial regression analysis.

RESULTS

Growth performances experimental fish

In the present study, growth performance results were significantly different in terms of FW, WG, SGR, HSI, VSI, and FCR (Table 5). The highest FW, WG, SGR, and HSI were recorded in treatment PP1. Experimental fish from treatment PP1 also presented significantly lower ($P < 0.05$) FCR whereas treatment PP2 exhibited the lowest VSI. Overall, experimental fish received dietary PP showed better growth performance compared to the control group. No mortality of experimental fish was recorded during the feeding trial.

Table 5 Growth performance parameters of experimental fish feeding trial

Parameters	Control	PP1	PP2	PP3
Initial weight, IW (g)	5.17±0.06	5.27±0.12	5.2±0.10	5.13±0.06
Final weight, FW (g)	$34.67±0.513^{c}$	$38.83±0.404^{a}$	$36.27±0.404^{b}$	$35.07±0.153^{c}$
Weight gain, WG (%)	$571.0±13.73^{d}$	$652.0±23.94^{a}$	$597.7±21.17^{b}$	$583.2±5.74^{c}$
Specific growth rate, SGR (%)	$1.48±0.016^{c}$	$1.56±0.025^{a}$	$1.51±0.023^{b}$	$1.49±0.007^{c}$
Viscerosomatic index, VSI	$4.52±0.105^{a}$	$4.29±0.109^{b}$	$4.14±0.005^{c}$	$4.47±0.147^{a}$
Hepatosomatic index, HSI	$3.36±0.121^{b}$	$3.43±0.147^{a}$	$3.40±0.191^{a}$	$3.42±0.290^{a}$
Feed conversion ratio, FCR	$1.36±0.025^{a}$	$1.19±0.018^{c}$	$1.29±0.021^{b}$	$1.34±0.005^{a}$

Data expressed as mean ± SD.
Different superscripts in the same row indicates significant difference ($P < 0.05$).
PP1 = *P. pellucida* powder 100 mg/kg; PP2 = *P. pellucida* powder 200 mg/kg; PP3 = *P. pellucida* powder 300 mg/kg

Hematology analysis

Blood parameters of experimental fish fed were presented in Table 6. The white blood cell (WBC), hemoglobin (HGB), platelet (PLT), and mean corpuscular hemoglobin concentration (MCHC) values of the experimental fish received dietary PP were significantly higher ($p < 0.05$) than the control group. In particular, treatment PP1 recorded the highest values of WBC, HGB, PLT and MCHC compared to other treatments that received PP diet. On the other hand, experimental fish received dietary PP generally exhibited significantly lower ($p <$

0.05) mean corpuscular volume (MCV) value, where the lowest MCV value was recorded by treatment PP1. Other blood parameters such as lymphocyte (LYM), monocyte (MON), red blood cell (RBC), and mean corpuscular hemoglobin (MCH) were no significant difference among the treatments in the present study.

Table 6 Blood parameters of experimental fish

Blood parameters	Control	PP1	PP2	PP3
WBC/ µL	5.8±0.06^{a}	7.27±0.15^{b}	7.17±0.25^{c}	6.97±0.21^{c}
LYM (%)	90.3±0.92^{a}	90.13±0.21^{a}	90.43±0.35^{a}	90.13±0.45^{a}
MON (%)	1.55±0.02^{a}	1.55±0.01^{a}	1.53±0.02^{a}	1.54±0.03^{a}
RBC/ µL	1.33±0.06^{a}	1.37±0.06^{a}	1.37±0.06^{a}	1.37±0.06^{a}
HGB (g/dl)	7.53±0.06^{a}	9.47±0.35^{b}	8.53±0.06^{c}	8.10±0.20^{c}
HCT (%)	23.13±0.06^{a}	23.13±0.25^{a}	23.70±0.20^{a}	23.47±0.21^{a}
MCV (µm^{3})	162.73±0.57^{a}	145.5±2.42^{b}	152.37±2.30^{c}	157.47±1.52^{c}
MCH (pg)	53.67±0.38^{a}	53.73±0.57^{a}	53.33±0.32^{a}	53.87±0.21^{a}
MCHC (g/dL)	28.53±0.06^{a}	37.8±0.3^{b}	35.83±0.25^{c}	35.1±0.2^{c}
PLT (10^{5}/µL)	1.35±0.01^{a}	1.57±0.03^{b}	1.49±0.03^{c}	1.41±0.02^{c}

Data expressed as mean ± standard deviation.
Different superscripts in the same row indicates significant difference ($P < 0.05$).
WBC = white blood cell, LYM = lymphocyte, MON = monocyte, RBC = red blood cell, HGB = hemoglobin, HCT = hematocrit, MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, PLT = platelet
PP1 = *P. pellucida* powder 100 mg/kg; PP2 = *P. pellucida* powder 200 mg/kg; PP3 = *P. pellucida* powder 300 mg/kg

Antioxidant enzyme activities

Antioxidant parameters namely catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) were shown in Figure 1. Overall, the values of CAT, SOD and GPx activities increased significantly ($P < 0.05$) in the groups that received dietary PP in comparison to control. Treatment PP1 resulted the highest values of CAT, SOD and GPx activities. This was followed by treatments PP2 and PP3.

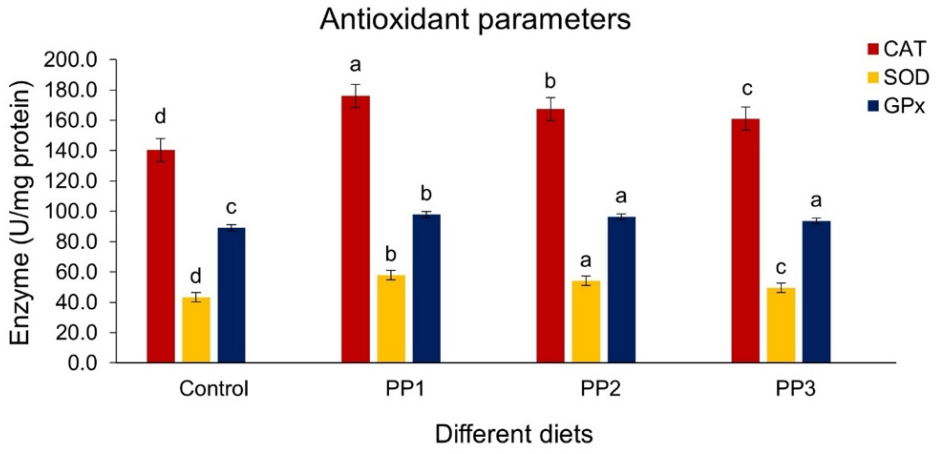

Figure 1 Catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) enzyme activities of the red hybrid tilapia fed with different diets at the end of feeding experiment. Different letters in each column show significant differences among the experimental treatments ($P < 0.05$). PP1 = *P. pellucida* powder 100 mg/ kg; PP2 = *P. pellucida* powder 200 mg/ kg; PP3 = *P. pellucida* powder 300 mg/ kg.

Histological examination

Micrographs of the histological examination of the experimental fish liver are presented in Figure 2. The liver tissues across all treatment groups, including the control, showed normal hepatic architecture with well-organized hepatocytes, centrally located nuclei, and intact sinusoids. Notably, no signs of fatty liver change, such as lipid vacuolation or hepatocyte degeneration, were observed in any of the treatment groups.

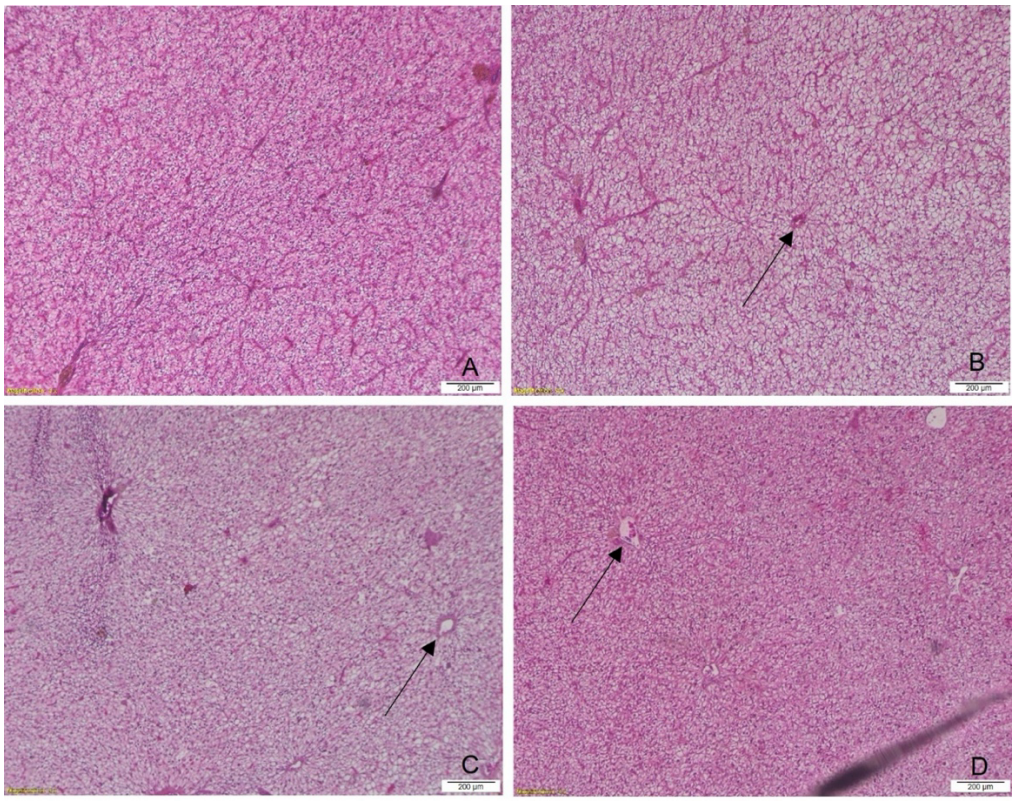

Figure 2 Histological examination of the experimental fish liver samples for (A); control, (B); PP1, (C); PP2, (D); PP3 treatments under the light microscope. Arrow showed the central vein of liver. All micrographs were captured at 40× magnification with the scale bar representing 200 µm. PP1 = *P. pellucida* powder 100 mg/ kg; PP2 = *P. pellucida* powder 200 mg/kg; PP3 = *P. pellucida* powder 300 mg/ kg.

Gene expression of growth and immune regulatory genes

Overall, growth and immune-related gene expression were significantly up-regulated ($P < 0.05$) in the experimental fish received different concentration of PP diets (Figure 3 and 4). The gene expression levels of TGF-β1 and HSP90a in the fish kidney tissue were significantly higher ($P < 0.05$) in treatment PP1 compared with the other diets. On the other hand, gene expression levels of NF-κB were significantly higher ($P < 0.05$) in all PP diets in comparison to the control group. LYZg1 gene expression levels were significantly higher ($P < 0.05$) in treatments PP2 and PP3 compared to PP1. Based on Figure 4, the expression of LYZg1, NF-κB, TGF-β1, and HSP90a genes in fish intestinal tissue were all significantly higher ($P < 0.05$) in treatment PP1 compared to other diets.

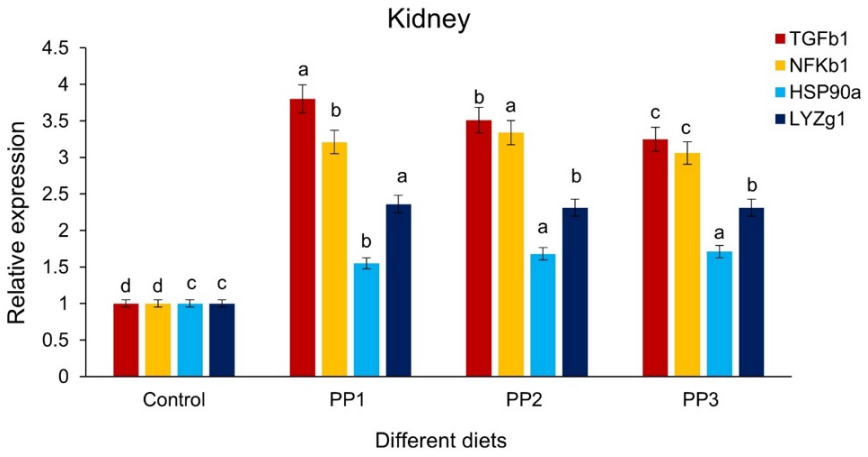

Figure 3 Growth and immune-related gene expression in the fish kidney samples in the present study. Different letters in each column show significant differences among the experimental treatments (P < 0.05). PP1 = *P. pellucida* powder 100 mg/kg; PP2 = *P. pellucida* powder 200 mg/kg; PP3 = *P. pellucida* powder 300 mg/kg.

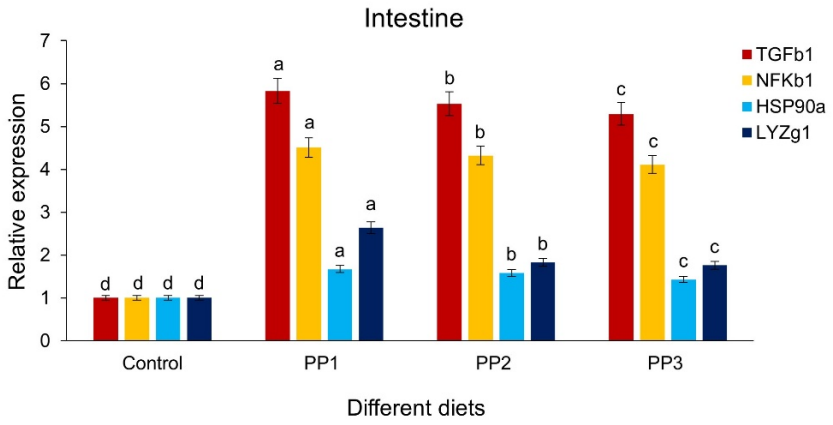

Figure 4 Growth and immune-related gene expression in the fish intestinal samples in the present study. Different letters in each column show significant differences among the experimental treatments (P < 0.05). PP1 = *P. pellucida* powder 100 mg/ kg; PP2 = *P. pellucida* powder 200 mg/ kg; PP3 = *P. pellucida* powder 300 mg/ kg.

Aeromonas hydrophila challenge

The cumulative survival rate of red hybrid tilapia challenged by *A. hydrophila* was presented in Figure 5. The survival rate of fish that received PP diets remain constant, i.e., 83.3% for treatment PP1, 63.3% for PP2, and 46.7% for PP3 throughout Week 1 to Week 4 after infected with *A. hydrophila*. Cumulative survival rate of fish in the treatment groups were significantly higher (P < 0.05) than the control group, in which cumulative survival rate of the fish in treatment PP1 was the best (83.3 ± 5.77%). This was followed by treatments PP2 (63.3 ± 5.77%) and PP3 (46.7 ± 5.77%)

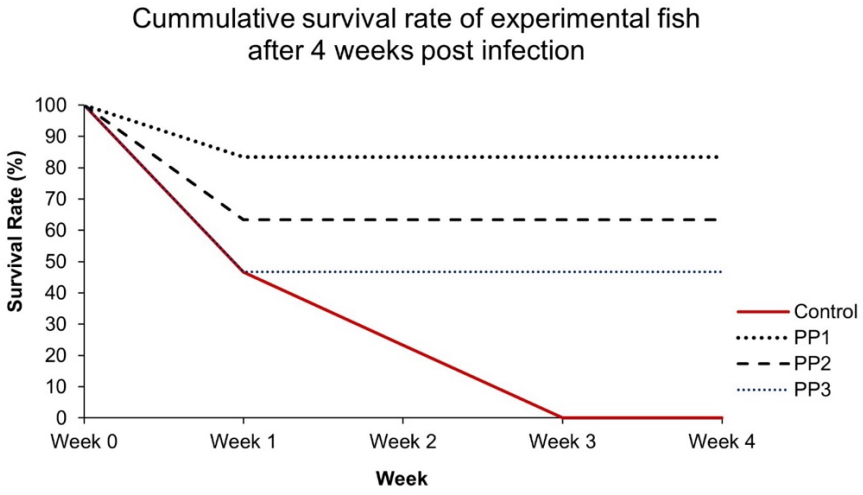

Figure 5 Cumulative survival rate of red hybrid tilapia, *Oreochromis* spp. post infection by *A. hydrophila*. PP1 = *P. pellucida* powder 100 mg/ kg; PP2 = *P. pellucida* powder 200 mg/ kg; PP3 = *P. pellucida* powder 300 mg/ kg.

Regression analysis

The regression analysis of selected parameters is shown in Table 7. Dietary PP is recommended in the red hybrid tilapia diets at 108 mg/kg, 151 mg/kg, and 123 mg/kg to show the best flesh quality, WG, and immune-related genes upregulation, respectively. Overall, dietary PP should be added at 108 to 151 mg/kg in the diets of red hybrid tilapia to show the best growth and health performances.

Table 7 Regression analysis of red hybrid tilapia fed dietary PP for eight weeks.

Parameter	Equation	R^2	PP dose (mg/kg)
Flesh protein content, %	$y = -0.0018x^2 + 0.2498x - 8.6337$	0.7676	108
Weight gain, WG (%)	$y = 0.0000x^2 + 0.017x - 5.1939$	0.5427	151
TGFb1 gene expression	$y = -0.0206x^2 + 0.0956x - 0.075$	0.6983	123

DISCUSSION

Present study evaluated the effects of PP at different inclusion levels on growth performance and health of *Oreochromis* spp. Several analyses conducted in the present study included fish flesh proximate composition, blood parameters, gene expression, liver histology, antioxidant capacity, and disease resistant against *A. hydrophila*. The proximate composition of the experimental fish flesh in all treatments was within permissible range by WHO. The fish flesh proximate composition was not only affected by water parameters such as salinity and dissolved oxygen level (Abdel-Tawwab et al., 2007; Gan et al., 2013; Syed et al., 2022), but also depends on the quality of feed given, especially the protein content (Abdel-Tawwab et al., 2010).

PP is an herb that reported to possess various medicinal properties such as antibacterial, anti-inflammatory, immunostimulatory and etc. (Alves et al., 2019; Ng et al., 2021). The bioactive compounds such as flavonoids, alkaloids, sterols, tannins, quinones, carbohydrates, and carotenoids were responsible to the medicinal values of the herb (Ng et al., 2020). Nevertheless, studies regarding the application of PP as feed additive in aquaculture were limited. In the present study,

PP was used as feed additive in red hybrid tilapia at the concentration 100 and 200 mg/kg, where treatment PP1 exhibited the best growth performance in terms of FW, WG, SGR, and FCR after eight weeks. The bioactive compounds in PP were reported to modulate gut microbiota and enhance digestive system of the fish (Barreto et al., 2008; Ajila et al., 2010; López-Cobo et al., 2017; Alañón et al., 2019). As a result, dietary PP helps to boost the growth performance of the fish. The usefulness of PP as feed additive to promote the growth performance of red hybrid tilapia was evidenced by the improvement in blood parameters, liver morphology, body indices and the flesh quality of the fish. Growth performance, blood parameters, immunological changing and histopathology can be used as the assessment tools in determining the potential use of plant material in aquafeed (Poleksic et al., 2010).

FCR is an important factor in estimating total feed needed for an aquaculture operation, in which it has a direct impact on the operational cost. Hence, low FCR is strongly associated with the profit margin of an aquaculture activity. In this study, FCR in the dietary PP group was significantly lower than the control, where treatment PP1 resulted the lowest FCR. FCR could have lowered due to the bioactive compounds in the dietary PP, which serve as prebiotics in digestive system to modulate the growth of gut microbiota and therefore enhance the digestion of the fish (Kari et al., 2022). On the other hand, VSI is used as a tool to evaluate the quality of feed (Abdul Kari et al., 2021). Fish with low VSI value was able to utilise carbohydrate wisely whereas fish with high VSI value was usually related to high carbohydrate feed intake (Abdul Kari et al., 2021). In present study, the carbohydrate level was almost similar in all treatment diets. Lower VSI value was observed in the experimental fish that received dietary PP, where treatment PP1 exhibited the lowest VSI value. Based on these findings, current study revealed that dietary PP contributed towards low VSI value and improved the health status of the fish.

SOD, CAT, and GPx are frontliners in protecting cells from oxidative stress by reactive oxygen species (ROS) formation (Ghafarifarsani et al., 2023). Antioxidant status of a fish can be assessed by monitoring the levels of these enzyme activities (Zhang et al., 2013). In present study, dietary PP was found to increase antioxidant capacity of red hybrid tilapia in comparison to the control. Bioactive compounds in the PP diets could have antioxidant role in the fish, as demonstrated by other phytobiotics such as oak (*Quercus castaneifolia*) leaf (Paray et al., 2020), papaya (*Carica papaya*) leaf extract (Hamid et al., 2022), and olive (*Olea europea*) leaf (Baba et al., 2018; Zemheri-Navruz et al., 2019) that having similar antioxidant capacity in aquatic animals.

Histological analysis showed that fish fed PP-supplemented diets exhibited normal liver cell structure. This indicates that the inclusion of *P. pellucida* did not cause hepatic lipid accumulation or impair liver health. The absence of pathological changes suggests that the experimental diets were well tolerated and did not adversely affect liver morphology, reinforcing the nutritional safety and compatibility of PP as a dietary supplement in fish.

HGB value is a good indicator that the iron-containing protein in the RBC is in good condition. It is also being interpreted that fish with high level of HGB is having normal respiration capacity and not suffering from anemia (Magnadóttir, 2006). Likewise, findings of the present study showed that HGB value of red hybrid tilapia in all the treatments that received PP diets were higher compared to the control. This is in agreement with the previous studies where dietary phytobiotics like coriander seed extract were beneficial to HGB of rainbow trout (Naderi Farsani et al., 2019) and Nile tilapia (Das et al., 2023). Moreover, the values of MCV, MCHC, and MCH were lower in the all the treatments that received PP diets in comparison with control group. According to (Witeska et al., 2022), fish are likely to develop increased values of MCV, MCHC, and MCH when suffered from anemia or infected by diseases. As for the total WBC count in fish, it is affected by many factors such as season, sex, feeding activity, diseases, stress and the presence of pollutant in

the fish environment (Ahmed et al., 2020). In this study, the value of WBC in the fish from all treatments that received PP diets were significantly higher compared to the control group. The presence of high total WBC count probably helps the experimental fish defend against *A. hydrophila* bacterial infection. This can be observed through high survival rate of the fish from treatments that received dietary PP after infection by *A. hydrophila*. In addition, total values of LYM and MON in the present study was almost similar with no significant difference. These values may also be used as indicator of health status of the fish (Witeska et al., 2022).

The head kidney and distal intestine segments of fish are regarded as immunologically important sites corresponding to the expression of growth and immune-related genes (Rombout et al., 2011; Kari et al., 2022). Findings from the present study showed that dietary PP significantly promoted ($P < 0.05$) growth performance and immune response, as well as the survival of red hybrid tilapia by up-regulating the growth and immune regulatory genes (LYZg1, NF-κB, TGF-β1, and HSP90a). This is in agreement with nutrigenomic principle that dietary signals found in the nutrients of a formulated feed can be picked up by a cellular sensor system, and further influence the gene expression and protein production for metabolites production. The metabolites, in turn, act as biological response modifiers in immunomodulation that trigger the B-cells, T-cells and NK cells to be ready against disease infection (Abdul Kari et al., 2022). It has also been widely established in previous studies that dietary phytobiotics can strengthen fish immune systems and promote disease resistance against *A. hydrophila* infections. For instance, dietary *Flos populi* extract in goldfish, *Carassius auratus*, (Zhang et al., 2022), dietary miswak, *Salvadora persica*, in Nile tilapia (Abd El-latif et al., 2021), dietary oregano essential oil in common carp, *Cyprinus carpio* (Abdel-Latif et al., 2020), dietary *Ginkgo biloba* leaf extract in common carp (Bao et al., 2019), and dietary *Euphorbia hirta* extract in sharptooth catfish, *Clarias gariepinus* (Sheikhlar et al., 2017). Therefore, the application of phytobiotics as feed additive is noteworthy in controlling fish disease due to *A. hydrophila*.

The lower performance observed in the PP3 group may be attributed to the higher inclusion level of *P. pellucida* powder, which could have exceeded the optimal dosage threshold. While PP contains bioactive compounds with potential health benefits, excessive levels may lead to anti-nutritional effects, reduced palatability, or interference with nutrient absorption. High concentrations of certain phytochemicals, such as tannins or alkaloids, can negatively affect digestive enzyme activity or cause metabolic stress, ultimately impairing growth and health performance. Therefore, the reduced effectiveness in the PP3 group may reflect a dose-dependent response where moderate inclusion is beneficial, but excessive supplementation becomes counterproductive.

From a practical standpoint, PP powder demonstrates strong potential as a natural and sustainable feed additive in aquaculture. The plant is widely available across tropical regions and can be easily cultivated or collected at minimal cost, making it an economically viable option for small- to medium-scale fish farmers. Its preparation is simple, involving only drying and grinding into powder form an approach that can be implemented without the need for advanced equipment or infrastructure. Importantly, the inclusion of PP in the diet did not adversely affect water quality, as no abnormal fish behavior or environmental concerns were observed during the trial. This is especially valuable for maintaining stable conditions in recirculating or static aquaculture systems. Owing to its known antimicrobial and immunostimulatory properties, PP may also help reduce dependence on synthetic antibiotics, thereby promoting more sustainable and environmentally responsible aquaculture practices. Nevertheless, further research is needed to develop standardized dosing protocols, assess long-term effects, and evaluate the shelf life and stability of PP powder for broader commercial adoption.

CONCLUSIONS

In conclusion, findings of the present study revealed that dietary *P. pellucida* powder improved growth performance, enhanced immune system, increased antioxidant capacity, up-regulated growth and immune-related gene expression, and stimulated disease resistant of red hybrid tilapia against *A. hydrophila*. Application of *P. pellucida* powder at the dose of 108 to 151 mg/kg as feed additive in current study demonstrated favourable outcomes which can be considered for commercial application in tilapia production. Nevertheless, extension research based on this study ought to be done in order to have a better understanding about the mode of action of *P. pellucida* powder in other aquaculture species.

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Lee Seong Wei: Project administration (Lead); Supervision; Conceptualization (Lead); **Formal analysis (Equal);** Writing –review & editing (Supporting).
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Mohamad Nor Azra: Data curation (Supporting); Resources (Supporting).
Wendy Wee: Visualization (Lead); Writing –original draft (Supporting). Data curation (supporting); formal analysis (lead); methodology (equal); resources (equal).

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