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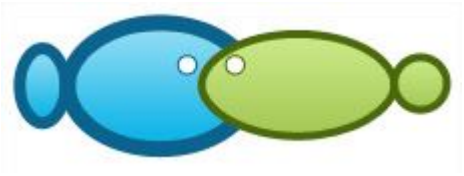

Effects of plant extracts on selected haematological parameters, digestive enzymes, and growth performance of striped catfish, *Pangasianodon hypophthalmus* (Sauvage, 1878) fingerlings

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Abstract. This study examined the effects of dietary supplementation with selected plant extracts on the haematology, enzymatic activities, and growth of *Pangasianodon hypophthalmus* fingerlings. A 60-day feeding trial was conducted with 11 diets: basal diet (basal diet); and basal diet supplemented with 0.4% or 2% *Euphorbia hirta* (Eh), 0.2% or 1% *Phyllanthus amarus* (Pa), 0.4% or 2% *Mimosa pudica* (Mp), 0.2% or 1% *Psidium guajava* (Pg), and 0.4% or 2% *Azadirachta indica* (Ai). On days 30 and 60, the haematocrit improved significantly in response to the Pg 0.2 diet (35.9±0.8% and 36.4±0.7%, respectively), while the haemoglobin level increased significantly in fish fed a diet with Mp or Pg at both concentrations. Chymotrypsin activity was highest (121±6.10 U min^{-1} mg^{-1} protein) under the Pa 0.2 diet followed by the Eh 0.4 and Pa 1.0 diets (94.1±7.20 and 92.2±7.80 U min^{-1} mg^{-1} protein, respectively) on day 30. The Pa 0.2 diet significantly enhanced chymotrypsin activity on day 60 compared to the other diets. Fish fed the Pa 0.2, Pa 1.0, or Pg 1.0 diet showed significantly increased pepsin activity than those fed the control diet on day 30, whereas there was not observed significant difference on day 60. Amylase activity was significantly enhanced in response to the Eh 0.4, Pa 0.2, Pg 0.2, and Ai 0.4 diets, but none of the diets led to a change in trypsin. Sixty days of oral administration of Pg 0.2 or Pa 0.2 extracts modulated haematological parameters and digestive enzyme activities of *P. hypophthalmus* fingerlings and resulted in higher growth performance.

Key Words: enzyme activity, glucose concentration, haematology, *Pangasianodon hypophthalmus.*

Introduction. The rapid growth of the striped catfish, *Pangasianodon hypophthalmus* (Sauvage, 1878), can be attributed to the intensification of the farming system in the Mekong Delta, Viet Nam. The intensification of farming has increased the incidence of stress-related diseases and mortality and compromised the immune system of the fish resulting in frequent outbreaks of diseases (Phan et al 2009). Bacillary necrosis of *Pangasius* (BNP) and motile *Aeromonas* septicaemia (MAS), caused by *Edwardsiella ictaluri* and *Aeromonas hydrophila*, respectively, commonly occur in farmed *P. hypophthalmus* (Crumlish et al 2010). Consequently, these diseases can be devastating to the aquaculture industry and cause significant economic losses. Evidence shows that stressors, such as localized environmental deterioration, agricultural waste, inadequate care, excessive stocking density, and poor seed quality, increase stock susceptibility to infectious diseases (Phuong et al 2007). Stressors in aquaculture can result in impaired metabolism (Santos et al 2010), inferior fillet product (Jittinandana et al 2003), higher disorder susceptibility (Wu et al 2013), and in severe cases, high mortality (McKenzie et al 2012).

Numerous efforts have been attempted to tackle the mortality problem, including the application of chemicals and antibiotics as prophylactic and therapeutic

pharmaceuticals. Rico et al (2013) reported that 100% of *P. hypophthalmus* farmers in Viet Nam used 17 types of pharmaceuticals (penicillin, aminoglycosides, cephalosporins, quinolones, tetracyclines, amphenicols, etc.) in the culture period. Nonetheless, because of the multiple negative impacts on the environment and human well-being (environmental pollution, residues in fish, and resistant bacteria), the use of pharmaceutical products is becoming more restricted (Andrieu et al 2015). In addition, vaccination is also being investigated as a potential approach for disease outbreak prevention in aquaculture. However, commercial vaccines are prohibitively expensive for widespread use by fish farmers, and each vaccine is specific to only one form of infection (Triet et al 2019).

As a result, researchers have made efforts to incorporate natural components into nutritional supplement formulas that enhance the health, growth, and immunity of fish sourced from less expensive sources without toxicity, which is crucial to the sustainability of aquaculture (Gabriel 2019; Gupta et al 2021). In aquaculture, the utilization of phytoconstituents is considered immune stimulants and anti-stressors (Chakraborty & Hancz 2011) and numerous plants have active substances such as flavonoids, alkaloids, phenolics, steroids, terpenoids, as well as essential oils that possess a wide spectrum of physiological functions (Ghosh et al 2019).

Haematology profiles are essential indicators of fish health and metabolism. Blood is the most often selected tissue in vertebrates, including fish, to assess health status (Fazio 2019); it offers adequate knowledge of fish's biological reactions to external conditions, which addresses homeostasis (Shahjahan et al 2018). Any imbalance in the formulation of or an inferior ingredient in the supplement may inadvertently impair the health status of fish and increase their susceptibility to diseases, and appropriate nutritional practices are crucial in maintaining a safe and healthy cultural environment and mitigating the risk of disease outbreaks (Kiron 2012). The measurement of digestive enzymes is crucial to understand the mechanism of digestion and how organisms respond to dietary modifications in response to adaptation to external environments (Sunde et al 2004) and to elucidate some elements in nutritional physiology (Uys & Hecht 1987).

Viet Nam has a high diversity of medicinal herbs distributed throughout the country's various ecological zones. Numerous botanicals have been used for medicinal purposes to strengthen the immunity and health status of *P. hypophthalmus*. According to Dao et al (2020), the most important antioxidant is *Phyllanthus amarus* Schumach. & Thonn. (Pa) extract, followed by extracts of *Psidium guajava* L. (Pg), *Euphorbia hirta* L. (Eh), and *Mimosa pudica* L. (Mp), in that order. Typically, Pa extracts showed the highest *in vitro* antibacterial properties against 2 different isolates of *Aeromonas hydrophila*, as revealed through its low minimum inhibitory concentration (MIC). In addition, extracts of *Azadirachta indica* (Ai), Eh, and Pa significantly stimulated the expression of pro-inflammatory, antiviral, and adaptive immune cytokines in striped catfish cells (Nhu et al 2019). However, farmers appear to be unaware of the existence of such bioactive compounds or their efficacy in fish, as well as the capacity of extracts of these plants to improve the haematological and digestive enzyme activities of aquatic species, with *P. hypophthalmus* receiving the most concern. This research attempts to objectively assess the effectiveness of five various plant extracts on haematology, enzymatic activities, and growth performance of *P. hypophthalmus*. The findings of this study could support farming productivity through the development of environmentally friendly biological products as a sustainable and practical approach to aquaculture development and minimizing the utilization of chemically synthesized therapeutic medicines, such as antimicrobial compounds, which are commonly applied in intensive fish farming systems.

Material and Method

Time and location of the study. The experiment was conducted at the College of Aquaculture and Fisheries, Can Tho University from April 2021 to July 2021.

Plant extract and feed preparation. Fresh Eh (leaves and twigs), Pa (twigs and leaves), Pg (leaves), Mp (twigs and leaves), and Ai (leaves) samples were manually

gathered from a location near Can Tho city, Viet Nam. The identity of the plants was then verified, and the plant samples were processed at the College of Natural Sciences, Can Tho University prior to being extracted with ethanol. After washing with sterile distilled water, damaged plant parts were eliminated. The plant parts were exposed to sunlight for some days before being dried at 60°C. Following that, the fine powder was stored at room temperature. To produce an ethanolic extract of the plants, 100 g of dried powder was immersed in 96% ethanol (800 mL) for 24 hours. Samples were decanted and screened; excess solvent was evaporated with a rotary evaporator under low pressure (Nhu et al 2019).

Fish diets supplemented with plant extracts were prepared; all experimental diets were iso-lipidic, iso-proteic, and iso-energetic (Table 1). The control diet was the basal diet without supplementation with plant extracts. All ingredients were thoroughly blended into a homogeneous mixture and then pelletized, air-dried, ground, and sieved to achieve the desired pellet size (2 mm). The pellets were preserved at -20°C in properly marked polythene bags until further usage.

Table 1

Experimental feed ingredients and formulation

Ingredients (100 g of feed)	*Basal diet*	*Supplementary plant extract diets*			
		0.2%	*0.4%*	*1%*	*2%*
Soybean meal (g)	32.6	32.6	32.6	32.6	32.6
Rice bran (g)	29.5	29.5	29.5	29.5	29.5
Cassava (g)	18.4	18.2	17.9	17.4	16.4
Fish meal (g)	15.0	15.0	15.0	15.0	15.0
Fish oil (g)	1.00	1.00	1.00	1.00	1.00
Premix* (g)	3.00	3.00	3.00	3.00	3.00
Carboxymethyl cellulose (CMC) (g)	0.50	0.50	0.50	0.50	0.50
Butylated hydroxytoluene (BHT) (g)	0.02	0.02	0.02	0.02	0.02
Plant extract (g)	0	0.20	0.40	1.00	2.00

*Premix: 1% attractant, 0.03% vitamin C, 0.5% CMC and mixture of vitamin and mineral. Fishmeal, soybean meal, cassava, rice bran was weighed and mixed and then sterilized at 110°C for 10 mins (the sterilization process can last from 60 to 90 mins due to the temperature reduction process of the machine to 70°C sterilization process eliminated bacteria, supports digestibility for fish). This is an important step in teed processing. BHT, premix, and CMC well mixed with the extract, powder (the powder was cooked for 30 minutes and stirred well) and later mixed with the sterilized mixture, fish oil. Afterward, the experimental feeds were dried at 60°C for an estimated 24 hours. All dried feed was stored at -20°C for further use. The control diet was following the same steps without plant extract.

Experimental fish acclimation, facilities, and feeding management. *P. hypophthalmus* fingerlings (14.1±0.46 g fish^{-1}) were sourced from a hatchery in Can Tho city and transported to the laboratory in oxygenated bags. The fish were acclimated to experimental tanks (2 m^3) with well-aerated water and a natural photoperiod. They were fed a basal diet twice a day (at 8:00 am and 4:00 pm) until satiation (up to 3-5% of body weight). After two weeks of acclimation, a total of 2,475 fish were randomly assigned to 33 separate 500-L fiberglass tanks containing 300-L water (11 treatments in triplicate) at a stocking density of 75 fish tank^{-1}. Each plant extract was administered in two doses corresponding to 0.4% and 2.0% Eh (Eh 0.4 and Eh 2.0, respectively), 0.2% and 1.0% Pa (Pa 0.2 and Pa 1.0, respectively), 0.4% and 2.0% Pg (Pg 0.4 and Pg 2.0, respectively), 0.4% and 0.2% Mp (Mp 0.4 and Mp 0.2, respectively), and 0.4% and 2.0% Ai (Ai 0.4 and Ai 2.0, respectively).

Throughout the experiment, all tanks were continuously supplemented with well-aerated filtered water. Fish were manually fed twice daily until they appeared to be satiated (3-5% of body weight). Daily siphoning was applied to restrict fouling from feed residues, as well as 30% of the water was weekly exchanged with fresh and dechlorinated water. Water pH values were recorded twice a week with a pH instrument (Metler Toledo SG2, USA); dissolved oxygen and temperature were determined with an Oxy Guard H04PP. The recorded water quality parameters, such as dissolved oxygen (3.95 to 4.90 mg L^{-1}), pH (7.41 to 7.63), and temperature (28.28 to 29.15°C), were all within the range for *P. hypophthalmus.* The experiment was continued for 60 days.

Accordingly, the experiment was performed in conformity with general legislation on the protection and experimental animal welfare in Vietnam (Law of animal health 2015).

Haematological and biochemical parameters. Three fish from each replicate were sampled on days 30 and 60. At the beginning of the experiment (day 0), 30 fish were randomly collected from the population. A cold moist cloth was placed on the head of each sampled fish to minimize stress throughout handly (Snellgrove & Alexander 2011). Blood samples from the caudal peduncle vein were obtained within 3 minutes of sampling using 1-mL heparin-coated syringes (Becker et al 2012). At least 300 µL of blood was drawn from each fish and transferred into a labeled 1.5-mL plastic tube. Before centrifuging for plasma glucose analysis, one part of the blood was used to determine haematological parameters. The blood was diluted and gently mixed with Natt and Herrick's solution (1:200). The cell suspension was placed in a Neubauer haemocytometer and red blood cells (RBCs) were counted at a magnification of 40× (Natt & Herrick 1952). Hematocrit (Hct) and blood samples were obtained in a glass capillary microcentrifuge for 6 minutes at 12,000 rpm in a microhaematocrit centrifuge (Sigma 201M, Hettich) (Larsen & Snieszko 1961). Drabkin's solution was used to quantify hemoglobin (Hb); 10 µL of the solution was diluted with 2.5 mL of Drabkin's reagent and colorimetric measurements were undertaken with a spectrophotometer (GENESYSTM 20, Thermo Scientific) to estimate cyanomethemoglobin formation (Zijlstra et al 1983). Then, the mean corpuscular volume (MCV) was calculated as Hct × 10 RBC^{-1}, mean corpuscular haemoglobin (MCH) was calculated as Hb × 10 RBC^{-1}, and mean corpuscular haemoglobin concentration (MCHC) was calculated as Hb × 100 Hct^{-1} (Ware 2020). The number of white blood cells (WBCs) was measured by smearing 5 µL of blood on a smeared slide (75 × 25 mm, Germany) under a microscope. The smearing slides were stained in methanol (95%, Merck, USA) for approximately 1-2 minutes followed by Wright's and Giemsa solutions. Samples were counted using a microscope with a 100× objective (Hrubec et al 2000). A portion of the blood was centrifuged for 6 minutes at 4°C and 6,000 g. The supernatant was then separated and refrigerated at -80°C for later analysis. A standardized glucose assay was used to quantify glucose concentrations (Huggett & Nixon 1957).

Digestive enzyme activities. Fish were fasted for 48 hours prior to sampling to ensure their stomachs were completely empty of contents. Fish were then euthanized with ice and dissected to obtain the stomach (for pepsin and amylase assays) and intestine (for analysis of amylase, trypsin, and chymotrypsin). A scalpel was used to remove the remnants of gut and stomach contents before collecting samples. Manipulation was carried out on the ice to minimize interfering with enzymatic activity. The intestine and stomach were cut longitudinally and carefully rinsed in distilled water; they were blotted dry using combining filter paper and transferred to labeled 1.5-mL tubes, which were then refrigerated at -80°C until homogenization. The stomachs and intestines were isolated after defrosting and weighed, followed by homogenization in a KH_2PO_4/NaCl buffer (pH of 6.9). After that, they were centrifuged for 30 minutes at 4,200 g, and the supernatant was separated for enzymatic activities analysis. Pepsin was quantified using Worthington & Manual (1982) method, which involved reacting 100 µL of the sample with bovine haemoglobin (Sigma-Aldrich) as a substrate in 1N HCl. Trichloroacetic acid (TCA; Sigma-Aldrich) was pipetted into the reaction solution. Each sample was centrifuged at 4,000 g for 10 minutes at 4°C to assess pepsin activity at 280 nm.

Trypsin activity was assayed by reacting 15 µL of the sample with 0.1 M BApNA solution (9.87 mg of N-benzoyl-D_L-arginine-p-nitroanilide in 250 µL of DMSO) and phosphate buffer solution at pH 8.2 followed by measurement of the optical density of trypsin at 407 nm after 5 minutes (Tseng et al 1982). Chymotrypsin activity was determined with 50 µL of the sample reacted with BTEE (N-benzoyl-L-tyrosine ethyl ester; Sigma-Aldrich) and buffer at pH 7.8 and then measured at 256 nm as described by Worthington & Manual (1982). The calibration curve for amylase activity was established using maltose, which was quantified at 540 nm (Bernfeld 1951). The total protein content was analyzed using diluted homogenates and bovine serum albumin for calibration

(Bradford 1976). The activity of each enzyme was quantified in units per milligram of protein (U min^{-1} mg^{-1} protein).

Growth performance and survival rate. At 30-day intervals of the experiment (days 30 and 60), weight gain (WG), daily weight gain (DWG), and survival rate (SR) were assessed. Fish were gathered from the corresponding tank and weighed with a digital balance and the number of fish was recorded to determine the survival rate. Fish were gently returned to respective tanks after measurement. Growth indicators were identified based on the formula: weight gain (WG, g) = (W_f – W_i); daily weight gain (DWG, g day^{-1}) = (W_f – W_i)/t; and survival rate (%) = No. of fish harvested × 100/No. of fish stocked. W_i and W_f are initial and final weight (g), respectively, and t is the duration of the experiment (days).

Statistical analysis. Statistical analyses were performed using SPSS software, version 20 (IBM Corp., Armonk, NY). The Levene test was used to determine the homogeneity of variance between groups. A one-way analysis of variance (ANOVA) and Duncan's multiple range test were performed to identify the differences between treatments at each sample interval. A p-value of less than 5% ($p < 0.05$) was considered significant. All data were presented as means and standard deviations of the mean (SEM).

Results

Effects of plant extracts on haematological parameters P. hypophthalmus. The treatment supplemented with 2.0% Mp showed the highest density of RBCs on both day 30 and day 60 (3.18±0.09 × 10^6 cells mm^{-3} and 3.70±0.28 × 10^6 cells mm^{-3}, respectively) but no significant difference was obtained among treatments ($p > 0.05$). Furthermore, the highest Hct was recorded as dose-dependent in all groups given the diet enriched with 0.2% Pg, with values differing significantly from the basal diet ($p < 0.05$) on both day 30 and day 60 (30.6±19% and 31.2±24%, respectively) of the trial. On day 30, Hb concentration was significantly highest in Mp 2.0 group (10.3±0.15 g 100 mL^{-1}) followed by the groups fed the Pg 0.2 diet (10.2±0.24 g 100 mL^{-1}). At the end of the trial, the obtained results indicated a difference in Hb in fish-fed diets supplemented with Mp or Pg at the two doses ($p < 0.05$; Table 2).

The change in MCV was not significantly different between the extract-based diets and the control on day 60, but MCV fluctuated significantly in the groups fed the Pg 0.2 diet (115±1 μm^3) among treatments. The Eh 0.4, Mp 2.0, Pg 0.2, and Pg 1.0 diets significantly affected MCH on day 30 compared to other treatments ($p < 0.05$). MCH showed the highest increase in fish fed the Pg 0.2 diet (31.6 ± 1.8 pg) on day 60. On day 30, MCHC presented a remarkable difference in Pg 1.0 and Ai 0.4 diets (31.3±0.4% and 31.3±0.5%, respectively; $p < 0.05$). On day 60, MCHC declined in a dose-dependent manner in fish fed various diets, with significantly higher values recorded for the fish fed the Mp 2.0 diet (29.5±0.8%) compared to the basal diet (Table 2).

The increase in WBCs was highest for the Pg 0.2 diet (245±19 × 10^3 cells mm^{-3}) followed by Pg 1.0, Eh 0.4, Mp 0.4, and Mp 2.0 diets which were significantly different from the remaining diets ($p < 0.05$) after 30 days of culture (Table 2). In addition, after 60 days, the WBC counts were significantly higher in fish fed the Pg 1.0 diet (238±23 × 10^3 cells mm^{-3}) than basal diet group. After 60 days on the plant extract-based diets, the treatment with the lowest observed glucose concentration was Ai 2.0 (57.1±0.91 mg 100 mL^{-1}), which was significantly different from Pa 0.2, Pa 1.0, Mp 0.4, Pg 0.2, Pg 0.1 and Ai 0.4 ($p < 0.05$; Figure 1).

Table 2

Haematological parameters of *P. hypophthalmus* fed extract-based diets in a 60-day experiment

Indicator	*Time*	*Control*	*Eh 0.4*	*Eh 2.0*	*Pa 0.2*	*Pa 1.0*	*Mp 0.4*	*Mp 2.0*	*Pg 0.2*	*Pg 1.0*	*Ai 0.4*	*Ai 2.0*
RBCs ($x10^6$ cells mm^{-3})	Day 0						2.57±0.07					
	Day 30	3.03±0.15	3.15±0.21	3.07±0.23	3.14±0.13	3.09±0.12	3.09±0.07	3.18±0.09	3.12±0.07	3.09±0.07	3.07±0.06	3.05±0.16
	Day 60	3.06±0.06	3.20±0.25	3.29±0.19	3.28±0.14	3.26±0.34	3.34±0.17	3.70±0.28	3.34±0.28	3.30±0.09	3.30±0.09	3.27±0.28
Hb	Day 0						6.57±0.17					
(g 100 mL^{-1})	Day 30	8.80±0.41	9.96±0.20	8.94±0.58	9.69±0.58	9.25±0.56	9.65±0.31	10.3±0.15*	10.2±0.24*	9.88±0.37	9.51±0.21	9.16±0.16
	Day 60	8.31±0.27	8.88±0.32	9.23±0.16	9.13±0.32	9.01±0.40	9.60±0.54*	10.0±0.19*	10.3±0.20*	9.66±0.08*	8.42±0.29	8.39±0.21
Hct	Day 0						27.1±0.60					
(%)	Day 30	31.8±1.90	33.7±1.30	31.9±1.80	33.1±1.50	32.2±1.30	32.8±1.40	34.1±1.80	35.9±0.8*	31.57±1.10	30.41±1.00	30.6±1.00
	Day 60	32.1±1.00	33.0±0.90	33.3±0.50	33.1±1.50	33.0±0.90	33.7±1.60	34.1±1.50	36.4±0.7*	34.0±1.30	33.3±1.00	29.1±1.40
WBCs ($x10^3$ cells mm^{-3})	Day 0						156±32.0					
	Day 30	154±13.0	212±54.0*	162±39.0	182±18.0	158±18.0	205±25.0*	203±34.0*	245±19.0*	236±48.0*	199±34.0	181±34.0
	Day 60	169±31.0	235±33.0*	210±40.0	226±39.0*	194±41.0	235±41.0*	209±28.0	228±24.0*	238±23.0*	176±11.0	195±17.0
MCV	Day 0						107±4.00					
(μm^3)	Day 30	105±4.00	108±3.00	105±2.00	105±1.00	105±2.00	105±2.00	107±4.00	115±1.00*	102±2.00	99±1.00	101±2.00
	Day 60	105±2.00	105±5.00	102±4.00	101±1.00	105±9.00	101±2.00	94±5.00	111±6.00	103±2.00	101±2.00	90±4.00
MCH	Day 0						26.1±1.00					
(pg)	Day 30	29.1±0.60	32.0±1.70*	29.2±0.60	30.8±0.90	29.9±0.80	31.2±0.50	32.4±0.70*	32.8±0.20*	31.9±0.50*	31.0±0.30	30.2±1.10
	Day 60	27.2±0.50	27.2±0.50	27.2±0.50	27.2±0.50	27.2±0.50	27.2±0.50	27.2±0.50	27.2±0.50	27.2±0.5	27.2±0.50	27.2±0.50
MCHC	Day 0						23.9±0.70					
(%)	Day 30	27.9±1.20	29.7±0.70	28.0±0.40	29.3±1.00	28.6±1.00	29.7±0.60	30.5±1.20	28.5±0.30	31.3±0.40*	31.3±0.50*	30.0±0.60
	Day 60	25.9±0.60	26.9±0.40	27.7±0.10*	27.7±0.50*	27.3±0.70	28.5±0.40*	29.5±0.80*	28.4±0.10*	28.5±0.90*	25.3±0.30	29.0±0.70*

Notes: value = mean±standard error, with the asterisk (*) in the row of each sampling period indicating the significant difference compared to basal diet ($p < 0.05$).

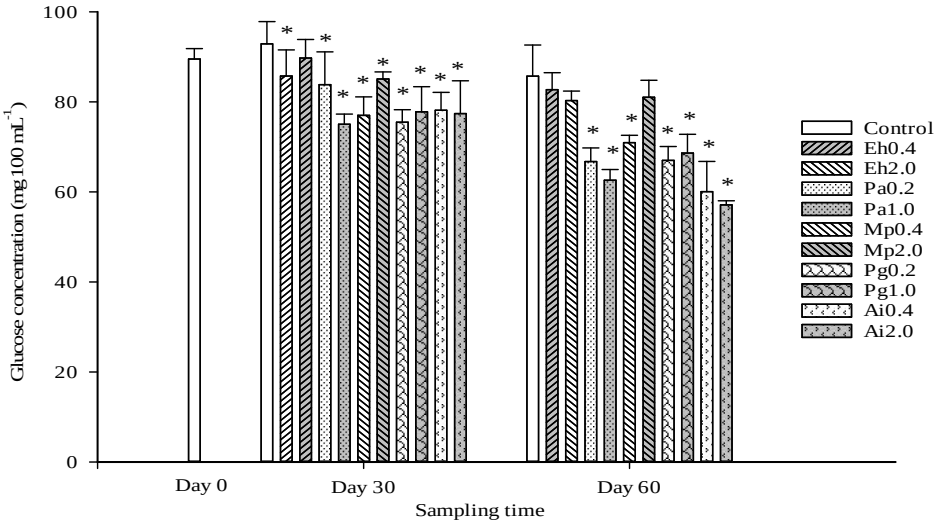

Figure 1. Plasma glucose concentrations (mg 100 mL^{-1}) of *P. hypophthalmus* fed extract-based diets in 60 days. Value = mean±standard error, with the asterisk (*) in the column of each sampling period indicating the significant difference compared to basal diet ($p < 0.05$).

Effects of plant extract on digestive enzyme activities of P. hypophthalmus. Generally, pepsin activity was highest on day 30 in the fish fed the Pg 1.0 diet, followed by those fed the Pa 0.2 and Pa 1.0 diets (0.840±0.153 U^{-1} min^{-1} mg^{-1} protein, 0.654±0.130 U^{-1} min^{-1} mg^{-1} protein and 0.602±0.049 U^{-1} min^{-1} mg^{-1} protein, respectively), and was significantly different from remaining groups fed extract-based diets ($p < 0.05$). After 60 days, pepsin activity remained higher, but not considerably compared to those in the basal diet group ($p < 0.05$; Figure 2).

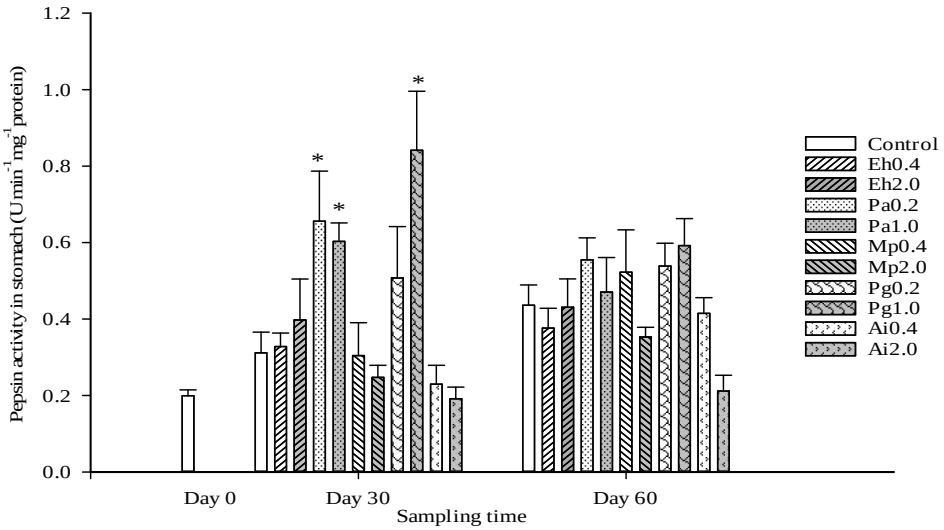

Figure 2. The activity of pepsin in the stomach (U min^{-1} mg^{-1} protein) of *P. hypophthalmus* fed extract-based diets in 60 days. Value = mean±standard error, with the asterisk (*) in the column of each sampling period indicating the significant difference compared to basal diet ($p < 0.05$).

Figure 3 indicated that the Ai 2.0 extract-based diet resulted in the highest trypsin activity (4.41±0.49 U^{-1} min^{-1} mg^{-1} protein) on day 30; however, there was no significantly different among other diets at 60 days ($p > 0.05$).

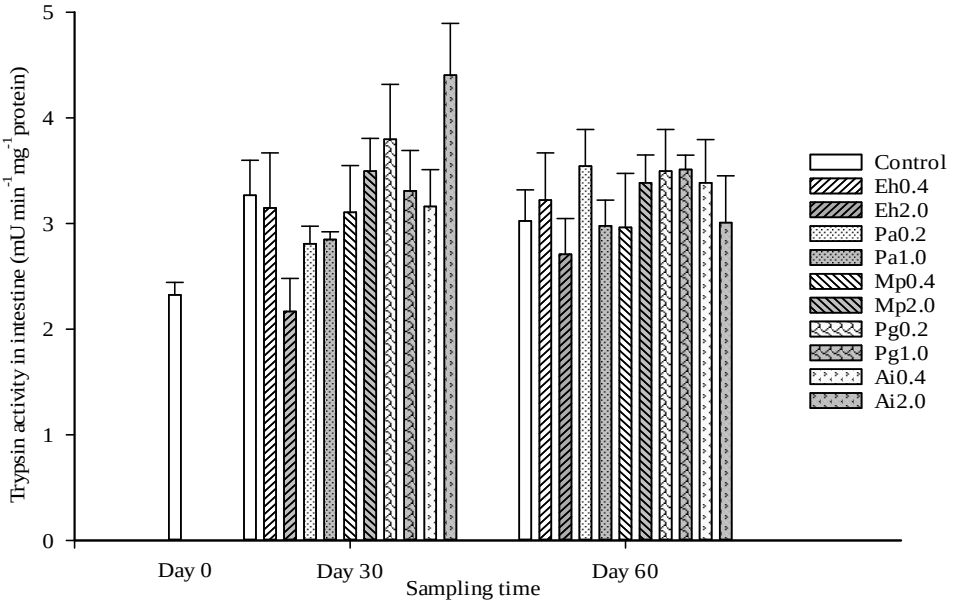

Figure 3. The activity of trypsin in the intestine (mU min^{-1} mg^{-1} protein) of *P. hypophthalmus* fed extract-based diets in 60 days. Value = mean±standard error.

On day 30, chymotrypsin activity showed significant differences ($p < 0.05$), with the highest response in fish fed the Pa 0.2 diet followed by those fed the Eh 0.4 and Pa 1.0 diets (121±6.1 U^{-1} min^{-1} mg^{-1} protein, 94.1±7.20 U^{-1} min^{-1} mg^{-1} protein and 92.2±7.80 U^{-1} min^{-1} mg^{-1} protein, respectively; $p < 0.05$). Chymotrypsin activity at the end of the trial was highest in Pa 0.2 ($p < 0.05$; Figure 4).

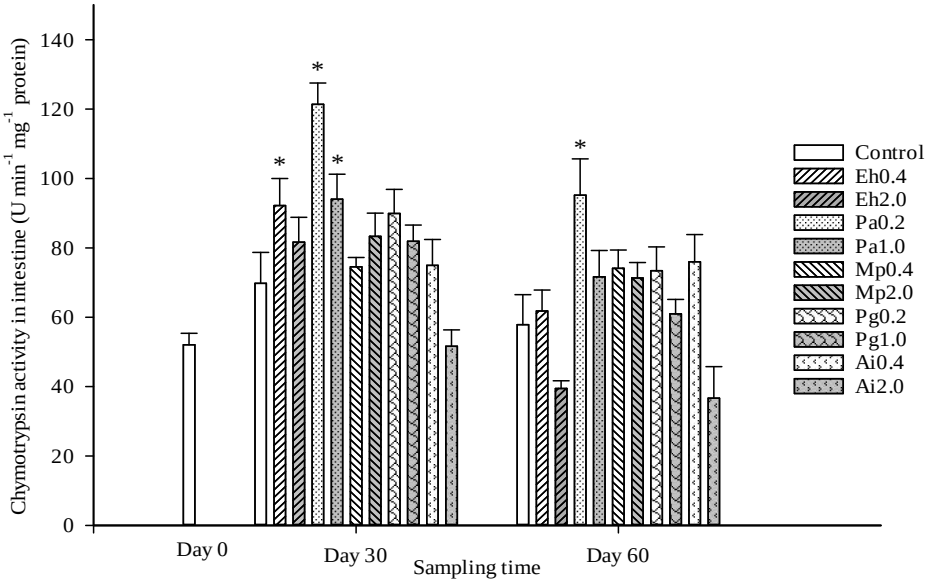

Figure 4. Chymotrypsin in the intestine (U min^{-1} mg^{-1} protein) of *P. hypophthalmus* fed extract-based diets in 60 days. Value = mean±standard error, with the asterisk (*) in the column of each sampling period indicating the significant difference compared to basal diet ($p < 0.05$).

After 60 days, the highest intestinal amylase activity was assayed in Ai 0.4 group (0.876±0.073 U^{-1} min^{-1} mg^{-1} protein); it was significantly different from activity in Ai 2.0, Mp 2.0, Pa 1.0, Eh 2.0 and the basal diet ($p < 0.05$; Figure 5).

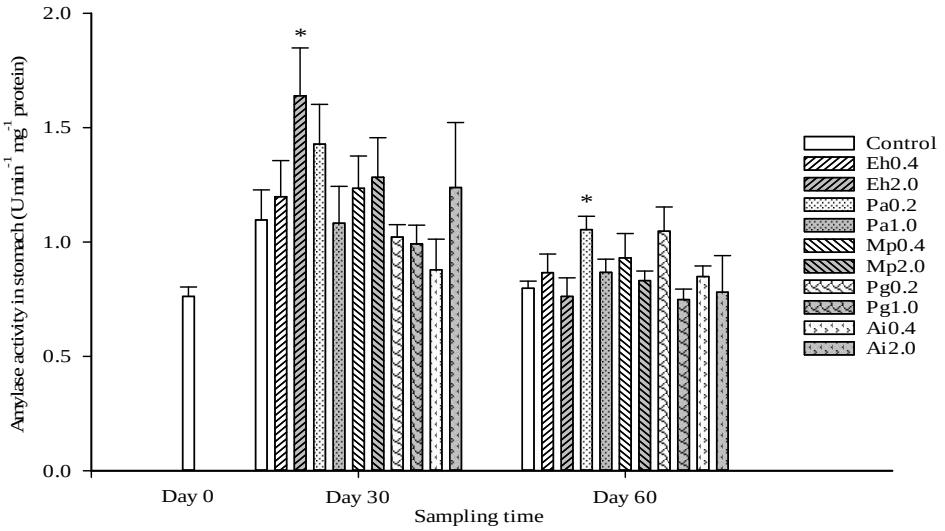

Figure 5. The activity of α-amylase in the intestine (U^{-1} min^{-1} mg^{-1} protein) of *P. hypophthalmus* fed extract-based diets in 60 days. Value = mean±standard error, with the asterisk (*) in the column of each sampling period indicating the significant difference compared to basal diet ($p < 0.05$).

After 30 days, significant and dose-dependent changes in amylase in the stomach were recorded for different plant extract-based diets, with the highest activity assayed for Eh 2.0 (1.64±0.21 U^{-1} min^{-1} mg^{-1} protein; $p < 0.05$). After 60 days, the highest gastric amylase activity was assayed in Pa 0.2 group (1.05±0.03 U^{-1} min^{-1} mg^{-1} protein; Figure 6).

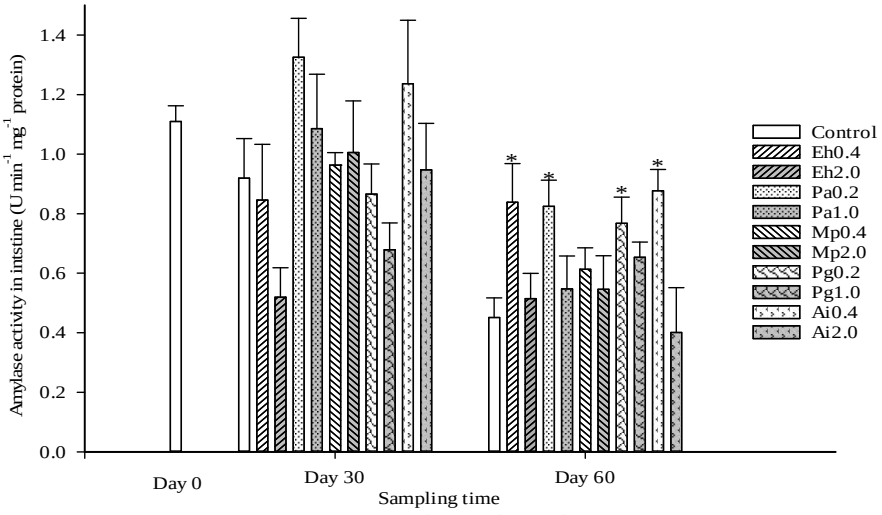

Figure 6. α-amylase activity in the stomach (U^{-1} min^{-1} mg^{-1} protein) of *P. hypophthalmus* extract-based diets in a 60-day experiment. Value = mean±standard error, with the asterisk (*) in the column of each sampling period indicating the significant difference compared to basal diet ($p < 0.05$).

Effects of plant extracts on growth performance and the survival rate. Weight gain (WG, g) and daily weight gain (DWG, g day^{-1}) were significantly higher ($p < 0.05$) in fish fed Pa 0.2 (25.0±0.5 g and 0.417±0.008 g day^{-1}, respectively), followed by those fed Pg 0.2 (24.1±0.3 g, 0.402±0.005 g day^{-1}, respectively), than the fish fed Ai-diets and the basal diet on day 60. In contrast, the survival was significantly lesser in the Ai 2.0-incorporated diet (59.1%) than in the remaining groups at the end of the experiment (Table 3).

Table 3

Growth performance of *P. hypophthalmus* fed plant extract-based diets in a 60-day experiment

Plant extract	*WG (g)*		*DWG (g day^{-1})*		*Survival rate (%)*	
	Day 30	*Day 60*	*Day 30*	*Day 60*	*Day 30*	*Day 60*
Control	5.70±0.30	13.8±0.60	0.19± 0.01	0.23±0.01	89.8±1.60	80.9±1.60
Eh 0.4	6.00±0.40	14.4±0.50	0.20±0.01	0.24±0.01	91.1±0.90	82.2±1.20
Eh 2.0	5.40±1.10	13.1±1.40	0.18±0.04	0.22±0.02	87.6±3.80	80.0±2.70
Pa 0.2	12.0±0.60*	25.0±0.50*	0.40±0.02*	0.42±0.01*	92.0±0.00	84.0±0.00
Pa 1.0	9.10±0.40*	18.7±0.60*	0.30±0.01*	0.31±0.01*	92.0±0.00	84.0±0.00
Mp 0.4	7.90±0.10*	17.7±0.30*	0.27±0.01	0.29±0.01*	92.0±0.00	84.0±0.00
Mp 2.0	7.50±0.50*	17.1±0.30*	0.25±0.02	0.29±0.01*	91.1±0.90	83.1±0.90
Pg 0.2	11.5±1.40*	24.1±0.30*	0.38±0.05*	0.40±0.01*	92.0±0.00	81.8±2.20
Pg 1.0	9.90±1.20*	21.2±1.20*	0.33±0.041*	0.35±0.02*	92.0±0.00	84.0±0.00
Ai 0.4	5.20±1.10	12.7±0.30	0.17±0.04	0.21±0.01	91.6±0.40	80.0±2.80
Ai 2.0	4.10±0.30	10.2±1.60	0.14±0.02	0.17±0.03	89.8±1.60	59.1±3.10*

Notes: value = mean±standard error, with the asterisk (*) in the column of each sampling period indicating the significant difference compared to basal diet ($p < 0.05$).

Discussion. Based on an *in vitro* screening of head-kidney white blood cells and peripheral blood mononuclear cells, Nhu et al (2019) suggested that Eh, Pa, Mp, Pg, and Ai probably enhanced the immunity of *P. hypophthalmus*. Furthermore, Dao et al (2020) classified antioxidant activities as high (IC_{50} < 30 µg mL^{-1}) followed by intermediate (30 < IC_{50} < 50 µg mL^{-1}) and absent (IC_{50} > 70 µg mL^{-1}) and antibacterial activity as high (MIC < 100 µg mL^{-1}), intermediate (100 < MIC ≤ 625 µg mL^{-1}) followed by weak (MIC ≥ 625 µg mL^{-1}). Based on these classifications, the five extracts were categorized into three active groups as follows: (i) Pa, Pg, and Eh with high antioxidant and moderate antimicrobial activity and eliciting a high immune response; (ii) Mp with high antioxidant and weak antimicrobial activity and inducing a low immune response; and (iii) Ai with no antioxidant or antimicrobial activity and eliciting a high immune response. Our study revealed that the dietary administration of extracts of these five plant species modified a large set of haematological parameters and digestive enzyme activities, as well as the growth performance of *P. hypophthalmus*.

Haematological parameters provide useful information on the status of RBCs and the ability to transport oxygen in the blood; therefore, the regular testing of these indicators is especially recommended for farming fish to assess stock's health (Haghighi et al 2014). In addition, Roberts & Rodger (2012) stated that the number and types of WBCs are critical factors in non-specific immune responses to adverse conditions (e.g. infection, nutritional deficiency, inappropriate stocking density, and environmental stress).

The improvement of Hb and Hct by plant extract-based diets was probably triggered by the activation of the erythropoiesis process, which enhances oxygen transport capacity and aids gas exchange through the gills of fish (Nugroho et al 2017). Nugroho et al (2019) administered a *Myrmecodia tuberosa* Jack leaves extract to *P. hypophthalmus* for 80 days and showed a significantly higher Hb level at 0.5-1% concentration of the extract. Supplementation with 0.02% commercial *Yucca schidigera* and *Quillaja saponaria* extracts (Nutrafito Plus®) of diets of *P. hypophthalmus* significantly elevated Hct and Hb (Güroy et al 2016). Similarly, Omitoyin et al (2019) performed that a *P. guajava*-based diet considerably stimulated Hb and RBCs of *Oreochromis niloticus*. Furthermore, a substantial dose-dependent elevation in Hct was also found for these diets, while values for the 1% Pg group were not significantly different from those in the basal diet group. Pandey & Shweta (2011) indicated that leaves contain high levels of anthraquinones, quercetin, phlobatannins as well as cardiac glycosides. Moreover, the improvement in these parameters may be attributed in part to the iron content of the plant extracts, as iron is an erythropoietic agent (Okwu 2005).

The improvement of WBCs in this research is in agreement with the significantly increased WBCs of *O. niloticus* fed *Camellia sinensis* L. in the diet which suggested that this plant enhanced haematological function (Abdel-Tawwab et al 2010). Rainbow trout

(*Oncorhynchus mykiss*) fed diets enriched by various doses of *Melissa officinalis* showed no differences in RBC, but WBC improved significantly at both doses of 0.1 g kg^{-1} and 0.5 g kg^{-1} of plant extract through 45 days intervals (Bilen et al 2020). Plant extracts contain active phytochemicals – flavonoids – which enhance immune function by preventing cell damage and strengthening immunological capacity through the employment of antioxidant enzymes (Saroja et al 2012). Lyu & Park (2005) found that flavonoids from herbal extracts regulate cytokines, such as interleukin and interferon, which can operate as biocatalysts in the formation of WBCs as part of non-specific cellular immunity to trigger via interacting with the immunological receptors system on the cell wall and promoting the expression of internal genes. Interestingly, another possible reason for the increase in WBCs in treated groups is leucopoiesis, particularly lymphopoiesis, as a reaction to boost immunity (Dada & Ikuerowo 2009).

The level of stress in fish has been assessed basing on plasma glucose concentration as it is altered in stressed fish (Cho et al 2009). A high plasma glucose concentration characterizes the metabolic profile of the blood of fish fed the control diet. In contrast to Hernández et al's (2015) results for gilthead seabream (*Sparus aurata*), the lowest glucose level in this treatment was 1,200 mg extract kg^{-1} feed (72.8 mg dL^{-1}). The hypoglycaemic influence of rosemary extract's antioxidative and protective activity on mammalian β cells, which maintains insulin production, has been attributed (Bakirel et al 2008). Bakirel et al (2008) obtained corresponding results for aquatic animals fed herbal immuno-stimulant diets; their glucose level declined probably due to improved biochemical and oxidative pathways, particularly those found in pancreatic cells resulting in variation in insulin production.

Digestion is a critical function in animal physiology because it regulates the absorption of nutrients essential for all biological mechanisms. Moreover, it is a key component in nutritional research and the adaptability of the organism to dietary changes (Gisbert et al 2009). A previous study found that chymotrypsin of *P. hypophthalmus* enhanced with supplementation of the diet with Eh 0.4, Pa 0.2, and Pa 1.0, while Pa 0.2, Pa 1.0, and Pg 1.0 improved pepsin activity in the stomach. In the case of amylase, the administration of Eh 0.4, Eh 2.0, Pa 0.2, Pg 0.2, and Ai 0.4 improved the digestibility of *P. hypophthalmus*; however, in the present study, no significant enhancement in trypsin activity were obtained in the intestine under all diets. The findings of these studies indicate that plant extracts may have beneficial implications on fish digestive enzyme activity and corroborate previous research. The results for *P. hypophthalmus* fed an *Eleutherine bulbosa*-based diet suggested that the diet with extract inclusion at concentrations of more than 30 g kg^{-1} was effective in enhancing amylase activity of fish over four weeks (Nugroho et al 2018). Awad et al (2012) studied rainbow trout, which were given three kinds of plant extract, 2% *Lupinus perennis*, 2% *Urtica dioica,* and 1% *Mangifera indica*; the fish showed improved amylase and pepsin activity in the stomach. Wang et al (2018) studied *Lateolabrax japonicus* given a mixture of Chinese herbs for four weeks and recorded the enhanced pepsin activity of 4 and 8 g kg^{-1} groups and amylase activity in 8 and 12 g kg^{-1} of herb enrichment. Amylase activity was also improved with the inclusion of *Apium graveolens* (0.1, 0.5, or 1%) in the diet of common carp (*Cyprinus carpio*; Mohamed et al 2018) and *Astragalus* extracts (polysaccharides) at a level of 1,500 mg kg^{-1} in *O. niloticus*'s diet (Zahran et al 2014). In these cases, high amylase activity in treatments with plant extract supplementation was possibly related to the active ingredients in the plant which stimulated the activity of the gastrointestinal tract and accelerated the duration substances passed through the tract and quickly discharged (Khan et al 2012).

Furthermore, this investigation revealed that high rates of inclusion of plant extracts may not necessarily show increased effectivity. Awad et al (2012) suggested that 1% mango extract led to higher amylase and pepsin activity in *O. mykiss* than the 2% dose. Generally, diets supplemented with *Aloe vera* powdered extracts (0.5% and 1.0% kg^{-1} feed) resulted in significantly improved intestinal and gastric amylase activity of *O. niloticus* (GIFT strain) juveniles compared to the control and 4% diet (Gabriel et al 2017). Similarly, the juvenile hybrid tilapia (*O. niloticus* × *O. aureus*) 's diet-related *Camellia sinensis* L. waste supplementation for eight weeks demonstrated that amylase

activity diminished in the intestines of fish fed a high dose of extracts (3.2% and 6.4%; Zheng et al 2017).

Although several plants extract might enhance amylase (Eh, Pa, Pg, and Ai), trypsin activity is not improved as shown by a study on ginger (*Zingiber officinale*) extract; amylase activity increased at a low level (0.1%) of supplementation but an effect on trypsin activity was not found in *Mesopotamichthys sharpeyi* fingerlings (Rahimi et al 2015). This may be due to the stimulation of the autonomous nervous system (ANS) by active compounds in plant extracts and the large changes in cholecystokinin activity stimulating the intestine and pancreas (Cudennec et al 2008). Furthermore, this leads to excessive secretion of amylase into the intestine and improved amylase activity, as well as the activity of amylase being altered more than that of proteases including trypsin (Tengjaroenkul et al 2000). Nevertheless, some bioactive compounds in plant extracts, saponins, phenols, and tannins, for instance, might trigger the release of digestive enzymes (Omitoyin et al 2019). The bioactive compound may be an anti-nutrient substance that inhibits digestive enzyme activity. Normally, anti-nutrients, which are ubiquitous in plants, can have both positive and detrimental health effects. Common types of anti-nutrient substances include polyphenols such as flavonoids and phenolics (tannins), terpenoids such as saponins, and amylase and protease inhibitors (Joseph & Priya 2011).

Significant improvements in essential digestive enzymes activities were observed in this study, indicating that supplementation with Pa, Mp, and Pg stimulated the secretion of these enzymes, which enhanced nutrient digestion, resulting in higher survival rates and growth of *P. hypophthalmus*. The delivery of extract-based regimens facilitating growth performance was previously documented in a fish in an interval- and dosage-dependent manner. Similar patterns have been observed in *P. hypophthalmus* given diets with 0.2% Pg and 0.5% Pa (Nhu et al 2020). Gobi et al (2016) indicated a substantial improvement of *Oreochromis mossambicus*'s final weight following administration of a 1% *P. guajava* extract-based diet for 30 days. The ultimate maximum weight of *O. niloticus* was achieved with a 0.75% *P. guajava*-augmented diet over an 84-day feeding period (Omitoyin et al 2019). The survival rate did not seem to vary considerably between the experimental and the control groups, providing compelling evidence for the plant extract's nontoxic effects on lower vertebrates, as also is the case for higher vertebrates as stated by Prasad & Priyanka (2011). The presence of anti-nutrient components, a characteristic of Ai which has high quantities of these compounds, may have contributed to the poor growth and survival rate of the groups fed the Ai-supplemented diet. Anti-nutrient substances have been demonstrated to alter animal gastrointestinal tracts. Moreover, they may have a detrimental influence on feeding efficiency and digestion in fish, resulting in a reduction in nutrient absorption. This leads to reduce pellets consumption, which culminates in the sluggish growth performance of fish (National Research Council 2011).

Conclusions. The enhancement of haematological parameters in conjunction with increased digestive enzyme activity may contribute to the improved health of *P. hypophthalmus* following the intake of a diet supplemented with plant extracts at different doses and feeding durations. A diet including Pg 0.2 and Pa 0.2 extracts administered for 60 days has the potential to modify hematology, enzymatic activity as well as the growth of *P. hypophthalmus*. Hence, the preference of fish for various plant extract-based diets should be assessed, as these results could demonstrate high use and intake of aquafeed which may lead to improve growth performance of fish.

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Conflict of interest. The authors declare that there is no conflict of interest.

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