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Effects of Psidium guajava and Phyllanthus amarus extracts on digestive enzyme activity and growth of *Pangasianodon hypophthalmus* fingerlings under high-temperature stress

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ABSTRACT

The rise in water temperature by global warming is of high concern to aquaculturists. In this study, the effects of extracts-based diets on digestive enzymes, and growth performance in Pangasianodon hypophthalmus fingerlings under elevated temperatures were investigated. Four distinct diets (control, Psidium guajava, (0.2%/kg) – Pg0.2, Phyllanthus amarus (0.5%/kg) – Pa0.5, and a mixture of Pg0.2 and Pa0.5 - Mix.) were administered to fish fingerlings for 42 days, followed by 4 days of temperature elevation. Fish were then continuously subjected for 42 days to temperatures of 27, 31, and 35°C to evaluate enzymatic activities and growth performance of fish. The results showed that although there is no interaction between two experimental factors on digestive enzyme activity and growth performance of fish, Pg0.2 followed by Mix groups accelerated digestive enzymes (trypsin, chymotrypsin, amylases and pepsin). Besides, enzymatic activities increased from 31°C to 35°C. The highest growth was observed from fish at 35°C followed by those at 31°C ($p<0.05$) which was significantly higher than the control (27°C); however, there was no significant difference in survival rate. In conclusion, these findings suggested 2 appropriate diets (Pg0.2 and Mix) for optimizing growth of this species and consequently contributing to the sustainable aquaculture under the global warming scenario.

1. INTRODUCTION

Having an export turnover of over $1.62 billion and a total production of 1.52 million tonnes in 2021, the striped catfish (*Pangasianodon hypophthalmus*) is one of the most significant species in the Vietnamese Mekong Delta (Ministry of Agriculture and Rural Development [MARD], 2022). Nonetheless, the production and export of *P. hypophthalmus* face numerous challenges, with climate change emerging as one of the most significant and difficult-to-manage obstacles due to its direct impact. According to Allen et al. (2018), the global mean surface temperature surpassed 1°C over pre-industrial levels in 2017. If significant greenhouse gas emissions are not reduced, the temperature is expected to exceed 1.5°C and 2°C during the twenty-first century (Intergovernmental Panel on Climate Change [IPCC] 2021). Fish make up poikilotherms which are hypersensitive to in the

different ambient temperature (Fry, 1971). The impacts of climate change resulting in escalating temperature levels are believed to be multifaceted, and the ambient temperature range encountered by each species can have substantial influences. Most fishes may adjust to some alterations, but substantially shifts may adversely influence fish growth performances, and survival rates, and trigger undesirable changes in biological response (Kemp, 2009; Tobin & Wright, 2011). Studies on fish digestive enzyme activity might give insight into some aspects of fish nutrition and assist in the alternative of nutritional issues including diet preference and fish nutritious potential (Furné et al., 2008).

The application of phyto-additives whether in single form or a mixture has been known to promote the activity of digestive enzymes in fish and might explain specific aspects of fish nutritional metabolism and assist in resolving nutritional concerns such as food suitability and fish nutritional aptitude, resulting in the greater economic expansion of the aquaculture industry (Gabriel et al., 2017; Shabana et al., 2019; Bilen et al., 2020). The potency using plant extracts such as *Psidium guajava L.* and *Phyllanthus amarus* Schum and Thonn in fish farming were reported, demonstrating improvements in antimicrobial activities and the immune response of fish species (Direkbusarakom et al., 1998, Pachanawan et al., 2008; Ferdous et al., 2017; Nhu et al., 2019; Dao et al., 2020; Nhu et al., 2020a, 2020b). Vietnam is a rich reservoir of herbal medicines, but publications on the benefits of commonly found medicinal herbs in stress reduction and improving the growth of farmed fish remain fragmented. There is currently rather restricted evidence on the activity of the digestive enzymes of fish and growth performance, notably *P. hypophthalmus* under increased stress temperature. To assess the potential correlation between temperature and diet in modifying digestion in *P. hypophthalmus*, a study on the effect of different rearing temperatures and plant extract-based diets on digestive enzyme activities and growth performance in fingerlings was carried out. The aim was to provide insights into enhancing growth performance and efficiency, thereby contributing to the mitigation of the impacts of climate change.

2. MATERIALS AND METHOD

2.1. Plant extracts and the processing of feed

Fresh specimens of *P. amarus* (leaves, twigs) and *P. guajava* (leaves) from a local farm in Can Tho City, Vietnam were authenticated and processed at the laboratory of the Department of Biology, College of Natural Science, Can Tho University. The extracting process was outlined in Nhu et al. (2020b), which were then preserved in screw-tight glass vials at -20°C until use.

Table 1. components for experimental feed

Ingredients (g in 100 g feed)	Basal diet	Supplementary plant extract diet		
		0.2%	0.5%	Mixture
Soybean meal	24	24	24	24
Rice bran	29.5	29.5	29.5	29.5
Cassava	17.9	17.8	17.5	17.3
Fish meal	24	24	24	24
Fish oil	1	1	1	1
Premix*	3	3	3	3
Phytase	0.02	0.02	0.02	0.02
Carboxymethyl cellulose (CMC)	0.5	0.5	0.5	0.5
Butylated hydroxytoluene (BHT)	0.02	0.02	0.02	0.02
Pg0.2 extract	0	0.2	-	0.2
Pa0.5 extract	0	-	0.5	0.5

** Premix: 0.5% MCP, 0.03% vitamin C, 1% attractant, and a combination of vitamins and minerals. After weighing and combining fishmeal, soybean meal, cassava, and rice bran, the mixture was sterilized for 10 minutes at 110°C (because the machine lowers its temperature to 70°C, the sterilizing process may take 60 to 90 minutes). Fish oil was blended with the extract, powder, BHT, premix, and CMC after they had been well mixed and sterilized. After that, the complete mixtures were extruded through a mini-extrusion machine (size 2 mm) at 70°C without steaming, all the feeds were mixed and cut manually. The trial feeds were later dried at 60°C until moisture content was 11-12%, the estimated drying time was 24 hours (the dryer has a capacity of 10kg). Before usage, all dried feed was kept at -20°C. The same procedures were followed, but without extract, in the control diet.*

The extract was added as a supplement to the fish diet including *P. guajava* (0.2%/kg) – Pa0.2, *P. amarus* (0.5%/kg) – Pa0.5, and a mixture of the two mentioned extracts - Mix. The experimental concentrations of extracts were based on the consequences on the effectiveness of these extracts in modifying the immune biological mechanisms and microbial activity of this species and suggested efficient synergistic implications of liver proteome profile associated with immune procedures (Nhu et al., 2020b). Plant materials for four above-mentioned treatments including control, Pg0.2, Pa0.5, and Mix were manually prepared, and all experimental diets were isoproteic, isolipidic, and isoenergetic (Table 1). The diet without supplementation was served as the control. All materials were well mixed and then pelletized, air-dried, processed, and sieved into pellet form (2 mm). The pellets were subsequently packed into well-labeled sample bags and maintained at -20°C till further usage.

2.2. Fish acclimation, facility, and feeding

This experiment was performed on a uniform fish group sourced from a local seed production farm (Can Tho, Vietnam). The fish were housed in fiberglass 2000-L tanks for acclimation for 14 days with well-aerated water on a natural photoperiod and supplied on an available prepared basal diet to satiation (to 3-5% of body weight). Before the initiation of the feeding trial, the fish were fasted for 24 hours. The experiment was conducted for two periods: (i) In the first period, after acclimation, 2700 healthy fingerlings (12.1±0.04 g) were randomly arranged in 36 composite tanks (300 L within 500 L of capacity) with 75 fish/tank and 9 replicates for each of four groups (P0 (control), Pg0.2, Pa0.5 and Mix). In this period, fish were fed twice a day to satiation with their experimental diets for 42 days before exposed to different temperature. After 42 days of being fed with different diets, fish weights were 18.9±0.38 g in the control treatment, 21±0.26 g in the Pg0.2 treatment, 22.1±0.4 g in the Pa0.5 treatment and 22.2±0.35 g in the Mix treatment; (ii) In the experimental temperature period: A 3 x 4 factorial design was distributed to evaluate the effects of 3 temperatures (27°C (control), 31°C, and 35°C) with 4 mentioned diets (Control, Pg0.2, Pa0.5, and Mix).

The temperature was progressively increased (Δ1°C per 24 hours on the groups of 31°C and Δ2°C per 24 hours on the 35°C groups) from 27°C (ambient temperature). The required temperature was maintained by using thermostats and heaters. After reaching the desired levels (0H), the fish from the feeding trial were cultivated under the three experimental temperatures for 42 days at the density of 45 fish/tank. Each treatment (n=3) was triplicated (36 tanks). Fish were fed twice a day with designated diets until satiation (fish stopped actively eating and leftovers remained at the bottom of the tanks for over 2 mins). Regarding digestive enzymes, the stomachs and intestines were obtained on the day which served as 0-hour (0H), day 7th (D7), day 14th (D14), and 42nd (D42).

Water in culture tanks was well-aerated and the environmental factors and routine management were the same as those during the acclimatization period. Fingerlings were fed to satiation twice a day (7:00 and 16:00). Culture water was replaced every week (approximately 30%) with fresh water, alongside daily siphoning. pH was measured weekly using a pH meter (Metler Toledo SG2, USA), while dissolved oxygen (DO) concentration was measured using a digital Oxy Guard H04PP device (Oxy Guard, Denmark). The DO (3.65 to 4.40 mg/L) and pH (6.72 to 7.29) remained in suitable range for *P. hypophthalmus* farming, whereas there were just slight variations in temperature (±0.1°C) throughout the trial.

2.3. Digestive enzyme activities

Sampling

After 48 days of fasting to ascertain that fish stomach and gut were empty, three fish from each tank were dissected using sterile surgical equipment for the stomach (pepsin, amylase assays) and anterior part of the fish intestine (amylase, trypsin, and chymotrypsin assays). Residues in stomach and intestine were carefully removed. The samples were kept in labeled 1.5-mL tubes and stored at -80°C until analysis.

Homogenization

The stomach and intestine samples were weighed after being carefully defrosted. The intestines and stomachs of fish were homogenized by IKA T10 basic Ultra-Turrax homogenizer (Germany) in a buffer solution including KH2PO4 (20 mM), NaCl (6 mM) at pH 6.9. They were then centrifuged at 4200 g for 30 minutes, and the supernatant was collected and refrigerated at -80°C before being assessed for digestive enzyme activities.

Digestive enzyme activity assays

Pepsin was assayed using Worthington's (1982) method, which involved interacting 100 µL of material with bovine hemoglobin (Sigma-Aldrich) as a substrate in HCl 1N. The reaction was stopped by pipetting trichloroacetic acid (TCA; Sigma-Aldrich). It was then centrifuged at 4000 g for 10 minutes at 4°C, and the pepsin activity was measured at 280 nm. Using 15 µL samples with 10 µL 0.1 M BAPNA solution (Nα-Benzoyl-DL-Arginine P-nitroanilide in DMSO solution) and pH 8.2 phosphate buffer solution, trypsin activity was assessed by colorimetric technique, and the optical density of trypsin was recorded at 407 nm (Tseng et al., 1982). Chymotrypsin activity was measured in a sample solution containing BTEE (N-Benzoyl-L-tyrosine ethyl ester, Sigma-Aldrich) and buffer pH 7.8. The activity of the enzyme was monitored for five minutes at 256 nm (Worthington, 1982). Using maltose as a calibration curve, amylase activity was calculated. Amylase activity was measured at 540 nm, and one unit was defined as the quantity of enzyme that released one mmol of maltose in one minute (Bernfeld, 1951). According to Bradford's (1976) instructions, the total protein content in the samples were calculated calculated using diluted homogenates and bovine serum albumin as a reference. Units of specific enzyme activity per milligram of protein (U/mL/mg protein) were used to measure it.

2.4. Growth performance of *P. hypophthalmus*

To examine the growth of fish fed with different treatments, weight gain (WG), daily weight gain (DWG), specific growth rate (SGR), and survival rate (SR) were assessed using an electronic balance. All growth indices were performed using a formula developed previously by Abdel-Tawwab et al. (2015).

- WG (g) = Wt (g) – W_0 (g);
- DWG (g) = WG(g)/T;
- SGR (%/day) = $[(\ln W_t - \ln W_0) / (T)] \times 100$
- SR (%) = (Final number of fish/initial number of fish) × 100

Where: W_0 is the fish weight at the beginning (g); Wt is the fish weight at the experimental termination (g); and T is the number of days).

2.5. Statistical analysis

All statistical tests were performed using the Statistical Package SPSS 20.0 (IBM Corporation, Armonk, New York, USA) with $\alpha = 0.05$. All data was collected and the differences among groups of extracts at various temperature settings were investigated and displayed as mean ± SE (standard deviation of the error) in each parameter ($p<0.05$) to indicate significant differences. The effect of temperature, feed, and their interaction over the 42-day exposed to temperature was tested two-way ANOVA analysis. The Duncan post-hoc test was utilized to determine significant differences between treatments when $p<0.05$ was reached.

3. RESULTS AND DISCUSSION

3.1. Results

3.1.1. Impact of plant extracts on the digestive enzyme activities of P. hypophthalmus

Fish fed plant extract-based diets for 42 days showed no interaction impact between temperature and the activity of digestive enzymes (Figure 1). However, both experimental factors had independent impacts on the digestive enzyme activities of fish at different sampling times. For the pepsin activity, all the groups with extract-based diets had higher pepsin activities than those in the control at all the given sampling times ($p<0.05$). There were no considerable changes in pepsin activity across the three extract-supplemented groups at all sampling times except 0H with the lower pepsin activity being shown in the Pa group ($p<0.05$). The effects of temperature were found in the two later sampling times (D14 and D42), in which the pepsin activities increased with respect to raised water temperature. Fish cultured at 31°C and 35°C had significantly higher pepsin activities than the control (0.401, 0.373, and 0.331 U/min/mg protein, respectively ($p<0.05$)).

The pattern of significant effects of temperature and plant extract-based diets detected with stomach pepsin activity was also observed in stomach amylase activity with the exception visible in D42 where the Pg-extract diets had the highest gastric enzyme activity (6.93 U/min/mg protein) and were significantly different from the other supplemental groups ($p<0.05$) (Figure 2).

Regarding intestinal enzyme activities, there was a similar trend detected in amylase activity in this organ where all the supplementary groups had significantly higher activities than the control. However, the significant difference in intestinal enzyme activities among supplement diets was only detected in the D42 sampling, which the Pg group showed considerably higher activities ($p<0.05$) than those in the other two supplement treatments. Regarding temperature, it was affected by

temperature from day 7th to the end of the experiment. Intestinal amylase activity was highest at the group of 35°C (9.23 U/min/mg protein) and differed significantly from the other two groups ($p<0.05$) (Figure 3).

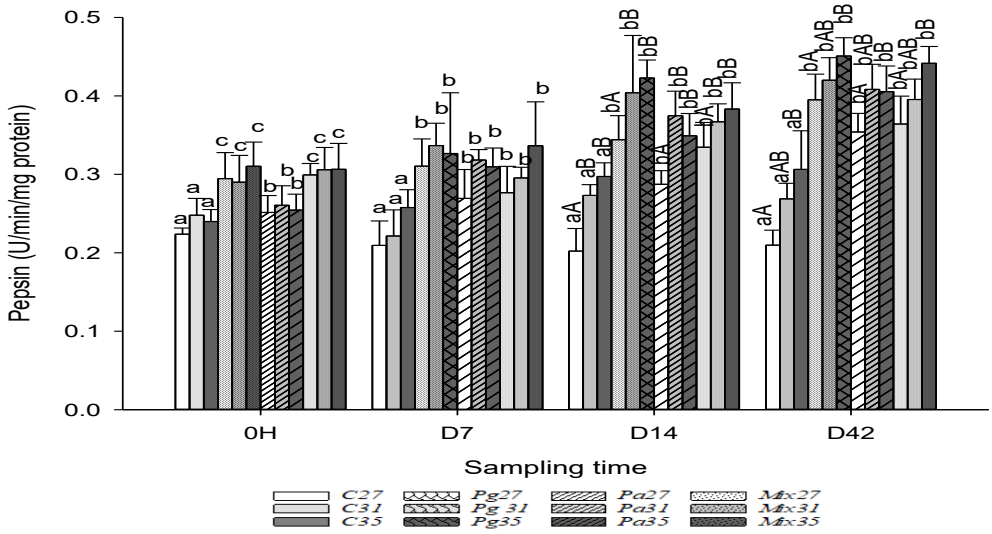

Figure 1. Pepsin activity in the stomach of *P. hypophthalmus* fingerlings fed extract-based diets and exposed to various temperatures for 42 days

Bars + SE with distinct uppercase alphabetic characters A, B... presented notable differences ($p<0.05$) between temperature levels while distinct lowercase alphabetic characters a, b,... showed notable differences within feeding groups ($p<0.05$).

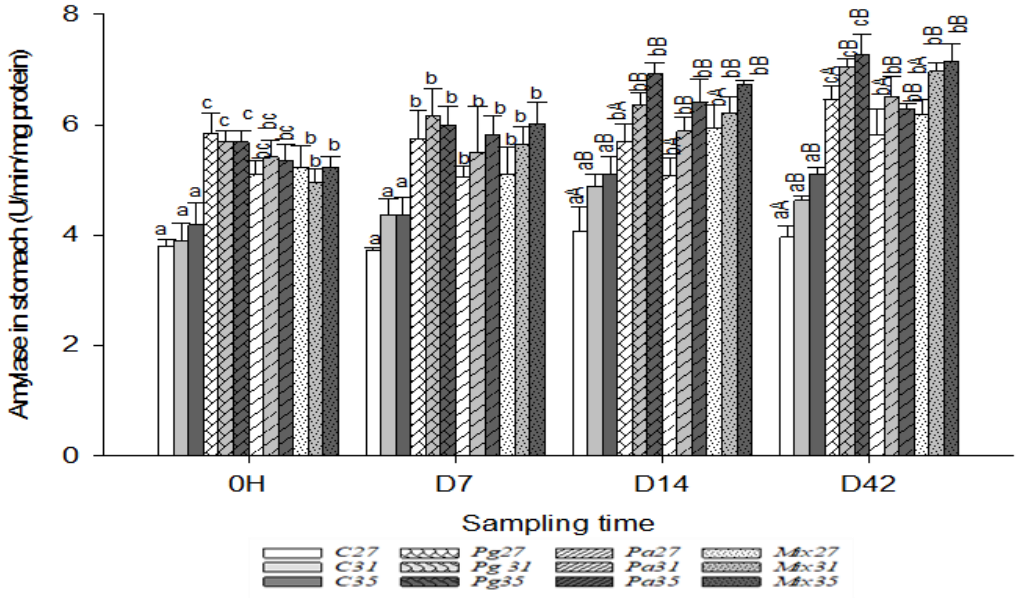

Figure 2. Amylase activity in the stomach of *P. hypophthalmus* fingerlings fed extract-based diets and exposed to various temperatures for 42 days

Bars + SE with distinct uppercase alphabetic characters A, B... presented notable differences ($p<0.05$) between temperature levels while distinct lowercase alphabetic characters a, b,... showed notable differences within feeding groups ($p<0.05$).

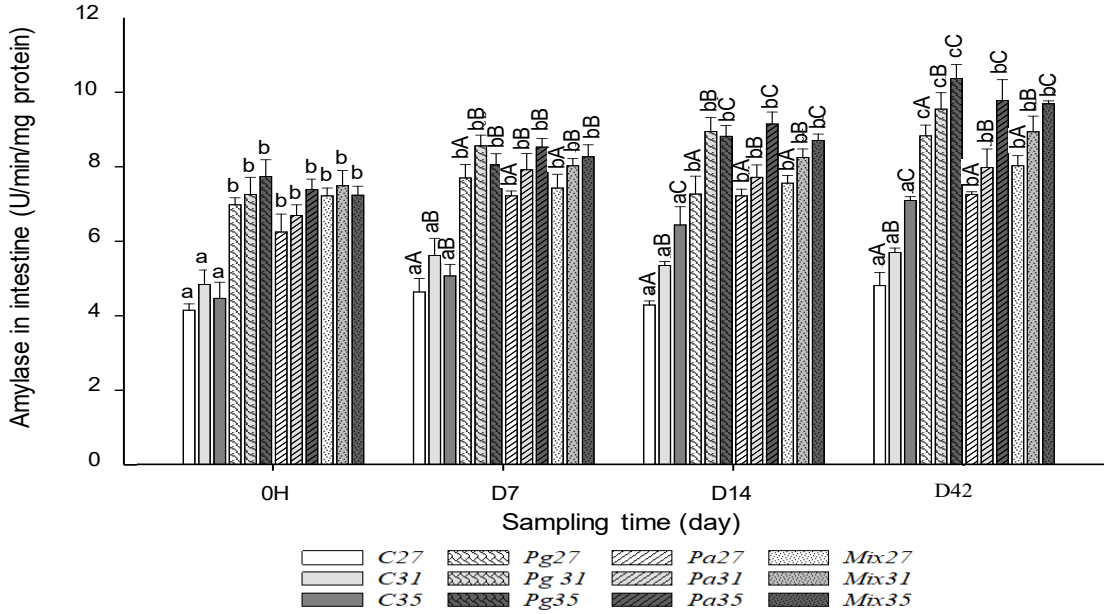

Figure 3. Amylase activity intestine of *P. hypophthalmus* fingerlings fed extract-based diets and exposed to various temperatures in 42 days

Bars + SE with distinct uppercase alphabetic characters A, B... presented notable differences ($p<0.05$) between temperature levels while distinct lowercase alphabetic characters a, b,... showed notable differences within feeding groups ($p<0.05$).

Trypsin and chymotrypsin activities in the fish intestine were also increased by plant-extract supplementation, which were all significantly higher than those in fish fed basal diets ($p<0.05$). However, differences in the intestinal amylase activities among the experimental diets were rather clear at all the sampling times except D14 (Figure 4, 5). Fish fed with Pg diets experienced enhancement in both intestinal trypsin and chymotrypsin activities.

With the only exception being visible in trypsin activities at 0H, trypsin, and chymotrypsin were influenced by raised water temperature at all given sampling times. Noticeably, both enzyme activities, after 42 days of being reared at elevated temperatures, were significantly higher in the 35°C group (chymotrypsin - 225 U/min/mg protein; 6.22 U/min/mg protein) and compared to 27°C and 31°C groups ($p<0.05$).

3.1.2. Effect of plant extracts on fish growth performance

The was also no evidence of an interaction between temperature and plant extract-based diets for growth rate and survival rate of striped catfish after 42 days ($p>0.05$). However, significantly higher values for WG (32.6 g), DWG (0.72 g/day), and SGR (2.08%/day) were achieved in the fish fed with a Pg0.2-based diet (Figure 6). In addition, the results revealed fish grew rapidly with WG, DWG, and SGR reaching 38.9 g, 0.864 g/day, and 2.36%/day, respectively at 35°C, followed by 31°C. These values were substantially greater than those of the control group ($p<0.05$).

The fish survival rates in all diets were over 83%, with the the highest survival rate being 89.4% (Pg0.2), while the lowest was in the control treatment (83.2%) ($p<0.05$, Figure 6). Two-factor ANOVA showed that all growth indices (WG, DWG, and SGR) and SR were unaffected by their interaction between feed and water temperature.

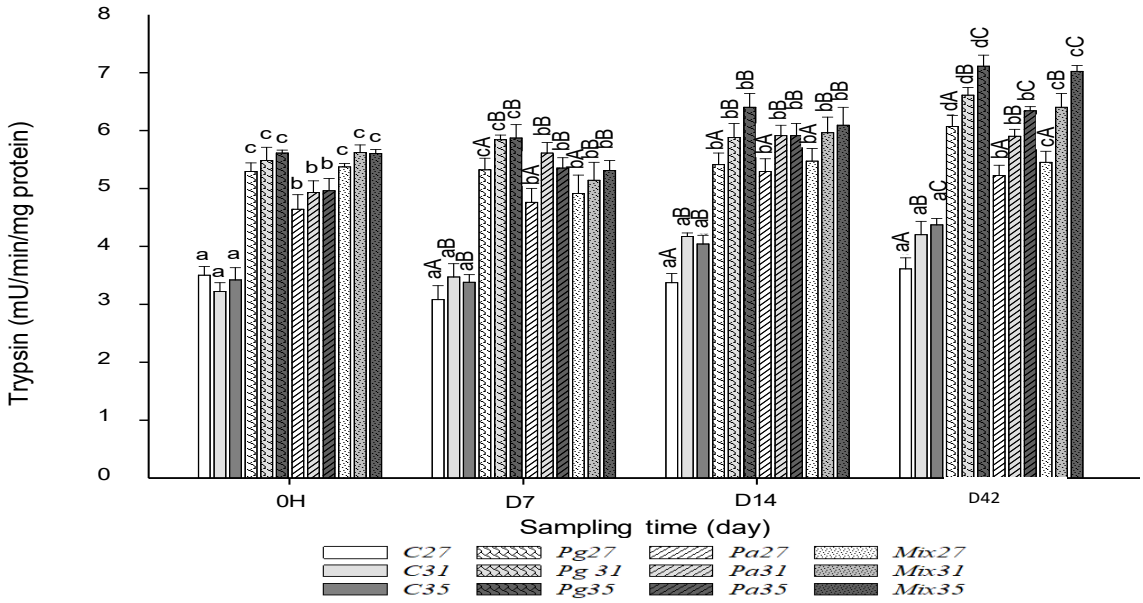

Figure 4. Trypsin activity intestine of *P. hypophthalmus* fingerlings fed extract-based diets and exposed to various temperatures in 42 days

Bars + SE with distinct uppercase alphabetic characters A, B... presented notable differences ($p<0.05$) between temperature levels while distinct lowercase alphabetic characters a, b,... showed notable differences within feeding groups ($p<0.05$).

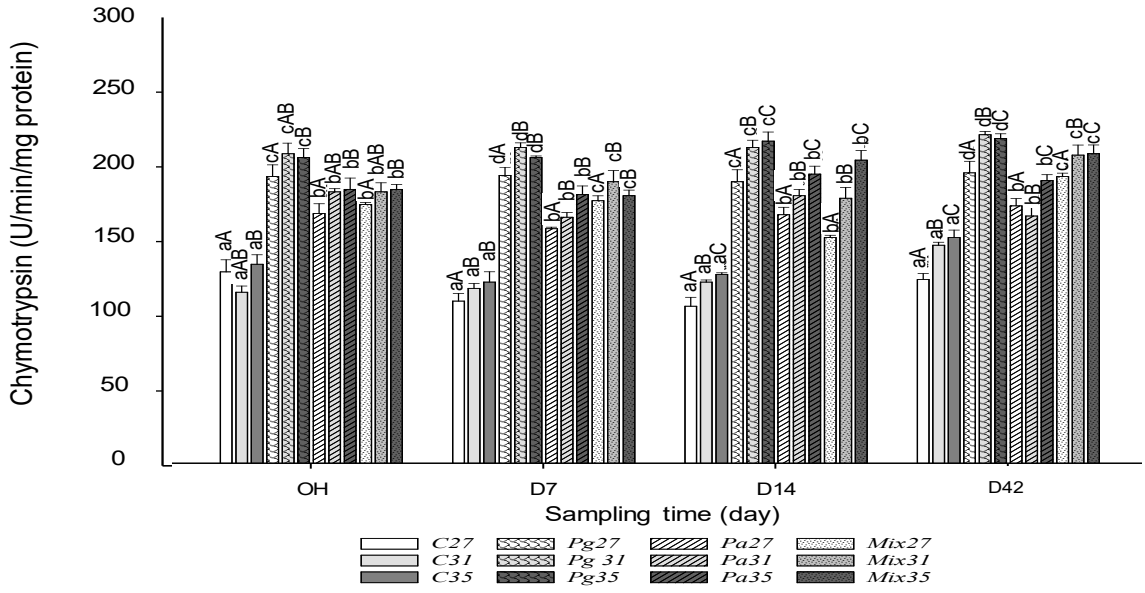

Figure 5. Chymotrypsin activity intestine of *P. hypophthalmus* fingerlings fed extract-based diets and exposed to various temperatures in 42 days

Bars + SE with distinct uppercase alphabetic characters A, B... presented notable differences ($p<0.05$) between temperature levels while distinct lowercase alphabetic characters a, b,... showed notable differences within feeding groups ($p<0.05$).

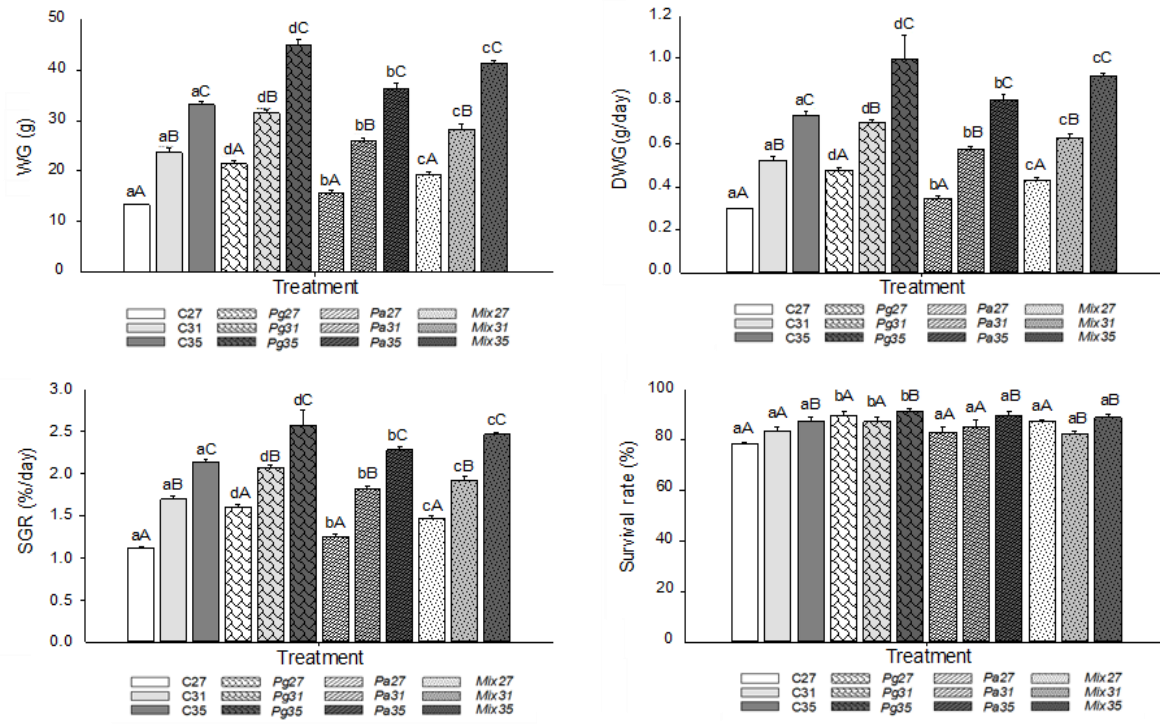

Figure 6. Growth performance (WG, DWG, and SGR) and survival rate of P. hypophthalmus fingerlings fed extract-based diets and exposure to various temperatures in 42 days

Bars + SE with distinct uppercase alphabetic characters A, B... presented notable differences ($p<0.05$) between temperature levels while distinct lowercase alphabetic characters a, b,... showed notable differences within feeding groups ($p<0.05$).

3.2. Discussion

Like other poikilothermic animals, temperature plays a significant role in the lives of fish, exerting both direct and indirect influences. They can be harmed by temperature shock, which can decrease their metabolic rate and impact swimming activities (Galloway & Kieffer, 2003; Hocutt, 1973), immune responses (Hurst, 2007), ability to catch prey (Ward & Bonar, 2003). These effects increase the likelihood of illness and death (Donaldson et al., 2008). Feed and temperature affect the activity of digestive enzymes, but there is no correlation among temperature levels and feeding diet on *P. hypophthalmus* under the effects of high temperature. The increase of digestive enzyme activity of *P. hypophthalmus* in this research was consistent with other research that digestive enzyme activity increased with the increase of temperature from 24°C to 33°C (Huong et al., 2020). Increased temperature, up to an optimum level, benefits aquaculture by shortening the time required to produce marketable size (Kita et al., 1996). According to Reece et al. (2014), enzyme activity increases as the temperature is elevated. The explanation for this is due to more collisions between substrates and active sites on the enzyme as the molecules move quickly. Moreover, amylase takes longer to break down carbohydrates at low temperatures (under physiological conditions) because food leaves the intestine more slowly (Munilla-Morán & Saborido-Rey, 1996). The ideal temperature conditions for digestive enzyme activities often coincide with the optimal thermal range for the fish (Volkoff & Ronnestad, 2020). In the current study, the activities pepsin in stomach, chymotrypsin and trypsin in intestines increased with the increase of temperatures from 27 to 35°C, which was consistent with the change of growth of fish. The current results are like the research of Gildberg and Raa (1983) with the highest pepsin activity in *Mallotus villosus* was reported at 38°C. The authors speculated that the efficiency of digestive enzyme activity improves under appropriate temperature, resulting in increases in fish growth performance.

In addition, it can be seen from the results that plant extract-based diets, especially Pg0.2 can positively influence the digestive enzyme activities. The effects of extracts on digestive enzyme activity were also showed in some previous results of Wang et al. (2017) on Japanese sea bass (*Lateolabrax japonicus*) and Gabriel et al. (2017) on GIFT tilapia. Another previous study revealed that the *P. guajava* extract's antimicrobial components, including phenols, saponins, and tannins, stimulate the production of digestive enzymes, which in turn improves *O. niloticus* biomass and specific growth (Abou-Zeid, 2002). According to Omitoyin et al. (2019), the increase in digestive enzyme activity exhibited enhanced capacity of fish in extract absorption, enhanced proliferation of liver cells, and normal cell functionality compared to the control. Moreover, the other research on *P. guajava* diets of Abdelhamid et al. (2012) indicated significantly gradual improvements in fish growth performance, and feed utilization by increasing *P. guajava* levels. Spontaneous contractions were suppressed by the P. guajava leaf extract (Lutterodt, 1989). In addition, potassium, which is found in guava leaves, is crucial for stoma motions, osmoregulation, enzyme stimulation, and cell proliferation (Fairbairn et al., 2000).

This study also conclusively revealed that high-temperature conditions had a beneficial effect on *P. hypophthalmus* growth indices such as WG, DWG, SGR, and SR. Based on the data, it appeared that *P. hypophthalmus* grew best at a temperature of 35°C, which produced the greatest WG, DWG, and SGR values and no appreciable drop in SR. The fish's growth rate was also at its highest when kept at 35°C. This is consistent with studies by some researchers and suggests that 35°C is close to the preferred or optimum temperature for this species. It is hypothesized that the increased temperature of 35°C led to the improved growth performance, which speeds up fish metabolism and allows them to consume more food than other treatments as would be expected under stressful circumstances. Killen (2011) revealed that in the desired temperature range, fish-catching behavior and digestibility are both enhanced. However, it was previously revealed that the majority of growth performance parameters at 36°C began to decline, indicating that energy may have been diverted away from growth to cope with thermal stress (Phuc, 2015). Thinh et al. (2014) had the same conclusion that the ability of the *P. hypophthalmus* to enhance as the temperature increases, but when the temperature exceeded (36°C), the fish's growth rate decreased in comparison to those at 34°C.

In addition, the Pg0.2-diet changes digestive enzyme activity between 31°C and 35°C. The diet supplemented with Pg0.2 and Mix improved digestive enzyme activity (trypsin, amylases and pepsin). Moreover, the highest values of the whole growth indices were indicated in the Pg0.2 groups after 42 days of feeding, asserting that *P. hypophthalmus* could grow faster when Pg was supplemented at the recommended dosage. This is consistent with the investigation of Nhu et al. (2020b) when *P. hypophthalmus* grew greatest in a Pg0.2-based diet, which was higher than Mix and Pa and significantly different from the basal diet. In addition, *P. guajava'*s effects have been proven in various studies. It has been shown that after just 30 days of feeding, a 1% P. guajava extract-based diet improved the growth performance of *O. mossambicus* (Gobi et al., 2016). Another research by Giri et al. (2015) concluded that rohu's growth performance in 60 days was significantly improved by *P. guajava* enriched diets at a high concentration (0.5%). After 84 days of feeding Nile tilapia *O. niloticus* fingerlings 0.5, 0.75, and 1% *P. guajava* extracts, the growth and nutritional indicators were improved significantly (Omitoyin et al., 2019).

4. CONCLUSION

This research again showed the effectiveness of supplementation of *P. amarus* and *P. guajava* into *P. hypothalamus* fingerlings diets by both single and mixed supplementation methods through increasing digestive enzymatic activities, resulting in improved fish growth performance. The present results also proved that this species can grow better at ambient temperatures of up to 35°C.

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