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Effects of Nevadensin from Rice Paddy Herb *(Limnophila aromatica)* on Growth and Physiological Parameters of Hybrid Catfish *(Clarias macrocephalus × C. gariepinus)*

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Abstract

This study examined the effects of dietary supplementation of nevadensin, a flavonoid isolated from rice paddy herb (*Limnophila aromatica*), on growth and physiological parameters of hybrid catfish (*Clarias macrocephalus* × *C. gariepinus*). Fish (initial weight of 5.00 ± 1.00 g) were fed with the diets containing 0 (control), 10, 30, and 50 mg nevadensin/kg feed for 8 weeks. Growth parameters, feed intake, and feed efficiency were significantly improved (P<0.05) by the diets containing 30 and 50 mg nevadensin. Survival rate and organosomatic indices for the intestine, gill, heart, kidney, and spleen were not significantly different (P>0.05) among the groups. However, the hepatosomatic index in fish fed with 30 mg nevadensin/kg diet was significantly increased (P<0.05) when compared with the control. A significant improvement (P<0.05) in villi heights, muscular thicknesses, goblet cells, and microvilli heights was detected in fish fed with nevadensin containing diets. Villi widths, total enterocyte heights and supranucleus heights decreased significantly (P<0.05) in fish fed with nevadensin supplemented diets. Red blood cells and white blood cells in fish fed with supplemented diets were significantly higher (P<0.05) than those in fish fed with the control diet. Hemoglobin and hematocrit significantly increased (P<0.05) in fish fed with 50 mg nevadensin/kg diet compared to the control. The highest level of lymphocyte was observed in fish fed with 10 mg nevadensin. Blood biochemical values were not significantly different (P>0.05) in any of the treated groups compared to the control. In conclusion, the results indicate that nevadensin could be used as a natural feed additive to improve somatic growth and physiological parameters of hybrid catfish. By using the second-order polynomial regression analysis, the optimal concentration of nevadensin for cultured catfish was estimated to be between 30.64 and 31.14 mg/kg diet.

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Introduction

Aquaculture products are recognized as a high-quality source of several essential nutrients for human consumption such as protein, omega-3, selenium, and vitamin D (Gupta, 2006). As an increase in worldwide demand for aquatic animals, intensified cultures have been developed to increase the production of fish (Van Hai, 2015). However, under intensive rearing conditions, farmed fish may expose to different stress conditions including water qualities, pathogens, and infectious diseases which have been reported to impair normal physiological functions (Pês et al., 2016). The use of antibiotics and synthetic compounds in aquaculture to improve fish growth and health is widely concerned due to their residues in fish products and surrounding environments (Gupta, 2006; Li et al., 2019). Additionally, inappropriate uses of drugs in fish cultivation could increase the relevance of drug-resistant organisms which may produce detrimental effects on both humans and animals (Abdel-Tawwab et al., 2018; Reverter et al., 2014; Zhou et al., 2015).

Natural additives obtained from plant-based products could be used to replace synthetic drugs or chemical compounds in aquaculture industries because of their lower side effects and toxicity (Adel et al., 2015; Amer et al., 2018; Hoseini et al., 2018; Wongtha et al., 2019). Herbal plants have long been used in folk medicine for a wide variety of many therapeutic proposes (Munglue, 2015; Munglue et al., 2019a). Several classes of bioactive compounds such as alkaloids, flavonoids, polyphenols, essential oils, terpenoids, and steroids were isolated and tested for their biological activities (Aanyu et al., 2018; Hoseinifar et al., 2020a; Jahazi et al., 2020; Villasante et al., 2016). In aquaculture, flavonoids from *Allium mongolicum,* curcumin, genistein, limonene, quercetin, cineole, thymol and rutin have positive effects on growth, nutrient assimilation and immunity of farmed fish (Aanyu et al., 2018; Amer et al., 2018; Hoseini et al., 2018; Jia et al., 2019; Li et al., 2019; Pês et al., 2015; Pês et al., 2016; Sruthi et al., 2018). However, the success of this approach depends largely on the correct doses, plant material forms, route of administration, optimal time of supplementation, life stages of fish and species-specific (Hoseinifar et al., 2020b; Van Hai, 2015; Zhou et al., 2015). Therefore, further investigations in this field are required.

Nevadensin (2-(4-methoxyphenyl)-5,7-dihydroxy-6,8-dimethoxy-4H-1-benzopyran-4-one) is a natural flavonoid that has been identified and isolated from many plant species including *Limnophila* species (Bui et al., 2004; Kukongviriyapan et al., 2007). It is considered as an effective substance that has been tested for its pharmacological potential and starting molecule in several drug development processes (Brahmachari & Gorai, 2006). It was indicated that nevadensin had an anti-hypotensive property in dog and cat models due to central and peripheral modulations (Song et al., 1985). Also, anti-inflammatory, anti-tumor, anti-cancer and anti-microbial properties of nevadensin have been identified by multiple scientific reports (Brahmachari et al., 2011; Chung & Geahlen, 1992; Yang et al., 1985; Reddy et al., 1991).

Hybrid catfish (*Clarias macrocephalus* × *C. gariepinus*) is an important freshwater fish that has been raised in several parts of Thailand because of its high growth rate and high market demand (Senanan et al., 2004). In 2017, the total production of catfish was around 105,144 tones and valued 5,126.6 million baht (Fishery Statistics Analysis and Research Group, 2019). Recently, catfish cultivation is now faced with uncontrollable disease outbreaks, various environmental changes and low water quality (Gabriel et al., 2019).

As the scientific data described above, nevadensin shows a variety of biological activities and more research on its pharmacological uses in both human and veterinary medicines are required. In aquaculture production sections, novel molecules that can be used as natural alternatives to synthetic compounds in the diets to improve growth performance, health, and general well-being of fish are also needed (Amer et al., 2018; Villasante et al., 2016). Thus, this research evaluated the effects of dietary nevadensin, a flavonoid isolated from rice paddy herb, on growth, feed efficiency, intestinal histology, hematology and serum biochemistry in hybrid catfish.

Materials and methods

1. Plant preparation, extraction, and isolation

Arial parts of rice paddy herb were harvested from Warinchumrap Distract, Ubon Ratchathani, Thailand, from August to September. A voucher specimen (Munglue 002) was kept at the Program of Biology, Faculty of Science, Ubon Ratchathani Rajabhat University, Thailand, for future reference. The plant samples were dried in a hot air oven at 60°C for 72 h and grounded into small pieces. Dried materials (1.8 kg) were macerated with

ethyl acetate for 72 h, filtrated with Whatman paper No.1, and the solvents evaporated using a rotary evaporator (Buchi, R-200, Switzerland) under reduced pressure, and dried using lyophilizer (Labconco Corporation, Missouri, USA). The crude extract (40.10 g) was chromatographed over silica gel (200 g). The column was eluted with *n*-hexane, *n*-hexane-ethyl acetate, ethyl acetate, ethyl acetate-methanol, and methanol, with a gradually increasing level of the more polar solvents, to give 17 groups of eluting fractions. The 6th subfraction (3.19 g) was then chromatographed over silica gel, eluted with *n*-hexane-ethyl acetate, ethyl acetate, ethyl acetate-methanol, and methanol, with a gradually increasing quantity of the more polar solvents, to give 6 subfractions. Subsequently, the 5th subfraction was subjected to column chromatography on silica gel and eluted with *n*-hexane-ethyl acetate, ethyl acetate, ethyl acetate-methanol, and methanol to afford 5 subfractions. The 3rd subfraction was further chromatographed to furnish nevadensin (0.0158 g) (Farkas et al., 1966; Suksamrarn et al., 2003). The characterization of nevadensin was performed by using ^{1}H and ^{13}C-Nuclear Magnetic Resonance (NMR). The data obtained from ^{1}H and ^{13}C-NMR by using dimethylsulfoxide as a solvent were ^{13}C: 182.2, 163.0, 162.3, 151.1, 148.3, 145.4, 131.6, 128.1, 128.0, 122.9, 114.7, 103.0, 102.8, 61.1, 60.0, and 55.5; ^{1}H NMR: 12.75 (s), 8.02 (d, J = 8.9 Hz), 7.14 (d, J = 8.9 Hz), 6.87 (s), 3.85 (s) and 3.76 (s). The chemical structure of nevadensin is presented in Fig. 1.

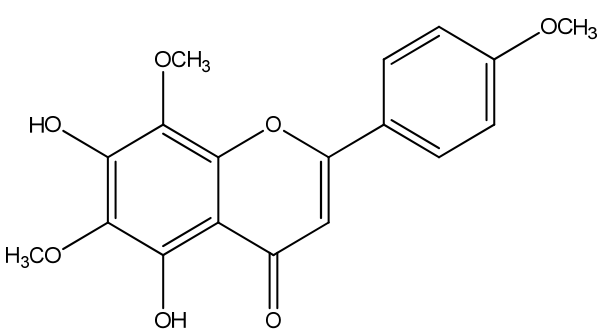

Fig. 1 Structure of nevadensin isolated from *L. aromatica*

2. Diet preparations

Fish diets containing 30% crude protein and 4% crude lipid were purchased from a local fish feed company (Centaco Group of Companies, Thailand). It is suggested that this protein level is recommended for the cultivation of catfish (Robinson et al., 2001). The range of nevadensin (0 (control), 10, 30, and 50 mg/kg diet) levels was determined according to the reports of Li et al. (2019) and Zhou et al. (2015). Each dose of nevadensin was mixed with the diets using cassava starch as a binder, moistened, minced, and oven-dried at 55°C for 24 h. The diet samples were kept in plastic bags at 4°C until use. The proximate composition of the experimental diets was analyzed according to the standard methods (Association of Official Agricultural Chemists [AOAC], 2012) and the results are shown in Table 1.

Table 1 Proximate analysis of the experimental diets

Nutritional composition expressed in dry weight basis (%)	Nevadensin levels in the experimental diets (mg/kg diet)			
	0	10	30	50
Moisture content	6.96	6.55	5.85	7.87
Ash	8.86	9.16	9.13	8.72
Crude protein	31.61	32.80	31.29	30.91
Crude lipid	5.21	5.26	5.06	5.13
Crude fiber	1.56	1.28	1.38	1.15
Nitrogen-free extract	45.80	44.95	47.29	46.22

3. Fish preparations

Animal procedures were performed according to the guidelines provided by the National Research Council of Thailand. Hybrid catfish were obtained from Ubon Ratchathani Fishery Cooperative, Ubon Ratchathani, Thailand and acclimatized under laboratory conditions for 2 weeks. The fish (initial weight 5.00 ± 1.00 g) were randomly divided into four treatments of three replications and cultivated in the circle cement tanks (90 cm in diameter and 50 cm in height) with 255 L of water (20 fish/tank). Fish were fed ad libitum twice a day at 08.00 and 16.00 h for 8 weeks. Water qualities were maintained in the optimal range for catfish farming (dissolved oxygen 7.00 ± 0.05 mg/L, temperature, 29.00 ± 2.00°C, and pH, 7.20 ± 0.50) and checked daily by using ExStik® EC500 (Extech Instrument Corporation, U.S.A.). Three-quarters of water in each tank was siphoned daily to remove fish waste and replaced by clean water from a storage tank. Dead fish were removed and noted.

4. Growth parameters and survival

After 8 weeks of the feeding period, four fish from each tank were collected and weighed. Growth parameters including weight gain (WG), specific growth rate (SGR), average daily gain (ADG), feed intake (FI), feed conversion ratio (FCR) and survival rate (SR) were quantified using the following equations (Gabriel et al., 2019):

WG (g) = final wet weight (g) – initial wet weight (g)

SGR (%/d) = 100 × [*In* final wet weight (g) – *In* initial wet weight (g)]/experimental days

ADG (g/d) = [final wet weight (g) – initial wet weight (g)] /experimental days

FI (g/fish) = feed intake (g)/number of fish

FCR = feed intake (g)/ weight gain (g)

SR (%) = 100 × (final number of fish/initial number of fish)

5. Organosomatic indices

In this present study, clove oil was used as an anesthetic for catfish. Due to clove oil is insoluble in water, it was firstly dissolved in ethanol at a ratio of 1:9 (clove oil:95% ethanol) to create a 100 mg/ml solution. This stock solution was then mixed with dechlorinated water in an anesthetic bath to obtain the final concentration of 100 mg/L (Fawole et al., 2020).

After 24 h of the fasting period, four fish from each replication were individually anesthetized in the anesthetic bath supplied with continuous aeration for 10 min and monitored for behavioral responses as previously described by Hamácková et al. (2006). After exposure, the abdominal wall of anesthetized fish was opened. The liver, intestine, gill, heart, kidney, and spleen samples of hybrid catfish were carefully obtained, cleared from adipose tissues, and weighed. Then, organosomatic indices including hepatosomatic index (HSI), intestinosomatic index (ISI), gill somatic index (GSI), renosomatic index (RSI), and spleen somatic index (SSI) were determined based on the following formula (Wongtha et al., 2019):

Organosomatic indices (%) = 100 × [organ weight (g)/fish wet weight (g)]

6. Blood collection and preparation

At the end of the experiment, fish fasted for 24 h before blood collection. Four fish from each replicate were individually anesthetized in the anesthetic bath with aeration for 10 min. The blood sample was collected from the caudal vessel of anesthetized fish using a sterile syringe and divided into 2 parts. One part of the blood was transferred to 3 ml tubes containing 10% EDTA (ethylenediaminetetraacetic acid) as an anticoagulant for hematological evaluation. Another part of the blood was allowed to clot for 3 h at room temperature in a 1.5 ml microcentrifuge tube and was centrifuged at 3000 rpm at 4°C for 10 min. The serum was collected and stored at -20°C for further analysis.

7. Hematology

Hematological studies were performed as the methods suggested by Campbell & Ellis (2007). Red blood cells (RBC), white blood cells (WBC), and platelets were counted by using a Neubauer hemocytometer. Blood smears were prepared and stained with Wright- Giemsa. Differential white blood cell counts were evaluated under a light microscope. Hematocrit (HCT) was determined by using the microhematocrit method. The hemoglobin concentration (HGB) was determined by using the cyanmethemoglobin method. The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were also calculated (Gabriel et al., 2015).

8. Serum biochemistry

The serum glucose level was determined by using Trinder's method (Barham & Trinder, 1972). Total protein content was measured by using the Biuret method (Gornall et al., 1949). Serum albumin was detected by using the Bromo Cresol Green (BCG) method (Leonard et al., 1971). Alkaline phosphatase (ALP) was analyzed using the method described by Tietz et al. (1983). Aspartate transaminase (AST) was evaluated according to Schumann et al. (2002). Triglyceride was estimated as reported by Cole et al. (1997). Creatinine content was tested using the Jaffe reaction (Bowers & Wong, 1980). Globulin content was calculated by subtracting the level of albumin from total protein (Valladão et al., 2017).

9. Intestinal histology

The intestinal samples were obtained from four fish of each replicate. They were cut at the end of the pyloric part of the stomach to 2 cm before the anus and subsequently divided into the proximal, middle, and distal parts. They were cleared from adjacent tissues, cleaned by using 0.90% normal saline, and preserved in 10% neutral buffered formalin. The specimens were subsequently dehydrated, embedded in the paraffin wax, cut into slices with a thickness of 5 µm, and mounted on the slides. The tissues were then stained by Hematoxylin and Eosin (H & E).

To evaluate the effects of nevadensin-enriched diets on intestinal macromorphology of hybrid catfish (Fig. 2A), villi height, villi width, total muscular thickness, inner circulatory smooth muscle, and outer longitudinal smooth muscle were observed by a light microscope and recorded on a computer using Dino Capture 2.0 software (Munglue et al., 2019a; Wongtha et al., 2019). Additionally, to study the effects on intestinal micromor-

phology (Fig. 2B), enterocyte height, supranucleus height, subnucleus height, nucleus height, nucleus width, goblet cell number, and microvilli height were measured as the method recommended by Escaffre et al. (2007).

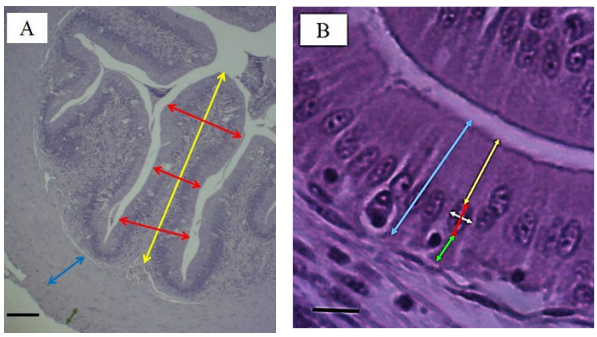

Fig. 2 Methodology for morphometric assessment of various parts of the intestine of hybrid catfish. (A) Intestinal macromorphology. Villi height (yellow line) was measured from the tip of the villi to the base, Villi width was measured from side to side of the villi at the top, the middle and the base and these values were then averaged to obtain a mean value of villus width, The thicknesses of the muscular layers including an inner circulatory layer (blue line) and an outer longitudinal smooth muscle (green line) were also evaluated. Scale bar = 200 µm. (B) Intestinal micromorphology. Measurement of enterocyte height (blue line), supranucleus height (yellow line), subnucleus height (green line), nucleus height (red line), and nucleus width (white line). Scale bar = 20 µm

10. Statistical analysis

Completely Randomized Design (CRD) was used in this research. The normal distribution of the data was evaluated using the Kolmogorov-Sminov test. To test the homogeneity of variances among the treatments, Levene's test was used (Pês et al., 2016). If the data were not normally distributed, the arcsin square-root transformation was established (Aanyu et al., 2018). Then, the data were analyzed by using one-way analysis of variance (ANOVA). To compare the differences between the treatments, Duncan's Multiple Range Test was used. The significant difference was set at $P<0.05$ and the data are represented as mean ± SEM. To estimate the suitable concentration of nevadensin for the cultivation of hybrid catfish, the second-order polynomial regression analysis was used (Yossa & Verdegem, 2015).

Results and discussion

1. Growth parameters and survival

As shown in Table 2, fish fed on 30 and 50 mg nevadensin/kg diet showed a significant increase ($P<0.05$) in the final weight, WG, SGR, ADG, and FI compared to the control. The final length of the treated fish was significantly higher ($P<0.05$) than that of the control fish. There was a significant improvement ($P<0.05$) in the FCR in fish fed nevadensin at 30 and 50 mg/kg compared with the control and those fed 10 mg nevadensin/kg diet. The SR levels were not significantly different ($P>0.05$) in all treated groups compared to the control and ranged from 99.16 to 100%. By using the second-order polynomial regression analysis on the FW ($y = -0.007x^2 + 0.436x + 21.755$, $R^2 = 0.133$, $P = 0.040$) and WG ($y = -0.007x^2 + 0.429x + 16.652$, $R^2 = 0.154$, $P = 0.023$), the optimal dosage of nevadensin for cultured catfish was found to be between 30.64 and 31.14 mg/kg diet (Fig. 3).

Table 2 Growth parameters and survival of hybrid catfish fed the diets supplemented with different levels of nevadensin for 8 weeks

Parameters	Nevadensin levels in the experimental diets (mg/kg diet)			
	0	10	30	50
IW (g)^{ns}	5.44 ± 0.17	6.22 ± 0.22	5.66 ± 0.16	5.11 ± 0.11
FW (g)	26.11 ± 1.84^{b}	28.55 ± 1.76^{ab}	32.66 ± 1.90^{a}	31.88 ± 1.67^{a}
IL (cm)^{ns}	8.77 ± 0.14	9.22 ± 0.12	8.88 ± 0.20	8.77 ± 0.16
FL (cm)	12.11 ± 0.77^{b}	16.22 ± 0.47^{a}	17.11 ± 0.51^{a}	17.22 ± 0.37^{a}
WG (g)	20.66 ± 1.78^{b}	22.33 ± 1.64^{ab}	27.00 ± 2.04^{a}	26.27 ± 1.68^{a}
SGR (%/day)	2.82 ± 0.11^{b}	2.75 ± 0.10^{b}	3.14 ± 0.15^{a}	3.28 ± 0.09^{a}
ADG (g/day)	0.36 ± 0.03^{b}	0.39 ± 0.02^{b}	0.48 ± 0.03^{a}	0.47 ± 0.03^{a}
FI (g/fish)	35.41 ± 1.02^{b}	37.14 ± 1.04^{ab}	38.44 ± 1.06^{a}	38.65 ± 1.01^{a}
FCR	1.72 ± 0.07^{a}	1.65 ± 0.09^{a}	1.42 ± 0.05^{b}	1.47 ± 0.08^{b}
SR (%)^{ns}	100.00 ± 0.00	100.00 ± 0.00	99.16 ± 0.83	100 ± 0.00

Remark: Data are represented as mean ± SEM. Different superscripts (^{a,b}) within a row are significantly different ($P<0.05$). Superscript ^{ns} indicates no statistical difference ($P>0.05$). IW = initial weight (g), FW = final weight (g), IL = initial length (cm), FL = final length (cm), WG = weight gain (g), SGR = specific growth rate (%/day), ADG = average daily gain (g/day), FI = feed intake (g/fish), FCR = feed conversion ratio, SR = survival rate (%).

The results of this study demonstrated that hybrid catfish fed different nevadensin diets showed a significant increase in growth performance, feed intake, and feed utilization compared to the control. No effects on SR values were observed in the tested fish compared to the control fish. Previous studies have shown that increased growth parameters of fish fed the diets supplemented with functional feed additives could be attributed to the improvement of nutrient digestion and absorption (Adel et al., 2015; Zheng et al., 2015). Additionally, the enhancement in feed intake and feed utilization efficiency by the feed additives supplemented diets could be associated with an increase of feed attraction and palatability in fish (Munglue, 2015; Reverter et al., 2014; Van Hai, 2015). Such reports suggested that medicinal

plants and their isolated phytochemicals have been reported to have positive effects on the growth and general health of several species of aquatic animals (Hoseini et al., 2018; Jahazi et al., 2020; Jia et al., 2019; Pês et al., 2015, 2016). It was demonstrated that juvenile northern snakehead fish (*Channa argus*) fed the diets incorporated with flavonoids from *Allium mongolicum* significantly improved growth rate, immunity, and antioxidant responses compared with the control (Li et al., 2019). Additionally, Nile tilapia (*Oreochromis niloticus*) fed dietary limonine showed a marked increase in FW and %WG (Aanyu et al., 2018). Jahazi et al. (2020) stated that common carp (*Cyprinus carpio*) fed the diets containing polyphenols extracted from chestnut and olive mill wastewater showed a significant improvement in growth indices including FW, SGR, WG and FCR compared with the control. The mechanisms of growth-promoting effects of herbal plants or their derivatives in fish have not yet been elucidated. It was speculated that phytochemicals could improve the expression of specific genes to modulate growth, feeding behavior, appetite regulation, digestive enzymes, nutrient digestion and metabolism of the fish including growth hormone, insulin growth factor (IGFs)-I and -II (Aanyu et al., 2018; Ahmadifar et al., 2019; Munglue, 2015; Sruthi et al., 2018). In this present research, fish that were fed with nevadensin diets showed significant increases in growth and feed utilization efficiency. As mentioned above, the growth-promoting property of nevadensin may be attributed to the improvement of feed palatability, feed intake, nutrient digestion and absorption as well as the modulation of some key genes (Ahmadifar et al., 2019; Jahazi et al., 2020; Munglue et al., 2019b; Sruthi et al., 2018). However, further research is required to clarify this hypothesis.

2. Organosomatic indices

Organosomatic indices of fish from different treatments are demonstrated in Table 3. It was found that HSI significantly increased ($P<0.05$) in fish fed 30 mg nevadensin/kg diet compared to the control and those fed 10 mg nevadensin/kg diet. There was no significant difference ($P>0.05$) in the HSI of fish fed nevadensin at 50 mg/kg diet when compared to the control. Additionally, ISI, GSI, CSI RSI and SSI did not differ significantly among the treatments.

Organosomatic indices are the indicators of nutritional and general health conditions in fish which can be calculated by the ratios of organs to body weight (Dekić et al., 2016). Studies of organosomatic indices in this research indicated that HSI of fish fed nevadensin at 30 mg/kg diet was significantly higher than that of the control fish. However, fish fed nevadensin supplemented diets did not show significant differences in ISI, CSI, RSI and SSI. The improvement in HSI could be due to increased hypertrophy and hyperplasia of hepatocyte, through improved cell metabolism, cell division and cell proliferation by nevadensin supplemented diet (Anderson et al., 1988; Strüssmann & Takashima, 1990). Thus, this could be the reason for an increase in the relative weight of the liver of fish fed nevadensin-enriched diets observed in this research.

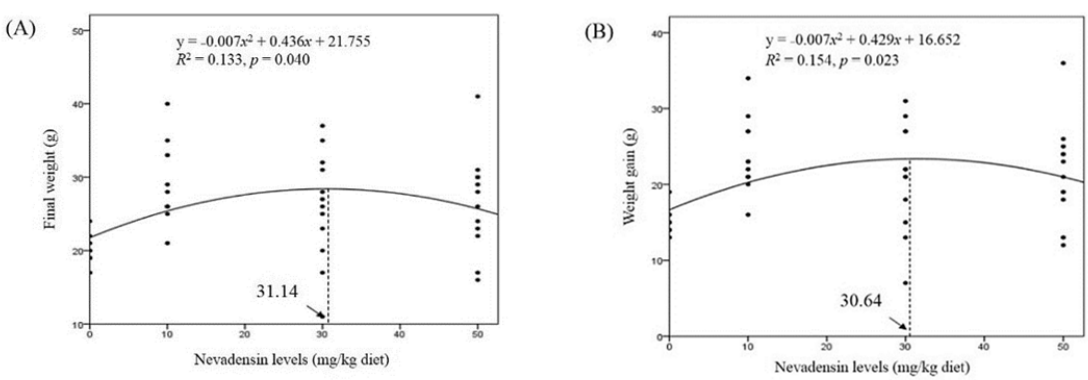

Fig. 3 The second-order polynomial regression analysis on (A) final weight (g) and (B) weight gain (g) of hybrid catfish fed the diets containing nevadensin at 0 (control), 10, 30, and 50 mg/kg diet for 8 weeks

Table 3 Organ somatic indices of hybrid catfish fed the diets supplemented with different levels of nevadensin for 8 weeks

Parameters	Nevadensin levels in the experimental diets (mg/kg diet)			
	0	10	30	50
HSI (%)	1.37 ± 0.05^{b}	1.43 ± 0.07^{b}	1.79 ± 0.01^{a}	1.61 ± 0.08^{ab}
ISI (%)^{ns}	2.70 ± 0.16	2.09 ± 0.25	2.24 ± 0.32	2.13 ± 0.221
GSI (%)^{ns}	4.20 ± 0.15	4.31 ± 0.50	4.63 ± 0.01	5.42 ± 0.18
CSI (%)^{ns}	0.14 ± 0.01	0.14 ± 0.01	0.16 ± 0.01	0.15 ± 0.01
RSI (%)^{ns}	0.48 ± 0.04	0.50 ± 0.03	0.43 ± 0.01	0.52 ± 0.02
SSI (%)^{ns}	0.32 ± 0.02	0.30 ± 0.28	0.32 ± 0.02	0.31 ± 0.12

Remark: Data are represented as mean±SEM. Different superscripts (^{a,b}) within a row are significantly different ($P<0.05$). Superscript^{ns} indicates no statistical difference ($P>0.05$). HSI = hepatosomatic index (%), ISI = intestinosomatic index (%), GSI = gill somatic index (%), CSI = cardiosomatic index (%), RSI = renosomatic index (%), SSI = splenosomatic index (%).

3. Intestinal macromorphology

The effects of different nevadensin diets on intestinal macromorphology of hybrid catfish are summarized in Table 4.

In the proximal part of the intestines (Fig. 4, A-D), villi heights of fish fed on diets supplemented with 10 to 50 mg nevadensin were significantly increased ($P<0.05$) when compared with the control. The total muscular thickness and inner circulatory muscle thickness were significantly enhanced in fish fed on a diet of 50 mg nevadensin compared with the control group and those fed with 10 and 30 mg levels of nevadensin. The outer longitudinal muscle thicknesses were significantly improved in fish fed diets supplemented with nevadensin at10 and 50 mg/kg in comparison to the control and fish fed with nevadensin at 30 mg/kg diet. Decreased villi widths were noticed in fish fed diets supplemented with 10 and 30 mg nevadensin compared with the control.

In the middle part of the intestines (Fig. 4, E-H), a significant increase in villi height was observed in fish fed nevadensin at 50 mg/kg diet compared with the other groups. Significant decreases in villi width and total muscular thickness were found in fish fed 30 mg nevadensin/kg diet compared to the control. The outer longitudinal muscle thickness was significantly improved in fish fed with a diet of 10 mg nevadensin compared to other treatments. However, the inner circulatory muscle thicknesses of fish fed nevadensin containing diets were similar to the control ($P>0.05$).

In the distal part of the intestines (Fig. 4, I-L), villi heights were significantly improved in all experimental groups compared with the control group. The total muscular thickness and inner circulatory muscle thickness were significantly enhanced in the fish fed with the diet containing 30 mg nevadensin compared to other treatments. Decreased villi widths were detected in fish fed nevadensin at 10 or 50 mg/kg diet compared with the control and those fed 30 mg nevadensin/kg diet. The outer longitudinal muscle thickness reduced significantly in fish fed nevadensin at 50 mg/kg diet compared to the control and those fed with 10 or 30 mg nevadensin/kg diet.

Table 4 Macromorphology of the intestines of hybrid catfish fed the diets supplemented with different levels of nevadensin for 8 weeks

Parameters	Nevadensin levels in the experimental diets (mg/kg diet)			
	0	10	30	50
Proximal portion				
Villi height (µm)	1418.76 ± 85.14^{c}	2414.42 ± 129.62^{a}	2641.12 ± 148.09^{a}	1931.40 ± 71.64^{b}
Villi width (µm)	627.03 ± 43.42^{a}	466.06 ± 28.37^{b}	479.06 ± 54.54^{b}	553.20 ± 49.84^{ab}
Total muscular thickness (µm)	204.78 ± 27.62^{b}	203.83 ± 15.92^{b}	197.92 ± 12.53^{b}	378.41 ± 43.93^{a}
Inner circulatory muscle thickness (µm)	124.38 ± 9.81^{b}	156.37 ± 12.53^{b}	157.22 ± 13.53^{b}	286.07 ± 33.14^{a}
Outer longitudinal muscle thickness (µm)	38.66 ± 3.68^{c}	47.46 ± 4.67^{b}	40.69 ± 3.58^{c}	92.33 ± 11.20^{a}
Middle portion				
Villi height (µm)	1345.54 ± 63.38^{b}	1343.54 ± 79.70^{b}	1150.25 ± 97.84^{b}	1429.32 ± 80.74^{a}
Villi width (µm)	551.95 ± 39.80^{a}	469.88 ± 31.09^{ab}	436.26 ± 20.41^{b}	502.57 ± 37.41^{ab}
Total muscular thickness (µm)	181.40 ± 20.86^{a}	194.17 ± 15.24^{a}	133.49 ± 19.40^{b}	160.18 ± 10.36^{ab}
Inner circulatory muscle thickness (µm)^{ns}	129.92 ± 18.15	124.04 ± 8.49	96.63 ± 14.12	109.37 ± 8.76
Outer longitudinal muscle thickness (µm)	51.47 ± 4.17^{b}	$70.12 \pm 8.07a$	36.87 ± 5.66^{b}	50.81 ± 4.12^{b}
Distal portion				
Villi height (µm)	1004.14 ± 50.79^{c}	1209.03 ± 43.94^{b}	1704.24 ± 110.05^{a}	1226.31 ± 53.77^{b}
Villi width (µm)	676.10 ± 41.62^{a}	440.86 ± 33.54^{b}	580.86 ± 68.96^{a}	522.48 ± 31.38^{b}
Total muscular thickness (µm)	162.03 ± 16.71^{b}	168.29 ± 22.89^{b}	215.37 ± 34.52^{a}	155.47 ± 12.75^{b}
Inner circulatory muscle thickness (µm)	121.55 ± 15.32^{b}	124.35 ± 18.36^{b}	161.99 ± 27.69^{a}	125.94 ± 12.77^{b}
Outer longitudinal muscle thickness (µm)	40.47 ± 3.78^{a}	43.94 ± 5.18^{a}	53.38 ± 8.35^{a}	29.53 ± 2.83^{b}

Remark: Data are represented as mean ± SEM. Different superscripts ($^{a-c}$) within a row are significantly different ($P<0.05$). Superscript^{ns} indicates no statistical difference ($P>0.05$).

It is well established that villi height is directly related to the abilities of the digestion and absorption of the gut (Boonanuntanasarn et al., 2018; Caballero et al., 2003). Intestinal muscular thickness plays a functional role in the maintenance of intestinal movement to support nutrient absorption as well as water reabsorption (Zhu et al., 2012). In this study, the application of nevadensin to the diets caused a significant increase in villi height and muscular thickness in fish intestines. These results are in agreement with the report of Ferreira et al. (2017) who found that the thickness of the muscular layer of *Astyanax aff. bimaculatus* fed the diets supplemented turmeric (*Curcuma longa*) significantly increased when compared to the control. The mechanisms

underlying of positive effects of dietary nevadensin on intestinal villi height and muscular thickness in this research are unclear. However, it is believed that nevadensin could regulate the expression of specific genes to modulate intestinal cell division and proliferation (Villasante et al., 2016). Additionally, nevadensin may induce the release of IGFs-I and II to enhance myogenesis in the intestine, leading to an increase in the muscular thickness (Crosnier et al., 2006). Thus, the findings of current work suggest that increased intestinal villi height and muscular thickness observed in fish fed the nevadensin diets could support feed digestion and water reabsorption in the gastrointestinal tract, increasing growth and nutrient utilization in fish (Munglue et al., 2019b; Zheng et al., 2015).

4. Intestinal micromorphology

The effects of dietary supplementation of nevadensin on intestinal micromorphology of hybrid catfish are demonstrated in Table 5.

In the proximal portion of the intestines, significant decreases (P<0.05) in total enterocyte heights and nucleus heights were obtained from fish received the diets supplemented with nevadensin compared to the control. Subnucleus heights in fish fed with the diets containing nevadensin were significantly higher than those fed the basal diet. A significant increase in microvilli heights was detected in fish fed diets supplemented with 30 or 50 mg of nevadensin. The goblet cell number of fish fed with 10 mg nevadensin/kg diet was significantly lower than fish fed with 30 mg nevadensin/kg diet. However, supranucleus heights and nucleus widths did not differ significantly (P>0.05) among the experimental groups.

In the middle portion of the intestines, total enterocyte heights and supranucleus heights significantly declined in fish fed with the diets containing nevadensin compared with fish fed the basal diet. A significant decrease in nucleus height was observed in fish fed the diet containing 10 mg nevadensin compared to the other treatments. Those fish fed 10 or 50 mg nevadensin/kg

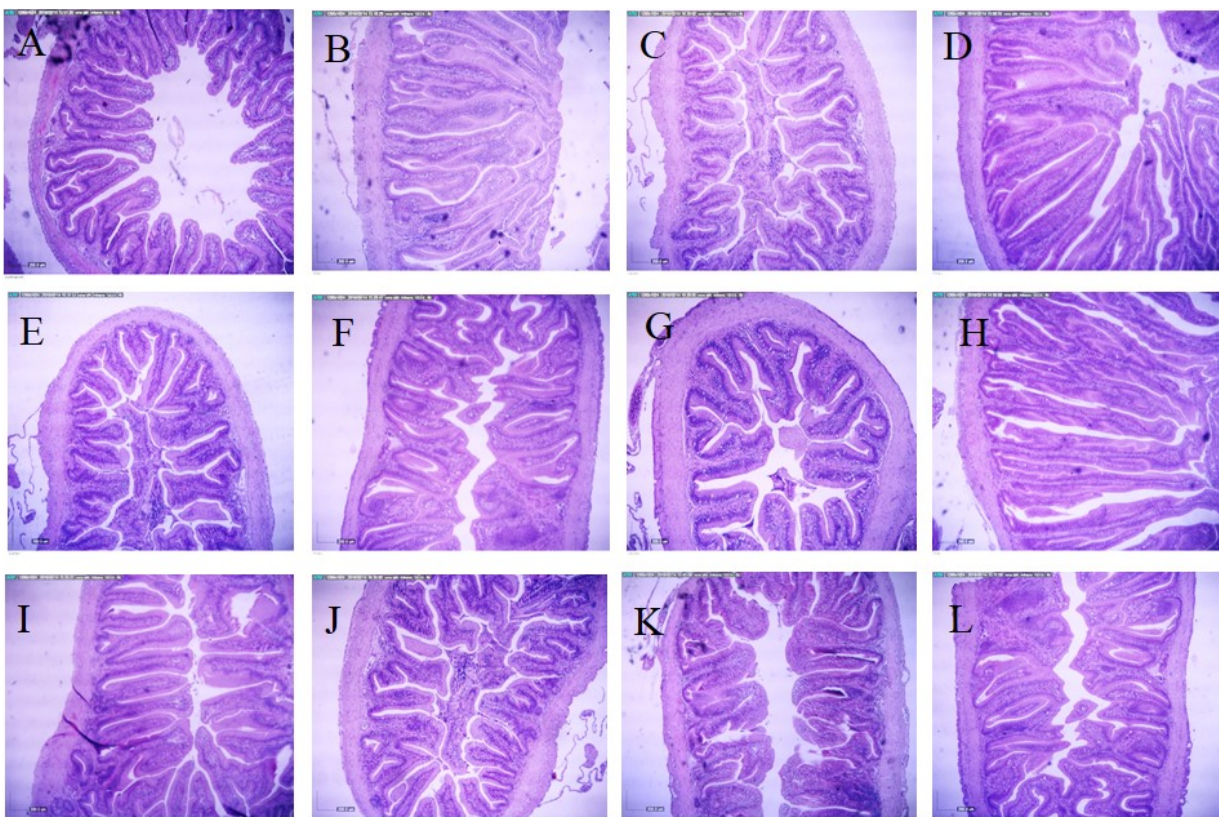

Fig. 4 Proximal (A - D), middle (E - H), and distal (I - L) portions of the intestines of hybrid catfish fed the diets containing nevadensin at 0 (A, E, and I), 10 (B, F, and J), 30 (C, G, and K), and 50 (D, H, and L) mg/kg diet for 8 weeks. Scale bar = 200 µm

diet produced a significant increase in goblet cells compared with the control. Significant increases in microvilli heights were observed in fish fed with nevadensin containing diets. Subnucleus heights and nucleus widths did not affect by nevadensin supplementation.

In the distal portion of the intestines, total enterocyte heights were significantly decreased in fish fed nevadensin containing diets compared with the control. A significant reduction of supranucleus height was found in fish fed 10 or 30 mg nevadensin/kg diet compared with other diets. Subnucleus height decreased significantly in fish fed 30 mg nevadensin diet compared to fish fed 50 mg nevadensin diet. The nucleus height in fish fed 10 mg nevadensin diet was significantly lower than that in fish fed the control diet. The goblet cell number and microvilli height increased significantly in the experimental groups compared to the control. Additionally, no significant differences were noted in the nucleus widths among the treatments.

Enterocyte characteristics, microvilli, and goblet cell could be used to demonstrate the physiological functions of fish intestines. Previous reports have shown that starvation and feed ingredients can affect the micromorphology of the intestines (Rašković et al., 2011; Savic et al., 2012). The longer enterocyte and microvilli would represent the effective nutrient absorption processes that occurred in the intestines, leading to the higher growth and feed efficiency of fish (Fonseca-Madrigal et al., 2006; Ostaszewska et al., 2005). Mucins, high-molecular-weight glycoproteins produced by goblet cell, play a key role in osmoregulation, protection of intestinal epithelial cells from mechanical and chemical damages and modulation of intestinal absorption processes (Kim & Ho, 2010; Kozarić et al., 2007). In this study, significant increases in goblet cell number, subnucleus height, and microvilli height were found in the tested fish. In contrast, enterocyte height, supranucleus height, and nucleus height decreased significantly in the experimental groups compared with the control. These findings were consistent with the report that lasia supplemented diets produced a marked decrease of enterocyte height and caused a significant increase of microvilli height in hybrid catfish (Munglue et al., 2019a). Similarly, sharpsnout seabream (*Diplodus puntazzo*) fed soybean meal-based diets containing mannanoligo-saccharides and inulin for 114 days significantly decreased enterocyte height values both in the proximal and distal part of intestines (Ferrara et al., 2015). As mentioned above, the increases in enterocyte height, microvilli height, and goblet cell numbers are related to better growth and feed utilization efficiency of fish (Akter et al., 2016; Boonanuntanasarn et al., 2018; Poleksić et al., 2014). Increased goblet cell numbers may in part be related to the enhancement of cell proliferation, cell differentiation, and cell division which might be modulated by nevadensin to improve intestinal functions (Ostaszewska et al., 2005). The longer enterocyte and microvilli would indicate the higher absorptive area of the intestines (Akter et al., 2016; Poleksić et al., 2014). In this current work, the decline in enterocyte height did not relate to the growth observed in fish fed nevadensin diets. Thus, it is hypothesized that other specific factors that were modulated by nevadensin would also have positive effects on the growth of hybrid catfish (Munglue et al., 2019a).

Table 5 Micromorphology of the intestines of hybrid catfish fed the diets supplemented with different levels of nevadensin for 8 weeks

Parameters	Nevadensin levels in the experimental diets (mg/kg diet)			
	0	10	30	50
Proximal portion				
Total enterocyte height (μm)	69.63 ± 4.57^{a}	40.74 ± 2.06^{c}	53.13 ± 1.63^{b}	47.22 ± 1.78^{bc}
Supranucleus height (μm)^{ns}	26.78 ± 2.44	20.83 ± 1.56	21.51 ± 2.41	27.73 ± 2.15
Subnucleus height (μm)	4.44 ± 0.26^{b}	12.80 ± 0.89^{a}	12.43 ± 0.65a	11.28 ± 0.90^{a}
Nucleus height (μm)	8.76 ± 0.27^{a}	5.37 ± 0.26^{c}	6.39 ± 0.41^{bc}	6.98 ± 0.46^{b}
Nucleus width (μm)^{ns}	11.49 ± 0.28	11.07 ± 0.26	10.33 ± 0.40	11.43 ± 0.21
Goblet cell number	8.00 ± 2.59^{ab}	6.33 ± 0.66^{b}	9.00 ± 0.64^{a}	8.86 ± 1.15^{ab}
Microvilli height (μm)	3.20 ± 0.26^{c}	4.96 ± 0.13^{bc}	4.60 ± 0.07^{b}	5.28 ± 0.14^{a}
Middle portion				
Total enterocyte height (μm)	72.82 ± 5.00^{a}	43.28 ± 2.15^{b}	46.43 ± 2.11^{b}	52.01 ± 2.08^{b}
Supranucleus height (μm)	31.47 ± 2.40^{a}	19.35 ± 1.54^{b}	19.15 ± 1.06^{b}	21.16 ± 0.87^{b}
Subnucleus height (μm)^{ns}	4.27 ± 0.33	4.39 ± 0.33	4.10 ± 0.42	3.79 ± 0.26
Nucleus height (μm)	7.58 ± 0.29^{a}	4.67 ± 0.35^{b}	6.35 ± 0.56^{a}	8.33 ± 0.75^{a}
Nucleus width (μm)^{ns}	11.74 ± 0.54	12.42 ± 0.66	10.39 ± 0.46	11.53 ± 0.36
Goblet cell number	8.86 ± 0.64^{b}	13.33 ± 1.52^{a}	11.40 ± 1.33^{ab}	12.20 ± 0.75^{a}
Microvilli height (μm)	3.12 ± 0.35^{b}	5.43 ± 0.10^{a}	5.20 ± 0.20^{a}	5.10 ± 0.18^{a}
Distal portion				
Total enterocyte height (μm)	96.65 ± 6.63^{a}	66.00 ± 4.12^{b}	69.09 ± 4.41^{b}	70.57 ± 5.68^{b}
Supranucleus height (μm)	43.10 ± 5.19^{a}	24.47 ± 1.44^{b}	30.76 ± 2.84^{b}	37.28 ± 4.46^{a}
Subnucleus height (μm)	4.13 ± 0.22^{ab}	4.16 ± 0.32^{ab}	4.67 ± 0.34^{a}	3.79 ± 0.16^{b}
Nucleus height (μm)	7.83 ± 0.27^{a}	6.63 ± 0.23^{b}	7.45 ± 0.46^{ab}	7.64 ± 0.21^{ab}
Nucleus width (μm)^{ns}	10.53 ± 0.62	10.40 ± 0.52	11.02 ± 0.53	11.07 ± 0.44
Goblet cell number	8.00 ± 0.66^{c}	12.04 ± 1.16^{b}	15.66 ± 1.57^{a}	13.80 ± 1.43^{ab}
Microvilli height (μm)	3.40 ± 0.16^{b}	4.83 ± 0.30^{a}	4.68 ± 0.30^{a}	4.63 ± 0.09^{a}

Remark: Data are represented as mean ± SEM. Different superscripts ($^{a-c}$) within a row are significantly different ($P<0.05$). Superscript ^{ns} indicates no statistical difference ($P>0.05$).

5. Hematology

The effects of nevadensin containing diets on the hematology of hybrid catfish are shown in Table 6. The levels of RBC and WBC of fish fed the diets containing nevadensin were significantly higher ($P<0.05$) than those of fish fed with the basal diet. HGB increased significantly in fish fed 50 mg nevadensin/kg diet

compared with the control, but it was not significantly different (P<0.05) from that of fish fed 10 or 30 mg nevadensin/kg diet. Additionally, fish fed a diet with 50 mg nevadensin showed the highest HCT level. The highest lymphocyte was noticed in fish fed with the diet containing 10 mg nevadensin/kg. No significant differences (P>0.05) were detected in PLT, MCV, MCH, MCHC, neutrophils, monocytes, basophils and eosinophils among the treatments.

Hematological indices can be useful for the evaluation of the general health status and well-being of fish (Campbell & Ellis, 2007; Clauss et al., 2008). Several intrinsic and extrinsic factors can affect cell morphology and the quantitative levels of the blood cells (Burgos-Aceves et al., 2019). The present study, it was demonstrated that the diets supplemented with nevadensin produced a significant increase in RBC, WBC, HGB, HCT and lymphocyte compared with the control diet. Many reports have been indicated the positive effects of medicinal plants and their derivative compounds on the hematological values of fish (Abdel-Tawwab et al., 2018; Awad & Awaad, 2017; Van Hai, 2015). Adel et al. (2015) noticed that fry Caspian white fish (*Rutilus frisii kutum*) fed peppermint-enriched diets for 8 weeks significantly enhanced RBC, WBC, HCT, HGB, and neutrophil compared with the control. Also, Giri et al. (2017) showed that RBC and WBC of *Labeo rohita* fed the diets mixed with *Hybanthus enneaspermus* aqueous extract for 6 weeks were significantly increased, while neutrophil and lymphocyte were significantly decreased compared with the control. Improved RBC count and HGB by dietary nevadensin may enhance the oxygen-carrying capacity and the response of fish against physiological stresses (Gabriel et al., 2019). Increased WBC and lymphocyte levels in fish fed dietary nevadensin could be demonstrated the enhancement of non-specific immune responses by this compound (Adel et al., 2015; Awad & Awaad, 2017). It is suggested that dietary nevadensin could help to protect fish from several infectious diseases and stressful conditions (Reverter et al., 2014). It is assumed that nevadensin may stimulate hematopoietic stem cells in hematopoietic organs to enhance RBC and WBC in the fish as demonstrated in the report of Gabriel et al. (2019). Thus, increased levels of WBC and lymphocyte in fish fed nevadensin supplemented diets would indicate the immunostimulatory potential of this natural compound.

Table 6 Hematological values of hybrid catfish fed the diets supplemented with different levels of nevadensin for 8 weeks

Parameters	Nevadensin levels in the experimental diets (mg/kg diet)			
	0	10	30	50
RBC ($\times 10^6$ cell/mm^3)	1.76 ± 0.40^b	2.74 ± 0.10^a	2.50 ± 0.09^a	2.64 ± 0.17^a
WBC ($\times 10^4$ cell/mm^3)	36.80 ± 3.98^b	49.16 ± 2.78^a	52.18 ± 3.85^a	48.76 ± 3.92^a
HGB (g/dL)	9.12 ± 1.49^b	11.70 ± 0.44^{ab}	11.12 ± 0.35^{ab}	12.98 ± 0.83^a
HCT (%)	25.66 ± 0.33^b	23.66 ± 0.88^b	22.33 ± 1.85^b	30.00 ± 3.05^a
PLT ($\times 10^3$ cell/µL)^{ns}	32.60 ± 4.40	49.00 ± 11.93	40.00 ± 9.11	46.75 ± 7.72
MCV (fL)^{ns}	134.38 ± 5.90	119.14 ± 2.30	122.30 ± 4.68	126.98 ± 6.12
MCH (pg)^{ns}	46.68 ± 0.49	42.60 ± 0.83	44.32 ± 0.51	49.08 ± 0.29
MCHC (g/dL)^{ns}	35.06 ± 1.45	35.84 ± 1.02	36.50 ± 1.26	39.00 ± 1.69
Lymphocyte (%)	64.33 ± 4.63^b	81.66 ± 2.02^a	65.66 ± 7.68^b	63.33 ± 2.33^b
Neutrophil (%)^{ns}	10.00 ± 0.00	3.50 ± 1.53	8.66 ± 6.02	12.33 ± 2.51
Monocyte (%)^{ns}	13.33 ± 8.08	5.50 ± 2.12	9.66 ± 5.13	13.33 ± 6.65
Basophil (%)^{ns}	8.00 ± 1.52	8.00 ± 1.15	10.66 ± 5.69	3.00 ± 1.00
Eosinophil (%)^{ns}	4.66 ± 2.72	4.33 ± 2.33	8.00 ± 0.00	8.00 ± 2.51

Remark: Data are represented as mean ± SEM. Different superscripts (^{a,b}) within a row are significantly different (P<0.05).
Superscript ^{ns} indicates no statistical difference (P>0.05). RBC = red blood cell ($\times 10^6$ cell/mm^3), WBC = white blood cell ($\times 10^4$ cell/mm^3), HGB = hemoglobin (g/dL), HCT = hematocrit (%), PLT = platelet ($\times 10^3$ cell/µL), MCV = mean corpuscular volume (fL), MCH = mean corpuscular hemoglobin (pg), MCHC = mean corpuscular hemoglobin concentration (g/dL).

6. Serum biochemistry

As shown in Table 7, dietary supplementation with nevadensin had no effects (P>0.05) on ALP, AST, albumin, creatinine, triglyceride, total protein and globulin levels in hybrid catfish compared with the practical diet. Fish fed the diets with nevadensin tended to have lower glucose levels compared to the fish fed the basal diet, although this effect did not reach statistical significance.

Serum biochemical values are very useful for monitoring stress, nutritional imbalance and the health status of fish (Yang & Chen, 2003; Zhai et al., 2016). In this study, it was found that nevadensin enriched-diets had no significant effects on the levels of AST, ALP, creatinine, glucose, triglyceride, total protein, albumin and globulin in hybrid catfish. Enzyme activities of AST and ALP are directly related to hepatic, gill and muscle functions (Yang & Chen, 2003). An increase in AST and ALP levels may indicate hepatic injuries due to malnutrition, toxins, infectious diseases and stress (Coppo et al., 2003). Creatinine is mainly a waste product produced by the breakdown of muscle cells and is generally used as an indicator of renal functions (Abdel-Tawwab et al., 2017; Coppo et al., 2003). Increased creatinine levels could indicate kidney injury and infectious diseases (Mutlu et al., 2015). In this study, dietary nevadensin did not produce any change in the levels of AST, ALP and creatinine in hybrid catfish. It is

suggested that nevadensin had no effects on the structural and functional integrity of the kidney, liver and muscle cells. Increased serum glucose level is an indicator of the environmental stress in fish (Adham et al., 2002; Güllü et al., 2016). In this present study, serum glucose levels tend to decrease in fish fed nevadensin diets compared with the control. This result suggested that nevadensin may have hypoglycemic action due to increased glucose uptake and glycogenesis in certain peripheral tissues of fish (Sruthi et al., 2018; Wang et al., 2018). It was postulated that serum triglyceride is directly correlated with the presence of lipid peroxidation and reactive oxygen species in fish (Gabriel et al., 2015). Increased triglyceride levels may indicate the prevalence of higher lipid peroxidation and oxidative stress. In this study, it was found that the levels of triglyceride in fish fed the diets containing nevadensin were similar to the control. Thus, it seems that nevadensin could have anti-lipid peroxidation and anti-inflammatory effects in fish (Gabriel et al., 2015; Reddy et al., 1991). The levels of total protein, albumin and globulin are associated with the innate immune response in fish (Güllü et al., 2016). The results showed that no significant differences in total protein, albumin and globulin were detected in fish fed dietary nevadensin. Therefore, it could be suggested that nevadensin supplementations at 10 to 50 mg/kg feed did not affect serum levels of total protein, albumin and globulin in hybrid catfish.

Table 7 Blood biochemical values of hybrid catfish fed the diets supplemented with different levels of nevadensin for 8 weeks

Parameters	Nevadensin levels in the experimental diets (mg/kg diet)			
	0	10	30	50
AST (U/L)^{ns}	170.09 ± 34.17	171.85 ± 46.26	105.87 ± 14.82	163.31 ± 12.15
ALP (U/L)^{ns}	31.73 ± 4.66	35.25 ± 4.43	37.02 ± 8.14	26.44 ± 3.05
Creatinine (mg/dl)^{ns}	0.95 ± 0.43	0.70 ± 0.10	1.04 ± 0.34	0.54 ± 0.14
Glucose (mg/dl)^{ns}	168.32 ± 42.22	163.39 ± 25.28	122.85 ± 11.95	123.33 ± 20.41
Triglycerides (mg/dl)^{ns}	176.92 ± 47.57	198.90 ± 16.31	285.37 ± 76.44	191.64 ± 9.03
Total protein (mg/dl)^{ns}	4.04 ± 0.18	3.36 ± 0.93	4.28 ± 0.31	2.65 ± 0.63
Albumin (mg/dl)^{ns}	1.59 ± 0.15	1.44 ± 0.03	1.41 ± 0.20	1.46 ± 0.07
Globulin (mg/dl)^{ns}	2.45 ± 0.34	1.91 ± 0.94	2.87 ± 0.10	1.18 ± 0.23

Remark: Data are represented as mean ± SEM. Superscript ^{ns} indicates no statistical difference (P>0.05). AST = aspartate transaminase (U/L), ALP = alkaline phosphatase (U/L).

Conclusion

To conclude, this research exhibited that dietary nevadensin produced the improvement in growth performance, feed intake, feed utilization, intestinal histology and hematology of hybrid catfish. By using the second-order polynomial regression analysis, the recommended dose of nevadensin for the cultivation of hybrid catfish observed in this present study was found to be between 30.64 and 31.14 mg/kg diet.

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