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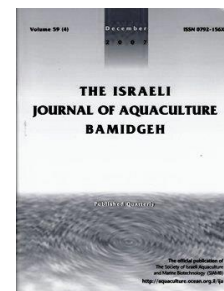
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Effects of Dietary Emodin Supplementation on Growth Performance, Non-Specific Immune Responses, and Disease Resistance to *Aeromonas hydrophila* in Juvenile Wuchang Bream (*Megalobrama amblycephala*)

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Key words: *Megalobrama amblycephala*; emodin; growth; non-specific immunity

Abstract

An 8-week feeding trial was conducted to investigate the effects of dietary emodin supplementation on growth, non-specific immunity, and protection against *Aeromonas hydrophila* infection in juvenile *Megalobrama amblycephala*. A basal diet was supplemented with 0 (control), 15, 30, 60 and 120 mg emodin/kg to formulate five experimental diets. Each diet was randomly allocated to triplicate tanks of fish in a circulating water system (initial average weight 3.49 ± 0.045 g, 25 fish per tank). At the end of the feeding trial, fish fed the diet supplemented with 0 and 120 mg emodin/kg had lower weight gain (WG) and specific growth rate (SGR) than those in the other treatment groups, but no significant differences were observed among diets supplemented with emodin from 15 to 60 mg/kg. A significant increase on feed conversion ratio (FCR) of fish fed diet supplemented with 120 mg emodin/kg was observed. The white blood cell count (WBC), respiratory burst activity, superoxide dismutase (SOD) activity, myeloperoxidase (MPO) activity, malondialdehyde (MDA) content, and tumor necrosis factor- α (TNF- α) activity first increased and then decreased with increase of the dietary emodin levels. Fish fed the 30 mg emodin/kg supplemented diet had higher WBC, respiratory burst activity, SOD and TNF- α activity, and lower MDA content, than fish fed diets supplemented with 0 and 120 mg emodin/kg. In the bacteria challenge experiment with *A. hydrophila*, fish fed a diet supplemented with 30 and 60 mg/kg had a lower cumulative mortality rate than the control group. These results indicated that appropriate dietary emodin supplementation (especially 30 mg emodin/kg diet) could enhance the growth and immune responses of fish and improve resistance to infection by *A. hydrophila*.

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Introduction

In aquaculture, due to high stocking density in intensive culture and deterioration of environmental conditions, fish often encounter high temperature, overcrowding, transport, handling, grading, and poor water quality. These conditions compromise the physiological environment increasing the susceptibility of fish to infectious agents thereby increasing the chance of disease outbreak due to an increasing range of pathogens. A large number of antibiotics and hormones are added in feed to control fish diseases. This may result in drug resistance, residues, and other issues, detrimental to the sustainable development of aquaculture, seafood safety, and human health (Ming et al., 2012). Therefore, the development of the non-chemical and natural therapeutics through nutrition, such as traditional herbal medicine, has become more important.

China's background is rich in the use of traditional medicines, most of which have been used as immunostimulants to treat human and animal diseases for thousands of years (Tan and Vanitha, 2004, Bhuvaneswari and Balasundaram, 2006; Ganguly et al., 2010). A large number of plant products are being investigated for immune-response-modifying activity in fish. Some of the medicinal plants shown to have an immunostimulatory activity in fish are *Astragalus membranaceus* (Yin et al., 2009), anthraquinone extract from *Rheum officinale* Bail (Liu et al. 2010), and *Lonicera japonica* (Ardó et al., 2008). Among the herbs mentioned above, emodin (1, 3, 8-trihydroxy-6-methyl- anthraquinone) is a promising one. It is an active anthraquinone constituent extracted from the rhizome of rhubarb *Rheum officinale* Baill. Emodin has been found to have many beneficial functions, such as anti-bacterial and anti-inflammatory (Chang et al., 1996), antioxidation and scavenging free radicals (Huang et al., 1995), reducing blood lipids (Zhou et al., 2006), liver protection (Lin et al., 1996), and regulating immunity (Wang et al., 1995). Anthraquinone extract (the main components being emodin, chrysophanol and rhein) can promote growth, enhance non-specific immunity, and increase resistance to high temperature of freshwater prawn (*Macrobrachium rosenbergii*) (Liu et al., 2010) and Wuchang bream (*Megalobrama amblycephala*) (Liu et al., 2012a). However, little information has been obtained to evaluate non-specific immunity, and disease resistance of fish in relation with the dietary emodin.

Wuchang bream (*Megalobrama amblycephala* Yih) is a major freshwater species cultured in China. Diseases of cultured Wuchang bream have increasingly occurred in the summer, resulting in high mortality both in China and in other areas of the world (He et al., 2006). The present study aims to determine the effects of dietary emodin levels on non-specific immunity and disease resistance of juvenile Wuchang bream. The outcomes of this study will improve our understanding on the immune ability to defend pathogens in fish, and also explore means to enhance non-specific immunity through nutritional modulation using emodin in fish diet.

Materials and methods

Experimental diets. The basal diet was supplemented with 0 (control), 15, 30, 60 and 120 mg emodin/kg diet, respectively. Emodin (with purity>99%) was provided by Feida Chemical Reagent Company (Xian, China). For preparation of experimental diets, the ingredients were ground into fine powder through a 60-mesh sieve. Diets were prepared by mixing dry ingredients, and then adding oil and water (40%, v/w) to form a soft dough. The dough was then pelleted using a laboratory pellet machine and dried in a forced-air oven at room temperature. After drying, the diet pellets were broken into smaller pieces and sieved into the required size. All diets were stored at -20 °C until use. Formulation and proximate composition of the basal diet are shown in Table 1.

Table 1 Formulation and proximate composition of the basal diet (g/kg)

Ingredients	% dry weight	
Fish meal	8	
Soybean meal	18	
Rapeseed meal	17	^a Vitamin premix (IU or mg per kg premix): Vitamin A, 900000 IU; Vitamin D, 250000 IU; Vitamin C, 5000 mg; Vitamin K ₃ , 220 mg; Vitamin B ₁ , 320 mg; Vitamin B ₂ , 1090 mg; Vitamin B ₆ , 5000 mg; Vitamin B ₁₂ , 116mg; biotin, 50 mg; Pantothenate, 1000 mg; Folic acid, 165mg; Choline, 60,000 mg; Inositol, 15000 mg; Niacin acid, 2500 mg.
Cotton meal	16.5	
Rice bran	8	
Wheat middling	22	
Soybean oil	4	
Lecithin	1	^b Mineral premix (per kg premix) : blue copperas, 2.5g; green vitriol, 28g; zinc sulfate heptahydrate, 22g; Manganese sulfate tetrahydrate, 9g; sodium selenate, 0.045g; potassium iodide, 0.026g; cobalt chloride hexahydrate, 0.1g.
Choline chloride	0.5	
Vitamin premix ^a	1	
Mineral premix ^b	1	
Powdered zeolite	1	^c Energy, calculated by using standard physiological fuel values of 16.7, 16.7 and 37.7 kJ/g for carbohydrate, protein and lipid, respectively.
Calcium dihydrogen phosphate	2	
Proximate composition (%)		
Crude protein	31.27	
Crude lipid	8.15	
Crude ash	2.79	
Gross energy (kJ/g) ^c	16.32	

Fish and animal husbandry. Wuchang bream were obtained from Nanquan fish farm of Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences. Prior to the experiment, fish were fed a commercial diet during the acclimation period. After acclimation, fish (initial weight, 3.49 ± 0.045 g) were selected and randomly distributed into 15 circular fiberglass tanks (each tank water volume=300 l) using a completely randomized design including five triplicate treatments. Flow rate in each tank was maintained at approximately 2 l/min. Each tank was stocked with 25 fish. Fish were hand-fed with the test diets three times per day (08:00, 12:00 and 16:00) until apparent satiation for 8 weeks. Throughout the experiment, the juveniles were kept under a natural photoperiod and water temperature was controlled at $26 \pm 1^\circ\text{C}$. pH ranged between 7.2-7.8, ammonia nitrogen < 0.05 mg/l and dissolved oxygen concentration > 6 mg/l.

Sample collection. At the end of the feeding trial, fish were not fed for 24 h to empty the digestive tract. They were then anesthetized in diluted MS-222 (tricaine methanesulfonate, Sigma, USA) at a concentration of 100 mg/l. The total number and weight of fish in each tank were determined later. The blood from three randomly chosen fish from each tank, was collected by the caudal venipuncture using 1 ml heparinized syringes. Blood samples for plasma were collected into anticoagulation tubes. After collection, 50 μl whole blood from anticoagulation tubes was used for analysis of respiratory burst activity. The other whole blood was centrifuged ($2000 \times g$ at 4°C for 10 min) and plasma stored at -20°C until further analysis (immunological assays).

The white blood cell count (WBC). After blood samples were obtained from the caudal vein using a heparinized syringe, white blood cell count (WBC) was measured using Auto Hematology Analyzer (BC-5300Vet, mindray, P.R. China) with a test kit from Shenzhen Mindray Medical International Co. Ltd. P.R. China.

Respiratory burst activity. The respiratory burst activity of the phagocytes was tested using nitroblue tetrazolium (NBT, Shanghai Reagent Corp., China) following the method described by Secombes et al. (1990) and Ai et al. (2007). Absorbance at 630 nm was measured with a Model Multiskan spectrum (Thermo, USA) using KOH/DMSO alone as a blank. Respiratory burst was expressed as NBT-reduction in 100 ml of cell suspension.

Plasma superoxide dismutase (SOD) and malondialdehyde (MDA) assay. SOD activity and MDA content were measured using xanthine oxides (Granelli et al., 1995) and barbituric acid reaction chronometry (Drape et al., 1993), respectively. These fish detection kits were purchased from Nanjing Jiancheng Bioengineering Institute of China.

Leucocyte myeloperoxidase (MPO) assay. The total myeloperoxidase(MPO) activity in the peripheral blood leucocytes was measured according to Palic et al. (2005) with the following minor modifications. 25 μ l of the cell suspension containing 1×10^6 cells per well (in triplicate) was incubated for 20 min with 125 μ l of 0.02% cetyltrimethylammonium bromide (CTAB; Sigma, USA) in microcentrifuge tubes. Then, 50 μ l of 20mM 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB; Amresco, USA) and 5 mM H_2O_2 were added. After 2 min of incubation, 50 μ l of 2M sulfuric acid was added to stop the reaction. The microcentrifuge tubes were centrifuged at $600 \times g$ for 15 min, and 200 μ l of the supernatant was transferred to 96-well plates. Optical density was measured at 450 nm in a plate reader (BioRad, USA).

Plasma tumor necrosis factor- α (TNF- α) assay. The activity of plasma tumor necrosis factor- α (TNF- α) was measured by the double antibody sandwich method using TNF- α elisa detection kit (IBL, Germany). The optical density was measured at 450 nm.

Challenge test. Gram negative bacteria *A. hydrophila* strain was originally isolated from infected fingerling *M. amblycephala*. The seven day LC_{50} was determined by intraperitoneal injection into 48 fish with graded concentrations of *A. hydrophila* ($10^6, 10^7, 10^8, 10^9$ and 10^{10} CFU/ml) at $24^\circ C$. The result showed that the LC_{50} on day 7 was 5×10^6 CFU/ml. Challenge tests were conducted in triplicate with 10 fish per replicate. *A. hydrophila* was diluted using sterile normal saline and the final concentration was set to 5×10^6 CFU/ml. Bacterial suspension (0.5 ml, per 50 body weight) was injected into the abdominal cavity. No diet was given to the fish during the test and mortality in each tank was observed daily over 4 days.

Statistical analysis. All data are presented as means \pm S.E. (standard error of the mean). Data were logarithmic transformed before being subjected to one-way analysis of variance (ANOVA) using SPSS 13.0. When the overall treatment effect was significantly different, Tukey's test was conducted to compare the means between the levels of emodin treatment. The level of significant difference was set at $P < 0.05$.

Results

Growth. After 8 weeks feeding period, fish fed the diets supplemented with 15 and 30 mg emodin/kg diet had significantly higher WG and SGR ($P < 0.05$) than fish fed diet supplemented with 0 and 120 mg emodin/kg diets. Although the WG and SGR of fish decreased as dietary emodin increased from 15 to 60 mg/kg, no significant differences between these three treatments were observed in the WG and SGR of fish. FCR was higher ($P < 0.05$) in fish fed diet supplemented with 120 mg emodin/kg than other treatment groups. There was no significant difference in the survival rate between the the control and the treatment groups (Table 2).

Table 2 Growth performance of *Megalobrama amblycephala* fed diets containing different levels emodin¹

Dietary emodin (mg /kg)	Initial body weight (g)	Final body weight (g)	WG ² (%)	SGR ³ (%/d)	FCR ⁴	Survival rate ⁵ (%)
0	3.5 \pm 0.01	11.54 \pm 1.14	229.51 \pm 3.38 ^a	2.13 \pm 0.02 ^a	1.78 \pm 0.06 ^a	98.67 \pm 1.33
15	3.51 \pm 0.02	12.04 \pm 0.15	245.9 \pm 5.1 ^b	2.21 \pm 0.03 ^b	1.69 \pm 0.12 ^a	100
30	3.44 \pm 0.05	11.95 \pm 0.2	245.36 \pm 1.11 ^b	2.22 \pm 0.08 ^b	1.58 \pm 0.04 ^a	94.67 \pm 5.33
60	3.49 \pm 0.02	11.84 \pm 1.08	240.26 \pm 1.08 ^{ab}	2.19 \pm 0.12 ^{ab}	1.84 \pm 0.09 ^a	100
120	3.49 \pm 0.01	10.95 \pm 0.3	214.13 \pm 4.25 ^c	2.04 \pm 0.05 ^c	2.18 \pm 0.07 ^b	100

¹ Values are means \pm S.E. of three replications, values with different superscript letters in the same column are significantly different in Tukey's test ($P < 0.05$).

² Weight gain (WG) = (Final body weight-initial body weight) \times 100/ initial body weight.

³ Specific growth rate (SGR) = (LnWt - LnW₀) \times 100/T, where W₀ and Wt are the initial and final body weights, and T is the culture period in days.

⁴ Feed conversion ratio (FCR) = total diet fed (g) / total wet weight gain (g).

⁵ Survival rate = (initial fish number-final fish number) \times 100/ initial fish number

Immune response. The respiratory burst and TNF- α activity of fish fed the diet supplemented with 30 mg emodin/kg was significantly higher ($P < 0.05$) than fish fed the diet supplemented with 0 and 120 mg emodin/kg (Fig. 1 & 2).

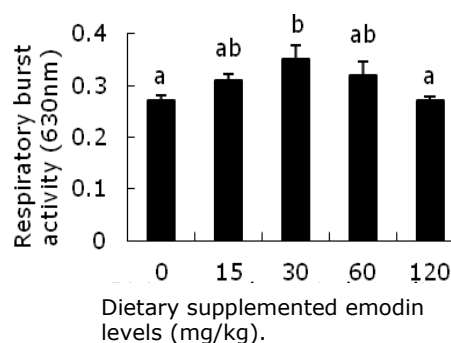


Fig. 1 Respiratory burst activity of *M. amblycephala* fed diets containing different levels emodin. Values are means \pm S.E. ($n=9$). Bars sharing different letters for each diet are significantly different determined by Tukey's test ($P < 0.05$).

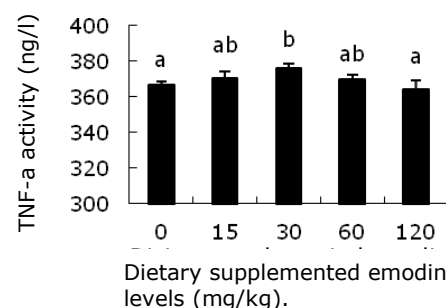


Fig.2. tumor necrosis factor- α (TNF- α) activity of *M. amblycephala* fed diets with diff. levels of emodin. Values are means \pm S.E. ($n=9$). Bars sharing diff. letters for each diet are significantly different determined by Tukey's test ($P < 0.05$).

The plasma MPO activity was also significantly affected by the dietary emodin levels. Fish fed 30 mg emodin/kg diet had higher MPO activity ($P < 0.05$) than fish fed diets supplemented with 0 and 120 mg emodin/kg, while no significant differences were observed on MPO activity of fish fed diets supplemented with 15, 30 and 60 mg emodin/kg diet (Fig. 3). The WBC count in 30 mg emodin/kg diet group was significantly higher ($P < 0.05$) than that of control and other treatment groups (Fig.4)

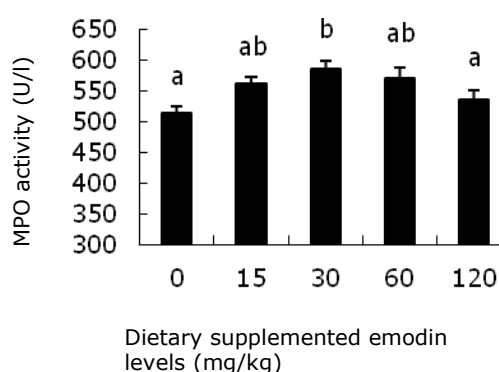


Fig. 3 Leucocyte myeloperoxidase (MPO) activity of *Megalobrama amblycephala* fed diets containing different levels emodin. Values are means \pm S.E. ($n = 9$). Bars sharing different letters for each diet are significant different determined by Tukey's test ($P < 0.05$).

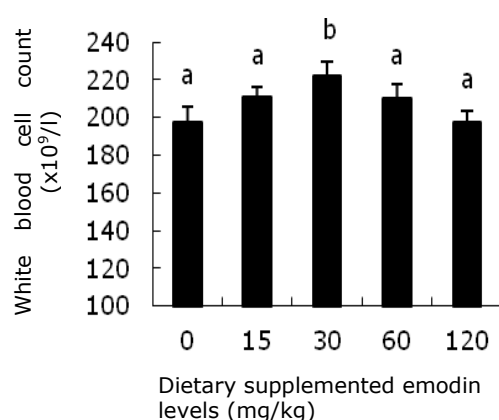


Fig. 4 The white cell count (WBC) of *Megalobrama amblycephala* fed diets containing different levels emodin. Values are means \pm S.E. ($n = 9$). Bars sharing different letters for each diet are significant different determined by Tukey's test ($P < 0.05$).

The plasma superoxide dismutase (SOD) activity of fish fed diets supplemented with emodin from 15 to 60 mg/kg was significantly higher ($P < 0.05$) than that of control and 120 mg emodin/kg group (Fig. 5). However, plasma malondialdehyde (MDA) content in the 15, 30 and 60 mg emodin/kg was significantly lower ($P < 0.05$) than that

of control and 120 mg emodin/kg diet group. The differences among the 15, 30 and 60 mg emodin/kg diet group were not significant (Fig. 6).

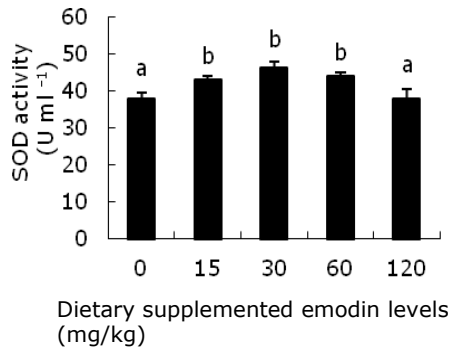


Fig. 5 Superoxide dismutase (SOD) activity of *M. amblycephala* fed diets containing different levels emodin. Values are means \pm S.E. ($n=9$). Bars sharing different letters for each diet are significantly different determined by Tukey's test ($P < 0.05$).

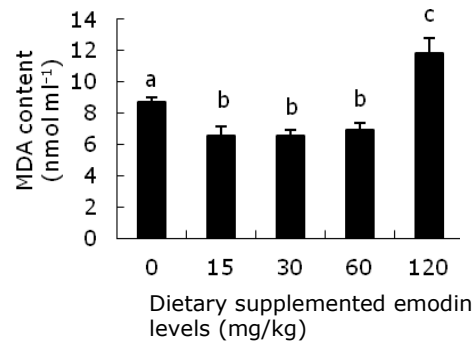


Fig. 6 Malondialdehyde (MDA) content of *M. amblycephala* fed diets containing different levels emodin. Values are means \pm S.E. ($n=9$). Bars sharing different letters for each diet are significantly different determined by Tukey's test ($P < 0.05$).

Challenge test. After being challenged with bacteria ($n=30$ for each dietary treatment), mortality of the fish was recorded daily for 4 days. The challenge test showed that dietary emodin significantly enhanced protection against infection for juvenile *M. amblycephala* (Fig. 7).

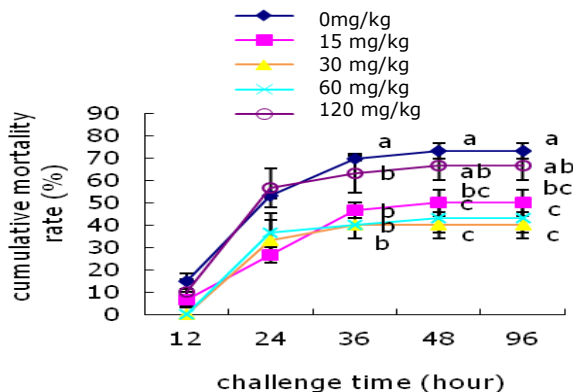


Fig. 7 Cumulative mortality rate of *Megalobrama amblycephala* fed diets containing different levels emodin. Values are means \pm S.E. ($n = 9$). Bars sharing different letters for each diet are significant different determined by Tukey's test ($P < 0.05$).

The cumulative mortality rate of fish in control group was significantly higher ($P < 0.05$) than the other groups 36 h after challenge. The cumulative mortality of fish fed diets supplemented with 15, 30 and 60 mg emodin/kg was significantly lower ($P < 0.05$) than that of the control group and 120 mg emodin/kg diet group 96 h after challenge.

Discussion

In this experiment, the basal diet supplemented with 15 and 30 mg emodin (with purity $> 99\%$)/kg increased the WG and SGR and lowered FCR of juvenile *M. amblycephala*. Similarly, a previous study (Ming et al., 2012) demonstrated that 60 mg/kg emodin in the dietary emodin (containing 40% emodin extracted from the rhubarb) could improve fish growth for *M. amblycephala*. However, in the present study, WG, SGR was lower, and FCR was higher, in fish fed 120 mg emodin/kg diet than in the control and other treatment groups. This inhibiting effect of dietary 120 mg emodin/kg on growth performance of fish suggests that an overdose of emodin probably causes an imbalance of intestinal flora, inhibits the growth of beneficial bacteria as also reported in a previous study (Liu et al., 2004a), and consequently decreases intestinal digestion and absorption (Liu et al., 2012). Secondly, in an unpublished follow up study, lower digestive enzymes activities were observed on juvenile *M. amblycephala* fed diet supplemented with 120 mg emodin/kg. (Zhang et al., 2013) The results from the present study conclusively

indicated that the growth performance as represented by the parameters tested was improved in *M. amblycephala* fed diets supplemented with 15 and 30 mg emodin/kg, but was inhibited in fish fed a diet supplemented with 120 mg emodin/kg.

Disease outbreaks in commercial fisheries may be controlled by enhancing non-specific immunity through the administration of natural immunostimulants. Anthraquinone derivatives from rhubarb have been tested as immunostimulants in aquaculture (Liu et al., 2010; Liu et al., 2012a). Emodin, one of the anthraquinone derivatives, is antimicrobial and anti-inflammatory (Chang et al., 1996), an antioxidant and scavenger of free radicals (Huang et al., 1995), and immunity regulator (Wang et al., 1995). The present study establishes the immunostimulatory activity of orally administered emodin as evidenced by the enhancement of non-specific immunity and disease resistance to *A. hydrophila* in Wuchang bream. The leukocytes protect against infectious agents caused by microbial and chemical factors (Harikrishnan et al., 2003). A number of studies in fish have provided indirect evidence that TNF- α is an important macrophage-activating factor (MAF) produced by leukocytes (Whyte, 2007), and this has been shown to induce a typical activated-macrophage response, evidenced by increases in respiratory burst activity, phagocytosis and nitric oxide production (Whyte, 2007). Results of this study confirmed the immunity pathway in which the group given 30 mg emodin/kg diet significantly increased the WBC, TNF- α activity and respiratory burst activity compared with control group. Similarly, an earlier study also indicated that TNF- α activity had a direct positive impact on respiratory burst activity (Whyte, 2007).

Neutrophils play a major role in innate immunity. Exocytosis of granules and secretory vesicles plays a pivotal role in most neutrophil functions from early activation to the destruction of phagocytosed microorganisms (Faurschou and Borregaard, 2003). There is evidence for phagocytic, chemotactic and bactericidal functions in fish neutrophils and intense respiratory burst, and these parameters can be used for health status assessment (Whyte, 2007). MPO is possibly released via the azurophilic granules of neutrophils during oxidative respiratory burst activity, as measured using peroxidase content (Menegazzi et al., 1992). In this study, MPO activity in fish given diets with supplemented optimal emodin levels was significantly improved. Similarly, MPO activity in tilapia (*Oreochromis niloticus*) increased after the oral administration of the Chinese herb *Sophora flavescens* (Wu et al., 2012). Results from our investigation indicate that non-specific immunity as represented by the parameters tested was improved in *M. amblycephala* juvenile fed diet supplemented with 30 mg emodin/kg.

In the course of normal metabolism of organisms, the production and elimination of reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radical ($\cdot OH$) and hydrogen peroxide (H_2O_2) maintain the dynamic balance. Excessive free radicals cause peroxidation of lipids. SOD is the first superoxide enzyme through catalyses dismutation of superoxide radicals to hydrogen peroxide and oxygen to relieve oxidative stress (Nordberg and Arner, 2001). Malondialdehyde (MDA), the main component of lipid peroxides, is produced through hydroxyl radical ($\cdot OH$) attack on the cell membrane, has a strong biotoxicity, and damages cell structure and function (Freeman and Crapo, 1982). In fish, dietary supplementation with Chinese and Indian herbal extracts can significantly enhance antioxidant activity (Bhuvaneswari and Balasundaram, 2006; Christyapita et al., 2007). Consistent with these studies, the present study revealed that oral administration of emodin at a medium dose (30 mg/kg) significantly increases plasma antioxidant status in the Wuchang bream. This might be related to the induction of SOD enzyme activity and reducing MDA contents. Our results indicate that oral optimal emodin can contribute to the antioxidant ability of Wuchang bream by decreasing oxidative damage.

A. hydrophila is a ubiquitous bacteria that is native to aquatic environments (Hazen et al., 1978). Our previous studies suggested that in common carp, resistance against *A. hydrophila* infection could be enhanced by dietary supplementation with Chinese herb extracts (Yin et al., 2009). Similarly, we have shown that improving non-specific immune response could increase resistance to pathogen infection in *M. amblycephala* fed an emodin diet.

In conclusion, this study provides evidence that optimal levels (30 mg emodin/kg) of dietary emodin can be an immunostimulant enhancer increasing WBC, respiratory burst activity, SOD activity, TNF- α activity, and the ability of disease resistance against pathogens and microbial infection in *M. amblycephala*.

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