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Comparison Study of the Effects of Anthraquinone Extract and Emodin from *Rheum officinale* Bail on the Physiological Response, Disease Resistance of *Megalobrama amblycephala* under High Temperature Stress

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Abstract

In order to evaluate the effects of anthraquinone extract and emodin from *Rheum officinale* Bail on the physiological response, disease resistance of *Megalobrama amblycephala* under high temperature stress, fish were randomly divided into three groups: one was the control group, fed with a basal diet, and the other two were the treatment groups, fed with the basal diet supplemented with 0.1% anthraquinone extract or 60 ppm emodin for 8 weeks. The results showed that compared with the control group, the two treatment groups significantly increased serum lysozyme activities, superoxide dismutase activities and the relative HSP70 mRNA levels of liver and reduced serum alanine aminotransferase activities before stress. After 12 h high temperature stress, the two treatment groups significantly increased serum triglycerides contents 6 h after stress, liver superoxide dismutase activities 2 and 6h after stress and the relative HSP70 mRNA levels of liver 2 h after stress compared with the control group. And there are lower in the serum cortisol content 2 and 12 h after stress, serum aspartate aminotransferase activity 12 h after stress and liver malondialdehyde content 6 h after stress in the two treatment groups than those of the control group. Artificial infection with *Aeromonas hydrophi1a* showed that the cumulative mortality in the control was higher than that of the treatment group. The results suggest that ingestion of supplements with 0.1% anthraquinone extract and 60 ppm emodin can increase immune ability, enhance resistance against high temperature stress and *A. hydrophi1a* infection.

Keywords: Stress; Anthraquinone extract; immune response; *Aeromonas hydrophila*; *Megalobrama amblycephala.*

Introduction

In intensive aquaculture, fish is constantly exposed to various stressors such as ambient temperature, stocking density, physicochemical parameters, transport, and confinement (Benfey, 2000; Chen *et al*., 2002; Fevolden *et al*., 2003; Xu *et al*., 2011). The feasibility of disease and infection is facilitated by various stress or when pathogens find an adequate environment for reproduction (Davis *et al*., 2002; Martins *et al*., 2011; Xu *et al*., 2011). Fish are poikilotherms and the immune response against invading pathogens depends much more on the environmental temperature. Temperature plays an important role in cold-blooded animals and temperatures below or above the thermal limit can affect physiological functions, including adaptive and innate immunity, increase susceptibility to infection and even cause death (Watts *et al*., 2001; Ainsworth *et al*., 1991 ; Le Morvan *et al*., 1998; Ndong *et al*., 2007). Therefore, how to prevent stress response and alleviate the harm caused by stress is currently one of the key subjects of research in this field. The present research falls on substances, which can strengthen immunity of organisms and alleviate high temperature stress, e.g. Lactoferrin (Yokoyama *et al*., 2005); Vitamin C (Chien *et al*., 2001; Hwang *et al*., 2002); extract of *Gracilaria tenuistipitata* (Yeh, 2010).

China has a rich background in traditional medicines, most of which have been used as immunostimulants to treat human and animal diseases for thousands of years (Tan and Vanitha, 2004). Chinese herbs can lead to an increase in non-specific immunity of fish (Xue *et al*., 2008; Ardó *et al*., 2008; Yin *et al*., 2009; Yeh *et al*., 2009; Jeney *et al*., 2009; Christybapita *et al*., 2007; Liu *et al*., 2010), anti-oxidization enzyme activity (Xie *et al*., 2008; Liu *et al*., 2010), and disease resistance (Xue *et al*., 2008; Ardó *et al*., 2008; Yin *et al*., 2009; Yeh *et al*., 2009; Yeh *et al*., 2010). In the case of some herbs, even the molecular mechanisms of their effects are known (Shao *et al*., 2004; Lei *et al*., 2008; Yuan *et al*., 2008; Liu *et al*., 2010). Anthraquinone extract from *Rheum officinale* Bail, containing emodin, chrysophenol

rhein and other compounds has been used as an immuno-stimulant (Huang *et al*., 1995; Pongnaravane *et al*., 2006; Lin *et al*., 1996; Wu *et al*., 2007; Ding *et al*., 2008; Chen *et al*., 2009). Recent studies have shown that anthraquinone extract from *R. officinale* Bail significantly elevated immunity, increased the resistance to stress, and enhanced the growth of common carp or the giant freshwater prawn (Xie *et al*., 2008; Liu *et al*., 2010).

Wuchang bream (*Megalobrama amblycephala* Yih) is a major freshwater species cultured in China. In recent years diseases of cultured Wuchang bream showed an increasing trend, especially in summer, high temperature causes disease outbreak, resulting in high mortality (He *et al*., 2006). However, few studies have been conducted on the effects of anthraquinone extract and emodin from *R. officinale* Bail on serum metabolites and hepatic anti-oxidization enzymes, and the expression of hepatic heat shock protein 70 of *M. amblycephala* under high temperature. The objectives of the present study were to evaluate and compare the effects of anthraquinone extract and emodin from *R. officinale* Bail on serum metabolites (alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose, total protein, Triglycerides(TG), Cholesterol (CHOL)), immune and stress parameters (cortisol, alkaline phosphatase activity (ALP), lysozyme) and hepatic oxidization indices (hepatic catalase(CAT), superoxide dismutase activities (SOD), malondialdehyde content (MDA)) and to further study the role of anthraquinone extract and emodin to help *M. amblycephala* prevent the high temperature stress so as to provide the theoretical basis for ichthyic pathology to prevent and control fish diseases.

Materials and methods

Fish, Chinese Herbal Medicines and Diets

1,170 fingerlings of *M. amblycephala* were obtained from the Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences. The fingerlings were selected to be of nearly the same size, with initial body weight of 131.5±4.4 g. They were allocated to 9 cement tanks (8 m × 2 m × 1 m) and acclimatized for 15 days. Then they were randomly divided into three groups: one control and two treatment groups. Triplicate groups (3 tanks, 130 individuals per tank) were fed with the basic diet (Table 1) and the basic diet supplied with 60 ppm emodin or 0.1% of anthraquinone extract from *R. officinale* Bail. The anthraquinone extract (1.4 mg of emodin per mL extract) was supplied by the Zhixin Pharmaceutical Co. Ltd., Nanjing, China. Emodin is effective content and emodin resource (containing 40% emodin extracted from *R. officinale* Bail) was provided by the Ju Bang Plant Raw Material Co. Ltd., Shifang, China. Diets were produced at the Wuxi Tongwei Feed Co. Ltd., China.

Rearing Management

Fish were acclimated in concrete tanks for 15 days and then fed by hand with the trial diet. The feeding amount was about 2.0~4.0% body weight, three times a day (06:^{00}-6:^{30}, 12:^{00}-12:^{30}, and 18:^{00}-18:^{30}) for each group. An underground water source was used. Water was oxygenated continuously using an aerator. Silt was siphoned daily, and one-third of the water was exchanged in each tank once a week; water temperature was measured every day, and water quality was checked every week. During the test period the water quality parameters on average were the following; water temperature 26.3±3.51°C, DO>5 mg L^{-1}, NH_3<0.05 mg L^{-1}, H_2S<0.01 mg L^{-1}, and pH 6.80-7.60. The feeding amount was increased every other week, and the amount was adjusted according to body weight measurement every 2 weeks. After the completion of the test period of 8 weeks, serum and

Table 1. The basal diet and nutrition levels of *M. amblycephala*

Ingredients (%)		Nutrition levels	
Fish meal	4.0	Dry matter (%)	88.68
Soybean meal	20.0	Crude protein (%)	30.89
Peanut meal	8.0	Ether extract (%)	4.58
Rapeseed meal	25.0	Calcium (%)	1.15
Cotton meal	8.0	Total phosphorous (%)	1.24
Rice bran	10.0	Available phosphorous (%)	0.84
Wheat middling	17.5	Methionine + Cystine (%)	1.13
Fish oil	2.0	Lysine (%)	1.48
Choline chloride	0.1	Gross energy (kJ·g^{-1})[a]	17.68
Vitamin premix[b]	1.0		
Mineral premix[b]	1.0		
Attapulgite	1.0		
Calcium dihydrogen phosphatee	2.0		
Salt	0.4		
Total	100.0		

[a] Gross energy (GE) kJ·g^{-1}: protein 23.64kJ·g^{-1}, fat 39.54k J·g^{-1}, carbohydrate 17.15k J·g^{-1}; And the others are measured in the nutrition levels. [b]Vitamin and mineral additives were provided by Nanjing Huamu Animal Research Institute.

liver samples were collected, and weight gain was measured.

Stress Experiment

After rearing period fish of almost similar sizes were selected for the experiment of acute high temperature stress at 34±1°C, imitating the methods described in Liu *et al*. (2010). Triplicate groups of *M. amblycephala* (25 individuals per tank; body weight about 200 g) with water temperature of 25±1°C were selected and transferred into the temperature controlled aquariums (2 m × 2 m × 1.2 m) with high temperature stress test of 34±1°C for 12 hours, with sufficient oxygen supply (DO>5 mg L^{-1}), in a quite environment with no human interference.

Challenge Experiments with *Aeromonas hydrophi1a*

At the end of the trial, a challenge test was done on two test groups and the control group in triplicate (3 rearing tanks, 20 individuals per tank) using the bacterial septicaemia pathogen *Aeromonas hydrophi1a*, Ah, BSK-10, provided by the Zhejiang Provisional Freshwater Fisheries Research Institute, China. According to the method described by Xie *et al*. (2008), *A. hydrophi1a* was activated twice and diluted by sterile saline, and the final concentration was set to 1×10^8 CFU cells ml^{-1}. Bacterial suspension (1.0 ml per 100 g body weight) was injected into the abdominal cavity, and mortalities were checked at 0 h, 12 h, 24 h, 48 h, 72 h, 96 h and 120 h after the challenge.

Serum and Liver Sample Collection and Measurement

Nine samples (3 tanks / group, 3 fish / tank) were taken from each group before stress and 2 h, 6 h, and 12 h after high temperature stress. Fish were netted quickly and immediately placed in MS-222 with a concentration of 150-200 mg L^{-1} for rapid and deep anesthesia. Then serum was sampled from the caudal vein and placed in a refrigerator at 4°C for 1-2 hours. The blood sample was centrifuged at 4°C and 3000 g for 10 minutes to prepare the serum. The supernatant was removed and the sample preserved at -20°C. After the collection of blood, nine liver samples of each group were collected and frozen in liquid nitrogen and stored at –80°C until further analysis.

Measurement of Serum and Liver Sample

The Serum Cortisol, Lysozyme, ALP Assay: Cortisol was measured by radioimmunoassay (RIA), according to Pickering *et al*. (1983) with a test kit bought from the Beijing Beifang Biotech Research Institute. Lysozyme activity was measured by the method of Muona and Soivio (1992) and test kits were bought from the Nanjing Jiancheng Biological Engineering Research Institute of China. The serum ALP activity was determined by the colorimetric method of Pinoni *et al*. (2004) (test kit from the Shanghai Fudan Zhangjiang Bio Medical Co., Ltd., China) in a Beckman Cx-4 type Auto Bio-chemical Analyzer (Beckman Coulter, USA).

The Serum Total Protein, ALT and AST assay: Serum total protein content was measured by Biuret method (kit was bought from Shanghai Fudan Zhangjiang Biopharmaceutical Co., Ltd., China) in a Beckman Cx-4 type Auto Bio-chemical Analyzer (Beckman Coulter, USA) and the bovine serum albumin was used as the standard protein (BSA, 66 kDa, Nanjing Jiancheng Biological Engineering Research Institute of China). Serum AST and ALT activities were determined by the colorimetric method (test kit from the Shanghai Fudan Zhangjiang Bio Medical Co. Ltd., China) in a Beckman Cx-4 type Auto Bio-chemical Analyzer (Beckman Coulter, USA).

The Serum Glucose, Triglycerides, Cholesterol Assay: Serum glucose, triglycerides, total cholesterol, and AST and ALT activities were determined by the colorimetric method (test kit from the Shanghai Fudan Zhangjiang Bio Medical Co. Ltd., China) in a Beckman Cx-4 type Auto Bio-chemical Analyzer (Beckman Coulter, USA).

Hepatic CAT, SOD, MDA assay: Hepatic samples were homogenized in ice-cold phosphate buffer (1:10 dilution) (phosphate buffer: 0.01 mol/L, pH 7.4). The homogenate was then centrifuged for 20 min (4°C, 3000×g). An aliquot of the supernatants were used to determine the hepatic CAT, SOD, and MDA. Hepatic CAT activity, SOD activity, and MDA content were measured by the Colorimetric method (Sinha, 1972), the xanthine oxides method (Marklund, 1974), and barbituric acid reaction chronometry (Drape *et al*., 1993), respectively. Protein content in hepatopancreas was measured by Folin method and the standard protein is the bovine serum albumin bought from Nanjing Jiancheng Biological Engineering Research Institute of China.

Measurement of Hepatic HSP70: HSP70 cDNAs were amplified by PCR using specific primers chosen in their cDNA sequences in *M. amblycephala* from GenBank (accession No EU884290.2). Primers of β-actin were based on *M. amblycephala* cDNA sequences in GenBank (accession No AY170122.2). Primers were as follows:

(1) 5'-CTTTATCAGGGAGGGATGCCAGC-3' and 5'-CCCTGCAGCATTGAGTTCATAAGGT-3' for the HSP70 cDNA;

(2) 5'- TCGTCCACCGCAAATGCTTCTA-3'; and

(3) 5'-CCGTCACCTTCACCGTTCCAGT-3' for the β-actin cDNA. All primers were synthesized by the Shanghai Generay Biotech Co., Ltd., China. The PCR products were all 100~150 bp long.

Total RNA was extracted from Megalobrama amblycephala liver of 100 mg using Trizol reagent (Dalian Takara Co. Limited, China.). The purified RNA generally had OD260/OD280 ratio of 1.8-2.0. RNA samples were treated with RQ1 RNase-free DNase (Dalian Takara Co. Limited, China) to avoid genomic DNA amplification. cDNA was generated from 500 ng DNase-treated RNA using the ExScriptTM RT-PCR Kit (Dalian Takara Co. Limited, China) and the reverse transcription PCR reaction solution consisted of 500 ng RNA, 2 µl 5×buffer, 0.5 µl dNTP mixture (10 mM each), 0.25 µl RNase Inhibitor (40 $U \cdot \mu l^{-1}$), 0.5 µl dT-AP primer (50 mM), 0.25 µl ExScript™ RTase (200 $U \cdot \mu l^{-1}$), and DEPC H_2O, up to 10 µl. The reaction conditions were as follows: 42°C for 40 min, 90°C for 2 min, and 4°C thereafter.

Real-time PCR was used to determine mRNA levels based on SYBR Green I fluorescence kit (Livak and Schmittgen, 2001). Real-time PCR was performed in a Mini Opticon Real-Time Detector (BIO-RAD, USA). The fluorescent quantitative PCR reaction solution consisted of 10.0 µl SYBR® premix Ex Taq™ (2×), 1.6 µl HSP70 primer or β-actin (10 µM), 2.0 µl RT reaction mix (cDNA solution), 6.4 µl dH_2O. The reaction conditions were as follows: 95°C for 3 min, followed by 43 cycles consisting of 95°C for 10 s and 60°C for 20 s. Then the florescent flux was recorded and the reaction continued at 72°C for 3 min. The dissolution rate was measured between 65°C and 90°C. Each increase of 0.2°C was maintained for 1 s, and the fluorescent flux was recorded. Relative quantification of the target gene transcript (HSP70) with a chosen reference gene transcript (β-actin) was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). This mathematical algorithm, which needs no calibration curve, computes an expression ratio based on real-time PCR efficiency and the crossing point deviation of the sample *versus* a control. PCR efficiency was determined with a standard curve, using serial dilution of cDNA; $\Delta\Delta C_T=(C_{T,Target}-C_{T,Bactin})_{time\ x}-(C_{T,Target}-C_{T,Bactin})_{time\ o}$.

Data Statistics and Analysis

We used SPSS (version 11.5) software Duncan's multiple range tests and Independent-Samples t-tests to determine the differences. In Duncan's multiple range test, diverse little letters show significant differences ($P<0.05$) in different dosage groups of each sampling point. In Independent-Samples t-tests, significant differences ($P<0.05$) between values obtained before and after stress are marked by asterisks. All the results were expressed as means ± standard error ($\overline{X}$ ±SE).

Results

The Effect of Anthraquinone Extract and Emodin on Serum Cortisol, Lysozyme, and ALP in *M. amblycephala*

Serum concentrations of cortisol increased significantly, relative to pre-stress levels, 2, 6 and 12 h after stress in control group, 0.1% anthraquinone extract group and 60 ppm emodin group ($P<0.05$) (Figure 1A). In addition, the serum cortisol concentration was significantly lower in the 0.1% anthraquinone extract group at 2 and 12 h after stress and 60 ppm emodin group prior to stress, at 2, 6 and 12 h after stress than the control group ($P<0.05$) (Figure 1A).

Serum lysozyme activity was significantly lower than pre-stress levels, in the control at 6h after stress, in the 0.1% anthraquinone extract group and 60 ppm emodin group at 12 h after stress ($P<0.05$) (Figure 1B). Serum lysozyme activity was significantly higher in the two treatment groups than the control group prior to stress ($P<0.05$) (Figure 1B). In addition, serum lysozyme activity was significantly higher in the 0.1% anthraquinone extract group at 6 h after stress than the control group ($P<0.05$) (Figure 1B).

There was no significant change in serum ALP activity prior to stress or at 2, 6 and 12h after stress ($P<0.05$) (Figure 1C). Serum ALP activity was only significantly higher in the 0.1% anthraquinone extract group than the control group at 2 h after stress ($P<0.05$) (Figure 1C).

The Effect of Anthraquinone Extract and Emodin on Serum Total Protein, AST and ALT in *M. amblycephala*

Serum total protein content was significantly lower than pre-stress level, 2 h after stress in the 0.1% anthraquinone extract group ($P<0.05$) (Figure 2A). Furthermore, the total protein concentration was significantly higher in the 0.1% anthraquinone extract group than the control group prior to stress, at 6 h after stress ($P<0.05$) (Figure 2A).

There was no effect on serum AST activity after stress in the control group and the two treatment groups compared to pre-stress level ($P>0.05$) (Figure 2B). However, serum AST activity was significantly lower in 0.1% anthraquinone extract group before stress and at 12 h after stress and 60 ppm emodin group at 12 h after stress than the control group ($P<0.05$) (Figure 2B).

Serum ALT activity was significantly higher than pre-stress activity, 2, 6 and 12 h after stress in the control group and the two treatment groups ($P<0.05$) (Figure 2C). Furthermore, serum ALT activity was significantly lower in the 0.1% anthraquinone extract group prior to stress and at 2, 12 h after stress, in the 60 ppm emodin group before stress than the control group ($P<0.05$) (Figure 2C).

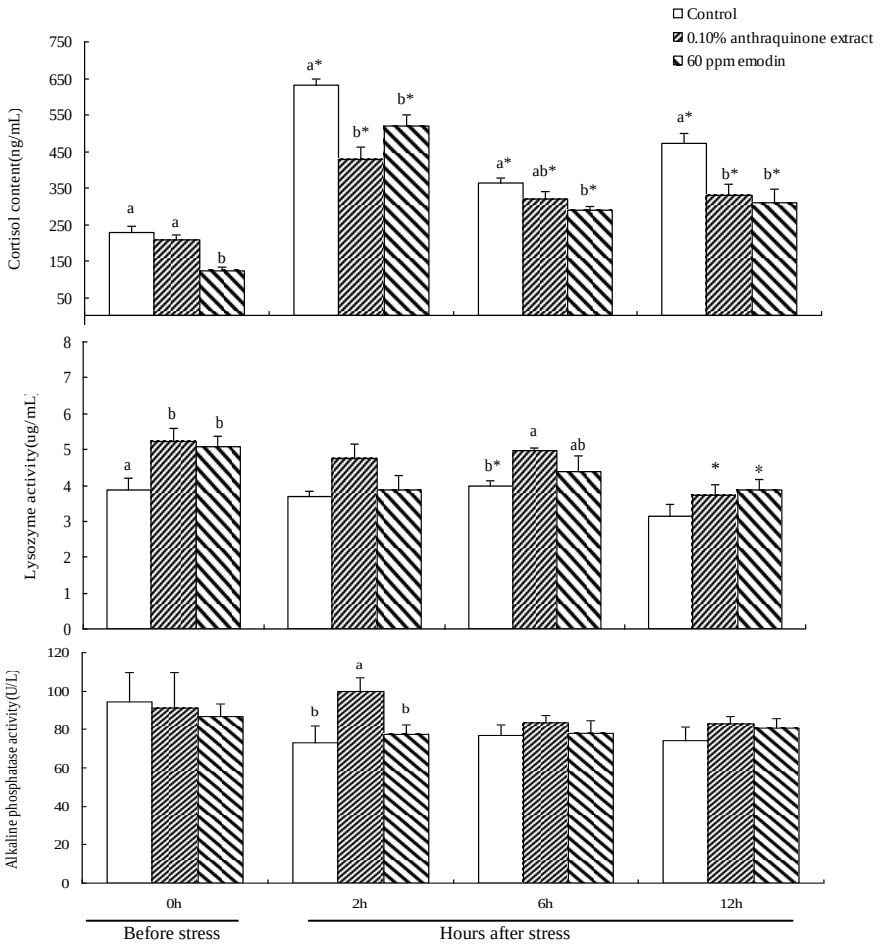

Figure 1. Effects of anthraquinone extract and emodin on serum cortisol (A), lysozyme (B) and ALP (C) o f *M. amblycephala.* Data are expressed as means ± SEM (n = 9).
Diverse little letters show significant differences (P<0.05) in different dosage groups of each sampling point in Duncan's multiple range test. Significant differences (P<0.05) between values obtained before and after stress are marked by asterisks in t-tests.

The effect of Anthraquinone Extract and Emodin on Serum Glucose, TG and CHOL in *M. amblycephala*

Serum glucose content was significantly higher than pre-stress levels, 2, 6, and 12 h after stress in the control group and 60 ppm emodin group, 12h after stress in the 0.1% anthraquinone extract group (P<0.05) (Figure 3A). Furthermore, the glucose concentration was significantly lower in the 0.1% anthraquinone extract group at 2h after stress and the 60 ppm emodin group at 6h after stress than the control group (P<0.05) (Figure 3A).

There was no effect on serum TG content in the control group and the two treatment groups compared with prior to stress (P>0.05) (Figure 3B). Serum TG content was significantly higher in the 0.1% anthraquinone extract group and 60 ppm emodin group at 6 h after stress than the control group (P<0.05) (Figure 3A).

There was no effect on serum CHOL concentration between the control group and the two treatment groups (P>0.05) (Figure 3C). Serum CHOL content was only significantly lower in the 60 ppm emodin group at 2 h after stress than pre-stress level (P<0.05) (Figure 3C).

The Effect of Anthraquinone Extract and Emodin on Hepatic Anti-oxidization Enzyme Activity in *M. amblycephala*

Hepatic catalase activity was significantly lower in the control at 12 h after stress, the 0.1% anthraquinone extract group and the 60 ppm emodin group at 6, 12 h after stress than pre-stress levels (P<0.05) (Figure 4A). Furthermore, hepatic catalase activity was significantly higher in the 60 ppm emodin group than the control group at 6 h after stress (P<0.05) (Figure 4A).

Hepatic superoxide dismutase activity was significantly lower in the 0.1% anthraquinone extract group and the 60 ppm emodin group at 6, 12 h after stress than pre-stress levels (P<0.05) (Figure 4B). Hepatic superoxide dismutase activity was significantly higher in the 0.1% anthraquinone extract group prior to stress, at 2, 6 h after stress and the 60

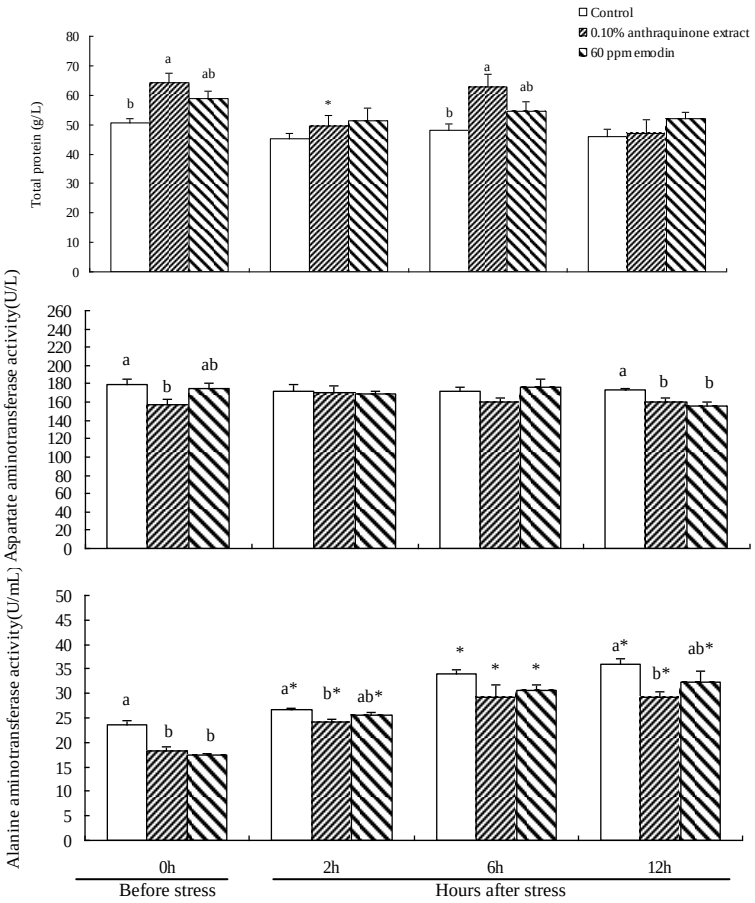

Figure 2. Effects of anthraquinone extract and emodin on serum total protein (A), AST (B) and ALT (C) of *M. amblycephala*
Note: Data are expressed as means ± SEM (n = 9). Legends are the same as on Figure 2.

ppm emodin group prior to stress, at 2, 6 and 12 h after stress than in the control group (P<0.05) (Figure 4B).

Hepatic malondialdehyde content was significantly higher in the control group at 6, 12 h after stress, in both treatment groups at 12 h after stress than pre-stress levels (P<0.05) (Figure 4C). Hepatic malondialdehyde content was significantly lower in the 0.1% anthraquinone extract group at 2, 6 h after stress, the 60 ppm emodin group at 6 h after stress than the control group (P<0.05) (Figure 4C).

Effect of Anthraquinone Extract and Emodin on the Relative Level of Hepatic HSP70 mRNA in *M. amblycephala*

The relative levels of hepatic HSP70 mRNA were significantly higher in the control group at 2, 6 and 12 h after stress, in the 0.1% anthraquinone extract group at 2, 12 h after stress and 60 ppm emodin group at 6, 12 after stress than pre-stress levels (P<0.05) (Figure 5). Furthermore, the relative level of hepatic HSP70 mRNA was significantly higher in the 0.1% anthraquinone extract group prior to stress, at 2 h after stress and 60 ppm emodin group prior to stress, at 2, 6 h after stress than in the control group (P<0.05) (Figure 5).

The Effects of Anthraquinone Extract on Effectiveness of Protection From Pathogenic Infection of *M. amblycephala*

After 8 weeks of feeding the anthraquinone extract, Wuchang bream were challenged with *A. hydrophila* infection. Accumulative mortality was calculated for 120 hours (Figure 6). The maximum cumulative mortality occurred between 24 h and 48 h after *A. hydrophila* infection. There were no significant differences in cumulative mortality among any of the groups at 0-12 h after *A. hydrophila* infection. However, after 24 h of infection, the cumulative mortality in the control group was about 75%, while the cumulative mortality in the 0.1% anthraquinone extract group was about 60%, and in the 60 ppm emodin group was about 55%. After 96 h, the cumulative mortality in the treated groups was about 80.00%, but in the control group it was about 95%. After 120 hours, the total accumulated percentages of mortalities were 100% in the control, 83.33% in the group with 0.1% anthraquinone extract, and 80.00% in the group with 60 ppm emodin.

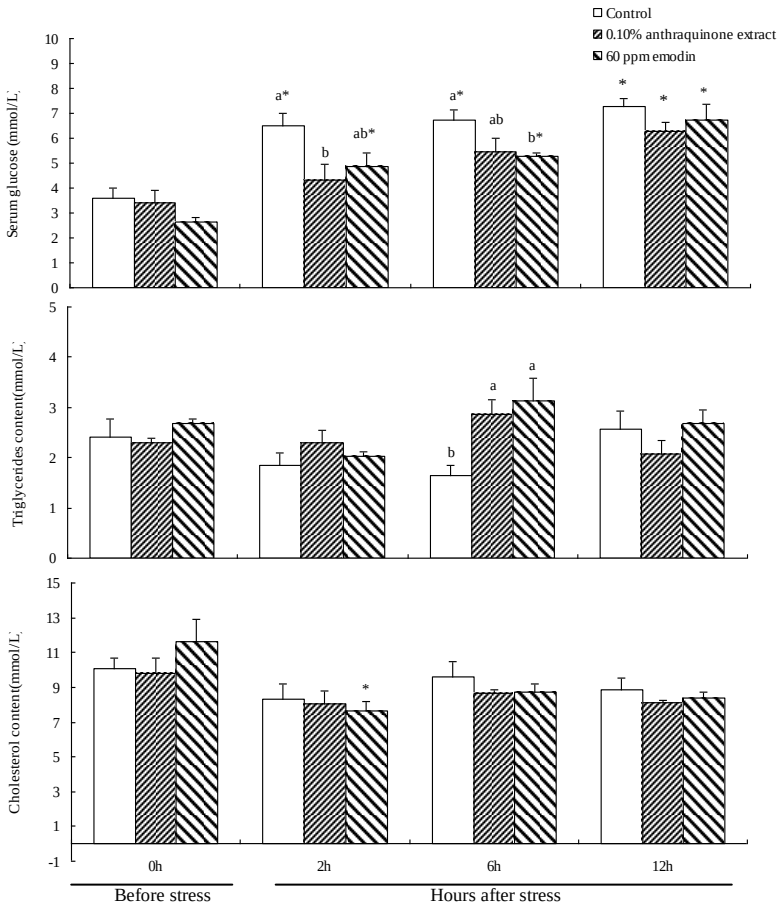

Figure 3. Effects of anthraquinone extract and emodin on serum glucose (A), TG (B) and CHOL (C) of *M. amblycephala*
Data are expressed as means ± SEM (n = 9). Legends are the same as on Figure 2.

Discussion

This study was conducted to evaluate how anthraquinone extract and emodin from *R. officinale* affect postprandial serum immune and stress parameters, serum metabolites, hepatic oxidization indices as well as the expression of HSP70 in *M. amblycephala* under the high temperature stress. Both herbal extracts were able to enhance resistance against high temperature stress and *A. hydrophila* infection of *M. amblycephala* to some degree.

In aquaculture fish are consistently affected by various stress factors such as high temperature, stocking density, transport and storage. If fish are in long- standing stress during culture process, the axis of hypothalamus - pituitary-interrenal axis of fish will be continuously stimulated so as to cause cortisol level upward (Benfey, 2000; Chen *et al*., 2002; Fevolden *et al*., 2003). Cortisol is considered a key response to stress in fish and in more general terms to an animal's welfare (Wendelaar Bonga, 1997; Mommsen *et al*., 1999). In rainbow trout, the plasma cortisol contents drastically decreased with concentration of 0.1% glucan compared with the control fish under stress (Jeney *et al*., 1997). A study of common carp treated with anthraquinone extract as an immunostimulant showed that anthraquinone extract in the diet can also reduce the plasma cortisol contents under stress (Xie *et al*., 2008). In the present study, we found that serum concentrations of cortisol increased significantly at 2, 6 and 12 h after high temperature stress in the two treatment groups and the control group. In addition, the serum cortisol concentration was significantly lower in the 0.1% anthraquinone extract group at 2 and 12 h after stress and 60 ppm emodin group prior to stress, at 2, 6 and 12 h after stress than the control group.

Stress made blood cortisol increase apparently and in turn caused some immune index change e.g. blood lysozyme, ALP activity (Sukhanova *et al*., 1996; Fevolden *et al*., 1999). Common carp or freshwater prawn fed with anthraquinone extracts under the high density stress or the high temperature stress (Xie *et al*., 2008; Liu *et al*., 2010) showed an increase of blood lysozyme activity. In *Labeo rohita*, the ALP activity drastically increased with doses of 0.01-0.05% *Achyranthes aspera* compared with the control fish (Rao *et al*., 2006). In the present study, the results showed that serum lysozyme activity was significantly lower than pre-stress level, in the control at 6h after stress and the two treatment groups at 12 h after stress and serum lysozyme activity was

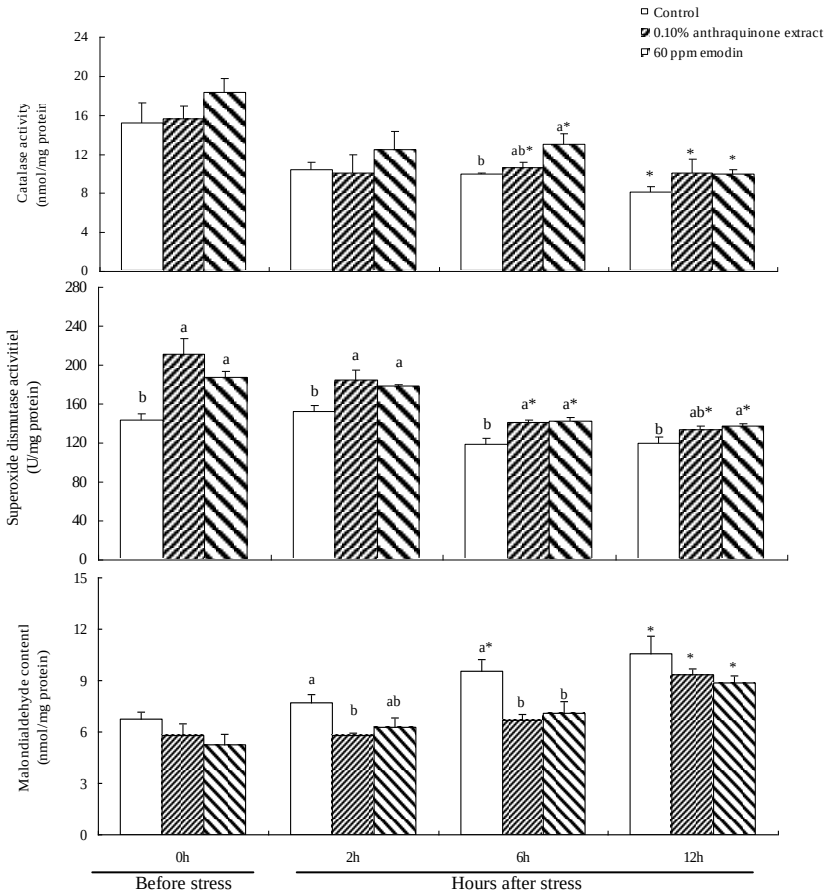

Figure 4. Effects of anthraquinone extract and emodin on hepatic catalase (A), superoxide dismutase activity (B) and malondialdehyde content (C) of *M. amblycephala*. Data was expressed as means ± SEM (n = 9). Significant differences (P<0.05) between values were marked by asterisks.

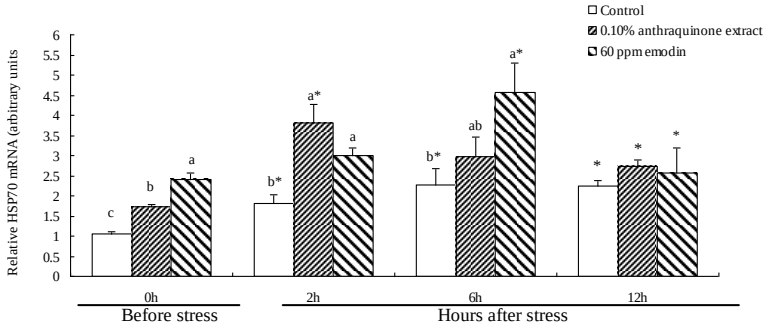

Figure 5. Effects of anthraquinone extract and emodin on the relative level of hepatic HSP70 mRNA of *M. amblycephala*. Data was expressed as means ± SEM (n = 9). Significant differences (P<0.05) between values were marked by asterisks.

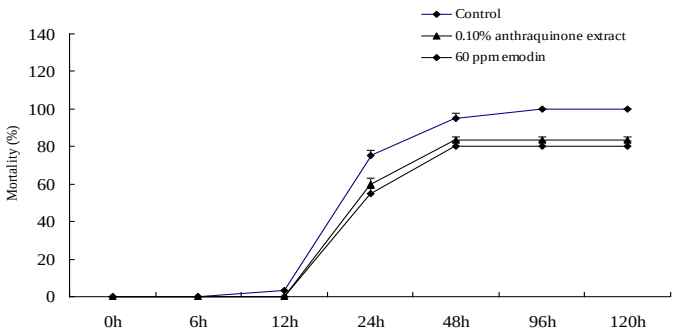

Figure 6. Effects of anthraquinone extract and emodin on the cumulative mortality after *A.hydrophi1a* infection of *M. amblycephala*. Data was expressed as means ± SEM (*n* = 9).

significantly higher in the two treatment groups than the control group prior to stress. Serum ALP activity was also significantly higher in the 0.1% anthraquinone extract group than the control group at 2 h after stress. Therefore the above results of the present study indicated that supplements of 0.1% anthraquinone extract or 60 ppm emodin could somewhat reduce the serum cortisol concentration, improve the serum lysozyme and ALP activities, and enhance the high temperature resistance ability of *M. amblycephala* to some degree.

The metabolism in their bodies will change, the demand of energy will increase, and some serum metabolites such as protein, AST and ALT, glucose, TG, CHOL will change to some extent under high temperature stress. Some scientists realized that blood protein will increase under acute stress conditions such as transportation, ammonia, pathogenic infection whereas chronic stress for a long period of time will cause decrease of blood total protein (Jeney *et al*., 1997; Chen *et al*., 1992). Rainbow trout treated with fructose as an immunostimulant showed that fructose in the diet can increase the plasma protein contents under stress (Jeney *et al*., 1997). The freshwater prawn fed with anthraquinone extracts under the stress showed an increase of blood total protein content (Liu *et al*., 2010). In the present study, serum total protein content was significantly lower than pre-stress levels, 2 h after stress in the 0.1% anthraquinone extract group and the total protein concentration was significantly higher in the 0.1% anthraquinone extract group than the control group prior to stress, at 6 h after stress.

Some studies have shown that various forms of stress can cause the increase of the plasma ALT and AST activities in the fish (Cho *et al*., 1994). Nakano *et al*. (1999) reported that AST and ALT activities in rainbow trout fed a diet containing astaxanthin rich red yeast (*Phaffia rhodozyma*) under oxidative stress were significantly lower than those of the control fish. Liu *et al*. (2010) also found the AST and ALT activities in prawn significantly decreased with a dose of 0.1-0.2% anthraquinone extracts compared with the control fish. In the present study, serum ALT activity was significantly higher than pre-stress levels, 2, 6 and 12 h after stress in the control group and the two treatment groups. Furthermore, 0.1% anthraquinone extract and 60 ppm emodin groups reduced the ALT activities before stress and AST activity at 12 h after stress compared with the control group.

In order to satisfy the requirement of energy during stress process, blood glucose would abruptly rise (Rotllant *et al*., 2003; Hsieh *et al*., 2003) and is an important parameter of fish under stress (Hsieh *et al*., 2003). Cholesterol is one of the components of cell membranes and an important raw material for synthesis of steroid hormones, while triglycerides can store energy (Chang *et al*., 2006). Common carp fed with different amounts of anthraquinone extract could alleviate the increase of glucose to some extent after high density stress (Xie *et al*., 2008). Liu *et al*. (2009) indicated that anthraquinone extract can increase haemolymph cholesterol and triglyceride contents to meet the demand for total cholesterol and triglycerides of freshwater prawn during stress. Consistent with these studies, this experiment showed that 0.1% anthraquinone extract group reduced the glucose concentration at 2h after stress and improved the serum TG content at 6h after stress compared to the control group. And the 60 ppm emodin group reduced the glucose concentration at 6h after stress and enhanced the serum TG content at 6h after stress compared to the control group.

The stress response might also increase free radical contents, which may lead to the increase of the lipid peroxidation content and lipid peroxidation injury (Zhou *et al*., 2003; Bagnyukovaa *et al*., 2007). The antioxidant enzyme system plays a prominent role in resisting lipid oxide damage (Holmblad *et al*., 1999; Lopes *et al*., 2001). In fish, dietary intake of Chinese herbal extracts can enhance the antioxidant ability and resisted the impact of the stress (Xie *et al*., 2008; Christybapita *et al*., 2007). Consistent with these studies, our study indicated that compared to the control, 0.1% anthraquinone extract group or 60 ppm emodin had increased activities of hepatic catalase and superoxide dismutase and decreased the malondialdehyde contents, thus decreasing lipidic superoxide harm to some extent under the high temperature.

Heat shock protein 70 (HSP70) is one of the most abundant proteins, maintains cellular homeostasis and plays a role in protecting an organism following stress (Iwama *et al*., 1998; Forsyth *et al*., 1997; Wang *et al*., 2009; Ming *et al*., 2010). White shrimp fed with Chinese herbs could improve the expression of HSP70 (Lei *et al*., 2008). Japanese flounder fed with bovine lactoferrin could increase the expression of HSP70 after high temperature stress (Yokoyama *et al*., 2005). Correspondingly in the present study, the relative levels of hepatic HSP70 mRNA were significantly higher in the control group at 2, 6, and 12 h after stress, in the 0.1% anthraquinone extract group at 2 and 12 h after stress and 60 ppm emodin group at 6 and 12h after stress than pre-stress levels. Furthermore, the relative level of hepatic HSP70 mRNA was significantly higher in the 0.1% anthraquinone extract group prior to stress, at 2 h after stress and 60 ppm emodin group prior to stress, at 2 and 6 h after stress than in the control group.

Earlier studies in our laboratory also revealed that common carp fed with Chinese herb extracts enhanced resistance to *A. hydrophila* infection (Xie *et al*., 2008; Yin *et al*., 2009). In the present study on *M. amblycephala* infected with *A. hydrophila*, the maximum cumulative mortality occurred between 1 and 2 days after infection. After 5 days, mortalities were significantly reduced in the groups with 0.1% anthraquinone extract and 67.5 ppm emodin

compared to the controls, which is the lowest mortality in fish fed with both herbs. These results indicate that supplements of 0.1% anthraquinone extract and 67.5 ppm emodin confer some protection for *M. amblycephala* from *A. hydrophila* infection.

Conclusions

In the present study, these results can be correlated with the increased serum lysozyme activity, ALP activity, total protein content, TG concentration or hepatic CAT and SOD contents and the decreased serum cortisol, glucose, AST, ALT or hepatic MDA in treated with the supplement of 0.1% anthraquinone extract and 60 ppm emodin to some degree under the high temperature stress. In summary, doses of 0.1% anthraquinone extract and 60 ppm emodin from *R. officinale* Bail can increase immune and anti-oxidation capability, enhance resistance to high temperature stress and *A. hydrophila* infection of *M. amblycephala*. It is worth mentioning that the 60 ppm emodin group only reduced the serum cortisol concentration before stress and improved the relative level of hepatic HSP70 mRNA prior to stress compared with the 0.1% anthraquinone extract group, and there were no significant differences in the other parameters. Furthermore, the cost of emodin is higher than that of anthraquinone extract, so anthraquinone extract are favored for prophylactic use in fish production.

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