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# Immune defense of emodin enriched diet in *Clarias batrachus* against *Aeromonas hydrophila*

Ramasamy Harikrishnan[a,*], Sundaram Jawahar[b], Subramanian Thamizharasan[b], Bilal Ahmad Paray[c], Mohammad K. Al-Sadoon[c], Chellam Balasundaram[d]

[a] Department of Zoology, Pachaiyappa's College for Men, Kanchipuram, 631 501, Tamil Nadu, India
[b] Department of Biotechnology, Bharath College of Science and Management, Thanjavur, 613-005, Tamil Nadu, India
[c] Zoology Department, College of Science, King Saud University, PO Box 2455, Riyadh, 11451, Saudi Arabia
[d] Department of Herbal and Environmental Science, Tamil University, Thanjavur, 613 005, Tamil Nadu, India

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ABSTRACT

This study investigates the effect of emodin enriched diet on growth, hematology, and immune response in walking catfish, *Clarias batrachus* against *Aeromonas hydrophila*. The basal (control) diet supplemented with emodin at 0.0, 0.1, 0.2, or 0.4 g $kg^{-1}$ was fed to the experimental groups for a period of four weeks. Feeding infected fish with 0.2 g $kg^{-1}$ and 0.4 g $kg^{-1}$ emodin enriched diets resulted in an overall weight gain, enhanced PER and FCR when compared to other diets. The survival rates were 98.3% and 96.7% in 0.1 g $kg^{-1}$ and 0.4 g $kg^{-1}$ emodin diet fed groups. Feeding with 0.2 g $kg^{-1}$ diet the RBC level significantly elevated on week 1 and with 0.4 g $kg^{-1}$ diet on weeks 2 and 4. The WBC, the percentage of globulin and neutrophils increased significantly with 0.2 g $kg^{-1}$ diet only on week 4; however with 0.4 g $kg^{-1}$ diet the increase was observed from week 1–4. The phagocytic activity increased significantly on being fed with 0.4 g $kg^{-1}$ diet on week 2 while with 0.2 g $kg^{-1}$ and 0.4 g $kg^{-1}$ diets the increase manifested only on week 4; the respiratory burst activity also significantly increased on week 4 whereas increased the complement activity on weeks 2 and 4. The superoxide dismutase (SOD) activity was high on being fed with 0.4 g $kg^{-1}$ diet on week 1; with 0.2 g $kg^{-1}$ or 0.4 g $kg^{-1}$ diets the increase was observed on weeks 2 and 4. The serum IgM level significantly increased when fed with 0.4 g $kg^{-1}$ diet whereas the lysozyme activity was enhanced with 0.2 g $kg^{-1}$ and 0.4 g $kg^{-1}$ emodin diets on weeks 2 and 4. The percentage cumulative mortality was 10% with 0.1 g $kg^{-1}$ or 0.2 g $kg^{-1}$ diets while with 0.2 g $kg^{-1}$ diet it was 15%. The results demonstrate that as a feed additive emodin acts as an immunostimulant enhancing the specific and nonspecific immune defense affording increased disease protection, enhances better growth and boosts hematology parameters in *C. batrachus* against *A. hydrophila* infection.

## 1. Introduction

Aquaculture as an industry has achieved significant strides in recent decades; this also impelled the need to boost the immunity of cultivated organisms against pathogens. Indeed intensive fish farming practice has triggered stress conditions that adversely affect the immune system, increasing the susceptibility to pathogens which contribute to economic loss. The walking catfish, *Clarias batrachus* a freshwater air breathing catfish, is an amenable species for intensive culture. Though, it is a native species of Southeast Asia and Africa due to its wide adaptability to varying conditions. *C. batrachus* has been successfully introduced outside its native range in several countries for intensive aquaculture. It is considered as one of the most popular and economically valuable freshwater food fish in the countries like India, Bangladesh, Sri Lanka, Myanmar, and Malaysia [1]. In the wild *C. batrachus* is becoming a critically endangered species due to over-exploitation and reduction of native habitat due to reclamation of wetlands coupled with excessive input of pesticides, herbicides, and inorganic fertilizers from agricultural farms [2]. In some parts of India, particularly in West Bengal, Tripura, and Tamil Nadu *C. batrachus* is valued as a medicinal fish used traditionally to boost the health of pregnant and lactating mothers, elders and children; it is an excellent source of aquaprotein ensuring nutritional enrichment in the regular diet of the people apart from being an export commodity. Besides it is a source for sustainable livelihood, employment generation. India has exported catfish products worth of 315.41 INR million in 2016 [3].

*Aeromonas hydrophila* is a Gram-negative rod-shaped bacterial pathogen commonly found in all aquatic habitats besides in sludge, soil,

* Corresponding author.
*E-mail address:* rhari123@yahoo.com (R. Harikrishnan).

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and food; its infection is commonly associated with several disease symptoms like hemorrhagic septicemia, epizootic ulcerative syndrome (EUS), fin and tail rot [4,5] and motile aeromonad septicemia (MAS) leading to high mortality worldwide [6]. In intensive culture practice *Aeromonas* inflicts mass mortalities and economic loss in catfish culture throughout world and is considered as the most common and troublesome pathogen in warm as well as cold water [1,7–10].

Application of traditional antibiotics and chemotherapeutics continue to remain as the standard measure to control fish diseases, though it leads to development of drug-resistant bacterial strains creating problems like increased toxicity, residues and possibly some public health as well as adverse environmental consequences; their application under culture conditions or in open-water systems remains questionable because of their bioaccumulation in sediments and organisms besides being costly; besides the farmers are often provided with ambiguous advice by feed and chemical companies [11]. Recently the application of natural immunostimulants such as herbals and probiotics is emerging as a safe alternative approach to enhance the immunity of cultured fish to provide better protection against pathogens. Prevention of fish diseases and reduction of the side effects are caused by chemotherapy demands promotion of organic aquaculture by using herbs and probioitcs [12–14]. Several natural plant have numerous active principles such as alkaloids polysaccharides, polypeptides, phenols, terpenoids, saponins, steroids volatile oils, tannins promote different biological activities like anti-stress, growth promotion, appetite stimulation, tonic, immunostimulation, and antimicrobial properties in both finfish and shellfish [15–18].

Emodin (1, 3, 8-trihydroxy-6-methyl-anthraquinone) is an important bioactive compound from plants that has a variety of pharmacological properties, such as anti-inflammatory [19], anti-oxidant [20], scavenging free radical [21], anti-microbial [22], immunity regulation [23], and anti-tumor [24] activities. As an immunostimulant it leads to an increase in nonspecific immunity and disease resistance [13] and anti-oxidation activity [13,25]. The anthraquinone extract contains main constituents, like emodin, chrysophanol, and rhein that promote growth, enhance immune response, and disease resistance in *Macrobrachium rosenbergii* [25]. However, the growth and immune response of emodin in fish against fish pathogens is yet to be documented. Hence, the present study examines for the first time the effect of dietary emodin on growth performance, hematology, immune response, and disease resistance in *C. batrachus* against *A. hydrophila*.

## 2. Material and methods

### 2.1. Diet

The basal diet comprised fish meal, crude fiber, soybean meal and maize powder as the protein sources; wheat bran, rice bran, and tapioca flour as carbohydrate and fish oil and mustard oil cake as lipid source in addition to premix vitamins and minerals as shown in Table 1. The emodin (Sigma-Aldrich, India) was incorporated separately in four experimental diets with basal control diet at 0.1, 0.2, and 0.4 g $kg^{-1}$ by mixing thoroughly. All the ingredients were passed through a grinder, and passed in a paste extruder (1 mm diameter) through mesh sieve. The prepared diets were stored at −20 °C until used and the proximate composition of the diets were quantified by following AOAC (1995); it comprised crude protein, crude lipid, crude carbohydrate, crude ash, and crude fiber as shown in Table 1.

### 2.2. Pathogen

*A. hydrophila* (MTCC 646) obtained from Institute of Microbial Technology in Chandigarh, India was isolated from infected fish. The bacterium was grown in a 250 ml conical flask containing tryptic soy broth (TSB; Merck) to log phase with agitation at 37 °C. The bacterial culture was harvested after centrifugation at 3500 × g for 20 min at 4 °C and the resulting bacterial pellets were washed twice with sterile 0.15 M phosphate buffered saline (PBS) at pH 7.2. The pellets were resuspended in PBS, divided into aliquots and stored in TSB supplemented with 15% (v/v) glycerol at −70 °C until used. The pathogenic of *A. hydrophila* was confirmed to inoculate and reisolation into *C. batrachus* according to Krieg and Hold [26] and Yogananth et al. [27]. The identity of *A. hydrophila* was confirmed by morphological, pictorial, biochemical characteristics, and PCR method [28,29].

**Table 1**
Feed ingredients and proximate composition (g/kg dry matter) of experimental diets.

| Ingredient | 0 g $kg^{-1}$ | 0.1 g $kg^{-1}$ | 0.2 g $kg^{-1}$ | 0.4 g $kg^{-1}$ |
|---|---|---|---|---|
| Fish meal | 15 | 15 | 15 | 15 |
| Crude fiber | 5 | 5 | 5 | 5 |
| Mustard oil cake | 15 | 15 | 15 | 15 |
| Fish oil | 5 | 5 | 5 | 5 |
| Rice bran | 15 | 15 | 15 | 15 |
| Soybean meal | 10 | 10 | 10 | 10 |
| Maize powder | 15 | 15 | 15 | 15 |
| Wheat bran | 10 | 10 | 10 | 10 |
| Tapioca flour | 5 | 5 | 5 | 5 |
| Molasses | 4 | 3.9 | 3.8 | 3.6 |
| Vitamins and minerals premix[a] | 1 | 1 | 1 | 1 |
| Emodin | 0 | 0.1 | 0.2 | 0.4 |
| Proximate composition (%) | | | | |
| Crude protein (%) | 16.4 | 16.2 | 16.6 | 16.5 |
| Crude lipid (%) | 8.2 | 8.0 | 8.4 | 8.6 |
| Crude carbohydrate (%) | 18.6 | 18.4 | 18.1 | 18.4 |
| Crude ash (%) | 11.3 | 11.5 | 11.1 | 11.6 |
| Crude fiber (%) | 6.5 | 6.3 | 6.6 | 6.1 |

[a] Vitamins and minerals premix (g/kg diet): Vitamin A, 1600 IU; vitamin D, 2400 IU; vitamin E, 160 mg; vitamin K, 16 mg; thiamin, 36 mg; riboflavin, 48 mg; pyridoxine, 24 mg; niacin, 288 mg; panthotenic acid, 96 mg; folic acid, 8 mg; biotin, 1.3 mg; cyanocobalamin, 48 mg; ascorbic acid, 720 mg; choline chloride, 320 mg; calcium 5.2 g; cobalt, 3.2 mg; iodine, 4.8 mg; copper, 8 mg; iron, 32 mg; manganese, 76 mg; zinc, 160 mg; Endox (antioxidant) 200 mg (Colborne Dawes Nutrition Ltd., UK).

### 2.3. Fish and experimental design

The healthy *C. batrachus* (35.2 ± 1.5 g) were purchased from a commercial fish farm. The fishes were screened for any pathological symptoms immediately upon arrival and the fish was dip treatment with 1% $KMnO_4$ solution. The fish were acclimation for 2 weeks in 60 L aerated fiber tanks prior to experiment conduct. Then fish were divided into five experimental groups of 25 each in triplicate (5 × 25 x 3 = 375 fish) into: Group I: uninfected fish fed with basal control diet (C), Group II: infected fish fed with basal control diet (I), Group III: infected fish, fed with 0.1 g $kg^{-1}$ emodin, Group IV: infected fish, fed with 0.2 g $kg^{-1}$ emodin, and Group V: infected fish, fed with 0.4 g $kg^{-1}$ emodin enriched diets at the rate of 5% of their body weight twice a day. Feeding with the respective diets continued till the end of experiment and unfed feed carefully collected for feed utilization conversion. About 50% of water was exchange daily. After 30 days of respective feeding, all groups were challenged intraperitoneally (i.p.) with 50 µl PBS containing *A. hydrophila* at $2.8 \times 10^7$ cfu $ml^{-1}$ as determined by using a Neubauer haemocytometer according to our previous study expect uninfected control diet group. End of weeks 1, 2, and 4 post-challenge, six fish were randomly collected from each experimental tank to collect blood samples by the caudal venipuncture with a 27 gauge needle fitted 1 ml syringe for hematological and immunological assays after fish anaesthetizing with MS-222 ($NaHCO_3$ and tricaine methane sulphonate; Sigma Chemicals, USA) 1:4000 in dechlorinated water for 2 min.

### 2.4. Sample collection

The blood samples were pooled from a random sample of six fish in each experimental tank and the samples were divided into non-heparinized and heparinized (K3EDTA vacuum) tubes (Becton