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Study the immunomodulation of anthracenedione in striped dwarf catfish, *Mystus vittatus* against pathogenic bacteria, *Aeromonas hydrophila*

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ABSTRACT

Anthracenedione is a derivative of anthraquinone aromatic organic natural pigments found in senna, aloe latex, rhubarb, cascara, lichens, and fungi having broad range of bioactivity, including anti-cancer, anti-inflammatory, anti-microbial, anti-fungal, anti-oxidant, anti-viral activities suggesting potential for clinical purpose of many diseases. The effect of anthracenedione enriched diet on growth, hematology, innate and adaptive immune parameters as well as protection from *Aeromonas hydrophila* in *Mystus vittatus* was reported. The weight gain, feed intake, specific growth rate (SGR), and feed conversion ratio (FCR) were significantly increased in uninfected groups fed with 5 mg kg^{-1} diet. The red blood cells (RBC) and white blood cells (WBC) count and the percentage of lymphocytes were significantly augmented in both infected and uninfected groups feeding with any diet. The percentage of monocytes, eosinophils, neutrophils and the biochemical profile such as total protein, albumin, and globulin also were significantly increased in the infected and uninfected groups fed with 5 mg kg^{-1} enriched diet. The innate and adaptive immune parameters such as phagocytic activity, immunoglobulin M (IgM), respiratory burst activity, complement activity, and lysozyme activity were significantly increased in uninfected and infected groups fed with 5 or 10 mg kg^{-1} diets but not with 1 mg kg^{-1} diet. The serum superoxide dismutase (SOD) activity is significantly increased in the uninfected and infected fish fed with 5 mg kg^{-1} diet but the increase was not significantly observed in 1 or 10 mg kg^{-1} diets. The nitric oxide (NO) production is significantly elevated in both uninfected and infected groups fed with 5 mg kg^{-1} diet. On the other hand, the lymphocyte proliferation and myeloperoxidase (MPO) activity were significantly increased the infected and uninfected groups fed with 5 and 10 mg kg^{-1} diets. The cumulative mortality was found 5% with 1 and 5 mg kg^{-1} diet groups while it was observed 10% mortality with 10 mg kg^{-1} diet group. Based on the results, it is observed that feeding the uninfected and infected groups with 5 mg kg^{-1} anthracenedione diet resulted in better improvement of growth, hematological, biochemical, and innate as well as adaptive immune parameters in *M. vittatus* against *A. hydrophila*.

1. Introduction

Asian striped dwarf catfish, *Mystus vittatus* (Bloch, 1794) is a freshwater species belonging to family Bagridae and order Siluriformes; it is a common inhabitant of estuarine and coastal waters, lakes and swamps in muddy substrates with marginal vegetation; it enjoys a wide distribution throughout the Indian subcontinent including Bangladesh, Bhutan, Cambodia, India, Laos, Nepal, Malaysia, Myanmar, Pakistan, Sri Lanka, Thailand, and Vietnam. *M. vittatus* is an omnivorous bottom dweller feeding mainly on cladocerans, rotifers, ostracods, insects, oligochaetes, *chlorophyceae*, *bascillariophyceae*, molluscs, crustaceans, diatoms, green algae, blue green algae, worms, insects, and plant materials. In fish markets, *M. vittatus* is available throughout the year; it is relished for its delicious taste and high nutrient content like protein, micronutrients, vitamins and many minor and trace elements, such as sodium, potassium, calcium, iron, iodine, zinc, magnesium, and phosphorus [1,2]. However, *M. vittatus* is infected by many bacterial, fungal, and parasitic pathogens including *Aeromonas hydrophila* leading to huge

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economic loss [3–10]. Some traditional antibiotics and chemotherapeutics agents are generally used to control of the infectious diseases in fish but its overuse resultant in development of drug-resistant bacteria, toxic residues, causing public health problems. On the other hand, fish vaccines though are efficient, a fish vaccine are not only pathogen specific but also are labor-intensive and expensive.

A number of herbals reported that act as immunostimulant in fish [11–15]; however, the biological and immunological activities of herbal bioactive compounds in fish limited [16–19]. Anthracenedione are a class of aromatic secondary metabolites compounds with a 9,10-dioxoanthracene core derived from Anthraquinones that possess a broad spectrum of bioactivities, including anti-cancer, anti-inflammatory, anti-microbial, anti-fungal, anti-oxidant, anti-tumor, anti-malarial, anti-viral, cathartic, diuretic, phytoestrogen, and vasorelaxing activities suggesting possible in clinical application of many diseases [20–27]. There was no any study on the effect of anthracenedione supplementation diet on growth performance or immunity in aquatic organisms. Therefore, this experiment was carry out to investigate for the first time the effect of diet enriched with anthracenedione on growth performance, hematology, physiology, immunity, and disease resistance in *M. vittatus* against *A. hydrophila*.

2. Material and methods

2.1. Diet

The formulated diet was incorporated with silkworm pupae and fish meal as protein sources; refined wheat flour (maida) as carbohydrate sources; oil cake, cod liver oil, and corn oil as lipid sources (Table 1). The ingredients were passed through an electric blender and hot water was added for paste condition, after cooling vitamins and minerals premix were added. The prepared of the feed ingredients were equally divided into four experimental diets and thoroughly mixing with or without anthracenedione obtained from sigma namely: (1) diet containing with anthracenedione and diet containing (2) 1 mg kg^{-1}, (3) 5 mg kg^{-1}, and (4) 10 mg kg^{-1} of anthracenedione and extruder mesh sieve (1 mm diameter). The anthracenedione purity and validation tested according to Mehta [28]. The diets were air dried and stored at −20 °C until used. The proximate composition of these experimental diets including crude protein, crude lipid, crude carbohydrate, crude ash, and crude fiber were analysis by the following method AOAC [29].

Table 1
Ingredients and the proximate composition (mg kg^{-1} dry matter) of experimental diets.

Ingredient	0 mg kg^{-1}	1 mg kg^{-1}	5 mg kg^{-1}	10 mg kg^{-1}
Silkworm pupae	500	500	500	500
Fish meal	150	150	150	150
Maida floor	150	150	150	150
Oil cake	150	150	150	150
Corn oil	20	19.01	19.05	19.1
Cod liver oil	20	20	20	20
Vitamin and mineral premix*	10	10	10	10
Anthracenedione (mg)	0	1	5	10
Total	1000	1000	1000	1000
Proximate composition (%)				
Moisture	8.2 ± 0.20	8.0 ± 0.24	8.1 ± 0.22	8.5 ± 0.26
Crude protein (%)	45.5 ± 1.32	45.2 ± 1.38	45.0 ± 1.82	45.3 ± 1.64
Crude lipid (%)	23.5 ± 1.22	23.1 ± 1.14	23.3 ± 1.18	23.6 ± 1.34
Crude carbohydrate (%)	16.2 ± 0.80	16.6 ± 0.74	16.4 ± 0.94	16.0 ± 0.75
Crude ash (%)	21.4 ± 0.76	21.0 ± 0.72	21.5 ± 0.64	21.2 ± 0.88
Crude fiber (%)	2.2 ± 0.26	2.0 ± 0.28	2.1 ± 0.22	2.5 ± 0.32

*Vitamin premix (g kg^{-1}): 19.2 α-cellulose, 5 choline chloride, 2 inositol, 1 ascorbic acid, 0.75 niacin, 0.5 calcium pantothenate, 0.2 riboflavin, 0.04 menadione, 0.05 pyridoxine hydrochloride, 0.05 thiamin hydrochloride, 0.015 folic acid, 0.005 biotin, 0.0001 vitamin B12, vitamin E (DL- α-tocopheryl acetate). Mineral mixture (g kg^{-1}): 135.7 calcium biphosphate, 326.9 calcium lactate, 29.7 ferric citrate, 132.0 magnesium sulphate, 239.8 potassium phosphate (dibasic), 87.2 sodium biphosphate, 43.5 sodium chloride, 0.154 aluminium chloride · $6H_2O$, 0.15 potassium iodide, 0.10 cuprous chloride, 0.80 magnus sulphate · H_2O, 1.0 cobalt chloride · $6H_2O$, 4.0 zinc sulphate · $7H_2O$; Loba Chemie India.

2.2. Pathogen

The pathogenic bacterium, *Aeromonas hydrophila* isolated from the kidney of infected fish showing common clinical signs like fin rot, gill necrosis, body lesion, protrusion of scale, and severe ulcerative lesions together with swollen in kidney and liver were brought from a local fish farm. The isolated kidney samples were grown on tryptic soy agar (TSA) plates for 24 h at 30 °C. The cultured bacterial cells were subcultured, purified, and resuspensions by using with 0.85% PBS (phosphate buffered saline). The concentration of the resultant bacterial cell suspension was 4.1×10^7 cfu ml^{-1} as calculated by a Neubauer haemocytometer [30]. The biochemical profiles of the bacterial cells were analysed by using microbe biochemical identification tube method (Hangzhou Microbial Reagent Co., Ltd) following the manufacturer's instruction. The genomic DNA was isolated from the bacterial cells that performed by polymerase chain reaction (PCR) using universal primers (ABI 3730): forward primer 27 F: 5′-agagtttgatcctggctcag-3′ and reverse primer 1492 R: 5′-tacggctaccttgttacgactt-3’ (Sangon Biotech, Shanghai Co., Ltd.) further confirmation according to manufacture's instructions [31,32]. The amplified 16 S genomic DNA gene sequences analysis based on BLAST search in GeneBank database [33].

2.3. Fish condition and experimental setup

Healthy *Mystus vittatus* (31.4 ± 1.4 g) were collected from a local fish market. The health status of the fish was examined immediately upon arrival. The fish were immersed with 1% $KMnO_4$ solution and acclimatized in 100 L aerated fiber tanks for 2 weeks prior to experiment. After fish were divided into eight groups of 25 fish each (8 × 25 = 200 fish) in triplicate groups (3 × 200 = 600 fish) as: i) uninfected control fish fed with control diet without incorporation of anthracenedione [C]; un-infected (UI) fish fed with (ii) 1 mg kg^{-1} [UI-1 mg] (iii) 5 mg kg^{-1} [UI-5 mg], and (iv) 10 mg kg^{-1} [UI-10 mg] of anthracenedione; (v) the *A. hydrophila* challenged/infected fish fed with control diet (I–C); infected (I) fish fed with (vi) 1 mg kg^{-1} [I-1 mg], (vii) 5 mg kg^{-1} [I-5 mg], and (viii) 10 mg kg^{-1} [I-10 mg] of anthracenedione. The respective diet was provided each group throughout the experimental period at the rate of 5%/day of their body weight two times in a day at 10.00 a.m. and 01:00 p.m. The water was renewed daily about 50%. After 30 days of respective feeding, groups v to viii were challenged intraperitoneally (i.p.) with 50 μl PBS suspension containing *A. hydrophila* at 4.1×10^7 cfu ml^{-1} while the other groups (i to iv) injected only with PBS suspension. There are six fish randomly chosen in each experimental group on 60th day for blood sampling. The blood samples done cardiac vein puncture in a sterile 20 gauge needle fitted 1 ml syringe for hematological and immunological study 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. Preparation of whole blood and their serum

The blood samples were mixed from a random sample of six fish in each experimental tank separately. Each sample were equally divided into nonheparinized and heparinized (K3EDTA vacuum) tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) for separation of serum and whole blood sample.

2.5. Growth, nutritional status, and survival rate

At the end of 60th day post-challenge, six fish randomly were collected in each experimental tank after fasted of 24 h and moderately anaesthetized with MS 222 and weighed. The mean weight gain (MWG) = [initial body weight] – [final body weight]; specific growth rate (SGR) = [(in final body weight - in initial body weight)/number of days x 100]; protein efficiency ratio (PER) = [(% protein in diet x weight of diet consumed)/100]; feed conversion ratio (FCR) = [(feed consumed)/(weight gain); protein intake (PI) = [(fish wet weight gain)/(fish consumed protein) x 100]; survival (%) = [(final number of fish)/(initial number of fish) x 100] was calculated according to Jawahar et al. [34].

2.6. Hematology

The red blood corpuscle (RBC) and white blood corpuscle (WBC) were enumerated by Neubaur's improved haematocytometer (Superior, Marienfeld, Germany) using Hyem's and Turk's diluting fluids. The percentages differential leucocytes such as lymphocytes, monocytes, neutrophils, and eosinophils were calculated by counting 100 cells from each smear under oil immersion microscope [35,36]. The blood biochemical profiles of serum total protein concentration was determined by Biuret colourimetric reactions [37] and the serum albumin and globulin concentrations were measured by bromocresol green colourimetric reaction [38,39].

2.7. Immunological assays

2.7.1. Isolation of head-kidney leucocytes

The nonheparinized whole blood samples allow clotting at room temperature (RT) for 4 h, than centrifuged at 3000 × *g* for 10 min at 4 °C. After centrifugation, the blood serums were separated and stored at −80 °C until used for immunological study. The head-kidney leucocytes (HKL) was separated and transferred into 8 ml of sRPMI [40]. The cell suspensions were acquired by forcing of the fragments through a nylon mesh (100 mm size), than the cell suspensions washed two times by centrifugation at 400*g* for 10 min. After, the leucocytes were count and adjusted to 10^7 cells ml^{-1} in sRPMI using trypan blue exclusion test.

2.7.2. Immunological assay

The complement activity was determined by using sheep red blood cells (SRBC, Biomedics) as targets [41]. The total serum immunoglobulin M (IgM) level was determined by enzyme-linked immunosorbent assay (ELISA) using mouse primary anti-fish IgM monoclonal antibody (Aquatic Diagnostics Ltd.) and secondary anti-mouse IgG-HRP antibody [42]. The respiratory burst activity of *M. vittatus* HKL was estimated by chemiluminescence method using phorbol myristate acetate (PMA, Sigma) and luminol (Sigma) [43]. The phagocytic activity of *M. vittatus* HKL was determined by flow cytometry at 488 nm [44]. The lysozyme activity of serum was determined by turbidimetric assay [45]. The superoxide dismutase (SOD) activity was estimated by its ability to inhibit superoxide anion generated through xanthine and xanthine oxidase reaction using an SOD detection kit (Nanjing Jiancheng Bioengineering Institute, China) [46]. The nitric oxide (NO) production was investigated by measuring absorbance of nitrite from the supernatant using the Griess reaction [47] and the leukocyte proliferate response of *M. vittatus* HKL was determined by the MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] colorimetric assay [48]. Total myeloperoxidase (MPO) content of serum was measured by Quade and Roth [49] and alpha-2 macroglobulin (α-2 M) was determined by Zuo and Woo [50].

2.8. Mortality, reisolation, and identification of the bacteria

A total of 20 fish in each group (20 × 8 = 160 fish) were used separately in triplicate (160 × 3 = 480 fish) for cumulative mortality. The challenge study, bacterial preparation, concentration, and the experimental conditions were same as mentioned previous section. The mortality was recorded for 30 days and the cause of mortality was determined and confirmed by reisolation of bacteria from infected fish kidney tissues [51,52] by streaked onto TSA agar plate. The characteristics of *A. hydrophila* was confirmed by morphological, pictorial, biochemical characterization, and PCR method [32,33]. The cumulative mortality (CM) and relative percent survival (RPS) values in each group were calculated over 30 days as follows: CM (%) = 100 - [(treatment mortality/control mortality)] x 100; RPS (%) = 1 – [(% mortality of challenged group)]/[(% mortality of unchallenged group)] x 100.

2.9. Statistical analysis

The obtained results are revealed as standard error of mean (mean ± SE). The variances between the means were analysed with Tukey's multiple range tests (SPSS 11.0 statistical software). The *P* value < 0.05 is considered for significant.

3. Results

3.1. Growth

Uninfected fish fed with anthracenedione enriched diets at 1, 5, and 10 mg kg^{-1} had better growth performance and feed utilization including SGR, PER, and FCR when compared to the infected group fed with the same diets. The infected fish fed with control diet, the values significantly low when compared with the uninfected group fed with control diet. The weight gain, feed intake, SGR, and FCR were significantly high in uninfected groups fed with 5 mg kg^{-1} diet than with other diets (1 mg kg^{-1} and 10 mg kg^{-1} diets) (Table 2).

3.2. Hematology

The RBC and WBC counts and the percentage of lymphocytes were significantly increased in the infected and uninfected groups fed with anthracenedione enriched diets at 1, 5, and 10 mg kg^{-1} diets. The

Table 2
Growth and feed utilization study of anthracenedione incorporation diets in *M. vittatus* against *A. hydrophila*.

Indices	C	UI-1 mg kg^{-1}	UI-5 mg kg^{-1}	UI-10 mg kg^{-1}	I–C	I-1 mg kg^{-1}	I-5 mg kg^{-1}	I-10 mg kg^{-1}
Initial weight (g)	31.3 ± 1.12^a	31.3 ± 1.12^a	31.7 ± 1.12^a	31.4 ± 1.14^a	31.5 ± 1.16^a	31.5 ± 1.12^a	31.5 ± 1.16^a	31.6 ± 1.22^a
Weight gain (g)	35.5 ± 1.14^a	35.3 ± 2.20^a	39.7 ± 2.12^b	38.4 ± 1.42^b	12.8 ± 1.74^c	33.3 ± 1.92^a	38.4 ± 2.14^a	37.4 ± 1.50^a
Feed intake (g)	41.4 ± 2.2^a	41.4 ± 1.6^a	43.5 ± 2.4^b	41.4 ± 2.4^a	36.5 ± 1.8^c	41.4 ± 2.2^a	42.4 ± 1.6^a	40.1 ± 2.2^a
SGR (%/day)	0.73 ± 0.04^a	0.72 ± 0.03^a	0.84 ± 0.03^b	0.75 ± 0.02^a	0.58 ± 0.03^a	0.72 ± 0.04^a	0.80 ± 0.04^a	0.75 ± 0.03^a
PER	0.83 ± 0.12^a	0.84 ± 0.10^a	0.94 ± 0.12^a	0.90 ± 0.15^a	0.72 ± 0.18^a	0.84 ± 0.14^a	0.85 ± 0.16^a	0.81 ± 0.12^a
FCR	1.34 ± 0.02^a	1.34 ± 0.03^a	1.54 ± 0.03^b	1.45 ± 0.03^a	1.25 ± 0.04^c	1.35 ± 0.04^a	1.56 ± 0.03^b	1.44 ± 0.03^a
SR (%)	96.0	95.5	98.0	96.2	91.8	93.5	95.5	93.0

SGR: specific growth rate, PER: protein efficiency ratio, FCR: feed conversion ratio, SR: survival rate, C: control, I–C: infected control, UI: uninfected, I: infected.

Table 3
Hematological changes of anthracenedione incorporation diets in *M. vittatus* against *A. hydrophila*.

Parameter	C	UI-1 mg kg^{-1}	UI-5 mg kg^{-1}	UI-10 mg kg^{-1}	I–C	I-1 mg kg^{-1}	I-5 mg kg^{-1}	I-10 mg kg^{-1}
RBC (million/m^3)	2.06^a	3.03^c	3.20^c	3.14^c	1.84^a	2.71^b	2.85^b	2.80^b
WBC (Per μl)	4124.2^a	4235.2^b	4381.0^c	4255.2^c	4134.4^a	4204.1^b	4367.0^b	4238.2^b
Lymphcytes (%)	28.6^a	32.4^c	33.7^c	33.5^c	29.1^a	30.2^b	32.0^c	30.2^b
Monocytes (%)	2.1^a	2.4^a	3.5^c	3.0^b	2.2^a	2.3^a	3.2^c	2.4^a
Eosinophils (%)	6.0^a	7.2^a	7.6^b	7.5^a	6.2^a	6.5^a	7.3^b	7.3^a
Neutrophils (%)	24.5^a	25.2^a	26.2^b	26.0^b	24.1^a	25.4^a	26.1^b	25.5^a
Total protein (mg/dl)	2.41^a	2.74^a	3.56^b	3.32^b	2.32^a	2.88^a	3.40^b	2.82^a
Albumin (mg/dl)	1.26^a	1.72^a	2.88^b	1.80^a	1.30^a	1.45^a	2.46^b	1.65^a
Globulin (mg/dl)	1.26^a	1.88^a	2.88^b	1.94^a	1.25^a	1.45^a	2.58^b	1.65^a

RBC: red blood cell, WBC: white blood cell, C: control, I–C: infected control, UI: uninfected, I: infected.

monocytes, eosinophils, and neutrophils percentage and the total protein, albumin, and globulin were significantly high in both groups fed with diet containing at 5 mg kg^{-1} of anthracenedione. However, though the percentage of leucocytes and blood biochemical parameters (total protein, albumin, and globulin) were not significantly increased in both groups fed with 1 mg kg^{-1} and 10 mg kg^{-1} of anthracenedione enriched diets. On the other hand, all the hematological and biochemical parameters decreased or did not show any significant difference in the infected group fed with control diet (Table 3).

3.3. Immune response

3.3.1. Phagocytic activity

The phagocytic activity was significantly high in the uninfected and infected groups fed with 5 mg kg^{-1} diet. However, the increase was not statistically significant in the uninfected and infected groups fed with others diet diets (1 mg kg^{-1} and 10 mg kg^{-1}). Similarly, the infected fish fed with control diet also was not significant as compared with control diet (Fig. 1).

3.3.2. Serum Ig level

The serum IgM level increased significantly in the infected and uninfected groups fed with 5 mg kg^{-1} and 10 mg kg^{-1} diets; but the increase was not statistically significant with 1 mg kg^{-1} diet as compared with control diet group. A similar result ($P > 0.05$) was observed in infected fish after fed with control diet group (Fig. 2).

3.3.3. Respiratory burst activity

The respiratory burst activity was significantly enhanced in the infected and uninfected groups fed with 5 mg kg^{-1} and 10 mg kg^{-1} diets. However, it was not significantly enhanced in the infected and uninfected groups fed with 1 mg kg^{-1} diet as compared with control diet group (Fig. 3).

3.3.4. Serum superoxide dismutase (SOD) activity

The SOD activity did not significantly increased the infected and uninfected groups fed with 1 mg kg^{-1} or 10 mg kg^{-1} diets, while it was observed significantly in both groups fed with 5 mg kg^{-1} diet as compared with control diet group (Fig. 4).

3.3.5. Nitric oxide (NO) production

The nitric oxide (NO) production did not significantly increase the infected and uninfected groups fed with 1 and 10 mg kg^{-1} diets. However, it was significantly increased in both groups fed with 5 mg kg^{-1} diet when compared to control group (Fig. 5).

3.3.6. Lymphocyte proliferation

Lymphocyte proliferation was significantly increase the infected and uninfected groups fed with 1 mg kg^{-1} diet; while it was not significantly high in both groups fed with 5 and 10 mg kg^{-1} diets when compared to control group (Fig. 6).

3.3.7. Myeloperoxidase (MPO) activity

The MPO activity did not significantly high in the infected and uninfected groups fed with 1 mg kg^{-1} diets. On the other hand, the MPO was significantly increased in both groups when fed with 5 mg kg^{-1} and 10 mg kg^{-1} diets as compared with control group (Fig. 7).

3.3.8. Alpha-2 macroglobulin (α-2 M) production

The α-2 M production did not affect the infected and uninfected groups fed with 5 mg kg^{-1} and 10 mg kg^{-1} diets. However, it was significantly low in both groups fed with 1 mg kg^{-1} diet group (Fig. 8).

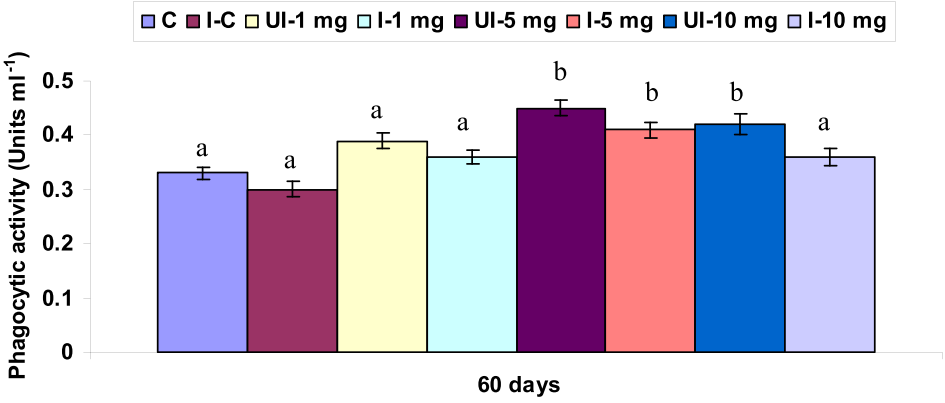

Fig. 1. Phagocytic activity of anthracenedione incorporation diets in *M. vittatus* against *A. hydrophila*. C: control, I–C: infected control, UI: uninfected, I: infected. Data expressed as standard error of mean (SEM) in same superscript letter did not statistically significant difference as determined by Tukey's test ($P > 0.05$).

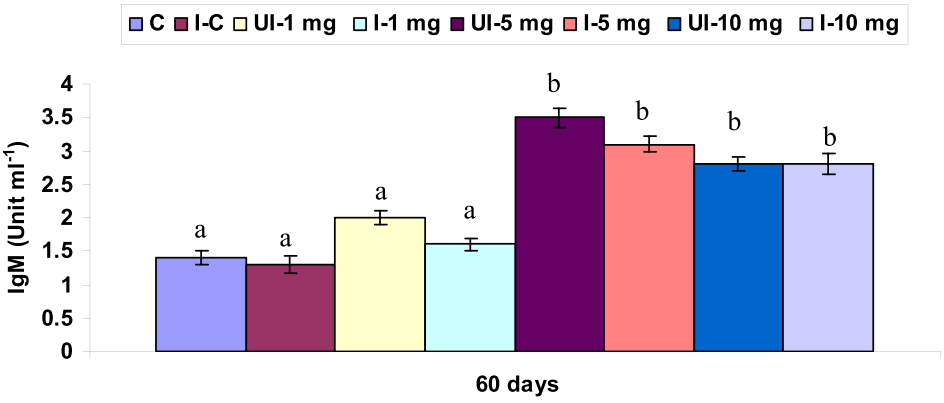

Fig. 2. Immunoglobulin M (IgM) of anthracenedione incorporation diets in *M. vittatus* against *A. hydrophila*. C: control, I–C: infected control, UI: uninfected, I: infected. The statistical information is shown in Fig. 1.

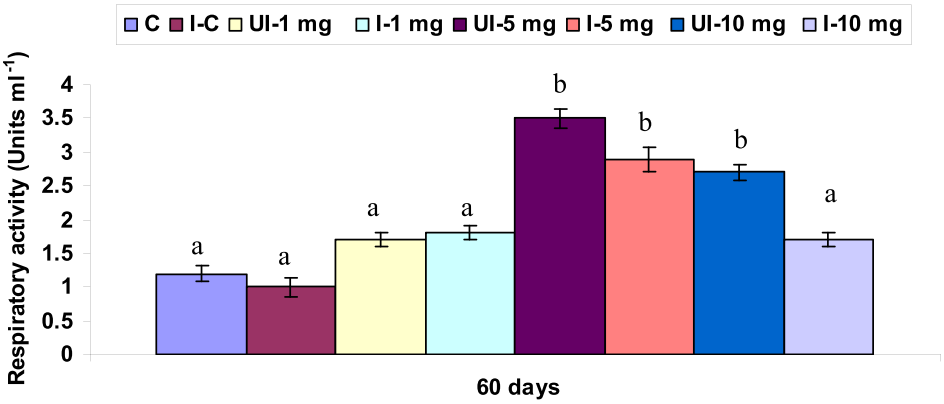

Fig. 3. Respiratory burst (RB) activity of anthracenedione incorporation diets in *M. vittatus* against *A. hydrophila*. C: control, I–C: infected control, UI: uninfected, I: infected. The statistical information is shown in Fig. 1.

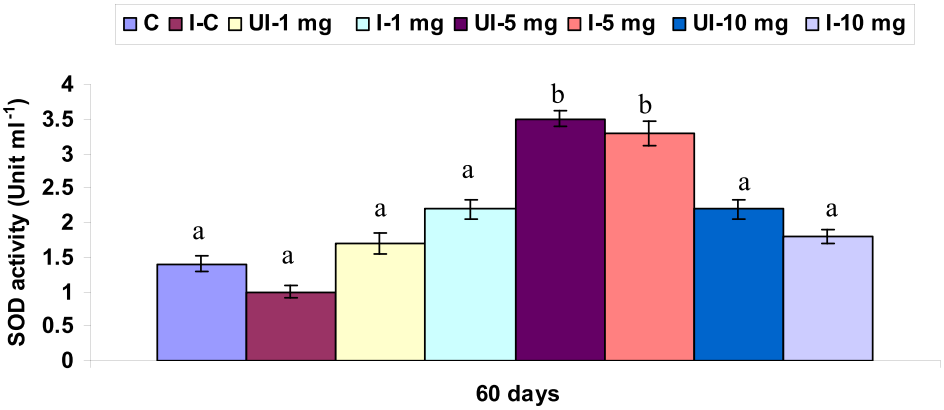

Fig. 4. Serum superoxide dismutase (SOD) activity of anthracenedione incorporation diets in *M. vittatus* against *A. hydrophila*. C: control, I–C: infected control, UI: uninfected, I: infected. The statistical information is shown in Fig. 1.

3.3.9. Complement activity

The complement activity was significantly enhanced in both groups fed with 5 mg kg^{-1} diet while the other diets (1 mg kg^{-1} diet and 10 mg kg^{-1} diet) did not significantly increase as compared with control group (Fig. 9).

3.3.10. Lysozyme activity

The lysozyme activity was found significantly increased in both groups fed with 5 mg kg^{-1} diet. However, this activity did not significantly increase in both groups with other diets (1 mg kg^{-1} diet and 10 mg kg^{-1} diet) as compared with control group (Fig. 10).

3.3.11. Disease resistance

The infected fish feeding the control diet was found 90% mortality. There was no mortality was found in uninfected groups feeding at 1, 5, and 10 mg kg^{-1} diets. However, the infected groups fed at 1 and

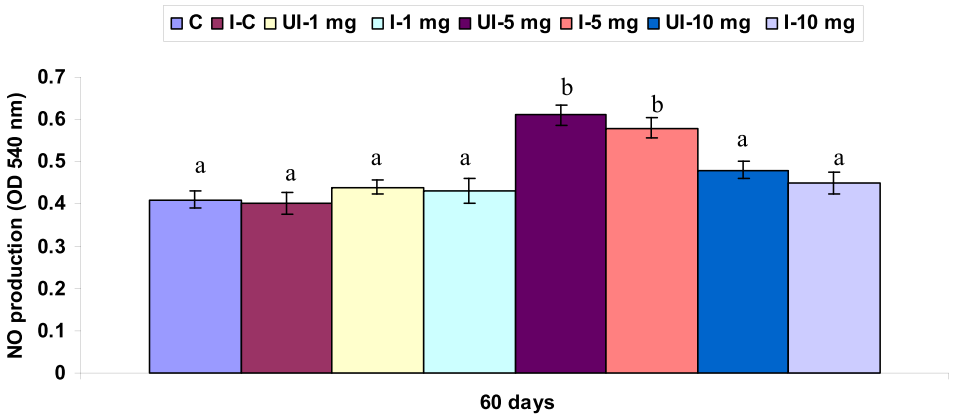

Fig. 5. Nitric oxide (NO) production of anthracenedione incorporation diets in *M. vittatus* against *A. hydrophila*. C: control, I–C: infected control, UI: uninfected, I: infected. The statistical information is shown in Fig. 1.

5 mg kg^{-1} diets were observed 5% mortality whereas with 10 mg kg^{-1} diet was observed 10% mortality (Fig. 11).

4. Discussion

M. vittatus is a delicious taste food fish that contain high protein, micronutrients, vitamins and many minor and trace elements as importance for human growth. *A. hydrophila* is a Gram-negative opportunistic bacterial pathogen that produces aerolysin cytotoxic enterotoxin in many fishes including *M. vittatus* as a results in tissue damage and shows a variety of clinical signs including ulcers, abdominal swelling, exophthalmia, fin rot, fin rot, hemorrhages in the gill and anal area. In aquaculture especially intensive farming rampant use of many antibiotics and chemotherapeutant that produce sedimentation, affect the human health, non target organisms, environmental issues, and leads to the emergence of drug resistant bacterial strains. Several herbal immunostimulants have been reported to improve growth, hematology, innate and adaptive immune response and protect from mortality due to infectious diseases [11,16–19,53–59].

Very few reports have been suggested that anthracenedione act as biological activities. But there was no any report of the anthracenedione enriched diet in fish and shellfish growth performance, physiology, and immunity against pathogens. In the present study for the first time reports on the effect of feeding with anthracenedione in *M. vittatus* have observed to improving growth, immunity, and disease protection against *A. hydrophila*. A better weight gain, feed intake, SGR, and FCR were observed in uninfected *M. vittatus* fed with anthracenedione at 5 mg kg^{-1} diet than with other diets (1 mg kg^{-1} and 10 mg kg^{-1}). This was supported in a previous study in *Clarias batrachus* when fed with 2 mg kg^{-1} and 4 mg kg^{-1} emodin enriched diets observed better growth performance against *A. hydrophila* [55]. It may suggest that anthracenedione influence for digestion process for growth. Total protein, albumin, and globulin levels were high in both groups fed with any enriched diet as compared to control. However, the blood biochemical parameters (total protein, albumin and globulin) were significantly high in both groups fed with 5 mg kg^{-1} diet than that of other diets (1 mg kg^{-1} and 10 mg kg^{-1} diets) as suggested that 5 mg kg^{-1} diet containing anthracenedione may be influence to production of total protein, albumin and globulin as well the percentage of leucocytes against *A. hydrophila*. However, the exact mechanisms of action of anthracenedione on the physiology and leucocytes production are unknown. The total protein, albumin, and globulin did influence significantly in both groups fed with 5 mg kg^{-1} diet but did not with 5 mg kg^{-1} and 5 mg kg^{-1} diets. The decreasing level of serum albumin indicates that the fluid may escape into tissues causing localized oedema reducing the delivery of nutrients to tissues [60]. On the other hand, a decrease in the level of serum albumin might be a reliable disease prognostic indicator increasing the risk of morbidity and mortality [61,62]. Similarly, the increase in the level of serum globulins values indicate that activation of B-lymphocytes differentiation and proliferation by IL-6 and TNF-α against parasites, viral, and bacterial infection [63,64].

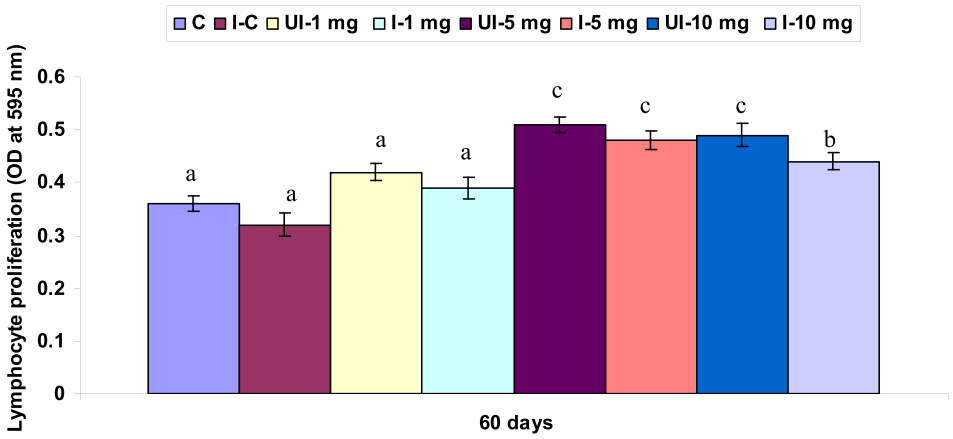

Fig. 6. Lymphocyte proliferation of anthracenedione incorporation diets in *M. vittatus* against *A. hydrophila*. C: control, I–C: infected control, UI: uninfected, I: infected. The statistical information is shown in Fig. 1.

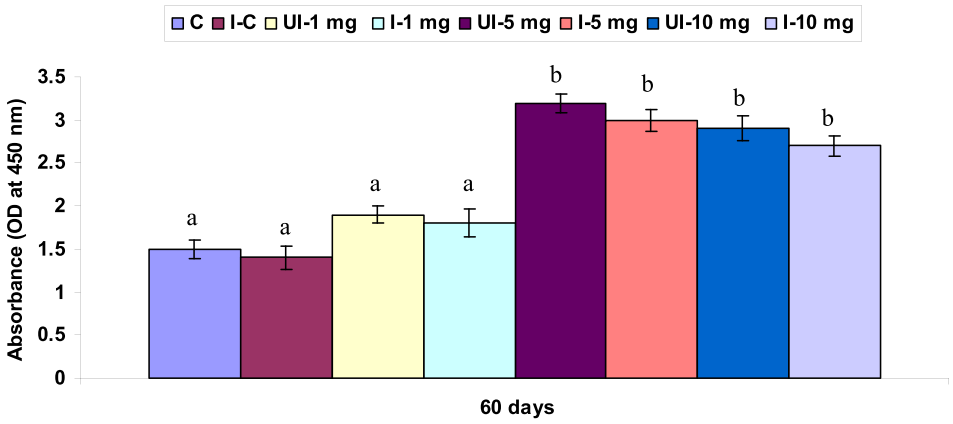

Fig. 7. Myeloperoxidase (MPO) activity of anthracenedione incorporation diets in *M. vittatus* against *A. hydrophila*. C: control, I–C: infected control, UI: uninfected, I: infected. The statistical information is shown in Fig. 1.

The RBC and WBC counts and the percentage of lymphocytes were significantly increased in both groups fed with anthracenedione incorporated diets at 1, 5, and 10 mg kg^{-1}. However, the percentage of monocytes, eosinophils, and neutrophils significantly increased only with 5 mg kg^{-1} diet but not with 1 mg kg^{-1} or 10 mg kg^{-1} diets. Hematology parameters provide valuable information for fishery biologists in the assessment of health status and monitoring the stress response. In fish an increase in total WBC counts is considered as the first line of defense in innate and adaptive immunity [56]. The present results are in agreement with other fishes (*Carassius auratus* and *Cirrhina mrigala*) against *A. hydrophila* and *Aphanomyces invadans* [57,58]. Differential leukocyte counts (lymphocytes, monocytes, eosinophils, and neutrophils) indicate significant uniqueness of the health status that helps in evaluating the immune status in fish. In line with the present findings an augmentation in monocytes, granulocytes, neutrophils, and macrophages has been reported in *C. mrigala* when fed with azadirachtin enriched diet against *A. invadans* [58].

Phagocytosis as innate defence function is mediated by phagocytic cells including monocytes, neutrophils, and macrophages which constitute the fundamental mechanism in fish defence against pathogens; this is widely used to assess the antimicrobial activity [65]. These constitute the majority of important cellular components that contribute to the innate immunity in fish against pathogens [66,67]. The increase of lymphocytes is may under the control of cytokine gene [68] or activated by macrophages [69]. The phagocytic activity significantly is enhanced in the present study of both groups when fed with 5 mg kg^{-1} diet. It was suggested that the phagocytic activity increased significantly in *Clarias batrachus* on being fed with 4 mg kg^{-1} diet on week 2 whereas with 2 mg kg^{-1} and 4 mg kg^{-1} of emodin enriched diets on week 4 against *A. hydrophila* [55]. However, the increase was not statistically significant in both groups fed with 1 and 10 mg kg^{-1} enriched diets in the present study.

The serum IgM values extensively modulate in the present study in both groups fed with 5 mg kg^{-1} and 10 mg kg^{-1} diets, but did not with 1 mg kg^{-1} diet. The IgM sometimes has been called a “natural antibody” found in normal serum; it binds to specific antigens even in the absence of prior immunization [70]. The decreasing levels of IgM describe some humoral immune-deficiencies whereas increasing IgM is related with diseases and haematological disorders [71]. This results agreement in *C. batrachus*, the serum IgM level significantly increased when fed with 4 mg kg^{-1} of emodin diet on week 4 [55]. The mechanism of action of anthracenedione on increasing level IgM in serum in *M. vittatus* against *A. hydrophila* is yet to be elucidated.

During phagocytosis, the phagocytic cells cause a spurt in the production of large quantities of reactive oxygen species (ROS), reactive nitrogen species (RNS), and antimicrobial enzymes (cathepsin G and elastase) [72–74] which enhancing the use of molecular oxygen for RB activity. Cytoplasmic membrane-associated NADPH oxidase enhance the oxidant production where molecular oxygen undergoes a one-electron reduction to form superoxide anions (O_2^-) and then forming hydrogen peroxide (H_2O_2) and several other ROS that provide a substrate for the myeloperoxidase (MPO) reaction which in turn generate a variety of highly toxic metabolites, including hypochlorous acid. This plays a major role in the immune system triggering a crucial reaction

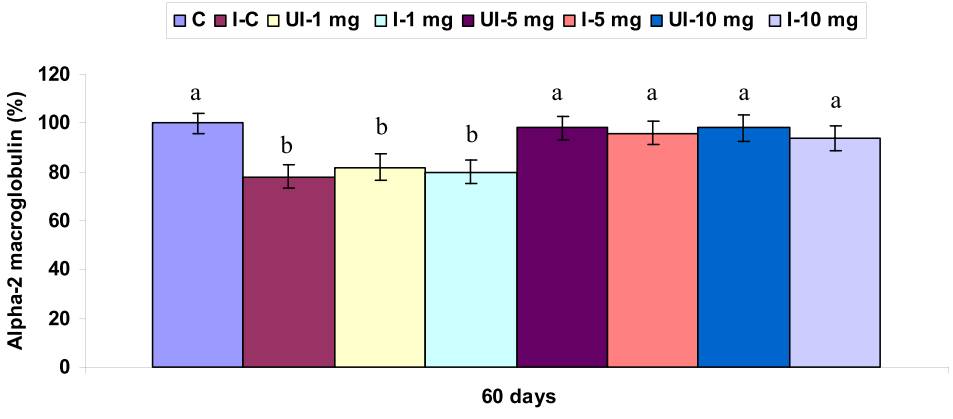

Fig. 8. Alpha-2 macroglobulin (α-2 M) of anthracenedione incorporation diets in *M. vittatus* against *A. hydrophila*. C: control, I–C: infected control, UI: uninfected, I: infected. The statistical information is shown in Fig. 1.

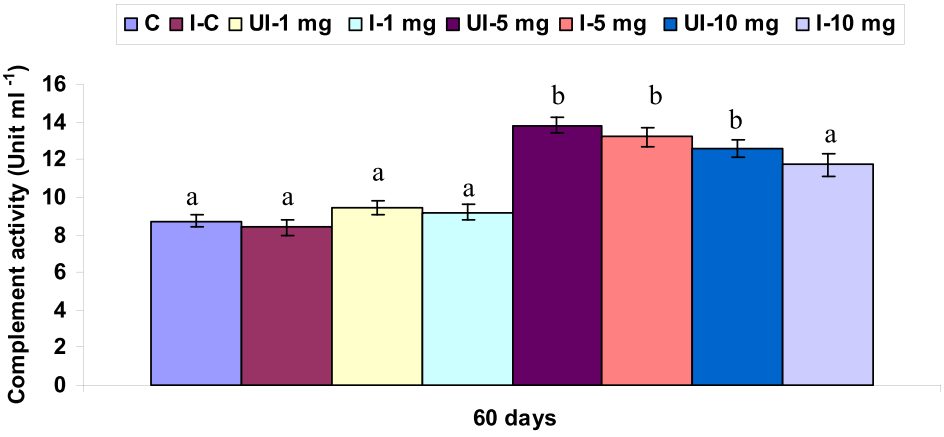

Fig. 9. Complement activity of anthracenedione incorporation diets in *M. vittatus* against *A. hydrophila*. C: control, I–C: infected control, UI: uninfected, I: infected. The statistical information is shown in Fig. 1.

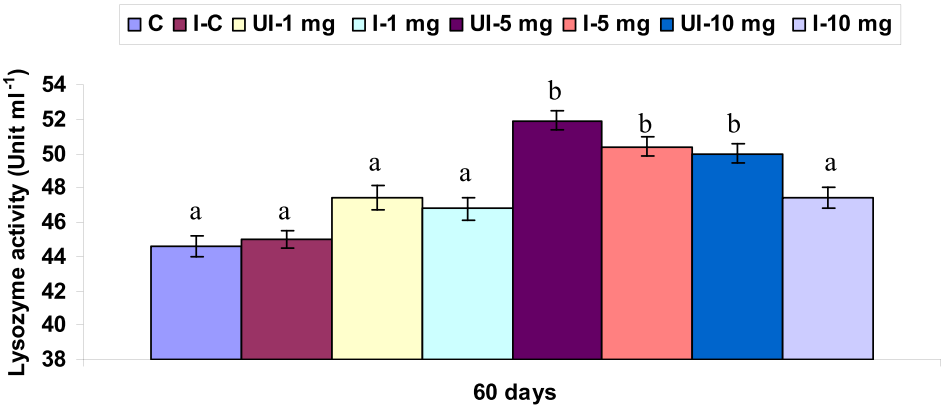

Fig. 10. Lysozyme activity of anthracenedione incorporation diets in *M. vittatus* against *A. hydrophila*. C: control, I–C: infected control, UI: uninfected, I: infected. The statistical information is shown in Fig. 1.

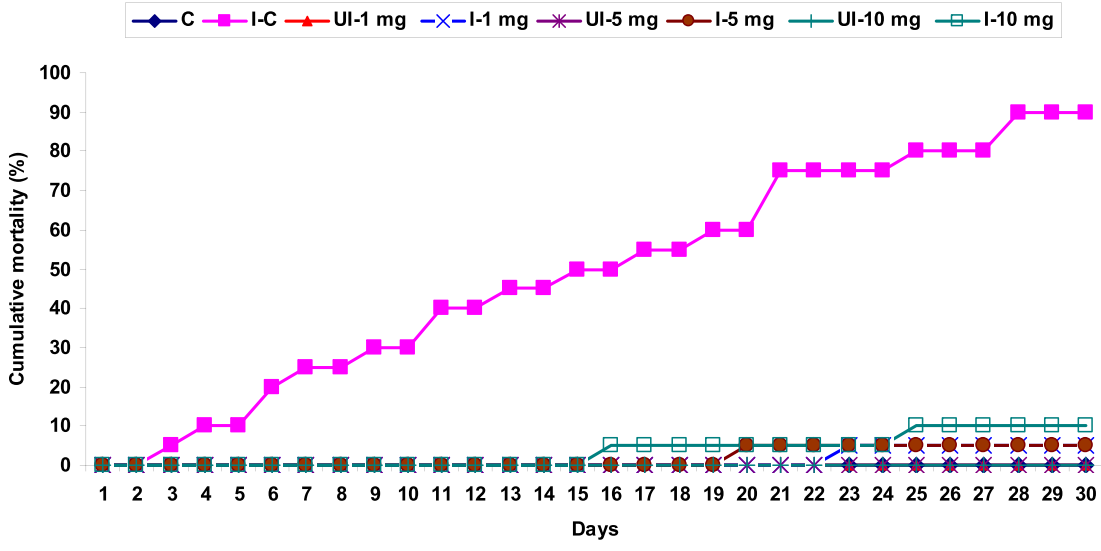

Fig. 11. Percentage of cumulative mortality of anthracenedione incorporation diets in *M. vittatus* (n = 20) against *A. hydrophila* for 30 days. C: control, I–C: infected control, UI: uninfected, I: infected.

that occurs in phagocytes to degrade internalized particles and bacteria. The RB activity is significantly enhanced in this study in both groups fed with 5 mg kg^{-1} diet. This result is in line with a recent study in *C. mrigala* administration with active compounds against *A. invadans* [75]. Harikrishnan et al. [55] also reported that in *C. batrachus* fed with diet containing 4 mg kg^{-1} emodin diet had increased RB activity significantly against *A. hydrophila* on week 4. The phagocytes produce RB required for the optimal killing of a wide variety of bacteria, virus, and fungi [76] and it can induce oxidative stress in host cells during infection [77,78]. However, it did not show significant phagocytic activity in the infected and uninfected groups fed with 1 mg kg^{-1} and 10 mg kg^{-1} diets.

Superoxide dismutase (SOD) catalyzes is conversion of superoxide radicals into hydrogen peroxide and molecular oxygen which play a significant role in the defense of cells against the toxic effects of oxygen radicals [79–81]. It competes with NO for superoxide anions, which react with NO to form peroxynitrite as measuring for antioxidant capacity [82]. The SOD activity was significantly increased in this study in both groups when fed with 5 mg kg^{-1} enriched diet. Similar result is reported in *C. batrachus* when fed with emodin diet with 4 mg kg^{-1} diet on week 1; with 2 g kg^{-1} or 4 mg kg^{-1} diets on weeks 2 and 4 against *A. hydrophila* [55]. However, in the infected and uninfected groups fed with 1 mg kg^{-1} or 5 mg kg^{-1} diets did not enhance the SOD activity in the present experiment.

The NO is a biologically active-free radical molecule that recognized for diversity of its physiological functions [82]. Lymphocytes are specifically recognized and respond to microbial agents form the core of the acquired immune system [83]. The MPO is an important enzyme involved in leukocyte mediated host defense function and it regulation of inflammation. This enzyme utilizes hydrogen peroxide and generated RB to produce hypochlorous acid with potent microbicidal action [84]. The α-2M is a largest repertoire of proteases in fish that believed to be potential virulent factors in attacking the host tissues [85]. The NO production significantly increased in both groups fed with 5 mg kg^{-1} diet. However, the lymphocyte proliferation and the MPO activity significantly increased in this study in both groups fed with 5 mg kg^{-1} and 10 mg kg^{-1} diets. On the other hand, α-2 M production did not significantly influence in both groups fed with 5 mg kg^{-1} and 10 mg kg^{-1} diets but the role anthracenedione on the production of NO, MPO, lymphocyte, and α-2 M is currently unknown.

Complement component of normal plasma augments the opsonization or destruction of bacteria during early stages of infection in the absence of antibodies; seems clearly that these components evolved first as a part of the innate immune system, where it still plays an important role. The complement system consists of a number of unique plasma proteins that react with one another to opsonize pathogens and induce a sequence of inflammatory response that help to fight infectious pathogen. The complement activity was significantly enhanced in both groups in the present study when fed with 5 mg kg^{-1} diet. In a recent study, *C. batrachus* is challenged with *A. hydrophila* reported that similar complement activity when fed with emodin enriched diet between weeks 2 and 4 [55].

Lysozymes active site can bind or attacks with peptidoglycan molecule present in Gram-positive bacterial cell walls as a prominent cleft between N-acetylmuramic acid (NAM) and the fourth carbon atom of N-acetylglucosamine (NAG). This antimicrobial enzyme produced by animals and major component of Gram-positive bacterial cell wall which is a part of the innate immune system that hydrolysis and breaking of 1,4-beta-linkages between NAM and NAG residues in peptidoglycan integrity of bacterial cell walls [72]. It was further confirmed an effective in attacking the cell wall polysaccharide of different bacterial species, leading to a break down and hydrolyzing a tetrasaccharide found most often in Gram-positive bacteria cell wall killing them. In the present study in both groups fed with 5 mg kg^{-1} diet significantly enhanced the lysozyme activity as compared with other diets (1 mg kg^{-1} diet and 10 mg kg^{-1}). *A. hydrophila* infected *C. batrachus* when fed with 2 and 4 mg kg^{-1} emodin has been reported similar results of lysozyme activity between weeks 2 and 4 [55]. On the other hand, dietary feeding of active compounds supplementation diet significantly increased the lysozyme activity [75].

The infected group when fed with 1 mg kg^{-1} and 5 mg kg^{-1} anthracenedione diets has observed only 5% mortality while infected group fed with 10 mg kg^{-1} diet the mortality was observed 10%. *C. mrigala* administration with active compounds resulted in very low mortality against *A. invadans* [74]. In *A. hydrophila* challenged *C. batrachus* the cumulative mortality was 10% with 1 mg kg^{-1} of emodin diet and 15% mortality when fed with 2 mg kg^{-1} and 4 mg kg^{-1} diets [55]. The infected fish fed without anthracenedione diet showed 90% mortality. The present study concludes that *M. vittatus* challenged with *A. hydrophila* could significantly modulate the innate and adaptive immune system resulting in decreased the mortality when fed with 5 mg kg^{-1} anthracenedione enriched diet. Further detailed immunological and molecular studies are needed the mechanisms and mode of action of anthracenedione before using it as feed additive in aqua feed for sustainable aquaculture production against pathogens.

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