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# Effect of cassic acid on immunity and immune-reproductive genes transcription in *Clarias gariepinus* against *Edwardsiella tarda*

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ABSTRACT

The present study was investigated the dietary administration of cassic acid (CA) on growth, innate immunity, transcription profiles of estrogen and follicle-stimulating hormones as well as lysozyme enzyme determined in *Clarias gariepinus* against *Edwardsiella tarda*. The weight gain (WG), protein efficacy ratio (PER), and feed conversion ratio (FCR) were significantly improved in infected fish fed dietary administration with CA at 5 and 10 mg $kg^{-1}$ diets. The survival is higher (96.7% and 98.3%) in the infected groups fed at 5 and 10 mg $kg^{-1}$ CA diets. The red (RBC) and white (WBC) blood cells, hemoglobin (Hb), and packed cell volume (PCV) was found significantly high in the infected fish feeding at 5 and 10 mg $kg^{-1}$ CA diets. Total protein and albumin were significantly increased with 5 and 10 mg $kg^{-1}$ CA diets among weeks 1–4 while the globulin and albumin: globulin ratio increased of these diet only after week 2. The phagocytic and respiratory burst activities were enhanced statistically the infected fish fed at 10 mg $kg^{-1}$ CA diet group whereas the production of superoxide anion (SOA) and nitric oxide (NO) were significantly increased at 5 and 10 mg $kg^{-1}$ CA diets. The lymphocyte proliferation and myeloperoxidase (MPO) activity were significantly high the infected fish when fed at 5 and 10 mg $kg^{-1}$ CA diets after 2nd week whereas the alternate complement activity (ACP), generation of reactive oxygen species (ROS), and lysozyme activity (Lyz) were observed at 5 and 10 mg $kg^{-1}$ CA diets among weeks 1–4. The accumulative mortality was 10% in infected fish fed at 1 and 5 mg $kg^{-1}$ CA diets whereas 15% mortality found with 10 mg $kg^{-1}$ CA diet. The highest levels of estrogen receptor alpha (ERα) mRNA expression found in gonad while the highest levels of follicle stimulating hormone-beta subunit (FSH-β) mRNA expression found in testis of the infected fish given at 5 and 10 mg $kg^{-1}$ CA diets. The up-regulation of chick-type lysozyme (c-Lyz) mRNA was observed at 5 and 10 mg $kg^{-1}$ CA diets after 2nd week while goose-type lysozyme (g-Lyz) mRNA was up-regulation amongst weeks 1–4 of these diets. The present study suggested that *E. tarda* challenged fish after feeding with 5 and 10 mg $kg^{-1}$ CA diets did not affect growth and hemato-biochemical parameter, but it enhanced nonspecific immune system and improving ERα, FSH-β, c-Lyz, and g-Lyz mRNA expression in *C. gariepinus* against *E. tarda*.

## 1. Introduction

Sharptooth African catfish, *Clarias gariepinus* (Burchell) is the most important tropical fish species cultured extensively in India. *C. gariepinus* is not a native species in Indian waters, but this catfish had buried first enter to West Bengal of India and later it spread other states of India. This introduction catfish has become culture many concerns, particularly this catfish negative effects on native fish fauna through predation and fast growth in village ponds, cement cisterns, tanks, and even run-down waters using chicken and slaughter house wastes in India [1]. The catfish has become an excellent aquaculture species in many countries because of its tolerance of extremes adaptability to tropical environmental conditions and high annual revenues, fast growth as well as feed conversion rate, suitability for monoculture and polyculture with other freshwater fish species, ability to withstand handling stress, disease resistance, high fecundity and weight gain, palatability and nutritional quality [2–4]. Catfish production in India has high economic returns; in India like West Bengal is top rank in

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catfish production range from 16 to 20% of total catfish production Indian since 2007 [5]. At the same time incidences of diseases in catfish culture are increasingly because the increase of culture under crowded condition result huge economic losses; among the bacterial diseases is major problem in tropical catfish culture systems that produce hemorrhagic septicemia by *Aeromonas* spp. [6], edwardsiellosis by *Edwardsiella tarda* [7], and columnaris by *Flavobacterium columnare* [8]. Among, the edwardsiellosis is known as septicemic disease showing characteristics extensive lesions in the muscle, skin, as well as internal organs of the economically important striped bass, channel catfish, eels, chinook salmon, flounder, mullet, tilapia, and carp species [9]. The edwardsiellosis is caused by *E. tarda* frequently under risky environmental circumstances, like highest water temperature, poor water conditions, and high organic load of *C. gariepinus* fingerlings culture leads to high economic loss [9].

Antibiotics and chemicals are traditionally used to control fish diseases, but it can develop negative impacts such as drug-resistant bacterial strains, toxicity, residues, public health and environment consequences. Recently an interesting alternative approach for immunoprophylactic control measures has been achieved remarkable success using of naturally available herbals, herbal active constituents, and probiotics immune-stimulants that enhance the immune system of fish and confer protection against different pathogens [10–12]. A number of natural plant active constituents including volatile oils, tannins, phenols, saponins, alkaloids polysaccharides, polypeptides, terpenoids, and steroids have been used because it stimulate various biological process including anti-stress, stimulation of appetite, boost of immune system, and anti-microbial activity in finfish and shellfish [13–16]. Therefore, the application of immune-stimulants looks to be an attractive alternate prevent diseases, influence of growth and immunity in fishes [11,17].

Cassic acid (CA) is known as rhein (1,8-dihydroxyanthraquinone-3-carboxylic acid) of anthraquinone group abundantly present in root and leaf of Rheum, Senna, and Cassia species; traditionally, the CA used as Chinese herbal medicine to cure or improve of parasite, anthelmintic, laxative, herpes, syphilis, scabies, ringworm problems, infant digestive problems, diarrhea, gastrointestinal hemorrhage, increase in the number of blood platelets, quick clotting for jaundice, prevent the progression of chronic renal failure [18,19]. Further, it has been reported that better anti-bacterial, anti-fungal, anti-viral, anti-tumor, anti-cancer, anti-oxidant, and anti-inflammatory properties [20–26]. Some recent study reported that herbal extracts and their active constituents improve or not affected growth performance, hematological parameters, and innate-adaptive immune response in fish against pathogens. There was no information of CA active constituents in fish on growth, reproductive performance and cytokine genes mRNA expression. For example, estrogens hormones are very important role for sexual and reproductive developmental process, especially female compared to male. It was enters passively into the cell where it binds and immediately mediated by a dimeric nuclear protein such as estrogen receptor (ER) that associated with DNA to controls gene expression, bone calcification, and memory. Follicle-stimulating hormone (FSH) is being involved in the initiation and early stages of spermatogenesis. Lysozyme (chicken- and goose-type) enzyme is one of the important fish bacterial defense components that hydrolysis of β-(1,4)-glycosidic bond in between sites of N-acetyl glucosamine and N-acetyl muramic acid of the peptidoglycan layer in both Gram-positive and Gram-negative bacterial bacteria cell walls. Therefore, the present research was conduct to explore as a preliminary study of CA supplementation diet on disease protection, growth response, immunity, mRNA expression of estrogens and FSH-β hormones as well as lysozyme (c- and g-type) enzymes in *C. gariepinus* against *E. tarda*.

**Table 1**
Supplementation feed ingredients and proximate composition (g $kg^{-1}$ dry matter).

| Ingredient | 0 | 100 mg $kg^{-1}$ | 250 mg $kg^{-1}$ | 500 mg $kg^{-1}$ |
|---|---|---|---|---|
| Fish meal | 40.00 | 40.00 | 40.00 | 40.00 |
| Maize gluten | 10.00 | 10.00 | 10.00 | 10.00 |
| Wheat meal | 30.00 | 30.00 | 30.00 | 30.00 |
| αCellulose | 6.00 | 5.009 | 5.005 | 5.010 |
| Corn oil | 4.00 | 4.00 | 4.00 | 4.00 |
| Cod liver oil | 4.00 | 4.00 | 4.00 | 4.00 |
| Binder | 3.00 | 3.00 | 3.00 | 3.00 |
| Blood meal | 2.00 | 2.00 | 2.00 | 2.00 |
| Vitamin and mineral premix[a] | 1.00 | 1.00 | 1.00 | 1.00 |
| Cassic acid (mg) | 0 | 0.001 | 0.005 | 0.010 |
| Proximate composition (%) | | | | |
| Crude protein (%) | 40.22 | 41.34 | 41.68 | 40.82 |
| Crude lipid (%) | 13.74 | 13.23 | 14.34 | 14.71 |
| Crude carbohydrate (%) | 17.62 | 18.14 | 17.84 | 18.48 |
| Crude ash (%) | 12.16 | 12.55 | 12.37 | 12.63 |
| Crude fiber (%) | 5.21 | 5.53 | 5.74 | 5.96 |

[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. Materials and methods

### 2.1. Preparation supplementation feed

To formulate the experimental feed with fish meal, maize gluten, wheat meal, and blood meal used for protein sources; αCellulose used for carbohydrate source; corn oil and cod liver oil used for lipid sources. The ingredients were powdered and mixed evenly in sterile water and steamed for 25 min to make them into a soft paste and sterilized for any microbes. After cooling at room temperature (RT), added with premix of vitamins and minerals evenly (Table 1). After thoroughly mixing of vitamins and minerals premix, the soft paste of feed ingredients were equally divided into four parts and mixed evenly with or without cassic acid (CA) for experimental diets namely: (i) basal diet without containing of CA (0 mg $kg^{-1}$), ii) basal diet containing with 1 mg $kg^{-1}$ of CA, iii) basal diet containing with 5 mg $kg^{-1}$ of CA, and iv) basal diet containing with 10 mg $kg^{-1}$ of CA. After preparing the experimental diet pastes were extruded through a manual noodle extruder with 0.5 mm diameter size. The prepared diets were air-dried then kept in oven at 40 °C for 18 h, packed in airtight sterile container, and stored −20 °C until used for the experiment. The proximate compositions of the formulated diets were determined in standard procedure.

### 2.2. Bacteriology

The morbid and healthy *C. gariepinus* with dropsy was collected from a disease-affected pond and brought into laboratory in oxygenated polythene bags. The fish was rinsed in sterile physiological saline (PBS; pH 7.2) immediately and wiped by sterile paper towels and than dissected out kidney aseptically. The kidney was inoculate on to brain heart infusion agar (BHIA) at 30 ± 2 °C for 24 h. Representative dominance colonies based on distinct colony morphology were picked randomly, repeated streaking on BHIA plates for purified, and kept on BHIA slants. A sequence of biochemical reactions were done for confirmation of *E. tarda* in automated bacterial identification system (BioMerieux, France) according to Austin and Austin [27]. Further the identification of *E. tarda* was confirmed through the following morphological, biochemical tests, and PCR methods [28,29]. The

pathogenic of *E. tarda* was confirmed to inoculate into *C. gariepinus* and re-isolation according to Abraham et al. [28].

### 2.3. Edwardsiella tarda

*E. tarda* streaked onto BHIA plates from the stock on BHIA slants and the plates were incubated for 24 h at 30 °C to achieve for sub-culture. One colony from the plate was aseptically select and transferred to 500 ml BHI broth (BHIB), incubated at 30 ± 2 °C for 24 h, centrifuged by 7500 rpm for 10 min at 20 °C than collect the bacterial pellet. The obtained pellets washed for thrice with PBS and re-suspended in 5 ml saline to count the numbers of bacterial cells by spread plating on BHIA.

### 2.4. Fish and experimental design

Health *C. gariepinus* (weight 35.6 ± 1.8 g) were procured from local fish farm. Then brought to the lab and immerged with 5 ppm $KMnO_4$ solution immediately for 5 min and the fish stocked in 500 l capacity fibreglass reinforced plastics (FRP) tanks. The fish were accustomed 2 weeks and this period was provided basal formulated control diet (Table 1) at a rate of 5% of their body weight/daily. Accrued wastes and feces were discarded every day and about 50% of the water was exchanged daily. After two weeks of acclimatization and feeding with basal formulated control diet, the fish were randomly distributed into 5 groups of 25 fish (5 × 25 = 125 fish) in three replicate groups (3 × 125 = 375 fish) namely: Group 1: health fish fed at basal control diet (without CA), Group II: *E. tarda* challenged fish given at control diet (without CA), Group III: *E. tarda* challenged fish fed diet containing 1 mg $kg^{-1}$ of CA, Group IV: *E. tarda* challenged fish fed diet containing 5 mg $kg^{-1}$ of CA, and V: *E. tarda* challenged fish fed diet containing 10 mg $kg^{-1}$ of CA. After 30 days of respective feeding (5% of their body weight/daily), Groups II to V were challenge (injected) intramuscularly (i.m.) with 50 μl PBS containing *E. tarda* at $3.6 \times 10^{-7}$ CFU $ml^{-1}$ while control group injected with 50 μl PBS alone at the dorsal fin after the fish were immersed under anaesthesia in 150 ppm buffered MS-222 solution (Sigma). The challenged and unchallenged groups were continued in their own tanks and fed with the corresponding diets during experimental period. The water quality parameters such as temperature 24 ± 2 °C, dissolved oxygen concentration 6.2 ± 2 mg $l^{-1}$, and pH at 5.7–6.8 were recorded during the experimental period.

### 2.5. Growth study

The growth performance, including percentage weight gain (RGR), specific growth rate (SGR) = [(log - final body weight – log - initial body weight)/time] x 100; feed conversion ratio (FCR) = (dry food intake/live weight gain); protein efficiency ratio (PER) = (live weight gain/protein intake); average daily gain (ADG) = (growth/experiment duration); percentage of weight gain (PWG) = [final weight - initial weight/initial weight] x 100; and survival rate (SR) = [(initial no. of fish/final no. fish) x 100 of each group were calculated by Jesu Arockiaraj et al. (1999). Relative percent survival (RPS) = (% mortality of control - % mortality of treatment)/(mortality of control) x 100.

### 2.6. Blood sampling

There are six fish from each experimental tank randomly selected for each sampling at weeks 1, 2, and 4 after post-challenge. The fish were then bled over their caudal vein thru a 24-gauge syringe needle. The collected blood samples were equally allocated and dispersed into heparinized and nonheparinized tubes. The nonheparinized blood samples were immediately kept at RT for 2 h than the sera were carefully isolated by centrifugation at 2700 rpm for 10 min and preserved at −20 °C until used for biochemical analysis and immunological assays.

### 2.7. Preparation of head kidney (HK) leukocytes

The HK leukocytes were isolated from each experimental fish samples according to Kamilya et al. [31]. The isolated HK leukocytes were enumerated and adjusted to $1 \times 10^6$ cells $ml^{-1}$ in RPMI-1640 after examination viability by trypan blue exclusion assay.

### 2.8. Hematology and biochemical study

The hemato-biochemical profiles such as red and white blood cells (RBC & WBC), hematocrit (Hct, %), hemoglobin (Hb, gd $l^{-1}$), total protein, albumin: globulin ratio, albumin, and globulin were determined by Blaxhall and Daisley [32]. The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were determined using the formulas described by Bain et al. [33]. The differential leukocytes were examined with May-Grunwald–Giemsa stained peripheral blood smears slides under oil-immersion at 100x. A 100 leukocytes were identified in each slides and calculated the lymphocytes (LYM), eosinophils (EOS), monocytes (MON), and neutrophils (NEU) according to Yılmaz et al. [34].

### 2.9. Immunological assays

The phagocytosis was estimated Sakai et al. [35] and Houwen [36]. The production of reactive oxygen species (ROS) of the intracellular respiratory bursts was measured by NBT assay [37] and the production of reactive nitrogen intermediate (RNI) of nitric oxide (NO) liberated by HK leucocytes using Griess reagent [38]. The synthesis of superoxide anion ($O_2^-$) and lymphocytes proliferation (LP) of HK leukocytes were determined according to Kamilya et al. [31] and Mosmann [39]. Total myeloperoxidase (MPO) content and alternative complement (ACP) activity of serum examined by Quade and Roth [40] and Yano [41]. The lysozyme (Lyz) activity was measured by turbidimetric assay [42].

### 2.10. Challenge study

Each experimental group were used 20 fish (5 × 20 = 100 fish) in three replicate groups (3 × 100 = 300 fish) and injected 50 μl PBS with or without *E. tarda* to observed cumulative mortality. The culture conditions, challenge methods and dose were the same as mentioned previously for the cumulative mortality (%) = Total mortality in each treatment group/Total number of fish in control group x 100 observed for 30 days [30].

### 2.11. Total RNA extraction for cDNA synthesis

HK, testis, and gonad were dissected out (n = 6) in each group at different time point (weeks 1, 2, and 4) and immediately frozen in liquid nitrogen and stored at −80 °C until used for RNS isolation. Total RNA was isolated from HK, testis, and gonad using GENEzol™ reagent (Genaid, Taiwan) according to the manufacturer's instruction. The concentration of total RNA was analysis using spectrophotometer at optical densities (O.D.) of 260 and 280 nm, respectively. The purification of RNA and synthesis of the first strand cDNA were performed from 1 μg of total RNA using RevertraAce® qPCR RT Mastermix with gDNA remover kit (Toyobo, Japan) following manufacturer's instructions. The RNA integrity was estimated by agarose gel electrophoresis method. The cDNA was diluted in nucleases free water and stored at −20 °C prior to use.

### 2.12. Gene cloning

The estrogen and FSH-β hormones, and chicken-type (*c-Lyz*) and groove-type (*g-Lyz*) lysozyme enzymes partial sequences were performed by PCR method (Table 2) in a 30 μl volume reaction that

**Table 2**
Forward and reverse primers nucleotide sequences of the reproductive and immune related genes used in this study.

| Gene | Acc. No. | Sequence (3'→5') | base pair (bp) |
|---|---|---|---|
| ERα | X84743 | F: TGTCCGGCCACCAATCA<br>R: GTGTCTCCTGCTGTGCTTCATC | 150 |
| FSH-β | AF324541 | F: CCATTGGACTGCGGTACGCTCT<br>R: ATGCGTGGCGTTGCCATGGTGT | 100 |
| C-Lyz | MK344777 | F: CGGTATGATCGGTGTGAGCTGG<br>R: CGGTTCTGGGCGTTGGTATTGA | 60 |
| G-Lyz | MH341528 | F: CCTAACTGGCCCAAAGAGCA<br>R: CCATACCCTCGTATGTGCGG | 60 |
| β-actin | KJ722166 | F: ACCGGAGTCCATCACAATACCAGT<br>R: GAGCTGCGTGTTGCCCCTGAG | 60 |

ERα: estrogen receptor alpha; FSH: FSH-β: follicle-stimulating hormone b subunit; C-Lyz: chicken-type lysozyme; G-Lyz: goose-type lysozyme.

consisting of 15 μl premix of MyTaq™ Red Mix (Bioline, UK), 1 μl (10 μM) of each primer, 12 μl nucleases free water, and 1 μl (50 ng/μl) of cDNA template prepared from HK, testis, and ovary. The PCR thermal cycling programs were set as follow: 95 °C for 3 min; 35 cycles of 95 °C for 30 s, each primer annealing temperature for 30 s and 72 °C for 30 s; and final elongation at 72 °C for 5 min. The final PCR products were isolated by 1.5% agarose gel, and then purify using Wizard® SV Gel and PCR Clean-Up (Promega, USA).

### 2.13. Statistical analysis

All the date results in this study were carryout ANOVA exhausted SPSS version 15.0 and the Duncan multiple range test used for compare differences between experimental groups.

## 3. Results

### 3.1. Growth performance

In this study was observed improved weight gain of infected fish after fed dietary supplementation of CA at 5 and 10 mg $kg^{-1}$ diets. PER and FCR were slightly improved in infected fish fed dietary supplementation of CA at 5 and 10 mg $kg^{-1}$ diets, but least PER and FCR value observed with 1 mg $kg^{-1}$ diet. The SGR was observed in this study did not significant difference among the experimental group. However, the survival rate has observed 96.7% and 98.3% with 5 and 10 mg $kg^{-1}$ CA diets whereas it was shown least survival rate 95.0% observed with 1 mg $kg^{-1}$ CA diet as compared to basal control diet group (Table 3).

### 3.2. Hematology

The RBC, WBC, Hb, and PCV augmented the infected fish fed at 5 and 10 mg $kg^{-1}$ CA supplementation diets in this study. There was no statistical difference of MCV, MCH, and MCHC among the experimental group. No statistical difference was observed between sampling time of the hematological parameters in this study. The percentage of lymphocyes improved significantly with 5 mg diet, but it did not shown with 1 or 10 mg $kg^{-1}$ CA diet groups. The percentage of neutrophils was significantly increased with 5 and 10 mg $kg^{-1}$ CA diet groups. However, the percentage of monocyes and eosinophils were significantly improved in 5 and 10 mg $kg^{-1}$ CA diet groups only after week 2. The lymphocytes and eosinophils percentage significantly varied between sampling time when the infected fish fed at 5 and 10 mg $kg^{-1}$ CA diets whereas the neutrophils and monocyes percentage differ between sampling time with 10 mg $kg^{-1}$ CA diet (Table 4).

### 3.3. Biochemistry

Total protein and albumin did not significantly differ on first week in 1 mg $kg^{-1}$ CA diet as compared to control diet group. However, it was significantly increased with 5 and 10 mg $kg^{-1}$ CA diet groups in this study. The globulin as well as albumin: globulin ratio significantly elevated in infected fish treated at 5 and 10 mg $kg^{-1}$ CA diet groups after week 2, whereas it was not shown with 1 mg $kg^{-1}$ CA diet group as compared to control diet group. The total protein and albumin level did not vary between sampling time when the infected fish fed with 1 and 10 mg $kg^{-1}$ CA diets whereas the globulin and albumin: globulin ratio significantly differed between sampling time with 5 and 10 mg $kg^{-1}$ CA diets (Table 5).

### 3.4. Immune response

The phagocytosis and respiratory bursts were not significantly enhances the infected provided at 1 mg $kg^{-1}$ CA diet group throughout the experiment. However, there were significant phagocytosis and respiratory bursts observed the infected given at 5 and 10 mg $kg^{-1}$ CA diet groups at any time to control group (Figs. 1 and 2). Production of SOA and NO did not significantly increase the infected fish feeding with any CA diet on 1st week as compared to control. It was significantly augmented when infected fish given at 5 and 10 mg $kg^{-1}$ CA diet groups as compared to other groups on weeks 2 and 4 (Figs. 3 and 4). The LP and MPO were not enhanced significantly among the groups on first week. These activities were observed significantly high the infected fish fed with 10 mg $kg^{-1}$ CA diet group as compared with other groups on second week; however, it was observed significantly high in both 5 and 10 mg $kg^{-1}$ CA diet groups when compared with other groups on fourth week (Figs. 5 and 6). The AC, ROS, and Lyz activity were observed did not significantly influenced by the infected fish feeding at 1 mg $kg^{-1}$ CA diet group whereas these activities were significantly enhanced the infected fish fed at 5 and 10 mg $kg^{-1}$ CA diet groups during the experiment as compared to control diet group (Figs. 7–9). The phagocytic, respiratory burst, and lysozyme activities were significantly differ with 5 and 10 mg $kg^{-1}$ CA diets between sampling period but the SOA and ACP activity significant differ between sampling time with 10 mg $kg^{-1}$ CA diet. However, there was statistical difference between sampling periods among the supplementation diet in this study. The highest g-Lyz and c-Lyz mRNA expression was found in 5 and 10 mg $kg^{-1}$ CA diets as determined by real-time quantitative PCR. However, there was tiny expression of g-Lyz and c-Lyz mRNA

**Table 3**
Growth performance and feed utilization of cassic acid enriched diets in *C. gariepinus* against *E. tarda* for 4 weeks.

| Indices | 0 mg $kg^{-1}$ (C) | 0 mg $kg^{-1}$ (I) | 1 mg $kg^{-1}$ | 5 mg $kg^{-1}$ | 10 mg $kg^{-1}$ |
|---|---|---|---|---|---|
| Initial weight (g) | 36.3 ± 1.10 | 36.1 ± 0.92 | 36.5 ± 1.30 | 36.4 ± 1.12 | 36.2 ± 1.38 |
| Weight gain (g) | 20.7 ± $0.8^a$ | 18.2 ± $0.6^b$ | 21.3 ± $0.6^a$ | 22.4 ± $0.8^b$ | 22.9 ± $0.8^b$ |
| Feed intake (g) | 24.8 ± $1.2^a$ | 22.8 ± $1.4^b$ | 24.6 ± $1.0^a$ | 26.0 ± $1.2^b$ | 26.6 ± $1.4^b$ |
| SGR (%/day) | 0.71 ± $0.08^a$ | 0.68 ± $0.06^a$ | 0.72 ± $0.08^a$ | 0.76 ± $0.06^a$ | 0.82 ± $0.08^a$ |
| PER | 0.82 ± $0.12^a$ | 0.86 ± $0.14^a$ | 0.87 ± $0.10^a$ | 0.89 ± $0.14^a$ | 0.92 ± $0.12^a$ |
| FCR | 1.36 ± $0.06^a$ | 1.20 ± $0.04^a$ | 1.34 ± $0.06^a$ | 1.42 ± $0.04^a$ | 1.48 ± $0.06^a$ |
| Survival rate (%) | 98.3 ± $3.2^a$ | 93.3 ± $3.28^b$ | 95.0 ± $2.8^b$ | 96.7 ± $4.0^a$ | 98.3 ± $3.25^a$ |

SGR: specific growth rate, PER: protein efficiency ratio, FCR: feed conversion ratio. Mean followed by the same letter is not significantly different ($P > 0.05$).

**Table 4**
Hematological parameters of cassic acid enriched diets in *C. gariepinus* against *E. tarda*.

| Parameters | Weeks | 0 mg (C) | 0 mg (I) | 1 mg | 5 mg | 10 mg |
|---|---|---|---|---|---|---|
| RBC ($x10^{12}$/L) | 1 | 2.38 ± 0.75$^a$ | 1.08 ± 0.32$^a$ | 2.41 ± 0.50$^a$ | 2.92 ± 0.54$^a$ | 3.57 ± 0.48$^b$ |
| | 2 | 2.41 ± 0.70$^a$ | 1.10 ± 0.38$^a$ | 2.55 ± 0.52$^a$ | 3.45 ± 0.56$^b$ | 3.86 ± 0.52$^b$ |
| | 4 | 2.43 ± 1.72$^a$ | 1.12 ± 0.40$^a$ | 2.79 ± 0.44$^a$ | 3.78 ± 0.62$^b$ | 3.98 ± 0.64$^b$ |
| WBC ($x10^9$/L) | 1 | 13,350 ± 14.12$^a$ | 11,762 ± 12.12$^a$ | 13,375 ± 13.14$^a$ | 15,384 ± 15.52$^b$ | 16,146 ± 12.48$^b$ |
| | 2 | 13,355 ± 11.14$^a$ | 11,723 ± 12.12$^a$ | 13,693 ± 12.10$^a$ | 15,871 ± 10.84$^b$ | 16,790 ± 11.73$^b$ |
| | 4 | 13,362 ± 10.25$^a$ | 11,684 ± 12.12$^a$ | 13,912 ± 11.72$^a$ | 16,126 ± 12.44$^b$ | 17,312 ± 13.34$^b$ |
| Hb (gm %) | 1 | 8.46 ± 0.82$^a$ | 7.72 ± 0.64$^a$ | 8.73 ± 0.76$^a$ | 9.25 ± 0.86$^a$ | 10.22 ± 1.14$^b$ |
| | 2 | 8.51 ± 0.77$^a$ | 7.47 ± 0.72$^a$ | 8.98 ± 0.76$^a$ | 10.31 ± 0.92$^b$ | 10.74 ± 0.75$^b$ |
| | 4 | 8.63 ± 0.68$^a$ | 7.17 ± 0.62$^a$ | 9.17 ± 0.81$^a$ | 10.63 ± 0.76$^b$ | 10.92 ± 0.84$^b$ |
| PCV (%) | 1 | 25.5 ± 2.2$^a$ | 24.5 ± 2.8$^a$ | 25.2 ± 2.6$^a$ | 26.4 ± 3.2$^a$ | 27.5 ± 2.7$^b$ |
| | 2 | 26.0 ± 2.8$^a$ | 24.8 ± 2.1$^a$ | 26.4 ± 2.8$^a$ | 27.6 ± 3.0$^b$ | 28.4 ± 2.7$^b$ |
| | 4 | 26.2 ± 2.6$^a$ | 25.0 ± 3.0$^a$ | 26.8 ± 3.2$^a$ | 28.6 ± 3.8$^b$ | 29.1 ± 2.6$^b$ |
| MCV (fl) | 1 | 101.2 ± 3.6$^a$ | 97.4 ± 3.2$^a$ | 102.6 ± 3.2$^a$ | 103.4 ± 2.6$^a$ | 106.2 ± 5.2$^b$ |
| | 2 | 102.6 ± 3.4$^a$ | 97.7 ± 3.0$^a$ | 103.2 ± 3.5$^a$ | 107.6 ± 4.6$^a$ | 108.2 ± 4.2$^b$ |
| | 4 | 102.8 ± 3.1$^a$ | 98.1 ± 3.8$^a$ | 103.8 ± 3.0$^a$ | 109.4 ± 4.2$^a$ | 112.2 ± 4.8$^b$ |
| MCH (pg) | 1 | 3.18 ± 0.6$^a$ | 3.05 ± 0.8$^a$ | 3.26 ± 0.6$^a$ | 3.43 ± 0.8$^a$ | 3.66 ± 0.6$^a$ |
| | 2 | 3.23 ± 0.7$^a$ | 3.24 ± 0.7$^a$ | 3.43 ± 0.8$^a$ | 3.64 ± 0.7$^a$ | 3.66 ± 0.8$^a$ |
| | 4 | 3.26 ± 0.8$^a$ | 3.31 ± 0.6$^a$ | 3.64 ± 0.7$^a$ | 3.70 ± 0.8$^a$ | 3.72 ± 0.8$^a$ |
| MCHC (%) | 1 | 30.00 ± 1.2$^a$ | 29.50 ± 1.4$^a$ | 30.42 ± 1.6$^a$ | 31.64 ± 1.7$^a$ | 32.34 ± 1.4$^a$ |
| | 2 | 30.50 ± 1.4$^a$ | 29.70 ± 1.2$^a$ | 30.86 ± 1.6$^a$ | 32.55 ± 1.6$^a$ | 33.48 ± 1.8$^b$ |
| | 4 | 30.82 ± 1.6$^a$ | 30.00 ± 1.6$^a$ | 31.12 ± 1.6$^a$ | 33.32 ± 1.5$^b$ | 33.62 ± 1.6$^b$ |
| Lym (%) | 1 | 66.2 ± 3.2$^a$ | 65.5 ± 3.2$^a$ | 66.2 ± 3.2$^a$ | 66.8 ± 3.6$^a$ | 68.9 ± 3.8$^b$ |
| | 2 | 66.8 ± 3.0$^a$ | 64.6 ± 3.4$^a$ | 66.9 ± 3.5$^a$ | 68.6 ± 4.0$^a$ | 69.8 ± 3.6$^b$ |
| | 4 | 67.2 ± 3.4$^a$ | 63.7 ± 3.0$^a$ | 67.4 ± 4.2$^a$ | 69.2 ± 3.8$^b$ | 70.2 ± 4.4$^b$ |
| Neutro (%) | 1 | 25.00 ± 2.2$^a$ | 24.76 ± 2.4$^a$ | 25.82 ± 2.6$^a$ | 28.64 ± 3.0$^b$ | 29.52 ± 2.4$^b$ |
| | 2 | 25.64 ± 2.5$^a$ | 24.32 ± 2.0$^a$ | 26.32 ± 2.8$^a$ | 29.42 ± 3.2$^b$ | 30.12 ± 2.8$^b$ |
| | 4 | 25.86 ± 2.8$^a$ | 24.00 ± 2.2$^a$ | 26.76 ± 3.0$^a$ | 29.86 ± 3.4$^b$ | 32.46 ± 3.4$^b$ |
| Mono (%) | 1 | 1.52 ± 0.42$^a$ | 1.34 ± 0.48$^a$ | 1.68 ± 0.40$^a$ | 1.74 ± 0.46$^a$ | 1.84 ± 0.54$^a$ |
| | 2 | 1.64 ± 0.47$^a$ | 1.28 ± 0.38$^a$ | 1.82 ± 0.42$^a$ | 2.32 ± 0.52$^b$ | 3.24 ± 0.56$^b$ |
| | 4 | 1.78 ± 0.38$^a$ | 1.16 ± 0.34$^a$ | 1.96 ± 0.44$^a$ | 2.62 ± 0.42$^b$ | 3.52 ± 0.50$^b$ |
| Eos (%) | 1 | 1.50 ± 0.12$^a$ | 1.46 ± 0.10$^a$ | 1.72 ± 0.14$^a$ | 1.86 ± 0.12$^a$ | 1.98 ± 0.16$^a$ |
| | 2 | 1.66 ± 0.18$^a$ | 1.42 ± 0.12$^a$ | 1.84 ± 0.18$^a$ | 2.86 ± 0.24$^b$ | 3.32 ± 0.26$^b$ |
| | 4 | 1.75 ± 0.22$^a$ | 1.53 ± 0.14$^a$ | 1.98 ± 0.20$^a$ | 3.12 ± 0.28$^b$ | 3.68 ± 0.28$^b$ |

RBC: Red Blood Cell, WBC: White Blood Cell, PCV: packed cell volume, Hb: Haemoglobin, MCV: Mean Cell Volume, MCH: Mean Cell Haemoglobin, MCHC: Mean Cell Haemoglobin Concentration, Lym: Lymphocytes, Neutro: neutrophils, Eos: Eosunophil, Mono: Monocytes. Mean followed by the same letter is not significantly different ($P > 0.05$).

**Table 5**
Biochemical parameters of cassic acid enriched diets in *C. gariepinus* against *E. tarda*.

| Parameters | Weeks | 0 mg (C) | 0 mg (I) | 1 mg | 5 mg | 10 mg |
|---|---|---|---|---|---|---|
| Total protein | 1 | 3.24 ± 0.16$^a$ | 2.96 ± 0.22$^a$ | 3.52 ± 0.18$^a$ | 3.98 ± 0.26$^a$ | 5.14 ± 0.32$^b$ |
| | 2 | 3.58 ± 0.30$^a$ | 2.52 ± 0.34$^a$ | 3.88 ± 0.28$^a$ | 5.10 ± 0.46$^b$ | 5.10 ± 0.40$^b$ |
| | 4 | 3.83 ± 0.36$^a$ | 2.28 ± 0.30$^a$ | 3.96 ± 0.30$^a$ | 5.38 ± 0.42$^b$ | 5.68 ± 0.44$^b$ |
| Albumin | 1 | 0.92 ± 0.32$^a$ | 0.96 ± 0.30$^a$ | 1.16 ± 0.36$^a$ | 1.48 ± 0.36$^a$ | 2.72 ± 0.28$^b$ |
| | 2 | 1.22 ± 0.30$^a$ | 1.14 ± 0.26$^a$ | 1.38 ± 0.32$^a$ | 2.56 ± 0.26$^b$ | 2.86 ± 0.38$^b$ |
| | 4 | 1.32 ± 0.38$^a$ | 1.28 ± 0.26$^a$ | 1.56 ± 0.28$^a$ | 2.74 ± 0.38$^b$ | 2.96 ± 0.34$^b$ |
| Globulin | 1 | 0.70 ± 0.06$^a$ | 0.83 ± 0.07$^a$ | 1.64 ± 0.06$^a$ | 1.77 ± 0.06$^a$ | 1.83 ± 0.07$^a$ |
| | 2 | 0.88 ± 0.06$^a$ | 0.80 ± 0.06$^a$ | 1.78 ± 0.07$^a$ | 2.38 ± 0.08$^b$ | 2.74 ± 0.08$^b$ |
| | 4 | 0.96 ± 0.07$^a$ | 0.74 ± 0.06$^a$ | 1.88 ± 0.07$^a$ | 2.67 ± 0.07$^b$ | 2.96 ± 0.08$^b$ |
| A:G ratio | 1 | 0.52 ± 0.06$^a$ | 0.66 ± 0.05$^a$ | 0.78 ± 0.06$^a$ | 0.87 ± 0.05$^a$ | 0.95 ± 0.07$^a$ |
| | 2 | 0.64 ± 0.05$^a$ | 0.70 ± 0.04$^a$ | 0.96 ± 0.05$^a$ | 1.44 ± 0.06$^b$ | 1.58 ± 0.06$^b$ |
| | 4 | 0.72 ± 0.05$^a$ | 0.73 ± 0.04$^a$ | 1.26 ± 0.06$^a$ | 1.66 ± 0.06$^b$ | 1.78 ± 0.08$^b$ |

A:G ratio: Albumin: Globulin ratio. Mean followed by the same letter is not significantly different ($P > 0.05$).

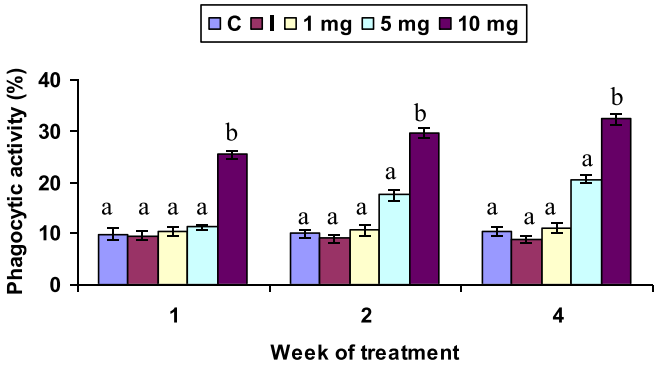


**Fig. 1.** Phagocytic activity of *C. gariepinus* (mean ± SEM, n = 6) fed with or without cassic acid supplemented diets against *E. tarda*. Mean followed by the same letter is not significantly different ($P > 0.05$).

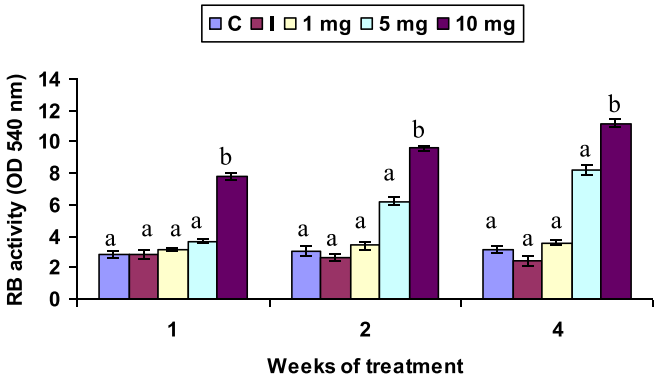


**Fig. 2.** Respiratory burst (RB) activity of *C. gariepinus* (mean ± SEM, n = 6) fed with or without cassic acid supplemented diets against *E. tarda*. Mean followed by the same letter is not significantly different ($P > 0.05$).

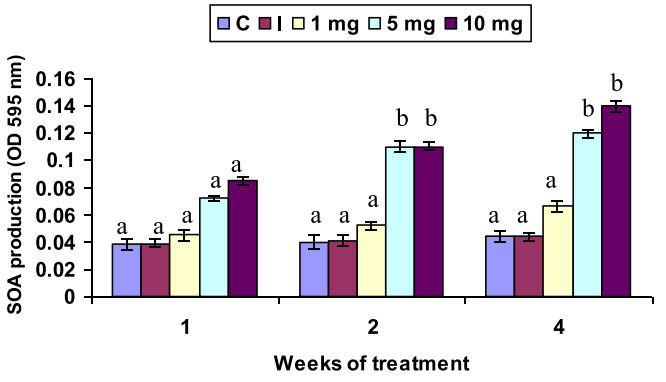


**Fig. 3.** Superoxide anion (SOA) production of *C. gariepinus* (mean ± SEM, n = 6) fed with or without cassic acid supplemented diets against *E. tarda*. Mean followed by the same letter is not significantly different ($P > 0.05$).

expression observed in uninfected and infected fish fed at 1 mg $kg^{-1}$ CA or without CA diets in the present study. There was no statistical difference observed with any diet between sampling time (Fig. 10 a & b).

### 3.5. Disease resistance

The infected fish when fed with 1 and 5 mg $kg^{-1}$ CA supplementation diet groups were found 10% cumulative mortality while it was shown 15% mortality with 10 mg $kg^{-1}$ CA supplementation diet group. It was shown 85% mortality in the infected fish when fed with basal control diet. There was no mortality shown in uninfected fish fed with control basal diet (Fig. 11).

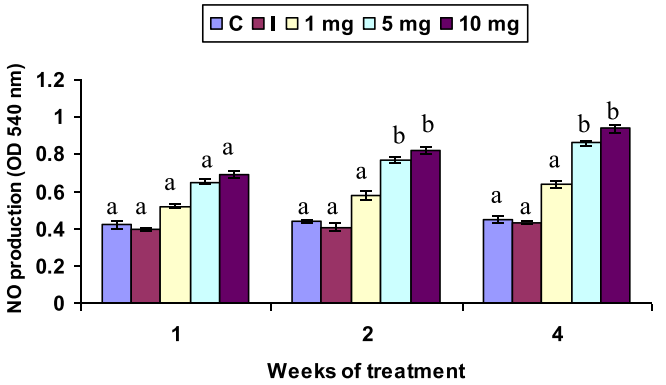


**Fig. 4.** Nitric oxide (NO) production of *C. gariepinus* (mean ± SEM, n = 6) fed with or without cassic acid supplemented diets against *E. tarda*. Mean followed by the same letter is not significantly different ($P > 0.05$).

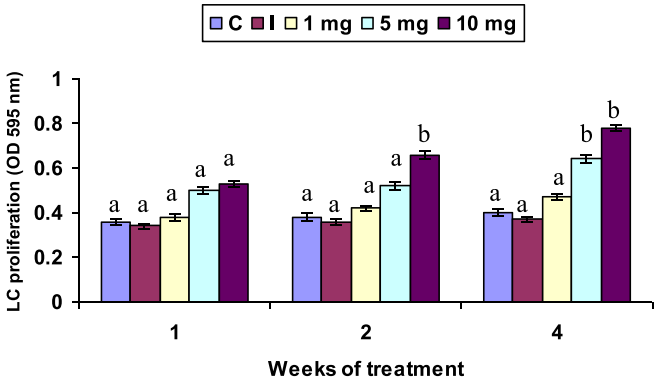


**Fig. 5.** Lymphocyte (LC) proliferation of *C. gariepinus* (mean ± SEM, n = 6) fed with or without cassic acid supplemented diets against *E. tarda*. Mean followed by the same letter is not significantly different ($P > 0.05$).

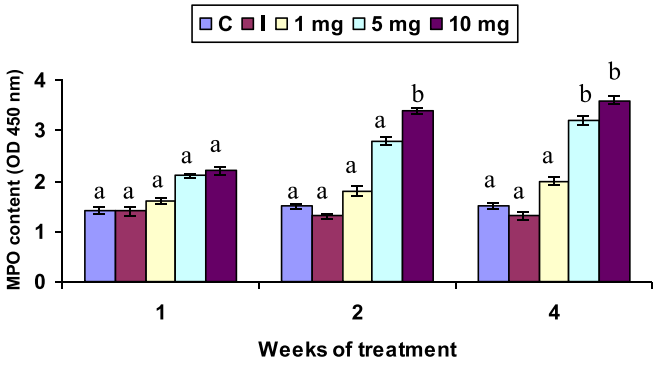


**Fig. 6.** Myeloperoxidase (MPO) activity of *C. gariepinus* (mean ± SEM, n = 6) fed with or without cassic acid supplemented diets against *E. tarda*. Mean followed by the same letter is not significantly different ($P > 0.05$).

### 3.6. Expression study

As shown in Fig. 12, the ERα mRNA expression was high in gonad of infected fish fed with 5 and 10 mg $kg^{-1}$ CA diets, but tiny expression was seen of these diet in testis as compared to other groups (Fig. 12 a & b). The FSH-β mRNA expression was high in testis of infected group given at 5 and 10 mg $kg^{-1}$ CA diets as compared with the group fed diet supplementation with 1 mg $kg^{-1}$ CA or without CA diet (Fig. 13 a & b).

## 4. Discussion

Edwardsiellosis is an important devastating fish pathogen caused by

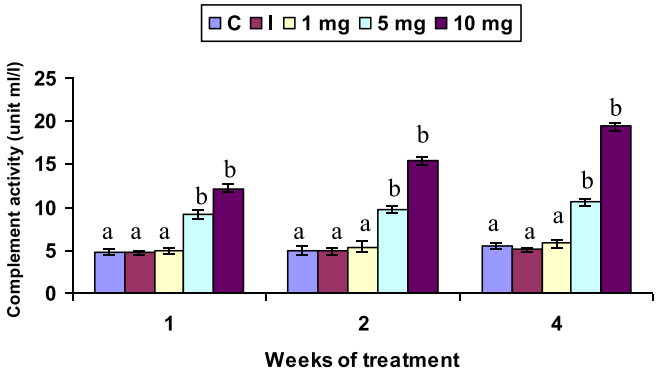


**Fig. 7.** Alternate complement activity (ACP) of *C. gariepinus* (mean ± SEM, n = 6) fed with or without cassic acid supplemented diets against *E. tarda*. Mean followed by the same letter is not significantly different ($P > 0.05$).

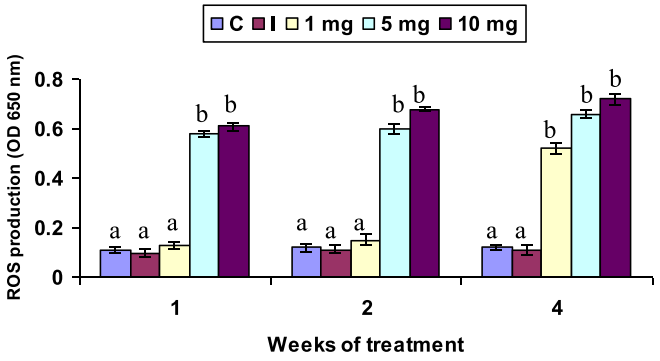


**Fig. 8.** Reactive oxygen species (ROS) production of *C. gariepinus* (mean ± SEM, n = 6) fed with or without cassic acid supplemented diets against *E. tarda*. Mean followed by the same letter is not significantly different ($P > 0.05$).

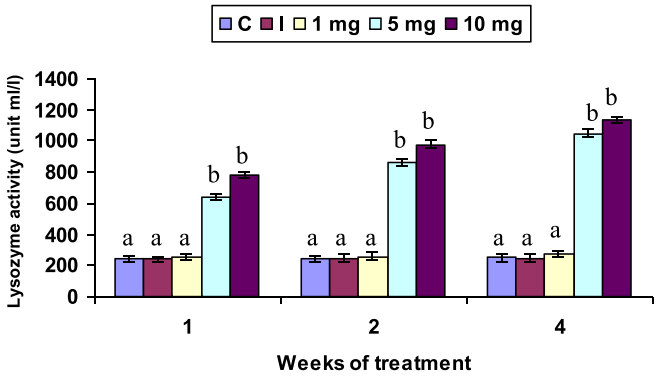


**Fig. 9.** Lysozyme activity of *C. gariepinus* (mean ± SEM, n = 6) fed with or without cassic acid supplemented diets against *E. tarda*. Mean followed by the same letter is not significantly different ($P > 0.05$).

*E. tarda* prevailing in worldwide aquaculture system that accounts severe economic losses in both cultured as well as wild fish species [7]. Chemotherapy (chemicals and antibiotics) is an effective prophylactic measures commonly used to protect fish from edwardsiellosis; however use of chemotherapy has some own disadvantages with origin of disease-resistant strains, cost effect as well as indiscriminate use by aqua farmers [7] thereby reducing drug efficacy [43]. Hence, there is an urgent need to develop, alternative approaches to control of edwardsiellosis in aquaculture. In this regards, a number for probiotics, herbals and their active constituents provide better growth performance, immunity, and disease protection in various fish species against different infectious pathogens [10–15]. To our knowledge, there is no or little effort on the effect of CA active constituents on growth performance, physiological disturbance, immunity, growth and reproductive hormones as well as lysozyme enzyme mRNA transcription in fish against pathogens.

This study was observed first time improved weight gain, PER, and FCR in the infected fish when fed dietary supplementation of CA at 5 and 10 mg $kg^{-1}$ levels as compared with 1 mg CA diet and control basal diet groups. A high survival rate of 96.7% and 98.3% were found the infected fish provided at 5 and 10 mg $kg^{-1}$ CA supplementation diets when compared with other groups. A similar result (PWG, FER, FCR, SGR, PER, and RPS) was reported recently in the *Cirrhinus mrigala* fed diet supplementation with 1.5% curcumin against *E. tarda* [44]. Soya saponins containing diet also reported similar results in *Scophthalmus maximus*, *Oreochromis niloticus*, and *Cyprinus carpio* [45–47]. It is strongly assumed that active constituents may improve digestion and nutrient metabolism. It has been reported that herbs may enhance the secretion of pancreatic enzymes, important factors in nutrient digestion and assimilation [48]. Based on the reason, this study the infected fish fed dietary supplementation of CA at 5 and 10 mg $kg^{-1}$ level may be the influence to secretion of pancreatic enzymes which may be improving digestion and nutrient metabolism in *C. gariepinus*.

The blood profiles are a fundamental tool in generally monitoring the diseases, health status, physiology conditions, and also assessing their immune system in natural and culture environments. Both the biochemical and hematological blood constituents had influenced by the quantity and quality of feed adjective or the level of anti-nutritional elements or factors [49]. The differential WBC is display of health status in fish [50]. The RBC, WBC, Hb, and PCV was found significantly high in this study the infected fish given at 5 and 10 mg $kg^{-1}$ CA enriched diets. The present result was similar to the results in *O. niloticus* when fed with garlic and chloramphenicol supplemented diets [51] who reported a significant increase in RBC, Hb, and Hct value. An increasing RBC, PCV, and Hb content had reported in *Oreochromis niloticus* fed garlic and ginseng [51], and allicin [52] supplementation diets. The infected fish feeding with basal control diet decreasing trend of the hematological parameters in this study. In line with the decreasing trends was observed in the RBC, Hb, and Hct values in fish may be accompanying with hypochromic microcytic anemia [53]. The decline in PCV concentration or RBC counts have possible the presence of a toxic factor results lower protein intake or mild anaemia and adverse effect on blood cell formation [54]. The increasing level of hematological constituents due to CA may be attributed to the fact that the active ingredients play a major role of physiological activity in fish and detailed mechanisms and role is currently unknown.

The blood indices such as MCV, MCH, and MCHC are particularly imperative for diagnosis of anaemia in the majority animals. This present study, the infected fish after feeding at 5 mg and 10 mg CA supplementation diets had no statically affected of MCV, MCH, and MCHC which is in conformity with the work in *O. niloticus* fed garlic and allicin supplementation diets [52]. The percentage of lymphocyes significantly increased with 10 mg $kg^{-1}$ CA diet whereas the percentage of neutrophils, monocyes, and eosinophils increased in 5 and 10 mg $kg^{-1}$ diets after 2nd week. The increasing level of leucocytes percentage may be influence by CA and the mechanisms of action is not understood. However, the infected fish was provide with control diet decreased of these parameters may consist of erythrocytosis, thrombocytosis, lymphopenia, neutrophilia, decrease in clotting time while increase in Hct due to erythrocyte swelling [55,56].

Serum total protein, globulin as well as the albumin levels are also believed to be associated to a stronger innate immune response in fish [57]. Total protein and albumin significantly increased the infected fish feeding at 5 and 10 mg $kg^{-1}$ diets in this study whereas globulin and albumin: globulin ratio elevated of these diets only after second week. This present findings were similar to the results obtained in *C. batrachus* when fed diet supplementation of emodin active constituent [58]. The mechanism of enhancement of total protein, albumin, globulin, and

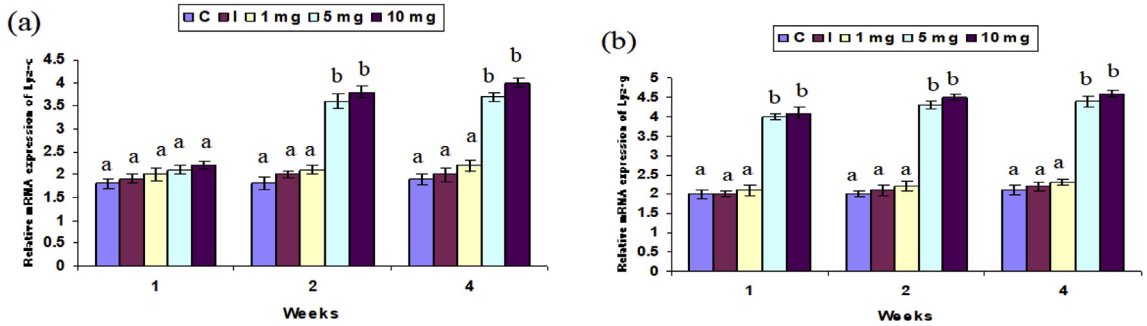


**Fig. 10.** Expression of (a) chick-type lysozyme (c-Lyz) and (b) goose-type lysozyme (g-Lyz) mRNA expression in head kidney (HK) of *C. gariepinus* (n = 6) fed with or without cassic acid supplemented diets against *E. tarda* as determined by real-time PCR. The asterisk indicates a statistically significant difference ($P < 0.05$).

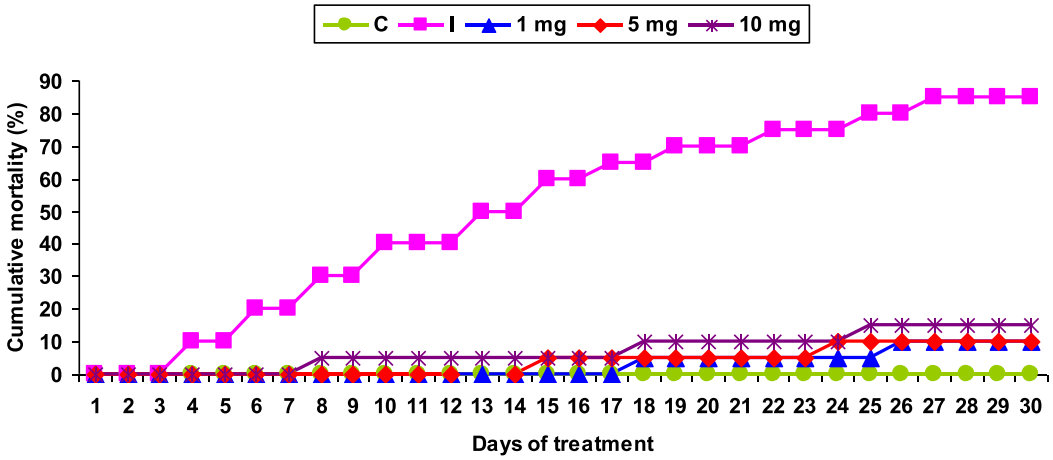


**Fig. 11.** Percentage of cumulative mortality of *C. gariepinus* (n = 20) fed with or without cassic acid supplemented diets against *E. tarda* for 30 days.

albumin: globulin ratio due to CA largely unknown.

The innate defense mechanisms play a key role in preserving effective disease conflict to a variety of fish pathogens [59]. The serum and plasma in aquatic species are considered by a number of proteins, including trypsin, Lyz, antibodies, C-protein, complement and other lytic factors, which are play an serious roles as antimicrobial agents and it act as first line of defense, primary barrier against invasion pathogens, and contain the proliferation of pathogens [60,61]. The phagocytic cells increase in their bactericidal activities and stimulate natural killer (NK) cells, complement system, Lyz activity, and antibody response in fish and shellfish which confer enhanced protection from invading pathogens [11]. Respiratory burst (sometimes called oxidative burst) plays an vital role in the immune system which is rapidly release chemicals from immune cells (e.g., neutrophils and monocytes) and reactive oxygen species (superoxide radical and hydrogen peroxide, HP) come into contact and they degrade internalized different foreign particles including bacteria and fungi. The phagocytic and RB activity significantly increase the infected fish when fed with 10 mg $kg^{-1}$ CA diet group in this experiment. These results are in agreement with some plant immunostimulant active compounds such as β-glucan, azadirachtin, garlic, andrographolide, polysaccharide extract, azadirachtin, camphor, curcumin, and saponin activate and significantly enhanced the phagocytic activity in *Carassius auratus*, *Oncorhynchus mykiss*, *Lates calcarifer*, and *L. rohita* against *Aeromonas hydrophila* [13,14,62–67]. The phagocytic and RB activity was increased in the infected fish when given at 10 mg $kg^{-1}$ CA diet due the enhancement of leucocytes. There was no effect the infected fish fed at 1 and 5 mg $kg^{-1}$ CA diets.

The role of superoxide anion ($O_2^-$) is one of the ROS, by the

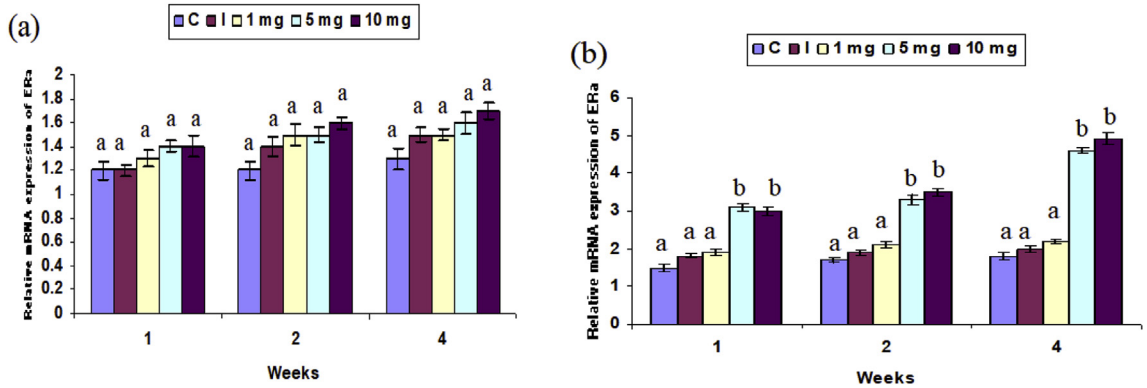


**Fig. 12.** Expression of estrogen receptor alpha (ERα) mRNA in (a) testis and (b) gonad of *C. gariepinus* (n = 6) fed with or without cassic acid supplemented diets against *E. tarda* as determined by real-time PCR. The asterisk indicates a statistically significant difference ($P < 0.05$).

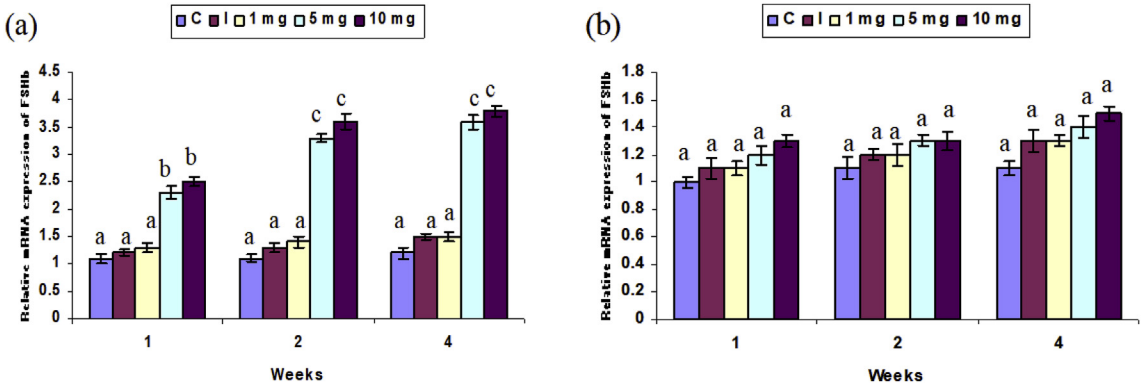


**Fig. 13.** Expression of follicle-stimulating hormone beta subunit (FSH-β) mRNA in (a) testis and (b) gonad of *C. gariepinus* (n = 6) fed with or without cassic acid supplemented diets against *E. tarda* as determined by real-time PCR. The asterisk indicates a statistically significant difference ($P < 0.05$).

resident as well as activated HK leucocytes necessary for the maintenance of redox homeostasis in organisms [68]. Fish phagocytes are capable to generate superoxide anion during a process called respiratory burst [37]. The ROS and also nitrogen species (RNS) are well-known as the RB or metabolic activation reaction; the consumed $O_2$ is converted into or reduced to $O_2^-$ or HP with the help of unique enzymes present in the cell wall of leucocytes which are involved in bactericidal activity [69–71]. Production of $O_2^-$ and NO significantly increased when infected fish provide at 5 and 10 mg $kg^{-1}$ CA diets on weeks 2 and 4 as compared with other groups. This was reported in previous study in *C. auratus* fed diet with azadirachtin against *A. hydrophila* [64]. Similarly, *C. mrigala* after feeding with azadirachtin, camphor, and curcumin containing diets had reported significantly enhanced the reactive oxygen and nitrogen species production against *Aphanomyces invadans* [13]. The present result of the production of $O_2^-$ and NO may suggest that the infected fish when administration at 5 and 10 mg $kg^{-1}$ CA diets. However, the details mechanism of actions of CA on the production of $O_2^-$ and NO is currently unknown.

The lymphocytes (B- and T-cells) generates by lymphoid organs such as bone marrow, thymus, liver which promote antibody against pathogens or toxins. The MPO is a peroxidase enzyme and lysosomal protein contains azurophilic granules of the neutrophil granulocytes that released hypohalous acids into the extracellular space during degranulation and carry out antimicrobial activity. The lymphocyte proliferation and MPO activity were observed significantly high the infected fish fed with 10 mg $kg^{-1}$ CA diet after second week while in both 5 and 10 mg $kg^{-1}$ CA diets only after fourth week. This result is correlated with ginger- and garlic-supplementation diets significantly elevated lymphocyte proliferation in *Huso huso* [72] and *Astragalus* polysaccharides -supplementation diet in *Pelteobagrus fulvidraco* [73]. The details role and mechanisms of action of CA and other active constituents on the lymphocyte proliferation and MPO activity is currently unknown.

The ACP is one of most another important serum factors since it activates the cellular defense and bactericidal activity recognized as one of the vital killing mechanisms of destroying bacteria in fish and other animals [74]. The Lyz is a humoral innate defense protein and well known to attack mostly Gram-positive bacteria in conjunction with complements widely distributed in fish [75]. The ACP and Lyz activity and ROS production are significantly enhanced the infected fish fed at 5 mg and 10 mg CA diets. The same result was reported in recent study in *C. auratus* increased ACP and Lyz activity when fish fed dietary azadirachtin against *A. hydrophila* [64], dietary andrographolide compound in *L. rohita* [66], dietary curcumin, camphor, and curcumin in *C. mrigala* [13,44] against pathogens. The present result suggested that dietary administration with 5 and 10 mg CA diets may involve to increasing of ACP, ROS production, and lysozyme activity; however, the exact mode of action of the CA in fish unknown.

The accumulative mortality was found 10% in the infected fish fed at 1 and 5 mg $kg^{-1}$ CA supplemented diets whereas it was shown 15% mortality with 10 mg $kg^{-1}$ CA diet. However, the infected fish fed with non-CA supplemented diet was shown 85% mortality in the present study. A similar result was reported in *C. auratus* fed azadirachtin containing diet [64] and *C. mrigala* fed azadirachtin, camphor, and curcumin containing diets against pathogen [13].

Estrogens are generally consider most significant role sex determination and differentiation and it necessary for induction of ovarian morphogenesis and development in fish [76,77]. The ERα mRNA expression was seen high in gonad but less expression in testis of infected fish fed at 5 and 10 mg $kg^{-1}$ CA diets. The ERα mRNA transcript was essentially the same as found in ovary or gonad of *Ictalurus punctatus*, *Oryzias latipes*, *O. niloticus*, and *Sparus aurata* ERα [78–81] but it expression was only detected in testis, liver and heart of in *S. aurata* [79]. To our surprise, we detected very high ERα mRNA expression in the infected fish given at 5 and 10 mg $kg^{-1}$ CA diets as suggested that CA may involve ovarian morphogenesis and development in *C. gariepinus* against *E. tarda*. It was implicit, the ERα mRNA expression was seen very low the infected fish fed without CA supplementation diet.

Gonad development in fish as compared to mammals is controlled by two gonadotropin hormones (GTHs), namely FSH and LH that mainly synthesized in the pituitary gland through gametogenesis and steroidogenesis. The FSH is a glycoprotein hormone produced by basophilic gonadotropin-producing cells (gonadotrophs) and stored in the anterior pituitary gland together with LH. The FSH-β mRNA expression in the testis was high of infected group fed with 5 and 10 mg $kg^{-1}$ CA diets as compared with the group fed diet supplementation with 1 mg $kg^{-1}$ CA or without CA. The FSH-β is already identified in *C. auratus* [82], *Anguilla japonica* [83], and *I. punctatus* [84] mRNA expression is same in the present study. Surprisingly, FSH-β mRNA was highly expressed in testis as compared to gonad in the infected provide at 5 and 10 mg $kg^{-1}$ CA diets as suggested that CA may be stimulate the pituitary gland and secreting gonadotropin hormone like FSH-β. There was no any detailed investigation of herbal and their active constituent on gonadotropin hormones regulation in aquatic organisms.

Lysozymes are a group of catalytic enzymes that hydrolyze between N-acetyl-D-glucosamine and N-acetylmuramic acid sites of 1, 4-beta-linkages peptidoglycan heteropolymers of bacteria cell walls, thus helping as innate immunity to invading pathogens [85,86]. In fish related to lower vertebrates, only c-Lyz and g-Lyz have been reported in different fish species [87,88] which act as catalytic mechanisms and enzymatic activities. The g-Lyz and c-Lyz mRNA expression was observed in high the infected fish provide at 5 and 10 mg $kg^{-1}$ CA diets as compare to uninfected and infected fish fed at 1 mg $kg^{-1}$ CA or without CA diet. This result suggested that the infected fish give at 5 and 10 mg $kg^{-1}$ CA supplementation diets may be influence the hydrolyze of bacterial cell wall of 1, 4-beta-linkages peptidoglycan layer between

N-acetyl-D-glucosamine and N-acetylmuramic acid sites. However, the detailed mechanisms of action of CA in other fish against pathogens are necessary to confirm.

The plant active compound CA used as dietary administration in this study considerably has not affect growth performance and hemato-biochemical parameters, but it enhanced the non-specific immunity and decreased mortality in *C. gariepinus* against *E. tarda*. Further, it influence to enhance or modulate of ERα, FSH-β, c-Lyz, and g-Lyz mRNA expression in testis, gonads, and HK as suggested that CA involved male and female reproductive development and limiting the bacterial infection through hydrolyzing of bacterial cell wall. However, the underlying molecular mechanism besides the timing, dosages, method of administration on health status of the CA in other fish species against different fish pathogen need to be taken into consideration.

## Credit author statement

Sharptooth African catfish, *Clarias gariepinus* (Burchell) is the most important tropical fish species cultured extensively in India its tolerance of extremes adaptability to tropical environmental conditions and high annual revenues, fast growth as well as feed conversion rate, suitability for monoculture and polyculture with other freshwater fish species, ability to withstand handling stress, disease resistance, high fecundity and weight gain, palatability and nutritional quality. At the same time incidences of diseases in catfish culture are increasingly because the increase of culture under crowded condition result huge economic losses; among the bacterial diseases is major problem in tropical catfish culture systems that produce hemorrhagic septicemia by edwardsiellosis by *Edwardsiella tarda* leads to high economic loss. Antibiotics and chemicals are traditionally used to control fish diseases, but it can develop negative impacts such as drug-resistant bacterial strains, toxicity, residues, public health and environment consequences. Recently an interesting alternative approach for immunoprophylactic control measures has been achieved remarkable success using of naturally available herbals, herbal active constituents, and probiotics immune-stimulants that enhance the immune system of fish and confer protection against different pathogens. Cassic acid (CA) is known as rhein of anthraquinone group abundantly present in root and leaf of Rheum, Senna, and Cassia species reported that better anti-bacterial, anti-fungal, anti-viral, anti-tumor, anti-cancer, anti-oxidant, and anti-inflammatory properties. There was no information of CA active constituents in fish on growth, reproductive performance and cytokine genes mRNA expression. Therefore, the present research was conduct to explore as a preliminary study of CA supplementation diet on disease protection, growth response, immunity, mRNA expression of estrogens and FSH-β hormones as well as lysozyme (c- and g-type) enzymes in *C. gariepinus* against *E. tarda*.

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