Article

Effects of Green Alga, *Chaetomorpha aerea* Extract on Non-Specific Immune Responses and Disease Resistance against *Edwardsiella tarda* Infection in *Labeo rohita*

Govindharajan Sattanathan [1], Vairakannu Tamizhazhagan [2], Nadeem Raza [3], Syed Qaswar Ali Shah [4], Muhammad Zubair Hussain [5] and Ki-Hyun Kim [6,*]

[1] Department of Zoology, Sacred Heart Arts and Science College, Perani, Tindivanam Taluk, Tamilnadu 605651, India; sattanathanphd@gmail.com
[2] Department of Zoology, Syed Ammal Arts and Science College, Ramanathapuram, Tamilnadu 623513, India; tamilzoon@gmail.com
[3] Department of Chemistry, Emerson University, Multan 60000, Pakistan; nadeemr890@gmail.com
[4] Department of Zoology, Cholistan University of Veterinary and Animal Sciences, Bahawalpur 63100, Pakistan; syedqaswaralishah@cuvas.edu.pk
[5] Department of Zoology, Emerson University, Multan 60000, Pakistan; zubairhussainpk1@gmail.com
[6] Department of Civil and Environmental Engineering, Hanyang University, 222 Wangsimni-Ro, Seoul 04763, Korea
* Correspondence: kkim61@hanyang.ac.kr

Abstract: The current study evaluated the effects of a methanol extract from *Chaetomorpha aerea* (a green alga) on non-specific immune responses and resistance against *Edwardsiella tarda* infection in *Labeo rohita*. Different doses of the extract (5, 50 and 500 mg/kg of body weight) were injected into the fish intraperitoneally while a control group was injected with 0.2 mL of sterile physiological salt solution. Variations in several immunostimulatory parameters (i.e., neutrophil, serum lysozyme, myeloperoxidase, serum antiprotease, and ceruloplasmin activity), reactive oxygen species (ROS) and reactive nitrogen species (RNS) were assessed after 7, 14, 21, and 28 days of post stimulation. *E. tarda* culture was injected into the fish after 28 days of post stimulation to induce infection to monitor fish mortality within 14 days. Interestingly, all doses of methanolic extract enhanced neutrophil, lysozyme, and myeloperoxidase activity, ROS and RNS, while a dose of 50 mg/kg was the most effective. Fish injected with this optimal dose were also protected against infection with virulent strain of *E. tarda*. The results of the study suggest that *C. aerea* extract is a potential prophylactic agent against bacterial infections in finfish.

Keywords: methanolic extract; immunological parameters; immunostimulant; aquaculture; fish mortality

Citation: Sattanathan, G.; Tamizhazhagan, V.; Raza, N.; Shah, S.Q.A.; Hussain, M.Z.; Kim, K.-H. Effects of Green Alga, *Chaetomorpha aerea* Extract on Non-Specific Immune Responses and Disease Resistance against *Edwardsiella tarda* Infection in *Labeo rohita*. *Appl. Sci.* **2021**, *11*, 4325. https://doi.org/10.3390/app11104325

Academic Editor: Cédric Delattre

Received: 22 March 2021
Accepted: 25 April 2021
Published: 11 May 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Aquaculture has drawn a great deal of attention during the last few decades due to the recognition on its potent role in meeting nutritional requirements, providing employment, and generating foreign trade [1]. Over the years, aquaculture practices have expanded into more diverse ways all across the globe. As a result, the incidence of infectious diseases in farmed fish has also been increased. In line with such situation, research efforts on defense mechanisms against microbial attacks in fish are increasing [2]. Various prophylactic measures (i.e., immunostimulation, vaccination, genetic improvement) and therapies (disinfectants and antibiotics) are in use [3]. As the application of antibiotics has become limited due to widespread incidences of antibiotic resistance and environmental pollution [4], immunostimulants have become promising alternatives for disease management in farmed fish [5]. A chemical, drug or action may be considered as an immunostimulant if it enhances the defense mechanisms or immune responses that improve resistance to diseases [6]. Both specific and non-specific defense mechanisms play crucial roles in control

of microbial fish infections. Fish metabolism in particular depends on non-specific defense mechanisms [7].

Use of plant-derived immunostimulants is increasing in animals including fish nowadays due to fewer side effects, lack of residual problems, greater economic feasibility and ease of administration [8,9]. Several studies have demonstrated an increase in various immunological parameters (e.g., respiratory burst, phagocytic complement and lysozyme activity) and plasma proteins (globulin and albumin) in different fish species after oral administration or intraperitoneal (IP) injection of plant-derived extracts [10].

Nitric oxide (NO) is an important molecule for regulation of immune function and has a direct antimicrobial effect [11]. Extracts of several herbs reportedly increased growth performance and improved immune response against bacterial infection in different fish species including *L. rohita* [12–14]. Natural metabolites produced by algae have been used for the production of bioenergy fuel [15] and removal of pollutants including heavy metals from the aquatic environment [16]. In addition, algal metabolites also have the potential to be used in medicine and pharmaceutics [17]. Fish diseases are one of the most serious constraints that reduce nearly half of the production, thus resulting in economic and food losses in aquaculture [18]. To control infectious diseases in aquaculture, health related measures should be addressed in a clinically proven and locally applicable manner [19]. Ethnoveterinary therapeutic practices, nonspecific immunostimulants, vaccine, probiotics, and prebiotics are the most effective approaches for controlling infectious diseases of fish, apart from management practices, genetically resistant stock and water disinfection [20].

Plant constituents may act on receptors that trigger gene activation, which results in the production of antimicrobial molecules. Methanolic extract of *Chaetomorpha antennina* has been reported to enhance the immunity of *L. rohita* against *E. tarda* infection [21]. *Chaetomorpha aerea* extract is thought to possess several antimicrobial characteristics that may be associated with its immunostimulation properties [22] and has the potential to activate innate defense mechanisms of the fish, either directly or indirectly [23]. Dietary supplement fortified with *C. aerea* extract has been reported to increase the growth of *L. rohita* and increase resistance against bacterial infection caused by *Aeromonas hydrophila* [24].

L. rohita (Rohu) is one of South-East Asia's more important carp species of farmed finfish due to its rapid growth and consumer preference [25]. *E. tarda* causes Edwardsiellosis in several important species used in aquaculture, including chinook salmon, eel, flounder, tilapia, and carps. Edwardsiellosis is characterized by septicemia, necrotic abscesses, abscesses on internal organs, lesions, pigment loss, and enlarged kidneys in fish [26]. According to the best of our knowledge, there are no previous studies on the immunostimulatory effects of IP administration of *C. aerea* methanolic extract and disease resistance against *E. tarda* in *L. rohita*. Therefore, the aim of the current study was to assess the effects of *C. aerea* methanolic extract on non-specific immunostimulatory responses and disease resistance against *E. tarda* infection in *L. rohita*.

2. Materials and Methods

2.1. Experimental Animals

Ninety healthy *L. rohita* specimens with no sign of disease or previous history of parasitic infestation were obtained from a private fish farm, Sakkangudi (11.4201° N, 79.6172° E) in Chidambaram, Cuddalore District, Tamil Nadu, India. Fish were bathed with a 3% sodium chloride solution to eradicate ectoparasites. Fish were acclimatized to experimental conditions in 150 L fiber-reinforced plastic tanks for 15 days prior to experimentation. The tanks were filled with dechlorinated tap water. During acclimatization, the temperature of the tanks was maintained at 28.0 ± 2.0 °C. A natural photoperiod was maintained and tank water was replaced after every three days.

After acclimatization, fish were distributed into 15 tanks, each tank containing six fish specimens. The experiment was conducted in triplicate. During the course of the experiment, fish were fed a balanced diet *ad libitum* as described earlier [27]. Fish with a mean weight of 25.0 ± 0.23 g were reserved for serological assays and disease-resistance

parameters. Fish with a mean weight of 50.0 ± 0.31 g were used for analysis of reactive oxygen species (ROS) and reactive nitrogen species (RNS).

2.2. Preparation of Extract

Methanol extract of *C. aerea* was prepared and employed according to a previously described protocol with slight modifications [28]. In brief, dried and coarse powdered algae were extracted by a cold maceration process that uses solvents with increasing polarity (i.e., petroleum ether, chloroform, ethyl acetate, methanol and 0.25% of chloroform:water [v/v]). Extracts were dried using a rotary vacuum evaporator (Buchi, Switzerland) and then stored at −20 °C until further use. Sterile water was used to reconstitute further dilutions of methanol extract.

2.3. Experimental Design

Reconstituted methanol extract (0.2 mL) was injected into fish in each experimental group (T1, T2 and T3) intraperitoneally with 10-fold increasing doses of 5, 50 and 500 mg/kg body weight, respectively. Controls were injected with 0.2 mL of sterile water. Blood was collected from each fish seven days before treatment and 7, 14, 21 and 28 days after treatment.

2.4. Blood Sampling and Serum Separation

Blood was collected using a 1 mL tuberculin syringe equipped with a 24-gauge needle through the common cardinal vein of fish [29]. To analyze serum, collected blood (200 µL) was stored in serological tubes at −4 °C overnight. Afterward, the blood was centrifuged at 400× g for 10 min. The resulting supernatant (serum) was transferred to sterilized Eppendorf tubes and stored at −20 °C until further analysis.

2.5. Immunological Parameters

2.5.1. Neutrophil Activity

A nitro blue tetrazolium (NBT) assay was adapted to measure neutrophil activity following Stasiacket al. [30] protocol with little modifications. A 100 µL sample of blood was added to each well of a flat-bottom microplate. For adhesion of cells, the microplate was incubated for 1 h at 37 °C. The supernatant was discarded and loaded wells were washed three times using phosphate buffered saline (pH 7.5). Afterward, 0.2% NBT (100 µL) was added to each well and the plate was incubated again for1 h. Methanol (100%) was added to the cells and mixed for 2–3 min. Methanol (70%) was employed to wash the mixture three times and plates were air-dried. Next, 120 µL of 2 N KOH and 140 µL of dimethyl sulfoxide were poured into each well, resulting in blue precipitate of formazone. Optical density (OD) of the mixture was measured using a microplate reader (Systronics, Kolkata, India) at 620 nm.

2.5.2. Serum Lysozyme Activity

Turbidity method was used to estimate serum lysozyme activity [31]. In brief, 0.03% of lyophilized *Micrococcus luteus* was added to 0.05 mM sodium phosphate buffer (PBS) (pH 6.2) and the suspension was treated as substrate. Blood serum of fish (10 µL) was poured into 250 µL of microbial suspension in a microplate, which was incubated for 0.5 and 4.5 min at 22 °C. Absorbance at 490 nm was determined at both time points using a microplate reader. The reduction in absorbance was the difference in absorbance values of the two time points. One unit of lysozyme activity was considered equal to a decrease in absorbance of 0.001 per min.

2.5.3. Myeloperoxidase Activity

Total serum myeloperoxidase activity was assessed following Sahoo et al. protocol [32] with slight modifications. In brief, 10 mL of serum was added to 90 mL of Hank's balanced salt solution without Ca^{2+} or Mg^{2+} in a 96-well microplate to which 25 µL of 20 mM 3,3,5,5-

tetra methyl benzidine hydrochloride (Genei, Bengaluru, India) and 5 mM H_2O_2 were added in a 1:20 dilution and incubated for 2 min. Reaction was stopped by adding 50 µL of 4 M H_2SO_4. A microtiter plate reader (Cyper lab) at 450 nm was used to determine OD.

2.5.4. Ceruloplasmin Activity

To measure ceruloplasmin activity in serum, p-phenylene diamine oxidase activity was utilized as described by Dautremepuits et al. [33] with slight alterations. In brief, 0.5 µL of 1.2 M acetate buffer (pH 5.0) with 0.1% p-phenylene diamine as a substrate was added to 25 µL of serum and shaken well to mix the contents. Similarly, 0.5 mL of 0.5% sodium azide was added to 25 µL of the same serum sample to prepare a blank. The solutions were incubated at 30 °C for 30 min. To stop the reaction, 0.5 µL of 0.5% NaN_3 was added. The quantity of oxidase that catalyzed reduction in absorbance of 0.001 min^{-1} at 550 nm was considered one unit of ceruloplasmin.

2.5.5. Serum Antiprotease Activity

Serum antiprotease activity was measured by employing an assay as described by Rao and Chakrabarti [34]. In brief, 10 µL of fish serum was mixed with 100 mL of bovine trypsin type I and incubated at 25 °C for 30 min. Two blanksamplescontaining 110 µL PBS and three reference samples containing 10 mL PBS/100 mL trypsin were also prepared. The reaction mixtures were incubated for 15 min with 1 µL of casein and the reaction was terminated by adding 500 µL of 10% trichloroacetic acid to each mixture. The mixtures were centrifuged at 10,551× *g* for 5 min and the OD of the supernatants at 280 nm was measured. The following formula was used to calculate percent inhibition:

$$\text{Percent inhibition } (\%) = \frac{\text{OD reference } \times \text{ OD sample}}{\text{OD reference}} \times 100 \quad (1)$$

2.5.6. Preparation of Viable Leukocytes from Peripheral Blood

To separate peripheral blood leukocytes, a 10 mL syringe with 2 mL of a blood-collecting medium (RPMI 1640 supplemented with 50,000 IU/L sodium heparin, 100,000 IU/L penicillin, and 100 mg/L streptomycin) was used. An equal volume of lymphocyte separation medium (Lymphosep, ICN Biomedicals, Inc., Irvine, CA, USA) was mixed with diluted blood. The cells were centrifuged at 800× *g* for 20 min. Leukocytes collected from the interface were washed twice using a wash medium (RPMI 1640 supplemented with 50,000 IU/L sodium heparin, 100,000 IU/L penicillin, and 100 mg/L streptomycin). The washed leukocytes were re-suspended in culture medium (RPMI 1640 supplemented with 3% [*v*/*v*] of pooled tilapia serum, 100,000 IU/L penicillin, 100 mg/L streptomycin and 4 mM/L glutamine, Biochrom AG, Berlin, Germany). The number of viable cells was estimated following the trypan blue exclusion method [35] and adjusted to 4×10^7 cells/mL using a culture medium.

2.6. Reactive Oxygen Species Production

For measurement of intracellular respiratory burst activity, a previously published method [36] with minor modifications was used. 25 µL of NBT (1 g/L) was added to 175 µL of culture medium and incubated at 28 °C with a fixed concentration (1×10^6 cells/well) of peripheral blood leukocytes for 2 h. The supernatants were then discarded and the remaining material with cells was mixed in absolute methanol for 5 min. Wells were washed twice with 125 µL of 70% methanol and the wells were air-dried overnight. Finally, 150 µL of dimethyl sulfoxide and 125 µL of 2 N KOH in each well were used to dissolve reduced NBT (in the form of formazan). OD was found using a microplate reader (at 650 nm).

2.7. Reactive Nitrogen Species Production

Leukocytes in peripheral blood generate NO, which is instantly converted into nitrite. Concentration of this nitrite extract can be estimated calorimetrically by adding a Griess reagent [37]. Briefly, peripheral blood leukocytes were added to 175 µL of culture medium

and incubated for 96 hat 28 °C. A solution of 1% copper sulfate was used to maintain moisture during incubation. 50 µL of culture supernatant was shifted to wells of a new microplate and 50 µL of Griess reagent (1% sulfanilamide, 2.5% phosphoric acid, and 0.1% N-naphthul-ethylenediamine) was added to each well with culture supernatant. After an incubation time of 10 min, the NO_2 concentration (in moles) was calculated following a pre-generated standard curve (formed from a graded series of $NaNO_2$ concentrations in the same culture medium).

2.8. Disease Resistance

To determine disease-resistance capability, *E. tarda* MTCC 2400 (IMTECH, Chandigarh, India) was used. Before initiation of the trial, LD_{50} of the pathogen was determined as 1.2×10^8 colony forming units (cfu) /fish employing a separate fish group. Brain–heart infusion broth was used to culture *E. tarda* for a period of 24 h at 28 °C, and then centrifuged at 3000× *g* for 10 min. The supernatant was discarded and the pellets were re-suspended in sterile PBS. After administration of methanol extracts, the fish were injected with 0.1 mL of *E. tarda* (1.2×10^8 cfu/mL) suspended in PBS intraperitoneally. OD of bacterial culture suspension absorbance was adjusted to 1.2 as it was previously calculated that 0.1 mL of the bacterial culture with an OD of 1.2 at 600 nm has a bacterial count of 1.2×10^8 cfu/mL. Ten fish from each tank of experimental and control groups were used to test resistance against *E. tarda* infection. Mortality of the fish was observed for 14 days. Disease resistance was calculated as relative percent survival (RPS) based on recorded mortality data, using the following formula [38]:

$$RPS = (n-1)\frac{number\ of\ surviving\ fish\ after\ bacterial\ injection}{number\ of\ dead\ fish\ due\ to\ bacterial\ injection} \times 100, \quad (2)$$

where, n = total number of fish.

2.9. Statistical Analyses

The significance of differences between means was evaluated by one-way analysis of variance following Duncan's multiple-range test (at 5% probability level) and using the statistical package SPSS, version 16.

3. Results and Discussion

Fish treated with methanolic extract of *C. aerea* at various doses showed significant ($p < 0.05$) differences for non-specific immune responses by the 7th, 14th, 21st and 28th day after treatment. Findings of the present study indicated that all doses of methanolic extract significantly increased neutrophil, lysozyme, and myeloperoxidase activity, as well as ROS and RNS. Injections of 50 mg/kg of methanolic extract were the most effective in increasing non-specific immune responses in *L. rohita* (Table 1).

3.1. Neutrophil Activity

The neutrophil activity (NBT activity; OD 620 nm) of the treated groups was higher ($p < 0.05$) than that of the controls. It was highest in the T2 group on the 14th and 21st days after treatment. Moreover, neutrophil activity showed a noticeably increasing trend from 7 to 14 days of post-treatment (Figure 1). Similarly, neutrophil activity has been reported to be significantly different in treated groups of *L. rohita* compared with controls, after treatment with methanolic extract of *C. antennina*. The NBT values of 75 mg/kg exhibited significantly higher values compared with controls (Sattanathan, 2020a). Gora, AH et al. (2018), has also reported that the highest and significantly different NBT activity was found in *L. rohita* treated with 2% dietary level of Fucoidan-rich seaweed (*Sargassum wightii*) extract [39].

Table 1. Values (Means ± SE) of various non-specific immune response parameters and relative percent survival of *Labeo rohita*, after treatment with extract of green algae (*Chaetomorpha aerea*) against *Edwardsiella tarda* infection.

Parameter	Groups	Days				
		0 Days	7 Days	14 Days	21 Days	28 Days
Neutrophil Activity	Control	0.098 ^{a} ± 0.013	0.134 ^{bc} ± 0.00632	0.156 ^{d} ± 0.00272	0.143 ^{c} ± 0.0034	0.123 ^{b} ± 0.0014
	T1	0.093 ^{a} ± 0.00033	0.185 ^{c} ± 0.0022	0.187 ^{c} ± 0.00323	0.164 ^{b} ± 0.0035	0.125 ^{b} ± 0.0014
	T2	0.096 ^{a} ± 0.0012	0.166 ^{b} ± 0.0023	0.226 ^{c} ± 0.00214	0.201 ^{a} ± 0.0033	0.143 ^{b} ± 0.00434
	T3	0.091 ^{a} ± 0.00134	0.145 ^{c} ± 0.00343	0.168 ^{c} ± 0.013	0.184 ^{b} ± 0.001854	0.132 ^{b} ± 0.00524
Lysozyme Activity	Control	609.4 ^{c} ± 0.39	565.4 ^{b} ± 0.43	678.3 ^{d} ± 0.582	545.3 ^{b} ± 0.34	464.3 ^{a} ± 0.212
	T1	612.4 ^{a} ± 0.423	745.54 ^{b} ± 0.48	798.45 ^{a} ± 0.743	612.43 ^{a} ± 0.85	600.3 ^{a} ± 0.88
	T2	603.5 ^{b} ± 0.65	689.3 ^{c} ± 0.24	995.35 ^{d} ± 0.17	690.43 ^{bc} ± 0.43	536.4 ^{a} ± 0.34
	T3	603.4 ^{ab} ± 0.64	678.3 b^{c} ± 0.83	789.32 ^{c} ± 0.13	546.43 ^{a} ± 0.54	453.2 ^{bc} ± 0.18
Myeloperoxidase Activity	Control	0.253 ^{a} ± 0.083	0.267 ^{a} ± 0.072	0.467 ^{b} ± 0.068	0.332 ^{a} ± 0.064	0.512 ^{c} ± 0.094
	T1	0.233 ^{a} ± 0.059	0.454 ^{b} ± 0.083	0.716 ^{a} ± 0.0783	0.564 ^{c} ± 0.085	0.703 ^{b} ± 0.084
	T2	0.254 ^{a} ± 0.082	0.503 ^{a} ± 0.074	0.954 ^{e} ± 0.074	0.696 ^{c} ± 0.093	0.605 ^{c} ± 0.0564
	T3	0.234 ^{a} ± 0.0734	0.534 ^{c} ± 0.0833	0.412 ^{bc} ± 0.083	0.624 ^{d} ± 0.0854	0.414 ^{b} ± 0.0824
Ceruloplasmin Activity	Control	0.137 ^{ab} ± 0.0083	0.129 ^{d} ± 0.0082	0.12 ^{a} ± 0.0079	0.139 ^{bc} ± 0.0084	0.119 ^{ab} ± 0.0094
	T1	0.133 ^{a} ± 0.0089	0.149 ^{b} ± 0.0093	0.173 ^{d} ± 0.0083	0.145 ^{b} ± 0.005	0.127 ^{a} ± 0.0034
	T2	0.135 ^{a} ± 0.0012	0.154 ^{ab} ± 0.0074	0.189 ^{b} ± 0.0057	0.138 ^{a} ± 0.0053	0.106 ^{a} ± 0.00634
	T3	0.133 ^{ab} ± 0.0086	0.159 ^{b} ± 0.0089	0.144 ^{b} ± 0.013	0.161 ^{b} ± 0.0077	0.111 ^{a} ± 0.00724
Antiprotease Activity	Control	0.353 ^{bc} ± 0.083	0.327 ^{b} ± 0.055	0.396 ^{d} ± 0.0538	0.395 ^{d} ± 0.0053	0.209 ^{a} ± 0.0084
	T1	0.334 ^{a} ± 0.0089	0.404 ^{bc} ± 0.0043	0.452 ^{c} ± 0.00623	0.365 ^{b} ± 0.05	0.308 ^{a} ± 0.034
	T2	0.332 ^{b} ± 0.008	0.35 ^{b} ± 0.0034	0.546 ^{c} ± 0.033	0.336 ^{b} ± 0.053	0.274 ^{a} ± 0.003
	T3	0.353 ^{a} ± 0.00454	0.367 ^{a} ± 0.0034	0.505 ^{b} ± 0.0153	0.353 ^{a} ± 0.00143	0.307 ^{a} ± 0.00254
Reactive Oxygen Species	Control	0.253 ^{c} ± 0.0123	0.267 ^{b} ± 0.0532	0.467 ^{d} ± 0.0332	0.332 ^{b} ± 0.0432	0.512 ^{a} ± 0.0412
	T1	0.233 b^{c} ± 0.0123	0.454 ^{c} ± 0.043	0.716 ^{d} ± 0.0543	0.564 ^{b} ± 0.035	0.703 ^{c} ± 0.0154
	T2	0.254 ^{a} ± 0.015	0.503 ^{a} ± 0.024	0.954 ^{d} ± 0.0214	0.696 ^{c} ± 0.0143	0.605 ^{b} ± 0.0234
	T3	0.234 ^{b} ± 0.0244	0.534 ^{a} ± 0.0143	0.412 ^{c} ± 0.0213	0.624 ^{d} ± 0.0254	0.414 ^{d} ± 0.0224
Reactive Nitrogen Species	Control	2.53 ^{a} ± 0.203	2.15 ^{b} ± 0.12	3.53 ^{c} ± 0.134	2.151 ^{d} ± 0.156	1.523 ^{d} ± 0.387
	T1	2.65 ^{a} ± 0.21	2.88 ^{b} ± 0.54	4.22 ^{c} ± 0.21	2.04 ^{c} ± 0.23	1.35 ^{c} ± 0.219
	T2	2.54 ^{a} ± 0.25	2.98 ^{b} ± 0.18	6.43 ^{d} ± 0.245	4.32 ^{a} ± 0.383	3.13 ^{c} ± 0.384
	T3	2.43 ^{a} ± 0.084	1.54 ^{c} ± 0.12	3.25 ^{b} ± 0.213	4.33 ^{a} ± 0.215	4.25 ^{b} ± 0.214
Relative Percent Survival	Control	-	-	-	-	58.4 ^{a} ± 4
	T1	-	-	-	-	70.54 ^{b} ± 7
	T2	-	-	-	-	85.3 ^{c} ± 12
	T3	-	-	-	-	60.43 ^{a} ± 16

Mean values with different superscript within a column for a parameter is significantly different ($p < 0.05$); (T1 = 5 mg/kg, T2 = 50 mg/kg, T3 = 500 mg/kg).

3.2. *Lysozyme and Myeloperoxidase Activity*

In the present study, serum lysozyme activity in fish treated with *C. aerea* extract significantly increased from 7 to 21 days ($p < 0.05$) post treatment. Maximum lysozyme activity was found in the T2 group on the 28th day after treatment compared with controls (Figure 2). Similar findings have also been reported in other studies. In one of our previous studies, treatment with methanolic extract of *C. antennina* significantly increased serum lysozyme level in *L. rohita* [24]. It has been reported that Fucoidan-rich seaweed (*Sargassum wightii*) extract at 2% dietary level significantly increased serum lysozyme activity in *L. rohita* [39]. Lysozymes constitute an important part of various defense molecules of

the immune system. They mediate resistance against the microbial attack of gram-positive bacteria [40] as well as gram-negative bacteria [41] by destroying cellular walls of bacteria. Lysozymes split the linkages between the β (1→4) linking N-acetyl muramic acid and N-acetyl glucosamine in gram-positive bacteria's cell walls. In the case of gram-negative bacteria, the lysozymes mediate other enzymes to interrupt the outer cell wall, thus exposing the inner peptidoglycan layer [40,41]. Lysozyme also plays a role to regulate immune cell differentiation and proliferation, and stimulates phagocytic activity [42]. Methanolic extracts of various herbs (i.e., *Ocimum sanctum*, *Myristica fragrans*, and *Withania somnifera* have significantly improved several immune parameters, including serum bactericidal and phagocytic activity, leukocrit and the albumin–globulin ratio, against bacterial infection induced by *Vibrio harveyi* in larvicultured juvenile *Epinephelus tauvina*, a grouper fish species [43]. It has been shown that *L. rohita* cultured on a diet with 0.5% *Achyranthes aspera* seeds exhibit enhanced lysozyme activity [44]. In addition, serum lysozyme activity increased significantly in rainbow trout (*Oncorhynchus mykiss*) cultured on food containing a marine algae, *Dunaliella salina* [45]. Divyagnaneswari and colleagues (2007) used various doses of water extract of *Solanum trilobatum* leaves (4, 40 and 400 mg/kg body weight) intraperitoneally to tilapia (*Oreochromis mossambicus*) and found that a dose of 40 mg/kg significantly increased lysozyme activity on days 4, 6, and 8 post-treatment [11].

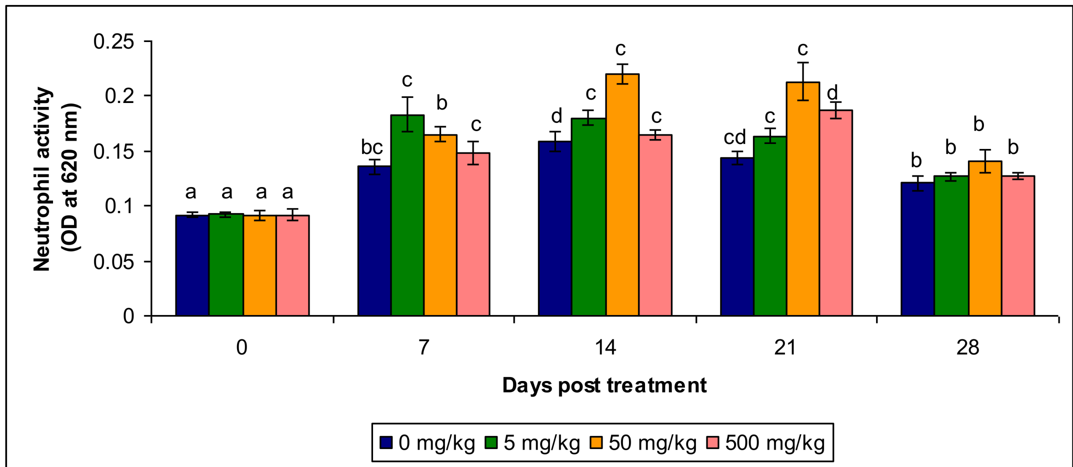

Figure 1. Neutrophil activity (OD at 620 nm) under different treatments administered with various levels of *C. aerea* extract in *L. rohita* during post-treatment days (values are mean ± SE). Mean values with different superscript within a column for a parameter is significantly different ($p < 0.05$).

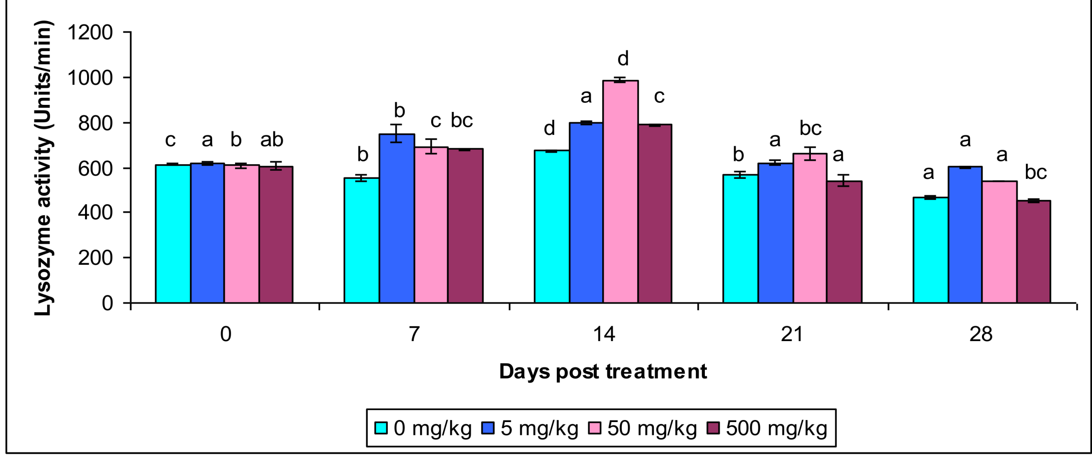

Figure 2. Serum lysozyme activity under different treatments administered with various levels of *C. aerea* extract in *L. rohita* during post treatment days (values are mean ± SE). Mean values with different superscript within a column for a parameter is significantly different ($p < 0.05$).

In the current study, myeloperoxidase activity of the experimental groups varied significantly ($p < 0.05$) compared with the controls (Figure 3). The enhanced myeloperoxidase (MPO) activity was observed in groups treated with 5 mg/kg (T1) and 50 mg/kg (T2) of *C. aerea* extract with the highest in T2 group (Figure 3). Myeloperoxidase has been reported to oxidize macrophages and neutrophil cells after invasion in the fish body [46]. In the study performed by Sattanathan, G 2020a [21] on the effect of methanolic extract of *Chaetomorpha antennina* on non-specific immune response in *Labeo rohita*, MPO activity increased in both 25 and 150 mg/kg of body weight. Previously, it was observed that rainbow trout (*O. mykiss*) treated with methanolic extract of nettle showed greater MPO activity [47]. In the current study, fish receiving methanolic extract of *C. aerea* showed significantly increased respiratory burst activity compared with the control group during the whole trial period. Rao et al. [44] reported an increase in superoxide anion production in leucocytes in *L. rohita* given feed fortified with *Allium sativum* and *Achyranthus aspera*. Production of superoxide anions during the respiratory burst of phagocytes is considered an important factor for limiting fish pathogens growth [48].

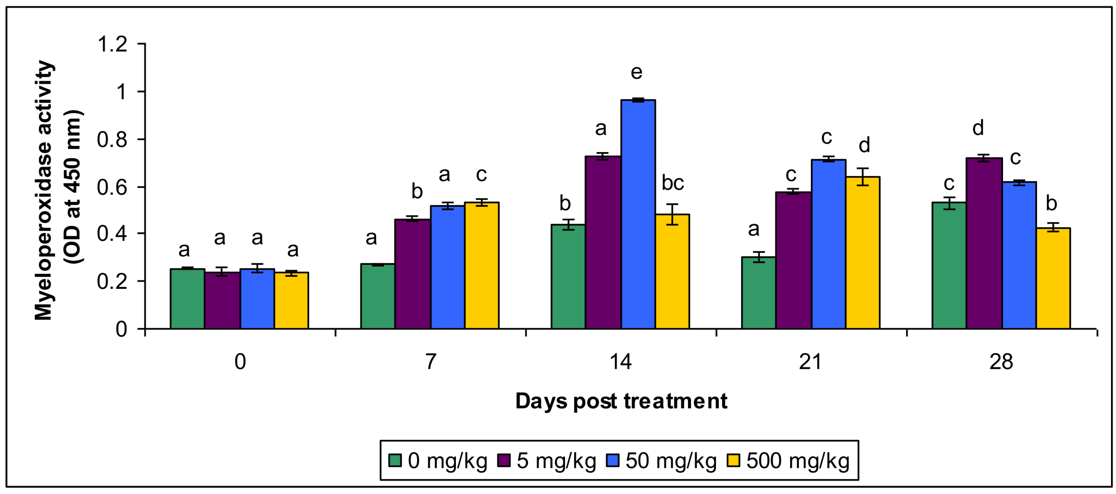

Figure 3. Serum myeloperoxidase activity under different treatments administered with various levels of *C. aerea* extract in *L. rohita* during post treatment days (values are mean ± SE). Mean values with different superscript within a column for a parameter are significantly different ($p < 0.05$).

3.3. Ceruloplasmin and Antiprotease Activity

Fourteen days after treatment, all groups showed relatively higher ceruloplasmin activity compared with the control. T2 group showed the highest ceruloplasmin activity ($p < 0.05$) among all groups. However, on the 7th day after treatment, ceruloplasmin activity was higher in T1 and T2 and lower in T3 (Figure 4). In another similar study, methanolic extract of *C. antennina* also significantly increased ceruloplasmin activity in treatment groups of *L. rohita* after induction of *E. tarda* infection [21]. In a previous study, a significantly positive (0.51) correlation was observed with all the studied immune parameters and survival in *L rohita* against aeromoniasis [49]. The plasma concentrations of several acute phase proteins including ceruloplasmin show a rapid and sharp rise against infections induced by bacterial pathogens. Such non-specific acute phase reactions are considered first-line defenses against pathogenic attack [49]. Ceruloplasmin performs its function in different ways depending on its activities like revamping tissue damage, preventing spread of infectious microbes, and invading pathogens [40].

All groups of fish exposed to *C. aerea* extract showed significantly higher ($p < 0.05$) antiprotease activity relative to controls on the 14th day after treatment. Antiprotease activity was highest in T2 compared with controls (Figure 5). Similarly, in another study, highest level of antiprotease activity, such as trypsin inhibition, was observed in T2 group (75 mg/kg of body weight) of *L. rohita* compared to controls after treatment with methanolic

extract of *C. antennina* [21]. Several antiproteases, including a1 antiprotease, a2 antiplasmin, and a2 macroglobulin, have been documented in fish plasma. These antiproteases have been shown to restrict bacterial invasion and growth in vivo [50]. We found significantly higher antiprotease activity in treatment groups. A higher inhibition (%) due to antiprotease activity was observed in the susceptible line compared with a resistant line, although this difference was not statistically significant [51]. In another study, serum antiprotease activity varied widely in several species of rohu. A negative correlation was found with survival against Edwardsiellosis [52].

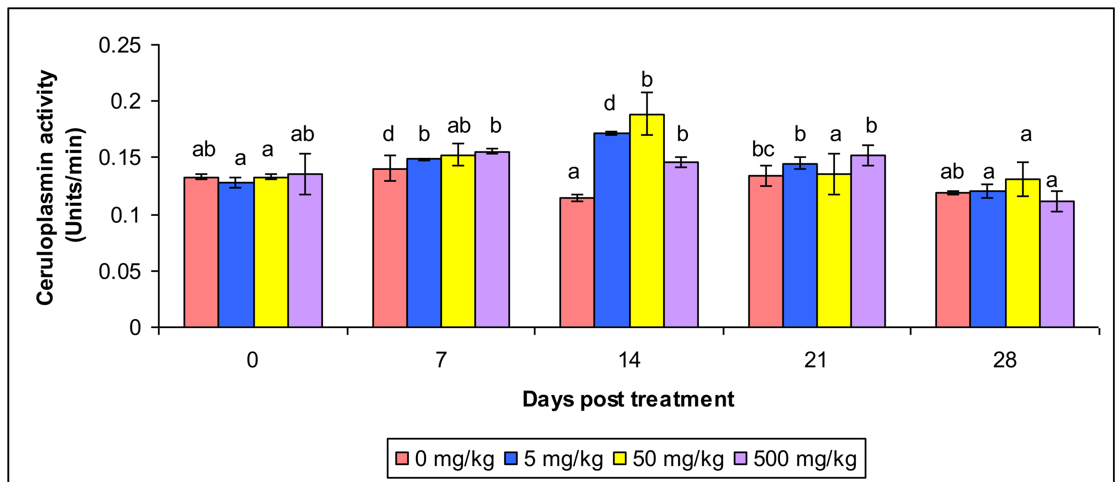

Figure 4. Ceruloplasmin activity under different treatments administered with various levels of *C. aerea* extract in *L. rohita* during post treatment days (values are mean ± SE). Mean values with different superscript within a column for a parameter are significantly different ($p < 0.05$).

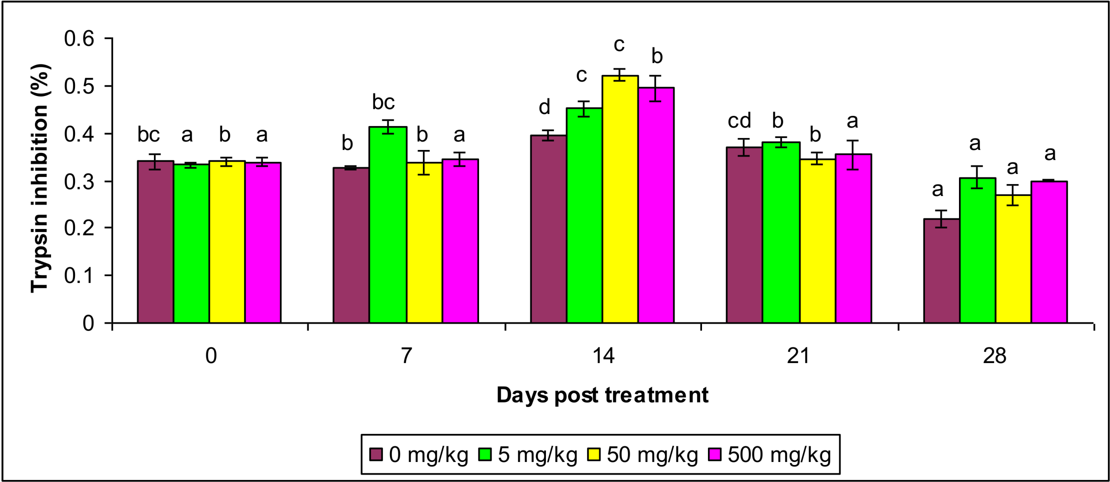

Figure 5. Serum antiprotease activity under different treatments administered with various levels of *C. aerea* extract in *L. rohita* during post treatment days (values are mean ± SE). Mean values with different superscript within a column for a parameter are significantly different ($p < 0.05$).

3.4. Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) Production

ROS production was higher ($p < 0.05$) in *C. aerea*-treated groups compared with controls (Figure 6). T2 fish treated with 50 mg/kg dose of methanolic extract of *C. aerea* showed the highest ROS production at all post-treatment time points, followed by T1 and T3. Similarly, RNS production also showed an increasing trend during the post-treatment period (Figure 7). Increased superoxide anion production in blood leukocytes has been observed in *L. rohita* fed with food containing 0.5% *Achyranthes* [44].

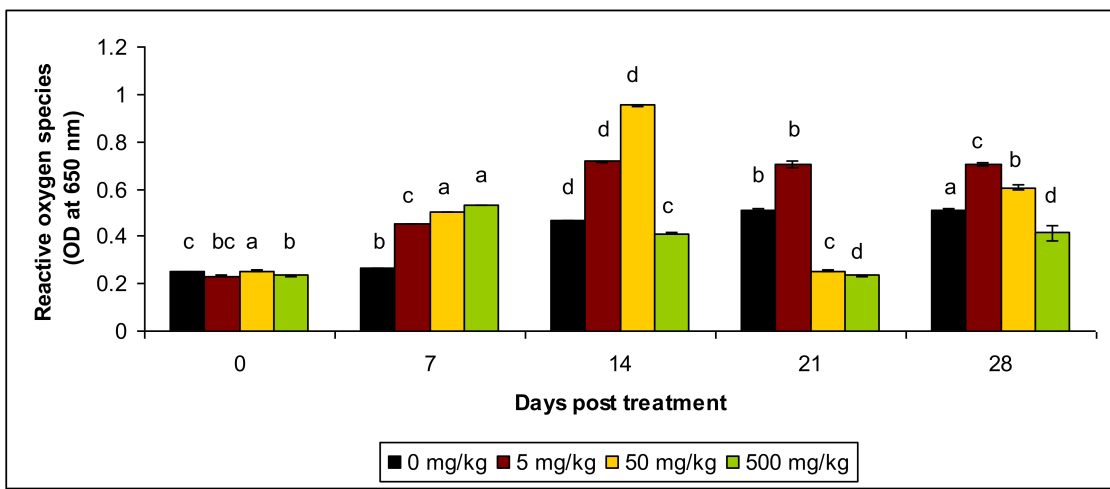

Figure 6. Effect of *C. aerea* extract on the reactive oxygen species production by peripheral blood leukocytes in *L. rohita* (values are mean ± SE). Mean values with different superscript within a column for a parameter are significantly different ($p < 0.05$).

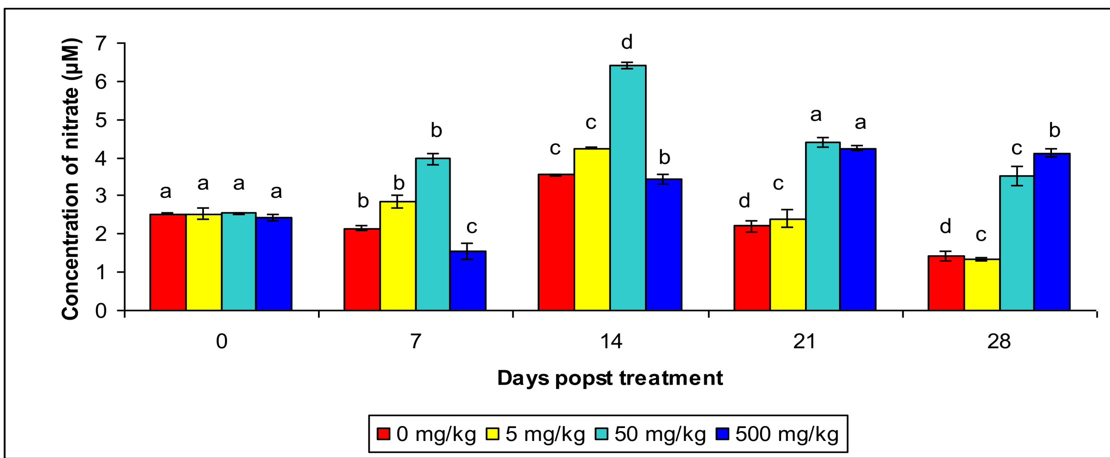

Figure 7. Effect of *C. aerea* extract on the reactive nitrogen species production by peripheral blood leukocytes in *L. rohita* (values are mean ± SE). Mean values with different superscript within a column for a parameter are significantly different ($p < 0.05$).

Respiratory burst, reflected by enhanced oxidation levels of phagocytes after stimulation with foreign agents, is regarded as an important indicator of non-specific immune mechanisms in fish [53]. In vitro treatment of rainbow trout with glycyrrhizin extracted from *Glycyrrhiza glabra* has been reported to increase macrophage levels. This stimulatory effect may be the result of the proliferative responses of leukocytes [54]. In Ergosan-injected rainbow trout, immunostimulatory effects had been suggested due to an increase in respiratory burst activity, the degree of phagocytosis and interleukin expression [55]. The reduction in NBT by intracellular superoxide radicals (produced by leukocytes) can be used to measure respiratory burst activity. It has been reported that treatment with low concentrations of immunostimulants yields higher respiratory burst activity of phagocytes in fish, while higher doses of immunostimulants yield lower NBT values, suppressing defensive mechanisms [56].

3.5. Relative Percent Survival

No mortality was observed up to 24 h after exposing fish to *E. tarda* infection. Fish treated with varied concentrations of methanolic extract of *C. aerea* exhibited higher survival ($p < 0.05$) compared with the control. The highest relative survival (83.5%) was shown in the T2 group, which received 50 mg/kg of algal extract, while it was lowest in the controls

(Figure 8). This may have resulted from an increase in non-specific immune responses. In several previous reports, various herbal medicinal plant extracts (*Cyanodon dactylon*, *Tinospora cordifolia*, *Aegle marmelos*, *Eclipta alba*, and *Picrorhiza kurooa*) exhibited a positive influence on survival rate (74%), and were associated with a decrease in viral load in shrimps infected with the white spot syndrome virus [57]. The results of the study demonstrated that the RPS was strongly correlated with functional assays as well as hematological parameters. An inverse relationship was found between the amount of extract administered and the mortality rate. In another similar study, methanolic extract of *C. antennina* significantly increased survival percentage in *L. rohita* after induction of *E. tarda* infection [21]. It has been found that various doses of fucoidan-rich extract of a seaweed (*Sargassum wightii*, a brown algae) has also reduced mortality in infected *L. rohita* compared to controls [39]. The low mortality rate in fish with algal extract treatment may also be attributed to antimicrobial properties. It has also been reported that intraperitoneal injections of protein-bound polysaccharides from *Coriolus versicolor* reduced mortality rate in *Oreochromis niloticus* against *E. tarda* infection [58]. Screening of antimicrobial activities of extracts from various algal species including *Chnoosporabic analiculata*, *Ulva fasciata*, *Cheilosporum spectabile*, *Bryopsisplumosa*, *Grateloupia filicina*, *C. antennina*, *Hypneapannosa*, *Centroceras clavulatum*, *Portieria hornemannii*, *Gracilaria corticata*, *Sargassum wightii*, *A. orientalis*, *Stocheospermum marginatum*, and *Padinate trastromatica* has been performed.It has been demonstrated that antimicrobial compounds extracted from fresh algae using methanol: toluene (3:1) solvents are more effective due to maintenance of lipophilic nature over wide temperature range (30–60 °C), whereas the extracts from dry algal species showed decreased level of bioactive compounds. Several of the extracts from algae like *Acrosiphonia orientalis* exhibited antibacterial properties against 70% of the microbes analyzed. The extracts from several algal species like *Stocheospermum marginatum* and *Gracilaria corticata* were found to be more effective against Gram-negative bacteria compared to Gram-positive bacteria [59].

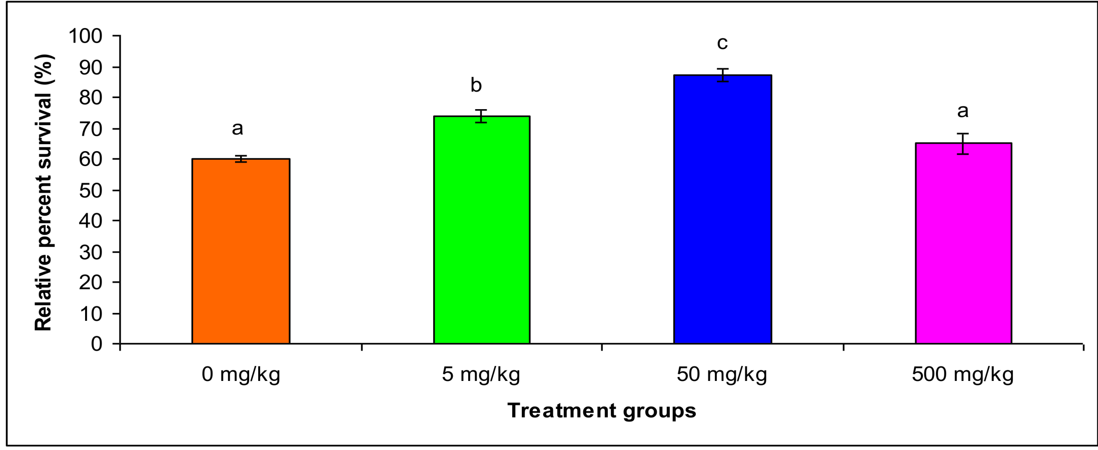

Figure 8. Effect of methanol extract of *C. aerea* algae administered on the percentage mortality in *L. rohita* challenged with virulent *E. tarda* (values are mean ± SE). Mean values with different superscript within a column for a parameter are significantly different ($p < 0.05$).

The findings of the current study suggest a novel insight into the immunostimulation capacity of the methanolic extract of *C. aerea* in *L. rohita* to reduce mortality caused by bacterial infection. Thus, the health of the fish can be improved by the use of ethnoveterinary therapeutic agents like algal extracts which boost the non-specific immune response. The immunostimulants obtained from algal extracts may replace the use of antibiotics in aquaculture. However, as a future perspective, antimicrobial activity of *C. aerea* extract against *E. tarda* needs to be assessed in vitro.

4. Conclusions

Outcomes of the study suggest that the methanolic extract of *C. aerea* exerts a protective effect in *L. rohita* against *E. tarda* infection. This protective effect could be mediated through non-specific immune mechanisms, as evidenced by the enhanced activity of the several non-specific immunostimulatory parameters, including neutrophil, lysozyme, ceruloplasmin, and serum antiprotease activity level, as well as elevated ROS and RNS levels. The results of our study also give a comparative insight into immunomodulatory effects of various doses of *C. aerea* in *L. rohita*. The 50 mg/kg body weight dose of *C. aerea* extract may be optimal, as indicated by increased levels of non-specific immune-response parameters and high survival rates.

The results of the present study suggest that methanolic extract of *C. aerea* administered as an IP treatment to fish could replace antibiotics. The immunostimulants present in *C. aerea* extract have a positive effect on fish health and therefore reduce aquaculture costs.

Author Contributions: Conceptualization, G.S., V.T. and K.-H.K.; methodology, G.S., V.T. and K.-H.K.; software, G.S., V.T. and K.-H.K.; validation, G.S., V.T. and K.-H.K.; formal analysis, G.S., V.T., S.Q.A.S., N.R., M.Z.H. and K.-H.K.; investigation, G.S., V.T., S.Q.A.S., N.R., M.Z.H. and K.-H.K.; resources, G.S., K.-H.K.; data curation, G.S., V.T., S.Q.A.S., N.R., M.Z.H. and K.-H.K.; writing—G.S., V.T., S.Q.A.S., N.R., M.Z.H. and K.-H.K.; writing—review and editing, G.S., V.T., S.Q.A.S., N.R., M.Z.H. and K.-H.K.; visualization, G.S., V.T., S.Q.A.S., N.R., M.Z.H. and K.-H.K.; supervision, K.-H.K.; project administration, K.-H.K.; funding acquisition, K.-H.K. All authors have read and agreed to the published version of the manuscript.

Funding: K.-H.K. acknowledges support made in part by grants from the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT, and Future Planning (No. 2016R1E1A1A01940995). This work was also supported by the Cooperative Research Program for Agriculture Science and Technology Development (Project No. PJ014297), Rural Development Administration, Republic of Korea.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Will be provided on request.

Acknowledgments: The authors would like to express their gratitude to the Dean of Marine Science, CAS in Marine Biology, Annamalai University, Parangipettai, Tamil Nadu, India, for their kind encouragement and provision of all necessary infrastructural facilities for carrying out the present work.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Ye, Y.; Barange, M.; Beveridge, M.; Garibaldi, L.; Gutierrez, N.; Anganuzzi, A.; Taconet, M. FAO's statistic data and sustainability of fisheries and aquaculture: Comments on Pauly and Zeller (2017). *Mar. Policy* **2017**, *81*, 401–405. [CrossRef]
2. Kar, B.; Mohanty, J.; Hemaprasanth, K.P.; Sahoo, P.K. The immune response in rohu, Labeo rohita (Actinopterygii: Cyprinidae) toArgulus siamensis (Branchiura: Argulidae) infection: Kinetics of immune gene expression and innate immune response. *Aquac. Res.* **2013**, *46*, 1292–1308. [CrossRef]
3. Van Hai, N. The use of medicinal plants as immunostimulants in aquaculture: A review. *Aquaculture* **2015**, *446*, 88–96. [CrossRef]
4. Giri, S.; Sukumaran, V.; Sen, S.; Jena, P. Effects of dietary supplementation of potential probiotic Bacillus subtilisVSG1 singularly or in combination with Lactobacillus plantarumVSG3 or/and Pseudomonas aeruginosaVSG2 on the growth, immunity and disease resistance of Labeo rohita. *Aquac. Nutr.* **2013**, *20*, 163–171. [CrossRef]
5. Ninawe, A. DNA vaccination and prophylactic measures in aquaculture health management. *Aquac. Asia* **2006**, *11*, 21.
6. Anderson, D.P. Immunostimulants, adjuvants, and vaccine carriers in fish: Applications to aquaculture. *Annu. Rev. Fish Dis.* **1992**, *2*, 281–307. [CrossRef]
7. Swain, P.; Dash, S.; Sahoo, P.K.; Routray, P.; Sahoo, S.K.; Gupta, S.D.; Meher, P.K.; Sarangi, N. Non-specific immune parameters of brood Indian major carp Labeo rohita and their seasonal variations. *Fish Shellfish. Immunol.* **2007**, *22*, 38–43. [CrossRef] [PubMed]
8. Harikrishnan, R.; Balasundaram, C.; Heo, M.-S. Impact of plant products on innate and adaptive immune system of cultured finfish and shellfish. *Aquaculture* **2011**, *317*, 1–15. [CrossRef]

9. Vaseeharan, B.; Thaya, R. Medicinal plant derivatives as immunostimulants: An alternative to chemotherapeutics and antibiotics in aquaculture. *Aquac. Int.* **2014**, *22*, 1079–1091. [CrossRef]
10. Yuan, C.; Li, D.; Chen, W.; Sun, F.; Wu, G.; Gong, Y.; Tang, J.; Shen, M.; Han, X. Administration of a herbal immunoregulation mixture enhances some immune parameters in carp (Cyprinus carpio). *Fish Physiol. Biochem.* **2007**, *33*, 93–101. [CrossRef]
11. Divyagnaneswari, M.; Christybapita, D.; Michael, R.D. Enhancement of nonspecific immunity and disease resistance in Oreochromis mossambicus by Solanum trilobatum leaf fractions. *Fish Shellfish. Immunol.* **2007**, *23*, 249–259. [CrossRef] [PubMed]
12. Citarasu, T.; Venkatramalingam, K.; Babu, M.M.; Sekar, R.R.J.; Petermarian, M. Influence of the antibacterial herbs, Solanum trilobatum, Andrographis paniculata and Psoralea corylifolia on the survival, growth and bacterial load of Penaeus monodon post larvae. *Aquac. Int.* **2003**, *11*, 581–595. [CrossRef]
13. Prasad, G.; Mukthiraj, S. Effect of methanolic extract of Andrographis paniculata (Nees) on growth and haematology of Oreochromis mossambicus (Peters). *World J. Fish Mar. Sci.* **2011**, *3*, 473–479.
14. Sahoo, P.K.; Mukherjee, S.C. The effect of dietary immunomodulation upon *Edwardsiella tarda* vaccination in healthy and immunocompromised Indian major carp (Labeo rohita). *Fish Shellfish. Immunol.* **2002**, *12*, 1–16. [CrossRef] [PubMed]
15. Shobana, S.; Kumar, G.; Bakonyi, P.; Saratale, G.D.; Al-Muhtaseb, A.H.; Nemestóthy, N.; Bélafi-Bakó, K.; Xia, A.; Chang, J.-S. A review on the biomass pretreatment and inhibitor removal methods as key-steps towards efficient macroalgae-based biohydrogen production. *Bioresour. Technol.* **2017**, *244*, 1341–1348. [CrossRef]
16. Daneshvar, E.; Zarrinmehr, M.J.; Kousha, M.; Hashtjin, A.M.; Saratale, G.D.; Maiti, A.; Vithanage, M.; Bhatnagar, A. Hexavalent chromium removal from water by microalgal-based materials: Adsorption, desorption and recovery studies. *Bioresour. Technol.* **2019**, *293*, 122064. [CrossRef]
17. Pulz, O.; Gross, W. Valuable products from biotechnology of microalgae. *Appl. Microbiol. Biotechnol.* **2004**, *65*, 635–648. [CrossRef]
18. Leung, T.L.F.; Bates, A.E. More rapid and severe disease outbreaks for aquaculture at the tropics: Implications for food security. *J. Appl. Ecol.* **2012**, *50*, 215–222. [CrossRef]
19. Peeler, E.J.; Taylor, N.G. The application of epidemiology in aquatic animal health -opportunities and challenges. *Vet. Res.* **2011**, *42*, 94. [CrossRef]
20. Swaminathan, T.R.; Kumar, R.; Dharmaratnam, A.; Basheer, V.S.; Sood, N.; Pradhan, P.K.; Sanil, N.K.; Vijayagopal, P.; Jena, J. Emergence of carp edema virus in cultured ornamental koi carp, Cyprinus carpio koi, in India. *J. Gen. Virol.* **2016**, *97*, 3392–3399. [CrossRef]
21. Sattanathan, G.; Tamizhazhagan, V.; Padmapriya, S.; Liu, W.-C.; Balasubramanian, B. Effect of Green Algae *Chaetomorpha antennina* Extract on Growth, Modulate Immunity, and Defenses against *Edwardsiella tarda* Infection in *Labeo rohita*. *Animals* **2020**, *10*, 2033. [CrossRef] [PubMed]
22. Newman, D.J.; Cragg, G.M.; Snader, K.M. Natural Products as Sources of New Drugs over the Period 1981−2002. *J. Nat. Prod.* **2003**, *66*, 1022–1037. [CrossRef]
23. Bricknell, I.; Dalmo, R.A. The use of immunostimulants in fish larval aquaculture. *Fish Shellfish. Immunol.* **2005**, *19*, 457–472. [CrossRef] [PubMed]
24. Sattanathan, G.; Palanisamy, T.; Padmapriya, S.; Arumugam, V.A.; Park, S.; Kim, I.H.; Balasubramanian, B. Influences of dietary inclusion of algae Chaetomorpha aerea enhanced growth performance, immunity, haematological response and disease resistance of Labeo rohita challenged with Aeromonas hydrophila. *Aquac. Rep.* **2020**, *17*, 100353. [CrossRef]
25. Talwar, P.K. *Inland Fishes of India and adjacent Countries*; CRC Press: New Delhi, India, 1991; Volume 2.
26. Plumb, J. Edwardsiella Septicaemias. In *Viral, Bacterial and Fungal Infections*; Woo, P.T.K., Bruno, D.W., Eds.; Fish Disease and Disorders Volume 3; CABI Publishing: Wallingford, UK, 1999.
27. Sattanathan, G.; Sournamanikam, V. Cow urine distillate as an eco safe and economical feed additive for enhancing growth, food utilization and survival rate in rohu, labeo rohita (Hamilton). *J. Fish. Aquat. Sci.* **2017**, *12*, 241–252. [CrossRef]
28. Kirubakaran, C.J.W.; Alexander, C.P.; Michael, R.D. Enhancement of non-specific immune responses and disease resistance on oral administration of Nyctanthes arbortristis seed extract in Oreochromis mossambicus (Peters). *Aquac. Res.* **2010**, *41*, 1630–1639. [CrossRef]
29. Dinakaran Michael, R.; Srinivas, S.; Sailendri, K.; Muthukkaruppan, V. A rapid method for repetitive bleeding in fish. *Indian J. Exp. Biol.* **1994**, *32*, 838.
30. Stasiack, A.; Bauman, C. Netrophil activity as a potential indicator immunomodulator Ecteinascida turbinata extract on Edwardsiella ictaluri infection of channel catfish. *J. Aquat. Anim. Health* **1996**, *7*, 141–146.
31. Parry, R.M., Jr.; Chandan, R.C.; Shahani, K.M. A Rapid and Sensitive Assay of Muramidase. *Proc. Soc. Exp. Biol. Med.* **1965**, *119*, 384–386. [CrossRef]
32. Sahoo, P.; Rauta, P.; Mohanty, B.; Mahapatra, K.; Saha, J.; Rye, M.; Eknath, A. Selection for improved resistance to Aeromonas hydrophila in Indian major carp Labeo rohita: Survival and innate immune responses in first generation of resistant and susceptible lines. *Fish Shellfish. Immunol.* **2011**, *31*, 432–438. [CrossRef]
33. Dautremepuits, C.; Betoulle, S.; Paris-Palacios, S.; Vernet, G. Humoral immune factors modulated by copper and chitosan in healthy or parasitised carp (*Cyprinus carpio* L.) by Ptychobothrium sp. (Cestoda). *Aquat. Toxicol.* **2004**, *68*, 325–338. [CrossRef]
34. Rao, Y.V.; Chakrabarti, R. Enhanced anti-proteases in Labeo rohita fed with diet containing herbal ingredients. *Indian J. Clin. Biochem.* **2004**, *19*, 132–134. [CrossRef]
35. Strober, W. Trypan blue exclusion test of cell viability. *Curr. Protoc. Immunol.* **1997**, *21*, A.3B.1–A.3B.2.

36. Secombes, C.J. Isolation of salmonid macrophages and analysis of their killing activity. *Tech. Fish Immunol.* **1990**, *1*, 137–154.
37. Palić, D.; Andreasen, C.B.; Menzel, B.W.; Roth, J.A. A rapid, direct assay to measure degranulation of primary granules in neutrophils from kidney of fathead minnow (Pimephales promelas Rafinesque, 1820). *Fish Shellfish. Immunol.* **2005**, *19*, 217–227. [CrossRef]
38. Amend, D.F. Potency testing of fish vaccines. *Fish Biol. Serodiagn. Vaccines* **1981**, *49*, 447–454.
39. Gora, A.H.; Sahu, N.P.; Sahoo, S.; Rehman, S.; Dar, S.A.; Ahmad, I.; Agarwal, D. Effect of dietary Sargassum wightii and its fucoidan-rich extract on growth, immunity, disease resistance and antimicrobial peptide gene expression in Labeo rohita. *Int. Aquat. Res.* **2018**, *10*, 115–131. [CrossRef]
40. Salton, M.R.J.; Ghuysen, J.M. The structure of di- and tetra-saccharides released from cell walls by lysozyme and streptomyces F1 enzyme and the β (1→4) N-acetylhexosaminidase activity of these enzymes. *Biochim. Biophys. Acta Bioenerg.* **1959**, *36*, 552–554. [CrossRef]
41. Glynn, A.A. The complement lysozyme sequence in immune bacteriolysis. *Immunology* **1969**, *16*, 463–471.
42. Ghiasi, F.; Mirzargar, S.S.; Badakhshan, H.; Shamsi, S. Leukocyte Count and Phagocytic Index in Cyprinus carpio under the Wintering Conditions. *J. Fish. Aquat. Sci.* **2010**, *5*, 113–119. [CrossRef]
43. Sivaram, V.; Babu, M.; Immanuel, G.; Murugadass, S.; Citarasu, T.; Marian, M. Growth and immune response of juvenile greasy groupers (Epinephelus tauvina) fed with herbal antibacterial active principle supplemented diets against Vibrio harveyi infections. *Aquaculture* **2004**, *237*, 9–20. [CrossRef]
44. Rao, Y.V.; Das, B.; Jyotyrmayee, P.; Chakrabarti, R. Effect of Achyranthes aspera on the immunity and survival of Labeo rohita infected with Aeromonas hydrophila. *Fish Shellfish. Immunol.* **2006**, *20*, 263–273. [CrossRef]
45. Amar, E.C.; Kiron, V.; Satoh, S.; Watanabe, T. Enhancement of innate immunity in rainbow trout (Oncorhynchus mykiss Walbaum) associated with dietary intake of carotenoids from natural products. *Fish Shellfish. Immunol.* **2004**, *16*, 527–537. [CrossRef]
46. Amat-García, E.C.; Amat-García, G.D.; Henaom, L.G. Diversidad taxonómica y ecológica de la entomofauna micófaga en un bosque altoandino de la cordillera oriental de Colombia. *Ecología* **2004**, *28*, 223–231.
47. Bilen, S.; Ünal, S.; Güvensoy, H. Effects of oyster mushroom (Pleurotus ostreatus) and nettle (Urtica dioica) methanolic extracts on immune responses and resistance to Aeromonas hydrophila in rainbow trout (Oncorhynchus mykiss). *Aquaculture* **2016**, *454*, 90–94. [CrossRef]
48. Olivier, G.; Evelyn, T.; Lallier, R. Immunity to aeromonas salmonicida in coho salmon (oncorhynchus kisutch) inducfd by modified freund's complete adjuvant: Its non-specific nature and the probable role of macrophages in the phenomenon. *Dev. Comp. Immunol.* **1985**, *9*, 419–432. [CrossRef]
49. Sahoo, P.; Mahapatra, K.D.; Saha, J.; Barat, A.; Sahoo, M.; Mohanty, B.; Gjerde, B.; Ødegård, J.; Rye, M.; Salte, R. Family association between immune parameters and resistance to Aeromonas hydrophila infection in the Indian major carp, Labeo rohita. *Fish Shellfish. Immunol.* **2008**, *25*, 163–169. [CrossRef]
50. Ellis, A. Innate host defense mechanisms of fish against viruses and bacteria. *Dev. Comp. Immunol.* **2001**, *25*, 827–839. [CrossRef]
51. Pradhan, J.; Das, S.; Das, B.K. Antibacterial activity of freshwater microalgae: A review. *Afr. J. Pharm. Pharmacol.* **2014**, *8*, 809–818.
52. Mohanty, B.; Sahoo, P.; Mahapatra, K.; Saha, J. Innate immune responses in families of Indian major carp, Labeo rohita, differing in their resistance to *Edwardsiella tarda* infection. *Curr. Sci.* **2007**, *92*, 1270–1274.
53. Miyazaki, T. A Simple Method to Evaluate Respiratory Burst Activity of Blood Phagocytes from Japanese Flounder. *Fish Pathol.* **1998**, *33*, 141–142. [CrossRef]
54. Jang, S.I.; Marsden, M.J.; Secombes, C.J.; Choi, M.S.; Kim, Y.G.; Kim, K.J.; Chung, H.-T. Effect of glycyrrhizin on rainbow trout Oncorhynchus mykiss leukocyte responses. *J. Korean Soc. Microbiol.* **1998**, *33*, 263–271.
55. Peddie, S.; Zou, J.; Secombes, C.J. Immunostimulation in the rainbow trout (Oncorhynchus mykiss) following intraperitoneal administration of Ergosan. *Vet. Immunol. Immunopathol.* **2002**, *86*, 101–113. [CrossRef]
56. Sakai, M. Current research status of fish immunostimulants. *Aquaculture* **1999**, *172*, 63–92. [CrossRef]
57. Citarasu, T.; Sivaram, V.; Immanuel, G.; Rout, N.; Murugan, V. Influence of selected Indian immunostimulant herbs against white spot syndrome virus (WSSV) infection in black tiger shrimp, Penaeus monodon with reference to haematological, biochemical and immunological changes. *Fish Shellfish. Immunol.* **2006**, *21*, 372–384. [CrossRef] [PubMed]
58. Park, K.H.; Do Jeong, H. Enhanced resistance against *Edwardsiella tarda* infection in tilapia (Oreochromis niloticus) by administration of protein-bound polysaccharide. *Aquaculture* **1996**, *143*, 135–143. [CrossRef]
59. Jacobson, D.M.; Andersen, R.A. The discovery of mixotrophy in photosynthetic species of Dinophysis (Dinophyceae): Light and electron microscopical observations of food vacuoles in Dinophysis acuminata, D. norvegica and two heterotrophic dinophysoid dinoflagellates. *Phycologia* **1994**, *33*, 97–110. [CrossRef]