

# Immuno-antioxidant Defense of Partially Purified *Rhizophora mucronata* in Clownfish (*Amphiprion sebae*) against *Vibrio alginolyticus*

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## Abstract

Dietary administration of *Rhizophora mucronata* (RM) enhance the growth response, innate immunity for example the activity of lysozyme (LYZ), alternative complement (ACP), respiratory burst (RB), and phagocytosis (PC), and the protective mechanisms like survival rate and mortality; antioxidant activities such as catalase (CAT), glutathione (GSH), malondialdehyde (MDA), nitric oxide (NO), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and glutathione peroxidase (GPx) are reported in clownfish, *Amphiprion sebae* against *Vibrio alginolyticus*. There are five experimental groups: (1) uninfected or (2) infected groups fed basal control diet without RM extract; infected or challenged fish fed at (3) 1 g kg<sup>-1</sup>, (4) 5 g kg<sup>-1</sup>, and (5) 10 g kg<sup>-1</sup> RM extracts enriched diets used in this study. After dietary administration with 5 and 10 g kg<sup>-1</sup> of RM extract the weight gain (WG), feed conversion ratio (FCR), and growth rate (SGR) increased significantly on weeks 4 and 8. The LYZ, RB, ACP, and PC activities increased significantly in infected fish treated at 5 and 10 g kg<sup>-1</sup> RM extract diets on weeks 4 and 8. A high rate survival or cumulative mortality was like 80% and 85% (20% and 15%) were found in the infected fish treated at 10 and 5 g kg<sup>-1</sup> diets, although the survival rate was 15% and 70% with 1 g kg<sup>-1</sup> or 0 (control) diets. This study confirm that 5 or 10 g kg<sup>-1</sup> of RM extract as dietary supplementation provide positive growth, boost the antioxidant and non-specific immune response in *A. sebae* to *V. alginolyticus* infection.

**Keywords:** Disease resistance, Innate immune parameter, Mortality, *Rhizophora mucronata*, *Vibrio alginolyticus*

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has been criticized negative effects such as tissue residues, drug resistance pathogens, and immunosuppression [2], besides the adverse side effects on the host [3]. Alternatively, for some diseases against specific pathogens, vaccines have been provided with varying degrees of accomplishment. The prevalence of a wide range of pathogens in fish farms also restrictions the efficacy of vaccines [4]. Hence, to promote sustainable fish culture there is a serious necessity to look into novel and eco-friendly disease defensive measures. Due to these reasons, in recent years, considerable attention has been attentive to find out alternatives, such as herbal and new traditional therapies in fish disease management.

Innate (non-specific) immune response in fish is considered initial defense mechanisms against pathogens. Additionally, pollutants are adversely affects the fish ability to protect them against pathogens. In this line, the use of herbal medicines, as immunostimulants in aquaculture has expected increasing attention last few decades, not only as immunomodulators but also enhancing the growth circumventing the side effects of traditional chemotherapy [5]. Herbal immunostimulants enhance immunity and decrease mortality against parasitic, bacterial, viral, and fungal infections in fishes [5-7]; herbs have the potential to enhance the proliferation of leukocyte cells and trigger phagocytic activity which is the most significant cellular mechanisms of the innate immune system of fish [8]. The phagocytic activity is a primeval defense mechanism and constitutes an essential characteristic of the non-specific immune system [5].

## Introduction

In 2008, the ornamental fish industry was export and import valued were US\$337 and US\$287 million. Among the various marine ornamental species, the fishes have a tremendous economic potential with a trade value of US\$43.8 million in 2006 [1]. Among, *Amphiprion sebae*, also known as the sebae clownfish originate from the northern Indian Ocean of Java to the Arabian Peninsula in association with sea anemones. This ornamental fish hit high market price and valuable foreign exchange than most other pomacentrids in the marine aquarium fish trade. Recently, the ornamental fish industry has been hit by out breaks of various diseases; among these, the most common infections are caused by Gram-negative organisms and are challenging in aquaculture production [1]. Antibiotics traditionally used to control or reduced the growth of bacteria long enough for the immune system. However, the use of antibiotics and chemotherapeutics under intensive aquaculture as prophylaxis and treatment

In mangrove ecosystem, *Rhizophora mucronata* is one of the common trees. Phytochemical analyses of the plant bark and other parts of this tree reveal content of alkaloids, hydrolysable tannins, terpenoids, sterols, saponins, flavonoids, proteins, anthocyanidins, carbohydrates, carotenoids, gibberellins, inositols, lipids, minerals, polysaccharides, polyphenols, procyanidins, and phenol acid. These bioactive compounds exhibit anti-viral, anti-bacterial, anti-inflammatory, anti-oxidant, activities, boost immune system, etc. *R. mucronata* has been used in the treatment of heamaturia, diabetes, diarrhea and has anti-HIV activity. Further, it presents the following properties astringent, anti-oxidant, anti-septic, hemostatic with anti-ulcerogenic, hepatoprotective, anti-bacterial, and anti-inflammatory activities [9-15].

Clownfish, *Amphiprion sebae* has been affected by bacterial disease like *Vibrio alginolyticus* leading to mass mortality [16,17]. Till date, there was no data available on the effect of dietary administration of *R. mucronata* on growth response, antioxidant activity, immune response, and disease resistance in aquatic species. Hence, the present study investigated first time on the effect of dietary augmentation of *R. mucronata* on growth response, non-specific response, antioxidant activity, and disease control in *A. sebae* to *V. alginolyticus*.

## Material and Methods

### Preparation of plant extraction

*Rhizophora mucronata* (RM) leaf were collected around the Vellar estuary mangroves, Parangipettai, Tamil Nadu, India (Latitude. 11° 29'N. Longitude. 79° 46'E). The leaves were cleaned with double distilled water and shadow desiccated for one week. The dried leaf were powdered in a automatic grinder, separated, and then preserved in an air tight vessel until used. A 100 g of dried leaf powdered sample were consecutively excavated with 85% ethanol. Then, it was filtered and performed by a cold marinating process with agitation twice a day for a period of seven days. The ethanol solvent was vaporized by a rotary vacuum evaporator (Buchi, Flawil, Switzerland). The residues were collected and stored at -20 °C until used for feed formulation.

### *Vibrio alginolyticus*

*Vibrio alginolyticus* (AUMOF2) strain was isolated from infected *Amphiprion sebae* and maintained on Zobell Marine Agar (ZMA, Himedia, Mumbai) in slopes at 5 °C [18]. The pathogenesis of *V. alginolyticus* was regularly confirmed by inoculation into *A. sebae*. The stock culture was preserved on Zobell Marine Broth (ZMB, Himedia, Mumbai) at -70 °C in 0.85% NaCl with 20% glycerol (v/v) which affords constant inoculate throughout the experiment. For challenge study, *V. alginolyticus* culture was centrifuged at 1000 x g for 10 min at 4 °C and then supernatant removed. The bacterial pellet was sterilized with phosphate-buffered saline (PBS) at pH 7.4 three times and re-suspended with PBS. The optical density (O.D.) of the bacterial solution was adjusted to 0.5 at 600 nm which is equal to 2.7 x 10<sup>7</sup> cells ml<sup>-1</sup>. The identification of *V. alginolyticus* was done by growing onto Tiosulphate Citrate Bile salt Sucrose (TCBS, Difco™) agar plates, selective to vibriaceae for 24 h at 30 °C and then isolated on Tryptone Soy Agar (TSA) to obtain a pure culture for identification using API 20E kit (Biomeriux™). The morphological features of bacteria and biochemical tests were confirmed by Bergey's manual of systematic bacteriology [19].

### Formulated diet preparation

The formulated diets and the proximate composition of the basal control diet were described in Table 1. All ingredients were evenly

mixed with oil and then added required water until rigid dough, and then extruded through a pellet extruder. From the basal diet, there are four experimental diets were prepared namely: (i) 0g (control basal diet without RM extract), and dietary enriched at (ii) 1, (iii) 5, and (iv) 10 g kg<sup>-1</sup> of RM extracts into the basal diet. The diets were shadow-dried, broken up, sieved into pellets, and stored in plastic bags at 4 °C until use. The control basal diet was added with the same volume of ethanol solvent without RM extract.

Ingredients (g/100 g)	0g	1g	5g	10g
Shrimp meal	52	52	52	52
Mytha powder	10	10	10	10
Ground net oil cake	22	22	22	22
Rice bran	10	9	5	0
Fish liver oil	2	2	2	2
Vitamina & Mineralb complex	1	1	1	1
Herbal extract	0	1	5	10
Proximate composition				
Crude protein	41.2	41.6	42.1	42.2
Crude lipid	7.4	7.3	7.3	7.2
Crude ash	7.6	7.7	7.7	7.8
Moisture	7	7.1	7.1	7.2
Fibre	3	3	3	2
<sup>a</sup> Vitamine premix (mg kg <sup>-1</sup> diet): thiamin, 25; riboflavin, 45; pyridoxine HCl, 20; vitamin B12, 0.1; vitamin K3, 10; inositol, 800; pantothenic acid, 60; niacin acid, 200; folic acid, 20; biotin, 1.20; retinol acetate, 32; cholecalciferol, 5; choline chloride, 2500; wheat middling, 18.52 g kg <sup>-1</sup> diet. <sup>b</sup> Mineral premix (mg kg <sup>-1</sup> diet): MgSO <sub>4</sub> •7H <sub>2</sub> O, 1200; CuSO <sub>4</sub> •5H <sub>2</sub> O, 10; ZnSO <sub>4</sub> •H <sub>2</sub> O, 50; FeSO <sub>4</sub> •H <sub>2</sub> O, 80; MnSO <sub>4</sub> •H <sub>2</sub> O, 45; CoCl <sub>2</sub> (1%), 50; Na <sub>2</sub> SeO <sub>3</sub> (1%), 20; Ca(IO <sub>3</sub> ) <sub>4</sub> (1%), 60.				

**Table 1:** Dietary ingredients of the formulated fish feed for *Amphiprion sebae*.

### Experimental fish

Clownfish (22.6 ± 2.3 g in weight) both sex have been purchased from Centre of Advanced Study in Marine Biology fish hatchery, Annamalai University of Parangipettai, India. The fish health were immediately check and stocked in 5000 l cement tanks containing 2500 l of UV treated seawater and provided aeration by an air stone throughout the experiment. Fish were acclimatization two weeks before experiment commencement. Fish were provided *ad libitum* twice daily at 5% formulated basal control diet (Table 1) at 09:00 h and 16:00 h. About 70% of water was exchanged once in two days during the experimental period. During the acclimatization period was observed no clinical signs. The water quality parameters such as temperature 28.8 °C ± 1.6, pH 8.3 ± 0.6, salinity 28.7 ± 1.8 ppt, dissolved oxygen concentration 5.7 ± 0.6 mg l<sup>-1</sup>, and photoperiod 14 h light : 10 h dark were recorded during the experiment. The fecal matter and unfed feed were tapped out daily to reduce ammonia content in water.

### Experimental design and challenge study

Fish were randomly distributed into five groups of 25 fish each in three replicate (5 x 25 x 3 = 375) such as: (1) uninfected or (2) infected groups fed basal control diet without RM extract; infected or challenged fish fed at (3) 1 g kg<sup>-1</sup>, (4) 5 g kg<sup>-1</sup>, and (5) 10 g kg<sup>-1</sup> RM extract enriched diets. After two weeks post-supplementation feeding, all groups except control group, were injected intramuscularly (i.m.) with 0.1 ml PBS containing *V. alginolyticus* (2.7 x 10<sup>7</sup> cells ml<sup>-1</sup>) whereas the control group were received the same volume of PBS alone. The LD<sub>50</sub> of the pathogen was accomplished from our earlier study [18]. The use

of goldfish for this study was approved by an official Ethics Committee of Annamalai University, Tamil Nadu, India.

## Growth performance

Fish were collected from each experimental tank at the end of weeks 1, 2, 4, and 8 and weighed out individually. The growth response and survival were calculated using the following formula: specific growth rate (SGR) =  $100 \times (\text{Final weight} - \text{Initial weight}) / (\text{Total duration of experiment})$ ; feed conversion ratio (FCR) =  $\text{Feed given (dried weight)} / \text{Weight gain (wet weight)}$ ; survival rate (%) =  $(\text{Total number of fish in experimental group}) / (\text{Total number of fish in control group}) \times 100$ .

## Preparation of samples

Six fish from each tank were randomly sampled at 1, 2, 4, and 8 weeks of post-supplementation feeding. About 0.5 ml of blood was collected from each fish through caudal vein puncturing using a 1 ml syringe with 23-gauge needle after euthanized buffered tricaine ( $200 \text{ mg l}^{-1}$  tricaine +  $400 \text{ mg l}^{-1}$  sodium bicarbonate) and equally divided into sterile microtubes (Eppendorf) containing with or without anticoagulant. The samples without anticoagulant were allowed to clot for 1 h at room temperature (RT) and then incubated 4 h at  $4^\circ\text{C}$ . Then, it was centrifuged at  $1500 \times g$  for 5 min at  $4^\circ\text{C}$  and collected serum to preserve at  $-20^\circ\text{C}$  until used for immunological tests.

## Hematology

The blood samples with anticoagulant were diluted and incubated 5 min with Hayem's fluid for complete haemolysis of white blood cells (WBCs) then loaded on the improved Neubauer haemocytometer by holding the pipette at  $45^\circ$  angle and the cover slip was placed. The blood cells were calculated and conveyed as cells  $\text{ml}^{-1}$ .

## Preparation of leukocytes

The leukocytes were separated from the peripheral blood according to MacArtain et al. [20]. The blood samples were mixed with 2 ml of Roswell Park Memorial Institute (RPMI) 1640 medium (RPMI 1640, Gibthai) and then prudently placed onto 3 ml of Histopaque (Sigma) in a sterile 15 ml tube. After, the tubes were centrifuged at  $400 \times g$  for 30 min at RT, then the white buffy coat of leukocytes cells floated on the top of the Histopaque. Impervious interfaces were discreetly extracted with a Pasteur pipette and transferred into a new sterile clean 15 ml tubes and added PBS in each tube to attain 6 ml then gently mixed by aspiration. Then, it was centrifuged three times at  $250 \times g$  for 10 min to remove any residual of Histopaque. The isolated leukocytes cells were then re-suspended with PBS and adjusted to the required cell numbers for phagocytic and respiratory burst activities.

## Immunological assays

The lysozyme (LYZ) activity was examined by Parry et al. [21] and the phagocytic (PC) activity assay was studied after improved from Yoshida and Kitao [22]. The phagocytic index (PI) was calculated as: Average number of beads per cell divided by number of phagocytizing cells. The respiratory burst (RB) activity is estimated in the peripheral blood leucocytes by the improved method of Secombes [23]. The alternative complement pathway (ACP) and bactericidal activity were investigated by Yanno [24] and Kampen [25].

## Antioxidant parameters

Liver and gills were dissected out and immediately washed in ice-cold saline. About 200 mg of liver and gills were taken in each fish and add cold PBS (pH 7.4) for grounded in glass homogenizer (pellet

pestle motor). The homogenate samples were then centrifuged at  $4000 \times g$  for 15 min at  $4^\circ\text{C}$ . The obtained supernatant samples were aliquot and preserved at  $-80^\circ\text{C}$  for oxidative stress and antioxidant enzyme study. Serum total antioxidant capacity (TAC) was estimated by the following method of Koracevic et al. [26] which accomplished by the reaction of antioxidants in the samples with a newly prepared exogenous  $\text{H}_2\text{O}_2$  residual measured calorimetric by enzymatic reaction. The glutathione peroxidase (GPx), malondialdehyde (MDA), and nitric oxide (NO) levels were determined in the tissue homogenate by spectrophotometric which enunciated as U/g tissue, nmol/g tissue, and  $\mu\text{mol l}^{-1}$ . Catalase (CAT) level was measured by the decrease of  $\text{H}_2\text{O}_2$  concentration at 240 nm [27]. The reduced glutathione (GSH) is analysis by Beutler et al. [28] and the malondialdehyde (MDA) concentration (nmol  $\text{ml}^{-1}$  of extract) measured by Ohkawa et al. [29]. The hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) is analysis by Aebi [27].

## Cumulative mortality

The bacteria culture, cell count, challenge study were same mentioned previously. To conduct the cumulative mortality, a group of 20 fish in each experimental group was injected i.m. with 0.1 ml of PBS containing *V. alginolyticus* ( $2.7 \times 10^7$  cells  $\text{ml}^{-1}$ ) except control group. The control group received 0.1 ml of PBS alone to each fish. The post-survival rate was calculated for 30 days of injection.

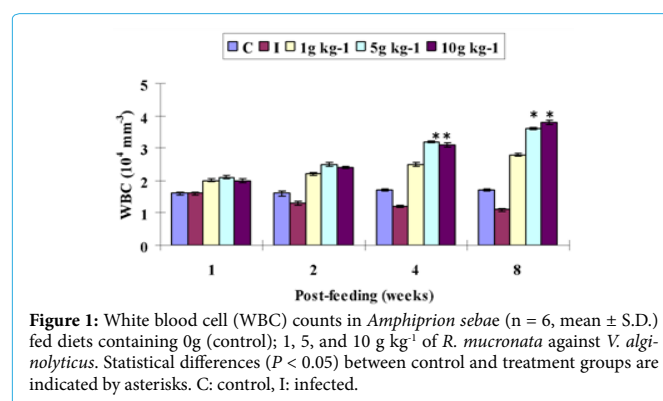
## Statistical analysis

The obtained results in this study were subjected to one-way analysis of variance (ANOVA) using the SPSS software (Windows version 17.0). Differences between means of control and experimental groups were calculated by Duncan's Multiple-Range Test and effects with a probability of  $P < 0.05$  were considered significant.

## Results

### Total white blood cell (WBC) counts

The WBCs did not increase significantly in infected fish feeding with any RM enriched diet on weeks 1 and 2 when compared with control. On the other hand, it was statically significant when infected fish were feeding with 5 or  $10 \text{ g kg}^{-1}$  RM diets between weeks 4 and 8, but not with  $1 \text{ g kg}^{-1}$  diet at any time (Figure1).



**Figure 1:** White blood cell (WBC) counts in *Amphiprion sebae* (n = 6, mean  $\pm$  S.D.) fed diets containing 0g (control); 1, 5, and  $10 \text{ g kg}^{-1}$  of *R. mucronata* against *V. alginolyticus*. Statistical differences ( $P < 0.05$ ) between control and treatment groups are indicated by asterisks. C: control, I: infected.

## Growth

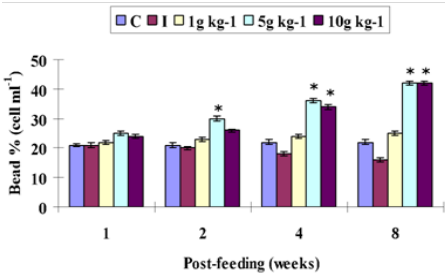
The MWG and SGR did not statically significant in infected fish when feeding with any RM diet between weeks 1 and 2 whereas it significantly augmented with 5 and  $10 \text{ g kg}^{-1}$  RM enriched diet on weeks 4 and 8. In contrast, the FCR progressively increased in infected fish feeding with all diets from weeks 1 to 4. But it was statistically significant with only 5 and  $10 \text{ g kg}^{-1}$  RM diets as compared with control on weeks 4 and 8 (Table 2).

Parameters	Weeks	C	I	Groups		
				1 g kg <sup>-1</sup>	5 g kg <sup>-1</sup>	10 g kg <sup>-1</sup>
MWG (g)	1	22.6±1.2	18.4±1.0	22.1±1.3	22.5±1.4	22.3±1.3
	2	22.8±1.0	18.2 ± 1.2	22.5 ± 1.1	23.3 ± 1.1	23.1 ± 1.3
	4	23.1±1.1	18.0 ± 1.1	22.9 ± 1.2	25.2±1.2*	25.6±1.3*
	8	23.4±1.3	18.3 ± 1.3	23.5 ± 1.0	26.8±1.3*	27.2±1.3*
SGR (%/day)	1	0.64 ± 0.03	0.62 ± 0.04	0.65 ± 0.03	0.66 ± 0.03	0.66 ± 0.04
	2	0.64 ± 0.04	0.58 ± 0.03	0.66 ± 0.04	0.72 ± 0.02	0.76±0.04
	4	0.64 ± 0.04	0.59 ± 0.03	0.69 ± 0.02	0.80 ± 0.04*	0.83 ± 0.02*
	8	0.6 6± 0.03	0.59 ± 0.02	0.72 ± 0.03	0.88 ± 0.04*	0.89 ± 0.04*
PER	1	0.78 ± 0.12	0.77 ± 0.11	0.79 ± 0.13	0.82 ± 0.11	0.80 ± 0.14
	2	0.79 ± 0.10	0.75 ±0 .11	0.82 ± 0.12	0.84 ± 0.11	0.85 ± 0.13
	4	0.81 ± 0.10	0.75 ± 0.12	0.85 ± 0.14	0.88 ± 0.11	0.85 ± 0.13
	8	0.83 ± 0.12	0.74 ± 0.11	0.88 ± 0.13	0.92 ± 0.12	0.91 ± 0.12
FCR	1	1.24 ± 0.04	1.21 ± 0.03	1.27 ± 0.04	1.29 ± 0.04	1.27 ± 0.03
	2	1.26 ± 0.03	1.22 ± 0.03	1.29 ± 0.02	1.37 ± 0.02	1.39 ± 0.03
	4	1.27 ± 0.03	1.21 ± 0.04	1.32 ± 0.04	1.54 ± 0.03*	1.58 ± 0.04*
	8	1.29 ± 0.02	1.20 ± 0.03	1.38 ± 0.02	1.59 ± 0.02*	1.63 ± 0.03*
SR (%)	1	98	82.6	95.3	97.2	96
	2	98.3	80	96	97.6	96.2
	4	98.3	76.2	96.2	98.4	97
	8	99.8	72.2	97.2	98.5	97.5
MWG: mean weight gain, SGR: specific growth rate, PER: protein efficiency ratio, FCR: feed conversion ratio, and fish survival rates (SR %)						

**Table 2:** Nutritional parameters of *Amphiprion sebae* fed with 0, 1, 5, and 10 g kg<sup>-1</sup> *R. mucronata* supplementation diets.

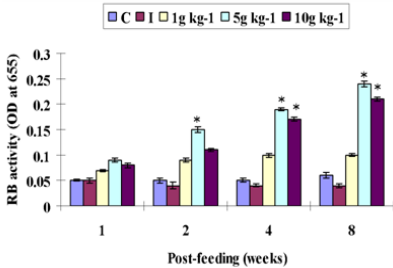
Immune protection

The PC activity did not significant when infected fish feeding with any RM enriched diet on weeks 1 and 2, except with 5 g kg<sup>-1</sup> diet where the increase manifested only after fourth week. However, the PC activity statistically significant with 5 and 10 g kg<sup>-1</sup> RM diets on weeks 4 and 8 as compared with control diet, but it did not with 1 g kg<sup>-1</sup> diet (Figure 2).



**Figure 2:** The phagocytic index in *Amphiprion sebae* (n = 6, mean ± S.D.) fed diets containing 0g (control); 1, 5, and 10 g kg<sup>-1</sup> of *R. mucronata* against *V. alginolyticus*. Statistical differences (P < 0.05) between control and treatment groups are indicated by asterisks. C: control, I: infected.

The RB activity did not statistically enhance on weeks 1 and 2 in the infected fish feeding with any RM diet except with 5 g kg<sup>-1</sup> diet on second week. Likewise, dietary administration of 5 and 10 g kg<sup>-1</sup> diets significantly increased the RB activity on weeks 4 and 8, but not with 1 g kg<sup>-1</sup> diet (Figure 3).



**Figure 3:** The respiratory burst (RB) activity in *Amphiprion sebae* (n = 6, mean ± S.D.) fed diets containing 0g (control); 1, 5, and 10 g kg<sup>-1</sup> of *R. mucronata* against *V. alginolyticus*. Statistical differences (P < 0.05) between control and treatment groups are indicated by asterisks. C: control, I: infected.

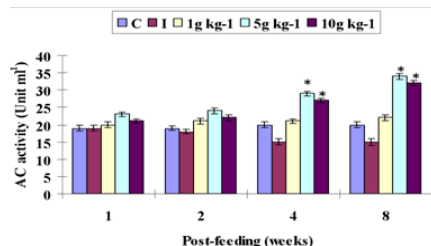
The ACP did not increase statistically with any RM enriched diet on weeks 1 and 2, but it was statistically significant with 5 and 10 g kg<sup>-1</sup> RM diets (Figure 4).

The LYZ activity did not increase significantly between weeks 1 and 2 in the infected fish feeding with any RM diet except with 5 g kg<sup>-1</sup> diet on second week when compared to control. On the other hand, all the enriched diets significantly increased the LYZ activity between weeks 4 and 8 as compared to control diet (Figure 5).

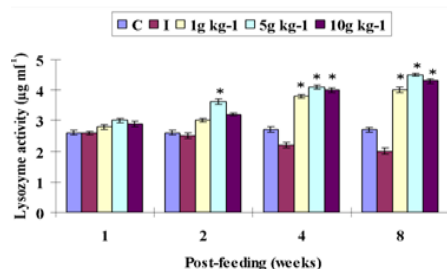
Bactericidal activity

The bactericidal activity did not statistically high with any RM diet from weeks 1 to 2, but did not shown with 1 g kg<sup>-1</sup> diet, while with any RM diet the bactericidal activity was found significantly better on weeks 4 and 8 in related to control diet (Figure 6).

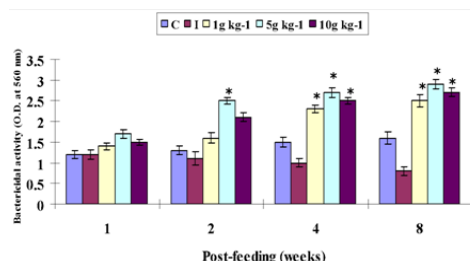




**Figure 4:** The alternate complement (ACP) activity in *Amphiprion sebae* (n = 6, mean ± S.D.) fed diets containing 0 g (control); 1, 5, and 10 g kg<sup>-1</sup> of *R. mucronata* against *V. alginolyticus*. Statistical differences ( $P < 0.05$ ) between control and treatment groups are indicated by asterisks. C: control, I: infected.



**Figure 5:** The lysozyme (LYZ) activity in *Amphiprion sebae* (n = 6, mean ± S.D.) fed diets containing 0 g (control); 1, 5, and 10 g kg<sup>-1</sup> of *R. mucronata* against *V. alginolyticus*. Statistical differences ( $P < 0.05$ ) between control and treatment groups are indicated by asterisks. C: control, I: infected.

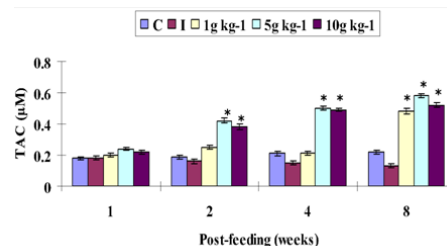


**Figure 6:** Bactericidal activity in *Amphiprion sebae* (n = 6, mean ± S.D.) fed diets containing 0 g (control); 1, 5, and 10 g kg<sup>-1</sup> of *R. mucronata* against *V. alginolyticus*. Statistical differences ( $P < 0.05$ ) between control and treatment groups are indicated by asterisks. C: control, I: infected.

## Antioxidant activity

The TAC showed no significant difference between treatment and control on first week. However, it was enhanced with 5 and 10 g kg<sup>-1</sup> RM diets, but did not shown with 1 g kg<sup>-1</sup> diet from weeks 2 to 4. On the other hand, all RM diets significantly high the TAC on week 8 (Figure 7).

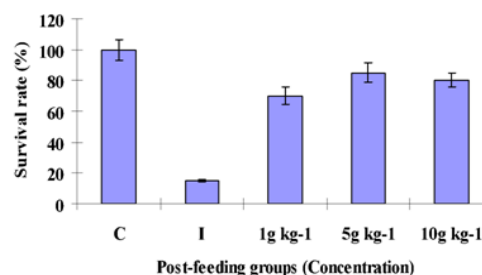
The NO activity in the liver and gills did not get significantly enhanced with any diet on weeks 1 and 2; but with 5 and 10 g kg<sup>-1</sup> diets it increased significantly on week 4 and all supplementation diet significantly increase on week 8 (Table 3). The CAT activity level in the liver and gills was moderately or did not significantly increased with all RM diet as compared with control diet (Table 3). The GSH and GPx levels did not increase significantly with any diet on weeks 1 and 2 whereas it was significantly enhanced with 5 and 10 g kg<sup>-1</sup> RM diets on weeks 4 or 8 (Table 3). The MDA and H<sub>2</sub>O<sub>2</sub> activities did not increased on first week with any RM enriched diet. However, it was significantly enhanced with all RM enriched diets on weeks 2, 4, and 8 in related to control diet (Table 3).



**Figure 7:** Total antioxidant capacity (TAC) activity in serum of *Amphiprion sebae* (n = 6, mean ± S.D.) fed diets containing 0 g (control); 1, 5, and 10 g kg<sup>-1</sup> of *R. mucronata* against *V. alginolyticus*. Statistical differences ( $P < 0.05$ ) between control and treatment groups are indicated by asterisks. C: control, I: infected.

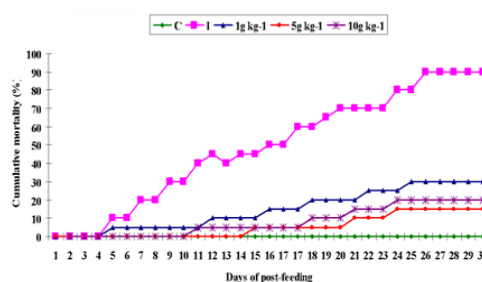
## Survival rate and cumulative mortality

A high survival percentage was observed in the infected fish feeding with 5 and 10 g kg<sup>-1</sup> RM enriched diets, i.e. 85% and 80%, respectively. It came down to 70% with 1 g kg<sup>-1</sup> diet. However, the survival rate was found 15% when infected fish after feeding with basal control diet (Figure 8).



**Figure 8:** The survival rate in *Amphiprion sebae* (n = 6, mean ± S.D.) fed diets containing 0 g (control); 1, 5, and 10 g kg<sup>-1</sup> of *R. mucronata* against *V. alginolyticus*. Statistical differences ( $P < 0.05$ ) between control and treatment groups are indicated by asterisks. C: control, I: infected.

Similarly, the cumulative mortality was found 15% and 20% in the infected groups were feeding with 5 and 10 g kg<sup>-1</sup> diets. It was shown 85% mortality in the infected un-treated group fed with control diet, while there was no mortality in un-infected group fed with basal control diet (Figure 9).



**Figure 9:** The cumulative mortality of *Amphiprion sebae* (n = 20) fed diets containing 0 g (control); 1, 5, and 10 g kg<sup>-1</sup> of *R. mucronata* against *V. alginolyticus* for 30 days. C: control, I: infected.

## Discussion

Aquaculture is one of the fast expanding income generating ventures providing many job opportunities in government and non-government sectors worldwide. Intensive aquaculture farms sometimes suffer sudden mass mortality of the cultivated organisms due to various adverse environmental factors and biological agents including microorganisms.

Parameters	Weeks			Liver					Gills			
		C	I	1gkg <sup>-1</sup>	5gk <sup>-1</sup>	10g kg <sup>-1</sup>		C	I	1gkg <sup>-1</sup>	5gkg <sup>-1</sup>	10gkg <sup>-1</sup>
NO (μmol l <sup>-1</sup> )	1	3.8 ± 0.18	3.8 ± 0.16	3.9 ± 0.14	4.2 ± 0.17	4.0 ± 0.12		1.8 ± 0.19	1.8 ± 0.17	1.9 ± 0.15	2.1 ± 0.14	2.0 ± 0.14
	2	3.8 ± 0.20	3.8 ± 0.14	4.0 ± 0.18	4.2 ± 0.15	4.1 ± 0.14		1.9 ± 0.22	1.7 ± 0.17	2.0 ± 0.13	2.3 ± 0.18	2.1 ± 0.17
	4	3.9 ± 0.21	3.7 ± 0.18	4.4 ± 0.20	9.2 ± 0.21*	7.2 ± 0.19*		1.9 ± 0.20	1.7 ± 0.15	2.2 ± 0.17	5.8 ± 0.16*	4.9 ± 0.21*
	8	4.0 ± 0.24	3.6 ± 0.16	8.2 ± 0.16*	9.8 ± 0.19*	9.1 ± 0.25*		2.0 ± 0.20	1.6 ± 0.14	5.5 ± 0.14*	6.6 ± 0.22*	6.2 ± 0.22*
CAT (U/g tissue)	1	3.1 ± 0.12	3.0 ± 0.11	3.1 ± 0.11	3.2 ± 0.11	3.0 ± 0.12		3.1 ± 0.10	3.1 ± 0.13	3.1 ± 0.12	3.2 ± 0.13	3.1 ± 0.12
	2	3.1 ± 0.14	2.9 ± 0.15	3.2 ± 0.12	3.4 ± 0.16	3.3 ± 0.14		3.2 ± 0.12	3.0 ± 0.14	3.1 ± 0.14	3.2 ± 0.11	3.1 ± 0.16
	4	3.2 ± 0.10	2.7 ± 0.12	3.2 ± 0.13	3.7 ± 0.12	3.6 ± 0.12		3.2 ± 0.14	2.8 ± 0.14	3.3 ± 0.16	3.6 ± 0.17	3.5 ± 0.12
	8	3.3 ± 0.16	2.5 ± 0.13	3.3 ± 0.16	3.9 ± 0.14	3.8 ± 0.16		3.2 ± 0.12	2.6 ± 0.11	3.3 ± 0.12	3.7 ± 0.12	3.6 ± 0.14
GSH (mg/g tissue)	1	12.1 ± 0.61	12.0 ± 0.44	12.2 ± 0.48	12.3 ± 0.48	12.2 ± 0.40		10.5 ± 0.56	10.4 ± 0.52	10.6 ± 0.61	10.8 ± 0.56	10.6 ± 0.56
	2	12.3 ± 0.53	11.9 ± 0.54	12.4 ± 0.54	12.5 ± 0.51	12.2 ± 0.44		10.6 ± 0.45	10.2 ± 0.60	10.8 ± 0.42	10.8 ± 0.42	10.6 ± 0.42
	4	12.5 ± 0.62	11.8 ± 0.64	12.5 ± 0.64	16.8 ± 0.66*	16.1 ± 0.62*		10.6 ± 0.54	10.1 ± 0.56	11.0 ± 0.46	15.7 ± 0.54*	15.4 ± 0.52*
	8	12.6 ± 0.54	11.6 ± 0.48	12.6 ± 0.56	18.2 ± 0.42*	17.7 ± 0.56*		10.8 ± 0.62	10.1 ± 0.64	11.2 ± 0.52	16.8 ± 0.62*	16.4 ± 0.63*
GPx (U/g tissue)	1	58.1 ± 3.8	57.3 ± 3.8	60.0 ± 3.6	61.2 ± 3.6	63.3 ± 3.1		36.2 ± 3.3	35.3 ± 2.6	37.2 ± 3.3	38.1 ± 3.3	37.0 ± 2.8
	2	60.4 ± 4.4	48.2 ± 4.4	61.1 ± 3.5	62.4 ± 4.2	64.4 ± 4.3		37.1 ± 4.4	33.4 ± 2.2	38.1 ± 3.8	39.2 ± 3.5	38.2 ± 3.4
	4	62.1 ± 4.8	50.0 ± 4.8	63.3 ± 2.4	87.1 ± 2.5*	78.3 ± 4.5*		38.4 ± 4.3	32.2 ± 2.4	40.0 ± 4.4	86.2 ± 3.8*	94.2 ± 3.8*
	8	63.0 ± 4.2	42.4 ± 3.2	64.3 ± 4.8	122.1 ± 3.8*	98.2 ± 4.4*		38.2 ± 3.1	30.3 ± 3.0	40.1 ± 4.2	40.1 ± 4.2	113.3 ± 4.2*
MDA (nmol/g tissue)	1	11.2 ± 0.5	11.0 ± 0.52	11.3 ± 0.58	11.6 ± 0.51	11.4 ± 0.56		8.8 ± 0.54	8.7 ± 0.62	8.8 ± 0.63	9.1 ± 0.66	8.9 ± 0.64
	2	11.4 ± 0.68	10.8 ± 0.62	11.4 ± 0.50	11.8 ± 0.54	11.6 ± 0.62		8.9 ± 0.58	8.6 ± 0.88	8.9 ± 0.78	9.3 ± 0.76	9.1 ± 0.82
	4	11.5 ± 0.62	10.6 ± 0.56	22.6 ± 0.66*	32.1 ± 0.64*	28.4 ± 0.68*		8.9 ± 0.56	8.5 ± 0.76	24.8 ± 0.66*	32.4 ± 0.86*	27.3 ± 0.80*
	8	11.8 ± 0.64	10.5 ± 0.6	30.8 ± 0.62*	35.6 ± 0.68*	33.2 ± 0.61*		9.1 ± 0.64	8.3 ± 0.72	31.8 ± 0.71*	38.5 ± 0.81*	34.5 ± 0.76*
H <sub>2</sub> O <sub>2</sub> (mM/g tissue)	1	0.66 ± 0.03	0.66 ± 0.03	0.68 ± 0.03	0.70 ± 0.04	0.69 ± 0.03		0.48 ± 0.04	0.48 ± 0.03	0.49 ± 0.03	0.52 ± 0.04	0.51 ± 0.04
	2	0.65 ± 0.03	0.67 ± 0.04	0.96 ± 0.04	1.19 ± 0.05	1.06 ± 0.05		0.48 ± 0.05	0.46 ± 0.05	0.92 ± 0.05	0.98 ± 0.05	0.96 ± 0.05
	4	0.67 ± 0.03	0.67 ± 0.04	1.04 ± 0.05*	1.30 ± 0.05*	1.16 ± 0.05*		0.49 ± 0.0	0.45 ± 0.06	0.96 ± 0.06*	1.10 ± 0.06*	1.00 ± 0.06*
	8	0.69 ± 0.04	0.68 ± 0.05	1.14 ± 0.04*	1.58 ± 0.05	1.34 ± 0.06*		0.44 ± 0.06	0.44 ± 0.06	1.00 ± 0.05*	1.20 ± 0.05*	1.10 ± 0.06*

NO: Nitric Oxide; CAT: Catalase activity; GSH: Glutathione activity; GPx: Glutathione peroxidase activity; MDA: malondialdehyde activity; H<sub>2</sub>O<sub>2</sub>: Hydrogen peroxide activity.

**Table 3:** Antioxidant activity in *Amphiprion sebae* fed diets containing 0, 1, 5, and 10 g kg<sup>-1</sup> *R. mucronata* supplementation diets (n = 6, mean ± S.D.) against *V. alginolyticus*. C: control, I: infected.

Traditional control measures were used with antibiotics and chemotherapy of fish diseases management creates resistant bacterial strains and aggravates immunosuppression in the host [2]. Very recently, herbal immunostimulants have been considered a promising option for fish disease management in aquaculture [2,5,7]. The dietary inclusion of herbs can modulate disease resistance by triggering up-regulation of the non-specific or specific immune defense mechanisms of the host against pathogens. In this regard, the non-specific immune defense mechanism is very important to boost immune system because aquatic animals are continually vulnerable to several opportunistic pathogens [8].

The WBCs gives protection in fish against infections and chemo toxicants [30]. This study, the WBC level slightly increased, but not statistically in the infected fish after dietary administration of any RM diet on weeks 1 and 2; on the other hand, 5 and 10 g kg<sup>-1</sup> RM diets increased significantly on weeks 4 and 8. Observing these results and collecting information on the profile of leucocytes can reveal on the overall immune prominence of fish. However, the poor relationship between differential composition of leucocytes and increase in the percentage of neutrophils in fish is correlated with viral or other infections [31].

The present study indicate that administration of 5 or 10 g kg<sup>-1</sup> enriched diet could better growth rate and feed effectiveness than with 1g kg<sup>-1</sup> RM diet; it appears that the effect of *R. mucronata* is dose independent. Further research is needed to address this issue. In fish, the non-specific defense system is most effective than the specific defense system, since the later required a longer time for antibody development and specific cellular stimulations [32].

In fish the phagocytes are regard a most significant constituent in the non-specific defense system which play an essential roles in both the initiation and regulation of immunity like in other vertebrates [33]. Further, it was early stage of fish innate immune response and plays an vital role in the non-specific defence mechanism of vertebrates against pathogens [34,35]; it establishes the key characteristic of innate immunity and first-line cellular defence mechanism. A number of immunostimulants are known to escalation the phagocytic activity in fish [36,37]. The present study indicate that the phagocytic activity did not statistically significant when infected fish were feeding with any enriched diet on weeks 1 and 2, but it was significant on weeks 4 and 8 when feeding with 5 or 10 g kg<sup>-1</sup> RM enriched diets.

RB an key indicator of innate defence mechanism in fish has been extensively used to assess the defence ability to pathogens [38,39]. The respiratory burst participates in the degradation of internalized microorganisms during phagocytosis, which is extensively used to estimate the host defence against pathogens. The respiratory burst activity did not statistically increase on 1 and 2 weeks with any RM enriched diet, but with 5 and 10 g kg<sup>-1</sup> RM diets it significantly better on weeks 4 and 8. This outcome is in contrast with earlier studies [6,7,40], which found that this activity was regulated by herbal supplementation diets against pathogens. Our results show that this activity did not increase with administration of *R. mucronata*, but no synergistic effect was observed.

Complement is a another key humoral constituent of the innate defence responses, which plays an critical role in alerting the host immunity in the existence of potential pathogens as well as in their clearance; it was possessed of more than 35 soluble plasma proteins that play key roles in innate and acquired immunity [41]. The complement is triggered by one or a combination of three pathways, i.e. alternative, lectin, and classical. Unlike in mammals, the complement in teleosts is active at very low temperatures; in several fish species the alternative complement pathway titers are relatively high and the ability to facilitate the lysis of target erythrocytes as suggesting their better capability to distinguish a wider range of foreign surfaces. Furthermore, the complement activity in fish is inclined by external factors including nutrition.

This study indicate that the infected fish dietary administration with 5 and 10 g kg<sup>-1</sup> RM enriched diets significantly enhanced the complement activity on weeks 4 and 8 but it did not with any RM enriched diet between weeks 1 and 2. The oral administration of individual or mixed herbal extract enriched diets can modulate ACP in fish to pathogens [32,42,43]. However, some of the herbal extract supplemented diets did not enhance the complement activity during the initial phases of experiment (weeks 1 to 2). The increasing some immunological parameters following the administration of RM diets did not relate with an enhancement in disease resistance. Moreover, the mortality in herbal-treated group was significantly higher than that of infected untreated group. The exact mechanism through which herbal extract exert their immunostimulant effects have not understood in fish.

Lysozyme is one very essential non-specific immune factor in fish that arising from neutrophils and macrophages. Lysozyme is secreted into blood and mucus for bacteriolytic effects [44]. Lysozyme is an cationic enzyme that degrade the peptidoglycan of bacterial cell walls which enables the lysozyme to lyse certain Gram-positive bacteria, and in conjunction with complement, even some Gram-negative bacteria. In this study, the infected fish feeding with 5 or 10 g kg<sup>-1</sup> RM enriched diet statistically improved the LYZ activity between weeks 4 and 8 but it did not on weeks 1 and 2. Previous studies indicate that the lysozyme activity is positively correlated with the administration of mono or triherbal extract enriched diets in grouper and olive flounder against diseases [7,40,45].

The bactericidal activity was significantly boosted in this study in fish feeding with any RM enriched diet from 4 to 8 weeks. In fish exposed to arsenic had reported higher bacterial load along with decreased bacterial clearance reported in *Clarias batrachus* against *A. hydrophila* [46]. Together with these results suggested that the arsenic exposure can compromises the host defense and decreases the ability to clearing of pathogen loading with the consequently increase in the susceptibility to pathogenic infection [47,48].

The antioxidant enzymes are very essential key elements in the animal defense to oxidative stress made by xenobiotic [49]. In the present study, there was no significant difference in the TAC on first week while it was significantly enhanced with 5 and 10 g kg<sup>-1</sup> RM enriched diets from weeks 2 to 8. A previous investigation on analyzing the effects of chemicals on zebrafish found a reduction of antioxidant competence [50]. In the liver and gills the NO activity was significantly enhanced with 5 and 10 g kg<sup>-1</sup> RM enriched diets on week 4 while all enriched diets the increase manifested on week 8. This present results are in line with Saha et al. [51]; a similar result was found by Chakraborty et al. [52] who had reported a significant enhancement in NO generation in the gills of *Lamellidens marginalis* after exposure to arsenic.

The CAT activity level in the liver and gills increased slightly with all supplementation diets during the experimental period. These results are in line with Aruljothi [53] who have found a down-regulation in CAT level in the brain and gill tissues of *L. rohita* exposed to arsenic. On the contrary, in zebrafish the CAT levels had no significant change when exposed to this chemical [54].

The GSH and GPx significantly enhanced with 5 and 10 g kg<sup>-1</sup> RM diets on 4 and 8 weeks. These results agree with Aruljothi [53] who had found a decrease of GSH and GPx levels in gill and brain tissues of *L. rohita* exposed to arsenic. A similar result was obtained in a previous work in goldfish [55]. GSH content may showed both increases and declines trends in fish tissues exposed to chemical due to their organ-specific responses; whereas, decreasing of GPx activity indicate that direct effects of metal ions on the active site of enzyme molecules [56]. The MDH and H<sub>2</sub>O<sub>2</sub> activities were significantly enhanced with all diet on weeks 2 to 8. LPO regards a biomarker for heavy metal toxicant or microbial infections, causing cell membrane structure damage or function with consequent imbalance between synthesis and degradation of enzyme protein [57,58]. Our results had shown a higher level of gills and liver MDA and H<sub>2</sub>O<sub>2</sub> in treated groups coincided with previous study showed that lead exposure to induction of LPO in the kidney, liver, and gills [59]. The LPO had been reported to elevate in gills and brain tissues of *L. rohita* after exposure arsenic [53]. In the present study, H<sub>2</sub>O<sub>2</sub> level was found a similar pattern to the MDA level, increasing in fish feeding with herbal diet compared to control diet. These results was confirm our present results; CAT enzyme catalyzes the removal of H<sub>2</sub>O<sub>2</sub> with production of H<sub>2</sub>O and O<sub>2</sub> [60,61]; in this study a decreasing level of CAT would have prevented the formation of radical intermediates into water and oxygen. It was probable through above results; chemical exposure can cause modifications in the innate immune response and antioxidant defense system in fish [62].

The aggregate mortality was 15% and 20% in the infected groups that were feeding with 5 and 10 g kg<sup>-1</sup> RM diets but it was 85% in the infected un-treated group feeding with control diet. This study was confirm the infected fish treated with *R. mucronata* extract enriched diet at 5 and 10 g kg<sup>-1</sup> dose can improved immune status and antioxidant ability to *V. alginolyticus* after 4th week. To our knowledge, this is the first investigation showing that RM extract can be used as a feed additive in fish, to enhance the growth parameter, immune system, and decrease the mortality. The detailed mechanism of action for enhanced growth response and involvement of the immune system with *R. mucronata* extract remains unclear. Our results have revealed that the potential of *R. mucronata* as an immunostimulant for fish, although further extensive research concerning the consequences and effects on immune system stimulating against pathogens, especially its protective ability against specific diseases. Further detailed immunol-

ogy and molecular research works are needed to address the relation between growth performance and involvement of the immune system in other fish against diseases.

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