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Effects of dietary supplementation of *Sonneratia alba* extract on immune protection and disease resistance in goldfish against *Aphanomyces invadans*

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Abstract. A 30-day study was conducted on the effects of diets supplementation with 0, 1.0%, 3.0%, and 5.0% *Sonneratia alba* leaf extracts on healthy goldfish, *Carassius auratus* against *Aphanomyces invadans*. Results showed that the numbers of white blood cell significantly increased in the infected fish fed with 3.0% and 5.0% supplementation diets after the second week of experiments. Whilst the numbers of red blood cell significantly decreased in the infected fish fed with 0 and 1.0% supplementation diets. After the third week of feeding trials, the total protein, albumin level and lysozyme activity were significantly increased in the infected fish fed with 3.0% and 5.0% supplementation diets. However, the myeloperoxidase activity significantly increased after two weeks in the infected fish were fed with 3.0% and 5.0% supplementation diets. The cumulative mortality rate of goldfish decreased up to 17% when the infected fish were fed with 3.0% supplementation diets. This study indicates that enriched fish feed with 3.0% and 5.0% *S. alba* leaf extracts enhanced the non-specific immunity and survival rate of the goldfish, suggesting that the extract may serve as a potential prophylactic treatment against *A. invadans*.

INTRODUCTION

The epizootic ulcerative syndrome (EUS) caused by the oomycete fungus, *Aphanomyces invadans* (David & Kirk, 1997) can be characterized histologically by its mycotic granulomas in infected fish. It is known to be one of the infectious fish diseases for farmed and wild fishes in fresh and brackish-water from the Asia-Pacific region, America and Africa (OIE, 2017; Kar, 2015). Fish such as *Channa* spp., barbs, major carps, gourami, catfish, goldfish and mullets are known as highly vulnerable to EUS (Afzali *et al.*, 2014, 2015; Bruno & Wood, 1999). Since EUS progressively spread throughout the world and caused huge economic losses (US$ 9 billion per year) (Harikrishnan *et al.*, 2011a), it had been documented by the Office International des Epizooties (OIE) in the infectious diseases list since 1995. Hitherto, there is neither standard chemical agents, which can be applied to treat this destructive infection in the time of outbreak, nor available vaccine for prevention (OIE, 2017).

Some toxic chemicals such as malachite green and formalin (Campbell *et al.*, 2001), and antibiotics including streptomycin, oxolinic acid (Lilley and Inglis, 1997) and oxytetracyclin (Saha and Pal, 2002) had been applied in EUS-infected fish ponds to minimize fish losses. These had caused several undesirable effects such as antibiotic resistance, environmental pollution, bioaccumulation and food toxicity issues (Maqsood *et al.*, 2011). Therefore, applying plant extracts as immunostimulants for EUS treatments are recently considered as an alternative strategy for fish disease

managements and controls (Pandey *et al.*, 2012). Plant-based immunostimulants are not only safe for the environment, but also easily biodegradable, and locally available in low cost, which make them a suitable choice for production (Valladao *et al.*, 2015). Plant-based extracts usually possess effective active compounds including phenols, polyphenols, alkaloids, quinones, terpenoids, lectines, and polypeptides that have shown to improve fish immune system against the pathogen and decrease fish mortality (Harikrishnan *et al.*, 2009a, b, 2011a; 2018; Citarasu, 2010).

Several plants with biomedicine properties have been applied as treatments against EUS. For examples, *Mikania cordata* (Burm) (see Kumar *et al.*, 2015), *Rauvolfia tetraphylla* (L.) (see Yogeshwari *et al.*, 2015), paddy husk extract (see Jahan *et al.*, 2014), *Lawsonia inermis* (L.), (see Uthayakumar *et al.*, 2014), neem (*Azadirachta indica* A. Juss) (see Alam *et al.*, 2014; Harikrishnan *et al.*, 2009; Harikrishnan *et al.*, 2005; Campbell *et al.*, 2001; Fairweather, 1999), turmeric (*Curcuma longa* L.) (see Chowdhury and Rahman, 2013; Campbell *et al.*, 2001), *Curcuma zedoaria* (Christm.), Indian sorrel (*Oxalis corniculata* L.), akand (*Calotropis gigantea* L.) (see Chowdhury and Rahman, 2013), and Kalojira (*Nigella sativa* L.) (see Alam *et al.*, 2014; Chowdhury and Rahman, 2013) have shown to be effective in inhibiting mycelial growth of *A. invadans in vitro* and *in vivo*. Among these plants, *A. indica* is known to be a more suitable candidate and a potential source of feedstuffs due to its broad spectrum activity and lower dose requirements (Immaraju, 1998).

Sonneratia alba J.E. Smith (also known as mangrove apple or "perepat" in Malaysia) belongs to the sonneratiaceae family (Backer and Van Steenis, 1951). Morphologically, *S. alba* can be identified with its rounded and leathery leaf, falcate shape seeds, smooth and shiny calyx and corolla width fruits (Tomlinson, 1994). It can be found from southeastern Africa to India and southern China to the western islands of the Pacific Ocean (Duke and Jackes, 1987) where these regions are prone to have EUS outbreak (OIE, 2017). Traditionally, the fruits and leaves of *S. alba* are applied to treat intestinal parasites, coughs, swellings and sprains (Peter and Sivasothi, 1999; Bandaranayake, 1998). The leaves of mangrove plants are rich in cyclitol, polyol, sucrose, glucose, fructose, condensed and hydrolysable tannins, minerals, nucleotides (Bandaranayake, 2002; Popp *et al.*, 1984). These compounds, particularly, tannins were reported to have an antimicrobial activity and may be act as a strong barrier against microbial attack by increasing resistance against pathogenic organisms (Balasooriya *et al.*, 1982). Saad *et al.* (2012) investigated the antimicrobial activity of *S. alba* against some infectious diseases caused by human fungal and bacterial pathogens. However, the authors concluded that *S. alba* leaf extracts were not effective against the tested fungal strain, *Candida albicans* (Robin) and yeast, *Cryptococcus neoformans* (Sanfelice) even though the extracts were used at a high concentration (10,000 ppm).

In a preliminary study, our scientific team proved that the organic extract of *S. alba* effectively inhibited the mycelial growth of *A. invadans in vitro* at a minimum concentration of 1000 ppm (Afzali and Wong, 2017). In the present study, we aimed to determine the effects of *S. alba* leaf extract on the immune response and survival of the EUS-infected goldfish *in vivo*. The goldfish, *Carassius auratus* L. was chosen to be used in the present study because it has been widely employed in EUS-related scientific studies for detection of *A. invadans* and for artificially EUS-infected fish (Afzali *et al.*, 2015; Phadee *et al.*, 2004).

MATERIALS AND METHODS

The present study was approved by UTAR Scientific and Ethical Review Committee (SERC) meeting on 19th October 2015 and UTAR Institutional Biosafety Committee (IBC).

Preparation herbal extract

Fresh leaves of *S. alba* were collected from Prai river, Penang, Malaysia that are shown in Figure 1. The plants were morphologically

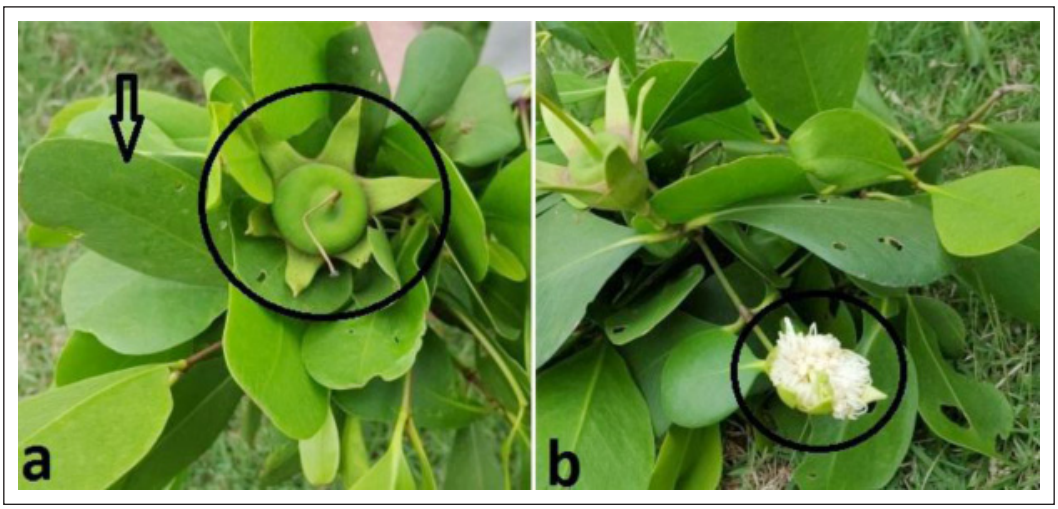

Figure 1. *Sonneratia alba* mangrove plant characterised with (a) rounded tips leaf (arrow), young fruit (circle), and (b) flower with small petals (circle).

identified to the species level according to Duke and Jackes (1987). The leaves were washed thoroughly with distilled water to remove any dirt and dust on the leaves. Then, the leaves were placed in the dark and air-dried for 48 hrs. Afterward, the leaves were placed at -20°C in a refrigerator overnight before transferring them to a freeze dryer device (Christ, Germany) for seven days. A total amount of 359.44 g dried leaves was obtained from 1 kg of fresh leaves. Dried leaves were ground to coarse powder with a mechanical grinder, weighed and then transferred into a thimble for organic extraction procedure using the Soxhlet extraction apparatus. In each extraction, 30 g of ground powder was soaked with 600 ml methanol solvent (GENE Chemicals) for 5-6 hr. The solvent was then distillated using a rotary vacuum evaporator (Buchi, Switzerland) to dryness under reduced pressure at 60 mbar and 40°C, and the residues were stored in airtight containers at -20°C until required. The final yield of extraction recorded was 8 g herbal powder per 30 g dried leaves.

Culturing of *Aphanomyces invadans*

Aphanomyces invadans isolate (NJM9701) was obtained from the Centre for Environment, Fisheries and Aquaculture Science (Cefas), U.K., (courtesy of Dr. Oidtmann B). The isolate was originally isolated from naturally infected Ayu in Japan. The cultures were stored on slopes of 'glucose-peptone' (GP) agar media (3 gL^{-1} glucose, 1 gL^{-1} peptone, 0.128 $gL^{-1}MgSO_4.7H_2O$, 0.014 gL^{-1} KH_2PO_4, 0.029 gL^{-1} $CaCl_2.2H_2O$, 2.4 mgL^{-1} $FeCl_3.6H_2O$, 1.8 mgL^{-1} $MnCl_2.4H_2O$, 3.9 mgL^{-1} $CuSO_4.5H_2O$, 0.4 mgL^{-1} $ZnSO_4.7H_2O$, 12 gL^{-1} technical agar, 10 mlL^{-1} Penicillin) in universal tubes, filled with sterile light paraffin oil and kept at room temperature according to the standard procedures by Lilley *et al.* (1998). The suspension of motile secondary zoospores was prepared and quantified using a Neubauer counting chamber and adjusted to the required concentration (100 spores/ml) according to Johnson *et al.* (2004).

Preparation supplementation feed

The experimental diets were prepared using fish meal, soya bean powder, coconut oil cake, corn flour, wheat flour, fish oil, vitamin mixture and different percentages of *S. alba* leaf extract, and the proximate compositions of the formulated diets were determined as shown in Table 1. Four experimental pellet diets, 0 (basal diet without *S. alba* extract), 1.0%, 3.0%, and 5.0% of *S. alba* extracts were prepared for the experiments. The ingredients were mixed well in hot water and steamed for 25 min to make them into a soft paste. After cooling at room temperature, vitamin mixture and plant extracts were

Table 1. Formulations and proximate compositions of the experimental fish feeds

Ingredients (g/kg^{-1})	*Sonneratia alba* extract in diets (%)			
	0.0	1.0	3.0	5.0
Fish meal	200	200	200	200
Soya bean powder	180	180	180	180
Coconut oil cake	180	180	180	180
Wheat flour	180	170	150	130
Corn flavor	180	180	180	180
Fish oil	50	50	50	50
Vitamin-mineral mix[1]	30	30	30	30
S. alba extract powder	0.0	10	30	50
Proximate Composition				
Crude protein	39.0	39.2	40.5	40.8
Crude lipid	8.6	8.6	8.5	8.4
Crude ash	7.5	7.5	7.6	7.7
Fiber	5.0	5.0	4.5	4.0
Moisture	8.5	8.3	8.1	5.0

[1] Vitamin and mineral mixture (mg/kg^{-1}): Vitamin A 5 000 I.U., Vitamin D 600 I.U., Vitamin B1 10, Vitamin B2 20, Pantothenic Acid 30, Niacin 50, Vitamin C 200, KI 0.1, $CaHPO_4$. $CuSO_4$ $5H_2O$ Cu 10, $FeSO_4$ $7H_2O$ 100, $MnSO_4$ H_2O 50, ZnO 50, $CaCI_2$ $6H_2O$ 0.05.

added to the paste and extruded through a manual noodle extruder with an opening diameter of 0.5 mm. The pellets were initially air-dried, placed in an oven at 40°C for 18 hrs, packed in airtight container and then stored in a freezer at -20°C until used.

Fish

Healthy goldfish (mean weight: 22 ± 3 g) obtained from a local fish farm in Ipoh, Perak, Malaysia were transferred to the laboratory in plastic bags filled with aerated clean water. The fish were acclimatized in 50 L plastic aquariums equipped with aeration and sponge filters for two weeks before initiating the experiments. Fish were fed twice per day (at 0900 and 1600) with the formulated basal diet at a rate of 2.0% of their body weight. The water quality parameters including temperature (23 ± 2 °C), concentration of dissolved oxygen (6.3 ± 2 mg l^{-1}) and pH (5.7–6.92) were maintained during the experiments.

Experimental design and challenge study

Fish were divided into five groups of 20 fish each in three replicates. The five groups were: i) uninfected fish (control) fed with basal diet (without *S. alba* leaf extract), (ii) infected fish fed with basal diet (without *S. alba* leaf extract), (iii) infected fish fed with enriched diet supplemented with 1.0% *S. alba* leaf extract, (iv) infected fish fed with enriched diet supplemented with 3.0% *S. alba* leaf extract, and (v) infected fish fed with enriched diet supplemented with 5.0% *S. alba* leaf extract. After the first week of feeding trial, the fish from all the groups except the control fish were injected intramuscularly at the left side of the body below the dorsal fin with 0.1 ml of *A. invadans* spore suspension (100 spores/ml) and the control was injected with 0.1 ml of autoclaved pond water (APW). In addition, a group of 20 fish in each group (not subjected to any blood sampling) were kept in a different tank for each experimental group in order to observe for daily mortality until the end of experiment. Fish in all the groups were fed with the experimental diets at a rate of 2.0% body weight twice a day within the experiment period. All the fish were monitored daily for EUS characteristic clinical signs. When ulcers were observed on moribund fish, tissues underlying the ulcers were isolated and screened if the isolated fungus fulfilled the Koch's postulates, thus confirming the infection of *A. invadans*.

Blood sampling

Six fish from each tank were sampled randomly in weeks 1, 2, 3, and 4 after treatments and were put under anaesthesia in 150 ppm buffered MS-222 (Sigma-Aldrich, St. Louis, MO, USA) solution. The anaesthetized fish were then bled through their caudal vasculature using a 24-gauge syringe needle and the collected blood was transferred into heparinized tubes. An aliquot of the blood, which was kept in serum collection tubes, were placed at room temperature for 1 hr. The sera were separated by centrifugation at 2700 rpm for 10 min and stored at -20°C for determining the biochemical parameters of blood.

Total blood cells counts

The blood samples were diluted by adding Turk's dilution fluid for white blood cell (WBC) and Hayem's solution fluid for red blood cell (RBC). After incubating the blood for 5 min at room temperature, the WBC and RBC cells were counted using a Neubauer haemocytometer chamber and expressed as cells ml^{-1}.

Total protein content

The total protein content of the serum was determined using a micro protein assay kit (Sigma-Aldrich, U.S.A) according to Bradford (1976). The procedure was based on employing the brilliant blue G dye and the amount of absorption at 595 nm was recorded using a spectrophotometer (Thermo Spectronic GENESYS 20 Visible Spectrophotometer, U.S.A). The concentrations of total proteins in the serum were expressed as mg/ml.

Albumin assay

The albumin level in the serum was measured using the bromocresol green albumin assay kit (Sigma-Aldrich, U.S.A) (Doumas *et al.*, 1971). The intensity of the color was measured at 620 nm using a spectrophotometric multi-well plate reader (Infinite® 200 PRO multimode reader, Switzerland). A standard curve was obtained for each set of the assays to determine the sample albumin concentration (g/dL). The globulin was calculated by subtracting the albumin value from the total plasma protein.

Serum lysozyme activity

The lysozyme detection kit (Sigma-Aldrich, U.S.A) was used to determine the presence of lysozyme activity in the serum according to Shugar (1952). A 0.01% (w/v) suspension of *Micrococcus lysodeikticus* cells was employed as the lysozyme substrate in a 2.6 ml reaction mixture (66 mM potassium phosphate, pH 6.24). The mixture was then incubated at 25°C. The decrease in absorbance at 450 nm was monitored and recorded for 5 min using a thermostated spectrophotometer (Thermo Spectronic GENESYS 20 Visible Spectrophotometer, U.S.A) to obtain the maximum linear rate (ΔA_{450}/minute). The lysozyme concentration was expressed in unit/ml enzyme.

Myeloperoxidase (MPO) assay

The MPO activity in the fish serum was measured using the MPO fluorometric activity assay kit (Sigma-Aldrich, U.S.A) according to the manufacturer's protocol (Nauseef, 2007). The procedure is based on the production of NaClO from H_2O_2 and NaCl which react with Aminophenyl fluorescein to generate fluorescein that can be detected at 525 nm using a fluorescence multi-well plate reader device (FLUOstar Omega Microplate Reader – BMG Labtech, MARS Data analysis software). Ten µl of serum was diluted with 40 µl of MPO assay buffer to reach to a final volume of 50 µl. The mixture was then added with the reaction buffer and incubated for 2 min. The initial fluorescence intensity (FLU) was measured at 525 nm, which required being in the linear range of the standard curve. The measurements of FLU were recorded every 5 min and the penultimate reading was considered as the final measurement for calculating the enzyme activity. The fluorescein standard curve was obtained for each assay. The FLU of each sample was compared to the standard curve to determine the amount of fluorescein generated by the MPO assay between the initial and final time.

Statistical analysis

To compare the significant differences in the hematological and immunological parameters data obtained from the experiments, the analysis of variance test was performed using the SPSS version 22. Prior to data analysis, normality test and homogeneity test of variances were applied. The Welch test and mean comparison was done using Tamhane test at a confidence level of 0.05.

RESULTS

Cumulative mortality

During the course of experiments, low cumulative mortality of 17% and 20% were recorded in the infected fish (n = 20) fed with 3.0% and 5.0% supplementation diets, respectively. However, the cumulative mortality rate of the infected fish fed with 1.0% of supplementation diet was higher (27%) than that of the infected fish fed with 3.0% and 5.0% supplementation diets. The untreated, infected fish group (0% *S. alba* extract supplementation feed) suffered a cumulative mortality of 90%, while there was no mortality in the untreated and uninfected fish (Figure 2).

Total blood cells counts

The WBC levels had slightly increased in all the groups from weeks 1 to 4 as compared to the control group shown in Figure 3. The increases were significant ($P < 0.05$) in weeks 2, 3, and 4 in the infected fish treated with 3.0% supplementation diet when compared to the control (Fig. 3). The RBC levels decreased during the post infection period as compared to the control in all the experimental groups. However, the reduction was significant ($P < 0.05$) in the untreated infected fish in weeks 2, 3 and 4, and in the infected fish treated with 1.0% supplementation diet on weeks 2 and 3 as are shown in Figure 4.

Total protein content

The total protein contents of serum increased in all the infected fish fed with *S. alba* leaf extract supplementation diets from weeks 2 to 4. However, the increases of the total protein contents were significant ($P < 0.05$) when the infected fish were fed with 3.0% and 5.0% supplementation diets on weeks 3 and 4 as compared to the control (Figure 5).

Albumin assay

The total albumin levels were observed to increase in all the infected fish treated with different percentages of supplementation diets as compared to the control. The increment was significant ($P < 0.05$) in the infected fish treated with 3.0% and 5.0% supplementation diets on weeks 3 and 4 (Figure 6). Whilst the albumin value was

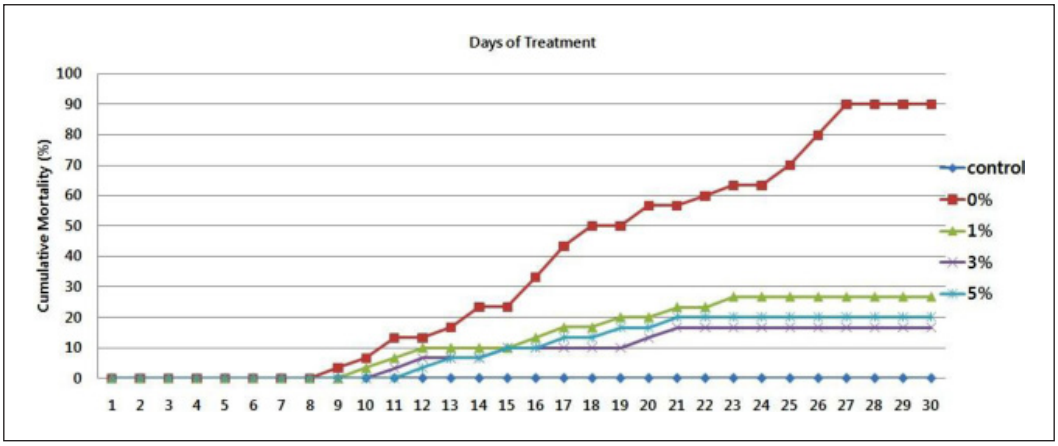

Figure 2. Effect of different dosages (0.0%, 1.0%, 3.0% and 5.0%) of supplementation diet with *Sonneratia alba* extract on the cumulative mortality in the goldfish, *Carassius auratus* L., against *Aphanomyces invadans* infection for 30 days.

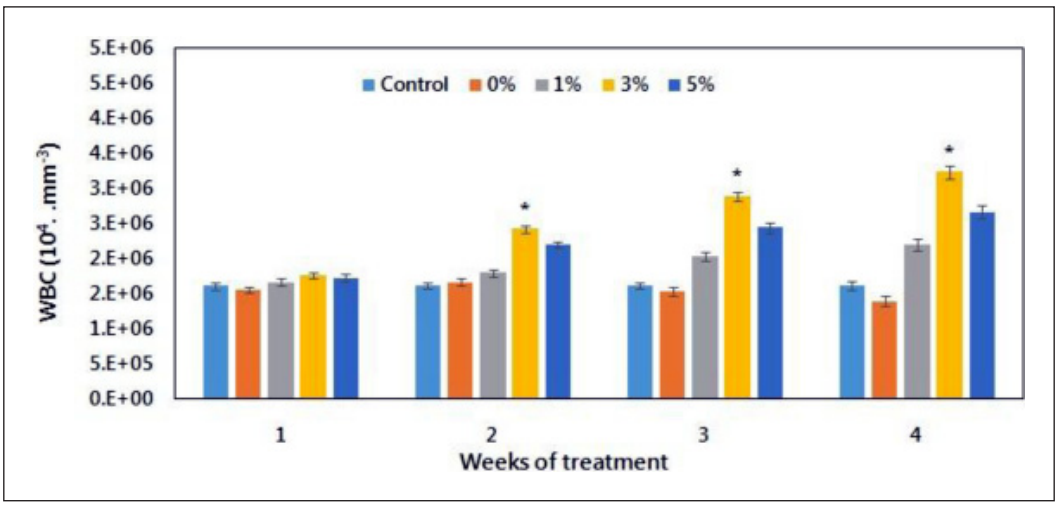

Figure 3. Effects of different dosages (0.0%, 1.0%, 3.0% and 5.0%) of supplementation diet with *Sonneratia alba* extract on total white blood cell (WBC) in the goldfish, *Carassius auratus* L., against *Aphanomyces invadans* infection for four weeks. Data are presented as mean ± S.E. and significant differences (*p < 0.05) are indicated by asterisk over the bars.

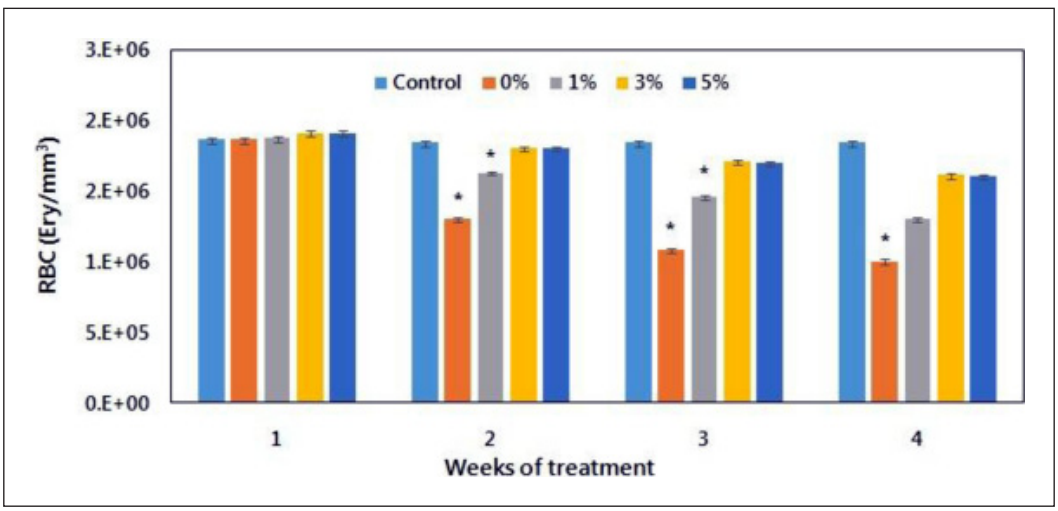

Figure 4. Effects of different dosages (0.0%, 1.0%, 3.0% and 5.0%) of supplementation diet with *Sonneratia alba* extract on the total red blood cell (RBC) in the goldfish, *Carassius auratus* L., against *Aphanomyces invadans* infection for four weeks. Data are presented as mean ± S.E. and significant differences (*p < 0.05) are indicated by asterisk over the bars.

slightly decreased in the untreated, infected group on weeks 2 and 3.

Serum lysozyme activity

The lysozyme activity of serum did not show any significant changes during weeks 1 and 2 in all the infected fish treated with different percentages of supplementation diets. Whilst in the infected fish treated with 3.0% and 5.0% supplementation diets, the lysozyme levels were significantly increased ($P < 0.05$) as compared to the control on weeks 3 and 4 (Figure 7).

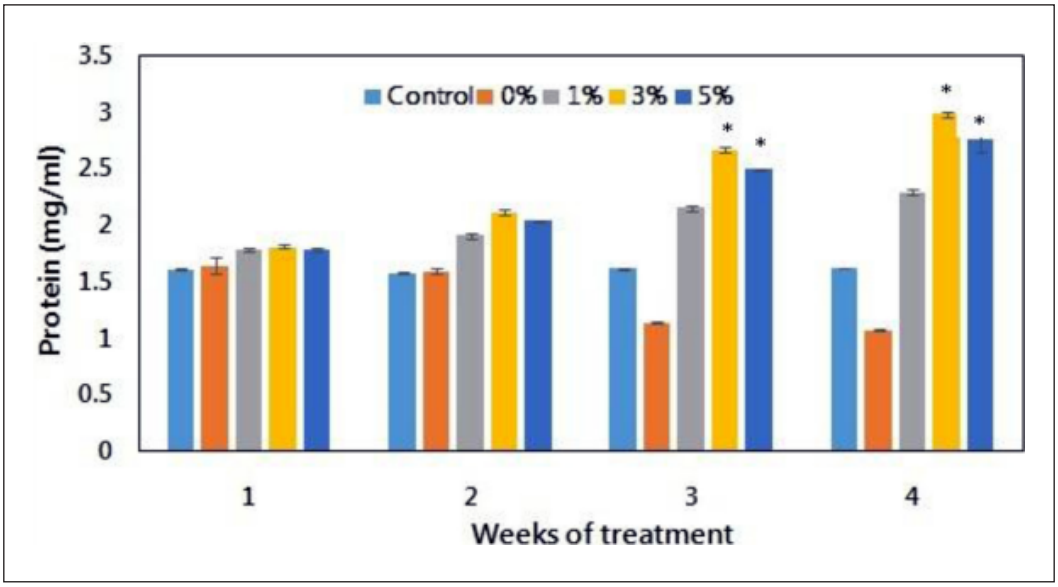

Figure 5. Effects of different dosages (0.0%, 1.0%, 3.0% and 5.0%) of supplementation diet with *Sonneratia alba* extract on total serum protein in the goldfish, *Carassius auratus* L., against *Aphanomyces invadans* infection for four weeks. Data are presented as mean ± S.E. and significant differences (*p < 0.05) are indicated by asterisk over the bars.

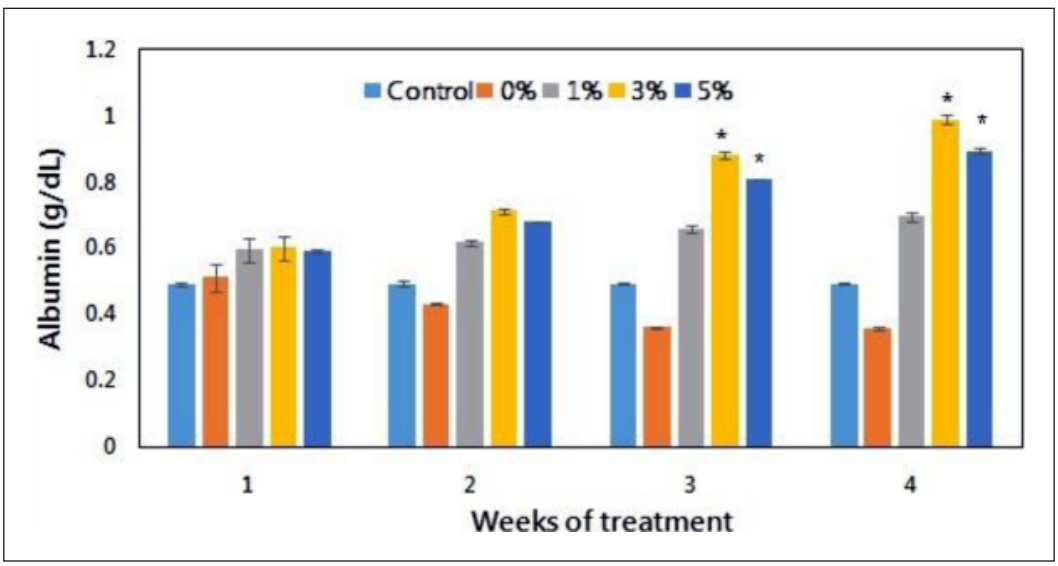

Figure 6. Effects of different dosages (0.0%, 1.0%, 3.0% and 5.0%) of supplementation diet with *Sonneratia alba* extract on total serum albumin in the goldfish, *Carassius auratus* L., against *Aphanomyces invadans* infection for four weeks. Data are presented as mean ± S.E. and significant differences (*p < 0.05) are indicated by asterisk over the bars.

Myeloperoxidase (MPO) assay

Myeloperoxidase activities were significantly increased when the fish group treated with supplementation diets containing 3.0% and 5.0% of *S. alba* extract on weeks 2, 3 and 4 as compared to the control. These values were not statistically changed in the infected fish treated with 1.0% supplementation diet (Figure 8).

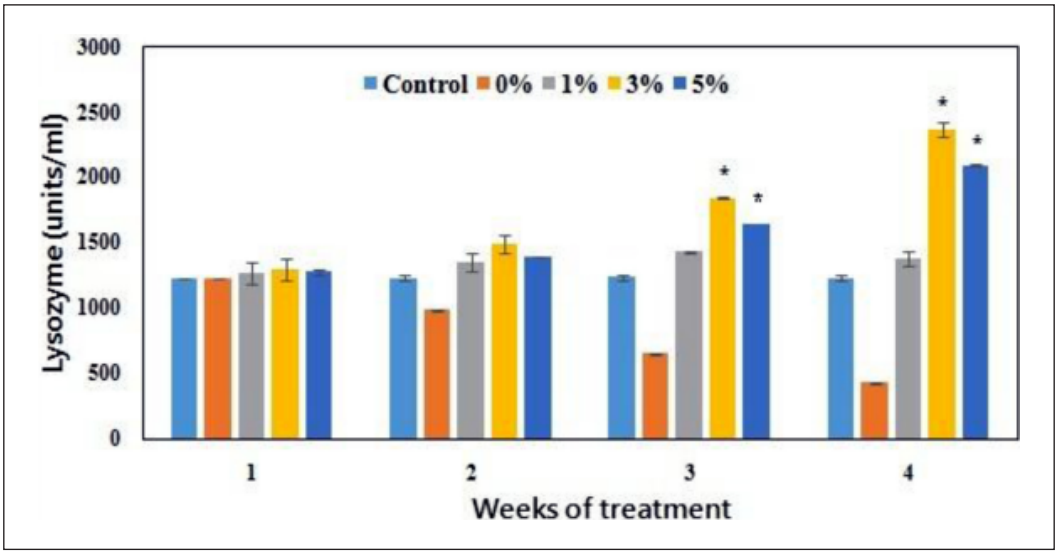

Figure 7. Effects of different dosages (0.0%, 1.0%, 3.0% and 5.0%) of supplementation diet with *Sonneratia alba* extract on serum lysozyme activity in the goldfish, *Carassius auratus* L., against *Aphanomyces invadans* infection for four weeks. Data are presented as mean ± S.E. and significant differences (*p < 0.05) are indicated by asterisk over the bars.

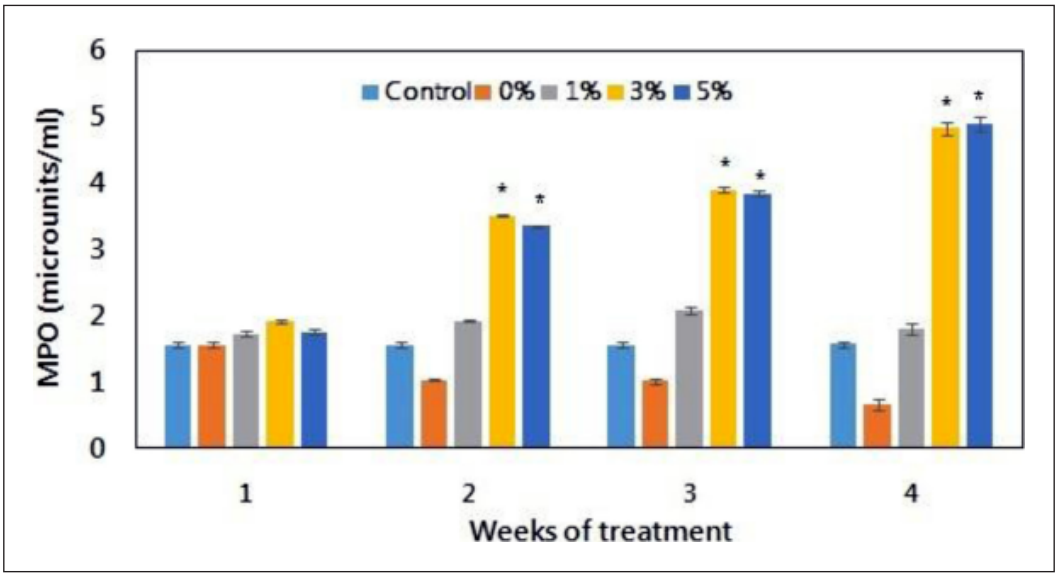

Figure 8. Effects of different dosages (0.0%, 1.0%, 3.0%, and 5.0%) of supplementation diet with *Sonneratia alba* extract on myeloperoxidase (MPO) activity in the goldfish, *Carassius auratus* L., against *Aphanomyces invadans* infection for four weeks. Data are presented as mean ± S.E. and significant differences (*p < 0.05) are indicated by asterisk over the bars.

DISCUSSION

Application of plant-based fish feed supplements are gaining much attention in fish diseases management and development of sustainable aquaculture (Gatlin *et al.*, 2007). In the present study, the antifungal properties of *S. alba* leaf extract against the fish oomycete pathogen, *A. invadans* was screened for the first time. The results

showed that goldfish fed diet enriched with *S. alba* leaf extract at concentrations of 3.0% and 5.0% may improve the immunity and disease resistance against *A. invadans*. The cumulative mortality was recorded lower (17% and 20%) in the infected fish fed with 3.0% and 5.0% supplementation diets, respectively as compared to the other treated groups. However, 90% of the fish died from EUS infection in the untreated groups. This has shown that *S. alba* leaf extract has probably improved the resistance of fish against EUS. Similarly, Yogeshwari *et al.* (2015) showed that the cumulative mortalities of the *A. invadans*-infected Indian major carp, *Labeo rohita* (Hamilton) administered with 5.0% and 10% supplementation diets of the herbal extract, *Rauvolfia tetraphylla* had lower to 15% and 25%, respectively. In addition, Harikrishnan *et al.* (2011b) found that Japanese snowbell, *Styrax japonicas* (Siebold and Zucc) enriched diets significantly increased the survival rate of Kelp grouper, *Epinephelus bruneus* (Bloch) fed for 30 days after being challenged with a marine bacterium, *Vibrio harveyi* (Johnson and Shunk) and a ciliate protozoan, *Uronema marinum* (Womersley). Furthermore, the lysozyme activity, total protein and myeloperoxidase levels of the blood in *E. bruneus* significantly increased when the fish were fed with 1.0% and 2.0% enriched diets, respectively, indicating an enhancement in the immune responses of the Kelp groupers against the target pathogens (Harikrishnan *et al.*, 2011b).

The first defense mechanism against pathogens in fish is the innate (non-specific) immune system (Ahilan *et al.*, 2010). The common components of the innate immune system of fish are physical parameters, cellular and humoral factors, which may influence the activity of innate immune parameters (Magnadottir, 2006; Secombes and Fletcher, 1992). White blood cells (leukocytes: neutrophils, lymphocytes, monocytes, eosinophils, and basophils) are an important part of immune systems, which protect fish body against infections pathogens (Abbas *et al.*, 2014). Red blood cells (erythrocytes) are responsible for blood oxygen transportation and alterations in this value reflect the health status of fish (Abbas *et al.*, 2014). In this study, the WBC level was significantly higher ($P < 0.05$) in the infected fish treated with 3.0% supplementation feed after the second week of treatment. This indicated a significant improvement in the general immune system status in the infected fish within a short period of time after the administration of *S. alba* leaf extract in the feed. On the other hand, the RBC levels decreased in all the infected fish after the EUS-infection, and the reduction of RBC was significant in the untreated infected fish and the infected fish treated with 1.0% supplementation feed. These indicate that the severity of EUS-infection may have been suppressed following the feeding of 3.0% and 5.0% *S. alba* leaf extract supplementation feeds. Similar observation was reported in a study of goldfish infected with the bacterial pathogen, *Aeromonas hydrophila* (Chester) (Harikrishnan *et al.*, 2010a). In their study, the WBC levels significantly increased in the fish group fed with 100 and 200 mg kg^{-1} of mixed herbal extract supplementation feeds while the RBC level decreased after the treatments. Harikrishnan *et al.* (2003) had also indicated that comparable changes in the WBC and RBC counts in the 'Common carp *Cyprinus carpio* L.' which had been artificially infected with *A. hydrophila* and dip-treated with neem (*A. indica*) leaf extract for 10 days. They reported that 30 days of oral administration of *A. indica* ethanol extract supplementation diet may protect the hematological and biochemical parameters in EUS-infected carp.

The total proteins in blood normally decreased when an animal is infected by pathogenic organisms or affected by any environmental stressors. This may due to a fall in the absorbed amino acids that are essential for protein synthesis. In present study, the total serum protein and albumin increased significantly in the infected fish treated with 3.0% and 5.0% supplementation feeds after the third week of infection. There were no remarkable differences in these values when the infected fish supplemented with 1.0% *S. alba* leaf extract during the

experimental period. The decreases in the total protein and albumin in the blood of the untreated infected group highlighted the positive effects of the administrated *S. alba* leaf extract. The results of the present study are in conformity with the finding by Harikrishnan *et al.* (2010a) who also observed an increase in the total serum protein in *A. hydrophila*-infected goldfish followed by the application of 400 and 800 mg kg^{-1} of mixed herbal enriched diets on week 4 of the treatments. In addition, Mari *et al.* (2014) suggested that the increases in the total protein and albumin, lysozyme activity in the infected Indian major carp, *Cirrhinus mrigala* (Hamilton) fed with 1.0% chitosan enriched diet may have enhanced the immunity of fish against *A. invadans* infection. In another Indian major carp, *Catla catla* (Hamilton), the level of total serum protein also increased when the fish was fed with enriched diet containing 'Chaff-flower *Achyranthes aspera* L.' seed on day 14 and 21 of post-injection (Vasudeva and Chakrabarti, 2005). However, the authors noted a significant increase in the globulin but not in the total protein or albumin.

Lysozyme activity has an important role in the innate immunity of fish and gives an indication of protection against invasive microbes (Saurabh and Sahoo, 2008). In the present study, the serum lysozyme concentration was significantly higher ($P < 0.05$) in the infected goldfish fed with 3% and 5% supplementation feeds on weeks 3 and 4, suggesting that the immune system of the infected goldfish may have been improved. There was no alteration in the treated group with 1.0% *S. alba* leaf extract. Whilst a sharp decrease was noted in the infected fish that did not have any *S. alba* extract in their diets. Dhayanithi *et al.* (2015) recorded similar lysozyme activity when the authors applied the Gray mangrove, *Avicennia marina* (Forsk.) extracts enriched diets in the Clownfish, *Amphiprion sebae* (Bleeker) against *Vibrio alginolyticus* (Miyamoto) infection on weeks 6 and 8. They concluded that administration of 4.0% and 8.0% *A. marina* extracts may strengthen the immune system of the infected clownfish. Also the tilapia, *Oreochromis niloticus* L. which was fed with 0.1% and 0.5% of traditional Chinese medicine (derived from Astragalus) for a week, had also showed an increment in the lysozyme activity (Yin *et al.*, 2006). Moreover, it was reported that intramuscular administration of the tri-herbal compounds (azadirachtin, camphor and curcumin) at a concentration of 100 ppm could significantly enhanced ($P < 0.05$) the serum lysozyme activity in *C. mrigala* against *A. invadans* (Harikrishnan *et al.*, 2009, 2010b). Similarly, the effects of Chinese herb (*Astragalus*) on the non-specific immunity of Jian carp, *Cyprinus carpio* (var. Jian) and large yellow croaker, *Pseudosciaena crocea* (Richardson) were reported (Jian and Wu 2003; 2004).

Myeloperoxidase is the most abundant neutrophil granule protein, which is synthesized during myeloid differentiation, that plays a key role in the various functions of neutrophils in innate and adaptive immunity (Odobasic *et al.*, 2016). The MPO values of the goldfish serum were significantly increased ($P < 0.05$) in the fish group treated with 3.0% and 5.0% of *S. alba* extract supplementation feeds on weeks 3 and 4 (present data). However, such changes in the MPO activity were not significant in the fish group treated with 1% supplementation feed. Moreover, an obvious decline in the MPO level was observed in the untreated infected fish, which may be attributed to the invasive spread of *A. invadans* in the goldfish. In *C. catla*, Kumar *et al.* (2015) showed that there is a significant increase in the MPO activity when the EUS-infected catla were fed with the leaf extract of heartleaf hempvine, *Mikania cordata* (Burm. f.).

Many plant-based products have been found to have non-specific immuno-modulatory effects in animals (Pandey *et al.*, 2012), to improve the innate system, and may serve as general immune prophylactic control in organic aquaculture (Magnadottir, 2010). Nowadays, crude plant extracts with antibacterial activity are potentially to be beneficial alternatives in aquaculture because they may provide cheaper treat-

ment sources, which are less toxic than chemotherapeutic agents (Maqsood *et al.*, 2011). The present study demonstrated that the immune system and survival rate of the *A. invadans*-infected goldfish may have been elevated when the fish were fed with 3.0% to 5.0% of *S. alba* leaf extract supplementation diets. This study may also provide an insight for upcoming research on the applications of different dosages of *S. alba* leaf extract in other EUS-susceptible fish, and the identification of specific bioactive compounds present in its leaf extract for potential development of antifungal compounds.

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Conflict of Interest

The authors declare no conflict of interests.

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