Enhancement of immune responses of rainbow trout (*Oncorhynchus mykiss*) fed a diet supplemented with *Aloe vera* extract

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Abstract

The effects dietary supplementation with *Aloe vera* extract (AE) on the immunity responses and hematological parameters of rainbow trout (*Oncorhynchus mykiss*) fry were evaluated in eight weeks trial. 600 rainbow trout (*O. mykiss*) fry with an average initial body weight of 13±0.05g were randomly allocated into two treatment groups including placebo-treated group (control) and *A. vera* extract-treated group, each with three replicates. The fish were hand-fed once a day with diet medicated AE or placebo (70 % lactose, 10 % starch and 20 % talc) at a rate of 1% of feed weight in the first feeding for 8 weeks. At the end of the identical every two weeks (2, 4, 6, and 8 weeks) 24 h after feeding, some of immunological and hematological parameters were analyzed. The results showed that serum total protein, albumin and globulin, respiratory burst activity, phagocytic activity and serum lysozyme activity vary among the two treatment groups which were found to be higher in the AE-treated group (p<0.05). However, there were no significant differences in none of hematological parameters between two groups. It was concluded that supplementation of AE at a rate of 1% in feed registered higher immunological responses in compared to placebo group. Therefore, supplementation of AE in fish diet would enhance the immunity responses in fish. It may use in fish diets particularly at time of outbreaks.

Keywords: Immune responses, *Aloe vera*, *Oncorhynchus mykiss*, Herbal medicine

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Introduction

In recent years, with worldwide fish production and intensive cultivation systems, fish are subjected to many diseases that lead to great losses and decreases in fish production. The lack of effective disease control has the potential of being the chief limiting factor of the realization of highly stable fish productions (Phillip *et al*., 2000). Rainbow trout (*Oncorhynchus mykiss*) is one of the cold water aquaculture species in Iran. Development of an economical artificial diet to accelerate growth and maintain the health status of this fish is of major importance for sustainable rainbow trout culture. Fish diseases are a serious threat to economic viability of any aquaculture practice. Currently, the commercial aquaculture industry prefers to reduce the cost of production due to the use of antibiotics for the prevention and treatment of diseases and excessive use of growth hormones to accelerate growth. However, the development of antibiotic resistant bacterial strains, accumulation of residues in cultured fish and environmental problems associated with the use of chemicals have led to the investigation of suitable methods of disease management. Therefore, a new approach to immunotherapy is actively used to prevent or treat fish diseases, increase disease resistance, feed efficiency and growth performance of fish in a sustainable aquaculture industry (Sakai, 1999). In this regard, extensive research has been carried out to test the new compounds leading to the development of the aquaculture industry. It has been proved that the use of medicinal herbs in fish diets enhance the immune system against infections from various bacteria, especially, *Aeromonas hydrophila* in different species of fish which is of the major bacterial pathogen, leading to heavy mortality rate (Karunasagar *et al.*, 1997; Kumar and Dey, 1988; Ahamad *et al.*, 2011) and a decrease in productivity efficiency, causing high economic loss to fish farmers (Castro *et al.*, 2008).

Aloe vera (synonym: *Aloe barbadensis* Miller) belonging to the family liliaceae is widely distribution in the tropical and subtropical regions of the world. Most *Aloe* species are indigenous to Africa, but now have wide distribution in the tropical and subtropical regions of the world (Haller, 1990). The genus *Aloe* contains over 400 different species and *A. barbadensis* Miller is considered to be the most biologically active. Cosmetics and some medicinal products are made from the mucilaginous tissue in the center of the *A. vera* leaf called *A. vera* gel. The peripheral bundle sheath cells of *A. vera* produce intensely bitter, yellow latex, commonly termed aloe juice, of sap, or aloes. *A. vera* sap is confused with *Aloe vera* gel. Unlike aloes, *A. vera* gel contains no anthraquinones, which are responsible for the strong laxative effects of aloes. However, total leaf extracts may contain anthraquinones. Although most commercially- available products are

based on the gel, the British Pharmacopoeia does not contain an entry for *A. vera* gel but it does describe aloes (Marshall, 1990). Alishahi *et al.* (2010) showed that dietary *A. vera* enhanced both specific and non-specific immune response in common carp when exposed to *A. hydrophila* pathogen. Reportedly, *A. vera* extracts improved growth performance in tilapia (Gabriel *et al.*, 2015), common carp (Mahdavi *et al*., 2013), and rainbow trout (Heidarieh *et al*., 2013). Moreover, Gabriel *et al*. (2015) reported dietary *A. vera* improves plasma lipid profile, antioxitant, and hepatoprotective enzyme activities in GIFT-tilapia (*Oreochromis niloticus*) after a *Streptococcus iniae* challenge. Farahi *et al*. (2012) also showed the protective effects of *A. vera* extract against lipid peroxidation in common carp and rainbow trout. The objective of the present study was to evaluate the effects of *A. vera* extract (AE) on the immune responses in rainbow trout (*O. mykiss*) to develop an alternative drug for the treatment of diseases in aquaculture.

Materials and methods

Preparation of Aloe vera extract

The *Aloe vera* plant was procured from the market and plant species was identified and confirmed by a botanist. The leaves were collected and washed in sterile distilled water and the gel was extracted. The leaves were separately shade-dried for 10 days till weight constancy was achieved. The sample was powdered in an electric blender. The extract was prepared with the standard method of percolation. To do this, chopped dried plant leaves in 80% ethanol were percolated for 72 hours. Then, the slurry was filtered through Whattman No. 1 filter paper and centrifuged for 5 min at 5000 rpm. The filtrate was obtained from ethanol using a rotary device, and the excess solvent was separated from the extract. These crude extract was stored at 4ºC until use (Sigei *et al*., 2015).

The formulated fish feed was prepared using the standard fish diet (Behparvar Co) (50% crude protein, 18% crude lipid, 1.9% fiber, 1.3% total phosphorus, 8.3% ashes, and 14.8% nitrogen free extract) with dried *A. vera* extract or placebo at a ratio 1% of food weight and mixed part by part in a drum mixer. Sufficient water along with the oil ingredients was then added to make a paste of each diet. Then it was pelleted and allowed to cool and dry. The pellets were air dried and stored in air tight containers until fed.

Fish and experimental conditions

600 rainbow trout, each weighing 13±0.05 g were used. All experiments were carried out in 1,000 liter circular concrete ponds with a continuous water flow of 2.5 liter per second. The fish were kept at ambient conditions, including uncontrolled water temperature of 15±1ºC, dissolved oxygen of 7.2±0.2 mg l^{-1} and pH 8±0.3. After 2 weeks adaptation, fish were randomly allotted in two groups

including an experimental group and a control group, each group was run in triplicate, and maintained in 6 concrete ponds each containing 100 fish. Each group was hand-fed once a day with diet medicated *A. vera* extract, or placebo (70% lactose, 10 % starch and 20 % talc) prepared in the laboratory at a rate 2% of body weight for 10 weeks and three times with standard diet.

Sampling and serum collection

During sampling, fish were rapidly netted, tranquillized with 50 mg/L of tricaine methane sulfonate (MS_{222}, Sigma chemical Co. St. Louis, MO, USA). Fish were bled from caudal vein using 1mL insulin syringe fitted with 24 gauge needle. To minimize the stress to fish, 1 mL of blood was drawn and the whole bleeding procedure was completed within 1 min. A total number of 15 blood samples were collected from 15 fish in each group (5 samples from each replicate) at the end of every 2 weeks, 24 h after final feeding period. The blood pooling of 5 fish from each replicate was divided into 2 half blood samples. The first half was collected in serological tubes containing a pinch of lithium heparin powder, shaken gently and kept at 4ºC to test hematological parameters. The other half was collected in tubes without an anticoagulant and allowed to clot at 4ºC for 2hrs to test serological parameters. The clot was the spun down at 2000g for 10 min to separate the serum. The sera were collected by micropipette and were stored in sterile Eppendorf tubes at -20ºC until used.

Biochemical assay

Serum total protein content was estimated photometrically by citrate buffer and bromocresol green (BCG) dye binding method (Dumas *et al.*, 1971) using the kit (total protein and albumin kit, Pars Azmun Company, Iran). Albumin was determined by BCG binding method. The absorbance of standards and tests were measured against a blank in a spectrophotometer at 546 nm. Globulin level was calculated by subtracting albumin values from total serum protein. Albumin/globulin (A/G) ratio was calculated by dividing albumin values by globulin values.

Immunological assay

Separation of leukocytes from the blood

Leucocytes for the assay were separated from each blood sample by density-gradient centrifugation. One milliliter of histopaque 1.119 (Sigma) containing 100µL of bactohemagglutination buffer, pH 7.3 (Difco, USA) was dispensed into siliconised tubes. 1 mL of a mixture of 1.077 density histopaque and hemagglutination buffer and 1 mL of blood was carefully layered on the top. The sample preparations were centrifuged at 850g for 15 min at 4°C. After centrifugation, plasma was collected and stored at 80°C for future analysis; separated leukocytes were gently removed and dispensed into siliconised tubes,

containing phenol red free Hanks Balanced Salt Solution (HBSS, Sigma). Cells were then washed twice in HBSS and adjusted to 2×10^6 viable cells/mL.

Respiratory burst activity

Respiratory burst activity of isolated leukocytes was quantified by reduction of ferricytochrome C (Secombes, 1990). Briefly, 100µL of leukocyte suspension and an equal volume of cytochrome C (2 mg/L in phenol red free HBSS) containing phorbol 12-myristate 13-acetate (PMA, Sigma) at 1µg/mL were placed in triplicate in micro titer plates. In order to test specificity, another 100µL of leukocyte suspensions and solutions of cytochrome c containing PMA and superoxide dismutase (SOD, Sigma) at 300 U/mL were prepared in triplicate in micro titer plates. Samples were then mixed and incubated at room temperature for 15 min. Extinctions were measured at 550nm against a cytochrome C blank in a multiscan spectrophotometer. Readings were converted to nmoles O_2 by subtracting the O.D. of the PMA/SOD treated supernatant from that treated with PMA given alone for each fish, and converting O.D. to n moles O_2 by multiplying by 15.87. Final results were expressed as nano moles O_2 produced per 10^5 blood leukocytes.

Phagocytosis assay

Phagocytosis activity of blood leukocytes was determined spectrophotometrically by the method of Seeley *et al*. (1990). This assay involves the measurement of congo red-stained yeast cells which have been phagocytised by cells. To perform the assay, 250 µL of the leukocyte solution was mixed with 500µL of the congo red-stained and autoclaved yeast cell suspension (providing a yeast cell: leukocyte ratio of 40:1). The mixtures were incubated at room temperature for 60 min. Following incubation, 1 mL ice-cold HBSS was added and1 mL of histopaque (1.077) was injected into the bottom of each sample tube. The samples were centrifuged at 850g for 5 min to separate leukocytes from free yeast cells. Leukocytes were harvested and washed two times in HBSS. The cells then were resuspended in 1 mL trypsin-EDTA solution (5.0g/l trypsin and 2.0g/l EDTA, Sigma) and incubated at 37°C overnight. The absorbance of the samples was measured at 510 nm using trypsin-EDTA as a blank.

Serum lysozyme assay

In this study, an assay based on the lysis of *Micrococcus lysodeikticus* was used to determine the lysozyme activity. Serum lysozyme activity was measured spectrophotometrically according to the method Parry *et al*. (15). Briefly, 0.02% (w/v) lyophilized *Micrococcus lysodeikticus* in 0.05 mM solution phosphate buffer (pH 6.2) was used as substrate. 10µL of fish serum was added to 250µl of bacterial suspension and reduction in absorbance at 490 nm was determined after 0.5 and

4.5 min of incubation at 25ºC using a microplate reader. A unit of lysozyme activity was defined as the amount of sample causing a decrease in absorbance of 0.001 per min.

A new standard curve was prepared for each assay. Standard solutions as well as samples were added to the substrate at 25°C. The results were expressed as mg/mL equivalent of hen egg white enzyme activity.

Statistical analysis

Results are presented as the average (Standard error mean) for five fish, and were compared at each time point using Student's t test for independent data. Values for each parameter measured were expressed as mean±standard error of mean. Significant differences between experimental groups were expressed at a significance level of $p<0.05$.

Results

Biochemical analysis

A. vera extract showed a significant ($p<0.05$) increase in total protein (TP), albumin (AL), and globulin (GL), at the end of every identical two weeks after feeding when compared to the placebo group (Table 1). The maximum level of total protein was recorded on week 2 of exposure duration. Similarly, albumin and globulin contents were significantly higher in the *A. vera* group as compared to the placebo group. Albumin/globulin ratio did not exhibit significant differences in comparison to the placebo group at the end of every identical two weeks after feeding ($p>0.05$; Table 1).

Immunological analysis

The respiratory burst activity significantly ($p<0.05$) enhanced in fish fed with 1% of *A. vera* extract supplemented feed at the end of the identical every two weeks after feeding in compared to placebo group (Fig. 1). Phagocytic activity of blood leucocytes significantly ($p<0.05$) enhanced in fish treated with 1% of *A. vera* extract supplemented feed at the end of every identical two weeks after feeding compared to that in the placebo group (Fig. 2). Lysozyme activity significantly ($p<0.05$) enhanced in fish fed with 1% of *A. vera* extract supplemented feed at the end of every identical two weeks after feeding compared to that in the placebo group (Fig. 3).

Table 1: Changes in the serum total protein, albumin, globulin and albumin/globulin ratio of rainbow trout after feeding with 1% placebo, or *Aloe vera* extract for 8 weeks.

Week	Groups	Total protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)	Albumin/globulin ratio (g/dL)
2	Placebo	3.26±0.03	1.31±0.01	1.95 ±0.02	0.67±0.01
	Aloe vera extract	3.58±0.03*	1.44±0.03*	2.14±0.01*	0.67±0.02
4	Placebo	3.59±0.05	1.49±0.04	2.10 ±0.02	0.70±0.04
	Aloe vera extract	3.73±0.05*	1.59±0.02*	2.14±0.03*	0.74±0.02
6	Placebo	3.87±0.09	1.53±0.02	2.34 ±0.03	0.65±0.03
	Aloe vera extract	4.28±0.07*	1.63±0.04*	2.65 ±0.04*	0.61±0.02
8	Placebo	3.88±0.05	1.60±0.05	2.28 ±0.04	0.70±0.03
	Aloe vera extract	4.82±0.02*	1.86±0.06*	2.96 ±0.02*	0.62±0.03

Data are expressed as mean±SE (n=15). *: $p<0.05$ compared with the placebo at the end of every identical two weeks**.**

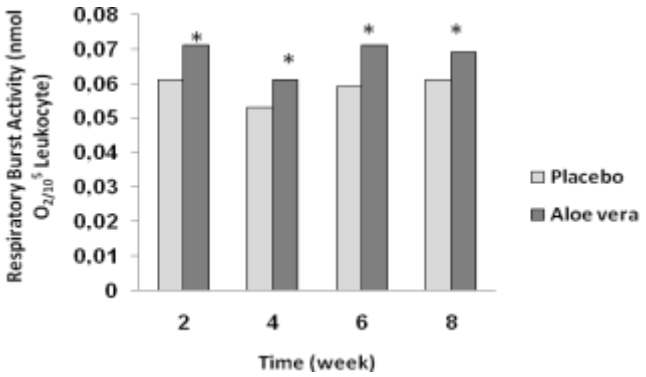

Figure 1: Respiratory burst activity of different experimental groups observed on different weeks. Data are expressed as mean±SE (n=15). Asterisks indicate significantly different from placebo in the same week. *$p<0.05$.

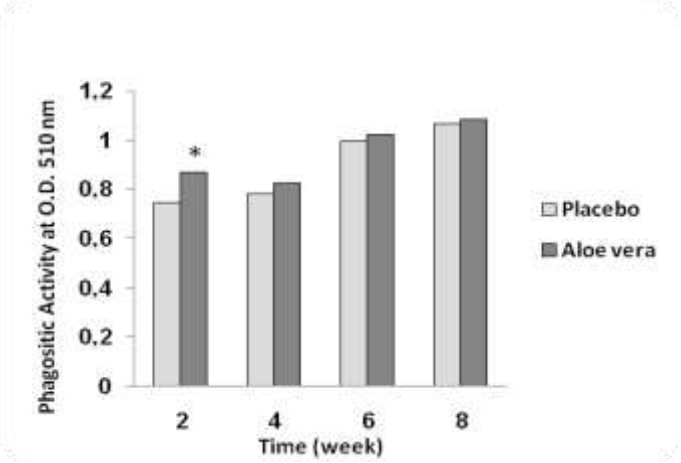

Figure 2: Phagocytic activity of different experimental groups observed on different weeks. Data are expressed as mean±SE (n=15). Asterisk indicates significantly different from placebo in the same week. *$p<0.05$.

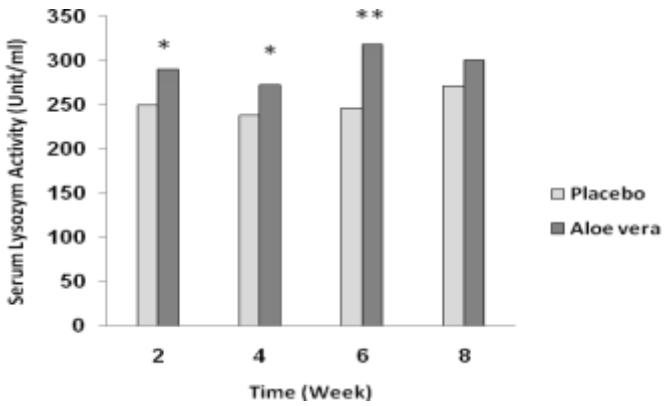

Figure 3: Serum lysozyme activity of different experimental groups observed on different weeks. Data are expressed as mean±SE (n=15). Asterisks indicate significantly different from placebo in the same week. *p<0.05; **p<0.001.

Discussion

The present study projects the impact of the dried *A. vera* extract on the immunological responses in rainbow trout (*Oncorhynchus mykiss*). The results of this study showed that feeding rainbow trout with 1% dose of dried *A. vera* extract for 8 weeks enhanced total plasma protein, albumin and globulin values in comparison with control group in the same weeks. Similar results were reported in rainbow trout fed garlic (Nya and Austin, 2009a), ginger (Nya and Austin, 2009b), lipopolysaccharide (Nya, 2009), *Laurus nobilis* (Bilen and Bulut, 2010), *Coggyria coggyria* (Bilen *et al.*, 2011), and *Nigella sativa* (Awad *et al.*, 2013). In addition, several medicinal herbs have been reported to significantly enhance serum total protein in different fish species such as *Pedalium murex* in *Labeo rohita* (Ojha *et al.*, 2014) and *Azadirachtra indica* (Neem) leaf in Asian seabass, *Lates calcarifer* (Talpur and Ikhwanuddin, 2013). Serum proteins are various humoral elements of the non-specific immune system, measurable total protein, albumin, and globulin levels suggesting that high concentrations of total serum protein, albumin and globulin are likely to be a result of the enhancement of the non-specific immune response of fish as a consequence of feeding dried *A. vera* extract. So, this study revealed that *A. vera* extracts incorporated in fish diet triggered the humoral elements in the serum. Globulin is the main resourse of immunoglobulin production, thus its enhancement in serum provides an immunostimulatory potential (Sahu *et al.*, 2006).

Respiratory burst activity is considered as an important indicator of non-specific defense in fish, which is a measure of the increase of oxidation level in phagocytes stimulated by foreign agents (Nematolahi *et al.*, 2005). Phagocytosis and respiratory burst response by phagocytes in blood present a major antibacterial defense

mechanism in fish (Secombes, 1996). The phagocytosis in fish is triggered by neutrophils and macrophages mainly by the production of reactive oxygen species (ROS) during a respiratory burst. The present study showed an enhancement of respiratory burst activity in the treated group in comparison with the placebo group which is in agreement with the results of some of studies dietary immunostimulants used in various fish species (Yadava *et al.,* 1992; Gopalakannan and Arul, 2006; Sahu *et al.,* 2007). It has also been shown that phagocytic activity of leucocytes was enhanced by *A. vera* in common carp (Alishahi *et al.,* 2010), dietary powdered ginger rhizome (Dügenci *et al.,* 2003; Haghighi and Sharifrohani, 2013), and zeranol (Keles *et al.*, 2002) in rainbow trout (*O. mykiss*), *Curcuma zedoaria* and *Zingiber zerumbet* in grouper *Epinephelus coioides* (Nan *et al.,* 2015), *Chlorella vulgaris* in *Salmo trutta Caspius* (Saberi *et al.,* 2017).

Lysozyme is an important enzyme in the blood that actively lyses bacterial cell wall (peptidoglycan), and it is known to act as an opsonin and activate the complement system as well as phagocytes (Magnadottir, 2006). The results of this study showed that lysozyme activity was significantly enhanced in fish fed with 1% of *A. vera* extract supplemented feed at the end of every identical two weeks after feeding which are in agreement with several reports indicating the role of herbal immunostimulants in enhancing lysosyme activity (Nan *et al.,* 2015; Hwang *et al.,* 2013; Talpur and Ikhwanuddin, 2013; Dotta *et al.,* 2014).

It was concluded that supplementation of *A. vera* extract at a rate of 1% in fish diets registered higher immunological responses compared to the placebo group. Therefore, supplementation of *A. vera* extract in fish diets enhances non-specific immune system in fish. *A. vera* extract may be used in fish diets particulary at the time of outbreaks.

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