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Haemato–immunological indices in rainbow trout (*Oncorhynchus mykiss*) fry fed with *Aloe vera* extract supplemented feed

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PEER REVIEW

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Comments

Paper is innovative suggesting preliminary study. The research presented in this paper is valuable, since this paper only shows the effect on immune rather than disease control after challenge. Over all the research has fascinating data.

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ABSTRACT

Objective: To evaluate the effects of *Aloe vera* extract on the immunity responses and haematological parameters in rainbow trout (*Oncorhynchus mykiss*) fry to develop alternative drug to chemotherapeutics in aquaculture.

Methods: Six hundred rainbow trout (*Oncorhynchus mykiss*) fry were randomly allocated into two treatment groups including 1) placebo–treated group (control), 2) *Aloe vera* extract–treated group, each of three replicates. The fishes were hand–fed once a day with diet medicated AE or placebo at the rate of 1% in feed in the first feeding for 10 weeks. At the end of the identical every two weeks 24 h after feeding, some of haematological and immunological parameters were analyzed.

Results: 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 *Aloe vera* extract–treated group ($P < 0.05$). However, there were no significant differences in none of haematological parameters between two groups ($P > 0.05$).

Conclusions: 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 diets enhances non–specific immune system in fish. It may use in fish diets particularly at time of outbreaks.

KEYWORDS

Herbal immunostimulant, Iranian medicinal plants, *Aloe vera*, Fish

1. Introduction

Rainbow trout [*Oncorhynchus mykiss* (*O. mykiss*)] is the most preferred coldwater species in aquaculture industry of Iran. Achieving sustainable development in rainbow trout culture, to maintain the health status of this fish is of major importance. Fish pathogenic organisms are serious

threats to economic viability of any aquaculture practice. Currently, the use of antibiotics for the prophylaxis and treatment of diseases leads to the development of antibiotic resistant bacterial strains, accumulation of residue in cultured fish and environmental problems. Therefore, a new approach to immunotherapy is actively used to prevent or cure fish diseases. In this regard, extensive research has

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been carried out to test various immunostimulants including medicinal plants which they have found to be effective in fish. It has been found that to use medicinal herbs in fish diets enhances the immune system against infections with various bacterial^[1–4].

Aloe vera (*A. vera*) (synonym: *Aloe barbadensis* Miller) belonging to family Liliaceae is widely distribution in the tropical and subtropical regions of the world. Most of *Aloe* species are indigenous to Africa, but now have wide distribution in the tropical and subtropical regions of the world^[5]. The genus *Aloe* contains over 400 different species and *Aloe barbadensis* Miller is considered to be the most biologically active^[6]. Cosmetic and some medicinal products are made from the mucilaginous tissue in the center of the *A. vera* leaf and called *A. vera* gel. The peripheral bundle sheath cells of *A. vera* produce intensely bitter, yellow latex, commonly termed *Aloe* juice, sap, and aloes. *A. vera* sap and gel are often confused. Unlike aloes, *A. vera* gel contains no anthraquinones, which are responsible for the strong laxative effects of aloes. However, extracts may contain anthraquinones which are commonly found in the total leaf. 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^[7]. Acemannan and the other constituents of *A. vera* have been found to improve macrophage activity as much as ten fold, to enhance macrophage effectiveness in modulating the entire immune system, in stimulating, producing, and releasing antibodies^[8]. This study was undertaken to evaluate the effects of *A. vera* extracts on the immunity responses and haematological parameters in rainbow trout (*O. mykiss*) to develop an alternative drug to chemotherapeutics in aquaculture.

2. Materials and methods

2.1. Preparation of *Aloe* extract

The plant of *A. vera* was procured from Medicinal Plants Production Cooperation of Havin and plant species was identified and confirmed by a botanist. The leaves were collected and washed in sterile distilled water and evacuated from gel. The leaves were separately shade-dried for 10 d 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 h. Then, the slurry was filtered with Whatman No. 1 filter paper (11 µm) and centrifuged for 5 min at 5000 r/min. The filtrate obtained from ethanol using a rotary device and the excess solvent was separated from the extract. These crude extracts were stored at 4 °C until use.

2.2. Supplementation of the normal diet with dried *A. vera* extract

The formulated fish feed was prepared using the normal fish diet (50% crude protein, 18% crude lipid, 1.9% fiber, 1.3% total phosphorus, 8.3% ashes, and 14.8% nitrogen free extract) with 1% dried *A. vera* extract or placebo in feed and mixing part by part in a drum mixer. Sufficient water along with the oil ingredients was then added to make a paste of each diet. After, it was pelleted (2.5 mm) and the pellets were allowed to cool dry. The pellets were air dried and stored in air tight containers until fed.

2.3. Fish and experimental conditions

Six-hundred rainbow trouts weighing (13.00±0.05) g were used. All experiments were carried out in 1000 L round ponds with a continuous water flow of 0.5 L per second. The fish were kept at an ambient, including uncontrolled water temperature of (15±1) °C, dissolved oxygen of (7.2±0.2) mg/L and pH (8.0±0.3). After 2 weeks adaptation, fish was randomly allotted in two groups including an experimental group and a control group, in triplicate and was maintained in 6 round ponds containing 100 fish for each. Each group was hand-fed once a day with diet medicated 1% of *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.

2.4. Bleeding and serum collection

During bleeding, fish were rapidly netted, tranquillized with 50 mg/L of tricaine methanesulfonate (MS222, Sigma chemical Co. St. Louis, MO, USA). Fish were bled from caudal vein using 1 mL insulin syringe fitted with 24 gauge needles. 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 halves. Half was collected in serological tubes containing a pinch of lithium heparin powder, shaken gently and kept at 4 °C to test hematological parameters. Other half collected in tubes without anticoagulant and allowed to clot at 4 °C for 2 h to test serological parameters. The clot was the spun down at 2000 r/min for 10 min to separate the serum. The serum was collected by micropipette and was stored in sterile Eppendorf tubes at –20 °C until used for assay.

2.5. Haematological assay

Blood samples were analyzed with routine methods adopted in fish haematology^[9]. The total red blood cell

counts ($\text{RBC} \times 10^6/\mu\text{L}$) were determined in a 1:200 dilution of the blood sample in Hayem's solution and total white blood cell counts ($\text{WBC} \times 10^3/\mu\text{L}$) in a 1:20 dilution of the blood sample with a Neubauer hemocytometer. The haematocrit (Hct) and leukocyte percentages were determined in duplicate by using micro Hct–heparinized capillary tubes of 75 μL volume and a micro Hct centrifuge at 15000 r/min for 5 min^[10]. The percentages of erythrocyte (Hct) and leukocyte volumes were calculated by overlaying the tubes on a sliding scale Hct reader. The haemoglobin (Hb, g/dL) concentrations were determined by the cyanmethemoglobin method using a Hb reagent set (Ziest Chem Diagnostics)^[11]. The values of red blood cell indices, the mean values of cell Hb (MCH, pg), cell Hb concentration (MCHC, %), and cell Hb volume (MCV, fL) were calculated according to Wintrobe formulae^[12]. The differential leukocytes count was carried out using blood smears stained with Wright–Giemsa. The percentage composition of leukocytes was determined based on their identification characters listed by Ivanava^[13].

2.6. Biochemical assay

Serum total protein content was estimated photo metrically by citrate buffer and bromocresol green (BCG) dye binding method using the kit (total protein and albumin kit, Pars Azmun Company, Iran)^[14]. Albumin was determined BCG binding method. The absorbance of standard and test were measured against blank in a spectrophotometer (Thermo Electron Corporation, HeLios α , UK) 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.

2.7. Immunological assay

2.7.1. Separation of leukocytes from the blood

Leukocytes for assay were separated from each blood sample by density–gradient centrifugation. One milliliter of histopaque 1.119 (Sigma) containing 100 μL of bacto hemagglutination buffer, pH 7.3 (Difco, USA) was dispensed into siliconized tubes. One mililiter 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 2500 r/min 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 siliconized 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^[15].

2.7.2. Respiratory burst activity

Respiratory burst activity of isolated leukocytes was

quantified by reduction of ferricytochrome C^[16]. Briefly, 100 μL of leukocyte suspension and an equal volume of cytochrome C (2 mg/L in phenol red free HBSS) containing phorbol 12–myristate 13–acetate (Sigma) at 1 $\mu\text{g}/\text{mL}$ were placed in triplicate in microtiter plates. In order to test specificity, another 100 μL of leukocyte suspensions and solutions of cytochrome C containing phorbol 12–myristate 13–acetate and superoxide dismutase (Sigma) at 300 U/mL were prepared in triplicate in microtiter plates. Samples were then mixed and incubated at room temperature for 15 min. Extinctions were measured at 550 nm against a cytochrome C blank in a multiscan spectrophotometer. Readings were converted to nano moles O_2 by subtracting the optical density of the PMA/SOD treated supernatant from that treated with PMA given alone for each sample, and converting optical density to nano moles O_2 by multiplying by 15.87. Final results were expressed as nano moles O_2 produced per 10^5 blood leukocytes.

2.7.3. Phagocytosis assay

Phagocytosis activity of blood leukocytes was determined spectrophotometrically according to Seeley *et al*^[17]. This assay involves the measurement of Congo red–stained yeast cells which have been phagocytosed by cells. To perform the assay, 250 μL of the leukocyte solution was mixed with 500 μL 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 and 1 mL of histopaque (1.077) was injected into the bottom of each sample tube. The samples were centrifuged at 2500 r/min for 5 min to separate leukocytes from free yeast cells. Leukocytes were harvested and washed twice in HBSS. The cells then were resuspended in 1 mL trypsin–ethylene diamine tetraacetic acid (EDTA) solution (5.0 g/L trypsin and 2.0 g/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 via spectrophotometer (Thermo Electron Corporation, HeLios α , UK).

2.7.4. Serum lysozyme assay

In this study, an assay based on the lysis of micrococcus lysodeikticus was used to determin the lysozyme activity. Serum lysozyme activity was measured spectrophotometrically according to the method of Parry *et al*^[18]. Briefly, 0.02% (w/v) lyophilized micrococcus lysodeikticus in 0.05 mmol/L solution phosphate buffer (pH 6.2) was used as substrate. Fish serum (10 μL) was added to 250 μL of bacterial suspension and reduction in absorbance at 490 nm was determined after 0.5 and 4.5 min of incubations at 25 °C using a microplate reader. One unit of lysozyme activity was defined as the amount of enzyme causing a decrease in absorbance of 0.001 per min.

2.8. Statistical analysis

All results for each parameter measured were expressed as means±standard errors, and were compared at each time point using Student's *t*-test for independent data. Significant differences between experimental groups were expressed at a significance level of *P*<0.05. All analyses were carried out on 15 fish per group.

3. Results

3.1. Hematological analysis

Dietary *A. vera* extract incorporated test diets had no significant (*P*<0.05) effect on RBC, WBC, differential leukocytes count (monocyte, lymphocyte and neutrophile), Hct, Hb, the all the values of red blood cell indices, MCH, MCHC, and MCV at the end of none of the identical two weeks after feeding in comparison with placebo group (Table 1).

3.2. Biochemical analysis

A. vera extract had significant (*P*<0.05) effect in increase of total protein, albumin, and globulin, at the end of the identical every two weeks after feeding in compared to placebo group (Table 2). The maximum level of total protein was recorded on Week 2 of exposure duration. Similarly, albumin and

globulin contents were significantly higher in *A. vera* group as compared to placebo group. However, albumin/globulin ratio was not exhibited significant differences in compared to placebo group at the end of the identical every two weeks after feeding in compared to placebo group (*P*>0.05, Table 2).

3.3. Immunological analysis

3.3.1. Respiratory burst activity

Respiratory burst activity significantly (*P*<0.05) enhanced in fish treated with 1% of *A. vera* extract supplementation feed at the end of the identical every two weeks after feeding as compared to placebo group (Figure 1).

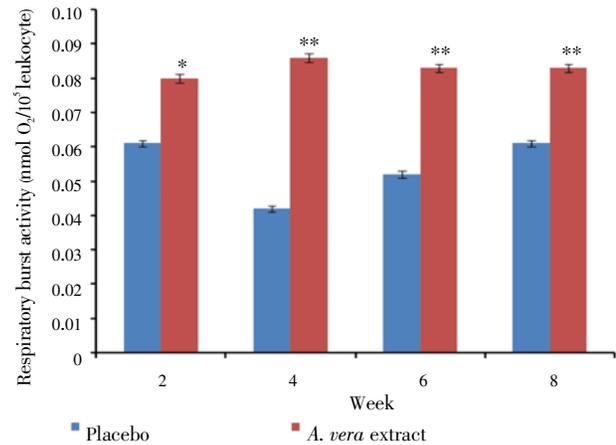


Figure 1. Respiratory burst activity of different experimental groups observed on different weeks.

Data are expressed as mean±SE (n=15). Astrisk indicate significantly different from placebo in the same week. **P*<0.05; ***P*<0.0001.

Table 1

The haematological parameters, WBC, RBC, Hct, Hb, MCH, MCV, MCHC, neutrophil, monocyte, and lymphocyte of rainbow trout fed with 1% *A. vera* exteact, or placebo in feed for 10 weeks.

Week	Groups	WBC (10 ³ /mL)	RBC (10 ⁶ /mL)	Hct (%)	Hb (g/dL)	MCH (pg)	MCV (fL)	MCHC (%)	Neut (%)	Mon (%)	Lymp (%)
2	Placebo	3.90±0.70	0.98±0.06	27.33±3.30	9.07±0.60	92.55±2.70	278.80±16.00	3.31±0.40	7.66±0.10	3.00±0.30	89.00±1.20
	<i>A. vera</i> exteact	4.11±0.10	1.02±0.07	28.33±1.90	9.77±0.70	95.78±2.10	277.70±13.00	3.44±0.20	7.33±0.40	2.66±0.30	90.30±0.90
4	Placebo	4.00±0.10	1.69±0.05	40.50±4.10	13.50±0.90	79.88±3.10	239.60±13.00	3.33±0.30	8.33±0.10	2.33±0.20	89.00±2.00
	<i>A. vera</i> exteact	4.00±0.20	1.77±0.05	40.50±3.20	13.10±1.10	74.01±3.50	228.80±10.00	3.23±0.50	7.66±0.40	2.33±0.40	90.00±1.00
6	Placebo	4.20±0.50	1.69±0.03	36.00±2.90	12.00±1.50	71.00±3.00	213.00±6.00	3.33±0.30	7.66±0.30	3.33±0.30	89.00±2.10
	<i>A. vera</i> exteact	4.56±0.40	1.79±0.03	38.66±3.60	12.66±0.60	70.72±2.60	215.90±9.00	3.27±0.30	8.66±0.50	3.66±0.10	88.00±1.30
8	Placebo	4.95±0.10	1.53±0.03	33.00±3.50	10.98±1.80	71.76±3.90	215.60±8.00	3.32±0.20	8.00±0.10	2.33±0.20	89.60±1.20
	<i>A. vera</i> exteact	5.03±0.30	1.55±0.05	38.33±2.80	12.68±1.80	81.80±3.60	247.20±11.00	3.30±0.20	7.66±0.30	2.00±0.40	90.30±0.50
10	Placebo	4.85±0.30	1.45±0.06	39.33±2.30	13.10±2.60	90.34±2.10	271.20±13.00	3.07±0.20	7.00±0.50	3.00±0.30	90.00±0.70
	<i>A. vera</i> exteact	4.18±0.30	1.51±0.03	39.66±3.00	13.16±1.60	87.15±5.30	262.60±12.00	3.31±0.20	7.33±0.50	2.66±0.10	90.00±0.80

Data are expressed as mean±SE (n=15). No significant differences were observed in the *A. vera* treated groups relative to the placebo group at the end of the identical every two weeks after feeding (*P*>0.05). Neut: neutrophil; Mon: Monocyte; Lymp: Lymphocyte.

Table 2

Changes in the serum total protein, albumin, globulin and albumin/globulin ratio of rainbow trout after feeding with 1% placebo, or *A. vera* extract for 10 weeks.

Week	Groups	Total protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)	Albumin/globulin ratio (g/dL)
2	Placebo	4.10±0.01	1.80±0.08	2.20±0.08	0.81±0.01
	<i>A. vera</i> exteact	4.47±0.03*	2.00±0.08*	2.47±0.05*	0.81±0.01
4	Placebo	4.03±0.01	1.80±0.04	2.23±0.08	0.80±0.02
	<i>A. vera</i> exteact	4.45±0.03*	1.99±0.03*	2.46±0.07*	0.80±0.01
6	Placebo	3.40±0.02	1.53±0.06	1.87±0.01	0.81±0.01
	<i>A. vera</i> exteactv	3.93±0.03*	1.75±0.05*	2.18±0.09*	0.80±0.02
8	Placebo	3.93±0.01	1.75±0.06	2.18±0.03	0.80±0.01
	<i>A. vera</i> exteact	4.33±0.01*	1.97±0.06*	2.36±0.02*	0.83±0.02
10	Placebo	3.26±0.03	1.50±0.03	1.76±0.07	0.85±0.03
	<i>A. vera</i> exteact	3.80±0.02*	1.77±0.05*	2.03±0.05*	0.87±0.02

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

3.3.2. Phagocytic activity

Phagocytic activity of blood leukocytes significantly ($P<0.05$) enhanced in fish treated with 1% of *A. vera* extract supplementation feed at the end of the identical every two weeks after feeding as compared to placebo group (Figure 2).

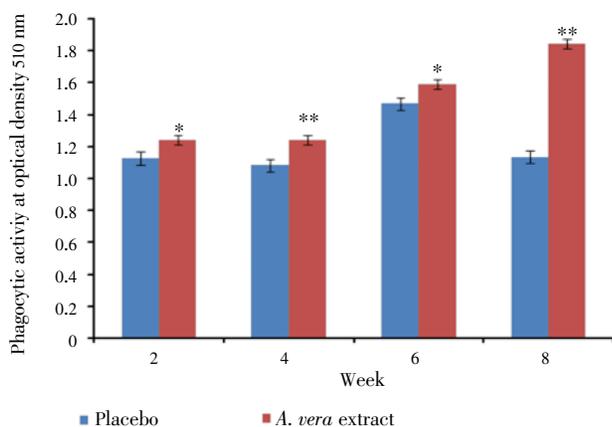


Figure 2. Phagocytic activity of different experimental groups observed on different weeks.

Data are expressed as mean±SE ($n=15$). Asterisk indicates significantly different from placebo in the same week. * $P<0.05$; ** $P<0.0001$.

3.3.3. Lysozyme activity

Lysozyme activity significantly ($P<0.05$) enhanced in fish treated with 1% of *A. vera* extract supplementation feed at the end of the identical every two weeks after feeding as compared to placebo group (Figure 3).

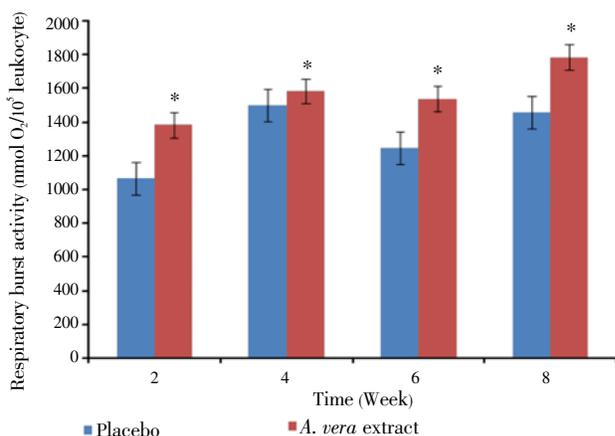


Figure 3. Serum lysozyme activity of different experimental groups observed on different weeks.

Data are expressed as mean±SE ($n=15$). Astrisk indicate significantly different from placebo in the same week. * $P<0.05$.

4. Discussion

Dietary medicinal plant extracts as immunostimulants elevate non-specific defenses against pathogens during period of stress. Haematological assay may provide an index of the physiology status of fish[19]. This study indicates the effects of dried *A. vera* extract on the haematological

parameters and immunological responses in rainbow trout (*O. mykiss*). In the present study, the haematological parameters such as RBC, WBC, differential leukocytes count, Hb, Hct, and the value of red blood cell indices including MCH, MCHC and MCV were no significant differences at the end of the identical every two weeks after feeding as compared to placebo group. These results are in consistent with the results obtained of Alishahi *et al.*, who reported common carp treated with dietary *A. vera* supplementation were no significant differences in RBC and Hct and this data also supports the study of Farahi *et al.* who reported no change in RBC and Hb in rainbow trout treated with dietary *A. vera* supplementation feed[20,21]. Also, in our study, there were no significant differences in WBC and differential leukocytes counts between experimental and placebo groups.

In the present study, dietary supplemented *A. vera* extract group enhanced total plasma protein, albumin and globulin values in comparison with placebo group. Similar results were reported in rainbow trout fed with garlic, ginger, lipopolysaccharide, *Laurus nobilis*, and *Coggyria coggyria*[22–26]. Serum proteins with various humoral elements of the non-specific immune system, measured total protein, albumin and globulin levels. This study suggests that high concentrations are likely to be a result of the enhancement of the non-specific immune response of fish as a consequence of feeding *A. vera*. This study revealed that *A. vera* extracts incorporated in fish diet triggered the humoral elements in the serum. Globulin is the main resource of immunoglobulin production, thus its enhancement in serum provide immunostimulatory potential[27]. Albumin/globulin ratio does not indicate significantly differences in treated group in compare to placebo group. Similar results were reported in *Cyprinus carpio* fingerlings after feeding levamisole diet[28].

The results obtained in this study showed an increasing trend in respiratory burst activity in treated group in comparison with placebo group which are in agreement the results of some of studies dietary immunostimulants used in various fish species[29,30]. Respiratory burst activity is considered as an important indicator of non-specific defense in fish, which is a measure of the increase oxidation level in phagocytes stimulated by foreign agents[31]. Respiratory burst and phagocytosis response by phagocytes in blood considered to have a major antibacterial defense mechanism in fish[32]. The phagocytosis in fish is triggered by neutrophils and macrophages mainly by the production of reactive oxygen species during a respiratory burst. In addition, neutrophils possess myeloperoxidase in their cytoplasmic granules which act as a defense barrier to kill the pathogenic bacteria by halogenations[33]. Moreover, these cells possess lysozymes and other hydrolytic enzymes

in their lysosomes^[33]. Similarly, macrophages can produce nitric oxide in mammals and can be as potent antibacterial agents, peroxy nitrates and hydroxyl groups^[34].

In the present study, the phagocytic activity of the blood leukocytes in *A. vera* extracted–treated group was significantly higher than the placebo group. Similar results were obtained in rainbow trout fed with other herbal immunostimulants^[35,36]. Also, in this study, an increasing trend in lysozyme activity has been shown in agreement with several reports indicating the role of herbal immunostimulants in enhancing lysozyme activity^[37,38]. Lysozyme is a humoral component of the non–specific defense mechanism which has the ability to prevent the growth of bacteria by splitting β –1, 4 glycosidic bonds in the peptidoglycan of bacterial cell walls.

In conclusion, supplementation of *A. vera* extract in fish diets would enhance non–specific immune system in fish. Therefore, further studies are necessary for effective use of *A. vera* extract with optimal dose, suitable duration, and method of administration.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

The immunostimulants increase the immune response, both in cell–mediate and humor. These are used as environmentally–friendly feed additives in the aquaculture. Increases in immune as a result of immunostimulant use, impart protection against disease.

Research frontiers

This paper describes the potent effects of *A. vera* on the immune response. Therefore immune primed feed receives attention in aquaculture. Effects of additive plant in this study depict preliminary data. Further research is needed for this plant with varied levels in fish diet.

Related reports

A. vera has been reported to trigger the immune in animals

due to activities of free radicals. This plant has worth of traditional medicine and commercial cosmetics due to its efficiency of beneficial compounds.

Innovations and breakthroughs

A. vera is considered as a useful medicinal plant because it contains lots of bioactive compounds. These bioactive compounds actively play a role for immune response. Finally health status of animal are enhanced. Therefore medicinal plants are gaining importance in aquaculture diet due to their positive effects with no harmful results.

Applications

A. vera has been used since centuries. It has no harmful effect both for human as well as for animals. Due to use full compounds contained in *A. vera*, its importance cannot be denied using as additive in aquaculture diet.

Peer review

Paper is innovative suggesting preliminary study. The research presented in this paper is valuable, since this paper only shows the effect on immune rather than disease control after challenge. Over all the research has fascinating data.

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