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Research Article

Immunological and Antistreptococcal Effects of *Salvia officinalis* and *Aloe vera* Extracts Supplemented Feed in Rainbow Trout *(Oncorhynchus mykiss)*

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Abstract

This study was conducted to determine the effect of *Salvia officinalis* (Sage) and *Aloe vera (Barbados aloe)* as immunostimulators on immune indices of Rainbow trout *(Oncorhynchus mykiss)* and resistance against *Streptococcus iniae* infection. A total of 945 rainbow trout (10±0.1 g) randomly distributed into seven groups with three replicates, were fed with 0% (control), 0.5, 1.0 and 1.5% of food *S. officinalis* and *A. vera* hydroethanolic extracts for 30 days. All fishes were fed with control diet for 15 next days. Total WBC, WBC drift, complement activity, lysozyme activity and total immunoglobulin were measured at 30th and 45th days of the trial. At days 30 and 45 samples from all trial groups were injected with *S. iniae* (3.66×10^8 CFU mL^{-1}), and their mortality was recorded at the end of next ten days period. Results showed that *A. vera* could significantly ($P<0.05$) increase total WBC, lymphocyte, neutrophil, complement activity, lysozyme activity and total Immunoglobulin in comparison to the control group. *S. officinalis* had no significant ($P<0.05$) effect on parameters under study except total Immunoglobulin at rate of 1.5%. Also *A. vera* (1.5%) decrease mortality and improves rainbow trout resistance against *S. iniae* beside control group significantly ($P<0.05$). Based upon findings of this study, *A. vera* ethanolic extract can enhance immune responses and improve resistance of rainbow trout against *S. iniae* infection and it can be used to replace synthetic immunostimulators for rainbow trout.

Keywords: *Rainbow trout, Aloe vera, Salvia officinalis, S. iniae, Immune responses*

Gökkuşağı Alabalığı *(Oncorhynchus mykiss)'*nda *Salvia officinalis* ve *Aloe vera* Ekstraktı ile Beslemenin İmmunolojik ve Antistreptokokal Etkileri

Öz

Bu çalışma Gökkuşağı alabalıklarında *(Oncorhynchus mykiss) Salvia officinalis* (Sage) ve *Aloe vera (Barbados aloe)'*nın immuniteyi uyarmada bağışıklığı uyarıcı etkisini ve *Streptococcus iniae* enfeksiyonuna karşı dirençte etkilerini araştırmak amacıyla gerçekleştirildi. Toplam 945 Gökkuşağı alabalığı (10±0.1 g) rastgele ve üç tekrar olmak üzere rastgele düzende yedi gruba ayrıldı ve balıklar %0 (kontrol), %0.5, %1.0 ve %1.5 *S. officinalis* ve *A. vera* hidroetanolik ekstraktı içeren yem ile 30 gün süresince beslendi. Sonrasında tüm balıklar 15 gün süresince kontrol diyet ile beslendi. Total alyuvar, Alyuvar dağılımı, komplemen aktivitesi, lizozim aktivitesi ve total immunglobulin denemenin 30. ve 45. günlerinde ölçüldü. Tüm deneme gruplarından 30 ve 45. günlerde örneklere *S. iniae* (3.66×10^8 CFU mL^{-1}) enjekte edildi ve sonraki 10 günlük sürede mortalite kayıt edildi. Elde edilen sonuçlar *A. vera* kullanımının kontrol grubuna kıyasla anlamlı oranda total alyuvar, lenfosit, nötrofil, komplemen aktivitesi, lizozim aktivitesi ve total immunglobulinde artmaya neden olduğunu gösterdi ($P<0.05$). %1.5 *S. officinalis* kullanımı araştırılan parametrelerden immunglobulin dışındakilerde anlamlı bir etkiye neden olmadı ($P<0.05$). Kontrol grubuna oranla *A. vera* (%1.5) kullanımı Gökkuşağı alabalıklarında mortalitede azalmaya ve *S. iniae*'ya karşı diençte iyileşmeye neden oldu ($P<0.05$). Çalışma sonuçları *A. ver*a etanolik ekstraktının Gökkuşağı alabalıklarında immun yanıtı ve *S. iniae* enfeksiyonuna karşı direnci artırdığını göstermiştir. *A. vera* etanolik ekstraktı Gökkuşağı alabalıklarında sentetik immun uyarıcılar yerine kullanılabilir.

Anahtar sözcükler: *Gökkuşağı alabalığı, Aloe vera, Salvia officinalis, S. iniae, İmmun yanıt*

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INTRODUCTION

Recently, due to intensive farming practices, infectious diseases pose a major problem in aquaculture industry, causing heavy loss to farmers. Fish are susceptible to several bacterial infections, mainly when reared in high density conditions [1]. In order to address this problem, several studies have been conducted on the modulation of fish immune system in order to prevent the outbreak [2].

The use of antibiotics is the main treatment applied to control bacterial illness in fish farms. Due to the use of a wide variety of antibiotics, aquaculture has been implicated as potential environment to the development and selection of resistant bacteria and a source of these pathogens to other animals and humans [1,3]. The adoption of same antibiotics in different fields (veterinary and human medicine) improves the emergence and occurrence of the resistance phenomenon. Some fish bacterial pathogens are also associated to diseases in humans, making the aquaculture products a potential risk to the customers (zoonotic or food borne diseases) [3]. *S. iniae* is a main zoonotic pathogen in cold and warm water fish, capable of causing invasive disease and outbreaks in marine and aquaculture farms.

Regarding the problem of microbial resistance in treatment of bacterial diseases, there is an urgent need to improve the fish immune system, especially in species with economic value to increase the natural resistance against infectious disease by using the immune stimulators [4].

The use of immune stimulators to enhancing fish resistance against diseases are involved and the immune stimulators has been investigated, including chemical agents, bacterial components, polysaccharides, animal and plant extracts [4-6] which will facilitate operation of immune factors [7-10].

Recently, there is an interest in using medical and aromatic herbs or spices as feed additive in fish diets instead of chemical products, to avoid side effects related to the currently used immunostimulants and the practice in organic aquaculture [11]. Herbal immunostimulants are substances which activate white blood cells (WBC) and may render fishes more resistant to infectious diseases, by stimulating phagocytic cells as well as complement, lysozyme and antibody responses of fish [12].

This study was examined to investigate effect of dietary hydroethanolic extracts of *S. officinalis* and *A. vera* on some immunological parameters and resistance against *S. iniae*, in rainbow trout *(Oncorhynchus mykiss)*.

MATERIAL and METHODS

Herbal Extracts Preparation

Aerial organs of two medicinal plants *(A. vera* and *S. officinalis)* were collected from Khuzestan province of Iran. Herbal samples were identified in the Department of Botany, Faculty of Agriculture, Urmia University, Iran. So plants were washed in running water and were air-dried and ground. Twenty grams of grinded powders from each plant was soaked in 100 mL solvent (mixture of ethanol: distilled water (50:50)%) for 15 min with occasional shaking at 60°C. The materials were filtered through Buchner funnel and Whatman No.1 filter paper. Then, the filtrates were evaporated using rotary evaporator and concentrated.

Fish and Husbandry Conditions

In this study, 945 fish (mean weight: 10±0.1 g) were obtained from a local farm in Urmia, Iran and transferred to Artemia and Aquaculture Research Institute, Urmia University, Urmia, Iran. The fish were immediately disinfected with 3% sodium chloride for 5 min and acclimatized to the laboratory conditions for one week. Fish were randomly distributed into each of 21 Polyvinyl Chloride (PVC) tanks (300 L capacity) filled with 200 liters of water. Each tank was continuously supplied with aerated free-flowing dechlorinated fresh water with the flow rate set at 3.5 L s^{-1}. During the trial, dissolved oxygen measured by Oxymeter (Spanish CRISON CO, model 45 P), and temperature and pH were measured by pHmeter (English Elmetron CO, model 411-CP). The mean of dissolved oxygen, water temperature and pH were 8.5 ppm, 14±1°C and 7.1±0.01 respectively.

Preparation of the Feed

The fishes were fed with a commercial feed pellet SFT2 (Faradaneh, Shahrekord, Iran) according to the water temperature and fish biomass [13]. Diets were supplemented by *S. officinalis* and *A. Vera* extract at levels of 0% (Control), 0.5%, 1.0% and 1.5% of diet. Feeds were prepared daily and stored at 4°C. All groups received related diets for one month and the study was continued for 15 days later and during this time fish were feed with control group diet (without any herbal extract).

Sample Collection and Preparation

At the end of 30th day (after herbal extracts feeding) and at the end of 45th day (the end of trial period) 30 fish were taken from each treatment randomly and blood was sampled from the caudal vein after euthanasia by immersion in solution containing clove powder 200 mg L^{-1} [14]. Blood samples were divided into two aliquots; one aliquot was allowed to clot at room temperature for 1 h. Then, samples were kept at 4°C for 5 h. The serum was separated by centrifugation (1500 g for 5 min at 4°C). The serums were used for assaying the lysozyme activity, complement activity and total immunoglobulin level. The other aliquot was mixed with heparin to count white blood cells (WBCs) [15].

Hematological Indices Assay

WBCs were counted under a light microscope using

Neubauer hemocytometer after dilution with phosphate-saline. Differential leukocyte counts (lymphocyte, monocyte, neutrophil, eosinophil and basophile) were determined using GIEMSA staining method of blood smears under a light microscope. Cells were identified on the basis of morphology and cell ultra-structure as documented in previous study [16].

Complement Activity Assay

Complement activity was assayed using Rabbit red blood cells (RaRBC) as target. RaRBC was provided in 1.5% agarose (pH= 7.2), containing 0.5 mM $MgCl_2$ and 1.5 mM $CaCl_2$. RaRBC in agarose were washed with PBS (0.1 M pH=7.0) by centrifugation at 750 g for 5 min and the cell concentration adjusted to 1×10^8 cell mL^{-1}. Agarose containing RaRBC was dispensed into plate, incubated at 4°C and punched (3 mm in diameter). Subsequently each hole was filled with 15 µL of serum and incubated at room temperature. After 24 h of incubation, the zone of lysis was measured and expressed in Arbitrary Unit mL^{-1} [17].

Arbitrary Unit (AU mL^{-1}) = (Zone of lysis / Volume of the sample loaded) × 1000

Lysozyme Activity Assay

Lysozyme activity was measured as described by Ellis [18]. Briefly, 10 µL of serum was mixed with 200 µL of a *Micrococcus lisodeichticus* (Sigma, ATCC: 4698) suspension at 0.2 mg mL^{-1} in 0.05 M sodium phosphate buffer (pH: 6.2). The mixture was incubated at 27°C, and its OD was detected after 1 and 6 min at 530 nm using an ELISA (enzyme-linked immunosorbent assay) plate reader. One unit of lysozyme activity was defined as the amount of enzyme that produced a decrease in absorbance of 0.001 min mL^{-1} of serum [18].

Total Immunoglobulin Assay

Total immunoglobulin was measured by the method of Panigrahi [19] and Bradford [20]. Briefly, the serum samples were diluted 100 times with 0.85% NaCl and the was employed to determining the total Immunoglobulin content, The bovine Plasma albumin (BSA) and reagents being sourced from Sigma (USA).

Bacterial Preparation and Challenge Test

The *S. iniae* (BCG/LMG 3740) bacterium was provided in a lyophilized vial and confirmation was done with biochemical tests. Bacteria was cultured in brain heart infusion broth (BHI) medium (QUELAB, Canada) under laminar flow hood in 28°C for 24 h. Then mass production of bacteria was done in a 200 mL Erlenmeyer flask containing 50 mL BHI medium. After culturing the bacteria, Erlenmeyer flask containing medium was incubated under aerobic conditions within incubated with Shaking (Company of INC, N-Biotech of South Korea) at 25°C with 75 rpm for 24-48 h period. Then the Erlenmeyer flask contents were centrifuged at 4°C for 15 min with distant 2500 rpm and washed twice with sterile PBS buffer. Finally bacterial suspension in sterile PBS buffer was prepared by standard MacFarlane density at constant concentration (10^7 CFU ml^{-1}) [21].

A bacterial challenges were designed at both of 30th (the end of herbal extracts administration) and 45th (the end of trial period) days of trial separately. For each bacterial challenge thirty fish from control group and each plant extract treatments (10 fish from each replication) were selected randomly and were anesthetized with 150 ppm of clove powder. So the fish were injected with 100 µL of *S. iniae* bacterial suspension containing 3.66×10^8 CFU ml^{-1} by insulin syringe, intraperitonally [22] and were entered in 21 PVC tanks and monitored for next ten days. Cumulative mortality of each injected fish group was recorded at the end of next ten days period in each bacterial challenge test (40th and 55th days of trial). During the bacterial challenges oxygen was supply by aeration with an air pump and 50 percent of tanks water was exchanged daily [23].

Statistical Analysis

All data were presented as means ± standard deviation (SD). One way analysis of variance (ANOVA) was used (SPSS, Ver. 16, Chicago, IL, USA) to determine the significant variation between the treatments with Tukey test ($P<0.05$).

RESULTS

According to the *Table 1,* briefly *A. vera* (1.5%) was exhibited significant difference ($P<0.05$) in total WBC, Lymphocyte and Neutrophil in both 30th and 45th days beside control group. *A. vera* (1.0%) was revealed significant difference ($P<0.05$) in total WBC and Neutrophil just at day 30th compared to the control group.

A. vera (1.5%) was revealed significant difference ($P<0.05$) in all serum immune parameters under study beside control group *(Table 2)*.

There are no significant differences ($P<0.05$) compared to the control group in the blood and serum immunity indices of the *S. officinalis* treatments on sampling days *(Table 1* and *Table 2)*.

A. vera (1.5%) was exhibited significant ($P<0.05$) minimal mortality compared to the control group in both series of bacterial challenges *(Fig. 1* and *Fig. 2)*.

DISCUSSION

The recent expansion of intensive aquaculture practices has led to high interest in understanding the various natural immunostimulators, so that they can be used to decrease side effects of antibiotics in treatment of infectious diseases.

Table 1. *Total WBC and WBC drift of treatments at sampling days (Mean±SD)*

Blood Index	Time (day)	Treatments						
		Control	*S. officinalis* (0.5%)	*S. officinalis* (1.0%)	*S. officinalis* (1.5%)	*A. vera* (0.5%)	*A. vera* (1.0%)	*A. vera* (1.5%)
Total WBC (10^3 Cell/µL)	30	0.515±11.03^{a}	0.48±11.34^{a}	0.93±11.40^{a}	0.95±11.34^{a}	0.75±11.35^{a}	0.31±11.37^{a}	0.27±12.99^{b}
	45	0.58±11.10^{a}	0.82±11.93^{a}	0.77±11.87^{a}	0.84±11.86^{a}	0.93±11.85^{a}	0.11±11.86^{a}	0.33±13.8^{b}
Lymphocyte (%)	30	0.71 ±86.33^{a}	0.83±86.78^{ab}	1.40±87.22^{ab}	1.22±87.33^{ab}	1.90±86.89^{ab}	1.12±88.00^{ab}	2.24±88.55^{b}
	45	1.00±86.33^{a}	1.24±87.44^{ab}	1.50±87.33^{ab}	1.24±87.44^{ab}	2.06±87.00^{ab}	1.12±88.00^{ab}	2.03±89.11^{b}
Monocyte (%)	30	0.73±2.25^{a}	0.73±2.44^{a}	1.13±2.55^{a}	0.73±2.55^{a}	0.53±2.56^{a}	0.50±2.33^{a}	0.50±1.67^{a}
	45	0.83±2.78^{a}	0.73±2.55^{a}	1.20±2.78^{a}	0.97±2.78^{a}	0.67±2.78^{a}	0.53±2.55^{a}	0.50±1.67^{a}
Neutrophil (%)	30	0.44±7.22^{a}	0.78±7.89^{ab}	0.60±7.89^{ab}	0.87±8.00^{ab}	0.50±8.00^{ab}	0.44±7.99^{ab}	0.53±8.55^{b}
	45	0.60±7.11^{a}	0.83±7.78^{ab}	0.78±7.89^{ab}	0.83±7.78^{ab}	0.44±7.78^{ab}	0.50±8.00^{ab}	0.71±8.33^{b}
Eosinophil (%)	30	0.67±2.22^{a}	0.87±2.00^{a}	0.86±2.00^{a}	0.87±2.00^{a}	0.60±1.89^{a}	0.53±1.44^{a}	0.44±1.22^{a}
	45	0.67±2.22^{a}	0.87±2.00^{a}	0.87±2.00^{a}	0.78±1.89^{a}	0.60±1.89^{a}	0.53±1.44^{a}	0.50±1.33^{a}

Data were subjected to analysis of variance (SPSS, One-Way ANOVA) followed by Tukey test. The different superscript alphabets in the same row are significantly different at P<0.05

Table 2. *Serum immune parameters of treatments during the study period (Mean±SD)*

Immune Parameters	Time (day)	Treatments						
		Control	*S. officinalis* (0.5%)	*S. officinalis* (1%)	*S. officinalis* (1.5%)	*A. vera* (0.5%)	*A. vera* (1.0%)	*A. vera* (1.5%)
Lysozyme (U min^{-1})	30	522.67±28.09^{a}	566.33±58.94^{ab}	576.00±33.00^{ab}	634.67±74.65^{ab}	624.00±28.21^{ab}	625.33±34.53^{ab}	669.33±29.50^{b}
	45	521.00±36.75^{a}	589.67±52.59^{ab}	606.00±13.08^{ab}	617.33±85.45^{ab}	603.67±27.15^{ab}	21.22^{b}±653.67	661.00±28.69^{b}
Complement (U mL^{-1})	30	467.33±82.53^{a}	459.67±44.96^{a}	548.00±53.11^{ab}	604.33±88.93^{ab}	707.67±64.44^{bc}	710.67±49.80^{bc}	782.67±68.65^{c}
	45	420.33±69.00^{a}	463.33±71.02^{ab}	514.67±18.17^{ab}	608.67±144.19^{ab}	507.33±133.66^{ab}	533.00±123.56^{ab}	733.33±76.05^{b}
Total Immuno-globulin (mg mL^{-1})	30	2.90±0.65^{a}	3.10±0.27^{ab}	3.12±0.83^{ab}	4.38±0.47^{b}	3.17±0.61^{ab}	3.12±0.32^{ab}	4.38±0.29^{b}
	45	2.91±0.32^{a}	3.14±0.07^{a}	3.15±0.74^{a}	3.58±0.39^{a}	2.98±0.63^{a}	3.16±0.13^{a}	3.56±0.37^{a}

Data were subjected to analysis of variance (SPSS, One-Way ANOVA) followed by Tukey test. The different superscript alphabets in the same row are significantly different at P<0.05

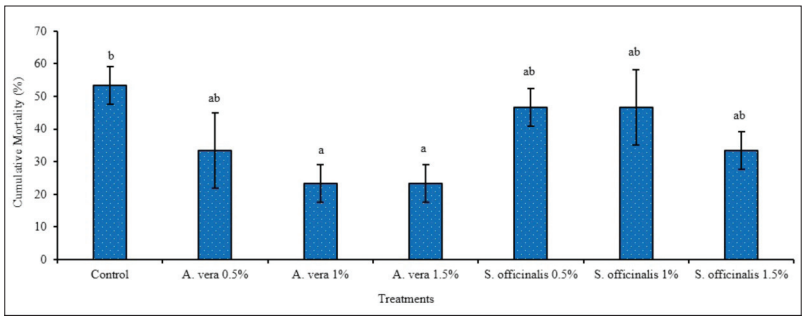
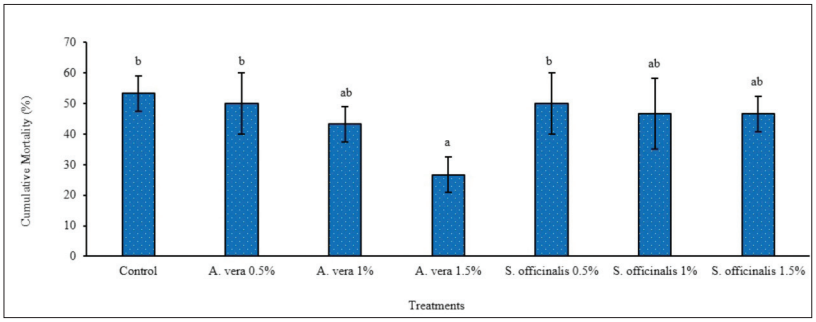


Fig 1. Cumulative mortality of trial groups after bacterial challenge with *S. iniae* at day 40th

Data were subjected to analysis of variance (SPSS, One-Way ANOVA) followed by Tukey test. The different alphabets above columns are significantly different at P<0.05

Fig 2. Cumulative mortality of trial groups after bacterial challenge with *S. iniae* at day 55th

Data were subjected to analysis of variance (SPSS, One-Way ANOVA) followed by Tukey test. The different alphabets above columns are significantly different at P<0.05



Recent advancement in various photobiotic immuno-nutritional studies revealed that some medical plants are linked to the immunological status of fish. This has drawn the attention of fish nutritionists to the immunoprotection of fish besides the growth [24].

It has been shown that herbal based immunostimulants are capable of enhancing nonspecific and specific defense mechanisms and reducing losses from viruses, bacteria and parasitic infections in fish [25-27]. Several plant materials/ products such as *Eclipta alba* [28], *Viscum album*, *Urtica dioica* and *Zingiber officinale* [6], *Solanum trilobatum* [29], *Achyranthes aspera* [30], *Astragalus radix* and *Scutellaria radix* [31], *S. officinalis* and *A. vera* [27] have been reported to enhance the immunity of fish.

In current study, *A. vera* has significant effect on immune responses in rainbow trout, whereas *A. vera* (1.5%) treatment enhanced total WBC, monocytes and neutrophils compared with control group at days 30th and 45th significantly (P<0.05). Any humoral immune parameters were not increased significantly in *A. vera* (0.5% and 1.0%) treatments *(Table 1)*. These results are in consistent with the results obtained of [16] who reported supplementation of *A. vera* ethanolic extract at a rate of 1.0% in rainbow trout had no significant (P<0.05) effect on white blood cell count and differential leukocytes count (monocytes, lymphocytes and neutrophils) in compared to control group.

Among hematological parameters under current study, no significant difference (P<0.05) was registered between *S. officinalis* treatments and control group *(Table 2)*. Unfortunately, there is lack of *in vivo* literature on the hematological and immunological effects of *S. officinalis* on fish, however antibacterial properties of *S. officinalis* were reported in many *in vitro* studies [32-35]. Asheg *et al.*[36] reported that there was no significant difference (P≤0.05) in total WBS, heterophils count and antibody titer of *S. officinalis* treatment beside control group in broiler chicken [36]. They just demonstrated that *S. officinalis* increased the lymphocyte count of broiler chicken compared to control group at the third week of life. This result could be attributed to the enhancement of cellular immune response by the natural boosting effects of such medicinal plant.

According to earlier studies, *A. vera* is able to induce some immune responses in fish [37,38]. Mesbah *et al.*[27] reported that Lysozyme, Complement and serum bactericidal activity enhanced in *Barbus grypus* fed with *A. vera* supplemented diet groups compared with control group (P<0.05) [27]. According to their obtained results, it might be concluded that the feeding of *Barbus grypus* by *A. vera* (specifically 0.2% of feed) extract could likely enhance immunological parameters. These results are in consistent with results of our study that show *A. vera* and *S. officinalis* can stimulate immune system of rainbow trout (specifically 1.5% of feed). *S. officinalis* increase total Immunoglobulin of rainbow trout at day 30th and *A. vera* enhanced Lysozyme activity, Complement and total Immunoglobulin of serum compared with control group significantly (P<0.05) *(Table 2)*.

Enhancement of immune responses in fish fed with medicinal herbal extracts supplemented diets lead to increase resistance fish against bacterial infections [11,39]. Dhayanithi *et al.*[40] reported that mangrove leaves *(Avicennia marina)* have potential to control the ornamental fish (Clownfish) infections caused by *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus* and *Vibrio anguillarum* [40].

S. iniae is one of the most serious aquatic pathogens causing high losses in farmed marine and freshwater finfish. This bacteria is capable of causing high rate of mortality in rainbow trout farms [41]. In current study the *A. vera* (1.0 and 1.5%) challenged groups showed reduced mortality against *S. iniae* when compared with the control group (P<0.05) *(Fig. 1* and *Fig. 2)*. These results indicate that hydroethanolic extract of *A. vera* was capable of activating the immune system of rainbow trout and enhance its resistance against *S. iniae*. Kaleeswaran *et al.*[42] demonstrated that ethanolic extract of plant *Cynodon dactylon* is very effective immunostimulant in *Catla catla* against *Aeromonas hydrophila* (which is one of the most important bacteria in aquatic animals, such as fish, shrimps and lobsters [42] infection [43]. This plant extract could develop or induce the specific antibody in fish against the antigen, especially at the higher (5%) concentration. The present study demonstrates that the *A. vera* extract supplemented diet (specifically 1.5% of feed) has positive effects in improving immune parameters of Rainbow trout fingerlings and enhance its resistance against *S. iniae* infectious. *A. vera* can be used to replace synthetic immunostimulator agents for rainbow trout.

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