The effects of different levels of *Aloe vera* extract on some of the hematological and non-specific immune parameters in Siberian sturgeon (*Acipenser baerii*)

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Abstract

In this study, *Aloe vera* extract was used to evaluate its effects on hematological and immune parameters of Siberian sturgeon (*Acipenser baerii*). A total of 360 Siberian sturgeon weighing on average 10.95±0.04 g were randomly distributed in four treatments including a control group and three experimental groups (each with three replications). *A. vera* extract powder at the rate of 0.5%, 1% and 1.5% were added to the food. The fish were fed for 60 days in fiberglass tanks. At the end of the first and second months of breeding, the necessary samplings for hematological studies were done. The physicochemical parameters of water were recorded every day**.** The results showed significant differences (p<0.05) in the amount of RBC, hemoglobin, MCV, WBC, lysozyme and ACH50 in the treatments compared to the control group at the end of the breeding period. The results showed that *A. vera* extract has the effect of a non-specific immune system booster and using the extract in particular at the level of 1.5% as an immune stimulant in the diet of Siberian sturgeon can be effective in preventing disease in this species.

Keywords: *Aloe vera* extract, Hematological, Non-specific immune, Siberian sturgeon

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Introduction

Along with the increasing development of the aquaculture industry in the world, emphasizing the principles of operation according to the economic and health aspects of aquaculture is of particular importance. In this regard, ensuring proper growth and preventing disease in aquatic animals are among the important issues that have attracted the attention of researchers in this industry more and more. Siberian sturgeon (*Acipenser baerii*) is a species of commercial value that easily adapts to the conditions of breeding and is resistant to changes in environmental conditions (Pyka and Kolman, 2003). Because of its fast growth, early sexual maturity and caviar production in a short time, as well as its diverse diet, this species was introduced as a candidate for freshwater culure for the production of sturgeon meat (Adamek *et al*., 2007). In recent years, in order to treat and the fight against bacterial infections in fish, chemical drugs such as antibiotics, have been used. However, in addition to the high costs involved, the administration of antibiotics has resulted in the development of drug resistance, destruction of natural bacterial flora, emergence and spread of bacterial resistance towards antimicrobial agents, and the transfer of antibiotic residues from aquatic animals to humans. Antibiotics also weaken the immune system against viral diseases and parasites.

The use of herbal immunostimulants in the aquaculture industry, in particular to strengthen the immune system and increase fish resistance against a variety of infectious diseases has spread world-wide. Many studies have shown that immunostimulants of plants are capable of enhancing specific and non-specific immune defense mechanisms, as well as increase or reduce fish losses against a variety of viral and bacterial or parasitic infections (Kim *et al*., 1999; Dügenci *et al*., 2003; Yin *et al*., 2006; Nya and Austin, 2009; Yin *et al*., 2009; Bilen and Bulut, 2010; Harikrishnan *et al*., 2010; Bilen *et al*., 2011; Begum and Navaraj, 2012; Ardo, 2013; Govind, 2013; Haghighi and Sharif Rohani, 2013; Sharif Rohani *et al*, 2013).

Aloe vera is one of the most valuable plants in the Liliaceae family, which is native to tropical regions. *A. vera* contains more than 75 nutrients, 200 active compounds, 20 minerals, 18 amino acids and 12 vitamins and compounds such as Aloin, famodin, Antrokinon and barbaloin (Shelton, 1991; Atherton, 1998; Mandrioli *et al*., 2011). This plant has a wide variety of pharmacological effects such as skin lesions and wound healing effects, anti-viral, antibacterial and other effects have been attributed to this plant. However, the effects of immune stimulation and growth of warm-blooded animals has not been proven. In addition, little information about the effects of immunogenicity, anti-toxicity and the effects on growth performance by *A. vera* in different fish species have

been found (Alishahi *et al*., 2010; Zodape, 2010; Wang *et al*., 2011; Alishahi and Abdi, 2013; Haghighi *et al*., 2014).

According to the characteristics and properties of this medicinal plant, this study was performed to evaluate the effects of three doses of *A. vera* on hematological and immune parameters of this valuable species (Siberian sturgeon).

Materials and methods

Preparation of A. vera extract

The plant of *A. vera* was procured from Medicinal Plants Production Cooperation of Havin and the 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 day 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 with Whattman No. 1 filter paper and centrifuged for 5 min at 5000 rpm. The filtrate obtained from ethanol using a rotary device, the excess solvent was separated from the extract. These crude extract was stored at -18ºC until use (Ozakan *et al*., 2007; Haghighi *et al*., 2014). At the end of the extraction process, to analyze compounds in *A. vera* extract gas chromatography (GC-MS) and liquid chromatography (HPLC) (Rouessac and Rouessac, 2007; Lakhsmi and Rajalakshmi, 2011) was used (Table 1).

Table 1: Types and amounts of compounds in *Aloe vera* extract.

Compounds	Amount (%)	Compounds	Amount (%)
Aloin	28.81	Comaric acid	7.62
Oleic acid	6.23	Squalene	13.8
B-Sitostrol	1.41	Limoene	10.26
Lupeol	4.7	n- Hexadecanoic acid	10.24
Campestrol	2.18	other components	6.3
Carvone	8.43		

Supplementation of the normal diet with dried A. vera extract

In this study, in order to produce food containing different percentages of *A. Vera* extract powder (0.5%, 1% and 1.5%), Biomar commercial feed containing 49% crude protein, 15% crude lipid, 2.5% cellulose, 1.24% phosphorus, 8.7% ash, 1.85% calcium, 0.55% sodium, Cu 1.5 mg kg^{-1}, Mn 12 mg kg^{-1}, Zn 75 mg kg^{-1} and I1.8 mg kg^{-1} was used. In order to add extract powder and prepare food diets containing different amounts of *A. vera* (0.5%, 1% and 1.5%), the the the required amounts of the extract powder was dissolved in water and sprayed on the food and then stirred. Feeds were

spread on separate trays (for 24 hours) and dried at room temperature (Noga, 2000). Feeds were stored in special containers with lids at a temperature of 4-6°C.

Fish and experimental conditions

A total number of 360 Siberian sturgeon (with average weight of 10.95±0.04 g), were randomLy divided into 12 fiberglass tanks of 500 liter (dewatering volume 350 liters, at 3 liters per minute water flow and permanent aeration) placed at the International Sturgeon Research Institute (Rasht, Iran) and cultured for two months.

Physico-chemical parameters of water including dissolved oxygen (WTW Oxi 330i digital devices made in Germany), pH and water temperature (WTW pH 330i digital devices made in Germany) were measured on a daily basis. Average water temperature (22.8±0.88˚C), dissolved oxygen (6.74±0.42 mg/L^{-1}) and 6.8±0.19 of pH were determined. Fish adaptation for 14 days was carried out in the fiberglass tanks. During the two months breeding, fish were fed the prepared feeds containing *A. vera* extract (0.5%, 1% and 1.5%) and Biomar basic diet (for control group) at a rate of 3% of body weight at 8:00, 15:00 and 22:00 hours.

Blood sampling

At the end of each month, to evaluate the effects of *A. vera* extract on hematological and immune parameters of Siberian sturgeon and to compare between different treatments, blood samples from the caudal vein of 6 fish from each group (18 samples in total) were taken. 0.5 cc of blood with anticoagulant heparin and 1.5 cc of blood without anticoagulant heparin were taken to measure immune parameters.

It should be noted that anesthetics were not used in the samplings because they probably have an impact on the blood indices (Torrecillas *et al*., 2007 and 2011). The samples were centrifuged using centrifugation at 3000 rpm for 10 minutes. They were then transferred using a sampler to a new vial, and stored at -80°C. Preparation and measurement of all hematological and serological parameters were carried out at the International sturgeon research Institute and Viromed laboratory.

Haematological assay

Blood samples were analyzed following routine methods adopted in fish hematology (Blaxhall and Daisley, 1973; Klontz, 1994). The total red blood cell counts (RBC×10^6/mL) were determined in a 1:200 dilution of the blood sample in Hayem's solution and total white blood cell counts (WBC×10^3/mL) in a 1:20 dilution of the blood sample with a Neubauer hemocytometer.The hematocrit (Hct) and leucocrit percentages were determined in duplicate using micro hematocrit-heparinized capillary tubes of 75µL volume and a micro hematocrit centrifuge at 15000 rpm for 5 min

(Houston, 1990; Klontz, 1994). The percentages of erythrocyte (hematocrit) and leucocyte (leucocrit) volumes were calculated by overlaying the tubes on a sliding scale hematocrit reader. The hemoglobin (Hb, g dL^{-1}) concentrations were determined by the cyanomethaemoglobin method (Valery *et al*., 1991; Klontz, 1994) using a haemoglobin reagent set (Pars Azmun Diagnostics). All the values of red blood cell indices, the mean values of cell haemoglobin (MCH pg), cell hemoglobin concentration (MCHC %), and cell hemoglobin volume (MCV fl) were calculated according to Wintrobe formulae (Anderson and Klontz, 1965).

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 (Ivanova, 1983).

Immunological assay

Alternative complement activity

Alternative complement activity (ACH50) was evaluated following the procedure of Yano (1992) by using rabbit red blood cells (RaRBC). Briefly, RaRBC were washed and adjusted to 2×10^8 cell/mL in EDTA-magnesium-gelatin veronal buffer (0.01 M). Precisely 100 µL of the RaRBC suspension was lysed with 3.4 mL of distilled water and the absorbance of the haemolysate was measured at 414 nm against distilled water to acquire the 100% lysis value. The test plasma was appropriately diluted, and different volumes ranging from 0.1 to 0.25 mL were made up to 0.25 mL total volume before being allowed to react with 0.1 mL of RaRBC in test tubes. After incubation at 20ºC for 90 min with occasional shaking, 3.15 mL of a 0.9% (w/v) saline solution was added to each tube with centrifugation at 1600 rpm for 10 min at 4ºC. The absorbance (A) of the supernatant was measured using a spectrophotometer at 414 nm. A lysis curve was obtained by plotting the percentage of haemolysis against the volume of plasma added. The volume of plasma producing 50% haemolysis (ACH50) was determined and the number of ACH50 units/mL was obtained for each fish.

Lysozyme activity

The turbidimetric assay for lysozyme was carried out according to Sahoo *et al*. (2008) with minor modifications. Thus, plasma (50 µL) was added to 2 mL of a suspension of *Micrococcus lysodeikticus* (0.2 mg/mL^{-1}) in a 0.05 M sodium phosphate buffer (pH 6.2). The reaction was carried out at 25ºC and absorbance was measured spectrophotometrically at 570 nm after 0.5 min and 4.5 min. PBS was used as a blank. A unit of lysozyme activity was defined as the sample amount causing a decrease in absorbance of 0.001 min^{-1}. Lysozyme of sample calibrated using a standard curve determined with hen's egg white lysozyme (Sigma) in PBS.

Statistical analysis

Statistical analyses were performed using SPSS (version 20) software. Data are presented as mean±SD. All the data were tested for normality (Kolmogorov-Smirnov test). Data were analyzed by one-way of variance analysis (ANOVA). The significant means were compared by Duncan test and a $p<0.05$ was considered statistically significant. Also using independent samples T-Test compares the average blood sampling between the two stages.

Results

The results showed significant differences ($p<0.05$) in the hematological parameters including Hct, Hb, MCH, MCV and WBC in fish fed *A. vera* compared with the control group (Table 2) in both samples (the first and second months ofbreeding). However no significant differences ($p>0.05$) were observed in the results of the first and second months of training in any of the hematological parameters.

Table 2: The haematological parameters of Siberian sturgeon fed with different levels of *Aloe vera* extract for two months (mean ±SD).

Parameters	*Aloe vera* extract levels (%)							
	First month				Second month			
	Control (0)	0.5	1	1.5	Control (0)	0.5	1	1.5
RBC (10^6 mL^{-1})	0.77 ± 0.06^a	0.78 ± 0.05^a	0.8 ± 0.06^a	0.78 ± 0.06^a	0.85 ± 0.07^a	0.86 ± 0.04^a	0.88 ± 0.05^a	0.92 ± 0.07^b
Hct (%)	26 ± 0.26^b	25 ± 0.52^a	25.17 ± 0.4^a	24.83 ± 0.17^a	30.67 ± 0.2^{ab}	29.17 ± 0.6^a	29.83 ± 0.48^a	31.5 ± 0.22^b
Hb (g dL^{-1})	5.1 ± 0.04^a	7.6 ± 0.07^b	5.98 ± 0.06^b	5.8 ± 0.07^b	6.68 ± 0.03^a	5.87 ± 0.07^b	7.75 ± 0.04^b	8.1 ± 0.07^c
MCH (pg)	65.33 ± 0.56^a	75.17 ± 0.54^b	75.17 ± 0.6^b	74.67 ± 0.33^b	78.17 ± 0.31^a	88.18 ± 0.65^b	88.17 ± 0.4^b	87.67 ± 0.22^b
MCV (fl)	338.7 ± 2.56^b	321 ± 5.53^{ab}	316.8 ± 3.18^a	320.5 ± 0.99^{ab}	360 ± 2.67^b	338.5 ± 5.49^a	339.7 ± 3.22^a	342.2 ± 1.11^{ab}
MCHC (%)	21.5±0.25	23.5±0.5	23.67±0.49	23.33±0.21	21.8±0.14	26.05±0.32	25.97±0.25	25.7±0.11
WBC(10^3 mL^{-1})	9.2 ± 0.8^a	11.6 ± 0.94^b	13.23 ± 1.12^c	13.51 ± 1.1^c	12.1 ± 0.93^a	15.78 ± 1.32^b	16.6 ± 1.41^c	16.68 ± 1.32^c
Neut (%)	9.83±0.98	10.5±1.05	14.33±0.82	15±1.89	10.17±1.12	10.83±0.98	14.33±1.21	14.33±1.21
Mon (%)	1.83±0.41	1.5±0.55	1.33±0.52	1.17±0.75	2.33±0.52	2±0.63	1.83±0.41	1.33±0.52
Lymp (%)	85.83±1.17	85.17±0.75	82.1±1.21	82±1.26	86.83±1.17	86±0.89	82.5±1.05	82.83±1.62
Eos (%)	1.5±0.55	2±0.69	1.83±0.41	1.67±0.52	1.67±0.52	2±0.69	1.83±0.41	1.67±0.52

Data are expressed as mean±SD (n=72). Neut: neutrophil; Mon: Monocyte; Lymp: Lymphocyte; Eos: Eosinophil.

Differences in Latin letters in each row shows a significant difference ($p<0.05$).

In this study, the results of the immune parameters (Table 3) showed that serum lysozyme levels and ACH50 in different *A. vera* treatments compared to the control in the tanks during the two months were significantly different ($p<0.05$). At the end of the breeding period, the highest values in the immune parameters were recorded in the 1.5% *A. vera* extract group.

Table 3: The immunological parameters of Siberian sturgeon fed with different levels of *Aloe vera* extract for two months (mean ±SD).

Parameters	*Aloe vera* extract levels (%)							
	First month				Second month			
	Control	0.5	1	1.5	Control	0.5	1	1.5
Lysozyme Activity (u mL^{-1})	8.67 ± 0.33^{a}	17.67 ± 0.33^{b}	19.33 ± 0.33^{b}	19 ± 0.58^{b}	11 ± 0.58^{a}	21.33 ± 0.67^{b}	25.33 ± 0.88^{c}	28 ± 0.58^{d}
ACH50 (u mL^{-1})	171 ± 1^{a}	175 ± 0.58^{a}	177.67 ± 0.88^{b}	180.67 ± 0.88^{b}	191.67 ± 1.45^{a}	217.67 ± 1.2^{b}	220 ± 1^{b}	224 ± 2.3^{c}

Differences in Latin letters in each row shows a significant difference ($p<0.05$; n=72).

Discussion

Attention to the prevention of disease and increase in survival rate can affect the security of investments and the development of sturgeon breeding. In this regard, medicinal plants have attracted much attention due to their therapeutic effects. Moreover they are also known to cause no or less biological and environmental risks and cost less compared to other antibiotics (Christybapita *et al*., 2007; Alishahi *et al*., 2010; Watanuki *et al*., 2010). Important indicators on health status of fish in intensive culture conditions can be non-specific resistance of different fish species, assessment of nutritional status to evaluate the impact of fish food additives, and the measurement hematologic parameters noted (Cataldi *et al*., 1998).

A number of studies have shown that herbal immune stimulants did not have a significant effect on red blood cell counts (Shalaby *et al*., 2006; Kumar Jha *et al*., 2007; Sahu *et al*., 2007; Harikrishnan *et al*., 2010). The study showed that the amount of hemoglobin in sampling stages (the first and second months of breeding) in the control group was significantly lower than that in all treatments. The amounts of hemoglobin in the second stage of sampling, in treatments and control, increased. Studies have shown that levels of hemoglobin, hematocrit and red blood cells are under the influence of different factors such as age, sex, race and environment (Ameri mahabadi, 1999).

Hematocrit values change in the presence of pathogens or imbalance in mineral density in blood. In addition, the very high volume of hematocrit may indicate the possibility of unfavorable environmental conditions associated with stress (Parker, 1974). The results of this study are consistent with that of some other studies regarding changes in the levels of red blood cells, hemoglobin, hematocrit and blood indices (Iwama and Nakanishi, 1996; Shalaby *et al*., 2006; Nya and Austin, 2009). Haghighi *et al*. (2014) observed significant changes in blood in levels of red blood cells and other indices at the end of the two months period in rainbow trout fed a diet containing *A. vera* extract. However, Alishahi *et al*. (2010) did not see any differences in the amount of red blood cells and related indices. Generally, an increase in white blood cells in the second month of breeding was evident. Increased levels of white blood cells in the study conducted by Pourgholam *et al*. (2013)

using *Echinacea purpura* extract on immune indices and survival of rainbow trout corresponded with the results of this study.

Hajibeglou and Sudagar (2010) reported an increase in the number of white blood cells in common carp fish with the use of dietary supplements containing herbal immune stimulants. Also, the results of Sahu *et al*. (2007) indicated an increase in the rate of WBC. Gopalakannan and Arul (2006) reported that WBC significantly increased after feeding carp with herbal stimulants such as chitin. Oskoii *et al*. (2012) also found that the use of herbal stimulants on blood indices, including white blood cells in rainbow trout was effective. The WBC increased in common carp blood by adding *Stachys lavandulifolia* (2% and 8%) to the diet of the fish (Bahrami Babahydari *et al*., 2015). In research conducted by Alishahi and Abdi (2013) amounts of WBC in the different treatments of *A. vera* rainbow trout showed statistically significant differences (p<0.05). However no significant differences (p>0.05) were observed in the lymphocytes, monocytes, neutrophils and eosinophils, which confirms the results of the present study.

Increase in the number of neutrophils after taking herbal stimulants, can be related to beta-glucan that are able to recognize specific receptors in their white blood cells. (Andrews *et al*., 2009). When these receptors are occupied by glucan, active white blood cells surround, kill and digest pathogenic bacteria more so that all these factors improve the host immune system (Andrews *et al*., 2009).

Lysozyme is an important component of non-specific immunity in fish that causes destruction of bacterial wall, enhances phagocytic activity and actives both of the complement pathways involved in stimulating the immune response of the fish. The level of serum lysozyme shows improvement in the immune status of the fish and an increase in the immune system of the fish to better fight against infectious agents and stress reduction. Lysozyme activity increases immune stimulants following administration of some vaccines and some prebiotics in fish (Alishahi *et al*., 2010). Results of this study showed that adding different levels of *A. vera* extract in lysozyme activity had significant effects (p<0.05) and the highest lysozyme activity was observed with 1.5% *A. vera*.

In this study, an increasing trend in lysozyme activity has been shown which is in agreement with several reports indicating the role of herbal immunostimulants in enhancing lysozyme activity (Rao *et al*., 2006; Choi *et al*., 2008; Haghighi *et al*., 2014). Lysozyme is a humoral component of the non-specific defense mechanism which has the ability to prevent the growth of bacteria by splitting â-1, 4 glycosidic bonds in the peptidoglycan of bacterial cell walls (Secombes, 1996; Choi *et al*., 2008).

In both sampling stages, ACH50 amounts between experimental and

control groups were statistically significant (p<0.05). Minimum and maximum ACH50 were observed in the control and 1.5% *A. vera* extract group, respectively. Awad *et al*. (2010) reported that the use of *Lupinus perennis*, *Urtica dioiaca* and *Managifera indica*, especially at a concentration of 1% and 2% in the diet of rainbow trout caused a significant increase in the complement activity. Feeding tilapia fish within two weeks using *Eclipta alba* extract could create a significant increase in complement activity (Christybapita *et al*., 2007). Alishahi and Abdi (2013) reported that the use of *A. Vera* extract (1%) in common carp diet is able to significantly increase ACH50 amounts in comparison with the control group and 0.5% *A. vera* extract group. Alternative complement pathway activity is a strong non-specific defense mechanism to protect fish against a wide range of pathogenic organisms such as bacteria, fungi, viruses and parasites (Chiu *et al*., 2010). In general, the results of this study indicate that the use of *A. Vera* extract (especially 1.5%) was more effective on some hematological and immune parameters in Siberian sturgeon.

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