Research Article

Effects of nanoparticles of dried *Aloe vera* extract on some of the hematological parameters, liver enzymes and immune responses in Siberian sturgeon (*Acipenser baerii*)

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Abstract

Today, nanotechnology is considered as a priority and strategic technology for all countries due to its wide range of applications. The present study was performed to evaluate the effects of various levels of dried *Aloe vera* extract nanoparticles on some hematological parameters, liver enzymes and immune responses of Siberian sturgeon (*Acipencer baerii*). Three hundred sixty *Acipenser baerii* (with mean weight of 10.95 ± 0.04 g), were randomly divided into three treatment groups including 0.5%, 1% and 1.5% of nanoparticles of dried *Aloe vera* extract and one control group (without any additive), each in three replicates, stored in twelve tanks of 500-liter fiberglass with 350 liters water. Water physicochemical parameters were recorded daily. Nanoparticles of dried *A. vera* extract in a ratio of 0.5%, 1%, and 1.5% were added to the treatment groups diet for 2 months. At the end of the first and second months of rearing, the necessary samplings for hematology and serology studies were done. The results of this study showed that the amounts of RBC, Hb, Hct, MCV, WBC, lysozyme, ACH_{50} and IgM were significantly different in the treatment groups compared with the control group at the end of the study ($p<0.05$). There were no significant differences in liver enzymes including ALT, AST and ALP between treatments and control groups ($p>0.05$). In conclusion the results have shown that use of nanoparticles of dried *A. vera* extract had the impact of immune function booster and the usage of this nanoparticle mainly at the level of 1% in the diet can be used as a safety stimulant for Siberian sturgeon.

Keywords: *A. vera*, Nanoparticles, Hematological parameters, Immune indices, Siberian sturgeon

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Introduction

Nanotechnology, according to experts, has grown exponentially in all aspects of science and industry in recent years. One of the favorite characteristics of drug nanoparticles is their biocompatibility. Today it is proved that the drug delivery system based on nanoparticles is more effective having fewer side effects, more acceptability, and more accumulation in the considered site (Douglas *et al*., 1987). Therefore, considering this science and exploitation its advantages is incredibly necessary to enhance productivity in aquaculture. In this regard, the use of nanotechnology in order to improve proper growth and aquatic health is important. Rapid growth, short sexual maturity and speedy caviar formation are important reasons for breeding Siberian sturgeon (Adamek *et al*., 2007).

The use of natural immune stimulants in aquaculture, it is developing to booster the immune responses and increase fish resistance against diffusion of diseases spread. Many researchers have proven that the use of immune stimulant is able to boost specific and non- specific mechanisms. (Dügenci *et al*., 2003; Yin *et al*., 2006; Yin *et al*., 2009; Nya and Austin, 2009a; Bilen and Bulut, 2010; Harikrishnan *et al*., 2010a; Bilen *et al*., 2011; Begum and Navaraj, 2012; Govind, 2013; Haghighi and Sharif Rohani, 2013).

Aloe vera is one of the most valuable plants in the Liliaceae family, which is native to tropical regions. *Aloe vera* contains more than 75 nutrients, 200 active compounds, 20 minerals, 18 amino acids, and 12 vitamins and compounds such as aloin, famotidine, antrokinon, and barbaloin (Atherton, 1998; Shelton, 1991; Mandrioli *et al*., 2011). Due to the valuable compounds of this plant, little information have been found about the effects of immunogenicity, anti-toxicity, and the effects of growth performance by *Aloe vera* in different fish species (Wang *et al*., 2011; Haghighi *et al*., 2014; Alishahi *et al*., 2010; Alishahi and Abdi, 2013; Bazari Moghaddam *et al*., 2017). The aim of this study was the impact of three doses of *A. vera* nanoparticles on some hematological parameters, liver enzymes and immune responses of Siberian sturgeon (*Acipencer baeri*i).

Materials and methods

Preparation of *Aloe Vera* extract

The extract of *Aloe vera* was prepared with the usual technique of percolation. For this purpose, chopped dried *Aloe vera* leaves in 80% ethanol was percolated for 72 hours. Then, the slurry became filtered with Whatman No. 1 and centrifuged for 5 min at 5000 rpm. The product was obtained using a rotary device. The crude extract was stored at -18 °C. (Haghighi *et al*., 2014; Ozakan *et al*., 2007).

Preparation of *Aloe vera* nanoparticles

In order to produce of *A. vera* nanoparticles, the prepared extract was transferred to Zanjan University (the Pharmaceutics Lab of Pharmacy College). Nanoparticles were produced according to patent no.73360 (Hamidi *et al*., 2011). Particle's size and confirm them was measured with Zetasizer

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(Fig. 1). The diameter of *A. vera* nanoparticles was 152nm and PDI=0.25 (Poly Dispersity Index). After confirming the size of nanoparticles, cryoprotector was added to nano extract suspension at 40°C to increase preservation time.

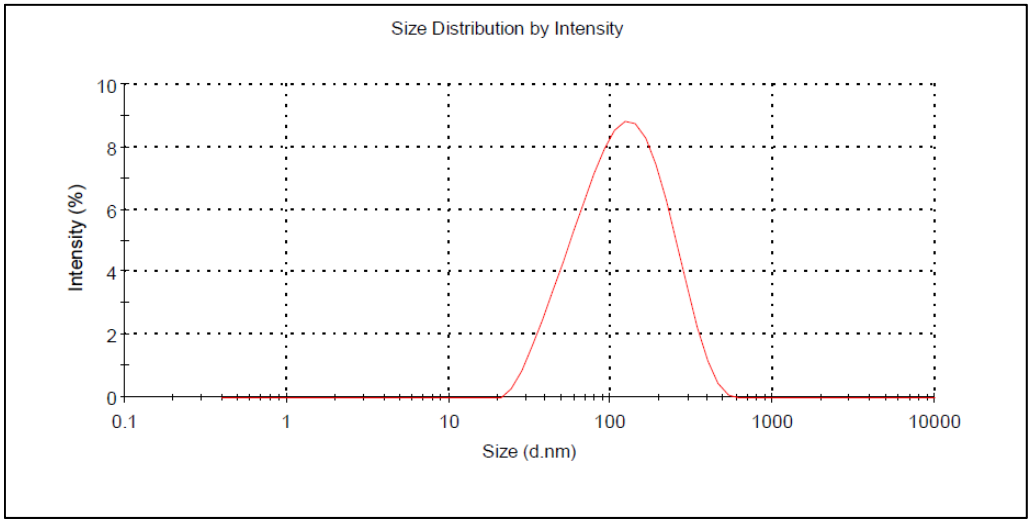

Figure 1. Size distribution by intensity

At the end of the process of producing *A. vera* nanoparticles, gas chromatography (GC-MS) and liquid chromatography (HPLC) were used to analyze compounds in *Aloe vera* nanoparticles based on methods of Rouessac and Rouessac (2007) and Lakhsmi and Rajalakshmi (2011), (Table 1).

Table 1. Compounds in *A. vera* nanoparticles

Types of compounds	Quantity (%)	Types of compounds	Quantity (%)
Aloin	28.81	Comaric acid	7.62
Oleic acid	6.23	Squalene	13.8
B-Sitostrol	1.41	Limonene	10.26
Lupeol	4.7	n- Hexadecanoic acid	10.24
Campestrol	2.18	other components	6.3
Carvone	8.43		

Experimental food preparation with dried *Aloe vera* nanoparticles

Firstly, dried *Aloe vera* nanoparticles were dissolved in distilled water and then sprayed on the fish food at the desired levels (0.5, 1 and 1.5%). The prepared foods were dried on separated trays at room temperature for 24 hours. Then, the oil was sprayed on the dried foods in method of Noga (2000). The prepared foods was placed in special closed dishes and preserved in the refrigerator at 4-6 °C for future use.

Fish farming in fiberglass tanks

This study was carried out at International Sturgeon Research Institute, Rasht, Iran. For this purpose, 360 *Acipenser baerii* (average weight of 10.95 ± 0.04 g), were randomly divided into twelve tanks of 500-liter fiberglass (350 liters, the flow 3 liters per minute and permanent

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aeration). The physicochemical 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 daily.

The average water temperature 22.8 ± 0.88˚C, dissolved oxygen 6.74 ± 0.42 mg/L and 6.8 ± 0.19 of pH were determined. Fish adaptation period for 14 days was carried out in the tanks. During the two months breeding, the fish were fed by using prepared foods containing *Aloe vera* nanoparticles (0.5%, 1% and 1.5%) and Biomar normal diet (control group) at a rate of 3% of body weight.

At the end of each month, hematological parameters, liver enzymes and immune responses of Siberian sturgeon were evaluated. For this purpose, from each group, 18 samples of blood (6 samples of each replication) were taken from the caudal vein (without anesthetic, Torrecillas *et al*., 2011). The 0.5 ml of blood with anticoagulant heparin and 1.5 ml of blood without anticoagulant heparin were taken to measure liver enzymes and immune responses. The samples were centrifuged with using centrifugation at 3000 rpm for 10 minutes. The supernatant was poured into new vials and stored at -80 ° C until final testing.

Hematology tests

Blood samples were analyzed with routine methods adopted in fish hematology (Blaxhall and Daisley, 1973; Klontz, 1994). The blood cellular counts (RBC×10^6/ml) were measured in a 1:200 dilution of the blood sample in Hayem's solution and white blood cell counts (WBC×10^3/ml) in a 1:20 dilution with a Neubauer hemocytometer. Hematocrit and leucocrit percentages were measured by using capillary tubes (Houston, 1990; Klontz, 1994). The erythrocyte and leucocyte volumes were determined by overlaying the tubes. Hemoglobin (g/dl) was measured by the cyanomethemoglobin method (Valery *et al*., 1991; Klontz, 1994). The volume percentages of erythrocyte (hematocrit) and leucocyte (leucocrit) were calculated by overlaying the tubes on a sliding scale hematocrit reader. The hemoglobin (Hb, g/dl) concentrations were determined by the cyanomethaemoglobin method (Valery *et al*., 1991; Klontz, 1994) using a haemoglobin reagent set (Pars Azmun Diagnostics). In this study, 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's formula (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).

Liver enzymes assay

Enzyme activities such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) were determined by using (www.parsazmun.com) diagnostic kit by BT-1500 model auto analyzer device (Instruments Biotecnica Italy), (Shahsavani *et al*., 2008).

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Immunological assay:

Lysozyme activity

Lysozyme level assay was accomplished according to Sahoo *et al*. (2008) with insignificant modifications. By turbidimetric method, gradual analysis of gram-positive *Micrococcus lysodeikticus* bacteria (Sigma, USA) was obtained. Then, plasma (50 µl) was added to 2 ml of a suspension of *M. 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 (Bazari Moghaddam *et al.*, 2017).

Immunoglobulin M (IgM)

The IgM was analyzed by using the (immunoturbidimetry kit) and according to the method described by Khoshbavar-Rostami *et al*. (2006). Briefly, in this method, IgM is complexed with polyclonal antibodies in the tampon solutions, causing the solution to become opaque. The turbidity intensity was directly related to IgM and was observed by a spectrophotometer (Model 2100 – VIS, Unico USA) at 320 nm with Planck (distilled water).

Alternative complement activity

Alternative complement activity (ACH_{50}) was evaluated by Yano (1992) method 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 tested 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 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 (ACH_{50}) was determined for each fish.

Statistical analysis

All statistical analyses were calculated using SPSS version 20.0. All the data has been examined for normality (Kolmogorov-Smirnov test). The means were analyzed by one-way variance analysis (ANOVA). The significant means were compared by Duncan's test at the level of $p<0.05$.

Results

The results of hematological parameters and liver enzymes in treatment groups fed with *A. vera* nanoparticles in compare to the control group are presented in Table 2. Based on the obtained results, Hct, Hb,

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MCH, MCV and WBC in the first and second months of breeding showed significant differences ($p<0.05$). However, there were not significant differences ($p>0.05$), in values of liver enzymes in the first and second months.

Table 2: The hematological parameters and liver enzymes after 2 months in Siberian sturgeon (Mean ±SD)

Indices	*A. vera* nanoparticles levels (%)							
	30 days				60 days			
	Control	0.5	1	1.5	Control	0.5	1	1.5
RBC(10^6/ml)	0.77±0.06^b	0.88±0.05^a	0.80±0.06^a	0.78±0.06^b	0.85±0.07^b	0.86±0.04^b	0.88±0.05^b	0.92±0.07^a
Hct (%)	26±0.26^a	25±0.52^b	25.17±0.4^b	24.83±0.17^b	30.67±0.2^{ab}	29.17±0.6^b	29.83±0.48^b	31.5±0.22^a
Hb (g/dl)	5.1±0.04^b	7.6±0.07^a	5.98±0.06^b	5.8±0.07^b	5.68±0.03^c	5.87±0.07^b	7.75±0.04^b	8.1±0.07^a
MCH (pg)	65.33±0.56^b	75.17±0.54^a	75.17±0.6^a	74.67±0.33^b	78.17±0.31^b	88.18±0.65^a	88.17±0.4^a	87.67±0.22^a
MCV (fl)	338.7±2.56^a	321±5.53^{ab}	316.8±3.18^b	320.5±0.99^{ab}	360±2.67^a	338.5±5.49^b	339.7±3.22^b	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)	9.2±0.8^c	11.6±0.94^c	13.23±1.12^b	13.51±1.1^a	12.1±0.93^c	15.78±1.32^b	16.6±1.41^a	16.68±1.32^a
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
ALT (u/l)	3.33±0.67	2±0.57	2.67±0.33	2.67±0.33	4.33±0.67	3±0.58	3.33±0.67	3.67±0.33
AST (u/l)	111.33±1.45	99±1.53	100±1	104.33±1.86	124.33±1.45	111±0.58	117±1.73	117.67±2.06
ALP (u/l)	181.67±2.85	158±1.53	170.33±2.18	181±3.46	247.67±3.76	222±1.53	234.33±3.67	244.33±2.73

Neut: neutrophil; Mon: Monocyte; Lymp: Lymphocyte; Eos: Eosinophil.
Data are expressed as Mean±SD (n=63). Significant differences were observed in the treatment groups relative to the control group at the end of every period (30 or 60 days) after feeding with nanoparticles of *Aloe vera*.
Superscript in a row with different letter represent significant differences over the control ($p<0.05$).

Table 3. The immunological indices after 2 months in Siberian sturgeon (Mean ±SD)

Indices	*A. vera* nanoparticles levels (%)							
	30 days				60 days			
	Control	0.5	1	1.5	Control	0.5	1	1.5
Lysozyme Activity (u/ml)	8.67±0.33^b	17.67±0.33^a	19.33±0.33^a	19.38±0.58^a	11±0.58^c	21.33±0.67^b	25.33±0.88^a	26.02±0.58^a
ACH_{50} (u/ml)	171±1^c	180±0.58^b	188±2.31^a	188±1.53^a	191.67±1.45^c	223.33±2.03^b	237.33±2.19^a	236.33±2.4^{ac}
IgM (mg/dl)	15.83±0.44^c	19.83±0.44^b	26±0.36^a	22.67±1.2^a	21.67±0.33^b	25.67±0.33^b	34.33±0.88^a	32.67±1.26^a

Superscript in a row with different letter represent significant differences over the control at the end of every period ($p<0.05$).

Also, the results of the immune indices (lysozyme and ACH_{50}) showed that have been significant differences in *A. vera* nanoparticles treatment groups in compared to control group during the two months ($p<0.05$). It should be noted that the end of the breeding period (60 days), the highest level of immunity, was measured in the 1.5% *A. vera* nanoparticle treatment.

Discussion

Disease prevention and survival rate are two important factors in the investment security of fish breeding. In this regard, various plant compounds have been used to improve survival rate, stimulate the immune system and growth performance of fish (Watanuki *et al*., 2006; Alishahi *et al*., 2010). The present study projects the impact of nanoparticles of dried

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Aloe vera extract on the hematological indices and immunological responses in Siberian sturgeon (*Acipenser baerii*)**.** The hematological parameters in the present investigation such as RBC, WBC, Hematocrit (Hct), Hemoglobin (Hb), and the values of red blood cell indices, MCH, and MCV in treatment groups were significant differences at the end of 60-day period after feeding when compared to control group, especially fed 1.5% nanoparticles of *Aloe vera*. However, there were no significant differences in differential leukocytes counts and liver enzyme activities (ALT, AST and ALP). Studies have shown that levels of hemoglobin, hematocrit and red blood cell counts are located under the influence different factors such as age, sex, race and environment (Ameri mahabadi, 1999). Haghighi *et al.* (2014) reported changes in red blood cell counts and its markers in rainbow trout fed 1% *Aloe vera* extract at the end of two-month period of the experiment. The results of the present study are in line with the results of the study of Haghighi *et al.* (2014). However, a number of studies have shown that herbal extract did not have a significant effect on red blood cell counts (Shalaby *et al*., 2006; Sahu *et al*., 2007; Kumar *et al*., 2007; Harikrishnan *et al*., 2010a). Hajibeglou and Sudagar (2010) were observed an increase in the number of white blood cells in the common carp with the use of dietary supplements containing herbal immune stimulants. Gopalakannan and Arul (2006) reported that the WBC after feeding carp with herbal stimulants such as chitin significantly increased. In the research conducted by Alishahi and Abdi (2013), they showed a significant increase ($p<0.05$) in amounts of WBC in rainbow trout fed *Aloe vera*. However, there were no significant differences in the lymphocytes, monocytes, neutrophils and eosinophils percentage which confirms the results of our study. Increase in the number of neutrophils after taking herbal stimulant, can be related to beta-glucans which are able to recognize specific receptors on their white blood cells (Andrews *et al*., 2009). When these receptors are occupied by glucan, activity white blood cells surround, kill and digest pathogenic bacteria more that all these factors improve the host immune system (Andrews *et al*., 2009).

In the present study, results of ALT activities were similar to those reported for *Thymallus thymallus*, and *Leuciscus cephalus* (Lusková, 1997), but were much lower than values reported for *Oreochromis niloticus* (Chen *et al*., 2002) and *Chondrorostoma nasus* (Lusková, 1997). The ALT, AST and ALP belong to the non-plasma specific enzymes which are localized within tissue cells of liver, heart, gills, kidney, muscle and other organs and in blood plasma. They may give specific information about organ dysfunction (Babalola *et al*., 2016). Elevated AST activity can be associated with the release of transaminase from the cytoplasm due to hepatic cellular damage (Kim *et al*., 2002).

The results of the present study showed an increase in lysozyme activity, ACH_{50} and IgM amounts at the end of the first and second months of breeding ($p<0.05$), which are in agreement with several reports

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indicating the role of herbal immunostimulants (Choi *et al*., 2008; Haghighi *et al*., 2014).

Awad (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 the tilapia 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 *Aloe Vera* extract (1%) in common carp dietary is able to increase ACH_{50} amounts in comparison with the control group significantly. The results of the present study on *Aloe vera* nanoparticles are in line with the results of the study of Alishahi and Abdi (2013). Lysozyme and ACH_{50} are humoral component of the non-specific defense mechanism that have the ability to prevent the growth of bacteria by splitting â-1, 4 glycosidic bonds in the peptidoglycan of bacterial cell walls (Choi *et al*., 2008).

Based on the present results, it was found that addition of *A. vera* nanoparticles could cause a significant increase in IgM levels. It seems that some biochemical compounds in *A. vera* have been able to increase the level of IgM immunoglobulins by stimulating the production and secretion of lymphocytes (Gannam and Schrock., 1999). In reality, serum immunoglobulins are the main constituent of humoral immunity and IgM is the principal immunoglobulin in fish (Wilson *et al*., 1995).

It is proven that consumption of some nutritional herbal dietary supplements, consist of peppermint, *Mentha piperita* (Adel *et al*., 2015), fenugreek, *Trigonella foenum graecum* (Guardiola *et al*., 2017) and myrtle, *Myrtus communis* (Taee *et al*., 2017) have been capable of enhancing the immune system of numerous fish species.

The results of the present study showed that the use of *A. vera* nanoparticles (especially 1%) in the diet can be used as a safety stimulant for Siberian sturgeon.

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Conflict of interest

Authors have no conflict of interest on this work.

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