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RESEARCH PAPER

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Dietary Administration of Common Sage (*Salvia officinalis*) and Coneflower (*Echinacea angustifolia*) Extracts Affects Growth, Blood Parameters and Immune Responses of Beluga, *Huso huso*

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Abstract

The purpose of the present study was to investigate the effects of *Salvia officinalis* and *Echinacea angustifolia* extracts on growth performance, blood parameters and innate immune responses of beluga, *Huso huso.* Six experimental diets were prepared using *S. Officinalis* (SAE-30, SAE-60, SAE-120) and *E. angustifolia* (EAE-30, EAE-60 and EAE-120) extracts containing concentrations of 30, 60 and 120 ml/kg feed, respectively. A control diet (CD) was prepared without extracts inclusion. The fish fed the diet containing 120 ml/kg of *E. angustifolia extract* (EAE-120) showed significant ($P < 0.05$) higher final weight (885.7 ± 11.5 g), body weight (318.5 ± 8.5%) and feed efficiency (72.7 ± 9.1) compared to those of other experimental diets. In addition, SGR (1.8 ± 0.14) and FCR (2.7 ± 0.18) were significantly improved in fish fed diet containing 30 ml/kg of *E. angustifolia* extract (EAE-30) compared to those of other experimental diets. All haematological parameters were influenced by inclusion of *Salvia officinalis* and *Echinacea angustifolia* extracts. The serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were significantly lower in supplemented diet groups compared to the control. The lysozyme activity (31.6 ± 3.3 µg/ml) and alternative complement activity (130.3 ± 10.9 U/ml) in the serum of fish fed diet containing120 ml/kg of *E. angustifolia* extract (EAE-120) showed significant difference compared to the other treatments ($P<0.05$). These results revealed remarkable beneficial effects of *E. angustifolia* extract on innate immune and growth performance of the beluga.

Introduction

In aquaculture, feeding is one of the most important factors influencing growth, feed utilization and tissue composition of fish in intensive culture (Nya & Austin, 2009; Talpur, Ikhwanuddin, Abdullah, & Bolong, 2013). The afore-mentioned chemicals have not been recently recommended in commercial aquaculture due to their residual effects on muscle of cultured species (Sambhu, 1996; Citarasu, Babu, Sekar, & Marian, 2002; Sagdic & Ozcan, 2003). In the recent years, the concern about bacterial resistance to antibiotics in livestock industry has led to minimizing application of such compounds. Other adverse impacts of antibiotics are development of resistant bacteria, presence of antibiotic residues in meat and destruction of the bacterial population in the cultivated aquatic environment (Marques *et al.*, 2005; Shalaby, Khattab, & Abdel Rahman, 2006). Thus, several studies have focused on natural products such as plants or their extracts as possible alternative to antibiotics (Sivaram, Babu, Immanuel, Murugadass, Citarasu, & Marian, 2004; Acar, Parrino, Kesbiç & Paro, 2018; Baba, Acar, Yılmaz, Ergün, Saoca, Abbate, Yılmaz, & Fazio, 2018). Plants and their extracts are known to play a significant role in preventing diseases via potential properties of antimicrobial and antioxidant activities (Buchanan, Hott, Cutlip, Rack, Asamer, & Moritz, 2008; Gabor, Sara, & Barbu, 2010). These bioactive components exert their beneficial effects by manipulating the intestinal microflora and improving digestibility. The natural plant origin products such as vegetables, herbs, spices, edible

plants, and their extracts are not explained as traditional feed additives for animal nutrition. However, various parts of these plants (fruit, leaves, oil) have been traditionally utilized in folk medicine since ancient times. These plant based feed additives act as growth promoters, immunostimulants or antimicrobial agents, representing the proper alternative to antibiotics and other chemotherapeutics with no detrimental impact on environment (Galindo-Villegas & Hosokawa, 2004). Since, herbal products enhance the activity of non-specific defense system (Dorucu, Colak, Ispir, Altinterim, & Celayir, 2009; Citarasu, 2010; Wang, Sun, Liu, & Xue, 2016), their effects on different fish species has become routine practice. Various types of substances are known to act as immunostimulants, but only a few are suitable to be used in fish farming. The immunostimulants have been categorized as natural (biological) and synthetic (chemical) substances that stimulate and enhance lysozyme activity, lymphocyte activity, antibody production and protection against pathogens (Citarasu, 2010; Van Hai, 2015). The common sage, *Salvia officinalis* is a plant endemic in Mediterranean countries with great medical importance. It has strong antioxidant activity due to the presence of excellent antimicrobial activity and hypoglycemic properties (Kamatou, Van Vuuren, Van Heerden, Seaman, & Viljoen, 2007; Eidi & Eidi, 2009). The main antioxidant and antimutagenic effects of *S. officinalis* are related to the presence of activecompounds such as: carnosic acid carnosol, rosmarinic acid and camphor (Cuvelier, Berset, & Richard, 1994; Miliauskas, van Beek, Venskutonis, Linssen, & de Waard, 2004). In addition, extract of Coneflower, *Echinacea purpurea* has been used traditionally for the treatment of various types of infections and wounds. Studies have shown the effect of E. *purpurea* as immune-modulator, comprising stimulation of certain immune functions such as phagocytic activity of macrophages and suppression of the proinflammatory responses of epithelial cells to viruses and bacteria (Vimalanathan, Kang, Amiguet, Livesey, Arnason, & Hudson., 2005; Hudson, 2009). Thus, the aim of the present study was to investigate the effect of different levels of *S. officinalis and E. angustifolia* extracts as feed additives on growth performance, haematological parameters and non-specific immune system in beluga

Materials and Methods

Preparation of Experimental Diets

The commercially available ethanolic extracts of *S. officinalis* and *E. angustifolia* (Zardband Pharmaceutical Company Ltd., Tehran, Iran) were purchased and prepared as a dietary additive. Six experimental diets were prepared based on *S. officinalis* (SAE-30, SAE-60, SAE-120) and *E. angustifolia* (EAE-30, EAE-60, EAE-120). A control diet (CD) was served without addition of extracts in diet. The designated concentrations of both herbs (30, 60 and 120 ml per 1 kg feed) were incorporated into the commercial diet (Skretting, 1.1 mm diameter, Puerto Montt, Chile) by first mixing with vegetable oil as carrier and then top-spraying. The control diet was also sprayed with vegetable oil (contains no extract). The proximate composition of main ingredients of commercial feed are measured as 57%, 9.5%, 0.9% and 0.4% for protein, ash, fat and fiber.The experimental diets were dried at room temperature for 2 h and then stored in sealed polythene bags at 4° C until further use.

Experimental Set up

The juveniles of great sturgeon (268.3 ± 0.41 g) were supplied from the Dafchah Sturgeon Fish Propagation and Rearing Centre (Rasht, Iran). Fish were stocked in acclimation for two weeks prior to beginning the experiment; during this period they were fed formulated diet. After the acclimation period, fish were stocked in rearing tanks (400 L) at density of 15 specimens per tank within an indoor flow-through system supplied with pumped-ashore filtered natural freshwater (100 L/h.) under constant 12:12 h (light/dark) schedule. The fish were hand-fed the experimental diets until apparent satiation twice daily (09:00 and 17:00 h) for 6 weeks. Water temperature, dissolved oxygen and pH were maintained at 15 °C, 7.3 and 8.0, respectively. Continuous aeration was provided to each tank through an air stone connected to a central air compressor.

Evaluation of the Growth Performance

Growth performance, feed utilization and survival rate were assessed according to average final weight gain, specific growth rate (SGR) and feed conversion ratio (FCR), based on the following formulae:

$$\text{Weight gain (\%)} = 100 - (W_2 - W_1)/W1;$$

$$\text{Specific growth rate (SGR, \%/day)} = 100 - (\text{Ln } W_2 - \text{Ln } W_1)/T;$$

$$\text{Feed conversion ratio (FCR)} = \text{feed intake (g)/weight gain (g)};$$

$$\text{Survival rate (\%)} = 100 - (Nf/Ni);$$

$$\text{Condition factor (CF)} = (\text{body weight (g)}/ (\text{standard length}))^3 \times 100$$

Where W_1, W_2, T, Ni and Nf are initial weight (g), final weight (g), number of days in the feeding period, initial and final number of fish, respectively.

FE: feed efficiency = 100 x (FBW–IBW)/TF where the TF indicated the total amount of feed given.

Blood Analysis

After 24 hours of the last experimental feeding, four fish from each replicate were sedated (200 mg MS 222/L of water), blood was collected from the caudal vein either with heparinized or non-heparinized syringes. Heparinized blood was transferred into heparinized tubes and kept on ice for analysis of whole blood parameter within 2 hours. The tubes contain non-heparinized blood (4 fish / tank; 12 fish / treatment) was allowed to clot at room temperature for 30 min and the serum separated by centrifugation (3000g for 10 min) at room temperature. Then, the serum was separated and stored at -70°C for later analysis. To measure hemoglobin (Hb) concentration, fresh blood was used according to the cyanmethemoglobin method (Jain, 1986). Red blood cells (RBC) and white blood cells (WBC) were counted using a Neubauer hemocytometer and hematocrit (Ht) was measured by routine microhematocrit technique. The smears obtained from heparinized samples were air-dried, fixed in 96% ethanol for 30 min, stained with Giemsa, and examined by light microscopy to determine differential leukocyte counts (neutrophils, lymphocytes, monocytes and eosinophils). The concentration of serum glucose, cholesterol, total protein and albumin (Alb) were measured by spectrophotometer at 546 nm (Jenway, 685-SC - UV/VIS, UK) using commercial kits (Pars Azmun, Tehran, Iran). Globulin content was calculated by subtracting albumin content from serum total protein content. Activities of ALT and AST were determined colorimetrically using kits from Pars Azmun Diagnostics (Karaj, Iran).

Immunological Parameters

The standard procedure suggested by Kumar *et al.* (2009) was followed for determination of serum lysozyme activity. A suspension of 150 ml lyophilized Micrococcus lysodeikticus (Sigma M-3770), with 0.2 mg/ml as substrate in 1M sodium acetate buffer adjusted to pH 5.5, was added to previously dispensed test serum (15 ml from each fish) in 96-well U-bottom microliter plate, and initial optical density (OD) was immediately measured at 450 nm. Final OD was assessed after 1 h incubation at 24 °C. A standard curve was prepared using lyophilized hen egg white lysozyme (HEWL, Sigma, USA). Serum lysozyme values were expressed as mg/ml.

Serum Immunoglobulin (Ig) and Alternative Complement Activity (ACH_{50}) Assay

Total immunoglobulin levels of blood plasma samples were measured by adding 50 µl blood plasma and equal amount of 12.0% solution of polyethylene glycol (Sigma) to each well of a 96-well microtiter plate. Samples were incubated for two hours at room temperature, and then the plates were centrifuged (5000 g at 4 °C) for 15 min. The supernatant was diluted 30 times with 0.85% NaCl and total protein levels were determined according to Bradford method. Samples were measured in triplicates. These values were subtracted from the total protein concentrations, and the results of immunoglobulin-concentrations were expressed as mg/ml. Also, the alternative complement activity (ACH_{50}) of samples was determined using sheep red blood cells (SRBC) as described by Yano (1992).

Statistical Analysis

Prior to analysis, homogeneity of variances and normality of the data were checked using Levene Kolmogorov-Smirnov test, respectively. Then, data with normal distribution were analyzed by one-way analysis of variance (ANOVA). Duncan test was applied to compare the significant differences among the treatments ($P < 0.05$). The nonparametric statistics using the Kruskal-Wallis test followed by the Mann-Whitney U-test were performed for comparison of non-normally distributed data. All analysis were conducted using SPSS version 18 (SPSS Inc., Chicago, IL, USA). Differences were considered significant at $P < 0.05$ for all analyses. Data were expressed as mean ± SD.

Results

The FBW and FE of fish were significantly ($P<0.05$) affected by the dietary treatments in the rearing period compared to the control (Figure 1). Feed conversion ratio, SGR and CF revealed significant differences among the dietary treatments (Figure 2). The changes in haematological indices are given in Figure 3 and 4. Significant differences were observed in WBC, Ht and Hb in the dietary treatments ($P<0.05$), but, there were no significant differences ($P>0.05$) in RBC levels among the dietary treatments. Also, the resutls showed significant differences in MCV, MCH and MCHC contents among the dietary treatments. Among leucocyte counts, only lymphocyte showed significant differences after 6 weeks feeding (Figure 5). Significant differences were observed in serum biochemical constituents among the dietary groups. There was significant difference in glucose level among dietary treatments and the highest were observed in the control and SAE-60 groups (Table 1). Moreover, significant changes were observed in serum total protein, albumin and globulin concentrations among the dietary treatments (Table 1). The effects of *S. officinalis and E. angustifolia* extracts on serum non-specific immune parameters of *H. huso* are shown in Table 2. Serum ALT and AST levels were higher in the control group than other dietary treatments (Table 2). The serum lysozyme activity and Ig levels showed significant differences among the dietary groups. The highest level of lysozyme was observed in EAE-30 and EAE-120 compared to the other dietary groups (Table 2). While, Ig level was significantly higher

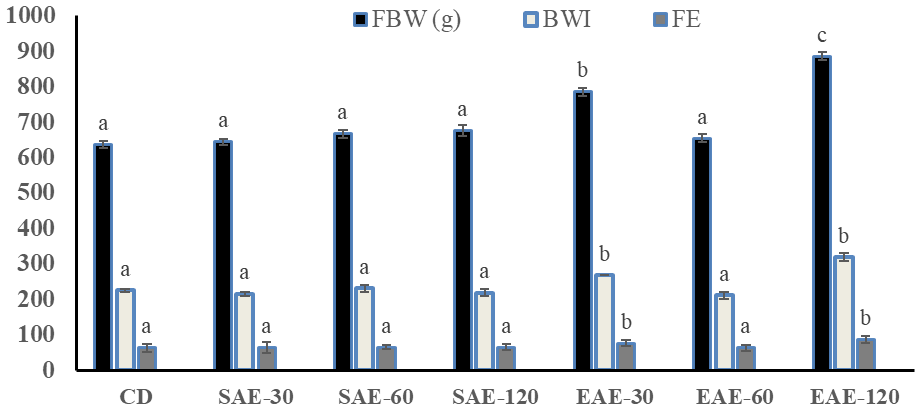

Figure 1. Mean ± SD of FBW, BWI and FE of the *Huso huso* fed diets inclusion of *Salvia officinalis* and *Echinacea angustifolia* extracts. Different superscript shows significant difference at P<0.05.

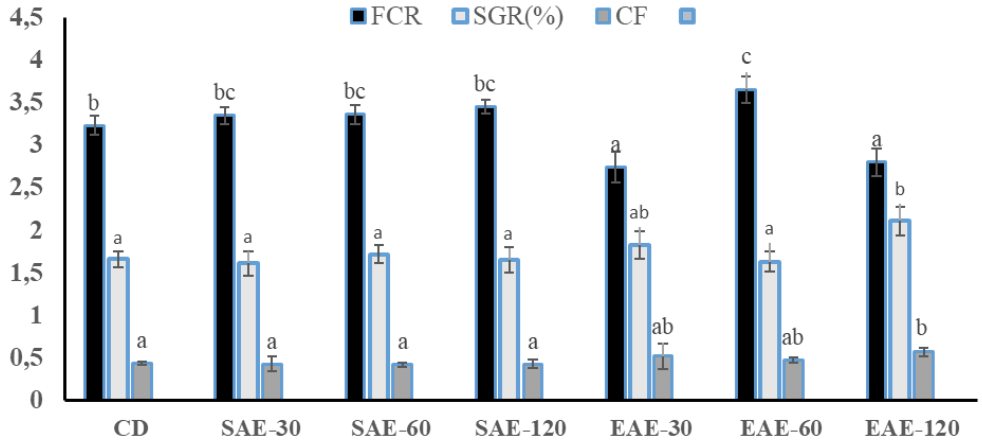

Figure 2. Mean ± SD of FCR, SGR and CF of the *Huso huso* fed diets inclusion of *Salvia officinalis* and *Echinacea angustifolia* extracts. Different superscript shows significant difference at P<0.05.

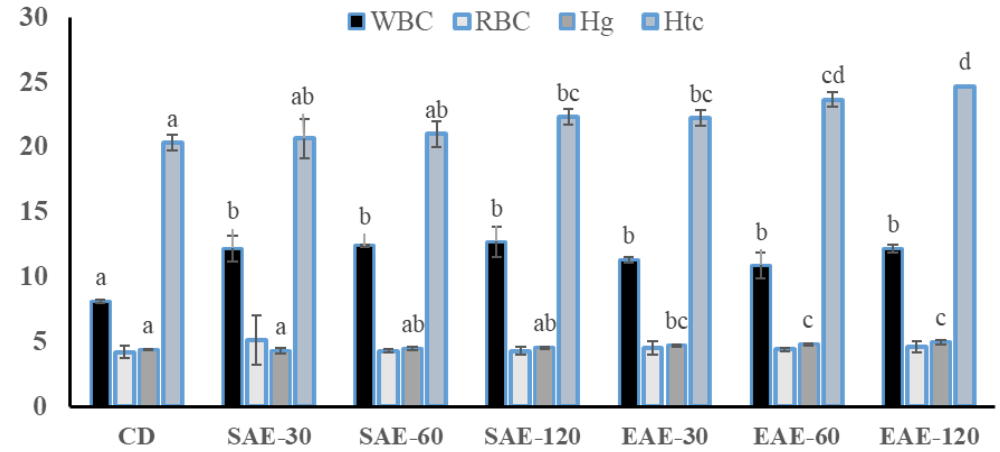

Figure 3. Mean ± SD of some haematological parameters (WBC, RBC, Hg and Htc) in *Huso huso* fed diets inclusion of *Salvia officinalis* and *Echinacea angustifolia* extracts. Different superscript shows significant difference at P<0.05.

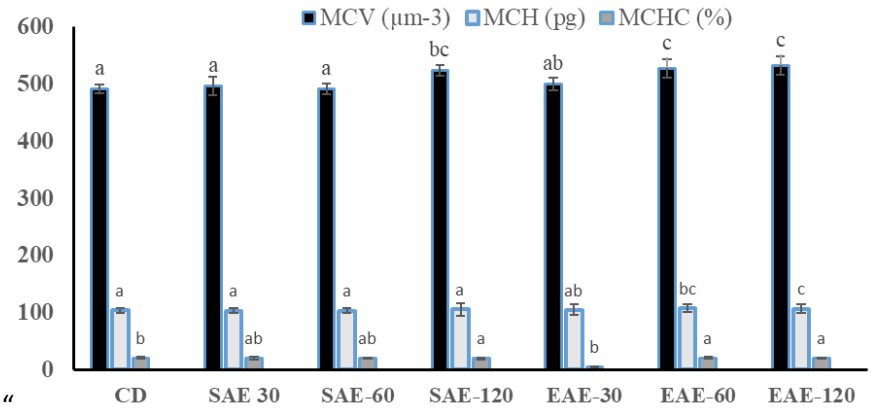

Figure 4. mean ± SD of MCV, MCH and MCHC in *Huso huso* fed diets inclusion of *Salvia officinalis* and *Echinacea angustifolia* extracts. Different superscript shows significant difference at P<0.05.

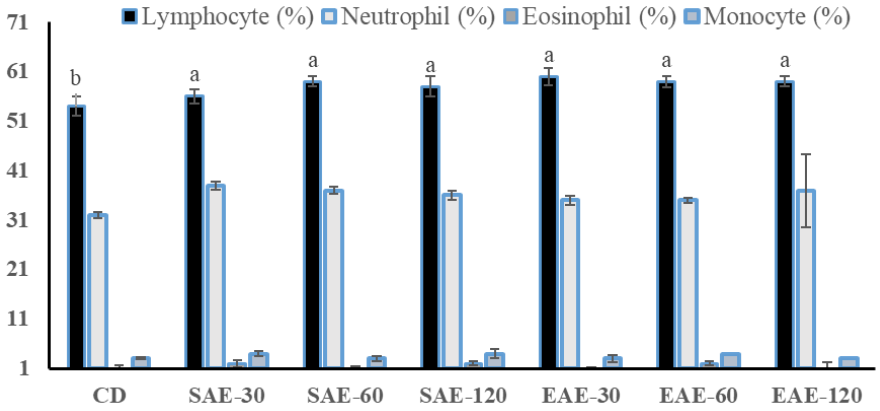

Figure 5. Mean ± SD of leucocyte counts in *Huso huso* fed diets inclusion of *Salvia officinalis* and *Echinacea angustifolia* extracts. Different superscript shows significant difference at P<0.05.

Table 1. Biochemical parameters of *Huso huso* following a 60 day diet supplemented with *Salvia officinalis* and *Echinacea angustifolia* extracts

Treatments	Glucose (g dL^{-1})	Total protein (g dL^{-1})	Calcium (g dL^{-1})	Globulin (g dL^{-1})	Albumin (g dL^{-1})
CD	35 ± 2.6^{c}	1.80 ± 0.10^{a}	8.1 ± 0.3	0.92 ± 0.09^{ab}	0.88 ± 0.02^{a}
SAE-30	27 ± 2.4^{ab}	1.83 ± 0.06^{a}	7.9 ± 0.1	0.94 ± 0.04^{ab}	0.91 ± 0.01^{ab}
SAE-60	35 ± 3^{c}	2 ± 0.05^{b}	7.3 ± 0.06	1.03 ± 0.02^{b}	0.96 ± 0.01^{ab}
SAE-120	28.6 ± 1.5^{b}	2.03 ± 0.06^{b}	7.4 ± 0.04	1 ± 0.01^{ab}	1.03 ± 0.06^{b}
EAE-30	41 ± 2^{d}	1.87 ± 0.06^{a}	7.3 ± 0.15	0.87 ± 0.03^{a}	0.97 ± 0.02^{ab}
EAE-60	22 ± 1^{a}	1.90 ± 0.10^{ab}	7.2 ± 0.06	0.94 ± 0.06^{ab}	0.96 ± 0.04^{ab}
EAE-120	26 ± 2^{ab}	2.07 ± 0.15^{b}	7.1 ± 0.10	1.03 ± 0.06^{b}	0.99 ± 0.09^{ab}

Means with different alphabetical characters in the same column are statistically different (P<0.05).

Table 2. Non-specific immune parameters of *Huso huso* fed diets containing *Salvia officinalis* and *Echinacea angustifolia* extracts

Treatments	AST (UL^{-1})	ALT (UL^{-1})	Lysozyme (µg/ml^{-1})	Ig (mg/ml^{-1})	ACH_{50} (U/mL)
CD	498.3 ± 15.5^{e}	91 ± 2^{c}	18.6 ± 1.5^{a}	10.8 ± 0.29^{a}	121.3 ± 15.5^{ab}
SAE-30	430.3 ± 12.4^{d}	87.3 ± 2.5^{c}	23.6 ± 3^{ab}	11.4 ± 0.25^{abc}	126 ± 11.5^{abc}
SAE-60	415 ± 7^{cd}	84.6 ± 2.2^{bc}	26.7 ± 1.3^{bc}	12.2 ± 0.10^{abc}	128.6 ± 10.5^{bc}
SAE-120	386.6 ± 13.6^{bc}	76 ± 3^{b}	28.3 ± 1.4^{bcd}	12.8 ± 0.76^{c}	134 ± 12^{c}
EAE-30	413.1 ± 14.5^{cd}	77.3 ± 5.5^{b}	34.3 ± 3.1^{d}	11.2 ± 0.55^{ab}	116.3 ± 9.5^{a}
EAE-60	373.1 ± 12.5^{ab}	78 ± 2^{b}	26 ± 4^{bc}	11.3 ± 0.85^{ab}	123.6 ± 5.4^{ab}
EAE-120	352.3 ± 15.3^{a}	63 ± 4^{a}	31.6 ± 3.3^{cd}	12.4 ± 0.40^{bc}	130.3 ± 10.9^{bc}

Means with different alphabetical characters in the same column are statistically different (P<0.05).

in SAE-120 and EAE-120 dietary treatments. The ACH_{50} activity was higher in SAE-120 and EAE-120 dietary treatments (Table 2).

Discussion

During the past decade, application of herbal extracts as immunostimulants enhances the innate defense mechanisms of fish during stressful periods such as intensive farming practices, grading, sea transfer, vaccination and reproduction (Galina, Yin, Ardo, & Jeney, 2009). In line with this, the growth-promoting effect was observed in fish fed diet containing *S. officinalis* and *E. angustifolia* extracts with increase of SGR and FCR in the present study. These results agree with the findings of Roohi, Imanpoor, Jafari, and Taghizadeh (2017) and Rufchaei, Hoseinifar, Mirzajani, and Van Doan (2017) who reported that the fenugreek (*Trigonella foenum graecum*) seed meal and *Pontogammarus maeoticus* extract as feed additives improved the growth parameters (weight gain, SGR and FCR) in common carp, *Cyprinus carpio* and Caspian roach, *Rutilus caspicus* respectively. There is no certain reason about enhancement of growth by inclusion of immunostimulants in fish diet, however it has been attributed to the response of local intestinal inflammatory against pathogens, leadin to weight gain (Dalmo & Bøgwald, 2008; Sirimanapong *et al.*, 2015). It has been proposed that plant based feed additives can modulate intestine microbiota and digestive process, and cause the improved growth performance of fish (MacLennan, Wilson & Taylor, 2002).

Evaluation of RBC, Hct, Hb values, and erythrocyte indices are useful to understand physiological status of the organs (Basusta, 2005). In our study, Htc and Hb levels for beluga fed supplemented diets showed significant differences compared to the control group. This suggests that herbs can improve the performance of the oxygen transport and promote a better tissue perfusion (Rummer and Brauner, 2015). Leucocyte count, erythrocyte count, hematocrit and hemoglobin are particularly recommended as useful indices in fish culture to monitor the health status of the stock (De Pedro, Guijaroo, Lopez-Patino, Martinez-Alvarez, & Delgado, 2005; Lin *et al.*, 2011). Several medicinal herbs such as ginger (*Zingiber officinale Roscoe*), garlic (*Allium sativum Linn*), curcumin and turmeric (*Curcuma longa*) have improved haematological parameters of fish during rearing (Dügenci *et al.*, 2003; Nya & Austin, 2009; Nya & Austin, 2011; Behera, Swain, Sahoo, Mohapatra, & Das, 2011; Alambra, Alenton, Gulpeo, Mecenas, & Miranda, 2012). In this study, extracts-supplemented diet improved the haematological indices (WBC, Hb and Ht) that agreed with findings of Kanani, Nobahar, Kakoolaki, and Jafarian (2014), showing the ginger and garlic feed additives improved haemagtological parameters in juvenile bluga. It has been assumed that the enhancement of hematological parameters in fish fed supplemented diets might be related to increase of cell nuclear contents of transcription factors (Sharma *et al.*, 2010). In the present study, the enhanced haematological indices proposed that active ingredients in *S. officinalis* and *E. angustifolia* extracts play stimulatory role on the level of many cells.

Total and differential leukocyte counts play prominent role in non-specific defense activities and their count can be considered as an indicator of the health status of fish (De Pedro *et al.*, 2005). Additionally, they are important indices with pivotal activity in response to bacterial, viral and parasitic challenges (Houston, 1990). In the current study, lymphocyte counts in bluga fed *S. officinalis* and *E. angustifolia* supplemented diets increased significantly. The proliferation of lymphocytes was observed in bluga fed diets supplemented with ginger, garlic and 2% safflower, *Carthamus tinctorius* (Kanani *et al.*, 2014; Dadras, Hayatbakhsh, Shelton, & Golpour, 2016). Since, concentrations of total protein, albumin and globulin in fish plasma are attributed to a potent innate immune response (Wiegertjes, Stet, Parmentier, & van Muiswinkel, 1996), the innate immune response can be associated with changes in blood biochemical indices.

In the present study, the total protein, albumin and globulin contents was increased using *S. officinalis* and *E. angustifolia* supplemented diets, indicating the improvment of immune system. Oral administration of herbal medicines have been commonly served as feed additives to enhance the immune response in intensive aquaculture system (Bagni *et al.*, 2005; Van Hai, 2015). Lysozyme is a major components of the innate immune system that prevents biofilm formation by adherence and colonization of microorganisms (Verlhac, Obach, Gabaudan, Schüep, & Hole, 1998; Magnadóttir, 2006). The immunomodulation of immune system through oral administration of medicinal plant extracts and their products has been attributed to their function in order to regulate the enhanced bactericidal activities, stimulated natural killer cells, complement, lysozyme activity and antibody responses in fish and shellfish (Harikrishnan, Balasundaram, & Heo, 2011). The results of the present study showed that diet supplemented with *S. officinalis* and *E. angustifolia* extracts can significantly modulate the innate immune responses of bluga (Table 2). Similar to other immunostimulants, a combination of herbs provides beneficial effects to hosts. A mixture of traditional Chinese herbs such as *Astragalus membranaceus* and *Angelica sinensis* in diet of common carp and large yellow croaker, *Pseudosciena crocea* increased plasma lysozyme activity (Jian & Wu, 2004). The plasma lysozyme activity in Nile tilapia, *Oreochromis niloticus* fed diets containing a mixture of *Astragalus membranaceus* and *Lonicerajaponica* extracts was significantly enhanced (Ardo´ *et al.*, 2008).

In conclusion, the present study showed that administration of *E. angustifolia* extract can stimulate innate immune response and feed intake leading to enhanced growth performance. Therefore, this study suggests that utilized herbs can be used as beneficial

feed additive to improve growth performance and health status (immune system) of bluga during rearing.

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