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Effects of dietary administration of purple coneflower on growth, hematology and non-specific immune parameters in juvenile sterlet (*Acipenser ruthenus*)

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Abstract: This study was conducted to evaluate the effects of purple coneflower (*Echinacea purpurea*) (EP) on growth and blood parameters in juvenile sterlet (*Acipenser ruthenus*). One hundred and eighty fish (mean weight 75±1SDg) divided into 12 tanks (n=15) after adaptation period. Base diet was supplemented with 0 (control), 0.5, 1 and 2g/Kg EP to formulate experimental diets. Fish were fed daily at a rate of 3% of body weight. After 60 days, all fish were weighted for growth analysis and blood samples were collected to assess hematological parameters. Results showed that growth indices were not significantly affected by EP extract ($p>0.05$). There was no notable differences in hematological parameters, including red blood cell, white blood cells, hemoglobin, lymphocyte and neutrophil percentage in treatments compared to the control group ($p>0.05$). Total protein, albumin, globulin and albumin to globulin ratio did not reveal any differences between experimental and control groups ($p>0.05$). Lysozyme and Immunoglobulin M in fish fed with EP were higher, compared to the control group ($p<0.05$). It could be concluded that diet supplementation with EP improved immune system function in *A. ruthenus*, but had no effect on growth in this species.

Keywords: Acipenseridae, blood, feeding, growth, lysozyme

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Introduction

Aquaculture has an undeniable role in improving the nutritional quality in most countries. The production rate of this industry has increased dramatically over the past decade. Immunostimulants enhance the activity of the non-specific defense mechanisms and increase disease resistance, both leading to production improvement, so their use in aquaculture is becoming popular (Raa 1996). Immunostimulant apply to any compound that modulates the immune system by increasing the host's resistance to disease, and include a wide range of chemical agents, bacterial components, polysaccharides, animal or plant extracts, nutritional factors and cytokines. Immunostimulants mainly facilitate the function of phagocytic cells and increase their bactericidal activities. Several immunestimulants also stimulate lysozyme and the antibody production of fish (Sakai 1999). Compared to chemicals, plants are natural, safer and cheaper sources. Plant products have various activities like antistress, growth

promotion, appetite stimulation and immunostimulation in aquaculture practices (Citarasu et al. 2002; Sivaram et al. 2004).

Purple coneflower or *Echinacea purpurea* (EP) is a member of the Asteraceae (daisy) family. It is estimated that 1-4% of the world population use herbs in a given year and purple coneflower is one of the most popular herbal medicines (Barrett 2003). It is claimed that purple coneflower has immunostimulatory effects, it is widely used around the world to treat common cold and other infections. Caffeic acid and alkamids are the main constituents of juices of Echinacea with reported bioactivity (Bauer & Wagner 1991). Echinacea treatment results in an increase in various cytokines, lymphocytes, and phagocytosis activities (Sasagawa et al. 2006). Improved growth rate, survival rate, resistance to bacterial infection and immune responses were reported in fish fed with EP (Aly et al. 2007; Aly & Mohamed 2010; Guzz et al. 2011; Bohlouli-Oskoii et al. 2013). Sturgeons are quite physiologically different from teleost species and there are less information about effect of EP on blood and immunological parameters in sturgeons (Gholipourkanani et al. 2013), thus, the aim of this study was to evaluate the effects of EP on growth indices, hematology and non-specific immune parameters of juvenile sterlet.

Material and Methods

Diets. The basal diet consisted of live daphnia, EXS2 (trout starter food), wheat flour, sunflower oil and Kilka (Table 1). The experimental diets were made by adding appropriate amounts of alcoholic EP extract (Zarband Company, Iran) to the basal diet, as follows: 0.5, 1 and 2g purple coneflower meal/kg, respectively. Purple coneflower was not added to diet of the control group. All diets kept at -20ºC till use.

Table 1. Ingredients and proximate composition of the base diet1. NFE: nitrogen-free extract.

Formulation of experimental diets (% dry weight basis)	Food Composition (%)
45	EXS2
22	Kilka
10	Wheat Flour
8	Sunflower oil
10	daphnia
proximate composition	
Dry matter (%)	90.4
Lipid (% DM)	18
Ash (% DM)	7.63
Crude protein (% DM)	46.02
NFE[1] (% DM)	16
Crude cellulose (% DM)	2.35

Experimental design. One hundred and eighty *A. ruthenus* (75±0.1g and 25±2cm) were obtained and used in this study. Fish were acclimatized for 1 week in fiberglass tanks. After this period, juvenile fish were distributed into 4 fiberglass tanks (1125 liter). Tanks were divided by mesh into 3 parts. Fish were randomly divided into 12 groups (n=15). Each treatment was done in triplicates. The fish were kept for 8 weeks in controlled condition (temperature 20.18±0.31ºC, pH= 7.4 and dissolved oxygen= 6.4±0.1mg/L) and were fed at 3% of body weight twice a day (7:00 and 20:00). At the end of the feeding trial, the fish in each treatment were individually weighed 24h after the last feeding. To determine 3% of

body weight following a 24h starvation period, batch weighing was done every 2 weeks.

Estimation of growth criteria. In order to analyze the growth indices of *A. ruthenus* and compare the possible effects of the different treatments, biometry was done. At the end of study all the fish in each group were weighed by a digital scale (to the nearest 0.01mg) after having been anesthetized in 2-phenoxyethanol 2%. Specific growth rate (SGR), feed conversion ratio (FCR) and condition factor (CF) were calculated by standard formula (Golestan et al. 2015).

$$WG \text{ (gained weight)} = W_i/100 - W_f$$

$$FCR = \text{Weight gain/feed intake}$$

$$CF = (W_f/L_f^3)\times 100$$

$$SGR = (\ln W_f - \ln W_i)\times 100$$

Where W_f is final weight mean, W_i is initial weight mean and L_f is final Length mean.

Blood parameters. For hematological analysis, blood samples were taken from 10 fish in each replicate (n=30 from each treatment) through the caudal vessels and divided into two parts. One part was stored in heparinized tubes, and the other was kept in intact tubes to separate serum. In the first part samples, red and white blood cells (RBC and WBC, separately) were counted after dilution with Natt & Herrick's staining solution using neubauer hemocytometer (Houston 1990). Hemoglobin (Hb) was analyzed by adding drabkin solution and photometric assay of cyanomethemoglobin. Hematocrit (Hct) was measured by microhematocrit method. Differential WBCs counts were done on giemsa stained blood smears. Blood indices including mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) calculated by using RBCs count, Hb and Hct values were measured by standard formulas (Houston 1990).

Immunological parameters. Serum lysozyme activity was assessed according to Demers & Bayne (1997), based on the lysis of the lysozyme sensitive Gram positive bacterium, *Micrococcus lysodeikticus* (Sigma, USA). The dilutions of hen egg white lysozyme (Sigma, USA) ranging from 0 to 20 mg ml^{-1} (in 0.1 M phosphate citrate buffer, pH 5.8) were taken as the standard. This along with the undiluted serum samples (25ml) were placed into wells of a 96-well plate in triplicate. One hundred and seventy five microliter of *M. lysodeikticus* suspension ($75mgml^{-1}$) prepared in the same buffer, was later added to each well. After rapid mixing, the change in turbidity of wells measured every 30s for 5min at 450nm at approximately 20ºC using a microplate reader.

Immunoglobulin M (IgM) level in sera was determined by an ELISA method (Cusabio, China). Briefly, samples were added to the plate wells with a biotin-conjugated polyclonal antibody preparations specific for IgM. Avidin conjugated to Horseradish Peroxidase (HRP) was added to each well and incubated. Then a TMB (3,3'5,5' tetramethyl-benzidine) substrate solution was added to each well. The enzyme-substrate reaction was terminated by the addition of a sulphuric acid solution and the color change was measured spectrophotometrically at 450nm. The concentration of IgM in the samples was then determined by comparing the O.D. of the samples to the standard curve.

Statistical analysis. Data were expressed as mean±SD. Data were tested for normality by Kolmogorov–Smirnov test and were analyzed by one way analysis of variance (ANOVA) test using SPSS 16, and where noticeable differences appeared, means were tested using Tukey's post hoc to compare the means. $p<0.05$ was the accepted significance level.

Results

As shown in Table 2, water quality parameters did not show any notable differences among experimental groups during the study period ($p>0.05$). No mortalities were recorded during the trial. As seen in Table 3, at the end of the feeding period, dietary EP did not affect growth indices in *A. ruthenus* and no marked changes were recorded between control and experimental groups ($p>0.05$). No significant

change was recorded in FCR in experimental groups compared to the control group ($p>0.05$). Similar results were recorded for CF and SGR, in which no pronounced changes were seen in experimental groups in comparison to control group.

There were no important differences in RBCs count ($p>0.05$). As seen in Table 4, the number of WBC revealed a considerable increase in fish fed with EP in their diet ($p<0.05$), as the highest value ($19369.33\pm202/mm^3$) was recorded in fish fed with 2g/Kg EP. Parameters related to RBC including Hb, Hct, MCV, MCH, and MCHC did not indicate any significant differences in experimental groups compared to control ($p>0.05$). In differential count of WBCs, lymphocytes percentages showed a marked increase in fish fed with EP, but neutrophils decreased reversely. Monocytes did not manifest any changes between experimental and control groups.

As described in Table 5, IgM values seriously increased in fish fed with EP compared to control group and highest value of IgM was seen in fish fed with 2g/Kg EP ($p<0.05$). Lysozyme activity displayed an increase in fish fed with EP, but this increase was only noteworthy in fish fed with 1 and 2g/kg EP compared to control group ($p<0.05$).

Table 2. Average water quality parameters during the 60 day of experiment.

pH	Temperature (°C)	Dissolved oxygen(ppm)
7.38 ± 0/49	20.18 ± 0/31	6.44±0.11

Table 3. Growth performance in *A. ruthenus* fed control diet supplemented with 0.5, 1 and 2g/kg EP for 8 weeks. Data are presented as mean±SD. WG: Weight gain, FCR: feed conversion ratio, SGR: special growth rate.

Growth index	Control	0.5 (g/kg)	1 (g/kg)	2(g/kg)
WG%	71.58±1.94	70.77±1.96	70.46±2.17	71.30±1.37
$W_{f\,(g)}$	128.39±0.95	128.15± 1.30	128.08 ± 0.88	128.42±0.48
FCR (g/ g)	3.53±0.08	3.58±0.09	3.55±0.04	3.49±0.06
SGR (% / day)	0.95±0.02	0.95±0.02	0.94±0.01	0.95±0.01
Cf	0.42±0.08	0.47± 0.05	0.42± 0.75	0.41± 0.09

Table 4. Hematological parameters of *A. ruthenus* fed for 8 weeks with diets containing different levels of EP. Data are presented as mean±SD. Means in the same row with different superscripts are significantly different ($p<0.05$).

Parameters	Control	0. 5 (g/kg)	1 (g/kg)	2 (g/kg)
RBC (cell/mm3)	679603.3±2366.2 [a]	679362.6±5956.6 [a]	668288.3±10981.5 [a]	676456.3±1959.24 [a]
WBC (cell/mm3)	18613±230.83[a]	18890±173.62[ab]	19169.33±166.76[ab]	19369.33±202.5[b]
Hb (g/dl)	3.73±0.2	3.73±0.12	3.86±0.12	3.66±0.17
Hct (%)	17.66±0.88	18.33±0.88	16.33±0.88	16.66±0.33
Lymphocyte (%)	61.66±1.45 [a]	70.66±2.4[b]	71.66±1.2 [b]	72.33±1.45 [b]
Neutrophil (%)	33.33±1.45 [a]	23±1.52 [b]	21.33±1.2 [b]	21.33±0.88 [b]
Monocyte (%)	3.6±0.33	4.6±0.88	4.6±0.33	4.6±0.33
MCV(fl)	260.04±13.83	269.68±10.71	244.1±9.11	246.41±5.58
MCH (pg)	54.19±2.79	54.94±1.48	57.84±1.09	54.19±2.48
MCHC (%)	21.35±2.26	20.43±0.96	23.74±0.8	22.05±1.5

Table 5. Effect of dietary administration of EP for 8 weeks on blood immunological parameters of *A. ruthenus*. Means in the same row with different superscripts are significantly different (P<0.05).

Parameter	Control	0.5 (g/kg)	1 (g/kg)	2 (g/kg)
Lysozyme (µg/ml)	41.70±0.6[a]	44.32±1.14[a]	48.93±0.7[b]	49.82±1.17[b]
Ig M(gd/l)	14.05±0.44[a]	15.81±0.27[b]	16.43±0.38[b]	16.52±0.39[b]

Discussion and Conclusions

In the present study, EP supplemented diets made no significant changes in growth parameters. Previous studies reported that EP made growth improvements in different teleost species. Aly et al. (2008) noted that in Nile tilapia (*Oreochromis niloticus*), body weight gain and SGRs were impressively higher in group fed with EP than in the control group. Also, in guppy (*Poecilia reticulate*) and rainbow trout (*Oncorhynchus mykiss*) EP added diet increased FCR and SGR (Guzz et al. 2011; Bohlouli Oskoii et al. 2012). Condition factor was affected in Nile tilapia, *Oreochromis niloticus* after being fed with EP enriched diet (Aly et al. 2008). Difference in condition coefficients may be affected by environment, age and feeding conditions (Guzz et al. 2011). Physiology of sturgeons is partially different compared to teleosts and controversial reports on effects of EP on *A. ruthenus* compared to other fish could be related to special phenomena of this species.

Hematological changes in fish supplemented with EP are shown in Table 3. EP treatment caused no significant changes in RBC count, Hb and Hct (P<0.05). This finding contrasts the findings of Bohlouli Oskoii et al. (2012), Aly & Mohamed (2010) and Aly et al. (2008a) in rainbow trout and Nile tilapia, but in other animals such as pigs dried EP did not significantly affect hematological parameters (Maass et al. 2005). No changes in RBCs could be related to no significant changes in growth, so fish do not need more oxygen capacity and therefore, more RBCs.

WBC counts increased in experimental groups compared to control (p<0.05) that was related to the increase in lymphocytes. Bohlouli Oskoii et al. (2012) and Aly et al. (2008) found similar results in rainbow trout and Nile tilapia fed with EP. Increase in WBC count had been reported in previous studied by adding EP to diets in hens, rats and pigs (Cundell et al. 2003; Böhmer et al. 2009). Bauer (1999) found pharmacological effects associated with aerial part or root of Echinacea. *In vitro* stimulatory effect of Echinacea extract on immune cells has been reported in mice and rat (Bauer 1998).

Dietary administration of EP stimulated some non-specific immune parameters of *A. ruthenus*. Lysozyme as an indicator of non-specific immunity in fish, increased in EP treated groups (p<0.05). This may explain the efficacy of Echinacea in improvement of the health status and non-specific immune response. Aly et al. (2008) found similar results in Nile tilapia. Enhanced disease resistance against *A. hydrophila* after 3 month dietary administration of EP was reported in Nile tilapia (Aly and Mohamed 2010). Immunostimulatory effects of EP are categorized as non-specific (Maass et al. 2005). Modulation of the non-specific cellular immune system by polysaccharides, glycoproteins, caffeic acid derivatives and alcylamides was reported as the main function of EP (Aly et al. 2008).

Previous studies have found that IgM showed various levels in different fish species, even among individuals of the same species. These variations could be related to size and age (Klesius 1990; Picchietti et al. 2001), environmental conditions (Klesius 1990) and health status (Magnadottir et al., 1999). Immunomodulators affect production of circulating IgM (Dorucu et al. 2009). In this study, IgM increased in EP fed groups that is in contrast with findings of Mishima et al. (2004) in which EP made a decrease in IgM production in mouse. They reported that EP

increased cell immune responses and decreased humoral responses (Mishima et al. 2004). Also, following administration of EP in rats, Rehman et al. (1999) claimed an increase in IgM content. Difference in results may, in fact, be caused by different species under study and concentration of the work. Increased blood IgM could be related to increase in lymphocyte percent as main source of production of IgM.

This study showed that dietary EP could be used as an effective immunostimulant in *A. ruthenus*, but could not affect growth indices, so more studies with different doses, time periods and environmental parameters should be performed to evaluate effects of EP on the growth performance of *A. ruthenus*.

Acknowledgment

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مقاله پژوهشی

بررسی عملکرد عصاره سرخارگل بر شاخص های رشد، خون‌شناسی و ایمنی بچه ماهی استرلیاد (*Acipenser ruthenus*)

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چکیده: در این پژوهش تأثیر عملکرد گیاه سرخارگل با نام علمی *Echinacea purpurea* بر شاخص‌های رشد و ایمنی در بچه ماهی استرلیاد (*Acipenser ruthenus*) مورد بررسی قرار گرفت. تعداد ۱۸۰ قطعه بچه ماهی استرلیاد با میانگین وزن اولیه ۱±۷۵ گرم انتخاب و پس از سازگاری با محیط بصورت تصادفی در ۳ تانک که توسط چارچوب به چهارقسمت مساوی تقسیم شده بودند، ذخیره شدند. گیاه سرخارگل (EP) در ۳ سطح ۰/۵ گرم در کیلوگرم (تیمار۲)، ۱(تیمار۳) و ۲ گرم در کیلوگرم (تیمار۴) به جیره غذایی اضافه گردید و جیره فاقد EP برای تغذیه گروه شاهد (تیمار۱) مورد استفاده قرار گرفت. هر تیمار در ۳ تکرار انجام گرفت. ماهیان روزانه به میزان ۳٪ از وزن بدن مورد تغذیه قرار گرفتند. در پایان۵۶ روز تعداد ۹ عدد ماهی از هرتیمار به صورت تصادفی انتخاب شده و پس از بیهوش شدن در محلول دو درصد ۲-فنوکسی اتانول خونگیری از ماهیان انجام گرفت. در پایان آزمایش، شاخص های رشد و ایمنی (فعالیت لیزوزیم، ایمنوگلوبین M، پروتئین کل و شمارش تفریقی سلول‌های خون) بچه ماهی استرلیاد در همه تیمارها مورد ارزیابی قرار گرفت. بر اساس نتایج به دست آمده، می‌توان بیان کرد که غلظت‌های ۱ و ۲ گرم در کیلوگرم عصاره سرخارگل بر روی فعالیت لیزوزیم، ایمنوگلوبین و تعداد گلبول‌های سفید تأثیر مثبت دارند، ولی بر پارامترهای رشد فاقد اثر می‌باشند.

کلیدواژه‌ها: تاس‌ماهیان، خون، تغذیه، رشد، لیزوزیم