Evaluation the effects of different levels of *Echinacea purpurea* extract on the immunity responses, biochemical and hematological indices and disease resistance against *Streptococcus iniae* in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

240 rainbow trout fingerlings weighting 16 gram were randomly allocated into three treatment groups including 0.5, 1.0and 1.5 gr/kg of *Echinacea purpurea* extract in food, and a control group (without EPE in food), each intriplicates for 60 days. At sampling times, some of hematological and biochemical parameters including C_3 and C_4 complements, oxygen free radicals, lysozyme activity, and other hemathological parameters were analyzed. Also, at the end of the experiment, fishes challenged with *Streptococcus iniae* and the mortality was analyzed. However, there were no significant differences in C_4, total immunoglobulin, lymphocyte and monocyte ($p>0.05$). The amount of glucose, serum total protein and albumin decreased at the end of the experiment significantly ($p<0.05$). In addition, the results showed that mortality percentage in Echinacea treatment group with 1.5 gr/kg concentration was significantly lower than other treatment groups ($p<0.05$). In conclusion the results have shown that Echinacea extract had the positive effect on immunity responses of rainbow trout especially in higher concentration (1.5gr/kg). Also, adding Echinacea extract in rainbow trout diet increased the resistance of rainbow trout against *S. iniae*.

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Introduction

Rainbow trout (*Oncorhynchus mykiss*) is the most preferred coldwater species in aquaculture industry of Iran. Achieving sustainable development in rainbow trout culture, to maintain the health status of this fish is of major importance. Fish pathogenic organisms are a serious threat to economic viability of any aquaculture practice. Currently, the use of antibiotics for the prophylaxis and treatment of diseases leading to the development of antibiotic resistant bacterial strains, accumulation of residue in cultured fish and environmental problems. Therefore, a new approach to immunotherapy is actively used to prevent ortreat fish diseases. In this regard, extensive research has been carried out to test various immunostimulants including medicinal plants which they have found to be effective in fish. It has beenfound that use of medicinal herbs in fish diets enhance the immune system against infections with various bacteria like *S. iniae,* especially (Leal, Freire, Carvalho, Oliveira & Figueiredo 2008; Ahamad, El Mesallamy, Samir & Zshran2011; Maqsood, Singh, Samoon & Munir 2011; Subeena Begum & Navaraj 2012).

Echinacea belonging to family echinaceadea is a grass form planet with 60-150cm high. Three species of this planet (*Echinacea purpurea, E. pallida, E. angustifolia*) have medical property. Underground rhizomes and aerial organs of this planet mostly used (Soudi, Hashemi, Zavaran Hosseini, Ghaemi & Asghari Jafarabadi 2007). Some of the known most important ingredients of *E. purpurea* extract (EPE) were caryophyllen-B, 8-pentadecadiene, germacrene-D and propyl paraben (Morrazoni, Cristoni, Di Pierro, Avanzini, Ravarino, Stornello , Zucca & Musso 2005; Xu, Xia, Wang & Wang 2008; Dahui, Wang, Zaigui & Yunhua 2011). Also Echinacea had the immunostimulant and anti inflammation effects especially against bacterial and viral disease (Melchart, Walther, Linde, Brandmier & Lersch1998; Galina, Yin, Ardo & Jeney 2009; Dahui *et al*., 2011). Furthermore, Echinacea extract increased humeral and cellular resistance in vertebrates (Stimpel, Proksch, Wagner & Lohman1984). The aim of the present study was to evaluate the effects of different levels of *E. purpurea* extract (EPE) on various parameters of non-specific immunity responses including respiratory burst activity, phagocytic activity of blood leukocytes, serum lysozyme activity, total plasma protein level and some of haematological indices in rainbow trout (*Oncorhynchus mykiss*) to develop alternative drug for the prevention or the treatment of diseases especially *Streptococcus iniae* in aquaculture.

Material and Methods

Preparation of *Echinacea purpurea* extract (EPE)

The plant of Echinacea was procured from Medicinal Plants Production Cooperation of Havin and plant species was identified and confirmed by a botanist. 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 (Morrazoni *et al*., 2005). To do this, chopped dried plant leaves in 75% 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 4ºC until use. The extract added to formulated fish diet in three different doses (0.5, 1.0 and 1.5 gr/kg food). The food was FFT-1 produced by an Iranian fish food factory (Mazandaran Fish Food Co, Mazandaran, Iran). The EPE added to diet using canola oil for coating in three different concentrations.

Fish, experimental conditions and sampling

240 rainbow trout weighing 16 gram were used. All experiments were carried out in 12 fiberglass tanks with water flow. The fish were kept at controlled water temperature of 15±1ºC. After 2 weeks adaptation, fish were randomly allotted in four groups including three experimental groups anda control group, in triplicate was maintained in 12 concrete ponds each containing 20 fish. Each group was hand-fed once a day with diet medicated different doses of *E. purpurea* extract (EPE) for 60days. In days of 30 and 60 after feeding, 9 fish from each replicate sampled and bleed. Half collected in serological tubes containing a pinch of lithium heparin powder, shaken gently and kept at 4ºC to test hematological parameters. Other half collected in tubes without of anticoagulant and allowed to clot at 4ºC for 2hrs to test serological parameters. The clot was the spun down at 2000 rpm for 10 min to separate the serum. The serum collected by micropipette and was stored in sterile Eppendorf tubes at -20ºC until used for assay.

Immunological assay

C_3& C_4 complements and total Immunoglobulin assayed by a commercial kit (Pars Azmoon, Iran) and authorizer (Eurolyser, Belgium) based on method described by (Shahsavani, Mohri & Gholipour Kanani2010) and (Johnson, Rohlfs & Silverman1999).

Serum Lysozyme and Respiratory burst activity

Respiratory burst activity was quantified by method described by Matheus(1990) using a luminoscanascent device (Thermo, Finland). Also an assay based on the method described by Ellis (1990) used to determine lysozyme activity.

Biochemical analysis

Serum total protein, glucose and albumin assayed by a commercial kit (pars Azmoon, Iran) and authorizer machine based on the method described by Whicher (1996).

White Blood Cell (WBC)

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 by Ivanava (1983).

Bacterial challenge

Bacteria separated from the fishes suspiciously tainted to Streptocuccos is identified by bacteria culture and cellular methods (Buller 2004; Austin & Austin 2007). Afterwards final identification was done. In order to challenge fishes with bacteria, 0.1 ml of bacteria suspension adjusted by McFarlaine tube No.1, injected in fishes belly. Then fishes analyzed for 14 days (Rodas, Angulo, Cruz & Garcia (2002). After the disease appearance kidney, liver and heart of fishes sampled and cultured in blood agar using Austin & Austin method (2007) and finally presence or in presence of colonies determined.

Statistical analysis

All results for each parameter measured were expressed as means±standard deviation, and were compared at each time point using student's t-test for independent data. Significant differences between experimental groups were expressed at a significance level of $P < 0.05$. All analyses were carried out on 9 fish per group.

Results

Immunological results are shown in table 1. The results have shown that there was no significant differences between treatment groups in total immunoglobulin in days of 30 and 60 ($p>0.05$). Also there was no significant differences between treatment groups in C_3 concentration, but C_3 concentration in treatment groups comparing to control, significantly increased ($p<0.05$). Nonetheless there was no significant differences between treatment groups in C_4, total protein, albumin and glucose concentrations in days of 30 and 60 ($p>0.05$). But, the other parameters including oxygen free radicals and lysozyme significantly changed in treatment groups. The results are shown that oxygen free radicals and lysozyme concentration in fishes feed with 1.5gr/kg of EPE were significantly higher than the other groups in days of 30and 60 ($p<0.05$). Hematological parameters results are shown in table 2. The results have shown that WBC in treatment groups feed with 1 and1.5 gr/kg of EPE, was significantly higher than 0.5 treatment group and control group in days of 30 and 60 ($p<0.05$). Also, there was significant differences between treatment groups and control group in neutrophils percentage days of 30 and 60 ($p<0.05$). However, there were no significant differences between control and treatment groups in monocyte and lymphocyte percentage in days of 30 and 60 ($p>0.05$).

Table 1 Immunological and biochemical results in fishes feed by *Echinacea purpurea* extract (EPE)(mean±SD)

Day	EPE Level (gr/kg)	Total Immunoglobulin (mg/dL)	C_3 (mg/dL)	C_4 (mg/dL)	Oxygen free radicals ($RLUs^{-1}$)	Lysozyme (mg/mL)	Glucose (g/dL)	Total Protein (g/dL)	Albumin (g/dL)
30	0.5	81.32±15.23	29.6±6.52	8.04±2.47	586.36±65.25	4.36±0.36	92.34±8.55	4.24±0.56	2.27±0.41
	1.0	86.06±14.67	33.33±5.23	8.11±2.75	585.19±56.85	4.46±0.44	95.44±7.21	4.50±0.48	2.32±0.25
	1.5	89.62±16.78	35.21±4.63	8.21±2.61	597.43±45.11	4.86±0.34	96.26±8.41	4.81±0.33	2.35±0.32
	Control	75.83±21.83	25.53±7.21	8.49±3.85	453.59±61.32	2.32±0.67	87.88±17.96	4.11±0.67	2.23±0.85
60	0.5	101.20±14.357	32.50±7.11	8.29±3.50	1100.38±121.25	5.24±0.44	65.24±7.45	4.11±0.54	2.12±0.35
	1.0	105.32±15.65	34.43±6.11	8.56±2.41	1213.53±156.35	5.39±0.65	74.64±8.15	4.21±0.45	2.14±0.26
	1.5	111.26±174.41	36.57±5.64	8.76±2.36	1315.26±314.2	5.67±0.47	75.56±9.31	4.51±0.38	2.21±0.31
	Control	73.23±18.25	21.25±6.25	6.30±2.62	486.23±56.32	2.42±0.56	73.62±14.54	3.20±0.56	2.08±0.56

The results are shown that oxygen free radicals and lysozyme concentration in fishes feed with 1.5gr/kg of EPE were significantly higher than the other groups in days of 30 and 60 ($p<0.05$). Hematological parameters results are shown in table 2. The results have shown that WBC in treatment groups feed with 1 and1.5 gr/kg of EPE, was significantly higher than 0.5 treatment group and control group in days of 30 and 60 ($p<0.05$). Also, there was significant differences between treatment groups and control group in neutrophils percentage days of 30 and 60 ($p<0.05$). However, there were no significant differences between control and treatment groups in monocyte and lymphocyte percentage indays of 30 and 60 ($p>0.05$).

Table 3 shows the survival results of bacterial challenge. The results have shown that there were significant differences between treatment groups and control group in survival rate after the bacterial challenge.

Table 2 Hematological parameters results (mean±SD)

Day	EPE Level (gr/kg)	WBC ($\times 10^3$)	Neutrophil (%)	Monocyte (%)	Lymphocyte (%)
30	0.5	10.50±0.35	2.32±0.97	1.56±0.12	**96.23±2.11**
	1.0	10.8±0.76	2.35±0.78	1.71±0.13	**95.94±2.86**
	1.5	10.50±0.83	2.21±0.70	1.77±0.17	**95.02±2.33**
	Control	9.70±0.73	1.44±0.53	0.67±0.15	**97.89±1.05**
60	0.5	14.70±0.65	5.43±1.16	1.77±0.14	**92.80±3.40**
	1.0	15.30±0.65	6.33±1.27	1.88±0.12	**91.79±4.96**
	1.5	16.70±0.87	8.11±1.83	1.91±0.18	**89.98±2.16**
	Control	13.20±0.88	2.11±0.32	1.11±0.12	**96.78±2.06**

Table 3 Total survival rate of controland treatmentgroups after the bacterial challenge(mean±SD)

Treatment groups	Survival rate (%)
0.0 gr/kg (C)	44.44±6.11[a]
0.5 gr/kg	84.44±3.39[b]
1.0 gr/kg	86.66±3.17[c]
1.5 gr/kg	91.11±2.23[d]

C=Control. Varied letters indicate significant differences between groups.

Discussion

Dietary medicinal plant extracts, like *E. purpurea* extract (EPE), as immunostimulants, elevate non-specific defenses during period stress (Yin, Wiegertjes, Li, Schrama, Verreth, Xu & Zhou 2004; Cao, Ding, Zhang, Jeney & Yin 2008). Hematological assay may provide an index of the physiology status of fish. This study indicates the effects of *E. purpurea* extracton the hematological parameters and immunological responses in rainbow trout (*O. mykiss*). In the present study, the hematological parameters such as WBC and Neutrophil indices were significant differences at the end of identical 30 and 60 days period after feeding when compared to control group. Also these parameters (WBC, Neutrophil) were higher in fishes feed with higher concentration of EPE. These results are in consistent with the results obtained of many researchers who reported common carp treated with dietary *E. purpurea* supplementation were significant differences in WBC and differential leukocytes counts (Gopulakannan & Arul 2006; Yin, Adro, Thompson, Adams, Jeney & Jeney 2009). Also,Oskoii, Kohyani, Parseh & Sadeghi. (2012) showed that EPE increased WBC in rainbow trout.

Serum proteins are various humoral elements of the non-specific immune system, measurable total protein, albumin and globulin levels suggest that high concentrations are likely to be a result of the enhancement of the non-specific immune response of fish. Globulin is the main resource of immunoglobulin production, thus its enhancement in serum provide immunostimularory potential. Albumin, total immunoglobulin and total protein do not indicate significantly differences in treated group in compare to control group. Similar result was reported in *Cyprinus carpio* fingerlings of treated levamisole (Maqsood, Samoon & Singh 2009). In our study, there were no significant differences in total immunoglobulin between experimental and control groups. Similar results were reported in

tilapia fed with *Astragalus membranaceus* extract (Ardo, Yin, Xu, Varadi, Szigeti, Jeney & Jeney 2008). But some herbal planets such as ginger (Dugenci, Arda & Candan 2003), mint and cinnamon (Hajibeglu & Sudagar 2010) and *Magnifera indica* (Rao, Das, Pradhan & Chakrabarti 2006) increase serum proteins such as albumin and globuline. This could be because of the differences in herbal extract ingredients.

Complements especially C_3 are produced by hepatic cells and they are very important in bactericidal effects of mucous and serum (Ellis 2001; Holland & Lambirs 2002).The results obtained of this study indicated an enhancement in complement C3 concentration at the end of the experiment. These results are in agreement with the results obtained from other studies. Awad et al. (2010) showed that using the extracts of *Lupinus perennis*, *Managifera indica* and *Urtica dioica* especially at the concentrations of 1 and 2% in fish diet corroborated the activity of complement ingredients especially C3 and C4 in rainbow trout. The same results obtained from the treatment with *Radix astragalinseu* and *R. Angelicae sinensis* in common carp and yellow croaker (Jian & Wu 2003, 2004). Furthermore *Eclipta alba*e xtract increased complement ingredients concentration in tilapia (Christy bapita, Divyagnaneswari & Michael 2007).

Also, the results obtained of this study indicated an enhancement in respiratory burst activity in treated groups in comparison with control group, which are in agreement the results of some of studies with dietary immunostimulants used in various fish species (Peddie & Secombes 2003; Yin et al., 2009; Bilen & Bulut 2010). Respiratory burst activity is considered as an important indicator of non-specific defense in fish, which is a measure of the increase of oxidation level in phagocytes stimulated by foreign agents (Liaghat, Akhlaghi, Hosseini, Nematollahi & Hosseini 2011). Respiratory burst and phagocytosis response by phagocytes in blood present a major antibacterial defense mechanism in fish (Harikrishnan, Balasundaram & Heo 2010). The main cells involved in phagocytosis in fish are neutrophils and macrophages. These cells remove bacteria mainly by the production of reactive oxygen species (ROS) during a respiratory burst. In addition, neutrophils possess myeloperoxidase in their cytoplasmic granules, which in the presence of halide and hydrogen peroxidase kills bacteria by halogenations of the bacterial cell wall. Moreover, these cells possess lysozymes and other hydrolytic enzymes in their lysosomes (Uribe, Folch, Enriquez & Moran 2011). Similarly, macrophages can produce nitric oxide in mammals and can be as potent antibacterial agents, peroxynitrates and hydroxyl groups (Secombes 1996).

But, in this study there was no significant difference between treatment groups in lysozyme activity nonetheless there was significant difference between treatment groups and control group. These results are in agreement with several reports indicating the

role of herbal immunostimulants in enhancing lysozyme activity (Rao *et al*., 2006; Choi, Park, Yoon, Kim, Jang & Choe 2008). 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.

The results of the bacterial challenge experiment have shown a significant difference between treatment groups and control in mortality rate. Other results of this study are shown that the treatment with EPE increased non specific immune system in fish. Lower mortality in treatment groups could be because of treatment with EPE. Other experiments have shown that herbal plantsextract treatment increased the resistance of common carp against *A. hydrophila* (Hajibeglu & Sudagar 2010). Same results obtained from treatment of tilapia with *Psidium guajava* extract against *A. hydrophila* (Pachanawan, Phumkhachorn & Rattanachaikunsopon 2008).

In conclusion, supplementation of EPE in rainbow trout diet enhances non-specific immune system and it's resistant against *S. iniae*. Also its upper concentrations have better results.

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بررسی اثر عصاره سرخارگل (*Echinacea purpurea*) بر ایمنی غیر اختصاصی قزل آلای رنگین کمان (*Oncorhynchus mykiss*) در برابر استرپتوکوکوزیس Streptococcosis

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چکیده

240 بچه ماهیان قزل آلا (باوزن متوسط 16گرم و در دمای 20-14 درجه سانتی گراد) در برابر استرپتوکوکوس اینیایی (*Streptococcus iniae*) استفاده گردید. سه غلظت از عصاره سرخارگل (0/5 ،1 و 1/5 گرم بر کیلوگرم غذا) به جیره غذایی ماهیان مذکور اضافه شد و با گروه کنترل(جیره فاقد سرخارگل) به مدت 60 روز مورد ارزیابی قرار گرفت. شاخصهای مورد بررسی شامل تغییراتاجزاکمپلمان (C_3، C_4) ، رادیکال آزاد اکسیژن، لیزوزیم، ایمنوگلوبولین تام، گلوکز، پروتین تام سرم، آلبومین و سایر فاکتورهای خونی و در انتهای کار نیز مواجهه سازي با باکتری استرپتوکوکوس اینیایی انجام و درصد بقاء ماهیان مورد آزمایش، ارزیابی گردید. نتایج نشان داد که مقادیر C_3، لیزوزیم، رادیکال آزاد اکسیزن، تعداد کل گلبولهای سفید و درصد نوتروفیل پس از 60 روز افزایش معنی داری در تیمارهای حاوی سرخارگل نسبت به گروه کنترل داشته ($p<0/05$) و غلظتهای بالاتر(1/5گرم) آن نیز نتایج بهتری به همراه داشته است. نتیجه گیری کلی آنکه گیاه مورد استفاده دارای اثرات تقویت کننده بر سیستم ایمنی ذاتی بوده و غلظتهای بالاتر آن (1/5گرم) نتایج بهتری به همراه داشته است.

کلمات کلیدی: سرخارگل، ماهی قزل آلا، استرپتوکوکوزیس، سیستم ایمنی، لیزوزیم

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