Effect of Dietary Sage (*Salvia officinalis* L.), Licorice Root (*Glycyrrhize glabra* L.), Blueberry (*Vaccinium myrtillus* L.) and Echinaceae (*Echinacea angustifolia* Hell) on Nonspecific Immunity and Resistance to *Vibrio anguillarum* Infection in Rainbow Trout, (*Oncorhynchus mykiss)*

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Abstract

In this study, the effects of blueberry (*Vaccinium myrtillus*), licorice root (*Glycyrrhize glabra*), echinaceae (*Echinacea angustifolia*) and sage (*Salvia officinalis*) on immune response, some haematological tests and disease resistant of rainbow trout were investigated. Fish were fed diets containing 0 (control), 0.1 and 1.0% herbs (w/w) for 45 days. Results of this study showed that rainbow trout the feeding with plants (0.1 %) enhanced NBT (nitroblue tetrazolium) and lysozyme activity and total leukocyte counts at 45th day of experiment ($p<0.05$). These results suggested that all plants used our study can be applied as fed supplement to elevate immunity in rainbow trout. However, RPS values only in the groups, bluberry (0.1, 1 %) and echinacea (0.1 %) were found to be over 50 following *V. anguillarum* infection.

Keywords: *Salvia officinalis, Vaccinium myrtillus, Glycyrrhize glabra, Echinacea angustifolia*, Non- specific immunity, *Vibrio anguillarum*

Adaçayı (*Salvia officinalis* L.), Meyan Kökü (*Glycyrrhize glabra* L.), Yaban Mersini (*Vaccinium myrtillus* L.) ve Ekinezyanın (*Echinacea angustifolia* Hell) Gökkuşağı Alabalıklarında (*Oncorhynchus mykiss*) Spesifik Olmayan Bağışıklığa ve *Vibrio anguillarum* Enfeksiyonuna Etkisi

Özet

Bu araştırmada, yaban mersini (*Vaccinium myrtillus*), meyan kökü (*Glycyrrhize glabra*), ekinezya (*Echinacea angustifolia*) ve adaçayının (*Salvia officinalis*) gökkuşağı alabalığının bağışıklık sistemi, bazı hematolojik testler ve hastalıklara direnç üzerindeki etkisi incelendi. Balıklar kontrol (0), % 0.1 ve 1 (g/kg) oranında diyete ilave edilen bitki ile 45 gün süre ile beslendi. Araştırma sonuçlarına göre denemenin 45. gününde % 0,1 oranında yemlerine bitki ilave edilen tüm gruplardaki balıklarda NBT ve lizozim kativiteleri ve toplam lökosit sayıları arttı ($p<0.05$). Araştırma sonuçlarına göre kullandığımız tüm bitkilerin gökkuşağı alabalıklarında spesifik olmayan bağışıklığı artırması nedeniyle yem katkı maddesi olarak kullanılabileceği tespit edilmiştir. Bununla birlikte, *V. anguillarum* ile deneysel enfeksiyon sonunda RPS değerleri sadece yaban mersini (%0,1,1) ve ekinezya (%0.1) gruplarında 50'nin üzerinde bulundu.

Anahtar kelimeler: *Salvia officinalis,Vaccinium myrtillus, Glycyrrhize glabra, Echinacea angustifolia*, Non-spesifik bağışıklık, *Vibrio anguillarum*

INTRODUCTION

The use of immunostimulants in fish culture for the prevention of disease is a promising new development. Immunostimulants enhance the innate immune response (Sakai, 1999). The use of medicinal herbs is an alternative to antibiotics in fish health management (Hermann et al., 2003; Abdel-Tawwab et al., 2008; Ashraf and Goda, 2008).

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Many studies have confirmed that the application of diet herbal additives has a positive impact on the health and resistance of the fish, and also improves their condition and growth rate.

Vibriosis is one of the most prevalent fish disease caused by bacteria belonging to the genus *Vibrio. Vibrio anguillarum* is reported in many fish species as a pathogen such as rainbow trout (Ekici et al., 2005), sea bream (Akayli and Timur, 2002), sea bass (Tanrikul et al., 2004).

In this study, blueberry (*Vaccinium myrtillus* L., Ericaceae), licorice root (*Glycyrrhize glabra* L., Fabaceae), Echinacea (*Echinacea angustifolia* Hell, Asteraceae) and sage (*Salvia officinalis* L., Laminaceae) were chosen because of their recorded pharmacological activities (Baydar, 2005). Blueberry (*V. myrtillus*) is used as traditional human medicine to treat circulatory problems (Martin-Aragon et al., 1998) and as anti-inflamatory agent (Turkben et al., 2008). Echinacea (*E. angustifolia*) is primarily used in medicine believed to stimulate the immune system. It also used as antibacterial agent (Barnes et al., 2005). *S. officinalis* L. (Lamiaceae) is an important and native the Mediterranean region. It is a rich source polyphenols. Rosmarinic acid, carnasol and carnosic acid were the prevalent compounds of *S. officinalis* methanolic extract. Polyphenolic compounds known to be responsible for the main antioxidant activity of *S. officinalis* (Baydar, 2005; Bayram and Sonmez, 2006; Ekren et al., 2007; Farhat et al., 2009).

G. glabra L. (Fabaceae) is native grow in Turkey. Liguorice is extracted from the root of the plant *G. glabra* and use pharmaceutical industry (Fenwick et al., 1990; Akan and Balos, 2008).

In the present study, the effect of sage, licorice root, blueberry and echinaceae on nitroblue tetrazolium (NBT) activity, lysozyme activity and some hematological parameters and disease resistance against a pathogenic bacteria *Vibrio anguillarum* in rainbow trout was investigated.

MATERIALS and METHODS

Fish and Experimental Design

The experiments were conducted in the flow-through system in the aquaculture laboratory at the fisheries faculty in Egirdir/Isparta. Healthy rainbow trouts, weighing approximately (15±2 g), were obtained from a commercial aquaculture farm in Isparta. Fish were randomly distributed into tanks (350 L), and acclimated to laboratory conditions for two weeks. The experimental fish were divided into twelve groups of 30 each, in triplicate. Water temperature and pH were constant (12° C; pH 7.2) during the experimental period and dissolved oxygen was maintained 7.5 mg l^{-1} and water flow rate of 1-1.5 min^{-1} with continuous aeration. The feed ingredients of diet were presented in Table1.

Table 1. Formulation (%) of the experimental diet

	Experimental Groups and Utilization Rates (mg kg^{-1})								
Ingredients	**1% Blueberry**	**0.1% Blueberry**	**1% Licorice Root**	**0,1% Licorice Root**	**1% Echinacea**	**0.1% Echinacea**	**1% Sage**	**0.1% Sage**	**Negative Control**
Fish meal[1]	35.00	35.00	35.00	35.00	35.00	35.00	35.00	35.00	35.00
Soybean[2]	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00
Wheat gluten[3]	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Wheat by-product[4]	13.999	13.9999	13.999	13.9999	13.999	13.9999	13.999	13.999	14.00
Fish oil[5]	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00
C vitamin[6]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Others[7]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Pellet binders[8]	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Antioxidant[9]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin premix [10]	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Mineral premix[11]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Herb (w/w)	0.001	0.0001	0.001	0.0001	0.001	0.0001	0.001	0.0001	0

[1-7]Abalioglu Feed Factory; Uzundere Location Torbalı-Izmir/Turkey.

[8]Aquacube

[9]Ethoxyquin

[10]Vitamin premix.; per kg, 4 000 000 IU vitamin A, 480 000 IU vitamin D_3, 40 000 mg vitamin E, 2400 mg vitamin K_3, 4 000 mg vitamin B_1, 6 000 mg vitamin B_2, 40 000 mg niacin, 10 000 mg calcium D-pantothenate, 4 000 mg vitamin B_6, 10 mg vitamin B_{12}, 100 mg D-biotin, 1 200 mg folic acid, 40 000 mg vitamin C and 60 000 mg inositol.

[11] Mineral premix.; per kg 23 750 mg Mn, 75 000 mg Zn, 5 000 mg Zn, 2 000 mg Co, 2 750 mg I, 100 mg Se, 200 000 mg Mg.

Four herbal supplement were obtained from local market. The dried, powdered plant materials were mixed with a certain proportion. The control diet contained no supplementation (0%). Herbal mixture was incorporated into diet at different rates of 0.1%, 1.0% then made into pellet feed. The experimental pellets were packed and stored at 4° C.

Gas Chromatography-Mass Spectroscopy Analyses of Plants Materials

The gas chromatographic analysis of plant materials was performed with Hewlett-Packerd G890 series gas chromatography (Perkin Elmer (PE) Auto System XL, USA), fitted with a flame ionization detector (FID). The PE Auto System XL gas chromatograph was employed under the following conditions: capillary column, CPW ax 52 CB (50M × 0.32 mm: film thickness ¼ 0.25 lm); oven temperature program. 40 °C raised to 230 °C at a rate of 4 °C/min and then held at 230 °C for 10 min; injector and detector temperatures, 250 °C; carrier gas, helium at flow rate of 1.5 ml min^{-1}; split ratio 1/20 ml min^{-1} Relative percentage amounts were calculated from chromatograms by the Turbo Crom. Navigator computer program (Baydar et al., 2004; Sarac et al., 2009) (Table 2).

Table 2. Gas chromatographic working condition

İnjection Column	250° C
Detector	250° C
Flow Rate (ml/min)	1,5
Detector	70 eV
Lonization Type	El
Carrier Gas	Helium
Capillary Column	Cp WAX 52 CB 50 m * 0.32 mm, 1,2 µm
Oven Temperature Programe	40 °C raised to 230 °C at a rate of 4 °C/min and then held at 230 °C for 10 min;
Used Library	Wiley, Nist, Tutor

Sample Collection, Haematological and Immunological Tests

Blood samples were collected from the caudal vein of rainbow trout for 20 and 45 days. Some of each blood sample was allocated for the haematological assays and the rest of the blood samples were added to heparin containing tubes for the other immunological analyses. Serums were separated by centrifugation at 5000 g for 5 min and immunological analyses were done.

For determining red blood cells (RBC) and white blood cells (WBC), Natt-Herrick diluting fluid was used. Counting was done by mixing the blood with the diluting fluids. Cell counting was performed using a Neubauer's counting chamber under a light microscope (Hoffman and Lommel, 1984).

Hematocrit (Hct) was determined using a capillary hematocrit tube and it was measured by hematocrit scale.

The turbidimetric assay for lysozyme was carried out according to Demers and Bayne, (1997). For this, 25 µl volumes of serum were added to 2 ml of suspension of *Micrococcus lysodeikticus* (0.2 mg ml^{-1}, Sigma-Aldrich) in sodium phosphate buffer (pH 6.2) and followed by 15 min reaction at 37 °C and the absorbance was measured at 530 nm in a microplate reader after 0.3 and 4.5 min on a spectrophotometer. Lysozyme activity was defined as µg per ml serum (Demers and Bayne, 1997).

NBT (Sigma N-6876) was used to determine the respiratory burst activity by following a modified method. Nitroblue tetrazolium (NBT) (Sigma No: N-6876) solution (0.2%) was freshly prepared in sterile 0.85% (w/v) saline and used in a modification of the method described by Anderson et al, (1992). Briefly, 50 µl of blood was dropped on to coverslip and incubated in 0.067 mM sodium phosphate buffer (pH 6.4) to remove the red blood cells. A drop of 0.2% NBT solution was placed on to a microscope slide and the coverslip was placed cell face down on the NBT solution. The cells were incubated for 30 min at 25°C. The positive, dark blue staining cells were counted under a microscope (×400 magnifications). Five coverslips were examined for each fish and five random fields were counted on each slide. The twenty five field were averaged and mean and standard error of values per field for fish were calculated (Anderson et al., 1992).

Challenge Test

V. anguillarum was obtained from Egirdir Fisheries Faculty. After 45 days of feeding, all fish from each group were injected inraperitoneally (i.p.) with 100 µl PBS containing 2.10^2 cfu ml^{-1} *V. anguillarum* strain cells (Ceylan and Altun, 2010; Ekici, 2010). *V.*

anguillarum was re-isolated to confirm the mortality during the experimental infection. Mortality was recorded for 10 days. Relative Percent Survival (RPS) was calculated according to Ellis (1998) as follows: RPS= (1- (%mortality treatment / %mortality control))×100

Statistical Analysis

The obtained data analyzed with one way analysis of variance (ANOVA) to determine if significant differences occurred among the herbal treatments and control group. Duncan's multiple-range test was used to compare differences among individual means. The data were analyzed with SPSS 11.05. All statistical computations were performed at the probability level of $P < 0.05$.

RESULTS

The results of the chemical analysis of *S. officinalis, G. glabra, E. angustifolia* and *V. myrtillus* were presented in Table 3. The major components of the *V. myrtillus, G. glabra, S. officinalis* and *E. angustifolia* were hexanal (32.84%) 1.8 cineol (31.62%), hexanal (36.66%), 1.8 cineole (56.98%) and 1.8 cineole (27.10%) respectively.

Table 3. Chemical composition of the plant materials

Bluberry (*V. myrtillus*)		**Licorice (*G. glabra*)**		**Sage (*S. officinalis*)**		**Echinacea (*E. angustifolia*)**	
Plant compenents	**(%)**	**Plant compenents**	**(%)**	**Plant compenents**	**(%)**	**Plant compenents**	**(%)**
Hexanal	32.84	Hexanal	36.66	Hexanal	0.43	Hexanal	10.64
1,8-cineole	31.62	1,8-cineole	19.84	1,8-cineole	56.98	1,8-cineole	27.10
1-hexanol	2.82	1-hexanol	6.39	2-hexanal	0.41	1-hexanol	3.26
7-octen-4-ol	2.82	7-octen-4-ol	1.07	7-octen-4-ol	0.39	7-octen-4-ol	1.70
Camphor	8.73	Camphor	11.08	Camphor	21.15	Camphor	15.27
Alpha-terpineol	1.84	Alpha-terpineol	1.77	Alpha-terpineol	1.31	Alpha-terpineol	2.53
1-pentanol	2.05	1-pentanol	2.92	α-cymene	0.91	α-cymene	4.95
Alpha pinene	8.39	Carvone	3.23	Alpha pinene	1.93	Camphene	3.24
α-cymene	2.41	Carvacrol	5.83	Camphene	1.74	Beta-pinene	1.94
2-heptanal	1.85	Borneol	1.14	Beta-pinene	0.39	Z-thujenol	3.39
Limonene	4.63	Trans-caryophyllene	0.30	Beta-myrcene	1.14	Beta-myrcene	1.44
		Bornyl acetate	1.31	Limonene	0.46	2-hexenal	4.57
		p-cymene	1.86	3-hexen-1-ol	0.20	Nerolidol	2.09
		2-pentyl-furan	6.60	Alpha-thujone	1.10	Limonene oxide	2.16
				Beta-thujone	0.69	Isoborneol	1.57
				Bornyl acetat	3.43	Berbenone	4.29
				4-terpineol	0.29	Alpha pinene	9.87
				Trans-caryophyllene	3.48		
				Aromadendrene	0.26		
				Linalyl oxide	0.42		
				Alpha-humulene	0.48		
				Terpinyl acetate	0.27		
				Borneol	2.13		

Immunostimulatory effects of plants were given Table 4. NBT and lysozyme activities and total leukocyte counts in the groups treated with plants (0.1 %) were significantly different compared to the control group ($P<0.05$) in 45th day of experiment.

Table 4. The effects of dietary herbal additives on the hematologic and immune parameters of rainbow trout

Blood Parameters	Days	1% Sage	0.1% Sage	1% Echinacea	0.1% Echinacea	1% Licorice Root	0.1% Licorice Root	1% Blueberry	0.1% Blueberry	Control
Hematocrit Values (%)	20	39.22±3.65^{a}	43.56±0.53^{ab}	45.89±1.65^{b}	42.22±0.95^{ab}	41.78±1.59^{ab}	44.33±1.65^{ab}	40.33±0.88^{a}	43.11±0.93^{ab}	46.89±1.49^{b}
	45	39.22±0.70^{a}	46.89±0.59^{bcd}	44.89±0.48^{b}	46.89±1.35^{bcd}	44.22±1.34^{b}	50.33±1.69^{d}	45.22±0.81^{bc}	49.11±2.03^{cd}	47.56±1.58^{bcd}
Erythrocyte Level ($\times 10^{6}$ μl^{-1})	20	0.24±0.005^{c}	0.18±0.004^{a}	0.18±0.011^{ab}	0.22±0.005^{bc}	0.19±0.011^{ab}	0.18±0.018^{ab}	0.20±0.006^{abc}	0.19±0.033^{ab}	0.16±0.005^{a}
	45	0.18±0.012^{ab}	0.18±0.007^{ab}	0.22±0.007^{c}	0.28±0.013^{e}	0.16±0.010^{a}	0.20±0.008^{bc}	0.19±0.011^{b}	0.22±0.009^{c}	0.18±0.008^{ab}
Total leukocyte Counts ($\times 10^{5}$ μl^{-1})	20	0.22±0.029^{bc}	0.13±0.005^{a}	0.17±0.012^{abc}	0.18±0.024^{abc}	0.19±0.012^{abc}	0.17±0.019^{abc}	0.19±0.012^{abc}	0.23±0.043^{c}	0.15±0.016^{ab}
	45	0.24± 0.013^{d}	0.23±0.013^{cd}	0.24±0.008^{d}	0.28±0.024^{e}	0.13±0.007^{a}	0.17±0.008^{b}	0.17±0.009^{b}	0.19±0.017^{bc}	0.10±0.005^{a}
Nitro Blue Tetrazolium (NBT)	20	0.31±0.10^{a}	0.04±0.01^{a}	1.61±0.74^{bc}	0.46±0.18^{a}	0.82±0.36^{ab}	0.60±0.23^{ab}	1.88±0.47^{c}	1.03±0.21^{abc}	0.22±0.11^{a}
	45	6.38±1.69^{d}	4.71±0.72^{bcd}	3.73±0.22^{bc}	5.09±0.44^{bcd}	4.96±0.59^{bcd}	3.58±0.76^{bc}	0.51±0.20^{a}	4.27±0.81^{bcd}	2.44±0.74^{a}
Lysozyme Activity (Unit ml^{-1})	20	666.60±2.84^{b}	1555.60±0.92^{e}	600.00±0.76^{ab}	1244.40±2.41^{c}	1333.30±2.09^{d}	533.30±2.84^{a}	1746.78±1.56^{g}	2133.30±2.58^{f}	675.00±0.70^{b}
	45	496.66±2.07^{a}	2010.53±3.61^{c}	823.67±2.03^{b}	1910.00±4.19^{bc}	1741.18±3.79^{bc}	1501.33±3.58^{bc}	1885.71±5.47^{bc}	2383.33±4.25^{c}	742.22±2.34^{a}

Values are provided as mean ± standard error. Values different letters are significantly different ($p<0.05$) within the lines.

It was observed that groups of rainbow trout fed with bluberry (0.1, 1 %) and Echinacea (0.1 %) had good RPS values following challenge infection with *V. anguillarum* (Table 5).

Table 5. The rate of mortality and RPS values offollowing challenge infection with *V. anguillarum*

Groups	Mortality (%)	RPS
1% Blueberry	30±2.00[a]	53.13
0.1% Blueberry	30±2.00[a]	53.13
0.1% Echinacea	32±2.00[ab]	50.00
0.1% Licorice Root	34±2.00[ab]	46.88
0.1% Sage	34±2.00[ab]	46.88
1% Licorice Root	38±2.00[bc]	40.63
1% Echinacea	44±2.00[cd]	31.25
1% Sage	48±2.00[d]	25.00
Control	64±1.00[e]	

Values are provided as mean ± standard error. Values different letters are significantly different ($p<0.05$) within the column.

DISCUSSION

The immunomodulatory effects of herbal medicines have been well reported in various fish species (Dugenci et al., 2003; Yin et al., 2006; Sahu et al., 2007; Aly et al., 2008; Ahilan et al., 2010; Awad and Austin, 2010; Tang et al., 2014). The immunostimulatory response and pathogen resistance of *V.myrtillus*, *G. glabra*, *E. angustifolia* and *S. officinalis* were investigated on rainbow trout in this study. Results of this study showed that the feeding rainbow trout with 0.1 and 1.0% licorice root and 0.1% blueberry enhanced NBT activity and 1.0% blueberry and licorice root enhanced lysozyme activity for 20 and 45 days. NBT activity was found higher in 0.1% fed with plants than the control group for 45 days. Lysozyme is a important antimicrobial effector in fish (Yeh et al., 2008), which also serve as on opsonin in complement system and phagocytes activation (Magnadottir, 2006). The present study indicated that 0.1% doses of plants enhanced lysozyme activity compared to the control for 45 day. Mesalhy et al., (2008) observed echinaceae (*E. purpurea*) as immunostimulatory agent in nile tilapia (*Oreochromis niloticus*). In addition, increased NBT value was also reported on koi carp (*Cyprinus carpio*) fed with tetra (*Cotinus coggygria*) (Bilen et al. 2013). Increased lysozyme activity reported by Nya and Austin, (2009) on rainbow trout fed with garlic (*Allium sativum*) bulb and Yin et al. (2006) on tilapia fed with Chinese herb (*Astragalus radix*).

It has been widely established that certain herbs can improve the resistance of fish to bacterial diseases. The antibacterial active principles of the herbals may lyse the cell wall, block the protein synthesis and DNA synthesis, inhibit the enzyme secretions and interfere with the signaling mechanism of quorum sensing pathway (Citarasu, 2010). Zilberg et al., (2010) found that feeding with dried rosemary leaves (*Rosmarinus officinalis*) significantly reduced mortality following infection with *Streptococcus iniae.* Mesalhy et al., (2008) observed echinaceae (*E. purpurea*) as immunostimulatory agent in nile tilapia (*O. niloticus*) and *E. purpurea* extract increased survival rate against the pathogen *Pseudomonas fluorescens*. The *V. anguillarum* is commonly considered halophilic pathogen (Taylor, 1989), but Fujiwara-Nagata and Eguchi, (2004) have shown that this pathogen can survive in freshwater conditions and it is also pathogenic for rainbow trout (Ekici et al. 2005). Alexander et al. (2010) reported that *Tinospora cordifolia* leaves gave protection in terms of reduced percent mortality which is reflected in the increased RPS values. In contrast, Huttenhuis et al. (2006) observed that a challenge with *V. anguillarum* resulted in an initially higher cumulative mortality in the group fed with lipopolysaccharide (LPS). In the present study, the groups fed with bluberry (0.1, 1 %) and echinacea (0.1 %) had good RPS values following challenge infection with *V. anguillarum*. As a result of this study, oral administration of plants at dose of 0.1% increased the non

specific immunity of rainbow trout. However, only the plants, bluberry (0.1, 1%) and echinacea (0.1%) protected the fish against *V. anguillarum.*

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