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Effect of mixed use of thyme and fennel oils on biochemical properties and electrolytes in rainbow trout as a response to *Yersinia ruckeri* infection

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Abstract

The aim of study was to compare the effects of supplementation of food with herbal oils (*Thymus vulgaris* and *Foeniculum vulgare*) on biochemical properties and electrolytes of rainbow trout infected with *Yersinia ruckeri*. In total, 120 healthy fish (mean weight 84 ± 1.02 g) were equally divided into four experimental groups. The experimental study was carried out for one week. The first group was control without supplementation and infection, the second group was infected and without oil supplementation, the third group was supplemented with oils (10 ml·100 g^{-1} rates) for one week and infected with *Y. ruckeri* and the last group was oil supplemented without infection. Results indicated that fish fed with dietary supplements showed enhanced bactericidal activity, total protein, albumin, cholesterol, triglyceride and bilirubin compared to the control ($P < 0.05$). As the value of herbal oils was increased in diets, the plasma glucose level decreased. The levels of K, Na, Ca, and Mg increased whereas Cl values decreased, compared to the control. It can be concluded that diet supplementation with herb oils used in this study can increase disease resistance by increasing levels of some biochemical parameters and electrolytes in rainbow trout to *Y. ruckeri* infection.

Thymus vulgaris, Foeniculum vulgare, Oncorhynchus mykiss, biochemical indices

The massive use of antimicrobials for disease control has suppressed the growth of aquatic animals since it leads to antibiotic and chemical resistance and consumer unwillingness (Smith et al. 1994; MacMillan 2001). To reduce or avoid the dependence of aquaculture on antibiotics, some products of natural plant origin have been considered as an effective alternative way to control bacterial and viral infections (Harikrishnan et al. 2010; Kirubakaran et al. 2010). Several antimicrobial, anti-stress, immunostimulant and growth-promoting plant products significantly influence the health of fish (Dada and Ikuerowo 2009). Plant phenolics, polysaccharides, proteoglycans, and flavonoids play a major role in preventing or controlling infectious microbes (Citarasu 2010).

Natural products are an important source of new chemical compounds and, hopefully, therapeutic agents for many bacterial diseases. Thyme (*Thymus vulgaris* Linnaeus), belonging to the Lamiaceae family, is a pleasant-smelling perennial shrub, which grows in several regions in the world (Davis 1982). *T. vulgaris* is a well-known aromatic plant and its essential oil and aromatic water are used in the mountainous areas of the Mediterranean Region in Turkey (Baytop 1984; Akgul 1993). Fennel (*Foeniculum vulgare* Miller) is an aromatic herb used in traditional medicine and as spice in Central Europe and the Mediterranean. In previous studies, natural plant extracts including essential oils (thymol, flavonoids and phenolic compounds) have been found to have antibacterial activity (Essawi and Srour 2000). The antifungal and antibacterial activity exhibited by the Thymus genus essential oil has been demonstrated by several researchers (Karaman et al. 2001; Rasooli and Mirmostafa 2003). Fennel is known as an excellent source of natural antioxidants and contributes to daily antioxidant diet (Shahat et al. 2011). In addition, the usage of herbal mixtures significantly improved the immune indicators (albumin-globin

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ratio etc.) and performance of haematological and biochemical properties in fish and shrimp (Sivaram et al. 2004; Citarasu et al. 2006).

Electrolytes are also required for normal life processes in fish. Some of them, e.g., magnesium (Mg), Zinc (Zn) and iron (Fe), are obtained from the diet or from water. Generally, electrolytes carry out several homeostatic functions, such as bone formation, polarization of membranes, integration of enzymatic systems, energy storage, acid-base balance, clotting and respiration (Coppo 2001). Moreover, mineral deficiencies may cause biochemical, structural and functional pathologies, which depend on several factors, especially on the duration and degree of mineral deprivation. Sugiura et al. (1998) suggested that the availability of minerals increased when diets became acidified, and decreased when minerals were added to diets.

The aim of this study was to evaluate the effects of mixed use of oils (*T. vulgaris* and *F. vulgare*) in diets on selected biochemical properties and electrolytes in plasma of rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) infected with *Yersinia ruckeri.*

Materials and Methods

Preparation of herbal oils

The herbal oils were obtained from a commercial company (Elazığ, Turkey) and their compositions were determined by gas chromatography-mass spectrometry (GC-MS) in laboratories of the TUBITAK Marmara Research Centre (Istanbul, Turkey). Gas chromatograph (GC) analyses were carried out on a Shimadzu GC-9A gas chromatograph equipped with Thermon-600 T (30 m × 0.25 mm × 0.25 μm film thickness) (Bagamboula et al. 2004).

Screening herbal oils

Screening of herbal oils for their antibacterial activity against *Y. ruckeri* was conducted using the disc diffusion method as described by Bauer et al. (1996). All the tests were replicated three times. Inhibition zone of per herbal oil was measured and recorded. Minimal inhibitory concentration (MIC) was determined as the lowest concentration and the highest dilution, which completely inhibited the growth of *Y. ruckeri* by an agar plate dilution method according to CLSI (2003). Minimal inhibitory concentration values of herbal oils were solved in 1 ml of the Mueller-Hinton broth medium. Similar to what was mentioned before, 3×10^7 cfu·ml^{-1} bacteria were added to each test tube and after 24-hour of incubation at 35 ºC, the MIC values for each of herbal oil were determined in μg·ml^{-1}.

The experimental diets were prepared by pulverization method. Diets were dried in open air, packed and stored to be used later in experiment.

Bacterial challenge

Yersinia ruckeri (ATCC 29473) was provided by the Pendik Veterinary Research Institute (Istanbul, Turkey) and stored at –80 °C. To determine the optimum bacterial cell concentration for the experiment, graded doses ranging from 10^{10} to 10^4 of cfu·ml^{-1} were utilized. The median lethal dose (LD_{50}) calculated by the method of Reed-Muench (1938) was 3×10^7 cfu·ml^{-1} and this concentration was used for the experiment.

Feeding and sampling

In total, 120 healthy fish (weight 84 ± 1.02 g) were divided into four experimental groups: Control - 30 fish without oil supplementation and infection, INF - fish infected with *Y. ruckeri* but without oil supplementation, OIL1 - fish with oil supplementation and infected with *Y. ruckeri*, OIL2 - fish with oil supplementation but without infection. Fish were stocked in tanks (450 l) with water re-circulated and aerated at 12 h light/12 h dark period for 1 week at 17 ± 1 °C. Each fish group was fed twice a day at 9:00 h and 17:00 h at the amount of 3% live body weight. Fish in CNT and INF groups were fed a commercial fish feed while fish in groups of OIL1 and OIL2 were orally fed feeds with oil mixture at a dose of 10 ml·100 g^{-1} for 1 week.

After acclimatization, each fish in group of INF and OIL1 were infected intraperitoneally (APHA 1998) with 0.1 μl of live *Y. ruckeri* (3×10^7 cfu·ml^{-1}). Mortality of challenged fish was noted up to 7 days but all fish of all groups were sampled for blood indices as per the method described earlier at the end of the feeding trial (Day 3). Previous studies have shown that the 3rd day showed peak levels of bacterial growth, therefore, Day 3 was chosen as sample time (Raida and Buchmann 2009).

Laboratory analyses

Blood samples were obtained from the caudal vein (vena caudalis) using syringes. Plasma was collected after centrifugation (3600 *g* for 5 min) and stored in the freezer at −70 °C.

Blood was used for the testing of biochemical indices. Plasma glucose concentrations were measured colorimetrically according to Trinder (1969), total protein was measured according to the method of Lowry et al. (1952) and albumin was measured following the method of Wotton and Freeman (1982). Albumin-globulin ratio was calculated by diving albumin values by globulin value.

The blood was digested with concentrated nitric acid (Aristar grade, BDH Ltd., UK) at 100 °C, and analyzed for concentrations of total sodium (Na), potassium (K), chlorine (Cl), calcium (Ca) and magnesium (Mg) by flame atomic absorption spectroscopy (AAS) using an instrumentation laboratory 157 atomic absorption spectrophotometer.

Statistical analyses

All data obtained from experimental groups were analyzed in Statistical Package for the Social Sciences (SPSS) 15.0 package program with Duncan's Multiple Range Test at 0.05 significance value to determine statistical differences among groups. Standard errors were also estimated.

Results

The most important components of *T. vulgaris* oils were phenol (40.95%) and 2-methyl-5-(1-methylethyl) (12.12%) whereas in *F. vulgare* oils the most important components were benzene (67.99%) and 1-methoxy-4-(1-propenyl) (16.03%). The results of disc diffusion test showed that both oils had inhibitor activity against *Y. ruckeri.* It was 24.6 ± 0.21 mm and 22.1 ± 0.18 mm for *T. vulgaris* and *F. vulgare*, respectively. The MIC values of the tested *T. vulgaris* and *F. vulgare* oils were determined as 40 $\mu g \cdot ml^{-1}$ and 60 $\mu g \cdot ml^{-1}$, respectively.

The total protein content increased in all experimental groups compared to the control group (Table 1). The highest content of total protein was determined in group OIL2 (14.56 ± 1.75 $g \cdot dl^{-1}$). Generally, albumin ratio was not significantly different from the control group throughout the whole experiment ($P > 0.05$). The cholesterol concentration was significantly ($P < 0.05$) higher in group INF compared to other groups. While the triglyceride concentration showed the highest value in group INF, in group OIL1 we determined a slight decreasing trend, and group OIL2 was observed as the best treatment group in the present study for triglyceride. The glucose concentration is shown in Table 1. Compared to the CNT, the glucose value showed a gradual significant increase ($P < 0.05$). There were no significant differences in the bilirubin concentrations among treated groups and the control group ($P > 0.05$).

Table 1. Effects of herbal oil supplemented diets on biochemical properties of *Oncorhynchus mykiss* infected with *Yersinia ruckeri*

Experimental group	Glucose ($mg \cdot dl^{-1}$)	Total protein ($g \cdot dl^{-1}$)	Albumin ($mg \cdot dl^{-1}$)	Cholesterol ($mg \cdot dl^{-1}$)	Triglyceride ($mg \cdot dl^{-1}$)	Bilirubin ($mg \cdot dl^{-1}$)
Control	55.6 ± 6.44^{b}	6.25 ± 2.45^{a}	3.46 ± 0.40^{a}	261.6 ± 32.04^{a}	384.0 ± 43.17^{a}	0.624 ± 0.24^{a}
INF	38.4 ± 6.19^{a}	12.30 ± 3.49^{b}	3.46 ± 0.40^{a}	471.20 ± 19.23^{b}	619.20 ± 127.15^{b}	0.62 ± 0.24^{a}
OIL1	39.00 ± 3.32^{a}	12.17 ± 1.31^{b}	3.42 ± 0.23^{a}	324.00 ± 35.61^{a}	523.40 ± 153.05^{ab}	0.66 ± 0.14^{a}
OIL2	37.80 ± 11.56^{a}	14.56 ± 1.75^{b}	4.04 ± 0.30^{a}	439.20 ± 79.50^{b}	445.00 ± 148.26^{ab}	0.57 ± 0.19^{a}

Fish groups: CNT - control group, INF – fish infected with *Yersinia ruckeri,* OIL1 - fish infected with *Yersinia ruckeri* and supplemented with the mixed oils, OIL2 - fish supplemented with the mixed oils. Values are expressed as mean± standard error (SE). Different superscripts in the same row indicate significant ($P < 0.05$) differences between weeks by the Turkey's test.

Concentration of Na was higher in group OIL1 (168.00 ± 5.57 $mmol \cdot l^{-1}$, $P > 0.05$) compared to OIL2. The highest value of K was in the OIL1 (34.93 ± 2.2 mmol l^{-1}, $P < 0.05$) and the lowest value in Ca was recorded in the control. The best effect in K concentrations was shown in group OIL1. Mg concentration in fish of group OIL2 was higher than in OIL1 ($P < 0.05$) when compared to control. Plasma Cl concentration in fish of group OIL2 was higher than that in group OIL1. The lowest significant value of Cl was recorded in fish of group INF ($P < 0.05$), whereas the highest value was found in the control (Table 2).

Table 2. Effects of herbal oils supplemented in diets on element status of *Oncorhynchus mykiss* infected with *Yersinia ruckeri*

Experimental group	K ($mmol \cdot l^{-1}$)	Cl ($mmol \cdot l^{-1}$)	Ca ($mg \cdot dl^{-1}$)	Na ($mmol \cdot l^{-1}$)	Mg ($mg \cdot dl^{-1}$)
Control	15.34 ± 4.93^{a}	134.80 ± 6.81^{b}	3.9 ± 0.12^{a}	159.8 ± 5.72^{a}	4.79 ± 1.06^{a}
INF	31.13 ± 6.25^{b}	97.80 ± 20.58^{a}	7.15 ± 1.98^{b}	154.20 ± 5.85^{a}	11.74 ± 3.61^{b}
OIL1	34.93 ± 2.21^{b}	112.00 ± 3.08^{a}	8.38 ± 1.19^{c}	168.00 ± 5.57^{a}	10.73 ± 1.62^{b}
OIL2	32.15 ± 6.41^{b}	105.20 ± 16.53^{a}	7.49 ± 0.85^{ab}	157.20 ± 13.05^{a}	13.16 ± 1.74^{b}

Fish groups: CNT - control group, INF – fish infected with *Yersinia ruckeri,* OIL1 - fish infected with *Yersinia ruckeri* and supplemented with the mixed oils, OIL2 - fish supplemented with the mixed oils. Values are expressed as mean ± standard error (SE). Different superscripts in the same row indicate significant ($P < 0.05$) differences between weeks by Tukey's test.

Discussion

Aquaculture fish production increased significantly over the past few decades. However, stressful environment also leads to consequent suppression of the immune system, increasing the susceptibility of fish to infectious diseases; infections routinely occur in aquaculture and lead to substantial economic losses. Massive use of antimicrobials and vaccination for disease control has suppressed the growth in aquatic animals due to spread of antimicrobial-resistant bacteria and the presence of antimicrobial residues in aquaculture products and the environment. One of the most promising methods of controlling diseases in aquaculture is by strengthening the defense mechanism of fish through prophylactic administration of natural plant products (Agarwal and Singh 1999; Devasagayam and Sainis 2002) which is considered as a promising alternative to chemotherapy and vaccines (Secombes 1994).

The present study demonstrated that diet supplementation with *T. vulgaris* and *F. vulgare* oils showed increases in all biochemical variables. Our results are in agreement with the report of Sahu et al. (2007) who reported that serum protein, albumin and bilirubin concentrations in *Labeo rohita* fingerlings fed *Magnifera indica* kernel were higher than in control. Similar results were reported by Rao et al. (2006). Different values in biochemical variables are likely to be a result of the enhancement of the non-specific immune response of fish. The plant oils used in this study could decrease glucose values in treatment groups compared to control. Our results for this variable showed similarity with the results of Sahu et al. (2007) and Citarasu et al. (2006). They found reduced glucose concentrations in the aquatic animals fed with herbal immunostimulant diets. Furthermore, Ji et al. (2007) reported low blood glucose concentrations and plasma glutamic oxaloacetic transaminase in juvenile Japanese flounder fed with herbal mixtures. The decrease of glucose concentrations might be due to the capability of plant oils to reduce the effects of stressors.

In our study, we also determined electrolyte content (Na, K, Mg, Cl, Ca) in fish serum. Among all experimental groups only plasma Na values were not significant. All other plasma electrolytes revealed significant differences, especially K and Mg. Chlorine contents in plasma were observed to decrease gradually. Our results are in agreement with the findings of Sivaram et al. (2004) in juvenile greasy groupers (*Epinphelus tauvina*) with *Ocimum sanctum* and *Withania somnifera*, and *E. tauvina* juveniles with herbal plant mixture diets.

This study showed that mixed using of *T. vulgaris* and *F. vulgare* oils in diet had the potential effect on enhancing biochemical properties and trace elements in *O. mykiss* infected with *Y. ruckeri.*

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