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Effects of commercial herbal oil mixture on some hematological, biochemical and immunological parameters of rainbow trout (*Oncorhynchus mykiss*) and its preventive efficacy against *Yersinia ruckeri* infection

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Abstract

This study was conducted to evaluate the effects of different levels of a commercial herbal oil mixture (*Mix-oil*®) on the hematological, biochemical and immunological responses of rainbow trout as well as the resistance against infection with *Yersinia ruckeri*. The *Mix-oil*® includes, *Thymus vulgaris*, *Origanum vulgare* and *Eucalyptus* sp. essential oils. Experimental diets were supplemented with *Mix-oil*® at 0 (control group), 50, 200 and 400 ppm. Fish with an initial weight of 31.0± 0.1 g were divided into four groups and reared for 56 days. The anti-bactericidal activity of *Mix-oil*® revealed that the minimum inhibitory concentration (MIC) value for *Streptococcus iniae*, *Aeromonas hydrophila* and *Lactococcus garviae* was 6.25 µl ml^{-1} while it was 12.5 µl ml^{-1} for *Y. ruckeri*. Significant improvements in red blood cell count, hematocrit, immunoglobulin, lysozyme, total protein, complement component C3 and C4, and alternative complement pathway (ACH_{50}) in fish fed with 400 ppm *Mix-oil*® were observed compared with the control group ($p<0.05$). No significant difference was observed between different treatments in terms of MCV, MCH, MCHC levels and hemoglobin percentage aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels ($p>0.05$). The results suggested that dietary administration of *Mix-oil*® at the level of 400 ppm could enhance hematological parameters and immune function of rainbow trout as well as the resistance against *Y. rukeri*. Thus, using *Mix-oil*® as an immunostimulant is recommended for farmed rainbow trout.

Keywords: Herbal oil mixture, (*Mix-oil*®), Non-specific immune responses, Rainbow trout, *Yersinia ruckeri.*

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Introduction

Rainbow trout (*Oncorhynchus mykiss*) is one of the most important cultured fresh water species. Due to the intensification strategies applied to increase the production of this fish, infectious bacterial diseases occur frequently which increase the demand for antibiotics (Miranda and Zemelman, 2002; Miranda and Rojas, 2007). Recently, the application of antibiotics has been banned due to the increased resistance of most of the infectious bacteria as well as serious environmental hazards (Bruun *et al.,* 2000; Schmidt *et al.,* 2000; Dawood *et al.,* 2016a). For this reason, the use of natural feed additives working as immunostimulants when supplemented in aquafeeds is highly recommended.

Mix-oil® is a commercial herbal mixture which includes herbal medicines such as *Thymus vulgaris, Origanum vulgare*, and *Eucalyptus* commonly identified as safe substances and are among the main candidates to combat bacterial diseases. *T. vulgaris*, is a rich source of thymol and is considered as an effective supplement against high antioxidant and antimicrobial activities (Marino *et al.,* 1999; Dorman and Deans, 2000; Rota *et al.,* 2008). Several studies reported that *O. vulgare* possesses radical-scavenging, antimicrobial, antioxidant and cytotoxic activities because of its high phenolic content (Carvacrol and Thymol), and monoterpene hydrocarbons of γ-terpinene, and p-cymene (Şahin *et al.,* 2004; Chun *et al.,* 2005; Faleiro *et al.,* 2005). Also, *Eucalyptus* sp. leaves are used as a natural antibiotic for the treatment of numerous infectious diseases (Ghalem and Mohamed, 2008; Elaissi *et al.,* 2011; Mulyaningsih *et al.,* 2011; Bachir and Benali, 2012). The main antibacterial components of *Eucalyptus* species are 1, 8-cineole followed by *α*-pinene, *p*-cymene, borneol, cryptone, spathulenol, viridiflorol and limonene (Elaissi *et al.,* 2011). Numerous studies have evaluated *Eucalyptus* herbal medicines as potent alternatives to antibiotics as therapeutic and prophylactic agents in aquaculture systems (De Rosa *et al.,* 1994).

The antimicrobial properties of *T. vulgaris, O. vulgare* and *Eucalyptus* sp. have been evaluated in numerous studies. For example, the effects of *O. vulgare* extract on serum antioxidant activities, as well as growth and resistance to *Vibrio alginolyticus* were evaluated in Nile tilapia (*Oreochromis niloticus*) (Abdel-Latif and Khalil, 2013). In addition, Navarrete *et al.* (2010) showed the inhibitory activity of *T. vulgaris* essential oil on the allochthonous microbial composition of rainbow trout. Also, another study revealed that *Eucalyptus* sp. had an effective role for the treatment of *Saprolegnia parasitica*–infected rainbow trout eggs in an aquaculture environment (Khosravi *et al.,* 2012). Moreover, the essential oils of *Eucalyptus globulus* exhibited antibacterial activity against *Streptococcus iniae* (Roomiani *et al.,* 2013).

Therefore, the main objectives of this study were to study the effects of dietary supplementation of herbal oil

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mixture (*Mix-oil*®) on immune responses and disease resistance of the rainbow trout following experimental challenge with *Yersinia ruckeri*.

Materials and methods

Bactericidal assay

In vitro bactericidal activity of *Mix-oil*® (including: *T. vulgaris*, *O. vulgare* and *Eucalyptus* sp. essential oils) were examined *versus* important fish pathogens including *S. iniae* (AF048773), *Y. ruckeri* (KC291153), *Aeromonas hydrophila* (RTCC1032) and *Lactococcus garviae* (X54262). These bacterial strains, which were prepared from lyophilized stocks, were obtained from the Faculty of Veterinary Medicine, Tehran University, Iran. The disc diffusion method as described by Wei *et al*. (2010) was used to determine the growth inhibition effect of mucus samples of fish. At first, selective bacteria were grown in Tryptic Soy Agar (TSA) for 24 h at 37 ºC, and then 0.1 ml of each broth culture medium (containing 1.5×10^6 CFU ml^{-1} bacteria) was cultured on Mueller Hinton agar. Afterward, 100 µl of *Mix-oil*® was added to sterile paper discs (5 mm in diameter), placed on the medium and newly incubated (25 ºC, 48 h). The antibacterial activities were determined by measuring the diameter of the zone of inhibition in millimeters (mm).

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values

Minimum Inhibitory Concentration (MIC) test for bacteria was carried out by the method described by Hammer *et al*. (1999). At first, different concentrations of *Mix-oil*® including 1.6, 3.12, 6.25, 12.5, 25 and 50 µl were placed in tubes containing 1.5×10^6 CFU ml^{-1} of the above-mentioned bacterial strains. Then, all the samples were incubated at 25 ˚C for 48 hours. The MIC value is defined as the lowest concentration of the *Mix-oil*® at which the bacteria does not demonstrate any visible growth. Also, the lowest concentration in which there was no bacteria is defined as the MBC value.

Animal and experimental conditions

Rainbow trout juveniles with an average initial weight of 31.0±0.1 g (mean±SD) were obtained from a commercial fish farm in Mazandaran Province, Iran and transferred to the Chalus farm (Mazandaran Province, Iran). The fish were acclimatized for two weeks under laboratory rearing conditions and were provided with a basal diet (Skretting, Italy, G2 size) 3 times a day at 3 % of body weight (Table 1). After the acclimatization period, 840 healthy fish were randomly distributed to 12 tanks of 3000 liters at a rate of 70 fish per tank. The tanks were divided into four groups of three tanks each (with three replicates). The physicochemical properties of water during acclimation and experiment periods were maintained as follows: dissolved oxygen at 8.26±0.1 mg L^{-1}, pH at 7.34±0.7, and temperature at 16.0±1.2 ° C (mean ± SE). The fish were subjected to a 16L: 8D photoperiod regime.

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Table 1: Proximate composition of the basal diet.

Proximate composition (% dry weight)	%
Crude protein	41
Crude lipid	12
Ash	8.2
Fiber	2.3
Moisture	4.76
NFE [a]	15.3

[a]Nitrogen-free extracts (NFE)=dry matter-(crude protein+crude lipid+ash+fiber).
[b]Gross energy (MJ kg $^{-1}$) calculated according to 23.6 kJ g $^{-1}$ for protein, 39.5 kJ g-^{1} for lipid and 17.0 kJ g $^{-1}$for NFE.

Diet preparation

Mix-oil® (including: *T. vulgaris* (%0.5), *O. vulgare* (2%) and *Eucalyptus* sp. (2.5%) essential oils as liquid form, glycol used as carrier) were prepared from Animal Wellness Products Company (Italy). Components of the basal diet (Table 1) were mixed with the obtained *Mix-oil*® in the appropriate concentrations to get four different experimental diets: with 0 (control group), 50, 200 and 400 ppm of *Mix-oil*®. The examined diets were prepared according to the protocol described by Sönmez *et al*. (2015). The diets were allowed to dry and were stored at 4 °C until use. During this study, fish were fed (3% of body weight) three times a day for 8 weeks.

Gas chromatography mass spectrometry (GC/MS) analysis of Mix-oil®

Analyses were performed using a Varian gas chromatograph (Varian Inc., Walnut Creek, California, model HP-6890) equipped with FID and MSD detectors, (Shimadzu, Japan, model 3600) with a DB5 fused silica column (methyl phenyl siloxane, 30 mm length, 0.25 mm id); the carrier gas was helium; split ratio was 1:15 and a flame ionization detector was used. The initial temperature of the column was 60 °C (for 2 min) rising to 240 °C at 5 °C min^{-1}, with the injector temperature at 250ºC and detector temperature at 260 ºC. GC-MS analysis was performed on a cross-linked 5% methyl phenyl siloxanef used silica capillary column (HP-5, 30m length, 0.25 mm id, 0.25μm film thickness). The carrier gas was helium with a constant flow rate of 1 ml min^{-1} constant pressure 35 Psi^{-1}, the split ratio was 1:15 and temperature program was from 60 ºC (3 min) to 220 ºC at 5 ºC min^{-1}, with injector temperature of 260 ºC and detector temperature of 270 ºC. A quadrupole mass spectrometer was operated in EI mode at 70 eV ionization energy. Chromatograms were recorded in TIC mode the m/z range was 50 to 500. The retention indices for all the components were calculated by using the retention times of n-alkenes (C8-C25 obtained from Sigma), which were injected after the essential oil under the same conditions (Saharkhiz *et al.*, 2012).

Sampling, hematology and biochemical assays

After 56 days, the fish were fasted for 24 h prior to sampling. Sampling strategy ensured that the fish were subjected to negligible handling stress as much as possible and they were humanely anesthetized with clove oil (100 mg L^{-1}, Sigma Aldrich, Germany) before sample collection (Saeidi Asl *et al.,* 2017). Blood samples (about 1 ml) were drawn from the caudal vein and

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immediately transferred to non-heparinized tubes for serum collection (30 fish per group). Serum was collected after centrifugation at 3000 g for 20 min, divided into several aliquots and stored at -20 °C for subsequent study (Saeidi Asl *et al.,* 2017). Blood samples of seven fish from each individual tank (21 fish per group) were immediately divided into two half parts. One half was transferred to a tube containing anti-coagulant (heparin) for hematological analyses, while the other half was transferred to non-heparinized tubes for biochemical and immunological studies. Sera samples were obtained from coagulated blood samples after centrifugation for 20 min at 3000×g at 4 °C and stored at -20 ˚C until use.

The total red blood cells (RBC: 10^6 mm^{-3}) and white blood cells (WBC: 10^3 mm^{-3}) were enumerated in an improved Neubauer hemocytometer using Hayem and Turck diluting fluids (Blaxhall and Daisley, 1973). Haematocrit (Ht %) was determined by the standard microhematocrit method and expressed as percentage. The haemoglobin (Hb, g dl^{-1}) level was determined according to cyanomethemoglobin procedure. Furthermore, differential leukocyte cells were measured by preparing Giemsa stained smears. Blood smears were studied using light microscopy in order to make blood cell counts.

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were calculated on fish sera using commercial kits (Pars Azmoon Company, Tehran, Iran) and a biochemical auto analyzer (Eurolyser, Belgium) (Saeidi Asl *et al.,* 2017).

Immune parameters

Serum total protein (TP) levels were measured using commercial kits (Pars Azmoon Co, Tehran, Iran). Serum IgM level was quantified by the microprotein determination method (C-690; Sigma). Precipitation was carried out by 12% polyethylene glycol solution and the difference in protein content before and after precipitation was considered as the IgM level (Siwicki and Anderson, 2000). Serum lysozyme activity was measured according to the method described by Ellis (1990) with some modifications. Briefly, 25 µl sera was added to 1 ml of a suspension of *Micrococcus lysodeikticus* (0.2 mg ml^{-1} in a 0.05 M sodium phosphate buffer (pH 6.2) and the absorbance was measured at 670 nm after 30 s and 180 s using a spectrophotometer (Biophotometer, Eppendorf). Complement components (C3 and C4) were estimated using commercial kits (Pars Azmoon Company, Tehran, Iran) by immuno turbidometry assay (Adel *et al.,* 2016). The alternative complement activity (ACH_{50}) was determined by methods described by Yano (1992).

Challenge test

Yersiniosis is one of the important bacterial diseases in coldwater fish farms in Iran and several epidemic outbreaks of *Y. ruckeri* with high economic losses were reported (Zorriehzahra *et al.,* 2017). For the challenge test, a lyophilized stock of *Y.*

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ruckeri (KC291153) was obtained from the Faculty of Veterinary Medicine, Tehran University, Iran and cultivated in tryptic soy broth (TSB, Merck, Darmstadt, Germany) at 25 ˚C for 48 h. At the end of the 8 week-feeding trial, fish from all treatment tanks (10 fish from each tank in triplicate) were challenged with an intraperitoneal injection of 0.1 mL of *Y. ruckeri* in 0.9% (w/v) saline containing 1×10^6 cells ml^{-1}. In the control group, instead, 0.1 ml of PBS was injected. All fish were fed with non-supplemented diets and kept under observation for 14 days to record any abnormal behavior, clinical signs and daily mortality. Finally, relative percentage survival (RPS) values were calculated using the following formula described by Tukmechi and Bandboni (2014): RPS= 100–[(treatment mortality/control mortality) ×100].

Statistical analysis

All the tests were performed in triplicate. The data were subjected to statistical analysis using the SPSS software version no. 20 (SPSS Inc., Chicago, IL, USA). After satisfying the assumptions of normality and equal variance, the data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. A $p<0.05$ was considered statistically significant.

Results

GC-Mas analysis of Mix-oil®

A total of 36 different components were identified in the *Mix-oil®*. For *Mix-oil®* Citraconic anhydride was the predominant compound followed by *1, 8-cineole* and Thymol.

MIC and MBC values

Results of this study revealed that MIC activity reached a value of 6.25 µl ml^{-1} for *S. iniae*, *A. hydrophila*, and *L. garvieae* while the value for *Y. ruckeri* was 12.5 µl ml^{-1}. Also, MBC quantities for *A. hydrophila*, and *S. iniae* were 6.25 µl $ml^{-1.}$ This value was 12.5 µl ml^{-1} for *L. garvieae* and 25 µl ml^{-1} for *Y. ruckeri*, respectively (Table 2).

Table 2: MIC and MBC values with different concentration of *Mix-Oil* against studied bacteria.

	Concentration (µl ml^{-1})					
Selected bacteria	**1.6**	**3.12**	**6.25**	**12.5**	**25**	**50**
Streptococcus iniae			MIC, MBC			
Yersinia ruckeri				MIC	MBC	
Aeromonas hydrophila			MIC, MBC			
Lactococcus garviae			MIC	MBC		

Hematological profile

The number of red blood cells significantly increased ($p<0.05$) in fish fed with the diets enriched with 200 and 400 ppm of *Mix-oil®* (Table 3). Similar results were observed regarding white blood cells. Hematocrit was also increased in fish fed with 400 ppm *Mix-oil®* enriched diets, although the increments observed in other groups

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were not statistically significant (Table 3). No significant differences were observed in MCV, MCH, MCHC levels and Hb percentage compared with the control group.

The percentage of blood neutrophils, monocytes and eosinophils among different groups were not statistically significant (Fig. 1). The number of blood lymphocytes was similar in all treatments (Fig. 1). There was no increase in the 400 ppm group (p>0.05).

Table 3: Hematological changes of rainbow trout fed diets enriched with different levels of *Mix-Oil* for 8 weeks.

Parameter	Control	50 ppm	200 ppm	400 ppm
RBC (10^6 mm-^{3})	1.19±0.11^b	1.24±0.05^{ab}	1.27±0.03^{ab}	1.33±0.04^a
WBC (10^3 mm-^{3})	22.11±0.78^b	21.78±0.65^b	23.30±0.51^a	23.58±0.47^a
Hct (%)	26.62±0.65^b	26.63±1.13^{ab}	28.07±1.44^{ab}	29.35±1.94^a
Hb (g dl^{-1})	8.55±0.7^a	8.6±0.38^a	8.9±0.34^a	8.93±1.1^a
MCV	224.6±14.4^a	216.2±13.9^a	224.5±4.6^a	221.3±8.9^a
MCH	71.09±0.17^a	67.42±4.58^a	70.8±0.27^a	69. 2±2.8^a
MCHC	31.9±2.0^a	31.1±0.3^a	31.5±0.5^a	31.2±0.1^a

Note: RBC, red blood cells; WBC, white blood cells; Hct, hematocrit; Hb, hemoglobin concentration. Data are presented as mean±S.D (n =21 fish from each group). Means in the same rows with different superscripts are significantly different (p<0.05).

Immune responses

Table 4 shows the profile of the various immunological parameters evaluated in the serum of the fish fed with various concentrations of *Mix-oil*® and in the control group. After feeding on the *Mix-oil*® for 56 days, the various immune response indices (IgM, lysozyme, complement component C3 and C4, and ACH_{50}) significantly increased (p<0.05) in the 400 ppm *Mix-oil*® fed group compared to other groups especially the control group. After 56 days, total serum protein, complement component C3 and C4, lysozyme activity, IgM levels and ACH_{50} were increased in the groups that received *Mix-oil*® (50, 200 and 400 ppm), compared to the control group (p<0.05). Also, groups receiving 400 ppm *Mix-oil*® had significantly higher levels of all measured immunological parameters (p< 0.05).

Table 4: Serum immune parameters of rainbow trout fed diets supplemented with different levels of *Mix-Oil* for 8 weeks.

Parameter	Control	50 ppm	200 ppm	400 ppm
Total protein (g dl^{-1})	3.71±0.07^b	3.73±0.05^b	3.75±0.05^b	4.11±0.1^a
Lysozyme activity (µg mg^{-1})	25.15±0.58^d	29.13±1.05^c	30.83±0.76^b	32.45±0.57^a
IgM (mg dl^{-1})	17.03±0.25^d	18.60±0.36^c	23.73±0.66^b	27.03±0.55^a
ACH_{50} (U ml^{-1})	58.93±2.34^b	64.75±3.23^b	79.10±4.15^a	79.98±6.30^a
C3 (mg dl^{-1})	13.30±0.78^c	15.50±1.67^{bc}	17.20±1.31^{ab}	19.35±2.02^a
C4 (mg dl^{-1})	18.63±1.77^c	21.50±0.77^b	22.15±1.50^b	25.45±1.55^a

*Data are presented as mean±S.D (n =21 fish from each group). Means in the same rows with different superscript are significantly different (p<0.05).

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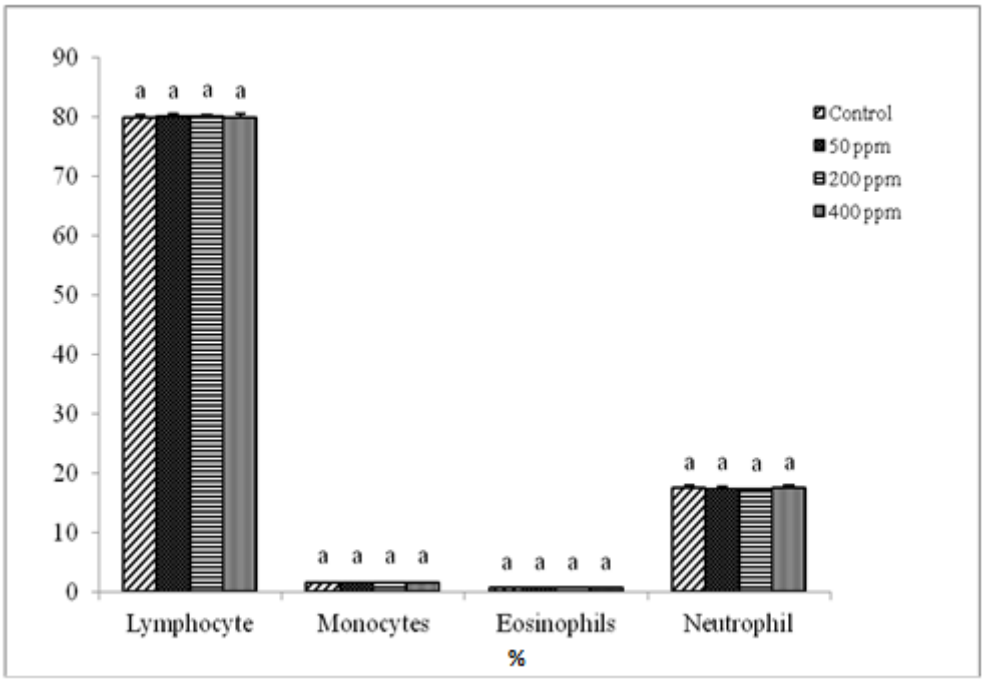


Figure 1: Changes in differential leukocyte counts of rainbow trout fed diets supplemented with *Mix-Oil* for 8 weeks. Similar letters indicate no significant difference between experimental groups ($p \geq 0.05$).

Biochemical analysis

Results of biochemical analysis are showed in table 5. Based on these results no significant differences were observed in the levels of the enzymatic activities of liver (AST and ALT) and in the levels of glucose and triglycerides between the different groups (Table 5).

Table 5: Biochemical parameters of rainbow trout fed diets supplemented with different levels of *Mix-Oil* for 8 weeks.

Parameter	Control	50 ppm	200 ppm	400 ppm
AST (U L^{-1})	323.0±18.4[a]	340.2±20.1[a]	345.5±21.5[a]	336.2±32.3[a]
ALT (U L^{-1})	15.0±0.82[a]	15.0±1.15[a]	16.0±0.82[a]	15.0±0.83[a]

*Data are presented as mean±S.D (n =21 fish from each group). Means in the same rows with different superscripts are significantly different (p<0.05).

Disease challenge

Results of the current study showed that administration of *Mix-oil*® enhanced the resistance of rainbow trout to *Yersiniosis* (Fig. 2). The first clinical signs of disease were observed in challenged fish 72 hrs. and the first mortality 96 hrs. after intraperitoneal injection. The highest relative percentage survival (80%) was recorded (day 14) in 400 ppm *Mix-oil*® group and the lowest survival rate (40%) was observed in the control group. However, no mortality was recorded in the control group.

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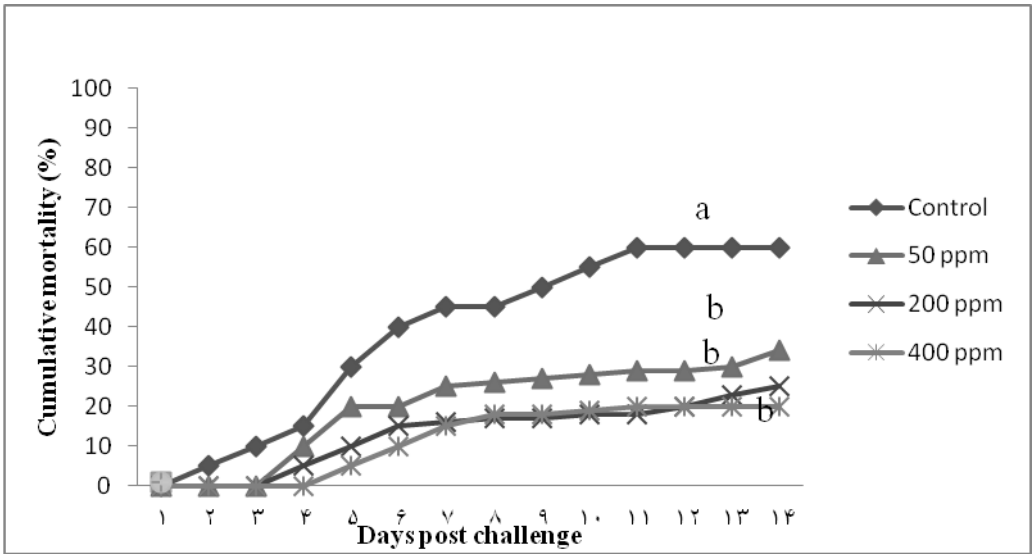


Figure 2: Cumulative mortality in rainbow trout fed with diets supplemented with *Mix-Oil* following experimental challenge with *Yersinia ruckeri.* Difference letters indicate significant difference between experimental groups ($p \leq 0.05$).

Discussion

Currently, the prevalence of microbial diseases acts as a major limiting factor for aquaculture. This situation has led to the use of extraneous chemicals (such as disinfectants and antibiotics) to control the mortality of fish and prevent economic losses (Dawood and Koshio, 2016b). The drug resistance of microorganisms, which is the result of over-the-counter medication, self-medication and drug administration by non-specialists, is one of the major problems in aquaculture (Lazado *et al.*, 2015). Therefore, the use of medicinal herbs or their related products can be used as a highly effective alternative to antibiotics, chemicals, vaccines and other artificial compounds due to the presence of various compounds such as phenols, polyphenols, alkaloids, quins, terpenes, lectins and polypeptides (Baser, 2008).

In this study, we investigated the antimicrobial properties of the *Mix-oil*® at different concentrations on pathogenic bacteria isolated from rainbow trout in *in vitro* conditions. Results of this study revealed that MIC activity reached a value of 6.25 µl ml^{-1} for *S. iniae*, *A. hydrophila* and *L. garvieae* bacteria while the value for *Y. ruckeri* was 12.5 µl ml^{-1}. For *Mix-oil*® Citraconic anhydride was the predominant compound followed by *1,8-cineole* and Thymol. The antibacterial effects of thymol was attributed to its ability to permeabilize and depolarize the cytoplasmic membrane (Xu *et al.*, 2008). The bacteria selected in this study are considered as the most important bacteria of the aquaculture industry in Iran. Because they can cause disease in humans and various complications, further attention to examine and adopt effective and practical policies to control this bacterium is needed.

In this study, oral administration of the *Mix-oil*® (50, 200 and 400 ppm) stimulated better and more effective immune responses (IgM, lysozyme,

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complement component C3 and C4, and ACH_{50}) in experimental treatments groups than in the control group. Based on the results, the concentration of 400 ppm of the mix had the highest effect. Carvacrol a key ingredient in the *Mix-oil*® (45.4%) is a monotonic phenol and is responsible for many biological activities such as antimicrobial activity, anti-inflammatory, anti-genotoxic, analgesic, anti-inflammatory, anti-thrombolytic (Baser, 2008). Similarly, Yilmaz *et al.* (2015) examined the effects of carvacrol on growth performance, hematological parameters, non-specific immunity, and serum biochemical parameters in rainbow trout. According to this study, serum lysozyme, serum total protein, globin level and glyceride levels increased in carvacrol-fed treatments. In the study of Ahmadifar *et al.* (2011), the addition of thymol and carvacrol to diets affected growth performance and bacterial flora of rainbow trout and increased the weight gain and lowered feed conversion in fish which can be attributed to the strong antibacterial properties of carvacrol and thymol in the severe containment of the anaerobic populations of the intestinal flora. The increase in the number of red blood cells in the fish fed 400 ppm of *Mix-oil*® was found to be due to immune stimulation agents that increase the metabolism of fish and ultimately increase the number and oxygen carrying capacity of the red blood cells (Irianto and Austin, 2002).

Lysozyme is one of the components of the body's non-specific defense system, which is responsible for destroying pathogens by breaking down the glycosylated bonds of the peptidoglycan layer of the bacteria. In addition, lysozyme is responsible for activating other important molecules of defense, including the complement system and phagocytic cells (Ellis, 1990). The level of lysozyme activity depends on environmental parameters (water temperature, pH, light period, season, and toxins) and intrinsic factors (size, age, sex, infections and stress) (Tukmechi and Bandboni, 2014). After 56 days of feeding, lysozyme activity was increased in three groups which received *Mix-oil*® compared to the control group. Similar results were observed on rainbow trout after feeding on feeds containing *T. vulgaris* L oil (Azizi *et al.*, 2016) and *Origanum vulgare* extract (Haghighi *et al.*, 2018). The studies conducted by the researchers showed that the amount of lysozyme increased following the use of herbal extracts in the fish diet. This increase was also significant in some cases, depending on the species of fish, the concentration of the plant extract and the type of extract used (Irianto and Austin, 2002).

In principle, the complement pathway is stimulated and activated by immunostimulants (Engstad *et al.*, 1992). Among them, the performance of medicinal plants has been proven to activate and stimulate complement activity. The results of this study are in line with the observations of other researchers, Awad and Austin (2010) showed that the use of *Lupinus perennis* indica *Managifera Urtica* dioica, especially in concentrations of 2 and 1

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percent in rainbow trout rations, significantly increases the complement activity after 14 days. Haghighi *et al*. (2018) illustrated that dietary inclusion of *O. vulgare* extract at a rate of 1% improves nonspecific immune parameters (respiratory burst activity, phagocytic activity and serum lysozyme activity) of juvenile rainbow trout. Further, Azizi *et al*. (2016) showed the effect of the diet containing essential oil of thyme (*T. vulgaris*) on blood and biochemical parameters of rainbow trout serum. The essential oil of this plant significantly increased both serum lysozyme and white blood cell count. These properties are mainly attributed to carvacrol and thymol. Ocana and Reglero showed that *T. vulgaris*, *T. zygis*, and *T. hyemalis* essential oils significantly decrease gene expression of the proinflammatory mediator's TNF-α, IL-1B, and IL-6 and significantly increased anti-inflammatory cytokine IL-10 (Ocaña and Reglero, 2012).

Results of the current study showed that administration of *Mix-oil*® enhanced the resistance of rainbow trout to yersiniosis in *in vitro* and *in vivo* conditions. Kucukgul Gulec *et al.* (2013) showed that supplementation of herbal oils (*T. vulgaris* and *Foeniculum vulgare*) can increase the disease resistance in rainbow trout to *Y. ruckeri* infection by increasing levels of total protein, albumin and some electrolytes (K, Na, Ca, and Mg). Adel *et al*. (2016) showed that the survival rate of rainbow trout fed with feeds containing 2% and 3% *Mentha piperita* plant extract were higher than those of the control after challenging with *Y. ruckeri*. Kucukgul Gulec *et al.* (2013) demonstrated that the use of nutritional supplements containing thyme jujube essential oil increased the activity of antibacterial, total protein, albumin, cholesterol, triglyceride and bilirubin in experimental treatment groups compared to that in the control.

The results of the present study demonstrated that *Mix-oil*® supplementation had no significant effect on ALT and AST. Generally, the increases in activities of alanine transaminase (ALT) and aspartate transaminase (AST) are commonly regarded as indicators of hepatic damage (Sheikhzadeh *et al.,* 2012). Therefore, these results suggest that *Mix-oil*® is not toxic to hepatic health.

Based on the results of this study, it could be concluded that oral administration of the *Mix-oil*® up to 400 ppm through feeds in rainbow trout is effective and beneficial. However, further studies on specific mechanisms for immune modulation and disease resistance should be conducted for exploring the feasibility of the commercial application of *Mix-oil*® in rainbow trout culture.

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