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MICROBIOLOGY

Effect of Dietary *Origanum onites* on Growth, Non Specific Immunity and Resistance against Yersinia ruckeri of Rainbow Trout (*Oncorhynchus mykiss*)

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Abstract: Natural substances has been identified to maintain health and improve growth performance in the aquaculture. The effect of *Origanum onites* on growth and immune response of rainbow trout was investigated. Experimental groups (A and B) of 70 fish were separated into 10 different treatments. A groups were fed with dietary administration of *O. onites* essential oil (0.5 mL kg^{-1} and 3.0 mL kg^{-1}) and crude powder (1.0 g kg^{-1} and 10.0 g kg^{-1}) for a period of 8 weeks. Other groups (B) were vaccinated against *Yersinia ruckeri* at the beginning of experiment and then fed the same diets described above. Results showed that feed conversion ratio in fish fed a combination of *O. onites* and vaccine was statistically better than the control. NBT-positive cells, phagocytic activity, serum lysozyme activity and immunoglobulin M level were stimulated in both non vaccinated and vaccinated fish ($p<0.05$). Cumulative mortality in fish fed *O. onites* was lower than controls following challenge with *Y. ruckeri*. No mortality was observed in vaccinated fish fed with 0.5 mL kg^{-1} of *O. onites*. These results indicated that dietary administration of *O. onites* could act as an enhanced non specific immune response, growth performance and resistance to *Y. ruckeri*.

Key words: growth, non specific immunity, *Oncorhynchus mykiss*, *Origanum onites*, *Yersinia ruckeri*.

INTRODUCTION

In recent years, medicinal plants have increased attention as feed additives provided strong evidence for stimulating immune response with improved growth performance in aquaculture facilities. Thus the use of plants as immunostimulants was found to prevent fish diseases (Ardo et al. 2008, Immanuel et al. 2009, Nya & Austin 2011, Harikrishnan et al. 2012).

Origanum onites is an endemic plant with a wide distribution throughout the eastern Mediterranean (Kokkini et al. 2004). *O. onites* mainly consists of phenolic compounds such as carvacrol, thymol, y-terpinene and p-cymene. Several studies have reported the antimicrobial agent of *O. onites*, showing a broad-spectrum of activities against bacteria (Kotan et al. 2014, Gormez & Diler 2017), fungi (Korukluoglu et al. 2008, Gormez & Diler 2014), virus (Orhan et al. 2012). Ekici et al. (2011) demonstrated that oregano (*Origanum vulgaris*) affects bacterial fish pathogens (*Yersinia ruckeri, Aeromonas hydrophila, Vibrio anguillarum, Vibrio alginolyticus, Flavobacterium psychrophilum* and *Lactococcus garvieae*). Starliper et al. (2015) determined that *O. vulgare* have a strong antibacterial effect against *Aeromonas salmonicida*. Bharti et al. (2013) noted that essential oil of *O. vulgare* had growth inhibition against fish pathogens, *Pseudomonas*

fluorescens and *A. hydrophila*. Afizi et al. (2013) reported the methanol extract of *O. vulgare* produced better activity against *A. hydrophila*, *A. sobria* and *A. caviae*.

Enteric Redmouth (ERM) disease, or yersiniosis causes acute, subacute or chronic, systemic infection of salmonids, particularly occurring in cultured rainbow trout *Oncorhynchis mykiss*. Vaccination which is widely used for the control of outbreaks has been reported to be variable under field conditions, and often does not completely prevent disease outbreaks when the level of infection is high, mainly under high-stress conditions (Barnes 2006). Immunostimulants used in combination of fish vaccines have been found to increase protective capabilities of fish against diseases (Maqsood et al. 2011).

The aim of the present study was to investigate the effect of different doses of *O. onites* crude powder and essential oil only - or in combination with vaccine on growth performance, immune response and disease resistance against *Y. ruckeri* in rainbow trout.

MATERIALS AND METHODS

Fish and experimental design

A total of 2100 healthy rainbow trout, (body weight of 14.08±2.74 - 16.81±3.89 g) were obtained from a commercial fish farm and carried to the aquaculture department at the Fisheries Faculty in Egirdir, Isparta, Turkey. All experiments were performed in 1.000 liter round concrete ponds with a water flow system of 12 liter per minute. The water quality parameters of temperature, dissolved oxygen and pH were measured as 12±2°C, 7.4 mg L^{-1} and 7.3, respectively. Experimental fish were fed twice daily with basal test diet ad libitum for two weeks. Then fish were randomly distributed in ten different groups with three replicates for each group consisting of 70 fish. The first experimental groups (A) were fed ad libitum twice a day with dietary administration of *O. onites* crude powder (1.0, 10.0 g kg^{-1}) and essential oil (0.5, 3.0 mL kg^{-1}) for eight weeks. The control group of nonvaccinated fish (A) was fed a basal diet without herbal supplementation. The second experimental groups (B) were initially vaccinated (i.p. 0.1 mL $fish^{-1}$) against *Y. ruckeri* and then fed the same diets for a period of 8 weeks. The control group (B) were vaccinated against *Y. ruckeri* and then fed basal diets without herbal supplements (Table I). The use of animals was performed under ethical standards of the national guidelines approved by the Suleyman Demirel University Animal Care and Use Committee (Republic of Turkey Suleyman Demirel University Ethics Committee of Experimental Animal Usage, 21438139-145).

Plant materials

O. onites were collected in the Mediterranean regions of Turkey in July 2015 during the flowering stage. The plant samples were identified by Prof. Dr. Hasan Ozcelik (Department of Biology, Suleyman Demirel University, Isparta - Turkey).

Isolation of essential oils

O. onites was dried at room temperature and ground using a mixer. The crude powder samples (200 g) were hydrodistillated for 3 hours by means of the Clevenger-type apparatus and the obtained essential oils were stored at -20°C until use.

Experimental diets

The formulation of the prepared diet is given in Table II. Different concentrations of *O. onites* crude powder powder (1.0, 10.0 g kg^{-1}) and essential oil (0.5, 3.0 mL kg^{-1}) were added into the diet and mixed with the inclusion of sunflower oil (0.05 mL kg^{-1}). Experimental diet was prepared weekly and stored at the 4°C.

Table I. Experimental design.

Experiment I (Groups A)		Fish / Number	Experiment II (Groups B)		Fish / Number
Control	*O. onites*, 0 mL kg^{-1}	210 (70×3)	Control	*O. onites*, 0 mL kg^{-1} + Vaccine	210 (70×3)
A1 (essential oil)	*O. onites*, 0.5 mL kg^{-1}	210 (70×3)	B1 (essential oil)	*O. onites*, 0.5 mL kg^{-1} + Vaccine	210 (70×3)
A2 (essential oil)	*O. onites*, 3.0 mL kg^{-1}	210 (70×3)	B2 (essential oil)	*O. onites*, 3.0 mL kg^{-1} + Vaccine	210 (70×3)
A3 (crude powder)	*O. onites*, 1.0 g kg^{-1}	210 (70×3)	B3 (crude powder)	*O. onites*, 1.0 g kg^{-1} + Vaccine	210 (70×3)
A4 (crude powder)	*O. onites*, 10.0 g kg^{-1}	210 (70×3)	B4 (crude powder)	*O. onites*, 10.0 g kg^{-1} + Vaccine	210 (70×3)

Table II. Formulation of the experimental diets.

	Experiment groups and utilization rate (%)				
Ingredients	**Control**	**0.5**	**3.0**	**1.0**	**10.0**
Fish meal	35.00	35.00	35.00	35.00	35.00
Soybean meal	30.00	30.00	30.00	30.00	30.00
Wheat gluten	5.00	5.00	5.00	5.00	5.00
Wheat by-product	14.00	13.95	13.7	13.9	13.0
Fish oil	8.00	8.00	8.00	8.00	8.00
Vitamin premix [1]	2.00	2.00	2.00	2.00	2.00
Mineral premix [2]	1.00	1.00	1.00	1.00	1.00
C vitamin	0.50	0.50	0.50	0.50	0.50
Pellet binders[3]	3.00	3.00	3.00	3.00	3.00
Antioxidant[4]	0.50	0.50	0.50	0.50	0.50
Others[5]	1.00	1.00	1.00	1.00	1.00
O. onites L. essential oils	0	0.05	0.3	0	0
O. onites L. crude powder	0	0	0	0.1	1.0

[1]Vitamin premix.; per kg, 4,000,000 IU vitamin A, 480,000 IU vitamin D3, 40,000 mg vitamin E, 2400 mg vitamin K3, 4,000 mg vitamin B1, 6,000 mg vitamin B2, 40,000 mg niacin, 10,000 mg calcium D-pantothenate, 4,000 mg vitamin B6, 10 mg vitamin B12, 100 mg D-biotin, 1,200 mg folic acid, 40,000 mg vitamin C and 60,000 mg inositol. [2]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. [3]Pellet binders; lignosulfonate. [4]Antioxidant; ethoxyquin. [5]Others; choline chloride, methionine+cystine.

Growth performance parameters

At the end of the growth trial experimental fish (20 fish/group) from each tank were anaesthetized with phenoxyethanol (0.1-0.5 mL L^{-1}) and weighed individually. Growth performance was calculated as follows:

Weight gain (WG, g)= Final weight – Initial weight

Specific growth rate (SGR % per day) = 100 x (In final weight – In initial weight) / days of the experiment

Feed conversion ratio (FCR) = (Feed intake, g) / (Weight gain, g)

Condition factor (CF) = 100 x (Body weight, g) / (Body lenght^3, cm)

Survival rate (SR %) = [(Final number of fish) / (Initial number of fish)] x 100

Non specific immun system parameters

Blood samples

At the 20th and 56th days of the experimental, blood samples (12 fish/group) were obtained from the caudal vein by using 1 mL hypodermal heparinized syringe after anesthetizing with phenoxyethanol (0.1-0.5 mL L^{-1}). Blood was centrifuged and then serum samples were stored at -20° C for further serum lysozyme activity and immunoglobulin M level analysis.

Nitroblue tetrazolium assay

The respiratory burst activity was determined using to NBT stain (Sigma Aldrich) method by Anderson et al. (1992). Briefly, 50 µL of blood was dropped onto a coverslip and incubated for 30 min at 22^{0}C and then the coverslip was washed with saline solution to remove the red blood cells. 0.2% NBT solution was dropped and placed onto the coverslip. The NBT-cells were incubated for 30 min at 22° C. Dark blue under the microscope (×40 magnification) were counted for NBT-positive cells. Five random microscopic fields were counted on from each blood sample and the fields were averaged.

Phagocytic activity

Leukocytes were initially isolated from blood using to the density-gradient centrifugation method by Rowley (1990). Phagocytic activity was determined spectrophotometrically according to Seeley et al. (1990). To perform the assay, yeast cell suspension was stained the congo red and was mixed with the leukocyte solution providing a yeast cell: leukocyte ratio of 40:1. The mixtures were incubated at room temperature for 60 min. Then 1 mL ice-cold HBSS was added and 1 mL of histopaque (1.077) was dropped into the bottom of each tube. The samples were centrifuged at 850 rpm for 5 min so that leukocytes were separated from free yeast cells. Leukocytes were picked up and washed two times in HBSS. The cells then were re-suspended in 1 mL trypsin-EDTA solution and incubated for 12 h at 37° C. The absorbance of the samples was measured at 510 nm.

Serum lysozyme activity

Serum lysozyme activity was determined by using the lysoplate technique according to the method of Ellis (1996). Briefly, 0.5% agarose gel was prepared containing 0.12% *Micrococcus lysodeikticus* in a phosphate buffer pH 6.2. The agar was cast onto petri dishes and punctured wells in 5 mm diameter when solidified, then 25 µL of the serum samples and standards were filled into the well. The plates were incubated at 36^{0}C for 20 h, diameter of the inhibition zones around the well were measured. The results for standards were marked on semilogarithmic graph paper and sample values extrapolated from this standard curve.

Immunoglobulin M level

Immunoglobulin M (IgM) level in serum was determined using enzyme-linked immunosorbent assay (ELISA) using a fish Immunoglobulin M (IgM) ELISA Kit (Cusabio Biotech Co. Ltd., CSB-E12045Fh, MD, USA) following the manufacturer's instructions. The absorbance of each well was read at a wavelength of 630 nm with a Bio-Tek FLX 800 plate reader.

Challenge test with Y. ruckeri

After the 8 weeks, fish fed with *O. onites* (groups A and B) were challenged with *Y. ruckeri*. Control diet included no feed additive supplementation and positive control diet supplemented with oxytetracycline (at a ratio of 1/100 w/w for 10 days). *Y. ruckeri* was obtained from the culture collection of the Egirdir Fisheries Faculty at Isparta Applied Sciences University. Fish were infected with *Y. ruckeri* by i.p. injection with 0.1 mL volumes of bacterial suspension adjusted to 3.10^5 cfu mL^{-1} (LD_{50} dose). Mortalities were recorded daily and any moribund fish were examined bacteriologically according to Austin & Austin (2007). The relative percent survival (RPS) was calculated according to Amend (1981).

RPS = (1 - % mortality in experimental group / % mortality in control) x 100

Statistical analyses

Results of the experiment are presented as average (±standard deviation) and were compared at groups using one way analysis of variance (ANOVA) tests (SPSS 18.0, Inc.) followed by Duncan' s test. Differences between groups were considered statistically at a significance level of $p<0.05$.

RESULTS

Growth performance analysis

Growth performance parameters in non vaccinated (A1-A4) and vaccinated (B1-B4) fish fed the experimental diets for 8 weeks were presented in Table III and IV. Non vaccinated fish fed with *O. onites* diets showed increased final weight, weight gain, specific growth rate, condition factor and survival rate when compared to the control ($p<0.05$) (Table III). Groups fed with 1.0 and 10.0 g kg^{-1} of *O. onites* crude powder performed better growth performance compared to those fed diets incorporated with *O. onites* essential oil at levels of 0.5 and 3.0 mL kg^{-1} in Experiment I. In the experimental groups fed diets with a combination of *O. onites* and vaccine (B1-B4) final weight, weight gain, specific growth rate and survival rate increased over the control group. Feed conversion rate was better in fish fed diets containing 0.5 mL kg^{-1}, 3.0 mL kg^{-1} and 1.0 g kg^{-1} than those fed 10.0 g kg^{-1} with *O. onites* in Experiment II ($p<0.05$) (Table IV).

Table III. Growth parameters in group A1-A4 (non vaccinated fish) fed diets containing different form of *O. onites* and control groups (Experiment 1).

	Experiment groups				
	Control	**A1 (0.5 mL kg^{-1})**	**A2 (3.0 mL kg^{-1})**	**A3 (1.0 g kg^{-1})**	**A4 (10.0 g kg^{-1})**
Initial weight	14.10±2.01	14.35±3.01	14.16±2.55	14.08±2.74	14.26±3.14
Final weight	36.66±9.71^b	50.65±18.03^a	50.80±17.36^a	51.46±17.70^a	55.51±18.72^a
Weight Gain	22.56±5.62^b	36.30±1.51^a	36.63±1.42^a	37.38±8.72^a	41.25±3.88^a
Specific Growth Rate	5.52±0.43^b	6.41±0.07^a	6.42±0.06^a	6.43±0.41^a	6.63±0.16^a
Feed Conversion Ratio	0.81±0.07	0.71±0.08	0.69±0.03	0.69±0.09	0.67±0.11
Condition Factor	0.99±0.14^b	1.11±0.17^a	1.14±0.16^a	1.09±0.18^a	1.11±0.18^a
Survival Rate	93.80±0.82^c	96.66±0.82^b	98.57±0.00^a	98.57±1.42^a	97.61±0.82^{ab}

Data are presented as the means ±SEM (n-3) values within the same row having different superscipts are significantly different ($p<0.05$).

Table IV. Growth parameters in group B1-B4 (vaccinated fish) fed diets containing different form of *O. onites* and control groups (Experiment 2).

	Experiment groups				
	Control	**B1 (0.5 mL kg^{-1})**	**B2 (3.0 mL kg^{-1})**	**B3 (1.0 g kg^{-1})**	**B4 (10.0 g kg^{-1})**
Initial weight	16.53±3.82	16.81±3.89	16.46±3.89	16.38±4.06	16.50±2.75
Final weight	44.05±11.62^{c}	65.46±18.18^{a}	57.75±14.29^{b}	63.70±20.31^{a}	54.60±14.95^{b}
Weight Gain	27.51±3.48^{d}	48.65±2.21^{a}	41.28±1.51^{bc}	47.31±6.10^{ab}	38.10±1.03^{c}
Specific Growth Rate	5.91±0.22^{d}	6.93±0.08^{a}	6.64±0.06^{bc}	6.87±0.22^{ab}	6.49±0.04^{c}
Feed Conversion Ratio	1.20±0.13^{a}	0.70±0.07^{b}	0.77±0.11^{b}	0.71±0.04^{b}	1.07±0.24^{a}
Condition Factor	1.44±0.20^{a}	1.38±0.19^{a}	1.29±0.15^{b}	1.43±0.24^{a}	1.42±0.34^{a}
Survival Rate	89.33±3.05^{b}	96.66±3.05^{a}	96.00±2.00^{a}	96.66±3.05^{a}	94.66±1.15^{a}

Data are presented as the means ±SEM (n-3) values within the same row having different superscipts are significantly different ($p<0.05$).

Immunological tests

The effect of non vaccinated and vaccinated groups on phagocytic activity is shown in Figure 1a, b. Phagocytic activity was significantly increased after 20 day in fish fed with crude powder and essential oil of *O. onites* (A1-A4) as compared to the control. The enhanced phagocytic activity was noted after 56 days in fish fed with crude powder and essential oil of *O. onites* (A1-A4) ($p<0.05$) (Figure 1a). When using the combination of *O. onites* and vaccine groups (B1-B4), elevation of phagocytic activity was noted compared to control at day 20, whereas phagocytic activity in the fish fed with 3.0 mL kg^{-1} essential oil (B2) of *O. onites* was significantly higher than the control group at day 56 ($p<0.05$) (Figure 1b).

Elevated immunoglobulin M level was noted after 20 days in groups fed with 0.5 mL kg^{-1} essential oil (A1) and 1.0 g kg^{-1} crude powder (A3) of *O. onites* when compared to the control diet whereas immunoglobulin M level was increased only in fish fed with 1.0 g kg^{-1} crude powder of *O. onites* compared to the control diet at day 56 ($p<0.05$) (Figure 2a). Vaccinated groups of fish fed with 3.0 mL kg^{-1} essential oil (B2) and 10.0 g kg^{-1} crude powder (B4) of *O. onites* showed significantly higher levels of immunoglobulin M compared to the control at day 20 ($p<0.05$). There were no significant differences between groups in immunoglobulin M levels at 56. days (Figure 2b).

Serum lysozyme activity of fish fed with *O. onites* (A1-A4) did not show significant differences between groups at day 20. A significant increase of serum lysozyme activity was observed in the group fed diets containing 0.5 mL kg^{-1} essential oil (A1) of *O. onites* comparing to control group after 56 days ($p<0.05$) (Figure 3a). When using the combination of *O. onites* and vaccine in fish (B1-B4), elevation of serum lysozyme activity was not significant among experimental groups at day 20 (Figure 3b). However serum lysozyme activity in vaccinated fish significantly increased in groups fed with 0.5 mL kg^{-1} essential oil (B1) and 1.0 g kg^{-1} crude powder of *O. onites* (B3) compared to control at day 56 ($p<0.05$) (Figure 3b).

Nitroblue tetrazolium positive cell was elevated in the group fed with 0.5 mL kg^{-1} essential oil (A1) and 1.0 g kg^{-1}, 10.0 g kg^{-1} of crude powder *O. onites* (A3-A4) compared to

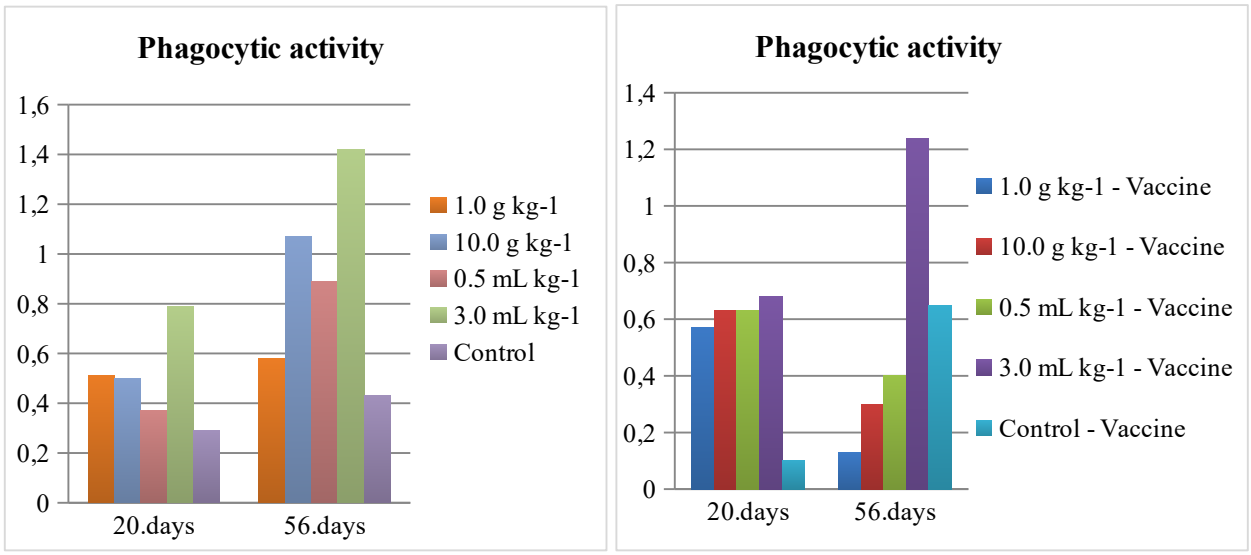


Figure 1. Phagocytic activity in groups fed diets containing different form of *O. onites* and control groups (a): non vaccinated fish groups (A1-A4) (b): vaccinated fish groups (B1-B4).

the control at day 20. After 56 days, fish fed the 10.0 g kg^{-1} crude powder of *O. onites* (A4) diet exhibited a significantly higher level of nitroblue tetrazolium positive cell when compared with the other groups and the control ($p<0.05$) (Figure 4a). Vaccinated groups in fed with 0.5 mL kg^{-1}, 3.0 mL kg^{-1} essential oil (B1-B2) and 1.0 g kg^{-1} crude powder of *O. onites* (B3), nitroblue tetrazolium positive cells showed elevated levels compared to the control at day 20 ($p<0.05$). There were no significant differences between groups (B1-B4) in nitroblue tetrazolium positive cells at day 56 (Figure 4b).

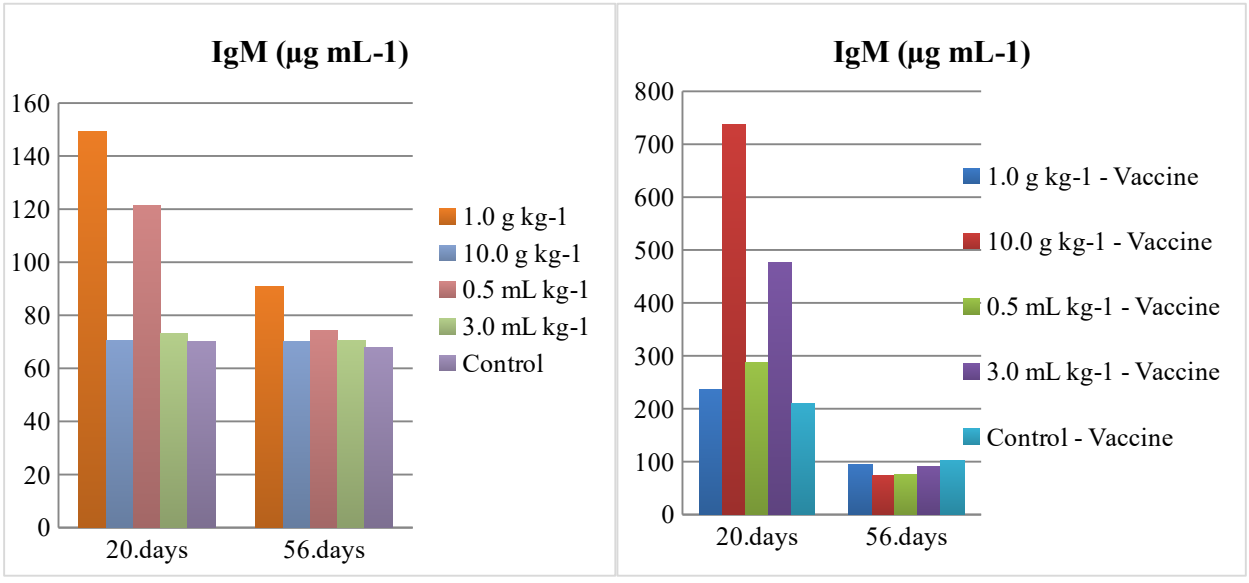
Challenge test

After eight weeks of feeding trial, experimental fish were challenged with *Y. ruckeri* and cumulative mortality was noted during 21 days. Cumulative mortalities in fish fed with crude powder and essential oil of *O. onites* (A1-A4) were significantly lower than the control group. The treatment group fed with 0.5 mL kg^{-1} essential oil of *O. onites* (A1) was the most effective with 12.5% mortality (Figure 5a). The 0.5 mL kg^{-1} (A1), 3.0 mL kg^{-1} (A2) and 1.0 g kg^{-1} (A3) diets showed better RPS than positive control group (oxytetracycline).

The lowest cumulative mortality ($p<0.05$) comparing to control was noted when using the combination of *O. onites* and vaccine in all treatment groups (B1-B4). Oral administration essential oil of *O. onites* to the vaccinated fish at the concentrations of 0.5 mL kg^{-1} diet (B1) enhanced their disease resistance against *Y. ruckeri* without mortality (Fig. 5b). The 0.5 mL kg^{-1} (B1) and 1.0 g kg^{-1} (B3) diets showed better RPS than positive control group (oxytetracycline).

DISCUSSION

Medicinal plants have been reported to promote various activities like growth-promoting, appetite stimulation, antimicrobial properties in aquaculture (Citarasu 2010, Ahmadifar et al. 2011, Yilmaz et al. 2012, Gormez & Diler 2014, Diler et al. 2017). Some natural products have positive effects on growth performance (Sonmez et al 2015, Diler et al 2017), while others do not make



(a) **(b)**

Figure 2. Immunoglobulin M levels in groups fed diets containing different form of *O. onites* and control groups (a): non vaccinated fish groups (A1-A4) (b): vaccinated fish groups (B1-B4).

significant changes of growth performance parameters (Volpatti et al. 2014, Yilmaz et al 2015, Bohlouli & Sadeghi 2016) in fish. The present study showed that rainbow trout fed with essential oil of *O. onites* (0.5 and 3.0 mL kg^{-1}) had significantly higher final weight, weight gain, specific growth rate, condition factor and survival rate compared to the control. Especially, dietary incorporation of *O. onites* crude powder (1.0 and 10.0 g kg^{-1}) showed higher final weight, weight gain and specific growth rate than *O. onites* essential oil supplemented groups. Growth parameters were elevated in rainbow trout fed with *O. onites* (0.5 mL kg^{-1}, 3.0 mL kg^{-1} and 1.0 g kg^{-1}) in combination with vaccine (p<0.05). Feed conversion rate was also positively affected when using *O. onites* combination with vaccine (p<0.05). Increase of the growth performance might be related to bioactive compounds in the *O. onites* such as thymol and carvacrol (Ahmadifar et al. 2011, Diler et al. 2017). These compounds may have metabolic properties that allow reducing free radical and pathogenic microorganisms and could increase the digestive potentials of beneficial microorganisms thereby a better use of the nutrients.

The use of plant - derived natural products alone or a combination with vaccine as immunomodulators stimulating the non specific and specific immune response for the prevention of fish diseases is a promising new development (Logambal et al. 2000, Yin et al. 2009). The present study showed that feeding both *O. onites* alone and in combination with vaccine significantly enhanced the non-specific defence parameters such as phagocytic activity, immunoglobulin M level, serum lysozyme activity and nitroblue tetrazolium positive cell of rainbow trout. Similar results have also been reported that feeding with combination of *Astragalus* and *Ganoderma* stimulated respiratory burst activity, phagocytosis and serum lysozyme activity in non vaccinated and vaccinated carp (Yin et al. 2009).

Lysozyme is a humoral component of the non-specific defense mechanism which is an effect of the peptidoglycan layer of bacterial

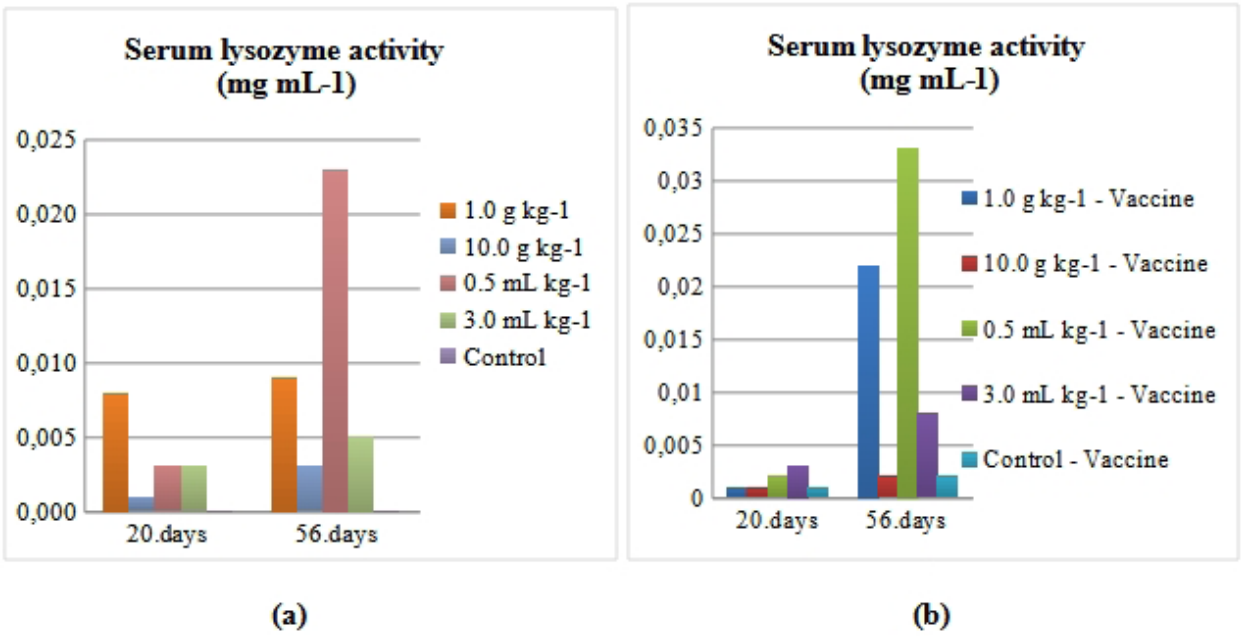
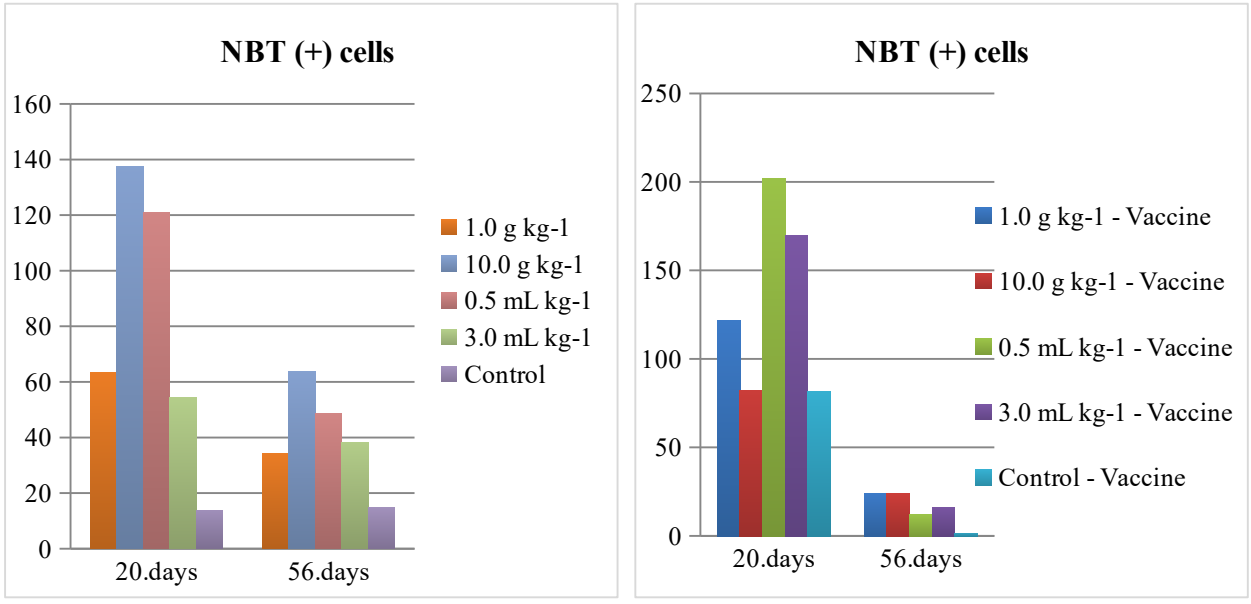


Figure 3. Serum lysozyme activity in groups fed diets containing different form of *O. onites* and control groups (a): non vaccinated fish groups (A1-A4) (b): vaccinated fish groups (B1-B4).

cell walls, leading to prevention of infection and disease (Ellis 1999). Several studies reported that dietary plant such as ginger (*Zingiber officinale*) (Nya & Austin 2009), garlic (Nya & Austin 2011), licorice root (*Glycyrrhize glabra*), blueberry (*Vaccinium myrtillus*), echinacea (*Echinacea angustifolia*), sage (*Salvia officinalis*) (Terzioglu & Diler 2016), oregano (*O. vulgare*) (Diler et al. 2015, Pourmoghim et al. 2015) and oregano (*O. onites*) (Diler et al. 2017) increased lysozyme activity in rainbow trout. Also in this study, serum lysozyme activity was significant increased in both vaccinated fish fed with 0.5 mL kg^{-1} and 1.0 g kg^{-1} *O. onites* and in non vaccinated fish feed containing 0.5 mL kg^{-1} of *O. onites*. Yin et al. (2009) reported that serum lysozyme activity was increased after one week in non vaccinated carp (*Cyprinus carpio*) fed with herbs whereas in vaccinated carp significant differences were measured in groups *Ganoderma* (second week), *Astragalus* (third week) and combination of herbs (fifth week). Alishahi et al. (2010) reported that serum lysozyme activity was enhanced in non vaccinated carp (*Cyprinus carpio*) with dietary *Aloe vera* after two weeks whereas elevated serum lysozyme activity in vaccinated fish after 2 and 4 weeks when compared to controls ($p<0.05$). In contrast, Volpatti et al. (2014) reported that fish fed with 0.025% carvacrol for 1–4 week exhibited a similar trend in the level of lysozymes but this parameter was significantly lower when compared with the control after 8 weeks in European seabass (*Dicentrarchus labrax*). Our results indicated that dietary herb combination with vaccine stimulated immune response fish (Yin et al. 2009, Alishahi et al. 2010). These data suggest that plant-derived natural products may interfere with activity of lysozyme and that its modulation depends on the dose and period of administration.

Phagocytic cells are the most important cellular components of the non-specific immune system of fish and play an important role in antibacterial defences. Phagocytic cells are the important parameters of innate immunity which includes neutrophils, monocytes and

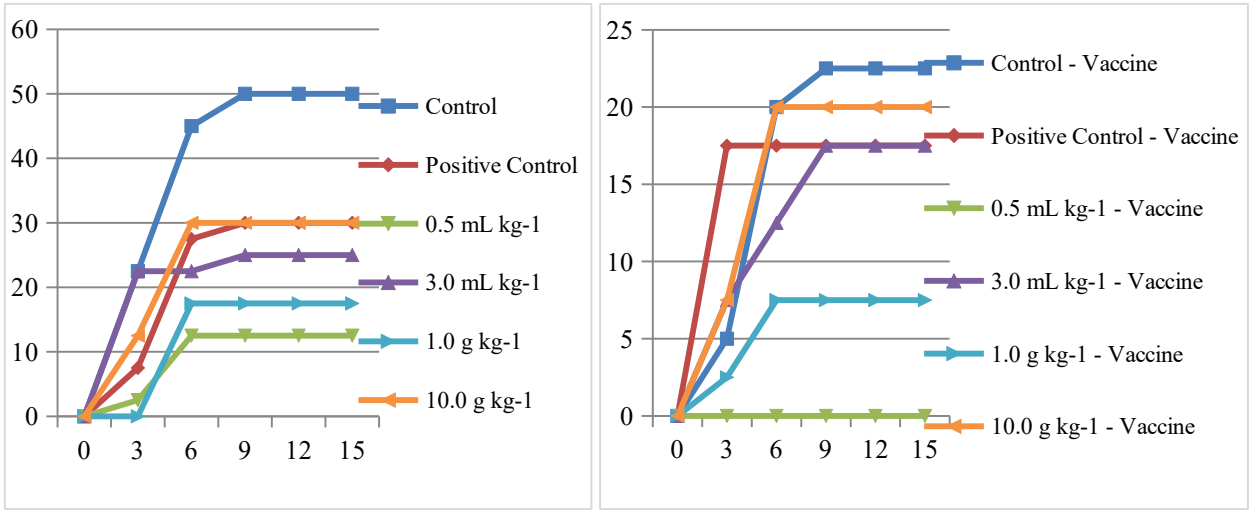


(a) **(b)**

Figure 4. Nitroblue tetrazolium positive cells in groups fed diets containing different form of *O. onites* and control groups (a): non vaccinated fish groups (A1-A4) (b): vaccinated fish groups (B1-B4).

macrophages. Herbal medicine such as misletoe (*Viscum album*), nettle (*Urtica dioica*), ginger (*Zingiber officinale*) (Karatas Dugenci et al. 2003, Nya & Austin 2009), laurel (Bilen & Bulut 2010) and *O. vulgare* (Pourmoghim et al. 2015) can also enhance phagocytosis in rainbow trout. In our experiments, elevated phagocytic activity was recorded only in vaccinated group fed with 3.0 mL kg^{-1} essential oil of *O. onites*, whereas in all non vaccinated groups fed with *O. onites*, phagocytic activity was significantly higher than the control group at day 56 ($p<0.05$). Yin et al. (2009) reported that phagocytic activity was elevated in non vaccinated carp fed with *Ganoderma* and *Astragalus* while in vaccinated groups fed with *Ganoderma* and the combination of two herbs, elevated levels were noted on week-1 and week-5 of experiment compared to the control. A possible mode of action of medicinal plants is in immunostimulation as a result of its bioactive constituent. Previous studies have reported that thymol is one of the bioactive compounds of *O. onites* and Astragalus polysaccharide modulates the functions of the macrophages (Kong et al. 2003, Chauhan et al. 2014).

Neutrophil activation is non-specific defense mechanism which serves the first line of defense against infiltrating pathogens (Anderson 1992). In this study, the nitroblue tetrazolium positive cell was significantly elevated both non vaccinated and vaccinated rainbow trout fed with diets containing *O. onites* compared to control at 20 days. Terzioglu & Diler (2016) showed significantly higher nitroblue tetrazolium positive cell in rainbow trout fed with *Glycyrrhize glabra*, *Vaccinium myrtillus*, *Echinacea angustifolia*, *Salvia officinalis*. Logambal et al. (2000) reported that the leaf extract of *O. sanctum* increased neutrophil activity when administered dietary in tilapia (*Oreochromis mossambicus*) vaccinated with *A. hydrophila*. The effects of *O. onites* on neutrophil degranulation have not been previously reported. Our data indicated components of the *O. onites* contribute to neutrophil activation.



(a) **(b)**

Figure 5. Cumulative mortalities (%) in groups fed diets containing crude powder and essential oil of *O. onites* and control groups (a): non vaccinated fish groups (A1-A4) (b): vaccinated fish groups (B1-B4).

Natural antibodies play a role in acquired immune defence, for example IgM is commonly the immunoglobulin class described in fish (Mashoof & Criscitiello 2016). Dorucu et al. (2009) reported that total immunoglobulin levels were high in rainbow trout fed with 1, 2.5 and 5% *Nigella sativa* incorporated diet. Similarly, Bohlouli & Sadeghi (2016) reported that the total immunoglobulin levels increased with 2 g kg^{-1} dose of *F. angulata* extract in rainbow trout. In addition, Alishahi et al. (2010) reported that IgM concentration were specially high in carp (*Cyprinus carpio*) vaccinated against *A. hydrophila* fed with dietary *Aloe vera* crude extract after 4, 6 and 8 weeks when compared to vaccinated controls (p<0.05). On the other hand, Ardo et al. (2008) found that feeding tilapia with two Chinese medicinal herbs (*Astragalus membranaceus* and *Lonicera japonica*) and boron alone or in combination no effect on total immunoglobulin level. This study reported that non vaccinated and vaccinated rainbow trout fed with dietary *O. onites* supplementation were significant different in the total immunoglobulin levels which is in agreement with earlier reports (Dorucu et al. 2009, Bohlouli & Sadeghi 2016) indicating the role of immunostimulants in fish. Our results indicated that plant essential oils likely represent a source of novel therapeutics that could be developed to modulate innate immune responses and either enhance defense against microbial infection.

Medicinal plants as well as their derived essential oils contain phenolic compounds that are known to restrict or inhibit the growth of bacteria, yeast, virus and moulds (Chorianopoulos et al. 2008). Several studies have also shown the inhibition against different microorganisms of *Origanum* species, damaging structural and functional in the cytoplasmic membrane by phenolic compounds such as thymol and carvacrol (Zheng et al. 2009, Abdel-Latif & Khalil 2014, Diler et al. 2017). When fish fed with *O. onites* supplemented diet and challenged with *Y. ruckeri*, the cumulative mortality was significantly reduced compared to control group in the present study. The best survival rate was determined in the group treated with 0.5 mL kg^{-1} *O. onites* essential oil (A1). Similar findings were reported by Diler et al.

(2017) underlining that dietary administration of *O. onites* essential oil significantly reduced fish mortality (p<0.05) and 3.0 mL kg^{-1} diet showed no mortality following challenge with *L. garvieae*. In other study, Volpatti et al. (2014) investigated that dietary carvacrol (0.025% and 0.05%) increased resistance of European seabass (*D. labrax*) to *Listonella anguillarum*. Cumulative mortality in fish fed 0.025% carvacrol was significantly lower than the control (75% RPS). Further, Abdel-Latif & Khalil (2014) recorded that Ropadiar powder plus® (Ropadiar) (*Origanum vulgare* essential oil) showed immune potentiating effects on Nile tilapia (*Oreochromis niloticus*). After eight weeks of experiment, fish were challenged with *Vibrio alginolyticus* and the cumulative mortality was reduced in fish fed Ropadiar. These observations are in close agreement with the other reports (Diler et al. 2017, Abdel-Latif & Khalil 2014, Volpatti et al. 2014).

The use of immunostimulants in combination with vaccine appears to show great potential for support of fish health (Anderson & Jeney 1992). Yin et al. (2009) observed that vaccined carp (*Cyprinus carpio*) fed a combination of *Ganoderma* (5%) and *Astragalus* (5%) showed the best survival following infection with *A. hydrophila*. Logambal et al. (2000) reported that the dietary incorporation of leaf extract from *Ocimum sanctum* enhanced disease resistance of vaccinated *Oreochromis mossambicus* against *A. hydrophila*. Alishahi et al. (2010) investigated the immunostimulatory effect of dietary *Aloe vera* (5%) crude extract in immunized *Cyprinus carpio* against *A. hydrophila* bacterin. The highest RPS were recorded in immunized group that also received *A. vera* (75%) and the lowest RPS was observed in the non-immunized non- *A. vera* -treated group (20%). In this study, mortalities were significantly reduced in all vaccinated fish fed with *O. onites*, after challenge with *Y. ruckeri*, and, no mortality was observed in the fish fed with 0.5 mL kg^{-1}. Definitely such profound enhancement in this non-specific immune parameters stemming from feed additives may have provided that protection against this pathogen. Especially given the critical role played by neutrophils, phagocytic cells and lysozyme activity against pathogens, our data supports the possible therapeutic effects of medicinal plants.

CONCLUSIONS

As a result, our study showed that administration of *O. onites* in both the vaccinated and non vaccinated fish enhanced non specific immune response and resistance against *Y. ruckeri*. This study was the first attempt to determine *O. onites* crude powder supplement and the combination of *O. onites* with vaccine groups significantly increased growth performance in rainbow trout. Both *O. onites* alone or in combination with vaccine reduced the mortality of rainbow trout after challenge with *Y. ruckeri*. The highest survival rate (100%) was observed in the vaccinated group fed with 0.5 mL kg^{-1} of *O. onites* essential oil. When using *O. onites* with vaccine the non specific immune response, growth performance and resistance against *Y. ruckeri* was also elevated compared to non vaccinated groups. It has been considered that the use of *O. onites* with vaccine increased efficacy of vaccine. Thus, it can be concluded that *O. onites* can be used as an immunostimulant to enhance growth performance, immune response and disease resistance of cultured fish species.

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