



Full length article

Therapeutic effects of beard lichen, *Usnea barbata* extract against *Lactococcus garvieae* infection in rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

In this study, therapeutic effects of aqueous methanolic extracts of beard lichen (*Usnea barbata*) against *Lactococcus garvieae* (ATCC 43921) in rainbow trout (*Oncorhynchus mykiss*) were investigated. Six different experimental groups [0 mg/100 µL (Control), 4 mg/100 µL, 8 mg/100 µL, 12 mg/100 µL, 6 mg/100 µL florfenicol (positive control), 6 mg/100 µL erythromycin (positive control)] were set up to determine effects of lichen extract on immune responses and survival rate. In the study, superoxide radical production was increased in fish treated with 12 mg beard lichen extract, florfenicol and erythromycin compared to that of control ($P < 0.05$). Lysozyme activity was generally decreased ($P < 0.05$) or no differences were observed in all experimental groups compared to that of control ($P > 0.05$). Myeloperoxidase was significantly increased in all antibiotic treated groups. No differences were observed in liver histology of experimental groups compared to control. Cytokine gene expressions were elevated in all experimental groups compared to that of control ($P < 0.05$), except IL-1 β expression at 10th day sampling time. Other immune related genes (IL-8, TGF- β , IL-12 Beta, TNF α 1, IL-10, COX-2, IL-6, TLR5, C3, IGM, MHC-II, iNOS, IgT, IFN1, IFN2, IFN reg) were also elevated in all experimental groups compared to that of control group. The survival rates obtained in 4 mg beard lichen treated group, 8 mg beard lichen treated group and erythromycin treated group were 73.08, 65.38 and 80.77% respectively. Our results suggest that beard lichen methanolic extract could be an effective therapeutic agent to be used against *L. garvieae* infection in rainbow trout at the dose of 4 mg/17.41 \pm 0.3 g body weight/day.

1. Introduction

In aquaculture industry, fish face serious disease problems, therefore, it is needed to identify new, effective and eco-friendly antimicrobials and improve farming techniques [1,2]. In recent years, production losses as a result of *Streptococcus iniae* and *Lactococcus garvieae* infections have become an important issue causing high economic losses [3]. *L. garvieae* is a Gram-positive coccus and a zoonotic pathogen, which is the causative agent of lactococcosis. It has been isolated from many fish species and even from human [4]. *L. garvieae* has caused significant losses in Japan, Italy, Israel and Europe [5]. Florfenicol [6] and erythromycin [7] are usually used for treatment of lactococcosis. However, resistance of fish pathogens to drugs is an important factor to be considered and it develops not only year by year but

also seasonally within a year [8]. In recent years, the use of vaccines and immunostimulants has become a widespread practice in preventing such diseases [9]. Despite all preventive measures, the disease can still occur. Medicinal plants have never been used in the treatment of such diseases until recently. Some medicinal plants have been shown to enhance the disease resistance against *Lactococcus garvieae* in fish species [10]. However, there is no report on the therapeutic effect of *Usnea barbata* against *L. garvieae* in fish. In this regard, the present study investigated the potential therapeutic use of beard lichen (*Usnea barbata*) [11], a member of Parmeliaceae family against the Gram-positive pathogen, *L. garvieae* infection in rainbow trout (*Oncorhynchus mykiss*).

Abbreviations: ATCC, American Type Culture Collection; IL-1 β , Interleukin 1 Beta; IL-8, Interleukin 8; TGF- β , Transforming Growth Factor Beta; IL-12 Beta, Interleukin 12 Beta; TNF α 1, Tumor Necrosis Factor; IL-10, Interleukin 10; COX-2, Cyclooxygenase-2; IL-6, Interleukin 6; TLR5, Toll Like Receptor 5; C3, Complement 3; IGM, Immunoglobulin M; MHC-II, Major Histocompatibility Complex II; iNOS, Inducible Nitric Oxide Synthase; IgT, Immunoglobulin T; IFN1, Interferon 1; IFN2, Interferon 2; IFN reg, Interferon regulatory; ROS, Oxidative radical production; qRT-PCR, Quantitative real time polymerase chain reaction; MPO, Myeloperoxidase; Flor, Florfenicol; Eryth, Erythromycin; BL, Beard lichen

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2. Materials and methods

2.1. Fish and experimental procedure

Rainbow trout (17.41 ± 0.3 g) used in the study were procured from Kastamonu University Inland and Marine Fish Research and Application Center, and after being brought to Kastamonu University Department of Aquaculture, Fish Diseases Laboratory, they were fed a standard trout feed for 2 weeks during the acclimation period. After the acclimation period, the fish were stocked in trial aquariums (18 number) and each fish tank contained 36 fish with 3 replicates per experimental group. Another 18 number of aquariums were also set up to determine survival rate of the experimental groups.

Aquariums were continuously aerated, water quality parameters were measured and maintained suitable for the fish species during the trial (18 °C, 8.5–8.7 pH, 7.7 mg/L O₂) with 20% of water in the tanks exchanged daily.

In order to determine the therapeutic effects of aqueous methanolic extracts of beard lichen against *L. garvieae* (ATCC 43921) infection, the fish were intraperitoneally infected with a predetermined LD₅₀ dose (10⁷ cells mL⁻¹) of pathogen at day 0 of the trial. In the same day, the fish were fed 0 (control), 4, 8 and 12 mg aqueous methanolic extract of beard lichen in 100 µL PBS twice daily, once in the morning and once in the evening, orally with feeding syringe after placentation using fenox-yethanol. In addition to the control group, two other positive control groups were also included that received florfenicol (6 mg/100 µL/fish) and erythromycin (6 mg/100 µL/fish), which are usually used in the treatment against *L. garvieae*. The fish in the control groups were fed relevant antibiotics orally twice daily. The study was continued for 10 days. At day 0, 3, 7 and 10, blood and tissue samples were obtained from 5 fishes from each experimental aquarium for immunological analysis. In order to determine the survival rate, another 18 aquariums were maintained with each having 36 fishes and none of these fish was used for any experimental analysis.

2.2. Beard lichen (*U. barbata*) and preparation of its extract

Beard lichen was collected from Kastamonu countryside forests (41° 25'50 N, 33° 45'19 E) in summer and was shade-dried. Fully dried plants were grinded in high-speed plant mills at Kastamonu University, Department of Aquaculture Laboratory and turned into powder. Aqueous methanolic extract of the pulverised plant was prepared according to method reported by Bilen et al. [12].

2.3. Immunological procedure

Blood and tissue samples were collected at day 0, 3, 7 and 10 of the study to determine the potential changes in immune response of fish experimentally infected with *L. garvieae* and treated with *U. barbata* extract. Oxidative radicals releasing and lysozyme activity of the fish were determined according to Siwicki et al. [13], and myeloperoxidase activity was determined according to Quade and Roth [14].

2.4. Histology

After completing 10 d of feeding, fish from each of experimental group were anesthetized with overdose of phenoxyethanol (as approved by the Ethics Committee of the Kastamonu University) and then liver was carefully removed and fixed in 10% buffered formalin solution for 48 h, followed by washing in 70% alcohol. After routine histopathological processing, the samples were embedded in paraffin blocks. Histopathological examinations were performed on 5 µm thick tissue sections placed on slides and all samples were stained with hematoxylin and eosin (H&E). The sections were examined under light microscope for histopathological evaluation. Olympus BX51 light microscope digital camera (Olympus DP74; Olympus, Tokyo, Japan) was used for

Table 1

Gene specific primers with their sequences and references used for qRT-PCR in the study.

Gene	Primer sequence (5'-3')	Amplicon Size (bp)	References
β-Actin	F5' ATGGAAGGTGAAATCGCC 3' R5' TGCCAGATCTTCCATG 3'	186	[16]
IL-1β	F5' ACCGAGTTCAAGGACAAGGA 3' R5' CATTTCATCAGGACCCAGCAC 3'	181	[17]
IL-6	F5' ACTCCCCTCTGTCCACACC 3' R5' GGCAGACAGGTCCTCCACTA 3'	91	[18]
IL-8	F5' CACAGACAGAGAAGGAAGAAAG 3' R5' TGCTCATCTTGGGGTTACAGA 3'	162	[17]
IL-10	F5' CGACTTTAAATCTCCCATCGAC 3' R5' GCATTGGACGATCTCTTTCTTC 3'	70	[19]
IL-12 Beta	F5' GAACCCAGACGATGATT 3' R5' GTTCAAACCTCAACCTCCA 3'	190	[20]
IFN-reg	F5' ACACCGACTACTGGTCACTGACAAC 3' R5' CAAGAAGTGGGCATGTGATCTGT 3'	76	[21]
IFN1	F5' AGAATGCCCCAGTCCCTTTCC 3' R5' GACTTTGTCTCAAACCTCAGCATCA 3'	71	[21]
IFN2	F5' GTTGAGGGCCATGGATGTG 3' R5' TCCAGCCCATCAAGCAGAA 3'	68	[21]
TGF-β	F5' AGATAAATCGGAGAGTTGCTGTG 3' R5' CCTGCTCCACCTTGTGTTGT 3'	275	[17]
TNFA1	F5' CAAGAGTTGAACCTTGTTCAA 3' R5' GCTGCTGCCGACATAGAC 3'	181	[22]
COX-2	F5' GGGCTTTGACATCCTCAAACA 3' R5' CATCGGACAAGAACCCTTGA 3'	73	[18]
C3	F5' AGCTTGCTGACTGGCTTTGT 3' R5' TCATAAACGGTGACCCCAAC 3'	227	[16]
IGM	F5' AGTTCCACAGCGTCCATCTG 3' R5' TACTGGCCATGCATCTCTG 3'	399	[16]
IgT	F5' AGCACCAGGGTGAAACCA 3' R5' GCGGTGGGTTTCAGAGTCA 3'	72	[23]
iNOS	F5' CGAATGGAGCTATCGTCAGACC 3' R5' CGGGAACGTTGTGGTCATAATACC 3'	234	[16]
MHC II	F5' ATGTGATGCCAATTCGCTTCTA 3' R5' TGTCTTGTCCAGTATGGCGCT 3'	336	[16]
TLR5	F5' GGCATCAGCCTGTTGAATTT 3' R5' ATGAAGAGCGAGACCTCAG 3'	89	[24]

Table 2

The ROS production (mg/ml) in ROS observed in rainbow trout of the experimental groups.

	0. Day	3. Day	7. Day	10. Day
Control	0.24 ± 0.11 ^a	0.23 ± 0.09 ^a	0.20 ± 0.06 ^a	0.28 ± 0.15 ^a
4 mg BL	0.35 ± 0.1 ^a	0.31 ± 0.06 ^b	0.19 ± 0.07 ^a	0.19 ± 0.11 ^a
8 mg BL	0.42 ± 0.07 ^a	0.25 ± 0.08 ^a	0.16 ± 0.04 ^a	0.25 ± 0.13 ^a
12 mg BL	0.44 ± 0.11 ^b	0.17 ± 0.05 ^a	0.23 ± 0.08 ^a	0.16 ± 0.04 ^a
Florfenicol	0.64 ± 0.19 ^c	0.37 ± 0.15 ^c	0.04 ± 0.02 ^b	0.22 ± 0.06 ^a
Erythromycin	0.73 ± 0.33 ^a	0.05 ± 0.02 ^d	0.13 ± 0.05 ^c	0.27 ± 0.16 ^a

The table shows mean values and standard deviations of the data. Different letters represent significant differences among groups (n = 5).

imaging the H&E staining.

2.5. Analysis of immune-related gene expression

A total of 54 fish were selected for gene expression studies in triplicate.

2.6. RNA extraction

Approximately 30 mg of kidney sample from each fish was collected and stored in RNAlater solution for RNA extraction after dissecting out the rainbow trout. Total RNA isolation was carried out using BIOLINE

Table 3

The variations in lysozyme activity (U/ml) observed in rainbow trout of the experimental groups.

	0. Day	3. Day	7. Day	10. Day
Control	0.10 ± 0.07 ^a	0.11 ± 0.05 ^a	0.44 ± 0.18 ^a	0.35 ± 0.07 ^a
4 mg BL	0.16 ± 0.09 ^b	0.80 ± 0.41 ^b	0.39 ± 0.18 ^a	0.25 ± 0.10 ^b
8 mg BL	0.24 ± 0.34 ^c	0.12 ± 0.07 ^a	0.09 ± 0.05 ^b	0.23 ± 0.08 ^b
12 mg BL	0.18 ± 0.26 ^b	0.19 ± 0.67 ^a	0.50 ± 0.2 ^a	0.05 ± 0.02 ^c
Florfenicol	0.20 ± 0.09 ^b	0.13 ± 0.11 ^a	0.25 ± 0.11 ^c	0.18 ± 0.06 ^d
Erythromycin	0.19 ± 0.28 ^b	0.12 ± 0.08 ^a	0.38 ± 0.17 ^c	0.38 ± 0.12 ^a

The table shows mean values and standard deviations of the data. Different letters represent significant differences among groups (n = 3).

kit (ISOLATE II RNA Mini Kit) according to manufacturer's protocol. The quantity and quality of all RNA samples were checked using a Multiscan GO (ThermoFischer Scientific, USA) spectrophotometer.

2.7. Complementary DNA (cDNA) synthesis

The extracted RNAs were subjected to treatment with 1 U DNase I (BIOLINE) in order to completely remove genomic DNA. The extracted total RNA was used for cDNA synthesis via reverse transcription from 1 µg mRNA using a BIOLINE kit (SensiFAST™ cDNA Synthesis Kit). cDNA reaction mixture included 1 µg of template RNA, 15 pmol/µL oligo dT primer, 4 µL 5× TransAmp Buffer, 1 µL of Reverse Transcriptase, oligo dT primer and up to 20 µL of nuclease free water. The reaction mixture was incubated for 10 min at 25 °C for primer annealing, 15 min at 42 °C for reverse transcription and 5 min at 85 °C for inactivation in thermal cycler (ThermoFischer Scientific).

2.8. Quantitative real-time PCR (qRT-PCR) analysis

After cDNA synthesis, qRT-PCR analysis was performed by utilising Rotor-Gene qPCR detection system (Qiagen, Germany) and SensiFAST SYBR No-ROX Kit PCR kit (BIOLINE, ABD). Gene specific primer sequences and references are enlisted in Table 1. qRT-PCR mixture included 12.5 µL of 2× SYBR Green Master Mix, 0.1 µg of template DNA, 0.4 µM of each gene specific forward and reverse primer (IL-1β, IL-6, IL-8, IL-10, IL-12, IFN-reg, IFN1, IFN2, TGF-β, TNFα, COX-2, C3, IgM, IgT, iNOS, MHC II, TLR5 and β-actin as reference) and distilled water to the final volume of 20 µL. qRT-PCR steps involved were as follows: denaturation at 95 °C for 5 s, then annealing and extension steps together at 60 °C for 10 s. Then, samples were denatured at 95 °C and held at 65 °C. Fluorescence signals were picked up at 530 nm wavelength from 60 °C to 95 °C at every 0.5 °C per second to implement melting curve analysis. qRT-PCR was achieved with 3 different samples from experimental and control groups, and 3 technical replicates were evaluated for each sample in all experiments. The ΔCT and ΔΔCT were estimated by $\Delta CT = CT_{\text{target gene}} - CT_{\text{reference}}$ and $\Delta\Delta CT = \Delta CT_{\text{treated sample}} - \Delta CT_{\text{control sample}}$. The results were analysed by $2^{-\Delta\Delta CT}$ method to estimate relative gene expression pattern [15]. The standard errors of mean between replicates were computed simultaneously.

Table 4

The variations in MPO activity (540 nm) observed in rainbow trout of the experimental groups.

	0. Day	3. Day	7. Day	10. Day
Control	20.23 ± 8.54 ^a	30.83 ± 18.34 ^a	40.77 ± 17.79 ^a	19.88 ± 9 ^a
4 mg BL	22.77 ± 10.31 ^a	332.20 ± 112.85 ^b	31.65 ± 11.71 ^a	126.98 ± 35.09 ^b
8 mg BL	28.05 ± 14.4 ^a	21.76 ± 12.38 ^a	73.88 ± 17.38 ^a	76.03 ± 35.63 ^c
12 mg BL	23.18 ± 4.70 ^a	257.54 ± 66.53 ^b	166.77 ± 38.52 ^b	30.42 ± 12.54 ^a
Florfenicol	41.83 ± 13.32 ^b	59.26 ± 20.88 ^c	185.36 ± 66.75 ^b	36.49 ± 7.04 ^a
Erythromycin	34.22 ± 11.61 ^b	59.83 ± 22.55 ^c	242.53 ± 93.52 ^c	103.31 ± 35.85 ^b

The table shows mean values and standard deviations of the data. Different letters represent significant differences among groups (n = 3).

2.9. Challenge test

Previously, to determine LD₅₀ dose of *L. garvieae*, the fish were intraperitoneally injected with different doses, such as 1×10^5 , 1×10^6 , 1×10^7 , 1×10^8 and 1×10^9 CFU mL⁻¹. Challenge test was performed as described in our previous study [12]. Briefly, *L. garvieae* (ATCC 43921) with 1×10^7 CFU mL⁻¹ mixed in 100 µL PBS was intraperitoneally injected to all fish at the beginning of the study and survival of groups was observed during 10 days post injection. Survival rate was determined using this formulae: SR (%) = (number of fish survived/number of fish injected) × 100.

2.10. Statistical analysis

All of the data obtained from the study was analysed using SPSS 22 software package. One-way ANOVA and then Duncan's test were performed at 95% confidence level to establish the differences between the groups.

2.11. Ethical approval

The present study was carried out and completed with the approval of Kastamonu University Animal Experiments Local Ethics with a decree number of 92495045-050-E.12086 dated 3 April 2017.

3. Results

Oxidative radical (ROS) production in rainbow trout of control group and infected with *L. garvieae* and treated with *U. barbata* extract or antibiotics are shown in Table 2. On day 0, ROS production did not significantly differ among the control, 4 mg, 8 mg and erythromycin treated groups ($P > 0.05$), whereas a remarkable increase in the 12 mg and florfenicol treated groups was detected ($P < 0.05$). On day 3, ROS production was similar in control, 8 mg and 12 mg group ($P > 0.05$), and significantly increased in 4 mg and florfenicol group compared to control ($P < 0.05$). On day 7 of the study, the ROS production was similar among fish treated with beard lichen extract and control group ($P > 0.05$), whereas florfenicol and erythromycin treated groups showed a significant decrease. On day 10 of the study, ROS production was similar among all groups ($P > 0.05$).

Changes in lysozyme activity are shown in Table 3. On day 0, fish treated with lichen extract and antibiotics showed a significant higher lysozyme activity compared to control fish ($P < 0.05$) and in particular the 8 mg beard lichen extract treated group showed a significantly higher lysozyme activity than that of other groups ($P < 0.05$). On day 3, lysozyme activity reached a significant higher level in the 4 mg beard lichen treated fish compared to all other experimental groups ($P < 0.05$). On day 7, no significant differences in lysozyme activity among the control, 4 mg and 12 mg beard lichen extract treated groups were observed ($P > 0.05$). However, the lysozyme activity was the lowest in the 8 mg beard lichen and florfenicol treated groups and it was significantly lower than that of other experimental groups. On day 10, the lysozyme activity in the erythromycin treated group was similar to that in the control group. In other experimental groups, a significant

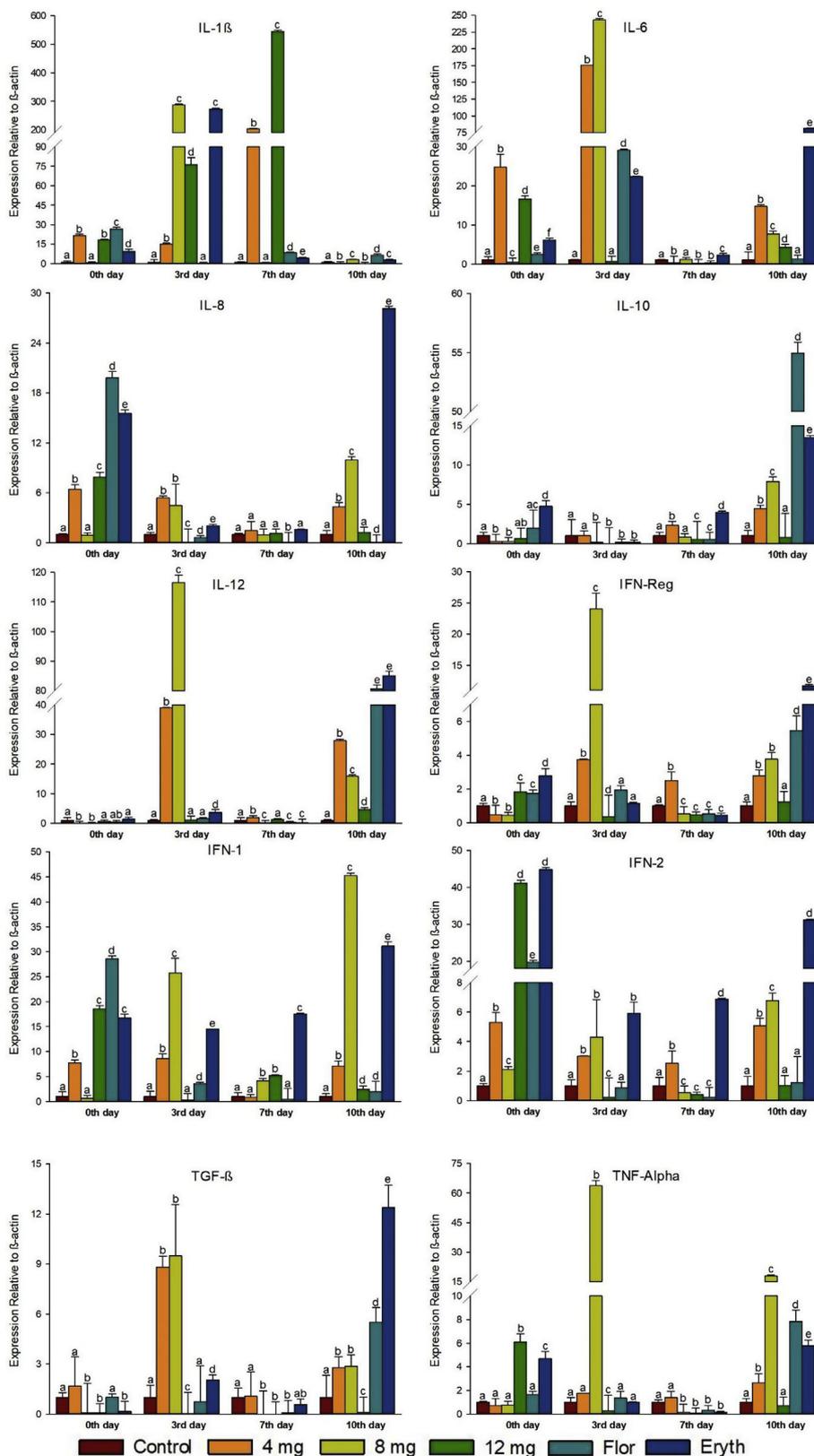


Fig. 1. Comparison of relative expression (mean ± SE; n = 3) levels of cytokines genes in the head kidney cells of rainbow trout treated with beard lichen against *Lactococcus garvieae* infection. Different letters on bars denote significant differences among groups (P < 0.05).

reduction in this parameter was observed (P < 0.05). The lowest lysozyme activity was observed in 12 mg beard lichen treated fish group.

During the trial, the changes detected in MPO activities of rainbow trout that were treated with aqueous methanolic extract of beard lichen

against *L. garvieae* infection are shown in Table 4. On day 0, a similar MPO activity was found in all groups treated with beard lichen compared to the control group (P > 0.05). Contrarily, antibiotic treated groups showed a significantly higher MPO activity levels than that in

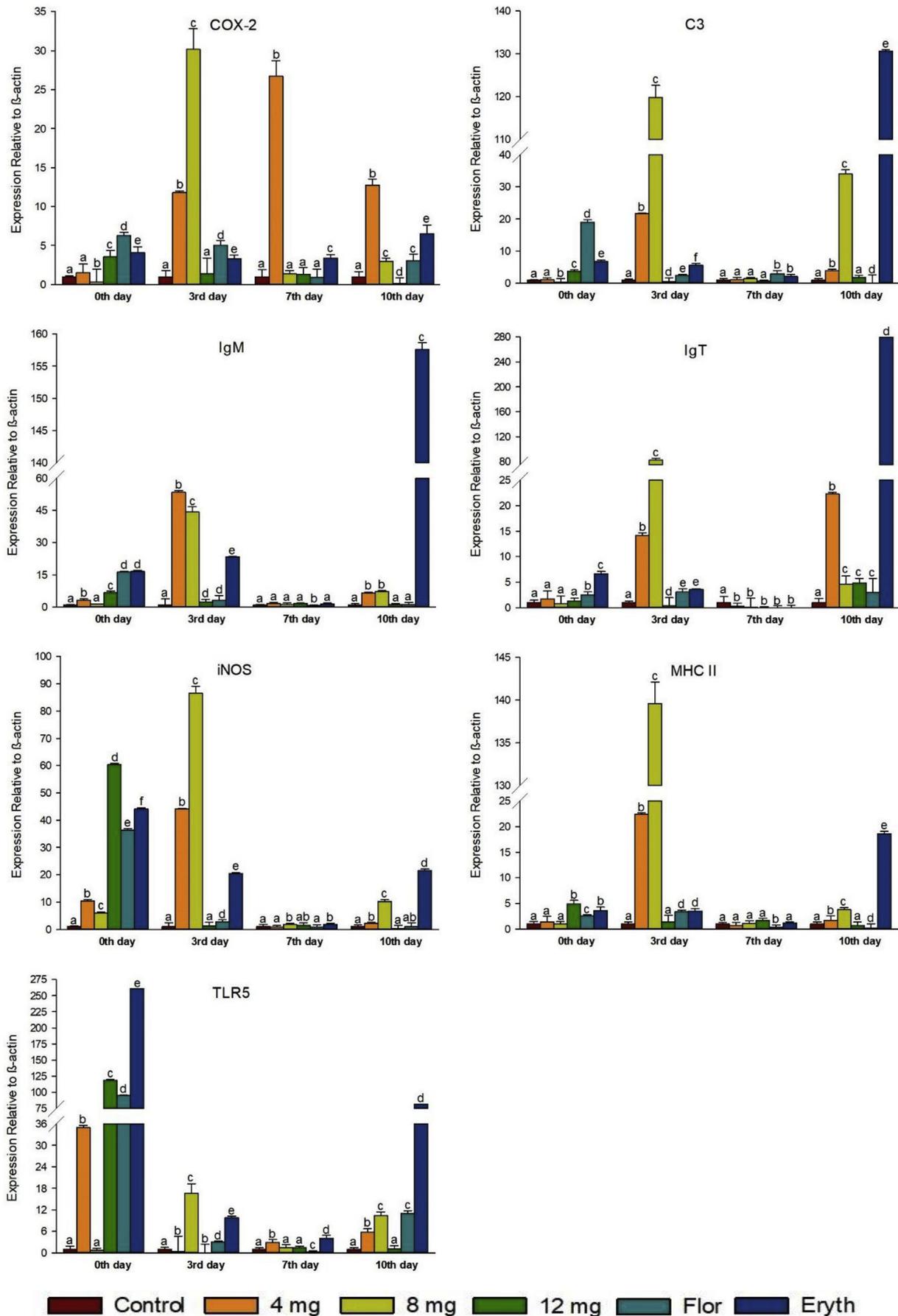


Fig. 2. Comparison of relative expression (mean ± SE; n = 3) levels of immune-related genes in the head kidney cells of rainbow trout treated with beard lichen against *Lactococcus garvieae* infection. Different letters on bars indicate significant differences among the groups (P < 0.05).

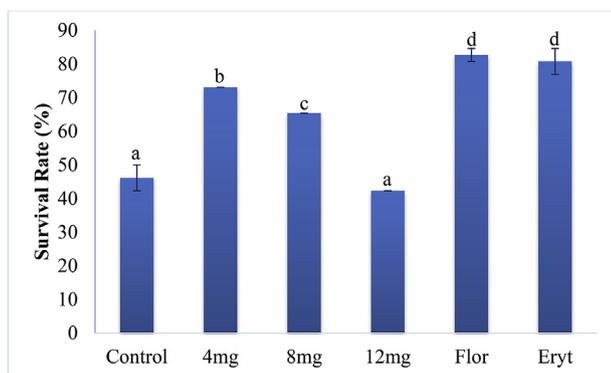


Fig. 3. Survival rate of the experimental groups. Different letters indicate significant differences among the groups ($P < 0.05$).

the lichen extract treated group and control group ($P < 0.05$). On day 3, there was a substantial increase in the 4 mg and 12 mg beard lichen extract treated group. In general, the MPO activities of all trial groups except that of the 8 mg beard lichen treated group were significantly higher than the control group ($P < 0.05$). Accordingly on day 7, there was no significant difference among the control, 4 mg and 8 mg beard lichen extract treated groups; however, there was a remarkable increase in the 12 mg beard lichen, florfenicol and erythromycin treated groups ($P < 0.05$). On day 10, no significant differences among the control group, 12 mg beard lichen and florfenicol treated groups were detected ($P > 0.05$); however, the MPO activities in other groups showed a significant increase ($P < 0.05$).

At the end of the study, from all experimental groups liver sample was taken and histological changes were given in Fig. 4. Those results suggested that no differences were observed in liver histology of any experimental group compared to control.

In the study, expressions of 10 different cytokines (IL-1 β , IL-6, IL-8, IL-10, IL-12, IFN-reg, IFN1, IFN2, TGF- β and TNF α) (Fig. 1) and 7 different immune-related (COX-2, C3, IgM, IgT, iNOS, MHC II and TLR5) (Fig. 2) genes were identified. All cytokine responses showed an elevation compared with the control group, but in general, this increase reached the highest level on day 3 ($P < 0.05$). Similar results were observed in other immune-related genes, but the expression of iNOS and TLR5 genes was also detected on day 0.

Survival of fish during the study is shown in Fig. 3. The survival rate was not different between the control and 12 mg beard lichen extract treated groups ($P > 0.05$). However, the survival rates in the 4-mg and 8-mg beard lichen treated groups were found to be significantly higher ($P < 0.05$). The highest survival rates were observed in rainbow trout of antibiotic treated groups.

4. Discussion

This study was carried out to determine the therapeutic effects of naturally harvested beard lichen (*U. barbata*) against *L. garvieae* infection in rainbow trout, compared to the use of currently available antibiotics. The beard lichen gathered from countryside forests was shade-dried under natural conditions, and aqueous methanolic extract was obtained and administered in a controlled manner twice daily to trout that were infected with *L. garvieae*. The fish in the control group were only administered 100 μ L PBS, and in order to compare the results, erythromycin and florfenicol used in the treatment of *L. garvieae* were administered as the positive control.

According to the results of the study, the optimum therapeutic effects against *L. garvieae* infection were observed in fish treated with florfenicol ($82.69 \pm 0.69\%$ survival) and erythromycin ($80.77 \pm 0.98\%$ survival) that are antibiotics which were effectively used in the treatment of this disease. However, the survival rate in fish treated with 4 mg beard lichen was determined to be $73.07 \pm 0.98\%$.

The survival rates in the 8 mg and 12 mg beard lichen treated groups were $65.38 \pm 0.01\%$ and $42.31 \pm 1.01\%$, respectively. Interestingly a dose-dependent survival rate was observed in rainbow trout treated with the beard lichen extract.

Antibiotic drugs are heavily used in aquaculture production and their use is gradually increasing. However, the major problems on the use of antibiotic drugs include antibiotic residues in fish, environmental impact and emergence of antibiotic-resistant bacteria [25,26]. Prophylactic treatment of fish is the simplest way to overcome the problems related to the use of antibiotics. For this purpose, fish are commonly subjected to vaccination, chemical treatments and immunostimulants. In recent years, there has been an increasing interest in the usage of medicinal plants as immunostimulants, and medicinal plants have been successfully used in several trials to limit the outbreaks of bacterial diseases in rainbow trout. However, until now no information is available concerning the potential use of medicinal plants for the treatment of *L. garvieae* infections.

Nya and Austin [27] have observed an increase in respiratory burst activity in ginger treatment. Moreover, an increase in superoxide radical production was observed in rainbow trout fed tetra powder [28]. Similarly, rainbow trout fed with diets containing garlic as a medicinal plant (in the form of garlic powder) showed an increase in the release of superoxide radicals [27].

In fish, lysozyme is an enzyme present in the mucous membrane, and it constitutes the first line of defence and is also responsible for the lysis of bacterial cell walls [29]. In our study, lysozyme activity increased in 0 day of the study in all the experimental groups compared to control. Baba et al. [30] have detected an increase in lysozyme activity of the rainbow trout that were fed *Lentinula edodes* extract. On the contrary, there was no change in lysozyme activity of rainbow trout fed laurel plant was reported [31]. However, Nya and Austin [27] detected an increased lysozyme activity in rainbow trout fed ginger. Moreover, Awad et al. [32] detected an increase in lysozyme activity of rainbow trout fed Quercetin, an active ingredient extracted from black cumin and nettle.

Myeloperoxidase is an important enzyme in fish immune system that has an important role in eliminating pathogens by producing H_2O_2 radicals [33]. In previous studies, in rainbow trout that were fed black cumin and nettle extracts, an increase in MPO activity was observed [32]. A similar increase in MPO activity was also observed in rainbow trout fed tetra methanolic extract [34]. An increase in MPO activity has also been reported in many fish that were fed medicinal plants [35–37]. In the present study, MPO activity was increased almost in all experimental groups at the sampling times except 0 day in lichen treated groups, 3. day 8 mg lichen treated group, 7 day 4 and 8 mg lichen treated groups and 10 day 12 mg and florfenicol groups.

IL-1 β is a pro-inflammatory cytokine activating the lymphocytes during immune response [38]. In our study, IL-1 β expression increased and reached the highest level on day 3 in all the experimental groups except florfenicol compared to control. A similar finding has been reported in trout fed caper [39], whereas a decrease in IL-1 β gene expression in rainbow trout fed black cumin was observed by other authors [40]. IL-6 is a pleiotropic cytokine and plays a regulatory role in processes, such as immunoglobulin synthesis, T-cell differentiation, acute phase reaction and haematopoiesis [41]. In the present study, there was a significant increase in IL-6 gene expression in all groups compared with that of the control group. This increase was highest in the 4-mg and 8-mg beard lichen groups on day 3. Similar to our study, an elevated expression of IL-6 gene was observed in trout that were fed *Lactobacillus rhamnosus*, *Enterococcus faecium* and *Bacillus subtilis*. IL-8 is a pro-inflammatory cytokine that showed an increase on day 0 and 3 in the 4 mg and 8 mg beard lichen extract treated groups compared to control group. IL-8 gene expression had a decrease in trout fed black cumin [41]. IL-10 is a multifaceted cytokine that is produced by and affects a variety of cell populations, including macrophages and T, B and NK cells [42]. In this study, IL-10 gene expression showed an

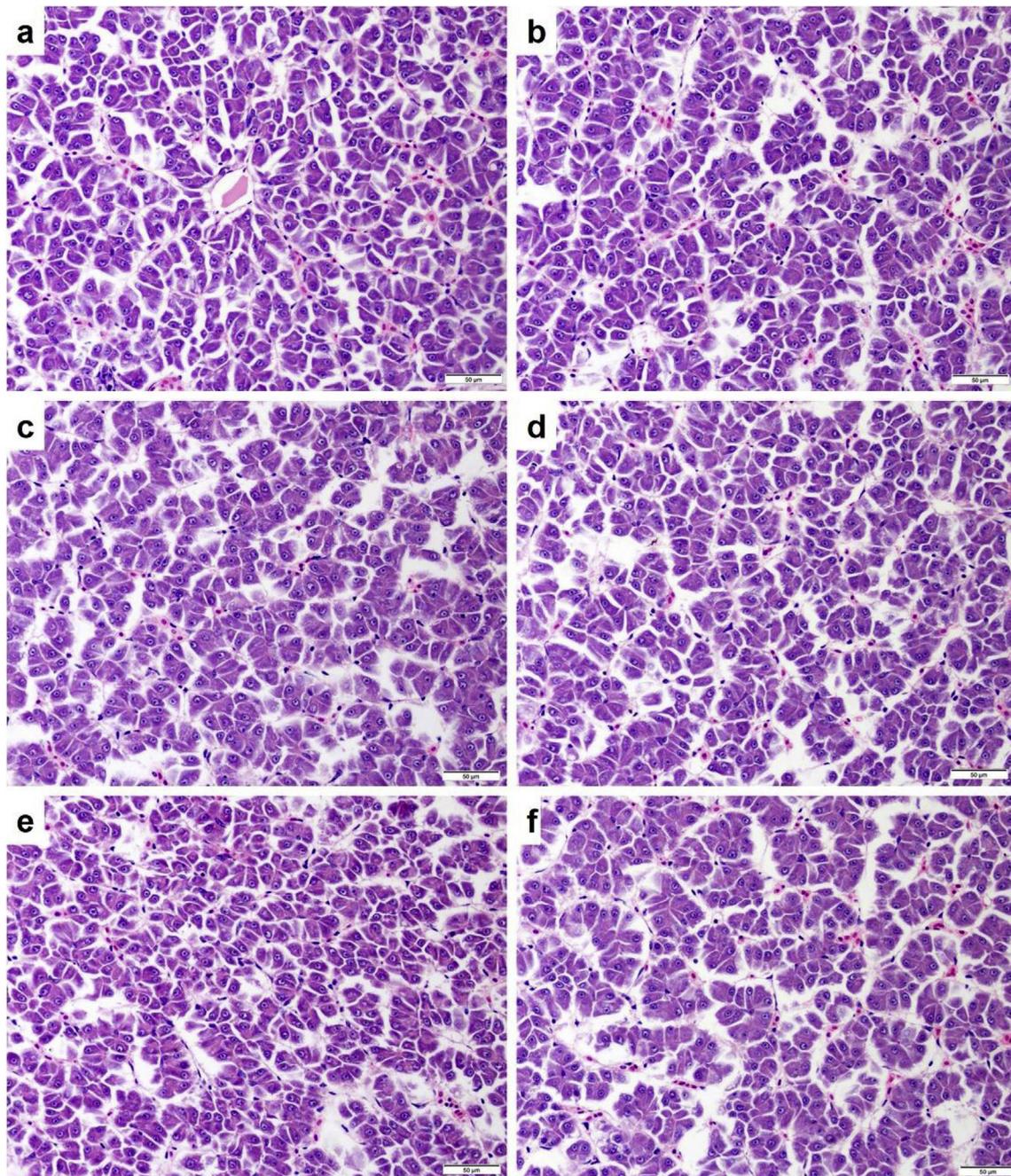


Fig. 4. Appearance of beard lichen extract on liver histopathology (a, b, c, d, e, f). a: Control group, b: 4 mg BL, c: 8 mg BL, d: 12 mg BL, e: Florfenicol, f: Erythromycin.

increase on day 10, especially in the 4 mg and 8 mg lichen extract and antibiotic treated groups compared to that of the control fish group. This result indicated that anti-inflammatory response was developed on day 10. IL-12 controls cell-mediated immune responses and provides immune protection against parasites, viruses and bacteria by producing IFN 2 (IFN- γ) from NK cells. IL-12 gene expression reached peak level on day 3 in the 4 mg and 8 mg lichen extract treated groups compared to control group, and these high levels were also observed on day 10. In this case, it is also possible that a significant immune response has been induced in the 4-mg and 8-mg beard lichen groups against *L. garvieae* infection. IFN-reg, IFN-1 and IFN-2 are responsible for the generation of immune response against viral infections [21]. In this study, an overall increase in expression of all IFN genes was observed compared to that in the control group. Similar to our study, Ooi et al. [21] reported an

increase in IFN-1 and IFN-reg gene expression in Atlantic salmon that received interferon- α 2. Along with its essential roles, such as those in lymphocyte proliferation and differentiation, TGF- β maintains immune system tolerance. TGF- β regulates immune responses by controlling chemotaxis in NK cells, dendritic cells, lymphocytes, mast cells, macrophages and granulocytes [43]. As with other gene responses, TGF- β gene expression also increased significantly on day 3 of the study in the 4 mg and 8 mg lichen extract groups. This finding shows that the inflammatory response has been regulated in groups that received 4 mg and 8 mg of lichen extract. In our previous study, TGF- β gene expression decreased in rainbow trout that received black cumin [40]. TNF- α is another cytokine that regulates respiratory burst activity. Increases in its activity were remarkable on day 10 of the present study. Awad et al. [44] reported an increased TNF- α expression in response to fenugreek

(*Trigonella foenum graecum*) in gilthead sea bream (*Sparus aurata* L.). Moreover, TNF- α expression was elevated by treatment with Chinese herbal medicine sinomenine and Liang Miao San [45] and caper bud extracts [46].

Other immune system-related genes, such as COX-2, C3, IgM, IgT, iNOS and MCH II were also upregulated on day 3 in the 4 mg and 8 mg lichen extract groups and on day 10 in the antibiotic treated groups, as was the case with cytokine responses. TLR5 markedly increased on day 0 of the study in all experimental groups except 8 mg lichen group compared to control. Cyclooxygenase gene COX-2 catalyses the initial reactions in prostanoid biosynthesis and produces the common prostanoids precursor [47]. It generally plays a role against parasitic infections [48]. Increased COX2 response may have been regulated by the immune system against secondary infections. Similarly, C3 activates the complement system in generating immune response against parasites [16]. Complement factor C3 markedly increased on day 3 in the 4-mg and 8-mg beard lichen treated groups compared to that in the control group. This finding indicates that the immune response is regulated against secondary infections. Inducible nitric oxide synthase (iNOS) is mainly present in macrophages but may also be found in hepatocytes, chondrocytes, retinal epithelial cells and osteoblasts [49]. It plays a role in production of nitric oxide against bacteria. *Usnea barbata*, especially in the 4 mg and 8 mg groups, elevated iNOS gene expression and consequently could indicate an increased activity against the bacteria. Major histocompatibility complex (MHC) molecules are the key players in initiating immune responses towards invading pathogens. In the present study, the increased MCH II gene expression shows that the groups receiving *Usnea barbata* have an elevated immune response against *L. garvieae* infection. In this study, beard lichen was used for the treatment of *L. garvieae* infection and it has been observed to increase survival rate significantly in the 4 mg and 8 mg lichen treated groups. From this study, it is concluded that aqueous methanolic extract of beard lichen can be used effectively as an organic product instead of antibiotics drugs against *L. garvieae* infection.

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