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THERAPEUTIC EFFECTS OF THYME (*THYMUS VULGARIS* LINNEAUS) AND FENNEL (*FOENICULUM VULGARE* MILLER) ESSENTIAL OILS IN INFECTED RAINBOW TROUT, *ONCORHYNCHUS MYKISS* (WALBAUM)

A. KUCUKGUL GULEC[a*], A. KUCUKGUL[b], D. DANABAS[a], M. URAL [a], E. SEKER [a], A. ARSLAN[a], O. SERDAR[a]
[a]*Tunceli University, Fisheries Faculty, TR62000, Tunceli, Turkey*
[b]*Mustafa Kemal University, Veterinary Faculty, TR31040, Hatay, Turkey*

The fish (84±1.02 g mean±SEM) were divided into 3 groups (N=15 in each group) as control (CNT), infected with *Yersinia ruckeri* (INF), and infected and essential oils (+EO). The CNT and INF groups were fed only with commercial fish feed. The +EO group was fed with feeds prepared with the rate of 10 ml herbal essential oils (mixed thyme and fennel) / 100 g feed during 21 days. The liver tissue (0.4 g) of fish was dissected in third day of the experimental period and mRNA transcription levels in liver tissue were determined by Real Time PCR analysis. INF and + EO groups exhibited decreased immunity against the challenging with Y. ruckeri infection (53.57 and 42.85 % mortality, respectively) when compared with CNT group (93.33 %). Gene mRNA transcription levels of tumour necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), inducible nitric oxide synthesis (iNOS), serum amyloid A (SAA), that are responsible during the initiation and continuation of the inflammation increased in 1.86, 2.46, 1.62 and 55.7 fold change, respectively in INF group, whereas mRNA expressions of haptoglobin (Hp) decreased (1.2–fold change) in 3th day of experimental period. The +EO group eliminated the increase in all of the gene expressions.

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1. Introduction

Yersiniosis or enteric redmouth disease (ERM), caused by *Yersinia ruckeri*, is a serious infectious disease in the rainbow trout that causes economic problems in aquaculture industry worldwide [1]. In aquaculture, broad-spectrum antibiotics have been used successfully to control of this fish diseases. However, because of the occurrence of resistant bacteria as a result of antibiotics usage, scientists recently focused on the usage experiments of natural antibacterial and antioxidants (spices, herbs, and vegetable extracts) due to their antioxidant properties and phenolic contents to control bacterial and viral infections [2].

The main constituents of thyme (*Thymus vulgaris* Linnaeus) belonging to the Lamiaceae family is a pleasant smelling perennial shrub, which grows in several regions in the world are thymol, carvacrol and flavonoids [3]. Fennel (*Foeniculum vulgare* Miller) belongs to the family Apiaceae has been used as spice to enhance the flavor, aroma of foods and their medicinal values for centuries [4-5-6]. Thyme and fennel also possesses various beneficial effects, like antiseptic, carminative, antimicrobial and anti-oxidative properties [7-8-9].

Initial reactivity to bacterial pathogens depends mainly on innate immunity mechanisms [10]. Innate immune mechanisms act as a first line of defence against infection and inflammation typifies an innate immune response [11]. Adaptive immune responses against *Y. ruckeri* bacteria has been studied by a number of authors [10-12] but the role of innate immune factors during the progress of *Y. ruckeri* infection in the rainbow trout is unknown. Also, acute phase proteins (APPs)

*Corresponding author: agulec@tunceli.edu.tr

and complement factors are one of important effectors molecules in the innate immune response against pathogens [13-14]. Positive APPs are haptoglobin (Hp), serum amyloid A (SAA) that they increase in serum concentrations during an acute phase response. The production of nitric oxide by iNOS is an important component of host immune responses and has beneficial antimicrobial, antiviral, antiparasital, and antitumoral effects [15]. That iNOS expression is regulated by cytokines in fish, it has been demonstrated that optimal release of nitric oxides from macrophages requires priming with macrophage activation factors and / or LPS [11]. Several studies have indicated the significance of Hp [16], iNOS [17-18] and SAA [16-19] in serum for identifying the occurrence and severity of inflammatory responses.

Cytokines are regulatory proteins secreted by immune cells that initiate and regulate cellular function 20. They exert a vital role in homeostatic mechanisms, such as the immune response, inflammation, acute phase response and tissue repair [21]. The level of a cytokine is a primary cause for a disease [21]. Amongst these mediators are a number of proinflammatory cytokines, such as tumor necrosis factor a (TNF- α), interleukin-1β (IL-1β), interleukin-6 (IL-6) and the chemokine IL-8 [22]. These molecules are synthesized by hepatocytes and their production is induced by the macrophage activating factor, and a combination of TNF-α, IL-1β, IL-6 [23] released by macrophages in the presence of pathogens such as bacteria [10]. Recently, a number of fish cytokine genes has been isolated and sequenced, including those for IL-1β [11-22-23-24-25-26] and TNF-α [26-27-28].

Despite in previous researches the importance and the use of plant oils in fish against bacterial agents was studied by examining effects of antibacterial and antioxidant, there is less data about the mechanisms of gene expressions in fish fed with diet supplements. In this study, it was evaluated the therapeutic effects of thyme and fennel essential oils by determining both cumulative mortality rates and the gene expressions related to the inflammatory process, in liver tissues of rainbow trout experimentally infected with *Y. ruckeri*.

2. Material and methods

2.1. Fish

Totally 300 rainbow trout (84±1.02 g, Mean±SEM) were provided from a commercial fish farm in September 2011 (Tunceli, Turkey). Fish were acclimatized to laboratory conditions (17±1 ^{o}C) for 20 days and fed twice time in a day with commercial pellet feed at 3 % rate of body weight (Kılıç, Turkey). Fish were maintained in three tanks (450 l), supplied with aerated flow-through well water, and at 12 h light/12 h dark period.

2.2. Bacterial Challenge

Yersinia ruckeri (ATCC 29473) isolates provided by Pendik Veterinary Research Institute (Istanbul, Turkey) were used in this study and stored at –80°C. Before usage in experimental infections, bacteria were sub cultured on triptych soy agar (TSA, Oxoid) to check purity, and then cultured in triptych soy broth (TSB) for 24 h at 22°C. Before experimental trials, this bacterium was used to infect rainbow trout intraperitoneally by bacterial suspension.

2.3. Essential Oils

Thyme (*Thymus vulgaris*) and fennel (*Foeniculum vulgare*) essential oils were provided from a commercial firm (Elazığ, Turkey). The compositions of the herbal essential oils used in this study were determined by gas chromatography–mass spectrometry (GC-MS) in Laboratory of Marmara Research Center Food Institute. GC-MS analyses were carried out on a Shimadzu GC-9A gas chromatograph equipped with Thermon-600 T (30 m × 0.25 mm × 0.25 μm film thickness) [29].

2.4. Diet

The essential oils mixture used in the study was added to feed via pulverization method. The feed was spread on aluminum folio and determined doses of the oils were pulverized onto the feed. The diets were dried in open air, packed and stored to be used in experimental process. Feed was given twice a day at 9:00 and 17:00 at a rate of 3 % body weight per day.

2.5. Screening Essential Oils

Screening of herbal oils to determine their antibacterial activity against *Y. ruckeri* was conducted using the disc diffusion method as described by Bauer et al. [30]. All the tests were

replicated three times. Inhibition zone of per herbal oil was measured and recorded. Minimal inhibitory concentration (MIC) was determined as the lowest concentration, and the highest dilution, which completely inhibited the growth of *Y. ruckeri* by an agar plate dilution method according to CLSI [31]. MIC dosage of herbal oils was solved in 1 ml of the Mueller-Hinton broth medium. Similar to what was mentioned before, 3 x 10^7 cfu ml^{-1} bacteria were added to each test tube and after 24 h of incubation at 35 ºC, the MIC dosage for per herbal oil was determined in µg ml^{-1}.

2.6. Estimation of Median Lethal Dose (LD_{50})

Healthy fish (N=120) were used for determining LD_{50} of *Y. ruckeri*. Graded doses ranging from 10^{10} to 10^4 of CFU/ml were utilized. Each dilution (0.1 ml) was injected intraperitoneally into each fish. 0.1 ml of 0.9 % of physiological saline was injected into the last group fish and served as a control group. Mortalities were observed over a period of 10 days. Dead fish were removed from the aquarium daily. The freshly dead fish were submitted for bacterial isolation to confirm specificity of mortality. The median lethal dose (LD_{50}) was calculated by the method of Reed-Muench [32].

2.7. Total RNA isolation, cDNA synthesis and real-time PCR

Rainbow trout were anaesthetized in frozen and dissected. Liver tissue samples (approximately 0.4 g) from all fish were removed aseptical conditions for RNA extraction, placed in Eppendorf tubes and immediately immersed in liquid nitrogen before storage in -80 ^oC until. After 25 mg of liver from each fish was homogenized by using TRIZOL Reagent (Sigma-Aldrich/GERMANY) total RNA was extracted. Two micrograms at microliter of total RNA were reverse transcribed in a reaction volume of 20 µl using reverse transcriptase in cNDA synthesis kit (Fermentase/USA). One microliter of each cDNA was used as templates for amplification using SYBER Green PCR amplification mastermix reagent (Fermentase/USA) and our target gene-specific primers. The specific primers for TNF-α, IL-1β, SAA, haptoglobulin and iNOS the binding lengths (base pair) of the PCR product size and annealing temperatures are showed in Table 1. The threshold cycle (Ct) values for the transcripts are normalized to β-actin by subtracting the average Ct value for each treatment [33]. Each PCR reaction was performed in triplicate. mRNA transcription values were determined as down- or up regulated for each gene.

Table 1. Primers used for RT-PCR

Gene	Primer	Oligonucleotide (5'-3')	Annealing temp. (^0C)
TNF-α	F R	CCT TCG TTC AGC TGG AAC AC GGC CTC TAC TTC GTC TAC	61.4
iNOS	F R	CGGGA ACGTT GTGGT CATAA TAC TTC CCG CTC TCT TGT CTT CC TTC	62
IL-1β	F R	CCGACTCCAACTCCAACACTA- TTGCTGGAGAGTGCTGTGGAAGAA	61.4
SAA	F R	TCA CTG TCC TCA AAC GTG GCT GTT CAA CGG AAA ACC TGT TT	60,1
Haptoglobulin	F R	GAG ATC CCA AAC AAT TCA GAC CTG CTT CCT GAC AAA CAA GTT CCT GCC	60,1
β-actin	F R	TGG CCC ATC CCA ACC ATC AC ATG GAA GAT GAA ATC GCC GCA C	59,2

2.8. Experimental Processing

Rainbow trout were divided randomly into three groups (N=15 in each group) as triplicate. The first group was control (CNT). The second group was infected with *Y. ruckeri* (INF). The last group was both infected and fed with feed containing the mixtures of essential oils (+EO) group. The CNT and INF groups were fed only with commercial fish feed. The +EO group was fed with

feeds prepared with the rate of 10 ml herbal essential oils (mixed thyme and fennel) / 100 g feed. All of the groups were fed during 21 days and mmortality and behavioral alterations of the fish were recorded. Dead fish were removed. The fish were monitored for their health status, cumulative mortality rates during this period. Tests were performed in triplicate. 3th day was chosen as sample time because of the reach peak levels of bacterial growth [10-22]. To examine the possible effects of mRNA transcriptions on infected fish in the end of this period, liver tissues of fish in each group were dissected and TNF-α, IL-1β, iNOS, SAA and Hp mRNA transcriptions were determined by Real time –PCR.

2.9. Statistical Analyses

All data obtained from experiments were analyzed by a one-way Analysis of Variance (ANOVA) using SPSS 11.5. When significant differences occurred, the group means were further compared with Duncan's multiple range tests.

3. Results

3.1. Analyses and Screening of Essential Oil

The most important components of thyme oil tested by GC-MS analyses in this study were phenol (40.95 %) and 2-methyl-5-(1-methylethyl) (12.12 %) while them of fennel oil were benzene (67.99 %) and 1-methoxy-4-(1-propenyl) (16.03 %) (Table 2 and 3).

Table 2. Major component of T. vulgaris essential oil

Chemical component	**Area (%)**
á-Myrcene	2.22
1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	2.42
Benzene, methyl(1-methylethyl)- (CAS)	12.12
ç-Terpinene	10.92
Linalool L	15.11
Carvacrol Methyl Ether	1.44
Phenol, 5-methyl-2-(1-methylethyl)- (CAS)	5.88
Phenol, 2-methyl-5-(1-methylethyl)- (CAS)	40.95
Caryophyllene	6.09
á-Bisabolene	1.37
(-)-Caryophyllene oxide	2.38

Table 3. Major components of F. vulgare essential oil

Chemical component	**Area (%)**
dl-Limonene	16.03
Benzene, 1-methoxy-4-(2-propenyl)- (CAS)	6.97
2-Cyclohexen-1-one, 2-methyl-5-(1-methylethenyl)-, (S)-	5.35
Benzaldehyde, 4-methoxy- (CAS)	3.66
Benzene, 1-methoxy-4-(1-propenyl)- (CAS)	67.99

According to disc diffusion test results, the highest values obtained for *T. vulgare* (24.6 ± 0.21 mm), *F. vulgare* (22.1 ± 0.18 mm). The MIC values of the tested thyme and fennel oils were determined as 40 µg ml^{-1} and 60 µg ml^{-1}, respectively (Table 4).

Table 4. Minimum inhibition concentrations (MIC) and zones of inhibition (mm) of herb oils

Botanical name	Antimicrobial Activity	
	Inhibition zone in diameter (mm)	MIC (µg/ml)
T.V	24.6 ± **0.21**	40
F.V	22.1 ± **0.18**	50

*Each result was expressed as the mean ± SD, **T.V**: *T. vulgaris* (10 µL), **F.V:** *F. vulgare* (10 µL)

3.2. Cumulative Mortality

Fish, for determination of LD_{50}, were intraperitoneally infected with different doses of *Y. ruckeri*, and the results were shown in Figure 1. All deaths occurred 5 days after bacterial infection and the pathogen was found in the livers and kidneys of all dead fish. No mortality or visible changes were observed in CNT group. Based on the mortality, the calculated LD_{50} of *Y. ruckeri* for rainbow trout was $3x10^7$ CFU ml^{-1}.

The fish mortalities kept increasing in time until the 5th day post-challenge (Figure 2). In the group receiving the control diet with no the oils, the cumulative mortality of the infected fish 50 %. INF and + EO groups exhibited decreased immunity against the challenging with *Y. ruckeri* infection (53.57 and 42.85 % mortality, respectively) when compared with CNT (93.33 %) (p<0.05).

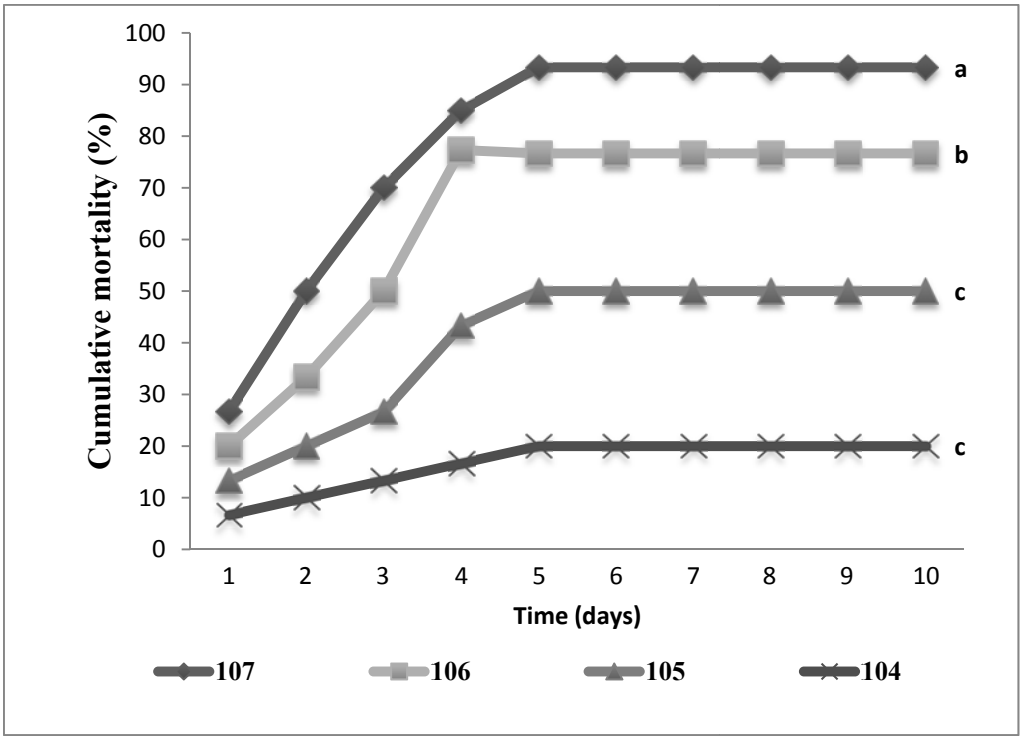


Fig. 1. Cumulative mortality of rainbow trout experimentally infected Y. ruckeri. Statistical differences (p<0.05) between groups are indicated by different letters (a-c)

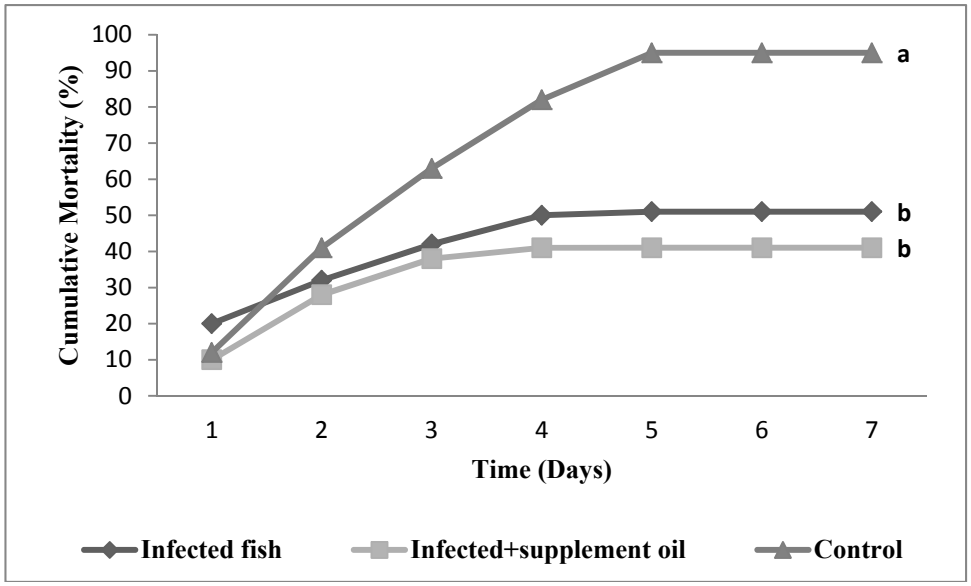
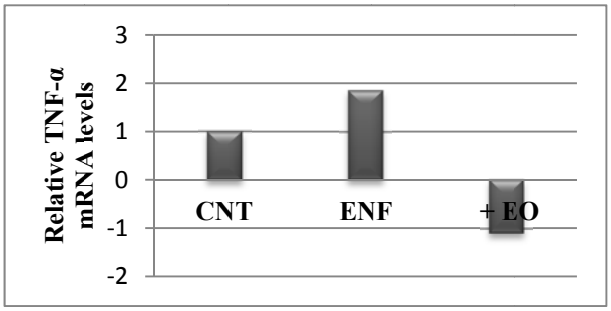


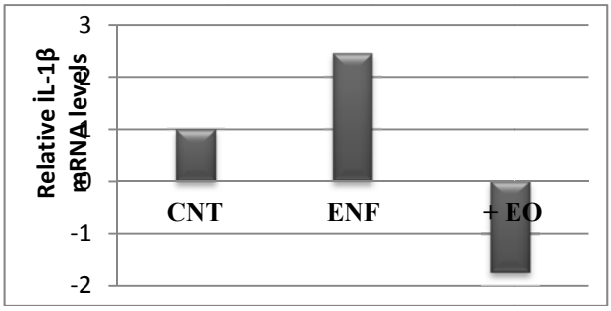
Fig. 2. Effects of fish diets supplemented with T. vulgaris and with F. vulgare on the cumulative mortality of rainbow trout against to Y. ruckeri. Data are expressed as mean S.E. Statistical differences ($p<0.05$) between groups are indicated by different letters (a-b)

3.3. RT-PCR analysis of mRNA levels

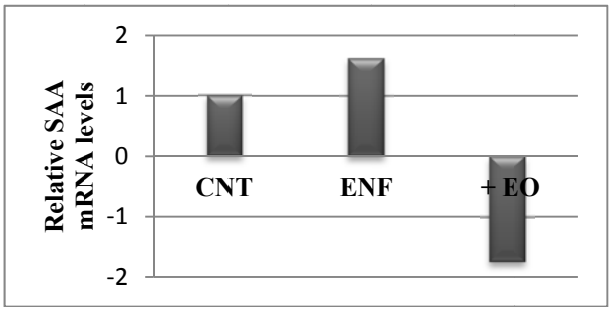
Gene mRNA transcription levels of TNF-α (Fig. 3A), IL-1β (Fig. 3B), SAA (Fig. 3C), iNOS (Fig. 3E), that are responsible during the initiation and continuation of the inflammation increased in 1.86, 2.46, 1.62 and 55.7 times (fold change), respectively in INF group according to CNT group whereas mRNA expressions of Hp (Fig. 3D) decreased (1.2 – fold change) in 3th day of experimental period which was showed in Fig. 3A-E. The +EO group eliminated the increase in all of the gene expressions.



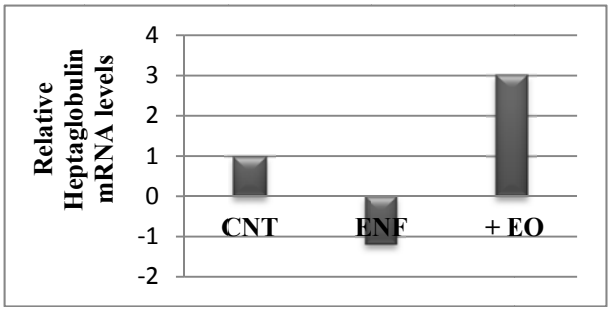
a



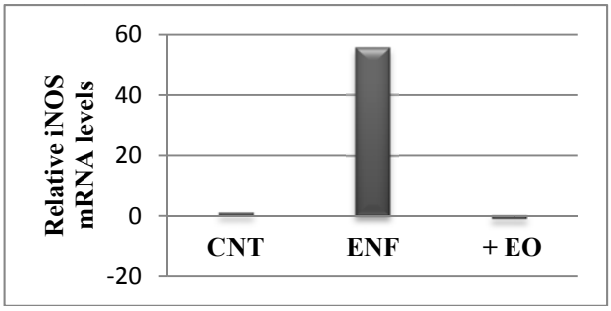
b



c



d



e

Fig. 3. RT-PCR analyses of mRNA levels for TNF-α, IL-1β, SAA, haptoglobulin and iNOS in liver tissues. The results were normalized with the house keep gene β-actin, respectively (A-E) show mRNA levels for TNF-α (A), IL-1β (B), SAA (C), Hp (D) and iNOS (E) were elevated in infected liver tissues treated with thyme and fennel essential oils.

4. Discussion

Infectious diseases are cause of serious fish deaths in both of wild and cultured trout [34]. *Y. ruckeri*, the causative agent of Enteric Red Mouth (ERM) is mainly observed in young rainbow trout. The previous studies show that therapeutic agents as antibiotics and various chemicals have been used against to the disease for many times but nowadays the use of entire plants or plant products, especially *Thymus* and *Fennel* sp essential oil, as therapeutic agents has been well known [4-7-8-9]. It was previously reported that the oil of *T. vulgaris* contained thymol (44.1-58.1 %), p-cymene (9.1-18.9 %) and carvacrol (2.4-4.2 %) [4]. It was determined in the present study that *F. vulgare* (67.99 %) contained benzene, while *T. vulgaris* essential oil contained phenol (40.95 %) in the trace amounts. The difference found in other reports on the antimicrobial activity of oils against bacteria generally varies depending on the source of an oil and strain of bacteria. In a study was reported by El-Adly et al. [35], the oil of *F. vulgare* had the diameter of inhibition zone ranging from 7-20 mm exhibited a very strong antibacterial activity against *Serratia marcescens*. The difference found in other reports on the antimicrobial activity of oils against bacteria generally varies depending on the source of an oil and strain of bacteria.

When *Y. ruckeri* was used to experimentally infect rainbow trout, a low LD_{50} value and short interval between bacterial infection and death of the fish were observed, indicating the severity of the infection. A similar finding has been reported by Rattanachaikunsopon and Phumkhachorn [36], who performed the experimental infection of tilapia (*Oreochromis niloticus*) with *Lactococcus garvieae*. Due to the severity of the disease caused by *L. garvieae* infection, a better strategy for using clove oil to control the disease is to use it as a protective agent rather than a therapeutic agent. In present study, fish diets supplemented with thyme and fennel oil (10 %) were given to fish 7 days before inducing the disease and, after bacterial infection, the diets were continuously given for 21 days. The diets were found to be able to reduce the cumulative mortality rate after challenging the fish with *Y. ruckeri* without any toxic effect on the fish. Similar a result demonstrated that there was a reduction in mortality when feeding goldfish (*Carassius auratus*) with tri-herbal extract supplementation feeds when the fish were challenged with *Aeromonas hydrophila* [37].

The acute phase response is stimulated by the release of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α from macrophages and monocytes at the site of inflammatory lesions or infection [13]. A number of APPs are likely to participate directly in the protection of the host. So, APPs analysis is critical to research on disease resistance. Many studies have indicated the significance of APPs, especially Hp and SAA, as a clinically useful parameter for measuring the occurrence and severity of inflammatory responses [16-19]. Research on rainbow trout has revealed that SAP-like pentraxin expression was reduced in the liver after confinement stress [16]. The results obtained in the study showed less pronounced changes in the SAA concentrations on liver of fish in INF group than those observed in the serum iNOS concentrations but fish in +EO group inhibited the increases completely. Hp gene was observed only in the opposite situation.

Cytokines exhibit both beneficial and pathologic effects on their target cells and are produced by many cell types [38]. Several natural compounds are known for their beneficial properties to some diseases or their derived complications and particularly concerning anti-inflammatory effects. Specially, IL-1β is one of the early response pro-inflammatory cytokines. A trout IL-1β derived peptide has been shown to stimulate macrophages by enhancing phagocytises and bactericidal activity against *A. salmonicida in vitro* [39]. Komatsu et al. [40] demonstrated that rainbow trout could produce several kinds of cytokines in response to bacteria. Ocaña and Reglero [26] studied inflammatory effects of thyme extracts from three different species on cytokine production and gene expression. They reported that TNF-*α*, IL-1B, IL-6, and IL-10 gene expression changes were dose dependent and thyme extracts had anti-inflammatory effects. Awad [23] recorded up/down regulation of IL-1ß, IL-8 and TGF-ß1 cytokines in rainbow trout fed for 2 months with dietary 1 % and 2 % lupin, mango and stinging nettle and challenged with *Aeromonas hydrophila,* in order to measure the expression of cytokines in response to pathogen. The worker reported that up-regulation of genes in the group fed with 1 % and both lupin and stinging nettle stimulated the expression of IL-1ß, IL-8 and TGF-ß1 in head kidney. Results of our study showed that mRNA transcription levels of TNF-α, IL-1β, SAA and iNOS on liver of fish in INF group

were greatly enhanced following the challenge with *Y. ruckeri,* only Hp except. Fish in + EO group fed the combination essential oils almost completely inhibited the enhancing the mRNA transcription of infected fish at 72h. Our results were in agreement with the ones regarding the expression of cytokines genes. However, the differences in the gene expression levels observed in the organs are to be expected in view of subtle functional and structural difference among organs.

In conclusion, it was examined antibacterial and therapeutic effects of thyme and fennel essential oils on gene expressions in liver of infected rainbow trout in this study. According to results of this experimental research, some diet supplements like thyme and fennel oils could be natural defenders against the infections depending on inflammation in liver tissue of fish. Nevertheless, more detailed studies using diet supplement feeding against infected fish are needed, especially on gene transcription levels.

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