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Immunomodulatory Role of Dietary Thyme against *Saprolegnia parasitica* Infection in Cultured Nile tilapia (*Oreochromis niloticus*)

Amal A. Al-Said[1*], Rehab A. Abed-elaziz[2], Asmaa M. Badawy[3]

[1]Mycology Department, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC).P.O. Box 12618, Gizza, Egypt.

[2]Fish Diseases Department, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC).P.O. Box 12618, Gizza, Egypt.

[3]Immunology Department, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC).P.O. Box 12618, Gizza, Egypt.

***Correspondence**
Corresponding author: Amal A. Al-Said
E-mail address: aml.gamal1972@gmail.com

Abstract

The fish industry has grown considerably worldwide, and fungal infections represent a significant aspect that increases economic losses and challenges through high mortality rates. The Egyptian aquaculture industry is particularly vulnerable to *Saprolegnia parasitica*, a deadly fish pathogen. Using phytobiotics as immunomodulators, antioxidants, and health promoters in aquaculture have been proven recently as an alternative strategy for banned malachite green. This research aimed to examine the thyme effect (*Thymus vulgaris*) on the immune status of cultured Nile tilapia against Saprolegniasis. A total of 50 fish (*Oreochromis niloticus*) with skin lesions were gathered from a private fish farm in Alexandria, Egypt. Skin swabs, gill swabs, and muscle tissue were obtained from each fish. After the mycological examination, results revealed that 35 isolates out of 150 examined samples (23.33%) were positive for fungal growth, of which 15 (10%) isolates were identified as *Saprolegnia* species. In addition, other fungi were detected; 5 (3.33%), 8 (5.33%), 4 (2.67%), and 3 (2%) isolates were identified as *Penicillium* species, *Aspergillus flavus*, *Alternaria* species, and *Fusarium* species, respectively. To evaluate the immunomodulatory effect of thyme, 300 healthy Nile tilapia fish with a mean weight of 30.0±5.0 g were brought to be experimentally designed; they were distributed into four groups (with three replicates) and were fed on an experimental diet including 0.0, 0.5, 1.0, and 1.5 thyme oil (gm/100gm diet) continuously for two months. Then fish were infected with *S. parasitica* zoospores, which were thoroughly mixed with their diet. According to results, after two months of feeding, catalase (CAT), serum lysozyme activity, and total protein dramatically increased according to the levels of thyme added; the acquisition was for the group fed on a 1.5 g/100g diet. Additionally, the expression of interleukin-10 (IL-10) and interleukin-1β (IL-1β) in liver tissues increased similarly. Hence, it is concluded that employing thyme would improve the well-being and yield of the farmed Nile tilapia.

KEYWORDS
Nile tilapia, *S. parasitica*, Thyme, Immunity, IL-10, IL-1β

INTRODUCTION

People in many countries rely heavily on freshwater fish as a source of protein (Hussain *et al*., 2011). However, freshwater and marine fish populations have drastically declined, and fish farming has risen globally during the past ten years (Iqbal *et al*., 2012); as a result, fish culture has become a critical industry commercially worldwide. One of the primary causes of financial losses in the ornamental and food fish farming businesses is fungal infections (Torto-Alalibo *et al*., 2005; Ali, 2009). Saprolegniasis is one of the significant infections leading to considerable economic losses and high mortalities in freshwater fish. Moreover, it affects fish eggs, influencing high mortality within the hatching stage (Hussain *et al*., 2013). Saprolegniasis is the leading cause of heavy winter mortalities in northern freshwater fish farms in Egypt (Emara *et al*., 2020), including tilapia, considered the most widely cultivated fish in Egypt and the second worldwide after carp (Zahran *et al*., 2017). *Saprolegnia* species are fungi-like organisms that fall within the oomycetes classification (Osman *et al*., 2008). Oomycetes represent a typical saprophytic opportunist, infecting injured, infected, or stressed fish; however, they have been reported in numerous reports as primary infection agents in fish (Roberts, 2012). *Saprolegnia parasitica* is linked to losses in various fish species, such as Nile tilapia (*Oreochromis niloticus*), where the mold is responsible for more than 95% of accumulative mortalities under experimental conditions (Ali *et al*., 2019). *S. parasitica* deaths are more common in fish farms than in natural settings because the farmed animals are frequently subjected to ongoing stress and a variety of contaminants, which ultimately raise the spread and risk of the infection.

In most cases, Saprolegniasis is a white, cotton-like lesion on the head or dorsal fin that spreads to cover the entire body over time before turning red, brown, or green. In untreated cases, *Saprolegnia* infection leads to death by osmoregulatory failure (Rezinciuc *et al*., 2018). The development of Saprolegniasis is significantly influenced by temperature; the majority of *Saprolegnia*-related fatalities are associated with the late autumn, winter, and early spring seasons, where outbreaks frequently occur at lower water temperatures (Kumar *et al*., 2020). Moreover, other stress factors, such as water quality, handling, or crowding, are recurrently accompanied by outbreaks of Saprolegniasis (Ali *et al*., 2013).

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Multiple virulence factors are employed by *S. parasitica*, with hyphae deploying virulence factors against a fish cell. In addition, *Saprolegnia* spores secrete adhesive materials that can bind to lectins (Almeida *et al*., 2009). The transfer of effector proteins into infected cells causes the oomycete infection. *S. parasitica* is a pathogen with long-haired hook bundles that are rapidly generated within the secondary cyst phase. These bundles may act as a possible pathogenic factor by enhancing the ability of the host epidermis cells to adhere to the extracellular matrix and proteins (thrombospondin and fibronectin), resulting in tissue injury and dehydration due to the release of bodily fluids and poisonous substances (Wawra *et al*., 2012; Rezinciuc *et al*., 2018).

Infections are often associated with immunosuppression (Shehata and Abdel-Hakim, 2016), and there is no proven therapy for this infection. The routine application of disinfectants is commonly followed, including anti-*Saprolegnia* agents such as malachite green and formalin, which have recently been banned in some countries (Madrid *et al*., 2015). Therefore, previous studies recommended utilizing non-synthetic, safe alternative treatments, such as feed additives, that are required to improve the immune response, improve growth, and enhance resistance to certain diseases (Ali *et al*., 2014; Heikkinen *et al*., 2016).

One of the crucial issues in aquaculture is the effective control of *S. parasitica*. Therefore, it is suggested to employ a variety of plant extracts while treating aquaculture. Due to its variant bioactivities, including antioxidant, antibacterial, antiprotozoal, antifungal, and antiviral properties, thyme (*Thymus vulgaris*), an aromatic plant in the *Lamiaceae* family, has been extensively utilized in traditional medicine (ALsafah and Faragi, 2017). In addition, 4-allylphenol, carvacrol (15%), eugenol, cymene, and thymol (40%) are particularly abundant in thyme, reflecting its strong antioxidant effect (Sönmez *et al*., 2015).

Thus, the present study aimed to isolate and identify *Saprolegnia* species from infected Nile tilapia (*Oreochromis niloticus*) and assess the effect of thyme (*Thymus vulgaris*) on oxidative biomarkers, transcription of immune-related genes and antifungal activity of cultured Nile tilapia (*Oreochromis niloticus*) by improving its immune status against Saprolegniasis.

MATERIALS AND METHODS

Study design and fish sampling

A total number of 50 Nile tilapia fish (*Oreochromis niloticus*) having a mean weight of 30.0±5.0 g were obtained from a private fish farm in Alexandria, Egypt, suffered from gray, white skin lesions, detached scales from the body surface, and necrosis of fins and membranous part of gills. Samples were transmitted to the Bacteriology Unit in AHRI (Damanhour Provincial Lab) to be examined. A gross examination of dead and diseased live fish was carried out for the presence of lesions and ulceration. To remove surface bacterial pollutants, fish covered in fungal mats resembling cotton wool were washed with double-distilled water.

Mycological examination

Skin lesions, infected gills, and infected muscles of the collected fish were swabbed and immersed in 0.1% sterile peptone water (Hashemi *et al*., 2012). The samples were inoculated and maintained at 25°C for up to 5 days on Sabouraud dextrose agar (SDA) containing chloramphenicol and chlortetracycline (100 mg of each), accompanied by routine daily monitoring for any anticipated fungal growth. A small amount of *Saprolegnia* species hyphae was sub-cultured on new plates of SDA media for purification. The fungal isolates were placed on slides with coverslips in a lactophenol cotton blue dye solution and microscopically inspected for vegetative bodies and spores. Fungal isolates were characterized on the basis of colonial features, pigment production, and the micro-morphology of the spores produced (Ellis *et al*., 2007; Pitt and Hocking, 2009).

Thyme extract preparation

A commercial product of thyme oil (Sigma-Aldrich, USA) was used to be incorporated into the experimental feed in the form of pellets (Dorojan *et al*., 2015). Four treatments (T2= 0.5, T3 = 1.0, and T4= 1.5 g/100 g diet) were used according to the thyme oil level. The control treatment (T1) did not get a thyme supplement. The pellets have been put in the air to dry well (Table 1).

Table 1. Ingredients and proximate chemical composition of experimental diet (g/kg on dry weight basis).

Ingredients	g/kg
Soybean meal (45% CP)	500
Fish meal (70.0% CP)	90
Wheat bran	200
Corn oil	15
Ground corn	100
Corn oil	20
Fish oil	45
Vitamin Premix[1]	15
Mineral Premix[2]	15

Vitamin Premix[1] (/kg in premix): vitamin A 67 IU, vitamin D 16.2 IU, vitamin E 7.4 g, vitamin K3 340 mg, vitamin B1 670 mg, vitamin B2 1000 mg, vitamin B6 800 mg, vitamin B12 1.4 mg, vitamin C 10 g, D-pantothenic acid 2.65 g, folic acid 330 mg, nicotinamide 5.35 g, choline chloride 35 g, biotin 34 mg, inositol 8g.
Mineral Premix[2]: Fe 14 g, Cu 350 mg, Zn 4 g, Mn 1.4 mg, Mg 10 g, Co 30 mg, I 40 mg, Se 35 mg.

Experimental fish rearing and management

A total of 300 Nile tilapia fish (*Oreochromis niloticus*) of 30.0±5.0 g average weight were brought from different farms in Alexandria, Egypt, and transmitted alive in polyethylene plastic bags supplemented with 2/3 air to the Fish Disease Unit in AHRI (Alexandria Provincial Lab). Then, they were acclimatized for 14 days and fed on a control diet with no feed additive supplementation (30% crude protein). After that, fish were put into prepared glass aquaria (100×80×60 cm) containing 100 L of water with continuous aeration with adjusted dissolved oxygen (5 mg/L) and an adjusted temperature of 25°C during the experimental period. Fish were divided into four groups (three replicates) and fed experimental diets containing thyme (0.5, 1.0, and 1.5 g/100 g diet) twice daily for two months. Clean, transparent water was applied all over the experiment, and the water was sampled every two weeks to monitor the quality. The water parameters were within normal limits for temperature (20–25°C), DO (5.4–5.7 mg/L), NH_3 (0.074–0.082 mg/L), and pH (7.4–7.6). The experiment settings were maintained within ranges that supported fish viability (Alagawany *et al*., 2020).

Challenge test

Isolated *Saprolegnia* strains were subcultured on fresh agar plates of SDA media with chloramphenicol and chlortetracycline (100 mg each). The tested *Saprolegnia* strains' zoospores were collected, counted, and adjusted to $1x10^3$ in a Neubauer chamber (ERMA, Tokyo, Japan) (Stueland *et al*., 2005). The challenge

test was performed on each experimental group with *S. parasitica* zoospores, which were thoroughly mixed with a diet and provided twice daily for one month.

For 24 h, fish were deprived of food. Then three fish were obtained from every tank. Blood samples were drawn from caudal veins and were centrifuged at 3000 rpm for 15 min, and a portion of the liver was extracted and preserved in 2 ml RNA later at -80°C in liquid nitrogen. Sampling was carried out before and after infection by *S. parasitica*.

Antioxidant stress markers and non-specific immunity

Lysozyme assay

The lysozyme activity was measured as described by Kumari *et al*. (2006). Lysoplates were prepared by dissolving 1% agarose in 0.06 M PBS (pH 6.3) with 500 mg of LG1Micrococcus lysodeikticus added to 1 L of agarose. The agarose mixture was distributed onto six plates, each with a diameter of 4 to 5 nm. 25 µL of serum samples and standard lysozyme were added to each well. After 18 h, the diameter of the cleared zones was measured, and the lysozyme concentration was estimated.

Serum total protein determination

The method reported by Siwicki and Anderson (2000) was employed to determine the serum total protein concentration colorimetrically, using a commercial kit supplied by Spectrum, Cairo, Egypt.

Determination of liver tissue catalase (CAT) activity

The technique developed by Yarahmadi *et al*. (2016) was applied to measure liver catalase activity.

Gene expression analysis

Fish were randomly selected on day 60 of the feeding trial to analyze immune-related genes, including interleukin-1β (IL-1β) and interleukin-10 (*IL-10*). The primers used were supplied by Metabion (Germany). Table 2 demonstrates the cycling conditions, primer sequences, amplicon sizes, and target genes for SYBR-Green RT-PCR.

RNA extraction

RNA was extracted from tissue samples using the QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH) by adding 30 mg of the tissue sample to 600 µl of RLT buffer containing ten µl of mercaptoethanol per ml. Tubes were inserted into adapter sets and clamps of the Qiagen tissue lyser to homogenize samples. For 2 min, high-speed (30 Hz) shaking steps were used to cause disruption. Finally, the cleaned lysate was mixed with one volume of 70% ethanol. The processes were carried out under the QIAamp RNeasy Mini Kit Purification of Total RNA from Animal Tissues Protocol (Qiagen, Germany, GmbH).

SYBR-Green RT-PCR

Primers were used in a 25 µl reaction, including 3 µl of the RNA template, 0.25 µl of RevertAid Reverse Transcriptase (200 U/µL), 8.25 µl of water, 0.5 µl of each primer at a concentration of 20 pmol, and 12.5 µl of the 2x QuantiTect SYBR-Green PCR Master Mix (Qiagen, Germany, GmbH). The reaction was performed utilizing a Stratagene MX3005P real-time PCR apparatus (RT-PCR).

SYBR-Green RT-PCR analysis

Amplification curves and CT values were calculated by the Stratagene MX3005P. The CT of the positive control group was compared with that of each sample utilizing the "CtΔΔCt" method (Yuan *et al*., 2006), employing the following ratio to determine the variance of gene expression on the RNA of the various samples:

ΔΔCt = ΔCt reference – ΔCt target

ΔCt target = Ct control –Ct treatment and ΔCt reference = Ct control- Ct treatment

ΔΔCt = ΔCt reference – ΔCt target

ΔCt target = Ct control – Ct treatment

Table 2. Primers sequences of the target genes and cycling conditions for SYBR-Green RT-PCR.

Target gene	Primers sequences	Reverse transcription	Primary denaturation	Amplification (40 cycles)			Dissociation curve (1 cycle)			Reference
				Secondary denaturation	Annealing	Extension	Secondary Denaturation	Annealing	Final denaturation	
EF-1α	CCTTCAACGCTCAGGTCATC TGTGGGCAGTGTGGCAATC	50°C 30 min	94°C 15 min	94°C 15 sec	62°C 30 sec	72°C 30 sec	94°C 1 min	62°C 1 min	94°C For 1 min	Gröner *et al.* (2015)
IL-10	CTGCTAGATCAGTCCGTCGAA GCAGAACCGTGTCCAGGTAA				60°C 30 sec			60°C 1 min		Staden *et al.* (2016)
IL-1 β	GCTGGAGAGTGCGTGGAAGAAGATATAG CCTGGAGCATCATGGCGTG				62°C 30 sec			62°C 1 min		Castro *et al.* (2011)

EF-1α: Eukaryotic translation elongation factor 1 alpha; IL-10: Interleukin-10; IL-1 β: interleukin-1β

Statistical analysis

SPSS V20 was utilized to analyze the data statistically. Data were shown as mean ± SE of three replicates. The Bartlett and Kolmogorov-Smirnov tests were applied to test the data for homogeneity of variances and normality of distribution prior to statistical analysis. The Duncan test was used as a post hoc test to analyze mean differences at a 5% probability level. Statistical significance was set at $P < 0.05$.

RESULTS

Incidence of Saprolegnia species

Mycological analysis of 50 inspected *Oreochromis niloticus* skins, gills, and muscles revealed 15 isolates of *Saprolegnia parasitica* (Table 3). The wet preparation of the gills, muscles, and skin revealed masses of immature and mature sporangia having a significant number of sporangia pores, and the hyphae emerged profusely branched and were non-septated, as shown in Figure 1. The positive colonies on SDA began with cysts of long hairs that were initially white and cottony in color before turning gray.

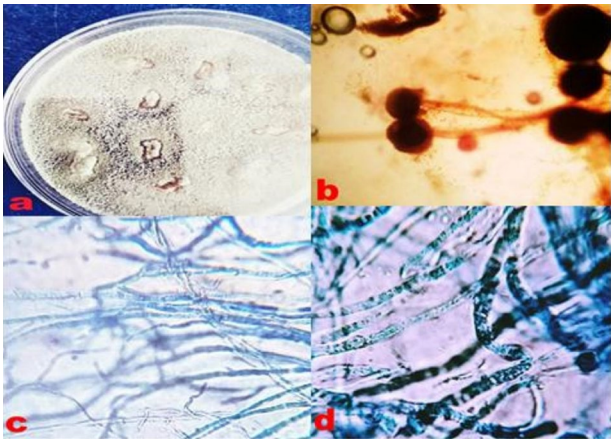

Fig. 1. a: *Saprolegnia* species cultures on SDA started as long hairs with whitish cottony color. b: The wet smear of skin showing masses of mature sporangia. c, d: The hyphae looked profuse, separated and were non-septated, stained with Lacto-phenol cotton blue, 400 X.

Immunity biomarkers

Serum lysozyme (LYZ) activities were influenced by thyme diet supplementation, and lysozyme activity was remarkably raised ($P<0.05$) in the thyme diet concluded group according to dose percentage in comparison with the control group (Figure 2).

Oxidative stress indicators

The serum catalase enzyme (CAT) and total protein activities were remarkably affected by dietary thyme supplementation ($P<$

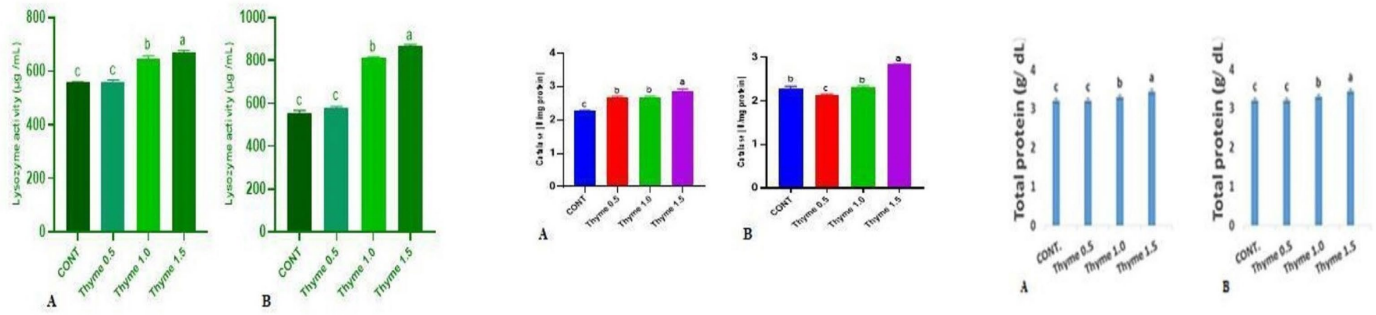

Fig. 2. Immune parameters in Nile Tilapia (*Oreochromis niloticus*) fed on diet with different levels of thyme (*Thymus vulgaris*) for 2 months (A: non-infected cases; B: infected cases). Data are expressed as ± SE. Different letters above bars indicate the significant difference among the treatments ($P < 0.05$).

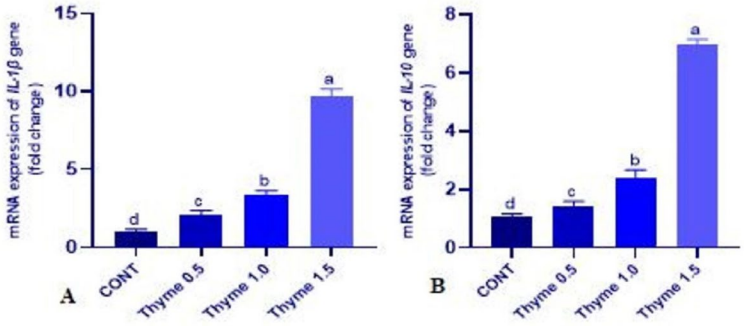

Fig. 3. mRNA levels of interleukin-1β (*IL-1 β*) (A) and Interleukin 10 (*IL-10*) (B) genes in the liver of Tilapia fish fed diets supplemented with thyme oil for 2 months.

Table 3. Incidence of molds in the examined Nile Tilapia fish

Sources of examined samples (50 for each)	*Saprolegnia parasitica* No. (%)	*Penicillium* species No. (%)	*Aspergillus flavus* No. (%)	*Alternaria* No. (%)	*Fusarium* No. (%)
Skin swabs	7 (14)	3 (6)	4 (8)	2 (4)	0 (0)
Gills swabs	5 (10)	1 (2)	3 (6)	2 (4)	1(2)
Muscles tissue	3 (6)	1 (2)	1 (2)	0 (0)	2 (4)
Total (150)	15 (10)	5 (3.33)	8 (5.33)	4 (2.67)	3 (2)

0.05) according to dose percentage in comparison with the control group (Figures 2).

Liver gene expression

Thyme-contained diets remarkably encouraged *IL-10* and *IL-1β* gene expression in all thyme groups (Figure 3), and the top expression levels were observed in the 1.5 g of thyme per 100 g diet, but the lowest levels were in the control group.

DISCUSSION

Tilapia species constitute the significant bulk of the Nile River, with more than 33.0% of the total catches (Gafard, 2015). The aquaculture industry has been considered one of the fastest-growing agribusinesses. The spread of *S. parasitica* infections in Nile tilapia has dramatically increased since malachite green, the most potent anti-*Saprolegnia* chemical, was prohibited in many regions worldwide. Mycological examination of 50 Nile tilapia fish (*Oreochromis niloticus*) with skin lesions revealed that isolated molds belonged to the following genera: *Saprolegnia*, *Penicillium*, *Aspergillus flavus*, *Alternaria*, and *Fusarium*. Similar results were recorded by Ammar (2001), El-Ahl (2010), and Refai *et al*. (2010). The fungal contamination of fish could be attributed to improper sanitation, contaminated feeds, and water supply, and workers' hands influencing the fish's health status (Kumar *et al*., 2020). Our findings about the infection rate of *S. parasitica* in Nile tilapia agreed with that obtained by Phillips *et al*. (2008) and Zahran *et al*. (2017), who described *S. parasitica* as one of the essential mycotic infections leading to economic loss in cultured ecosystems.

Robert *et al*. (2003) revealed that rapid decreases in water temperature impaired the fish's immune system and temporarily suppressed mucus production by goblet cells in the dermal layers of the skin, which acted as a physical barrier preventing fungal spores from contacting the fish skin. Additionally, mucus has antimicrobial elements that can eliminate invasive zoospores, including proteolytic enzymes, complement, lysozyme, C-reactive protein, and immunoglobulin. Without mucus, the skin is exposed, and fungi start growing in numbers and extending their hyphae into the muscular tissue.

One of the most promising strategies for disease management in aquaculture is enhancing fish defense mechanisms through the prophylactic administration of natural plant products. Plant extract is more valuable and eco-friendly, and its application has increased significantly after malachite green treatment was forbidden worldwide. Thyme (*Thymus vulgaris*) is a herb that has been used in traditional medicine for a variety of purposes, including antitussive, antioxidant, antispasmodic, bronchodilator, anti-asthmatic, expectorant, carminative, anthelmintic, antimicrobial, and antiseptic (Ocaña and Reglero, 2012; Alsafah and Al-Faragi, 2017; Soliman *et al*., 2021). Additionally, essential thyme oil has a higher efficacy as an antifungal against *Saprolegnia* species.

Regarding the immunomodulatory effect of *Thymus vulgaris*, the current study confirmed that it had many benefits for Nile tilapia health by increasing the level of Lysozyme, a crucial bactericidal enzyme, with abundance in epithelial secretions. Lysozymes are fish's most essential immunity factors to resist infections (Mirghaed *et al*., 2020). In this study, lysozyme secretion in fish was increased by thyme treatment. According to Farsani *et al*. (2019), the dietary herbal treatment improved lysozyme activity, which aided fish in resisting infections. Several studies have approved this theory, which showed that thyme treatment stimulated fish immune responses through increasing lysozyme secretion (Perez-Sánchez *et al*., 2015; Diler *et al*., 2016; Hoseini and Yousefi, 2019; Zargar *et al*., 2019; Yousefi *et al*., 2022).

In this study, thyme treatment stimulated the immune response by increasing total serum protein, constituting a significant measure of the fish's nutritional condition and overall health (Hoseini and Tarkhani, 2013). To the same extent, Hoseini and Yousefi (2019) and Yousefi *et al*. (2022) proved that thyme had hepatoprotective effects.

Natural antioxidant catalase is one of the most important antioxidant enzymes that protect fish from oxidative damage due to free radicals, nitrogen species, and reactive oxygen (Halliwell and Gutteridge, 2007). The present results showed that thyme administration elevates catalase levels in the same manner that Zheng *et al*. (2009) approved after using thyme to control *S. parasitica* in freshwater fish. Similarly, in rainbow trout, thyme oil administration significantly increased the catalase enzyme (Giannenas *et al*., 2012).

Interleukin (IL) represents a cytokine that promotes inflammation and is crucial for innate immunity. In humans, there are 100 different forms of IL. Recent genetic investigations have revealed that the first cytokine in fish was IL-1 (Wang *et al*., 2009). In mammals, the *IL-1α* and *IL-1β* genes are located on the same chromosome and are close to each other. However, only the *IL-1β* gene has been discovered in fish thus far.

In this study, significant increases in IL-10 and IL-1β were achieved by increasing the concentration of thyme. IL-1β influenced a fish's immune system by enhancing phagocytosis, stimulating the lysozyme activities of macrophages (Hoseini and Yousefi, 2019), and modulating IL-17 family members' expression, which was an essential defense against infections (Kono *et al*., 2011). The current results were in line with Zargar *et al*. (2019) and Yousefi *et al*. (2022), who found that *Thymus vulgaris* essential oil considerably upregulated the degrees to which immune-related genes such as the cluster of differentiation 4 (CD4), the lysozyme gene, complement 3 (C3), and *IL-1β* were expressed. According to previous research, bacterial infection, LPS stimulation, and the administration of immune stimulants could enhance *IL-10* expression (Zhang *et al*., 2009), and this agreed with our finding of a significant increase in IL-10 compared with the control in the case of elevated thyme concentration. IL-10 represented a pleiotropic regulatory and a crucial anti-inflammatory cytokine that regulated the immune response, preventing the severe consequences of inflammation (Moore *et al*., 2001).

In this study, the highest levels of lysozyme (LYZ), total protein activities, and serum catalase enzyme (CAT), as well as a significant increase in *IL-10* and *IL-1β* genes, were at doses of 1.5 g/100 g and 1.0 g/100 g *Thymus vulgaris* diet; similar findings were observed by (Zaki *et al*., 2012).

CONCLUSION

In general, our study suggests that *Thymus vulgaris* has the potential to be used as a healthy control for Nile tilapia through increasing immunity. This reflects their future potential application as preventive measures against winterkill through frequent application in commercial aquaculture, including broodstock and fingerling overwintering.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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