



Effect of *Oreganum (Origanum vulgare L.)* essential oil on some immune parameters of the Nile tilapia (*Oreochromis niloticus*)

Salah M. Aly¹, Heba M. A. Abdelrazek², Nada H. Eidaros³, Samar I. Mostafa⁴,
Samar S. Marzouk⁵, Mai Nashaat⁵, Doaa S. Farid⁶, Noha I. ElBanna^{4*}

¹Pathology Department, College of Veterinary Medicine, Suez Canal Univ., Egypt.

²Physiology Department, Faculty of Veterinary Medicine, Suez Canal University, Ismailia 41522, Egypt.

³Microbiology and Immunology Department, Faculty of Veterinary Medicine, Suez Canal University, Ismailia 41522, Egypt.

⁴Aquaculture Diseases Control Department, Fish Farming & Technology Institute, Suez Canal University, Egypt.

⁵National institute of oceanography and Fisheries (NIOF), Egypt

⁶Environmental protection Department, Faculty of Environmental Agriculture science, Arish University, Egypt.

*Corresponding Author: Noha.ElBanna.fish@suez.edu.eg

ARTICLE INFO

Article History:

Received: Oct. 30, 2022

Accepted: Nov. 7, 2022

Online: Nov. 15, 2022

Keywords:

Oreganum,
Nile Tilapia
Hematology,
Immunity,
Histopathology.

ABSTRACT

This study provided a preliminary framework for the effects of Ropadiar (*Origanum vulgare L.*) on Nile tilapia (*Oreochromis niloticus*) on some immune parameters over a 60-day feeding trial and after bacterial challenge with *Aeromonas hydrophila*. A total of 60 *O. niloticus* 14.5-15.3 g were divided into two groups (10 fish/ aquarium/3 replicate). The first group was fed a basal diet, which served as control, the second group was fed 2% Oreganum oil added to the diet for 60 days, then they were inoculated with *Aeromonas hydrophila*. Hematological parameters and cytokines levels, phagocytic index and nitric oxide level (NO) as well as serum lysozyme activity were determined. Histopathology for the spleen was done. Results revealed significant improvement in hemoglobin (HB), red blood cells (RBCs) and white blood cells (WBCs) count, lysozyme, phagocytic activity, nitric oxide levels and cytokines up-regulation in 2% oreganum oil supplemented group than the control. Histopathology for the spleen was significantly improved in 2% of the oreganum oil-supplemented group compared to the control. The incorporation of Ropadiar 2% in the diet of Nile tilapia is recommended to improve hematological, histopathological and immune parameters in Nile tilapia.

INTRODUCTION

Tilapia is the second most farmed fish worldwide, and its production has multiplied over the past decade because of its relative resistance to diseases, good marketability, and stable prices (Wang and Lu, 2016). The Nile tilapia, *Oreochromis niloticus*, is widely distributed in different freshwater habitats. It constitutes the largest percentage of fish production in Egypt (Soliman and Yacout, 2016). Farmed tilapia production in Africa

has dramatically increased from 644,403 tonnes in 2010 to 1,220,320 tonnes in 2017, with an annual growth rate of 20%. Farmed Tilapia output in Africa was dominated by a single country, Egypt, accounting for 79% of the total production of farmed Tilapia in Africa in 2017 (**El-Sayed, 2019**). Among the commercially important cultured finfish, Nile tilapia (*O. niloticus*) is considered as one of the top-most candidate species for freshwater aquaculture due to its meat quality, market demand, and well-established rearing protocol (**Souza *et al.*, 2019**).

The use of immunostimulants can enhance activity in a non-specific defence mechanism and increase resistance to infectious disease by enhancing innate humoral and cellular defence mechanisms (**Akrami *et al.*, 2013**). According to **Elsheikh *et al.* (2016)**, immunostimulants, which are naturally occurring compounds and effective alternatives for controlling fish and shellfish diseases, modulate pathogens by facilitating the function of phagocytic cells while also stimulating fish natural killer cells (NK), complement, lysozyme, and antibody responses.

Plant-derived products or phytochemicals have been shown to stimulate appetite and promote weight gain in farmed animals, act as immunostimulants and possess potent anti-pathogenic properties in fish. Their potency is mediated by the presence of bioactive molecules including alkaloids, terpenoids, saponins, and flavonoids, which have a great role in modulating immune and physiological responses, as well as promoting optimum health and a microbial community in the gastrointestinal tract of fish (**Caipang, 2020**). **Effendi *et al.* (2022)** stated that some plants have the potential to improve fish immunity, enhance the antimicrobial activity in fish bodies, and could replace the role of antibiotics in fish health management. **Gupta *et al.* (2021)** reported that the application of medicinal plants and essential oils was reported to improve the immunological parameters in many fishes, including lysozyme, phagocytic, and respiratory burst, as well as complement activities, peroxidase, and anti-protease activities. The activity of these medical plants depends on dose, types of medicinal plants, as well as their active compounds and fish size. **Ahmadifar *et al.* (2021)** revealed that essential oils contain high concentrations of polyphenols and natural antioxidants that remove the free radicals that cause lipid peroxidation and immune cell damage. **Sahin *et al.* (2014)** concluded that antioxidants containing essential oils as positive supplements provided a good solution to the impaired growth and the stress responses related to intensive culture conditions as well as the oxidative stress-related immune deficiencies.

Thus, The aim of this study is to investigate the possible immunostimulant effect of dietary oregano essential oil on Nile tilapia. by examining hematological parameters, cytokines levels, phagocytic index, Nitroblue Tetrazolium assay (NBT), nitric oxide level

(NO) as well as serum lysozyme activity both before and after *Aeromonas hydrophila* challenge.

MATERIALS AND METHODS

Diet preparation

According to the NRC (AOAC, 1990), fish fed on commercial floating pellets containing 30% crude protein, 5.5% crude fat, and 3.4% crude fibre met the basic dietary requirements of Nile tilapia. They were fed twice daily at a rate of 3% of body weight, as described by Eurell *et al.* (1978). *Oreganum* essential oil was provided in the form of Ropadiar® 20%, which contained 120.4 g carvacrol and 8.0 g thymol as active components. It was purchased from Ropapharm International BV., India. The experimental basal diet was ground into a fine powder, supplemented with 2% Ropadiar, thoroughly mixed with 350 mL of cold water/kg diet, and pelleted (5 mm diameter) using a pelleting electrical mincer. The pellets were dried at room temperature (26°C) for 24 hours before being stored in plastic containers at 4°C for daily use.

Experimental design

A total of 60 apparently healthy *O. niloticus* with an average body weight of 14.5-15.3 g were obtained from a private fish farm at El-Kantara, Ismailia Governorate, Egypt, and then transferred to the Fish Farming and Technology Institute, Suez Canal University, where the experimental and analytical procedures were performed. Prior to the experiment, fish were acclimatised for 2 weeks in two tanks (1 m³ for each) and were fed a basal diet. After acclimation, fish were divided into two groups and reared in six glass aquaria, each containing 10 fish with triplicate (10 fish/ aquarium/3 replicate) where the first group was fed the basal diet as control (C) and the second group was fed the basal diet supplemented with 2% Ropadiar®. The fish were fed for 60 days, at a rate of 3% of their body weight.

All aquaria were supplied with automatic aerators. Water parameters were maintained throughout the experimental period according to the requirements of *O. niloticus* as follows: (temperature 22 ± 1°C, pH 7.6, dissolved oxygen 7.06 mg/L with photoperiod of 12 h light/12 h dark).

Bacterial culture preparation and challenge trail

A pathogenic reference strain of *A. hydrophila* was obtained from the Animal Health Research Institute and was subcultured on tryptone soya agar (Oxoid, UK) for 24 hours at 28°C before being harvested and suspended in tryptone soya broth (Oxoid, UK) to prepare the bacterial suspension.

At the end of the feeding treatment, fish from the group were anaesthetized with clove oil solution (12.5 mg/L) and intraperitoneally injected with 0.5 mL of the pathogenic *A. hydrophila* suspension that contained 1.5×10⁸ CFU/mL (Carraschi *et al.*, 2012). Fish were monitored daily over 15 days to record any abnormalities.

Sampling

Five fish groups were assigned randomly for sampling at 30 days, 60 days, and 75 days (15 days post infection). The blood was collected from the caudal vein of fish under the effect of clove oil anaesthesia (12.5 mg/L) and divided into three portions. One portion was collected into EDTA tubes for haematological analysis. The second portion was transferred to plain tubes for sera separation, then stored at -80°C in screw-capped vials for immunological studies. The third portion was obtained in lithium heparinized tubes for phagocytic index. After blood sampling, the fish were euthanized by overdose anesthesia. Specimens from the spleen were collected and preserved in 10% formalin for histopathological examination.

Hemogram

RBCs count was done according to **Stoskopf (1993)**. Total and differential white blood cells (WBCs) count and hemoglobin were determined according to Wintrobe (2008). They were performed at 30 days, 60 days, and 15 days post challenge (75 days) of the experimental period, except differential WBCs count was performed after challenge (75 days).

Phagocytic index, lysozyme activity and nitric oxide

Blood samples were obtained from 5 fish/ group using puncture of the caudal vein at day 15 post exposure with a heparinized syringe, 1/4-inch needle. These fish were discarded after use. The phagocytic activity was evaluated using a microscoping counting technique as described by **Ghiasi *et al.* (2010)**. Formalin killed *E. coli* was used. The number of *E. coli* engulfed was determined and a phagocytic index was calculated as the number of bacteria engulfed/number of phagocytes counted.

The lysozyme activity was measured using the turbidity assay. Chicken egg lysozyme (Sigma) was used as a standard, and 0.2 mg/mL of lyophilized *Micrococcus lysodeikticus* in 0.04 M sodium phosphate buffer (pH 5.75) was used as a substrate. Serum (50 mL) samples were added to 2 mL of bacterial suspension and the reduction in the absorbance at 540 nm was determined after 0.5 and 4.5 min of incubation at 22°C . One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 min^{-1} (**Ellis, 1990**). Serum nitric oxide was determined colorimetrically according to **Montgomery (1971)**.

Cytokines

Tumor necrosis factor alpha (TNF alpha), interleukin 1 beta (IL-1 beta), interleukin 10 (IL-10), interleukin 6 (IL-6) and interleukin 2 (IL-2) were measured in serum after bacterial challenge using the ELISA technique using specific ELISA kits. The kits were obtained from MyBio- Source, Inc., San Diego, California, USA. The procedures were followed according to manufacturer instruction.

Histopathological examination

For histopathological investigations, spleen tissue specimens of *Aeromonas hydrophila* infected fish groups were dissected out and fixed in 10% buffered formalin. According to the method described by **Humason (1962)** specimens were processed for paraffin wax embedding, cut into five μm thick sections, and stained with haematoxylin and eosin (H & E). Microscopic examination and photography were done using a light microscope (Carl Zeiss, Germany) equipped with a digital camera.

Statistical analysis

Data of the present study were analyzed using student-T test according to **Snedecor and Cochran (1989)** for testing of significance among the studied groups. Statistical analyses were conducted by SPSS for windows (SPSS version 20) and Statistical Analysis System (SAS Institute 2003) software.

Ethical statement

All experimental procedures were conducted according to the Ethics Committee on Animal Research at Suez Canal University, Egypt. The procedures were approved by the local Administrative Panel on Laboratory Animal Care Committee.; **code:** REC8/10/2022.

RESULTS

Hemogram

The RBCs count and HB values exhibited significant ($P \leq 0.05$) elevation in Ropadiar© group than control after 30 and 60 days of feeding period. Also, after experimental infection the RBCs count HB values showed significant ($P \leq 0.05$) elevation in Ropadiar© group than control. Total leukocytes count (TLC) significantly increased ($P \leq 0.05$) in Ropadiar © group than control after 30 and 60 days of feeding period and after experimental infection (**Table 1**).

Concerning DLC after bacterial challenge with *Aeromonas hydrophila*, the neutrophils % was significantly ($P \leq 0.05$) decreased in Ropadiar © group than the control group. Lymphocytes % exhibited significant ($P \leq 0.05$) increase in Ropadiar groups than the control group. The N/L ratio revealed significant ($P \leq 0.05$) reduction in Ropadiar © group than the control group (**Table 2**).

Phagocytic index, lysozyme activity and nitric oxide

After 30 and 60 days of feeding period as well as after bacterial challenge with *Aeromonas hydrophila*, the phagocytic index and lysozyme activity were significantly ($P \leq 0.05$) increased in Ropadiar© group than the control group. Nitric oxide value showed no significant difference between Ropadiar© group and control one after 30 and 60 days of feeding period. After bacterial challenge with *Aeromonas hydrophila*, nitric oxide

value was significantly ($P \leq 0.05$) increased in Ropadiar© group than the control (**Table 3**).

Table 1. Hematological parameters of Nile tilapia fed oreganum essential oil during feeding period (60 days) and post challenge with *Aeromonas hydrophila* (at 75 days of experiment).

| Parameters | Challenge | Days | Control (C) | Ropadiar ©(R) |
|--|------------------|------|--------------|---------------|
| HB (g/dL) | Before challenge | 30 | 5.98 ± 0.14 | 6.30 ± 0.11* |
| | | 60 | 4.91± 0.19 | 6.59 ± 0.18* |
| | After challenge | 75 | 4.22± 0.25 | 5.23 ± 0.32* |
| RBCs (X10⁶/mm³) | Before challenge | 30 | 1.73 ± 0.04 | 2.61 ± 0.08* |
| | | 60 | 1.58 ± 0.03 | 2.48 ± 0.03* |
| | After challenge | 75 | 1.02± 0.01 | 1.29± 0.04* |
| TLC(/µl) | Before challenge | 30 | 9.83 ± 0.31 | 19.17 ± 0.48* |
| | | 60 | 10.33 ± 0.42 | 17.67 ± 0.71* |
| | After challenge | 75 | 12.01± 0.31 | 20.24± 3.10* |

The data was represented as mean ± SE. superscript *was statistically significant at ($P \leq 0.05$) within the same row.

Table 2. Differential leukocytes count of Nile tilapia fed oreganum essential oil for 60 days then post challenged with *Aeromonas hydrophila* (at 75 days of experiment).

| Parameters | Control (C) | Ropadiar (R) |
|------------------------|-------------|--------------|
| Neutrophils (%) | 38.50±0.43 | 28.83±1.25* |
| Lymphocytes (%) | 53.83±0.75 | 64.17±0.70* |
| Monocytes (%) | 5.00±0.89 | 4.33±0.21 |
| Eosinophils (%) | 1.50±0.22 | 2.00±0.36 |
| Basophils (%) | 0.50±0.22 | 0.33±0.21 |
| N/L ratio | 0.72±0.01 | 0.45±0.02* |

The data was represented as mean ± SE. superscript *was statistically significant at ($P \leq 0.05$) within the same row.

Table 3. Phagocytic index, lysozyme activity and nitric oxide (NO) level of Nile tilapia fed different essential oils and Ropadiar© during feeding period (60 days) and post challenge with *Aeromonas hydrophila* (at 75 days of experiment).

| Parameter | Challenge | Days | Control (C) | Ropadiar(R) | |
|-------------------|---------------------------|------------------|---------------|----------------|--------------|
| Phagocytic index | Before challenge | 30 | 55.21± | 58.87± | |
| | | | 1.65 | 4.21* | |
| | After challenge | 60 | 58.21± | 64.23± | |
| | | | 2.21 | 3.54* | |
| | | 75 | 62.00 ± 2.35 | 70.50 ± 2.45* | |
| | Lysozyme activity (µg/ml) | Before challenge | 30 | 242.51± 3.34 | 243.72± 1.63 |
| 60 | | | 200.13± 0.25 | 228.28± 0.79* | |
| After challenge | | 75 | 173.37± 5.79 | 300.70± 1.41* | |
| | | | | | |
| Nitric oxide (µM) | | Before challenge | 30 | 68.52 ± 7.34 | 66.53 ± 5.82 |
| | | | 60 | 83.29 ± 4.58 | 77.89 ± 4.21 |
| | After challenge | 75 | 110.99 ± 2.92 | 285.25 ± 3.95* | |

The data was represented as mean ± SE. Superscript *was statistically significant at ($P \leq 0.05$) within the same row.

Cytokines level

After bacterial challenge with *Aeromonas hydrophila*, TNF α , IL-1 β , IL-10 and IL-6 levels were significantly ($P \leq 0.05$) increased in Ropadiar© group than the control. However, IL-2 exhibited non-significant variation among the two groups (**Table 4**).

Table 4. Cytokines value of Nile tilapia fed Ropadiar© after challenge with *Aeromonas hydrophila* (at 75 days of experiment).

| Cytokines | Control (C) | Ropadiar (R) |
|----------------------|----------------|-----------------|
| TNF- α (ng/L) | 355.92 ± 7.45 | 415.05 ± 13.68* |
| IL-1 β (ng/L) | 290.92 ± 5.82 | 363.58 ± 14.53* |
| IL-10 (ng/L) | 454.64 ± 27.36 | 750.25 ± 72.11* |
| IL-6 (ng/L) | 99.46 ± 5.12 | 165.67 ± 8.07* |
| IL-2 (pg/mL) | 650.33 ± 28.80 | 693.92 ± 23.35 |

The data was represented as mean ± SE. Superscript *was statistically significant at ($P \leq 0.05$) within the same row.

Histopathological examination:

The normal histological structure of the splenic white and red pulps was noticed by the histopathological analysis of the splenic tissue from the control group. On the other hand, the spleen displayed focal depletion of lymphoid tissue along with substantial activation and proliferation in Melanomacrophage centers, which are dark brown in colour, post infection with *A. hydrophila*. While infected Ropadiar group displayed activation and infiltration of melanomacrophages surrounding the splenic ellipsoids with mild depletion of some lymphoid tissue (**Fig.1**).

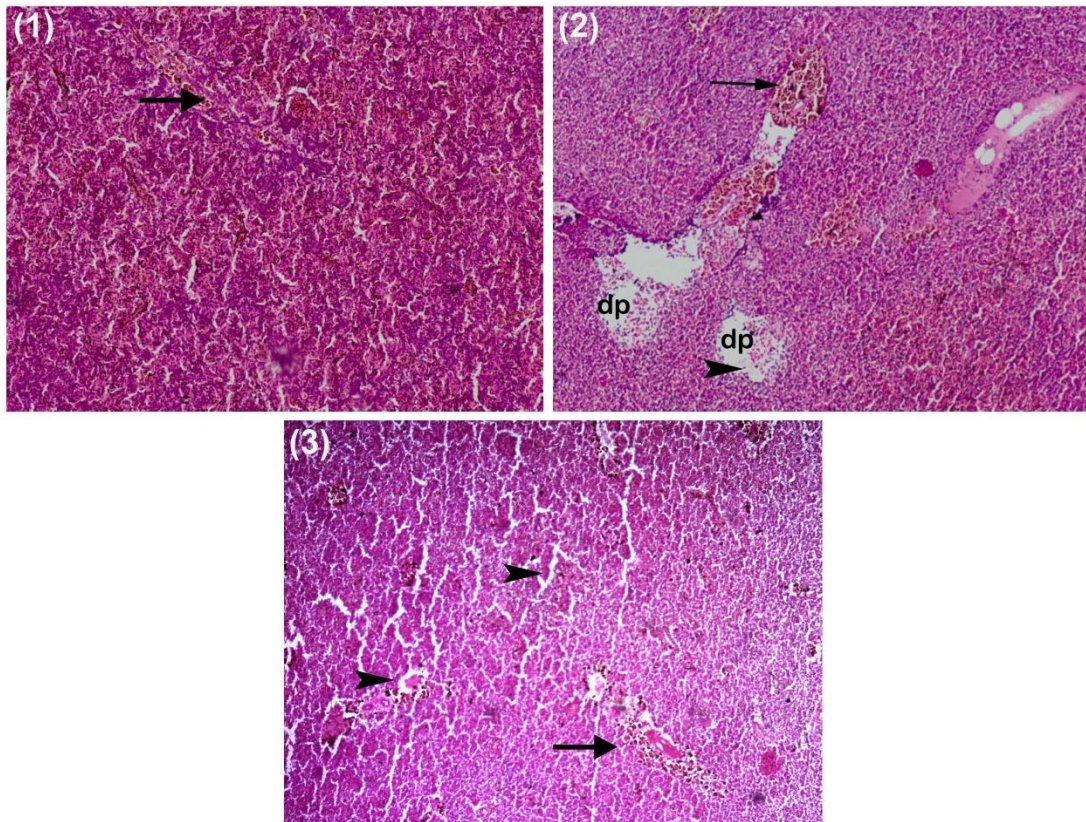


Fig.1. Histopathology of spleens of *O. niloticus* experimented groups post *A. hydrophila* infection. (H&E staining). (1) Reference spleen; (2) spleen of control fish infected with *A. hydrophila*; (3) spleen of Ropadiar group post infection. Melanomacrophages (arrow), focal depletion of lymphoid tissue (arrow head, dp, depletion). Scale bar = 200µm.

DISCUSSION

As the increased incidence of drug-resistant bacteria, antibiotic residues in fish and in the environment pose significant risks; an alternative approach to solve disease problems is the use of immunostimulants, including vitamins and some herbs to enhance the non-specific immunity of fish. It was seen that some farmers had applied herbs for

disease prevention and treatment (**Chitmanat et al., 2016**). Phytogetic compounds can be effective alternatives especially for the prevention of diseases caused by bacteria and parasites in aquatic animals and are also believed to improve feed utilization, growth performance and immune system in aquatic animals (**Bhujel et al., 2017; Sivaram et al., 2004; Ardó et al., 2008; Sahu et al., 2007**).

Hematological parameters are useful stress or disease indicators for fish; and the changes in RBCs count and HB value are important to detect organ health status (**Başusta, 2005**). Therefore, they can determine any abnormalities or promotion owing to the use of immunostimulants (**Talpur and Ikhwanuddin, 2013**). Hemoglobin is a very important iron containing protein which occupies most area of the RBCs. It is the key for blood oxygen (O₂) transport as it can optimize tissue O₂ delivery by increasing the total O₂ that can be transported in the blood (**Rummer and Brauner, 2015**). Therefore, HB is a good indicator of fish O₂ transportation capacity allowing establishing a relationship between the oxygen available in the environment and fish health (**Amrevuawho et al., 2014**). Ropadiar© group revealed a significant elevation in HB value than control group. These results suggested that dietary supplementation of Ropadiar© could improve the performance of the oxygen transport. Similarly, **Ahmadifar et al. (2011)** reported that the HB concentrations in rainbow trout, *Oncorhynchus mykiss* fed thymol- carvacrol supplemented diet were slightly higher than the control. **Yilmaz and Ergün (2015)** found that HB concentration in rainbow trout fed dietary carvacrol were significantly higher in comparison to the control group.

Concerning RBCS count, Ropadiar© group exhibited a significant increase in RBCs count than the control group. This was in agreement with **Mohammadi et al. (2020)** who noted that Nile tilapia (*Oreochromis niloticus*) juveniles fed *Origanum vulgare* extract showed a significant increase in RBCs compared to the control. In contrary, **Cararo et al. (2017)** noted that dietary concentrations of oregano essential oil had no effects on hematology of silver catfish juveniles. The administration of dietary oreganum oil in the form of Ropadiar© seemed to improve the fish welfare and oxygen carrying capacity that were positively reflected on the final body weights of Nile tilapia in the herein study. Moreover, *Oreganum* may play a role in regulation of digestive enzyme and hormones secretion, immune stimulation, synergistic interactions with the gastro-intestinal microbiota, and antibacterial and antioxidant activities. All these effects could result in increased digestibility, nutrients absorption and protein conversion (**Castañeda-Monsalve et al., 2019**).

Total leukocytes count is considered a significant marker on determination of immune response of fish (**De Pedro et al., 2005**). The innate immune system has both cellular and humeral components, through which it carries out its protective function; leukocytes are the major component of the innate immune system at the cellular level (**Vallejos-Vidal et al., 2016, Secombes, 1996**). Our result revealed that Ropadiar © group recorded a significant elevation in TLC compared to the control. Similarly,

Panigrahi *et al.* (2005) reported that WBCs increase could result from improvement of the non-specific immune system and/or enhanced phagocytosis and cytotoxic activity (**Picchietti *et al.*, 2007**). Ropadiar © group showed decreased neutrophil number compared to the control. However, lymphocytes, showed significant elevation in Ropadiar © groups than the control. Similar results obtained by **Youssef (2019)**. Lymphocytosis may be attributed to the antigenic stimulation in Ropadiar © treated groups which enhanced T-lymphocytes by bacterial infection (**Medway *et al.*, 1969**). The reduced N/L ratios in Ropadiar © group may be attributed to the antioxidant effect of oreganum that alleviated stress (**Abdel-Latif and Khalil, 2014**).

Concerning phagocytic index, Ropadiar© group showed the highest phagocytic index value that denoted an immune response enhancement especially non-specific immune response to engulf the pathogenic bacteria. **Magouz *et al.* (2022)** reported that dietary oregano essential oil 0.25, 0.5, and 1 g/kg to Nile tilapia for eight weeks increased phagocytic index. Our results could be attributed to the improvement in innate immunity by the action of active principles in oreganum oil (**Kirimer *et al.*, 1995; Foudah *et al.*, 2022**) involved with high lymphocyte proliferation and phagocytic rates (**Khalil *et al.*, 2020**).

Lysozyme is a humeral component of the non-specific defense mechanism which has the ability to prevent the growth of bacteria by splitting β -1,4 glycosidic bonds in the peptidoglycan of bacterial cell walls, resulting in bacteriolysis (**Ellis, 1999, Magnadóttir, 2006**), in addition to increasing the capacity for activating the complement system (**Ragland and Criss, 2017**) therefore lysozyme has been frequently used as an indicator of non-specific immune infection (**Watts *et al.*, 2001**). In this study, Ropadiar© group significantly increased serum lysozyme activity than the control. on the same line, (**Diler *et al.*, 2017**) recorded a significant increase in serum lysozyme activity in rainbow trout fed *Oreganum onites* essential oil after 60 days. Similar study obtained by **Magouz *et al.* (2022)** reported that dietary oregano essential oil 0.25, 0.5, and 1 g/kg to Nile tilapia for eight weeks increased lysozyme activity improving the immune and antioxidative responses (**Shourbela *et al.*, 2021**). The elevated lysozyme activity could be correlated with enhanced phagocytic activity and serum bactericidal activity (**Jagruthi *et al.*, 2014**). Also, elevation in lysozyme activity may be associated with increase in TLC (**Jagruthi *et al.*, 2014**) as observed in the present study or antibody titer (**Jha *et al.*, 2007**) due to essential oil active ingredients (**Yousefi *et al.*, 2020**).

Nitric oxide is an intracellular mediator produced in various live cells if exposed to stress factors, while the unregulated production of nitric oxide can cause nitrosative stress, leading to damages of proteins/DNA, cell injury and death and its concentration in serum can be used as an inflammatory marker for disease status and progression (**Murphy, 1999**). Several potent oxidizing reactive nitrogen intermediates (such as peroxynitrite) that are produced from the reaction between NO and reactive oxygen intermediates (such as superoxide) can efficiently kill pathogens (**Shiloh and Nathan,**

2000). Inducible NO synthase in macrophages, upon response to cytokines and microbial products, catalyses L-arginine and yields nitric as non-specific immune response (**Clem *et al.*, 1985**). The present study elucidated that Ropadiar© group significantly increased NO production after challenge compared to control. This was in agreement with **El-Hawarry *et al.* (2018)** who observed an increase in NO as the rearing density increased together with the inclusion of oreganum EO. Our results may be attributed to the potential activity of essential oils suggesting the improvement of fish innate immune response activated by inflammatory cytokines such as $\text{IFN}\gamma$, $\text{IL-1}\beta$, and $\text{TNF}\alpha$ that produce NO, a source of free radicals that effectively kill pathogens (**Rashidian *et al.*, 2021**).

Cytokines are critical players in the generation and resolution of inflammation and hence are generally grouped into 2 categories proinflammatory and anti-inflammatory (**Stoycheva and Murdjeva, 2005**). $\text{TNF-}\alpha$ is a fundamental mediator of cell death, differentiation and initiation of inflammation and immune modulation. T Helper cells in turn activate various other type of cells (monocytes, B cells etc.) by releasing various cytokines, as $\text{TNF-}\alpha$, $\text{IL-1}\beta$, IL-2 , which affect the progression of inflammation. In the present study, Ropadiar© exhibited a significant high $\text{TNF-}\alpha$, $\text{IL-1}\beta$, IL-10 and IL-6 and IL-2 up-regulation in Ropadiar© group than the control. This could be attributed to the improvement in innate response against *Aeromonas hydrophila* by the active ingredient in Ropadiar, oreganum oil; carvacrol (**Kucukgul and Gulsafak, 2019**). The current results may be attributed to the immunomodulatory effect of active principles in essential oils on macrophages, natural killer cells and lymphocytes (**Stow *et al.*, 2009**). Cytokines, like $\text{TNF-}\alpha$ and interleukins are used in determination of immune response in macrophage cell-based models, since macrophages as a barrier of the immune system produce those cytokines. Our results suggest the stimulation of pro-inflammatory cytokines which plays an important role in fish disease resistance against bacteria by the action of the active ingredients in essential oils (**Ocaña and Reglero, 2012**). $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ act synergistically to mediate resistance to infections by controlling intracellular pathogen replication (**Haugland *et al.*, 2007**). These cytokines as the key molecules in initiating inflammation and immune response activate T cells, B cells, natural killer cells and phagocytic cells, induction of other cytokines (IL-6 , IL-2 and IL-10) and stimulation of specific adaptive immunity (**Dash *et al.*, 2015**).

Histopathological findings revealed hyperplasia of melanomacrophage centers in spleen. This result came in parallel to the hematological and immunological results in the current study, confirming the improvement in the hematopoietic system and the innate immune response expressed by the up-regulation of $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ proving the role of essential oils as immunostimulants and disease resistance (**Dawood *et al.*, 2022**).

CONCLUSION

The present study revealed that dietary supplementation of 2% oreganum EO in the form of Ropaiar© offered a pronounced immunostimulant effect in Nile tilapia. This was clear from positive impacts on hematological parameters and immune performance especially after *Aeromonas hydrophila* challenge. Therefore, oreganum can be considered as a good candidate to be alternative to the use of antibiotics in feed.

REFERENCES

Abdel-Latif, H. M. and Khalil, R. H. (2014). Evaluation of two phytobiotics, *Spirulina platensis* and *Origanum vulgare* extract on growth, serum antioxidant activities and resistance of Nile tilapia (*Oreochromis niloticus*) to pathogenic *Vibrio alginolyticus*. *Int. J. Fish. Aquat. Stud*, 250: 250-255.

Ahmadifar, E.; Falahatkar, B. and Akrami, R. (2011). Effects of dietary thymol-carvacrol on growth performance, hematological parameters and tissue composition of juvenile rainbow trout, *Oncorhynchus mykiss*. *Journal of Applied Ichthyology*, 27: 1057-1060.

Ahmadifar, E.; Yousefi, M.; Karimi, M.; Fadaei Raieni, R.; Dadar, M.; Yilmaz, S.; Dawood, M. A. and Abdel-Latif, H. M. (2021). Benefits of dietary polyphenols and polyphenol-rich additives to aquatic animal health: an overview. *Reviews in Fisheries Science & Aquaculture*, 29: 478-511.

Akrami, R.; Iri, Y., Rostami, H. K. and Mansour, M. R. (2013). Effect of dietary supplementation of fructooligosaccharide (FOS) on growth performance, survival, lactobacillus bacterial population and hemato-immunological parameters of stellate sturgeon (*Acipenser stellatus*) juvenile. *Fish & shellfish immunology*, 35: 1235-1239.

Amrevuawho, o. M.; akinyemi, a. A.; ezeri, o. G. N.; bankole, o. M. and Takeet, o. V. (2014). Pathological study of *Clarias gariepinus* (Burchell, 1822) sub-adult artificially infected with *Pseudomonas aeruginosa*. *Brazilian Journal of Aquatic Science and Technology*, 18: 65-69.

AOAC, A. (1990). Official methods of analysis of the aoac. International, vol. I.

Ardó, L.; Yin, G.; Xu, P., Váradi, L.; Szigeti, G.; Jeney, Z. and Jeney, G. (2008). Chinese herbs (*Astragalus membranaceus* and *Lonicera japonica*) and boron enhance the non-specific immune response of Nile tilapia (*Oreochromis niloticus*) and resistance against *Aeromonas hydrophila*. *Aquaculture*, 275: 26-33.

Başusta, G. (2005). Fish hematology and hematological techniques. *Research Techniques in Fish Biology* (in Turkish). Nobel Publications, Ankara, 275-300.

Bhujel, R. C.; Poolsawat, L. and Gonçalves, R. A. (2017). Phyto-genic Feed Additive (Pfa) Improved Survival And Growth Of Nile Tilapia (*Oreochromis Niloticus*) Fry During Subsequent Nursing. *Journal of Aquaculture in the Tropics*, 32.

Caipang, C. M. A. (2020). Phytochemicals in aquaculture: a short review of their effects on gut health and microflora in fish. *The Philippine Journal of Fisheries*, 27: 11-22.

Castañeda-Monsalve, V. A.; Junca, H.; García-Bonilla, E.; Montoya-Campuzano, O. I. and Moreno-Herrera, C. X. (2019). Characterization of the gastrointestinal bacterial microbiome of farmed juvenile and adult white Cachama (*Piaractus brachypomus*). *Aquaculture*, 512: 734325.

Carraschi S.P.; Da Cruz C.; Goncedil J.; De Moraes F.R.; Júnior O.D.R.; Neto A.N. and Bortoluzzi N.L. (2012) Evaluation of experimental infection with *Aeromonas hydrophila* in pacu (*Piaractus mesopotamicus*)(Holmberg, 1887). *International Journal of Fisheries and Aquaculture* 4: 81-4.

Chitmanat, C.; Lebel, P.; Whangchai, N.; Promya, J. and Lebel, L. (2016). Tilapia diseases and management in river-based cage aquaculture in northern Thailand. *Journal of Applied Aquaculture*, 28: 9-16.

Clem, L.; Sizemore, R.; Ellsaesser, C. and Miller, N. (1985). Monocytes as accessory cells in fish immune responses. *Developmental & Comparative Immunology*, 9: 803-809.

Dash, P.; Patel, S.; Dixit, A.; Garg, L. and Sahoo, P. (2015). Four pro-inflammatory cytokines of rohu (*Labeo rohita*) during early developmental stages, their tissue distribution and expression by leucocytes upon in-vitro stimulation. *Fish & shellfish immunology*, 47: 913-922.

Dawood, M. A.; El Basuini, M. F.; Yilmaz, S.; Abdel-Latif, H. M.; Alagawany, M.; Kari, Z. A.; Abdul Razab, M. K. A.; Hamid, N. K. A.; Moonmanee, T. and Van Doan, H. (2022). Exploring the Roles of Dietary Herbal Essential Oils in Aquaculture: A Review. *Animals*, 12: 823.

De Pedro, N.; Guijarro, A. I.; López- Patiño, M. A.; Martínez- Álvarez, R. and Delgado, M. J. (2005). Daily and seasonal variations in haematological and blood biochemical parameters in the tench, *Tinca tinca* Linnaeus, 1758. *Aquaculture research*, 36: 1185-1196.

Diler, O.; Gormez, O.; Diler, I. and Metin, S. (2017). Effect of oregano (*Origanum onites* L.) essential oil on growth, lysozyme and antioxidant activity and resistance against *Lactococcus garvieae* in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Aquaculture nutrition*, 23: 844-851.

Effendi, I.; Yoswaty, D.; Syawal, H.; Austin, B.; Lyndon, A. R.; Kurniawan, R.; Wahyuni, S. and Al-Harbi, A. (2022). The Use of Medicinal Herbs in Aquaculture Industry: A Review. *Current Aspects in Pharmaceutical Research and Development* Vol. 7: 7-20.

El-Hawarry, W. N.; Mohamed, R. A. and Ibrahim, S. A. (2018). Collaborating effects of rearing density and oregano oil supplementation on growth, behavioral and stress response of Nile tilapia (*Oreochromis niloticus*). *The Egyptian Journal of Aquatic Research*, 44: 173-178.

El-Sayed, A.F. M. (2019). *Tilapia culture*, Academic Press.

Ellis, A. (1999). Immunity to bacteria in fish. *Fish & shellfish immunology*, 9: 291-308.

Ellis, A. I. (1990). Lysozyme assays. *Techniques in fish immunology*, 1: 101-103.

Elsheikh, M.; Mohamed, A.; Hassanin, A. and Aly, S. (2016). Role of Immunostimulants in improving Mariculture.

Foudah, A. I.; Shakeel, F.; Alqarni, M. H.; Ali, A.; Alshehri, S.; Ghoneim, M. M. and Alam, P. (2022). Determination of Thymol in Commercial Formulation, Essential Oils, Traditional, and Ultrasound-Based Extracts of *Thymus vulgaris* and *Origanum vulgare* Using a Greener HPTLC Approach. *Molecules*, 27: 1164.

Ghiasi, F.; Mirzargar, S.; Badakhshan, H. and Shamsi, S. (2010). Lysozyme in Serum, Leukocyte Count and Phagocytic Index in *Cyprinus carpio* under the Wintering Conditions. *Journal of fisheries and Aquatic Science*, 5: 113-119.

Gupta, N.; Rani Kar, S. and Chakraborty, A. (2021). A Review on Medicinal Plants and Immune Status of Fish. *Egyptian Journal of Aquatic Biology and Fisheries*, 25: 897-912.

Haugland, Ø.; Mercy, I. S.; Romøren, K.; Torgersen, J. and Evensen, Ø. (2007). Differential expression profiles and gene structure of two tumor necrosis factor- α variants in Atlantic salmon (*Salmo salar* L.). *Molecular immunology*, 44: 1652-1663.

Humason, G. L. (1962). Animal tissue techniques. *Animal tissue techniques*.

Jagruthi, C.; Yogeshwari, G.; Anbazahan, S. M.; Mari, L. S. S.; Arockiaraj, J.; Mariappan, P.; Sudhakar, G. R. L.; Balasundaram, C. and Harikrishnan, R. (2014). Effect of dietary astaxanthin against *Aeromonas hydrophila* infection in common carp, *Cyprinus carpio*. *Fish & shellfish immunology*, 41: 674-680.

Jha, A. K.; Pal, A.; Sahu, N.; Kumar, S. and Mukherjee, S. (2007). Haemato-immunological responses to dietary yeast RNA, ω -3 fatty acid and β -carotene in *Catla catla* juveniles. *Fish & shellfish immunology*, 23: 917-927.

Khalil, S. R.; Abd Elhakim, Y.; Abd El-Fattah, A. H.; Farag, M. R., Abd El-Hameed, N. E. and Abd Elhakeem, E.-M. (2020). Dual immunological and oxidative responses in *Oreochromis niloticus* fish exposed to lambda cyhalothrin and concurrently fed with Thyme powder (*Thymus vulgaris* L.): Stress and immune encoding gene expression. *Fish & shellfish immunology*, 100: 208-218.

Kirimer, N.; Başer, K. and Tümen, G. (1995). Carvacrol-rich plants in Turkey. *Chemistry of Natural Compounds*, 31: 37-41.

Kucukgul, A. and Gulsafak, İ. (2019). Bio-functions of carvacrol-supplemented feeds on lipopolysaccharide-induced rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792).

Magnadóttir, B. (2006). Innate immunity of fish (overview). *Fish & shellfish immunology*, 20:137-151.

Magouz, F. I.; Amer, A. A.; Faisal, A.; Sewilam, H.; Aboelenin, S. M. and Dawood, M. A. O. (2022). The effects of dietary oregano essential oil on the growth

performance, intestinal health, immune, and antioxidative responses of Nile tilapia under acute heat stress. *Aquaculture*, 548: 737632.

Medway, W.; Prier, J. E. and Wilkinson, J. S. (1969). A textbook of veterinary clinical pathology. A textbook of veterinary clinical pathology.

Mohammadi, G.; Rafiee, G.; El Basuni, M. F.; Van Doan, H.; Ahmed, H. A.; Dawood, M. A. O. and Abdel-Latif, H. M. R. (2020). Oregano (*Origanum vulgare*), St John's-wort (*Hypericum perforatum*), and lemon balm (*Melissa officinalis*) extracts improved the growth rate, antioxidative, and immunological responses in Nile tilapia (*Oreochromis niloticus*) infected with *Aeromonas hydrophila*. *Aquaculture Reports*, 18: 100445.

Montgomery, H. (1971). The determination of nitrite in water. *Analyst*, 1: 123-130.

Murphy, M. P. (1999). Nitric oxide and cell death. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, 1411: 401-414.

Ocaña, A. and Reglero, G. (2012). Effects of thyme extract oils (from *Thymus vulgaris*, *Thymus zygis*, and *Thymus hyemalis*) on cytokine production and gene expression of oxLDL-stimulated THP-1-macrophages. *Journal of obesity*.

Panigrahi, A.; Kiron, V.; Puangkaew, J.; Kobayashi, T.; Satoh, S. and Sugita, H. (2005). The viability of probiotic bacteria as a factor influencing the immune response in rainbow trout *Oncorhynchus mykiss*. *Aquaculture*, 243: 241-254.

Picchietti, S.; Mazzini, M.; Taddei, A. R.; Renna, R.; Fausto, A. M.; Mulero, V.; Carnevali, O.; Cresci, A. and Abelli, L. (2007). Effects of administration of probiotic strains on GALT of larval gilthead seabream: Immunohistochemical and ultrastructural studies. *Fish & Shellfish Immunology*, 22: 57-67.

Ragland, S. A. and Criss, A. K. (2017). From bacterial killing to immune modulation: Recent insights into the functions of lysozyme. *PLoS pathogens*, 13: e1006512.

Rashidian, G.; Boldaji, J. T.; Rainis, S.; Prokić, M. D. and Faggio, C. (2021). Oregano (*Origanum vulgare*) Extract Enhances Zebrafish (*Danio rerio*) Growth Performance, Serum and Mucus Innate Immune Responses and Resistance against *Aeromonas hydrophila* Challenge. *Animals*, 11: 299.

Rummer, J. L. and Brauner, C. J. (2015). Root Effect Haemoglobins in Fish May Greatly Enhance General Oxygen Delivery Relative to Other Vertebrates. *PLoS One*, 10, e0139477.

Sahin, K.; Yazlak, H.; Orhan, C.; Tuzcu, M.; Akdemir, F. and Sahin, N. (2014). The effect of lycopene on antioxidant status in rainbow trout (*Oncorhynchus mykiss*) reared under high stocking density. *Aquaculture*, 418: 132-138.

Sahu, S.; Das, B. K.; Pradhan, J.; Mohapatra, B.; Mishra, B. and Sarangi, N. (2007). Effect of *Magnifera indica* kernel as a feed additive on immunity and resistance to *Aeromonas hydrophila* in *Labeo rohita* fingerlings. *Fish & shellfish immunology*, 23: 109-118.

Secombes, C. (1996). The nonspecific immune system: cellular defenses. The fish immune system: organism, pathogen and environment, 15: 63-103.

Shiloh, M. U. and Nathan, C. F. (2000). Reactive nitrogen intermediates and the pathogenesis of Salmonella and mycobacteria. Current opinion in microbiology, 3: 35-42.

Shourbela, R. M.; El-Hawarry, W. N.; Elfadadny, M. R. and Dawood, M. A. (2021). Oregano essential oil enhanced the growth performance, immunity, and antioxidative status of Nile tilapia (*Oreochromis niloticus*) reared under intensive systems. Aquaculture, 542: 736868.

Sivaram, V.; Babu, M.; Immanuel, G.; Murugadass, S.; Citarasu, T. and Marian, M. P. (2004). Growth and immune response of juvenile greasy groupers (*Epinephelus tauvina*) fed with herbal antibacterial active principle supplemented diets against *Vibrio harveyi* infections. Aquaculture, 237: 9-20.

Snedecor, G. and Cochran, W. (1989). Statistical methods 8th ed., Ames, IA: Iowa State University Press, 235-236.

Soliman, N. and Yacout, D. (2016). Aquaculture in Egypt: status, constraints and potentials. Aquaculture International, 24.

Souza, C. D. F.; Baldissera, M. D.; Verdi, C. M.; Santos, R. C.; Da Rocha, M. I. U.; Da Veiga, M. L.; Da Silva, A. S. and Baldisserotto, B. (2019). Oxidative stress and antioxidant responses in Nile tilapia *Oreochromis niloticus* experimentally infected by *Providencia rettgeri*. Microbial pathogenesis, 131: 164-169.

Stoskopf, M. (1993). Fish medicine WB Saunders Company. Harcourt Brace Jovanovick Inc. The Curtis centre, independence square, west Philadelphia, Pennsylvania, 19106: 214-216.

Stow, J. L.; Low, P. C.; Offenhäuser, C. and Sangermani, D. (2009). Cytokine secretion in macrophages and other cells: pathways and mediators. Immunobiology, 214: 601-612.

Stoycheva, M. and Murdjeva, M. (2005). Serum levels of interferon- γ , interleukin-12, tumour necrosis factor- α , and interleukin-10, and bacterial clearance in patients with gastroenteric Salmonella infection. Scandinavian journal of infectious diseases, 37: 11-14.

Talpur, A. D. and Ikhwanuddin, M. (2013). *Azadirachta indica* (neem) leaf dietary effects on the immunity response and disease resistance of Asian seabass, *Lates calcarifer* challenged with *Vibrio harveyi*. Fish & shellfish immunology, 34: 254-264.

Vallejos-Vidal, E.; Reyes-López, F.; Teles, M. and Mackenzie, S. (2016). The response of fish to immunostimulant diets. Fish & Shellfish Immunology, 56: 34-69.

Wang, M. and Lu, M. (2016). Tilapia polyculture: a global review. Aquaculture research, 47: 2363-2374.

Watts, M.; Munday, B. and Burke, C. (2001). Immune responses of teleost fish. Australian Veterinary Journal, 79: 570-574.

Wintrobe, M. M. (2008). *Wintrobe's clinical hematology*, Lippincott Williams & Wilkins.

Yilmaz, E. and Ergün, S. (2015). Influence of carvacrol on the growth performance, hematological, non-specific immune and serum biochemistry parameters in rainbow trout (*Oncorhynchus mykiss*). *Food and Nutrition Sciences*, 6: 523.

Yousefi, M.; Shabunin, S. V.; Vatnikov, Y. A.; Kulikov, E. V.; Adineh, H.; Hamidi, M. K. and Hoseini, S. M. (2020). Effects of lavender (*Lavandula angustifolia*) extract inclusion in diet on growth performance, innate immunity, immune-related gene expression, and stress response of common carp, *Cyprinus carpio*. *Aquaculture*, 515: 734588.

Youssef, F. M. A. (2019). Clinicopathological Studies on African Catfish *Clarias gariepinus* Infected with Motile *Aeromonas* Septicemia. *EC Veterinary Science*, 4: 498-510.