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Effect of dietry *Origanum vulgare* extract on Immunological responses of Basa fish (*Pangasius hypophthalmus*) against *Aeromonas hydrophila* infection.

Rehab A. Abd-Elaziz* and Safaa H. Aboolo**

*Fish Diseases Dept., Alexandria Provincial Lab, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Egypt
**Pathology Dept., Damnhour Provincial Lab, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Egypt

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ABSTRACT

Origanum vulgare essential oil (OEO) was added in diets of Basa fish (*Pangasius hypophthalmus*) at levels of 0.0, 5.0, 10.0, 15.0, and 20.0 g /kg feed to determine its effects on antioxidative capacity, immunity, histopathology, expression of immune-related genes and resistance to *Aeromonas hydrophila.* After 2 months of feeding experiment, serum lysozyme, superoxide dismutase and catalase enzymes have been dramatically increased according to the levels of added (OEO) , the highest level was at group fed on 20 gm /kg feed and 15gm/kg feed while, the lowest level at the control group also the expression of interleukin-1beta and toll-like receptor 2 genes in kidney tissues have been increased as the same manner .OEO-supplemented diet pereserves the normal architecture of liver, gills, intestines, and kidneys in all experimental groups. The scores of the histopathological lesions were significantly decreased with presence of regeneration to damaged cells in groups fed 20 gm OEO/kg feed &15 gm OEO/kg feed. After pathogenic *A. hydrophila* challenge the mortality percent after 10 days of the challenge experiment promptly reduced and the percent of survival of Basa fish was relatively increased by OEO administration according to the given dose . Findings show that the OEO in diet has improved the antioxidative status, immunological responses, histopathology, evaluation of immune related genes expression of Basa fish against *A. hydrophila* infection.

INTRODUCTION

Lately, there is shortage in the global food basket, so the aquaculture industry is very important to fill this gap (Newman 2000), thus the application of the intensive aquaculture system in farms is adopted to face the highly needs for the fish and shellfish . Basa fish (*Pangasius hypophthalmus*) is an omnivore fish that feeds on higher plants, zooplankton, algae and insects. It is a member of the *Pangasiidae* family. This fish can live in wide range of environmental conditions and can grow rapidly in high stocking densities **(Jahan et al. 2019).** Basa fish is suitable for markets due to its low price and its good tasty white

Corresponding Author: Rehab A. Abd-Elaziz, Fish Diseases Dept., Alexandria Provincial Lab, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Dokki, Giza, Egypt
E-mail address:
DOI:

flesh ,therefore it can be used as a food source instead of highly prices fish **(Guimarães et al. 2016).** Antibiotics have been utilized for controlling bacterial infections in fish, but the uncontrolled use produce new pathogenic bacterial strains and antibiotic residues in fish flesh **(Romero et al. 2012).** Plant-derivatives, also known as "phytogenics" **(Dügenci et al. 2003)** or "phytobiotics" and essential oils derived from aromatic plants have been widely usedto enhance the fish growth **(Hoseinifar et al. 2020).** Among these natural phytobiotic products there is potent growth-promoting factor called oregano (*Origanum vulgare*). Oregano has potent fragrant and therapeutic benefits as well as its antibacterial action, so it is natural phytobiotic with growth promoting effect **(Oniga et al. 2018).** For many years, the usage of the oregano extract improved the immunity and growth in catfish **(Zheng et al. 2009),** rainbow trout **(Ahmadifar et al. 2011),** Tilapia zillii **(Mabrok and Wahdan 2018).** Moreover, the leaves of oregano plant have been proved to promote the immune system level and antioxidative biomarkers of gilt-head seabream **(Beltrán et al. 2020)** Therefore the goal of the current study is to judge the effect of dietary OEO extract on non-specific immunity, antioxidant level, histopathology, immune-related genes of Basa fish when challenged with pathogenic *Aeromonas hydrophila.*

2. MATERIALS and METHODS

2.1. Essential oil of *Origanum vulgare* and Tested diets preparations

A commercial product of oregano essential oil (OEO) from *O. vulgare* (Ropapharm International BV, Netherlands) known as Ropadiar powder plus®. One kg of this product has about 602.0 g carvacrol (60.2%), calcium carbonate and 40 g thymol as a carrier. The Five experimental treatments diet have been made (Table 1) using several supplementation levels of OEO at 0.0, 5.0, 10.0, 15.0, and 20.0 OEO gm/kg diet. All diets constituents were grounded to be small pellets then well covered with OEO to make the five experimental diets including 0.0, 5.0, 10.0, 15.0, and 20.0 OEO gm/kg diet (Table 1), it has been air dried to be used in the experiment which lasted for 2 months .

Table (1). Ingredients and proximate chemical composition (g/kg on a dry weight basis) of experimental diets of Basa fish containing different levels of oregano essential oil.

Ingredients	Oregano essential oil (g/kg diet)				
	0.0	5.0	10.0	15.0	20.0
Fish meal (72% crude protein)	85	85	85	85	85
Soybean meal (45% crude protein)	400	400	400	400	400
Wheat bran	190	190	190	190	190
Ground corn	230	230	230	230	230
Cod fish oil	30	30	30	30	30
Corn oil	15	15	15	15	15
Vitamins premix1	15	15	15	15	15
Mineral premix2	15	15	15	15	15
Starch	20	20	20	20	20
Oregano essential oil	0	5	10	15	20
Total	1000	1000	1000	1000	1000
Proximate chemical analysis (g/kg)					
Dry matter	922	927	924	925	923
Crude protein	294	294	297	292	294
Ether extract	70	72	73	77	73
Total ash	69	67	63	68	71
Crude fiber	49	48	47	46	45
Nitrogen-free Extract3	518	519	520	517	517
GE (KJ/g diet)4	18.58	18.68	18.81	18.86	18.75

1 Vitamin premix (per kg of premix): thiamine, 2.5 g; riboflavin, 2.5 g; pyridoxine, 2.0 g; inositol, 100.0 g; biotin, 0.3 g; pantothenic acid, 100.0 g; folic acid, 0.75 g; para-aminobenzoic acid, 2.5 g; choline, 200.0 g; nicotinic acid, 10.0 g; cyanocobalamin, 0.005 g; a-tocopherol acetate, 20.1 g; menadione, 2.0 g; retinol palmitate, 100,000 IU; cholecalciferol, 500,000 IU.

2 Mineral premix (g/kg of premix): CaHPO4.2H2O, 727.2; MgCO4.7H2O, 127.5; KCl 50.0; NaCl, 60.0; FeC6H5O7.3H2O, 25.0; ZnCO3, 5.5; MnCl2.4H2O, 2.5; Cu (OAc)2.H2O, 0.785; CoCl3.6H2O, 0.477; CaIO3.6H2O, 0.295; CrCl3.6H2O, 0.128; AlCl3.6H2O, 0.54; Na2SeO3, 0.03

3 Nitrogen-Free Extract (calculated by difference) = 100 – (protein% + lipid% + ash% + fiber%).

4 Gross energy (GE) was calculated from NRC (1993) as 16.7, 37.4, and 16.7 kJ/g for protein, lipids, and carbohydrates, respectively.

2.2. Fish rearing and management

A total of 150 Basa fish (*Pangasius hypophthalmus*) with average weight (12.3± 0.8 g) have been brought from fish farm (Alexandria governorate, Egypt) and transported alive in polyethylene plastic bags supplemented with Oxygen to fish disease unit in AHRI (Alexandria Provincial Lab) .They were acclimatized for 14 days and fed on a control feed (percent of crude protein 30%). After acclimatization fish have been put into prepared glass aquaria (100 × 80 × 60 cm) contain 100 L water with continuous aeration with (dissolved oxygen adjusted 5gm / L) water temp adjusted at 28◦C, during the experimental period. Fish have been split up into 5 groups (with 3 replicates). The experimental diets were applied twice per day continuously for 2 months. Clean transparent dechlorinated water was adjusted throughout the experiment.

2.3. Water quality measurements

Every two weeks water was sampled by 15 cm pipette from each aquarium to asses the water quality. The water parameters were within normal limits as Temp. (27.5 –29.2 °C), DO (5.4 – 5.7 mg/L), NH_3 (0.01 - 0.03 mg/L) and, pH (7.4 –7.6), The parameters during the experiment are kept-up within acceptable ranges for fish viability (Boyd and Tucker 2012).

2.4. Blood and tissues sampling

Before sampling, the fish have been deprived of food for 24 h. Blood has been taken from caudal veins, and serum has been centrifuged (3000 × rpm for 15 minutes) .

A portion of Kidney from three fish in each group was extracted and preserved in 2 ml RNA latter at 80°C liquid nitrogen.

2.5. Antioxidant stress markers and non-specific immunity

Lysozyme activity was evaluated by turbidimetric test (Ellis 1990). Superoxide dismutase (SOD) and catalase activity (CAT) in fish serum was done using (diagnostic kits Biodiagnostics, Giza, Egypt) according to the manufacturer's instructions, using the **Nishikimi et al. (1972) and Aebi (1984)** methodologies.

2.6. Histopathological examination

After the feeding experimental period, (7 fish /group) were necropsied for assemblage of livers, gills, intestines, and kidneys of fish in the control group and OEO-fed groups were gathered. Tissue specimens were processed through the paraffin embedding technique as the methodology described by **Suvarna et al. (2013).**

Dehydration of tissues in ascending grade of ethyl alcohol, cleared by xylene, and finally embedded in paraffin wax to prepare 5 µm thick sections stained with hematoxylin and eosin stain for microscopic examination. Later, several representative photomicrographs were captured with a digital camera (Labomed LC-1 CMOS, Labomed, USA) connected to a microscope (Labomed LB-212).

2.7. Gene expression analysis

At day 60 of the feeding experiment, fish were sampled in a random way for gene expression analysis including interleukin-1 beta (IL-1β) and toll-like receptor 2 (TLR-2) genes were measured. Primers used were purchased from Metabion (Germany). Primer sequences, target genes, amplicons sizes, and cycling conditions for SYBR green rt-PCR were listed in Table 2.

Table (2). Primers sequences, target genes, amplicon sizes 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	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 1 min	(Gröner et al. 2015)
	TGTGGGCAGTGTGGCAATC									
TLR-2	CCCACAATGGATTCACCAG									
	AAAGATCAAGACTCAAGGCACTG									
IL1ß	GCTGGAGAGTGCTGTGGAAGAACATATAG									(Castro et al. 2011)
	CCTGGAGCATCATGGCGTG									

2.7.1. RNA extraction

RNA extraction from tissue samples was done using QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH) when 30 mg of the tissue sample was mixed with 600 µl RLT buffer with ten µl β-mercaptoethanol per 1 mL. For homogenization of samples, tubes were put into the adaptor sets, fixed into the clamps of the Qiagen tissue Lyser. Disruption was performed in 2 minutes high-speed (30 Hz) shaking step. One volume of 70% ethanol was added to the cleared lysate. The steps were ended depending on the Purification of Total RNA from Animal Tissues, protocol of the QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH).
N.B. On column DNase digestion was done to remove residual DNA.

2.7.2. SYBR green RT-PCR

Primers were used in a 25- µl reaction containing 12.5 µl of the2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 0.25 µl of Revert Aid Reverse Transcriptase (200 U/µL) (Thermo Fisher), 0.5 µl of each primer of 20 pmol concentration, 8.25 µl of water, and 3 µl of RNA template. The reply was performed in a Stratagene MX3005P real-time PCR machine.

2.7.3. Analysis of the SYBR green RT-PCR results

The Stratagene MX3005P software determined amplification curves and ct values. To calculate the variation of gene expression on the RNA of the different samples, the CT of each sample was compared with that of the positive control group according to the ΔΔCt method **(Yuan et al. 2006).**

2.8. Challenge test

The bacterial strains were isolated and fully identified using standard modern molecular techniques (RT- PCR) from Basa by the unit of

Bacteriology ,AHRI (Damnhour provincial lab.) ,The LD50 of *Aeromonas hydrophila* for Basa fish was evaluated as the following, fish were IP injected with different doses of 24-h live bacteria, and mortalities have been calculated for 5 days post-infection. LD50 was 1 × 10^7 CFU/ ml. After feeding experiment from each fish group 10 fish were collected per 100-L tank. Then, fish have been injected with 200 µL of phosphate-buffered saline (PBS) containing 1×10^6 CFU of *A. hydrophila*, which IP injected in Basa fish (**Azad et al. 2004).** The cumulative fish mortality curve and their survival rates were recorded after 10 days. Calculating the relative percent of survival (RPS %) according to the following formula by Amend (1981): - RPS % = [1- (% of mortality in experimental group / % of mortality in control group) × 100]

2.9. Data analysis

Results recorded from the experiment were statistically analyzed by usage of one-way ANOVA to determine the influences of OEO on the biological activities of Basa fish. The varieties between means were calculated at a 5% probability level using the Duncan test as a post-hoc test.

3. Results

3.1. Oxidative stress indicators

The serum CAT and SOD enzyme activities were remarkably affected with dietary OEO supplementation ($P < 0.05$) in OEO groups according to dose percent in correlation with the control group (Fig. 1).

3.2. Immunity biomarkers

Serum LYZ activities were influenced by OEO diet supplementation (Fig. 1), LYZ activity was remarkably raised ($P < 0.05$) in OEO diet concluded groups in according to dose percent in comparison with the control group.

Fig.1 (A)

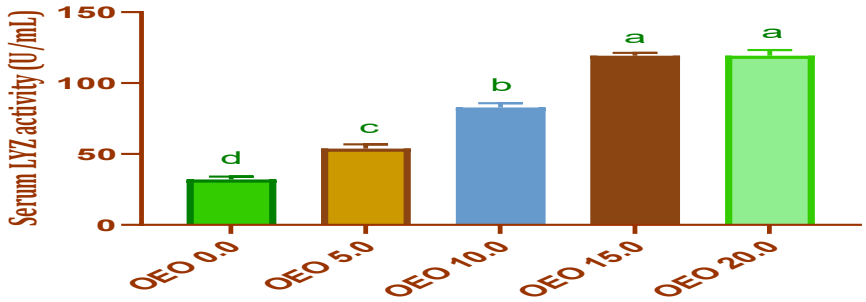

Fig.1 (B)

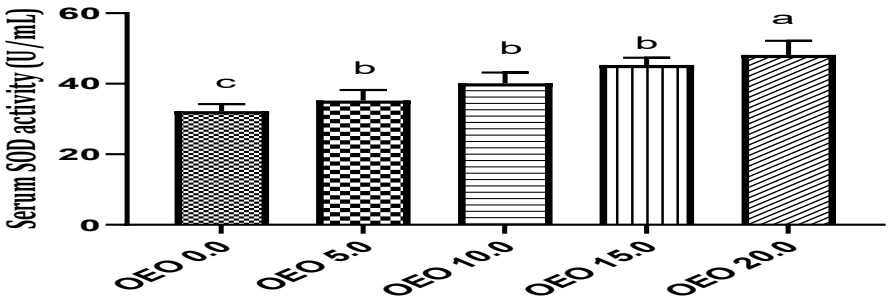

Fig.1 (C)

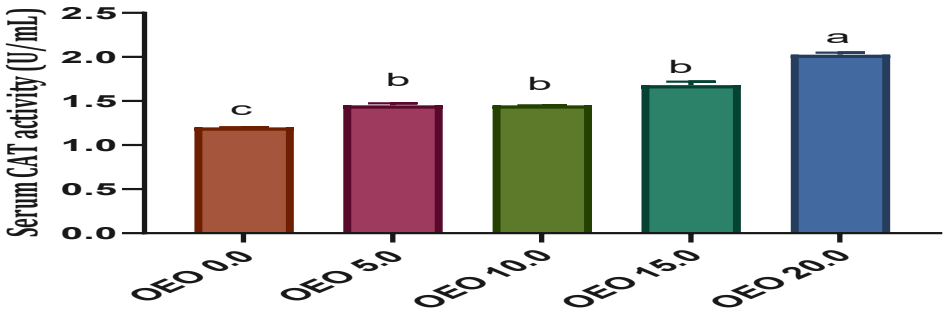

Fig. (1). Serum lysozyme (LYZ) (**Fig. 1A**), superoxide dismutase (SOD) (**Fig. 1B**), and catalase (CAT) (**Fig. 1C**) of Basa fish (*Pangasianodon hypophthalmus*) fed diets supplemented with oregano essential oil for 2 months. Data were expressed as means±SEM. Different letters indicate significant differences at $P < 0.05$.

3.3. Histopathology

In the current study the histopathological examination of gills, liver, kidney and intestine of fish fed diets contain different doses of OEO showed normal architecture and tissues compared to control group of fish that fed basal control diets, while fish groups that experimentally infected with virulent *A. hydrophila* and fed on diets contain different doses of OEO gills, liver, kidney and intestine showed less histopathological changes than fish groups that experimentally infected with virulent *A. hydrophila* and fed on basal control diets. The scores of the histopathological lesions in infected groups with *A. hydrophila* and fed on OEO basal diets were significantly decreased compared to the infected groups with *A. hydrophila* and fed basal control diets (Table 3).

Table (3). Histopathological scoring of Basa fish infected with Ah and fed basal diet compared to Groups of Basa fish fed on OEO (5.0, 10.0, 15.0 and 20.0 g OEO per kg diet) and post-challenged with *Aeromonas hydrophila*

Histopathological findings	Control infected with *A. hydrophila*	Groups of Basa fish fed on OEO and infected with *A. hydrophila*			
		5.0 g OEO per kg diet	10.0 g OEO per kg diet	15.0 g OEO per kg diet	20.0 g OEO per kg diet
Gills					
Ballooning of primary gill lamellae	-	-	+	+	-
Curling of secondary gill lamellae	+	+	+	+	+
Hyperplasia of lamellar epithelium	-	-	-	+	+
Dilatation of central venous sinus	+	-	-	-	-
Congestion of primary lamellae	-	-	-	+	-
Loss of secondary lamellae	-	-	-	+	-
Liver					
Congestion of blood vessels	+	+	+	+	+
Vacuolation of hepatocytic cytoplasm	+	+	+	+	±
Regeneration	-	-	-	-	+
Effusion of blood into tissue	-	-	+	+	-
Kidney					
Vacuolation of epithelium lining of proximal and distal renal tubules	+	++	+	++	-
Necrosis of glomerular corpuscles	-	+	+	-	-
Regeneration	-	-	-	-	+
Intestine					
Hyperplasia of villus epithelium	-	-	+	+	+
Necrosis	+	+	-	-	-
Thickening of muscularis mucosa	-	-	-	-	-
Vacuolation of epithelium lining of intestinal villi	+	+	-	-	-

+ means present, ++ means sever, ± means less, - means absent

3.3.1 Liver:
Livers of Basa fish showed vacuolation of hepatocytic with the normal architecture in groups infected with *Aeromonas hydrophila* and fed basal control diet, while normal hepatopancreatic tissue with congestion of hepatic blood vessels were noticed in groups of fish fed OEO-based diet, vacuolation of hepatocytic cytoplasm & presence of bacteria in the lumen of the blood vessel were demonstrated in fish groups infected with *A. hydrophila* & fed OEO-based diet 5 gm. Vacuolation of hepatocytic cytoplasm with congestion of blood vessel & effusion of blood into hepatic tissue were the major lesion in fish groups infected with *A. hydrophila* & fed OEO -based diet 10 gm & 15 gm, regeneration of hepatic tissue, less vacuolation of hepatocyte and congestion of hepatic vessels were present in fish groups fed OEO-based diet 20gm (Fig.2).

3.3.2Intestine :
Intestinal villi showed presence of *Aeromonas hydrophila* (Ah) in submucosa with vacuolation and sloughing of villus epithelium in fish groups infected with *Aeromonas hydrophila* (Ah) and fed basal control diet ,while intestinal villi showed numerous goblet cells in fish groups fed OEO-based diet. Necrosis and vacuolation of villus mucosa were seen in fish groups fed OEO-based diet 5 gm and infected with Ah, while hyperplasia of intestinal villi with presence of Ah in submucosa were evident in fish groups fed OEO-based diet 10 gm and infected with Ah. Hyperplasia of villus mucosa in fish groups fed OEO-based diet 15 gm and infected with Ah were evident , hyperplasia of villus mucosa with presence of bacteria in the submucosa and thickening of muscularis mucosa were present in fish groups fed OEO-based diet 20 gm and infected with Ah. (Fig.3).

3.3.3 Kidney :
Fish groups infected with *Aeromonas hydrophila* (Ah) and fed basal diet showed vacuolated cytoplasm of lining epithelium of proximal and distal renal tubules with presence of *Aeromonas hydrophila* (Ah) bacteria in the lumen of renal blood vessel, while normal proximal and distal renal tubules, large renal corpuscle were seen in fish groups fed OEO-based diet and infected with Ah . Fish infected with *A. hydrophila* and fed OEO-based diet 5 gm showed sever vacuolation of lining epithelium of proximal and distal renal tubules, wide lumen of distal renal tubules with necrotic renal corpuscles, lining epithelium of proximal and distal renal tubules are vacuolated , some renal corpuscles are shrieked and degenerated were present in fish groups infected with *A. hydrophila* and fed OEO -based diet 10 gm . Fish infected with *A. hydrophila* and fed OEO-based diet 15 gm showed severe vacuolation and degeneration of lining epithelium of proximal and distal renal tubules ,while some renal tubules were regenerated and others degenerated in fish groups infected with *A. hydrophila* and fed OEO-based diet 20 gm (Fig.4).

3.3.4 Gills:
Fish infected with *Aeromonas hydrophila* and fed basal control diet showed dilatation of central venous sinus of primary gill lamellae with curling of secondary lamellae, while fish groups fed OEO–based diet showed normal primary gill lamellae and increased number of secondary gill lamellae. Primary gill lamellae , ballooning dilatation and curling were noticed in fish groups infected with *A. hydrophila* and fed OEO-based diet 5 gm & 10 gm. Fish groups infected with *A. hydrophila* and fed OEO-based diet 15 gm showed congestion of primary gill lamellae ,curling of secondary gill lamellae and hyperplasia of epithelial cells of secondary gill lamellae which led to its fusion and loss of secondary gill lamellae. Fish groups infected with *A. hydrophila* and fed OEO-based diet 20 gm showed hyperplasia of epithelial cells of secondary gill lamellae resulted in its fusion, leukocytic infiltration and curling of secondary gill lamellae (Fig.5).

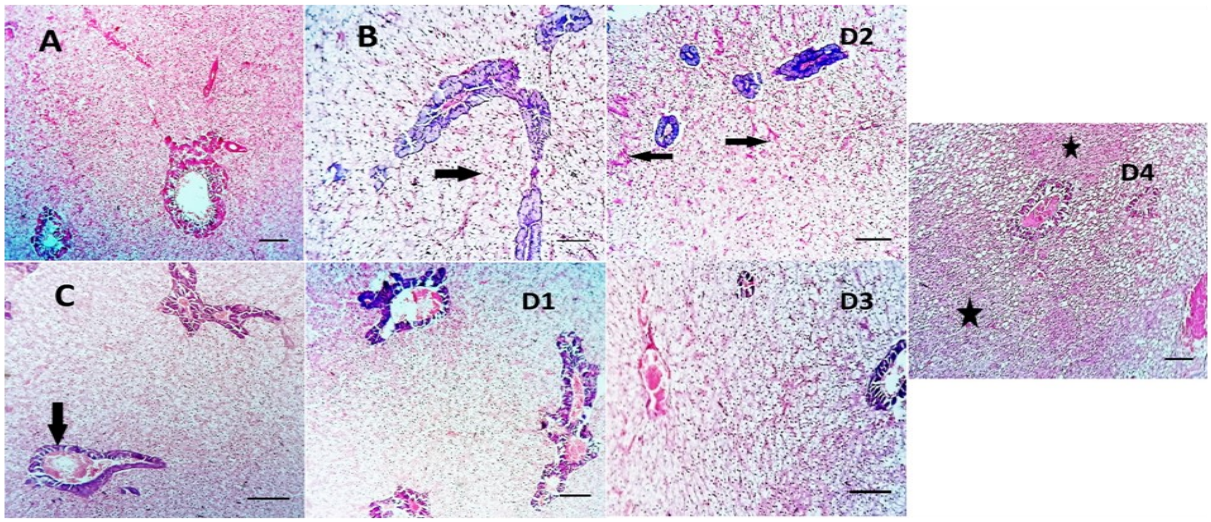

Fig .2 Liver of Basa fish showing normal hepatopancreatic tissue of fish fed basal control diet (A; H & E, X100), vacuolation of hepatocytic cytoplasm (black arrow) with the normal architecture of fish infected with *Aeromonas hydrophila* and fed basal control diet (B; H & E, X250), normal hepatopancreatic tissue with congestion of hepatic blood vessels (black arrow)of fish fed OEO-based diet (C; H & E, X100), vacuolation of hepatocytic cytoplasm & presence of bacteria in the lumen of the blood vessel of fish infected with *A. hydrophila* & fed OEO-based diet 5 gm (D1; H & E, X100), vacuolation of hepatocytic cytoplasm with congestion of blood vessel &effusion of blood into hepatic tissue (black arrows) of fish infected with *A. hydrophila* & fed OEO-based diet 10 gm (D2; H & E, X100), vacuolation of hepatocytic cytoplasm with congestion of hepatic vessels in fish fed OEO-based diet 15 gm (D3; H & E, X100), regeneration of hepatic tissue (stars), less vacuolation of hepatocyte and congestion of hepatic vessels in fish fed OEO-based diet 20 gm (D4; H & E, X100).

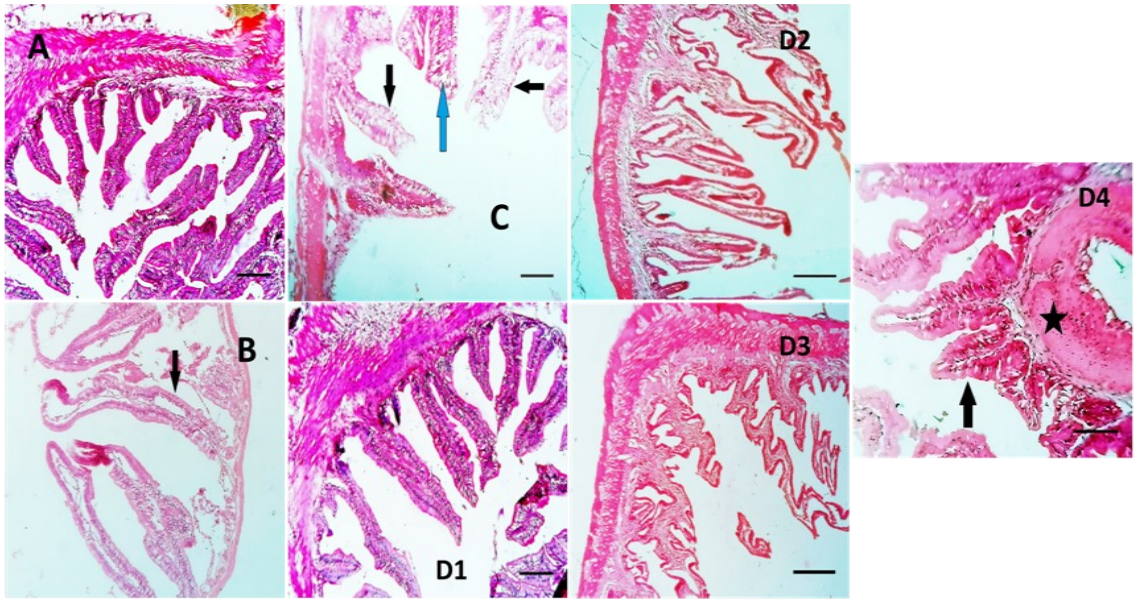

Fig 3 The intestine of Basa fish showing normal intestinal villi, mucosa and submucosa of fish fed basal control diet (A; H & E, X250), intestinal villi showing presence of *Aeromonas hydrophila* (Ah) submucosa with vacuolation and sloughing of villus epithelium (black arrow) of fish fed basal control diet (B; H & E, X400), intestinal villi with numerous goblet cells of fish fed OEO-based diet (C; H & E, X250), necrosis and vacuolation of villus mucosa (black arrows) of fish fed OEO-based diet 5 gm and infected with Ah and numerous goblet cells (blue arrow) (D1; H & E, X250), hyperplasia of intestinal villi with presence of Ah in submucosa of fish fed OEO-based diet 10 gm (D2; H & E, X250), hyperplasia of villus mucosa of fish fed OEO-based diet 15 gm (D3; H & E, X250), hyperplasia of villus mucosa (black arrow) of fish fed OEO-based diet 20 gm with the presence of bacteria in the submucosa and thickening of muscularis mucosa (star) (D4; H & E, X400).

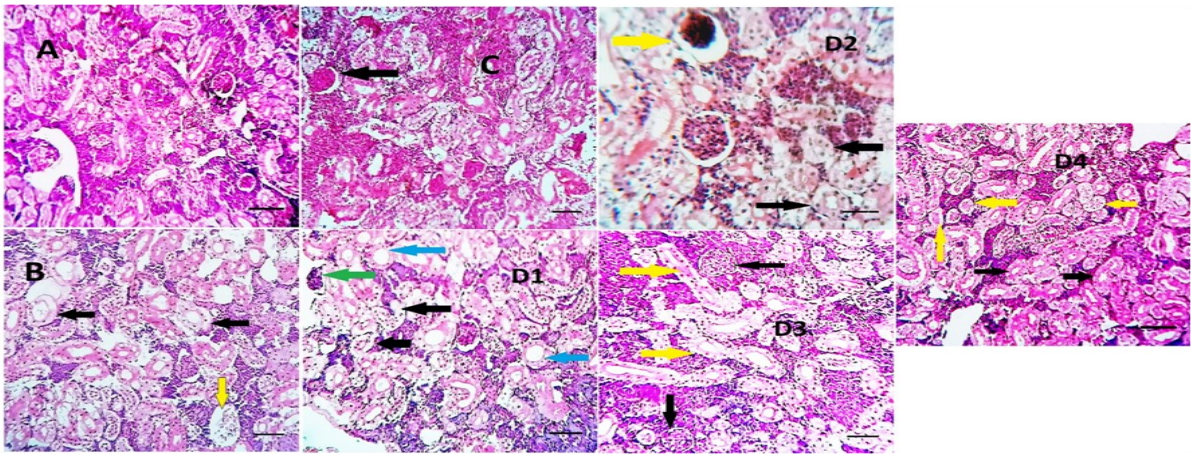

Fig 4. Kidney of Basa fish showing normal renal tubules and renal corpuscles of fish fed basal control diet (A; H & E, X100), vacuolation of cytoplasm of lining epithelium of proximal and distal renal tubules (black arrows), presence of *Aeromonas hydrophila* (Ah) bacteria in the lumen of renal blood vessel (yellow arrow) of fish infected with *Aeromonas hydrophila* (Ah) and fed basal diet (B; H & E, X100), normal proximal and distal renal tubules, large renal corpuscle (black arrow) of fish fed OEO-based diet (C; H & E, X100), sever vacuolation of lining epithelium of proximal and distal renal tubules (black arrows), wide lumen of distal renal tubules (blue arrows) with necrotic renal corpuscles (green arrow) of fish infected with *A. hydrophila* and fed OEO-based diet 5 gm (D1; H & E, X100), lining epithelium of proximal and distal renal tubules are vacuolated (black arrow), some renal corpuscles are shrinked and degenerated (yellow arrow) of fish infected with *A. hydrophila* and fed OEO -based diet 10 gm (D2; H & E, X400), lining epithelium of proximal and distal renal tubules are severely vacuolated and degenerated (yellow arrows), normal renal corpuscles (black arrows) of fish infected with *A. hydrophila* and fed OEO-based diet 15 gm (D3; H & E, X100), some renal tubules was regenerated (black arrows) and others degenerated (yellow arrows) of fish infected with *A. hydrophila* and fed OEO-based diet 20 gm (D4; H & E, X100).

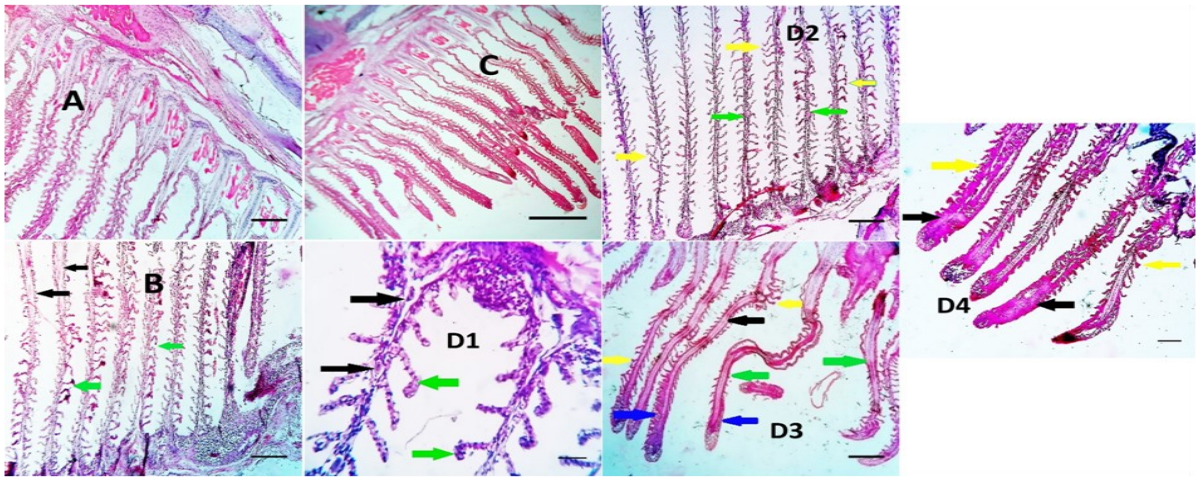

Fig. 5 Gills of Basa fish showing normal primary gill and secondary gill lamellae of fish fed basal control diet (A; H & E, X100), primary gill lamellae showing dilatation of central venous sinus (black arrows) and curling of secondary lamellae (green arrows) of fish infected with *Aeromonas hydrophila* and fed basal control diet (B; H & E, X100), normal primary gill lamellae and increased number of secondary gill lamellae of fish fed OEO–based diet (C; H & E, X100), primary gill lamellae showing ballooning dilatation (black arrow) and curling (green arrows) of fish infected with *A. hydrophila* and fed OEO-based diet 5 gm (D1; H & E, X400), ballooning dilatation (green arrows) and curling of secondary gill lamellae (yellow arrows) of fish infected with *A. hydrophila* and fed OEO-based diet 10 gm (D2; H & E, X250), congestion of primary gill lamellae (black arrow), curling of secondary gill lamellae (yellow arrow) and hyperplasia of epithelial cells of secondary gill lamellae leading to its fusion (blue arrow), loss of secondary gill lamellae (green arrow) of fish infected with *A. hydrophila* and fed OEO-based diet 15 gm (D3; H & E, X250), hyperplasia of epithelial cells of secondary gill lamellae leading to its fusion (black arrows) with leukocytic infiltration and curling of secondary gill lamellae (yellow arrows) of fish infected with *A. hydrophila* and fed OEO-based diet 20 gm (D4; H & E, X 250).

3.4. Kidney gene expressions

OEO concluded diet remarkably encourage IL-1β and TLR-2 genes expression ($P < 0.05$) in all OEO diet groups (Fig. 6), and the top expression levels have been noticed in 15 - 20 gm OEO per kg diet furthermore, the lowest levels in the control group

Fig 6 (A)

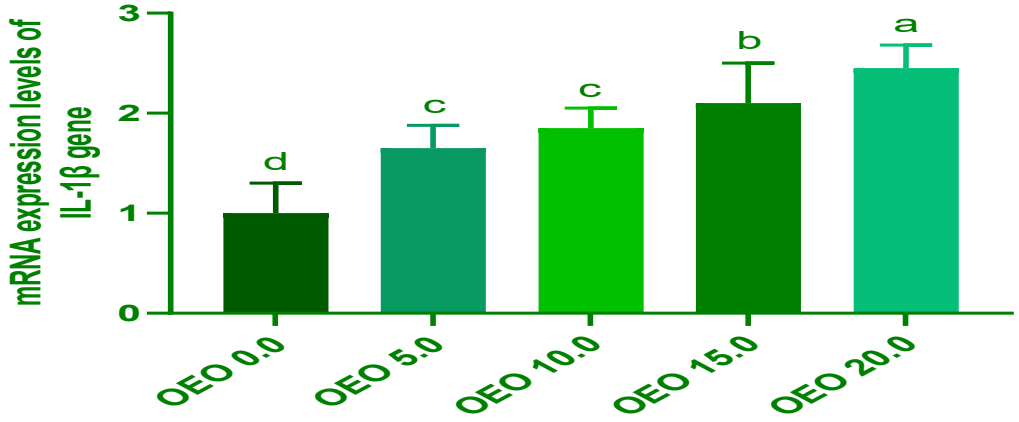

Fig 6 (B)

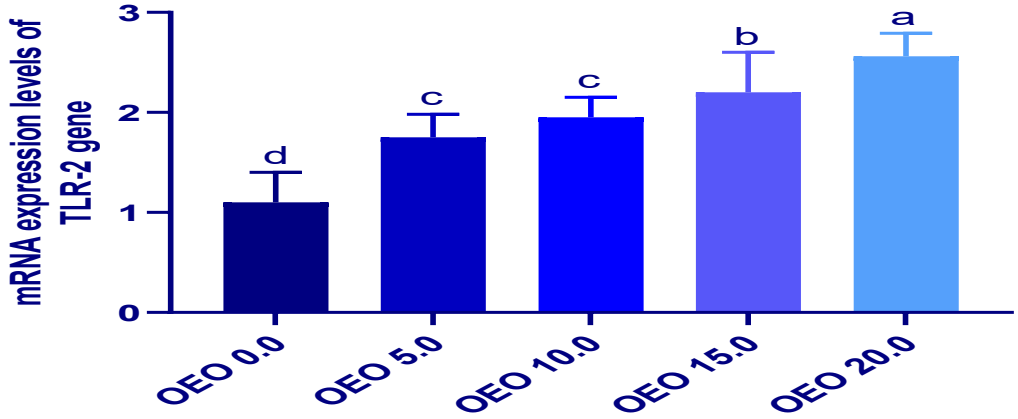

Fig 6 mRNA expression levels of interleukin 1 beta (IL-1β) (**Fig. 6A**) and toll like receptor 2 (TLR-2) (**Fig. 6B**) genes in liver of Basa fish (*Pangasianodon hypophthalmus*) fed diets supplemented with oregano essential oil for 2 months. Data were expressed as means ± SEM. Different letters indicate significant differences at $P < 0.05$.

3.5. RPS and resistance to bacterial challenge

The OEO containing feed leads to a remarkable improvement of the survivability percent of Basa fish after pathogenic *A. hydrophila* challenge. The survivability within 10 days has been elevated ($P < 0.05$) in all OEO fish groups to the control one (Fig. 7). After challenge, the control group mortality percent remarkably increased and sharply decreased in OEO concluded diet fish according to dose. The RPS (Fig. 8) was 85.0, 65.0, 55.0, and 40.0% in 5, 10, 15 and 20 g OEO/kg diet respectively, meanwhile it was 20.0% in the control fish.

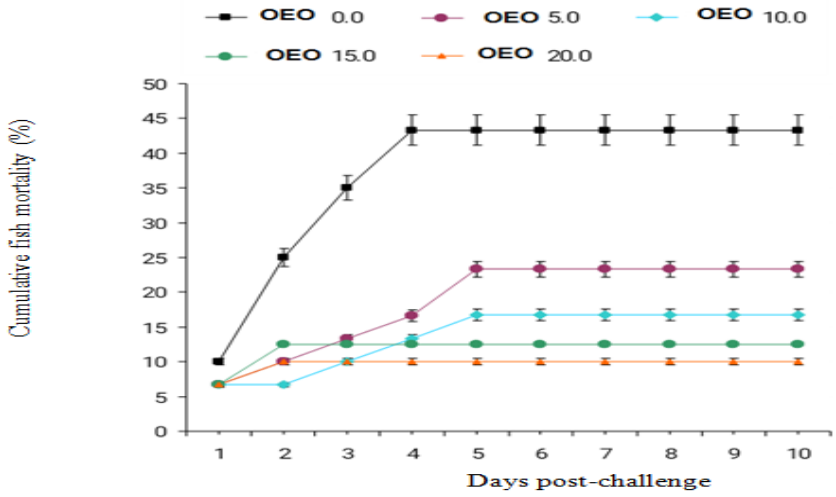

Fig 7 The cumulative mortality rate (%) of Basa fish (*Pangasianodon hypophthalmus*) fed diets supplemented with oregano essential oil for 2 months and post-challenged by *Aeromonas hydrophila* infection and observed for 10 days. Data were expressed as Standerd Error Mean (±SEM). Different letters indicate significant differences at $P < 0.05$.

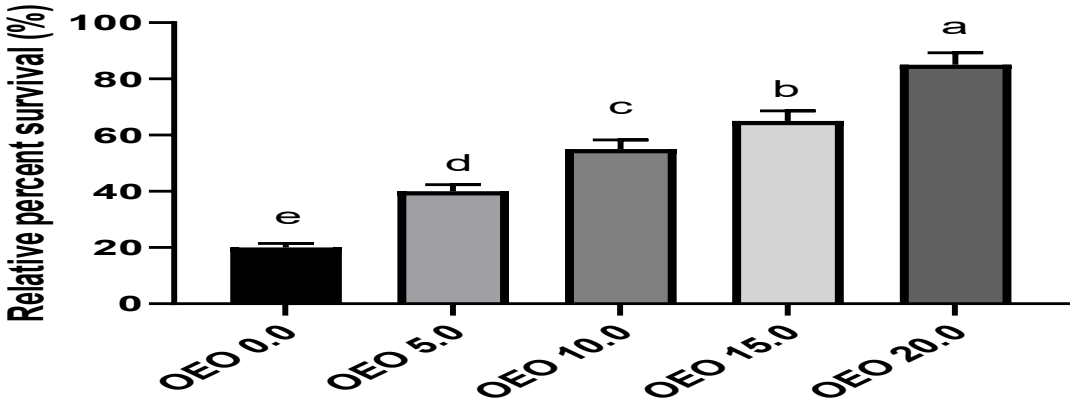

Fig 8 Relative percent of survival (RPS; %) of Basa fish (*Pangasianodon hypophthalmus*) fed diets supplemented with oregano essential oil for 2 months and experimentally challenged with pathogenic *Aeromonas hydrophila*. Data were expressed as Standerd Error Mean (±SEM) . Differenet letters indicate significant differences at $P < 0.05$.

Discussion

The growth enhancing and immunomodulatory benefits of OEO in the experimental diets as been proved against bacterial diseases this agree with **(Ahmadifar et al. 2019).** OEO has proved that it is a viable cross relationship to dietary antibiotics with biological parameters **(Zhang et al. 2009).** Oxidative stress causes oxidative damage on fish health (**Jooyandeh and Aberoumand 2011).** It has been proved that medicinal plants act as perfect way to decrease the oxidative stress in fish **(Adeshina et al. 2019).** The findings show that OEO concluded diet can be beneficial to health of Basa fish via increasing SOD and CAT enzymes as natural antioxidants (**García-Beltrán and Esteban 2016),** in this study the highest result were at doses 15 gm/kg feed &20 gm/kg feed, it was found that feeding OEO diet increased serum antioxidant activity levels, **(Zhang et al. 2020)** indicated a significant increase in serum SOD activities in koi carp after OEO supplemented diet was recorded **(García-Beltrán et al. 2020)** dietary oregano leaves powder has no effect on antioxidant activities in gilt-head seabream. This differ according to fish species, exposure time and the type of oregano used in fish feeds.

4.2. Non-specific immunity

Basa non-specific immune responses have

been improved by including of The OEO in fish diet, via significantly increasing serum LYZ activity, due to the highly beneficial components of OEO as thymol and carvacrol (**Sivropoulou et al. 1996).** Several researchers have proven that dietary OEO improve of non-specific immunity in fish **(Mabrok and Wahdan 2018, Zheng et al. 2009).** As, **Giannenas et al. (2012),** there is increase of LYZ in rainbow trout fed diets supplemented with carvacrol, **(Yilmaz et al. 2015, Ran et al. 2016),** This is differ from **Zheng et al. (2009)** illustrated that channel catfish fed on diets supplemented with carvacrol or thymol has no significant differences in LYZ activities.

4.3. Histopathological studies

The general health status of fish can be evaluated via Histopathology **(Rašković et al. 2013**). According to the Histopathology of hematopoietic tissues of Basa fish in our study, results showed that Histopathological aspect of hematopoietic tissues of the anterior kidneys were normal in all OEO included diet compared to the control one. In contrast to results reported by **Yigit et al. (2017),** who recorded necrosis in kidneys, liver, and spleen in rainbow trout with different doses of essential oil of *O. onites*. In this study thehighest results were at doses 5gm OEO/ kg diet and 15gm OEO/ kg diet.

Liver and kidneys in groups experimentally infected with *A. hydrophila* showed hepatitis and necrosis which occurred with different degrees of intensity in liver of groups that fed on diets contain OEO. Various degrees of intrahepatic pancreatic exocrine cell necrosis are common with gram-negative bacterial infection in catfish (**Abdelhamed et al. 2017).** Kidneys histopathological changes ranged from vacuolation to degeneration of lining epithelium of renal tubules with shrinkage of renal corpuscles in fish groups experimentally infected with *A. hydrophila* and fed on basal control diets while regeneration and healing of kidney tissue took place in fish groups fed on diets contain OEO and experimentally infected with *A. hydrophila*. The liver and kidneys are considered target organs for *A. hydrophila* infections (**Abdelhamed et al. 2017).**

4.4. Expression of immune-related genes

IL-1β and TLR-2 genes are pro-inflammatory cytokines for evaluation of the immune system of fish (Guzmán-Villanueva et al. 2014). In this study the fish fed OEO included diets have been elevated levels of kidney IL-1β and TLR-2 in according to dose, the immunostimulants incorporation in fish diets help increase both IL-1β and TLR-2 (Pionnier et al. 2014 , Wang et al. 2015). The role of cytokines (IL-1β and TLR-2) keeping good histopathological findings of the spleen and anterior kidney tissues of Basa fish .

4.5. Resistance to challenge with *A. hydrophila*

The results cleared that after challenge, the Relative percent of survival has been increasesd **(Zheng et al. 2009)** according to the applied dose, the highest results were at doses 20gm/kg feed and the lowest results were at dose 5 gm/ kg feed proved there is an elevation in survivability percent of koi carp fed OEO included diet after being challenged with *A. hydrophila* **(Rattanachaikunsopon and Phumkhachorn 2010)** prophylactic feeding of Nile tilapia with a diet included 200 ppm carvacrol after challenge with pathogenic *Edwardseilla tarda* resulted in low mortalities, also feeding fish with combination of 200 ppm carvacrol and 200 ppm cymene resulted in no fish mortality.

5. CONCLUSIONS:

In conclusion, feed supplementation with Oregano Essential Oils (OEO) differently affect general health status of Basa fish depending on the dose of OEO in feed. By increasing the dose of the OEO in Basa fish diets (5 gm/kg feed, 10gm/kg feed, 15 gm/kg feed and 20 gm /kg feed) their non-specific immunity, antioxidant capacity were promoted, the expression of immune-related genes were triggered, the resistance of Basa fish to challenge with pathogenic *A. hydrophila* were enhanced. Therefore using of OEO as feed additives improving aquaculture production to compliance with standard food safty regulations and increasing economic income.

Conflict of interest: Authors declare they have no confliction of interest.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of fish have been followed by the authors.

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