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OREGANO LEAF EXTRACT DIETARY ADMINISTRATION MODULATES PERFORMANCE, REDOX STATUS, INTESTINAL HEALTH, AND EXPRESSION OF SOME RELATED GENES OF NILE TILAPIA (*OREOCHROMIS NILOTICUS* L.)

Ahmed F. Fath El-Bab[1], Asem A. Amer[2], Mohamed M. El-Nawsany[3], Ibrahim H. Ibrahim[4], Ali H. Gouda[2], Ahmed A. El-Bahlol[3], Mohammed A.E. Naiel[5]♦

[1]Department of Animal Production, Faculty of Agriculture, Damietta University, Damietta, P.O. Box 34517, Egypt
[2]Department of Fish Nutrition, Sakha Research Unit, Central Laboratory for Aquaculture Research (CLAR) Abbassa, Sharkia, Agriculture Research Center (ARC), P.O. Box 12619, Giza, Egypt
[3]Department of Fish Production, Faculty of Agriculture, Al-Azhar University, Cairo, P.O. Box 11651, Egypt
[4]Department of Fish Genetics and Breeding, Central Laboratory for Aquaculture Research (CLAR), Abbassa, Sharkia, Agriculture Research Center (ARC), Giza, P.O. Box 12619, Egypt
[5]Department of Animal Production, Faculty of Agriculture, Zagazig University, Zagazig, P.O. Box 44519, Egypt
♦Corresponding author: mohammednaiel.1984@gmail.com

Abstract

The main objective of the current trial was to evaluate the beneficial influences of *Origanum vulgare* leaf extract (OVLE) on growth, some blood indices, redox status, and the expression of some growth and immune-related genes. Three thousand seven hundred and eighty Nile tilapia fingerlings were allocated to six equal groups. The first group served as a control and received a basal diet, while the other five groups were fed diets containing graded amounts of OVLE (0.1, 0.2, 0.3, 0.4, and 0.5%, respectively) and defined as $OVLE_{0.1}$, $OVLE_{0.2}$, $OVLE_{0.3}$, $OVLE_{0.4}$, and $OVLE_{0.5}$ for 98 days. Growth performance and feed efficiency parameters were significantly improved in $OVLE_{0.4}$ and $OVLE_{0.5}$ groups compared with the control group. The hematological indices, specifically hemoglobin, red blood cell, and white blood cell count were significantly increased in the fish group fed a 4% OVLE-supplemented diet compared to other groups. Fish fed higher dietary inclusion levels of OVLE significantly increased total protein and albumin concentrations compared to other experimental groups. The $OVLE_{0.4}$ and $OVLE_{0.5}$ supplemented groups promoted innate immune response and phagocytic activity and stimulated the lipase enzyme activity of Nile tilapia. All estimated redox markers were significantly improved in the fish groups that received 4 or 5% OVLE compared with the control and other OVLE groups. The fish groups fed higher levels of OVLE significantly upregulated the expression of *GH*, *IL-8*, and *IL-1β* genes and downregulated the expression of the *Hsp70* gene. Furthermore, the intestinal histological features demonstrated normal structure in all OVLE-administered fish groups, with improved intestinal villus length compared to the untreated group. Finally, it could be concluded that including OVLE in the Nile tilapia diet at higher levels (0.4 up to 0.5%) could improve performance and general health status.

Key words: herbal extract, immune-related genes, blood indices, antioxidative markers, histomorphology, growth performance

Aquaculture is one of the primary food industries that ensures humanity's nutritional requirements all around the globe (Naiel et al., 2022 d). This has resulted in greater attention towards aquaculture sustainability with improved productivity and a desire to endorse highly intensive production systems (Naiel et al., 2022 c) and is a farming activity under constant expansion. The growing expansion of aquaculture led to its intensification, which has been achieved through a reduction in production areas and an increase in fish stocking density (Heluy et al., 2020). The most prevalent disadvantages associated with highly intensive aquaculture systems are increased power needs, poor water quality, stress triggered by overcrowding disease outbreaks (Gharib et al., 2022), weak immune system, and insufficient resistance to pathogens (Shourbela et al., 2021). Thus, successfully reducing the risk of disease breakout requires stress reduction via several different husbandry practices throughout production that may create chronic stress in aquatic animals, leading to an increase in glucocorticoid levels, making the fish more vulnerable to infection (Naiel et al., 2021; Raza et al., 2022). Also, technical experience, management procedures, and feeding practices affect the success of intensive systems. A formulated, balanced aquafeed incorporating functional feed additives are a practical way to sustain intensive systems (Shourbela et al., 2021; Sönmez et al., 2022 a). Since the use of antibiotics was prohibited in many countries, natural feed additives such as herbs, algae, and their derivatives have continued to be of significant interest in aquatic animals owing to their positive benefits on growth as well as the general health status (Abdelghany et al., 2020; Naiel et al., 2020; Negm et al., 2021; Abdel-Latif et al., 2022; Sheikhzadeh et al., 2022; Abd-Elaziz et al., 2023).

Particularly, medicinal herbs and their extracts are commonly employed in farmed fish as effective feed additives (Naiel et al., 2019; Sönmez et al., 2021; Amoush et al., 2022; Sönmez et al., 2022 b; Abdel-Latif et al., 2023). In particular, *Origanum vulgare* L., as a leaf powder or essential oil, is well known for its potency as a therapeutic medication in aquaculture (Khafaga et al., 2020; Özel et al., 2022). *Origanum vulgare* L. leaf extracts (OVLE) with a high amount of carvacrol are being investigated for their potential application in nutrition as well as their influence on the performance and immunity of fish such as zebrafish (Rashidian et al., 2021) and rainbow trout (Haghighi et al., 2018). Aside from their ease of extraction and application, several findings show that when they are incorporated into diets, they may behave as growth promoters, digestion enhancers, anti-inflammatory agents (Ahmadifar et al., 2021 b; Dawood et al., 2022), antibacterials (Pongsak and Parichat, 2010), antioxidants (Zheng et al., 2009) and stimulate immune responses of fish (Alagawany et al., 2020). As previously indicated, the essential oils of *Origanum vulgare* are enriched with a diversity of bioactive components that have efficient biological effects, mainly carvacrol and thymol, which account for around 78 to 82% of the total oil constituents, respectively (Diler et al., 2017; Ahmadifar et al., 2021 a). These compounds are potent for their free radical scavenger properties (Eid et al., 2021), anti-inflammatory characteristics (Harikrishnan et al., 2021), antibacterial, antiparasitic, anesthetic, and antistress features (Rattanavichai and Cheng, 2014), as well as stimulating the innate immune response (Salem et al., 2022), which would finally lead to sustainable aquaculture.

Besides, it has been well established that histological examination of the digestive system is a reliable indicator of fish nutritional status (Filogh et al., 2023). At the same trend, Magouz et al. (2022) showed that Nile tilapia diets enriched with *O. vulgare* essential oil may affect the amount and quality of the intestinal mucosa's secretions and the physical and chemical properties of the overall intestinal environment. Even though essential oils from *O. vulgare* have been extensively studied in several fish species, few researches have examined the influences of its plant extract on aquatic animals. In addition, tilapia are frequently applied in different culture systems because they are easily spawned, can be fed a range of natural and artificial foods, tolerate poor water quality, and grow rapidly at warm temperatures (Naiel et al., 2022 a). To the best of our knowledge, no data are available on the impacts of OVLE on tilapia growth, redox status, and immunity. Also, we believed that the *in-vitro* experiment was insufficient for assessing the biological effects of studied feed additives. Thus, the current research aims to investigate the effect of different dietary OVLE levels in fish feed on *Oreochromis niloticus* growth, efficiency of consumed feed, antioxidant activity, immunological response, and intestinal digestive enzyme activities and histomorphometric features.

Material and methods

Plant extract and diet preparation

Dried oregano leaves were purchased from the Egyptian B2B Ecommerce Platform market (El-Sabaq ST, Roxy, Cairo, Egypt). The ultrasonic-assisted extraction procedure was applied following Rashidian et al. (2021) protocol with small modifications. Briefly, 100 mL of 95% ethanol (Sigma-Aldrich) was applied to dissolve 25 g of the oregano leaves dry powder, and then sonication was performed for 15 min, three times at 30 kHz. The obtained solution was then carefully stirred for 24 h under dark conditions at 50°C and 150 revolutions per min to avoid sedimentation. The resulting solution was then condensed using a rotary evaporator and finally filtered using a Whatman qualitative filter paper (diam. 25 mm, Grade 1 circles), maintained at 4°C until further examinations. According to Oroian et al. (2020) and Sakr et al. (2022) procedures, the extract's total phenolic and flavonoid contents were estimated, and they were found to be 156.12 and 86.75 mg g^{-1} of extract, respectively. The diet components were well mixed before adding 400 ml of water per kg of diet. The homogenous combination (tested additive, water, and components) was mixed to create a mixture of each diet. The blended material was processed via a laboratory pellet machine (average 1–2 mm diameter) during the pelleting process. The obtained wet pellets were air-dried at ambient room temperature. The experimental diets were stored in dark plastic bags until they were administered in the refrigerator at 4°C. The analysis of experimental diet samples was performed following the AOAC (2005) procedure.

Trial conditions

Fingerlings were purchased from a private hatchery (Tollumbat No. 7, Riyad City, Kafr El-Sheikh Governorate, Egypt) and transported in plastic bags containing two-thirds oxygen and one-third water. Fish were acclimated to the laboratory environment and administered a control diet for around 15 days before the feeding trial. The apparent healthy 3780 fingerlings (with initial weights of 5.24±0.06 g) were randomly assigned in triplicate into 18 concrete ponds (3 × 7 × 1 m) at a rate of 210 fingerlings/pond (10 fingerlings/m^3). Fish were administered the tested diets twice daily (08:00 and 15:00) until apparent satiation for 98 days. The experimental groups were as follows: CTR (control group fed un-supplemented diet), while the other experimental groups were offered oregano leaf extract (OVLE) supplemented diets at doses of 0.1, 0.2, 0.3, 0.4, and 0.5%, respectively. Table 1 illustrates the experimental diet formulation and chemical composition.

Parameters of water quality

Water samples were taken biweekly from each pond for chemically assessing total alkalinity and total hardness, as described by Diana et al. (2017), and daily for measuring temperature and dissolved oxygen using

a portable oxygen meter (YSI model 58, Yellow Spring Instrument Co., Yellow Springs, Ohio, USA). The un-ionized ammonia levels were measured using the Multi-parameter Ion Analyzer (HANNA Instruments, Rhodes Island, USA). Also, a pH meter (Digital Mini-pH-Meter, model 55, Fisher Scientific, Denver, USA) was used to assess the pH value. According to Boyd and Tucker (2012), all water quality assessments were within acceptable ranges for fish health and growth. The average water quality parameters of the rearing water were 26±1.00°C, 7±0.5 mg/L, 0.11±0.03 mg/L, 188.17±0.97mg/ L, 226.06±0.49 mg/L and 7.3±0.5 for temperature, dissolved oxygen, un-ionized ammonia total alkalinity, total hardness and pH, respectively, during the whole experimental period.

Growth performance and feed utilization

Initial and final body weights of *O. niloticus* were recorded at the beginning and end of the experiment. Twenty-four hours before the final sampling, all experimental fish were starved to avoid any stress during sampling. The following formulas were employed to estimate all of the growth performance and feed efficiency parameters:

Weight gain (WG, g) = (weight gain / initial weight) × 100

Specific growth rate (SGR%/day) = 100 × (LnW_2− LnW_1) / T

Where: Ln = the natural log; W_2 = final weight at a certain period (g); W_1 = initial weight at the same period (g); T = period per day.

Feed conversion ratio (FCR) = consumed feed (g)/ WG (g).

Protein efficiency ratio (PER) = WG (g)/protein intake (g).

Blood sampling

At the end of the experimental period, blood samples were taken from the caudal vertebral vein of an anesthetized fish according to Duman et al. (2019) procedure. A collected blood sample was divided into two tubes. The first tube contained EDTA as an anticoagulant agent for hematological examination. While the other tube was clean without any anticoagulant and allowed to clot blood sample for 2 h to obtain serum after separation via centrifugation of clotted blood at 4000 rpm for 15 min, then stored at −20°C until further analysis.

Hematological analysis

The leukocytes and erythrocytes were counted according to the method described by Inoue et al. (2002) using a hemocytometer and Natt-Herrik solution. At the same time, the hemoglobin (Hb) concentration was determined with a spectrophotometer (Model RA 1000, Technicon Corporation) at 540 nm by applying Van Kampen and Zijlstra (1983) procedure. The packed cell volume percentage (PCV%) was calculated using the microhematocrit method as described by Jain and Kono (1975). The white blood cell differentiation was assessed according to the method described by Weinstein et al. (1996).

Table 1. Diet formulation and chemical composition of the experimental diets

Ingredients (%)	CTR	$OVLE_{0.1}$	$OVLE_{0.2}$	$OVLE_{0.3}$	$OVLE_{0.4}$	$OVLE_{0.5}$
Fish meal (62% crude protein)	11	11	11	11	11	11
Soybean meal (44% CP)	37	37	37	37	37	37
Wheat bran	13	12.9	12.8	12.7	12.6	12.5
Yellow corn	21	21	21	21	21	21
Corn gluten meal (60% CP)	10	10	10	10	10	10
Fish oil	5	5	5	5	5	5
Vitamin and mineral mix[1]	3	3	3	3	3	3
OVLE	0	0.1	0.2	0.3	0.4	0.5
Sum	100	100	100	100	100	100
Chemical analysis						
Dry matter	90.1	89.8	89.5	89.7	89.1	89.2
Crude protein	30.8	30.76	30.82	30.94	30.98	30.86
Crude lipid	7.3	7.5	7.8	7.9	8.1	8.2
Fiber	5.1	5.2	5.1	5.2	5	5.4
Ash	6.3	6.1	6.4	6.2	6.5	5.1
NFE	50.5	50.44	49.88	49.76	49.42	50.44
GE (MJ/kg)[2]	263.966	266.041	268.804	270.838	272.132	274.043

[1]Providing, per kg of mixture: vitamin E, 5.8 g; vitamin K_3, 3.3 g; thiamin, 3.3 g; riboflavin, 6.6 g; pyridoxine (as pyridoxine hydrochloride), 3.3 g; niacin, 16.6 g; folic acid, 3.3 g; vitamin B_{12} (cyanocobalamin), 0.01 g; D-biotin, 0.1 g; vitamin C (ascorbic acid), 33.3 g, calcium pantothenate, 13.3 g; Cu as copper sulfate, 3 g; I as calcium iodine, 0.4 g; Co as cobalt carbonate, 0.3 g; Mn as manganese sulfate, 10 g; zinc oxide, 30 g; sodium selenite, 0.08 g; calcium, 0.8 g.

[2]Estimations of GE were based on protein, lipid and carbohydrate content analyzed in feed raw materials multiplied by the energy content of the previous: GE = protein × 23.62 kJ/g + lipid × 39.52 kJ/g × carbohydrates × 17.2 kJ/g.

Table 2. Specific primer sequences applied for RT-qPCR analysis

Target gene	Forward 5' → 3'	Reverse 5' →3'	Accession No.
β-actin	CGACGGACAGGTCATCACCA	AGAAGCATTTGCGGTGGACG	AF384096.1
Hsp70	TGGAGTCCTACGCCTTCACA	CAGGTAGCACCAGTGGGCAT	KP645179
GH	CTGGTTGAGTCCTGGGAGTT	CAGGTGGTTAGTCGCATTGG	KT387598.1
IL-1β	AGAGCAGCAATTCAGAGCCT	GTGCTGATGTACCAGTACGT	XM_005457887.3
IL-8	GCACTGCCGCTGCATTAAGA	GCAGTGGGAGTTGGGAAGAA	XM_003447521

Serum biochemistry, antioxidative and immunity measurements

Serum biochemistry was determined colorimetrically according to the manufacturer's instructions using readymade chemicals (kits). Specifically, serum total proteins and albumins were determined following Debro et al. (1957) protocol. In contrast, globulin content was calculated mathematically. Blood digestive enzymes (lipase and amylase), liver function enzymes [aspartate aminotransferase (AST), alanine aminotransferase (ALT)], immunoglobulin M (IgM), total cholesterol, and triglycerides levels were detected by RA-50 Chemistry Analyzer (Bayer) using readymade chemicals (kits) supplied by Spinreact Co. Spain, following the manufacturer's instructions.

The quantification of serum lysozyme was estimated using turbidimetric assay (Fogelson et al., 1954) based on the analysis of the Gram-positive bacterium *Micrococcus lysodeikticus* (Sigma, USA). Whereas, leukocyte phagocytic activity was assessed following Allen (1986) procedure. Briefly, leukocytes that were consumed by bacteria were counted in proportion to the total number of leukocytes found in the smear after the phagocytosis experiment. After that, the phagocytic index and activity were calculated as described by Kawahara et al. (1991).

Superoxide dismutase (SOD), catalase (CAT), glucose, and lipid peroxide (malondialdehyde) (MDA) levels in serum were measured using diagnostic reagent kits following the manufacturer's (Cusabio Biotech Co., Ltd., China) instructions.

Intestinal histology

The intestinal samples were collected randomly from each treatment (3 fish from each pond). The intestine was dissected and directly fixed within Bouin's solution for 24 h according to Suvarna et al. (2013) method. Then, samples were dehydrated and embedded in paraffin wax. The wax blocks were sectioned to six microns; afterward, the sections were stained by hematoxylin and eosin. Thereafter, the examination was performed under light microscopy at a high magnification level (scale bar 100 µm) and using Image J software.

Gene expression

Following the manufacturer's instructions, RNA was separated and extracted from the liver samples for real-time PCR (RT-PCR) analysis using Trizol reagents (iNtRON Biotechnology). 2 µl of RNase was blended with 20 µl of DNA dissolved in Tris-buffer solution (pH = 8.0) and incubated for 3 to 4 hours at 37°C to prevent any RNA contamination. Then, Nanodrop was applied to estimate the RNA absorbance at 260 to 280 nm (Quawell, USA). While mRNA quality has been determined by electrophoresis of whole RNA followed by staining with ethidium bromide. The generated cDNA was diluted 5 to 10 times with nuclease-free water before being kept at –20°C for further analysis. Selected genes, such as interleukin-8 (*IL-8*), interleukin-1β (*IL-1β*), heat shock protein-70 (*Hsp70*), and growth hormone (*GH*), were detected using RT-qPCR. The primers employed in the present study are presented in Table 2. RT-PCR amplifications were performed following the Pereira-Gomez et al. (2021) procedure using SensiFast SYBR Lo-Rox kit (Bioline) in 20 µl reaction mixtures containing 2 µl of cDNA, the gene specific primers (0.5 µM each), and 10 µl of SYBR green as a fluorescent dye and SYBR 10 µl. The conditions for the thermal cycling were initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The estimated genes were performed in triplicate. The fold change for the gene expression of mRNA level was estimated using the $2^{-\Delta\Delta CT}$ formula (Vaerman et al., 2004).

Statistical model and analysis procedure

Data were edited in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). A MIXED procedure (PROC Mixed; SPSS 2006) was used to assess growth performance, feed utilization, hemato-biochemical parameters, immunity and antioxidative traits, immune-related genes, heat shock protein gene and growth hormone gene of Nile tilapia. The statistical analyses were determined using SPSS Ver. 22. The data are presented as the mean ± standard error (n = 3). All variables were calculated using a one-way analysis of variance (ANOVA) followed by Tukey's test. The differences between individual means were subjected to a significance level of $P \leq 0.05$.

Results

Growth performance

Nile tilapia showed higher FBW, WG, and SGR ($P<0.05$) in fish fed 0.3, 0.4, and 0.5% OVLE than the control (Table 3). The PER was higher in fish fed 0.3, 0.4, and 0.5% OVLE ($P<0.05$) than in those fed 0, 0.1, and 0.2% OVLE (Table 3). However, the FCR markedly declined in fish fed 0.4 and 0.5% OVLE ($P<0.05$) than the control without differences with those fed 0.1, 0.2, and 0.3% (Table 3).

Table 3. Effects of dietary administration of various *Origanum vulgare* leaf extract % (OVLE) on performance and feed efficiency of Nile tilapia (*O. niloticus)*

Items	CTR	$OVLE_{0.1}$	$OVLE_{0.2}$	$OVLE_{0.3}$	$OVLE_{0.4}$	$OVLE_{0.5}$	P-value
IW (g)	5.04±0.15	5.28±1.02	5.35±0.15	5.14±0.11	5.31±0.15	5.35±0.16	0.653
FW (g)	62.72±0.34 d	67.66±0.39 c	68.02±0.68 c	71.42±0.78 b	76.57±0.65 a	76.87±0.75 a	<0.001
WG (g)	57.68±0.34 d	62.38±0.42 c	62.67±1.11 c	66.27±1.28 b	71.26±0.68 a	71.52±0.78 a	<0.001
SGR (%/day)	2.57±0.93 b	2.60±0.33 ab	2.58±0.65 b	2.67±0.14 ab	2.74±0.23 a	2.73±0.03 a	0.012
FCR (g/g)	1.32±0.12	1.29±0.22	1.31±0.28	1.32±0.17	1.31±0.91	1.29±0.01	0.360
PER (g/g)	2.51±0.01 b	2.57±0.01 b	2.57±0.06 b	2.74±0.06 a	2.86±0.41 a	2.85±0.0 3 a	<0.001

CTR, the control group fed an un-supplemented diet; OVLE*,* the fish group fed various levels of *Origanum vulgare* leaf extract (0.1, 0.2, 0.3, 0.4 and 0.5%). IW, initial body weight; FW, final body weight; WG, weight gain; SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency ratio. a, b, c – values with different letters in the same row differ significantly (P<0.05).

Table 4. Effects of dietary administration of various *Origanum vulgare* leaf extract % (OVLE) on the hematological indices of Nile tilapia (*O. niloticus*)

Items	CTR	$OVLE_{0.1}$	$OVLE_{0.2}$	$OVLE_{0.3}$	$OVLE_{0.4}$	$OVLE_{0.5}$	P-value
Hb (g/100 ml)	9.41±0.04 d	9.48±0.06 d	9.94±0.03 c	10.16±0.03 b	10.76±0.08 a	9.99±0.03 c	<0.001
RBCs ($\times 10^3$/mm)	3.08±0.03 d	3.15±0.02 d	3.27±0.01 c	3.35±0.01 b	3.56±0.03 a	3.33±0.01 bc	<0.001
PCV (%)	30.33±0.33 c	31.0±0.57 bc	32.31±0.61 b	33.00±0.57 ab	34.66±0.88 a	32.33±0.66 b	0.006
MCV (µm/cell)	94.90±0.05	95.45±1.32	96.93±0.43	96.53±0.63	95.92±0.19	96.08±0.08	0.313
MCH (pg/cell)	30.25±0.18	30.01±0.02	30.14±0.09	30.35±0.09	30.14±0.08	30.02±0.12	0.321
MCHC (%)	31.67±0.17	31.61±0.42	30.89±0.06	31.13±0.29	31.18±0.14	30.93±0.13	0.153
WBCs ($\times 10^6$/mm)	10.21±0.26 c	10.50±0.35 c	11.98±0.22 bc	12.52±0.30 ab	13.25±0.20 a	11.62±0.24 b	<0.001
Heterophils (%)	18.00±0.57 a	13.00±0.57 b	10.00±0.57 c	12.66±0.33 b	9.00±0.57 c	14.33±1.20 b	<0.001
Lymphocytes (%)	69.33±0.88 d	73.66±0.33 bc	78.00±0.57 a	75.33±0.88 b	78.00±0.57 a	72.00±0.57 c	<0.001
Monocytes (%)	8.33±0.33	8.66±0.33	9.33±0.66	8.66±0.88	9.66±0.33	8.33±0.88	0.588
Eosinophils (%)	2.00±0.00	2.33±0.33	2.33±0.88	2.33±0.88	2.66±1.20	2.66±0.66	0.989
Basophils (%)	2.33±0.33	2.33±0.88	2.33±0.88	2.33±0.33	2.33±0.88	2.66±0.33	0.999

CTR, the control group fed an un-supplemented diet; OVLE*,* the fish group fed various levels of *Origanum vulgare* leaf extract (0.1, 0.2, 0.3, 0.4, and 0.5%). Hb, hemoglobin; RBCs, red blood cells; PCV, packed cell volume; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; WBCs, white blood cells. a, b, c – values with different letters in the same row differ significantly (P<0.05).

Table 5. Effects of dietary administration of various *Origanum vulgare* leaf extract % (OVLE) on biochemical blood indices of Nile tilapia (*O. niloticus*)

Items	CTR	$OVLE_{0.1}$	$OVLE_{0.2}$	$OVLE_{0.3}$	$OVLE_{0.4}$	$OVLE_{0.5}$	P-value
ALT (IU)	38.36±0.83 a	36.23±0.28 b	35.25±0.45 b	35.80±0.37 b	35.54±1.24 b	36.09±0.13 b	0.008
AST (IU)	29.90±0.08	28.88±0.40	29.50±0.24	28.83±0.67	28.87±0.17	29.65±0.21	0.203
TP (g/dL)	3.90±0.00 b	3.94±0.01 b	3.92±0.00 b	3.82±0.00 b	4.02±0.02 b	4.35±0.02 a	<0.001
Albumin (g/dL)	2.02±0.02 b	2.02±0.00 b	1.98±0.00 b	1.86±0.02 b	2.06±0.02 b	2.19±0.03 a	<0.001
Globulin (g/dL)	1.90±0.01	1.93±0.02	1.97±0.00	1.97±0.02	1.99±0.00	1.84±0.33	0.971
Glucose (mg/dL)	13.34±0.37	13.61±0.25	13.61±0.17	13.34±0.37	12.50±0.67	12.77±0.21	0.231
T-CHO (mg/dL)	79.47±0.25	84.71±3.17	81.05±0.92	81.73±1.30	80.12±0.41	81.76±2.41	0.426
TG (mg/dL)	91.33±0.95	92.97±1.03	93.10±0.42	94.39±0.33	92.38±0.84	92.86±1.00	0.421

CTR, the control group fed an un-supplemented diet; OVLE*,* the fish group fed various levels of *Origanum vulgare* leaf extract (0.1, 0.2, 0.3, 0.4 and 0.5%). ALT, alanine aminotransferase; AST, aspartate aminotransferase; TP, total protein; T-CHO, total cholesterol; TG, triglyceride. a, b, c – values with different letters in the same row differ significantly (P<0.05).

Blood indices

Generally, blood hematological and biochemical indices were significantly affected by OVLE inclusion (P<0.05), as displayed in Tables 4 and 5. Most of the measured parameters revealed insignificant (P>0.05) differences except for Hb, RBCs, PCV, ALT, WBCs, total protein, albumin, and globulin, which were significantly affected by the inclusion of OVLE (P<0.05). Significantly the highest Hb, RBCs, PCV, and WBCs levels were noticed in fish fed 0.4 and 0.5% OVLE, whereas ALT and globulin levels were significantly boosted in fish fed OVLE at 0.4 and 0.5% when compared to the other groups (P<0.05). Moreover, total protein was significantly increased with the increasing dietary level of OVLE when compared to the control (P<0.05) diets (Table 4).

Immune responses

Immune indicators in fish showed significant differences between groups. Moreover, immune response and antioxidant levels showed improvement in groups of fish fed with increased levels of OVLE (Table 5). Lysozyme activity, phagocytic activity, and phagocytic index showed a higher value in *O. niloticus* fed 0.4 and 0.5% dietary OVLE compared to the other groups (P<0.05).

Table 6. Effects of dietary administration of various *Origanum vulgare* leaf extract % (OVLE) on immune activity, digestive enzymes, and redox status of Nile tilapia

Items	CTR	$OVLE_{0.1}$	$OVLE_{0.2}$	$OVLE_{0.3}$	$OVLE_{0.4}$	$OVLE_{0.5}$	P-value
LPS (U/mL)	82.98±1.11 c	83.85±3.98 bc	86.11±1.74 b	82.74±0.51 c	90.90±0.28 a	92.64±1.66 a	0.013
AMY(U/mL)	103.83±3.12	105.08±2.33	104.01±2.98	105.03±0.41	103.88±0.87	104.42±2.40	0.448
Phag. activity	9.20±0.02 c	9.46±0.03 b	9.94±0.06 b	9.55±0.09 ab	10.35±0.13 ab	10.90±0.07 a	<0.001
Phag. index	1.09±0.14 b	1.12±0.06 b	1.14±0.02 b	1.06±0.03 b	1.09±0.02 b	1.25±0.02 a	0.024
LYZ (µg/mL)	8.29±0.42 d	8.91±0.11 cd	9.76±0.17 bc	9.35±0.10 c	10.41±0.28 b	11.34±0.42 a	<0.001
IgM (µg/ mL)	4.14±0.07 c	5.10±0.02 ab	5.22±0.37 a	5.07±0.02 b	4.97±0.37 b	5.70±0.23 a	0.002
SOD (IU/L)	10.69±0.26 c	11.91±0.34 b	12.89±0.26 ab	11.53±0.52 b	12.40±0.65 ab	13.30±0.26 a	0.009
CAT (IU/L)	13.03±0.07 c	14.23±0.03 b	14.45±0.31 b	14.34±0.31 b	14.16±0.21 b	15.52±0.25 a	<0.001
MDA (IU/L)	16.77±0.73 a	14.68±0.21 b	13.61±0.31 c	15.61±0.64 ab	11.50±0.02 cd	10.94±0.21 d	<0.001

CTR, the control group fed an un-supplemented diet; OVLE, the fish group fed various levels of *Origanum vulgare* leaf extract (0.1, 0.2, 0.3, 0.4 and 0.5%). LPS, lipase activity; AMY, amylase activity; Phag.; phagocytic; LYZ, lysozyme; IgM, immunoglobulin M; SOD, superoxide dismutase; CAT, catalase; MDA, malondialdehyde. a, b, c – values with different letters in the same row differ significantly (P<0.05).

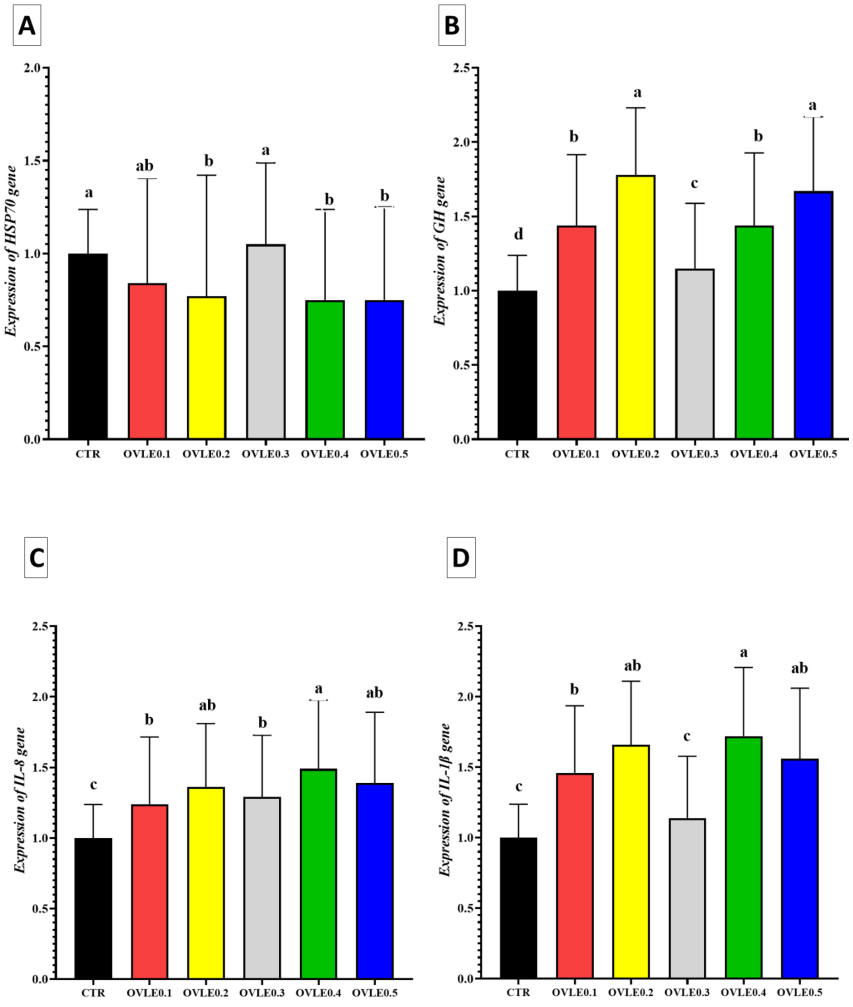

Figure 1. Effect of OVLE supplementation on the expression of (A) *Hsp70*, (B) *GH*, (C) *IL8*, and (D) *IL-1β* genes in *O. niloticus*. *Hsp70*: heat shock protein; *GH*: growth hormone, *IL-8*: interleukin-8, *IL-1β*: interleukin1-β. Data are mean ± SE; values with different superscripts in the same column are significantly different (P<0.05)

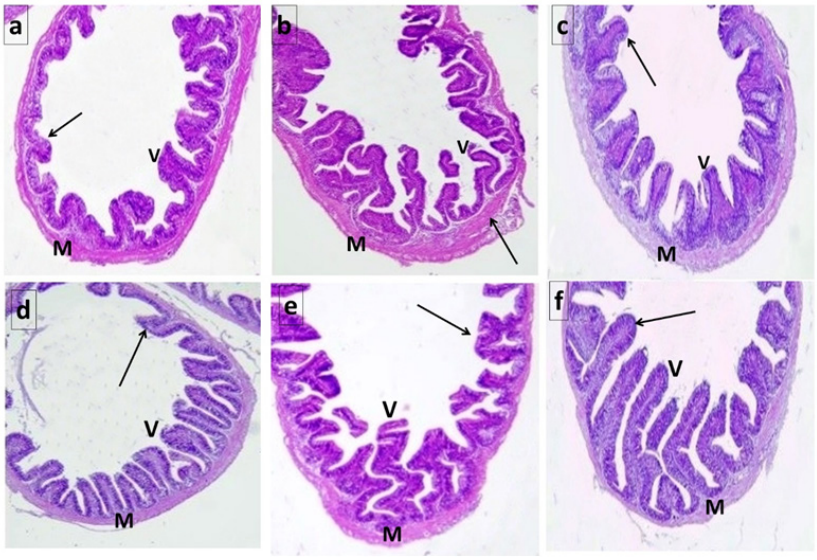

Figure 2. Photomicrograph at high magnification (scale bar 100µm) showing H&E stained sections from Nile tilapia fish intestinal samples fed diets supplemented with several doses of *Origanum vulgare* leaf extract (OVLE). a) control group fish fed un-supplemented diets; showing thickened and expansible intestinal mucosa (M) with normal and shortened intestinal villi (V and arrow); b) fish group fed diets supplemented with 0.1% OVLE; showing normal structure (arrow) admixed and widely separated intestinal mucosa and submucosal connective tissue with thickness of epithelial layer and normal villus length with appearance of some branchial villus structure (V); c) fish group fed diets supplemented with 0.2% OVLE; showing normal histological appearance of intestinal mucosa and moderate improvement of the intestinal villi absorption area (arrow); d) fish group fed diets supplemented with 0.3% OVLE; showing normal, intact intestinal villi (V), lamina propria sub mucosa, tunica muscularis (M) and tunica serosa; e) fish group fed diets supplemented with 0.4% OVLE; showing normal mucosal appearance with increments in the submucosal folds and showing slight improvements in the intestinal length and seems to be more brachiated (arrow); f) fish group fed diets supplemented with 0.5% OVLE; showing normal histological appearance of intestinal mucosa and showing high increments in the villus length (arrow) and showing low thickness of muscular layer with low mucosal folds (M)

Antioxidative status and gene expression

The superoxide dismutase (SOD) and catalase (CAT) were markedly improved in *O. niloticus* treated with OVLE compared with the control, and the group of fish treated with increasing dietary levels of OVLE had the highest SOD and CAT ($P<0.05$). Immunoglobulin M (IgM) was significantly higher in fish fed with dietary OVLE compared with the control, and fish fed 3% OVLE showed the highest IgM value ($P<0.05$; Table 6). The concentration of lipid peroxide malondialdehyde was moderately lowered in *O. niloticus* fed OVLE at varying levels ($P<0.05$; Table 6). The relative expression of heat shock protein 70 (*Hsp70*) was markedly decreased ($P<0.05$) in fish treated with OVLE, with the lowest level in fish fed 0.1% (Figure 1). The gene expression of *Hsp70* at mRNA level was significantly lowered in all supplemented diets, with the maximum significance decrease observed under the impact of OVLE dietary supplementation compared to the control group (Figure 1). In contrast, feeding the mRNA level of the *Hsp70* gene was significantly lowered in all supplemented diets, with the maximum significance decrease. In contrast, feeding OVLE elevated *GH*, *IL-1β*, and *IL8* transcription compared to the control group. However, high levels of *GH* transcription were identified in fish fed low levels of OVLE, whereas *IL-1β* levels increased under the impacts of OVLE compared to the control group and other treated groups. Finally, *IL-8* transcription was activated at a higher level in fish groups that received any amount of OVLE compared to other experimental groups.

Histomorphology

Histological examination of the intestines revealed a significant effect by the inclusion of oregano leaf extract (OVLE) in a dose-dependent manner, and the appearance of intestinal villi was improved in OVLE-treated groups (Figure 2). As well as the intestinal walls of Nile tilapia consist of four layers: mucosa, lamina propria sub-mucosa, tunica muscularis, and serosa which were normal and intact (Figure 2). The intestinal mucosa, lamina propria, tunica muscularis, and tunica serosa were normal and intact in all groups fed OVLE supplemented diets. The highest villus length with lower epithelial thickness appears in the fish group fed high levels of OVLE.

Discussion

The current study sought to determine the effect of *Origanum vulgare* leaf extract (OVLE) dietary supplementation on growth performance, hematological indices, antioxidant activity, and immunological responses of Nile tilapia (*Oreochromis niloticus*). The growth trial results indicated that the final body weight (FBW), weight gain (WG), specific growth rate (SGR), and protein ef-

ficiency ratio (PER) were significantly improved in the fish groups fed with high levels of OVLE (0.4 and 0.5%) compared to the other experimental groups. Similarly, Rashidian et al. (2021) demonstrated that supplementing the zebrafish diet with 1% OVLE significantly enhanced FBW, WG, and SGR compared to all other experimental groups. In addition, numerous previous studies have shown that oregano essential oil (OEO) positively impacts the growth and feed efficiency of several fish species. For instance, Abdel-Latif et al. (2020) illustrated that dietary OEO could improve performance indices and feed utilization parameters in common carp. Furthermore, Shourbela et al. (2021) demonstrated that fish diets containing OEO might markedly diminish the impact of high stocking density on Nile tilapia growth and feed utilization indices via improving redox status and immunological responses. It has previously been discovered that the aerial parts of OV contain a diverse range of medicinally active molecules, including phenolic glycosides, flavonoids, tannins, sterols, and high levels of terpenoids (Pezzani et al., 2017). These active molecules have been shown to perform a variety of biological functions. For instance, it has been investigated that a small dosage of tannins improves growth performance, immunity, intestinal health, and antioxidative stress of pacific white shrimp (Gong et al., 2022). Thus, the presence of tannins in fish diets may be the primary reason for improved fish growth indices and increased protein efficiency ratio in the fish group given a high examined dose of OVLE (0.5%).

It is previously known that normal hemoglobin (Hb) levels indicate no anemic features with normal metabolic function (Hrubec et al., 2001). Whereas high red blood cell counts (RBCs) have been shown to improve feed utilization and, as a result, immunity (Dawood et al., 2020). In this investigation, fish administered 0.4% OVLE had the highest levels of Hb, RBCs, PCV, and WBCs compared to other experimental groups. Similarly, Ahmadifar et al. (2011) stated that supplementing the *Oncorhynchus mykiss* juvenile diet with thymol and carvacrol significantly enhanced the leucogram and erythrogram. Another study illustrated the positive effect of the hematic test by demonstrating higher Hb, PCV, RBCs, and WBCs in great sturgeon juveniles who received a blend of thymol and carvacrol compared to the control (Ahmadifar et al., 2014). Thus, including carvacrol or thymol (the main components of OVLE) in aquafeed has been recognized as a blood metabolic regulator and immunomodulator under normal conditions on several finfish species (Valladão et al., 2019; Firmino et al., 2021).

The alteration in alanine aminotransferase (ALT) levels in the blood indicates the liver health of aquatic animals. Specifically, low blood ALT levels normally indicate a healthy liver, but high levels indicate liver disorder (Naiel et al., 2022 b). While total protein and albumin are essential for transporting organic molecules between cells and blood (Abd El-hameed et al., 2021). Furthermore, their presence in appropriate quantities maintains the fish's proper physiology and health status (Ismael et al., 2021). Hence, the present research findings showed that fish fed OVLE dietary supplementation significantly improved blood protein profile (total protein and albumin) compared to control diets. Furthermore, ALT levels were markedly lower in all OVLE-supplemented groups compared to the control. The acquired results were similar to Rashidian et al. (2021), who reported that feeding zebrafish a diet supplemented with 0.5% OVLE markedly increased total protein levels in the blood and, as a result, boosted innate immune responses. Nonetheless, research has revealed that terpenes found in oregano and thyme, such as carvacrol and thymol, have antibacterial and antioxidant characteristics (Gavaric et al., 2015), as well as bacteriostatic and bactericidal activities against a variety of infections (Ahmadifar et al., 2011). Thus, the antioxidant characteristics of OVLE provide healthy cellular conditions associated with higher blood protein levels and alter liver enzymes and renal metabolite activities. Similarly, Abdel-Latif et al. (2020) showed that adding oregano essential oil to the diet of common carp improved biochemical blood indices, renal function, and hepatocyte enzyme activities.

The inclusion of oregano essential oils or their bioactive molecules in aquafeed, particularly thymol and carvacrol, balances and stabilizes the intestinal microbial population and permits beneficial bacteria to excrete digestive enzymes involved in feed digestion in rainbow trout (Giannenas et al., 2012) and common carp (Zhang et al., 2020). In fact, dietary oregano might perform as an antibacterial agent by breaking down pathogen cellular walls, decreasing toxic secretions that can damage the intestinal mucosal layer (Magouz et al., 2022). The present study's main results include improving lipase activity and intestine histological characteristics in *O. niloticus* fed dietary OVLE. Furthermore, fish fed diets supplemented with graded amounts of OVLE significantly enhanced intestinal villus length while maintaining normal and undamaged intestinal mucosa, lamina propria, tunica muscularis, and tunica serosa. This obtained result agrees with Ferreira et al. (2016) findings that the oregano essential oil improved villus absorption areas, modified the number of goblet cells, and increased cell cytoplasmic intensity in yellow tail tetra. Similar results were also indicated in largemouth bass (*Micropterus salmoides*) fed tea tree essential oil (Liu et al., 2022).

In this investigation, we found that fish administered OVLE-supplemented diets had significantly boosted innate immune response activities. Compared to the other experimental groups, *O. niloticus* fish fed diets supplemented with high doses of OVLE (0.4 and 0.5%) seemed to have the maximum lysozyme activity, phagocytic activity, and phagocytic index. These findings agree with those of Magouz et al. (2022), who revealed that oregano essential oil increased lysozyme and phagocytic activity in Nile tilapia fish under heat stress conditions. Feeding fish diets supplemented with 1 g OEO per kg significantly raised phagocytic index and lysozyme activity compared

to fish fed low OEO (0.25 or 0.5 g/kg). In addition, similar cell line model studies with other dietary plants have demonstrated an increase in phagocytosis as well as the generation of cytokines required for immune response modulation (Shao et al., 2004; Schepetkin et al., 2008; Vattem et al., 2013). Plant secondary metabolites found in oregano extract have been demonstrated to stimulate the upregulation of the NRF_2 (nuclear factor E2-related factor)-antioxidant response element (ARE)-mediated expression of antioxidant genes, enabling effective oxidant removal and antioxidant recycling and may be involved in reducing the oxidant formation rate within the cell (Vattem et al., 2013). All of these elements may have contributed to the stimulation of innate immune activity in the fish administered OVLE.

The total immunoglobulins (IgM) are another biochemical marker involved in regulating the immune-related derivatives in fish blood as well as some redox indices such as SOD, CAT and GPx enzyme activities which were significantly improved in fish groups fed OVLE supplemented diets, indicating an enhanced metabolic and immune response. The result agrees with several previous reports (Dawood et al., 2018; Shourbela et al., 2021; Magouz et al., 2022). Furthermore, Aanyu et al. (2018) have shown that feeding Nile tilapia a diet supplemented with limonene and thymol significantly increased CAT activity. El-Hawarry et al. (2018) revealed markedly elevated SOD in Nile tilapia fish groups administered oregano-supplemented diets. Meanwhile, MDA levels were considerably reduced in all Nile tilapia fish groups receiving OVLE-supplemented diets. Similar results were found in black seabream (Jin et al., 2017). The antioxidative properties of OVLE might be attributed to the presence of thymol. In the same context, Alagawany et al. (2021) reported that thyme extract might contain many antioxidant elements with radical scavenging features, which might stimulate antioxidant defensive mechanisms and reduce oxidative stress, modulating the lipid and protein oxidations in fish tissues.

In studying the influence of functional feed additives on aquatic organisms, the transcription of some growth, immunological, and stress-related genes is often used to determine the genetic mechanism of action (Dawood et al., 2018). Stress causes fish cells to secrete high levels of heat shock protein 70 (*Hsp70*), which increases protein integrity and decreases apoptosis (Fath El-Bab et al., 2022). This study exhibited *Hsp70* downregulated in Nile tilapia fish fed diet supplemented with 0.4 and 0.5% OVLE, which is related to sustaining fish health status. Whereas *GH* gene showed overexpression in fish that received diets supplemented with 0.2 and 0.5% OVLE, and that may be due to the distinct variations between fish and their different response to the feed supplement under study, which point to alteration in metabolic activities. Furthermore, it is well recognized that growth hormone (GH) regulates various key physiological activities in fish. These findings were consistent with other previous reports (Jin et al., 2017; Shourbela et al., 2021). Also, Magara et al. (2022) showed that enriched rainbow diets supplemented with the waste derived from the supercritical fluid extraction of basil (*Ocimum basilicum*) at low and moderate doses led to enhancing immune-related gene expressions in comparison to the control group. A similar result has been reported in largemouth bass (*Micropterus salmoides*) feeding with a diet containing tea tree essential oil supplementation in a low fish meal diet (Liu et al., 2022). In the same context, the current trial estimated some cytokines related to immunity, such as interleukin genes, *IL-Iβ* and *IL-8* that contribute to maintaining growth, differentiation, and activation during inflammatory and immunological responses of fish. The findings of immune-associated genes showed that fish fed 0.2 and 0.4% OVLE represented higher expression levels of interleukin-1 beta (*IL-1β*) while the fish fed 0.4 and 0.5 OVLE had higher expression levels of interleukin-8 (*IL-8*). For this reason, the activation of the *IL-Iβ* and *IL-8* genes in response to OVLE-supplemented diets confirmed the synergistic protective potential function of oregano essential oil in attracting and stimulating neutrophils in inflammatory regions, promoting the immune response function and overall health status of fish (Pilarski et al., 2017). In general, including growth and immunostimulants such as essential oils or extracts in the diets of various fish species can improve the expression of *IL-8*, *IL-1β,* and *GH* (Giannenas et al., 2012; Yu et al., 2020; Zhang et al., 2020).

Finally, the heat shock protein 70 (*Hsp70*) is released from cells to decrease the homeostasis of proteins and preserve the immune and antioxidative responses (Ozogul et al., 2020), in that way protecting cell functionality (Giannenas et al., 2012; Ozogul et al., 2020; Yu et al., 2020). In the current study, the fish fed un-supplemented diet had upregulated Hsp70 gene expression, whereas the other fish fed OVLE enriched diets had downregulated *Hsp70*, indicating that using OVLE at the mentioned concentrations does not cause oxidative stress but was very useful in raising immunity and boosting the efficiency of the essential physiological functions of tilapia fish in this study. In this regard, Li et al. (2021) discovered that *O. niloticus* fish given diets containing medicinal plant *Yucca schidigera* downregulated Hsp70 expression under heat stress conditions.

Conclusion

Finally, our findings showed that *O. vulgare* leaf extract (OVLE) might improve redox status, hematological and biochemical indices, and immunological responses in treated Nile tilapia. It is important to note that a highly tested level of OVLE resulted in maximum productive performance and increased feed efficiency. Thus, it would be recommended that OVLE be incorporated into the Nile tilapia diet at high levels (0.4−0.5%). More studies are required to incorporate more dietary OVLE (more than 0.5%) and assess its influence *in-vivo* on fish health and growth.

Author contributions

All authors contributed equally to the writing of this paper. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this manuscript.

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