Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 29(4): 5121 – 5146 (2025)
www.ejabf.journals.ekb.eg

The Efficiency of Dietary Nano-Formulation of *Laurencia obtusa* Extract in Terms of the Growth Performance, Antioxidant Activity, and Immune Response of Juvenile Nile Tilapia (*Oreochromis niloticus*)

Adel S. A. Mekhaimar[1, 2*], Samar S. Negm[3], Sameh H. Ismail[4], Adel A. Shaheen[1], Amel M. El Asely[1]

[1]Department. of Aquatic Animal Medicine, Faculty of Veterinary Medicine, Benha University, 13736, Benha, Qalyubia, Egypt

[2]General Services Organization, Armed Forces, Cairo, Egypt

[3]Fish Biology and Ecology Department, Central Lab for Aquaculture Research Center, Giza, 12622, Egypt

[4]Faculty of Nanotechnology for Postgraduate Studies, Sheikh Zayed Campus, Cairo University, 6th October City, Giza 12588, Egypt

***Corresponding author: elsalam.clinic21@gmail.com**

ARTICLE INFO

Article History:
Received: June 3, 2025
Accepted: Aug. 5, 2025
Online: Aug. 11, 2025

Keywords:
Seaweeds,
Laurencia obtuse,
Nanoparticles,
Growth,
Immune gene expression

ABSTRACT

This study evaluated the effects of using *Laurencia obtusa* (*L. obtusa*) extract nanoparticles as a feed additive for the Nile tilapia (*Oreochromis niloticus*) juveniles. Four diets (30% protein) were prepared; three diets were supplemented with *L. obtusa* extract nanoparticles (LENPs) at concentrations of 0.25, 0.5, and 1 g kg−1, represented by LENP1, LENP2, and LENP3, respectively. The fourth diet served as the control. The fish, averaging 19.85 ± 1.35 g, were fed experimental diets for a duration of 60 days. The growth performance, antioxidant activity, immune parameters, digestive enzymes, and antioxidant and immune genes expression were evaluated. Results indicated that growth parameters were not affected by LENP incorporation compared to the control. All antioxidant biomarkers showed a significant increase in all groups compared to control, particularly LENP2 and LENP3, while levels of MDA and nitric oxide exhibited a considerable decrease in all groups than control. Concurrently, in terms of immunity impact, immunoglobulin levels exhibited substantial enhancement, as levels of IgM demonstrated significant increases, recorded maximum values in LENP2 and LENP1, respectively. Total protein, albumin, and globulin recorded the highest level in LENP3. Regarding blood indices, particularly RBC,s count, hemoglobin, packed cell volume (PCV) and mean corpuscular volume (MCV), all experimental groups showed a significant increase ($P< 0.05$) compared to the control. Liver and kidney function exhibited non-significant changes compared to the control. Digestive enzymes values showed a significant rise compared to the control group. A substantial upregulation of GPX and SOD gene expression levels was recorded in LENP3 fed groups in both liver and spleen. A similar pattern was observed for interleukin1 beta (IL-1β) and interleukin-10 (IL-10), with elevated levels in the spleen compared to the liver. In conclusion, *L. obtusa* nanoparticles at different concentrations were effective in positively modulating *O. niloticus* physiological and immune parameters, with a non-significant effect on fish growth after 60 days feeding trial.

INTRODUCTION

The aquaculture industry is a crucial contributor to global high-yield animal protein resources and significantly influences the economic base of numerous countries. The Nile tilapia is the third most cultured freshwater fish globally **(FAO, 2024)** due to its rapid growth, high tolerance to a variety of environmental conditions, high palatability, resistance to diseases and stress, and quick acceptance of artificial feed **(Webster & Lim, 2024)**. Tilapia, a highly productive African fish, plays a vital role in the aquaculture industry by providing high-quality protein and offering opportunities for sustainable nutrition and aquaculture, thereby contributing to global food security **(Arumugam *et al.*, 2023)**.

There are over 80 species of red algae belonging to the genus *Laurencia*, which occurs in tropical, subtropical, and warm-temperate climates worldwide **(Machín-Sánchez *et al.*, 2018)**. Members of this genus can produce secondary metabolites, with *Laurencia obtusa* being particularly rich in phenols and exhibiting excellent antioxidant activity **(El-Shenody *et al.*, 2019; Peñalver *et al.*, 2020)**. *L. obtusa* contains ten essential and six non-essential amino acids, including aspartic acid, glutamic acid, proline, and alanine. Furthermore, *L. obtusa* has a high content of ascorbic acid (334mg g−1), along with significant amounts of polyphenols and flavonoids **(El-Shenody *et al.*, 2019)**.

Numerous studies have demonstrated the beneficial effects of seaweed and seaweed-derived functional metabolite supplementation on fish growth, immune response, and physiological stress resilience, in addition to enhancing antioxidant and serum immune status **(Akbary & Aminikhoeis, 2018; Ashour *et al.*, 2020; Salem *et al.*, 2021; Siddik *et al.*, 2023)**. However, few studies have evaluated the use of red algae, specifically *L. obtusa*, as a feed additive. *L. caspica* extract has been shown to improve non-specific immunity and antioxidant activity in the rainbow trout (*Oncorhynchus mykiss*), thereby increasing resistance to *Aeromonas hydrophila* **(Kiadaliri *et al.*, 2020)**. In another experiment, **Salem *et al.* (2021)** incorporated *L. obtusa* into diets at 0, 2, 4, and 8g kg−1 for 70 days, resulting in improved growth. Similarly, *L. caspica* extract acted as an immunostimulant for *O. niloticus*, significantly increasing antioxidant enzyme activities, white blood cell count, hemoglobin levels, and immunoglobulin concentrations compared to the control group **(Khanzadeh *et al.*, 2023)**.

The incorporation of natural feed additives, such as nano-formulated seaweed extract, can enhance fish health, reduce the need for synthetic chemicals, and promote ecologically sustainable farming practices **(Hossain *et al.*, 2024)**. Nanomaterial delivery technologies can also improve feed nutritional characteristics and feed conversion efficiency **(Bhattacharyya *et al.*, 2015)**. The objective of this study was to assess the potential of using a nano-formulation of *L. obtusa* as a functional feed additive to improve *O. niloticus* growth and overall health.

MATERIALS AND METHODS

1. Experimental fish

One hundred twenty healthy juveniles of *O. niloticus*, averaging 19.85 ± 1.35g in weight and 10 ± 1.80cm in length, were obtained from the freshwater hatchery at the Central Laboratory for Aquaculture Research (CLAR), Abbassa Abo-Hammad, Al-Sharqia, Egypt. The fish were immediately transported alive in fully equipped vessels to the wet laboratory of the Department of Fish Biology and Ecology at CLAR, where they were acclimated for 14 days in a well-equipped polyethylene tank (1 × 1 × 1 m^3). During this period, they were fed a basal diet twice daily, and freshwater was renewed at a daily rate of 10%. All fish were visually inspected and confirmed to be healthy, showing no lesions or injuries.

2. Preparation of *L. obtusa* extract nanoparticles

L. obtusa was collected from the intertidal zone along the Red Sea shore near Hurghada City, with preparation and taxonomic identification carried out following **Mekhaimar *et al.* (2024)**. Solvent extraction of *L. obtusa* was performed using 95% ethyl alcohol and Whatman filter paper No. 1, following **Sivakumar and Kannappan (2013)**, with some modifications.

The nanoparticles of the ethanolic extract were prepared following **Hinman and Suslick (2017)**. Briefly, one gram of the *L. obtusa* extract was solubilized in 100mL of deionized water. The solution was treated in an ultrasonic bath, followed by sonication for 30 minutes at a frequency of 40 kHz and a power output of 400 W. After sonication, the solution was centrifuged at 10,000 rpm for 15 minutes to isolate the synthesized *L. obtusa* nanoparticles. The resulting nanoparticle solution was washed three times with deionized water to remove residual impurities, and then dried in an oven at 40°C.

2.1. Characterization of L. obtusa nanoparticles

Key characteristics of the synthesized *L. obtusa* nanoparticles (LENP) were determined using TEM (JEM-2100F, JEOL) to assess morphology, size, and size distribution. AFM (Multimode 8, Bruker) was used to analyze surface roughness and topography. Hydrodynamic size, size distribution, surface charge, and colloidal stability were measured using DLS and zeta potential analysis (Zetasizer Nano ZS, Malvern Panalytical). The crystalline structure and phase composition of the nanoparticles were examined using XRD (X'Pert PRO MPD, PANalytical) following **Al-Qasmi *et al.* (2022)**.

3. Preparation of experimental feeds

Table 1. Feed composition and chemical analysis of the basal diet

Ingredient	%
Ingredient	%
Fish meal (72.0% CP)	9.80
Soybean meal (48% CP)	42.20
Yellow corn	20.50
Wheat flour	5.70
Wheat bran	15.20
Vegetable oil	3.50
Cod liver oil	2.00
Di-calcium phosphate	0.87
Vitamin and mineral mixture	0.20
Vitamin C	0.03
Total	100.00
Chemical *Composition (%)*	
Moisture	7.70
Dry matter	92.30
Crude protein	30.64
Ether extract	5.37
Ash	7.82
Crude fibers	4.92
Gross energy (kcal/kg)	1,768.9

The basal diet consisted of commercial fish feed (Aller Aqua, Egypt) containing 30% protein, as detailed in Table (1). The experimental diets were prepared by finely grinding the basal feed using a mincer, then incorporating the required amount of LENP according to the respective experimental group: 0.25, 0.5, 1, or 0 g kg^{-1} feed (V/W) into 100 mL of distilled water. This mixture was sprayed onto the fish feed powder and thoroughly mixed. The moistened mixture was then pressed into pellets and dried for 24 h at 60 °C in a forced-air oven. All feeds were stored at −20 °C until use.

4. Experimental design

One hundred twenty fish were randomly allocated into four groups in triplicate (n= 10) and maintained in well-equipped glass aquaria (60 × 40 × 40cm) containing 72L of water. The fish remained in the aquaria for 60 days until the conclusion of the experiment. Aquaria were filled with dechlorinated tap water, adjusted to meet water quality standards, and monitored before siphoning using water quality instruments and commercial kits (HANNA). Biweekly water sampling from each tank recorded the following parameters: water temperature (26.7 ± 1.2°C), dissolved oxygen (5.89 ± 0.46 mg/L), total ammonia

nitrogen (1.02– 1.04mg/ L), and pH (7.4 ± 0.5). A 12h light/12h dark photoperiod was maintained throughout. All water quality parameters remained within acceptable culture standards **(Boyd & Tucker, 2014)**.

The three supplemented diets—LENP1, LENP2, and LENP3—were based on the basal diet enriched with 0.25, 0.5, and 1 g of LENP per kg of diet, respectively, while the control group (LENP0) received only the basal diet. Fish were hand-fed twice daily at 9:00 a.m. and 3:00 p.m., at 4% of body weight, six days per week. Water was replaced three times weekly with fresh, dechlorinated tap water. Over the 60 days, daily records of feed consumption and mortality were kept for each tank. All fish were weighed biweekly, with biomass and feed intake calculated accordingly.

5. Blood and tissue sampling

At the end of the trial, fish were fasted for one day prior to sampling. Five fish from each replicate were sedated with tricaine methanesulfonate (MS-222) (Merck, Germany). Blood was collected from caudal vessels using a 1mL syringe fitted with a 26G needle and containing sodium heparin (0.2mL per mL of blood) for complete blood count analysis **(Sayed & Moneeb, 2015)**. Additional blood samples were collected without anticoagulant for serum separation, allowed to stand at room temperature for four hours, and then centrifuged at 1500g for five minutes. Serum samples were stored at −20°C.

For antioxidant enzyme and lipid peroxidation assays, 50mg of liver tissue was preserved in cold PBS (pH 7.4) and stored at −20°C. The anterior intestine from all groups was collected for digestive enzyme (amylase and lipase) analysis. Each specimen was homogenized in 20mL (v/w) of ice-cold distilled water, then centrifuged at 10,000g for 15min at 4°C. The resulting supernatants were stored at −20°C until analysis **(Jun-sheng *et al.*, 2006)**.

For mRNA expression analysis, liver and spleen samples were preserved in RNAlater and stored at −80°C.

6. Evaluation of fish growth parameters

Each fish from every tank was carefully weighed using a precise weighing scale to ascertain their starting and ultimate body weight. Weekly weight data were kept until the completion of the experiment. The calculation of growth parameters was carried out by **Lovell (2001)** using the subsequent equations:

- Weight gain (WG, g) = FW (g)/ IW (g).
- Average daily gain (ADG, g/fish/day) = WG (g) / T
- Relative growth rate (RGR, %) = (TWG (g) / IW (g)) × 100
- Specific growth rate (SGR, %/day) = [(Ln FW – Ln IW) / T] × 100

Where, FW: Final weight (g); IW: Initial weight (g); T: The experimental period (day).

Feed intake (FI, g) was recorded, and then feed utilization parameters were calculated according to the following equations;

- Feed conversion ratio (FCR) = FI / WG

- Feed efficiency (%) = (WG / FI) × 100
- Protein efficiency ratio (PER) = live weight gain (g) / protein intake (g).
- Percentage weight gain (WG%) and specific growth rate (SGR) were determined according to **Dedeke *et al.* (2013)**. Feed conversion ratio (FCR) and feed conversion efficiency (FCE) were calculated according to the formula of **Craig *et al.* (2017)**. Protein efficiency ratio (PER) was calculated according to the formulae described by **De Silva and Anderson (1994)**.

7. Oxidative stress biomarkers and nitric oxide (NO)

Glutathione peroxidase (GPx) was evaluated using NADPH oxidation at 340nm according to **Cribb *et al.* (1989)**. Catalase activity (CAT) was determined using the method of **Clairborne *et al*. (1985)**, with hydrogen peroxide (30%) as a substrate. Superoxide dismutase (SOD) was measured using the **Marklund and Marklund (1974)** method. The TAC was measured as described by **Akinrinde *et al*. (2018)**.

8. Determination of hematological indices

The red blood cell count (RBCs) and white blood cell count (WBCs) were determined optically with a Neubauer chamber according to **Dacie and Lewis (2001)**. The hematological indices of mean corpuscular hemoglobin concentration (MCHC), mean cell hemoglobin (MCH), and mean cell volume (MCV) were calculated using the total red blood cell count (RBCs) and hemoglobin concentration (Hb) according to **Dacie and Lewis (2001)**, following the formulae:

MCHC (g/dl) = [Hb (gdl^{-1}) x 10]/Hct x 100.

MCH (pg) = [Hb ($g\ dl^{-1}$) x 10]/RBC ($10^6\ \mu l^{-1}$).

MCV (fl) = Hct/RBC ($10^6\ \mu l^{-1}$).

The hemoglobin concentration was determined with Drabkin's reagent at an absorbance of 540nm.

9. Serum chemistry and immunoglobulin M

Assays for total protein (TP), albumin, globulin, immunoglobulin, creatinine, urea, ALT, AST were conducted as detailed below: -

Serum total protein was estimated following **Wootton (1964)** using a total protein assay kit by Biolabo manufacturer; France. Albumin was assessed by the bromocresol green binding method **(Doumas *et al*., 1972)**. A spectrophotometer was used to measure the absorbance of the standard and test against a blank at 550 and 578nm, respectively, for total serum protein and albumin. The albumin readings were subtracted from the total serum protein levels to determine the globulin values. The Ig level was determined using a microprotein determination method and precipitation using a 12% polyethylene glycol solution, comparing protein content before and after precipitation **(Siwicki &**

Anderson, 2000). Blood aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were estimated by the colorimetric method explained by **Reitman and Frankel (1957)**. Creatinine and urea were determined according to **Lausen (1972)** and **Whitehead *et al*. (1991)**, respectively.

10. Digestive enzymes assays

Fish's anterior intestine was rinsed, homogenized in a buffer, centrifuged, and stored. Lipase and amylase activity were measured using methods from **Bernfeld (1951)** and **Iijima *et al*. (1998)**, using starch as substrate. Supernatants were collected and stored at -80°C.

11. mRNA analysis of liver and spleen tissues

11.1. Real-time PCR

Tissue homogenization of the samples was performed by homogenization of 50mg of tissue with 1mL EASY RED Reagent by WiseTis HG-15D homogenizer (Daihan Scientific Co., Seoul, Korea).

RNA extraction from tissue was applied, including precipitating the RNA, then RNA concentration determination using a Spectrophotometer at 260nm. For the RNA quality evaluation, analysis of the A260/A280 ratio using the NanoDrop® ND–1000 Spectrophotometer (NanoDrop Technologies; Wilmington, Delaware, United States). The estimated purity of the isolated RNA was between 1.8 and 2.0 according to **Bustin *et al*. (2009)**.

11.2. Synthesis of complementary DNA (cDNA)

cDNA synthesis was performed using a HiSenScript™ RH (−) cDNA Synthesis Kit (iNtRON Biotechnology Co., South Korea). A total of 1μg of RNA sample was added to 10μL of 2× reverse transcriptase (RT) master mix. The RT reaction mixture was incubated at 45°C for 60min, followed by incubation at 85°C for 10min to inactivate the enzyme, using an Applied Biosystems Veriti 96-well thermal cycler (Applied Biosystems). The synthesized cDNA was then stored at −20°C until use, following prior study **(Bustin *et al.*, 2009)**.

Table 2. Primers used in the determination of gene expression of the selected genes

Gene	Primer sequences (5′–3′)	Reference
SOD	F-CTCCAGCCTGCCCTCAA R-TCCAGAAGATGGTGTGGTTAATGTG	Varela-Valencia *et al*. (2014)
GPx	F-GGAACGACAACCAGGGACTA R-TCCCTGGACGGACATACTTC	El-Barbary (2016)
IL-1β	TGGTGACTCTCCTGGTCTGA	
IL-10	CTGCTAGATCAGTCCGTCGAA	
GAPDH	F: 5`- GGCACAGTCAAGGCTGAGAATG -3` R:5`- ATGGTGGTGAAGACGCCAGTA -3`	Yang *et al*., (2015)

11.3. cDNA reaction procedures

cDNA Synthesis mixture was prepared and stored at -20°C until used in PCR according to **Bustin *et al*. (2009)**.

11.4. Real-time PCR amplification

Using 5x HOT FIRE Pol EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia), the real-time RT-PCR was performed in a Mx3005P Real-Time PCR System (Agilent Stratagene, USA) following the manufacturer's instructions. The oligonucleotide-specific primers were synthesized by Sangon Biotech (Beijing, China). A melting curve analysis was performed following PCR amplification with primer sequences as the procedure was performed according to **Bustin *et al*. (2009)** (Table 2). Housekeeping genes (GAPDH) were used as a normalizing control to calculate the relative gene expression or fold change **(Livak & Schmittgen, 2001)**.

12. Ethical approval

The experiment was conducted following the ethics of animal welfare, reviewed and approved by the Faculty of Veterinary Medicine, Benha University committee of Animal Ethics number (BUFVTM 04-02-24).

13. Statistical analysis

The data was subjected to analysis of variance (ANOVA), and the software used is SPSS (Version 22.0). Duncan's multiple range test was used to compare between means of the control and supplemented groups.

RESULTS

1. Characterization of LENP

- **X-ray diffraction (XRD)**

  The XRD pattern (Fig. 1A) showed a broad hump, indicating an amorphous structure that may influence the nanoparticles' chemical reactivity and bioavailability.

- **Atomic force microscopy (AFM) and transmission electron microscopy (TEM)**

  Atomic force microscopy (AFM) (Fig. 1B) and TEM (Fig. 1C) analyses revealed predominantly spherical to sub-spherical shapes with a uniform size distribution, which is advantageous for applications like drug delivery.

- **Dynamic light scattering (DLS)**

  DLS measurements (Fig. 1D) yielded an average hydrodynamic diameter of approximately 65nm, which is optimal for various nanomedical applications and can benefit from the enhanced permeability and retention (EPR) effect.

- **Zeta potential measurement**

  The zeta potential (Fig. 1E) of the nanoparticles was -31 mV, suggesting a high degree of surface charge negativity, which can prevent aggregation and contribute to stability in circulation.

- **Brunauer–Emmett–Teller (BET) method**

  The BET (Fig. 1F) is the surface to volume ratio, which indicates a high surface area of LENP per mass, as the porous form of particles, which can be measured by BET analysis, resulting in more than $1.2 m^2/ g$.

2. Effect of LENP on growth performance

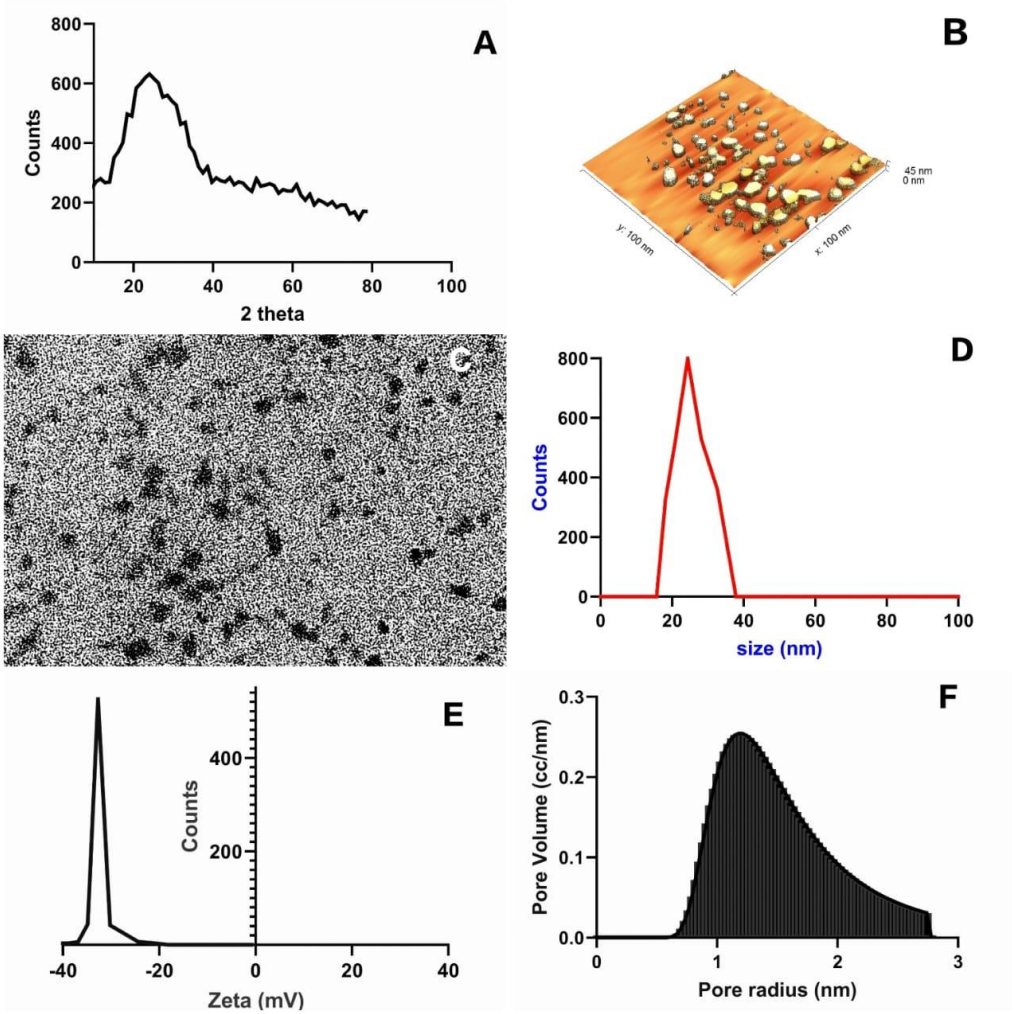

Fig. 1. explaining the results of different characterization tests used for LENP where (A) is the XRD test and (B) is AFM test and (C) is TEM test and (D) is DLS test and (E) is Zeta potential test, and (F) is BET test

No mortalities were observed in both control and LENP-supplemented groups. The effect of LENP supplementation in the fish diet at different concentrations on the growth performance is presented in Table (3). A significant decrease in FW, DWG, SGR, WG%, and RGR was observed in groups LENP1 and LENP3. PER, TFI, and FCR showed a significant reduction in all experimental groups compared to the control. In contrast, FER and PI exhibited a significant increase in all experimental groups relative to the control. WG showed no significant difference between the experimental groups and the control.

Table 3. Growth parameters of different experimental groups of *O. niloticus* fed basal diet enriched with *L. obtusa* ethanolic extract nanoparticles

	FW (g)	*DWG (g)*	*WG (g)*	*FCR*	*IFI (g)*	*SGR (%)*	*PER (g/g)*	*WG%*	*FER (g/g)*	*RGR (%)*	*PI*
LENP1	41.07 ± 0.01 ^{b}	0.489 ± 0.00 ^{b}	22.01 ± 0.02 ^{b}	1.4 ± 0.018 ^{bc}	30.77 ± 0.43 ^{b}	0.74 ± 0.001 ^{b}	2.14 ± 0.028^{bcd}	115.39 ± 0.28 ^{b}	0.65 ± 0.001 ^{ab}	53.57 ± 0.06 ^{b}	10.26 ± 0.14 ^{b}
LENP2	42 ± 0.17 ^{a}	0.51 ± 0.004 ^{a}	22.92 ± 0.19 ^{b}	1.34 ± 0.01 ^{c}	30.69 ± 0.11 ^{b}	0.76 ± 0.005 ^{a}	2.24 ± 0.01 ^{b}	120.13 ± 1.11 ^{a}	0.71 ± 0.009 ^{a}	54.57 ± 0.23 ^{a}	10.23 ± 0.04 ^{b}
LENP3	40.85 ± 0.43 ^{b}	0.48 ± 0.009 ^{b}	21.76 ± 0.41 ^{b}	1.44 ± 0.036 ^{b}	31.22 ± 0.2 ^{b}	0.73 ± 0.002 ^{b}	2.09 ± 0.04 ^{bcd}	113.98 ±2.01 ^{bc}	0.75 ± 0.003 ^{b}	53.26 ± 0.44 ^{b}	10.41 ± 0.07 ^{b}
LENP0	42.04 ± 0.09 ^{a}	0.51 ± 0.002 ^{a}	22.98 ± 0.1 ^{b}	1.53 ± 0.01^{a}	35.23 ± 0.09 ^{a}	0.76 ± 0.003 ^{a}	1.96± 0.01 ^{a}	120.53 ± 0.57 ^{a}	0.73 ± 0.03 ^{c}	54.65 ± 0.12 ^{a}	11.74 ± 0.03 ^{a}

FW: Final Weight, DWG: Daily Weight Gain, WG: Weight Gain, FCR: Feed Conversion Ratio, SGR: Specific Growth Rate, PER: Protein Efficiency Ratio, WG%: Weight Gain %, FE: Feed Efficiency, RGR: Relative Growth Rate, and PI: Protein Efficiency. Results presented as mean ± standard Error. Different letters indicate significant differences between groups (P< 0.05).

3. Effect of LENP on oxidative stress biomarkers

As presented in Table (4), the results of MDA showed that the experimental group exhibited a significant reduction in LENP2 and LENP3 compared to the control group. Values GPx exhibited a significant increase in LENP2 and LENP3 compared to the control group. The results for CAT, SOD, and TAC indicated a significant increase in all experimental groups compared to the control, with the highest values observed in LENP2. The values of NO were significantly lower than those of the control group, particularly LENP2.

Table 4. Antioxidant biomarkers of various experimental groups of *O. niloticus* fed a basal diet supplemented with LENP

	MDA (nmol/mL)	**GPX (U/g tissue)**	**CAT (U/g tissue)**	**SOD (U/g tissue)**	**TAC (U/mg prot)**	**Nitric oxide (nmol/L)**
LENP1	5.06 ± 0.36^{a}	3.6 ± 0.21^{b}	29.18 ± 0.76 ^{abc}	11.85±0.16 ^{a}	2.22 ± 0.14 ^{a}	16.99 ± 1.08 ^{ab}
LENP2	3.83 ± 0.14 ^{b}	4.73 ± 0.31^{a}	30.93 ± 1.38 ^{a}	12.16±0.39 ^{a}	2.66 ±0.2 ^{a}	13.05 ± 0.2 ^{c}
LENP3	4.54 ± 0.21 ^{b}	4.48± 0.05 ^{ab}	30.37 ± 0.4^{a}	11.81± 0.24 ^{a}	2.38 ± 0.18 ^{a}	13.45 ± 1.19 ^{bc}
LENP0	5.94 ± 0.16 ^{a}	3.42± 0.11 ^{b}	25.15 ± 0.75 ^{b}	10.1 ±0.6 ^{b}	1.55 ± 0.17 ^{b}	18.47 ± 1.54 ^{a}

Values with different numeric superscripts in a column differ significantly (P< 0.05 (between LENP applications. Values are mean ± SE (n= 3/replicate).

4. Effect of LENP on blood indices

Results are presented in Table (5). The highest RBC, Hb, and PCV values were observed in LENP1, showing a significant increase ($P< 0.05$) compared to the control group. MCV values were significantly higher in all experimental groups than in the control, with LENP1 showing the highest value. The highest TLC value was recorded in LENP3, which was significantly greater ($P< 0.05$) than the control. No significant differences were detected among experimental groups for MCH, MCHC, and PC.

Table 5. Blood indices of different experimental groups of *O. niloticus* fed basal diet supplemented with LENPs

	RBCs ($\times10^{12}$ /L)	Hb (g /dL)	PCV (%)	MCV (fL)	MCH (pg)	MCHC (g(dl))	TLC ($\times10^{9}$ /L)	PC ($\times10^{9}$ /L)
LENP 1	3.9 ± 0.36 ^{a}	11.47 ± 1.0^{a}	33.067 ± 2.89 ^{a}	84.99± 0.48 ^{a}	29.4 ± 0.16 ^{a}	34.6 ± 0 ^{a}	2.73 ± 0.29 ^{ab}	1093.33± 70.5 ^{a}
LENP 2	3.43 ± 0.29 ^{ab}	9.97± 0.85 ^{ab}	28.73± 2.45^{ab}	83.77± 0.23 ^{a}	28.96 ± 0.07 ^{a}	34.6 ± 0 ^{a}	2.53± 0.29 ^{abc}	1043.33± 165.7^{a}
LENP 3	3.53 ± 0.18 ^{ab}	10.38± 0.46^{ab}	29.93± 1.31^{ab}	84.86± 0.56 ^{a}	29.34 ± 0.17 ^{a}	34.6 ± 0 ^{a}	3.2 ± 0.23 ^{a}	1333.33± 70.5 ^{a}
LENP0	3.013 ± 0.06 ^{b}	8.77 ± 0.2 ^{b}	25.27± 0.58 ^{b}	15.67± 1.76 ^{b}	29.03 ± 0.13 ^{a}	34.6 ± 0 ^{a}	1.87 ± 0.12 ^{cd}	1046.67± 40.5 ^{a}

Data are presented as mean ± SE (n=3 fish from each treatment). RBC: red blood cells, Hb: hemoglobin concentration, PCV: packed cell volume, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, TLC: total leukocytic count, PC: protein concentration. Significant differences ($P<0.05$) are indicated by different letters.

5. Blood chemistry parameters

5.1. Effect of LENP on blood protein

Results are presented in Table (6). The highest values for blood protein, albumin, and globulin were observed in LENP3, showing a significant increase ($P< 0.05$) compared to the control group.

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Table 6. Plasma protein, albumin, and globulin of different experimental groups of *O. niloticus* fed a basal diet supplemented with LENPs

	Protein (g /dL)	Albumin (g /dL)	Globulin (g /dL)
LENP1	2.83 ± 0.18 [b]	1.37 ± 0.15 [c]	1.47± 0.12 [b]
LENP2	3.37 ± 0.2 [b]	1.79 ± 0.06 [b]	1.57± 0.17 [b]
LENP3	6.6 ± 0.47 [a]	2.93 ± 0.15 [a]	3.67 ±0.34 [a]
LENP0	2.67 ± 0.23 [b]	1.15 ± 0.12 [c]	1.52 ± 0.15 [b]

The values are means ± SEM with (n = 3/replicate). Different letters denote significant differences (P<0.05).

5.2. Effect of LENP on serum immune parameters

Results presented in Table (7), show that the highest IgG values were recorded in LENP2, showing a significant increase (P< 0.05) compared to the control group. The highest IgM values were observed in LENP1, also showing a significant increase (P< 0.05) compared to the control group.

Table 7. Immunoglobulin values (mg/ dl) for IgM of *O. niloticus* fed a basal diet supplemented with LENPs.

	IgM (µg/l)
LENP1	3.33 ± 0.45 [a]
LENP2	2.69 ± 0.08 [ab]
LENP3	1.86 ± 0.39 [b]
LENP0	1.98 ± 0.19 [b]

Values expressed as means ± SEM, n = 3/replicate. Different letters indicate significant differences (p< 0.05).

5.3. Effect of LENP on liver and kidney function

Results demonstrated in Table (8), show that serum creatinine values recorded variable values compared to the control group, as LENP1 recorded a significant increase and LENP3 recorded a significant decrease compared to the control. Serum urea levels showed no difference from control. While AST values recorded the highest value in LENP3. ALT values showed the highest values in LENP1 and LENP3, which were significantly higher than the control.

Table 8. Liver and kidney function of different experimental groups of *O. niloticus* fed a basal diet supplemented with LENPs

	Creatinine(mg/dL)	Urea (mg/dL)	AST (U/L)	ALT (U/L)
LENP1	1.19 ± 0.06 [a]	28.67 ±11.92 [a]	31 ± 1.73 [b]	27.67 ± 1.76 [a]
LENP2	0.7 ± 0.04 [b]	31.67 ± 2.91[a]	31.67 ± 2.33 [b]	17.33 ± 0.88 [b]
LENP3	0.42 ± 0.03 [c]	37 ± 1.53 [a]	45.67 ± 5.04 [a]	27 ± 2.31 [a]
LENP0	0.7 ± 0.04 [b]	26 ± 8.62 [a]	33.67 ± 3.38 [b]	15.67 ± 1.76 [b]

Values expressed as means ± SEM, n= 3/replicate. Different letters indicate significant differences ($P< 0.05$).

6. Digestive enzymes

Results illustrated in Table (9), show that amylase and lipase values recorded the highest significant increase in LENP2 compared to the control.

Table 9. Amylase and Lipase values of different experimental groups of *O. niloticus* fed feed enriched with LENP

	Amylase (U/L)	Lipase (U/L)
LENP1	33.65 ± 0.09 [c]	13.21 ± 0.11 [b]
LENP2	37.31 ± 0.38 [a]	16.61 ± 0.2 [a]
LENP3	28.55 ± 0.34 [d]	13.09 ± 0.27 [b]
LENP0	36.41 ± 0.23 [b]	12.13 ± 0.14 [c]

Values are Means ± SEM, n = 3/replicate. Different letters indicate significant differences ($P< 0.05$).

7. Gene expression

7.1. Hepatic gene expression

Our results (Table 10) show that LENP-supplemented feed did not affect hepatic mRNA expression of superoxide dismutase, whereas glutathione peroxidase was downregulated in LENP1 and LENP2. Interleukin analysis revealed downregulation of IL-10 in all groups and a significant increase in IL-1β expression across all groups.

Table 10. Hepatic gene expression values of different experimental groups of *O. niloticus* fed a basal diet supplemented with LENPs

Liver	SOD	GPX	IL-10	IL-1B
LENP1	0.85 ± 0.01 [a]	0.72 ± 0.01 [b]	0.47 ± 0.02 [d]	3.46± 0.02 [b]
LENP2	0.91 ± 0.02 [a]	0.83 ± 0.02 [b]	0.65 ± 0.02 [c]	2.13 ± 0.02 [c]
LENP3	1.12 ± 0.14 [a]	1.05 ± 0.08 [a]	0.88 ± 0.03 [b]	3.97 ± 0.04 [a]
LENP0	1.0 [a]	1.0 [a]	1.0 [a]	1.0 [d]

Values are Means ± SEM, n = 3/replicate. Different letters indicate significant differences ($P< 0.05$).

7.2. Splenic gene expression

Results presented in Table (11) show that SOD and GPX mRNA expression levels were significantly decreased in LENP1 and LENP2 compared to the control. The interleukin expression pattern revealed downregulation of IL-10 in LENP1 and LENP2, while IL-1β expression was significantly upregulated in all groups relative to the control.

Table 11. Levels of splenic gene expression in various experimental groups of *O. niloticus* fed a basal diet supplemented with LENPs

Spleen	SOD	GPX	IL-10	IL-1B
LENP1	0.72 ± 0.03 [b]	0.68 ± 0.02 [b]	0.64 ± 0.02 [b]	4.35 ± 0.14 [a]
LENP2	0.75 ± 0.03 [b]	0.7 ± 0.02 [b]	0.59 ± 0.03 [b]	3.41 ± 0.24 [b]
LENP3	0.99 ± 0.06 [a]	0.97 ± 0.01 [a]	0.94 ± 0.02 [a]	4.66 ± 0.41 [a]
LENP0	1.0 [a]	1.0 [a]	1.0 [a]	1.0 [c]

Values are Means ± SEM, n = 3/replicate. Different letters indicate significant differences ($P< 0.05$).

DISCUSSION

The present study provides a comprehensive assessment of LENP effects on *O. niloticus*, addressing several gaps in the existing literature. This research integrates physiological and molecular parameters, including growth performance, immune

function, antioxidant capacity, digestive enzyme activity, and gene expression, offering a holistic view of LENP's impact on tilapia health and performance.

The administration of nano-*L. obtusa*-supplemented feed resulted in a significant reduction in FCR, suggesting that the nano-seaweed diet may enhance feed efficiency despite not affecting growth parameters. These findings are consistent with **Baki *et al.* (2018)**, who reported that incorporating *L. obtusa* into *O. mykiss* feed reduced growth performance and feed utilization. Similarly, **Hoseinifar *et al.* (2018)** observed no significant differences in growth performance among *Danio rerio* fed red seaweed (*Gracilaria gracilis*) powder at 0.25, 0.5, and 1% for 8 weeks. Conversely, **Khalafalla and El-Hais (2015)** found that *Pterocladia capillacea* significantly enhanced growth rate and feed efficiency in *O. niloticus* at inclusion rates of 2.5% and 5%. This discrepancy may be attributed to differences in algal species and supplementation rates. Furthermore, **Salem *et al.* (2021)** reported optimal growth performance in the red tilapia fingerlings fed *L. obtusa* at 2g kg^{-1}, followed by 4g kg^{-1}, for 70 days.

The observed growth reduction in some cases may be due to the antifouling properties and chemical defense mechanisms of seaweed, including active metabolites such as halogenated terpenoids (e.g., elatol) with potential biological effects, or substantial levels of antinutritional compounds **(König & Wright, 1997; Pereira *et al.*, 2003; O'Sullivan *et al.*, 2010)**. Conversely, growth enhancement may be linked to bioactive metabolites in *Laurencia* spp., such as fatty acids, including oleic acid, palmitoleic acid, palmitic acid, and linolenic acid **(Cvitković *et al.*, 2021; Čagalj *et al.*, 2022)**.

Regarding antioxidant activity, CAT, SOD, and TAC values were significantly higher ($P< 0.05$) than the control, while GPX showed its highest value at the highest LENP concentration. MDA values decreased significantly or non-significantly compared to the control. Similar antioxidant effects were reported by **Zhang *et al.* (2003)** and **Rocha De Souza *et al.* (2007)**, where extracts from *Porphyra haitanensis* and *Padina gymnospora* exhibited strong *in vitro* antioxidant activity and markedly inhibited MDA production by scavenging free radicals such as hydrogen peroxide and superoxide anion, thereby reducing lipid peroxidation products in tissues. **Magnoni *et al.* (2017)** also found elevated CAT levels in sea bream fed *Gracilaria vermiculophylla* under normal experimental conditions. Likewise, **Hossam-Elden *et al.* (2024)** reported that *O. niloticus* fed chitosan nanoparticles for two months had reduced MDA levels and increased TAC compared to fish fed chitosan macromolecules.

Nitric oxide plays a key role in cardiovascular regulation, protecting organisms from hypoxia through vasodilation under low oxygen conditions **(Hansen & Jensen,**

2010). Its significantly lower values in our study further reflect the antioxidant and stress-mitigating potential of LENP diets.

Hematological indices showed marked improvements in RBC count, Hb, and PCV, with the highest significant values recorded in the lowest LENP concentration group. These findings are consistent with those of **Khanzadeh *et al.* (2023)**, who reported significant increases in WBCs and Hb in *O. niloticus* supplemented with *L. caspica* extract at 1% and 2% after 50 days. Similar improvements were observed by **Hossam-Elden *et al.* (2024)** with chitosan nanoparticles.

Protein profile results (albumin, globulin, total protein) improved in a dose-dependent manner, aligning with **Yazdanpanah *et al.* (2021)**, who found elevated albumin levels in *O. mykiss* fed *Gracilaria pulvinata*. Likewise, **Hossam-Elden *et al.*, (2024)** observed significant increases in total protein, globulin, and albumin in *O. niloticus* after 60 days of feeding on nanochitosan-supplemented diets.

The highest IgM levels were found in LENP1 ($P< 0.05$ vs. all groups), while the highest IgG levels occurred in LENP2. Similar immune-enhancing effects have been documented by **Kiadaliri *et al.* (2020)** with *L. caspica* extract in the rainbow trout, **Salem *et al.* (2021)** with *L. obtusa*-supplemented red tilapia diets, and **Radwan *et al.* (2022)** with seaweed extract mixed with CA/bio-AgNPs.

Liver and kidney function analysis showed the highest creatinine values in the lowest LENP concentration, with no significant differences in urea levels among groups. AST was significantly elevated in LENP3, while ALT increased in LENP1 compared to the control. **Salem *et al.* (2021)** reported lower AST and ALT in red tilapia receiving low *L. obtusa* inclusion, likely due to polyphenols improving hepatic function.

For digestive enzyme activity, amylase and lipase were significantly higher in LENP2 (0.5g/ kg) compared to the control. This may be due to bioactive compounds—such as carotenoids, fatty acids, polysaccharides, and amino acids—that enhance feed palatability, stimulate digestive enzyme secretion, and improve nutrient assimilation **(Sattanathan *et al.*, 2020; Abd El-Hamid *et al.*, 2021)**.

In terms of gene expression, hepatic SOD expression was unaffected, GPX was downregulated, IL-10 expression was reduced, and IL-1β was significantly upregulated in all groups. In the spleen, SOD and GPX were significantly downregulated in LENP1 and LENP2, IL-10 was downregulated, and IL-1β was significantly upregulated. These trends are consistent with **Negm *et al.* (2021)**, who observed IL-10 downregulation in *O. niloticus* treated with *Sargassum aquifolium*, and **Elabd *et al.* (2022)**, who reported IL-1β upregulation in *O. niloticus* fed nano–iron oxide diets.

Overall, these findings support the hypothesis that nanoparticle-based seaweed supplementation can enhance immune signaling through improved bioavailability and cellular uptake.

CONCLUSION

In summary, the inclusion of *Laurencia obtusa* extract nanoparticles in the diets of the Nile tilapia juveniles demonstrated notable enhancements in antioxidant activity, immune parameters, and digestive enzyme levels, without adversely affecting growth performance. The significant increases in immunoglobulin levels and various blood parameters, alongside the upregulation of key antioxidant and immune-related gene expression, highlight the potential benefits of LENPs as feed additives. However, further research is needed to examine different inclusion rates to accurately test the effect of LENPs on growth performance for maximizing health benefits while maintaining growth efficiency.

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