2-4-2023

Dietary Methanolic Extract of Fenugreek Enhanced the Growth, Haematobiochemical, Immune Responses, and Resistance against Aeromonas hydrophila in Nile Tilapia, Oreochromis niloticus

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Hindawi
Aquaculture Research
Volume 2023, Article ID 3055476, 13 pages
https://doi.org/10.1155/2023/3055476

Research Article

Dietary Methanolic Extract of Fenugreek Enhanced the Growth, Haematobiochemical, Immune Responses, and Resistance against *Aeromonas hydrophila* in Nile Tilapia, *Oreochromis niloticus*

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Received 19 September 2022; Revised 1 November 2022; Accepted 11 January 2023; Published 4 February 2023

Academic Editor: Hamed Ghafarifarsani

Fenugreek seed (FS) contains abundant functional biomolecules that activate the antioxidative and immunity system of aquatic animals. In this study, the methanolic extract of FS was included in Nile tilapia diets at 0, 0.05, 0.1, 0.15, and 0.2% levels and fed to fish for 90 days. Nile tilapia (10.57 ± 0.14 g) were randomly divided into five triplicate groups to check the growth performance, haematobiochemical profile, immunity, antioxidative response, and tolerance to *Aeromonas hydrophila* infection. The results revealed that the methanolic extract of FS significantly improved the growth performance while reducing the feed conversion ratio (FCR). Methanolic extract of FS also modulated the haematobiochemical profile. Markedly, the lysozyme and phagocytic activities were activated by the dietary methanolic extract of FS. The superoxide dismutase (SOD) activity was improved, while the malondialdehyde (MDA) level was decreased by the dietary methanolic extract of FS. The SOD was markedly increased in the fish-fed dietary methanolic extract of FS at 0.1, 0.15, and 0.2%, while the MDA decreased in the fish fed 0.15 and 0.2%. The transcription of *IL-1β*, *TNF-α*, and *TGF-β* genes showed upregulated expression by ethanolic extract of FS. Accordingly, Nile tilapia showed high resistance against the *A. hydrophila* infection. The regression analysis revealed that the inclusion of 0.09% is recommended to improve the specific growth rate and FCR. In conclusion, the study revealed that an ethanolic extract of FS is recommended to enhance the growth performance, immunity, antioxidative response, and tolerance to *A. hydrophila* in Nile tilapia.

1. Introduction

Nile tilapia (*Oreochromis niloticus*) is one of the most important economic finfish species worldwide, and its production is increasing steadily [1, 2]. However, significant economic losses have been observed in the tilapia aquaculture sector in recent years due to the spread of infectious diseases [3]. As a result of the spread of diseases in tilapia farms, a group of chemical treatments were used on a large scale to prevent pathogens, such as antibiotics [4, 5]. The continued use of these chemicals led to the emergence of what is known as advanced antibiotic resistance and the accumulation of remnants of these chemotherapies in the fish body [6]. Hence, it is necessary to resort to an alternative treatment, such as using natural functional substances to limit the spread of infectious diseases and control the pathogen [7].

Medicinal herbs and their extracts are used as an alternative to antibiotic therapy as an environmentally friendly component that contributes to increasing

productivity in aquaculture [8]. Medicinal herbs and their extracts effectively contribute to digestibility, improvement of growth, improving feed efficiency, and increasing the immune response of aquatic animals [9, 10]. Besides, medicinal herbs and their extracts have been defined as growth promotors with a noticeable effect on increasing growth rates, possibly because it increases the appetite of aquatic animals [11]. Many studies have investigated the potential role of medicinal herbs and their extracts on the growth performance, feed utilization, immunity, and stress resistance of Nile tilapia [12–15]. Besides, Nile tilapia fed dietary medicinal herbs, and their extracts displayed enhanced immunity and disease resistance.

Fenugreek (*Trigonella foenum-graecum*) belongs to the *Fabaceae* family with various functional properties [16]. Fenugreek seeds contain various bioactive compounds, including medicinal alkaloids, sapogenins, and steroid compounds [17]. Pharmaceutically, fenugreek seeds exhibited many valuable effects, such as anti-inflammation, pain reduction, antimicrobial, anticancer, carminative, heart tonic, triglyceride, cholesterol level reduction, and hypertension reduction [17, 18]. Recently, the inclusion of fenugreek seed powder has improved the growth performance and antioxidative and immune responses of Nile tilapia [19, 20]. Further, Yu et al. [21] reported that fenugreek seed extract enhanced the biochemical blood indices, antioxidative capacity, and immune response of blunt snout bream (*Megalobrama amblycephala*).

Aeromonas hydrophila is a Gram-negative fish pathogen causing economic losses in Nile tilapia farming in Belém-Costa and Cyrino [22]. *A. hydrophila* infection is induced by Motile *Aeromonas septicaemia*, which is clinically characterized by the induction of ulcerations, abscesses, ascitic fluid, hemorrhages, and anaemia features [23]. Hence, it is necessary to control the septicaemia infection in Nile tilapia using novel immunostimulants with environmentally friendly effects [24]. In this context, the methanol extract of fenugreek has antibacterial activity against fish pathogens such as *A. hydrophila* and *Pseudomonas liquefaciens* [25]. However, fenugreek seed extracts are still not well investigated in tilapia aquaculture. Hence, the present work proposes using fenugreek seed extracts as novel growth promotors and immune stimulant supplements in Nile tilapia diets.

2. Materials and Methods

2.1. Ethical Approval. The Committee of Aquatic Animal Care and Use in Research, Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University, Egypt, reviewed and approved the experiments (approval number: IAACUC-KSU-26-2021).

2.2. Chemical Extraction of Fenugreek Seeds and Test Diet Preparation. The fenugreek (*Trigonella foenum-graecum*) seeds (FSs) were collected from a private market in Gharbia governorate, Egypt, in 2020, and then FSs were ground to a fine powder. Fenugreek seed extraction was conducted according to [26]. In brief, the FS extract was extracted in methanol (70% methanol aqueous solution, Merck) and agitated in an agitator (Gerstel) for 24 h at 120 × g. Then, the methanolic extract of FS was filtered to remove the insoluble components using a Whatman filter (1 mm). Subsequently, a rotary evaporator (RV4) at 40°C was used to filtrate the methanolic filtered FS before being reconstituted with 1 mL methanol HPLC grade for chromatographic analysis. The FS extract was stored in dark glass bottles at 4°C until use. The methanolic extract of FS was analyzed using gas chromatography-mass spectrometry (GC-MS-MS) (5977A MSD, American), following the method described by Sørensen et al. [27]. The GC-MS framework (Agilent Advances) was prepared with a gas chromatograph (7890B) and mass spectrometer finder (5977A) at Nawah-Scientific Research Centre, Al Mokattam, Cairo, Egypt. The test diets were formulated as indicated in Table 1, with 30.1% protein content and 6.3% lipid content. The diets were mixed with a methanolic extract of FS at doses of 0, 0.05, 0.1, 0.15, and 0.2% of the feed. The chemical composition of the diets was confirmed by following the AOAC [29].

2.3. Fish and Rearing Conditions. Fish was obtained from a private tilapia hatchery in Kafrelsheikh, Egypt, and transported to the laboratory of the Aquaculture Department, Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University, Egypt. Each aquarium has a mechanical filter (cleaned daily) and air stones; half of the aquarium's water was changed daily with new dechlorinated tap water to maintain the water quality parameters within the typical range for Nile tilapia; temperature (26.42 ± 0.36°C), pH (6.67 ± 0.12) and dissolved oxygen (6.62 ± 0.37 mg/l). Water quality parameters were detected daily by using multiparameters probe meter (HI9829-03042-HANNA® instruments, https://www.hannainst.com), and the total ammonia nitrogen (0.26 ± 0.04 mg/L) was estimated using a portable colorimeter (Martini Instrument MI 405, Romania). The light/dark cycle was 12 hours and 12 hours every day. During the adaptation period (two weeks), the fish were fed on a manually formulated basal diet (30.1% crude protein) (Table 1). Fish leftover food and waste produced by fish was siphoned out of the aquaria daily.

Healthy Nile tilapia (*Oreochromis niloticus*) (10.57 ± 0.14 g, $n = 300$) were randomly divided into five groups, three replicates each (15 glass aquaria (70 × 45 × 35 cm), 90 L/aquarium) at 20 fish/aquarium. The control group (T1) received a diet free from the methanolic extract of FS. The 2nd, 3rd, 4th, and 5th groups fed on diets containing extract powder of FS at doses of 0.05, 0.1, 0.15, and 0.2% of feed, respectively (Table 1). The suggested doses of methanolic extract of FS were decided by following Wang et al. [18]. Methanolic extract of FS was dissolved in double-distilled water to get a concentration of 10% w/v, and then sprayed on feed [30]. The fish feeding rate initially was 6% of the fish's body weight until it reached 3% at the end of the study [31]. Feeding rates were recalculated every two weeks based on changes in fish biomass. Nile tilapia were hand-fed twice daily (8:00 and 16:00) for six days a week for 90 days. Daily registers of fish feed intake (FI) were kept.

Table 1: Feed ingredients and proximate analysis of different diets.

Feed ingredients (%)	Methanolic extract of FS (%)				
	0	0.05	0.1	0.15	0.2
Soybean meal (47% Cp)	37	37	37	37	37
Fishmeal (65% Cp)	4	4	4	4	4
Yellow corn (7.5% Cp)	19.4	19.4	19.4	19.4	19.4
Fish oil	0.7	0.7	0.7	0.7	0.7
Soy oil	0.7	0.7	0.7	0.7	0.7
Rice bran	11.2	11.2	11.2	11.2	11.2
Wheat bran	16.5	16.45	16.4	16.35	16.3
Corn gluten	6	6	6	6	6
Di calcium phosphate	0.6	0.6	0.6	0.6	0.6
Poultry-by meal (60% CP)	3.5	3.5	3.5	3.5	3.5
Vitamins and minerals mixture*	0.3	0.3	0.3	0.3	0.3
Choline	0.05	0.05	0.05	0.05	0.05
STAY c-35**	0.05	0.05	0.05	0.05	0.05
Fenugreek seed extract	0	0.05	0.1	0.15	0.2
Proximate composition (%) (on DM basis)					
Dry matter	90.15	90.22	90.28	90.23	90.31
Crude protein	30.1	30.08	30.11	30.13	30.14
Ether extract	6.38	6.31	6.34	6.37	6.39
Fiber	5.12	5.14	5.12	5.13	5.12
Ash	6.15	6.13	6.12	6.14	6.15
Carbohydrate	42.4	42.56	42.59	42.46	42.51
Gross energy (KJ/g)***	18.61	18.59	18.61	18.61	18.62

*Vitamin and mineral mixture (mg/kg of the premix): vitamin B_1 (150 mg), vitamin B_2 (700 mg), vitamin B_6 (500 mg), vitamin B_{12} (65 mg), biotin (8000 mg), vitamin A (3000 IU), vitamin D3 (550 IU), vitamin E (2950 mg), inositol (300 mg), para-aminobenzoic acid (7500 mg), niacin (30 mg), pantothenic acid (2500 mg), manganese (400 mg), copper (60 mg), iron (300 mg), cobalt (8 mg), iodine (8 mg). **STAY c-35: extrusion stable vitamin c for DSM Company (the phosphorous salt of ascorbic acid). ***Gross energy was calculated based on the values for protein, lipid, and carbohydrate as 23.6, 39.5, and 17.2 KJ/g, respectively, according to [28].

2.4. Final Sampling and Growth Performance Calculation. At the end of the experiment, fish were anesthetized with tricaine methane sulfonate (MS222, 25 mg/L, Argent Laboratories, Redmond, Washington) to get the individual weight and length (L) of each fish. Fish growth performance and somatic indices were estimated according to Ghazi et al. [32]. The other growth performance parameters and feed utilization were calculated as follows:

$$\text{Weight gain ratio (WG\%)} = (W1 - W0)/W0 \times 100$$

$$\text{Feed conversion ratio (FCR)} = \text{feed intake (g)/BWG (g)}$$

$$\text{Specific growth rate (SGR\%/day)} = 100 \times (\ln W1 - \ln W0)/t$$

$$\text{Protein efficiency ratio (PER)} = (\text{BWG(g)/protein intake}) \times 100$$

$$\text{Condition factor (K)} = 100 \times (W1/L^3)$$

$$\text{Viscerosomatic index (VSI)} = 100 \times (\text{intestine weight}/W1)$$

$$\text{Hepatosomatic index (HSI)} = 100 \times (\text{liver weight}/W1)$$

$$\text{Survival rate (SR\%)} = (\text{total number of fish at the end of the experiment/total number of fish at the start of the experiment}) \times 100$$

where ln = natural log

$W1$ = final weight at the end of the experiment (g)

$W0$ = initial weight (g)

L = length of fish (cm)

"t" is the experimental period (days)

Six fish per treatment were selected for proximate analysis of the body tissues from the same fish used for gene sampling (dry matter, crude protein, total lipid, crude fiber, ash, and energy content) according to the standard methods of the AOAC [29].

Blood samples (9 fish/treatment) were obtained using plastic syringes from the caudal vein. Half the blood samples were ejected into heparinized tubes, and the second part was kept in a 2-mL sterile Eppendorf tube without anticoagulants for serum separation after centrifugation at 3000 rpm for 15 minutes at 4°C [33].

2.5. Hematology and Blood Biochemical Analyses. An automatic blood cell counter was used to determine the number of red blood cells (RBCs), haemoglobin content, and packed cell volume (PCV). The numbers of leukocytes in the blood smear × erythrocytes quantified in the haemocytometer/7000 erythrocytes in the blood smear [34]. For the assessment of differential white blood cell count, two thin smears from each blood sample were made and air-dried on clean microscope slides. A modified version of Wright's stain was used to stain smears before slipping the cover. A total of 100 cells were counted under × 100 oil immersion lenses, and the percentages of heterophils, lymphocytes, and monocytes were computed.

Total proteins were assessed using a commercial colorimetric kit (TP0100, Sigma-Aldrich, St. Louis, MO, USA). Albumin was appreciated using the bromocresol green binding method [35], globulin was estimated mathematically, creatinine levels were determined by the colorimetric method [36], the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were estimated by the colorimetric method at the wavelength of 540 nm according to Diab et al. [37], and alkaline phosphatase (ALP), serum triglyceride, and total cholesterol were determined according to Reitman [38].

2.6. Immune and Oxidative Stress Responses. Phagocytic activity and index were determined according to Wang et al. [39]; whereas phagocytic activity = phagocytic cell containing yeast/total number of phagocytic cell × 100 and phagocytic index = number of cells phagocytized/number of phagocytic cells. The serum lysozyme activity was estimated by ELISA, according to Demers and Bayne [40]. Superoxide dismutase (SOD) and malondialdehyde (MDA) were determined using ELISA kits (Inova Biotechnology, China) at the wavelength of 450 nm using the microplate ELISA reader [41].

2.7. Expression of Studied Immune-Related Genes. RNA extraction was performed using liver tissue samples (6 fish/treatment) from the same fish used for blood sampling using

TriZol® reagent (iNtRON Biotechnology, South Korea). Quality and quantity assurance of the obtained RNA were checked with a nanodrop spectrophotometer (BioDrop®, USA) depending on the A260/A280 ratio, and then the integrity of the RNA was checked by gel electrophoresis. Random RNA samples were run on an agarose gel with an ethidium bromide (EtBr) stain to assure its integrity [42]. The presence of sharp, high-intensity bands of 28S and 18S rRNA was considered evidence of integrity, with the 28S rRNA band being approximately twice as intense as the 18S rRNA band [43].

For cDNA synthesis, two μg of RNA from each sample was reversely transcribed using Maxime RT PreMix™ (iNtRON Biotechnology, South Korea) following the manufacturer's protocol [44]. Gene expression profile was conducted in the Mic-qPCR® thermocycler (Bio-molecular systems, Australia) using the SensiFast SYBR No-Rox kit™ (Bioline, UK) with *Oreochromis niloticus* specific primers (Table 2) according to the manufacture instructions. Dissociation curve analyses were detected for the specificity of the PCR product determination. Reaction efficiency was calculated based on the slope using the formula: efficiency = $10^{(-1/\text{slope})} - 1$. A geometric average of two reference genes (18s rRNA and β-actine) was used to standardize quantitative RT-PCR data. The relative expression of profiled genes was calculated based on the $2^{-\Delta\Delta CT}$ method [45].

2.8. Experimental Bacterial Challenge. After 90 days feeding experiment, 15 fish per treatment (5 fish/aquarium) were challenged by *Aeromonas hydrophila* (ATCC-13037, Microbiological Resources Centre (Cairo MIRCEN). Fish were intraperitoneally injected (IP) with 0.2 ml of a suspension containing 1×10^8 CFU/ml of *A. hydrophila* [19]. During the 14 days of the observation period the mortality was recorded according to Naiel et al. [48]. During the observation period, fish were fed their corresponding diets according to their nutritional trail. The commutative survival rate was calculated according to the following equation:

Survival% = (Total number of fish at the end of the experimental bacterial challenge/total number of fish at the start of the experimental bacterial challenge) × 100.

2.9. Statistical Analysis. The Shapiro–Wilk normality test checked data distribution normality. Before processing percentage data, an arcsine transformation was employed. GraphPad Prism 9 (GraphPad Prism v9.0, San Diego, CA, USA) was used for data analysis, and all present results were expressed as means with a standard error of the mean (SEM). A one-way ANOVA was used to compare between different treatments. Tukey's multiple comparisons were used as a post hoc test where appropriate. The significance level was established at $P \leq 0.05$. The survival data distribution from the challenge trial was evaluated using Kaplan–Meier curves (Mantel–Cox test) along the two-week challenge period, as well as significant differences between groups at P value ≤0.05. To interpolate the optimally performing doses of methanolic extract of FS to promote the SGR and FCR in Nile tilapia, polynomial regressions were conducted.

3. Results

3.1. Methanolic Extract of Fenugreek Seeds Characterization. Methanolic extract of fenugreek seeds analysis by GC-MS-MS revealed the presence of 36 bioactive compounds, with the most superior compounds illustrated in Table 3 and Figure 1. The highest peak area (%) of MEFS components was methyl sulfate, while the lowest was decanoic acid. The detected primary phytochemical components have a lot of biological activities, but there have been few studies on fish.

3.2. Growth Performance and Feed Utilization. Growth performance and feed utilization parameters revealed significant differences between different treatments and control groups in all measured parameters except for fish length, liver weight, and the hepatosomatic index (Table 4). The best growth performance results were demonstrated in fish fed 0.1% of methanolic extract of FS (final weight, body weight gain, weight gain ratio, FCR, ADG, SGR, and PER) (P value are 0.0001) and condition factor (P value is 0.029) but 0.2% of methanolic extract of FS demonstrated the worse results compared to the other treatments. Polynomial regression curves interpolate the optimum growth-performing dose in terms of FCR and SGR are 0.09 and 0.085%, respectively (adjusted $R^2 = 0.96$ and 0.90, respectively) (Figure 2).

3.3. Chemical Analysis of Fish. Fish whole-body proximate analysis (as %) of all Nile tilapia treated groups at day zero and after 90 days of the experiment revealed no significant difference compared to the control group except for moisture content, dry matter, and crude protein (P values are 0.018, 0.036 and 0.038, respectively). The best results were demonstrated in fish fed 0.2% of a methanolic extract of FS for both dry matter and crude protein (Table 5).

3.4. Haematological and Biochemical Blood Analyses. The analyses of hematological parameters are tabulated in (Table 6), revealing that fish fed 0.2% of methanolic extract performed ultimately for RBCs, Hb, PCV, WBCs, and lymphocytes (P values are 0.007, 0.003, 0.003, 0.0005, and 0.0001, respectively), comparing to the control group. MCV, MCHC, monocytes, basophile, and eosinophil counts revealed no significant differences between the experimental groups.

The haematobiochemical analysis of Nile tilapia fed different doses of MEFS are not significantly differ ($P > 0.05$) compared to the control group in all measured parameters except for total protein, albumin, and globulin (P values are 0.002, 0.028, and 0.010, respectively), with the ultimate result being demonstrated in fish fed 0.15 and 0.2% of methanolic extract of FS for both total protein and globulin (Table 7).

3.5. Immune and Oxidative Stress Responses. Lysozyme activity, phagocytic activity as well as superoxide dismutase (SOD) and malondialdehyde (MDA) significantly differ among groups (P values are 0.019, 0.009, 0.0003, and 0.0004, respectively), with the higher values were reported in fish fed 0.2% of a methanolic extract of

Table 2: Primer used for qPCR amplification.

Gene	Sequence (5′–3′)	A.S.* (bp)	A.T.** (°C)	Amp. eff*** (%)	Accession no.	References
Housekeeping genes						
β-actin F	CAGCAAGCAGGAGTACGATGAG	135	61.3	94.4	XM_019351010.2	[44]
β-actin R	TGTGTGGTGTGTGGTTGTTTTG					
18s rRNA F	GGACACGGAAAGGATTGACAG	110	58.8	96.1	JF698683.1	[42]
18s rRNA R	GTTCGTTATCGGAATTAACCAGAC					
Immune-related genes						
TNF-α F	GGAAGCAGCTCCACTCTGATGA	137	61	93.4		[46]
TNF-α R	CACAGCGTGTCTCCTTCGTTCA					
IL-1β F	CAAGGATGACGACAAGCCAACC	149	59	96.7	XM_019365844.2	[43]
IL-1β R	AGCGGACAGACATGAGAGTGC					
TGF-β F	TATCTGGGATGCCGAAAAC	120	55	93.8	NM_001311325.1	[47]
TGF-β R	GCAGTGGCTCTAGTGTCTGT					

A.S.*: amplicon size, A.T.**: annealing temperature, Amp. Eff***: amplification efficiency.

FS compared to other treatments, while there were no significant differences between all treatments in the phagocytic index (Table 8).

3.6. Expression of Immune-Related Genes. Gene expression profiling demonstrated a significant difference ($P < 0.05$) between the transcription levels of the studied immune-related genes in the liver of fish fed different dietary levels of MEFS compared to the control group (Figure 3). The highest upregulated expression level of interleukin 1 beta (*IL-1β*) and tumor necrosis factor alpha (*TNF-α*) genes was observed in fish fed 0.2% of methanolic extract of FS with (P value are 0.0001 and 0.027, respectively) fold increase relative to the control group. *TGF-β* showed a significant difference between the methanolic extract of FS-treated groups and the control group (P value <0.0001).

3.7. Bacterial Challenge and Survival Rates. During the bacterial challenge trial, the survival rates of different groups of fish were recorded (Figure 4). Mortalities of *O. niloticus* challenged with *Aeromonas hydrophila* were significantly lower in all treated groups than in the control group (P value is 0.015). Fish fed 0.2% of methanolic extract of FS showed the highest commutative survival rate (100%) compared with other treatments.

4. Discussions

In aquaculture, herbal extracts showed powerful growth-promoting, antistress, and immune-stimulation activities [49]. It contains functional bioactive compounds, steroids, essential fatty acids, essential oils, flavonoids, glycosides, and phenolics [50]. Moreover, herbal extracts are more ecofriendly and cost-effective than synthetic antibiotics because they are less likely to induce drug resistance in environmental and intrinsic microbes [51]. The present study showed significant improvements in the growth performance and feed efficiency in Nile tilapia treated with a methanolic extract of FS in a dose-dependent manner. The results also showed improved immune and antioxidative responses as well as high resistance to *Aeromonas hydrophila* infection in Nile tilapia. The obtained results confirm the hypothesis of the necessity of adding herbal extracts to sustain the productivity of finfish species [52]. In the same line, Yu et al. [21] reported that fenugreek seed extract enhanced the biochemical blood indices, antioxidative capacity, and immune response of blunt snout bream.

Dietary supplementation of methanolic extract of FS significantly improved the growth performance of Nile tilapia. These results are in line with Abbas et al. [11]; who reported an improvement in growth parameters in Nile tilapia fed the fenugreek seed powder. Further, Awad et al. [53] stated that gilthead seabream (*Sparus aurata*) fed dietary fenugreek had enhanced growth performance. The feed conversion ratio (FCR) is a good tool to measure the acceptability of fish for formulated feed. The results showed improved FCR in fish treated with methanolic extract of FS, which implies enhanced feed utilization and absorption by fish intestines. Hence, fish treated with methanolic extract of FS could exhibit high metabolic function and, thereby, growth performance. Indeed, including fenugreek seed revealed enhanced intestinal functionality and high antibacterial activity that cope with harmful intestinal microorganisms [16]. Fenugreek seeds contain high protein and mineral contents that stimulate fish's digestive and absorption properties [54]. In addition, Awad et al. [53] reported that fenugreek seed powder could enhance the feed utilization of sea bream by protecting intestinal morphology. Moreover, the methanolic extract of FS contains flavoring agents such as furfuol, trisulfide, dimethyl, 2,2,6-trimethyl-6-(4-methyl-3-tetrahydro-2H-pyran-3-Ol, bisabolol oxide L), 2-methylamino, benzoic acid, methyl ester [21] which may enhance feed palatability.

The hematological profile is an essential indicator for assessing a fish's nutritional and health status [33]. The results showed rising RBCs, Hb, PCV, and MCH in fish-fed diets supplemented with a methanolic extract of FS. The improved values of these parameters revealed the ability of the methanolic extract of FS to stimulate hematopoiesis. The

Table 3: GC-MS-MS profile of FSME.

No.	The compound	Molecular formula	Retention time (min)	Peak area (%)	Molecular weight (*m*/*z*)
1	Dimethoxysulfone, methyl sulfate	$C_2H_6O_4S$	8.02	12.25	126
2	2-Furanmethanol, furfuol	$C_5H_6O_2$	9.26	9.65	98
3	Benzo[B]thiophen-2-amine, N,N-dimethyl-3-phenyl-	$C_{16}H_{15}NS$	7.67	8.63	253
4	Propanedioic acid, [2-[(4-methylphenyl) sulfonate Yl] ethylidene]-, dimethyl ester	$C_{14}H_{16}O_6S$	6.64	5.16	312
5	10,11-Dihydroxy-3,7,11-time thyl-2,6-dodecadienyl acetate	$C_{17}H_{30}O_4$	23.08	5.14	298
6	Trisulfide, dimethyl	$C_2H_6S_3$	6.02	4.64	126
7	2,2,6-Trimethyl-6-(4-meth Yl-3- tetrahydro- 2H-pyran-3-Ol, bisabolol oxide L)	$C_{15}H_{26}O_2$	25.08	4.37	238
8	1,2-Benzenedicarboxylic acid, diethyl ester	$C_{12}H_{14}O_4$	21.69	4.29	222
9	Propanedioic acid, [2-[(4-methylphenyl) sulfonate Yl] ethylidene]-, dimethyl ester	$C_{14}H_{16}O_6S$	6.25	4.05	312
10	2-Methylamino, benzoic acid, methyl ester	$C_9H_{11}NO_2$	17.08	3.96	165
11	Late 4-aminoestradiol-3-methyl ether	$C_{19}H_{27}NO_2$	14.51	2.64	301
12	Cyclohexasiloxane, dodecamethyl-	$C_{12}H_{36}O_6Si_6$	15.20	2.33	444
13	4-4Methylphenyl pentanal	$C_{12}H_{16}O$	22.09	1.51	176
14	Decanoic acid	$C_{10}H_{20}O_2$	16.16	0.64	172

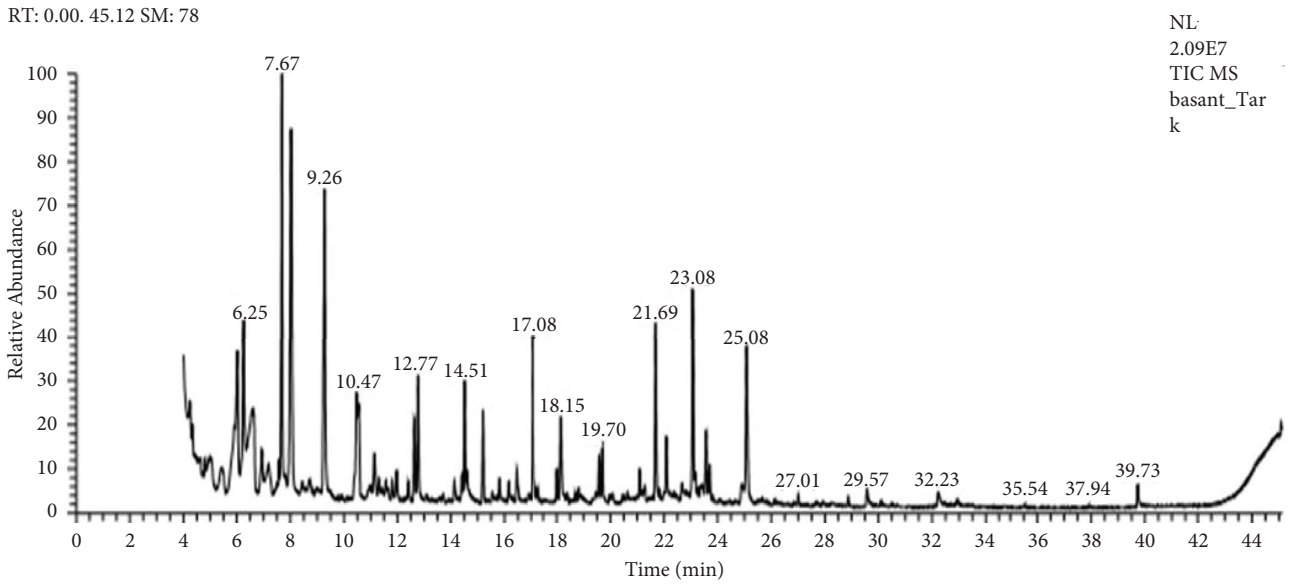

Figure 1: GC-MS-MS chromatogram of MEFS.

Table 4: Growth performance and somatic indices (mean ± SEM) of Nile tilapia after 90 days of feeding on methanolic extract of fenugreek seed (FS).

Items	Methanolic extract of FS (%)					*P* value
	0	0.05	0.1	0.15	0.21	
Initial body weight (g)	10.39 ± 0.14	10.71 ± 0.14	10.53 ± 0.15	10.58 ± 0.14	10.65 ± 0.14	0.5547
Final body weight (g)	39.26 ± 0.20c	43.51 ± 0.22b	46.42 ± 0.18a	42.31 ± 0.19b	36.77 ± 0.17d	<0.0001
Weight gain ratio (%)	277.86 ± 3.57c	306.26 ± 4.17b	340.84 ± 5.66a	299.91 ± 4.65b	245.26 ± 3.71d	<0.0001
Feed conversion ratio	1.50 ± 0.01b	1.32 ± 0.01c	1.21 ± 0.01d	1.37 ± 0.01c	1.66 ± 0.01a	<0.0001
Specific growth rate (%/day)	1.48 ± 0.01c	1.56 ± 0.01b	1.65 ± 0.01a	1.54 ± 0.01 b	1.38 ± 0.01d	<0.0001
Protein efficiency ratio	2.21 ± 0.01c	2.51 ± 0.11db	2.75 ± 0.01a	2.43 ± 0.01 b	2.00 ± 0.01d	<0.0001
Condition factor	1.670 ± 0.041	1.63 ± 0.03	1.515 ± 0.07	1.484 ± 0.06	1.531 ± 0.02	0.0293
Visceralsomatic index (%)	3.97 ± 0.02b	3.74 ± 0.02c	3.74 ± 0.03c	4.23 ± 0.02a	4.29 ± 0.02a	<0.0001
Hepatosomatic index (%)	1.99 ± 0.0	1.88 ± 0.0	1.79 ± 0.0	1.82 ± 0.0	2.23 ± 0.0	0.9296
Survival rate (%)	100	100	100	100	100	—

Means within each raw of different superscripts are significantly different at $P < 0.05$.

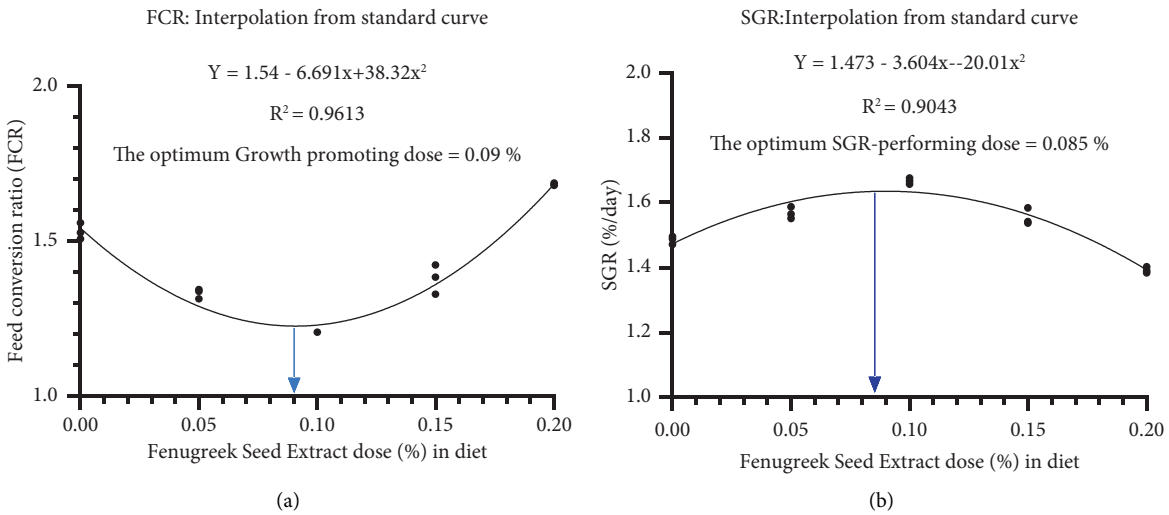

Figure 2: Interpolation of optimum growth-performing doses (A: FCR, B: SGR) of Nile tilapia fed methanolic extract of fenugreek seed as demonstrated by second-order polynomial regression curve. Optimum-performing dose, regression equation, and adjusted R^2 are given for each curve.

Table 5: Whole body composition analysis (mean ± SEM) of Nile tilapia at zero-day and after 90 days of feeding on methanolic extract of fenugreek seed (FS).

Proximate composition (%)	At day zero	Methanolic extract of FS (%)					P value
		0	0.05	0.1	0.15	0.2	
Moisture	75.78	75.81 ± 0.16a	74.87 ± 0.11b	75.02 ± 0.13ab	74.95 ± 0.23ab	74.58 ± 0.21b	0.018
Dry matter	24.22	24.19 ± 0.22b	25.13 ± 0.06ab	24.98 ± 0.073ab	25.05 ± 0.17ab	25.42 ± 0.16a	0.0364
Crude protein	13.08	13.11 ± 0.17ab	12.88 ± 0.12b	13.33 ± 0.133ab	13.61 ± 0.18ab	13.68 ± 0.16a	0.0381
Crude fat	2.58	2.60 ± 0.26	2.61 ± 0.16	2.64 ± 0.19	2.69 ± 0.14	2.680 ± 0.12	0.9948
Ash	3.8	3.89 ± 0.21	3.83 ± 0.06	3.85 ± 0.1	3.62 ± 0.04	3.780 ± 0.1	0.137
Carbohydrate	4.75	4.63 ± 0.12	4.89 ± 0.12	4.40 ± 0.11	4.30 ± 0.16	4.080 ± 0.16	0.0513

Means within each raw of different superscripts are significantly different at $P < 0.05$.

Table 6: Hematological indices (mean ± SEM) of Nile tilapia after 90 days of feeding on methanolic extract of fenugreek seed (FS).

Items	Methanolic extract of FS (%)					P value
	0	0.05	0.1	0.15	0.2	
RBCs ($10/mm^3$)	2.58 ± 0.02c	2.76 ± 0.04b	2.77 ± 0.07b	2.78 ± 0.05b	2.91 ± 0.03a	0.0073
Hb (g/100 ml)	7.76 ± 0.05c	8.21 ± 0.17b	8.34 ± 0.12b	8.41 ± 0.21b	8.92 ± 0.10a	0.003
PCV (%)	24.67 ± 0.33c	26.67 ± 0.33b	27.00 ± 0.58b	27.33 ± 0.67b	28.33 ± 0.33a	0.0034
MCV (fL)	95.60 ± 0.59	96.50 ± 0.67	97.34 ± 0.37	98.33 ± 0.60	97.26 ± 0.51	0.0557
MCH (pg)	30.11 ± 0.04a	29.71 ± 0.29b	30.10 ± 0.11a	30.28 ± 0.22a	30.64 ± 0.07a	0.0386
MCHC (g/dL)	31.28 ± 0.05	30.78 ± 0.33	30.93 ± 0.22	30.79 ± 0.05	31.51 ± 0.15	0.0832
WBCs ($10/mm^6$)	15.64 ± 0.34c	16.79 ± 0.24b	16.82 ± 0.29b	17.08 ± 0.57b	19.40 ± 0.36a	0.0005
Lymphocyte (10^3)	11.31 ± 0.19b	13.14 ± 0.12b	12.84 ± 0.18ab	13.60 ± 0.41b	15.84 ± 0.28a	<0.0001
Monocyte (10^3)	1.20 ± 0.05	1.39 ± 0.05	1.12 ± 0.06	1.37 ± 0.14	1.49 ± 0.18	0.1997
Basophile (10^3)	0.26 ± 0.05	0.17 ± 0.10	0.28 ± 0.15	0.17 ± 0.01	0.06 ± 0.07	0.479
Eosinophil (10^3)	0.15 ± 0.01	0.11 ± 0.06	0.22 ± 0.06	0.11 ± 0.11	0.13 ± 0.07	0.7676
Neutrophil (10^3)	2.71 ± 0.16a	1.960 ± 0.17b	2.35 ± 0.09b	1.82 ± 0.17c	1.88 ± 0.09c	0.0051

Means within each raw of different superscripts are significantly different at $P < 0.05$.

Table 7: Biochemical analysis (mean ± SEM) of Nile tilapia after 90 days of feeding on methanolic extract of fenugreek seed (FS).

Items	Methanolic extract of FS (%)					P value
	0	0.05	0.1	0.15	0.2	
ALT (U/L)	28.38 ± 0.42	28.78 ± 0.54	27.34 ± 0.45	28.68 ± 0.38	27.88 ± 1.09	0.4381
AST (U/L)	30.48 ± 0.50	31.99 ± 1.96	29.12 ± 0.89	29.60 ± 0.33	29.19 ± 0.79	0.3419
ALP (U/L)	39.99 ± 0.12	40.52 ± 0.37	39.04 ± 0.89	37.49 ± 1.79	35.50 ± 2.67	0.1983
Total protein (g/dL)	3.19 ± 0.11d	3.38 ± 0.06b	3.55 ± 0.04b	3.61 ± 0.02a	3.68 ± 0.07a	0.0023
Albumin (g/dL)	1.32 ± 0.03c	1.35 ± 0.03c	1.48 ± 0.01a	1.41 ± 0.02b	1.42 ± 0.05b	0.0289
Globulin (g/dL)	1.87 ± 0.10c	2.02 ± 0.05b	2.07 ± 0.05b	2.19 ± 0.03a	2.26 ± 0.05a	0.0106
Triglyceride (mg/dL)	78.83 ± 4.04	79.34 ± 5.68	88.75 ± 3.47	88.62 ± 6.07	94.75 ± 5.60	0.2031
Cholesterol (mg/dL)	102.2 ± 0.85	99.90 ± 0.71	94.33 ± 2.04	94.90 ± 7.72	93.03 ± 3.06	0.4279
Urea (mg/dL)	4.93 ± 0.11	3.87 ± 0.5	3.93 ± 0.11	4.34 ± 0.58	4.05 ± 0.62	0.4806
Creatinine (mg/dL)	0.31 ± 0.02	0.29 ± 0.02	0.24 ± 0.02	0.30 ± 0.01	0.28 ± 0.01	0.0701

Means within each raw of different superscripts are significantly different at $P < 0.05$.

Table 8: Immune and antioxidative stress responses (mean ± SEM) of Nile tilapia after 90 days of feeding on methanolic extract of fenugreek seed (FS).

Items	Methanolic extract of FS (%)					P value
	0	0.05	0.1	0.15	0.2	
Lysozyme activity (unit/mL)	16.64 ± 0.35c	17.33 ± 0.58c	17.81 ± 0.77b	18.02 ± 0.10b	19.49 ± 0.26a	0.019
Phagocytic activity (%)	11.07 ± 0.12c	11.12 ± 0.51c	12.04 ± 0.83b	13.77 ± 0.77a	13.91 ± 0.16a	0.0096
Phagocytic index	0.96 ± 0.06	0.99 ± 0.08	1.08 ± 0.05	1.20 ± 0.03	1.12 ± 0.1	0.6961
Superoxide dismutase (SOD) (IU/L)	8.75 ± 0.27c	9.53 ± 0.28bc	9.80 ± 0.42b	10.06 ± 0.11b	11.67 ± 0.21a	0.0003
Malondialdehyde (MDA) (IU/L)	17.73 ± 0.57a	13.44 ± 0.15b	13.37 ± 0.50b	11.98 ± 0.73c	11.67 ± 0.97c	0.0004

Means within each raw of different superscripts are significantly different at $P < 0.05$.

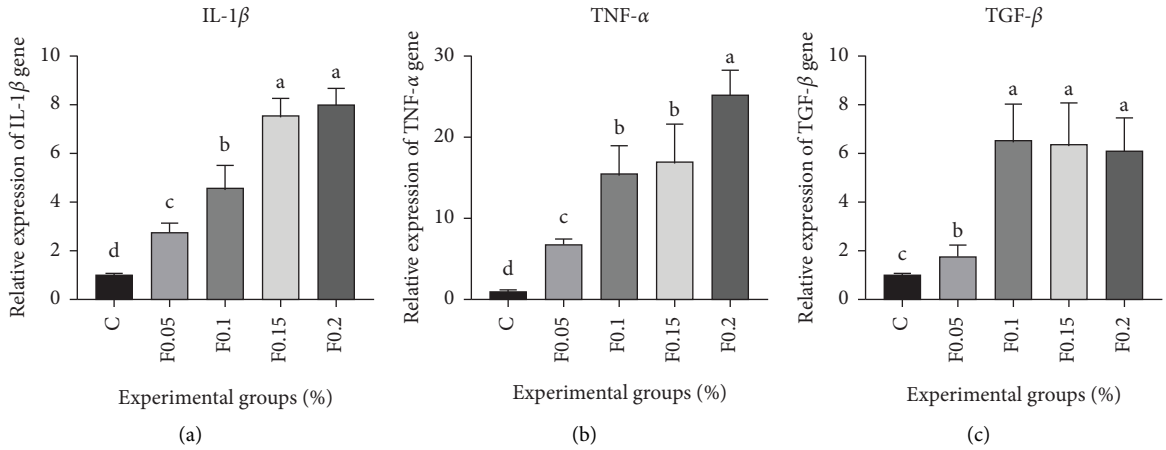

Figure 3: The relative expression profile of the immune-related genes interleukin-*lβ* (*IL-1β*), tumor necrosis factor-*α* (*TNF-α*), and transforming growth factor-*β* (*TGF-β*) genes of Nile tilapia fed different dietary levels of methanolic extract of fenugreek seed (FS). Values are expressed as mean ± SE from triplicate groups and significance letters demonstrated the significant statistical differences at $P < 0.05$.

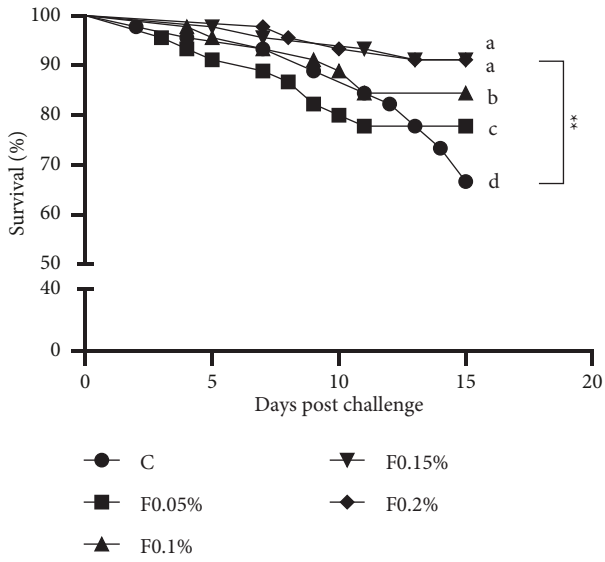

Figure 4: Kaplan–Meier (log-rank Mantel–Cox) curves demonstration of cumulative survival of Nile tilapia fed methanolic extract of fenugreek seed (FS) at 2 weeks postchallenge with 0.2 ml and 1×10^8 CFU of *Aeromonas hydrophila*. Each curve represents the average results of three parallel tanks holding 5 fish/tank, small letters indicate difference significance between groups ($P < 0.05$) as determined by log-rank (Mantel–Cox) test.

results are compatible with Basha et al. [55] and Kumar et al. [56]; who found that fenugreek powder-enriched diets significantly increased PCV, HB, RBCs, and MCV in juvenile tilapia and carp, respectively. Besides the hematological indices, the biochemical blood variables are necessary to reveal the metabolic and physiological condition of fish treated with feed additives. In this study, the biochemical profile suggested the safe use of the methanolic extract of FS for Nile tilapia due to the absence of undesired effects of the methanolic extract of FS on ALT, AST, triglyceride, cholesterol, urea, and creatinine biomarkers. The results agree with Mousallamy and Samir [57]; who stated that Nile tilapia-fed fenugreek seeds meal displayed normal blood biochemical values. Further, the results indicated that methanolic extract of FS improved serum total protein and globulin levels, and this improvement increased by increasing the methanolic extract of FS concentration in the fish diet. These results are consistent with Zenhom and Ibrahim [30]; who reported that common carp fed on dietary supplementation with fenugreek seeds had significantly high levels of serum total protein and globulin. The high blood protein profile in Nile tilapia indicates enhanced protein metabolism and entire-body immunity [58].

The literature is rich in reports indicating fenugreek's immunostimulation and antioxidative roles [59–61], which are illustrated in the current study. The study also revealed a marked enhancement of the phagocytic and lysozyme activities in Nile tilapia by a dietary methanolic extract of FS. Similarly, Moustafa et al. [19] reported that dietary fenugreek seed powder improved the lysozyme activity in Nile tilapia. These results may be related to the immune-boosting functionality of fenugreek-derived biomolecules such as 4-aminoestradiol3-methyl ether [62], cyclohexasiloxane [63], decanoic acid [64], propenoic acid [65], and dicarboxylic acid [66].

The extracts of medicinal herbs known for their antioxidative capacity are involved in mitigating the oxidative stress impacts on fish [12, 67]. The high formation of free radicals results from biotic and abiotic stressors in aquatic animals and can be expressed by detecting malondialdehyde (MDA) levels [68]. Over formation of lipid peroxides causes the activation of antioxidative enzymes such as superoxide dismutase (SOD) to overcome the lipid peroxidation involved in the cellular damage [69, 70]. Concurrently in the current study, we evaluated the MDA level and SOD activity to detect the antioxidative capacity of an ethanolic extract of FS in Nile tilapia. The results revealed that SOD was increased while MDA was decreased by the dietary ethanolic extract of FS in Nile tilapia. Similarly, Yu et al. [21]; who stated improved antioxidative capacity in blunt snout bream by dietary FS extract. Further, enhanced antioxidative

capacity was reported in rainbow trout (*Oncorhynchus mykiss*) fed oak acorn extract [71] and channel catfish (*Ictalurus punctatus*) fed oregano essential oil [72]. Indeed, FS contains several functional nutrients, such as trisulfide [73], bisabolol oxide l [74], diethyl ester [75], and propanedioic acid [76], which are involved in eliminating free radicals and activating the antioxidative capacity [77]. Interestingly, the activated antioxidative capacity of Nile tilapia by dietary methanolic extract of FS may explain the enhanced immunity since increased-free radicals involved in oxidative stress disrupts the immune cells. However, enhanced SOD in this study may lead to high lysozyme and phagocytic activities due to the methanolic extract of FS.

The detection of immune-related genes may draw a precise overview of the effects of the methanolic extract of FS on fish immunity. In the current study, anti-inflammatory (*IL-1β* and *TNF-α*) and proinflammatory-related genes (*TGF-β*) were upregulated in tilapia-fed methanolic extract of FS. The enhanced expression of *IL-1β*, *TNF-α*, and *TGF-β* genes indicated that dietary methanolic extract of FS can regulate the immune response of Nile tilapia [78]. The results agreed with Moustafa et al. [19]; who reported that fenugreek seed powder improved the gene expressions of *IL-1β* and *TNF-α*. Further, Yu et al. [21] indicated that dietary extract of FS upregulated the *TGF-β* in blunt snout bream. The enhancement of inflammation-related gene expression is related to the anti-inflammatory and antiallergic roles of flavonoids and polyphenols, which are abundantly present in fenugreek seed and its extract.

It is necessary to evaluate the tolerance of finfish species against possible pathogenic infections to have a clear overview of the functionality of additives [79, 80]. *A. hydrophila* severely hits tilapia farms and causes massive mortality and economic loss [81]. In this study, the tolerance of Nile tilapia against *A. hydrophila* infection was increased by a dietary ethanol extract of FS. In the same manner, fenugreek supplementation increased the tolerance of Nile tilapia to *A. hydrophila* [11, 19]. The high resistance of Nile tilapia is related to activated immunity and antioxidative responses resulting from ethanolic extract of FS supplementation. The increased lysozyme activity in the current study could deactivate the peptidoglycans of *A. hydrophila*, leading to high resistance [82, 83]. Besides, phagocytosis develops high resistance through the production of anti-inflammatory and proinflammatory factors [84], which is fully recognized in the current study. Fenugreek is rich in antioxidative molecules such as dimethoxysulfone, 10,11-dihydroxy-3,7,11-trimethyl-2,6-dodecadienyl acetate [85], and 3-octyn-2-one [86] involved in the antioxidation and immunity of fish and thereby result in high resistance to infection.

5. Conclusion

It could be concluded that inclusions of 0.09% feed of ethanolic extract of FS could be a better choice to improve *O. niloticus* growth performance. Further, the inclusion of ethanolic extract of FS can be a practical choice to enhance the haematobiochemical profile, immune responses, oxidative status, and resistance against *A. hydrophila* infection. Thus, the present study suggests that using an ethanolic extract of FS is recommended for improving the productivity and tolerance against *A. hydrophila* infection in Nile tilapia.

Data Availability

The data supporting findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

All authors equally contributed to this work (conception, acquisition, sample analysis, statistical analysis, data interpretation, manuscript drafting, and manuscript revision).

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