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Growth Performance, Immune Response, Antioxidative Status, and Antiparasitic and Antibacterial Capacity of the Nile Tilapia (*Oreochromis niloticus*) After Dietary Supplementation With Bottle Gourd (*Lagenaria siceraria*, Molina) Seed Powder

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Medicinal plants are a potential safe source of bioactive compounds. Fish diet supplemented with the medicinal plant bottle gourd (*Lagenaria siceraria*) seed powder was evaluated in this study for the potential effect on growth performance, antioxidative status, immunological response, and resistance to infectious pathogens in the Nile tilapia (*Oreochromis niloticus*). Nile tilapia fingerlings with mean weight (± SD)= (25.64 ± 0.17 g), were fed four *L. siceraria* seed supplemented diets (LSSD) at 0.0, 1, 2, and 3% for 60 days. Specimens were then challenged with the bacterium *Aeromonas hydrophila* for 10 days. Also, three different concentrations (2.5, 5, and 10 ml/L) of *Lagenaria siceraria* ethanolic extract (LSEE) were tested for their antibacterial and antiparasitic efficacy on four selected bacterial and one parasitic species. All parameters' values generally improved with elevating the content of the *L. siceraria* seed powder in the diet. Dietary administration of LSSD-3% enabled significant ($P < 0.05$) higher growth performance, and feed utilization efficiency. It reduced the mortality induced by *A. hydrophila* infection, increased crude protein content in the fish body and exhibited the highest *in vitro* antibacterial and antiparasitic efficiency. RBCs, WBCs, Hb, PCV, MCV, MCH, and total serum protein values in pre- and post-challenge groups were significantly higher ($P < 0.05$) in the LSSD-3% group. While MCHC, ALT, AST and glucose levels were significantly lower ($P < 0.05$) than those of the other groups. Lysozyme and antioxidant enzyme activities in pre- and post-challenge groups were also higher ($P < 0.05$) in the LSSD-3% group compared to

the other groups. LSEE provided good efficacy against Gram-negative bacterial strains, mild efficacy against Gram-positive bacterial strains, and an antagonistic effect on the parasite *Cichlidogyrus Tilapiae*. The 10 ml/L concentration was the most effective against the pathogens followed by the 5 ml/L concentration and then 2.5 ml/L. Our findings suggest the feasibility of supplementation of Nile tilapia (*O. niloticus*) diet with *L. siceraria* seed powder by 3% to improve the growth performance, immunity, and vital parameters.

Keywords: dietary supplements, cucurbits, hematological and biochemical parameters, growth promoters, immunomodulatory diet, Nile tilapia feed

INTRODUCTION

The challenges of food security considering the global population increase require concerted efforts between researchers and animal protein producers in the fish sector to implement scientific solutions in line with the global trend to reduce pressure on wild stocks and increase production through aquaculture. This leads to new concerns related to the production of fish that are safe for human consumption in so-called "green fish" or fish raised in an antibiotic-free environment. Antibiotics are among the most widely used chemicals in the aquaculture sector, especially in intensive systems to control outbreaks of fungal, bacterial, and parasitic pathogens (Sapkota et al., 2008). The use of several compounds classified as antibiotics has been reported in the majority of aquaculture producing countries (Lulijwa et al., 2019). Despite the remarkable positive effect of these antibiotics in resisting infection, they have various concerns related to human health such as the development of antibiotic resistance to bacterial pathogens and adverse drug reactions (ADR), as well as possible chronic toxicity in the case of bioaccumulation of residues in the consumer's body (Lulijwa et al., 2019). Therefore, there was a need for natural immunostimulants as an alternative to antibiotics for infectious diseases in cultivated fish (Meena et al., 2013; Song et al., 2014; Soares et al., 2020). Medicinal plants are one of those sources that have shown effectiveness in improving the immune status, stress resistance, growth performance, and nutrient utilization in cultivated fish as well as deterring infection from pathogens during early life stages (Newaj-Fyzul and Austin, 2015; Hoseinifar et al., 2017; Mansouri Taee et al., 2017; Safari et al., 2017; Nawaz et al., 2018; Hoseinifar et al., 2019; Kesbiç et al., 2020; Soares et al., 2020; Fazio et al., 2022). The bottle gourd *Lagenaria siceraria* (Molina) is a big pubescent, annual, prostrate, or ascending plant native to Africa and Asia. The plant is a member of the Cucurbitaceae family that is widely dispersed throughout the world's warmer climates (Yetişir et al., 2008). This plant has traditionally been used to treat asthma, fever, hypertension, jaundice, ulcer, trypanosomiasis, myiasis, and some ectoparasite infestations in tropical regions (Habibur Rahaman, 2003; Tadeg et al., 2005; Hussain et al., 2008; Yirga et al., 2012). Various studies have revealed good therapeutic potential of the fruits and seeds of *L. siceraria* as an effective antibiotic, antioxidant, antihyperglycemic, anticancer, analgesic, antidiabetic, antihepatotoxic, anti-inflammatory, anthelmintic, antimicrobial, cardioprotective, and diuretic (Prajapati et al., 2010; Saha et al., 2011; Gill et al., 2012; Ahmed et al., 2014; Dar et al., 2014; Ahmed et al., 2017; Ferdaus et al., 2020). Furthermore, the biochemical composition of different parts of the plant presents a good nutritional profile due to the presence of important nutrients and bioactive compounds such as triterpenoids, saponins, polyphenols, flavonoids (Chen et al., 2008), pectin, β-carotene, amino acids, vitamin B, vitamin C (Ogunbusola et al., 2010; Roopan et al., 2016), and cucurbitacin I (Attar and Ghane, 2018).

In aquaculture, there is a scarcity of studies dealing with dietary supplementation with cucurbits in aquatic feeds, hence the novelty of this study, especially as it investigates the effect of this supplement on improving growth performance and enhancing immune resistance toward bacterial and parasitic pathogens in the Nile tilapia (*Oreochromis niloticus*) as a species of high economic importance. The Nile tilapia is the third most produced species globally, representing more than 8% of the total species produced globally by aquaculture (FAO, 2020). Many communities, especially Africans, depend on this species specifically for their daily sustenance as a cheap source of animal protein, encouraging farmers to cultivate it to meet the great demand.

Aeromonas hydrophila is a very common bacterium pathogen that is involved in a disease condition inducing hemorrhage and septicemia and may lead to significant economic losses in cultivated fish (Bailone et al., 2010). Thus, the present study aims to investigate whether dietary supplementation with *L. siceraria* seed powder has a significant effect on growth performance and immune response to bacterial and parasitic pathogens in juveniles of the Nile tilapia *O. niloticus*.

MATERIALS AND METHODS

Preparation of the Experimental Diet

Different concentration levels (0, 1, 2, and 3%) of *Lagenaria siceraria* seed powder (LSSD) were selected according to Fay et al. (2014). All ingredients of the 4 experimental diets shown in **Table 1** were mingled with oil, water was added until a solid paste was obtained, and then each formulated diet was extruded using a mincer. The obtained diet pellets were air-dried and stored at 4°C in plastic bags until use.

Feeding Trial

There were 350 juvenile specimens of *Oreochromis niloticus* with mean weight (± SD)= 25.64 ± 0.17 g) obtained from a private fish

TABLE 1 | Ingredients and chemical composition (%) of the diet.

Ingredients	LSSD levels (%)			
	0	1	2	3
Fish meal (72.0% CP[a])	10.00	10.00	10.00	10.00
Soybean meal (48% CP)	44.00	44.00	44.00	44.00
Yellow corn	20.00	20.00	20.00	20.00
Wheat flour	5.00	4.90	4.51	4.01
Wheat bran	15.00	14.10	13.49	12.99
Vegetable oil	2.85	2.85	2.85	2.85
Cod liver oil	1.84	1.84	1.84	1.84
Dicalcium phosphate	0.81	0.81	0.81	0.81
Vitamin and mineral mixture[b]	0.20	0.20	0.20	0.20
Vitamin C	0.30	0.30	0.30	0.30
LSSD powder	0.00	1.00	2.00	3.00
SUM	100.00	100.00	100.00	100.00
Proximate chemical analysis (%)				
Moisture (%)	7.70	8.20	8.60	8.70
Crude protein	28.30	28.00	27.60	27.40
Ether extract	7.50	7.50	7.40	7.40
Total ash	7.60	7.60	7.70	7.80
Crude fiber	4.90	4.80	4.90	4.90
Nitrogen free extract (NFE[c])	44.00	43.90	43.80	43.80
Gross energy[d] (MJ/Kg)	18.30	18.00	17.90	17.80

[a]CP, crude protein. [b]Composition of each kilogram of the vitamin and mineral mixture premix containing vitamin D3, $8x10^5$ IU; A, $48x10^5$ IU; E, 4 g; K, 0.8 g; B1, 0.4 g; riboflavin, 1.6 g; B6, 0.6 g, B12, 4 mg; pantothenic acid, 4.4 g; nicotinic acid, 10 g; folic acid, 0.5 g biotin, 20 mg, Mn, 22 g; Zn, 22 g; Fe, 12 g; Cu, 4 g; I, 0.4 g, selenium, 0.4 g and Co, 4.8 mg. [c]NFE = 100 – (% protein + %EE + %ash + %fiber). [d]Gross energy content was calculated using the values 5.65, 4.2, and 9.45 Kcal/g for protein, carbohydrate, and lipid, respectively. The Kcal was transferred to Joule by multiplying by 4.184.

farm, Abbassa, Sharkia Governorate, Egypt. They were transferred in polyethylene bags filled with dechlorinated water and supplied with aeration to an experimental unit of a private fish farm at Abbassa, Sharkia Governorate, Egypt. Once they arrived at the experimental unit, they were allocated into (1 × 1 x 1) m^3 fiberglass tanks during 14 days for acclimatization. All specimens appeared healthy, and no injuries or lesions were recorded on the visual examination (Schmitt et al., 2004).

After acclimatization, a total of 320 specimens were randomly separated into 4 groups corresponding to the 4 formulated diets. Each group consisted of 80 specimens and was evenly quadrupled (20 specimens/replicate). Specimens were then placed in (1.50 x 1.50 x 1.10) m^3 experimental concrete ponds and fed with the formulated diet twice daily, at 9:00 and 14:00 for 60 days during June and July 2021. The ponds were provided with dechlorinated water (10% daily exchange) and aeration and were siphoned daily to remove solid waste. The feeding trial was conducted under natural photoperiod (12L:12D) and water temperature of 26 ± 2°C. Water quality parameters such as pH, DO, and total ammonia values were stable throughout the trial and were found to be within the normal ranges reported by Boyd and Tucker (2012).

Growth Performance and Feed Efficiency Indices

At the end of the feeding trial, the fish were collected, counted, and the final wet weight of all specimens was recorded. Weight Gain (WG), Specific Growth Rate (SGR), and Feed Conversion Ratio were estimated according to the formulas:

$$WG\ (g) = W_f - W_i$$

$$SGR\ (\%.day^{-1}) = 100 * (\ln W_f - \ln W_i)/\text{number of days}$$

$$FCR = FI/WG$$

where W_i is the fish initial wet weight (g), W_f is the fish final wet weight (g), and FI is the feed intake (g).

Chemical Analysis

The chemical composition of the formulated diet and fish (8 specimens/replicate) was measured according to standard methods of Thiex et al. (2012) to estimate the dry matter, crude protein, total lipids, crude fiber, and ash content as illustrated in **Table 1**. The nitrogen-free extract (NFE) was calculated using the following formula: NFE ($g.kg^{-1}$) = 1000 – (crude protein + crude lipids + ash + crude fiber). In addition, the gross diet energy was estimated based on the values of protein, lipid, and carbohydrates as 5.65, 9.45, and 4.2 Kcal/g, respectively. All analyses were performed in triplicate.

The Pathogenic Bacterium Challenge Test

A bacterial strain (*Aeromonas hydrophila*) was used in the challenge test and was obtained from the Department of Microbiology, Faculty of Science, Al-Azhar University, Egypt. It was cultured using Tryptic Soy Broth (Himedia, Mumbai, India) and then incubated for 24 h at 25°C. The culture broth solution was centrifuged at 3000 rpm for 10 min. The supernatant was discarded, and the obtained pellets were washed twice with phosphate-buffered saline as described by Naiel et al. (2020). The optical density (OD) was then measured using the prepared solution at 456 nm, which parallels 1×10^7 $cells.ml^{-1}$ (Bailone et al., 2010).

Twenty-four hours after the end of the feeding trial, the pathogenic bacterium challenge test was performed on 40

specimens of each diet group (10 specimens/replicate). The specimens were injected intraperitoneally with 0.1 ml of bacterial suspension (Zahran et al., 2018a; Zahran et al., 2018b). During the 10-day trial period, the fish were fed once daily on the control diet (LSSD-0). Dead specimens were removed continuously and recorded daily.

The mortality data were used to calculate the relative live percentage (RLP) according to the Amend (1981) equation:

RLP = 1 - [(recorded mortality percentage in treated groups (%))/(recordedmortalitypercentageinnon-treatedgroups(%))]×100.

Blood Samples Collection

After 60 days of the feeding trial (Phase I), fish were starved for 24 h before being removed from the holding ponds and immersed in an anaesthetic solution (Shah and Altindag, 2004). Using a plastic syringe, 2 ml of blood samples was collected from the caudal peduncle of the fish specimens in each group replicate (n=3). A small portion was mixed with EDTA dipotassium salt as an anticoagulant and placed in Eppendorf tubes, while the other portion was allowed to coagulate at room temperature for serum in a plain centrifuge tube. Serum was extracted from a blood sample and centrifuged at 4°C for 10 min at 3000 rpm before being stored in Eppendorf tubes at -20°C until analysis. Red blood cells (RBCs) and white blood cells (WBCs) were counted under the recommendations of Blaxhall and Daisley (1973). The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentrations (MCHC) were estimated as described by Dacie and Lewis (1991). While samples for enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose, and total protein were preserved in bottles without anticoagulants, they were also estimated using the standard Dacie and Lewis (1975) formula. The turbidimetric assay (Ellis, 1990) based on the lysis of *Micrococcus lysodeikticus* (Sigma Chemical Co.) was used to measure serum lysozyme activity, with some modifications as described elsewhere (Zahran et al., 2018a; Zahran et al., 2018b). All these procedures have been repeated after completing the bacterial challenge test (Phase II).

Determination of Lipid Peroxidation

Lipid peroxidation serum was measured by estimation of the hepatic malondialdehyde (MDA), following procedures of the Diamond Diagnostic Co. of Modern Chemicals Laboratory, Egypt. In a test tube, 1 ml of thiobarbituric acid reagent was mixed with 0.2 ml of each sample and standard. The mixture was thoroughly mixed and heated for 30 min at 95°C in a boiling water bath to form thiobarbituric acid reacting substances measured at 534 nm. MDA was measured as nmol/g tissue.

Hepatic Oxidative Stress Biomarkers

The activity of superoxide dismutase (SOD) was measured using commercial kit procedures (Bio-diagnostic, Egypt). According to Aebi (1984), catalase (CAT) activity was measured spectrophotometrically (spectrophotometer, 510 nm) by measuring the decrease in H_2O_2 concentration at 240 nm. The activity of glutathione peroxidase (GPx) was measured using commercial kits (Biodiganostic, Egypt) according to the manufacturer's instructions. In addition, the activity of glutathione peroxidase (GPx) was determined using commercial kits (Biodiganostic, Egypt), under the manufacturer's instructions with reading absorbance at 340 nm. The activity of all enzymes was expressed as U/g tissue.

Preparation of *L. siceraria* Ethanolic Extract

The fruits of the bottle gourd, *Lagenaria siceraria*, were collected from a private vegetable and fruit farm in Kafr El-Sheikh Governorate, Egypt, and were identified by the research staff of the botany department, Faculty of Science, Al-Azhar University, Egypt. The fruits were cut lengthwise to reveal the seeds. The seeds were collected, washed, and then shade-dried for 3 days and crushed with the peel. The pulverized powder of the seeds was defatted by maceration using petroleum ether for 48 h. The solution was then subjected to Soxhlet extraction with ethanol (95%) for 72 h. The extract was concentrated in vacuo to obtain the *L. siceraria* ethanolic extract (LSEE). LSEE was kept in the refrigerator in a closed glass flask. The dose-calculated amount of extract was dissolved in distilled water for desired experimental use (Rajput et al., 2014).

GC-MS of *L. siceraria* Seed Extract

The composition of the prepared concentrated seed extract was analyzed and identified by gas chromatography-mass spectrometer method (GC-MS) (**Table 2** and **Figure 1**). The analysis was done with standard specification by using GC-MS Agilent 7890A Series GC system interfaced to 5975C inert MSD with a Triple-Axis detector with a 7697A autosampler (Agilent Technologies, US), and an HP-5MS 5% phenyl methyl silox-bonded phase column (30 m in length × 250 µm in diameter × 0.25 µm in thickness of film) (Agilent Technologies, US). The total GC run time was 62 min and the carrier gas was helium at a flow rate of 1.22 ml/min at a constant pressure of 22.231 psi. The initial oven temperature was held at 90°C for 1 min and then raised to 205°C at a rate of 8 ml/min for 1 min, followed by raising to 240°C at a rate of 5 ml/min for 1 min and finally held at 300°C at a rate of 8 ml/min for 30 min. An injection volume of 1 µl was used. Compound identification was performed by comparison with chromatographic retention characteristics, a mass spectral library of the GC-MS data system (Sigma-Aldrich), and quantified using total ion peak area and calibration curves of the external standards (Agricultural Research Center, Dokki, Giza).

In Vitro Antibacterial Activity of LSEE

The 3 LSEE levels (2.5, 5, and 10 ml/L) were tested for their antibacterial ability on 4 selected bacterial species. First, the weight of each selected level was extracted using 100 ml of ethanol (80%) by distillation and stored in a dark Durham's bottle. The bottles were then incubated at <24°C for 24 h in a 130-rpm shaking water bath (Hashemi Karouei et al., 2012). After obtaining the solution, it was separated by centrifugation at 5000 rpm for 15 min and then filtered by Whatman filter paper (150 mm, Ashless). The solvent was evaporated to one-fifth of

TABLE 2 | Identified components by GC-MS of *L. siceraria* seed extract.

No.	Compound identified	M.wt	Formula	RT (min)	Area (%)
1	Palmitic acid	256.40	$C_{16}H_{32}O_2$	6.381	97.20
2	Oleic acid	895.00	$C_{18}H_{34}O_2$	7.056	77.40
3	Tetradecanoic acid	228.38	$C_{14}H_{28}O_2$	7.443	90.60
4	Glutamic acid	147.13	$C_5H_9NO_4$	8.338	26.40
5	β-Sitosterol	414.71	$C_{29}H_{50}O$	9.449	76.90
6	Eicosene	280.50	$C_{20}H_{40}$	10.556	58.30
7	p-Coumaric acid	164.05	$C_9H_8O_3$	12.808	95.80
8	Hydroxybenzoic acid	138.12	$C_7H_6O_3$	14.992	33.30
9	7.22-ergostadienone	397.00	$C_{28}H_{44}O$	20.241	9.91
10	Kaempferol	286.23	$C_{15}H_{10}O_6$	21.662	55.10
11	Apigenin	270.05	$C_{15}H_{10}O_5$	22.153	30.90
12	22-deoxoisocurcubitacin-D	502.70	$C_{30}H_{46}O_6$	22.713	24.90
13	4-methoxymethyl phenol	168.19	$C_9H_{12}O_3$	25.399	16.20
14	Spinasterol	412.70	$C_{29}H_{48}O$	25.728	21.50
15	Catechin	290.27	$C_{15}H_{14}O_6$	25.809	67.00
16	Oxacycloheptadec-8-en-2-one	252.39	$C_{16}H_{28}O_2$	28.130	22.60
17	Squalene	858.00	$C_{30}H5_0$	28.280	12.60
18	Neophytadiene	278.50	$C_{20}H_{38}$	37.082	6.52

M.wt, Molecular weight; RT, retention time.

the original volume and the extract was stored in closed bottles at 4°C. The four bacterial strains that were tested are *Aeromonas hydrophilia*, *Aeromonas sobria*, *Streptococcus agalactiae*, and *Streptococcus iniae*, and they were obtained from the Department of Microbiology, Faculty of Science, Al-Azhar University, Egypt.

In Vitro Anti-Parasitic Activity of LSEE

Infected Nile tilapia (*O. niloticus*) with parasite *Cichlidogyrus tilapiae* was obtained from Abbassa, Sharkia Governorate, Egypt, in July 2021. The target parasite was identified according to Radwan (2022). The fish were sacrificed by a blow to the head, then the gill arches were extracted and placed in Petri dishes containing distilled water. Parts of gill filaments holding parasites were cut and placed in 12-well plates, each containing 10 ml of distilled water and 10 parasites. The three LSEE concentrations (2.5, 5, and 10 ml/L for 120 min, 4 replicates each), were applied against *C. tilapiae* when the time was defined as zero. Each treatment had control wells containing distilled water without the addition of the LSEE. The parasites were observed every 10 min using a dissecting microscope and mortality was recorded. Parasites were considered dead if they did not respond to touch and did not show any reaction when being transferred to clean wells containing distilled water. According to

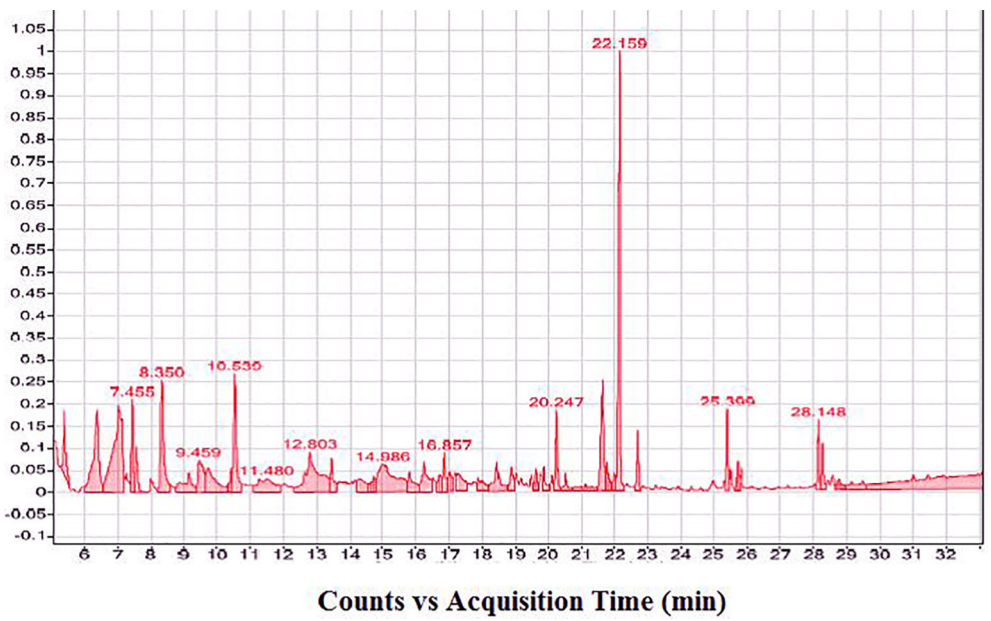

FIGURE 1 | GC-MS analysis of the identified compounds of *Lagenaria siceraria* seed extract.

Zhang et al. (2014), treatment can be considered effective if 100% parasite mortality is achieved within 24 h. Finally, the anti-parasitic efficacy of each treatment and control group was calculated using the Wang et al. (2009) formula:

$$AE = [B - T] \times 100\%/B$$

where AE is the anti-parasitic efficacy, B is the mean number of survival in control, and T is the mean number of survival in the treatment group.

Data Analysis

The obtained data were examined for normality and homogeneity distribution using Levene's test. All the data expressed in percentage was arcsine transformed before performing the statistical analysis. One-way ANOVA was applied to test the differences between LSSD levels in 95% confidence value ($P < 0.05$) using the SPSS software (v.22.0). Tukey's HSD was used when significant differences ($P < 0.05$) between data were identified. Data were expressed as mean ± SE, except for the antibacterial and antifungal data that were expressed as mean ± SD.

RESULTS

Growth and Survival Performance

The growth performance of *O. niloticus* presented in **Table 3** and **Figure 2** reveals increases in all parameters with a significant difference ($P < 0.05$) between all supplemented groups, where the values improved with the increasing proportion of the LSSD in the diet. The LSSD-3% diet showed the best growth performance, followed by LSSD-2%, then LSSD-1%, with a significant difference ($P < 0.05$) from the control group. Consistently, the survivability represented by SR and RPS increased in the supplemented groups with the increasing proportion of LSSD in the diet, after a challenge with *A. hydrophila*. The RPS of LSSD-1, LSSD-2, and LSSD-3% was 75%, 90%, and 95%, respectively, compared to the 70% LSSD-0 control group.

Biochemical Composition of Fish Body

The results of the body biochemical analysis showed that the crude protein content in the fish was significantly higher ($P < 0.05$) in the supplemented groups with higher LSSD than in those fed with a lower level of LSSD and the control group. In contrast, the ash content was significantly lower ($P < 0.05$) in the supplemented groups with higher LSSD than in those fed with a lower level of LSSD and the control group. While no significant difference ($P < 0.05$) was observed in the crude fat content among all groups as observed in **Table 4**.

Blood Parameters

Table 5 reveals that WBCs were significantly higher ($P < 0.05$) in the LSSD supplemented diet groups in both phases I and II in agreement with the elevated LSSD content compared to the control diet group without LSSD supplementation. Likewise, the values of other indices including RBCs, Hb, PCV, MCV, and MCH were significantly higher ($P < 0.05$) in the diet groups with LSSD supplementation at both phases compared to the control diet. On the other hand, MCHC decrease significantly ($P < 0.05$) in the LSSD supplemented diet groups compared to the control group in phases I and II.

The values of other blood parameters such as ALT, AST, and glucose were generally lower in the diet groups with the highest LSSD content than in the lower and control groups in the first and second phases, dissimilar, the total serum protein value increased in the diet groups with the highest LSSD content than in the lower and control groups (**Table 6**).

Stress Biomarkers and Lipid Peroxidation

The lysozyme activity was found to differ significantly ($P < 0.05$) between the supplemented diet groups in phases I and II with the best activity observed in the LSSD-3% group and the lowest in the LSSD-0 group in both phases. In parallel, the enzymes SOD, GPx, and CAT had a similar trend with the highest activity observed in the LSSD-3% group and the lowest in the LSSD-0

TABLE 3 | Growth performance of *O. niloticus* fed 0, 1, 2, and 3% diet of *L. siceraria* seeds supplemented diets (LSSD) for 60 days, and relative survival after bacterial challenge with *A. hydrophila* for 10 days.

LSSD levels (%)	Final weight (g)	Weight gain (g)	FCR	SGR (%.g/day)	SR (%)	RPS (%)
LSSD-0	57.59 ± 0.58^{d}	32.43 ± 0.59^{d}	1.80 ± 0.03^{a}	1.38 ± 0.02^{d}	95 ± 1.21^{b}	70 ± 2.35^{d}
LSSD-1	67.41 ± 1.12^{c}	41.30 ± 1.25^{c}	1.71 ± 0.02^{b}	1.58 ± 0.04^{c}	99 ± 1.00^{a}	75 ± 1.53^{c}
LSSD-2	73.56 ± 0.48^{b}	47.54 ± 0.74^{b}	1.52 ± 0.01^{c}	1.73 ± 0.03^{b}	100 ± 0.2^{a}	90 ± 1.65^{b}
LSSD-3	80.67 ± 0.60^{a}	54.68 ± 0.56^{a}	1.35 ± 0.07^{d}	1.89 ± 0.01^{a}	100 ± 0.1^{a}	95 ± 2.11^{a}

Data were expressed as mean ± SEM. Different superscript letters in the same column are significantly different (ANOVA, P < 0.05). LSSD, Lagenaria siceraria seed supplemented diet; SGR, specific growth rate; FCR, feed conversion ratio; SR, survival rate; RPS, relative percent survival.

TABLE 4 | Biochemical composition of *O. niloticus* (%, dry weight basis) fed 0, 1, 2, and 3% diet of *L. siceraria* seeds supplemented diets (LSSD).

LSSD levels	Dry matter	Crude protein	Crude fat	Ash content
LSSD-0	27.74 ± 0.55^{b}	57.49 ± 0.30^{d}	18.73 ± 0.32	23.13 ± 0.02^{a}
LSSD-1	27.99 ± 0.29^{b}	60.93 ± 0.53^{c}	18.86 ± 0.16	19.23 ± 0.29^{b}
LSSD-2	28.14 ± 0.16^{a}	64.68 ± 0.45^{b}	19.05 ± 0.12	15.37 ± 0.34^{c}
LSSD-3	28.94 ± 0.27^{a}	67.53 ± 0.51^{a}	19.16 ± 0.18	12.51 ± 0.10^{d}

Data were expressed as mean ± SE. Different superscript letters in the same column are significantly different (ANOVA, P < 0.05). LSSD, Lagenaria siceraria seed supplemented diet.

TABLE 5 | Hematological indices of *O. niloticus* fed 0, 1, 2, and 3% diet of *L. siceraria* seeds supplemented diets (LSSD) for 60 days (Phase I) and after exposure to *A. hydrophila* (Phase II).

	LSSD levels (%)	RBCs($\times 10^6$ cell/mm^3)	WBCs($\times 10^3$ cell/mm^3)	Hb (g/dl)	PCV (%)	MCV (fL)	MCH (pg)	MCHC (%)
Phase I	0	1.77 ± 0.07^{dB}	16.86 ± 0.28^{cB}	5.85 ± 0.44^{cB}	19.91 ± 0.49^{cB}	111.71 ± 1.72^{dA}	36.33 ± 0.57^{dB}	34.71 ± 0.60^{aB}
	1	2.11 ± 0.09^{cB}	17.39 ± 0.71^{bB}	6.42 ± 0.04^{bB}	21.65 ± 0.39^{bB}	120.96 ± 1.26^{cA}	38.74 ± 0.44^{cB}	32.05 ± 0.37^{bB}
	2	2.21 ± 0.07^{bB}	17.65 ± 0.31^{bB}	6.58 ± 0.18^{bB}	22.00 ± 0.30^{bB}	129.02 ± 0.48^{bA}	40.56 ± 0.36^{bB}	31.79 ± 0.39^{cB}
	3	2.29 ± 0.06^{aB}	18.82 ± 0.24^{aB}	6.96 ± 0.09^{aB}	23.04 ± 0.08^{aB}	142.16 ± 1.55^{aA}	42.63 ± 0.40^{aB}	30.02 ± 0.27^{dB}
Phase II	0	2.20 ± 0.13^{bA}	18.18 ± 0.53^{cA}	6.51 ± 0.14^{cA}	22.73 ± 0.39^{cA}	105.59 ± 1.04^{dB}	42.25 ± 0.78^{cA}	37.46 ± 0.46^{Aa}
	1	2.41 ± 0.09^{aA}	19.14 ± 0.56^{cA}	7.36 ± 0.14^{bA}	24.13 ± 0.12^{bA}	114.01 ± 1.27^{cB}	42.36 ± 0.18^{cA}	36.19 ± 0.27^{bA}
	2	2.44 ± 0.02^{aA}	20.05 ± 0.62^{bA}	6.93 ± 0.08^{bA}	23.84 ± 0.13^{bA}	123.55 ± 1.61^{bB}	44.29 ± 0.31^{bA}	34.41 ± 0.33^{cA}
	3	2.46 ± 0.08^{aA}	22.62 ± 0.68^{aA}	7.76 ± 0.20^{aA}	24.98 ± 0.10^{aA}	131.53 ± 2.42^{aB}	45.27 ± 0.74^{aA}	33.02 ± 0.06^{dA}

Data were expressed as mean ± SE. Different superscript letters in the same column are significantly different (ANOVA, P < 0.05). LSSD, Lagenaria siceraria seed supplemented diet; RBCs, red blood cells; WBCs, white blood cells; Hb, hemoglobin; PCV, packed cell volume; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.

group in both phases. The CAT activity in phase II was significantly higher ($P < 0.05$) in the higher supplemented LSSD groups (LSSD-2 and LSSD-3%) than in the lower groups (LSSD-0% and LSSD-1%) (**Table 7**). Clearly, the increase in the LSSD content was accompanied by a decrease in the production of the MDA, as the lowest MDA was found in LSSD-2 and LSSD-3 supplemented diet groups in both phases.

In Vitro Antibacterial Activity of LSEE

The result of the antibacterial examination is represented in **Figure 3**. It showed good efficacy of the LSEE against gram-negative bacterial strains (*Aeromonas hydrophilia* and *A. sobria*), and mild efficacy against gram-positive bacterial strains (*Streptococcus agalactiae* and *Staphylococcus iniae*). The higher concentration of the LSEE exhibited higher antibacterial activity in both gram-negative and gram-positive bacteria than the lower concentration groups of LSEE.

In Vitro Anti-Parasitic Activity of LSEE

An antagonistic effect was observed on *C. tilapiae* parasite following the addition of the LSEE. The parasites were initially noticed moving turbulently, after which time they began to contract and twitch violently. This was followed by a detachment of the parasite from the host. Then the parasites continued to contract, but at a slower rate until their final death. Parasites in the control group were not affected to the same degree as those in the LSEE treated groups, as the number of dead parasites was increasing with increasing LSEE concentration over time. In the control group, the number of dead parasites increased from about 12% after 30 min to 50% after 60 min, while at the higher concentration of the LSEE 10 ml/L, about 41% of the parasites were reported to die after only 10 min while the complete parasite death occurred after 40 min (**Table 8**).

DISCUSSION

The current study contributes to enhancing research studies on the potential use of medicinal plants in aquaculture for growth promotion, immunity stimulation, and disease resistance. The results of this study demonstrate that *O. niloticus* diets supplemented with *L. siceraria* seed powder can improve feed utilization efficiency, fish growth, and fish body biochemical composition. This advantage of supplemented feed can be attributed to the distinct nutritional profile of the bottle gourd seed powder being rich in amino acids, dietary fibers, carbohydrates along with several bioactive compounds such as saponins and triterpenoid glycosides that have been reported to actively promote growth and feed utilization in Nile tilapia (Goda, 2008). The presence of flavonoids, di- and triterpenes found in the seed extract suggests antimicrobial capability that may explain the better feed and growth efficiency due to potential inhibition of pathogens and thus the increased beneficial microbial activity leading to improved feed digestibility and nutrient absorption as well as better resistance of fish specimens to the pathogenic *A. hydrophila* bacteria. A study conducted on possible alternatives to fish meal has also reported a better growth performance of *O. niloticus* fingerlings when fed on a diet supplemented with squash

TABLE 6 | Biochemical indices of *O. niloticus* fed 0, 1, 2, and 3% diet of *L. siceraria* seeds supplemented diets (LSSD) for 60 days (Phase I) and after exposure to *A. hydrophila* (Phase II).

	LSSD levels (%)	AST (U/ml)	ALT (U/ml)	Total serum protein (g/dl)	Glucose (mg/dl)
Phase I	0	32.45 ± 0.57^{aB}	27.39 ± 0.71^{aB}	1.79 ± 0.06^{cB}	76.50 ± 0.48^{aB}
	1	31.32 ± 0.82^{aB}	24.79 ± 0.49^{bB}	2.25 ± 0.18^{bB}	74.72 ± 0.31^{bB}
	2	29.90 ± 0.24^{bB}	24.31 ± 1.27^{bB}	2.88 ± 0.02^{bB}	72.62 ± 0.35^{cB}
	3	29.32 ± 0.62^{bB}	22.40 ± 0.23^{cB}	3.51 ± 0.09^{aB}	71.08 ± 0.55^{cB}
Phase II	0	38.95 ± 0.53^{aA}	39.87 ± 0.49^{aA}	2.38 ± 0.24^{cA}	87.75 ± 1.49^{aA}
	1	35.04 ± 0.98^{bA}	34.46 ± 0.65^{bA}	$3.28 \pm 0.\ 12^{bA}$	81.98 ± 1.00^{bA}
	2	34.75 ± 0.66^{cA}	30.49 ± 0.45^{cA}	3.33 ± 0.13^{bA}	78.66 ± 0.79^{cA}
	3	32.27 ± 0.67^{dA}	26.84 ± 0.34^{dA}	3.79 ± 0.09^{aA}	76.64 ± 0.36^{cA}

Data were expressed as mean ± SE. Different superscript letters in the same column are significantly different (ANOVA, P < 0.05). LSSD, Lagenaria siceraria seed supplemented diet; AST, alanine aminotransferase; ALT, aspartate aminotransferase.

TABLE 7 | Variations in lysozyme activity, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) activities and malondialdehyde (MDA) value and of Nile tilapia, O. niloticus, fed 0, 1, 2, and 3% diet of *L. siceraria* seeds supplemented diets (LSSD) for 60 days (Phase I) and after exposure to A. hydrophila (Phase II).

	LSSD levels (%)	Lysozyme activit (µg ml -1)	SOD(U g tissue-1)	CAT (U g tissue-1)	GPX (U g tissue-1)	MDA (nmol tissue-1)
Phase I	0	12.68 ± 0.29^{dB}	1.76 ± 0.10^{dB}	228.15 ± 10.49^{dB}	31.53 ± 0.52^{dB}	8.23 ± 0.10^{aB}
	1	15.25 ± 0.71^{cB}	1.87 ± 0.04^{cB}	285.19 ± 11.06^{cB}	39.01 ± 0.67^{cB}	7.54 ± 0.80^{bB}
	2	18.12 ± 0.46^{bB}	2.26 ± 0.10^{bB}	326.12 ± 10.04^{bB}	43.12 ± 0.82^{bB}	6.36 ± 0.05^{cB}
	3	19.68 ± 0.31^{aB}	2.77 ± 0.22^{aB}	350.79 ± 4.19^{aB}	49.27 ± 0.65^{aB}	5.10 ± 0.10^{dB}
Phase II	0	9.98 ± 0.40^{dA}	1.82 ± 0.01^{dA}	356.20 ± 0.86^{bA}	39.62 ± 0.39^{dA}	9.60 ± 0.32^{aA}
	1	16.86 ± 0.37^{cA}	2.03 ± 0.06^{cA}	365.41 ± 2.33^{bA}	45.87 ± 1.26^{cA}	8.82 ± 0.49^{bA}
	2	20.55 ± 0.38^{bA}	2.74 ± 0.04^{bA}	397.20 ± 3.76^{aA}	54.22 ± 0.83^{bA}	8.07 ± 0.56^{cA}
	3	22.58 ± 0.30^{aA}	3.25 ± 0.09^{aA}	402.43 ± 0.86^{aA}	61.86 ± 0.39^{aA}	7.79 ± 0.32^{cA}

Data were expressed as mean ± SE. Different superscript letters in the same column are significantly different (ANOVA, P < 0.05). LSSD, Lagenaria siceraria seed supplemented diet.

meal (Ajuru and Nmom, 2017). Ahmed et al. (2011) stated that *L. siceraria* is a rich source of prebiotics that are believed to enhance probiotic populations that affect intestinal enteric metabolism and physiology, leading to the synthesis and release of various beneficial biomolecular substances.

Dietary supplementation with *L. siceraria* seeds may also contribute to improving hematological parameters that mainly reflect fish health status and their stress responses (Osman et al., 2018; Fazio, 2019). WBCs, RBCs, Hb, and PCV values in this study increased significantly in all groups of LSSD diets compared to the control group, suggesting an immunostimulant and protective ability of LSSD against stressors. This may be due to the content of bioactive compounds including saponins, glycosides, flavonoids, terpenoids, tannins, and phenols that are reported to boost hematological parameters in fish as immunostimulants (Talpur and Ikhwanuddin, 2012; Antache et al., 2014; Roohi et al., 2017; Abd El-Gawad et al., 2020). Fish diet supplemented with ginseng herb was found to improve hematological indices, increasing Hb and WBCs in the Nile tilapia *O. niloticus* and the effect was also attributed to similar bioactive compounds to that reported in the current study (Goda, 2008). Recent studies have shown that a variety of medicinal plants are rich in secondary metabolites that may induce immune-modulating activities during stress events (Hoseinifar et al., 2021). Similarly, elevated serum total protein levels can be linked to the innate immune response in fish that are fed on diets typically enhanced with immunostimulants (Wiegertjes et al., 1996; Choudhury et al., 2005; Rodneva and Kovyrshina, 2011). Thus, the significant elevated total serum protein in fish groups fed on LSSD supplemented diets pre- and post-challenge with *A. hydrophila* in this study suggests that LSSD-based diets increase innate immunity in Nile tilapia. Other herbal plants such as the ginseng herb were found to elevate total plasma protein and globulin in fish when used as a supplementation in the feed diet (Goda, 2008). When mixes of sterols and flavonoids were isolated from *L. siceraria* fruit and tested on experimental rats, they presented increased WBCs and hemagglutination antibody titers and inhibited delayed-type hypersensitivity response which indicate an immunomodulatory activity and phagocytosis action of this plant (Deshpande et al., 2008; Gangwal et al., 2008; Gangwal et al., 2009). Regarding alanine aminotransferase (ALT) and aspartate aminotransferase (AST), they are liver enzymes that have the function of transferring the amino group from alpha-amino acids to alpha-keto acids and are often released in large quantities into the blood during liver cell damage (Ye et al., 2011). Clearly, LSSD has an influence in reducing the release of these two enzymes in the blood, likely due to the presence of phenolic and flavonoid groups, which act as hepatoprotective agents (Deshpande et al., 2008; Owais, 2018). Lin et al. (2012) had similar observations on the values of ALT and AST of the pompano *Trachinotus ovatus* fish when fed on a fermented soybean diet without presenting a possible mechanism

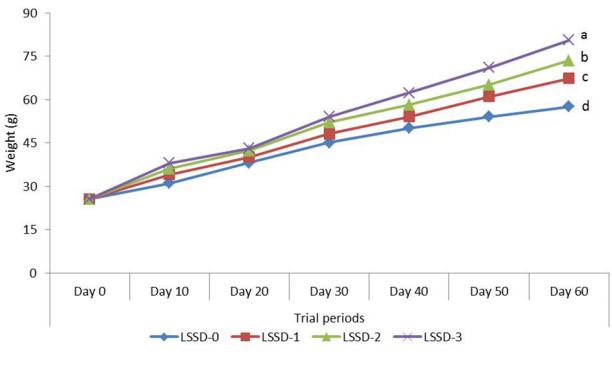

FIGURE 2 | Growth performance of *O. niloticus* fed 0, 1, 2, and 3% diet of L. siceraria seeds supplemented diets (LSSD) for 60 days. Different superscript letters indicate significantly different (ANOVA, *P < 0.05*).

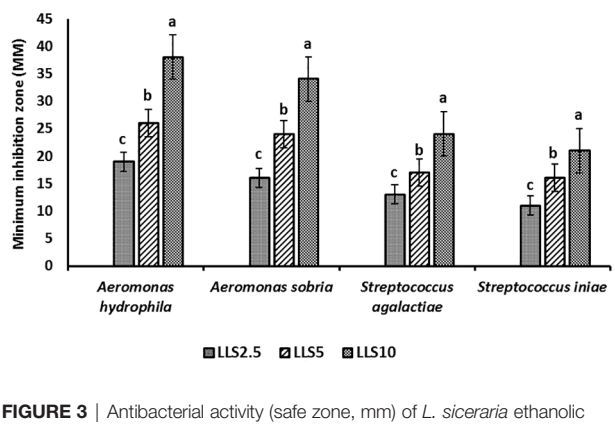

FIGURE 3 | Antibacterial activity (safe zone, mm) of *L. siceraria* ethanolic extract (LSEE) against 4 selected bacterial strains. Data were presented as mean ± SD. Different superscript letters indicate significantly different (ANOVA, P < 0.05).

TABLE 8 | Anti-parasitic efficacy of different concentrations of *L. siceraria* ethanolic extract on time after treatment (min) to death of *C. tilapiae* (%).

Time (min)	LSEE levels			
	0	2.5	5	10
10	–	18.21 ± 1.06^{f}	32.11 ± 2.61^{f}	41.26 ± 2.54^{c}
20	–	28.61 ± 4.34^{e}	53.02 ± 2.72^{d}	78.47 ± 4.34^{b}
30	12.04 ± 2.14^{c}	35.31 ± 3.11^{d}	74.41 ± 1.82^{c}	91.11 ± 1.54^{a}
40	27.11 ± 1.98^{c}	54.05 ± 6.23^{c}	83.67 ± 3.40^{b}	100
50	46.35 ± 3.25^{b}	4.11^{b} ± 63.54	3.64^{a} ± 92.11	100
60	50.12 ± 2.44^{a}	3.69^{a} ± 78.11	100	100

Data were expressed as mean ± SE. Different superscript letters in the same column are significantly different (ANOVA, P < 0.05).

or explanation for their findings. On the other hand, the lower glucose level associated with LSSD supplemented diets in pre- and post-challenge conditions compared to the control group, appears to be due to the hypoglycemic effect of *L. siceraria* seeds caused by the presence of bioactive components, particularly phenols, flavonoids, and saponins. Flavonoid and phenolic compounds are well known for their ability to reduce blood glucose levels, which may be attributed to their potent antidiabetic activity (Parwata et al., 2018). Saponins are also phytochemicals that exhibit a wide range of biological activities, including lowering blood glucose by inhibiting the enzymes that break down disaccharides into monosaccharides (Oishi et al., 2007). Many authors have reported that several different parts of the bottle gourd have efficacy against hyperglycemia and dyslipidemia (Deshpande et al., 2008; Kumar et al., 2012; Bhattacharya and Das, 2012; Sharmin et al., 2012; Charu et al., 2013; Rajasree et al., 2016; Juee and Naqishbandi, 2020). Thus, the current findings present a good scientific base to use bottle gourd in the diet of diabetic patients.

Lysozyme is a cationic enzyme that has phagocytic and antimicrobial activity through the splitting of peptidoglycan in bacterial cell walls which allows bacterial cell lysis (Alexander and Ingram, 1992; Magnadottir, 2010). Thus, serum lysozyme is used as an indicator of the innate immune response in fish (Magnadottir, 2010; Uribe et al., 2011), and therefore, the increased level of serum lysozyme activity as observed in the current study is an indicator of beneficial use of LSSD in Nile tilapia diet to promote the innate immunity against potential bacterial infections and pathogens. These findings support other studies investigating chemical extracts of plant origin to raise immunity in cultivated fish where several bioactive molecules and phytochemicals including alkaloids, flavonoids, pigments, phenolics, terpenoids, and steroids promote several biological activities such as immunostimulant, anti-stress, phagocytic, and complement system activation in cultivated fish (Citarasu, 2010; Chakraborty and Hancz, 2011; Chakraborty et al., 2014). The benefits of herbal supplements extend to providing appropriate oxidative conditions for animals and preventing oxidative stress by scavenging free radicals and/or stimulating antioxidant enzymes (Sönmez et al., 2015; Bilen et al., 2020; Elbesthi et al., 2020; Yousfi et al., 2020). Among those enzymes, SOD, CAT, and GPx represent the first line of defense against oxidative stress (Farombi et al., 2007), and their values are used as biomarkers that reflect animals' physical health (Ding et al., 2015). As for MDA, it is formed as an end product of lipid peroxidation and is used as an indicator of the toxic process caused by reactive oxygen species (ROS) (Lushchak, 2011). In the present study, LSSD significantly reduced hepatic MDA and raised enzymatic antioxidant levels pre- and post-challenge compared to the control group. These results can also be explained considering the bioactive compounds present in *L. siceraria* seeds, phenols, flavonoids, tannins, and terpenoids that play a significant role in the antioxidant and lipid peroxidation potential (Attar and Ghane, 2019). These phytochemicals are excellent reducing agents, hydrogen donors, singlet oxygen quenchers, and metal chelators (Sharma et al., 2013; Sulaiman et al., 2013; Nagarani et al., 2014; Attar and Ghane, 2017a; Attar and Ghane 2017b; Ghane et al., 2018; Patel et al., 2018). They can react with the stable free radicals by donating electrons or hydrogen atoms and converting them to non-radical form molecules (Benzie and Strain, 1996; Gill et al., 2012; Antia et al., 2015).

Regarding the results of *in vitro* antimicrobial trials, the ethanolic extract of *L. sicereria* showed an anti-parasitic and anti-bacterial efficacy against the tested strains. Apparently, the higher concentration of LSEE enabled better antimicrobial efficacy, thus suggesting an optimum concentration of 0.5% for the ideal anti-parasitic and antibacterial activity. Indeed, the general antimicrobial activity of LSEE in this study was attributed to constituents, phenols, sterols, terpenoids, flavonoids, and saponins, which exist in the seed extract. These results were generally consistent with several studies revealing a potent efficacy of *L. sicereria* seed extract against gram-positive and gram-negative bacteria including *Staphylococcus aureus*, *Pseudomonas* sp., *Escherichia coli*, and *Bacillus subtilis* in addition to some fungal strains such as *Candida* sp. and *Aspergillus niger* (Goji et al., 2006; Essien et al., 2015). Moreover, Smita et al. (2009) and Ramalingam and Patel (2010) both reported the anthelmintic activity of *L. sicereria* seed and leaf extract against the earthworm *Pheretima posthuma* as well as antibacterial and antifungal activity. Joseph et al. (2001) stated that the plant's medicinal properties and biological activities are usually due to its phytochemical profile and bioactive molecules. This may explain the antimicrobial effects exhibited in this study for the constituents, saponins, alkaloids, or terpenoids in the potent diethyl ether extract of *L. siceraria*.

CONCLUSION

Based on these findings, Nile tilapia diets supplemented with different levels of *Lagenaria siceraria* seed powder can promote growth and enhance feed utilization and resistance against stressors. High levels of LSSD may be ascribed to activate

antioxidant and immune status and exhibited potential anti-bacterial and anti-parasitic properties against common infected pathogens. LSSD up to 1% in this study presented the best performance in the Nile tilapia *Oreochromis niloticus* in all parameters. Our findings support the use of medicinal plants as dietary supplementation in fish feeds as an eco-friendly approach for sustainable aquaculture.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MR: Writing – original draft, formal analysis, methodology, investigation, visualization. MA: Formal analysis, statistical analysis, methodology, editing. AM: Formal analysis, methodology, conceptualization, editing. JA: Formal analysis, methodology, visualization, editing. SM: Chemical analysis, GC-MS data interpretation, editing. MM: Writing – original draft, methodology, visualization, writing – review and editing. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.901439/full#supplementary-material

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