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Physiological and immunological responses of Nile tilapia fed dietary supplementation of sweet basil ethanolic and aqueous extracts

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The use of phytotherapy in aquaculture is rapidly increasing for more environmentally sustainable measures. The present work aimed to investigate the effects of different dietary levels of sweet basil, *Ocimum basilicum*, leaves ethanol (BEE) or aqueous (BAE) extracts (0, 200, 300, and 500 mg/kg) on *Oreochromis niloticus* growth, digestive enzyme activities, hemato-biochemical profile, antioxidant status, immune responses, and resistance against *Streptococcus agalactiae*. *Oreochromis niloticus* fingerlings (40.00 ± 1.00 g/fish, n = 210) were randomly divided into seven triplicated groups (control, BEE₂₀₀, BEE₃₀₀, BEE₅₀₀, BAE₂₀₀, BAE₃₀₀, and BAE₅₀₀) and fed the experimental diets for 8 weeks. The results revealed that dietary inclusion of BEE and BAE significantly improved final weight, weight gain, survival, and digestive enzyme activities. The growth response revealed a dose-dependent in favor of fish fed BEE. The hemato-biochemical biomarkers showed a significant improvement in RBCs, WBCs, hemoglobin, and lymphocyte, and a significant decrease in aminotransferases, creatinine, and cholesterol levels with dietary basil extracts. The cellular and humoral immune responses (phagocytic activity, phagocytic index, lysozyme activity, immunoglobulin) were significantly improved with increasing BEE and BAE in a dose-dependent manner. The expression of *IL-1β* and *TNF-α* genes were increased, while *TGF-β* was decreased in a dose-dependent manner and BEE₅₀₀ have the highest expression. The antioxidant balance was improved with increasing basil extracts supplementation, and the BEE₅₀₀ group showed the best antioxidant status. Dietary BEE and BAE increased Nile tilapia resistance to *S. agalactiae*. In conclusion, the dietary supplementation of both ethanolic and

aqueous extracts could improve the growth performance and physiological, and immune-antioxidant status of Nile tilapia.

KEYWORDS

sweet basil, phytotherapy, Nile tilapia, physiological performance, bacterial challenge

Introduction

The aquaculture industry recorded the highest annual growth among different animal production sectors during the last few decades. Aquaculture can extend aquatic products with reasonable prices to countries or regions that have limited access to natural fisheries and improve nutrition and food security (Stead, 2019). Nile tilapia, *Oreochromis niloticus*, is considered one of the predominant farmed freshwater fish species in the world (Diab et al., 2022; Mansour et al., 2022a). It accounted for 8% of total finfish produced globally in 2018 (FAO, 2020). Due to the fast-growing rates, efficient feed utilization, disease resistance, robustness, and adaptability to different environmental conditions, and systems, tilapia could lead freshwater fish farming (El-Sayed, 2019). Nevertheless, in recent years, high mortalities have been reported among tilapia farms due to the spreading of different infectious diseases that threaten the global tilapia industry (Behera et al., 2018; Legario et al., 2020). Accordingly, improving fish health, immunity, and/or pathogen resistance by dietary supplementation, especially with eco-friendly and natural additives, is a very attractive research strategy.

Due to their numerous vital properties, such as immune stimulation, antimicrobial, growth promotion, and anti-stress, many studies have suggested using herbal extracts in aquaculture as a safer, more effective, and less expensive alternative to chemotherapeutic agents (Sallam et al., 2017; Mansour A. et al., 2018; Mansour A. T. et al., 2018; Mansour et al., 2020; Mansour et al., 2021; Mansour et al., 2022b). Sweet basil (*Ocimum basilicum*) is a very common, annual aromatic plant (herb) worldwide and belongs to the family Lamiaceae (Chung et al., 2020). Basil grows in tropical, sub-tropical, and Mediterranean regions (Sipos et al., 2021). Basil has a long history of use in traditional medicine as an antimicrobial, anti-inflammatory, anti-aging, antiviral, antioxidant, and anticancer (Shahrajabian et al., 2020). The therapeutic activities of *O. basilicum* are related to its bioactive compounds, including alkaloids, flavonoids, saponins, essential oils, phytosterols, phenolic compounds, terpenoids, anthraquinones, and tannins (Sakr and Al-Amoudi, 2012; Andayani et al., 2020). In addition, basil contains several volatile components, including linalool, methyl chavicol, eugenol, bergamotene, and methyl cinnamate (Klimankova et al., 2008; Sonmezdag et al., 2018).

The leaves and seeds of basil have several applications in the animal diet. Basil leaves can be used as a feed ingredient (Tolay, 2021). Basil leaf mucilage extract, as a natural polymer, was used as a binder in the diets of *Cyprinus carpio* fingerlings and improved the physical properties of the diet, palatability, and enhanced growth performance (Al-Hamdani et al., 2021). In addition, basil essential oil was used as an anesthetic for several fish species, tambaqui (*Colossoma*

macropomum) and silver catfish (*Rhamdia quelen*) (Silva et al., 2012; de Lima Boijink et al., 2016). In addition, basil essential oils and extracts revealed several beneficial effects as dietary supplement. It could improve the growth performance, feed utilization, immune responses, and antioxidant status of hybrid Nile tilapia (El-Dakar et al., 2008) gilthead seabream (*Sparus aurata*) (El-Dakar et al., 2015), common carp (*C. carpio*) (Amirkhani and Firouzbakhsh, 2015) pirarucu (*Arapaima gigas*) juveniles (Chung et al., 2020), Indian shrimp (*Penaeus indicus*) (Abdel-Tawwab et al., 2021), and rainbow trout (*Oncorhynchus mykiss*) (Magara et al., 2022).

Streptococcus agalactiae is a gram-positive spherical or ovoid-shaped bacteria, arranged in chains or pairs, and a non-motile, non-spore former (Delfani et al., 2017). *S. agalactiae* is one of the fish-streptococcosis main causative agents, which is characterized clinically by high morbidity, severe mortality, erratic swimming, low growth rate, exophthalmia, and abdominal distension (Evans et al., 2006; Bowater et al., 2012). Streptococcosis is a dangerous veterinary disease as well as a zoonotic disease that causes significant economic losses worldwide (Pereira et al., 2010; Diab et al., 2019). The use of herbs and their natural extracts for the treatment of various fish diseases or as immunostimulants has been commonly used (Delfani et al., 2017). Therefore, the present study aimed to evaluate the use of sweet basil, *O. basilicum*, ethanolic and aqueous extracts as health-improving and immune stimulant feed supplements in *O. niloticus* diet by investigating their effects on growth performance, digestive enzyme activities, hemato-biochemical profile, immune and antioxidant status, some immune-related genes expressions, and resistance against *S. agalactiae* challenge.

Materials and methods

Basil extract preparation

The basil, *O. basilicum*, leaves were obtained from a local market in Alexandria governorate, Egypt. Basil leaves were washed, dried, and ground into a fine powder. Basil ethanol extract (BEE) was prepared according to Zhang et al. (2018). Briefly, the basil leaves fine powder was immersed in 70% ethanol solution at a ratio of (1:1) for 48 h. The basil leaves residue was filtered using filter paper and the obtained extract was concentrated at 40°C using a rotatory evaporator. The obtained BEE extract was kept at 4°C until use. Basil aqueous extract (BAE) was conducted according to Zhang et al. (2018). Briefly, samples of 50 g of the dried fine-ground basil leaves powder were immersed in 500 ml sterile distilled water and shaken at room temperature for 4 h. The BAE was filtered twice through a 100-µm pore size nylon net, then concentrated using a rotatory evaporator and stored at -20°C until it was used.

Experimental fish and rearing conditions

A total number of 210 apparently healthy *O. niloticus* (40.0 ± 1.0 g/fish) were obtained from a private farm, Kafr El-Sheikh, Egypt. The fish was transferred to the wet laboratory, Faculty of Veterinary Medicine, Alexandria University, Egypt, and randomly allocated into 21 glass aquaria ($90 \times 50 \times 35$ cm, 10 fish per aquarium). Each aquarium has continuous aeration by using an electric air-pumping compressor. Wastes were daily siphoned with water exchange at a daily rate of 30% using dechlorinated tap water. The water temperature was kept at $27^\circ\text{C} \pm 2^\circ\text{C}$. Fish were adapted for 2 weeks in the laboratory conditions before the start of the experiment. Fish were manually fed twice a day at 9:00 and 15:00 on 32% crude protein commercial tilapia diet (Aller-Aqua Egypt Co, 6th of October, Giza, Egypt). The feeding rate was 3% of the total fish body weight. The actual feed intake was adjusted bi-weekly according to the change in fish body weight.

Experimental design

The fish were randomly divided into seven groups (three replicates each). The control group was fed basal diets free of BEE and BAE extracts. The second, third, and fourth groups were fed diets containing basil ethanol extract (BEE) at 200, 300, and 500 mg/kg of feed (BEE₂₀₀, BEE₃₀₀, and BEE₅₀₀). The fifth, sixth, and seventh groups were fed diets that contained basil aqueous extract (BAE) at 200, 300, and 500 mg/kg of feed, respectively (BAE₂₀₀, BAE₃₀₀, and BAE₅₀₀) (Table 1). The BEE or BAE powder was separately dissolved in distal water to have a concentration of 10% w/v and was sprayed on the diet of the respected treatment and then dried in an air-force oven at a temperature of $45\text{--}50^\circ\text{C}$ (Motlagh et al., 2020; Zenhom and Ibrahim, 2020). The selected doses of basil extract were done according to the study of (Amirkhani and Firouzbakhsh, 2015).

The fish diet was kept at 4°C until use. Fish were fed the experimental diets for 8 weeks before the bacterial challenge, followed by 2 weeks of the observation period after the challenge by *S. agalactiae* for recording the mortality.

Fish growth performance

At the end of the eighth week, the fish from each replicate were anaesthetized with tricaine methane sulfonate (MS222, 25 mg/L, Argent Laboratories, Redmond, WA, USA) to get fish weight (individually). Fish growth performance was evaluated, including final weight (g), weight gain (%; $[100 \times \text{initial weight} - \text{final weight}]/\text{initial weight}$), and survival (%).

Blood and serum collection

After 8 weeks of dietary supplementation of BEE or BAE, blood samples (15 fish/treatment) were collected from the caudal vein by a disposable plastic syringe. Blood samples were divided into two parts; one part was added to heparinized tubes, while the other part of blood samples was added to plain tubes without anticoagulants for serum collection after centrifugation at 3000 rpm for 15 minutes at 4°C .

Haemato-biochemical analysis

The red and white blood cells (RBCs and WBCs) count, the differential leukocyte count, packed cell volume (PCV), and hemoglobin content, were determined as described by Mansour and Esteban (2017). The activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) (Reitman and Frankel, 1957), in addition to the serum total proteins (Gornall et al., 1949), albumin (Doumas et al., 1971), globulins, and creatinine levels (Henry, 1964) were estimated using commercial kits (Biodiagnostic Co., Cairo, Egypt). Cholesterol and triglyceride were determined according to Reitman (1957).

Immune and oxidative stress responses

The cellular immune response was determined, including blood respiratory burst activity, which was examined by the nitroblue tetrazolium (NBT) assay (Secombes, 1990) and phagocytic cells

TABLE 1 The used gene primers.

Gene		Sequence (5'-3')	Accession No.
Housekeeping genes			
B-actin	F	CAG CAT CAT GAA GTG CGA CG	KJ126772
	R	ACT CCT GCT TGC TGA TCC AC	
Immune related genes			
IL-1 β F	F	GCA TTG TGG AAG CAC AGT ATAA	DQ061114.1
	R	GTC TCA GCG ATG GGT GTA GG	
TGF- β F	F	GGA TCC ATA AGC CAA CGG GT	XM_025897821
	R	CTC CAA CAC GTC TTC TTT CCT	
TNF- α F	F	CAG GAT CTG GCG CTA CTC AG	AY428948
	R	TAG CTG GTT GGT TTC CGT CC	

activity and index (Kawahara et al., 1991). The humoral immune response was determined, including serum lysozyme activity, which was determined by a turbidimetric assay according to Ellis (1990). Bactericidal activity was detected as described by Rainger and Rowley (1993). Immunoglobulin M (IgM) was estimated by ELISA using a commercial kit (Bio Diagnostic Co., Cairo, Egypt). (Siwicki and Anderson, 1993).

The antioxidant status parameters include catalase (CAT) (Aebi, 1984), superoxide dismutase (SOD) (Nishikimi et al., 1972), total antioxidant capacity (TAC) (Bartosz, 2003), and malondialdehyde (MDA) (Uchiyama and Mihara, 1978) were determined calorimetrically (Bio diagnostic Co., Cairo, Egypt).

Some immune genes examination

Interleukin 1 beta (*IL-1β*), transforming growth factor-beta 1 (*TGF-β*), tumor necrosis factor (*TNF*), and β-actin expression in the head kidney of *O. niloticus* were quantitatively examined by real-time PCR amplifications. The PCR primer sequences of the studied genes are presented in (Table 1). Briefly, total RNA was extracted from the head kidney tissue after 8 weeks of treatment. The concentration and purity of the extracted RNA were determined *via* spectrophotometry with absorption at 260 and 280 nm. The following thermal cycling conditions were used for the expressed genes: initial denaturation at 95°C for 5 min, 40 cycles of amplification (DNA denaturation at 95°C for 15 s, annealing at 60°C for 15 s, extension at 72°C or 15 s), and final extension at 72°C for 5 min. The comparative CT method ($2^{-\Delta\Delta C_t}$) (Livak and Schmittgen, 2001) was used to calculate the gene expression values using β-actin as a housekeeping gene.

Activities of some digestive enzymes

The intestinal samples were collected from the anesthetized fish after blood collection then washed with PBS, then the intestinal samples were homogenized in PBS (1 g/10 ml), centrifuged (5000 rpm/10 min), and the supernatant was stored at 4°C until it was used for determination of the activities of lipase, amylase, and protease enzymes as reported in by Dawood et al. (2019).

Bacterial challenge

At the end of the experiment, fish were bacteriologically examined to be free from bacterial infection. Twenty fish from each treatment were challenged by *S. agalactiae* (Kindly provided by Prof. Dr./Khalil H.R., Department of Poultry and Fish Diseases, Faculty of Veterinary Medicine, Alexandria University, Alexandria, Egypt). Fish was intra-peritoneally injected with 0.2 ml of a suspension containing 2×10^7 CFU/ml of *S. agalactiae* (Moustafa et al., 2020). During 14 days of the observation period, the mortality was recorded and death specificity was estimated by *S. agalactiae* re-isolation from freshly dead fish (Zahran et al., 2018). During the observation period, fish was fed their corresponding diets according to their groups. The cumulative mortality was reported and the relative level of protection (RLP)

among the challenged fish was determined according to Ruangroupan et al. (1986):

$$\text{RLP \%} = 100 - (\text{treatment mortality \%} / \text{control mortality \%}) \times 100$$

Statistical analysis

The data was expressed as means ± pooled standard error. The results were checked for homogeneity and normal distribution before conducting the parametric analysis of the two-way ANOVA test using SPSS 21 (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.). The *post hoc* Tukey test was used to identify the significant differences among means at significant levels of 0.05. Some selected parameters were used to identify the fit regression trend of increasing supplementation levels of basil extracts and fish response to compare the dose-response of each extract. In addition to comparing the significant difference between slopes of the two extracts using GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA, USA).

Results

Growth performance

The growth performance of fish-fed ethanolic and aqueous basil extract-supplemented diets revealed significant differences among treatments. The FBW, WG, and survival were improved with increasing supplementation levels of BEE and BAE with the superiority of groups supplemented with BEE (Table 2). The two-way ANOVA analysis revealed significant effects of extract and dose on the growth response of treated fish.

The regression analysis of FBW and survival with increasing dietary supplementation of BEE and BAE showed a fit linear regression model and the difference between the slopes of both extracts is highly significant in favor of BEE (Figure 1).

Activities of some digestive enzymes

The protease, amylase, and lipase results revealed significant differences in their activities in relation to extracts, dose of supplementation and their interaction. The highest protease and amylase activities were observed in BEE₅₀₀, while the best lipase activity was detected in BAE₅₀₀ (Table 3).

Hemato-biochemical examination

Hematological results were tabulated in Table 4. All dietary-supplemented groups had significantly higher RBCs, WBCs, hemoglobin (Hb), and PCV values compared to the control. The hematological status of groups fed BEE-supplemented diets was better than BAE-treated groups, except for BAE₅₀₀, which had non-significant results compared to BEE₂₀₀. The lymphocytes increased

TABLE 2 Effect of dietary supplementation of basil ethanol and aqueous extracts (mg kg⁻¹ of feed) on growth performance and survival of Nile tilapia, *Oreochromis niloticus*.

Variable	BEE				BAE				Pooled SEM	p-Value		
	0	200	300	500	0	200	300	500		Extract	Dose	E × D ¹
Initial body weight (g)	40.67	41.00	40.50	41.00	40.67	40.67	41.00	40.83	0.53	1	0.917	0.71
Final body weight (g)	64.00 ^f	68.53 ^d	75.83 ^b	78.20 ^a	64.00 ^f	66.17 ^e	68.07 ^d	71.50 ^c	0.39	<0.001	<0.001	<0.001
Weight gain (%)	57.40 ^d	67.20 ^c	87.30 ^a	90.80 ^a	62.80 ^{cd}	66.00 ^c	75.20 ^b	57.40 ^d	0.79	<0.001	<0.001	<0.001
Survival (%)	89.00 ^d	93.33 ^{bc}	97.67 ^a	98.00 ^a	89.00 ^d	91.33 ^c	94.00 ^b	95.33 ^b	0.60	<0.001	<0.001	0.004

Values are means and pooled SEM. BEE: basil ethanol extract; BAE: basil aqueous extract.

^{a-f}Means in a row without a common superscript letter differ ($p < 0.05$) as analyzed by two-way ANOVA and the TUKEY test.

¹E × D = Extract × Dose interaction effect.

significantly with all supplementation treatments compared to the control and the highest levels were reported in the group BEE₅₀₀. No significant differences were recorded in monocytes, eosinophil, basophil, and neutrophil counts neither for extracts nor doses among the experimental groups.

Different biochemical parameters of Nile tilapia fed experimental diets were evaluated (Table 5). Total protein and globulin were improved significantly with both BEE- and BAE-supplemented groups compared to the control, and the best results were recorded in the BEE₅₀₀ group. Meanwhile, the transaminase activities (ALT and AST), creatinine, cholesterol, and triglyceride levels were significantly decreased in BEE- and BAE-supplemented groups than in the control.

Innate immune parameters

The cellular immune response showed a significant improvement in the NBT, phagocytic activity, and index in all BEE and BAE supplemented groups in a dose-dependent manner (Table 6). The lyzosome activity and IgM as non-specific humoral immune indicators showed significant improvement with different basil extracts and the best results with BEE₅₀₀. The bactericidal activity affected significantly with both basil extracts and dietary doses of application.

The dose response study showed a dose-dependent increase in phagocytosis activity and lysozyme with increasing dietary supplementation of both basil extracts. The difference between the effects of both extracts on immune response is highly significant, with the highest response in groups feed BEE (Figure 2).

Immune related genes expression

The two-way analysis of variance of immune-related gene expressions in the head kidneys of *O. niloticus* fed different dietary levels of BEE or BAE showed significant effects of both basil extracts and dietary doses (Table 7). The expression of *IL-1β* and *TNF-α* genes increased in a dose-dependent manner and the highest expression was recorded in BEE₅₀₀. Meanwhile, the expression of *TGF-β* was down-regulated with increasing different extract supplementation levels.

Antioxidant status

The antioxidant status of fish that received different basil extracts showed an improvement in oxidant/antioxidant balance *via* significant improvement of SOD and CAT activities associated with a significant reduction of MDA levels (Table 8). The significant differences were reported among different extracts, dietary levels, and interaction. The BEE₅₀₀ group showed the best antioxidant status among studied groups. The fit regression trend was positive linear regression with total antioxidant capacity and negative linear regression with MDA levels in favor of increasing BEE supplementation levels (Figure 3).

Bacterial challenge

After bacterial challenge, the cumulative mortality and RPL in different groups of fish were recorded (Figure 4). The RPL of *O.*

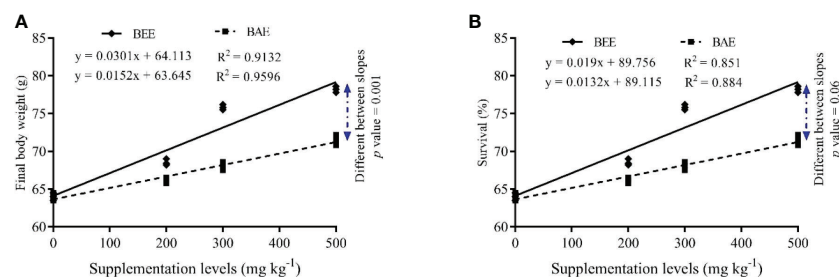


FIGURE 1

The fit regression model and slope differences between dietary supplementation of basil ethanol (BEE) and aqueous extracts (BAE) on final body weight (A) and survival (B) of Nile tilapia, *Oreochromis niloticus*.

TABLE 3 Effect of dietary supplementation of basil ethanol and aqueous extracts (mg kg⁻¹ of feed) on digestive enzyme activities of Nile tilapia, *O. niloticus*.

Variable	BEE				BAE				Pooled SEM	p-Value		
	0	200	300	500	0	200	300	500		Extract	Dose	E×D ¹
Alkaline protease activity (U mg ⁻¹)	47.67 ^e	75.67 ^c	123.30 ^b	153 ^a	47.67 ^e	52.33 ^{de}	57.33 ^{ce}	74.67 ^{cd}	6.635	<0.001	<0.001	<0.001
Amylase activity (U mg ⁻¹)	27.67 ^d	53.00 ^c	95.67 ^b	124.70 ^a	27.67 ^d	31.33 ^d	35.33 ^d	56.00 ^c	4.173	<0.001	<0.001	<0.001
Lipase activity (U mg ⁻¹)	17.67 ^b	25.67 ^b	39.00 ^a	38.67 ^a	17.67 ^b	19.67 ^b	24.00 ^b	46.67 ^a	2.828	0.035	<0.001	<0.001

Values are means and pooled SEM. BEE: basil ethanol extract; BAE: basil aqueous extract

^{a-f}Means in a row without a common superscript letter differ ($p < 0.05$) as analyzed by two-way ANOVA and the TUKEY test.

¹E × D = Extract × Dose interaction effect.

niloticus challenged with *S. agalactiae* was improved in all basil extract groups than the control group. In addition, the BEE₅₀₀ recorded the lowest mortality (20%); meanwhile the control recorded the highest cumulative mortality (93%).

Discussion

In aquaculture, herbal extracts revealed the potential to be used as cheap, safe, non-toxic, biocompatible, and biodegradable immunostimulants and/or antimicrobial agents (Amirkhani and Firouzbakhsh, 2015; García-Beltrán et al., 2020). Sweet basil is one of the most common medicinal plants worldwide due to its rich phytochemicals and essential oil constituents with multiple purposes (Shahrajabian et al., 2020). Basil is rich in substances that could improve fish growth and health as natural antioxidants, including flavonoids (quercetin, rutin, and kaempferol), phenolic acids (caftaric

acid, caffeic acid, and p-coumaric acid), steroids, and vitamins A, E, C, and K (Amirkhani and Firouzbakhsh, 2015; Chung et al., 2020).

The present findings revealed a significant improvement in Nile tilapia growth performance with dietary supplementation of either BEE or BAE compared to the control group, with the superiority of fish-fed BEE-supplemented diets. In accordance, dietary dried basil leaves at 20 g kg⁻¹ diet improved the growth performance of hybrid tilapia fingerling over the control (El-Dakar et al., 2008). Common carp, *C. carpio*, fed BEE-supplemented diets experienced higher growth performance than the non-supplemented group (Amirkhani and Firouzbakhsh, 2015). In addition, the use of 2.0 ml of basil essential oil/kg diet improved the FBW, WG, and feed conversion ratio of pirarucu, *A. gigas*, juveniles (Chung et al., 2020). Dietary supplementation of basil oil at levels of 1–5 g/kg significantly improved FBW, WG (%), and feed intake of *P. indicus* in a dose-dependent manner (Abdel-Tawwab et al., 2021). The growth-stimulating effects of basil extracts could be attributed to the

TABLE 4 Effect of dietary supplementation of basil ethanol and aqueous extracts (mg kg⁻¹ of feed) on hematological parameters of Nile tilapia, *O. niloticus*.

Variable	BEE				BAE				Pooled SEM	p-Value		
	0	200	300	500	0	200	300	500		Extract	Dose	E×D ¹
Red blood cells (10 ⁶ μl ⁻¹)	2.09 ^e	2.27 ^d	2.40 ^b	2.55 ^a	2.09 ^e	2.23 ^d	2.31 ^{cd}	2.39 ^{bc}	0.03	<0.001	<0.001	0.002
Wight blood cells (10 ⁴ μl ⁻¹)	25.42 ^d	35.91 ^b	40.45 ^a	38.72 ^a	25.42 ^d	31.01 ^c	34.18 ^b	35.55 ^b	0.55	<0.001	<0.001	<0.001
Hemoglobin (g dL ⁻¹)	9.37 ^c	11.95 ^b	13.72 ^a	13.23 ^a	9.37 ^c	10.16 ^c	11.35 ^b	11.83 ^b	0.23	<0.001	<0.001	<0.001
Packed cell volume (%)	18.35 ^f	27.82 ^c	32.12 ^a	30.78 ^b	18.35 ^f	20.19 ^e	24.34 ^d	27.78 ^c	0.33	<0.001	<0.001	<0.001
The differential leukocyte count												
Lymphocyte (10 ³ μl ⁻¹)	52.00 ^e	62 ^c .00	65.67 ^b	69.00 ^a	52.00 ^e	54.67 ^e	58.67 ^d	63 ^b .00 ^c	0.82	<0.001	<0.001	<0.001
Monocyte (10 ³ μl ⁻¹)	1.33	1.33	1.00	1.67	1.33	2.00	1.67	1.67	0.41	0.122	0.468	0.468
Basophils (10 ³ μl ⁻¹)	10.67	11.33	9.00	8.33	10.67	10.00	9.67	9.00	1.88	1.00	0.368	0.86
Eosinophils (10 ³ μl)	13.33	11.67	7.00	7.33	13.33	10.33	12.00	7.33	2.08	0.392	0.007	0.188
Neutrophils (10 ³ μl)	22.00	13.67	17.33	13.67	22.00	20.33	18.00	19.00	3.48	0.088	0.132	0.456

Values are means and pooled SEM. BEE: basil ethanol extract, BAE: basil aqueous extract.

^{a-f}Means in a row without a common superscript letter differ ($p < 0.05$) as analyzed by two-way ANOVA and the TUKEY test.

¹E × D = Extract × Dose interaction effect.

TABLE 5 Effect of dietary supplementation of basil ethanol and aqueous extracts (mg kg⁻¹ of feed) on serum biochemical examination of Nile tilapia, *O. niloticus*.

Variable	BEE				BAE				Pooled SEM	p-Value		
	0	200	300	500	0	200	300	500		Extract	Dose	ExD ¹
Total protein (g dl ⁻¹)	3.14 ^e	3.71 ^c	4.18 ^b	4.67 ^a	3.14 ^e	3.22 ^{de}	3.43 ^d	3.67 ^c	0.07	<0.001	<0.001	<0.001
Albumin (g dl ⁻¹)	2.17 ^{ab}	2.03 ^b	2.27 ^a	2.10 ^b	2.17 ^{ab}	1.11 ^c	2.17 ^{ab}	2.17 ^{ab}	0.04	<0.001	<0.001	<0.001
Globulin (g dl ⁻¹)	0.97 ^e	1.677 ^{bc}	1.91 ^b	2.567 ^a	0.97 ^e	1.11 ^c	1.237 ^{de}	1.507 ^{cd}	0.08	<0.001	<0.001	<0.001
AST (U l ⁻¹)	22.23 ^a	19.70 ^{bc}	16.50 ^{de}	15.37 ^e	22.23 ^a	20.40 ^{ab}	19.47 ^{bc}	17.83 ^{cd}	0.65	<0.001	<0.001	0.015
ALT (U l ⁻¹)	28.83 ^a	25.50 ^{ab}	21.70 ^c	20.8 ^c	28.83 ^a	27.73 ^a	25.90 ^{ab}	24.03 ^{bc}	0.98	<0.001	<0.001	0.045
Creatinine	0.79 ^a	0.72 ^{bc}	0.64 ^e	0.54 ^f	0.78 ^a	0.75 ^{ab}	0.69 ^{cd}	0.65 ^{de}	0.01	<0.001	<0.001	<0.001
Cholesterol	155.70 ^a	137.30 ^{bc}	122.70 ^{cd}	87.67 ^e	155.70 ^a	147.70 ^{ab}	137.00 ^{bc}	118.70 ^d	4.56	<0.001	<0.001	0.002
Triglyceride	133.70 ^a	127.30 ^{ab}	114.70 ^c	102.00 ^d	133.70 ^a	130.00 ^a	122.70 ^{ac}	117.30 ^{bc}	3.40	0.002	<0.001	0.028

Values are means and pooled SEM. BEE: basil ethanol extract, BAE: basil aqueous extract, AST: aspartate aminotransaminase, ALT: Alanine aminotransaminase.

^{a-f}Means in a row without a common superscript letter differ (p < 0.05) as analyzed by two-way ANOVA and the TUKEY test.

¹E × D = Extract × Dose interaction effect.

TABLE 6 Effect of dietary supplementation of basil ethanol and aqueous extracts (mg kg⁻¹ of feed) on cellular and humoral immune parameters, and antioxidant status of Nile tilapia, *O. niloticus*.

Variable	BEE				BAE				Pooled SEM	p-Value		
	0	200	300	500	0	200	300	500		Extract	Dose	ExD ¹
Nitroblue tetrazolium (OD 450 nm)	0.23 ^c	0.26 ^a	0.21 ^e	0.23 ^c	0.23 ^c	0.26 ^a	0.24 ^b	0.22 ^d	0.001	<0.001	<0.001	<0.001
Phagocytic activity (%)	20.67 ^f	31.33 ^c	34.33 ^b	37.33 ^a	20.67 ^f	23.67 ^e	27.00 ^d	31.00 ^c	0.58	<0.001	<0.001	<0.001
Phagocytic index (%)	1.61 ^f	2.69 ^b	2.81 ^b	3.73 ^a	1.61 ^f	1.87 ^e	2.10 ^d	2.46 ^c	0.07	<0.001	<0.001	<0.001
Lysozyme (unit ml ⁻¹)	0.22 ^f	0.24 ^e	0.35 ^b	0.43 ^a	0.22 ^f	0.22 ^{ef}	0.27 ^d	0.29 ^c	0.001	<0.001	<0.001	<0.001
Bactericidal activity (%)	42.60 ^c	45.30 ^a	29.60 ^e	42.60 ^c	42.60 ^c	45.30 ^a	43.10 ^b	41.70 ^d	0.001	<0.001	<0.001	<0.001
Immunoglobulins (IgM) (µg ml ⁻¹)	1.15 ^f	1.36 ^e	2.13 ^b	2.38 ^a	1.16 ^f	1.75 ^c	1.54 ^d	1.67 ^c	0.04	<0.001	<0.001	<0.001

Values are means and pooled SEM. BEE: basil ethanol extract, BAE: basil aqueous extract.

^{a-f}Means in a row without a common superscript letter differ (p < 0.05) as analyzed by two-way ANOVA and the TUKEY test.

¹E × D = Extract × Dose interaction effect.

aromatic flavor, which enhances the olfactory and palatability of the experimental diets (El-Dakar et al., 2015). The stimulation of fish appetite could improve voluntary feed intake, and consequently, provide enough nutrients to improve fish weight (Sallam et al., 2017). In addition, basil extract has been shown to improve digestive system functions and enhance feed utilization in fish (de

Souza et al., 2019) as well as modulate intestinal microbiota (Reverter et al., 2014).

Regarding the difference in Nile tilapia response to both basil extracts (BAE and BEE) in the present study, fish fed BEE gained higher final weight than those fed BAE supplemented diet. Nguyen et al. (2021) reported that the extraction techniques could affect the

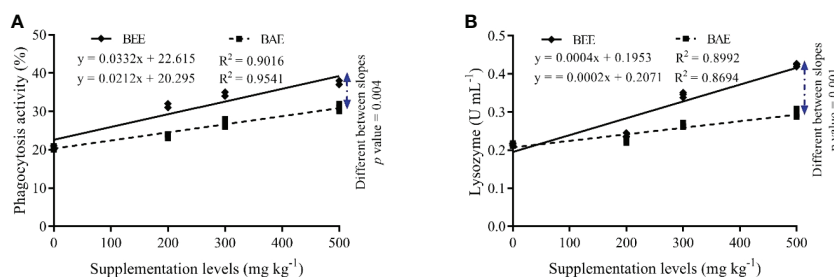


FIGURE 2

The fit regression model and slope differences between dietary supplementation of basil ethanol (BEE) and aqueous extracts (BAE) on phagocytosis activity (A) and lysozyme activity (B) of Nile tilapia, *Oreochromis niloticus*.

TABLE 7 Effect of dietary supplementation of basil ethanol and aqueous extracts (mg kg⁻¹ of feed) on relative expression of some immune related genes in head kidney of Nile tilapia, *O. niloticus*.

Variable	BEE				BAE				Pooled SEM	p-Value		
	0	200	300	500	0	200	300	500		Extract	Dose	ExD ¹
IL-1 β	1.13 ^e	1.533 ^d	1.99 ^b	2.73 ^a	1.13 ^e	1.27 ^e	1.52 ^d	1.74 ^c	0.06	<0.001	<0.001	<0.001
TNF- α	1.18 ^e	1.57 ^c	1.96 ^b	2.36 ^a	1.183 ^e	1.28 ^{de}	1.46 ^{cd}	1.65 ^c	0.06	<0.001	<0.001	<0.001
TGF-B	1.67 ^a	1.48 ^{bc}	1.41 ^c	1.18 ^d	1.67 ^a	1.57 ^{ab}	1.51 ^{bc}	1.46 ^{bc}	0.03	<0.001	<0.001	0.001

Values are means and pooled SEM. BEE: basil ethanol extract, BAE: basil aqueous extract.

^{a-f}Means in a row without a common superscript letter differ ($p < 0.05$) as analyzed by two-way ANOVA and the TUKEY test.

¹E \times D = Extract \times Dose interaction effect.

phytochemical constituents of the extract and consequently the biological properties. Basil ethanolic and aqueous extracts have several compounds as alkaloids, flavonoids, saponins, coumarins, reducing sugar, terpenoids, and tannins (Nguyen et al., 2021). However, reducing sugar and saponin were not detected in BEE as well as alkaloids were not present in BAE, which could interpret the difference in fish responses with both extracts.

In addition, in the present study, the inclusion of BEE, as well as BAE to *O. niloticus* diets relatively improved protease, amylase, and lipase activities compared to the control. These findings were in coordination with El-Dakar et al. (2015), where dietary supplementation of basil in gilthead seabream, *S. aurata*, diet increased the concentrations of both lipase and amylase. Moreover, the use of 1 ml of basil essential oil/kg diet of Nile tilapia improved digestive enzyme activities, including protease, amylase, and lipase (de Souza et al., 2019). Also, several medicinal plant supplements to fish feed improve digestion by increasing digestive enzyme secretion and activity (Bilen et al., 2018; Mohamed et al., 2018). The enhancement of digestive systems reported herein could participate in the improvement of fish growth.

Hematological and biochemical evaluations are an important analyses for assessing the health status of the animal in relation to nutritional and cultural conditions (Bicudo et al., 2009). In the present study, both BEE and BAE improved RBCs, WBCs, Hb, PCV, and lymphocytes of Nile tilapia in a dose-dependent manner. The increase in RBCs and Hb in fish fed with basil extract reflects providing a higher oxygen supply for fish growth. The significant increase in lymphocyte count may be related to the increase of white

blood cells synthesis, which may be due to the immunostimulating effect of *O. basilicum* extracts which matches the observed results of Flores et al. (2008) in *O. basilicum* aqueous extract. Also, Nile tilapia-fed basil essential oils reported higher hematological indices than the control (de Souza et al., 2019).

In addition, plasma total proteins reveal the status of nitrogen synthesis and degradation in the fish body, whereas good nutritional status increases plasma proteins and promotes protein deposition (Higuchi et al., 2011). The current findings revealed a significant increase in total protein, albumin, and globulin in BEE and BAE-supplemented groups. This improvement was associated also with higher growth performance. In line with this, high plasma total protein concentrations were linked to rainbow trout growth performance and protein utilization (Rumsey et al., 1994). Moreover, increasing dietary concentrations of basil essential oils in the diet increased plasma total proteins and reduced triglycerides, and ALT levels of Nile tilapia (de Souza et al., 2019) and pirarucu (*A. gigas*) (Chung et al., 2020). Total protein, albumin, and globulin levels were significantly increased but AST, ALT, creatinine, and urea were significantly decreased in *P. indicus* fed 2.5–5.0 g basil oil/kg diet (Abdel-Tawwab et al., 2021).

The serum level of transaminases revealed liver integrity status (Xia et al., 2022). The serum AST and ALT levels of Nile tilapia-fed basil extracts supplemented diet decreased in the current study, indicating the safety of basil ethanolic and aqueous extracts up to 500 mg/kg diet. The hepato-protective activity of BEE or BAE was due to their antioxidant effects, which are related to BEE or BAE phenolic compounds high contents, which act as free radical scavengers, and reducing agents, metal chelators (Jayasinghe et al., 2003).

TABLE 8 Effect of dietary supplementation of basil ethanol and aqueous extracts (mg kg⁻¹ of feed) on serum antioxidant status of Nile tilapia, *O. niloticus*.

Variable	BEE				BAE				Pooled SEM	p-Value		
	0	200	300	500	0	200	300	500		Extract	Dose	ExD ¹
CAT activity (IU l ⁻¹)	1.63 ^e	2.17 ^c	2.44 ^b	2.87 ^a	1.63 ^e	1.847 ^d	2.09 ^c	2.21 ^c	0.04	<0.001	<0.001	<0.001
SOD activity (IU l ⁻¹)	0.22 ^e	0.38 ^{bc}	0.43 ^{ab}	0.47 ^a	0.22 ^e	0.28 ^d	0.32 ^d	0.38 ^c	0.01	<0.001	<0.001	<0.001
MDA level (IU l ⁻¹)	30.74 ^a	25.26 ^c	22.17 ^{de}	19.44 ^e	30.74 ^a	29.10 ^{ab}	26.90 ^{bc}	24.67 ^{cd}	0.84	<0.001	<0.001	0.002
TAC (mM ml ⁻¹)	0.81 ^d	1.37 ^c	1.57 ^b	1.80 ^a	0.81 ^d	0.92 ^d	1.33 ^c	1.42 ^{bc}	0.05	<0.001	<0.001	<0.001

Values are means and pooled SEM. BEE: basil ethanol extract, BAE: basil aqueous extract, CAT: catalase, SOD: superoxide dismutase, MDA: malondialdehyde, TAC: total antioxidant capacity.

^{a-f}Means in a row without a common superscript letter differ ($p < 0.05$) as analyzed by two-way ANOVA and the TUKEY test.

¹E \times D = Extract \times Dose interaction effect.

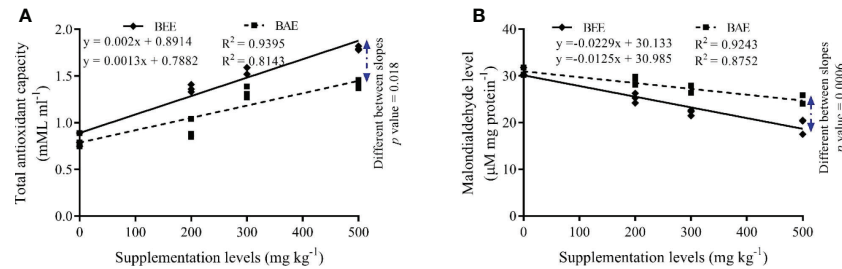


FIGURE 3

The fit regression model and slope differences between dietary supplementation of basil ethanol (BEE) and aqueous extracts (BAE) on total antioxidant capacity (A) and malondialdehyde levels (B) of Nile tilapia, *Oreochromis niloticus*.

Furthermore, the effect of dietary supplementation of BEE and BAE on kidney filtration activities showed a decrease in creatinine levels with increasing supplementation levels. In accordance, dietary basil extract decreased creatinine levels in the serum of rainbow trout after 15 days of feeding (Pastorino et al., 2022). In addition, plasma urea levels decreased with 2.0 ml basil essential oil/kg (Chung et al., 2020). This could indicate better kidney functions, together with decreasing urea levels (Ajeniyi and Solomon, 2014).

The oxidant/antioxidant balance is a normal biological process among the continuously produced free radicals and antioxidant defense systems (Di Giulio et al., 1989). However, under stressful conditions, including farming practices or during competing for infectious diseases, this balance could be interrupted, leading to lipid peroxidation and several oxidative stress implications (Ji, 1995; Guo et al., 2015). The present findings revealed an increase in CAT and SOD activities and decreasing in MDA levels with increasing both BEE and BAE concentrations in *O. niloticus* diets compared to the control. Similarly, the oxidant/antioxidant balance was improved in terms of high SOD, CAT, and glutathione peroxidase activities, and low MDA and nitric oxide levels with increasing dietary levels of basil oils. (Abdel-Tawwab et al., 2021). Rainbow trout, *O. mykiss*, fed a diet supplemented with a basil supercritical extract at a level of 0.5% experienced higher SOD, CAT, and glutathione activities, and reduced the MDA levels in liver and kidney of supplemented fish (Magara et al., 2022). Basil extract's showed high antioxidant properties, likely due to the presence of polyphenols recognized as antioxidant molecules (Perron and Brumaghim, 2009). The anti-oxidative activity of basil extract may be related to the high content of some bioactive compounds, such as rutin, epicatechin (Rezzoug

et al., 2019), flavonoid content of BEE or BAE (Nguyen et al., 2021), and their antioxidant ability (Chin and Lindsay, 1994).

The innate immune response is the first defense line of fish against pathogens. In the present study, dietary supplementation of both BEE and BAE stimulated cellular and humoral immune responses of Nile tilapia, whereas phagocytosis activity and index, lysozyme, and Ig significantly improved compared to the control. In the same line, various herbal compounds triggered the increase of plasma lysozyme activity (Abdel-Tawwab et al., 2021; Mansour et al., 2022b) as an important component of the innate immune system, as lysozyme is a mucolytic enzyme excreted by leukocytes and found in most body fluids and mucus (Motlagh et al., 2020). It increases in the present study associated with increased lymphocyte count. In addition, dietary basil oil up to 1% increased respiratory burst activity, serum lysozyme, and serum bactericidal activity of Nile tilapia after 42 days of the experiment (El-Ashram et al., 2017). Basil oil supplementation stimulated the humoral innate immune response, including lysozyme and phenol oxide activities in *P. indicus* (Abdel-Tawwab et al., 2021). Furthermore, basil aqueous extract is a powerful natural immunomodulatory substance and could directly affect the lymphocytes and modulate several immune-related genes expression (Jaw-Ming, 2011). Also, the *O. basilicum* immunostimulant effect could be due to the high flavonoid content (El-Ashram et al., 2017), a phenolic compound content that enhances both non-specific and specific immune response (Nahak and Sahu, 2014).

In addition, the balanced cytokine secretion orchestrated the immune response, including *IL-1β* and *TNF-α* as pro-inflammatory and *TGF-β* as an anti-inflammatory cytokine, which acts on enhancing the defense against pathogens and regulates the balanced

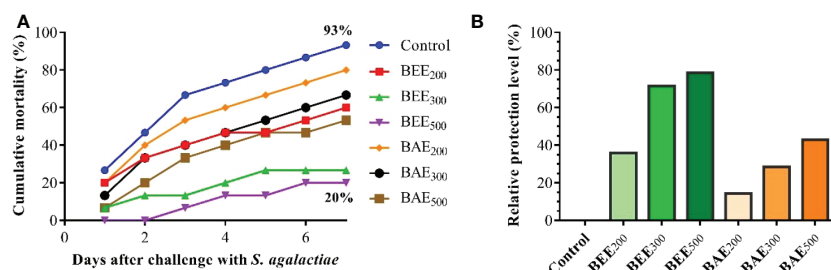


FIGURE 4

Cumulative mortality curve (A) and relative protection level (B) of Nile tilapia, *Oreochromis niloticus*, fed different dietary supplementation of basil ethanol (BEE) and aqueous extracts (BAE) and challenged with *S. agalactiae*.

inflammatory response (Goldstein et al., 2006). In the present study, supplementation with BEE or BAE up-regulated *IL-1 β* and *TNF- α* and down-regulated *TGF- β* in a concentration-dependent manner in both BEE and BAE-treated groups compared to the control group. In accordance, oregano essential oil increased the transcription levels of *IL-1* and *IL-10* and downregulated *TNF* and *TGF* (Abdel-Latif et al., 2020). Meanwhile, Güez et al. (2017) did not report any difference in pro-inflammatory cytokines (*TNF- α* and *IL-6*) in response to *O. basilicum* hydro-alcoholic.

The wide use of antimicrobial agents in modern food animal production has led to the emergence of antimicrobial resistance worldwide (Park et al., 2012; Behera et al., 2018; Legario et al., 2020). It is worth noting that in the present study dietary basil extract improved the resistance and decreased mortality of Nile tilapia against *S. agalactiae* infection. *O. basilicum* was effective against *S. agalactiae* and *Pseudomonas fluorescens* pathogens of particular concern for farmed fish (El-Ekiaby, 2019). In the same line, the mortality rate of Nile tilapia was decreased significantly with dietary basil essential oils (El-Ashram et al., 2017). The relative percentage of survival was improved in *P. indicus* fed basil oil and challenged with *Vibrio parahaemolyticus* (Abdel-Tawwab et al., 2021). Also, Mozambique tilapia, *O. mossambicus*, fed with diets supplemented with citrus limon peel extract experienced improved non-specific immune parameters and decreased mortality (Baba et al., 2016). Amirkhani and Firouzbaksh (2015) found that BEE improved *C. carpio* resistance against *A. hydrophila*. These results might be attributed to the antibacterial activities of basil flavonoids (Rezzoug et al., 2019), and BAE was reported to have enhancement effects on the immune responses (Nahak and Sahu, 2014). Moreover, dietary basil oil revealed stress releasing effect against high stocking density (Chung et al., 2020).

Conclusion

The dietary supplementation of both ethanolic and aqueous extracts of sweet basil (*Ocimum basilicum*) significantly improved the growth performance, digestive enzyme activities, and physiological responses of Nile tilapia after eight weeks of treatment. The cellular and humoral immune responses, resistance against *Streptococcus agalactiae*, and antioxidant balance were improved with increasing dietary supplementation of basil extracts. The dose-response study revealed a significant superiority of ethanolic over aqueous extract in most of the investigated parameters. Accordingly, the use of 500 mg ethanol extract/kg diet of Nile tilapia could promote growth performance, improve antioxidant status, and stimulate the immune response. However, higher levels of the ethanolic extract could be evaluated to determine the optimum dietary levels of basil extract.

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Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Ethics Committee of Animal Use in Research Committee, Faculty of Veterinary Medicine, Alexandria University.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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