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RESEARCH

Diets supplemented with phytobiotics *Calopogonium mucunoides*, *Ocimum gratissimum*, and *Tridax procumbens* improve growth, immunity, and *Oreochromis niloticus* resistance to *Streptococcus agalactiae*

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Abstract

This research examined how three phytobiotics impact the growth, utilization of feed, immune response, and resistance of Nile tilapia (*Oreochromis niloticus*) to disease. A total of 180 fish, with an average initial weight of 31.2 g (number = 15 per 150-L tank), were randomly divided into four groups, with each group replicated three times. Fish were fed isoenergetic (15.7 kJ g^{-1} gross energy) or isonitrogenous (262 g/kg^{-1} crude protein) control diets supplemented with 1% *Calopogonium mucunoides*, *Ocimum gratissimum*, or *Tridax procumbens* leaf meals, and the feeding trial lasted 10 weeks. The growth performance, feed utilization, and innate immunity of the fish were measured. Ten fish from each replicate were intraperitoneally injected with *Streptococcus agalactiae*, and mortality was recorded for 18 days. The *O. niloticus* fed phytobiotic-enriched diets exhibited significantly greater weight gain (68.6 ± 3.7; 70.3 ± 1.6; 71.6 ± 2.9) and specific growth rates (1.70 ± 0.05; 1.71 ± 0.01; 1.73 ± 0.06) than did those in the control group (55.3 ± 1.1; 1.50 ± 0.05). Additionally, phytobiotic supplementation in Nile tilapia diets positively impacted feed conversion efficiency, protein efficiency ratio, feed conversion ratio, hematocrit, hemoglobin, red blood cells, lymphocyte count, serum protein profile, glucose level, kidney-liver function, and bactericidal and lysozyme activities. Following the challenge with *S. agalactiae*, the survival rates were significantly greater ($p < 0.05$) in the fish fed a diet supplemented with *O. gratissimum* (70%), *T. procumbens* (63.3%), or *C. mucunoides* (53.3%) than in those in the control group (26.3%). The abovementioned phytobiotics incorporated into Nile tilapia feed can boost production and enhance growth performance, feed efficiency, immunity, and resistance to disease.

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Introduction

The Nile tilapia (*Oreochromis niloticus*) ranks as the second most economically valuable fish species in aquaculture globally owing to its high growth rate, high nutritional value favored by consumers, and adaptability to different environmental conditions (FAO 2020; Mengistu et al. 2020). However, outbreaks of diseases, particularly those caused by bacterial pathogens, pose major challenges to sustainable production (Jansen et al. 2019). *Streptococcus agalactiae* is a bacterial pathogen that affects various freshwater and marine fish species, including Nile tilapia, resulting in severe economic losses (He et al. 2021). The abnormally high fish mortality, which sometimes exceeds 70% of fish stocks linked to *S. agalactiae*, is forcing some fish farmers to use chemicals or antibiotics to treat infections to maintain tilapia production. Nevertheless, the misuse of antibiotics in fish culture has sparked concerns about public health and the safety of consumers. Scientists have expressed opposition to chemical treatment in fish culture due to adverse consequences, including the increase in pathogenic resistance strains, residual chemical accumulation in fish tissues, and potential harm to both human health and aquatic organisms (Gholamhosseini et al. 2020; Han et al. 2020; Lulijwa et al. 2020; Shen et al. 2020). In addition to the drawbacks associated with antibiotics (Shakya 2017), their exorbitant price increases the cost of production (Abdel-Tawwab and El-Araby 2021). Investigating more ecologically friendly and affordable approaches is necessary to enhance fish health and disease resistance. Recently, numerous studies have explored the potential benefits of phytobiotic plant-based feed additives for promoting growth performance, immunity, and disease resistance in various fish species (Saccol et al. 2018; Abidemi-Iromini and Kolawole 2019; Hoseini et al. 2021; Yousefi et al. 2021).

Currently, research on the use of dietary supplements, including phytobiotics, as fish disease prevention measures in aquaculture has increased (Gupta et al. 2021). Phytobiotics are incorporated into fish feed to protect fish from prevalent infections in aquaculture. Previous studies have indicated that the addition of phytobiotics or medicinal herbs such as moringa (*Moringa oleifera* Lam.) (Zhang et al. 2020), common fig (*Ficus carica*) (Wang et al. 2022), and miswak (*Salvadora persica* L.) (Naiel et al. 2021) improves growth performance, reduces stress, increases the innate immune response, and protects fish from pathogenic infections. Compared with antibiotics, they are less expensive and exhibit a reduced environmental footprint. In addition, the inclusion of phytobiotics in the diet improved the flavor, palatability, and acceptability of the feed (Shakya 2017).

The wild peanut (*Calopogonium mucunoides*), mantle button (*Tridax procumbens*), and clove basil (*Ocimum gratissimum*) are three plant species that have shown promising bioactive properties in various contexts. These plants are known for their immunostimulant, antimicrobial, anti-inflammatory, and antioxidant properties, which are essential for maintaining fish health and fighting bacterial infections (Beck et al. 2018; Fadeyi et al. 2020; Ugbogu et al. 2021). Following Hoseinifar et al. (2019, 2020) and Jahanjoo et al. (2018), with the addition of 1% phytobiotic powder meals to common carp (*Cyprinus carpio*) and sobaity sea bream (*Sparidentex hasta*) diets, it was hypothesized that the inclusion of the same amount of the abovementioned phytobiotic leaf meals in the feed would significantly improve growth, hematological, and blood biochemistry, as well as *O. niloticus* resistance to disease. To test the abovementioned hypothesis, this research was carried out to investigate how phytochemicals present in

leaf meals of *O. gratissimum*, *T. procumbens*, and *C. mucunoides* influence the enhancement of growth, blood health, and the ability of *O. niloticus* to better resist *S. agalactiae* infection. The powdered form of the above phytobiotics was used instead of the extraction method. The reason for this is that the preparation is simple and requires fewer resources (Naliato et al. 2021). Moreover, local fish farmers, especially in developing countries, can easily adapt to these practices. This study provides novel information on how these phytobiotics help improve growth and how they affect serum biochemistry parameters, especially liver-kidney functions and other immunological parameters, in *O. niloticus* before and after *S. agalactiae* infection. Understanding the effects of these phytochemicals in phytobiotics on fish health and disease resistance may provide valuable insights into their potential application as natural and sustainable alternatives to chemicals in tilapia aquaculture.

Study materials and methods

Collection of phytobiotic and leaf meal preparation

Fresh *T. procumbens*, *O. gratissimum*, and *C. mucunoides* were harvested from Mamponteng, a recently developed residential estate in the Kwabre East Municipality of the Ashanti Region. The plants were verified and authenticated at the Forestry Department, Kwame Nkrumah University of Science and Technology (KNUST), before being processed. Following thorough washing under running tap water, distilled water was used to rinse the leaves of each plant. After being air-dried to a moisture content of 10% at room temperature, the leaves were ground into a fine powder with an electric blender and stored at 4 °C until use.

Analysis of phytochemicals in the leaf powders

The leaf powder phytochemical analysis was performed using the Ezeonu and Ejikeme (2016) method. Analysis was conducted on triplicate samples of each leaf powder. Briefly, to assess the alkaloid content, a 2.50 g sample of a powder or supplement was added to 200 cm^3 of 10% acetic acid in ethanol, reduced, filtered, and subsequently added to concentrated ammonium hydroxide. To obtain a constant weight, the mixture was then cleaned, filtered, and oven-dried, after which the supernatant was discarded. Additionally, the flavonoid content of the leaf samples was determined using a 250-cm^3 beaker that contained 2.50 g of each sample and 50 cm^3 of 80% aqueous methanol. Three separate extractions of the residue were performed, after which the residue was filtered, dried in a crucible, and weighed. Additionally, 5 g of each leaf powder sample was put into 100 cm^3 of 20% aqueous ethanol in a conical flask measuring 250 cm^3, which was subsequently heated and agitated to measure the saponin content. Extraction and evaporation of the residue were performed. The mixture was agitated with diethyl ether, and the aqueous layer was collected. Using 5% sodium chloride and 60 cm^3 of n-butanol, two extractions were carried out to obtain the residual solution. The residual solution was heated and put into an oven for weighing. The percentage of each phytochemical (alkaloid, flavonoid, or saponin) was determined.

$$\% \text{ Phytochemical} = \frac{\text{Weight of phytochemical}}{\text{Weight of samples}} \times 100$$

Tannin was determined by mixing 25 cm^3 of orthophosphoric acid (H_3PO_4) and 10 g of phosphomolybdic acid ($H_3PMo12O_{40}$) with Folin–Denis reagent. Before being used in a color development procedure, the solution was heated, cooled, and diluted. A Spectrum Lab 23A spectrophotometer was used to determine the optical density at 700 nm, and the results were compared to a normal tannic acid curve. The optical density was then plotted after the reagents were added to test tubes containing different tannic acid concentrations. The computation was performed using the following formula:

$$\text{Tannic acid}\left(\frac{\text{mg}}{100\text{g}}\right) = \text{C} \times \text{Extract volume} \times \frac{100}{\text{Aliquot volume}} \times \text{Sample weight}$$

where *C* is the concentration of tannic acid detected in the graph.

Proximate chemical analysis of the leaf meals

The proximate chemical composition of the leaf meals was analyzed according to the methodology of Association of official analytical chemists (AOAC) (2005). In brief, each leaf meal sample underwent a 24-h oven-drying process at 105 °C (conducted in a Gallenkamp hot air oven CHF097 XX2.5, Gemini B.V., Apeldoorn, The Netherlands) to measure the dry matter. Subsequently, to evaluate the ash content, the samples were burned in a muffle furnace (K1252, Heraeus Instruments GmbH, Hanau, Germany) for 6 h at 550 °C. The crude protein concentration was measured using the Kjeldahl method (Foss Kjeltec 2200, Hillerod, Denmark), while the method described in Bligh and Dyer (1959) was also employed to estimate the crude lipid concentration. The chemical composition of the leaf meals is presented in Table 1.

Experimental diet preparation

A control diet and three test diets were formulated to obtain four isonitrogenous diets. *T.* procumbens, *C. mucunoides*, and *O. gratissimum* powders were added to the test diets at a 1% level of a basal diet. The control diet was a basal diet without any leaf meals. The mixture was formed into pellets by using a meat grinder with a 3-mm perforated plate attached after being thoroughly mixed with 300 mL of water to moisten it. It was then dried at room temperature. Before use, the feeds were stored at 4 °C in clearly labelled zip-lock plastic bags. The chemical analysis of the experimental feeds was also performed according to the methodology of AOAC (2005) as described in "Proximate chemical analysis of the leaf meals." The experimental diet ingredients and the proximate composition are also listed in Table 1.

Experimental design and fish culture conditions

All-male Nile tilapia juveniles, weighing between 19 and 22 g, were procured from a well-established hatchery located in Akosombo, within the Eastern Region of Ghana. The fish, enclosed in plastic bags enriched with oxygen, were transported to the fish farm of the Faculty of Renewable Natural Resources at KNUST, where the study was conducted. They

Table 1 Experimental diet ingredients and proximate compositions (g/kg)

Ingredients	Control	CMD	TPD	OGD
Fishmeal	100	100	100	100
Soybean meal	320	320	320	320
Wheat bran	410	400	400	400
Soybean oil	50	50	50	50
Cassava flour	80	80	80	80
Vitamins—premix	20	20	20	20
Minerals—premix	20	20	20	20
C. mucunoides meal	0	10	0	0
T. procumbens meal	0	0	10	0
O. gratissimum meal	0	0	0	10
Proximate composition				
Crude protein (%)	26.2	26.2	27.0	27.0
Lipid (%)	9.6	9.8	9.5	10.2
Ash (%)	11.8	11.8	12.0	11.5
Dry matter (%)	93.8	93.1	93.7	93.4
Gross energy (KJ g^{-1})	15.7	15.7	15.7	16.7
Leaf meals' proximate	Composition	*C. mucunoides*	*T. procumbens*	*O. gratissimum*
Crude protein (%)		25.5	20.9	21.3
Lipid (%)		3.4	3.6	3.1
Ash (%)		8.6	12.5	15.6
Dry matter (%)		92.2	90.7	91.3

CMD, *Calopogonium mucunoides*-supplemented diet; *TPD*, *Tridax procumbens*-supplemented diet; *OGD*, *Ocimum gratissimum*-supplemented diet. Mineral premix (per kg of premix): manganese (II) oxide—manganese 3b502, 30,000 mg; zinc oxide—zinc 3b405, 20,000 mg; iron (III) sulfate monohydrate—iron 3b103, 20,000 mg; copper (II) sulfate (pentahydrate)—copper 3b405, 4000 mg; calcium iodate (anhydrous)—iodine 3b202, 600 mg; and sodium selenite 3b801 – selenium, 80 mg. [5]Vitamin premix (per kg of premix): vitamin A/Retinyl acetate (3a672a, 3,400,000 IU; vitamin D 3a671, 1,000,000 IU; choline chloride 3a890, 80,000 mg; vitamin E 3a700 (all-rac-alfa-tocopheryl acetate), 4000 mg; Niacinamide 3a315, 6000 mg; calcium D-pentothenate 3a841, 2420 mg; vitamin B2 3a825, 800 mg; vitamin B6/pyridoxine hydrochloride 3a831, 400 mg; vitamin B1/thiamine mononitrate 3a821, 400 mg; folic acid 3a316, 80 mg; and vitamin B12/cyanocobalamin, 4 mg; Gross energy (KJ g^{-1}), estimated as follows: Crude protein% × 23.66 + Crude lipid% × 39.5 + Nitrogen–free extract × 17.2 (NRC, 1993); Nitrogen − free extract, calculated as follows: 100 − (Crude protein% + Crude lipid% + Ash% + Moisture%)

were routinely examined clinically, parasitologically, and bacteriologically at random to determine the presence of infection, infestation, or pathological lesions, respectively. However, to ensure that fish were free of *streptococci* and other infections, 20 fish were selected randomly from the original population, and their livers, brains, and skin were dissected and streaked onto Tryptic Soy agar plates for bacterial isolation and inoculation. For a 2-week period, they were acclimated in 12-cylindroconical tanks within a recirculating aquaculture system holding 150 L and fed the control diet. A total of 180 fish with an average weight of 31.2 ± 0.8 g were weighed in bulk, counted, and randomly allotted into four groups (45 fish per group) with three replicates each (15 fish per replicate) in the recirculating aquaculture system (RAS)-12 tanks after the acclimatization period. During the experimental period, the fish were hand-fed until they appeared to be satiated and were administered two times a day, at 9:00 am and 4:00 pm. The treatment groups comprised of fish that fed a basal diet

without phytobiotic supplementation (control) and those that fed a control diet enriched with 1% *O. gratissimum*, 1% *C. mucunoides*, or 1% *T. procumbens* leaf meals (Table 1). The quantity of feed fed to each fish group was recorded to calculate the feed utilization and efficiency parameters. The study was conducted in an indoor RAS and filtered both biologically and mechanically. The digital timer switch regulated the fluorescent light in the RAS, enabling a 12-h alternation between light and darkness daily. The fish in each tank were weighed every fortnight using an electronic scale (Constant 5000 g/11LB, China). The fish were not fed on the days they were weighed during the experimental period. Fish waste was siphoned out of the tanks daily. Water quality variables such as pH, temperature, and dissolved oxygen were measured twice a week in each treatment tank using an HACK multiparameter probe (HQ40D, Loveland, Colorado, USA). Concurrently, water samples for ammonia analysis were assessed photometrically within the laboratory. All fish mortalities during the grow-out period were recorded. The trial lasted 70 days, a duration shown to be sufficient for the effect of supplemental feeding on fish (Hoseinifar et al. 2015; Yeganeh et al. 2015; Al-Khalaifah et al. 2020; Lee et al. 2020; Amenyogbe et al. 2022).

The following formulae were used to determine the following growth indices:

$$\text{Fish weight gain (g)} = [\text{Fish final weight (g)} - \text{Fish initial weight (g)}]$$

$$\%\ \text{Fish weight gain} = \frac{[\text{Final weight(g)} - \text{Initial weight(g)}]}{\text{Fish initial weight (g)}} \times 100$$

$$\text{Specific growth rate } (\%\text{day}^{-1}) = \frac{[\text{Ln fish final weight(g)} - \text{Ln fish initial weight (g)}]}{\text{Experimental days}} \times 100$$

$$\text{Average daily growth (g)} = \frac{\text{Weight gain (g)}}{\text{Experimental days (g)}}$$

$$\text{Feed conversion ratio} = \frac{\text{Feed intake (g)}}{\text{Weight gain (g)}}$$

$$\text{Feed conversion efficiency} = \frac{\text{Weight gain of fish (g)}}{\text{Feed intake of fish (g)}} \times 100$$

$$\text{Protein efficiency ratio} = \frac{\text{Weight gain (g)}}{\text{Protein intake (g)}}$$

$$\text{Survival rate} = \frac{\text{Final fish number}}{\text{Initial fish number}} \times 100$$

Blood sampling for the osmotic fragility test and hematological analyses

At the end of the 70-day period, three fish per replicate (for a total of nine per treatment group) were randomly selected. These chosen fish were sedated in aerated water using propofol buffer (10 mg/L) from NEOROF® Laboratory Limited, India, following a 24-h period of feed deprivation. Blood samples from the fish were collected with the use of a sterile plastic syringe and needle through a nonlethal puncture of the caudal vein. A set of blood samples was collected and immediately placed in test tubes coated with a solution of ethylenediaminetetraacetic acid (EDTA). This set of blood samples was used to determine the osmotic fragility test and full blood count. The fish were placed in several tanks of aerated freshwater to recover after blood sampling.

The rates of hemolysis in NaCl solutions with varying osmotic pressures (0.1 to 0.7% NaCl), as reported previously by Yang et al. (2013), with minor modifications, were used to examine the osmotic fragility of red blood cells. Twenty (20) microliter blood samples were gently diluted with 500 µL of NaCl solution in 1.5-mL glass tubes, incubated for 30 min at room temperature, and centrifuged for 5 min at 7000 rpm. After collection, the supernatants were transferred into a 96-well microplate with a flat bottom (Micro Plate Read, Senior No.: RT0400814GDM, Germany), and the optical density was measured at 492 nm. Prior to this, a map indicating the various treatment replicates was placed in a

flat-bottom 96-well microplate, and the corresponding numbers were drawn on a plane sheet of paper for easy tracing and recording of the optical density. The hemolysis rates (100 and 0%) of the samples were determined and corrected in accordance with their hematocrit levels in both deionized water and a 0.85% NaCl solution.

The blood samples were subjected to analysis using an automated blood analyzer (Sysmex XP 300 model, Japan). The measured parameters included hemoglobin levels, hematocrit levels, platelet counts, red blood cell counts, white blood cell counts, mean corpuscular volume, mean corpuscular hemoglobin levels, mean corpuscular hemoglobin concentration, and differential white blood cell counts, such as lymphocyte counts, mixed difference counts (basophil, monocyte, and eosinophil counts), and neutrophil counts.

Blood sampling for serum biochemical and immunological analyses

A second batch of blood samples, three fish per replicate (nine fish per treatment group), was taken from another batch of fish samples. The samples were placed in gel-containing serum tubes, allowed to coagulate, and subjected to 4000 rpm centrifugation (Eppendorf 5804) for 10 min to separate the serum from the cellular components. The serum was subsequently kept at a temperature of − 20 °C until use. Using a spectrophotometer (Jenway 6305, Cole Palmer, Staffordshire, UK), photometric analysis was performed on the serum samples to assess the levels of total proteins; albumin, glucose, cholesterol, and triglyceride; kidney function test parameters, including total bilirubin, urea, and creatinine; and liver function indicators, such as alanine aminotransferase and aspartate aminotransferase. The concentrations of the serum samples were assessed according to the guidelines provided by the manufacturer using commercially available diagnostic reagent kits (ELITech Diagnostics, ELITech Group, Puteaux, France). Some of the serum samples were also used to measure the activities of lysozyme and bactericide.

The lysozyme activity was assessed utilizing the turbidimetric procedure outlined by Kumari et al. (2006), with slight modifications. A volume of 25 µL of blood serum was dispensed into a flat-bottomed 96-well microplate, followed by the addition of 175 µL of bacterial suspension (*Micrococcus lysodeikticus*, Sigma M3770). After 0 and 30 min of incubation at 25 °C, the optical density was then measured at 450 nm. Using Sigma's hen egg white lysozyme, a standard curve was generated.

Isolation of *S. agalactiae* for bactericidal activity and disease challenge

Tissue samples were collected aseptically from the spleen, liver, brain, and kidney of Nile tilapia showing clinical signs of streptococcosis, sourced from a cage farm located at Akosombo on Volta Lake in Ghana. The bacteria were isolated and quantified using the pour plate method, with growth observed on tryptic soy agar (TSA; Oxoid Ltd., Basingstoke, UK) supplemented with 2% NaCl, prepared on sterile petri dishes. The plates were incubated at 30 °C for 24 h at 110 rpm in a rotating shaker incubator. Initially, the samples were inoculated onto tryptic soy broth, followed by streaking the cultured broth onto tryptic soy agar for a 24-h incubation period at 30 °C. Following the incubation period, all isolates exhibiting *Streptococcus* characteristics, such as gram-positive cocci arranged in pairs or chains and non-motility, underwent gram staining and biochemical assays for identification and characterization. For this purpose, the bacteria were cultured on brain heart infusion agar (BHIA; Oxoid Ltd., UK) supplemented with 2% NaCl and then incubated at 35 °C for 24 h. Identification of the bacterial isolates (*S. agalactiae*) was conducted using the API 20

Strep Kit (bioMerieux Inc., Durham, NC) system, where results were compared with the analytical profile index as per the manufacturer's instructions.

The bactericidal activity was measured on the basis of the serum's ability to kill *S. agalactiae*. The *S. agalactiae* bacterial culture was centrifuged at 15,000 rpm for 15 min at 4 °C, and the obtained pellet was purified before diluting in phosphate-buffered saline until the optical density reached 0.5 at a wavelength of 546 nm. The samples were serially diluted five times with phosphate-buffered saline (1:10). The serum's bactericidal activity was assessed by combining 2 µL of the diluted bacterial solution with 20 µL of each fish treatment group serum and then incubating the mixture for 1 h at 37 °C. In the group of bacterial controls, phosphate-buffered saline was used instead of serum. Following the incubation period, the colonies were inoculated on tryptic soy agar plates at 37 °C for 24 h to determine the viable bacteria count.

The inoculum of *S. agalactiae* was used to perform the disease challenge. Before its use, the bacterial suspensions underwent dilution using a sterile saline solution (0.75% NaCl solution) until they reached a concentration of 1.0×10^7 CFU/mL, employing a ten-fold serial dilution. Before calculating the cell density using standard plate count methods, the cells were counted on Tryptic Soy Agar plates. The bacteria inoculum was initially administered to a cohort of ten naïve Nile tilapia with an average weight of 50 g to validate its pathogenicity. Additionally, a control group consisting of ten fish was injected intraperitoneally with 0.1 mL of a 0.9% saline solution to act as a sham treatment. Following the experiment, ten fish randomly chosen from each replicate group were transferred to 12 plastic tanks within a confined facility. In this setting, the plants were infected through intraperitoneal injection of 0.1 mL of solution containing 10^7 CFU mL^{-1} live *S. agalactiae* in a 0.85% normal saline solution. The fish were fed their respective treatment diets, and the degree of clinical signs, either mild, moderate, or severe, was assessed following the modality outlined by Haenen et al. (2023). Additionally, mortalities were meticulously documented. Each aquarium underwent daily manual renewal of half of its water volume. Daily siphoning of fish waste from the tanks was also performed. To prevent cross-contamination, different siphons and equipment were used for each treatment. The plants were disinfected with an iodine solution after each use. The cumulative survival of the fish for 18 days was considered. Necropsies were performed on moribund or freshly dead fish, and samples from the brain, liver, kidney, and trunk were streaked on Slanetz and Bartley Agar plates to isolate bacteria. Blood samples from the eight fish that survived were collected from each treatment group for serum biochemical and lysozyme activity analysis using the methods already described. The equation below was used to calculate the percentage of fish that survived after being infected with *S. agalactiae*:

$$\% \text{ Survival} = \frac{\text{Survived fish number after disease challenge}}{\text{Fish challenged with disease number}} \times 100$$

Analysis of the data

GraphPad Prism version 8 was used to construct the graphs and conduct the data analysis. Variations among the four treatment groups across all parameters were assessed through one-way analysis of variance (ANOVA), followed by Tukey's test for multiple comparisons. A significance level of $p < 0.05$ was used to measure differences between treatment groups. Before analysis, percentages underwent arcsine transformation, and data normality

Table 2 Water quality in the recirculating aquaculture system tanks during experimental periods

Water quality parameter	Control-T	TP-T	CM-T	OG-T	*p* value
Dissolved oxygen (mg/L)	5.29 ± 0.5	5.34 ± 0.5	5.21 ± 0.4	5.29 ± 0.5	0.87
Ammonia (mg/L)	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.96
pH	6.90 ± 0.9	6.90 ± 0.9	6.80 ± 0.9	6.90 ± 0.8	0.94
Temperature (°C)	28.24 ± 0.7	28.24 ± 0.7	28.23 ± 0.7	28.31 ± 0.7	0.98

Control-T, Control fish group RAS-tank; *TP-T*, *Tridax procumbens* treated fish group RAS-tank; *CM-T*, *Calopogonium mucunoides* treated fish group RAS-tank; *OG-T*, *Ocimum gratissimum* treated group RAS-tank

Table 3 Phytochemical composition of the leaf powders

Phytochemical	*Tridax procumbens*	*Calopogonium mucunoides*	*Ocimum gratissimum*	*p* value
Alkaloids (%)	9.3 ± 0.21^{a}	9.4 ± 0.02^{a}	12.3 ± 0.04^{b}	< 0.0001
Flavonoids (%)	4.8 ± 0.11^{b}	4.4 ± 0.30^{b}	3.5 ± 0.10^{a}	0.0001
Saponins (%)	3.1 ± 0.06^{b}	2.1 ± 0.10^{a}	4.4 ± 0.10^{c}	< 0.0001
Tannins mg/100 g	2.1 ± 0.10^{b}	1.6 ± 0.08^{a}	5.6 ± 0.11^{c}	< 0.0001

Significant differences are indicated by different superscript letters in the row ($p < 0.05$) based on analysis of variance followed Tukey's multiple comparison test

was verified by the use of the Kolmogorov−Smirnov test. The results are displayed as the mean ± standard deviation of the mean.

Results

Water quality

The water quality variables of the RAS tanks are displayed in Table 2. The temperature, ammonia, pH, and dissolved oxygen content were similar among all the treatment tanks. The values ranged from 28.23 to 28.31 °C, 0.03 to 0.04 mg/L, 6.80 to 6.70, and 5.21 to 5.34 mg/L, respectively, throughout the trial.

Phytochemical composition of the phytobiotics

The phytochemicals examined in the leaf powders are detailed in Table 3. Alkaloid content was significantly higher in *O. gratissimum* compared to *C. mucunoides* and *T. procumbens* ($p < 0.05$). In contrast, flavonoid levels were notably higher in both *C. mucunoides* and *T. procumbens* than in *O. gratissimum*. Additionally, the quantities of saponin and tannin were significantly higher in *O. gratissimum*, followed by *T. procumbens*, when compared to *C. mucunoides*.

Fish growth performance

The growth performance and feed utilization of the Nile tilapia fed the supplemented diets were significantly better than those of the control fish ($p < 0.05$) (Table 4). Improved

Table 4 Effect of phytobiotic-enriched diets on the growth performance and feed utilization of *O. niloticus* cultured for 70 days

Parameters	Control	TPFG	CMFG	OGFG	*p* value
Fish initial weight (g)	31.2 ± 0.4	30.9 ± 0.4	30.6 ± 0.6	31.1 ± 0.5	0.5
Fish final weight (g)	86.4 ± 1.0^{a}	102.7 ± 2.9^{b}	100.6 ± 1.4^{b}	99.6 ± 4.2^{b}	0.0003
Fish weight gain (g)	55.3 ± 1.1^{a}	71.6 ± 2.9^{b}	70.3 ± 1.6^{b}	68.6 ± 3.7^{b}	0.0002
Fish weight gain (%)	177.3 ± 5.3^{a}	232.3 ± 9.9^{b}	230.1 ± 4.7^{b}	220.6 ± 8.8^{b}	< 0.0001
Average daily growth (g)	0.789 ± 0.02^{a}	1.030 ± 0.04^{b}	1.010 ± 0.02^{b}	0.980 ± 0.05^{b}	0.0002
Specific growth rate (%/day)	1.50 ± 0.05^{a}	1.73 ± 0.06^{b}	1.71 ± 0.01^{b}	1.70 ± 0.05^{b}	0.0001
Feed intake (g/fish)	78.9 ± 2.1^{a}	93.9 ± 2.1^{c}	87.7 ± 1.4^{b}	87.3 ± 3.3^{b}	0.0004
Feed conversion ratio	1.43 ± 0.03^{b}	1.31 ± 0.04^{a}	1.30 ± 0.02^{a}	1.30 ± 0.06^{a}	0.003
Feed conversion efficiency	70.03 ± 1.2^{a}	76.4 ± 2.3^{b}	80.2 ± 1.1^{b}	78.5 ± 2.5^{b}	0.0008
Protein efficiency ratio	2.7 ± 0.04^{a}	3.0 ± 0.07^{b}	3.0 ± 0.08^{b}	3.0 ± 0.05^{b}	0.004
Survival rate (%)	91.1 ± 3.8	93.3 ± 0.0	91.1 ± 3.8	93 ± 6.7	0.85

TPFG, *Tridax procumbens*-supplemented fish group; *CMFG*, *Calopogonium mucunoides*-supplemented fish group; *OGFG*, *Ocimum gratissimum*-supplemented fish group. Significant differences are indicated by different superscript letters in the same row ($p < 0.05$) based on analysis of variance followed by Tukey's multiple comparison test

growth performance was shown by increased fish final weight, weight gain, average daily growth, and a specific growth rate, while enhanced feed utilization manifested as a low feed conversion ratio, high feed conversion efficiency, and protein conversion efficiency. Throughout the course of the experiment, the feed intake of the fish groups fed a phytobiotic-containing diet was considerably greater than that of the control fish group ($p < 0.05$). The fish survival rates did not significantly differ among the treatments ($p > 0.05$).

Blood hemolysis in *O. niloticus* osmotic fragility test

Table 5 presents the results of the Nile tilapia osmotic hemolysis test after feeding the fish diets supplemented with phytobiotics. Compared to those in the control group, the fish in the phytobiotic-enriched diet group exhibited significantly lower hemolysis rates ($p < 0.05$) at sodium chloride concentrations ranging from 0.3 to 0.7%. Nonetheless, among the Nile tilapia groups fed phytobiotic-containing diets, the hemolysis rates did not significantly differ.

Hematological parameters of *O. niloticus* fed phytobiotic-supplemented diets

Oreochromis niloticus that were fed diets enriched with phytobiotics presented higher red blood cell counts, hemoglobin levels, and hematocrit levels. However, significantly greater values than those of the control group were observed for the fish fed a diet supplemented with *T. procumbens* and *O. gratissimum* ($p < 0.05$) (Table 6). Only for hemoglobin did the fish fed a *C. mucunoides*-supplemented diet exhibit a substantially greater hemoglobin level ($p < 0.05$) than the control fish. In addition, white blood cells, platelets, or blood indices, such as the mean corpuscular volume and mean corpuscular hemoglobin, did not significantly vary ($p > 0.05$) among the treatment fish groups. Significant differences were found ($p < 0.05$) between the mean corpuscular hemoglobin

Table 5 Effect of phytobiotic-enriched diets on blood hemolysis of *O. niloticus* according to the osmotic fragility test

Sodium chloride concentration (%)	Control	TPFG	CMFG	OGFG	*p* value
0.1	91.1 ± 1.4	88.6 ± 3.4	89.6 ± 2.3	89.0 ± 1.7	0.601
0.2	74.1 ± 2.5	69.1 ± 1.9	68.2 ± 4.9	66.4 ± 2.4	0.076
0.3	63.8 ± 2.2^{b}	40.6 ± 1.9^{a}	35.3 ± 2.1^{a}	38.4 ± 2.1^{a}	< 0.0001
0.4	49.8 ± 3.0^{b}	21.7 ± 1.0^{a}	24.0 ± 1.6^{a}	21.2 ± 2.0^{a}	< 0.0001
0.5	27.8 ± 1.4^{b}	15.1 ± 1.8^{a}	17.0 ± 2.0^{a}	14.4 ± 1.6^{a}	< 0.0001
0.6	15.0 ± 2.3^{b}	9.6 ± 1.4^{a}	9.0 ± 1.4^{a}	9.3 ± 1.9^{a}	0.0098
0.7	8.7 ± 1.2^{b}	3.5 ± 0.8^{a}	3.3 ± 1.2^{a}	3.5 ± 1.3^{a}	0.0008

TPFG, *Tridax procumbens*-supplemented fish group; *CMFG*, *Calopogonium mucunoides*-supplemented fish group; *OGFG*, *Ocimum gratissimum*-supplemented fish group. Significant differences in the rows are indicated by different superscript letters ($p < 0.05$) based on analysis of variance followed by Tukey's multiple comparison test

Table 6 Effect of phytobiotic-supplemented diets on the hematology of *O. niloticus* cultured for 70 days

Parameters	Control	TPFG	CMFG	OGFG	*p* value
Red blood cells ($\times 10^6$/μL)	1.5 ± 0.2^{a}	1.9 ± 0.2^{b,c}	1.7 ± 0.2^{a,b}	1.89 ± 0.1b^{c}	0.0002
Hemoglobin (g/dL)	7.1 ± 0.7^{a}	9.7 ± 1.0^{b}	9.3 ± 1.2^{b}	9.7 ± 1.2^{b}	< 0.0001
Hematocrit (%)	25.0 ± 3.5^{a}	32.2 ± 5.4^{b,c}	26.2 ± 4.7^{a,b}	31.3 ± 5.2^{b,c}	0.0056
MCV (fL)	173.0 ± 28.8	174.0 ± 36.2	158.9 ± 28.8	166.0 ± 28.6	0.708
MCH (pg)	52.2 ± 10.7	52.8 ± 14.4	56.3 ± 6.6	51.2 ± 8.2	0.751
MCHC (g/dL)	29.1 ± 4.8^{a}	30.2 ± 3.0^{a,b}	36.6 ± 8.4^{b}	31.2 ± 5.0^{a,b}	0.038
Platelets ($\times 10^3$/μL)	63.9 ± 17.9	50.8 ± 5.3	54.2 ± 16.1	56.2 ± 21.8	0.396
White blood cells ($\times 10^3$/μL)	45.8 ± 4.0	49.4 ± 5.0	46.7 ± 8.3	48.3 ± 3.6	0.537
Lymphocytes (%)	92.4 ± 2.8^{a}	96.5 ± 1.6^{b}	96.7 ± 1.5^{b}	96.7 ± 1.0^{b}	< 0.0001
Basophils, monocytes, and eosinophils (%)	4.3 ± 2.0^{b}	1.8 ± 1.0^{a}	1.5 ± 0.9^{a}	1.6 ± 0.7^{a}	< 0.0001
Neutrophils (%)	3.3 ± 0.9^{b}	1.7 ± 0.7^{a}	1.8 ± 0.8^{a}	1.7 ± 0.5^{a}	< 0.0001

TPFG, *Tridax procumbens*-supplemented fish group; *CMFG*, *Calopogonium mucunoides*-supplemented fish group; *OGFG*, *Ocimum gratissimum*-supplemented fish group; *MCV*, mean corpuscular volume; *MCH*, mean corpuscular hemoglobin; *MCHC*, mean corpuscular hemoglobin concentration. Different superscript letters in the same row indicate significant differences ($p < 0.05$) based on analysis of variance followed by Tukey's multiple comparison test

concentrations of the fish in the group fed a diet supplemented with *C. mucunoides* and those in the control group, with the values for the fish fed the *C. mucunoides*-supplemented diet (36.6 ± 8.4 g/dL) being greater than those in the control group (29.1 ± 4.8 g/dL). Among the fish in all the treatment groups, white blood cells had a greater percentage of lymphocytes and very low levels of neutrophils, basophils, monocytes, and eosinophils. However, compared with those in the control group, the percentages of lymphocytes in the *O. niloticus*-fed diet enriched with *T. procumbens* (96.5 ± 1.6), *C. mucunoides* (96.7 ± 1.5), and *O. gratissimum* (96.7 ± 1.0) were substantially greater ($p < 0.05$) (92.4 ± 2.8). Conversely, the proportions of mixed differences and neutrophils

in the control group were significantly greater ($p < 0.05$) than those in the supplemented diet group.

Oreochromis niloticus serum biochemistry and bactericidal and lysozyme activities after feeding supplemented diets

All the fish groups fed diets supplemented with phytobiotics had substantially greater serum protein and albumin levels than did the control group ($p < 0.05$) (Table 7). However, no statistical significance ($p > 0.05$) was detected in those fed a *C. mucunoides*-enriched diet, in which the total protein concentration was 48.3 ± 4.9 g/L, compared to that in the control group (42.6 ± 4.4 g/L). The addition of phytobiotics to the diet did not affect the total cholesterol or total bilirubin levels ($p > 0.05$). Furthermore, no significant difference in glucose levels was noted between the fish groups supplemented with phytobiotics and the control group. However, in comparison to those of the *O. niloticus* group fed a diet supplemented with *O. gratissimum*, those of the *O. niloticus* fed a diet supplemented with *C. mucunoides* had significantly lower glucose levels. Moreover, in comparison with those in the control group, the Nile tilapia in the phytobiotic-incorporated diet group exhibited improved levels of creatinine, urea, alanine aminotransferase, and aspartate aminotransferase ($p < 0.05$). Fish fed *T. procumbens* and *O. gratissimum*-enriched diets had considerably greater triglyceride levels than did those fed diets supplemented with *C. mucunoides* or the control diet ($p < 0.05$). In addition, compared with those in the control group, the fish in the phytobiotic diet group exhibited significantly greater bactericidal and lysozyme activities.

Table 7 Effect of phytobiotic-supplemented diets on the biochemical profile, bactericidal, and lysozyme activities of *O. niloticus* cultured for 70 days

Parameters	Control	TPFG	CMFG	OGFG	*p* value
Total protein (g/L)	$42.6 \pm 4.4^{a,b}$	50.7 ± 4.2^{c}	$48.3 \pm 4.9^{b,c}$	51.5 ± 5.4^{c}	0.0015
Albumin (g/L)	14.4 ± 1.9^{a}	17.7 ± 1.9^{b}	19.7 ± 1.2^{b}	22.4 ± 2.3^{c}	< 0.0001
Glucose (mmol/L)	$2.43 \pm 0.7^{a,b}$	$2.5 \pm 0.9^{a,b}$	1.7 ± 0.7^{a}	2.6 ± 0.5^{b}	0.041
Cholesterol (mmol/L)	4.7 ± 1.7	4.03 ± 1.3	4.1 ± 1.6	4.5 ± 1.4	0.764
Urea (mmol/L)	25.3 ± 2.3^{a}	$16.1 \pm 1.3^{b,c}$	18.0 ± 2.1^{c}	15.7 ± 1.3^{b}	< 0.0001
Creatinine (μmol/L)	22.04 ± 3.2^{b}	13.2 ± 3.5^{a}	16.4 ± 4.8^{a}	11.3 ± 1.5^{a}	< 0.0001
Alanine aminotransferase (U/L)	27.8 ± 5.3^{b}	21.0 ± 4.0^{a}	20.7 ± 3.3^{a}	18.3 ± 2.7^{a}	< 0.0001
Aspartate aminotransferase (U/L)	49.0 ± 9.3^{b}	33.4 ± 5.1^{a}	37.1 ± 5.8^{a}	36.0 ± 4.0^{a}	< 0.0001
Triglyceride (mmol/L)	1.12 ± 0.5^{a}	2.6 ± 1.0^{b}	$1.8 \pm 0.9^{a,b}$	2.9 ± 1.4^{b}	0.0027
Total bilirubin (μmol/L)	26.2 ± 2.0	24.8 ± 2.2	25.3 ± 1.5	23.9 ± 2.8	0.406
Bactericidal activity (CFU)	12.3 ± 1.8^{a}	3.8 ± 1.4^{c}	5.5 ± 1.1^{b}	4.2 ± 0.8^{bc}	< 0.0001
Lysozyme activity (U/mL)	144.6 ± 5.5^{a}	$184.8 \pm 6.4^{b,c}$	$166.6 \pm 5.1^{b,c}$	182.2 ± 6.9^{b}	< 0.0001

TPFG, *Tridax procumbens*-supplemented group; *CMFG*, *Calopogonium mucunoides*-supplemented group; *OGFG*, *Ocimum gratissimum*-supplemented group. Different superscript letters in the same row indicate significant differences ($p < 0.05$) based on analysis of variance followed by Tukey's multiple comparison test

Disease challenge

The survival rates of *O. niloticus* following *S. agalactiae* infection in fish fed diets containing *O. gratissimum* (70%), *T. procumbens* (63.3%), and *C. mucunoides* (53.3%) were significantly greater ($p < 0.05$) than those in fish fed the control diet (26.7%) (Fig. 1). Pop eyes, isolation, sluggish movement, reluctance to eat, and skin hemorrhages were common signs exhibited by the fish preceding death. In addition to exhibiting loss of orientation, some fish died without showing any other signs of the disease. The fish displayed varying degrees of signs after disease infection. Severe signs were observed in the control group; moderate signs, in *C. mucunoides*-treated fish; and mild signs, in those fed diets supplemented with *T. procumbens* and *O. gratissimum*. There was no mortality in the control fish group injected with the saline solution as a sham treatment. However, a high percentage of the 20 deaths associated with severe clinical signs of disease, similar to what has already been described above, was observed in those injected with the inoculum.

Serum biochemical parameters and lysozyme activity of *O. niloticus* after disease challenge

After *S. agalactiae* infection, fish fed diets supplemented with phytobiotics exhibited notably elevated total protein and albumin levels and decreased glucose, urea, creatinine, alanine aminotransferase, and aspartate aminotransferase levels in comparison to those in the control group ($p < 0.05$) (Table 8). For cholesterol levels, Nile tilapia fed the supplemented diets exhibited lower but not significant values compared to the control. Nevertheless, for those fed a *T. procumbens*-enriched diet, the value was significantly lower than that of the control ($p < 0.05$). Furthermore, compared with those in the control group, the fish fed diets enriched with phytobiotics exhibited notably elevated lysozyme activity ($p < 0.05$).

Discussion

The study showed that phytobiotic-enriched diets improve the growth and utilization of feed in *O. niloticus*. Growth improvement leads to higher production yields, while efficiently utilizing feed not only reduces production costs but also promotes sustainable aquaculture practices by reducing resource waste. The enhancements in fish growth and feed conversion might be due to the saponins, flavonoids, alkaloids, and tannins present in the phytobiotics

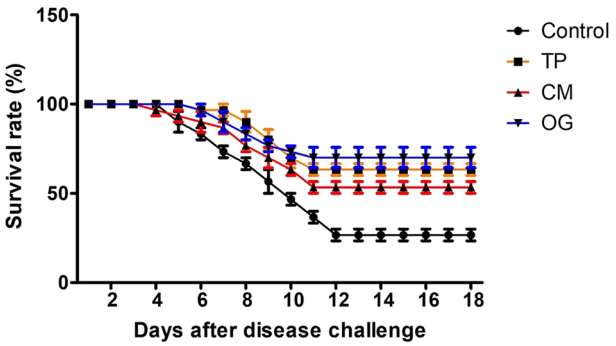

Fig. 1 *O. niloticus* survival rate after feeding on *C. mucunoides* (CM), *T. procumbens* (TP), or *O. gratissimum* (OG)-supplemented diets and infection with *S. agalactiae*. The values represent the means of triplicate samples

Table 8 Effects of *Streptococcus agalactiae* infection on the serum biochemical profile and lysozyme activity of *O. niloticus* fed phytobiotic-supplemented diets

Parameters	Control	TPFG	CMFG	OGFG	*p* value
Total protein (g/L)	29.9 ± 5.7^{a}	$46.7 \pm 2.8^{b,c}$	40.5 ± 4.4^{b}	49.6 ± 4.4^{c}	< 0.0001
Albumin (g/L)	10.8 ± 1.5^{a}	$15.7 \pm 1.3^{b,c}$	13.6 ± 2.4^{b}	18.4 ± 2.2^{c}	< 0.0001
Glucose (mmol/L)	4.43 ± 0.5^{a}	$3.14 \pm 0.6b^{b}$	3.40 ± 0.4^{b}	3.23 ± 0.5^{b}	< 0.0001
Cholesterol (mmol/L)	5.9 ± 1.0^{a}	4.7 ± 0.7^{b}	$5.0 \pm 0.6^{a,b}$	$5.4 \pm 0.7^{a,b}$	0.024
Urea (mmol/L)	37.6 ± 5.1^{a}	27.5 ± 2.9^{b}	30.3 ± 2.4^{b}	26.2 ± 2.0^{b}	< 0.0001
Creatinine (µmol/L)	$36.7.7 \pm 6.3^{b}$	23.5 ± 3.8^{a}	25.2 ± 6.0^{a}	21.7 ± 6.0^{a}	< 0.0001
Alanine aminotransferase (U/L)	52.4 ± 2.0^{c}	$35.5 \pm 2.7^{a,b}$	37.4 ± 2.4^{b}	33.2 ± 2.0^{a}	< 0.0001
Aspartate aminotransferase (U/L)	63.5 ± 7.2^{b}	50.1 ± 2.3^{a}	54.2 ± 4.7^{a}	51.2 ± 2.8^{a}	< 0.0001
Lysozyme activity (U/mL)	192.9 ± 5.9^{a}	241.5 ± 6.6^{c}	226.0 ± 5.8^{b}	244.3 ± 5.5^{c}	< 0.0001

TPFG, *Tridax procumbens* supplemented group; *CMFG*, *Calopogonium mucunoides* supplemented group; *OGFG*, *Ocimum gratissimum* supplemented group. Significant differences are indicated by different superscript letters in the same row ($p < 0.05$) based on analysis of variance followed by Tukey's multiple comparison test

added to the fish feed. In addition, the concurrent presence of the above-mentioned herbal bioactive compounds in phytobiotic leaf meals might potentially induce synergistic effects on the growth and feed conversion efficiency of Nile tilapia. Alkaloids, tannins, and saponins growth-promoting effect and feed conversion ratio improvement have been manifested in fish species such as blunt snout bream (*Magalobrama amblycephala*) (Ye et al. 2019), beluga sturgeon (*Huso huso*) (Safari et al. 2020), and olive flounder (*Paralichthys olivaceus*) (Taştan and Salem 2021). According to Rashidian et al. (2020), the aforementioned phytochemicals also enhance the digestion and absorption of feed while stimulating digestive enzymes, hence promoting growth and feed utilization. Studies have shown comparable outcomes in terms of better utilization of feed and improved growth performance when fish are fed diets enriched with phytobiotics. The growth rate and feed efficiency were strongly enhanced when African catfish (*Clarias gariepinus*) were fed *T. procumbens*-incorporated diets for 8 weeks (Abidemi-Iromini and Kolawole 2019). Feeding *C. gariepinus* on an *O. gratissimum* leaf powder-enriched diet for 12 weeks also enhanced feed efficiency and growth rates (Abdel-Tawwab et al. 2018). Although comparative research on the utilization of *C. mucunoides* as a dietary supplement for fish is lacking, it has been demonstrated that incorporating *C. mucunoides* into the diet of *O. niloticus* leads to improvements in both growth and feed utilization. These improvements are attributed to the presence of bioactive compounds within *C. mucunoides*. These findings align with those of other studies that have shown that supplementing feed with phytobiotics such as Aloe vera and piperine, an alkaloid compound derived from black pepper (*Piper nigrum*), can similarly enhance better growth and feed conversion efficiency in fish (Mehrabi et al. 2019; Shin et al. 2023).

In the present study, phytobiotic-mediated enhancement of immune responses was observed in the blood parameters, which helps the fish maintain their overall health and vitality. The elevated hematocrit, hemoglobin, and red blood cell levels observed in the *O. niloticus* groups fed the supplemented diets could be attributed to the presence of flavonoids, tannins, alkaloids, and saponins found in the leaf meals of the phytobiotics. These hematological variables play a crucial role in evaluating the overall health condition of fish (Hoseini et al. 2021). Higher hematocrit, hemoglobin, and red blood cell levels facilitate more efficient tissue oxygenation in fish, thereby promoting growth and overall health

(Hoseini et al. 2021). Previous studies have reported similar findings, suggesting that diets supplemented with phytobiotics contribute to increased fish growth accompanied by elevated hematocrit, red blood cells, and hemoglobin levels (Adeshina et al. 2021; Hoseini et al. 2021). Adeshina et al. (2021) and Hoseini et al. (2021) explained further that better antioxidant capacity after phytobiotic intake contributed to improvements in hematological indices. Antioxidants are essential for protecting the lipids of erythrocyte membranes from stress resulting from oxidation, thereby inhibiting red blood cell hemolysis. This protective effect was demonstrated by the results of the osmotic fragility test conducted in this study. The dietary inclusion of antioxidant compounds such as amino acids and phytobiotics in diets has been shown to enhance antioxidant defenses and mitigate red blood cell hemolysis in fish species like grass carp (*Ctenopharyngodon idella*) and rainbow trout (*Oncorhynchus mykiss*) (Gao et al. 2016; Adeshina et al. 2021; Hoseini et al. 2021). Alkaloids, flavonoids, saponins, and tannins present in the phytobiotic leaf meals might synergistically work to provide antioxidant protection against oxidative stress. Taştan and Salem (2021) contended that the antioxidant efficacy of phytochemicals stems from their synergistic interactions rather than being attributed to any singular compound. This protective mechanism could promote fish welfare and reduce the occurrence of diseases.

Furthermore, supplementation with the three phytobiotics was found to significantly increase lymphocyte levels in white blood cells. This might be due to the aforementioned bioactive substances present, which may induce higher lymphocyte counts in white blood cells and consequently enhance immune defense against inflammation. Lymphocytes, comprising the largest proportion of white blood cells in fish, play a vital role in enhancing innate immunity by producing antibodies that act as a defense mechanism against infections (Osman et al. 2019).

Increased protein profiles are indicative of an enhanced humoral defense system and strong non-specific immunity in fish (Esmaeili 2021). The favorable effects of tannins, saponins, alkaloids, and flavonoids found in *C. mucunoides*, *T. procumbens*, and *O. gratissimum* might have contributed to the heightened total protein and total albumin levels in fish-fed diets enriched with these phytobiotics during the prechallenge period. In line with these findings, Yonar et al. (2019) noted that the dietary inclusion of turmeric (*Curcuma longa*) enhances total protein and albumin levels in fish blood. Despite the considerable increase in triglyceride levels in the fish groups fed diets incorporated with *O. gratissimum* and *T. procumbens*, the values remained within the normal range for healthy fish, indicating no serious concerns (Mona et al. 2015). Elevated levels of urea, creatinine, and total bilirubin in the serum are commonly associated with kidney dysfunction (Salah et al. 2020). However, Nile tilapia fed the supplemented diets exhibited reduced urea and creatinine levels during the prechallenge period, suggesting that the phytobiotics had a positive impact on *O. niloticus* metabolism, which was potentially linked to decreased stress levels. Phytobiotic intake did not affect total bilirubin levels. This study demonstrated that the dietary incorporation of the phytobiotics enhances *O. niloticus* serum alanine aminotransferase and aspartate aminotransferase levels, indicating improved liver function in these fish. The improvement in liver function observed in fish fed phytobiotic-enriched diets during the prechallenge period may be attributed to increased antioxidant capacity and decreased hemolysis (Mirghaed et al. 2018). Similarly, Abdel-Tawwab and El-Araby (2021) and Yousefi et al. (2021) reported that dietary intake of phytobiotics modulates ALT and AST enzymatic activities.

Research has indicated that diets supplemented with phytobiotics enhance the ability of fish to resist *S. agalactiae*. This was due to the enhanced immune response of the Nile tilapia, as evidenced by the significantly greater activities of bactericides and lysozymes in the

phytobiotic-supplemented diets. The enhanced bactericidal activity, increased survival rate, and reduced signs of streptococcal infection observed in fish fed *T. procumbens*, *O. gratissimum*, and *C. mucunoides*-enriched diets might be linked to the positive impacts of the phytonutrients found in the phytobiotics. Plant-derived compounds, like saponins, exhibit antibacterial properties (Dong et al. 2020). Saponins can adhere to bacterial cell walls, thereby preventing adherence and improving cell wall permeability (Dong et al. 2020). Additionally, they form complexes with bacteria, leading to lysis and subsequent destruction (Khan et al. 2018). Other bioactive compounds, such as flavonoids, tannins, and alkaloids, can also inhibit microbial growth by interfering with microbial enzymes, proteins responsible for transporting cell envelopes, adhering to microbial communities, and binding polysaccharides to form complexes (Castro et al. 2019; El-Beltagi et al. 2019). Only a limited number of studies have delved into the combination of multiple phytochemicals. Nonetheless, it is plausible that such combinations could give rise to synergistic effects, potentially resulting in more favorable outcomes (Taştan and Salem 2021). Saponins, alkaloids, flavonoids, and tannins might synergistically interact to modulate the fish immune system, potentially bolstering disease resistance and overall health.

After the fish were infected with *S. agalactiae*, there was a notable decrease in both total protein and total albumin levels across all treatment groups. However, in the *O. niloticus* groups that were fed phytobiotics, the decreases in total protein and albumin levels were altered, indicating an increase in the humoral defense system of the fish. This protein profile improvement could be attributed to the combined beneficial effect of the phytobiotic constituents, which appeared to restore protein production in liver tissues. In addition, after disease infection, there was an increase in the serum glucose, urea, creatinine, alanine amino transferase, and aspartate aminotransferase levels in the fish groups fed phytobiotic-enriched diets, albeit significantly greater than those in the control group. The enhanced immune response of infected fish may be due to the positive effects of the above bioactive compounds in the phytobiotics. Previous studies have shown that diets enriched with phytobiotics such as milk thistle, *Silybum mari*anum (Owatari et al. 2018), and *Aloe barbadensis* (Gabriel et al. 2015) protect the liver and immune system in *O. niloticus* during infection with *S. iniae* or *S. agalactiae*.

Conclusions

The results of this research indicate that the addition of *T. procumbens*, *C. mucunoides*, and *O. gratissimum* to Nile tilapia diets improves growth, feed utilization, immunity, and resistance to *S. agalactiae* infection. The aquaculture sector can utilize the potential of phytobiotics to support sustainable and healthy fish production while addressing concerns about antibiotic-resistant microbes and chemical residues in seafood products. Based on these results, the phytobiotics mentioned above could be recommended as additives for aquafeed for fish growth improvement, immunity enhancement, and disease resistance.

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Author contributions IOK, KAO, and DAB contributed to the study's conception and design. IOK, KAO, and DAB performed material preparation, data collection, and analysis. IOK wrote the first draft of the manuscript and KAO and DAB commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethical approval The authors followed all applicable international, national, and/or institutional guidelines for the care and use of animals.

Competing interests The authors declare no competing interests.

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