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Stimulatory Effects of *Phyllanthus amarus* Extract on the Growth Performance, Hematobiochemical Activity, Antioxidative Status and Immune Response of *Clarias gariepinus*

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Abstract

This study investigated the antibacterial activity of *Phyllanthus amarus* extracts and its influence on the performance of *Clarias gariepinus* fingerlings. The fish were fed diets containing 0.0, 0.5, 1.0, 1.5 or 2.0 g *P. amarus* methanol extract (PAE) / kg basal diet for 84 days. Thereafter, blood samples were collected for hematological and biochemical analyses. At the end of the 84-day feeding trial, fish were challenged with *Aeromonas hydrophila*, after which immune response parameters were measured. The data obtained were analyzed using one-way analysis of variance at $p<0.05$. The results showed significant antibacterial activity of PAE against *A. hydrophila*, and its application at 0.5-1.5 g significantly promoted growth performance, with the highest at 1.0 g. Hematocrit, hemoglobin and lymphocytes were enhanced at 0.5-1.5 g PAE. All the fishes fed PAE-supplemented diets had lower concentrations of serum liver enzymes; while the values of creatinine, glucose and total bilirubin did not differ among the treatments. Glutathione peroxidase, glutathione-S-transferase, lysozyme, phagocytic and respiratory burst activities increased in all PAE-fortified treatments, with highest fish survival in 1.0 g PAE treatment. Therefore, the inclusion of 1.0 g *Phyllanthus amarus* extract is recommended as a dietary supplement for *Clarias gariepinus*.

Introduction

Aquaculture is growing rapidly as a result of its intensification and diversification to complement capture fisheries, and interestingly, approximately 83% of the aquaculture production in 2020 was from the inland sector (Food Agriculture Organization, 2022) to meet the increasing demand for high quality protein. The global production of aquatic animals from both capture fisheries and aquaculture was estimated to be 178 million tonnes in 2020, 89 percent (157 million tonnes) of which were utilized as food for human consumption. However, intensive aquaculture is associated with stress, which can result to disease infections, especially bacterial diseases, one of which is motile aeromonad septicaemia caused by *Aeromonas hydrophila,* which is widespread in freshwater finfish (Hardi *et al*., 2019; Ngo *et al*., 2020; Adeniyi 2021a) and a serious threat in Clarias gariepinus culture (Adah *et al*., 2021; Mulia *et al*., 2023; Adah *et al*., 2024). Morbidity and mortality resulting from disease outbreaks prompt farmers to utilize synthetic chemotherapeutic drugs to prevent or treat diseases, thereby enhancing the healthy production of farmed fish and preventing economic loss (Reverter *et al*., 2014; Gabriel *et al*., 2015). However, the application of

synthetic chemicals has created substantial problems such as development of drug resistance, residue in fish, negative impacts on consumers' health and aquatic animals, and environmental pollution (Gullberg *et al*., 2011; Lim *et al*., 2013; Santos and Ramus, 2018). Synthetic chemicals are not sustainable for aquaculture use and therefore efficacious alternatives are of necessity.

Herbal supplements have been increasingly utilized in fish culture in the recent decade due to their beneficial effects, including growth-promotion, immune-stimulation, antioxidative status enhancement, as well as cheaper, widely available, relatively safe and have not been reported to cause drug resistance or environmental pollution (Saleh *et al*., 2014; Kareem *et al*. 2016; Abdel-Tawwab *et al*., 2018; Hoseinifar *et al*., 2020; Adesina *et al*., 2021; Adeniyi *et al*., 2023). *Phyllanthus amarus* is a small herb in the family Euphorbiaceae, that has fused leaves and flowers, and is widely distributed in tropical, subtropical and temperate regions worldwide. The plant is well known for its medicinal value in treating many ailments such as gastropathy, diarrhea, dysentery, gonorrhea, menorrhagia, dropsy, hepatitis B, and jaundice, as well as intermittent fever, oxidative stress, scabies, ulcers and wounds (Calixto *et al*., 1998, Verma *et al.,* 2014, Mao *et al*., 2016; Ghosh *et al*., 2022). The plant has many valuable compounds such as lignans (phyllanthin and hypophyllanthin), flavonoids (kaempferol, quercetin, quercitrin, and rutin), hydrolysable tannins (amariin, amariinic acid, furosin, geraniin, and geraniinic acid), triterpenes (phyllanthenol, phyllanthenone, and phyllantheol), and alkaloids (securinine, epibubbialine, isobubbialine, and allosecurine); with a wide spectrum of pharmacological activities, including antibacterial, antidiabetic, antioxidant, and hepatoprotective effects (Verma *et al*., 2014; Ghosh *et al*., 2022). Previous *in vitro* bacteriostatic observations showed significant inhibitory effects of *P. amarus* extracts against some pathogenic bacteria such as *Bacillus subtilis, Staphylococcus aureus*, *Enterococcus fecalis*, *Salmonella typhi*, *Proteus vulgaris*, *Streptococcus* spp*., Escherichia coli, Candidas albican, Aspergillus flavus, Klebsiella pneumonia, Proteus mirabilis*, *Enterobacter species, Serratia marcescens* (Okigbo and Igwe, 2007; Olufemi and Debiri, 2008; Verma *et al*., 2014; Biswas *et al*., 2020). Despite the great medicinal potential of this herb, there is scant scientific information as to its application in fish culture. Therefore, this study aimed to investigate the effect of *P. amarus* on the performance of *Clarias gariepinus*.

Materials and Methods

Plant extraction and Antibacterial Screening

Phyllanthus amarus foliage was obtained, rinsed to remove dirt, and air-dried for 7 days, after which the stems and stalks were removed, while both the leaves and flowers were ground together into fine powder using a kitchen blender. Ethanol, methanol or distilled water was used as solvents for extraction by mixing the blend plant and solvent at a ratio of 1:10 (weight/volume) and thereafter concentrated using a rotary evaporator as described by Adeniyi *et al*. (2023). The antibacterial activity of the plant extracts on *Aeromonas hydrophila*, *Pseudomonas fluorescens,* and *P. aeruginosa* isolates was determined in accordance with the agar-well diffusion method of Clinical and Laboratory Standards Institute (CLSI, 2012) described in Adeniyi *et al*. (2021b).

Experimental Diets

Five isonitrogenous (40% crude protein) and isocaloric (≈405 kcal/100 g gross energy) diets were formulated using fishmeal, ground nut cake, soybean meal, maize flour, fish oil, and vitamin/mineral premix (Table 1). Air-died *Phyllanthus amarus* methanol extract (PAE), which has the highest antibacterial activity at 0.0 (control), 0.5, 1.0, 1.5 or 2.0 g/kg basal diet. The feed ingredients were thoroughly mixed, pelletized and dried using a pelletizer machine (CAP, China).

Experimental Setup and Feeding

Fingerlings of *Clarias gariepinus* were obtained and acclimated for two weeks, during which they were fed commercial feed (Skretting, 1.8 mm). Thereafter, a total of 450 fingerlings (4.37±0.21 g) were randomly stocked at 25 fish per tank (100 L capacity) in triplicate, using an indoor static water-renewal system, in which regular culture water exchange occurred at 48-hour intervals. The fish were starved overnight before the commencement of the feeding trial and thereafter fed to apparent satiation twice daily for 84 days. The fish in each tank were monitored for mortality; dead fish, if any, were removed, and the number of dead fish was recorded. The water quality was determined and monitored during the study, and the dissolved oxygen content, temperature and pH were within 4.5-5.5 mg/L of water, 25.6-27 7^{o}C and 6.9-7.1, respectively.

Growth Performance and Nutrient Utilization

The following parameters were calculated for fish growth performance and feed utilization using the data obtained for each replicate during the study:

Weight gain (WG, g) = Final weight (FW) – Initial weight (IW)

Weight gain (%) = 100 (WG / IW)

Specific growth rate (%/day) = 100 (Ln FW- Ln IW) / Duration of experiment (days)

Feed intake (g) = Sum of feed fed during the experiment

Feed conversion ratio (FCR) = Feed intake / WG

Table 1. Gross composition of the experimental diets containing *Phyllanthus amarus* extract (PAE)

Ingredients	Composition (g/kg diet)				
	0.0	0.5	1.0	1.5	2.0
Fish meal	200.0	200.0	200.0	200.0	200.0
Soya bean meal	260.0	260.0	260.0	260.0	260.0
Groundnut cake	300.0	300.0	300.0	300.0	300.0
Corn flour	206.0	205.5	205.0	204.5	204.0
Fish oil	20.0	20.0	20.0	20.0	20.0
PAE	0.0	0.5	1.0	1.5	2.0
Di calcium phosphate	2.5	2.5	2.5	2.5	2.5
[a]Fish premix	2.5	2.5	2.5	2.5	2.5
Acidifier	1.0	1.0	1.0	1.0	1.0
Toxin binder	1.0	1.0	1.0	1.0	1.0
Methionine	1.0	1.0	1.0	1.0	1.0
Lysine	1.0	1.0	1.0	1.0	1.0
Common salt	5.0	5.0	5.0	5.0	5.0
Total	1000.0	1000.0	1000.0	1000.0	1000.0
Proximate Composition (g/kg)					
Moisture	86.5	86.5	86.7	87.3	87.1
Ash	100.2	100.2	100.2	100.2	100.2
Ether extract	94.7	94.7	94.8	94.8	94.8
Crude fiber	45.0	44.8	44.8	45.3	45.0
Crude protein	400.2	400.2	400.2	400.2	400.2
[b]Nitrogen-free extract	359.8	360.2	360.0	359.6	359.8
[c]Gross energy (kj/kg)	19309.9	19307.2	19308.5	19301.1	19305.7

[a]Fish premix (Miconsult International Ltd.; per kg premix): vitamin A, 22,000 IU; vitamin B1, 20 mg; vitamin B2, 25 mg; vitamin B6, 10 mg; vitamin B12, 0.05 mg; vitamin C, 300 mg; vitamin D3, 5,000 IU; vitamin E, 300 mg; vitamin K3, 10 mg; niacin, 120 mg; folic acid, 5 mg; biotin, 1 mg; inositol, 50 mg; calcium pantothenate, 60 mg; choline chloride, 500 mg; manganese, 30 mg; iron, 35 mg; zinc, 45 mg; copper, 3 mg; cobalt, 2 mg; iodine, 5 mg; selenium, 0.15 mg

[b]Nitrogen - free extract = 1000 – (Crude protein + crude fiber + ether extract + ash)

[c]Gross energy was calculated as 23.6, 39.5, and 17 kJ/g for protein, lipids, and carbohydrates, respectively (NRC, 1993).

Protein efficiency ratio (PER) = WG / Protein intake

Fish survival (%) = 100 (Number of survived fish / Initial number of fish stocked)

Condition factor (CF) = 100 (FW / Final standard length^3)**.**

Blood Collection and Analyses

After the feeding trial blood samples were collected from anesthetized fish in each treatment randomly, through the caudal vein using a 2 mL disposable plastic syringe and 21-gauge disposable hypodermic needle. Two sets of blood samples were dispensed into both ethylenediaminetetraacetic acid (EDTA) and non-EDTA bottles for hematological and serum biochemical analyses. The hematological parameters were analyzed with an automated hematological analyzer, while erythrocytic indices were calculated from the data obtained as described by Adeniyi *et al*. (2023).

Blood samples in non-EDTA bottles were allowed to coagulate and were centrifuged at 3000 rpm at an ambient temperature of 28°C to obtain the serum samples. The samples were stored in a freezer and total protein, albumin, total blood cholesterol, blood bilirubin, creatinine, urea nitrogen, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were subsequently analyzed using a Randox kit, following the standard procedure of the manufacturer. Globulin (Total protein - albumin) level, and the albumin globulin ratio (AGR, albumin / globulin) were calculated. The concentrations of malondialdehyde (MDA) (Varshney and Kale, 1990) and reduced glutathione (GSH) (Jollow *et al*., 1974), as well as the activities of glutathione-S-transferase (GST), glutathione peroxidase (GPx), and superoxide dismutase (SOD) (Rotruck *et al*., 1973), were measured spectrophotometrically.

Fish Challenge Protocols and Nonspecific Immune Responses

Fish (n=8/replicate) were randomly selected, aseptically challenged through intraperitoneal injection of 0.2 mL (1×10^8/mL) *A. hydrophila* as described by Wang *et al*. (2014) and observed for 14 days for survival. Thereafter, blood samples were obtained from the fish and examined for phagocytic (Yoshida and Kitao, 1991), respiratory burst (Secomebs, 1990) and lysozyme (Ellis, 1990) activities spectrophotometrically (UV/VIS, Mettler Toledo, USA) as described previously (Adeniyi *et al*., 2023). Briefly, the number of phagocytic cells was counted and expressed as the percentage of cells with phagocytic activity**;** respiratory burst activity was expressed as the NBT reduction of the sample**,** while one unit of lysozyme activity was defined as the amount of the enzyme producing a decrease in absorbance of 0.001/minute.

Results

Antibacterial Activity

Figure 1 shows the antibacterial activity of PAE against *A. hydrophila*, *P. aeruginosa* and *P. fluorescens*. The methanol extract had the highest antibacterial effect among the three extracts (ethanol, methanol and aqueous) of *P. amarus*. The zone of inhibition of the synthetic antibiotic used did not significantly differ ($p>0.05$) from those of the ethanol and methanol extracts against *P. fluorescens,* while the methanol and ethanol extracts had greater ($p<0.05$) inhibitory effects against *P. fluorescens* than did the synthetic antibiotic. Additionally, the zone of inhibition of *P. amarus* methanol extract against *A. hydrophila* was similar to that of the synthetic antibiotic and greater than the values obtained for the other two extracts.

Growth Performance and Nutrient Utilization

The growth performance and feed utilization of *Clarias gariepinus* fed diets supplemented with different concentrations of *P. amarus* extracts are presented in Table 2. The final weight, weight gain and specific growth rate increased significantly ($p<0.05$) in the fish fed 0.5-1.5 g PAE, with the greatest increase occurring in the 1.0 g treatment group in the present study, compared to those in the control and 2.0 g PAE/kg diet groups. The fish fed 2.0 g PAE had lower ($p<0.05$) feed intake, than did those fed lower levels of PAE and control diets. Lower ($p<0.05$) FCRs were obtained at 1.0 and 1.5 g PAE, while PER of the experimental fish increased significantly in the groups of fish fed PAE-supplemented diets, compared with the control group. The highest ($p<0.05$) fish survival was obtained in the 0.5 g PAE treatment group, compared to the value obtained in the control group, although the survival did not significantly differ from the values obtained at higher concentrations of PAE. Higher values of condition factor were obtained for the 0.5-1.5 g PAE treatment groups, than for the 2.0 g PAE and control groups.

Hematological Profile

The hematological profile of *C. gariepinus* fed the experimental diets is shown in Table 3. Dietary supplementation with 0.5-1.5 g PAE/kg diet enhanced ($p<0.05$) the pack cell volume (PCV) and hemoglobin (Hb). However, significantly greater red blood cell (RBC) counts were obtained in all the groups of fish fed PAE-supplemented diets, than in the control group. The MCH (mean corpuscular hemoglobin) and MCV (mean corpuscular volume) values were greater ($p<0.05$) in the fish fed 0.5-1.5 g PAE/kg diet, than in those fed the control diet, while a higher MCHC (mean corpuscular hemoglobin concentration) was obtained in the fish fed 2.0 g PAE/kg diet. Dietary PAE stimulated higher ($p<0.05$) white blood cell production in the treatment groups fed 0.5-1.5 g/kg concentration, compared with that of the control group. The lymphocyte counts of the fish fed PAE-based diets were higher ($p<0.05$) than in the fish fed control diet and opposite was true for heterophils. There were no significant differences among eosinophil, basophil, or monocytes among the experimental treatments. A lower heterophil lymphocyte ratio was obtained in fish fed 1.0 g PAE, than in the other groups.

Serum Biochemical Profile

Dietary supplementation with 1.0 g PAE enhanced ($p<0.05$) the synthesis of total protein, albumin and AGR in *C. gariepinus,* although the globulin concentration at this inclusion level did not differ from the control group (Table 4). All the fish fed PAE-supplemented diets had lower ($p<0.05$) concentrations of serum AST, ALT and ALP with the lowest in the groups fed 1.0-1.5 g PAE diets. Blood urea nitrogen levels were also lower ($p<0.05$) in fish fed 1.0-1.5 g PAE diets, than in fish fed the control diet. The creatinine, glucose and total bilirubin levels did not differ ($p>0.05$) among the treatments. The total cholesterol concentration seemed to be higher than in the control group, although it did not differ from the values obtained for the 1.0 and 2.0 g PAE dietary inclusion levels.

Antioxidative Status

There were significant differences in the levels of MDA, which were lower ($p<0.05$) in the fish fed 1.0-2.0 g PAE, than in those fed the control or 0.5 g PAE diet (Table 5). Dietary PAE also enhanced ($p<0.05$) GSH levels in all the groups of fish fed PAE-supplemented diets, with the highest GSH level occurring at the 1.0 g inclusion level. Supplementing the diets of *C. gariepinus* with 1.0-1.5 g PAE increased SOD levels with the greatest increase occurring at 1.0 g level. Moreover, the activities of GST and GPx in the experimental fish increased ($p<0.05$) in all PAE treatments, compared to the control, in an increasing order from the 0.5 to 1.5 g concentration.

Immune Response

Table 6 shows the immune response and survival of the experimentally *A. hydrophila*-challenged *C. gariepinus* individuals in the present study. Dietary fortification with PAE boosted ($p<0.05$) lysozyme and phagocytic activities in all the PAE-fortified treatments, with the highest ($p<0.05$) obtained at 1.0 g and 1.5 g, respectively, among the PAE treatments. Respiratory burst activity and post-challenge survival were greater in fish fed 1.0-2.0 g PAE diets groups, than in the fish fed control diet.

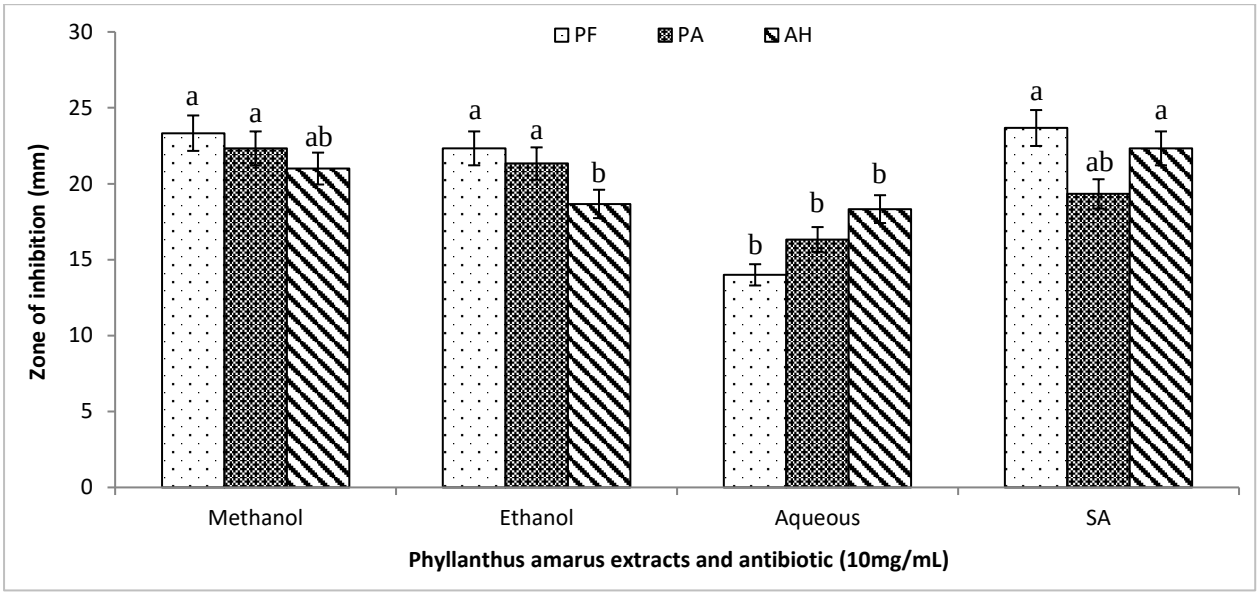

Figure 1. Zone of inhibition of *Phyllanthus amarus* extracts (PAE) against some bacteria
Similar bars with different letters are significantly different at p<0.05.
PF, *Pseudomonas fluorescens*; PA, *P. aeruginosa*; AH, *Aeromonas hydrophila*; SA, synthetic antibiotic

Table 2. Growth performance of *Clarias gariepinus* fed diets supplemented with different concentrations of *Phyllanthus amarus* extract (PAE) for 12 weeks

Parameters	Dietary inclusion levels of PAE (g/kg diet)				
	0.0	0.5	1.0	1.5	2.0
IW (g)	4.31±0.01	4.33±0.03	4.33±0.01	4.30±0.01	4.33±0.03
FW (g)	30.60±0.62^{c}	35.47±0.41^{b}	38.52±0.64^{a}	36.87±0.37^{ab}	30.00±0.48^{c}
WG (g)	26.29±0.63^{c}	31.14±0.40^{b}	34.19±0.65^{a}	32.57±0.37^{ab}	25.67±0.49^{c}
WG (%)	710.60±15.32^{c}	818.54±8.35^{b}	890.37±16.17^{a}	858.05±9.25^{ab}	692.54±14.08^{c}
SGR (%/day)	2.33±0.03^{c}	2.50±0.01^{b}	2.60±0.02^{a}	2.56±0.01^{ab}	2.30±0.02^{c}
FI (g)	39.08±1.07^{a}	40.1±0.62^{a}	42.12±0.58^{a}	40.09±0.66^{a}	34.77±1.56^{b}
FCR	1.49±0.01^{a}	1.29±0.02^{bc}	1.23±0.03^{c}	1.23±0.01^{c}	1.36±0.06^{b}
PER	1.68±0.01^{b}	1.94±0.03^{a}	2.03±0.05^{a}	2.03±0.02^{a}	1.85±0.10^{a}
FS (%)	90.67±1.33^{b}	98.67±1.33^{a}	96.00±2.31^{ab}	96.00±2.31^{ab}	94.67±1.33^{ab}
CF	1.02±0.02^{b}	1.23±0.06^{a}	1.30±0.02^{a}	1.24±0.05^{a}	1.05±0.07^{b}

The values are presented as the means ± standard errors; values with different superscripts within the same row are significantly different at p<0.05.
IW, initial weight; FW, final weight; WG, weight gain; SGR, specific growth rate; FI, feed intake, FCR, feed conversion ratio; PER, protein conversion ratio; FS, fish survival; CF, condition factor

Table 3. Hematological profile of *Clarias gariepinus* fed diets supplemented with different concentrations of *Phyllanthus amarus* extract (PAE) for 12 weeks

Parameters	Dietary inclusion levels of PAE (g/kg diet)				
	0.0	0.5	1.0	1.5	2.0
PCV (%)	43.25±0.25^{c}	48.25±0.65^{b}	51.75±0.75^{a}	48.75±0.75^{b}	43.56±0.65^{c}
Hb (g/dL)	14.25±0.03^{d}	15.65±0.29^{b}	16.85±0.16^{a}	16.00±0.09^{b}	14.76±0.18^{c}
RBC (x 10^{6}/uL)	3.36±0.01^{c}	3.52±0.04^{ab}	3.56±0.03^{a}	3.50±0.04^{ab}	3.46±0.03^{b}
MCV (fL)	128.91±0.90^{d}	135.33±0.84^{c}	147.21±0.73^{a}	139.27±0.49^{b}	128.86±1.21d
MCHC (%)	32.89±0.13^{b}	32.43±0.19^{b}	32.57±0.20^{b}	32.88±0.33^{b}	33.98±0.38^{a}
MCH (pg)	42.40±0.20^{d}	43.89±0.49^{c}	47.99±0.16^{a}	45.72±0.32^{b}	42.73±0.25^{d}
WBC (x 10^{6}/uL)	16.88±0.07^{c}	17.70±0.05^{b}	18.71±0.06^{a}	17.60±0.05^{b}	17.30±0.10^{bc}
Lymphocytes (%)	71.75±0.25^{c}	73.25±0.25^{b}	74.25±0.25^{a}	73.25±0.25^{b}	73.50±0.50^{b}
Heterophil (%)	21.25±0.48^{a}	20.75±0.25^{ab}	18.75±0.00^{d}	19.75±0.25^{c}	20.25±0.25^{bc}
Monocytes (%)	3.50±0.29^{a}	3.25±0.25^{a}	3.25±0.25^{a}	3.50±0.29^{a}	3.25±0.25^{a}
Eosinophil (%)	3.50±0.29^{a}	2.75±.0.25^{a}	3.75±0.25^{a}	3.50±0.29^{a}	3.25±0.25^{a}
Basophil (%)	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.25^{a}
HLR	0.30±0.01^{a}	0.28±0.00^{ab}	0.25±0.00^{c}	0.27±0.00^{b}	0.28±0.00^{b}

The values are presented as the means ± standard errors; values with different superscripts within the same row are significantly different at p<0.05.
PCV, packed cell volume; Hb, hemoglobin; RBC, red blood cell; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; MCH, mean corpuscular hemoglobin; WBC, white blood cell; HLR, heterophil- lymphocyte ratio

Discussion

Infection of farm fishes with *Aeromonas hydrophila* (wang *et al*., 2014; Adeshina *et al*., 2019; Adeniyi *et al*., 2021a; Mulia *et al*., 2023), *Pseudomonas fluorescens* and *P. aeruginosa* (Magdy *et al*., 2014; Thomas *et al*., 2014; Duman *et al*., 2021; Olusola *et al*., 2021) resulting to high economic losses arising from high mortality (Plumb & Hanson, 2011; Noga, 2012; Hardi *et al*., 2019) as well as contamination of fish and fish products with *Pseudomonas* spp (Hussein *et al*., 2023) are of pivotal concern. The present study demonstrated the antibacterial activities of crude PAE against *A. hydrophila*, *P. aeruginosa* and *P. fluorescens*, with the methanol extract exhibiting similar activity to that of the synthetic antibiotics used in aquaculture. Our observations in the present study are similar to those of previous authors (Okigbo & Igwe, 2007; Oluwafemi & Debiri, 2008; Bhat *et al*., 2015; Biswas *et al*., 2020; Adeniyi *et al*., 2021b), who report significant *in vitro* antimicrobial effects of genus *Phyllanthus*.

The growth-promoting potential of PAE was demonstrated in the present study. The enhanced growth performance might have been due to the ability of *P. amarus* to stimulate nutrient digestibility through the presence of several phytochemical compounds, including the lignin glycoside called phyllanthostatin (Pettit *et al*., 1990); flavonoids like quercetin and kaempferol (El-Mekkawy *et al*., 1995); ellagitannins, such as geraniin and furosin (Miguel *et al*., 1996); niruroidine (Quian-Cutrone *et al*., 1996); and nirtetralin A (Wei *et al*., 2012). Although there are few studies on the influence of *P. amarus* on fish performance, growth-promoting effects of this species have been reported in

Table 4. Serum biochemical profile of *Clarias gariepinus* fed diets supplemented with different concentrations of *Phyllanthus amarus* extract (PAE) for 12 weeks

Parameters	Dietary inclusion levels (g/kg diet)				
	0.0	0.5	1.0	1.5	2.0
TP (g/dL)	5.10±0.05^{b}	5.08±0.10^{b}	5.70±0.07^{a}	4.70±0.08^{c}	4.95±0.18^{bc}
Albumin (g/dL)	1.20±0.06^{b}	1.25±0.06^{b}	1.63±0.05^{a}	1.25±0.03^{b}	1.30±0.04^{b}
Globulin (g/dL)	3.90±0.01^{ab}	3.83±0.05^{bc}	4.08±0.03^{a}	3.50±0.04^{ab}	3.46±0.03^{b}
AGR	0.31±0.01^{c}	0.33±0.01^{bc}	0.40±0.01^{a}	0.36±0.01^{b}	0.36±0.01^{b}
AST (IU/L)	292.75±6.28^{a}	269.00±3.78^{c}	236.50±1.50^{d}	233.50±2.36^{d}	278.75±1.03^{b}
ALT (IU/L)	65.75±0.95^{a}	62.75±1.10^{b}	57.25±1.03b^{c}	54.25±0.85^{c}	58.25±1.32^{bc}
ALP (IU/L)	700.00±2.48^{a}	654.50±2.90^{c}	644.00±1.25^{d}	648.00±2.34^{cd}	667.75±2.89^{b}
BUN (mg/dL)	7.10±0.10^{a}	7.00±.0.08^{a}	6.50±0.07^{b}	6.28±0.11^{b}	7.10±0.09^{a}
CREA (mg/dL)	0.58±0.03^{a}	0.55±0.03^{a}	0.58±0.03^{a}	0.57±0.01^{a}	0.55±0.03^{a}
GLU (mg/dL)	317.05±1.80^{a}	313.00±1.47^{a}	315.50±2.22^{a}	314.00±2.08^{a}	315.25±1.25^{a}
CHOL (mg/dL)	188.25±2.50^{a}	180.50±2.10^{b}	183.30±1.85^{ab}	181.50±1.71^{b}	185.25±0.95^{ab}
TB (mg/dL)	0.40±0.03^{a}	0.38±0.03^{a}	0.45±0.03^{a}	0.38±0.03^{a}	0.38±0.03^{a}

The values are presented as the means ± standard errors; values with different superscripts within the same row are significantly different at p<0.05. TB, total protein; AGR, albumin globulin ratio; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; CREA, creatinine; GLU, glucose; CHOL, cholesterol; TB, total bilirubin

Table 5. Antioxidative status of *Clarias gariepinus* fed diets supplemented with different concentrations of *Phyllanthus amarus* extract (PAE) for 12 weeks

Parameters	Dietary inclusion levels of PAE (g/kg diet)				
	0.0	0.5	1.0	1.5	2.0
MDA	0.54±0.00^{a}	0.53±0.00^{a}	0.52±0.00^{b}	0.52±0.00^{b}	0.52±0.00^{b}
GSH	149.61±2.10^{d}	152.29±2.49^{c}	160.52±2.59^{a}	157.33±3.07^{b}	155.65±2.64^{b}
SOD	322.67±3.95^{c}	322.49±4.57^{c}	334.60±4.85^{a}	329.02±2.46^{b}	324.45±4.62^{bc}
GST	522.02±4.32^{e}	573.29±3.44^{d}	607.08±3.59^{c}	656.22±5.28^{a}	634.90±3.56^{b}
GPx	56.48±0.63^{d}	57.66±0.82^{c}	65.51±0.95^{b}	72.61±2.02^{a}	67.65±0.55^{b}

The values are presented as the means ± standard errors; values with different superscripts within the same row are significantly different at p<0.05. MDA, malondialdehyde; GSH, reduced glutathione; SOD, superoxide dismutase; GST, glutathione-s-transferase; GPx, glutathione peroxidase

Table 6. Immune response and survival of *Clarias gariepinus* experimentally challenged with *Aeromonas hydrophila* and fed *Phyllanthus amarus* extract

Parameters	Dietary inclusion levels (g/kg diet)				
	0.0	0.5	1.0	1.5	2.0
Lysozyme (U/mg protein)	16.64±0.21^{e}	17.98±0.04^{c}	19.64±0.1^{a}	18.52±0.27^{b}	17.29±0.01^{d}
Phagocytic activity (%)	13.20±0.01^{e}	13.34±0.01^{d}	13.42±0.01^{c}	13.72±0.01^{a}	13.57±0.22^{b}
Respiratory burst (OD)	0.77±0.00^{c}	0.78±0.00^{bc}	0.79±0.00^{b}	0.81±0.01^{a}	0.79±0.00^{b}
Survival (%)	46.67±3.33^{c}	66.67±3.33^{b}	83.33±3.33^{a}	76.67±3.33^{ab}	76.67±3.33^{ab}

The values are presented as the means ± standard errors; values with different superscripts within the same row are significantly different at p<0.05

aquatic crustaceans (Kalaiselvi *et al*.., 2019; Ngo *et al*.., 2020) while Sunitha *et al*. (2017) and Olusola *et al*. (2021) also observed growth-promoting effects of the leaf of another species, *P. niruri*, on common carp and African catfish. Adeniyi *et al* (2021a, 2023) also reported that the growth performance and/or nutrient digestibility of *C. gariepinus* increased with dietary tamarind and *Euphorbia heterophylla* foliage extracts. The greater survival and growth exhibited by the fish fed PAE-supplemented diets in the present study were similar to the observations of Sunitha *et al*. (2017). The enhanced survival might also be associated with the improved condition factor, which could be attributed to the enhanced uptake of nutrients and the antimicrobial activities of the supplement.

Red and white blood cell counts were enhanced in *Clarias gariepinus* fed fortified *P. amarus* in the present study. Annalakshmi *et al*. (2013) and Olusola *et al*. (2021) also observed increased RBC and WBC counts in *Labeo rohita* fed P. *amarus* and *P. niruri* infected with *Aeromonas hydrophila* and *P. aeruginosa*, respectively. Hematological parameters such as Hb, PCV, MCV, MCH and MCHC are particularly known to indicate erythrocytic status and oxygen carrying capability in fish. An increase in the levels of these parameters might indicate the stimulation of erythropoiesis, hence increasing the capacity for oxygen transport and strengthening of defense mechanisms in the fish against physiological stress (Gabriel *et al*., 2015). The increase in the WBC and lymphocyte counts in fish fed PAE-supplemented diets might be attributed to the immunostimulatory property of the plant. Medicinal herbs and their active metabolites have potent therapeutic and prophylactic effects on a wide array of immunological disorders by modulating the immune response (Mohamed *et al*., 2017); the aforementioned phytoconstituents of PAE could have been responsible for the physiological enhancement of *C. gariepinus* in the present study.

Blood biochemical parameters are widely used tools for evaluating the safety of feed additives in animal and fish nutrition. The increase in protein synthesis and decrease in AST, ALT, ALP and BUN in fish fed 1.0 and/or 1.5 g PAE in the present study could indicate the protective effect of these diets on hepatocytes and glomeruli (Brien & Walterson, 2009; Reda *et al*., 2013). The results obtained in the present study are in line with those of earlier studies on hepatoprotective effects of genus *Phyllanthus* leaf in mammals (Igwe et al., 2007; Faremi et al., 2008; Bakhtiary et al., 2012) in relation to reduced ALT and AST. The increase in antioxidant enzymes (SOD, GPx and GST) and reduction in MDA in the present study are consistent with the earlier *in vitro* reports of Kumaran and Karunakaran (2007) and Devi *et al*. (2016) as well as the *in vivo* antioxidative activity of this plant in mice (Karuna et al., 2009; Akporowhe & Onyesom, 2016) and crustacean (Ngo *et al*., 2020). The antioxidant activity of PAE could be attributed to the presence of flavonoids (rutin, astragalin, kaemferol, quercetin) and ellagitannins (amariin, repandusinic, phyllanthusiin D (Ghosh *et al*., 2022; Motou *et al*., 2023) in the plant.

Lysozyme, phagocytic and respiratory burst activities have been used widely to evaluate the immunostimulatory effects of dietary herbal additives in both farmed fishes and shrimps (Punitha *et al*., 2008; Sunitha *et al*., 2017; Ngo *et al*., 2020, Adesina *et al*., 2021, Adeniyi *et al*., 2023). Lysozyme is an important enzyme that contributes immensely to the innate immune system in animals. The enzyme plays an antimicrobial role by preventing the invasion of bacteria through disruption of the bacterial wall and promoting destruction of the wall through phagocytic cells, while respiratory bursts cause the release of reactive oxygen species that kill pathogenic bacteria (Saurabh & Sahoo, 2008; Abarike *et al*., 2019). The enhanced activities of these immune parameters in the fish fed PAE-fortified diets further support the basis for utilization of the plant in traditional medicine. The enhanced immune responses and post challenge survival obtained in the present study are in line with earlier reports on responses of fishes and shrimps fed herbal-supplemented diets due to their antimicrobial agents and immune-stimulating effects (Hardi *et al*., 2019; Ngo *et al*., 2020; Adesina *et al*., 2021; Adeniyi *et al*., 2023). The *in vivo* antibacterial activity observed in the present study could be attributed to the aforementioned lignans, flavonoids, alkaloids, and terpenoids, among others, in the *P. amarus* extract, which might justify the wide utilization of this plant in traditional medicine to treat different ailments.

Conclusions

In conclusion, the observations in this study have shown that feeding *Clarias gariepinus* with diets containing methanol extract of *Phyllanthus amarus* leaf and flower significantly promoted growth performance; increased hemoglobin and lymphocytes levels; decreased liver enzymes (ALP, ALP, AST); and enhanced SOD, GPx, GST, lysozyme, phagocytic and respiratory burst activities at the 1.0-1.5 g concentration. Therefore, the inclusion of 1.0 g of the *P. amarus* methanol extract is recommended in the diet of *C. gariepinus* for enhanced growth and physiological status. Further study on the antimicrobial and growth-promoting effects of isolated active components of *P. amarus* in fish is also recommended.

Ethical Statement

This study was conducted according to applicable national and international guidelines for the Care and use of laboratory animals of the National Institutes of Health. The protocol was approved and carried out in accordance with the ethical guidelines of the Ethical Committee under the general directorate of Center for

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Author Contribution

OVA: Conceptualization, Methodology, Supervision, Data curation, Writing - review and editing; AOA: Investigation, Project administration, Writing – Original draft preparation**;** GHT: Resources, Investigation, Writing -original draft. All authors read and approved the published version of the manuscript.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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