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Stimulatory effect of *Phyllanthus amarus* extract on the growth performance, hemato-biochemical activity, antioxidative status and immune response of African catfish, *Clarias gariepinus*

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Abstract

This study investigated the antibacterial activity of *Phyllanthus amarus* extracts and influence of best extract on the growth; physiological and immune status of *Clarias gariepinus* fingerlings. The fish were fed diets containing 0.0 (control), 0.5, 1.0, 1.5 or 2.0 g *P. amarus* methanol extract (PAE) / kg basal diet to apparent satiation for 84 days. Thereafter, blood samples were collected from the fish and used for hematological and biochemical analyses. After the 84 days of feeding, the fish were challenged with *Aeromonas hydrophila* by intraperitoneal injection; and observed for survival, 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 *P. amarus* methanol extract against *A. hydrophila*, and its application at 0.5–1.5 g PAE significantly promoted weight gain and specific growth rate, with the highest at 1.0 g. Moreover, compared with that of the control group, the feed conversion ratios of the fishes fed PAE diets significantly decreased. Hematocrit, hemoglobin and lymphocytes were enhanced at 0.5–1.5 g PAE. All the fishes fed PAE-based diets had lower concentrations of serum liver enzymes; the lowest concentration was detected in fish fed 1.0–1.5 g PAE; while the values of creatinine, glucose and total bilirubin did not differ among the treatments. Glutathione peroxidase and glutathione-S-transferase activities increased in all PAE-fortified treatments. Lysozyme, phagocytic and respiratory burst activities also increased in the challenged fish fed PAE diets. Post-challenge survival was highest in the 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 (FAO, 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 in diseases, especially bacterial diseases, one of which is motile aeromonad septicaemia caused by *Aeromonas hydrophila*, which is widely spread in freshwater fish culture (Hardi et al., 2019; Ngo et al., 2020; Adeniyi 2021a). 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 the development of drug resistance, residue in fish, negative impacts on consumer health and aquatic animals; and environmental pollution (Gullberg et al., 2011; Lim et al., 2013; Santos and Ramus 2018) and hence it's not sustainable. Therefore, there is need for sustainable alternatives.

Herbal products have been increasingly utilized in the recent decade in fish culture due to their beneficial effects, including growth-promotion, immune-stimulation, antioxidative status enhancement, low cost

and wide availability. Additionally, these products are relatively safe; and have not been reported to have drug resistance or contribute to 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 Phyllanthaceae 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 values in treating many ailments associated with the gastrointestinal system, genitourinary system, respiratory system, liver, kidney and spleen, 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).

Phyllanthus amarus has many valuable compounds such as lignans (phyllanthin and hypophyllanthin), flavonoids (kaempferol, quercetin, quercitrin, and rutin), hydrolysable tannins (ellagitannins such as amariin, amariinic acid, furosin, geraniin, and geraniinic acid), triterpenes (phyllanthenol, phyllanthenone, and phyllantheol), and alkaloids (securinine, epibubbialine, isobubbialine, and allosecurine). The extracts and the compounds isolated from *P. amarus* exhibit a wide spectrum of pharmacological activities, including antiviral, antibacterial, antiplasmodial, anti-inflammatory, antimalarial, antimicrobial, anticancer, antidiabetic, hypolipidemic, antioxidant, hepatoprotective nephroprotective and diuretic effects (Verma et al., 2014; Ghosh et al., 2022). Despite the great medicinal potential of this herb, there is scant scientific information on its application in fish culture. Therefore, this study aimed t0 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 form 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 described by CLSI (2012). The isolates were grown on nutrient agar for 24 hours before use and standardized to 0.5 Mcfarland standards. Approximately one hundred microliters of the standardized cell suspension were spread on Mueller-Hinton agar plates, after which approximately 100 µL of each concentrated crude extract at 10 mg/mL was introduced into wells bored with a sterile 6 mm diameter core borer and incubated at 37°C in triplicate for 24 hours. Controls were established in parallel using the solvent that was used to reconstitute the extract as a negative control and with antibiotic agent (containing oxytetracycline and erythromycin) as a positive control. The plates were observed for zones of inhibition (mm) after incubation at 37°C.

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). *Phyllanthus amarus* methanol extract (PAE), which has the highest antibacterial activity, was added 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 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 in triplicate using an indoor static water-renewal system during which culture water was renewed 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°C and 6.9–7.1, respectively.

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

[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, 300mg; 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)

Ingredients	Composition (g/kg diet)				
[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, 300mg; 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).

Growth Performance and Nutrient Utilization

At the end of the twelve-week feeding trial the following parameters were calculated for on fish growth performance and feed utilization from the data obtained for each treatment 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 = Feed intake/WG

Protein efficiency ratio = WG/Protein intake

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

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

Collection of Blood and Analyses

Blood was collected from sampled anesthetized fish in each treatment after the feeding trial 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 hematocrit (also known as packed cell volume [PCV]), hemoglobin concentration, red blood cell (RBC) counts, white blood cell (WBC), and lymphocyte, neutrophil, eosinophil, monocyte and platelet counts were analyzed with an automated hematological analyzer. Thereafter, the following erythrocytic indices were calculated from the data obtained:

1. Mean corpuscular volume (MCV, fl) = 10 (PCV / RBC)
2. Mean corpuscular hemoglobin (MCH, pg) = 10 (Hemoglobin / RBC)
3. Mean corpuscular hemoglobin concentration (MCHC, %) = 100 (Hemoglobin / PCV)

Blood samples in non-EDTA bottles were allowed to coagulate and were centrifuged at 3000rpm and 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 analysed using a Randox kit, following the standard procedure of the manufacturer. Globulin (Total protein - albumin), 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 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* (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*. It was discovered that 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 the synthetic antibiotic against *A. hydrophila* did not differ from that of methanol extract of PAE, while the later showed greater inhibition against *A. hydrophila*, compared with the value obtained for the other two extracts. Generally, the aqueous extract exhibited the least antibacterial activity against the test organisms.

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 increased significantly ($P < 0.05$) in the fish fed 0.5–1.5 g PAE, with the greatest increase occurring in the 1.0g treatment group, compared to the groups fed control and 2.0 g PAE/kg diets. The fish fed 2.0 g PAE had lower ($P < 0.05$) feed intake, than 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 fish fed the control diet had the highest FCR. The 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 that 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 at 0.5–1.5 g PAE inclusion levels, than at 2.0 g PAE and control groups.

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 represented 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

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 PCV and hemoglobin. However, while significantly greater RBC counts were obtained in all the groups of fish fed PAE-supplemented diets, than in the control group. The MCH and MCV 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 was obtained in the fish fed 2.0 g PAE/kg diet. Dietary PAE stimulated higher ($P < 0.05$) WBC production in the treatment groups fed 0.5–1.5 g/kg levels, compared with that in the control group. The lymphocyte count 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 eusinophil, basophil, or monocytes among the experimental treatments. A lower heterophill lymphocyte ratio was obtained in fish fed 1.0 g PAE, than in the other groups.

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 ± 0.75b	43.56 ± 0.65c
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}	$2.75 \pm .0.25^{a}$	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 represented 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

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 ($P < 0.05$) concentration 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 those 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 in the control group, although it did not differ from the values obtained at the 1.0 and 2.0 g PAE dietary inclusion levels.

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}	$57.25 \pm 1.03b^{c}$	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}	$7.00 \pm .0.08^{a}$	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 represented 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

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 diets (Table 5). Dietary PAE also significantly enhanced 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 also 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 treatment groups, compared to the control group, in an increasing order from the 0.5 to 1.5 g concentration.

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 represented 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, gluthathione-s-transferase; GPx, glutathione peroxidase

Immune response

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

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 represented as the means ± standard errors; values with different superscripts within the same row are significantly different at $P < 0.05$.

DISCUSSION

Infections resulting from *A. hydrophila and Pseudomonas fluorescens* in fish have been reported to lead to high mortality, which is a pivotal concern in fish culture, due to high economic losses (Plumb and Hanson 2011; Noga 2012; Hardi et al., 2019). 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 and Igwe, 2007; Oluwafemi and Debiri, 2008; Bhat et al., 2015; Biswas et al., 2020; Adeniyi et al., 2021b) who observed significant antimicrobial effects of *Phyllanthus amarus* against several pathogenic and food poisoning organisms.

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 glycosides 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 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 was 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, while the white blood cell differential counts did not differ significantly. Annalakshmi et al. (2013) and Olusola et al. (2021) also observed increased RBC and WBC counts in *Labeo rohita* fed with *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. The increased in the levels of these parameters might indicate the stimulation of erythropoiesis, hence increasing the capacity for oxygen transport and strengthening the defense mechanisms 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).

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 diets in the present study could indicate the protective effect of these diets on hepatocytes and glomeruli (Brien and Walterson, 2009; Reda et al., 2013). Our the results obtained in this study is in line with several studies that have demonstrated hepatoprotective effects of genus *Phyllanthus* leaf in mammals (Bhattacharjee and Sil, 2007; Naaz et al., 2007; Pramyothin et al., 2007; Igwe et al., 2007; Faremi et al., 2008; Bakhtiary et al., 2012) and fish (Olusola et al.., 2021) in relation to reduced ALT and AST. The increase in antioxidant enzymes (SOD, GPx and GST) and reduction of MDA in the present study coincide with the earlier reports on the *in vitro* (Kumaran and Karunakaran, 2007; Devi et al., 2016) and *in vivo* antioxidative activity of this plant in terrestrial animal (Harikumar and Kuttan, 2007; Karuna et al., 2009; Akporowhe and Onyesom, 2016) and shrimp (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 (Londhe et al., 2008; 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 burst causes release of reactive oxygen species that kill pathogenic bacteria (Saurabh and Sahoo, 2008; Abarike et al., 2019; Hampton et al., 2020). 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 are in line with the response of grouper (Punitha et al., 2008) and shrimp (Ibrahim et al., 2015; Ngo et al., 2020) to diets containing *Phyllanthus* extract and might have contributed to the enhanced fish survival. The enhanced survival is consistent with earlier reports on fishes fed herbal-supplemented diets due to their antimicrobial agents and immune-stimulating effects against fish pathogens (Hardi et al., 2012, 2019; 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.

In conclusion, the current study investigated the effects of dietary *Phyllanthus amarus* on the growth, blood profile, antioxidative status and immune response of *Clarias gariepinus*. It could be concluded that dietary *Phyllanthus amarus* leaf and flower mixture extract significantly promoted growth performance; hemoglobin and lymphocytes levels; and SOD, GPx, GST, lysozyme, phagocytic and respiratory burst activities at the 1.0-1.5 g inclusion level. Therefore, the inclusion of 1.0 g *Phyllanthus amarus* is recommended in the diet of *Clarias gariepinus* for enhanced growth and physiological status.

Declarations

Authors contribution

Olarinke Victoria Adeniyi contributed to the study's conceptual design, statistical analysis and general supervision. The test ingredient preparation, experimentation, data collection and first draft of the manuscript were performed by Adijat Omowunmi Alade and Gafar Hammed Tijani. Olarinke Victoria Adeniyi revised the manuscript, while all the authors read and approved the final manuscript.

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Data availability

All the data used in this study are included in this manuscript.

Ethical approval

This study was approved and carried out in accordance with the ethical guidelines of the Ethical Committee of Kwara State University, Malete, Nigeria.

Informed consent

Not applicable because our study did not involve humans.

Conflict of interest

The authors declare that they do not have any relevant financial or nonfinancial interests to disclose.

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Figures

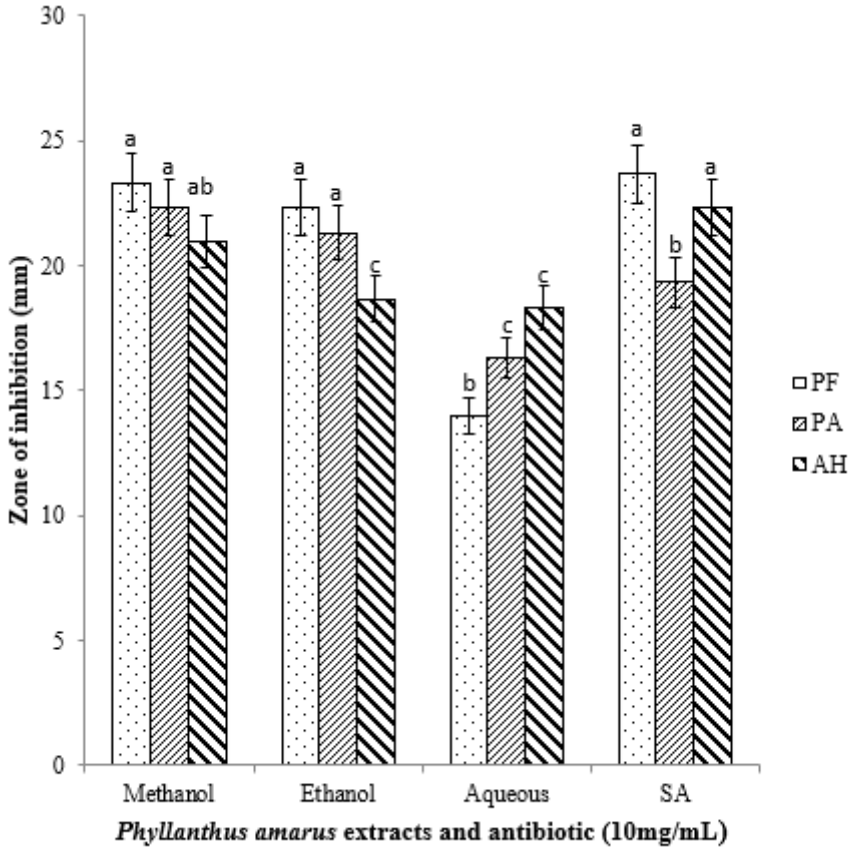


Figure 1

Zone of inhibition of *Phyllanthus amarus*extracts (PAE) against some bacteria

PF, *Pseudomonas fluorescens*; PA, *P. aeruginosa*; AH, *Aeromonas hydrophila*; SA = synthetic antibiotic