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Haematological and Blood Biochemical Changes in African Catfish, *Clarias gariepinus* Fed Walnut (*Tetracarpidium conophorum* Mull Arg) Leaf and Onion (*Allium cepa* Linn) Bulb Supplemented Diets

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Authors' contributions

This work was carried out by three authors. Authors OSB, FEO and BOE designed the study and wrote the protocol, authors FEO and BOE supervised the study. Author OSB performed the experiment and the statistical analysis as well as managed the literature searches. Also, author OSB wrote the first draft of the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Juvenile *Clarias gariepinus* were fed diets containing Onion Bulb (OB) and Walnut Leaf (WL) residues at different graded levels: control (0%), OB2 (0.5%), OB3 (1.0%), OB4 (1.5%), OB5 (2.0%), WL6 (0.5%), WL7 (1.0%), WL8 (1.5%) and WL9 (2.0%). Packed Cell Volume (PCV), Haemoglobin (Hb) content, Red Blood Cells (RBC) and White Blood Cells (WBC) counts were measured in test fish after 84 days of feeding. Biochemical indices such as total protein, Albumin as well as blood serum, aspartate amino-transferase and alanine amino-transferase were investigated before and after the experiment. Data were analyzed using descriptive statistics and ANOVA at $p = 0.05$. The results obtained showed that packed cell volume and haemoglobin content were significantly different ($p<0.05$) among the treatments while red blood cell, white blood cell and mean cell volume and mean cell haemoglobin were not significantly different ($p>0.05$) among the dietary groups.

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There were increases in total protein and albumin but values of aspartate amino-transferase and alanine amino-transferase decreased though not significantly ($p>0.05$) among the treatments. However, fish fed the walnut leaves and onion bulbs residue- based diets recorded higher values in PCV, Hb, WBC and total protein compared to the values obtained before experiment and the control. The results of this study suggested that the dietary supplementation of walnut leaves and onion bulbs residues could be a potential, less expensive and positively affected haematological factor and boost immune response of cultured *Clarias gariepinus* juveniles.

Keywords: Walnut leaf; onion bulb; haematology; Clarias gariepinus.

1. INTRODUCTION

Aquaculture production has increased tremendously in Nigeria and *Clarias gariepinus* is an economically important cultured fish species. Nutrition is a major factor that determines the physiological well being of an animal. Use of natural feed additives is becoming more important for fish feeding rather than chemical feed additives due to the cumulative effects of the chemical components on animal and human health. Therefore, using immunostimulants seems to be an attractive alternative to control fish diseases and enhance growth [1,2]. These substances play a promising role in aquaculture by enhancing the resistance of cultured fish against diseases [1]

Recent studies have shown successful use of medicinal plants and natural herbs in fish nutrition including marjoram, licorice roots, black seeds, peppermint, caraway seed, fennel seed and fenugreek seeds [3-11]. Also, a number of studies have shown that differences in blood cell formation and function can also be indicative of dietary manipulations.

Use of natural herbs, medicinal and aromatic plants in fish feeding is still limited both on the experimental and commercial scales and there is scarce information on walnut leaf and onion bulb as plants immunostimulants for treating fish diseases or boost immune response in fish as well as feed supplements. This study is aimed at investigating the effects of different levels of inclusion of walnut leaf and onion bulb residues in the diets and haematology of *Clarias gariepinus* juveniles.

2. MATERIALS AND METHODS

2.1 Plant Collection, Identification, Preparation and Extraction

Onion (*A. cepa*) bulbs were purchased from a market in Ibadan while walnut (*T. conophorum*) leaves were harvested from a farm at Oka -Akoko, Ondo State in Nigeria. They were authenticated at the herbarium of the Forestry Research Institute of Nigeria (FRIN), Ibadan, where a voucher specimen was deposited under FHI 107515.

2.2 Onion Extraction

The onion bulbs were washed with distilled water and air-dried at ambient temperature (25^{o}C) for one hour. The dry outer coverings of the onions were manually peeled off, washed and extracted as described by [12]. 200g of the fresh onion bulbs were blended into fine pulp and soaked in 100ml of 95% ethanol for 24hrs. It was left in a clean, sterile glass container,

shaken vigorously to allow proper extraction and filtered using a sterile muslin cloth. The residue obtained after the removal of the liquid was air-dried and stored in a refrigerator at 4ºC until required.

2.3 Walnut Leaf Extraction

The extraction of walnut leaves was as described by [13]. The air – dried walnut leaves were ground with a hammer mill to fine powder and 200 g of the powder mixed thoroughly with methanol and filtered using a sterile muslin cloth. The residue obtained after extraction was air – dried and stored in a refrigerator at (25ºC) until required.

2.4 Experimental System

The experiment was carried out in twenty seven plastic experimental tanks (50x34x27cm) for 12 weeks in the Fisheries Laboratory, University of Ibadan, Nigeria. The water level in each tank was maintained at volume of 35 litres throughout the study period. Water in each tank was replaced every three (3) days throughout the period to maintain relatively uniform physiochemical parameters and prevent fouling resulting from feed remnants. The source of water was from University of Ibadan water station and each tank was well aerated using aerators [14]. The mean water temperature, pH and dissolved oxygen were recorded as 26.79±1. 25ºC, 7.83±0.26 and 6.97±0.09 mg/l respectively.

2.5 Experimental Procedure & Feeding Trials

There were nine experimental diets during this study. Each treatment had three replicates with 20 fish per replicate and mean initial body weight of 7.39±0.02g. The fish were acclimatized for fourteen days before the experiment and fed at 3% body weight daily. Daily feed was divided into two rations of 50% of total feed weight and was given in the morning (between 8.00am and 9.00am) and in the evening (at 5.00p.m.).

2.6 Preparation of Experimental Diets

The mean proximate composition of the experimental diet was 40.00±0.01% crude protein, 15.90±0.04% ether extract, 15.70±0.02% ash, 7.40±0.08% moisture, and 20.90±0.01% Nitrogen Free Extract. Nine experimental diets were prepared by incorporating walnut leaf and onion bulb residues at the following inclusion levels; 0 (control), 0.5%, 1.0%, 1.5% and 2.0% respectively. Feed ingredients such as fishmeal, soybean, maize, starch, vegetable oil, Di-calcium phosphate (DCP), salt and vitamin- mineral premix were added. The dry ingredients were mixed thoroughly in a mixer, water was added and the resulting dough pelletized. The pellets were sun –dried, and stored in airtight containers at room temperature until required.

2.7 Haematological and Biochemical Analyses

Haematological analysis of the fish was carried out before and after the experiment at the Haematological Laboratory of Veterinary Pathology Department, University of Ibadan within 30 minutes of sampling. 3 – 4cm from the genital opening of each fish was punctured and wiped with dry tissue paper to avoid contamination with mucus. The needle was inserted at right angle to the vertebral column of the fish. The blood was taken under gentle aspiration until about 3ml had been obtained. The needle was gently withdrawn and the blood

transferred into heparinized plastic containers and complete haematology was done as described by the methods of [15] using modified hyme's dilution fluid. The collected blood was introduced into Neubauer Counting Chamber (Neubauer improved bright line Marienfield, Germany 0.100mm, 0.0025mm^2) and the cells were counted under the microscope at 100 x objective.

.Blood samples for biochemical analysis were centrifuged for 5 minutes at 3000rpm with Hawsley minor bench centrifuge (P spectra, Centromix no 231254 CD7000549, Spain). The derived was stored at – 20ºC and analyzed at the Haematology Laboratory of Veterinary Pathology Department, University of Ibadan for total protein, albumin, globulin, albumin – globulin ratio and blood serum enzymes such as Aspartate amino transferase (AST) and Alanine amino transferase (ALT) as described by the methods of [15].

2.8 Statistical Analysis

Haematology, biochemical indices and blood serum resulting from the experiment were subjected to one-way analysis of variance (ANOVA) using SPSS (Statistical Package for Social Sciences 2006 version 15.0). Duncan multiple range test was used to compare differences among individual means.

3. RESULTS

3.1 Haematology of Clarias Gariepinus before and After Feeding Trials

There was increased in values of some haematological parameters after the feeding experiment when compared with the values obtained before the experiments. Packed cell volume and haemoglobin content were significantly different ($p<0.05$) among the treatments while red blood cell, white blood cell and mean cell volume and mean cell haemoglobin were not significantly different ($p>0.05$) among the dietary groups (Table 1).

3.2 Plasma Biochemistry of *Clarias Gariepinus* before and After Feeding Trials

The result of plasma biochemistry (total protein, albumin, globulin and albumin and globulin ratio) shows variation in the values. Albumin values was not significant ($p>0.05$) difference among the treatments but, total protein, globulin and albumin and globulin ratio were significantly different ($p<0.05$) among the treatments (Table 2).

3.3 Blood Serum Enzymes of *Clarias Gariepinus* before and After Feeding Trials

The result of the experiment shows that highest value of AST was recorded in OB2 and lowest in WL 8 while highest ALT was recorded in OB3 and lowest in WL 8, these values were lower than the one obtained before the experiment (Table 3).

Table 1. Mean Values of Some Haematological Parameters of African Catfish *Clarias gariepinus* Juveniles before and after the feeding experiment

Parameters	Before experiment	After feeding experiment								
		CONTROL	OB2	OB3	OB4	OB5	WL6	WL7	WL8	WL9
PCV (%)	12.50±2.50^{a}	26.00±4.00^{bc}	24.00±1.41^{bc}	28.00±2.83^{bc}	22.00±0.89^{bc}	20.00±1.70^{b}	29.50±2.12^{bc}	25.00±4.24^{bc}	28.00±1.10^{bc}	34.50±0.71^{c}
Hb (gm %)	4.10±0.45^{a}	8.30±1.98^{bc}	7.25±0.64^{bc}	8.80±1.41^{bc}	6.70±2.97^{bc}	5.65±0.07^{b}	9.35±0.92^{bc}	7.70±1.41^{bc}	9.10±3.25^{bc}	10.65±0.07^{c}
RBC x10$^{12/L}$	1.07±0.05^{a}	2.47±1.12^{a}	2.02±0. 50^{a}	2.90±0.59^{a}	2.38± 1.75^{a}	1.75±0.05^{a}	2.90±0.52^{a}	2.20±0.53^{a}	2.55±1.26^{a}	3.39±0.03^{a}
WBC x10$^{9/L}$	15,250±5.50^{a}	11,650±2.10^{a}	16,025±3.64^{a}	17,325±8.24^{a}	14,875±3.14^{a}	13,400±4.10^{a}	17,050±2.68^{a}	16,050±9.68^{a}	13,625±1.87^{a}	13,500±3.82^{a}
Platelet	133,000±1.10^{ab}	99,500±4.94^{a}	129,000±3.50^{ab}	203,000±4.10^{b}	135,500±1.06^{ab}	112,000±4.24^{a}	123,000±1.55^{a}	128,500±2.89^{ab}	160,000±4.81^{ab}	107,000±9.89^{a}
ESR (mm/hr)	0.20±0.14^{ab}	0.3±0.14^{b}	0.15±0.07^{ab}	0.10±0.00 ^{a}	0.25±0.07^{ab}	0.15±0.07^{ab}	0.20±0.00^{ab}	0.20±0.14^{ab}	0.10±0.00^{a}	0.10±0.00^{a}
MCV (Fl)	118.17±0.38^{a}	111.49±0.62^{a}	122.02±0.33^{a}	97.60±0.70^{a}	95..24±0.75^{a}	114.66±0.23^{a}	102.88±0.60^{a}	113.84±0.13^{a}	112.57±0.40^{a}	101.76±0.68^{a}
MCH (Pg)	3.91±0.86^{a}	3.55±0.80^{a}	3.68±0.60^{a}	3.05±0.14^{a}	2.76±0.0.50^{a}	3.24±0.05^{a}	3.26±0.26^{a}	3.50±0.20^{a}	3.71±0.56^{a}	3.15±0.07^{a}
MCHC (%)	34.00±0.04^{b}	32.00±0.00^{b}	31.00±0.01^{ab}	32.00±0.02^{b}	31.00±0.01^{ab}	29.00±0.01^{a}	32.00±0.04^{b}	31.00±0.01^{ab}	33.00±0.01^{b}	31.00±0.00^{ab}
Lym (%)	69.00±1.00^{a}	66.00±0.07^{a}	68.00±0.00^{a}	66.00±0.07^{a}	67.50±0.71^{a}	67.00±0.41^{a}	67.00±0.65^{a}	68.00±5.66^{a}	70.00±0.07^{a}	68.50±0.49^{a}
Hetero (%)	25.00±2.00^{a}	29.50±2.12^{a}	25.00±1.40^{a}	30.00±8.49^{a}	30.00±1.41^{a}	32.00±2.83^{a}	31.00±2.83^{a}	36.00±7.07^{a}	33.50±7.78^{a}	31.00±8.49^{a}
Mono	3.00±0.00^{a}	1.50±0.71^{a}	2.50±0.07^{a}	2.00±1.41^{a}	1.50±0.71^{a}	1.50±0.71^{a}	2.50±0.07^{a}	1.50±0.11^{a}	2.00±1.41^{a}	1.50±0.71^{a}
Eos	3.00±1.00^{a}	2.50±2.12^{a}	4.50±0.71^{a}	2.00±0.00^{a}	1.00±0.00^{a}	1.50±2.12^{a}	1.50±2.12^{a}	1.50±0.71^{a}	2.50±0.71^{a}	2.50±2.12^{a}

Key: Mean followed by the same letter is not significantly different (p > 0.05).

NOTE: *PCV = packed cell volume, Hb =Haemoglobin, RBC = Red Blood Cell, WBC = White Blood Cell, ESR = Erythrocytes sedimentation rate, MCV =Mean Cell Volume, MCH = Mean Cell, Haemoglobin, MCHC = Mean Cell Haemoglobin Concentration, Lym =Lymphocytes, Hetero =Heterophil, Eos = Eosunophil, Mono = Monocytes.*

Table 2. Mean Plasma Biochemistry Parameters of African Catfish *Clarias gariepinus* Juveniles before and after the feeding experiment

Parameters	Before experiment	After feeding experiment								
		CONTROL	OB2	OB3	OB4	OB5	WL6	WL7	WL8	WL9
Total protein	3.10±0.14^{a}	2.85±0.49^{a}	4.60±0.05^{ab}	3.95±1.06^{ab}	2.60±0.85^{a}	4.00±0.57^{ab}	2.90±0.14^{a}	5.70±0.99^{b}	4.15±0.92^{ab}	3.60±1.99^{ab}
Albumin	0.95±0.35^{a}	2.35±0.21^{a}	2.25±0.07^{a}	2.30±0.00^{a}	1.90± 1.27^{a}	2.60±0.57^{a}	2.50±0.28^{a}	2.75±0.07^{a}	2.45±0.64^{a}	1.95±1.06^{a}
Globulin	2.15±0.49^{bc}	0.50±0.28^{a}	2.35±0.07^{bc}	1.65±1.06^{abc}	0.70±0.42^{a}	1.40±0.00^{ab}	0.40±0.14^{a}	2.95±1.06^{c}	1.50±0.00^{abc}	1.65±0.92^{abc}
A.G Ratio	0.50±0.28^{ab}	0.20±0.14^{a}	1.00±0.00^{b}	0.65±0.49^{ab}	0.55±0.64^{ab}	0.55±0.07^{ab}	0.15±0.07^{a}	1.00±0.42^{b}	0.65±0.21^{ab}	0.80±0.00^{ab}

Key: Mean followed by the same letter is not significantly different (p > 0.05).

Table 3. Blood Serum of African Catfish *Clarias gariepinus* Juveniles before and after the feeding experiment

Parameters	Before experiment	After feeding experiment								
		CONTROL	OB2	OB3	OB4	OB5	WL6	WL7	WL8	WL9
AST(IU/l)	151.00±1.31^{a}	132.00±4.24^{a}	136.00±2.83^{a}	141.50±9.19^{a}	135.00±9.90^{a}	131.00±9.89^{a}	131.00±4.14^{a}	139.00±9.90^{a}	129.50±6.26^{a}	137.50±10.61^{a}
ALT(IU/l)	68.00±8.49^{b}	24.00±4.14^{a}	22.00±7.07^{a}	26.00±5.66^{a}	19.00±2.83^{a}	20.00±2.83^{a}	20.00±7.07^{a}	21.50±2.12^{a}	18.00±2.83^{a}	22.50±3.84^{a}

Key: Mean followed by the same letter is not significantly different (p > 0.05).
Note: AST =Aspertate amino transferase; ALT =Alanine amino transferase.

4. DISCUSSION

As immunological studies continue to include dietary manipulation, haematology becomes a necessary research tool for further interpretation of dietary effects. Examination of the fish before and after the experiment revealed that the fish were in good condition free from disease and infections. However, there was decrease in some haematological and biochemical parameters after the experiment when compared with the values recorded before the experiment. This could be attributed to stress encountered during sampling, capture and handling procedure, which must have increased the cathecholamine secretion. The mean PCV(%) values of *Clarias gariepinus* juveniles fed different graded level of onion bulb (OB) and walnut leaf (WL) of 0.5%, 1.0%, 1.5% and 2.0% respectively showed general increase in values when compared with the values obtained before the experiment.

This study revealed that use of onion bulb and walnut leaf extract residues in fish feed induced increases in the blood parameters like (erythrocyte count, heamoglobin content and hematocrit values. From the result obtained in PCV, Hb and RBC during the study showed that the onion bulb and walnut leaf residues inclusion does not result into anaemia and this could support its use in aquaculture as safe plant immunostimulants. Reduction in packed cell volume and red blood cell values are indicative of low protein intake or mild anaemia [16]. Reduction in the concentration of PCV in the blood usually suggests the presence of a toxic factor (e.g. haemagglutinin) which has adverse effect on blood formation [17]. Also, [18] reported that both the biochemical and haematological blood components are influenced by the quantity and quality of feed and also the level of anti-nutritional elements or factors present in the feed.

This suggests that walnut leaf and onion bulb residues could enhance non- specific immune responses. The result also, shows that white blood cell recorded in the treated groups were higher in values compared to the control which reflected that fish fed walnut leaf and onion bulb diet were able to build immunity against pathogens. The findings of this study were similar to the results of [19] who reported a significant ($p<0.05$) increase in erythrocyte count, heamoglobin content and hamatocrit value in *O. niloticus* fed different graded levels of garlic (*Allium sativum*) and chloramphenicol. Also, the results of [20] showed that addition of *Allium sativum* to fish diets increased erythrocyte number, heamoglobin content, hematocrit value, leucocytes and thrombocytes. Also, [21] reported that the erythrocyte-count was significantly higher in *Oreochromis niloticus* fed with probiotics (*Micrococcus luteus* and *Pseudomonas species*) than the control. [22] evaluated the uses of probiotics (*Bacillus subtilis* and *Lactobacillus acidophilus)* in the diets of *Oreochromis niloticus* and reported that the hamatocrit values were significantly higher in the group fed on diet supplemented with probiotic, when compared with the control group. [23] evaluated the uses of *Euglena viridis* in the diets of *Labeo rohita and* reported that a significant ($p<0.05$) increase in WBC and RBC was observed in group fed treated diet of different graded level of *Euglena viridis* compared to the control. Onion bulb (*Allium cepa)* and walnut leaves *(Tetracarpidium conophorum)* have some constituents (alkaloids, flavonoids, tannin and thiosulfinates) that may play a role in the immune system stimulation and in the function of organs related to blood cell formation such as thymus, spleen and bone marrow.

Blood indices (MCV, MCH and MCHC) are particularly important for the diagnosis of anaemia in most animals [24]. There were decreases of MCV, MCH and MCHC in fish fed onion bulb and walnut leaves residue –based diets which is in agreement with the work of [18] who reported decrease of MCV, MCH and MCHC in *Oreochromis niloticus* fed graded levels of *Allium sativum*. The differential leucocytic-count is an indicator of health in fish [25].

The current study showed insignificant changes in the counts of the large lymphocytes: heterophils and monocytes among the experimental groups. This is in agreement with the results of [26] who reported insignificant change in the count of the large lymphocytes, heterophils and monocytes among the experimental groups. [27] reported that water and ethanolic-extracts of propolis increased the percentage of phagocytes (monocyte-macrophages and acidophilic granulocytes) of gilthead seabream.

Result obtained from this study shows an increase in total protein, albumin, globulin and albumin – globulin ratio. These findings were similar to results obtained by [28] in which total protein levels remained unchanged irrespective of aflatoxin / levamisole exposure, although the increased albumin / globulin ratio noted due to aflatoxin treatment was restored by levamisole feeding. Another study by [29] in African catfish reported that levamisole was found to restore the total protein level in immune-compromised fish and increasing γ-globulin levels in healthy fish.

However, [23] reported that the serum total protein after long term feeding with *E. viridis* increased in comparison to the control diet which supported the present findings. Also, [30] observed an increase in total protein content after feeding of b-glucan (0.2%) and chitosan (0.5%) in the diet. Serum albumin and globulin values in fish fed with *E. viridis* were higher than the control. [31] reported that increases in serum protein, albumin and globulin levels may be a result of immune response to certain constituents of the extracts. [32] also showed that serum total protein content was elevated in male Albino rats after administration of garlic oil. Blood serum protein is a fairly labile biochemical system, precisely reflecting the condition of the organism and the changes happening to it under influence of internal and external factors [19].

Results of this study showed that serum enzymes Aspertate Amino Transferase (AST) and Alanine Amino Transferase (ALT) activities decreased in the fish group fed on all levels of inclusion of *A. cepa* and *T. conophorum* compared to control. There were no significant differences ($P<0.05$) among the treatments in terms of AST and ALT. These findings were similar to [19] who reported that serum AST and ALT activities decreased significantly in the fish group fed on all levels of *Allium sativum* and chloramphenicol. This work also agreed with those reported by [33, 34], who found that the lipid parameters and enzyme activities (AST, ALT, and ALP) in serum of rats decreased significantly when they were fed on a diet containing 5% *Allium sativum*. The results of this study revealed *Allium cepa and T. conophorum*, which may cause stabilized cell membrane and protect the liver against deleterious agents and free radical-mediated toxic damage to the liver cells. This is reflected in the reduction of liver enzymes. *Allium cepa and T. conophorum* help the liver to maintain its normal function by accelerating the regenerative capacity of its cells.

5. CONCLUSION

In conclusion, this work revealed that the immune response studied in fish can be affected by dietary feed intake. Therefore, dietary supplementation of walnut leaf and onion bulb residues in African catfish, *Clarias gariepinus* feeds should be encouraged since the values obtained were within acceptable limits for cultured fish and no traces of infection such as anaemia were observed in the cultured fish, *Clarias gariepinus.* The extracts of walnut leaf and onion bulb will be useful in fish diseases to stimulate immune response.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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