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THE PROTECTIVE EFFECT OF WALNUT (*TETRACARPIDIUM CONOPHORUM*) LEAF AND ONION (*ALLIUM CEPA*) BULB RESIDUES ON THE EXPERIMENTAL *PSEUDOMONAS AERUGINOSA* INFECTION IN *CLARIAS GARIEPINUS* JUVENILES

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Abstract

The study assessed the effect of Walnut Leaf (WL) and Onion Bulb (OB) residues on disease resistance of Clarias gariepinus juveniles against infection with the bacteria pathogen Pseudomonas aeruginosa. Clarias gariepinus juveniles were fed with diets containing 0 (control), 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%). Biochemical (serum total protein, albumin, globulin, albumin: globulin ratio) and haematological indices of the fish were investigated. Fish were exposed to 0.5ml of 107 *Pseudomonas aeruginosa* of 24h old culture with the percentage mortality and relative level of protection recorded for 4 weeks post – infection. The results demonstrated that the fish fed with treated diets showed increased in biochemical and *haematological indices* ($P<0.05$) compared with the control. The challenge infection showed an improvement from treated groups with percentage mortalities and relative level of protection highest in WL8 (3.33%, 90%) and OB2 (3.33%, 90%) and least (33.33%, 0%) in control respectively. The results suggest that walnut leaf residue at 1.5% inclusion for one month could be a potential, less expensive and promising dietary supplement that would positively affect growth, haematology and make *C. gariepinus* more resistant to *Pseudomonas aeruginosa*

Keywords: *Pseudomonas aeruginosa, Clarias gariepinus, Onion bulb,* Walnut leaf Mortality, *Haematology*

L'EFFET PROTECTEUR DES RESIDUS DE FEUILLES DE NOYER *(TETRACARPIDIUM CONOPHORUM)* ET DE BULBES D'OIGNON *(ALLIUM CEPA)* CONTRE L'INFECTION EXPERIMENTALE AUX PSEUDOMONAS AERUGINOSA CHEZ DES *CLARIAS GARIEPINUS* JUVENILES

Résumé

L'étude a évalué l'effet des résidus de feuilles de noyer (WL) et de bulbes d'oignons (OB) sur la résistance des Clarias gariepinus juvéniles à l'infection aux bactéries pathogènes Pseudomonas aeruginosa. Des Clarias gariepinus juvéniles ont été nourris avec des régimes contenant 0 (témoin), OB2 (0,5%), OB3 (1,0%), OB4 (1,5%), OB5 (2,0%), WL6 (0,5%), WL7 (1,0%), WL8 (1,5%) et WL9 (2,0%). Les indices biochimiques (la teneur totale en protéines sériques, l'albumine, la globuline, le ratio albumine/globuline) et hématologiques des poissons ont été étudiés. Les poissons ont été infectés avec 0,5 ml de 107 *Pseudomonas aeruginosa* issues d'une culture de 24 h ; et le taux de mortalité et le niveau relatif de protection ont été enregistrés pendant 4 semaines après l'infection. Les résultats ont montré une augmentation des indices biochimiques et hématologiques ($P<0,05$) chez les poissons nourris avec les régimes traités par rapport à ceux nourris avec le régime témoin. L'infection d'épreuve a montré une amélioration chez les groupes traités ; le pourcentage de mortalité et le niveau relatif de protection étaient élevés respectivement chez les poissons nourris aux régimes WL8 (3,33%, 90%) et OB2 (3,33%, 90%) et faibles (33,33%, 0%) chez le groupe témoin. Les résultats font penser que les résidus de feuilles de noyer à une inclusion de 1,5% pendant un mois pourrait être un supplément alimentaire potentiel, moins cher et prometteur, susceptible d'avoir des effets positifs sur la croissance et l'hématologie et augmenter la résistance des *C. gariepinus* aux bactéries *Pseudomonas aeruginos.*

Mots-clés : *Pseudomonas aeruginosa, Clarias gariepinus, bulbe d'oignon, feuille de noyer, mortalité, hématologie*

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Introduction

Disease outbreaks were recently identified as the major constraint to aquaculture production with consequent adverse effects on the industry's economic development (Yunxia *et al.*, 2001). Attempts to control or prevent such devastating outbreaks using conventional antimicrobials and other chemotherapeutants have been generally unsuccessful (Jadhav *et al.*, 2006). The uncontrolled and repeated uses of antibiotics to treat bacterial infections have in some cases led to the development of antibiotic- resistant pathogens (Flores *et al.*, 2003; Food and Agriculture Organization, 2006). Considering the potential threat of diseases on human and animal health, issues associated with the use of antibiotics, disease management aspect should therefore focus on environmental-friendly, preventive methods such as the use of immunostimulants and natural products with antimicrobial properties.

Immunostimulants, which have been effective in enhancing immune responses in salmonids, carp, channel catfish and giant fresh water Macrobrachium rosenbergii include lavamisole (Siwicki *et al.*, 1990), glucans (Yano *et al.*, 1989; Robertson *et al.*, 1990; Niki *et al.*, 1991; Mishra *et al.*, 2004) and chitin (Sakai *et al.*, 1992; Anderson and Siwicki, 1994). Das *et al.*, 2009 reported that immunostimulants could be administering orally or in the feed.

As an hypothesis, onion (Allium cepa) bulb and walnut (Tetracarpidium conophorum) leaf, as plant immunostimulants, can be used as a growth promoter and in health management in African catfish (Clarias gariepinus) in which it could increase body weight gain, feed intake and feed efficiency. Onion (A. cepa) bulb and walnut (T. conophorum) leaf potentially display broad spectrum activities against bacterial agents (Gram positive and Gram negative) both in vitro and as well as in vivo studies (Abd-Elallatif and Ebraheem, 1996) and also antihelmintics and anti-fungal properties. The aim of the current study was therefore to evaluate the possible protective effect of walnut leaf and onion bulb residues as a potential antimicrobial in the farming of Clarias gariepinus against an experimental challenge infection using Pseudomonas aeruginosa

Materials and Methods

Plant collection and identification

Onion bulbs were purchased from Bodija market in Ibadan, Nigeria. Walnut leaf was obtained from a farm at Oka -Akoko, 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.

Preparation and Extraction of Plant Materials

Onion extraction

The onions bulbs were washed with distilled water and allowed to air dry at ambient temperature (25oC) for one hour. The dry outer coverings of the onions were manually peeled off, washed and extracted as described by Azu and Onyeagba, (2007). 200g of the fresh onion bulbs were blended into fine powder and soaked in 100ml of 95% ethanol for 24hrs. The pulp obtained was left in a clean, sterile glass container, shaken vigorously to allow for proper extraction, filtered using a sterile muslin cloth after which the residue was obtained, air-dried and stored (4°C) until required.

Walnut leaf extraction

The extraction was as described by Ajaiyeoba and Fadare (2006). The air – dried walnut leaf were ground with a hammer mill to fine powder. 200g of the powder of walnut leaves was soaked in 100ml of 80% methanol for 72 hours. Walnut leaf were properly mixed with methanol, filtered using a sterile muslin cloth after which the extract was obtained, air – dried and stored at (25°C) until required.

Preparation of Experimntal Diets

The proximate composition of the experimental diet was 40.0%crude protein, 15.9% ether extract, 15.7% ash, 7.4% moisture, and 20.9% NFE. 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, soyabean, maize, starch, vegetable oil, Di calcium

phosphate (DCP), salt and vitamin- mineral premix were added and the dry ingredients were mixed thoroughly in a mixer. Water was added and the resulting dough was pelleted. The pelleted diets were sun –dried and stored in airtight containers at room temperature to prevent mycotoxin formation until required.

Culture Of Pathogen

P. aeruginosa was collected from the Laboratory stock of the Department of Microbiology, University of Ibadan, Nigeria. The pure cultures were sub-cultured on Nutrient slants and preserved in the refrigerator at 4°C until required for the study

Collection of Blood and Serum

This was carried out before and after the experiment at the haematological laboratory ofVeterinary Pathology Department, University of Ibadan within 30 minutes of sampling. a distance of 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, which was gently aspirated during penetration. The blood was taken under gentle aspiration until about 1cm3 had been obtained. Thereafter the needle was gently withdrawn and the blood gently transferred into heperarinized plastic containers. The samples were then mixed gently but thoroughly. The haematology were taken according to the methods of Blaxhall and Daisley (1973). Plasma was obtained from blood samples by centrifugation and then drawn into 1cm3 plastic syringe transferred into a universal bottle in refrigerator to be later used for biochemical analysis.

Challenge Test

270 Clarias gariepinus (30 from each treatment) were challenged by intraperitoneal route with 0.5ml of 107 Pseudomonas aeruginosa of 24h old culture. The challenged fish were kept under observation for 30 days. The mortalities were recorded and the relative level of protection (RLP) among the challenged fish was determined as described by Azza and Abd-El-Rhman (2009), Ibrahem *et al.,* (2010)

$$\text{RLP} = 1 - \frac{[\text{percentage of mortality in treated group}] / \times 100}{[\text{percentage of mortality in control group}]}$$

Statistical Analysis

Challenge test, haematology and biochemical analysis results 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 between individual means.

Results

Challenge test of *Pseudomonas aeruginosa* injected by intraperitoneal route and relative level of protection among Clarias gariepinus treated with onion bulb and walnut leaf was given in Table 1. Mean post challenge test haematological parameters of African Catfish Clarias gariepinus Juveniles fed treated onion bulb and walnut leaf (Table 2). Mean post challenge test plasma biochemistry parameters of African Catfish (Clarias gariepinus) Juveniles fed onion bulb and walnut leaf are shown in Table 3. Post challenge test blood serum of African Catfish Clarias gariepinus Juveniles fed onion bulb and walnut leaf are given in Table 4

Discussion

A properly functioning immune system is critical in maintaining the fitness and health of an organism; thus the opportunity to determine the effect of exposure to bacteria on the performance and immunity of the fish species and on their natural habitats (AraKoosh, *et al.,* 2005). The results showed that infected fish in the present study were weak in the 1st week of the experiment with decrease in feed intake and weight gain (see Table 1). However, feed intake and weights increased in the second week. The first mortality was recorded in the control experiment on the 5th day. The observed symptoms of this infection included lack of appetite, swimming abnormalities,

Table 1: Challenge test of Pseudomonas aeruginosa injected by intraperitoneal route and relative level of protection among Clarias gariepinus treated with onion bulb and walnut leaf

	Control	OB2	OB3	OB4	OB5	WL6	WL7	WL8	WL9
Weight:									
Initial	45.12±2.47	46.67±1.75	50.35±2.94	42.43±2.69	46.24±2.07	47.97±3.25	41.19±0.83	44.19±1.01	37.42±1.06
Week 1	44.22±1.99	46.57±1.04	49.95±2.80	43.05±3.56	45.89±1.29	48.08±3.46	40.94±0.37	44.37±0.62	36.80±3.29
Week 2	47.68±1.83	51.14±0.94	54.62±2.90	46.30±4.62	47.69±0.77	53.63±2.40	45.68±1.10	48.99±0.54	41.22±1.52
Week 3	50.33±1.23	52.41±0.39	56.76±2.64	48.28±2.81	50.65±0.69	54.47±2.27	48.74±1.08	51.15±0.21	41.76±1.04
Week 4	50.66±3.69	55.87±5.18	59.77±0.38	53.92±1.07	52.08±0.64	57.91±0.54	52.96±1.47	55.93±1.00	43.46±1.30
Weight gain	5.54±1.22[a]	9.30±3.43[d]	9.42±2.56[e]	11.49±1.62[g]	5.84±0.43[b]	9.94±2.71[f]	11.77±0.64[h]	11.74±0.01h	6.04±0.30c
Number of fish injected	30	30	30	30	30	30	30	30	30
Mortality (N)	10[b]	1[a]	4[a]	2[a]	3[a]	3[a]	2[a]	1a	3a
Mortality (%)	33.33[e]	3.33[a]	13.33[d]	6.67[b]	10.00[c]	10.00[c]	6.67[b]	3.33a	10.00c
Relative level of protection	0[a]	90[e]	60[b]	80[d]	70[c]	70[c]	80[d]	90e	70c

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

blotted appearance, skin alteration, anorexia, shaking head, and mouth tumidity. These syndromes were similar to those reported by Zhang *et al.*, (2009).

Results of the challenge test shown in Table 1 revealed that the mortality rate following challenge with P. aeruginosa was reduced in the groups of fish fed with onion bulb and walnut leaves incorporated diets. OB 2 and WL 8 showed highest rate of survival as compared with the control; the values were 3.33%, 90% for percentage mortality and relative level of protection (RLP) in OB 2 and WL 8 respectively and the control value was 33.33%, 0% for percentage mortality and relative level of protection. It can be inferred from the challenge study that the increased protection against the pathogen could be due to the enhancement in the defence system as evidenced from the increase in different immune parameters such as lymphocytes and white blood cell in post-challenge fish.

The findings of this present study is in accord with the work of Shalaby *et al.*, (2006) who reported that diets with Allium sativum and chloramphenicol showed decrease in the mortality rate of O. niloticus challenged intraperitonealy with A. hydrophila. Das *et al.*, (2009) reported that mortality following challenge with A. hydrophila was decreased in the group of fish fed with Euglena incorporated diets compared with the control. The report also by Ibrahem *et al.*, 2010 that the showing relative level protection (RLP) after challenge infection using Aeromonas hydrophila was higher in treated groups (41.67% and 33.33% for vitamin c and insulin respectively) than the control (0%) supported the findings of the present study.

Similarly in their study, Aly *et al.*, (2008) reported that the group that was treated with probiotics (Bacillus subtilis and Lactobacillus acidophilus) showed higher levels of protection against the test pathogens than the control (without probiotics). Sharma *et al.*, 2010 also reported that the challenge test with A. hydrophila proved that increased per cent survival rate was highest (42.85%) in the treatment containing 2 g /kg

Table 2: Mean Post Challenge Test Haematological Parameters of African Catfish Clarias gariepinus Juveniles fed treated onion bulb and walnut leaf

Parameters	Pre – challenge	Post challenge			
		CONTROL	OB2	OB3	OB4
PCV (%)	12.50±2.50	29.00±0.00^{a}	37.00±3.00^{a}	28.50±2.50^{a}	26.00±1.00^{a}
Hb (g/dl)	4.10±0.45	9.30±1.00^{ab}	11.95±0.75^{b}	9.10±0.90^{ab}	8.30±0.50^{ab}
RBC x1012/ l	1.07±0.05	2.75±0.06^{a}	4.05±0. 35^{a}	3.19±0.66^{a}	2.63± 0.02^{a}
WBC x109/ l	15,250±5.50	15,250±5.50^{a}	18,500±2.10^{a}	17,375±8.25^{a}	14,250±0.14^{a}
Platelet (m/µl)	133,000±1.10	131,000±5.00^{a}	195,000±3.50^{a}	190,500±5.95^{a}	140,000±3.40^{a}
MCV (Fl)	118.17±0.38	105.50±2.30^{a}	91.50±0.03^{a}	91.75±1.15^{a}	99.05±4.35^{a}
MCH (Pg)	3.91±0.86	3.88±0.11^{a}	2.96±0.50^{a}	2.92±0.32^{a}	3.16±0.0.21^{a}
MCHC (g/dl)	34.00±0.04	32.00±0.00^{a}	32.50±0.05^{a}	32.00±0.00^{a}	32.00±0.10^{a}
Lym x109/ l	69.00±1.00	63.00±3.00^{a}	85.50±2.50^{b}	72.50±4.50^{ab}	71.50±0.01^{ab}
Hetero x109/ l	25.00±2.00	33.00±0.10^{b}	13.50±3.50^{a}	23.00±3.00^{ab}	26.00±0.50^{ab}
Mono x109/ l	3.00±0.00	2.00±0.50^{a}	0.50±0.00^{a}	2.00±0.50^{a}	0.50±0.01^{a}
Eos x109/ l	3.00±1.00	2.00±0.05^{ab}	0.50±0.00^{a}	1.00±0.01^{ab}	1.00±0.00^{ab}

	Post challenge				
	OB5	WL6	WL7	WL8	WL9
PCV (%)	31.00±1.00a	29.50±1.50a	29.00±1.00a	32.50±3.50a	26.10±0.10a
Hb (g/dl)	9.70±2.10ab	9.15±0.65ab	9.10±0.40ab	10.30±1.30ab	8.05±1.55a
RBC x1012/ l	3.34±0.58a	3.19±0.43a	2.87±0.55a	3.53±0.76a	2.44±1.11a
WBC x109/ l	17,550±2.50a	17,750±1.95a	15,725±1.38a	17,500±2.00a	16,750±4.45a
Platelet (m/µl)	111,000±1.00a	119,500±5.00a	125,500±2.50a	157,000±3.90a	148,500±6.00a
MCV (Fl)	91.95±0.20a	93.50±0.90a	104.20±1.60a	94.40±1.03a	125.05±4.00a
MCH (Pg)	2.88±0.13a	2.90±0.19a	3.27±0.49a	2.98±0.27a	3.80±1.09a
MCHC (g/dl)	31.50±0.05a	31.00±0.05a	31.50±0.05a	31.50±0.02a	31.00±0.10a
Lym x109/ l	71.50±1.50ab	65.50±4.50a	73.00±2.00ab	74.00±1.00ab	70.50±4.50ab
Hetero x109/ l	25.50±1.50ab	31.00±0.03ab	22.50±1.50ab	22.50±0.05ab	27.00±0.09ab
Mono x109/ l	1.50±0.10a	1.50±0.43a	1.50±0.02a	2.00±0.05a	1.50±0.01a
Eos x109/ l	1.50±0.02ab	2.00±0.00ab	3.00±0.71b	1.50±0.01ab	1.00±0.00ab

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, MCV =Mean Cell Volume, MCH = Mean Cell Haemoglobin, MCHC = Mean Cell Haemoglobin Concentration, Lym =Lymphocytes, Hetero =Heterophil, Eos = Eosunophil, Mono = Monocytes

W. somnifera followed by 1 g /kg W. somnifera (14.28% survival) and 9.92% survival in the diet containing 3 g/ kg W. somnifera root when compared with the control which is also in agreement with the present study.

The decrease in mortality rate, in this study, with dietary onion bulb and walnut leaves after injection of bacteria, P. aeruginosa, is in agreement with previous studies conducted in *O. mossambicus* fed with diet containing *Ocimum sanctum* (Logambal et *al.*, 2000), L. rohita fed with the diet containing herb Achyranthes aspera (Rao et *al.*, 2006) and *O. mossambicus* treated with Eclipta alba leaf extract (Christybapita et *al.*, 2007). It may be deduced that the plants constituents may directly initiate activation of the innate defence mechanisms acting on receptors and triggering intracellular gene activation that may result in the production of antimicrobial molecules (Bricknell and

Table 3: Mean Post Challenge Test Plasma Biochemistry Parameters of African Catfish Clarias gariepinus Juveniles fed onion bulb and walnut leaf

Parameters	Pre – challenge	Post challenge			
		CONTROL	OB2	OB3	OB4
Total protein (g/dl)	3.10±0.14	4.40±0.10^{a}	4.95±0.35^{a}	4.50±0.20^{a}	3.90±0.85a
Albumin (g/dl)	0.95±0.35	1.15±0.50^{abc}	1.90±0.30^{cd}	2.80±0.50^{e}	1.10±0.00^{ab}
Globulin (g/dl)	2.15±0.49	3.25±0.05^{a}	3.05±0.25^{a}	1.65±0.15^{a}	2.30±0.90^{a}
A.G Ratio	0.50±0.28	0.30±0.00^{a}	0.60±0.50^{a}	1.70±0.50^{b}	0.50±0.40^{a}
	3.00±1.00	2.00±0.05^{ab}	0.50±0.00^{a}	1.00±0.01^{ab}	1.00±0.00^{ab}

	Post challenge				
	OB5	WL6	WL7	WL8	WL9
Total protein (g/dl)	4.25±0.55^{a}	3.90±0.50^{a}	3.90±0.30^{a}	4.35±0.45^{a}	4.80±1.20^{a}
Albumin (g/dl)	0.95±0.15^{a}	1.10±0.50^{ab}	2.00±0.30^{d}	1.45±0.15^{abcd}	1.85±0.45^{bcd}
Globulin (g/dl)	3.30±0.40^{a}	2.80±0.40^{a}	1.90±0.60^{a}	2.90±0.30^{a}	2.95±1.65^{a}
A.G Ratio	0.50±0.00^{a}	0.55±0.05^{a}	1.20±0.50^{ab}	0.50±0.10^{a}	1.00±0.40^{ab}

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

Table 4: Post Challenge Test Blood Serum of African Catfish Clarias gariepinus Juveniles fed onion bulb and walnut leaf

Parameters	Pre – challenge	Post challenge			
		CONTROL	OB2	OB3	OB4
AST(IU/l)	151.00±1.31	124.00±1.20^{a}	111.00±0.00^{a}	122.00±2.00^{a}	106.00±1.50a
ALT(IU/l)	68.00±8.49	38.50±1.50^{a}	32.00±0.00^{a}	31.50±0.50^{a}	25.50±0.50^{a}

	Post challenge				
	OB5	WL6	WL7	WL8	WL9
AST(IU/l)	115.50±2.50^{a}	105.50±2.50^{a}	115.60±3.00^{a}	122.50±0.20^{a}	109.00±0.01^{a}
ALT(IU/l)	28.00±2.00^{a}	28.50±2.50^{a}	32.50±0.10^{a}	27.50±0.50^{a}	33.00±0.02^{a}

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

Dalmo, 2005). The stimulation of specific and non-specific immune defence observed in the present study might be due to the presence of one or more components present in Allium cepa and Tetracarpidium conophorum. The antimicrobial properties present in OB and WL might contribute to suppress the growth of bacteria and reduces the microbial load and inhibit pathogenic infection in the fish.

The values of Packed Cell Volume (PCV) and Red Blood Cell (RBC) generally increased in the treatments except WL9; OB4, WL9 respectively in post challenge test compared with the values obtained in pre challenge and the control of the post challenge test. The value of White Blood Cell (WBC) increased in the treated groups as compared with the control in post challenge test, although the differences were insignificant ($P > 0.05$) among the treatments. The results of the present findings supported the work of Das *et al.*, 2009 that also found increased WBC and RBC after 10 days challenge with Aeromonas hydrophila as compared with control. Das *et al.*, 2009 also reported decreased in Hb after 10 days challenge which agrees with present findings as decreased Hb was reported in the treatments except OB2, OB5 and WL8 as compared with control.

The values of lymphocytes recorded in post challenge test were higher than the one obtained in pre- challenge and the control of post challenge and the values were significantly higher ($P< 0.05$) in all the treated groups compared with the control in post challenge test. The value recorded in control were also lower than the one recorded in the pre – challenge, the reason for this might be due to lower immune functions against fish pathogen, Pseudomonas aeruginosa. This suggests that walnut leaf and onion bulb residues could enhance non – specific immune response. Also, the findings support the report of Dugena *et al.,* (2003) that fish fed medicinal plants harbour a variety of specific and non –specific defence mechanism against invading pathogens. The results of the present study revealed that increased in White Blood Cells (WBC) and lymphocytes following feeding of walnut leaf and onion bulb residues diets supported the notion of antimicrobial properties of walnut leaves and onion bulb (Ajaiyeoba and Fadare, 2006; Azu and Onyeagba, 2007) and traditional herbal medicines (Blumanthal *et al.,* 2000; Kumar and Anantharaja, 2007).

Results obtained from the post challenge test; there were lower levels of total protein in treated groups except OB2, OB3 and WL9 as compared with the control, there were no significant different ($P > 0.05$) among the treatments. The albumin level was reduced in OB4, OB5 WL6 and increased in OB2, OB3 WL7, WL8 and WL9 as compared with the control after the challenge test. The globulin were generally reduced in the treated groups except OB5 as compared with the control for post challenge test and the values obtained were insignificantly ($P> 0.05$) in the treated groups as compared with the control in post challenge test. The albumin and globulin ratio were higher in the treated groups as compared with the control in post challenge test and the variation of albumin and globulin ratio was insignificant ($P> 0.05$) among the treated groups as compared with the control.

Results of the present findings were in accord with Das *et al.,* 2009 that reported a reduction in values of total protein, albumin level and globulin content and there was no significant decrease in the globulin content of post challenge as compared with the control, this were applicable in the present research. Das *et al.,* 2009 reported increase in values of albumin and globulin ratio at 10 days post challenge with A. hydrophila as compared with the control which also support the present findings that revealed higher values in the treated groups compared with the control.

Increases in values of total protein, albumin, globulin level and albumin and globulin ratio of treated groups of post challenge test compared with pre – challenge and the control of post challenge are thought to be associated with a stronger innate immune response of fish. The reason for these results might be due to the presence of constituents in walnut leaves and onion bulb that have stimulatory effect on the immune mechanisms of C. gariepinus.

Transamination is considered to be important in assessing the state of the liver and some other organs (Verma *et al.,* 1981). Attention has been focused on the changes in AST and ALT activities which promote gluconeogenesis from amino acid as well as on the changes in aminotransferase activities in the liver (Hilmy *et al.,* 1981; Rashatuar and Ilyas, 1983). AST and ALT activities might be altered by a variety of chemical, biological and physiological factors or by a disturbance in the Kreb's cycle. Decreased activities of the Kreb's cycle cause a decrease in its intermediates, thereby letting AST and ALT compensate by providing a – Ketoglutarate (Salah El-Deen and Rogers, 1993).

The result of the present study in post challenge test showed that AST and ALT activities decreased in all the treated groups as compared with the pre – challenge and control of the post challenge test. This report agrees with those reported by Shalaby *et al.,* 2006 who found decrease in AST and ALT fed Oreochromis niloticus at different graded level of Allium sativum and chloramphenicol. The possible role of reduction of AST and ALT can be attributed to the presence of constituents of walnut leaves and onion bulb that may stabilize the cell membrane and protect the liver against deleterious agents and free – radical- mediated toxic damages to the liver cell. This is reflected in the reduction of liver enzymes. Allium cepa and T. conophorum helps the liver to maintain its normal function by

accelerating the regenerative capacity of its cells. Thus, from the present experiment, it is clear that dietary supplementation of walnut leaf and onion bulb residues at all inclusions have enhancing effects on innate immunity and disease resistance of P. aeruginosa.

Conclusion

In conclusion, walnut leaf and onion bulb residues appear to be useful tools that can be used to enhance growth, survival and non- specific immune functions against fish pathogens such as Pseudomonas aeruginosa and the inclusion of walnut leaf residue may significantly enhance the productivity in aquaculture industry.

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