PERFORMANCE AND ANTIMICROBIAL POTENTIALS OF ONION (*Allium cepa* Linn) BULB AND WALNUT (*Tetracarpidium conophorum* Mull. Arg) LEAF IN THE DIET OF *Clarias gariepinus* Burchell, 1822

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ABSTRACT

High cost of conventional feedstuffs and competition between livestock and fish for ingredients necessitate research into low cost, non-conventional feedstuffs for profitable fish farming. In aquaculture, the emphasis is on producing feeds that promote growth and health of fish. There is scanty documentation of the potential of onion (*Allium cepa*) bulbs and walnut (*Tetracarpidium conophorum*) leaves in fish production. The growth performance of *Clarias gariepinus* on diets containing Onion Bulb (OB) and Walnut Leaf (WL) and their antibacterial activities were therefore investigated.

Growth experiments, replicated 3 times with 20 fish per replicate, were carried out for 18 weeks on *C. gariepinus* juveniles. Experimental diets composed of 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%) were fed twice daily at 3% body weight. Mean Weight Gain (MWG), Specific Growth Rate (SGR) and Feed Conversion Ratio (FCR) were measured. Also, Packed Cell Volume (PCV), Haemoglobin (Hb), Total Protein (TP), Amino Alanine Transferase (ALT) and Aspartate Amino Transferase (AST) contents were determined using standard methods. Antibacterial activities and inhibition of diameter of ethanol and methanol extracts of OB and WL were evaluated against four clinical strains of bacteria isolates from *C. gariepinus* and *Oreochromis niloticus* using agar well diffusion method. Microbial loads of water and fish tissues (skin, gill, intestine and liver) were determined using American Public Health Association (APHA) methods. *Clarias gariepinus* juveniles were innoculated with *Pseudomonas aeruginosa* at $5.0x10^{-6}$cfu/mL intraperitoneally and fed the different diets to assess their Level of Protection (LP). Wound ($1cm^2$) was created on lateral and caudal parts of the *C. gariepinus* and percentage Dermal Wound Healing (DWH) was investigated using standard methods. Data were analysed using descriptive statistics and ANOVA at p=0.05

Fish on OB and WL based diets had higher growth rates than the control diet but *C. gariepinus* fed WL8 had significant higher MWG, SGR and FCR of 53.81±1.20g, 1.09±0.11g and 2.16±0.01 respectively. The PCV (34.5±0.7%), Hb (10.65±0.07g/dL), TP (5.70±0.99g/dL), ALT (22.50±3.40 μ/L) and AST (139.00±9.90 μ/L) in WL were

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significantly higher than those of OB. The OB and WL extracts had inhibition zones of 10±0.01mm and 12±0.01mm diameter respectively against *P. aeruginosa;* 11±0.00mm and 11±0.01mm diameter against *Pseudomonas fluorescens;* 11±0.01mm and 13.5±0.01mm diameter against *Staphylococcus aureus*. Microbial loads in water (log_{10}cfu/mL) 4.37±0.02, skin, gill, intestine and liver (log_{10}cfu/g) were 3.35±0.05, 3.20±0.06, 3.27±0.04 and 3.25±0.07 respectively in fish on WL diets. These values were significantly lower than the corresponding values for OB. The LP against *P. aeruginosa* were higher in WL8 (90%) and OB2 (90%) than uninnoculated fish. The DWH of *C. gariepinus* was better on lateral and caudal parts (100%, 100%) in WL8 compared to the control (98%, 80%) respectively.

Fish fed with onion bulb and walnut leaf diets had improved mean weight gain, specific growth rate and feed conversion ratio, and were more resistant to *Pseudomonas aeruginosa* infection.

Keywords: *Clarias gariepinus*, Onion bulb, Walnut leaf, Fish growth, Fish wound healing

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To the four main pillars upon which the structure of my joy rests.

Pillar One: To the Everlasting Pillar; the Pillar of the pillars, the Almighty God (my creator), Jesus Christ (my saviour) and Holy Spirit (my companion) whose goodness has enabled this work, whose mercy and grace has preserved my life. Unto Him the Entire glory belongs.

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CERTIFICATION

I certify that this project was carried out by Mr Olusola Sunday **BELLO** in the Department of Aquaculture and Fisheries Management, University of Ibadan, Ibadan.

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CHAPTER ONE

1.0 INTRODUCTION

Fish and fishery products represent a very valuable source of protein and essential micronutrients for balanced nutrition and good health (Food and Agriculture Organization, 2012). Fish depends on protein and minerals supplied through feed and from the water environment for growth. The rise in global awareness of the importance of fish led to increase progress in aqua feeds with diets being specifically designed to meet the nutritional requirements of species, life cycle and health condition of fish (Rawling *et al*., 2012).

Capture fisheries and aquaculture supplied the world with about 148 million tonnes of fish in 2010 worth US $ 217.5 billion of which about 128 million tones was utilized as food for people, and preliminary data for 2011 indicate increased production of 154 million tonnes of which 131 million tonnes was destined as food (Table 1.1). With sustained growth in fish production and improved distribution channels, world fish food supply has grown dramatically in the last five decades, with an average growth rate of 3.2 percent per year in the period 1961 – 2009, outpacing the increase of 1.7 percent per year in the world's population. World per capita food fish supply increased from an average of 9.9kg (live weight equivalent) in the 1960s to 18.4kg in 2009, and preliminary estimates for 2010 point to a further increase in fish consumption in 2009, fish consumption was lowest in Africa (9.1 million tonnes, with 9.1kg per capita). In 2009, fish accounted for 16.6 percent of the world population's intake of animal protein and 6.5 percent of all protein consumed. Globally, fish provides about 3.0 billion people with almost 20 percent of their intake of animal protein, and 4.3 billion people with about 15 percent of such protein (FAO, 2012).

Nigeria's population was projected to be over 140 million with 12.5 kg per capita consumption that would require 1,890 million metric tonnes of fish in order to meet the protein needs of the populace in 2007 (FDF, 2003; Omitoyin, 2007). The need to increase fish production from aquaculture sector has led to increase in the

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artificially formulated aqua feeds for fin fish and shell fish culture. Farm – made feeds constituted 69.75% of the fish feed produced in Nigeria in 2000 (Fagbenro *et al*., 2003). Production of aqua feeds is obviously one of the expanding agricultural industries in the world with an annual growth rate in excess of 30% per year (Tacon, 1996).

Feed formulation accounts for more than 50% of the total production costs in modern intensive aquaculture (Ibrahem *et al*., 2010). In other to optimize the fish feed there is a need to increase feed efficiency, especially by improving the assimilation of dietary nutrients (Ibrahem *et al*., 2010). Artificial feeds supplemented with antibiotics have been used to prevent the spread of diseases and improve feed conversion ratio (FCR) (Reilly and Käferstein, 1997), thereby promoting growth and health in most fish including carp, trout and Nile Tilapia (Essa *et al*., 1995).

It is well understood that harmful microbes (bacteria, fungi, viruses and parasites), nutritional disorders and poor water quality or environmental disorders cause diseases which in turn have been major obstacles to aquaculture worldwide (Kumar and Aanatharaja, 2007). Stressors including overcrowding, high or sudden change of temperature, handling, low dissolved oxygen, poor nutritional status and fungal (*Aspergillus niger*, Aflatoxin B_1 etc) or parasitic damage of the epidermis, contribute to physiological changes and heighten susceptibility to infection (Bastardo *et al*., 2012). 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; FAO, 2006). Considering the potential threat of diseases on human and animal health, issues associated with the use of antibiotics in disease management should therefore focus on environmental-friendly, preventative methods such as the use of natural immunostimulants.

Imunnostimulants are chemical compounds that stimulate the non specific immune system when given alone or the specific immune mechanism when given with an antigen, thereby making the animal more resistant to microbial and parasitic infections (Cuesta *et al*., 2005). Immunostimulants can be grouped under chemical agents, bacterial preparations, polysaccharides, animal or plants extracts, nutritional factors and cytokines (Sakai, 1999). Therefore, using immunostimulants seems to be an attractive alternative to control fish diseases and enhance growth (Raa, 1996;

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Secombes, 1994). In fish, several immunostimulants such as levamisole (Siwicki *et al.,*1990), chitin (Sakai *et al*., 1992; Esteban, *et al*., 2001), lactoferrin (Sakai *et al*., 1993), nisin (Villamil *et al*., 2003), recombinant transferrin (Stafford *et al*.,2004), modified carbohydrate (Mishra *et al*., 2006), b-glucan (Das *et al*., 2009a; EL –Boshy *et al*., 2010), chitosan (Geng *et al*.,2011), and various kinds of probiotics (Chiu *et al*., 2010; Harikrishnan *et al*., 2010; Harikrishnan *et al*., 2011) have been reported. These substances play a promising role in aquaculture by enhancing the resistance of cultured fish to diseases. Most of these studies have demonstrated the use of immunostimulants by injection or dietary administration.

The effect of immunostimulant depends on various factors such as time, dosage, method of administration and the physiological condition of fish. A large number of plants have been used in traditional medicine for the treatment and the control of several diseases (Chakrabarti *et al., 2012*). Recent studies showed that the incorporation of medicinal plants in the diets of carps stimulated the immune system of fish and enhanced their disease resistant properties (Chakrabarti *et al.,* 2012).

Moreover, Onion (*A. cepa*) bulb and walnut (*T. conophorum*) leaf as herbal remedies could reduces a multiple of risk factors that plays decisive roles in the genesis and progression of arteriosclerosis (Siegel *et al*, 1999). *Allium cepa* has been reported to decrease both the total cholesterol and low density lipoprotein (LDL-C) in addition to reducing blood pressure (Adler and Holub, 1997). Walnut leaves (*T. conophorum*) on the other hand has panacea for stress, infections, infertility in men and animals and it has high potential as an antimicrobial medicinal plant.

Onion (*A. cepa*) bulb and walnut (*T. conophorum*) leaf appears to have 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. These series of investigations were carried out to assess the effect of *Allium cepa* and *Tetracarpidium conophorum* on growth performance, bacterial growth, dermal wound healing, haematology and tissue pathology of African catfish (*C. gariepinus*).

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Table 1.1: World Fisheries Aquaculture Production and Utilization

	2006	2007	2008	2009	2010	2011
Production	Million tonnes					
Capture						
Inland	9.80	10.00	10.20	10.40	11.20	11.50
Marine	80.20	80.40	79.50	79.20	77.40	78.90
Total capture	90.00	90.40	89.70	89.60	88.60	90.40
Aquaculture						
Inland	31.30	33.40	36.00	38.10	41.70	44.30
Marine	16.00	16.60	16.90	17.60	18.10	19.30
Total aquaculture	47.30	49.90	52.90	55.70	59.90	63.60
Total world fisheries	137.30	140.30	142.60	145.30	148.50	154.00
Utilization						
Human consumption	114.30	117.30	119.70	123.60	128.30	130.80
Non- food use	23.00	23.00	22.90	21.80	20.20	23.20
Population (billions)	6.60	6.70	6.70	6.80	6.90	7.00
Per capita food fish supply (kg)	17.40	17.60	17.80	18.10	18.60	18.80

Source: FAO, 2012

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1.1 JUSTIFICATION

Continuous use of synthetic antibiotics in aquaculture to promote growth and health can lead to the emergence of drug – resistant strains and residual effects on the fish which can create serious public health hazards (Aly *et al*., 2008; Das *et al*., 2009 and Harikrishnan *et al*., 2011). This problem led to the use of natural products such as onion bulb and walnut leaves that have antimicrobial effects. Other potentials of plants immunostimulants/ herbs in aquaculture are as follows:

- Suitable for boosting immune system
- Effective against a number of opportunistic pathogens
- Enhance immune response to conventional vaccines
- Safe and non –toxic (toxin binders)
- No resistance problems
- Environmental friendly, fully biodegradable
- Materials for correcting digestive tract disorders
- Growth promoter through efficient digestion and assimilation
- Growth promoter through efficient feed conversion
- Stress relievers
- Ammonia binders for water quality management
- Liver stimulants and liver protectants
- Rejuvenators and neural stabilizers (Jadhav *et al*., 2006; Kumar *et al.,* 2007).

Although, the use of immuno-stimulant/ herbs by aquatic animals is not a new concept in countries like India, Malaysia, Britain, and USA (Kumar *et al.,* 2007), little knowledge about its use is available in developing countries like Nigeria. Hence, the need to investigate the uses of herbal substances like onion bulb and walnut leaf on the indigenous fish species like *C. gariepinus*.

1.2 OBJECTIVES

This study was carried out to investigate the performance and antimicrobial potentials of onion bulb and walnut leaf on *C. gariepinus*. Hence the need;

- To assess the phytochemical properties of onion (*A. cepa*) bulb and walnut (*T. conophorum*) leaf

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- To evaluate onions (*A. cepa*) and walnut (*T. conophorum*) leaf residues as dietary ingredients as well as assess the growth performance and nutrient utilization of *C. gariepinus* juveniles fed diets containing various inclusion level of onions and walnut leaves meal residues.
- To examine the effect of onion (*A. cepa*) bulb and walnut (*T. conophorum*) leaf residues on bacteriological characteristics, survival and disease resistance of African catfish, *C. gariepinus* challenged with *Pseudomonas aeruginosa.*
- To evaluate the effect of onion (*A. cepa*) bulb and walnut (*T. conophorum*) leaf residues on the haematology, plasma biochemistry and histology of African catfish.
- To determine the dermal wound healing pattern in *C. gariepinus* and provide baseline data for future research in area of wound healing in *C. gariepinus*

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CHAPTER TWO

2.0 LITERATURE REVIEW

2.1. FAMILY CLARIIDAE

The family Claridae is divided into two genera – *Clarias* and *Heterobranchus*. Genus *Heterobranchus* has rayed dorsal fin followed by an adipose fin, while Genus *Clarias* has a single rayed dorsal fin extending almost to the tail. Both genera have four pairs of barbels on the flattened strongly depressed head.

2.1.2 HABITAT PREFERENCE

Clarias gariepinus occurs mainly in quiet waters, lakes and pools and prefer rather shallow and swamp areas with a soft muddy substrate and calmer water. They may also occur in fast flowing rivers. They are widely tolerant of extreme environmental conditions (Teugels, 1986). The presence of an accessory breathing organ enables this species to breath air when very active or under very dry conditions. They remain in the muddy substrates of ponds and occasionally gulp air through the mouth. They can leave the water at night using its strong pectoral fins and spines in search of land-based food or can move into the breeding areas through very shallow pathways. *Clarias gariepinus* are omnivorous bottom feeders which occasionally feed at the surface. *Clarias gariepinus* feed at night on a wide variety of prey like insects, plankton, invertebrates and fish but also take in young birds, rotting flesh and plants. Migrate to rivers and temporary streams to spawn. During intra-specific aggressive interactions, this species was noted to generate electric organ discharges that were monophasic, head-positive and lasting from 5-260 ms (Teugels, 1986).

2.1.3 TAXONOMY AND MORPHOLOGY OF *Clarias gariepinus*

The two most important *Clarias* species in aquaculture are *Clarias batranchus* and *Clarias gariepinus*, (Haylor, 1991). Body depth of *C. gariepinus* is 6-8 times in standard length, the head 3-3.5 times. The head is somewhat between rectangular and pointed in dorsal outline; the snout is broadly rounded. The eyes have a supero-lateral position and are relatively small. Teeth on the premaxilla and lower jaw are small, fine and arranged in several rows.

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Nasal barbels from 1/5 to 1/2 times as long as the head in fishes longer than 12 cm, and from 1/2 to 4/5 of the head length in smaller individuals; maxillary barbels rarely shorter than the head, usually somewhat longer and reaching to a point midway between the origin of the dorsal fin and the insertion of the pelvic fins; outer mandibular barbel longer than the inner pair. Contrary to other *Clarias* species, *Clarias gariepinus* has a high number of gill rakers varying from 24 to 110, the number increasing with the size of the fish; these gill rakers are long, slender and closely set (Teugels**,** 1986, Kamthorn and Jim, 2006) The distance between the occipital process and the base of the dorsal fin is short; the dorsal fin almost reaches the caudal fin. The anal fin origin is closer to the caudal fin base than to the snout; it nearly reaches the caudal fin.

The pelvic fin is closer to the snout than to the caudal fin base. The pectoral fin extends from the operculum to below the first dorsal fin rays; the pectoral spine is robust, serrated only on its outer face, the number of serrations increasing with age. Dorsal spines (total): 0; Dorsal soft rays (total): 61-80; Anal spines: 0; Anal soft rays: 45 - 65; Vertebrae: 56 - 63 (Viveen *et al.*, 1986). The lateral line appears as a small, white line from the posterior end of the head to the middle of the caudal fin base; the openings to the secondary sensory canals are clearly marked. Two colour patterns can be discerned: the uniform and the marbled pattern. In the uniform pattern, the dorsal surface and the flanks of the body and the dorsal parts of the pectoral and the pelvic fins are generally dark greyish-greenish black, while the belly and the ventral parts of the paired fins are lightly coloured (Teugels**,** 1986, Viveen *et al.,* 1986).

In the marbled pattern, the specimens show irregular dark blotches on a light coloured background above and laterally; the belly and the ventral parts of the paired fins are whitish. Most specimens show pigmentation bands on both sides of the lower surface of the head. A series of light and dark bands may occur on the caudal fin; the proximal third of the fin is lightly coloured while its other part is dark; occasionally, irregular black spots may occur on the caudal fin (Teugels**,** 1986). It is omnivorous, predatory and cannibalistic in nature. *C. gariepinus* has accessory respiratory structure to breathe on land (Kamthorn and Jim, 2006). This accessory breathing organ is located under the operculum.

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2.1.4 REPRODUCTION

Clarias gariepinus awaits suitable environmental condition for spawning, gonadal maturation starts during rainy season. The species is a gonochorist. Size and age at first maturity vary greatly (150–750 mm TL between one and four years), although the average size at sexual maturity is around 300–350 mm TL. The elongate and pointed urinogenital papilla of the male and the more rounded papilla of the female are the only external features upon which the sexes can be distinguished from each other. Average relative fecundity is in the region of 20 000–25 000 eggs/kg body weight. Fecundity is related exponentially to total length in mm (Fecundity = 0.000004TL mm3.563) and linearly to weight in grams (Fecundity = 45.18W (g) + 5 786) (Viveen *et al.,* 1986).

2.2 NUTRIENT REQUIREMENT OF FISH

The major nutrients required by fish for growth, maintenance, reproduction and other physiological functions are proteins and amino acids, lipids and fatty acids, carbohydrate, minerals, vitamins and energy source (FAO, 1990).

2.2.1 PROTEIN

Protein is the major organic matter in fish tissue, constituting about 65-75% of the total on a dry weight basis. It therefore constitutes a great part of fish diet (Wilson, 1991). Protein are complex, organic compounds composed of many amino acids linked together through peptide bonds and cross-linked between chains of sulfhydryl bonds. It can be classified into: simple proteins, conjugated proteins and derived proteins. Protein has a metabolizable energy value of about 4.5kcal/g in fish comparatively higher than that of mammals and birds (Pillay, 1993).

Studies on feed requirements of fry and fingerlings have shown that gross protein requirements are highest in fry and decrease as fish size increases. To grow at maximum rate, fry must have a diet in which nearly half of the digestible ingredients consist of balanced protein (Eyo, 1995). The optimum protein requirement in fish is influenced by water temperature, body size, stocking density and oxygen level (Lovell, 1984).

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AMINO ACIDS

The amino acids are the building blocks of protein and about twenty-three amino acids have been isolated from natural protein. Ten of these are indispensable for fish they include isoleusine, Histidine, Arginine, Lysine, Leucine, Methionine, Phenylalanine, Threonine, Tryptophan and valine. Animals are incapable of synthesizing these amino acids, therefore they must be included in the diets (Lovell, 1990). Table 2.1 shows the dietary requirements of essential amino acids by African catfish.

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Table 2.1: The essential amino acids requirements (g/kg protein) of African Catfish, *Clarias gariepinus*

Amino acids, % minimum of dietary protein	Requirements (g/kg)
Arginine	4.50
Histidine	1.39
Isoleucine	1.56
Leucine	4.87
Threonine	2.04
Tryptophan	2.59
Valine	2.08
Lysine	4.49
Methionine	3.2
Phenylalanine	4.56

Source: Pantazis, 1999

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2.2.2 CARBOHYDRATE

Carbohydrates make up about 75% of the biomass of plants but are present only in small quantities in the animal body as glycogen, sugars and their derivatives. Metabolizable energy value of carbohydrate may be up to 3.8kcal/g for easily digestible sugar, and about zero in indispensable cellulose (FAO, 1990).

The products of digestion of carbohydrates are assimilated into the blood stream where their function is to provide energy (Pillay, 1993) which gives them a protein-sparing function and the excess is partially stored in the liver as glycogen and partly converted into visceral and muscular fat. It also has the physical function of texturizing manufactured feeds and acting as a binder in the formulation of diets (Wilson, 1985).

2.2.3 LIPID AND FATTY ACIDS

Lipids are the generic names assigned to a group of fat soluble compounds found in the tissues of plants and animals. They are broadly classified as fat, phospholipids, sphingolipids, waxes and sterols (Kanazawa, 1985) Fats are the fatty acid esters of glycerol and are the primary energy depots of animals. These are used for long-term energy requirements during periods of extensive exercise or during periods of inadequate food and energy intake (NRC 1991; 1993).

Fish have the unique capability of metabolizing these compounds readily and as a result can exist for long periods of time under conditions of food deprivation (Rammarine, 1995). The metabolizable energy for lipids is 8.5kcal/g (Pillay, 1993). Researches show that the omega-3 (ω^3) series are considered to be non-essential fatty acid or only have a sparing action on EFA deficiency while ω^6 (omega-6) series of fatty acids are the essential fatty acids, it is assumed by many that fish also require ω^6 fatty acids. The polyunsaturated fatty acids (PUFA) of the ω^3 series which are present in relatively large concentration in fish oil play the role of essential fatty acid for fish (Kanazawa, 1985)

The EFA requirement of *C. gariepinus* can be supplied by suitable lipid source such as corn oil, soybean oil, and sunflower oil (Takeuchi *et al.*, 1983; Stickney and Handy, 1989). The EFA and saturated fatty acids are all equally utilized by fish for energy production (Stickney and Handy, 1989).

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2.2.4 MINERALS

Mineral elements have a great diversity of uses within the animal body for the formation of skeletal tissue, respiration, digestion and osmoregulation. The following mineral elements are recognized as essential for body functions in fish: calcium, phosphorus, sodium, molybdenum, chlorine, magnesium, iron, selenium, and iodine, manganese, copper, cobalt, zinc, fluorine and chromium (Jauncey and Barbara, 1982).

Dietary mineral requirements for *Clarias gariepinus* are phosphorus (0.45/kg diet) calcium (0.45g/kg), magnesium (0.04g/kg), sodium (0.03g/kg), potassium (0.26g/kg) Sulphur (0.025g/kg) selenium (0.25g/kg), Iron (30g/kg), Copper (5g/kg), manganese (2.40g/kg), Iodine (0.05g/kg) (Wilson and Moreau, 1996). Dietary symptoms of mineral deficiencies include poor growth, loss of appetite, tetany, microcytic, homocronic anemia and goitre (NRC, 1991; Luquet, 1991)

2.2.5 VITAMINS

Vitamins are organic compounds required in small amounts and they are essential for normal growth, reproduction and health of most animal species (Luquet 1991). Vitamins are classified into two groups namely: the fat-soluble and water-soluble vitamins. The fat-soluble, which include vitamin A, D, E and K and the water-soluble vitamins including eight well-recognized members of the vitamin B complex: Thiamine, riboflavin, Pyridoxine, Pantothenic acid, niacin, biotin, folic acid and vitamin B12 (Cyanocobalamin). The water-soluble essential nutritional factors: choline, inositol, and ascorbic acid vitamins with less-defined activity for fish: Paminobenzoic acid, lipoic acid and citrin (NRC, 1993). Table 2.2 shows the recommended amounts of vitamins for African catfishes

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Table 2.2: Recommended amounts of vitamins for African catfishes

Vitamin	Amount mg/kg of diet
Vitamin A (I.U)	1000 -2000
Vitamin D_3 (I.U)	500 -1000
Vitamin E (I.U)	25 -50
Vitamin K	5 -10
Choline	400
Niacin	33.1
Riboflavin	9
Pyridoxine	3
Thiamine	1
D-calcium pantothenate	10 - 15
Biotin	2.49
Folacin	1.2
Ascorbic acids	11 -60
Vitamin B_{12}	-
Inositol	-

Source: Wilson and Moreau, 1996

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2.2.6 ENERGY SOURCES

This can be classified into two types of energy namely: heat energy and free energy. Heat is utilized for maintaining body temperature and free energy available for biological activity and growth, the latter is more important to fish. The free energy is needed for growth, reproduction and maintenance (Wilson 1985; Lovell, 1984). The energy requirement of fish and other cultured organisms are generally supplied by carbohydrate, protein and fats (Pillay, 1993).

Most cultured fish digest protein and fat satisfactorily, but use starch less efficiently than land animals (Lovell, 1984). Warm water fishes such as tilapia, carp and catfish utilize starch better than cold-water fish. Approximately 70% of energy is used for maintenance and growth while 30% is lost to the environment. The digestion of energy by fish is influenced by temperature, age or size, oxygen, carbon (iv) oxide, pH and salinity of water (Wilson, 1991). The various nutrients requirements of fish have been studied and there is need for cheap and available alternatives in our environment of such are walnut leaf and onion bulb due to the possible high cost in today's aquaculture production.

2.3 WALNUT (*Tetracarpidium conophorum /Plukenetia conophora*)

The plant *T. conophorum* belongs to the family Euphorbiaceaae. *Tetracarpidium conophorum /Plukenetia conophora is* a climber found in Southern Nigeria and West Africa in general (see plate 2.1).. The fruits are greenish with four round seeds in each fruit. The testa of the seed is hard and the cotyledons white in colour. The fruits are edible and the plant is medicinal and used for various purposes. In Nigeria, the plant is known as "Ukpa" in Ibo, "Asala" in Yoruba, Okhue or Okwe in Edo and "Kaso" in Cameroon. It produces significant amount of chemical form of aspirin, antioxidants and essential fatty acids. Extract of walnut trees are effective anti-microbial agents, it could be used to boost sperm count and fertility as well as cure dysentery. (Ajaiyeoba and Fadare, 2006). Some phytochemical constituents present in *T. conophorum* are tannins, proteins, oil, carbohydrates and fibre (Enujiugha, 2003). Most studies on walnut leaf focus on its antimicrobial properties with less information on its growth promoting effect especially in fish.

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Plate 2.1: The walnut plant with the leaves (8.2 mega pixels, 3x optical zoom)

2.3.1 HABITAT AND CULTIVATION

Walnut growth / thrive well in cavernous well-drained soil with adequate sunlight. However, the walnut needs to be protected from strong winds, which other wise tend to uproot the vegetation or even rummage the branches. The tree grows well in a mildly alkaline heavy soil, but also flourishes in damp soils. Studies have shown that the walnut trees can withstand an annual rainfall in the range of 31 cm to 147 cm and annual temperature fluctuations from 7.0 to 21.1°C and relative pH in the range of 4.5 to 8.2 (Duke, 1997). The latent or dormant walnut plant can endure much cold, so much so that it remains alive even in freezing temperatures up to -27°C without sustaining any damage. However, the young spurs coming out in spring are very sensitive to cold and may be harmed by late frostiness (Duke, 1997; Foster and Duke, 1990).

Scientists have developed some late-leafing cultivable varieties of the walnut which are not only capable of avoiding damage from spring frosts, but also yield better quality of tree. In the temperate climes of the globe, different varieties of the walnut tree are often grown for its seeds that are edible (Foster and Duke, 1990).

2.3.2 USES OF WALNUT LEAF

The walnut leaf has various benefits for different health conditions. These leaves are useful for treating acne, eczema and ringworm. Astringent tannins are important ingredients of walnut leaves and these tannins cross-link with the skin cells enabling them to be resistant to allergies and diseases caused by micro-organisms. It may be noted that the walnut leaf possess two anti-bacterial constituents - walnut essential oil and juglone - that directly initiate steps against contagious micro-organisms. In addition, large concentration of vitamin C found in the walnut leaf also enables them to contain/ reduce the effect of infectious diseases. People suffering from excessive sweating too benefit from walnut leaves by cleansing the sweat pores and also shrink the sweat glands in order to reduced perspiration. Tannins found in walnut leaves cross-link with the proteins found in the cells coating the sweat glands and forming an effective obstruction to prevent excessive sweat secretion (Duke, 1997).

Walnut tea may be prepared by boiling walnut leaves in water and this is used in baths, bandages as well as skin washes with a view to cleansing the skin and also to reducing the bacterial load in wound. Walnut is believed to have the following

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traditional uses; Anthelminthic, Antifungal, Antibacterial, Anti-inflammatory, Antiviral, Hemorrhoids, Hypothyroidism, Immune System, Gallbladder Problems, Liver Health Maintenance, Poultice, Scabies (Blumenthal *et al.,* 2000). Walnut hulls are blended with other herbs in tinctures and are used as intensive laxatives. It is always best to use herbal products made from walnut leaf as they are not only more effective, but also do not have any side effects (Blumenthal *et al.,* 2000). This application in aquaculture is yet to be exploited.

2.4 ONION (*Allium cepa*)

2.4.1 ORIGIN AND GEOGRAPHIC DISTRIBUTION

Onion forms a large genus of about 700 species of strong – smelling, bulbous or rhizomatous biennials and perennials (Deni, 1996). It probably originated from Central Asia (between Turkmenistan and Afghanistan) where some of its relatives still grow in the wild (Currah, 2002) (see plate 2.2). The closest among them are *Allium vavilovii* Popov and Vved. from southern Turkmenistan and Northern Iran, with which it gives 100% fertile hybrids, and *Allium asarense* R.M. Fritsch and Matin from Iran. *Allium oschaninii* O. Fedtsch. (Uzbekistan and neighbouring countries), which used to be considered the ancestor of *Allium cepa* cannot be crossed successfully with the cultivated onion, but its domestication seems to be the origin of some European 'shallots' ('échalote grise' in France, 'Scalogno di Romagna' in Italy). From Central Asia, the supposed onion ancestor probably migrated first towards Mesopotamia, where onion is mentioned in Sumerian literature (2500 BC), then to Egypt (1600 BC), India and South-East Asia. From Egypt, *Allium cepa* was introduced into the Mediterranean area and from there to all the Roman Empire (Holland *et al.*, 1991).

Traditional tropical African cultivars may have been introduced either from southern Egypt, or from India via Sudan to Central and West Africa, as genetically heterogeneous seed or bulb lots, then bred by local farmers into better adapted seed-propagated onions, or selected to become shallots (Abdalla, 1967). *Allium cepa* as bulb onion and/or shallot is probably cultivated in all countries of tropical Africa. Important production areas for bulb onion are Senegal, Mali, Burkina Faso, Ghana, Niger, Nigeria, Chad, Sudan, Ethiopia, Kenya, Tanzania, Uganda, Zambia and Zimbabwe. In the lowlands between 10°N and 10°S shallots replace onions because the temperature is too high for vernalization and seed production, and the climate too

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humid. The short vegetative cycle of shallots (60–75 days) gives the possibility of two crops a year, especially in the four-season climate along the Gulf of Guinea. Yellow or red/purple shallots are grown in Guinea, Côte d'Ivoire, Ghana, Benin, Nigeria, Sudan, Ethiopia, Uganda, Kenya, Tanzania, and on both banks of the Congo River near Brazzaville (Congo) and Kinshasa (DR Congo). The spicy taste and high dry matter content (15–18%) of shallots have made them attractive for growers farther from the equator, in many areas where common onions are also produced, e.g. by the Dogon in Mali, or in Cape Verde (Currah and Proctor, 1990) and it varies in hardness according to origin (Deni, 1996).

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Plate 2.2: Red onion bulb (8.2 mega pixels, 3x optical zoom)

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2.4.2 USES

Bulbs of *Allium cepa* are popular vegetable all over the World. They can be used raw, sliced for seasoning salads, boiled with other vegetables, or fried with other vegetables and meat (Holland *et al.*, 1991). They are an essential ingredient in many African sauces and relishes, they can be considered as a condiment. The pungency varies from species to species and few are almost odourless (Deni, 1996). The leaves, whole immature plants (called 'salad onions' or 'spring onions'), or leafy sprouts from germinating bulbs (called 'cébettes' in southern France) are used in the same way. Locally, immature flower heads are also a popular food item. In parts of West Africa, leaves still green at bulb harvest are pounded, then used to make sun-dried and fermented balls, which are used later for seasoning dishes. Sliced raw onions have antibiotic properties, which can reduce contamination by bacteria, protozoa or helminths in salads. In traditional medicine onion is used externally to treat boils, wounds and stings, and internally to relieve coughs, bronchitis, asthma, gastro-intestinal disorders and headache (Diah *et al.*, 1999).

Onion is used as a diuretic, expectorant and antiseptic. They appear to be at least somewhat effective against colds, heart disease, diabetes and other diseases and contains anti-inflammatory, anticholesterol, and anticancer component. Onions contain many active compounds that appear to inhibit the growth of cancerous cells, help combat heart disease, inhibit strokes, lower blood pressure & cholesterol, and stimulate the immune system. Alliums are also antibacterial and anti-fungal, so they can relieve stomach upset and other gastrointestinal disorders. As with Garlic, Onions help prevent thrombosis and reduce hypertension, according to the American Heart Association.

2.4.3 ECOLOGY

Optimal vegetative growth and good bulb maturation are generally obtained under dry and cool conditions. Bulb maturity is promoted by a combination of increasing day length and rising temperatures, whereas withdrawal of irrigation also hastens bulb maturation. Onions and shallots can grow on any soil with pH above 5.6, but adequate calcium nutrition is essential for good vegetative development and disease tolerance. Onion crops in the tropics are often grown under irrigation between rains fed crops of cereals (Van der Meer, 1994; Rabinowitch and Currah, 2002)

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2.4.4 PROPERTIES OF ONION BULB AND WALNUT LEAF

The composition of the dry matter is rather constant; the variation in the composition of fresh onion bulb and walnut leaf is mainly caused by variation in moisture content.

Table 2.3 shown the nutritional value of onion bulb and walnut leaf per 100g.

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Table 2.3: Nutritional value of onion bulb and walnut leaf per 100g

	Raw onion bulb	Walnut leaf
Energy	166KJ (40Kcal)	2,738KJ (654Kcal)
Carbohydrates	9.34g	13.71g
• Sugars	4.24g	2.61g
• Dietary fibre	1.7g	6.7g
• Starch	-	0.06g
Fat	0.1g	65.21g
• Saturated	0.042g	6.125g
• Monounsaturated	0.013g	8.933g
• Polyunsaturated	0.017g	47.174g
Protein	1.1g	15.23g
Water	89.11g	4.07g
Vitamin A (equiv.)	0μg	1 μg
Vitamin A	-	20IU
• Beta- carotene	-	12 μg
• Lutein and zeaxanthin	-	9 μg
Thiamine (vit. B1)	0.046mg	0.341mg
Riboflavin (vit B2)	0.027mg	0.15mg
Niacin (vit 3)	0.012mg	1.125mg
Pathothenic acid (vit. B5)	-	0.570mg
Vitamin B6	0.12mg	0.537mg
Folate (vit. B9)	19 μg	98 μg
Vitamin B12	0 μg	0 μg
Vitamin C	7.4mg	1.3mg
Vitamin E	0.02mg	0.7mg
Vitamin K	0.4 μg	2.7 μg
Calcium	23mg	98mg
Iron	0.21mg	2.91mg
Magnesium	0.129mg	158mg
Phosphorus	29mg	346mg
Potassium	146mg	441mg
Sodium	4mg	2mg
Zinc	0.17mg	3.09mg

Source: USDA nutrient database, 2011

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There is a dearth of information on the use of onion bulb and walnut leaf as a growth promoter or as feed additive in aquaculture. With the antimicrobial properties of walnut leaf and onion bulb enumerated, there is need to exploit this property in aquaculture production which in Nigeria is challenged by numerous and emerging bacterial diseases.

2.5 BACTERIA

Bacteria are tiny, single-celled organisms which are continually present in the water, soil, and air. Most bacteria are beneficial (e.g. Lactobacillus, probiotics etc they help digest foods and breakdown ammonia, nitrite, and organic debris in the environment). There are a number of pathogenic bacteria in cultured freshwater food fish such as catfish, salmon, and trout. In aquaculture, many bacterial diseases of fish can be successfully treated with medicated feeds. However, prevention through good management practices is the best control measure for bacterial diseases. Bacterial diseases of fish are usually a result of stress such as overcrowding. Avoiding these stressors often reduces disease incidence. Failing to correct stressful conditions while treating sick fish with medicated feed will usually either prevent the medication from being effective or will cause the disease to recur after the treatment is completed. Hence there is need for alternatives such natural product that has antimicrobial properties.

2.5.1 BACTERIAL DISEASE

Some bacteria are considered opportunistic pathogens. These bacteria are often present in water and inside the fish, and they usually cause no problem. In nature fish are resistant to these pathogens and can seek the best living conditions available. In aquaculture, however, food fish are weakened by stress conditions including increased fish density, inadequate nutrition, poor water quality (i.e., low dissolved oxygen, or high ammonia and nitrite), parasite infestation, and handling. Stress suppresses the immune system, increasing the fish's susceptibility to bacterial infections. As a result, cultured fish are more susceptible to disease than free ranging animals. Common examples of opportunistic bacteria which can cause disease and death of fish include: *Aeromonas hydrophila*, *Cytophaga (Flexibacter) columnaris*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*. Some bacteria are considered obligate pathogens. They can be the sole cause of disease even in the

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absence of stressors. *Aeromonas salmonicida, Edwardsiella ictaluri, Renibacterium salmoninarum,* and *Yersinia ruckeri* are considered by some to be obligate pathogens (Durborow and Francis-Floyd, 1996). In Nigeria, the most important fish bacterial diseases are those caused by *Pseudomonas aeruginosa*

2.5.2 *Pseudomonas aeruginosa*

2.5.2.1 CLASSIFICATION

SCIENTIFIC CLASSIFICATION

Kingdom:	Bacteria
Phylum:	Proteobacteria
Class:	Gamma proteobacteria
Order:	Pseudomonales
Family:	Pseudomonadaceae
Genus:	Pseudomonas
Species:	Pseudomonas aeruginosa

Binomial name

P. aeruginosa

The genus *Pseudomonas* contains about sixty different types of species. The species *Pseudomonas aeruginosa* is classified as a gram-negative bacterium (Willey *et al.*, 2008). Most *Pseudomonas aeruginosa* are categorized as obligate aerobes, however, in certain environmental conditions, the bacteria act as facultative anaerobe. Furthermore, because of the way it obtains its energy, it is considered to be a chemoheterotroph (Cooper *et al.*, 2003; Ryan and Ray, 2004; Willey *et al.*, 2008).

2.5.2.2 DESCRIPTION AND SIGNIFICANCE

The *Pseudomonas* genus includes bacteria that are straight or slightly curved rods. *P. aeruginosa* is a rod-shaped bacterium. Its size ranges from 0.5 to 1.0mm by 1.5 to 5.0mm in terms of its length and width. Almost all types of strains are motile by means of a polar flagellum (Todar, 2008). *P. aeruginosa* was first isolated from green pus in 1882 by Gessard. He described the bacterium as a pathogen which he linked with wound infections, often seen as blue/green colors underneath bandages of wounded soldiers (Rozwadaowski *et al.*, 2005). *P. aeruginosa* is a well-studied

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species due to its high level pathogenicity and its significant role in human disease. The organism can affect humans, animals as well as plants, and can thrive under many environmental conditions, such as in soil, water and even hospital environments. *P. aeruginosa* is primarily a nosocomial pathogen. The bacterium is the fourth most commonly isolated nosocomial pathogen accounting for 10.1 percent of all hospital-acquired infections (Todar, 2008). Gene mapping studies of *Pseudomonas aeruginosa* have helped researchers and clinicians better understand local gene expression and the evolution of *P. aeruginosa* as it has adapted to the CF lung. Because of its ability to resist many antibiotics and due to its high level of adaptability to most environments, *P. aeruginosa* genome sequencing proved to be crucial.

2.5.2.3 GENOMIC STRUCTURE

Pseudomonas aeruginosa has the largest genome of the 25 bacteria that scientists have sequenced so far (Cornelis, 2008). The genome of *P. aeruginosa* has an unusually large number of genes for nutrient transport, metabolic regulation and catabolism; this may be why the bacterium has the ability to grow in a wide range of environments and resist antibiotics (Willey *et al.*, 2008). This microbe's genome is a single circular chromosome that is made up of 6,264,403 base pairs (Bp), which is 6.3 million bases (Mb) and contains 5,570 predicted genes on one chromosome. Also, *P. aeruginosa* has metabolic plasmids that are about 75-230 kbp in size and are involved in degrading substances such as sugars (Cornelis, 2008). These plasmids may have genes that code for factors that make the bacteria drug resistant (Todar, 2008).

The large genome sequence of *P. aeruginosa* shows that their low levels of outer membrane permeability impart in them an all-rounded, "intrinsic" drug resistance to many antibiotics and also an efflux system of organic compounds. A multi-drug efflux system has been identified and is probably the strongest in any of the other gram-negative bacteria that have been sequenced. Due to its large genome size, *P. aeruginosa* has tremendous genetic density, allowing it to form biofilms. It also utilizes quorum sensing (group symbiosis) to achieve its resistance against microbial agents in most cases.

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2.5.2.4 CELL STRUCTURE AND METABOLISM

Pseudomonas aeruginosa has a cell wall that is gram-negative as it is composed of three layers; the plasma membrane, a thin peptidoglycan layer, and an outer membrane. Gram-negative bacteria generally have seven different pathways of protein secretions, and three of them are seen in *P. aeruginosa*. These protein secretion pathways include transport proteins that are embedded in lipid membranes, and are involved in the translocation of many different substrates across the membrane (Cornelis, 2008).

Pseudomonas aeruginosa secretes virulence factors that include potent toxins and degradative enzymes. The toxins cause extensive tissue damage and also interfere with the human immune systems defense mechanisms. They also enter and kill host cells at or near the sites of colonization. Moreover, the bacterium's degradative enzymes permanently disrupt the cell membranes and connective tissues in various organs; these enzymes include lipases and proteases (Hachem *et al.*, 2007)

Since *P. aeruginosa* is a chemoheterotroph, it can grow on the least amount of media available, and it only needs a carbon source to build up a biofilm in various types of environments (Willey *et al.,* 2008). Its metabolism is respiratory, as it almost always functions as an obligate aerobe, but it is able to grow in the absence of O_2 if NO_3 is available as a terminal electron acceptor (Todar, 2008).

2.5.2.5 ECOLOGY

Pseudomonas aeruginosa can thrive in a wide range of environmental conditions and the bacteria have developed adaptability to minimal nutritional requirements (Todar, 2008). They are found almost everywhere, including the most unlikely places such as distilled water and many sterile environments such as in hospitals. *P. aeruginosa* has been isolated from very sick individuals who have decreased immune responses, such as patients suffering from AIDS, cancer (undergoing chemotherapy) and especially those suffering from cystic fibrosis. Because *P. aeruginosa* is an opportunistic pathogen, it has a permanent detrimental effect on sick individuals as the bacterium almost always kills its host cells.

2.5.2.6 PATHOLOGY

Pseudomonas aeruginosa can infect animals, plants and also humans. They cause disease immunosuppressed individuals. *Pseudomonas aeruginosa* are opportunistic pathogens and cause infection in patients who are diagnosed with cystic fibrosis, have lower respiratory tract infections, surgical wounds, urinary tract

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infections, skin infections i.e. (dermatitis), and even in patients who have cancer and are undergoing chemotherapy (Willey *et al.*, 2008). It causes nosocomial infection in patients as this bacterium can grow anywhere where enough nutrients and enough moisture are found. An opportunistic pathogen, *P. aeruginosa* produces a thick biofilm and due to its dense colonization, it is able to resist many antibiotics, disinfectants, as well as ultra violet (UV) light and infected patients can therefore be difficult to treat. Another factor that contributes to *P. aeruginosa* resistance is its gram negative cell wall that is composed of three layers; the inner plasma membrane, peptidoglycan, and its outer membrane. This high level of resistance in *P. aeruginosa* can be of consequence and dangerous to a patient's health. Pseudomonas maintains also antibiotic resistance plasmids, R-factors and RTFs, and it is able to transfer these genes by horizontal gene transfer (HGT), mainly by transduction and conjugation (Todar, 2008).

Pseudomonas aeruginosa bacterium is naturally resistant to many antibiotics due to the permeability barrier afforded by its Gram-negative outer membrane. Also, its tendency to colonize surfaces in a biofilm form makes the cells even more resistant to antibiotics. Biofilms form organized and specialized bacterial communities that mediate bacterial attachment to surfaces and provide protection. Biofilms are made of micro colonies that are buried in a dense matrix of exopolysaccharides. The mechanism by which the bacterial colony initiate biofilm formation is yet to be understood (Cornelis, 2008).

One of the major factors that makes *P. aeruginosa* infections difficult to treat is their overproduction of a sugar-like substance, called alginate, an exopolysaccharide. The AlgR protein, which is found to be one factor that regulates alginate production, has recently been shown to be involved with *P. aeruginosa's* pili function (pili mediate attachment in bacteria). Pili are involved in the initial stages of *Pseudomonas aeruginosa* infection of CF lungs. Thus, it is thought that the AlgR protein might be regulating not only genes controlling alginate production, but also other *P. aeruginosa* virulence genes involved in the infection process (Prithiviraj *et al., 2005).*

Furthermore, two extra cellular proteases have been associated with *P. aeruginosa* virulence; elastase and alkaline protease, which cleave collagen and interfere with fibrin formation and lyse fibrin in host cells, respectively. The bacterium produces three other soluble proteins which aid in the invasion/infection

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process; a cytotoxin which is a "pore-forming" protein, and two hemolysins called phospolipase and lecithinase which work synergistically to break down lipids and lecithin. *P. aeruginosa* produces two extra cellular protein toxins called Exotoxin A and Exoenzyme S and it has been suggested that Exoenzyme S protects the bacterium against phagocytic cells, whereas Exotoxin A affects protein synthesis in the host cell and is thought to contribute to the colonization process (Todar, 2008). It is obvious that because *P. aeruginosa* secretes quite a lot of extra cellular proteins, the genes that regulate production of these proteins and various protein-secretion mechanisms may be an important evolutionary adaptation that has led this bacterial species to be so pathogenic and virulent.

As far as symptoms are concerned, cystic fibrosis patients that are infected with *P. aeruginosa* may not show symptoms at first. However, as the infection grows and becomes severe, patients may notice symptoms of infection and inflammation as well as decreased tolerance for exercise and shortness of breath. The inflammation usually leads to blockage of breathing passages and can permanently damage airways in the lungs (Todar, 2008). Some more symptoms include salty-tasting skin, persistent coughing with phlegm, poor growth or even weight gain and also difficulty in bowel movements (Prithiviraj *et al, 2005).*

2.6 MEDICINAL PLANTS IN FISH FEED

Medicinal plants possess therapeutic properties; exert beneficial pharmacological effects on the animal body, widely available in nature and eco-friendly. Some medicinal plants / herbs had been evaluated experimentally in fish by various researchers, some with the use of specific part of the plant at different concentration / inclusion rate. Some of the recent ones were listed in the Table 2.4.

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Table 2.4: Botanical classification of some plants and parts used in fish feed

Scientific name	Common name	Part of the plants used	Concentration/inclusion rate	References
Allium sativum	Garlic	Bulb	0.10, 20, 30, 40g/kg diet	Shalaby *et al.*, 2006
		Bulb	0, 0.5 and 1.0g/100g diet	Nya and Austin, 2009
		Bulb	2,4,6 and 8mg/ml	Muniruzzanian and Chowdhury, 2004
Tetraselmis chuii	Microalgae	Whole	0, 100g/kg diet Synergy (100g/kg T. C + 10^7cfu/g *B. subtillis*)	Cerezuela *et al.*, 2012
Phaeodactylum tricornutum	microalgae	Whole	0. 100g/kg diet Synergy (100g/kg P.T + 10^7cfu/g *B. subtillis*)	Cerezuela *et al.*, 2012
Euglena viridis	Microalgae	Whole	0, 0.1, 0.5 and 1.0g/kg diet	Das *et al.*, 2009
Andrographis paniculata	Nees	Leaves and shoots	0, 500, 1000, 2000 and 3000mg/kg	Prasad and Mukthiraj, 2011
Lonicera japonica	Honey suckle	Leaves	1.0%	Yin *et al.*, 2008
Ganoderma lucidium	lacquered	Leaves	1.0% Synergy (0.5% L. J + 0.5% G. L)	Yin *et al.*, 2008
C. gigentia	akand	Leaf	2, 4, 6 and 8mg/ml	Muniruzzanian and Chowdhury, 2004
C. gigentia + A. Indica	Akand + neem	Leaf	2, 4, 6 and 8mg/ml	Muniruzzanian and Chowdhury, 2004
Curcuma longa	turmeric	Bulb/rhizomes	2, 4, 6 and 8mg/ml	Muniruzzanian and Chowdhury, 2004
Viscum album	mistletoe	Leaf, trunks and fruits	0, 10, 50 and 200mg /kg	Park and Choi, 2012
Ficus benghalensis	-	Root	5g/kg diet	Verma *et al.*, 2012

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2.6.1 EFFICACY OF MEDICINAL PLANTS ACTING AGAINST FISH DISEASES

Various plants and herbs have found their use in aquaculture and most are found in the tropics, one of such is *Eclipta alba* (false daisy, bhringaraja) which belongs to the family Compositae. It is a bitter/sweet herb that has tonic effect on the circulatory, nervous and digestive systems. Christybapita *et al* (2007) observed the immunostimulatory effect of aqueous extract (AqE) of *Eclipta alba* leaf (oral administration as feed supplement) in tilapia (*Oreochromis mossambicus).* It was noted that *E. alba* extract enhanced non-specific immune responses and disease resistance of *O. mossambicus* against *Aeromonas hydrophila* infection.

Ginger (Zingiber *officinale*) belongs to the family of Zingiberaceae, it is a sweet pungent, aromatic, warming herb and was also found (Yin *et al*., 2008) when administered orally to increase the phagocytic capability of cells in rainbow trout (fish), while the extracts of 4 Chinese herbs (*Rheum officinale, Andrographis paniculata, Isatis indigotica* and *Lonicera japonica*) increased the phagocytosis of white blood cells of Carp, *Cyprinus carpio*. The immunostimulant effects of the dietary intake of 3 plants (*Viscum album, Urtica dioica* and *Zingiber officinale*) extracts on rainbow trout (*Oncorhynchus mykiss*) have been reported (Dügenci *et al.,* 2003)

Azadirachta indica (Neem) tree which belong to the family Meliaceae is a bitter, tonic herb that acts as antipyretic and anti-inflammatory agent (Deni, 1996) The leaves of this plant had been shown to contain nimbin, azadirachtin and meliantroil which possess insecticidal and antiviral properties (Chitmanat *et al.,* 2005). The insecticidal and antibacterial effects have been explored in aquaculture (Biswas *et al*., 2002, Winkaler *et al.,* 2007, Abdul Kader and Haniffa, 2011) with very little information on the use of its antiviral properties in fish. Ravikumar *et al.*, (2011) reported that among 15 coastal medicinal plants/ parts of plants, *A. indica, Cinnamomum verum* and *Eupatorium odoratum* exhibited excellent antibacterial activity against 10 bacterial pathogens from diseased ornamental fishes. The antimicrobial activity of Aqeuous extract of 3 medicinal plants: *A. indica* (leaf), *Solanum torvum* (Sundakai fruit coat) and *Curcuma longa* (rhizome) against the *in vitro* growth of *A. hydrophila*, isolated from infected fresh-water fish, *Channa striatus* (Abdul Kader and Haniffa, 2011). Kraus (1995) found that the extract of neem fruit,

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seeds, seed kernel, twigs, stem bark and root have fungicidal and bactericidal properties.

Other plant that has been extensively studied include Garlic (*Allium sativum*) which belongs to the family of Liliaceae. A pungent, warming herb has been reported to inhibit bacterial growth, lowers fever, reduced blood pressure, cholesterol and blood sugar levels. It has been used as rejuvenatives, detoxicant and aphrodisiac in ayurvedic medicine (Deni, 1996). Studies abound on its medicinal and culinary purposes (Rahman *et al*., 2009). The bulbs which contained an acrid volatile oil (0.25%), propyl disulphide which is a powerful germicide (Anawer, 2001). Garlic or onion has been mixed to the shrimp pellet and fed every day to protect the bacterial infection (Direkbusarakom, 1992). Externally garlic (*A. sativum*) is used as disinfectant and it is applied to indolent tumors, ulcerated surface and wounds (Dastur, 1977). Chowdhury *et al*., (1991) reported that extract obtained from garlic was also highly effective against two tested bacteria, *A. hydrophilla* and *P. fluorescens* (MIC 0.6mg/ml). Garlic, Turmeric, Akand and Neem could be used as an alternative therapeutic measure against bacterial infection of fish (Rahman, 2005).

Some investigations revealed the growth promoting, antibacterial effect of garlic and enhancement of blood parameters, erythrocytes counts (RBC) and haemoglobin (Hb) content in fish fed on diets containing *A. sativum* were higher than the control while the total plasma protein content was significantly higher in fish fed on diets containing *A. Sativum* (Shalaby *et al*., 2006). The liver enzymes: Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) decreased significantly with increasing levels of *A. sativum*. The combination containing *A. sativum*, *A. indica* and *Curcuma longa* (turmeric) in spawn had been shown to resist disease in *Catla catla* fish while the combination of Indian almond (*Terminalia catappa*) and garlic (*Allium sativum*) have been useful as an ectoparasiticidal alternatives in fish especially *Trichodina* sp. infections in tilapia (*O. niloticus*) fingerlings.

On the effect of medicinal plants on the oxidative stress parameters, various medicinal plants had been reported to increase the levels of superoxide anion production, lysozome, serum bactericidal activity, serum protein and albumin ($P< 0.05$) when challenged with *Aeromonas hydrophila* (Das *et al*., 2009). Herbal mixture containing (*A. koreanum, G. Liralensis* and *P. Ginseng*) was found also found to enhance the total protein, glucose, phagocytic, respiratory burst activities,

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complement and lysozome activities significantly in infected fish fed with supplementation diet from week 4 to 12. This suggested that the herbal mixture supplemented diet enhanced growth, blood chemical constituents, and non- specific immunity in *Olive flounder* against *Streptococcus parauberis* (Harikrishnan *et al*., 2011). However the possible role in disease resistance has not been accorded due attention in fish.

Another very important plant of interest is *Aloe vera* which belongs to the familly Liliaceae/ Aloeaceae. It is an intensely bitter, purgative herb that have shown prospect as an antifungal, anti- inflammatory, healing promoting agent. It also has antihelmintic property. The growth promoting effect and disease resistant property was proven in gold fish *Carassius auratus* when experimentally challenged by *A. hydrophila* (Ahilan *et al*., 2010). Harikrishnan *et al*., (2010) reported that mixed herbal extracts supplemented diets restored the altered haematological parameters and triggered the innate immune system of goldfish (*C. auratus*) against *A. hydrophila* infection. This synergistic effect of herbs has been reported in other fishes, including Japanese flounder and *Clarias gariepinus* (Ahilan *et al*., 2010). *A. vera* has also been used as a disease suppressing agent and showed antibacterial effect in juvenile rock fish. The growth increase in *Labeo rohita* fish fed with herbal supplemented diet was due to improved food utilization and high protein synthesis. The benefit of herbal growth promoters as an additive in the carp feed has also been the focus of many researchers.

Withania somnifera (winter cherry) is bitter – sweet, astringent, warming herb with a horse- like smell, it acts mainly on the reproductive and nervous systems. It has sedative, rejuvenative and aprodisiac effects. Sharma *et al.,* 2010 observed the stimulatory effect of dietary doses of *Withania somnifera* (Ashwagandha) root on immunity and disease resistance against *A. hydrophila* infection in Indian major carp, *L. rohita* fingerlings. Kolkovski and Kolkovski (2011) also reported that some herbal extracts are very effective against gill and skin fukes like *Benedenia seriolae*. Nargis *et al.,* (2011) reported the immunostimulant effects of the dietary intake of *A. sativum* and *Vitex negundo* extracts on fingerlings of *L. rohita* fish.

Viscum album (mistletoe) belongs to the family Viscaceae. It is a pungent, bitter- sweet warming herb that lowers blood pressure, stimulates the immune system, slows heart beat, relaxes sperms and has sedative, diuretic anti- cancer effects (Deni, 1996). The study of Park and Choi, (2012) showed that the dietary supplemented

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mistletoe extract appeared to significantly elicit non-specific immune responses in terms of the respiratory burst activity, lysozyme activity, phagocytic activity, and ACH50 activity in tilapia. 50 mg mistletoe-treated group showed the highest survival rate compared to control and the challenged test suggests decreased mortality rate following experimental infection with *A. Hydrophila.* The dietary supplementation of an appropriate concentration of mistletoe extract had an enhancing effect on disease resistance of tilapia. Medicinal plants: ginger, nettle and mistletoe act as adjuvant therapy in rainbow trout through enhanced phagocytosis, cellular and humoral defense mechanisms against pathogens. The traditional Chinese medicines in yellow croaker elevated the non specific defense mechanism and increased the disease resistance of fish against bacterial pathogens.

Achyranthes aspera was studied by Rao *et al*. (2006) who reported that *Achyranthes aspera* in the diet increased the non-specific immunity and significantly decreased mortality when *L. rohita* were experimentally infected with *Aeromonas hydrophila*, a bacterial pathogen. Achyranthes can be used as a prophylactic to reduce mortalities associated with disease. Besides stimulating immunity, Achyranthes also enhanced the growth rate of the fish as well as increased serum protein, lysozyme, total serum protein and A/G ratio which are good indicators of health status. Results indicate that Achyranthes improves phagocytosis and killing activity by neutrophils and macrophages. It also enhances superoxide anion when fish fed with Achyranthes incorporated diets.

Some other plants that look promising in aquaculture include *Lonicera japonica* which belongs to the family Caprifoliaceae. It has been reported to have anti- bacterial, diuretic, antipyretic and anti inflammatory properties. It has been shown to also relax spasms (Lee *et al.,* 1998). It has been reported that *Lonicera* significantly increased blood neutrophil activity and promoted phagocytosis by the neutrophils in bovine at the correct concentration (Hunn *et. al*., 1992). Also *Ganoderma lucidum* has anti- allergenic, anti – viral and anti- bacterial effects (Deni, 1996). It has been found to promote phagocytosis and stimulate proliferation of lymphocytes (Wang *et al.,* 1997).

The combination of the extracts of two Chinese herbs (*Lonicera japonica* and *Ganoderma lucidum*) in diets of tilapia fish, (*Oreochromis niloticus*) acts as immunostimulants and appears to improve the immune status and disease resistance. When used alone or in combination, increased the survival of fish after challenge with

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Aeromonas hydrophila (Yin *et al.,* 2008). The results of the experiment conducted showed that both *Ganoderma* and *Lonicera* were able to enhance phagocytosis and stimulate lysozyme activity after two weeks, but not respiratory burst activity of phagocytic blood cells, total protein or total immunoglobulin in plasma.

Datura metel (Thorn apple) belongs to the family Solanaceae. It was studied by Ravikumar *et al.* (2010) who observed that the chloroform extract of *Datura metel* plant had wide range of antimicrobial activities against many fish pathogens. *D. metel* could be used as a putative antimicrobial drug in the aquaculture maintenance. The chloroform extract of *D. metel* could be effectively used as a potential antimicrobial agent to overcome the problem of mass mortality of ornamental fish in aquarium. Turker *et al*., (2009) also reported that the alcoholic and aqueous extracts of *Nuphar lutea, Nymphaea alba, Stachys annua, Genista lydia, Vinca minor, Fragaria vesca, Filipendula ulmaria* and *Helichrysum plicatum* had antibacterial activities against *A. hydrophila*, *Yersinia ruckeri*, *Lactococcus garvieae*, Str. *agalactae* and *Enterococcus faecalis* bacteria isolated from fish. This observation provides the aquaculturists with a promising management tool with the use of medicinal plants for control or treatment of fish diseases especially in resource poor settings.

Apart from the antimicrobial properties of medicinal plants, some had been reported to have some immunomodulating, toxic effects and haematological changes in fish, these properties have not being fully elucidated for walnut leaf and onion bulb.

2.7 IMMUNE SYSTEM

Fish is a heterogeneous group of organisms that include the agnathans (lampreys and myxines), condryctians (sharks and rays) and teleosts (bony fish) (Nelson, 1994). As in all vertebrates, fish have cellular and humoral immune responses, and central organs which function in immune defence. Fish and mammals show some similarities and some differences regarding immune function (Table 2.5).

Immune organs vary by type of fish (Zapata *et al.*, 1996) in the jawless fish (lampreys and hagfishes), true lymphoid organs are absent. These fish rely on regions of lymphoid tissue within other organs to produce immune cells. For example, erythrocytes, macrophages and plasma cells are produced in the anterior kidney (or pronephros) and some areas of the gut (where granulocytes mature.) They resemble primitive bone marrow in hagfish. Cartilaginous fish (sharks and rays) have a more

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advanced immune system. They have three specialized organs that are unique to chondrichthyes; the epigonal organs (lymphoid tissue similar to mammalian bone) that surround the gonads, the Leydig's organ within the walls of their esophagus, and a spiral valve in their intestine.

These organs house typical immune cells (granulocytes, lymphocytes and plasma cells). They also possess an identifiable thymus and a well-developed spleen (their most important immune organ) where various lymphocytes, plasma cells and macrophages develop and are stored. Chondrostean fish (sturgeons, paddlefish and bichirs) possess a major site for the production of granulocytes within a mass that is associated with the meninges (membranes surrounding the central nervous system.) Their heart is frequently covered with tissue that contains lymphocytes, reticular cells and a small number of macrophages. The chondrostean kidney is an important hemopoietic organ; where erythrocytes, granulocytes, lymphocytes and macrophages develop. The major immune tissues of bony fish (or teleostei) include the kidney (especially the anterior kidney), which houses many different immune cells (Anderson, 1977).

In addition, teleost fish possess a thymus, spleen and scattered immune are as within mucosal tissues (e.g. in the skin, gills, gut and gonads). Much like the mammalian immune system, teleost erythrocytes, neutrophils and granulocytes are believed to reside in the spleen whereas lymphocytes are the major cell type found in the thymus(Chilmonczyk, 1992), Hansen and Zapata, (1998).

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Table 2.5: Relevant immune differences between jawed fishes and mammals

	Jawed Fishes	Mammals
Biotic constrictions		
Temperature range	-2 to 35^{0}C	36.5 to 37.5^{0}C
Primary environment	Water	Air
Metabolism	Poikilothermia Endothermia (e.g. bluefin,tuna and some pelagic fishes)	Homeothermia
External interfaces	Mucous skin, gills	Respiratory tree
Humoral diversity		
Ig isotypes	IgM, IgD?(Teleostei) IgM, IgX/IgR, IgW, NAR (C) (Chondrichthyes) IgM redox forms	IgM, IgA, IgD, IgE, IgG
Ig gene rearrangement	Multiduster (Chondrichthyes and Teleostei)	Translocon
Non –specific diversity	Several C3 isoforms (Teleostei)	No C3 isoforms
Overall performance		
Antibody affinity	Low	High
Antibody response	Slow	Fast
Memory response	Weak	Strong
Affinity maturation	Low or absent	High
Low temperature	High dependence ,immunosuppressive response (only in poikilothemic fish)	Low dependence
Lymphoid organs		
Haematopoietic tissue	Head kidney (Teleostei) Epigonal and Leydig organs, meningeal tissue, Orbital and sub-cranial hematopoietic tissue (Chondrichthyes)	Bone marrow
Thymus	Involution species – dependent, influenced by seasonal changes and hormonal cycles	Involution with age
Lymphoid nodes	Absent	Present
Gut-associated lymphoid tissues	Not organized, lymphoid aggregates Leydig organ and spiral valve (Chondrichthyes)	Organized, Peyer patches
Geminal centres	Absent (melanomagrophage centre?), dendritic cells probably present	Present

Source: Tort *et al*., 2003

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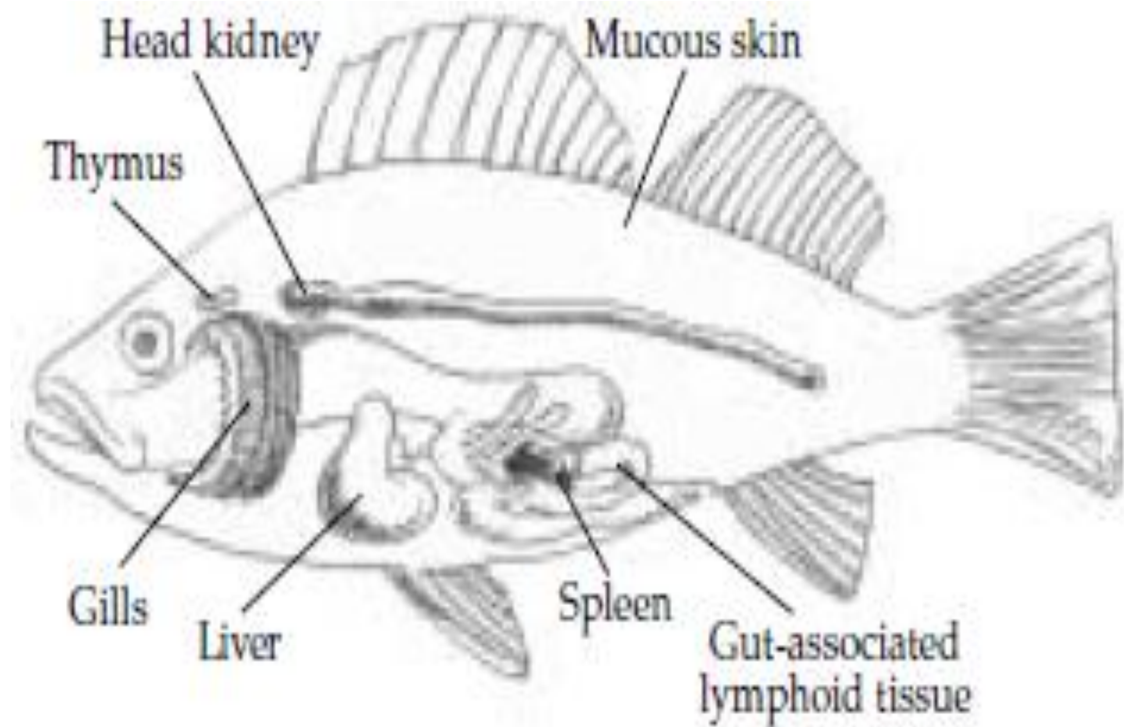

Fig. 2.1: Immune structures in teleost fish (mag x10)

Source: Tort *et al*., 2003

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2.8 HISTOPATHOLOGICAL CHANGES IN FISH

Histopathological changes have been widely used as a means in the evaluation of the health of fish exposed to contaminants, both in the laboratory (Wester and Canton, 1991; Thophon *et al.,* 2003) and field studies (Hinton *et al.,* 1992; Schwaiger *et al.,* 1997; Teh *et al.,* 1997). One of the great advantages of using histopathological changes in environmental monitoring is that this category of changes allows examining specific target organs, including gills, kidney and liver, that are responsible for vital functions, such as respiration, excretion, the accumulation and biotransformation of xenobiotics in the fish (Gernhofer *et al.,* 2001).

Furthermore, the alterations found in these organs are normally easier to identify than functional ones (Fanta *et al.,* 2003), and serve as warning signs of damage to animal health (Hinton and Laurén, 1990). Apart from the tissue pathology, haematological changes in live fish can also be a good health indicator.

2.9 HAEMATOLOGY OF FISH

This is widely used in clinical diagnosis in aquatic and terrestrial animals. The application of haematological techniques is therefore valuable in fish biology in the assessment of fish health and stress response (Olukunle, 1996). Haematological examination is an essential tool for the diagnosis of infection in animals. Decrease in haemoglobin content and haematocrit values are said to cause reduction in fish activity (Adeyemo, 2005). The decrease in both haemoglobin and haematocrit are possibly the consequence of anaemia and haemodilution caused by massive erythrocytosis.

Svobodova *et al.*, (1991) recommended that ichthyo – haematology would be useful in the assessment of feeds and feed mixture, evaluation of fish condition and determination of toxic effect of substances as well as diseases. A study on total and differential leucocytes counts has equally suggested an indication of stress in fish (Blaxhell and Daisely, 1973). Increase in the number and distribution of small lymphocytes have been recorded during stress condition in fish (Adeyemo, 2005).

2.9.1 Erythrocytes

The main function of the erythrocyte is to carry haemoglobin – the respiratory pigment. The whole of mass red blood cell plus their precursors (erythropoitic) in the bone marrow is known as the erythron. Qualitative and quantitative changes in the

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erythron may occur as a sign of or consequence of certain disease processes. Evaluation of erythrocyte number and morphology, haemoglobin concentration and haematocrit values are important in speculating on the nature of disease and impact on the affected animals (Ihedioha and Chineme, 2004). A greater than the normal number of erythrocyte circulation is termed polycythemia while a decrease below the normal in the erythrocyte or haemoglobin concentration is referred to as anaemia (Ihedioha and Chineme, 2004). Red blood cells carry oxygen to and remove waste products from the body's tissues. These cells also contain hemoglobin. Red blood cells are measured in millions per cubic millimeter (mil/uL) of blood (National Institutes of Health Clinical Centre, 2008).

2.9.2 Haemoglobin (Hb)

This is known as respiratory pigment, is a conjugated protein consisting of heme (protoporphyrin plus ferrous iron) and globin. It functions as a carrier of oxygen and carbon dioxide in circulating blood. The main function of erythrocyte is to serve as a carrier of haemoglobin, and haemoglobin constitutes about 30 – 36% of erythrocytes (Ihedioha and Chineme, 2004). Free haemoglobin in the blood is quickly disposed of by oxidation to forms that are lost through the kidney, destroyed by the reticuloendothelial system or degraded by the liver mainly and to a lesser extent the spleen and bone marrow (Ihedioha and Chineme, 2004). Haemoglobin gives red blood cells their colour. Haemoglobin carries oxygen from the lungs to the tissues and takes carbon dioxide (the waste products) from the tissues to the lungs. From the lungs, carbon dioxide is exhaled. Haemoglobin is measured in grams per decilitre (g/dL) of blood (NIHCC, 2008)

2.9.3 White Blood Cells (WBC)

These cells are the mobile units of the body's infection-fighting system. White blood cells travel in the bloodstream to areas of infection and destroy the responsible bacteria. However, the WBC lab value is not meaningful unless the "differential" is also known. The differential measures each of the five types of white blood cells:

Neutrophils/ Heterophils (polymorphonuclear and bands)

Basophils

Eosinophils

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Lymphocytes

Monocytes

The differential is usually based on 100 cells counted in a laboratory sample. They are measured in thousands per cubic millilitre (K/uL) of blood (NIHCC, 2008)

2.9.3.1 Neutrophils / Heterophils

Neutrophils/ Heterophils are the most numerous white blood cells. They make up about 56 percent of white blood cells. Neutrophils/ heterophils are the "soldiers" that fight infections. They phygocytose infectious particles (bacteria) in body. Polymorphonuclear are mature neutrophils. Bands are young polys, which also fight infections. The absolute neutrophils count (ANC), also called absolute granulocyte count (AGC) are the measure of the number of infection-fighting white blood cells in the blood (NIHCC, 2008)

2.9.3.2 Eosinophils

Eosinophil serves as detoxifier, inactivate histamine or histamine like toxic materials. They also play an important role in inflammation as they inhibit oedema – inducing properties of serotonin and bradykinin. They possess phagocytic abilities to certain limits, they phagocytize antigen – antibody complexes, certain bacteria and fungi, antibody - coated erythrocyte and inert properties (Ihedioha and Chineme, 2004). A decrease below the normal range or even complete disappearance of eosinophil from the circulating blood is known as eosinopaenia while eosinophilia is an increase beyond normal in the number of circulating eosinophils in the peripheral blood. Eosinophil is seen on condition of stress (Ihedioha and Chineme, 2004).

2.9.3.3 Lymphocytes

These function mainly immunological activities on exposure to an antigen, lymphocytes proliferate and differentiate into thymus – dependent cell (T lymphocytes) and bursa – dependent cell (B lymphocytes). The T – lymphocytes are responsible for antigen recognition and serve the cell – mediated responses of the animals while the B – lymphocytes are precursors for antibody – producing cells (Ihedioha and Chineme, 2004). Lymphocytosis is an increase in the absolute numbers of circulating lymphocyte above the normal range for the species and lymphopaenia is a decrease below the normal range for the species in the absolute lymphocyte count in the blood (Ihedioha and Chineme, 2004).

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2.9.3.4 Monocytes

They are capable of developing into macrophages. They function mainly in phagocytosis of larger particles such as fungi and protozoa. They have the capability of ingesting and removing large particles of cellular debris that may accumulate in tissue. They also play an important role in processing of antigens (Ihedioha and Chineme, 2004).

2.9.3.4 Platelet count

Platelets help to stop bleeding by forming blood clots. They are measured in thousands per cubic millimeter (m/uL) of blood (NIHCC, 2008). They are often exhausted in exhaustive coagulipathy.

2.10 Plasma

Plasma is a fluid of blood left after removal of the cellular elements. Serum is the fluid which is obtain after blood has been allowed to clot and the clot removed. Serum and plasma differ only in their content of fibrinogen and several minor components which are removing in the process. Plasma is composed of over 90% water, contains a mixture of protein which include albumin, globulin and fibrinogen. Albumin forms the main bulk of the plasma proteins and is of considerable importance in maintaining osmotic homeostasis, as it prevents the accumulation of excess fluid in the body tissue (Oyewale, 2011).

Globulins are subdivided into α_1, α_2, β and δ- globulin fractions. The δ-globulin fraction contains the antibodies. Many substances circulating in the blood (e.g. hormones, vitamins, electrolytes, metabolites etc) are partially or wholly bound to albumin or globulin fraction while fibrinogen participates in the blood clotting mediation (Oyewale, 2011). There are many other classes of compound circulating in blood plasma. Most of these are smaller molecules which diffuse freely through cell membranes and are therefore more similarly distributed throughout all the fluids of the body. They primarily regulate the osmotic pressure of plasma and contribute also to the control of pH.

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CHAPTER THREE

3.0 RESEARCH METHODOLOGY

3.1 PLANT COLLECTION AND IDENTIFICATION

Onion bulbs were purchased from Bodija market in Ibadan, Nigeria. Walnut leaf was obtained from a farm at Oka -Akoko, Ondo State, Nigeria. They were identified at the herbarium of the Forestry Research Institute of Nigeria (FRIN), Ibadan, where a voucher specimen was deposited under FHI 107515.

3.2 PREPARATION AND EXTRACTION OF PLANT MATERIALS

3.2.1 Onion extraction

The onions bulbs were washed with distilled water and allowed to air dry at room temperature for one hour. The dry outer coverings of the onions were manually peeled off and washed. 200g of the fresh onion bulbs were blended into fine slurry 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^oC) until required (Azu and Onyeagba, 2007).

3.2.2 Walnut leaf extraction

Walnut leaves obtained were air – dried in a room for 3 weeks. The walnut leaves (200g) were extracted by maceration at room temperature (30^0C) in 100ml aqueous methanol (20:80) for 72 hours. After removal of solvents, yields of extracts were obtained and the extracts stored in the refrigerator until required (Ajaiyeoba and Fadare, 2006).

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3.3 MEDIA PREPARATION

All media used were prepared according to manufacturer's instruction as follows:

A. **MacConkey agar:** This agar was prepared by suspending 52g in 1 litre of distilled water. It was brought to boil to dissolved completely then, sterilized by autoclaving at 121^0C for 15 minutes.

B. **Nutrient agar:** This agar was prepared by suspending 28g in 1 litre of distilled water and then sterilized by autoclaving at 121^0C for 15 minutes.

C. **Mueller Hinton agar:** This agar was prepared by suspending 36g in 1 litre of distilled water and then sterilized by autoclaving at 121^0C for 15 minutes.

D. **Nutrient broth:** This broth was prepared by suspending 25g in 1 litre of distilled water and then sterilized by autoclaving at 121^0C for 15 minutes.

E. **Peptone water:** This was prepared by suspending 15g in 1 litre of distilled water and then sterilized by autoclaving at 121^0C for 15 minutes.

All these media were allowed to cool after sterilization to about 45^0C before pouring into Petri dishes.

3.4 ISOLATION OF MICROORGANISM/COUNTS

One gramme (1g) of each of the gills, skin, intestine and liver samples of the African catfish, *Clarias gariepinus* were aseptically separated, macerated and put into sterile capped test tube containing sterilized peptone water and homogenized (Shalaby *et al.*, 2006). Serial dilution was carried out and 1ml each from 10^{-1} to 10^{-6} dilution factors were dispensed into Petri dishes that were appropriately labelled and molten sterile medium was poured aseptically into each Petri dish. The plates were swirled gently for even distribution of inocula and allowed to set /gel and then incubated at 37^0C for 24-48 hours. The organisms grew into visible different colonies after 24 hours. Total viable count and enterobacteriacea were determined, the results were expressed in cfu/g.

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3.5 ANTIMICROBIAL ASSAY

Pseudomonas aeruginosa, *Escherichia coli, Pseudomonas fluorescens, Staphyloccocus aureus, Salmonella typhi, Bacillus subtilis* and *Aspergillus niger* were 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.

3.6 DETECTION OF ANTAGONISTIC ACTIVITY

A well diffusion assay as described by Schillinger and Lucke, (1989) was used. Pre- poured indicator {pathogen (4 mm depth)} was overlaid with a 10ml soft agar (0.7%) lawn of indicator culture (thus generating a potential mat for the indicator bacterium). The indicator lawn was prepared by adding 250 µl of a 10^{-1} dilution from an overnight culture to 10ml of indicator organism soft agar. Wells of 5mm diameter were cut into these agar plates by using cork borer and 100µl of walnut leaf or onion bulb extract was placed into each well (Takashiro *et al.*, 1991). The plates were incubated aerobically at 37^0C for 24 hours. The plates were examined for zones of inhibition which were scored positive, if the width of the clear zone was 5mm or longer. The diameter of the inhibition zones was taken to be proportional to the logarithm of the antimicrobial compounds in walnut leaf and onion bulb (Maria *et al.*, 1994).

3.7 MINIMUM INHIBITORY CONCENTRATION OF WALNUT LEAVES AND ONION BULB

Doubling dilution of 2000 µg/ml of walnut leaves and onion bulb extract were made in 5ml volume of broth to 3. 912 µg/ml. One row of the test was inoculated with 0.02 ml of 1 in 100 dilution of the overnight broth culture of the test organism (Stokes and Ridgeway, 1980). The test was incubated at 37^0C for 24 hours aerobically. The minimum inhibitory concentration was the lowest concentration that prevented the growth of bacteria after 24 hours incubation (Osoba, 1979)

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3.8 EXPERIMENTAL SYSTEM AND DESIGN

The feeding trial was carried out in twenty seven plastic experimental tanks (50x34x27cm) for 18 weeks (12 weeks for feeding trials, 4 weeks for challenge test and 2 weeks for wound healing experiment) in the Fisheries Laboratory and Research Farm of the University of Ibadan, Ibadan. The water level in each tank was maintained at 35 litres throughout the experimental period. Water in each tank was replaced every three (3) days throughout the period of the experiment to maintain relatively uniform physiochemical parameters and also to prevent fouling that may resulting from feed residues. The source of water was from University of Ibadan (U.I) water station and each experimental tank was well aerated using air stone and aerator pumps (Cosmos aquarium air pump, double type 3500 50 Hz, 2.5 – 3 W) as described by Lawson, (1995). The dissolved oxygen content of the experimental tanks was monitored using dissolved oxygen metre (Jenway 3015pH metre, 0.01 accuracy, Genway, Staffordshire, UK). The water temperature of the experimental tanks was monitored by mercury-in-glass thermometer (producer Paragon Scientific Ltd, Birkenhead, Wirral, UK). While the pH value will be measured by using pH metre (Jenway 3015pH metre, 0.01 accuracy, Genway, Staffordshire, UK) after standardizing the metre. The experimental design for this research work is factorial experiment in completely randomized design (CRD).

3.9 FEED INGREDIENTS AND DIET FORMULATION

Fish feed ingredients were analyzed for proximate composition before the formulation (Table 3.1). The value obtained was used to formulate 40% crude protein diets using Pearson's square method to determine individual ingredient contribution at 40% crude protein per g / 100g diet. Each ingredient was weighed using sensitive weighing balance (OHAUSLS model 2000) and dry ingredients were mixed thoroughly in a mixer (ASEFAC, model 1989). Onion bulb and walnut leaves were added as feed additive in the study as a partial replacement for vitamin – mineral premix. Each diet mixture was treated separately. Water was added and the resulting dough was pelleted through a 1/4mm die mincer of Hobart A-200T pelleting machine (Hobart GmbH, Rben-Bosch, Offenbug, Germany) to form a noodle like strand which was mechanically broken into suitable sizes for the *Clarias gariepinus* juveniles. The pelleted diets were sun dried and store in airtight containers at room temperature until required (Table 3.2).

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Table 3.1: Showing fish feed ingredients and their crude protein.

Ingredient	Crude protein (%)
Fish meal	72
Soybean	42
Maize	10
Onion bulb	08
Walnut leaf	15.10
Starch	-
Vegetable oil	-
Vit – min premix	-
Salt	-
Di- calcium phosphate	-
Chromium oxide	-

Note:

Vit –min premix = vitamin – mineral premix

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Table 3.2: Gross ingredient composition (g/ 100g diet) of onion bulb and walnut leaf diets in partial substitution for vit – min premix at different inclusion levels for *C. gariepinus*

INGREDIENTS	Control	OB2	OB3	OB4	OB5	WL6	WL7	WL8	WL9
Fishmeal	21.25	21.25	21.25	21.25	21.25	21.25	21.25	21.25	21.25
Soybean	42.49	42.49	42.49	42.49	42.49	42.49	42.49	42.49	42.49
Maize	28.26	28.26	28.26	28.26	28.26	28.26	28.26	28.26	28.26
Vit-min*	2.00	1.50	1.00	0.50	-	1.50	1.00	0.50	-
Starch	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Vegetable oil	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Chromium oxide	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
DCP	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Salt	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Onion	-	0.50	1.00	1.50	2.00	-	-	-	-
Walnut leaves	-	-	-	-	-	0.50	1.00	1.50	2.00
TOTAL	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Note:

DCP = Di- calcium phosphate, Vit –min = vitamin – mineral premix

* vit-min premix (vitamin and minerals premix) each 2.5kg of premix contains; vitamin A, 12.5 million international unit (MIU); D3,2.5 MIU; E, 40g; K3, 2g; B1, 3g; B2, 5.5g; B6, 5g; B12, 0.25g; Niacin 55g; Calcium pantothenate 11.5g; Choline chloride, 500g; folic acid, 1g; Biolin, 0.08g; Manganese,120g; Iron,100g; Zinc,80g; Copper, 8.5g ; Iodine,1.5g ; Cobalt, 0.3g ; Selenium, 0.12g ; Anti- oxidant,120g.

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3.10 EXPERIMENTAL PROCEDURE AND FEEDING TRIALS

Each treatment had three replicates, 20 fish (7.39± 0.02g) per replicate was selected from 700 juveniles. Weighed and distributed in experimental tanks. The fish were acclimated for 2 weeks in glass aquaria before the experiment. The experiment lasted for 18 weeks during which the fish was fed at 3% body weight daily; 1.5% given in the morning by 8.00 – 9.00 am and 1.5% in the evening by 5.00 pm. Measurement of the weight changes was performed forthnightly and the feeding rate adjusted forthnightly according to the new body weight.

3.11 BIOLOGICAL EVALUATION AND ANALYTICAL METHODS

3.11.1 Biological evaluation

Weight gain = final body weight - initial body weight

$$\text{Weight gain (\%)} = \frac{100\,(\text{final body weight - initial body weight})}{\text{Initial body weight}}$$

Increase in standard length (CM) = $L_2 - L_1$

Where: L_2 = Final standard length

L_1 = Initial standard length

$$\text{Specific growth rate (SGR)} = \frac{100\,(\log_e \text{final body weight} - \log_e \text{initial body weight})}{\text{Time (days)}}$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Dry weight of feed fed (g)}}{\text{Fish weight gain (g)}}$$

$$\text{Protein efficiency ratio (PER)} = \frac{\text{Wet body weight gain (g)}}{\text{Crude protein fed}}$$

$$\text{Protein productive value (PPV)} = \frac{(\text{Final fish body protein - initial body protein})}{\text{Crude protein intake}} \times 100$$

$$\text{Survival rate (\%)} = \frac{\text{Initial Number of Fish Stocked} - \text{Mortality}}{\text{Initial number of fish stocked}} \times 100$$

Condition factor (K) = $100W/L^3$

Where: W =Weight of fish (g)

L = Standard length (cm)

$$\text{Protein intake: } \frac{\text{Feed intake x percentage protein in diet}}{100}$$

$$\text{Nitrogen metabolism} = \frac{(0.549)\,(a + b)\,h}{2}$$

Where, a = initial mean weight of fish

b= final mean weight of fish

h = experimental periods in days (Nwanna, 2003)

3.11.2 ANALYTICAL METHODS

This was carried out in Livestock Research Laboratory of the Institute of Agriculture Research and Training (IAR&T) Moor Plantation, Ibadan. Feed ingredients, experimental diets and fish carcass were analyzed for proximate composition before and after the experiment using the methods of A.O.A.C, (2005).

3.11.2.1 Crude protein

Crude protein of the samples was determined using microkjeldahl distillation method of the A.O.A.C (1990). The percentage protein was calculated by multiplying the nitrogen content of the sample by a factor of 6.25.

3.11.2.2 Crude fibre

This was determined by subjecting the residual sample from ether extraction (lipids) to a successive treatment with boiling acid (0.25N sulphuric acid) and alkali of defined concentration (0.313N sodium hydroxide) under controlled condition.

3.11.2.3 Ash content

The ash content of the oven-dried sample was determined by burning each of the samples in the muffle furnace at 550^{0}C for three hours, these samples was allowed to cool and weighed as percentage ash content.

3.11.2.4 Lipid content

The lipid content was determined by using the soxhlet method of extraction using petroleum ether (40-60^{0}C) for three hours, the ether extract (lipids) is the residue obtained after evaporating the solvent.

3.11.2.5 Nitrogen free extract

It was obtained as a difference between hundred and the sum of percentages of ash, lipid, crude protein and crude fibre.

3.12 Organ index

The fish were killed by rapid cervical chopping, and the fish were weighed (after killing) at the end of experiment. The liver, kidney intestine and spleen were removed and weighed. Moreover, the hepatosomatic and splenosomatic indices were calculated according to Fox *et al.,* (1997)

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Organ-somatic index = [organ weight (g)/body weight (g)] $\times$100.

3.13 MICROBIOLOGICAL ANALYSIS

Water samples from the aquaria were collected monthly in sterile glass bottles. Peptone water (0.1%) was used for serial dilution; 1ml of water sample was added to 9ml sterile peptone water to 10^{-1} and then serially diluted to 10^{-4}. Each diluent (1ml) was poured in two Petri dishes; one received plate count agar for total bacterial count using the pure plate count method according to the standard methods for the examination of water and wastewater (APHA, 1985), the second Petri dish received MacConky agar for total coliforms count according to Hitchins *et al.,* (1995). Petri dishes were gently tapped on the sides for a few times. Petri dish of total coliforms count and that of the dishes of total bacterial count were incubated at 37^0C for 24h.

Fish samples (skin, liver, gill and intestine) were collected monthly during the experimental period for bacteriological examination with through asepsis (medical examinations or procedures that prevent contamination or infection of microbes). 1g of fish sample was macerated in 9ml sterile peptone water in the mortar. 1ml of the suspension was diluted by peptone water to 10^{-4}. Each diluent (1ml) was poured in two Petri dishes; one received plate count agar and the other received MacConky agar (APHA, 1985). The incubation period was 24h at 37^0C. After incubation of water and fish sample dishes the colonies were counted using colony counter.

3.14 CHALLENGE TEST

At the end of the feeding experiment, 270 *Clarias gariepinus* (30 from each treatment) were induced by intraperitoneal route with 0.5ml of 10^7 *Pseudomonas aeruginosa* of 24h living. The induced fish were kept under observation for 30 days. The mortalities were recorded and the level of protection (LP) among the challenged fish was determined as described by Salah *et al.*, (2008), Azza and Abd-El-Rhman (2009)

LP= 1− [percentage of mortality in treated group] / x 100
[percentage of mortality in control group]

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3.15 WOUND HEALING EXPERIMENT

The experiment was conducted at the Department of Wildlife and Fisheries Teaching and Research Farm, University of Ibadan. Fifty four (54) adults' male and female *Clarias gariepinus* (27 each) with mean body weight of 1kg of the same age were studied. The fish were randomly distributed into nine treatments with 6 fish per treatment. They were purchased from a known farm in Ibadan, Nigeria. The fish were acclimatized for six weeks before the experiment and judged to be of good general health based on complete physical examination before the commencement of the experiment.

The lateral and caudal regions of fish were aseptically prepared (medical examinations or procedures that prevent contamination or infection of microbes) and this was conducted to determine if there differences were exist in the rate of healing at different locations (lateral and caudal part) of fish. The wound location was chosen because the tissue of these locations varies from one another. A standard template (a transparent paper, drawing the site on a well uniform 1 mm cubic square quadrant) was placed on the fish and the cuts were made on the lateral and caudal regions of the fish to draw the wound area and was replicated twice on the fish body (Abo *et al*., 2004, Bell, 2002) (see plate 3.2 -3. 4).

Immediately after cutting, the wound was photographed with a digital camera (Kodak Easy Share C813, 8.2 mega pixels, 3x optical zoom). The fish were taken out of water on daily basis (everyday) to examine the wound closure. Photograph and measurement was done using measuring scale at 0, 7 and 14 days in wound area in two-dimensional healing. Percentage healing was calculated as;

$$\% \text{ healing} = \frac{\text{change in wound area (area of regenerated tissue)}}{\text{initial wound area,}} \times 100$$

$$\text{Daily healing rates} = \frac{\text{total percentage of area healed}}{\text{number of day's measurement taken.}} \qquad \text{(Bell,2002)}$$

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***Clarias gariepinus* cut on lateral and caudal part for dermal wound healing experiment**

Plate 3.1: *C. gariepinus* with 1cm^2 template

Plate 3.2: *C. gariepinus* with 1cm^2 cut on Lateral part

Plate 3.3: *C. gariepinus* with 1cm^2 cut on caudal part

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3.16 HISTOPATHOLOGY ANALYSIS

The organs (muscle, liver, intestine, kidney, gill and testis) of the fish were taken for histological examination and this was carried out in Histopathology Laboratory of Department of Veterinary Pathology, University of Ibadan, Ibadan. The slides were prepared for histopathology according to Culling (1974) and Drury *et al* (1967) method for the organs or tissues after the experiment. Each slide was observed under microscope (Olympus CX21, Japan). The procedure for the preparation of slides for histopathological study is outlined below:

(1) Techniques of fixation

The organ of fish was fixed in 10% buffered formalin for 24 hours. The pH of the buffered formalin was 7 and the bottles were kept at room temperature.

(2) Techniques of dehydration

Following fixation, the tissues was dehydrated with ascending grade of alcohol in glass-stoppered jars to prevent evaporation. The volume of the alcohol is 100 times the bulk of the specimens. The tissues were first put in 70% ethyl alcohol for 3hours. After this they were transferred into 90% ethyl alcohol for 16 hours and absolute alcohol I (75%) for 1 hour, followed by absolute alcohol II (85%) for 1hour and lastly in absolute alcohol III (100%) for 1hour.

(3) Techniques of clearing

After dehydration, the organs were transferred into xylene in glass-stoppered jar for 16 hours. The volume of xylene is 100 times the bulk of the specimens.

(4) Techniques of wax impregnation

The tissue was transferred from the clearing agent to molten paraffin wax impregnation with paraffin was done in an oven [Baird and Tatlock (London) Limited, Chadwell Heath Essex Y151299/48 England} heated to 63^0C. The volume of the tissue and the paraffin was changed 3 times. This change was effected by simply lifting the tissue from one pot of wax to the next with warmed forceps and each lasted for 39 minutes. The first wax was discarded by tipping it in the used wax bowl. The container was then refilled with fresh wax.

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(5) Techniques for casting

Tissues were blocked by transferring it from the final wax bath to moulds filled with molten wax. The mould to be used consisted of two L-shaped pieces of metal that were laid on a metal plate to form rectangular shape. With forceps previously warmed to prevent the wax setting on them, the tissues was then lifted from the final wax and placed in the bottom of the mould. The label was fixed in position by pressing one edge against the solidification wax. When the block has cooled by forming a skin on the surface it is then immersed in cold water to cool it rapidly. The block wax having set quite hard was removed from mould.

(6) Techniques of section cutting

A rotary microtome (Mvtex, Senior Rotary Microtome, model MT-820, India) was used for cutting. The blocks is first trimmed and fixed to wooden fillets, using the following stepwire procedure:

- The block was fixed in the block holder on the microtone in such a position that the knife is cleared when positioned.
- The feed mechanism on the microtone was turn back as far it would go
- A blade was appropriately inserted in the blade holder and screwed it tightly in position.
- The block holder was moved forwards or upwards until the wax block was almost touching the knife.
- The adjusting screws on the microtone was well tightened
- The block set was trimmed and the section thickness gauze to about 15 microns and with the rough knife, operate the microtone until complete sections of the tissue is being cut.
- The rough knife was replaced by a sharp one.
- The thickness gauge is set.
- The microtone was now operated until complete sections are cut.

(7) Procedure for attaching section to slide

- Some slides were cleared with methylated spirit.
- The surface was rubbed with liberal glycerine albumen to receive the section.
- Single ribbons are cut from arranged section.

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- Forceps were used to transfer section to a slide containing some drops of 20% alcohol to flatten out.
- Transfer to a water of 5-6 ^{0}C below the melting point of wax is used.
- The section is picked from water bath using the side of the slide covered with glycerine albumen drain off excess water and arranges the section in the center of the slide.

(8) Procedure for staining of slides with haematoxylin and eosin stains

- Dewaxing was done by using xylene for 2 minutes, twice
- It was hydrate through graded alcohol that was 100%, 95% and 70% for 2 minutes each.
- It was stained in Erhlick haematoxylin for 15 minutes.
- Excess stains are washed off in water.
- 1% acid alcohol was used for differentiation until only the nuclear is blue and the rest of tissue is colourless.
- Stains were put in tap water for 10 minutes.
- Counter stain in alcoholic eosin for 30 minutes.
- Dehydrate through 70% -95% -100% alcohols for 2 minutes each.
- Clear in xylene.
- Mount in DPX.

3.17 HAEMATOLOGICAL ANALYSIS

This was carried out before and after the experiment at the haematological laboratory of Veterinary Medicine Department, University of Ibadan within 30 minutes of sampling. Needle and syringe was inserted 3 – 4cm from the genital opening, each fish was punctured and wiped with dry tissue paper to avoid contamination with mucus. The needle was inserted at a right angle to the vertebral column of the fish, which was gently aspirated during penetration. The blood was taken by gently aspirating until about 1cm^3 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. Plasma was obtained from blood samples by centrifugation and then drawn into 1cm^3 plastic syringe transferred into a universal bottle in refrigerator to be later used for biochemical analysis. The standard haemocytometer were used in both erythrocyte and leucocyte

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counts according to the methods of Blaxhall and Daisley (1973) using modified hyme's dilution fluid. The collected blood was introduced into an improved Neubaeur Counting Chamber (Neubauer improved bright line Marienfield, Germany 0.100 mm, 0.0025 mm^2) and the cells were counted under the microscope at 100 x objective.

3.17.1 Pack Cell Volume (PCV)

This was determined after drawing freshly collected blood samples into microhaematocrit tubes (75 mm long, 1.1 – 1.2 mm internal diameter) sealed with plasticine (cristaseal) at one end. The blood was centrifuged for five minutes at 3000rpm using a Hawskley micro-haematocrit centrifuge, England. The actual value was obtained using haematocrit reader (Blaxhall and Daisley 1973)

3.17.2 Erythrocyte Sedimentation Rate (ESR)

Erythrocyte sedimentation rate (ESR) was determined using the Micro-Wintrobe Method (Blaxhall and Diasley, 1973). A microhaematrocrit tube (75mm long, 1.1 – 1.2mm internal diameter) was placed at an angle 60°. The blood was allowed to flow into the tube to about 50mm. One end was sealed with cristaseal (plasticine) and the tube was allowed to stand in a vertical position for one hour at room temperature. Millimeter graph paper mounted on the card was used to take the reading. Measurement was made from the top column of sedimented erythrocytes to the surface of the plasma.

3.17.3 Red Blood Cells (RBC)

The blood was diluted 1: 200 with Hyme's diluting fluid using the method by Jain (1986). Few drops was expelled out, and then introduced under cover slip on an improved Neubaeur counting chamber (Neubauer improved bright line Marienfield, Germany 0.100 mm, 0.0025 mm^2) count with x 10 objective.

Calculation,

Numbers of cell counted x Depth x Dilution x Area

= X blood x 10^{12}L (Dacie and Lewis, 1975)

3.17.4 White Blood Cells (WBC)

The blood was diluted 1: 200 with WBC diluting fluid (2% Acetic Acid tinted with dye). Few drops were expelled out and introduced under cover slip on an improved Neubaeur Counting chamber (Neubauer improved bright line Marienfield, Germany 0.100 mm, 0.0025 mm^2), count with x 10 objective.

Calculation,

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Average number of cell counted per square mm x Depth x Dilution

= X blood x 10^9/L (Dacie and Lewis, 1975)

3.17.5 Haematic indices

Mean Cell Volume (MCV): The diluted blood was centrifuged for 200 rpm for 10 minutes to remove the red cell nuclei that cause turbidity.

Calculation,

This was the average volume of a single cell exposed in fentolitres (fl) or µ.m

$$MCV = \frac{PCV \times 1000}{RBC}$$

Mean Cell Haemoglobin Concentration (MCHC): The diluted blood was centrifuged for 200 rpm for 10 minutes to remove the red cell nuclei that cause turbidity.

Calculation,

This was referring to the percentage haemoglobin content in g/dl by the PCV and the result expressed as percentage.

$$MCHC = \frac{\text{Hb content}}{PCV}$$

Mean Cell Haemoglobin (MCH): The diluted blood was centrifuged for 200 rpm for 10 minutes to remove the red cell nuclei that cause turbidity.

Calculation,

This is expressed the average Haemoglobin (Hb) content in picograms (pg) of a single red blood cell (RBC)

$$MCH = \frac{Hb}{RBC}$$

3.17.6 Haemoglobin (Hb)

2.0 µl of blood was added to 5 ml of drabkin solution mixed and was allowed to stand for 3 – 5 minutes. The same was done to standard haemoglobin. The optical density (O.D) of standard and test was obtained on spectrophotometer (Spectrum lab 23A, spectrophotometer QLYXL 23 – 1996) at wavelength 540 nm

Calculation

$$\frac{\text{O.D. test}}{\text{O.D standard}} \times \text{Concentration of Standard} \times \text{Dilution}$$

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= X gm % of Haemoglobin

3.18 BIOCHEMICAL ANALYSIS

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 blood was stored at – 20°C and analysis at the Haematology Laboratory of Veterinary Pathology Department, University of Ibadan for glucose concentration, total protein, total lipid and blood serum such as Aspartate amino transferase (AST) and Alanine amino transferase (ALT).

3.19 DETERMINATION OF PHYTOCHEMICALS IN ONION AND WALNUT LEAF

Phytochemical tests for bioactive constituents were carried out on portions of the residual material using standard phytochemical procedures:

3.19.1 Colour tests for alkaloids: 500 mg of plant material was extracted with 500 mls of methanol for 20 minutes, on a water bath. The extract was then filtered off and allowed to cool. This extract was dispensed in 2 ml of portions into four different test tubes. Either the Dragendorff's or Hager's or Mayer's or Wagner's alkaloidal reagent was added to each tube or the presence or absence of colours of any precipitates was noted in each test tube.

3.19.2 Frothing test for saponins: Water extract was obtained by boiling on the water bath. The extract was transferred into a test tube and shaken vigorously then was left to stand for 10mins and the result noted. A thick persistent froth indicated saponins.

3.19.3 Ferric chloride solution test for tannins: Water extract was treated with 15 % ferric chloride test solution. The resultant colour was noted. A blue colour indicates condensed tannins, a green colour indicated hydrolysable tannins.

3.19.4 Test for Flavonoid*s*: Water extract of the sample was reduced to dryness on the boiling water bath. The residue was treated with dil. NaOH, followed by addition of dilute HCl, solubility and colour were noted. A yellow solution with NaOH, which turns colourless with dil HCl confirm flavonoids.

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3.19.5 Borntrager's test for anthraquinone derivatives: Chloroform extract of the material was obtained by boiling on the water bath. To 2 ml of this extract, 1ml of dilute (10 %) ammonia was added and the mixture was shaken. Any colour change was recorded. A pink-red colour in the ammoniacal (lower) layer shows anthracene derivatives.

3.20 HISTOMORPHOMETRIC EVALUATION

The organ (intestine) of *C. gariepinus* was taken for histological examination and this was carried out in the Histopathology Laboratory of the Department of Veterinary Pathology, University of Ibadan. The slides were prepared for histology based on Culling (1974) and Drury *et al*., (1967) method for the organs and tissues. Measurements of cryptal depth, villi length and width were taken using microscope (Olympus CX21, Japan) with a micrometer rule as described by Joaquim *et al*., (2005), Spadoni *et al.* (2005) and Eyarefe *et al*., (2008). Four different villi were measured in each slide per parameter, recorded and an average value calculated.

3.21 ECONOMIC ANALYSIS

Economic analysis of producing catfish using walnut leaf and onion bulb was carried out with emphasis on profit index and incidence of cost determination as described by Vincke, (1969).

Profit index = Value of fish produced (₦/kg)/
Cost of feed used in production (₦/kg)

Incidence of cost = Cost of feed used in production (₦/kg)/
Total weight of fish produced (kg)

Assumption:

The cost of feeds formulated was based on prevailing market prices at the feed mill while the cost of walnut leaf and onion treatment was based on market prices and cost of processing.

The value of fish was based on the selling price of fish / kg (₦ 400.00) in fish market around Ibadan as at the end of the experiment.

Total weight of the fish produced was got from the total weight of fish recovered at the end of the experiment.

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Also, Economic Conversion Ratio (ECR) was determined as described by Piedecausa *et al*., 2007

$$\text{Economic Conversion Ratio (ECR)} = \frac{\text{Cost of diet}}{\text{Feed conversion ratio}}$$

3.22 STATISTICAL ANALYSIS

Growth performance and nutrient utilization, bacteriological characteristics, haematology and biochemical analysis 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.

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CHAPTER FOUR

4. O RESULTS

The results of this study were presented as follows:

4.1 DETERMINATION OF PHYTOCHEMICALS IN ONION BULB AND WALNUT LEAF

Premilinary phytochemicals screening of onion bulbs and walnut leaves for secondary metabolites showed the presence of saponins, tannins, alkaloids, cyanogenic glycosides while anthraquinones was not detected in both plant; flavonoids was present in onion bulbs while flavonoids was not detected in walnut leaves. The values of these metabolites were in moderate quality (+) in both plants as shown in Table 4.1.

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Table 4.1: Determination of important phytochemicals of ethanol and methanol extracts of onion bulb and walnut leaf

Samples	Parameters	Value
Onion bulb	Alkaloids	+
	Cardenolides	+
	Antraquinones	-
	Saponins	+
	Tannins	+
	Flavoniod	+
Walnut leaf	Alkaloids	+
	Cardenolides	+
	Antraquinones	-
	Saponins	+
	Tannins	+
	Flavoniod	-

Legends

+ = present and available in normal quality

- = absent

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4.2 MICROBIAL LOAD OF *Clarias gariepinus*

The microbial loads of fish tissues (skin, gills, intestine and liver) were determined and the results show that highest enterobacteriacea counts were recorded in control. Also, the highest total viable counts were recorded in skin and least in liver while no enterobacteriacea and total viable counts were recorded in the control as shown in Table 4.2.

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Table 4.2: Microbial load of liver, intestine, skin and gills of *Clarias gariepinus*

Fish sites	Organisms	Microbial load (log_{10}cfu/g)
Liver	Enterobacteriacea counts	2.04±0.03
	Total viable counts	2.61±0.05
Intestine	Enterobacteriacea counts	2.60±0.01
	Total viable counts	3.00±0.10
Skin	Enterobacteriacea counts	6.48± 1.50
	Total viable counts	6.93± 1.90
Gills	Enterobacteriacea counts	2.78±0.08
	Total viable counts	3.11±0.02
Control	Enterobacteriacea counts	-
	Total viable counts	-

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4.3 DETECTION OF ANTAGONISTIC ACTIVITIES OF ONION BULB AND WALNUT LEAF

Ethanol and methanol extracts of onion bulbs and walnut leaves respectively shows antibacterial and anti-fungal properties in the present study. The walnut leaves extracts exhibited the highest activities with all the pathogens investigated. Although, the onion bulbs extract had highest anti- fungal property, while no antibacterial activity were recorded for *B. subtilis* and *E. coli* in onion bulbs and walnut leaves respectively (Table 4.3)

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Table 4.3: Antibacterial activities (diameter of inhibition zone, mm) of walnut leaf and onion bulb.

Pathogens	Diameter of zone of inhibition (mm)		
	Onion bulb	Walnut leaves	Control
Pseudomonas aeruginosa	10±0.01	12±0.00	-
Bacillus subtilis	-	12±0.02	-
Pseudomonas fluorescens	11±0.00	11±0.01	-
Staphyloccocus aureus	11±0.01	13.5±0.03	-
Escherichia coli	9±0.02	-	-
Salmonella typhi	10±0.00	10±0.01	-
Aspergillus niger	16.5±0.02	10±0.00	-

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4.4 DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC) OF ONION BULB AND WALNUT LEAF EXTRACTS

The minimum inhibitory concentration of the ethanolic and methanolic extracts onion bulbs and walnut leaves against 6 pathogenic bacteria isolated from aquatic animals were examined in the present study and their potency were assessed by MIC and it was recorded that both plants shows 500µg/ml against all the tested pathogens (Table 4.4)

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Table 4.4: Minimum inhibitory concentration of onion bulb and walnut leaf extracts

		Minimum inhibitory concentration in µg/ml									
Onion	Isolates	2000	1000	500	250	125	62.5	31.3	15.65	7.825	3.912
	Pseudomonas aeruginosa	-	-	-	+	+	+	+	+	+	+
	Pseudomonas fluorescens	-	-	+	+	+	+	+	+	+	+
	Staphyloccocus aureus	-	-	-	-	-	+	+	+	+	+
	Escherichia coli	-	-	-	-	-	-	-	+	+	+
	Salmonella typhi	-	-	-	+	+	+	+	+	+	+
	Control (without isolates)	-	-	-	-	-	-	-	-	-	-
Walnut leaves	*Pseudomonas aeruginosa*	-	-	-	+	+	+	+	+	+	+
	Bacillus subtilis	-	-	-	+	+	+	+	+	+	+
	Pseudomonas fluorescens	-	-	-	+	+	+	+	+	+	+
	Staphyloccocus aureus	-	-	-	-	+	+	+	+	+	+
	Salmonella typhi	-	-	-	+	+	+	+	+	+	+
	Control (without isolates)	-	-	-	-	-	-	-	-	-	-

Key:
+ = indicating growth showed by turbidity of the broth - = no growth

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4.5 PROXIMATE COMPOSITION OF EXPERIMENTAL DIETS FED TO *C. gariepinus* FOR 84 DAYS

The proximate composition of the diets showed highest moisture content in diet 6 and the least in diet 9, the highest crude protein and ash content were recorded in diet 8 and the least was recorded in diet 5 and diet 1 (control), there were significantly difference ($p<0.05$) among the treatments respectively. The highest ether extract was obtained in diet 2 and the least in diet 5 and the highest NFE was recorded in the control diet and least in the diet 6 (Table 4.5).

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Table 4.5: Proximate composition of experimental diets (DM)

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9
Moisture	6.80 ± 0.02^{a}	6.98 ± 0.02^{c}	7.55 ± 0.01^{d}	7.78 ± 0.01^{e}	6.90 ± 0.03^{b}	8.10 ± 0.02^{g}	7.98 ± 0.04^{f}	7.50 ± 0.06^{d}	6.78 ± 0.01^{a}
Crude protein	40.08 ± 0.24^{ab}	40.08 ± 0.27^{ab}	40.10 ± 0.11^{b}	40.08 ± 0.09^{ab}	40.04 ± 0.18^{a}	40.10 ± 0.05^{b}	40.18 ± 0.01^{c}	40.20 ± 0.08^{c}	40.13 ± 0.24^{b}
Ether extract	15.38 ± 0.10^{c}	16.62 ± 0.01^{h}	16.40 ± 0.06^{f}	15.10 ± 0.02^{b}	14.92 ± 0.00^{a}	16.20 ± 0.12^{e}	16.00 ± 0.03^{d}	15.98 ± 0.05^{d}	16.50 ± 0.06^{g}
Ash	14.80 ± 0.02^{a}	15.10 ± 0.03^{b}	15.40 ± 0.01^{c}	16.10 ± 0.50^{ef}	15.98 ± 0.04d	16.02 ± 0.01^{d}	16.08 ± 0.11^{e}	16.15 ± 0.08^{f}	15.99 ± 0.10^{d}
NFE	22.94 ± 0.17^{e}	21.22 ± 0.18^{bcd}	20.55 ± 0.10^{cd}	20.94 ± 0.32^{bc}	22.16 ± 0.13^{de}	19.58 ± 0.11^{a}	19.76 ± 0.09^{a}	20.17 ± 0.15^{ab}	20.60 ± 0.21^{abc}

Key: The above values are means of triplicate data, mean values in each row with similar superscripts are not significantly different ($p > 0.05$)

Note:

NFE= Nitrogen Free Extract

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4.6 BODY CARCASS COMPOSITION OF *C. gariepinus* FED EXPERIMENTAL DIETS FOR 84 DAYS

Moisture content was highest in the group fed control diet compared to the treated groups after the experiment and the moisture taken before the experiment, except for WL 9. There were significant differences ($P < 0.05$) among the treatments. The crude protein level of the fish increased significantly ($P < 0.05$) during the experiment with highest value recorded in treated group, WL 9 compared to the value of control. Higher ether extract contents were observed after the feeding trial than before the experiment. There was a general decline in the value recorded in the treated groups except OB 2 and the value recorded in the control. There were no significant difference ($P < 0.05$) between OB 2 and control but there were significant differences ($P < 0.05$) among other treatments. The ash content recorded in the treated groups were higher than the control with highest value in OB 4 and least in control, they were significant difference ($P < 0.05$) among the treatments (Table 4.6).

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Table 4.6: Body carcass composition of *C. gariepinus* fed experimental diets for 84 days

Parameters	Initial	Control	OB 2	OB 3	OB 4	OB 5	WL 6	WL 7	WL 8	WL 9
Moisture	73.37±0.02	77.22±1.64^{i}	75.94±0.90^{e}	76.81±0.16^{h}	76.41±0.02^{f}	75.81±0.24^{d}	75.74±0.33^{c}	75.57±0.62^{b}	76.58±0.59^{g}	75.46±0.33^{a}
Crude protein	47.46±0.01	70.32±0.04^{a}	71.15±0.08^{f}	70.77±0.04^{e}	70.46±0.05^{c}	70.76±0.07^{e}	71.14±0.04^{f}	70.38±0.06^{b}	70.70±0.02^{d}	71.25±0.04^{g}
Ether extract	2.87±0.02	4.89±0.00^{e}	4.95±0.04^{e}	3.65±0.02^{bc}	3.90±0.11^{d}	3.45±0.03^{a}	3.67±0.07^{bc}	3.62±0.09^{b}	3.61±0.00^{b}	3.71±0.05^{c}
Ash	12.75±0.08	15.50±0.03^{a}	18.65±0.02^{c}	17.95±0.01^{b}	19.20±0.10^{f}	18.99±0.06^{e}	18.89±0.12^{d}	18.65±0.06^{c}	19.00±0.15^{e}	18.85±0.13^{d}
NFE	36.92±0.06	9.29±0.04^{i}	5.25±0.07^{a}	7.63±0.01^{h}	6.44±0.13^{d}	6.80±0.08^{e}	6.30±0.12^{c}	7.35±0.11^{g}	6.89±0.09^{f}	6.18±0.11^{b}

Key: The above values are means of triplicate data, mean values in each row with similar superscripts are not significantly different ($p > 0.05$)

Note:

NFE= Nitrogen Free Extract

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4.7 WATER QUALITY PARAMETERS OF EXPERIMENTAL TANKS OF CULTURED *C. gariepinus* FED ONION BULB AND WALNUT LEAF FOR 84 DAYS

The highest temperature was recorded in WL7 and WL8 while the least value was recorded in control. Highest dissolved oxygen was recorded in WL 7 and the least in control while highest pH was recorded in WL 8 and the least in control. The water quality parameters; temperature, dissolved oxygen and pH values recorded were closely related (table 4.7).

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Table 4.7A: Mean bi-weekly water quality parameters of the experimental tanks in onion treatment

Treatments	Parameters	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12	Mean
Control	Temp (^{OC})	25.07±0.05	25.50±0.01	26.70±0.01	26.80±0.05	26.82±0.01	26.87±0.00	26.29±0.79
	DO (mg/l)	6.58±0.01	6.72±0.03	6.60±0.05	6.70±0.02	6.71±0.10	6.76±0.01	6.68±0.07
	pH	7.80±0.01	7.20±0.10	7.93±0.07	7.20±0.01	8.10±0.09	8.20±0.12	7.74±0.44
OB2	Temp (^{OC})	25.27±0.10	26.17±0.02	26.80±0.12	27.10±0.00	27.50±0.11	27.80±0.02	26.77±0.93
	DO (mg/l)	6.68±0.01	6.80±0.02	6.78±0.04	6.81±0.04	6.75±0.06	6.81±0.01	6.77±0.05
	pH	7.80±0.02	7.50±0.02	7.95±0.08	7.55±0.01	8.10±0.02	8.10±0.01	7.83±0.26
OB3	Temp (^{OC})	25.33±0.15	26.53±0.05	26.90±0.01	27.70±0.11	27.70±0.02	27.90±0.06	27.01±0.98
	DO (mg/l)	6.75±0.02	6.89±0.01	6.80±0.05	6.96±0.05	6.87±0.02	6.97±0.03	6.87±0.09
	pH	7.84±0.10	7.78±0.10	7.97±0.01	7.90±0.10	8.15±0.01	8.20±0.10	7.97±0.12
OB4	Temp (^{OC})	25.50±0.00	25.67±0.20	26.97±0.04	27.80±0.01	27.80±0.01	28.01±0.06	26.96±1.12
	DO (mg/l)	6.86±0.05	6.91±0.01	6.90±0.50	7.09±0.02	7.02±0.03	7.05±0.20	6.97±0.09
	pH	7.84±0.01	7.90±0.01	7.95±0.01	7.95±0.03	8.50±0.04	8.55±0.01	8.12±0.32
OB5	Temp (^{OC})	25.10±0.02	25.60±0.01	26.90±0.00	26.80±0.02	26.89±0.11	26.95±0.05	26.37±0.81
	DO (mg/l)	6.72±0.01	6.79±0.03	6.72±0.01	6.78±0.02	6.82±0.07	6.89±0.06	6.79±0.06
	pH	7.87±0.01	7.39±0.00	7.95±0.08	7.30±0.02	8.16±0.07	8.22±0.05	7.82±0.39

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Table 4.7B: Mean bi-weekly water quality parameters of the experimental tanks of the walnut leaf treatment

Treatments	Parameters	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12	Mean
Control	Temp (ᴼᶜ)	25.07±0.05	25.50±0.01	26.70±0.01	26.80±0.05	26.82±0.01	26.87±0.00	26.29±0.79
	DO (mg/l)	6.58±0.01	6.72±0.03	6.60±0.05	6.70±0.02	6.71±0.10	6.76±0.01	6.68±0.07
	pH	7.80±0.01	7.20±0.10	7.93±0.07	7.20±0.01	8.10±0.09	8.20±0.12	7.74±0.44
WL6	Temp (ᴼᶜ)	25.17±0.06	26.07±0.04	26.70±0.05	27.02±0.00	27.35±0.01	27.70±0.01	26.67±0.92
	DO (mg/l)	6.60±0.07	6.70±0.02	6.70±0.02	6.71±0.04	6.69±0.01	6.71±0.09	6.69±0.04
	pH	7.75±0.06	7.41±0.04	7.85±0.01	7.43±0.09	8.03±0.02	8.05±0.03	7.75±0.28
WL7	Temp (ᴼᶜ)	26.03±0.05	26.53±0.75	27.20±0.01	27.70±0.01	27.70±0.03	28.90±0.01	27.34±1.00
	DO (mg/l)	6.90±0.02	6.97±0.04	7.10±0.01	7.05±0.02	7.07±0.02	7.27±0.01	7.06±0.13
	pH	7.84±0.01	7.98±0.01	7.99±0.03	7.99±0.08	8.10±0.05	8.20±0.01	8.02±0.12
WL8	Temp (ᴼᶜ)	25.55±0.03	25.70±0.20	27.00±0.02	27.85±0.02	27.83±0.01	28.10±0.03	26.79±1.25
	DO (mg/l)	6.90±0.01	6.95±0.02	6.92±0.00	7.13±0.05	7.07±0.08	7.09±0.10	7.01±0.10
	pH	7.88±0.02	7.94±0.01	7.97±0.04	7.98±0.02	8.53±0.02	8.90±0.05	8.20±0.42
WL9	Temp (ᴼᶜ)	25.10±0.02	25.52±0.01	26.73±0.02	26.84±0.01	26.85±0.05	26.90±0.10	26.32±0.80
	DO (mg/l)	6.62±0.01	6.75±0.05	6.65±0.04	6.75±0.02	6.75±0.07	6.80±0.01	6.72±0.07
	pH	7.82±0.03	7.25±0.03	7.96±0.01	7.25±0.03	8.14±0.01	8.25±0.02	7.78±0.44

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4.8 MICROBIOLOGICAL ANALYSES OF WATER

The control diet recorded highest enterobacteriacea and total viable counts at 4, 8, and 12 weeks. The lowest enterobacteriacea counts were recorded in OB5 among onion bulb containing residue treatments and WL9 in walnut leaf residue treatments for weeks 4, 8 and 12. The lowest TVC was recorded in OB5 in onion bulb residue treatments and WL9 in walnut leaf residue treatments for water during the same period of time. The enterobacteriacea and total viable counts recorded were significantly different ($p<0.05$) among the treatments (Table 4.8).

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Table 4.8: Enterobacteriacea and total viable counts (log $_{10}$CFU/ml) of water samples in tanks with fish fed with onion bulb and walnut leaf for 84 days

Treatment	4 Weeks		8 Weeks		12 Weeks	
	Enterobacteriacea counts	Total viable counts	Enterobacteriacea Counts	Total viable counts	Enterobacteriacea Counts	Total viable counts
Control	5.29 ± 0.01^{f}	5.48 ± 0.01^{c}	5.28 ± 0.05^{f}	5.40 ± 0.02^{f}	5.28 ± 0.00^{e}	5.41 ± 0.01^{g}
OB2	5.02 ± 0.02^{e}	5.11 ± 0.03^{a}	4.88 ± 0.00^{d}	5.28 ± 0.01^{e}	5.00 ± 0.04^{d}	5.19 ± 0.03^{f}
OB3	4.70 ± 0.05^{c}	5.08 ± 0.00^{a}	4.88 ± 0.07^{d}	5.16 ± 0.00^{d}	5.00 ± 0.02^{d}	5.12 ± 0.02^{e}
OB4	4.60 ± 0.02^{b}	5.50 ± 0.00^{c}	4.78 ± 0.04^{c}	4.93 ± 0.03^{b}	4.70 ± 0.00^{c}	4.90 ± 0.06^{c}
OB5	4.40 ± 0.03^{a}	5.56 ± 0.02^{c}	4.18 ± 0.02^{a}	4.74 ± 0.02^{a}	4.54 ± 0.04^{b}	4.70 ± 0.02^{ab}
WL6	5.02 ± 0.01^{e}	5.22 ± 0.01^{b}	5.04 ± 0.01^{e}	5.06 ± 0.04^{c}	5.00 ± 0.07^{d}	5.04 ± 0.01^{d}
WL7	4.78 ± 0.01^{d}	5.11 ± 0.03^{a}	5.04 ± 0.05^{e}	5.04 ± 0.10^{c}	$4.98\pm .0.08^{d}$	5.02 ± 0.00^{d}
WL8	4.60 ± 0.02^{b}	5.26 ± 0.01^{b}	4.65 ± 0.03^{b}	4.95 ± 0.03^{b}	4.54 ± 0.02^{b}	4.78 ± 0.00^{b}
WL9	4.54 ± 0.05^{b}	5.56 ± 0.00^{c}	4.18 ± 0.02^{a}	4.78 ± 0.00^{a}	4.40 ± 0.04^{a}	4.65 ± 0.02^{a}

Key: The above values are means of triplicate data, mean values in each row with similar superscripts are not significantly different ($p > 0.05$)

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4.9 MICROBIOLOGICAL ANALYSES OF *Clarias gariepinus* FED ONION BULB AND WALNUT LEAF RESIDUES FOR 84 DAYS

The control diet recorded highest enterobacteriacea and total viable counts in skin, gills, liver and intestine at 4, 8, and 12 weeks. The lowest enterobacteriacea and total viable counts was recorded in OB5 (0.5% inclusion) in onion bulb residue treatments for skin, gill, liver and intestine at 4, 8 and 12 weeks and WL9 (2.0% inclusion) in walnut leaf residue treatments for skin, gill, liver and intestine at 4, 8 and 12 weeks respectively. The enterobacteriacea and total viable counts recorded were significantly different ($p<0.05$) among the treatments (Table 4.9A and 4.9B).

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Table 4.9A: Enterobacteriacea and total viable counts (log $_{10}$CFU/g) of *Clarias gariepinus* fed with onion bulb for 84 days

Treatment	Fish sites	4 Weeks		8 Weeks		12 Weeks	
		Enterobacteriacea Counts	Total viable counts	Enterobacteriacea counts	Total viable counts	Enterobacteriacea Counts	Total viable counts
Control	Skin	4.04± 0.02^{g}	4.11± 0.10^{g}	4.02± 0.00^{f}	4.10± 0.02^{h}	3.71± 0.00^{g}	3.74± 0.02^{g}
	Liver	3.88± 0.04^{g}	3.89±0.00^{g}	3.85± 0.02^{g}	3.87± 0.01^{h}	3.65± 0.02^{g}	3.70± 0.04^{f}
	Gill	3.97± .002^{g}	4.09± 0.04^{g}	3.93± 0.01^{f}	4.06± 0.01^{g}	3.46± 0.01^{h}	3.49± 0.02^{g}
	Intestine	3.85± 0.00^{h}	3.85± 0.05^{a}	3.79± 0.00^{f}	3.82± 0.03^{g}	3.52±0.02^{f}	3.58± 0.06^{f}
OB2							
	Skin	3.76± 0.00^{f}	3.91± 0.07^{f}	3.72± 0.00^{e}	3.89± 0.01^{g}	3.58± 0.09^{f}	3.70± 0.02^{f}
	Liver	3.80±0.02^{f}	3.80± 0.06^{f}	3.74± 0.05^{f}	3.77± 0.00^{g}	3.62±0.10^{e}	3.66± 0.05^{ef}
	Gill	3.72± 0.02^{f}	3.92± 0.02^{f}	3.68± 0.02^{e}	3.91± 0.05^{f}	3.24± 0.01^{h}	3.46± 0.03^{f}
	Intestine	3.81± 0.01^{g}	3.77± 0.00^{a}	3.77± 0.04^{f}	3.74± 0.00^{f}	3.42±0.04^{e}	3.56± 0.01^{ef}
OB3							
	Skin	3.65±0.01^{e}	3.76±0.00^{e}	3.59± 0.01^{d}	3.73± 0.00^{f}	3.49± 0.03^{e}	3.62± 0.00^{e}
	Liver	3.60±0.02^{e}	3.67±0.08^{d}	3.54± 0.01^{e}	3.71± 0.00^{f}	3.43± 0.02^{d}	3.62± 0.01^{e}
	Gill	3.62±0.00^{e}	3.64±0.08^{d}	3.58±0.00^{d}	3.62± 0.01^{d}	3.11± 0.04^{g}	3.44± 0.01^{e}
	Intestine	3.65± 9.09^{f}	3.69±0.01^{a}	3.61± 0.02^{e}	3.68± 0.03^{e}	3.20± 0.09^{d}	3.40± 0.05^{d}
OB4							
	Skin	3.53± 0.00^{d}	3.63±0.01^{d}	3.48± 0.05^{c}	3.59±0.04^{e}	3.41± 0.09^{d}	3.56± 0.04^{d}
	Liver	3.51±0.05^{d}	3.62±0.01^{c}	3.46±0.02^{d}	3.57±0.00^{d}	3.32± 0.07^{d}	3.52± 0.03^{d}
	Gill	3.52±0.06^{d}	3.61±0.02^{d}	3.42±0.02^{c}	3.59± 0.01^{d}	3.09± 0.01^{e}	3.28± 0.00^{c}
	Intestine	3.54±0.01^{e}	3.69±0.01^{a}	3.50±0.01^{d}	3.64± 0.02^{e}	3.13± 0.00^{c}	3.39± 0.05^{d}
OB5							
	Skin	3.48± 0.07^{c}	3.49± 0.02^{b}	3.39±0.04^{b}	3.45± 0.03^{b}	3.18±0.06^{b}	3.46± 0.08^{b}
	Liver	3.46± 0.09^{c}	3.53± 0.02^{b}	3.34±0.03^{c}	3.53± 0.13^{c}	3.28±0.05^{b}	3.41± 0.00^{c}
	Gill	3.40± 0.02^{c}	3.53±0.03^{c}	3.33±0.01^{b}	3.51± 0.02^{c}	2.81± 0.00^{d}	3.24± 0.02^{b}
	Intestine	3.48±0.06^{d}	3.54±0.00^{a}	3.42± 0.05^{c}	3.52± 0.01^{d}	3.06± 0.10^{b}	3.31± 0.01^{c}

Key: The above values are means of triplicate data, mean values in each row with similar superscripts are not significantly different ($p > 0.05$)

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Table 4.9B: Enterobacteriacea and total viable counts (log $_{10}$CFU/g) of *Clarias gariepinus* fed with walnut leaf for 84 days

Treatment	Fish sites	4 Weeks		8 Weeks`		12 Weeks	
		Enterobacteriacea	Total viable counts	Enterobacteriacea	Total viable counts	Enterobacteriacea	Total viable counts
Control	Skin	4.04± 0.02^{g}	4.11± 0.10^{g}	4.02± 0.00^{f}	4.10± 0.02^{h}	3.71± 0.00^{g}	3.74± 0.02^{g}
	Liver	3.88± 0.04^{g}	3.89±0.00^{g}	3.85± 0.02^{g}	3.87± 0.01^{h}	3.65± 0.02^{g}	3.70± 0.04^{f}
	Gill	3.97± .002^{g}	4.09± 0.04^{g}	3.93± 0.01^{f}	4.06± 0.01^{g}	3.46± 0.01^{h}	3.49± 0.02^{g}
	Intestine	3.85± 0.00^{h}	3.85± 0.05^{a}	3.79± 0.00^{f}	3.82± 0.03^{g}	3.52±0.02^{f}	3.58± 0.06^{f}
WL6							
	Skin	3.54±0.01^{d}	3.61± 0.00^{d}	3.48±0.02^{c}	3.59±0.07^{e}	3.51±0.04^{e}	3.63±0.08^{e}
	Liver	3.48±0.00^{cd}	3.70± 0.05^{e}	3.43±0.06^{d}	3.66±0.08^{e}	3.39±0.01^{f}	3.57±0.05^{g}
	Gill	3.60± 0.01^{e}	3.69± 0.07^{e}	3.57±0.04^{d}	3.66±0.00^{e}	3.31±0.03^{f}	3.56±0.03^{d}
	Intestine	3.48± 0.02^{d}	3.53± 0.03^{a}	3.45±0.01^{cd}	3.48±0.02^{c}	3.39±0.09^{e}	3.50±0.01^{e}
WL7							
	Skin	3.46± 0.05^{bc}	3.53± 0.08^{c}	3.41±0.01^{b}	3.50±0.02^{c}	3.32±0.02^{c}	3.51±0.02^{c}
	Liver	3.36± 0.03^{b}	3.53±0.00^{b}	3.29±0.02^{b}	3.48±0.01^{b}	3.11±0.07^{c}	3.38±0.05^{d}
	Gill	3.44± 0.00^{c}	3.57± 0.01^{c}	3.41±0.09^{c}	3.53±0.07^{c}	3.06±0.00^{c}	3.31±0.01^{b}
	Intestine	3.37± 0.04^{c}	3.50± 0.02^{a}	3.31±0.00^{b}	3.46±0.02^{bc}	3.22±0.02^{d}	3.28±0.09^{bc}
WL8							
	Skin	3.43± 0.03^{b}	3.43± 0.01^{a}	3.41±0.03^{b}	3.38±0.06^{a}	3.11±0.01^{b}	3.32±0.02^{a}
	Liver	3.35± 0.00^{b}	3.51± 0.00^{a}	3.36±0.01^{c}	3.46±0.05^{b}	2.93±0.02^{a}	3.31±0.08^{b}
	Gill	3.30± 0.01^{b}	3.46± 0.04^{b}	3.33±0.01^{b}	3.42±0.04^{b}	2.70±0.05^{b}	3.11±0.03^{a}
	Intestine	3.43± 0.01^{b}	3.40± 0.02^{a}	3.24±0.02^{b}	3.37±0.01^{a}	3.11±0.02^{c}	3.26±0.06^{ab}
WL9							
	Skin	3.35± 0.07^{a}	3.42± 0.01^{a}	3.33±0.02^{a}	3.53±0.09^{d}	2.90±0.01^{a}	3.29±0.06^{a}
	Liver	3.20± 0.08^{a}	3.35± 0.09^{a}	3.16±0.05^{a}	3.33±0.04^{a}	2.88±0.01^{a}	3.28±0.04^{a}
	Gill	3.04± 0.01^{a}	3.15± 0.00^{a}	2.93±0.07^{a}	3.06±0.03^{a}	2.70±0.03^{a}	3.06±0.02^{a}
	Intestine	3.18± 0.03^{a}	3.37±0.03^{a}	3.11±0.02^{a}	3.45±0.13^{b}	2.98±0.04^{a}	3.23±0.00^{a}

Key: The above values are means of triplicate data, mean values in each row with similar superscripts are not significantly different ($p > 0.05$)

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4.10 GROWTH PERFORMANCE AND NUTRIENTS UTILIZATION OF *Clarias gariepinus* FED ONION BULB AND WALNUT LEAF BASED DIETS FOR 84 DAYS

The results of the experiment revealed that the highest weight gain, percentage weight gain, protein efficiency ratio, nitrogen metabolism and specific growth rate were recorded in WL 8 and the least in control diet. There were no significant differences ($p>0.05$) among the treatments. The feed conversion ratio was best in WL 8 and least in control diet while highest protein intake was recorded in OB 4 and the least in WL 9. Also, highest protein productive value was recorded in WL 9 and the least in control diet, there were significant differences ($p<0.05$) among the treatments (Table 4.10).

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Table 4.10: Growth performances and nutrients utilization of *Clarias gariepinus* fed onion bulb and walnut leaf based diets for 84 days.

Parameters	Control	OB2	OB3	OB4	OB5	WL6	WL7	WL8	WL9
Initial body weight(g)	7.39±0.29[a]	7.41±0.58[a]	7.38±0.20[a]	7.39±0.29[a]	7.39±0.29[a]	7.41±0.58[a]	7.39±0.29[a]	7.40±0.50[a]	7.39±0.29[a]
Final body weight (g)	52.83± 2.18[a]	55.17± 4.68[a]	55.67± 1.28[a]	56.70± 4.61[a]	53.22± 0.04[a]	53.70± 4.10[a]	56.80±5.28[a]	61.21±11.20[a]	53.08±7.96[a]
Body weight gain (g)	45.44±2.18[a]	47.76±4.68[ab]	48.08±0.92[ab]	49.31±4.61[ab]	45.83±0.04[a]	46.29±4.18[ab]	49.41±05.24[ab]	53.81±11.20[b]	45.69±7.96[a]
Body weight gain (%)	614.93±0.19[a]	644.58±0.25[a]	651.45±0.40[a]	667.30±0.50[a]	620.16±0.49[a]	624.65±0.26[a]	668.56±0.02[a]	727.16±1.40[a]	618.27±0.30[a]
Feed conversion ratio	2.64±0.03[a]	2.50±0.01[a]	2.56±0.02[a]	2.57±0.00[a]	2.52±0.01[a]	2.46±0.00[a]	2.25±0.01[a]	2.16±0.02[a]	2.40±0.03[a]
Protein efficiency ratio	1.13±0.05[a]	1.19±0.12[a]	1.20±0.03[a]	1.23±0.12[a]	1.14±0.01[a]	1.15±0.11[a]	1.23±0.38[a]	1.34±0.28[a]	1.14±0.20[a]
Protein productive value	57.00±0.11[a]	58.95±0.19[e]	58.02±0.09[d]	57.38±0.12[b]	58.20±0.18[d]	58.98±0.00[e]	57.04±0.14[a]	57.80±0.06[c]	59.30±0.09[f]
Protein intake (g)	480.72±0.87[ab]	478.95±4.55[ab]	492.83±6.45[ab]	506.81±0.22[b]	462.26±0.85[ab]	457.04±2.54[ab]	446.66±0.62[ab]	467.43±1.46[ab]	440.43±1.42[a]
Nitrogen metabolism	1218±0.20[a]	1272.19±0.91[ab]	1278.72±0.17[ab]	1307.47±0.19[ab]	1227.15±0.83[a]	1238.14±0.39[ab]	1309.77±0.75[ab]	1582.01±0.34[b]	1223.92±0.47[a]
Specific growth rate	1.02±0.02[a]	1.04±0.05[a]	1.05±0.01[a]	1.05±0.04[a]	1.02±0.00[a]	1.02±0.04[a]	1.04±0.13[a]	1.09±0.11[a]	1.02±0.08[a]
Condition factor:									
A Initial	0.59±0.01	0.60±0.00	0.59±0.01	0.59±0.01	0.59±0.01	0.60±0.00	0.59±0.01	0.60±0.00	0.59±0.00
B Final	3.30±1.29	3.91±0.47	3.05±0.47	2.94±0.44	3.16±0.001	2.59±0.39	2.84±0.61	3.34±0.74	3.18±0.37
C Difference	2.71±0.65[a]	2.59±0.24[a]	2.46±0.24[a]	2.35±0.23[a]	2.57±0.11[a]	1.99±0.19[a]	2.25±0.32[a]	2.74±0.37[a]	2.59±0.18[a]

Key: The above values are means of triplicate data, mean values in each row with similar superscripts are not significantly different ($p > 0.05$)

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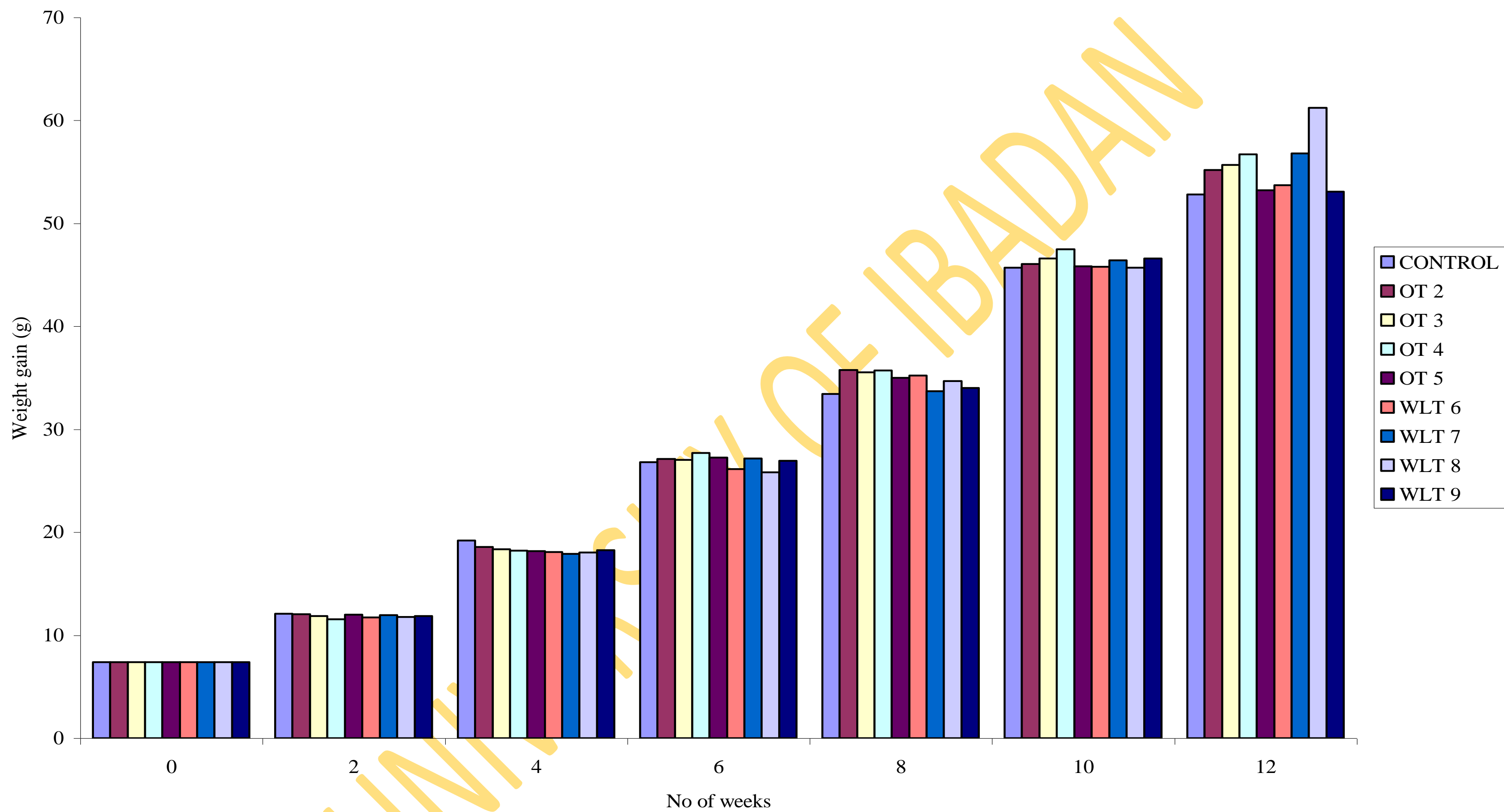

Fig. 4.1: Weight gain of *Clarias gariepinus* fed experimental diets for 84 days.

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4.11 HAEMATOLOGICAL PROFILE OF *Clarias gariepinus FED* ONION BULB AND WALNUT LEAF DIETS IN INITIAL AND AFTER FEEDING TRIAL FOR 84 DAYS

There were increased values of some haaematological parameters after the feeding experiment when compared with the values obtained before (initial) experiments. Packed cell volume and haemoglobin contents were significantly different ($P<0.05$) among the treatments with highest value for WL 9 and the least in OB 5 while red blood cells, while blood cells, mean cell volume and mean cell haemoglobin were not significantly different ($p>0.05$) among the dietary groups with highest value recorded in WL 9, OB 3, OB 2, WL 8 and the least in OB 5, control, OB 4 respectively (Table 4.11).

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Table 4.11: Haematological Parameters of *Clarias gariepinus* Juveniles in initial and after the feeding experiment fed onion bulb and walnut leaf diets for 84 days.

Parameters	Initial	After feeding experiment								
		CONTROL	**OB2**	**OB3**	**OB4**	**OB5**	**WL6**	**WL7**	**WL8**	**WL9**
PCV (%)	12.50±2.50	26.00±4.00^{ab}	24.00±1.41^{ab}	28.00±2.83^{ab}	22.00±0.89^{ab}	20.00±1.70^{a}	29.50±2.12^{ab}	25.00±4.24^{ab}	28.00±1.10^{ab}	34.50±0.71^{b}
Hb (g/dl)	4.10±0.45	8.30±1.98^{ab}	7.25±0.64^{ab}	8.80±1.41^{ab}	6.70±2.97^{ab}	5.65±0.07^{a}	9.35±0.92^{ab}	7.70±1.41^{ab}	9.10±3.25^{ab}	10.65±0.07^{b}
RBC x10^{12}/ l	1.07±0.05	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	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 (m/µl)	133,000±1.10	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)	0.2±0.14	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	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	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 (g/dl)	34.00±0.04	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 x10^{9}/ l	69.00±1.00	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 x10^{9}/ l	25.00±2.00	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 x10^{9}/ l	3.00±0.00	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 x10^{9}/ l	3.00±1.00	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}
Hetero:Lym ratio	0.36±0.00	0.45±0.02^{b}	0.37±0.05^{a}	0.45±0.01^{b}	0.44±0.04^{b}	0.48±0.00^{bc}	0.46±0.02^{b}	0.53±0.01^{c}	0.48±0.01^{bc}	0.45±0.05^{b}

Key: The above values are means of triplicate data, mean values in each row with similar superscripts are 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, Hetero:Lym ratio = Heterophil/lymphocytes ratio

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4.12 HAEMATOLOGICAL PROFILE OF *Clarias gariepinus* IN PRE – CHALLENGE AND POST CHALLENGE TEST FED ONION BULB AND WALNUT LEAF RESIDUES FOR 28 DAYS

There were increased values of some haaematological parameters at the post challenge test when compared with the values obtained in pre – challenge. The packed cell volume, haemoglobin, red blood cell, white blood cell, mean cell volume and mean cell haemoglobin were not significantly different ($p>0.05$) among the dietary groups with highest values recorded in OB 2, WL 9, control and the least in OB 4,WL 9,OB 4, OB2 and OB 5 respectively. The lymphocytes were significantly different ($P<0.05$) among the treatments with highest value recorded in OB 2 and the least in control (Table 4.12).

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Table 4.12: Haematological Parameters of Post – Challenge Test of *Clarias gariepinus* Juveniles Fed Onion Bulb and Walnut Leaf Residues for 28 days

Parameters	Pre – challenge	Post challenge								
		CONTROL	OB2	OB3	OB4	OB5	WL6	WL7	WL8	WL9
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}	31.00 ± 1.00^{a}	29.50 ± 1.50^{a}	29.00 ± 1.00^{a}	32.50 ± 3.50^{a}	26.10 ± 0.10^{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}	9.70 ± 2.10^{ab}	9.15 ± 0.65^{ab}	9.10 ± 0.40^{ab}	10.30 ± 1.30^{ab}	8.05 ± 1.55^{a}
RBC x10^{12}/ l	1.07 ± 0.05	2.75 ± 0.06^{a}	$4.05\pm0.\ 35^{a}$	3.19 ± 0.66^{a}	$2.63\pm\ 0.02^{a}$	3.34 ± 0.58^{a}	3.19 ± 0.43^{a}	2.87 ± 0.55^{a}	3.53 ± 0.76^{a}	2.44 ± 1.11^{a}
WBC x10^{9}/ l	$15{,}250\pm5.50$	$15{,}250\pm8.20^{a}$	$18{,}500\pm2.10^{a}$	$17{,}375\pm8.25^{a}$	$14{,}250\pm0.14^{a}$	$17{,}550\pm2.50^{a}$	$17{,}750\pm1.95^{a}$	$15{,}725\pm1.38^{a}$	$17{,}500\pm2.00^{a}$	$16{,}750\pm4.45^{a}$
Platelet (m/µl)	$133{,}000\pm1.10$	$131{,}000\pm5.00^{a}$	$195{,}000\pm3.50^{a}$	$190{,}500\pm5.95^{a}$	$140{,}000\pm3.40^{a}$	$111{,}000\pm1.00^{a}$	$119{,}500\pm5.00^{a}$	$125{,}500\pm2.50^{a}$	$157{,}000\pm3.90^{a}$	$148{,}500\pm6.00^{a}$
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}	91.95 ± 0.20^{a}	93.50 ± 0.90^{a}	104.20 ± 1.60^{a}	94.40 ± 1.03^{a}	125.05 ± 4.00^{a}
MCH (Pg)	3.91 ± 0.86	3.88 ± 0.11^{a}	2.96 ± 0.50^{a}	2.92 ± 0.32^{a}	$3.16\pm0.0.21^{a}$	2.88 ± 0.13^{a}	2.90 ± 0.19^{a}	3.27 ± 0.49^{a}	2.98 ± 0.27^{a}	3.80 ± 1.09^{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}	31.50 ± 0.05^{a}	31.00 ± 0.05^{a}	31.50 ± 0.05^{a}	31.50 ± 0.02^{a}	31.00 ± 0.10^{a}
Lym x10^{9}/ 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}	71.50 ± 1.50^{ab}	65.50 ± 4.50^{a}	73.00 ± 2.00^{ab}	74.00 ± 1.00^{ab}	70.50 ± 4.50^{ab}
Hetero x10^{9}/ 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}	25.50 ± 1.50^{ab}	31.00 ± 0.03^{ab}	22.50 ± 1.50^{ab}	22.50 ± 0.05^{ab}	27.00 ± 0.09^{ab}
Mono x10^{9}/ 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}	1.50 ± 0.10^{a}	1.50 ± 0.43^{a}	1.50 ± 0.02^{a}	2.00 ± 0.05^{a}	1.50 ± 0.01^{a}
Eos x10^{9}/ 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}	1.50 ± 0.02^{ab}	2.00 ± 0.00^{ab}	3.00 ± 0.71^{b}	1.50 ± 0.01^{ab}	1.00 ± 0.00^{ab}
Hetero:Lym ratio	0.36 ± 0.00	0.52 ± 0.04^{d}	0.16 ± 0.01^{a}	0.32 ± 0.05^{bc}	0.36 ± 0.02^{bc}	0.36 ± 0.01^{bc}	0.47 ± 0.03^{d}	0.31 ± 0.04^{b}	0.30 ± 0.00^{b}	0.38 ± 0.02^{c}

Key: The above values are means of triplicate data, mean values in each row with similar superscripts are 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, Hetero:Lym ratio = Heterophil/lymphocytes ratio

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4.13 PLASMA BIOCHEMISTRY PARAMETERS OF *Clarias gariepinus* JUVENILES IN INITIAL AND AFTER THE FEEDING EXPERIMENT OF ONION BULB AND WALNUT LEAF SUPPLEMENTED DIETS FOR 84 DAYS

The results of the study showed that the highest value of total protein was obtained in WL 7and least in OB 4 after the experiment. There were significant differences ($p<0.05$) among the treatments. Albumin value obtained was higher than the initial with the highest value obtained in WL 7 and the least in OB 4 after the feeding trial, though not significantly differences ($p>0.05$). The highest globulin value was recorded in WL 7 and the least in WL 6, there were significantly different ($p<0.05$) among the treatments and the highest value of albumin/ globulin ratio were recorded in OB 2 and WL 7 and the least in WL 6 after the experiment. There were significant different ($p<0.05$) among the treatments (Table 4.13).

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Table 4.13: Plasma Biochemistry Parameters of *Clarias gariepinus* Juveniles in initial and after the feeding experiment of onion bulb and walnut leaf supplemented diets for 84 days.

Parameters	Initial	After feeding experiment								
		CONTROL	OB2	OB3	OB4	OB5	WL6	WL7	WL8	WL9
Total protein (g/dl)	3.10±0.14	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 (g/dl)	0.95±0.35	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 (g/dl)	2.15±0.49	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	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: The above values are means of triplicate data, mean values in each row with similar superscripts are not significantly different ($p > 0.05$)

A.G ratio = Albumin /Globulin ratio

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4.14 PLASMA BIOCHEMISTRY PARAMETERS OF *Clarias gariepinus* JUVENILES IN PRE- CHALLENGE AND POST CHALLENGE TEST FED ONION BULB AND WALNUT LEAF SUPPLEMENTED DIETS FOR 28 DAYS.

The results revealed that the highest value of total protein was obtained in OB 2 and the least in OB 4, WL 6 and WL 7. These values were higher than pre-challenge value. There were not significantly different ($p>0.05$) among the treatments. Globulin was highest in OB 5 and least in OB 3, but there were no significant differences ($p>0.05$) among the treatments. Albumin recorded highest value in WL 7 and the least in OB 5 and highest albumin /globulin ratio was recorded in OB 3 and the least in control (Table 4.14).

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Table 4.14: Plasma Biochemistry Parameters post challenge test of *Clarias gariepinus* Juveniles fed onion bulb and walnut leaf diets for 28 days.

Parameters	Pre - challenge	Post challenge								
		CONTROL	OB2	OB3	OB4	OB5	WL6	WL7	WL8	WL9
Total protein (g/dl)	3.10±0.14	$4.40±0.10^{a}$	$4.95±0.35^{a}$	$4.50±0.20^{a}$	$3.90±0.85^{a}$	$4.25±0.55^{a}$	$3.90±0.50^{a}$	$3.90±0.30^{a}$	$4.35±0.45^{a}$	$4.80±1.20^{a}$
Albumin (g/dl)	0.95±0.35	$1.15±0.50^{abc}$	$1.90±0.30^{cd}$	$2.80±0.50^{e}$	$1.10±0.00^{ab}$	$0.95±0.15^{a}$	$1.10±0.50^{ab}$	$2.00±0.30^{d}$	$1.45±0.15^{abcd}$	$1.85±0.45^{bcd}$
Globulin (g/dl)	2.15±0.49	$3.25±0.05^{a}$	$3.05±0.25^{a}$	$1.65±0.15^{a}$	$2.30±0.90^{a}$	$3.30±0.40^{a}$	$2.80±0.40^{a}$	$1.90±0.60^{a}$	$2.90±0.30^{a}$	$2.95±1.65^{a}$
A.G Ratio	0.50±0.28	$0.30±0.00^{a}$	$0.60±0.50^{a}$	$1.70±0.50^{b}$	$0.50±0.40^{a}$	$0.50±0.00^{a}$	$0.55±0.05^{a}$	$1.20±0.50^{ab}$	$0.50±0.10^{a}$	$1.00±0.40^{ab}$

Key: The above values are means of triplicate data, mean values in each row with similar superscripts are not significantly different ($p > 0.05$)

Note:

A.G ratio = Albumin /Globulin ratio

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4.15 BLOOD SERUM OF *Clarias gariepinus* IN INITIAL AND AFTER FEEDING TRIAL OF ONION BULB AND WALNUT LEAF DIET FOR 84 DAYS

The highest values of AST and ALT were recorded in OB 3 and lowest in WL 8 respectively, these values were lower than those obtained before the experiment. There were no significant differences ($p>0.05$) among treatments (Table 4.15).

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Table 4.15: Blood Serum of *Clarias gariepinus* in initial and after feeding trial of onion bulb and walnut leaf diet for 84 days

Parameters	Initial	After feeding experiment								
		CONTROL	**OB2**	**OB3**	**OB4**	**OB5**	**WL6**	**WL7**	**WL8**	**WL9**
AST(IU/l)	151.00±1.31	132.00±4.24[a]	136.00±2.83[a]	141.50±9.19[a]	135.00±9.90[a]	131.0±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	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: The above values are means of triplicate data, mean values in each row with similar superscripts are not significantly different ($p > 0.05$)

Note: AST =Aspertate aminotransferase ALT =Alanine aminotransferase

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4.16 BLOOD SERUM OF POST CHALLENGE TEST OF *Clarias gariepinus* JUVENILES FED ONION BULB AND WALNUT LEAF RESIDUES FOR 28 DAYS

The result of the experiment shows that highest values of AST and ALT were recorded in control and lowest in WL 6 and OB 5 respectively. These values were lower than those obtained before the experiment. There were no significant differences ($p>0.05$) among the treatments (Table 4.16).

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Table 4.16: Blood Serum of post challenge test of *Clarias gariepinus* Juveniles fed onion bulb and walnut leaf residues for 28 days

Parameters	Pre - challenge	Post challenge								
		CONTROL	OB2	OB3	OB4	OB5	WL6	WL7	WL8	WL9
AST(IU/l)	151.00±1.31	124.00±1.20[a]	111.00±0.00[a]	122.00±2.00[a]	106.00±1.50[a]	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)	68.00±8.49	38.50±1.50[a]	32.00±0.00[a]	31.50±0.15[a]	25.50±0.50[a]	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: The above values are means of triplicate data, mean values in each row with similar superscripts are not significantly different ($p > 0.05$)

Note: AST =Aspertate aminotransferase ALT =Alanine aminotransferase

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4.17 ORGAN INDEX OF *Clarias gariepinus* FED ONION BULB AND WALNUT LEAF RESIDUES FOR 84 DAYS

The results of the experiment shows that highest liver was recorded in OB 3 and least in OB 4, spleen and heart recorded highest in OB 5 and lowest in OB 4 and other treatments respectively. There were no significantly different ($p>0.05$) among the treatments while kidney recorded highest value in WL 6 and lowest in OB 3 and OB 4 (Table 4.17).

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Table 4.17: Organ index of *Clarias gariepinus* fed onion bulb and walnut leaf residues for 84 days

Treatments	Liver	Spleen	Kidney	Heart
Control	0.007 ± 0.00^{ab}	0.002 ± 0.01^{a}	0.004 ± 0.00^{ab}	0.002 ± 0.01^{a}
OB2	0.008 ± 0.01^{ab}	0.002 ± 0.01^{a}	0.003 ± 0.02^{a}	0.002 ± 0.00^{a}
OB3	0.011 ± 0.00^{b}	0.002 ± 0.00^{a}	0.002 ± 0.02^{a}	0.002 ± 0.00^{a}
OB4	0.004 ± 0.01^{a}	0.001 ± 0.02^{a}	0.002 ± 0.00^{a}	0.002 ± 0.02^{a}
OB5	0.009 ± 0.02^{b}	0.003 ± 0.00^{a}	0.003 ± 0.01^{a}	0.003 ± 0.00^{a}
WL6	0.009 ± 0.01^{b}	0.002 ± 0.01^{a}	0.007 ± 0.00^{b}	0.002 ± 0.01^{a}
WL7	0.008 ± 0.00^{ab}	0.002 ± 0.00^{a}	0.005 ± 0.00^{ab}	0.002 ± 0.01^{a}
WL8	0.007 ± 0.01^{ab}	0.002 ± 0.01^{a}	0.004 ± 0.01^{ab}	0.002 ± 0.00^{a}
WL9	0.007 ± 0.00^{ab}	0.002 ± 0.01^{a}	0.005 ± 0.01^{ab}	0.002 ± 0.01^{a}

Key: The above values are means of triplicate data, mean values in each row with similar superscripts are not significantly different ($p > 0.05$)

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4.18 CHALLENGE TEST OF *Clarias gariepinus* FED ONION BULB AND WALNUT LEAF RESIDUES FOR 28 DAYS

The highest weight gain was recorded in WL 7 and lowest in control diet. The highest mortality was recorded in control diet and lowest in OB 2 and WL 8. The treated groups were significantly different ($p<0.05$) from the control diet. The highest level of protection was obtained in OB 2 and WL 8 and least in control diet. There were significant differences ($p<0.05$) among the treatments (Table 4.18).

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Table 4.18: Challenge test of *Pseudomonas aeruginosa* injected by intraperitoneal route and level of protection among *Clarias gariepinus* fed onion bulb and walnut leaf residues for 28 days

	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±0.01^{h}	6.04±0.30^{c}
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}	1^{a}	3^{a}
Mortality (%)	33.33^{e}	3.33^{a}	13.33^{d}	6.67^{b}	10.00^{c}	10.00^{c}	6.67^{b}	3.33^{a}	10.00^{c}
Relative level of protection	0^{a}	90^{e}	60^{b}	80^{d}	70^{c}	70^{c}	80^{d}	90^{e}	70^{c}

Key: The above values are means of triplicate data, mean values in each row with similar superscripts are not significantly different ($p > 0.05$)

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4.18.1 Some clinical observations in *Clarias gariepinus* after intraperitoneal injection with *Pseudomonas aeruginosa*

Plate 4.1: *C. gariepinus* with bloated/ speared head appearance

Plate 4.2: *C. gariepinus* with skin alterations

Plate 4.3: *C .gariepinus* with bleached skin

Plate 4.4: *C. gariepinus* with haemorrhage

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4.19 DERMAL WOUND HEALING OF *Clarias gariepinus* FED WITH ONION BULB AND WALNUT LEAF FOR 14 DAYS

The highest percentage healing and daily healing rate on lateral and caudal regions were observed in WL 8 and least in control at 7 days. There were significant differences ($p<0.05$) among the treatments (Table 4.19A).

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Table 4.19A: Wound healing of *Clarias gariepinus* fed with onion bulb and walnut leaf for 14 days

Treatment	Lateral part (area) 7 days				Caudal part (area) 7 days			
	Initial wound area (cm^2)	Change in wound area(cm^2)	% Healing	Daily healing rates	Initial wound area (cm^2)	Change in wound area (cm^2)	% Healing	Daily healing rates
Control	1.00	0.40	40^{a}	5.71^{a}	1.00	0.19	19^{a}	2.71^{a}
OB2	1.00	0.64	64^{cd}	9.14^{d}	1.00	0.44	44^{c}	6.29^{c}
OB3	1.00	0.70	70d^{e}	10.00^{e}	1.00	0.49	49^{d}	7.00^{d}
OB4	1.00	0.79	79^{f}	11.29^{g}	1.00	0.75	75^{f}	10.71^{f}
OB5	1.00	0.58	58^{c}	8.29^{c}	1.00	0.36	36^{b}	5.14^{b}
WL6	1.00	0.51	51^{b}	7.29^{b}	1.00	0.44	44^{c}	6.29^{c}
WL7	1.00	0.58	58^{c}	8.29^{c}	1.00	0.44	44^{c}	6.29^{c}
WL8	1.00	0.99	99^{g}	14.14^{h}	1.00	0.95	95^{g}	13.57^{g}
WL9	1.00	0.75	75^{ef}	10.71^{f}	1.00	0.64	64^{e}	9.14^{e}

Key: The above values are means of triplicate data, mean values in each row with similar superscripts are not significantly different ($p > 0.05$)

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DERMAL WOUND HEALING OF *Clarias gariepinus* FED WITH ONION BULB AND WALNUT LEAF FOR 14 DAYS

The highest percentage healing and daily healing rate on lateral region were observed in all the treatments at 14 days, there were no significant differences ($p>0.05$) among the treatments. The highest value on caudal region was recorded in WL 8, WL 9 and lowest in control and there were significantly different ($p<0.05$) among the treatments (Table 4.19B).

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Table 4.19B: Wound healing of *Clarias gariepinus* fed with onion bulb and walnut leaf for 14 days

Treatment	Lateral part (area) 14 days				Caudal part (area) 14 days			
	Initial wound area (cm^2)	Change in wound area(cm^2)	% Healing	Daily healing rates	Initial wound area (cm^2)	Change in wound area (cm^2)	% Healing	Daily healing rates
Control	1.00	1.00	100^a	7.14^a	1.00	0.80	80^a	5.71^a
OB2	1.00	1.00	100^a	7.14^a	1.00	0.91	91^b	6.50^b
OB3	1.00	1.00	100^a	7.14^a	1.00	0.97	97^c	6.93^c
OB4	1.00	1.00	100^a	7.14^a	1.00	1.00	100^c	7.14^e
OB5	1.00	1.00	100^a	7.14^a	1.00	0.99	99^c	7.07^d
WL6	1.00	1.00	100^a	7.14^a	1.00	0.99	99^c	7.07^d
WL7	1.00	1.00	100^a	7.14^a	1.00	0.99	99^c	7.07^d
WL8	1.00	1.00	100^a	7.14^a	1.00	1.00	100^c	7.14^e
WL9	1.00	1.00	100^a	7.14^a	1.00	1.00	100^c	7.14^e

Key: The above values are means of triplicate data, mean values in each row with similar superscripts are not significantly different ($p > 0.05$)

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DERMAL WOUND HEALING OF *Clarias gariepinus* (MALE AND FEMALE) FED WITH ONION BULB AND WALNUT LEAF FOR 14 DAYS

The highest percentage healing and daily healing rate on lateral and caudal regions were observed in WL 8 and least in control at 7 days. These values were significantly different ($p<0.05$) among the treatments (Table 4.19C).

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Table 4.19C: Wound healing of Male and Female *Clarias gariepinus* fed with onion bulb and walnut leaf

Treatment	Male (*Clarias gariepinus*) 7 days				Female (*Clarias gariepinus*) 7 days			
	Initial wound area (cm^2)	Change in wound area(cm^2)	% Healing	Daily healing rates	Initial wound area (cm^2)	Change in wound area (cm^2)	% Healing	Daily healing rates
Control	1.00	0.38	38^a	5.43^a	1.00	0.22	22^a	3.14^a
OB2	1.00	0.58	58^c	8.29^c	1.00	0.44	44^b	6.29^b
OB3	1.00	0.70	70^d	10.00^e	1.00	0.49	$49b^c$	7.00^c
OB4	1.00	0.84	84^e	12.00^f	1.00	0.75	75^d	10.71^f
OB5	1.00	0.58	58^c	8.29^c	1.00	0.44	44^b	6.29^b
WL6	1.00	0.52	52^b	7.43^b	1.00	0.44	44^b	6.29^b
WL7	1.00	0.58	58^c	8.29^c	1.00	0.52	52^c	7.43^d
WL8	1.00	0.98	98^f	14.00^g	1.00	0.94	94^e	13.43^g
WL9	1.00	0.59	59^c	8.43^d	1.00	0.73	73^d	10.43^e

Key: The above values are means of triplicate data, mean values in each row with similar superscripts are not significantly different ($p > 0.05$)

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DERMAL WOUND HEALING OF *Clarias gariepinus* (MALE AND FEMALE) FED WITH ONION BULB AND WALNUT LEAF FOR 14 DAYS

The highest percentage healing and daily healing rate on lateral region were observed in all the treatments at 14 days, there were not significantly different ($p>0.05$) among the treatments. The highest value on caudal region was recorded in WL 8, WL 9 and lowest in control and there were significantly different ($p<0.05$) among the treatments (Table 4.19D).

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Table 4.19D: Wound healing experiment of Male and Female *Clarias gariepinus* fed with onion bulb and walnut leaf for 14 days

Treatment	Male (*Clarias gariepinus*) 14 days				Female (*Clarias gariepinus*) 14 days			
	Initial wound area (cm^2)	Change in wound area(cm^2)	% Healing	Daily healing rates	Initial wound area (cm^2)	Change in wound area (cm^2)	% Healing	Daily healing rates
Control	1.00	1.00	100^a	14.29^a	1.00	0.82	82^a	11.71^a
OB2	1.00	1.00	100^a	14.29^a	1.00	0.90	90^b	12.86^b
OB3	1.00	1.00	100^a	14.29^a	1.00	0.96	96^c	13.71^c
OB4	1.00	1.00	100^a	14.29^a	1.00	0.99	99^c	14.14^f
OB5	1.00	1.00	100^a	14.29^a	1.00	0.97	97^c	13.86^d
WL6	1.00	1.00	100^a	14.29^a	1.00	0.98	98^c	14.00^e
WL7	1.00	1.00	100^a	14.29^a	1.00	0.99	99^c	14.14^f
WL8	1.00	1.00	100^a	14.29^a	1.00	1.00	100^c	14.29^g
WL9	1.00	1.00	100^a	14.29^a	1.00	1.00	100^c	14.29^g

Key: The above values are means of triplicate data, mean values in each row with similar superscripts are not significantly different ($p > 0.05$)

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4.20 GUT MORPHOMENTRY OF *Clarias gariepinus* FED ONION BULB AND WALNUT LEAF DIETS FOR 84 DAYS

The results of the experiment revealed that the treated groups had better area of absorption compared to the control. The highest area of absorption was recorded in WL 8 and least in OB 5, there were significantly different ($p<0.05$) among the treatments while the highest cryptal depth was recorded in OB 4 and least in WL 7. There were significant differences ($p<0.05$) among the treatments (Table 4.20). The regression relationship between weight gain and surface area of absorption (villi length and width) of walnut leaf and onion bulb residues based diets revealed that the linear regression coefficient of walnut leaf and onion bulb treatment were 0.9877 and 0.7758 respectively of the surface area of absorption (appendix 1- 4). The result showed that walnut leaf diets positively correlated with the weight gain when compared to the onion bulb treatment.

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Table 4.20: Changes in villi length, villi width and cryptal depth of *C. gariepinus* fed onion bulb and walnut leaf residues for 84 days.

Parameters	Villi length (μm)	Villi width (μm)	Area of absorption (villi length x villi width) (μm^2)	Cryptal depth (μm)
Control	69.00±1.15^c	10.33±1.53^{ab}	712.77±0.00^c	19.00±1.41^{bc}
OB2	69.50±0.00^c	10.85±0.10^{abc}	754.08±2.82^d	16.00±0.00^{ab}
OB3	69.00±0.04^c	13.50±1.50^{def}	931.50±0.00^g	15.60±0.00^{ab}
OB4	67.50±0.05^c	16.00±1.73^g	1080.00±2.82^h	30.00±0.00^d
0B5	54.00±1.41^a	10.00±0.00^a	540.00±0.00^a	20.00±0.00^c
WL6	61.25±2.50^b	12.50±1.32^{cde}	765.63±0.00^e	18.00±0.82^{bc}
WL7	58.00±2.40^{ab}	15.00±0.00^{fg}	870.00±2.82^f	13.00±0.02^a
WL8	78.75±0.96^d	14.00±1.00^{ef}	1102.50±2.80^i	20.00±0.00^c
WL9	58.00±0.00^{ab}	12.00±0.00^{bcd}	696.00±0.00^b	20.00±0.00^c

Key: The above values are means of triplicate data, mean values in each row with similar superscripts are not significantly different ($p > 0.05$)

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4.21 HISTOPATHOLOGICAL CHANGES IN *Clarias gariepinus* JUVENILES FED ONION BULB AND WALNUT LEAF DIETS FOR 28 DAYS

The result of the experiment shows no visible lesion in intestine and testis of C. gariepinus in all the treatments, generalized fatty congestion and multifocal mild necrosis with mononuclear infiltration were observed in all the treatments while focal necrosis on the skin were observed in control, OB 2 and WL 6 and degeneration with vacuolation was observed in gill of control diet (Table 4.21).

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Table 4.21: Histological changes observed in the organs and tissues of *C. gariepinus* juveniles fed walnut leaf and onion bulb at the end of the challenge test experiment.

Organs and tissues	Histological changes	Control	OB2	OB3	OB4	OB5	WL6	WL7	WL8	WL9
Skin + muscle	Focal necrosis on the skin, More aggregate and focal SALT	½	½	-	-	-	½	-	-	-
Intestine	No visible lesion	-	-	-	-	-	-	-	-	-
Gill	Degeneration with vacuolation	½	-	-	-	-	-	-	-	-
Liver	Generalized fatty degeneration, multifocal mild necrosis with mononuclear infiltration.	½	½	½	½	½	½	½	½	½
Kidney	Highly congested	½	-	-	-	-	-	-	-	-
Testis	No visible lesion	-	-	-	-	-	-	-	-	-

Legends
½ = present but less marked (mild) than usual
- = no lesion and morphological changes in organ and tissue

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4.22 ECONOMIC ANALYSIS OF THE EXPERIMENTAL DIETS FED *Clarias gariepinus* FOR 84 DAYS

The results of the experiment shows that highest cost of producing 1 kg of feed was obtained in OB 5 and least in WL 6. There were significant differences ($p<0.05$) among the treatments. The highest cost of producing 1 kg of fish was recorded in WL 8 and least in control, there were no significant differences ($p>0.05$) among the treatments while economic conversion ratio was highest in WL 8 and least in control, there were significant differences ($p<0.05$) among the treatments. Also, highest cost of incidence was recorded in WL 9 and lowest in WL 8. There were significantly different ($p<0.05$) among the treatments (Table 4.22).

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Table 4.22: Economic analysis of differently graded level of onion bulb and walnut leaf fed *Clarias gariepinus* juveniles

INGREDIENTS	Price(#)/kg	Control	OB2	OB3	OB4	OB5	WL6	WL7	WL8	WL9
Fishmeal	350.00	74.38	74.38	74.38	74.38	74.38	74.38	74.38	74.38	74.38
Soybean	70.00	29.74	29.74	29.74	29.74	29.74	29.74	29.74	29.74	29.74
Maize	58.00	16.39	16.39	16.39	16.39	16.39	16.39	16.39	16.39	16.39
Vit-min*	180.00	7.20	5.40	3.60	1.80	-	5.40	3.60	1.80	-
Starch	50.00	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Vegetable oil	210.00	4.20	4.20	4.20	4.32	4.20	4.20	4.20	4.20	4.20
Chromium oxide	500.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
DCP*	250.00	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50
Salt	90.00	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Onion		-	3.90	7.80	11.70	15.60	-	-	-	-
Walnut leaves		-	-	-	-	-	2.30	4.60	6.90	9.20
Total feed intake (g)		57.67	61.97	61.64	62.17	61.15	60.62	60.72	60.23	58.58
Cost of feed (₦/kg feed)		139.61^{a}	141.71^{f}	143.81^{g}	145.91^{h}	148.01^{i}	140.11^{b}	140.51^{c}	141.11^{d}	141.62^{e}
Cost of flesh gain (₦/kg)		162.29^{a}	170.57^{a}	171.71^{a}	176.11^{a}	163.68^{a}	165.32^{a}	175.46^{a}	192.18^{a}	163.18^{a}
Profit index		1.16^{ab}	1.20^{bc}	1.19^{bc}	1.21^{bc}	1.11^{a}	1.18^{b}	1.25^{c}	1.36^{d}	1.15^{ab}
Incidence of cost		3.07^{ef}	2.97^{c}	2.99^{cd}	2.96^{c}	3.23^{g}	3.03^{de}	2.84^{b}	2.62^{a}	3.10^{f}
Economic conversion ratio		52.88^{a}	56.68^{c}	56.18^{b}	56.77^{d}	58.73^{f}	56.96^{e}	62.45^{h}	65.33^{i}	59.00^{g}

Note:
* Ingredients purchased at half (½) kg
Cost of feedstuffs at the prevailing markets prices in Nigeria (March, 2010): 1 US $ = ₦ 140.00

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CHAPTER FIVE

5.0 DISCUSSION

5.1 DETERMINATION OF PHYTOCHEMICALS IN ONION BULB AND WALNUT LEAF

Plants generally contain chemical compounds (such as saponins, tannins, oxalates, phytates, trypsin inhibitors, flavonoids and cyanogenic glycosides) known as secondary metabolites, which are biologically active (Soetan and Oyewole, 2009). Secondary metabolites may be applied in nutrition and as pharmacologically-active agents (Soetan and Oyewole, 2009). They have antibacterial and anti-parasitic properties. Plants are also known to have high amounts of essential nutrients, vitamins, minerals, fatty acids and fibre (Gafar and Itodo, 2011).

Flavonoids (quercetin) have inhibitory activity against disease - causing organisms in animals. Preliminary research indicates that flavonoids may modify allergens, viruses and carcinogens and so may be biological response modifiers. In vitro studies show that flavonoids also have anti allergic, anti – inflammatory, anti-microbial, anti – cancer and anti – diarrheal activities (Cushnie and Lamb, 2011). Tannins are plant polyphenols, which have ability to form complexes with metal ions and with macro-molecules such as proteins and polysaccharides (Dei *et al.,* 2007). Dietary tannins are said to reduce feed efficiency and weight gain in animal (Dei *et al.,* 2007). Environmental factors and the method of preparation of samples may influence the concentration of tannins present. Tannin presence influences protein utilization and build defense mechanism against micro organism (Cushnie and Lamb, 2011).

Saponins are glycosides, which include steroid saponins and triterpenoid saponins. High levels of saponins in feed affect feed intake and growth rate in animal (Dei *et al.,* 2007). Saponins (in excess), causes hypocholestrolaemia because it binds cholesterol making it unavailable for absorption (Soetan and Oyewole, 2009). Saponins also have haemolytic activity against red blood cell (RBC) (Ogbe and Affiku, 2011). Saponin-protein complex formation can reduce protein digestibility

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(Ogbe and Affiku, 2011). Saponins reduced cholesterol by preventing its reabsorption after it has been excreted in the bile. Proper food processing would reduce anti-nutrients (Akinyeye *et al.,* 2011).

The results obtained in this study showed the presence of alkaloids, cyanogenic glycosides, saponins, tannins and flavonoids in onion bulb and walnut leaves but anthraquinones were not detected in onion bulb and walnut leaves. The concentrations of these metabolites in the onion bulb and walnut were moderately available (+) (Table 4.1). Although, Ajaiyeoba and Fadare (2006) described that these secondary metabolites were present in higher concentration in walnut leaves (++). These variations can be explained by differences in agro-climatic conditions, age of trees, genotype, environmental factors, post-harvest treatments, the season of harvesting and maturation stage of the leaves have a strong influence on the phytochemical content of walnut leaves

Azu and Onyeagba, (2007) also ascribed the antimicrobial properties to the presence of flavonoid in onion bulb. Haniffa and Shanthi (2012) reported that the phytochemical screening of some medicinal plants revealed the presence of alkaloids, carbohydrates, flavonoids, saponnins and phenolic compounds which are associated with antimicrobial activities and curative properties against pathogen which are similar to the findings of this study.

5.2 MICROBIAL LOADS OF LIVER, INTESTINE, SKIN AND GILLS OF *Clarias gariepinus*

In fish health management, the most useful index of success is prevention of disease and systematic physicochemical analysis of the water and monitoring of the micro organism in the aquaculture system (Krishna *et al*., 2012). The epithelial surfaces of fish such as those of skin, gill or gastrointestinal tract are the first contact areas for potential pathogens (Narvaez *et al*., 2010). The result of this work revealed that the microbial counts in the liver, intestine, skin and gill of *C. gariepinus* varied with the skin and gills having the highest values of enterobacteriacea and total viable counts. This agrees with Shalaby *et al.,* (2006) that bacterial load is greater on the skin and gills than any part of fish as these parts are the constantly exposed to challenges. However, higher bacterial loads were observed in the guts of fish than surrounding water. This finding was similar to Ampofo and Clerk, (2010).

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5.3 DETERMINATION OF ANTAGONISTIC ACTIVITIES OF WALNUT LEAVES AND ONION BULB (DIAMETER OF INHIBITION ZONE, MM AND BACTERIAL COUNT)

The use of herbal extracts is widely expected to become an alternative therapy in aquaculture as a prophylactic and to control fish diseases. Studies on antimicrobial properties of herbal extracts against bacteria of fish culture important *in vitro* and *in vivo* are still limited, hence this study revealed that the extracts of walnut leaves and onion bulb (methanol and ethanol) respectively had antimicrobial activities against the tested pathogenic bacteria (*P. aeruginosa*, *B. subtilis, P. fluorescens, S. aureus, E. coli, S. typhi*). For walnut leaves, the widest zone of inhibition was obtained with *S. aureus* (13.5mm), followed by *P. aeruginosa* (12mm) and *B. subtilis* (12mm) while the least zone of inhibition was obtained in *S. typhi* (10mm) and *E. coli* did not show zone of inhibition. With onions, *B. subtilis* (12mm) had the widest zone of inhibition, followed by *P. fluorescens* (11mm), *S. aureus* (11mm), while the least was obtained from *E. coli* (9mm). These corroborate earlier reports that plant extracts are usually more active against gram positive bacteria than gram negative bacteria (Abu Shanab *et al.,* 2004; Basri and Fan, 2005).

The walnut leaves had better antibacterial activities with higher diameter of inhibition zone than the onion while onion had a better anti –fungal activity against *Aspergillus niger* than walnut leaves. The factors responsible for high value of antimicrobial activities in walnut leaves were not fully elucidated but could be due to the presence of two antibacterial constituents – walnut essential oil and juglore – that directly initiate steps against contagious microorganism, large concentration of vitamin C and presence of secondary plant metabolites (tannins, saponins, flavonoids, alkaloids etc). The fact that the methanolic extracts of walnut leaves and onion bulbs inhibited the growth of the tested bacteria (*P. aeruginosa*, *B. subtilis, P. fluorescens, S. aureus, E. coli, S. typhi*) was similar to the observations by Abu Shanab *et al.,* (2005) where the methanolic extract of the dried ripe berries of *Rhus coriaria* inhibited the bacteria studied. The results showed that the bacterial counts of the tested organism during antibacterial activities ranged from 5 - 48 with B. *subtilis* having the least bacterial count and *E. coli* the highest. This result was similar to that observed by Ajaiyeoba and Fadare, (2006), Azu and Onyeagba, (2007), Panghal *et al*., (2011) and Selvamohan *et al*., (2012).

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5.4 DETECTION OF MINIMUM INHIBITORY CONCENTRATION OF ONION BULB AND WALNUT LEAF

The minimum inhibitory concentration of walnut leaves and onion bulb revealed that 500 µg/ml was the least concentration that prevented the growth of bacteria after 24 - hour incubation. *S. aureus* and *E. coli* had 125µg/ml and 31.3µg/ml, respectively for onion while *S. aureus* had 250µg/ml for walnut leaves.

Muniruzzaman and Chowdhury (2004) also reported that the extracts obtained from bulb of onion (*Allium cepa*) had inhibitory effect on *Pseudomonas fluorescens* at minimum inhibitory concentration (MIC) 1.2mg/ml. This report agreed with the present study in which *Allium cepa* showed inhibitory activity against *Pseudomonas fluorescens*. The ethanol extract of medicinal plants screened showed antibacterial activity and inhibited the growth of bacterial strains, *Pseudomonas aeruginosa, Bacillus cereus* and *Escherichia coli* (Yadav and Khan, 2012), which was similar to that observed in this present study.

Ethanol extract of onion bulb and methanol extract of walnut leaf showed very high potentials as antimicrobial agents and 500µg/ml minimum concentrations prevented the growth of bacteria. This could be further explored for improve productivity in aquaculture industry. These strong antimicrobial activities could be due to the presence of various phytoconstituents such as alkaloids, flavonoids, tannins, saponins and sterols (Ravikumar *et al*., 2010).

5.5 PROXIMATE COMPOSITION OF EXPERIMENTAL DIETS FED *C. gariepinus* FOR 84 DAYS

Experimental diets were formulated with different levels of walnut leaf and onion bulb residues for *C. gariepinus* juveniles. The proximate composition of the diets showed highest moisture content in diet 6 and the least in diet 9, the highest value of crude protein was recorded in diet 8 and least in diet 4. The values of crude protein recorded in all treatments of this study were similar to findings of Degnani *et al*., (1989) who concluded that gross protein requirement for *C. gariepinus* juveniles were 38 – 42% crude protein respectively for warm water fish. Also, Olaifa and Bello, (2011), Olaifa *et al.,*(2012) who reported 35 – 40% crude protein diet for *Clarias gariepinus* juveniles agreed with the present study and the proximate composition of the experimental diets of this study supports the growth of *C. gariepinus* juveniles.

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Eyo, (1995) stated that for growth at maximum rate, fry and juveniles must have a diet in which nearly half of the digestible ingredients consist of balanced protein.

5.6 BODY CARCASS COMPOSITION OF *C. gariepinus* FED EXPERIMENTAL DIETS FOR 84 DAYS

The proximate composition of *Clarias gariepinus* juveniles before and after experiments is presented in table 4.6. The crude protein content of the fish varied between the initial and end of the experimental period. The crude protein level of the fish increased significantly ($P < 0.05$) during the experiment with highest value recorded in treated group, WL 9 (71.25 ± 0.04) compared to the value 70.32 ± 0.04 of control and 47.46 ± 0.01 of initial. The reason being that fish fed onion bulb and walnut leaf residue - based diets showed increased growth response and high protein deposition compared to control and the value recorded before experiment might be that 'free' amino acid was better utilized or growth promoting constituents present in onion bulb and walnut leaf and amino acid profile in the combine ingredients might have formed a better balanced diet for the juvenile catfish, *Clarias gariepinus* or enhancement of hormones and repatriating agents that alter the physiology and bio-metabolites in the fish. The plant component such as tannin, flavonoids, and saponins were beneficial in promoting growth, antimicrobial activities, healing wounds and to stimulate the immune response in fish.

The results indicate that the diets supported the growth of fish as increased body protein levels were recorded in all the treatments. This also showed that the protein requirement for the African catfish was met for body maintenance and growth. The reason for this might be as a result of presence of growth promoting constituents in walnut leaf and onion bulb. The higher body protein deposition and increased weight gain is indicative of the adequacy of the protein content and higher protein intake. This result agrees with the findings of Fagbenro *et al.*, 1992 of higher body deposition and weight gain at 40% crude protein for *Heterobranchus bidosalis* fingerlings fed compound diets and Dada *et al.*, 2001.

Furthermore, the ash content recorded was significantly increased in all the diet treatments compared to the control. The result of the experiment is similar to Oresegun and Alegbeleye (2001) who reported highest ash level in the diet 6 treated with methionine and the least in diet 1 (control). Similarly, Dada *et al.*, 2001 reported

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that the ash content of fingerlings after 84 days of feeding were significant (P< 0.05) higher than the initial ash content.

5.7 WATER QUALITY PARAMETERS OF EXPERIMENTAL TANKS OF CULTURED *C. gariepinus* FED ONION BULB AND WALNUT LEAF FOR 84 DAYS

The water quality parameters of the experimental tanks, temperature, dissolved oxygen and pH were closely related. The highest temperature was recorded in WL7 and WL8 while the least value was recorded in control. The temperature, dissolved oxygen and pH measured during the experiment (Table 4.7) were within recommended limits for warm water fishes (Boyd, 1981). Hogendoorn *et al.*, (1983) reported that the optimum temperature for the growth of small *C. gariepinus* between (0.5 – 5 g) was 30^0C and for large (25g) was 25^0C. Olaifa and Bello, (2011) reported 25^0C, 6.4mg/l and 7.10 for temperature, dissolved oxygen and pH respectively for *C. gariepinus* juveniles. Also, Olaifa *et al.* (2012) reported 25^0C -26^0C, 6 -7mg/l and 7.0 for temperature, dissolved oxygen and pH respectively for *C. gariepinus* juveniles. From the result obtained walnut leaf and onion bulb residues could be used in aquaculture as they did not significantly alter the water quality.

5.8 MICROBIOLOGICAL ANALYSES OF WATER

Microbiological analyses showed that enterobacteriacea populations in water were higher in the control than treated groups containing onion bulb and walnut leave residues at 4, 8 and 12 weeks. The values decreased in all the treated groups with increasing inclusion levels. The lowest enterobacteriacea populations were recorded in OB5 among onion bulb containing residue treatments and WL9 in walnut leaf residue treatments for weeks 4, 8 and 12.

Total viable count (TVC) of bacteria from the water of *Clarias gariepinus* fed onion bulb and walnut leaves showed that the TVC was higher in the control diets at 4, 8 and 12 weeks; the lowest TVC was recorded in OB5 (1.5% inclusion) in onion bulb residue treatments and WL9 in walnut leaf residue treatments for water during the same period of time. The total viable counts of bacteria obtained from this study decreased in all the treated groups with increasing inclusion levels of OB and WL.

Observations revealed that the bacterial loads in the water of the experimental tanks were affected by the presence of *A. cepa* and *T. conophorum* in the diet than the

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control. Also, the enterobacteriacea and total viable count in water sample for 4, 8 and 12 weeks were significantly different ($P < 0.05$) from the control. This agree with Shalaby *et al.* (2006) who observed decreases in bacterial load of water with which *O. niloticus* was fed different graded levels on diet containing garlic and chloramphenicol. Sugita *et al*. (1989) found that bacterial count in growing water of puffer fish (*Fugu niphobles*) housed in glass aquaria ranged from 10^4 – 10^5 CFU/ml which is in also agreement with the present work.

The total viable counts (TVC) of bacteria from water with *C. gariepinus* fed diets with onion bulb and walnut leaf residues were lower than the water from the control. The TVC was lower than that reported by McKeon *et al.* (2001) in pre-filtered water of recirculating systems (10^6 cfu/100 ml), but in filtered water it was 4.20 $\log_{10}$ cfu/100 ml which also agree with result obtained in 8 weeks and 12 weeks of the study. The antimicrobial effects of walnut leaf and onion bulb could lead to reduction in the microbial load of water in experimental tanks, these effects inhibited the growth of microorganisms or pathogenic bacteria that could cause infection.

5.9 MICROBIOLOGICAL ANALYSES OF *Clarias gariepinus* FED ONION BULB AND WALNUT LEAF RESIDUES FOR 84 DAYS

Enterobacteriacea loads in skin, liver, gills and intestines of *C. gariepinus* on control diet were higher than the treated groups. The lowest enterobacteriacea was recorded in OB5 (0.5% inclusion) in onion bulb residue treatments for skin, gill, liver and intestine at 4, 8 and 12 weeks and WL9 (2.0% inclusion) in walnut leaf residue treatments for skin, gill, liver and intestine at 4 weeks, 8 weeks and 12 weeks. The values decreased in treated groups as the level of inclusion (0.5%, 1.0%, 1.5% and 2.0%) increased and as the months increased.

The total viable counts of bacteria in skin, liver, gills and intestine of *C gariepinus* of the control fish were higher than for onion bulb and walnut leaf residues – based diets at 4, 8 and 12 weeks. The lowest TVC values were recorded in OB5 among onion bulb residue treatments and WL 9 at 4, 8 and 12 weeks.

TVC values decreased in treated groups as the levels of inclusion (0.5%, 1.0%, 1.5% and 2.0%) of OB and WL increased and as the months increased. The results of enterobacteriacea counts from this present study revealed that bacteria load in skin, liver, gills and intestine of *C. gariepinus* fed *A. cepa* and *T. conophorum* were lower than the control. Enterobacteriacea and total viable count of (skin, liver, gill and

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intestine) for 4, 8 and 12 weeks were significantly different ($P< 0.05$) from the control. The reason for this might be due to the presence of antimicrobial properties (flavonoids, tannin, saponins, alkaloids etc) present in walnut leaf and onion bulb. This finding was different from the report of Al- Harbi and Uddin, (2003) that total bacteria count from intestine of Nile tilapia in earthen pond was 6.53 – 7.76 $\log_{10}$ CFU/g which was higher than the present findings. Treatment with *T. conophorum* was more effective in reducing bacteria in skin, liver, gills and intestine than onion bulb residue treatment and control. The findings of this study is in accord with the work of Shalaby *et al*., (2006) that muscles and intestine of *O. niloticus* fed *Allium sativum* and chloramphenicol on different graded levels were decreased as the inclusion level increased. Also, Shalaby *et al*., (2006) revealed that coliform count from the intestine of fish fed garlic diet was 4.78 – 5.69 $\log_{10}$ CFU/g and in fish fed on chloramphenicol diet was 3.48 – 5.45 $\log_{10}$ CFU/g this report was in agreement with the present findings.

Since antimicrobial effects of walnut leaf and onion bulb residues resulted in reduction in microbial loads in water and on fish, the inclusion of these plants as a replacement or additive in fish feed could aid productivity in aquaculture industry. Their use in aquaculture industry is safe since it is highly biodegradable and do not have any side effects (Blumenthal *et al*., 2000) such as drug resistance that have been generally reported in synthetic antibiotics.

5.10 GROWTH PERFORMANCE AND NUTRIENT UTILIZATION OF *Clarias gariepinus* FED ONION BULB AND WALNUT LEAF DIETS FOR 84 DAYS

The initial mean body weight and initial length of *C. gariepinus* were 7.39±0.29g and 10.75±0.02cm respectively. At the end of the experiment there was general increase in weight (fig. 4.1), with highest weight gain in WL 8 and lowest in control. The fish showed good appetite to all the diet treatments manifested to by the increase in body weight and standard length. The highest growth performance was observed in fish fed 1.5% walnut leaves (61.21±11.20g) and 1.5% onion bulb (56.70±04.61g). The treated groups had a better growth than the control diet. There were no significant differences ($P< 0.05$) in the final body weight among the fish fed on diet containing onion bulb and walnut leaf – based diets and the control.

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The result of the experiment shows that the onion bulb and walnut leaf residue – based diets increased the body weight than the control. Such increase in the body weight of fish fed on onion bulb and walnut leaf supplemented diets could be attributed to the improved digestive activity by enhancing the synthesis of vitamins, cofactors, enzymatic activity and hormones in the fish. The higher values obtained in the treated groups could also be due to the presence of growth stimulants or constituents in onion bulb (flavonoids and thiosulfinates) and walnut leaves (alkaloids and tannins) as reported by Azu and Onyeagba (2007) and Kumar and Anantharaja (2007). These properties could contribute to improving the digestion and nutrient absorption with a subsequent increase in the fish-weight. This result agrees with Shalaby *et al.*, (2006) who obtained highest growth performance in *Oreochromis niloticus* with 30g garlic /kg diet. Also, Diab *et al.,* (2002) obtained the highest growth performance in *O. niloticus* with 2.5% garlic /kg diet. Abou- Zeid, (2002) showed that *Allium sativum* supplementation positively affected *O. niloticus* biomass and specific growth rate (SGR).

Specific growth rate and condition factor reflect the health status in fish (Ibrahem *et al.*, 2010). The results of specific growth rate revealed that WL 8 (1.09±0.11g) had better growth rate compared to the control, there were no significant differences among the treatments ($p > 0.05$).

Feed Conversion Ratio (FCR) is used to assess feed utilization and absorption (conversion of feed to flesh). FCR was best (2.16 ± 0.02) with WL 8 (1.5% inclusion) and least recorded in control (2.64 ± 0.03). The result revealed that diet containing walnut leaves at 1.5% inclusion (WL 8) was better utilized by *C. gariepinus* juveniles than onion bulb and control diets. There were no significant differences ($P > 0.05$) among the treatments.

The result of the experiment shows that the group fed WL 8 recorded the highest value of protein efficiency ratio and nitrogen metabolism of 1.34±0.28g and 1582.01±0.34g respectively. The best growth performance was observed in WL 8 (1.5%) followed by WL 7 (1.0%) of walnut leaves, followed by OB4 (1.5%) of onion bulb while the lowest was recorded in the control. Feed Efficiency Ratio (FER) and Protein Efficiency Ratio (PER) are used as quality indicators for fish diet and amino acid balance. These parameters are used to assess protein utilization and turnover.

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These results are also in agreement with those obtained by Shalaby *et al.*, (2006) who recorded increase in FCR, FER and PER on *O. niloticus* with 30g garlic/kg diet compared to the control which had the least value and Khattab *et al.*, (2004) had increased feed intake, FCR, PER, and body composition (crude protein, ether extract, ash, and moisture) in fish.

However, the survival rate in control, OB2 - OB4 and WL8 showed a similar trend (100% survival) while the least value of 90% was recorded in OB5, WL6, WL7 and WL9. The robustness and general well – being of the fish fed different graded levels of onion bulb and walnut leaf residues – based diets as expressed by the condition factor (k) was best in WL 8 (1.5% inclusion) with a gain of 2.74 ± 0.37 from the initial body status while the least gain of robustness (1.99 ±0.19) was recorded in WL 6 (0.5% inclusion). There were no significant differences (P> 0.05) among the treatments. All the fish were in better condition at the end of the experiment than they were at the beginning (see Table 4.10). Ibrahem *et al.*, (2010) reported that the body weight gain, specific growth rate, condition factor and survival rate were significantly higher (P< 0.05) in fishes fed basal diet supplemented with insulin and vitamin C than the control which is in accord with the present study.

The presence of vitamins such as vitamin C in walnut leaf and onion bulb could promote growth as well as having antioxidant property in the treated groups compared to the control. The uses of walnut leaf and onion bulb residue in fish feed could be a novel approach to aid productivity in aquaculture industry.

5.11 HAEMATOLOGICAL PROFILE OF *Clarias gariepinus* FED ONION BULB AND WALNUT LEAF DIETS IN INITIAL AND AFTER FEEDING TRIAL FOR 84 DAYS

Haematology is widely used in clinical diagnosis in aquatic and terrestrial animals. The application of haematological techniques is therefore valuable in fish biology in the assessment of fish health and stress response (Olukunle, 1996). Examination of the fish before and after the experiment revealed that the fish were in good conditions free from disease and infections. However, there were decreases in few haematological and biochemical parameters at the end of the experiment when compared with the value recorded before the experiment. This could be attributed to stress encountered during sampling, capture and handling procedures, which tended to increase the cathecholamine secretion which may result into haemaconcentration in

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the fish. However, Osuigwe *et al*., (2005) reported that MCV, MCH and MCHC could be used as a toxicological tool to monitor health status of animals.

This study revealed that supplementation of feed with onion bulb and walnut leave residues induced increases in the blood parameters (erythrocyte count, heamoglobin content and hematocrit value) in treated fish. The increased value of erythrocytes count, haemoglobin, hematocrit at the end of the experiment of the feeding trial, indicated the safety of onion bulb and walnut leaf residues supplemented diets used and their efficacy in improving the health status as a reduced hematocrit can indicate that the fish are not eating or suffering infections (Blaxhall, 1972). Improvement in the haematological parameters observed in the study indicated positive contribution of walnut leaf and onion bulb diets protein content in blood formation and improving immunity in the fish. This suggests that walnut leaf and onion bulb residues could enhance non- specific immune responses. The result also, shows that white blood cell (WBC) recorded in the treated groups were higher in values compared to the control which reflected that fish fed walnut leaf and onion bulb residue - based diets were able to build immunity against pathogens.

There was an increase in the heterophils/lymphocytes ratio in treated groups compared to the control and the value obtained before the experiment which indicates the protective function of the onion bulb and walnut leaf diets in *C. gariepinus*. The findings of the study support the results of Shalaby *et al.*, (2006) who reported a significant ($P < 0.05$) increase in erythrocyte count, heamoglobin content and hamatocrit value in *O. niloticus* fed graded level of garlic (*Allium sativum*) and chloramphenicol. Also, the results of Martins *et al*. (2002) showed that addition of *Allium sativum* and *Piaractus mesopotamicus* to fish diets increased erythrocytes number, heamoglobin content, hematocrit value, leucocytes and thrombocytes.

Azza *et al*., (2009) recorded that the erythrocyte-count was significantly higher with treated groups than the control. Das *et al.*, (2009b) reported that a significant ($P < 0.05$) increase in WBC and RBC were observed in groups 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 (flavonoids, alkaloids, tannins) that may play a role in the immune system stimulation and in the function of organs related to blood cell formation such as thymus and spleen (Tort *et al*., 2003).

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Furthermore, Osuigwe *et al*., (2005) recorded that depressed erythrocyte value (PCV, RBC and Hb counts) were found to characterize anaemia. From the result obtained in PCV, Hb and RBC during this study showed that the onion bulb and walnut leaf residues inclusion did not result in anaemia and this could support their use in aquaculture as safe plant immunostimulants. Blood indices (MCV, MCH and MCHC) are particularly important for the diagnosis of anemia in most animals (Coles, 1986). There was a decrease of MCV, MCH and MCHC in fish fed on onion bulb and walnut leaf residue – based diets at different inclusion rates which agree with Shalaby *et al*., (2006).

The differential leucocytic-count is an indicator of health in fish (Fox *et al*., 1997). The current study showed insignificant change in the count of the large lymphocytes, heterophils (increased) and monocytes (decreased) among the experimental groups. This is in agreement with the results of Shalaby *et al*., (2006) that reported insignificant change in the count of the large lymphocytes, heterophils and monocytes among the experimental groups. Cuesta *et al*., (2005) reported that water and ethanolic-extracts of propolis increased the percentage of phagocytes (monocyte-macrophages and acidophilic granulocytes) of gilthead Seabream. The neutrophil-count was significantly increased in blood of rainbow trout with infected bacterial or intoxicated with heavy metal (Witesta, 2005).

However, this result agrees with the work of Das *et al*., (2009b) that the feeding of algal and herbal diets supports the presence of antimicrobial properties of the algae *Euglena viridis*. The result of erythrocytes count of this research also showed decrease in all treated groups compared to the control. Changes in the physiological state often reflect alteration of hematologic and blood biochemical values. Clinical chemical analysis is a fundamental tool used to diagnose and predict the outcome of diseases and to monitor the effects of therapeutic, nutritional and environmental management in human and veterinary medicine (Shalaby *et al*., 2006).

5.12 HAEMATOLOGICAL PROFILE OF *Clarias gariepinus* IN PRE – CHALLENGE AND POST CHALLENGE TEST FED ONION BULB AND WALNUT LEAF RESIDUES FOR 28 DAYS

The values of PCV and RBC were generally increased in the treatments except WL9; OB4, WL9 respectively in post challenge test compared to the values obtained before experiment and the control of the post challenge test. The released RBC count

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recorded during the study might be due to the release of new RBC from the erythropoietic tissue to improve the oxygen – carrying capacity of the fish blood with resultant higher values of erythrocyte count as observed by Alkahem *et al*., (1998). The value of WBC was increased in the treated groups as compared with the control in post challenge test, although the variation was insignificant (P> 0.05) among the treatments. This was similar to the report of Das *et al*., (2009b) that reported an increase in WBC and RBC after 10 days challenge with *Aeromonas hydrophila* as compared with control and decreased in haemoglobin (Hb) content after 10 days challenge which is in accord with present findings as decreased haemoglobin (Hb) content was reported in the treatments except OB2, OB5 and WL8 as compared to control. Ajani, (2006) and Kori –Siakpere and Ubogu, (2008) reported that high WBC counts means a release of more cells to maintain homeostasis while low WBC counts is a common stress response. Therefore, increasing or decreasing numbers of WBC were normal physiological reactions to toxicant/ infections and these show the response of immune system under infections.

The report of the present study showed decrease in MCV, MCH and MCHC in post challenge test and it could be concluded that fish fed with onion bulb and walnut leaf supplemented diets were not anaemic and this was similar to the report of Osuigwe *et al*., (2005). The values of lymphocytes recorded in post challenge test were higher than in pre- challenge and the control of post challenge and the values were significantly higher (P< 0.05) in all the treated groups compared to the control in post challenge test.

The value recorded in control of post challenge test 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 Dugenci *et al*., (2003) that fish fed medicinal plants harbour a variety of specific and non – specific defence mechanisms against invading pathogens. The results of the present study revealed that increase in White Blood Cells (WBC) and lymphocytes following feeding of walnut leaf and onion bulb residues diets supported immunomodulatory 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).

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5.13 PLASMA BIOCHEMISTRY PARAMETERS OF *Clarias gariepinus* JUVENILES IN INITIAL AND AFTER THE FEEDING EXPERIMENT OF ONION BULB AND WALNUT LEAF SUPPLEMENTED DIETS FOR 84 DAYS

Result obtained from the present study shows an increase in total protein, albumin, globulin and albumin – globulin ratio. These findings were similar to results obtained by EL-Boushy and EL-Ashram, (2002) in African catfish reported that levamisole restored the total protein level in immunocompromised fish and increased γ-globulin levels in healthy fish.

However, Das *et al.*, (2009) reported that the serum total protein after long term feed with *E. viridis* increased in comparison to the control diet which supported the present findings. Siwicki (1989) 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. Wiegertjes *et al.*, (1996) reported that increases in serum protein, albumin and globulin levels are thought to be associated with a stronger innate immune response of fish. Hussein *et al.*, (2001) 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 (Shalaby *et al.*, 2006).

From present study, it could be inferred that the plants, onion bulb and walnut leaf – based diets had beneficial effects of improving the biochemical parameters of the *Clarias gariepinus*. Adequate quantity/ inclusion of these plants when incorporated in the regular feed of the fish will definitely improve its nutritive value and there by its growth, as well.

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5.14 PLASMA BIOCHEMISTRY PARAMETERS OF *Clarias gariepinus* JUVENILES IN PRE- CHALLENGE AND POST CHALLENGE TEST FED ONION BULB AND WALNUT LEAF SUPPLEMENTED DIETS FOR 28 DAYS.

Results obtained from the post challenge test showed lower levels of total protein in treated groups except OB2, OB3 and WL9 as compared to the control, there were no significant differences ($P > 0.05$) among the treatments. The albumin level was reduced in OB4, OB5 WL6 and increased in OB2, OB3 WL7, WL8 and WL9 as

compared to the control after the challenge test. The globulin were generally reduced in the treated groups except OB5 as compared to the control for post challenge test and the values obtained were insignificantly (P> 0.05) in the treated groups as compared to the control in post challenge test. The albumin and globulin ratio were higher in the treated groups compared to 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 to the control. Similar trend was observed by Alkahem *et al*., (1998) who attributed the reduction in total protein as a means of meeting an increased energy demand by fish to cope with detrimental conditions imposed by a toxicant/infections.

Results of the present findings agree with Das *et al*., 2009 that reported a reduction in values of total protein, albumin level and globulin content and non significant decrease in the globulin content of post challenged fish as compared to the control, increase in values of albumin and globulin ratio at 10 days post challenge with *A. hydrophila* as compared to the control which also support the present findings that revealed higher values in the treated groups compared to the control.

Increases in values of total protein, albumin, globulin level and albumin and globulin ratio of treated groups of post challenge test compared to pre – challenge and the control of post challenge are thought to be associated with a stronger innate immune response of fish. In this study, higher serum protein along with higher bactericidal activity in treatment groups might be associated with higher synthesis of active protein which resulted in a stronger innate response in *C. gariepinus*.

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*. The result of this study shows that walnut leaves and onion bulb can be added/ included in fish diets without compromising the growth performance, survival, haematology and boost immunity against pathogenic organism in fish culture.

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5.15 BLOOD SERUM OF *Clarias gariepinus* IN INITIAL AND AFTER FEEDING TRIAL OF ONION BULB AND WALNUT LEAF DIET FOR 84 DAYS

Results of this study showed that serum aspertate aminotransferase (AST) and alanine aminotransferase (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 AST and ALT. This result was similar to Shalaby *et al*., (2006) 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 El-Shater *et al*., (1997) and Augusti *et al*., (2001), 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*. These results can be attributed to *Allium cepa and T. conophorum*, which may cause stabilized cell membrane and protect the liver against deleterious agents and free radical-mediated toxic damages to the liver cells. This is reflected in the reduction of liver enzymes, as AST and ALT activities often used in the assessment of the state of liver as well as some other organs. Velisek *et al*., (2006) reported that high value of ALT and AST when compared with control shows destruction of liver cells. *Allium cepa and T. conophorum* help the liver to maintain its normal function by accelerating the regenerative capacity of its cells.

5.16 BLOOD SERUM OF POST CHALLENGE TEST OF *Clarias gariepinus* JUVENILES FED ONION BULB AND WALNUT LEAF RESIDUES FOR 28 DAYS

Transamination is considered 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 AST and ALT compensate by providing α – Ketoglutarate (Salah El-Deen and Rogers, 1993).

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The result of the present study in post challenge test showed that AST and ALT activities decreased in all the treated groups as compared to the pre – challenge and control of the post challenge test. This report agrees with those of Shalaby *et al*., (2006) who found decreases in AST and ALT of *Oreochromis niloticus* at different inclusion levels of *Allium sativum* and chloramphenicol. Reduction in the values of AST and ALT of onion bulb and walnut leaf supplemented diets can be due to present of constituents (tannin, alkaloids, saponnins etc) of walnut leaves and onion bulb that may cause stabilized cell membrane and protect the liver against deleterious agents and free – radical- mediated toxic damages to the liver cell. Thus, from the present experiment, it is clear that dietary supplementation of walnut leaf and onion bulb residues at all dosages have enhancing effects on innate immunity and disease resistance against *P. aeruginosa.*

5.17 ORGAN INDEX OF *Clarias gariepinus* FED ONION BULB AND WALNUT LEAF RESIDUES FOR 84 DAYS

Observations during this study on organ index showed that the liver, heart, kidney and spleen i.e. the hepatosomatic and splenosomatic indices were not significantly increased in all the treated groups. This result was similar to Azza and Abd-El-Rhman, (2009) that crude propolis and its ethanolic-extract fed for 28 days on hepato-somatic index (HSI) (hepatosomatic and splenosomatic indices) of *Oreochromis niloticus* produced no significant variation among treatments and the control group. Fox *et al*., (1997) reported that the organosomatic indices are indicators of health (hepatosomatic index and splenosomatic index) which could be used to predict the health status of fish. There were no traces of oedema and high variation (increased in the values) of the intestinal organs, the inclusion of walnut leaf and onion bulb residues in the diet of *C. gariepinus* could be said to be safe and non-toxic

5.18 CHALLENGE TEST OF *Clarias gariepinus* FED ONION BULB AND WALNUT LEAF RESIDUES FOR 28 DAYS

A properly functioning immune system is critical in maintaining the fitness and health of an organism, thus the disease challenge is a way that provides an 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).

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The challenge infection was performed by injection of a pathogenic strain of *P. aeruginosa* with a dose of 0.5x 10^{-7} intraperitonealy. The infected fish in the present study were weak in the 1st week of the experiment and showed decrease in feed intake and weight gain (see table 4.15) however, feed intake and weights increased in the second week. This might be as a result of the action of the plants that stimulated digestion and assimilation of the feeds as well as prevent the growth and multiplication of microorganisms. Also, the plants enhance immune response to infection. The first mortality was recorded in the control experiment on the 5th day. The symptoms of this infection observed included lack of appetite, swimming abnormalities, bloated appearance, skin alteration, anorexia, shaking head, and mouth tumidity. These symptoms observed in the study were similar to those reported by Wang and Wang, (1997) and Zhang *et al*. (2009).

Results of the challenge test (Table 4.15) revealed decreased following challenge with *P. aeruginosa* were decreased 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 mortality percentage and level of protection (LP) in OBR 2 and WLR 8 respectively and the control value was 33.33%, 0% for mortality percentage and level of protection. Plant constituents such as flavonoids, saponins and tannins, once absorbed, influence many biological functions including protein synthesis, making them beneficial in a variety of disorders. 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 is evidenced with the increase in different immune parameters such as lymphocytes, heterophils and white blood cells in post-challenged fish.

The results of this study is in agreement with 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.*, (2009b) reported that mortality following challenge with *A. hydrophila* decreased in the group of fish fed with *Euglena viridis* incorporated diets compared to the control. Also, Ibrahem *et al.* (2010) reported that the level of protection (LP) 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%) their findings supported the present study that revealed treated groups had higher relative level protection than the control

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Moreover, Sharma *et al.*, 2010 reported that the challenge test with *A. hydrophila* showed that increased percent survival rate was highest in the treated groups when compared with the control which is also in agreement with the present study. The decrease in mortality rate with dietary onion bulb and walnut leaves after injection of bacteria, *P. aeruginosa* is in agreement with previous study 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). *O. mossambicus* treated with *Eclipta alba* leaf extract (Christybapita *et al.*, 2007).

Furthermore, mortality of fish challenged with *A. hydrophila* decreased in all mistletoe-treated groups compared to the control (Park and Choi, 2012). Similar reports regarding the reduced mortalities on challenge with *A. hydrophila* were reported (Chu, 2006; Abd El-Rhman, 2009), these reports support the present study that showed decrease mortalities in onion bulb and walnut leaf based supplemented diets compared to the control. 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 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 in onion bulb and walnut leaves helps in prevent the growth of bacterial and reduce the microbial load and inhibit pathogenic infection in the fish.

5.19 DERMAL WOUND HEALING OF *Clarias gariepinus* FED WITH ONION BULB AND WALNUT LEAF FOR 14 DAYS

Wound healing is a complex and dynamic process of restoring cellular structures and tissue layers in damaged tissues as closely as possible to its normal state. Wound contracture is a process that occurs throughout the healing process, commencing in the fibroblastic stage whereby the area of the wound undergoes shrinkage. In the maturational phase, the final phase of wound healing, the wound undergoes contraction resulting in a smaller amount of apparent scar tissue. Granulation tissue formed in the final part of the proliferative phase is primarily composed of fibroblasts, collagen, edema and new small blood vessels (Douglas and Alan, 2003).

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Results of the study shows that *C. gariepinus* cut on lateral and caudal parts, had better healing percentages and daily healing rates in onion bulb treatments and walnut leaf treatments than the control. WL 8 recorded better percentage healing and daily healing rate (99%, 14.14; 100%, 14.29) on the 7 days and 14 days for lateral part and (95%, 13.57; 100%, 14.29) for caudal part of *C. gariepinus* for 7 days and 14 days respectively compared to the control (40%, 5.71; 100% 14.29) for lateral part and caudal part (19%, 2.71; 80%, 11.43) on the 7 days and 14 days respectively. Also, the findings revealed that walnut leaves treatment were more effective in dermal wound healing than the onion bulb and the control which might be as result of the presence of vitamin C in the walnut leaf that might aid the wound healing (closure) .

This study shows that healing percentage and daily healing rates were better on the lateral than the caudal part. The reason may be due to presence of more flesh on the lateral part than the caudal part which had less flesh. The finding shows that the onion bulb and walnut leaf supplemented diets healed faster than the control. These results were similar to Omale and Isaac, (2010) that higher percentage of wound closure was observed in the group of animals treated with *Saba florida* on day 24 of the experiment compared to the control. Omale and Emmanuel, (2010) also reported that the wound closure rate was rapid in groups treated with *Euphorbia heterophylla* (Euphorbiaceae) leaf extract when compared with control rats. In addition, the study of Subhashini and Arunachalam, (2010) demonstrated that the *Adhatoda vasica leaves* promote wound healing activity in mice when compared with the control which support the present study.

Success of wound healing depends on sufficient nutrients being supplied to the wound site. Essential trace elements, especially zinc, selenium, copper and iron also influence the process of wound repair. They act as co-factors or co-enzymes in a number of metabolic functions involved in wound healing (Bhar *et al*., 2003).

Zinc, vitamin C contents and flavonoids derivatives of T. *Conophorum* and *A. cepa* might have contributed to the wound healing process, along with other phytochemical contents of the plant. It seems that the residue of *T. Conophorum* and *A. cepa* is effective in both inflammation and proliferation phases. Since Zinc is a co-factor of collagenase, a member of the metalloproteinase group, which assists to remove fibrinogen at the beginning of the healing process in inflammatory phase (MacKay and Miller, 2003). Zinc also contributes to DNA synthesis, cell division and protein synthesis, which occur in the proliferation phase. It also provides resistance to

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epithelial apoptosis via cytoprotection, probably through antioxidant activity of the cysteine-rich metallothioneins, against reactive oxygen species and bacterial toxins. Deficiency of zinc in body, either hereditary or dietary, can lead to delayed wound healing (Lansdown *et al*., 2007). Furthermore, improves the moisture holding capacity of skin, complexion, cell migration and cell regeneration, and thus speeds up the wound healing process (Datta *et al*., 2011). Vitamin C content of the extract (residue) might have a role in enhancing neutrophil migration and lymphocyte transformation along with collagen synthesis.

The walnut leaf (1.5% and 2.0% inclusions) and onion bulb (1.5% inclusion) residues demonstrated a significant increase in wound closure compared to the control. One of the phytochemical constituents (flavonoids, tannins, thiosulfinates and alkaloids) present in *T. conophorum* and *A. cepa* may be responsible for the wound-healing activity. Studies on *T. conophorum* and *A. cepa* have shown that phytochemical constituents like flavonoids, tannins, saponins and alkaloids were present and these are known to promote wound healing process mainly due to their astringent and antimicrobial properties, which appears to be responsible for wound contraction and increased rate of epithelization.

Flavonoids have therapeutic uses due to their anti-inflammatory, antifungal, antioxidant and wound healing properties (Okuda, 2005, Nayak *et al*., 2009). Moreover, flavonoids and their derivatives are known to decrease lipid peroxidation by improving vascularity and preventing or slowing down the progress of cell necrosis. Hence, any drug that inhibits lipid peroxidation is supposed to increase the viability of collagen fibrils by increasing the circulation and strength of collagen fibers, encouraging the DNA synthesis and preventing cell damage (Shetty *et al*., 2008). Flavonoids are also known to endorse wound healing processes primarily owing to their antimicrobial and astringent properties, which appear to be responsible for wound contraction and elevated rate of epithelization (Pesin *et al*., 2011).

The quicker process of wound healing in the treated group compared to control could be a function of either the individual or the additive effects of the phytochemical constituents (Subhashini and Arunachalam, 2010). The early tissue closure of the wound observed in the present study may have been contributed by the tannin content of *T. conophorum* and *A. cepa.* Tannins are the main components of many plant extracts and they facilitating healing of wounds. When a wound occurs and is exposed to external environment, it is more prone to attack by microbes which

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gain entry through the skin and delay the natural wound healing process (Patil and Sunil, 2008). Therefore, if a compound or a plant extract/ residue possess antioxidant potentials and antimicrobial activities, it can be a good therapeutic agent for accelerating the wound healing process (Subhashini and Arunachalam, 2010).

Moreover, male and female *C. gariepinus* dermal wound healing were investigated and the results revealed that male *C. gariepinus* healing percentage and daily healing rate were better in treated groups than the control. For the male *C. gariepinus,* the best performance was recorded in the group fed 1.5% inclusion of walnut leaf for percentage healing and daily healing rate (98%, 14.00; 100%, 14.29) on 7 days and 14 days respectively compared to the control (38%, 5.43; 100%, 14.29) in the 7 days and 14 days respectively while the female showed that WL 8 recorded better wound closure for percentage healing and daily healing rate (94%,13.43; 100%, 14.29) on 7 days and 14 days respectively than control (22%, 3.14; 82%, 11.71)

This study revealed that dermal wound healing in male *C. gariepinus* was better and faster than the female. This may be due to anabolic effects of the male and growth hormones. Presence of hormone such as androgens, estrogens and testosterones enhance wound healing in animals and in the remodeling phase of wound healing, estrogens can affect collagen content, tensile strength, and macroscopic appearance of scar tissue (Pesin *et al*., 2011). Percentage healing and daily healing rate at 7 and 14 days on lateral part and caudal part were significantly higher (P< 0.05) than the control. Also, the male and female percentage healing and daily healing rate respectively at 7 and 14 days on lateral part and caudal part were significantly higher (P< 0.05) than the control. Among the treated groups, walnut leaf were more effective than onion bulb treatment most especially in the group fed 1.5% inclusion of walnut leaf which had complete healing on 6th day and without any traces of scar on the 9th day while other treated groups had their complete healing from 10th day with or without scar, but the control had complete healing on 12th day with slight traces of scar on the 14th day. The reason for the performance (wound closure) might be due to high quality of wound healing properties, antimicrobial properties and growth promoting effect present in walnut leaf than the onion bulb treatment and the control.

The wound-healing property of *T. conophorum* and *A. cepa* may be attributed to the phytoconstituents present in the plant and the faster process of wound healing in walnut leaf residue could be a function of either the individual or the additive effects

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of the phytoconstituents (tannins and saponins) and antimicrobial in property, hence it can be inferred that the wound healing activity of the *T. conophorum* and *A. cepa* observed is due partly to its tannin and flavonoids contents, which seems to be responsible for wound contraction and increased rate of epithelization. The enhanced rate of wound contraction and reduction in healing time in treated fish, *C. gariepinus* when compared with control might be due to enhanced epithelization aciliated by *T. conophorum* and *A. cepa* compositions. The enhanced wound healing activity of *T. conophorum* and *A. cepa* could possibly be made use of clinically in healing of open wounds.

From the study carried out showed that the ethanol and methanol extract (residues) of *A. cepa* bulb and *T. Conophorum* leaf possesses a definite prohealing activity, there by justifying its use in the indigenous system of medicine in fish farming

5.20 GUT MORPHOMENTRY OF *Clarias gariepinus* FED ONION BULB AND WALNUT LEAF DIETS FOR 84 DAYS

Gut morphometry is a tool that can be used to evaluate the absorption of nutrients in animals and the assessment of the gut morphometry of the *C. gariepinus* fed different level of inclusions of walnut leaf and onion bulb residues – based diets showed significant values of mean villi length and width (area of absorption) ($p < 0.05$) in the treated groups compared to the control. This represents an increase in the absorptive surface area of the intestine and thus an increased absorptive capacity with the resultant higher body weight gain and FCR in the treated groups compared to the control. Also, there was significant mean cryptal depth (regeneration) in the treated groups than the control, which in turn enhanced digestion as cryptal cells had been reported to be responsible for secretion of the electrolytes which enhance water secretion into the intestinal lumen for the purpose of digestion (Bowen, 2011).

It could be suggested that digestibility which was enhanced in the treated groups compared to the control with highest body weight gain, percent weight gain and FCR of 53.81± 5.85, 727.16±1.40 and 2.16±0.02 in WL 8 could be due to the enhanced absorptive area and the increased cryptal depth recorded which is an indication of a higher mucosal proliferation activity and greater intestinal glandular activity. The report is similar to that of Zhous *et al*., (2000) that higher cryptal depth revealed more efficient digestibility and absorption in ingested feed.

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Although, the relationship between the treated groups revealed that the diets were better utilized and enhanced growth as a result of better surface area of absorption recorded in treated groups compared to the control, this may be as a result of growth promoting properties present in walnut leaf and onion bulb. The result of gut morphometry observed in *C. gariepinus* fed with onion bulb and walnut leaf residues compared with the control further showed the benefit of walnut leaf and onion bulb in enhancing and maintaining the intestinal mucosa.

It could be inferred that walnut leaf and onion bulb have the ability to increase the digestive and absorptive capacities of the intestine of *C. gariepinus* juveniles by increasing the cryptal depth, as well as absorptive surface area of the intestine (villi length and width).

5.21 HISTOPATHOLOGICAL CHANGES IN *Clarias gariepinus* JUVENILES FED ONION BULB AND WALNUT LEAF DIETS FOR 28 DAYS

The organ most associated with the detoxification and biotransformation process is the liver, and due to its function, position and blood supply (Van der Oost et al., 2003) it is also one of the organs most affected by contaminants in the water (Rodrigues and Fanta, 1998). Rahman *et al.,* (2002) also observed degenerative changes like hypertrophy, pyknosis, necrosis and vacuolation of hepatocytes in *Anabas testudineus*, *Chana punctatus* and *Barboids gonionotus* exposed to sub concentration of Diazion 60EC for 70 days. Gross and microscopic appearances of the liver, kidney, testis, intestine, gill and skin as presented:

5.21.1 LIVER

The liver of *C. gariepinus* in this experiment had brown colouration which revealed marked diffuse (fatty change) vacuolation of hepatocytes among the treatments (control – WL 9) after the experiment. This observation may be due to environmental stress and age of fish may cause changes in cellular function that alter the physiology of organ systems in the fish as reported by Vanvuren *et al.,* (1994). The lesion being mild showed that treatment with the plant does not have severe toxic effect as observed in fingerlings of *C. gariepinus* exposed to sub lethal concentration of the different part of the fruits of *Raphia hookeri* aqueous extracts where intense vacuolar degeneration of hepatocytes, periportal hepatic necrosis and fibrosis were observed (Adeogun, 1994).

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5.21.2 &3 TESTIS AND INTESTINE

There was no visible lesion in testis and intestine in all the treatments (control – WL9). This was in accord with Pyle *et al.*, (2002) that homeostatic regulation of intestinal diets by fish may not be toxic to fish. The result of the experiment revealed that onion bulb and walnut leaf residues – based diets could not alter the gastrointestinal tract and testis of *C. gariepinus* which mean that inclusion of onion bulb and walnut leaf in the diet of *C. gariepinus* may not have residual effect on the fish.

5.21.4 SKIN/MUSCLE

The observed focal and more aggregate of skin associated lymphoid tissue (SALTS), in the treatments except OB2 and WL6 is suggestive of an enhanced mucosal immunity response which can be explored in enhancing vaccination.

5.21.5 KIDNEY

Kidney of teleost fish is mainly involved with the excretion of toxicant. It maintains the delicate osmotic balance between the fish and its environment (Awaad, 1991). Kidney receives about 20% of cardiac blood output and it is mainly involved in the excretion of toxicants (Awaad, 1991). It also plays an important role as a haemopoietic organ. The result of the experiment shows that there is no visible lesion in the kidney of the fishes among the treatments which further strengthens the safety of the plants being used as feed additives.

5.21.6 GILL

The gill is a sensitive organ constituting as much as 50% of total body surface area (Adeogun, 2004). The gill epithelium is thin to allow gas exchange and this also renders it particularly vulnerable to invasion by pathogens (Roberts, 1978). The gill filaments had a reddish coloration due to suffusion with blood capillaries. Histopathological examination of the gills fish, *C. gariepinus* from the treatment groups showed no visible lesion except OB 2 that showed slight degeneration with vacuolation. The observed changes were similar to Shaw and Handy, (2006) who reported no visible lesion (NVL) in tilapia fed 2000Cu/kg diet for 42 days.

The result of the experiment shows no alteration in the organs and tissues. Also, since no visible lesion were observed in the treatments, it mean that walnut leaf and onion bulb residues are safe to be used as feed additives in the production of *C. gariepinus*

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5.22 ECONOMIC ANALYSIS OF THE EXPERIMENTAL DIETS FED *Clarias gariepinus* FOR 84 DAYS

Values of fish produced followed a similar trend as mean feed intake and mean weight gain. From the results of the experiment, it was observed that the treated groups had the highest mean feed intake compared to the control. 1.5% inclusion level of walnut leaf produced the highest weight gain which consequently resulted in the highest value of fish (that is, more income to the fish farmer). This observation has been corroborated by a similar observation by Nwanna (2003).

The findings of the study shows that WL 8 had the highest profit index (1.40), followed by WL 7 (1.30) and the least was obtained in OB5 (1.18), the reason for these observation may be due to high inclusion rate which increase the cost of producing the feed such as (OB5 at 2.0% inclusion). However, the best economic conversion ratio was recorded in WL 8 (80.70) and least in control (65.97). This result showed that more profit will be made when 1.5% inclusion of walnut leaf is added to fish feed. It is therefore recommended that walnut leaf residue, WL 8 (1.5% inclusions) in the diet of *C. gariepinus* should be absorbed by farmers, aquaculturists and other stakeholders since inclusion of 1.5% of walnut leaf residue promote growth and had highest profit index, lowest incidence of cost and best economic conversion ratio.

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CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 SUMMARY AND CONCLUSION

The results obtained from the study on nutritional and antimicrobial potentials of walnut (*T. conophorum*) leaf and onion (*A. cepa*) bulb residues to C. gariepinus have provided base line information on the potentials of the plants and their utilization by fish farmers, aquaculturist and scientists involved in fish health management. The phytochemicals screening of the plants revealed secondary metabolites; tannins, saponin, alkaloids, cyanogenic glycosides, anthraquinones and flavonoids. The preliminary in vitro antibacterial activities of the methanol and ethanol extracts of the walnut leaf and onion bulb were conducted against six (6) fish pathogens and their potency was assessed by the presence or absence of inhibition zone and zone diameters (Table 4.3), from the result, walnut leaf and onion bulb inhibited the growth of six pathogens and higher inhibition was obtained in walnut leaf.

However, minimum inhibitory concentration (MIC) of walnut leaf and onion bulb of doubling dilution of 2000µg/ml of walnut leaf and onion bulb extracts were made in 5ml volume of the broth to 3.192 µg/ml against six pathogens; *P. aeruginosa, S. aureus, S. typhi, B. subtilis, E .coli and P. fluorescens*, the results showed that walnut leaf and onion bulb inhibited the growth of the pathogens at 500 µg/ml (Table 4.4). Growth performance and nutrient utilization of walnut leaf and onion bulb were assessed on *C. gariepinus* for 12 weeks and at the end of the experimental phase, treatment with 1.5% inclusion of walnut leaf (WL 8) had better growth performance indices than one of the onion bulb and the control. The bacteriological study revealed that walnut leaf and onion bulb reduced the microbial load of the water sample and fish (skin, liver, gill and intestine) as the inclusion level increased and as the post inoculation days increased. Also, the challenge test was conducted on *C. gariepinus* {0.5ml of 10^7 *P. aeruginosa* of 24 h old culture (7.63 $\log_{10}$ cfu/ml)}, the results revealed that WL 8 had higher relative protection level and survival, 90%, 3.33% compared to the control that had 0%, 33.33% respectively.

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An investigation on dermal wound healing was conducted on *C. gariepinus* using walnut leaf and onion bulb at varying inclusion levels. The experiment showed that WL 8 had better healing rate compared to the control and other treated groups. The study further showed that walnut leaf and onion bulb residues did not adversely affect the haematological, biochemical, organ index and histological indices in *C. gariepinus* which can be used to predict the health status of fish. It also indicated that walnut leaf and onion bulb supplemented diets improved the growth, nutrient utilization, bacteriological, haematological indices and survival of *C gariepinus*. WL 8 showed the best performance out of the treated groups in terms of growth, nutrient utilization and relative protection level against *P. aeruginosa*, followed by WL 7 and OB 4 and the least in the control. It could be suggested that WL 8 (1.5% inclusion) or OB 4 (1.5% inclusion) could be added to fish feed to enhance healthy growth. The relative abundance and availability of walnut leaf and onion bulb makes them cheap natural nutritional and antimicrobial products to be explored in aquaculture.

6.2 RECOMMENDATIONS

Walnut leaf and onion bulb have potential nutritional and antimicrobial natural benefits for aquaculture as they are eco- friendly and environmentally acceptable. Their application or use as plant preparation for therapeutic, prophylactic or curative purposes, have great advantages over other methods of medication and treatments as they appear free from any side effects and their use in aquaculture could be said to be safe.

The active ingredients associated with the effects of walnut leaf and onion bulb could be further explored in tablet form so that fish farmers, aquaculturists and other stakeholders can use it at anytime to enhance healthy growth.

Further research should be carried out on interaction / synergetic effects of walnut leaf and onion on C. gariepinus.

Further research should be carried out to explore the potential of walnut leaf and onion bulb on other species of fish.

Further research should be carried out on oxidative stress status of fish fed with onion bulb and walnut leaf.

Further research should be carried out on effects of the plants on mucosal immunity of fish which could be explored in enhancing vaccination in fish.

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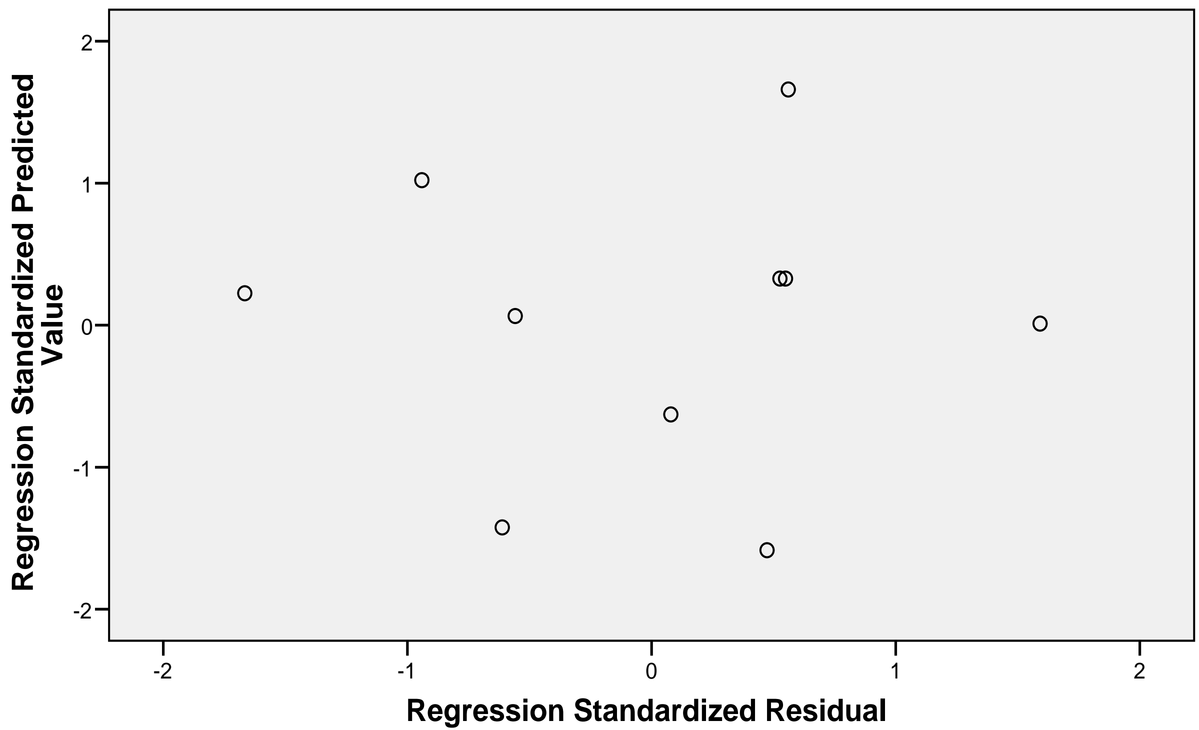
APPENDIX 1:

Regression analysis of onion bulb residue showing regression coefficient and scatter plot diagram of surface area of absorption (length of villi multiply by width of villi)

R^2=0.7758



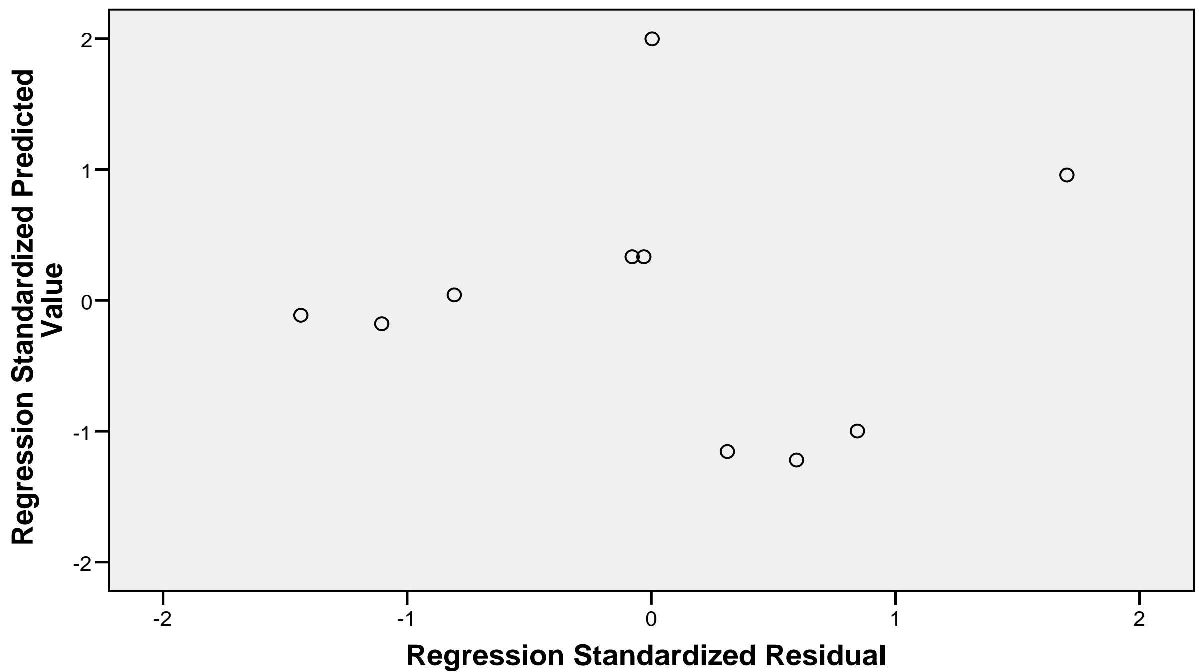
KEY

OTSAAB =Onion bulb residue surface area of absorption

APPENDIX 2:

Regression analysis of walnut leaf residue showing regression coefficient and scatter plot diagram of surface area of absorption (length of villi multiply by width of villi)

$R^2 = 0.9877$



KEY

WLTSAAB =walnut leaf residue surface area of absorption

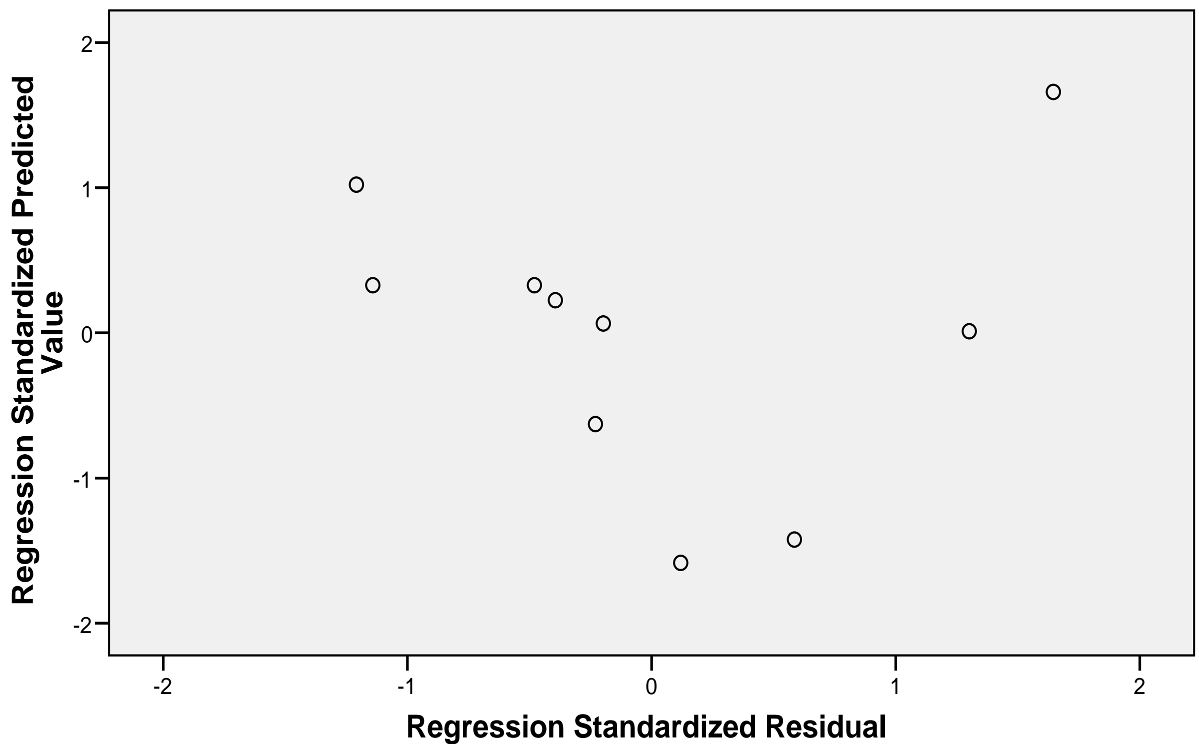
APPENDIX 3:

Regression analysis of onion bulb residue showing regression coefficient and scatter plot diagram of regenerative ability (crystal depth)

R^2=0.1952

Scatterplot

Dependent Variable: OTREG

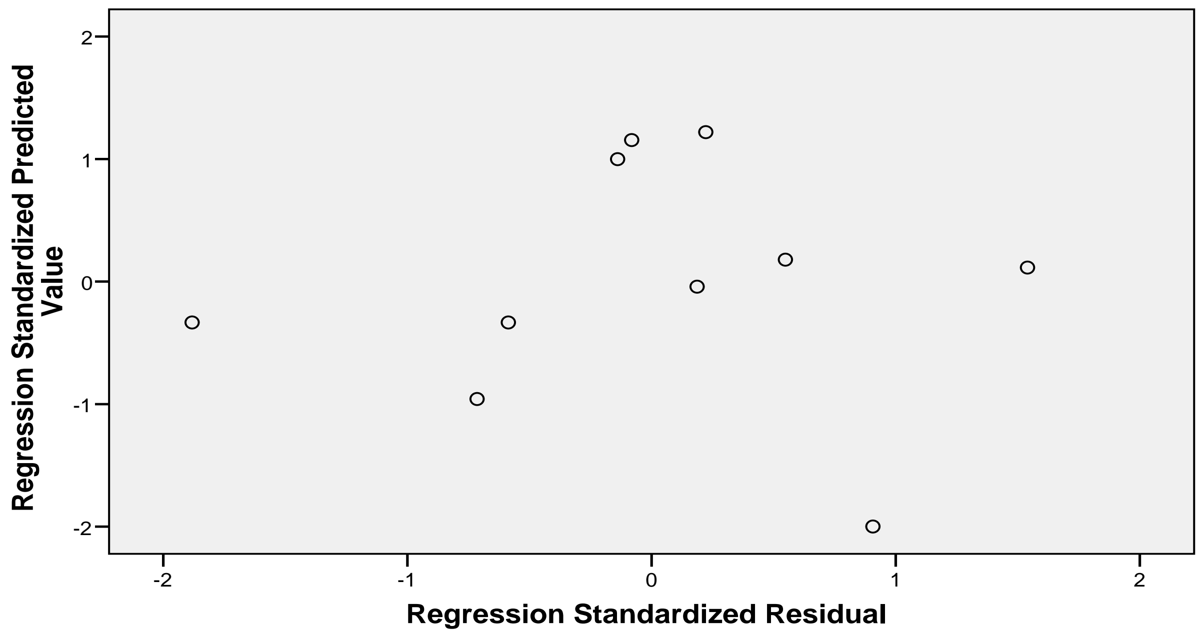


Key:
OTREG: onion bulb residue regeneration

APPENDIX 4:

Regression analysis of walnut leaf residue showing regression coefficient and scatter plot diagram of regenerative ability (crystal depth)

R^2=0.3991



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Key:
WLTREG: walnut leaf residue regeneration

APPENDIX 5:

Analysis of variance (ANOVA) table for biological evaluation (condition factor, specific growth rate, nitrogen metabolism, feed conversion ratio, percentage weight gain and weight gain) of *Clarias gariepinus* fed onion bulb and walnut leaf diet for 84 days

		Sum of Squares	Df	Mean Square	F	Sig.
CF	Between Groups	1.385	8	.173	.438	.882
	Within Groups	7.106	18	.395		
	Total	8.491	26			
SGR	Between Groups	.013	8	.002	.330	.943
	Within Groups	.087	18	.005		
	Total	.099	26			
NM	Between Groups	306951.128	8	38368.891	1.180	.363
	Within Groups	585375.891	18	32520.883		
	Total	892327.019	26			

		Sum of Squares	Df	Mean Square	F	Sig.
FCR	Between Groups	13.120	8	1.640	.309	.953
	Within Groups	95.468	18	5.304		
	Total	108.588	26			
PWG	Between Groups	30653.753	8	3831.719	.343	.937
	Within Groups	201272.615	18	11181.812		
	Total	231926.368	26			
WG	Between Groups	580.097	8	72.512	1.188	.359
	Within Groups	1098.493	18	61.027		
	Total	1678.591	26			

APPENDIX 6:

Analysis of variance (ANOVA) table for biological evaluation (final weight gain, protein efficiency ratio, protein productive value and protein intake) of *Clarias gariepinus* fed onion bulb and walnut leaf diet for 84 days

		Sum of Squares	Df	Mean Square	F	Sig.
FWG	Between Groups	58.461	8	7.308	.119	.998
	Within Groups	1101.906	18	61.217		
	Total	1160.367	26			
PER	Between Groups	.102	8	.013	.336	.940
	Within Groups	.681	18	.038		
	Total	.782	26			

		Sum of Squares	Df	Mean Square	F	Sig.
PPV	Between Groups	11.766	8	1.471	99.153	.000
	Within Groups	.134	9	.015		
	Total	11.900	17			
PI	Between Groups	7450.614	8	931.327	1.538	.267
	Within Groups	5450.750	9	605.639		
	Total	12901.364	17			

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APPENDIX 7:

Analysis of variance (ANOVA) table for blood biochemical (total protein, albumin, globulin, albumin/globulin ratio, aspertate aminotransferase, and alanine aminotransferase) of *Clarias gariepinus* fed onion bulb and walnut leaf diet for 84 days

		Sum of Squares	Df	Mean Square	F	Sig.
TP	Between Groups	15.250	8	1.906	2.094	.146
	Within Groups	8.195	9	.911		
	Total	23.445	17			
A	Between Groups	1.258	8	.157	.393	.899
	Within Groups	3.605	9	.401		
	Total	4.863	17			
G	Between Groups	11.424	8	1.428	3.803	.031
	Within Groups	3.380	9	.376		
	Total	14.804	17			

		Sum of Squares	Df	Mean Square	F	Sig.
AGR	Between Groups	1.460	8	.183	1.815	.196
	Within Groups	.905	9	.101		
	Total	2.365	17			
AST	Between Groups	272.111	8	34.014	.312	.942
	Within Groups	981.500	9	109.056		
	Total	1253.611	17			
ALT	Between Groups	101.444	8	12.681	.306	.945
	Within Groups	373.000	9	41.444		
	Total	474.444	17			

APPENDIX 8:

Analysis of variance (ANOVA) table for wound healing experiment (percentage healing rate and daily healing rate on lateral and caudal regions) of *Clarias gariepinus* fed onion bulb and walnut leaf diet at 7 and 14 days

		Sum of Squares	Df	Mean Square	F	Sig.
LPPH1	Between Groups	4776.000	8	597.000	81.409	.000
	Within Groups	66.000	9	7.333		
	Total	4842.000	17			
LPDHR1	Between Groups	97.417	8	12.177	36531.217	.000
	Within Groups	.003	9	.000		
	Total	97.420	17			
CPPH1	Between Groups	8135.111	8	1016.889	457.600	.000
	Within Groups	20.000	9	2.222		
	Total	8155.111	17			
		Sum of Squares	df	Mean Square	F	Sig.
CPDHR1	Between Groups	166.327	8	20.791	101145.041	.000
	Within Groups	.002	9	.000		
	Total	166.329	17			
LPPH2	Between Groups	.000	8	.000	.	.
	Within Groups	.000	9	.000		
	Total	.000	17			
LPDHR2	Between Groups	.000	8	.000	.	.
	Within Groups	.000	9	.000		
	Total	.000	17			
		Sum of Squares	df	Mean Square	F	Sig.
CPPH2	Between Groups	713.778	8	89.222	57.357	.000
	Within Groups	14.000	9	1.556		
	Total	727.778	17			
CPDHR2	Between Groups	14.570	8	1.821	4553.056	.000
	Within Groups	.004	9	.000		
	Total	14.573	17			
MPH1	Between Groups	5089.778	8	636.222	110.115	.000
	Within Groups	52.000	9	5.778		
	Total	5141.778	17			

APPENDIX 9:

Analysis of variance (ANOVA) table for wound healing experiment (percentage healing rate and daily healing rate on lateral and caudal regions) of *Clarias gariepinus* (male and female) fed onion bulb and walnut leaf diet at 7 and 14 days

		Sum of Squares	Df	Mean Square	F	Sig.
MDHR1	Between Groups	103.795	8	12.974	36490.422	.000
	Within Groups	.003	9	.000		
	Total	103.798	17			
FPH1	Between Groups	7483.111	8	935.389	183.011	.000
	Within Groups	46.000	9	5.111		
	Total	7529.111	17			
FDHR1	Between Groups	152.684	8	19.085	45202.382	.000
	Within Groups	.004	9	.000		
	Total	152.687	17			

		Sum of Squares	df	Mean Square	F	Sig.
MPH2	Between Groups	.000	8	.000	.	.
	Within Groups	.000	9	.000		
	Total	.000	17			
MDHR2	Between Groups	.000	8	.000	.	.
	Within Groups	.000	9	.000		
	Total	.000	17			
FPH2	Between Groups	572.000	8	71.500	20.109	.000
	Within Groups	32.000	9	3.556		
	Total	604.000	17			

FDHR2

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11.710	8	1.464	5987.864	.000
Within Groups	.002	9	.000		
Total	11.712	17			

APPENDIX 10:

Analysis of variance (ANOVA) table for microbiological analysis (enterobacteriacea counts in skin, liver, gills) of *Clarias gariepinus* fed onion bulb and walnut leaf diet for 84 days (4, 8 and 12weeks).

		Sum of Squares	Df	Mean Square	F	Sig.
EBS1	Between Groups	.706	8	.088	396.850	.000
	Within Groups	.002	9	.000		
	Total	.708	17			
EBS2	Between Groups	.746	8	.093	381.273	.000
	Within Groups	.002	9	.000		
	Total	.748	17			
EBS3	Between Groups	1.100	8	.138	386.859	.000
	Within Groups	.003	9	.000		
	Total	1.104	17			

		Sum of Squares	Df	Mean Square	F	Sig.
EBL1	Between Groups	.755	8	.094	472.028	.000
	Within Groups	.002	9	.000		
	Total	.757	17			
EBL2	Between Groups	.761	8	.095	463.000	.000
	Within Groups	.002	9	.000		
	Total	.763	17			
EBL3	Between Groups	1.159	8	.145	815.062	.000
	Within Groups	.002	9	.000		
	Total	1.161	17			

		Sum of Squares	Df	Mean Square	F	Sig.
EBG 1	Between Groups	1.078	8	.135	336.847	.000
	Within Groups	.004	9	.000		
	Total	1.082	17			
EBG 2	Between Groups	1.229	8	.154	768.028	.000
	Within Groups	.002	9	.000		
	Total	1.231	17			
EBG 3	Between Groups	1.198	8	.150	481.500	.000
	Within Groups	.003	9	.000		
	Total	1.201	17			

APPENDIX 11:

Analysis of variance (ANOVA) table for microbiological analysis (enterobacteriacea counts in intestine and total viable counts in skin and liver) of *Clarias gariepinus* fed onion bulb and walnut leaf diet for 84 days (4, 8 and 12weeks).

		Sum of Squares	Df	Mean Square	F	Sig.
EBI1	Between Groups	.800	8	.100	899.900	.000
	Within Groups	.001	9	.000		
	Total	.801	17			
EBI2	Between Groups	.909	8	.114	115.602	.000
	Within Groups	.009	9	.001		
	Total	.918	17			
EBI3	Between Groups	.525	8	.066	196.817	.000
	Within Groups	.003	9	.000		
	Total	.528	17			

		Sum of Squares	df	Mean Square	F	Sig.
TVCS1	Between Groups	.869	8	.109	444.295	.000
	Within Groups	.002	9	.000		
	Total	.871	17			
TVCS2	Between Groups	.845	8	.106	679.179	.000
	Within Groups	.001	9	.000		
	Total	.847	17			
TVCS3	Between Groups	.397	8	.050	279.281	.000
	Within Groups	.002	9	.000		
	Total	.399	17			
		Sum of Squares	df	Mean Square	F	Sig.
TVCL1	Between Groups	.413	8	.052	331.857	.000
	Within Groups	.001	9	.000		
	Total	.414	17			
TVCL2	Between Groups	.460	8	.058	235.386	.000
	Within Groups	.002	9	.000		
	Total	.463	17			
TVCL3	Between Groups	.480	8	.060	269.725	.000
	Within Groups	.002	9	.000		
	Total	.482	17			

APPENDIX 12:

Analysis of variance (ANOVA) table for microbiological analysis (total viable counts in gills and intestine) of *Clarias gariepinus* fed onion bulb and walnut leaf diet for 84 days (4, 8 and 12weeks).

		Sum of Squares	df	Mean Square	F	Sig.
TVCG1	Between Groups	1.145	8	.143	373.384	.000
	Within Groups	.003	9	.000		
	Total	1.148	17			
TVCG2	Between Groups	1.297	8	.162	521.214	.000
	Within Groups	.003	9	.000		
	Total	1.300	17			
TVCG3	Between Groups	.384	8	.048	154.304	.000
	Within Groups	.003	9	.000		
	Total	.387	17			

		Sum of Squares	Df	Mean Square	F	Sig.
TVCI1	Between Groups	61716.995	8	7714.624	1.002	.493
	Within Groups	69273.865	9	7697.096		
	Total	130990.860	17			
TVCI2	Between Groups	.384	8	.048	308.821	.000
	Within Groups	.001	9	.000		
	Total	.386	17			
TVCI3	Between Groups	.291	8	.036	148.909	.000
	Within Groups	.002	9	.000		
	Total	.293	17			

APPENDIX 13:

Analysis of variance (ANOVA) table for microbiological analysis (enterobacteriacea counts and total viable counts in water ample) of *Clarias gariepinus* fed onion bulb and walnut leaf diet for 84 days (4, 8 and 12weeks).

		Sum of Squares	Df	Mean Square	F	Sig.
WSE1	Between Groups	1.296	8	.162	214.397	.000
	Within Groups	.007	9	.001		
	Total	1.303	17			
WSE2	Between Groups	2.282	8	.285	712.972	.000
	Within Groups	.004	9	.000		
	Total	2.285	17			
WSE3	Between Groups	1.363	8	.170	319.500	.000
	Within Groups	.005	9	.001		
	Total	1.368	17			
		Sum of Squares	df	Mean Square	F	Sig.
WSTVC 1	Between Groups	.665	8	.083	71.957	.000
	Within Groups	.010	9	.001		
	Total	.676	17			
WSTVC 2	Between Groups	.760	8	.095	224.855	.000
	Within Groups	.004	9	.000		
	Total	.763	17			
WSTVC 3	Between Groups	1.010	8	.126	177.477	.000
	Within Groups	.006	9	.001		
	Total	1.016	17			

APPENDIX 14:

Analysis of variance (ANOVA) table for organ index (kidney skin, liver and heart) of *Clarias gariepinus* fed onion bulb and walnut leaf diet for 84 days

		Sum of Squares	Df	Mean Square	F	Sig.
OIL	Between Groups	.000	8	.000	2.078	.148
	Within Groups	.000	9	.000		
	Total	.000	17			
OIS	Between Groups	.000	8	.000	.750	.652
	Within Groups	.000	9	.000		
	Total	.000	17			
OIK	Between Groups	.000	8	.000	2.938	.065
	Within Groups	.000	9	.000		
	Total	.000	17			

OIH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	8	.000	.200	.983
Within Groups	.000	9	.000		
Total	.000	17			

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APPENDIX 15:

Analysis of variance (ANOVA) table for haematological parameters (packed cell volume, haemoglobin, red blood cell, white blood cell, platelets and lymphocytes) of *Clarias gariepinus* fed onion bulb and walnut leaf diet for 84 days

		Sum of Squares	Df	Mean Square	F	Sig.
PVC	Between Groups	297.000	8	37.125	1.148	.417
	Within Groups	291.000	9	32.333		
	Total	588.000	17			
Hb	Between Groups	36.800	8	4.600	1.449	.295
	Within Groups	28.580	9	3.176		
	Total	65.380	17			
RBC	Between Groups	4.027	8	.503	1.029	.478
	Within Groups	4.402	9	.489		
	Total	8.429	17			

		Sum of Squares	Df	Mean Square	F	Sig.
WBC	Between Groups	58900000.000	8	7362500.000	.286	.954
	Within Groups	231805000.000	9	25756111.111		
	Total	290705000.000	17			
PLT	Between Groups	16021444444.444	8	2002680555.556	2.156	.137
	Within Groups	8361500000.000	9	929055555.556		
	Total	24382944444.444	17			
LYM	Between Groups	89.000	8	11.125	.419	.883
	Within Groups	239.000	9	26.556		
	Total	328.000	17			

APPENDIX 16:

Analysis of variance (ANOVA) table for haematological parameters (heterophils, eosunophil, monocytes, erythrocytes, mean cell haemoglobin and mean cell haemoglobin concentration) of *Clarias gariepinus* fed onion bulb and walnut leaf diet for 84 days

		Sum of Squares	df	Mean Square	F	Sig.
HETERO	Between Groups	144.778	8	18.097	.584	.770
	Within Groups	279.000	9	31.000		
	Total	423.778	17			
MONO	Between Groups	3.000	8	.375	.450	.863
	Within Groups	7.500	9	.833		
	Total	10.500	17			
EOS	Between Groups	17.000	8	2.125	.981	.505
	Within Groups	19.500	9	2.167		
	Total	36.500	17			

		Sum of Squares	df	Mean Square	F	Sig.
ESR	Between Groups	.081	8	.010	1.659	.233
	Within Groups	.055	9	.006		
	Total	.136	17			
MCH	Between Groups	1.583	8	.198	1.051	.466
	Within Groups	1.694	9	.188		
	Total	3.277	17			
MCHC	Between Groups	.002	8	.000	2.776	.075
	Within Groups	.001	9	.000		
	Total	.003	17			

MCV

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1287.702	8	160.963	.625	.741
Within Groups	2319.676	9	257.742		
Total	3607.378	17			

APPENDIX 17:

Analysis of variance (ANOVA) table for proximate composition (moisture, ether extract, crude protein, ash and nitrogen free extract) of experimental diets fed *Clarias gariepinus* for 84 days

		Sum of Squares	df	Mean Square	F	Sig.
DMOIST	Between Groups	4.336	8	.542	1016.354	.000
	Within Groups	.005	9	.001		
	Total	4.341	17			
DEE	Between Groups	6.211	8	.776	1746.900	.000
	Within Groups	.004	9	.000		
	Total	6.215	17			
DNFE	Between Groups	19.732	8	2.466	11.523	.001
	Within Groups	1.926	9	.214		
	Total	21.658	17			
DCP	Between Groups	.042	8	.005	11.925	.001
	Within Groups	.004	9	.000		
	Total	.046	17			
DASH	Between Groups	4.041	8	.505	811.777	.000
	Within Groups	.006	9	.001		
	Total	4.046	17			

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APPENDIX 18:

Analysis of variance (ANOVA) table for proximate composition (moisture, ether extract, crude protein, ash and nitrogen free extract) of *Clarias gariepinus* fed onion bulb and walnut leaf diets for 84 days

		Sum of Squares	df	Mean Square	F	Sig.
FMOIST	Between Groups	5.951	8	.744	1106.525	.000
	Within Groups	.006	9	.001		
	Total	5.957	17			
FCP	Between Groups	1.904	8	.238	382.563	.000
	Within Groups	.006	9	.001		
	Total	1.910	17			
FEE	Between Groups	5.176	8	.647	850.139	.000
	Within Groups	.007	9	.001		
	Total	5.183	17			
FASH	Between Groups	21.055	8	2.632	4229.795	.000
	Within Groups	.006	9	.001		
	Total	21.061	17			
FNFE	Between Groups	20.540	8	2.567	4126.339	.000
	Within Groups	.006	9	.001		
	Total	20.546	17			

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