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RESEARCH ARTICLE

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ANTIBACTERIAL ACTIVITIES OF NEEM LEAVE (*Azadirachta indica*) EXTRACTS ON AFRICAN MUD CATFISH *Clarias gariepinus* (Burchell, 1822)

Abstract:
Large-scale settings of aquaculture have resulted in an increased antibiotic resistance in bacteria potentially pathogenic to fish. Hot water method was used in extracting the Neem leaves (*Azadirachta indica*) and bacteria were isolated from *Clarias gariepinus.* Twenty post juveniles *Clarias gariepinus* were infected with the isolated bacteria (*Aeromonas hydrophila*) and assessed at different concentrations of 25mg/l, 50mg/l, 75mg/l, and 100mg/l to confirm the inhibitory effects of the species and later treated with leave extracts to determine the antibacterial activities. The result shows bactericidal effects of the leave extracts against the *Aeromonas hydrophila*. All the haematological indices i.e. Packed Cell Volume (PCV), Red blood cell count (RBC), White Blood Cell (WBC), Haemoglobin (Hb), Mean Corpuscular Haemoglobin Concentration (MCHC) and Mean Corpuscular Volume (MCV) of fishes inoculated with *Aeromonas hydrophila* deviated to an abnormal range due to the inhibitory effects of the bacteria on the fishes. After treatment with Neem leave extracts, significant differences were noticed at a concentrations of 50mg/l and 75mg/l when compared to the control group as the values rises to the normal ranges. This implies that the antibacterial activities of the *Azadirachta indica* leaves extract is concentration dependent and one can therefore say that *Azadirachta indica* leaves extract is very active in treating *Aeromonas hydrophila*.
Keywords: Aquaculture, antibacterial activities, blood indices, *Clarias gariepinus,* haematological indices, Neem leave, pathogen

Introduction:

Aquaculture provides a good opportunity for developing countries (Manoj and Vasudevan, 2009), and is most rapidly developing sector in the world (Harikrishnam *et al;* 2011). It has become a key component of the animal health industry, due to the continued expansion of cultured expansion of cultured fish and shellfish species (Kolkovski and Kolkovski 2011).

Fish and fishery products represent a very valuable source of protein and essential micronutrients for balanced nutrition and good health. In 2009, fish accounted for 17% of the world population intake of animal protein and 6.5% of all protein consumed (FAO, 2012).

In Africa, catfish has replaced tilapia as the most cultured fish since 2004 and Nigeria is ranked the highest producer of catfish in Africa (FAO, 2012). The progressive dominance of this species in Nigeria and many African countries is due to its fast growth rate, high resistance to diseases and tolerance to environmental extremes and high consumer preference.

Aquaculture production is vulnerable and an increase of disease outbreaks has been reported due to culture intensification, resulting in partial or total loss of production (Bondad-Reantaso *et al*., 2005). Therefore, to avoid economic losses related to disease outbreak, several veterinary drugs are commonly used in aquaculture to prevent or treat disease outbreaks. Antimicrobials and other veterinary drugs are administered regularly in baths and injections and are used as prophylactics (prevent diseases before they occur), therapeutics (treat sick animals) or growth promoters (Rico *et al*., 2013). Nevertheless, the use of veterinary drugs is becoming more restricted since its

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present numerous side-effects for the environment and health safety. Mirand and Zemelman, 2002; Seyfried *et al*., (2010) reported that massive use of antibiotics have resulted in the development of resistant bacteria strains or presence of residual antibiotics in the muscle of commercialized fish and thus has potential consequences on human health (Cabello, 2006; Romero Ormazábal *et al*., 2012).

Considering the potential harm of veterinary drug treatments on the environment and human health and in some cases their limited efficacy, disease management should concentrate on harmless, preventive and lasting methods. Moreover, disease outbreaks are frequently associated with fish fitness and health, most pathogens being opportunistic and taking advantage of immune compromised or stressed fish, thus alternate solutions should maximize fish immunity and fitness to avoid and face pathogen infections (Ashley, 2007; Davis *et al*., 2002; Iguchi *et al.*, 2003; Ruane *et al*., 1999). Some of the proposed solutions are the use of natural products (plant extracts) in the culture of fish and shrimp (Citarasu, 2010; Lee *et al*., 2009; Makkar *et al*., 2007; Mohapatra *et al*., 2013; Panigrahi and Azad, 2007).

Material and methods:

Fish Sample collection

Samples of healthy catfish were collected from the Lagos State University, Lagos, Nigeria hatchery Centre and taken aseptically to Department of Microbiology Laboratory, LASU for Microbial analysis.

Isolation of pathogens

Samples were taken from the skin, Gills and intestine for isolation of bacteria. These samples were homogenized, serially diluted and inoculated on Nutrient agar plates. After being cultured for 24h at 30°C, the uniform colonies were sub cultured for further processing (Dhayanithi *et al*., 2010).

Characterization and identification of pathogens

The isolated bacteria were identified in concurrence with Bergey's Manual of Systematic Bacteriology. They were characterized based on colony morphology, gram nature, shape, motility. Further, the following biochemical techniques were performed on the isolates to identify the bacteria based on their reactions: Indole, methyl red, voges proskeur,citrate utilization, urease, catalase, oxidase and fermentation of glucose, galactose, fructose, lactose, maltose, sucrose, starch hydrolysis and coagulase tests. The results of biochemical characterization were compared with those appearing in previous reports (Austin and Austin, 2010) and subsequently with standard data of Bergy's Manual of Systemic Bacteriology.

Organisms Identified

The following organisms were identified: *Pseudomonas aeruginosa, Aeromonas sp, Bacillus subtilis, Staphylococcus* sp. *Sarcina* sp.

Collection and extraction of Neem leaves

Leaves of Neem plant (*Azadirachta indica*) were collected from the back of hatchery at LASU. Spoilt parts of the leaves were removed while the remaining parts were washed with sterile distilled water. 40g of Neem fresh leaf was extracted using solvents (ethanol and water). After 48hrs of incubation in the shaker, the supernatant was collected, dried in vacuum desiccators and stored in sterile containers (Dhayanithi *et al*., 2012).

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Antimicrobial activity

The antimicrobial potential of the Neem plant extracts was seen against the test organisms using the agar-gel diffusion susceptibility test. Sterile Mueller–Hinton plates were taken one plate/organism tested. Two wells of about 3.0 mm diameter were aseptically punched on each agar plate using a sterile cork borer, with at least 30 mm distance between adjacent wells and the periphery. According to the standard technique of Opara and Anasa (1993), 2 to 4 colonies of the test organisms were inoculated in sterile broth and these inoculums was swabbed using sterile swab on the surface of punched Mueller - Hinton agar plates. A fixed volume (0.1ml) of the plant extract was then introduced into the wells in the increasing concentration and then incubated at 37^0C for 24 hours. The resulting zones of inhibition were measured.

Determination of minimum inhibitory concentration (MIC)

The MIC of the plant extracts was determined using the micro broth dilution technique (Murray *et al.*, 1999). It was performed in 96-well microtiter plates for determining the minimum inhibitory concentration (MIC). Standardized suspensions of the test organisms (*Pseudomonas aeruginosa and others*) were inoculated into a series of 96-well microtiter plate, including one growth and one sterility control. Brain Heart Infusion(BHI) and Sabouraud dextrose broth containing plant extracts in increasing concentration of 1.0, 2.5, 5, 7.5, 10,12.5, mg/ml. and incubated at 37◦ C for 24 hours. After overnight incubation these tubes were observed for turbidity. The microtiter plate showing the minimum turbidity was noted for MIC.

Fish collection and acclimatization

A total of 200 healthy, sub-adult Mud catfish (*Clarias gariepinus*), each weighing around 120-175g, were obtained from the LASU fish hatchery and transferred to a plastic tank with a water capacity of 100 litres. This tank was filled with water from the borehole. The fish were maintained in the same tank for days with continuous aeration and were fed with coppens feed twice a day. During the period of the experiment the water was maintained at a temperature of 25.2 °C, pH at 7.5, Dissolved oxygen level of 5.4 mg/L. Ammonia and nitrite levels in the water were 0.00 mg/L.

Re-infection and experimental infection

100 specimens of active fish were stocked in a 30lts capacity plastic tanks filled with 10lts of sterilized water. The bacterial culture was centrifuged at 1000g for 10 min. The supernatant was discarded and the isolated bacterial pellets were washed thrice and re-suspended in distilled water at a pH of 7.4. The optical density of the solution was adjusted to 0.5 at 456nm using spectrophotometer equivalent to 3.0 x 10^8. Same amounts of bacterial pellets were dissolved in experimental groups of 10 fish each in duplicate. The control group was exposed only to distilled water. The fish were carefully observed for any behavioural changes or mortality.

The experimental treatment

Healthy fish, numbering 100 were divided into 5 groups. Each group contained 10 fish each in duplicate. The water extract from Neem leaves were used to treat infected fish at different concentration of 25%, 50%, 75% and 100% while the control group (0%) was treated with antibiotics (oxytetracycline).

Sampling Techniques and estimation of blood count

Infected fish were randomly selected from each group and treated with plant extracts for haematological analysis. Blood sample was collected from the caudal vein of the fish using plastic syringe treated with an anticoagulant (EDTA). Part of the blood was added to an equal volume of 10% tri sodium citrate and stored at 40°C until further

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analysis. Blood samples were analyzed using Auto Haematology Analyzer BC-3200, Shenzhen Mindray Bio-Medical Electronics Co. Ltd 2005-2008. The parameters measured include: PVC%, RBC (10^{12}mm), WBC (mm), HB (g/dl), MCHC/100ml, MCH (pg), MCV (F), Neutrophils % and Lymphocytes % (Hammed *et al.*; 2015).

Clinical trial and disease resistance experiment

After one and half month, fish in the experimental tanks were exposed to a 24h culture of the same bacterial pathogens, *Aeromonas hydrophila*. This was done with a standardized concentration of bacterial isolates in pellet form under aseptic conditions. The appropriate doses of the virulent bacteria were standardized based on the results of the infected fish and used for clinical trial and the disease resistance experiment. The control group was exposed to the same amount of buffer without the pathogenic bacteria. Subsequently, the fish were observed for specific symptoms. All groups were maintained in duplicate.

Results:

The major symptoms observed in the fish in the aquarium tanks were: Fin rot, hemorrhages at the base of fins and sloughing of fins/ skin peeling.

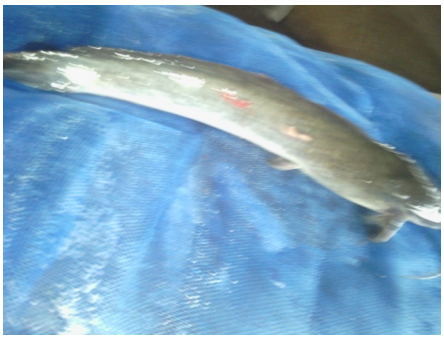

Plate 1: Picture showing fin rot, hemorrhages at the base of fins and sloughing of fins/ skin peeling. (Source: Dr. A.M. Hammed

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Table 1: Biochemical Characterization of isolated bacteria from the catfish

Gram staining	-
shape	Bacilli
Motility	-
Catalase	+
Oxidase	+
Coagulase	-
Glucose	-
Fructose	-
Sucrose	+
Ribose	-
Starch hydrolysis	-
Gelatin liquefaction	-

Table 2: Colonial characteristics of Isolate

Forms/shape	irregular
Surface texture	smooth and glistening
Colour/Pigmentation	creamy
Elevation	raised
Margin	curled
Optical characteristics	opaque

Table 3: Antimicrobial activity of Neem leaves against bacterial isolates

Bacteria	Solvent					
Aeromonas hydrophila	Ethanol			Water		
	0.375	0.375	0.35	0.30	0.32	0.33

Table 4: Percentage of mortality caused by bacterial isolates

Name of the bacteria	**% mortality**	**% survival**
Aeromonas	30	70

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Table 5: Haematological and blood profile indices of *Aeromonas* spp. Infected adults *Clarias gariepinus,* treated with Neem (*Azadirachta indica*) plant extracts at different concentrations.

Blood indices	Experimental Treatments					
	Control	A ($25mg.l^{-1}$)	B ($50mg.l^{-1}$)	C ($75mg.l^{-1}$)	D ($100mg.l^{-1}$)	Mean
PCV%	26.9	23(32)	20(37)	25(26)	26(33)	23.5(32)
RBC(10^{12}mm)	2.3	1.5(2.75)	1.7(3)	1.9(1.95)	1.8(2.6)	1.725(2.575)
WBC(mm)	7	6000(125)	6000(150)	6100(100)	6300(200)	6100(143.75)
HB(g/dl)	9.6	8.6(11)	8.4(12.3)	8.3(9.3)	8.6(11.4)	8.475(11)
MCHC/100ml	35.7	34.8(34.4)	35.8(33.2)	35.1(35.8)	36(34.5)	35.425(34.475)
MCH(Pg)	41.7	45.3(40)	44(41)	46(48)	43(43.8)	44.575(43.2)
MCV(F)	116	127(116)	124.5(123)	126(133)	130(127)	126.875(124.75)
Neutrophils%	1.8	3.9(44)	4.8(36)	5.5(36)	3.9(40)	4.525(39)
Lymphocytes%	98,2	95.1(56)	95.8(64)	95.4(64)	96(60)	95.575(61)

Figures in bracket () are blood profile indices

The Packed Cell Volume (PCV) of the fish at the start of the experiment was 26.9% which was higher than the PCV of the infected fish of 23.5%. The Red blood cell count (RBC) of the fish at the start of experiment was $2.3x10^{12}$mm and was higher compared to the infected fish of $1.7x10^{12}$mm. The White Blood Cell (WBC) of the infected fish was 6100mm compared to a lower value of 7m at the start of the experiment. The Haemoglobin (Hb) of the initial fish of 9.6g/dl was higher to the Haemoglobin (Hb) after infection with bacteria with a value of 8.48g/dl. Mean Corpuscular Haemoglobin Concentration (MCHC) 35.7/100ml of the initial was higher than the Mean Corpuscular Haemoglobin Concentration (MCHC) 35.4/100ml after infection. The Mean Corpuscular Haemoglobin of 44.6Pg after infection was higher than the Mean Corpuscular Haemoglobin 41.7Pg at the start of the experiment. The Mean Corpuscular Volume (MCV) value of the initial fish of 112F was lower to the Mean Corpuscular Volume (MCV) value of 126.9F after infection of fish. Neutrophils of the initial of 1.8% were lower to the Neutrophils 4.5% after infection of fish. Finally, the Lymphocytes of the initial was 98.2% and this was higher than the Lymphocytes after infection with a value of 95.6%.

As shown above, the Packed Cell Volume (PCV) of 37% at 50% concentration was recorded to be the highest compared to other concentrations, and the lowest of 26% was recorded at 75% of the concentration. 50% of the concentrations showed that the highest Red Blood Cell count (RBC) was at $3x10^{12}$mm and the lowest was recorded at $1.95x10^{12}$mm of 75% of the concentrations. The White Blood Cell (WBC) was greatest at 100% at 200mm compared to the lowest WBC of 125mm at 25mg/l of the concentrations. The Haemoglobin (Hb) was lowest at 75% concentration with a value of 9.3g/dl and was highest at 50% concentration of 12.3g/dl. 75% of the concentrations has the highest Mean Corpuscular Haemoglobin Concentration (MCHC) of 35.8g/100ml, while it was lowest at 33.2g/dl at 25% concentration. The Mean Corpuscular Haemoglobin (MCH) was highest at 75% concentrations with a value of 48Pg while 40Pg was recorded as the lowest MCH at 25% concentrations. The Mean Corpuscular Volume (MCV) of 133F was highest at 75% concentrations while the lowest was at 116F at 25% of the concentration. 25% concentration has the highest Neutrophils at 44% compared to others, while 50% and 75% of the concentrations have the lowest Neutrophils. The Lymphocytes were highest at 50 and 75% concentrations with values of 64% and 56% respectively while the lowest value was at 25% concentration (Table 5).

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The Packed Cell Volume (PCV) of the initial fish was 26.9% which was lower than the mean PCV of 32% after treatment with plant extracts and 50% concentration had 37%. The Red blood cell count (RBC) of the initial fish was $2.3x10^{12}$mm and was lower compared to the mean value of treated fish of $2.575x10^{12}$mm with 50% concentration having highest value of 3. The White Blood Cell (WBC) of 143.75mm as the mean value of the treated fish was higher than the initial fish of 7mm WBC. The Haemoglobin (Hb) of the initial fish at 9.6g/dl was lower to the Haemoglobin (Hb) of the treated fish, of 11g/dl. Mean Corpuscular Haemoglobin Concentration (MCHC) 35.7g/100ml of the initial fish was lower than the Mean Corpuscular Haemoglobin Concentration (MCHC) 34.475g/100ml of the treated fish. The Mean Corpuscular Haemoglobin 43.2Pg of the treated fish was higher than the Mean Corpuscular Haemoglobin 41.7Pg of the initial fish. The Mean Corpuscular Volume (MCV) of the initial fish 116F was lower to the Mean Corpuscular Volume (MCV) 124.75F of the treated fish. Neutrophils of the initial of 1.8% was lower to the Neutrophils 39% of the treated fish. The Lymphocytes of the initial fish was 98.2% and higher than the Lymphocytes of the treated fish at 61% (Table).

Generally, the antibacterial effects of *Azadirachta indica* leaves extract on catfish, *Clarias gariepinus* post juveniles were shown in Tables. Differences were noticed in the inoculated and the treated group when compared to the initial not infected.

There were differences in the values of the Packed Cell Volume (PCV), Red Blood Cell (RBC) count, Haemoglobin Concentration (Hb), and Lymphocytes (L) with significance increase in the values of the White Blood Cell (WBC) count, Mean Corpuscular Haemoglobin Concentration (MCHC), Mean Haemoglobin Concentration (MCH), Mean Corpuscular Volume (MCV) and Neutrophils (N).
There were significance increase in the values of Haematological Indices with decrease in the values of MCHC, MCH and Lymphocytes while that of MCV remains unaffected when the fish were treated with 25% conc. of the extract. Similar trends was noticed when the fish were treated with 50% conc. of the extract except for the MCV value which increases significantly. There were significance decreases in the values of the PCV, RBC, Hb, and Lymphocytes with an increase in other Hematological Indices.
The MCHC and Lymphocytes showed that the values decrease following the treatment of the fish with 100% conc. of the extract.

Discussion:
The result of the leaves extract on *Clarias gariepinus* post juvenile at the four different concentrations (25, 50, 75, and 100mg) indicates that the leaves extract of *Azadirachta indica* is very active against *Aeromonas hydrophila* on *Clarias gariepinus* post juvenile. This is agreement with the findings of Chitmanat *et al*., 2005, which confirms that the different parts of Neem trees are the best alternative to preserve and protect the environment and animals. Previous research also reported that Neem leaves containing nimbin, azadirachtin and miliantriol possess a variety of properties including antiviral, antimicrobial, insecticidal and antibacterial properties (Govind *et al*., 2012).
Some recent studies have addressed the hematological changes related to different plant extract (Dan-Ologe and Sogbesan, 2007). In coincidence with the present findings, a clear decrease in Hb content in a study on the effect of leaf extract of *Khaya senegalensis* on channel catfish, *Clarias gariepinus* was reported (Obadiah, 2012).

Contrary, some researchers reported that the hematological parameters such as haemoglobin and RBC are significantly higher in groups treated with herbal immune stimulants over control group (Sahu *et al*., 2007, and Sudagar and Hajibeglou, 2010). The findings of the present study is in agreement with the work of Sahu *et al.*, Sahu *et al* 2007, Subeena Begum and Navaraj reported that White Blood Cell counts are higher in *Labeo rohita* fingerlings treated with *Magnifera indica* when compared to control.

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Further, these observations of our study also verify the findings of other investigations by Gopalakannan and Arul (Gopalakman and Arul, 2006) who found that there is an increase in the WBC count in the common carp after exposure to chitin extract. On the other hand, Sudagar and Hajibeglou stated that after feeding the common carp with plant extracts and challenging with *Aeromonas* with cyprinus carpio showed higher hemoglobin, WBC and RBC compared to the control. These alterations could possibly be due to activation of the immune system in the presence of herbal extracts, which in turn may be an adaptive response of the organism resulting in a more effective immune defense (Barreto-Medeiros, 2005).

Neem *Azadirachta indica* (*A. indica*), is one of the most promising medicinal plant, having a wide spectrum of biological activity, well known for its insecticidal properties (ICAR, 1993). Every part of Neem tree have been known to possess a wide range of pharmacological properties, especially as antibacterial, antifungal, antiulcer, antifeedant, repellent, pesticidal, molluscicidal, ecdysone inhibitor and sterilant and is thus commercially exploitable (Biswas *et al.*, 2002; Das *et al.*, 2002), and hence, traditionally used to treat large number of diseases (Van Der Nat *et al.*, 1991). This eco-friendly native tree of India is perhaps most researched tree in the world. Water soluble extract of *A. indica* leaves was found to possess significant hypoglycemic, hypolipidemic, hepatoprotective, anti-fertility and hypotensive activities.

One of the most promising natural compounds is azadirachtin (AZA), an active compound extracted from the neem tree (*Azadirachta indica*), whose antiviral, antibacterial and antifungal properties have been known for several years (Isman *et al.*, 1990; Harikrishnan *et al.*, 2003). The chemistry and biological activity of both Neem extracts and purified AZA have been investigated in various countries (Biswas *et al.*, 2002).

Neem has been used successfully in aquaculture systems to control fish predators (Dunkel and Ricilards, 1998). Martinez (2002) stated that aqueous extract of Neem leaves and other Neem-based products have been extensively used in fish-farms as alternative for the control of fish parasites and fish fry predators such as dragon-fly larvae. Although Neem extract is considered of low toxicity towards non-target aquatic life, water extracts of the bark of the Neem plant caused respiratory problems in *Tilapia zilli* (Omoregie and Okpanachi, 1997), while long exposure to low concentrations of the crude extract of *A. indica* delayed the growth of this cichlid fish (Omoregie and Okpanachi, 1992).

PCV after infection revealed lower values compared to the start of the experiment. The values increased in treatment at 25, 50 and 100 % concentrations and lower at 75% concentration. The PCV was highest at 50% concentration. of 37%. This result shows that the PCV of the fish was affected by the invasion of the bacteria as other studies had proved that the PCV of animals is greatly reduced. Thus this result corroborates the findings of Sebastiao *et al* (2011) who reported a reduction in the PCV of Nile Tilapia naturally infected with *Aeromonas hydrophila*. The RBC and Hb values reduces after inoculation with bacteria and the values increased tremendously after treatment with Neem extracts with highest value of RBC of 3 at 50% conc.; Hb value of 12.3 at 50% conc. which was the highest and decrease further at higher concentration of 75 and 100%. This is line with findings of Bektas and Ayik (2009) who recorded lower erythrocytes and heamoglobin. This may be due to the bacterial infection which causes destruction to the heamatopoietic tissue in the kidney and spleen and decrease blood cell production. The WBC increased from 7mm at the start of experiment to 6300mm after infection, the WBC is to counter any form of invasion to fight the foreign body invading the system. This also corroborates the findings of Sebastiao *et al* (2011) which reported significant increase in the number of WBC in fish infected with different bacteria species. The lymphocyte was lower after infection and after treatment than at the initial stage; this is in line with the scientific findings that in tissues, mononuclear cells (lymphocytes and macrophages) predominate in the organism's defense reactions. But, in stressful situations, the number of circulating lymphocytes decreases. The MCH and MCV increases after infection with

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bacteria compare to at the initial stage. However, the value decreased slightly after treatment with Neem plant extracts. The MCHC increase after infection at 50% conc. and decrease after treatment with extracts except at 75% conc. which shows higher values. This result does not agreed with the findings of Sebastiao *et al* (2011) that recorded higher values in MCV, MCH and MCHC in the control fish than in the infected fish.

Conclusion:

The study revealed that, blood parameters exhibited response to the invading bacteria and the subsequent treatment with Neem plant extracts. It is realized that changes in haematological parameter due to unfavourable exogenous factors like disease is an index of the ill health of cultured fish.

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