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RESEARCH ARTICLE

EFFECTS OF *MORINGA OLEIFERA* LEAF EXTRACTS ON BACTERIA (*AEROMONAS HYDROPHILA*) INFECTED ADULTS AFRICAN MUD CAT FISH *CLARIAS GARIEPINUS* (BURCHELL, 1822)

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ABSTRACT

Investigation into the effects of *Moringa oleifera* leaf extract on bacteria (*Aeromonas hydrophila*) infected adult African mud cat fish (*Clarias gariepinus*) was conducted using twenty infected adults *Clarias gariepinus* and treated with *M. oleifera* leaf extract at concentrations of 25%, 50%, 75% and 100% respectively. Blood samples were collected (pre-inoculation, post-inoculation and after treatment) from fish in replicate and analyzed for haematological parameters vis-à-vis RBC (Red blood cell), WBC (White blood cell), PCV (Packed cell volume), MCH (mean corpuscular haemoglobin), MCHC (mean corpuscular haemoglobin concentration), MCV (mean corpuscular volume), Hb (haemoglobin), L (lymphocyte) and N (Neutrophil). It was observed that upon inoculation with *A. hydrophila*, all the haematological indices deviated to an abnormal range due to the inhibitory effects of the bacteria on the fishes. Results indicated no significant differences ($P<0.05$) among the parameters except white blood cell which shows significant difference at 50% treatment with *Moringa oleifera* extract. Infected fish responded to treatment better at 50% concentration *Moringa oleifera* leaf extract.

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INTRODUCTION

Fish production has increased significantly over the past few decades leading to intensive fish culture practices where overcrowding, transport, handling, size grading and poor water quality are common problems (Li *et al*., 2004). It has been widely demonstrated that farmed fish are more susceptible to various disease causing agents in intensive farming. Therefore, to improve health conditions in the rearing of aquatic organisms, several alternatives such as improved husbandry, nutrition, water quality, optimal stocking density, use of vaccines, immune-stimulants (Gastesoupe, 1999) and probiotics (Sakai, 1999) have been proposed. The enhancement of the immune system of fish is considered to be the most promising method of preventing fish diseases in aquaculture. This enhancement can be achieved through application of vaccines, which activate the specific immune response of the fish and are considered to be the most effective agents. Fish diseases have been classified as infectious which fish can acquire from the environment and non-infectious which are normally due to deficiencies in nutritional requirements of fish. Infectious diseases are caused by pathogenic organisms (parasites, bacteria, virus and fungi) present in the environment. Leong and Fryer (1993) noted that 10% of all cultured aquatic animals are lost as a result of infectious disease. Francis-Floyd, (2005) opined that diseases in fish are not in balance with itself or with its environment. Stress in fish results in poor feeding, deformity and cannibalism, reduced growth and survival rate. All these predispose the fish to infection and disease leading to a reduction in fish health status and eventual mortality will occur.

The major importance of fish to human is majorly to serve as a source of protein, and they are being converted to different forms for different purposes (Adewunmi, 2015). However there are a number of constraints to its growth/expansion, which include among others, infection by bacteria. Aquatic bacteria that infect fish belong to three groups: the Gram-negative bacteria (most common), Gram-positive bacteria and acid-fast bacteria, which are obtained from food or from the environment. Gram-negative bacteria cause most diseases in tropical fish (Ducenci and Candan, 2003; Kar and Ghosh,

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2008); some of which are opportunistic pathogens (Schmidt *et al*., 2000) while others are obligatory pathogens (Tendencic, 2004). *Clarias gariepinus* is a popular fish for aquaculture because of its hardiness, ease of larval production in captivity and good market price (Ferguson *et al*., 2001; Crumlish *et al*., 2002; Dung *et al*., 2004; Sarter *et al*., 2007; El-Yazeed and Ibraheem, 2009).

Study of bacteria disease and its effect on catfish (*Clarias gariepinus*) and other species of fish have been a serious concern in the fishing industries and its effect on the consumers. Fishes are found all over the world, basically in marine and fresh water bodies (Colwel *et al*., 1960). Bacteria disease in marine fish come in bewildering array, and studies about fish diseases have accelerated enormously in recent times, largely because of urgent problems in marine aquaculture and occasional epizootics in natural populations. Fish living in natural environment are known to habour pathogenic enterobacteriaceae (Pillay, 1990). Bacterial diseases are more acute in cold than warm water aquaculture, and may be aggravated by unfavorable conditions such as crowding, malnutrition and unstable water temperature (Fafioye, 2011). Fish dependency on water is crucial; hence the source, volume and quality of physico-chemical parameters such as dissolved oxygen, total hardness, pH, alkalinity, carbonate and ammonia are salient factors to consider in relation to fish health (Fafioye, 2011). Water of poor physico-chemical quality has adverse effects on fish and fish consumers, thereby, resulting in serious economic and human losses (Amadi, 2009). Although vaccines are being developed and marketed, these cannot be used as a universal disease control measure in aquaculture.

During the last decades, antibiotics were not only used as traditional strategy for fish diseases management but also for the improvement of growth and efficiency of feed conversion (SCAN 2003; Kim *et al*., 2004; Cabello 2006; Sørum 2006). There is a risk associated with the transmission of resistant bacteria from aquaculture environments to humans, and risk associated with the introduction in the human environment of nonpathogenic bacteria, containing antimicrobial resistance genes, and the subsequent transfer of such genes to human pathogens (FAO, 2005). On the other hand antibiotics inhibit or kill beneficial micro-biota in the gastrointestinal (GI) ecosystem but it also made antibiotic residue accumulated in fish products to be harmful for human consumption (WHO, 2006). Bacteria can enter the fish body through the gills or skin or it can stay on the surface of the body (Douglas, 2007). There are four types of bacterial infections. Bacterial gill disease: The gills are the primary target, Systemic bacterial disease: bacteria invades the fish's body and damages internal organs, bacterial body ulcers: Lesions on the fish body that can be shallow or deep and fin rot: Most likely resulting from environmental stress. (Douglass 2007).

Antibiotics are utilized as growth promoters at sub-therapeutic levels and for treatment of diseases. The beneficial effects of antibiotic in combating bacterial problems and as growth promoters are well documented. Antibiotic helps to recover from certain diseases of bacterial origin. However, there may be problems associated with usage of antibiotics such as drugs toxicity, residual effects and development of microbial resistance. Animal scientists and veterinarians are now turning attention towards alternative sources of natural ingredients such as herbs or plants (phytobiotic) to replace antibiotic (Ogbe, 2008; Ogbe *et al.,* 2008; Ogbe *et al.,* 2009). Plants are known to have high amounts of essential nutrients, vitamins, minerals, fatty acids and fibre (Gafar and Itodo, 2011). Plant oil from seeds and leaves such as *Moringa oleifera* are in high demand for their medicinal value. Apart from the medicinal uses, *Moringa oleifera* was reported to be a good source of vitamins and amino acids (Olugbemi *et al*; 2010). *Moringa oleifera* was claimed to boost immune systems (Jayavardhanan *et al.,* 1994; Olugbemi *et al.,* 2010). The leaves and green fresh pods are used as vegetables by man and are rich in carotene and ascorbic acid (vitamin C) with a good profile of amino acids (Makkar and Becker, 1996). They are also used in livestock feed and the twigs are reported to be very palatable to ruminants (Sutherland *et al.,* 1990; Kimoro, 2002; Sarwatt *et al.,* 2002; Kakengi *et al.,* 2007). The edible leaves are very nutritious and are consumed in Nigeria. The Moringa seed oil is high in (80.4%) polyunsaturated fatty acid (Ogbunugafor *et al.,* 2011). *Moringa oleifera* extract was reported to have antibacterial properties and conclusion was made to investigate it as a phytotherapeutic agent to combat infectious agents (Patel, 2011).

The moringa tree is native to northeastern India. It is rich in nutrients and, apart from a range of industrial and medicinal applications, is used to purify water for human consumption. Moringa is of economically important in the production of several commodities, such as oils, foods, condiments and medicines (Makkar and Becker 1997). It is found in most of tropical America and, though not native to Brazil, is grown extensively in the northern and northeastern regions of the country (Souza *et al.,* 2003). Muyibi and Evison (1995) noted that *M. oleifera* seeds have been used in the treatment of hard water, and proved that hardness removal efficiency of *M. oleifera* increased with increasing dosage. *Moringa* seed powder is a natural alternative to imported alum (Jahn, 1986; Ndabigengesere and Nasarasiah, 1998). (Aluminum sulphate, the conventional synthetic coagulant) used in purifying turbid water in fish culture enclosures (earthen ponds, farm dams and irrigation canals). The study was aimed at determining the effect of bacteria (*Aeromonas hydrophila*) on the health status of Adults *Clarias gariepimus* and the potency of moringa leave extract as antibiotic agents.

MATERIALS AND METHODS

Plant sample collection, preparation and specimens' sterilization

Moringa (*Moringa oleifera*) leaves were collected from the Staff Quarters garden of the Lagos State University Main Campus, Ojo and taken to the Fisheries laboratory for extraction. 40g of the plant leaves was weighed using electronic sensitive analytical balance. It was then soaked in a conical flask of 250ml distilled water for 24hour. The plant was then crushed and the extracts were filtered using sieve. The extracts were collected into a beaker and heated up with an electric heater at 5^0c for 20minutes to reduce the volume to concentrated forms of 25mls which was stored in air-tight bottles and preserved in a refrigerator till the next day. Working tables were swabbed with ethanol to disinfect them. All the glass-wares were washed and air dried before they were sterilized in the hot air oven at 160^oc for 1hour. Petri dishes were sterilized in Petri-dish cans in the hot air ovens at 100^oc for 1hour, cotton plugged and aluminum foil covered conical flask were sterilized in the hot air oven at 160^oc for 1hour

Table 1. Morphological Characteristics of the Isolate

Source	Motility	Shape
Skin	-ve	Bacilli

-ve: Not motile
The isolate which was gotten from the skin of the fish shows that the isolate is not motile i.e. it doesn't possess flagellate for movement and the shape gotten from the Morphological Characteristics is Bacilli (Table 1).

Table 2. Colonial Characteristics of the Isolate

Forms/ Shapes	Surface Texture	Colour Pigment	Elevation	Margin	Optical Characteristics
Irregular	Smooth and Glistering	Creamy	Raised	Curled	Opaque

From the colonial characteristics of the Isolate gotten, it shows that the Isolate has an irregular shape or form and it has a glistering and smooth surface texture, creamy colour pigmentation and the elevation of the Isolate was raised. The margin was curled with an opaque optical characteristic (Table 2).

Table 3. Biochemical Characteristics of the Isolate

			Sugar Fermentation							
Starch hydrolysis	Gelatin liquefaction	Gram reaction	Catalase	Coagulase	Fructose	Ribose	Lactose	Sucrose	Glucose	Suspected organism
-	-	-	+	-	-	-	+	+	-	*Aeromonas hydrophila*

-: Negative reaction; +: positive reaction
After all this analysis was done, the suspected organism was *Aerosomonas hydrophila* according to the Biochemical, Colonial and Morphological characteristics analyzed on the Bacteria Isolate gotten from *Clarias gariepinus* (Table 3).

Table 4. Haematological indices of Adult *Clarias gariepinus* infected with bacteria (Aeromonas sp.) and treated with *Moringa oleifera* leave extract

Blood parameters	Control	Infected	Treatment			
			25%	50%	75%	100%
RBC($\times 10^{12}$)	2.3±0.1	1.90±0.6	1.94±0.7	2.8±0.9	2.5±0.7	2.25±0.7
WBC(10^7)	700 ±0.3	610 ± 334.8	400 ±236.6	700 ±442.7	300±118.3	200±105.0
PCV(%)	26.9±7.8	24±8.6	26±13.5	33±12.1	30±11.8	27±11.3
MCH(pg)	41.7±27.5	45.3±11.0	47±6.8	40.7±19.4	41.2±7.1	42.7±12.5
MCHC(g/l)	35.7±10.2	35.8±20.7	35.8±7.6	34.5±13.7	34.3±15.1	35.6±9.7
MCV(fl)	116±52.4	126±60.2	134±27.7	118±53.3	120±51.0	120±51.0
Hb(g/l)	9.6±5.5	8.6±5.0	9.2±4.2	11.4±0.9	10.3±0.8	9.6±5.5
N(%)	1.8±0.3	4.6±2.2	32±14.1	44±10.5	28±11.8	48±7.7
LYMPH(%)	98.2±77.9	95.4±16.6	68±49.3	56±40.3	72±44.4	52±43.6

Legend: RBC (Red blood cell), WBC (White blood cell), PCV (Packed cell volume), MCH (mean corpuscular haemoglobin), MCHC (mean corpuscular haemoglobin concentration), MCV (mean corpuscular volume), Hb (haemoglobin), LYMPH (lymphocyte), N (Neutrophil).

Table 5. Water Quality Parameters

Parameters	Tank A	Tank B	Tank C	Tank D	Tank E
Temperature ^oC	27.5±0.06	26.4±0.37	27±0.07	28±0.11	27.4±0.19
P^H	6.9±0.08	7±0.11	7.1±0.07	6.8±0.07	6.9±0.06
DO (mg/l)	6.1±0.10	5.9±0.21	6.0±0.31	6.9±0.12	6.0±0.18

while the wire loop was sterilized by flaming it red-hot using a spirit lamp.

Isolation of pathogens

Sample was taken from the skin of the procured fish and weighed. The weighed skin was dropped in 10ml sterile distilled water to release the bacteria organisms into it for serial dilutions (Dhayanithi *et al*., 2010). The infected sample was thoroughly mixed in McCartney bottle in order to have microorganism evenly distributed. A series of test tubes containing 9ml sterile distilled water as diluents was set up 1ml of stocked culture was first added to initial 9ml test tube to give 10^{-1}. This was done serially into other tubes to give 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}.

Preparations of Media

Nutrient broth: this was prepared according to manufacturer's specification 1.3g/100ml. This was soaked in 100ml distilled water for 10minutes. It was well mixed, later sterilized by autoclaving at 151b pressure, at 121^{o}C for 15minutes. It was allowed to cool to 47^{o}C, well mixed before being used for sugar fermentation tests.

Nutrient agar: 7g/250ml of nutrient agar was weighed out according to the manufacturer's specification, using an Electric

Figure 1. A *Clarias gariepinus* infected with *Aeromonas hydrophila* showing external symptoms

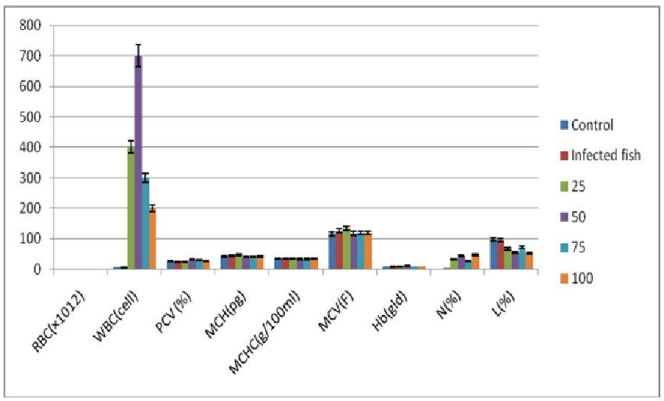

Figure 2. Graph indicating haematological indices of Adult *Clarias gariepinus* infected with bacteria (*Aeromonas* spp.) and treated with *Moringa oleifera* leave extract

Measuring Balance; it was weighed in a conical flask. It was soaked in 250ml of distilled water for 10minutes. It was well mixed by shaking, and then sterilized by autoclaving at 15Ib pressure, at 121°c for 15minutes. It was allowed to cool to 47°C, and was well mixed before pouring into the plates. It was soaked for 10minutes, and was well mixed by shaking and it was autoclaved for 15minutes at 121°C. It was allowed to cool to 47°C and was mixed before pouring into Petri dishes and the agar surface was dried in an oven.

Mueller-Hinton agar: 3.8g was dispersed in 100ml of distilled water. It was soaked for ten minutes, and was well mixed by shaking and it was autoclaved for 15minutes AT 121°C. It was allowed to cool to 47°C and was mixed before pouring into Petri dishes and agar surface was dried in an oven.

Bacterial Isolation: This was done using pour plate techniques. 1ml of the diluents in the test tube (10^{-5}) was ascetically transferred into sterile Petri dishes. The cold sterile nutrient agar (200ml) was poured into the diluents in the Petri dish containing the organism. The plates were swirled to evenly distribute the medium and the organism was allowed to stand for some minutes. After the medium solidifies, the plates were incubated at 37°C for 18-24 hours inverted upside down for bacteria growth.

Purification of Microorganisms: The resultant bacteria growths were streaked on fresh sterile nutrient agar medium. Several sub-culturing were done to get pure colonies. The pure culture were inoculated on nutrient agar slants, labeled and incubated at 37°C for growth and later stored in the refrigerator at 4°C to preserve the pure cultures until required for identification.

Identification of microorganisms: The Isolated organism on nutrient agar slants was sub-cultured on nutrient agar plates to activate the pure culture. Characterization of the organism was based on colonial characteristic and biochemical tests.

Colonial characteristics: The macroscopic examination of the surface colonies on nutrient agar was used to determine their size in (mm), shaped and elevation (flat, low convex, high convex, plateau), edge (entire, wavy, irregular, serrated), texture (smooth, rough), colour and pigmentation, translucent or opaque and effect on culture medium.

Biochemical tests: Gram staining, catalase, coagulase, starch hydrolysis, sugar fermentation, gelatin liquefaction and motility tests were carried out following the procedure of Konemman *et al,(2001*)

Preparation of Fish Sample for Treatment

Collection and preparation of Fish Samples: Twenty (20) Adults *C. gariepinus* were used for this research; they were purchased from a private fish hatchery at Badagry area of Lagos state. The fishes were taken to LASU hatcheries and acclimatized in a circular concrete tank for a week during which the physicochemical parameters were measured. The fish were later stocked at 4 fish/tank in five 50 liters water capacity plastic tanks at different concentrations (25mg/l, 50mg/l, 75mg/l, 100mg/l and control respectively).

Hematological Indices: The Hematological analyses were performed at the Hematology and Blood Transfusion Department of the Lagos University Teaching Hospital, Idiaraba, Lagos. The samples were collected from the fish and were then analysed for different hematological indices. The WBC, RBC Hb, PCV, L, MCV, MCH and MCHC were determined following the process and procedures of Klinkon *et al.,* (2015).

RESULTS AND DISCUSSION

Fish is widely accepted because of its high palatability, low cholesterol and tender flesh (Onyia *et al*., 2010). However, less number of consumers eats fish because of its nutritional value. Aquaculture urges for more accurate information on stress control, in order to ensure health of fish under culture environment. Medicinal plants have been known to synthesize active secondary metabolites with established potent anti-microbial activities, which indeed have formed the basis for their applications in pharmaceuticals, alternative medicines and natural therapies (Fabricant and Famsworth, 2001; Hammer *et al.,* 1999). Haematological parameters are routinely used for the evaluation of physiological environment and husbandry stressors in fishes (Rainza-Paiva *et al.,* 2000).

Reduction in RBC (Red blood cell) count was observed in bacteria infected fish (Figure 1) but showed a significant ($p<0.05$) improvement when treated with 50% *M. Oleifera* leave extract (Figure 2). Improvement in Erythrocyte count was considered an indicative of high oxygen carrying capacity of the blood, which is characteristic of fishes capable of aerial respiration and with high metabolic activity (Lenfant and Johansen, 1972). There were significant differences in WBC (leucopomia) values recorded across the treatment with an

increase in 50% treated fish, same value with the controlled samples. It has been observed (Ayoola, 2011) that an increase in WBC can be attributed to increase in the production of leucocytes in the haematopoietic tissue of the kidney and perhaps the spleen. Thus at 50% treatment there was an increase in WBC leading to suppression of infection while at 100% treatment less WBC were produced and the fish was observed to be seriously affected. As reported by Wedemeyer and Wood (1974), the primary consequence of observed changes in leucocytes count in stressed fish is suppression of the immune system and increased susceptibility to disease (Ayoola, 2011).

The parked cell volumes (PCV) have been suggested as test that can be carried out on routine basis in fish hatchery as check on fish health (Olapade and Kargbo, 2015). The increase observes however, in control and Moringa leaf extract treated fish can be liken to the findings of Joshi *et al.,* (2002b) that survival of fish can be correlated with increase in antibody production which helps in the survival and recovery. The MCH, MCHC and MCV showed a significant ($P<0.05$) difference with increase in Moringa leaf extract treatment. The values recorded ranges from 34.3±15.1 (MCHC), 40.7±19.19.4 (MCH) and 118±53.3 (MCV) respectively. The haemoglobin (Hb) result indicated that fishes treated with 50% *Moringa oleifera* leave extract had an increase haemoglobin concentration though not significantly ($P>0.05$) different from control but shows significant difference ($P<0.05$) from the infected fishes. The lymphocyte (LYMPH) count indicated a decrease as the level of *M. oleifera* leaf extract for treatment increases except 75% inclusion which shows higher percentage (72±44.4). No significant difference ($P>0.05$) between fish treated with 50% and 100% inclusions. Ayoola (2011) noted that Lymphocytes are the most numerous cells comprising the leucocytes, which function in the production of antibodies and chemical substances serving as defense against infection. The result of this study provides values for some haematological parameters for *C. gariepinus* which had initially been infected with Bacteria, *Aeromonas hydrophila* and to assess the health status of the fish were further treated with *M. Oleifera* leaf extract at different concentrations.

Conclusion

The study revealed *C. gariepinus* infected with bacteria (*Aeromonas* spp.) can be effectively treated with *M. oleifera* leaf extract at 50% concentration without adverse effect.

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