Treatment of Nile tilapia, *Oreochromis niloticus*, using Neem leaf extract against *Aeromonas hydrophila* infection

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Abstract : Nile tilapia *Oreochromis niloticus* was injected 1x 10^8 cfu/ml with a strain of the Gram- negative bacterium, *Aeromonas hydrophila*. After inoculation, the disease signs began on the 5th day as a haemorrhagic spot at the site of injection and the lesion subsequently progressed in size, inflammation of the anal opening and asitis**.** After this period, the mortality of infected group was 10±5% daily; hence, they were dip treated with an aqueous *Azadirachta indica* leaf extract at 1g/l for 10 min. daily for 30 days until the lesions healed completely. The hematological and biochemical parameters of infected and control fishes were monitored on the 10th,20thand 30thday. The white blood cells WBCs:10^4mm^{-1}counts significantly increased on the10th day of treatment and in treated fish on the 30th day. The red blood cells count RBCs: 10^6mm^{-1}significantly decreased on the 10th day. The hemoglobin Hb and hematocrit PCV decreased significantly in infected fish and in treated fish on the 10th day and this value returned to the normal value on the 30th day. serum protein level significantly increased in treated fish. In infected fish it decreased sinficantly.serum glucose, cholesterol and serum calcium levels were significantly lower in control fish when compared with treated fishes. In infected fishes levels of them continued to decrease significantly, The results indicate that after dip treatment A. indica aqueous leaf extract fishes exhibited a significant increase in serum glucose, cholesterol, total protein, RBC, Hb and PCV.the fish treated and nearly become normal after infection with Aeromonas hydrophila these for the treatable and immunestimulant action of *A. indica* aqueous leaf extract .

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1. Introduction

Aeromonas hydrophila causes disease in fish known as "Motile Aeromonas Septicemia" (MAS), "Hemorrhagic Septicemia," "Ulcer Disease," or "Red-Sore Disease." The many synonyms of this disease relate to the lesions caused by this bacterium which include septicemia where the bacteria or bacterial toxins are present within numerous organs of the fish, and ulcers of the fish's skin. *Aeromonas hydrophila* is a ubiquitous gram-negative rod-shaped bacterium which is commonly isolated from fresh water ponds and which is a normal inhabitant of the gastrointestinal tract. The disease caused by this bacterium primarily affects freshwater fish such as catfish, several species of bass, and many species of tropical or ornamental fish. Many have considered *Aeromonas hydrophila* to be an opportunistic pathogen. This seems like a contradiction in terms, since most bacteria which are termed"opportunistic" usually do not cause disease unless other factors are involved, and those bacteria which are considered a "pathogen" cart cause disease regardless of other factors. However, the term "opportunistic pathogen" conveys that *Aeromonas hydrophila* always is capable of producing disease if given the chance.wound or abrasions facilitate infection by opportunistic pathogens such as Aeromonas hydrophila (Ventura and Grizzle, 1998; Elliott and Shotts, 1980). Generally, the external symptoms of disease are hemorrhagic spots in the body. This requires information on severity of problem in aquaculture then mention of potential for antibiotic resistance using current treatments (Aoki and Kgusa, 1971). Boremann (1989) reports the occurrence of an antibiotic resistant strain of A. hydrophila in mirror carp (Cyprinus carpio) isolated from skin, organs (mixed samples of heart, liver, pancreas and spleen) and intestinal tracts against 50 mg/l ampicillin, 30 mg/l chloramphenicol, 30 mg/l kanamycin or 20 mg/l chloretetracycline. Nevertheless, despite

various treatment methods (Das and Das, 1993) to date no effective control measure is available (Anbarasu et al., 1998) for septicaemic conditions caused by A. hydrophila.

Neem, is known for its antiviral, antibacterial and antifungal properties and has been aptly known as the village dispensary for the past 2000 years (Biswas et al., 2002). It is referred to by the US National Academy of Sciences as "a tree for solving global problems" (Schmutterer, 1995; Singh et al., 1996) since it is a rich source of unique natural products for development of medicines against various diseases (Govindachari, 1992). The neem leaves contains nibin, nimbinene desacetylnimbinase, nimbandial, nimbolide and quercentin. Oral administration has even been attempted to treat fish infected with epizootic ulcerative syndrome (EUS) (Lilley et al., 2000). Consequently, the present study describes the potential recovery of O.niloticus infected with A. hydrophila after herbal treatment with neem leaves water extract and associated hematological and some biochemical change**s.**

2. Materials and methods :

2.1. Bacterial strain :

A. hydrophila was obtained from the Hydrobilogy Department National Research Center. It had been identified after . Subcultures were maintained on tryptone soya agar slopes at 5 ˚C and routinely tested for pathogenesis (Joseph and Carnahan, 1994) by inoculation into apparently healthy Oreochromis niloticus . A Stock culture in tryptone soya broth was stored at-20 ˚C .

2.2. Fish

Cultured Nile tilapia *O.niloticus* (average weight = 40 ± 10 g) collected from a private fish farm at Kafer El-Sheikh Governorate. The fish were transported to the laboratory in plastic bags (5 l) filled with oxygenated water and acclimatized in a stock tanks to laboratory conditions for 2 weeks under normal conditions (23 ± 2 ˚C). They were fed with commercial fish ration throughout the period of study and water was changed once a day.

2.3. Growth of *A. hydrophila* :

A. hydrophila was cultured on tryptone soya agar and harvested in tryptone soya broth . The broth was incubated overnight in a shaker for 12 h at 20°C and centrifuged at 10,000 rpm for 20 min at 4 °C; the supernatant was discarded and the bacterial pellet was washed three times with phosphate buffered saline (pH 7.2) and prepared to 10^8 cfu/ml as determined using a Neubauer hemocytometer slide (Yadav et al., 1992).

2.4. Infectivity experiments

After 2 weeks of acclimation, fish (100) were injected intraperitoneally IP with 100 µl of *A. hydrophila* at a concentration of 10^6–10^{10} cfu/ml to induce ulcers in order to determine the LC50 value for experiments.

2.5. Preparation of aqueous Azadirachta indica (Neem) leaf extract: :

Azadirachta indica (A. indica) leaves were obtained from the nurseries of the Ministry of Agriculture , dried and finely chopped, then dissolved in tap water, at a concentration of 500 g of dried leaves per liter of water, for 24 h at room temperature (as described by Cruz et al., 2004). The mixture was filtered and the extract (500 g/l) was used immediately in the experiments.

2.6. Experimental design :

Fish were divided into three groups of 10 each in triplicate, as follows:

Group 1: control fish injected with distilled water.
Group 2: ulcer induced fish, non treated.
Group 3: ulcer induced and dip treated with 1 g/l aqueous neem extract (15 min /day for 30 days).

2.7. Collection of blood samples

Approximately 0.05 ml of blood was collected in with a 20-gauge needle from six fish in each group caught randomly on the 10th, 20th and 30th day . The temperature of the samples was kept at 4 °C; EDTA and an aqueous solution of heparin were used as anticoagulants . To allow complete healing of the site, the samples were collected from either the right or left side of the fish on a given day. Half of the blood sample was used for hematological examination and the remaining half was stored at 4 °C for further biochemical analyses.

2.8. Hematology and biochemical indices:

The red blood cell counts (RBC: 10^6 mm^{-3}) were determined in a 1:20 dilution of the blood sample in Hayem's solution and the white blood cell counts (WBC: 10^4 mm^{-3}) from a 1:200 dilution of the blood sample in Turke's solution with a Neubauer

hemocytometer. The average of triplicate microhematocrits were used to determine the red blood cell volume at 10,000 rpm for 5 min (PCV: %) (Larsen and Snieszko, 1961). Hemoglobin (Hb: g/dl) was determined by the cyanhemoglobin method. A 20 µl blood sample was drawn from a heparinized capillary tube and mixed in 5.0 ml of cyanhemoglobin reagent (Hycel). Hemoglobin concentrations were determined at 540 nm with a Beckman DU spectrophotometer (Yokoyama, 1960; Larsen and Snieszko, 1961; Larsen, 1964; Hesser, 1960; Houston, 1990). The packed cell volume counts (PCV: %) were read after centrifugation for 10 min. After reading the hematocrit, the packed erythrocytes were discarded and the plasma was stored at — 12 °C, and subsequently the biochemical indices were determined with a Hitachi 704C instrument. These included total protein (TP: g/dl), glucose (GLO: mg/dl) and cholesterol (CHO: mmol/l) which were determined spectrophotometrically in the UV area, whereas the calcium contents (CAL: mmol/l) were determined by flame emission photometry (Hawk et al., 1954).

2.9. Statistical analysis

Data are presented as mean ± S.D. of the number of fish per group. Hematological and biochemical parameters were analyzed using the student's t-test to compare the difference in values between infected, herbal treated and the normal (control) fish

3. Results :

3.1. Clinical signs of *Oreochromis niloticus* after infection:

At the site of administration (10^8 cfu / ml) of A. *hydrophila* pathogen, ulceration commenced as sloughing off of scales, followed by the occurrence of a hemorrhagic spot all over the body which progressed to form an epidermal lesion (Fig 1). The lesion expanded in diameter and depth affecting the muscles (Fig 2). and infected fish died within 20 days. After A. indica dip treatment, the lesion decreased in diameter before healing completely treated after 30 days. Fish dipped in aqueous *Azadirachta indica* (Neem) leaf extract showing some nervous manifestations and respiratory distress expressed as increased opercular movement surfacing and gulping the atmospheric air .

Fig (1) *Oreochromis niloticus* showing hemorrhagic spot all over the body with slouphing of scales after IP injection of *A. hydrophila*

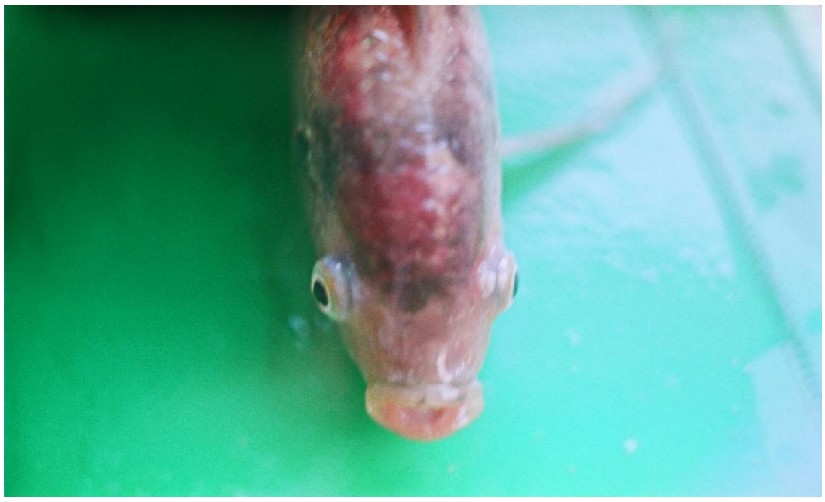

Fig (2) *Oreochromis niloticus* showing abrasions on the dorsal muscles after injection of A. hydropfila with exthiophalmia

Table 1: the results of the hematological parameters of infected and treated *O.niloticus*

Groups	WBCs (10^4mm^{-3})			RBCs (10^6 mm^{-3})			Hemoglobin (g/dl)			PCV (%)		
	10 days	20 days	30 days	10 days	20 days	30 days	10 days	20 days	30 days	10 days	20 days	30 days
Control	3.15 ± 0.31	3.15 ± 0.31	3.15 ± 0.31	2.31 ± 0.16	2.31 ± 0.16	2.31 ± 0.16	10.37 ± 0.61	10.37 ± 0.61	10.37 ± 0.61	33.60 ± 3.20	33.60 ± 2.20	33.60 ± 3.20
Infected	3.86 ± 0.31	4.16 ± 0.32	4.72 ± 0.22	1.75 ± 0.10	1.62 ± 0.10	1.67 ± 0.12	5.63 ± 0.60	6.09 ± 0.75	5.60 ± 0.42	18.57 ± 0.54	18.18 ± 0.66	18.83 ± 1.60
Treated	3.60 ± 0.20	3.07 ± 0.27	3.32 ± 0.30	1.68 ± 0.21	1.85 ± 0.15	3.37 ± 0.30	5.77 ± 0.74	8.43 ± 0.37	10.43 ± 0.67	20.23 ± 3.8	21.90 ± 3.47	32.37 ± 1.99

Table 2 : results of the biochemical parameters of infected and treated *O.niloticus.*

Groups	Total protein (g/dl)			Glucose (mg/dl)			Cholesterol (mmol/l)			Plasma calcium (m mol/l)		
	10 days	20 days	30 days	10 days	20 days	30 days	10 days	20 days	30 days	10 days	20 days	30 days
Control	3.34 ± 1.32	3.34 ± 1.63	3.33 ± 1.34	119.0 ± 16.93	119.2 ± 14.82	118.6 ± 13.71	10.0 ± 2.25	10.2 ± 2.55	10.0 ± 2.23	4.88 ± 0.34	4.74 ± 0.23	4.34 ± 0.34
Infected	2.76 ± 0.69	2.33 ± 0.61	2.38 ± 0.39	64.0 ± 11.97	60.07 ± 11.31	56.10 ± 9.56	4.37 ± 1.03	3.92 ± 1.07	4.31 ± 1.21	3.30 ± 0.46	2.93 ± 0.54	2.88 ± 0.32
Treated	3.61 ± 0.96	4.09 ± 0.88	6.11 ± 0.88	77.63 ± 15.57	86.70 ± 14.38	121.27 ± 18.95	6.11 ± 1.55	6.95 ± 1.01	10.56 ± 1.04	3.62 ± 0.39	4.55 ± 0.70	5.02 ± 0.75

3.2. Progression and healing of ulcers with *A. indica* extract dip treatment :

At a concentration of 10^6 cfu of *A. hydrophila*/ml, the mortality was 10% while at 10^{10} cfu/ml the mortality was 90% after an incubation period of 10 days. Hence, 10^8 cfu/ml, the LC50 calculated over a period of 10 days, was chosen since it was found to be optimal and ensured 50% survival (Brenden and Huizinga, 1986). The hematological and biochemical changes were monitored after 10, 20 and 30 days, after manifestation of the disease and they were compared with the control values.

3.3. Hematology results :

The values of the various indices for the A. hydrophila infected, A. indica aqueous leaf extract dip treatment and control fish are indicated in table (1.) The WBC level of infected nontreated fish initially increased from a control value of 3.15 ± 0.14 to 3.86 ± 0.31 on the 10th day. After the 20th and the 30th day, the WBC level in infected fish significantly increased to a maximum of 4.16 ± 0.31 and 4.72 ± 0.22, respectively. The treated fish registered a slight decrease in the level of WBC on the 10th day (3.60 ± 0.20) and on the 20th and the 30th day further decreased to 3.07 ± 0.27 and 3.32 ± 0.30, respectively. On the other hand, the RBC count in infected fish came down from the control value of 2.31 ± 0.16 to 1.75 ± 0.10 on the 10th day and attained the maximum decrease on the 20th and 30th day as 1.62 ± 0.10 and 1.67 ± 0.12, respectively. In the A. indica treated fish, the RBC increased from the minimum of 1.68 ± 0.12 (10th day) to a maximum of 3.37 ± 0.30 (30th day). The hemoglobin level in infected fish came down from the control value of 10.37 ± 0.61 to a minimum of 5.63 ± 0.60 on the 10th day and registered a further slight decrease on the 30th day (5.60 ± 0.42). On the other hand, the Hb level in the treated fish slightly increased from the 10th day to a maximum of 10.43 ± 0.67 (30th day). Hemoglobin contents also appeared to show a decreasing trend in infected fish and in treated groups it increased slightly. The hematocrit level in infected fish initially decreased from the control value of 33.60 ± 3.20 on the 10th day (18.57 ± 0.54). The hematocrit level in infected fish significantly decreased ($P < 0.001$) to a minimum of 18.18 ± 0.66 and 18.83 ± 1.60 (20th and 30th day), respectively. Although the hematocrit level decreased in infected fish, in the treated fish it increased slightly ($P < 0.05$) on the 10th day (20.23 ± 3.8) whereas on the 20th day it further increased ($P<0.01$) to 21.90 ± 3.47 and reached near control value (32.37 ± 1.99) on the 30th day ($P>0.05$).

3.4. Biochemical results :

. The serum total protein level in infected fish initially decreased from the control value of 3.34 ± 1.32 to 2.76 ± 0.69 on the 10th day table (2), whereas it significantly decreased ($P<0.01$) to a minimum of 2.33 ± 0.61 and 2.38 ± 0.39 on the 20th and 30th day. The treated fish registered a slightly increased level of serum protein on the 10th day 3.61 ± 0.96, whereas on the 20th and 30th day it further increased to 4.09 ± 0.88 and 6.11 ± 0.88, respectively. The serum glucose level in infected fish decreased significantly ($P<0.01$) from the control value of 119.0 ± 16.93 to 64.6 ± 11.97 on the 10th day. After the 20th and 30th day, the glucose level further significantly decreased ($P<0.001$) to a minimum of 60.07 ± 11.31 and 56.10 ± 9.56, respectively. The treated fish registered a significantly increased ($P<0.01$) level of glucose on the 10th day (77.63 ± 15.57) and on the 20th and 30th day it further increased to 86.70 ± 14.38 and 121.27 ± 18.95 ($P<0.01$ and $P>0.05$), respectively. The cholesterol level in infected fish initially decreased from the control value of 10.0 to 4.37 on the 10th day. After the 20th and 30th day, the cholesterol level significantly decreased ($P<0.001$) to a minimum of 3.92 and 4.31, respectively, in the infected fish. The treated fish registered a slightly increased level of cholesterol on the 10th day (6.11 ± 1.55) and on the 20th and 30th day it further increased to 6.95 ± 1.01 and 10.56 ± 1.04, respectively. The serum calcium level in infected fish initially decreased from the control value of 4.88 ± 0.34 to 3.302 ± 0.46 on the 10th day. After the 20th and 30th day, the plasma calcium level significantly decreased ($P<0.001$) to a minimum of 2.932 ± 0.54 and 2.875 ± 0.52, respectively. In the treated fish, the value increased slightly on the 10th day (3.62 ± 0.39), whereas on the 20th and 30th day it further increased 4.55 ± 0.70 and 5.020 ± 0.75, respectively.

4. Discussion

The clinical signs of fish injected with *A.hydrophyla* were At the site of administration of *A. hydrophila* pathogen, ulceration commenced as sloughing off of scales, followed by the occurrence of a hemorrhagic spot all over the body which progressed to form an epidermal lesion. The lesion expanded in diameter and depth affecting the internal muscles these results nearly agree with Sharifuzzaman, and Austin, (2009). Medicinal plants are environment friendly containing diverse biologically active principles. Comparisons of the sensitivity of different fish species to neem are questionable, since the amount of active compounds in a given weight of neem varies widely with the part of the plant, its place of origin or even the individual tree (Luo *et al.*, 1999 and Winkler *et al.*, 2007) The WBC levels in infected fish initially increased from the control level and after the 20th and 30th day the WBC count significantly increased to a maximum whereas in treated fish then decreased during the same period.

Erythrocytic necrosis virus (ENV) infected fish have also shown abnormal, dense, compact WBCs that reached the highest level for 72 h (Haney *et al.*, 1992). In almost all infected fishes, the homeostatic processes are extended beyond the normal limits due to stress (Pickering, 1981). In the A. indica treated fish, the RBC count increased (P>0.01) from the 10th day to the 30th day. The hemoglobin level in infected fish came down from the control value on the 10th day to the 30th day but the Hb level in the treated fish increased slightly by the 30th day. The decreased hemoglobin content may be brought about as a result of the swelling of RBC as well as poor mobilization of hemoglobin from the spleen and other hemopoeitic organs in Ictalurus punctatus (Scott and Rogers, 1981). These facts support the present finding that the significant decrease in erythrocyte and hemoglobin content is possibly due to hypochromic microcytic anemia caused by the bacteria. In the A. indica treated groups, reversible changes occurred since the levels recovered after 30 days. Scott and Rogers (1981) showed a significant increase of hemoglobin at Stressed-Sampled 48 h (SS48) and Stressed-Sampled 72 h (SS72) hypoxia leading to elevated oxygen carrying capacity of the individual erythrocyte I. punctatus. Decreased RBC counts, hematocrit and hemoglobin concentration indicate that RBCs are being destroyed by the leucocytosis activity in an erythrocytic anemia with subsequent erythroblastosis (Haney *et al.*, 1992). An increase in hematocrit has been reported as a result of oxygen deficiency (Holeton and Randall, 1967; Wood and Johansen, 1972; Swift and Lloyd, 1974; Kirk, 1974). In our experiments, the hematocrit level significantly decreased (P < 0.001) in infected fish on the 20th and 30th day and in the treated fish increased. In addition, other studies have reported that there is a significant reduction in many other parameters as well. For instance, the pearl spot fish Etroplus suratensis when infected with EUS becomes anemic followed by a significant reduction in RBC, Hb and PCV (Pathiratne and Rajapakshe, 1998). Mitra and Varshney (1994) obtained Catla catla and Labeo rohita with fungal infection from fish farms and the infection resulted from ulceration followed by hemorrhage on the dorsal surface of the body. Chemical treatments with copper sulphate, potassium permanganate and common salt solution did not yield positive results. Significant recovery was achieved with repeated intramuscular injections of the homeopathic drugs heaper sulfer and arnica spray.

The serum protein level initially decreased in infected fish from the control value on the 10th, 20th and 30th day. Total plasma protein also increased due to the destruction of RBCs and the resultant release of cell contents into the blood stream (Haney *et al.*, 1992). Scott and Rogers (198 1) reported that the plasma protein values did not vary significantly (P>0.05) from that of the control in infected fish. The total erythrocyte and leukocyte counts in Stressed-Sampled (SS) and stressed-reacclimatized (SR) fish did not vary significantly from the control. The treated fish in our experiment registered a slightly increased level of serum protein between the 10th day to the 30th day. The treated fish registered a significantly increased level of glucose on the 30th day, which was similar to the control fish values. The cholesterol and calcium levels significantly decreased from the 10th day to the 30th day in infected fish but the treated fish significantly increased.

Herbal medicines employed to dip treat fish against *A. hydrophila* pathogens typically contain soluble and particulate components, both of which may generate protective immune responses. The results indicate that after dip treatment (A. indica aqueous leaf extract) fishes exhibited a significant increase in serum glucose, cholesterol, total protein, RBC, Hb and PCV.the fish treated and nearly become normal these for the treatable and immunestimulant action of A. indica aqueous leaf extract.

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