Trials for Neem leaf extract treatment against MAS in Nile tilapia, *Oreochromis niloticus*

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Abstract : Nile tilapia *Oreochromis niloticus* was injected experimentally with *Aeromonas hydrophila*. After inoculation, the disease signs began on the 5th day as a haemorrhagic spots 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 5% daily; hence, they were dip treated with an aqueous *Azadirachta indica* leaf extract. daily for 30 days until the lesions healed completely. The hematological and biochemical parameters of infected and control fish were monitored on the 10th,20th and 30thday. The white blood cells counts were significantly increased on the10th day of treatment and in treated fish on the 30th day. The red blood cells count significantly decreased on the 10th day. The hemoglobin and hematocrit 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 levels were significantly increased in treated fish. In infected fish it decreased signficantly.serum glucose, cholesterol and calcium levels were significantly lower in control fish when compared with treated fish. In infected fish levels of them continued to decrease significantly, The results indicate that after dip treatment of *A. indica* aqueous leaf extract fishes exhibited a significant increase in serum glucose, cholesterol, total protein, RBC, Hb and PCV.The treated fish nearly became 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), The disease related to the lesions caused by this bacterium which include septicemia where the bacteria and bacterial toxins are present within numerous organs of the fish, and ulcers of the fish's skin. *A. 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 *A. 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, The term "opportunistic pathogen" conveys that *A. hydrophila* always is capable of producing disease if given the chance espcialy wound or abrasions facilitate infection (Elliott and Shotts, 1980;Ventura and Grizzle, 1998). Generally, the external symptoms of disease are hemorrhagic spots on the body.

Neem is known for its antiviral, antibacterial and antifungal properties and has been known as the village dispensary for the past 2000 years (Biswas *et al.*, 2002;Girish and Shankara 2008). It is referred to by the US National Academy of Sciences as "a tree for solving global problems" (Schmutterer, 1995; Singh *et al.*, 1996; Saravanan *et al.*,2010) 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 des-acetylnimbinase, 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 try to describe the potential recovery of *O.niloticus* infected with *A. hydrophila* after herbal treatment with neem leaves water extract and associated with some hematological and 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 25 ˚C and routinely tested for pathogengity (Joseph and Carnahan, 1994) by inoculation into apparently healthy *Oreochromis niloticus.*

2.2. Fish

A total number of 190 Cultured Nile tilapia *O.niloticus* (average body weight = 100 ± 10 g) were collected from a private farm at Kafer El-Sheikh Governorate. The fish were transported to the laboratory in plastic bags (5 L) filled with oxygenated water and acclimiated in a stock tanks at 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 acclimiation, fish (100) were injected intraperitoneally IP with 100 µl of *A. hydrophila* at a concentrations of 10^6,10^7,10^8,10^9 and10^{10} cfu/ml to induce spiecimeia (clinical sings of infection) 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. The mixture was filtered and the extract (500 g/l) was used immediately in the experiments (Cruz et al., 2004).

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: experimentally infected fish, (non treated).

Group 3: experimentally induced and diped treated with aqueous neem extract (15 min /day for 30 days).

2.7. Blood sampling :

Approximately 1ml of blood was collected from the caudal vein from six fish in each group caught randomly on the 10^{th}, 20^{th} and 30^{th} day . The temperature of the samples was kept at 4 °C; EDTA was used as anticoagulant . Half of the blood sample was used for hematological examination and the remaining half was used for separation of serum and stored at -20 ℃ for further biochemical analyses.

2.8. Hematology and biochemical indices:

The red blood cell count was determined in a 1:20 dilution of the blood sample in Hayem's solution and the white blood cell counts (WBC) 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 (PCV) (Larsen and Snieszko, 1961). Hemoglobin was determined by the cyanhemoglobin method (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 — 20 °C, and subsequently the biochemical indices were determined. These included total protein, glucose and cholesterol were determined spectrophotometrically, whereas the calcium contents 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*, ulceration commenced as sloughing off of scales, followed by the occurrence of a hemorrhagic spots all over the body which progressed to form an epidermal lesion (Fig 1). The lesion expanded in diameter and depth affecting the muscles making erosions (Fig 2). Infected fish (control) died within 20 days after infection. After A. indica dip treatment, the lesion decreased in diameter gradually before healing completely, treated after 30 days. Fish dipped in aqueous *Azadirachta indica* (Neem) leaf extract in the begining showing some nervous manifestations and respiratory distress expressed expressed as increased opercular movement surfacing and gulping the atmospheric air.

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

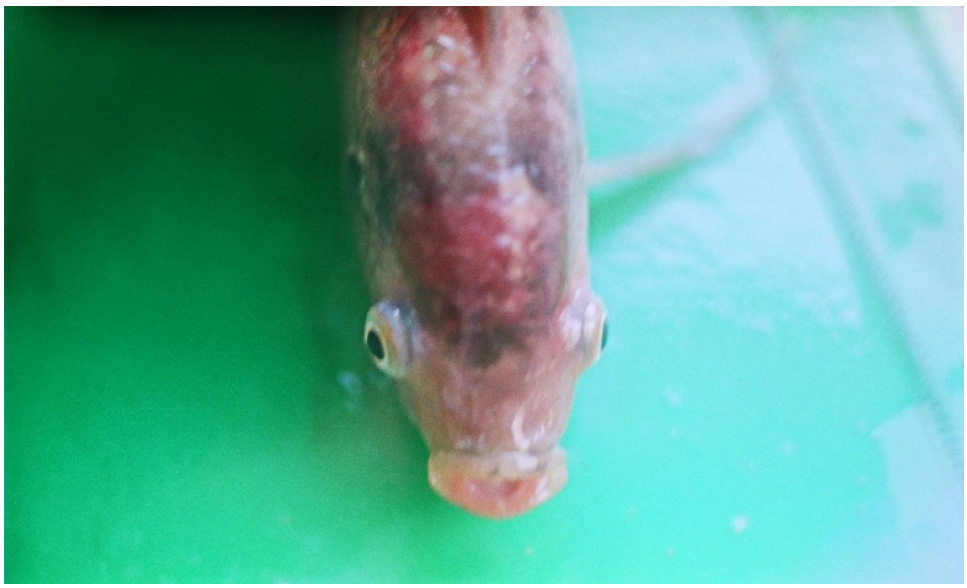

Fig (2) *Oreochromis niloticus* showing erosions on the dorsal musculature after injection of *A. hydropfila* with bilateral pop eyes

Table 1: Showing 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

Data are presented as mean ± S.D. of the number of fish per group - * Significance (P<0.001)

Table 2: Showing serum biochemical parameters of infected and treated *O.niloticus*

Groups	Total protein (g/dl)			Glucose (mg/dl)			Cholesterol (mmol/l)			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

Data are presented as mean ± S.D. of the number of fish per group - * Significance (P<0.001)

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%. 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.

3.3. Results Hematological examinations :

The values of the various blood parameters for the *A. hydrophila* infected, *A. indica* aqueous leaf extract dip treatment and control fish are indicated in table (1.).

3.4. Biochemical results:

The serum parameters levels in *A. hydrophila* infected fish In the *A. indica* aqueous leaf extract dip treatment treated fish, and control fish are indicated in table (2.).

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 spots 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 (1981) reported that the plasma protein values did not vary significantly 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 the 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 in ifected fish and returned to the normal in treated fish, 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 these may be due to osmoregulation disturbance in infected untreated fish.

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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