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EVALUATION THE ANTIBACTERIAL EFFECT OF NEEM PLANT LEAVES ON *CLARIAS GARIEPINUS*

By

Zinab M. Abou-Talb[1], Eman S. Abd El-hamid[2], Soad A. Mekawy[3], and Sally H. Abou-Khadra[4]

[1] Fish diseases department, Animal health research institute, Zagazig branch.

[2] Biochemistry department, Animal health research institute, Zagazig branch.

[3] Clinical pathology department, Animal health research institute, Zagazig branch.

[4] Bacteriology department, Animal health research institute, Zagazig branch.

ABSTRACT

The present study aimed evaluates the antibacterial, biochemical, and heamatological activities of neem (*Azadirachta indica*) leaves on the health statues of catfish (C*larias gariepinus*) experimentally infected with *Aeromonas hydrophila.* The bacteriological examination of naturally infected *Clarias gariepinus* revealed that,the most prevalent isolates were *Aeromonas hydrophila* 13% followed by *Pseudomonas fluorescens* 12%. Two hundred and forty apparently healthy *Clarias gariepinus* were divided into 8 subgroups for experimental purpose. Experimental infection was done by intraperitoneal (IP) injection of *Aeromonas hydrophila* and examination of the infected and control fish in the 1st, 2nd and 3rd weeks post infection. The antibacterial activity of neem leaves reduced the mortality rate from 80% in an infected nontreated subgroup to 26.6% and 30% in subgroups that infected and treated with neem and oxytetracycline combination and treated with neem only respectively. Subgroup infected and treated with neem had lower total bacterial count (TBC) ($1x10^4$CFU/ml) than subgroup infected and treated with oxytetracycline ($7x10^5$CFU/ml). Heamatological parameters and serum biochemical index in both infected subgroups treated with neem or treated with neem and oxytetracycline combination were approximately similar to noninfected nontreated subgroups (negative control normal subgroup). We could conclude that neem leaves-supplemented diets had a potential effect in reduce mortality and bacterial infection in *Clarias gariepinus*.

Keywords:

Clarias gariepinus, neem plant, *Azadirachta indica*, *Aeromonas hydrophila.*

INTRODUCTION

African catfish (*Clarias gariepinus)* is one of high economic, nutritional value and one of the most promising freshwater fish species in African aquaculture because of its tolerance to hard rearingconditions,easy marketability,high fecundity,and hatchability**(Kemigabo *et al.,* 2018).** Bacteria are the major group of pathogens; possess one of the most significant threats to successful fish production throughout the world. Bacterial diseases are responsible for heavy mortalities in both culture and wild fish throughout the world and most of the causative microorganisms are opportunist pathogens which invade the tissue of fish host rendered it susceptible to infection **(Roberts, 2004).**

The majority of bacterial infections are caused by Gram - negative (*Aeromonas spp, Pseudomonas spp., and Vibrosis spp*) and considered to be the major bacteria incorporated in severe outbreaks among catfish **(Ashiru *et al.,* 2011).**

Motile *Aeromonas* species (MAS) namely *Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas sobria* have been reported to be indigenous (authochtonous) in the aquatic environment, hence they are associated with the fish as normal commensal flora **(Janda and Abbott, 2010; Yardimic and Aydin,2011).** *Aeromonas hydrophila* is the most common bacteria in freshwater habitat throughout the world, and this bacterium frequently causes diseases among cultured and feral fishes **(Cipriano 2001). Sarder *et al.,* (2016)** mentioned that *Aeromonas hydrophila* is an opportunistic microorganism.

Till now, the traditional use of huge amounts of an antimicrobial agent for control strategies has been used in aquaculture. However, the use of antimicrobial compounds is less favored due to adverse impacts like bioaccumulation and the emergence of drug resistant strains in the environment. Recently, all attentions were focused on immunostimulants and herbal plants which could have a good effect on fish diseases management **Hari Krishnan *et al.,* (2011).**

Neem is an important member of herbal medicine. The anti-bacterial properties of neem *(Azadirachta indica*) have been recognized in Indian tradition medicine **(Jegede and Fagbenro, 2007). Biswas *et al.,* (2002)** recorded that Neem leaves compounds have many biological activities as anti-inflammatory,antipyretic, antifungal, antitumor,diuretic, immune-modulatory, and hepatoprotective effects. Neem leaves also contain fatty acids, Vitamin E and other essential amino acids. The Biological active compounds isolated from different

parts of the plant include azadirachtin, meliacin, gedunin, salanin,nimbin,valassin, margosine, eriterperenoid, azatin, rotinine and quinine among other active ingredients **(Adewole *et al.*, 2002). Schmutterer and Singh (1995)** said that most important bioactive principal of neem is azadirachtin has at least 10 other limonoids. **Duke (2003)** mentioned that, the chemical constituents in different crude extracts of the leaves of neem are normal hydrocarbons, phenolic compounds, terpeniods, alkaloids, and glycosides. Neem plant is used as a good source of antitumor, antiviral and antimicrobial agents **(Biswas *et al.*, 2002).**

Bandyopadhyay*et al.,*(2002)andVerma,(2009)who saidthat when neem orally administered, it absorbed through intestinal villi to blood and then to the liver while its excretion is through bile to fecal contents. Oral administration of neem leaves aqueous extract has been reported to produce an increase in red blood cell, white blood cell and lymphocyte counts hence enhancing cellular immune response, increasing antibody production thus most pathogens (particularly intestinal organisms) were eliminated before causing symptoms of the disease **(NRC, 1992).**

So, the aim of this research was to undergo a bacteriological examination of naturally infected catfish (*Clarias gariepinus*). Assessment of antibacterial activity of neem leaves and their effect in the hematological parameters, liver and kidney functions on experimentally infected catfish (*Clarias gariepinus*) with *Aeromonas hydrophila*.

MATERIAL AND METHODS

Fish:

A- Naturally infected fish.

A total number of (25) *African catfish* (*Clarias gariepinus*) were collected from different localities at El-Sharkia governorate. Fish were transported alive to Animal health research institute, Zagazig branch. Fish examined clinically in glass aquaria, supplied with aerated chlorine free tapwater.Fish examined clinically for anyabnormal lesions and bacteriologically according to **Austin and Austin (2012).**

B- Experimental Fish.

Two hundred and forty apparently healthy *Clarias gariepinus* with an average body weight (150-200 gm) were collected from the Fish Farm of Central Laboratory for Aquaculture Research, Abbassa,Abo-Hammad, El-Sharkia, Egypt and acclimated in indoor tanks supplied with dechlorinated tap-water and continuous aeration for 2 weeks during which they were fed

commercial feed at a daily rate of 5% of biomass. Fish were classified into eight equal subgroups as shown in (Table 1). Any dead fish was rapidly removed to avoid contamination. The fishes were accepted as being adapted to the laboratory conditions when the percentage of death recorded during acclimatization was less than 2%.

Table (1): Experimental design of fish under study.

Groups	No. of fish	Description
Group I: non-infected		
Subgroup (1)	**30**	**non-treated (control negative)**
Subgroup (2)	**30**	**Treated with Neem leaves (5%)**
Subgroup (3)	**30**	**Treated with oxytetracycline (0.1%)**
Subgroup (4)	**30**	**Treated with Neem (5%) and oxytetracycline (0.1%)**
Group II: infected with *A. hydrophila*		
Subgroup (5)	**30**	**non-treated (control positive)**
Subgroup (6)	**30**	**Treated with Neem leaves (5%)**
Subgroup (7)	**30**	**Treated with oxytetracycline (0.1%)**
Subgroup (8)	**30**	**Treated with Neem (5%) and oxytetracycline (0.1%)**

Sampling and isolates identification.

A total of 100 Examined samples included (gills, kidney, liver, and intestine) were aseptically collected from natural infected *Clarias gariepinus*. Samples inoculated in tryptic soy broth and incubated for 24 hrs at 30˚C. A loopful of tryptic soy broth were streaked onto nutrient agar, trypticase soy *agar*, Rimler - Shotts medium (RS), blood agar, baird parker and Pseudomonas agar medium (Oxoid,England), plates then incubated at 28˚C for 24-48 hr. The growing colonies were picked up in pure form and identification of all isolates was done by cultural, morphological and biochemical characters according to **(Quinn *et al.,* 2002; Austin and Austin (2012).** The *salmonella* isolates were serotyped in the Serology Unit, Animal Health Research Institute, Dokki, and Giza, Egypt using commercial antisera **(Difco, Detroit, and MIUSA).**

Plant and feed preparation:

Fresh leaves of neem (*Azadirachta indica*) were collected from a garden in El-Asher Men Ramadan City. The samples identified in Botany Department, Faculty of Science, Zagazig University. The leaves were washed in sterile distilled water, dried in shade, coarsely powdered, leaves contain several chemical and biological active ingredients previous determined by **(Adewole *et al.*, 2002).** The powder was added to fish diet constituent during preparation at proportion 5% of diet **(Temitope and Oyedapo, 2008).**

Preparation of inoculum:

Preparation of the inoculum (bacterial suspension) of *A. hydrophila* that confirmied previously to be sensitive for Oxytetracycline by in vitro suseptability test. *A. hydrophila* freshly cultured onto TSA (tryptic soy agar) plate, incubated at 25°C for 48 hours and then bacterial colonies were mixed with sterile physiological saline (6×10^6 cfu/ml) and adjusted using Mcferlend standers **(McFarland, 1907).** Fish were experimentally infected intraperitoneally with 0.5 ml of the prepared inoculum according to **(Emeish *et al.*, 2018).** Injections were given with utmost care to avoid puncture of internal organs. At the injection period care also was taken so that, the inoculum would not come out after pushing back of the syringe.

All the injected fish were then transferred to the aquaria then clinical signs, post mortem lesions,and mortalities were recorded daily for up 3 weeks (experimental period).

Experimentally infected Moribund Fishes within experimental period were examined for re-isolation of *A. hydrophila* from internal organ. Positive culture was confirmed by conventional bacteriological techniques as re-isolated strain was identical with those of the isolates used in the experimental infection.

Total bacterial count (TBC):

Gills, liver, and kidney of each fish collected in the 1st, 2nd, and 3rd weeks post infection and treatment were dissected out aseptically and placed in sterilized separate plastic petri dishes. After pooling and weighting, the samples were homogenized and suspended in sterile physiological saline (1 part of sample: 9 parts of PS) to obtain a stock solution.

Two consecutive decimal dilutions, from the stock solution, were made.

The different dilutions were cultured onto duplicate TSA plates. All such plates were incubated at 25°C for 48 hours. The growing colonies were counted and their numbers were used to interpret the effect of treatment **(Anonymous, 2004).**

Blood Sampling:

Collection of Blood samples:

Blood samples were collected by the caudal vein method which is considered the suitable way **(Lied *et al*., 1975).** Two blood samples were taken from each fish for hematological and biochemical studies. Firstly, the sample of blood was collected in a test tube mixed with EDTA as an anticoagulant, the second blood sample was collected in a plain centrifuge tube, clotted and serum was separated by centrifugation at 3000 R.P. M. for 20 minutes.

Clear serum was separated carefully and stored in Eppendorf tube at-20 ^{o}C until used for biochemical analysis.

Hematological studies:

Total erythrocytic count was performed according to **Dacie and Lewis (1984).** Heamoglobin concentration was determined according to **Baker and Silverton (1976)** while the packed cell volume percent was determined according to **Cohen (1967).** The total leucocytic count was counted according to **Harrison and Harrison (1986).**

Serum biochemistry parameters:

All biochemical parameters were carried out using commercial kits, the used protocol for each parameter was done as recommended by the manufacture manual.

Liver function tests:

Estimation of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined according to **Reitman and Frankel (1957).** Estimation of serum total protein was determined according to **Grant *et al*., (1987).**

Kidney function tests:

Determination of serum urea level was performed according to **Patton and Crouch (1977) and** estimation of serum creatinine was according to **Henry *et al.,* (1974).**

Statistical analysis:

The results were subjected to one-way analysis of variance (ANOVA). The Duncan multiple range tests were applied to rank treatment means ($p< 0.05$) with the aid of SPSS version22 according to **Corp, (2013).**

RESULTS

Clinical and Post-mortem Examination of naturally infected Fishes.

Naturally infected fishes showed hemorrhages all over the fish body especially at the base of fins, tail and skin ulceration, abdominal distention, liver paleness and enlargement in some fishes and congestion. Spleen was congested with enlarged and hemorrhagic enteritis in some fishes (photo 1, 2, 4, 5).

Bacteriological examination and identification of pathogens.

The screening of bacterial isolates was carried out based on their colony morphology, biochemical and serological characterization from different samples of naturally infected *Clarias gariepinus*. The most prevalent isolates were *Aeromonas spp.* (18%) followed by *Pseudomonas fluorescens* (12%) and the lowest prevalent isolates were *Staph. Epidermidis* (4%) and *salmonella Essen* (4%) (Photo 6 -10).

The highest isolation rates were from gills (80%) and liver (68%) as showed in (Table 2).

Table(2): Prevalenceandserotypes of bacterial isolates in naturally infected*Clarias gariepinus.*

Isolates	Isolation Site (n)				Prevalence %
	Gills (25)	Kidney (25)	Liver (25)	Intestine (25)	
S. epidermidis	**1**	**2**	**--**	**1**	**4 (4%)**
S. aureus	**5**	**1**	**1**	**2**	**9 (9%)**
A.hydrophila	**5**	**3**	**5**	**--**	**13 (13%)**
A.sobria	**1**	**2**	**2**	**--**	**5 (5%)**
K. pneomoniae	**3**	**2**	**2**	**--**	**7 (7%)**
P. fluorescens	**4**	**3**	**4**	**1**	**12 (12%)**
C. freundii	**1**	**2**	**2**	**1**	**6 (6%)**
S. essen	**--**	**--**	**1**	**3**	**4 (4%)**
Total	**20 (80%)**	**15 (60%)**	**17 (68%)**	**8 (32%)**	**60 (60%)**

n:number of samples,*S. epidermidis:Staphylococcus epidermidis,S.aureus: Staphylococcus aureus, A. hydrophila: Aeromonas hydrophila, A. sobria: Aeromonas sobria. K. pneomoniae: Kelbsilla pneomoniae, P. fluorescens: Pseudomonus fluorescens, C. freundii: Citobacter freundii, S. essen: Salmonella essen.*

Experimental infection by *A. hydrophila* and mortality rate:

Clarias gariepinus fish in all the infected subgroups post-infection became very pale with hyperaemic spots at the base and tips of the fins with or without skin lesions.

In a subgroup (5) showed extensively distributed haemorrhagic skin ulcers and severe

hyperaemic patches (photo.3).Bacterial agentsrecoveredfromthe skin lesionsof experimentally -infected fish were found to be the same as those used in the experimental infection.

The highest mortality rate (11.66%) was recorded in all subgroups in first week post infection. The highest cumulative mortality was observed in the infected nontreated subgroup (5) with mortality reached to (80%) as shown in (Table 3).

Table (3): Percentages of mortality in all subgroups of tested *Clarias gariepinus.*

Subgroups	1st week	2nd week	3rd week	Total
(1)non-infected non-treated	**0**	**1**	**1**	**2 (6.66%)**
(2) non-infected treated with neem	**0**	**0**	**1**	**1 (3.33%)**
(3)non-infectedtreated with oxytetracycline	**1**	**0**	**0**	**1 (3.33%)**
(4)non-infected treated with neem and oxytetracycline	**0**	**0**	**0**	**0 (0%)**
(5) infected non-treated	**13**	**9**	**2**	**24 (80%)**
(6) infected and treated with neem	**6**	**2**	**1**	**9 (30%)**
(7) infected and treated with oxytetracycline	**4**	**6**	**1**	**11 (36.66%)**
(8)infected and treated with neem and oxytetracycline	**4**	**3**	**1**	**8 (26.66%)**
Total	**28 (11.66%)**	**21 (8.75%)**	**7 (2.91%)**	**56 (23.33%)**

Total bacterial count (TBC) in infected and noninfected groups.

In the infected group II, we found that, the subgroup infected and treated with neem and oxytetracycline showed the lowest TBC (1.77 x 10^2 CFU/ml) that was nearly similar to TBC in noninfected nontreated subgroup (1) (1.22 x 10^2CFU/ml). Subgroup infected and treated with neem had TBC lower than subgroup infected and treated with oxytetracycline in 1st, 2nd and 3rd week post infection (Table 4).

Table (4): Total bacterial count 1st,2nd and 3rd weeks post treatment and experimental infection.

Subgroups	1st week	2nd week	3rd week
Group I	**CFU/ml**		
(1) non-infected non-treated	**2.67×10^2**	**1.43×10^2**	**1.22×10^2**
(2) non-infected treated with neem	**4.2×10^2**	**2.1×10^2**	**2×10^2**
(3) non-infected treated with oxytetracycline	**2.57×10^2**	**1.60×10^2**	**1×10^2**
(4)non-infectedtreated withneemand oxytetracycline	**2×10^2**	**1.6×10^2**	**0.9×10^2**
Group II	**20×10^6**	**18×10^5**	**8×10^5**
(5) infected non-treated			
(6) infected and treated with neem	**1×10^4**	**2×10^3**	**2×10^2**
(7) infected and treated with oxytetracycline	**7×10^5**	**3×10^4**	**3×10^2**
(8)infectedand treated withneem and oxytetracyclines	**5×10^4**	**9×10^2**	**1.77×10^2**

Hematological Changes:

The hematological indices results of experimental fishes (Table 5) showed that, the Packed Cell Volume (PCV), Haemoglobin (Hb) and Red blood cells (RBCs) and the white blood cells count (WBCs) were had no significant differences ($P<0.05$) between noninfected nontreated subgroup (1) which considered as control negative and noninfected treated with neem subgroup (2). Also, we recorded that, the Packed Cell Volume (PCV), Haemoglobin (Hb) and Red blood cells (RBCs) were significantly decreased and the white blood cells count (WBCs) were significantly increased ($P<0.05$) in infected and treated subgroups (6, 7, 8) in the 1st week post infection in compared with noninfected non-treated (control) subgroup (1) and returned to be nearly similar to it in the 2nd week, then in the 3rd week recorded no significant differences. But infected nontreated subgroup (5) showed that,the Packed Cell Volume (PCV), Haemoglobin (Hb) and Red blood cells (RBCs) were highly significantly decreased while white blood cells count (WBCs) was highly significantly increased ($P<0.05$) in 1st,2nd and 3rd weeks post infection in compared with infected and treated subgroups (6, 7, 8) and noninfected non-treated control subgroup (1).

Interestingly, we found that in subgroup (2) non-infected and treated with neem was significantly increased in the white blood cells count (WBCs) in compared with a subgroup (3) noninfected treated with oxytetracycline and subgroup (4) non-infected treated with neem and oxytetracycline.

Serum biochemical parameters Changes:

Changes in some Liver function parameters.

In the 1st and 2nd weeks post infection the infected nontreated subgroup (5) showed that Alt and AST significantly increased while serum total proteins were significantly decreased in comparison with the rest of infected and treated subgroups (6,7,8), noninfected nontreated subgroup (1), and noninfected treated subgroup (2,3,4).

The levels of ALT and AST after the 1st and 3rd weeks post infection and treatments showed no significant differences between both of subgroups (2,4) and subgroup (8) but in subgroups (3,7) were significantly increased.

All liver function parameters examined in the subgroup (6) in the 3rd week post infection had no significant differences with a subgroup (1), showed that liver factor in the infected and neem treated subgroup (6) returned to the normal level of liver function parameters (Table 6).

Changes in some Kidney function parameters:

In the 1st, 2nd and 3rd weeks post infection and treatments the infected nontreated subgroup (5) showed that serum urea and serum creatinine were significantly increased in comparison with the rest of infected and treated subgroups (6,7,8), noninfected nontreated subgroup (1), and noninfected treated subgroup (2,3,4) .

In the 1st and 2nd weeks post treatment no significant differences were noticed in serum urea and serum creatinine between subgroup (1) and subgroup (2).

All the kidney function parameters which were examined in the infected treated subgroups (6, 8) in the 3rd week post infection had no significant difference with a subgroup (1), this showed that kidney function returned as normal (Table 6).

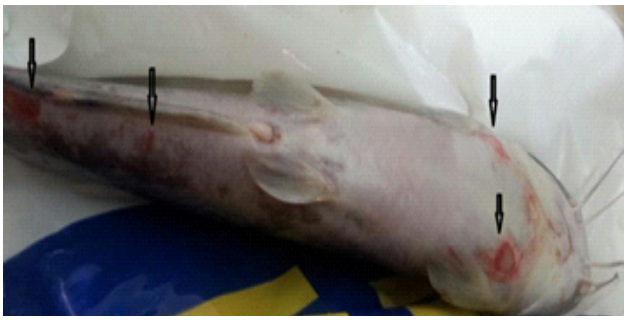


Photo. (1): Naturally infected *Clarias gariepinus* showing skin ulceration with haemorrhagic patches on different parts of body surface.

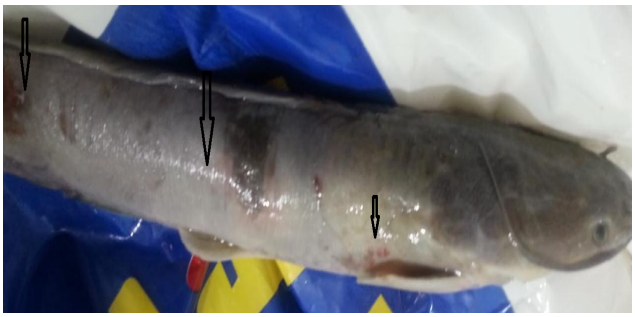
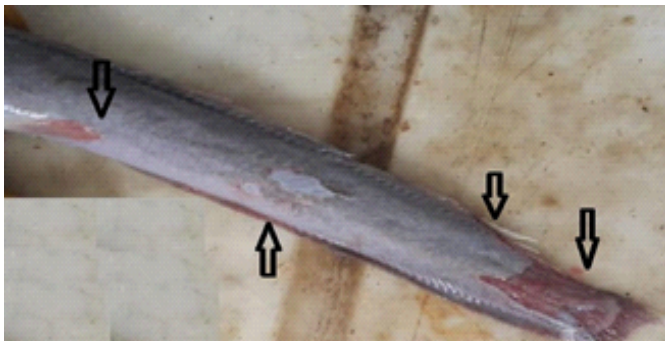


Photo. (2): Naturally infected *Clarias gariepinus* showing skin ulceration and abdominal distention.



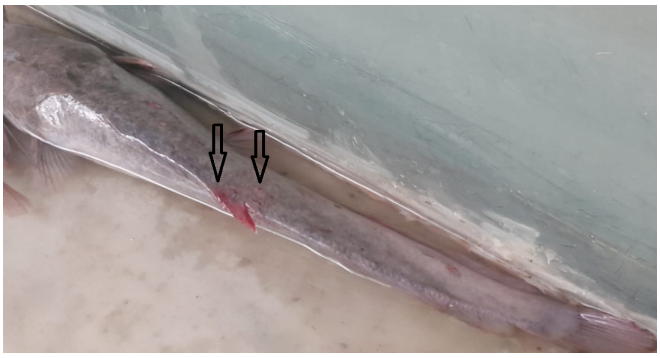


Photo. (3): Experimental infected *Clarias gariepinus* with *A. hydrophila* showing hemorrhage and ulceration base of the fins.

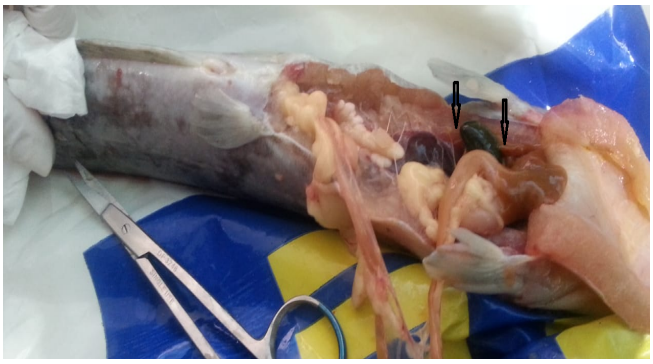


Photo. (4): Naturally infected *Clarias gariepinus* showing enlarged and congested Spleen.

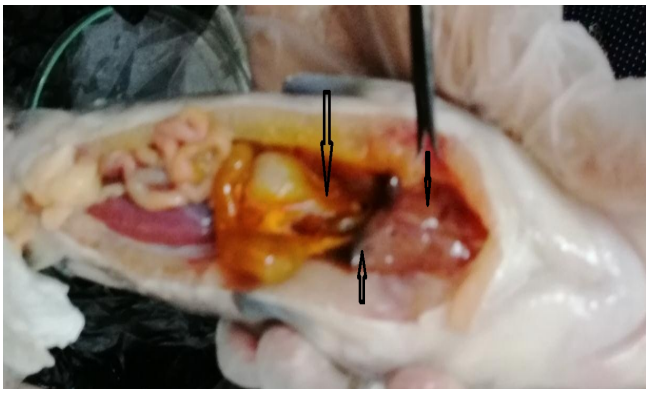


Photo. (5): Naturally infected *Clarias gariepinus* showing distended abdomen with ascetic fluid and paleness and anemic liver with necrosis and hemorrhage on its edge.

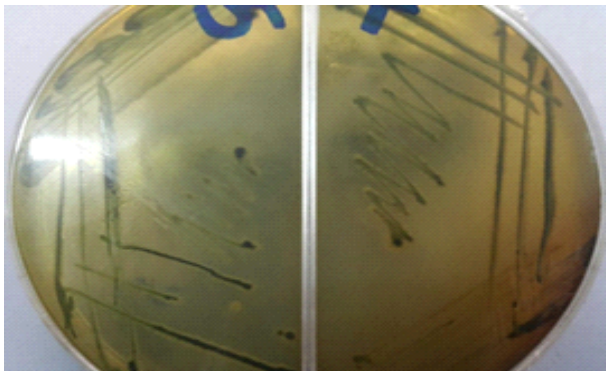


Photo (6): *Aeromonas hydrophila* colonies on Rimler- Shotts agar.

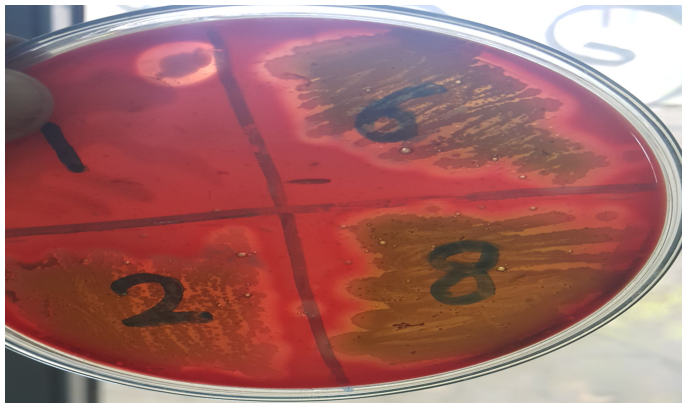


Photo (7):*Aeromonas hydrophila* colonies on blood agar (β-heamolysis).

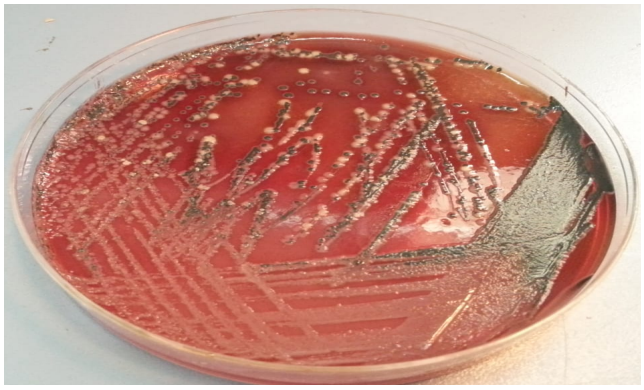


Photo. (8): *Salmonella Spp.* colonies on XLD agar.

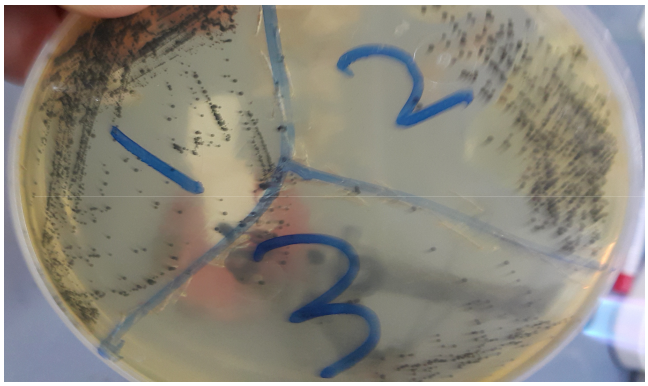


Photo. (9): Staphylococcus *Spp.* on baird parker agar.

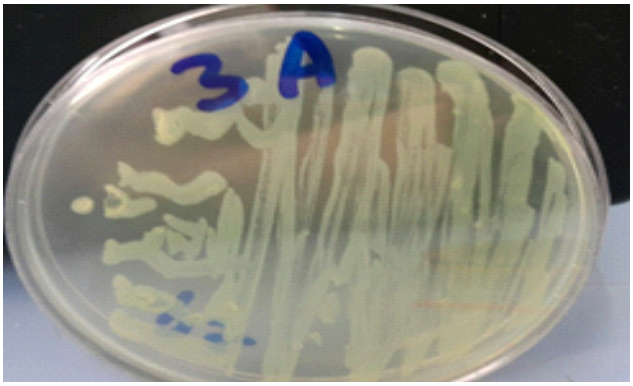


Photo. (10): *Pseudomonas Spp.* colonies on Pseudomonas agar.

Table (5): The effect of supplementation diet with Neem (*Azadirachta indica*) leaves (5%) and oxytetracycline (0.1%) on heamatological parameters of *Clarias gariepinus*. (M± SD) (n = 5).

Parameter	Subgroups							
1st week	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
RBCs (10^6/µL)	3.19 ± 0.71^{d}	2.63 ± 0.79^{cd}	2.16 ± 0.33^{bc}	1.78 ± 0.12^{b}	0.84 ± 0.04^{a}	1.62 ± 0.03^{b}	1.56 ± 0.10^{b}	1.64 ± 0.20^{b}
Hb (g/dL)	12.06 ± 1.3^{d}	10.93 ± 0.52^{d}	11.60 ± 0.51^{c}	9.34 ± 0.56^{b}	4.68 ± 0.58^{a}	7.35 ± 0.33^{b}	6.88 ± 0.29^{b}	7.51 ± 0.48^{b}
PCV%	31.69 ± 1.7^{cd}	32.03 ± 2.7^{cd}	32.32 ± 2.3^{d}	29.13 ± 1.4^{c}	13.17 ± 0.85^{a}	16.00 ± 0.87^{b}	16.30 ± 0.88^{b}	16.30 ± 0.45^{b}
WBCs (10^3/µL)	15.57 ± 0.77^{c}	$15.91\pm.24^{c}$	11.12 ± 0.93^{a}	12.64 ± 1.07^{b}	25.57 ± 1.23^{e}	22.53 ± 0.41^{d}	22.39 ± 1.13^{d}	21.36 ± 0.55^{d}
2nd week RBCs (10^6/µL)	2.49±0.48d	2.39±0.55d	1.99±0.08bd	1.54±0.46b	0.81±0.19a	1.89±0.02bd	1.90±0.10bd	1.98±0.02bd
Hb (g/dL)	10.66 ± 0.57^{cd}	10.59 ± 0.69^{cd}	10.86 ± 0.40^{d}	9.51 ± 1.3^{bc}	6.04 ± 0.44^{a}	9.17 ± 0.30^{b}	9.24 ± 0.42^{b}	9.51 ± 0.50^{bc}
PCV%	30.63 ± 2.28^{d}	32.96 ± 5.0^{d}	30.87 ± 0.24^{d}	23.93 ± 0.94^{c}	14.60 ± 0.87^{a}	18.46 ± 0.51^{b}	19.00 ± 0.30^{b}	19.78 ± 0.17^{b}
WBCs (10^3/µL)	12.48 ± 0.47^{b}	$13.14\pm.89^{b}$	9.79 ± 0.36^{a}	10.31 ± 0.78^{a}	22.24 ± 0.80^{d}	17.57 ± 1.3^{c}	17.23 ± 1.03^{c}	15.90 ± 1.40^{c}
3rd week RBCs (10^6/µL)	2.57 ± 0.51^{cd}	2.43 ± 0.51^{cd}	2.62 ± 0.30^{cd}	1.68 ± 0.22^{b}	1.0 ± 0.10^{a}	2.37 ± 0.22^{cd}	2.08 ± 0.15^{bc}	2.74 ± 0.07^{d}
Hb (g/dL)	11.64 ± 0.52^{c}	10.83 ± 0.30^{bc}	11.56 ± 0.49^{c}	10.04 ± 0.05^{b}	6.51 ± 0.84^{a}	11.11 ± 0.71^{c}	11.20 ± 0.43^{c}	11.48 ± 0.46^{c}
PCV%	32.36 ± 2.6^{c}	34.70 ± 4.08^{c}	33.98 ± 1.06^{c}	27.13 ± 1.2^{b}	17.13 ± 0.31^{a}	27.80 ± 0.75^{b}	27.60 ± 1.4^{b}	33.46 ± 1.4^{c}
WBCs (10^3/µL)	13.48 ± 0.56^{bc}	14.14 ± 1.1^{c}	9.69 ± 0.42^{a}	10.20 ± 0.43^{a}	17.90 ± 0.73^{d}	12.90 ± 0.94^{bc}	12.88 ± 0.84^{bc}	12.14 ± 1.05^{b}

(1) non infected non treated, (2) non infected treated with neem, (3) non infected treated with oxytetracycline, (4) non infected treated with neem and oxytetracycline, (5) infected non treated, (6) infected and treated with neem, (7) infected and treated with oxytetracycline, (8) infected and treated with neem and oxytetracycline

Table (6): The effect of supplementation diet with Neem (*Azadirachta indica*) leaves (5%) and oxytetracycline (0.1%) on some liver and Kidney function parameters *Clarias gariepinus*. (M± SD) (n = 5).

Parameter	Subgroups							
1st week	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
ALT(U/L)	20.66±1.5a	23.33±1.5ab	27.66±1.5bc	27.66±4.9bc	39.33±5.8e	31.00±3.6cd	37.33±2de	27.66±4.9bc
AST(U/L)	17.62±4.04a	26.85±3.2b	26.46±2.1b	26.00±3.6b	40.00±1d	33.00±4.3c	31.33±1.5bc	27.66±1.5b
Total protein (gm/dl)	6.87±.22b	6.96±.05b	7.31±.54b	6.87±.15b	5.26±.30a	7.20±.43b	7.46±.64b	7.43±0.45b
Serum urea (mg/dL)	20.33±4.5a	23.00±3.6ab	29.00±2.6bc	24.33±5.8ab	47.33±4.09d	28.66±4.9bc	34.66±4.9c	28.33±3.2bc
Serum creatinine (mg/dL)	0.81±.01a	0.84±.15a	0.94±.15a	0.90±.10a	1.38±.10b	0.94±.11a	0.88±.22a	0.93±.11a
2nd week ALT(U/L)	19.33±1.5a	20.83±0.7b	29.43±1.5d	17.00±1.ab	71.00±1e	24.00±2.6c	29.33±2.5d	21.00±1b
AST(U/L)	22.00±1c	18.00±1b	32.00±1d	15.00±1a	67.00±1e	21.00±1c	30.00±1d	22.66±2.5c
Total protein (gm/dl)	7.26±.25c	7.13±.32c	6.73±.25bc	7.30±.30c	4.86±.58a	6.16±.28b	7.43±.55c	6.83±.47bc
Serum urea (mg/dL)	24.0±1b	22.33±.5b	26.33±1.5c	23.66±1.5b	62.00±1d	20.00±1a	22.66±1.5b	18.66±1.5a
Serum creatinine (mg/dL)	0.85±.05a	0.91±.01a	0.93±.02a	0.77±.05a	2.10±.10b	0.83±.15a	0.92±.14a	0.73±.21a
3rd week ALT(U/L)	19.66±1.5ab	21.00±1b	25.00±1c	17.66±2.5a	25.66±.57c	21.00±1b	23.66±1.5c	18.33±1.5ab
AST(U/L)	22.00±1cd	19.33±1.5bc	24.33±3.2d	16.00±4ab	66.00±2.6e	13.66±2.08a	21.33±1.5cd	15.66±2.08ab
Total protein(gm/dl)	7.13±.15b	6.73±.25b	6.76±.20b	7.03±.45b	5.30±.91a	6.70±.26b	7.23±.55b	6.83±.47b
Serum urea (mg/dL)	16.33±.5a	21.00±1b	17.00±1a	16.00±1a	26.33±1.5c	17.33±2.5a	21.66±1.5b	16.66±1.5a
Serum creatinine (mg/dL)	0.83±.05a	0.77±.06a	0.88±.03a	0.67±.11a	1.90±.1b	0.70±.17a	0.88±.20a	0.70±.18a

(1) non infected non treated, (2)non infected treated with neem, (3)non infected treated with oxytetracycline, (4)non infected treated with neem and oxytetracycline, (5) infected non treated, (6) infected and treated with neem, (7) infected and treated with oxytetracycline, (8) infected and treated with neem and oxytetracycline.

DISCUSSION

Bacteria are one of the important causative agents of fish diseases in both wild and cultured fish and are responsible for serious economic losses **(Chowdhury and Baqui, 1997; Thomas *et al* 2013).** The excessive use of antibacterial drugs in fish aquaculture increases the resistance in bacteria that can infect both humans and animals **(Burridge *et al.,* 2010; Kümmerer, 2010; Defoirdt *et al.,* 2011)**. Drug choices for the treatment of common infectious diseases are becoming increasingly limited, expensive and in some cases, unavailable due to the emergence of drug resistance in bacteria **(FAO, 2005)**. Therefore it is very important to find the alternative agent for the prevention and control of fish diseases as an herbal plant. Our results showed that Gram-negative bacterial isolates (47%) were higher than Gram-positive bacterial isolates (13%). This finding is similar to earlier reported by **(Meyer, 1991; Rodger, 2010; Roberts, 2004).**

In the present study, the most prevalent bacterial isolates in naturally infected *Clarias gariepinus*, were *Aeromonas spp.* (18%) followed by *Pseudomonas fluorescens* (12%). This result contradicted with those reported by **Rahayu *et al.,* (2017); Elhady and Ahmed**, **(2014**). They stated that, the percentage of catfish infected with *Aeromonas hydrophila* was 95%. The isolation and identification results illustrated that *Aeromonas hydrophila* percent was higher than *Aeromonas sobria*. This result agreed with some authors **(Paniagua *et al*., 1990; Janda and Abbott,2010;Daood, 2012).** The isolation rate of *Pseudomonas fluorescens* was (12%).This finding was lower than that mentioned by **Masbouba, (2004); El-Hady and Samy, (2011)** and higher than that reported by **Akinobowale *et al,* (2006)**. *Klebsiella pneomoniae* was isolated in a percentage of 7%. This finding was nearly in accordance with the result previously reported by **Adeshina *et al.,* (2016).***Citrobacter freundi* isolation rate was (6%) which higher than recorded by **Akinobowale *et al.,*(2006),**but *Salmonella Spp.* Isolates (4%) was lower than stated by **Budiat *et al.,* (2013).**

Distribution of bacterial isolates through organs and tissues of clinically diseased *clarias gariepinus* indicated that,the most commonly isolated bacteria were from gills (80%) followed by liver (68%), Kidney (60%) and intestine (32%).**Abd El-Aziz, (1996)** found that,the highest rate of isolation was obtained from the intestinal contents followed by liver, and kidney.

In the present study, the mortality rate reached (11.66%) post intraperitoneal infection with *A. hydrophila* in the first week post infection and the highest cumulative mortality rate (80%)

was observed in the infected non-treated subgroup (5). These findings were consistent with the results previously cited by **(Sarkar and Rashid, 2012; Austin and Austin, 2012; Hossain *et al.* 2013).**

The cumulative mortality rate varies in different infected and treated subgroups, either infected and treated with neem (30%), infected and treated with oxytetracycline (36.66%) or infected and treated with neem and oxytetracycline combination (26.66%).Our findings agree with other observations recorded by **Noga, (2000).**

Addition of 5% neem and 5% neem plus 0.1% Oxytetracyclin to fish feed was reduced mortality rate better than addition of 0.1% oxytetracycline alone.These results were consistent with those reported by **Das *et al.,* (1999); Mona *et al.,* (2015).**

The highest TBC was found in an infected nontreated subgroup (5) (20×10^6 CFU/ml). This record agreed with the result previously mentioned by **Rahman and Chowdhury, (1996)**.

The subgroup (8), infected and treated with neem and oxytetracycline combination showed the lowest TBC which approximately similar to the TBC in the noninfected nontreated subgroup. Also, subgroup (6) infected and treated with 5% neem had TBC lower than subgroup (7) infected and treated with 0.1%oxytetracycline alone in the 1st, 2nd and 3rd weeks post infection. This result was supported by the results previously reported by **Harikrishnan *et al.,* (2003)**; **Mona *et al.,* (2015).**

The hematological study is an important tool that can be used as an effective index to monitor physiological and pathological changes in fishes**.**

The present investigation showed that, the Packed Cell Volume (PCV), Hemoglobin (Hb) and Red blood cells (RBCs) and the white blood cells count (WBCs) had no significant differences ($P<0.05$) between non-infected non-treated subgroup (1) which considered as control negative and subgroup (2) non-infected treated with neem (Table 5) which ensure that neem leaves had no toxic effect on hematological parameters. These findings were supported by the results previously recorded by **Annune and Ahuma,(1998); Kori-Siakpere and Egor, (1999); Adeyemo, (2007); Winkaler *et al*., (2007).**

Our findings showed that,the (PCV), (Hb) and (RBCs) in the infected nontreated subgroup (5) were high significantly decreased while the WBCs was high significantly increased ($P<0.05$) in the 1st,2nd and 3rd weeks post infection when compared with infected and treated subgroups (6,7,8) and noninfected nontreated control subgroup (1). These results were in

agreement with mentioned by **Nya and Austin, (2011); Mona *et al*., (2015)**. In contrary, the present results disagreed with that published by **Oniovosa *et al.,* (2017).**

Interestingly, we found in the noninfected and treated with neem subgroup (2) had a significant increase WBCs than the noninfected treated with oxytetracycline subgroup (3), this suggestive the immune-stimulant effect of the neem plant in fish. This finding coincided with other observations **(Mona *et al*., 2015)** and disagreed with results previously reported by **(Saravanan *et al*., 2011)**.

The Infected non-treated fish in subgroup (5)showed significant differences in heamatological parameters when compared with non-infected non-treated subgroup (1). This attributed to the harmful effect of *A. hydrophila*. Examination of ALT and AST after the 1st and 3rd weeks post infection and treatments showed no significant differences between both subgroup (2, 4) and subgroup (8) but subgroups (3, 7) were significantly increased this may be due to side effects of oxytetracycline. These findings were consistent with that previously mentioned by **Elema *et al.* (1996).**

In the 1st and 2nd week post infection, the infected nontreated subgroup (5) showed that alt and AST were significantly increased while serum total protein recorded was significantly decreased. A similar result was obtained by **Bo Liu *et al.,* (2012)** which concluded that, the exposure of fish to bacterial infection stimulates the activities of ALT and AST enzymes.

In this study, no significant differences ($P<0.05$) were recorded in the activities of alanine aminotransferase (ALT),and total protein (TP) in the infected and treated with neem subgroup (6) when compared with control (noninfected nontreated subgroup (1) in 3rd week post treatment. These findings were similar to that earlier reported by **Svoboda, (2001)**.

The low levels of ALT and AST recorded in this study (Table 6) in response to neem treatments contradicted with that mentioned by **Saravanan *et al*., (2011)** in Indian Carp. A progressive decrease in AST and ALT levels has been reported to be suggestive of the hepato-protective activity of neem leaves meal as well as the lower levels of creatinine and urea recorded in fish fed on neem leaves indicate that neem had no adverse effect on kidney.

In the 1st and 2nd weeks post infection and treatment no significant difference in serum urea and creatinine between subgroup (1) and subgroup (2) were recorded. This attributed that neem leaves powder had no harmful effect on kidney.

In the 1st, 2nd and 3rd weeks post infection the infected nontreated subgroup (5) showed that

serum urea and creatinine were significantly increased due to the effect of *A. hydrophila* on renal tissues. Similar findings were documented by **Murray *et al.*, (1990).**

CONCLUSION

This work found that addition of neem leaves to fish diet decreased the mortality rates in experimentally infected *Clarias gariepinus* with *A. hydrophila* and reduced total bacterial counts with no harmful or toxic effects on liver and Kidney and without adverse action on heamatological parameters.

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تقييم التأثير المضاد للبكتيريا لأوراق نبات النيم في اسماك القرموط.

زينب محمد ابوطالب[1]- ايمان سعود عبدالحميد[2]- سعاد احمد مكاوى[3]- سالى حامد ابوخضره[4]

قسم امراض الاسماك - معهد بحوث الصحه الحيوانيه- معمل فرعى الزقازيق[1]

قسم الكيمياء الحيويه - معهد بحوث الصحه الحيوانيه- معمل فرعى الزقازيق[2]

قسم كلينكال باثولوجى- معهد بحوث الصحه الحيوانيه- معمل فرعى الزقازيق [3]

قسم البكتريولوجيا - معهد بحوث الصحه الحيوانيه- معمل فرعى الزقازيق[4]

الملخص

تهدف هذه الدراسة إلى تقييم أوراق النيم (*Azadirachta indica*) من حيث الخواص المضادة للبكتيريا وتأثيرها على بعض المعايير الدموية والوظائف الخلويه من خلال دراسة فعاليتها على سمك القرموط المعدى صناعيا (*Clarias gariepinus*) بميكروب الايرمونس هيدروفيلا(*Aeromonas hydrophila*). تم فحص سمك القرموط المريض طبيعيا بالطرق البكتريولوجية وجد أن المعزلات الأكثر انتشارا كانت الايرمونس هيدروفيلا 13% تليها السيديمونس فلوريسنس 12% وكانت أقل المعزلات انتشارا الاستافيلو كوكس ابيدرميدس 4% وسالمونيلا ايسن 4%. تم استخدام مائتان وأربعون سمكه قرموط سليم ظاهريا للدراسة التجريبية وتم تقسيمة إلى 8 مجموعات فرعية. تم إجراء العدوى عن طريق الحقن داخل البطن (IP) وتم فحص المعايير الدموية والكيميائية الحيوية بعد 1 و 2 و 3 أسابيع من الإصابة. أدى النشاط المضاد للبكتيريا لأوراق النيم إلى خفض معدل الوفيات من 80٪ في مجموعة فرعية غير معدية مصابة إلى 26.6٪ و 30٪ في كلتا المجموعتين المصابتين اللتين تم علاجهما باوراق النيم مضافه إلى أوكسي تتراسايكلين والاخرى التى تم معالجتها باستخدام النيم فقط على التوالي .وجد ان العد البكتيري(TBC) كان فى المجموعه الفرعيه المصابة والمعالجة باوراق النيم (1×10^4 CFU / مل) أقل من المجموعة الفرعية المصابة والمعاملة المعالجه بأوكسي تتراسايكلين (7×10^5 CFU / مل). وقد وجد ان المؤشرات الدمويه ومؤشرات الكيمياء فى كل من المجموعات الفرعية المعديه والمعالجة باوراق النيم و بالأوكسيتراسيكلين مع اوراق النيم. الحيوية مساويه تقريبا للقياسات في المجموعات الفرعية الغير معديه. من خلال ذلك يمكننا التاكيدعلى أن اضافه أوراق النيم لاعلاف الاسماك ادى الى تقليل نسبه الوفيات والاصابه بالعدوى البكتيريه فى اسماك القرموط.