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### Effect of some immunostimulants on growth performance , survival and disease resistance of Nile tilapia (*Oreochromis niloticus*) fry.

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#### ABSTRACT

This study was carried out to evaluate the comparative effects of some immunostimulants and antibiotic supplementation on growth performance parameters, survival rate, diseases resistance (*Aeromonas hydrophila*) and immune status of Nile tilapia fry. A total number of 500 *Oreochromis niloticus* fry, with an average initial weight ( $0.35 \pm 0.05$  g) were stocked at 5 treatments with 2 replicate, control group (Gr1) and from Gr2:Gr5 provided with dietary supplementation as following: Garlic 40g/kg *purpurea* extract 4gm/kg, yeast 4 g/kg and ciprofloxacin 1gm/kg of the diet, for a three month period. The results revealed that groups treated with Echinacea, Garlic and yeast, significantly improved the growth performance parameters, but *Echinacea purpurea* gave the best feed conversion ratio. In addition, increasing the relative protection levels against *Aeromonas hydrophila* which was previously isolated from naturally infected farms depending on mortalities, clinical signs and PM lesions, and biochemically identified and molecular screened for detection of virulence genes. Hematological, biochemical and Immune parameters revealed that all immunostimulant supplemented groups were significantly better than control and antibiotic groups. Histopathological examination within 7 days post challenge revealed necrosis of internal organs, inflammatory reaction, associated with hemosiderosis in positive control and antibiotic groups, but protection were higher in immunostimulant groups. In conclusion, the addition of immunostimulants especially garlic, *Echinacea purpurea* and yeast to the diet of *O. niloticus* fry could improve growth performance, fish survival, immunological parameters. Further, they could be suggested as an effective alternative to antibiotics with respect that Echinacea extract is the most potent one of them.

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## INTRODUCTION

Nile tilapia (*O. niloticus*), considered as one of the most productive and internationally traded fish food in the 21st century (Hernández et al. 2014) because of their enormous adaptability to a wide range of physical and environmental conditions, ability to reproduce in captivity, relative resistance to handling stress and diseases compared to other cultured finfish species, as well as their excellent growth rate on a wide variety of natural and artificial diets (Wang and Lu 2016).

As aquaculture facing the problem of massive loss caused by diseases and the negative effects of chemicals and antibiotics on the environment, followed by the development of mutagenic microbial strains and adversely affected fish health, their application to control disease outbreaks is no longer recommended (Cabello, 2006). Therefore one of the main challenges in achieving productive, feasible, sustainable aquaculture with the lowest production cost is to develop alternative prophylactics that could improve the survival, growth performance, disease resistance and immune response (Dawood and Koshio 2016). Fish diets should not only provide the essential nutrients that are required for normal physiological functioning, but also serve as the medium by which fish receive other components that affect their health and disease resistance (Goda et al. 2018).

Recently, fish farmers give more attention to the use of immunostimulants as a dietary supplementation to increase fish production and resistance against many fish pathogens (Sakai, 1999). Many of immunostimulants have been used in Egyptian tilapia farms as garlic, *Echinacea purpurea*, and probiotics as *Lactobacillus* or *Saccharomyces cerevisiae* (*S. cerevisiae*) and have been proved to be good feed additives (Aly et al. 2008).

*Aeromonas hydrophila* is one of the most ubiquitous bacterial pathogen causing clinical disease known as Motile Aeromonas Septicemia (MAS), in immune-compromized fresh water fish and causes a serious problems to the fish farming industry in Egypt (Noga 2010). The cumulative mortality that persists for a week can be high (Zhang et al. 2014), in which

skin ulcers, hemorrhage and congestion of the visceral organs are the major symptoms (Mesalhy et al. 2010). The main virulence factors that effect on *A. hydrophila* pathogenicity are, aerolysin, enterotoxins and hemolysin, in addition to other factors such as mucinase and adhesion 8 production (Youser et al. 2007), these virulence factors create pores in the membrane of target cells leading to osmotic lysis by destruction of the membrane permeability barrier (Parker et al. 2010).

Histopathological investigations have been recognized to be reliable biomarkers of stress in fish (Van der Oost, 2003) and evaluation of the fish health exposed to infection, both in the laboratory and field studies by examining specific target organs, including gills, liver, and kidney that are responsible for vital functions and accumulation and biotransformation of toxins which serve as warning signs of damage to the fish (Gernhofer, 2001). Gills and gastrointestinal tract are considered the main passage and the first target of waterborne pollutants and infections to different body organs through the blood (Thophon et al. 2003).

So, the aim of this study is to evaluate the effect of some immunostimulants on the growth performance, survival, haematological, biochemical, histological findings and disease resistance of Nile tilapia fries as a natural feed additives in comparison with antibiotics supplementation. Also, to isolate and identify *A. hydrophila* and its virulence genes. Further to detect the potential pathogenicity of the organism and subsequent histological Alterations in Some Vital Organs.

## MATERIALS AND MEHODS

### Sampling from naturally infected farms:

A total number of 240 fish (60 fish/farm) alive and freshly dead cultured *O. niloticus* were sampled from four private farms in two governorate, Kafr El Sheikh and Dakahlia, suffered from mass mortalities, fish weights ranged from 150:200 g. Alive samples were packed in a labeled plastic bag, and freshly dead fish were kept in ice tanks then transported to Mansoura Lab, Animal Health Research Institute for clinical, post-mortem examination and swabbing preparation (Noga et al. 2011).

### Bacteriological examination

A loopful from liver, spleen, kidney, and skin ulcers were obtained from the collected samples under complete sterile condition and inoculated on tryptic soya broth (TSB), incubated at 25°C for 18-24 hrs, then streaked on different laboratory media, as tryptic soya agar (TSA) and Rimler-Shotts media (R-S media, CM0833, Oxoid, England). The inoculated plates were incubated at 25 °C for 18-24 hrs (Austin and Austin , 2012).

### Identification of the obtained bacterial isolates

The morphological characteristics of the bacterial isolates were examined under a microscope after by stained with Gram stain (Bergmans et al. 2005). Detection of motility on semisolid media was done (Buller, 2004).

### API 20E Kits

Final identification of the retrieved isolates were achieved by using an analytical profile index system API 20 E system (Bio Mérieux) according to Eissa (2016).

### DNA extraction

DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was

incubated with 20 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit. Oligonucleotide Primer. Primers used were supplied from Metabion (Germany) are listed in table (1).

PCR amplification. Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an Appliedbiosystem 2720 thermal cycler.

### Analysis of the PCR Products:

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the products was loaded in each gel slot. Gelpilot 100 bp plus ladder (Qiagen, GmbH, Germany) and Generuler 100 bp ladder (Fermentas, Thermo) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table (1) Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)				Reference
				Secondary denaturation	Annealing	Extension	Final extension	
<i>alt</i>	TGACCCAGTCCTGG-CACGGC	442	94°C 5 min.	94°C 30 sec.	55°C 40 sec.	72°C 45 sec.	72°C 10 min.	Nawaz et al., 2010
	GGTGATCGATCAC-CACCAGC							
<i>hly</i>	CTATGAAAAAACTA AAAATAACTG	1500	94°C 5 min.	94°C 30 sec.	55°C 40 sec.	72°C 1.2 min.	72°C 12 min.	Yousret et al., 2007
	CAGTATAAGTGGG-GAAATGGAAAG							
<i>aero</i>	CACAGCCAA-TATGTCGGTGAAG	326	94°C 5 min.	94°C 30 sec.	52°C 40 sec.	72°C 40 sec.	72°C 10 min.	Singh et al., 2008
	GTCAC-CTTCTCGCTCAGGC							

### Antibiotic sensitivity test:

The antibiotic sensitivity test was carried out using the disc diffusion method on Muller-Hinton Agar (Oxoid) and the results were interpreted in accordance with the Clinical and Laboratory Standards Institute guidelines (CLSI, 2016). Antibiotic discs, Ampicillin (AM, 10µg), Streptomycin (S, 10 ug), Tetracycline (TE, 30µg), Erythromycin (E,15 ug), Sulphamethoxazole/Trimethopri (STM, 25 µg) and ciprofloxacin, were all used for antibiogram. After 24 hrs incubation, the inhibition zone were measured using a digital caliper and compared with the breakpoints. The multi-antibiotic resistance (MAR) index was determined as described by Vivekanandhan et al. (2002). The MAR index is equal to the number of antibiotics resisted by the tested isolate divided by the total number of tested antibiotics.

### Experimental setup:

#### Experimental Fish

A total of 500 *O. niloticus* fry (0.35±0.05g) were obtained from Kafr El-shiekh governorate, and transported to Mansora Laboratory of Animal Health Research Institute and were kept in two ponds for two weeks to be acclimatized before the start of the experiment.

### The immunostimulants

The immunostimulants used in the present study were added to the basal diet according to Mesalhy et al. (2010) as follow: Garlic (*Allium sativum* L) was purchased from the local market, crushed and mixed with the formulated diet at a rate of 40 g of garlic /kg. Echinacea (*Echinacea purpurea*) extract that contains polysaccharides, caffeic acids (echinacosides), 1.5% chicoric acid was obtained from SEKEM Company, Egypt. It was mixed to the balanced diet at 4 g of Echinacea/ kg feed. Yeast is manufactured locally in Egypt, one gram of the product contains  $1 \times 10^8$  *S.cervisiae* dried cells according to the manufacturers, it was mixed with the balanced ration at 4 g / kg feed.

### Experimental fish design

As shown in Table 2, The total of 500 (*O. niloticus*) fry were divided into 5 equal groups, fish of each group (100 fry) were subdivided into 2 equal sub-groups (50 fry each) reared in ponds and fed on diet containing 35% protein at a rate of 5% of body weight per day . Fish were fed three times and cleaned daily., and extended for 3 months in the summer season .

Table (2) Experimental fish design

Diet	Group				
	Gr (1)	Gr (2)	Gr (3)	Gr (4)	Gr (5)
	100	100	100	100	100
Basal diet (35% protein)	+ve	+ve	+ve	+ve	+ve
Garlic powder (40 g/kg diet)	-ve	+ve	-ve	-ve	-ve
Echinacea extract (4 gm/kg diet)	-ve	-ve	+ve	-ve	-ve
Yeast (4 g/kg diet)	-ve	-ve	-ve	+ve	-ve
Ciprofloxacin (1 g/kg)	-ve	-ve	-ve	-ve	+ve

### Diets and feeding

A balanced dietary ration formulation was prepared to meet the requirements of Nile tilapia according to Mesalhy et al. (2010) every two weeks. The pellets were left for 24 h to air dry, and stored in a refrigerator (4 °C) for daily

use. The amount of feed (on dry matter basis) delivered per day was adjusted.

### Measurement of survival, growth performance and feed utilization parameters:

These parameters were calculated biweekly till

the end of the experiment in all treated groups and control according to Abdelhamid(2003) as following:

Average body weight (initial and final) = the total weight of fish / the number of fish in each group.

weight gain % = average final weight (g) – average initial weight (g) / average initial weight × 100.

Specific growth rate (g) SGR =  $\text{Ln} [(Final\ weight\ (g) - Initial\ weight\ (g)) \div time \times 100]$ .

Feed intake was calculated as the total weight diet offered in a given period divided by the number of survival fish.

Feed conversion ratio = Feed given/ Weight gain.

The condition factor (K) used to shows the degree of well being of the fish in their habitat was determined by using the equation,  $K = 100 W / L^3$  according to Gomiero and de Souza Braga (2005). Where by K = condition factor W = the weight of the fish in gram (g). L = the total length of the fish in centimeters (cm). b = the value obtained from the length/weight equation.

#### Blood and serum samples:

Blood and serum samples were taken after three months of the feeding experiment, ten fish were randomly taken from each group, and were anaesthetized with 50 mg/L benzocaine (Sigma-Aldrich). Blood samples were collected from the caudal vein and were divided into two portions. The first portion was collected with anticoagulant 10% EDTA. The second portion was allowed to clot overnight at 4°C, and then, it was centrifuged at 3,000rpm for 10 min (Bricknell et al. 2000) to obtain the serum and stored at -20°C for the biochemical analysis.

#### Hematological and biochemical parameters

To measure red blood cells (RBCs), haemoglobin (Hb) and haematocrit (HT) According to Guimaraes (2009). Liver function test including aspartate aminotransferase (AST) and alanine aminotransferase (ALT), glucose according to Henry (1974), total lipids according to Tietz (1990). In addition, the serum total protein was measured according to the method of Grant et al. (1987). The serum albumin level was estimated according to the method of Dumas et al. (1981). The serum globulin was

calculated by subtracting the albumin from the obtained total protein as described by Dumas and Biggs (1972).

#### Evaluation of immunological parameters:

Total leukocytic count: TLC were done by using the improved Neubaur chamber, Natt and Herrick's solution as diluting fluid and 1:100 diluted blood according to the method described by Goda et al. (2008).

Lysozyme activity: for determination the Lysozyme concentrations in all groups which have the ability to lyse *Micrococcus lysodeikticus* cells (Nakanishi, 1987). Through a plotted standard curve against the corresponding clear zone ring diameter on the linear axis (Rao et al. 2006).

Serum bactericidal activity: SBA examines the ability of the fish's serum of different groups to kill the pathogenic organisms (Maqsood et al. 2010). The bactericidal activity of the tested serum was expressed as the percentage of colony forming units in test group to that in the control group (Lim et al. 2009).

**Serum IgM:** the quantitative determination of IgM was made by using nephelometry technique, according to Barta (1984).

#### Challenge test :

At the end of the experiment, a number of 10 (*O. niloticus*) fish with an average weight of (51±0.11) g were collected from all groups for disease resistance challenge test with virulent strain of *A. hydrophila* that were isolated previously and fully identified from naturally infected fish farm. The collected fish were divided into equal groups (10 fish/group), Gr1 (negative control, Gr2(positive control), and from Gr3: Gr6 were infected and supplemented with immunostimulants: garlic, echinecea, yeast and antibiotic (ciprofloxacin), respectively. Gr1 was I/P injected by sterile normal saline as a sham control, and from Gr2:Gr6 were IP injected with 0.1 ml of  $1.4 \times 10^8$  CFU/ml 18hrs culture of pathogenic *A. hydrophila* according to Brook et al.(2015). Clinical signs, post mortem and mortalities were recorded daily for 7 days post infection and the bacterial re-isolation was confirmed from the dead fish.

The relative protection level (RPL) among the

challenged fish was determined according to **Ruangpan et al. (2001)** with the following equation:

$$\text{RPL} = 1 - (\text{mortality percent in stimulated group} \div \text{mortality percent in control group}) \times 100.$$

### Histopathological Examination

Tissue specimens from gills, liver, spleen, intestine and kidney were collected from freshly dead or alive fish of all groups after 48 hour post challenge (HPC). The tissue specimens were fixed in 10% neutral buffered formalin for 72h. The fixed tissue were rinsed in tap water, dehydrated through graded series of alcohols, cleared in xylene and embedded in paraffin wax, 5 µm thick sections were cut and stained with hematoxylin and eosin (H and E) and then examined by light microscopy, according to **Bancroft et al. (2003)**.

### Absorptive Capacity of Intestine (villi parameters):

Intestinal villi were examined at the end of experiment. H&E stained paraffin sections and documented photographically with a digital camera (DCM 130E/1.3 megapixels, CMOS

Software Scopephoto, China) connected to a light microscope (Leica).

### Statistical analyses:

The statistical analysis was performed to all experimented fish and collected samples using a one way analysis of variance (ANOVA) and Duncan's Multiple Range Test (Duncan, 1955) was used to determine differences between treatment (mean at significance level of  $P < 0.05$ ).

### Results

#### Bacteriological Isolation and Identification:

Ten *A. hydrophila* isolates characterized by, gram-negative short rods, Oxidase positive, resistant to Vibrio-static reagent, and small, translucent, circular, pinpoint colonies on TSA and on RS media was large flattened yellow colonies, and arabinose acid production. Consequently, showed growth at 37 °C and the optimum at 24 °C but no growth at 4°C and 40 °C. Additionally, they could grow in 1% NaCl, however, no growth in 2-4% NaCl media.

#### API 20E profile system for *A. hydrophila* isolated from naturally diseased Nile tilapia:

Table (3). Biochemical reactions of the identified *A. hydrophila* isolates by API 20E kits.

Item	<i>A. hydrophila</i>	Item	<i>A. hydrophila</i>
Sorbitol fermentation "SOR"	-ve	Rhamnose fermentation RHA	+ve
Melibiose fermentation MEL	-ve	Amygdalin fermentation AMY	+ve
Sucrose fermentation SAC	-ve	Arabinose fermentation ARA	+ve
Lysine decarboxylase LDC	-ve	Cytochrome oxidase OX	+ve
Citrate utilization CIT	-ve	o-Nitro phenyl Galactoside ONPG	+ve
Urease production URE	-ve	Arginine dihydrolase ADH	+ve
Tryptophanedeaminase TDA	-ve	Glucose fermentation "GLU"	+ve

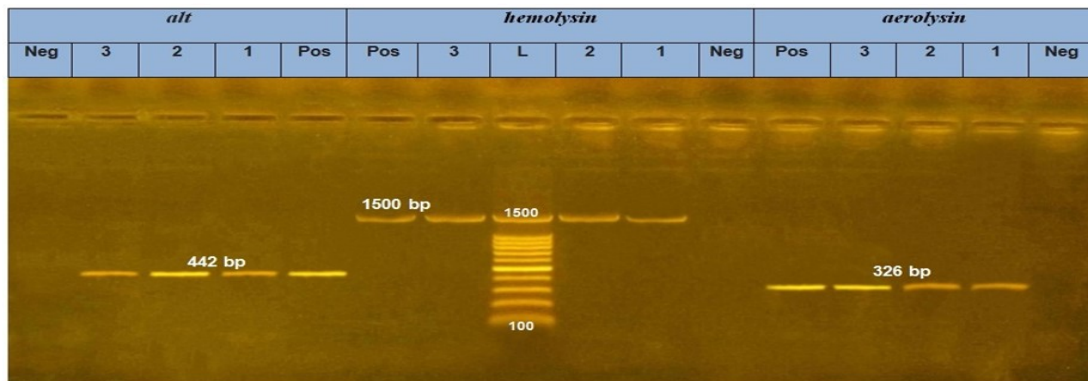
The identification percentage of the analytical profile index of *A. hydrophila* isolates is (id% = 98.6).

**Molecular identification of the isolates:**

A total of 3 *A. hydrophila* strains out of the tested 6 isolates carried aerolysin gene at

the expected product size (326bp), hemolysin gene at (1500)pb and cytotoxic enterotoxin at (442) pb as showed in (Fig. 1).

Fig (1) Molecular identification of *A. hydrophila* for detection of aerolysin, hemolysin and enterotoxin virulence genes.



sample	alt	aerolysin	hemolysin
1	+	+	+
2	+	+	+
3	+	+	+

**Antibiotic and antibacterial susceptibility profile:**

Sensitivity test was performed to determine the effective antibiotics against *A. hydrophila*. Isolates were highly sensitive to ciprofloxacin, trimethoprim and Tetracyclin, but less sensitive to trimethoprim and intermediate susceptible to neomycin and nalidixic acid. The multi-antibiotic resistance index (MAR) was ranged from 0.2 to 0.41. MAR index, MAR indices greater than 0.2 In all except three isolates, had an indicating the existence of multiple-antibiotic resistance.

**Growth performance**

The different growth parameters after 3 months of experiment have been shown in table (4).

**Average weight gain (AWG):** all treated groups showed an increase in AWG and significantly improve with of immunostimulants and antibiotic supplemented group compared to control.

**Specific growth rate (SGR) and the condition factor (K):** were significantly the best in garlic and yeast supplemented groups compared to the control and ather groups.

**Feed conversion ratio (FCR):** The FCR was better (lower) in *Echinacea purpurea* supplemented group compared to the at herimmunostimulant groups.

**Survival Rate (SR) and Relative Level of Protection (RLP):** against *A. hydrophila* infection were significantly improved in allimmunostimulents groups compared to control and antibiotic group, but it mainly increased in *Echinacea purpurea* and garlic supplemented group compared to other immunostimulants.

Table (4) Growth performance and feed utilization of Nile tilapia fry fed a 35%-CP diet containing different immunostimulants and antibiotic for 3 months.

Parameter	Treatments Group				
	Gr 1	Gr 2	Gr 3	Gr 4	Gr 5
Initial weight (g)	0.33 ± 0.14 <sup>a</sup>	0.34 ± 0.09 <sup>a</sup>	0.34 ± 0.17 <sup>a</sup>	0.33 ± 0.14 <sup>a</sup>	0.34 ± 0.53 <sup>a</sup>
Final weight (g)	50.5 ± 0.29	57.9 ± 2.57	57.51 ± 0.12	56.27 ± 1.14	52.65 ± 0.19
Weight gain %	16.07 ± 0.05 <sup>f</sup>	17.19 ± 0.05 <sup>e</sup>	20.14 ± 0.15 <sup>d</sup>	18.57 ± 0.13 <sup>d</sup>	17.8 ± 0.02 <sup>b</sup>
Specific growth rate (SGR)	1.62 ± 0.05 <sup>a</sup>	2.08 ± 0.01 <sup>a</sup>	1.92 ± 0.12 <sup>a</sup>	2.04 ± 0.05 <sup>a</sup>	1.51 ± 0.06 <sup>a</sup>
Feed intake (FI)	41.65 ± 0.49 <sup>bc</sup>	43.73 ± 0.74 <sup>a</sup>	42.98 ± 0.28 <sup>ab</sup>	36.24 ± 0.11 <sup>d</sup>	41.87 ± 0.13 <sup>bc</sup>
Feed conversion ratio	3.69 ± 0.08 <sup>a</sup>	2.03 ± 0.05 <sup>b</sup>	1.48 ± 0.04 <sup>cd</sup>	2.95 ± 0.02 <sup>d</sup>	3.36 ± 0.08 <sup>c</sup>
Condition factor(K-factor)	1.63 ± 0.01 <sup>c</sup>	1.88 ± 0.02 <sup>b</sup>	1.75 ± 0.02 <sup>a</sup>	1.82 ± 0.00 <sup>c</sup>	1.60 ± 0.02 <sup>b</sup>
Survival %	90 ± 2.31 <sup>a</sup>	96 ± 2.31 <sup>a</sup>	95.33 ± 1.33 <sup>a</sup>	96.67 ± 1.33 <sup>a</sup>	94.33 ± 1.33 <sup>a</sup>
A.hydrophila, RLP%	0	48.5	60	46	35

\*Rows with the same litter are not significant different (P<0.05). Gr 1: control, Gr2: Garlic, Gr3: Echinacea, Gr4: vet-yeast, Gr 5 : ciprofloxacin.

#### Haematological and Biochemical blood parameters :

significant increasing were noted in RBCs, Hb, and Ht values, among all immunostimulant supplemented groups, especially in yeast followed by *Echinacea purpurea* compared to control and antibiotic group. Garlic and *Echinacea purpurea* supplemented groups exhibited significantly lower AST and ALT activity in comparison with the values noted in control and the antibiotic group. That could indicate improved liver function in im-

munostimulant groups. A significant increase were noted in the total protein, albumin and globulin levels in immunostimulant supplemented groups, mostly in Echinacea and Yeast groups. There are a significant decrease in total lipid parameters in Echinacea group comparing to other group. The immunostimulant supplemented groups showed significant decrease in serum glucose compared with the other group, especially Echinacea group.

Table(5) Hematological and biochemical parameters of Nile tilapia (*O. niloticus*) fry fed on immunostimulants and antibiotic for 3 months.

Items	Treatments				
	Gr 1	Gr 2	Gr 3	Gr 4	Gr 5
RBCs ( $\times 10^6/\text{mm}^3$ )	1.64 ± 0.138 <sup>a</sup>	1.91 ± 0.087 <sup>b</sup>	2.69 ± 0.045 <sup>b</sup>	2.04 ± 0.142 <sup>b</sup>	1.78 ± 0.170 <sup>a</sup>
Haemoglobin (g/dl)	10.67 ± 0.53 <sup>a</sup>	11.52 ± 0.46 <sup>b</sup>	11.78 ± 0.56 <sup>b</sup>	11.83 ± 0.63 <sup>b</sup>	10.89 ± 0.43 <sup>a</sup>
Haematocrit (%)	14.67 ± 0.917 <sup>a</sup>	15.43 ± 0.921 <sup>c</sup>	16.93 ± 0.984 <sup>a</sup>	16.23 ± 0.322 <sup>a</sup>	14.80 ± 0.153 <sup>a</sup>
Glucose (mg/dl)	196.2 ± 9.8 <sup>d</sup>	146.3 ± 9.8 <sup>c</sup>	122.7 ± 16.3 <sup>b</sup>	140.4 ± 18.8 <sup>a</sup>	210.7 ± 11.7 <sup>b</sup>
Total lipid (mg/dl)	10.97 ± 0.09 <sup>a</sup>	11.45 ± 0.31 <sup>b</sup>	13.84 ± 0.98 <sup>c</sup>	11.51 ± 1.37 <sup>b</sup>	10.21 ± 0.31 <sup>b</sup>
Total protein (g/dl)	3.45 ± 0.9 <sup>a</sup>	4.23 ± 0.23 <sup>b</sup>	5.11 ± 0.2 <sup>c</sup>	4.87 ± 0.51 <sup>b</sup>	3.10 ± .25 <sup>a</sup>
Albumin (g/dl)	1.22 ± 0.9 <sup>c</sup>	1.58 ± 0.8 <sup>b</sup>	1.93 ± 0.9 <sup>b</sup>	1.67 ± 0.8 <sup>c</sup>	1.47 ± 1.5 <sup>ab</sup>
Globulin (g/dl)	1.90 ± 2.3 <sup>d</sup>	2.12 ± 0.7 <sup>c</sup>	2.84 ± 2.1 <sup>b</sup>	2.29 ± 4.5 <sup>a</sup>	1.78 ± 2.6 <sup>b</sup>
Creatinine (mg/dl)	0.87 ± 0.15 <sup>a</sup>	0.79 ± 0.04 <sup>b</sup>	0.78 ± 0.12 <sup>b</sup>	0.77 ± 0.13 <sup>b</sup>	1.3 ± 0.9 <sup>c</sup>
AST(U/l)	21.81 ± 2.8 <sup>a</sup>	15.36 ± 1.15 <sup>b</sup>	14.87 ± 0.77 <sup>b</sup>	15.56 ± 1.05 <sup>b</sup>	19.45 ± 1.6 <sup>c</sup>
ALT(U/l)	90.58 ± 0.66 <sup>a</sup>	84.26 ± 1.39 <sup>b</sup>	78.21 ± 0.15 <sup>c</sup>	83.26 ± 1.39 <sup>b</sup>	89.77 ± 1.61 <sup>a</sup>

\*Rows with the same litter are not significant different (P<0.05). Gr1:control, Gr2: Garlic, Gr3:Echinacea, Gr4:Yeast, G5:ciprofloxacin.



**Immunological parameters:**

Total leukocytic count were significant increase in all immunostimulant supplemented group comparing to control group, except Ciprofloxacin group, showed a significant decrease, *Echinacea* and garlic groups showed significant increase in serum lysozyme activity comparing to other groups, Serum bactericidal activity was significantly higher in *Echinacea* and garlic groups in comparison with other

groups. After challenge with pathogenic *A. hydrophila*, all treated groups showed a significant increase in the amount of IgM in comparison with control untreated group, but more significant in *Echinacea* as shown in Table (5).

After challenge with pathogenic *A. hydrophila* the mortalities were recorded for 7 days, groups received *Echinacea* or Garlic showed a significant decrease in mortalities than that treated with Ciprofloxacin or con-

Table (6) immune parameters of *O. niloticus*, 12 weeks supplemented with garlic, *Echinacea*, yeast and antibiotic.

Parameter	Treatments				
	Gr 1	Gr 2	Gr 3	Gr 4	Gr 5
Total leukocytic count ( $\times 10^3$ / $\text{mm}^3$ )	66 $\pm$ 1.15 <sup>a</sup>	88 $\pm$ 1.44 <sup>b</sup>	89.5 $\pm$ 1.5 <sup>b</sup>	79.17 $\pm$ 0.72 <sup>d</sup>	65.5 $\pm$ 1.76 <sup>a</sup>
Lysozyme activity ( $\mu\text{g/ml}$ )	110.13 $\pm$ 1.72 <sup>c</sup>	130.28 $\pm$ 1.34 <sup>a</sup>	135.69 $\pm$ 1.23 <sup>a</sup>	120.81 $\pm$ 1.82 <sup>b</sup>	113.38 $\pm$ 1.24 <sup>c</sup>
Serum bactericidal activity (% of CFU/ control)	34 $\pm$ 0.15 <sup>b</sup>	68 $\pm$ 0.18 <sup>a</sup>	72 $\pm$ 0.08 <sup>c</sup>	69 $\pm$ 1.5 <sup>c</sup>	35 $\pm$ 0.23 <sup>b</sup>
Ig M (mg/l) (After challenge)	0.159 $\pm$ 0.003 <sup>a</sup>	0.249 $\pm$ 0.001 <sup>b</sup>	0.258 $\pm$ 0.005 <sup>b</sup>	0.219 $\pm$ 0.005 <sup>c</sup>	0.165 $\pm$ 0.008 <sup>a</sup>

\*Rows with the same litter are not significant different ( $P < 0.05$ ). Gr1: control, Gr2: Garlic, Gr3: *Echinacea*, Gr4: Yeast, Gr5: Ciprofloxacin

**Pathological Investigation:****Clinical and post Mortem findings**

With in 7 days post challenge by *A. hydrophila*, all infected immunostimulant supplemented groups showed slightly abnormal behavior, swimming closer to surface and aquarium wall, loss of balance, mild hyperemia in skin, eyes and fin base and dullness. But in comparison with Ciprofloxacin and positive control group showed superficial and deep ulcers in skin and pectoral fin base and fin rot (**fig2. A**), off food, bilateral exophthalmia (**fig2. B**), abdominal ascites with high morbidity and mortality by

the 7th day. The common gross pictures observed in the diseased fish supplemented with immunostimulants were abdominal ascites (**Fig2 C**), congestion and enlargement in the internal organs (**fig2. D**). While, fish exposed to septicemia showed congestion and enlargement of kidney and spleen, hemorrhagic spots on liver, bloody exudate in abdominal cavity and intestine is devoid of food and filled with bloody mucus.

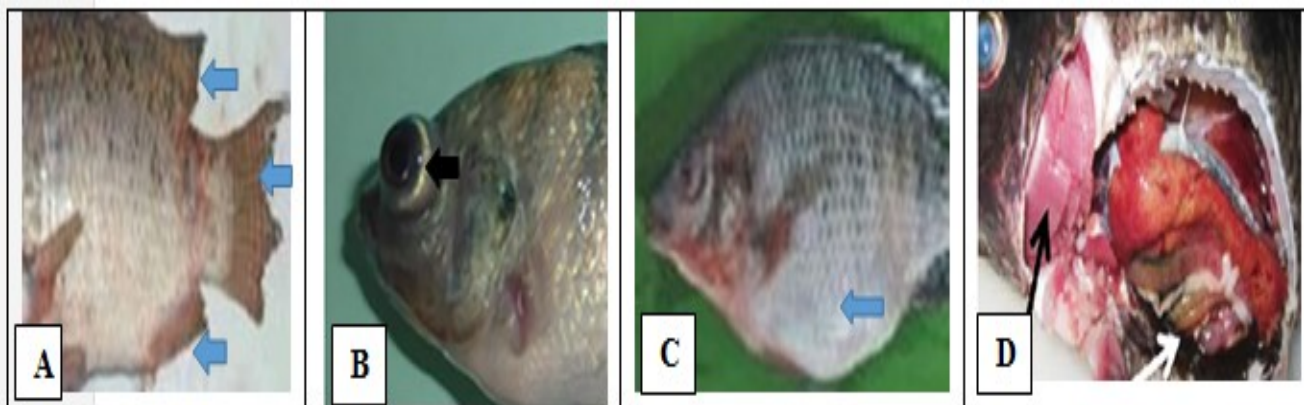


Figure (2) clinical and postmortem findings in *O. niloticus* challenged with *A. hydrophila* Fig 2.A: hyperemia and erosions in skin and fin rot. Fig 2. B: exophthalmia. fig2.C: abdominal ascites. fig2. D: congestion and enlargement in the internal organs.

### Histopathological findings:

In this study, nearly all examined organs of *O. niloticus* exhibited histopathological lesions including, gills, liver, kidney, spleen and intestine, but More significant in positive control and antibiotic groups in comparison with immunostimulants supplemented group which showed mild to moderate histological ultra-tion, especially in Echinacea and garlic groups.

### Gills:

In group 1 (negative control), showed normal gill arches, each arch composed of two rows of secondary lamellae covered with pillar epithelial cells, and run perpendicular to the primary filament that covered with chloride and mucous epithelial cells (**fig3.A**). In group 2 (positive control), showed necrosis of primary

filament epithelium, epithelium rupture of Secondary lamellae and severe congestion (**fig3.B**). In group 3 (infected and supplemented with garlic) showed cellular hypertrophy, multifocal fusion of gills lamellae and blood congestion (**fig3.C**). In group 4 (infected and supplemented with Echinacea) showed congestion of the central venous sinus, and mild cellular hypertrophy (**fig3. D**). In group 5 (infected and supplemented with yeast) showed diffuse thickening of lamellar epithelium and complete fusion of secondary lamellae (**fig3. E**). In group 6 (infected and supplemented with antibiotic) showed moderate telangiectasis at the tips of secondary lamellae with cellular hypertrophy (**fig3.F**).

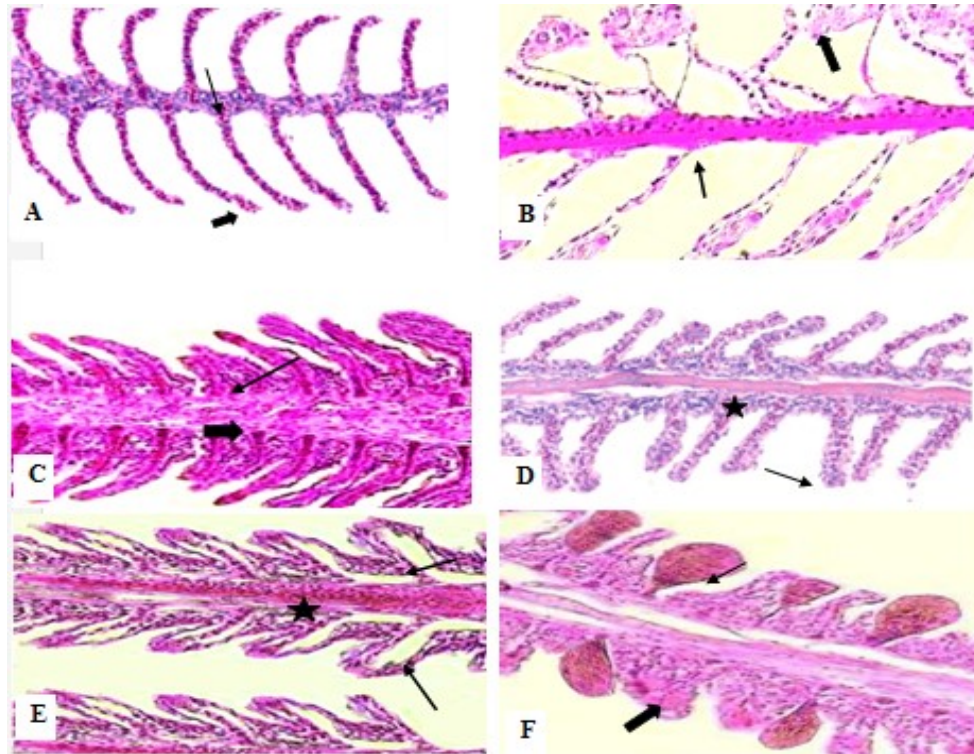


Figure (3) **Histopathological changes in *O. niloticus* gills:**

**A:** negative control group, showing normal aspect of gill arch, Primary filament (thin arrow) covered with pillar cells and Secondary lamellae (thick arrow) covered with chloride and mucous cells, H&EX 400.

**B:** positive control, showing necrosis of primary filament epithelium (thin arrow) and epithelium rupture of Secondary lamellae (thick arrow), H&E, X 400.

**C:** Infected - garlic supplemented group, showing hypertrophy of lamellar epithelium (thin arrow), vasodilation of central venous sinus (thick arrow), H&E, X400.

**D:** Infected- Echinacea supplemented group, showing congestion of the central venous sinus (star), and mild cellular hypertrophy (arrow), H&E, X 400.

**E:** Infected- yeast supplemented group showing congestion of central venous sinus (star), cellular hypertrophy with separation of lamellar epithelium (lifting) and edema (arrow), H&E, X 400.

**F:** Infected-antibiotic supplemented group showing rupture of pillar cells at the tip of secondary lamellae and capillaries (thin arrow) with cellular hypertrophy (thick arrow), H&E, X 400.

#### **Liver:**

In group1, liver of control fish exhibits normal hepatocytes have homogenous cytoplasm and a large spherical nucleus, arranged in indistinct lobules with two cell thickness separated by blood sinusoids and central vein (**fig4. A**). In group2, liver showed dilatation of blood vessels surrounded by focal migrated lymphocytic aggregation, liver hemorrhage and multi focal areas of lytic necrosis within the liver parenchyma and cytoplasmic vacu-

ation (**fig4. B**). In group3, characterized by mild hepatocytic vacuolation (**fig4. C**). In group 4, showed normal hepatocytes with mild congestion in sinusoids and normal pancreatic cells (**fig4. D**). In group5, liver exhibited normal hepatocytes with mild congestion in sinusoids (thin arrow) and normal pancreatic cells (thin arrow) (**fig4. E**). In group 6, marked dilatation and congestion of central vein and hepatic sinusoids were seen, in addition to diffuse hepatocytic vacuolation (**fig4. F**)



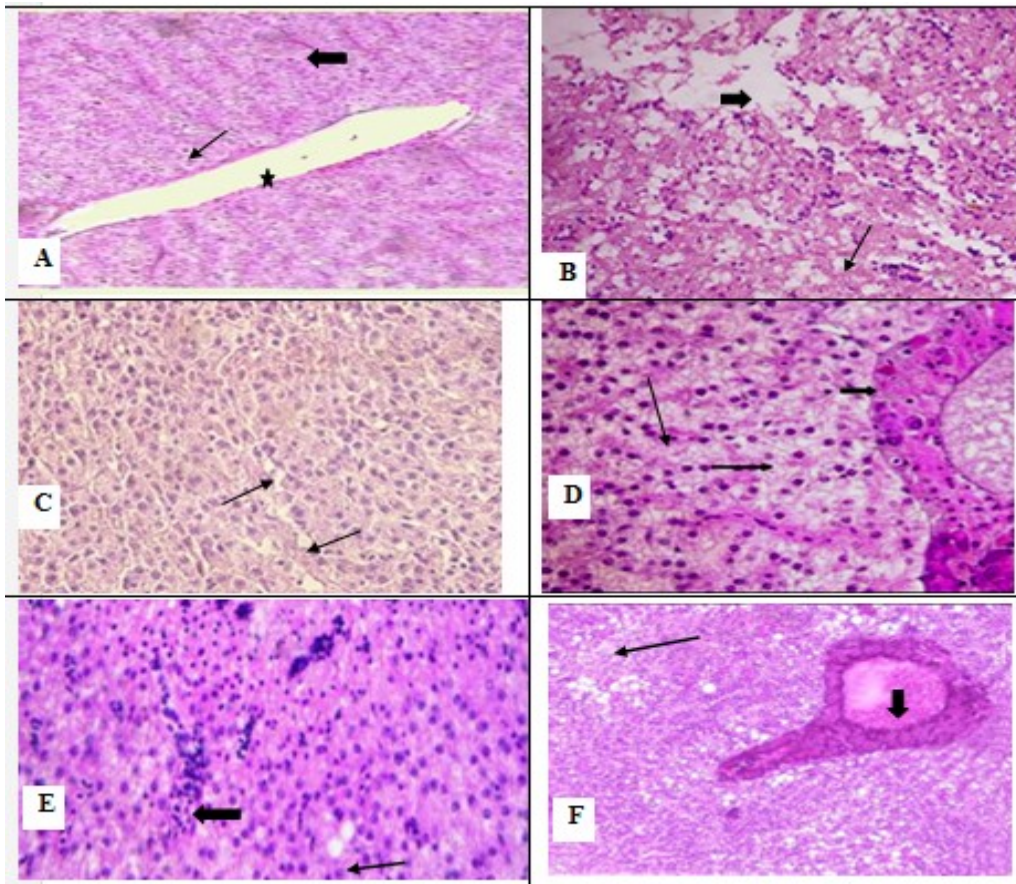


Figure. (4) **Histopathological changes in *O. niloticus* liver:**

**A:** Negative control group, showing normal hepatic tissue, hepatocytes (thin arrow), blood sinusoid (thick arrow) and central vein (star), H&E, X100.

**B:** positive control, showing lytic necrosis within the liver parenchyma (thick arrow) and hepatic vacuolation (thin arrow), H&E, X400.

**C:** infected- garlic supplemented group, showing, mild vacuolation of hepatocytes (thin arrow), H&E, X200.

**D:** infected-*Echinacea* supplemented group, showing normal hepatocytes with mild congestion in sinusoids (thin arrow) and normal pancreatic cells (thick arrow). H&E, X400.

**E:** infected - yeast supplemented group, showing normal hepatocytes with mild congestion in sinusoids (thin arrow) and normal pancreatic cells (thick arrow), H&E, X400.

**F:** infected- antibiotic supplemented group, showing degenerative changes in liver (thin arrow), congestion and diffuse hepatocytic vacuolation (thick arrow) (HE, 200x).

#### **Kidney:**

In group 1, kidney showed normal renal corpuscle, the glomerulus and the Bowman's space well defined, normal renal tubules and normal intertubular hematopoietic tissue (**fig5. A**). In group 2, showed severe interstitial nephritis with diffuse coagulative necrosis of tubular epithelium, edematous fluid, occlusion of tubular lumen and shrinkage of renal corpuscle (**fig5. B**). In group 3, focal degeneration of tubules and lymphocytic infiltration in in-

terstitial tissue were seen (**fig5. C**). In group 4, showed mild hyaline droplets accumulation in epithelium lining renal tubules (**fig5. D**). In group 5, showed perivascular oedema, inflammatory infiltration and deformation in renal tubules (**fig5. E**). In group 6, showed interstitial infiltration of mononuclear inflammatory cells and degeneration of tubular epithelium (**fig5. F**).

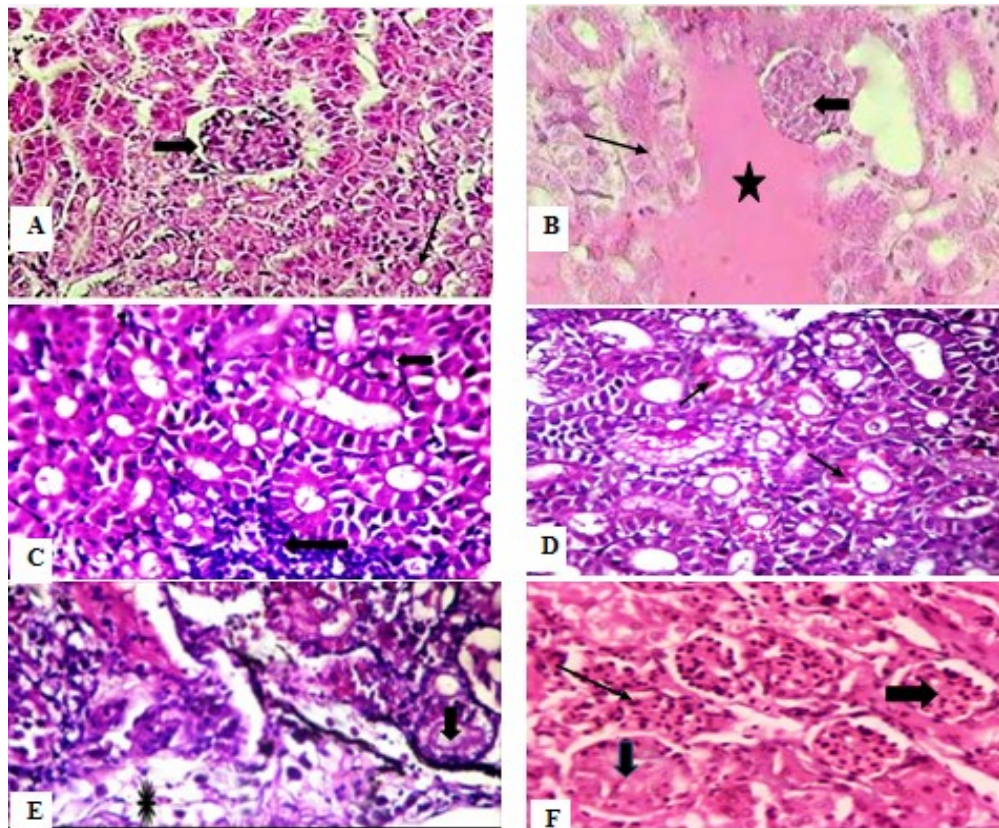


Figure. (5) **Histopathological changes in *O.niloticus* kidney:**

**A:** negative control group, showing normal renal corpuscle, the glomerulus and the Bowman's space well defined (thin arrow), normal renal tubules (thick arrow), H&E, X400.

**B:** positive control group, showing edematous fluid (star), cellular degeneration in tubules and occlusion of tubular lumen (thin arrow) shrinkage of renal corpuscle (thick arrow), H&E, X400.

**C:** infected - garlic supplemented group, showing normal renal tubules and mild lymphocytic infiltration in interstitial tissue (thick arrow) H&E, X400.

**D:** infected- Echencica supplemented group, showing mild hyaline droplet degeneration in renal tubules (thin arrow), H&E, X400.

**E:** ) infected- yeast supplemented group, showing per vascular oedema and inflammatory infiltration (thin arrow) and deformation in renal tubules (thick arrow), H&E, x400.

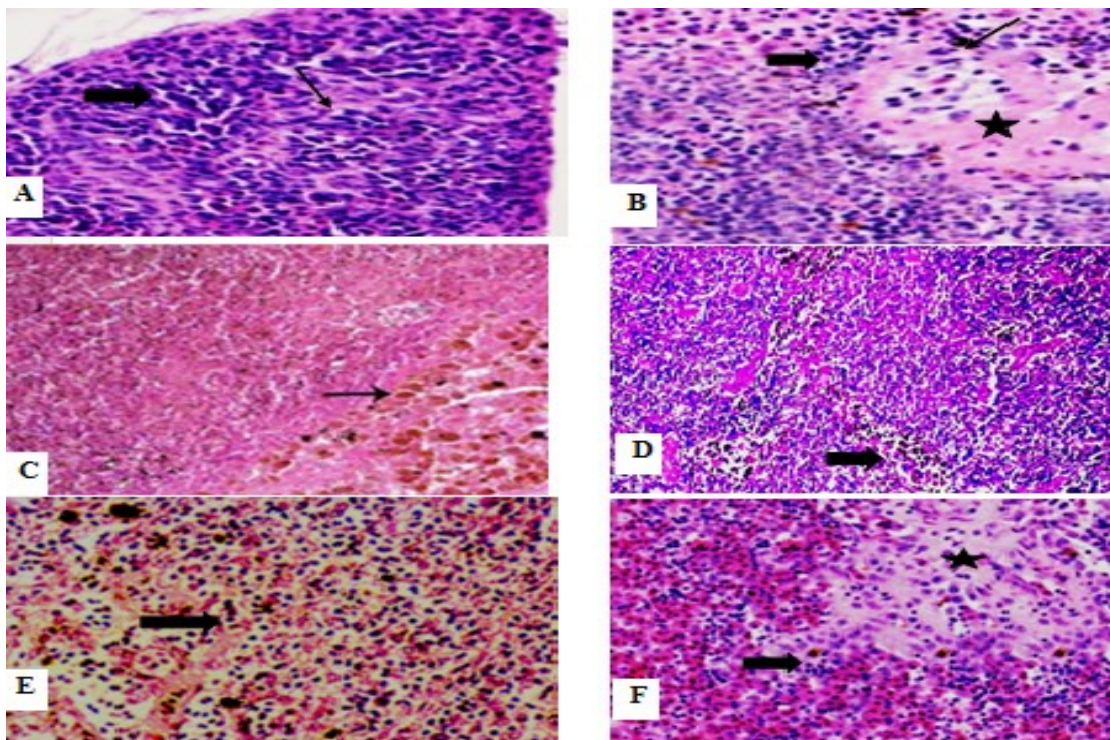
**F:** infected-antibiotic supplemented group showing , interstitial infiltration of mononuclear inflammatory cells (thin arrow) and degeneration of tubular epithelium (thick arrow), H&E, X400.

### Spleen:

In group1, revealed normal architecture with mixed red and white pulp (**fig6.A**). In group2, showed severe splenitis characterized by diffuse fibrinous necrosis of ellipsoidal area, depletion of erythrocyte within red pulp, which is replaced by pink fibrinous material (**fig6.B**). In group3, showed mild focal lymphoid depletion and hyperplasia of melanomacrophages (**fig6.C**). In group 4, showed mild aggregation of macrophages and lymphocytes around ellipsoids in addition to hemosiderosis

(**fig6.D**). In group5, spleen showed hemorrhages characterized by aggregation of Dark brown pigment of hemoglobin and nucleated erythrocytes (**fig6.E**). In group 6, showed, degenerative changes associated with loss of cellular structure with macrophages and lymphocytes aggregation around ellipsoids in addition to hemosiderosis. (**Fig6.F**).





**Figure.6: Histopathological changes in *O. niloticus* spleen:**

**A:** negative control group showing normal red pulp structure (thin arrow) with normally compacted ellipsoids (thick arrow). H&E, X100.

**B:** positive control group showed severe splenitis characterized by diffuse fibrinoid necrosis of ellipsoids (star), inflammatory cells aggregation (thick arrow), and hemosiderosis (thin arrow). H&E, X400.

**C:** infected - garlic supplemented group, showing mild focal lymphoid depletion replaced by hyperplasia of melanomacrophages (arrow). H&E, X100.

**D:** infected- Echencica supplemented group, showing mild aggregation of macrophages and lymphocytes around ellipsoids in addition to hemosiderosis (thick arrow). H&E, X400 .

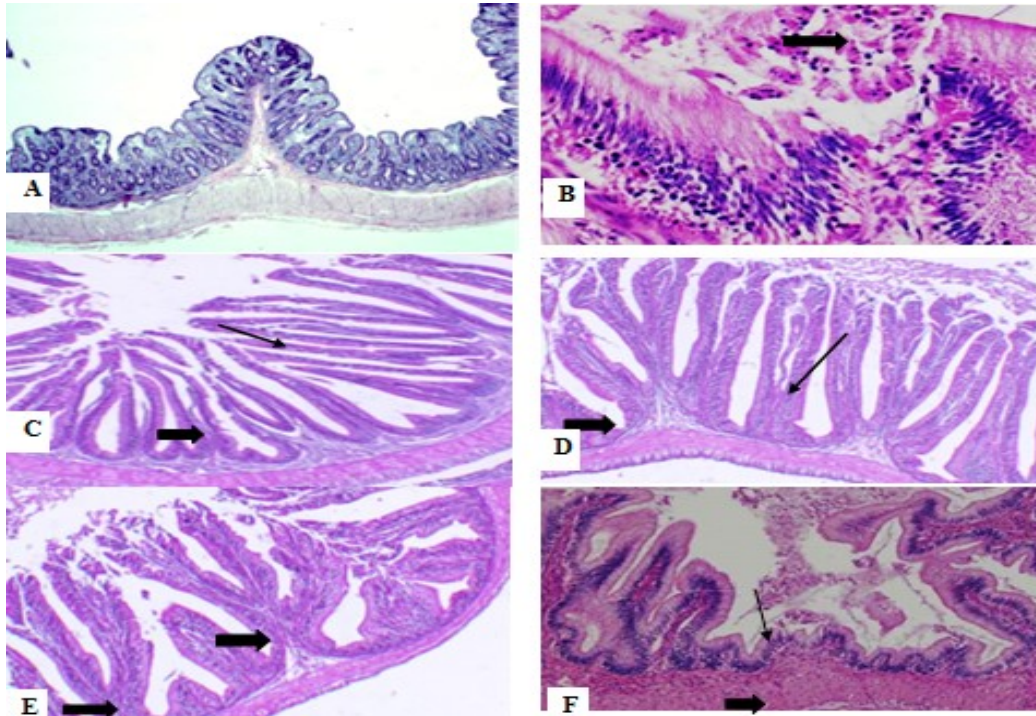
**E:** infected- yeast supplemented group, showing hemorrhages characterized by aggregation of dark brown pigment of hemoglobin and nucleated erythrocytes (arrow). H&E, X400

**F:** infected-antibiotic supplemented group showing, degenerative changes associated with macrophages and lymphocytes aggregation around ellipsoids (arrow) in addition to hemosiderosis. (arrow), H&E, X400.

### Intestine:

In group 1, the intestinal layers were normal including mucosa, submucosa, muscularis and serosa (**Fig7. A**). In group2, showed extensive necrosis of mucosa of the villi, with sloughed necrotic debris in the lumen (**fig7. B**). Group3, showed normal short villi with intact lining epithelium and decrease the villus width (**Fig7. C**). Group4 showed increase in

villi length, width and branches (**Fig7. D**). Group5, showed increase the villus branches, length and width (**Fig7. E**). Group6, showed decrease the villus length and width, destruction of lining epithelium (**Fig7. F**).



**Figure.7: Histopathological changes in *O. niloticus* intestine:**

**A:** negative control group showing its normal characteristic layers: mucosal epithelium, submucosa, muscularis, and serosa, H&E, X200.

**B:** positive control group showing severe epithelial necrosis of villi with sloughed necrotic debris in the lumen and hemorrhage (arrows), H&E, X400.

**C:** infected - garlic supplemented group, showing normal and short villi (thin arrow) with intact lining epithelium and decrease the villus width (thick arrow), H&E, X400.

**D:** infected- *Echinacea* supplemented group, showing normal villi and increase in villi length, width and branches. H&E, X400.

**E:** infected- yeast supplemented group, showing normal villi with increasing the villus branches and width H&E, X400.

**F:** infected-antibiotic supplemented group showing decrease the villus length and width, destruction of lining epithelium and submucosal hemorrhage (arrows), H&E, X400.

#### **Effect of immunostimulants on absorptive Capacity of Intestine (villi parameters):**

As shown in table (7), *Echinacea* and yeast supplemented groups showed significant increase in villi length with intact lining epithelium and increase villus branches and width comparing to control and other groups. Garlic

group showed normal villi but shorter and less in width compared to yeast and *echinecea*. Control positive and antibiotic groups showed significant decrease in length and width of villi due to erosion and destruction.

Table (7) Impact of some immunostimulants and antibiotic on villi parameters of *O. niloticus* intestine challenged by *A. hydrophila* (absorptive capacity of intestine):

parameters	Treatments					
	Gr1	Gr2	Gr3	Gr4	Gr5	Gr6
<b>Duodenum</b>						
Length	500±3 <sup>a</sup>	405±23 <sup>b</sup>	536±33 <sup>c</sup>	560 ±6 <sup>d</sup>	579 ±14 <sup>d</sup>	480±10 <sup>a</sup>
width	120±6 <sup>b</sup>	90 ±15 <sup>c</sup>	131±13 <sup>a</sup>	150±10 <sup>b</sup>	149±22 <sup>b</sup>	115 ±123 <sup>b</sup>
<b>Jejunum</b>						
length	660±21 <sup>a</sup>	510±20 <sup>c</sup>	800±30 <sup>b</sup>	810±27 <sup>b</sup>	804±25 <sup>b</sup>	600±35 <sup>a</sup>
width	110±14 <sup>a</sup>	60±33 <sup>a</sup>	133±23 <sup>b</sup>	142±10 <sup>c</sup>	149±35 <sup>c</sup>	130±20 <sup>b</sup>

\*Rows with the same litter are not significant different (P<0.05). Gr1:negative control, Gr2:positive control, G3 Garlic, Gr4:Echinacea, Gr5:Yeast, Gr5:Ciprofloxacin.

## DISCUSSION

*Aeromonas* sp. is one of the main bacterial problems facing the growth of aquaculture in Egypt, where it causes mass mortalities and a significant reduction in the production rates (Eissa et al. 2010 Elsheshtawy et al. 2019). Based on the convention of biochemical identification and PI20E characterization of the bacterial isolates, *A. hydrophila* strains were identified. The characteristic colonies of *A. hydrophila* were indicated by yellow colonies due to maltose fermentation on RS Media these results agreed with Cantas et al.(2012) who stated that RS Media was 94% efficient for isolation of *A. hydrophila*. In the current study, the possibility of detecting the virulence genes of *A. hydrophila* (i.e. aerolysin, haemolysins and enterotoxins) was promoted during a single PCR amplification from clinical isolates, the sequence of the universal 16SrRNA gene was used to identify *A. hydrophila* with a typical homology at 625 pb. Additionally, the PCR analysis for the detection of virulence genes revealed that all bacterial isolates contain aerolysin (*AerA*) and haemolysin (*hly*) and cytotoxic enterotoxin (*alt*) at their specific segment sizes of 326 pb and 1,500 pb and *at* (442) pb genes respectively. This finding could explain and supported by the detected haemorrhagic, ulcerative and septicaemic signs of infected fish in the current study. According to the phylogenetic tree of the *Aeromonas* strains, we observed that our outbreak isolates clearly belonged to *A. hydrophila* (Singh et al. 2008). Recently, several investigations reported the pathogenic potential of *A. hydrophila* associated with the production of haemolysin and aerolysin genes (Wang et al. 2003).

In the current study, the susceptible, intermediate and resistance rates of the examined *A. hydrophila* isolates with 12 antibiotics reported that it was highly sensitive to Ciprofloxacin, which may indicate the production of beta lactamase, which is consistent with the findings of Revina et al. (2017). The multi-antibiotic resistance index (MAR) is a good tool for risk assessment; generally, the acceptable value is 0.2, with high values indicating a high risk (Krumpelman, 2000).

The main challenge facing aquaculture industry is how to increase growth rate, feed conversion, survival and disease resistance with the lowest production cost (Aly et al. 2015). In current study, addition of immunostimulants to fish diet (garlic, echenicea and yeast) could significantly increase weight gain and growth rate compared to control and antibiotic group. Since, garlic contain allicin which enhance the performance of intestinal flora and improves digestion with better energy utilization, in addition improving feed conversion ratio leading to increased growth rates (Lee et al. 2014).

Yeast has been used as a probiotic in aquaculture due to high stability, low cost and fast effect on growth rate (Irianto and Austin, 2002). The results of current study are similarly with El-Boshy et al. (2010) who indicate that dietary supplementation with *S.cerevisiae* induced the best growth rate, immune response and disease resistance of *O.niloticus*.

In the current study, *Echinecea* supplemented



diet significantly improved the feed conversion ratio (FCR), comparing to control and other groups, that may be attributed to the mode of action of echinecea, through enhancement the digestive function and immune response of *O.niloticus* (Mohamed, 2010). In this study, survival rate and relative level of protection against *A.hydrophila* infection were significantly improved with immunostimulants supplemented group compared to control and antibiotic group, but significance was clearly high in garlic and yeast supplemented groups. Similarly with Abdelkhalek et al. (2015). That may be due to many bioactive component of garlic therapeutic action which applied as antibacterial and antifungal agent (Amagase et al. 2001). This results were supported by Aly et al. (2008) who reported that feeding tilapia on diet with 10g/kg diet for amonth, significantly induced high survival rate and protection even with highly pathogenic *A. hydrophila*. In addition Yeast, *S. cerevisiae* has a polysaccharide cell wall which contains glucan, mannon and chitin, the presence of glucan receptors on macrophages, monocytes and neutrophils which facilitating the pathogen recognition, moreover, chitin also increase immune response of head kidney and phagocytosis (Cook et al. 2001).

Biochemical analyses often provide vital information for health assessment and management of cultured fish (Rhulka et al. 2014). In this study, a significant increasing were noted in RBCs, Hb, and Ht values, among all immunostimulant supplemented groups, especially in yeast and *Echinacea purpurea* compared to control and antibiotic group, that could be attributed to improvement and activation of the erythropiote tissue and enhancement the iron metabolism leading to increase the serum iron, ferritin and hemoglobin (Ferreira et al. 2010). In this study, Garlic and *Echinacea purpurea* supplemented groups exhibited significantly lower AST and ALT activity in comparison with the values noted in control and the antibiotic group. That could indicate improved liver function in immunostimulant groups, that in agreement with Davis et al. (2018) who reported that ALT and AST are important indicator for amino group transformation to alpha keto acids, so the high level of ALT and AST

indicate liver cell damage. In the current study, a significant increase were noted in the total protein, albumin and globulin levels in immunostimulant supplemented groups, mostly in Echinacea and Yeast groups, albumin and globulin are essential for cellular and humeral immune system, and consider a diagnostic value in fish, as it relates to general nutritional status as well as the integrity of the vascular system and liver function. as proved by Goda et al. (2012). In contrast, (Barros et al. 2013) proved that dietary yeast had effect on plasma total proteins albumin and globulin. There are a significant decrease in total lipid parameters in Echinacea group comparing to ather group. Which may be attributed to activation the synthesis of long chain poly unsaturated fatty acids under control of hepatic and intestinal desaturase enzymes (Gill et al. 1988). The immunostimulant supplemented groups showed significant decrease in serum glucose compared with the other group, that may be related to the regulatory action of natural immunostimulant especially Echinacea on the concentration of circulatory cortisol and glucose tolerance factor (Ringo et al. 2012).

Immunological parameters in the current study revealed that, total leukocytic count were significant increase in all immunostimulant supplemented group and a significant decrease of Ciprofloxacin group comparing to control group, these results were correspond with Yonar et al. (2011). Echinacea and garlic groups showed significant increase in serum lysozyme activity comparing to other groups, that agree with Meshaly et al. (2008). Serum bactericidal activity was significantly higher in Echinacea and garlic groups in comparison with other groups, these finding agree with Zhai et al.(2007). In this study, after challenge with pathogenic *A. hydrophila* all treated groups showed a significant increase in the amount of IgM in comparison with control untreated group. These findings were consistent with Hayashi et al. (2010).

In the current study After challenge with pathogenic *A. hydrophila* the mortalities were recorded for 7 days, groups received garlic, Echinacea or Garlic showed a significant decrease in mortalities comparing with antibiotic

and positive control group. That indicates a good disease resistance, these findings was explained by **Zhai et al. (2007)**, and may be due to the effect of Ciprofloxacin and other similar compounds that may interfere with normal immunological processes in fish, as mentioned by **Grondel et al. (1986)**.

In the current study clinical and post Mortem findings with in 7 days post challenge, significantly decreased in immunostimulants groups than positive control and antibiotic group, these results may be attributed to septicemic reaction of *A. hydrophila*, (**Shoemaker et al. 2000**). The sluggish movement associated with *A. hydrophila* infection was probably as a result of frayed and sloughed tail, beside hemorrhagic edematous and ulcerated fins, in addition to anorexia which affected the vital activities of the diseased fish, as mentioned by **Noor El Deen et al. (2014)**.

In the current study, nearly all examined organs of *O. niloticus* including, gills, liver, kidney, spleen and intestine, exhibited histopathological lesions which more significant in positive control and antibiotic group in comparison with immunostimulants supplemented group that showed mild to moderate histological ultra-structure, especially in *Echinacea* and garlic groups. These results were similar to those observed in channel catfish (**Abdelhamid et al. 2017**), crucian carp and rainbow trout (**Harikrishnan et al. 2010**) infected with *A. hydrophila*.

In the current study, histopathological changes of the gills varied from hyperplasia and hypertrophy of lamellar epithelium, vasodilatation and congestion in immunostimulant group, to lamellar fusion, interstitial edema, telangiectasis, epithelium rupture and cellular necrosis in antibiotic and positive control groups. Hyperplasia and hypertrophy may be due to increase mitotic division and increase epithelial thickness, these changes considered as a defense mechanism, thus serve as a barrier against entrance of infection by increase the distance between contaminated water and blood (**Karlsson et al. 2001**), but it may be inversely causes impaired oxygen intake, hypoxia and respiratory failure (**Mazon, 2003**). La-

mellar vasodilatation may be attributed to pillar cells damage which have the main support function of gill lamellae, thus could increase blood flow inside lamellae causing dilatation of blood channel and congestion (**Carrasco et al. 2002**). Lamellar fusion may be due to cell proliferation and thickening of filament epithelium which induced by bacterial toxins thus alerts the mucus glycoproteins to cover cells and effect on its negative charge causing adhesion to the adjacent lamellae (**Santos et al. 2007**). Lifting or separation of lamellar epithelium probably induced by severe edema which is the most frequent gill lesions in fish exposed to highly pathogenic toxins as this separation increase the distance to prevent toxins and pollutants from diffusing to blood stream (**Wood et al. 2004**). Telangiectasis induced by rupture of pillar cells at the tip of secondary lamellae and capillaries under effects of pollution and infection (**Romano, 2000**).

In the current study, The histopathological appearance of liver within 7 days post challenge with highly pathogenic *A. hydrophila* showed important alterations in immunostimulants supplemented groups as hepatocytic and nuclear hypertrophy, congestion in the central veins. In addition cellular degeneration, necrosis and cytoplasmic vacuolization in positive control and antibiotic groups. Hypertrophy of hepatocytes may be due to exposure to highly pathogenic infection which induce proliferation of endoplasmic reticular membrane and hydropic swelling, or due to high content of lipids (**Peebua et al. 2006**). On other hand nuclear hypertrophy induced by high metabolic activity or pathologic origin (**fernandes et al. 2007**). Vacuolization in hepatocytes cytoplasm attributed to accumulation of lipids and glycogen, that indicates an imbalance between rate of synthesis and rate of their release into systemic circulation, and when increased considered as a signals of degeneration and metabolic damage (**Santos 2002**), so vacuolization is considered as acellular defense mechanism by collecting the hepatocytes injuries and prevent them from interfering with the biological activities of hepatic cells (**Mollendroff 1999**).

Hepatic degeneration and necrosis may be due to anoxia from gills degeneration and tis-

sue hypoxia, also may be due to accumulation of toxins and inhibition of DNA synthesis needed for liver growth and maturation (Oyejobi et al. 2005).

In the current study, histopathological changes of kidney within 7 days post *A. hydrophila* challenge varied according fish diet supplementation. Immunostimulants supplemented group showed congestion, hyaline droplet and vacuolization of renal tubules and interstitial infiltration of mononuclear cells. While in positive control and antibiotic groups showed acute degeneration and necrosis of renal tubules, shrinkage of renal corpuscles and occlusion of the tubular lumen. Similarly with Noor El Deen et al.(2014) Abdelhamid et al. (2017) who reported the same lesion in the kidney of crucian carp, Nile tilapia and catfish. kidneys are target organs of an acute septicemia, attacked by bacterial toxins and lose their structural integrity (Huizinga et al.1999).

Shrinkage of glomeruli may be return to increase the amount of edematous fluids in the interstitial tissue as well as cellular degeneration (Santos, 2002). Congestion and hemorrhage of kidney referred that bacterial toxins affect on cell membrane permeability and inhibit the ion transporting system leading to disturbance of fluid transportation of the cells, causing disperse of red blood cells and serum albumin in the interstitial space due to destruction of endothelium lining capillaries (Hadi and Alwachi, 1999). Increase the size of renal tubules refers to hydropic swelling of tubular epithelium and accumulation of eosinophilic granules in the cells which mainly formed by reabsorption the plasma proteins in the urine thus indicates damage in renal corpuscles (Takashima, 1995).

One of the major pathological changes in the current study included hemosiderosis, especially in the spleen. The reason of the hemosiderosis was b-hemolysin that cause hemolysis inside the fish body followed by deposition of hemosiderin (Noor El Deen et al. 2014 Abdelhamid et al. 2017). *A. hydrophila* toxins including, haemolysins, enterotoxins, aerolysins, and - proteases, haemagglutinins and adhesions (Rey et al. 2009).

The intestinal lesions recorded in the current study showed improvement in villus lining epithelium, length, width and thickness of intestinal mucosae of immunostimulant groups, while in positive control and antibiotic groups showed destruction of intestinal villi and accumulation of necrotic debris in intestinal lumen. These observations may be due to extensive bacterial invasion and the immunity state, and were agree with Poline et al. (2014). And could be attributed to the different bacterial toxins expressed by *A. hydrophila* (Roberts , 2001).

### In conclusion

using of natural immunostimulant (Echinacea, garlic or yeast) in fish diets based on low cost and the immunostimulative effect, is more useful and effective alternative than antibiotics that cause adversely side effects for fish, environment and consumers health. In addition, immunostimulants improved the weight gain, survival rate, immunity and resistance to *A. hydrophila*. with respect that *Echinacea* extract is the most potent one of them.

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