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Comparative studies about the effect of *Gracilaria spp* on *Clarias gariepinus* fish infected with *Aeromonas hydrophila*

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Abstract

Antibacterial effect of both *Gracilaria* and oxytetracycline against *Aeromonas hydrophil*a was evaluated through conduction an experiment on one hundred and fifty apparently healthy *Clarias gariepinus* fish divided equally into five groups with two replicate in each, control one without any special treatments while the other four groups infected with *Aeromonas hydrophil*a. Both the third and fifth group fish were fed on balanced diet mixed with *Gracilaria spp.* (5%) for a week before infection and a month post infection while fish of both the fourth and fifth groups were subjected to oral administration of oxytetracyclin in the diet, three days before infection and seven days post infection. Hematological &biochemical analysis, phagocytic activity and phagocytic index, growth and feed utilization parameters and pathological examination were applied on all experimental groups at both **7th** and **30th** days post infection. Re isolation of *Aeromonas hydrophil*a from previously infected groups were done at **7th** day post infection. Adverse results were demonstrated clearly in *A. Hydrophil*a infected group (second group) and ameliorated in other treated groups with various degrees, while the fifth group had the more effective results among those treated groups.

Keywords: *Gracilaria spp., clarias gariepinus, aromonus hydrophila.*

Introduction

Aeromonas hydrophilla causes disease condition termed "Motile *Aeromonas* Septicemia" (MAS) is considered as one of the most dangerous disease-causing bacteria in fresh water fish farming. It can mainly attack *Clarias gariepinus* resulting in high death rates that can reach **80%** producing huge losses **Sanoesi, (2008).**

Antibiotic resistance for different bacterial pathogens in fish aquaculture has received more attention in last few years and that enforces finding possible antibiotics alternatives, from those were products from marine organisms which are considered as pharmaceutical agents with good antibacterial activities **Shannon and Abu-Ghannam, (2016).**

Aeromonas hydrophila infection represented severe hepatic and renal lesions as degenerative necrotic changes, hemosiderosis, hepatic hemorrhages and renal coagulative necrosis **Dalia, (2013).**

Recently selective utilization of marine algae as a source of pharmaceutical agents has been increasing. Many of the seaweeds have bioactive components which inhibit the growth of some bacterial pathogens. The algal extracts were used as a curative and preventive agent for various diseases such as antibiotics, anti-helminthics, antitumour and anti-diarrhea. The maximum antibacterial and antifungal activity were observed in the red algae (*Gracillaria spp*) 37%, brown algae 33.3% and green algae 8.3% activity **Kolanjinathan *et al.*, (2009)**.

Gracilaria spp. is a type of sea weed red algae commonly found in marine water that had moderate antibacterial activity against *Aer-*

omonas hydrophila, Pseudomonas aeruginosa, Pseudomonas putida and had weak antibacterial activity against *Vibrio harveyi* and *Vibrio algynoliticus* bacteria **(Maftuch *et al.,* 2016a).** *Gracilaria spp* at 5% supplementation level in diet exhibited higher growth performance and immunity enhancements than the non-supplemented and a 10% supplemented diet **Araújo *et al.,* (2016).**

Antibiotics are very important tools for the control of fish bacterial diseases. Yet, there are strict regulations controlling the use of antibiotics in aquaculture. The efficacy of oxytetracycline dehydrate given to *A. hydrophila* infected fish could bring out improved functioning of fish kidneys that carry infectious agents **(Julinta *et al.,* 2017).** Oxytetracycline is considered the drug of choice for treatment of *A. hydrophila* in fish **(Romero *et al.,* 2012)**. Oxytetracyclin is a broad spectrum antibiotic, belonging to tetracycline group. It is produced from *Streptomyces spp*, it interfere with bacterial protein synthesis. However, as the global trend in antibiotic use becomes more restricted in the aquaculture industry, it is necessary to find suitable prophylactic and growth-promoting alternatives **Rigos and Smith, (2015).**

The objective of the present study was to qualify the antibacterial activity of *Gracillaria spp* (**5%**). Against *Aeromonas hydrophila* infection in *Clarias gariepinus* in comparison with oxytetracycline with regarded to pathological, biochemical and microbiological assessment**.**

Materials and Methods

Bacteriological examination:

I-Pre-experimental stage

A total of **50** *Clarias gariepinus* were captured from El-Sharkia fish' markets and transported to lab. It was dissected under complete aseptic conditions and tissue specimens were collected including apparently pathological lesions in gills, kidney, liver and intestine, samples from above mentioned organs were submitted for bacteriological examination according to method citied by **(Markey *et al.,* 2013).** Surface of organs was seared by hot spatula and a sterile loop was deeply introduced in the affected organ and cultured to tryptic soya broth and incubated at 28°C/24h then a loopful was streaked to different isolation media as (tryptic soya agar, *Aeromonas* agar, blood agar with Ampicillin, and Ma Cconkey agar) then incubated at 28°C/24-48h, suspected colonies were subjected for further presumptive identification.

Phenotypic identification:

Phenotypic characterization was conducted according to **Austin and Austin, (2016)** and it depends on a series of morphological, biochemical and metabolic activities, the applied tests include gram staining, oxidase test, catalase test, indole production, methyl red, vogues preskour, simmon citrate, TSI, Motility test and H_2S production.

Molecular identification:

Bacterial DNA was extracted from isolates using QIA amp DNA extraction Mini kit (Qiagen, Germany, GmbH) and then PCR reaction was conducted aiming amplification of *16S*rRNA gene **(Gordon *et al.,* 2007)**. Primers used and PCR cycling protocol were listed in **Table (1).**

Amplified PCR products was electrophoresed on 1**.5%** agrose gel in tris acetate EDTA and visualized by UV transilluminator. **Sambrook *et al.,* (1989).**

II-Experimental design:

A total of **150** apparently healthy *Clairas gariepinus* fish with average body weight **140 ± 0.5**g were obtained from the Central Laboratory for Aquaculture Research. Abbassa, Sharkia., Egypt and pre experimentally acclimated for a week in indoor tanks in glass aquaria (**40x 50x 100**) of **200 liter** capacity with **180 liter** net water volume and maintained in aerated de-chlorinated fresh water at 25 ± 2ºC. Fish were divided into five equal groups with two replicate in each group, control one without any special treatments while the other four infected groups were injected by isolated *A. hydrophilla* strain with a single dose of **0.5ml of ($6x10^6$ CFU/ml)** intraperitoneal **(Emeish *et al.,* 2018)**. Both the third and fifth groups fish were fed on balanced diet

mixed with *Gracilaria spp* (**5%**) **50g / kg** diet **(Peixoto *et al.*, 2016)** prepared in faculty of veterinary medicine (Zagazig university) for a week before infection and a month post infection while fish of both the fourth and fifth groups were subjected to oral administration of **75** mg/kg body weight oxytetracycline mixed with diet **(Plumb, 1999)** three days before infection and seven days post infection**.** Experimental design was demonstrated in **table (2).**

Ingredients and chemical composition of the basal diet (air/dry basis, %).

Ingredients	%
Fish meal	11
Corn flour	33
Soybean meala	29
Corn gluten meala	12
Wheat bran	8
Vegetable oil	4
Vitamin premixb	1.5
Mineral premix	1.5
Total	100
Analyzed composition	**(%) as fed basis**
Crude protein	**(N×6.25) 30.79**
Crude lipids	7.43
Ash	5.22
Crude fiber	5.20
Nitrogen free extractd	51.36
Gross energy (kcal/kg) e	4553

a-Soybean meal, crude protein 44%; corn gluten meal, crude protein 60%.
b-Vitamin premix (per kg of premix): vitamin B1, 700 mg; vitamin B2, 3500 mg; vitamin B6, 1000 mg; vitamin B12, 7 mg; biotin, 50 mg; folic acid, 700 mg; nicotinic, 20,000 mg; pantothenic acid,7000 mg; vitamin A, 2400 mg; vitamin E, 7000 mg; vitamin D3, 50 mg; vitamin K3,1500 mg;
c-Mineral premix (per kg of premix): zinc sulfate, 4.0 g; iron sulfate, 20 g; manganese sulfate, 5.3 g; copper sulfate, 2.7 g; calcium iodine, 0.34 g; sodium selenite, 70 mg; cobalt sulfate, 70 mg, and CaHPO4·2H2O up to 1 kg.
d-Nitrogen free extract (NFE)=100 – (protein%+lipids %+ash%+crude fiber %).
e-Gross energy (GE) was calculated as 5.65, 9.45 and 4.11 kcal/g for protein, lipid and NFE, respectively **(NRC, 2011).**

Preparation of Seaweeds (*Gracilaria spp*)
Gracilaria spp (Red algae) from the phyla Rhodophyta was collected from the Egyptian Mediterranean coast of Alexandria. The collected samples washed consequently in sea water, tap water and distilled water to separate potential contaminants then dried for **2** weeks and finely ground through 60 mesh using a laboratory mill according to **(Peixoto *et al.*, 2016).**

Drug:
Oxytetracyclin (Terramycin) ® was purchased from commercial Egyptian veterinary pharmacy and used in a dose **75**mg /kg/day body weight orally for ten days **(Plumb, 1999)**, three days before infection and seven days post infection.

Growth and feed utilization parameters
After the feeding trial, fish were collected,

counted, and bulk weighed. Growth performance was determined and feed utilization was Calculated as follows:
Weight gain= (W2 – W1) while, W2 is a final weight and W1 is an initial weight
Feed intake=the summation of the offered feed to fish throughout the experiment
Feed conversion ratio (FCR)= feed intake/ weight gain.

Blood samples:
Three types of pooled blood samples were collected from each group from caudal vein under complete aseptic condition at both 7^{th} and 30^{th} days post infection. The first blood sample was collected on EDTA for hematological examination (1ml). The second blood sample was collected in a sterile plastic tube containing heparin to be used for phagocytic activity investigation (2 ml), while the third blood sample was taken without anticoagulant in a clean and dry centrifuge tube (3 ml), left to clot at room temperature and centrifuged at 3000 rpm for 5 min. Serum was collected, labeled, placed in dry clean-capped tubes and frozen at -20^{o}C for biochemical analysis of glutathione peroxidase (GPx) and malondialdehyde (MDA).

The hematological and biochemical study:
The erythrocytic count **(Wintrobe, 1934)**, hemoglobin concentration, packed cell volume and total leucocytic count were carried out manually **(Blaxhall & Daisley, 1973)**. Differential leucocytic counts were calculated according to **(Cole, 1986).** Glutathione peroxidase GPx activity was assayed according to (**Miller and Slebodzinska, 1993).** And Malondialdehyde (MDA) was calculated according to **Esterbauer (1982).**

Phagocytic activity and index
Blood sample were collected on heparinized tube for phagocytic activity:
(a)-Measurement of phagocytic activity of Peripheral Blood Monocyte (PBM) using *Candida albicans* was performed **Anthony *et al.*, (1985) and Chu & Dietert, (1989).**
(b)-Separation of Peripheral Blood Mononuclear Cells (PBMC) using ficoll-plaque density gradient was carried out **Boyum, (1986) and Godeeris *et al.*, (1986).**
(c)- Phagocytic assay **(Wilikinson, 1976).**
Pathological examination
Tissue specimens*:*
Specimen from liver, kidneys, intestine, skin and brain were collected from sacrificed ***Clarias gariepinus*** at both **7^{th}** & **30^{th}** days from the beginning of the experiment and fixed in **10%** buffered neutral formalin. Paraffin sections **5** micron thick were prepared and stained with hematoxylin and eosin stain **(Survarna *et al.*, 2013)** and examined microscopically. Lesion score of treated groups were applied as mild **(+),** moderate (++) and Severe and were demonstrated in **table (4**).

Statistical analysis:
Statistical analysis was performed using the analysis of variance (**ANOVA**). Duncan's Multiple Range (**Duncan, 1955)** was used to determine differences among treatments mean at significance level of 0.05. All statistics were run on the computer using the SPSS program **SPSS, (2004).**

Results
I-Bacteriological results
The results of isolation and identification have revealed that **26** ***A. hydrophila*** isolates were successfully recovered from examined tissue specimens in **50** examined *Clarias gariepinus* with total recovery rate **52% (**each isolate was from separate fish regardless to number of examined organs), the recovered isolates had grown well on tryptic soya agar producing white, round, creamy colonies, pale colonies on MaCconkey agar, whereas it produce green colonies with dark center on *Aeromonas* agar and finally on blood agar it produce large grayish glistening colonies mostly surrounded by ß-heamolysis.
Biochemical and morphological characterization of presumptively identified *A. hydophila* were summarized in **Table (3)**
PCR was applied on seven randomly selected *A. hydrophila* isolates for the detection of ***16S*** rRNA gene and results showed that this gene was detected in all examined isolates and gave a characteristic band at **625** bp as shown in **Fig (1)**.
A. hydrophila was reisolated from gills, liver, kidney and intestine of experimentally infected

Clarias gariepinus by the same isolation technique.

II-Biochemical results

The effect of *A. hydrophila* infection on *Clarias garipeinus* growth and feed utilization parameters was presented in **(table 5).** Second group fish revealed significant decrease in body weight and FCR when compared with the first group fish, while there were significant increase in third, fourth and fifth groups fish when compared with the second group fish. Body weight gain was significantly decrease in the second group all over the experimental period while it was significantly increased in the third, fourth and fifth groups fish when compared with fish of the second group.

Results for hematological parameters in **(table 6, 7)** showed that fish infected with *A. hydrophila* (second group) revealed a significant decrease in the erythrocytic count, Hb concentration and packed cell volume; on the other hand, there was a significant increase in the leucocytic count when compared with control group (first group). Infected fish supplemented with *Gracilaria spp*. in diet (third group) showed a significant increase in RBCs count, Hb concentration and PCV% when compared with infected non-treated group. Also, infected fish treated with oxytetracyclin (fourth group) and that received both of *Gracilaria spp.* and oxytetracyclin (fifth group) revealed increase in erythrocytic count, Hb concentration and packed cell volume when compared with infected non-treated group. Regarding to the immunological parameters our results in table **(6 & 7)** revealed that phagocytic activity of fish infected and supplemented with *Gracilaria spp*. or oxytetracyclin or that received both of them showed a significant increase in phagocytic activity comparing with infected non treated fish. Results for oxidative stress after infection with *A. hydrophila* were presented in Table (**6 & 7**) the results showed that fish infected with *A. hydrophila* showed a significant increase in malondialdehyde activity. Treatment with *Gracilaria spp.*, oxytetracyclin and both of them in(third, fourth and fifth groups) consequently revealed a significant decrease in malondialdehyde activity when compared with fish in the second group. On the other hand infection with *A. hydrophila* resulted in a significant decrease in GPx all over the experimental period while treatment with *Gracilaria spp.* or oxytetracyclin or both revealed a significant increase in this enzyme when compared with infected non treated group.

III- Clinical signs, mortalities and pathological results:

A-Clinical signs and mortalities.

Only fish of second group (infected not treated) revealed variable degrees of clinical signs including loss of vitality, swimming closer to surface and defecate free mucus few hours post infection. While no signs observed among fish of other groups except mild weakness and loss of vitality among fish of the third and fourth group comparing with control. Nine fish out of thirty from of the second group were died with **26.67%** mortalities, three fish out of thirty from the third group fish with **10%** mortalities and one fish out of thirty from the fourth group also died with **3.33%** mortalities while no mortalities exhibited among fish of the first and fifth group. Totally thirteen fish out of one hundred and fifty **(9.3%)** were died **28** hours post infection till **10^{th}** day post infection.

B- Postmortem examination:

As shown in **table (**7), macroscopical findings revealed variable degrees of skin laceration during the experimental period in the second group (infected non treated) associated with partial fin sloughing in some cases **(Fig. 2A)** especially at **7^{th}** day post infection. Pale enlarged livers accompanied with distended gall bladder in addition to intestinal necrosis **(Fig. 2B)** and partial hemorrhagic areas. Pale whitish areas (supposed to be necrotic foci) were also seen on some visceral organs particularly livers and kidneys at **7^{th}** day post infection. Mild macroscopical lesions were detected either externally or internally on fish of other groups particularly those of third and fourth groups represented as small ulcers and /or erosions at **7^{th}** day post infection and scares formation in most fish at **30^{th}** day post infection, while fish of fifth group exhibited mild to non-gross lesions throughout the experimental period.

C– Pathological results:

The microscopical examination of the second group *Clarias gariepinus* showed marked histopathological ulceration especially at **7th** day post infection; Gills showed mild deformity of primary lamellae and partial absence of secondary one **(Fig 3a)**. Areas of necrosis were seen in the intestine of this group and these were associated with partial denuded epithelia and necrotic epithelia filling the intestinal lumen **(Fig. 3b)**, few cases showed necrosis of the intestinal glands and hemorrhages. Most cases of this group showed variable degrees of skin ulceration **(Fig. 3c)** which persist without healing till 30th day post infection. Histopathological examination of pathology revealed focal gliosis in many cases during the experimental period **(Fig. 3d),** some cases showed areas of demylination and variable degrees of haemorrhages. Vacuolation of the epithelial lining the renal tubules were the most prevalent lesions observed among this group at **7th** day post infection and these were accompanied with atrophy of glomerular tuft in some cases **(Fig. 4a),** some other cases showed marked interstitial round cells aggregation **(Fig. 4b)** in addition focal areas of hepatocellular necrosis were seen also in some cases**.** Vacuolation of the hepatocytes was clearly exhibited mostly at **7th** day post infection**. (Fig. 4c**). Fishes of both third and fourth groups exhibited marked tissue improvement when compared with those changes noticed on the second group fish. The livers of these group showed just mild congestion and very mild leucocytic infiltration at **7th** day post infection **(Fig. 4d**) and these were associated with interstitial edema. also renal congestion and tissues embolism were seen especially in the kidney of fourth and fifth groups fish **(Fig. 5a).** These amelioration of fourth and fifth groups was not detected in third group fish as compared with other treated groups. The most obvious histopathological alterations were seen on the intestine of this group which showed partial epithelial sloughing accompanied with fusion of intestinal villi **(Fig. 5b).** As shown in **table (7)**, fish of group 5 were the most apparently normal fish as compared to control one with the exception of appearance of mild skin ulceration (few hours to five days post infection). At 7th day post infection granulation tissue and scare formation was detected **(Fig. 5c).** The kidneys and liver of the fifth group fish showed just haemosiderosis in the liver and mild tubular degeneration within renal tubules **(Fig. 5d).**

Discussion

Genus ***Aeromonas*** causes serious problems in various fish and shellfish species. The disease is characterized by septicemia and mass mortalities **(Noga, 2010)**. Genus ***Aeromonas*** is considered the most dominant species infecting *C. gariepinus* in Egypt **(Emeish *et al.*, 2018).** In the present investigation the occurrence of *A. hydrophila* in **50** diseased *C. gariepinus* was studied. Relying on a succession of morphological, cultural and biochemical tests**, 26** isolates were confirmed to belong to *A. hydrophila* with a total recovery rate **(52%),** This wide spread distribution of *A. hydrophila* may be attributed to its high affinity to adapt to environmental stress factors (**Fowoyo and Achimugu, 2019),** nearly similar incidence rate of *A.hydrophila* isolation in *C. gariepinus* was reported by **(Abd El Tawab *et al.,* 2017 and Emeish *et al.,* 2018)** as it was **50.4%** and **50%**, respectively from examined *C. gariepinus* , lower recovery rate was reported by (**El-Barbary & Hal., 2016 and Fowoyo & Achimugu**, **2019**) who isolated *A. hydrophila* from *C. gariepinus* with incidence rate **21.8% and 30%,** respectively whereas very high isolation rate was reported by **(Kusdarwati *et al.* 2017)** who found that **95%** of examined *C. gariepinus* in their study were infected with *A. hydrophila.* The occurrence of *A. hydrophila* in the intestine, kidney, liver, skin and muscles of fish is in agreement with previous studies which isolated this microbe from the same isolation sites factors (**Fowoyo and Achimugu, 2019)**. Some researchers believe that *A. hydrophila* is a primary fish pathogen while others considers it as secondary invader as immune suppressive in fish **(Hayes, 2007),** it was recorded that high prevalence in diseased *C. gariepinus* may suggest that *A. hydrophilla* is a part of normal intestinal flora of freshwater fish and under certain conditions it became pathogenic; therefore it is considered as an opportunistic fish pathogen **(Plumb, 1999)**. The morphological characteristics of the colonies,

gram staining and biochemical profile of the obtained isolates was quite in consonance with results of previous studies **(Sabur, 2006 and Mostafa *et al.,* 2008).** The molecular identification is considered the most perfect identification tool for fish pathogens even in early infection phases **(Buller, 2004)** as it is more accurate than phenotypic and biochemical identification which can be modified by the variability of expression of characters **(Hossain, 2008)**. Genotypic identification of *A. hydrophilla* by targeting its highly conserved gene (***16S*** rRNA) showed positive amplification of target gene at 625bp in all examined isolates, this was in accordance with **(Gordon *et al.,* 2007)** who made amplification of the same gene by specific primers and on the basis of the conventional PCR results. The potential hazards of using antibiotics in aquacultures are the development of antibiotic resistant bacteria, chemical residues in fish products and contamination of surrounding ecological system (**Serano *et al.,* 2005 and Cabello, 2006)** and therefore it represents a priority for exploring and developing alternatives with better potential, good bioavailability, minimal toxicity and less-side effects than antibiotics on human farmed organisms and environments. Hence the main objective of the existing study was to evaluate the capability of *Gracilaria spp.* to inhibit the growth of fish pathogenic *A. hydrophilla* with the aim of assessing them as possible alternatives to common antibiotics in aquaculture. Isolates of *A. hydrophilla* could be reisolated from liver, kidney, intestine and skin lesions of experimentally infected fish by same technique of isolation and identification; this is in accordance with **Mostafa *et al.,* (2008)** who reported that injected *A. Hydrophilla* was mostly re isolated from liver, kidney and intestine of challenged *C. gariepinus*.

In recent years, several studies have been carried out to evaluate the feasibility of using plant products and seaweeds as ingredients in fish feed. The results showed that partial or total replacement of fishmeal with plant proteins showed similar growth performance and did not affect nutritional value and health of fish **(Cheng *et al.,* 2010).** In our study fish infected with *A. hydrophila* and supplemented with *Gracilaria* spp. alone or with oxytetracyclin revealed increase in body weight compared with the second group. Also there were improvement in the food intake and FCR. Our results were in accordance with those provided by **Peixoto *et al.,* (2016)** who showed a similar weight increase for all dietary treatments, suggesting that seaweeds can be included in fish diets without compromising growth rates. Similar values have also been reported by **Valente *et al.,* (2006),** they showed that *Gracilaria bursapastoris* can use as alternative ingredients in diets for European sea bass with no adverse effects on growth performance and feed utilization efficiency, meanwhile **Al-Asgah *et al.,* (2016)** reported that *C. gariepinus* supplemented with 20%and 30% *G. arcuata* showed poorer growth and feed utilization than the first (control) group and recommended that *C. gariepinus* can accept *Gracilaria* up to 10% in their diets**.** Our results also are parallel to the results obtained by **(Sanchez-Martinez *et al.,* 2008 and Koh *et al.*, 2016)** who recorded significant improvements in the growth performance of Channel *C. gariepinus* and tilapia fed diets supplemented with oxytetracyclin in diet. Haematological parameters have been studied in many fish species to determine the normal range and any variations from these ranges were indicative of pathophysiology **(Ranzani-paiva *et al.,* 2000)**. In the present study infected non-treated fish with *A. hydrophila* (second group) revealed a significant decrease in the erythrocytic count, Hb concentration and packed cell volume. In the mean time there was a significant increase in the total leucocytic count; in my openion these changes may be due to *A. Hydrophila* enterotoxins and alterations in biological activity due to production of extracellular products and enzymes including cytotoxins, and proteases **(Allan and Stevenson, 1981)**. Our results in accordance with results recorded by **(Amer *et al.,* 2009)** who found that *Clarias lazera* infected with *A. hydrophila* induced a significant decrease in the Rbcs, Hb and pcv. Fish experimentally infected and treated with *Gracilaria spp.* showed a significant increase in RBCs count, Hb concentration and PCV% when compared with that infected group, which were in agreement with **Singh, (2014) and Mag-**

noni *et al., (*2017)] they stated that *Gracilaria* has positive impact on haematological parameters. This improvement in the haematological results may be due to *Gracilaria spp*. has antibacterial activity against *A hydrophila* **(Maftuch *et al.,* 2016)**. This antibacterial activity is due to its disabling antibacterial compounds such as, Alkaloid, Flavonoid, Tannin, Phenolic compound and Quercetin-7-methylether which is a dominant group of its antibacterial activity **(Maftuch *et al.,* 2016).** Our studies showed that treatment of infected fish with oxytetracyclin revealed an increase in Rbcs, Hb and pcv this was in agreement with (**El-Adawy *et al.,* 2018)** who recorded improvement in the hematological parameters on Nile tilapia experimentally infected with *A. hydrophila* and treated with oxytetracyclin. In the current work, administration of infected fish to *Gracilaria* spp. alone or in a combination with oxytetracyclin induced a significant increase in total leucocytic count comparing with control group, these results parallel to that reported by **Ramazan *et al.,* (2005) and Singh, (2014)**. Here in, we concluded that *Gracilaria spp*. does not only enhance the antibacterial effect of oxytetracyclin, but it also improves the immune response and resistance against pathogens. This conclusion is based on our findings and those obtained by **Zahra *et al.,* (2017) and Jasmanindar *et al.,* (2018)** who reported that *G. verrucosa* extract supplementation can increase the total hemocytes in shrimp. Hemocyte is a cell that plays an important role in cellular and humoral immune defense of crustacean, and that indicated increase in body reactive defense. The increase in total leucocyte count in the present study was also followed by increase in phagocytic activity, after supplementation of *Gracilaria spp.* to infected fish. In fish, phagocytic activity is the main mediators of innate immune to the pathogens **(Nonakaand Smith 2000)**. Phagocytic activity produce bactericidal product, which is part of innate immune systems and increase the resistance of fish against pathogens **(Chen *et al.,* 2012)**. Several researchers suggested that *Gacilaria spp* has immunostimulant effect modulates the immunity via increase Immune responses such as total hemocytes and phagocytic activity **(Zahra *et al.,* (2017) and Jasmanindar *et al.,* (2018)]**. In the present work infected fish and treated with oxytetracyclin showed significant increase in phagocytic activity this disagree with **(Taffalla *et al*.,1999)** who reported that administration of oxytetracyclin to *turbot* fish for 12 days has no significant effect on the phagocytic activity. However, when oxytetracyclin was added with *Gracilaria evokeda* maximal immune response and resistance against infection. These results parallel to that reported by **(El-Adawy *et al.,* 2018)** who recorded that Supplementation of oxytetracyclin with propionic acid to Nile tilapia challenged with *A. hydrophila*, enhanced tilapia immunity and this indicated by increase phagocytic activity. In our study infected fish with *A. Hydrophila* revealed alteration in antioxidant status, represented by significant increase in the malondialdhyde (MAD) comparing with control and that was in accordance with results reported by (**Abdel-Magid *et al.,* 2018).** While GPX level was significantly decreased, similar results were reported by **(Junming *et al.,* 2013)**. In the present study addition of *Gracilaria spp.* alone or with oxytetracyclin restored MDA and GPx to its normal levels. The improvement in MDA and GPx may be due to large amounts of bioactive molecules in ***Gracilaria spp.*** (tannins, sterols, triterpenes, saponins, and flavonoids) which have antioxidant properties **(Ebrahimzadeh *et al.,* 2018).** Our results parallel to these results reported by **[Peixoto *et al.,* (2016) Magnoni *et al.,* (2017)]** who reported that dietary seaweed supplementation of *Gracilaria spp.* have protective role against oxidative stress. As an important tool, histopathology was applied to evaluate the qualitative changes in the targeted affected organs in the infected groups and the patterns of recovery in the treated groups. Clinically, fish infected with *A .hydrophyla* in the second group exhibited clinical signs similar to that obtained by **{(Afifi *et al.,* 2000) & (Banu and Yılmaz, 2011)}** except exophthalmia that could be attributed to the difference in the species of experimental fish .Our studies showed that mortality rate was **9.3%**, which was in contrast with most of previous studies who reported mortalities less than our percent **{(Afifi *et al.,* 2000) & (Banu and Yılmaz, 2011)}** in my opinion it could be explained on a base of

differences in virulence and dose of administered strain, and its route of administration.

Macroscopical Our studies showed variable degrees of macroscopical changes in the viscera which could be attributed to the virulence of the injected strains and route of administration (intra peritoneal) as bacterial virulence in fish intra peritoneal injected were higher than that fish intramuscularly injected. The reason why the virulence was higher in intra peritoneal injection group than the intramuscular was the faster and higher morbidity rate of intra peritoneal injection **Afifi *et al.,* (2000)**. All previously mentioned gross lesions were in harmony with those mentioned by several authors, **(Roberts, 2001)** and **(Dalia, 2013).**

Microscopically

The microscopical alteration seen in gills of the second group was matching with those obtained by **(Maha *et al*., 2017)** who commented that the gills was affected by *A. hydrophila* infection .Our studies showed skin ulceration this was agree with most authors **as {(Julinta *et al*., (2017) and Laith & Najiah, (2013)}**. Many organs like kidney, liver, intestine and brain were affected and showed necrosis, hypremia and degenerative changes and/or without hemorrhage in addition to leucocytic cells infiltration similar result was found by **(Cipriano, 2001).** These results are parallel to those obtained by **(Samnejhad *et al.,* 2016**) and **(Afifi *et al.,* 2000)** which could be explained on a base of the effect of associated toxin and extracellular proteins (hemolysin and elastase) produced by *A. hydrophila* **(Rodriguez *et al.,* 1992)**. Hemosiderosis in the visceral organs was seen and this was the same as reported by **(Miyazaki & Kaige, 1985)** due to the effect of *A .hydrophila* toxin which cause hemolysis of blood. Kidney lesion was the most prominent visceral lesions in all infected fish that support the nephropathy effect of *A. hydrophila* **(Laith & Najiah, 2013**). *Gracillaria spp*. have an ameliorative alterations effect appeared in different tissues of *C. Gariepinus* infected with A. *hydrophila* due to its antibacterial effect that reported by **Maftuch *et al.,* (2016)**. Antibiotic (oxytetracycline) treated group score a good target and minimize the previously mentioned lesion in all organs except those in kidneys those could be attributed to the demonstrated negative effect of OTC intramuscularly administration at high concentration on the renal tissue damage **(Soler *et al.,* 1996).**

Using both *Gracillaria spp.* and oxytetracycline in the fifth group was the most effective treatment which demonstrated minimal and milder lesion when compared with the other treated groups which confirm the biochemical results about the synergistic effect between both of them.

Conclusion

Applying macro algae **5%** *Gracilaria spp.* in diet ameliorate the adverse effect of *A. hydrophila* infected *Clarias garipeinus* and improve the influenced growth performance and feed utilization, hematological parameters and immune response with concomitant increase of phagocytic activity with pathological screening, this improvement became higher when using oxytetracyclin. This may be the first study to indicate the synergistic effect between *Gracilaria spp.* and oxytetracyclin when supplemented to diets of *Claria sgaripeinus*.

Recommendation

Adding *Gracillaria spp.* in fish meal as a natural antibacterial, immunostimulant agent and applying in all concerned fish cultures through the whole life cycle of the cultured fish accompanied with usage of oxytetracyclin in heavy infection.

Table (1). PCR cycling protocol and primers of *16S*rRNA gene of *A. hydrophila*

Target gene	Primers sequences	(bp)	Primary denaturation	Amplification (35 cycles)			Final extension
				Denaturation	Annealing	Extension	
A. hydrophila 16S rRNA	GAAAGGTTGATGCCTAATAC-GTA CGTGCTGG-CAACAAAGGACAG	625	94°C/ 5 min.	94°C/ 30 sec.	50°C / 40 sec.	72°C/ 45 sec.	72°C 10 min.

Table (2). Experimental design of the study.

Treatments / Groups	*Aeromonas Hydrophylla* I/P $6x10^6$ CFU/ml	Medicated feed with *Gracilaria spp.* 5g/ kg.orally	Medicated feed with Oxytetracycline 75mg/kg orally
Group (1)	--	--	--
Group.(2)	+	--	--
Group.(3)	+	+	--
Group.(4)	+	--	+
Group.(5)	+	+	+

Table (3). Biochemical and morphological characterization of presumptively identified *A. hydophila*

Test	Reaction
Gram's stain	Gram – bacilli
Motility	Motile
Oxidase	Positive
Catalase	Positive
H2s	Positive
SimmonCitrate	Positive
Indole	Positive
M. R.	Negative
V. P.	Positive
T. S. I	Y/Y (gas)

M.R.: methyl red, **V. P.:** VogeusPreskour **T.S.I:** triple sugar iron **Y/Y:** yellow slant, yellow butt

Table (4). Lesion score of all treated groups at both 7th day* and 30th **day post infection

Lesions	Treated groups and lesion score							
	G. (2)		G. (3)		G. (4)		G. (5)	
Macroscopical lesions	*	**	*	**	*	**	*	**
Skin ulceration and /or necrosis	++	+	+	+	+	++	--	--
Scare formation	+	++	+	+	++	++	--	--
Fins sloughing	++	+	+	+	-	-	-	-
Congested gills	+++	+	++	+	+	-	+	-
Pale liver& distended gall bladder	+	++	+	+	+	+	+	-
Necrotic foci presence in liver	++	+	+	+	-	-	-	-
Necrotic foci presence in kidney	++	+	++	+	+	-	-	-
Microscopic lesions	*	**	*	**	*	**	*	**
Skin ulceration	++	+	+	--	+	+	--	--
Abnormalities of 2^{dry} lamellae	++	+	+++	--	++	+	+	+
Deformities in the primary lamellae	+	+	+	+	-	-	-	-
Congestion of gills	+++	+	++	+	++	+	--	-
Intestinal sloughing of mucosa	++	+	++	++	+	+	--	-
Submucosal degeneration	++	+	++	+	+	+	--	--
Neuronal demylination	+	+	+	+	--	--	--	--
Focal gliosis	++	+	+	+	--	--	--	--
Hepatic vacuolation	+++	++	++	+	+	+	+	-
Hepatic necrosis	++	+	+	++	--	+	--	--
Renal degeneration	+++	+	++	+	+	+	+	-

(+++): Severe (++): Moderate (+): Mild (-):Absent

Table (5). Growth performance parameters of *Clarias garipeinus* in all groups (mean±SE)

Parameter / Group	Initial average b.wt	Final average b.wt	Wt. gain	Feed intake	Feed conversion ratio
Group 1	140.6±5.8	181.6±2.6 a	41±2.8 a	63.3±1.6 a	1.6±0.21 a
Group 2	138.3±3.3	156.6±4.4 b	18.3±1.6 b	19.6±1.4 c	1±0.33 b
Group 3	138.5±5	179±1.6 a	40.6±2.2 b	56.6±3.4 ab	1.3±0.11 ab
Group 4	143.3±5.1	180±3.6 a	36.6±3.3 b	55±2.8 ab	1.4±0.88 ab
Group 5	145±2.8	176±2.8 a	31.6±4.3 ab	48.3±2.5 b	1.5±0.11 a

(n=5) Different letters at the same column means that there was a significant change at **p<0.05.**

Table (6). The effect of treatment with *Gracilaria spp*. and oxytetracyclin in 7 days post infection with *Aeromonas hydrophila* on erythrogram and some biochemical parameters, phagocytic ratio and phagocytic index of clinically healthy and infected *Clarias garpennius.*

Groups	RBCs (10^6×mm3)	Hb (g/dL)	PCV%	WBCs (10^3×mm3)	Lymphocyte	Neutrophil	Monocyte	Phagocytic ratio	Phagocytic index	GPX (mg %)	MDA3 (mg %)
Group1	1.67± 0.014a	7.15± 0.02a	25± 0.57 a	12.7± 0.14c	63± 0.57a	31.3± 0.33 c	3.2± 0.14b	75.4± 0.30a	3.7± 0.05a	3.1± 0.81a	6.5± 0.17d
Group2	1.16± 0.035d	5.26± 0.12d	17± 0.57 c	23± 0.57a	59.4± 0.30 c	35.1± 0.60 a	3.8± 0.08a	51± 0.57d	1.3± 0.08d	1.5± 0.57d	16.2± 0.16a
Group3	1.23± 0.08 c	5.96± 0.12c	19.5± 0.23b	17.4± 0.24b	60.4± 0.23 b	33.6± 0.60 b	3.3± 0.06b	71.2± 0.44b	3.1± 0.05b	2.3± 0.75c	8± 0.11 b
Group4	1.29± 0.02 c	6.1± 0.05 c	19.8± 0.44b	15.5± 0.74b	60.9± 0.43 b	32.1± 0.30bc	3.2± 0.03b	69.2± 0.46c	2.6± 0.05c	2.6± 0.3bc	7.6± 0.26 bc
Group5	1.47± 0.014b	6.5± 0.08 b	21.4± 0.74b	13.5± 0.23c	62.3± 0.18 a	31.8± 0.39bc	3± 0.03 b	74.8± 0.40a	3.6± 0.02a	2.8± 0.3ab	6.8± 0.28 cd

RBCs: Red blood corpuscle Hb: Haemoglobin PCV%: Packed cell volume WBCs: White blood corpuscle MDA: Malondialdhyde GPX: glutathioneperoxidase Means with different letters at the same column were significant P<0.05.

Table (7). The effect of treatment with *Gracilaria spp.* and oxytetracyclin in 30 days post infection with *Aeromonas hydrophila* on erythrogram and some biochemical parameters, phagocytic ratio and phagocytic index of clinically healthy and infected *Clarias garpennius*

Groups	RBCs (10^6×mm3)	Hb (g/dL)	PCV%	WBCs (10^3×mm3)	Lymphocyte	Neutrophil	Monocyte	Phagocytic ratio	Phagocytic index	GPX (mg %)	MDA3 (mg %)
1	1.65± 0.02a	7.12± 0.06a	26± 0.57a	12.9± 0.3 c	62.4± 0.30 a	31.5± 0.29b	3.2± 0.08b	76.1± 0.58a	3.8± 0.12 a	3.3± 0.14a	6.4± 0.18b
2	1.08± 0.04c	5.1± 0.057 b	16± 0.57c	23± 0.51 a	59.3± 0.24 b	34.5± 0.34a	3.7± 0.17a	52.6± 0.45c	1.2± 0.14 d	1.5± 0.18d	16.1± 0.23a
3	1.54± 0.02a b	6.5± 0.21a	22.6± 0.72b	15± 0.52 b	61.1± 0.15 a	32.2± 0.37b	3.2± 0.05b	74.3± 0.38ab	3.4± 0.15bc	2.8± 0.15b c	7.1± 0.33b
4	1.49± 0.03b	6.6± 0.23a	23± 0.57b	13.8± 0.21bc	62.2± 0.14 a	31.9± 0.48b	3.1± 0.08b	72.7± 0.38b	3.3± 0.18bc	2.9± 0.12b c	7± 0.13b
5	1.55± 0.03a b	6.8± 0.2a	24.3± 0.88ab	13± 0.20bc	62.6± 0.25 a	31.8± 0.44b	3.2± 0.12b	75.2± 0.37ab	3.6± 0.15ab	3.1± 0.18a b	6.5± 0.17b

RBCs: Red blood corpuscle Hb: Haemoglobin PCV%: Packed cell volume WBCs: White blood corpuscle MDA: Malondialdhyde GPX: glutathione peroxidase Means with different letters at the same column were significant P<0.05. (n =5)

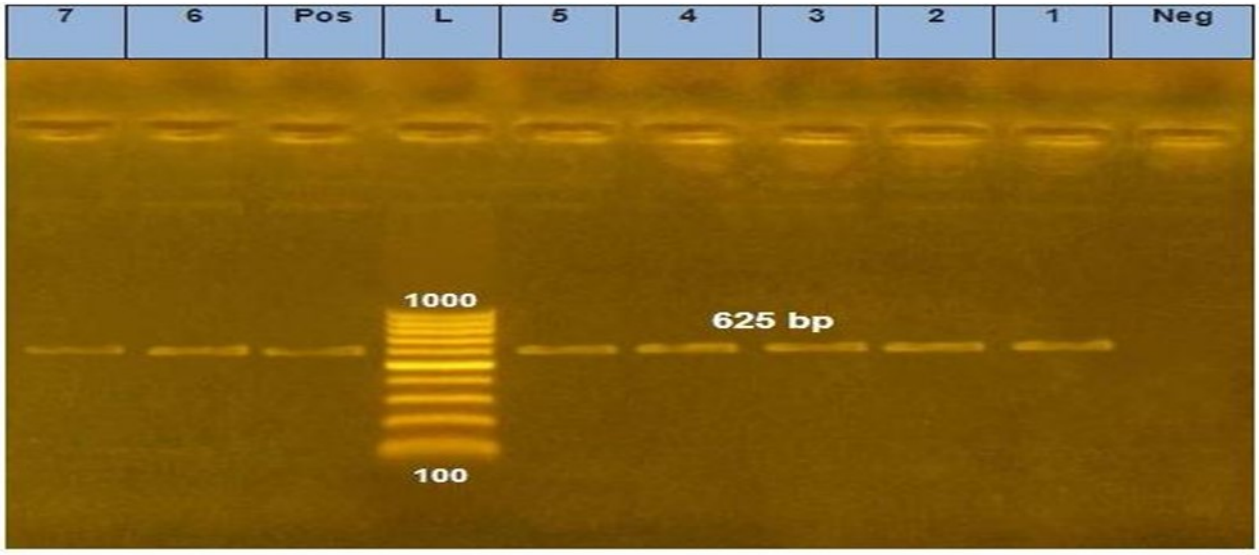

Figure (I): Agarose gel electrophoresis of ***16S***rRNA gene *A. hydrophila* isolates, 100 bp marker, **Lanes (1-7):** Positive amplification of ***16S***rRNA gene in all examined isolates at 625 bp, **Pos:** Positive control, **Neg:** Negative control.

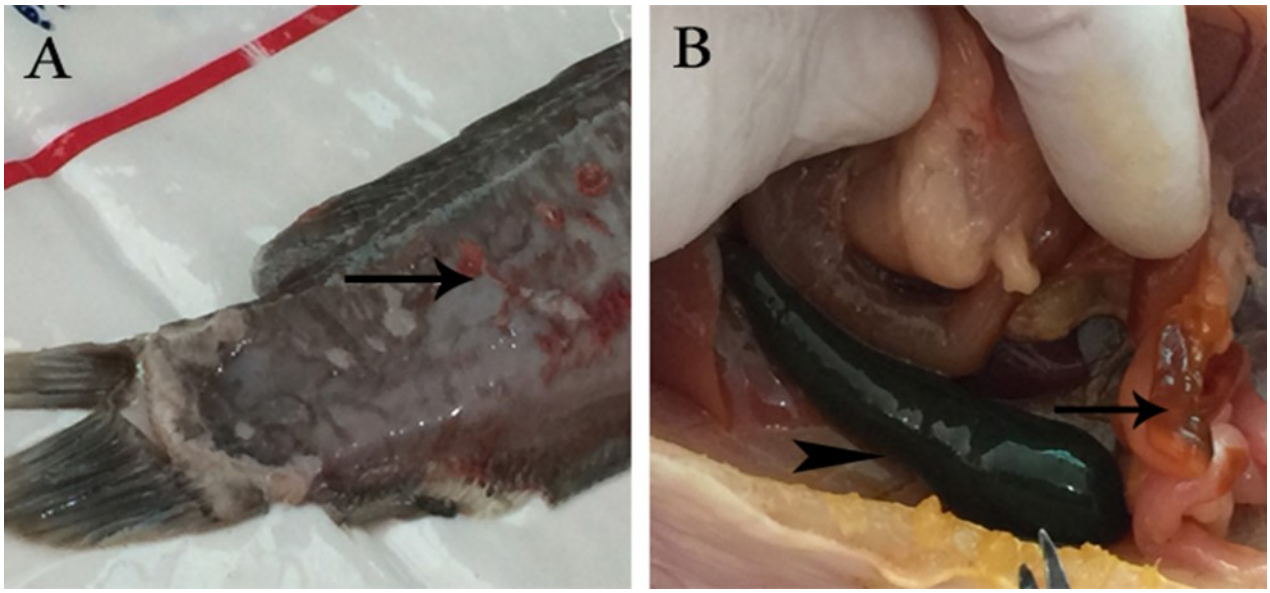

Figure (2): Macroscopical changes in *C. gariepinus* infected non treated group (second group)
(A):-Clarias gariepinus infected with *Aeromonas hydrophila* non treated at 7th day from infection showing skin laceration (arrow)
(B):-Clarias gariepinus infected with *Aeromonas hydrophila* non treated at **7th** day from infection showing pale liver, distended gall bladder (head arrow) with partial necrotic intestine (thin arrow)

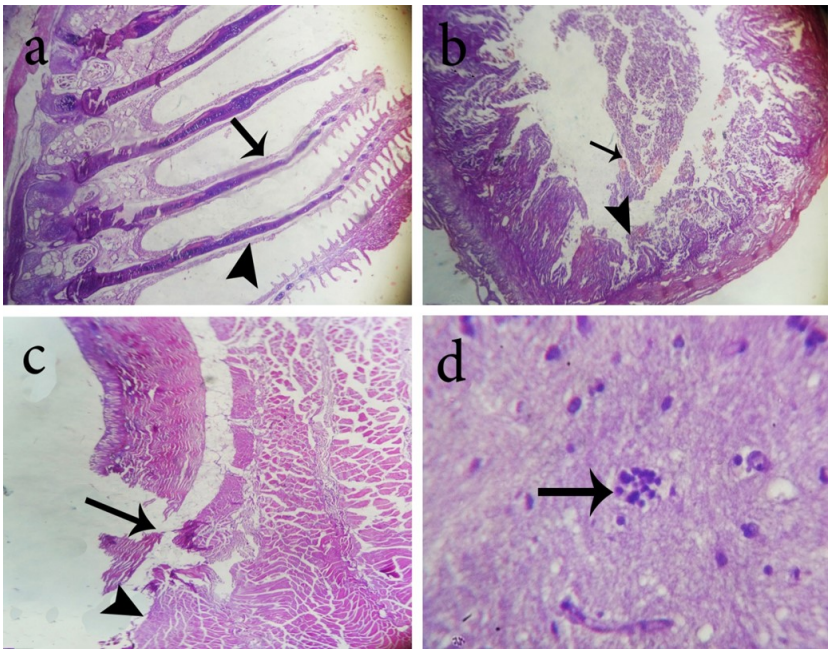

Figure (3): ***Clarias gariepinus infected with Aeromonas hydrophila non* treated (second group)**
(a)- gills showing partial absence of secondary lamellae (head arrow) with mild deformity of primary lamellae (arrow) *at 7th* day post infection (H&Ex200).
(b)- intestine showing partial denuded epithelial villi (head arrow) with sloughed necrotic villus epithelia filling the lumen (arrow) *at 7th* day post infection (H&Ex100).
(c)- skin showing complete skin layers removal (arrow) with absence of basement membrane (ulceration) (head arrow) *at 7th* day post infection (H&Ex100).
(d)- Brain showing focal gliosis (arrow) *at 30th* day post infection. (H&Ex100).

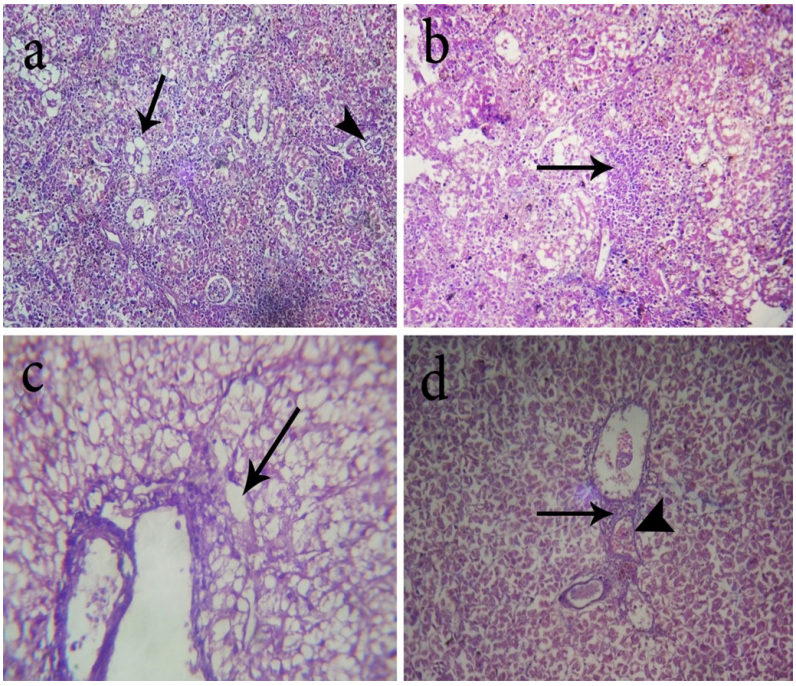

Figure (4): *Clarias gariepinus* infected with *Aeromonas hydrophila* non treated group (second group a, b, c)
(a)- kidney showing vacuolation of renal epithelium (arrow) and atrophy of glomerular tuft ***at 7th*** day post infection (head arrow) **(H&Ex100)**.
(b)- kidney showing marked interstitial lecuocytic cells infiltration (arrow) ***at 7th*** day post infection **(H&E x100).**
(c)- liver showing marked hepatocellular vacuolation ***at 7th day*** post infection (**H&E x200**).
(d)- liver of *C. gariepinus* of third group showing mild congestion (head arrow) associated with interstitial edema and perivascular leucocytic cells infilltration (arrow**) *at 7th day*** post infection **(H&E x100)**

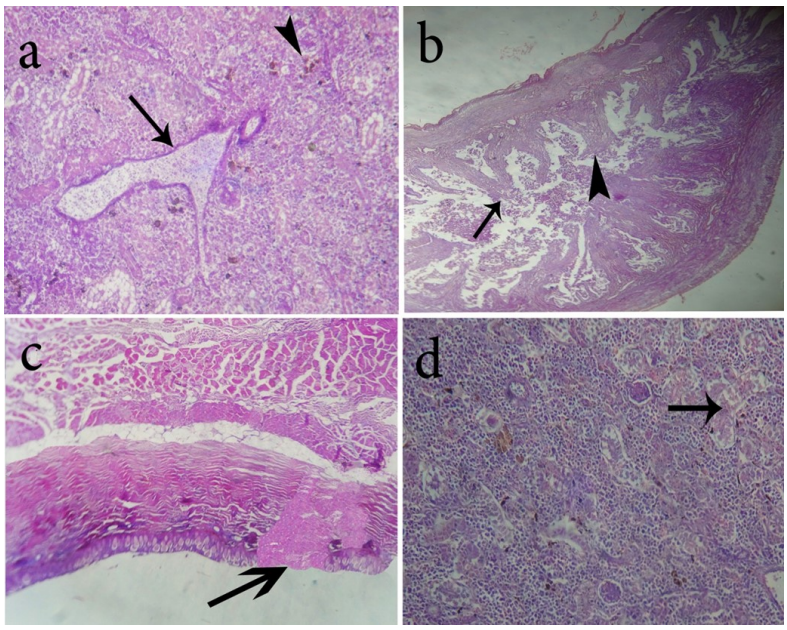

Figure (5): ***Clarias gariepinus*** **of the fourth and fifth group of the fourth * and fifth **group**
(a)- kidney of *C. gariepinus* *showing congestion with **tissue** cell embolism within renal vasculature ***at 7th day*** post infection **(H&Ex100)**
(b)- intestine of *C. gariepinus* *showing mild sloughing of intestinal mucosa (head arrow) with fusion of some intestinal villi (arrow) ***at 7th day*** post infection **(H&Ex100)**.
(c) –skin of *C. gariepinus* **showing granulation tissue replacing ulcerated area (scare formation) (arrow) **at 30th** post infection **(H&Ex100).**
(d) Kidney of *C. gariepinus* **showing apparently normal renal tissues just **hemosiderosis** and degeneration of some renal tubules **(arrow) at 7th** post infection **(H&Ex100)**.

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