Egypt. J. Exp. Biol. (Zool.), 13(2): 321 – 329 (2017)
DOI: 10.5455/egysebz.20170915064526

RESEARCH ARTICLE

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Histopathological changes in diseased catfish (*Clarias gariepinus)* treated by ciprofloxacin and clove extract

ABSTRACT:

Bacteria pathogens cause a serious loss in aquaculture and health hazards to humans. Based on biochemical characteristics and API-20E system, the highest prevailing isolate was identified as *Aeromonas sobria*. Infection with *Aeromonas sobria* in African catfish (*Clarias gariepinus*) induced focal haemorrhage at the end of gill, generalized oedema and ulcers on skin surface. Internal signs of infection include congestion of the hepatic blood vessels, areas of coagulative necrosis in the liver and hydropic degeneration of renal tubules according to the histopathological examination. In-vivo the combination of ciprofloxacin and clove extract was the choice as an alternative bacterial therapeutic agent in infected *Clarias gariepinus* with *Aeromonas sobria*. Moreover, histopathological changes have been widely used as biomarkers in the evaluation of the health of fish exposed to contaminants.

KEY WORDS:

Aeromonas, Catfish, Ciprofloxacin, Clove, Histopathology.

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ARTICLE CODE: 24.01.17

INTRODUCTION:

The most important pathogen in channel catfish is *Aeromonas spp*, which is the primary causative agent of Motile *Aeromonas* Septicemia (MAS) and can infect many fish species including tilapia, catfish, gold fish and common carp (Hariknishnan *et al.*, 2003). Motile *Aeromonas* Septicemia is considered one of the most common diseases of cultured warm-water fish in freshwater environments caused by motile *Aeromonas* species (Francis-Floyd, 2002). Nam and Joh (2007) noticed that the dominant strain at the farm during all four seasons was *Aeromonas sobria. A. sobria* was detected in 100% of intestinal samples from diseased trout. It was reported that epizootic ulcerative syndrome (EUS) caused by *Aeromonas sobria* resulted in great damage to fish farms (Rahman *et al.,* 2002). *Aeromonas sobria* was also the causative agent of fish disease in the farm of perch, *Perca fluviatilis* L, in Switzerland (Wahli *et al.,* 2005).

Chronic infections could lead to ulceration, inflammation and dermal lesions with focal haemorrhages (Cipriano, 2001) and during acute septicaemia, the liver and kidney are the common target organs (Huizinga, 1979). In human, *Aeromonas* species cause diarrhoea, gastroenteritis and extra enteritic conditions such as septicaemia, wound infection, endocarditis, meningitis and pneumonia (Buckely and Howard, 1999). Considering the susceptibility of fish to various potential stressors, it would be advisable to conduct regular health studies on the fish.

The isolated *A. sobria* was sensitive to Vofloxacin, Norfloxacin, Rifampicin, Ciprofloxacin and Leucomycin (Min and Ying, 2005). Also, Hatha *et al.* (2005) confirmed that none of the *Aeromonas* strains were resistant to Streptomycin, Ciprofloxacin and Nalidixic acid. Negm (2010) demonstrated that clove oil could reduce total bacterial counts in water and muscle of fish toxicated by cadmium, also inhibited the growth of *Pseudomonas spp* and *Aeromonas spp*. Gülçin *et al*. (2012) concluded that clove oil was found to be effective antioxidant in different in-vitro assays including reducing power. DPPH radical, ABIS radical and superoxide anion radical scavenging, it can be used for preventing lipid oxidation in food and pharmaceutical products retarding the formation of toxic products.

The present investigation was planned to study the symptoms and the histopathological alterations of *Aeromonas* in infected African catfish (*Clarias gariepinus*) collected from different localities in Sharkia governorate of Egypt.

MATERIAL AND METHODS:

Fish Collection:

One hundred and fifty (150) clinically health *Clarias gariepinus* were collected randomly with an average body weight of 100 to 150 gm and length of 20 to 22 cm. Fish were kept in glass aquaria (100 x 50 x 50 cm) and supplied with chlorine free water at 25º ± 1ºC with continuous aeration using electric air pumping compressors (RINA, Italy).

Bacterial isolation and identification:

Under sterilizing conditions, samples were collected from (skin, gill, liver, spleen, kidney and intestine) then cultivated on Tryptic Soya Agar (TSA) and incubated at 30ºC for 24 hours. The separated colonies were picked up and inoculated into Rimler-shotts agar (RSA) plate for further identification. Biochemical tests were carried out according to (Austin and Austin, 2007), and API-20E strips (BioMerieux) were used as confirmatory identification.

Pathogenicity test:

Five hundred and seventy specimens of healthy *Clarias gariepinus* (125 ± 5 g average body weight) were collected randomly to study the experimental infection of some bacterial isolates of *Aeromonas sobria, A. jandaei, A. caviae and A. veronii* isolated from naturally infected fish. The fish were acclimated for two weeks, and then divided into 19 equal groups, each group in three replicates (each of 10 fish).

1- Groups (1 - 9) were injected by 0.2 ml of 10^{10} *Aeromonas. sobria* cells of different isolates.

2- Groups (10 - 12) were injected with 0.2 ml of 10^{10} *A. caviae cells.*

3- Groups (13 - 15) were injected with 0.2ml of 10^{10} *A. jandaei cells.*

4- Groups (16 - 18) were injected by 0.2ml of 10^{10}*A.veronii cells.*

5- Groups (19) were injected with 0.2ml sterile saline (NaCl 0.85%) as control (Brook *et al*., 1988). All experimentally injected fish were observed daily for 3-5 weeks to record any clinical signs, mortalities, abnormalities. Specimens were seeded for bacterial re-isolation as determined by Miles and Misra (1938).

Effect of ciprofloxacin and clove extract on mortality rate (%) of experimentally infected *Clarias gariepinus* with *A. sobria*:

To determine in vivo efficacy of ciprofloxacine and clove extract (which was detected in the previous study) against *Aeromonas sobria* infection, 120 Nile catfish (*Clarias gariepinus*) were divided into four equal groups each of 30. They were kept in well aerated glass aquaria measuring 100 x 50 x 50 cm to be acclimated on dechlorinated tap water for 50 days.

Group 1: Fish inoculated intra-peritoneal (IP) with 0.2 ml of 24 hours broth culture of *Aeromonas sobria* (2.5 x 10^8) / ml and kept without medication (positive control).

Group 2: The same as experimentally infected fish and treated with therapeutic dose of ciprofloxacin (25 mg/ litter of water).

Group 3: The same as experimentally infected fish and treated with therapeutic dose of clove extract (13.25 µl).

Group 4: The same as experimentally infected fish and treated with therapeutic dose of both ciprofloxacin and clove extract.

1	30	Infected non-treated
2	30	Infected and treated with ciprofloxacin (25mg /L)
3	30	Infected and treated with clove extract (13µ/L)
4	30	Infected and treated with ciprofloxacin &clove extract (25 mg/l and 13 µ/l)

Histopathological Examination:

For histopathological studies, tissue specimens were obtained from skin, gills, liver and kidney. The tissue specimens were fixed in 10% natural buffered formalin. Dehydration and infiltration of tissue were carried on using automatic tissue processor. Samples were embedded in paraffin and sectioned using rotary microtome (4 to 5 µm) and stained with Haematoxylin and Eosin (H&E) according to the method described by Carleton *et al.* (1967).

RESULTS AND DISCUSSION:

Mortality rate was among the experimentally infected *Clarias gariepinus* with 0. 2 ml of saline containing 10^{10} cells/ ml of 24 hrs *A. sobria* by intra-peritoneal rout (I/P) were showed in table 1. The mortality rate differed from 20 – 100% of *Aeromonas sobria* isolates. While, the recorded mortality rate with one isolate of *A. jandaei* was 90% and two isolates of *A. veronii* was 20% also mortality rate in *A. caviae* ranged from 10 - 80%. Fish were found dead without any clinical signs while the post-mortem findings were congestion in liver and kidneys with haemorrhages in the intestine. *A. jandaei, A. veronii*, and *A. caviae* were re-isolated from different organs of moribund and recently dead fish.

Table 1. Mortality rate of *Clarias gariepinus* due to experimental infection intra-peritoneal (I/P) with 0.2ml of 10^{10} cells/ml of different isolates of *Aeromonas spp*.

Items	Bacterial isolates																		
	Aeromonas sobria									*Aeromonas caviae*			*Aeromonas jandaei*			*Aeromonas veronii*			*Control*
Groups	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
No of fish	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30
Organs	K	L	S	S	L	L	K	K	K	L	K	S	L	K	K	L	S	L	*Sterile saline*
Mortality	90	100	100	90	100	60	100	20	90	0.0	80	10	0.0	0.0	90	0.0	20	20	0.0

L: liver k: kidney S: spleen A: ascites

This variation of mortality rate was due to differences in the virulence among individual strains. the results were in accordance with (Sahoo *et al.*, 2004; Kai-yu, 2004; Pan *et al*., 2004; Shome *et al*., 2005; Jun *et al*., 2010) who recorded that *A. hydrophila* produced mortality ranging from 0 to 100%, while Dhanaraj *et al*. (2008) recorded mortality rate in experimental infected carp fish by *A. hydrophila* (45%) and reached to 100% in other fish species with the same isolates.

Ciprofloxacin, clove extract and both of them (Table 2) successfully reduced mortality rate from 83% in GP (1) to 20%, 16%, and 13%, respectively by the 5th day post treatment with extract and ciprofloxacin while it completely disappeared by 7th day of treatment, which this means that ciprofloxacin and clove extract was effective against MAS and the treated fish returned to the normal stats of health. The present results were confirmed with those reported by Abdalla and Selim (2004), Rahman *et al.* (2009), Thanikachalama *et al*. (2010), and Wafaa and Ismail (2013).

The fish infected with *A. sobria* then treated by ciprofloxacin (group 2) showed few petechial haemorrhages and depression during the 1st week but some fish showed recovery at the end of experimental period this indicates that ciprofloxacin increased the survival rates of fish challenged with *A. sobria* and in turn decrease the mortalities (Nouws *et al.,* 1988; Maisa, 1999).

Fish infected with *A. sobria* then treated by clove extract (group 3) showed lesions but they were milder than those in group 1 (Pérez and Lewis, 2006). Fish infected with *A. sobria* and treated with both Ciprofloxacin and Clove extract (group 4) The lesions of such group were ameliorated than those described with the infected (group 1), treated with Clove extract (group 3), or with Ciprofloxacin (group 2) alone. These results were nearly agreed with those reported by Nascimento *et al*. (2000) and Abd El-Raouf *et al*. (2011) who clarified that the association of antibiotic and plant synergistic antibacterial activity especially with Ciprofloxacin and Erythromycin on *Pseudomonas aeruginosa,* and *Staphylococcus aureus* respectively.

The clinical signs and histopathological examination:

Group 1: Catfish infected with *Aeromonas sobria* and non-treated:

The skin shows areas of ulceration with sloughing of the epidermis, inflammatory oedema and leukocyte aggregation extending to the dermis (Fig. 1. A). The mucous cells appeared hypertrophied with formation of intra-epidermal mucous cyst. Ballooning, hydropic and vacuolar degeneration are shown in some epidermal cells while the others show mucous metaplasia with melanomacrophages infiltration. The dermis revealed extensive aggregations of lymphocytes and oedema (Fig. 1. B). The gills showed extensive necrosis in the covering epithelium of secondary lamellae with leukocytes replacement (Fig. 2. A & B).

Congestion of the lamellar blood capillaries was noticed. Oedema of the gill arch and focal haemorrhage was also seen. The suprabronchial organ also showed necrosis and leukocytes infiltration in the lining epithelium (Fig. 2. C). The liver revealed irregular areas of coagulative necrosis (Fig. 3. A). Congestion of hepatic blood vessels with few leukocyte infiltration and haemorrhage especially around the hepatoportal anastomosis (Fig 3 B). The pancreatic acini were necrotic and infiltrated with lymphocytes. The kidney showed coagulative necrosis in the tubules, congestion of the blood vessels and depletion of the hemopoietic elements (Fig. 4). The glomeruli were contracted with dilated Bowman's capsule, similar results were obtained by Austin and Austin (1993), Plumb *et al.* (1994), Deepak *et al.* (2002), Yildiz *et al.* (2005), and Laith and Najiah (2013).

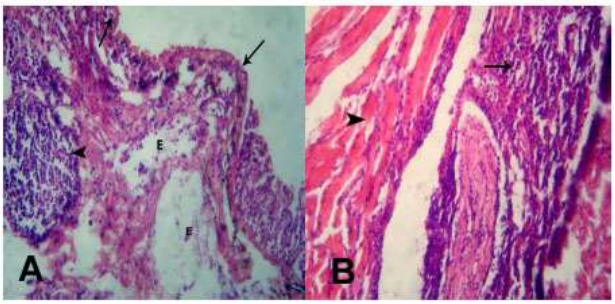

Fig. 1. (A) cross Skin section of *C. gariepinus* from group 1 showing area of ulceration (arrow) and aggregation of leukocytes (arrowhead)

(B) vertical showing aggregation of lymphocytes (arrow) and Zenker's necrosis in skeletal muscles (arrowhead) in the dermis. (H&E stain X 400).

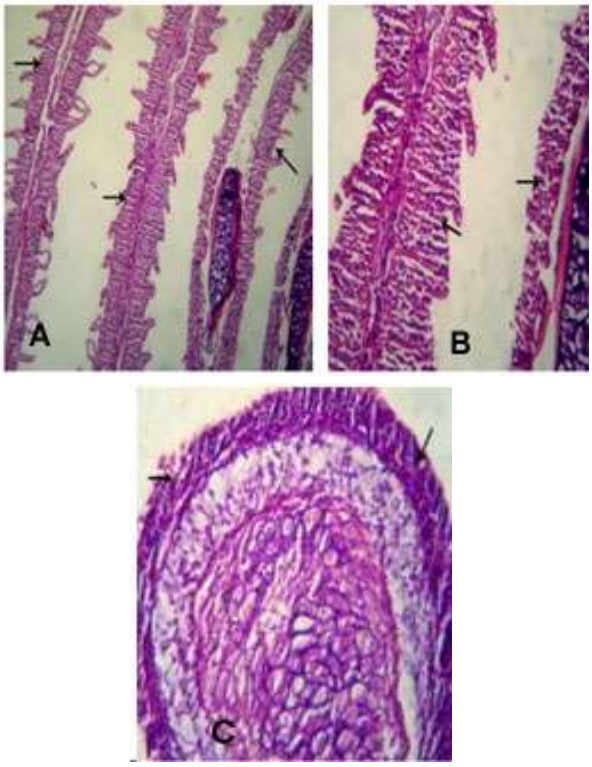

Fig. 2. vertical Gill section of *C. gariepinus* from group 1

A & B. showing extensive necrosis in the covering epithelium of the secondary lamellae with leukocytes aggregation (arrows). (H & E stain X 100 & 200).

C. showing necrosis and leukocytes infiltration in the epithelium of suprabronchial organ (arrows). (H & E stain X 400)

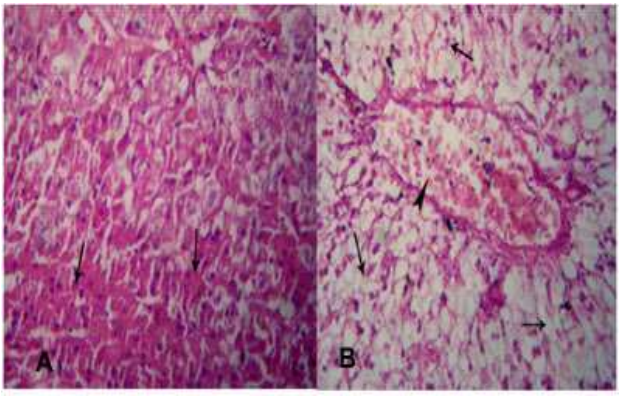

Fig. 3. Liver section of *C. gariepinus* from group 1

A. showing coagulative necrosis (arrows)

B. showing congestion (arrowhead) and diffuse vacuolation of hepatocytes (arrows). (H&E Stain).

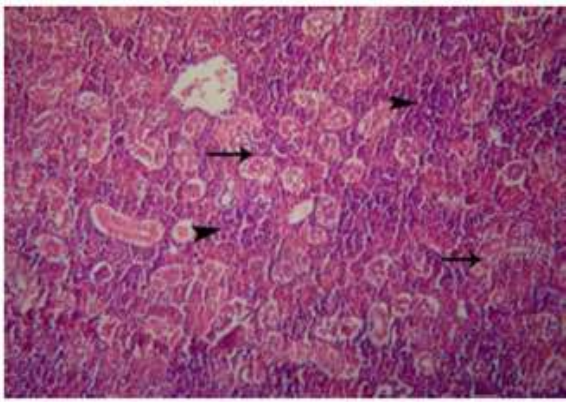

Fig. 4. Kidney section of *C. gariepinus* from group 1 showing severe coagulative necrosis in the renal tubules (arrows) and depletion of hemopoietic elements (arrowheads). (H & E stain)

Group 2: Catfish infected with *Aeromonas sobria* and treated with ciprofloxacin:

The skin shows intact epidermis with slight increase in mucous cells and few leukocytes infiltration extending to the dermis (Fig. 5). The underlying muscles were rarely suffering from necrosis. The gill showed congested blood capillaries and focally desquamated epithelium at the tips of the primary lamellae (Fig. 6). The liver showed hydropic degeneration and vacuolations in the hepatocytes (Fig. 7). Congestion of the hepatic blood vessels and haemorrhage were rarely detected. The kidney revealed focal vacuolations in the tubular epithelium with activation in the hemopoietic cells (Fig. 8). Hyper-cellularity in the glomerular tufts was noticed. Few PNLs were seen in the interstitial tissue. Cellular and hyaline casts were observed in the lumina of some renal tubules, this result corresponded with (Nouws *et al.*, 1988; Maisa, 1999) which indicated the ciprofloxacin increases the survival rate of fish challenged with *A. sobria* and in turn decrease the mortalities.

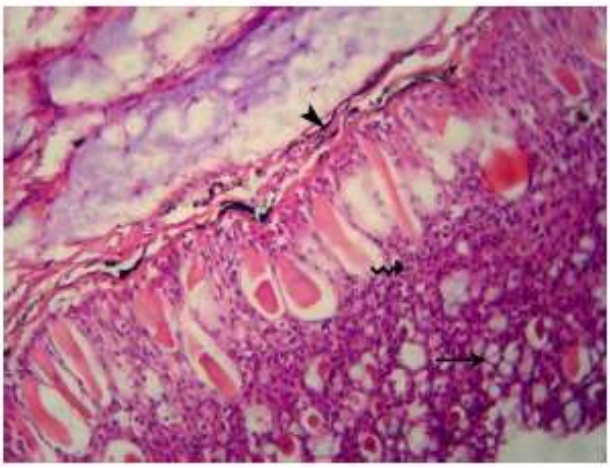

Fig. 5. cross Skin section of *Clarias gariepinus* from group 2 showing intact epidermis and slight increase in mucous cells, ballooning degeneration in the epidermal cells and few leukocytes infiltration. (H & E stain).

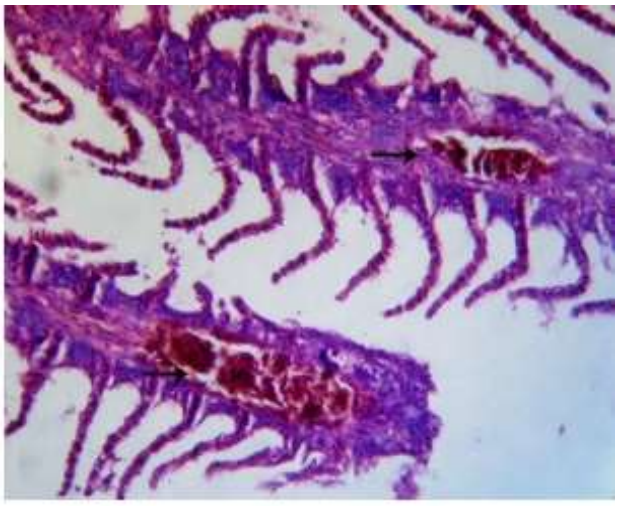

Fig. 6. vertical Gill section of *Clarias gariepinus* from group 2 showing congested blood capillaries (arrow) and primary lamellae. (H & E stain ×100).

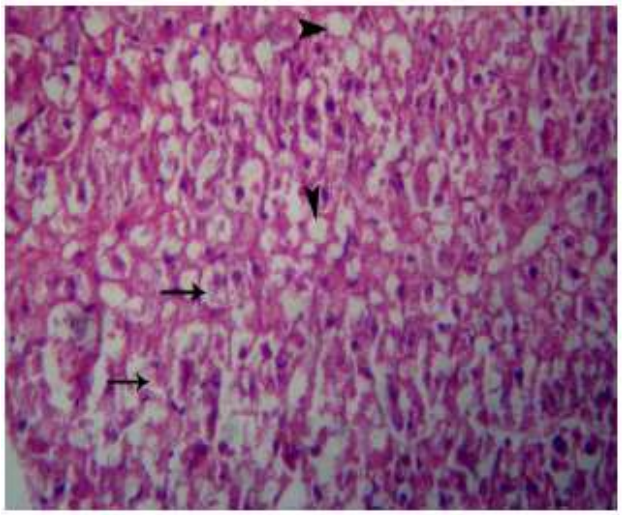

Fig. 7. Liver section of *Clarias gariepinus* from group 2 showing (A) hydropic degeneration and (B) vacuolations in the hepatocytes (arrow). (H & E stain × 400).

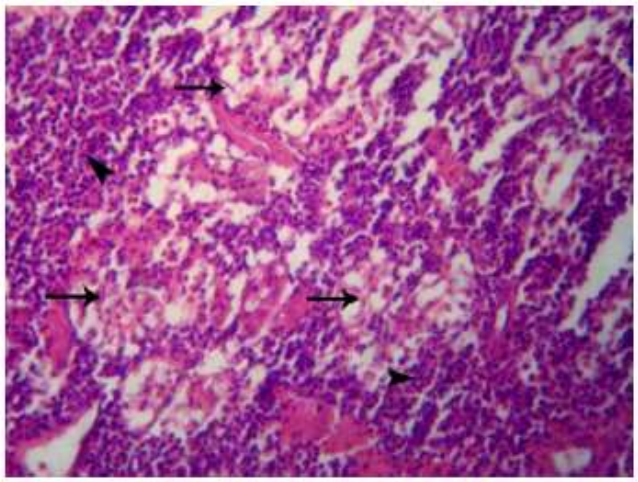

Fig. 8. Kidney section of *Clarias gariepinus* from group 2 showing focal vacuolations in the tubular epithelium (arrow) and activation in the hemopoietic cells (arrowheads). (H & E stain × 400).

Group 3: Catfish infected with *Aeromonas sobria* and treated with clove extract:

The lesions in this group were similar to those described in group 1 but they were milder when treated with clove extract. The skin showed extensive necrosis in the epidermis with intense lymphocytic infiltration. The remaining epidermal cells undergo spongiosis and ballooning degeneration with increased and hypertrophied mucous cells (Fig. 9 A). The epidermis, dermis and skeletal muscles were invaded by numerous granulocytes and few lymphocytes (Fig. 9 B). The skin was oedematous and the muscle bundles were degenerated and focally necrotic. The gill showed hypertrophy and hyperplasia of the mucous cells with fusion of the gill filaments was focally noticed with leukocytes infiltration (Fig. 10 A). Congestion of branchial blood vessels and extravasation of erythrocytes (Fig. 10 B). The lining epithelium of the secondary lamellae undergoes focal proliferation, frequent desquamation, focal destruction and lymphocytes infiltration (Fig. 10 C). The liver revealed congestion of hepatic sinusoids beside diffuse hydropic changes in the hepatocytes and few leukocytes infiltration (Fig. 11 A). Focal macro-vesicular steatosis and coagulative necrosis were also noticed in the hepatocytes (Fig. 11 B). The pancreatic acini were focally necrotic with depletion of zymogene-granular acini. The kidney showed focal areas of coagulative necrosis in the tubules with activation of the hemopoietic elements (Fig. 12). The glomeruli were destructed with multifocal areas of haemorrhage (Pérez and Lewis, 2006).

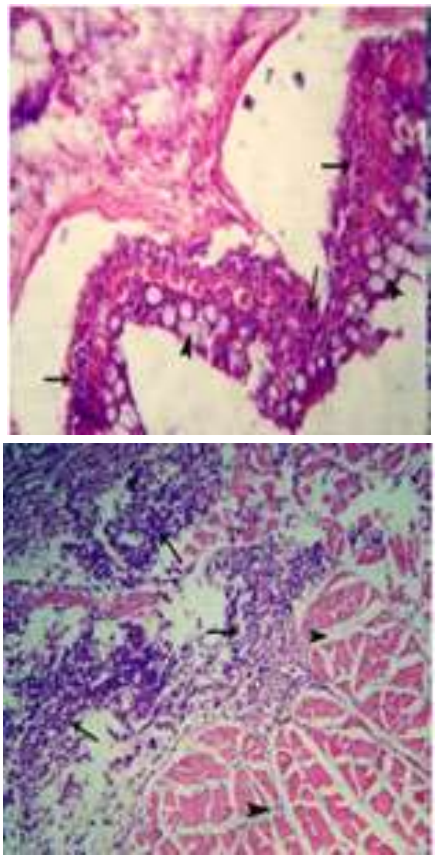

Fig. 9. V.S. Skin section of *Clarias gariepinus* from group 3

(A) showing extensive necrosis in the epidermis with intense lymphocytic infiltration (arrow) and increased mucous cells (arrowheads). (H & E stain × 400)

(B) showing intense aggregation of lymphocytes in the dermis (arrows) and among the skeletal muscles. (H & E stain × 200).

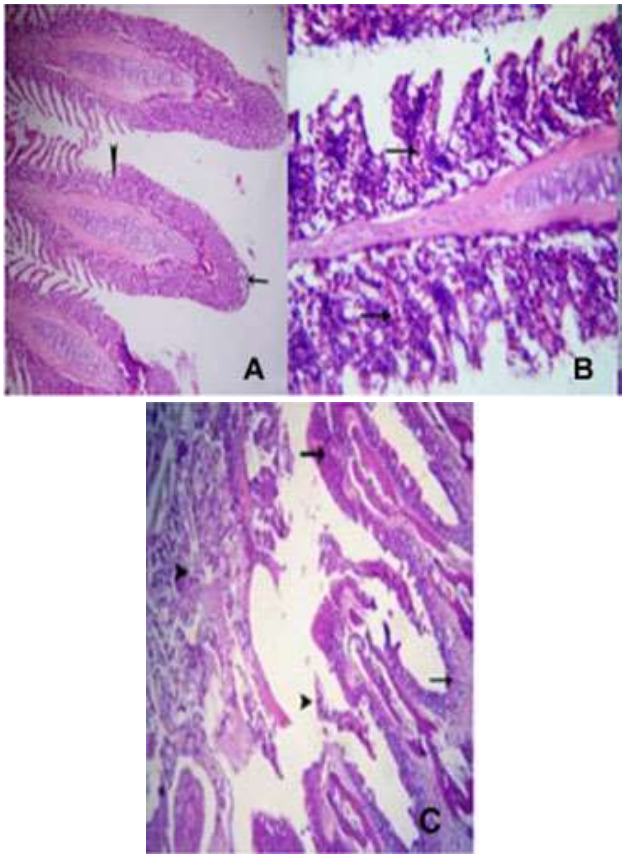

Fig. 10. Gill sections of *Clarias gariepinus* from group 3

(A) Showing proliferation of the epithelial covering of the secondary lamellae (arrow head) and leukocytic infiltration (arrow). (H & E stain × 100).

(B) Showing congestion and hemorrhage (arrows). (H & E stain × 200).

(C) Showing Gill rakers with severe inflammation, mucinous degeneration (arrows) and extensive desquamation in the lining epithelium and leukocytic infiltration (arrowheads). (H & E stain × 400).

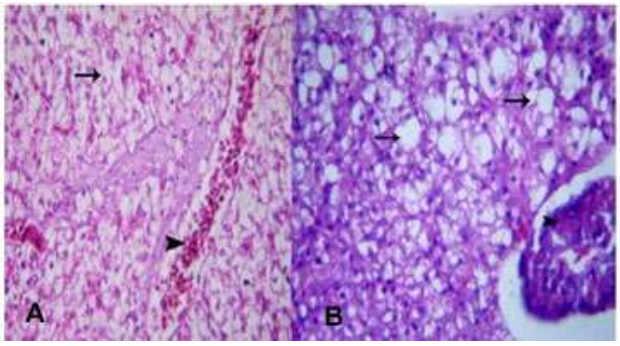

Fig. 11. Liver section of *Clarias gariepinus* from group 3

(A) Showing severe congestion (arrowhead) and vacuolations in the hepatocytes (arrows).

(B) showing macrovesicular steatosis (arrows). (H & E stain × 400).

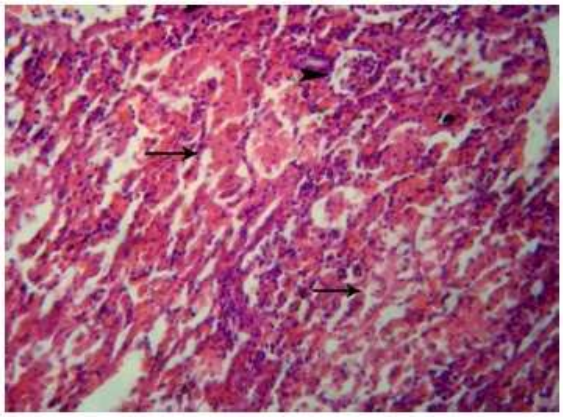

Fig. 12. Kidney section of *Clarias gariepinus* from group 3 showing focal coagulative necrosis in the renal tubules (arrows) and activation of the hemopoietic elements. (H & E stain × 400).

Group 4: Catfish infected with *Aeromonas sobria* and treated with the synergistic effect of ciprofloxacin and clove extract:

The skin showed intact epidermis with no evidence of erosion and ulceration while the dermis was infiltrated by few lymphocytes and the muscle fibres were widely separated by oedema (Fig. 13). The gills revealed mild proliferation of the covering epithelium particularly at the base of the secondary lamellae and few lymphocytes infiltration (Fig. 14). Congestion and haemorrhage are rarely visualized. The liver showed intact parenchyma with slight congestion, few interstitial and portal lymphocytes infiltration, focal hydropic degeneration and vacuolation of some hepatocytes (Fig. 15). The kidney was normal except few focal vacuolation in the tubular epithelium (Fig. 16). The lesions of fish infected with *A. sobria* and treated by

both ciprofloxacin and clove extract were ameliorated than those infected with *A. sobria* and treated by either ciprofloxacin or clove as described by Nascimento *et al.* (2000) and Deivasigamani (2008) concluded that infected catfish mortality is due to typical histopathological lesions such as necrosis and atrophy of hepatocytes, necrosis of sheathed arteries in the spleen and necrosis of renal tubules and glomeruli in the kidney has occurred.

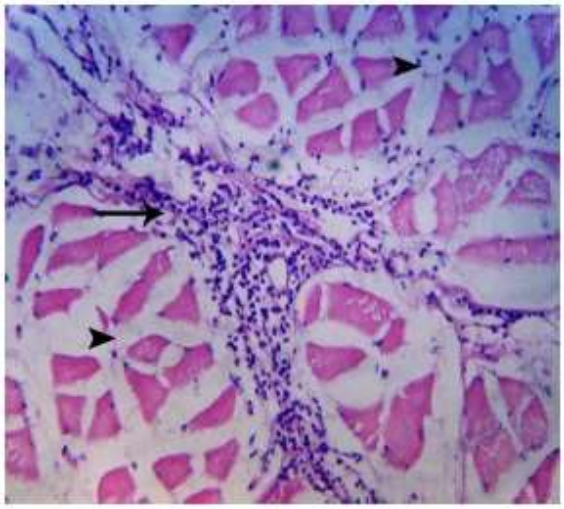

Fig. 13. Skin section of *Clarias gariepinus* from group 4 showing few lymphocytes infiltration among the muscle in the dermis (arrow). (H & E stain × 200).

Fig. 14. Gill section of *Clarias gariepinus* from group 4 showing mild proliferation of the covering epithelium at the base of the secondary lamellae (arrows). (H & E stain × 100).

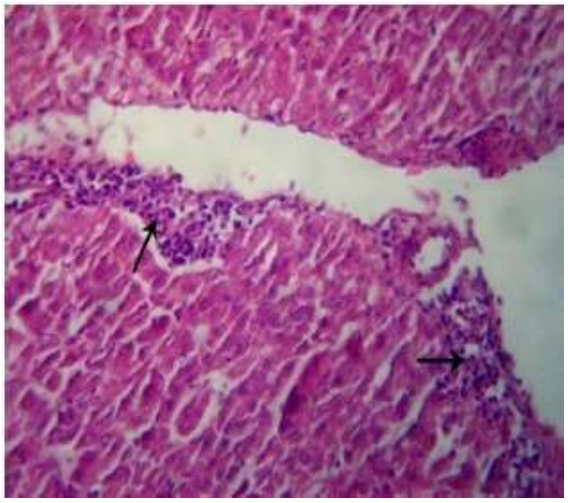

Fig. 15. Liver section of *Clarias gariepinus* from group (4) showing few lymphocytes in the portal area (arrows). (H & E stain × 400).

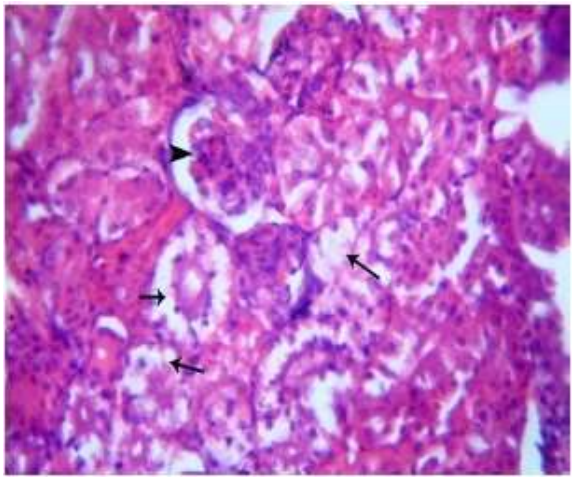

Fig. 16. Kidney section of *Clarias gariepinus* from group 4 showing mild vacuolation in the tubular epithelium (arrows) and congested glomerulus (arrowheads). (H & E stain × 400).

CONCLUSION:

This result shows both ciprofloxacin and clove extract was the best choice as an alternative bacterial therapeutic agent in infected *Clarias gariepinus* with *A. sobria.* Since *Aeromonas sobria* may threaten human health, transmission of the reduced susceptibility may have negative consequences for humans.

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التغيرات النسيجية المرضية في القراميط (*Clarias gariepinus*) المصابة التي يعالجها Ciprofloxacin ومستخلص القرنفل

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البكتريا المسببة للمرض أدت الى خسارة فادحة في الاستزراع المائي للأسماك وكذلك المخاطر الصحية على البشر. تم تحديد البكتريا الأعلى انتشارا من المجموعات المعزولة وهي*Aeromonas sobria* في سمك القراميط الأفريقي (*Clarias gariepinus*) على أساس الخصائص البيوكيميائية ونظام API-20E. تظهر أعراض مرضيه على الأسماك المصابة وتتضمن نزيف البؤري في نهاية الغشاء الخيشومي، واستسقاء بشكل عام وقرح على سطح الجلد. وتشمل الأعراض الداخلية للعدوى احتقان الأوعية الدموية في الكبد، ومناطق التليف في الكبد والتحلل المائي في الأنابيب الكلوية وفقا للفحص النسيجي. استخدمت التغيرات المرضية النسيجية على نطاق واسع كعلامات حيوية في تقييم صحة الأسماك المعرضة للملوثات. تم المعالجة بمستخلص القرنفل وكذلك مضاد حيوي Ciprofloxacin كلا على حده وجد ان المزيج من Ciprofloxacin ومستخرج القرنفل كانت الخيارات العلاجية الأفضل في*Clarias gariepinus* المصابة بواسطة *Aeromonas sobria*.