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ANTIBACTERIAL ACTIVITY OF SAND CRAB (EMERITA ASIATICA) HEMOLYMPH AGAINST NILE TILAPIA (OREOCHROMIS NILOTICUS) INFECTED WITH AEROMONAS SALMONICIDA AND EDWARDSIELLA TARDA

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ABSTRACT

Crustaceans have been identified as abundant sources of bioactive chemicals with promising uses in nutraceuticals and medicine. Since 1980s, the amount of fish produced worldwide has surged from 7% to nearly 40% and with a 6.3% share of the total global fish output, India now ranks second in the world for aquaculture production. Tilapias are the second most produced inland fish in the world, behind carps. The Nile tilapia is a species that is commonly cultivated because of its resilience to a variety of environmental circumstances, however one of the most significant elements influencing the success of Nile tilapia production is bacterial infections. Therefore, the hemolymph from *Emerita Asiatica*, a sand crab, was tested for its antibacterial properties. A sample of hemocoel from *the Emerita Asiatica* crab was taken, and the growth inhibition at various zones was used to examine the hemolymph's anti-bacterial properties. It was noticed that the Relative Percentage Survival (RPS) for the single and double immunisation groups against *A. salmonicida* and *E. tarda* were 60% and 100%, respectively, while for the combined group against both diseases, it was 34% and 100%. On healthy control subjects, a histological examination was performed to look at the structural changes in several organs, such as the kidney, liver, gills, and muscles. The treated fish displayed far fewer symptoms and reduced cellular damage to the organs, demonstrating the hemolymph's antibacterial action. Hence the present study revealed that *Emerita Asiatica's* hemolymph is a prospective source of very effective antibacterial agents and may be employed as a readily available natural immunostimulant material to combat bacterial infections in aquaculture.

***IndexTerms*: Antibacterial, Hemolymph, *Emerita asiatica, Edwardsiella tarda, Aeromonas salmonicida,* Histopathology.**

I. INTRODUCTION

The Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758), is a commonly cultivated species as it develops and reproduces in a variety of environmental circumstances and can withstand stress brought on by handling (Tsadik and Bart 2007). In many tropical and subtropical nations across the world, it is extensively practiced (Lin et al. 2008). It is an ideal fish for culture because to its quick growth rates, strong tolerance to challenging environmental circumstances, rapid feed conversion, simplicity of spawning, disease resistance, and favourable customer acceptability (Coimbra and Henriques 2005).

An estimated 179 million tonnes of fish were produced globally in 2018, with a total first-sale value of USD 401 billion, of which 82 million tonnes, worth USD 250 billion, were produced through aquaculture (Liti et al. 2005).India presently ranks second in the world for aquaculture output, contributing 6.3% to the overall fish production (FAO 2018). This is mostly due to the effective use of "induced fish breeding," which provides the farmers with high-quality fish seeds for cultivation throughout the whole nation. More than 1,500 hatcheries were built by India around the country, producing more than 32 billion carp fry (FAO 2015).The freshwater aquaculture is mainly dominated by Indian major carps, namely catla (*Catlacatla*), rohu (*Labeorohita*), and mrigal (*Cirrhinus mrigala*) (Marx et al. 2020). They contribute a majority of the total Indian aquaculture production. In 1987, the Nile tilapia was introduced to India for aquaculture. Nile tilapia contributes a total of more than 7.17% to the output of inland fish. Tilapias are the

second most produced inland fish in the world, behind carps. (Singh and Lakra 2006; Ridha 2006).The Nile tilapia is a species that is commonly cultivated because of its resilience to a variety of environmental circumstances, however illnesses are one of the most significant variables determining the success of Nile tilapia production (Tsadik and Bart 2007). *Aeromonas hydrophila* and *A. sobria*-caused hemorrhagic septicemia, *Aeromonas salmonicida*-caused furunculous, and *Flavobacterium branchiophilum*-caused bacterial gill illness all cause significant losses to the aquaculture industry (Govind et al. 2012). Crustaceans are said to have peptide defences against microorganisms, according to popular belief. The antibacterial activities of hepatopancreas and lobster plasma from *Homarus americanus* were discovered in 1972 (Stewart and Zwicker 1972; Ramesh et al. 2009). In order to combat the bacterial diseases that are severely harming the aquaculture industry's bottom line, the current effort focuses on examining the hemolymph's possible antibacterial action.

The large-scale aquaculture environments have led to an increase in antibiotic resistance in bacteria that may be harmful to fish and the surrounding environment. According to the hemolymph research of *O senex senex* and *Liagore Rubromaculat*, crabs are a fantastic source of bioactive proteins with a variety of antibacterial and antioxidant capabilities (Sumalatha et al. 2016).Potential novel antibiotic sources include the hemolymph of sand crabs. In wealthy nations, the hunt for antimicrobial compounds has clearly taken a new turn (Bulet et al. 1999). The hemolymph of Sand crabs must be properly screened in order to discover bioactive chemicals because no adequate research on the antibacterial activity of the species have been conducted. The goal of the current experiment was to examine the antibacterial properties of an *Emereita asiatica* sand crab hemolymph extract and to offer early evidence that the hemolymph does indeed have antimicrobial, primarily antibacterial, properties.

II. MATERIALS AND METHODS

2.1 Experiment Animal Collection

The Sand Crabs, *Emerita asiatica* were collected from the Injambakkam coastline ECR, Chennai-600115, Tamil Nadu, India. The Sand Crab had a mean weight of 15g.

Fingerling tilapia (1.0-3.0 cm and 0.28-1.3 g on average) were sourced from kelambakkam, Tamil Nadu. Fingerlings were inspected for signs of disease before being chosen for the study.

2.2 Bacterial isolation and identification:

Aeromonas salmonicida and *Edwardsiella tarda* pure strains were obtained from Aquatic Animal Health Laboratory, C. Abdul Hakeem College. Strains of bacteria were confirmed by Catalase, Oxidase, Motility test (Reiner 2010; Shields and Cathcart 2010; Shield and Cathacart 2011).The media that were used for isolation includes TCBS (Thiosulphate citrate bile salt) Agar, Blood Agar, SCDA Agar, Nutrient Agar, Nutrient Broth and Mueller Hinton Agar Medium.

2.3 PCR-based 16S rRNA gene amplification

The 16S ribosomal RNA (16S rRNA) genes of *A.salmonicida* and *E.tarda* were amplified by polymerase chain reaction (PCR) using a primer set developed based on the nucleotide sequences of *A.salmonicida* and *E.tarda*, respectively (Yanagi and Yamasato 1993). The sequence of the primers and the appropriate annealing temperatures are provided in Table 1, and the optimal PCR conditions are indicated in Tables 2 and 3.

Table 1: Optimized PCR parameters for amplification of 16s rRNA of *A.salmonicida* and *E.tarda.*

PCR parameters	**Temperature (ºC)**	**Minutes**
i) Denaturation	95°C	5 minutes
ii) 30 cycles of Denaturation Annealing Elongation	95ºC 58°C 72°C	40 seconds 40 seconds 50 seconds
iii) Final Extension	72°C	10 minutes

Table 2 : Primers used for detection of 16S rRNA of *E. tarda.*

Primer	Product size	Sequence (5'-3')	Annealing temperature
16s rRNA – F 16s rRNA – R	427bp	TAC TTC AAG CCA CGA CACC AGC CCA GAA CGA CGA CAG	58°C

Table 3 : Primers used for detection of 16S rRNA of *A.salmonicida.*

Primer	Product size	Sequence (5'-3')	Annealing temperature
16s rRNA – F 16s rRNA – R	421bp	GGC TGA TCT CTT CAT CCT CAC CCC CAG AGT GAA ATC TAC CAG CGG TGC	58°C

2.4 Collection of hemolymph:

3-5 animals were used to collect hemolymph from the crabs' hemocoel by passing a sterilized 2 ml syringe through the arthroidal membrane. The hemolymph was then collected and diluted to a final level of 2 ml. The hemolymph was collected using an ice-cold citrate EDTA buffer to prevent hemocyte degranulation and coagulation (Soderhall and Smith 1983).

Fig.1: Hemolymph collection

2.5 Antibacterial activity by agar well diffusion method:

The agar well diffusion test is used to evaluate plant or microbial extracts antibacterial activity (Chavez et al. 2021).The broth culture inoculum was dipped using a sterile cotton swab in this study. To remove additional inoculum, the cotton swab was rotated against the tube's inner wall above the fluid level. Inoculated with *Aeromonas salmonicida*. Swabs were used to inoculate the plate's agar surface three times, rotating the plate 60° between swabs. To dry the inoculum, the petri dish cover was replaced and the plate was left at room temperature for 5–10 minutes. Five 8 mm wells were cut into the agar plate using a sterile well cutter. Each well received 100 µl of *Emerita asiatica* haemolymph at 5, 10, 15, and 20 µl concentrations. In the other well, 100µl saline was introduced. Two hours were allotted for solution diffusion. At 28 °C for 24-48 hours, the plates were incubated. Antibacterial activity was measured by measuring the zone of inhibition around the well. The same method was used for the other bacterium *E.tarda.*

2.6 Experimental setup and design

Experimental pathogenicity was conducted based on standard protocols (Ducklow et al. 1980; Egidus 1987; Hameed et al. 1996).The healthy Tilapia fingerlings were maintained at the rate of five fish per tub with each having a capacity of 5 liters. Artificial fish meal was given to the animals twice daily Twelve groups of 60 *O.Niloticus* in total were created (5 fish each) and were maintained in 12 tubs respectively, containing freshwater at 29°C ± 1. They were further divided into 4 batches, each batch containing 3 tubs. The experiment was conducted in duplicates and a duplicate was maintained for every single tub, (Table 4).

Table 4: Experimental setup.

Batch (a) ***A.salmonicida***	Negtive control	Positive control	Immunized and Challenged
Batch (b) ***E.tarda***	Negative control	Positive control	Immunized and Challenged
Batch (c) ***A.salmonicida***	Negative control	Positive control	Double Immunized and Challenged
Batch (d) ***E.tarda***	Negative control	Positive control	Double Immunized and Challenged

Two times each day, animals were examined for clinical symptoms of sickness and death. Animal carcasses were removed. Affected tissues and organs were isolated and removed from these dead animals and were stored to perform histopathological analysis.

2.7 Hemolymph immunization

Hemolymph extract was injected through intramuscular injection using a sterilized1ml insulin syringe, near the dorsal fin; the hemolypnh concentration was 60g. Each fish was injected manually under aseptic laboratory conditions and one syringe was used per batch, which was sterilized periodically after every single injection using Bunsen flame and then immediately cooled before proceeding (Liu et al.2006; Hurton et al. 2009).

2.8 Bacterial Challenge

The intramuscular injection of bacteria, at a concentration of 60 µl, was used to challenge untreated and treated (immunised) Tilapia fingerlings (Soto et al. 2016; Situmorang et al. 2014). The fish were separated into three groups: group I was a positive control, group II was a treated or challenged group, and group III was a normal control. Every batch had a duplicate. The Tilapia fingerlings were put in 5 litre plastic water tubs containing freshwater with constant aeration. The tubs were covered with plastic nets to prevent external intervention and also to prevent the fishes from accidentally jumping out.

Fig.2 : Intramuscular injection of bacterium into the fishes

After immunization,the fishes were challenged with *A.salmoicida* and *E.tarda* by intramuscular injection at a concentration of 0.1 ml of E. tarda cells (1.2 X 10’ CFU/mL perfish) in batches B and D and 0.1 ml of *A. salmonicida* cells (1.5×106 CFU/mL per fish) in batches A and C. The same bacteria was also administered to control fish. Calculations were made for RPS and cumulative percent mortality (CPM).

2.9 Histopathological investigation of Tilapia fingerlings

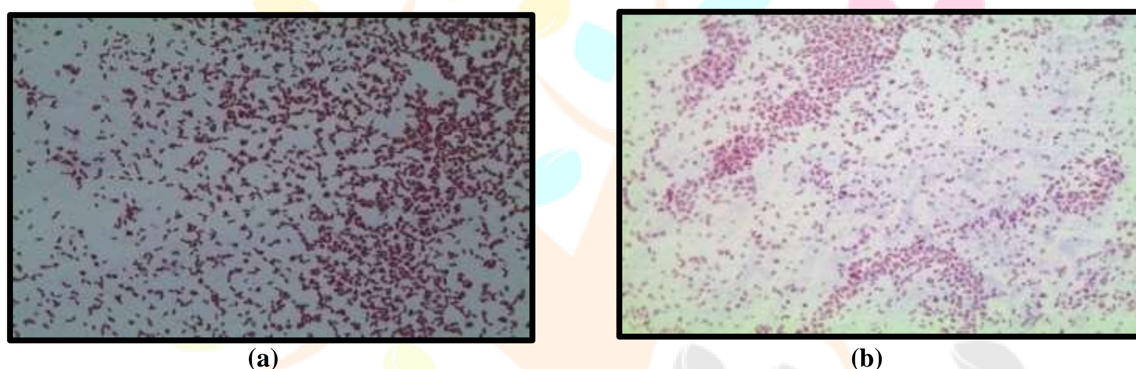
The kidney, liver, and gills of experimentally infected Tilapia fingerlings were removed for histological analysis. Additionally, the histology of these organs in normal fish was studied for this study. Fixative with 10% neutral buffered formalin (NBF) was used to keep organs in good condition. After that, the organs such as the kidney, liver, and gill were sliced and preserved in screw cap bottles containing the fixative in accordance with a conventional approach that was reported in prior study (Ma et al. 2016). After an initial fixation duration of 48 hours with 10% neutral buffered formalin (NBF) fixative, the materials were then moved to 70% alcohol to be preserved.

Fig.3: Samples stored in eppendorf tubes

III. RESULTS AND DISCUSSION

3.1 Gramstaining

Bacteria obtained from the SCDA's colony morphology agar media were identified as *A. salmonicida and E.tarda*. Both types of bacteria share characteristics of being Gram-negative, curved rod-shaped, motile bacteria.



(a) **(b)**

Fig.4 : (a) *Aeromonas salmonicida* and (b) *Edwardsiella tarda*

3.2 Biochemical Analysis

The Enzyme oxidase and catalase are produced by both *A. salmonicida* and *E. tarda*. Both are motile bacteria**.**

Table 5 : Biochemical Analysis of *A. Salmonicida,* and *E. tarda.*

TestAnalysis	**Bacteria – *A. salmonicida* and *E. tarda***
Motility	+
Catalase	+
Oxidase	+
Hemolysis	+

A modest quantity of growth from each culture was placed on clean microscope slides. Then the smear was blended with a few drops of H2O2. The presence of catalase enzyme was demonstrated by the rapid evolution of O2 during bubbling.

3.3 PCR Analysis

3.3.1 *Aeromonas salmonicida*

By employing primers unique to bacteria, PCR analysis was used to validate *Aeromonas salmonicida*, and the findings are displayed in Fig.6. The results revealed that positive samples displayed the emergence of a noticeable band of a PCR amplified product (421 bp), whereas negative samples did not show this same band.

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3.3.2 *Edwardsiella tarda*

Using bacteria-specific primers, PCR analysis confirmed *Edwardsiella tarda*. The results are in Fig.7. The findings revealed that positive samples had a clear band of PCR amplified product (427 bp), whereas negative ones did not.

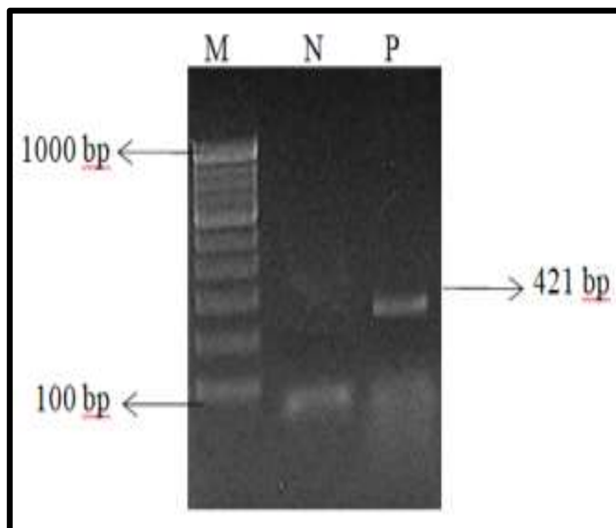


Fig.5 : *Aeromonas salmonicida* was confirmed by PCR utilising a set of bacterial-specific primers in the current investigation. Lane – M 100 DNA marker; Lane N – Negative control, Lane P–Inoculum of *A. salmonicida*

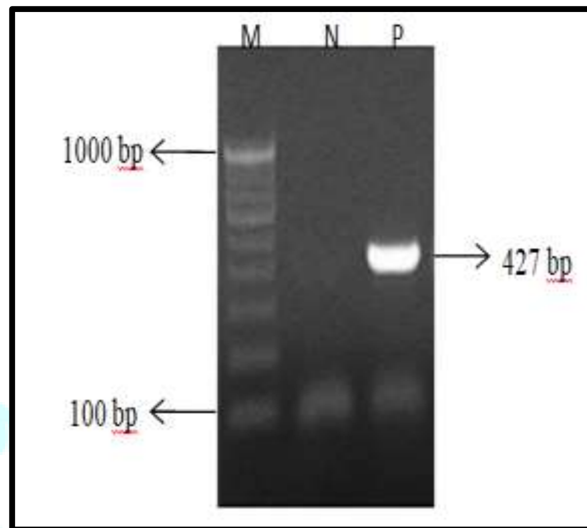


Fig.6 : Employing a bacterial-specific primer set, PCR was performed to validate the presence of the *Edwardsiella tarda*. Lane – M 100 DNA marker; Lane N – Negative control, Lane P –Inoculum of *E.tarda*

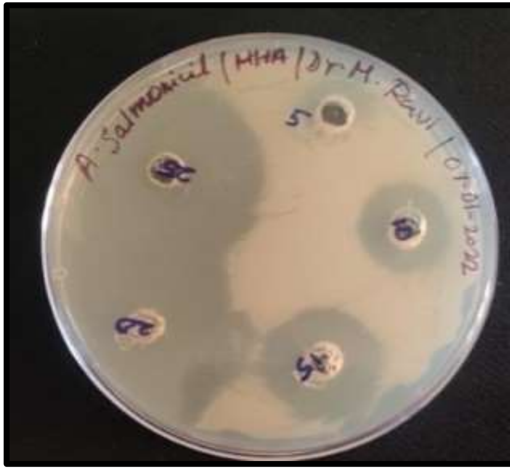
3.4 Zone of inhibition

Table 6: Mean inhibition zones of Hemolymph (sandcrab) against *A.salmonicida.*

Sl. no	**Sample**	**Against *Aeromonas salmonicida***					
		5µg	**10µg**	**15µg**	**20µg**	**25µg**	**P.C**
1.	Hemolymph	0.6cm	0.66cm	0.88cm	1.1cm	3.33cm	6.45cm

Table 7 : Mean inhibition zones of Hemolymph (sandcrab) against *E.tarda.*

Sl. no	**Sample**	**Against *Edwardsiella tarda***					
		5µg	**10µg**	**15µg**	**20µg**	**25µg**	**P.C**
1.	Hemolymph	0.77cm	0.95cm	1.17cm	1.23cm	0.60cm	4.73cm



(a) **(b)**

Fig. 7 : Growth inhibition, (a) ***A. salmonicida and (b) E. tarda***

3.5 Test for Pathogenicity

Aeromonas salmonicida challenged Tilapia fingerlings demonstrated odd behaviour including moving around corners, relaxing at the bottom, and floating vertically. The clinical signs of infected Tilapia fingerlings include skin ulcer seen in dorsal side.

Fig.8 : Infected fish showing visible symptoms

Challenged by *Edwardsiella tarda*, Tilapia fingerlings also demonstrated sluggishness and odd behaviour, including as wandering in circles, taking breaks at the bottom, and swimming slowly. Lesions on the body's surface and skin ulceration were discovered on the dorsal side of infected Tilapia fingerlings.

3.5.1 Batch A

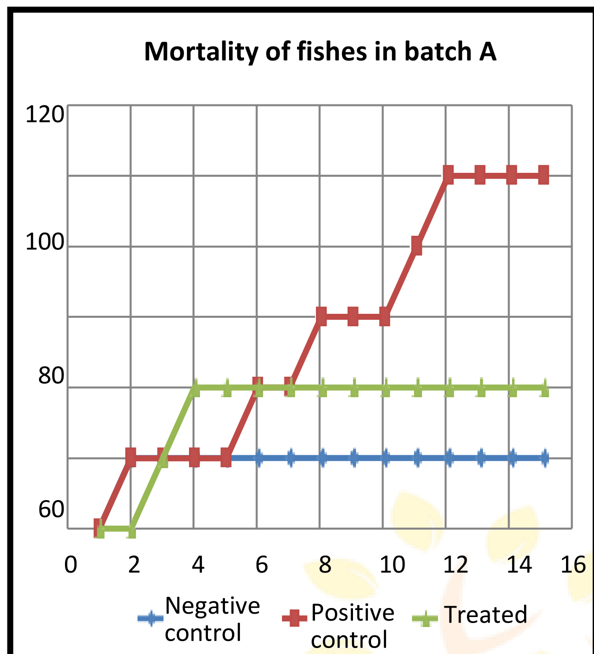


Fig.9 : Cumulative mortality of Tilapia fingerlings of batch A, observed till 15 dpi.

Table 8 : Relative percentage survival(RPS) treated and non-treated fishes.

Fish injected with *Aeromonas salmonicida*	Cumulative percentage mortality(Dead fish/total injected fish)	RPS
Negative control	20.00% (1/5)	-
Positive control	100.00% (5/5)	
Treated fish	40.00% (2/5)	60%

3.5.2 Batch B

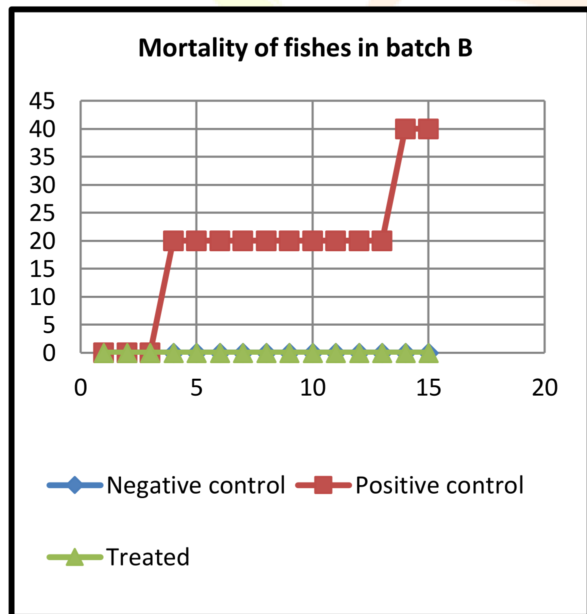


Fig.10 : Cumulative mortality of Tilapia fingerlings of batch B, observed till 15dpi.

Table 9 : Relative percentage survival (RPS) treated and non-treated fishes.

Fish injected with *E. tarda*	Cumulative percentage mortality(Dead fish/total injected fish)	RPS
Negative control	0.00% (0/5)	-
Positive control	40.00% (2/5)	-
Treated fish	0.00% (0/5)	100%

3.5.3 Batch C

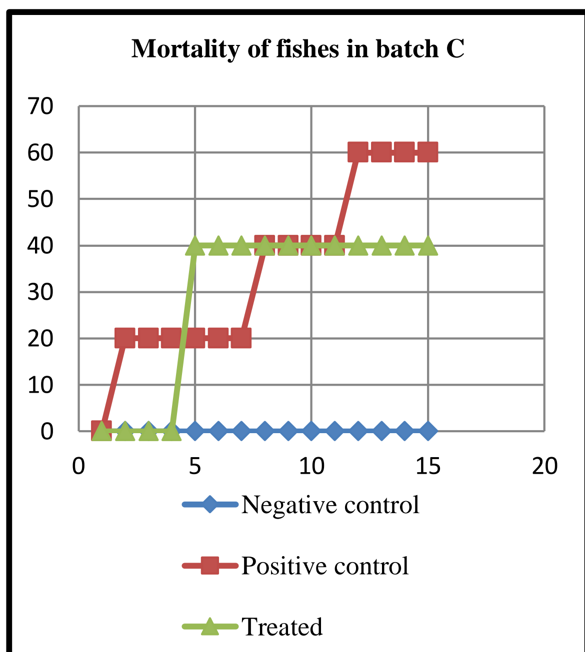


Fig.11 : Cumulative mortality of Tilapia fingerlings of batch C, observed till 15dpi.

Table 10 : Relative percentage survival (RPS) treated and non-treated fishes.

Fish injected with *Aeromonas salmonicida*	**Cumulative percentage mortality(Dead fish/total injected fish)**	**RPS**
Negative control	0.00% (0/5)	-
Positive control	60.00% (3/5)	-
Treated fish	40.00% (2/5)	34%

3.5.4 Batch D

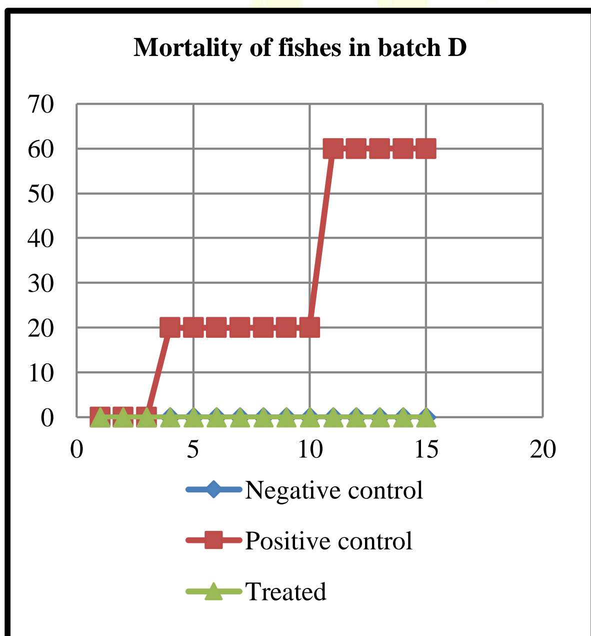


Fig.12 : Cumulative mortality of Tilapia fingerlings of batch D, observed till 15dpi.

Table 11 : Relative percentage survival (RPS) treated and non-treated fishes.

Fish injected with *E. tarda*	**Cumulative percentage mortality(Dead fish/total injected fish)**	**RPS**
Negative control	0.00% (0/5)	-
Positive control	60.00% (3/5)	-
Treated fish	0.00% (0/5)	100%

3.6. Histopathological studies: Batch A against *A.salmonicida* (Single Immunization Dose)

3.6.1.Gills

Control Tilapia had normal lamellar epithelial organisation. Branchitis at the lamellae and hyperplasia were both visible in infected gills. Normal gill lamellae were visible in treated gills. The epithelium covering the gills showed modest growth, especially at the base of the secondary lamellae, where there was hemocytic infiltration and congestion (Fig.no 13).

3.6.2 Liver

Hepatocytes from Tilapia fish that had been experimentally infected showed a lack of coordination in the tissue. Hepatocyte vacuolation and hemorrhage were brought on by bacterial intervention. Treated Tilapia showed normal structure with mild hemocytic inflammation (Fig. 14).

3.6.3 Kidney

In experimentally infected Tilapia fingerlings, the A. salmonicida bacteria induced haematological atrophy, hyaline droplet degeneration with vacuolations, and glomerular degeneration in the kidneys. Treated Tilapia showed normal renal tubules, glomeruli, and hematopoitic tissue(Fig. 15).

3.6.4 Muscle

The tissues of infected Tilapia fingerling fish showed a lack of coordination. The tissues experienced significant deterioration and hemorrhage as a result of bacterial interaction. Tilapia fingerlings that had been treated had normal structure with just some necrosis and inflammation (Fig. 16).

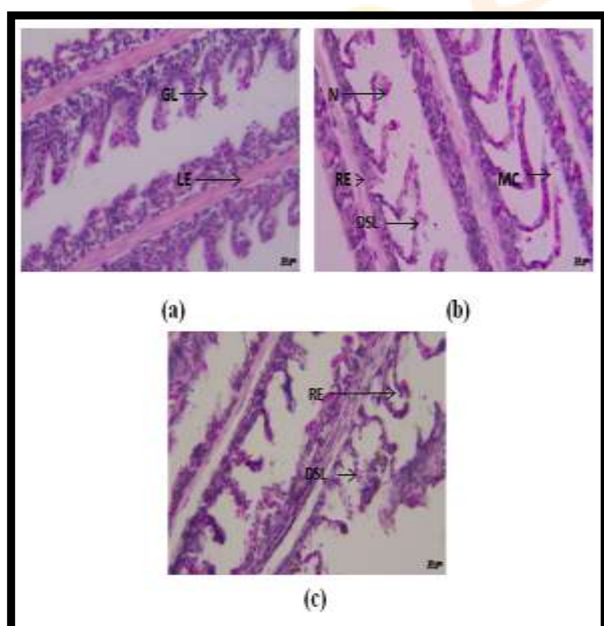


Fig.13 : Histology of Tilapia gills stained with H&E. Magnification 40X. (a) Negative control , (b) Positive control, (c)Treated

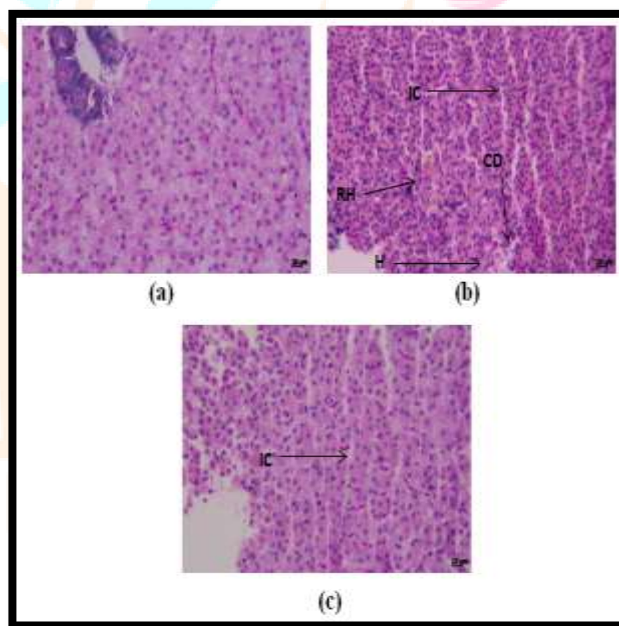


Fig. 14 : Histology of Tilapia Liver stained with H&E. Magnification 40X. (a) Negative control, (b) Positive Control, (c) Treated

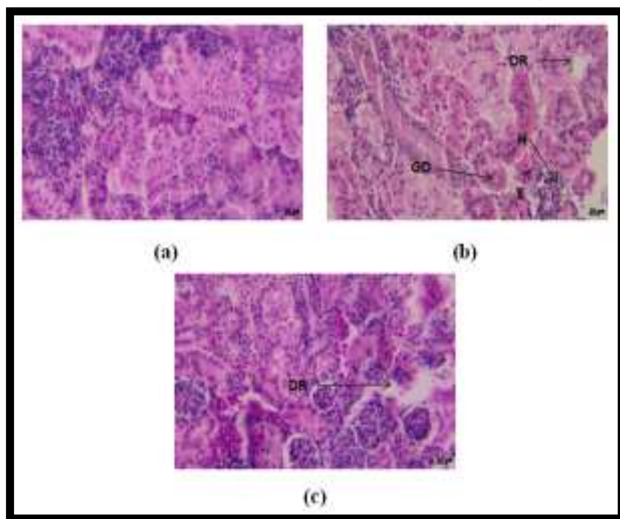


Fig.15 : Histology of Tilapia Kidney stained with H&E. Magnification 40X. (a) Negative control , (b) Positive control, (c) Treated.

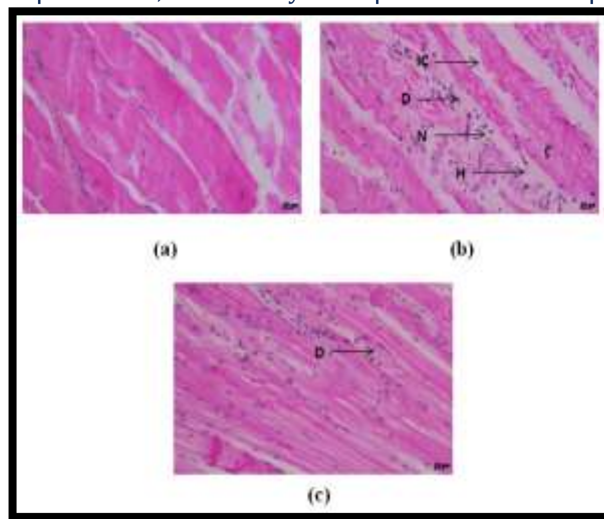


Fig.16: Histology of Tilapia muscle stained with H&E. Magnification 40X. (a) Negative control , (b) Positive control, (c) Treated.

3.7 Batch (B) against *E.tarda* (Single Immunization Dose)

3.7.1Gills

The Gill lamellae that were infected had significant degeneration and necrosis. The treated gill lamellae displayed some necrosis and degeneration (Fig. 17).

3.7.2 Liver

Hepatocytes from the infected liver displayed necrosis and minimal granulomatous lesions. There was barely any necrosis in the treated liver (Fig. 18).

3.7.3 Kidney

The kidney of the Tilapia that had been given treatment had normal tubular organisation. Glomeruli and hematopoietic tissue were positioned properly (Fig. 19).

3.7.4 Muscle

Muscle tissue that was infected had significant necrosis and degeneration. The treated muscle tissue exhibited some necrosis and degeneration (Fig. 20).

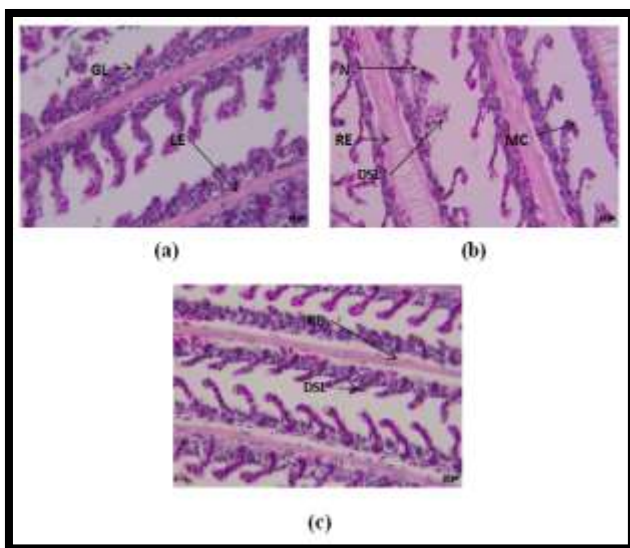


Fig.17 : Histology of Tilapia gills stained with H&E. Magnification 40X. (a) Negative control , (b) Positive control, (c) Treated.

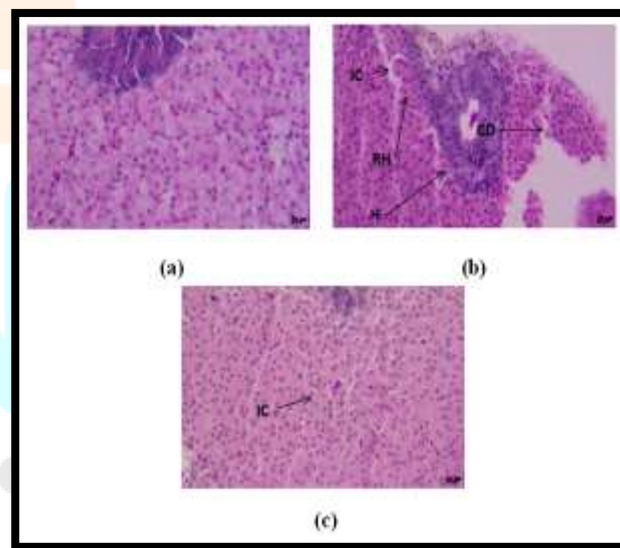


Fig.18 : Histology of Tilapia liver stained with H&E. Magnification 40X. (a) Negative control , (b) Positive control, (c) Treated.

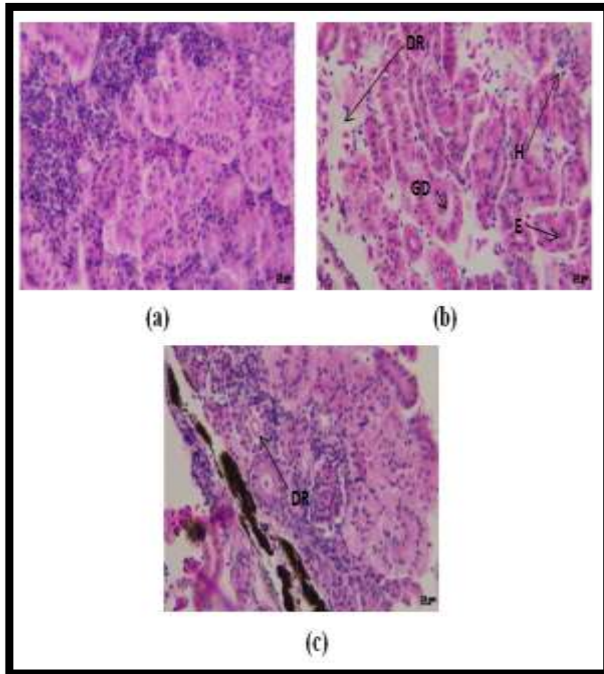


Fig.19 : Histology of Tilapia Kidney stained with H&E. Magnification 40X. (a) Negative control , (b) Positive control, (c)Treated.

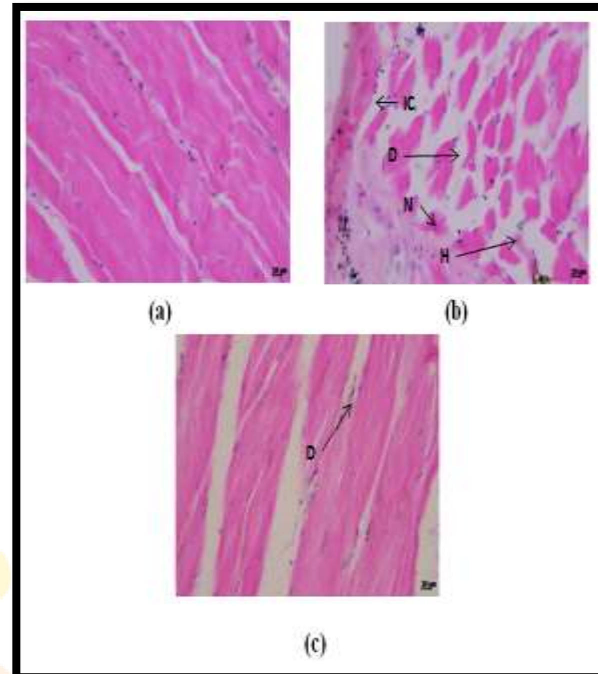


Fig.20 : Histology of Tilapia muscle stained with H&E. Magnification 40X. (a) Negative control , (b) Positive control, (c) Treated.

3.8 Batch (C) against *A. Salmonicida* (Double Immunization Dose)

3.8.1 Gills

Branchitis and hyperplasia were visible in infected gills' lamellae. The gill lamellae in treated gills seemed normal. The gills showed some growth at the epithelium's surface, especially at the base of the secondary lamellae where there was also congestion and hemocytic infiltration (Fig. 21).

3.8.2 Liver

The incoordination of the tissue was visible in the hepatocytes of the experimentally infected Tilapia fish. Vacuolation and hemorrhage in the hepatocytes were brought on by bacterial intervention. Tilapia that had been treated had normal structure and just minimal hemocytic irritation (Fig. 22).

3.8.3 Kidney

The kidneys of experimentally infected Tilapia fingerlings showed glomerular degeneration, hyaline droplet degeneration with vacuolations, and hematological shrinkage. Furthermore, the treated Tilapia's kidney's renal tubule configuration was normal (Fig. 23).

3.8.4 Muscle

The tissues of infected Tilapia fingerling fish showed a lack of coordination. The tissues experienced significant deterioration and hemorrhage as a result of bacterial interaction. Tilapia fingerlings that had been treated had normal structure with a light necrotic inflammation (Fig. 24).

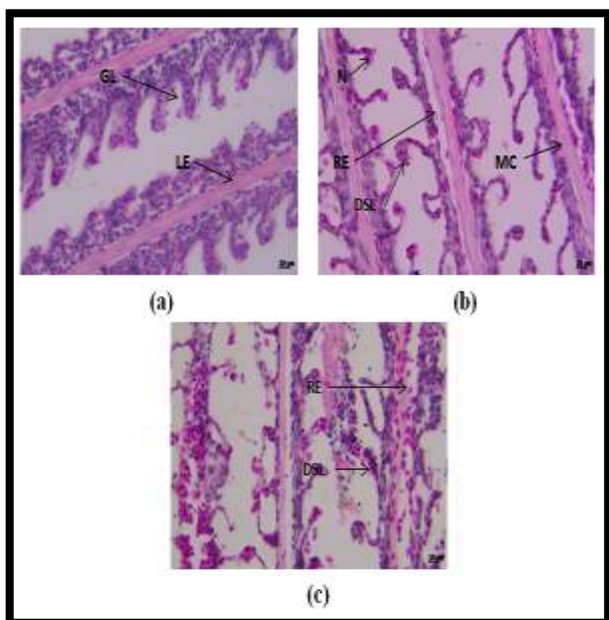


Fig.21 : Histology of Tilapia gills stained with H&E. Magnification 40X. (a) Negative control, (b) Positive control, (c) Treated.

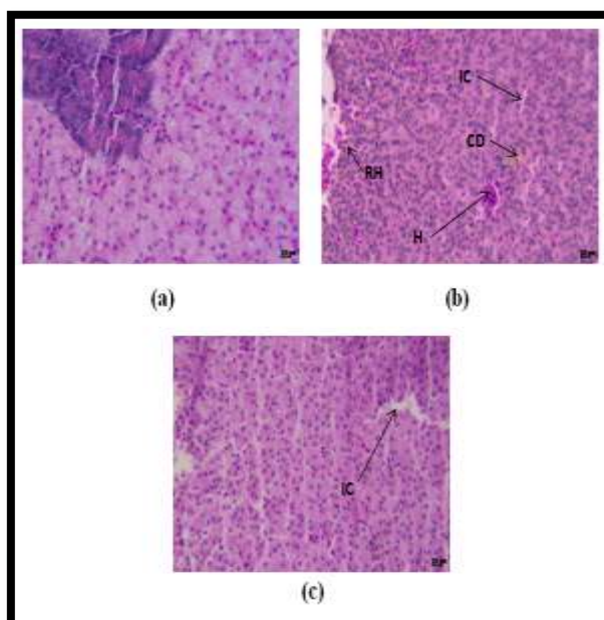


Fig.22 : Histology of Tilapia liver tained with H&E. Magnification 40X. (a) Negative control , (b) Positive control, (c)Treated.

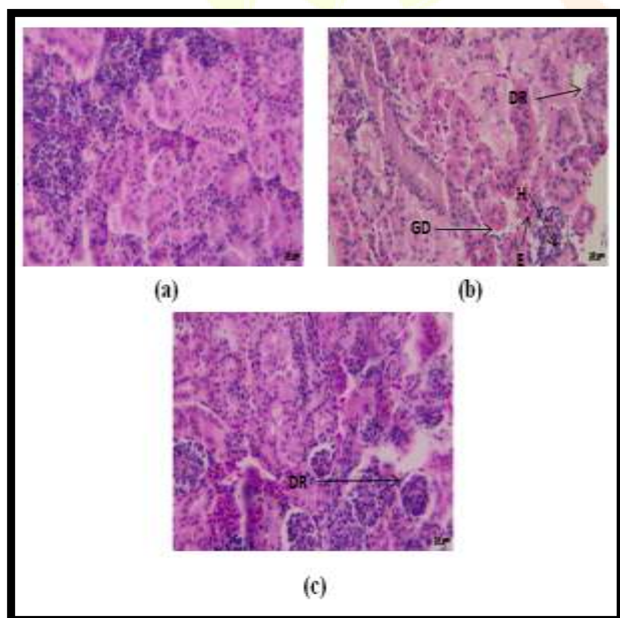


Fig.23 : Histology of Tilapia Kidney stained with H&E. Magnification 40X. (a) Negative control , (b) Positive control, (c)Treated.

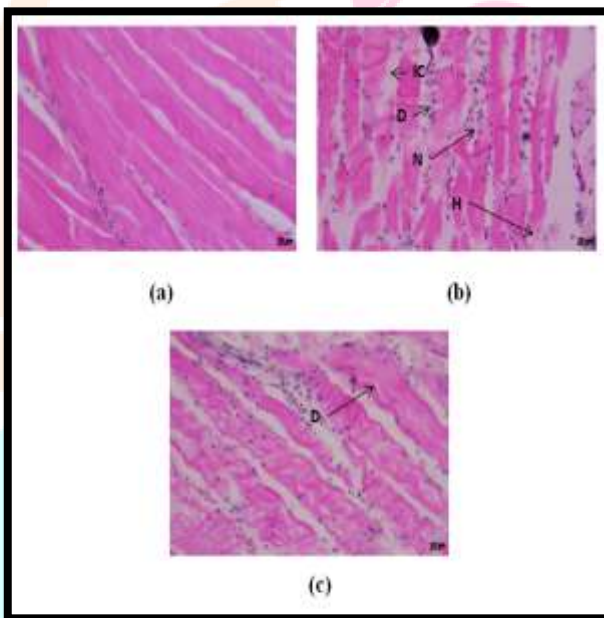


Fig.24 : Histology of Tilapia muscle stained with H&E. Magnification 40X. (a) Negative control , (b) Positive control, (c)Treated.

3.9 Batch (D) against *E.tarda* (Double Immunization Dose)

3.9.1 Gills

The Gill lamellae that were infected had significant degeneration and necrosis. The treated gill lamellae displayed some necrosis and degeneration (Fig. 25).

3.9.2 Liver

Infected liver hepatocytes had necrosis and minimal granulomatous lesions.The treated liver had minor necrosis (Fig. 26).

3.9.3 Kidney

The kidneys of experimentally infected Tilapia fingerlings showed glomerular degeneration. Furthermore, the treated Tilapia's kidney's renal tubule configuration was normal (Fig. 27).

3.9.4 Muscle

Muscle tissue that was infected had significant necrosis and degeneration. The treated muscle tissue exhibited some necrosis and degeneration (Fig. 28).

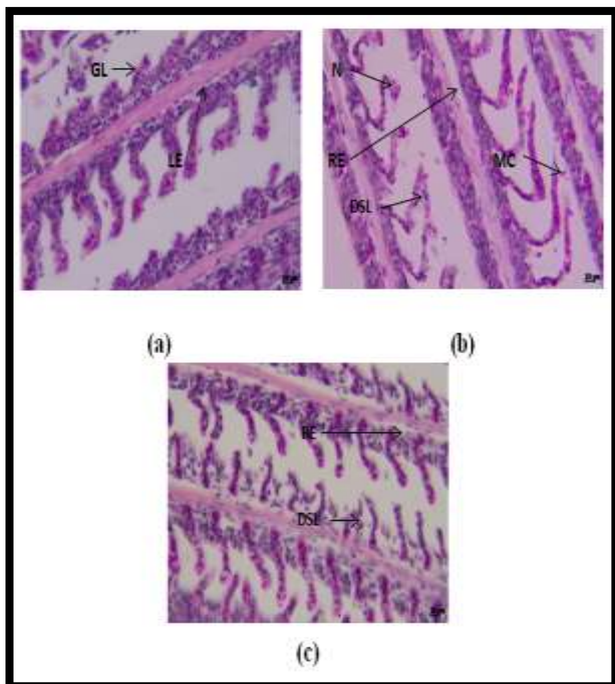


Fig.25 : Histology of Tilapia gills stained with H&E. Magnification 40X. (a) Negative control , (b) Positive control, (c) Treated.

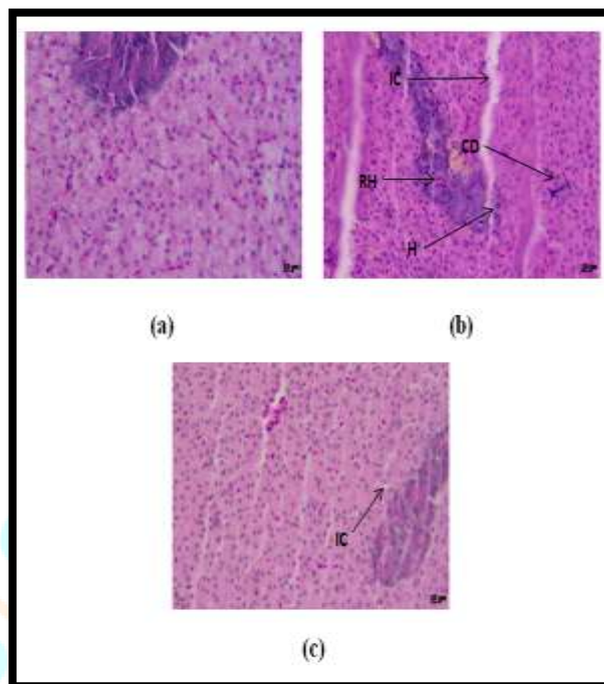


Fig.26: Histology of Tilapia liver stained with H&E. Magnification 40X. (a) Negative control , (b) Positive control, (c) Treated.

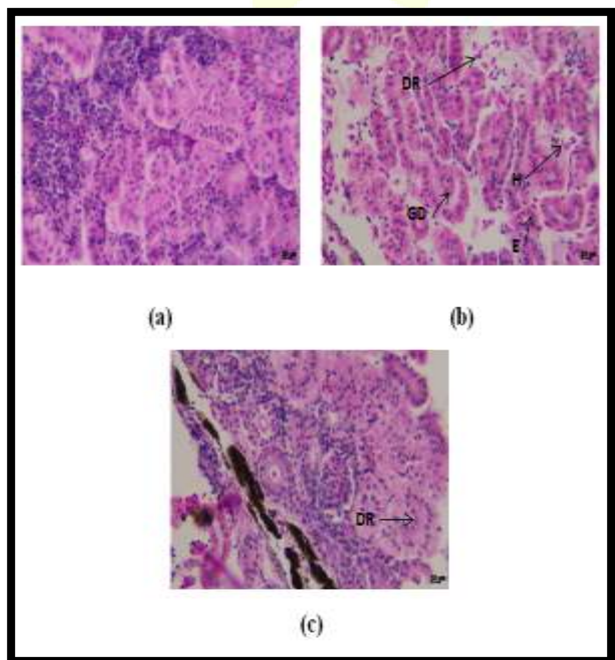


Fig.27 : Histology of Tilapia Kideny stained with H&E. Magnification 40X. (a) Negative control , (b) Positive control, (c) Treated.

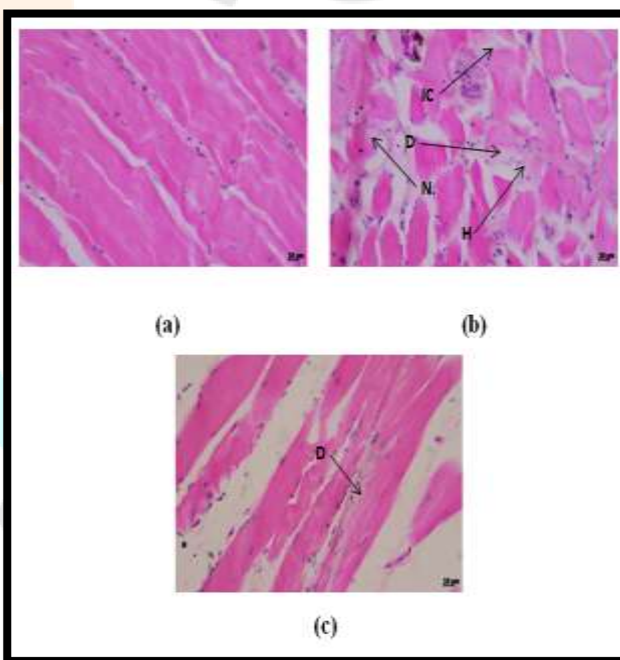


Fig.28 : Histology of Tilapia muscle stained with H&E. Magnification 40X. (a) Negative control , (b) Positive control, (c) Treated.

IV. Discussion

Bacteria were obtained from infected fish and tentatively identified as either *Aeromonas salmonicida* or *Edwardsiella tarda* based on their unique biological, biochemical, physiological, and structural properties. A further piece of evidence was uncovered by polymerase chain reaction (PCR) studies of the 16S rRNA gene. Hemolymph was collected from the *Emerita asiatica* crab's hemocoel and its antibacterial properties were evaluated by measuring the zone of inhibition of bacterial growth. After first immunising four groups of fish (Nile Tilapia) with *Emerita asiatica* hemolymph, two of the groups received a booster immunisation. Among the three groups, one was challenged with *A.salmonicida* and two with *E.tarda* after receiving either a single or double dose of vaccination. Infected fish exhibited irregular swimming, the development of skin lesions, and a listless demeanour. In addition, skin ulcerations were found. The results showed that the RPS for *A.salmonicida* and *E.tarda* were 60% and 100% in the single immunisation group, and 34% and 100% in the double immunised group. Histopathological analysis was performed on fish at three different stages of the experimental infection and treatment process to compare the structural changes in the kidney, liver, gills, and muscles. Most severely impacted were the gills. A number of spots on the affected gill were damaged. There was branchitis and hyperplasia seen. Evidence of infiltration by leucocytes was seen. The treated fish had far less symptoms and reduced organ cell damage, suggesting the hemolymph's anti-bacterial efficacy. This research proved that the hemolymph of the *Emerita Asiatica* plant might be a rich source of effective antibacterial compounds.

4.1 Conclusion

Antibiotic resistance poses a severe threat to human health, with the problem growing and becoming global. Resistance to the antimicrobial agents used in aquaculture has increased in many countries in recent years. A wide range of antimicrobial compounds are now being used in aquaculture. The current study looks at a natural immunostimulant with antibacterial characteristics that may aid in the fight against bacterial infections in aquaculture. In this study, the crude hemolymph of the sand crab (*Emerita Asiatica*) was evaluated for its antibacterial activities in this study. The results of this research imply that *Emerita Asiatica's* hemolymph is a potential source of very powerful antibacterial compounds and may be used as an easily accessible natural immunostimulant to battle bacterial infections in aquaculture. Future study might focus on extracting, separating, and identifying the bioactive components. To ascertain their chemicals composition and evaluate their potential as novel medications, the bioactive compounds must be refined further. It may have the potential to replace conventional antibiotics with a more effective alternative and may also be effective against antibiotic-resistant bacterial strains.

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