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EXTRACELLULAR PRODUCTS OF *Aeromonas hydrophila*

AND ITS EFFECTS ON INDIAN WHITE PRAWN,

Penaeus indicus H.Milne Edwards

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PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

MASTER OF FISHERIES SCIENCE
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CENTRAL INSTITUTE OF FISHERIES EDUCATION
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BY

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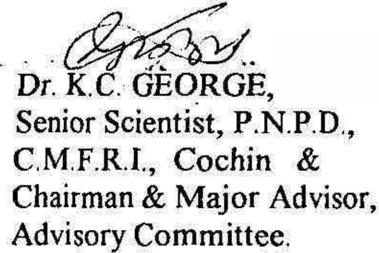
CERTIFICATE

Certified that the dissertation entitled **"EXTRACELLULAR PRODUCTS OF *Aeromonas hydrophila* AND ITS EFFECTS ON INDIAN WHITE PRAWN, *Penaeus indicus* H. Milne Edwards"** is a bonafide record of work done by **Ms. RADHIKA GOPINATH** under our guidance at the Central Marine Fisheries Research Institute during the tenure of her M.F.Sc. (Mariculture) Programme of 1997-'99 and that it has not previously formed the basis for the award of any other degree, diploma or other similar titles or for any publication.



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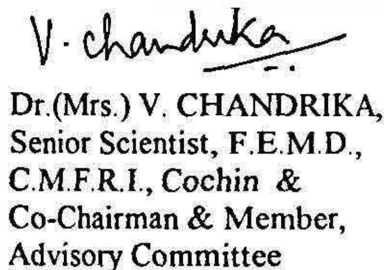

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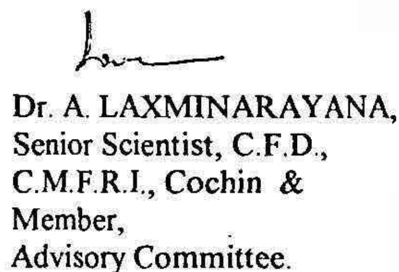

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DECLARATION

I hereby declare that this dissertation entitled " **EXTRACELLULAR PRODUCTS OF *Aeromonas hydrophila* AND ITS EFFECTS ON INDIAN WHITE PRAWN, *Penaeus indicus* H.Milne Edwards.**" is based on my own research and has not previously formed the basis of any degree, diploma, associateship, fellowship or other similar titles or recognition.

RADHIKA GOPINATH

COCHIN-682014.

July, 1999.

Dedicated to
my Parents &
Rajesh

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सारांश

इंडिकस झींगे में रोगकारक बाक्टीरिया एरोनोमस हाइड्रोफिला के प्रभाव पर किया अध्ययन का सारांश इस प्रकार है । परीक्षण केलिए पहले बाक्टीरिया से एक्स्ट्रासेल्लुलार प्रोडक्ट माने ई सी पी की तैयारी की । ई सी पी सान्द्रक की तैयारी केलिए बाक्टीरिया का पालन प्रयोगशाला में ही किया था । ट्रिप्टिक सोया अगर प्लेटों में सेल्लेफीन प्लेट लगाके पाले गए इस बाक्टीरिया संवर्धन का उपकेन्द्रण (सेन्ट्रिप्फ्यूगेरन) और निस्यंदन करके सूपरनेटन्ट निकाल दिया था । ई सी पी में प्रोटीन प्रोफाइल का निर्धारण एस डी एस-पेज के जरिए किया । पेनिअस झींगों में ई सी पी सान्द्रकों के प्रभाव समझने के लिए पेनिअस झींगे के छह समूहों का अनुरक्षण किया जिनके पाँचों में विभिन्न सान्द्रतावालों ई सी पी का प्रयोग किया और एक को कन्टौल ग्रूप के रूप में इसके प्रयोग किए बिना रख लिया । ई सी पी के प्रयोग किए झींगा समूहों में उनके अभिलक्षणों और अशनरीतियों में द्रुत व्यक्तियान दिखाए दिए । क्लोम व उदर के भागों में कालिमा, उपांग, बाह्य कवच और पूँछ में गलन आदि रोग लक्षण भी दिखाए पडे । सब से अधिक ई सी पी सान्द्रक के प्रयोग किए झींग समूह के कवच नरम हो गए थे । प्रत्येक सान्द्रक में हुए मृत्यु दर का भी निर्धारण किया । रुधिरलसिका के रुधिरकोशों की संख्या का आकलन सभी समूहों में किया गया । प्रत्येक सान्द्रकों के झींगा समूहों के रुधिरकोशों की संख्या में अन्तर दिखाई पडी । हेपाटोपान्क्रियास व पेशियों में ऊतकविज्ञानीय अध्ययन चलाया । हेपाटोपान्क्रियास के कोशों में अपोटोसिस व मृत्युता दिखाये पडे ; ट्यूब्यूल के बाह्य ऊतकों में छीलन दूसरा लक्षण था । पेशियों में अलगाव, नाश व मृत्युता दिखाई पडी । रुधिरलसिका व ऊतकों में दिखाए पडे नाशक लक्षण एयरोनोमस बाक्टीरिया से बनाए ई सी पी से उत्पादित विषाक्त घटकों से हुए हैं ।

PREFACE

I. PREFACE

The near exhaustion of the commercially important species, which fetch global export value and enormous market demand, have caused sudden decrease in the catches from wild. Diminishing returns and depleting resources has made Man to ponder on the development of an alternative to exhilarate the production through aquaculture. The developments scaled by aquaculture has become an answer to the multitudes of demands, like reducing returns from capture sector, global food production, export earnings, rural development, and problem of unemployment.

Aquaculture is no doubt a solution to the existing problem of augmentation of protein, utilization of natural resources and conservation of natural fishery resources. Aquaculture which in the past decade was mainly a traditionally practice has been upgraded to a capital intensive industry in the recent era because of the midas touch of scientific, technical and economic advancements.

Among the commercially important marine organisms, prawns occupy the pinnacle position regarding great demand in the international market and feasibility of culture in marine and brackishwater sectors. In view of both,

capture and culture fisheries, the Indian white prawn, *Penaeus indicus* deserves a predominant position. Considering the growing need to elaborate our food production it is high time that some strategies are enforced to promote the culture of *Penaeus indicus* by improving upon existing practices, introduction of new techniques and effective management of viable culture systems.

The increasing returns from semi-intensive and intensive prawn culture operations have attracted investment from greedy industrialists, who have not paid any attention to the horrors of environment damages. Not only such culture operations cost irreparable damages to the environment but due to improper management practices resulted in disease in the farms themselves. Irrational stocking rate, improper feeding, ineffective husbandry practices have caused the rapid spread of disease in the culture systems like wild fire.

In this study emphasis is laid on the infection caused by *Aeromonas hydrophila* in *Penaeus indicus* and its virulence agents. However, information on *Aeromonas* infection in marine sector is much inadequate. Attempt has been made to study the effects using histology as a tool to unlock the critical problem and to reveal the probable effects of the infection on the normal histological architecture of the animal.

Objectives of the present investigation were:

- To obtain extracellular products (ECP) of *Aeromonas hydrophila* and quantify its total protein content.
- To enumerate the haemolymph changes in *Aeromonas* infections.
- To visualize the histopathological changes caused by ECP in *Penaeus indicus*.

Significance of ECP:

- ✓ The ECP form the virulent determinants of the bacterium which coupled with environmental stressors contribute to the development of disease.
- ✓ The ECP were able to cause the same lesions of the disease when injected into the test animal.
- ✓ The ECP can serve as immunogens in the susceptible stock when exposed to predetermined doses.

INTRODUCTION

II. INTRODUCTION

The sky rocketing progress of penaeid shrimp culture due to the genesis of scientific techniques and technical contributions has been lined by the dark clouds of disease manifestations. Development of disease has been assigned to virulence of the pathogen, degree of infectivity and immune status of the host.

Disease to a great extent in recent years has hampered aquaculture production. The health's of aquaculture animals are under constant threat from bioagressors such as viruses, bacteria, parasites and fungi. Indeed it is generally recognised that disease problems follow the development of techniques for animal production.

To increase production, the aquaculturists have been resorting to intensification with high stocking densities, excessive feeding etc. A natural consequence of this is the deterioration of the water quality and the outbreak of various diseases. In recent years, there is hardly any shrimp industry in the world that has not suffered a serious loss due to disease outbreak·

Generally bacterial disease in a culture system is found in conjunction with other disease or reflect an outcome of a breakdown in the ecological balance

within the culture system. Many of the bacteria considered to be responsible for disease are normal inhabitants of the marine environment and are generally regarded as opportunistic pathogens. Among the bacterial groups of prime importance in causing disease in shrimps are *Vibrio* spp, *Pseudomonas* spp, and *Aeromonas* spp. Fresh water, especially with a high organic load is normally considered to be the natural habitat of *Aeromonas hydrophila*. Although not commonly associated with disease in marine sector it has caused losses in *Chryophrys major* in South East Asia by extension from wounds to the spinal cord. There are also reports of *Aeromonas* infection in Penaeid species.

The pathogenesis of *Aeromonas hydrophila* is multifactorial. A majority of virulence factor works in concert to contribute to overall virulence of this bacterium. In the study of pathogenic mechanisms of the bacterium *Aeromonas*, there has been much interest in the role of extracellular substances as toxins or aggressins. In our conditions fall in salinity in culture ponds due to frequent rains will lead to change in microflora of the water.

Disease of penaeid shrimps have been reviewed a number of times by Overstreet (1973), Sindermann (1974), Johnson (1975), Lightner (1977,1983). Almost all the stages of cultured penaeid shrimps especially larval, Post larval and juvenile stages are affected by different groups of bacteria, majority are of a

secondary etiology. (Sindermann, 1971; Lightner, 1977, 1983, 1988; Johnson, 1976; Sindermann, 1990). In most of the cases, bacterial infection in penaeids is due to gram negative, motile, oxidase positive and fermentative rods (Barkate, 1972; Lewis, 1973: AQUACOP, 1977; Lightner, 1977; Zheng, 1986). Most of the disease outbreaks are caused by *Vibrio* spp., *Pseudomonas* spp., *Aeromonas* spp., *Flavobacterium* spp. (Lightner 1977, 1983, 1988; Sindermann, 1990).

Many of the penaeid shrimp disease are related to the type of culture system viz.; extensive, semi-intensive and intensive; the animal, its environment and the invasive pathogens (Tareen, 1982; Lightner, 1983; Liu, 1989). *Vibrio* spp., *Pseudomonas* and *Aeromonas* have been identified as chitinoelastic bacteria from crustaceans in general and penaeid shrimps in particular. Chitinoelastic bacteria form a normal part of the microflora of both living and dead crustaceans and hence they are opportunistic pathogens (Brisou *et al.*, 1965; Lightner, 1977, Lewis *et al.*, 1982; Soni, 1986).

Aquatic environments are the primary habitat of *Aeromonas* spp. (Hazen, 1978). The wide distribution of *Aeromonas* species is probably a consequence of its high capacity to adopt to different aquatic environments. It seems that these bacteria have basically survival capacity in different aquatic environments

(Kersters *et al*, 1996). The survival of the pathogen for a long period of time in the environmental waters is thought to contribute to an outbreak of the disease; however, infectivity of the pathogen surviving in the water has not been elucidated (Chowdhury and Wakabayashi, 1988). Rehman, *et al* 1977 studied the survival of *Aeromonas hydrophila* in a variety of waters containing different salt concentrations.

REVIEW OF LITERATURE

III. REVIEW OF LITERATURE

1. ***AEROMONAS* IN SHRIMPS.**
2. **IN OTHER MARINE ORGANISMS.**
3. **EXTRACELLULAR PRODUCTS (ECP) OF *AEROMONAS*.**

Aeromonas hydrophila is a common bacterial pathogen found in variety of aquatic environments. The development of experimental and commercial culture of penaeid shrimp has been accompanied by the occurrence of diseases of infectious and non-infectious etiologies. A number of diseases caused by bacteria have been reported from penaeid shrimp. While bacterial diseases of primary bacterial etiology have been observed in penaeids, the majority is of a secondary etiology. In every reported case of bacterial infections in penaeid shrimp; motile, Gram negative, oxidase positive, fermentation rods have been isolated. Most isolates have been *Vibrio* spp. and other Gram negative rods including *Aeromonas* spp. and *Pseudomonas* spp. *Aeromonas* spp. was also found to be part of the normal microflora of these animals.

Aeromonas spp. has been isolated from the haemolymph and tissues of cultured penaeid shrimp. It was associated with shell disease consisting of melanized erosions of cuticle on body, gill or appendages and Haemocytic enteritis. Few isolates of *Aeromonas* spp. were obtained from Black Death and Blue shrimp syndrome. *Aeromonas* spp. has also been reported in cultured penaeid shrimp in association with septicemia of juvenile and adult, brown spot disease and infected wounds.

Yasuda *et al.*, (1980) investigated the bacterial flora in the gut of wild cultured prawns, *Penaeus japonicus* and the isolated strains were classified to genera. Prawns that grew poorly had large numbers of *Aeromonas* and *Vibrio* groups in the digestive tract.

According to the Lewis *et al.*, (1982); *Pseudomonas piscicida*, *Aeromonas formicans* and *Flavobacterium* spp. were involved in the aggregation of hatchery reared *Penaeus stylirostris* larva.

Singh *et al.*, (1985) enumerated the total viable aerobic heterotrophic bacteria associated with egg, nauplius, zoea, mysis and postlarvae of *Penaeus indicus* and sea water in a hatchery system. The bacteria isolated included *Aeromonas* spp.

Chitinoelastic bacteria, *Vibrio* and *Aeromonas* were isolated from soft-shelled prawns (Baticadas *et al.*, 1986). Owens *et al.*, (1992) reported the presence of black spot caused by *Aeromonas hydrophila* in cultured marine *Penaeus esculentus*.

Hameed and Rao (1993) observed the gradual increase in the bacterial flora of penaeid groups from egg to post larvae. *Vibrio* was found to be dominant in all stages followed by *Aeromonas* and *Pseudomonas.* In the rearing medium also, *Aeromonas* was encountered. The strains which were obtained from an epizootic septicaemia of *Penaeus chinensis* were identified as *Aeromonas hydrophila* and *Aeromonas caviae* (fan *et al*, 1994).

Huang *et al.*, (1994) investigated the microflora in tank waters of *Penaeus monodon* culture at different salinities. *Aeromonas* sp. occupied dominant position in 20 ppt. After 50 days, *Aeromonas* sp. increased in 10 and 20 ppt.

The bacterial strains observed in diseased prawn samples belonged to *Vibrio* spp and *Aeromonas* spp. (Yang *et al.*, 1994, Yang, 1995). Chang *et al.*, (1996) isolated from the hepatopancreas of *Penaeus monodon* and *Penaeus chinensis*, several strains of *Aeromonas salmonicida* and *Aeromonas* sp.

2. IN OTHER MARINE ORGANISMS.

Aeromonas sp. was also found in other marine organisms. *Aeromonas hydrophila* was identified from pompano, *Trachinotus carolinus* and striped bass, *Morone saxatilis* that were reared in brackish water ponds in the Southeastern United States (Hawke, 1976). Riqueleme *et al.*, (1976) reported that the bacterial population of dying larvae of *Argopecten purpuratus* (Scallop) comprised of only *Aeromonas hydrophila* which proved to be resistant to most of the chemotherapeutic agents tested. Coexisting infections with *Vibrio anguillarum* were found in both species. Similar infections with the two pathogens had been reported from the cod, *Gadus morhua,* from Denmark associated with ulcer disease (Larsen and Jenson; 1977). *Aeromonas* sp. had been implicated as etiological agent of shell disease of lobsters (Malloy, 1978). Black disease caused by *Aeromonas hydrophila* was reported for first time in brine shrimp Artemia by Hernandorena (1987), Austin and Austin (1993) reported Aeromonas infection in oysters. Doukas *et al.*, (1998) reported *Aeromonas hydrophila* infection in cultured sea bass, *Dicentrarchus labrax*.

3. ECP IN *AEROMONAS.*

The pathogenesis of most of the members of the family Vibrionaceae were attributed to the extracellular products secreted by them. Griffin *et al.*, (1953) reported beta-haemolysis and gelatin liquefaction when *Aeromonas salmonicida* were grown on rabbit blood agar and nutrient gelatin plates. The extensive tissue destruction characteristic of furunculosis was assigned to the production of protease enzymes. The first attempt to purify and characterise the extracellular protease was described by Dahle (1971). The haemolysin from *Aeromonas hydrophila*, aerolysin has been partially purified and resolved into two components by isoelectric focussing (Wretlind, *et al.*, 1971).

Bernheimer *et al.*, (1974) reported Beta haemolysin in *Aeromonas hydrophila.* Wadstrom *et al.*, (1976) observed enterotoxins in *Aeromonas hydrophila.* Fuller *et al.*, (1977) purified a leucocytolytic factor which caused a transient leucopania after injection into fish. The surface of many pathogenic bacteria had been shown to be of key importance in pathogenecity (Smith, 1977).

A multienzyme complex with acyl transferase and phospholipase activity has been described in *Aeromonas hydrophila* culture filtrates (MacIntyre and Buckley 1978). Atkinson *et al.*, (1980) described haemoagglutinins in the

pathogenesis of *Aeromonas hydrophila.* Extracellular products (ECP) produced by growing the bacterium *Aeromonas salmonicida* on cellophane overlays were shown to have potent lethal activity as well as proteolytic, haemolytic and leucocytolytic activities (Munro *et al.*, 1980).

Seidler *et al.*, (1980) reported that 40% of the strains from environmental sources produced cytotoxin. It was found that *Aeromonas hydrophila* produced extracellular substances, which were capable of causing pathological effects when injected into trout. Proteolytic and haemolytic activity of the ECP and the effect on fish were lost on heating (Allan *et al.*, 1981). Ellis (1981) reported that all the lesions associated with furunculosis were induced when ECP was injected into fish. Complete lysis of trout erythrocytes (T-lysin) appeared to be due to the combined effects of caseinase and another substance T_1 activity which probably made the erythrocyte membrane susceptible to proteolytic digestion. Studies on the histopathology of furunculosis in salmonids had shown that the disease was accompanied by extensive proteolysis of both muscle and connective tissue.

An important virulence factor for *Aeromonas salmonicida* appeared to be the A-protein which was visible by electron microscopy as a layer covering the outer surface of most virulent strains but was absent from attenuated strains (Kay *et al.*, 1981).

Aeromonas hydrophila was a prolific producer of extracellular products and many of them had been described as important factors in pathogenesis. Alpha haemolysin was described in *Aeromonas hydrophila* by Ljungh *et al.*, (1981). Titball and Munn (1981) detected two haemolytic activities produced by *Aeromonas salmonicida*, an H-lysin which had maximal activity against horse erythrocytes and a T-lysin which was active only against trout red blood cells.

Thune *et al.*, (1982) observed gross pathologies associated with *Aeromonas hydrophila* infections in the young of channel catfish, when sublethal doses of ECP were administered. The characterisation of ECP suggested that the lethal factors were proteases. Mellergaard (1983) purified a serine protease with fibrinolytic and caseinolytic activities in the ECP of *Aeromonas salmonicida.*

Cytotoxins (Asao *et al* 1984) Lipopolysacharide structure (Dooley *et al.*, 1985) Siderophores (Barghouthi *et al.*, 1986) Surface proteins (Dooley *et al.*, 1986) were reported to be the virulence factors of *Aeromonas hydrophila.*

Nieto and Ellis (1986) reported Proteases in the ECP of *Aeromonas hydrophila* as one of the important factors in pathogenesis. Thune *et al.*, (1986) purified Beta haemolysin and examined it's role in the virulence of zero group channel catfish.

Majority of *Aeromonas* strains isolated from catfish culture were found to be proteolytic, amylolytic and produced DNA ase. Elastase and staphytolytic activities were present only in *Aeromonas hydrophila* (Santos *et al.*, 1987). It had been reported that proteolytic enzymes secreted by *Aeromonas* sp. played an important role in invasiveness and establishment of infection by overcoming initial host defences (Leung and Stevenson, 1988).

Dooley and Trust (1988) confirmed the presence of S layer in *Aeromonas hydrophila*, which helps the bacteria in resisting attack by the host *phagocytes* and other innate immune factors. Santos *et al.*, (1988) observed enterotoxins in *Aeromonas hydrophila*. It was found that a prerequisite for the initiation of infection was the initial adherence of bacteria to host epithelial cells. Electron microscope demonstrated the presence of fimbriae (pili) on *Aeromonas* cells regardless of virulence potential (Corral *et al.*, 1990).

An extracellular lethal toxin produced by *Aeromonas salmonicida* composed of glycerophospholipids: cholesterol acyltransferase (GCAT) was purified by fast protein liquid ion – exchange chromatography. The Toxin, which had haemolytic, leukocytolytic and cytotoxic activities was lethal for Atlantic salmon (Lee and Ellis 1990).

Chabot *et al.*, (1991) identified and partially characterised three proteases P1, P2, P3 produced by *Aeromonas hydrophila* by biochemical techniques and polyacrylamide gels. An extracellular acetylcholinesterase produced by *Aeromonas hydrophila* had been identified as a major lethal toxin for fish. (Nieto *et al.*, 1991).

In Isoelectric focussing, the ECP of various strains of *Aeromonas hydrophila* and *Aeromonas sobria* possessed between 19 and 31 proteins and the number of extracellular proteases ranged from one to twelve (Nieto and Ellis 1991). He *et al.*, (1992) isolated pathogenic strains of *Aeromonas hydrophila* from silver carp that produced haemolysin and cytotoxin.

Three different lethal extracellular toxins – metalloprotease, serine protease and haemolysin of *Aeromonas hydrophila* were isolated from rainbow trout, which were lethal to fish (Rodriguez *et al* 1992). Cytotoxin producing strains were more frequently associated with Epizootic ulcerative syndrome (EUS) infected fish compared to normal fish. Haemolysin producing strains were equally present in healthy and EUS positive fish (Yadav *et al.*, 1992)

Yan (1992) observed that the ECP of *Aeromonas punctata*.obtained from fish in an epizootic outbreak in Shanxi province had haemolytic, caseinase and lethal activities. The author also described the histopathological signs.

Powell *et al.*, (1993) reported the rapid reduction in the number of Eosinophilic granule cells (EGC) in the gut of ECP injected fish, but there was no apparent change in the number of EGC of control fish. Lygren et aL, (1994) described the purification and characterisation of two Norwegian strain of *Aeromonas salmonicida* sub sp. *salmonicida.*

Arnesen *et al.*, (1995) reported that *Aeromonas salmonicida* were shown to produce several extracellular products having gelatinolytic activity. The pathogenic activities of live bacteria and extracellular products of motile *Aeromonas* were investigated invitro and invivo. Elastases, haemolysins and exotoxins play a leading role in the pathogenecity of motile *Aeromonas* for eels.

Dong (1995) reported the haemolytic activity and virulence of *Aeromonas hydrophila*, which caused fish bacterial septicaemia in *Carassius auratus.* A metallo–caseinase was detected in ECP of *Aeromonas salmonicida* sub spp. *achromogenes* (Gudmundsdottir 1996).

Toxins and enzymes together with some structural features were considered to be important virulence factors for *Aeromonas hydrophila.* Most of the virulence factors were regulated by environmental conditions. Temperature

greatly influenced the rate of growth of some serotypes as well as the production of enzymes, toxins and cell surface composition (Mateos and Paniagua 1996).

The production of haemolysins, caseinases, elastases and growth yields of environmental strains of *Aeromonas hydrophila* decreased sharply during cultivation at 37 °C but cytotoxins were produced to same extent or slightly less than at 28 °C (Mateos 1996).

Shome *et al.*, (1996) elaborated that the abdominal transudate of dropsy affected fishes contained heat stable haemolytic, proteolytic, amylolytic and dermonecrotic exotoxin activity. Ammonium sulphate precipitated partially purified ECP's were highly cytotoxic when tested in same cell lines.

In an experimental challenge, ECP's of *Aeromonas hydrophila* were found to elicit better protection than whole bacteria (Loghothetis *et al.*, 1996). Uddin *et al.*, (1996) described that the peak growth of *Aeromonas hydrophila* was favoured at 34.5 $\pm$ 1.0 °C while the protease production at 27.6 $\pm$ 4.9 °C.

The pathogenecity of ECP's from *Aeromonas salmonicida* strains were studied with respect to lethality in Atlantic salmon, pathogenic effect on muscle, haemolytic, cytotoxic and proteolytic activities. An extracellular metallo–

caseinase was linked with lethal toxicity and a strong pathogenic effect. (Gunnlaugsdottir, 1997).

Khalil *et al.*, (1997) observed that the highest production of the haemolysin product was achieved when *Aeromonas hydrophila* was grown at 35°C for 30 hrs. *Aeromonas hydrophila* was found to produce haemolytic and proteolytic exotoxin lethal to tilapia. Some heat stable unknown virulent factors were responsible for 20 % mortality.

Aeromonas isolates from the hepatopancreas of giant fresh water prawn (*Macrobrachium rosenbergii*) showed activities of five extracellular enzymes: amylase, lipase, protease, gelatinase and chitinase and also haemolysins (Sung *et al.*, 1997).

MATERIALS & METHODS

III. MATERIALS AND METHODS

3.1 EXPERIMENTAL PROTOCOL

Healthy *Penaeus indicus* (Indian white prawn) with mean body weight of 5-7gm caught from KVK, Narakkal, were used in this study. About 50 shrimps were maintained in a set of plastic tubs. About 6 numbers of 50 litre plastic tubs equipped with air supply were stocked with 8 shrimps. The shrimps caught from brackishwater farms were gradually acclimatised and the salinity was maintained between 15 and 20 ppt and fed with pelleted feed 2-3% of body weight twice daily.

For the experiment, triplicates were maintained. Soon after stocking, the animals were starved for 24 hours. 80% of water was exchanged daily. Extreme care was maintained by providing appropriate food and removing the faecal matter. Water quality parameters were checked daily. The mean temperature during the trial was 28 ± 1°C. Prior to the experiment, a set of animals was sacrificed for standardising the experiment.

3.2 BACTERIAL STRAINS

A pathogenic strain of *Aeromonas hydrophila* isolated from fish sample was obtained from CIFT, Kochi. The strain obtained was inoculated in Tryptic Soya Broth (TSB) and routinely cultured in Tryptic Soya Agar (TSA) at 28°C for 24-48 hrs and also stored on TSA slants for further use at 4°C.

3.3 EXTRACELLULAR PRODUCTS (ECP) PREPARATION

The ECP was prepared as described in Liu (1957) Nieto & Ellis (1986). The culture of *A. hydrophila* in TSB were inoculated onto sterilized cellophane sheets overlying TSA plates and incubated at 28°C for 24 hrs. The bacterial culture in log phase were suspended in about 5-7 ml of normal saline and centrifuged at 6000 rpm for 20 min and the supernatant (ECP) was sterilized by filtration by passing through 0.22 μm Millipore filter. The ECP was freshly prepared each time for the experiment.

3.4 TOTAL PROTEIN ASSAY

The folin-ciocalteau phenol method of Lowry *et al.*, (1951) was adopted for the estimation of total proteins in the ECP.

To the ECP (0.05 ml, 0.1 ml) freshly prepared 5 ml alkaline reagent was added (50 ml of 2 % sodium carbonate in 0.1 NAOH + 1 ml of 0.1 NAOH + 1 ml of 0.5 % of $CUSO_4.5H2O$ sodium tartarate). After 10 minutes 0.5 ml of 1 N folin-ciocalteau reagent was added and mixed rapidly.

A standard stock solution was prepared using Bovine Serum Albumin at a concentration of 25 mg / 5 ml in 1 N NAOH. Different dilutions in the range of 0.25-2.5 mg/ml were prepared from this stock solution. The alkaline reagent and folin-phenol reagent were added. Blank was prepared with 1 N NAOH and the remaining agents added.

The test tubes were kept for 30 minutes at room temperature and optical density of the blue colour developed measured against the blank at 660 nm.

3.5 DETERMINATION OF PROTEIN PROFILE OF ECP

The protein profile of saline washings of bacterial extracellular products were studied by SDS-Poly acrylamide gel electrophoresis as per the methods of Laemmli (1970). The molecular weights of standard SDS-PAGE molecular marker were 97.4 KDa, 68 KDa, 43 KDa, 29 KDa, 14.3 KDa. A semi-log graph was plotted using the Rf values. The Rf values of unknown samples were

calculated and extrapolated using the standard graph to determine the molecular weight.

3.6 STANDARDISATION

For standardizing the experiment, the shrimps maintained in plastic tubs were exposed to minimum concentration of the ECP. The ECP was injected at the ventral side of the abdomen of the animal between the segments.

3.7 STUDY OF MORTALITY PATTERN

After estimating the protein constant in the ECP, the level of administration of ECP was divided into five concentrations.

1 ml of normal saline = 2.5 μg of ECP

Group I	control
Group II	0.5 μg ECP /ml of normal saline
Group III	1.0 μg ECP /ml of normal saline
Group IV	1.5 μg ECP /ml of normal saline
Group V	2 .0 μg ECP /ml of normal saline
Group VI	2.5 μg ECP /ml of normal saline

3.8 HAEMOLYMPH STUDY

The five groups of shrimps and 1 control were reared in triplicates for determining the total haemocyte count of *Penaeus indicus*. Samples were taken from each group at 24 hrs, 48 hrs and 72 hrs respectively. Haemolymph was taken from the heart with a heparinised 28-G needle and 2 ml sterilized heparinised plastic syringes for each treatment. The haemolymph was extracted along with filtered May-Grunwald's Giemsa stain (Merck chemicals) and taken on the haemocytometer and incubated for 20 minutes and the haemolymph counted under the light microscope.

3.9 HISTOLOGICAL STUDY

The organs used for the study were Hepatopancreas and muscle tissues, which were separated from the animals showing disease condition from each group. The tissues were taken from live animals and fixed in Davidson's fixative (George Clark, 1981) for 24 hrs. After the required period of fixation, tissues were transferred directly to 70 % ethanol and then to ascending series of alcohol for complete dehydration. The tissues were cleared by immersing in alcohol – chloroform (1:1) mixture for 2 hrs. After that two changes in pure chloroform were done and left in saturated chloroform – wax mixture overnight.

Paraffin infiltration was carried out by transferring the tissues to pure wax kept in an oven at 59°C to 60°C, followed by three changes in wax. Paraffin molded blocks were prepared and sectioning at 6µ was carried out by using a Rotary Microtome. The deparaffinised sections were stained with Haemotoxylin – Eosin stain (Culling *et al.*, 1985). After the staining procedure, the slides were cleared in xylene, mounted in DPX and observed under the light microscope.

3.10 PHOTOMICROGRAPHY

The stained sections were photomicrographed using 35 mm, Kodak 100 ISO color film in Nikon microscope AFX-II A with automatic exposure unit and colour temperature metering.

RESULTS

V. RESULTS

1. BEHAVIOUR AND GROSS PATHOLOGICAL CHANGES

The shrimps injected with extracellular products (ECP) of *Aeromonas hydrophila* showed abnormal swimming behaviour and loss of appetite. The shrimps in Group II injected with lowest concentration of ECP (0.5 µg) showed normal behaviour and consumed feed at reduced rate (1% of body weight). A few shrimps in Group III and Group IV also consumed less feed and showed erosions on the appendages and telson (PLATE 3.a & 3.b).

Complete loss of appetite was noticed in Group V and Group VI that received the highest concentration of ECP (2 µg and 2.5 µg). Melanisation and erosions on exoskeleton was observed in Group IV and Group V in about 40 % of the animals (PLATE 4.a & 4.b). In Group VI that received the highest concentration of ECP, soft shelling was observed in the moribund shrimps.

2. STUDY OF MORTALITY PATTERN

The shrimps injected with different concentrations of ECP were assayed to find the percentage of mortality. In Group II and Group III there was 50%

mortality in 24 hours. Group IV showed 80 % mortality in 72 hours. Group V showed 50 % mortality in 24 hours and remaining died in about 72 hours. Maximum mortality was noticed in Group VI with complete death in about 48 hours (Table I).

3. STUDY OF PROTEIN PROFILE OF ECP

The protein profile of ECP of *Aeromonas hydrophila* revealed three bands in lane 1.The molecular weights of the bands were in the range of 98.4 KDa–68 KDa and 43 KDa-29 KDa with that of molecular marker. Lane 2 revealed 15 bands and the molecular weights of the prominent bands were in between 68 KDa and 43 KDa , 43 KDa-29 KDa and 29 KDa-14.3 KDa. In lane 3; 5 bands were observed, the molecular weights of the major ones were in the range of 43-29 KDa of that of molecular marker (PLATE 2).

4. HAEMATOLOGICAL STUDIES

Sampling was done from the injected shrimps in the five groups and control groups at 24 hours, 48 hours and 72 hours. The mean values of different treatment groups and control group are given in table II and graphs.

Group I

The control was injected with normal saline. The total haemocyte count ranged from 206 x 10^4 cells / ml. to 603 x 10^4 cells/ ml. The mean value of the group was 300 x 10^4 cells / ml.

Group II

There was gradual reduction in the number of haemocytes from 24 hours to 72 hours. The range of haemocyte count was 155 x 10^4 to 240 x 10^4 cells/ ml. The mean value observed in 24 hours was 217 x 10^4 cells/ ml, 173 x 10^4 cells/ ml. in 48 hours and 155 x 10^4 cells/ ml. in 72 hours respectively.

Group III

The total haemocyte count was found to show rapid decrease in numbers. The haemocyte counts varied from 177 x 10^4 cells/ml to 120 x 10^4 cells/ml .The mean value was 164 x 10^4 cells/ml, 150 x 10^4 cells/ml, 124 x 10^4 cells/ml at 24, 48 and 72 hours respectively.

Group IV

The total haemocyte count ranged from 146 x 10^4 cells/ml to 56 x 10^4 cells/ml. The mean value at 24 hours was 135 x 10^4 cells/ml; at 48 hours were 123 x 10^4 cells and 77 x 10^4 cells/ml in 72 hours.

Group V

Since the shrimps did not survive to 72 hours of the experiment, all the samplings were done within 24 hrs of observation. The total count varied from 109 x 10^4 cells/ml to 125 x 10^4 cells/ml. The mean value observed was 117 x 10^4 cells/ml.

Group VI

Most of the shrimps died within 6-7 hours after the administration of ECP. The haemocyte count ranged from 86 x 10^4 cells/ml to 105 x 10^4 cells/ml. The mean value of count was found to be 97 x 10^4 cells/ml.

5. HISTOLOGICAL STUDY.

The study was carried out on Hepatopancreas and muscle tissues of the different groups.

5.1 HEPATOPANCREAS

1. Group I

The hepatic tubules showed normal structure with F cells and R cells. F cells with basophilic appearance and R cells with small vacuoles were observed. There was also secretory B cell. The luminal surface of cells contained brush border appearance (PLATE 5.a and 5.b).

Group II

Rounding of F cells seen occasionally (Apoptosis). A few haemocytes were seen infiltrated into the mesenchymal tissue. Minor disorganization of the tubules was observed. The number of secretory B cells was less and extensive vacoulation was noticed in the R cells (PLATE 6.a and 6.b).

Group III

More number of apoptotic cells in F cells were observed compared to the previous group. Nuclei of F cells became pyknotic. R cells became coalesced in some areas (PLATE 7.a and 7.b).

Group IV

The brush border of R cells has disappeared. In many areas F cells became rounded and there was disappearance of cells from the lining of the tubules. The lumen occasionally contained multinucleated cells. The lumen contained desquamated cells and also a few haemocytes (PLATE 8.a and 8.b).

Group V

In the tubules, there was complete loss of architecture. No nuclei were observed in the cells. Lytic changes were observed in a few areas (PLATE 9.a and 9.b).

Group VI

Complete loss of architecture of the cells and severe lysis of the tubules were observed (PLATE 10.a and 10.b).

5.2. MUSCLE

1. Group I

No significant changes were observed in the control group (PLATE 11).

2. Group II

When compared to the control, there were not much significant alterations. However occasional areas of lytic changes were noticed (PLATE 12).

3. Group III

Muscle fibres showed separation, lysis and necrotic regions.

4. Group IV

In this group, there was loss of structure and necrosis in muscle tissue.

5. Group V

There was loss of cell architecture and severe necrosis in this group (PLATE 13).

6. Group VI

Extensive necrosis and lysis of muscle fibres were observed in many areas (PLATE 14).

Table I: Study of Mortality Pattern in the control and experimental groups at different intervals.

Groups	24 hours	48 hours	72 hours
G I	1	-	-
G II	3	-	-
G III	3	-	-
G IV	1	3	1
G V	3	2	1
G VI	5	1	-

Table II: Mean haemocyte counts in the control and experimental groups at different intervals

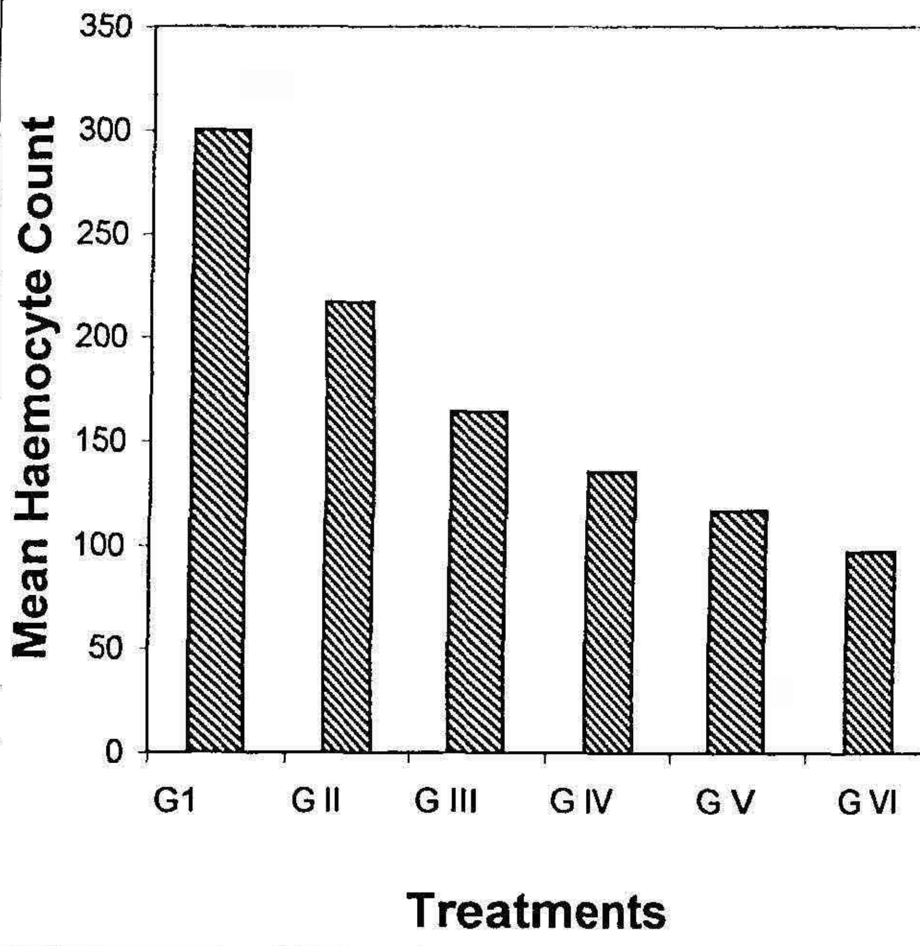
GROUPS	24 HOURS	48 HOURS	72 HOURS
G I	$300X10^4$ cells/ml	-	-
G II	$217X10^4$	$173X10^4$	$155X10^4$
G III	$164X10^4$	$150X10^4$	$124X10^4$
G IV	$135X10^4$	$123X10^4$	$77X10^4$
G V	$117X10^4$	-	-
G VI	$97X10^4$	-	-

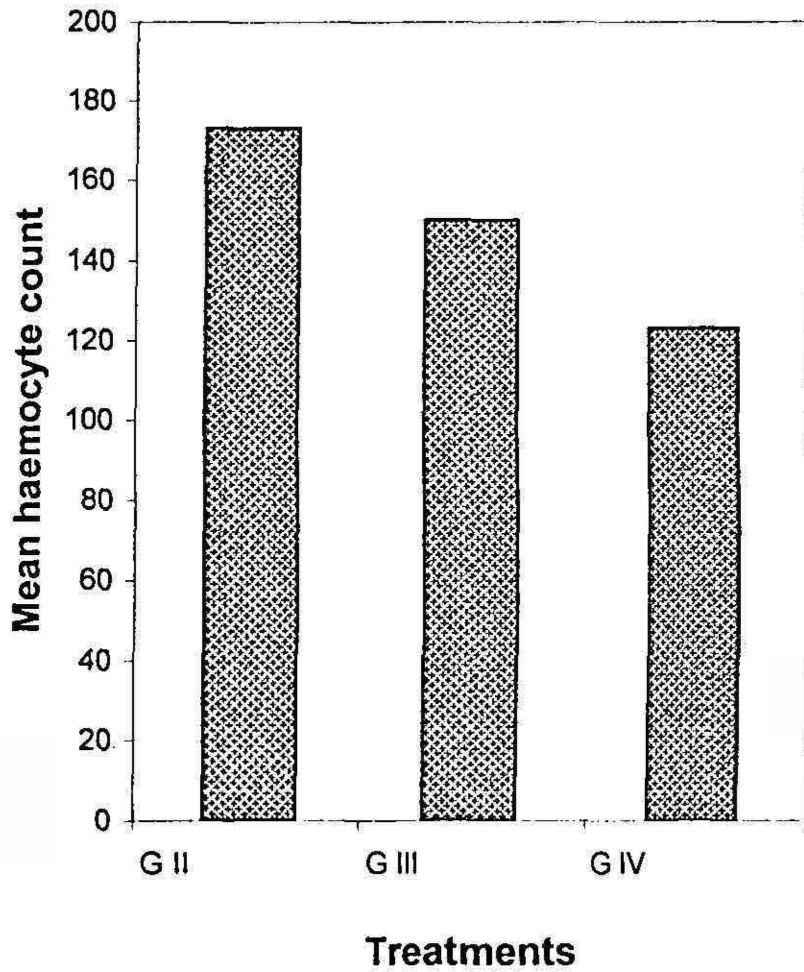
ANOVA to find the level of significance between the groups

F_{cal} =49.576*

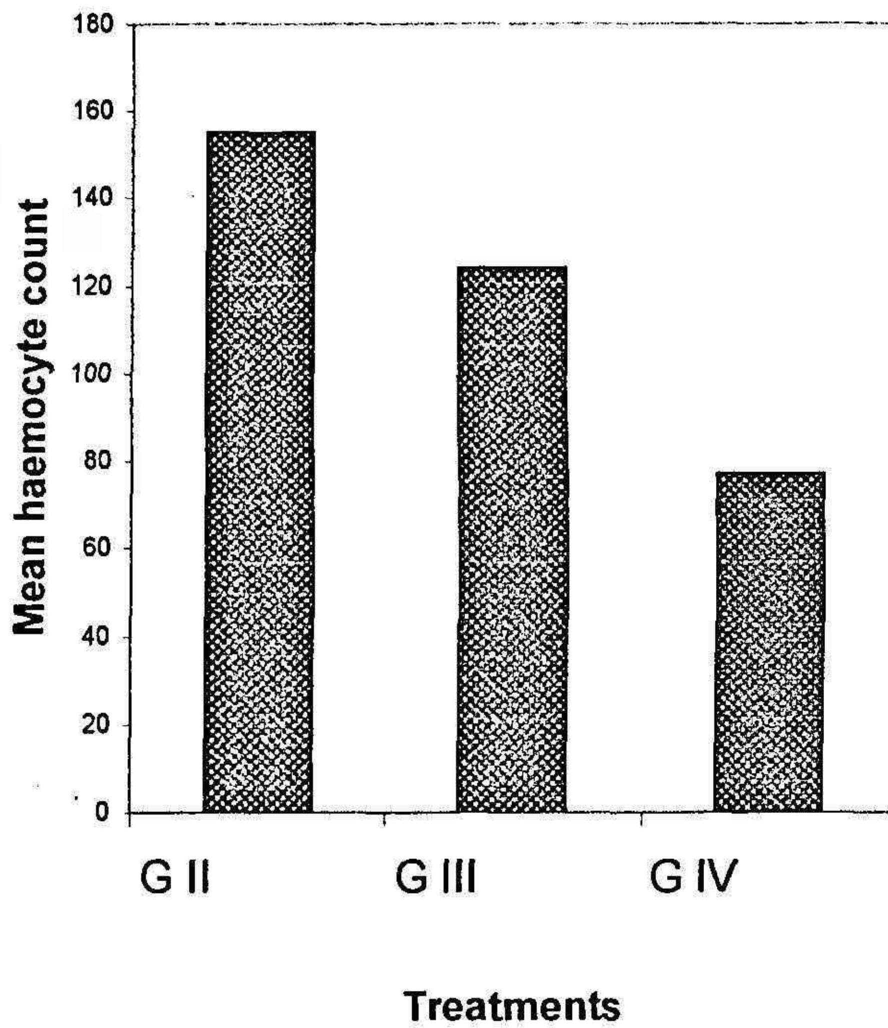
From the F table, at 5 and 31 df = 3.7 at 1% level of significance.

Since F_{cal} * is greater than table value, there is significant variation between the groups.


GRAPH I THE MEAN HAEMOCYTE COUNT FOR CONTROL AND TREATMENT GROUPS AT 24hours
Mean Haemocyte Count
350
300
250
200
150
100
50
0
G1
G II
G III
G IV
G V
G VI
Treatments


GRAPH II THE MEAN HAEMOCYTE COUNT FOR CONTROL AND TREATMENT GROUPS AT 48 hours
Mean haemocyte count
200
180
160
140
120
100
80
60
40
20
0
G II
G III
G IV
Treatments

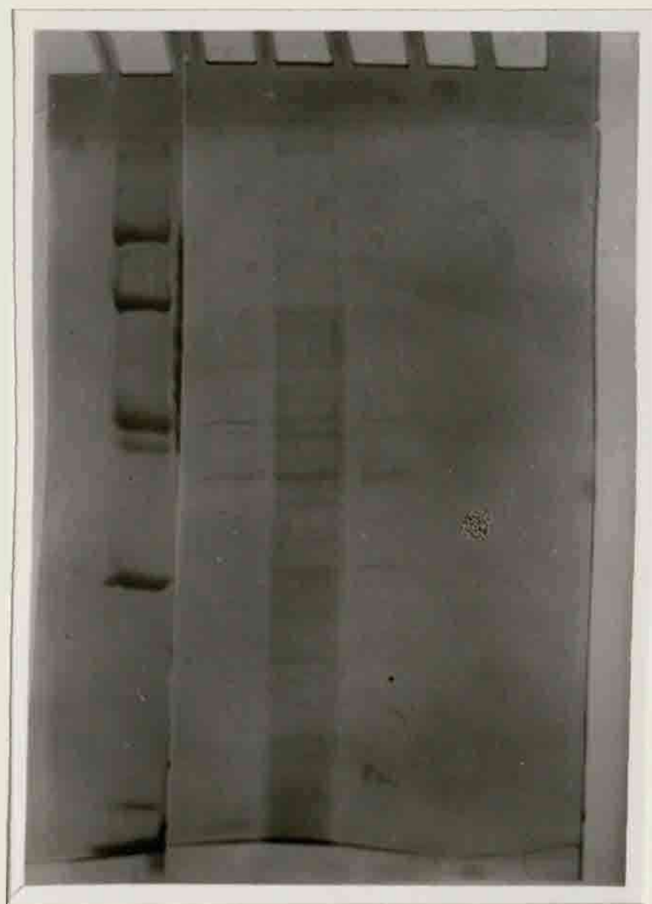
GRAPH III THE MEAN HAEMOCYTE COUNT FOR CONTROL AND TREATMENT GROUPS AT 72 hours



PLATES

PLATE 1: Log phase culture of *Aeromonas hydrophila* on cellophane sheets over tryptic soya agar plates.

PLATE 2: The protein profile of ECP of *Aeromonas hydrophila* resolved by SDS-PAGE.

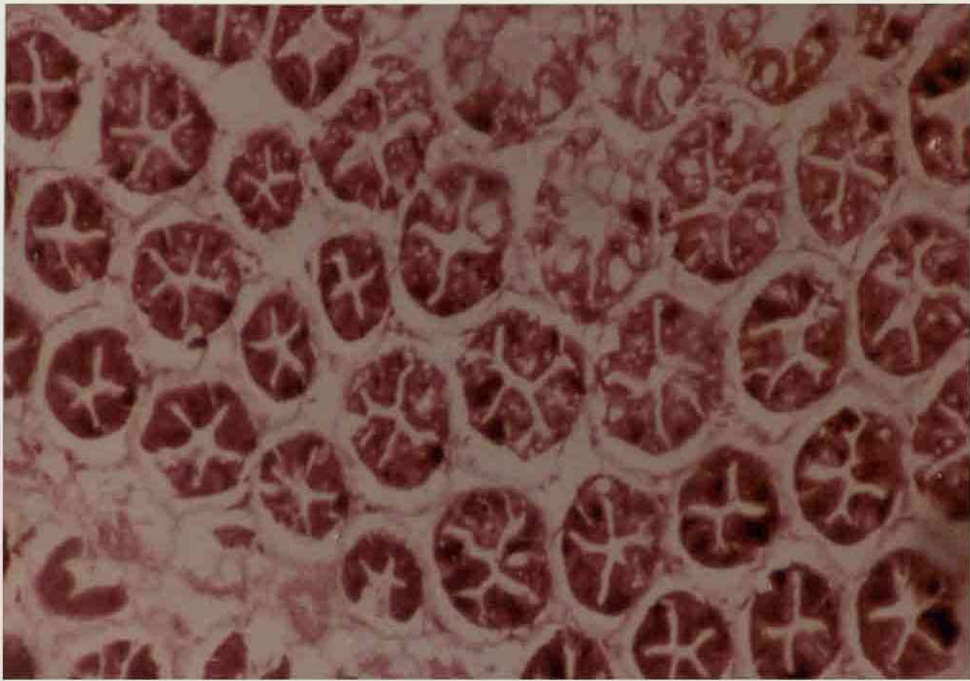


PLATES 3.a & 3.b: Animals in Group III and Group IV showing erosions in gill region and telson.

PLATES 4.a & 4.b: Animals in Group IV and Group V revealing Melanisation on gill region, exoskeleton and telson.

PLATE 5.a: Section of Hepatopancreas of control group, H&E, 100 X , showing the tubular structure of F cells and R cells with vacoules.

PLATE 5.b: Section of Hepatopancreas of control group, H&E, 400 X.



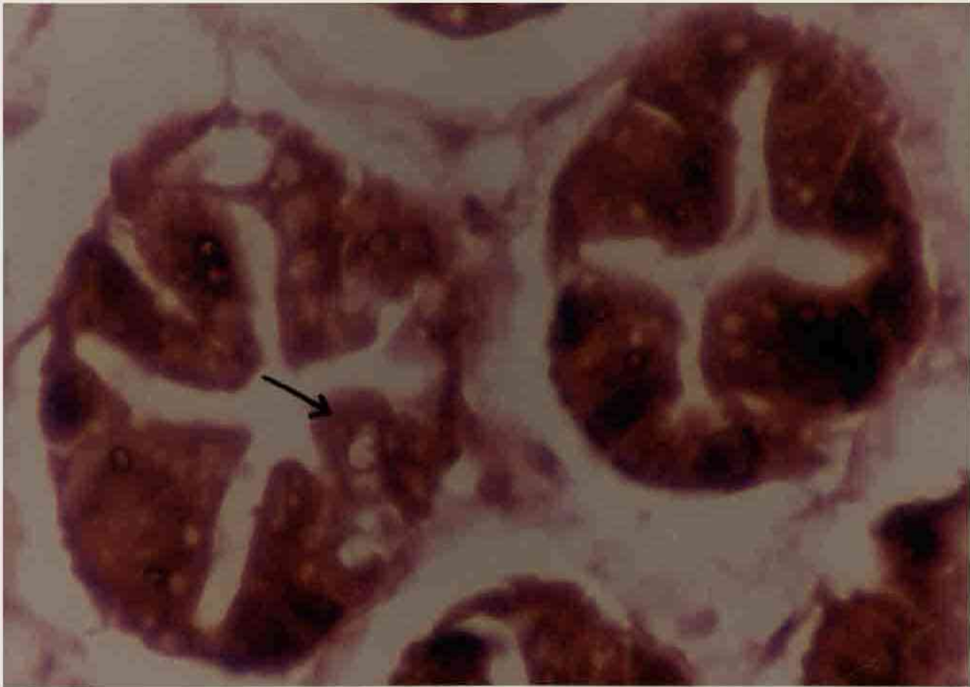
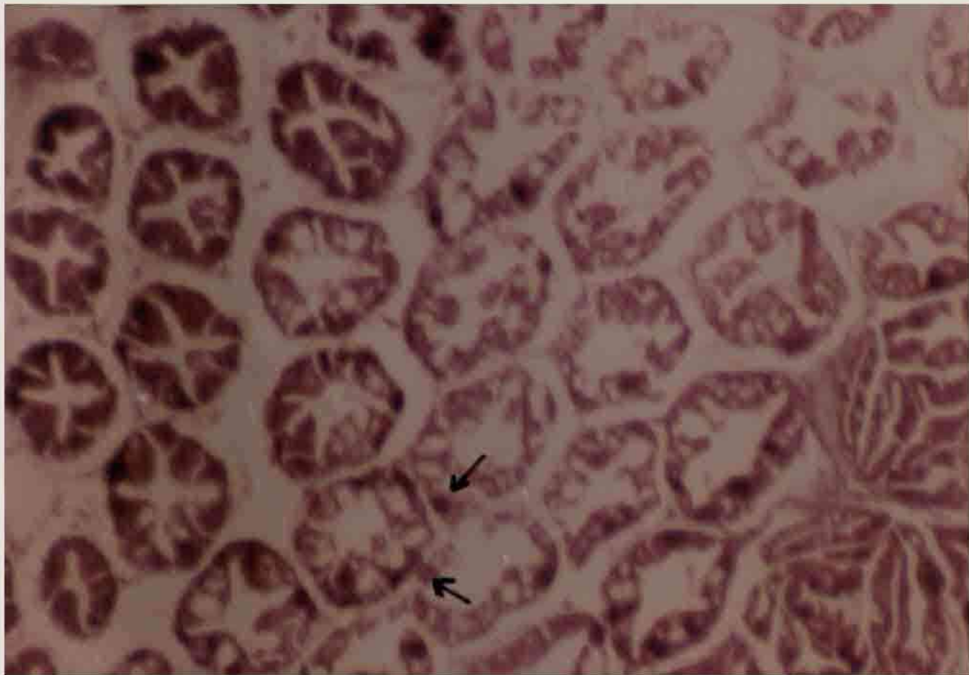


PLATE 6.a: Section of Hepatopancreas of treatment Group II, H&E, 100 X , showing occassional Apoptosis of F cells.

PLATE 6.b: Section of Hepatopancreas of treatment G II, H&E, 400 X.



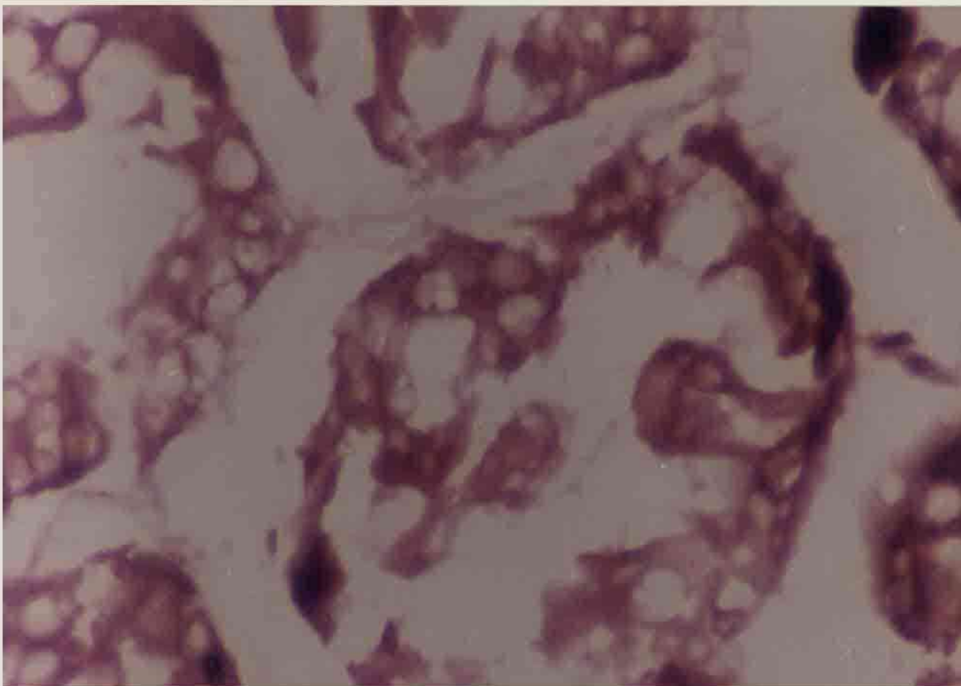
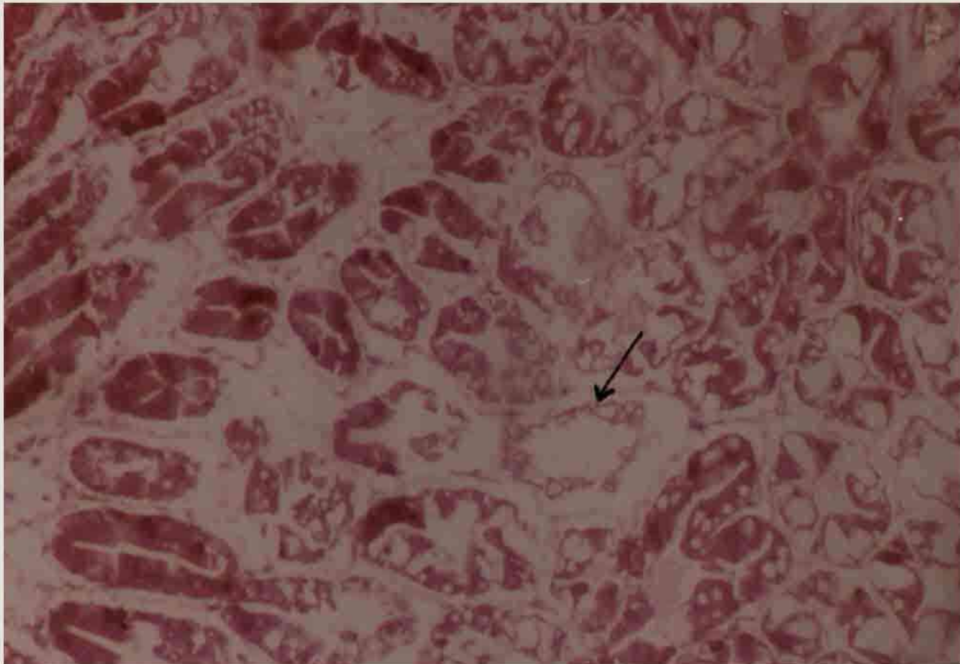


PLATE 7.a: Section of Hepatopancreas of treatment G III revealing more destuction of cells, H&E, 100 X

PLATE 7.b: Section of Hepatopancreas of Treatment G III, H&E, 400 X.



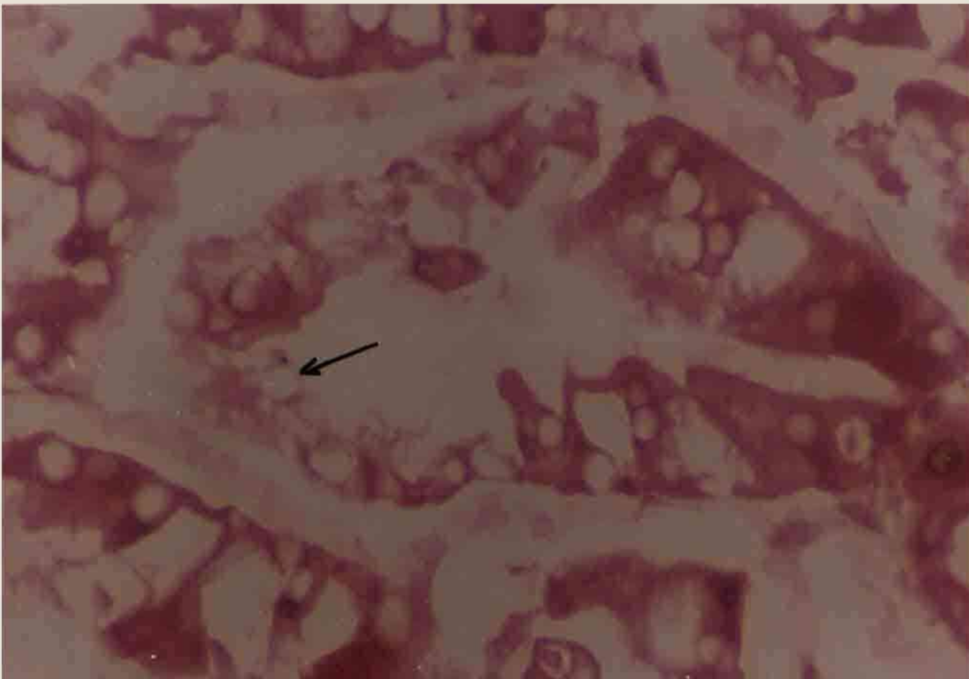
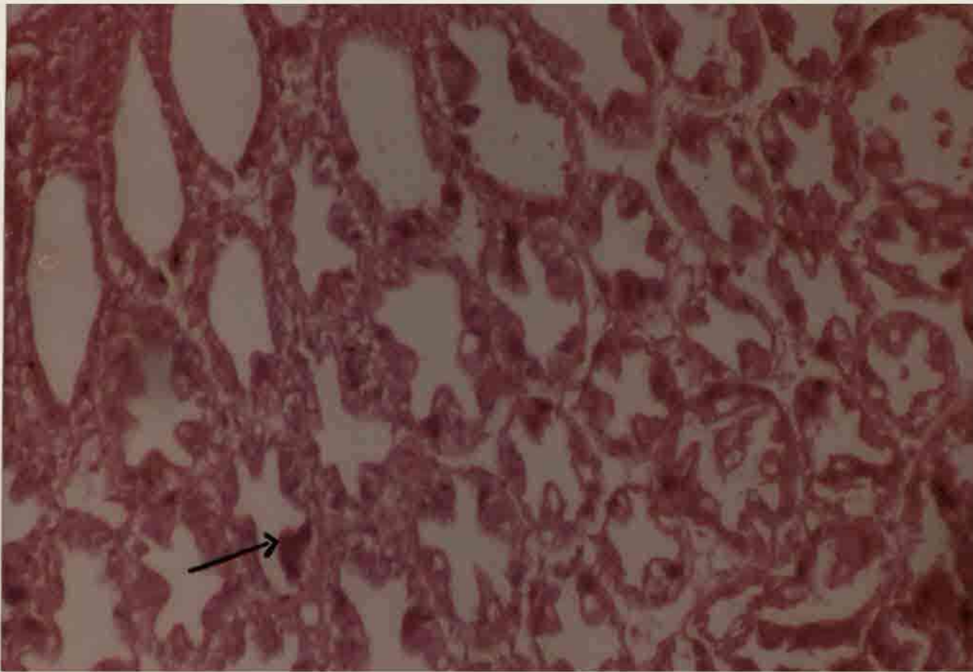


PLATE 8.a.: Section of Hepatopancreas of Treatment Group IV revealing desquamated multinucleated cells into the lumen and loss of brush border appearance of R cells,H&E, 100X.

PLATE 8.b.: Section of Hepatopancreas of Treatment G IV, H&E, 400 X.



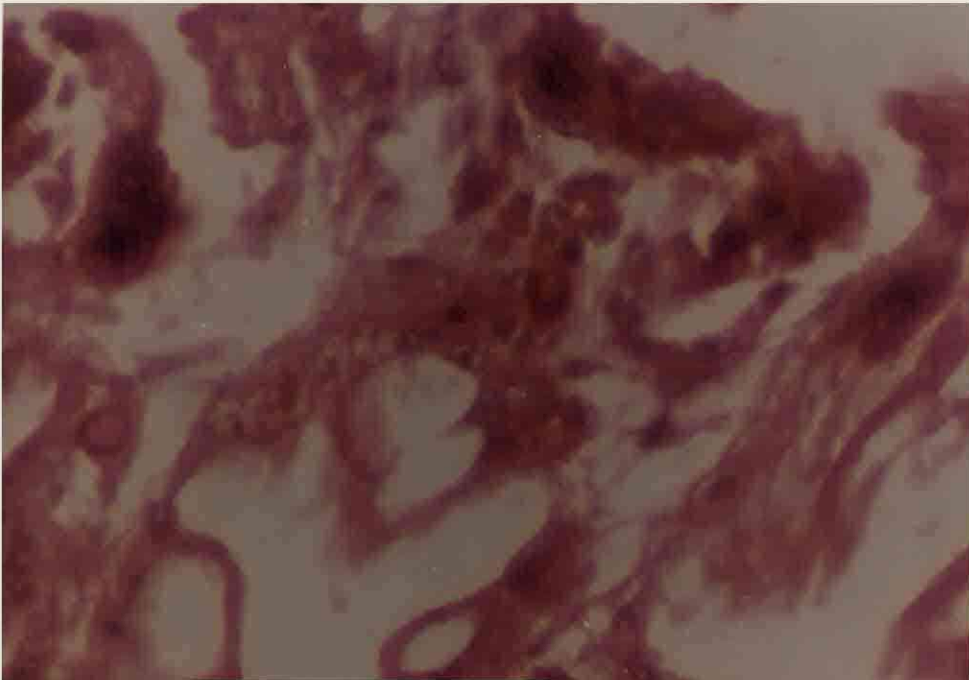
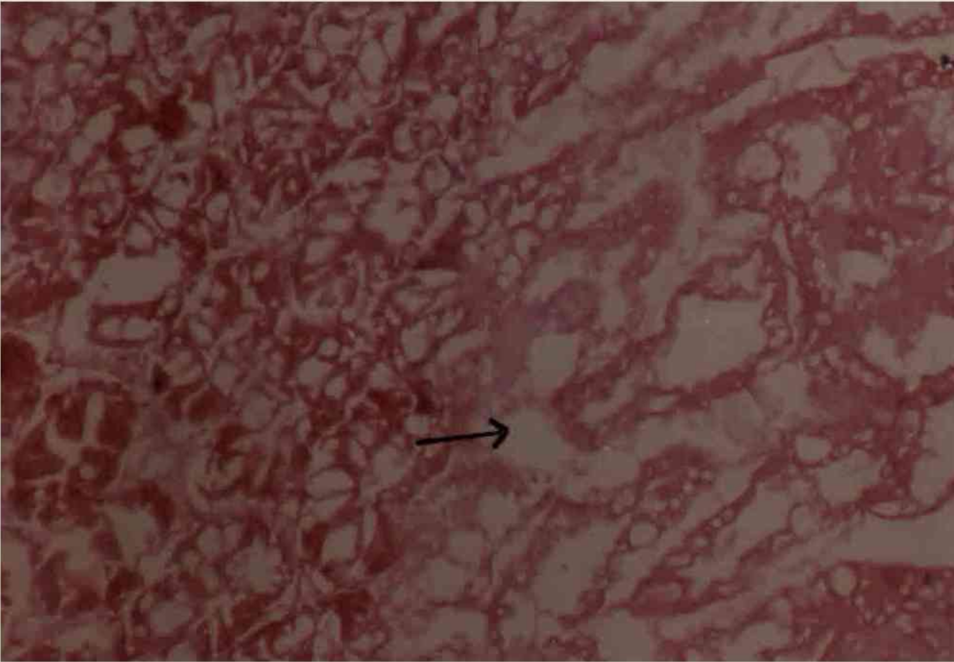


PLATE 9.a: Section of Hepatopancreas of treatment G V revealing loss of architecture and many cells without nuclei, H&E, 100X.

PLATE 9.b: Section of Hepatopancreas of treatment G V showing lytic changes. H&E, 400 X



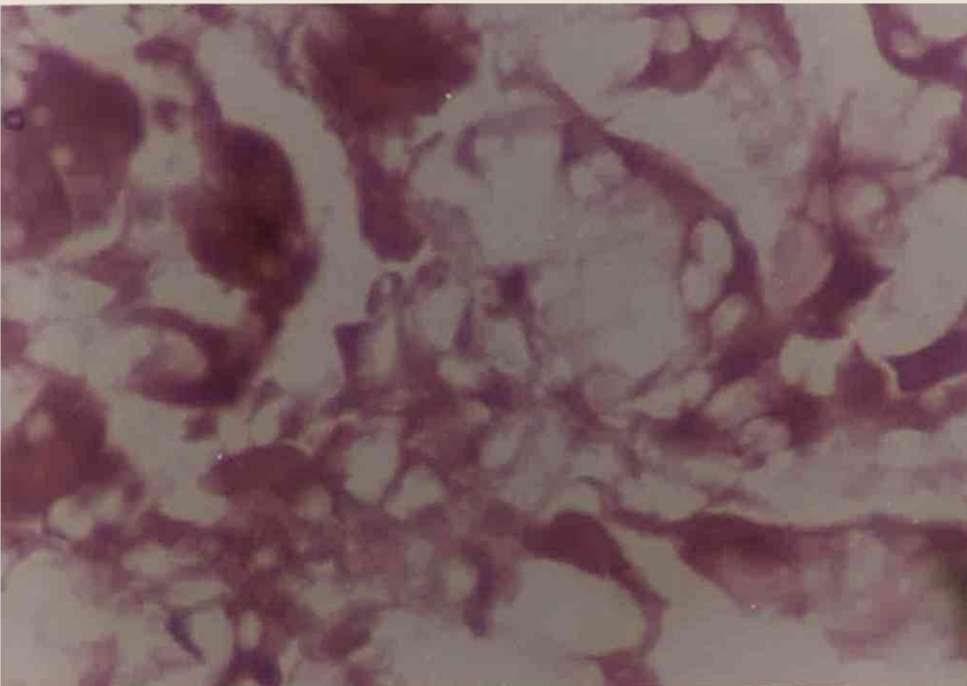
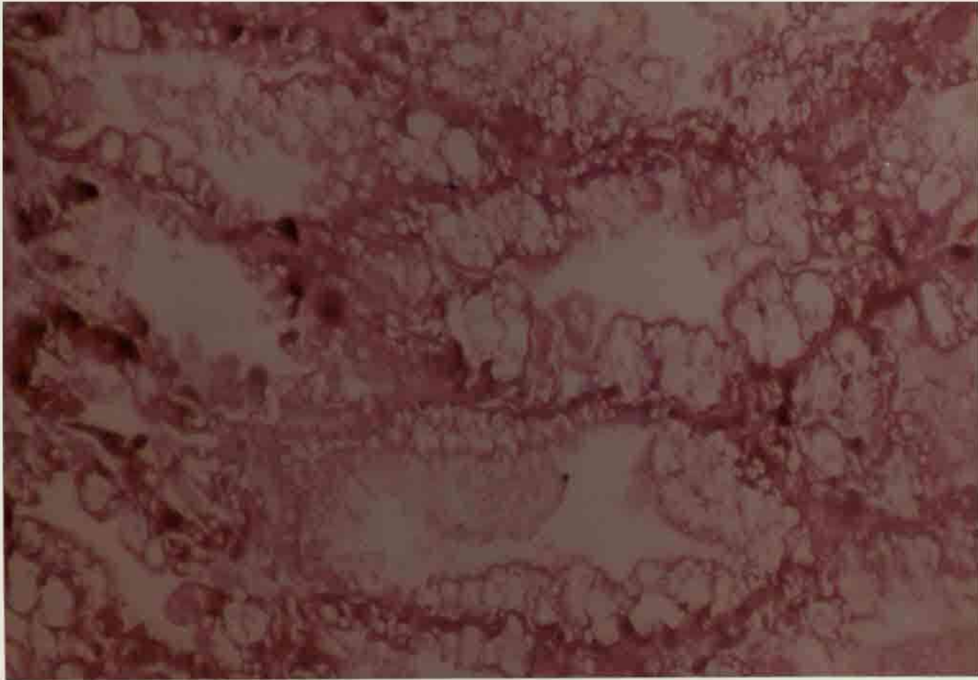


PLATE 10.a: Section of Hepatopancreas of treatment G VI revealing severe lysis of tubules and loss of architecture, H&E,100X.

PLATE 10.b: Section of Hepatopancreas of treatment G VI, H&E, 400 X.



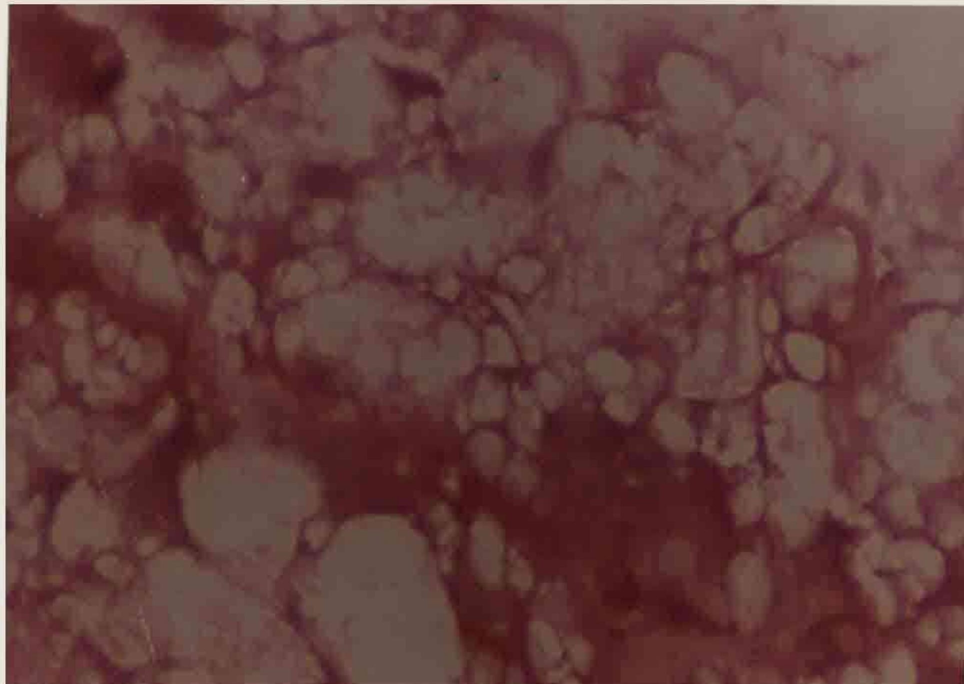
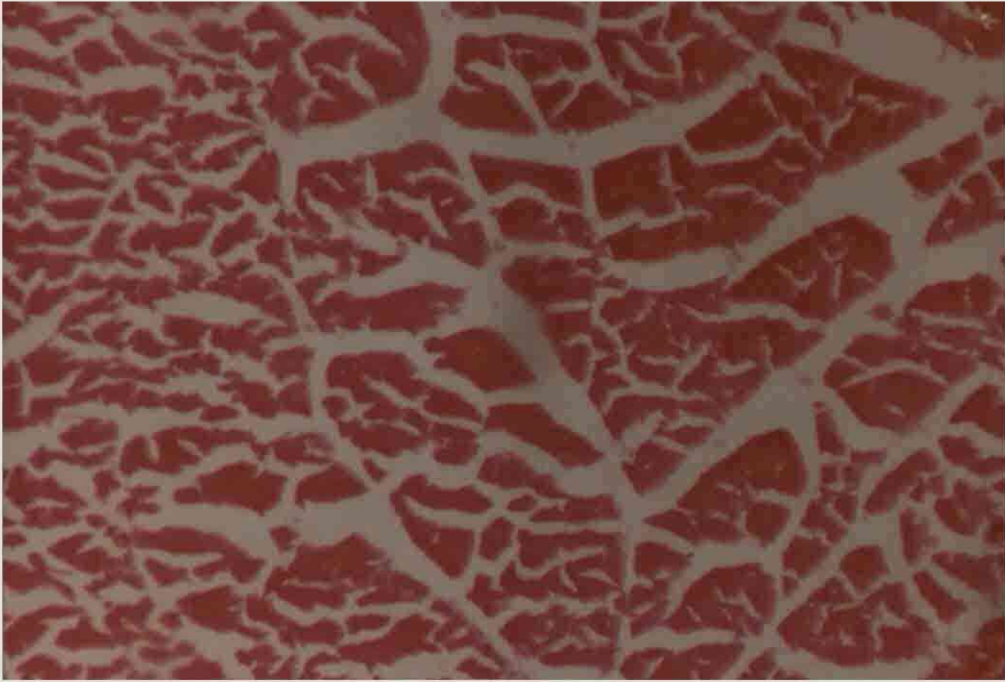


PLATE 11: Section of muscle tissue showing normal architecture, H&E, 100 X

PLATE 12: Section of muscle tissue of G II showing focal area of lytic change.H&E, 100 X.



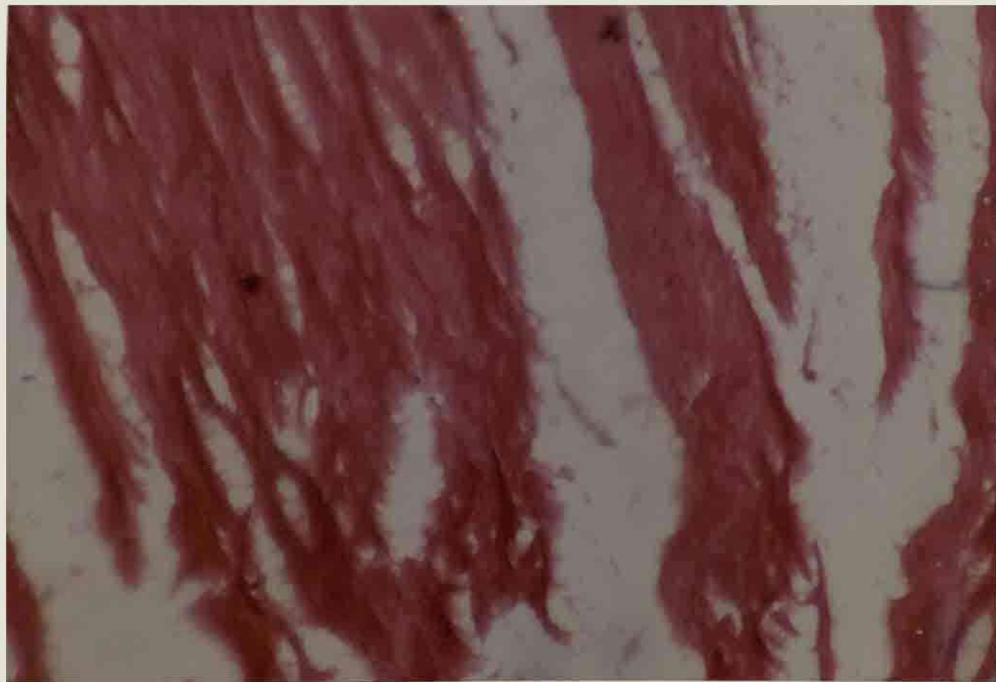
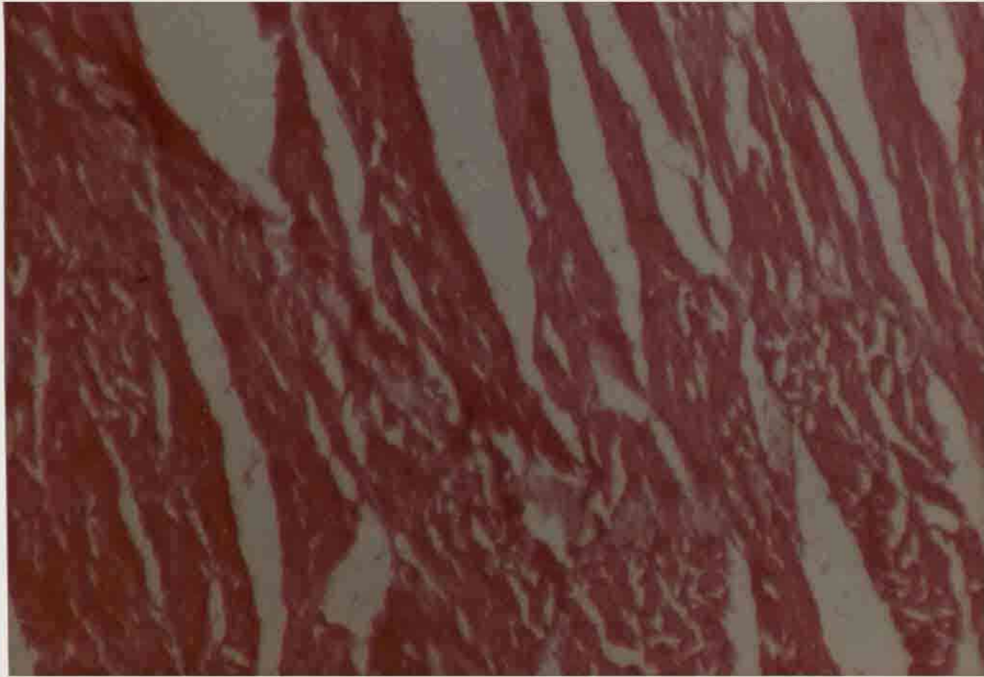
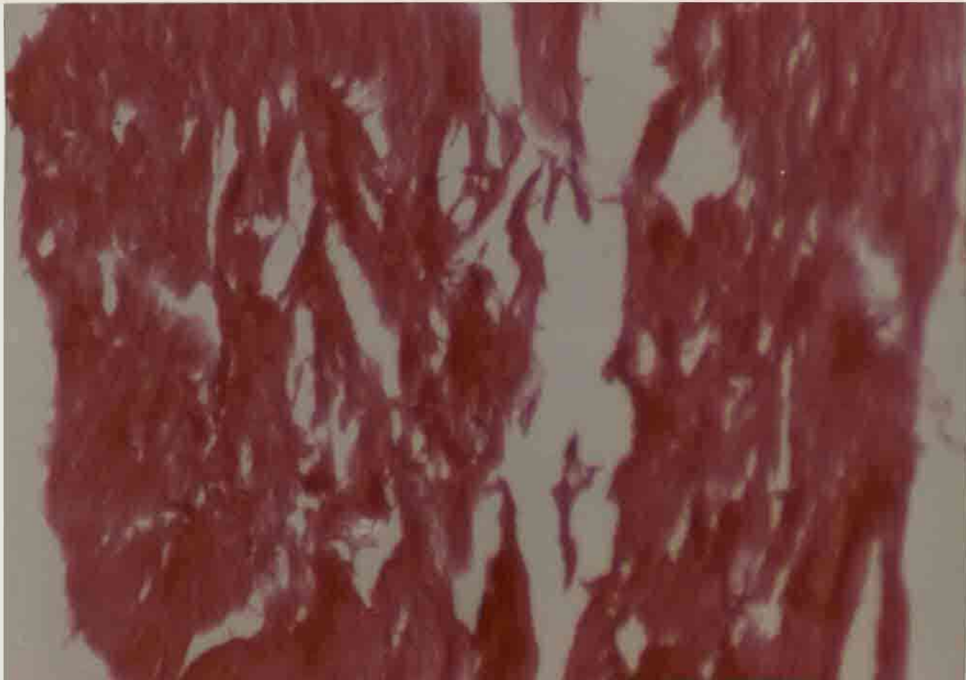


PLATE 13: Section of muscle tissue from G IV showing loss of architecture and necrosis. H&E, 100 X.

PLATE 14: Section of muscle tissue from G V revealing extensive necrosis. H&E, 100 X.





DISCUSSION

VII. DISCUSSION

The study involved changes in the morphological, behavioural, haematological and histopathological alterations in *Penaeus indicus* at different concentrations of administration of extracellular products of *Aeromonas hydrophila.*

Occurrence of *Aeromonas* sp. has been observed by Lakshmanaperumalsamy *et al;* (1982) who isolated a few *Aeromonas* strains from the blackened lesions of *Penaeus indicus* caught from Kochi backwaters. Several workers have observed *Aeromonas* infections in *Penaeus* species (Yasuda *et al.*, 1980, Lewis *et al.*, 1982, Owens *et al.*, 1992, Huang *et al.*, 1994, Chang *et al.*, 1996). However the pathogenecity studies were limited. Hence the present investigation was taken up.

The protein profile of ECP of Aeromonas hydrophila was analysed by SDS-PAGE. Thune *et al* reported three extracellular proteases which had molecular weights of 56 KDa, 34-35 KDa and 19.5 KDa respectively. In our study ,bands corresponding to 50-55 KDa, 32-36 KDa and 29-14.3 KDa approximately were seen. Further studies are needed to confirm the result of the present study.

In the present study an attempt was made to find out the virulence and pathogenic factors involved in the *Aeromonas* infection of *P. indicus.* The Extracellular products of *Aeromonas* culture were injected into the shrimps at various doses. The behavioural changes observed were abnormal swimming and loss of appetite. Sis *et al.*, (1980) and Jasmine (1996) had reported similar changes in *Vibrio* infections of penaeid prawns. *Vibrio, Pseudomonas* and *Aeromonas* were reported to be producing ECP containing: proteases, lipopolysaccharides, haemolysins and acetylcholinesterase.

Our studies also indicate that the extracellular products of *Aeromonas* are responsible for the behavioural changes observed in *Penaeus indicus* .The gross pathological change noticed in the present study was melanisation and erosion on appendages, exoskeleton and telson in Group IV and Group V that were given 1.5 µg ECP and 2 µg ECP respectively. Melanin appears during the host inflammatory response to the injury that has a bacteriostatic, clotting and localising function. This has been reported by Unestam and Weiss (1970), Soderhall and Smith (1986).

In crustaceans infected with shell disease, the lesions on exoskeleton typically begin as small, dark brown or black pits. Melanin spots were observed in areas where injury to skeleton had occurred (Rosen 1970). In group VI, which

when compared to the control animals. The haemocyte studies in relation to diseases in crustaceans are very limited, though the haemocytes are the major effector cells in the defence mechanism of crustaceans. In Gaffkemia of lobsters the haemocytes were reported to undergo reduction in number (Stewart and Rabin, 1970).

Our present study is a remarkable one because probably this is a first report about the reaction of haemocytes in a disease condition. Since our observation are limited to a few experiments further studies are required to draw definite conclusion about haemocyte dynamics in disease/ disease related condition.

Histological study was carried out on hepatopancreas in order to assess the effect of ECP of *A. hydrophila* on the various cells of hepatopancreas. In the control group we have observed four types of cells and the normal architecture of the tubules were maintained. The structure was similar to those of other decapod hepatopancreas (Young, 1959; Al-Mohanna, *et al.*, 1985; Vogt *et al.*, 1985).

In the present investigation, when the animals were injected with 0.5 μg ECP in Group II, the hepatopancreas revealed rounding of F cells (Apoptosis) and infiltration of haemocytes. As the dose of ECP increased it was noticed that the tubules were disorganised and there was a decrease in the number of B cells.

The R cells had extensive vacoulation and the brush border appearance of R cells had been lost. F cells became pyknotic. Multinucleated, desquamated cells were observed in the lumen. In Group V and Group VI, severe necrosis and complete loss of architecture of tubules of hepatopancreas were noticed.

Earlier workers had observed similar changes in hepatopancreas of penaeid prawns in association with Vibriosis, Soft-shell syndrome, Brown spot disease and other pathological conditions. This included necrosis of the cuticular lining, focal necrosis, haemocyte infiltration, hyperchromatisation of nuclei and vacoulation of hepatic cells (Lightner, 1983; Lee, 1985; Soni, 1986; Remesh, 1988; Jasmine, 1996). Our study also revealed same changes. The toxins liberated in bacterial infections like Vibriosis effect the same pathological changes. This may be the reason for the similar changes we have observed in the present study.

The histopathological changes in muscles were of mild in nature in Group II that received low dose of 0.5 µg ECP. The muscle fibres showed separation, loss of striations, severe necrosis and lysis when the dose of ECP increased. Similar pathological changes in muscle were observed in Vibriosis by Soni and Hameed (1988), in soft-shell syndrome by Remesh (1988) and in Brown spot disease by Jasmine (1996). The ECP of Vibrio sp. were reported to produce necrosis in *P. japonicus* (Chiang *et al* 1992).

Since bacterial diseases effect their pathogenecity through the exotoxins, similar changes were also observed in other bacterial diseases. *A.hydrophila* toxins also produced similar changes in the present study. In the present investigation, it was shown that the pathogenic effect of *Aeromonas hydrophila* infection was probably due to the production of exotoxins by the virulent bacteria. This was evident from the behavioural and haematological changes and also from the histopathological alterations in hepatopancreas and muscle.

However there is only limited information on the various aspects of toxin production and its effects on the vital system of prawns. It is necessary to elucidate these factors for devising proper control measures against the bacterial infections.

SUMMARY

VIII. SUMMARY

- Healthy Indian white prawn, *Penaeus indicus* collected from KVK, Narakkal was used to study the effects of Extracellular products (ECP) of *Aeromonas hydrophila.*

- The investigation focussed to evaluate the survival, behaviour, gross pathological, haematological and histopathological aspects followed by the introduction of ECP of *A.hydrophila* into the animal.

- The protein profile of ECP was determined by SDS-PAGE.

- Gross pathological changes observed included abnormal swimming behaviour, reduction in feed consumption. Melanisation was noticed on gill region and abdomen. Erosion was observed on the telson and appendages.

- Haematological study revealed a rapid reduction in the total haemocyte count showing the effect of virulent agents involved in the extracellular products of the bacterium.

- Histopathological study was carried out on Hepatopancreas and muscle of the diseased and control groups.

- Hepatopancreas from the diseased shrimps showed Apoptotic cells, infiltrating haemocytes, necrosis in many areas. Desquamated cells were observed in the lumen. Complete loss of architecture of the hepatopancreas was also noticed.

- Muscle from the diseased shrimps showed separation, severe necrosis and lytic changes.

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IX. REFERENCES

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