SALIVARY GLAND TOXINS OF THE OLD WOMAN OCTOPUS CISTOPUS INDICUS (ORBIGNY, 1840) FROM MUMBAI WATERS

Review and dissertation submitted in partial fulfillment of the requirements for the award of degree of

MASTER OF FISHERIES SCIENCE
in
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by

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(1997-1999)

UNDER THE GUIDANCE OF

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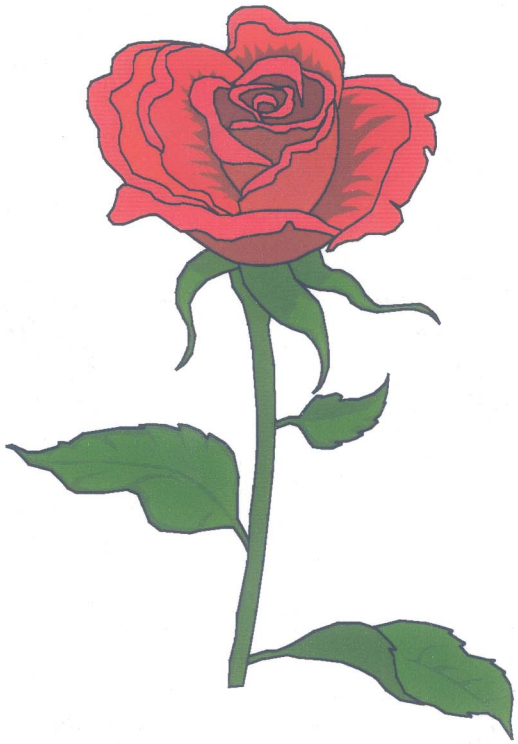

dedicated to my beloved parents....

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(Deemed University) Indian Council of Agricultural Research

CERTIFICATE

This is to certify that the dissertation entitled **Salivary Gland Toxins of the Old Woman Octopus, *Cistopus indicus* (Orbigny, 1840) from Mumbai Waters** is a record of bonafide research work done by **Mr. K.V.L.Bharat Kumar,** of the 1997 - 99 batch of M.F.Sc (FRM) programmme under our supervision and guidance and that no part of the thesis has been submitted for the award of any other degree, diploma, fellowship or other similar titles.

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DECLARATION

I do hereby declare that the dissertation entitled **SALIVARY GLAND TOXINS OF THE OLD WOMAN OCTOPUS, *CISTOPUS INDICUS* (ORBIGNY,1840) FROM MUMBAI WATERS** is a record of bonafide research work done by me during the tenure of my MFSc (FRM) program of 1997-99 and that the dissertation has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other University.

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ABSTRACT

The venomous nature of the salivary gland secretions of the old woman octopus, *Cistopus indicus* (Orbigny, 1840) had been established. The extracts from salivary glands of 16 specimens (average size 397 g/40 cm) were lyophilized to obtain 6.2 mg/ml of crude extract which was then partially purified on HPLC using a linear gradient of Phosphate Buffer Saline. The MLD of the crude venom for 20±2 g male albino mice was 0.5 ml at 90 seconds. Two lethal factors with death times of 23 seconds and 58 seconds were detected from among the partially purified venom. At autopsy, hemorrhage and pale discoloration of liver were discernible. Tubular necrosis and pyknotic nuclei were the histopathological changes induced by the venom in the kidney and liver and, to a moderate extent, in the heart. There was no hemolytic or hemagglutinating activity. The venom appears to be a potent neurotoxin with a pronounced blocking of the peripheral nerve conduction by interfering with excitable membranes. The observed symptoms of toxicity strongly suggest that tetrodotoxin (TTX) might be one of the toxic components.

सारांश

प्रौढ़ मादा ऑक्टोपस (सीस्टोपस इण्डीकस) (ऑरबिगनी 1840) के लार ग्रान्थि से स्त्रवित विष स्वरुप को रखा गया । 16 नमूनों (औसत आकार 397 g/40 cm) के लार ग्रन्थि से सत्त निकाला गया जिससे कच्चा सत्त 6.2 mg/ml प्राप्त हुआ जो फॉस्फेट बफर स्लाइन के रैखीय ढाल (लाइनियर ग्रैडियन्ट) का इस्तेमाल कर HPLC पर उसका अंशतः शुद्धिकरण किया गया । 20 $\pm$ 2g नर अल्बिनो चूहा के लिए कच्चे विष का MLD 90 सेकेन्ड तक 0.5 ml था । दो घातक तथ्य के साथ 23 सेकेण्ड और 58 सेकेण्ड के मृत्यु समय पर अंशतः शुद्धित विष को देखा गया ।

ऑटोप्सी, खून के टुकड़े (हेमोरहेज) तथा विवर्ण यकृत के विपर्णन पर इन्द्रियगोचर हो रहे थे । नलिकाकार उत्तकक्षय एवं पिकनोटिक न्यूक्लिई में वृक्क एवं यकृत में विष द्वारा उत्तक रोग विज्ञान में परिवर्तन देखे गए तथा हृदय में संतुलित फैलाव देखे गए । किसी प्रकार की रक्त कोशिका का टुकड़ा (हेमोलेटिक), हेमोग्लूटिनेटिंग अभिक्रिया नहीं था । विष शक्तिशाली तंत्रिका विष के साथ पेरीफेरल नर्भ चालन के स्पष्ट रोध (ब्लौकिंग) द्वारा उत्तेजनशील झिल्ली दिखाई दिया । इस लक्षण को ध्यान में रखते हुए जोर देकर कहा जा सकता है कि टैट्रोडौटॉक्सीन (TTX) विष का समूह एक महत्वपूर्ण उपकरण है ।

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TITLES


Introduction

1. INTRODUCTION

Among the earth's animal species of over 10,00,000 in the 30 phyla nearly 5,00,000 species are estimated of which a few thousand species or either poisonous or venomous marine animals, among which only a few have been investigated (Hashimoto, 1979).

Not more than 50 marine toxins are known whose molecular structure has been elucidated and whose pharmacological activities are known (Halstead and Courville, 1985). Research on marine toxins has focussed mainly on those species harmful to human beings and domestic animals. In recent years, ever increasing awareness of ocean exploration and of natural conservation have caused us to pay greater attention to aquatic organisms that have harmful substances, which should become a driving force to expose new toxic species.

1.1 DEFINITION OF MARINE TOXINS:

A ""toxin"" is an entity that has an adverse physiological effect on a living organism even when applied in a small quantity. In addition "poison" and "venom" are the terms very often used, but these three terms are defined differently from each other and the term 'toxin' has been used in the broadest sense. It was once suggested at an international symposium of Toxinology that the term 'Toxin' should be restricted to proteinaceous and antigenic substances (Hashimoto, 1979). Against their suggestion Vogt (1970) opinioned that a compound that could be called a toxin when the following premises are fulfilled:

1. It should occur in plants, animals, bacteria, etc.
2. It should be foreign to the victims , e.g. Human beings and
3. It should be predominantly toxic and adverse to the well being or life of the victim.

A substance, however should not be called a toxin when used as a remedy and viewed under therapeutic aspects. Venoms are usually proteinaceous substances that are secreted from venom glands or highly specialized secretory cells and delivered by a parenteral mechanism into other animals. On the other hand, poisons are causative agents of human intoxication from ingestion of marine organisms, which possess toxic substances in the entire body or in specific organs.

1.2 BIOLOGICAL SIGNIFICANCE OF MARINE BIOTOXINS:

Halstead and Courville (1965) observed that the scientific importance of biotoxins was frequently misunderstood. The most popular attitude is that poisons are lethal substance causing intoxication and death and are, therefore substances to be avoided. Ethological studies of biotoxins entail that they appear to serve as defensive or offensive mechanism, in food procurement, for e.g. the nematocyst present in the tentacles of the jellyfish and sea anemones.

Some marine toxins have been believed to have a physiological significance for the host organisms. Some fish are found to possess toxic substance in their eggs, tetrodotoxin in the tetrododontid fish, wax ester in mullet roe, and dinogunellin in ichthyotooxic fish. These

toxins may serve a physiological function for embryonic development. However, the biological functions of many toxins remain unknown and present as challenging research topic.

1.3 PUBLIC HEALTH SIGNIFICANCE:

There are indications that some of the marine biotoxins yet to be explored may rank among the most toxic substance known. Food poisoning from ingestion of marine organisms not only threatens human life and health, but also is an obstacle to the exploitation of marine resources. Outbreaks of food poisoning makes people worried of all seafoods and this greatly affect the fishing industry.

1.4 EXPLOITATION OF FOOD RESOURCES FROM THE SEA AND MARINE TOXINS:

Fishing activity has been expanding both horizontally and vertically for the exploitation of protein resources from the aquatic environment. It is not easy to recognize whether a particular fish is safe to eat or not. There is no established rule at present. Generally, an unexploited organism is not marketed until the fishing company decides to do so after listening to local people and after crewmembers and then the staff of the company have eaten the organism. This procedure is reasonable for organisms, which show no geographical seasonal and individual variation in toxicity, but for organisms such as ciguatoxic fish (Concon, 1988). In recent years, fishing companies, fishing crew and fish markets have become aware of ciguatera, and no outbreaks of poisoning have occurred that were caused by fish taken

from such seas. However many people regret that such an abundant fishery resource cannot be utilized.

1.5 DRUGS FROM THE SEA:

A toxin is a substance that has a specific functional group properly arranged in the molecule and that which shows a strong physiological activity. It also has the potential to be applied as a drug or as a pharmacological reagent.

Recently attempts have been made to develop useful drugs by screening marine organisms for pharmacologically active substances, e.g. anticarcinogenic, antibiotic, growth promoting or inhibiting, hemolytic, analgesic, antispasmodic, hypotensive, and hypertensive agents. This activity has led to the following two successful results: the first is tetradotoxin, which has been used as a pharmaceutical reagent. The second example is a new insecticide, which has been developed from *Neries* toxin. Among anglers it has been a well-known fact that flies die from contact flies die from contact with the dead marine annelid, Lumbri Nereis *(Lumbriconeries brevicirra)* which is commonly used as bait. The toxic constituent was first isolated and named nereistoxin in 1934 (Hashimoto, 1979).

Cartaphydrochloride was selected as the most favourable compound to be developed on the basis of its activity against the rice stem borer and other insect pests, its safety to man and because of other requirements for an agricultural insecticide, It has been marketed

since 1966, and has attracted much attention, because classical insecticides such as DDT and r-HCH (BHC), have been criticized because of their toxicity to warm blooded animals and since resistant strains of insects have been developed. This suggests that thousand of toxic marine organisms may be regarded as precious treasure for man.

1.6 VENOMOUS MARINE INVERTEBRATES:

Venomous marine invertebrates that cause injuries by stinging can be grouped into five major categories, following Halstead (1980):

1. Poriferans : Sponges
2. Coelenterates : Hydroids, Jellyfish Corals and Sea anemones
3. Molluscs : Univalve shellfish and Octopii
4. Annelid worms : Stinging or bristle worms
5. Echinoderms : Sea urchins

1.7 VENOMOUS CEPHALOPODS:

Cephalopods are of interest to the biotoxinologists because on occasion they may be poisonous to eat, and most of them possess a well-developed venom apparatus involving their so-called salivary glands. Bellesme (1879) was among the first to publish regarding the toxic effects of cephalopod saliva on crustaceans.

The history of intoxications resulting from the ingestion of poisonous cephalopods is brief. One of the few references to appear in the literature is by Read (1939), who stated that the oriental cuttlefish;

Sepia is sometimes poisonous to eat. The only reports of any actual outbreak of cephalopod poisoning were made by Motohiro and Tanikawa (1952 a,b), and Kawabata *et al.* (1957). From 1952 through 1955, persons in Japan poisoned from eating squid and octopus totaled 2, 874 (Halstead and Courville, 1965).

Haplochlaena macualosa is a species of octopus whose occurrence is widespread in the southern coastal waters of Australia. It secretes in its posterior salivary glands a potent neurotoxin, maculotoxin. It was responsible for the deaths of at least three people in Australia (Freeman and Turner, 1970) A number of cases are known in which human victims have received sublethal doses of venom which has produced neurotoxic symptoms followed by complete recovery. Victims have described symptoms of weakness, nausea and respiratory difficulties. The other species of octopus found in Australian waters have been shown to contain no neurotoxin (Sutherland and Lane, 1969).

1.8 CEPHALOPOD RESOURCES – WORLD SCENARIO:

There is general consensus that cephalopods constitute potentially an important marine living resource where future exploitation to a high magnitude is possible. Estimates are that the global production of cephalopods which stood at 1.5 million tons in 1985 (Silas, 1985) can be increased many fold. Nearly 70 percent of presently exploited resources of squids, cuttlefishes and octopus come from the neritic waters where directed fisheries for this resource are

sparse. While cephalopods are considered a nonconventional resource in many areas, its high-protein and low fat content makes it an important item of human diet.

1.8.1 OCTOPOD RESOURCES:

Among cephalopod resources, octopods are the least exploited in India. It is known that octopods occur in fair quantities in different parts of Indian coasts (Hornell, 1917).

As many as 200 species of Octopodidae are known to occur in the world oceans (Worms, 1983). Of these about 60 species are known from the Indian Ocean (Roper *et al*. 1984). Thirty-eight species of octopods belonging to the family Octopodidae, Tremoctopodidae, and Argonautidae abound the Indian seas. Of these the common species are *Octopus dollfusi, Octopus globosus* and *Cistopus indicus.*

Many octopods such a *O.dollfusi, O.cyaneus, O.aegina* and *Cistopus indicus* occur in shallow coastal waters in the intertidal and subtidal areas among rocks, stones or corals hiding themselves among crevices. Unlike squids and cuttle fishes octopods lead a solitary life and do not form schools. Some species such as *Berrya* spp occur in deep waters along the continental shelf edge and upper continental slope. A few others such as *Ocythoe* and *Tremoctopus* are pelagic (Voss, 1973) Some octopods are known to make seasonal migrations, which are influenced by breeding activity. Octopods are

exclusively carnivorous and they feed on crustaceans, fishes and molluscs.

At present there is not much demand for octopods within the country except in the bait fishery and where they are caught as subsistence fishery. There appears to be scope for developing an octopus fishery particularly in the Andaman Nicobar islands, the Lakshadweep, the Gulf of Mannar and Plakbay and the Gulf of Kutch (Silas,1985). The present status and future prospects of the Cephalopod fishery in India, based on (Silas, 1985), are presented in Fig. 1.

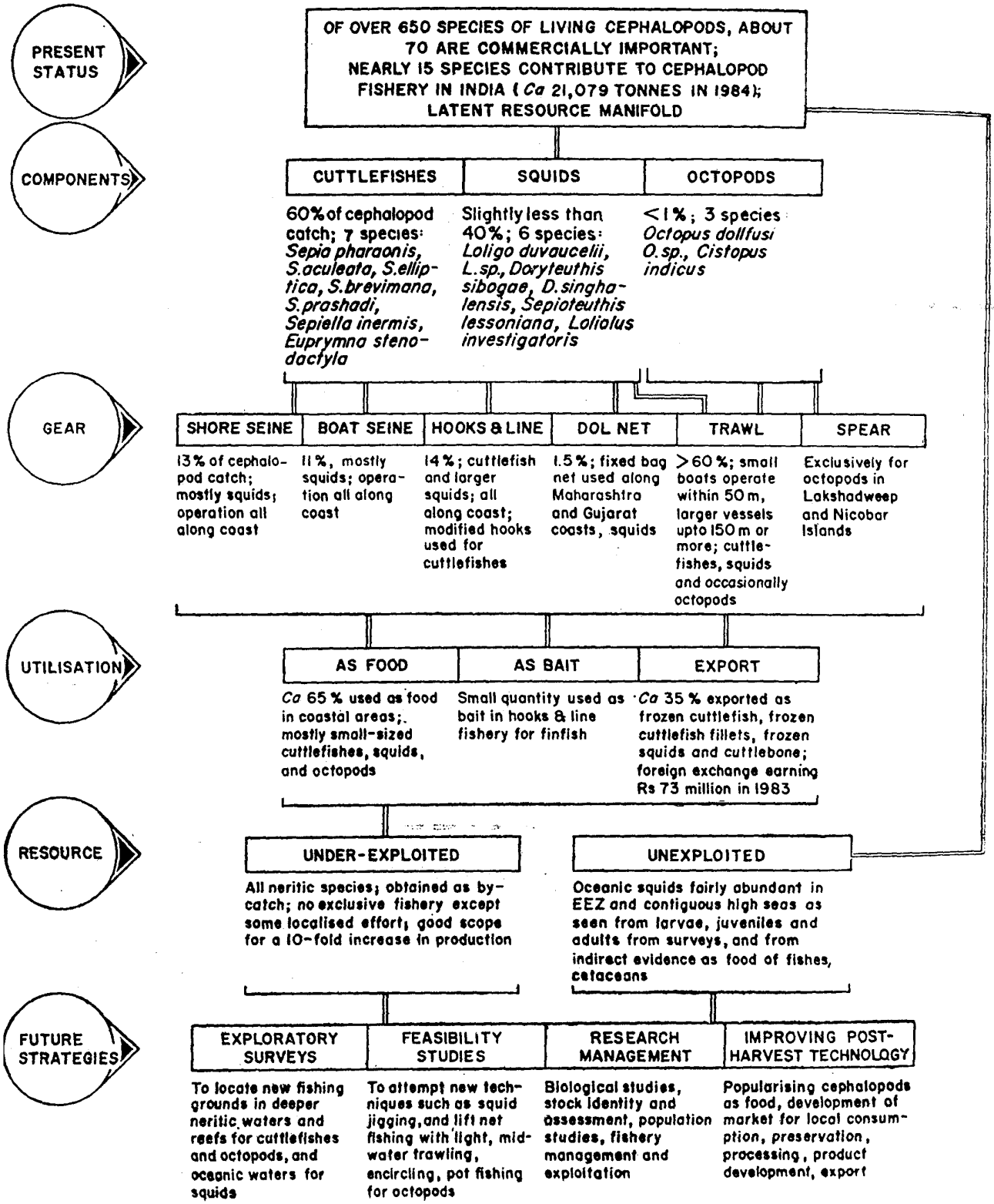
1.9 SCOPE OF THE PRESENT STUDY:

Cephalopods are being used as food for human consumption and as bait in the fishery for a wide verity of predators, viz., large perches, tunas, etc. and are also exported.

Other than this, cephalopods have been used in neurophysiological studies (Rosenburg, 1973). A good amount of effort has gone into the study of the giant nerve fibre system and the stellate ganglion of squids to understand split second body movements and reactions. The basic processes connected with the nerve excitation and nerve conduction have been clarified by the study of squid gaint axons (Hodgkin, 1964; Tassaki, 1968,1968). More significant has been the outcome of the study of the nervous system of the *Ocotopus* by Wells (1962,1966,1978), Young (1971,1977) and of the nautilus by Young

FIG. 1

CEPHALOPODS: RESOURCES AND UTILISATION



(From Silas, 1985)

(1965). We have made no use of such excellent material available in our waters for biochemical and neuropharmacological research. Further more, Octopus toxins are being used as pharmacological tools. The possibility of synthesizing new and more effective anesthetic agents need much attention.

There were no reported cases of fatalities due to the bite of this species from our waters; perhaps as fishermen instinctively know it from its distinctive coloration and know that it is an undesirable species to handle. According to Silas (1985) a lot of basic research need to be done on the toxin of this and the other cephalopods. The present study was, therefore, taken up to assess the venomous nature of the salivary gland secretion of the Old woman octopus, *Cistopus indicus* (Orbigny, 1840) from Mumbai waters, whose systematic position and other details, based on Roper *et al.* (1984,) are as follows:

Species: *Cistopus indicus* (Orbigny, 1840)

Octopus indicus Orbigny,1840,*in* 1834-1848,*Hist.Nat.Ceph.Acetab.* **24.**

Synonomy: *Octopus indicus* Orbigny, 1840; *Cistopus bursarius* (Steenstrup MS) Hoyle, 1886.

FAO names: English - Old woman octopus; French - Poulpe vieille femme; Spanish - Pulpo per forado

Diagnostic features: Mantle elongate; neck constricted; head narrow. Arms long, slender, attenuate tips; dorsal arms (I) always longest and stoutest, IV shortest; dorsum covered with fine, low, widely-spaced warts; a small pouch occurs on each segment of the web between the bases of the arms; these 8 water pouches communicate

with the sea water through small pores that open on the oral surface of the web; right arm III hectocotylized with a very small ligula (3% of arm length) that appears smooth and poorly developed; 10 or 11 lamellae on demibranch of gill.

Geographical distribution: Indo-Pacific: mostly Indo-Malayan region, the Philippines, China, Bangladeh, India and Pakistan, and recorded off Mozambique.

Habitat and Biology: A benthic species, occuring from 0 to 50 m depth on mud bottom in Hong Kong.

Size: Maximum total length 60 cm; maximum mantle length 18cm; weight 2kg.

Interest to Fisheries: The species is a primary commercial octopod in most Asain markets. It also supports localized and subsistence fisheries in the Philippines. About 50 metric tons landed in Hong Kong per year.


Review

2. REVIEW

2.1 HISTORY OF RESEARCH:

The cephalopod octopods and squids have attracted the attention of biologists since the time of Aristotle. The "salivary" or "venom" glands, whose full function is still unknown completely, have been studied since the time of Paul Bert (1867).

One of the earliest references to the nature of the salivary glands of cephalopods was by Bert (1867), who attributed to them a digestive function. Kraukenberg (1878) regarded them as simply mucous glands. In his monograph on the physiology of octopus, Frederiq (1878) emphatically stated that octopod's saliva does not possess any digestive properties. Bourquelot (1882,1885) came to a similar conclusion in his studies on digestion in cephalopods.

Krause (1895,1897) performed a unique experiment in which he electrically stimulated the buccal ganglion, inserted a minute canula into the salivary duct and thereby obtained pure saliva from the posterior salivary gland of *Octopus macropus*, which he injected into a crab and frog and observed the resulting paralysis. Krause was apparently the first to test the cephalopod saliva on warmblooded vertebrates. Pelseneer (1897) believed that the secretion of the posterior salivary gland had both venomous and digestive properties. Rouville (1910 a, b, c) pointed out that the anterior salivary glands do produce a poison but it is less toxic than that obtained from the

posterior salivary gland. Calmette (1908) and Bauer (1909) referred to the role of salivary glands as venom organs.

Bellesme (1879) was among the first to describe the paralytic effect of cephalopod saliva on crustaceans. Lo Bianco (1888) for the first time that there is a substance in the posterior glands of octopus that is toxic for crabs. He observed that the octopus did not kill with the use of either its beak of tentacles but apparently injected a poison believed to be secreted by the posterior salivary glands.

Livon and Briot (1906) working chiefly with *Eledone,* claimed that the poison from the posterior salivary glands is a substance of complex nature, probably a kind of albuminoids, as proteins were called at that time. According to them, the poison is specifically active against the central nervous system of crustaceans. Curiously enough, these observations did not receive any attention at that time

Henze (1913), succeeded in isolating the tyramine from the posterior salivary gland of *Octopus vulgaris* and claimed immediately that this was the crab-paralyzing agent present in the glands and saliva.

In his interesting account of dangerous giant cephalopods, Bartsch (1917) cited that case of Montfor was the first to record in 1802 an otopus biting a human being. Useful general reviews of this early period have been published by Phisalix (1922), and Pawlowsky (1927).

Bottanzzi (1921) suggested that another factor, histamine might be present and was responsible for the paralyzing actin against crustaceans. Since then a number of substances have been found in the posterior salivary gland of cephalopods. In addition to tyramine, histamine, acetylcholine and taurine, two new amines were discovered in the glands, viz. i) p-hydroxyphenyl - ehanol amine (octopamine); and ii) 5- hydroxytryptamine (5-HT, serotonin, enteramine) by Erspamer (1948 a, b) and Erspamer and Asero (1953).

In *H.maculosa*, octopamine was shown to be absent. It was identified in *O, vulgaris, O. macropus* and *Eledone moschanta* (Erspamer, 1948 a; Erspamer and Boretti, 1951). Afterwards it was found to be present in *H. maculosa* (Simon *et al.* 1964). Erspamer (1948 b) found tyramine content was quite low in *H. maculosa* compared to other species of *Octopus*. However the serotonin present in *H. maculosa* was about half that reported much later in *O.vulgaris* (Welsh and Moorhead, 1960).

Enzymes found in the salivary gland include amine oxidases and fluvanic enzymes. Because tyramine (Tya) and histamine (HM) were present, it was first postulated by Bottazzi (1921) and later by Bacq and Ghiretti (1953) that tyrosine (Tya) and histidine ("Hd") decarboxylases might be present. This was experimentally verified later by Hartman *et al.* (1960).

Other pharmacologically and physiologically active substances found in the glands include the guanidine bases, octopine and agmatine, acetycholine, nordrenaline ("NA") and possibly even 11-hydroxysteroids. Later Bacq and Ghiretti (1953) compared posterior salivary glands to adrenal glands of vertebrates.

Polyphenols, phenolamines and indoleamines have been suspected as endogenous substance of these glands since 1909, from histochemical evidence, colour reactions and pharmacological assays, particularly Tya, which was first isolated by Henze in 1905, and the role and function of which has been studied and speculated by many investigators (Hartman *et al.* 1960). Meta - tyramine ("m-tya") also has been invoked (Bacq and Ghiretti, 1953). Serotonin (5-Hydroxytryptamine, Enteramine, "5-HT") was suspected as an endogenous constituent of the posterior glands by Erspamer (1948 a, b) who subsequently isolated it from the glands and showed it to be identical to authentic material obtained by synthesis; it was postulated that it was a constituent of the "venom", a neurohumoral transmitter, and an endocrine determining, among other things, the nervous control of the melanophore system.

Hartman *et al.* (1960) found out 6 amino acids in the posterior salivary glands of *H.maculosa* by chromatography; viz., glycine, histidine, asparagine, phenylalanine, tyrosine and tryptophan. In addition, three physiologically active amines were shown to be present: serotonin tyramine and histamine. However, it was easily demonstrated

neither tyramine nor any other amine present in glands was able to kill a crab, when injected into the animal in an amount equal to that present in a lethal quantity of saliva (Ghiretti, 1949) These amines, of course, have their own physiological activity, but the paralysis produced by salivary gland extracts could not be fully explained by presence of amines (Ghiretti, 1960) Later the principal toxins were found to be proteins.

2.2 VENOMOUS OCTOPODS:

2.2.1 Cephalotoxin:

Ghiretti (1960) demonstrated the toxic activity of saliva from *Octopus vulgaris* against Crustacea; while the neurotoxic component, cephalotoxin, was a protein, exciltory responses observed were due to octopamine, tyramine and serotonin. The biosynthesis of these amines was dependent on specific decarboxylases (Hartman *et al.*1960). The same toxin showing strong toxicity in crustaceans was also obtained from *O.macropus*. However, the toxin seemed to differ in chemical composition from one species to another (Ghiretti, 1960).

2.2.2 Maculotoxin:

The toxin from *H.maculosa* was believed to contain at least two toxic compounds (Sutherland and Lane, 1969; Simon *et al.* 1964) but Freeman and Turner (1970) were only able to detect one major toxic substance, designated maculotoxin (MTX). Croft and Howden (1972) found that one major toxic compound was present in *H. maculosa* as well as a minor one, which appeared to possess chemical properties

very similar to that of maculotoxin; comparative death times for mice were considerably longer for the second toxic compound than that of maculotoxin. These toxins were very similar compounds since their IR spectra were identical and they had same RF on a TLC plate.

Freeman and Turner (1970) have emphasized a close similarity between maculotoxin and tetrodotoxin. It was found that, maculotoxin was pharmacologically very similar to tetrodotoxin (TTX) and saxitoxin (STX). Evidence was presented which suggested that respiratory failure after intravenous injection was due to blockage of muscular nerve axons. The toxin blocked transmission in the sciatic nerve of toad and rat. At low dose levels, it appeared to have a neuromuscular blocking activity. While at higher dose levels the muscle membrane also became inexcitable. After intravenous injection of the toxin, mice became agitated, and the hind limbs splayed and became paralyzed. Breathing was labored and ceased after 60-90 sec. Terminally there were brief but violent convulsions. At autopsy the heart was found to be beating irregularly. But there was no macroscopically evident pathology.

Crone *et al.* (1976) concluded that MTX was very similar to TTX since the pharmacological (Freeman and Turner, 1970) and chromatographic (Jarvis *et al.* 1975) properties were similar to those of tetradotoxin (TTX) from the puffer fish.

Trethewie (1965) found that the main effect of the venom of *H.maculosa* was respiratory paralysis. Cardiac effects were largely

secondary, possibly due to anoxia, and appeared in the intact animal but to a significant degree in the isolated organ. In the intact animal respiration failed before the blood pressure fell significantly. The respiratory excursion fell and the rate slowed within a few seconds of the intravenous injection of 0.5 ml of gland extract. With 1.5ml of gland extract, they found lack of respiration in 1 to1.5 min of injection. Further, they tested Maculotoxin on isolated perfused heart of cat, phrenic nerve diaphragm of rat and isolated jejunum of guinea pig and found a reduced coronay blood flow and relatively unchanged heart beat in isolated perfused heart. The jejunum of guinea pig showed immediate contraction with delayed relaxation. Neuromuscular transmission was inhibited in diaphragm preparation.

Simon *et al.* (1964) have found that isolated phrenic diaphragm preparation was at least ten times more sensitive than isolated gastrocnemius preparation of toad to the salivary gland venom of *O.maculosus*. The paralysis brought about by the sting of octopus would suggest that the venom had some neuromuscular blocking activity. Simon *et al.* (1964) and Trethewie (1965) revealed that the venom of *H.maculosa* contains a low molecular weight neurotoxin. Sutherland and Lane (1969) have shown that macualotoxin was a relatively low molecular weight molecule which did not elicit an antigenic response in the body. This was consistent with their findings of its rapid diffusion into body.

Gel permeation chromatography results of Croft and Howden (1972) confirmed that maculotoxin had a low molecular weight probably less than 700. Dulhunty and Gage (1971) extracted MTX with a molecular weight less than 540 salivary gland toxins of *H.maculosa*.

The small blue-ringed octopus, *Octopus* (*Haplochlaena*) *maculosus* Hoyle, has been responsible for a number of cases of temporary paralysis among people who have handled the animal on Australian beaches. Victims have described symptoms, which included weakness, nausea and respiratory difficulty. There have been 3 fatalities. Death has been ascribed to respiratory failure due to flaccid paralysis, with a time of onset of 5-10 min (Freeman and Turner, 1970).

Sheumack *et al.* (1978) reported maculotoxin (mol wt >5000) secreted by salivary glands contain at least one fraction identical to TTX, which blocks peripheral nerve conduction by interfering with sodium conductance in excitable membranes.

Botazzi and Valentini (1924) studied the effect of *Octopus macropus* venom on dogs and observed that extensive subserous hemorrhages of stomach, intestines and throughout the omentum, as well as congestion of spleen.

2.2.3 Eledoisin:

The posterior salivary glands from Mediterranean species of *Eledone altrovandi* contained a substance that caused contraction of

smooth muscle and hypotension in mammals (Erspamer, 1949). The active principle, first called moschatin, was later renamed as eledoisin, in1962. Erspamer and Anastasi (1962). isolated this substance and presumed that it has polypeptide character it was also showed that eledoisin was fifty times more potent than acetylcholine, histamine or bradykinin in its ability to provoke hypotension in dog.

2.2.4.Indian Scenario:

Octopus herdomani, O. globossus, O. rogosus, O. dolfusi, and O. hongkongenisis are reported to be common in Indian waters (Bal and Rao, 1984). In Lakshadweep area, the octopii are regularly hunted and used for food but along coasts of the mainland their utilization as food is comparatively less (Bal and Rao, 1984)

However, there had been no reports of poisoning due to consumption of octopii or due to bites. The only reference available is that of Bal and Rao (1984) who mentioned as follows: "Occasionally in the fishermen's net along the Palk Bay, a small slender species of octopus is reported to occur and is know to be poisonous and is locally called as 'VISHAKANAVAI' meaning ""poisonous octopus"". Its bite on the limbs was reported to be very painful, as the sting of scorpion.

Lendhe (1995) reported the salivary gland extracts of *Octopus globosus* from Mumbai waters to be neurotoxic and hepatotoxic to experimental mice.

2.3 POISONOUS CEPHALOPODS:

The history of intoxications resulting from the ingestion of poisonous cephalopods is brief. One of the few references to appear in the literature is by Read (1939), who stated that the Oriental cuttle fish *Sepia* is sometimes poisonous to eat. The only reports of any actual outbreak of cephalopod poisoning were made by Motohiro and Tanikawa (1952a,b) and Kawabata *et al.* (1957). From 1952 through 1955,persons in Japan poisoned from eating squid and octopus totaled 2,874 (Halstead and Couriville, 1965).

Material and Methods

3. MATERIAL AND METHODS

3.1 LOCATION:

The study was carried out in the Aquatic Biotoxinology Laboratory of Central Institute of Fisheries Education, Mumbai.

3.2 SPECIMEN COLLECTION:

Dead specimens of the octopus, *Cistopus indicus* (Orbigny 1840) (Plate 1), which were frozen on board immediately upon capture, were procured from Ferry Warf, Mumbai, and brought to the laboratory in ice and stored at -20°C until use. Taxonomic identification was done following Roper *et al.* (1984).

3.3 EXTRACTION OF CRUDE TOXIN :

Crude toxin was extracted from both male and female specimens following Shiomi *et al.* (1987). The salivary glands (Plates 2&3) were dissected out, ground in saline phosphate buffer on a motorized homogenizer and centrifuged at 15,000 rpm for 5 minutes at 4°C. The supernatant was collected and again centrifuged at 15,000 rpm for 5 minutes. The supernatant was collected and lyophilized (LabConco Freeze Dry System) and maintained at 4°C until use, as crude toxin.

3.4 HPLC FRACTIONATION OF THE CRUDE TOXIN:

20 mg of the crude toxin was dissolved in 1ml of phosphate buffer. This was filtered using 0.45µ Millipore filter paper. 20 µl of the

filtered toxin was used for fractionation on HPLC (Hewlett Packard Series 1050 System).

The run characteristics were as follows:

Column used	: H.P.Hypersil BDS - C18.
Mobile phase	: 0.02M Phosphate buffer (pH 7.5) and 1M NaCl gradient
Flow rate	: 1ml/min.
Wave length	: 400nm.

The fractions were collected manually at 1-minute intervals.

3.5 ESTIMATION OF PROTEIN:

Protein estimation was done following Peterson (1977) using Bovine Serum Albumin as standard. Ten concentrations of the standard ranging from 0.1 - 1.0 mg were taken, made up to 1 ml with distilled water and added with 1 ml reagent A prepared by mixing equal parts of Copper tartarate carbonate (CTC), 10% Sodium Dodecyl Sulphate (SDS), 0.8N NaOH and triple filtered water and allowed to stand for 10min at room temperature, a which 0.5ml of reagent B (Folin Ciocalteu Phenol) was added. The test tubes were shaken gently to mix the solutions and incubated for 10 min in the dark. The absorbance at 750nm was read spectrophotometrically.

3.6 HEMOLYSIS AND HEMAGGLUTINATION ASSAYS :

3.6.1 Hemolysis :

Chicken blood was collected in sterile glass vials precoated with 2.7% EDTA as anticoagulant @ 5mg/ml blood from a nearby slaughter

house and brought to the laboratory. The blood sample was washed thrice with normal saline and centrifuged at 5,000 rpm for 7 minutes at 4^0 C. The supernatant was discarded in each case and 3ml or 4ml of the final residue (packed RBC) was taken and 97 or 96 ml of normal saline was added to get a 3% or 4% erythrocyte suspension respectively.

The hemolytic activity of the crude toxin and HPLC fractions on chicken blood was estimated following by Pani Prasad and Venkateshvaran (1997) in V-bottom Laxbro microtitre plates. 100µl of normal saline was transferred to each well. 100µl of the toxin was added to the 1st well in a row, mixed thoroughly and from it 100µl was transferred to the next well. This process was repeated till the last well and from the last well 100µl was discarded. 100µl of 4% erythrocyte suspension was added to all the wells. The plates were incubated at room temperature for 2 hours. Appropriate positive and negative controls were set up using distilled water and normal saline respectively. Formation of button in wells was taken as negative and complete red coloration was taken as positive. Reciprocal of the highest dilution of the mucus toxin showing the hemolytic pattern was taken as 1 Hemolytic Unit (HU). Specific hemolytic activity was also calculated as number of Hemolytic Unit per mg of protein (HU/mg).

3.6.2 Assay For Hemagglutination:

The hemagglutinating activity of crude toxin and HPLC fractions were tested following the method of Liang and Pan (1995) in U - bottom

microagglutination plates (96 wells, Tarsons India). 25µl of the sample were used for the test on 3% erythrocyte suspension of chicken blood. The plates were shaken for 1min after addition of the erythrocytes. The plates were subsequently incubated for 1 hour at 4°C. Formation of clumped RBCs was taken as positive and button formation was taken as negative.

3.7 MOUSE BIOASSAY:

Kasauli strain male albino mice, 20 ± 2 g in weight, were procured from M/s. Haffkine Biopharma Ltd., Mumbai and were maintained in laboratory. Two sets of bioassays were conducted, one using the crude toxin and the other using the fractions. Triplicate sets of mice were injected intraperitoneally (i.p) with varying doses of crude mucus toxin and fractions. Appropriate controls received an equal volume of 0.02M phosphate buffer (pH 7.5) in each case. The time of injection, activities of the injected mice and time taken for death were noted.

Convulsion, vigorous jumping, rolling over, paralysis of hind limbs, apathy, excessive urination, dragging of the hind limbs, ataxia, palpitation, arching the back and gasping were taken as indicative of toxicity.

3.8 AUTOPSY:

The mice, which died upon envenomation, were autopsied for assessing any abnormalities like hemorrhage, pale or dark discoloration

of heart, kidney and liver or granular appearance on these organs, septicemia /dropsy, and other gross anatomical changes, if any.

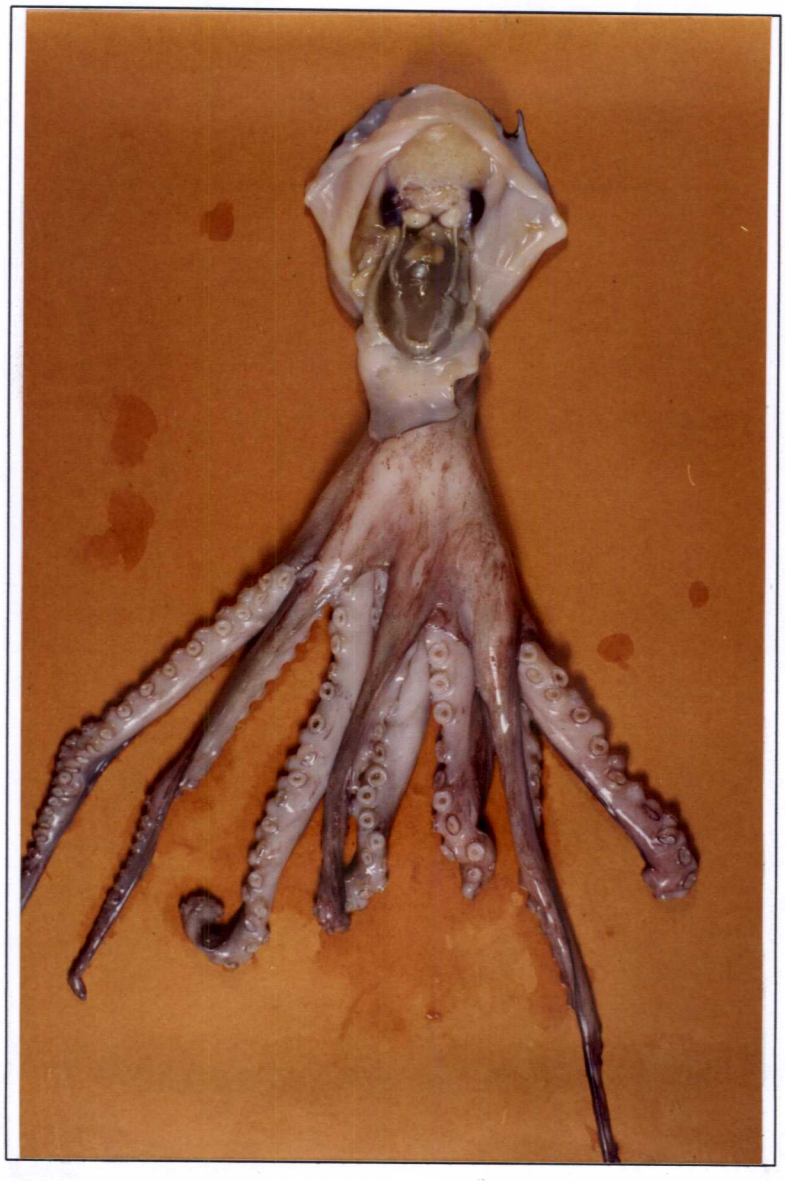
3.9 HISTOPATHOLOGICAL STUDY:

Kidney, heart and liver from the mice dead upon envenomation were excised and fixed in neutral buffer (pH 7.0) fixative overnight, and then washed thoroughly to remove the fixative. The tissues were dehydrated in 50%, 70% and 90% alcohol for 45 minutes separately, cleared in xylene for 2 hours and embedded in paraffin wax. They were then blocked, allowed to cool, cut on a rotary microtome at 7μm and mounted on to glass slides.

Sections were dewaxed in xylene and dehydrated serially in alcohol and were stained in Delafield's haematoxylene for 7 min. Stained sections were washed in tap water followed by Scott's tap water substitute and then in distilled water. They were stained in eosin for 5 min. The sections were then washed in water and dehydrated through 70%, 90% and 100% alcohol for 1 min each and finally mounted with DPX. Prepared sections were examined and photographed on an Olympus microscope.

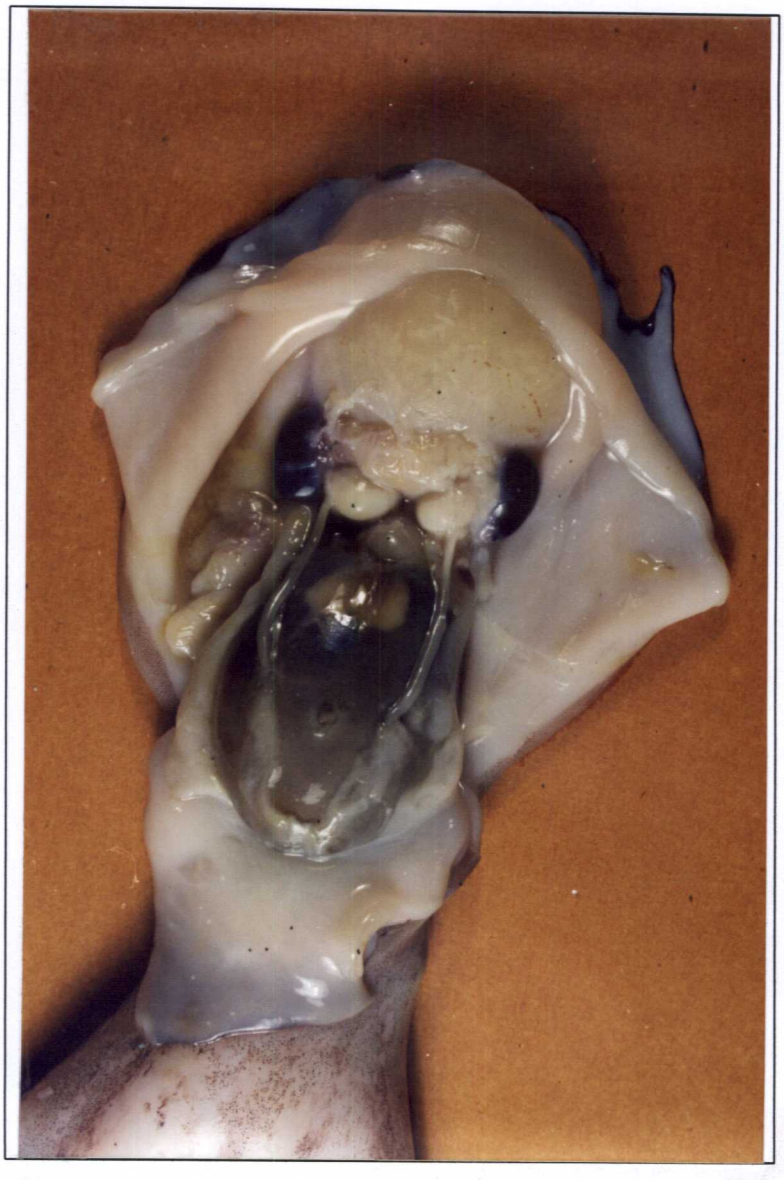
Showing the Old woman octopus *Cistopus indicus* (Orbigny, 1840)

Plate 1



Salivary Glands of Old woman octopus
***Cistopus indicus* (Orbigny, 1840)**

Plate 2



Salivary Glands of Old woman octopus
***Cistopus indicus* (Orbigny, 1840)**

Plate 3


RESULTS

4. RESULTS

4.1 EXTRACTION OF CRUDE TOXIN:

Salivary gland extracts were obtained from 16 specimens of *Cistopus indicus* of an average size of 397 g / 40 cm and upon lyophilization yielded a crude extract of 6.2 mg/ml of extract (=6.98mg/g of extract).

4.2 PARTIAL PURIFICATION BY HPLC ANALYSIS:

A total of 6 peaks were attained at 1.61, 2.28, 2.74, 3.5, 4.51, 5.64 minutes. The fractions so collected were designated F1-F6 and stored at -20^0 C until use.

4.3 MOUSE BIOASSSAY:

4.3.1Crude Toxin :

The following symptoms were commonly observed upon intraperitoneal injection of the crude extract: Convulsions (particularly of hind limbs), palpitation, defecation, urination, escape reaction, and bleeding from the mouth. The minimum lethal dose (MLD) was 0.5 ml at 2 minutes and 30 seconds. Results of the bioassays are presented in Table 1 and Fig. 2.

4.3.2 Fractions:

The following symptoms were commonly observed upon intraperitoneal injection: apathy, excessive defecation, and dragging of hind limbs. F2 at 0.5 ml was the most lethal fraction killing the mice in

23 seconds, at which dose frothing from mouth was observed. F6 killed the mice in 58 sec at 0.5 ml. The remaining fractions F1, F3, F4, F5 were not lethal but produced symptoms of toxicity from which the mice recovered within 20 minutes. The results are presented in Table 2.

4.4 AUTOPSY:

Autopsy revealed hemorrhage inside the body cavity and pale discoloration of heart in all envenomated mice.

4.5 HISTOPATHOLOGY:

Microscopic examination of the prepared sections of heart, liver and kidney revealed the following:

4.5.1 Heart: Pathological changes in the heart was comparatively less pronounced than in the liver and kidney restricted to moderate muscular necrosis.

4.5.2 Liver: Vacuolization, nuclear condensation (pyknosis) and degeneration of hepatocytes as also occasional hemorrhages were observed. Necrosis was pronounced at certain areas.

4.5.3 Kidney: Tubular necrosis and pyknotic nuclei were more prominent in certain areas.

The results are presented in Plates 4-9.

4.6 HEMOLYTIC ACTIVITY:

Both the crude toxin and fractions failed to elicit any hemolytic activity on chicken blood. In both the cases the erythrocytes got deposited and button cells were formed at the well bottom.

4.7 HEMAGGLUTINATION:

The crude toxin (concentration: 1mg/ml) failed to elicit any hemagglutinating activity on chicken blood. The erythrocytes got deposited and button cells were formed at the well bottom.

4.8 PROTEIN ESTIMATION:

The amount of protein in the crude toxin of *C. indicus* was found to be 36.57 μg/ml. The details are presented in Table 4.

TABLE 1

MOUCE BIOASSAY WITH THE CRUDE TOXIN SALIVARY GLAND EXTRACTS OF *CISTOPUS INDICUS* ON MALE ALBINO MICE (20±2g)

S.No	DOSE (ml)	DEATH TIME (Seconds)	REMARKS
1	0.25	-	NON-LETHAL
2	0.4	-	NON -LETHAL
3	0.5	90	LETHAL
4	0.6	75	LETHAL
5	0.7	62	LETHAL
6	0.8	58	LETHAL
7	0.9	54	LETHAL
8	1.0	42	LETHAL

TABLE 2

SHOWING THE EFFECT OF THE PARTIALLY PURIFIED SALIVARY GLAND TOXIN OF *Cistopus indicus* ON MALE ALBINO MICE (20±2g)

FRACTION	DOSE (ml)	DEATH TIME (SECONDS)	REMARKS
1	0.5	-	NON-LETHAL
2	0.5	23	LETHAL
3	0.5	-	NON-LETHAL
4	0.5	-	NON-LETHAL
5	0.5	-	NON-LETHAL
6	0.5	58	LETHAL

TABLE 3

SHOWING ABSORBANCE VALUES OF THE STANDARD BSA OBTAINED AT 750 nm (CONC: 0.5mg/ml).

S.NO	CONCENTRATION OF BSA (μg/ml)	REAGENT A (ml)	REAGENT B (ml)	ABSORBANCE
1	50	1	0.5	0.595
2	100	1	0.5	0.765
3	150	1	0.5	1.097
4	200	1	0.5	1.250
5	250	1	0.5	1.352
6	300	1	0.5	1.526
7	350	1	0.5	1.547
8	400	1	0.5	1.699
9	450	1	0.5	1.808
10	500	1	0.5	1.926

TABLE 4

SHOWING THE AMOUNT OF PROTEIN IN THE PARTIALLY PURIFIED FRACTIONS OF THE SALIVARY GLAND TOXINS FROM *Cistopus indicus*

S.No	FRACTION	ABSORBANCE AT 750 nm	CONCENTRATION (μg/ml)
1	F-1	0.432	27.79
2	F-2	0.288	12.69
3	F-3	0.302	13.91
4	F-4	0.233	8.43
5	F-5	0.228	8.08
6	F-6	0.219	7.48
7	F-7	0.227	8.08
8	F-8	0.270	11.21

FIG 2.
SHOWING THE EFFECT OF THE CRUDE SALIVARY GLAND TOXIN OF *CISTOPUS INDICUS* ON MALE ALBINO MICE (20+2g).

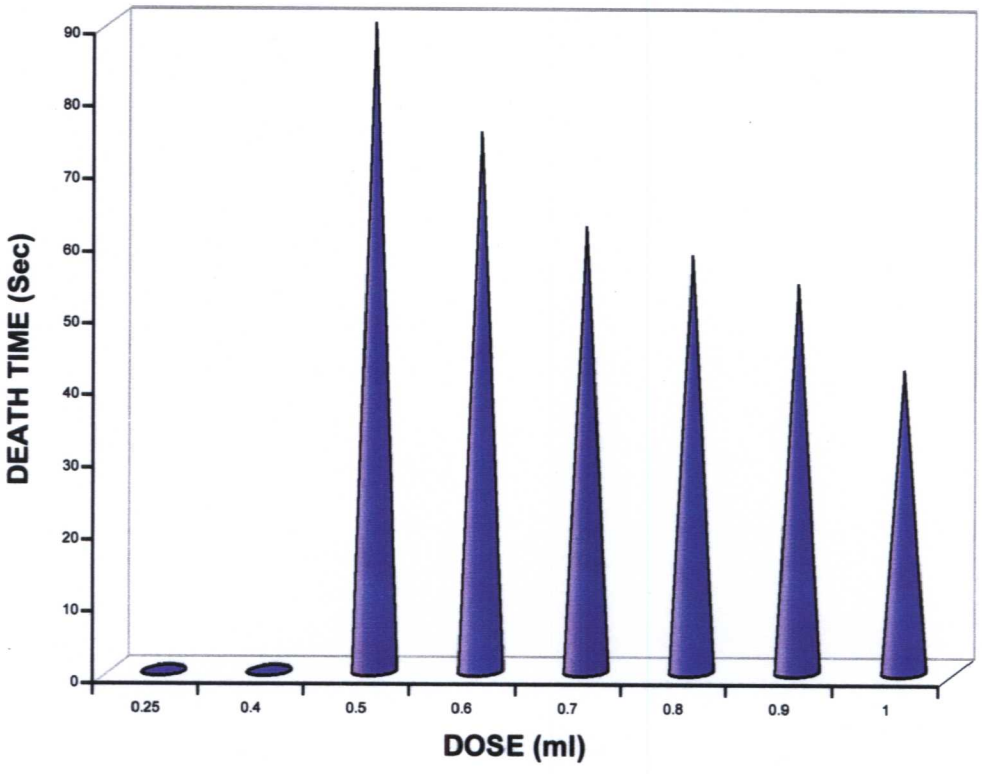
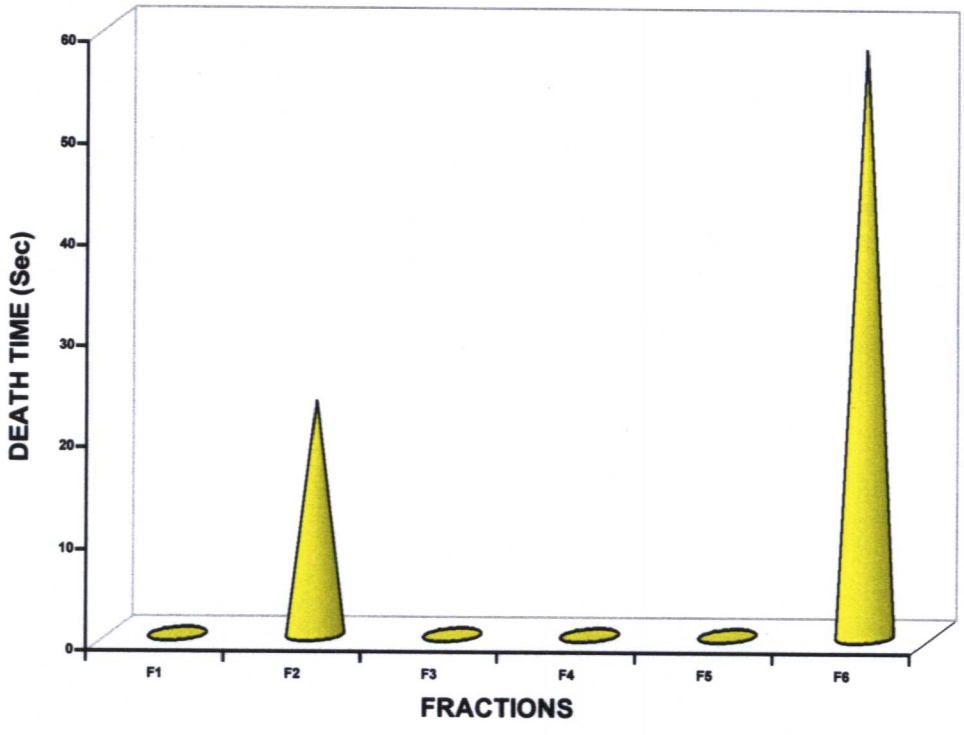


FIG 3.
SHOWING THE EFFECT OF THE PARTIALLY PURIFIED SALIVARY GLAND TOXIN OF *CISTOPUS INDICUS* ON MALE ALBINO MICE (20+2g).



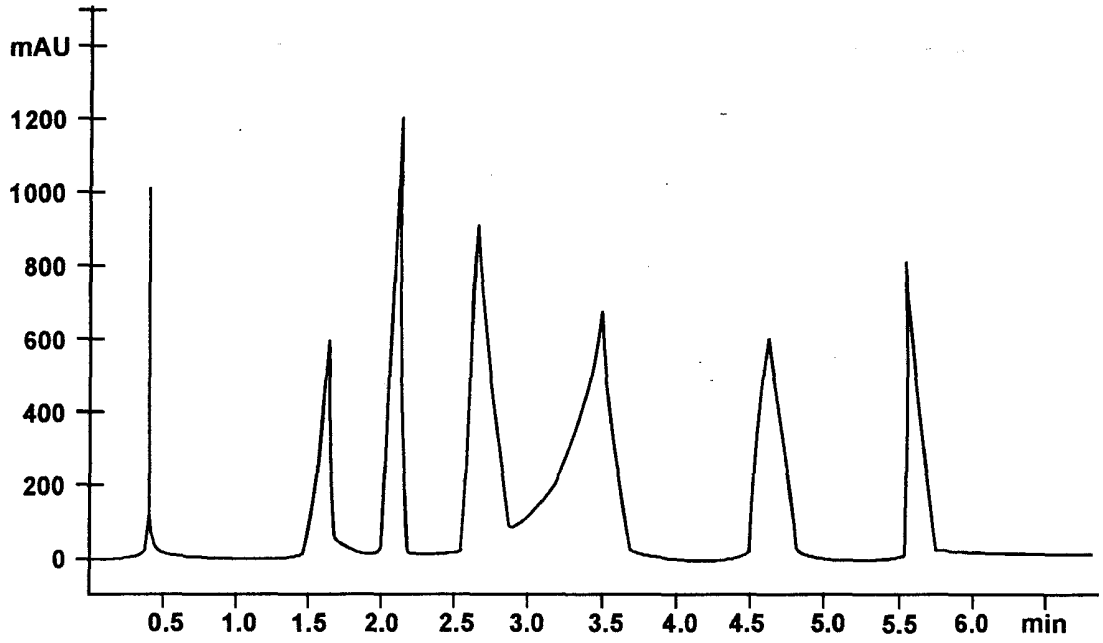
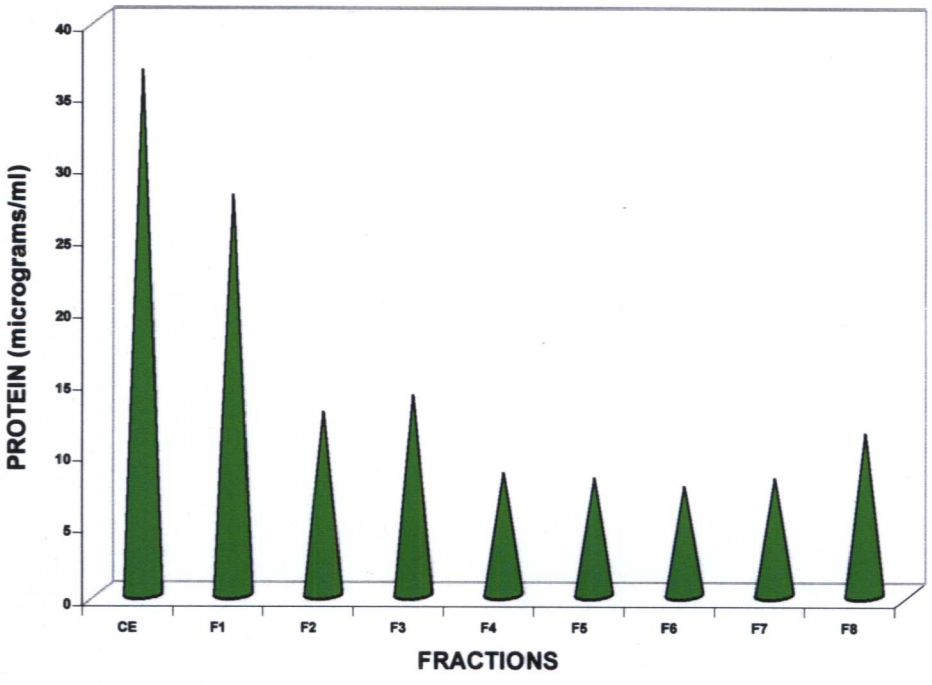


Fig:4 Chromatogram of salivary gland extract of *C.indicus*

FIG 5.
SHOWING THE AMOUNT OF PROTEIN IN THE CRUDE AND PARTIALLY PURIFIED FRACTIONS OF THE SALIVARY GLAND TOXINS FROM *CISTOPUS INDICUS*.



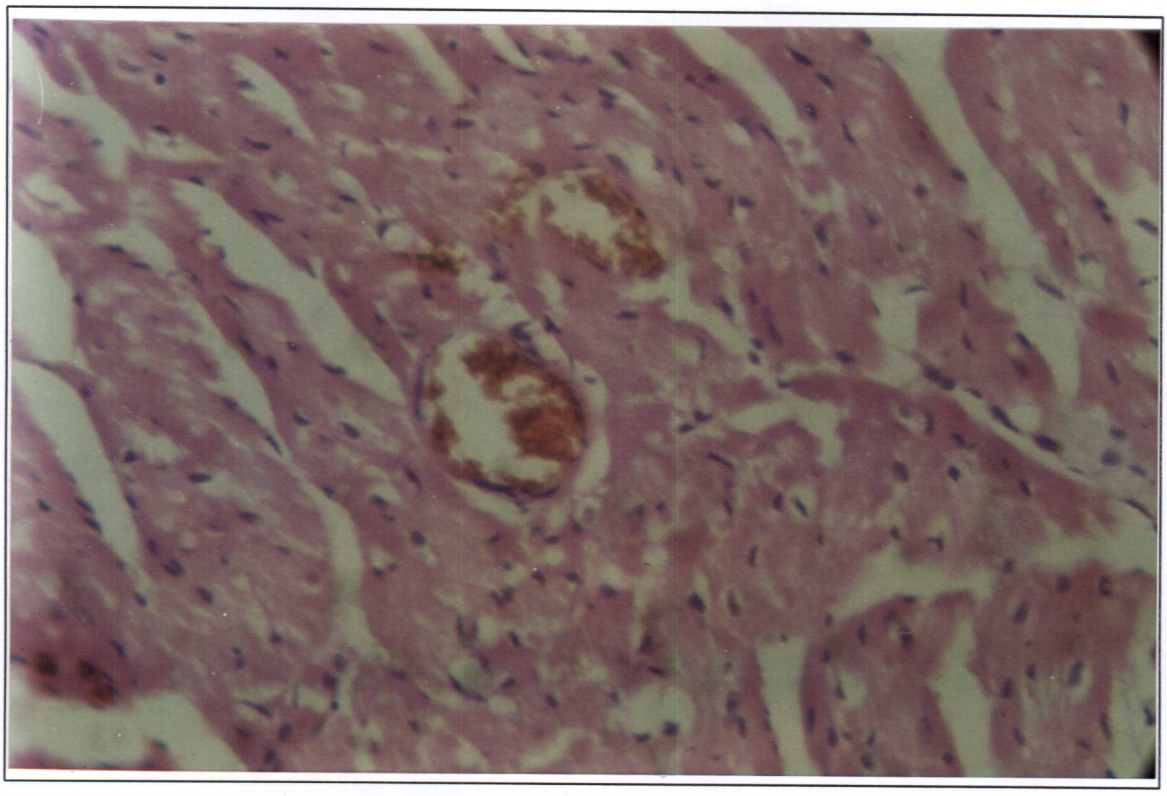


Plate 4

Cross Section of heart of control mouse Haematoxylin-Eosin (X-100)

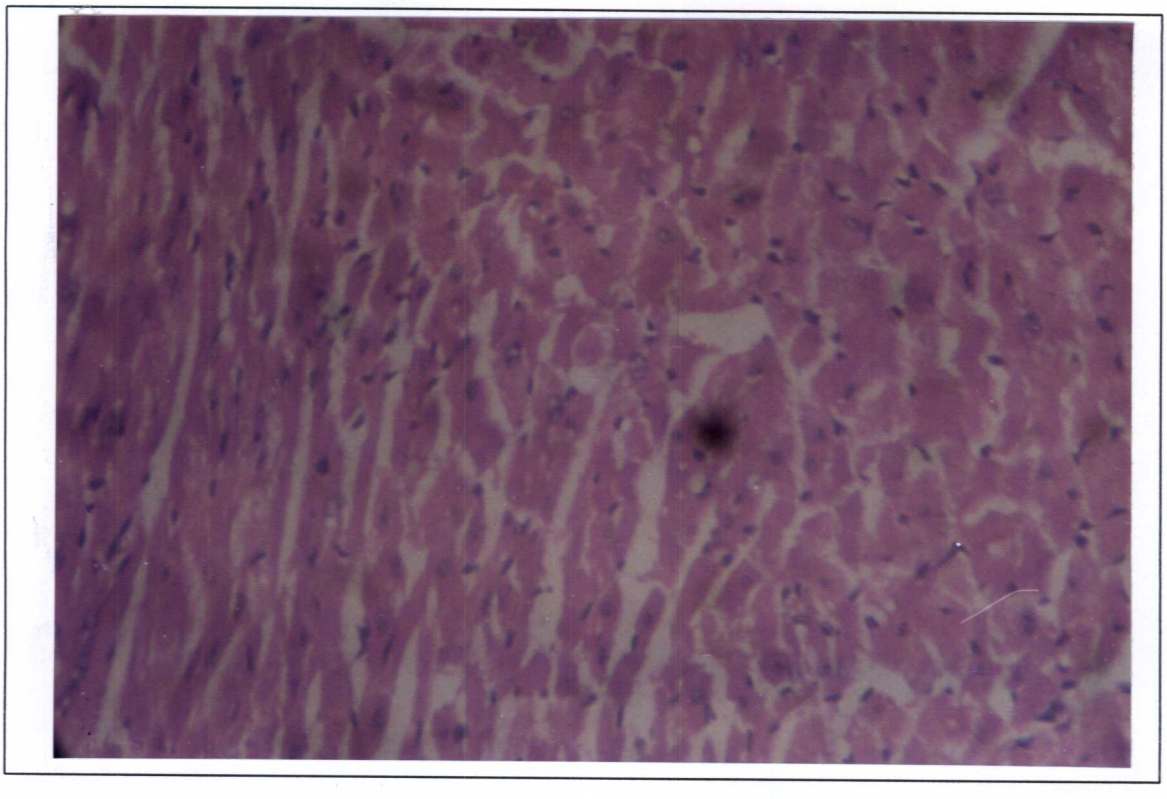


Plate 5

Cross Section of heart of mouse envenomated with purified fractions of *Cistopus indicus* (Orbigny, 1840) Haematoxylin-Eosin (X-100)

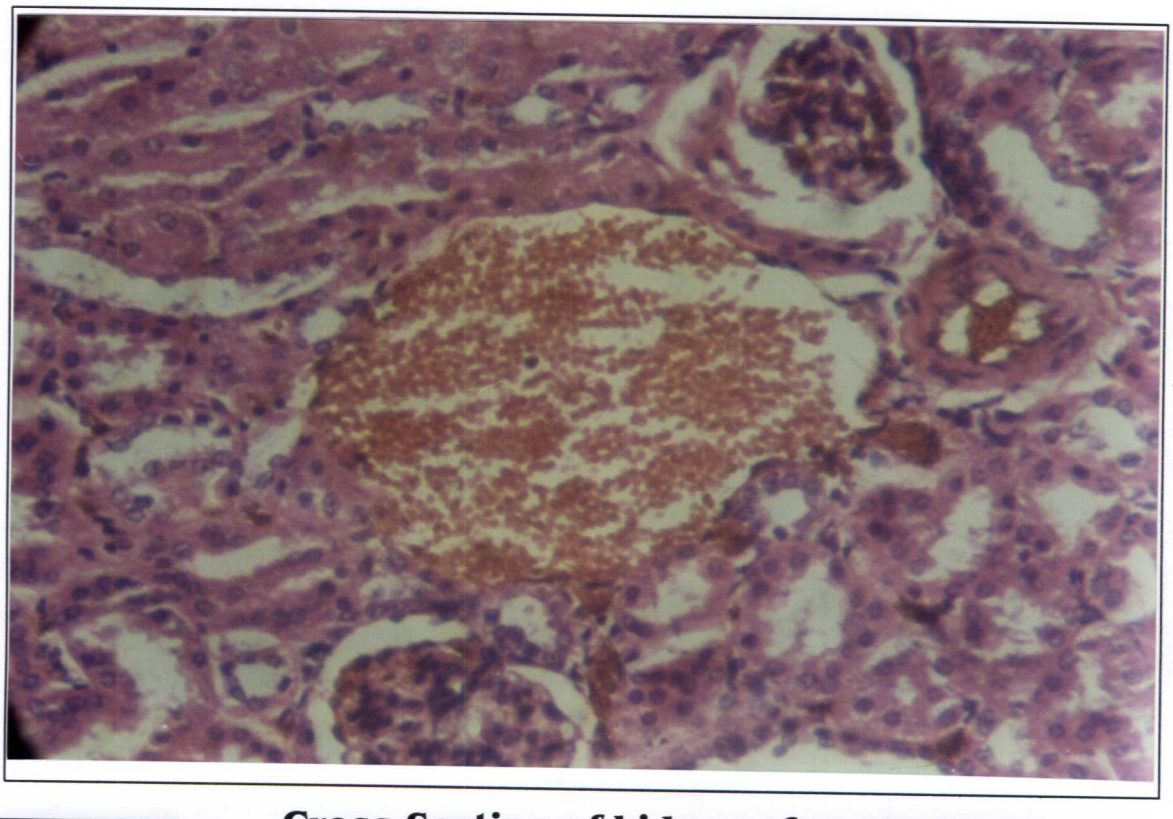


Plate 6

Cross Section of kidney of control mouse Haematoxylin-Eosin (X-100)

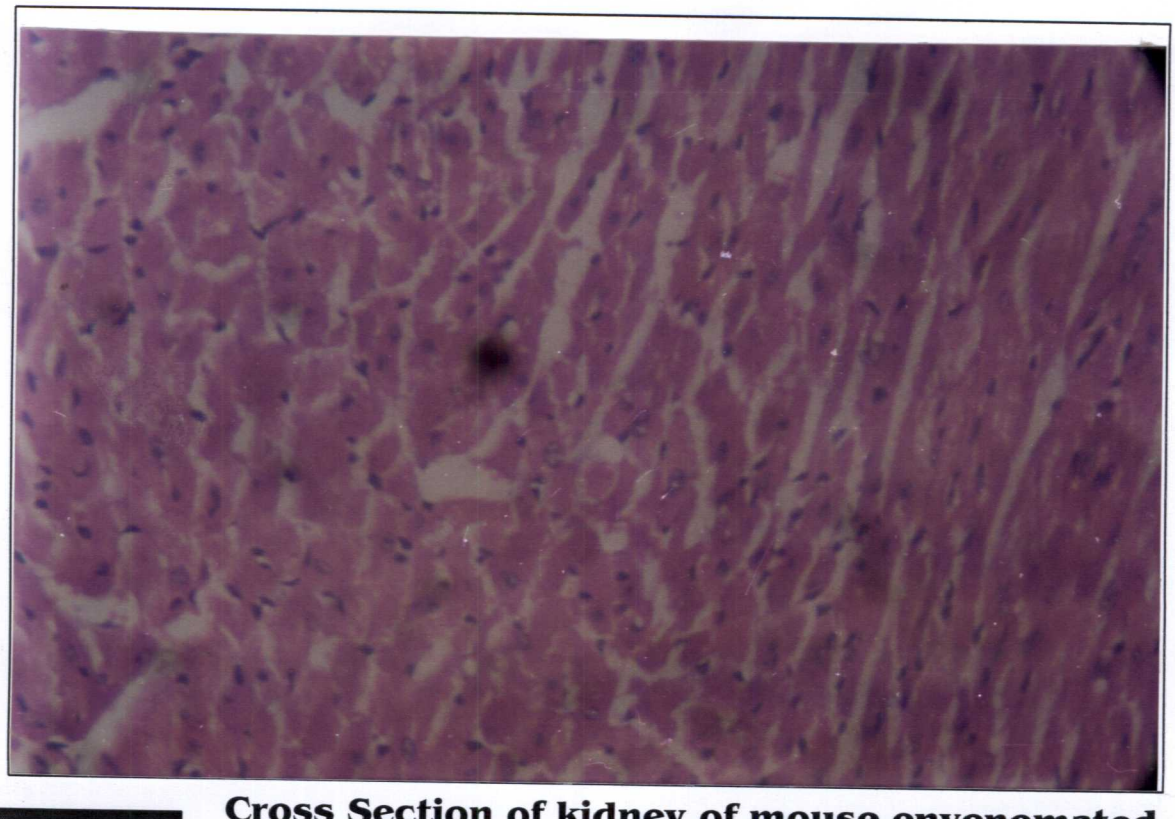


Plate 7

Cross Section of kidney of mouse envenomated with purified fractions of *Cistopus indicus* (Orbigny, 1840)Haematoxylin-Eosin (X-100)

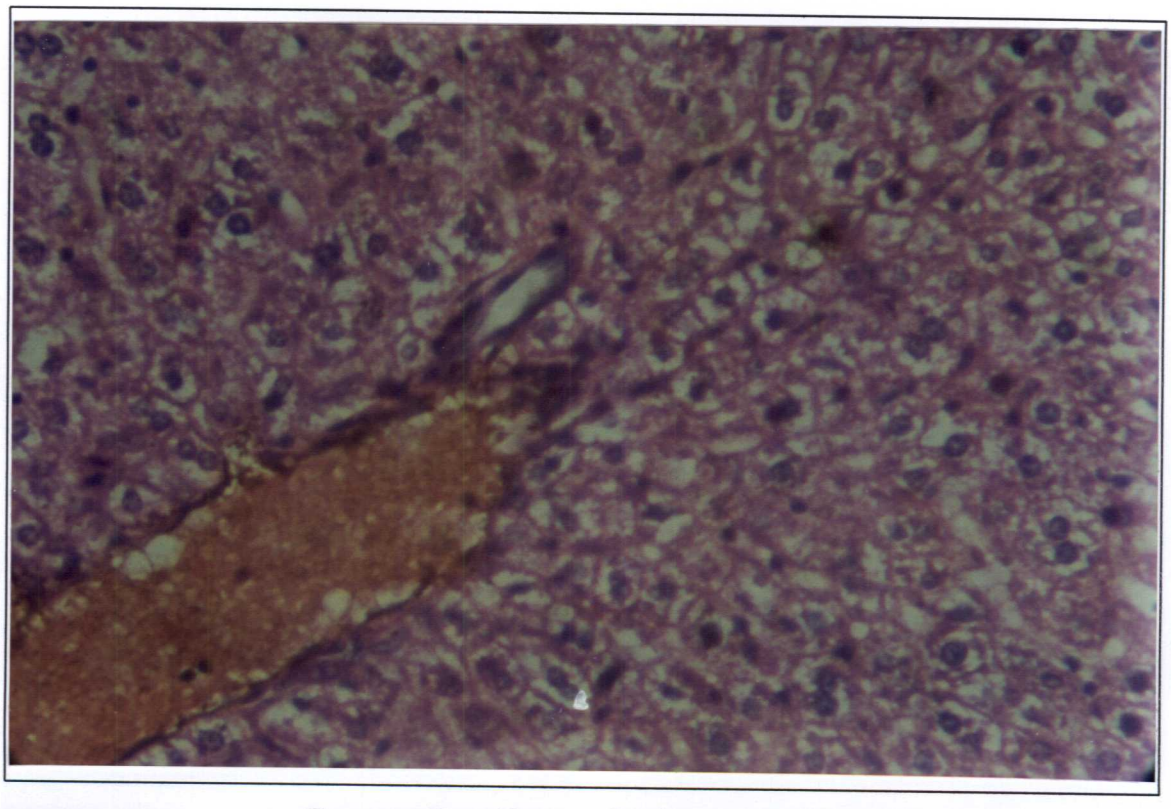


Plate 8

Cross Section of Liver of control mouse Haematoxylin-Eosin (X-100)

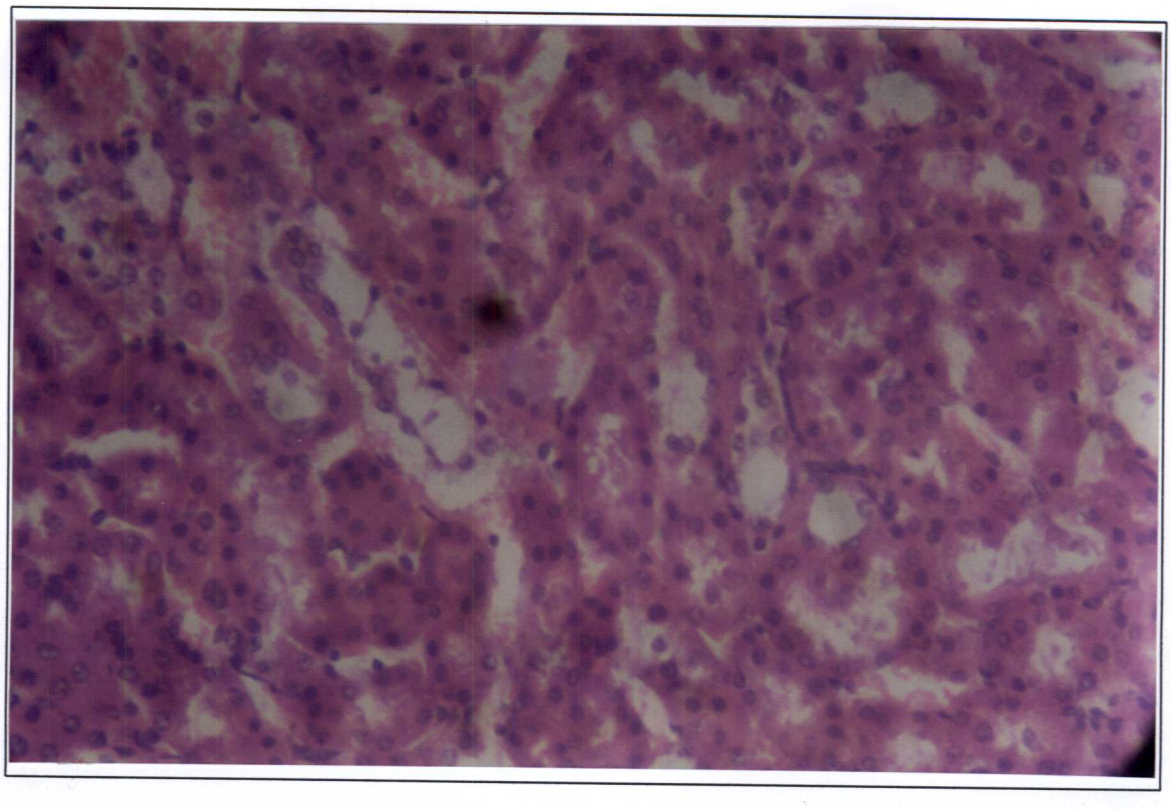


Plate 9

Cross Section of Liver of mouse envenomated with purified fractions of *Cistopus indicus* (Orbigny, 1840)Haematoxylin-Eosin (X-100)


Discussion

5. DISCUSSION

5.1 LETHALITY:

The crude as well as partially purified fractions of toxin from the salivary gland Cistopus indicus exhibited lethality to mice. The MLD in the case of crude toxin was found to be 0.5ml / 20g mouse. The observed symptoms, viz. violent jumping and convulsion of hind limbs prior to death of mice indicate the venom to be neurotoxic. Trethewie (1978) found that main effect of venom was respiratory paralysis, which also has been seen, in the present case. Trethwie (1978) also observed that cardiac effects are largely secondary and may be due to anoxia.

In the experiments of Trethwie (1978) mice, which were given a dose of 1.5 ml of gland extract intravenously, died after 1.5 minutes, in one case, mice injected with 0.5 ml recovered. Despite being administered i.p. as compared to the faster acting of i.v. Administration in case of Trethwie (1978), the MLD in the present case was 0.5 ml for both the crude and partially purified toxins. It may, therefore, be deduced that the toxic components in case of *C.indicus* is more virulent than that of *H.maculosa*.

Frothing from the mouth was also observed in mice, which received i.p. F2 and died in 23 sec. Although this symptom has not been reported in the case of any cephalopod venom so far, it has been reported to occur in mice envenomated with TTX from the puffer fish *Lagocephalus lunaris* (Reddy, 1997). The similarity of MTX to TTX

(Crone *et al.* 1976) has been well established based on their chromatographic (Jarvis *et al.* 1975) and pharmacological (Freeman and Turner, 1970) similarities. Sheumak *et.al.* (1978) mentioned that MTX secreted by salivary glands of *Haplochlaena maculosa* contained at least one fraction of TTX which blocks the peripheral nerve conduction by interfering with sodium conductance in excitable membranes. Thus it seems possible that a toxic component identical to TTX might be present in *Cistopus* toxin. Further studies as to the identical nature or otherwise of the *Cistopus* toxin with MTX and TTX would be interesting. It is also possible that a CNS activity leading to neuromuscular block of the diaphragm thus producing respiratory arreset, as in the case of sea snake venoms (Arenson and Nistri,1975) could have caused this symptom.

5.2 AUTOPSY:

Autopsy revealed hemorrhages inside the body cavity of mice injected with both the samples. Botazzi and Valentini (1924) found that hemorrhages almost all over the gastrointestinal tract. Pale discoloration of heart was observed in all the cases.

5.3 HISTOPATHOLOGY:

Histopathological sections of heart did not reveal much damage to the heart tissues other than slight to moderate necrosis. Trethwie (1978) also reported that there was no apparent cardiotoxicity in cases of octopus envenomation. However, there had been repots to the contrary: autopsy of rabbit envenomated with *E.moschata* venom

(Rouville 1910 a, b, c) revealed diastolic cardiac arrest. Rouville believed, therefore, that octopus venom affected the respiratory and other medullary centres of rabbit. Observing a distinct hypotensive effect, which persisted after severing the vagus nerve, he believed it to be due to both a cardiac effect and a peripheral vasodilatation. Results of the present study, however, indicate the cardiotoxic effects are only secondary but a detailed study on the cardiovascular effect of the venom of *C.indicus* would be rewarding.

The present study however revealed extensive damage to the liver and kidney tissues, especially Pyknosis (nuclear condensation), and hemorrhage and degeneration of hepatocytes. Necrosis was pronounced at certain areas in the case of liver tissue. It can be assumed that the damage to liver may be due to storage of toxins for the detoxification. In the kidney tissue tubular necrosis, pyknotic nuclei were more prominent in certain areas.

Erspamer and Erspamer (1962) have indeed shown that eledoisin, a polypeptide in posterior salivary gland secretion of *Eledone* species of Mediterranean region, manifest a powerful stimulating effect on isolated digestive tract of various animals. However, such a marked reaction was not recorded in rat or rabbit. The present results on the cellular level damage to liver, kidney might therefore indicate the involvement of elidoisin in the salivary gland secretions *C. indicus*.

5.4 HEMOLYSIS:

Results of the present study ruled out any hemolytic property in the venom of *C.indicus*, contrary to Rouville's (1910 a, b, c) findings of hemolytic properties of the venom of *Eledone moschata*.

5.5 HEMAGGLUTINATION:

Rouville (1910 a, b, c) working on Eledone *moschata* venom presented the evidence of its blood coagulation properties. In the present study, the venom, either crude or purified, failed to show hemagglutination of chicken blood.

5.6 CONCLUSION:

The venom from the salivary gland of *Cistopus indicus* thus appears to be a potent neurotoxin having a primary impact on the peripheral neuromuscular transmission leading to cardiovascular damage. Nephrotoxic properties are also exhibited by the venom. Although cytolytic effects were observed in the kidney and liver, hemolytic activity was totally absent; the observed cytolytic effect may thus be mediated through mechanisms other than membrane disruption. The toxic symptoms observed also indicate that tetrodotoxin (TTX) could be involved as a component in the venom. Further chemical characterization of the toxin would shed much more light on the neuropharmacological and pharmaceutical applications of the venom

References

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