STUDIES ON THE VENOM OF THE FISH *SCATOPHAGUS ARGUS* FROM COCHIN ESTUARY – A BIOCHEMICAL APPROACH

Thesis submitted to the

COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY

In partial fulfilment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

IN

MARINE BIOLOGY

UNDER THE FACULTY OF MARINE SCIENCES

BY

GISHA SIVAN

DEPARTMENT OF MARINE BIOLOGY, MICROBIOLOGY AND BIOCHEMISTRY

SCHOOL OF MARINE SCIENCES

COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY

KOCHI – 682016, INDIA

March - 2007

DECLARATION

I hereby do declare that the thesis entitled "STUDIES ON VENOM OF THE FISH SCATOPHAGUS ARGUS FROM COCHIN ESTUARY- A BIOCHEMICAL APPROACH" is a genuine record of research work done by me under the supervision and guidance of Dr.C.K.Radhakrishnan, Reader, Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology in partial fulfillment of the requirements for the PhD degree in Marine Biology of the Cochin University of Science and Technology and that no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or other similar title of any University or Institution.

Gisha Sivan

Kochi-16
March 2007

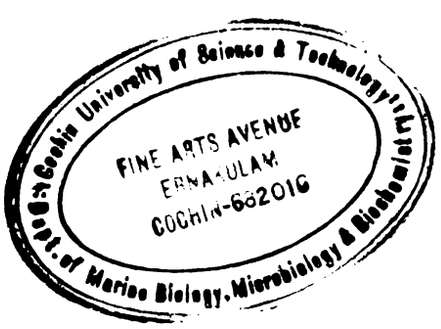
CERTIFICATE

I hereby declare that the thesis entitled "STUDIES ON VENOM OF THE FISH SCATOPHAGUS ARGUS FROM COCHIN ESTUARY- A BIOCHEMICAL APPROACH".) an authentic record of research work carried out by Ms. Gisha Sivan under my supervision and in the School of Marine Sciences, Cochin University of Science and Technology in partial fulfillment of the requirements for the degree of Doctor of Philosophy and that no part thereof has been presented for the award of any degree, diploma or associateship in any University.

Dr. C.K. Radhakrishnan
(Supervising Teacher)

Dr.C.K.Radhakrishnan
Retd. Professor
Dept of Marine Biology, Microbiology and Biochemistry
School Of Marine Sciences
Cochin University of Science and Technology
Kochi - 682016

Kochi-16
March 2007



ACKNOWLEDGEMENT

I am deeply indebted to my supervising guide Dr. C.K. Radhakrishnan, Retd. Professor, Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology (CUSAT) for his meticulous guidance, constant encouragement and moral support. I take this opportunity to express my deep sense of gratitude for suggesting this problem and valuable suggestions.

My thanks are due to Dr. A.V. Saramma, Head, Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology.

I wish to record my sincere thanks to Dr.K.T.Damodaran, Director, Faculty of Marine Sciences, Cochin University of Science and Technology. I am indebted to Dr. Mohan Kumar, Dean, Faculty of Marine Science, Cochin University of Science and Technology.

For whatever merit this book may possess I am indebted beyond measure to Dr. K. Venketashvaran, Senior Scientist, Central Institute of Fisheries Education, Mumbai for his inspiring guidance, constant encouragement, critical evaluation and unfailing moral support which has always instilled in me the energy and strength to face and overcome several hurdles on the way during this research programme.

I am beholden to Dr. G. Malarvannan, Ehime University, Japan for his benevolent support, unfailing guidance, intellectual inputs, critical assessment and invaluable suggestions at crucial junctures of this work.

I am grateful to Dr. Bijoy Nandan, Reader Department of Marine Biology, Microbiology and Biochemistry, CUSAT for his timely help. I thank Dr. (Prof) Babu Philip, Dr. Rossama Philip, Department of Marine Biology, Microbiology and Biochemistry, CUSAT for all help and valuable suggestions rendered throughout the period of study.

I acknowledge with thanks the wholehearted assistance and valuable suggestions of Dr. K.C. George, Principal Scientist, Central Marine Fisheries Research Institute, Kochi. I acknowledge with thanks the help rendered by Dr. T.V. Sankar, Senior Scientist, Central Institute of Fisheries Technology, Kochi.

I am extremely thankful to Dr.Prabha Balaram, Head, Department of Cancer Research, Regional Cancer Centre, Thiruvananthapuram. I gratefully acknowledge Mr.

Vino T. Cherian of the Department of Cancer Research, Regional Cancer Centre, Thiruvananthapuram for his valuable help.

I would like to place on record my sincere thanks to Dr. Nandini Menon, Selvan.S and Remya Varadarajan for their timely help and support.

My sincere thanks to Jayanthi. T.T. for her friendship and wholehearted support. I thank Sanitha. B and Shivappa. M.U. for their contributions for completion of this work.

My special thanks are due to Arun.A.U, Harikrishnan. E, Jehosheba Mathews, Meera Venugopal and Renjith.G.

I consider it a great privilege to be part of wet lab. Mere words of thanks will be inadequate to those who walked along with me through the years helping me to weather every storm, cheering me when I was down, chiding me when I was wrong and turning little moments of joy into celebrations. I thank Jaleel, Bindhya, Nikitha, Anupama, Swapna, Roja, Anil, Smitha, Nousher, Beena, Sreeja, Jini, Soja, Cilla and Chitra for their love, care, affection and wholehearted support throughout my tenure.

I deeply appreciate the extreme sincerity, assistance and love rendered by Priyalakshmi, Jisha, Anila Devi, Smitha Bhanu, Lakshmi and Simi.

I express my sincere thanks to all the teachers and office staff of the Department of Marine Biology, Microbiology and Biochemistry, CUSAT.

Words are still before the deep, dedicated and unparallel love of my sisters Linsha and Nisha without whose sincere efforts it would have been difficult for me to complete the work in time. Their constant encouragement and unfailing moral support have always instilled in me the energy and strength to face and overcome several hurdles on the way during the research programme.

My deepest feelings for the love and care of my beloved parents who had been the inspiration of all my endeavours, cannot be contained in words and I am indebted to them for all my achievements. I fondly cherish their encouragement and wholehearted support as my fortune.

I have been extremely privileged to have the love, encouragement, support and care from my sister Sanju, to whom I am deeply indebted. I extend my deepest feelings of gratitude for her.

I am grateful to the care, love and affection I received from all my family members and friends.

GISHA SIVAN

PREFACE

Aquatic biodiversity enthralls bountiful resources of flora and fauna providing food, medicine and livelihood since time immemorial. Marine natural products have attracted the attention of biologists and chemists the world over for the last five decades. Marine organisms not only elaborate pharmaceutically useful compounds but also produce toxic substances. Among them are a number of toxins found in vertebrate venom that have evolved to target vital processes in the body and they do have a lot more selectivity than many drugs. Over 1200 species of fish are known or thought to be venomous. As fish are now known to be the dominant venomous group among vertebrates, they represent a massive untapped resource of potentially medically beneficial compounds. They have a wide range of pharmacological effects on human nervous, muscular and cardiovascular systems, among others. The venom proteins offer a source for the development of drugs for the treatment of pain, cancer, infectious diseases, auto-immune diseases, allergies, and hypertension. The deadliness of the venomous fish is well written by the ancient Greek poet Oppain in his poem Halieutica.

"Cruel spines defend some fishes, as the Goby fond
Of sands and rocks, the scorpion, swallows fleet,
Dragons and Dog fish, from their prickly mail
well named the spinous. These in the punctures sharp,
A fatal poison from their spines inject."

- Oppian

Accidents caused by spotted scat (*S.argus*) envenomation are characterized by intense pain, throbbing sensation, and edema at the site of sting. The present piece of work strives to investigate the biochemical aspects of *Scatophagus argus* venom. The preliminary screening of bioactivity of the *S. argus* venom is carried out. The mode of action and biochemistry of venoms are obviously complex and require a better knowledge and investigation to explore the toxic action and resulting biochemical changes. Even though this study reveals the conundrums of *S. argus* venom, it opens new vistas of research on the venom components and the application and design of the venom as a drug.

Gisha Sivan

CONTENTS

ABBREVIATIONS

ROS	-	Reactive Oxygen species
SOD	-	Super Oxide Dismutase
CAT	-	Catalase
GPx	-	Glutathione Peroxidase
GR	-	Glutathione Reductase
GST	-	Glutathione-S-Transferase
GSH	-	Glutathione
CD	-	Conjugated dienes
MDA	-	Malondialdehyde
ALP	-	Alkaline Phosphatase
ACP	-	Acid Phosphatase
AST	-	Aspartate Transaminase
ALT	-	Alanine Transaminase
AChE	-	Acetyl Cholinesterase
LDH	-	Lactate Dehydrogenase
SDS	-	Sodium dodecyl sulphate
EU	-	Enzyme Unit
µg	-	microgram
ml	-	millilitre
µl	-	microlitre
kg	-	kilogram
SNTX	-	Stonustoxin
CK	-	Creatine Kinase
PBS	-	Phosphate Buffer Saline
H_2O_2	-	Hydrogen peroxide
SD	-	Standard Deviation
PAGE	-	Poly Acrylamide Gel Electrophoresis
KDa	-	Kilo Dalton
SE	-	Standard Error
TLY	-	Trachynilysin
%	-	Percent
mM	-	millimoles
µM	-	micromoles
nm	-	nanomoles

Chapter 1

GENERAL INTRODUCTION

Ocean, the vast and the least understood ecosystem, its relentless and impredictable moods, the myriad life forms above and below its restless surface, the vast treasure of energy sources hidden under the deep trenches, have all fascinated and lured man. The potent resources of the oceans that lie unexplored and unexploited perceptibly influence the health and wealth of a nation.

The oceans are unique sources of diverse array of natural products. Apart from viewing oceans as the avenue for cheap source of protein, other usage like the pharmaceutical importance for producing drugs against many vagaries sounds encouraging. It is increasingly recognized that a huge number of natural products and novel chemicals entities exist in the oceans, with biological activities that may be useful in the quest for drugs with greater efficacy and specificity for the treatment of many human diseases.

The marine environment is an exceptional reservoir of bioactive natural products, many of which exhibit structural/chemical features not found in terrestrial natural products. Marine animals fight daily for both food and survival and this underwater warfare is waged with chemicals which are potent sources of many therapeutic compounds. Biomolecules extracted from the aquatic organisms have been proved to have powerful bioactive and biomedical properties. Attempts are being made to develop drugs from the sea which have the potential as anticarcinogenic, antibiotic, growth regulatory, haemolytic, haemagglutinating, analgesic, antispasmodic, hypotensive or hypertensive agents, and even anti-HIV agents. To date approximately 16,000 marine natural products have been isolated from marine organisms and reported in approximately 6,800 publications (Bhakuni and Rawat 2005).

Biotoxins from marine organisms usually come to the attention of biologists because they exert a striking effect on the organisms in marine community. Many sea creatures can directly injure, envenom, and poison their victims. Biotoxins are found throughout the entire phylogenetic series of marine animals. There is substantial practical motivation for studying marine biotoxins, for like other toxins they are highly active in biological sense and therefore useful in biomedical research. Chemically the toxins make the most challenging targets because of their structural complexity and extremely limited availability. To pharmacologists and biochemists marine toxins are attractive because of their potent and specific action.

Marine toxins have a wide range of biological activities and often these activities are induced by minute amounts of toxin, i.e., by nature toxins are highly potent molecules. The biological activities exhibited by the toxins are harmful to the target organisms, since the function of the toxin is either to protect the toxic species from attack by a predator or to immobilize potential prey. Despite the general harmful effects in the target organisms, toxins have a great potential to be harnessed for favorable effect on non-target organisms and in particular humans for therapeutic purposes. Biological significance of marine biotoxins is rather an important subject in medical science. The scientific importance of biotoxins is frequently misunderstood, because the most popular attitude among the public is that poisons are lethal substances, causing intoxications and death and are therefore substances to be avoided. Research activities on biotoxins are justified because of the fact that knowledge on their pharmacological and chemical properties can be useful in developing an antitoxin or decephering their clinical characteristics.

The perspective science of Biotoxinology imbibes to have absolute knowledge of any organism by solving a number of complex biological and

biochemical conundrums yielding in several tangible results in terms of human health security and economic benefits. Natural toxins are unique toxins, which possess some common properties whether they are obtained from plants, microorganisms, or animals. One common characteristic is that they exert a prominent effect on the metabolism and biological functions of the intoxicated animals with just a minute quantity.

Biotoxins are mainly of two types; Phytotoxins or plant poisons and Zootoxins or animal poisons. A large variety and number of marine creatures inhabiting the ocean waters pose threat to humans by virtue of their ability either to bite, lacerate or sting or contain toxic substances within their flesh, blood etc. In the past several decades the research carried out in the field of Fish Biotoxinology has acquired new dimensions. The types of toxic fish, the nature and source of toxins and the conditions governing their toxicity are all topics of interest in this field. The information regarding the source of adventitious toxicity of fish is not only vital for development of antidotes and rational assessment of the usefulness of the fish species as food source, but it also opens an arena for the exploration of new biologically active chemical substances or biodynamic compounds of therapeutic value.

Halstead and Courville (1970), Concon (1988), and Halstead (2001) have classified ichthyotoxic fish into two groups: a) Poisonous or Phenerotoxic fish which when ingested cause a biotoxication in humans due to a toxic substance present in the fish and b) Venomous or Acanthotoxic fish that produce poison by means of glandular structures that are equipped with a traumatogenic device to purvey their venoms. Intermediate to the poisonous and venomous fish are the crinotoxic fish that produce a poison by means of glandular structures independent of true venom apparatus.

Poisonous Fish: Research in the past several decades has increased our knowledge on the types of toxic fish, the nature and source of toxins and the conditions governing their toxicity. The major problem facing the marine biotoxinologist is the variability and frequent unpredictability of the toxicity of this segment of marine life. The degree of toxicity of these fish may fluctuate periodically. The poisonous substances in fish may be concentrated in specific tissues or organs. Based on the tissue in which the toxin is present in the fish the poisonous fish are further classified into Ichthyosarcotoxic (flesh, musculature, viscera or skin) eg: herrings, anchovies, tarpons and pufferfish, Ichthyootoxic (roe or gonad) eg: The most dangerous forms are the genera *Barbus*, *Schizothorax*, *Tinca* (Cyprinidae) and *Stichaeus* (Stichaeidae). Ichthyohaemotoxic (blood or serum) eg: Those fish having poisonous blood, members of the anguilliform families Anguillidae, Congridae, Muraenidae, and Ophichthidae Ichthyohepatotoxic (liver) eg: sharks

Crinotoxic fish: Crinotoxic fish are intermediate to poisonous and venomous fish and include filefish, pufferfish, trunkfish, boxfish, toadfish, gobyfish, catfish etc. They are able to secrete substances from their skin, known as icthyocrinotoxins, that are capable of repelling or incapacitating other marine animals (Klaassen and Watkins, 1999). These secretions are also thought to possess antibiotic activity, protecting fish from the myriad of invading microorganisms in the marine environment, a fact that has prompted recent research on them. These have specialized cells or glands in their skin but lack a parenteral mechanism (Perriere and Perriere 2003). Glandular secretions are normally released into the surrounding medium, the water. Crinotoxins are often called mucus toxins. The poison glands of ichthyocrinotoxic fish assist in the defensive mechanism of the fish by producing warning or repellent substances especially under stress conditions (Deo, 2000).

Venomous fish: Fish constitute almost half the number of vertebrates on earth (Nelson, 1984), and approximately 22,000 species of fish are contained in some 50 orders and 445 families. Of these, nearly 1,200 species of marine fish, including stingrays, scorpionfish, zebrafish, stonefish, weeverfish, toadfish, stargazers, and some species of shark, ratfish, catfish, surgeonfish and blenny, are known or suspected to be venomous (Smith and Wheeler, 2006). The vast majority of these fish is non-migratory, slow moving, and tend to live in shallow waters in protected habitats (Maretic, 1988). It has been suggested that this tendency towards inactivity is closely linked with the evolution of venom apparatus (Cameron and Endean, 1973).

Although only a handful of species of venomous fish are thought to be capable of causing human mortality, many other species of fish can produce severe envenomation. While not considered life threatening, envenomation by these fish is associated with considerable pain, and their venoms contain many pharmacologically active components. Therefore, these species are important as sources of pharmacological compounds that may be useful as research tools or lead compounds for drugs.

Venomous or Acanthotoxic fish produce their poison by means of glandular structure and are equipped with a traumatogenic device (teeth, spine, sting, etc.) to purvey their venom (Church and Hodgson, 2002). The venom apparatus invariably consists of spines that may be located on the dorsal, pectoral, opercular, shoulder, pelvic, anal and caudal areas of the fish depending on the species (Williamson, 1995). Venom is delivered when the spine pierces the tissue of the victim, the integumentary sheath enclosing the spine and venom is ruptured, and the venom enters the wound (Church and Hodgson 2002).

It is thought that venom apparatus evolved relatively recently in the development of these fish, because fish despite being on a higher level of

development than some other groups of venomous creatures (e.g. spiders), their venom apparatus are much more primitive, relying on a completely involuntary mechanical action, rather than a voluntary expulsion of venom (Maretic, 1988). Venomous spines can be observed in species from many evolutionary classes of fish, from the primitive cartilaginous fish like stingrays, to the more advanced bony fish such as stonefish.

As venoms from various animals including fish have a wide range of pharmacological effects on human nervous, muscular, and cardiovascular systems, the venom proteins offer a source for the development of drugs for the treatment of pain, cancer, infectious diseases, auto-immune diseases, allergies, and hypertension. Most pharmaceutical drugs have been derived from natural products (compounds discovered in nature). However, most organisms, including fish, have only recently been examined for biologically active molecules (or compounds that affect the biochemistry of living things) that could be developed into potential drugs. In addition, pharmaceutical companies have recently de-emphasized natural products research and search for biologically active molecules by screening mass produced combinations of compounds. Neither strategy has met expectations, and the number of new registered drugs continues to decrease dramatically. To date, most venom bioprospecting has focused on snakes, resulting in six-stroke or cancer treatment drugs that are nearing U.S. Food and Drug Administration review (Smith and Wheeler, 2006). However, as fish, not snakes, are now known to be the dominant venomous group among vertebrates, they represent a massive untapped resource of medically beneficial compounds.

Studies on Acanthotoxic fish from the subcontinent of India are very scarce. The present investigation aims at elucidating the bioactivities of the spine extract of *Scatophagus argus* (Family: Scatophagidae) from Cochin estuary that would qualify them as a potential source of biopharmaceutical compounds. Injuries

inflicted by the fish cause pain disproportionate to the resultant wound even in the absence of notable secondary tissue injury. Secondly the quality and duration of the pain caused by the venom and its and heat labile nature suggest potential soluble protein-venom–nocicepter interaction. The study of biological properties and chemistry of the venom would make an impact in developing new detoxification techniques. In the light of the above the main objectives of the present study are:

- To elucidate a method for extraction of the venom.
- To find out the toxicity of the spine extract of *Scatophagus argus* in mice.
- To analyze the gross anatomical changes and histopathological changes caused by the toxin in mice models.
- To elucidate the effect of the *S. argus* venom on the antioxidant enzymes, antioxidants, detoxifying enzymes in mice tissues.
- To assess the impact of *S. argus* venom on cell line.
- To study the neuromuscular toxicity of the *S. argus* venom.
- To find out the biomedical application of the *S. argus* venom.
- To understand the lysosomal membrane stability against the *S. argus* venom.
- To characterize the toxin and to assay the different enzymes and biochemical molecules present in the *S. argus* venom.

SCATOPHAGUS ARGUS

Scatophagus argus belonging to the family Scatophagidae are widely distributed in brackish water and marine habitats of Indo-Pacific, the Malay Archipelago, the Philippines, Australia, South and Southeast Asia especially India where they inhabit estuaries, coastal mud flats, mangrove swamps, harbors and upstream swamps. They are commonly known as butterfish, argus fish, spade fish, spotted spade fish [Barry and Fast, 1988], leopard pomfret [Mookerjee *et al.*, 1949] and spotted scat [Bardach *et al.*, 1972]. The word Scatophagus means "offal eater" or "eater of feces" and the word argus means "thousand eyed" which refers to the spot found on all juvenile and larger fish. Scats are well adapted to live in ever changing environment that endows them with many biological attributes highly desired in cultured finfish. The euryhaline nature and the beautifully spotted rhombic body rank it as a fascinating aquarium fish while the quality and taste ranks it as a highly priced edible fish.

Phylum	: Chordata
Class	: Osteichthyes
Subclass	: Neopterygii
Infraclass	: Teleostei
Super Order	: Acanthopterygii
Order	: Perciformes
Suborder	: Acanthuroidei
Family	: Scatophagidae
Genera	: Scatophagus
Species	: *argus*

Scats are greenish brown in colour with dull white belly and black blotches of varying sizes all over the body. Body is rhombic and well compressed with firm skin and small ctenoid scales. Rostrodorsal profile strongly ascending and followed by a deep concavity above the eyes. Mouth is small, terminal, and

horizontal with bands of fine teeth in jaws but not on palate. It is generally believed that the fish spawn in the neighbourhood of coral reefs but the young migrate to the fresh water at the mouth of rivers and estuaries, until they grow large enough well adapted to live in the sea. (Pic1.1)

Scats are venomous fish and the venom apparatus is constituted by 11 dorsal spines, a pair of ventral spine and 4 anal spines, elongated venom glands and an integumentary sheath enveloping all [Cameron and Endean, 1977]. The spines are very sharp and pointed each possessing a pair of antero-lateral grooves one on each side accommodating the paired venom glands in each spine. The venom glands are irregularly shaped tending to be columnar in the deepest portion of the gland. The glands are aggregations of large gland cells in the thickened epidermis of the integumentary sheath. The venom glands are not encapsulated in connective tissue sheath. Elongate supporting cells occur among the venom glands cells some of which possess unusual rod like bodies in their cytoplasm.

Scats are not aggressive and do not actively attempt to inflict wound. However they frequently induces accidents in fishermen while handling it. Of the family Scatophagidae *S. argus* is said to inflict more painful wounds than do allied species [Marshall, 1964]. During envenomation the spines are erected and the mechanical pressure on the spine tears or pushes down the integumentary sheath over the spine as the venom passes into the wound. Though the puncture is painful which aches for many hours [Barry and Fast, 1988] it is rarely life threatening. The butterfish envenoming appears within 5-10 min as excruciating and persistent local pain disproportionate to the size of injury, redness, swelling and throbbing sensation that extend to the limbs followed by dizziness. Most patients are treated with empirical procedure such as emersion of the wounded area in hot water.

Scatophagus argus

Scatophagus argus male and female

Chapter 2

EXTRACTION OF VENOM FROM *S.ARGUS* SPINE AND MICE BIOASSAY

2.1. INTRODUCTION

The word fish usually brings to our minds, the silhouette of gentle and delicate creatures that mean no harm to anybody at least not to the human beings. While salmon, tuna and many others have been an inseparable part of culinary traditions across the world, there are a few species of fish, which are enlisted among the deadliest creatures in the world. They are loaded with toxins ready to unleash on anyone who dares to intimidate them. Poisonings caused by bites and stings of venomous fish are a common health hazard worldwide (Isbister 2000). The vast majorities of the venomous fish are non-migratory, slow moving, and tend to live in shallow waters in protected habitats (Maretic, 1988). It has been suggested that this tendency towards inactivity is also linked with the evolution of venom apparatus (Cameron and Endean, 1973).

Traumatization of the enemy by venomous spines, through which a toxin is injected directly into its body is a well known form of chemical defence in some fish, though it is associated with mechanical defence. It is thought that the venom apparatus in fish have evolved relatively recently in the development of fish, because while fish are on a higher level of development than some other groups of venomous creatures their venom apparatus are much more primitive relying on a completely involuntary mechanical action, rather than a voluntary expulsion of venom (Maretic, 1988).

The production of venom is an important strategy that guarantees the survival of the venomous forms in a highly competitive ecosystem. These animals produce an enormous number of metabolites whose combinations result in a great variety of chemical structures and complex molecules with chemical and pharmacological properties. Though several studies have been carried out on venomous fish, only very few of the venoms have been characterized. Many of these substances still remain to be investigated, even for the confirmation of their venomous status.

2.2. REVIEW

A venomous fish secretes noxious substances from specialized secretary glands through a specialized delivery apparatus (Church and Hodgson, 2002). The venom apparatus consists of spines in whose grooves are venom glands and the whole being covered by the integumentary sheath (Maretic, 1988). In some species the venomous apparatus is highly developed with distinct venom glands and venom ducts associated with the spine while in others the venom apparatus is loosely associated with the venomous secretary products.

The crude venom extract of *S.horrida* has been subjected to lethality in mice (Khoo *et al.*, 1992) and the lethal factor stonustoxin has been purified to homogeneity (Poh *et al.*, 1991). Shiomi *et al.*, (1989) studied the venoms of six species of marine fish *Synanceja verrucosa, Inimicus japonicus, Pterois lunulata, P. volitans, P. antennata* and *Dendrochirus zebra*. All were potentially lethal to mice, *S. verrucosa* assuming to be the most venomous of all. Later Garnier *et al.*, (1995) purified *S. verrucosa* venom and isolated the lethal factor verrucotoxin. LD_{50} for Scorpanaeid fish, *Synanceja plumeiri, S. guttata* and *S. trachynis* were also studied in mice that showed comparable values (Kreger 1991; Schaeffer 1971 and Carrijo *et al.*, 2005). Studies for LD_{50} values in toadfish venom have been carried out for *Thalassophryne nattereri* and *T. maculosa*. (Lopes Ferriera *et al.*, 1998; Sosa Rosales *et al.*, 2005). *Trachinus draco* and *T. vipera*, the greater weever fish and the lesser weever fish respectively have shown to be lethal to mice (Carlisle, 1962;Chhattwaal and Dryer, 1992).

2.3. MATERIAL AND METHODS

2.3.1. Collection of specimen

Specimens of *Scatophagus argus* were caught by Chinese net from the barmouth region of Cochin estuary, Kerala (Pic 2.1). The backwaters of Kerala

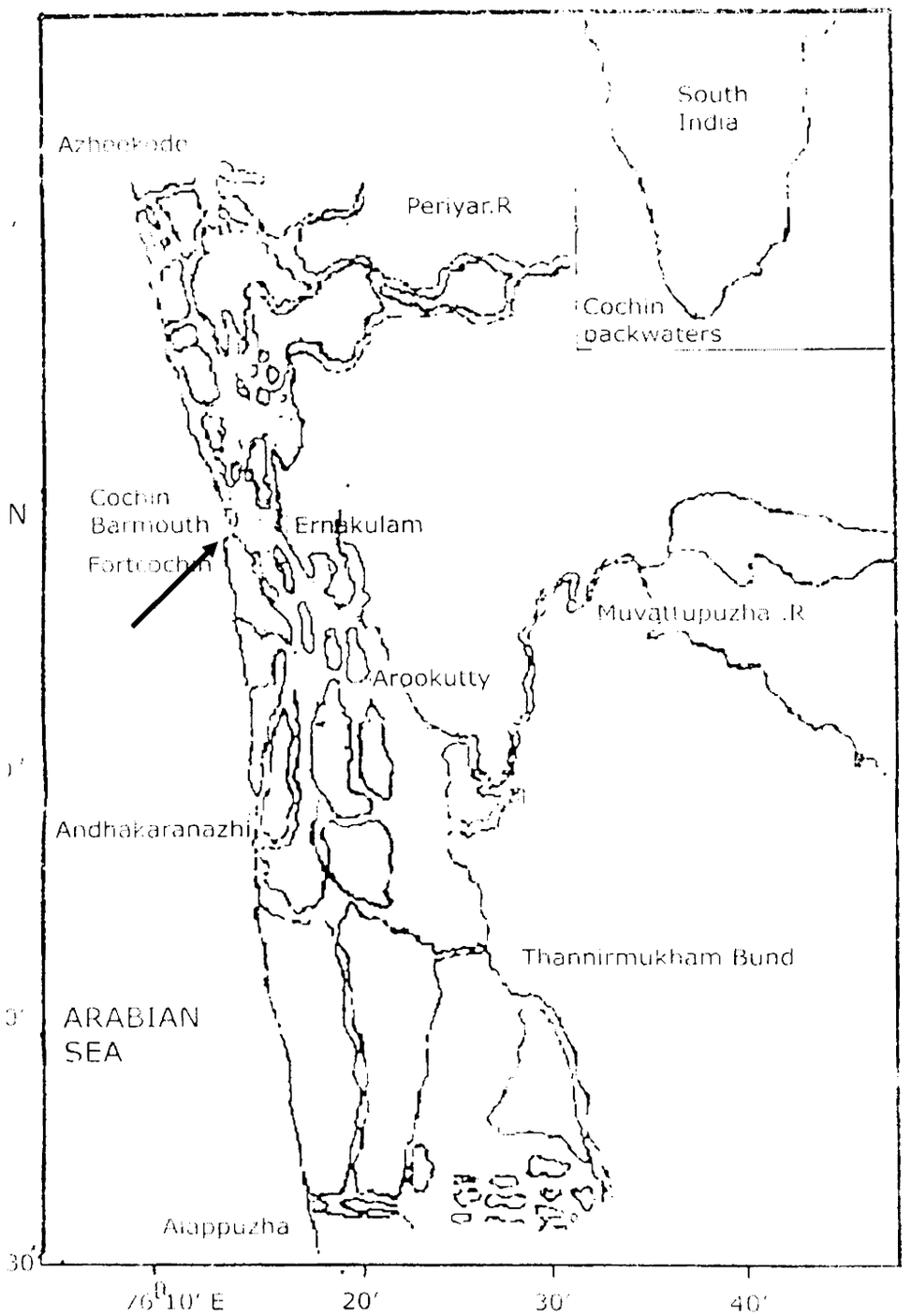
support as much biological productivity and diversity as tropical rain forest. The Cochin estuary is one of the largest estuaries in the west coast. It is about 96km long and extends from about 9°30' to 10°20' Lat N and 76°13' to 76°5' Long E. The backwaters occupy an alluvial plain, lying parallel to the coast between the Arabian Sea to the west and the Western Ghats to the east in Peninsular India. It is permanently connected to Arabian Sea by a 450 mile channel, which is about 5-15 m deep. The bottom of the estuary is predominantly muddy. The major hydrological variable in the Cochin Estuary is salinity. The salinity gradient in the Cochin backwaters support diverse species of flora and fauna. The changes in the hydrology controlled by the season play an important role in regulating the migrant fauna of the estuary. The fish were transported live back to the laboratory, where they were sacrificed and the dorsal, anal and pectoral spines removed.

2.3.2 Extraction of crude venom

Venom extraction was carried out using saline solution, 10% glycerol and 0.1 M phosphate buffer pH 7.4.

2.3.2.1.Saline

The extraction was done using saline according to the method of Cohen and Olek (1988) at a temperature of 4-10°C. Fish were cooled for 10-20 minutes and then decapitated and all spines were cut approximately 3-5 mm from their base. The integumentary sheath and tissue residing in the grooves of spines were removed from the fish and then homogenized in a chilled mortar and pestle in 0.9% NaCl. After centrifugation at 10000 rpm for 7 minutes, the supernatant was collected and the pellet was resuspended again in 0.9% NaCl and centrifuged at 10000 rpm for 7 minutes. The supernatant was combined and again centrifuged for 30 minutes. The resulting supernatant was designated saline extract and used on the same day or stored at -25°C.



Pic 2.1. Map showing the sampling site.

2.3.2.2. Buffer

The spines removed from the fish were homogenized in a chilled mortar and pestle using 0.1M phosphate buffer (pH 7.4). The homogenate was centrifuged for 15 minutes at 6000g to remove the insoluble materials. The resulting supernatant was designated buffer extract. All steps were performed in a cold room at 4-10^{0}C.

2.3.2.3. Glycerol

The venom was extracted using 10% glycerol as described by Church and Hodgson (2002). The spines were removed and stored in 10% glycerol at –80°C to prepare a batch of venom. Stored spines were ground in a chilled mortar and pestle in 10% glycerol. This was then centrifuged (5000 rpm 10min), the supernatant removed, the pellet resuspended in approximately 2ml of 10% glycerol, recentrifuged and the final supernatants pooled to form the 10% glycerol venom extract.

Venom concentrates were subsequently expressed as μg venom protein. The protein quantification of the venom was determined by the method of Lowry *et al.*, (1951) using bovine serum albumin as standard.

2.3.3. Partial purification of the crude extract

Partial purification of the crude extract was carried out using DEAE Cellulose Anion Exchange chromatography according to the procedure of Shiomi *et al.*, (1987).

2.3.3.1. Preparation of DEAE Cellulose Column:

25g of DEAE Cellulose were swelled in 625ml of distilled water for three hours and the supernatant was discarded. The sediment was stirred with 500ml of 1M NaOH for 30 minutes with gentle stirring and allowed to stand for 30 minutes. The supernatant was discarded again. The sediment obtained was again stirred

gently with 500ml of 1M HCl for.30 min, soaked with 0.02 M EDTA for 30 minutes and washed with distilled water, and again with Tris-HCl buffer till pH 7.4.

2.3.3.1. Preparation of DEAE Cellulose Column:

25g of DEAE Cellulose were swelled in 625ml of distilled water for three hours and the supernatant was discarded. The sediment was stirred with 500ml of 1M NaOH for 30 minutes with gentle stirring and allowed to stand for 30 minutes. The supernatant was discarded again. The sediment obtained was again stirred gently with 500ml of 1M HCl for 30 min, soaked with 0.02 M EDTA for 30 min and washed with distilled water, and again with Tris-HCl buffer till pH 7.4.

2.3.3.2. Packing of Column:

A properly cleaned glass column (45 x 2cm) was mounted vertically on a stand and filled to about 8cm from the bottom with Tris-HCl buffer (pH 7.4) and the outlet was closed. Care was taken to remove air bubbles from below the sintered glass disc and from the flow-regulating device. The slurry of DEAE-Cellulose in Tris-HCl buffer was poured into the column and was allowed to settle under gravity until about 5 cm of cellulose was packed. The outlet was opened to give a moderate flow rate and the process repeated till the end height of packed material reached the required level of 30cm. The flow rate was adjusted to 25ml/hr and washing with Tris-HCl buffer was continued until the pH of the eluant was the same as that of the buffer.

2.3.3.2. Sample Application and Elution:

Lyophilized crude venom extract was dissolved in PBS @ 5mg/ml and was dialyzed against 0.01M Tris-HCl buffer pH 7.4. The level of buffer in column was allowed to run down to the level of packed DEAE-Cellulose and the outlet was closed. A circular disc of filter paper was placed at the top of the cellulose layer. 5ml of sample was applied carefully on the bed of the column and the outlet opened till the entire sample had entered the bed. An unadsorbed fraction of crude

toxin were eluted with Tris-HCl buffer and then 10 adsorbed fractions were eluted with a linear gradient of 0.1-1.0M NaCl in 0.01 M Tris-HCl buffer. Thus a total of 11 fractions each of 15 ml were collected and stored at -4°C for further use.

2.3.4. Mice Bioassay for LD_{50} Study

Mice bioassay was done to assess the lethality of *S. argus* crude venom. Assessment of LD_{50} was carried out by standard method of AOAC (AOAC, 1984) for which male albino mice (Kasauli strain) of 20±2g weight were used. 6 groups of 12 mice each was selected, one of which served as control. Each experimental group of mice were injected intraperitonially (i.p) with *S. argus* crude venom in the range from 3μg/ml to 15μg/ml (3,6,9,12,15 μg/ml). The mice were observed for 24 hours and behavioural changes were noted. The LD_{50} for *S. argus* venom was calculated using probit analysis.

2.3.5. Mice Bioassay for lethality

Lethality studies of the partially purified fractions of *S. argus* crude venom, was carried out on mice. Each group consisted of 4 mice of which one was control. The partially purified fractions of saline, buffer and 10% glycerol spine extract were injected i.p. at doses of 0.1 ml to triplicate sets of mice. The symptoms of toxicity such as modifications of normal activities of injected mice and death time were recorded in all the sets. . An appropriate control in each case was maintained. The mice were observed for 24 hours.

2.4.RESULTS

Mice receiving sub lethal doses of venom exhibited behavioural changes such as labored breathing, nociception, limb weakness, palpitation, grooming, hurdling, violent jumping and increase in urination. When lethal doses where employed mice exhibited paralysis, convulsions, respiratory distress and foaming from mouth prior to death. LD_{50} value for *S. argus* venom using saline extract was found to be (i.p.) 9.8 mg protein / kg body weight after around 24 hours (Fig 2.1)

while the buffer extract showed to possess an LD_{50} of 9.97 mg protein / kg body weight (Fig 2.2). The 10% glycerol extract showed an LD50 of 9.92 after around 24 hours (Fig 2.3).

2.4.1. Mice Bioassay Involving Partially Purified Venom

Eleven fractions were obtained by DEAE cellulose column chromatography of the crude toxin, of which one was unadsorbed. The toxins adsorbed by the column were eluted by a linear grade of 0 – 1.0 M NaCl. The three extractions of saline, buffer and glycerol were eluted by Ion Exchange Chromatography. Studies with the eluted fractions have shown lethality and symptoms of toxicity within certain fraction. Lethality was exhibited by the partially purified fractions upon i.p injection to mice at a dose of 0.1 ml/ 20g body weight.

2.4.1.1. Effect of Saline venom extract when injected into mice.

The behavioural changes such as labored breathing, violent jumping, paralysis following death were observed for lethal fractions F1 and F2. The toxic fractions showed palpitation, exploring followed by limb paralysis. The F1 fractions and the F2 fractions were lethal above a concentration of 0.45 μg/g and 0.94 μg/g respectively. F3 and F6 exhibited symptoms of toxicity. Mild toxicity was also observed for fractions, F4, F5, F7 and F8 (Table 2.4). No lethal activity or toxicity was observed for fractions F9 and F10.

2.4.1.2. Effect of Buffer venom extract when injected into mice.

Labored breathing, paralysis, arching of body, foaming from mouth following total paralysis and death were observed in mice exposed to lethal fractions while the toxic fractions showed palpitation, violent jumping and convulsions. The lethal activity was observed for F1, F4, F5 and F6 fractions. The F4, F5 and F6 fractions were lethal above a concentration of 0.53 μg/g, 0.78 μg/g and 0.97 μg/g respectively. Toxicity was exhibited for fractions F7, F8, F9 and F10. (Table 2.5).

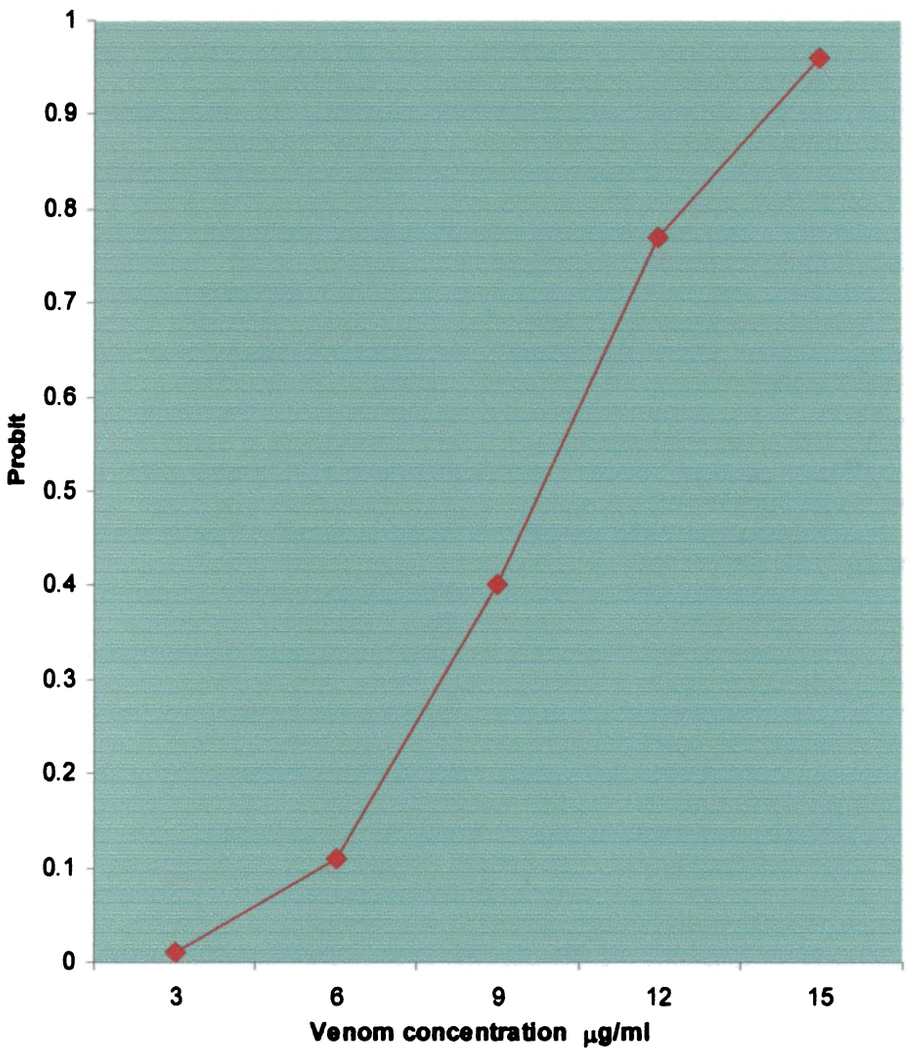


Fig 2.1 : Graph showing LD_{50} values of *S.argus* saline extract in mice/12 group.

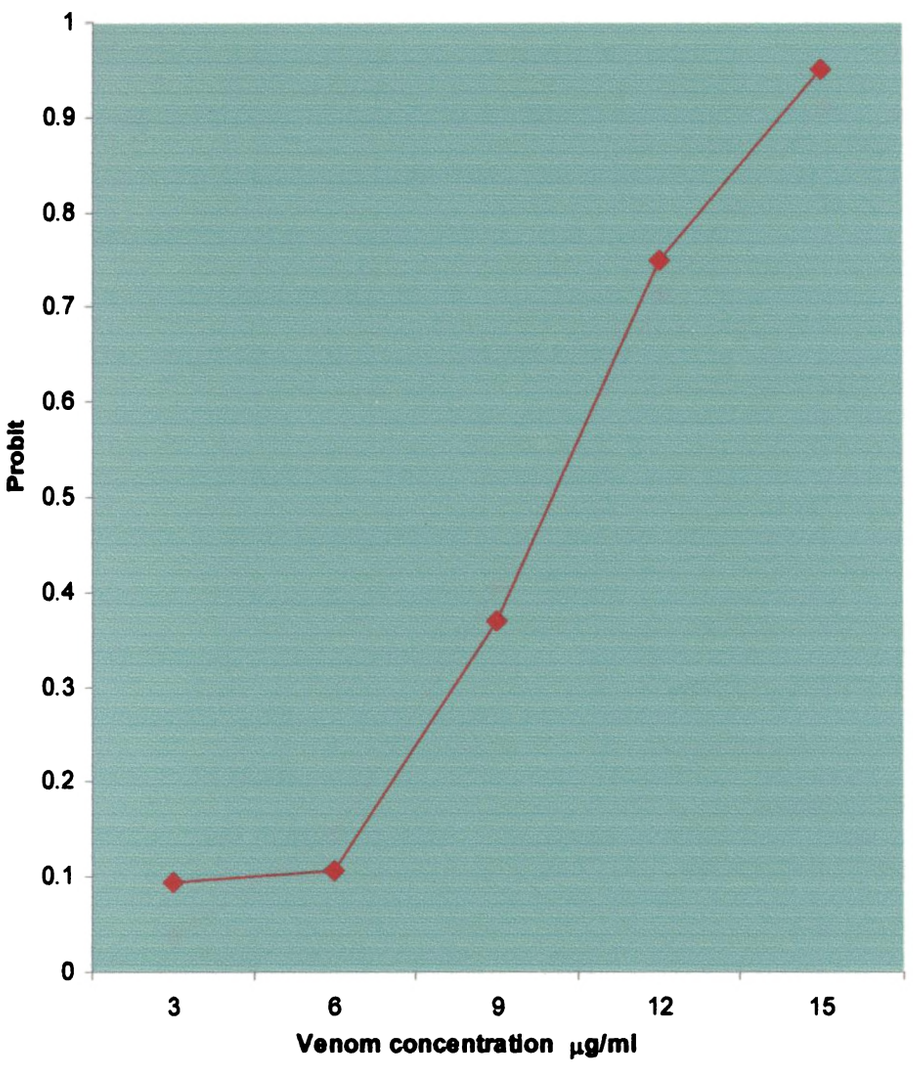


Fig 2.2 : Graph showing LD_{50} values of *S.argus* buffer extract in mice/12 group.

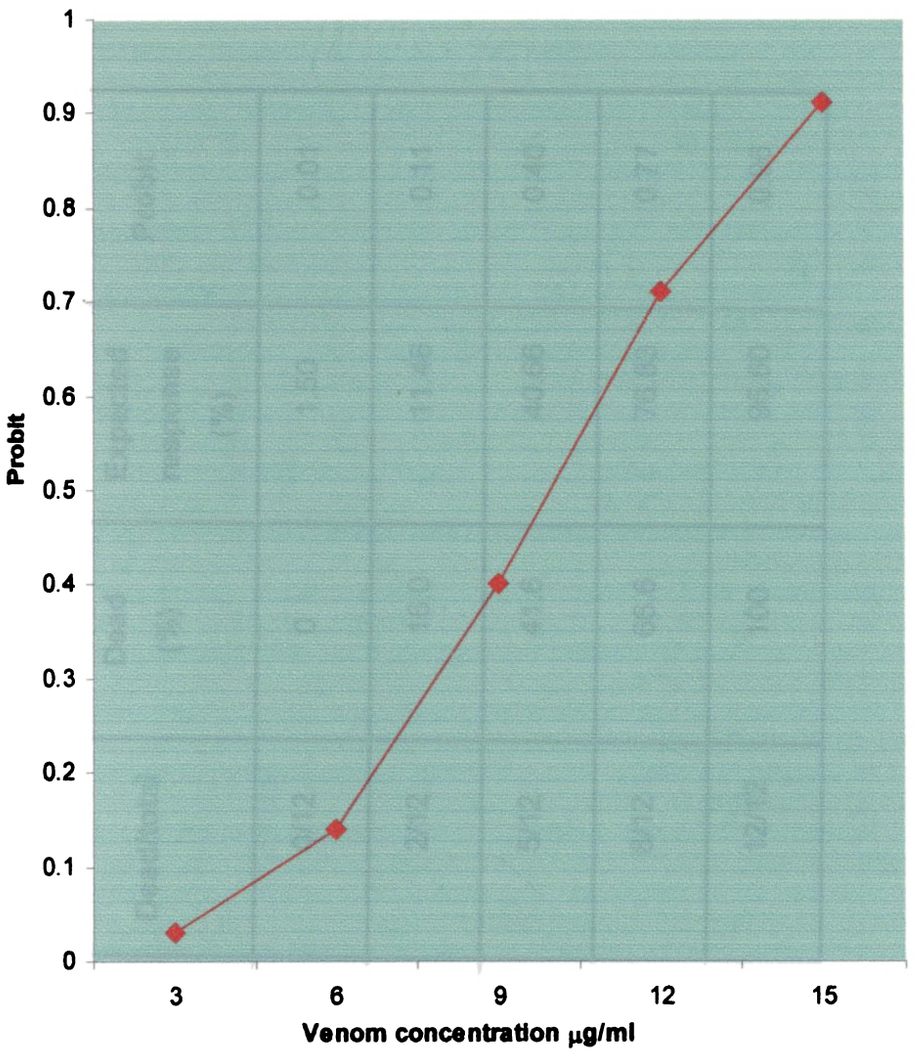


Fig 2.3 : Graph showing LD_{50} values of *S.argus* 10% glycerol extract in mice/12 group.

Group	Dose (mg/kg)	Dead/total	Dead (%)	Expected response (%)	Probit
1	3	0/12	0	1.50	0.01
2	6	2/12	16.0	11.46	0.11
3	9	5/12	41.6	40.66	0.40
4	12	8/12	66.6	76.83	0.77
5	15	12/12	100	95.60	0.96

Table 2.1. : LD_{50} values of S. argus saline extract in mice/12 group.

Group	Dose (mg/kg)	Dead/total	Dead (%)	Expected response (%)	Probit
1	3	0/12	0	1.06	0.094
2	6	2/12	16.0	9.47	0.106
3	9	4/12	33.3	37.39	0.37
4	12	8/12	66.6	74.84	0.75
5	15	12/12	100	95.16	0.95

Table 2.2. : LD_{50} values of S. argus buffer extract in mice/12 group.

Group	Dose (mg/kg)	Dead/total	Dead (%)	Expected response (%)	Probit
1	3	1/12	8.30	3.11	0.03
2	6	2/12	16.0	14.53	0.14
3	9	3/12	25.0	40.14	0.40
4	12	8/12	66.6	71.15	0.71
5	15	12/12	100	91.38	0.91

Table 2.3. : LD_{50} values of S. argus 10% glycerol extract in mice/12 group.

Sl No:	Fractions	Toxicity	Protein Concentration (µg/g)
UA	Unadsorbed	xxx	1.06
F1	0.1 M	xxx	0.45
F2	0.2 M	xxx	0.94
F3	0.3 M	xx	0.74
F4	0.4 M	x	0.53
F5	0.5 M	x	0.26
F6	0.6 M	xx	0.25
F7	0.7 M	x	0.27
F8	0.8 M	x	0.26
F9	0.9 M	-	0.31
F10	1.0 M	-	0.21

Table 2.4. : Toxicity of partially purified saline fractions using DEAE cellulose column on male albino mice of 20 ± 2g weight. All results are average of 3 independent experiments.

xxx – Lethality xx – High Toxicity x – Mild Toxicity - - No Activity

Sl No:	Fractions	Toxicity	Protein Concentration(µg/g)	
UA	Unadsorbed	xxx	0.30	
F1	0.1 M	xxx	1.32	
F2	0.2 M	-	1.35	
F3	0.3 M	-	0.61	
F4	0.4 M	xxx	0.53	Lethal Fraction
F5	0.5 M	xxx	0.78	
F6	0.6 M	xxx	0.96	
F7	0.7 M	xx	0.96	
F8	0.8 M	xx	0.95	
F9	0.9 M	xx	1.01	
F10	1.0 M	xx	0.92	

Table 2. 5. : Toxicity of partially purified buffer fractions using DEAE cellulose column on male albino of 20 ± 2g weight. All results are average of 3 independent experiments.

xxx – Lethality xx – High Toxicity x – Mild Toxicity - - No Activity

Sl No:	Fractions	Toxicity	Protein Concentration (µg/g)
UA	Unadsorbed	xxx	0.62
F1	0.1 M	xx	0.61
F2	0.2 M	xx	0.70
F3	0.3 M	xxx	1.20
F4	0.4 M	xx	1.30
F5	0.5 M	-	0.77
F6	0.6 M	-	0.71
F7	0.7 M	xxx	0.71
F8	0.8 M	x	0.64
F9	0.9 M	xx	0.72
F10	1.0 M	x	0.60

Table 2. 6. : Toxicity of partially purified glycerol fractions using DEAE cellulose column on male albino of 20 ± 2g weight. All results are average of 3 independent experiments.

xxx – Lethality xx – High Toxicity x – Mild Toxicity - - No Activity

2.5. DISCUSSION

While piscine venom differs markedly in its chemical and pharmacological properties from the venom of the terrestrial animals (Russell, 1996) they do share a number of important similarities with each other (Church and Hodgson, 2002) Venomous fish produce venom primarily for defensive purpose therefore it is not surprising that all piscine venoms produce similar symptoms in envenomated humans. Animal venoms have been recognized as potential pharmacological agents and physiological tools. The biological properties of the venoms of terrestrial animals (eg: snakes, spiders and scorpions) have been extensively investigated. However less research has been undertaken on marine creatures, particularly venomous fish. Generally this is due to the difficulties in obtaining and storing venom extracts and their extreme liability.

The behavioural changes of mice on i.p. injection of *S. argus* venom are comparable to that of the changes reported as a result of envenomation of other venomous fish. Crude venom of *S.verrucosa* induced immediate death with convulsions, pulmonary oedema, muscular discordination and paralysis, urination and respiratory failure in mice (Garnier *et al.*, 1995). Subcutaneous injection of *T.nattereri* fish venom induced systemic effects consisting of jerking motion, paralysis of hind limbs, erection of hair, rotational movements and violent convulsions followed by death (Lopez – Ferreira *et al.*, 1998).

Symptoms like paralysis and convulsions were observed for *S. argus* venom, which are suggested to be due to neurotoxic effects of the venom. Similar studies suggesting neurotoxicity have also been carried out for other fish venoms. Kelynack (1977) noted neurotoxic symptoms of *G.marmoratus* envenomation in mice including paralysis of hind limbs, muscular weakness and at higher doses, coma, and respiratory cessation. *S.plumieri* venom showed uncontrollable

movements, limb weakness and increase in urination and defecation in mice following injection of the crude venom (Carrijo *et al.*, 2005) which was similar to the behavioral changes observed in mice injected with sub lethal concentration of *S. argus* crude venom. Venom obtained from freshly captured specimens of *S. argus* was tested in animal models for a better characterization of the toxicity of the venom. Symptoms of toxicity and lethality of mice revealed the venomous nature of the spinuous extract of *S.argus.*

In the present study the LD_{50} i.v. of the venom was found to be 9.8 mg/kg of venom in mice with saline extract as well as buffer extract. The LD_{50} for the glycerol extract of venom was relatively low when compared with that obtained for stonefish and scorpion fish. The lethal studies in Scorpaenid fish for S. *horrida*, S. *trachynis*, S. *plumeiri*, S. *verrucosa*, S. *guttata* (Kreger 1991; Carrijo *et al.*, 2005; Garnier *et al.*, 1995; Khoo *et al.*, 1992; Shiomi *et al.*, 1993) show that the LD_{50} for the crude toxins in mice are somewhat comparable to each other. The stonefish *S.horrida* has an LD_{50} value of around 0.36 μg/g mice after 24 hour of i.v. injection (Poh *et al.*, 1991) and less than 125 mg protein /g of crude extract of *S. verrucosa* venom induced lethality (Garnier *et al.*, 1995).

The i.p. LD_{50} of *S. trachynis* venom in mice was approximately 1.6 mg/kg (Kreger, 1991), while 2.6mg/kg was the LD_{50} i.v for *Scorpaena guttata* (Schaeffer *et al.*, 1971). *S. plumieri* recorded an LD_{50} of 0.28 mg/kg (Carrijo *et al.*, 2005). The LD_{50} values of toadfish venom are well comparable to the values obtained for S. *argus* venom indicating that scat venoms have a critical influence on local manifestations. Apart from the stonefish the toadfish *T. nattereri* and T.*maculosa* have been shown to produce venoms which are lethal to mice with lethality of 4.93 mg/kg for *T. maculosa* and 4.54 mg/kg for *T.nattereri* (Lopes Ferriera *et al.*, 1998

and Sosa Rosales *et al.*, 2005). The crude venom extract of greater weever fish T. *draco* has a minimum lethal dose of 1.8mg/g mouse (Chattwaal and Dreyer 1992).

The lethal factor stonustoxin has been purified to homogeneity (Poh *et al.*, 1991) from the crude venom extract of S.*horrida* (Khoo *et al.*, 1992). The lethal factors isolated from the crude extracts of other species of stonefish are verrucotoxin from S.*verrucosa* (Garnier *et al.*, 1995) and trachynilysin from S.*trachynis* venom (Kreger *et al.*, 1991). Stonustoxin, the purified lethal fraction of *S.horrida* venom is highly toxic than the crude venom having a lethality of 17ng/g (Poh *et al.*, 1991). The purified fraction of *S.verrucosa* verrucotoxin was lethal at less than 40 ng/g (Garnier *et al.*, 1995).

The LD_{50} value obtained for *S. argus* crude venom is comparable to the systemic manifestations induced by the scat venom on humans when compared with symptoms of envenomation of stonefish and scorpion fish. Lethality studies for these fractions revealed lethality at lesser concentrations nearly 20 times than the crude venom (0.45 μg/g). Some of the fractions though did not possess lethality exhibited the symptoms of toxicity. The symptoms of lethality observed for the partially purified fractions are well in conformity with those observed for crude fractions.

The LD_{50} values obtained for *S. argus* venom are different for different extracts. In the three types of extraction carried out the lethal compound in the *S. argus* venom was found to be more stable in saline extract and the LD_{50} obtained for this was well below other solvents. The difference in extraction techniques and the instability of the venom may reduce the lethality of the venom. Lyophilization plays an important role in loss of potency, since Russel and Brodie (1974) reported that on lyophilisation as much as half of the lethal property may

sometimes be lost. The route of administration for the fish venoms studied depends on the authors and this difference in administration also results in different LD_{50} values obtained. Studies have shown that among the three types of extraction procedures it is convenient to use saline extract for further studies as it is found to have high lethal activity and is a suitable vehicle for the venom for all further experiments to be carried out.

Findings

- Saline extract gives an LD_{50} value of 9.8 mg /kg body weight for *S. argus* venom in mice.
- The LD_{50} value for buffer extract of *S. argus* venom is 9.97 mg /kg for mice.
- 9.92mg/kg is the LD_{50} value in mice obtained for *S. argus* venom extracted in 10% glycerol.
- Of the 11 partially purified fractions obtained for each of the above said extracts using DEAE Ion Exchange chromatography some of the fractions exhibited lethality.
- Lethality was observed for concentrations less than 45μg / g body weight for the partially purified fractions of saline extract which is 20 times more than the crude extracts.
- One of the partially purifeied fraction of buffer extact exhibited lethality at a concentration of 0.53μg / g body weight.
- The least concentration at which the glycerol fractions showed lethality was at a concentration of 0.71μg / g body weight.

Chapter 3

HISTOPATHOLOGICAL EFFECTS OF *S.ARGUS* VENOM ON MICE

3.1. INTRODUCTION

Histology is concerned with the organization of tissues and Pathology is the study of disease. Histopathology is that branch of pathology that deals specifically with tissue abnormalities. The study of cytological and histopathological alterations is an integral part of toxicology. Histopathological techniques are reliable, inexpensive, sensitive, and rapid and have the ability to provide a presumptive diagnosis of the result as well as demonstrating the tissue reaction for the assessment of damage due to xenobiotics.

Cell damage is a result of persistent or irreversible biochemical and sub cellular dysfunction caused by stress. Though the cell has a great adaptability in responding to changes in internal and external environment by undergoing reversible alterations in both cellular structure and function often the stressed cells undergo irreversible structural and biochemical changes, which in turn result in alterations in the physiology of the animal. Thus assessment of histopathological manifestation provides insight into the degree of stress, susceptibility and adaptive capability of the stressed organism and is one of the major tools for diagnosis of disease.

3.2. REVIEW

Studies involving histopathological effects of fish venoms are very few or limited in number. Studies with spine extracts on gastrocnemius muscle of mice injected with *T. natte*reri venom have been carried out [Lopes-Ferriera *et al.*, 2001]. Balasubashini *et al.*, [2006] studied the effect of *P. volitans* venom on the vital organs of mice. Studies have been conducted on the neuropathological alterations in mice brain occurring on administration of verrucotoxin, the purified fraction of fish venom from *S. verrucosa* [Breton *et al.*, 1999].

Histopathological studies on the effects of crinotoxins have been carried out. Notable among these is that of Deo [2000] on the epidermal secretions of two marine catfish *Arius disumieri* and *Osteogeneosus militaris* and that of Al- Hassan *et al.*, [1985] on the skin toxin of *Arius thalassinus*. Histopathological studies on rabbits injected with crude skin toxin of Arabian Gulf catfish *A.bilineatus* have shown that the toxin causes liver and lung damage [Alnaqeeb *et al.*, 1989].

3.3. MATERIAL AND METHODS

The gross anatomical changes upon autopsy and histopathological changes in various organs of the mice that succumbed to the spine extracts of *S. argus* were studied.

3.3.1. Gross Anatomical Changes

Autopsy was carried out on mice, which died upon envenomation to observe the gross anatomical changes. The colour changes if any of the brain, pale or dark discoloration as the case may be of the heart, liver, lungs and kidney and also other abnormalities such as internal haemorrhage, retention of fluids were examined.

3.3.2. Histopathology

The metabolic pathway traversed by the toxin often dictates the choice of organs of study. Liver [being the major detoxifying organs directly receiving materials from intestine], kidney [the major excretory organ involved in xenobiotic excretion], brain [the major organ controlling CNS] and heart [the effect of the toxin on the cardiac system and the cardiotoxic nature of xenobiotic] being the cardinal organs were selected for study.

Brain, heart, liver and kidney were excised from the envenomated mice and fixed in 10% formalin. The tissues were dehydrated in ascending grades [30%, 50%, 70%, 90% and 100%] of alcohol for 1 hour each. The samples were then cleared in xylene for two hours and impregnated in paraffin wax thrice each time for 45 minutes. The samples were embedded in wax and were allowed to solidify and the surplus amount was trimmed off. Sections of 4 µm thickness were made using a hand rotary microtome. The sections were mounted on microslides and dried. The sections were deparaffinised in xylene and hydrated in descending grades of alcohol.

The best sections among them were picked up on a microscopic slide. The excess water was removed using a blotting paper. Dewaxing was done by drying the slides in hot plate for 2-3 hours and by clearing them in xylene. Samples were then hydrated in descending grades of alcohol. Staining was done by using Delafield's hematoxylene for 7 minutes. These were then passed through descending grades of alcohol [3 minutes each], followed by eosin stain [3 minutes] and absolute alcohol [1 dip] and finally cleared in xylene. These were mounted on DPX. Prepared sections were examined and photographed under a Leica microscope.

3.4. RESULTS

3.4.1. Gross Anatomical changes

Autopsy revealed non-specific heamorrhage inside the body cavity and discoloration of heart and liver in mice injected with crude *S. argus* venom whereas no discoloration was observed for brain and kidney.

3.4.2. Histopathological Effects

The effect of *S. argus* venom on the vital organs are very well confirmed by the histopathological changes of the vital organs liver, kidney, brain and heart. In the case of liver moderate degenerative changes were noticed and the hepatic cells lost their structural integrity. Extremely vacuolated areas and haemolysis were observed in addition to marked pycnotic nuclei [Fig.3.1.]. Microvesicular types of fatty changes [Microvesicular steatosis] were also seen in hepatocytes. Blood sinusoids were distended, congested, or disrupted with partially haemolysed blood; in most of the cases hepatic cells were disrupted and also cells were occluded by haemolysed blood. Focal areas in the liver revealed hepatic cells undergoing degenerative changes and coagulative necrosis. Occasionally oedematous fluids were also seen at some places.

Noted histopathological changes were observed in the kidney. Histopathological analysis indicated that the venom and its fraction acted on the renal tubules and glomeruli. Blood vessels were highly congested with haemolysed blood and haemorrhage was observed [Fig.3.2.]. Cloudy swellings in the lining of renal tubules were noted. In addition to tubular necrosis, pycnotic nuclei were also seen. The parietal epithelium of Bowman's capsule was found to be prominent. Proteinaceous /foreign material were found accumulated with the glomerulus and often shrinkage of the glomerular tuft could be seen. Moderate degenerative changes were noticed and the cells had lost their normal structure.

In the case of brain focal area lysis [Encephalomalacia] were noticed. Demylinated areas were observed. Coagulative necrosis and pycnotic nuclei were also seen [Fig.3.3.]. Blood sinusoids were highly congested with haemolysed blood and haemorrhage was observed. Glial nodule formation was observed in

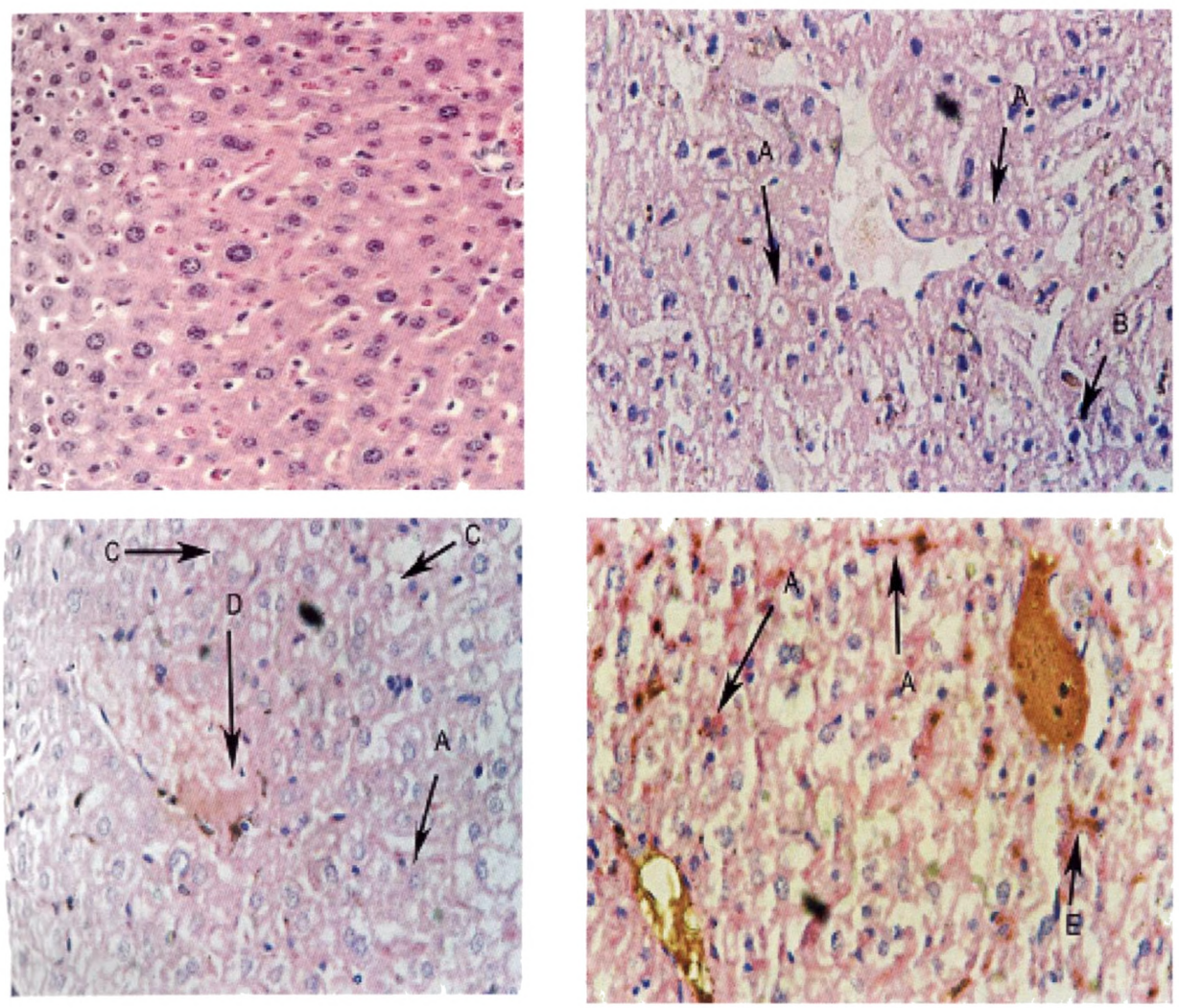


Fig. 3.1. Histopathological effects of S. argus venom on mice liver. Control and sections showing A.Vacoulation B.Pycnotic nuclei C.Congested blood sinusoids D.Necrosis E. Blood clot.

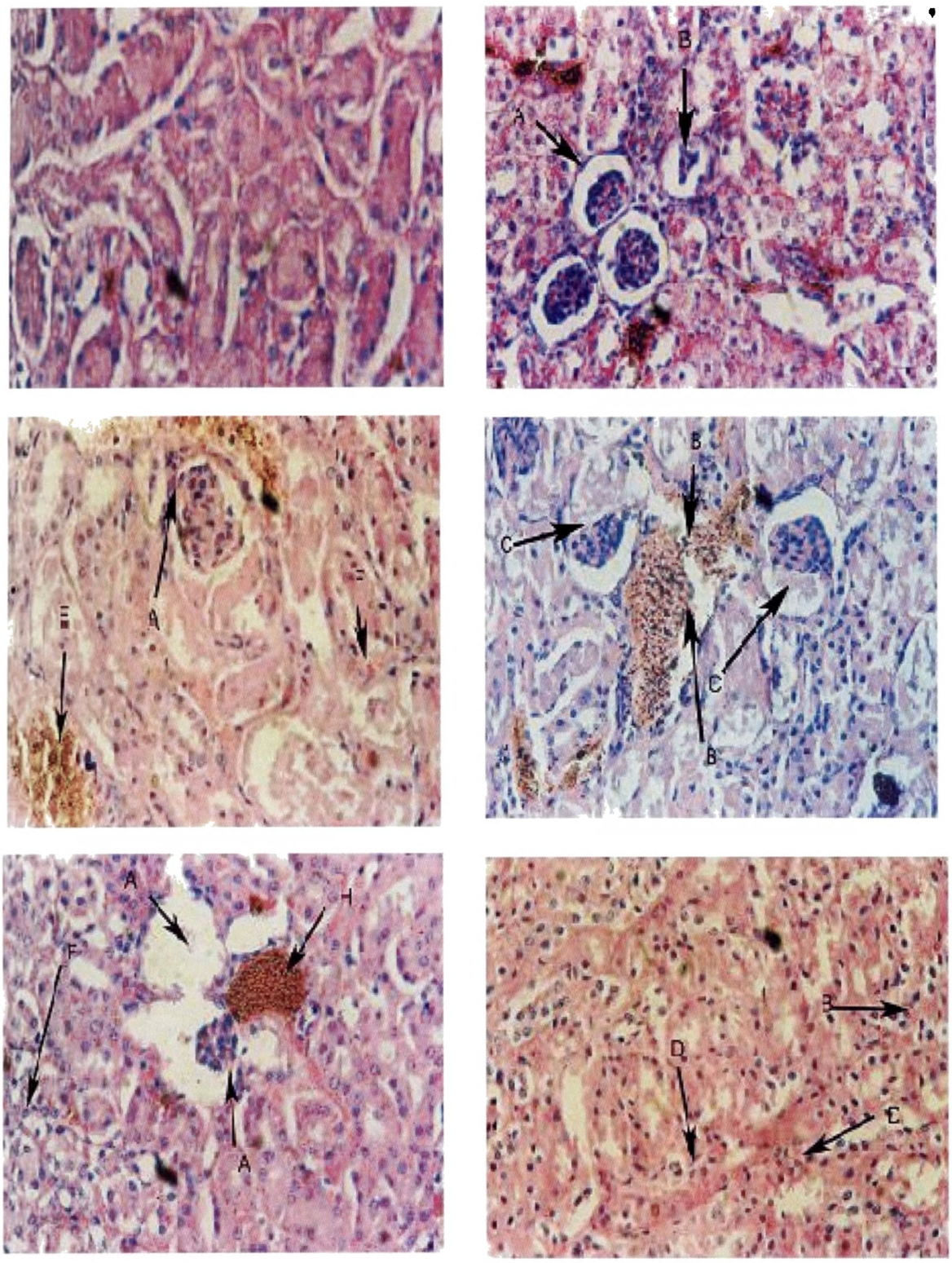


Fig. 3.2. Histopathological effects of S. argus venom on mice kidney. Control and sections showing A.Thickening of bowmans capsule B.Shrinkening of glomeruli C.Proteinaceous or foreign material present D. Tubular necrosis E.Lysis F.Congested blood sinusoids H.Blood clot.

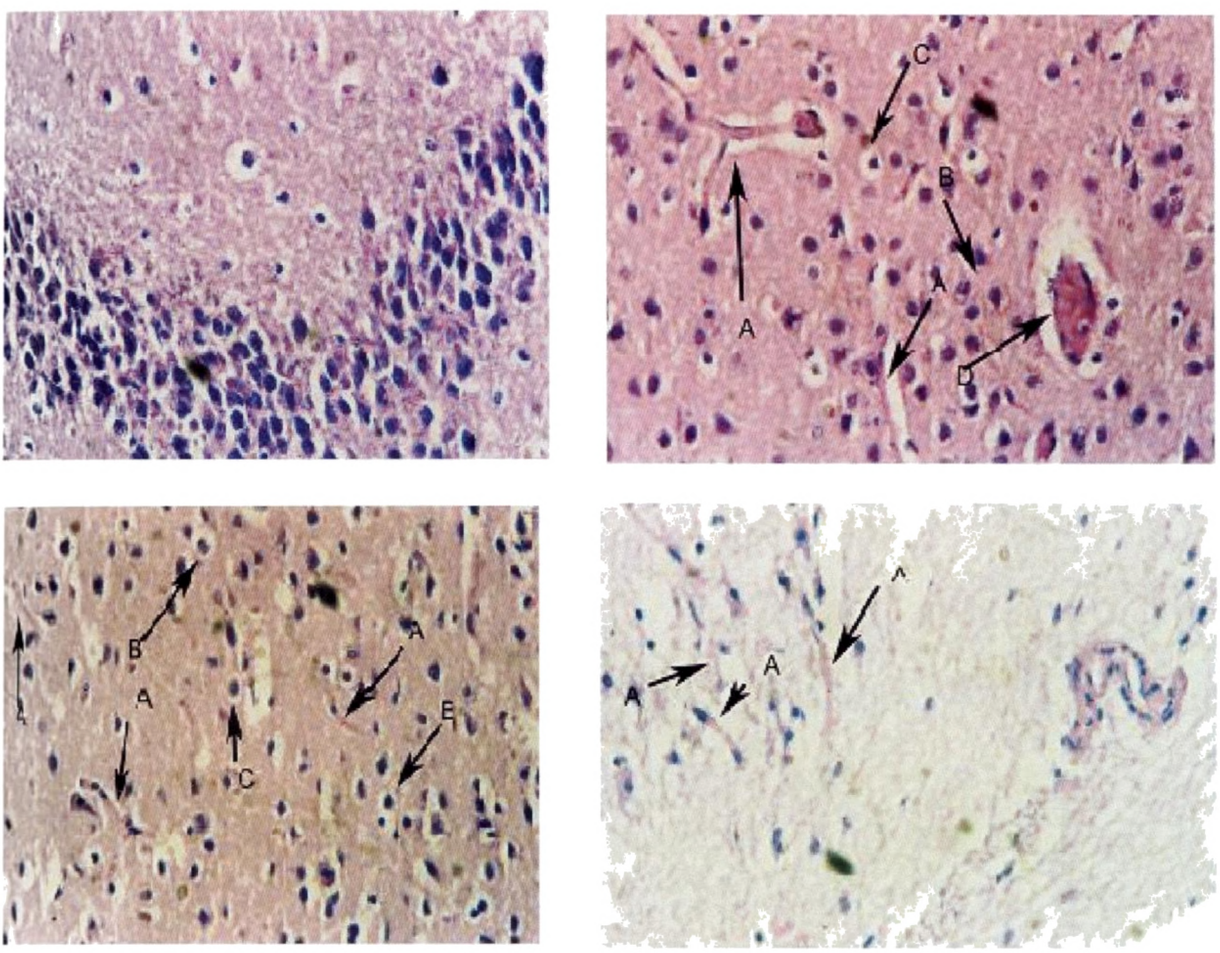


Fig. 3.3. Histopathological effects of S. argus venom on mice brain. Control and sections showing A. Congested blood sinusoids B.Pycnotic nuclei C.Vacoulation D.Necrosis.

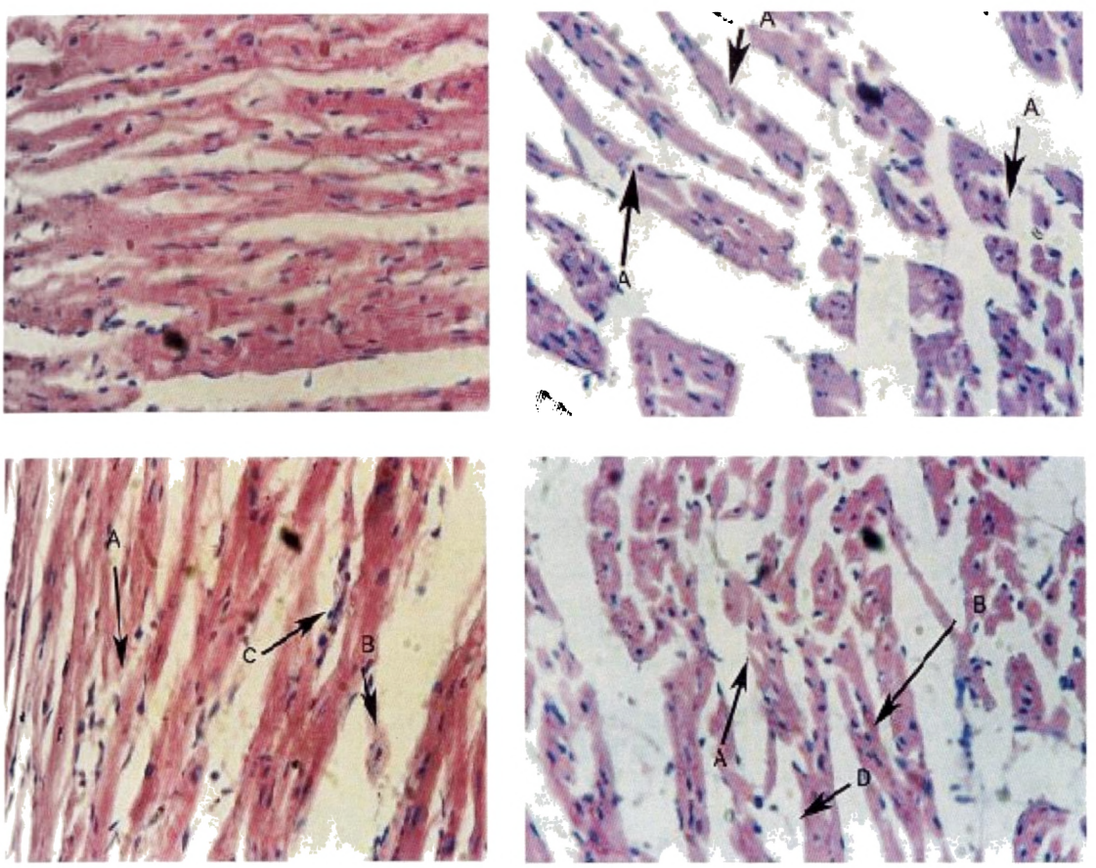


Fig. 3.4. Histopathological effects of S. argus venom on mice heart. Control and sections showing A. Myofibrillar degeneration B. Blood clot C.Pycnotic nuclei D.Vacoulation.

some areas of the cerebrum. Brain tissue showed spongiosis [oedema] throughout the parenchyma. Prominent histopathological changes of the heart were degeneration of myofibrils, and focal areas of necrosis [Fig.3.4.]. Cloudy areas of swelling were also observed in addition to pycnotic nuclei. Coagulative necrosis was also seen.

3.5. DISCUSSION

The histopathological changes revealed the effect of *S. argus* venom on the vital organs like liver, kidney, brain, and hear. The venom appeared to be injurious to the vascular endothelium and caused congestion of blood vessels and cloudy swellings in liver, kidney, brain, and heart. Liver of *S. argus* venom treated mice showed congestion, cloudy swelling, microvesicular fatty changes, and infiltration of inflammatory cells around the portal vein. The damage to the hepatocytes in the present study could be attributed to the storage of the toxin in liver for detoxification.

Brain tissue of *S. argus* venom treated mice showed spongiosis throughout parenchyma. This is in similarity to the effect of *P. volitans* venom on rat brain [Balasubashini *et al.,* 2006]. Areas of haemorrhage, vascular congestion, and cloudy swelling in renal tubules were observed that revealed the pathological alterations caused in the kidney of *S. argus* venom treated mice. Heart showed the presence of oedema and degeneration of myofibrils when compared to that of the normal animal.

The effects of *S. argus* venom on the vital organs like liver, kidney, brain, and heart are in accordance with that of *P. volitans* venom on rats [Balasubashini *et al.,* 2006]. *P. volitans* venom caused congestion, microvesicular fatty changes, and infiltration of inflammatory cells around the portal vein. Brain tissue showed

spongiosis [oedema] throughout the parenchyma, whereas lungs show areas of haemorrhage, congested blood vessels and inflammatory cells in parenchyma. Areas of haemorrhage, vascular congestion and cloudy swelling in renal tubules were observed in the kidney. Heart of the envenomated mice had presence of oedema and degeneration of myofibrils when compared to the normal animal. [Balasubashini *et al.*, 2006] The oedema of brain and the cloudy swelling in lining cells of renal tubule suggest that the venom might contain oedema-causing factors that could have crossed over the blood–brain barrier [BBB] and damaged the brain [Saminathan *et al.*, 2006]. Cloudy swellings were observed in all the vital organs under study. The effects of *S. argus* venom on the vital organs are very well confirmed by the histopathological changes of the venom treated mice. Similar results were observed during administration of venom from *T. nattereri* [Fonesca and Lopes Ferriera, 2000] *Tityus serrulatus* [Correa *et al.*, 1997] and *Conus lorreossi* [Saminathan *et al.*, 2006].

Liver of venom treated mice showed congestion, micro vesicular fatty changes and infiltration of inflammatory cells around the portal vein. Absuma and Venketashvaran [1999] have reported that administration of crude extract of epidermal secretion of *Boleophthalmus dentatus* causes discoloration of heart, liver and kidney. Alnaqeeb *et al.*, [1989] observed extensive haemorrhage in liver tissue which was attributed to tissue destruction, due to blocking of blood flow in turn leading to necrosis. Toxic effect on liver was also observed for the skin toxin of *Arius thalassinus* [Al-Hassan *et al.*, 1985]. Skin toxin of the giant slender moray eel *Thyrsoidea macrura* showed extensive necrosis and haemorrhage in kidney and liver of mice [Raju and Venketashvaran, 1999].

Similar effects were also observed for skin toxins from the three arid catfish *Arius caelatis, A. dissumieri* and *Osteogeneiosus militaris* [Variath and

Venketashvaran, 1999]. Histopathological studies of mice exposed to *A. dissumieri mucus* extract showed pycnotic nuclei [Deo, 2000]. Studies on the histological evaluation of rat kidney perfused with *Thallasophryne nattereri* venom showed moderate deposits of proteinaceous material in the renal tubule [Faco *et al.*, 2003].

Kidney cells can release prostaglandins, cytokinins, bradykinin, complement fractions and platelet activating factors [Barraviera *et al.,* 1995]. The histopathological alterations noticed in the kidney cells in the present study could be due to the direct action of the venom in renal glomeruli and tubules or an indirect release of mediators.

Intravital microscopic study with *T. nattereri* fish venom showed pronounced alterations on microvesicular haemodynamics represented by fibrin depots and thrombus formation followed by complete venular and transient arteriolar stasis [Lopes-Ferriera *et al.,* 2002]. Kristensen et al., [2000] described that intact endothelium is non-thrombogenic. Damage to the endothelial lining of microvessels promotes procoagulant events activating platelets and coagulation cascade resulting in thrombosis.

From the present study it is concluded that the *S. argus* venom is hepatotoxic, nephrotoxic, cardiotoxic and neurotoxic to the test mice, as it showed pronounced histopathological effects on liver, kidney, heart and brain.

Findings

- Histopathological changes revealed the effect of *S. argus* venom on the vital organs of mice.

- Venom appeared to be injurious to the vascular endothelium of mice and caused congestion of blood vessels.
- Tubular necrosis in kidney, coagulative necrosis in liver and myofibrillar degeneration of cells in heart and haemolysis in all organs shows the cytolytic activity of venom.
- Microvesicular fatty changes were noted in liver tissue of envenomated mice. Foreign / Proteinaceous material was observed in Bowman's capsule.
- Vacoulation and pycnotic nuclei were observed throughout the cells in all organs.
- Spongiosis in brain, infiltration of inflammatory cells around the portal vein and the cloudy swellings in the renal tubule all points towards the oedematic activity of the venom.
- Congested blood vessels, spongiosis, pycnotic nuclei, glial cell accumulation, focal area necrosis all state the neurotoxicity of *S. argus* venom.
- Thickening of Bowman's capsule, shrunken glomeruli, proteinaceous / foreign material within Bowman's capsule, haemolysis, blood clot, tubular necrosis all revealed the nephrotoxicity of *S. argus* venom.
- Myofibrillar degeneration, pycnotic nuclei, blood clot emphasise the cardiovascular toxicity of *S. argus* venom.
- The hepatotoxicity of the *S. argus* venom is revealed by the alterations in liver tissue. Micro vesicular fatty changes were noted. In addition pycnotic nuclei and vacoulation were observed. Congested blood vessels, coagulative necrosis and infiltration of inflammatory cells around the portal vein were observed.

Chapter 4

EFFECT OF *S.ARGUS* VENOM ON MICE SERUM AND TISSUE ENZYMES

4.1. INTRODUCTION

Enzymes are the primary indicators of the health of animals and can be easily and accurately quantified when compared to other indicators of health. Venom induced changes in enzyme activity could represent the symptoms of metabolic impairments [Jackim *et al.,* 1970]. Differential tissue enzyme sensitivity to venom could be attributed to the ability of the compounds to alter the cellular membrane configuration by binding with lipid protein of membrane and thus blocking the movement of substances by active transport. Toxins are known to alter the physiological and biochemical stage of the animals by inducing marked changes in the activities of several enzymes

Chemical compounds and reactants capable of generating potential free radicals are referred to as pro-oxidants. On the other hand, compounds and reactions disposing off these species, scavenging them, suppressing their formation, or opposing their actions are called antioxidants. An appropriate balance between the pro-oxidant anti-oxidant is vital for normal functioning. The increase in intracellular levels of reactive oxygen species [ROS] to such a level that cellular antioxidant defences are insufficient to maintain these harmful molecules below a toxic threshold level is generally referred to as oxidative stress, the basis of many physiological aberrations.

Free radicals are formed by cleavage of a covalent bond of molecule, by the loss of a single electron from a normal molecule or by the addition of a single electron from a normal molecule. Most of the molecular oxygen consumed by aerobic cells during metabolism is reduced to water by using cytochrome oxidase in mitochondria. When oxygen is partially reduced it becomes activated and reacts readily with a variety of bio-molecules. This partial reduction occurs in single electron steps by addition of one, two or four electrons to O_2, which leads to

successive formation of ROS. There are five possible species: O_2^- [Superoxide Anions], HO_2 [hydroperoxyl radical], peroxide ion [HO_2^-], hydrogen peroxide [H_2O_2] and hydroxyl radical [-OH].

Lipid Peroxidation

Lipid peroxidation is otherwise known as oxidative deterioration of polyunsaturated lipids. It is one of the several mechanisms by which reactive oxygen species turn toxic to cells and tissues. It induces alteration in the structure and function of cellular membrane, which could lead to cell injury. Lipid peroxidation arising from the direct reaction of free radicals with lipids is considered as a feature of cellular injury. This progresses by three operationally defined processes: initiation, propagation and termination. The initiation phase of peroxidation usually proceeds with the formation of conjugated diene bonds generated by abstraction of hydrogen atoms from the methylene group of polyunsaturated lipids. Propagation of lipid peroxidation relies on the interaction of molecular oxygen with carbon-centered free radicals to propagate the lipid peroxidation. Through this chain reaction, one initiating radical may lead to the peroxidation of hundreds of fatty acids. The resulting hydroperoxides are unstable and decomposed by chain leavage to very complex mixtures of aldehydes [such as malondialdehyde] ketones, alkanes, carboxylic acids and ploymerisation products [Esterbauer *et al.*, 1982].

Antioxidant Defence against Oxyradicals

Antioxidant defense mechanism operates by detoxifying or scavenging these ROS. The antioxidant system comprises of different types of functional components classified as first line, second line and third line defences.

Preventive Antioxidants – First Line Defence

The first line defence comprises preventive antioxidants that act by quenching of O_2^-, decomposition of H_2O_2 and sequestration of metal ions. This is the catalytic removal of ROS by antioxidant enzymes and free radical scavenger [antioxidants]. Superoxide dismutase [SOD], Catalase [CAT] and peroxidases constitute mutually supportive team of defence against ROS. While SOD lowers the steady state level of O_2^-, catalase does the same for H_2O_2.

a] Superoxide dismutase [SOD]

The biosynthesis of Superoxide dismutase [SOD] is controlled by its substrates super oxide [O_2^-] the active oxygen radical produced at different stages of aerobic metabolism [Kizaki *et al.*, 1997; Liy *et al.*, 1995; MacMillan – Crow *et al.*, 1998;Meier *et al.*, 1998; Yamakura *et al.*, 1998].

SOD catalyses the reaction

$$2O_2^- + 2H \xrightarrow{SOD} H_2O_2 + O_2$$

SOD is a metalloprotein found in both prokaryotic and eukaryotic cells in different forms. The iron containing [Fe SOD] and Manganese containing [Mn – SOD] enzymes are characteristic of prokaryotes while copper containing and zinc containing enzymes are characteristic of eukaryotes.

b] Glutathione Peroxidase [GPx]

Glutathione peroxidase is the most important peroxidase for the detoxification of hydroperoxides. These selenoenzymes catalyse the reduction of

hydroperoxides [Flohe, 1989] with reduced glutathione [GSH] to form glutathione disulphide [GSSG] and the reduction product of the hydroperoxide.

$$2GSH + ROOH \xrightarrow{GPx} GSSG + ROH + H_2O$$

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O$$

GPx are present in the cytosol and mitochondrial matrix [Mills, 1960].

c] Catalase [CAT]

Catalase a ubiquitous heme protein detoxifies hydrogen peroxide to oxygen and water and thus protects the cell from oxidative damage by H_2O_2 and OH.

$$2H_2O_2 \xrightarrow{CAT} 2\,H_2O + O_2$$

Catalase present in almost all mammalian cells is localized in the peroxisomes and is an enzyme of high molecular weight.

d] Glutathione Reductase [GR]

Glutathione reductase, a flavoprotein, holds key role in the reduction of oxidized GSSG at the expense of NADPH [Schirmer and Siegel, 1989]

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

e] Glutathione – S – Transferase [GST]

Glutathione – S – Transferase [E.C.2.5.1.18] is a multifunctional enzyme system catalyzing the conjugation of biologically active electrophiles to the endogenous tripeptide glutathione [GSH] [Fahey *et al.*, 1984].

Radical Scavenging Antioxidants – Second line of defence

The antioxidants belonging to second line defense include glutathione [GSH], ascorbic acid, uric acid, albumin, bilirubin, vitamin E, carotenoids and flavanoids.

a] Glutathione [GSH]

Glutathione is a tripeptide [gamma glutamyl cysteinyl glycine] and the most abundant thiol in most tissues. Its unique structure holds the key for the two-fold function. It acts as an antioxidant, which scavenges O_2^- and a cofactor for enzymatic reactions. Glutathione play a key role in detoxification by reacting with H_2O_2 and organic peroxides, the harmful byproducts of aerobic life.

b] Ascorbic Acid

Ascorbic acid directly reacts with O_2^- and OH. When compared to other water soluble antioxidants Vitamin C offers the most effective protection against free radical damage [Yu, 1994].

Repair and De-Novo Enzymes, Third line of defence

Third line antioxidants are complex group of enzymes for repair of damaged protein, oxidized lipids and peroxide and also to stop chain propagation of peroxyl

lipid radical eg: lipase, proteases, DNA repair enzymes, transferase, methionine sulpoxide reductase, etc. [Henle and Linn, 1997].

Conjugates dienes, Malondialdehyde and Hydroperoxides

The quantification of oxidative stress is complicated by the short half-lives of free radicals and many of the products initially produced by free radical attack on electron rich substrates such as PUFA's [Esterbauer, 1996]. Consequently the detection of oxidative stress has relied largely on the quantification of compounds such as conjugated dienes, hydroperoxides as well as malondialdehyde [MDA] which are formed by degradation of initial products of free radical attack [Janero, 1990]. The reaction of MDA with thiobarbituric acid [TBA] is one of the most widely used methods of estimation of oxidative stress [Liu *et al.*, 1997].

Phosphatases

Phosphatases catalyse the hydrolytic cleavage of phosphoric acid esters. They are designated either as acid phosphatase [ACP] [E.C.3.1.3.2] or alkaline phosphatase [ALP] [E.C.3.1.3.1] depending on their pH optima. Phosphatases are concerned with carbohydrate metabolism [Milter and Grave, 1961] oxidative phosphorylation and growth and differentiation. Phosphatases serve as indicators of stressful situation [Gupta and Dhillon, 1988].

Alkaline phosphatase is a brush border enzyme involved in membrane transport and transphosphorylation process and is a good indicator of stress in biological systems. They are intrinsic plasma membrane enzymes found on the membranes of almost all animal cells. Acid phosphatases act as marker enzymes for the detection of lysosomes in cell fractions and can be altered by the presence of venom. Acid phosphatase is a lysosomal enzyme that hydrolyses the ester

linkage of phosphate esters and helps in autolysis of cell after its death. These enzymes are involved in a variety of metabolic processes such as molecular permeability, growth and cell differentiation and steroidogenesis [Ram and Sathayanesan, 1985].

Transaminases

Transaminases are widely distributed enzymes which play important role in metabolic pathways. Transamination serves as a pathway of conversion of alpha-ketoacids to L-aminoacids and as an alternate means of replenishing pyruvate pool. Glutamate Oxaloacetate Transaminase [GOT] or Aspartate Transaminase [AST] [E.C.2.6.1.2] and Glutamate Pyruvate Transaminase [GPT] or Alanine Transaminase [ALT] are two widely studied transaminases. ALT catalyses the transfer of amino group from L-alanine to 2-oxoglutarate with the formation of pyruvate and L-glutamate. AST catalyses the transfer of aminogroup from L-aspartate to 2-oxaloglutarate forming oxaloacetate and L-glutamate. Transamination allows interplay between carbohydrate, fat and protein metabolism, favouring gluconeogenesis an activity which can serve the changing demands of organisms.

ALT and AST are active under stress, starvation or any altered physiological condition. Liver parenchyma is a rich source of AST and has a very high concentration of ALT. Under normal conditions there are a baseline activity of these enzymes. But when the organism is subjected to stress, the levels of these enzymes are significantly increased in order to meet the increase in ATP demands. Thus the activity of transaminase would convert amino acids to keto acids like pyruvate and oxaloacetate which would be used as intermediate in Kreb's cycle or directed into gluconeogenic pathway.

4.2. REVIEW

Natural toxins are unique even though they possess some common properties irrespective of the source beings plants, microorganisms or animals. One common characteristic is the ability of very low concentration of these toxins to disrupt the metabolism and biological functions of the intoxicated animals with just minute quantity. The cardiac and the liver enzymes of rabbit envenomated with the crude extract as well as the purified toxic factor from Arabian Gulf catfish have been studied [Al-Hassan *et al.,* 1985; Thomson *et al.,* 1998]. Serum enzymes of mice have been studied for the lionfish *P. volitans* [Balasubashini *et al.,* 2006].

Studies on the relationship of lethality of the venom and enzymes of mice have been carried out with the venom of *Naja naja* and *Leiurus quiquestriata* [Omran *et al.,* 1997; Omran and Rahman 1992]. Saminathan *et al.,* [2006] studied the effect of conus venom on the serum enzymes of mice. The animals have the capacity to regulate and modulate the inherent diversions in their metabolism to meet the altered physiological conditions [Hoar, 1976]. This is easily done to meet the energy demand under attenuated or imposed stress conditions to facilitate synthesis of extra energy to overcome such impeding situations. In the light of the above literature, some aspects of enzyme system in selected tissues were determined.

4.3. MATERIAL AND METHODS

4.3.1. Experimental Design

Inbred Swiss albino male mice of two months age, weighing 20 ± 2g were used for the study. The mice were obtained from the stock inbred colony which was maintained on dry pellets of rat feed and water ad libitum.

Of the four groups of mice three groups were injected with crude *S. argus* venom in saline at concentrations of 0.98 μg/ml, 1.47 μg/ml and 1.97 μg/ml. One group of mice was injected only with saline [control]. Mice were sacrificed at different time intervals of 2, 4, 6, 12 hours. Blood was collected and serum was separated from blood cells by centrifugation at about 2000 rpm for 30 min. Serum was used for enzyme assay. Liver and kidney were dissected out, blotted, weighed, minced, and stored in aliquot at -80°C for enzyme assay. The activity of the enzymes SOD, CAT, GPx, GSH, GR, GST and the content of MDA, CD, Ascorbate and Hydroperoxides present in liver and kidney were assayed. In addition to liver and kidney for the enzymes AST, ALT, ACP, and ALP level in serum was also assayed.

4.3.2. Detoxifying and antioxidant enzymes

a] Estimation of Superoxide Dismutase

Reagents		
Sodium pyrophosphate buffer	-	0.052M [pH8.3]
Tris-HCl buffer	-	0.0025M [pH7.4]
Sucrose	-	0.25M
Phenazine methosulphate	-	186μM
Nitro blue tetrazolium	-	300 μM
NADH	-	780 μM
Glacial acetic acid		
n-butanol		

Superoxide dismutase activity in tissues was determined using the method of Kakkar *et al.,* [1984]. 100mg tissue [liver and kidney] were homogenized in 2ml, 0.25M sucrose and differentially centrifuged to get cystosol fraction. This fraction was then dialysed against 0.0025M Tris HCl buffer [pH7.4] overnight before using

for enzyme assay. Assay mixture contained 1.2ml of the sodium pyrophosphate buffer, 0.1ml of phenazine methosulphate, 0.3ml nitroblue tetrazolium, 0.2ml NADH and 1.2ml of the enzyme source. Reaction was initiated by the addition of NADH and incubated at 30^0C for 90 sec. The reaction was stopped by the addition of 1ml glacial acetic acid. Reaction mixture was shaken vigorously with 4.0ml of n-butanol. The mixture was allowed to stand for 10 min. and was centrifuged. The upper butanol layer was taken out. Colour intensity of the chromogen in butanol was measured at 560nm, against n-butanol blank. A system devoid of enzyme served as control. Protein estimation was carried out on the same enzyme source by the method of Lowry et al. The values were expressed as 50% inhibition of nitroblue tetrazolium / min/mg protein.

b] Estimation of Catalase

Reagents

Phosphate buffer	-	0.5M [pH 7.5]
H_2O_2	-	30 µl / 10 µl buffer

Catalase level in tissues was determined using the method of Machly and Chance [1974]. 100mg of tissue [liver and kidney] were homogenized in 2ml of phosphate buffer and centrifuged. To 30µl of the supernatant, added 3ml of buffer and 0.75ml of H_2O_2. Change in OD was measured at 240 nm at 0 sec, 30 sec, 60 sec respectively. The control system devoid of tissue extract was used as blank. Values are expressed in µ moles of H_2O_2 consumed/min/mg protein.

c] Estimation of Glutathione peroxidase [GPx]

Reagents

Tris buffer	-	0.4M [pH7.0]
Sodium azide solution	-	10mM

Trichloro acetic acid [TCA]	-	10%
Ethylene diaminetetra acetic acid [EDTA]	-	0.4mM
Hydrogen peroxide [H_2O_2]	-	0.2mM
Reduced Glutathione solution	-	2mM

Glutathione peroxidase was estimated by the method of Rotruck [1973]. Weighed sample of tissue [liver and kidney] were homogenized in a known volume of Tris buffer. To 0.2ml of Tris buffer, 0.2ml EDTA, 0.1ml Sodium azide and 0.5ml tissue homogenate were added and mixed well. To this mixture 0.2ml of GSH followed by 0.1ml H_2O_2 solution were added. The contents were mixed well and incubated at 37^0C for 10 min along with a control containing all reagents except tissue homogenate. After 10min the reaction was arrested by the addition of 0.5ml of 10% TCA. Tubes were centrifuged and the supernatant was assayed for GSH by the method of Beutler and Kelley [1986].

GSH estimation by the method of Beutler and Kelley [1986]

1ml of the supernatant from the above test tubes was taken, 0.5ml of Ellman's reagent and 3ml of phosphate buffer were added. The yellow colour developed was read at 412nm with a blank containing 3.5ml of phosphate buffer. A series of standards were also treated similarly. The amount of GSH was expressed in mg/100g tissue.

d] Estimation of Glutathione-S-transferase [GST]

Reagents

Phosphate buffer	-	0.5M [pH 6.5]
CDNB	-	25mM in 95% ethanol
GSH	-	20mM

Glutathione-S-transferase [GST] in tissues was determined using the method of Beutler *et al.*,[1984]. Tissues were homogenized in phosphate buffer. The reaction mixture containing 200µl phosphate buffer, 20µl CDNB, and 730µl distilled water were taken in the control tubes and 200µl phosphate buffer, 20µl CDNB, and 680µl distilled water were taken in the sample test tubes. Then the tubes were incubated at 37^0C for 10min. After the incubation, added 50µl of GSH in both set of tubes. After mixing well, added 50µl of tissue extract in the test sample tubes. Absorbance was noted at 340nm for 5 minutes in a quartz cuvette of 1cm length path in a spectrophotometer. Values were expressed as n moles of CDNB complexed/min/mg protein.

e] Estimation of Glutathione reductase [GR]

Reagents		
Phosphate buffer	-	0.5M [pH 6.5]
EDTA	-	8.25 mmol/l
NADPH	-	9.6 mmol/l
GSSG	-	65.3 mmol/l

Glutathione reductase was estimated by the method of Goldberg [1983]. Weighed sample of tissue was homogenized in a known volume of phosphate buffer, 2.6ml of buffer, 0.1ml EDTA, and 0.1ml GSSG acts as reagent mixture to which was added 0.1ml of the homogenized sample and kept for 5 min. Then 0.05ml of NADPH was added, mixed thoroughly and read at 340nm for 5 min against buffer blank.

4.3.3. Antioxidants

a] Estimation of Ascorbic acid

Reagents

Trichloro acetic acid	-	6%
Thiourea agent	-	50% in alcohol
2,4,dinitrophenylhydrazine	-	2% in 9N sulphuric acid
Con. Sulphuric acid	-	85%
Ascorbic acid standard		
Activated charcoal		

Ascorbic acid level in tissues [liver and kidney] was determined using the method of Roe [1954]. Immediately after sacrificing the animals a weighed sample of tissue was homogenized in 5ml ice cold 6% TCA in a pre-chilled mortar. The extract was shaken well in a test tube, added activated charcoal and allowed to stand for 15min. The clear supernatant was filtered through Whatman filter paper No.1. To 4ml of supernatant, added a drop of Thiourea reagent [50% in alcohol] and 1ml of 2%, 2, 4 dinitrophenyl hydrazine in 9N H_2SO_4 and incubated for 3h at 37^0C in a water bath. At the end of the incubation, place the test tubes in an ice bath and added carefully 4ml of 85% Con. H_2SO_4. Kept for 30 minutes in refrigerator. Centrifuged and OD of the supernatant measured at 540 nm in a spectrophotometer. The values are expressed in mg/100g tissue.

b] Estimation of Glutathione [GSH]

Reagents

Alloxan	-	0.1M
Phosphate buffer	-	0.5M [pH 7.5]
NaOH	-	0.5N

NaOH	-	1N
GSH standard		

Glutathione level in tissue was determined using the method of Patterson and Lazarrow [1955]. Weighed sample of tissue [liver and kidney] were homogenized in phosphate buffer. The reaction mixture containing 50µl tissue extract, 50µl alloxan, 50µl phosphate buffer, and 50µl NaOH [0.5N] was incubated at 25^0C for 6 minutes. The reaction was stopped by the addition of 50µl 1N NaOH. Absorbance was noted at 305nm in a quartz cuvette of 1cm length path in a spectrophotometer. A control tube was maintained with phosphate buffer instead of extract. The values were expressed in mg/100g tissue.

4.3.4. Lipid peroxidation products such as Malondialdehyde [MDA], Conjugated Dienes [CD] and Hydroperoxides

a] Estimation of Malondialdehyde

Reagents		
Sodium Phosphate buffer	-	50mM [pH 7.4]
SDS [Sodium dodecyl Sulphate]	-	8.1%
Glacial acetic acid	-	20%
Thiobarbituric acid	-	0.8%
n-butanol and pyridine	-	15:1 [v/v]

Malondialdehyde was estimated by the method of Ohkawa *et al.*, [1979]. Weighed sample of the tissue [liver and kidney] were homogenized in a known volume of the buffer. 0.1ml homogenate was taken and mixed with 0.2ml SDS, 1.5ml glacial acetic acid and 1.5ml thiobarbituric acid. The mixture was heated at 95^0C for 1hr on a water bath and cooled under tap water. Then added 1ml distilled water and 5ml mixture of n-butanol and pyridine. The mixture was shaken

vigorously and centrifuged at 2000 rpm for 5minutes and the upper organic layer was read at 532nm. The blank contained all the reagents except the sample. The result was expressed as moles of MDA/mg protein.

b] Estimation of Conjugated dienes

Reagents

Tris HCl buffer	-	0.025M [pH 7.5]
Chloroform :Methanol	-	2:1 [v/v]
Cyclohexane AR		

Conjugated dienes were estimated by the method of Beuge J.A [1978]. Weighed sample of tissue [liver and kidney] were homogenized in a known volume of the buffer. An aliquot of the homogenate was shaken with Chloroform: Methanol [2:1] and the lower layer were recovered. Then evaporated this layer to dryness and re-dissolved in a known volume of cyclohexane. Read the absorbance at 233nm against cyclohexane as blank. The amount of conjugated dienes was expressed in terms of millimoles/100g tissue.

c] Estimation of Hydroperoxides

Reagents

Potassium Iodide	-	6 mg in 5 ml distilled water
Cadmium Acetate 0.5%	-	500 mg in 100 ml distilled water
Chloroform : Methanol	-	2:1
Acetic acid : Chloroform	-	3:2

Hydroperoxides were estimated by the method of Mair and Hall [1977]. Weighed sample of tissue [liver and kidney] were homogenized in a known volume of buffer. 1 ml of tissue homogenate was mixed with 5 ml of chloroform : methanol [2:1] followed by centrifugation at 1000 g for 5 min to separate the phases. 3 ml of

lower chloroform layer was recovered using a syringe and placed in a test tube and dried in a 45°C water bath under a stream of nitrogen. 1ml of acetic acid : chloroform [3:2] mixture followed by 0.05ml of KI was quickly added and the test tubes were stoppered and mixed. The tubes were placed in the dark at room temperature exactly 5 min followed by the addition of 3 ml of cadmium acetate. The solution was mixed and centrifuged at 1000 g for 10 min. The absorbance of the upper phase was read at 353 nm against a blank containing the complete assay mixture except the tissue homogenate.

4.3.5. Phosphatases

a] Estimation of Acid phosphatase

Reagents

Paranitrophenyl phosphate	-	400 mg%
Citrate buffer	-	0.1M [p^H4.8]
Sodium hydroxide	-	0.1N
Paranitrophenol		

Acid phosphatase was measured according to the method of Anon [1963]. 0.5 ml of p-nitrophenyl phosphate [400 mg%] was mixed with an equal volume of 0.1M citrate buffer of pH4.8. The enzyme was added and incubated for 30 minutes at room temperature. At the end of 30 minutes, reaction was stopped by the addition of 4ml of 0.1N NaOH. The absorbance of the solution was measured at 410nm in UV spectrophotometer [Hitachi]. The amount of p-nitrophenyl liberated by the acid phosphatase / hour / gm gives the specific activity.

b] Estimation of Alkaline Phosphatase

Reagents

Disodium phenyl phosphate [Merck]: 0.01 M, Sodium carbonate and bicarbonate Buffer: 0.1 M, 3.18 g anhydrous sodium carbonate and 1.68 g Sodium bicarbonate dissolved in 500ml distilled water.

Buffer substrate for use:

Prepared by mixing equal volumes of the above two reagents:

Sodium hydroxide	-	0.5 N
Sodium bicarbonate	-	0.5 M
4-amino antipyrene [sigma]	-	0.6%
Potassium ferricyanide	-	2.4%

Alkaline phosphatase was assayed following the method of King and Jagatheesan, [1959]. Measured 2 ml of buffered substrate into each of the two test tubes and was incubated at 37°C for few minutes in a water bath. Then 0.1 ml of serum or 0.1 ml tissue [Liver and Kidney] homogenate were added to the test tubes [test] and further incubated for 15 min. At the end of incubation, the tubes were removed from the bath. To it added 0.8 ml of sodium hydroxide, 1.2 ml of sodium hydroxide, and 1.2 ml of sodium bicarbonate to both tubes. Then 0.1 ml of serum or 0.1ml tissue was added to the second tube to respective tubes as [Blank]. To both tubes, added 1 ml of amino antipyrene reagent and 1 ml potassium ferricyanide solution. For standard 1.1 ml of the buffer and 1 ml of phenol solution containing 0.01 mg of phenol, and for the standard blank 1.1 ml buffer and 1 ml distilled water were taken, instead of buffered substrate and serum. All other procedures were same as in the case of test samples. The absorbance was read at 520 nm. The amount of p-nitrophenyl liberated by the acid phosphatase per hour per gm gives the specific activity.

4.3.6. Transaminases

a] Estimation of Aspartate aminotransferase [AST]

Reagents

Buffered substrate

Phosphate buffer	-	0.1 M [pH 7.4]
Aspartic acid	-	1 M
2-oxoglutarate	-	2 mM
NaOH	-	0.4 N

Pyruvate standard

Sucrose.	-	0.25 M

2, 4-Dinitro phenyl hydrazine [DNPH]

Aspartate aminotransferase [AST] was assayed by the method of Mohun and Cook, [1957]. Homogenate of kidney and liver tissues and serum were prepared in 0.25 M cold sucrose solution and centrifuged at 1000 g for 15 minutes. The supernatant obtained was used as the enzyme source. Pippetted out 1 ml each of buffered substrate into two test tubes labeled 'test' and 'control'. Added 0.2 ml of the enzyme / serum to the tube labeled 'test' and incubated the tubes at 37°C for 60 minutes. After incubation, 0.2 ml of the enzyme / serum was added to the control tube. 1 ml of 2, 4-DNPH reagent was added to each tube and kept at room temperature for 20 minutes. The reaction was stopped by the addition of 10 ml of 0.4 N NaOH, vortexed and kept at room temperature for 5 minutes. The absorbance was measured at 520 nm in a spectrophotometer against blank. The blank preparation was the same as that of the experimental, except that the corresponding volume of distilled water replaced the supernatant. The AST activity in tissue and serum were expressed as units / min/ mg protein.

b] Estimation of Alanine transaminase [ALT]

Reagents

Buffered substrate

Phosphate buffer	-	0.1 M [pH 7.4]
DL-alanine	-	0.2 M
2-oxoglutarate	-	2mM

2, 4-Dinitro phenyl hydrazine [DNPH]

Sodium hydroxide	-	0.4 N

Standard pyruvate solution.

Alanine transaminase [ALT] was estimated by the method of Mohun and Cook, [1957]. 10% homogenate of liver and kidney tissues were prepared in 0.25 M cold sucrose solution and centrifuged at 1000 g for 15 minutes. The supernatants were used for assay. Pippetted out 1 ml each of buffered substrate into two test tubes labelled 'test' and 'control'. Added 0.2 ml of the supernatant / serum to the tube labeled 'test' and incubated the tubes at 37°C for 30 minutes. After incubation, 0.2 ml of the enzyme / serum was added to the control tube. 1 ml of 2, 4-DNPH reagent was added to each tube and kept at room temperature for 20 minutes. The reaction was stopped by the addition of 10 ml of 0.4 N NaOH, vortexed and kept at room temperature for 5 minutes. The absorbance was measured at 520 nm in a spectrophotometer. The values for tissue and serum ALT were expressed as units / min/ mg protein.

4.3.7. Statistical Analysis

The SPSS® statistical software for windows, version 13.0 [SPSS Inc., Chicago, USA] was used in all data analyses. To examine the significant differences in tissue enzymatic activity of the venom treated mice over the periods of observations [2,4, 6, and 12 hour] GLM [General linear model] doubly

multivariate repeated measures procedure was applied. After which a post hoc test, Bonferroni *t*-test was done for multiple comparisons.

4.4. RESULTS

The toxic effect of *S. argus* venom on mice was studied. Antioxidant enzymes like superoxide dismutase [SOD], Catalase [CAT], Glutathione peroxidase [GPx], Glutathione reductase [GR], Glutathione –S- transferase [GST]; Antioxidants like Glutathione and ascorbate and lipid peroxidation products like malondialdehyde [MDA], conjugated dienes [CD] and hydroperoxides were assayed in liver and kidney of mice envenomated with different concentration of *S. argus* venom are given in Fig 4.1 – 4.10. Enzyme acid phosphatase [ACP], alkaline phosphatase [ALP], alanine transaminase [ALT], and asparate transaminase [AST] were analysed in tissues of liver and kidney and in serum of mice envenomated with *S. argus* venom and the results are given in Fig 4.11 – 4.14.

4.4.1. Antioxidant enzymes and Detoxifying enzymes

The activity of antioxidant enzymes namely, superoxide dismutase [SOD], catalase [CAT], glutathione peroxidase and detoxifying enzymes glutathione reductase and glutathione -S- transferase in mice envenomated with *S. argus* venom are given in fig 4.1. – 4.5. The statistical analysis for each enzyme is given in table 4.15. – 4.20. [see appendix]

The enzyme SOD showed an increase in activity both in liver and kidney with increase in time and increase in concentration. An increase in SOD was observed for liver and kidney tissues at 2 hours. The values increased again when compared to that of the control in kidney tissues by 4 hours. The increase in activity was observed in liver by 6 hours, which was retained for about 12 hours.

Activity was more in liver than in kidney. Doubly multivariate repeated measures revealed an overall significant increase in SOD activity both in liver [F=6.2] [p < 0.05] and kidney [F=6.2][p < 0.05] of mice envenomated with different concentrations of venom in comparison to the control group] over time. Multiple comparisons by Bonferroni t – test revealed that all the concentrations caused a significant increase in SOD both in liver [F=426.2] and kidney [F=531.4] [p < 0.05] when compared to control.

Doubly multivariate repeated measures revealed an overall significant increase in CAT activity both in liver and kidney [p < 0.05] when mice exposed to different concentrations of venom were compared with control group over time. An increase in catalase activity was evident with increase in concentration for liver [F=15.6] and kidney [F=5.8]. As time elapsed the activity increased, consistently for about 6 hours after which a decrease in activity was observed by 12 hours. Multiple comparisons by Bonferroni t-test revealed that all the concentrations caused a significant increase in CAT both in liver and kidney [p <0.05] when compared to control.

The GPx showed an elevation in activity, which was more prominent with time. The increase in activity was peaked in 4 hours, after which it was stable for about 6 hours. By 12 hours the GPx level in kidney and liver were restored. An overall significant increase in GPx activity both in liver [F=426.9] and kidney [F=313.9] was revealed by doubly multivariate repeated measures [p < 0.05] between different concentrations of venom with control group over time. Multiple comparisons by Bonferroni t-test revealed that all the concentrations caused a significant increase in GPx both in liver and kidney [p < 0.05] when compared to control.

The increment observed for GST was dose and time dependent. An overall significant increase in GST activity both in liver and kidney was revealed by doubly multivariate repeated measures [$p < 0.05$] between different concentrations of venom with control group over time. Multiple comparisons by Bonferroni t-test revealed that all the concentrations caused a significant increase in GST both in liver [F=90.6] [$p < 0.05$] and kidney [F=65.5] [$p < 0.05$] when compared to control. The increase was peaked at about 4 hours and remained up to 6 hours. By 12 hours the activity decreased.

With increased concentration an increase was observed for GR both in liver and kidney. An overall significant increase in GR activity both in liver and kidney was revealed by doubly multivariate repeated measures [$p < 0.05$] between different concentrations of venom with control group over time. Multiple comparisons by Bonferroni t-test revealed that all the concentrations caused a significant increase in GR both in liver [F=5.2] [$p < 0.05$] and kidney [F=35.3] [$p < 0.05$] when compared to control.

4.4.2. Antioxidants

Glutathione [GSH] and ascorbate [Vitamin C] levels were studied in the liver and kidney of mice envenomated with different concentration of *S. argus* venom and the results are given in table 4.6. and 4.7. The statistical analysis is represented in the table 4.21 and 4.22.

The glutathione levels both in kidney and liver increased with increase in dose and time. The increase in concentration of ascorbate levels in tissues was prominent for liver. In liver the increase was dose and time dependent. An overall significant increase in GSH activity both in liver and kidney was revealed by doubly multivariate repeated measures [$p < 0.01$] between different concentrations of

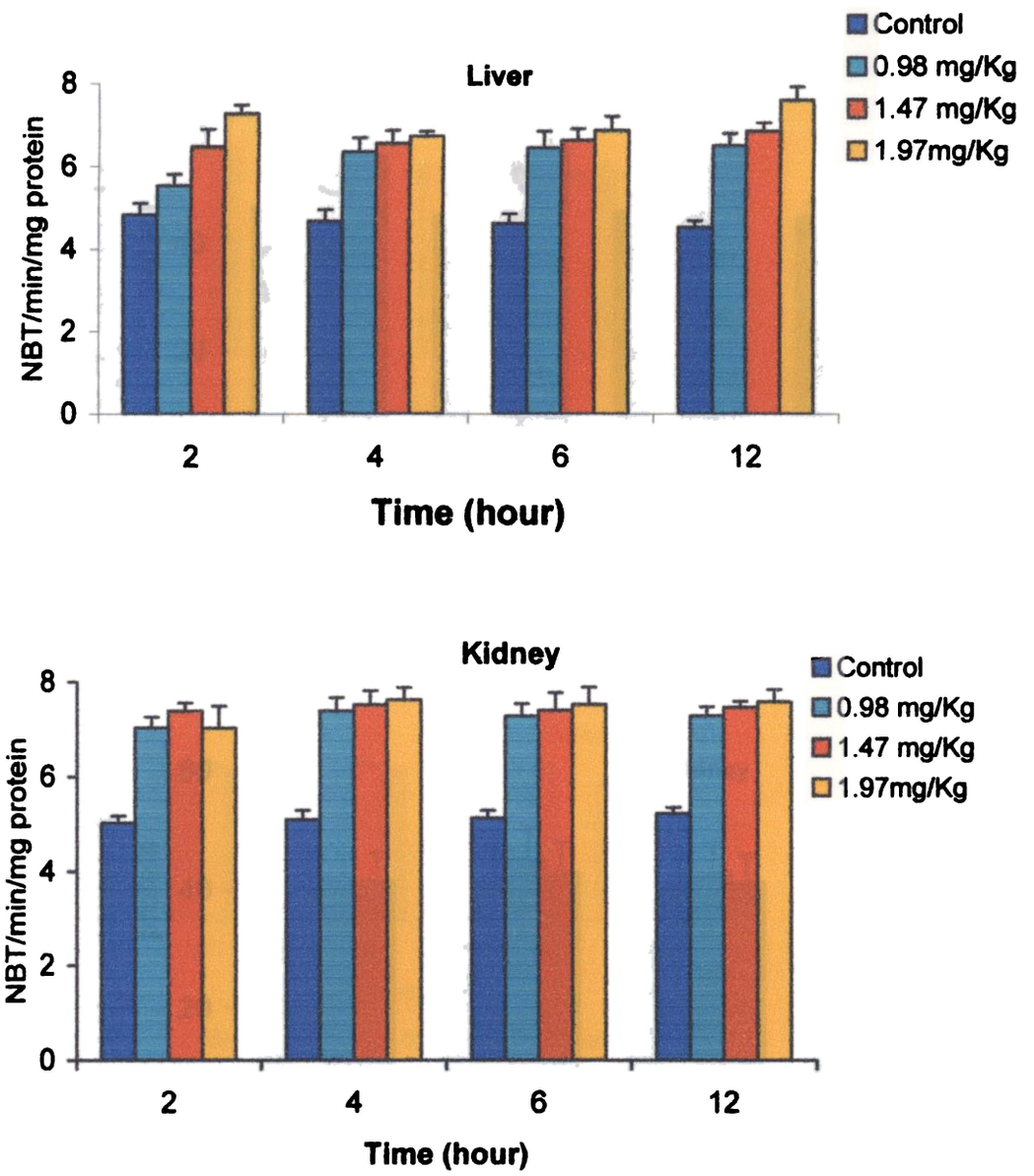


Fig 4.1: Superoxidedismutase [SOD] activity in tissues of mice envenomated with S. argus venom. Values are expressed as 50% inhibition of NBT/min/mg protein. Results are mean± SD of six separate determinations.

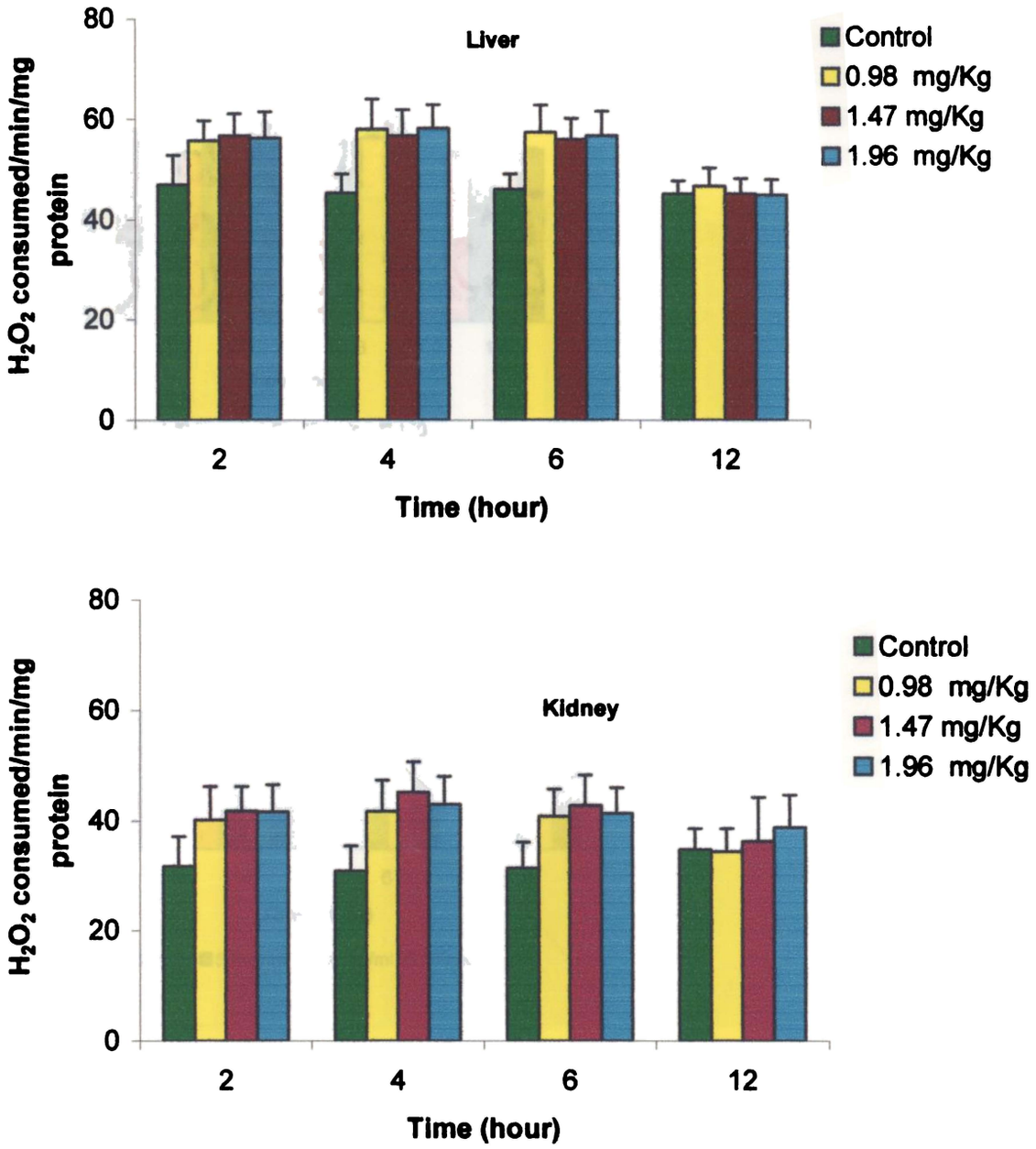
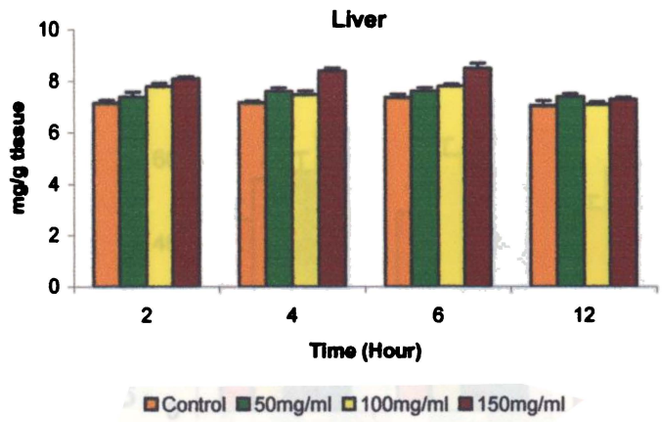


Fig 4.2.: Catalase activity in tissues of mice envenomated with S. argus venom. Values are expressed as H_2O_2 consumed/min/mg protein. Results are mean± SD of six separate determinations.



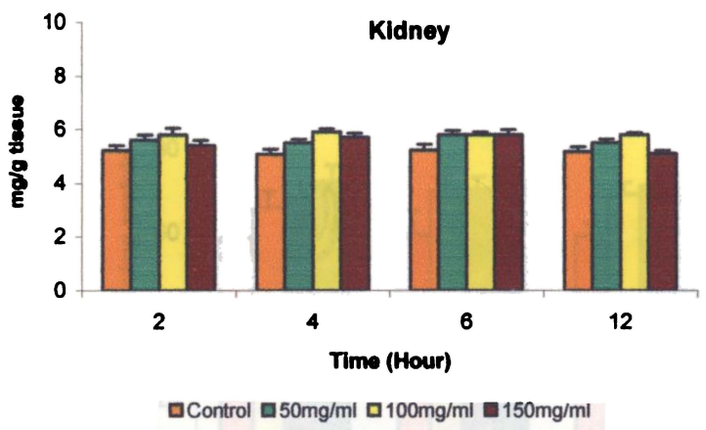


Fig 4.3.: Glutathione peroxidase [GPX] activity in tissues of mice envenomated with S. argus venom. Values are expressed as µg of glutathione /min/mg protein. Results are mean± SD of six separate experiments.

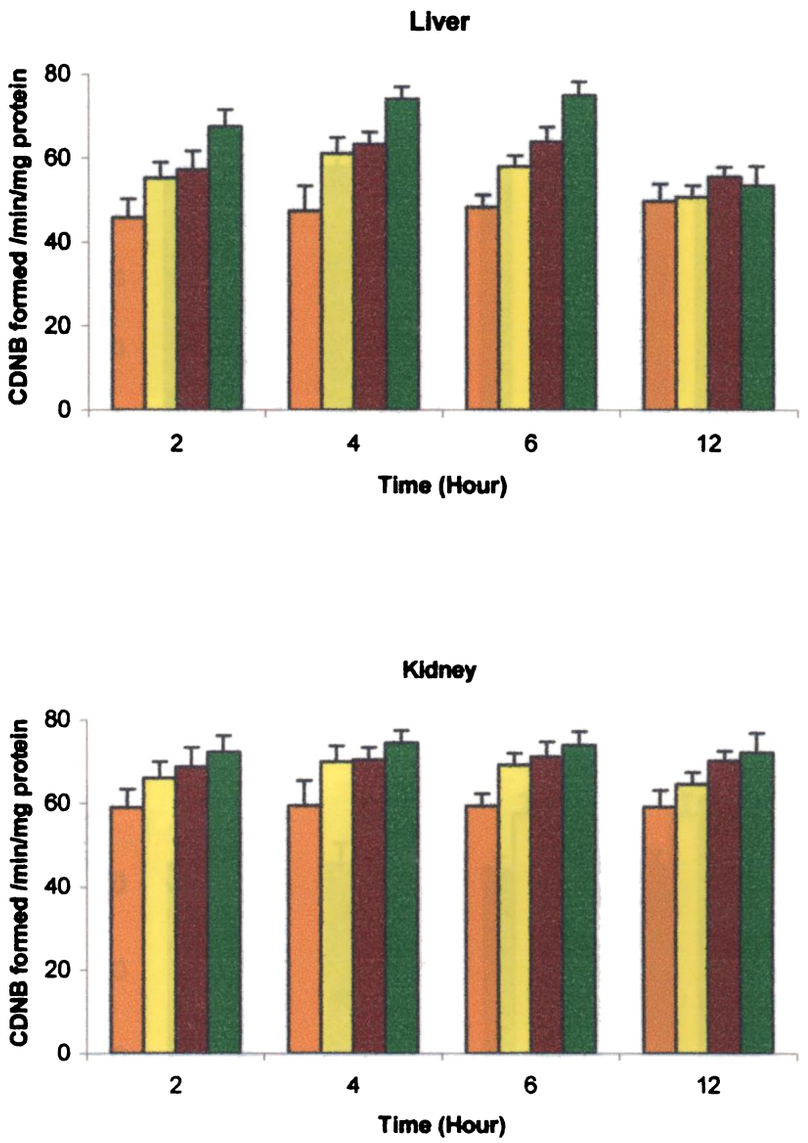


Fig 4.4. Glutathione –S-transferase activity in tissues of mice envenomated with S. argus venom. Values are expressed as n moles of CDNB formed /min/mg protein. Results are mean± SD of six separate experiments.

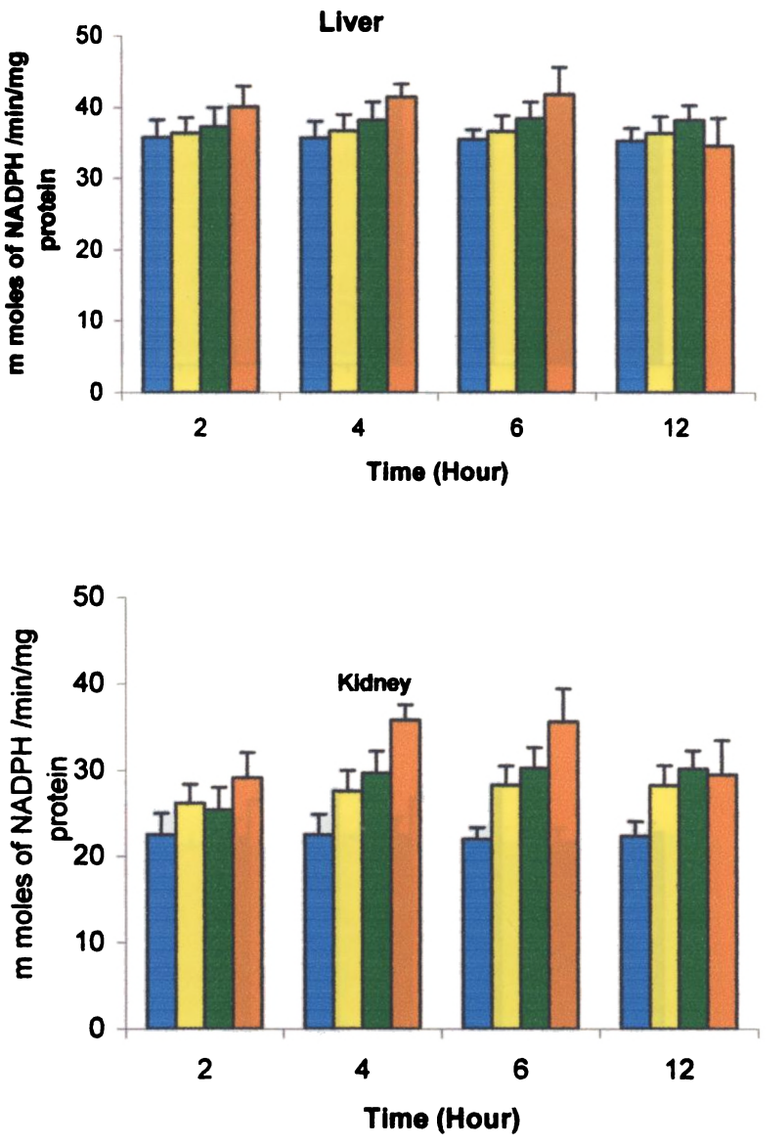
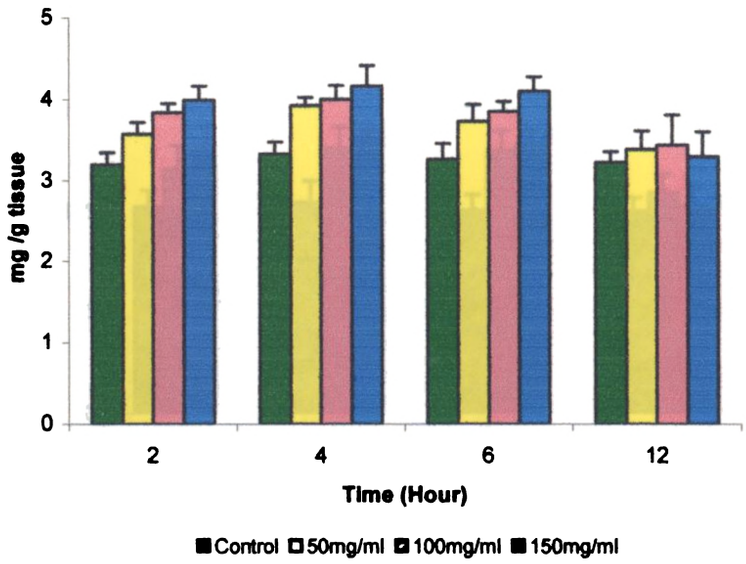


Fig 4.5: Glutathione reductase activity in tissues of mice envenomated with S. argus venom. Values are expressed as m moles of NADPH /min/mg protein. Results are mean± SD of six separate experiments.



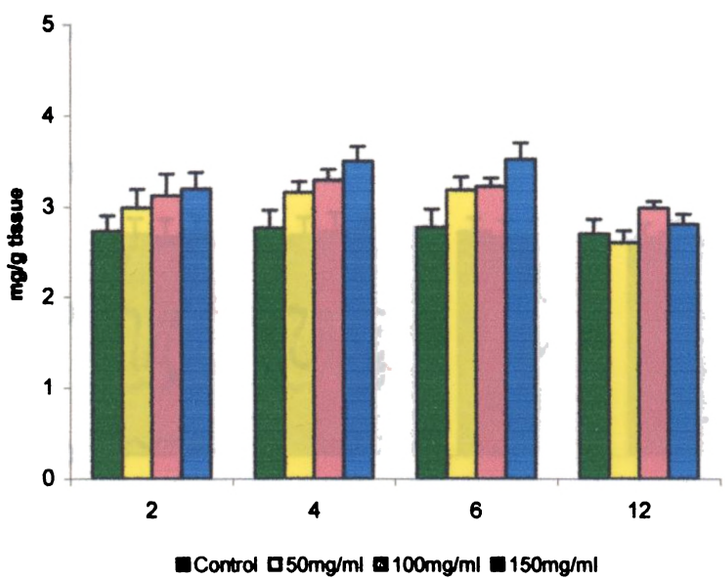


Fig 4.6.: Glutathione levels in tissues of mice envenomated with S. argus venom. Values are expressed as mg /g tissue. Results are mean± SD of six separate experiments.

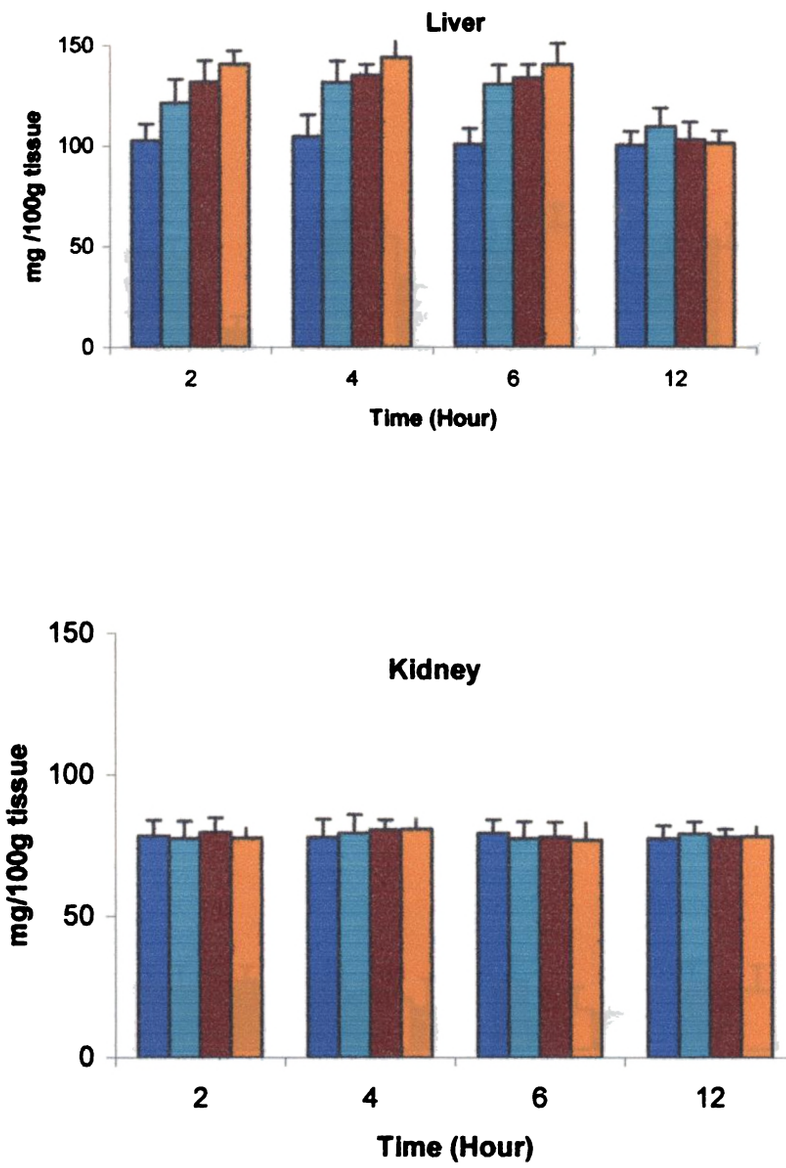


Fig. 4.7.: Ascorbate [Vitamin C] levels in tissues of mice envenomated with S. argus venom. Values are expressed as mg /100g tissue. Results are mean± SD of six separate experiments.

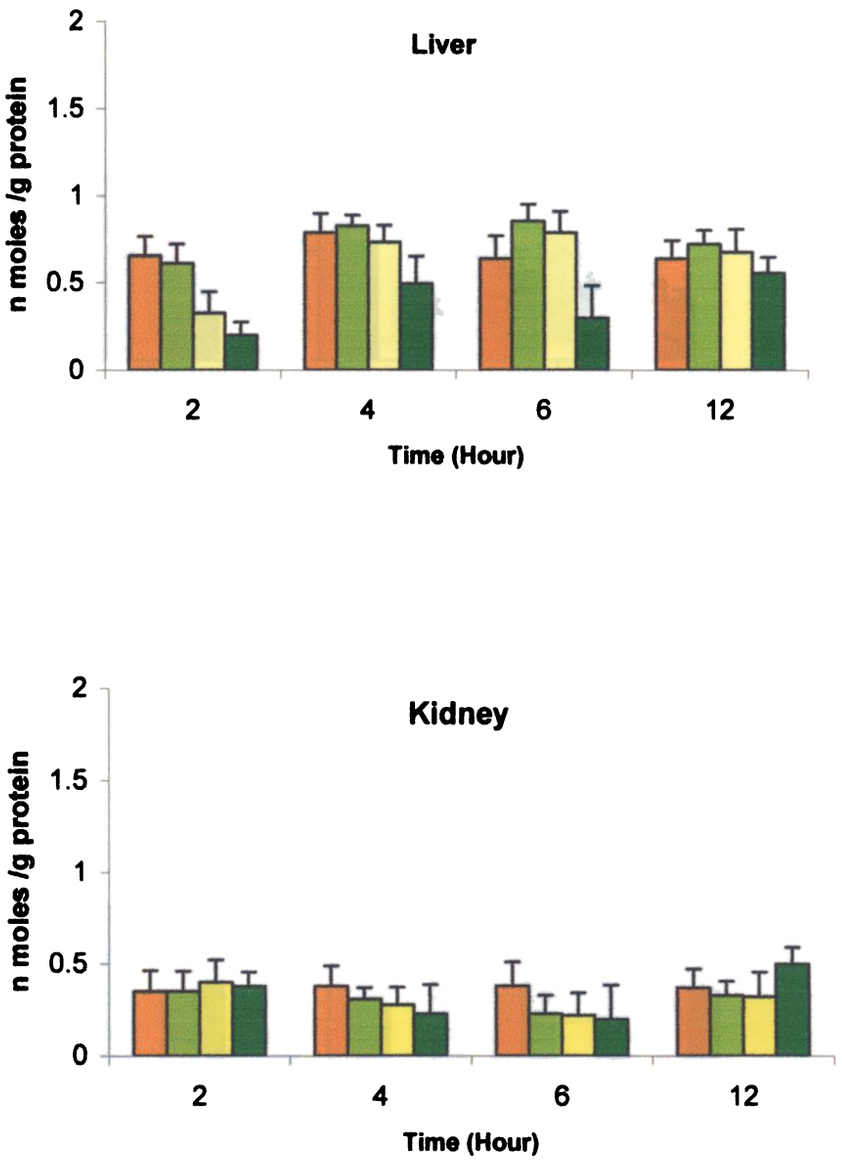
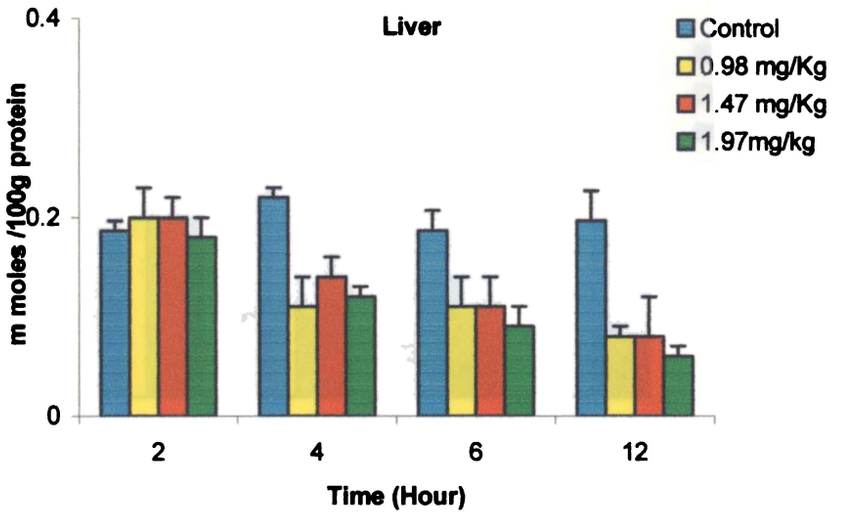


Fig. 4.8.: Malondialdehyde [MDA] levels in tissues of mice envenomated with S. argus venom. Values are expressed as n moles /g protein. Results are mean± SD of six separate experiments.



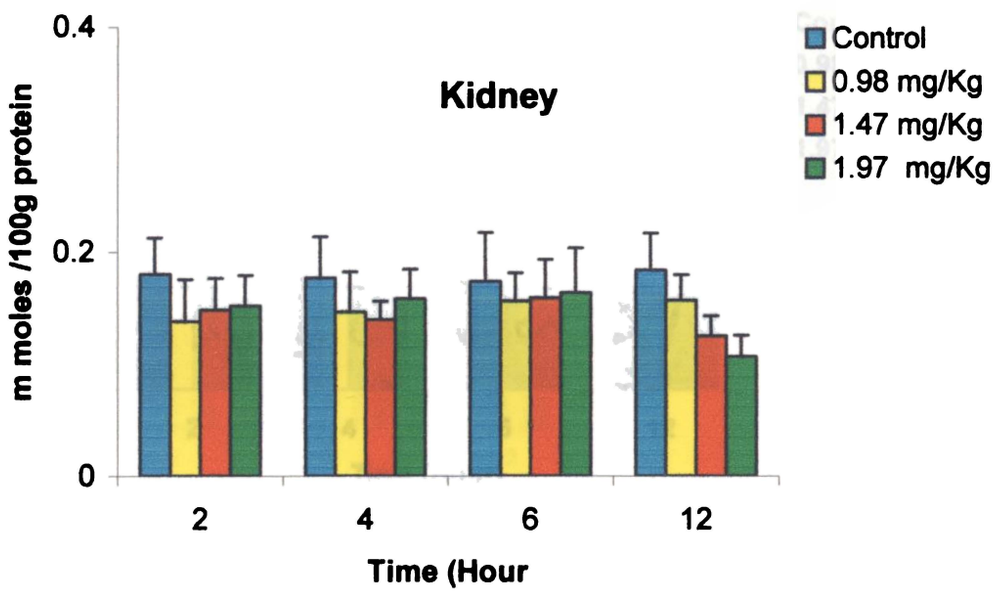


Fig. 4.9.: Conjugated dienes [CD] levels in tissues of mice envenomated with S. argus venom. Values are expressed as m moles /100g protein. Results are mean± SD of six separate experiments

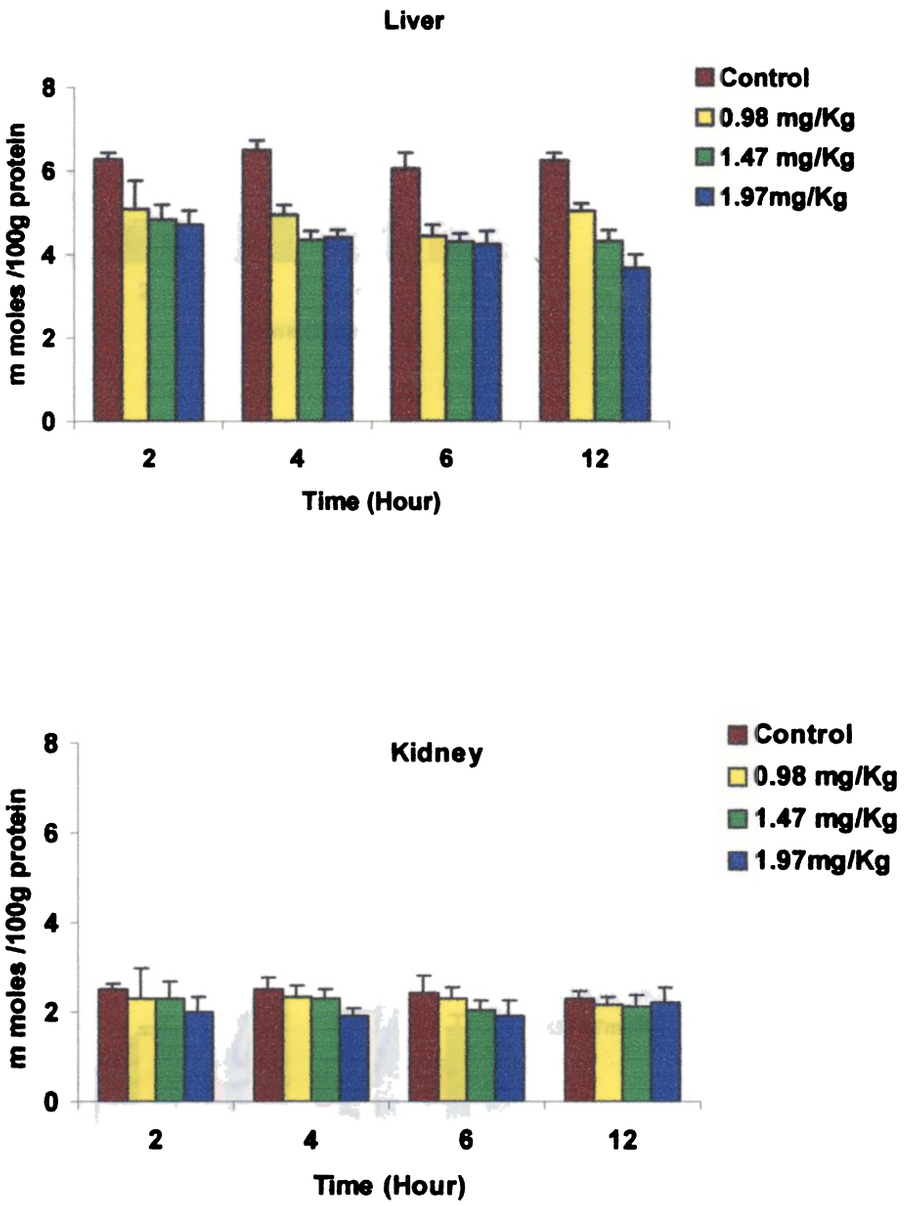


Fig. 4.10.: Hydroperoxide levels in tissues of mice envenomated with S. argus venom. Values are expressed as m moles /100g protein. Results are mean± SD of six separate experiments.

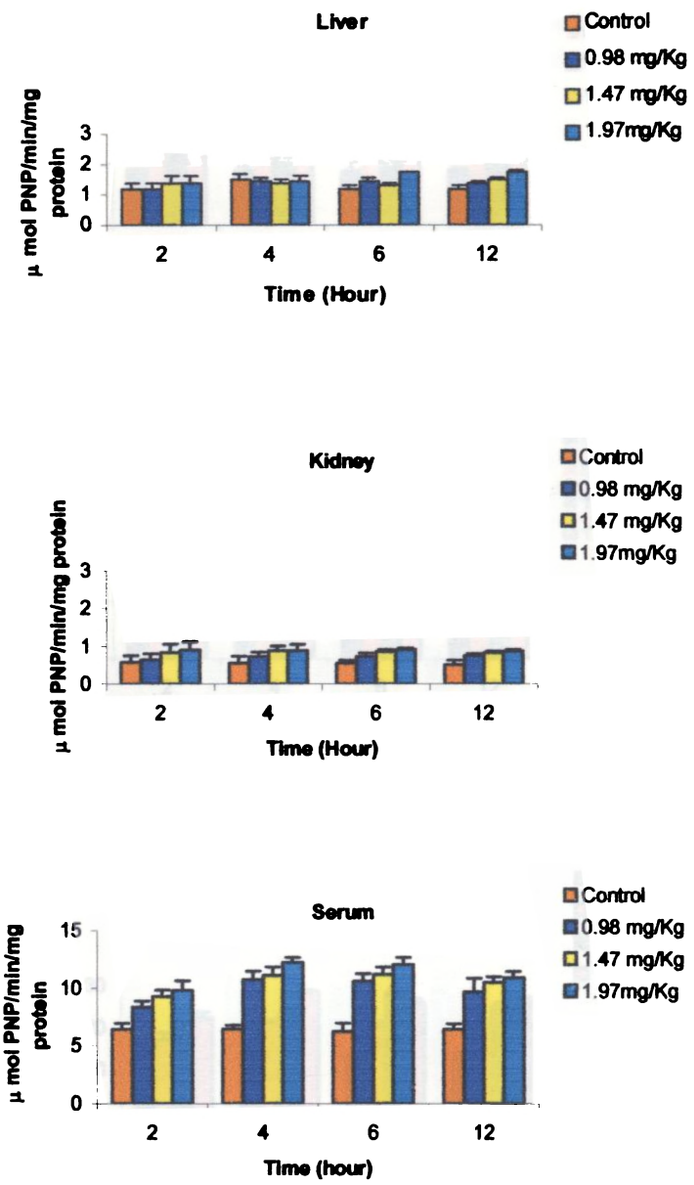


Fig. 4.11.: Acid phosphatase activity in tissues [Liver and Kidney] and serum of mice envenomated with S. argus venom. Values are expressed as µmolPNP/min/mg protein for tissue and serum. Results are mean± SD of six separate determinations

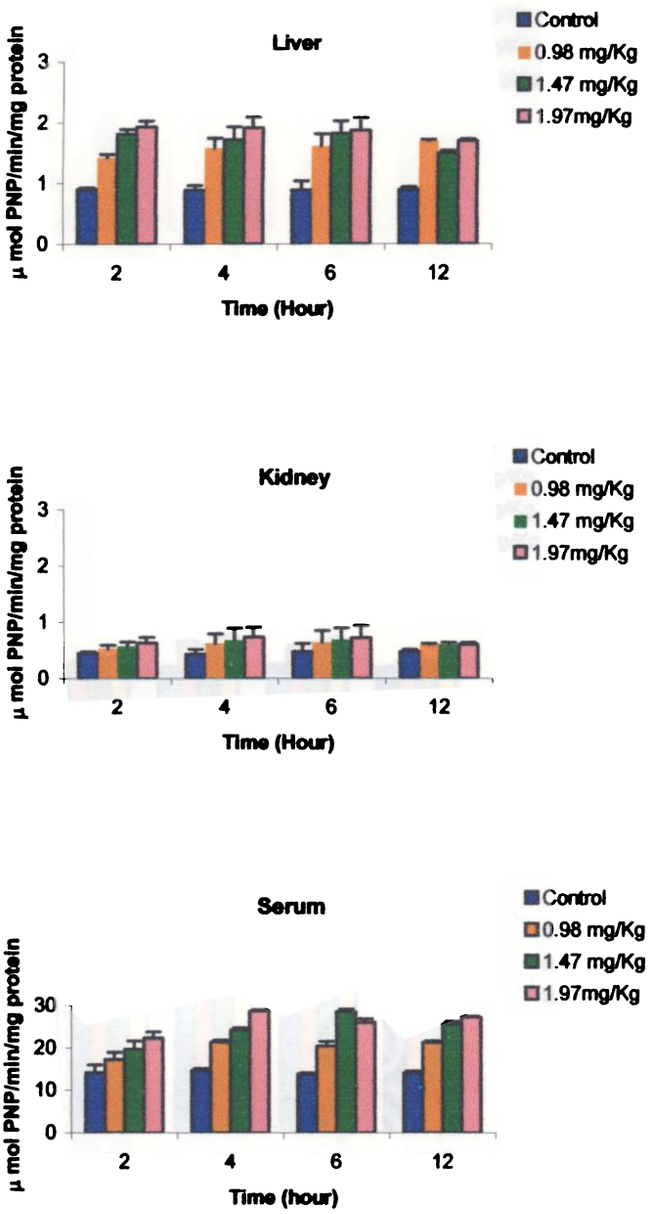


Fig.4.12.: Alkaline phosphatase activity in tissues [Liver and Kidney] and serum of mice envenomated with S. argus venom. Values are expressed as µmolPNP/min/mg protein for tissue and serum. Results are mean± SD of six separate experiments.

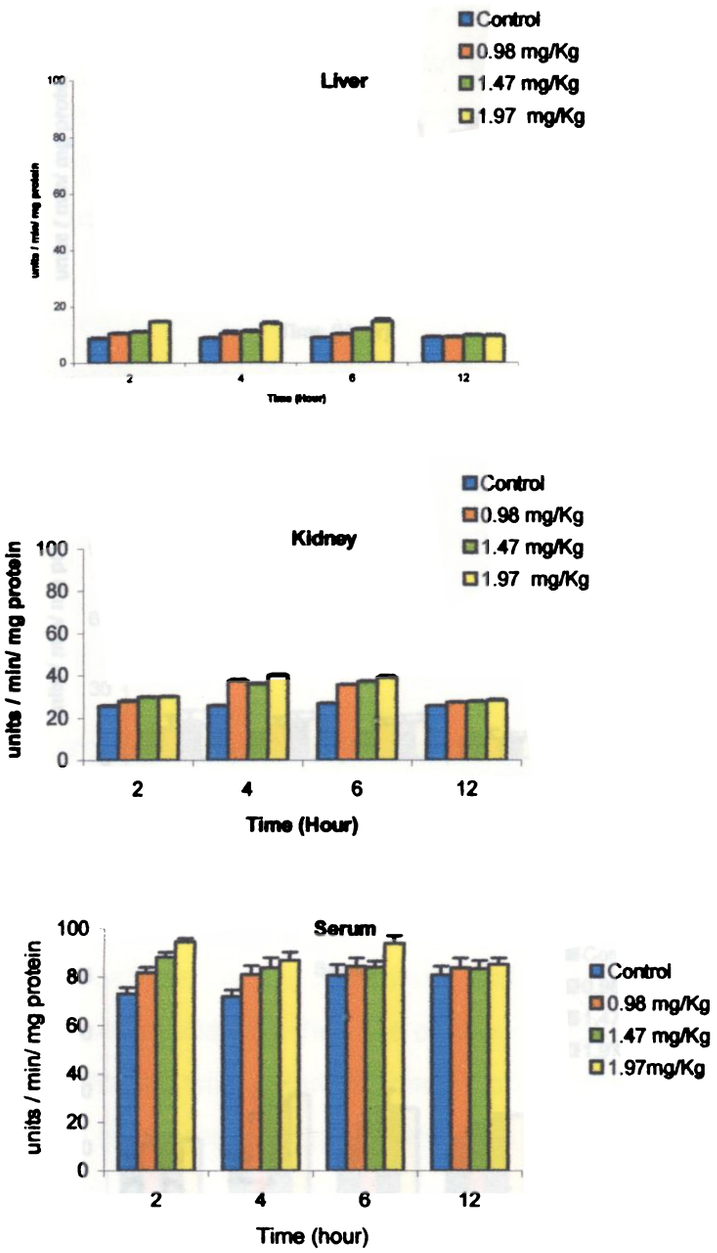


Fig. 4.13.: Alanine transaminase activity in tissues [Liver and Kidney] and serum of mice envenomated with S. argus venom. Values are expressed as units / min/ mg protein for tissue and serum. Results are mean± SD of six separate experiments.

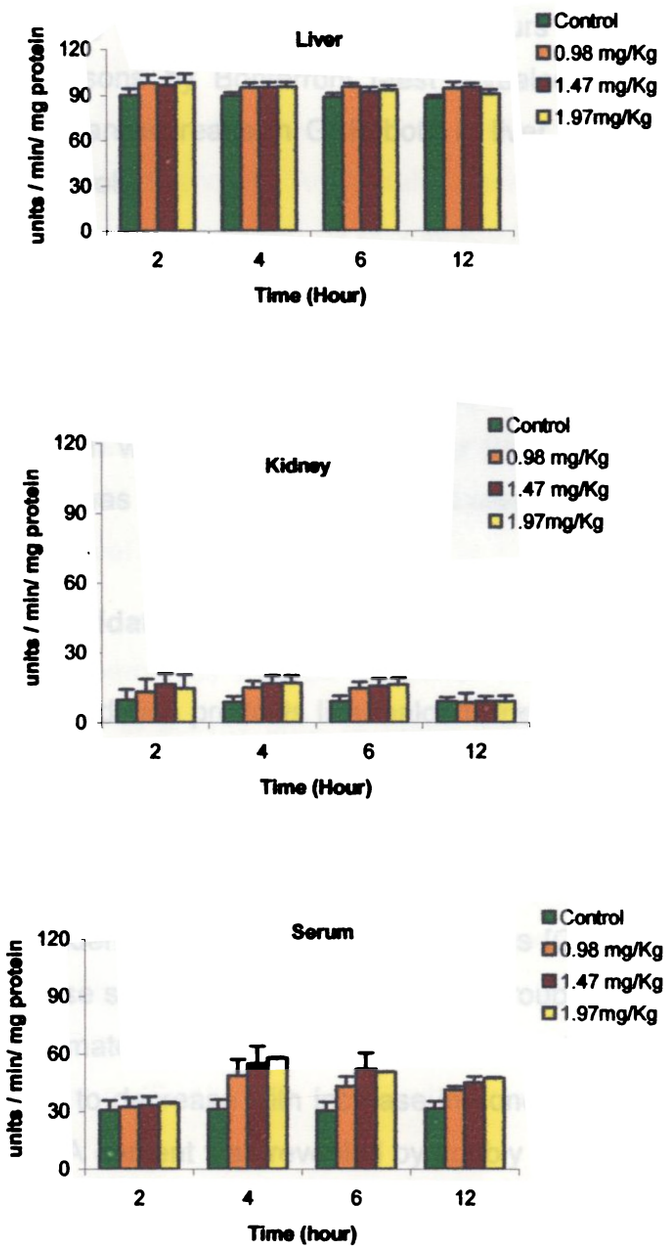


Fig.4.14.: Aspartate transaminase activity in tissues [Liver and Kidney] and serum of mice envenomated with S. argus venom. Values are expressed as units / min/ mg protein for tissue and serum. Results are mean± SD of six separate experiments.

venom with control group for liver [F=39.2] and kidney [F=22.1] over time. The increase in activity was observed to about 6 hours both in liver liver and kidney. Multiple comparisons by Bonferroni t-test revealed that all the concentrations caused a significant increase in GSH both in liver and kidney [$p < 0.001$] when compared to control.

The increase in concentration of ascorbate levels in tissues was prominent for liver. An overall significant increase in ascorbate levels was revealed by doubly multivariate repeated measures [$p < 0.05$] between different concentrations [F=19.9] of venom with control group in liver [F=39.0] over time. No change in ascorbate levels was observed for kidney and was shown not to be significant.

4.4.3. Lipid peroxidation products

Lipid peroxidation products like malondialdehyde [MDA], conjugated dienes [CD] and hydroperoxides in tissues [liver and kidney] of mice envenomated with different concentration of *S. argus* venom were assessed and the results are given in fig 4.8. to 4.10.

Malondialdehyde [MDA], conjugated dienes [CD] and hydroperoxides were found to decrease significantly than the control groups in tissues [liver and kidney] of mice envenomated with different concentration of *S. argus* venom. The MDA level was found to decrease with increase in concentration. An overall significant decrease in MDA content was revealed by doubly multivariate repeated measures [$p < 0.05$] between different concentrations of venom with control group both in liver [F=10.8] and kidney [F=47.5] over time. The decrease in MDA content was observed in liver for about 6 hours. The decresead activity was more pronounced with increase in concentration both in liver [F=5.9] and kidney [F=45.9]. Kidney also showed decrease in activity for about 6 hours. Multiple comparisons by

Bonferroni t-test revealed that all the concentrations caused a significant decrease in MDA level both in liver and kidney [$p < 0.05$] when compared to control.

A decrease in CD was observed for both liver and kidney tissue. Decrease in activity was dose dependent. An overall significant decrease in CD content both in liver and kidney was revealed by doubly multivariate repeated measures [$p < 0.05$] between different concentrations of venom with control group both in liver [F=0.8] and kidney [F=1.8] over time. The decrease was observed at about 6 hours which was observed to 12 hours in liver [F=7.9]. The decrease in CD level in kidney was observed even at 2 hours upto 12 hours [F=5.9]. Multiple comparisons by Bonferroni t-test revealed that all the concentrations caused a significant decrease in CD level both in liver and kidney [$p < 0.05$] when compared to control.

The hydroperoxides showed decreased level both in liver and kidney with time. A decrease in activity upto 12 hours was observed in liver. An over all significant increase in hydroperoxide content both in liver and kidney was revealed by doubly multivariate repeated measures [$p < 0.05$] between different concentrations of venom with control group in liver [F=19.8] and not significant kidney [F=1.5] over time. The decrease in activity was also observed upto 6 hours in kidney. Multiple comparisons by Bonferroni t-test revealed that all the concentrations caused a significant increase in hydroperoxide content both in liver [F=257.6] and kidney [F=13.3] [$p < 0.05$] when compared to control.

4.4.4. Phosphatases

The phosphatases, acid phosphatase and alkaline phosphatase were studied in tissues [liver and kidney] of mice envenomated with different concentration of *S. argus* venom and the results are given in fig 4.11. to 4.12. The increased concentration of ACP and ALP were observed for serum upto 6 hours

after envenomation. An increase in serum ACP and ALP was observed which was dose dependent. The peak enzyme activity was observed for about 6 hours. By 12 hours a decrease in enzyme activity was observed.

An increased activity was observed for about 2 hours for ALP in liver and peaked at about 4 hours. ACP content for liver showed an increased concentration for 4 hours after which a decrease was observed, which was dose dependent. The increased concentration of ACP was observed for kidney upto 6 hours after envenomation. An increase in ACP and ALP activity in kidney was observed which was dose dependent. The peak enzyme activity was observed for about 6 hours. The ACP level showed a decrease after 6 hours. By 12 hours a decrease in enzyme activity was observed for ALP.

An overall significant increase in ACP and ALP activity both in liver and kidney was revealed by doubly multivariate repeated measures [$p < 0.05$] between different concentrations of venom with control group over time. Multiple comparisons by Bonferroni t-test revealed that all the concentrations caused a significant increase in ACP and ALP both in liver and kidney [$p < 0.05$] when compared to control.

4.4.5. Transaminases

The transaminases, alanine transaminase and aspartate transaminase were studied in tissues [liver and kidney] of mice envenomated with different concentration of *S. argus* venom and the results are given in fig 4.13. to 4.14. In liver the ALT and AST activity showed an increase, which peaked by 4 hours and the increase in activity, was also observed at 6 hours. The increase in ALT activity observed for liver remained as such upto 12 hours.

An increase in AST activity was observed for serum and kidney with time and increase in concentration. Both in kidney and serum the activity was increased throughout the exposure period. An overall significant increase in AST and ALT activity both in liver and kidney was revealed by doubly multivariate repeated measures [$p < 0.01$] between different concentrations of venom with control group over time. Multiple comparisons by Bonferroni t-test revealed that all the concentrations caused a significant increase in AST and ALT both in liver and kidney [$p < 0.001$] when compared to control.

4.5. DISCUSSION

In this study mice envenomated with *S. argus* crude venom of different concentrations and at different time intervals showed pronounced influence of ROS as well as the other enzymes studied. It was found that in the mice envenomated by *S. argus* venom, the antioxidant enzymes like SOD, CAT, GPx, GR, GST and antioxidants like GSH and ascorbic acid were found to be seriously affected.

SOD plays an important role in the body defence mechanism against the deleterious effects of oxygen free radicals in biological systems. Its significance in various types of pathogenic response especially toxic chemical injury to cells makes the study of the functional status of SOD an important aspect of research in bio-medical studies. SOD and CAT are considered as the primary antioxidant enzymes, since they are involved in the direct elimination of active oxygen species. The biological importance of catalase is more evident in light of the fact that hydrogen peroxide is the main cellular precursor of the hydroxyl radical, the most reactive and toxic form of oxyradicals [Halliwell and Aruoma 1991]; due to the relative poor efficiency of antioxidants towards this ROS, the removal of

hydrogen peroxide has been indicated as an important strategy for counteracting the toxicity of hydroxyl radicals [Regoli *et al.*, 1997,Regoli *et al.*, 2000]. Halliwell and Gutteridge [1989] reported that free radical production in cells could be greatly increased by certain toxic foreign compounds. Although the free radicals have short half life and most are impermeable, some leakage of superoxide, hydroperoxides and other ROS is inevitable [Cheeseman and Slater, 1993]. The increase in CAT and SOD in the present study could be attributed to the increased activity of these enzymes to counteract the toxic effects of the venom at sublethal concentration or they might be due to the antioxidant activity of venom. Ramanaiah and Venkaiah [1992] have reported that, scorpion venom has the SOD activity whose action is inhibited by specific antivenom. Apart from this, in the past few years several peptides have been reported to exert different mechanisms of action in free radical mediated oxidative sequences by radical scavenging and metal ion chelation [Niranjan *et al.*, 2004].

There was a concomitant increase in GPx with increase in time in the present study. Several investigators have emphasized the role of glutathione peroxidase as the primary mechanism for degrading low levels of H_2O_2 in cells. Since glutathione peroxidase acts on hydroperoxides of unsaturated fatty acids, the enzyme plays an important role in protecting membrane lipids and thus the cell membranes from oxidative disintegration.

The increase in tissue SOD activity suggests an increased generation of intracellular hydrogen peroxide that could be adequately detoxified by CAT and GPx activities, which was also significantly higher in the tissues. Thus the increased activities of SOD, CAT and GPX in the present study are known to serve as protective responses to eliminate reactive free radicals formed from venom components and thereby protect the cell from further injury.

Both in liver and kidney tissues there was an increase in GST activity, which showed a dose dependent increase. Induced GST activity indicates the role of this enzyme in protection against the toxicity of the [venom] induced lipid peroxidation. The activity was found to increase with time. GR activity in liver and kidney tissues showed an increment by activity, which was, dose dependent and the peak activity was at 6 hours after envenomation. Elevated GST activity may reflect the possibility of better protection against the venom.

Antioxidants such as GSH and ascorbic acid, which directly scavenge the free radicals and the detoxifying enzymes like GR, GPx, and GST, which play an important role in glutathione metabolism, were studied. The enzyme glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase take part in the defence against reactive oxygen species of O_2 which are responsible for increased oxidative stress. Glutathione reductase is an important enzyme in maintaining glutathione as well as protein sulfyhydryls in reduced state. NADH required in the glutathione reductase reaction is regenerated from NADPH by glucose - 6- phosphate dehydrogenase through the HMP shunt [Bhat *et al.*, 1991]. These reactions constitute the glutathione cycle. Ascorbic acid showed an increased activity in liver tissues, while no significant activity was detected in kidney tissues.

Glutathione acts as intracellular and extracellular antioxidants, supplying reducing equivalent in the form of electrons that reduces peroxides. It plays a very important role in scavenging H_2O_2 and is also involved in protecting against lipid peroxidation. Varied trends of enzyme activity in different time intervals are solely dependent on the membrane stability. This stability in turn depends on the availability of the venom in the system and on the performance of the detoxicant system. Thus it is conceivable that higher activities of GSH, GR and GST in

different dosed experimental mice may represent a first line defence against tissue damage.

Lipid peroxidation and free radical generation are complex and deleterious process which are closely related to toxicity. An increase in MDA content was observed for both liver and kidney tissues indicating lipid peroxidation. The increased activity showed a decrease by 6 hours, which was, restored to the control values by 12 hours. Lipid peroxidation is a well known mechanism of cellular injury in vertebrates and is an indicator of an oxidative damage in cells and tissues. When a cell or tissue is not capable of preventing oxidative damage an increase of lipid peroxidation measured as increase in MDA is the net result. Measurement of MDA is widely used as an indicator of lipid peroxidation [Wheatly, 2000]. MDA can react readily with amino groups on proteins and other molecule to form a variety of adducts [Esterbauer *et al.*, 1991; Marnett, 1999]. MDA is usually formed in most tissues in small amount. But larger amounts are formed during the peroxidation reactions of cell membranes by free radicals that have been reported to attack all major classes of biomolecules, of which lipids are the most susceptible. Lipid peroxidation process leads to a very destructive chain reaction that can directly damage other cell component by the production of reactive aldehydes. A decrease in CD was observed for both liver and kidney tissue. Decrease in activity was dose dependent. The hydroperoxides showed decreased level both in liver and kidney with time. A decrease in activity at 12 hours was observed in liver.

An elevation in the acid phosphatase activity following *S. argus* venom administration may be due to necrotic changes in tissues. The strong toxic action of venom probably ruptures the cellular and lysosomal membrane that contains the hydrolytic enzymes resulting in their increase. The increase in ACP activity may be due to the intracellular digestion of damaged materials by lysosomes that

could be prompted by venom. The higher the concentration of the venom the greater will be the tissue damage and it is reflected as an increase in ACP activity in serum. Alkaline phosphatase activity was significantly increased indicating involvement of plasma membrane. ALP is basically a membrane bound enzyme and any perturbation in the membrane property caused by the interaction with venom could lead to alteration in ALP activity.

Increase in AST and ALT activity is reported to be an indication of stress. In the present study, elevation in activity of both the transaminases may be an immediate response to the venom stress. This elevation indicates enhanced hepatic injury. Hepatotoxicity and tissue damage are strong possibilities. The general trend of enhanced activity may be due to cellular degradation or tissue injury. Another noteworthy feature is that the ALT activity is found to be relatively higher than AST activity in the liver, kidney and serum suggesting that pyruvate contribution is slightly more than oxaloacetate formation in these tissues. In the liver and kidney tissues ALT predominates over AST where the feeding of amino acids is slightly more than oxaloacetate formation in these tissues. The level of serum ALT and serum AST were found to be increasing with time, which showed seepage of liver and kidney transaminases into the blood stream. The peak activity of ALT may be due to increased availability of pyruvate formed due to lactate dehydrogenase activity. The stimulation of activity of enzyme may in turn disturb the metabolic processes. The generally high enzyme activity particularly ALT in serum in the present study indicate large-scale gluconeogenic activity. Enzyme activity was more or less dose dependent.

Hepatic tissue damage leads to elevated serum concentration of both transaminases. Elevated levels of hepatic enzymes are indicative of increased metabolism and tissue damage following hepatotoxicity [Rao, 2006]. Changes in

activities of amino transferases brought about by an internal or external factor are associated with changes in many other metabolic functions. The altered metabolic activities in turn affect the organism's physiology. The generally high enzyme activity particularly ALT in liver, kidney as well as serum in the present study indicate cellular damage or tissue injury. The elevated activities of AST and ALT in serum are indicative of cellular leakage and loss of the functional integrity of cell membrane in liver [Drotman and Lawhorn, 1978; Rao, 2006]. Under conditions of severe tissue injury the necrosis of the tissue occurs and the enzymes get released into the blood stream. Thus the levels of transaminases in tissues and blood stream serve as an indicator of stressful situations. Mild stress only elevates tissue levels of these enzymes whereas severe injury of the tissues would result in release of these enzymes into the blood stream by the damage of the tissue [Wieser and Hinterleitnar, 1980]. This accounts for the increased activity of transaminases with time. Alterations in the activity of alanine and aspartate transaminase enzymes will be reflected on the energy yielding TCA cycle and nitrogen metabolism. They also influence the gluconeogenic process and any change in the transaminase activity and can be correlated with the protein and carbohydrate metabolism and thereby help in analyzing the metabolic shifts [Beyer *et al.,* 1996]. Studies have shown that *S. argus* venom induces significant changes in the enzyme system.

An increase in antioxidants Ascorbic acid, GSH and also an increase in antioxidant enzymes CAT, GR, GPx and SOD were observed. The lipid peroxidation products, MDA, CD and hydroperoxides showed a decrease in level. This trend shows an activation if the antioxidant enzymes against the oxidative damages in cells and tissues. The elevation in ACP activity observed may be due to necrotic changes in tissues. The increase in ALP activity can be attributed to

alterations in membrane property caused by interaction with venom. The enhanced AST and ALT shows hepatic injury and tissue damage.

Findings

- Mice envenomated with different concentration of *S. argus* crude venom at different time intervals showed pronounced influence on enzymes studied.
- The ALT activity was found to be higher than the AST activity suggesting that pyruvate contribution is slightly more than oxaloacetate formation in these tissues.
- Elevated GST activity may reflect the possibility of better protection against the venom.
- The increase in CAT activity indicates an activation of the antioxidant defensive system indicating direct or indirect ROS generation after *S. argus* envenomation.
- Increased levels of SOD indicated the increased ability of tissues to handle O_2^- radicals.
- Increased GSH and GPx activities may be attributed to adaptive response of the tissues to the oxidant challenge due to exposure to *S. argus* venom.
- The elevation in ACP activity observed may be due to necrotic changes in tissues.The increase in ALP activity can be attributed to alterations in membrane property caused by interaction with venom.
- The enhanced AST and ALT shows hepatic injury and tissue damage.
- *S. argus* venom induced changes in enzymes represent the initial disorders of impairment or they may be symptoms of metabolic alterations.

Chapter 5

NEUROMUSCULAR MODULATORY ACTIVITY OF *S.ARGUS* VENOM

5.1. INTRODUCTION

The activity of neuromuscular system is vital to normal behaviour and muscular function and it represents a prime target on which some toxicants can produce a detrimental effect. · The excitability of sensory cells, neurons and myocytes depends on ion channel and signal transducers that provide a regulated path for the movement of inorganic ions across the plasma membrane in response to various stimuli. Ion channels in plasma membrane are primary targets of marine toxins. These channels are important regulators of cell physiology and many of the pathological effects of toxins result from their modulation of the ion channels.

Nerve cells are different from other cells in that they conduct bioelectrical signals for long distances and possess intercellular connections with neurons and other tissues. For proper functioning of the neuron, it is essential that neurotransmission is maintained undisturbed. Drugs and toxins that alter the normal processes of the neurotransmission are known to be neuromodulators yielding beneficial or deleterious effects on neuronal function. Neuromodulatory effects of the venom are worth studying because of their profound usefulness in pharmacological and neurophysiological studies.

ATPase activity is associated with the active transport system, which is responsible for the extrusion of Na^+ from animal cells and the accumulation of K^+ within these cells. This enzyme is fundamental to such function as the generator of cell volume and electrolyte composition. The levels of acetylcholine, the primary excitatory neurotransmitter at the vertebrate neuromuscular junction must be carefully regulated and this role is fulfilled by the enzyme acetylcholinesterase, which degrades acetate. This is reabsorbed and used as raw material for the

continued production of acetylcholine. Venoms and toxins represent useful tools to investigate muscle degeneration and regeneration since synchronic lesion can be induced by these agents [Harris, 1992]. A variety of myotoxins directly affect the integrity of muscle plasma membrane, promoting a calcium influx, which results in cellular derangements.

Lactate dehydrogenase is a widespread cytosolic enzyme, found in greatest concentrations in heart, skeletal muscle, liver, kidney and red blood cells. Many of these tissues rely on anaerobic glycolysis for energy. LDH catalyses the conversion of pyruvate to lactate, and vice versa. In the forward reaction, NADH is oxidized to produce NAD. NAD is vital as an oxidizing agent to facilitate flux through the glycolytic pathway.

5.2.REVIEW

The neuromuscular properties of fish venom have been well studied. Most of the piscine venom shows neurotoxic symptoms such as paralysis, convulsions, and muscular weakness and at higher doses respiratory cessation, which leads to death, when injected into mice [Kelynack, 1977; Breton *et al.*, 1999; Carrijo *et al.*, 2005; Fahim *et al.*, 1996].

Neuromuscular modulations have been studied for stonefish venom [Saunders 1959a; b; Church and Hodgson, 2000b; Austin *et al.*, 1961, 1965; Breton *et al.*, 1999; Colasante *et al.*, 1996; Kreger *et al.*, 1993; Low *et al.*, 1974; Mattei *et al.*, 1999; Meunier *et al.*, 1999; 2000; Saunders *et al.*, 1962; Weiner, 1959]. Signs of neurotoxicity of lionfish venom have been observed both in mice and other fish [Saunder and Taylor, 1959; Nair *et al.*, 1985; Cohen and Olek, 1989]. Studies on muscle degeneration in mice have been carried out for toadfish

T. nattereri [Lopes-Ferreira *et al.*, 2000] while myotoxicity has been detected in *T. maculosa* [Sosa-Rosales *et al.*, 2005]. Studies on neuromuscular activity of the soldierfish *G. marmoratus* have been carried out [Church *et al.*, 2003]. The present work is a preliminary study of the neuromuscular effects of *S. argus* venom.

Snake venoms and toxins have been used as tools to develop experimental models of muscle degeneration and regeneration [Ownby 1990; Harris, 1992; Gutiérrez & Lomonte, 1997], including local myonecrosis as well as systemic myotoxicity associated with myoglobinuria [Gopalakrishnakone *et al.*, 1997]. It is however, necessary to find new experimental models of acute muscle damage that may help to elucidate the mechanisms underlying basic degenerative and regenerative processes in mammalian skeletal muscle. It is important to develop models in which muscle cell injury occurs concomitantly with thrombosis, since these two pathological findings occur along with muscle damage in a number of clinical examples. This study was carried out in order to describe the morphological and biochemical aspects of neuromuscular damage and regeneration after experimental injections of *S. argus* venom, in order to investigate the pathogenesis of local tissue damage in this envenomation.

5.3. MATERIAL & METHODS

5.3.1. *Invitro* Evaluation of the Effect of Toxin on Mouse Brain Na^+K^+ ATPase Enzyme

a] P2 Fraction Preparation

P_2 fraction [mitochondrial nerve endings] from male albino mice [20$\pm$2g] brain was prepared by the method of Green *et al.*, [1957]. Brain isolated from the mice was homogenized in ice-cold sucrose solution [0.32M] and centrifuged [Sorvall Super T 20 Refrigerated centrifuge] at 2500 rpm for 15 minutes at 4°C

temperature to remove cell debris, nuclei and plasma membrane fragments. Again the supernatant was centrifuged at 15000 rpm for 20 minutes at 4°C. Then the supernatant was discarded and the pellet dissolved in sucrose solution and again respun at 15000 rpm for 20 minutes. It was washed once again in the same method and the resultant pellet was dissolved in the sucrose solution depending upon the pellet size and kept in deep freezer as enzyme source. Protein content of enzyme source was measured by the method of Lowry *et al.*, [1951].

b] ATPase Assay

The procedure for ATPase assay for inorganic phosphate method as laid down by Lowry and Lopez [1946] was followed. For total ATPase reaction mixture, 0.8ml of Imidazole buffer [0.135mM] with 100mM NaCl, 20 mM KCl and 5mM MgCl, were taken in each test tube and 0.1ml enzyme [this quantity depends on the protein mg/hr of enzyme source] was added and stirred. The test solution of 0.1 ml of venom with different concentrations [100, 200, 400, 600, 800 and 1000 µg/ml] were added immediately using micropipettes. For Mg^{++}ATPase reaction mixture, 0.07 ml Oubain [1mM] was added as inhibitor for Na^{+} K^{+} ATPase in addition 0.8 ml of Imidazole buffer [0.135mM] to the above mixture;0. 1 ml of triple distilled water was added to the total ATPase reaction mixture and 0.03ml of triple distilled water was added to Mg^{++}ATPase mixture to bring the reaction mixture to a total volume of 1.05ml. The reaction was started by adding 50 µl of ATP substrate [4.5mM] in each tube. All the tubes were gently shaken and incubated at 37°C for 30 min. in a water bath. By adding 0.5 ml of 10%TCA, the reaction was stopped and the contents of all tubes were centrifuged and the supernatant taken. To each of this supernatant, 0.3ml of 0.1N sodium acetate solution followed by 0.4 ml of ammonium molybdate [1%] and Sulphuric acid [0.05N] solution were added .The colour developed was read at 800nm in a spectrophotometer after 15 min. Control experiments were also run simultaneously with 100 µl of triple distilled water instead of toxin.

$Na^+ K^+$ ATPase Activity = The total ATPase activity - Mg^{++}ATPase activity

Enzyme activity has been expressed in terms of μ moles of inorganic phosphate per min per mg protein.

5.3.2.*Invitro* Evaluation of the Effect of Toxin on Mouse Brain AChE Enzyme

Reagents

Dithionitrobenzoate	-	0.01M [pH8.0]
Acetylthiocholine iodide	-	0.075M
Phosphate buffer	-	0.1 M [pH 8.0]

The assay for mouse brain acetylcholine esterase [AChE] activity was carried out according to the method of Ellmann et al [1961]. Brain isolated from the albino rat [Wistar] each weighing 150 ± 20 g was homogenized in 0.25 M ice-cold sucrose solution and 20% [w/v] tissue homogenate was prepared in the sucrose solution and kept as enzyme source. Three ml phosphate buffer [8.0] was taken in each test tube to which 100 μl of 2% blood suspension was added and stirred. Then 100μl of 0.01M DTNB [5-5 dithiobis-2 nitro benzoic acid] was added and the initial color was measured spectrophotometrically at 412nm.The test solution of venom extract of different concentration such as 100, 200, 400, 600, 800 and 1000 μg were added. To start the reaction 20 μl of Acetylthiocholine Iodide [ATCI] [0.075 M] was added to each tube as substrate and then the reaction was allowed to continue for 15 minutes at room temperature. The color developed was measured at 412 nm spectrophotometrically. Enzyme activity has been expressed in terms of μM ACh hydrolyzed /mg protein/ hour

5.3.3. Estimation of Myotoxic Activity

Groups of six mice [20 ± 2 g body weight] were injected intra muscularly in the right gastrocnemius muscle with 100 μg venom in 100 μl saline. Control mice

received vehicle of same amount. Blood was collected from tail at different time intervals [2, 4, 6, 12 hours] into heparinized capillary tubes. Creatine kinase [CK, EC 2.7.3.2] activity in plasma was determined according to Forster *et al.*, [1974] and Lactate Dehydrogenase activity according to the method of Bergmeyer and Bernt [1974].

5.3.4. Quantification of wet weight and CK activity in muscle

Group of five mice were injected with 100μg venom, as described. After 4 hour or one, two, six and 10 days, mice were sacrificed and both gastrocnemius muscles were dissected out, weighed and immediately homogenized in 5 ml of PBS containing 0.1% Triton X-100 and centrifuged at 5000 X g. Supernatants were diluted 1:30 with PBS and the CK activity determined according to Forster *et al.*, [1974]. Muscle CK content was expressed as a percentage, taking the values of the left, non-envenomated gastrocnemius as 100%.

5.3.5. Quantification of lactic acid, pyruvate and LDH in muscle

For determining Lactic acid, pyruvate and LDH in muscle, groups of four mice [18-20g] were injected intramuscularly in the right gastrocnemius with 100μg venom, dissolved in 100μl PBS. Control mice received 100μl PBS alone. At various time intervals [2,4,6 and 12 hours] mice were sacrificed and their injected muscles excised.

5.3.5.1. Estimation of Lactic acid

For assay of lactic acid content of muscle, the gastrocnemius muscle dissected out was homogenized in 2ml distilled water. 4ml of 10% Trichloro acetic acid was added and after mixing, tubes were incubated for 5 min at 4°C and centrifuged at 2000 x g for 10 min and supernatant assayed for lactic acid content

according to the method of Barker and Summerson, [1941] as modified by Huckabee, [1961].

Reagents

Trichloro acetic acid [TCA]	-	10%
Copper sulphate, calcium hydroxide	-	20%
Analar sulphuric acid		
p-hydroxy diphenyl.		

To 1.0 ml of supernatant, 1.0 ml of 20% copper sulphate solution was added. The contents were mixed and the volume was made up to 10.0 ml with distilled water. To it 1 gram of powdered calcium hydroxide was added and contents were mixed vigorously till the calcium hydroxide set dissolved. The tubes were kept aside for 1 hour at room temperature giving intermittent shaking and later centrifuged at 3000g for 10 minutes. To 1.0 ml of the supernatant, 0.05 ml of 4% copper sulphate solution and 6.0 ml of analar sulphuric acid were added. The contents were mixed well by lateral shaking and then boiled in a water bath for 6 minutes. After cooling 0.1 ml of p-hydroxy diphenyl was added to the solution and kept at room temperature for 30 minutes. Again, the contents were boiled in a water bath for 1 minute. After cooling, the absorbance was read against a reagent blank at 560 nm in a spectrophotometer. The blank received the same treatment as that of the samples, except that distilled water replaced the homogenate. The values were expressed as mg lactic acid / g wet wt of tissue.

5.3.5.2.Estimation of Pyruvate [Frioedemann and Haugen, 1943]

Reagents

Trichloroacetic acid [TCA]	-	10%
2,4 – Di nitro phenyl hydrazine [DNPH]	-	[0.1%]
NaOH	-	2.5N

Pyruvate Stock standard: Dissolved 125mg of sodium pyruvate in 0.1N H_2SO_4 and diluted to 100ml with 0.1N H_2SO_4. 1ml of this solution contains 1.25mg of sodium pyruvate.

Working Standard: Diluted 5ml of Stock to 100ml with 0.1N H_2SO_4 such that 1ml of it contains 0.0625mg of sodium pyruvate.

Muscle homogenate was prepared separately in 10% TCA and centrifuged at 1000g for 15min. To 2ml of the supernatant, 0.5ml of 0.1% 2,4 di nitro phenyl hydrazine was added and the tubes were kept for 5 min at room temperature and 3ml of 2.5N NaOH solution was added. After 10min the colour was read in a spectrophotometer at 540 nm against a reagent blank. The blank consisted of 2ml of 10% TCA, 0.5ml of 0.1% 2,4 DNPH and 3ml of 2.5N NaOH solution. The values were expressed as μ moles of pyruvate.

5.3.5.3. Estimation of Lactate dehydrogenase

Reagents		
Phosphate buffer [pH 7.5]	-	50mM
Pyruvate	-	0.6mM
NADH	-	0.18mM

Lactate dehydrogenase was estimated by the method of Bergmeyer and Bernt, [1974]. The tissue was homogenized in 50 mM phosphate buffer [pH 7.5]. The homogenate was centrifuged at 20,000 g for 30 min in a refrigerated centrifuge at 0°C. The supernatant obtained was used as the enzyme source. The reaction mixture consisted of 50mM phosphate buffer [pH 7.5], 0.06 mM pyruvate, 0.18 mM NADH and the enzyme preparation. The reaction was initiated by the addition of enzyme and the assay was carried out at 30°C. The activity was determined from the rate of oxidation of NADH. The change in absorbance at 340 nm was measured in a spectrophotometer. A standard graph of NADH was

prepared and the activity of the enzyme was expressed as mg of NADH oxidized per hour per gram protein in the sample.

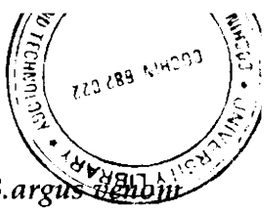
5.3.6. Statistical Analysis

The SPSS® statistical software for windows, version 13.0 [SPSS Inc., Chicago, USA] was used for all data analyses. All responses were expressed as Mean ± Standard Deviation. To examine the significant differences in the response of the venom treated mice over the periods of observations were analyzed using GLM [General linear model] repeated measures procedure. One-way ANOVA was used to analyse the dose response effect over concentration after which an appropriate post hoc test was performed [Tukeys test].

5.4. RESULTS

5.4.1. *Invitro* Evaluation of the Effect of Toxin on Mouse Brain Na^+K^+ ATPase

With increase in concentration of *S. argus* venom an increase in Na^+K^+ ATPase activity was observed [Fig.5.1.]. At about 15 µg/ml concentration of *S. argus* venom the value was 9.25 ± 0.39 of µ moles /min / mg protein. The value increased to about 13.53 ± 0.92 at 30µg/ml concentration of *S. argus* venom and 15.33 ± 1.0 at 45µg/ml concentration of *S. argus* venom. At about 60 µg/ml concentration of *S. argus* venom the value was high as 17.18 + 0.62 µ moles /min / mg protein. One-way ANOVA comparing ATPase activity with concentration revealed an overall significant change [$P<0.05$] [Table 5.1.1.] when compared to the control [$F=229.67$]. Multiple comparison with Tukeys test reflected significant differences [$P<0.05$] ie., there was an increase in enzyme activity with increase in concentration. [Fig. 5.3]



5.4.2. *Invitro* Evaluation of the Effect of Toxin on Mouse Brain AChE Enzyme

An elevated level of AChE was observed in brain of mice injected with different doses of crude *S. argus* venom. The increase in activity observed was dose dependent. [Fig. 5.2.] One-way ANOVA comparing ATPase activity with concentration revealed an overall significant change [$P<0.05$] when compared to the control [$F=74.11$] [Table 5.2.1]. Multiple comparison with Tukeys test reflected significant differences [$P<0.05$] ie., there was an increase in enzyme activity with increase in concentration.

5.4.3. Muscle Wet Weight

5.4.3.1. Macroscopic Observations: Mice injected with saline did not show locomotion problems or any type of distress. In contrast tot his the mice receiving *S. argus* venom had difficulties in mobilizing their right hind leg within the first few minutes after injection and evident swelling observed which persisted for more than 24 hours.

Nearly 4 hours after injection of venom, muscle wet weight showed an increase of 121±9.2% with respect to contralateral gastrocnemius, which was persistent for 12 hours [Fig 5.3.]. At 48 hour the wet weight decreased to about 92.8±6.9% of contralateral muscle. The muscle mass continued decreasing in envenomated muscle and remained significantly lower than in control muscle by day 6 [41.6±3.2] and day 10 [21.96±5]. [Table 5.3.]. To study the relation of muscle-wet weight with time, GLM repeated measure was done. The values showed an overall significance [$p<0.05$; $F=415.48$] in the experimental groups.

5.4.4. Muscle and Plasma Creatine Kinase

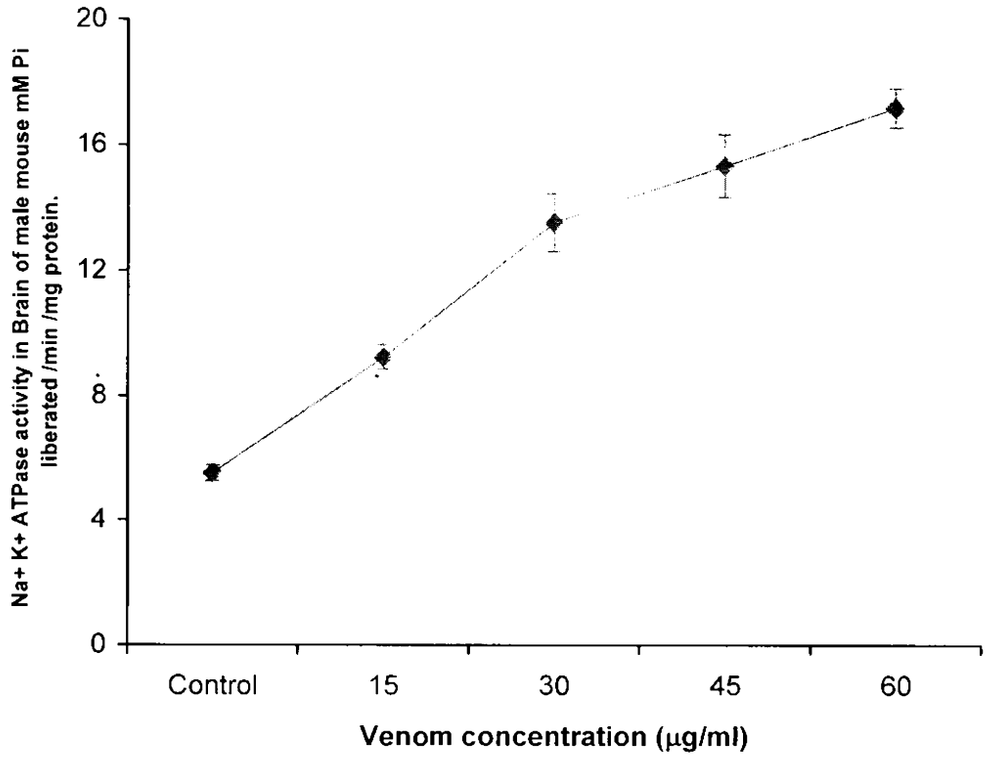
There was a muscle CK content decrease observed in creatine kinase activity in gastrocnemius muscle by 2 hours to 85.3 ± 3.3% of activity of

contralateral non-injected gastrocnemius muscle [Fig 5.4.]. By 4 hours the activity reached 73.8 ± 4.2%. The creatine kinase activity decreased drastically by 24 hours to 38.1 ± 2.6 %. The activity decreased to 30.1 ± 4.8 % by day 6. By day 10 the creatine kinase activity of injected gastrocnemius muscle showed an increment of 55.3 ± 5.12 %.

A rapid and prominent increment in plasma creatine kinase activity was observed after injection of *S. argus* venom. The activity peaked by 2 hours to 965.08 ± 61.67U/Lwhich increased to 983.28 ± 52.32 U/L by 4 hours. A decline to 1048.58 ± 51.87 U/L was observed by 6 hours. By 24 hours creatine kinase level in plasma reached to 414.18 + 43.13 [Fig 5.5.]. To examine the significant differences in the plasma and muscle creatine kinase activity of the venom treated mice over the periods of observations were analyzed using GLM [General linear model] repeated measures procedure. Analysis showed significant differences over time both for plasma [$p<0.001$; $F=891.921$] and muscle [$p<0.05$; $F=314.346$] creatine kinase activity.

5.4.5. Muscle Lactic Acid

Lactic acid concentration in muscle of mice injected with *S. argus* venom showed a decreased activity by 2 hours to a concentration of 25.70 ± 3.33 μmol/g tissue. Lactic acid levels in muscles after 4 hr and 6 hr are 23.78± 3.87μmol/g and 23.84 ± 3.93μmol/g respectively. The lactic acid content in muscle of mice injected with PBS was 33.48 ±2.6μmol/g tissue [Fig 5.6]. To examine the significant differences in the muscle lactate content of the venom treated mice over the



Venom concentration µg/ml	Na+ K+ ATPase activity µM Pi liberated /min /mg protein.
Control	5.51 ± 0.25
15	9.25 ± 0.39
30	13.53 ± 0.92
45	15.33 ± 1.00
60	17.18 ± 0.62

Fig. 5.1 In vitro effects of S. argus venom on mouse brain Na+K+ ATPase. Results are expressed as mean ± SD [n = 6].

Source Of Variation	Sum of Squares	df	Mean Square	F	P value
Between Concentration	448.951016	4	112.237754	229.679	< 0.001
Error	9.77344	25	0.488672		
Total	4156.8238	30			

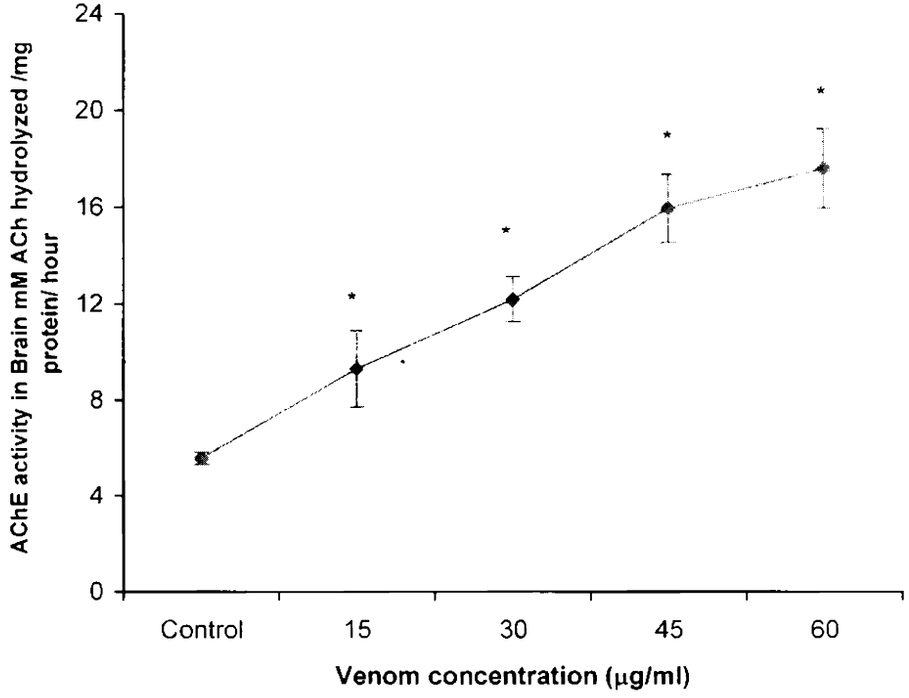
Table 5.1.1: One way ANOVA for Na+ K+ ATPase.

Source Of Variation	Sum of Squares	df	Mean Square	F	P value
Between Concentration	0.648	4	0.162	74.11	< 0.001
Error	5.47E-02	25	2.19E-03		
Total	17.294	30			

Table 5.2.1: One way ANOVA for Acetylcholinesterase.

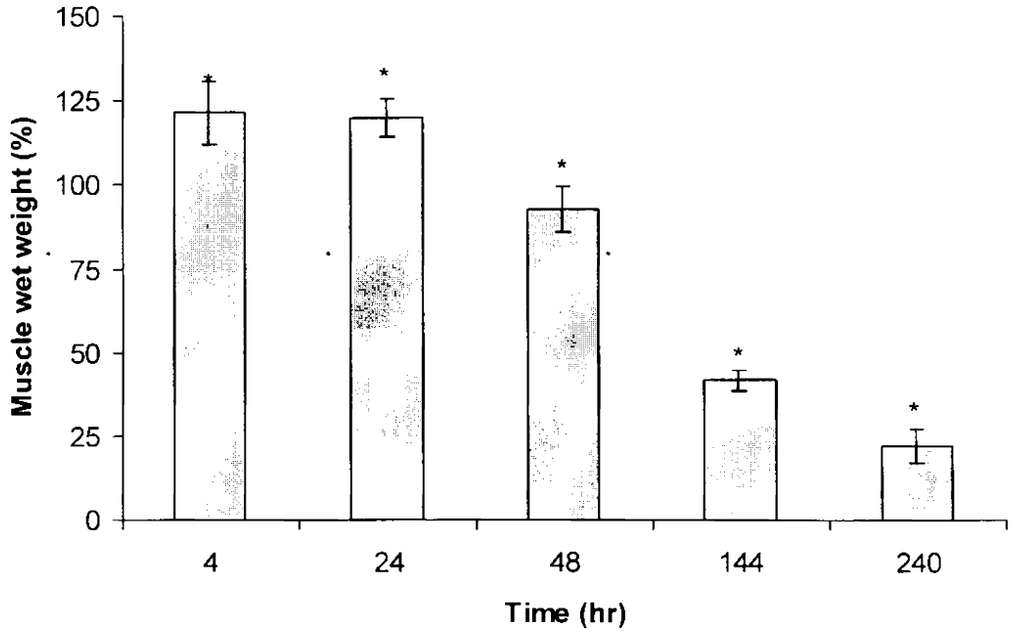
Groups	Parameters	
	Na+ K+ATPase	Acetylcholinestrase
Control vs 15	p < 0.001	NS
Control vs 30	p < 0.001	p < 0.001
Control vs 45	p < 0.001	p < 0.001
Control vs 60	p < 0.001	p < 0.001

Table 5.3: Results for Tukeys test.



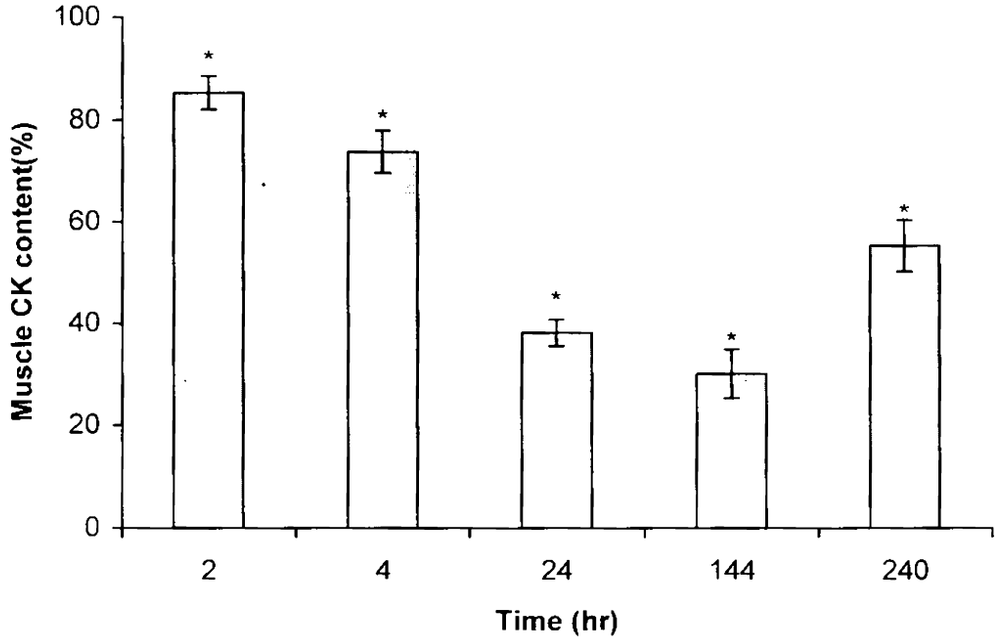
Venom concentration µg/ml	AChE activity in Brain µM ACh hydrolyzed /mg protein/ hour
Control	0.55 ± 0.05
15	0.63 ± 0.07
30	0.73 ± 0.03
45	0.83 ± 0.03
60	0.97 ± 0.02

Fig. 5.2 In vitro effects of S. argus venom on mouse brain acetylcholinesterase [AChE] Results are expressed as mean ± SD [n = 6].



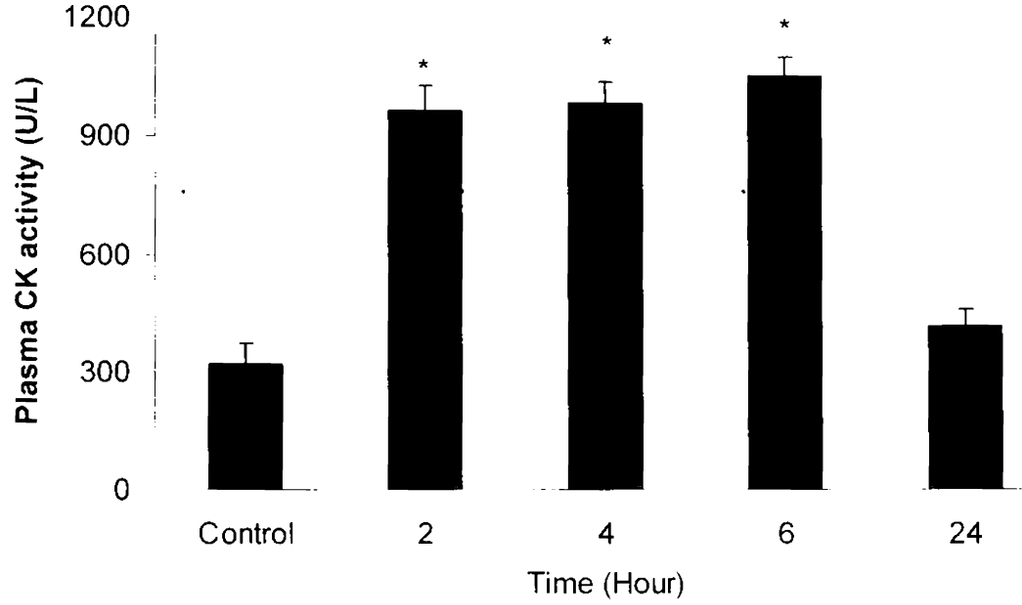
Time (Hrs)	Muscle wet weight (%)
4	121.43 ± 9.16
24	120.07 ± 5.70
48	92.80 ± 6.85
144	41.63 ± 3.19
240	21.97 ± 5.07

Fig 5.3: Changes in wet weight of mouse gastrocnemius muscle with respect to contralateral non-injected muscle after intramuscular injection of S. argus venom of 100μg venom protein.



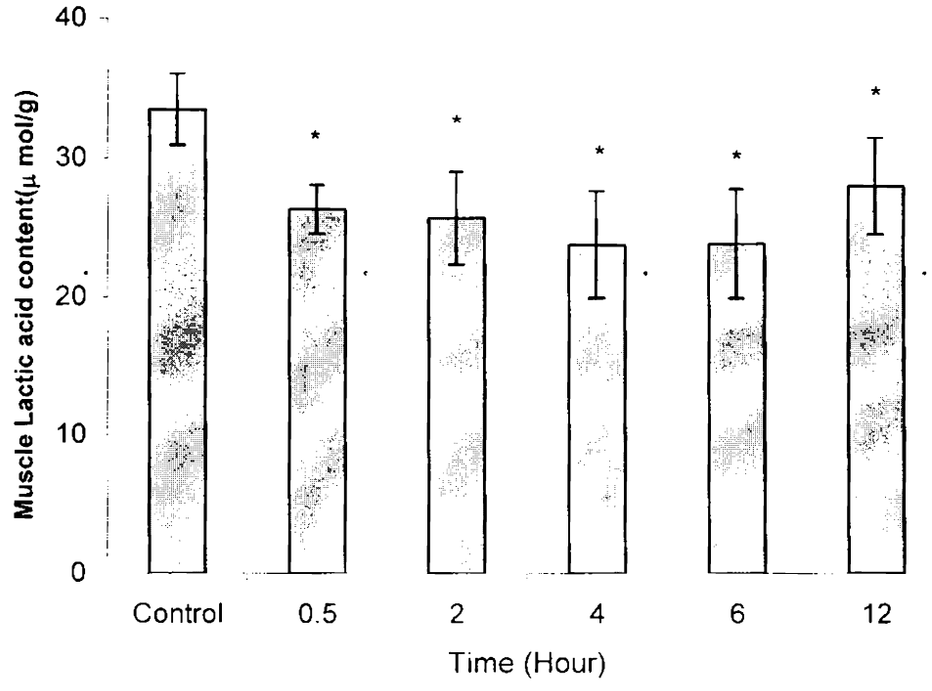
Time (Hour)	Muscle Creatine kinase content (%)
2	85.33 ± 3.27
4	73.83 ± 4.17
24	38.17 ± 2.56
144	30.17 ± 4.79
240	55.32 ± 5.12

Fig 5.4: CK content of mouse gastrocnemius muscle at different time intervals after intramucular injection of 100μg S. argus venom dissolved in 100μL PBS. CK activity in envenomated muscle is expressed as a percentage, taking as 100% the CK activity of contralateral, noninjected gastrocnemius



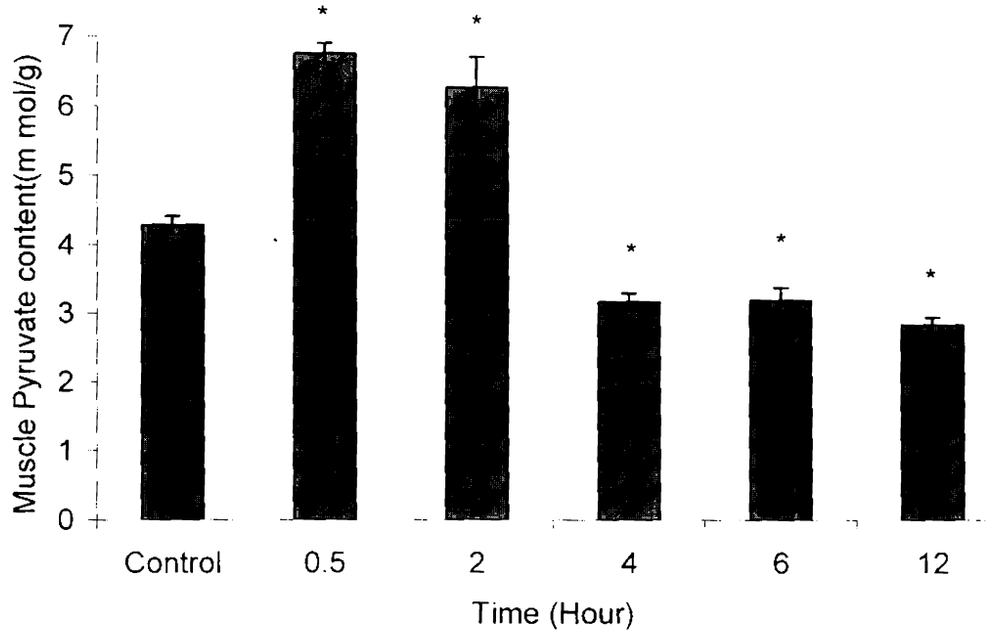
Time(Hour)	Plasma Creatine kinase content (U l/mg)
Control	32.10 ± 4.97
2	125.08 ± 7.11
4	143.28 ± 5.91
6	106.58 ± 5.20
24	28.98 ± 4.05

Figure 5.5: Changes in plasma CK activity of mice injected intramuscularly in the gastrocnemius with 100μg S. argus venom dissolved in 100 μL PBS at different time intervals.



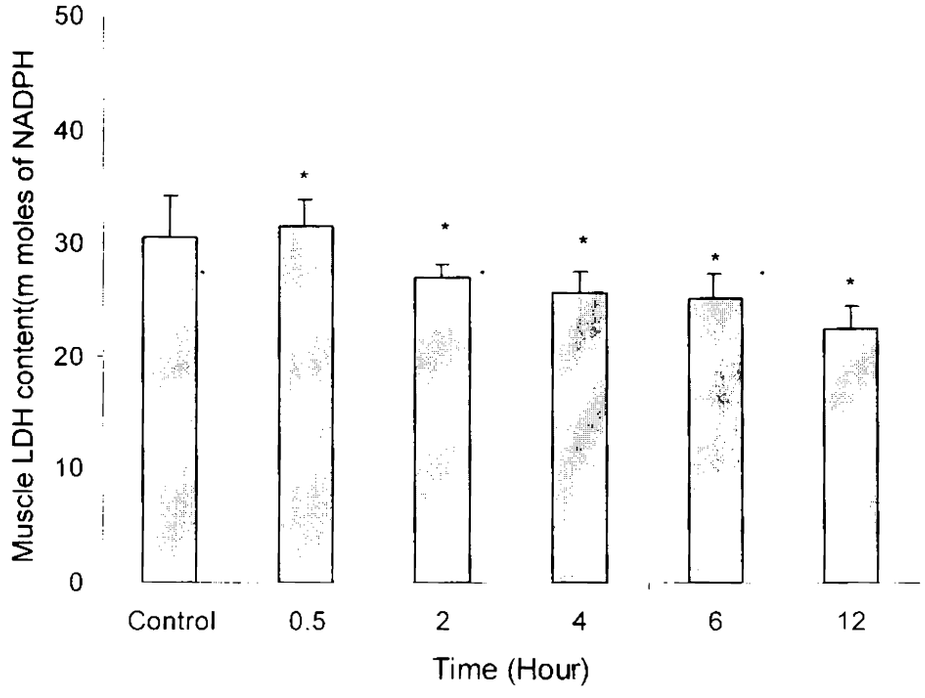
Time (Hour)	Lactate content in muscle (μ mol/g)
Control	33.48 ± 2.55
0.5	26.34 ± 1.75
2	25.70 ± 3.33
4	23.78 ± 3.87
6	23.84 ± 3.93
12	28.00 ± 3.47

Fig 5.6 : Lactic acid content of mouse gastrocnemius muscle at different time intervals after intramucular injection of 100μg S. argus venom dissolved in 100μL PBS Lactic acid activity in envenomated muscle is expressed as a percentage, taking as 100% the Lactic acid activity of contralateral, noninjected gastrocnemius. Results are presented as mean ±SD [n = 6].



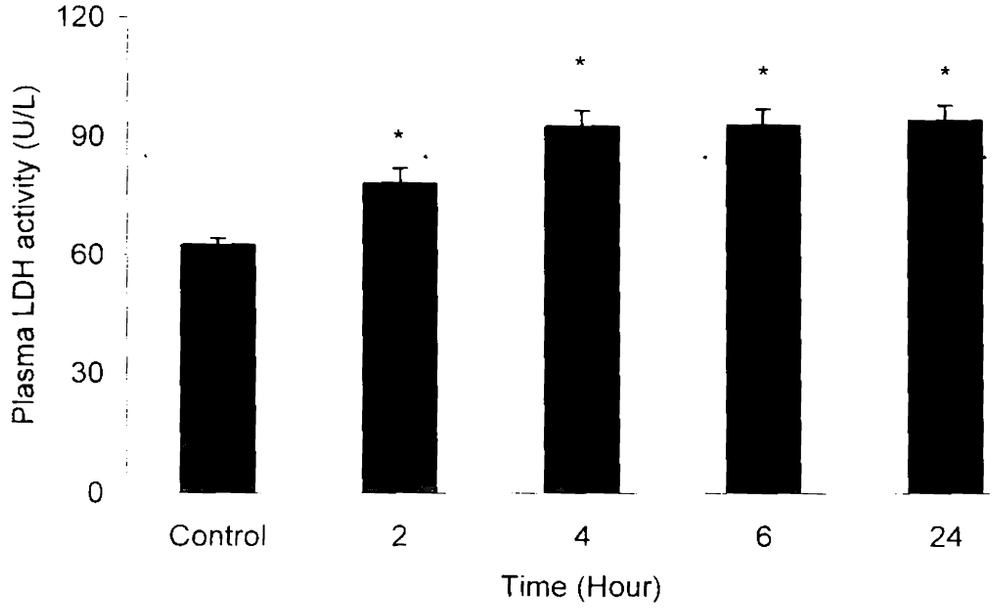
Time (Hour)	Pyruvate Content in muscle (μ mol/g wet weight tissue)
Control	4.28 ± 0.14
0.5	6.76 ± 0.14
2	6.27 ± 0.44
4	3.17 ± 0.13
6	3.19 ± 0.18
12	2.83 ± 0.11

Fig 5.7 : Pyruvate content of mouse gastrocnemius muscle at different time intervals after intramucular injection of 100μg S. argus venom dissolved in 100μL PBS.



Time (Hours)	Lactate dehydrogenase Content in muscle (μ moles
Control	30.47 ± 3.79
0.5	31.53 ± 2.33
2	27.05 ± 1.09
4	25.69 ± 1.87
6	25.18 ± 2.15
12	22.55 ± 1.99

Fig 5.8 : Lactate dehydrogenase activity of mouse gastrocnemius muscle at different time intervals after intramucular injection of 100μg S. argus venom dissolved in 100μL PBS. Results are presented as mean ±SD [n = 6].



Time (Hour)	Plasma Lactate dehydrogenase Activity (U l/L)
Control	62.58 ± 1.57
2	78.30 ± 3.82
4	92.65 ± 3.98
6	93.03 ± 4.05
24	94.27 ± 3.75

Fig 5.9 : Plasma Lactate dehydrogenase activity at different time intervals after intramucular injection of 100μg S. argus venom dissolved in 100μL PBS. . Results are presented as mean ±SD [n = 6].

periods of observations were analyzed using GLM [General linear model] repeated measures procedure. Analysis showed no significant differences over time for lactate content.

5.4.6. Muscle Pyruvate

Pyruvate content in muscle of mice injected with *S. argus* venom showed no significant activity with increase in time. The activity increased by 2 hours to a concentration of 6.27 $\pm$ 0.14 μ mol/g wet weight tissue [Fig. 5.7]. Later a decrease in activity was observed after 4 and 6 hours, the values being 3.17 $\pm$ 0.13 μ mol/g wet weight tissue and 3.19 $\pm$ 0.18 μ mol/g wet weight tissue respectively. By 12 hours the pyruvate level was 2.83 $\pm$ 0.11 μ mol/g wet weight. The significant differences in the muscle pyruvate content of the venom treated mice over the periods of observations were analyzed using GLM [General linear model] repeated measures procedure. Analysis showed significant differences over time [$p<0.05$; $F=662.053$]

5.4.7. Muscle and Plasma Lactate Dehydrogenase [LDH]

A decrease in LDH activity was observed in plasma of mice injected with *S. argus* venom. The activity decreased by 2 hours to 27.05 $\pm$ 1.1 μ moles of NADPH, and was still lower by 4 hours [25.69 $\pm$ 1.87 μ moles of NADPH]. The activity again decreased by 6 hours [25.18 $\pm$ 2.15 μ moles of NADPH] and reached 22.55 $\pm$ 2.0 μ moles of NADPH by 12 hours [Fig. 5.8].

As in the case of CK a rapid and prominent increment in plasma LDH activity was observed after injection of *S. argus* venom. The activity peaked by 2

hours to 78.3± 3.81U/L and to 92.65 ± 3.98 U/L by 4 hours. A decline to 93.03 ± 4.05 U/L was observed by 6 hours. By 24 hours LDH level in plasma reached up to 94.2 + 3.75 [Fig 5.9]. To examine the significant differences in the plasma and muscle LDH activity of the venom treated mice over the periods of observations were analyzed using GLM [General linear model] repeated measures procedure. Analysis showed significant differences over time both for plasma [$p<0.05$; $F=263.036$] and muscle [$p<0.05$; $F=873.102$] LDH activity.

5.5. DISCUSSION

Various lines of evidence strongly suggest the neuromyotoxicity of *S. argus* venom. The increased plasma creatine kinase and the decreased muscle creatine kinase activity in mice points towards the integrity of skeletal muscle plasma membrane. An increase in muscle wet weight was observed after injection of *S. argus* venom. An inflammation in affected muscle was detected. The increments in muscle lactic acid content were prominent. Studies on skeletal muscle necrosis induced by *T.nattereri* venom showed an increased plasma creatine kinase and decreased muscle creatine kinase activity [Lopes-Ferriera *et al.*, 2001] well in conformity with present observations.

The membrane-bound enzyme ATPase plays an important role in the maintenance of membrane potentials, and it has been estimated that it accounts for up to 50% of oxidative metabolism in the brain and is deeply involved in cellular function [Ratnakumari *et al.* 1995]. Membrane Na^+ K^+ ATPase plays an important role in active transport of Na+ and K+ ions across the plasma membrane. The enzyme is present in high concentration in brain and muscle. Na^+ K^+ ATPase is ubiquitous in nature, and in the mammalian central nervous system it is found

predominantly in glial and nerve terminals [Chen *et al.* 2005]. The sodium gradient is important for the uptake of neurotransmitters into nerve cells and glia, which suggests that changes in Na^+ K^+ ATPase activity result in the modulation of neurotransmission [Fighera *et al.*, 2006]. This enzyme activity has been used as a potential indicator for membrane toxicity [Engelke *et al.*, 1992]. The observed increase in the activity of Na^+ K^+ ATPase by *S. argus venom* suggests the presence of antinociceptive substance in the venom. The increase observed was dose dependent .A similar increase in the enzyme activity was observed by morphine and *P.volitans* venom in the mouse brain [Masocha *et al.*, 2002; Balasubashini *et al.*, 2006]

Acetyl cholinesterase [AChE], the enzyme involved in the hydrolysis of the neurotransmitter acetlycholine, contributes to the integrity and permeability of the synaptic membrane that occurs during neurotransmission and conduction [Grafius *et al.* 1971]. This enzyme has been implicated in cholinergic and noncholinergic actions, which may play a role in neurodegenerative diseases [Cummings 2000; Law *et al.*, 2001]. The effect of *S. argus* venom on the AChE activity in vitro in brain revealed that fish venom increases the activity of enzyme in a dose-dependent manner. This may be either due to presence of acetlycholine [Garnier *et al.*, 1996], the substrate for the AChE in the fish venom, or by the massive release of acetylcholine from the nerve terminal that potentiates the activity of the enzyme in mouse brain [Church and Hodgson 2002].

Studies have shown the endogenous release of neurotransmitter acetylcholine from the neuromuscular junctions. Studies using SNTX have shown that it acts directly on muscle cells [direct myotoxic effect] to inhibit contracture rather than through any inhibition of neurotransmission and the contracture relying on Ca^{2+} release and/or activation [Low *et al.*, 1990; Low *et al.*, 1994]. The

prejunctional effects of SNTX and the direct depolarizing effect of SNTX were distinct from each other [Cheah *et al.*, 1992]. Studies on crude venom of *G. marmoratus, P. volitans* and *S.trachynis* have shown to act by direct depolarization effect on skeletal muscle [Church *et al.*, 2003]. Studies on lionfish toxin [Cohen and Olek, 1989] have concluded that the cellular action of toxin is by inducing massive release and subsequent depletion of acetylcholine from the nerve terminal. The soldierfish *G.marmoratus* stimulate the release of ACh to act at muscarinic receptors on guinea – pig gastrointestinal smooth muscle [Hopkins *et al*, 1997]. Trachynilysin [TLY] isolated from *S.trachynis* venom enhances the release of acetylcholine from atrial cholinergic nerve terminals [Colasante *et al.*, 1996; Sauviat *et al*, 2000; Ouanounou *et al.*, 2000]. Experiments with rat synaptosomes revealed that stonefish venom affects neurotransmission and has demonstrated the stimulation of release of the neurotransmitter acetylcholine [Khoo *et al.*, 1992].

Creatine kinase catalysis the conversion of creatine to phosphocreatine, a high-energy molecule. The phosphocreatine is burned as a quick source of energy by cells. Studies on plasma creatine kinase showed an increment. Elevation of creatine kinase is an indication of damage to muscles. The creatine kinase level in muscle was less when compared to control. The *in vivo* assay of plasma creatine kinase showing increased activity is further evidence for the myolytic activity of *S. argus* venom. The decrease in muscle creatine kinase activity may be due to degeneration of muscle fibres. The increase in muscle CK by day 6 and 10 points towards the fact that there was regenerating muscle fibres have started getting regenerated after their degeneration of muscle fibres by envenomation. The myotoxic activity of venoms can be monitored by the increase of plasma creatine kinase [CK] activity and morphological analysis. The increase

in plasma CK levels results from sarcolemmal damage due to myotoxic components of the venom [Ownby *et al.*, 1982; Mebs *et al.*, 1983; Ownby and Coldberg 1986; Melo and Suarez- Kurtz 1987; 1988; Melo *et al.*, 1984; 1993;Lomonte *et al.*, 1993].

Increases in lactate concentration occur under conditions where the rate of energy demand by tissues cannot be met by aerobic respiration. Under these conditions pyruvate dehydrogenase cannot convert pyruvate to Acetyl CoA and pyruvate begins to build up. This would normally inhibit glycolysis and reduce ATP production.

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{Lactate} + NAD^+$$

In the present study the increment of lactate content within the envenomated gastrocnemius muscle was of significance suggesting that anaerobic respiration had taken place.

A decrease in lactate dehydrogenase and lactic acid content with increase in concentration were observed in the present study. The decrease in LDH in the present studies suggests a lack in anaerobic respiration. In conasance with decrease in LDH activity decrease in lactic acid was observed. The decrease in LDH activity with a consequent decrease in the levels of lactate suggests aerobic oxidation, Krebs cycle [Skidmore, 1970]. Increases in lactate concentration occur under conditions where the rate of energy demand by tissues cannot be met by aerobic respiration. Under these conditions pyruvate dehydrogenase cannot convert pyruvate to Acetyl CoA and pyruvate begins to build up. This would normally inhibit glycolysis and reduce ATP production. The reason for the observed decrease in pyruvate level indicates its forming a precursor for many

metabolic products. Increase of LDH activity in plasma may occur in any injury that causes loss of cell cytoplasm.

Cellular enzymes in the extracelluar space, although of no further metabolic function in this space, are still of benefit because they serve as indicators suggestive of disturbances of the cellular integrity induced by pathological conditions. Lactate dehydrogenase [LDH] is a cytoplasmic enzyme present in essentially all major organ systems. The extracellular appearance of LDH is used to detect cell damage or cell death. Due to its extraordinarily widespread distribution in the body, serum LDH is abnormal in a host of disorders. It is released into the peripheral blood after cell death caused by, *e.g.* ischaemia, excess heat or cold, injury, after ingestion of certain drugs, and from chemical poisonings. If cell lysis occurs, or cell membranes are damaged, cytoplasmic enzyme, LDH get released.

The lack of increment of lactic acid concentration in muscle might be due to the fact that the majority of muscle fibres might have been affected by the direct action of myotoxic components of venom. Consequently, cells were already irreversibly damaged by the time blood flow was reduced and therefore there was no increment in the glycolytic pathway and no lactic acid accumulation in necrotic muscle cells.

In conclusion it is found that the *S. argus venom* induces neuromuscular modulatory activity. The muscular damage is evident by the elevation of creatine kinase in plasma and decrease in muscle. *S. argus* venom induces a complex pattern of muscle damage characterized by direct Myotoxic effect.

Findings

- An elevated Na+ K+ ATPase activity observed in mouse brain treated with *S. argus* venom *in vitro* shows nociceptive neuronal activity of the venom.
- An increased cholinesterase activity in mouse brain treated with *S. argus* venom *in vitro* may be due to the presence of acetylcholine in the venom or some compound inducing release of acetylcholine in the venom or some compound inducing release of acetylcholine.
- The decrease in LDH with decrease in lactate shows inability for anaerobic respiration of cells or the impartial death of cells.
- The decrease in the pyruvate content shows that it is a precursor for many other reactions taking place.
- The increase in plasma creatine kinase indicates the myotoxicity of *S. argus* venom.
- The increase in plasma LDH shows cellular damage [muscle damage] to gastrocnemius muscle on exposure to *S. argus* venom.
- The decrease in creatine kinase indicates the damage of muscle cells. The creatine kinase released is sweeped into the plasma resulting in an increase in plasma creatine content.
- *S. argus* venom induces a complex pattern of muscle damage characterized by direct myotoxic effect.

Chapter 6

IN VITRO AND *IN VIVO* LYSOSOMAL MEMBRANE STABILITY AGAINST *S.ARGUS* VENOM

6.1. INTRODUCTION

Lysosomes are intracellular unit membrane bound organelles enclosing hydrolytic enzymes like acid phosphatase, Cathepsin D, β - glucoronidase, β. N – glucosominidase, etc. Lysosomes play an important role in catabolic process of the cell and their main functions are intracellular and extracellular digestion. Lysosomes have been implicated in the defence mechanisms of the cell and in the pathogenesis and progression of different disorders. In certain pathological conditions the lysosomal membranes may rupture releasing the hydrolytic enzymes into the cell. It is well established that many agents such as various disease conditions, stress, hormone and drugs can induce destabilizing alterations in lysosome. The release of the enzymes serves as an index of lysosomal membrane damage.

6.2. REVIEW

Studies on the effects of Russell's Viper Venom on renal lysosomal functions in experimental mice was carried out employing the three typical marker enzymes N – acetyl – β – D- glucosaminidase [NAG] , Cathepsin D and acid phosphatase [Wing Aung *et al.,* 1998]. The effects of fish venom upon the lysosomal membrane have not been tested until now. The aim of the present work was to study the functional alterations produced by *Scatophagus argus* venom *in vivo* and *in vitro* on renal lysosomal membrane.

6.3. MATERIALS AND METHODS

6.3.1. Isolation of renal lysosomal enriched fraction

Inbred Swiss albino male mice of two months age weighing 20±2g were anaesthetized by ether inhalation in a tightly closed bottle. The kidneys of mice were excised, blotted, immediately weighed minced with scissors and then diluted

with 1:8[W/V] with 0.3M ice-cold sucrose and homogenized in a motor driven Teflon Potter Elvehjem glass homogenizer. The renal homogenate was centrifuged at 143g for 10 minutes in a Hitachi refrigerated centrifuge to sediment nuclei and unbroken cell debris. The procedure was repeated with the supernatant. These two sediments were combined and reconstituted in the same volume of 0.3M sucrose to form the "nuclear fraction". Then the supernatant was centrifuged at 9000g for 3min. The sediment was reconstituted with same volume of 0.3M sucrose to form "lysosome enriched fraction" consisting of mitochondria, lysosome and brush border of renal tubular cell. The supernatant formed during this centrifugation is the "cytosol fraction"[Maunsbach, 1974].

6.3.2. Viability Conformation

For viability conformation each of the three fractions were assayed for activities of respective marker enzymes namely proteinase [EC.3.4.21.14] for cytosol, succinic dehydrogenase [EC.1.3.99.1] for mitochondria, acid phosphatase [ACP] [EC.3.1.3.2] for lysosome and alkaline phosphatase [EC.3.1.3.1] for brush border of renal tubular cell. All steps were carried out at 4°C whenever necessary and freshly prepared lysosome enriched fraction was used for subsequent experiments.

a] Estimation of Proteinase

Reagents

Haemoglobin
Trichloroacetic acid - 8%
Lowry reagent
Folin –cocalteau reagent

The method of Mc Donald and Chen [1965] was followed. 200µl of cell free supernatant were added to 2ml volume of haemoglobin substrate solution

prepared by dissolving 1.2g of denatured haemoglobin [standardized for proteinase assays] in 100µl of buffer. Assay tubes were attemperated to temperature appropriate for determinants before enzyme additions. Blanks were prepared in the same manner as assay tubes, except that 4ml of precipitating agent [8% TCA] was added before addition of enzymes. After 1 hour of incubation in a circulating water bath at 40 °C a 4ml volume of precipitating agent was combined with the contents of each assay tube. The resulting precipitates of non-hydrolyzed haemoglobin were removed by filtration through Whatman No.1 filter paper and 1ml samples of filtrate were assayed. A 5ml volume of Lowry reagent was added to each sample. After 10min at room temperature 0.5ml of Folin – Cocalteau reagent diluted 1:1 with deconized glass distilled water was added. Tubes were immediately vortexed stored in the dark for 1hour and then read against blanks at 700nm in a spectrophotometer. One unit enzyme activity [EU] is defined as that amount of enzyme, which released the color equivalent of 1µg of tyrosine in 1 minute.

b] Estimation of Succinate dehydrogenase [SDH]

Reagents		
Sucrose	–	0.25M
Sodium Succinate	–	40µM
Pot. PO_4 buffer	–	100µM [pH7.0]
INT [2-p-idodophenyl] –3-[p-nitrophenyl]		
5 phenyl tetrazolium chloride	–	4µM Toluene

Succinate dehydrogenase activity was estimated by the method of Nachlas *et al.,* [1960] using sodium succinate as substrate. Homogenate of kidney was prepared in cold 0.25M sucrose solution and centrifuged at 1000g for 15min. The supernatants were used for the assay. The reaction mixture of 2.0ml contained:

40μ moles of sodium succinate, 100μ moles of potassium phosphate [p^H7.0] buffer, 4μ moles of INT [[2-p-idodophenyl] – 3-[p-nitrophenyl] – 5 phenyl tetrazolium chloride and 0.5ml supernatant. The contents were incubated for 30min at 37°C and the reaction was stopped by adding 5.0ml of glacial acetic acid. The iodoformazan formed was extracted overnight in 5ml of toluene at 5°C. The absorbance of colour was measured in a spectrophotometer at 495nm against a blank of toluene. The enzyme activity is expressed as μ moles of formazan formed /mg protein / hour.

c] Estimation of Acid phosphatase

Acid phosphatase was measured according to the method of Anon [1963]. The procedure for Acid phosphatase is presented in 4.3.5.a

d] Estimation of Alkaline Phosphatase

Alkaline phosphatases were assayed following the method of King and Jagatheesan, [1959] using 4-amino antipyrene. The procedure for Acid phosphatase is presented in 4.3.5.b

6.3.3. *In vivo* effects of crude extracts on renal lysosome

(A) Different Time Interval [*In vivo* I]

Four groups of mice each consisting 6 animals were injected intraperitonially with venom extract of *S. argus* at a concentration of 100μg/0.1ml and then sacrificed at 2,4,6 and 24 hours post injection. A set of 6 mice, which received 0.1ml of saline, served as control. At each time interval mice were sacrificed, kidneys excised, homogenized and differentially centrifuged as mentioned above. The lysosome enriched pellet fraction of each animal was

subjected to enzyme assay. The lysosome-enriched fraction was divided into two equal portions marked Maximal [Complete lysis of lysosomal membrane to release intralysosomal enzymes] and Basal [hypotonic solution which facilitates the limited release of intralysosomal enzymes]. To the 'Maximal' 0.2% Triton-X 100 was added [Win Aung *et al.,* 1998] while the same volume of distilled H_2O was added to the 'Basal'. They were incubated at 37°C for 1 hour with gentle shaking, followed by centrifugation to remove unlysed lysosome and then the enzymes released into supernatants were assayed. The ratio between maximal and basal enzyme activities expressed the lysosomal membrane integrity Bjorkerud *et al.,* [1967]

(B) Different Concentration [*In vivo* II]

Four groups of mice each consisting of 6 animals were injected intraperitonially with phosphate buffered saline 0.1ml [control] and crude extracts of *S. argus* venom in three different concentration [ie.10, 50, and 100µg/0.1ml]. After 2 hours mice were sacrificed, kidneys excised, homogenized followed by differential centrifugation as mentioned above and the fraction obtained was subjected to enzyme assay.

6.3.4. *In vitro* effects of crude extracts on renal lysosome

[A] Different Concentration

For the study of *in vitro* effects of S. argus venom on mice renal lysosome, mice were sacrificed, their kidney excised, weighed, homogenized and differentially centrifuged to obtain lysosome-enriched fraction. 1ml of lysosome enriched fraction was incubated in equal volumes of 0.1M acetate buffer pH5 with [1] 0.2%Triton X-100 marked as "maximal" [2] distilled water marked as "basal" and [3] crude venom extract in different concentrations [50,100,200 and 300 µg/ml]

for 1 hour. After incubation it was centrifuged at 9000g for 20 min to remove the unlysed lysosome. The supernatants were measured for Cathepsin D and Acid Phosphatase released from ruptured lysosomes.

[B] Different time Interval

Another experiment was set up to find the activity of enzymes at different time intervals [ie. 15-60 min]. 1ml of lysosome enriched fraction was incubated in equal volumes of 0.1M acetate buffer pH5 and [1] 0.2% Triton X-100 [2] Distilled water and [3] S. argus venom extract [100µg/ml]. The activities of enzymes released into supernatants were assayed after centrifugation as mentioned above.

6.3.5. Enzyme assay

6.3.5.1. Estimation of Cathepsin D

Cathepsin D was measured using haemoglobin as substrate according to the method of Barrett and Heath [1977]. Cathepsin D activity was measured in the medium [total vol 0.5ml] containing 50mM Sodium acetate buffer pH 3.8 and 0.25M Sucrose. After pre incubating 0.05ml of enzyme at 37°C the reaction was initiated by adding 0.5mg of haemoglobin. At the end of 10min of incubation period the reaction was terminated by the addition of 2.5ml of 5% TCA. The tubes were kept on ice and the contents were filtered using Whatman No.1 filter paper. The tyrosine positive materials in the filtrate were estimated.

6.3.5.2. Estimation of Acid phosphatase

Acid phosphatase was measured by using P-nitrophenol as substrate according to the method of Anon [1963] as given under section 4.3.5.a.

Protein was determined by the method of Lowry *et al., [1951]* using bovine serum albumin as standard. The specific activities of the enzymes were expressed as enzyme unit [EU] per mg protein
where I EU per protein is equal to 1 nmole of substrate transformed or product produced per min per mg protein.

6.3.6. Statistical Analysis

The SPSS® statistical software for windows, version 13.0 [SPSS Inc., Chicago, USA] was used in all data analyses. Statistical analyses were performed using ANOVA followed by Fisher's LSD procedure [Zar 1996].

6.4. RESULTS

The studies of the marker enzymes proteinase, acid phosphatase, alkaline phosphatase and succinic dehydrogenase were carried out for the three subcellular fractions. Table 6.1 illustrates the distribution of the marker enzymes. The activity of proteinase was highest in the cytosol fraction as expected while the activity of the alkaline phosphatase [the marker enzyme for brush border of renal cells] acid phosphatase [marker enzyme for lysosome] and succinic dehydrogenase [marker enzyme for mitochondria] were high in the lysosome-enriched fraction of the kidney. [Table 6.1] The study of lysosome enriched fractions for lysosomal function thus proved to be viable.

Studies show that the renal lysosomal integrities of mice gradually reduced with different time intervals after envenomation as shown in Fig 6.1 [*in vivo* test I]. The integrities expressed as maximal and basal activity for the two enzymes acid phosphatase and Cathepsin D were found to reduce gradually and reached their minima at 4 to 6 hour after envenomation after which a restoration of the integrity was observed at 24 hour.

Specific activities [a]			
Marker Enzymes	**Nuclear**	**Cytosol**	**Lysosome enriched**
Succinic dehydrogenase	0.39±0.08[9.1%]	0.77±0.07[17.9%]	4.3±0.3[100%]
Proteinase	1.2 ±0.2[11.7%]	10.2±0.5[100%]	1.05±0.06[10.3%]
Alkaline phosphatase	20.0 ±0.7[10.3%]	17.2±0.5[8.9%]	193.9±3.2[100%]
Acid phosphatase	2.2 ±0.02[2.4%]	4.9±0.2[5.3%]	93.6±2.0[100%]

Table 6.1: Distribution of marker enzymes among three sub-cellular fractions of mice kidney. Results are expressed as mean ± SEM [n=6]. Percentage distribution of enzyme activity in respective fractions is shown in paranthesis.

[a] :Specific activities is expressed as enzyme unit per milligram protein where I EU per mg protein is equal to 1 nmol substrate transformed or produced per minute per mg protein.

6.4.1. *In vivo* studies on the effects of crude extracts on renal lysosome

One way ANOVA comparing membrane stability with time revealed an overall significant change [P<0.05] in the release of acid phosphatase [F = 17.960] [Table 4.1] and Cathepsin D [F = 51.14] [Table 4.2] from lysosomes of the experimental groups when compared to the control. Subsequent Fisher's LSD test reflected significant differences in membrane integrity for Cathepsin D [P<0.05] and for acid phosphatase [P<0.05] ie., there was a decrease in membrane integrity from 2 hours to 6 hours [Table 4.3].

In vivo studies involving different concentration showed a dose dependent response in the membrane stability with the membrane integrity heading towards a minima with increase in concentration of venom as shown in Fig 6.2 [*in vivo* test II]. One-way ANOVA comparing membrane stability with time revealed an overall significant change [P<0.05] in the release of ACP [F = 25.46] [Table 4.4] and Cathepsin D [F = 17.653] [Table 4.5] from lysosomes of the experimental groups when compared to the control. Subsequent Fisher's LSD test reflected significant differences in membrane integrity for cathepsin D [P<0.05] and for acid phosphatase [P<0.05] [Table 4.6].

6.4.2. *In vitro* studies on the effects of crude extracts on renal lysosome

The two enzymes ACP and Cathepsin D showed dose dependent responses to various increasing concentration of the venom as illustrated in Fig 6.3. Fig 6.4 depicts the response to the venom at various time intervals. The enzymatic activities were found to increase with increase in time. One-way ANOVA [comparing ACP [Table 4.7] and Cathepsin D [Table 4.8] with time intervals of 0-15, 15-30, 30-45 and 45-60] revealed an overall significance [p<0.05] in release of enzymes from the experimental groups. Subsequent Fisher's LSD test reflected significant difference in the release of marker enzymes with time. An increase in release of enzymes was observed with increase in time [Table 4.9].

To study the release of ACP and Cathepsin D with concentration of venom, one-way ANOVA was done. The values showed an overall significance [p<0.05] in the experimental groups for both ACP [Table 4.10] and Cathepsin D [Table 4.11]. Fisher's LSD test reflected significant difference with venom concentration where with increase in concentration there was an increase in release of enzymes. [Table 4.12].

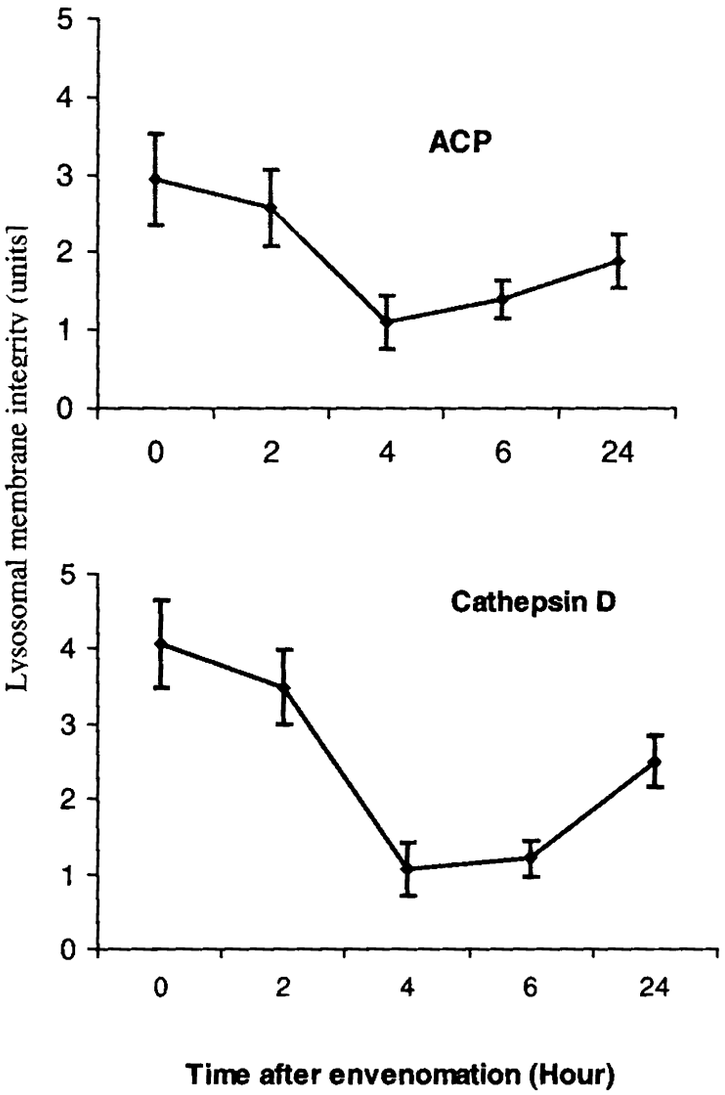


Fig 6.1. Renal lysosomal membrane integrities expressed in term of ratios of maximal basal activities of lysosomal enzymes Cathepsin D and acid phosphatase in in vivo condition at different time intervals after envenomation with S. argus venom. Each value represents mean ± SEM [n=6].

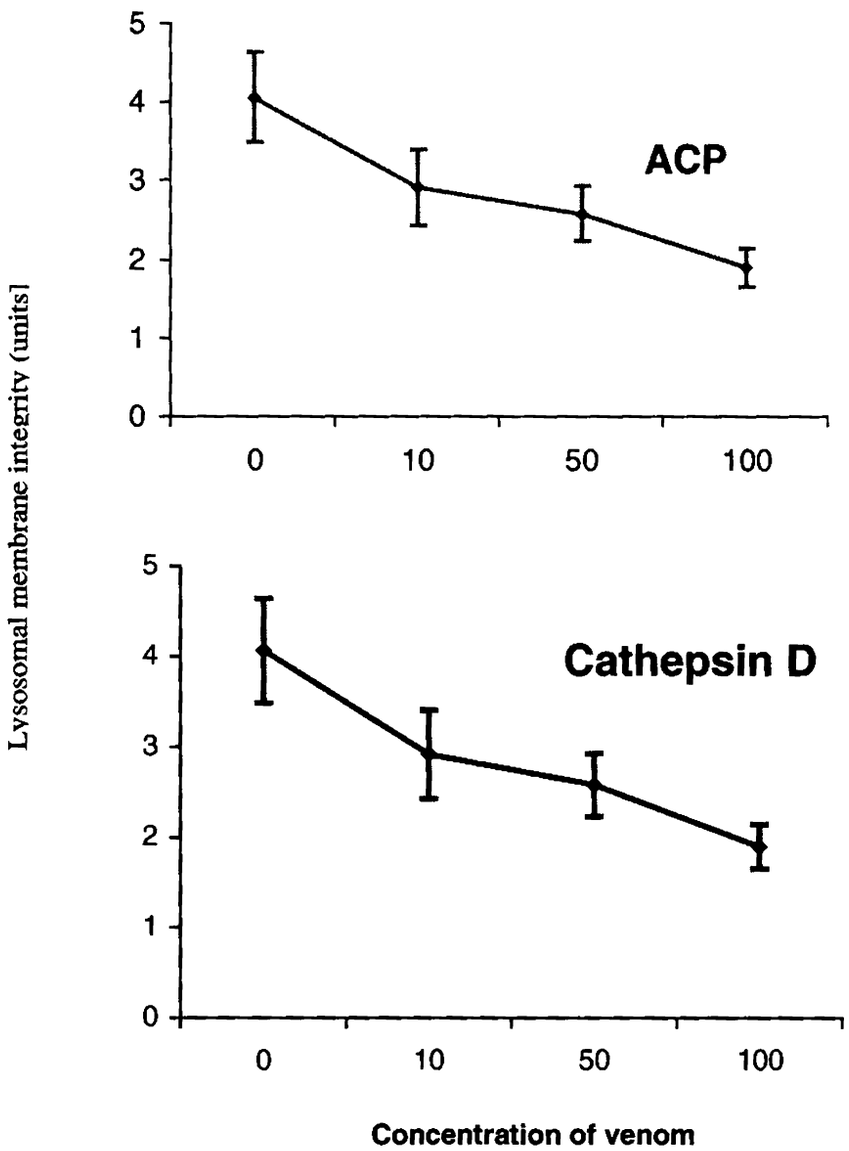


Fig 6.2. Renal lysosomal membrane integrities expressed in term of ratios of maximal basal activities of lysosomal enzymes Cathepsin D and acid phosphatase in in vivo conditions.

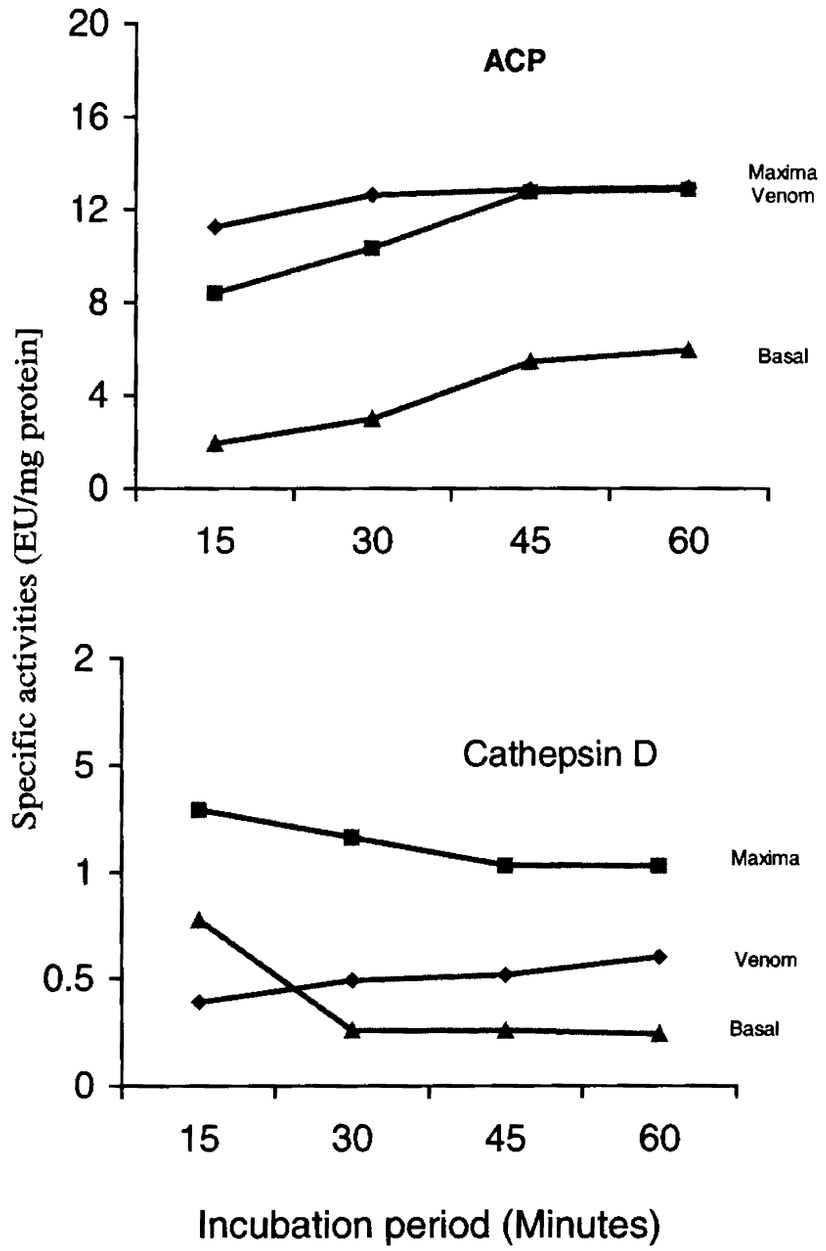


Fig 6.3. Marker enzymes released from the renal lysosome enriched fraction under in vitro condition in response to incubation at various time intervals with 0.2% Triton X-100 [maximal] distilled water [basal] and S. argus venom [100μg/ml].

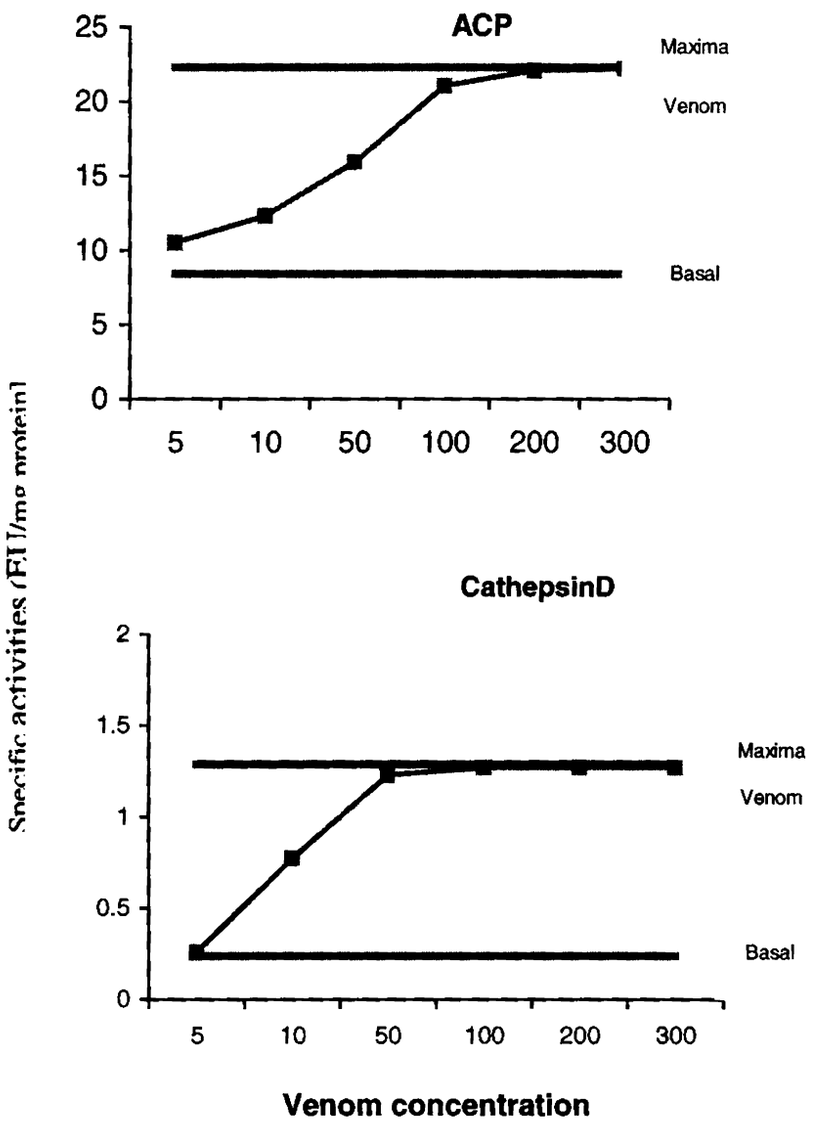


Fig 6.4. Marker enzymes released from the renal lysosome enriched fraction under invitro condition in response to incubation with 1 hour with 0.2% Triton X-100 [maximal] distilled water [basal] and various concentration of S. argus venom [5,10,50,100,200 and 300µg/ml].

Table 4.1.One way ANOVA for ACP (In vivo study for membrane stability with time)

Source of Variation	Sum of Squares	Degree of Freedom	Mean Square	F	P-value
Between intervals	42.90	4	10.72	51.13	P< 0.05
Error	5.24	25	0.20		
Total	230.34	30			

Table 4.2.One way ANOVA for Cathepsin D (In vivo study for membrane stability with time)

Source of variation	Sum of Squares	Degree of Freedom	Mean Square	F	P-value
Between intervals	42.90	4	10.727	51.13	P< 0.05
Error	5.244	25	0.209		
Total	230.3	30			

Table 4.3. Results of LSD analysis for the enzymes ACP and Cathepsin D for in vivo study for membrane stability with time.

Groups	Parameters	
	ACP	Cathepsin D
Time 0 vs 2 hours	P<0.05	P<0.05
Time 0 vs 4 hours	P<0.001	P<0.001
Time 0 vs 6 hours	P<0.001	P<0.001
Time 0 vs 24 hours	P<0.001	P<0.001

Table 4.4.One way ANOVA for ACP (In vivo study for membrane stability with different concentration of S. argus venom)

Source of variation	Sum of Squares	Degree of Freedom	Mean Square	F	P-value
Between intervals	42.90	4	10.727	51.13	P< 0.05
Error	5.244	25	0.209		
Total	230.3	30			

Table 4.5. One way ANOVA for Cathepsin D (In vivo study for membrane stability with different concentration of S. argus venom)

Source of variation	Sum of Squares	Degree of Freedom	Mean Square	F	P-value
Between intervals	14.72	3	4.907	17.65	P< 0.05
Error	5.559	20	0.277		
Total	217.13	24			

Table 4.6. Results of LSD analysis for the enzymes ACP and Cathepsin D for In vivo study for membrane stability with different concentrations of S. argus venom.

Groups	Parameters	
	ACP	Cathepsin D
Control vs 10 µg/ml	P<0.05	P<0.001
Control vs 50 µg/ml	P<0.001	P<0.001
Control vs 100 µg/ml	P<0.001	P<0.001

Table 4 .7. One way ANOVA for ACP (In vitro study for membrane stability with different concentration of S. argus venom)

Source of Variation	Sum of Squares	Degree of Freedom	Mean Square	F	P-value
Between intervals	14.35	4	3.589	17.95	P< 0.05
Error	4.997	25	0.199		
Total	137.44	30			

Table 4.8. One way ANOVA for Cathepsin D (In vitro study for membrane stability with different concentration of S. argus venom)

Source of Variation	Sum of Squares	Degree of Freedom	Mean Square	F	P-value
Between intervals	0.135	3	0.045	16.05	P< 0.05
Error	0.056	20	0.002		
Total	6.201	24			

Table 4.9. ***Results of LSD analysis for the enzymes ACP and Cathepsin D for In vivo study for membrane stability over time.***

Groups	Parameters	
	ACP	Cathepsin D
Time 0 vs 2 hours	NS	P<0.005
Time 0 vs 4 hours	P<0.001	P<0.001
Time 0 vs 6 hours	P<0.001	P<0.001
Time 0 vs 24 hours	P<0.001	P<0.001

Table 4.10. One way ANOVA for ACP (In vitro study for membrane stability with different concentration of S. argus venom)

Source of variation	Sum of Squares	Degree of Freedom	Mean Square	F	P-value
Between intervals	18.07	3	6.02	25.4606	P< 0.05
Error	4.73	20	0.23		
Total	121.02	24			

Table 4.11. One way ANOVA for Cathepsin D (In vitro study for membrane stability with different concentration of S. argus venom)

Source of variation	Sum of Squares	Degree of Freedom	Mean Square	F	P-value
Between intervals	1.285	3	0.42	536.8	P< 0.05
Error	0.015	20	0.007		
Total	4.83	24			

Table 4.12. Results of LSD analysis for the enzymes ACP and Cathepsin D for in vivo study for membrane stability with different concentrations of S. argus venom.

Groups	Parameters	
	ACP	Cathepsin D
Control vs 50µg/ml	NS	P<0.001
Control vs 100 µg/ml	P<0.001	P<0.001
Control vs 200µg/ml	P<0.001	P<0.001
Control vs 300µg/ml	P<0.001	P<0.001

6.5. DISCUSSION

Lysosomes have been implicated in the defence mechanisms of the cell and in the pathogenesis and progression of different disorders. In certain pathological conditions the lysosomal membranes may rupture releasing the hydrolytic enzymes into the cell. Lysosomal damage is well established as a biomarker of stress in a wide range of vertebrates [Tabata *et al.*,., 1990] and that many agents such as various disease conditions, stress, hormone and drugs can induce destabilizing alterations in lysosome.

Acid phosphatases act as marker enzymes for the detection of lysosomes in cell fractions and can be altered by the presence of venom. Acid phosphatase is a lysosomal enzyme that hydrolysis the ester linkage of phosphate esters and helps in autolysis of cell after its death. These enzymes are involved in a variety of metabolic processes such as molecular permeability, growth and cell differentiation and steroidogenesis [Ram and Sathayanesan, 1985].

Cathepsin D is the major lysosomal aspartic proteinase and is widely distributed in almost all mammalian cells. Together with Cathepsin B it forms the most abundant of the lysosomal proteinases although the concentration of Cathepsin L can be in the similar range. Cathepsin D is considered to be involved in physiological processes such as intracellular protein catabolism and antigen presentation also in a number of pathological conditions.

With increase in time and concentration of venom the rate of activity of the two enzymes cathepsin D and acid phosphatase increased which showed a decrease in membrane stability. A linear relationship of the enzymes acid phosphatase and cathepsin D with time and concentration was observed in both *in vitro* and *in vivo* studies. The lysosomal membrane stability has been proved to be

a useful index of cellular condition and correlates significantly with physiological condition of organism.

Most current evidence supports the hypothesis that the cytolytic activity by piscine venom is the result of pore formation in cell membranes [Church and Hodgson 2002]. Studies on cytolytic activity of stonustoxin – the fish venom from stonefish *Synanceja horrida* found that it does produce haemolysis by forming hydrophilic pores in cell membranes, which then result in cell lysis [Chen *et al.*, 1997]. Trachylysin from *S.trachynis* also show to form pores in cell membranes [Ouanounou *et al.*, 1999]. The presence of elevated levels of toxicants in lysosomes can result in the breakdown of their limiting membrane and leakage of the acid hydrolases and toxic contents into the cytosol resulting in cell injury or death.

Studies suggest that the *S. argus* venom is cytolytic to renal lysosomal membranes allowing enzymes to be released into the intracellular medium. The enzymes Cathepsin D and ACP prove to be useful marker enzymes of renal lysosomal membrane integrity in response to *S. argus* venom.

Findings

- Both for *in vivo* and *in vitro* studies with increase in concentration the enzymes ACP and Cathepsin D showed increased activity.
- The enzymes ACP and Cathepsin D showed increase in activity both in *vivo* and *in vitro* studies over time.
- The elevated activity of the enzymes shows the decrease in stability of the lysosomal membrane both *in vivo* and *in vitro* studies.

Chapter 7

BIOMEDICAL APPLICATIONS OF *S.ARGUS* VENOM

7.1. INTRODUCTION

The increased demand for effective, newer and better drugs has made man turn towards sea as a perennial source largely because marine natural products continue to be viewed as one of the few de novo sources of drug discovery yielding unorthodox and often unexpected chemical structure that offer novel points of departure for molecular modification leading to clinically available drugs.

The scientific community is focusing its efforts on the isolation and characterization of biologically active compounds derived from marine organisms with various pharmacological activities. Marine toxinologists in the past decade were involved in the search for potential pharmaceuticals from marine resources. Although it is virtually difficult to single out a particular bioactive molecule that will find place in medicine, many compounds have shown promise. During the past three decades there had been an increase in awareness of the remarkable potential of marine flora and fauna in areas as diverse as health, food additives, material for orthopedics, thermostable polymerases for polymerase chain reaction and bioactive material.

Toxicity being indicative of potent physiological activity it is quite possible that most of the toxic substance could yield valuable biomedical compounds. Although all of them may not enter the realm of material medica as such, they could at least serve as useful models to the synthesis of valuable medical compounds. The present investigation was aimed at elucidating some of the bioactivities of *S. argus* venom that would qualify them as potential biopharmaceutical compounds.

Oedematic Activity

Oedema formation is a common feature of the cutaneous inflammatory processes and is dependent on a synergism between mediators that increase vascular permeability and those that increase blood flow. The mediators include

prostaglandins, bradykinin, histamine, ATP and acetylcholine that cause the classical signs of inflammation i.e., swelling, redness, hyperthermia and pain.

Haemolytic Activity

Haemolysis is the breakdown of red blood cells and in the final stage of breakdown haemoglobin is released from the red cells. Haemolysis in small amounts is a normal body process. About 0.8 – 1% of all red cells in the body are haemolysed every day. It is usually balanced by red cell production in the marrow of the bones. But sometimes, so many cells breakdown so that marrow production is insufficient and anemia may result. Many biotoxins are known to cause haemolysis of RBC and they do have considerable potential as anticancer agents.

Blood Clotting

Animal venoms are usually complex mixtures of bioactive molecules which mainly include proteins and peptides. These toxins interact with physiological targets causing immobilization, death, or digestion of tissues. The vasculature and the blood coagulation system constitute relevant targets owing to their prominent role in homeostasis.

Platelet Lysis

Platelets play a key role in normal blood clotting. During the clotting process, platelets clump together to plug small holes in damaged blood vessels. Platelets also activate factor VIII and release phospholipids as part of the blood clotting process.

Cytolysis

Cytolytic proteins and peptides play an important role in performing offensive and defensive actions in a number of organisms by lysing cells through enzymic and non-enzymic mechanisms. Many cytolytic toxins lyse cells directly or

make cells more susceptible to damage by hydrolyzing membrane lipids through enzymic action. On the other hand a wide variety of non-enzymic proteins and peptides that possess cytolytic activity have been isolated. Most of these cytolysins lyse these cells by formation of discrete transmembrane pores. Through these pores osmoticants can move in or out whereas the large molecules such as protein cannot. Thus the cell interior is hyperosmotic, attracting a net influx of water, which results in sustained cell swelling and subsequently cell lysis. Pore forming toxins bind to either lipids or proteins in the cell membrane.

7.2. REVIEW

Fish venoms are known to induce intense and sustained oedematogenic responses space. Envenomations by venomous fish are associated with oedema observed both clinically and experimentally. The acute inflammatory responses in experimental animal after envenomation with the toadfish venoms have been well demonstrated in *Thalasophryne nattereri* and *T.maculosa* [Lopez-Ferriera *et al.*, 1998; Lima *et al.*, 2003; Sosa-Rosales *et al.*, 2005]. The stonefish *S.horrida* and the lethal factor stonustoxin have been studied for oedematic activity [Poh *et al.*, 1991; Khoo *et al.*, 1992]. Studies have been carried out on the Potamotrygon stingrays and scorpion fish, *S.plumeiri* for oedematic activity [Carrijo *et al.*, 2005 and Magalhaes *et al.*, 2006].

Considerable work has been done on the haemolytic properties of fish venoms [Auerbach *et al.*, 1987]. Most of the piscine venom exhibit haemolytic activity. The venom of the catfish *Plotosus canius* and *Heteropneustes fossilis* both produce haemolysis [Datta *et al.*, 1982; Auddy *et al.*, 1994]. Studies on the weeverfish *Trachinus draco* and dracotoxin the lethal toxin from it have shown to be potent haemolytes [Chhatwal and Dreyer, 1992 a; b]. Haemolytic studies on crude venom of stonefish have been carried out in *S. horrida, S. trachynis* and *S. verrucosa* as well as for the lethal factors stonustoxin, verrucotoxin and trachylysin [Kreger, 1991; Poh *et al.*, 1991; Khoo *et al.*, 1992; Garnier *et al.*, 1995;

Ouanounou *et al.,* 1999]. The toadfishes, *Thallassophryne nattereri* and *T. maculosa* have also shown haemolytic activity [Lopes-Ferriera *et al.,* 1998; Sosa-Rosales *et al.,* 2005]. Species specific haemolytic activity has been observed in the six species of fishes *S. verrucosa, Pterois lunulata, P. volitans, P. antennata, Dendrochirus zebra* and *Inimicus japonica* [Shiomi *et al.,* 1989]. Stonustoxin, the lethal factor isolated from stonefish venom was studied for platelet lytic activities [Khoo *et al.,* 1995]. Lysis of human platelets by the toadfish *T. nattereri* was also studied [Lopes-Ferriera *et al.*, 2002].

Several marine peptides have displayed high order of antitumor activity. The approach of testing venoms as antitumour agents dates back to the beginning of the last century, when Calmette et al. [1933] reported on the antitumour activity of snake venom, [Naja species venom] in adenocarcinoma cells. Since then many anticancer compounds have been isolated from toxins of marine sponges e.g. Sesterstatin, Dolastatin 10, Crellastatin etc. and Ecteinascidin 743 from tunicate is under Phase II clinical trial [Mayer 1999]. It was also demonstrated that purified protein from cobra venom was selectively cytotoxic to cancer cells [Baldi *et al.,* 1988; Braganaca 1976]. Particularly, proteinacious venom from several animals like snake, scorpion, spider etc. was reported to have excellent cytotoxicity in cultured cancer cell lines and also reduced tumor growth in mice [Abu-Sinna *et al.,* 2003; Orsolic *et al.,* 2003]. Among all, snake venom has been studied extensively, however, the marked curative properties of the snake venoms are always hindered by their high toxicities, and hence less toxic species, fish venom are proving to be a promising drug for research. The role of active component of the venom, its molecular target and signaling pathways through which it cause apoptosis in cancer cells are still in their early stages of study.

Cell lysis was determined for *G. marmoratus, P. volitans and S.trachynis* venom by measurement of Propidium Iodide Fluorescence [Church *et al.,* 2003]. Mitogenic and cytotoxic effects of *S.verrucosa* and *Hypodytes rubripinnis* on

normal and tumor cell lines have been carried out using flow cytometer [Satoh *et al.*, 2002]. The oriental catfish *Plotosus lineatus* was cytotoxic to cultured Ehrilisch ascites tumor cells, are a common model of tumor growth [Fahim *et al.*, 1996]. The cytolytic effect of *T. nattereri* venom on cultured myoblast, endothelial cells and mononuclear cell lines [J774AI] was studied [Lopes-Ferriera *et al.*, 2001; 2002]. The present study tries to emphasise the biomedical applications of *S. argus* venom.

7.3. MATERIAL AND METHODS

7.3.1. Oedematic activity

The ability of *S. argus* venom to induce oedema was studied in mice. 30µl of sterile 0.9 % [W/V] saline solution with venom in different concentrations [12.5,25 and 50µg/paw] were injected in the sub plantar region of the right hind paw. The left hind paw received an equal volume of sterile saline alone and served as the control. Prior to injection the venom solutions were filtered through 0.22µm Millipore filters. The volumes of both paws were measured using calipers at 0.5, 2,4, 6,24 and 48 hours after venom administration.

Percent oedema was calculated as follows:

$$= \frac{\text{Right paw thickness} - \text{initial thickness}}{\text{Right paw initial thickness}} \times \frac{\text{Left paw volume} - \text{initial volume}}{\text{Left paw initial volume}} \times 100$$

Each point represents mean ± SEM.

The effect of the antihistamine, pheniramine maleate on oedema inducing activity was tested by injecting the pheniramine maleate [50mg/kg] 30min before injection of 25 µg of *S. argus* venom. After two hours the volume of the paws were measured using calipers.

7.3.2. Estimation of Nociceptive activity

For nociceptive tests each mouse was kept in an adapted chamber. After 10 minutes adaptation period, the animals were injected with the venom [12.5, 25, 50 μg of protein] into the intraplantar region of the hind foot paw in a fixed volume of saline. The control group was injected only with sterile saline. Each animal was then returned to the observation chamber and the amount of time spent licking or biting each hind paw was recorded for 30 minutes and taken as index of nociception [Hunskaar *et al.*, 1985]. Each point represents mean ± SD of six independent experiments.

7.3.3. Haemolytic activity

Haemolytic activity was assayed on washed human, mice, goat, chicken and rat erythrocytes as described earlier by Garnier *et al.*, [1995]. To samples, 1ml containing various concentrations of venom protein in 150mM NaCl were added, 200μl of re-suspended 2% erythrocytes of each of the above mentioned organisms and kept for 30 minutes at room temperature. The suspensions were centrifuged [5min, 3000 rpm in Hitachi refrigerated centrifuge]. The absorbance of the supernatant was measured at 540nm to detect released haemoglobin. A negative control [erythrocyte suspension in 150mM NaCl] and a positive control [erythrocyte suspension in distilled water] were prepared, to enable calculation of percentage haemolysis. All assays were carried out in triplicates.

7.3.4. Blood Clotting

The assay was performed according to the protocol described in the United States Pharmacopoeia [1985]. Fresh human blood was collected directly into a test tube containing 8% sodium citrate solution in a proportion of 1:19 [v/v]. It was mixed immediately by gentle agitation. The plasma was then separated by

centrifugation of the sample. The separated plasma was pooled together and kept under refrigeration for the subsequent clotting assay. About 1.0ml of plasma was incubated at 37°C in a water bath. About 0.2ml of calcium chloride [1%] was added into the test tube and mixed. A solid clot observed within 5 min gave the indication that the plasma is suitable for the following test. The test sample *S. argus* venom was prepared in normal saline at the following concentrations 10, 20, 30, 40 and 50 x 10^2 μg/ml to determine the effect on blood coagulation. The test samples were added to the test tubes containing 1.0ml of plasma with and/or without addition of 0.2ml calcium chloride concentration and observed for changes. The experiments were repeated three times to confirm every observation.

7.3.5. Platelet lysis

Fresh citrated human blood obtained by forearm venepuncture was centrifuged for 10 min at room temperature at 160g to obtain the supernatant platelet rich plasma [PRP]. The effects of *S. argus* venom on platelets were evaluated by incubating increasing concentration of venom with 100μl PRP for 5 min at 37°C. Controls of 0 and 100% cytotoxicty were prepared incubating platelets with saline and 0.1% Triton X-100 respectively. The mixture was centrifuged at 2000 g for 10 min and lactic dehydrogenase activity was measured according to Wroblewski *et al.*, [1955] with slight modifications.

7.3.6. Cytolytic activity

MTT [3-4, 5 – dimethythiazol –2- yl] – 2,5 diphenyl tetrazolium bromide assay was used to evaluate the cell viability after treatment with venom. The assay detects living cells and the signal generated is dependent on the degree of activation of the cells [Mosmann, 1983]. HeLa cells were cultivated in RPMI 1640 medium containing 10% FCS at 37°C and 5% carbon dioxide. HeLa cells [5x10^4

cells per well] were incubated for 24 hours in a 96 well microplate. After changing the medium in each well, to the cells were added sterile PBS or venom diluted in medium in different concentrations [0.1, 0.5, 1, 2.5, 5 and 10μg of protein venom / ml]. After 72 hrs the microplates were centrifuged at 1000 rpm for 10min at 4°C. The medium was removed and 20μl of MTT [5mg/ml] in PBS/ well was added. The microplate was incubated for 3h at 37°C and 5% CO_2. After incubation 100μl of SDS [10%] diluted in PBS were added to each well [18h at 37°C and 5% CO_2]. The results were read on a multiwell scanning spectrophotometer [ELISA Reader] at 570nm. The results were expressed as the percentage of viable cells compared with PBS treated cells. Similar results were obtained in six separate experiments.

7.3.7. Statistical Analysis

The SPSS® statistical software for windows, version 13.0 [SPSS Inc., Chicago, USA] was used in all data analyses. One-way ANOVA was used to determine the levels of difference between all groups. GLM repeated measure was used to determine the significance of change in oedematic activity over time.

7.4. RESULTS

For determination of oedematogenic response induced by *S. argus* venom, concentration of 12.5, 25 and 50 μg venom/paw were used. The thickness of right different doses of venom produced a dose dependent oedema. The maximal response was observed from 1-3 hours oedema remaining significantly elevated compared with control [Table7.1]. Oedematic activity in mice persisted for more than 24 hours.

In sets of mice administered with highest dose of venom, haemorrhage was observed in the injected paw after 4 hours and tissue necrosis was observed after 24 hours. The time course of oedematic activity for *S. argus* venom at a concentration of 25μg/paw showed an increased activity during the initial 4 hours, though a decrease in oedema was observed compared to control. The oedema

Time (Hours)	Concentration of Venom		
	12.5µg/paw	25µg/paw	50µg/paw
0.5	65.00 ± 3.16	89.17 ± 6.65	118.33 ± 5.16
2	76.67 ± 5.16	103.33 ± 6.06	132.50 ± 4.47
4	75.83 ± 3.76	102.50 ± 6.12	131.67 ± 5.16
6	63.33 ± 4.08	91.67 ± 4.08	102.50 ± 2.74
24	40.83 ± 3.76	70.00 ± 4.47	69.17 ± 7.36
48	31.67 ± 2.58	49.17 ± 5.85	59.17 ± 4.92

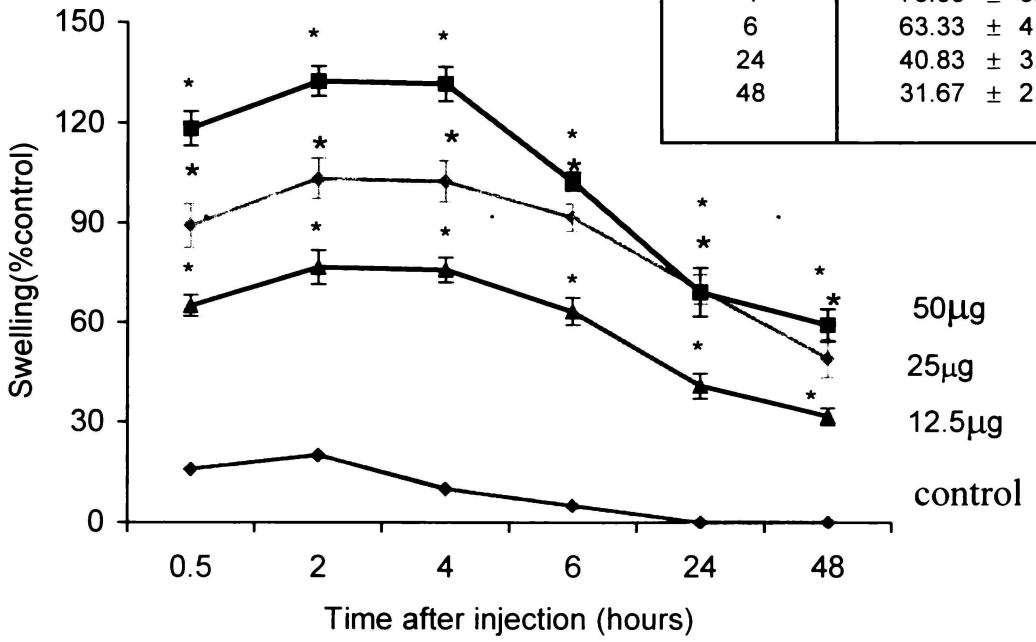
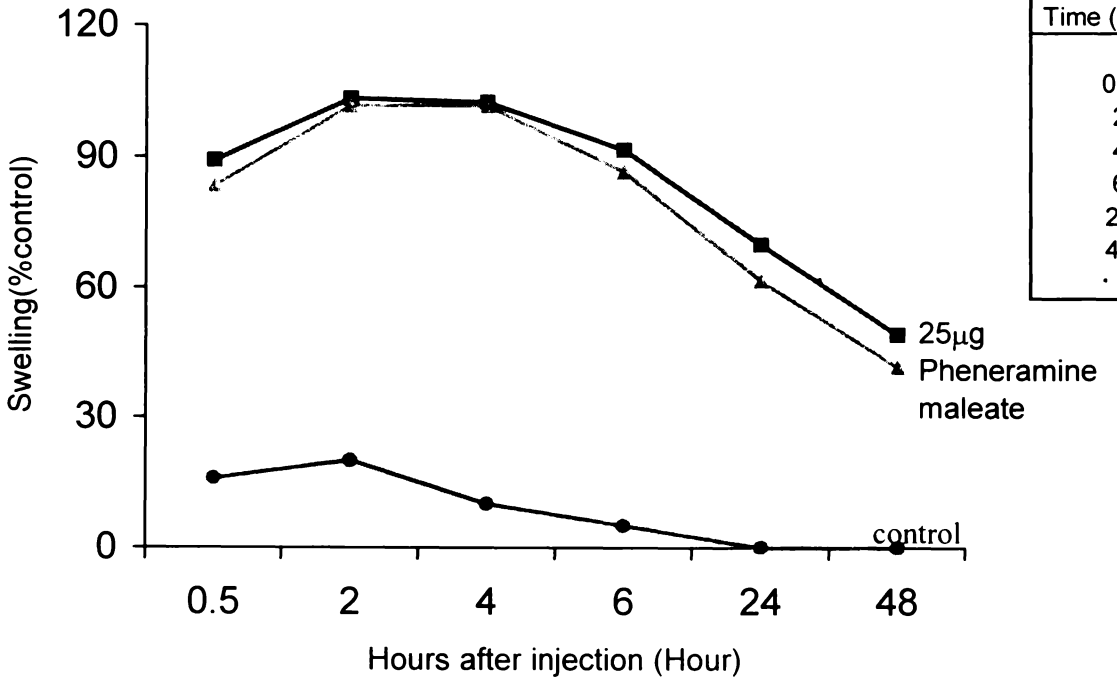


Fig 7.1: Oedematogenic activity of S. argus venom on mouse hind paw. Different doses of S. argus venom and normal saline were injected into the right footpad of mice [subplantar]. Each point represents mean [± SE] of six mice.



Time (Hours)	Concentration of Venom	
	25µg/paw	Pheniramine Maleate
0.5	89.17 ± 6.65	83.33 ± 5.16
2	103.33 ± 6.06	101.67 ± 6.83
4	102.50 ± 6.12	101.67 ± 6.83
6	91.67 ± 4.08	86.67 ± 4.08
24	70.00 ± 4.47	61.67 ± 7.53
48	49.17 ± 5.85	41.67 ± 5.16

Fig 7.2: Effect of antihistamine, pheniramine maleate on oedema formation induced by S. argus venom injected into mouse hind paw. Pheniramine maleate [50mg/kg] was administered 30 min before S. argus venom injection [25µg in 0.025ml]. Each point represents the mean [± SE] of six mice.

Table 7.1.Results of GLM repeated measure for oedematic activity .

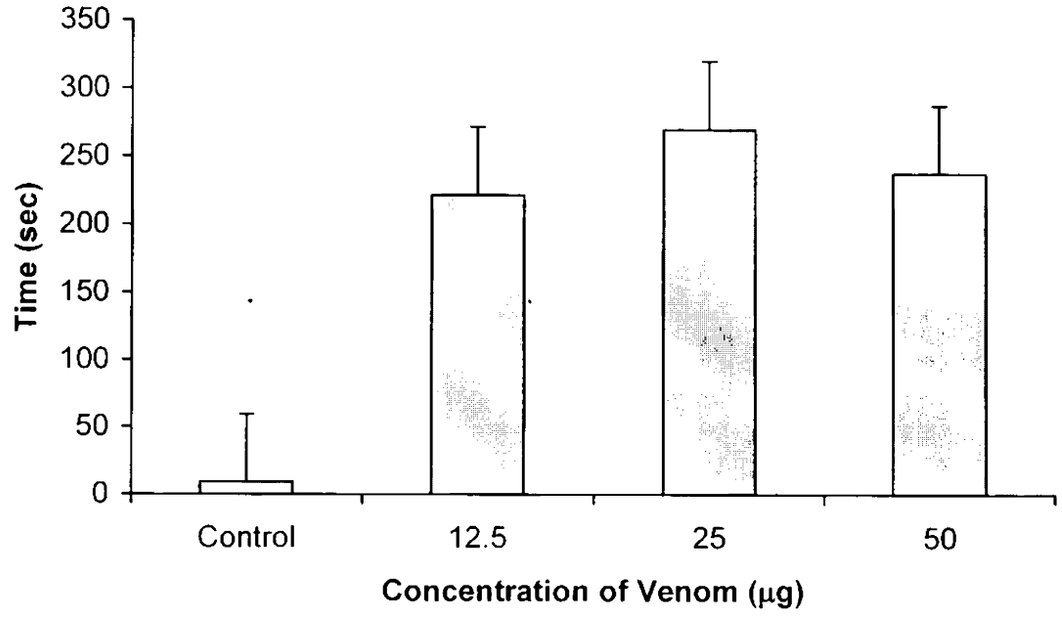
Effect		Value	F	Hypothesis df	Error df	Sig.
TIME	Wilks' Lambda	0.005	618.699	5	16	0.000
TIME * CONCN	Wilks' Lambda	0.004	18.528	15	44.6	0.000

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
CONCN	181435.021	3	60478.340	2248.033	0.000
Error	538.056	20	26.903		

Table 7.2.Results of GLM repeated measure showing the effect of Pheneramine maleate on oedematic activity .

Effect		Value	F	Hypothesis df	Error df	Sig.
TIME	Wilks' Lambda	0.013	162.639	5	11	0.000
TIME * CONCN	Wilks' Lambda	0.036	9.436	10	22	0.000

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
CONCN	131602.796	2	65801.398	1719.050	0.000
Error	574.167	15	38.278		



Venom concentrat	Nociception (%)		
Control	9.33	±	1.34
12.5	221.80	±	9.07
25	270.03	±	15.46
50	237.68	±	11.82

Fig 7.3: Estimation of nociceptive inducing activity. Each point represents mean ± SD of six independent experiments

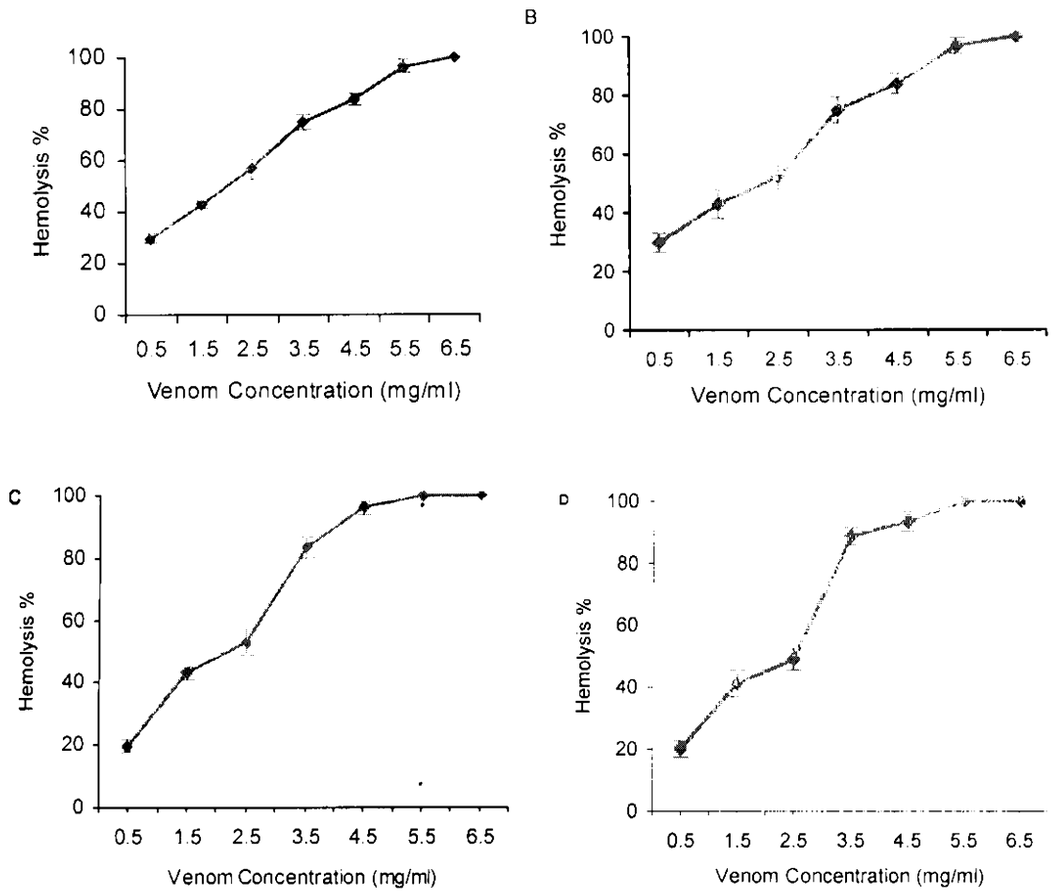


Fig 7.4: Haemolytic activity of S. argus venom on [A] Rat erythrocytes [B] Human erythrocytes [C] Mice erythrocytes [D] Goat erythrocytes. The 100% control for cell lysis was determined by addition of distilled water value represents the mean ± SE of six experiments.

Group	Dose [µg/ml]	LDH activity [% Response]	Expected response [%]	Probit
1	0.1	28	33.02	0.33
2	0.5	41	46.77	0.46
3	1	80	64.31	0.64
4	2.5	92	95.63	0.95
5	5	100	99.99	0.99

Table 7.3.: Probit analysis for effect of S. argus venom on platelet lysis [LDH activity].

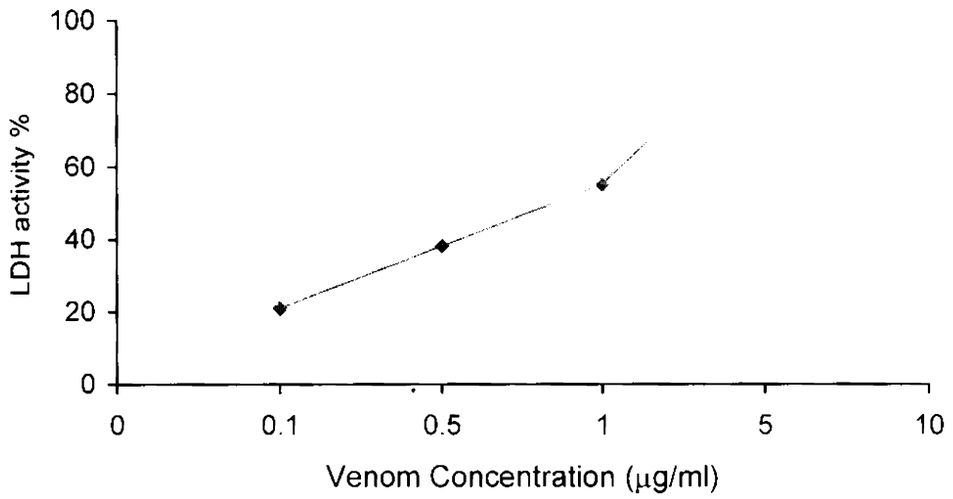


Fig 7.5 : Effect of S. argus venom on platelet lysis [LDH activity]. The release of LDH from washed platelets incubated with different concentrations of S. argus venom results are expressed as percentage of activity in relation to that evoked by 0.1% Triton X-100 in assay medium and represented as mean ± SE of six independent experiments.

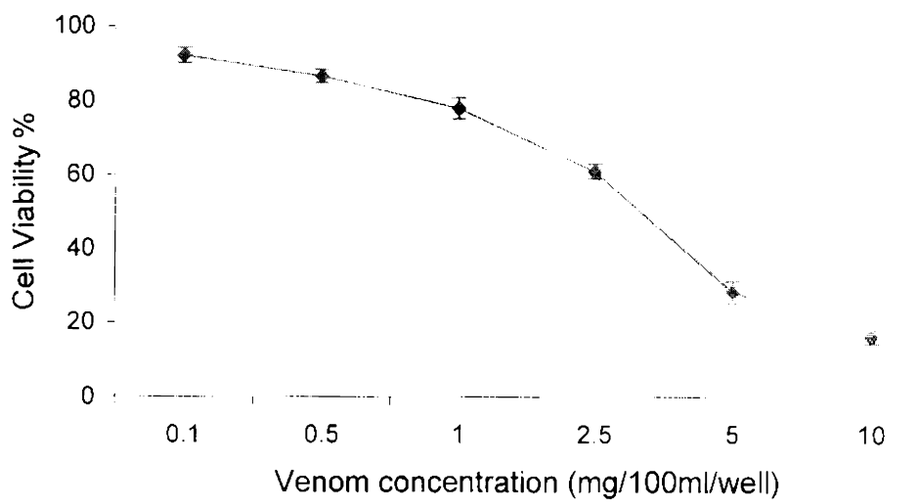


Fig 7.6: Effect of S. argus venom on cell viability of HeLa cells. Increasing concentrations of S. argus venom were incubated with HeLa cells for 72hours at 37°C. The cell viability as estimated by a colorimetric test with MTT and expressed as percentage of viable cells compared with cells cultured with medium alone. Results are represented as mean ± SE of six independent experiments.

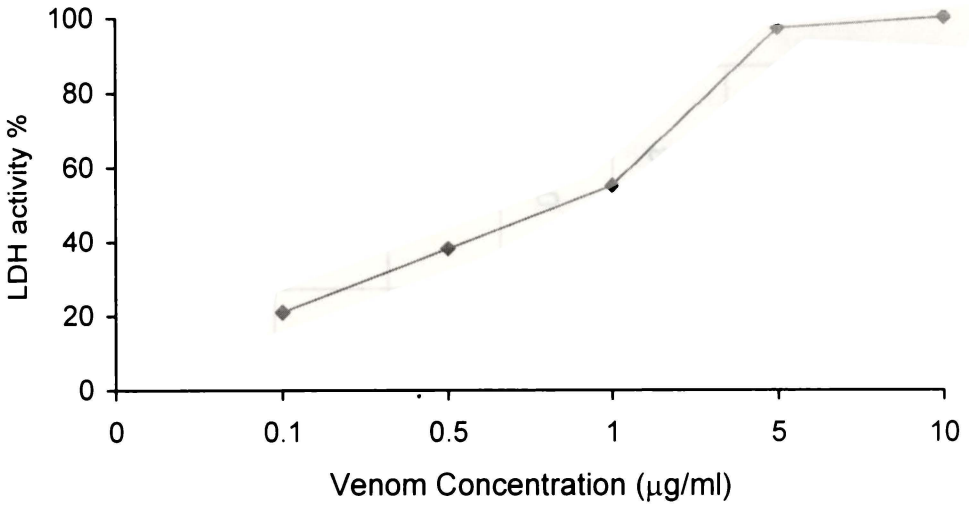


Fig 7.5 : Effect of S. argus venom on platelet lysis [LDH activity]. The release of LDH from washed platelets incubated with different concentrations of S. argus venom results are expressed as percentage of activity in relation to that evoked by 0.1% Triton X-100 in assay medium and represented as mean ± SE of six independent experiments.

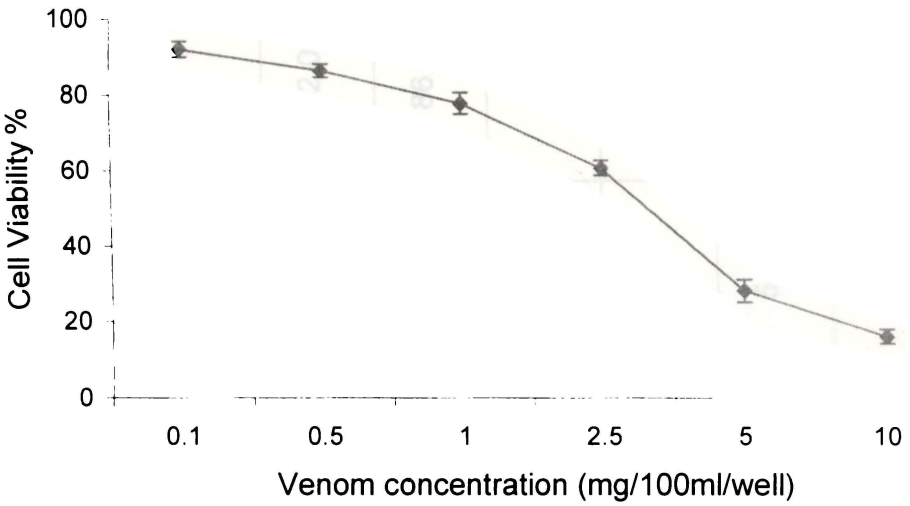


Fig 7.6: Effect of S. argus venom on cell viability of HeLa cells. Increasing concentrations of S. argus venom were incubated with HeLa cells for 72hours at 37°C. The cell viability as estimated by a colorimetric test with MTT and expressed as percentage of viable cells compared with cells cultured with medium alone. Results are represented as mean ± SE of six independent experiments.

Group	Dose [μg/ml]	Cellviability [%]	Lysis [%]	Expected response [%]	Probit
1	0.1	92.03	8.0	15.76	0.16
2	0.5	86.07	13.9	18.17	0.18
3	1	77.78	22.2	21.48	0.21
4	2.5	60.52	39.5	33.25	0.33
5	5	28.12	71.9	56.43	0.56
6	10	16.13	83.9	91.17	0.91

Table 7.4.: Probit analysis for cell viability of HeLa cells on exposure to S. argus venom. The cell viability as estimated by a colorimetric test with MTT and expressed as percentage of viable cells compared with cells cultured with medium alone.

persisted beyond 48 hours [Fig.7.2]. The antihistamine pheneramine maleate did not have any profound influence on the oedematic activity suggesting that histamines were not involved in *S. argus* venom oedema formation. [Table7.2]. footpad was measured 2hr after injection. Fig.7.3 shows that an intra plantar injection of the venom induced an increase in paw licking duration that reached its maximum with 25 μg.

S. argus venom exhibited haemolytic activity on washed erythrocytes of human, rat, mice, and goat. Lysis of erythrocytes was dose dependent. The activity was found to be species specific showing a high activity for rat erythrocytes followed by human > mice and finally goat erythrocytes which showed mild activity. The ED_{50} for human erythrocytes was 1.97 μg/ml [Fig: 7.4 A], ED_{50} for rat erythrocytes was [1.28 μg/ml] [Fig:7.4 B], ED_{50} for mice and goat erythrocytes were 2.2μg/ml [Fig:7.4 C] and [2.7μg/ml] [Fig :7.4 D] respectively. No clotting was observed when blood plasma was incubated with different doses of venom nor did it cause any lysis of clot formed.

Incubation of platelets with venom resulted in their rapid clearance from the suspension. A dose dependent lysis of platelets as shown in Fig.7.5 was observed. At a dose 0.59 μg venom/ml, 50% of platelet lysis occurred, while 5.8μg/ml venom resulted in almost complete lysis of platelets [Table7.3].

To study the cytotoxic ativity of *S. argus* venom HeLa cells were incubated with different doses of venom [0.1, 0.5, 1, 2.5, 5 and 10μg/protein of venom/ 100μl/ well]. Results obtained are shown in [Table7.4]. The crude extract of *S. argus* venom adversely affected the viability of HeLa cells when compared with the control [with PBS] [Fig.7.6]. Phase Contrast Microscope studies of HeLa cells

incubated with different doses of venom showed rounding, distension and lysis of cells.

7.5. DISCUSSION

The discoveries of toxins from venoms, especially from marine resources, are racing ahead because of their extremely complex and unique action upon various mammalian physiological systems. After all, the venom secretion is part of an organism's defense and/or predatory mechanism, whose specificity is horned over a million years of evolution.

The oedematogenic response observed for *S. argus* venom in mice footpad was persistent even after 24 hours and the oedema formed was intense suggesting the potentiality of *S. argus* venom in producing oedematic activity. The role of histamines released from mast cells causing vasodilation and increasing vascular permeability is ruled out as pheneramine maleate, an antihistamine was unable to block or reduce the oedematic activity. Studies on stonustoxin have shown that the increase in vascular permeability is not mediated by histamine release as the antihistamine diphenylhydramine, did not inhibit the oedema effect in mice [Poh *et al.*, 1991]. The inflammatory cellular influx into intraplantar region of mice induced by *T. nattereri* venom have shown to be due to a consequence of an impaired blood flow in venules at injured tissue and the cytotoxic effect of the venom on inflammatory cells contribute to this impairment [Lima *et al.*, 2003].

The pretreatment of mice with either indomethacin [a cyclo-oxygenase inhibitor] dexamethasone [a steroid anti-inflammatory inhibitor] or L-NAME [inhibitor of nitric oxide synthase] did not affect the *T. nattereri* venom induced oedematogenic responses [Lopes-Ferriera *et al.*, 2004]. These studies suggest an alternate mechanism of inflammation. Such venom-induced modifications of vascular permeability may account for the potent hypotension associated with

envenomation [Low *et al.,* 1993]. Significant nociceptive response was observed for *S. argus* venom injected into the mouse right hind paw. The maximum nociceptive response was observed for 25 μg venom.

S. argus venom exhibited haemolytic activity for human, mice, goat, and rat erythrocytes. The haemolytic activity for chicken erythrocytes was mild. This suggest that the haemolytic activity was species specific being selective for erythrocytes of each species. Studies have shown that most of the piscine venom exhibit species specific haemolysis. The stone fish *S.trachynis* venom show potent haemolysis which is lytic *in vitro* for guinea pig [Weiner, 1959a] rabbit, dog, rat and pig, while activity is less against sheep, cow, human, monkey, mouse, goat, horse, burro and cat erythrocytes [Kreger, 1991]. The verrucotoxin and stonustoxin are potently haemolytic being specific for rabbit erythrocytes [Garnier *et al.,* 1995; Khoo *et al.,* 1992]. Dracotoxin, the lethal factor from *T.draco* is species specific being very potent on rabbit erythrocytes, less potent on rat erythrocytes and weakly effective against mouse and bovine erythrocytes [Chhatwal and Dreyer, 1992a; b]. No phospholipase activity was found in *S. argus* venom [Chapter 8] suggesting an alternate mechanism for lysis. Similarly though *P. volitans, T. natterei, T. maculosa* and *S. plumeiri and N. robusta* possess haemolytic activity, no phospholipase activity is detected in the extracts of the venoms of these fishes supporting the present findings [Carrijo *et al.*2005; Hahn and Oconnor, 2000;Lopes-Ferriera *et al.,* 1998; Shiomi *et al.,* 1989]. Studies on cytolytic activity of SNTX found that it does produce haemolysis by forming hydrophilic pores in cell membranes, which then result in haemolysis [Chen *et al.,* 1997].

No clotting was observed when blood plasma was incubated with different doses of venom nor does it cause any lysis of the clot formed. The findings show that the amount of bleeding from the puncture wound appears to be similar to that from a similar non-venomous injury. The *S. argus* venom does not have any anticoagulant activity as observed for other vertebrate venoms. This is well in relation to the effects observed in envenomated patients where no reports of bleeding have been reported after stung by venomous spine of *S. argus*.

LDH is a cytosolic enzyme present in platelets that are intact under normal activation and release reactions of platelets. Lysis of platelets results in release of this enzyme. Hence LDH assay served as an induction of platelet lysis. 2% Triton X-100 caused complete platelet lysis. Studies have shown that *S. argus* venom induces platelet lysis. It has an ED_{50} of 0.7 μg/ml. *S. argus* venom presented a marked lytic effect towards platelets. Adhesion, activation, and aggregation of platelets are integrated phenomena that trigger thrombus formation [Kristensen *et al.*, 2000]. The lytic effect of *S. argus* on platelets leads to the liberation of potent products contained in their granules [Ca+, ADP, Thromboxane A_2] that induce aggregation, thrombus formation and further contribute to vasoconstriction effects. However the significance of the observed platelet lysis in the pathogenesis of envenoming is still unclear. Lysis *invivo* may occur but only in a limited number of platelets or since the number of platelets are more in *invivo* condition and the haemolytic ED_{50} observed is more than for platelets, RBC's were lysed more than platelets. Studies have shown that *T.nattereri* venom induced total lysis of platelet at a concentration of 5μg/ml and the effect was dose dependent [Lopes-Ferriera *et al.*, 2002]. SNTX was shown to induce a dose dependent [ED_{50} = 0.038 μg/ml] lysis of platelet in rabbit platelet rich plasma [Khoo *et al.*, 1992].

Nearly 4 hours after incubation of HeLa cells with *S. argus* venom of different concentration, the cells were distended [a morphological feature common to necrosis [Moldrich *et al*, 2000] when compared to preexposure on examination under phase contrast microscope. Degradation or lysis of the cell had taken place. Vehicle [PBS] had no effect on the morphology of cells. Studies on HeLa cells have shown that *S. argus* venom have potent cytolytic activity. The viability of cells was dose dependent. When cell death was measured in cultured neurons using propidium iodide fluorescence, *G.marmoratus* venom [30μg protein/ml], *S.trachynis* venom [4μg protein/ml] and *P.volitans* venom [20μg protein/ml] all produced an increase in fluorescence to 35,50 and 55% of pretreatment fluorescence respectively [Church *et al.*, 2003].

The flow cytometric analysis for *S.trachynis* [stonefish] and *Hypodytes rubripinnis* [redfin velvette fish] have shown that the venom are capable of differentiating between normal and tumor cells as mitogenic effects were observed towards cell lines. There was significant cytotoxicity induced by crude venom as well as certain isolated fractions [Satoh *et al.*, 2002]. The incubation of murine endothelial cell line of capillary origin with *T.nattereri* venom resulted in a dose dependent increment of LDH [Lopes-Ferriera, 2002]. *T.nattereri* venom also affects the viability of mononuclear cells [J774AI] [Lopes-Ferriera, 2003]. Studies on TLY have found evidence of an ability to form pores in the membrane of neuroblastoma cells [Ouanounou *et al.*, 1999] suggesting that the cytolytic activity of the venoms is also dependent on extra-cellular Ca^{2+}, and most likely due to ionic imbalances caused by the entry of extra cellular Ca^{2+} through pores in the cell membrane [Church *et al.*, 2003]. The ionic imbalances in the cells produce an influx of fluid, cell swelling, and necrosis.

The haemolytic activity, platelet lysis and cell line studies have all pointed out the cytolytic activity of the venom. Further studies are required to trace the

pathway of oedematic activity of *S. argus* venom. The present results open up new vistas for research on the effects of *S. argus* venom on cytolytic activities. *Scatophagus argus* venom stands out as an extremely encouraging material for future studies.

Findings

- *S. argus* venom showed oedematic activity in mouse hind paw which was peak during 1-3 hours and persisted for more than 24 hours.
- The antihistamine Pheneramine maleate did not have any profound influence on the oedematic activity suggesting that histamines were not involved in *S. argus* venom oedema formation.
- *S. argus* venom induced an increase in paw licking duration.
- The haemolytic activity exhibited by *S. argus* venom was species specific.
- No clotting was observed when blood plasma was incubated with different doses of venom.
- The ED_{50} for the platelet lysis induced by *S. argus* venom was 0.75 μg / ml.
- *S.argus* venom showed cytolytic activity towards HeLa cells.

Chapter 8

VENOM COMPONENTS OF *S.ARGUS*

8.1. INTRODUCTION

Nature's wonder of overwhelming biological diversity of marine organisms form a unique resource that provide a diverse array of natural products and novel chemical entities which when coupled with man's latest inventory techniques of separation prove to be useful in the quest for finding drugs with greater efficacy and specificity.

Marine biotoxins are complex and relatively dissimilar mixtures. The nature of marine toxins is complicated by the fact that qualitative as well as quantitative differences in toxin exist not only from species to species within the same genus but also from individual to individual within the same species. They have received increased attention from chemists and pharmacologists during the last two decades. Natural product chemists have probed marine organisms as a source of new and unusual organic molecules, while synthetic chemists have targeted these novel structures for development of new synthetic methodologies and strategies. The isolation and structure elucidation of new structurally complex compounds have provided immense gratification to marine toxinologists and natural product chemists.

Chemical defense is a protective measure employed by living organisms to evade predation or infection employing chemical substances, which affect the physiology of potential predators. Venomous creatures have a sophisticated mechanism for prey capture which includes a vast array of biologically active compounds such as enzyme, proteins and low molecular weight compounds. These substances target an immense number of receptors and membrane proteins with high affinity, selectivity, and potency. Venoms are a milieu of substances, interacting with one another to produce an overall response in an

animal or tissue although studies on the mechanism of action of venoms are often simplified by the isolation of individual toxins from the venom.

A toxin is a substance possessing a specific functional group arranged in the molecules and showing strong physiological activity. Biotoxins represent a somewhat more restrictive category, partly obscuring the fact that many toxins have the potential to be applied as a drug or pharmacological reagent particularly after careful extraction, isolation and characterization. Furthermore even if direct use is not feasible because of potent or harmful side effects, the toxin can serve as a pharmaceutical lead.

The process of isolating toxins from venomous fishes, of separating and purifying the compounds responsible for that bioactivity and of assigning chemical structures to compounds available in very small amounts is often time consuming, difficult and has sometimes proved to be impossible. The difficulty in obtaining adequate amounts of compounds for clinical trials or commercialization has long been regarded as a major hindrance to the development of marine pharmaceuticals. Technical advances in analytical instrumentation and methodology have greatly enhanced the productivity of the toxins.

8.2. REVIEW

Progress in the purification of fish venoms has closely followed advances in biochemical separation procedure. The initial studies employed dialysis paper electrophoresis, paper chromatography, and ultra centrifugation [Carlisle, 1962;Saunder, 1959; 1960; Haavaldsen and Fonnum, 1963;Russel and Emery, 1960; Austin *et al.*, 1965; Carlson *et al.*, 1973;Deakins and Saunders, 1967; Schaeffer *et al.*, 1971]. Several fish venom proteins have now been successfully

purified by FPLC or HPLC [*Plotosus lineatus*: Shiomi *et al.*, 1987; *Synanceja horrida* [Stonustoxin]: Poh *et al.*, 1991; Trachylysin: Kreger, 1991; Hyaluronidase: Poh *et al.*, 1992; *Trachinus draco* [dracotoxin]: Chhatwal and Dreyer, 1992; *Synanceja verrucosa* [verrucotoxin]: Garnier *et al.*, 1995; *N.robusta* [Nocitoxin]: Hahn and Connor, 2000; *Scorpaena plumeiri* [Gelatinase]: Carrijo *et al.*, 2005.

Most of these toxins in their pure form are high molecular weight proteins that retain all the lethal activities and usually all the biological activities of the crude extract. Though the number of toxin present in the fish venom is relatively low when compared to other venomous animals piscine venom also possess some enzymes and non-proteinaceous compounds. The esterase, amino peptidase, 5' nucleotidase, arginine esterase, arginine amidase, acid phophatases, alkaline phosphatases, phosphodiesterases, hyaluronidase, amylases, proteases, proteinases, acetylcholine esterase, alkaline phosphomonoesterases are some of the enzymes detected in piscine venoms. [Haaveledsen and Fonnum, 1963; Hopkins & Hodgson, 1998; Russel & Van Harreveld, 1954; Carrijo *et al.*, 2005; Garnier *et al.*, 1995; Khoo *et al.*, 1992]. In addition to these enzymes some other active components like norepinephrine, biogenic amines, histamine, catecholamines, 5'hydroxytryptamine, acetylcholine or cholinomimitic have also been detected. [Rodrigues, 1972; Cohen and Olek, 1989; Church and Hodgson, 2000; Haaveledsen and Fonnum, 1963; Hopkins and Hodgson, 1994; Garnier *et al.*, 1996].

In contrast to studies on venoms from a variety of venomous fish, the venom components of *S. argus* fish remains unexamined. The present study was carried out to detect the presence of few enzymes present in the venom.

8.3. MATERIALS AND METHODS

8.3.1 Phosphodiesterase

Reagents

Tris HCl Buffer – 200 mM [p^H 8.9 at 25°C]

bis [p – Nitrophenyl] phosphate – 6mM

Phosphodiesterase was assayed according to the method of Landt and Butler [1978]. To bis [p nitrophenyl] phosphate added equal amount of Tris HCl buffer and incubated at 37°C for 5 min. After incubation added 60µl of enzyme source [extract] and immediately mixed by inversion. Recorded the increase at 405 nm in a UV visible spectrophotometer [Hitachi] for 5 minutes at intervals of 30 seconds each. To the blank added deionised water instead of enzyme. Enzyme activity was expressed as 1.0 µ mole of bis [p-nitrophenyl] phosphate hydrolysed per minute at pH 8.9 at 25°C per milligram protein.

8.3.2. Acid phosphatase

The procedure for Acid phosphatase given in section4.3.5.a

8.3.3. Alkaline phosphatase

The procedure for Alkaline phosphatase given in section4.3.5.b

8.3.4. Proteinase

The procedure for Proteinase given in section 6.3.2.a

8.3.5. Acetylcholinesterase

The procedure for Acetylcholinesterase is given in section 5.3.2.

8.3.6. Acetylcholine

Reagents

Hydroxylamine HCl	–	20%
Ferric Chloride	–	10%
Hydrochloric acid		

Acetylcholine was estimated by the method of Hestrin as described by Augustinson [1957] with slight modification. The venom extract was kept in a boiling water bath for 10 minutes to inactivate the enzyme acetylcholinesterase and to release the bound acetylcholine. The tubes were cooled and the contents were homogenized in 2.0ml distilled water. 20% hydroxylamine hydrochloric acid and diluted HCl [1:1 with distilled water] was added. The contents were centrifuged and 10% ferric chloride was added to the supernatant and measured at 540 nm against a blank in a UV spectrophotometer. . The values were expressed as µM of Ach/mg protein.

8.3.7. Caseinase

Reagents

Casein	-	1%
Calcium chloride	-	0.008M
Trichloroacetic acid	-	5%

Caseinolytic activity was determined according to the method of Mandelbaum *et al* [1990]. One ml of 1% Casein was incubated for 2 hours at 37°C with 400µl of test solutions containing different concentrations of venom in the presence of 0.008M calcium chloride at pH 8.8. To the reaction mixture was added 5% trichloroacetic acid to stop the reaction and the hydrolyzed peptides contained in the supernatants were quantified according to Lowry *et al* [1951].

One unit was defined as the amount of enzyme yielding an increase in absorbance of 1.0 per min at 750nm. Results were expressed in U/mg of venom.

8.3.8. Gelatinase

Reagents

Sample buffer [2x]

0.5M Tris HCl, p^H6.8	-	2.5ml
Glycerol	–	2 ml
10% [W/V] SDS	–	4 ml
0.1% Bromophenol blue	–	0.5ml
Distilled Water	-	10 ml

Running buffer [1x]

Tris Base	-	2.9 g
Glycine	–	14.4 g
SDS	–	1.0 g
Distilled Water	–	1000 ml

Adjust pH to 8.3

Retaining buffer [10x]

Triton x – 100 - 25% [V/V] in water

Developing buffer [1x]

Tris Base	-	1.21 g
Tris – HCl	-	6.3 g
NaCl	-	1.7 g
$CaCl_2$	–	7.4 g
Brij - 35	–	0.02%
Distilled Water	–	1000 ml

Prepared gels [12%] according to the procedure of Heunssen and Dowdle [1980] to which added gelatin stock solution [10mg/ml in distilled water] to get the gelatin concentration of 0.1%. Mixed one part sample with one part Tris-Glycine SDS sample buffer [2x] and kept for 10 minutes at room temperature. The samples were applied and the gel run with 1 x Tris – Glycine SDS Running buffer according to the standard running conditions [~ 125V, constant voltage]. The run was completed when the bromophenol blue tracking dye reached bottom of the gel. After running, diluted the zymogram renaturing buffer [10x] 1:9 with deionized water and incubated the gel in the buffer with gentle agitation for 30 minutes at room temperature. Decanted the Zymogram Renatuing Buffer and replaced with 1x Zymogram Developing Buffer. Equilibrated the gel for 30 minutes at room temperature with gentle agitation and then replaced with fresh 1x Zymogram Developing Buffer and incubated at 37°C overnight. Stained with 0.5% Coomassie Blue R-250 fro 30 minutes and thereafter destained with appropriate Coomassie R-250 destaining solution.

8.3.9. Sodium dedecyl Sulphate – Polyacrylamide Gel Electrophoresis – [SDS–PAGE]

Principle

SDS is an anionic detergent that wraps around the protein giving it a net negative charge depending on its molecular weight. In SDS-PAGE the movement of protein molecule depends on the molecular weight of the protein. The protein SDS complex carries a net negative charge, hence moves towards the anode and the separation is based on the size of the protein. The number of SDS molecule bound to the polypeptide chain is approximately half the number of amino acid residues in that chain. Crude extract was subjected to electrophoresis following the method of Lammeli et al., [1970] in 12% Polyacrylamide slab gels.

8.3.9.1. Reagents for electrophoresis

Seperating Gel: [12%]

Distilled Water	–	3.2 ml
30% Acrylamide	–	4.0 ml
1.5M Tris Buffer [p^H 8.8]	–	2.6 ml
10% SDS	–	0.1 ml
10% APS	–	0.1ml
TEMED	–	0.004 ml

Stacking Gel: [5%]

Distilled Water	–	2.8 ml
Acrylamide	–	0.61 ml
1.5M Tris Buffer [p^H 6.8]	–	0.5 ml
10% SDS	–	0.04 ml
10% APS	–	0.04 ml
TEMED	–	0.04 ml

SDS Running Buffer [p^H 8.3] :

Glycine	–	14.4 g
Tris	–	3 g
Distilled Water	–	1000 ml
SDS	–	1 g

The glass plates were cleaned with ethanol and assembled on the casting stand. The separating gel components were mixed and poured into plates leaving about 2cm at the top. Few drops of butanol were overlayed to prevent meniscus formation and the gel was left undisturbed to set for 30 minutes. After polymerisation of the separating gel butanol was removed, washed and wiped with

filter paper. The prepared stacking gel was overlayed on the separating gel. Inserted the comb and was allowed to set for 20-30 minutes. Generally the stacking gel should not be prepared until the samples are ready as there is a pH difference between the two gels, which will diffuse with time. After the gel had solidified the comb was removed without distorting the shape of the well. The running unit was assembled after removing the clips, bottom spacers, etc. The bubbles and liquid were aspirated from wells. The electrode buffer was added to the tanks.

Sample buffer with SDS

Glycerol	–	1ml
β-Mercaptoethanol	–	0.5ml
Stacking gel buffer	–	0.9ml
Bromophenol blue 5%	-	0.3ml
SDS 10%	-	0.5ml

10µl of the sample were mixed with 40µl of sample buffer with SDS and boiled for 5 minutes. The prepared samples were loaded into the wells of the stacking gel. Molecular markers of 6.5 KDa – 68 KDa were used. SDS running buffer flooded the upper chamber. Both upper and lower tanks of the apparatus were filled with electrode buffer and constant current of 80V applied for pre-running. After about 15minutes of pre-running the dye front crossed the stacking gel. Voltage was increased to 100V and electrophoresis continued until the dye reached the bottom of the gel.

8.3.9.2. Staining the Gels:

Immediately after completion of electrophoresis the gels were carefully separated from the trays and washed in tap water to remove excess SDS. After

staining the gels for two hours in Coomassie Brilliant Blue R250, the excess stains were washed off and the gels were destained.

8.3.9.3. Determination of Molecular weight:

Molecular weights of standard SDS-PAGE molecular markers used ranged from 6.5 KDa – 68 KDa. Albumin [68KDa], ovalbumin [43 KDa], glyceraldehydes-3-dehydrogenase [36 KDa], anidrase carbonic [29 KDa], trypsinogen [24 KDa], trypsin inhibitor [20 KDa], α-lactoalbumin [14.2 KDa] and aprotinin [6.5 KDa] [Sigma chemical company, St. Louis, MO, USA] were used as molecular markers. Rf values of the standard markers were calculated by using the following formula.

$$\text{Relative front [Rf]} = \frac{\text{Solute front}}{\text{Dye front}}$$

Using these Rf values calculated for standard markers, a graph was drawn with Rf and log of the molecular weights of the standard proteins on a semi-log graph. The Rf values of unknown samples were calculated and extrapolated on a standard graph to determine the molecular weight.

Staining Solution:

Coomassie Brilliant Blue R250	–	100mg
Methanol	–	40ml
Glacial Acetic acid	-	10ml
Distilled Water	–	40ml

Destaining Solution:

Methanol	–	40ml
Acetic acid	-	10ml
Distilled Water	–	50ml

8.4. RESULT

The crude venom showed the presence of phosphodiesterase [156 ± 8 nmol PNP/ h/ mg venom protein]. Studies have shown the presence of alkaline and acid phosphatase in the concentration of 1.33 ± 12.8 μ mol PNP/min/mg and 629.4 ± 11.2 μ mol PNP/min /mg respectively. Mild proteinase activity of 0.416 U/mg was detected in *S. argus* crude venom. Acetyl cholinesterase activity was present in the crude *S. argus* venom at a concentration of 1.56 ± 0.02 μ mol of ACh hydrolysed/mg/h. Micromolar concentration of acetylcholine was 1.21 ± 0.04 μmol of ACh/mg protein was found to be present in the venom.

ENZYMES	ACTIVITY
Phosphodiesterase[nmolPNP/h/mg]	156 ± 8.2
Acid phosphatase[nmolPNP/min/mg]	629 ± 11.2
Alkaline phoshatase[μmolPNP/min/mg]	1.36 ± 0.12
Proteinase [U/mg]	0.416 ± 0. 01
Acetylcholinestrase [μmol of ACh hydrolysed /mg/hr]	1.56 ± 0.02
Acetylcholine[μmol of ACh/mg protein]	0.121 ± 0.04
Caseinase [U/mg]	3.74 ± 0. 3

Table 8.1.: Enzyme activity of Scatophagus argus venom. Results are expressed as mg venom protein

Fig. 8.1. SDS PAGE Gelatin Zymography of S.argus crude venom using resolution gel of 12%. After electrophoresis the gel was rinsed in 2.5% (v/v) of Triton X- 100 for 1 hour and incubated overnight at 37oC in 0.1M sodium citrate buffer pH 8.0

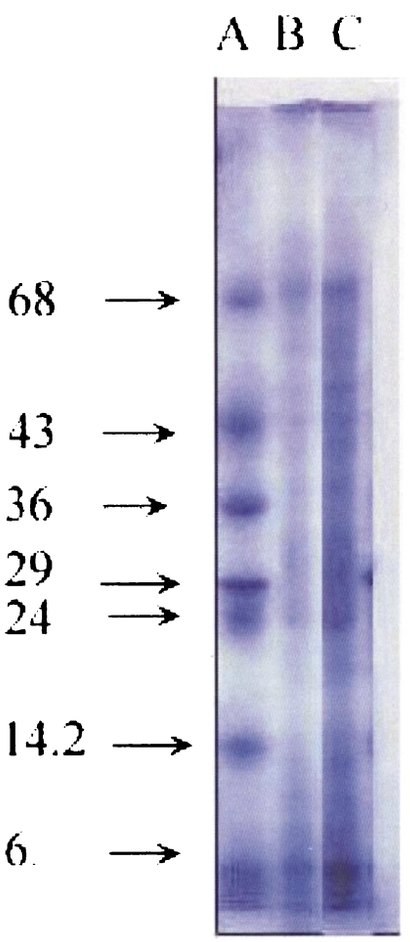


Fig. 8.2. Electrophoretic profile of the S. argus venom. The venom was analysed by SDS PAGE using 12% polyacrylamide gel and was stained with Comassie blue. Numbers at left corresponds to position of molecular markers.

Caseinolytic activity assayed showed a positive indication of a mild caseinolytic activity of 3.74 ± 0.3 U/mg. Zymographic studies for gelatinolytic activity showed the presence of a gelatinase. The white band formed revealed the presence of gelatinase [Fig.8.1.]. Studies for phospholipase activity was below detectable level.

The electrophoretic profile of S. argus venom presented prominent bands: One band located near 68 KDa, two bands between 45 and 36 KDa and one near 24 KDa. In addition weakly stained bands were also observed [Fig.8.2.].

8.5. DISCUSSION

Animal venoms are usually complex mixtures of bioactive molecules recognized as potential sources of pharmacological agents and physiological tools. Enzymes are an important and common component of the venom of many animals including bees, snakes, spiders and scorpions, with several functions probably involved in the toxic action [Nget-Hong and Ponnudurai, 1992]. They are responsible for some pathological activities triggered by these venoms. In this respect, the venom of fishes is no different, containing various enzymatic activities.

The present study detected the presence of phosphodiesterase [Table I] in S. argus venom with levels being significantly comparable to that of *G. marmoratus* venom [183±nmol PNP/U/mg] [Hopkins and Hodgson, 1998]. Phosphodiesterase has been detected in the venom of *Synanceja horrida* [Khoo *et al.*, 1992], *S. trachynis* [Hopkins and Hodgson, 1998] and in the sting rays [Fenner et al., 1989]. Acid phosphatase has been detected in *S. argus* venom. High levels of acid phosphatase have been detected in *S. argus* venom [629.4±11.2µmol PNP/min/mg] when compared to *S.trachynis* [75 nmol PNP/min/ mg] and *G.marmoratus* venom [320 nmol PNP/min/mg]. The presence of alkaline phosphatase has been detected in higher levels in *S. argus* venom [1.36 µmol

PNP/min/mg] than in *G.marmoratus*, [471 nmol PNP/min/mg] and *S.trachynis* [8 nmol PNP/min/mg] with levels lower than in *S.horrida* venom [3 µmol PNP/min/mg][Khoo *et al.*, 1992;Hodgson and Hopkins, 1998;]. The presence of thrombin like proteinase has been reported in *Synanceja horrida* [Khoo *et al.*, 1992]. Though proteinase was present in *S. argus* the activity was mild. [Table I]

S. argus venom has displayed the presence of micromolar concentration of acetylcholine [Table I] .The presence of micromolar concentration of acetylcholine has been reported for lionfish, *Pterois volitans* [Cohen and Olek, 1989]. In addition the venom of the fresh water sting ray *Potamatrygon motoro* and *S.trachynis* are believed to contain either acetylcholine or cholinomimetic compounds [Rodrigues, 1972; Church and Hodgson, 2000a]. Acetylcholine could enhance the action of the toxic component, perhaps by inducing local vasodilation at the injection site or by producing pain by direct or indirect action on sensory neurons [Cohen and Olek, 1989].

Phospholipase activity in *S. argus* venom showed values below the detection threshold indicating that the venom does not possess this enzyme. Studies for PLA activity in fish venom have demonstrated no activity for this enzyme in *Pterois volitans, S.trachynis, G.marmoratus, T.nattereri* nor *Nothesthes robusta* venom. [Shiomi et al., 1989; Hopkins and Hodgson, 1994; Hopkins and Hodgon, 1998; Lopez Ferriera et al., 1998; Hahn and O'Connor, 2000].

The proteolytic activity of *S. argus* venom on casein and gelatin were assayed based on the probable involvement of proteases in the instability of biological activities of the fish venom. Caseinolytic activity detected in *S. argus* venom [3.74 ± 0.3 U/mg] compared well with proteolytic activity in *Thalassophyrne maculosa* [2.0 – 4.4 U/mg] and stingrays, *Potamotrygon cf. scobina* and *P. gr. orbignyi* [Sosa Rosales *et al.*, 2005; Magalhaes et al., 2006]. Mild proteolytic activity has been observed in bullrout, *Notesthes robusta* venom [Hahn and

O'Connor, 2000]. In *Scorpeana plumeri* caseinolytic activity has been detected. Zymography has revealed the presence of gelatinolytic proteases *in S. argus* venom [Fig I]. A gelatinase [Sp-GP Scorpion fish gelatinase] with molecular weight around 80,000 Da under reducing and 72,000 Da under non-reducing conditions has been purified from the Scorpion fish *S.plumeri* [Carrijo *et al.,* 2005] and *Thalassophryne nattereri* has also shown the presence of a gelatinase [Lopes – Ferreira *et al.,* 1998].

The electrophoretic profile of *S. argus* venom presented important bands: One band located near 68 KDa, two bands between 45 and 36 KDa and one near 24 KDa. In addition weakly stained bands were also observed. Similar studies with other fish venom have shown that stonustoxin [SNTX] isolated from stonefish *S. horrida* showed a single protein peak with molecular weight of 148 kDa. SDS PAGE determined this protein peak to comprise of two subunits designated as α and β with weights of 71 and 79 kDa respectively [Poh *et al.,* 1991] while the cytolysin, trachynlysin purified by from the stonefish species *S.trachynis* is a monomeric protein having m.w of 158 kDa [Kreger, 1991]. Verrucotoxin, a tetrameric glycoprotien having a m.w of 322kDa comprising 2 α and 2 β subunits with m.w 83 kDa and 78kDa respectively with lethal cytolytic and hypotensive activities [Garnier *et al.,* 1995] and a monomeric lethal protein of 98 kDa has also been purified from *S. verrucossa* [Shiomi *et al.,* 1993].

The polypeptide toxin, Dracotoxin is characterized from the greater weeverfish, *T. draco* with m.w 105 kDa [Chhatwal and Dreyer, 1992b] while trachinine, a 324 kDa protein having four identical subunits has been isolated from lesser weeverfish, *T. vipera* [Perriere *et al.,* 1998]. The 15 kDa venom, Toxin-PC is isolated from the Indian catfish, *Plotosus canius* [Auddy and Gomes, 1996]. Purification of *Notesthes robusta* venom yielded Nocitoxin, a single monomeric soluble protein of m.w 170kDa [Hahn and Connor, 2000]. The Brazilian niquim,

Thallassophryne nattereri venom yielded a 47 kDa toxin [Lopes-Ferreira *et al.*, 1998].

Studies reveal the presence of enzymes and different proteins in the venom of *S.argus*. Though exact relationships of these enzymes and proteins in envenomation are not traced yet the involvement of enzymes in envenomation cannot be ruled out. Further studies are required to find the mechanism of action of these enzymes and proteins present in *S. argus* venom. The present study opens new dimensions for isolation of the lethal compound present in *S. argus* venom.

Findings

- The phosphatases ACP and ALP are found to be present in the *S. argus* venom.
- Micromolar concentration of the neurotransmitter acetylcholine is detected in the venom of *S. argus.*
- The enzyme phosphodiesterase is present in the *S. argus* venom.
- Caseinase and gelatinase the two proteases were detected in *S. argus* venom.
- Phospholipase the lytic enzyme present in most snake venoms is absent in *S. argus* venom.
- Mild activity of the proteinase enzyme is present in *S. argus* venom.
- The electrophoretic profile of *S. argus* venom showed few major and minor bands to be present between 6.5 KDa – 68 KDa

Chapter 9

SUMMARY AND CONCLUSION

Scatophagus argus also known as spotted scat is widely distributed in the coastal mud flats, mangrove swamps, harbors, upstream swamps and the estuaries of India. Scats are greenish brown in colour with dull white belly and black blotches of varying sizes all over the body. The euryhaline nature and the beautifully spotted rhombic body rank it as a fascinating aquarium fish while the quality and taste ranks it as a highly priced edible fish. Scats are well adapted to live in a fluctuating environment that endows them with many biological attributes highly desirable for cultured finfish.

Scats are venomous fishes and the venom apparatus is constituted by 11 dorsal spines, a pair of ventral spine and 4 anal spines, elongated venom glands and an integumentary sheath enveloping all (Cameron and Endean, 1977). The spines are very sharp and pointed, each possessing a pair of antero-lateral grooves one on each side accommodating the paired venom glands in each spine. The venom glands are irregularly shaped tending to be columnar in the deepest portion of the gland. The glands are aggregations of large gland cells in the thickened epidermis of the integumentary sheath. Scats are not aggressive and do not actively attempt to inflict wound. However the fishermen get stung accidentally and frequently while handling them. The butterfish envenoming appears within 5-10 min as excruciating and persistent local pain disproportionate to the size of the injury, redness, swelling and throbbing sensation that extend to the limbs followed by dizziness. Most patients are treated with empirical procedure such as immersion of the wounded area in hot water.

The crude extract of *S. argus* spine was obtained with saline, phosphate buffer (pH 7.4) and also with 10% glycerol. LD_{50} was assayed with these three extracts and it was found to have an LD_{50} of 9.8 mg / kg for saline extract. The LD_{50} value for buffer extract of *S. argus* venom is 9.97 mg /kg for mice and 9.92mg/kg is the LD_{50} value in mice obtained for *S. argus* venom extracted in 10%

glycerol. The crude extracts were further purified using ION exchange chromatography using DEAE cellulose. The partially purified fractions were also assayed for mice lethality. From the fractions it was found that a concentration less than 0.44 µg/g was able to induce lethality.

The effects of *S. argus* venom on the vital organs are very well confirmed by the histopathological changes of the venom treated mice. Liver of *S. argus* venom treated mice congestion, cloudy swelling, microvesicular fatty changes and infiltration of inflammatory cells around the portal vein. The damage to the hepatocytes in the present study could be attributed to the storage of the toxin in liver for detoxification. Brain tissue of *S. argus* venom treated mice showed spongiosis of the entire parenchyma. Areas of haemorrhage, vascular congestion, and cloudy swelling in renal tubules revealed the pathological alterations caused in the kidney of mice by *S. argus* venom. Heart showed the presence of oedema and degeneration of myofibrils when compared to that of the normal animal. Venom appeared to be injurious to the vascular endothelium and caused congestion of blood vessels.

The oedema of brain and the cloudy swelling in lining cells of renal tubule suggest that the venom might contain oedema-causing factors that could have crossed over the blood–brain barrier (BBB) and damaged the brain. The alterations described here in can be due to a direct action of the venom in renal glomeruli and tubules or an indirect release of mediators. Tubular necrosis in kidney, coagulative necrosis observed in liver and myofibrillar degeneration of cells in heart and haemolysis in all organs shows the cytolytic activity of venom. Thickening of Bowman's capsule, shrunken glomeruli, proteinaceous / foreign material within Bowman's capsule, haemolysis, blood clot, tubular necrosis all revealed the nephrotoxicity of *S. argus* venom. Myofibrillar degeneration, pycnotic nuclei, blood clot etc emphasise the cardiovascular toxicity of *S. argus* venom. The

hepatoxicity of the *S. argus* venom was revealed by the alterations in liver tissue. Mice envenomated with different concentration of *S. argus* crude venom at different time intervals showed pronounced influence on enzymes studied. The ALT activity was found to be higher than the AST activity suggesting that pyruvate contribution is slightly more than oxaloacetate formation in these tissues. Elevated GST activity may reflect the possibility of better protection against the venom. An increase in CAT activity was observed which indicates activation of the antioxidant defensive system indicating direct or indirect ROS generation after *S. argus* envenomation. Increased levels of SOD indicated the increased ability of tissues to handle O_2^- radicals. Increased GSH and GPx activities observed in present study may be attributed to the adaptive response of the tissues to oxidant challenge caused by *S. argus* venom. The elevation in ACP activity observed may be due to necrotic changes in tissues. The increase in ALP activity can be attributed to alterations in membrane property caused by interaction with venom. The enhanced AST and ALT shows hepatic injury and tissue damage. *S. argus* venom induced changes in enzymes represent the initial disorders of impairment as well as symptoms of metabolic alterations.

An elevated cholinesterase and Na+ K+ ATPase activity was observed for mouse brain treated with *S. argus* venom *in vitro* in mouse brain. The decrease in LDH with decrease in lactate shows lack of anaerobic respiration of cells or the impartial death of cells. The decrease in the pyruvate content shows that it is a precursor for many other reactions taking place. The increase in plasma creatine kinase indicates the myotoxicity of *S. argus* venom. The increase in plasma LDH shows cellular damage (muscle damage) to gastrocnemius muscle on exposure to *S. argus* venom. The decrease in muscle creatine kinase indicates the damage to muscle cells. The creatine kinase released is sweeped into the plasma that results in an increase in plasma creatine content. *S. argus* venom induces a complex pattern of muscle damage initiated by direct myotoxic effect, together with

vascular alterations characterized by blood stasis and thrombosis. The combination of these effects results in acute muscle damage followed by a partially impaired regenerative process. The isolation and characterization of venom components responsible for causing these effects is a relevant task.

Toxicity being indicative of potent physiological activity, it is quite possible that most of the toxic substances could yield valuable biomedical compounds. The oedematogenic response observed for *S. argus* venom in mice footpad was persistent even after 24 hours and the oedema formed was intense suggesting the potential for oedematic activity of *S. argus* venom. The role of histamines released from mast cells causing vasodilation and increasing vascular permeability is ruled out, as pheneramine maleate, an antihistamine was unable to block or reduce the oedematic activity. Significant nociceptive response was observed for *S. argus* venom injected into the mouse right hind paw. The maximum nociceptive response was observed for 25 μg venom.

S. argus venom exhibited haemolytic activity for human, mice, goat, and rat erythrocytes. The haemolytic activity for chicken erythrocytes was mild. This suggest that the haemolytic activity was species specific being selective for erythrocytes of each species. No clotting was observed when blood plasma was incubated with different doses of venom nor did it cause any lysis of clot formed. The findings show that the amount of bleeding from the puncture wound appears to be similar to that from a non-venomous injury. The *S. argus* venom does not have any anticoagulant activity as observed in the case of other venoms. This is well in relation to the effects observed in envenomated patients who do not report bleeding after stung by venomous spine.

Studies have shown that *S. argus* venom induces a marked lytic effect towards platelets. The lytic effect of *S. argus* on platelets leads to the liberation of potent products contained in their granules (Ca^{+}, ADP, Thromboxane A_2) that

induce aggregation, thrombus formation and further contribute to vasoconstriction effects. However the significance of the observed platelet lysis in the pathogenesis of envenoming is still unclear. Lysis *in vivo* may occur but only in a limited number of platelets i.e., since the number of platelets are more in *in vivo* condition and the haemolytic ED_{50} observed is more for RBC's than for platelets, RBC's are lysed more than platelets.

The incubation of HeLa cells with *S. argus* venom of different concentration showed that the cells were distended a morphological feature common to necrosis. Degradation or lysis of the cell had taken place. Studies on HeLa cells have shown *S. argus* venom to have potent cytolytic activity. The viability of cells was dose dependent. The haemolytic activity, platelet lysis and cell line studies have all pointed out the cytolytic activity of the venom. Further studies are required to trace the pathway of oedematic activity of *S. argus* venom. The present results open up new vistas for research on the effects of *S. argus* venom on cytolytic activities.

Studies reveal the presence of enzymes and different proteins in the venom of *S.argus*. The present study detected the presence of phosphodiesterase in *S. argus* venom. *S. argus* venom has displayed the presence of micromolar concentration of acetylcholine. Phospholipase activity in *S. argus* venom shows values below the detection threshold indicating that the venom does not possess this enzyme. The proteolytic activity of *S. argus* venom on casein and gelatin were assayed due to the probable involvement of proteases in causing the instability of biological activities of the fish venom. Caseinase and gelatinase enzymes were detected in *S. argus* venom. Though exact relationships of these enzymes and proteins in envenomation are not traced, the involvement of enzymes in envenomation cannot be ruled out. Further studies are required to find the mechanism of action of these enzymes and proteins present in *S. argus*

venom. The present study opens new dimensions for isolation of the lethal compound present in *S. argus* venom. The preliminary study carried out here shows the presence of a lethal factor between 6.5 KDa – 68 KDa.

Studies conclude that fish venom possesses many bioactive substances, especially peptides, proteases and enzymes that bind with high affinity to physiological targets and can be trapped for therapeutic purposes in the near future. Even though this study reveals the conundrums of *S. argus* venom, it opens new vistas of research on the venom components and the application and design of the venom as a drug.

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APPENDIX

Time	Control		0.98 mg/Kg	
	Liver	Kidney	Liver	Kidney
2 Hour	4.8 ± 0.3	5.0 ± 0.2	5.5 ± 0.3	7.0 ± 0.2
4 Hour	4.7 ± 0.3	5.1 ± 0.2	6.4 ± 0.3	7.4 ± 0.3
6 Hour	4.6 ± 0.2	5.1 ± 0.2	6.5 ± 0.4	7.3 ± 0.3
12 Hour	4.5 ± 0.2	5.2. ± 0.1	6.5 ± 0.3	7.3 ± 0.2
Time	1.47 mg/Kg		1.97 mg/Kg	
	Liver	Kidney	Liver	Kidney
2 Hour	6.5 ± 0.4	7.4 ± 0.2	7.3 ± 0.2	7.0 ± 0.5
4 Hour	6.6 ± 0.3	7.5 ± 0.3	6.7 ± 0.1	7.6 ± 0.3
6 Hour	6.6 ± 0.3	7.4. ± 0.4	6.9 ± 0.3	7.5 ± 0.4
12 Hour	6.9 ± 0.2	7.5 ± 0.1	7.6 ± 0.3	7.6 ± 0.2

Table 4.1.: Superoxide dismutase [SOD] activity in tissues of mice envenomated with S. argus venom. Values are expressed as 50% inhibition of NBT/min/mg protein. Results are mean± SD of six separate determinations.

Time	Control		0.98 mg/Kg	
	Liver	Kidney	Liver	Kidney
2 Hour	47.0 ± 5.8	31.8 ± 5.4	55.7 ± 4.0	40.2 ± 6.0
4 Hour	45.4 ± 3.8	31.0 ± 4.6	58.0 ± 6.0	41.8 ± 5.6
6 Hour	46.1 ± 3.0	31.4 ± 4.7	57.5 ± 5.4	40.9 ± 4.9
12 Hour	45.2 ± 2.6	34.8· ± 3.8	46.8 ± 3.6	34.5 ± 4.1
Time	1.47 mg/Kg		1.96 mg/Kg	
	Liver	Kidney	Liver	Kidney
2 Hour	56.8 ± 4.3	41.8 ± 4.4	56.3 ± 5.2	41.7 ± 4.9
4 Hour	56.8 ± 5.1	45.2 ± 5.5	58.2 ± 4.6	43.0 ± 5.0
6 Hour	56.0 ± 4.2	42.8· ± 5.5	56.8 ± 4.8	41.4 ± 4.6
12 Hour	45.2 ± 3.0	36.3 ± 8.0	45.0 ± 3.0	38.9 ± 5.8

Table 4.2.: Catalase activity in tissues of mice envenomated with S. argus venom. Values are expressed as H_2O_2 consumed/min/mg protein. Results are mean± SD of six separate determinations.

Time	Control		0.98 mg/Kg	
	Liver	Kidney	Liver	Kidney
2 Hour	7.2 ± 0.1	5.2 ± 0.1	7.4 ± 0.2	5.6 ± 0.1
4 Hour	7.2 ± 0.1	5.1 ± 0.2	7.6 ± 0.1	5.5 ± 0.1
6 Hour	7.4 ± 0.1	5.2 ± 0.4	7.6 ± 0.2	5.8 ± 0.1
12 Hour	7.0 ± 0.2	5.2 ± 0.2	7.4 ± 0.1	5.5 ± 0.1
Time	1.47 mg/Kg		1.96 mg/Kg	
	Liver	Kidney	Liver	Kidney
2 Hour	7.8 ± 0.1	5.8 ± 0.1	8.1 ± 0.1	5.4 ± 0.2
4 Hour	7.5 ± 0.1	5.9 ± 0.1	8.4 ± 0.1	5.7 ± 0.1
6 Hour	7.8 ± 0.1	5.8 ± 0.1	8.5 ± 0.2	5.8 ± 0.2
12 Hour	7.1 ± 0.1	5.8 ± 0.0	7.3 ± 0.1	5.1 ± 0.0

Table 4.3: Glutathione peroxidase [GPX] activity in tissues of mice envenomated with S. argus venom. Values are expressed as µg of glutathione /min/mg protein. Results are mean± SD of six separate determinations.

Time	Control		0.98 mg/Kg	
	Liver	Kidney	Liver	Kidney
2 Hour	45.9 ± 4.4	59.1 ± 2.1	55.3 ± 3.8	66.1 ± 2.6
4 Hour	47.4 ± 5.9	59.5 ± 2.1	61.1 ± 3.8	70.1 ± 2.5
6 Hour	48.3 ± 2.9	59.4 ± 1.7	58.0 ± 2.7	69.3 ± 1.7
12 Hour	49.8 ± 4.0	59.2 ± 2.4	50.7 ± 2.8	64.7 ± 3.1
Time	1.47 mg/Kg		1.97 mg/Kg	
	Liver	Kidney	Liver	Kidney
2 Hour	57.2 ± 4.6	68.8 ± 4.5	67.5 ± 4.0	72.3 ± 4.0
4 Hour	63.3 ± 3.0	70.5 ± 2.8	74.1 ± 2.9	74.6 ± 3.7
6 Hour	63.9 ± 3.5	71.2 ± 3.2	74.9 ± 3.2	74.0 ± 3.1
12 Hour	55.5 ± 2.4	70.2 ± 3.0	53.5 ± 4.6	72.2 ± 2.5

Table 4.4. Glutathione-S-transferase activity in tissues of mice envenomated with S. argus venom. Values are expressed as n moles of CDNB formed /min/mg protein. Results are mean± SD of six separate determinations.

Time	Control		0.98 mg/Kg	
	Liver	Kidney	Liver	Kidney
2 Hour	35.8 ± 2.5	22.5 ± 1.6	36.4 ± 2.2	26.2 ± 1.8
4 Hour	35.7 ± 2.4	22.6 ± 1.3	36.7 ± 2.3	27.7 ± 2.5
6 Hour	35.5 ± 1.4	22.0 ± 1.5	36.7 ± 2.2	28.4 ± 2.7
12 Hour	35.3 ± 1.7	22.4 ± 1.4	36.4 ± 2.3	28.3 ± 2.8
	1.47 mg/Kg		1.97 mg/Kg	
Time	Liver	Kidney	Liver	Kidney
2 Hour	37.3 ± 2.6	25.4 ± 1.4	40.1 ± 2.9	29.2 ± 2.3
4 Hour	38.2 ± 2.5	29.7 ± 2.8	41.4 ± 1.8	35.8 ± 2.5
6 Hour	38.4 ± 2.3	30.3 ± 2.4	41.8 ± 3.8	35.6 ± 3.2
12 Hour	38.2 ± 2.1	30.2 ± 2.6	34.6 ± 3.9	29.5 ± 1.4

Table 4.5.: Glutathione reductase activity in tissues of mice envenomated with S. argus venom. Values are expressed as m moles of NADPH /min/mg protein. Results are mean± SD of six separate determinations.

Time	Control		0.98 mg/Kg	
	Liver	Kidney	Liver	Kidney
2 Hour	3.2 ± 0.2	2.7 ± 0.2	3.6 ± 0.1	3.0 ± 0.2
4 Hour	3.3 ± 0.1	2.8 ± 0.2	3.9 ± 0.1	3.2 ± 0.1
6 Hour	3.3 ± 0.2	2.8 ± 0.2	3.7 ± 0.2	3.2 ± 0.1
12 Hour	3.2 ± 0.1	2.7 ± 0.2	3.4 ± 0.2	2.6 ± 0.1
	1.47 mg/Kg		1.97 mg/Kg	
Time	Liver	Kidney	Liver	Kidney
2 Hour	3.8 ± 0.1	3.1 ± 0.2	4.0 ± 0.2	3.2 ± 0.2
4 Hour	4.0 ± 0.2	3.3 ± 0.1	4.2 ± 0.2	3.5 ± 0.2
6 Hour	3.9 ± 0.1	3.2 ± 0.1	4.1 ± 0.2	3.5 ± 0.2
12 Hour	3.4 ± 0.4	3.0 ± 0.1	3.3 ± 0.3	2.8 ± 0.1

Table 4.6.: Glutathione levels in tissues of mice envenomated with S. argus venom. Values are expressed as mg /g tissue. Results are mean± SD of six separate determinations.

Time	Control		0.98 mg/ml	
	Liver	Kidney	Liver	Kidney
2 Hour	102.9 ± 8.2	78.5 ± 5.5	121.8 ± 11.5	77.6 ± 6.2
4 Hour	104.9 ± 10.7	77.9 ± 6.6	131.8 ± 10.8	79.4 ± 6.7
6 Hour	101.1 ± 7.8	79.4 ± 4.9	130.7 ± 9.7	77.6 ± 5.9
12 Hour	100.4 ± 6.9	77.6 ± 4.5	109.8 ± 9.2	79.2 ± 4.3
Time	1.47 mg/ml			
	Liver	Kidney	Liver	Kidney
2 Hour	132.2 ± 10.6	79.8 ± 5.2	141.0 ± 6.5	77.8 ± 3.5
4 Hour	135.3 ± 5.3	80.7 ± 3.6	144.2 ± 12.3	80.9 ± 3.7
6 Hour	134.0 ± 6.7	78.2 ± 5.2	140.6 ± 10.4	77.1 ± 6.0
12 Hour	103.3 ± 8.9	78.1 ± 2.8	101.5 ± 6.1	78.4 ± 3.3

Table 4.7.: Ascorbate [Vitamin C] levels in tissues of mice envenomated with S. argus venom. Values are expressed as mg /100g tissue. Results are mean± SD of six separate determinations.

Time	Control		0.98 mg/Kg	
	Liver	Kidney	Liver	Kidney
2 Hour	0.7 ± 0.1	0.4 ± 0.1	0.6 ± 0.4	0.4 ± 0.0
4 Hour	0.8 ± 0.0	0.4 ± 0.1	0.8 ± 0.1	0.3 ± 0.1
6 Hour	0.6 ± 0.1	0.4 ± 0.1	0.9 ± 0.1	0.2 ± 0.1
12 Hour	0.6 ± 0.1	0.4 ± 0.1	0.7 ± 0.1	0.3 ± 0.1
Time	1.47 mg/Kg		1.97mg/Kg	
	Liver	Kidney	Liver	Kidney
2 Hour	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0.4 ± 0.2
4 Hour	0.7 ± 0.3	0.3 ± 0.0	0.5 ± 0.4	0.2 ± 0.2
6 Hour	0.8 ± 0.3	0.2 ± 0.1	0.3 ± 0.4	0.2 ± 0.1
12 Hour	0.7 ± 0.1	0.3 ± 0.1	0.6 ± 0.1	0.5 ± 0.1

Table 4.8.: Malondialdehyde [MDA] levels in tissues of mice envenomated with S. argus venom. Values are expressed as n moles /g protein. Results are mean± SD of six separate determinations.

Time	Control		0.98 mg/Kg	
	Liver	Kidney	Liver	Kidney
2 Hour	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
4 Hour	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
6 Hour	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
12 Hour	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
Time	1.47 mg/Kg		1.97 mg/Kg	
	Liver	Kidney	Liver	Kidney
2 Hour	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
4 Hour	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
6 Hour	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
12 Hour	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0

Table 4.9.: Conjugated dienes [CD] levels in tissues of mice envenomated with S. argus venom. Values are expressed as m moles /100g protein. Results are mean± SD of six separate determinations.

Time	Control		0.98 mg/Kg	
	Liver	Kidney	Liver	Kidney
2 Hour	6.3 ± 0.2	2.5 ± 0.3	5.1 ± 0.3	2.3 ± 0.2
4 Hour	6.5 ± 0.2	2.5 ± 0.3	5.0 ± 0.2	2.4 ± 0.1
6 Hour	6.1 ± 0.2	2.4 ± 0.2	4.4 ± 0.3	2.3 ± 0.2
12 Hour	6.3 ± 0.2	2.3 ± 0.1	5.1 ± 0.2	2.2 ± 0.1
Time	1.47 mg/Kg			
	Liver	Kidney	Liver	Kidney
2 Hour	4.8 ± 0.3	2.3 ± 0.2	4.7 ± 0.3	2.0 ± 0.2
4 Hour	4.3 ± 0.2	2.3 ± 0.1	4.4 ± 0.3	1.9 ± 0.4
6 Hour	4.3 ± 0.1	2.1 ± 0.1	4.2 ± 0.1	1.9 ± 0.2
12 Hour	4.3 ± 0.3	2.1 ± 0.1	3.7 ± 0.5	2.2 ± 0.4

Table 4.10.: Hydroperoxide levels in tissues of mice envenomated with S. argus venom. Values are expressed as m moles /100g protein. Results are mean± SD of six separate determinations.

Time	CONTROL			0.98 mg/Kg		
	SERUM	LIVER	KIDNEY	SERUM	LIVER	KIDNEY
2 Hour	6.4 ± 0.5	1.2 ± 0.2	0.6 ± 0.1	8.4 ± 0.5	1.2 ± 0.1	0.7 ± 0.0
4 Hour	6.5 ± 0.3	1.5 ± 0.4	0.6 ± 0.0	10.7 ± 0.7	1.4 ± 0.4	0.7 ± 0.0
6 Hour	6.3 ± 0.7	1.2 ± 0.1	0.5 ± 0.1	10.6 ± 0.6	1.5 ± 0.2	0.7 ± 0.0
12 Hour	6.4 ± 0.5	1.2 ± 0.1	0.5 ± 0.1	9.7 ± 1.2	1.4 ± 0.1	0.8 ± 0.1
Time	1.47mg/Kg			1.97 mg/Kg		
	SERUM	LIVER	KIDNEY	SERUM	LIVER	KIDNEY
2 Hour	9.3 ± 0.6	1.4 ± 0.2	0.8 ± 0.0	9.8 ± 0.8	1.4 ± 0.2	0.9 ± 0.0
4 Hour	11.1 ± 0.7	1.4 ± 0.1	0.9 ± 0.0	12.2 ± 0.5	1.5 ± 0.2	0.9 ± 0.0
6 Hour	11.2 ± 0.6	1.3 ± 0.2	0.9 ± 0.0	12.0 ± 0.6	1.7 ± 0.0	0.9 ± 0.0
12 Hour	10.5 ± 0.5	1.5 ± 0.3	0.8 ± 0.1	10.9 ± 0.5	1.7 ± 0.0	0.9 ± 0.1

Table 4.11.: Acid phosphatase activity in tissues [liver and kidney] and serum of mice envenomated with S. argus venom. Values are expressed as μmolPNP/min/mg protein for tissue and serum. Results are mean± SD of six separate determinations.

Time	CONTROL			0.98 mg/Kg		
	SERUM	LIVER	KIDNEY	SERUM	LIVER	KIDNEY
2 Hour	14.2 ± 1.9	0.9 ± 0.0	0.5 ± 0.0	17.3 ± 1.8	1.4 ± 0.1	0.5 ± 0.0
4 Hour	14.7 ± 0.6	0.9 ± 0.1	0.4 ± 0.0	21.5 ± 0.4	1.6 ± 0.2	0.6 ± 0.1
6 Hour	13.9 ± 0.2	0.9 ± 0.1	0.5 ± 0.0	20.5 ± 1.0	1.6 ± 0.2	0.6 ± 0.0
12 Hour	14.1 ± 0.5	0.9 ± 0.0	0.5 ± 0.1	21.3 ± 0.4	1.7 ± 0.0	0.6 ± 0.1
Time	1.47 mg/Kg			1.97 mg/Kg		
	SERUM	LIVER	KIDNEY	SERUM	LIVER	KIDNEY
2 Hour	19.7 ± 1.9	1.8 ± 0.1	0.6 ± 0.1	22.3 ± 1.4	1.9 ± 0.1	0.6 ± 0.0
4 Hour	24.3 ± 0.4	1.7 ± 0.2	0.7 ± 0.1	28.7 ± 0.3	1.9 ± 0.2	0.7 ± 0.0
6 Hour	28.5 ± 0.6	1.8 ± 0.2	0.7 ± 0.1	26.0 ± 0.8	1.9 ± 0.2	0.7 ± 0.0
12 Hour	25.6 ± 0.6	1.5 ± 0.0	0.6 ± 0.0	27.2 ± 0.3	1.7 ± 0.0	0.6 ± 0.1

Table 4.12.: Alkaline phosphatase activity in tissues [liver and kidney] and serum of mice envenomated with S. argus venom. Values are expressed as μmolPNP/min/mg protein for tissue and serum. Results are mean± SD of six separate determinations.

Time	CONTROL			0.98 mg/Kg		
	SERUM	LIVER	KIDNEY	SERUM	LIVER	KIDNEY
2 Hour	73.2 ± 2.5	8.7 ± 0.3	25.8 ± 4.8	82.0 ± 2.2	10.5 ± 0.9	28.1 ± 3.6
4 Hour	72.0 ± 2.7	8.9 ± 0.2	25.8 ± 3.2	81.2 ± 3.5	10.5 ± 1.6	37.7 ± 4.5
6 Hour	80.8 ± 4.4	9.0 ± 0.3	26.9 ± 1.8	84.5 ± 3.3	10.2 ± 1.0	35.7 ± 5.0
12 Hour	81.0 ± 3.5	9.0 ± 0.4	25.6 ± 1.9	83.8 ± 3.9	9.2 ± 0.3	27.3 ± 1.3
Time	1.47mg/Kg			1.97 mg/Kg		
	SERUM	LIVER	KIDNEY	SERUM	LIVER	KIDNEY
2 Hour	88.4 ± 2.0	11.0 ± 1.7	29.9 ± 3.3	94.7 ± 1.3	14.8 ± 1.4	30.2 ± 4.2
4 Hour	83.9 ± 4.2	11.1 ± 1.2	35.9 ± 4.4	87.1 ± 3.3	14.1 ± 2.0	40.2 ± 5.9
6 Hour	84.2 ± 2.5	11.8 ± 0.7	37.2 ± 5.3	94.0 ± 3.2	14.7 ± 0.9	39.1 ± 4.8
12 Hour	83.4 ± 3.4	9.8 ± 0.3	27.9 ± 1.4	85.4 ± 2.4	9.6 ± 0.4	28.4 ± 1.8

Table 4.13.: Alanine transaminase activity in tissues [Liver and Kidney] and serum of mice envenomated with S. argus venom. Values are expressed as units / min/ mg protein for tissue and serum. Results are mean± SD of six separate determinations.

Time	CONTROL			0.98 mg/Kg		
	SERUM	LIVER	KIDNEY	SERUM	LIVER	KIDNEY
2 Hour	30.8 ± 5.0	90.2 ± 3.0	9.8 ± 0.5	32.7 ± 4.8	98.2 ± 5.5	13.3 ± 0.4
4 Hour	31.2 ± 5.0	89.6 ± 0.9	9.0 ± 0.3	48.8 ± 8.4	95.3 ± 3.6	15.0 ± 0.7
6 Hour	30.4 ± 4.5	88.9 ± 0.9	9.2 ± 0.3	43.2 ± 4.9	95.4 ± 3.6	14.9 ± 0.3
12 Hour	31.4 ± 3.9	88.2 ± 1.6	9.1 ± 0.4	41.2 ± 1.9	94.7 ± 3.7	8.8 ± 0.4
Time	1.47mg/Kg			1.97 mg/Kg		
	SERUM	LIVER	KIDNEY	SERUM	LIVER	KIDNEY
2 Hour	33.3 ± 4.6	96.7 ± 2.5	16.5 ± 0.6	34.4 ± 5.0	98.5 ± 2.5	14.9 ± 0.7
4 Hour	55.1 ± 9.2	95.2 ± 3.7	16.7 ± 0.4	57.9 ± 7.9	95.4 ± 3.4	17.0 ± 0.4
6 Hour	52.1 ± 8.5	92.1 ± 7.0	15.7 ± 0.4	50.6 ± 5.5	93.6 ± 2.9	16.4 ± 0.4
12 Hour	45.0 ± 3.1	95.3 ± 3.7	9.2 ± 0.5	47.4 ± 2.2	91.0 ± 2.0	9.1 ± 0.5

Table 4.14.: Aspartate transaminase activity in tissues [Liver and Kidney] and serum of mice envenomated with S. argus venom. Values are expressed as units / min/ mg protein for tissue and serum. Results are mean ± SD of six separate determinations.

Table 4.15. Table showing the Univariate analysis and the effect of different concentration of S. argus venom on the tisuue (liver and kidney SOD activity).

Source	Measure		Type III Sum of Squares	df	Mean Square	F	Sig.
TIME	LIVER	**Sphericity Assumed**	1.691	3	0.564	6.210	0.001
	KIDNEY	**Sphericity Assumed**	1.334	3	0.445	6.261	0.001
TIME * CON	LIVER	**Sphericity Assumed**	5.652	9	0.628	6.918	0.000
	KIDNEY	**Sphericity Assumed**	0.745	9	0.083	1.166	0.333
Error(TIME)	LIVER	**Sphericity Assumed**	5.446	60	0.091		
	KIDNEY	**Sphericity Assumed**	4.261	60	0.071		

Source	Measure	Type III Sum of Squares	df	Mean Square	F	Sig.
CON	**LIVER**	81.736	3	27.245	426.164	0.00
	KIDNEY	92.733	3	30.911	531.392	0.00
Error	**LIVER**	1.279	20	0.064		
	KIDNEY	1.163	20	0.058		

Table 4.16. Table showing the Univariate analysis and the effect of different concentration of S. argus venom on the tisuue (liver and kidney CAT activity).

Source	Measure		Type III Sum of Squares	df	Mean Square	F	Sig.
TIME	LIVER	Sphericity Assumed	1369.741	3	456.58	26.324	0.000
	KIDNEY	Greenhouse-Geisser	725.471	1.658	437.433	14.432	0.000
TIME * CON	LIVER	Sphericity Assumed	397.658	9	44.184	2.547	0.015
	KIDNEY	Greenhouse-Geisser	604.999	4.975	121.598	4.012	0.006
Error(TIME)	LIVER	Sphericity Assumed	1040.666	60	17.344		
	KIDNEY	Greenhouse-Geisser	1005.352	33.169	30.31		

Source	Measure	Type III Sum of Squares	df	Mean Square	F	Sig.
CONCN	LIVER	1210.303	3	403.434	15.603	0.000
	KIDNEY	1049.542	3	349.847	5.790	0.005
Error	LIVER	517.118	20	25.856		
	KIDNEY	1208.533	20	60.427		

Table 4.17. Table showing the Univariate analysis and the effect of different concentration of S. argus venom on the tisuue (liver and kidney GPx activity).

Source	Measure		Type III Sum of Squares	df	Mean Square	F	Sig.
TIME	LIVER	Sphericity Assumed	21.384	3	7.128	426.982	0.000
	KIDNEY	Greenhouse-Geisser	20.875	1.931	10.808	313.948	0.000
TIME * CONCN	LIVER	Sphericity Assumed	11.273	9	1.253	75.032	0.000
	KIDNEY	Greenhouse-Geisser	8.27	5.794	1.427	41.459	0.000
Error (TIME)	LIVER	Sphericity Assumed	1.002	60	0.017		
	KIDNEY	Greenhouse-Geisser	1.33	38.627	0.034		

Source	Measure	Type III Sum of Squares	df	Mean Square	F	Sig.
CONCN	LIVER	1210.303	3	6.767	341.007	0.000
	KIDNEY	1049.542	3	7.200	197.816	0.000
Error	LIVER	517.118	20	0.020		
	KIDNEY	1208.533	20	0.036		

Table 4.18. Table showing the Univariate analysis and the effect of different concentration of S. argus venom on the tisuue (liver and kidney GST activity).

Source	Measure		Type III Sum of Squares	df	Mean Square	F	Sig.
TIME	LIVER	Sphericity Assumed	1358.512	3	452.837	35.070	0.000
	KIDNEY	Greenhouse-Geisser	94.822	1.683	56.335	4.583	0.022
TIME * CONCN	LIVER	Sphericity Assumed	1135.168	9	126.130	9.768	0.000
	KIDNEY	Greenhouse-Geisser	67.087	5.049	13.286	1.081	0.389
Error(TIME)	LIVER	Sphericity Assumed	774.735	60	12.912		
	KIDNEY	Greenhouse-Geisser	413.787	33.663	12.292		

Source	Measure	Type III Sum of Squares	df	Mean Square	F	Sig.
CONCN	LIVER	4814.354	3	1604.785	90.610	0.000
	KIDNEY	2573.978	3	857.993	65.470	0.000
Error	LIVER	354.218	20	17.711		
	KIDNEY	262.104	20	13.105		

Table 4.19. Table showing the Univariate analysis and the effect of different concentration of S. argus venom on the tisuue (liver and kidney GR activity).

Source	Measure		Type III Sum of Squares	df	Mean Square	F	Sig.
TIME	LIVER	Greenhouse-Geisser	60.194	1.25	48.156	5.140	0.026
	KIDNEY	Sphericity Assumed	162.475	3	54.158	20.952	0.000
TIME * CONCN	LIVER	Greenhouse-Geisser	146.950	3.75	39.187	4.182	0.011
	KIDNEY	Sphericity Assumed	198.147	9	22.016	8.517	0.000
Error(TIME)	LIVER	Greenhouse-Geisser	234.235	25	9.369		
	KIDNEY	Sphericity Assumed	155.091	60	2.585		

Source	Measure	Type III Sum of Squares	df	Mean Square	F	Sig.
CONCN	LIVER	213.143	3	71.048	5.244	0.008
	KIDNEY	1275.381	3	425.127	35.288	0.000
Error	LIVER	270.946	20	13.547		
	KIDNEY	240.948	20	12.047		

Table 4.20. Table showing the Univariate analysis and the effect of different concentration of S. argus venom on the tisuue (liver and kidney GSH level).

Source	Measure		Type III Sum of Squares	df	Mean Square	F	Sig.
TIME	LIVER	Greenhouse-Geisser	3.579	2.002	1.788	32.885	0.000
	KIDNEY	Greenhouse-Geisser	2.624	2.150	1.220	54.045	0.000
TIME * CON	LIVER	Greenhouse-Geisser	1.400	6.007	0.233	4.288	0.002
	KIDNEY	Greenhouse-Geisser	1.030	6.450	0.160	7.070	0.000
Error (TIME)	LIVER	Greenhouse-Geisser	2.177	40.045	0.054		
	KIDNEY	Greenhouse-Geisser	0.971	43.002	0.023		

Source	Measure	Type III Sum of Squares	df	Mean Square	F	Sig.
CONCN	LIVER	5.530	3	1.843	39.221	0.000
	KIDNEY	3.649	3	1.216	22.124	0.000
Error	LIVER	0.940	20	0.047		
	KIDNEY	1.100	20	0.055		

Table 4.21. Table showing the Univariate analysis and the effect of different concentration of S. argus venom on the tisuue (liver and kidney Ascorbate content).

Source	Measure		Type III Sum of Squares	df	Mean Square	F	Sig.
TIME	LIVER	Sphericity Assumed	10217.233	3	3405.744	43.221	0.000
	KIDNEY	Sphericity Assumed	39.588	3	13.196	0.528	0.664
TIME * CON	LIVER	Sphericity Assumed	3846.137	9	427.349	5.423	0.000
	KIDNEY	Sphericity Assumed	66.347	9	7.372	0.295	0.973
Error (TIME)	LIVER	Sphericity Assumed	4727.871	60	78.798		
	KIDNEY	Sphericity Assumed	1498.261	60	24.971		

Source	Measure	Type III Sum of Squares	df	Mean Square	F	Sig.
CON	LIVER	11844.743	3	3948.248	42.467	0.000
	KIDNEY	9.861	3	3.287	0.131	0.940
Error	LIVER	1859.436	20	92.972		
	KIDNEY	500.506	20	25.025		

Table 4.22. Table showing the Univariate analysis and the effect of different concentration of S. argus venom on the tisuue (liver and kidney MDA content).

Source	Measure		Type III Sum of Squares	df	Mean Square	F	Sig.
TIME	LIVER	Greenhouse-Geisser	0.938	1.908	0.492	5.863	0.007
	KIDNEY	Sphericity Assumed	0.996	3	0.332	45.902	0.000
TIME * CON	LIVER	Greenhouse-Geisser	0.654	5.723	0.114	1.363	0.256
	KIDNEY	Sphericity Assumed	3.093	9	0.344	47.532	0.000
Error (TIME)	LIVER	Greenhouse-Geisser	3.201	38.151	0.084		
	KIDNEY	Sphericity Assumed	0.434	60	0.007		

Source	Measure	Type III Sum of Squares	df	Mean Square	F	Sig.
CONCN	LIVER	1.814	3	0.605	10.644	0.000
	KIDNEY	1.122	3	0.374	47.529	0.000
Error	LIVER	1.136	20	0.057		
	KIDNEY	0.157	20	0.008		

Table 4.23. Table showing the Univariate analysis and the effect of different concentration of S. argus venom on the tisuue (liver and kidney Conjugated diene content).

Source	Measure		Type III Sum of Squares	df	Mean Square	F	Sig.
TIME	LIVER	Greenhouse-Geisser	0.003	1.729	0.002	0.753	0.460
	KIDNEY	Sphericity Assumed	0.005	3	0.002	1.838	0.150
TIME * CON	LIVER	Greenhouse-Geisser	0.011	5.186	0.002	0.780	0.575
	KIDNEY	Sphericity Assumed	0.013	9	0.001	1.591	0.138
Error (TIME)	LIVER	Greenhouse-Geisser	0.092	34.573	0.003		
	KIDNEY	Sphericity Assumed	0.053	60	0.001		

Source	Measure	Type III Sum of Squares	df	Mean Square	F	Sig.
CON	LIVER	0.062	3	0.021	7.897	0.001
	KIDNEY	0.020	3	0.007	5.986	0.004
Error	LIVER	0.052	20	0.003		
	KIDNEY	0.022	20	0.001		

Table 4.24. Table showing the Univariate analysis and the effect of different concentration of S. argus venom on the tisuue (liver and kidney Hydroperoxide level).

Source	Measure		Type III Sum of Squares	df	Mean Square	F	Sig.
TIME	LIVER	Sphericity Assumed	3.293	3	1.098	19.783	0.000
	KIDNEY	Sphericity Assumed	0.205	3	0.068	1.540	0.213
TIME * CON	LIVER	Sphericity Assumed	3.450	9	0.383	6.910	0.000
	KIDNEY	Sphericity Assumed	0.727	9	0.081	1.817	0.084
Error(TIME)	LIVER	Sphericity Assumed	3.329	60	0.055		
	KIDNEY	Sphericity Assumed	2.665	60	0.044		

Source	Measure	Type III Sum of Squares	df	Mean Square	F	Sig.
CON	LIVER	59.385	3	19.795	257.575	0.000
	KIDNEY	2.205	3	0.735	13.323	0.000
Error	LIVER	1.537	20	0.077		
	KIDNEY	1.103	20	0.055		

Table 4.25. Table showing the Univariate analysis and the effect of different concentration of S. argus venom on the tisuue (liver and kidney) and serum acid phosphatase activity.

Source	Measure		Type III Sum of Squares	df	Mean Square	F	Sig.
TIME	SERUM	Sphericity Assumed	42.149	3	14.050	35.759	0.000
	LIVER	Sphericity Assumed	0.425	3	0.142	3.010	0.037
	KIDNEY	Greenhouse-Geisser	0.010	2.039	0.005	1.154	0.326
TIME * CON	SERUM	Sphericity Assumed	16.539	9	1.838	4.677	0.000
	LIVER	Sphericity Assumed	0.984	9	0.109	2.322	0.026
	KIDNEY	Greenhouse-Geisser	0.055	6.117	0.009	2.207	0.061
Error (TIME)	SERUM	Sphericity Assumed	23.574	60	0.393		
	LIVER	Sphericity Assumed	2.824	60	0.047		
	KIDNEY	Greenhouse-Geisser	0.166	40.777	0.004		

Source	Measure	Type III Sum of Squares	df	Mean Square	F	Sig.
CON	SERUM	332.542	3	110.847	216.223	0.000
	LIVER	1.292	3	0.431	12.344	0.000
	KIDNEY	1.760	3	0.587	212.714	0.000
Error	SERUM	10.253	20	0.513		
	LIVER	0.698	20	0.035		
	KIDNEY	0.055	20	0.003		

Table 4.26. Table showing the Univariate analysis and the effect of different concentration of S. argus venom on the tisuue (liver and kidney) and serum alkaline phosphatase activity.

Source	Measure		Type III Sum of Squares	df	Mean Square	F	Sig.
TIME	SERUM	Greenhouse-Geisser	980.657	1.44	679.514	384.993	0.000
	LIVER	Greenhouse-Geisser	8.053	1.96	4.114	169.675	0.000
	KIDNEY	Greenhouse-Geisser	0.583	2.35	0.248	83.382	0.000
TIME * CON	SERUM	Greenhouse-Geisser	535.653	4.33	123.721	70.097	0.000
	LIVER	Greenhouse-Geisser	3.187	5.87	0.543	22.381	0.000
	KIDNEY	Greenhouse-Geisser	0.251	7.06	0.036	11.979	0.000
Error (TIME)	SERUM	Greenhouse-Geisser	50.944	28.86	1.765		
	LIVER	Greenhouse-Geisser	0.949	39.15	0.024		
	KIDNEY	Greenhouse-Geisser	0.140	47.03	0.003		

Source	Measure	Type III Sum of Squares	df	Mean Square	F	Sig.
CON	SERUM	1073.178	3	357.726	249.700	0.000
	LIVER	8.021	3	2.674	107.954	0.000
	KIDNEY	0.393	3	0.131	51.672	0.000
Error	SERUM	28.652	20	1.433		
	LIVER	0.495	20	0.025		
	KIDNEY	0.051	20	0.003		

Table 4.27. Table showing the Univariate analysis and the effect of different concentration of S. argus venom on the tisuue (liver and kidney) and serum Alanine transaminase activity.

Source	Measure		Type III Sum of Squares	df	Mean Square	F	Sig.
TIME	SERUM	Sphericity Assumed	302.083	3	100.694	10.650	0.000
	LIVER	Greenhouse-Geisser	64.117	2.27	28.227	22.634	0.000
	KIDNEY	Greenhouse-Geisser	1173.541	1.94	606.171	33.777	0.000
TIME * CON	SERUM	Sphericity Assumed	657.216	9	73.024	7.723	0.000
	LIVER	Greenhouse-Geisser	67.856	6.81	9.958	7.985	0.000
	KIDNEY	Greenhouse-Geisser	362.503	5.81	62.415	3.478	0.008
Error (TIME)	SERUM	Sphericity Assumed	567.316	60	9.455		
	LIVER	Greenhouse-Geisser	56.656	45.43	1.247		
	KIDNEY	Greenhouse-Geisser	694.876	38.72	17.946		

Source	Measure	Type III Sum of Squares	df	Mean Square	F	Sig.
CON	SERUM	2262.776	3	754.259	70.250	0.000
	LIVER	248.570	3	82.857	61.092	0.000
	KIDNEY	982.579	3	327.526	13.217	0.000
Error	SERUM	214.734	20	10.737		
	LIVER	27.125	20	1.356		
	KIDNEY	495.614	20	24.781		

Table 4.28. Table showing the Univariate analysis and the effect of different concentration of S. argus venom on the tisuue (liver and kidney) and serum Aspartate transaminase activity.

Source	Measure		Type III Sum of Squares	df	Mean Square	F	Sig.
TIME	SERUM	Greenhouse-Geisser	3071.060	2.01	1524.913	37.608	0.000
	LIVER	Sphericity Assumed	380.588	3	126.863	10.111	0.000
	KIDNEY	Sphericity Assumed	458.851	3	152.950	949.905	0.000
TIME * CON	SERUM	Greenhouse-Geisser	1168.031	6.04	193.326	4.768	0.001
	LIVER	Sphericity Assumed	207.372	9	23.041	1.836	0.080
	KIDNEY	Sphericity Assumed	161.569	9	17.952	111.492	0.000
Error (TIME)	SERUM	Greenhouse-Geisser	1633.204	40.28	40.548		
	LIVER	Sphericity Assumed	752.814	60	12.547		
	KIDNEY	Sphericity Assumed	9.661	60	0.161		

Source	Measure	Type III Sum of Squares	df	Mean Square	F	Sig.
CON	SERUM	4129.525	3	1376.508	29.302	0.000
	LIVER	820.726	3	273.575	23.682	0.000
	KIDNEY	426.769	3	142.256	346.273	0.000
or	SERUM	939.527	20	46.976		
	LIVER	231.039	20	11.552		
	KIDNEY	8.216	20	0.411