Studies on Anticoagulant Potential of Moon Jellyfish (*Aurelia aurita*) and Barrel Jellyfish (*Rhizostoma pulmo*) Tentacle Extracts

THESIS

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by

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CERTIFICATE

This is to certify that the thesis entitled "**Studies on Anticoagulant Potential of Moon Jellyfish (*Aurelia aurita*) and Barrel Jellyfish (*Rhizostoma pulmo*) Tentacle Extracts**" submitted by **Akriti Rastogi**, ID No **2009PH290004G** for award of PhD degree of the Institute embodies original work done by her under our supervision.

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"I can no other answer make, but thanks and thanks and ever thanks…"

-William Shakespeare

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Akriti Rastogi

"Double, double toil and trouble;

Fire burn, and cauldron bubble.

Fillet of a fenny snake,

In the cauldron boil and bake;

Eye of newt and toe of frog,

Wool of bat and tongue of dog,

Adder's fork and blind-worm's sting,

Lizard's leg and owlet's wing,

For a charm of powerful trouble,

Like a hell-broth boil and bubble.

Double, double toil and trouble;

Fire burn and cauldron bubble."

-Act 4, Scene 1, Macbeth - William Shakespeare

Shakespeare's recipe for the witches in Macbeth is fiction, but animals and plants are the source and inspiration for the most potent poisons and medicines. The complexity of these chemicals is matched only by the complexity of behavior, anatomy, and biology that organisms have evolved to employ these natural weapons.

ABSTRACT

Natural products have long been explored as invaluable sources of inspiration for drug design. Over the last two decades, protein bio-therapeutics have gained popularity and are fast becoming the major part of the pharmaceutical market. The high specificity binding to target receptors/ion channels, high affinity, consequent low toxicity and side effects of protein drugs are extremely attractive as lead molecules in drug development.

Along with the toxins of snakes, scorpions, spiders and cone snails, many cnidarian toxins have become valuable research tools. Cnidarians may negatively impact human activities and public health but concomitantly their venom represents a rich source of bioactive substances. Cnidaria is a large, diverse and ecologically important group of marine invertebrates that includes over 11,000 extant species. In the last decade, over 3000 marine natural products have been described from this phylum alone.

Thrombosis and related cardiovascular disorders have been and continue to be a major health problem leading to mortality. Anticoagulants are pivotal for the prevention and treatment of these disorders. Coumarins and heparin are the most well known clinically used anticoagulants. However, the nonspecific mode of action of these anticoagulants accounts for their therapeutic limitations in maintaining a balance between thrombosis and hemostasis. These limitations have provided the impetus for the development of new anticoagulants that target specific coagulation enzymes or a particular step in the clotting process. This has further encouraged the scientific community to venture into new resources like the marine animals.

This study was aimed at the assessment of anticoagulant and platelet aggregation inhibiting activities in the tentacle extracts of two commonly found jellyfish species on the Goan coast, i.e., *Aurelia aurita* (Moon jellyfish) and *Rhizostoma pulmo* (Barrel jellyfish). Both the tentacle extracts were capable of inhibiting the blood coagulation cascade at three different levels: 1) Platelet aggregation 2) Fibrinogen digestion 3) Fibrin degradation

Moon jellyfish tentacle extract (JFTE) could digest fibrinogen and fibrin clots *in vitro*. It could also inhibit ADP and collagen dependent platelet aggregation in a dose dependent manner. JFTE showed mild hemolytic activity and no phospholipase activity.

Barrel jellyfish tentacle extract (BJFTE) could also digest fibrinogen in dose and time dependent manner. It could digest clots, made with fibrinogen but not the ones made with essentially plasminogen free fibrinogen. Fibrinogenolytic activity as well as fibrinolytic activity of BJFTE was significantly reduced on treatment with EDTA and on exposure to heat. BJFTE showed dose and time dependent hemolytic activity against human RBCs. The RBCs on exposure to BJFTE initially showed swelling of the cells followed by gradual crenation. It lacked phospholipase activity and could also inhibit ADP dependent platelet aggregation in a dose dependent manner. BJFTE exhibited strong proteolytic activity on fibrinogen, casein, gelatin and azocaesin, which was significantly lost on treatment with EDTA.

Owing to the highly unstable and aggregation prone nature of the jellyfish proteins, various attempts were made using combinations of purification techniques to purify active protein/peptides from BJFTE. As a result of a combination of ammonium sulphate precipitation and SE-HPLC, a semi-pure anticoagulant fraction was obtained, which showed strong fibrinogenolytic activity but no fibrinolytic activity. A 95 kDa metalloproteinase was identified in this fraction and was named Rhizoprotease. Atomic absorption spectroscopy studies suggested the presence of Zn^{+2} in BJFTE as well as in the semi-pure fraction containing Rhizoprotease. Protein mass fingerprinting analysis revealed that Rhizoprotease is a novel protein and was found to have similarity with invertebrates like *Dinoponera quadriceps* (giant ant), *Taenia saginata* (beef tapeworm), *Helobdella robusta* (freshwater jawless leech), *Lepeoptheirus salmonis* (hookworm), *Ancyclostoma ceylanicum* (sea louse), *Aedes albopictus* (mosquito). All these invertebrates are known to have anticoagulant and antiplatelet activities.

BJFTE was found to be mildly toxic to lung adenocarcinoma cells (A549). However, it could inhibit wound healing in A549 cells in a dose dependent manner. BJFTE could also completely digest laminin, a major basement

membrane component, involved in cell adhesion.

This work reveals, for the first time, the anticoagulant potential of jellyfish tentacle extracts. It also details a novel anticoagulant metalloproteinases, Rhizoprotease from *Rhizostoma pulmo*. The work presented here increases the bio-discovery potential of these jellyfish species and provides direction for future research to consider them as a source for designing anticoagulant/antiplatelet/thrombolytic drugs.

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LIST OF SYMBOLS AND ABBREVIATIONS

ACD: Acid citrate dextrose

ACE: Angiotensin-converting enzyme

ADP: Adenosine diphosphate

AMP: Antimicrobial peptide

BJFTE: Barrel jellyfish tentacle extract

BSA: Bovine serum albumin

C: Centigrade

CM: Carboxy methyl

cm: centimeters

Da: Daltons

DEAE: Diethyl amino ethyl

DMEM: Dulbecco’s modified eagle medium

DMSO: Dimethyl sufloxide

DNA: Deoxyribonucleic acid

DTT: Dithiothreitol

EDTA: Ethylenediaminetetraacetatic acid

GPa: Gigapascal

HMWK: High molecular weight kininogen

FBS: Fetal bovine serum

FDA: Food and drug administration

h: Hour(s)

HPLC: High performance liquid chromatography

IEC: Ion exchange chromatography

JFTE: Jellyfish tentacle extract

kDa: Kilo daltons

LDH: Lactate dehydrogenase

M: Molar

mg: Milligram

mm: Millimeter

mM: Millimolar

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MW: Molecular weight

MWCO: Molecular weight cut off

NOACs: New oral anticoagulants

PAS: Periodic acid schiff's base

PBS: Phosphate buffer saline

PLA: Phospholipase A

PMSF: Phenylmethyl sulfonyl fluoride

PPP: Platelet poor plasma

PRP: Platelet rich plasma

RBC: Red blood cell

RP HPLC: Reverse phase high performance liquid chromatography

rpm: Rotations per minute

rRNA: Ribosomal ribonucleic acid

SDS PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SE-HPLC: Size exclusion high performance liquid chromatography

SEM: Scanning electron microscope

SVMP: Snake venom metalloproteinase

tPA: Tissue plasminogen activator

UV: Ultra violet

UH: Unfractionated heparin

LMWH: Low-molecular-weight heparin

VKA: Vitamin K antagonists

VTE: Venous thromboembolism

α: Alpha

β: Beta

γ: Gamma

µg: Microgram

µl: Microliter

CHAPTER 1

INTRODUCTION

1.1. Wonders of the Seas: Cnidarians

Phylum Cnidaria (corals, sea pens, sea anemones, jellyfish and hydroids) contains about 13,000 diverse, intricate and colorful marine organisms living in marine habitats worldwide. They range in size from the tiny hydra *Psammohydra nanna* with a height of only 0.02 in (0.5 mm) to the massive Lion's Mane Jellyfish, *Cyanea arctica*, which has a bell diameter of up to 1.7 meters and a tentacle length of 36.6 m. Toxicity also displays an extreme range, from non-toxic to the infamous *Chironex fleckeri* (Box Jellyfish), a contender for the crown of having the most toxic venom within venomous animals.

With thousands of species, cnidarians are diverse in their habitat and are distributed in all the world's oceans, in polar, temperate and tropical waters. While only 0.2% of cnidarians live in freshwater, the other 99.8% can be found in shallow or deep marine habitats. Cnidarians inhabit all depths, from the sandy substrate up to the surface. They can be found from the Great Barrier Reef rising off the coast of northeastern Australia to the continental shelves of bone-chilling arctic oceans and just about every saltwater marine habitat in between (Figure 1).

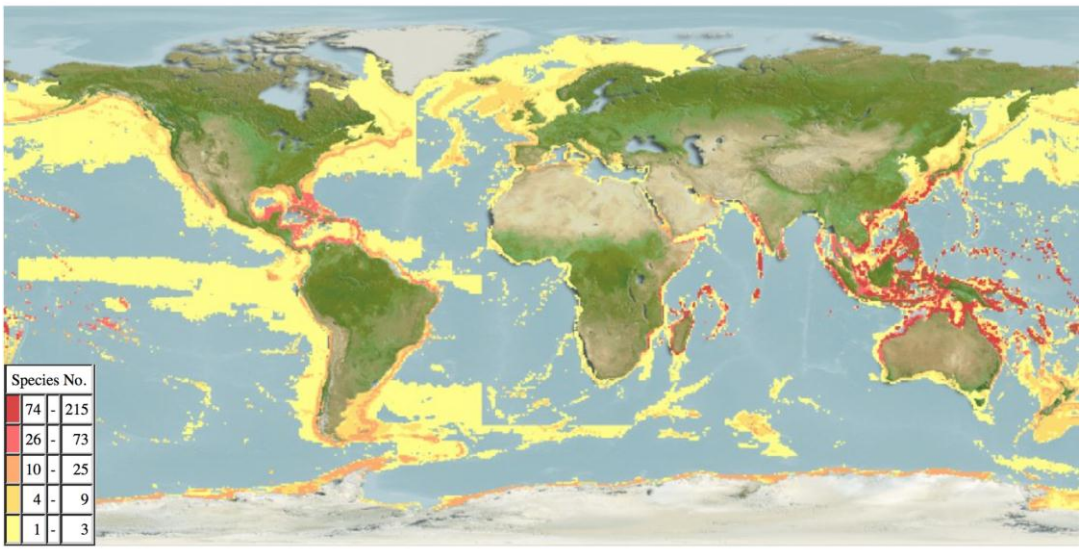


Figure 1. Species richness map for cnidaria. Computer Generated Richness Map for Cnidaria. www.aquamaps.org, version Aug. 2013. Web. Accessed 2 Mar. 2017.

1.1.1. Cnidarians: Simple Animals with a Sting

Cnidarians are radially symmetrical. Located at the center of one end of the body, the mouth opens into a gastro-vascular cavity, which is used for digestion and distribution of food; an anus is lacking. Cnidarians are further characterized by having a body wall composed of three layers: an outer epidermis, an inner gastro-dermis and a middle mesoglea. Tentacles encircle the mouth that is used in part for food capture. They do not have specialized excretory or respiratory organs but do have a nervous system. Gaseous exchange takes place directly across the surface of their body and waste is released either through their gastro-vascular cavity or by diffusion through their skin. Cnidarians have no brains or central nervous system but they do have decentralized nerve nets consisting of sensory neurons to generate signals in response to various types of stimuli. They also have motor neurons that signal the muscles to contract. All of them are connected by many intermediate neurons, which also form ganglia and act as local co-ordination centers. All cnidarians can regenerate, enabling them to recover from injury and to reproduce asexually. Polyps can regenerate from small pieces or even collections of separated cells but medusa have limited ability to regenerate.

Cnidarians are carnivorous and their prey ranges from plankton to animals several times larger than themselves. Some cnidarians are parasites on jellyfish and some are major pests of fish. Others obtain most of their nourishment from end symbiotic algae or dissolved nutrients. Predators of cnidarians include: a) sea slugs, which can incorporate nematocysts into their own bodies for self-defense; b) starfish, notably the crown of thorns starfish, which can devastate corals; c) butterfly fish and parrot fish, which eat corals; and d) marine turtles, which eat jellyfish. Some jellyfish and sea anemones have a symbiotic relationship with some fish; for example clown fish live among the tentacles of sea anemones and each partner protects the other against predators.

Cnidarians, as simple marine-living metazoans, include two extant subphyla: Anthozoa and Medusozoa. Using modern systematic methods, the distinction between these classes and their subclasses has been well studied by rRNA

data (Odorico et al., 1997), mitochondrial DNA data (Kayal et al., 2013) and life cycle (Schuchert, 1993). Anthozoa possess circular mitochondrial DNA, similar to other metazoans, while Medusozoans have an atypical linear genome (Bridge et al., 1995). Four classes of phylum Cnidaria are currently represented by systematics as toxic animals. Three members of these classes, Hydrozoa, Schyphozoa and Cubozoa, have a free-swimming sexual medusa stage. The fourth class, Anthozoa (which includes sea anemones, corals and sea pens), has a sessile adult stage (Figure 2 and Table 1.).

Two body forms and two lifestyles are characteristic of the Cnidaria. The sessile hydroid or polyp form is more or less cylindrical, attached to its substratum at its aboral (opposite the mouth) end, with the mouth and surrounding tentacles at the upper, oral, free end. Colonies of hydroids comprise several different types of individuals: some function in feeding, some in defense and some in reproduction. The motile jellyfish or medusoid form is flattened, with the tentacles usually located at the body margin. The medusoid's convex aboral surface is oriented upward and the concave oral surface is oriented downward.

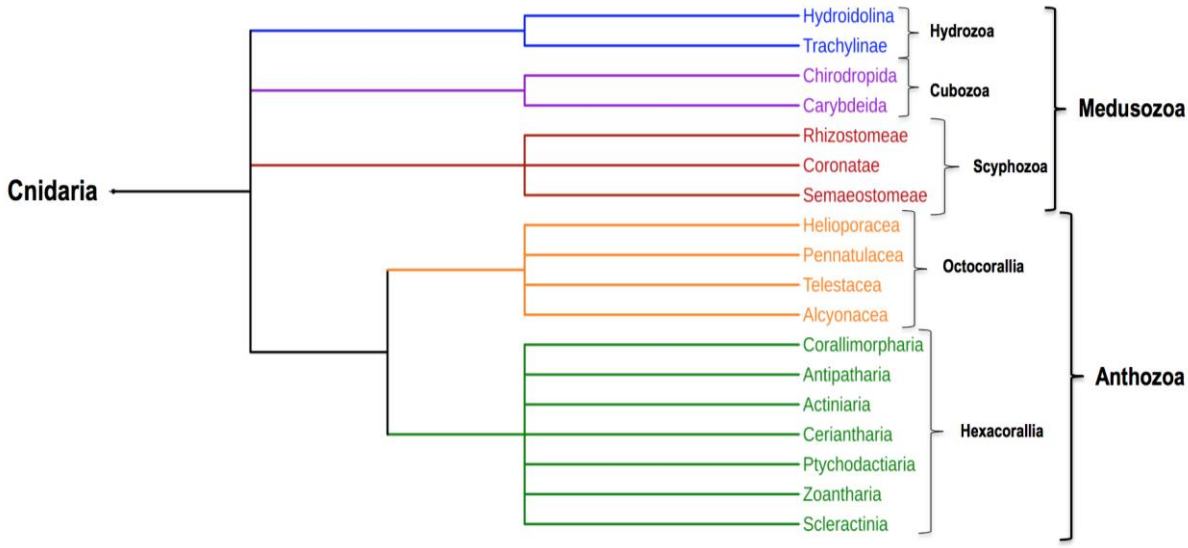


Figure 2. Phylogenetic tree of phylum Cnidaria. This phylogenetic tree was constructed using Interactive Tree Of Life (http://itol.embl.de), a web-based tool for the display, manipulation and annotation of phylogenetic trees.

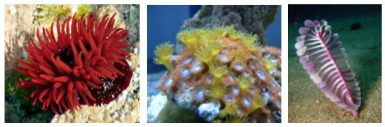
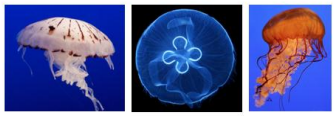
Class	Examples	Features
Anthozoa	Sea anemones, most corals and sea fans 	All marine, medusa stage completely absent; mostly sessile and colonial
Scyphozoa	Jellyfishes and sea nettles 	All marine; polyp stage reduced; free swimming; medusae up to 2 m in diameter
Cubozoa	Box jellies/sea wasps 	All marine; box shaped medusae; complex eyes
Hydrozoa	Portuguese man-of-war, hydras, Obelia and some corals 	Mostly marine with few exceptions of freshwater; Both polyp and medusa stage present in most of the species; polyp stage often colonial

Table 1. Cnidarian diversity. The four generally recognized classes within phylum Cnidaria and its features. (All images used are licensed under creative commons by 4.0)

The typical cnidarian life cycle is an alternation between a medusa and a polyp: the former is sexually reproductive stage and the latter asexual stage. Cnidarian species cluster in two distinct classes (Bridge et al., 1995; Collins et al., 2006); the anthozoans that live exclusively as polyps (sea pens as *Renilla*, stony corals as *Acropora*, sea anemones as *Aiptasia*, *Anthopleura*, *Nematostella*) and the medusozoans that display a complex life cycle with a parental medusa stage and a sessile polyp stage. Among those, the cubozoans (*Tripedalia cystophora*) and scyphozoans (*Aurelia aurita*, *Cassiopeaxam achana*) predominantly live as medusae, whereas the hydrozoans (*Podocoryne*, *Clytia*, *Cladonema*, *Eleutheria*) usually follow a life cycle where they alternate between these two forms. However some hydrozoan species have lost the medusa stage as the marine *Hydractinia* and the freshwater *Hydra* polyps (Galliot and Schmid, 2002). Similarly the staurozoans that were only recently characterized as a group (Collins et al., 2006) live exclusively as polyps (Figure 3).

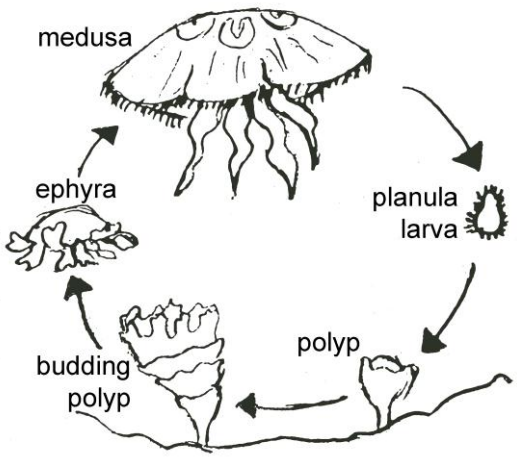


Figure 3. Generalized life cycle of a Cnidarian. (Adapted from tree of life web project)

The name for the phylum Cnidaria is derived from the Greek root “knide” (meaning “nettle”) and the Latin root “aria” (meaning “like” or “connected with”). In other words, the cnidarians are named for the fact that they possess cnidocytes. These cnidocytes allow cnidarians to be effective predators, despite their very simple bodies. They are concentrated mostly within the epidermis of the tentacles. Each cnidocyte produces one of over 30 different types of distinctive organelles known as cnidae. A cnida is a cup-shaped organelle that can contain any of several different substances. They act according to a common principle but serve various functions, following an appropriate stimulus perceived by its sensory organelle, the cnidocil. When something brushes against a cnidocil, the cnida rapidly ejects whatever substance it contains to the outside of the cell, in an explosive exocytosis. Each cnidocyte can discharge only once, hence absorbed and replaced after it discharges.

Cnidocytes can be broadly divided into four different categories, depending on what the cnidae contain and eject. A **penetrant** is a cnidocyte that ejects a harpoon-like structure that penetrates the skin of potential attackers or prey. A **glutinant** ejects a sticky fluid onto the surface of the cell, which traps prey. A **volvent** ejects a lasso-like thread that wraps around and captures prey. A **ptychocyst** is a unique type of cnidocyte found in burrowing (tube) anemones. Lacking a coelom of any sort, a cnidarian cannot burrow, at least not directly but a tube anemone’s ptychocysts eject sticky threads that the

animal can use to construct its burrow. The most notable type of penetrant cnida is known as a nematocyst. A nematocyst contains a hollow, coiled, threadlike filament inside itself. This filament is often barbed. When triggered, a nematocyst ejects the filament with enough force to penetrate the skin of a small animal. Different cnidaria have characteristic types of nematocysts and their shape, size and internal structures/arrangements can be used to identify individual species (Marsh and Slack- Smith, 1986).

1.1.2. Cnidarian Venom Apparatus and Venom Composition

The jellyfish tentacles, within its surface layers, have mature nematocysts containing nematocytes, which are surrounded by several epidermal cells called accessory cells (Rifkin, 1996b; Endean et al., 1991). Each nematocyte has a cnidocil (triggering apparatus) attached to its apical end, which is linked by fibres to the operculum of its associated nematocyst. The cnidocil of the nematocyte initiates nematocyst discharge when mechanically and/or chemically stimulated (Rifkin, 1996b; Kass-Simon and Scappaticci, 2002). Injection of the toxins requires an effective release mechanism that breaks the physical barrier of the prey's outer-surface tissue. The discharge kinetics of nematocysts in Hydra was observed to be as short as 700 nanoseconds, creating an acceleration of up to 5,410,000 g. The researchers calculated that although the accelerated mass was very small (~1 nanogram), a pressure generated at the site of impact was more than 7 GPa, which was in the range of that generated by some bullets, and sufficient to penetrate the cuticle of crustacean prey. This high speed of discharge is caused by the release of energy stored in the stretched configuration of the collagen-polymer of the nematocyst capsule wall. This ingenious solution allows the cellular process of vesicle exocytosis to release kinetic energy in the nanosecond range by a powerful molecular spring mechanism (Nüchter et al., 2006).

When discharge is initiated, the operculum of the nematocyst opens rapidly and the spine-covered butt of the tubule bursts explosively through the hole. As the butt emerges, its spines temporarily form a piercing structure, which facilitates penetration of the butt into the tissue of the prey/victim. The spines of the butt fan outwards, presumably to anchor the butt securely in place. As

the tightly coiled thread everts, it rotates in a manner that successive thread spines cut a path through the tissue of the prey/victim (Rifkin and Endean, 1983). During tubule eversion, venom is translocated from the capsular matrix to the external environment via hollow, outward-facing spines or barbs located along the surface of the everted tubule (Lotan et al., 1996). The progressive release of venom along the tubule facilitates venom delivery to both vascular and non-vascular tissue. This may explain the rapid onset of venom-induced systemic effects as well as the severe pain, damage and inflammation of the skin at the site of envenomation (Rifkin, 1996b) (Figure 4).

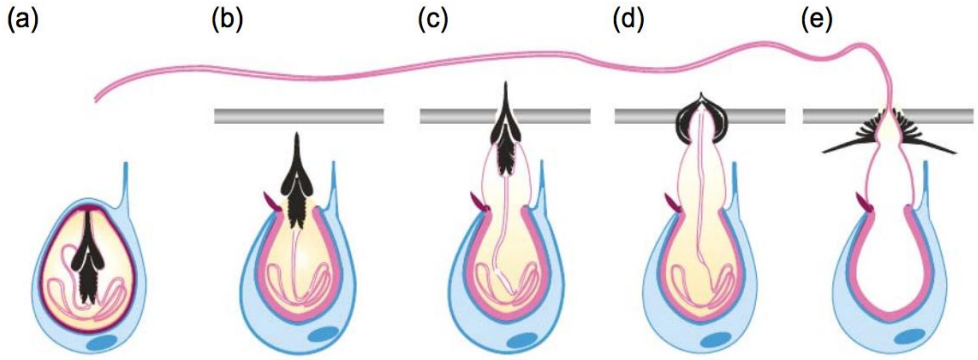


Figure 4. Schematic diagram of nematocyst discharge. The nematocyte (blue) harbours a single nematocyst (pink walls, tubule and operculum; black stylets (large spines)) (a) undischarged stenotele nematocyst from *Hydra attenuate* (b) operculum opens ejecting stylets (c) stylets puncture cuticle of prey/victim; (d) stylets expand; (e) tubule everts into subsurface tissue and venom is discharged. (Reproduced from Nüchter et al., 2006, with permission from Elsevier)

Cnidarians inject the nematocyst content or "venom" into their prey, which initiates toxic and immunological reactions in the envenomed organism. Previous investigations have revealed that cnidarian venoms contain diverse cocktail of non-protein bioactive compounds such as 5-hydroxytryptamine (5-HT), catecholamines, histamine and histamine liberators; neuro-active compounds such as quaternary ammonium compounds; protein and peptide compounds with enzymatic activity and non-enzymatic activity (Hessinger and Lenhoff, 1973; Tamkun and Hessinger, 1981;Halstead, 1988; Long and Burnett, 1989; Endean et al., 1993; Macek, 1992; Gusmani et al., 1997). Enzymes include lipolytic and proteolytic proteins that catabolize prey tissues and non-enzymatic components include cytolysins, hemolysins, cardiotoxins and neurotoxins. In addition, certain cnidarian venoms contain or induce the

release of host vasodilatory biogenic amines such as serotonin and histamine accelerating the pathogenic effects of other venom enzymes and porins.

Cnidarian venoms are hydrophobic, relatively thermo-labile and have the ability to aggregate/disaggregate especially under *in vitro* conditions (Othman and Burnett, 1990). The range of potency between venoms of different cnidarian species is quite diverse. The venom components vary in antigenic potency with some of the more active compounds capable of binding antibody within few minutes. Cross reactivity of epitopes present in the polypeptides of different venoms is quite common.

Although literature on venoms of terrestrial organisms provide a number of clear examples of the association of chemical elements in venom components, the problems associated with obtaining large amounts of pure nematocyst venom have made it difficult to do similar studies with cnidarians. Thus, much of what is known of cnidarian venoms (or perhaps, more correctly, cnidarian toxins) comes from studies in which the whole animal or its tentacles are homogenized and then analyzed for toxic properties. It is often assumed, probably correctly in many cases, that nematocyst venoms are responsible for the observed toxic effects, it is very difficult to prove. The reason is that some cnidarians are known to possess inherent tissue toxicity in addition to whatever toxic effects are provided by nematocyst venoms (Mariscal, 1974; Blanquet, 1977; Burnett and Calton, 1977, 1987a; Beress 1982 a & b; Russell, 1984)

The cnidarian attacking/defending mechanism is fast and efficient and massive envenomation of humans may result in death, in some cases within a few minutes to an hour after sting. As depicted by Sir A. C. Doyle in one of the Sherlock Holmes famous short stories “The Adventure of the Lion’s Mane”, the killer was not a jealous suitor but Lion’s Mane (*Cynae capillata*), one of the largest known jellyfish species. The venomous sting of this deadly “medusae” in the cold waters of Sussex town killed the protagonist. The complexity of venom components represents a unique therapeutic challenge. Thus, they are invaluable as a therapeutic target for sting treatment or as lead

compounds for drug design.

1.1.3. Clinical Profiling of Cnidarian Envenomation

The medical significance of cnidarians, in particular jellyfish, is underestimated throughout the world with respect to both morbidity and mortality. With the exception of a few regions, knowledge on the distribution of cnidarians and their medical significance is quite fragmentary. Moreover, in many accidents it is only possible to speculate about the cause, as the victim can often only give an insufficient description of the animal involved or may not even have seen it.

Victims of cnidarian stings are most frequently swimmers or divers in coastal waters and cnidarians are among the most common causes of accidents with venomous marine animals. Contact with sessile cnidarians and many types of jellyfish usually only results in unpleasant local symptoms. The animal and its tentacles are typically quite friable and broken-off tentacle fragments in the surf or washed up on the beach can retain their toxicity and stinging capacity even if they appear dried out and withered. There are several species that quite frequently cause more serious local effects, such as skin and soft tissue alterations that heal slowly and can result in scarring or even lasting damage (above all on the extremities). The severity is related to the season and species (venom potency and configuration of the nematocyst), the number of nematocysts triggered and the size of the animal (venom inoculum), the size and age of the victim (the very young and old and the smaller person tend to be more severely affected), the location and surface area of the sting and the health of the victim.

Treatment of jellyfish envenomation is directed at: alleviating the local effects of venom, preventing further nematocyst discharges and controlling systemic reactions, including shock.

1.2. Toxins to Therapeutics

Animal venom toxins have evolved over millions of years to target diverse and key biological functions with extreme precision. Venom is a cocktail of different components with widely differing functions. Venom proteins play a

number of adaptive roles: immobilizing, paralyzing, killing, liquefying prey and deterring competitors. The synergistic action of other venom proteins may enhance the activity or spreading of toxins. Venoms can be part of the feeding system, may serve in digestion or can play a defensive role. The efficiency of venom is crucial for survival of the animals. Each venomous animal is usually highly specialized with respect to its prey. Toxins affect the vital functions of another organism through specific interaction with their target. These toxins have adapted to their molecular targets during evolution. A response to selective pressure during the evolution of animal toxins was their strict target selectivity, discrimination between closely related receptor subtypes and hence distinguishing between physiologically relevant and irrelevant targets. They interact with each other through rather small but specific portions of their surfaces, which confer selectivity and specificity between complimentary proteins. That is exactly what is needed when looking for a pharmaceutical compound. Researchers are looking for something that affects our bodies in a very specific manner. Venoms already do what man-made drugs need to do: target and modulate key molecules in our cells. Just like pharmaceuticals, venoms can alter core physiological processes that are disrupted by diseases. By controlling the dosage or slightly altering the chemical composition, scientists can turn toxins into treatments. The advantage of exploring specific toxins is how selective they are when attaching to their targets within the body thus minimizing the potential for unwanted side effects.

1.2.1. Animal Toxins as Medicine from Past to Present: A Historical Perspective

It is remarkable that 21st century medicine, advanced as it undoubtedly is, has started seriously considering remedies once regarded as myths and folklore, with a far more open eye. What ancient Greeks, Indians, Chinese, Egyptians and Romans were happy to use for curing ailments may have seemed unpalatable to us but science is coming to appreciate how well they actually work. History is replete with stories of the earliest humans using animal venoms for treating diseases for millennia.

Since very ancient times, snake was a constant companion of medicine men and magicians. In mythical stories, snakes were often said to have knowledge of healing and there was a widespread belief that wisdom and medical knowledge could be obtained from the snake in various ways. The snake became a sign of medicine and healing both by virtue of its godly associations, and also through the metaphorical immortality represented by the shedding of its skin. Greeks were thought to have discovered that snake venom in low doses could be used to treat various illnesses (Figure 5). The ancient Greeks aptly used the snake as a symbol for medicine. The caduceus, a short staff, entwined by two symmetrically opposite serpents, carried by Hermes in Greek mythology is perhaps the best-known representation of the Greek sacred snake, one that persists as a symbol of the art of medicine to this day (Retief and Cilliers; 2010). This is due to its association with Asclepius, Greek god of medicine. As per Indian history of *Rasa Grantha*, hindu communities were pioneers in the use of snake venom in medicine. Earlier, it was known that snake venom, at a very low dose, applied orally is a very powerful stimulant and if it is mixed with bile of animals, its action becomes totally different. *Suchikabharana rasa*, a dry mixture of cobra (*Naja naja*) venom containing metals, nonmetals and animal derived components, was administrated orally in small doses. It was effective in plague, fever, coma and tuberculosis*. Ardhanarisvara* was processed from *Naja naja* venom used for the treatment of obstinate fever. In *Charak Samhita*, *Sushruta Samhita* and *Vagbhata Astanghryday samhita*, the three classic doctrines in Ayurveda, the cobra venom has been mentioned for curing *Dushyodara*, *Jalodara* (ascites) and *Sannipatik Jwara* (a type of fever) respectively. In the Unani system of medicine, cobra venom is referred as a tonic, aphrodisiac, hepatic stimulant and was used for revival in collapsed conditions. Some homeopathy medicines are prepared from venoms of *Vipera russelli (Daboia russelli)*, *Crotalus atrox*, *Naja naja* and *Lachesis muta*. Snake venoms have also been used in allopathic medicine for more than a century to treat thrombosis, arthritis, cancer, immune dysfunction, viral infections, delirium, hallucinations, cholera and melancholia.

Using venom to treat cancer is not a totally new idea; one such method in traditional Chinese medicine, known as *huachansu*, uses the venom secreted from a toad's skin glands and has been around for 1,000 years. Scorpion venom has been surprisingly effective the treatment of nervous system diseases, cerebro-vascular diseases, malignant tumors, stubborn viruses, etc. It was considered one of the best medicines for a dozen of challenging or difficult-to-diagnose diseases. In certain rural corners of India, whole scorpions are dipped in mustard oil and then rubbed on arthritic joints. Bee venom therapy was practiced in ancient Egypt, Greece, and China. Hippocrates recognized the healing virtues of bee venom for treating arthritis and other joint problems. In 14 BC, the Greek writer Pliny the Elder described the use of bee venom as a cure for baldness. Doctors used bee stings to treat the Emperor Charlemagne's gout in the 700s. Today growing scientific evidence suggests that various bee products promote healing by improving circulation, decreasing inflammation and stimulating a healthy immune response.

The use of leeches in medicine dates as far back as 3,500 years ago, when they were used for bloodletting in ancient Egypt. Leech therapy is one of the oldest medical practices, having been practiced among the diverse ancient people, including the Mesopotamians, the Egyptians, the Greeks, the Mayans and the Aztecs. In Greece, Hippocrates, the father of medicine, first introduced leech therapy. The popularity of this therapy was reinforced by the ideas of Roman philosopher Galen, who believed that blood was the dominant humour and the one in most need of control. In order to help cure a person of their sickness, dirty or too much blood needed to be removed from the body. One of the ways to remove blood was by attaching leeches to the skin. When the leech was full it would simply fall off from the skin. Yet it was not the leech that sucked most of the blood from the body, once it fell off the anticoagulants in its saliva would keep the wound open and doctors would allow more blood to drain from the open wound. Leeches were also used in ancient medicine to aid in drawing blood to damaged/dying body parts. It was also known in Ayurvedic medicine, described in *Sushruta Samhita*. *Dhanvantari*, the Indian god of Ayurveda holds a leech in one of his four hands (Figure 5).

Contemporary leech therapy is based on the rationale that the leech bite creates a puncture wound that bleeds for hours. Today medicinal leeches are used in the treatment of various diseases such as thrombophlebitis, hypertension, varicose ulcer, skin and musculoskeletal diseases.

Since ancient times extracts from marine organisms were also used for medicinal purposes in China, India and Europe. Hippocrates documented that juices from various species of mollusks were commonly used as laxatives. Extracts from the sea hare, *Aplysia* were used as depilatory. Before the invention of synthetic sponges, natural ones were used for absorbent purposes in medical settings. Blood flow was contained with sponges. As late as the 1800's, many of these were a part of standard pharmacopeias.

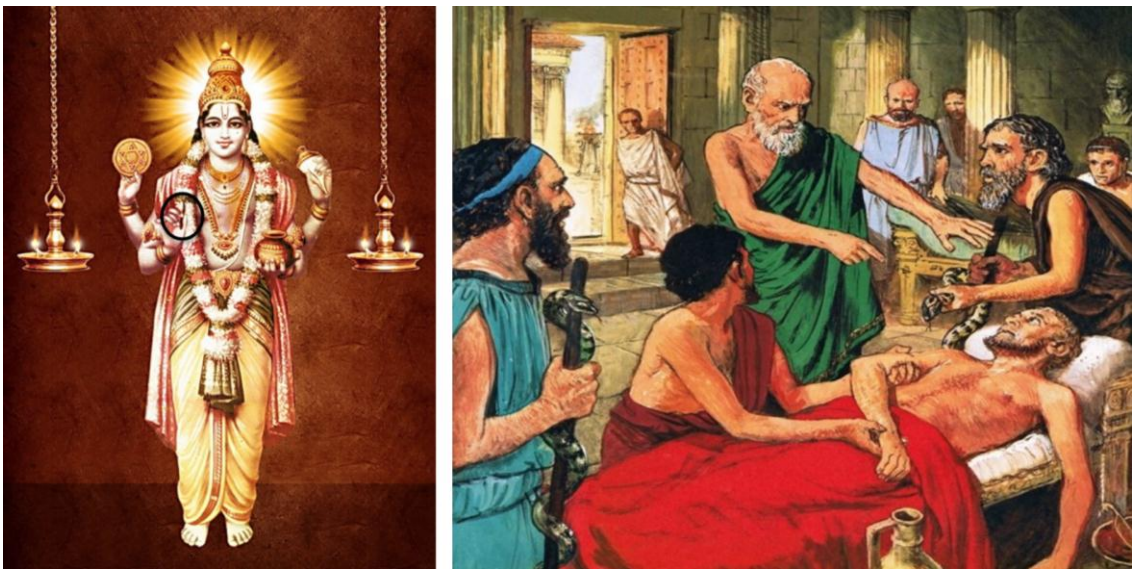


Figure 5. Premier ancient evidences. *Dhanvantari* the Indian god of Ayurveda holding a leech in one of his hands (L). A Greek physician using snake venom to treat a patient (R). (Images used are licensed under creative commons by 4.0)

Jellyfish have been long known for their medicinal value (Omori, 1981). It is believed to be an effective cure for arthritis, hypertension, back pain and ulcers, while softening skin and improving digestion. Jellyfish was also used as a remedy for fatigue and exhaustion, stimulate blood flow during the menstrual cycle of women and ease any type of swelling. Most of these claims regarding the medicinal value of jellyfish are described in in Chinese traditional literature. Aborigine shamans in Australia used dried jellyfish powder as a remedy for burns. In Southern Korea they were used to improve the beauty of skin and for weight loss (Hsieh and Rudloe, 1994; Hseih, 2001).

However, no scientific research was carried out to document the medicinal efficacy of jellyfish. Collagen was hypothesized to be the ingredient in jellyfish contributing to the beneficial health effects: as it is the essential building material of muscle tissue, cartilage and bone and has a great medicinal promise (Hsieh & Rudloe, 1994). The antiquity in the use of medicinal animals and its persistence through times are a testimony to the importance of these therapeutic resources to mankind.

1.2.2. Medical Menagerie: Terrestrial Animals and their Venoms as a Source for Development of Human Therapeutics

When venoms are used to make medicine, it's the toxins that are actually used and not the whole venoms themselves. Any given venom, contains a mixture of twenty to hundred toxins, each of which must be identified structurally and then studied to reveal the relevant receptors on human cells (Figure 6).

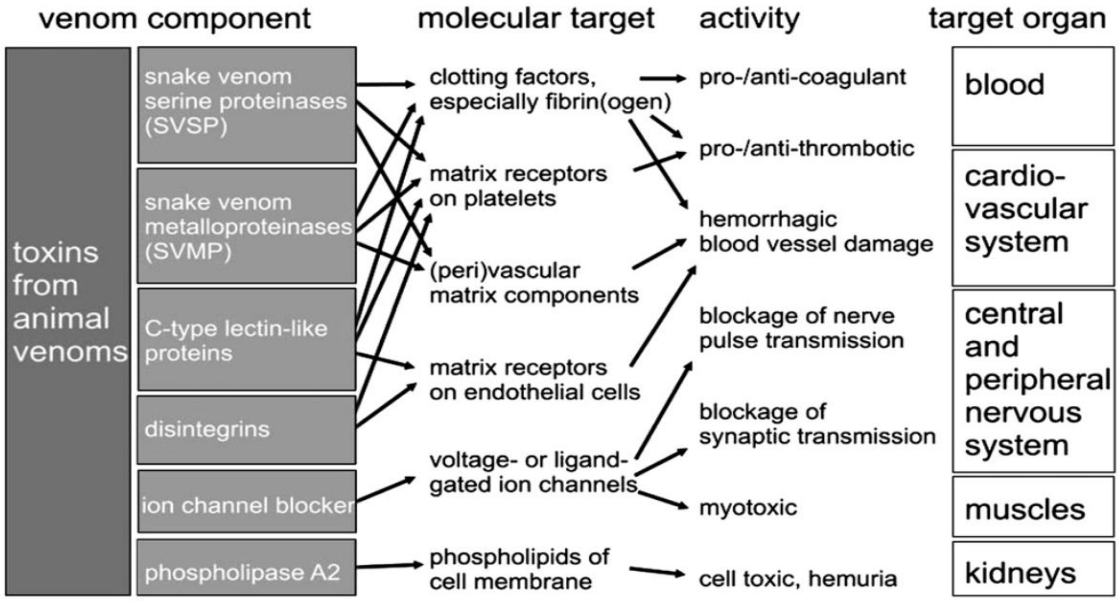


Figure 6. Venom components and their associated interactions. Animal venoms contain a plethora of pharmacologically active compounds, which can be grouped structurally and functionally into subgroups. Venom components are directed to different molecular targets of the envenomed organism with high specificity and affinity. (Reproduced from Johannes A. Eble, 2010, with permission from Elsevier).

Modern medicine's relationship with venom-based drugs began in the 1970s with the development of the blockbuster antihypertensive drug captopril based on the venom of the Brazilian viper, *Bothrops jaracaca*. The FDA approved it

in 1981 and its approval pushed the idea that venoms could be used to create modern medicines. The field of venom-based medicine has flourished ever since and teams across the world have been exploring the most exotic animals in search of potent drugs that could emerge from their highly evolved venom. The field, as a whole, spans beyond lethal snakes to other venom-producing animals such as leeches, cone snails, scorpions, spiders, bees and lizards. But as snakes primarily target warm-blooded animals, their venoms are more fruitful for human drugs and have drawn major share of attention. Some of the animal toxins and animal toxin-based compounds that have already received US Food and Drug Administration approval for use as drugs for treatment of specific indications like hypertension, coagulopathies, chronic pain and diabetes have been enumerated in Table 2.

Generic Name (Trade Name)	Species of origin	Mechanism of Action	Indication
Captopril (CAPOTEN®)	*Bothrops jararaca* (Jararaca Pit Viper Snake)	Angiotensin-converting enzyme inhibitor	Hypertension, Cardiac failure
Eptifibatide (INTEGRILIN®)	*Sistrurus miliarius* (Pigmy Rattlesnake)	Prevents binding of fibrinogen, von Willebrand factor, and other adhesive ligands to GPIIb/IIIa	Acute coronary syndrome; Percutaneous coronary intervention
Tirofiban (AGGRASTAT®)	*Echis carinatus* (Saw-scaled Viper Snake)	Antagonist of fibrinogen binding to GPIIb/IIIa receptor	Acute coronary syndrome
Bivalirudin (ANGIOMAX®)	*Hirudo medicinalis* (European Medicinal Leech)	Reversible direct thrombin inhibitor	Anticoagulant in percutaneous coronary intervention
Ziconotide (PRIALT®)	*Conus magnus* Magical Cone Snail	$Ca_v2.2$ channel antagonist	Management of severe chronic pain
Exenatide (BYETTA®)	*Heloderma suspectum* (Gila Monster Lizard)	Glucagon-like peptide-1 receptor agonist	Type 2 Diabetes mellitus

Table 2. Toxin-Based Drugs Approved for Use by the U.S. Food and Drug Administration. (Adapted from Takacs and Nathan, 2014; Fox and Serrano, 2007)

Snake venom toxins have served as a starting material for drug design to combat several patho-physiological problems such as cardiovascular disorders, neurological problems and cancer therapy. Several isolated snake venom proteins (*e.g.,*phopholipases A_2, metalloproteinases, serineproteases, L-amino acid oxidases, lectins and others) and peptides (bradykinin potentiators, natriurectic, analgesic peptides) have found practical application as pharmaceutical agents. Angiotensin-converting enzyme (ACE) inhibitors are perhaps the best examples of a drug class that has been developed from peptide leads derived from snake venom. For years, these have been used in the treatment of hypertension. A large number of snake venom proteins affect

the hemostatic system and can have procoagulant, anticoagulant, fibrinolytic or platelet aggregation inhibiting/promoting activities. More recently, exenatide, a synthetic analogue of exendin-4, which is a venom peptide of the Gila Monster, from the deserts of the Southwest USA and Mexico, is now available as a prescription medicine for the treatment of Type 2 diabetes. Additionally, chlorotoxin, a peptide derived from the venom of a desert scorpion, has now been proven to be effective treatment for malignant glioma. Only a small number of spider venom peptides have been pharmacologically characterised but the array of known biological activities is impressive. In addition to the well known neurotoxic effects of spider venoms, they contain peptides with antiarrhythmic, antimicrobial, analgesic, antiparasitic, cytolytic, haemolytic and enzyme inhibitory activity (Vassilevski et al., 2009). Today, with the increasing knowledge of the human physiology, the pipeline "from-fang-to-pharmacy" is growing even bigger (Figure 7).

1.2.3. Cures from the Deep

Oceans cover 70% of the Earth's surface and depending on their location, temperature and salinity, a great diversity of beings inhabit them, forming a much more extensive phylogenetic diversity than in the terrestrial environment. Marine toxins have lagged behind in the exploration for bioactive compounds as compared to the terrestrial organisms due to limited accessibility to most marine organisms. Approximately out of 34 fundamental phyla, 17 occupy terrestrial habitats, whereas 32 inhabit marine biotopes. This emphasizes the richness and uniqueness of ocean as a source of active compounds of interest to pharmaceutical industry. Most of the compounds that have attracted the interest of the pharmaceutical industry till now are those possessing anti-microbial, anti-tumor, anti-inflammatory and immunosuppressive activity.

Researchers have found a cancer cell-killing molecule called Ecteinascidin 743 (*Ecteinascidia turbinate*) in a type of sea squirt living in the reefs and swamps of the West Indies. This molecule was subsequently utilized outside the U.S. for soft-tissue sarcomas and has entered U.S. clinical trials for several cancers. The first effective remedy against AIDS in the 1990s,

Azidothymidine was modeled on a compound isolated from a sponge, *Cryptotethya crypta* discovered in the Caribbean in 1949. The cone snail venom has shown to be 1,000 times more powerful than morphine in treating certain kinds of chronic pain. The cone snail-derived drug Prialt® (ziconotide) blocks the nerve transmission in the spinal cord and blocks certain pain signals from reaching the brain (Table 3).

Compound Name (Trade Name)	Marine Organism	Chemical Class	Indication
Cytrabine, Ara-C (Cytosar-U®)	*Cryptotheca crypta* (Sponge)	Nucleoside	Acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), chronic myelogenous leukemia (CML), and non-Hodgkin's lymphoma
Brentuximab vedotin, SGN-35 (Adcetris®)	*Dorabella auricularia* (Mollusk/cynobacterium)	Antibody drug conjugate-Monomethyl auristatin E (ADC-MMAE)	Directed to the protein CD30, which is expressed in classical Hodgkin lymphoma (HL) and systemic anaplastic large cell lymphoma (sALCL)
Vidarabine, Ara-A	*Tethya crypta* (Sponge)	Nucleoside	Anti-viral drug which is active against herpes simplex and varicella zoster viruses
Ziconotide (Prialt®)	*Conus magus* (Cone snail)	Peptide	Analgesic agent for the amelioration of severe and chronic pain
Eribulin mesylate, E7389 (Halaven®)	*Halichondria okadai* (Sponge)	Macrolide	For treating breast cancer and liposarcoma. Also used for variety of other solid tumors, including non-small cell lung cancer prostate cancer and sarcoma
Trabectedin, ET-743 (Yondelis®)	*Ecteinascidia turbinata* (Tunicate)	Alkaloid	Treatment of advanced soft tissue sarcoma

Table 3. List of marine origin drugs approved for human use in different parts of the world. (Adapted from Harshad Malve, 2015)

Bryozoans produce a substance called bryostatin that is believed to be a potential treatment for Alzheimer's disease. Didemnin-B from the Caribbean tunicate *Tridemnum solidum* was the first marine compound to enter human cancer clinical trial as a purified natural product (Carte, 1996), but was unsuccessful in further trials (Davidson, 1993). Nevertheless, this class of cyclic peptides provides important structural lead for a variety of antiviral, anticancer and immuno-suppressant activities (Sakai et al., 1995). Neosurugatoxin isolated from *Babylonia japonica* is useful in characterizing two classes of acetylcholine receptors (Ireland et al., 1993). Dolastatin, a cytotoxic peptide from *Dolabella auricularia* is an anti-neoplastic substance (Pettit et al., 1989).

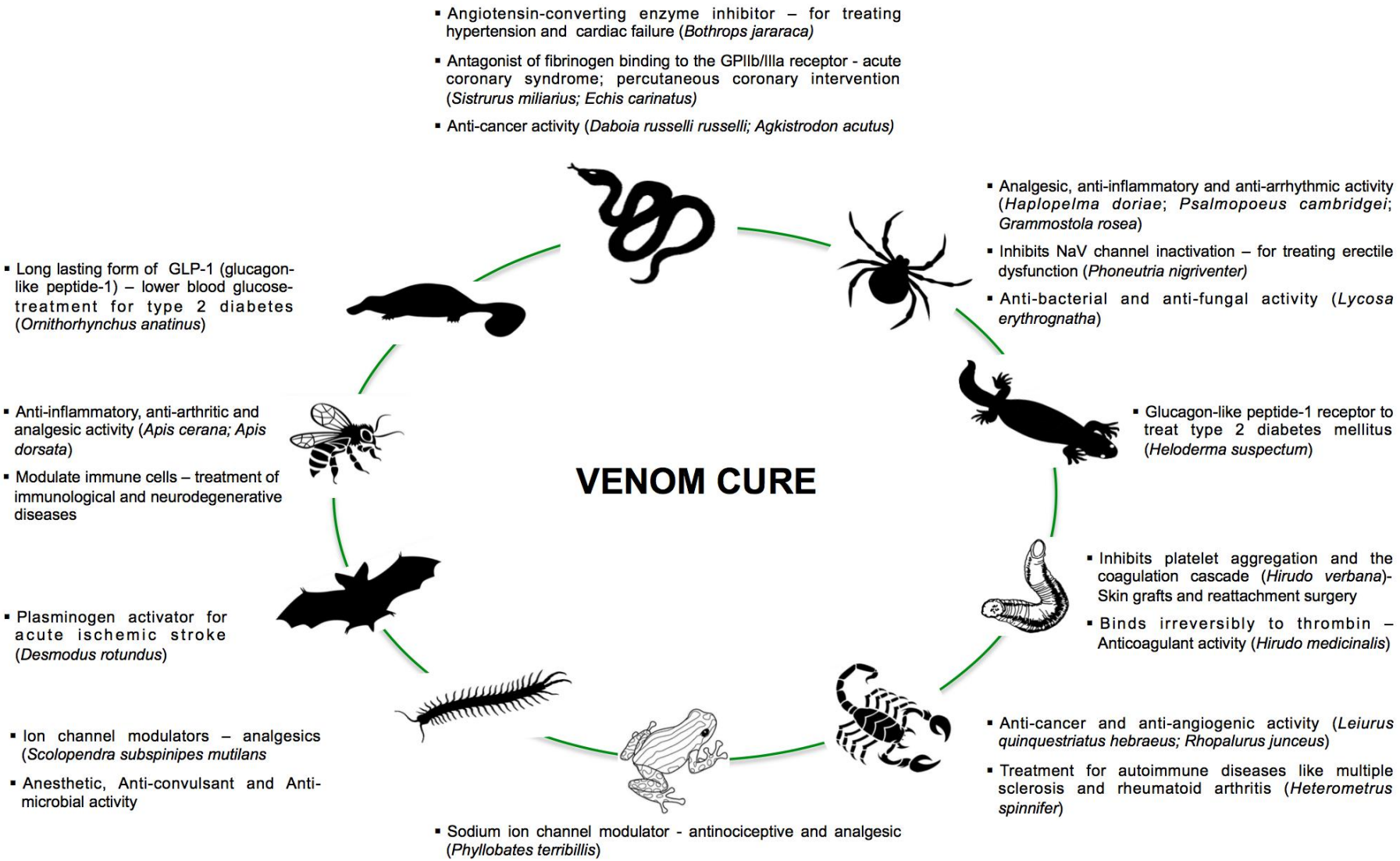


Figure 7. Venom cure. Terrestrial organisms and their venoms as a source of various kinds of bioactive compounds.

Ulapualide-A, a sponge-derived macrolide isolated from the nudibranch *Hexabranchus sanguineus* exhibits cytotoxic activity against leukemia cells and antifungal activity, which exceeds that of clinically useful amphotericin-B (Rorsener and Scheuer, 1986). Chromodorolide-A isolated from *Chromocloris cavae* exhibits *in vitro* antimicrobial and cytotoxic activities (Morris et al., 1990). Onchidal from *Onchidellabieyi* is a useful probe for identifying the active site residues that contribute to binding and hydrolysis of acetyl cholinesterase (Ireland et al., 1993) A major metabolite convolutamide-A from *Anthia convolut* exhibited *in vitro* cytotoxicity against murine leukemia cells and human epidermoid carcinoma cells (Zhang et al., 1994). *Cribricellina cribreria* yielded a β-carboline alkaloid, which exhibited cytotoxic, antibacterial, antifungal and antiviral activities (Princep et al., 1991). Indole alkaloids isolated from *Flustrafoliacea* showed strong antimicrobial activity (Holst et al., 1994). Halichondrin-B, a polyether macrolide from Japanese sponge *Theonella* spp. generated much interest as a potential anticancer agent (Carte, 1996; Fuestani et al., 1992). Theopederins are structurally related to mycalamide-A from marine sponge, *Mycale* spp. collected in New Zealand (Perry et al., 1988) and onnamide-A from marine sponge, *Theonella* spp. collected in Okinawa (Sakemi et al., 1988) showed *in vitro* cytotoxity and *in vivo* antitumour activity in many leukemia and solid tumour model systems (Burres and Clement, 1989). The most spectacular substance of pharmacological importance extracted from fish is tetradotoxin (TTX), the puffer or fugu poison. Other toxins isolated include ciguatoxin from electric rays, which is served as a potent antidote for pesticide poisoning (Oliviera et al., 2003). TTX isolated from puffer fish and many other marine organisms has become a useful tool for researchers studying the voltage-gated sodium channels and it also plays an important role in many biological experiments (Auyoung, 1999). An anti-cancer drug, namely “Fu-anntai”, which has anti-blastic effects on cervical carcinoma, stomach cancer, rhinocarnoma and leukemia cells, has been extracted from sea snakes (family Hydrophiidae) in China (Figure 8).

Marine toxins, whose immense genetic and biochemical diversity is only beginning to be appreciated, are likely to become a rich source of novel chemical entities for the discovery of more effective drugs.

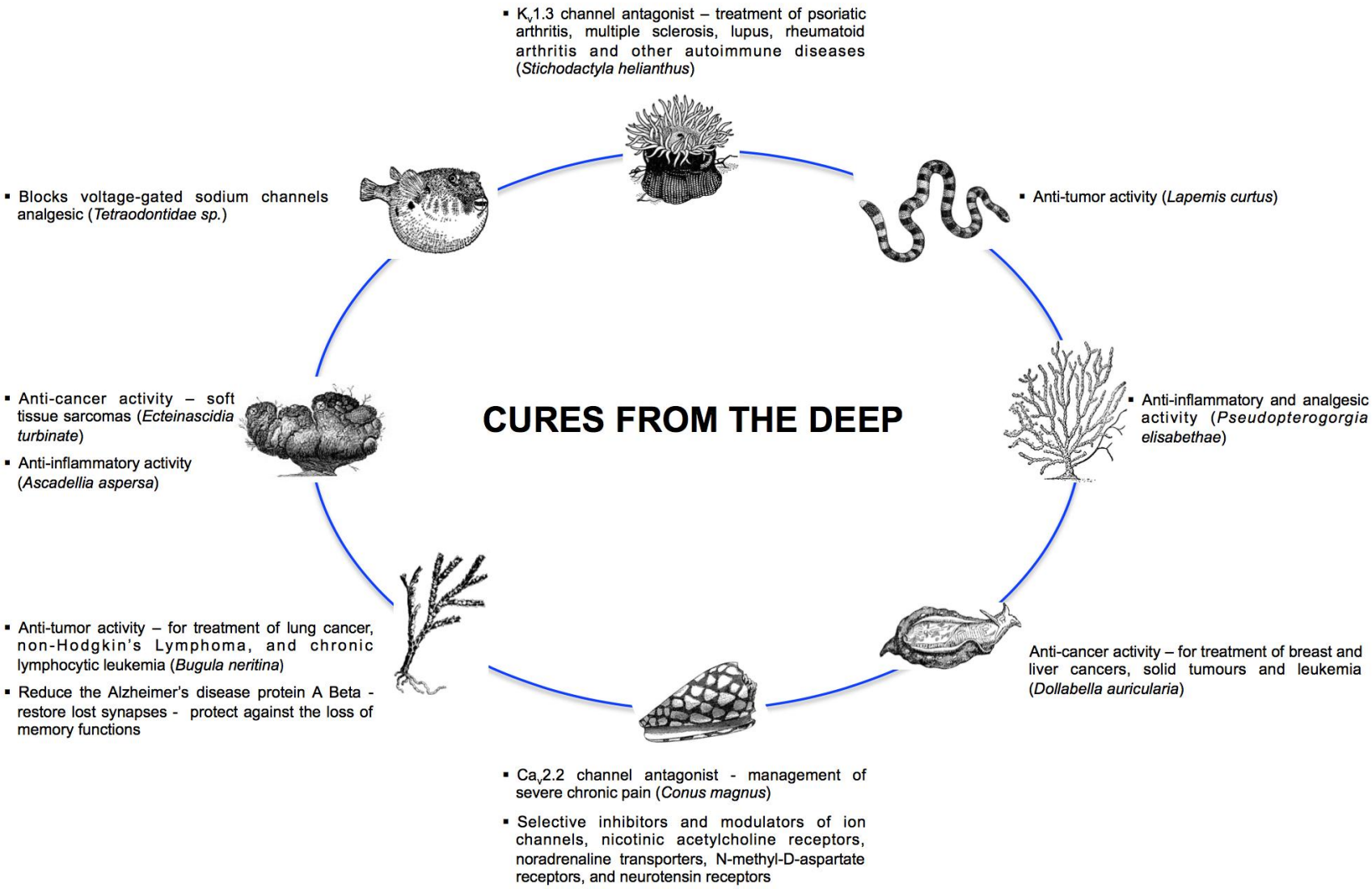


Figure 8. Cures from the deep. Marine organisms as a source of various bioactive compounds.

1.2.4. Therapeutic Virtues of Cnidarian Venom and Toxins

Phylum Cnidaria is a large, diverse and ecologically important group of marine invertebrates that includes over 11,000 extant species (Daly et al., 2007). Over 3000 marine natural products have been described from this phylum alone (Rocha et al., 2011).Toxic compounds isolated from Cnidaria have been viewed to produce several serious implications to human health due to their neurotoxicity, cytotoxicity and tissue damage. However, novel findings have demonstrated that their toxins might be used as a tool to study cell physiology (Morabito et al., 2015). This in turn may be promising sources of pharmacological lead molecules/active agents for therapy of human diseases.

Palytoxin is a highly potent non-protein toxin originally isolated from the zoantharians of the genus Palythoa is also found in marine organisms ranging from dinoflagellates to fishes. (C. H. Wu, 2009). It has a unique action on the Na, K-ATPase, converting the pump into an ion channel and resulting in K^+ efflux, Na^+influx and membrane depolarization. As a result, palytoxin causes a wide spectrum of secondary pharmacological actions. By acting like a key to unlock the internal structure of the Na, K-ATPase, palytoxin holds promise as a useful tool for investigation of the pump molecule. Palytoxin has been reported for anti-cancer activity against head and neck carcinoma cells (Gorogh et al., 2013), Ehrlich ascites tumour and P-388 lymphocytic leukaemia cells (Quinn et al., 1974). The mechanism of action of its cytotoxicity and tumour suppressor activity has been established by actin filament distortion and apoptosis (Louzao et al., 2011). Conversely, palytoxin has been identified as a tumour promoter by disrupting the regulation of cellular signalling cascades (E.V.Wattenberg, 2011). Over the past decade, several cytolysins and protease inhibitors have been extracted from the sea anemone *Actinia equina*. Equinatoxin II (EqT II) is a pore-forming protein that has been shown to have significant toxicity against Ehrlich ascites tumour and L1210 leukaemia cell lines (Giraldi et al., 1976) and diploid lung fibroblasts of the Chinese hamster (Batista et al., 1990). Equistatin is a potent inhibitor of papain-like cysteine proteinase and aspartic proteinase cathepsin D (Lenarcic and Turk, 1999). Over expression and hyper secretion of cathepsin-D has been reported in breast carcinoma cells (Liaudet-Coopman et al., 2006) and

papain-like cysteine proteases are involved in diseases of the central nervous system (Brömme and Petanceska, 2002). Recently anti-butyrylcholinesterase activity was detected in the crude venom extracted from the tentacle material of the Mediterranean jellyfish *Pelagia noctiluca*. Inhibition of butylcholinesterase in the central nervous system may prove useful in the treatment of neurodegenerative diseases such as Alzheimer's disease and senile dementia (Ayed et al., 2012).

Another venom-derived drug comes from the sun anemone *Stichodactyla helianthus* found in reefs of the Caribbean called ShK, which is a potent inhibitor of a T-lymphocyte potassium channel called Kv1.3. Since this channel is crucial in the activation of human effector memory T cells, it could act as a valuable immunosuppressant for the treatment of autoimmune diseases mediated by T cells (Chi et al., 2012). Kv1.3 blockers are also considered as therapeutic targets for the treatment of obesity, thus highlighting the potential use of ShK in treatment of obesity and insulin resistance (Upadhyay et al., 2013) (Table 4).

Research on cnidarian toxins has increased considerably in magnitude and sophistication during the past two decades. Both peptide and non-peptide toxin groups are being studied more intensively. Still, since there are >10,000 cnidarian species, this phylum is still a relatively under exploited source of toxins acting on an increasing number of ion channels and other receptor targets. Along with the toxins of snakes, scorpions, spiders and cones, many cnidarian toxins may become valuable research tools. In the last two decades there have been many new discoveries: a plethora of new toxins have been identified, but many more surely await discovery.

Compound	Species	Activity
Palytoxin	*Palythoa toxica*	Local anesthetic and vasoconstrictive agent; induced ion currents in mouse neuroblastoma cells (Castle et al., 1988).
Prostanoid compounds	*Clavularia virdis*	Inhibited the growth of HL-60 leukemic cells (Jha et al., 2004).
Equinatoxin II	*Actinia equina*	Pharmacological activities like platelet aggregation, cardiotoxicity, cytotoxicity and ability to cause pulmonary edema; at cellular level, causes formation of pores in various lipid membranes (Teng et al., 1988; Maček, 1994).
Jellyfish tentacle extract	*Acromitus rabanchatu*	Caused glycaemic alteration in fasting rabbits (Ghosh et al., 1990).
Crude venom	*Cassiopea xamachana*	Incidence and growth of SNC tumors induced by *N-Ethyl-N-Nitrosourea* were affected (Orduña-Novoa et al., 2003).
Aurelin	*Aurelia aurita*	Exhibits antimicrobial activity on gram positive as well as gram-negative bacteria (Ovchinnikova et al., 2006).
Lipidic α-amino Acids (LAAs)	*Protopalythoa variabilis*	Completely inhibited tumor cell growth in four tumor cell lines HCT-8, SF-295, MDA-MB-435, and HL-60 (Wilke et al., 2009).
Norzoanthamine	*Zoanthus sp.*	Promising candidate for an osteoporotic drug as an IL-6 inhibitor (Yamaguchi et al., 1999).
Gigantoxin 1	*Stichodactyla gigantea*	First example of EGF-like toxins of natural origin and its activity was evidenced by rounding of A431 cells and tyrosine phosphorylation of the EGF receptor in the cell (Shiomi et al., 2003)
Gigantoxin 4		Could make pores in cellular, lipid or artificial membranes, especially when cell membranes contained sphingomyelin, - increasing membrane permeability or causing leakage of cell contents, leading to cell death (Hu et al., 2011).
Pseudopterosin	*Pseudoptero-gorgia elisabethae*	Potent anti-inflammatory and analgesic agent- appear to inhibit eicosanoid biosynthesis by inhibition of both PLA2 and 5-lipoxygenase; Extracts available in the market as a cosmetic skin-care product (Look et al., 1986).
Eleutherobin	*Eleutherobia* sp.	Potent cancer cell inhibitor- breast, renal, ovarian and lung cancer cell lines (Long et al., 1998)

Table 4. List of a few bioactive compounds reported from the phylum Cnidaria.

1.3. In Focus: Anticoagulant Activity of Jellyfish Tentacle Extracts from the Jellyfish found on the Goan Coast of India

Hemostasis is critical to normal health by preventing blood loss following injury and contributing to the maintenance of the vascular system, which supplies oxygen and nutrients to all parts of the body as well as performing other essential tasks. The integrity of the system is compromised during injury and processes such as vasoconstriction, platelet aggregation, blood coagulation and fibrinolysis play a vital role in restoring the balance (Boon, 1993). Any sort of imbalance in its regulation can lead to either unwanted clot (thrombosis) or excessive bleeding (hemorrhage). Thrombosis leads to blockage of vessels and cutting off the blood supply to vital organs. There is significant interest in the development of new drugs due to these functions that can prevent or cure thrombosis. Platelet malfunction is also a major contributor to thrombus formation, thus inhibition of platelet aggregation is a prime target for developing new drugs (Garcia et al., 2010).

Thromboembolic disorders are of major clinical concern due to their high occurrence and consequences, which are often fatal. Venous thromboembolism (VTE) is projected to be the third most common cardiovascular disorder after coronary heart disease and stroke (S. Z. Goldhaber; 1992). Treatment of arterial and venous thrombotic phenomena represents a major medical challenge and the development of anticoagulant drugs denotes a revolution in medicine.

1.3.1. Hemostasis

Hemostasis is a complex system of reactions that are normally held in check by a dynamic interplay between the normal blood vessel endothelial surface and certain regulatory plasma proteins that prevent activation of platelets and the prothrombin pathway. It involves intricate chemical interactions among soluble clotting factors, blood elements and vascular tissues all working in concert to stop bleeding when blood vessels are damaged. Normal hemostatic reactions can be classified into several overlapping and sequential events: localized vasoconstriction at the site of vessel injury; adhesion of circulating platelets to the damaged vessel wall; development of a platelet

aggregate or plug; activation of the coagulation cascade leading to formation of fibrin which fortifies the platelet plug and finally activation of the fibrinolytic system, which digests the hemostatic plug, re-establishes vascular patency and allows growth of new vascular endothelial cells to complete the repair process. To control and limit any excessive or inappropriate activation of the hemostatic system, a complex interplay of physiological inhibitors and control mechanisms also exist (Figure 9).

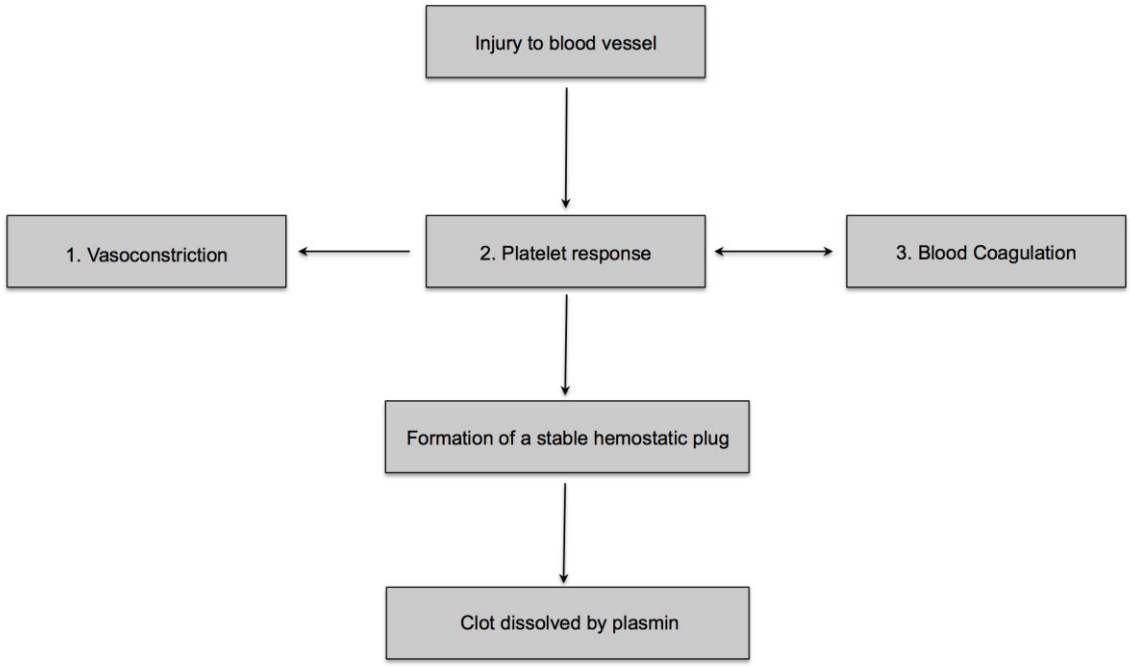


Figure 9. Three stages of hemostasis

First, localized vasoconstriction occurs as a response to a reflex neurogenic mechanism and to the secretion of endothelium-derived vasoconstrictors such as endothelin. Immediately after vasoconstriction, primary hemostasis occurs. At this stage, platelets are activated and adhere to the exposed sub-endothelial matrix. Platelet activation implicates both a change in shape of the platelet and the release of secretory granule contents from the platelet. The granule substances thus secreted result in the recruitment of other platelets, causing more platelets to adhere to the sub-endothelial matrix and to aggregate with one another at the site of vascular injury (Figure 10). Primary hemostasis ultimately results in the formation of a primary hemostatic plug.

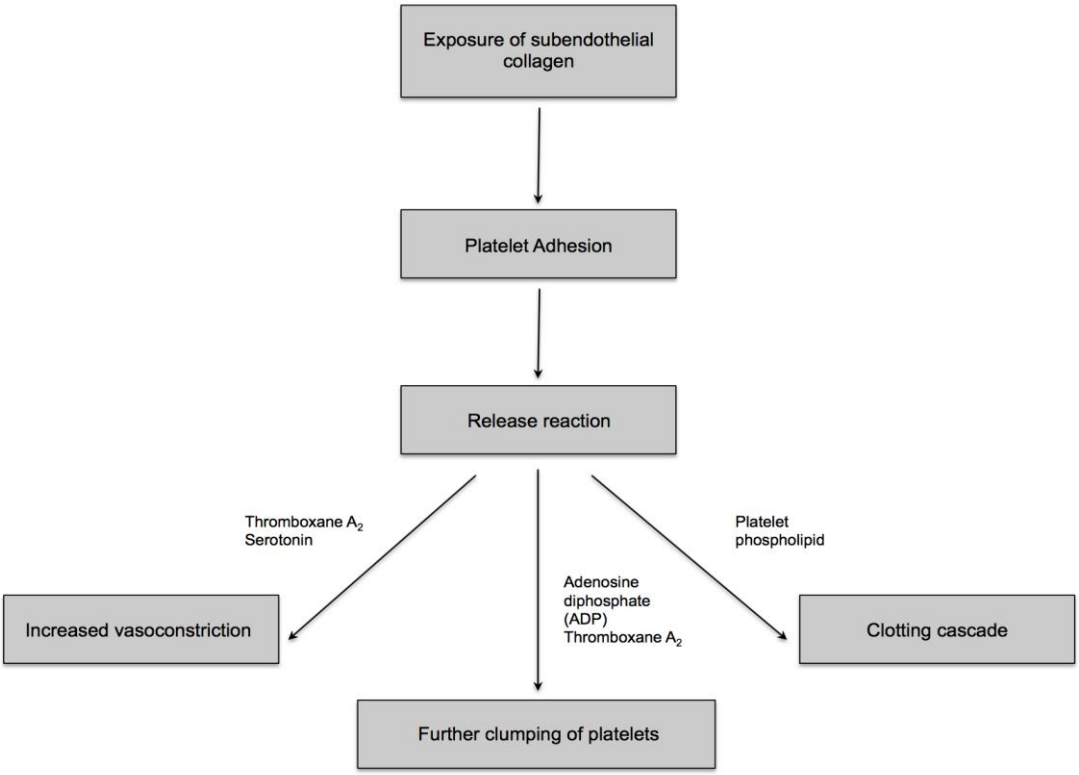


Figure 10. **Platelet response in hemostasis**

The goal of the final two stages of hemostasis is to form a stable, permanent plug. During secondary hemostasis, also known as the coagulation cascade, the activated endothelium and other nearby cells express a membrane-bound procoagulant factor called tissue factor, which complexes with coagulation factor VII to initiate the coagulation cascade. The final result of this cascade is the activation of a critical enzyme: thrombin. Thrombin serves two major functions in hemostasis: (i) it converts soluble fibrinogen to an insoluble fibrin polymer that forms the matrix of the clot and (ii) it induces more platelet recruitment and activation. Recent evidence indicates that fibrin clot formation overlaps temporally with platelet plug formation (secondary hemostasis overlaps primary hemostasis) and that each process reinforces the other. During the final stage, platelet aggregation and fibrin polymerization lead to the formation of a stable and permanent plug. The fibrinolytic system comes into play next. Fibrinolysis is actually initiated by vessel injury but inhibited by thrombin produced through secondary hemostasis. Thus, first the balance shifts towards procoagulation, i.e. fibrin production and then once the initiating stimulus for clot formation (vessel injury) is reduced or eliminated (i.e. the

injury is sealed by the clot), thrombin generation decreases and the balance then shifts to fibrinolysis. Tissue plasminogen activator (tPA) is the main plasminogen activator that is released by the injured endothelial cells. Factor XII also gets activated on exposure to sub-endothelial matrix proteins and forms a complex with high molecular weight kininogen (HMWK) and pre-kallikrein. This auto-activating complex yields kallikrein from pre-kallikrein and bradykinin (a potent vasoactive mediator) from HMWK. Bradykinin is a potent inducer of tPA release from endothelial cells and also stimulates release of nitric oxide and prostacyclin from endothelial cells (platelet inhibitors). Furthermore, both FXIIa and kallikrein are direct plasminogen activators but are weaker than tPA. Plasminogen binds to fibrin through lysine residues. Plasminogen activators (tPA, FXIIIa and kallikrein) cleave this bound plasminogen to plasmin, which also remains bound to the clot. The binding of plasminogen to the clot intensifies its conversion to plasmin (fibrin acts like a cofactor for tPA) and serves to localize fibrinolysis to the clot (Figure 11a and 11b). Plasmin cleaves and breaks down the fibrin clot thus restoring normal blood circulation. An imbalance in anti-fibrinolytic factors results in a hemorrhagic or thrombotic disorder (Rasche, 2001; Gale, 2011; Veersteeg, 2013)

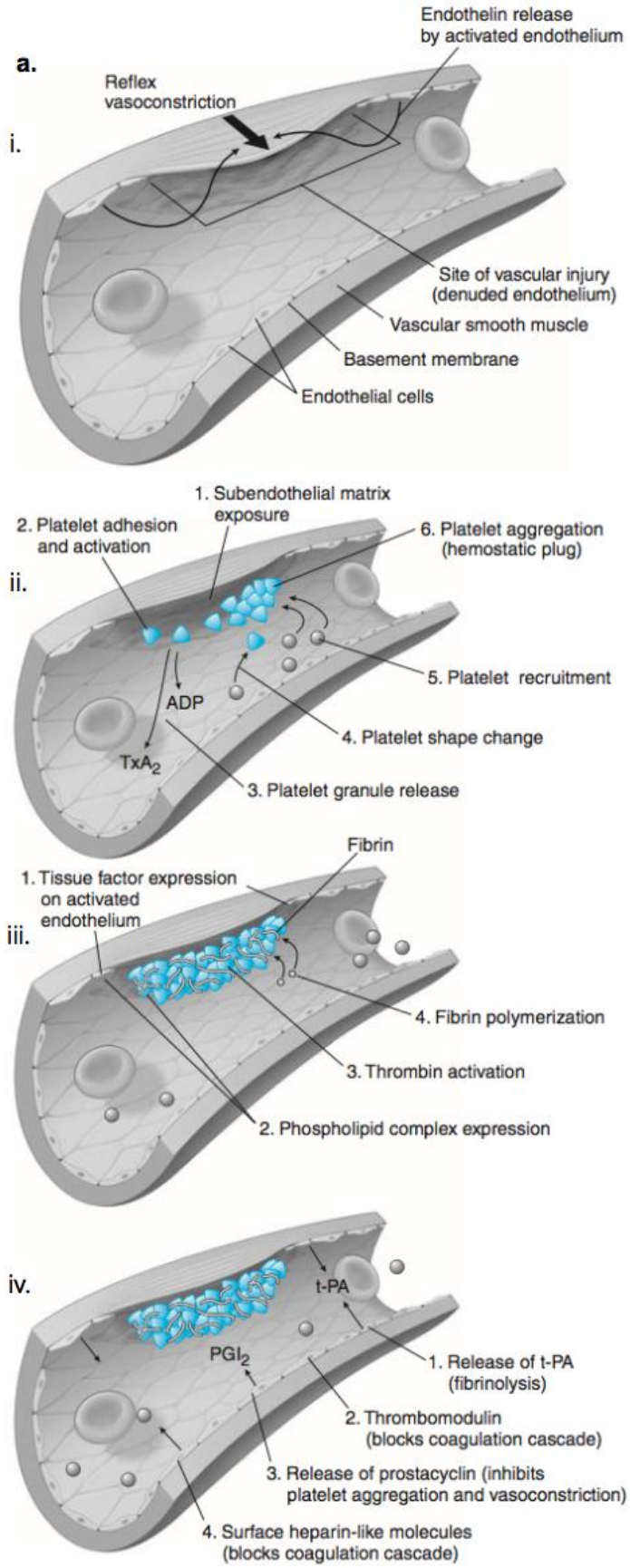


Figure 11a. Sequence of events in hemostasis. (Reproduced from Armstrong and David, "Pharmacology of hemostasis and thrombosis", *Principles of Pharmacology*. Ed. David E. Golan, 2008. 387-415, with permission from Lippincott Williams & Wilkins).

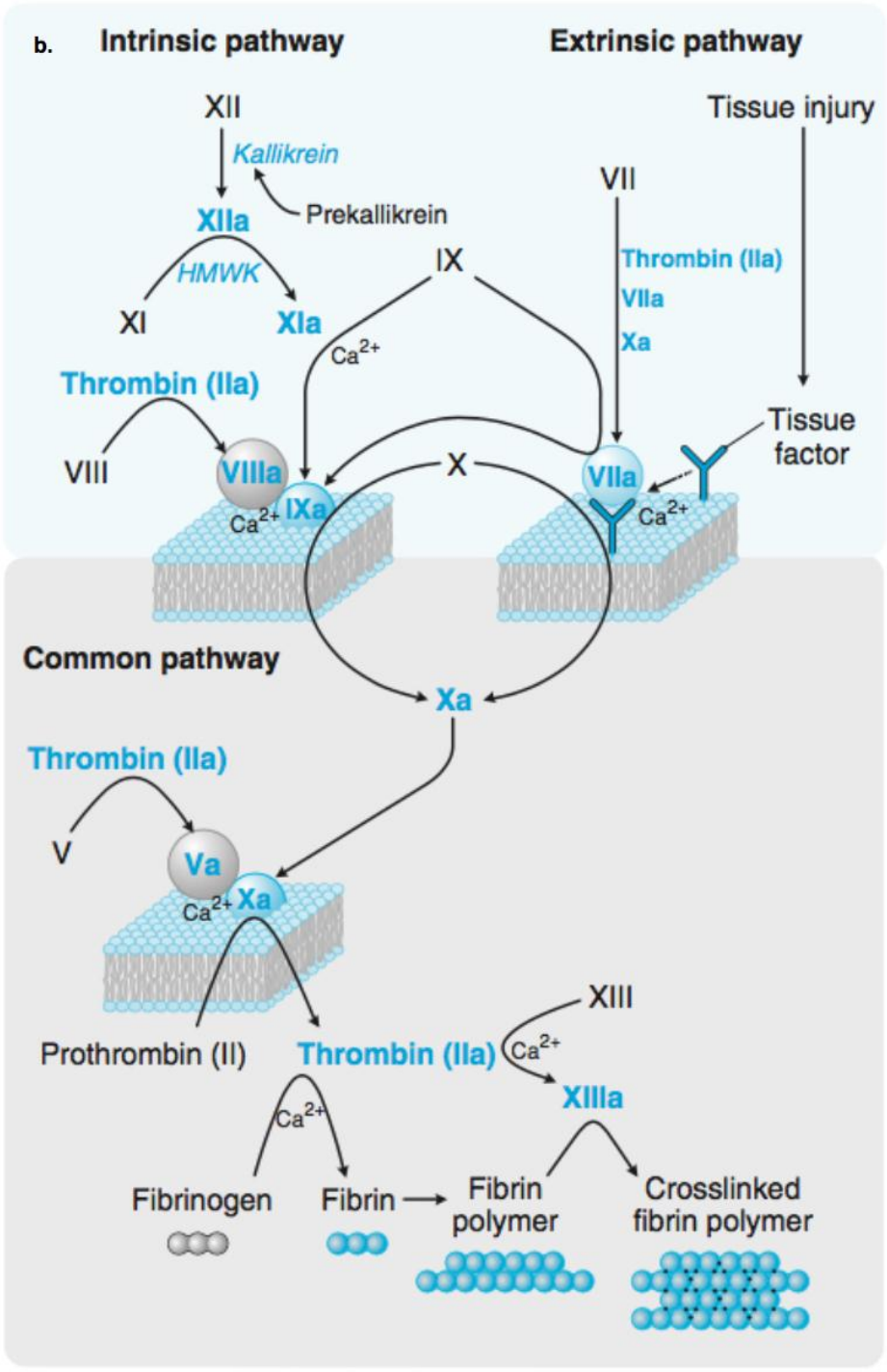


Figure 11b. Coagulation cascade. The intrinsic and extrinsic pathways converge at the level of factor X activation. The intrinsic pathway is an *in vitro* pathway, while the extrinsic pathway accounts for the majority of *in vivo* coagulation. Activated coagulation factors are shown in *blue* and indicated with a lower case ''a''. (Reproduced from Armstrong and David, "Pharmacology of hemostasis and thrombosis", *Principles of Pharmacology*. Ed. David E. Golan, 2008. 387-415, with permission from Lippincott Williams & Wilkins)

1.3.2. Drugs used in Disorders of Blood Coagulation

Hemostasis is achieved through a balance between endogenous procoagulant (e.g. thrombin) and anticoagulant influences (e.g. antithrombin) as well as mechanisms regulating fibrinolysis (e.g. plasmin and antiplasmin).

All of which originate from proteins of blood plasma, platelets and extravascular environment that could be altered due to a variety of medical conditions. Thrombosis could be driven by: (i) blood hypercoagulability, (ii) blood stasis and (iii) vessel wall injury with differential mechanisms being engaged under low shear (venous) or high shear (arterial) conditions. This pathogenetic diversity requires a matching spectrum of precise medical strategies, optimized for each specific condition. Thrombosis can be arterial or venous; the major difference between the two is the underlying rheology. Arterial thrombosis occurs under high flow velocity and platelets have developed unique mechanisms to interact with damaged blood vessels under high shear stress. Antiplatelet drugs are the mainstay of treatment of arterial thrombosis. Venous thrombosis occurs in areas of stasis and activation of soluble coagulation factors play a major role in the pathogenesis. Anticoagulants rather than antiplatelets are the main therapeutic option in venous thrombosis. Drugs used to decrease hemostatic activity in the blood can be divided into three categories (Rang et al., 1999):

- That interfere with coagulation (Anticoagulant Drugs)
- That alter platelet function (Antiplatelet Drugs)
- That cause or increase the rate of breakdown of stabilized fibrin (Thrombolytic Drugs)

1.3.2.1. Anticoagulant Drugs

These are used to reduce coagulability of blood and are classified into two groups: (i) Heparin (low molecular weight Heparin, Danaparoid, Lepirudin and Heparan sulfate) and (ii) Oral anticoagulants (Warfarin, Dicumoral, Acenocoumarol and Phenindione). Heparin class of drugs act by inhibiting the thrombin mediated conversion of fibrinogen to fibrin, the aggregation of platelets by thrombin, activation of fibrin stabilizing enzyme, activated factors XII, XI, IX, X and II. Warfarin and other oral anticoagulants act as vitamin K antagonist and impair the generation of active vitamin K, decreasing the amounts of vitamin K dependent coagulation factors. Warfarin, a coumarin derivative is the most commonly used oral anticoagulant (De Caterina R., 2009).

1.3.2.2. Antiplatelet Drugs

Platelet function is regulated by three categories of substances: (i) agents generated outside the platelet interact with platelet membrane receptors, eg, catecholamines, collagen, thrombin and prostacyclin (ii) agents generated within the platelet interact with membrane receptors, *eg*, ADP, prostaglandin D2, prostaglandin E2 and serotonin (iii) agents generated internally in the platelet that act within the platelet, eg, prostaglandin endoperoxides and thromboxane A2, the cyclic nucleotides cAMP and cGMP and calcium ion (Ji X., 2011; DeVile and Foëx, 2010). From this list of agents, several targets for platelet inhibitory drugs have been identified; inhibition of prostaglandin synthesis (aspirin), inhibition of ADP- induced platelet aggregation (clopidogrel and ticlopidine) and blockade of glycoprotein IIb/IIIa receptor on platelets (abciximab, tirofiban and eptifibatide).

1.3.2.3. Thrombolytic Drugs

These agents activate the conversion of plasminogen to plasmin, a serine protease that hydrolyzes fibrin and dissolves the clots. Conventional nonselective fibrinolytic agents include streptokinase and urokinase, which act by converting inactive plasminogen to active plasmin. Recombinant tissue plasminogen activator (tPA), Ateplase, Reteplase and Tenectaplas are fibrin selective agents that act by endogenously activating plasminogen (Ali et al., 2014).

1.3.2.4. New Oral Anticoagulants (NOACs)

In recent years, several new oral anticoagulants (NOACs) have been introduced and more drugs are currently under development. These drugs have given patients alternatives to heparin and warfarin. The NOACs fall into two broad categories: (i) the oral direct factor Xa (FXa) inhibitors (rivaroxaban and apixaban) and (ii) the oral direct thrombin inhibitor (dabigatran etexilate, the prodrug of dabigatran). The NOACs differ from vitamin K antagonists (VKAs) in their action mechanism because of direct inhibition of proteins of the coagulation cascade (Meisbach and Seifried, 2012; Altman R., 2014). They have more predictable pharmacokinetics leading to fixed and convenient dosing regimens, obviating the need for routine monitoring and rapid onset of

action (Eriksson et al., 2009) and more importantly high efficacy and low risk of bleeding (Poulsen et al., 2012). Some of their limitations are the higher cost, limited monitoring and the lack of a specific antidote (Miesbach and Seifried, 2012).

1.3.3. Need for New Anticoagulants

Anticoagulants have been known for many years to produce a striking decrease in the rate of stroke. Heparin products, which include unfractionated heparin (UH), low-molecular-weight heparin (LMWH) and vitamin K antagonists (VKA) like warfarin, are the most commonly used treatments and they have shown good efficacy and cost effectiveness. While these agents have been used for many years, each class has its drawbacks and are far from being "ideal" anticoagulants (Table 5). They also prevent clotting in locations and situations where clotting is desirable. In other words, they can cause bleeding. The anticoagulant effect of these drugs must be carefully monitored with periodic blood tests. If the effect is too small, it will fail to prevent strokes; if the effect is too high, it will cause excess bleeding. So the dosage of these drugs must be carefully adjusted to keep the *blood thinning* effect in the appropriate range. Although several promising new anticoagulants have been evaluated, the role of many of these agents remains to be delineated. Despite the widespread use of anticoagulant drugs for the prevention and treatment of arterial and venous thrombosis, thrombotic diseases continue to be a major cause of death and disability. For this reason, the search for new anticoagulants that may be able to maintain the balance between efficacy and safety continues.

Characteristic(s)	Benefits
Orally active	Ease of administration
Rapid onset of action	Obviates need for overlap with a parenteral anticoagulant
No food or drug interactions	Simplified dosing
Predictable anticoagulant effect	No routine coagulation monitoring
Extra-renal clearance	Safe in patients with renal insufficiency
Rapid offset of action	Simplifies management in case of bleed or need for intervention and minimizes need for an antidote
Availability of a safe antidote	Provides rapid reversal in case of hemorrhagic event or need for an intervention
Reasonable cost	Improved access
Favourable net clinical benefit	Treatment benefit outweighs risk

Table 5. Characteristics of an ideal anticoagulant. (Adapted from Bounameaux, 2009 and Eikelbloom et al., 2010)

1.3.4. Anticoagulants from Venomous Animals

Various animals have developed mechanisms to target the hemostatic system and exploit this vulnerability for their own ends. In some snake species, such mechanisms are used to immobilize and kill the prey/victim and defensive strategy against attacks by larger animals. In leeches or insects like mosquitoes and ticks, they are used to feed continuously on blood as food. These mechanisms are not limited to procoagulant and anticoagulant agents that affect the coagulation cascade and platelet aggregation but also alter the vasodilatory responses. In all these animals, the mechanisms have evolved to perfection over millions of years, in parallel with the development of their hosts, to allow their survival. Many of the components, present in saliva and venom, target critical hemostatic pathways. They present area of great interest to study the mechanisms of these pathways and their possible applications to modify deviant behavior. Over the last few decades, significant progress has been made in understanding the structure-function relationships as well as the mechanism of action of a number of exogenous factors from various animals that affect blood coagulation, platelet aggregation and vasodilation. These exogenous factors have contributed notably to the development of research tools as well as providing new therapeutic agents.

Venomous creatures are abundant sources of anticoagulants and thrombolytic agents that include direct thrombin inhibitors, disintegrins, fibrinolytic compounds and plasminogen activators (Weitz and Crowther, 2003). Integrilin (eptifibatide, barbourin) is a disintegrin from the venom of the Pygmy rattlesnake (*Sistrurus miliarus barbouri*) that inhibits platelet aggregation by binding to the fibrinogen receptor (integrin αIIbβ3) via a Lys-Gly-Asp recognition sequence. It was approved in 1998 by the U.S. FDA as an anticoagulant in patients with acute coronary syndrome and for patients undergoing angioplasty. Other snake venom disintegrins use the more common Arg-Gly-Asp recognition sequence to interact with their target receptor. A mimetic of echistatin (Aggrastat®) was observed to reduce the rate of thrombotic cardiovascular events. It belonged to the class of antiplatelet called glycoprotein IIb/IIIa inhibitors. It obtained FDA approval for anticoagulant use in 1998. Viprinex was being evaluated as a late stage

therapy for the management of acute ischemic stroke but was recently discontinued due to adverse side effects. Fibrolase from the Southern copperhead snake, belonging to the class of nonglycosylated metalloproteinases is a fibrinolytic enzyme that cleaves fibrin (Stocker and Bode W, 1995). Alfimeprase, a recombinant fibrinolytic enzyme derived from fibrolase, is in clinical trials for the treatment of peripheral arterial occlusions (Jones et al, 2001). Desmoteplase, a salivary plasminogen activator from the vampire bat *Desmodus rotundus* (DSPA alpha 1), which demonstrates 85% sequence similarity with human tissue plasminogen activator. It was under clinical trials for use in acute ischemic stroke but was terminated in 2014 (W. D. Shleuning, 2003; Reddrop et al., 2005). Hirudin, a polypeptide from the saliva of the medicinal leech, exemplifies antithrombin anticoagulants. It forms a complex with thrombin via its acidic C-terminus and thereby prevents thrombin's activity. A recombinant form of hirudin -Desirudin is effective in the treatment of heparin-induced thrombocytopenia. A variety of derivatives of hirudin are available such as hirugen (a synthetic C-terminal peptide fragment of hirudin), hirulog (a derivative of hirugen) and argatroban (heterocyclic peptidomimetic) (D. Kikelj, 2003). A novel anticoagulant was isolated from the nematode, *Ancylostoma canium* called nematode anticoagulant protein c2 (NAPc2) that binds to factor X and Xa at a noncatalytic site (G. J. Broze, 1995; Cappello et al., 1996; Duggan et al., 1999; Lee at al., 2001). Factor Xa bound NAPc2 is an effective inhibitor VIIa-tissue factor complex, akin to tissue factor pathway inhibitor (TFPI) and has a long half-life of almost 50 hours. NAPc2 is currently being evaluated in clinical trials (Table 6).

The increasing life expectancy of world population, coupled with recent changes in life style, has led to significant increase in cardiovascular and hematological disorders. This in turn has given impetus to the search for novel agents from various sources that interfere with cardiovascular and hematological processes and could be used to regulate these. Thus, exogenous factors are excellent sources of such novel therapeutic lead molecules and offer exciting field of research.

Name	Source	Target	Use
Integrilin (Barbourin, Eptifibatide)	*Sisturus milliarus barbouri* (Snake)	Disintegrin/ Fibrinogen receptor (integrin αIIbβ3)	Anticoagulant in acute coronary syndrome
Salmosin	*Agkistrodon halys brevicaudus* (Snake)	Disintegrin	Anticoagulant
Rhodostomin (Kistrin)	*Calloselasma rhodostoma* (Snake)	Disintegrin	Anticoagulant
Ancrod (Viprinex™)	*Calloselasma rhodostoma* (Snake)	Disintegrin	Anticoagulant for acute ischemic stroke
Echistatin	*Echis carinatus* (Snake)	Disintegrin	Anticoagulant
Dendroaspin (Mambin)	*Dendroaspis jamesoni kaimose* (Snake)	Disintegrin	Anticoagulant
Crotavarin	*Crotalus viridis* (Snake)	Disintegrin	Anticoagulant for infectious endocarditis
Hirudin and Desirudin (recombinant hirudin)	*Hirudo medicinalis* (Medicinal leech)	Thrombin	Heparin-induced thrombocytopenia
Fibrolase	*Agkistrodon contortrix contortrix* (Snake)	Fibrinolytic enzyme	Peripheral arterial occlusions
Desmoteplase	*Desmodus rotundus* (Bat)	Plasminogen activator	Acute ischemic stroke

Table 6. Anticoagulant and thrombolytic agents from venom peptides. (Adapted from Takacs and Nathan, 2014)

1.3.5. Anticoagulants from Jellyfish – Is it Possible?

Envenomation by jellyfish of some species is known to cause hemolysis in severe sting cases. Some hemolytic toxins have also been isolated from jellyfish venom or tentacle extracts. Coagulopathy in case of jellyfish envenomation is rarely noted and least studied and literature on hemotoxic components of jellyfish venoms is scanty.

The hemolytic, pore-forming toxin equinatoxin III (a cardiotoxic protein from the sea anemone *Actinia equina*) has demonstrated the ability to pass through a large tissue mass in an animal model of envenoming (Suput et al. 2001). Pore- forming toxins in the venom of the box jellyfish *C. fleckeri* have been demonstrated to form pores of 50–80 nm on cell membranes of myocytes and are very similar to those observed for *Physalia physalis* venom-treated cells (Edwards et al. 2002). Reports of hemolytic activity in some jellyfish venoms initiated attempts to purify the responsible toxins. Brinkman and Burnell (2007) reported purification of two of the most abundant hemolytic toxins from the nematocysts of box jellyfish, *Chironex fleckeri*. They were named *C.*

fleckeri toxin-1 (CfTX-1) and *C. fleckeri* toxin-2 (CfTX-2). Both toxins have similar molecular weights of around 43 kDa. The amino acid sequences of the mature CfTX-1 and CfTX-2 share homology with the CrTXs, CaTX-A, and CqTX-A isolated from the deadly Okinawan sea wasp *Chiropsalmus quadrigatus* (also known as *Chironex yamaguchii*).

Coagulopathy is not a frequently reported phenomenon in marine envenomation and reports on coagulopathy in jellyfish envenomation are not available. However, intracranial hemorrhage is one of the symptoms in severe Irukandji syndrome, named after the Irukandji tribe who live in north Queensland, Australia. Whether this hemorrhage is due to action of hemorrhagic components of the venom or due to severe hypertension, is not clear. The most predominant toxins found in jellyfish venom are members of SVMP disintegrins. These share ancestral genetic relationship with endogenous matrix metalloproteinases (Moura-da-Silva et al. 2007). The members of this family are known to disrupt blood vessel integrity and cause blood coagulation and local tissue damage. Lee et al. (2011) reported the proteolytic activity in the venoms of four scyphozoan jellyfish species, including *Nemopilema nomurai*, *Rhopilema esculenta*, *Cyanean ozakii* and *Aurelia aurita*. Venoms from all these species showed gelatinolytic, caseinolytic and fibrinogenolytic activity. These activities varied qualitatively and quantitatively based on the species. It also suggested that all of these proteases in the venoms were metalloproteinases as 1,10-phenanthroline could inhibit them. They demonstrated that the relative cytotoxic potency of jellyfish venom appears to be closely associated with their proteolytic activity, suggesting the metalloproteinase in jellyfish venom may contribute to its cytotoxicity. These observations and reports are suggestive of the possibility of presence of anticoagulant components in jellyfish.

1.4. Jellyfish and Goa

Every year after the monsoon (October-November) jellyfish blooms are a common site on the Goan coast. But in past few years their sighting and incidence has increased manifold. In 2016, a longer stretch of the state's coastline was hit by an invasion of these gelatinous animals (Figure 12). These mass occurrences severely affect swimmers, fisheries, aquaculture,

tourism and shipping industry. Jellyfish blooms are on the rise, with the past several decades seeing a dramatic increase and temporal shift in jellyfish distributions around the world. They survive best in warmer, nutrient-rich and oxygen-poor water. The apparent increase in size and frequency of such blooms is convincingly linked to human activity, from global warming to overfishing, habitat destruction to introduction of fertilizers, toxic chemicals and trash. Jellyfish blooms occur when ocean currents, nutrients, prey availability and water temperature bring the jellyfish together. Changes in these factors can cause jellyfish blooms to form more frequently (Mills, 2001; Hays et al., 2005).

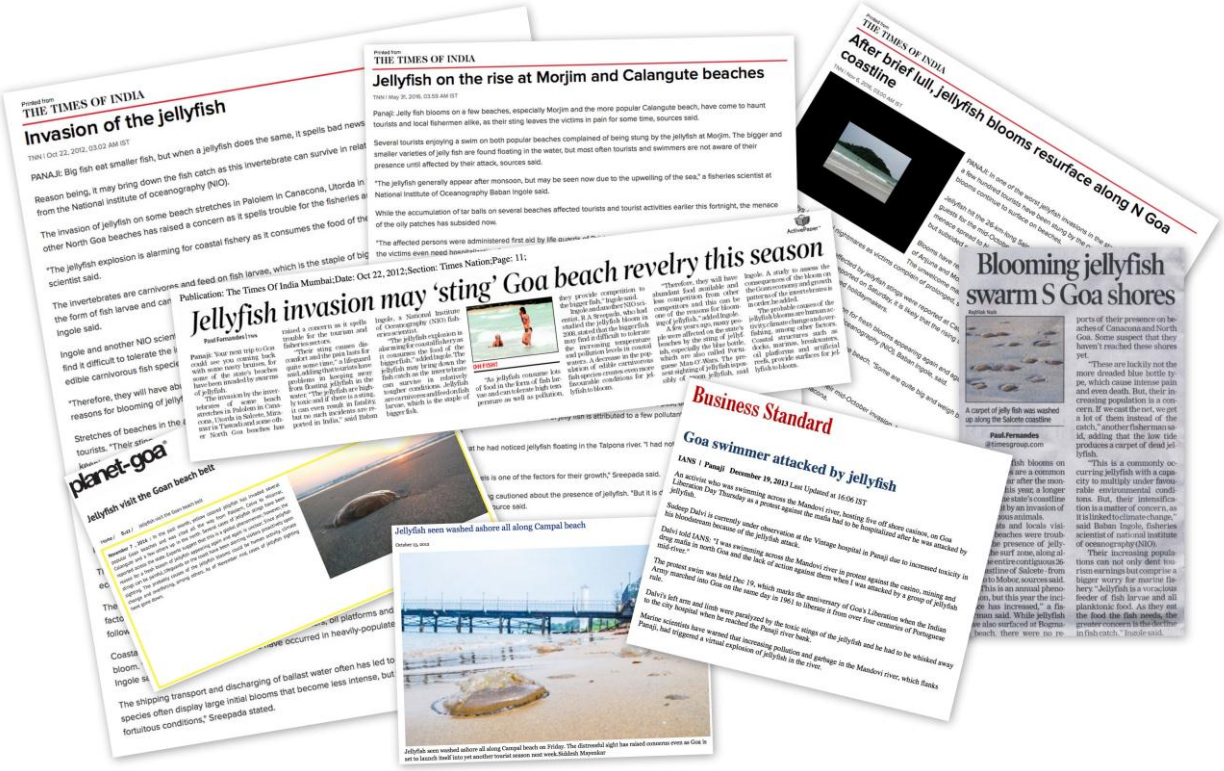


Figure 12. Local newspaper snippets reporting the jellyfish invasion on the Goan coast and its implications. (2012-2017)

Some of the most commonly sighted jellyfish on the Goan coast are *Aurelia aurita* (Moon jellyfish), *Phsalia physalis* (Portuguese Man o' War/Bluebottle jellyfish) and *Rhizostoma pulmo* (Barrel/dustbin lid jellyfish) (Figure 13).

Figure 13. Sample collection sites. *Rhizostoma pulmo* sighted at Mobor beach (L) and Velsao beach (R). (Photo courtesy: Akriti Rastogi)

1.4.1. Gaps in Existing Research

Thrombosis has been and continues to be a major health problem leading to mortality. Anticoagulants are pivotal for the prevention and treatment of thromboembolic disorders. However, the nonspecific mode of action and continuous monitoring after consumption of the current anticoagulants accounts for their therapeutic limitations in maintaining a balance between thrombosis and hemostasis. These limitations have opened up new frontiers for research and development of new anticoagulants that target specific coagulation enzymes or a particular step in the clotting process.

Oceans cover more than 70% of earth's surface. The marine environment covers a wide thermal range (from the below freezing temperatures in Antarctic waters to about 350°C in deep hydrothermal vents), pressure range (1-1000 atm), nutrient range (oligotrophic to eutrophic) and it has extensive photic and non-photic zones. This all-encompassing variability has facilitated extensive speciation at all phylogenetic levels, from microorganisms to mammals. The biodiversity in the marine environment far exceeds that of the terrestrial environment. Because of the immense biological diversity in the sea as a whole, it is increasingly recognized that a huge number of natural products and novel chemical entities exist in the oceans, with biological activities that may be useful in the quest for finding drugs with greater efficacy and specificity for the treatment of many human diseases. With the continuous exploitation of the marine environment, cnidarians being the

largest phylum of generally toxic animals, have not received as much scientific attention as those of some marine animals (i.e. cone snails, sponges, molluscs, tunicates, marine microorganisms).

Biochemical and pharmacological profile of jellyfish venom is not well studied. Very little effort has been made till now to discover potential drugs from jellyfish. If purified jellyfish proteins have significant anticoagulant activity then they could be used as a cure/treatment for thrombosis.

1.4.2. The Idea

Researchers are currently involved in exploiting the untapped potential of the marine ecosystems for finding cures for varied life threatening diseases (Haefner, 2003; Wallace, 1997). Review of literature has revealed no reports of anticoagulant properties of jellyfish extracts. The paucity of information in this field and the recent trends of exploring the marine ecosystems for cures formed the basis of this research. It encapsulates exploration, and establishment of the anticoagulant activity in the tentacle extracts of moon jellyfish and barrel jellyfish.

1.4.3. Species of Interest

This study was conducted on Moon jellyfish (*Aurelia aurita*) and Barrel jellyfish (*Rhizostoma pulmo*). These jellyfish belong to class Scyphozoa of phylum Cnidaria (Figure 14). Scyphozoa represent true jellyfish and are most frequently responsible for human-marine animal conflict on the beach.

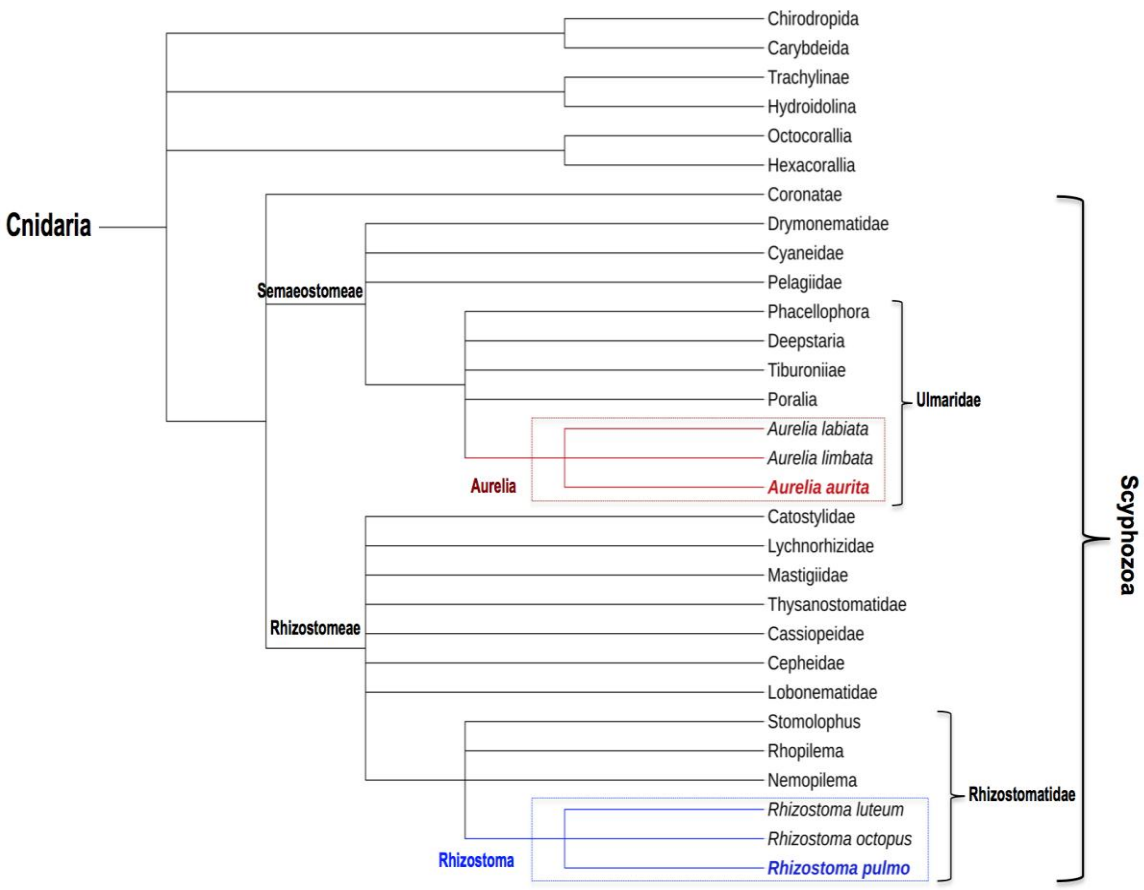


Figure 14. Phylogenetic tree of phylum Cnidaria highlighting the species of interest. This phylogenetic tree was constructed using Interactive Tree Of Life (http://itol.embl.de), a web-based tool for the display, manipulation and annotation of phylogenetic trees.

1.4.4. Objectives

To address some of the gaps in existing research on study of jellyfish tentacle extract the following objectives are proposed:

1. To study anticoagulant activity in jellyfish tentacle extract.
2. To investigate other biological activities in jellyfish tentacle extract
3. Characterization of purified toxins on cultured cell lines.

CHAPTER 2

ANTICOAGULANT ACTIVITY OF MOON JELLYFISH TENTACLE EXTRACT

2.1. Introduction

Most jellyfish species are free-moving planktons that drift with ocean currents and might appear at one location only for a few weeks in a given year. Jellyfish blooms appear in irregular numbers all along the coast of India. There is a bloom during October and November along the Goan coast. Global warming and consequent increase in temperature has been linked with an increase in sightings as well reports of jellyfish reaching the shores of Goa. Some of the most common species that infest the Goan shores are *Aurelia aurita* (Moon Jellyfish) and *Rhizostoma pulmo* (Barrel Jellyfish).

Aurelia aurita belongs to the phylum *Cnidaria*, which are characterized by having a polyp and medusa stage, radial symmetry, planula larvae, cnidia and tentacles around the mouth. Classified under the class of *Scyphozoa*, it qualifies as a “true jelly”, that is primarily found in medusoid morphology, with asexual reproduction in polyp stage via strobilation (Dawson and Martin, 2001). It is of the order *Semaeostomeae*, family *Ulmaridae*, genus *Aurelia* and species *aurita*. It is often called “moon jelly”, after its milky, translucent colour and shape.

Moon jellies are the most common and widely recognized type of jellyfish, which can be found in the Atlantic, Arctic, Pacific and Indian Oceans near the coasts. The Moon jelly ranges in size from 5 cm to 40 cm across. It is easily recognizable by its four pink or purple interfolded gonads that form a horseshoe or circular shape in the center of the bell. Its bell is thicker towards the middle, thinning towards the edge. It has short tentacles, which generally do not produce a sting in most people. These tentacles are located along the edge of the umbrella and four short arms situated around the mouth for catching food (Figure 15). Like many other species of jellyfish, the moon jelly’s ability to move by itself is limited, so it is subjected to the water currents of the ocean. This makes them more susceptible to being washed ashore by currents and tidal action. Moon jellyfish stinging cells, though poisonous for small marine animals but are relatively benign to human and cause only mild stinging sensation in a

sting victim. The discomfort is fairly localized and does not persist for long. The colour of the moon jellyfish depends on its diet. Brine shrimps give it a yellow tinge, while other crustaceans give it a pinkish or purplish tinge.

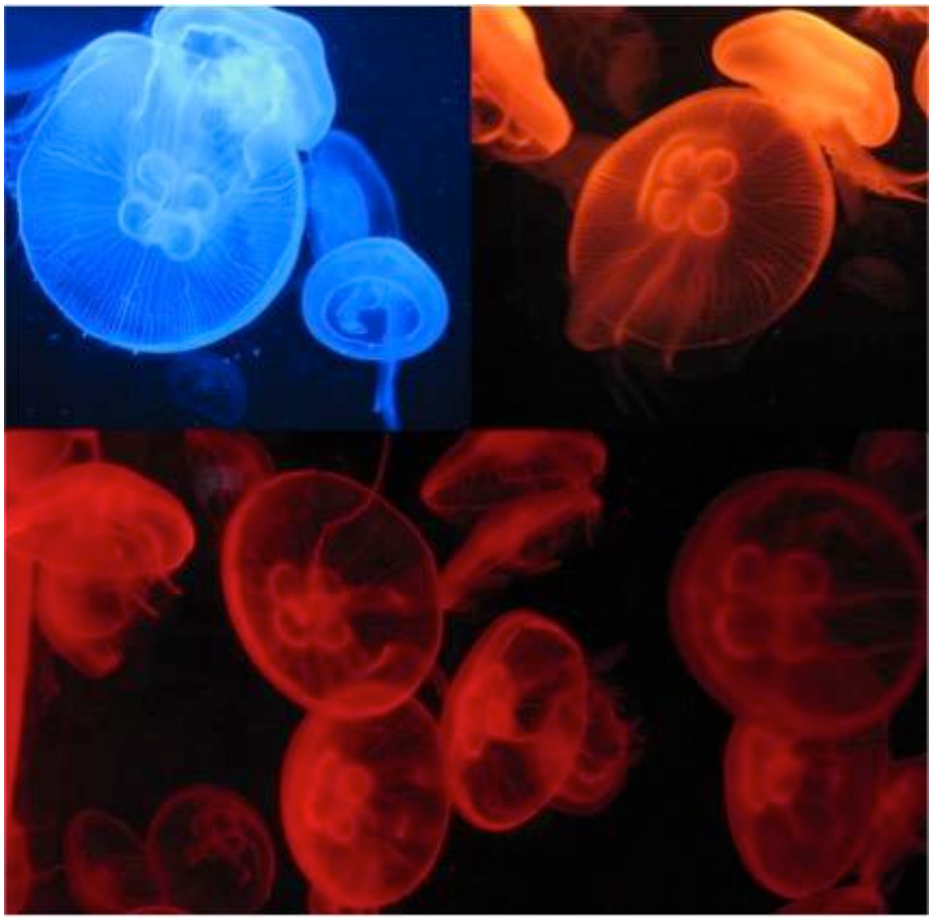


Figure 15. Moon jellyfish under artificial lighting in a marine aquarium. (Photo courtesy: Dibakar Chakrabarty at S.E.A aquarium, Singapore)

Two marine natural products have been purified from the jellyfish *Aurelia aurita* so far: *Aurelin* a novel antimicrobial peptide (AMP) that exhibited activity against gram-negative and gram-positive bacteria. It was found to have β-sheet structure and partial similarity with defensins and K^+channel-blocking toxins of sea anemones (Ovchinnikova et al., 2006). A novel mucin, Qniumucin, was isolated from moon jellyfish and was found to exhibit three times more moisturizing and hygroscopic activity than hyaluronic acid, which is extensively used as a moisturizing agent in cosmetics (Ushida et al., 2007). The Chemical and biophysical properties of Qniumucin make it a useful protective biomaterial with lubrication and moisturizing effects as well as a starting material for large-scale industrial synthesis of bioactive mucin-like glycoproteins (Kim and Karadeniz, 2015).

Tentacle extract from moon jellyfish (*Aurelia aurita*) was screened for anticoagulant activity and its effects on ADP and collagen dependent platelet aggregation.

2.2. Sample Collection

Specimens of *A. aurita* were collected from Mobor and Agonda beaches of the Goan coast during the month of October (Figure 16).

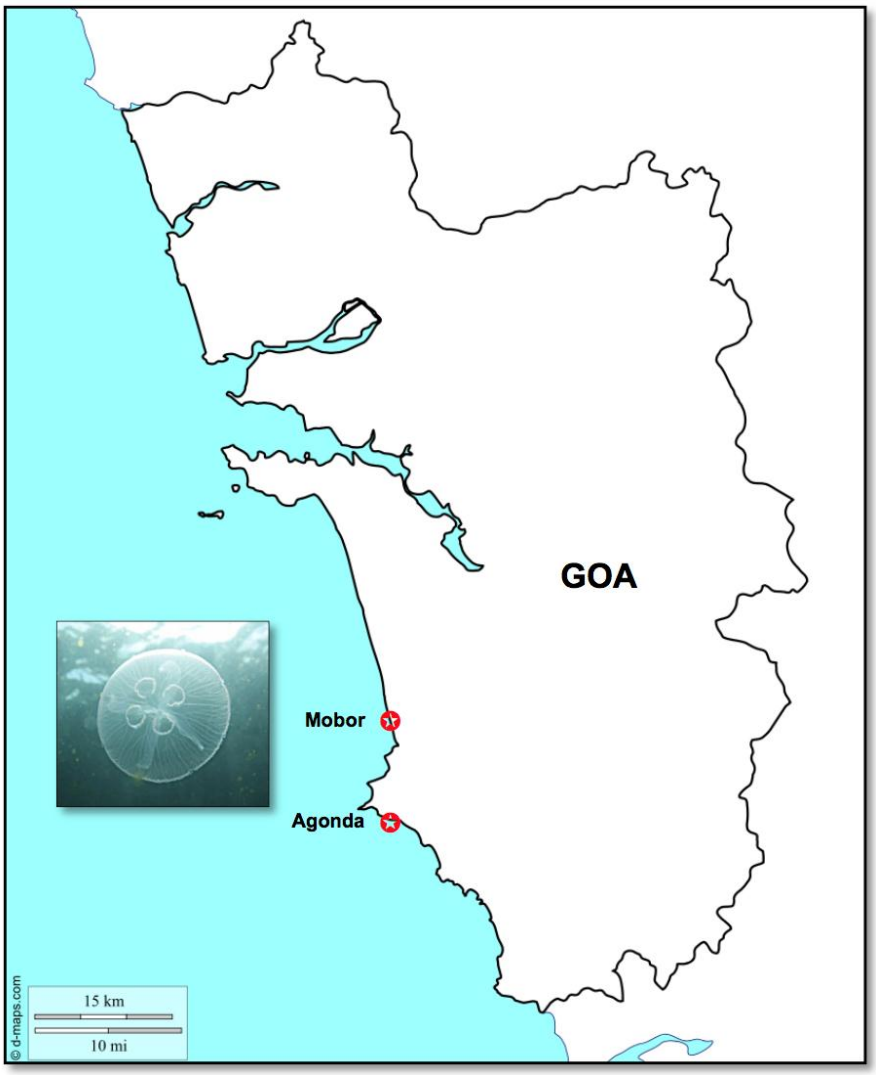


Figure 16. Sample collection sites for *Aurelia aurita* on the South-Goan coast of India.

2.3. Materials and Methods

Fibrinogen and thrombin from bovine plasma, Phenylmethylsulfonyl fluoride (PMSF) and Ethylenediaminetetraacetatic acid (EDTA), were purchased from Sigma-Aldrich, India. All fine chemicals used, were purchased from Fisher Scientific and rest of the reagents used were of analytical grade.

2.3.1. Preparation of Tentacle Extract

The tentacles were manually excised within 1 h of collection and stored in 0.85% saline at -20°C. Frozen tentacles were autolyzed at 4°C in 0.85% saline (9:1 v/v) for 4 days. Resultant fluid was clarified by centrifugation at 20,000 g for 1 h at 4°C. This clarified fluid was called Jellyfish Tentacle Extract (JFTE) and was used as the working material for further investigations. It was stored at -80°C in 2 ml aliquots, till used.

2.3.2. Protein Estimation

The protein concentration of JFTE was estimated by Bradford's method involving the binding of Coomassie brilliant blue G-250 to the protein molecules (Bradford, 1976). A standard curve was constructed using Bovine Serum Albumin (BSA) as standard protein.

2.3.3. Component Proteins and their Molecular Weight Determination

Different component proteins of JFTE and their molecular weights were assessed by electrophoresis (SDS-PAGE) using 12% resolving gel and 5% stacking gel. JFTE was prepared in non-reducing sample buffer (4% SDS, 125 mM Tris–HCl, pH 6.8, 20% glycerol, 0.01% bromophenol blue). The extract (5µg) was electrophoresed at 50V and 70V for stacking and resolving gel, respectively. The molecular weight ladder, 29–205 kDa (GeNeI unstained protein molecular weight marker - higher range) was run along side JFTE for molecular weight estimation. The protein bands were stained with 1% Coomassie brilliant blue R250 in 7.5% acetic acid and 10% methanol overnight. The gel was then destained by repeated washings with 7.5% acetic acid and 10% methanol.

2.3.4. Assay of Fibrinolytic Activity

Fibrinolytic activity of JFTE or its protein fractions was monitored by a modification of the fibrin plate method of Astrup and Mullertz, 1952. Bovine Fibrinogen fraction I (3.3 mg) was dissolved in 0.2 ml of 20 mM potassium phosphate buffer, pH 7.4. Ammonium sulphate was added to above solution to a final concentration of 70 mM. Five microliters of thrombin was added to the above solution and transferred immediately to a 0.5 ml microfuge tube. The solution was allowed to clot by incubating for 2 h at 25°C (Chandrasekhar and Chakrabarty, 2011). JFTE was applied on the surface of the clot and incubated at 37°C for 24 h. Fibrinolytic activity of the sample was observed by the liquefaction of the clot. 0.85% saline was used as negative control.

2.3.5. Assay of Fibrinogenolytic Activity

Fibrinogenolytic activity was confirmed by incubating fibrinogen fraction I (2 mg/ml) with JFTE and 0.85% saline (control) for different time intervals and doses at 37 ºC. The incubated mixtures were subjected to SDS-PAGE on 12% separating gel with a 5% stacking gel. The protein bands were viewed by staining with 1% Coomassie brilliant blue, R250. Fibrinogenolytic activity was monitored by comparing position and appearance of specific bands with that of fibrinogen incubated with 0.85% saline only (Bos *et.al.,* 1997).

Above activity of JFTE was also estimated after treatment with 2 mM Ethylenediaminetetraacetatic acid (EDTA) a metalloprotease inhibitor, freshly prepared 1mM Phenylmethylsulfonyl fluoride (PMSF) a serine protease inhibitor or exposure to 100°C for 1 min in a boiling water bath.

2.3.6. Assay of Hemolytic Activity

Blood was collected aseptically from healthy volunteers in 0.85% saline and centrifuged at 3000 rpm for 3 min. Supernatant was discarded and the pellet containing RBCs were washed thrice with normal saline. RBC suspension (0.3 ml) was taken in each tube to which 0.2 ml (50 µg) of JFTE was added. Distilled water and 0.85% saline were added to RBC suspension as positive and negative control, respectively. All the tubes were then incubated for 1 h at 37 °C and centrifuged at 3000 rpm for 5 min. Absorbance of the supernatants were

measured at 540 nm. Values obtained with positive control represented 100% hemolysis (Chakrabarty, *et al.,* 2000).

2.3.7. Assay of Phospholipase Activity

Presence of phospholipase A (PLA) activity was tested using egg yolk as substrate (Neumann and Habermann, 1954). Five micrograms of JFTE was added to 2 ml of egg yolk suspension, mixed well and incubated at 37 °C for 1 h. Incubated samples were then placed in a boiling water bath and time required for coagulation of the samples was then noted. 0.85% saline and 5 µg Russell's viper venom were used as negative and positive controls, respectively.

2.3.8. Platelet Aggregation Studies

Blood was freshly collected from healthy 'O' positive human volunteers. Blood was collected in vials containing nine parts of blood and one part of 3.8% sodium citrate. Platelet aggregation was stimulated with 10 µM Adenosine di-phosphate (ADP) and 2µg/ml of collagen, respectively. Dose dependent effect of JFTE on ADP and collagen induced aggregation was monitored by adding JFTE at the time of incubation. Platelet aggregation was expressed as the change in electrical impedance and ~~is~~ expressed in ohms. Aggregation was recorded for 7 min and analyzed using AGGROLINK® software.

2.3.9. Assay of Anticoagulant Activity

Anticoagulant activity of JFTE was determined by calculating recalcification and thrombin time. 100 µl of 50 mM Tris-HCl buffer (pH 7.4), 100 µl plasma and 50 µg JFTE were pre-incubated for 2 min at 37 °C. Clotting was then initiated by adding 50 µl of 50mM $CaCl_2$ (Langdell et al., 1953). 50 µg JFTE was incubated with 100µl of plasma and 100 µl of 50 mM Tris-HCl buffer (pH 7.4) for 2 min at 37 °C. Standard thrombin reagent (0.001 NIH unit in 50µl) was added to initiate clotting (Jim, 1957). Thrombin time is a direct measurement of functional fibrinogen as it is the time taken for a standardized thrombin solution to convert fibrinogen to fibrin to form a clot. Clot formation is decreased if there is abnormal fibrinogen, which in turn prolongs the thrombin time. Furthermore, any other factors inhibiting fibrin polymerization will prolong this time, without there being any abnormalities in fibrinogen.

2.4. Results

2.4.1. SDS-PAGE of JFTE

Protein concentration of JFTE was found to be 2.5 mg/ml by Bradford's Method. SDS-PAGE of JFTE revealed six prominent protein bands between 160 and 50 kDa. Multiple low molecular weight bands were also observed (Figure 17).

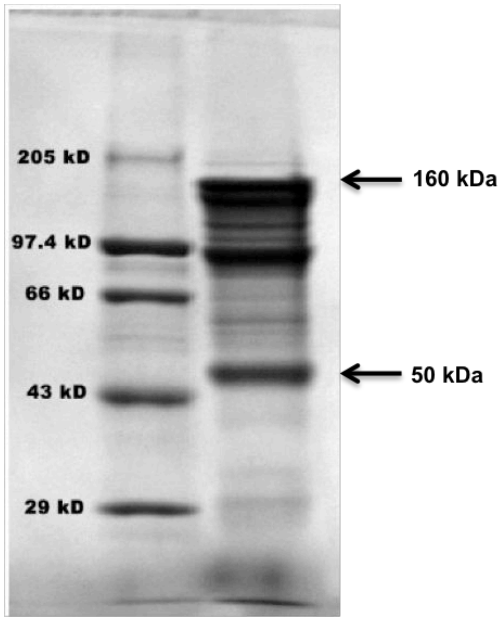


Figure 17. Band pattern for JFTE on 12% SDS-PAGE

2.4.2. Fibrin(ogen)olytic Activity

JFTE showed preferential digestion of Aα chain of fibrinogen, followed by Bβ chain in a dose and time dependent manner. Fibrinogen solution (2 mg/ml) was incubated with 2.5 mg of JFTE for different time periods at 37°C. Immediate digestion of the Aα chain followed by Bβ chain was observed (Figure 18a). JFTE, at a dose of 2.5 mg caused almost complete digestion of Aα and Bβ chains within 3 h. Fifteen micrograms of JFTE caused significant digestion of γ chain also (Figure 18b).

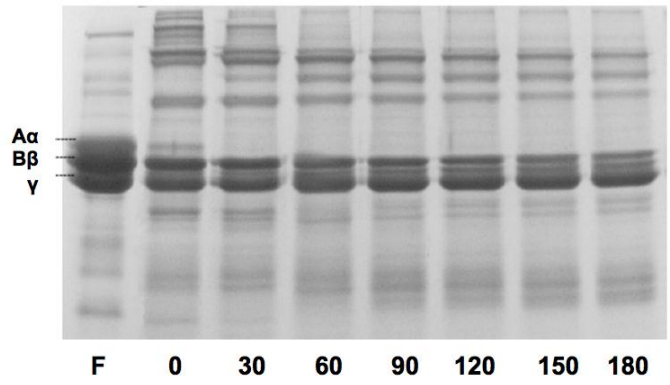


Figure 18a. Time-dependent fibrinogenolytic activity of JFTE. Fibrinogen (2mg/ml) was incubated independently with JFTE (2.5µg) for different time intervals at 37°C. Samples were stored at −80°C after their incubation period till run on SDS-PAGE. (F) Fibrinogen alone after 180 min incubation. (JFTE) JFTE alone after 180 min incubation. Numbers at the bottom of each lane periods of incubation in min.

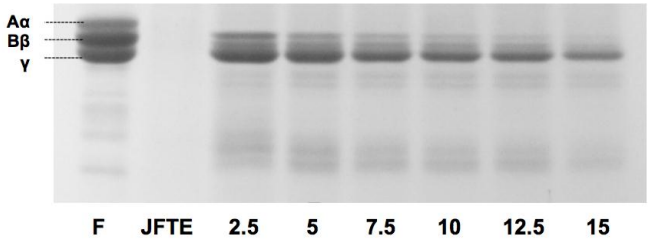


Figure 18b.Dose dependent fibrinogenolytic activity of JFTE. Fibrinogen (2mg/ml) was incubated independently with different concentrations of JFTE at 37°C for 180 min. (F) Fibrinogen alone, (JFTE) JFTE alone. Numbers at the bottom of each lane indicates dose of JFTE in µg.

JFTE (15 µg) completely liquefied 200 µl fibrin clots *in vitro* when incubated for 24 h at 37°C (Figure 19).

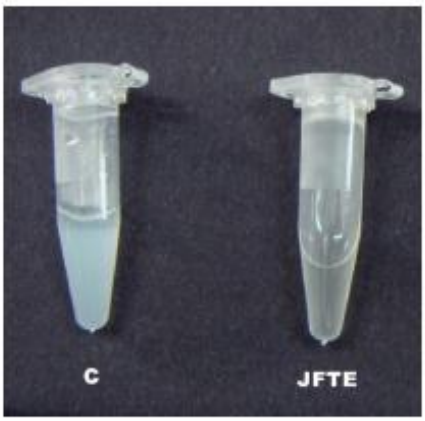


Figure 19. Fibrinolytic activity of JFTE. 0.85% saline and JFTE (15µg) were incubated with fibrin clot developed in the microcentrifuge tube at 37°C. The fibrinolytic activity was visualized after 24 h. Fibrin clot incubated with (C) 0.85% saline (JFTE) Jellyfish Tentacle Extract.

Fibrinogenolytic activity of JFTE was totally inhibited on exposure to 100°C for 1 min. Pre-treatment of JFTE with 1 mM PMSF caused almost complete inhibition of fibrinogenolytic activity, whereas, pre-treatment with 2 mM EDTA delayed fibrinogenolysis (Figure 20).

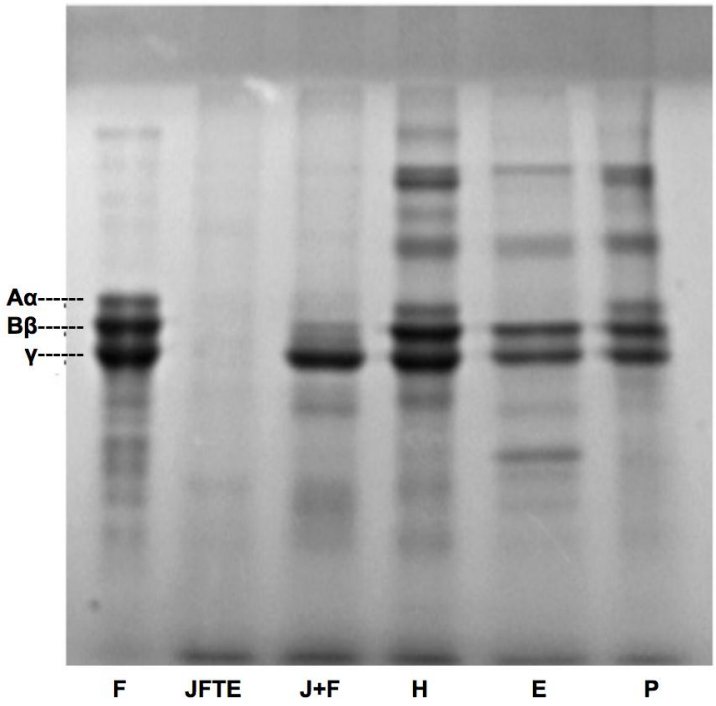


Figure 20. Inhibition of fibrinogenolytic activity of JFTE by 12% SDS-PAGE. (F) Fibrinogen (2mg/ml), (JFTE) Jellyfish Tentacle Extract, (J+F) Fibrinogen incubated with 2.5µg of JFTE, (H) Fibrinogen incubated with 2.5µg JFTE pretreated with heat at 100°C for 1min, (E) Fibrinogen incubated with 2.5µg JFTE pretreated with 2mM EDTA and (P) Fibrinogen incubated with 2.5µg JFTE pretreated with 1 mM PMSF.

2.4.3. Hemolytic and Phospholipase Activity

JFTE (15 µg) showed approximately 25.6% hemolysis on human RBCs compared to positive control (Figure 21). JFTE was found to be devoid of phospholipase activity.

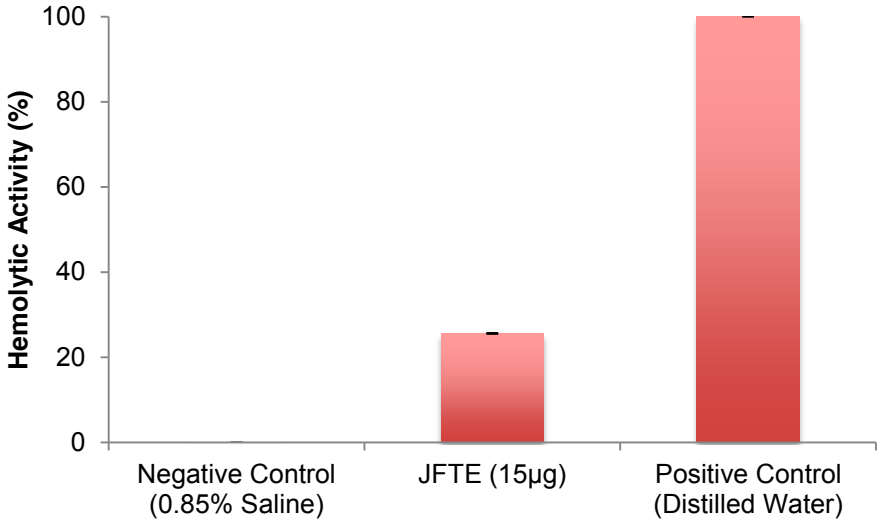
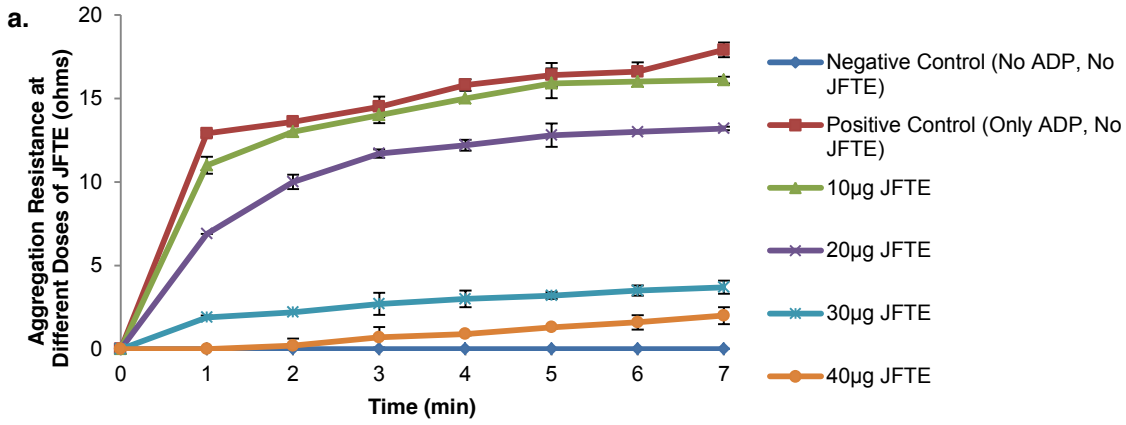


Figure 21. Hemolytic activity of JFTE. Positive Control: RBC incubated with distilled water. JFTE: incubated with 15µg JFTE. Negative Control: incubated with 0.85% saline. Values represent mean ± SD of three independent experiments.

2.4.4. Platelet Aggregation

ADP induced platelet aggregation was inhibited by JFTE in a dose dependent manner. ADP induced platelet aggregation dropped by 9.4% with 10 µg of JFTE. The drop in ADP induced platelet aggregation was as high as 82.4% with 40 µg of JFTE (Figure 22a, 22b). Similar inhibition of 82.63% was observed for 40 µg of JFTE in collagen induced platelet aggregation (Figure 22c & 22d).



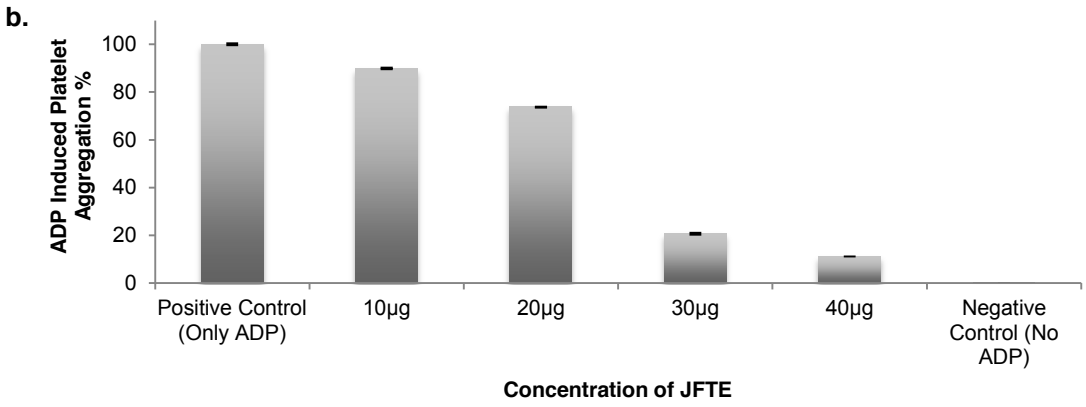


Figure 22a & 22b. Effect of different doses of JFTE on ADP dependent platelet aggregation.

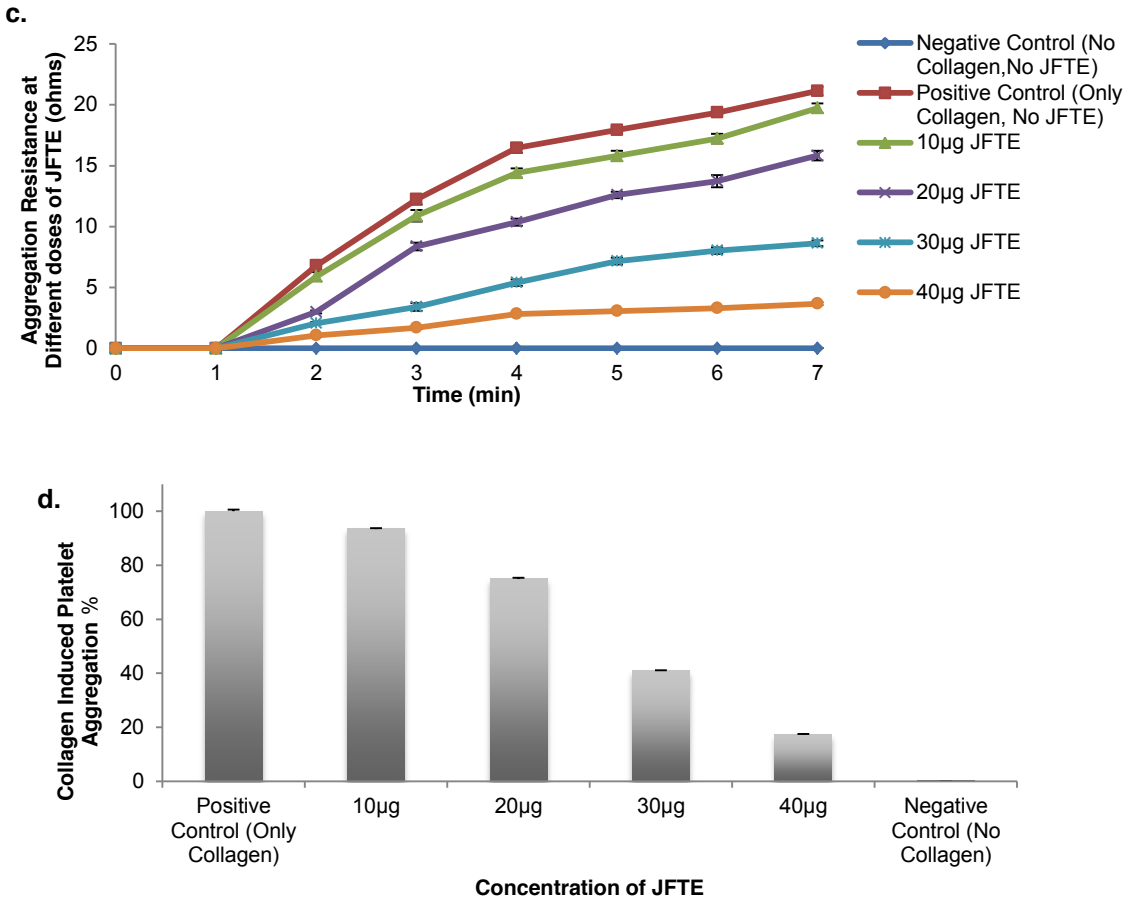


Figure 22c & 22d. Effect of different doses of JFTE on Collagen dependent platelet aggregation.

2.4.5. Assay of Anticoagulant Activity

Recalcification time and thrombin time for JFTE were recorded to be lesser than the negative control suggesting presence of some pro-coagulant components in the extract (Figure 23a and 23b).

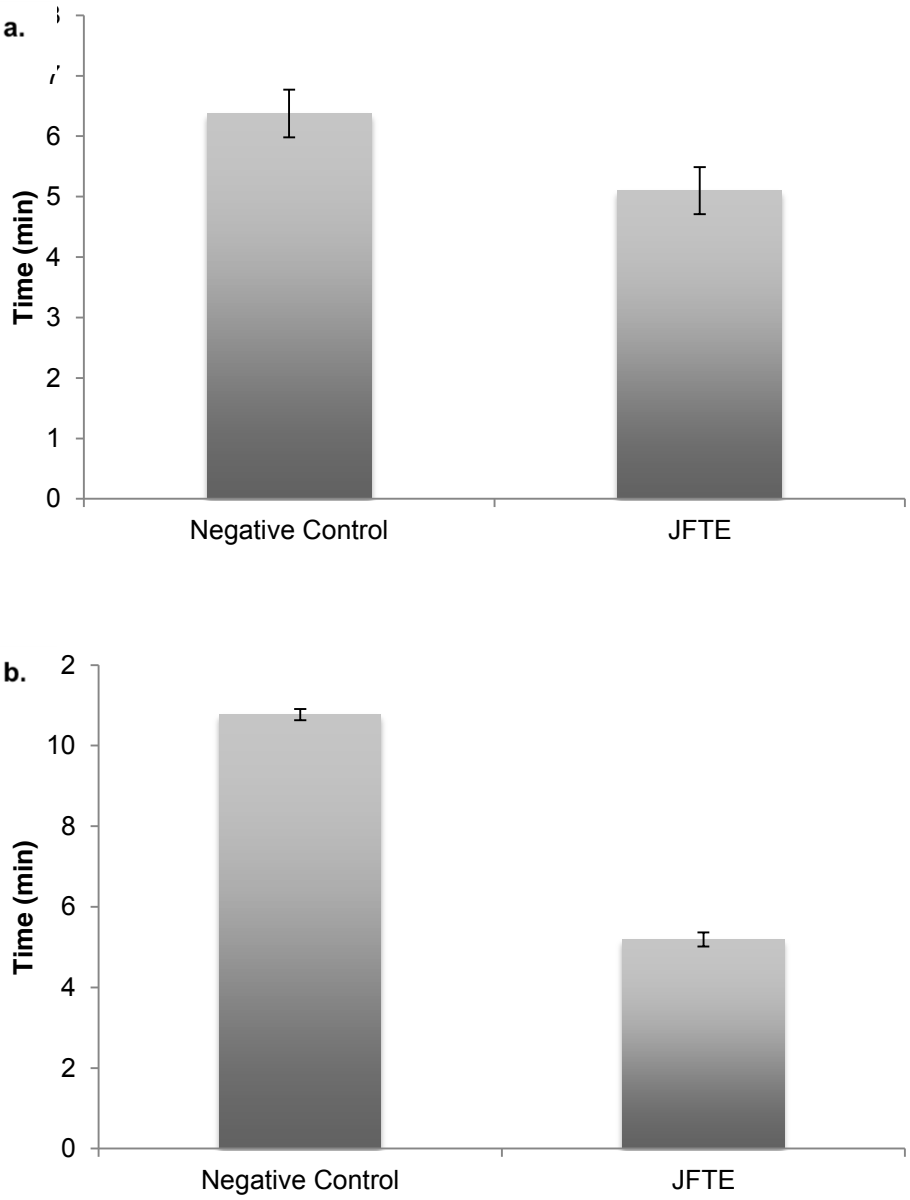


Figure 23a and 23b. Anticoagulant activity of 50µg JFTE on human plasma. Effects of JFTE on recalcification time and thrombin time. The results were mean ± SD of three independent experiments.

2.4.6. Autodegradation

Autodegradation of JFTE was observed in a time-dependent manner. It was observed that 1 mM PMSF inhibited auto-degradation of high molecular weight proteins along with fibrinogenolytic activity. However, 2 mM EDTA inhibited autodegradation without affecting fibrinogenolytic activity.

2.5. Discussion

Moon jellyfish tentacle extract was studied for its anticoagulant activities *in vitro*. JFTE was incubated with bovine fibrinogen and two major chains, namely Aα and Bβ were found to be digested after 3 h. JFTE also completely liquefied fibrin clots in 24 h. Proteolytic activity of jellyfish venom has been reported earlier by Lee et al. (2011), where one of the substrates used was fibrinogen but it was not described whether specific fibrinogenolytic activity was observed or fibrinogen digestion is a reflection of wide spectrum proteolytic activity.

Rapid degradation of jellyfish toxins has been reported earlier (Bloom et al., 1998). JFTE proteins were also found to undergo rapid auto-degradation. It was observed that high molecular weight protein bands of JFTE gradually disappeared with time. EDTA delayed the digestion of Bβ chain, but digestion of Aα chain continued. PMSF significantly inhibited digestion of Aα and Bβ chains by JFTE. It is possible that different proteins are involved in the digestion of different chains of fibrinogen or even smaller peptides, resulted from auto-digestion, also retained the fibrinogenolytic activity. PMSF inhibited toxin-induced digestion of fibrinogen chains but did not abolish it. It is possible that fibrinogenolytic toxins of different types are present in the sample, i.e. serine proteases as well as metalloproteases.

Hemolytic activity of jellyfish venom is a well-documented phenomenon (Nagai et al., 2000; Radwan et al., 2000; Chung et al., 2001; Gusmani et al., 1997; Rottini et al., 1995). JFTE showed significant hemolytic activity on human RBCs.

Inhibition of ADP and collagen dependent platelet aggregation by JFTE was observed to follow dose dependence. However, linearity of aggregation was not observed for mid range concentrations in case of ADP dependent platelet aggregation. Whereas, a linear pattern of inhibition was observed for different

doses of collagen as agonist of aggregation. The presence of many proteins in JFTE may be the reason for this observation. The recalcificaton and thrombin time were found to be lesser than that of the negative control thus indicating the presence of some procoagulant factors in the extract.

Attempts were made to purify the active proteins in the extract using different combinations of ion-exchange chromatography. Due to the unstable nature of the component proteins and limited sample availability, further characterization of the active components of JFTE was not possible. The tentacle extract from *A. aurita* showed presence of very strong fibrinogenolytic factors. Whether all of these factors are part of the venom or some are simply present as normal tissue constituents remain unanswered.

CHAPTER 3

ANTICOAGULANT ACTIVITY OF BARREL JELLYFISH TENTACLE EXTRACT

3.1. Introduction

A pelagic jellyfish abundantly found along the coast of Goa is *Rhizostoma pulmo* commonly known as the barrel jellyfish, the dustbin-lid jellyfish or the frilly-mouthed jellyfish. It is a schyphozoan, of the order *Rhizostomae*, family *Rhizostomatidae*, genus *Rhizostoma* and species *pulmo*. The juveniles are produced in spring, while the largest adults can be seen towards the end of summer and early autumn. Compared to other species, instead of having trailing tentacles around the bell, it has eight thick arms underneath that are covered in frilly tissue. The frills are tentacles around hundreds of little mouths used to catch/eat prey. The edge of the bell-shaped body is lined with blue organs called statocysts, which help with balance. Young jellyfish tend to have a more transparent color whereas adults are much more opalescent. The sex is determined by a difference in colour of the gonads. Mature males have blue gonads, contrasting with the reddish-brown colour of the female gonads. They can grow upto 80-90 cm in diameter and 35 kg in weight (Figure 24). *Rhizostoma pulmo* has a typical rhizostome life cycle that alternates between pelagic and benthic phases. It begins with a fertilized egg that develops into a planula and hence into a sessile and asexually reproducing scyphistoma, which strobilates and releases swimming ephyrae that develop into medusae (Fuentes et al., 2011).

Irrespective of their large size, barrel jellyfish cause little harm to humans, as their sting is not considered powerful enough. In water it releases some stinging substance or toxin that causes small itchy and mild burning abrasions, which are due to the release of irritants in the area immediately around the jellyfish for defensive purposes. Irritations like that can cause swelling and numbness, but cures automatically in a matter of hours.

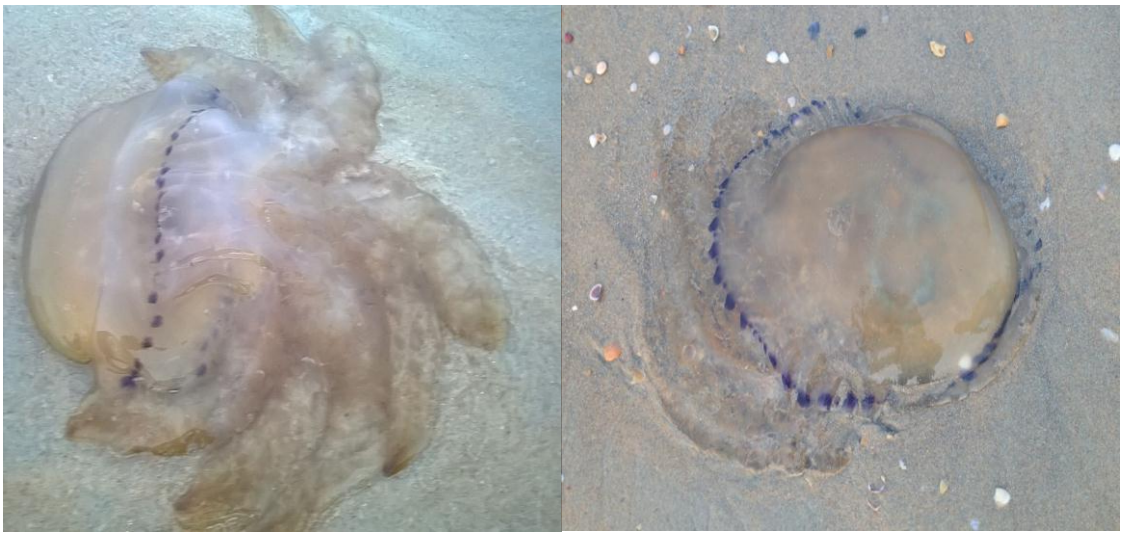


Figure 24. ***Rhizostoma pulmo* from Mobor and Velsao.** (Photo courtesy: Akriti Rastogi)

So far two proteins have been identified from the barrel jellyfish. Rhizolysin, a high molecular weight cytolysin of 260 kDa was isolated from *Rhizostoma pulmo* (Cariello et al., 1988). It showed only hemolytic activity and no phospholipase activity. Later it was also reported to be present in nematocyst free tissue and showed both hemolytic as well as cytotoxic effects but no clastogenic effects (Allavena et al., 1998). A 30kDa chromoprotein named *rpulFKz1* was isolated from the barrel jellyfish with a Frizzled cystein-rich domain and a Kringle domain (Bulina et al., 2004). Kringles are found throughout the blood clotting factors and fibrinolytic proteins and these domains are believed to play a role in binding mediators and in the regulation of proteolytic activity (Patthy et al., 1984; Atkinson and Williams, 1990).

There is no report on anticoagulant or pro-coagulant toxins from jellyfish venom, except those reported by this laboratory that showed strong fibrinogenolytic and platelet-inhibiting activities in the tentacle extracts of moon jellyfish (Rastogi et al., 2012). Isolation and characterization of hemolytic and anticoagulant toxins from marine venoms is expected to provide novel molecules of therapeutic interest. Attempts were made to study the same in barrel jellyfish tentacle extract.

3.2. Sample Collection

Specimens of *Rhizostoma pulmo* were collected from Mobor, Utorda and Velsao beaches of the South-Goan coast during the month of October in 2013, 2014 and 2015 (Figure 25).

Figure 25. Sample collection sites for *Rhizostoma pulmo* on the South-Goan coast of India.

3.3. Materials and Methods

Fibrinogen and thrombin from bovine plasma, Fibrinogen from human plasma, PMSF, EDTA and Azocasein were purchased from Sigma-Aldrich, India. All fine chemicals used were purchased from Thermo Fisher Scientific India Private Limited. All the other reagents used were of analytical grade.

3.3.1. Preparation of Tentacle Extract

The tentacles were manually excised within 1 h of collection and stored in 0.85% saline at -20°C. Frozen tentacles were autolyzed at 4°C in 0.85% saline (9:1 v/v) for 4 days. Resultant fluid was clarified by centrifugation at 20,000 g for 1 h at 4°C. This clarified fluid was called Barrel Jellyfish Tentacle Extract (BJFTE) and was used as the working material for further investigations. This was stored at
-80°C in 2 ml aliquots till used.

3.3.2. Protein Components of BJFTE and their Molecular Weight Determination

The protein concentration of BJFTE was estimated by Bradford's method involving the binding of coomassie brilliant blue G-250 to the protein molecules (Bradford, 1976). Bovine Serum Albumin (BSA) was used as standard protein for constructing the standard curve. Different component proteins of BJFTE and their molecular weights were assessed by electrophoresis (SDS-PAGE) using 12% resolving gel and 5% stacking gel. BJFTE was prepared in reducing sample buffer (10% SDS, 250 mM Tris–HCl, pH 6.8, 30% glycerol, 0.02% bromophenol blue, 5% β-mercapitalethanol). The extract (5µg) was subjected to electrophoresis at 50V and 70V for stacking and resolving gels, respectively. The molecular weight ladder, 29–205 kDa (GeNeI unstained protein molecular weight marker) was run parallel with BJFTE for molecular weight estimation. The protein bands were stained with 1% coomassie brilliant blue R250 in 7.5% acetic acid and 10% methanol overnight. The gel was then destained by repeatedly washing with 7.5% acetic acid and 10% methanol.

3.3.3. Assay of Fibrinogenolytic Activity

Fibrinogenolytic activity was confirmed by incubating fibrinogen fraction I (2 mg/ml) with BJFTE and 0.85% saline (control) for different time intervals and doses at 37ºC. The incubated mixtures were subjected to SDS-PAGE on 12% separating gel with a 5% stacking gel. The protein bands were viewed by staining with 1% coomassie brilliant blue, R250. Fibrinogenolytic activity was monitored by comparing position and appearance of specific bands with that of fibrinogen incubated with 0.85% saline only (Bos et al., 1997).

Above activity of BJFTE was also estimated after treatment with 2 mM EDTA (metalloprotease inhibitor), freshly prepared 1mM PMSF (serine protease inhibitor) or exposure to 100°C for 1 min in a boiling water bath.

3.3.4. Assay of Fibrinolytic Activity

Fibrinolytic activity of BJFTE or fractions was monitored by a modified fibrin plate method (Astrup and Mullertz, 1952). Fibrinogen fraction I (16.5 mg) was dissolved in 1 ml of 20 mM potassium phosphate buffer, pH 7.4. Ammonium sulphate was added to the above solution to a final concentration of 70 mM. Twenty-five microliters of thrombin was added to this solution and transferred immediately to five, 0.5 ml microfuge tube (200 µl each). The solution was allowed to clot by incubating for 2 h at 25°C (Chandrasekhar and Chakrabarty, 2011). Fifty micrograms of BJFTE, BJFTE pretreated with 2 mM EDTA, BJFTE pretreated with 1 mM PMSF and heat treated BJFTE at 100 °C for 1 min were applied on the surface of the clot and incubated at 37°C for 24 h. Fibrinolytic activity of the sample was observed by liquefaction of the clot. 0.85% saline was used as negative control. Similar experiment was performed using fibrinogen from human plasma (essentially plasminogen free). These clots were further referred to as post-treated clots.

Effect of BJFTE on clot formation was studied. BJFTE and BJFTE pretreated with EDTA, PMSF and heat were mixed with fibrinogen/essentially plasminogen free fibrinogen, ammonium sulphate and thrombin mixture before the clot is formed. These clots were further referred to as pre-treated clots.

3.3.5. Assay of Hemolytic Activity

Blood was collected aseptically from healthy volunteers in 0.85% saline and centrifuged at 3000 rpm for 3 min. Supernatant was discarded and the pellet containing RBC were washed thrice with normal saline. One percent RBC suspension (0.3 ml) was taken in each tube to which different concentrations of BJFTE (5, 10, 25, 50 µg) were added. 1% SDS and 0.85% saline were added to RBC suspension as positive and negative controls, respectively. All

the tubes were then incubated for different time periods (30 min, 60 min and 120 min) at 37 °C and centrifuged at 3000 rpm for 5 min. Absorbance of the supernatants were measured at 540 nm. Values obtained with positive control represented 100% hemolysis (Chakrabarty, et al., 2000). The percentage of hemolysis was determined at the end of the assay using the following equation:

$$Hemolysis\ \% = \frac{(Abs.of\ Sample - Abs.of\ Negative\ Control)}{(Abs.of\ Positive\ Control - Abs.of\ Negative\ Control)} \times 100$$

1% RBC suspension treated with 10 µg BJFTE was also observed under 100X magnification using Olympus BX41 compound microscope upto 45 min.

3.3.6. Whole Blood Preparation for Scanning Electron Microscopy

Effect of BJFTE on human RBCs was also studied using Scanning Electron Microscopy (SEM). Samples were prepared by making smears for blood and blood treated with 10 µg BJFTE on glass cover slips coated with poly-L-lysine. Blood smears were fixed with 0.4% glutaraldehyde/4% formaldehyde in sodium phosphate buffer solution, pH of 7.2 for 10 min. The samples were rinsed 3 times in distilled water for 10 min before being fixed for 1 h with 1% osmium tetroxide (OsO_4). This was followed by another 3 rounds of rinsing with distilled water for 10 min each and then followed by serial dehydration with 30%, 50%, 70%, 80%, 90%, 96% and 100% ethanol for 10 min each. The samples were then dried using Leica critical point drier. This was followed by coating with gold/palladium and was examined using a FEI QUANTA 250 SEM.

3.3.7. Assay of Phospholipase Activity

Presence of phospholipase A (PLA) activity was tested using egg yolk as substrate (Neumann and Habermann, 1954). Different doses (5, 10, 25, 50 µg) of BJFTE were added to 2 ml of egg yolk suspension, mixed well and incubated at 37 °C for 1 h. Incubated samples were then placed in a boiling water bath and time required for coagulation of the samples were then noted.

Saline (0.85%) and 5 µg Russell's viper venom were used as negative and positive controls, respectively.

3.3.8. Assay of Anticoagulant Activity

Coagulation tests were performed *in vitro* on human platelet poor plasma (PPP) (Mustard et al., 1972). Briefly nine volumes of human blood was mixed with one volume of acid citrate dextrose (ACD) and centrifuged at 1100 rpm for 10 minutes at room temperature. The supernatant obtained was platelet rich plasma (PRP). PRP was then centrifuged at 4000 rpm for 15 min at room temperature and the supernatant obtained was PPP.

For calculating recalcification time, 100 µl of 50 mM Tris-HCl buffer (pH 7.4), 100 µl PPP and 50 µg BJFTE were pre-incubated for 5,10,15 min at 37 °C. Clotting was then initiated by adding 50 µl of 50mM $CaCl_2$ (Langdell et al., 1953). 50 µg BJFTE was incubated with 100µl of PPP and 100 µl of 50 mM Tris-HCl buffer (pH 7.4) for 5,10 and15 min at 37 °C. Standard thrombin reagent (0.001 NIH unit in 50µl) was added to it to initiate clotting and the thrombin time was calculated for different times of incubation of BJFTE (Jim, 1957).

3.3.9. Assay of Proteolytic Activity

3.3.9.1. Substrate Zymography

For zymography assays SDS-PAGE (12% stacking and 4% resolving) were prepared and co-polymerized with casein (1.5mg/ml), fibrinogen (1.5mg/ml) and gelatin (2mg/ml), respectively (Heussen, et al., 1980). BJFTE (25µg), to be analyzed, was prepared in non-reducing sample buffer (4% SDS, 125 mMTris–HCl, pH 6.8, 20% glycerol, 0.01% bromophenol blue) in 3:1 ratio, then run on 12% gels at 15 mA/gel at 4°C. After electrophoresis, the gels were washed twice for 20 min in 2.5% Triton X-100 to remove SDS. Then the gels were incubated in 20 mMTris (pH 7.4), 0.5 mM calcium chloride at 37°C for 16 h and stained with 0.125% coomassie blue R250. Clear zones in the gels indicated regions of proteolytic activity. Protease inhibition was observed by adding the protease inhibitors, EDTA and PMSF. They were added to all the gel wash and incubation buffers and the gels were stained as usual. PMSF was prepared as a 200 mM stock in methanol and added fresh to each

incubation buffer to give a final concentration of 1 mM. EDTA was prepared as a 500 mM stock and added fresh to each incubation buffer to give a final concentration of 2 mM.

3.3.9.2. Azocasein Assay

Proteolytic activity of BJFTE was also determined using azocasein as substrate. Different doses of BJFTE (25, 50, 75 and 100 µg) were incubated with 250 µl of 0.25% azocasein in 20 mM potassium phosphate buffer, pH 7.4 at 37°C for different time intervals from 0-120 min. This was followed by adding 10% trichloroacetic acid to stop the reaction. The samples were then centrifuged at 4000 rpm for 15 min at 37°C. The supernatant was diluted with an equal volume of 0.5 N NaOH and absorbance was read at 440 nm (Chakrabarty, et al., 2000). The azocasein hydrolysis by BJFTE was further characterized using protease specific inhibitors. The inhibition percentage of azocasein hydrolysis was calculated.

3.3.10. Preparation of Washed Human Platelets and Platelet Aggregation Studies using Photo-Optical Method

Blood was collected from healthy human volunteers who had not taken any medicines for at least 2 weeks and immediately mixed with Acid Citrate Dextrose (2.5g sodium citrate, 1.4g citric acid, 2g anhydrous glucose pH.4.5). Blood was then promptly centrifuged at 90g for 10 min at room temperature. The supernatant platelet rich plasma (PRP) was retained. PRP was incubated at 37°C for 15 minutes and centrifuged at 4500g for 20 min. The pellet obtained was suspended in Tyrode-albumin buffer (pH 6.5), mixed well and centrifuged again for 20 min at 4500g. Platelets were washed twice by repeating the above step. Washed platelets thus obtained were re-suspended in Tyrode buffer (pH 7.35) containing 2mM $CaCl_2$. 2 H_2O. This suspension was used for the platelet aggregation studies. ADP induced platelets aggregation studies were performed using the washed platelets. Platelet count was adjusted to $5x10^8$/ml. Platelet suspension (400µl) was incubated with BJFTE (25µg and 50 µg) at 37°C for 5 minutes under constant stirring. ADP (10mM) was added to the platelet suspension and incubated for different time intervals 2 min, 5 min and 10 min. An aggregated platelet suspension will absorb less light as compared to a homogeneous platelet suspension. Note

that platelet poor plasma was used as blank. Therefore in this experiment, absorbance is inversely proportional to the degree of platelet aggregation. Absorbance was checked immediately at 697 nm using Shimadzu UV/VIS Spectrophotometer, UV- 1800 (Born and Cross, 1963).

3.3.11. Antimicrobial Activity Assay

The agar (1.8%) plate surface was inoculated by spreading microbial inoculum of gram-positive (*Staphylococcus aureus MTCC737*) and gram-negative (*Escherichia coli MTCC723*) bacteria over the entire agar surface. Then holes (8 mm diameter) were punched aseptically with a sterile well borer and 100 µL of BJFTE at concentrations of 25 µg, 50 µg and 100 µg was introduced into the wells. Then, agar plates were incubated for 24 h at 37 °C.

3.3.12. Periodic Acid Schiff Staining

Protein glycosylation, i.e. the attachment of a saccharide moiety to a protein, is a modification that occurs either co-translationally or post-translationally. The two major types of glycosylation, *N*-linked and *O*-linked, are both involved in the maintenance of protein conformation and activity, in protein protection from proteolytic degradation and in protein intracellular trafficking and secretion (Varki et al., 1993). BJFTE was run on 12% SDS-PAGE, thereafter the gel was washed continuously with 40% methanol and 7% acetic acid overnight. The solution was changed and the gel was put in 7.5% acetic acid and kept at room temperature for 1 hr. The gel was transferred to 1% periodic acid, kept immersed for 1 h in dark at 4°C. The gel was subsequently washed 6 times in 7.5% acetic acid for 10 min. The gel was incubated in Schiff's reagent at 4°C in dark for 1 h and was washed in 0.5% sodium metabisulphate and preserved in 7.5% acetic acid.

3.4. Results

3.4.1. Protein Components of BJFTE on SDS PAGE

Protein concentration of BJFTE was found to be 3.5 mg/ml by Bradford's Method. SDS-PAGE of BJFTE revealed twelve prominent protein bands between 300 and 43 kDa. It mainly consisted of many high molecular weight proteins (Fig 26).

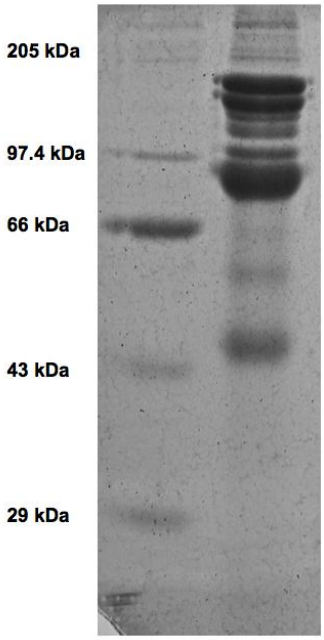


Figure 26. Band pattern for 5 μg BJFTE on 12% SDS-PAGE gel.

3.4.2. Fibrinogenolytic Activity

BJFTE preferentially digested Aα chain of fibrinogen, followed by Bβ chain in a dose and time dependent manner. Fibrinogen solution (2 mg/ml) was incubated with 2.5 μg of BJFTE for different time periods at 37 °C. Immediate digestion of the Aα chain followed by very slow digestion Bβ chain was observed (Figure 27a). BJFTE, at a dose of 18 μg caused almost complete digestion of Aα and Bβ chain within 5 h (Figure 27b).

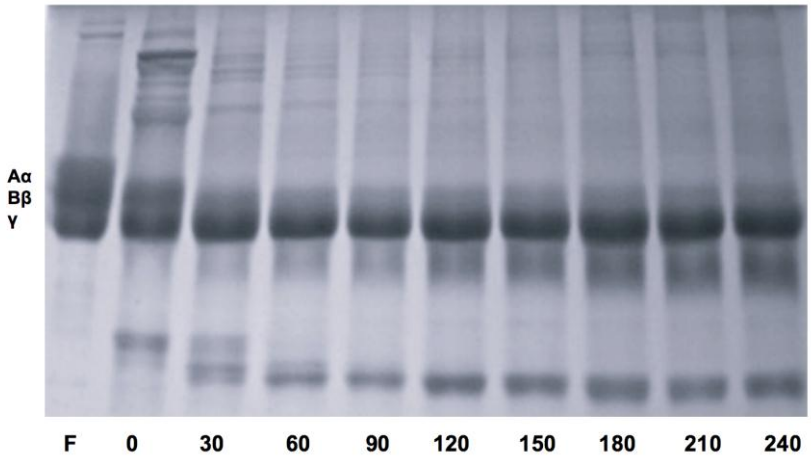


Figure 27a. Time-dependent fibrinogenolytic activity of BJFTE. Fibrinogen (2 mg/ml) was incubated independently with BJFTE (2.5 µg) for different time intervals at 37 °C. (F) Fibrinogen alone after 240 min incubation. Numbers at the bottom of each lane indicate minutes of incubation with BJFTE.

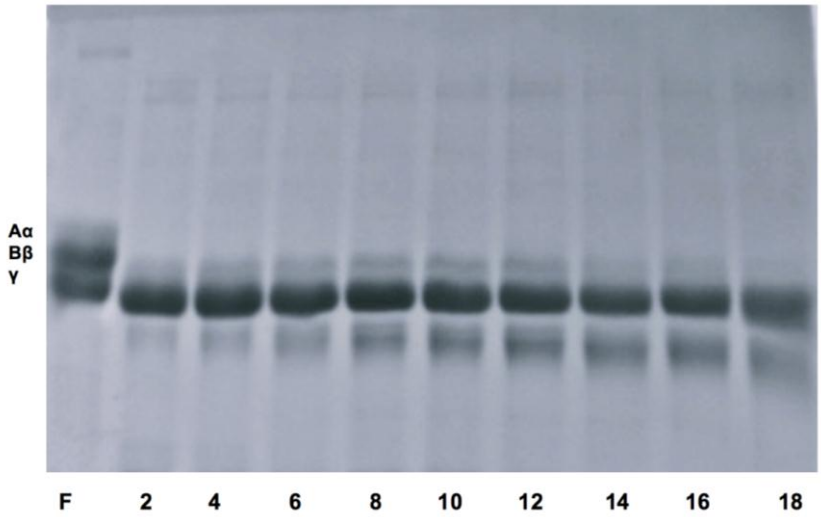


Figure 27b. Dose dependent fibrinogenolytic activity of BJFTE.Fibrinogen (2 mg/ml) was incubated independently with different concentrations of BJFTE at 37 °C for 5 h. (F) Fibrinogen alone, (BJFTE) BJFTE alone. Numbers at the bottom of each lane indicate dose of BJFTE in microgram.

Fibrinogenolytic activity of BJFTE was significantly inhibited on exposure to 100 °C for 1 min and on pre-treatment with EDTA whereas, it remained unaffected on pre-treatment with 1 mM PMSF (Figure 28).

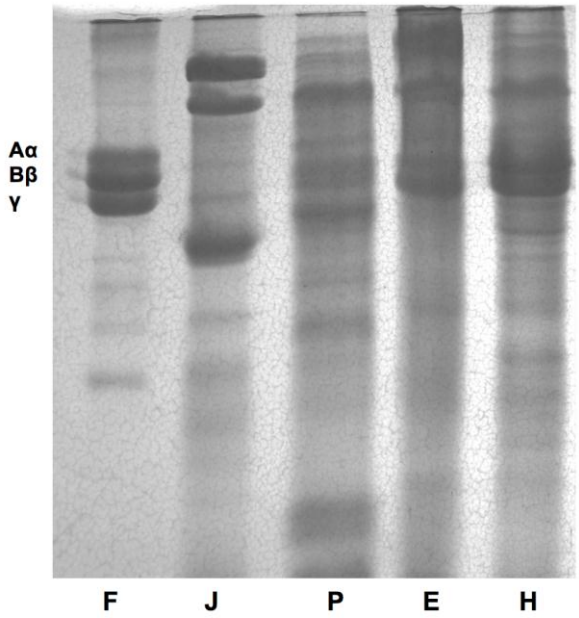


Figure 28. Inhibition of fibrinogenolytic activity of BJFTE by 12% SDS-PAGE. (F) Fibrinogen (1mg/ml), (J) Fibrinogen incubated with 2.5µg of BJFTE, (P) Fibrinogen incubated with 2.5µg BJFTE pretreated with 1 mM PMSF, (E) Fibrinogen incubated with 2.5µg BJFTE pretreated with 2mM EDTA, (H) Fibrinogen incubated with 2.5µg BJFTE pretreated with heat at 100°C for 1 min.

3.4.3. Fibrinolytic Activity

BJFTE almost liquefied 200 µl fibrin clots *in vitro* when incubated for 24 h at 37°C. The fibrinolytic activity of BJFTE was completely inhibited by EDTA treatment. However, exposure to 100 °C for 1 min and pre-treatment with PMSF delayed clot dissolution (Figure 29a). These observations indicate that major components of the BJFTE responsible for the clot digestion are metalloproteinases and fibrinolytic activity of BJFTE was only partially affected by exposure to heat. In case of fibrin clots made using essentially plasminogen free fibrinogen from human plasma, no digestion was observed even up to 48 h (Figure 29b).

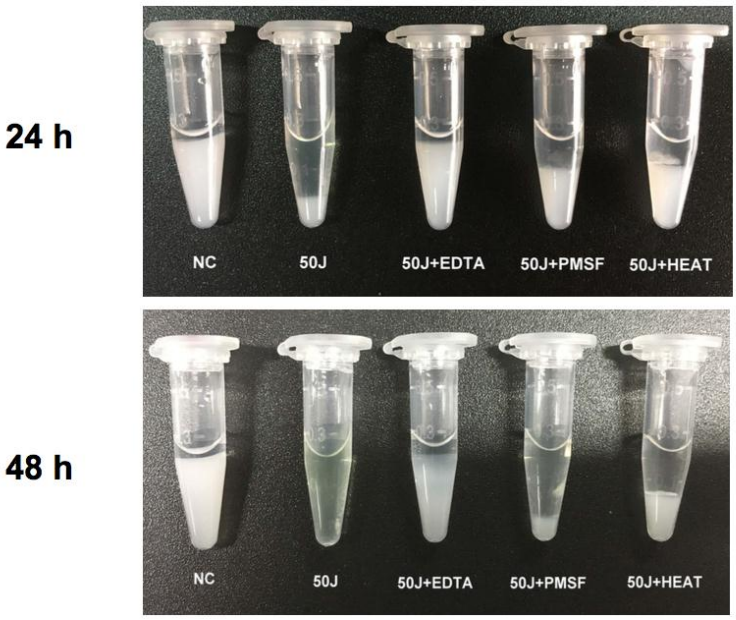


Figure 29a. Fibrinolytic activity and inhibition studies of BJFTE (fibrinogen). BJFTE (50 µg) was pretreated with metalloprotease and serine protease inhibitors for 1 h at 37 °C or exposed to heat at 100 °C for 1 min before incubating with fibrin clot developed in the microfuge tube. The fibrinolytic activity was visualized after 24 and 48 h. Fibrin clot incubated with NC) saline alone, 50J) BJFTE, 50J+EDTA) pretreated BJFTE with 2 mM EDTA, 50J+PMSF) BJFTE pretreated with 1mM PMSF, 50J+HEAT) BJFTE exposed to heat**.**

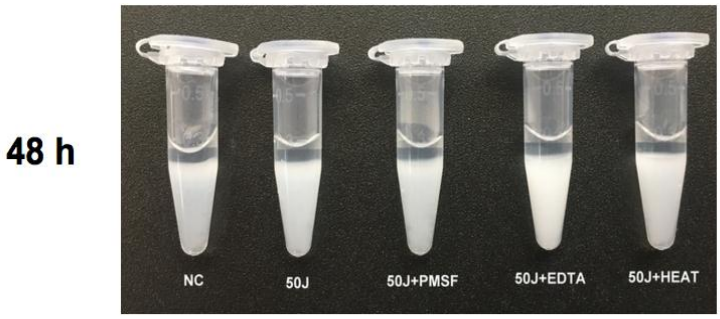


Figure 29b. Fibrinolytic activity and inhibition studies of BJFTE (essentially plasminogen free fibrinogen). BJFTE (50 µg) was pretreated with metalloprotease and serine protease inhibitors for 1 h at 37 °C or exposed to heat at 100 °C for 1 min before incubating with fibrin clot developed in the microfuge tube. The fibrinolytic activity was visualized after 24 and 48 h. Fibrin clot made with essentially plasminogen free fibrinogen incubated with NC) saline alone, (50J) BJFTE, (50J+PMSF) BJFTE pretreated with 1mM PMSF (50J+EDTA) pretreated BJFTE with 2 mM EDTA, (50J+HEAT) BJFTE exposed to heat**.**

It was observed that normal clots were formed within 2-3 h in pre-treated clots. Pre-treated clots made with fibrinogen showed clot digestion after 24 h and the clot digestion further increased up to 48 h (Figure 30a). But in case of heat-treated and EDTA treated BJFTE sample the activity was significantly

lost. In case of pre-treated clots made with essentially plasminogen free fibrinogen again no clot digestion was noticed (Figure 30b). These observations suggest that the toxins in the extract do not interfere with the clot formation and work more like tissue plasminogen activator and thus no clot dissolution was observed in case of essentially plasminogen free fibrinogen.

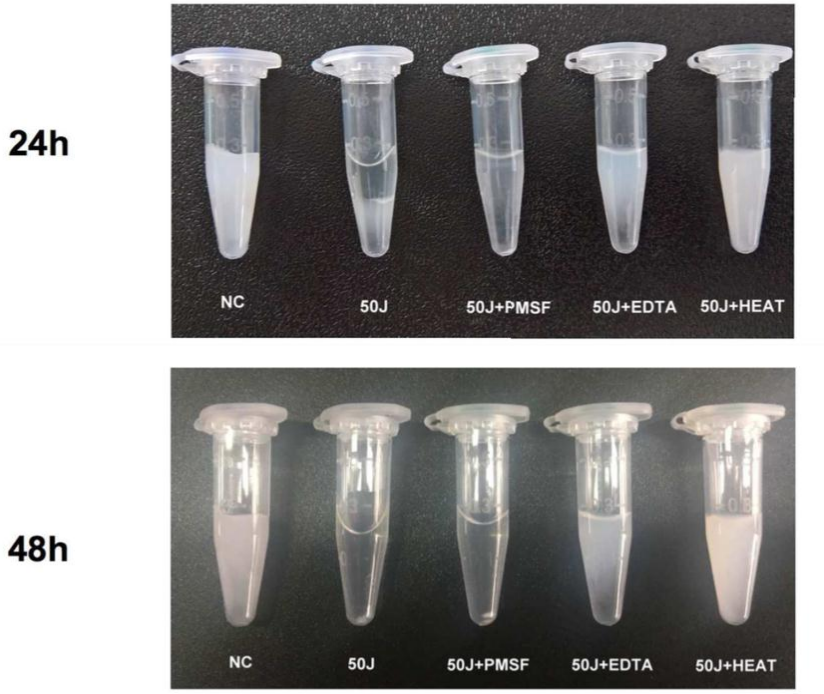


Figure 30a. **Effect of BJFTE on clot formation (fibrinogen).** BJFTE (50 µg) was pretreated with metalloprotease and serine protease inhibitors for 1 h at 37 °C or exposed to heat at 100 °C for 1 min before mixing with fibrinogen and thrombin mixture in the microfuge tube. The activity was visualized after 24 and 48 h. This mixture incubated with NC) saline alone, 50J) BJFTE, 50J+EDTA) pretreated BJFTE with 2 mM EDTA, 50J+PMSF) BJFTE pretreated with 1mM PMSF, 50J+HEAT) BJFTE exposed to heat

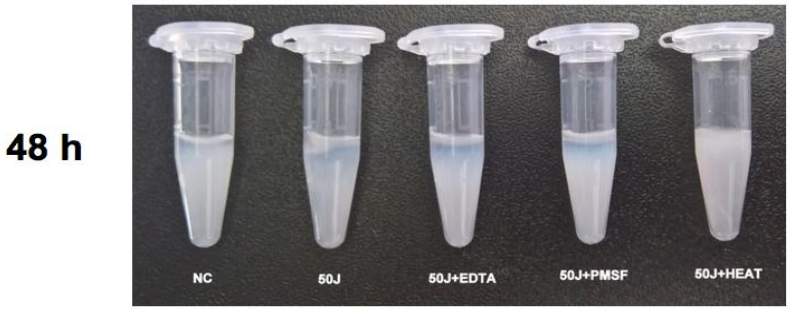


Figure 30b. **Effect of BJFTE on clot formation.(essentially plasminogen free fibrinogen).** BJFTE (50 µg) was pretreated with metalloprotease and serine protease inhibitors for 1 h at 37 °C or exposed to heat at 100 °C for 1 before mixing with essentially plasminogen free fibrinogen and thrombin mixture in the microfuge tube. The activity was visualized after 24 and 48 h. This mixture was incubated with NC) saline alone, (50J) BJFTE, (50J+PMSF) BJFTE pretreated with 1mM PMSF (50J+EDTA) pretreated BJFTE with 2 mM EDTA, (50J+HEAT) BJFTE exposed to heat.

3.4.4. Hemolytic and Phospholipase Activity

BJFTE was assessed for its hemolytic activity using human erythrocytes. The extract showed concentration and time dependent hemolytic activity. It was observed that the difference in hemolytic activity at 30 min and 60 min for different doses of the extract was not as drastic as in the case of 60 min and 120 min (Figure 31).

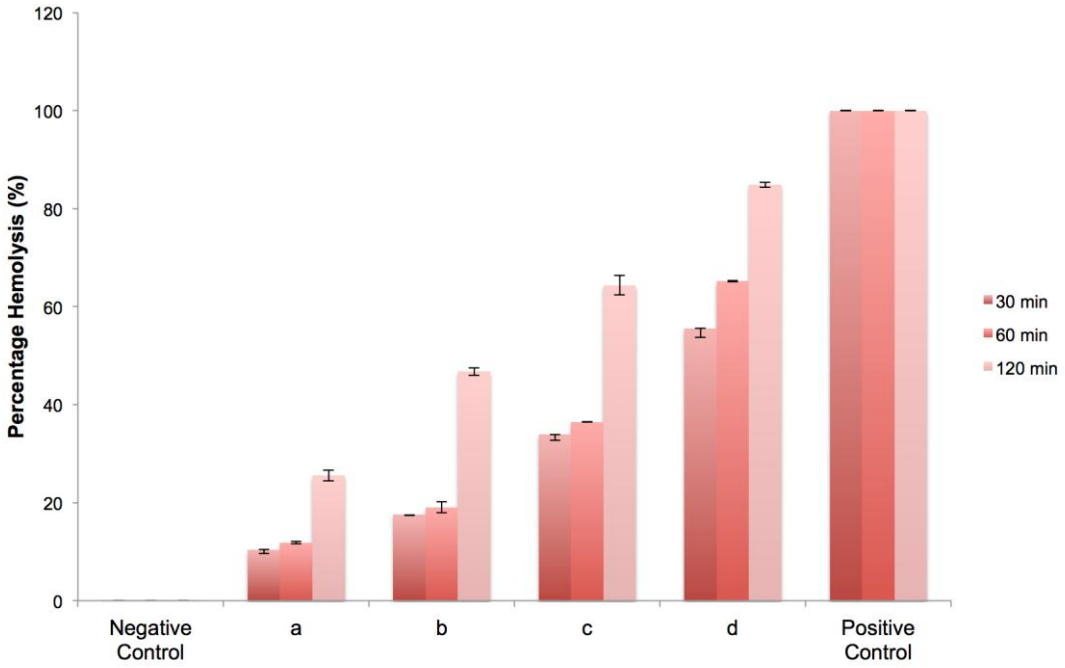


Figure 31. Hemolytic activity of BJFTE. RBC suspension incubated with: Negative Control = 0.85% saline; a = 5 µg of BJFTE, b = 10 µg of BJFTE, c = 25µg, d = 50 µg of BJFTE, Positive Control = 0.1% of SDS. Values represent mean ± SD of three independent experiments.

The effect of BJFTE on RBC was also visualized under 100X magnification of a compound light microscope. There were very significant changes in the size and morphology of the RBCs in a time dependent manner (Figure 32). These changes were further verified by SEM. SEM analysis showed significant surface membrane changes (Figure 33). BJFTE was found to be devoid of phospholipase activity.

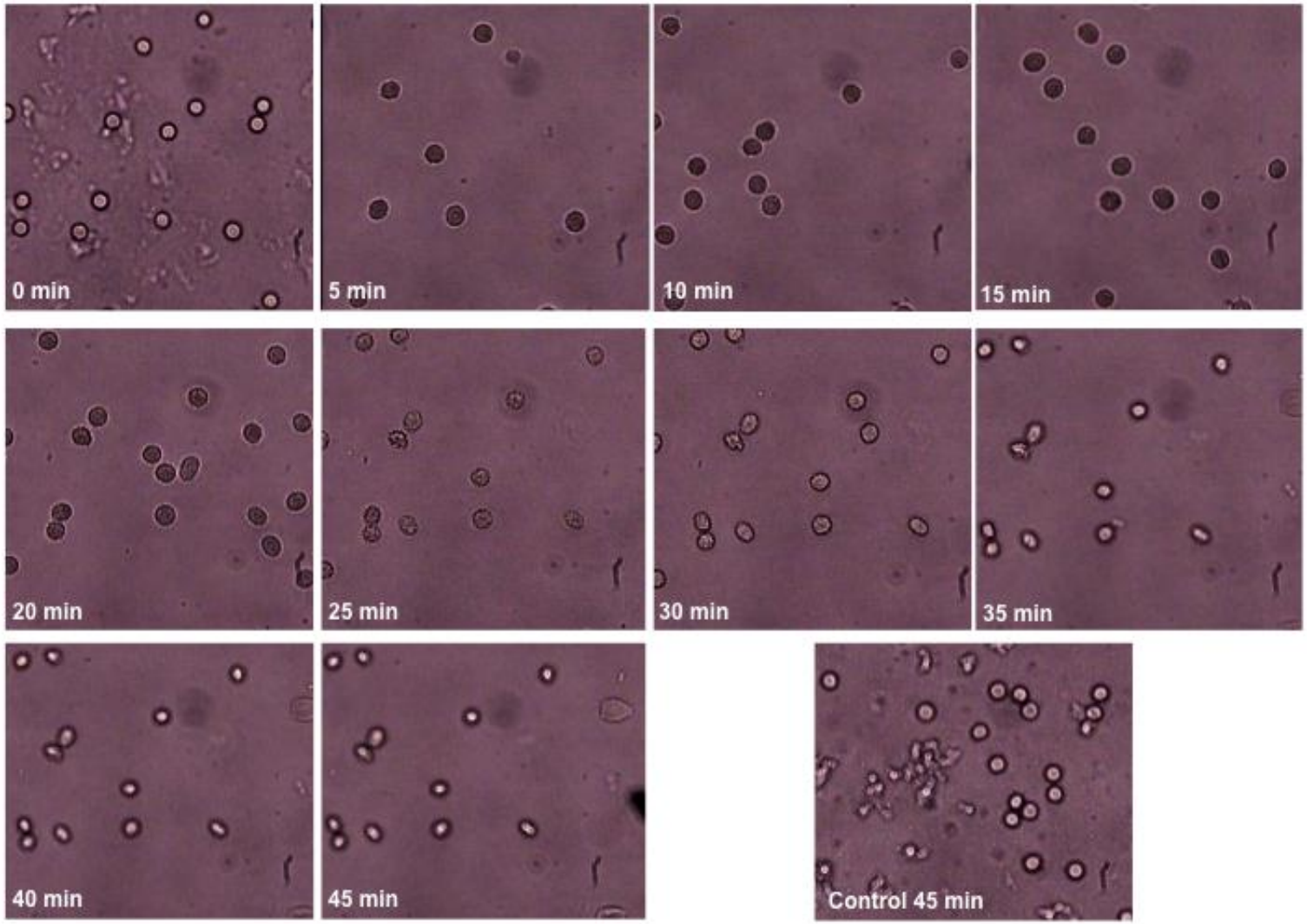


Figure 32. Time dependent effects of 10 µg BJFTE on 1% human RBC suspension as observed under 100X magnification by Olympus BX41.

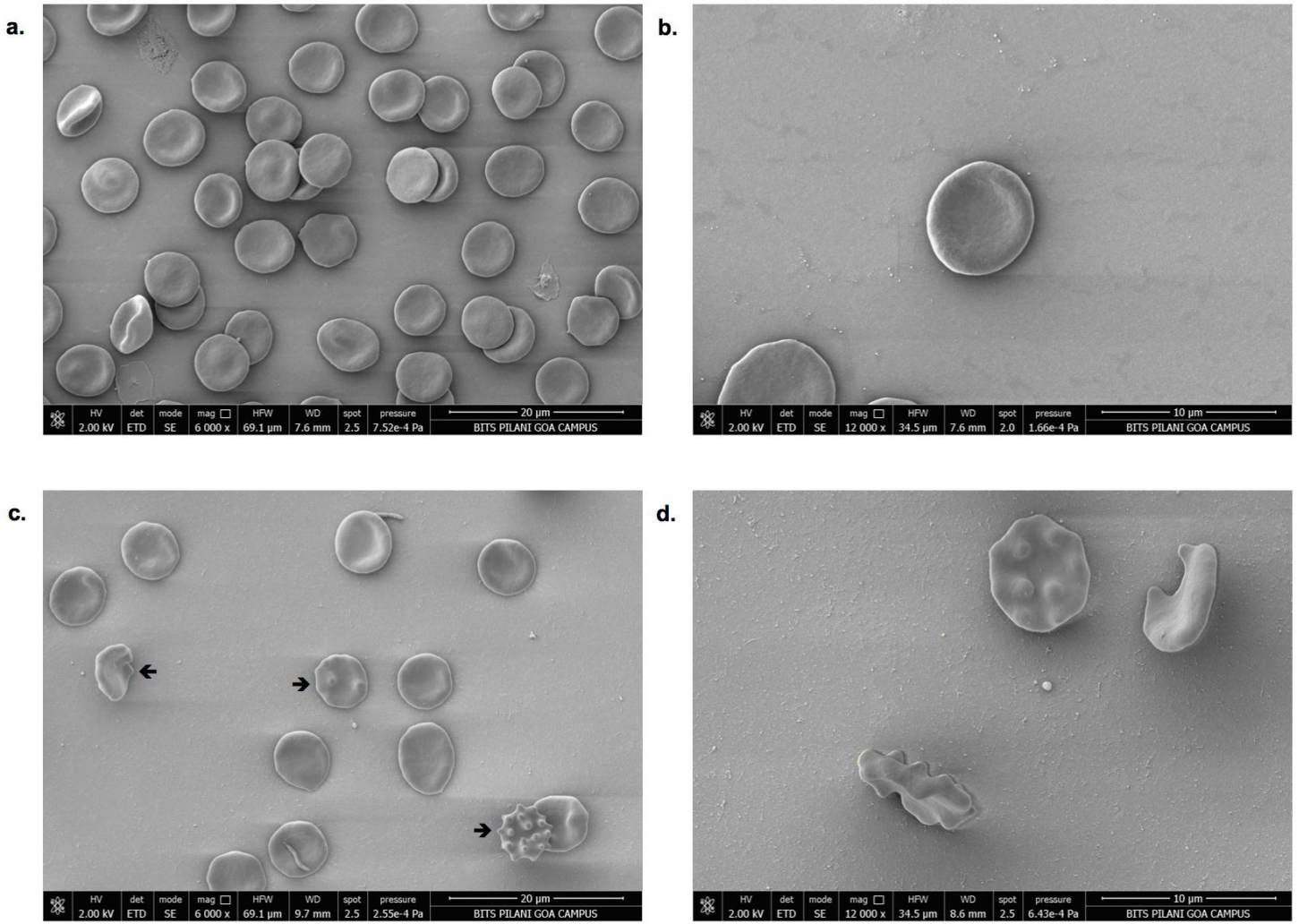


Figure 33. Effects of 10 µg BJFTE on 1% human RBC suspension as observed under Scanning Electron Microscope FEI QUANTA 250. (a and b) Control RBCs without treatment with BJFTE at 6000 and 12000 magnifications respectively. (c and d) RBCs after treatment with 10µg of BJFTE for 45 min at 6000 and 12000 magnifications respectively.

3.4.5. Anticoagulant Activity of BJFTE

Recalcification time was prolonged as compared to the control and increased as the incubation time of BJFTE with PPP increased (Figure 34a). Thrombin time is a direct measurement of functional fibrinogen as it is the time taken for a standardized thrombin solution to convert fibrinogen to fibrin. Clot formation is decreased if there is abnormal fibrinogen, thus in turn prolonging the thrombin time. Furthermore, any other factors inhibiting fibrin polymerization will prolong this time, without there being any abnormalities in fibrinogen. Thrombin time also increased with increasing time of incubation of BJFTE with PPP (Figure 34b).

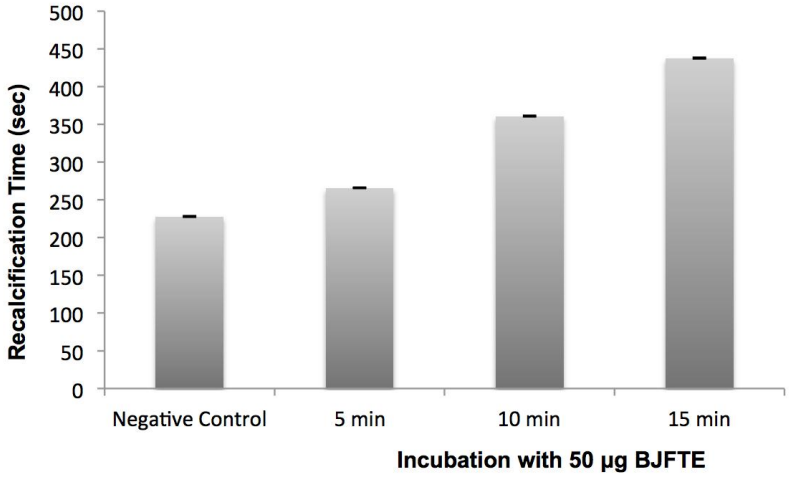


Figure 34a.Incubation time dependent recalcification time of BJFTE. Effect of 50 µg of BJFTE on recalcification time was observed for different incubation times of the extract with PPP (5, 10, 15 min) and 20mM potassium phosphate buffer, pH 7.4, was used as negative control. The results were mean ± SD of three independent experiments.

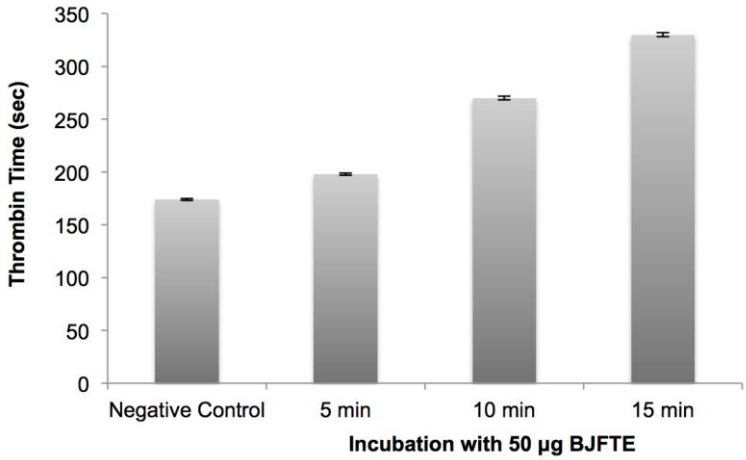


Figure 34b. Incubation time dependent thrombin time of BJFTE. Effect of 50 µg of BJFTE on thrombin time was observed for different incubation times of the extract with PPP (5, 10, 15 min) and 20mM potassium phosphate buffer, pH 7.4, was used as negative control. The results were mean ± SD of three independent experiments.

3.4.6. Substrate Zymography

Proteolytic activity of BJFTE was investigated using substrate zymography, where fibrinogen, casein and gelatin were used as substrates. It was observed that BJFTE had very strong fibrinogenolytic, caseinolytic and gelatinolytic activity (Figure 35a). Most of these activities were restricted to the high molecular weight proteins (250-65 kDa). When the zymograms were treated with 2mM EDTA, most of the fibrinogenolytic, caseinolytic and gelatinolytic activity disappeared (Figure 35b). However, on treatment with 1mM PMSF all the above-mentioned activities remained unaffected (Figure 35c).

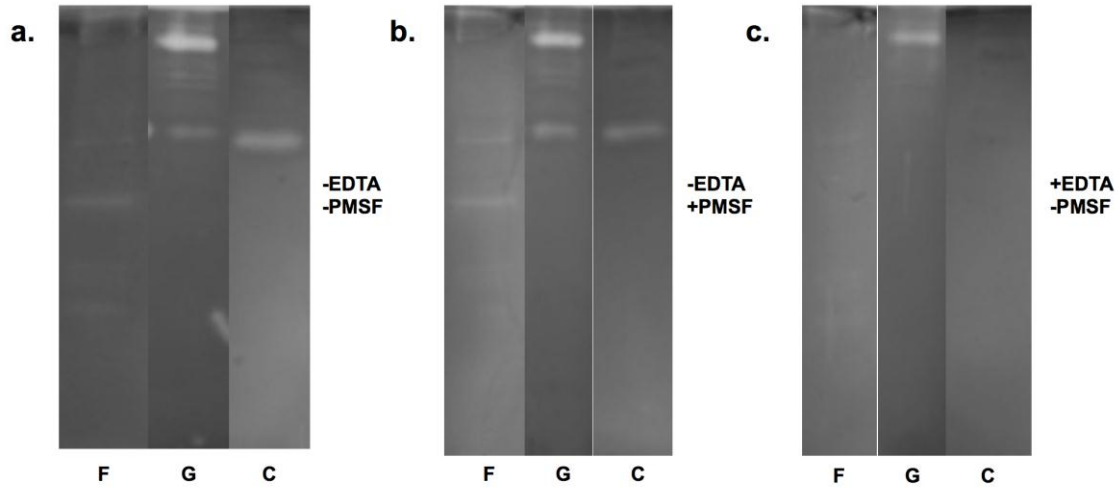


Figure 35. Analysis of proteolytic activity by zymography (a.) Fibrinogen (F), gelatin (G) or casein (C) was used as a substrate and copolymerized in non-reducing 12% SDS-PAGE for analyzing the proteolytic activities of BJFTE (b.) Same sets of zymography assays were performed in the presence of 2mM EDTA during the enzymatic reaction time (c.) Same sets of zymography assays were performed in the presence of 1mM PMSF during the enzymatic reaction time.

3.4.7. Azocasein Activity

BJFTE hydrolyzed azocasein in a dose as well as time dependent manner. Azocasein hydrolysis reached saturation after 4h for a dose of 100 µg of BJFTE (Figure 36). Inhibition percentage of azocasein hydrolysis, in the presence of various inhibitors, provided information about the relative contribution of the inhibited class of proteases to total BJFTE protease activity. EDTA caused almost complete inhibition of hydrolysis of azocasein by BJFTE whereas PMSF caused mild inhibition of activity (Figure 37).

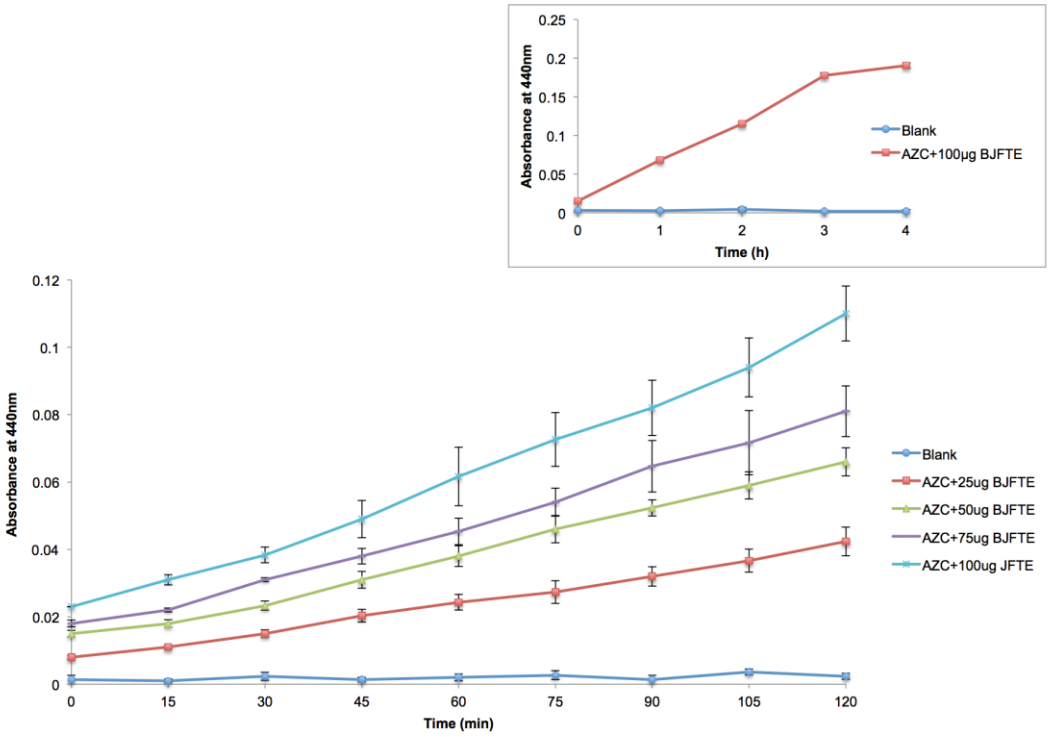


Figure 36. Dose and time dependent azocasein assay (n=3). Inset**.**Hourly hydrolysis of azocasein by 100 µg of BJFTE, where the activity reaches saturation after 4 h.

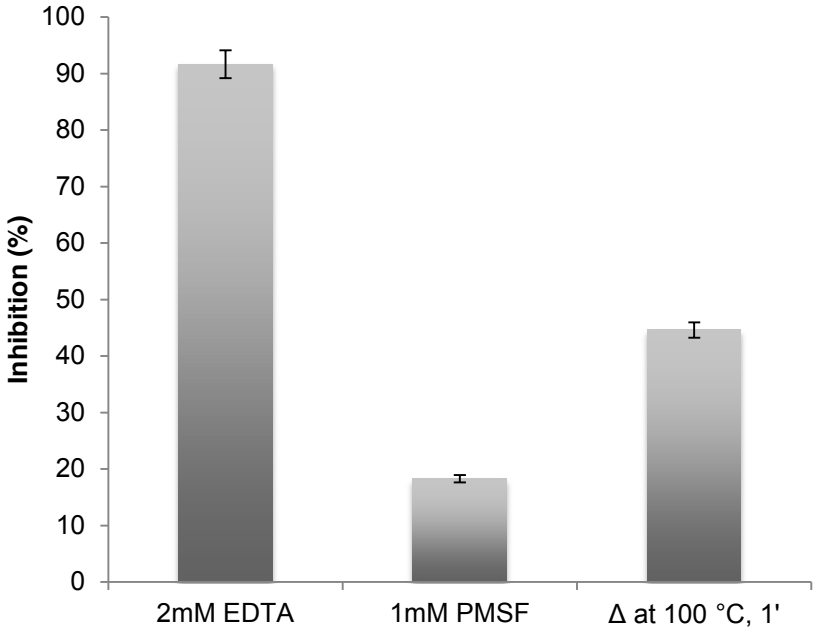


Figure 37. Percentage inhibition of azocasein hydrolysis by BJFTE in presence of various protease inhibitors. Protease activity without inhibitor (control) was considered 100%. The results were mean ± SD of three independent experiments.

3.4.8. Platelet Aggregation

Inhibition of aggregation was observed with purified platelets by photo-optical method using UV/VIS Spectrophotometer UV-1800 at 697 nm. Two-fold increase in absorbance was observed when washed platelets were treated with 50 µg of BJFTE, indicating inhibition of aggregation (Fig 38).

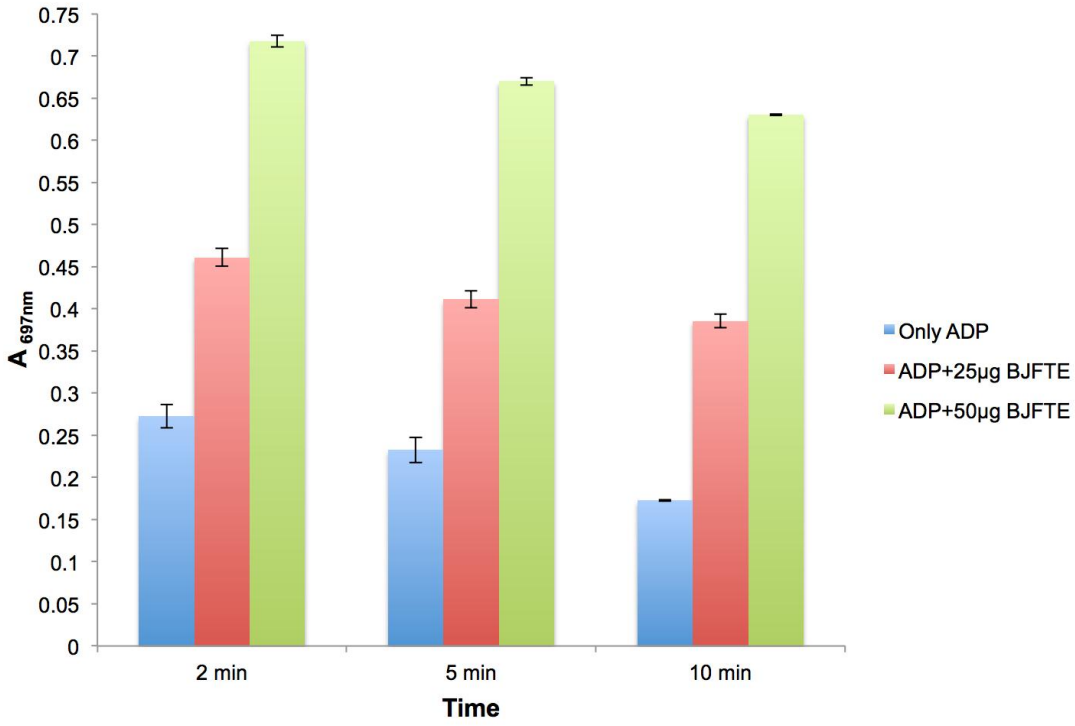


Figure 38. Washed platelet aggregation studies using photo-optical method. Washed human blood platelets were incubated with BJFTE (25 and 50 µg) and aggregation was induced with 10 mM ADP. OD at 697 nm was noted at different time intervals. Increase in absorbance values for the BJFTE treated samples suggests the inhibition of ADP induced platelet aggregation.

3.4.9. Antimicrobial Activity

No zone of inhibition was observed for both gram-negative as well as gram-positive culture for all the doses of BJFTE, indicating the absence of antimicrobial activity.

3.4.10. PAS Staining

It was a basic method to determine whether the component proteins of BJFTE are glycosylated by resolving them on SDS-PAGE and followed by staining the gel using the periodic acid-Schiff (PAS) reaction, in which periodic acid

oxidizes two vicinal diol groups to form an aldehyde, which reacts with the Schiff reagent to give a magenta color (Clarke et al., 1964). No glycoproteins or glycosylated protein containing bands were observed.

3.5. Discussion

Barrel jellyfish (*Rhizostoma pulmo*) tentacle extract was studied for its anticoagulant activity *in vitro*. BJFTE was able to digest the Aα chain and Bβ chain completely after 5 h of incubation with 2 mg/ml of bovine fibrinogen at a dose of 18µg. Digestion of fibrinogen by BJFTE was much slower than that reported for the moon jellyfish tentacle extract which could digest the Aα chain and Bβ chain completely within 3 h of incubation at a dose of 2.5 µg (Rastogi et. al., 2012). The fibrinogenolytic activity was slowed down in presence of EDTA (Fig. 28), whereas the fibrinolytic activity in case of pre-treated and post-treated clots formed with fibrinogen was completely abolished (Fig. 29a and 30a). On treatment with PMSF, the fibrinogenolytic activity remained unaffected, whereas the fibrinolytic activity was found to be slower than untreated BJFTE, which caused complete dissolution of the clot within 48 h. It was observed that in case of the heat-treated BJFTE; the fibrinogenolytic activity was significantly reduced but not abolished suggesting that the active components of the extract are not completely thermolabile. But in case of fibrinolytic activity, the heat-treated extract could dissolve the post-treated clots but not the pre-treated clots. This could be because the components that act on post-treated clot are different from the ones acting on pre-treated clot. Also these components could be differently sensitive to heat. The results of the assay showed that BJFTE did not interfere with the clotting process as the clots were formed within 3 h of addition of thrombin. However, it was earlier observed that BJFTE could digest both Aα and Bβ chains of fibrinogen and increased the recalcification and thrombin time. Consequently, this is indicative of the fact that the fibrinogenolytic activities of BJFTE are much slower compared to the fibrin polymerization pathway. The pre-treated and the post-treated clots made with essentially plasminogen free fibrinogen were not digested even up to 48 h. This implied that there was some correlation between plasminogen and BJFTE with respect to fibrin clot digestion. Possibly, the active components in the extract act as tissue plasminogen

activators and absence of plasminogen inhibits their ability to digest the fibrin clot just like *Desmoteplase* purified from the saliva of vampire bats (Hawkey C, 1966).

Some phospholipases induce degradation of bilayer phospholipids or channel-forming agents embedded into the RBC membrane while lytic peptides can alter cell membrane permeability resulting in altered ion transport leading to cell swelling and osmotic lysis. Hemolysis is a frequent effect of cnidarian stinging and a number of cnidarian species have been reported to have active hemolytic proteins/peptides. BJFTE also showed strong hemolytic activity in a dose and time dependent manner. This observation was in accordance with the previous reports of presence of hemolytic factor Rhizolysin from the nematocysts as well as from the tissue devoid of nematocysts of *Rhizostoma pulmo* (Cariello et al., 1988; Allavena et al., 1998). Research by Allavena et al. has revealed that jellyfish tissue devoid of nematocysts is still able to destroy cells including erythrocytes, though the toxic effects of this tissue are not as strong as pure nematocyst. In addition, the tissue surrounding nematocysts has a different chemical composition than the fluid of nematocysts. This discovery raises the possibility that jellyfish toxins or toxin precursors are produced in the tissue surrounding the nematocysts and later transported into It. Phospholipase activity was absent in BJFTE and the drastic increase in the hemolytic activity between 60 min and 120 min as compared to the increase between 30 to 60 min suggests that the active components of the extract belong to the category of lytic peptides, which bind to the surface and induce change in ion flux across the membrane causing cell lysis through ion and solution imbalance, following the formation of pore-forming complexes in cell membranes. This was in sync with the time dependent morphological changes that were spotted on treating 1% RBC suspension with 10 µg of BJFTE at 100X magnification of the compound light microscope (Figure 32). SEM images for RBCs treated with BJFTE also showed distinct surface membrane morphology changes (Figure 33).

BJFTE prolonged the recalcification and thrombin time in a time dependent manner suggesting that BJFTE cleaves fibrinogen in such a manner that the resultant fibrino-peptides delay the clotting process. BJFTE also inhibited ADP

dependent platelet aggregation in a dose dependent manner. BJFTE is thus capable of affecting the haemostatic system at three different levels: platelet aggregation, fibrinogen digestion and fibrin clot digestion.

There are substantial reports on the presence of proteolytic enzymes in cnidarian venoms (Lee et al., 2011; Hessinger and Lenhoff, 1973; Tamkun and Hessinger, 1981;Halstead, 1988; Long and Burnett, 1989; Endean et al., 1993; Macek, 1992; Gusmani et al., 1997). BJFTE also showed strong proteolytic activity for fibrinogen, casein, gelatin and azocasein. Majority of the proteolytic proteins present in the extract were metalloproteinases as treatment with EDTA completely abolished the activity. Proteolytic enzymes are one of the major contributors to inducing diverse toxicological effects, including hemorrhage, edema and necrosis (Takeya et al., 1990). The protease-mediated local tissue damage involves the degradation of extracellular matrix and connective tissue surrounding blood vessels (Dunn, 1989). In case of snake venom metalloproteinase, they cause fibrinogenolysis, fibrinolysis, apoptosis, activation of prothrombin and Factor X and the inhibition of platelet aggregation (Fox and Serrano, 2005; Rucavado et al., 1998). Thus venom proteases play a significant role in the pathogenesis of venom-induced local tissue damages.

Several antimicrobial compounds have been isolated from cnidarians but unfortunately with the exception of few, which have been evaluated in pre-clinical evaluations, no compound originating from cnidarian have, to date, been seriously considered for utilization (Mariottini and Grice; 2016). BJFTE was also tested for antimicrobial activity. It did not show any significant antimicrobial activity against gram-positive or gram-negative bacteria.

CHAPTER 4

PURIFICATION OF ACTIVE ANTICOAGULANT COMPONENT(S) FROM BARREL JELLYFISH TENTACLE EXTRACT

4.1. Introduction

Previous experiments with BJFTE revealed its ability to affect the hemostatic system at three different levels: platelet aggregation, fibrinogen digestion and fibrin clot digestion was observed. The next logical step was to purify the active protein/peptide components from the extract. Historically, the isolation, characterization and purification of the bioactive components in jellyfish venom have been challenging and results have varied significantly among research groups (Bloom et al., 1998; Tibballs, 2006). Problems encountered during isolation and characterization included thermolability, adhesion to preparative surfaces and aggregation/disaggregation of components, which further complicated the investigations (Bloom et al., 1998; Endean et al., 1993; Othman and Burnett, 1990).

The methods used during the purification depended on the nature of the component proteins. The initial state of the protein mixture, the required sample size and the reason for the extraction were the factors that contributed to decide which protein purification technique(s) should be used. Each protein purification step usually resulted in some degree of product loss. Therefore, an ideal protein purification strategy was the one in which the highest level of purification was reached in least number of steps. For protein purification, visualization and assessment of purification following techniques were used:

A. Buffering component

When purifying a protein for activity assays, it's important that the protein should be soluble and active. For such experiments, it is crucial to use a buffer that prevents unfolding and aggregation. The following factors were considered during the designing of the buffer:

Buffer system: The buffering compound, nature and pH of the buffer depended on the stability of the target protein and the purification procedure. Most

experiments are done at pH 7.4 to mimic biological conditions. When BJFTE was tested for its activity over a pH range from 6.8 to 9, maximum activity was observed in case of pH 7.4.To avoid time and protein loss caused by an additional buffer exchange step, it was preferred to choose a buffer that was compatible with most of the chromatography steps.

Additives: Various agents or additives are added to the buffer to augment protein solubility and stability. For example: salts (maintain ionic strength of medium), reducing agents (reduce oxidation damage), detergents (solubilization of poorly soluble proteins) and glycerol (stabilization).

One of the important classes of additives is the protease inhibitor. They are chemical compounds used to protect protein samples from the digestive function of proteases, which is a major problem faced during fractionation of natural protein mixtures, e.g., venom. Mostly a wide spectrum protease inhibitor cocktail is used and is recommended to be added to the protein extraction buffer. The protein inhibitor cocktail used in our study was cOmplete™ ULTRA Tablets, Mini, EDTA-free EASYpack (Roche). It was used during the tentacle extract preparation and storage for a longer duration. All inhibitors in the protease inhibitor cocktail tablets could be removed via dialysis by using a dialysis membrane with cutoff >10 kDa (as recommended by the manufacturer).

Buffer	Concentration	Additive
Potassium Phosphate Buffer	20mM	Nil
Potassium Phosphate Buffer	20mM	100mM NaCl; 200mM NaCl
Tris-HCl	20mM	10mM $CaCl_2$
Phosphate Buffer Saline	1X	Nil
Acetonitrile-Trifluoroacetic Acid (0.1% TFA)	0-80% (Gradient)	Nil

Table 7. Different buffers used in different attempts attempted for purification of active components. A pH of 7.4 was maintained for all buffers as maximum activity was observed at this pH (except for ACN/TFA)

B. Chromatography techniques

Chromatographic methods were applied using bench-top columns or automated HPLC equipment. Both, manually packed as well as pre-packed columns, were used in case of bench-top columns. These columns were mainly ion-exchange columns. Separation by HPLC was done by reverse-phase or size-exclusion methods:

Ion-exchange Chromatography: Separation in ion-exchange chromatography depends upon the reversible adsorption of charged solute molecules to immobilized ion-exchange groups of opposite charge. Anion-exchange columns contain a stationary phase with a positive charge that attracts negatively charged proteins. Cation-exchange columns are the reverse, negatively charged beads, which attract positively charged proteins. Separation is obtained as different substances have different degrees of interaction with the ion-exchanger due to differences in their charges, charge densities and distribution of charge on their surfaces. These interactions can be controlled by varying conditions such as pH and ionic strength. The differences in charge properties of biological compounds are often considerable. The ion-exchange chromatography is capable of separating species with very minor differences in properties, e.g. two proteins differing by only one charged amino acid, hence it is a very powerful and most commonly used separation technique.

Reverse Phase Chromatography (RPC): Reverse-phase chromatography separates proteins based on their relative hydrophobicities. This technique is highly selective and requires the use of organic solvents. These solvents cause permanent denaturation and loss in functionality of many proteins, during chromatography. Therefore this method is not recommended for all applications, particularly if it is necessary for the target protein to retain activity.

Size Exclusion Chromatography (SEC): Size exclusion chromatography is the separation of mixtures based on the molecular size of the components. Separation is achieved by the differential exclusion or inclusion of solutes as they pass through stationary phase consisting of heteroporous (pores of different sizes) cross linked polymeric gels or beads. It is based upon different permeation rates of each solute molecule in the interior of gel particles. Size

exclusion chromatography involves gentle interaction with the sample which enables high retention of biomolecular activity. For separation of organic polymers in non-aqueous systems is called gel permeation chromotagraphy (GPC) and for separation of biomolecules in aqueous systems, SEC is referred to as gel filtration chromatography (GFC).

Column	Type of Column	Company
CM Sephadex C50	Ion Exchange Chromatography (Weak Cation Exchanger)	Sigma Aldrich (Manually Packed)
DEAE Sephadex	Ion Exchange Chromatography (Weak Anion Exchnager)	Sigma Aldrich (Manually Packed)
Q Sepharose High Performance	Ion Exchange Chromatography (Strong Anion Exchanger)	GE Life Sciences
Q Sepharose FF	Ion Exchange Chromatography (Strong Anion Exchanger)	GE Life Sciences
Protein-Pak SEC 300 (10µm; 300X7.5mm)	Size Exclusion Chromatography	Waters
Yarra 3µm SEC-2000 (300X7.8mm)	Size Exclusion Chromatography	Phenomenex
BioSep SEC-s2000 (300X7.8mm)	Size Exclusion Chromatography	Phenomenex

Table 8. Different columns that were used during the purification process.

C. Protein precipitation, dialysis and sucrose concentration

Most recognized mechanism is salting proteins out of solution which occurs when the water molecules are titrated away from the solvent shells around the protein to the solvent shells around the ions that make up the salt. The cations and anions are ordered by their ability to precipitate proteins in the Hofmeister series. Salts high in the Hofmeister series are the most efficient at protein precipitation because of the large and stable solvent shells they sustain. This increases the surface tension of the solution that effectively increases the hydrophobic effect, which in turn stabilizes the protein structure while encouraging the hydrophobic regions on the surfaces of different molecules to interact, affecting aggregation. Once the proteins start aggregating into larger structures, the amount of water per protein drops, enhancing the density differences between the proteins and the solute. In addition, the density differences increase enough for the aggregates to be readily pelleted in the centrifuge. The efficiency of this method depends on the concentration of the protein being precipitated – the lower the concentration, harder it is to form aggregates. Proteins with a larger amount of hydrophobic surface character

precipitate at lower salt concentrations than one with little hydrophobic surface character and these protein-to-protein differences are exploited during protein purification procedures.

Ammonium sulfate is the most commonly used salt to precipitate proteins selectively since it is very soluble in water; it allows high concentration about 4M. NH_4^+ and SO_4^{2-} are both favourable, non-denaturing and end of the Hofmeister series.

However, even though salting out occurs via a phase transition mechanism, some quantity of salt will come down with the protein that is removed by dialysis or a de-salting column. Dialysis was performed using a benzoylated cellulose membrane with a pore size of 2000 NMWCO from Sigma Aldrich.

D. SDS-PAGE

SDS-PAGE can be used to determine the molecular mass of the mixture of proteins by comparing the positions of the bands to those produced by proteins of known size. SDS used in electrophoresis resolves mixture of proteins according to the length of individual polypeptide chains (Karp, 2008; Cseke et al., 2004). SDS-PAGE is often used to test the purity of a protein after each step in a series.

The partially purified protein from the chromatography separation can be further purified with reducing polyacrylamide gel electrophoresis (PAGE) or native gel electrophoresis. As unwanted proteins are gradually removed from the mixture, the number of bands visualized on the SDS-PAGE gel, is reduced, until there is only one band representing the desired protein.

Zymography: Another electrophoretic technique based on SDS PAGE and a substrate (e.g. gelatin, casein, albumin, hemoglobin etc.) co-polymerized with the polyacrylamide matrix, is zymography which is used to measure enzyme activity. The technique is particularly useful for analyzing the proteinase composition of complex biological samples. Proteins are prepared by the standard SDS-PAGE buffer under non-reducing conditions (no boiling and no reducing agent). They are separated by molecular mass in the standard

denaturing SDS-PAGE co-polymerized with a protein substrate. After electrophoresis, peptidases are re-natured by the removal of the denaturing SDS by a non-ionic detergent, such as Triton X- 100. This is followed by incubation in conditions specific for each peptidase activity (temperature, time, ions, ionic strength), when the enzymes hydrolyze the embedded substrate. Then proteolytic activity can be visualized as cleared bands on a Coomassie stained background (Heussen and Dowdle, 1980). With substrate zymography, proteases with different molecular weights showing activity towards the same substrate can be detected and quantified on a single gel.

4.2. The Attempts

Different permutation and combinations of the above mentioned techniques were used and numerous attempts were made to purify the active components of the tentacle extract.

Initial screening was done for choosing the ideal buffer pH for purification and also with respect to the binding of the tentacle extract proteins to different ion-exchange matrices (CM Sephadex C50/DEAE Sephadex). It was observed that maximum activity was obtained at a pH of 7.4. Also the binding of BJFTE proteins was higher in case of CM Sephadex C50 as compared to DEAE Sephadex.

4.2.1. Attempt # 1

CM Sephadex C50 was mixed with several volumes of double distilled water and broken beads (fine) were discarded by decantation. Matrix suspension was then boiled at 100°C for 30 min to open the pores on the beads. Matrix was then allowed to swell by soaking in dd H_2O over night at 4°C. A 5 ml syringe was fixed to a stand using a clamp in cold chamber (4°C). A thin layer of moist glass wool was placed at the base of the syringe to stop the matrix from flowing out. Matrix suspension was then poured into the column without air bubble formation. Equilibration of the column was done by passing several bed volumes of 20 mM potassium phosphate buffer (pH 7.4) into the column so as to obtain constant pH throughout the column matrix.

BJFTE (0.7mg/200µl) was then subjected to cation-exchange chromatography on a column of dimensions (6cm x 1.25 cm). The column was first washed with two column volumes of running buffer only and unadsorbed fractions were collected in microfuge tubes (1 ml each) at a flow rate of 0.5 ml/min. The adsorbed fractions were then eluted with a linear gradient of NaCl (0 - 0.5 M) in 3 column volumes of running buffer. The fraction size was limited to 1ml and absorbance was measured at 280 nm.

The peak resolution was not very sharp and the absorbance values were low. This could be because of i) Low concentration of loading sample and ii) small column size.

4.2.2. Attempt # 2

Earlier reported attempts at purification of proteins from different jellyfish venoms and extracts using size exclusion chromatography encouraged on attempting the same. The required facility was provided by the Structural Biology and Bio-Informatics Laboratory, Indian Institute of Chemical Biology, Kolkata.

Fractionation was attempted using Waters Protein-Pak SEC 300 (10 µm; 300 X 7.5 mm) size exclusion chromatography column. BJFTE was fractionated using 20 mM potassium phosphate buffer+100 mM NaCl as the buffer and a flow rate of 0.5 ml/min was maintained. Only peak fractions were collected for further studies. Later fractionation was also attempted using 200 mM NaCl with the potassium phosphate buffer. However, not much difference was observed in such cases. The protein peaks/shoulders obtained were checked for both fibrinogenolytic as well as fibrinolytic activities. It was observed that the shoulder fraction, obtained at retention time of 14.5 min, showed fibrinogenolytic as well as fibrinolytic activity (Figure 39). Mild fibrinogenolytic activity was also observed in fractions collected at retention time 9.2 and 12 min. Attempts were made to visualize the protein components of this fraction. No bands were visible on coomassie or silver staining. Only when the gel was stained with silver blue, a very faint band was visible. This band corresponded to an apparent molecular weight of ~95 kDa (Figure 40).

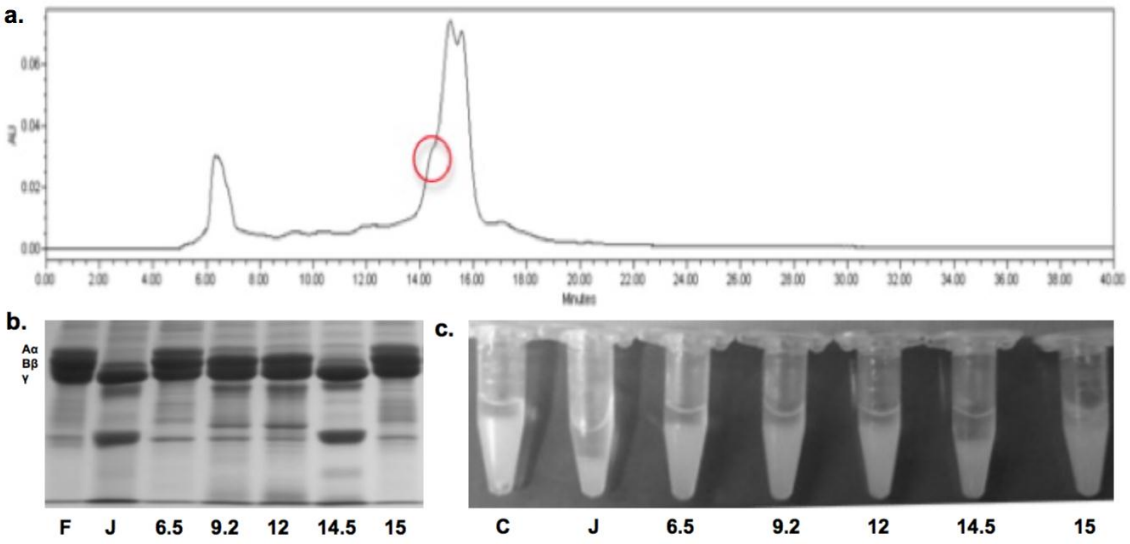


Figure 39. Size-exclusion chromatogram for BJFTE, fibrinogenolytic activity and fibrinolytic activity of all the fractions obtained. (a) Size exclusion chromatogram of BJFTE. (b) Fibrinogenolytic activity of fractions collected at different retention time after 5h of incubation with 2mg/ml of fibrinogen. (F) Only fibrinogen (J) Fibrinogen with BJFTE (6.5, 9.2, 12, 14.5, 15) Different fractions incubated with fibrinogen (c) Fibrinolytic activity of the fractions obtained after SEC, after 12h.

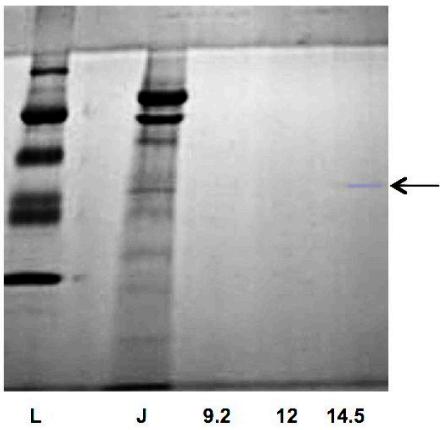


Figure 40. Silver blue stained 12% SDS PAGE gel of the fractions that showed fibrinogen-olytic activity. (J) BJFTE (9.2, 12, 14.5) fractions obtained after size-exclusion chromatography that showed fibrinogenolytic activity.

The proteolytic activity for this fraction was assessed by substrate zymography (fibrinogen, casein and gelatin). In case of gelatin and casein zymograms, proteolytic activity was observed for this 95 kDa band and the activity was lost on treatment with EDTA but not PMSF. In case of substrate fibrinogen no proteolytic activity was observed corresponding to 95 kDa band but proteolytic activity was observed for a band of 66k Da. This suggested two things, one that the fraction obtained at retention time 14.5 min had more than one proteins present in it and secondly that the fibrinogenolytic activity was dependent on its concentration, as the same protein in the BJFTE lane showed fibrinogenolytic activity. It was also observed that both the 95 kDa (active in BJFTE) and 66 kDa lost their fibrinogenolytic activity in presence of EDTA. 66 kDa band showed proteolytic activity only for fibrinogen (Figure 41). The concentration was extremely low for any further characterization.

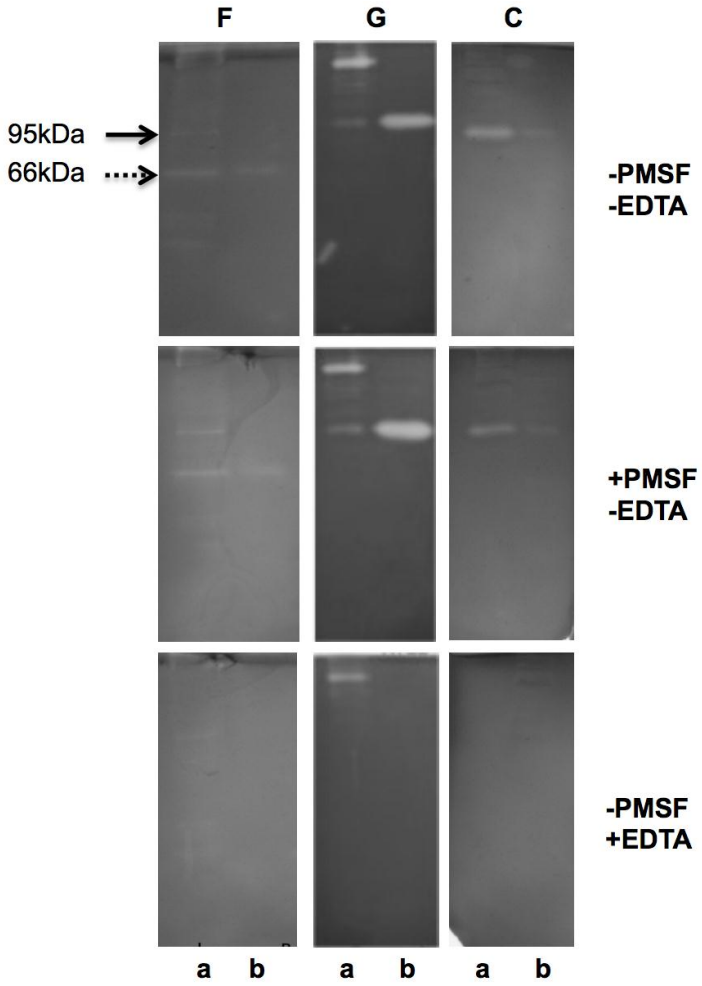


Figure 41. Analysis of proteolytic activity of fraction 14.5 by substrate zymography. Fibrinogen (F), gelatin (G) or casein (C) was used as a substrate and copolymerized in non-reducing 12% SDS-PAGE for analyzing the proteolytic activities. (a) BJFTE (b) Fraction 14.5. Same set of zymography assays were performed in the presence of 2mM EDTA and 1mM PMSF during the enzymatic reaction time.

4.2.3. Attempt # 3

To overcome the problem of extremely low concentration of semi-pure fraction faced in the previous attempt, another concentration step of ammonium sulphate precipitation was added. First varying percentages (20%, 40%, 60% and 80%) of saturation of ammonium sulphate were screened for optimal activity and substantial protein enrichment. Both the pellets and supernatants obtained for these four saturation concentrations were tested for their fibrinogenolytic activity and protein composition. The ideal saturation concentration was decided to be 40% and the pellet obtained was used as the loading sample/starting sample for the next step of purification. The pellet was suspended in buffer and then dialyzed against 20 mM potassium phosphate buffer, pH 7.4. This was further referred to as 40P, which was then fractionated using a CM-Sephadex C50 column (12cm X 1.5cm). The column was first washed with two column volumes of running buffer only and unadsorbed fractions were collected in microfuge tubes (1 ml each) at a flow rate of 0.5 ml/min. The adsorbed fractions were then eluted with a linear gradient of NaCl (0 - 0.5 M) in 3 column volumes of running buffer. The fraction size was restricted to 1ml and absorbance was measured at 280 nm. Fractions 63 and 67 showed very strong fibrinogenolytic activity (Figure 42).

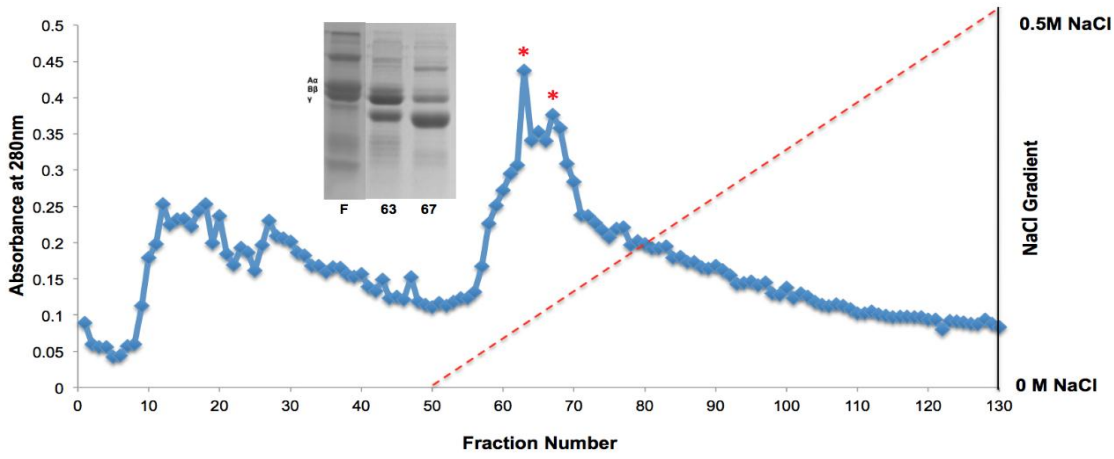


Figure 42. Ion exchange chromatogram for 40P. Inset: Fraction 63 and 67 showed strong fibrinogenolytic activity.

Peaks containing fractions 63 and 67 were pooled and subjected to further fractionation on a linear gradient (0.2 - 1 M) of NaCl on CM Sephadex C-50 column of dimensions (12 cm x 1.5 cm). One milliliter fractions were collected at a flow rate of 0.5 ml/ min. Fractions 14 and 16 showed strong fibrinogenolytic activity (Figure 43).

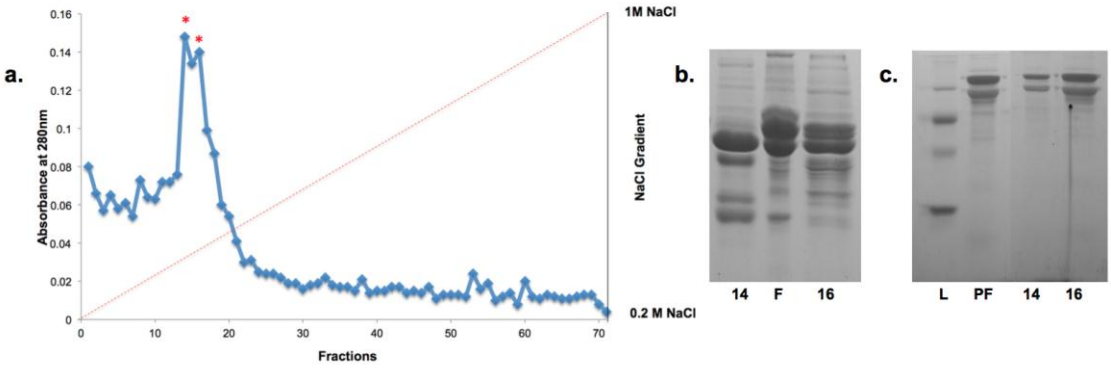


Figure 43. Ion-exchange chromatogram for pooled fraction, fibrinogenolytic activity and protein composition of the active fractions. (a) Ion exchange chromatogram for pooled fraction (peaks containing fractions 63 and 67; PF). (b) Fraction 14 and 16 showed strong fibrinogenlytic activity. (c) Purity profile for the active fractions. High molecular weight ladder, 205-29 kDa (L) pooled fractions (PF) fraction 14 (14) and fraction 16 (16).

Attempts were made to further purify fractions 14 and 16 by size exclusion chromatography using Yarra 3µm SEC-2000. The mobile phase used was 20mM potassium phosphate buffer, pH 7.4, and a flow rate of 0.5ml/min was maintained. Fractions were collected as and when a peak was obtained. The chromatogram obtained was not in synchrony with the principle of size exclusion, as the peaks obtained were beyond a retention time of 20 mins. Fractions 14 and 16 consisted of high molecular weight proteins (150 kDa-65 kDa) and as per the standards provided with the column, proteins of this molecular weight range should have been eluted out at retention time between 14-17 mins for a flow rate of 0.5ml/min. Also the concentration of proteins was extremely low (undetectable) for any further analysis (Figure 44).

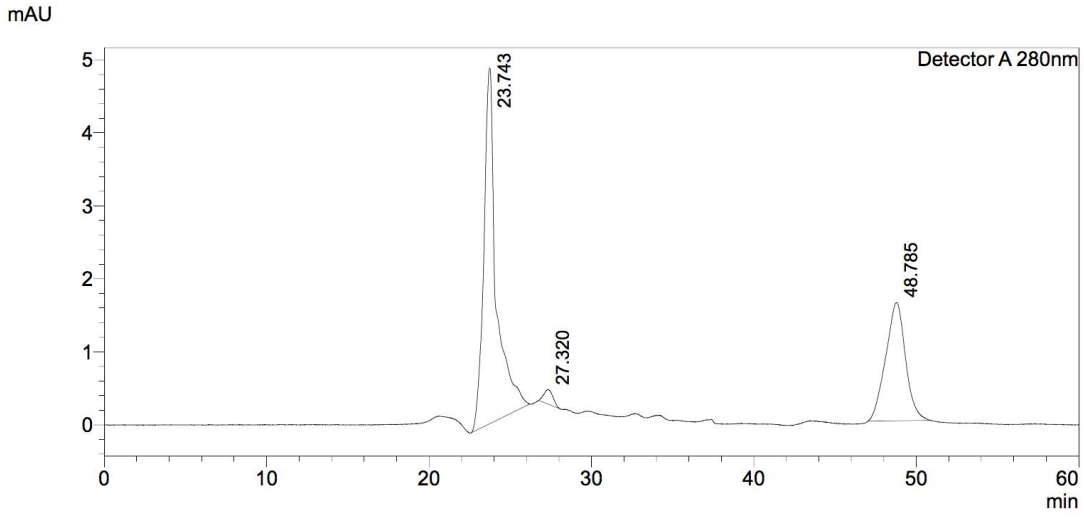


Figure 44. Chromatogram obtained after running fraction 16 on Yarra 3µm SEC-2000.

4.2.4. Attempt # 4

Next, attempts were made by directly using 40P as the loading sample for size exclusion chromatography. 40P resulted in clogging of the column and the purification profile that was obtained was yet again not in synchrony with the principle of size exclusion chromatography.

4.2.5. Attempt # 5

Yarra 3µm SEC-2000 column was replaced with BioSep SEC-s2000 column and attempts were made to purify using 40P. There were signs of clogging yet again so 40P was centrifuged once again before loading it on the column. After centrifugation the supernatant was used and referred to as $40P_s$. Based on previous experiments, it was observed that some of the active components were metalloproteinases. Calcium chloride was added in the eluting buffer for the purification. Addition of $CaCl_2$ is known to be favourable for the purification of metalloproteinases .

Next round of fractionation was performed using $40P_s$ as the loading sample on BioSepSEC-s2000 column with 20mM Tris + 10mM $CaCl_2$, pH 7.4 as the mobile phase. Decent amount of separation was obtained but the profile was not in synchrony with the principle of size exclusion chromatography and the fibrinogenolytic activity was completely lost.

4.2.6. Attempt # 6

Next attempt of purification was carried out with $40P_s$ as the loading sample and 20mM phosphate buffer as the mobile phase. Multiple runs were given and the peaks obtained at the same retention time for each run were pooled and rerun on BioSep SEC-s2000 column at different flow rates (0.5 ml/min, 1ml/min and 2ml/min). This was done to try and further purify the multiple protein bands that were obtained in the previous round of purification with $40P_s$.The results thus obtained were not very conclusive, as intensity of the protein peaks was very low.

4.2.7. Attempt # 7

A combination of ion exchange and size exclusion chromatography was tried next. $40P_s$ was used as the loading sample for ion-exchange column chromatography (CM Sephadex C 50; 16X1.7 cm). Active fractions were obtained in the adsorbed region at a NaCl gradient of 0.2-0.3 M. The peak that consisted the active fractions was pooled and this pooled fraction mixture was loaded on the size exclusion column (BioSepSEC-s2000). The purification profile thus obtained had very low intensity peaks suggesting low concentrations of protein. To overcome this limitation of low concentration of proteins scale up was done at two levels:

i. Scale up I: The amount of $40P_s$ that was loaded on the CM Sephadex C-50 column was doubled with respect to the protein concentration, while keeping the loading volume unchanged (protein concentration: 2 mg; loading volume: 1ml) (Figure 45).
ii. Scale up II: IEC was repeated twice with scaled up sample loading concentration. The active fractions for each round of IEC were compared and found to have similar protein composition. All these fractions were pooled together and concentrated against sucrose (mixed pooled fractions; M). This was used for SE-HPLC (Figure 46).

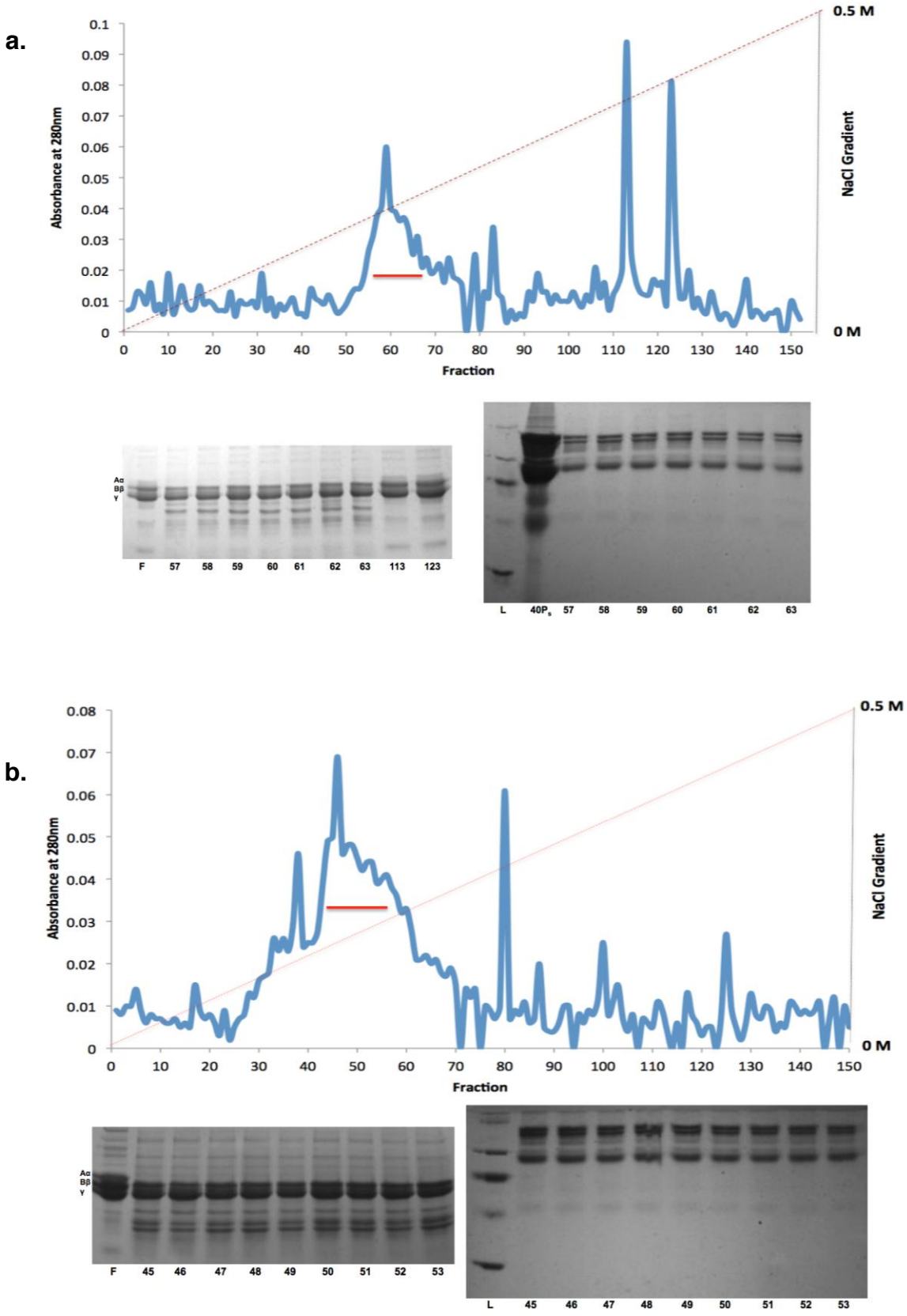


Figure 45. Ion-exchange chromatogram, fibrinogenolytic activity and protein composition of the active fractions. (Two consecutive rounds of ion exchange chromatography using CM Sephadex C-50)

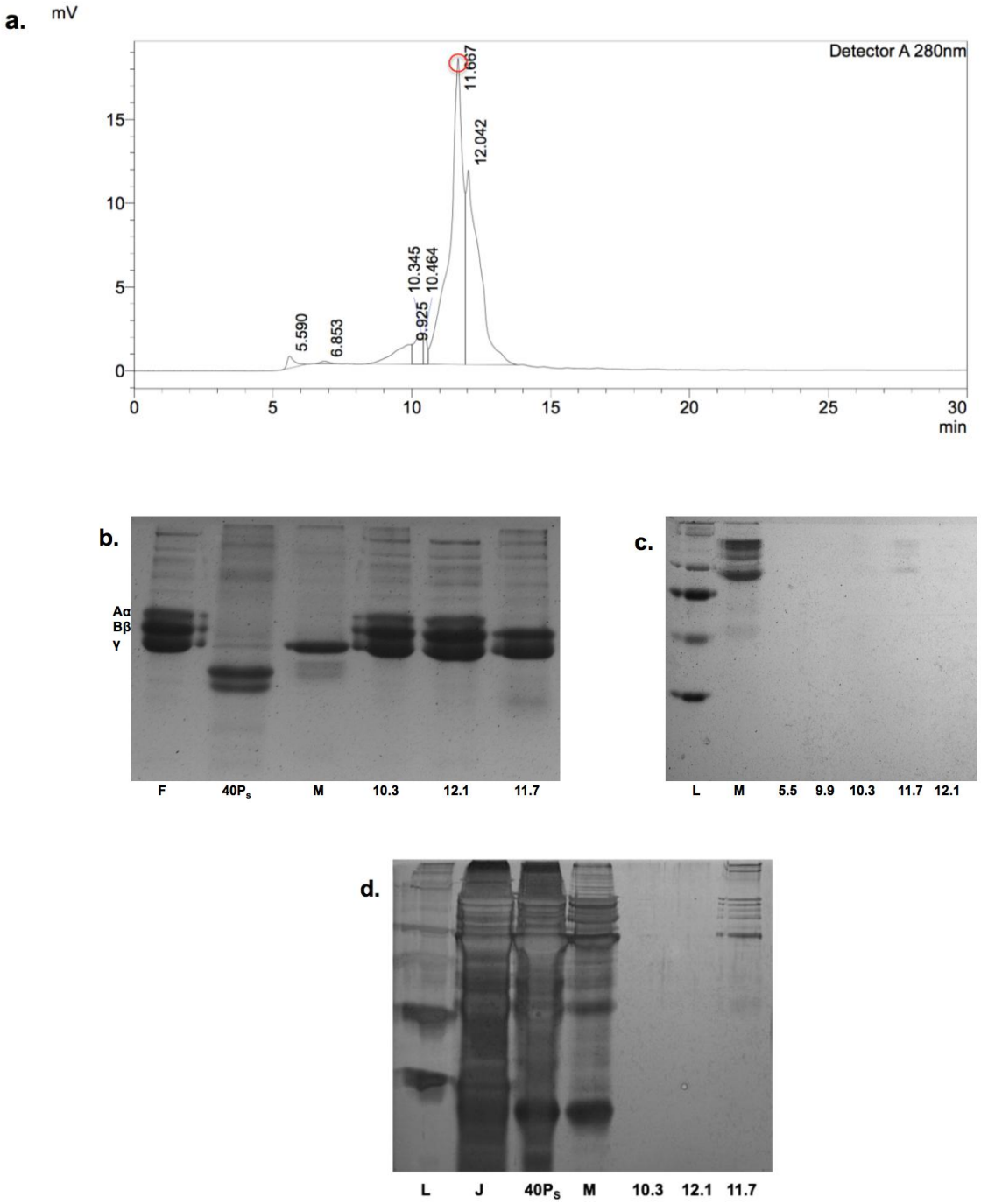


Figure 46. Ion-exchange chromatogram for mixed pooled fraction, fibrinogenolytic activity and protein composition of the active fractions. (a) Chromatogram obtained after SE-HPLC (flow rate 1ml/min) using mixed pool fraction "M" as the loading sample. (b) Fraction obtained at a retention time of 11.7 min could digest Aα chain of fibrinogen and (c) consisted of high molecular weight protein bands. (d) a silver stained gel that shows the level of purification achieved after every step of purification (10% SDS PAGE).

After scaling up at both these levels, an active fraction was obtained at a retention time of 11.7 minutes. This fraction consisted of six/seven high molecular weight proteins and thus did not show any coherence with the principle of size exclusion. This may be caused by aggregation of proteins.

Attempts were made to address this problem of protein aggregation. The active fraction obtained at the retention time of 11.7 min was treated with 8M urea and further purification was attempted using Biosep SEC-s2000 column. The results obtained were inconclusive. Mixed pooled fractions (M) was treated with 8M urea and run on Biosep column. The separation thus obtained was inconclusive yet again.

Fraction 11.7 was run on Q Sepharose FF (5ml), Q Sepharose High Performance (5ml) and CM Sephadex C-50 (6cm x 1.25 cm) columns as an attempt to further purify the proteins but none of the columns could resolve the proteins any further. The chormatograms obtained remained inconclusive.

It was observed that the mobile phase used for RP-HPLC, a mixture of acetonitrile and TCA caused a loss in fibrinogenolytic activity in BJFTE. Still RP HPLC was attempted to separate the proteins in fraction 11.7 and check the exact number of proteins that were present. The results for this chromatography were inconclusive as well.

4.2.8. Attempt # 8

Next direct SE-HPLC of $40P_s$ was tried by changing the buffer system, 1X phosphate buffer saline pH 7.4 was used instead. An injection volume of 200 µL was used and eluted at a flow rate of 1 mL/min for 30 min. The elution of protein/ peptide fractions was recorded with a UV detector set at A_{280nm}. The eluate corresponding to each peak was collected in 1 mL fractions (Figure 47). An active fraction at retention time 5.643 min (Fraction 5.6) was obtained. It corresponded to a molecular weight of 528.68 kDa when plotted against the SE-HPLC BioSep-SEC-s2000 standard at a flow rate of 1ml/min with phosphate buffer saline as the mobile phase (Figure 48a). For the first time a chromatogram was obtained where the peaks were in sync with the principle of

SE-HPLC. The collected fractions were concentrated against sucrose at 4°C. The protein content of each peak was estimated by Bradford assay. The final concentrated samples were stored in -80 °C till further use.

Fraction 5.6 showed fibrinogenolytic activity but no fibrinolytic activity (Figure 48c and 49). Fibrinolytic activity was observed in fractions obtained at retention time 7.2 min and 8.7 min. An over-developed silver stained gel showed presence of lower molecular weight proteins in the fibrinolytic fractions (Figure 50). Fraction 5.6 revealed four protein bands in SDS-PAGE, out of which only one showed very strong proteolytic activity for all the three substrates, i.e. fibrinogen, casein and gelatin in zymographic assays (Figure 48b and 51). This proteolytic activity corresponded to a protein band of 95 kDa (approx.). This was named as Rhizoprotease. The proteolytic activity was completely lost on treatment with EDTA. However, this activity was retained on treatment with PMSF. This data was further supported by detection of Zn^{2+} with Frac 5.6 by atomic absorption spectroscopy on an AA-7000F Shimadzu Spectrophotometer. Samples were dissolved in Millipore filtered water and parallel water control was also analyzed for Zn^{2+} content.

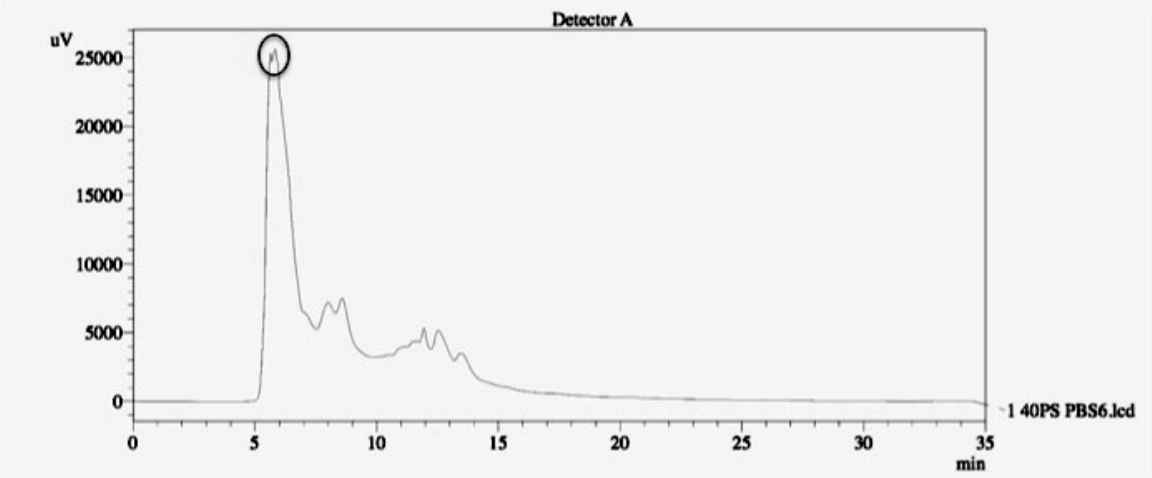


Figure 47. SE-HPLC of $40P_s$ with 1X PBS pH 7.4 as the mobile phase and a flow rate of 1ml/min.

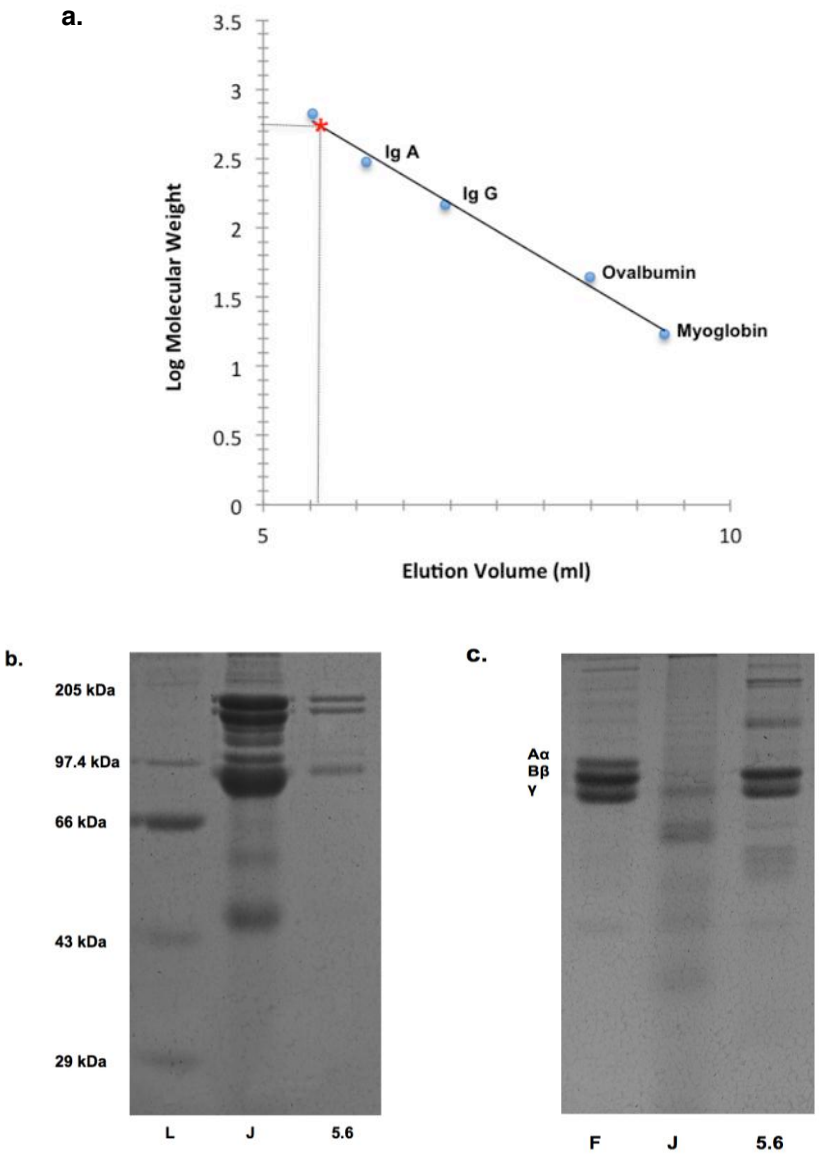


Figure 48. Column calibration curve, fibrinogenolytic activity and protein composition of the active fraction. (a) Calibration curve for the SE-HPLC column; * on the calibration curve denotes the active fraction (b) comparative protein profile of fraction 5.6 and BJFTE (c) fibrinogenolytic activity of Frac. 5.6 compared to BJFTE for 1mg/ml of fibrinogen. The concentration of fibrinogen was reduced, as the concentration of the active fraction was very low.

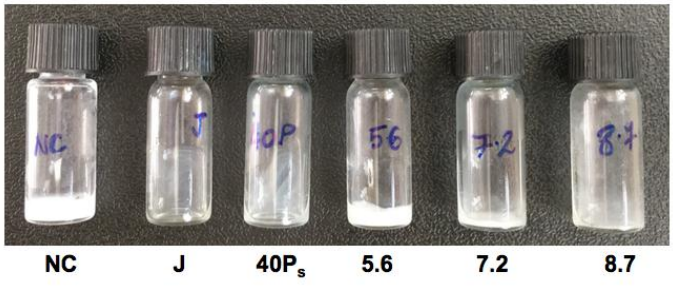


Figure 49. Fibrinolytic activity of different fractions obtained after SE-HPLC of $40P_s$. The volume of clot made *in vitro* was reduced and the thickness was reduced by making them in glass ampoules as the concentration of fractions obtained was low.

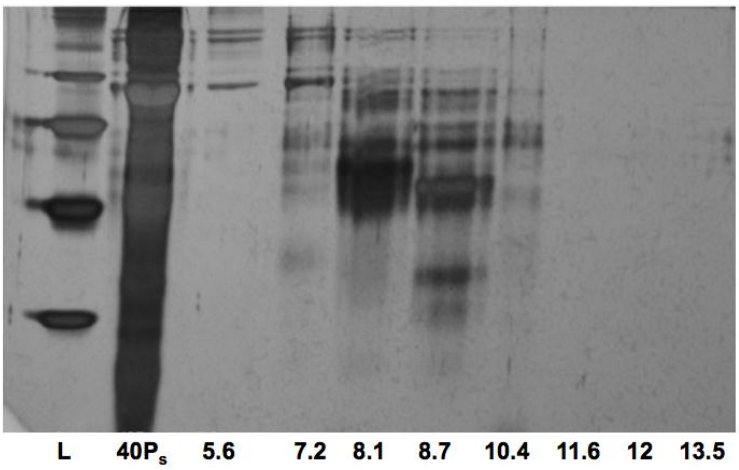


Figure 50. Silver stained gel of different fractions obtained after SE-HPLC of $40P_s$. An over developed silver stained gel which shows the presence of comparatively lower molecular weight protein bands in fraction 7.2 and fraction 8.7 which showed fibrinolytic activity.

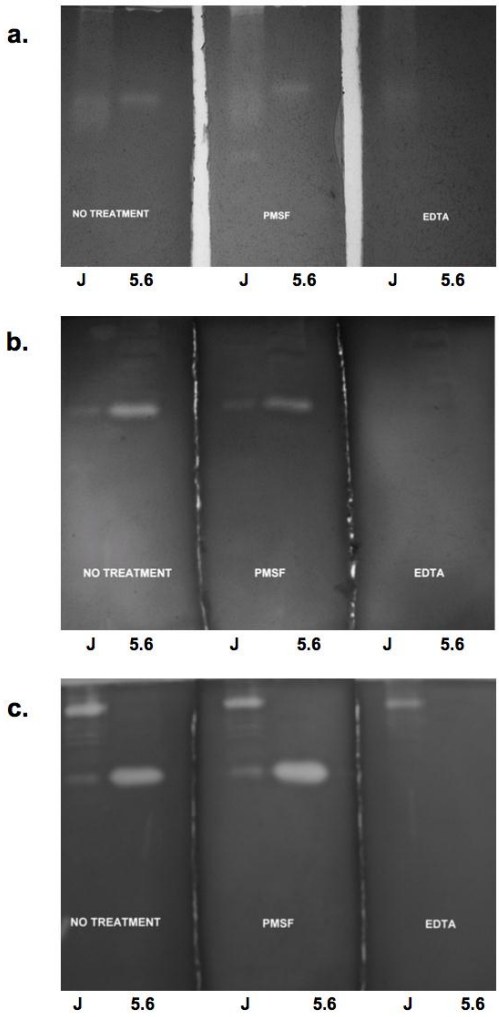


Figure 51. Analysis of proteolytic activity of fraction 5.6 by substrate zymography (a) Fibrinogen (b) Casein and (c) Gelatin were used as substrate and copolymerized in non-reducing SDS-PAGE for analyzing the proteolytic activity of (J) BJFTE and (5.6) Semi-pure fraction obtained after SE HPLC. Same sets of zymography assays were performed in the presence of EDTA and PMSF.

Rhizoprotease (95 kDa) protein band was excised from a coomassie stained gel and was subjected to Protein Mass Fingerprinting (PMF). The analysis was done by Sandor Life Sciences Private Limited, Hyderabad, India. The sample was analyzed using MALDI TOF/TOF ULTRAFLEX III instrument. Further analysis was done with flex analysis software for obtaining the peptide mass fingerprint. The masses obtained in the peptide mass fingerprint were submitted for Mascot search in concerned database for identification of similarities in sequences.

Several peptides of mass numbers ranging from 905 to 3700 resulted from digestion in PMF analysis. However, no previously described protein was found to match Rhizoprotease, which indicated that it is a novel protein. The closest hit to Rhizoprotease was found to be gi|951575371, PREDICTED: cytosolic carboxypeptidase 2 [*Dinoponera quadriceps*], with a protein score of 88 (where protein score greater than 78 was significant). It is a zinc metalloproteinase of the M14 family of metallocarboxypeptidases (Appendix I).

4.3. Discussion

Different combinations of fractionation techniques were attempted over extended period to purify the active components from the extracts. Owing to the highly unstable nature of jellyfish toxins and tendency to aggregate, most of the attempts for purification were not very fruitful. Proteomic studies of the venom of creatures such as cone snails, scorpions and spiders that are often dominated by the presence of relatively small disulfide-rich peptides: explaining the proteome of jellyfish species has been complicated because of the presence of more number of high molecular weight proteins in the venom (Brinkman et al., 2009). Using ammonium sulphate precipitation and SE-HPLC, partial purification of a protein (Frac 5.6) with significant fibrinogenolytic activity was achieved. However, it was found to be contaminated with four high molecular weight proteins. Further purification of this fraction was attempted, but these four proteins always eluted out together. Out of these four proteins a 95 kDa protein showed very strong fibrinogenolytic, caesinolytic and gelatinolytic activity. All these proteolytic activities were lost on treatment with EDTA. This 95 kDa protein was called Rhizoprotease.

Peptide mass fingerprinting of Rhizoprotease did not show any match, suggesting it to be a novel protein. But the most significant hit was that of PREDICTED: cytosolic carboxypeptidase 2 from *Dinoponera quadriceps* (giant ant). M14 family of metallo-carboxypeptidases is Zn^{2+} dependent metalloproteinases and is known to play a role in blood coagulation. Atomic absorption spectral analysis of Frac 5.6 also revealed the presence of Zn^{2+}. The giant ant venom is also known to possess anticoagulant and antiplatelet activities (Madiera et. al., 2015). The next few hits were with parasitic beef tapeworm which is from the same family as the pork tapeworm and inhibits extrinsic blood coagulation pathway and platelet aggregation (Wang et al., 2006); Hypothetical protein HELRODRAFT_100443 from freshwater jawless leech from which Leech Antiplatelet Protein (LAPP) is derived which is an anticoagulant that specifically inhibits collagen-stimulated platelet aggregation. Recent phylogenetic work has suggested that this non-blood-feeding leech has a hematophagus ancestry (Kvist et al., 2011); tRNA-dihydrouridine synthase 4-like from sea-louse are known to have aspartic- cysteine- and metallo-peptidases that play a key role in blood and hemoglobin digestion (Williamson et al., 2003); Hypothetical protein Y032_0742g1987 from hookworm, which secretes potent anticoagulants that have been shown to target coagulation factors Xa and the factor VIIa/Tissue Factor complex (Cappello et al., 1995); and Hypothetical protein RP20_CCG013479 from *Aedes albopictus* which has FXa-directed anticoagulant activity (Calvo et al., 2011). These results suggest that Rhizoprotease has some sequences similar to several anticoagulant proteins of invertebrate origins.

Major challenges for purification of active components from BJFTE like complexity of the sample, seasonal availability of the sample and the absence of databases for the determination of peptide and protein sequences in jellyfish still remain.

Rhizoprotease is probably the first anticoagulant protein reported from any jellyfish, albeit in a semi-pure form.

CHAPTER 5

EFFECT OF BARREL JELLYFISH TENTACLE EXTRACT ON HUMAN LUNG ADENOCARCINOMA CELLS CULTURED *IN VITRO*

5.1. Introduction

Cytotoxicity describes the detrimental effects of substances or environmental changes on cell health. Exposure of cells to a cytotoxic stimulus may compromise metabolic activity, inhibit cell growth/division or ultimately produce cell death. Upon exposure to cytotoxic compound, cells respond to stress in a variety of ways, ranging from activation of pathways that promote survival to eliciting programmed cell death, which eliminates damaged cells. The cell's initial response to a stressful stimulus is geared towards helping it to defend against and recover from the insult. However, if the deleterious stimulus is unresolved, then cells activate death signalling pathways (Pathan et al., 2015). The mechanism by which a cell dies, depends on various exogenous factors as well as the cell's ability to handle the stress, it is exposed. On cell death induction, it can undergo different cell fates and morphological alterations including apoptosis, autophagy and necrosis (Fiers et al., 1999).

Apoptosis: Cells undergoing apoptosis reveal characteristic changes in nuclear morphology (including chromatin condensation and fragmentation), overall cell shrinkage, blebbing of the plasma membrane and formation of apoptotic bodies containing nuclear or cytoplasmic material (Kerr, 2002; Elmore et al., 2007).

Autophagy: Cells undergoing autophagy are characterized by massive accumulation of double-membrane containing vacuoles, called autophagosomes, which originate from phagophores or isolation membranes and encapsulate cytoplasmic material. The autophagosomes subsequently fuse with lysosomes to form autolysosomes, which cause the degradation of the autophagosomal contents (Gozuacik and Kimchi, 2004).

Necrosis: Cells undergoing necrosis are morphologically defined by cytoplasmic swelling, dilation of organelles, which causes cellular vacuolation

and rupture of the plasma membrane, resulting in the pro-inflammatory leakage of the intracellular content (Trump, et al., 1997).

These responses can be measured with various assays. Most cytotoxicity assays work on the premise that dying cells have highly compromised cellular membranes, which allow the release of cytoplasmic components or the penetration of dyes within the cell structure. Cytotoxicity assays are widely used in fundamental research and in drug discovery to screen toxic compounds. Some of the commonly used cytotoxicity assays are MTT assay, Resazurin assay and LDH assay. MTT is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide by mitochondrial succinate dehydrogenase to its insoluble formazan, which has a purple color. Cellular reduction is only catalyzed by living cells, hence it is possible to quantify the percentage of living cells in a solution (Cory et al., 1991). Resazurin is a cell permeable redox indicator, which can be used to monitor viable cell number. It is a deep blue colored solution; when added directly to cells in culture, viable cells with active metabolism can reduce resazurin into the resorufin product, which is pink and fluorescent (Ahmed at al., 1994). Lactate dehydrogenase (LDH) is a soluble cytosolic enzyme present in most eukaryotic cells. LDH is released into culture medium on cell death, due to damage of plasma membrane. The increase of the LDH activity in culture supernatant is directly proportional to the number of lysed cells (Decker et al., 1988).

Effect of jellyfish venoms on *in vitro* cultured cells has been studied since early 1980s. There are a few reports on the effects of different jellyfish venoms/extracts on different cell lines. High doses of crude or fractionated venom from sea nettle *Chrysaora quinquecirrha* (Semaeostomeae) were seen to produce morphological changes in CHO K-1 cells, to inhibit cell growth and interfere with intracellular uridine and thymidine incorporation, while low doses induced mitogenic activity (Neeman et. al., 1980). Venom from *Chrysaora quinquecirrha* also induced nuclear alterations on K-1 cells (Chinese hamster ovary) with production of multi-nucleated cells and multiple nucleoli as well as loss of peripheral chromatin and dissolution of intercellular collagen as a consequence of its enzymatic activity (Cao et. al., 1998). Purified extracts

from cnidocysts of *Cyanea capillata* and *Cyanea lamarckii* jellyfish induced strong damage to hepatoma cells HepG2. These extracts also demonstrated PLA2-like activity (Helmholz et. al., 2007). The venom from the nematocysts of *Cyanea nozakii* Kishinouye was assessed for cytotoxicity on Bel-7402, SMMC-7721 human hepatoma cells and on H630 human colon cancer cells. The venom was able to damage cell membrane, which was demonstrated by lactate dehydrogenase (LDH) release that increased with time and venom concentration (Li et. al., 2012). Crude venom fractions extracted from *Pelagia noctiluca* showed time-dependent anti-proliferative activity. These also caused a dose-dependent inhibition of cell adhesion to fibrinogen. This activity was due to the interaction of venom with integrins, suggestive of the fact that *Pelagia noctiluca* venom may play a role in the development of anti-cancer drugs (Ayed et. al., 2012). Cytotoxicity of four Scyphozoan jellyfish (*Nemopilema nomurai*, *Rhopilema esculenta*, *Cyanea nozakii*, and *Aurelia aurita*) was assessed on NIH 3T3 cells, following cytotoxic potency scale, in ascending order *C. nozakii*, *N. nomurai*, *A. aurita*, *R. esculenta* was reported. The metalloproteinases were indicated to play an important role in jellyfish toxicity. Cell-damaging activity of scyphozoans has been related with the proteolytic activity of their venoms (Lee et. al., 2011). Crude toxin from *Rhizostoma pulmo was reported to* produce cytotoxic effects and growth inhibition on V79 cells, both after short and long time treatments (Mazzei et. al., 1995).

A549 cells are human alveolar basal epithelial cells. These cells are squamous in nature and responsible for the diffusion of substances, such as electrolytes and water, across the alveoli of lungs. They grow adherently *in vitro* as a monolayer. It was shown that cytotoxins from some snake species could penetrate into living adenocarcinoma cells (A549) and markedly accumulate in lysosomes. This internalization of cytotoxins, most likely explained the higher sensitivity of A549 cells to cytotoxin-induced toxicity as compared to normal human cells (Gasanov et. al., 2014).

Based on the cytotoxic effects of different jellyfish extracts and toxins on varied cell lines, effect of BJFTE was also studied on human lung

adenocarcinoma cells cultured *in vitro*.

5.2. Materials and Methods

5.2.1. Reagents

Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), phosphate buffer saline (PBS), trypsin, antibiotic-antimycotic solution and MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) were purchased from HiMedia Laboratories, India. DMSO (dimethyl sulfoxide) was obtained from Merck Life Science Private Limited, India. A549 cell lines were obtained from National Center for Cell Science (NCCS), Pune, India. For cell culture, all the plastic wares were procured from Corning, USA. All other chemicals used were of analytical grade.

5.2.2. In vitro culture of human A549 cells

A549 cells were grown exponentially in 25cm^{2}culture flasks, containing Dulbecco's modified eagle medium (DMEM) supplemented with heat inactivated 10% FBS, 1% antibiotic-antimycotic solution and were maintained at 37°C in a humidified atmosphere of 5% CO_2 and passaged every third day.

5.2.3. Effect of BJFTE on A549 Cells

Following 24 h and 48 h incubation of the cells with BJFTE, an inverted microscope was used to observe any morphological alterations and cell damage qualitatively. The photos were taken with a digital camera (Nikon-Eclipse, TS 100F, USA), connected to the inverted microscope.

5.2.4. Determination of Cell Viability (MTT assay)

A549 cells were cultured in DMEM medium, in the presence of FBS 10% plus antibiotic-antimycotic 1%, and incubated in presence of 5% CO_2 at 37°C. The cytotoxicity of BJFTE was evaluated using MTT assay. A549 cells were seeded in a 96 well plate at 2-4 × 10^4 cells/ well and incubated for 24 h to adhere. After discarding the old medium, the cells were exposed in the medium containing various concentrations (2.5, 5, 10, 20, 50, 100 µg) of BJFTE. 20 µL MTT (5 mg/ ml) was added to each well after 24 h, 48 h and 72 h exposure and cells were incubated for another 4 h. Finally, the culture

medium containing MTT solution was removed and the formazan crystals were dissolved in 200 µL of dimethyl sulfoxide solvent (DMSO). Absorbance was measured at 570 nm with an automated microplate reader (Multiskan Go, Thermo Scientific). The percentage of inhibition was determined by comparing the absorbance values of treated cells with that of untreated controls.

$$Cell\ Viability\ Percentage\ (\%) = \frac{Absorbance\ of treated\ cells}{Absorbance\ of\ untreated\ cells} \times 100$$

5.2.5. Monolayer Cell Migration Assay

Cell migration was studied *in vitro* by wound healing or cell scratch assay. This method mimics cell migration during wound healing *in vivo*. It is commonly used to study the effects of cell-matrix and cell-cell interactions on cell migration (Lampugnani, 1999).

A549 cells were seeded in 6-well plates at a density of 3×10^6 cells per well. Once the cells reached 90% confluence, a wound was created by scraping the cell monolayer with a sterile 200 µl pipette tip, from one end of the well to the other. The detached cells were removed by washing with PBS. Subsequently, the cells were incubated at different concentrations of BJFTE (50 and 100 µg). Migration of cells into the wounded region was observed using digital camera (Nikon- Eclipse, TS 100F, USA), connected to the inverted microscope and images were captured at 0 h, 24 h and 48 h at 10X magnification. The wound area was measured using the image-processing program ImageJ (NIH, Bethesda, Maryland, USA). The cell wound closure rate was calculated using the following equation:

$$Wound\ Closure = \left[1 - \frac{Wound\ area\ at\ T_t}{Wound\ area\ at\ T_0}\right] \times 100$$

Where T_t is the time passed since wounding and T_0 is the time the wound was created. The experiments were performed in triplicate.

5.2.6. Laminin Digestion by BJFTE

Laminin was incubated with BJFTE, $40P_s$ and Frac 5.6 for 5 h at 37°C. After incubation, samples were prepared in reducing sample buffer (10% SDS, 250

mMTris–HCl, pH 6.8, 30% glycerol, 0.02% bromophenol blue, 5% β-mercaptoethanol). The samples were then subjected to SDS-PAGE on 7.5% separating gel with a 4% stacking gel. The protein bands were viewed by staining with 1% Coomassie brilliant blue, R250. Laminin digestion was monitored by comparing position and appearance of specific bands with that of laminin incubated with 0.85% saline only.

5.3. Results

5.3.1. Effect of BJFTE on A549 cells

There was no significant change in the morphology of the cells on treatment with BJFTE, even at a dose as high as 100 µg (Figure 52).

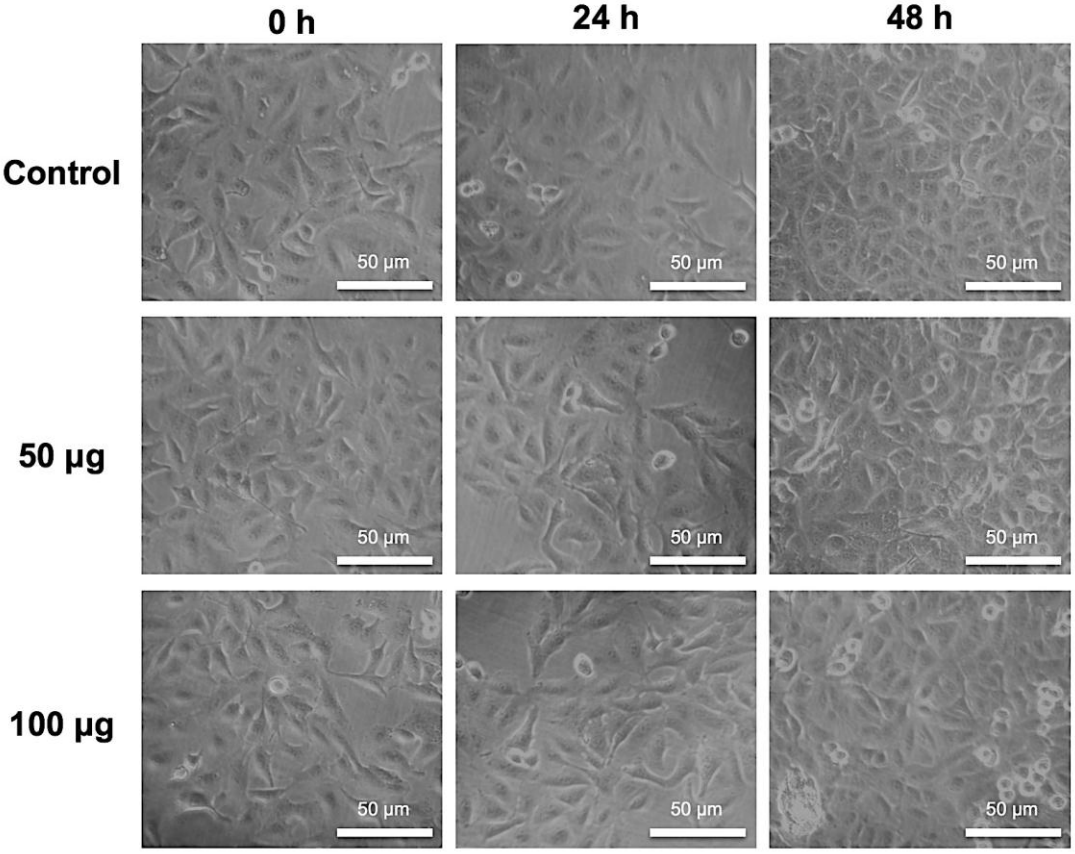


Figure 52. Effect of BJFTE on A549 cells. 1) Control cells 2) treated with 50 µg of BJFTE and 3) treated with 100 µg of BJFTE for duration of 24 h and 48 h.

5.3.2. MTT assay

The results of MTT assay showed that IC_{50} for 24 h and 48 h were 82.5 µg and 89.64 µg respectively (Figure 53). Therefore, it is reasonable to interpret that BJFTE is not that toxic to the cells, for the concentrations we have used. Though there is a sudden increase after 48 h of exposure, which may be because the cells are trying to overcome the stress, induced by the extract.

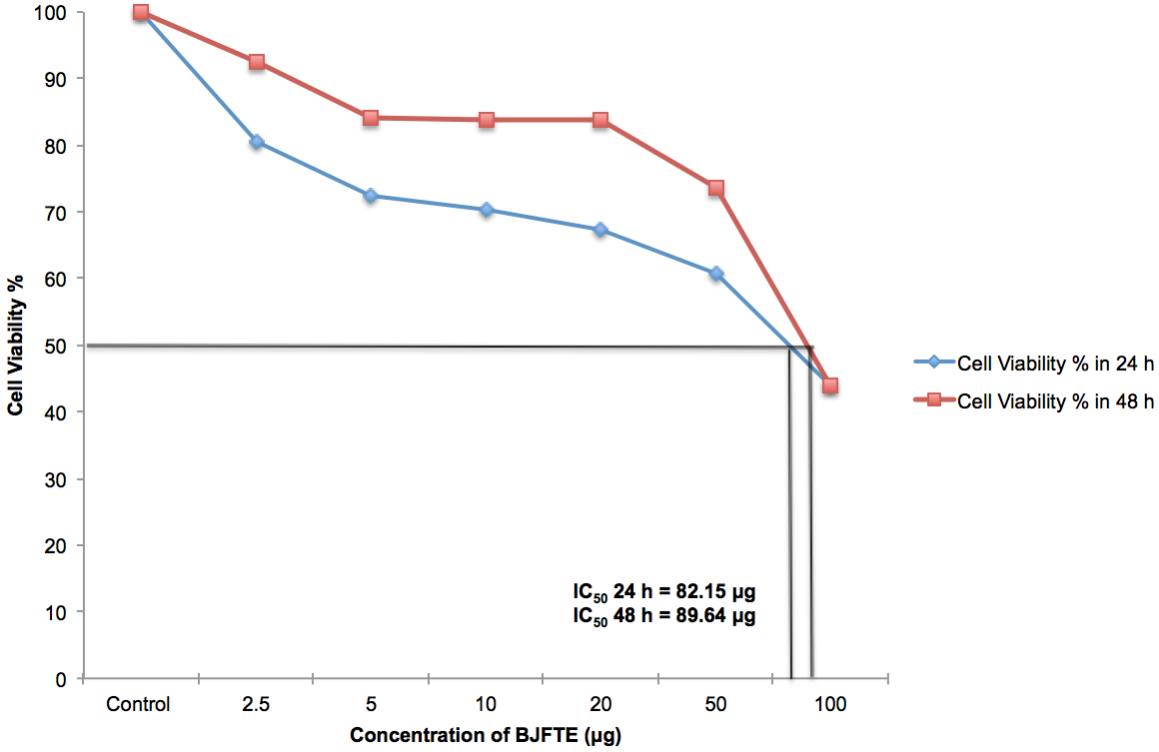


Figure 53. Cytotoxic effect of BJFTE in A549 cells. Cells were treated with BJFTE in concentration-dependent manner for 24 h and 48 h. The ratios of cell viability were measured by MTT assay.

5.3.3. Wound Healing Assay

Wound healing assay was conducted to investigate the effects of BJFTE on the migration ability of A549 cell lines. The untreated group showed complete migration ability (Figure 54). However, treatments with 50 and 100 µg of BJFTE for 24 h and 48 h retarded the migrating ability of A549 cells. In presence of BJFTE, the wound closure was reduced to 35% and 14% respectively after 24 h; while 56% and 23% reduction was observed after 48 h for the same (Figure 54). The reduction of cell migration in BJFTE treated group was found to be statistically significant.

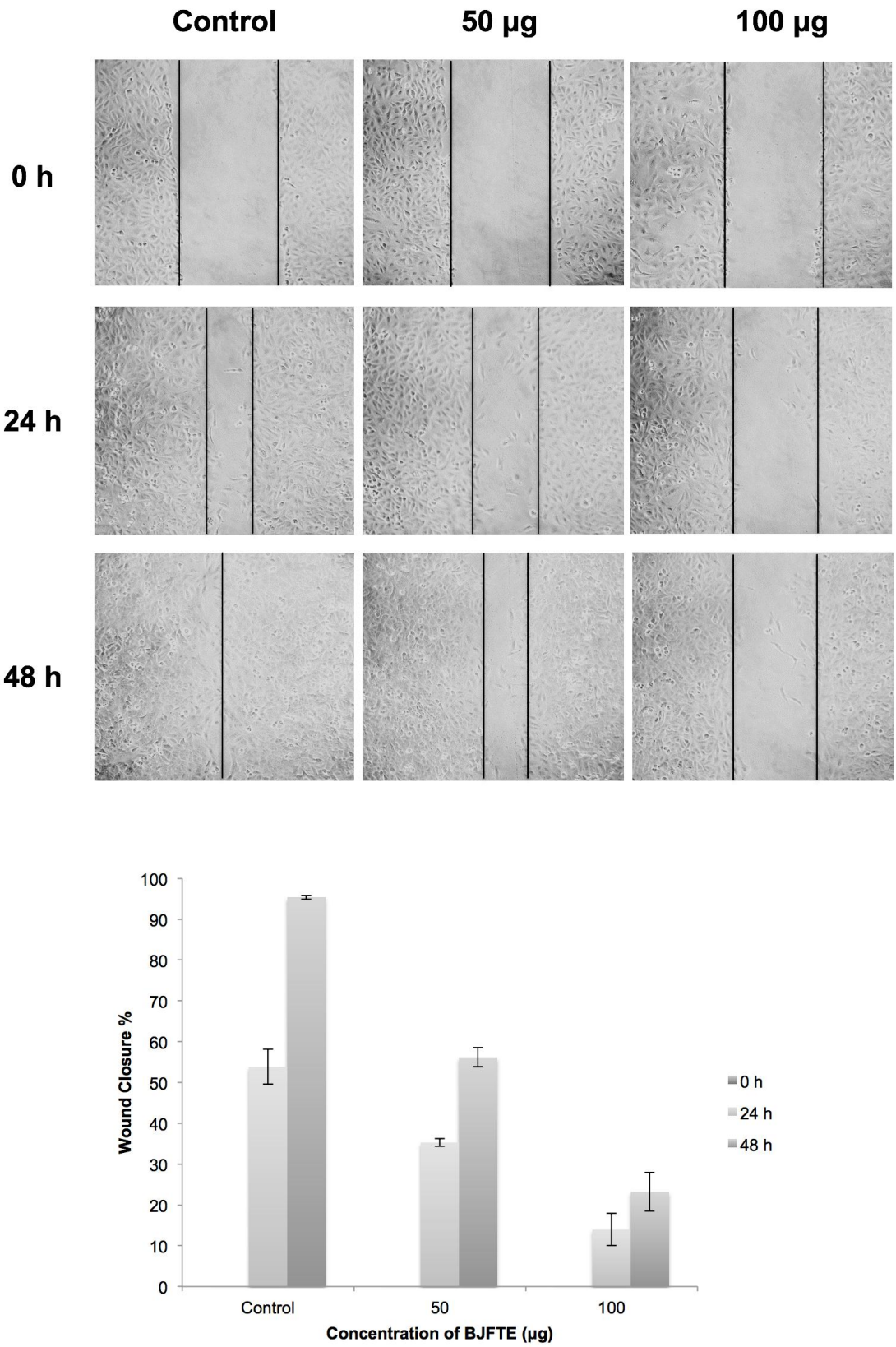


Figure 54. Effect of BJFTE on migratory capacity of A549 cells. Wound healing assay was initiated by making a uniform scratch in a six well plate containing A549 cells grown to confluence. Scratch was monitored under a microscope. The width of the scratch was measured and the percentage of closure was estimated. Results were expressed as mean ± SE (n = 3) that showed significant inhibition of wound closure by BJFTE.

5.3.4. Laminin Digestion by BJFTE

Both BJFTE and 40P could digest laminin, which was evident from extensive degradation of both the A and B chains (Figure 55).

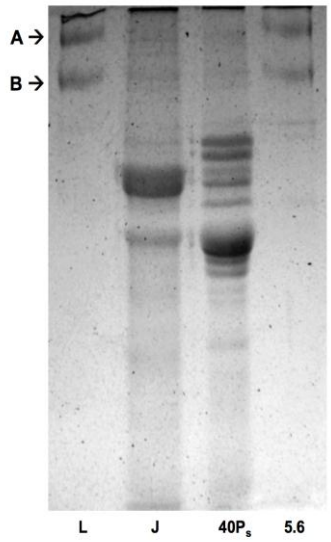


Figure 55. Coomassie blue stained 7.5 % SDS PAGE showing electrophoretic patterns of laminin incubated with BJFTE, 40P$_s$ and Frac 5.6. Samples in lanes L, J, 40P$_s$ and 5.6 were incubated at 37°C for 5 h. Lane L contains 20µg of only laminin and A and B chains are indicated. Lanes J, 40P$_s$ and Frac 5.6 contain laminin incubated with 4 µg of BJFTE, 40P$_s$ and Frac 5.6, respectively.

5.4. Discussion

In recent years, cytotoxicity assays have been used to assess the toxicity of jellyfish venoms and extracts on different cells. Crude venoms from different sea anemones like *Heteractis magnifica*, *Stichodactyla haddoni* and *Parachondylactis sinensis* induced a decrease in the viability on mouse fibroblast cell line L929 and leukemia cell line P388 in a concentration-dependent manner (Ravindran et al., 2010). Crude venom of *B. globulifera* induced cytotoxicity in A549 human lung adenocarcinoma cell line (Monroy-Estrada et al., 2013), exposure at a dose of 50 µg/ml induced a reduction of approximately 50% in cell viability. Crude venom from the giant jellyfish *Nemopilema nomurai* (Rhizostomeae) was found to be hemolytic and cytotoxic *in vitro* and showed high cytotoxicity against H9C2 heart myoblasts with a $LC_{50}$of 2 µg/ml. The fishing tentacle plus lappets nematocyst venom of *Chrysaora achlyos* showed minimal damage to human liver cells (STCC CCL-13) after 30 s exposure to 10 mg protein/ml (Radwan at al., 2000). Carli et al.

(1996) reported that *Aequorea aequorea* venom greatly affected the growth rate of V79 cells during long-term experiments. *Rhizostoma pulmo* venom showed remarkable cytotoxicity on V79 cells and killed all treated cells at the concentration of 15,000 nematocysts/ml within 2 and 3 h. The crude venom extracted from *Pelagia noctiluca* showed time-dependent, anti-proliferative activity and also caused a dose-dependent inhibition of cell adhesion to fibrinogen. The authors attributed this activity to the interaction of venom with integrins (Ayed et al., 2012) and concluded that *Pelagia noctiluca* venom may play a role in the development of anti-cancer drugs.

Barrel jellyfish tentacle extract was found to have an IC_{50} of 83 and 89 µg for 24 and 48 h, respectively for A549 cells. It did not show any significant morphological changes even at doses as high as 100µg. Thus, BJFTE showed mild cytotoxicity towards A549 cells. However, it could significantly inhibit the wound healing in A549 cells in a dose dependent manner.

According to Lee et al. (2011) proteolytic activities in scyphozoan jellyfish played a very important role in the toxicity of the jellyfish venom. The relative cytotoxic potency of jellyfish venom appeared to be closely associated with their proteolytic activity. Their results also showed a significant positive correlation between metalloproteinase activity and cytotoxicity of the venom extracts. BJFTE proteolytically digested laminin. It has been reported that certain snake venom derived metalloproteinases act on ECM component proteins in a manner that the integrin binding sequences are detached from rest of the ECM protein. However, cell integrins remain bound to those detached ligand fragments. Therefore, cells lose their contact with extracellular membrane (Radis-Baptista et al., 2005)

Based on the data of the present work, more studies are needed to investigate the cytotoxicity mechanism of BJFTE.

CHAPTER 6

CONCLUSION

Cnidarians in spite of their toxicity have long been indicated as a potential source of natural bioactive compounds of pharmacological concern useful to develop new drugs or biomedical materials. A number of metabolites, anti-cancer, anti-microbial and anti-oxidant compounds have been isolated in the interest of human health and have been found to be active at the cellular level. This makes them possible sources of new drugs. The Scyphozoans, or 'true' jellyfish, have been relatively less explored for toxin studies, compared to the more dangerous box jellyfish.

Thrombosis and related cardiovascular disorders are major life threatening diseases of mankind. Currently used drugs to counter these disease conditions have various limitations including uncontrolled activity leading to haemorrhage or lower efficacy and sometimes, allergic reactions. These limitations drive the continual and intense efforts to develop new anticoagulants targeting specific coagulation factors. Several snake venom-derived toxins and hematophagous animal toxins have shown strong anticoagulant properties. Many of these toxins can dissolve blood clots at very low dose. Search for the ideal anticoagulant with minimal side effects have become a challenging field for the researchers. The aim of this study was to explore jellyfish tentacle extracts for anticoagulant activity followed by purification and biochemical characterization of active components.

Jellyfish tentacle extract from moon jellyfish (*Aurelia aurita*) and barrel jellyfish (*Rhizostoma pulmo*) were studied for their anticoagulant activity *in vitro*. Both the extracts were able to affect the haemostatic system at three different levels: platelet aggregation, fibrinogen digestion and fibrin clot digestion. These extracts could immediately digest Aα chain of fibrinogen and then could gradually digest Bβ and γ chain in time and dose dependent manner. They could also completely liquefy fibrin clots within 24 and 48 h, respectively. In case of BJFTE, it could not digest the clots made using essentially plasminogen free fibrinogen suggestive of the fact that there is possible correlation between plasminogen and BJFTE with respect to fibrin clot

digestion. Fibrinogenolytic and fibrinolytic activities of JFTE and BJFTE were significantly reduced in presence of EDTA and on pre-exposure to heat, but not on pre-treatment with PMSF. This observation indicates the involvement of thermolabile metalloproteinases in the above-mentioned activities. Phospholipase activity was below detectable limits in both the extracts.

JFTE reduced the plasma recalcification and thrombin time, whereas BJFTE prolonged them. JFTE showed mild hemolytic activity but BJFTE showed very strong hemolytic activity. The human RBCs on exposure to BJFTE initially exhibited swelling of the cells followed by gradual crenation. JFTE inhibited both ADP and collagen dependent platelet aggregation and BJFTE inhibited ADP dependent platelet aggregation. Due to the unstable nature of the component proteins and limited sample availability, further characterization of the active components of JFTE was not possible.

BJFTE demonstrated very strong proteolytic activity for substrates like fibrinogen, casein, gelatin and azocasein. These proteolytic activities were almost completely abolished in presence of EDTA. BJFTE even at a dose as high as 100µg showed no evident morphological changes in A549 cells. However BJFTE significantly delayed the wound healing process. BJFTE was also found to digest laminin, a major basement membrane component involved in cell adhesion. Attempts were made to purify the active components present in the barrel jellyfish tentacle extract using different techniques. A 95 kDa metalloproteinase was identified after ammonium sulphate precipitation and SE-HPLC of BJFTE. It was named Rhizoprotease. Atomic absorption spectroscopy studies suggested the presence of Zn^{+2}. PMF analysis revealed that no previously described protein was found to completely match Rhizoprotease, which indicated that it is a novel protein. Some of the closest hits revealed similarity with proteins from organisms like giant ant, parasitic beef tape worm, freshwater jawless leech, sea louse, hookworm and mosquito. Reports show the presence of some anticoagulant and antiplatelet activities in some protein components of these invertebrates. Rhizoprotease is the first anticoagulant protein reported from any jellyfish, albeit in a semi-pure form.

While the work presented here provides the first comprehensive insight into anticoagulant activity of cnidarian tentacle extract, it also increases bio-discovery potential and provides direction for future research.

FUTURE SCOPE

Despite being one of the most ancient venomous animals, cnidarians are largely uninvestigated. Cnidarians harbor an interesting pool of unstudied toxins that may represent an attractive resource in the search for leading therapeutics. The aim of this thesis was to provide a comprehensive insight into the bio discovery potential of these distinctive organisms.

In the present study, tentacle extracts of moon jellyfish (*Aurelia aurita*) and barrel jellyfish (*Rhizostoma pulmo*) have been investigated for anticoagulant activities. Characterization of these two tentacle extracts was performed using several biochemical assays and their mechanisms of action were studied *in vitro* systems.

Both the extracts could affect the hemostatic system at three different levels: platelet aggregation, fibrinogen digestion and fibrin clot digestion. The active components showed strong proteolytic activity and were majorly metalloproteinases. Given the thermolability, adhesion to preparative surfaces and aggregation/disaggregation of components of the tentacle extracts, purification of active components was extremely difficult. However, a novel 95 kDa metalloproteinase with strong fibrinogenolytic activity (Rhizoprotease) was identified in a semi pure fraction obtained after ammonium sulphate precipitation and SE-HPLC. Further purification of Rhizoprotease and other active components will help in developing natural/synthetic molecules of therapeutic importance in future.

Also the PMF studies conducted for Rhizoprotease suggested that it had similarity with some peptides and proteins found in other organisms. Comprehensive characterization of these jellyfish tentacle extracts is likely to provide insight into the diversification of toxins and might be a valuable resource in drug design.

Detailed studies on the structure and pharmacology of active components in the jellyfish tentacle extracts will not only help us in discovering newer classes of drugs but also will contribute immensely to decipher various molecular mechanisms involved in the physiological processes. In addition, these

studies might help in the development of various new therapeutic agents for the treatment of cardiovascular and hematological disorders.

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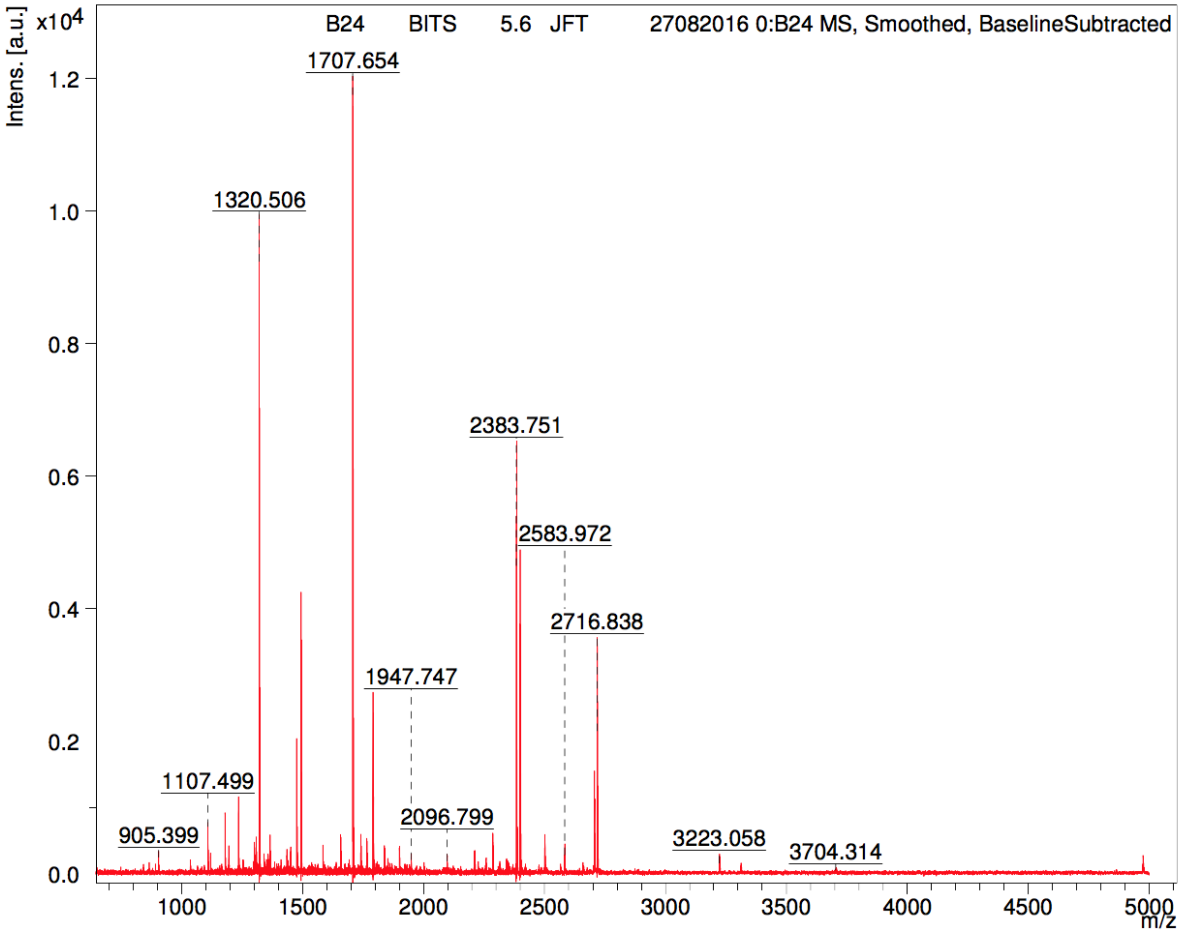
APPENDICES

MALDI Spectra and MASCOT Search Result



m/z	S/N	Quality Fac.	Res.	Intens.	Area
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866.601	3.0	128	2056	113.34	84
891.380	2.5	57	2863	96.50	54
905.399	6.0	351	2745	231.20	138
1037.478	3.1	214	2988	123.68	84
1065.484	2.1	65	2508	86.36	73
1107.499	16.5	4941	4545	690.05	348
1109.446	3.5	214	2588	147.46	129
1118.463	6.8	483	4438	285.38	149
1157.541	2.6	57	8091	113.81	36
1179.548	19.5	12784	5293	850.53	411
1194.545	7.4	900	4051	325.92	206
1234.597	24.2	3005	4733	1078.99	618
1252.510	3.3	62	5149	147.68	79
1297.496	2.6	103	4155	122.13	87
1300.530	8.3	1891	5301	394.62	224
1305.631	4.5	131	3038	214.98	215
1307.600	7.8	415	5075	374.35	222
1309.580	2.9	205	4607	136.83	89
1320.506	192.9	27946	5455	9215.79	5228
1340.603	4.2	135	4393	200.87	144
1351.571	3.0	289	5565	144.07	82
1357.632	3.6	301	2943	172.37	187
1365.570	9.5	2013	6151	458.54	244
1383.748	2.1	198	1615	100.57	210
1410.608	2.4	122	4121	115.23	95
1434.669	5.2	492	4609	252.94	195
1438.596	2.2	62	4491	109.85	87
1450.554	6.1	954	4411	299.27	245
1475.656	38.3	15955	6156	1894.90	1161
1493.649	82.8	57710	6407	4086.06	2499
1584.627	7.5	2674	6209	368.55	258
1638.716	2.7	128	7863	132.45	77
1657.677	10.3	3376	5887	505.36	402
1674.690	3.1	99	7834	152.53	97
1707.654	240.2	84667	6625	11732.83	9015
1740.618	9.6	1404	6895	467.26	356
1765.628	8.0	1321	5002	386.93	411
1791.610	50.5	39616	7061	2440.63	1940

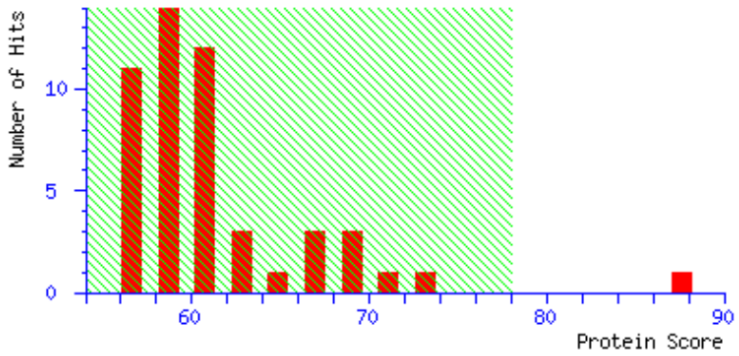
m/z	S/N	Quality Fac.	Res.	Intens.	Area
1808.664	2.3	77	3757	112.02	165
1838.768	6.8	1750	6423	327.31	295
1851.755	3.1	299	4160	148.25	207
1899.755	6.3	953	5488	292.90	325
1947.747	4.1	686	4866	187.37	241
2000.807	2.6	284	5006	110.74	142
2096.799	4.3	534	5087	171.35	230
2210.873	6.5	1414	5659	230.87	299
2257.791	3.7	294	6073	124.63	153
2285.857	13.1	9581	5664	437.38	607
2314.800	2.9	138	6199	94.81	118
2342.779	4.6	522	6304	147.36	186
2349.811	3.2	183	5006	102.13	161
2383.751	148.0	40047	7649	4629.75	5019
2398.795	111.7	58898	7919	3465.69	3662
2420.786	2.4	111	5954	73.91	103
2500.956	12.2	1381	4715	349.05	641
2566.039	2.4	187	3703	66.36	159
2583.972	9.7	3598	7284	264.99	334
2659.025	3.6	369	6136	94.98	151
2704.917	36.4	26850	7838	921.00	1170
2716.838	84.5	26806	7961	2134.38	2668
3223.058	7.3	1115	7868	138.59	238
3312.102	4.2	448	6661	74.66	162
3704.314	2.2	113	6211	32.94	87
4973.292	6.7	1215	9842	62.49	175

MATRIX SCIENCE Mascot Search Results

User : joy
Email : joyprashant@sandor.co.in
Search title :
MS data file : DATA.TXT
Database : NCBInr 20160821 (93482448 sequences; 34454162062 residues)
Taxonomy : Other Metazoa (3835002 sequences)
Timestamp : 27 Aug 2016 at 08:52:24 GMT
Warning : A Peptide summary report will usually give a much clearer picture of MS/MS search results.
Top Score : 88 for gi|951575371, PREDICTED: cytosolic carboxypeptidase 2 [Dinoponera quadriceps]

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 78 are significant (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Index

	Accession	Mass	Score	Description
1.	gi|951575371	128487	88	PREDICTED: cytosolic carboxypeptidase 2 [Dinoponera quadriceps]
2.	gi|1046537654	334107	72	nucleoprotein TPR [Taenia saginata]
3.	gi|675869340	101738	72	hypothetical protein HELRODRAFT_100443 [Helobdella robusta]
4.	gi|225712694	33674	70	tRNA-dihydrouridine synthase 4-like [Lepeophtheirus salmonis]
5.	gi|597889745	15167	69	hypothetical protein Y032_0742g1987 [Ancylostoma ceylanicum]
6.	gi|1000212992	13360	68	hypothetical protein RP20_CCG013479 [Aedes albopictus]
7.	gi|1046521088	334404	67	nucleoprotein TPR [Taenia asiatica]
8.	gi|1005456042	34917	66	PREDICTED: neurocan core protein-like isoform X2 [Acropora digitifera]
9.	gi|1005456040	34988	66	PREDICTED: neurocan core protein-like isoform X1 [Acropora digitifera]
10.	gi|358256655	26922	65	hypothetical protein CLF_113313 [Clonorchis sinensis]
11.	gi|769845287	209819	64	PREDICTED: sporulation-specific protein 15-like, partial [Pogonomyrmex barbatus]
12.	gi|918315422	12953	63	hypothetical protein OCBIM_22021113mg, partial [Octopus bimaculoides]
13.	gi|957853061	30793	63	PREDICTED: S-adenosylmethionine mitochondrial carrier protein-like [Priapulus caudatus]
14.	gi|585711654	73907	61	PREDICTED: dual specificity tyrosine-phosphorylation-regulated kinase 4-like [Saccoglossus kow
15.	gi|954295388	108842	61	hypothetical protein T06_3684, partial [Trichinella sp. T6]
16.	gi|985391267	38065	61	PREDICTED: cytoplasmic tRNA 2-thiolation protein 1 [Diuraphis noxia]
17.	gi|597889747	18989	60	hypothetical protein Y032_0742g1987 [Ancylostoma ceylanicum]
18.	gi|732831659	45957	60	fibronectin type III domain protein [Oesophagostomum dentatum]
19.	gi|1048050070	12155	60	PREDICTED: uncharacterized protein LOC108369503 [Rhagoletis zephyria]
20.	gi|759057091	18823	60	PREDICTED: muscle-specific protein 20 [Cerapachys biroi]
21.	gi|607360558	19001	60	Muscle-specific protein [Cerapachys biroi]
22.	gi|1026562806	68418	60	hypothetical protein WN48_08122 [Eufriesea mexicana]
23.	gi|954295391	109186	60	hypothetical protein T06_3684, partial [Trichinella sp. T6]
24.	gi|1035613013	94884	60	PREDICTED: uncharacterized protein LOC107999074 [Apis cerana]
25.	gi|985384563	57349	60	PREDICTED: fatty acyl-CoA reductase 1-like [Diuraphis noxia]

Results List

1. gi|951575371 **Mass:** 128487 **Score:** 88 **Expect:** 0.0067 **Matches:** 12

PREDICTED: cytosolic carboxypeptidase 2 [Dinoponera quadriceps]

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Ions	Peptide
1107.4985	1106.4912	1106.5390	-43.19	1025	1036	0	---	R.GAGAVTSTCVGK.V
1179.5484	1178.5412	1178.4436	82.8	303	312	0	---	R.NNDSSNDEER.E
1194.5451	1193.5378	1193.5822	-37.22	1	11	1	---	-.MTEAASSSRVR.E
1320.5058	1319.4985	1319.6582	-121.03	614	624	1	---	K.VTSNPFSRQER.L
1365.5699	1364.5626	1364.7962	-171.13	797	807	0	---	K.CLILPEIVRPR.S
1493.6493	1492.6420	1492.8912	-166.91	796	807	1	21	K.KCLILPEIVRPR.S
1707.6536	1706.6463	1706.8111	-96.55	911	925	1	---	K.KPLAYDEAGEEDSRK.S
1740.6155	1739.6082	1739.7573	-85.71	583	597	0	---	R.CGTHFSAQDYEQIGK.A
2210.8726	2209.8653	2210.0603	-88.24	670	689	1	---	R.IEYTDAAWSSEGGELLASRR.R
2398.7946	2397.7873	2398.2290	-184.18	691	711	1	---	R.YLCVPPPSPTFVPPRSVGLCR.R
2583.9720	2582.9647	2583.2816	-122.67	266	287	1	---	K.EESLYNEGLKPLLYSTEDARTR.S
3223.0576	3222.0503	3221.5928	142	882	910	1	---	K.TPLSWGVSRHALMHYPTDGEDATLKPQSK.K

No match to: 905.3990, 1118.4630, 1234.5973, 1300.5300, 1307.5996, 1450.5537, 1475.6560, 1584.6269, 1657.6771, 1765.6278, 1791.6101, 1838.7684, 1899.7546, 2285.8566, 2383.7510, 2500.9563, 2704.9174, 2716.8381, 4973.2922

2. gi|1046537654 **Mass:** 334107 **Score:** 72 **Expect:** 0.22 **Matches:** 17

nucleoprotein TPR [Taenia saginata]

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Ions	Peptide
905.3990	904.3917	904.4138	-24.43	256	263	0	---	R.DQASDIEK.K
1107.4985	1106.4912	1106.4961	-4.39	2399	2407	1	---	R.SMPVRDACR.T + Oxidation (M)
1118.4630	1117.4558	1117.5801	-111.28	294	304	0	---	K.ASVVNAMVAEK.D
1179.5484	1178.5412	1178.5675	-22.35	271	280	0	---	K.AVTEMQEMLK.Q
1234.5973	1233.5901	1233.6070	-13.76	2649	2658	1	---	R.VVANCRICSR.Q
1300.5300	1299.5227	1299.7146	-147.66	305	316	1	---	K.DKLISENAALAR.E
1320.5058	1319.4985	1319.5888	-68.43	1562	1572	1	---	R.MRGQAEEQSER.L
1450.5537	1449.5464	1449.7034	-108.30	2877	2889	1	---	R.MSPSYPTATPRSR.C
1475.6560	1474.6487	1474.7562	-72.84	734	745	1	0	K.QEISILREMNAR.Y + Oxidation (M)
1493.6493	1492.6420	1492.7270	-56.95	1058	1071	0	7	K.LESSAHSEVAAHQK.T
1584.6269	1583.6196	1583.6919	-45.64	147	159	1	5	K.KMSECEQTSAELR.E + Oxidation (M)
1765.6278	1764.6205	1764.8067	-105.51	2938	2952	0	---	R.SFQPNPNTTDDVFQR.G

2210.8726	2209.8653	2210.0242	-71.89	2544 - 2563	1	---	R.IANSDTCITCGSRMVVNANK.C
2383.7510	2382.7437	2383.1186	-157.31	230 - 249	1	---	R.TDELANMEQLHANELEAQRR.L + Oxidation (M)
2398.7946	2397.7873	2398.0859	-124.52	47 - 65	1	---	R.CEEEQSKNQQNFNNFLIEK.E
2716.8381	2715.8308	2716.3306	-183.99	2826 - 2849	1	---	R.LGKLMFADVDESQPEEVAISFTFK.R + Oxidation (M)
4973.2922	4972.2850	4972.3228	-7.61	889 - 940	0	---	R.AAIAASKPSSPTSNAGPSDTQAQASSLSASDQPTSADATSGDGAQPVAMQLR.N

No match to: 1194.5451, 1307.5996, 1365.5699, 1657.6771, 1707.6536, 1740.6155, 1791.6101, 1838.7684, 1899.7546, 2285.8566, 2500.9563, 2583.9720, 2704.9174, 3223.0576

3. gi|675869340 **Mass:** 101738 **Score:** 72 **Expect:** 0.26 **Matches:** 9

hypothetical protein HELRODRAFT_100443 [Helobdella robusta]

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Ions	Peptide
905.3990	904.3917	904.3999	-9.04	688 - 695		1	---	R.ASDDRADR.L
1300.5300	1299.5227	1299.7034	-139.03	617 - 627		0	---	R.IALQTELEDLR.A
1365.5699	1364.5626	1364.6320	-50.84	710 - 721		0	---	R.AQENYAQADSLR.K
1493.6493	1492.6420	1492.7270	-56.94	710 - 722		1	15	R.AQENYAQADSLRK.G
1584.6269	1583.6196	1583.7865	-105.33	383 - 395		1	---	R.LKQSESMTIEYQK.R
1791.6101	1790.6028	1790.8356	-129.98	364 - 381		0	13	R.DIAAELEAANMSGGDLAK.R + Oxidation (M)
1838.7684	1837.7611	1837.8442	-45.22	87 - 103		1	---	R.LEEAEGGSSAQSEFNRK.R
2500.9563	2499.9490	2500.2842	-134.06	358 - 381		1	---	K.LTIEIRDIAAELEAANMSGGDLAK.R
2583.9720	2582.9647	2583.2636	-115.72	397 - 420		1	---	R.AEDLNASLANNSGELQRLQAENAR.L

No match to: 1107.4985, 1118.4630, 1179.5484, 1194.5451, 1234.5973, 1307.5996, 1320.5058, 1450.5537, 1475.6560, 1657.6771, 1707.6536, 1740.6155, 1765.6278, 1899.7546, 2210.8726, 2285.8566, 2383.7510, 2398.7946, 2704.9174, 2716.8381, 3223.0576, 4973.2922

4. gi|225712694 **Mass:** 33674 **Score:** 70 **Expect:** 0.42 **Matches:** 4

tRNA-dihydrouridine synthase 4-like [Lepeophtheirus salmonis]

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Ions	Peptide
905.3990	904.3917	904.4800	-97.67	150 - 156		1	---	R.RTIDLCK.V
1450.5537	1449.5464	1449.6956	-102.88	6 - 18		1	---	R.EGELTKMGAPMVR.Y + 2 Oxidation (M)
1475.6560	1474.6487	1474.7562	-72.88	210 - 223		1	45	R.VREVTGVDGVMAAR.G + Oxidation (M)
2500.9563	2499.9490	2500.1870	-95.18	54 - 75		1	---	R.DNEFTSDPKEDHPLVVQFAANK.V

No match to: 1107.4985, 1118.4630, 1179.5484, 1194.5451, 1234.5973, 1300.5300, 1307.5996, 1320.5058, 1365.5699, 1493.6493, 1584.6269, 1657.6771, 1707.6536, 1740.6155, 1765.6278, 1791.6101, 1838.7684, 1899.7546, 2210.8726, 2285.8566, 2383.7510, 2398.7946, 2583.9720, 2704.9174, 2716.8381, 3223.0576, 4973.2922

5. gi|597889745 **Mass:** 15167 **Score:** 69 **Expect:** 0.47 **Matches:** 5

hypothetical protein Y032_0742g1987 [Ancylostoma ceylanicum]

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Ions	Peptide
1107.4985	1106.4912	1106.5754	-76.02	1 -	10	1	---	-.MSDSAKIIAR.H + Oxidation (M)
1300.5300	1299.5227	1299.6605	-106.03	56 -	66	0	8	R.IVQPMIDQSNR.A
1365.5699	1364.5626	1364.6109	-35.36	21 -	31	0	---	R.NHHPYPSEEQK.K
1493.6493	1492.6420	1492.7059	-42.79	21 -	32	1	20	R.NHHPYPSEEQKK.Q
1584.6269	1583.6196	1583.8202	-126.62	56 -	69	1	---	R.IVQPMIDQSNRAGR.A

No match to: 905.3990, 1118.4630, 1179.5484, 1194.5451, 1234.5973, 1307.5996, 1320.5058, 1450.5537, 1475.6560, 1657.6771, 1707.6536, 1740.6155, 1765.6278, 1791.6101, 1838.7684, 1899.7546, 2210.8726, 2285.8566, 2383.7510, 2398.7946, 2500.9563, 2583.9720, 2704.9174, 2716.8381, 3223.0576, 4973.2922

6. gi|1000212992 **Mass:** 13360 **Score:** 68 **Expect:** 0.55 **Matches:** 3

hypothetical protein RP20_CCG013479 [Aedes albopictus]

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Ions	Peptide
1234.5973	1233.5901	1233.6605	-57.06	3 -	13	0	---	K.AISEPSYLNLK.Q
1493.6493	1492.6420	1492.7959	-103.11	1 -	13	1	37	-.MKAISEPSYLNLK.Q
1584.6269	1583.6196	1583.8341	-135.42	102 -	115	1	5	K.RAITEVCLLPLDGQ.-

No match to: 905.3990, 1107.4985, 1118.4630, 1179.5484, 1194.5451, 1300.5300, 1307.5996, 1320.5058, 1365.5699, 1450.5537, 1475.6560, 1657.6771, 1707.6536, 1740.6155, 1765.6278, 1791.6101, 1838.7684, 1899.7546, 2210.8726, 2285.8566, 2383.7510, 2398.7946, 2500.9563, 2583.9720, 2704.9174, 2716.8381, 3223.0576, 4973.2922

Publications

Research Articles

- **Rastogi, A.**, Sarkar, A., Chakrabarty, D., 2017. Partial purification and identification of a metalloproteinase with anticoagulant activity from *Rhizostoma pulmo* (Barrel Jellyfish). Toxicon 132, 29-39.

- **Rastogi, A.**, Biswas, S., Sarkar, A., Chakrabarty, D., 2012. Anticoagulant activity of Moon jellyfish (*Aurelia aurita*) tentacle extract, Toxicon 60, 719-723.

Book Chapter

- Chakrabarty, D., and **Rastogi, A.** Hemotoxic Activity of Jellyfish Venom, In: Handbook of Clinical Toxinology in Asia Pacific and Africa, P Gopalakrishnakone ed., Springer, 2014. Chapter 26, pp. 539-552. *(doi 10.1007/978-94-007-6288-6_3-1)*

Conference Proceedings

- **Rastogi, A.**, Chakrabarty, D., 2016. Anticoagulant Activity of Barrel Jellyfish (*Rhizostoma pulmo)* Tentacle Extract, Toxicon 116; Special Issue – Toxins: New Targets and New Functions, 85.

- **Rastogi, A.**, Biswas, S., Sarkar, A., Chakrabarty, D., 2012. Anticoagulant activity of Moon jellyfish (*Aurelia aurita*) tentacle extract, Toxicon 60 (2); Special Issue – 17th World Congress of the International Society on Toxinology and Venom Week 2012. 4th International Scientific Symposium on All Things Venomous, 133-134.

Oral Presentations

- **Rastogi, A.,** Sarkar, A., and Chakrabarty, D., 2017. Studies on Anticoagulant Potential of Barrel Jellyfish (*Rhizostoma pulmo*) Tentacle Extract. Venoms 2017, Oxford, United Kingdom.

- **Rastogi, A.,** and Chakrabarty, D., 2014. Anticoagulant Protein from Barrel Jellyfish, *Rhizostoma pulmo* from the Goan Coast. 4th Annual conference of the Toxinological Society of India at Calcutta School of Tropical Medicine, Kolkata. (**Best oral presentation**)

- **Rastogi, A.,** and Chakrabarty, D., 2013. Thrombolytic and Antiplatelet Avtivities of Semi-Purified Moon Jellyfish Tentacle Extract. 3rd Annual conference of the Toxinological Society of India and 1st International Conference on Biology of Natural Toxins at BITS Pilani K K Birla Goa Campus, Goa.

Poster Presentations

- **Rastogi, A.,** and Chakrabarty, D., 2014. Anticoagulant Activity of Barrel Jellyfish (*Rhizostoma pulmo*) Tentacle Extract. 22nd Meeting of the French Society of Toxinology, Toxins: New Targets and New Functions" at Pasteur Institute, Paris, France. **(Travel grant received from Department of Science and Technology, Government of India)**

- **Rastogi, A.,** Biswas, S., Sarkar, A., Chakrabarty, D., 2012. Anticoagulant Activity of Moon Jellyfish Tentacle Extract. 17th World Congress of International Society on Toxinology and Venom Week 2012 at Honolulu, Hawaii. **(Travel grant received from Department of Biotechnology, Government of India)**

- **Rastogi, A.,** and Chakrabarty, D., 2012. Moon Jellyfish Tentacle Extract Contains Mixture of Anticoagulant Factors. National Seminar on Advances in Zoology and Life Processes at Goa University, Goa. (**Best poster**).

*(First page of each publication is attached hereafter)

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Partial purification and identification of a metalloproteinase with anticoagulant activity from *Rhizostoma pulmo* (Barrel Jellyfish)

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ABSTRACT

Rhizostoma pulmo (Barrel Jellyfish) is one of the commonly found jellyfishes on the South-Goan coast of India. Here we present characterization of *R. pulmo* tentacle extract. The tentacle extracts were found to be capable of affecting the hemostatic system at three different levels, as it exhibited fibrinogenolysis, fibrinolysis and inhibition of ADP induced platelet aggregation. It preferentially cleaved the Aα chain of fibrinogen, followed by the Bβ chain and the γ chain. The tentacle extract also showed significant hemolytic activity against human RBCs and strong proteolytic activity for substrates like (azo) casein and gelatin. However, this proteolytic activity was completely inhibited by EDTA (metalloproteinase inhibitor) but not by PMSF (serine proteinase inhibitor). The extract was devoid of phospholipase activity. A semi-purified protein possessing fibrinogenolytic activity was obtained by a combination of ammonium sulphate precipitation and size exclusion HPLC. Atomic absorption analysis of this protein indicated presence of Zn^{2+} and treatment with metalloproteinase inhibitor caused complete loss of activity. A 95 kDa metalloproteinase was identified in this fraction and was named Rhizoprotease. Protein Mass Fingerprinting of Rhizoprotease indicates it to be a novel protein.

1. Introduction

Jellyfish are generally known for their negative impact on human activities such as fishing, aquaculture and tourism. But recent advances in research on venom components from a range of jellyfish species have found them to possess useful bioactivities that may be exploited for therapeutic purpose. Studies also suggest that few species of jellyfish contain toxins related to peptides and proteins found in other venomous creatures (Weston et al., 2013). Most jellyfish species are free-moving planktons that drift with ocean currents and appear in irregular quantities all along the coast of India. There is a bloom between October and November along the Goan coast. Global warming and consequent increase in temperature has been linked with increase in sightings and reports of jellyfish reaching the shores of Goa. One of the most abundantly found jellyfish on the Goan coast is the *Rhizostoma pulmo* commonly known as the barrel jellyfish, the dustbin-lid jellyfish or the frilly-mouthed jellyfish. Irrespective of their large size, barrel jellyfish cause little harm to humans, as their sting is not considered powerful enough to cause serious injury. It is similar to a nettle sting and can cause a rash. Rhizolysin, a high molecular weight cytolysin of 260 kDa was isolated from *Rhizostoma pulmo* (Cariello et al., 1988). It showed hemolytic activity but no phospholipase activity. Later it was also reported to be present in nematocyst free tissue and showed both hemolytic as well as cytotoxic effects but no clastogenic effects (Allavena et al., 1998). A 30 kDa chromoprotein named *rpulFKz1*was isolated from the barrel jellyfish with a Frizzled cystein-rich domain and a Kringle domain (Bulina et al., 2004). Kringles are found throughout the blood clotting factors and fibrinolytic proteins. Kringle domains are believed to play a role in binding mediators and in the regulation of proteolytic activity (Patthy et al., 1984; Atkinson and Williams, 1990).

In spite of the presence of kringles, there is no report on anticoagulant or procoagulant toxins from jellyfish venom. However, strong fibrinogenolytic, fibrinolytic and platelet-inhibiting

Abbreviations: AAS, Atomic Absorption Spectrophotometry; ACD, Acid citrate dextrose; ADP, Adenosine di-phosphate; BJFTE, Barrel jellyfish tentacle extract; BSA, Bovine serum albumin; EDTA, Ethylenediaminetetraacetic acid; PLA, phospholipase A; PMF, Peptide Mass Fingerprinting; PMSF, Phenylmethylsulfonyl fluoride; PPP, Platelet poor plasma; PRP, Platelet rich plasma; RBC, Red blood cells; SDS-PAGE, Sodium dodecyl sulphate polyacrylamide gel electrophoresis; SE-HPLC, Size exclusion high performance liquid chromatography; SVMPs, Snake venom metalloproteinase.

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Anticoagulant activity of Moon jellyfish (*Aurelia aurita*) tentacle extract

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ABSTRACT

Moon jellyfish (*Aurelia aurita*) tentacle extract was studied for its anticoagulant activity *in vitro*. The Jellyfish Tentacle Extract (JFTE) showed very strong fibrinogenolytic activity by cleaving Aα and Bβ chain of fibrinogen molecule. The fibrinogenolytic activity was found to be stronger than some snake venom derived anticoagulants. JFTE also completely liquefied fibrin clots in 24 h. JFTE was found to contain both high and low molecular weight proteins/peptides. The fibrinogenolysis appears to be caused by high molecular weight fractions of the extract. It has been also noted that PMSF significantly reduced fibrinogenolytic activity and heating totally abolished it. Autolytic degradation of the high molecular weight protein was also noted. Autolysis slowed down, but did not abolish the fibrinogenolytic activity of the extract.

1. Introduction

Thrombosis has been and continues to be a major health problem leading to mortality. Anticoagulants are pivotal for the prevention and treatment of thromboembolic disorders. Coumarins and heparin are the most well known clinically used anticoagulants. However, the nonspecific mode of action of these anticoagulants accounts for their therapeutic limitations in maintaining a balance between thrombosis and hemostasis. These limitations have provided the impetus for the development of new anticoagulants that target specific coagulation enzymes or a particular step in the clotting process.

Potent anticoagulants have been discovered in snake venoms, earthworm secretions, dung beetles, food-grade microorganisms, marine creatures, herbal medicines, and fermented food products like Japanese *Natto* and Korean *Chungkook-Jang* soy sauce (Nikai et al., 1984; Sumi et al., 1987, 1992; Mihara et al., 1991; Kim et al., 1996; Chang et al., 2000; Choi and Sa, 2000; Jeong et al., 2001; Ahn et al., 2004).

Anticoagulant components from snake venoms have inspired the design and development of a number of therapeutic agents or lead molecules. For example, inhibitors of platelet aggregation, such as Eptifibatide and Tirofiban, were designed based on disintegrins, a large family of platelet aggregation inhibitors found in viperid and crotalid snake venoms (O'Shea and Tcheng, 2002; Marcinkiewicz, 2005; Huang and Hong, 2004; Plosker and Ibbotson, 2003; Kondo and Umemura, 2002; McClellan and Goa, 1998). Ancrod/Viprinex (extracted from the venom of the Malayan pit viper) reduces blood fibrinogen levels and has been successfully tested in a variety of ischemic conditions, including stroke (Sherman, 2002).

In recent years, cnidarians like jellyfish have become an attractive source of physiologically active compounds. Their extracts have been reported to exert hemolytic (Kang et al., 2009), insecticidal (Yu et al., 2005a), cardiovascular (Ramasamy et al., 2005), antioxidant (Yu et al., 2005b), and cytotoxic (Kang et al., 2009) effects. Anticoagulants from marine organisms have rarely been isolated, except for several anticoagulant proteoglycans and polysaccharides from marine algae (Changaff et al., 1936; Kindness et al.,

Abbreviations: BSA, bovine serum albumin; EDTA, ethylene di-amine tetra acetic acid; JFTE, Jellyfish tentacle extract; MW, molecular weight; PLA, phospholipase A; PMSF, phenyl methyl sulfonyl fluoride; RBCs, red blood cells.

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Hemotoxic Activity of Jellyfish Venom

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Dibakar Chakrabarty and Akriti Rastogi

Contents

Abstract

Jellyfish are marine invertebrates of the phylum Cnidaria. All jellyfish species are venomous. Human victims of jellyfish sting each year are 120 million. However, most victims do not require hospitalization. Severe cases of envenomation may sometimes be fatal. Among the symptoms of envenomation, hemotoxicity constitutes a small fraction. Hemolysis has been reported in severe envenomation cases. On the other hand, coagulopathy in jellyfish envenomation is almost absent in scientific literature. Some hemolytic pore-forming toxins have been isolated from venom and tentacle extracts of a few jellyfish species. These toxins show some degree of variation in size and structure. However, many of them cause hemolysis by disturbing the transmembrane ion concentrations. It is also claimed that lipid peroxidation

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ANTICOAGULANT ACTIVITY OF BARREL JELLYFISH *RHIZOSTOMA PULMO* TENTACLE EXTRACT

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Marine venomous organisms have attracted less interest compared to the terrestrial animals from toxinologists in spite of their abundance and variety, till recently. Although investigations on marine envenomation have a long history, particularly in Australia and the USA, research on the use of marine venoms and toxins for therapeutic use was rare. The good news is extremely specific activities of certain marine toxins on different physiological systems have finally succeeded in attracting scientific attention. Several marine compounds are now recognized to be active against pathological conditions involving the cardiovascular, endocrine, immune, and nervous systems. Marine toxins with anti-inflammatory, antiplatelet, antitumor, or cytotoxic activities are now being reported at regular intervals. A few of these toxins are active against infectious diseases also and some of these are currently in preclinical phase trials and/or under phase I and II clinical studies. Biochemical and pharmacological profile of the venom of jellyfishes found along the western coast of India is not well studied. These venoms are expected to contain novel pharmacologically active molecules of therapeutic importance. In this study, *Rhizostoma pulmo* tentacle extract was investigated for its anticoagulant activity *in vitro*. The JellyFish Tentacle Extract (JFTE) showed very strong fibrinogenolytic activity by cleaving Aα and Bβ chains of fibrinogen molecule. JFTE completely liquefied fibrin clots. Inhibition studies suggested the active component was heat labile and was a metalloprotease. Attempts were made to purify the active component by SE-HPLC. The active fraction consisted of two proteins of molecular weights ~95 kDa and 65 kDa. Substrate zymography showed that ~95 kDa protein possesses strong caesinolytic and gelatinolytic activity with mild fibrinogenolytic activity whereas ~65 kDa protein showed very strong fibrinogenolytic activity only. Both these proteins lost their activities on treatment with EDTA, suggesting them to be metalloproteases. JFTE reduced the recalcification time and thrombin time in human plasma. However, the semi-pure fraction prolonged it. The extract and semi-pure fraction also inhibited ADP dependent platelet aggregation, in a dose dependent manner. JFTE and the semi-purified fractions are capable of affecting the haemostatic system at three different levels: platelet aggregation, fibrinogen digestion and fibrin clot digestion. Strong gelatinolytic activity of JFTE and semi-purified fractions also suggests that it could be playing a significant role in inducing diverse toxicological effects associated with jellyfish envenomations like ulceration of the skin and appearance of hemorrhagic spots. Further characterization of semi-purified fraction is currently being pursued.

RELATIONSHIP BETWEEN BINDING CHARACTERISTIC OF A NON-NATURAL PEPTIDE, LEI-DAB7, AND DISTRIBUTION OF SK SUBUNITS IN THE RAT CENTRAL NERVOUS SYSTEM

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Small-conductance calcium-activated potassium channels (SK), among which, three subunits were identified (SK1-3), were initially studied using apamin and to know the structure-function relationship studies of this toxin. Apamin, a neurotoxin blocker, extracted from bee venom, includes 18 amino acid residues reticulated by two disulfure bridges and reported to be the reference ligand of SK2 and SK3 channels because of its high affinity for both of them. More recently, using the radiolabeled apamin competition assays, other blockers were found in different scorpion venoms, such as Leirotoxin1 (LTX1) and P05. These toxins of 31 residues of amino acids, reticulated by three disulfure bridges, have 87 to 90 % of sequence homology. Sequence alignment has shown that LTX1 and PO5 contain a motif (RXCQ) reported to be important for binding to SK channels. Apamin contains a RRCQ sequence, having a spatial arrangement (α helix) similar to that of the RXCQ motif in LTX1 and P05. LTX1 and PO5, potently blocked human SK2 and SK3 but not SK1. By replacing Met(7) in the RXCQ motif of LTX1 with the shorter, unnatural, positively charged diaminobutanoic acid (Dab), we generated Lei-Dab7, a selective SK2 inhibitor (Kd 3.8 nM). This study evaluates the relationships between selectivity and affinity of apamin and Lei-Dab7. Competitive binding of radio-iodinated apamin to rat brain regions, in presence of native apamin and Lei-Dab7, has shown dissociation constants significantly different by a factor of a thousand, signifying that ligand affinity is as important as selectivity for binding a specific receptor. To evaluate the differences of the binding characteristics in rat brain structures, we used a message-passing clustering techniques. Five groups of brain structures were identified reflecting a singular profile of affinity and selectivity of Lei-Dab7 in comparison with apamin. A multiple correspondences analysis allowed us to analyze the correspondences between Lei-Dab7 binding and expression of SK subunits in these brain structures groups. The comparison between the selectivity of Lei-Dab7 and the composition in SK subunits in brain regions suggest functional heteromeric SK channels. Our analysis contributes to a better understanding of the molecular combination of SK channel in the native tissue involved in specific information processes.

IN VITRO EFFECTS OF THREE AZASPIRACID ANALOGUES ON HEPATOCYTES

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Azaspiracids (AZAs) are polyether marine toxins of dinoflagellate origin that can contaminate edible shellfish and induce foodborne poisonings in humans, characterized by gastrointestinal symptoms. AZAs include more than 30 analogues, but only AZA-1, -2 and -3 are regulated by the European Union. Acute oral administration in mice of these compounds was shown to induce intestinal and liver damages, the latter visible as swollen pale liver. Moreover, fat droplets in hepatocytes as well as cell vacuoles in the central part of the hepatic lobuli and subcapsular liver region were observed after oral AZA-1 administration in mice. To investigate the effects of AZA-1, -2 and -3 on hepatocytes, an *in vitro* study was carried out using the IHH non-tumoral and the HepG2 tumoral human hepatic cell lines. On IHH cells, AZA-1, -2 and-3 induced a concentration-dependent reduction of mitochondrial activity (MTT assay) only after 72 h exposure (EC_{50} 1.2×10^{-11} M, 7.0×10^{-11} M and 3.8×10^{-11} M, respectively). While the effect was almost undetectable at 48 h, a significant concentration-dependent increase of mitochondrial activity was observed after 24 h. This effect was not due to an increased cell proliferation, as evidenced by cell mass evaluation [sulforhodamine B (SRB) assay]: no effect was observed at 24 h, whereas 72 h exposure to AZA-1, -2 or -3 reduced cell mass with EC_{50} values of 1.3×10^{-11} M, 4.5×10^{-11} M and 2.1×10^{-11} M, respectively. These effects were confirmed on HepG2 cells, which were more sensitive to AZAs than IHH cells: after 72 h exposure, mitochondrial activity (MTT assay) was reduced with EC_{50} of 4.1×10^{-12} M, 8.6×10^{-12} M and 4.3×10^{-12} M, while cell mass (SRB assay) was decreased with EC_{50} values of 6.5×10^{-12} M, 2.5×10^{-11} M and 6.3×10^{-12} M, respectively. Thus, 24 h exposure to AZAs induced a metabolic stimulation at mitochondrial level in both cells rather than a proliferative effect. In fact, mitochondrial electron transport chain complex I inhibition by rotenone (5 M) completely abolished the effect observed by MTT assay at 24 h. This evidence is in line with preliminary flow cytofluorimetric observations in cells exposed to the metabolic-sensitive DiOC6 fluorescent probe indicating mitochondria hyperpolarization. These results gain new insights

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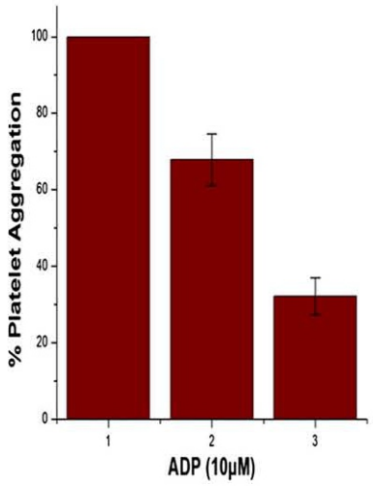

Fig. 2. 1) Control Human whole blood 2) 20 μg Lahirin treated blood and 3) 40 μg Lahirin treated blood (n=10).

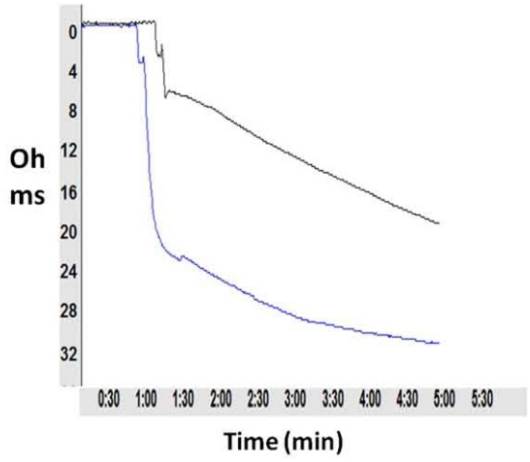

Fig. 3. Thrombin induced platelet aggregation process. Blue represents Control Human whole blood platelet aggregation after thrombin treatment. Black represents thrombin induced aggregation of Lahirin (40 μg) treated blood.

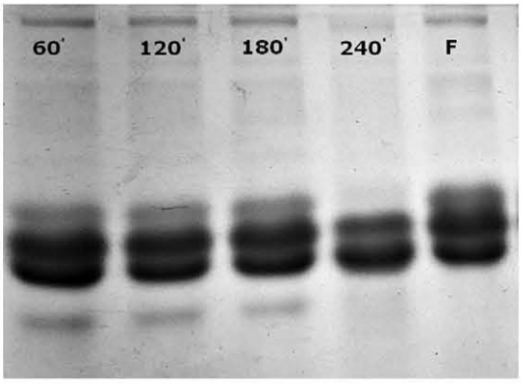

Fig. 4. Time dependent fibrinogenolytic activity of the Lahirin by 10% SDS-PAGE.

Keywords: fibrinogenolytic, antiplatelet, peptide
10.1016/j.toxicon.2012.04.077

77. Anticoagulant Activity of Moon Jellyfish (*Aurelia aurita*) Tentacle Extract

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Background: Biochemical and pharmacological profile of jellyfish venom is not well studied in jellyfishes found along the western coast of India. The venom is expected to contain novel pharmacologically active molecules of therapeutic importance. In this study, *Aurelia aurita* tentacle extract was investigated for its anticoagulant activity *in vitro*.

Methods: Fibrinogenolytic Assay, Fibrinolytic Assay and Platelet Aggregation Studies using Whole Blood Aggregometer.

Results: The Jellyfish Tentacle Extract (JFTE) showed very strong fibrinogenolytic activity by cleaving Aα and Bβ chains of fibrinogen molecule. JFTE completely liquefied fibrin clots. JFTE appears to contain several anticoagulant proteins or peptides. Some of which appear to be metalloproteases and some serine proteases. JFTE also inhibited ADP and collagen dependent platelet aggregation, in a dose dependent manner.

Discussion: Purification and characterization of the protein(s) responsible for anticoagulation is under progress.

Conclusion: JFTE affects the haemostatic system at three different levels: Platelet aggregation, Fibrinogen digestion and Fibrin clot digestion.

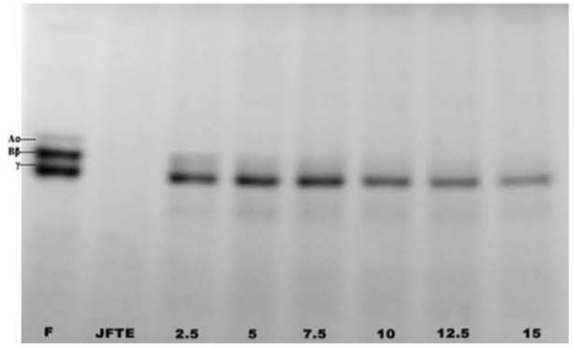

Fig. 1. Dose dependent fibrinogenolytic activity of JFTE. Fibrinogen (2 mg/mL) was incubated independently with different concentrations of JFTE at 37°C for 180 min. (F) Fibrinogen alone. (JFTE) JFTE alone. Numbers at the bottom of each lane indicates dose of JFTE in μg.

Anticoagulant Targeted Venom Discovery Array™

Venomtech®, Drug Discovery Solutions, United Kingdom, has recently introduced an anticoagulant array library under the category of targeted venom discovery array which has been designed to screen novel anticoagulant compounds. It uses *Aurelia aurita* as one of the control venoms based on the research described in this thesis.

VENOMTECH®
Drug Discovery Solutions™

Target:	**Anticoagulant**
Format:	**Targeted Venom Discovery Array**
Code:	**T-VDA^{acog}**

Product Description

The **Anticoagulant Targeted Venom Discovery Array™** is specifically designed to maximise discovery of new tools. Anticoagulants are important drug tools for a range of **cardiovascular disorders** including **heart attack and stroke**. Alongside leeches, venoms from snakes and jelly fish are also rich sources of new anticoagulants. Our targeted arrays contain pure venom fractions from 12, 24, 48 or 96 species optimised for identification of novel tools. Every array contains characterised venoms with anticoagulant activity from the literature to act as positive controls. The control venoms for T-VDA^{acog} include *Naja kaouthia* (monocled cobra) which contains **fibrinogenolytic toxins**[1]; *Aurelia aurita* (moon jellyfish) where the fibrinogenolytic activity can completely liquefy clots[2]; and *Hirudo verbana* (medicinal leech) which famously also contains a diverse collection of **anticoagulants**[3]. The other venom fractions making up the library have been specially selected by our drug discovery scientists to maximise novel hit potential.

- Venoms are supplied lyophilised in Echo® qualified acoustic source plates (Labcyte Inc) and are useable on any SBS footprint liquid handling device or by hand.
- 384-well format has 1µg venom fraction per well, re-suspension with 30µl will produce ~1.6µM-16µM stock concentration of peptides.
- 1536-well format has 300ng venom fraction per well, re-suspension with 10µl will produce ~1.5µM-15µM stock concentration of peptides.

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3. Kvist, S., Min. G.S. Siddall, M.E. (2013). Diversity and selective pressures of anticoagulants in three medicinal leeches (Hirudinida: Hirudinidae, Macrobdellidae). *Ecology and Evolution* **3**, 918.

Data compiled from UniProt: Reorganizing the protein space at the Universal Protein Resource (UniProt), Nucleic Acids Res. 40: D71-D75 (2012).

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- Masters of Engineering in Biotechnology (2011), Birla Institute of Technology and Sciences, Pilani, India.
- PG Diploma in Patents Law, one year non-residential web based programme, (2012), NALSAR, Hyderabad, India.

Awards, Scholarships and Memberships

- Recipient of CSIR Senior Research Fellowship. (2013-2017)
- Recipient of best oral presentation award. (December 2014)
- Recipient of Department of Science and Technology and Department of Biotechnology, Government of India, travel grants. (December 2014 and July 2012)
- Recipient of best poster presentation award. (March 2012)
- Recipient of full academic scholarship during M.E. (2009 –2011)
- Member of International Society on Toxinology (IST) and Toxinological Society of India (TSI)

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Academic Records:

- MSc in Physiology (1985), University of Calcutta, India.
- PhD, Jadavpur University (1993) for thesis entitled **'**Studies on Haemorrhagic Principle of Russell's Viper venom'.
- Currently engaged in teaching Animal Physiology, Developmental Biology and Reproductive Physiology as theoretical courses. Teaching Experimental techniques as laboratory based course for post-graduate biotechnology students.

Major research interest: Animal toxins as drug leads.

Publications:

http://universe.bits-pilani.ac.in/goa/dibakarchakrabarty/Publications

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Position: Associate Professor

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Academic Records:

- PhD, Department of Biotechnology, University of Pune (2004), for thesis entitled **'**The heme-regulated eukaryotic initiation factor 2alpha kinase: Expression and regulation of protein synthesis in human cells *in vitro* during heat-shock and lead toxicity'.

- Post Doctoral Experience (2004-2009)
  - Cold Spring Harbor Laboratory, New York, USA, Molecular Developmental Biology.
  - School of Micro Biology and Molecular Biology, Case Western Reserve University, Cleveland, OH, USA.
  - Department of Cancer Biology, Cleveland Clinic Foundation, Cleveland, OH, USA.

- Currently engaged in teaching animal cell technology and cancer biology as theoretical courses. Teaching Experimental techniques as laboratory based course for post-graduate biotechnology students.

Major research interest: *In vitro* cell biology.

Career Highlights:

- Awarded fellow from All India Society of Cell Biology, 2016.

- Awarded 'Young Investigator' Pilot Project Grant by DBT 'Cancer Biology Mission' in 2015.

- Awarded "Appreciation Certificate" by Memorial Sloan Sloan-Kettering Cancer Center, USA, in 2008 for outstanding leadership and commitment in the Cancer Research in 2009.

- Awarded Research Associateship by Department of Cell and Molecular Biology [CWRU, Cleveland, USA in 2007.

- Offered a Fellowship by Cold Spring Harbor Laboratory (CSHL), New York, USA for Post Doctoral Training in 2004.

Publications:
http://universe.bits-pilani.ac.in/goa/asarkar/Publication