Study on Coelomic Fluid of *Astropecten indicus* and Isolation and Characterisation of a Bioactive Agent

THESIS

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By

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CERTIFICATE

This is to certify that the thesis entitled **"Study on Coelomic Fluid of *Astropecten indicus* and Isolation and Characterisation of a Bioactive Agent"** submitted by **MANSI BAVEJA**, ID No. **2010PH290405G** for award of Ph.D. degree of the Institute embodies original work done by her under our supervision.

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DECLARATION

I hereby declare that this Ph.D. thesis entitled "**Study on Coelomic Fluid of *Astropecten indicus* and Isolation and Characterisation of a Bioactive Agent**" was carried out by me for the degree of Doctor of Philosophy in Biological Sciences under the guidance and supervision of Prof. Dibakar Chakrabarty and Prof. Angshuman Sarkar, Birla Institute of Technology and Science Pilani, K K Birla Goa campus.

Mansi Baveja

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Abstract

The marine environment covers more than 70 percent of the earth's surface and contains more than 87 percent of all the life forms available on earth. Despite its vast expanse, the marine fauna remain largely unexplored for their therapeutic potential. Of all the marine animals present, echinoderms especially sea stars (starfish) are least studied for their bioactive potential. Sea stars have a remarkable regenerative potential and are model organisms for regenerative studies. Their coelomic fluid is a rich reservoir of immunocytes, coelomocytes, cytokines and secondary metabolites which have shown to have various bioactivities.

Our research group is predominantly focussed on investigating novel thrombolytic agents from natural sources, both terrestrial and marine. This study was aimed at exploring these therapeutic agents from the marine source: sea star coelomic fluid. In the process of this investigation, a biomolecule, AiP1 was isolated which also exhibited great wound healing and fibrinogenolytic potential. Sea star *Astropecten indicus* were collected from the coast of Goa, India during low tides. Sea star Coelomic Fluid (SCF) extracted from the sea stars was processed and tested for its effects on fibrinogen, fibrin, platelets and on viability of cultured cells *in vitro*. Sodium dodecyl Sulphate poly-acrylamide gel electrophoresis (SDS-PAGE) analysis showed that SCF contains various proteins and peptides. In this thesis, purification and partial characterisation of one of these proteins, AiP1 is described.

The coelomic fluid of sea star *Astropecten indicus* was fractionated using ion-exchange chromatography and size exclusion high performance liquid chromatography (HPLC) to yield AiP1. The purified protein showed fibrinogenolytic and fibrinolytic activity in a dose dependent manner. It affected ADP and collagen induced platelet aggregation. Proteolytic activity of SCF and AiP1 was inhibited post-treatment with a metal chelator, Ethylene diamine tetra acetic acid (EDTA), indicating its metallo-proteinase nature. The protein AiP1 showed no toxicity on A549, HaCaT or HEK293 cells, *in vitro.* It was also devoid of hemolytic, laminin digesting and phospholipase activities. Scratch wounds created on surface of A549, HaCaT and HEK293 cells showed faster wound closure rates

post treatment with AiP1 as compared to untreated cells. Experimental data was substantiated with peptide mass fingerprinting analysis of AiP1 peptides which revealed homology to the von Willebrand factor superfamily-A like domain.

In conclusion, the coelomic fluid of sea stars contains various bioactive compounds. One such compound is the protein AiP1 which was partially characterised in this study. This protein showed significant wound healing and thrombolytic activities *in vitro*. These investigations reveal the bioactive potential of an unexplored marine invertebrate, the *Astropecten indicus,* its coelomic fluid and a protein component, AiP1 isolated from the coelomic fluid. Thus it is proposed that SCF and AiP1 are effective thrombolytic and wound healing agents.

List of Abbreviations

ADP Adenosine di-Phosphate

BSA: Bovine serum albumin

CBB R-250: Coomassie Brilliant Blue R-250 Dye

CM Sephadex: Carboxy Methyl Sephadex

Da Daltons

DEAE-Sephadex: diethyl amino ethyl

DMEM Dulbecco's Modified Eagle's Medium

DMSO dimethyl sulfoxide

DNA Deoxyribonucleic acid

DTT Dithiothreitol

DVT Deep vein thrombosis

ECM: Extra Cellular Matrix

EDTA Ethylene diamine tetra acetic acid

FBS Fetal Bovine Serum

FDA Food and drug Administration

GPCR G Protein Coupled Receptors

IEC Ion-Exchange Chromatography

KPB Potassium phosphate buffer

M Molar

MALDI TOF Matrix assisted laser desorption/ionization Time of flight

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

MW Molecular weight

MWCO Molecular weight cut off

NaCl: Sodium Chloride

PBS Phosphate Buffer Saline

PE Pulmonary embolism

PLA Phospholipase A

PMSF Phenylmethyl sulfonyl fluoride

PPP Platelet poor plasma

PRP Platelet rich plasma

RBC: Red blood cell

RPM Rotations per minute

SCF Sea-star Coelomic Fluid

SDS-PAGE Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SE HPLC Size exclusion High Performance Liquid Chromatography

SEM Scanning electron microscopy

tPA Tissue plasminogen activator

UV Ultra violet

VTE Venous thromboembolism

vWF von Willebrand factor

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1 Introduction and Review of Literature

1.1 Background

1.1.1 Marine environment: a useful bioresource

The marine environment covers an area of about 360 million square kilometer, which accounts for more than 70% of surface of the earth. Out of the 36 phyla, 34 are present in the oceans, thus comprising approximately 87% of the total biosphere. With this vast expanse, the marine environment embodies abundant biological and chemical diversity and hence is a promising source for investigating novel compounds of pharmaceutical, cosmetic, dietary and agro-chemical importance (Hu et al., 2011; Minh et al., 2005).

Despite their vast extent and importance, the oceans and the life they contain are seriously understudied. It has been estimated that only about five percent of the oceans have been explored by humans. Marine microbes, plants and animals have yielded more than 16,000 novel compounds with few hundreds being discovered every year (Subramanian et al., 2011). Different marine sources with bioactive compounds that have been isolated from them is summarized in Figure 1.1 (Sable et al., 2017).

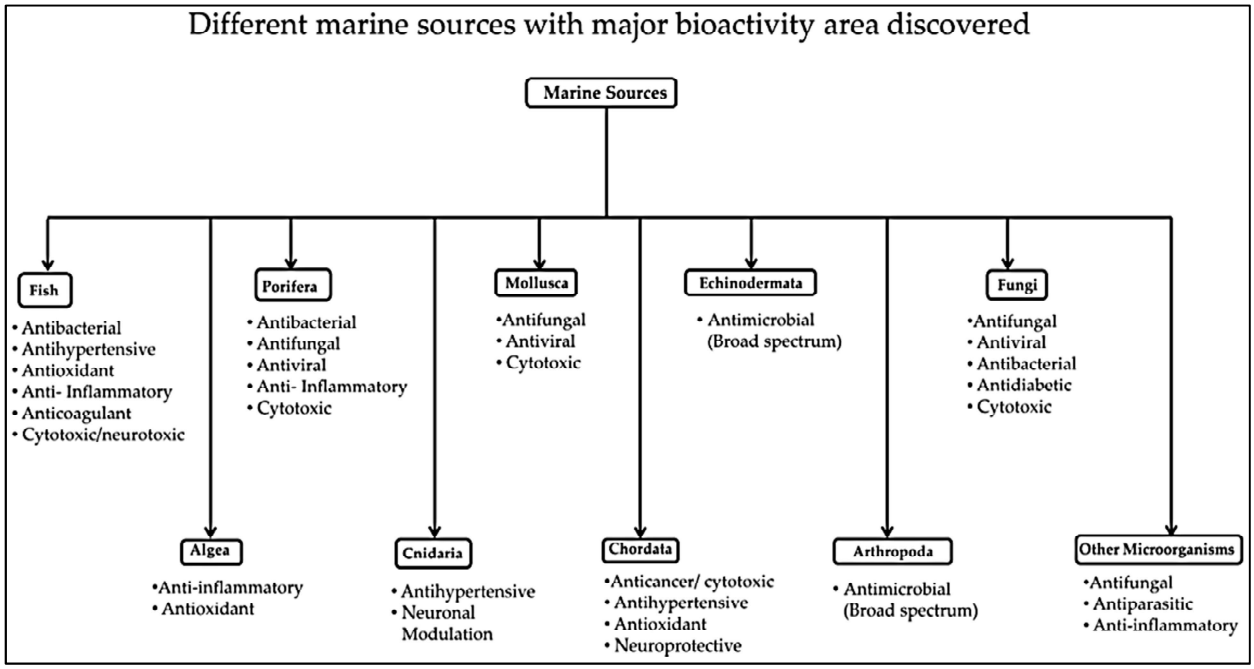

Figure 1.1 Different marine sources with major bioactivity area discovered.

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Out of all bioactive compounds obtained from nature, peptides are most crucial pharmacophores for drug design studies owing to their high selectivity and high binding efficiency to carry out signaling processes. Nearly 7000 peptides or peptidomimetics from natural resources have been identified till the year 2017 and there is a dire need for many more. Given the vast yet unexplored expanse of oceans, it is desirable to isolate novel peptides from them (Sable et al., 2017). The present work aimed to isolate novel proteins/peptides produced by lesser exploited marine resources - the sea stars.

1.1.2 Why do marine organisms produce bioactive compounds?

Marine organisms, unlike their terrestrial counterparts, need to adapt to intense environmental conditions like high salt concentration, high pressure, low nutrient concentration, low but steady temperature, and low oxygen content and limited sunlight. Additionally, there are ecological pressures like competition for survival, space, nutrition and self-defense. Marine organisms adapt to these extreme conditions owing to the presence of distinct characteristics in them such as their metabolism, behavioral patterns, chemical strategies, information transfer and adaptation strategies (Avila et al., 2007; Hu et al., 2011). The marine organisms produce secondary metabolites for countering the environmental stresses. The secondary metabolism in marine organisms is responsible for the diverse bioactive compounds that are absent in terrestrial species. (Datta et al., 2015a; Khattab et al., 2018).

Some drugs of marine origin in different phases of clinical trials are summarized in Table 1.1 (Malve, 2016).

1.1.3 Marine invertebrates

Marine invertebrates cover about 60% of all the marine fauna. They mainly belong to the phyla Annelida, Arthropoda, Bryozoa, Cnidaria, Echinodermata, Mollusca, Platyhelminthes, Porifera and sub-phylum Tunicata (Leal et al., 2012). Given their rich diversity and secondary metabolite production, marine invertebrates are common targets for investigation of novel bioactive compounds (Sheean et al., 2007).

Marine invertebrates have contributed to the discovery of majority of novel marine derived natural products since 2005 (Leal et al., 2012). In the last two decades, Poriferans and Cni-

darians have been extensively studied, leaving echinoderms a primarily under-explored invertebrate till date as seen in statistics shown in Figure 1.2.

Table 1.1 A perspective of pipeline of marine drugs

Clinical status	Compound name	Marine organism	Chemical class	Disease area
Approved	Cytarabine, ara-C	Sponge	Nucleoside	Cancer, leukemia
	Brentuximab vedotin (SGN-35)	Mollusk/cyanobacterium	ADC (MMAE)	Cancer, lymphoma
	Vidarabine, ara-A	Sponge	Nucleoside	Anti-viral
	Omega-3-acid ethyl esters	Fish	Omega-3 fatty acid	Hypertriglyceridemia
	Ziconotide	Cone snail	Peptide	Pain
	Eribulin mesylate (E7389)	Sponge	Macrolide	Breast cancer
	Trabectedin (ET-743)	Tunicate	Alkaloid	Cancer
Phase III	Plitidepsin	Tunicate	Depsipeptide	Cancer
	Tetrodotoxin	Pufferfish	Guanidinium alkaloid	Chronic pain
	Soblidotin (TZT 1027)	Bacterium	Peptide	Cancer
Phase II	DMXBA (GTS-21)	Worm	Alkaloid	Cognition, Alzheimers disaese, schizophrenia
	Plinabulin (NPI-2358)	Fungus	Diketopiperazine	Cancer
	Glembatumumab vedotin	Mollusk/cyanobacterium	ADC (MMAE)	Breast cancer, melanoma
	Elisidepsin	Mollusc	Depsipeptide	Cancer
	PM1004	Nudibranch	Alkaloid	Cancer
	Tasidotin, synthadotin (ILX-651)	Bacterium	Peptide	Cancer
	Pseudopterosins	Soft coral	Diterpene glycoside	Wound healing
Phase I	Bryostatin 1	Bryozoa	Polyketide	Cancer
	Pinatuzumab vedotin (DCDT-2980S) and (DCDS-4501A)	Mollusk/cyanobacterium	ADC (MMAE)	Non-Hodgkin lymphoma, chronic lymphocytic leukemia
	Hemiasterlin (E7974)	Sponge	Tripeptide	Cancer
	HuMax®-TF-ADC	Mollusk/cyanobacterium	ADC (MMAE)	Cancer for ovary, endometrium, cervix, prostate
	Marizomib (salinosporamide A)	Bacterium	Beta-lactone-gamma lactam	Cancer
Preclinical	Chrysophaentin A	Alga *Halobacillus salinus*	Shikimate	Bacterial infections
	Phenethylamine	Bacterium lyngbyoic acid	Shikimate	Bacterial infections
	Geodisterol sulfates	Sponge	Peptide	Fungal infections
	Pseudoalteromonas sp. metabolites	Bacteria	Polyketide	Bacterial infections
	Peziza vesiculosa β-carboline	Bryozoa	Alkaloid	Fungal infections
	Bromophycolides	Alga	Terpene	Malaria
	Plakortin	Sponge	Polyketide	Malaria
	Homogentisic acid	Sponge	Shikimate	Malaria
	Cladonia cervicornis diterpene	Alga	Terpene	Protozoal infections
	Hymenidin	Sponge	Alkaloid	Tuberculosis
	Ggyrosanols	Soft coral	Terpene	Viral infections
	Dysidine	Sponge	Terpene	Diabetes
	Arenamides A and B	Bacteria	Peptide	Inflammation
	Capnellene	Soft coral	Terpene	Inflammation
	Floridosides	Alga	Glycolipid	Inflammation
	Grassystatins A-C	Bacteria	Peptide	Immunity
	Callyspongidiol	Sponge	Polyketide	Immunity
	Calyculin A	Sponge	PKS/NRPS	Nervous system
	Pulicatin A	Bacteria	Alkaloid	Nervous system
	Dysideamine	Sponge	Terpene	Nervous system

ADC: Antibody drug conjugate, MMAE: Monomethylauristatin E, PKS: Polyketide synthases, NRPS: Nonribosomal peptide synthases, DMXBA: 3-(2,4 dimethoxy) benzylidene-anabaseine

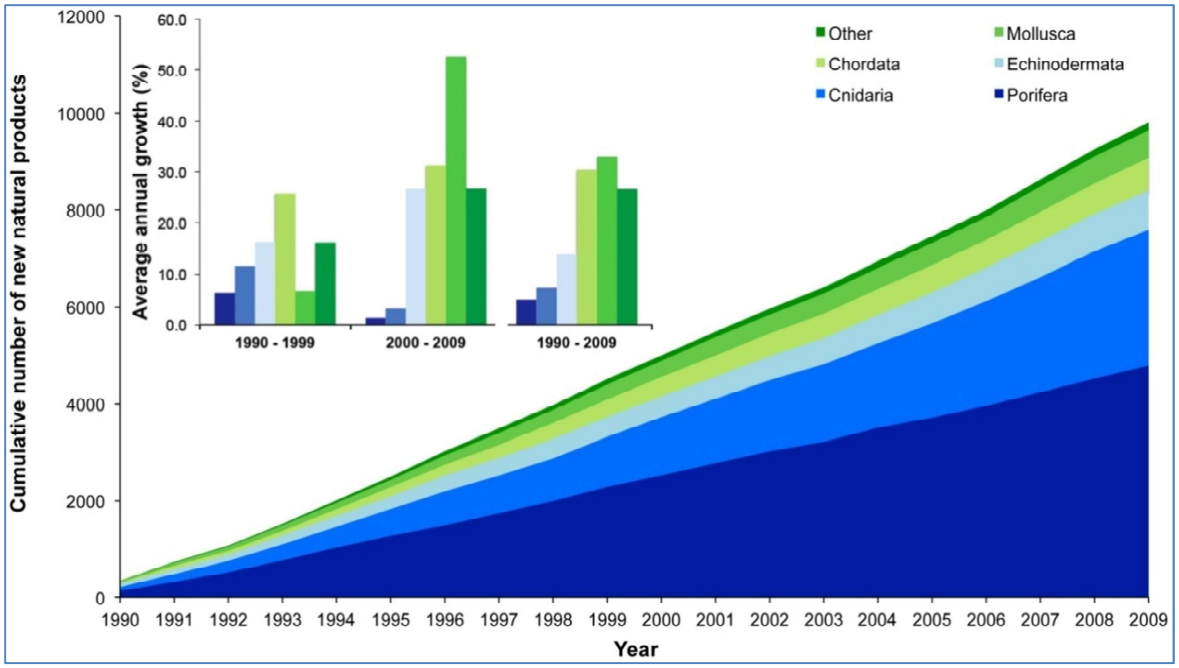

Figure 1.2 New natural products from marine invertebrate phyla

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1.1.4 Echinoderms

The phylum Echinodermata encompasses about 7000 species and is an unexploited source for novel bioactive compounds (Stabili *et al*., 2018).

In the last two decades, majority of the new marine derived natural products from this phylum belonged to the sub-phyla Asterozoa (54.9%) and Echinozoa (33.7%). The classes Asteroidea (Sea stars) and Holothuroidea (Sea-cucumbers) of Phylum Echinodermata accounted for 91.7% of new marine natural products having 529 and 213 new marine natural products, respectively (Walag, 2017).

Extracts from echinoderms have exhibited a wide range of biological properties in the areas of cell biology, microbiology, immunology and oncology. While Echinoidea (Sea-urchins) is the most extensively studied class of echinidermata, the most popular bioactive compounds are triterpene glycosides and steroids extracted from the Asteroidea (sea stars) and Holothuroidea (sea-cucumbers) (Gomes, Freitas, Duarte, & Rocha-Santos, 2016; Ramírez-Gómez &

García-Arrarás, 2010). Sea urchins from the phylum Echinodermata are one of the classical animal models for studies of fertilization and developmental biology. The immune systems of echinoderms display the same basic responses which most multicellular animals (including vertebrates) display. They can recognize self from non-self and, if a foreign material (e.g., microorganism/pathogen) enters the body cavity, they can readily neutralize and dispose it (Dybas and Fankboner, 1986; Gliński and Jarosz, 2000; Yui and Bayne, 1983). Furthermore, echinoderms exhibit remarkable wound healing capabilities, a key feature that also plays a pivotal role in one of the best known characteristics of the group: regeneration of ravaged body parts (Ramírez-Gómez and García-Arrarás, 2010).

1.1.5 Sea stars and their bioactive potential

The sea stars (class Asteroidea) have about 2,000 species in habitats from tropical coral reefs, kelp forests to the cold deep oceans (greater than 6 km). They have been widely studied for their antimicrobial activities against an array of human pathogens. Some species like *Astropecten polycanthus* have also been reported for their anti-inflammatory activities. Other sea stars like Tremasternovaecaledoniae, Asteriasamurensis, Styracastercaroli and Echinasterbrasiliensis contain sterol compounds which can inactivate HIV (Walag, 2017). Other activities reported from sea stars are anti-cancer and anti-oxidant activities. A few bioactive compounds isolated from sea stars are listed in Table 1.3 (Banu, 2017).

Table 1.2 Summary of coelomocyte types reported for echinoderm classes. E: Echinoidea, H: Holothuroidea, A: Asteroidea, C: Crinoidea, O: Ophiuroidea

Cell type	Present in class	Role	Reference
Discoidal cell	E, H	Phagocytosis, clotting, encapsulation, chemotaxis, opsonisation, graft rejection	(Coteur*et al.*, 2002; de Faria and da Silva, 2008; Eliseikina and Magarlamov, 2002; Endean, 1966; Matranga*et a*l. 2005; Ramirez-Gomez *et al.*, 2010; Smith *et al.*, 2006)
Polygonal cell	E		
Small phagocyte	E, H		
Amebocytes /Phagocytes	E, H, A, C, O		
Colored spherule	E, H, C	Antibacterial activity	(de Faria and da Silva, 2008; Endean, 1966; Smith *et al.*, 2006)

Colorless spherule	E, H, A, C, O	Antibacterial, inflammation, Wound healing, ECM remodeling	(Coteur*et al.*, 2002; de Faria and da Silva, 2008; Eliseikina and Magarlamov, 2002; Endean, 1966; Garcia-Arraras*et al.*, 2006; Ramirez-Gomez *et al.*, 2010; Smith *et al.*, 2006)
Lymphocyte	E, H, A	Progenitor cells	(Coteur*et al.*, 2002; Eliseikina and Magarlamov, 2002; Endean, 1966; Ramirez-Gomez *et al.*, 2010; Xing *et al.*, 2008)
Vibratile	E, H, A, O	Celomic fluid movement, clotting	(de Faria and da Silva, 2008; Eliseikina and Magarlamov, 2002; Endean, 1966; Matranga *et al.*, 2005; Pinsino *et al.*, 2008; Ramirez-Gomez *et al.*, 2010; Smith *et al.*, 2006; Xing *et al.*, 2008)
Crystal cells	H	Osmoregulation	(Eliseikina and Magarlamov, 2002; Endean, 1966; Ramirez-Gomez *et al.*, 2010; Xing *et al.*, 2008)
Hemocytes	H, A, O	Oxygen transport	(Eliseikina and Magarlamov, 2002; Endean, 1966; Pinsino*et al.*, 2008)

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1.1.6 Species of interest for this study: sea star *Astropecten indicus*

It is the plain sand star from the family Astropectinidae that is commonly found along the Indo-Pacific region and along the coast of South East Asian countries. These sea stars are characterized by five arms, a bluish grey dorsal surface and an aboral pore on the ventral surface.

Table 1.3 Natural products from sea stars and their activity

S.No	Sea Star	Origin	Compounds/ Crude Extracts in Solvents	Activity	References
1.	Leptasterias ocholensis	Far east coast	Asterosaponins & Glycosides Methanolic extract	Cytotoxic activity	Malyarenko et al. [1]
2.	Ctenodiscus crispatus	Sea of Okhotsk	Steroidal compound (polyhydroxylated steroidal derivative) Methanolic extract	Cytotoxic activity & Antitumor against Hepatocellular carcinoma & Glioblastoma cells	Tranhong et al. [2]
3.	Ophiocoma erinaceus	Oeshn island Persian gulf	Saponins Ethanolic fractions	Hemolytic and cytotoxic activity	Elaheh Amini et al. [11]
4.	Luidia maculata	Center of Mandapam South East Tamilnadu	Partially purified compound Ethanolic extract	Antioxidant, Antifungal & Antibacterial	Suguna et al. [13]
5.	Astropecten polyacanthus	Coast of Vietnam	Asteropectinol (steroidal compound) Methanolic extract	Cytotoxic activity (potent compound against Leukemia)	Nguyen Phuong et al. [5]
6.	Acanthaster planci	Indo pacific region (Australia)	Glycoprotein (toxic veneom)	Cytotoxicity	Chi-Chiu Lee et al. [12]
7.	Protoreaster linckii	Center of Mandapam South East Tamilnadu	Crude Compound Methanolic extract	Antimicrobial, Hemolytic, Antinociceptive & Cytotoxic activity	Suguna et al. [13]
8	Stellaster equestris	Center of Mandapam South East Tamilnadu	Crude and fractioned compound (steroidal compound)	Antibacterial	Bragadeeswaran et al. [13]
9.	Acanthaster planci	Indo pacific region coast of Pulau Redang (Malasia)	Homogenized crude extract	Potent Cytotoxic and apoptotic effect on Human breast cancer	Ahmed Faisal et al. [10]
10.	Astropecten indicus	Center of Mandapam South East Tamilnadu	Crude compound Methanolic extract	Antibacterial	Chamundeeswari et al. [17]
11.	Archaster typicus	Coast of Vietnam	Asterosaponins a. Archasterosides b. Regularosides	Cytotoxic activity against human and mouse myeloma	Kicha et al. [8]
12.	Archaster typicus	Queensland, Australia	Water borne compound	Antifouling	Jana Guenther et al. [25]
13	Linckia laevigata	John Brewer Reef	Water borne compound	Antifouling	Jana Guenther et al. [25]
14	Fromia indica	John Brewer Reef	Water borne compound	Antifouling	Jana Guenther et al. [25]
15	Cryptasterina pentagona	Kissing Point in Townsville	Water borne compound	Antifouling	Jana Guenther et al. [25]
16.	Asterina pectinifera	Coast of Pohang Korea	Polysaccharides	Antitumor against colon cancer	Kyung et al. [7]
17.	Culcita novaeguineae	Far east coast	Asterosaponins	Cytotoxic activity & Antitumor and chemotherapeutic agent	Guang et al. [3]
18.	Asterias rubens	Coast of Norway	Coelomocytes fluid (Antimicrobial peptides)	Antibacterial	Maltseva et al. [19]
19.	Anasterias minuta	Californian coast	Steroidal Glycosides	Antifungal	Chludi et al. [21]
20.	Asterina pectinifera	East coast of Korean Peninsula	Crude compound Methanolic extract	Antifungal	Choi et al. [23]
21	Dermasterias imbricate	Coast of North America	Saponins, Sulfated steroidal compound	Antifungal	Bruno et al. [24]

Adapted from: R Sumithaa, N Banu, V Deepa P. Novel Natural Products from Marine Sea stars. Curr Trends Biomedical Eng&Biosci. 2017; 2(4): 555592. DOI: 10.19080/CTBEB.2017.02.555592

The *Astropecten indicus* species are abundantly found along the coast of Goa during the mid and post monsoon season. The newspaper clipping in Figure 1.3 shows how these sea stars get washed ashore during low tides each year.

Figure 1.3 *Astropecten indicus* washed ashore and reported in Newspaper dated 10th July 2018, Times of India. Image adapted from Times of India, Goa edition with permission.

1.1.7 Hemostasis and blood coagulation system

Hemostasis is derived from Greek words "Heme", meaning blood and "stasis", meaning to stop. Hemostasis thus means arrest of blood flow. It is a complex physiological process that maintains the circulating blood in fluid phase by maintaining a sensitive balance between thrombogenic and anti-thrombogenic mechanisms in the body (De Caterina et al., 2013; Khazaei et al., 2008). The cardiovascular system maintains this balance by various components of the vascular wall and blood as follows:

- Promotion of anti-thrombotic state is associated with a normal endothelial cell layer lining all luminal surfaces of the vascular system.

- Promotion of pro-thrombotic state are events that are associated with vascular damage i.e. the improper production of antithrombotic factors by the endothelial cells or the physical removal or injury of endothelial cells, which permits the thrombogenic factors resting beneath the endothelium to come in contact with blood. The activation of platelets by any of the ligands that bind to platelet receptors also promotes a pro-thrombotic state.
-

Any disturbance in this equilibrium may cause excessive bleeding or thromboembolism (Palta et al., 2014). Hemostasis is maintained by a sequential occurrence of following sequential steps: 1) Vasoconstriction, 2) Increased tissue pressure, 3) Platelet plug formation in case of capillary bleeding and 4) Clot formation.

The thrombus propagation is controlled by certain inhibitors or factors that ensure the fluid nature of blood. This delicate balance is disturbed whenever the pro-coagulant activity of the coagulation factors is increased, or the activity of naturally occurring inhibitors is decreased (Previtali et al., 2011). Some of the thrombogenic and anti-thrombogenic components are listed in Table 1.4 below (Palta et al., 2014).

Table 1.4 Thrombogenic and anti-thrombogenic components of blood coagulation system

Site	Thrombogenic	Antithrombogenic
Vessel wall	Exposed endothelium	Heparin
	TF	Thrombomodulin
	Collagen	Tissue plasminogen activator
Circulating elements	Platelets	Antithrombin
	Platelet activating factor	Protein C and S
	Clotting factor	Plasminogen
	Prothrombin	
	Fibrinogen	
	vWF	

vWF – Von Willebrand factor; TF – Tissue factor

1.1.7.1 Blood coagulation factors

Blood coagulation is a complex physiological process by which blood forms clots. Blood clots contain a meshwork of fibers running in different directions and capturing plasma, blood cells and platelets. The fibrin fibers enwrap the accumulated and aggregated platelets, forming a hemostatic plug which adheres to the vascular opening and thus prevents further loss of blood. A series of factors participate in the coagulation cascade in an orchestrated manner. All the blood coagulation factors are enlisted in Table 1.5 below (Palta et al., 2014).

Table 1.5 Blood coagulation factors

Coagulation Factor	Name
I	Fibrinogen
Ia (a=activated)	Fibrin
II	Prothrombin
IIa	Thrombin
III	Thromboplastin
IV	Calcium
V	Proaccelerin
VI	Accelerin
VII	Proconvertin
VIII	Antihemophilic factor A
IX	Antihemophilic factor B
X	Stuart-Prower factor
XI	Antihemophilic factor C
XII	Hageman factor
XIII	Fibrinase

Blood coagulation occurs in three essential steps:

I) Coagulation begins with the formation of the prothrombin activator:

Prothrombin activator is formed in two ways - the extrinsic and intrinsic pathways - which constantly interact with each other to ultimately form a blood clot.

The extrinsic pathway: It initiates with a strain to the vascular wall and its surrounding tissues. This pathway gets activated as blood comes in contact with material from impaired cell membranes. When tissue suffers from trauma the Tissue factor (Factor III/ Tissue Thromboplastin) is exposed. The Tissue factor is a crucial membrane protein having one trans-membrane protein domain. It is expressed at low levels in endothelial cells but is expressed at much higher levels in the sub-endothelial cells (e.g., smooth muscles). When a vessel wall is damaged, the tissue factor from the sub-endothelial cells comes in contact with the plasma proteins. Tissue factor, being a trans-membrane protein, it is unlikely to be released into circulation unless there is a considerable tissue damage.

The extrinsic pathway is a very rapid process, i.e., it occurs within 12 to 15 seconds. The production of thrombin during the extrinsic pathway however is little and the subsequent clot formed is small. Tissue Thromboplastin activates Factor VII which is a calcium dependent step. In turn Factor VII activates Factor X (Prothrombin Activator) in a calcium dependent manner.

The intrinsic pathway: It becomes activated as the blood gets in contact with collagen or a negatively charged surface (glass test tube). A cell adhesion protein called the von Willebrand Factor (vWF) is produced by the endothelial cells. It helps in adherence of the endothelial cells to collagen in the basement membrane.

A sequence of different plasma proteins called blood-clotting factors play major roles in both extrinsic and intrinsic pathways as seen below in Figure 1.4. Most of these factors are zymogens of proteolytic enzymes. Upon conversion to their active forms, their enzymatic actions cause the successive, cascading reactions of the coagulation process. When the endothelial layer is injured, collagen and vWF from the sub endothelial layer become exposed to the bloodstream.

The intrinsic pathway is considerably slower than the extrinsic pathway. Yet, it produces larger yields of thrombin, thus enabling the larger clot formation.

i. Factor XII gets activated upon contacting the exposed collagen lying within the endothe lium of the ruptured vessel. Factor XII activates Factor XI.
ii. Factor IX gets activated by Factors XII and XI (contact activation product) jointly.
iii. Factor VIII gets activated by Factor IX
iv. Factor X (also known as the Prothrombin activator) gets activated by a combination of Factor VIII, calcium ions and Factor III from platelets (Platelet Thromboplastin).

The completion of the intrinsic pathway largely depends on the presence of activated platelets as the Factor III is released from activated platelets.

Activated Factor X participates in a series of reactions with Factor V, calcium ions and phospholipids derived from platelets. This amalgamation of clotting factors and their reactions is referred to as the Factor V Complex or prothrombin activator

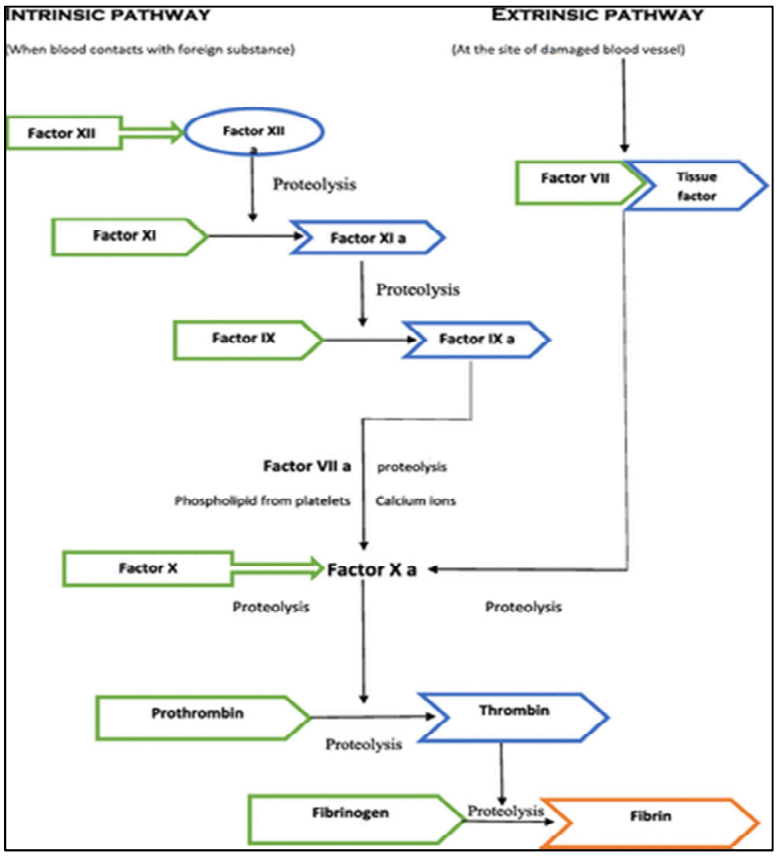

Figure 1.4 Intrinsic and extrinsic pathway associated with the blood clotting process

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II) Conversion of prothrombin to thrombin:

The prothrombin activator in the presence of sufficient Ca^{2+} causes the conversion of prothrombin to thrombin. Platelets also play an important role.

III) Thrombin acts on fibrinogen to form fibrin monomers which then polymerize:

In the early stages of coagulation fibrin monomers are held together by weak non-covalent hydrogen bonds. They are not cross-linked with each other. But as the process continues fibrin-stabilizing factor (produced by the entrapped platelets), helps in the formation of covalent bonds between monomers and hence cross-linkage is established. This process gives strength to the fibrin meshwork.

1.1.7.2 Thrombosis and platelet aggregation

Thrombosis is a condition wherein excess coagulation occurs, leading to formation of intravascular clots (Spronk et al., 2003). It is the principal pathology of the three main cardiovascular disorders namely, ischemic heart disease, stroke and venous thromboembolism (Raskob, 2014). There are two main kinds of blood clots: Venous Thromboembolism (VTE) wherein the blood clot forms within a vein and Arterial Thrombosis wherein a blood clot forms within an artery. In Deep vein thrombosis (DVT), a blood clot forms within a deep vein of the body. A fragment of a newly formed clot may sometimes travel through the blood stream and lodge itself in a smaller blood vessel, completely blocking the same. This condition is called embolism. When a clot lodges in the lungs, it is called pulmonary embolism (PE). Arterial thrombosis is usually associated with atherosclerosis which narrows down the arteries. In case a blockage occurs in the artery leading to the heart or brain, it causes heart attack or a stroke respectively. Most of the intravascular clots occur after a major surgery, trauma, immobilization, in cancer patients or women using birth control or estrogen treatment.

Fibrin is an essential component of the blood coagulation system and hemostasis; being the primary product of the coagulation cascade and also the ultimate substrate for fibrinolysis (Chapin and Hajjar, 2015).

Platelets are anucleate cell fragments present in the blood that play a predominant role in managing vascular integrity, thrombus formation, hemostasis and wound healing (Li et al., 2010). The α and dense granules of platelets secrete molecules like GPIIb , IIIa, fibrinogen, vWF, catecholamines, serotonin, calcium, ATP, ADP, etc., which are involved in aggregation (Ghoshal and Bhattacharyya, 2014). Platelet aggregation is the initial step in hemostasis. Platelet receptors, blood cells and blood coagulation factors act in an orchestrated manner with the vascular endothelium to initiate the tethering of platelets over a vascular injury to promote formation of a platelet plug (Rivera et al., 2009).

1.1.7.3 The von Willebrand factor

The von Willebrand factor (vWF) is a large multimeric glycoprotein produced in the endothelium and megakaryocytes (the precursors of platelets) and present in the blood plasma (Stockschlaeder et al., 2014). It is one of the first factors that contribute to inter platelet response heterogeneity during the phases of thrombus formation. Each of its many monomers has many domains. The domains are arranged in the order: D1-D2-D′-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK, with the D1-D2 domains representing the propeptide and the rest corresponding to the mature vWF subunit (Lenting et al., 2015).

- A1 domain, binds to platelet GP Ib-receptor and collagen.
- A3 domain, binds to collagen.
- C1 domain, within which the RGD domain binds to platelet integrin $\alpha_{IIb}\beta_3$ when this is activated.
- D'/D3 domain, which binds to Factor VIII. This prevents Factor VIII degradation.

The vWF plays a central role in hemostasis by forming a bridge between collagen and platelets. It complexes to collagen present in the vessel wall and mediates platelet adhesion to damaged vascular subendothelium via the receptors on platelet surface, viz., GPIb, IX and V and the activated GPIIb/IIIa complex (Kehrel et al., 1998; Peyvandi et al., 2011).This binding facilitates platelet aggregation and activation at the site of vascular injury and carries the coagulation factor VIII in circulation (Lenting et al., 2015).

1.1.8 Mechanism of platelet aggregation

Platelets form majority of the blood cells in the circulation and are the primary responders at the site of vascular injury. Platelet adhesion to the extra cellular matrix is the initial step in primary hemostasis. Upon occurrence of a vascular injury, the collagen and vWF of the sub endothelial layer get exposed. The circulating platelets adhere and aggregate at the site of injury to form a platelet plug and eventually a thrombus to stop blood flow (Jennings, 2009). A number of pathways lead to platelet activation including the ones stimulated by collagen, adenosine diphosphate (ADP), thromboxane A2, epinephrine, serotonin and thrombin. The cumulative action of all these activators leads to recruitment of the circulating platelets, which ultimately results in platelet activation. Some of these include change in platelet morphology, expression of P-selectin and soluble CD40 ligand (sCD40L) which are pro-inflammatory molecules, expression of procoagulant activity of platelets and conversion of GPIIb/IIIa (αIIβ3-integrin) to its active form, which allows platelet aggregation and the potential for pathologic thrombosis. A stable hemostatic plug is formed as a result of recruitment of the circulating platelets by accumulation of these agonists. Thrombin-mediated generation of fibrin from fibrinogen also helps in consolidation of the hemostatic plug.

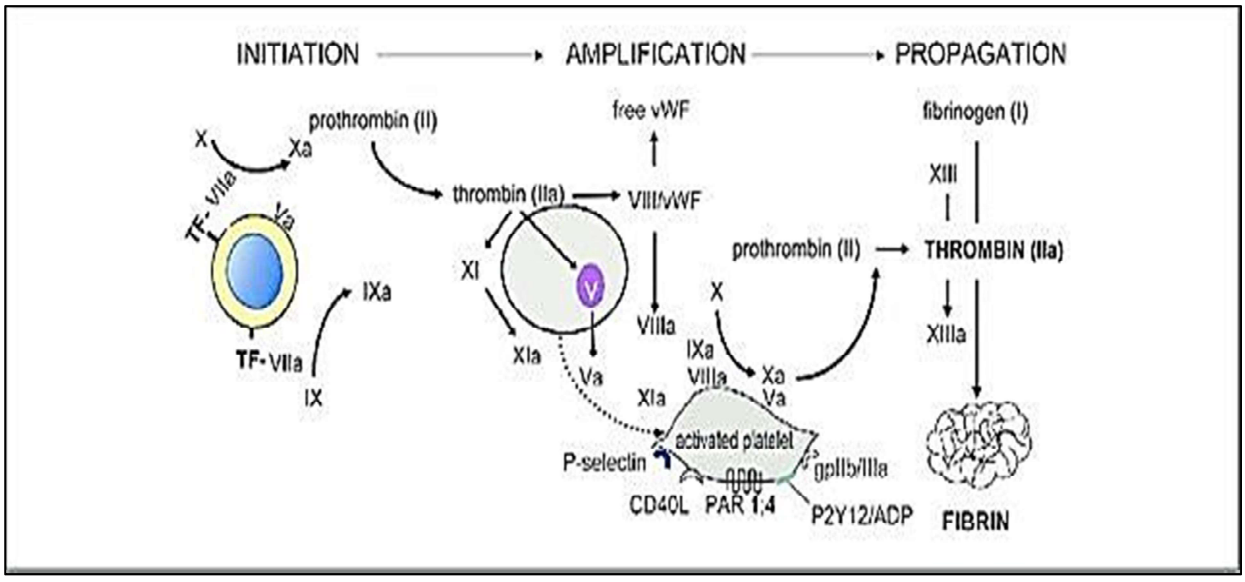

Figure 1.5 A scheme of current concepts on the coagulation process

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Platelet Receptors and activation pathways

Platelet aggregation involves a number of receptors. These receptors are the primary initiators of platelet activation at the site of vascular injury. Some platelet aggregation receptors are discussed in brief along with their function below:

1.1.8.1 Platelet ADP receptors:

The agonist ADP plays an essential role in normal physiological hemostasis and thrombosis. It is well established that ADP activates platelets through 3 purinergic receptors, namely $P2Y_1$ (Gq coupled) , $P2Y_{12}$ (Gi-coupled) and P2X1(Murugappan and Kunapuli, 2006). Detailed studies of the $P2Y_{12}$ receptor has resulted in the development of thrombotic drugs like clopidogrel and ticlopidine which are being successfully used clinically (Li et al., 2010; Murugappan and Kunapuli, 2006).

1.1.8.2 Platelet collagen receptors

Platelets express several collagen receptors of which integrin α2β1 is pivotal for platelets adhesion to collagen and GPVI (Li et al., 2010). Collagen interacts with vWF via receptors GPIb and integrin αIIb3. GPVI, the major receptor for collagen on platelets, mediates cellular activation and is essential for effective platelet adhesion, aggregation, degranulation and also coagulant activity on the matrix protein. The first platelet collagen receptor, integrin α2b1 (GPIa/IIa, VLA-2, or CD49b/ CD29) serves primarily as an adhesion receptor (Savage et al., 1999). These are also the targets for development of anti-thrombotic drugs (Surin et al., 2008). The complex GPIb-V-IX, acts via vWF as bridging molecule to mediate platelet-collagen interaction at high shear rates (Clemetson and Clemetson, 2001).

1.1.8.3 Platelet thrombin receptors

Thrombin plays a pivotal role in the coagulation cascade by being one of the most potent platelet activators. Platelets respond to thrombin via surface G protein-coupled receptors (GPCRs) known as thrombin receptors or Protease activated receptors (PARs). Thrombin directed cleavage of PAR-1 causes the activation of heterotrimeric G proteins of the Gα12/13, Gαq and Gαi/z families that further connect a number of intracellular signaling pathways to the various phenotypic effects on platelets by thrombin. Additionally, activated

PAR-1 stimulate platelet procoagulant activity, resulting in enhanced thrombin production and conversion of fibrinogen to fibrin (Angiolillo et al., 2010; Vretenbrant et al., 2007).

1.1.8.4 Integrins

The ubiquitous transmembrane receptors, the integrin, bridge the cell-matrix and cell-cell interactions. Thus one of their prime functions is to couple the ECM present outside cells, to the cytoskeleton inside the cell. Other functions of integrins include tissue migration during embryogenesis, cellular adhesion, cancer metastases and lymphocyte helper and killer cell functions. Integrin receptors are obligate heterodimers, composed of two non-covalently linked α and β subunits (Bennett, 2005; Chen et al., 2016).

Platelets express three β1 integrins, i) $\alpha_2\beta_1$ (collagen receptor), ii) $\alpha_5\beta_1$ (fibronectin receptor) and iii) $\alpha_6\beta_1$ (laminin receptor) as well as two β3-integrins, $\alpha_2\beta_3$ and $\alpha_v\beta_3$, the latter only being present in very low amounts. The most prevalent glycoprotein on the platelet surface is the integrin $\alpha_2\beta_3$ (GPIIb/IIIa). It has about 60,000 to 80,000 copies present per platelet and an additional intracellular pool that gets exposed on the surface up on activation (Perutelli et al., 1992). The affinity of $\alpha_2\beta_3$ for fibrinogen, fibronectin and vWF is highly variable and once activated, it mediates platelet adhesion, aggregation and spreading on the exposed ECM of the injured vessel wall and thrombus formation (Chen et al., 2015; Nieswandt et al., 2007). Upon binding to its ligands, $\alpha_2\beta_3$ induces spreading and clot retraction and contributes to the procoagulant activity of the platelets. There are other integrins like $\alpha_v\beta_3$, $\alpha_v\beta_1$ and $\alpha_6\beta_1$, apart from the major integrin $\alpha_2\beta_3$ and $\alpha_2\beta_1$, which are expressed on the platelet surface. They bind to different proteins in the ECM; i) Vitronectin is the ideal ligand of $\alpha_v\beta_3$ which can, however, also bind to fibronectin and osteopontin; ii) $\alpha_v\beta_1$ serves as a receptor for fibronectin and iii) $\alpha_6\beta_1$ is the major laminin receptor on platelets. Despite being established adhesion receptors, their role in platelet attachment has not been studied in detail. Laminin-integrin $\alpha_6\beta_1$ interactions stimulate platelet spreading through GPVI. Integrins may thus mediate platelet responses and their role in adhesion, activation and spreading is determined by the nature of the exposed ECM, which may vary between individual regions of the body and the type and severity of injury (Avraamides et al., 2008; Humphries, 2006; Lowell and Mayadas, 2011).

1.1.9 Need for novel anticoagulants

Deep vein thrombosis (DVT) and pulmonary embolism (PE) are leading causes of death worldwide. Approximately, 10 million humans are diagnosed of DVT and PE annually (Goldhaber and Bounameaux, 2012).

Together, DVT and PE are referred to as venous thromboembolism (VTE), the third largest cardiovascular killer worldwide after heart attack and stroke. The pathogenesis of venous thrombosis is complex and deregulation of coagulation is the main cause of deep vein thrombosis (Groot et al., 2005). Fibrinolytic and thrombolytic therapy is the primary treatment to cure DVT (Karim, 1994). Anticoagulants prevent thrombosis by inhibiting one or many steps in the coagulation cascade. However, present day anticoagulants have a serious drawback of unanticipated bleeding leading to hemorrhage or trauma (Ansell, 2016). Presently, anticoagulant drugs like warfarin (Coumadin) and heparin and anti-platelet drugs like aspirin are being used for its treatment and there is an increasing demand for safer and more efficient drugs. In the recent years newer anticoagulants have been introduced like dabigatran, rivaroxaban, apixaban and edoxaban which overcome some of the limitations associated with warfarin and heparin therapy. However, these cannot satisfy the global need for anticoagulants due to their renal dependence for elimination, absence of a specific reversal agent and non-uniform dose regimen to name a few (Schulman, 2013). Arterial thrombosis on the other hand is treated by administering medication such as alteplase or by surgery like coronary angioplasty.

Warfarin has been in use over 60 years to treat VTE. It is highly effective, but, owing to certain limitations such as excessive bleeding and need for constant monitoring makes it imperative to search for an ideal anticoagulant drug with much less side effects and high efficacy. This study hence focussed on searching for a novel anticoagulant from the marine environment.

1.1.10 Need for novel wound healing agents

Skin is the largest organ of the body and is constantly exposed to various stress conditions. In response to dermal injuries, the epidermis initiates a wound healing response in response to skin damage (Choi et al., 2015). Wound healing is a complex mechanism which comprises of

following steps: hemostasis, inflammation, proliferation and remodeling. All these four steps must occur sequentially and within a proper time frame for the wound to heal successfully (Guo and DiPietro, 2010). Perturbation/intrusion in any of these steps results in impaired wound healing, which pose major health complications such as predispositions to infections, long term morbidity, inflammation, necrosis, ulcers and quickened mortality (Choi et al., 2015; Han and Ceilley, 2017). Sea stars being model organisms for regenerative studies, its coelomic fluid was also assessed for its wound healing activities *in vitro* during the course of this study.

1.2 Gaps in existing research and scope of this study

India has a coastline of about 7500 km, yet the potential of Indian marine bioresource remains largely unexplored (Malve, 2016). More than 33% of the commercial drugs are of natural origin, majorly from terrestrial resources. Despite the vastness of the oceans, very few drugs of marine origin are in clinical trial. Most of them are for treatment of cancer, pain and infectious diseases (Minh et al., 2005). By exploring more untapped geographical sources, the search for newer and more effective drugs to treat a spectrum of diseases can be maximized. It is imperative that the nations possessing these biological resources benefit from the potential economic revenues associated with these findings (Leal et al., 2012).

The major limitation of working with marine resources is their poor accessibility, storage and maintenance. This leaves marine chemical ecology several decades behind its terrestrial counterpart.

In the year 1999, marine resources contributed to a larger percentage of bioactive natural products than those provided by the terrestrial counterparts. Nevertheless, there is still a great population of potential organisms to be explored, particularly marine invertebrates (Harvey, 2008; Leal et al., 2012). Of all the marine invertebrates, echinoderms are widely prevalent but only few species from this phylum such as sea-urchins and sea-cucumbers have been fairly studied. However, the potential of most sea stars species is still unexplored. Sea stars and their coelomic fluid is a rich source of secondary metabolites having anti-cancer, antimicrobial, anti-helminthic activities (Datta et al., 2015b). However, the anti-coagulant and throm-

bolytic potential of coelomic fluid has not yet been studied. Sea stars are also model organisms for regenerative studies due to their immense regenerative potential. However, no wound healing agent has been isolated from them till date. Thrombosis and thromboembolic disorders are leading causes of mortality worldwide and the existing thrombolytic drugs are associated with side effects like toxicity, unanticipated bleeding, hemorrhage and non-specific site of action. There is a growing need for novel thrombolytic drugs from natural sources which could overcome these problems.

1.3 Aims and objectives of this study

In view of the aforementioned lacunae in existing research, this study was aimed at finding the thrombolytic and wound healing potential of sea star *Astropecten indicus* by proposing the following objectives:

1. To study sea star (*Astropecten indicus*) coelomic fluid (SCF) for its hemotoxic activity.
2. To study SCF for its cytotoxic activity.
3. Purification and characterization of the active compound(s).

2 Materials and Methods

For any scientific experiment, it is imperative that the results be consistent. This chapter of my thesis covers in-depth details of procurement of all material, protocols and the molecular biology methods of experiments performed in this study.

2.1 Procurement of sample and extraction of coelomic fluid

Sea stars *Astropecten indicus* are commonly found along the coast of South-East Asian countries. In our study, the animals were collected from the Arabian Sea at Goa, India. Sample collection for the entire duration of the thesis was scheduled in the months of September to November (post monsoon season) every year, especially during low tides, when thousands of sea stars get washed along the sea-shore. Live sea stars were collected in tanks containing sea-water and they were transported to our laboratory. The sea stars were stored in our laboratory in trays where sea-water was replenished at regular time intervals. Sea stars have a water vascular system. The coelomic fluid of sea stars bathes all the internal organs and comprises of secondary metabolites, signal peptides, secretory molecules, peptides and many more biomolecules (da Silva Laires, 2012a). In this study, we partially characterized the crude coelomic fluid of *Astropecten indicus* and a novel protein isolated from it. The sea stars were released back into the sea after extraction of coelomic fluid from them. It is noted that no animals were sacrificed for this study.

The coelomic fluid was extracted from the sea stars by syringe aspiration. Care was taken to only extract maximum 500µl of Coelomic fluid from each animal to avoid harming the animals. The extracted coelomic fluid from each sea-star was pooled and centrifuged at 10,000 rpm for 15 min. at 4˚C, to remove cell debris and other contaminants from sea water. The supernatant was pooled and subject to ultra-filtration using a 10 kDa membrane. Permeate and retentate were collected separately and stored at 4ºC overnight. The retentate was dialysed against milliQ water for 2h followed by dialysis against 50mM Potassium Phosphate Buffer, pH 7.4 (KPB) at 4˚C overnight with buffer changes after 3h, 5h and 10h. The dialysed fluid was concentrated against sucrose and stored in aliquots of 10ml in -20ºC until further use. This processed fluid was referred to as SCF.

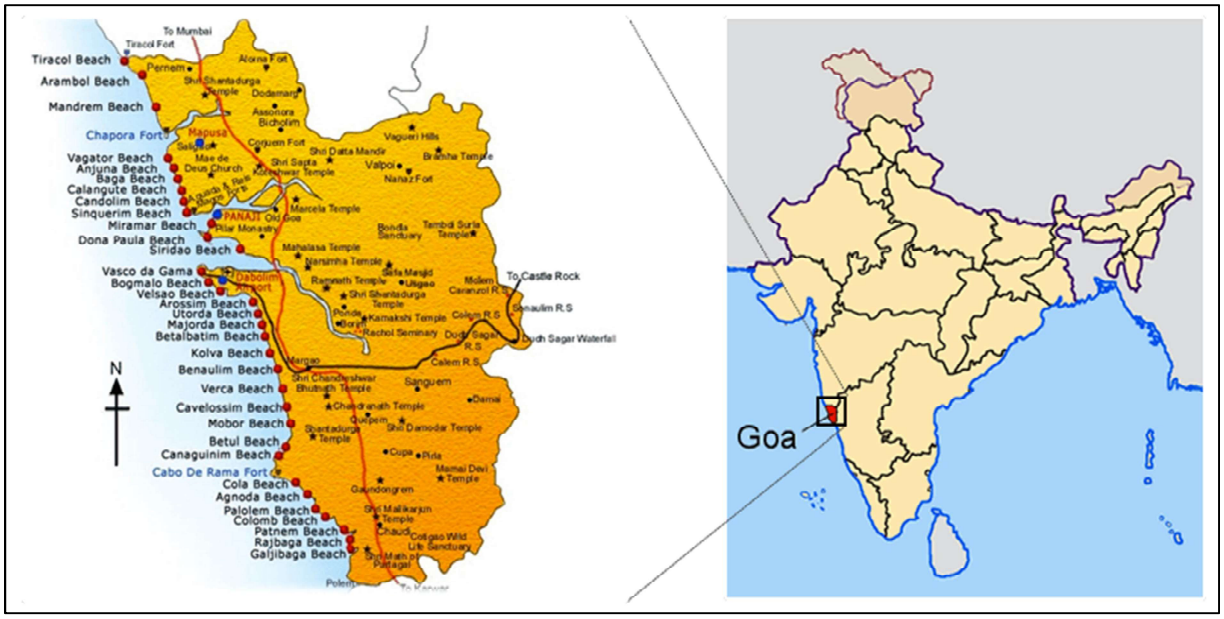


Figure 2.1 Site for sample collection of *Astropecten indicus*

(Image Source: open source image link URL: http://www.aljanh.net/goa-india-wallpapers/985866310.html)

Figure 2.2 Collection of *Astropecten indicus* from Velsao beach in Goa, India

Sea stars were collected in abundance from the sea shore of Goa in tanks containing sea water.

Figure 2.3 *Astropecten indicus* collected for extraction of SCF

2.2 Reagents and chemicals

All Chemicals for biochemical assay were of analytical grade and purchased from Sigma Aldrich, USA. Protein molecular weight markers were purchased from GeneI, Bangalore, India. Reagents for platelet aggregation studies were purchased from Wheecon Instruments Pvt. Ltd., Chennai, India. Reagents for cell culture were purchased from Hi-Media and Sigma Aldrich, USA. Plastic wares were purchased from Tarsons, India and Corning, USA.

2.3 Instruments

Spectrophotometric assays were performed using a UV-1800 240V spectrophotometer purchased from Shimadzu Corporation, Japan. The Fractional collector for chromatography (Frac-920) was purchased from GE Life sciences, Bangalore, India. The High Performance Liquid Chromatography (HPLC) system UFLC LC 20AD Prominence system was purchased from Shimadzu. The Microkin SDS-PAGE apparatus was purchased from Tech-Resource, India. Whole blood platelet aggregometer was purchased from Wheecon instruments Pvt. Ltd, India. ELISA plate reader for 96 wells plate was purchased from Thermo Scientific, India.

Various chromatography columns as well as resins were used for fractionation of SCF. Carboxy methyl (CM) Sephadex C-50 and Diethyl amino ethyl (DEAE) Sephadex A-25 and A-50 were purchased from Sigma Aldrich, USA. Pre-packed ion exchange columns of Sepharose XL and Q-Sepharose were purchased from GE Life sciences, Bangalore, India. For HPLC, size exclusion BioSep SEC s-2000 (300 x 7.8mm) and Yarra 3µm columns were purchased from Phenomenex, India.

2.4 Methods

2.4.1 Selection of buffer system

The choice of buffer system and its pH were chosen on the basis of optimal fibrinogenolytic activity observed with the protein fractions in various buffers. The crude SCF was found to be fibrinogenolytically active at pH of 6.0 to 9.0. Potassium phosphate buffer (KPB), being one of the most common buffer system for protein studies, was chosen as the buffer system for all extractions and experimentations in this study. A buffer concentration of 50mM at pH 7.4 showed optimal activity.

2.4.2 Estimation of protein concentration using Bradford's Assay

Protein content in SCF was estimated using Bradford protein assay (Bradford, 1976) The protein profile of SCF was studied by running SCF in a 12% SDS-PAGE followed by coomassie brilliant blue R-250 (CBB-R250) staining or silver staining.

2.4.3 Estimation of protein purity using SDS-PAGE

The protein purity of SCF and protein samples eluted after chromatographies were assessed by electrophoresis using 12% SDS-PAGE followed by visualization of bands using CBB R-250 and silver staining technique.

2.4.4 Silver staining of polyacrylamide gels

The entire silver staining procedure was carried out on a gel rocker. The electrophoresed gel was removed from the SDS-PAGE apparatus and washed in a container of milliQ water to remove excess SDS. The washing was carried out for 30 mins. Washing was followed by fixing the gel in 50% ethanol and 10% glacial acetic acid for 10 min. The gel was then rinsed in 50 percent

ethanol for 5 min. Rinsing step was followed by sensitizing the gel in 0.02% sodium thiosulphate solution. The gel was washed for 2 min. in milliQ water and cold staining solution (0.1% silver nitrate) was poured onto it. The gel was kept immersed in staining solution for 20 min. The excess stain was removed for washing the gel two times in milliQ water for 1 min each. Bands were visualized by addition of the developing solution (2% sodium carbonate, 0.004% formaldehyde and 0.04% sensitizer) into the gel. Developer was changed every 5 min. until protein bands were visible on the gel. Finally, the developer was washed and the gel was stored in stop solution (1% acetic acid) until documentation of result.

2.4.5 Estimation of effect on platelet aggregation using whole blood platelet aggregometer

SCF was studied for its effects on ADP and collagen induced platelet aggregation using Chronolog whole blood platelet aggregometer. Human blood (9 ml) was freshly collected from 'O' positive, healthy volunteers with prior and informed consent in 3.8% sodium citrate (1 ml) under expert medical supervision. All studies were carried out using the whole blood platelet aggregometer. Blood was incubated with 0.85% saline (1:1 dilution) in a cuvette for 5min at 37°C. Platelet aggregation agonists ADP (10µM) or collagen (2µg/ml) added to the above mixture served as positive controls. As a platelet agonist is added to the mixture, platelets aggregate as a monolayer on the electrical probes immersed in the blood samples kept in the cuvettes. Accumulation of platelets offers a resistance (or impedance) to the electrical circuit which is quantified. Change in impedance is expressed as a function of time. Protein fraction(s) incubated with blood, saline and agonist mixture served as test samples. Blood incubated with saline served as negative control. Positive control showed maximum resistance. Negative controls were devoid of any agonists for aggregation and hence offered negligible resistance (Chanda et al., 2013).

2.4.6 Visualization of platelet aggregation using Scanning Electron Microscopy (SEM)

Blood was withdrawn with prior consent from physiologically healthy individuals under medical supervision in vials containing 3.8% sodium citrate. Platelet rich plasma (PRP) was prepared by the method of differential centrifugation (Dhurat and Sukesh, 2014). The PRP was washed overnight in alcohol and was incubated with 5µg and 20µg SCF. Each sample was fixed with 2% glutaraldehyde for 1h, rinsed with cacodylate buffer solution and fixed for 1h with 0.1% osmium tetraoxide. The samples were serially dehydrated using 30, 50, 70, 90 and 100% ethanol solu-

tions for 10 min each. Critical drying of the samples was performed followed by analysis of the sample using SEM (Rodella et al., 2011).

2.4.7 Assay of fibrinogenolytic activity

Human fibrinogen was suspended in potassium phosphate buffer (KPB), pH 7.4 and different doses of SCF were incubated with 2 mg/ml of it for 5h at 37ºC. Fibrinogenolytic activity was assessed by running the pre-incubated samples on a 12% SDS-PAGE. Fibrinogenolytic activity was confirmed by disappearance of one or more chains of fibrinogen observed by coomassie staining of the gel followed by de-staining. Fibrinogen (30µg) yielded 3 protein bands viz. Aα, Bβ and γ when run on a 12% SDS-PAGE. SCF upon incubation with fibrinogen led to the sequential degradation of Aα, Bβ and γ bands in a dose and time dependent manner (Chanda et al., 2016a). Protein bands were visualized by staining the gel with CBB R-250.

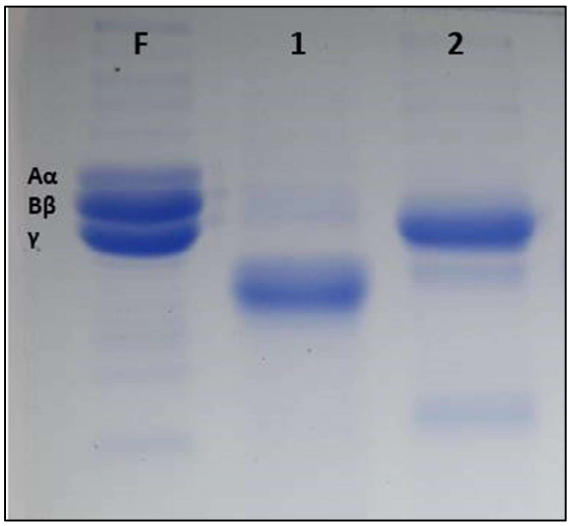


Figure 2.4 Estimation of fibrinogenolytic activity using SDS-PAGE

Lane F is fibrinogen incubated with saline (negative control); Lane 1 is fibrinogen incubated with a strong fibrinogenolytic agent; Lane 2 is fibrinogen incubated with a mild fibrinogenolytic agent.

2.4.8 Inhibition of fibrinogenolytic activity

SCF was treated with various protein inhibitors such as ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF) and dithiothreitol (DTT) for 2h at 37ºC and then assayed for fibrinogenolytic activity using zymogram assay as well as by SDS-PAGE. This determined the nature of protease present in the test compound.

2.4.9 An assay of fibrinolysis by a novel spectrophotometric real time method

A novel method for estimation of fibrinolytic activity was developed in our laboratory.

The method is based on monitoring the change in absorbance of light in the visible spectrum while passing through a medium undergoing gradual change in density. Fibrinogen starts to polymerize by enzymatic action of thrombin and forms semi-solid fibrin clots. Therefore, absorbance of light passing through a mixture of fibrinogen and thrombin will gradually increase as polymerization of fibrin progresses. Absorbance will stabilize, as polymerization is completed. Similarly, absorbance will gradually decrease in presence of a fibrin digesting (and thereby liquefying) factor in the fibrinogen-thrombin mixture and stabilize finally after complete digestion of the fibrin clot. Therefore, it can be said that the gradual rise in absorbance signifies clot formation and fall in absorbance represents clot dissolution. The concentrations of thrombin and fibrinogen were chosen in such a way, that formation of fibrin clot is moderately rapid. This facilitates monitoring of the process at an appropriate pace. The absorbance curves obtained may be used for real-time monitoring of clot formation and digestion. Parallel assays without a fibrinolytic material may be used as negative control.

Fibrin clots were formed in glass cuvettes by adding 6.5µl of bovine thrombin (1mg/ml) to 1 ml of bovine fibrinogen (2mg/ml). Ten micro litres of BPB (6mg/ml) was added to the above solution. The above mixture served as the reaction mixture in all tests. Compounds with known anticoagulant activity and suspected thrombolytic action from various sources were incubated separately with the above reaction mixture and the formation of clot and its digestion was observed by measuring gradual rise in absorbance at 619nm.

A fibrinolytically inactive compound such as Bovine serum albumin or heat inactivated urokinase was added to the reaction mixture and the time dependent changes in absorbance was monitored at 619nm. A gradual rise in absorbance was observed with progress in clot formation, which stabilized upon complete formation of the clot. The absorbance curve obtained served as the negative control in all experiments (Figure 2.5).

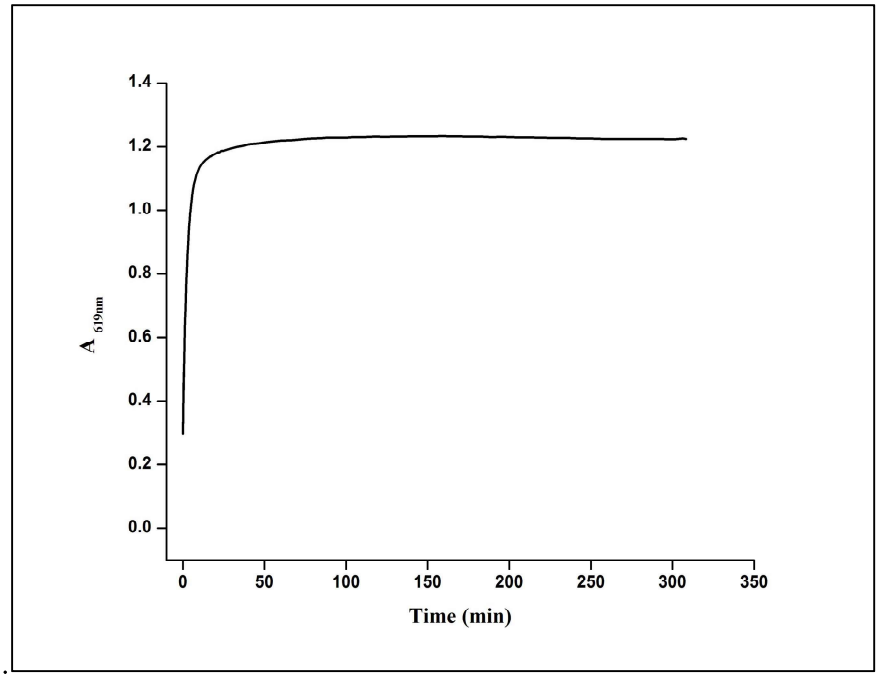


Figure 2.5 Absorbance versus Time plot for heat inactivated urokinase (negative control)

Absorbance values corresponding to progress in clot formation upon addition of inactivated urokinase to the reaction mixture. The increase in absorbance represents the process of fibrin clot formation. The plateau phase represents stabilization of clot formation.

Addition of a fibrinolytic factor to the reaction mixture resulted in clot formation followed by dissolution of the clot which is marked by gradual decrease in absorbance at 619nm (Figure 2.6). The absorbance at various times of the assay were subtracted from the maximum absorbance (due to clot formation) and plotted against time, showing clot digestion by the fibrinolytic factor used (Figure 2.7 and Figure 2.8). This is carried out separately for different fibrinolytic compounds to determine their fibrinolytic potency in a dose and time dependent manner. Urokinase was used as a standard fibrinolytic factor for the assay.

All results were expressed as % clot digestion by the fibrinolytic factor. This was calculated by subtracting the individual absorbance values (y_n) at different time intervals from the maximum absorbance (y_{max}) in the absorbance versus time plot as (y_{max}-y_n) and calculating the percent change by using peak absorbance as the reference. i.e. [(y_{max}-y_n)/y_{max}]*100.Y_{max} represents the maximum absorbance attained at the time of clot formation and y_n represents the absorbance at time interval 'n', taken as the subscript of y (e.g. y_{20} represents the absorbance at 20^{th}min.). This gave the percent clot digestion at time interval n (represented as y_n). Similar values were calcu-

lated at different time intervals for (y_1, y2…..y_n) and plotted against time. Any time interval can be chosen as per the investigator's choice for plotting the % clot digestion curve

There is an initial increase in absorbance indicating the formation of a solid fibrin clot followed by a significant decrease in absorbance due to enzymatic activity of urokinase which lyses the clot. Urokinase takes some time to exhibit its activity as a tissue plasminogen activator (t-PA), during the course of which the solid clot forms marked by rise in absorbance. Digestion of fibrin clot starts with formation of plasmin, causing a dip in absorbance which causes flattening of the curve after maximum clot dissolution. Percent clot digestion was calculated using the formula:

% clot digestion = $[(y_{max} - y_{n)} / y_{max}]*100$

Where y_{max} indicates the maximum absorbance, corresponding clot formation and y_n is the absorbance at n^{th} time interval.

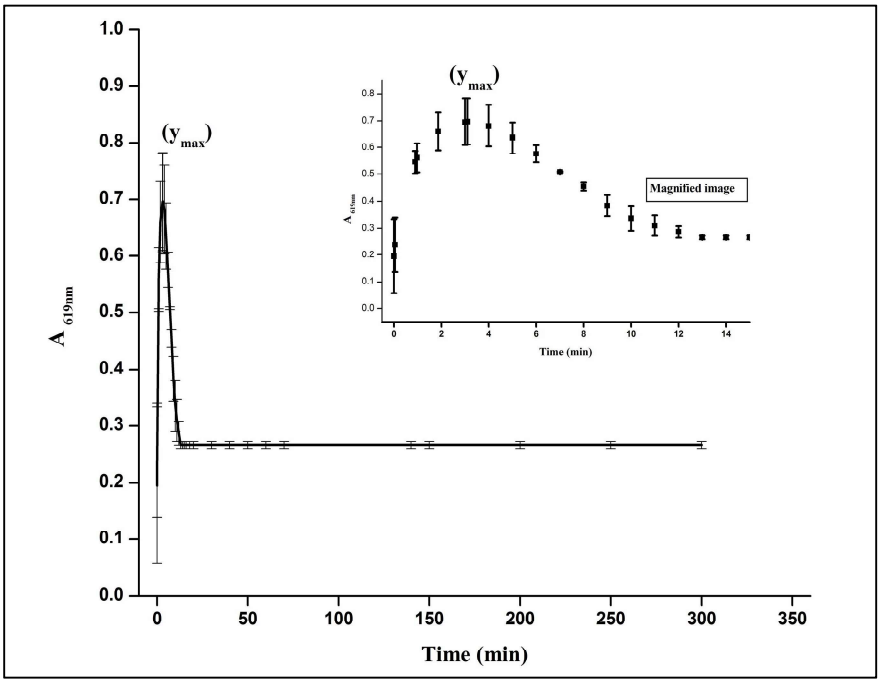


Figure 2.6 Absorbance versus time plot for fibrinolysis by urokinase

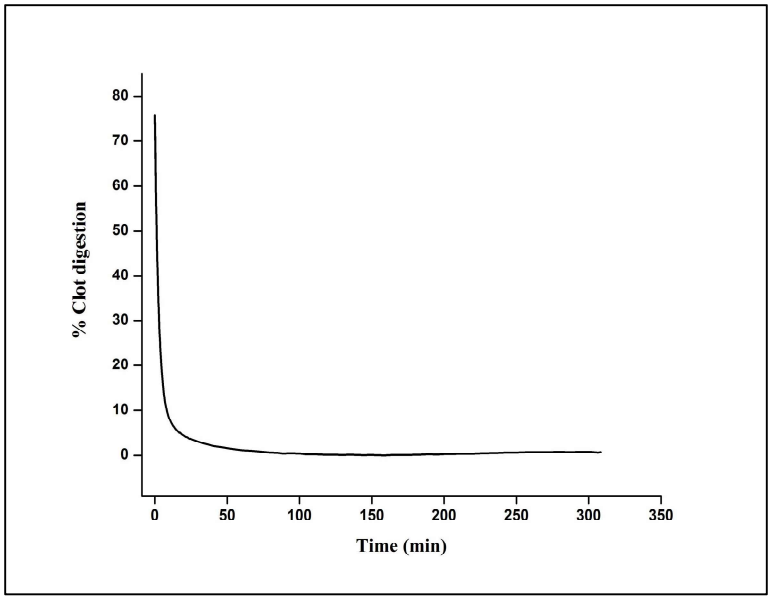


Figure 2.7 Clot digestion by heat inactivated urokinase (negative control)

Absorbance values at different time intervals were subtracted from the peak absorbance value as obtained in Figure 2.6 (corresponding to clot formation) and was plotted against time to yield percent clot digestion.

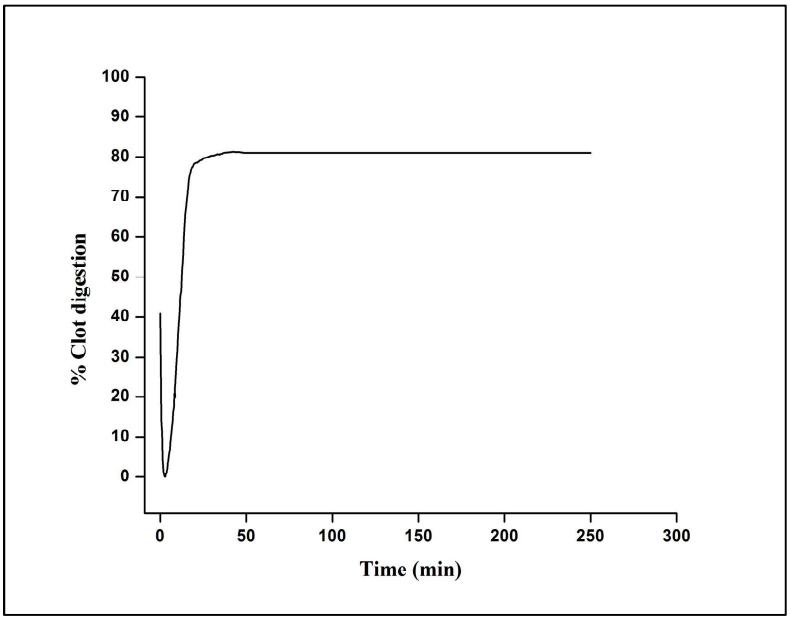


Figure 2.8 Percent Clot digestion by Urokinase

Percent clot digestion by urokinase plotted versus time. Urokinase causes maximum lysis of the clot within 20 min. after which the absorbance and hence digestion of clot becomes constant as seen in the plot.

2.4.10 Assay of proteolytic activity using substrate zymography

Zymogram protease assay was performed to estimate different kinds of protease activities (fibrinolytic, fibrinogenolytic and caseinolytic) of the protein components of SCF. The protein substrate (10mg) was co-polymerized with acrylamide during preparation of the polyacrylamide gel, followed by electrophoresis of SCF (4µg/ml) on the same gel using native page running conditions. The gel was washed with Triton-X (2 times) to remove the SDS and followed by incubation in an incubation buffer Tris $Cacl_2$, pH 7.4 at 37ºC for pre-determined time periods. Protease activity of SCF was observed by staining the gel with 1.25% coomassie brilliant blue, R-250 (CBB) followed by destaining with 10% acetic acid and 10% methanol in water. The entire gel was stained with CBB due to presence of protein substrate embedded in it. Proteolytic activity of proteases present in SCF were observed as regions of destained areas on the gel due to digestion of substrate by them (Leber and Balkwill, 1997; Masure et al., 1991).

2.4.11 Estimation of enzyme activity using Azocasein substrate

Non-specific proteolytic activity of SCF was estimated by using azocasein as substrate. Briefly, 125µl of suitable dilutions of SCF were incubated with 125µl of 0.25% (w/v) azocasein and incubated for 30 min. at 37ºC. The reaction was terminated by adding 500µl of 10% Trichloroacetic acid to the reaction mixture and vortexed. This was followed by centrifugation at 4000 rpm at 4ºC for 15 min. Five hundred microliters of the supernatant was neutralized by adding 500µl of 1.8N NaOH and absorbance was measured at 440 nm using a spectrophotometer. One unit of enzyme activity was defined as the amount of protease which yielded an increase in absorbance of 0.01 in 30 min. at 37ºC (Secades and Guijarro, 1999).

2.4.12 Assay of laminin digestion

Laminin (1mg/ml) was reconstituted in KPB. SCF and AiP1 isolated from SCF were incubated with 15µg laminin at 37ºC incubator for 5h. Laminin degradation activity was estimated by comparing the protein band pattern of untreated Laminin with protein bands of SCF or AiP1 treated Laminin using SDS-PAGE.

2.4.13 Molecular weight determination using SDS-PAGE

Molecular weight of the protein peaks obtained after HPLC was estimated by comparing the retention times of eluted protein peaks with that of protein standards using a graph.

2.4.14 Fractionation of protein using ion-exchange chromatography

The crude Coelomic Fluid (SCF) was fractionated using cation as well as anion exchange chromatography. Fractionation profile of SCF using cation exchange resins Carboxy Methyl (CM) Sephadex C-50 and CM Sepharose did not yield distinct peaks upon chromatography. Anion exchange chromatography was performed using diethyl-aminoethyl (DEAE) Sephadex A-50 and DEAE-Sephadex A-25 resins. Protein peaks eluted post DEAE A-25 based fractionation were distinct and this resin was thus selected for further fractionation of SCF. SCF (1mg/ml) was loaded on a 5ml column of DEAE-Sephadex A-25 resin. Mobile phase used was 50mM potassium phosphate buffer pH 7.4. Proteins were eluted using a linear gradient of sodium chloride (0 to 1M) and 1ml fractions were collected.

2.4.15 Fractionation and purification of protein using size exclusion HPLC

The protein peak obtained after ion-exchange chromatography was subjected to size exclusion chromatography using the BioSep SEC s2000 column. The mobile phase was 50mM potassium phosphate buffer, pH 7.4. One sharp protein peak was eluted which was studied further.

2.4.16 Assay of hemolytic activity

Hemolytic activity was assayed by incubating various doses of SCF with 1% human or goat erythrocyte suspension in 0.85% saline at 37ºC for 1h. Hemolysis was estimated by measuring absorbance at 540nm and comparing it with positive control (1% SDS) which showed 100% lysis; 0.85% saline served as negative control, (Chakrabarty et al., 2000).

2.4.17 Assay of phospholipase activity

Phospholipase activity was assayed using egg yolk as substrate. Egg yolk suspension (1ml) was taken in microfuge tubes and incubated with various dosages of SCF at 37˚C for 1h. The incubates were placed in boiling water bath and the time for coagulation to occur was noted. Egg yolk suspension incubated with saline and Russell's viper venom served as negative and positive controls, respectively.

2.4.18 Culture of animal cells *in vitro*

A549 cells (human lung carcinoma epithelial cells) and HaCaT cells (human epithelial keratinocytes) were procured by from National Centre for Cell Science (NCCS), Pune, India. HEK293 cells (Human Embryonic Kidney) stock was gifted by Dr. Indrani Talukdar, Dept. of Biological Sciences, BITS Pilani K K Birla Goa campus. The cells were cultured *in vitro* in T-25 flasks in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS (Fetal Bovine Serum) and 0.01% antibiotic and anti-mycotic solution. The effects of SCF and AiP1 on cultured cells were studied by observing changes in morphology of cells, change in viability of the cells after treatment with different doses of SCF or AiP1 using, MTT cytotoxicity assay (Amini et al., 2014; Pathan et al., 2017) and Resazurin reduction assay (Allshire et al., 2005; Pathan et al., 2015).

2.4.18.1 Culture of A549 cells *in vitro*

The human human lung carcinoma epithelial cells (A549 cells) were grown in $25cm^2$ culture flasks in DMEM supplemented with 10% FBS and 1x antibiotic and anti-mycotic solution at 37ºC in a humidified CO_2 incubator with 5% carbon dioxide (CO_2). Cells were passaged every third day upon reaching confluence.

2.4.18.2 Culture of HaCaT cells *in vitro*

The human keratinocyte (HaCaT) cells were grown in $25cm^2$ culture flasks in DMEM supplemented with 10% FBS and 1x antibiotic and anti-mycotic solution at 37ºC in a humidified CO_2 incubator with 5% carbon dioxide (CO_2). Cells were passaged every third day upon reaching confluence. HaCaT cells were seeded in 96 well plates for cytotoxicity studies and in 6 well plates for wound healing studies at a seeding density of $2X10^4$ cells/ml.

2.4.18.3 Culture of HEK293 cells *in vitro*

The human embryonic kidney (HEK293) cells were obtained as a generous gift from Dr. Indrani Talukdar, Dept. of Biological Sciences, BITS Pilani K K Birla Goa campus. The cells were revived in $25cm^2$ culture flasks in DMEM supplemented with 10% FBS and 1X antibiotic and anti-mycotic solution at 37ºC in a humidified CO_2 incubator. Cells were passaged every third or

fourth day upon reaching confluence. Cells were seeded for cytotoxicity studies and wound healing studies at a seeding density of 2 X 10^4 cells/ml.

2.4.19 Cell counting by Trypan blue exclusion assay

This assay relies on the ability of the dye to enter dead cells or cells with ruptured membranes. It is a direct indicator of cell death. Upon reaching confluence, cells were detached using trypsin and counted using a hemocytometer. Viable cells were counted and 2-3 X 10^4 cells/ml were seeded into culture plates for further experimentation (Baveja et al., 2018; Strober, 2015).

2.4.20 Estimation of cell cytotoxicity by MTT assay

The effect of SCF and AiP1 on cell viability was assessed by the MTT assay, which is an indicator of the metabolic activity of cells. A549, HaCaT and HEK293 cells were assessed separately for the effect of SCF and AiP1 on their viability. Cells were seeded into 96 well plates at a seeding density of 2*10^4 cells/ ml. Upon reaching confluence, the used media was decanted and fresh media containing varying doses of AiP1 were added to different wells of the 96 well plate and incubated for the desired time intervals in 37ºC humidified incubator. Once the incubation period was completed, the complete media was removed and 100µl of MTT (1mg/ml) was added to the cells and covered with a sterile aluminium foil and incubated at 37ºC for 3-4h until formazan crystals were formed. The formazan crystals were dissolved by adding DMSO and absorbance was measured at 550nm using a 96 well plate reader. Cell cytotoxicity was estimated at % loss in cell viability taking untreated cells as control (Berridge et al., 2005; Pathan et al., 2017; Patravale et al., 2012).

2.4.21 Estimation of cell viability by Resazurin reduction assay

This assay estimates the metabolic activity of the cells. Resazurin is converted to resorufin by the dehydrogenase enzymes present in the metabolically active cells (viable cells). The amount of resorufin formed is estimated spectrophotometrically using a microplate reader (Anoopkumar-Dukie et al., 2005; Pathan et al., 2017).

2.4.22 Estimation of wound healing activity using *in vitro* scratch assay

A549, HEK293 and HaCaT cells were cultured *in vitro* in T-25 flasks as described in section 2.15. Cells were seeded at 70% confluence in 6-well plates at a seeding density of 30,000 cells/ ml and were left overnight in sterile humidified conditions. Upon 60% cell confluence, the spent media was discarded from the wells followed by washing with phosphate buffer saline (PBS) to remove unattached cells. A longitudinal scratch was made in each well using a sterile 200µl pipette tip. The cells were again washed with PBS and 2ml of DMEM supplemented with 10% FBS and 0.01% antibiotic solution was added to each well of the culture plate. SCF or AiP1 at different doses was added to the well such that the final volume in each well was 2ml. Images were taken for each well at time intervals of 24h till 72h to observe effect of SCF on the wounds. Area of the wound was calculated using Image J software and wound healing activity of SCF and AiP1 was expressed as the % wound healed at given time period using initial wound area (0^{th} hour) as reference (Baveja et al., 2018).

2.4.23 Total RNA extraction from treated cells

Total RNA was isolated from HEK293 cells at intervals after 24h and 48h post treatment of cells with AiP1 (protein isolated from *Astropecten indicus*; described in chapter 4). Total RNA extraction was performed using TRIzol® LS Reagent (Invitrogen, USA) as per the instructions in the manufacturer's protocol. Briefly, 1ml of the TRIzol® LS reagent was added directly to the monolayer after removing the media and the cells were homogenized by repeated pipetting. The homogenate was then transferred to a new sterile vial and was incubated with chloroform for 10 min followed by centrifugation at 12000 rpm for 10 min. The separated aqueous layer was carefully transferred to a new vial and RNA was precipitated using isopropanol. The precipitated RNA was further washed with 70% ethanol and dissolved in sterile double distilled water followed by incubation at 60^0C for 3 min. After the incubation, total cellular RNA was quantified at 260 nm and 280nm using a nano-drop and then stored at -80^0C for *c*DNA synthesis.

2.4.24 Reverse transcription polymerase chain reaction (RT-PCR) for *c*DNA synthesis

RNA isolated from AiP1 treated cells was reverse transcribed and *c*DNA was synthesized using the *c*DNA synthesis kit for RT PCR (Tetro *c*DNA synthesis kit from Bioline, USA) as per instructions provided in the kit. Amplification of *c*DNA was performed using PCR using specific primers. The primers utilized for RT-PCR were as follows: *GAPDH* (forward: 5'AAG CCT

GCC GGT GAC TAA C-3', reverse: 5'-GCG CCC AAT ACG ACC AAA TC-3'); *E-cadherin* (forward: 5' GAT CCA TTT CTT GGT CTA CGC 3', reverse: 5' TGG TGA TTA TTA CGA CGT TAG CC 3'); *N- cadherin* (forward: 5'-CAA CTT GCC AGA AAA CTC CAG G-3', reverse: 5'-ATG AAA CCG GGC TAT CTG CTC-3'); Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (forward: 5'-AGA ACA TCA TCC CTG CCT CTA C-3', reverse: 5'-CTG TTG AAG TCA GAG GAG ACC A-3'). All PCR conditions were normalized to the *GAPDH* expression. The relative expression of E-cadherin and N-cadherin in untreated as well as in cells treated with AiP1 was measured via densitometry analysis (Choi et al., 2009; Pathan et al., 2017).

3 Effect of Sea star Coelomic Fluid on Blood Coagulation System and Cultured Cell Lines *in vitro*

3.1 Introduction

Oceans cover more than 70 percent of the surface of the earth's surface and have an enormous biomass (Montaser and Luesch, 2011; Newman and Cragg, 2014). Despite having an infinite scope for exploration, the marine environment has been one of the most under-studied biological resources. It contains a vast array of organisms with unique biological properties which could provide important clues to novel therapeutic tools (Cardoso et al., 2016; Vinothkumar and Parameswaran, 2013).

Research on marine derived therapeutics burgeoned in the last 15 years owing to the advances in technology, expertise and machinery making deep sea explorations possible and popular. Terrestrial animals, on the other hand, have been exploited for many decades as resources for the treatment of a range of human disorders and diseases (Costa-Neto, 2005; Montaser and Luesch, 2011). Marine derived natural products have yielded promising therapeutics with high specificity and low toxicity and are hence the current focus of scientific investigation to discover novel bioactive molecules (Bordbar et al., 2011).

Members of the class Echinodermata such as the sea stars (or starfish) have become symbolic of the sea life. They were used as a source of bioactive compounds in ancient oriental medicines and are now used for isolation and purification of novel cytotoxic, hemolytic, antiviral, antifungal, antifouling, antimicrobial and anti-tumoral compounds (Bordbar et al., 2011; Cardoso et al., 2016; Lee et al., 2013; Sharmin, 2017; Thao et al., 2014).

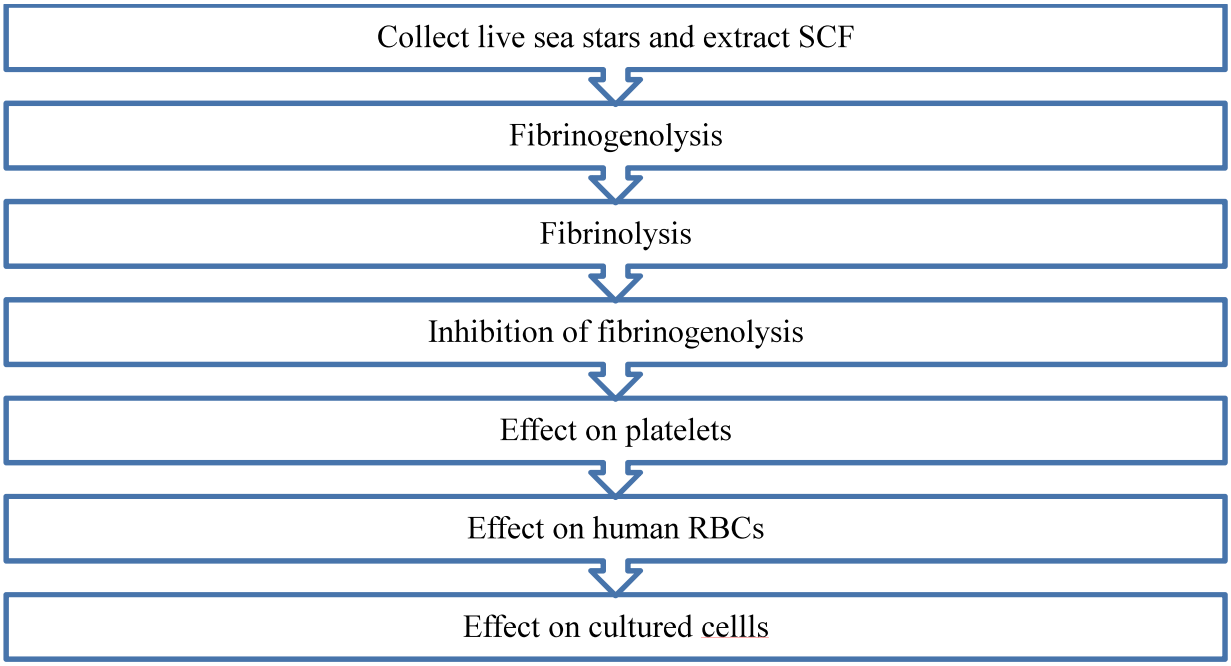
India has a coast line of more than 7,500 km and sea stars are abundantly available along the coast after the monsoon season. The most common sea stars found along the Indian coast are the sand stars *Astropecten indicus.* The pharmacological profiles of the coelomic fluid of *Astropecten sp.* are however not well studied and little effort has been made till now to dis-

cover potential drugs from sea stars (Dong, 2011; Prabhu, 2013). Anticoagulants and platelet aggregation inhibitors are in high demand due to heavy death toll by thromboembolic disorders in modern stressful life style (Engelmann and Massberg, 2013; Key et al., 2016; Lau, 2003).

Keeping in mind the abundant availability of *Astropecten sp*. and yet their limited investigation reports, this chapter of my thesis focussed on studying the coelomic fluid of *Astropecten indicus* for its effects on blood coagulation system and on cultured cell lines *in vitro*.

3.2 Materials and Methods

The following methodology was followed for characterization of SCF



3.2.1 Protein profile of SCF

Protein content in SCF was estimated using Bradford protein assay (Bradford, 1976). The protein profile of SCF was studied by running SCF in a 12% SDS-PAGE followed by silver staining as described in section 2.4.3.

3.2.2 Effects on platelet aggregation

Effects of SCF on ADP and collagen induced platelet aggregation was assessed using Chronolog whole blood platelet aggregometer as described in section 2.4.5. Effect of SCF on platelets was also assessed using SEM as described in section 2.4.6.

3.2.3 Assay of fibrinogenolytic and fibrinolytic activities

Proteolytic activities of SCF using fibrinogen, fibrin and casein substrates was assayed using SDS-PAGE and substrate zymography as described in sections 2.4.7, 2.4.9 and 2.4.10.

3.2.4 Estimation of proteolytic activity using Azocasein

Non-specific proteolytic activity of SCF was estimated by using azocasein as substrate as method described in section 2.4.11. One unit of enzyme activity was defined as the amount of protease which yielded an increase in absorbance of 0.01 in 30 min. at 37ºC (Secades and Guijarro, 1999).

3.2.5 Assay of hemolytic activity

Hemolytic activity was assayed by incubating varying doses of SCF with 1% human erythrocyte suspension as described in section 2.4.16. Hemolysis was estimated by measuring absorbance at 540nm and comparing it with positive control (1% SDS) which showed 100% lysis; 0.85% saline served as negative control.

3.2.6 Assay of laminin digestion

Laminin (20µg) was incubated with various dosages of SCF for 5h at 37˚C. Laminin digestion was observed as appearance/ disappearance of protein bands on 12% SDS-PAGE as compared to untreated Laminin which served as control (section 2.4.12).

3.2.7 Assay of Phospholipase activity

Phospholipase activity was assayed using egg yolk as substrate. Egg yolk suspension (1ml) was taken in microfuge tubes and incubated with various dosages of SCF at 37˚C for 1h. The incubates were placed in boiling water bath and the time for coagulation to occur was noted. Egg yolk suspension incubated with saline and Russell's viper venom served as negative and positive controls, respectively (section 2.4.17).

3.2.8 Effect on plasma recalcification time and thrombin time

Anticoagulant potential of SCF was estimated by monitoring the change in plasma recalcification time upon treatment with SCF. For this assay, 100µl of 50Mm Tris-HCl (pH 7.4), 100µl of platelet poor plasma (PPP) and various dosages of SCF were incubated at 37˚C for 30 min in glass vials. Clotting was initiated by the addition of 50µl of 50mM Calcium chloride and the time for plasma recalcification were monitored (Chanda et al., 2016).

3.2.9 Effect of SCF on cultured cell lines *in vitro*

A549, HaCaT and HEK293 cells were cultured *in vitro* in T-25 flasks in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS (Fetal Bovine Serum) and 0.01% antibiotic and anti-mycotic solution. The effects of SCF and AiP1 on cultured cells were studied by observing changes in morphology of cells, change in viability of the cells after treatment with different doses of SCF or AiP1 using, MTT cytotoxicity assay (Amini et al., 2014; Pathan et al., 2017) and Resazurin reduction assay (Allshire et al., 2005; Pathan et al., 2015).

3.3 Results

3.3.1 Protein profile of SCF

Protein profile of SCF was studied by electrophoresis of on a 12% polyacrylamide gel followed by silver staining the gel. Many protein bands were observed in crude SCF as seen in Figure 3.1.

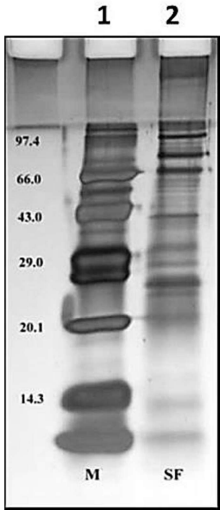


Figure 3.1 SDS-PAGE showing protein profile of SCF

Silver stained SDS-PAGE gel (12%) for visualizing proteins present in SCF. Lane 1: protein molecular weight marker; Lane 2: crude SCF. SCF was found to contain various proteins and peptides.

3.3.2 Fibrinogenolytic activity of SCF

Fibrinogenolytic activity of SCF was estimated by incubating different dosages with fibrinogen as substrate for varied time periods. It is noted that at low concentrations (4µg/ml), SCF could digest only A-α chain of human fibrinogen. However, at concentrations of 10µg/ml and higher, SCF could digest B-β and γ chains of fibrinogen as well. The fibrinogenolysis could be observed within 30 min. of incubation with the substrate indicating its prompt activity at lower dosages Figure 3.2 and Figure 3.3.

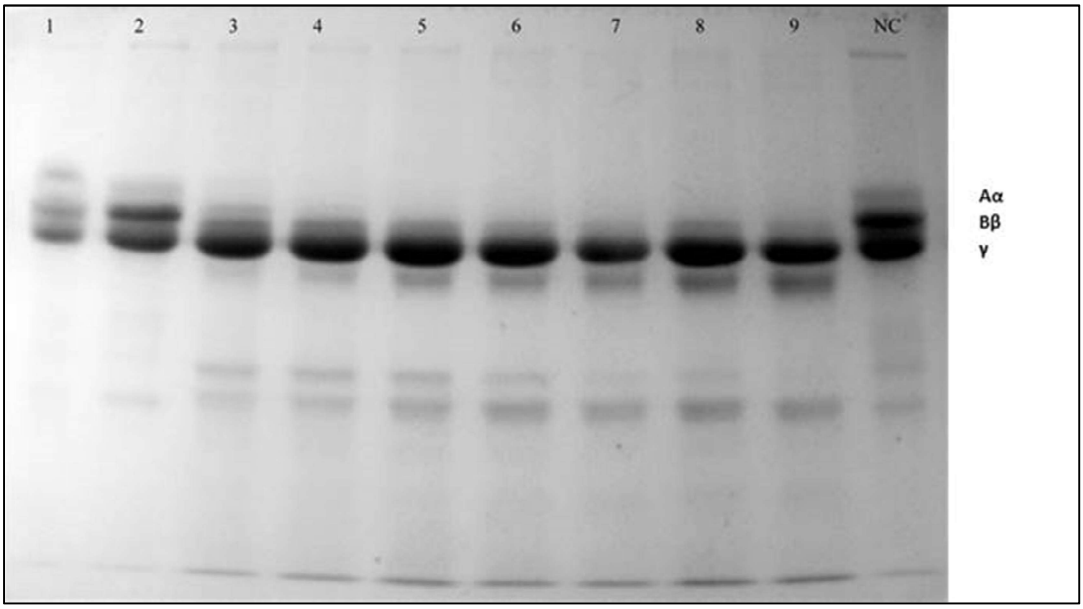


Figure 3.2 Dose dependent fibrinogenolysis by SCF

Varying dosages of SCF were incubated with fibrinogen as substrate. Numbers on each lane indicate the concentration of SCF (in µg/ml) incubated with 15 µl of 2mg/ml of fibrinogen at 37ºC for 5h. NC is negative control (fibrinogen incubated with KPB).

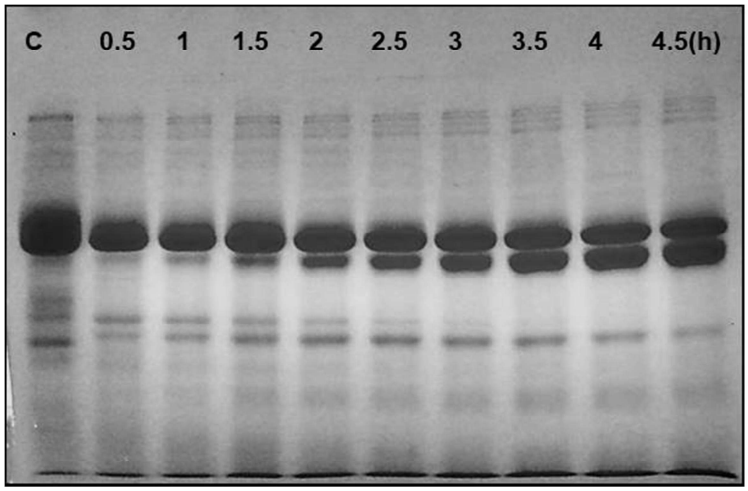


Figure 3.3 Time dependent fibrinogenolysis by SCF

SCF (4µg/ml) was incubated with 2mg/ml of fibrinogen for varied time periods to test the time required by 4µg/ml SCF to digest fibrinogen. Numbers on each lane indicate incubation time periods in hours. C: fibrinogen incubated with KPB.

3.3.3 Zymogram assays for protease activity

The substrates (fibrin, fibrinogen and casein) were co-polymerized separately with the polyacrylamide gel, followed by electrophoresis of SCF (4µg/ml). The gel was cut in 3 parts and incubated for 1, 2 and 5h in the incubation buffer at 37ºC. Active proteolytic proteins showed clear digestion of the substrate within 1h of incubation. SCF was confirmed to possess fibrinolytic, fibrinogenolytic and caseinolytic activities (Figure 3.4 a, b and c, respectively). Proteolytic activity was detected around 66 kDa and 45 kDa region of the gel as evident from the figures.

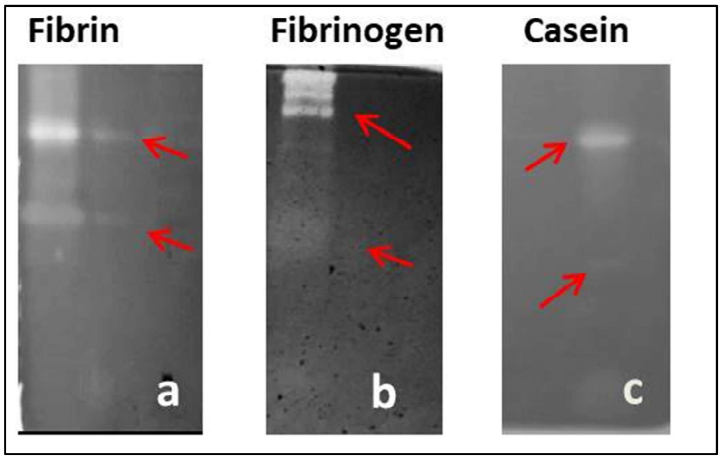


Figure 3.4 Proteolytic activity of SCF using different substrates assayed using Zymogram protease assay

Arrows in red indicate zones of digestion of protein substrates. a) Fibrinolytic activity of SCF; b) Fibrinogenolytic activity of SCF; c) Caseinolytic activity of SCF
Fibrinolytic activity of SCF (4µg/ml) was assayed by co-polymerizing fibrinogen and thrombin to yield fibrin in the acrylamide gel followed by incubation of the gel in incubation buffer for 1, 2 and 5h incubation period at 37ºC. Fibrinolytic activity was observed at all three incubation times, seen as digestion of the substrate. The figure shows fibrinolysis by SCF after 1h of incubation. SCF (4µg/ml) digested fibrinogen copolymerized with the acrylamide gel within 1h of incubation at 37˚C. SCF (4µg/ml) digested casein present in the polyacrylamide gel seen as destained region of the zymogram gel. Fibrinolytic activity was seen within 1h of incubation at 37ºC.

3.3.4 Inhibition of fibrinogenolytic activity

SCF was treated with various inhibitors such as EDTA, PMSF and DTT for 2h at 37ºC and then assayed for fibrinogenolytic activity using zymogram assay as well as by SDS-PAGE. EDTA was found to completely inhibit fibrinogenolytic activity by chelating the metal ions, indicating the fibrinogenolytically active protein components to be metalloproteinases (Figure 3.5).

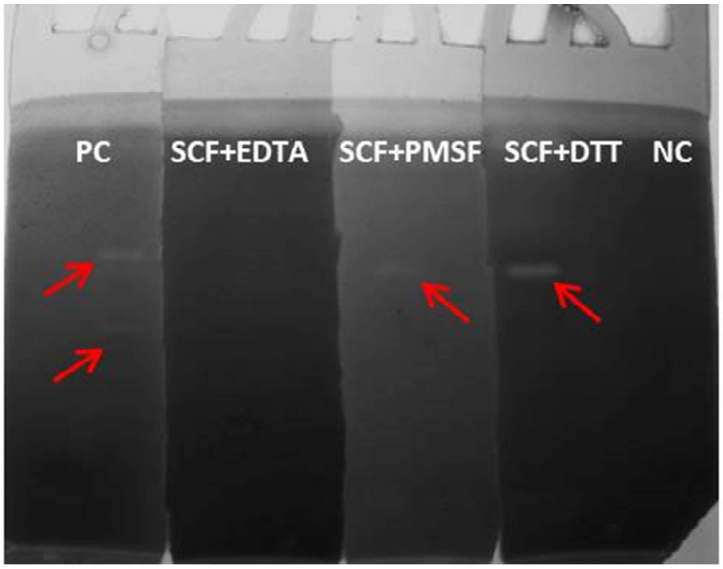


Figure 3.5 Inhibition of fibrinogenolytic activity of SCF using Zymogram protease assay. Arrows in red indicate zones of digestion of fibrinogen as substrate

SCF (4µg/ml) was treated with protease inhibitors PMSF, EDTA and DTT for 2h. and fibrinogenolytic activity of treated SCF was estimated post incubation. PC is positive control (untreated SCF), NC is negative control (20mM KPB). Fibrinogenolytic activity was inhibited upon incubation of SCF with EDTA.

3.3.5 Hemolytic activity of SCF

Hemolytic activity of SCF was studied on human RBCs. SCF showed hemolytic activity below 5% on human RBCs at all dosages (Figure 3.6). Low hemolytic effects of SCF on human RBCs make it a safe candidate for drug discovery and development.

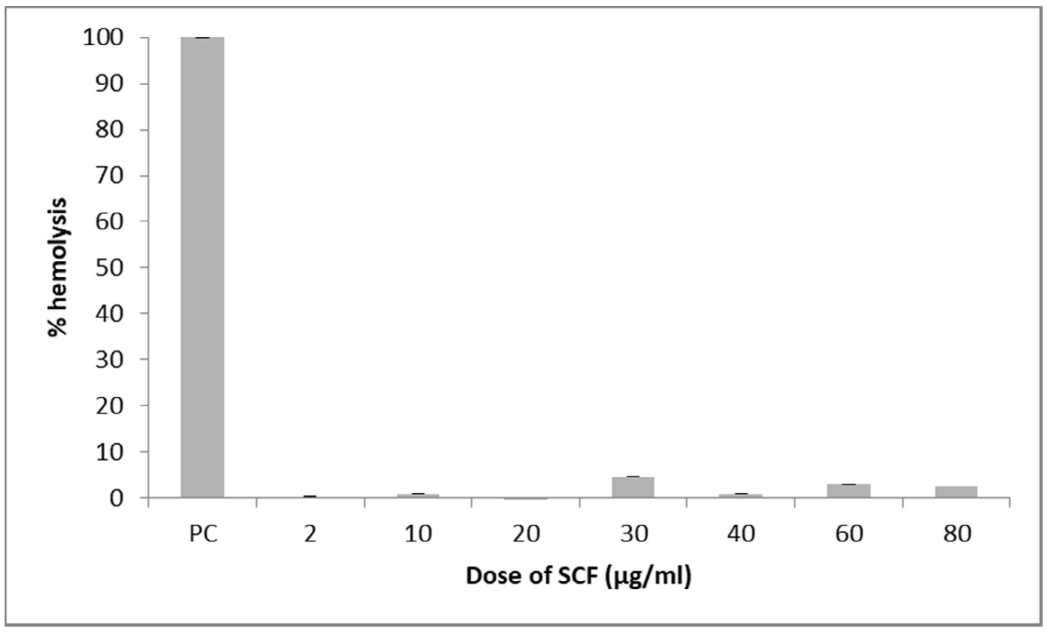


Figure 3.6 Hemolytic activity of SCF on Human RBCs

Hemolytic activity of various dosages of SCF were tested on human RBCs in vitro.
PC: 1% SDS; NC: 0.85% saline. SCF showed negligible hemolysis of human erythrocytes. (n=3 replicates).

3.3.6 Effect of SCF on ADP and collagen induced platelet aggregation

SCF was found to promote ADP induced aggregation of platelets in a dose dependent manner. SCF could not initiate the activation of platelets themselves. However, they could further aggregate the ADP induced platelets in a dose dependent manner. It is noted that SCF inhibited ADP induced platelet aggregation at doses below 10µg/ml and caused aggregation at higher doses (Figure 3.7). Similar effect was seen in case of collagen induced platelet aggregation in which aggregation of platelets was observed at SCF concentrations 2µg/ml and higher (Figure 3.8).

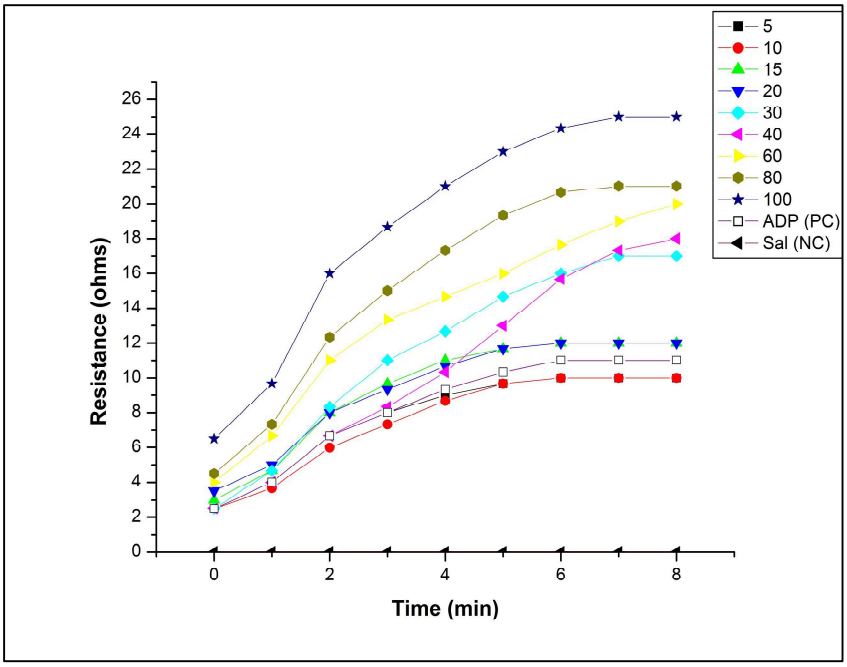


Figure 3.7 Effect of SCF on ADP induced platelet aggregation

Blood was treated with different dosages of SCF and platelet aggregation was induced by ADP and measured using whole blood platelet aggregometer. PC= Blood incubated with saline and ADP; NC= Blood incubated with saline only (no agonist added). SCF inhibited platelet aggregation till a dosage of 10µg/ml and promoted aggregation at higher dosages in a dose dependent manner. (n=3 replicates).

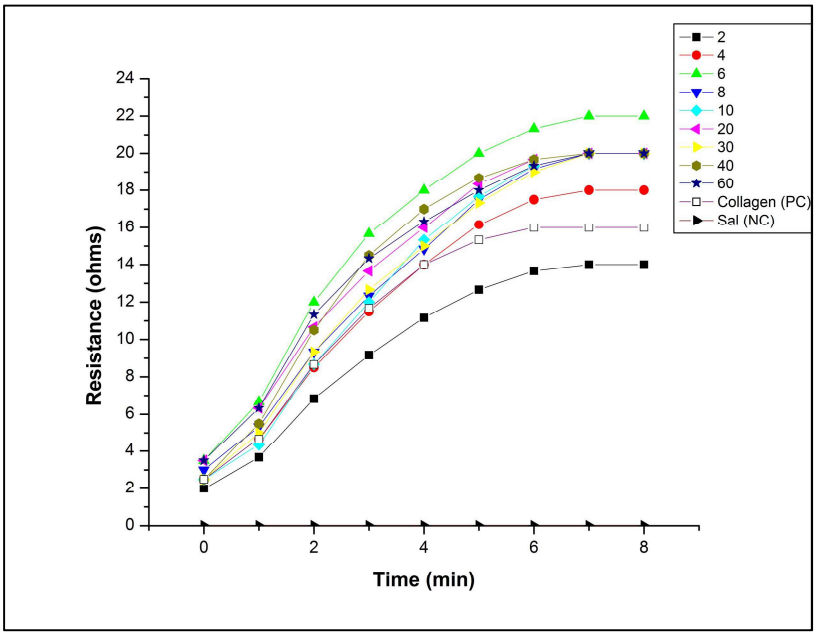


Figure 3.8 Effect of SCF on collagen induced platelet aggregation

Blood was treated with different dosages of SCF and platelet aggregation was induced by collagen. PC: Blood incubated with saline and collagen; NC: blood incubated with saline only (no

agonist added). SCF promoted collagen induced platelet aggregation in a dose dependent manner starting from 2µg/ml. (n=3 replicates).

3.3.7 SEM analysis of SCF treated platelets

The effect of SCF on platelets was observed using scanning electron microscopy. The platelets aggregated in a dose dependent manner as are visible in Figure 3.9.

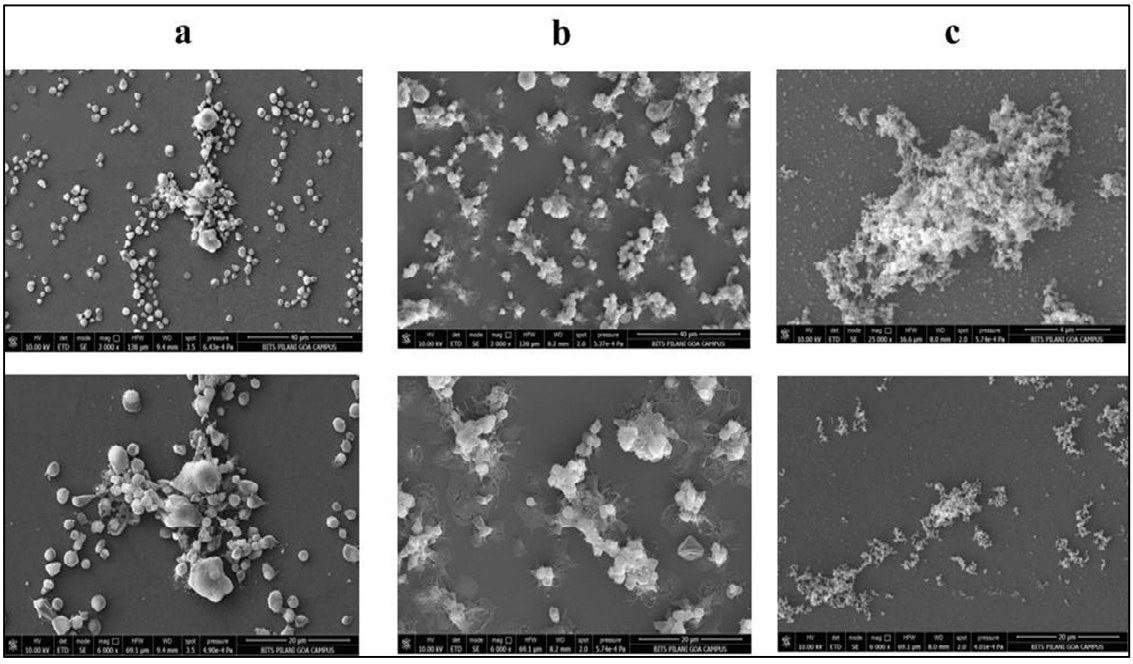


Figure 3.9 Effect of SCF on platelets as seen under SEM

Lane a: untreated platelets extracted from PRP; Lane b: platelets treated with 5µg of SCF and Lane c: platelets incubated with 20µg SCF.
SCF clearly promoted aggregation of platelets as seen under SEM.

3.3.8 Effect of SCF on laminin digestion

SCF was assayed for its effect on Laminin digestion. No digestion was observed in Laminin pre-incubated with SCF as seen in Figure 3.10.

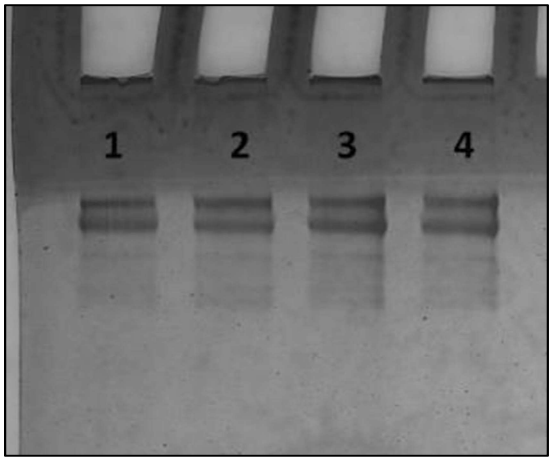


Figure 3.10 Effect of SCF on laminin digestion

Lane 1: laminin (control); Lane 2 to 4: laminin incubated with 5,10 and 20µg of SCF respectively.

3.3.9 Effect of SCF on Phospholipase activity

The effect of SCF on membrane phospholipids was assayed using egg yolk as substrate (described in section 2.5.14). SCF had no effect on phospholipid just like negative control (0.85% saline).

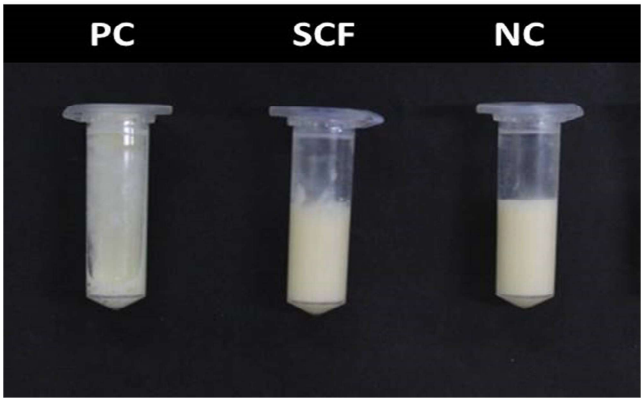


Figure 3.11 Effect of SCF on phospholipase A

SCF had no effect on phospholipids. Positive control (PC) is Russell's Viper venom; Negative control (NC) is 0.85% saline.

3.3.10 Effect of SCF on plasma recalcification time

Time taken for plasma recalcification was estimated by incubating various doses of SCF with platelet poor plasma (PPP) followed by adding calcium chloride to initiate clot formation. Plasma recalcification time prolonged in SCF.

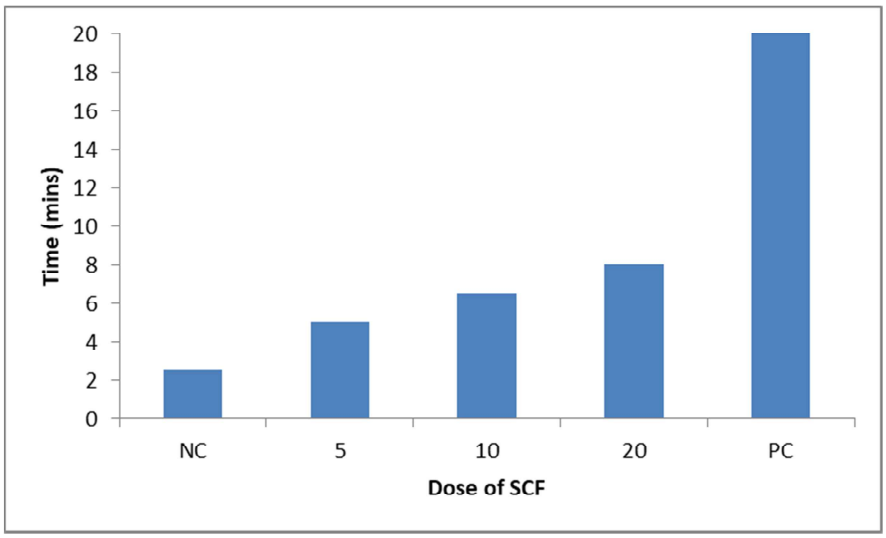


Figure 3.12 Estimation of effect of SCF on plasma recalcification time

Platelet poor plasma incubated with saline served as negative control NC; PPP incubated with Russell's viper venom served as positive control PC.

3.3.11 Effects of SCF on morphology of cultured cells *in vitro*

A549 cells were seeded *in vitro* in 6 well plates at a density of 30,000 cells/ml and were left overnight in 2 ml of DMEM supplemented with 10% FBS and antibiotic and antimycotic solution at 37˚C in a humidified 5% CO_2 environment. Different dosages of SCF were added to each well, and subsequently morphology of the cells was observed after every 24h up to 72h post treatment. There was no significant change in morphology of the cells at less than 10µg/ml dosage of SCF (Figure 3.13).

3.3.12 Effects of SCF on viability of cells *in vitro*

Effects of SCF on cell viability and proliferation was observed in a dose and time dependent manner. Cell treated with SCF did not cause decrease in viability (Figure 3.14). On the other hand, significant increase in cell proliferation was observed in treated cells as compared to untreated cells.

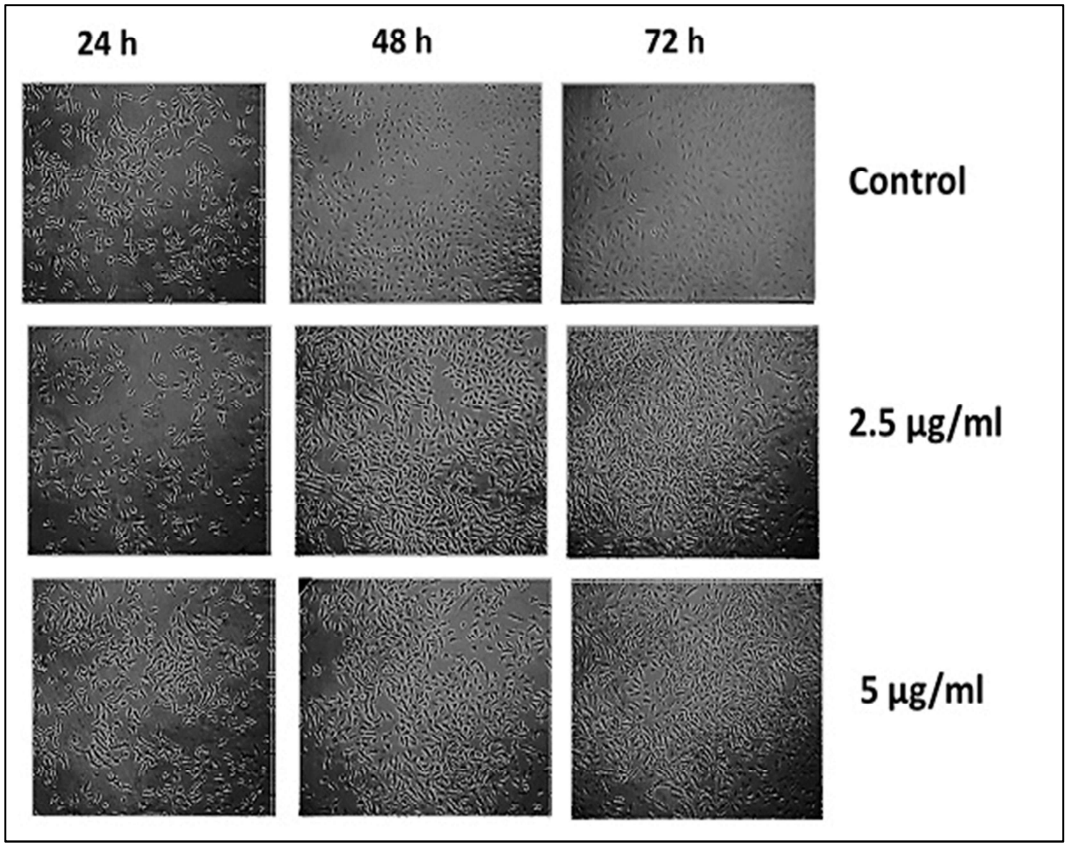


Figure 3.13 Effect of SCF on morphology of A549 cells *in vitro*

Cell morphology of A549 cell was observed after treating them with SCF for 24, 48 and 72h. Untreated cells served as control. SCF treatment did not cause any significant change in the morphology of cells.

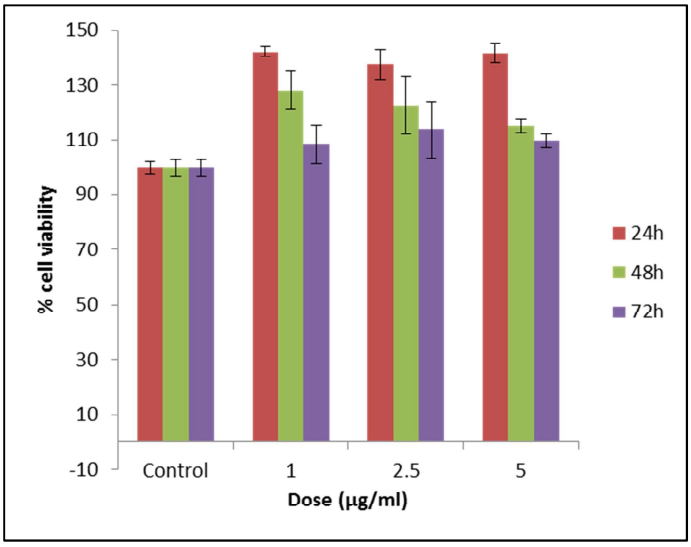


Figure 3.14 Cytotoxicity of SCF on A549 cells using MTT assay

Effect of SCF on cell viability was assessed for for 24, 48 and 72h. Untreated cells served as control (regarded as 100% viable cells).

Resazurin reduction assay was used to estimate increase in metabolic activity of the cells post treatment with various dosages of SCF (Figure 3.15). It was found that SCF treated cells were more viable as compared to control (untreated cells). This was estimated by conversion of resazurin to resorufin by metabolically active cells and quantification of resorufin formation by measuring absorbance at 610nm. Untreated cells were considered as 100% viable.

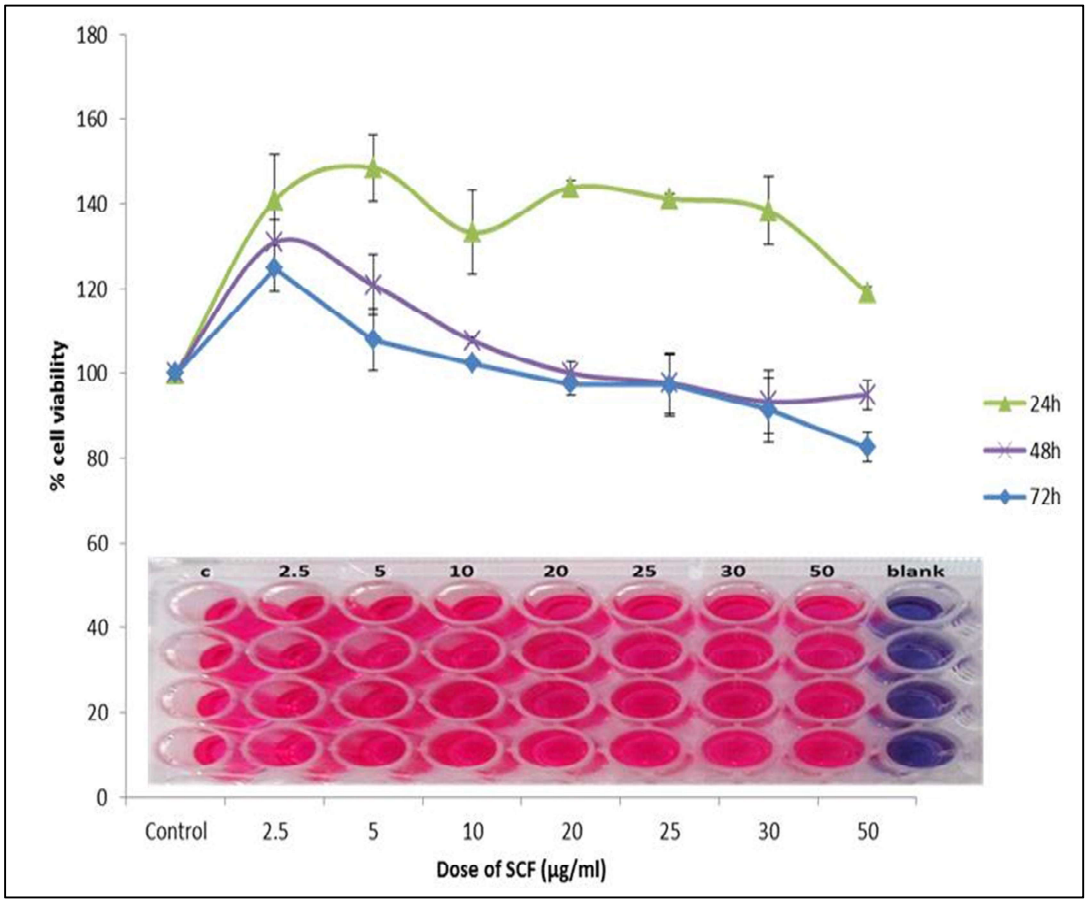


Figure 3.15 Estimation of cell viability using Resazurin reduction assay on A549 cells post treatment with various dosages of SCF.

Dose dependent effect of SCF on viability of A549 cells using Resazurin reduction assay. Live and viable cells reduce Resazurin to resorufin (blue to pink). There was no loss in viability of cells post treatment with SCF indicating that SCF does not affect the metabolic activity of cells. Numbers on the wells indicate dose of SCF in µg/ml. C: control (untreated cells considered as 100% viable). Blank: wells containing resazurin and DMEM only (no cells).

3.3.13 *In vitro* scratch assay to study effect of SCF on wound closure

Scratch wounds were created on A549 cells cultured in 6 well plates *in vitro*. SCF was added to the wells to assess its effects on wound closure. It was observed that wounds healed faster in SCF treated cells as compared to untreated cells which served as control as seen in Figure 3.16.

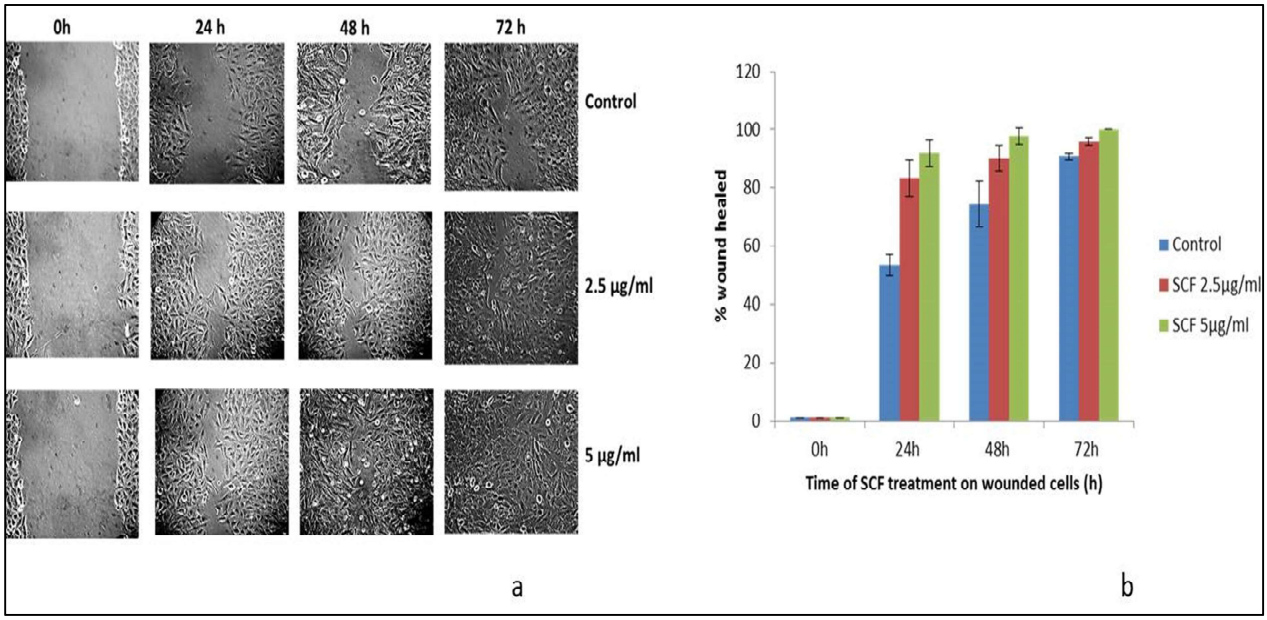


Figure 3.16 Effect of SCF on scratch wounds *in vitro*

a. Effect of SCF on wounds created on A549 cells. Wounds healed faster in SCF treated cells as compared to untreated cells (control). **b.** Wound healing ability of SCF was quantified as the % area of the wound healed by different dosages of SCF using ImageJ software. Wounded cell layer treated without any treatment served as control.

3.4 Discussion

Sea stars, like other invertebrates have diverse bioactivities owing to their secondary metabolites (Ferguson, 1964**;** Schillaci and Arizza, 2013). Whole body extracts of the sea star *Astropecten indicus* has been previously reported for their antimicrobial potential (Chamundeeswari, 2012). However, their coelomic fluid has not been studied for hemotoxic and anticoagulant activities. The coelomic fluid of sea stars bathes all the internal organs and is thus a reservoir of immunocytes, secondary metabolites, cytokines, growth factors, hormones, proteins and peptides involved in cell signaling.

Coelomic fluid from *Astropecten indicus* was studied for its effects on the blood coagulation system *in vitro*. It was found to digest human fibrinogen (30µg) at a concentration of 4µg/ml within 30 min. of incubation with the substrate. Other proteolytic activities of SCF such as fibrinolytic, fibrinogenolytic and caseinolytic activities were also observed at this concentration (Figure 3.4). Significant fibrinogen digesting potential of SCF at such low concentration indicates the presence of potent anticoagulants which need to be further purified and characterised. Fibrinogenolytic activity of SCF was lost in the presence of metal ion chelators like EDTA, indicating the active protein components to be metalloproteinases (Figure 3.5). Specific protease activity of SCF was estimated to be 2.5 µg/ml in 30 min. at 37ºC as evidenced by azocasein assay. Hemolytic, laminin digestion and phospholipase activities were below 5% rendering it a probable safe candidate for drug discovery. The first physiological response upon occurrence of a vascular injury is platelet aggregation, activation and adhesion. This initiates thrombus formation and also wound healing. Platelets aggregate post-vascular injury when the circulating platelets in the blood come in contact with collagen, fibronection, vWF (von Willebrand factor) A1 domain and the membrane glycoproteins GPIbα of the endothelial membrane. This in turn initiates recruitment of more platelets to form a platelet plug at the site of injury. Fibrinogen binds to the integrin receptors on activated platelets, thereby acting as bridges between platelets to bring about platelet aggregation. Interestingly, SCF at concentrations above 5µg/ml showed pro-aggregation of platelets with ADP as agonist. Whereas, pro-aggregation activity of SCF was observed above 2µg/ml concentration in collagen activated platelet (Figure 3.7 and Figure 3.8). Scanning electron microscopy also confirmed the pro-aggregation ability of SCF on blood platelets (Figure 3.9). SCF is a mixture of several proteins and peptides. Probably, at higher concentrations of SCF, some minor protein components could be reaching a threshold concentration at which they are able to show platelet aggregation activity. This could explain both anti-platelet and pro-platelet aggregating activities of SCF at different dosages. Further purification of these active proteins/peptides would be useful to decipher the exact mechanism of anticoagulant activity and effects on platelet aggregation. This was performed in the subsequent chapter.

Results of MTT and Resazurin reduction assays confirmed that SCF produced no cytotoxic effects on A549 cells cultured *in vitro* (Figure 3.14 and Figure 3.15). In fact, exposure of cells to SCF showed increase in their metabolic activity and viability. Increased resorufin production in

SCF treated cells coincided with SCF induced proliferative activity seen in A549 cells (Riss et al., 2016). Complete healing of the wounds created on A549 cells *in vitro* was observed at the same dose. The wound healing process occurs in four phases: hemostasis, inflammation, proliferation and maturation (Michael Mercandetti, 2015; Patrick E. Simon, 2015). Platelets play a major role in hemostasis and vasoconstriction which is the first phase of wound healing (Italiano et al., 2008). On activation of platelets, many factors are released from dense granules of the aggregating platelets which influence cell migration and proliferation (Golebiewska and Poole, 2015); Nurden et al., 2008).

The two major pharmacological activities found in SCF namely promotion of platelet aggregation, cell proliferation and migration may be either caused by a single factor in SCF or due to synergistic effect of several factors. It would thus be interesting to study the proteins present in SCF separately in different *in vitro* and *in vivo* systems to decipher the exact contributor to these bioactivities in SCF.

4 Purification of a Protein, AiP1, from SCF and its Biochemical Characterisation

4.1 Introduction

The marine environment covers more than 70% of the earth's surface and marine organisms constitute more than 50% of the biodiversity (Montaser and Luesch, 2011). Oceans, thus remain the largest resource of novel bioactive compounds and useful leads for drug development (Bordbar et al., 2011; Datta et al., 2015; Hamed et al., 2015). Out of the many unexplored marine bioresources, one such resource is the coelomic fluid of sea star *Astropecten indicus.* This species of sea stars is commonly found around the coast of South-East Asian countries especially India. The coelomic fluid is a reservoir of bioactive compounds of diverse therapeutic as well as cosmetic potential (Kijjoa and Sawangwong, 2004). This study focussed on searching for novel bioactive molecules to treat thrombosis and thromboembolic disorders which are highest causes of mortality worldwide. Present day thrombolytic agents have adverse side effects like unanticipated bleeding, hemorrhage and lack of specific site of action (Engelmann and Massberg, 2013; Key et al., 2016; Lau, 2003). This makes the search for novel and potent thrombolytic drugs from natural sources imperative.

Skin is the largest organ of the body and is constantly exposed to various stress conditions. In response to dermal injuries, the epidermis initiates a wound healing response in response to skin damage (Choi et al., 2015). Wound healing is a complex mechanism which comprises of following steps: hemostasis, inflammation, proliferation, and remodeling. All these four steps must occur sequentially and within a proper time frame for the wound to heal successfully (Guo and DiPietro, 2010). If any one of these phases is affected, impaired wound healing occurs, which pose risk like inflammation, infection, necrosis or ulcers (Han and Ceilley, 2017).

Keeping in mind the aforementioned gaps in existing literature, this study focused on the isolation of thrombolytic and wound healing agent(s) from the coelomic fluid of the sea star *Astropecten indicus.* Protein extraction was performed using a combination of anion exchange chromatography and size exclusion HPLC to yield AiP1. This protein was found to be thrombo-

lytic with its fibrinogen and fibrin digesting activities. It also showed cell proliferative and wound healing activities *in vitro.* The role of AiP1 in cell migration was assessed by treating HEK293 cells with AiP1 and studying the change in expression of E and N cadherin. E and N cadherin are calcium dependent trans-membrane adhesion proteins that form hemophilic interactions with similar molecules present on other cells, thus forming cell-cell interactions. Increase in expression of N cadherin in cultured cells post treatment with AiP1 confirmed its role in cell migration (Maître and Heisenberg, 2013; Zuppinger et al., 2000). The protein AiP1 was devoid of cytotoxic, hemolytic, phospholipase and laminin digestion activities. Other biological activities present in AiP1 are currently being explored.

4.2 Methodology

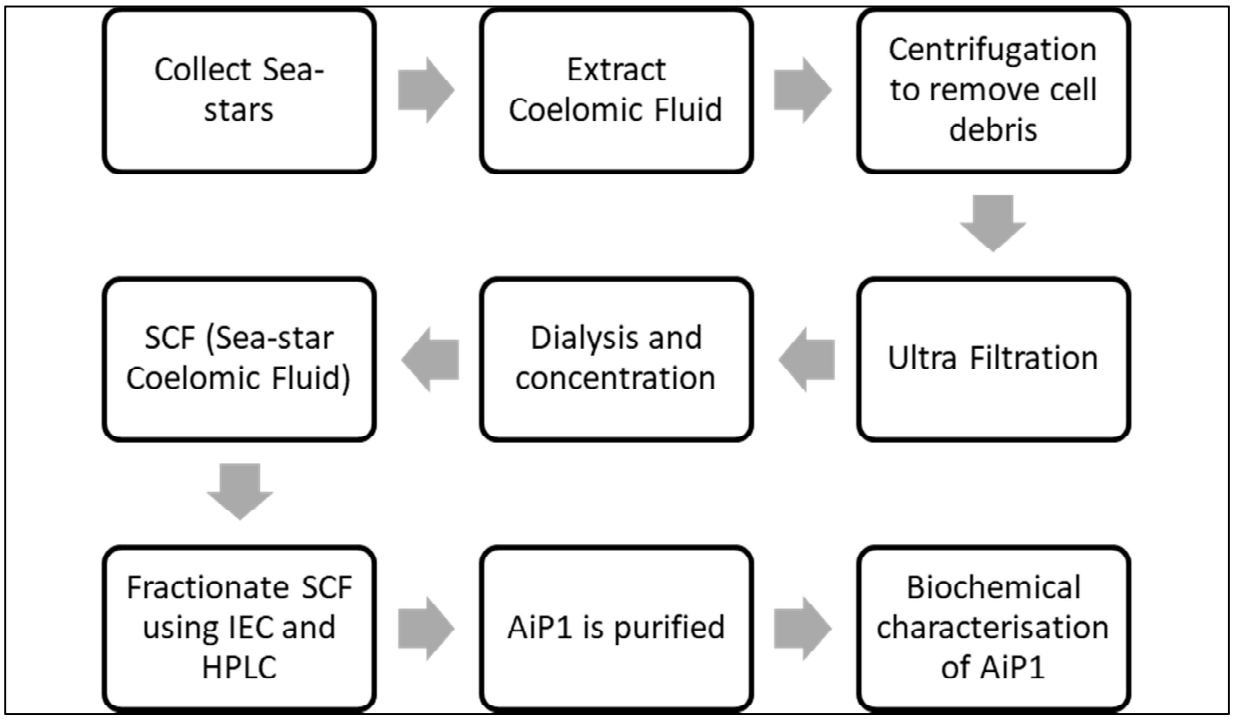


Figure 4.1 Methodology followed in purification of AiP1 from SCF

4.2.1 Fractionation of SCF using DEAE Sephadex A-25

Sea star coelomic fluid was extracted from *Astropecten indicus* using syringe aspiration as described before. This SCF was dialysed overnight against 50mM KPB, pH 7.4 at 4˚C. The dialysed fluid was concentrated using sucrose to yield concentrated SCF (2mg/ml). This SCF was

fractionated on a column of DEAE Sephadex A-25 (5ml) and bound proteins were eluted using a linear NaCl gradient (0 to 0.5M). Fractionation profile in Figure 4.2 shows one major peak followed by two minor protein peaks eluted from SCF. Protein purity of the peaks was estimated by electrophoresis followed by silver staining of the SDS-PAGE gel (Figure 4.3).

4.2.2 Fractionation of P-A using Size Exclusion HPLC

The proteins comprising P-A were pooled, concentrated and subject to fractionation of a size exclusion column (SEC s2000) using HPLC. The chromatogram obtained after HPLC showed one sharp protein peak which eluted after 23min (Figure 4.5). This protein peak yielded AiP1.

4.2.3 Characterisation of AiP1 for its protease activity

The pure protein AiP1 obtained after repeated fractionation of SCF was studied for its proteolytic activities using substrates like fibrinogen, fibrin, laminin, phospholipase A2, casein as per methods described in chapter 2. Effects of AiP1 on fibrinogenolysis, fibrinolysis, hemolysis and platelet aggregation are shown in figures 4.6 to 4.13.

4.2.4 Characterisation of AiP1 using bioinformatics tools

The purified protein AiP1 was outsourced to the Protein Facility Laboratory of Iowa State University, Iowa, USA to assess its partial sequence. The protein was digested using trypsin followed by MS/MS analysis. The peptide fragments of AiP1 were scanned using BLAST-P (version 2.8.0) against the protein NCBI nr data-bases to find homology with non-redundant proteins (Johnson et al., 2008) accessed on June 29, 2018.

The analysed peptide fragments of AiP1 and their similarity search results are summarized in Table 4.1.

4.2.5 *In vitro* effects of AiP1 on cell viability and cell migration

HEK293 and HaCaT cells were cultured *in vitro* as described in section 2.4.18. Effect of AiP1 on cell viability was assessed in a dose dependent manner using MTT cytotoxicity assay (Figure 4.16). The effect of various dosages of AiP1 on cell migration was studied using the monolayer scratch assay described in 2.4.22. The effects of AiP1 on cell migration gave an estimate of its wound healing activity *in vitro* as seen in Figure 4.17.

4.3 Results

4.3.1 Fractionation of SCF using DEAE-Sephadex A-25 column

Fractionation of SCF was performed using a 5ml column of DEAE-Sephadex A-25 using mobile phase of 50mM KPB pH 7.4. Proteins were eluted using a linear gradient of NaCl (0 to 0.5 M). One major protein peak (P-A) and two minor peaks were eluted as seen in Figure 4.2.

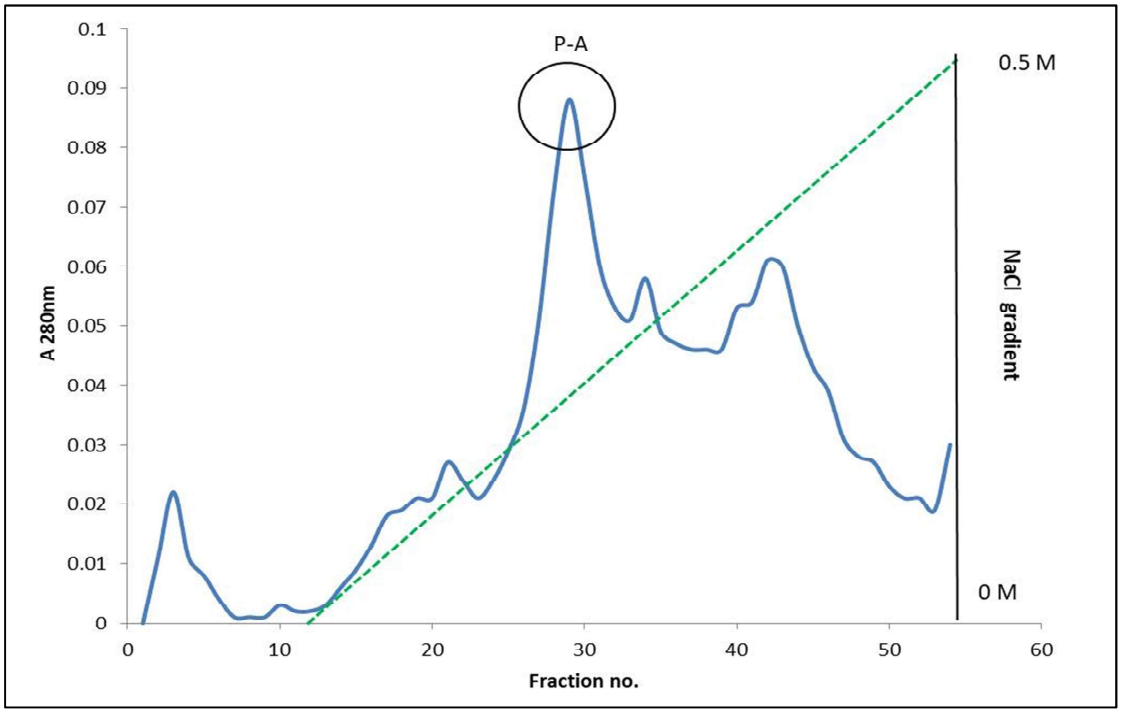


Figure 4.2 Fractionation of SCF using DEAE-Sephadex A-25 column (5ml)

SCF was fractionated using anion exchange chromatography on DEAE-Sephadex. Mobile phase was 50mM KPB, pH 7.4. One major peak was eluted using a gradient of 0 to 500mM sodium chloride. This protein peak was designated as P-A and was studied further.

4.3.2 Protein purity of P-A

Protein fractions comprising P-A were assessed by running on 12% SDS-PAGE. Protein bands were visualized using silver staining of the gel (Figure 4.3).

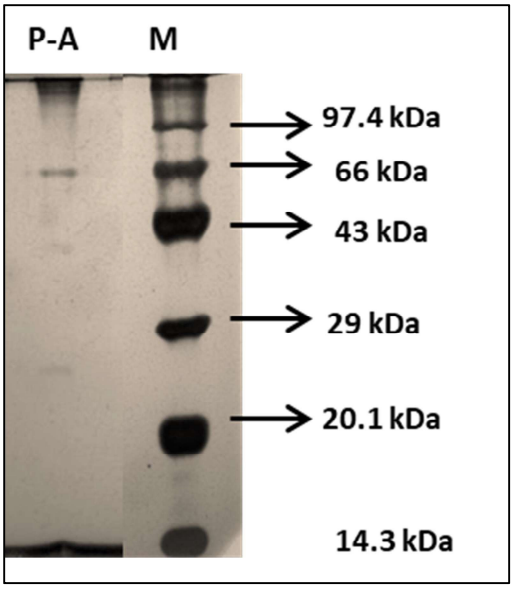


Figure 4.3 Purity of P-A estimated using 12% SDS-PAGE

Protein peak P-A showed 3 major protein bands as seen in SDS-PAGE gel followed by silver staining.

4.3.3 Fibrinogenolytic activity of fractions of P-A

Protein fractions comprising P-A were incubated with fibrinogen to study their effect on fibrinogen digestion. 2.4.10. All fractions comprising P-A digested Aα chain of fibrinogen as seen in Figure 4.4

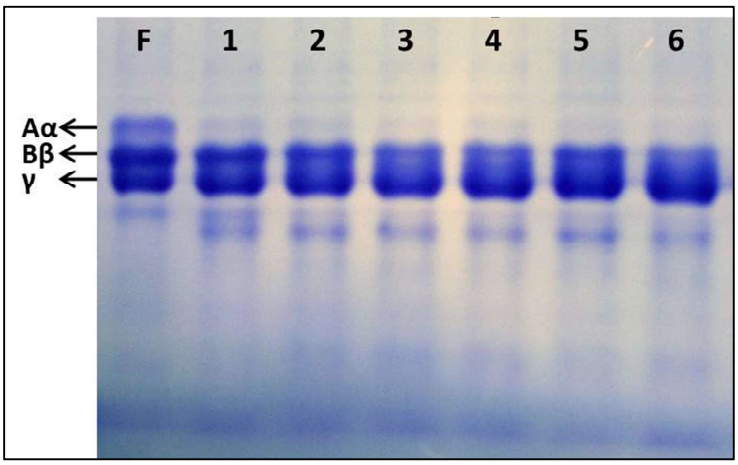


Figure 4.4 Fibrinogenolysis by protein fractions of peak P-A

F is fibrinogen which served as negative control; Lanes 1 to 6 are protein fractions comprising peak P-A incubated with fibrinogen at 37˚C for 5h. All fractions of protein peak P-A lysed fibrinogen as seen as disappearance of the Aα chain in lanes 1 to 6.

4.3.4 Size Exclusion HPLC of P-A

The protein fractions comprising P-A were pooled, dialysed, concentrated and fractionated using HPLC on a size exclusion column BioSep SEC s-2000 (300 x 7.8mm). One major protein peak and several smaller peaks were eluted (Figure 4.5).

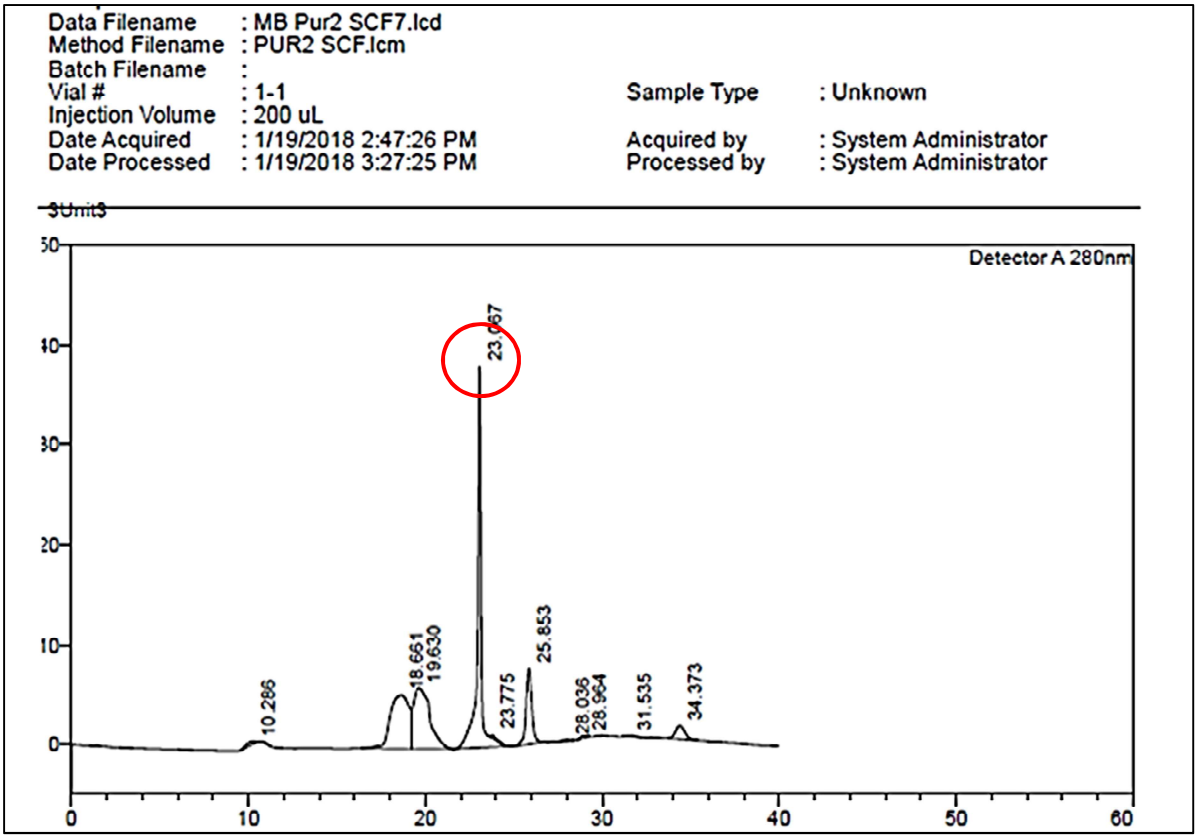


Figure 4.5 Size Exclusion HPLC of P-A

Protein peak P-A was fractionated using HPLC on a size exclusion column. Flow rate = 0.5 ml/min; mobile phase = 50mM KPB, pH 7.4. One sharp protein peak was eluted at retention time of 23 min. This peak was studied in detail in further sections.

4.3.5 Protein purity of eluted fractions

The protein fraction eluted at 23rd min was assessed for its purity by SDS-PAGE. A single protein band was obtained at molecular weight of 66 kDa. This protein would be referred to as AiP1 (first protein from *Astropecten indicus*) in further sections (Figure 4.6).

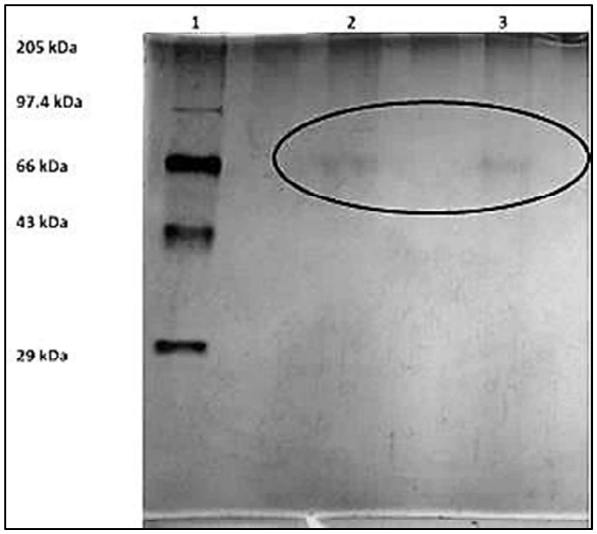


Figure 4.6 Protien purity and molecular weight estimation of AiP1 using 12% SDS-PAGE

Lane 1 is high range protein molecular weight marker; lanes 2 and 3 are AiP1. Molecular weight of AiP1 was estimated to be 66 kDa.

4.3.6 Fibrinogenolytic activity of AiP1

Fibrinogenolytic activity of AiP1 was estimated in a dose and time dependent manner (Figure 4.7). AiP1 digested fibrinogen at a dose of 10 µg within a time period of 3.5h. Fibrinogen incubated with saline served at negative control.

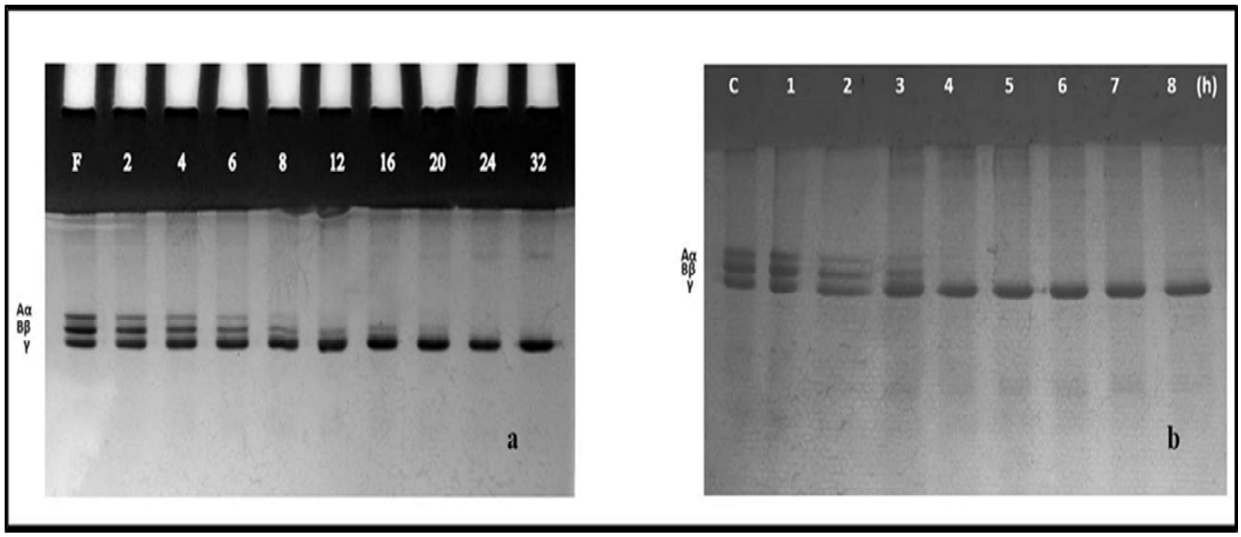


Figure 4.7 a) Dose dependent fibrinogenolysis by AiP1 and b) Time dependent fibrinogenolysis by AiP1

a) F is fibrinogen incubated with 0.85% saline (negative control); other lanes represent the dose

of AiP1 (in µg) incubated with fibrinogen; **b)** C =fibrinogen incubated with 0.85% saline (negative control); other lanes represent the time period of AiP1 (10 µg) incubated with fibrinogen at 37˚C.

4.3.7 Hemolytic activity of AiP1

AiP1 did not lyse human RBCs even at dosage of 100µg as seen in Figure 4.8. The low hemolysing potential of AiP1 makes it a safe candidate for further drug development.

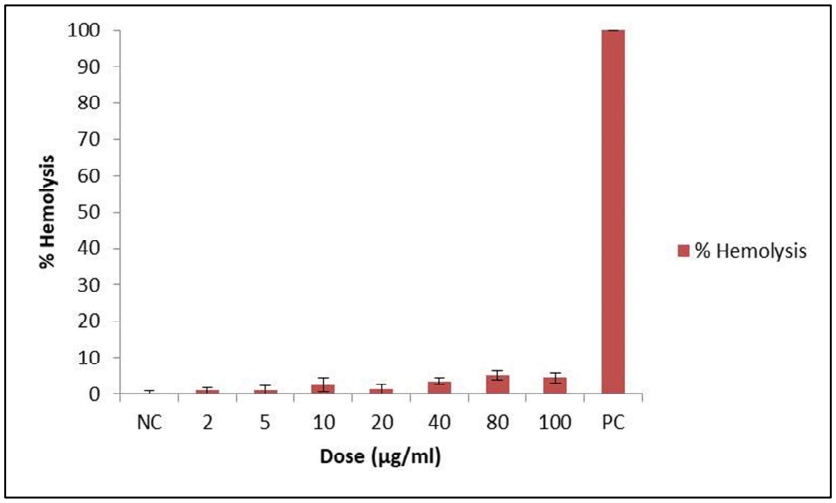


Figure 4.8 Hemolytic activity of AiP1 on human RBCs

Protein AiP1 showed negligible hemolytic activity in human RBCs even at dose of 100µg.

4.3.8 Phospholipase A activity of AiP1

Phospholipase activity of AiP1 was assayed using egg yolk suspension as substrate. There was no phospholipase activity detected in crude SCF, semi-pure protein P-A or AiP1.

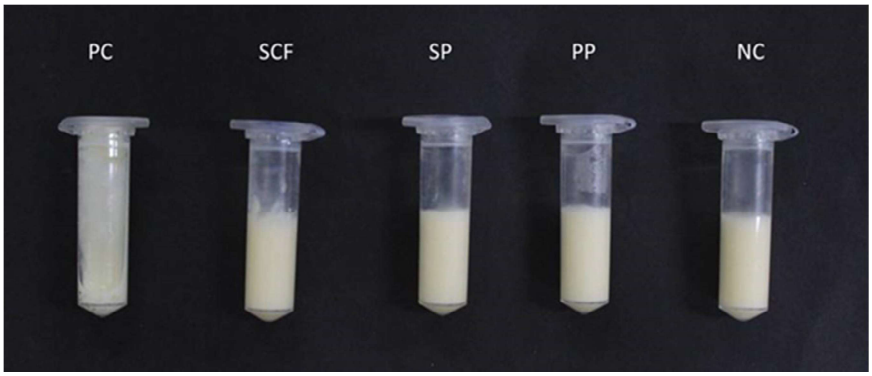


Figure 4.9 Effect of AiP1 on phospholipids

Phospholipase activity was absent in AiP1. Egg yolk suspension incubated with Russell's' viper

venom (RVV) served as positive control (PC); SP is semi-pure SCF (P-A) incubated with yolk suspension; PP is pure protein AiP1 isolated from SCF and incubated with yolk suspension and NC is yolk suspension incubated with 0.85% saline.

4.3.9 Effect of AiP1 on laminin Digestion

Protein AiP1 was assayed for its effect on laminin digestion. No digestion was observed in laminin preincubated with AiP1 as seen in Figure 4.10.

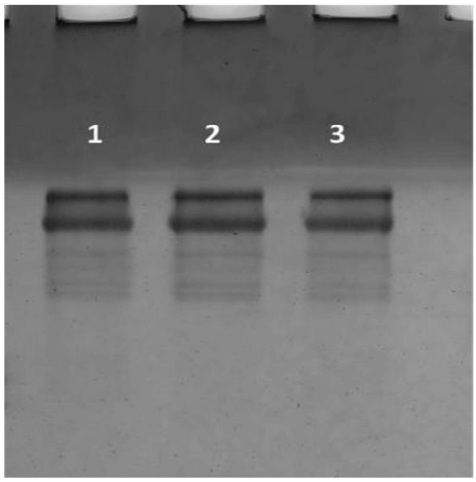


Figure 4.10 Effect of AiP1 on laminin digestion

Lane 1 is Laminin (control); Lane 2 and 3 are laminin incubated with 10 and 20µg of AiP1 respectively. AiP1 was devoid of laminin digestion activity.

4.3.10 Inhibition of fibrinogenolytic activity of AiP1

The nature of protease activity was assayed by incubating AiP1 with EDTA (metallo-protease inhibitor) or PMSF (serine protease inhibitor) for 1h followed by assessing its fibrinogenolytic activity. It was observed that treatment of AiP1 with EDTA for 30 min. reduced its fibrinogenolytic activity. Furthermore, incubation with EDTA for 1h completely inhibited the fibrinogenolytic activity of AiP1 as seen in Figure 4.11. This indicated the metalloproteinase nature of AiP1.

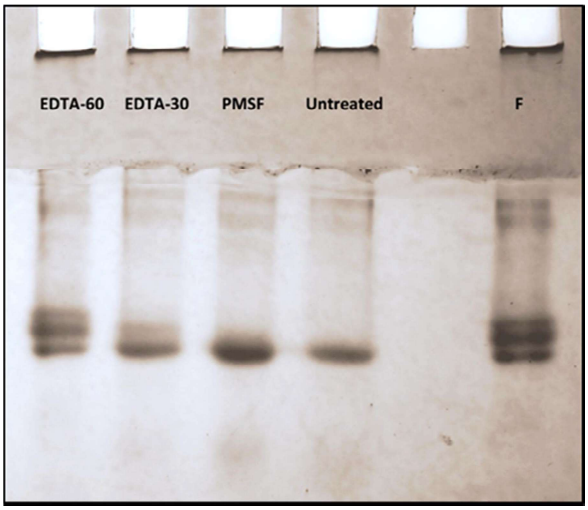


Figure 4.11 Inhibition of fibrinogenolytic activity of AiP1 by EDTA

Metalloproteinase nature of AiP1 was confirmed by estimating fibrinogenolytic activity of AiP1 post treatment with different inhibitors followed by incubating with fibrinogen to assess presence or loss of fibrinogenolytic activity post treatment. Lane 1: AiP1 treated with EDTA for 60 min; Lane 2: AiP1 treated with EDTA for 30 min; Lane 3: AiP1 treated with PMSF for 60 min; Lane 4: Untreated AiP1; Lane 5: Fibrinogen control.

4.3.11 Thermal stability of AiP1

The effect of temperature on fibrinogenolytic activity of AiP1 was assayed by exposing AiP1 to various temperatures for 1h followed by assessing the fibrinogen digesting ability of the samples post heat exposure. The protein AiP1 was found to withstand temperature of up to 70˚C, beyond which its fibrinogenolytic activity was inhibited.

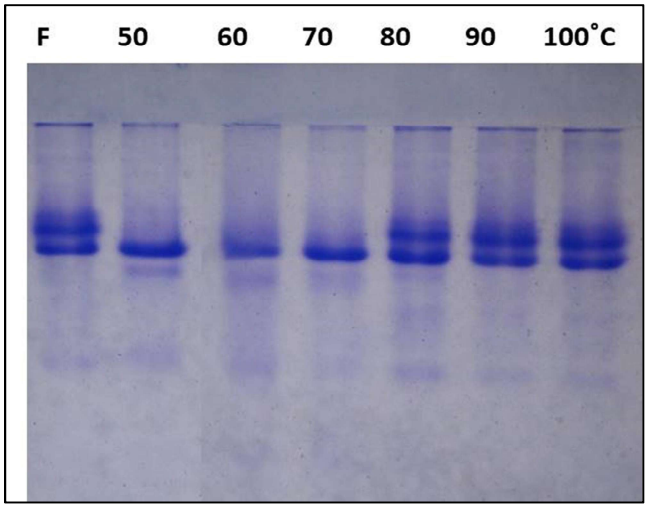


Figure 4.12 Effect of temperature on AiP1

The protein AiP1 was assessed for its fibrinogenolytic activity post exposure to different temperatures for 30min. Numbers above each lane indicate the temperature exposure in ˚C; F is fibrinogen control. The activity of AiP1 was retained till 70˚C beyond which it was inhibited as seen in the figure.

4.3.12 Fibrinolytic activity of AiP1

Fibrinolytic activity of AiP1 was estimated using the zymogram assay. Fibrin was co-polymerized with the polyacrylamide gel, followed by electrophoresis of SCF (4µg/ml). The gel was incubated for 8h in the incubation buffer at 37ºC. Fibrinolytic proteins showed clear digestion of the substrate as seen in Figure 4.13.

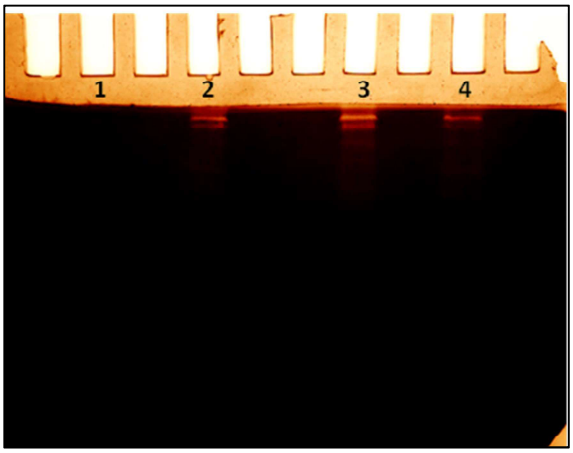


Figure 4.13 Fibrinolytic activity of AiP1

Lane 1 = AiP1 treated with EDTA; lane 2= SCF (2.5 µg); Lane 3= SCF (5µg); Lane 4 = AiP1 (2.5 µg). AiP1 showed digestion of fibrin as observed by zones of substrate digestion in the zymogram.

4.3.13 Effect of AiP1 on platelet aggregation

The effect of AiP1 on blood platelets was assessed by a Chronolog Whole Blood Platelet Aggregometer using ADP and collagen as agonists. An increase in aggregation of ADP activated platelets was observed post treatment with 2.5 and 5µg of AiP1 followed by a sequential inhibition of platelet aggregation at AiP1 doses higher than 10µg (Figure 4.14a). For collagen induced platelet aggregation, AiP1 showed a dose dependent inhibition of platelet aggregation as seen in Figure 4.14b below.

Loss of platelet inhibiting activity of AiP1 was observed upon its treatment with EDTA as expected of its metalloproteinase nature.

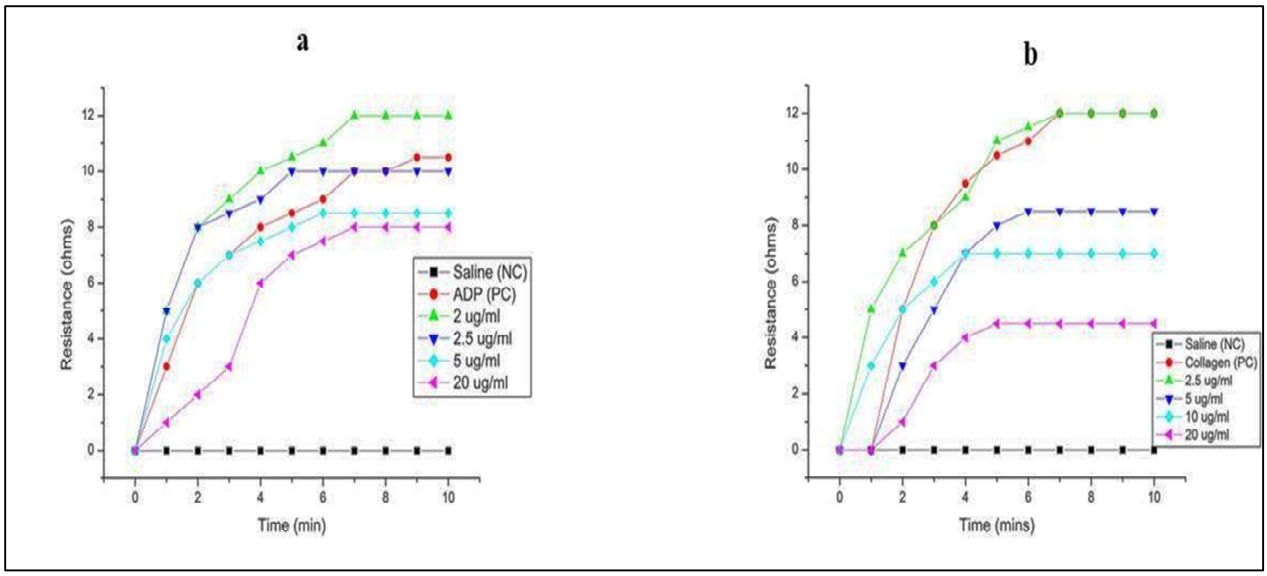


Figure 4.14 Effect of AiP1 on a) ADP and b) Collagen induced platelet aggregation

a: Effect of AiP1 on ADP induced platelet aggregation. An increase in platelet aggregation was observed in platelets treated with 2.5 and 5µg of AiP1. At higher doses, a dose dependent inhibition of platelet aggregation was observed; **b:** Effect of AiP1 on collagen induced platelet aggregation. Inhibition of collagen induced platelet aggregation was observed upon treatment with AiP1 in a dose dependent manner.

4.3.14 Peptide mass fingerprinting of AiP1

The protein isolated from SCF post HPLC was outsourced to the Protein Facility Laboratory of Iowa State University, Iowa, USA to assess its partial sequence. The protein was digested using trypsin followed by MS/MS analysis. The peptide fragments of AiP1 were scanned using BLAST-P (version 2.8.0) against the protein NCBI nr data-bases to find homology with non-redundant proteins (Johnson et al., 2008) accessed on June 29, 2018.

The analysed peptide fragments of AiP1 and their similarity search results are summarized in Table 4.1. Only in one of the peptides of AiP1, which had the amino acid sequence "RIM-LFMGGPPTQGPGMVVGEELKT", the matched region was identical to a functional domain, the von Willebrand factor A (vWFA)-like domain superfamily (InterPro id IPR036465) from the organism *Strongylocentrotus purpuratus*. This peptide would be referred to as peptide1 in subse-

quent sections. All other peptides showed similarity to uncharacterized proteins from the purple sea urchin *Strongylocentrotus purpuratus.*

Table 4.1 Peptide Fragments of AiP1 and their top NCBI BLAST hits scanned[i] against NCBI nr database

([1] Hits means peptides with query coverage = 100% and identity = 100% to the available databases)

Analysed Fragments	Accession no. of matched protein and source organism
RIMLFMGG-PPTQGPGMVVGEELKT	XP_780042.1 from *Strongylocentrotus purpuratus*
KAVTISIDRN	XP_003724743.2 from *Strongylocentrotus purpuratus*
RDPIQVPEVTGNFASSTSDDLRG	XP_780590.1 from *Strongylocentrotus purpuratus*
KRDVRVGKR	XP_001189839.1 from *Strongylocentrotus purpuratus*
KKSNSATPARW	XP_011669910.1 from *Strongylocentrotus purpuratus*
	XP_011669897.1 from *Strongylocentrotus purpuratus*
KVVTSGIAQKF	XP_011682725.1 from *Strongylocentrotus purpuratus*
	XP_001178826.1 from *Strongylocentrotus purpuratus*
RLQQAYLRMEQGEAPTPE-AARE	XP_011683542.1 from *Strongylocentrotus purpuratus*
KNRFQI-LAELETDNQPNMDADTDNVNTNWEQVKT	No significant match obtained
RALAWKRQRI	XP_788209.4 from *Strongylocentrotus purpuratus*
	WP_073916543.1 from *Strongylocentrotus purpuratus*
RQIKNVRN	XP_003728172.2 from *Strongylocentrotus purpuratus*
	XP_018832541.1 Juglans regia
RTQLEIAQEPNSKT	XP_011663628.1 from *Strongylocentrotus purpuratus*
RAAQTFSMDKY	XP_003723710.1 from *Strongylocentrotus purpuratus*
	XP_798150.3 from *Strongylocentrotus purpuratus*

This peptide (peptide1) search was extended to another recently sequenced sea star, the crown of thorns or *Acanthaster planci* (Hall et al., 2017). The Figure 4.15 shows peptide1 aligned with homologous domains present in *Strongylocentrotus purpuratus* and *Acanthaster*

planci. These three organisms belong to the same phylum Echinodermata. Similarity of peptide1 from *Astropecten indicus* with other echinoderms such as purple sea urchin (*Strongylocentrotus purpuratus)* and the Crown of thorns starfish (*Acanthaster planci)* indicates that peptide1 may have functional significance in echinoderms in their various biological processes.

The vWFA-like domain from *Strongylocentrotus purpuratus* was modeled using homology modeling to understand the potential role of peptide1 matched region. The peptide1 was observed to be an integral part of the central β sheet of vWFA-like domain and some part of it was overlapped with the predicted PPI residues and thus it may be structurally and functionally significant for echinoderms (Figure 4.15 B and C*).*

These results suggest that AiP1 may contain vWFA-like domain. Proteins incorporating the vWFA-like domain are known to participate in various biological events (like cell adhesion, migration, homing, pattern formation, and signal transduction), involving interaction with a large array of ligands (Colombatti et al., 1993).

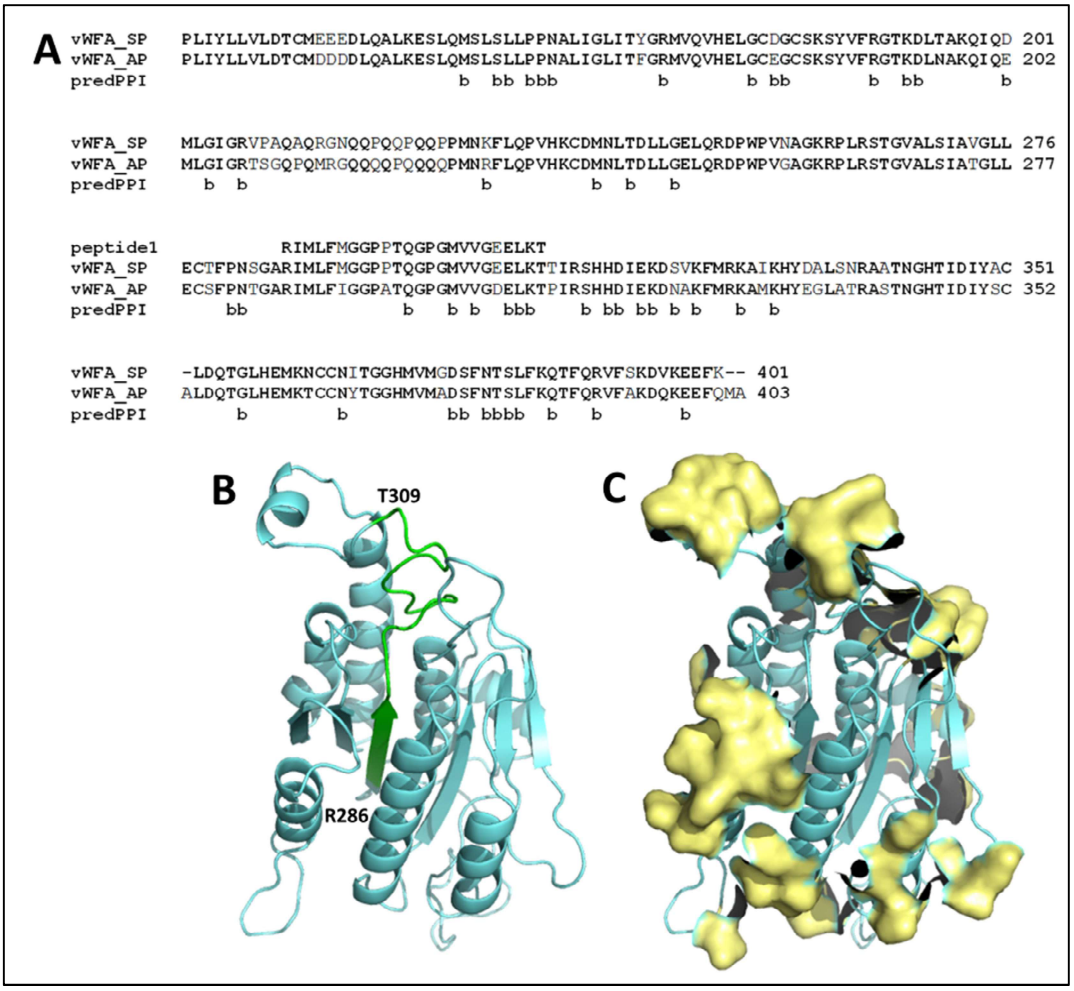


Figure 4.15 vWFA-like domain from the organism *Strongylocentrous purpuratus* and its putative functional sites.

(A) Multiple sequence alignment of peptide1 and vWFA-like domain from the organisms *Strongylocentrous purpuratus* (vWFA_SP, NCBI RefSeq accession numbers XP_780042.1) and *Acanthaster planci* (vWFA_AP, NCBI RefSeq accession numbers XP_022096018.1). *The alignment was generated using CLUSTAL Omega version 1.2.4 (*/www.ebi.ac.uk/Tools/msa/clustalo/*).* Predicted solvent accessible protein-protein interacting residues (predPPI) are highlighted ('b'). Solvent accessibility and PPI residues were predicted using NetSurfP (version 1.1, http://www.cbs.dtu.dk/services/NetSurfP/) and LORIS (Dhole et al., 2014), respectively. (B) Ribbon representation of the modeled vWFA_SP (5kyn_A as template with sequence identity 77.5%) generated using Swiss-Model (Bienert et al., 2017). Highlighted position (green, R286 to T309) on this model is identical with peptide1. (C) Predicted solvent accessible PPIs (predPPI) mapped on the modeled vWFA_SP. The figures (B) and (C) were generated using PyMol (version 2.2, https://pymol.org/).

4.3.15 Effect of AiP1 on cell viability

The effect of AiP1 on viability of HEK293 and HaCaT cells were assessed using the MTT assay. No loss in cell viability was observed in cells post treatment with AiP1 for 24h, 48h and 72h. However, AiP1 treated cells showed an increase in viability as compared to those in untreated cells which served as control.

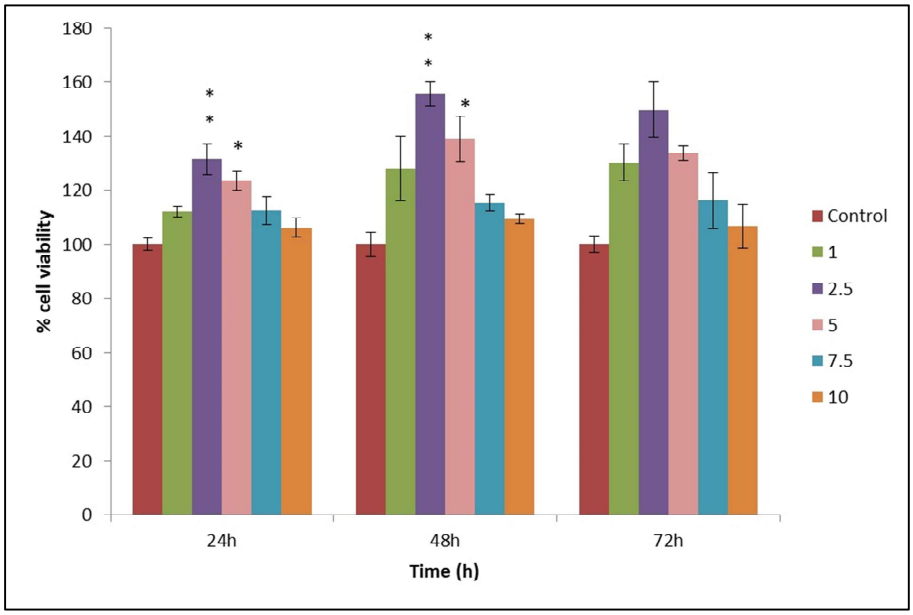


Figure 4.16 Effect of AiP1 on viability of HEK293 cells *in vitro*

An increase in cell viability was observed post treatment with AiP1 compared to untreated cells (control). Doses up-to 5µg/ml AiP1 showed maximum increase in cell viability *=$p<0.05$; **=$p<0.01$.

4.3.16 *In vitro* scratch assay to study effect of AiP1 on wounds created on HEK293 and HaCaT cells

Wound healing activity of AiP1 was assessed using the monolayer scratch assay as described in section 2.4.22. Wounded cells treated with AiP1 showed faster wound closure rates compared to untreated cells which served as control. HaCaT cells showed complete wound closure within 48h of incubation with AiP1, whereas HEK293 cells showed wound closure in about 60h as seen in Figure 4.17.

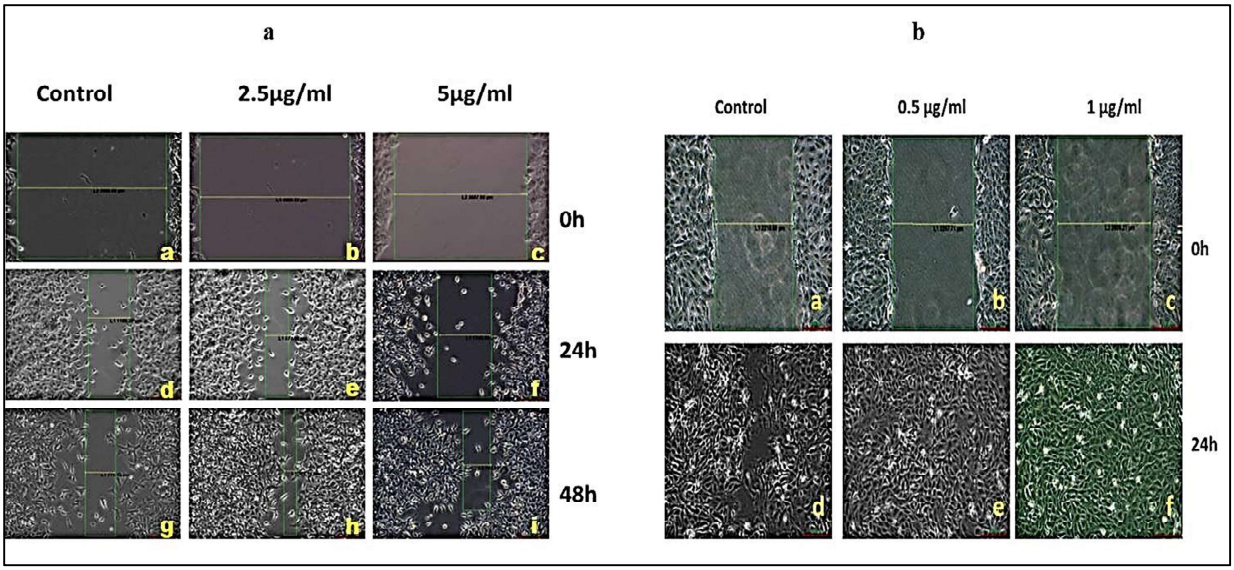


Figure 4.17 Wound healing activity of AiP1 on a) HEK 293 cells and b) HaCaT cells

a. Wound healing was accelerated in AiP1 treated cells compared to untreated cells which served as control. Significant wound healing was observed at concentrations 2.5 and 5µg/ml of AiP1 (as seen in sections e,f,h and i). **b.** Wound healing accelerated in HaCaT cells treated with AiP1 (sections b,c,e and f) compared to untreated cells (section a and d) which served as control.

4.3.17 RT-PCR and *c*DNA synthesis

Total cell RNA was extracted from HEK293 cells treated with 2.5µg/ml and 5µg/ml of AiP1 for 24h and 48h. RNA concentration and purity was estimated by measuring absorbance at 260nm and 280nm. The *c*DNA was synthesized from the RNA template using PCR conditions:

The *c*DNA synthesized was stored in aliquots in -20˚C. PCR conditions for expression studies were normalized using GAPDH. PCR conditions were: Initial denaturation: (2 min. at 97˚C); denaturation (35 cycles for 1 min at 94˚C); annealing (1min 30s at 57˚C) for GAPDH; extension (1 min at 70˚C) and final extension (5 min. at 70˚C).

4.3.18 Effect of AiP1 on expression of E and N Cadherin

The effect of AiP1 on RNA expression was assessed by electrophoresing the PCR products on 1.2% agarose gel. PCR conditions were: Initial denaturation: (2 min. at 97˚C); denaturation (35 cycles for 1 min at 94˚C); annealing (1 min 30s at 56˚C) for N Cadherin and (1 min 30 s at 52˚C) for E Cadherin; extension (1 min at 70˚C) and final extension (5 min. at 70˚C).

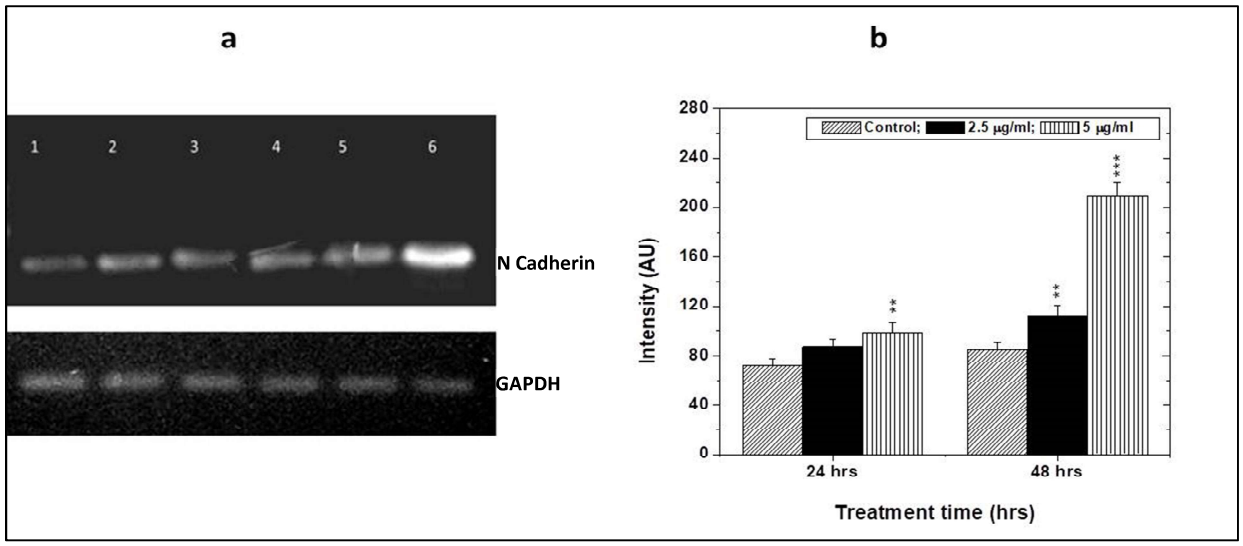


Figure 4.18 Expression of N cadherin post treatment with AiP1

An increase in N cadherin expression was observed in cells treated with 5µg/ml AiP1 after 48h as seen in Fig 15 a and b. This indicated that AiP1 promoted cell migration *in vitro.*
Lane 1 = Control (24h); 2 = 2.5µg/ml AiP1 (24h); 3 = 5µg/ml AiP1 (24h); 4 = Control (48h); 5 = 2.5µg/ml AiP1 (48h); 6 = 5µg/ml AiP1 (48h). *=$p<0.05$; **=$p<0.01$; ***=$p<0.001$.

4.3.19 Purification table of AiP1 from SCF

The SCF extracted was fractionated using anion exchange chromatography followed by size exclusion HPLC to yield AiP1. The fibrinolytic activity of AiP1 during its various purification stages was estimated using the quantitative spectrophotometric assay developed as a part of this study and described in 2.4.9. The stepwise specific activity and yield for each step of purification is summarized in Table 4.2 below. The protein AiP1 had a yield of 7% .

Table 4.2 Purification table of AiP1 from SCF

Sample	Purification step	Volume (ml)	Activity (U/ml)	Protein Conc (mg/ml)	Specific activity (U/mg)	Purification fold	Total activity (U)	Yield (%)
SCF	Ultra Filtration	200	5.6	2	2.8	1	1120	100
P-A	Ion Exchange Chromatography	10	19.89	1	19.89	7.103571	198.9	17.76
AiP1	HPLC	2	39.4411	0.2	197.2055	70.43054	78.8822	7.043

SCF is Sea star Coelomic fluid obtained after ultra-filtration using 10kDa membrane; P-A is the protein peak obtained after SCF was fractionated on a DEAE Sephadex A-25 column; AiP1 was the protein obtained after protein fractions comprising P-A were fractionated using size exclusion HPLC.

4.4 Discussion

Echinoderms, especially sea stars have been used as model organisms for regenerative studies due to their enormous regenerative potential (Dai et al., 2016). The biomolecules responsible for this property are predominantly the coelomocytes which are the immune cells present in the coelomic fluid (da Silva Laires, 2012). Sea stars have been studied extensively in the past few years due to their diverse bioactivities and the secondary metabolites that they produce. (Ferguson, 1964; (Schillaci and Arizza, 2013). Despite their immense regenerative potential, not many wound healing agents have been extracted from sea stars, especially from the Astropecten genus. The only reported bioactivity of *Astropecten indicus* hitherto is their antimicrobial potential (Chamundeeswari, 2012).

The coelomic fluid of invertebrates bathes all the internal organs of sea stars and is a rich source of growth factors, hormones, proteins and peptides involved in cell signaling. Coelomic fluid from sea star *Astropecten indicus* was studied for its effects on hemostasis *in vitro.* It lysed human fibrinogen (30µg) at a concentration of 4µg/ml within 30 min. of incubation with the substrate. Other proteolytic activities of SCF such as fibrinolytic, fibrinogenolytic and caseinolytic activities were also observed at this concentration. Significant fibrinogen digesting potential of SCF at such low concentration indicates the presence of potent anticoagulants which need to be further purified and characterized. EDTA inhibited fibrinogenolytic activity of SCF by chelating the metal ions, indicating the active protein components to be metalloproteinases. Specific protease activity of SCF using azocasein as a substrate was estimated to be 2.5 µg/ml in 30 min at 37ºC. Hemolytic activity of SCF on human RBCs was below 5% and no effect of SCF was observed on phospholipase and laminin, suggesting it to possibly be a safe candidate for drug discovery. These results show that the anticoagulant activity of SCF is optimum at a concentration of 4µg/ml at which it shows fibrinogenolytic as well as anti-platelet activity (for ADP induced platelet aggregation). Platelet adhesion and aggregation are considered as the first physiological

responses towards vascular injury which initiate thrombus formation and permit wound healing. Platelet aggregation is initiated in response to vascular injury wherein the circulating platelets come in contact with collagen, fibronection, vWF (von Willebrand factor) A1 domain and the membrane glycoproteins GPIbα causing platelets to adhere to basement membrane and further initiating recruitment of more platelets to the site of injury to form a platelet plug. Fibrinogen binds to the integrin receptors on activated platelets, thereby acting as bridges between platelets to bring about platelet aggregation. Interestingly, at concentrations above 5µg/ml, SCF showed increased aggregation of platelets with ADP as agonist. Aggregation of Collagen-induced platelets was seen to increase at a concentration above 2µg/ml. Scanning electron microscopy also confirmed the pro-aggregation ability of SCF on blood platelets. SCF is a mixture of several proteins and peptides. This might suggest that, at higher concentrations of SCF, some minor protein components reach the threshold concentration required by them to exhibit platelet aggregation activity. This could explain both anti-platelet and pro-platelet aggregating activities of SCF at different dosages.

SCF was found to have no cytotoxic effects on A549 cells cultured *in vitro.* Time dependent increase in cell viability was noted in A549 cells post-treatment with SCF as confirmed by MTT assay and Resazurin reduction assay. Exposure of A549 cells to SCF showed increase in their metabolic activity by conversion of Resazurin to resorufin. Increased resorufin production in SCF treated cells coincided with SCF induced proliferative activity seen in A549 cells (Riss et al., 2016). Complete healing of the wounds created on A549 cells was observed in SCF treated cells. SCF is comprised of various proteins, polypeptides and factors which were responsible for its bioactivities. In the present study, one of the many bioactive proteins from SCF was isolated and partially characterized. This protein designated as AiP1, was fibrinogenolytically active at concentration of 10µg/ml. The proteolytic activity of AiP1 was persistent till a temperature of 70˚C. Protein AiP1 promoted ADP induced platelet aggregation till dose of 5µg/ml. At higher doses, inhibition of aggregation was observed for ADP as well as collagen induced platelets. Metalloproteinase nature of AiP1 was confirmed by the loss of proteolytic and platelet inhibiting activity post treatment of AiP1 with EDTA.

The cell proliferative and wound healing activity of AiP1 was observed *in vitro* in human keratinocytes (HaCaT cells) and human embryonic kidney epithelium (HEK293) cells. Cadherins are calcium dependent cell adhesion molecules that play an important role in cell-cell adhesion by forming adherent junctions. N-Cadherin is important for cell migration by forming N-Cadherin – actin linkage. Cadherin switching *i.e.* down-regulation of E-Cadherin and up-regulation of N-Cadherin, plays a pivotal role in EMT transition (Priya and Yap, 2015). The change in expression of E- Cadherin post treatment with AiP1 could not be assessed due to low expression levels of E-Cadherin in HEK293 cells (INADA et al., 2016). An increase in N-cadherin expression in HEK293 cells post treatment with AiP1 further confirmed its role in cell migration and further in wound healing.

AiP1 was found to contain a vWFA-like superfamily domain. The von Willebrand factor is a plasma glycoprotein which aids in hemostasis and thrombosis by binding to blood coagulation factor VIII (FVIII) and tethers platelets to injured blood vessel wall (Butera et al., 2018; Peyvandi et al., 2011). It is an indispensable adhesive substrate in platelet-platelet aggregation via the A1 domain binding to the GPIbα (Hassan et al., 2012; Matsushita and Sadler, 1995; Ruggeri, 1997). The vWF plays an instrumental role in hemostasis by forming a complex with collagen in the sub endothelial wall and concurrently binding to specific platelet receptors, the glycoprotein (GP) Ib/V/IX complex and the activated GPIIb/IIIa complex; hence forming a bridging collagens and platelets (Kehrel et al., 1998). Majority of vWFA-containing proteins are all intracellular proteins involved in DNA repair, transcription, ribosomal and membrane transport. Proteins incorporating vWFA-like domains contribute in numerous biological processes like blood coagulation, platelet adhesion and aggregation, cell adhesion, migration, homing, pattern formation, and signal transduction (Bork, 1991; Colombatti et al., 1993; Perkins et al., 1994). Combination of experimental results and peptide mass fingerprinting analysis of AiP1 helped us hypothesize the function of AiP1 in following ways:

1) The wound healing activity of AiP1 could be attributed to the presence of vWFA-like domain in it, which is known to aid in cell migration and adhesion (Hassan et al., 2012). AiP1 treated cells also showed an increase in N-Cadherin levels (Figure 4.18), indicating the acceleration of cell migration and eventually faster wound closure compared to untreated cells.

2) There may also be an unidentified metalloproteinase domain in AiP1 which contributes to its thrombolytic activity (section 0). Since no other protein contaminations were detected by HPLC, the same protein AiP1 may have another domain responsible for its metalloproteinase activity. The presence of a metalloproteinase domain and the vWFA-like domain in the central β sheet of AiP1 could be responsible for its effects on hemostasis. vWF promotes fibrin formation in response to arterial injury in a site-directed manner as a result of its function as a chaperone for factor VIII and ligand for sub-endothelial collagen. It forms covalent crosslinks with fibrin and modifies its structure (increasing its diameter) which enhances fibrinolysis and fibrin polymerization (Marchi and Rojas, 2015; Spiel et al., 2008). The von Willebrand Factor may have a dual role in hemostatic process: Under high shear stress, the vWF enhances platelet aggregation and thrombus formation; at low concentrations it causes thrombolysis and wound healing (Miszta et al., 2014). Adhesion of platelets and collagen is enhanced via binding of vWF at high shear rates (Laduca et al., 1987). The initial binding occurs between vWF and GPIb, which is further supported by interaction of platelet receptor GPIIa/IIIb with fibrinogen (Miszta et al., 2014). This could possibly explain the effects of AiP1 on platelet aggregation, fibrinogenolysis and fibrinolysis (Delaney et al., 2014; Skipwith et al., 2010; Wu et al., 1997).

The increase in platelet aggregation upon treatment with AiP1 at doses up to 5µg/ml might be due to the attachment of the vWFA-like domain present in it, which binds platelets to collagen and promotes aggregation. Time and dose dependent fibrinogenolysis and fibrinolysis of AiP1 could be due to the dual role of the vWFA-like domain present in it. However, the exact mechanism needs to be deciphered. The absence of hemolytic and phospholipase activities in AiP1 render it safe for further drug development.

These findings suggest that vWFA-like domain is the most important region of the AiP1 protein for its thrombolytic and wound healing activities. It remains to be seen whether this domain is also responsible for other biological activities.

The fibrinogenolytic, fibrinolytic and particularly wound healing activity of AiP1 could further be explored using *in vivo* models. This would also help us compare the wound healing activity of

AiP1 with other known wound healing drugs like placentrex, mevastatin, methyluracil and myramistin and the like.

The bioactive potential of an unexplored marine invertebrate, *Astropecten indicus* was thus established and a bioactive peptide, AiP1 was isolated and partially characterised in this study.

5 Summary and Conclusion

The potential of marine derived compounds is as vast as the expanse of the oceans. Marine invertebrates especially sea stars are potential yet unexplored sources of numerous bioactive agents. Of the many sea stars that the Arabian Sea harbors, the *Astropecten indicus* species is most commonly found along the west coast of India. Despite its abundant occurrence along the coast, the *Astropecten indicus* remains largely unexplored.

This study focussed on the use of the coelomic fluid (SCF) of *Astropecten indicus* as a source of novel thrombolytic drugs and wound healing agents. Thrombosis is a cardio-vascular disorder and a leading cause of mortality world-wide. Present day thrombolytic and anticoagulant drugs are associated with side effects such as uncontrolled bleeding leading to hemorrhage or non-specific mode of action. There is thus a demand for novel anticoagulants with controlled activity that target specific coagulation factors.

It was observed that crude SCF contained many proteins and polypeptides, which affected the human cardiovascular system. The crude coelomic fluid (SCF), promoted platelet aggregation as observed using whole blood platelet aggregometer and scanning electron microscopic analysis. It lysed fibrinogen and fibrin in a dose and time dependent manner. The SCF thus affected the human blood coagulation system by affecting the platelets, fibrinogen and fibrin. The major proteases that imparted these activities to SCF were metallo-proteinase in nature. The SCF did not alter the morphology of A549, HEK293 and HaCaT cultured cell lines *in vitro* and rather increased cellular proliferation upon treatment with SCF. Scratch wounds drawn on the surface of cultured cells showed faster wound closure rates post treatment with SCF compared to untreated cells. The thrombolytic and wound healing potential of SCF was thus discovered.

A novel protein AiP1 was purified from SCF by a combination of anion exchange chromatography and size exclusion HPLC. This is the first protein isolated from the sea-star *Astropecten indicus* and was found to sustain the fibrinogenolytic and fibrinolytic activities. It inhibited ADP and collagen induced platelet aggregation. The proteolytic activity of AiP1 sustained till a temperature of 70˚C and was inhibited at temperatures above 70˚C and in presence of EDTA. AiP1

promoted cell proliferation and accelerated wound healing in cancerous as well as normal cell lines cultured *in vitro*. Additionally, AiP1 was devoid of hemolytic, phospholipase and laminin digestion activities. This renders AiP1 a possibly safe candidate to be explored in drug design and cosmetic industries. Characterisation of trypsin digested AiP1 using bio-informatics tools revealed homology of one of its peptides (peptide1) to the vWFA type domain. This protein family is important for various biological processes such as platelet aggregation, blood coagulation, DNA repair, cell adhesion and cell migration. Some vWFA domains are known to contain a metal ion dependent binding site and a binding site for plasma protease ADAMTS13 (a disintegrin and a metalloproteinase with thrombospondin type 1 motif, member 13).

A combination of experimental and computational analysis of AiP1 confirms its potential as a thrombolytic and wound healing agent. This is the first report on the potential of coelomic fluid of *Astropecten indicus* as a thrombolytic and wound healing agent and AiP1 is the first protein isolated from this species and charaterised for various bioactivities.

Some important contributions emerging from this study are as follows:

1. A novel spectrophotometric method for the real estimation of fibrinolytic activity of a compound was developed which is simple, fast and economic.
2. An unexplored marine organism, the *Astropecten indicus* was reported for its thrombolytic and wound healing potential
3. A novel protein, AiP1, was isolated from SCF which aided in thrombolysis and wound healing.
4. AiP1 contained a vWF-A like domain which could be responsible for its bioactivities.

Future Prospects of this Study

Given that the marine biodiversity is much greater than that recorded on land, the discovery of marine derived natural products will increase multifold in the coming years. This may provide novel and better therapeutics for human ailments and other products for industrial and biotechnological applications like nutraceutics and cosmetics (Leal et al., 2012).

Of all the marine natural products that are being investigated, peptides and polypeptides have a broad spectrum of applications from pharmaceuticals to cosmetics. With significant advancements in the sensitivity of analytical techniques, spectroscopy and high-throughput screening, pure peptides from natural resources can now be easily extracted and characterized. The characterization can be followed by a synthesis scheme to produce them on a large scale for pharmaceutical applications. Furthermore, some of these active peptides can be modified for intravenous or oral administration (Leal et al., 2012; Sable et al., 2017).

This study focussed on studying an unexplored bioresource: the coelomic fluid of *Astropecten indicus.* We were successful in extracting SCF without harming the animals and report it's thrombolytic and wound healing activities. A novel protein AiP1 was isolated and partially characterized.In the process of this study, a novel method was also developed for the estimation of fibrinolysis in an effective and real time manner.

Given that the parameters for SCF extraction and AiP1 isolation were standardized and optimized in this report, this protein could be further studied for a spectrum of applications ranging from its anti-diabetic potential to cosmetic application. A complete biochemical characterization, structural analysis and finally *in vivo* studies of AiP1 could help us decipher its exact mode of action as a thrombolytic and a wound healing agent.

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Appendix I: List of publications and conference proceedings

1. Patent published with the Indian Patent Office in April 2013: *Spectrophotometric Real Time method for Assessment of Fibrinolytic Activity*: (Ref. no. 1370/MUM/2013); presently at final stage of patent application.

2. Hemotoxic and wound healing potential of coelomic fluid of sea-star Astropecten indicus **M Baveja**, A Sarkar, D Chakrabarty. The Journal of Basic and Applied Zoology 79 (1), 27.

3. Purification and partial characterization of AIP1: A novel protein from sea-star (Astropecten indicus) coelomic fluid. **M Baveja**, A Sarkar, S Mondal, D Chakrabarty. Toxicon 159, S10-S11.

4. Hemotoxic activity of sea-star coelomic fluid. **M Baveja**, KN Kiran, D Chakrabarty. Toxicon, 80.

5. Anticoagulant and wound healing properties of sea-star (Astropecten indicus) coelomic fluid. D Chakrabarty, **M Baveja**, A Sarkar. TOXICON-OXFORD- 158 (1), S37-S37.

6. AiP1, a Protein from the Coelomic Fluid of Sea star Astropecten indicus Promotes Wound Healing and Fibrinogenolysis in vitro **M Baveja**, A Sarkar, S Mondal, J Pathan and D Chakrabarty. accepted for publication (after minor revisions suggested) in the Journal of Basic and Applied Zoology, April 2019

Appendix II: Accolades received by this work in national and international conferences and seminars

1. Awarded the Prestigious Young Researcher Award for Excellence in the Field of Life Sciences for the year 2018 by the Society of Young Biomedical Scientist.

2. Awarded 1st prize in Poster presentation (Life Science Category) at the National Biomedical Research Competition held at AIIMS Rishikesh on 15th October 2018.

3. Awarded 4th prize in Poster Presentation (Innovations and Patents category) at the National Biomedical Research Competition held at AIIMS Rishikesh on 15th October 2018.

4. Awarded 1st prize in Oral Presentation at the DBT sponsored National Conference Bioactive Marine Natural Products and their Therapeutic Application, held at Satyabhama Institute of Science and Technology, Chennai, from 24-25th January 2018.

5. Awarded 2nd prize in Three Minute Thesis competition held in BITS Pilani, K K Birla Goa campus, Goa, May 2017

6. Presented a poster in the 22nd Meeting on Toxinology, The French Society of Toxinology (SFET) held in Pasteur Institute, Paris, France in December 2014.

7. Won best presentation award for presenting poster titled "Spectrophotometric Real Time Method for Assessment of Fibrinolytic Activity" in an International conference TSICON 2013 held in Goa, December 2013.

8. Won best poster award in a national seminar on "Advanced Chromatographic techniques" held in Mysore, February 2013.

9. Won best poster award for presenting a poster titled “Anticoagulants from Russell’s viper venom” in a conference on “Advances in Biological Sciences” held in Goa University, Goa, February 2012.

Appendix III: Brief Biographies of the candidate and supervisors

Brief Biography of the Candidate

Name	Ms. Mansi Baveja
Education	M.E. (Biotechnology), BITS Pilani, K K Birla Goa campus, Zuarinagar – 403726, Goa. B.Tech. (Biotechnology), GITAM College of Engineering, Andhra University, Vishakapatnam.
Email	baveja.mansi@gmail.com

Research Experience:

Ms. Mansi Baveja joined BITS Pilani K K Birla Goa campus as a research scholar in department of Biological Sciences in January 2013. She worked as a DST INSPIRE from 2013 to 2018 on her Ph.D. Thesis titled "Study on Coelomic Fluid of *Astropecten indicus* and Isolation and Characterisation of a Bioactive Agent". Her work encompassed collection of sea star *Astropecten indicus* from the coast of Goa in India, extraction of coelomic fluid (SCF) from them, isolation of bioactive peptides from SCF and partial biochemical characterisation of isolated peptides. During the course of her research work, she was the primary inventor of a novel spectrophotometric method for the estimation of fibrinolytic activity of a compound which is presently under examination at the Indian patent office. She has published one research article and has communicated one article as the first author. Her research work has been presented in numerous conferences and seminars for which she has been awarded the best presentation award many times. She has also been the recipient of the Young Researcher Award in the field of Life Sciences by the Society of Young Scientists for the year 2018.

Brief Biography of Prof. Dibakar Chakrabarty

Name	Dr. Dibakar Chakrabarty
Designation	Professor
Education	Ph.D., Jadavpur University, Kolkata
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Research Experience

Dr. Dibakar Chakrabarty completed his PhD under the guidance of Prof. Antony Gomes, a pioneer pharmacologist in India. Dr. Chakrabarty followed his research interest with his Post Doctorate at the Indian Institute of Science, Bangalore, India after which he started teaching Physiology at reputed medical colleges. His major research interest lies in the search of novel thrombolytic agents from natural sources, both marine and terrestrial. He has already reported novel anticoagulants from Indian cobra venom, Russell's viper venom, Jellyfish tentacle extract and many more. He has innumerable research articles and book chapters which can be found on the university website link: http://universe.bits-pilani.ac.in/goa/dibakarchakrabarty/Publications.

Brief Biography of Dr. Angshuman Sarkar

Name	Dr. Angshuman Sarkar
Designation	Associate Professor
Education	Ph.D., NCCS, Pune
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Dr. Angshuman Sarkar completed his Ph.D. in the subject of Biotechnology under the guidance of Prof. Jayanta K Pal. He pursued further research experience as a Post Doctorate candidate in various reputed institutes such the Molecular Development Laboratory, Cold Spring Harbour in New York, USA; School of Micro Biology and Molecular Biology , Case Western Reserve University in Cleveland, Ohio, USA; and also in the department of Cancer Biology, Cleveland Clinic Foundation, Cleveland, Ohio, USA.

He is presently engaged in *in vitro* cell biology and is an Awarded fellow from the All India Society of Cell Biology in 2016.

He has more than 20 research publications which can be found at http://universe.bits-pilani.ac.in/goa/asarkar/[ublication.

Appendix IV

Annexures

1. Institute Human Ethical clearance form for this work
2. Published Patent related to this work
3. Published Research Articles related to this work
4. Published Abstracts in Special issue.
5. Newspaper report in The Times of India based on this work
6. Newspaper article in Kuriocity supplement of The Navhind Times about this work and the authors

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI, RAJASTHAN

INSTITUTIONAL HUMAN ETHICS COMMITTEE

Ref. No: IHEC/17/ M/1 Date: 12/12/2017

To
Dr. Dibakar Chakrabarty
Associate Prof. Dept. of Bio.Sciences
BITS Pilani, K. K Birla- Goa Campus

Subject: Approval of submitted research proposal

This is to inform that your proposal submitted to the Institutional Human Ethics Committee has been approved as mentioned below, at the 16th meeting of the committee held on Tuesday, December 12, 2017 at 2.30 pm, HOD office Department of Pharmacy, BITS, Pilani

Name of PI	Dr. Dibakar Chakrabarty
Proposal No.	T- IHEC-R-43/17-1
Approval No.	IHEC-43 /17-1
Title	Study of Hemotoxic and Cytotoxic Agents from Sea star (Starfish) Coelomic Fluid
Date of Approval	12/12/17
Date of Expiry	11/12/18

You are hereby directed to

* Quote the above approval No. for all further correspondence with the committee.
* Also maintain, and submit the records of subject requirement and subject data sheet of the human participants in the study.
* Ensure that all the studies are done under the supervision of the person (s) whose name have been approved under the proposal submitted.
* Reports any unusual events, accidents or emergencies encountered during the course of the study to the committee.
* PIs have to submit an interim progress report on a six month, ~~or yearly~~ basis and project completion report should be submitted within one month from the end of the project period.

Yours truly,

Dr. Rajeev Taliyan
(Member Secretary, IHEC)
BITS-Pilani, Rajasthan

Prof. Hemant R.Jadhav
(Chairman, IHEC)
BITS-Pilani, Rajasthan

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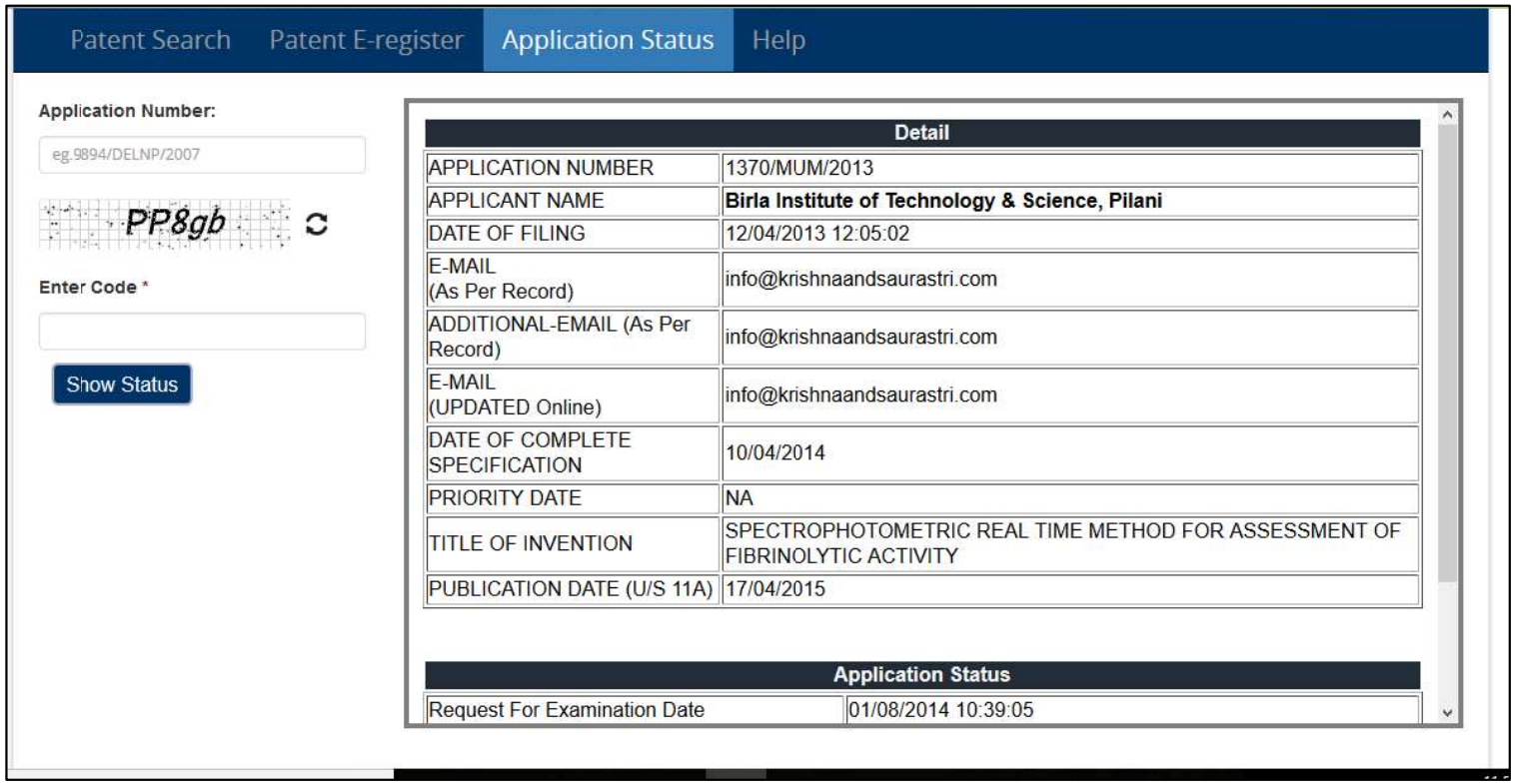
Non-compliance of IHEC guidelines may lead to cancellation of an approval

Patent publication report on Indian Patent Office website



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The Journal of Basic and Applied Zoology

RESEARCH Open Access

Hemotoxic and wound healing potential of coelomic fluid of sea-star *Astropecten indicus*

Mansi Baveja, Angshuman Sarkar and Dibakar Chakrabarty*

Abstract

Background: The coelomic fluid of echinoderms bathes all the internal organs and is a natural reservoir of various bio-active compounds. However, coelomic fluid from Indian sea-star *Astropecten indicus* is not well characterized for its biochemical and pharmacological profiles. Sea-star (or starfish) *Astropecten indicus* was collected from the coast of Goa, India during low tides. Sea-star coelomic fluid (SCF) extracted from the sea-stars was concentrated, filtered, and assayed for various hemotoxic activities such as fibrinogenolysis, fibrinolysis, hemolysis, and effects on platelet aggregation.

Results: Studies on effects of SCF on A-549 human lung cancer cell line showed its non-cytotoxic and wound healing abilities in vitro. SCF was found to possess fibrinogen and fibrin digesting ability at a dose of 4 μg/ml within 30 min of incubation with the substrate. Additionally, SCF could hemolyze goat RBC by 50% and human RBC by only 5%. SDS-PAGE analysis showed that SCF contains various proteins and peptides. Two protein components of SCF yielded strong fibrinolytic, fibrinogenolytic, caseinolytic, and gelatinase activities as revealed by zymography. SCF enhanced the aggregation of ADP and collagen-activated platelets in a dose-dependent manner and had a specific proteolytic activity of 2.5 μg/ml in 30 min at 37 °C as assayed using azocasein as substrate.

Conclusion: This study focuses on the anticoagulant, pro-platelet aggregation and cell proliferative potential of SCF in vitro and is the first report on hemotoxic and wound healing potential of coelomic fluid of *Astropecten indicus*.

Keywords: Coelomic fluid, Fibrinogenolysis, Fibrinolysis, Hemolysis, Wound healing, Cell proliferation

Background

More than 70% of the surface of the earth is covered with water and has an immense biomass (Montaser & Luesch, 2011; Newman & Cragg, 2014). It is well known that the marine environment, despite having an infinite scope for exploration, has been one of the most under-utilized biological resources. It contains a vast array of organisms with unique biological properties which could provide a vast resource to combat major diseases (Cardoso, Costa, & Mano, 2016; Vinothkumar & Parameswaran, 2013).

Terrestrial animals, however, have been exploited for many decades as medicinal resources for the treatment and relief of a myriad of illnesses and diseases in practically every human culture (Costa-Neto, 2005; Montaser & Luesch, 2011). Marine natural products are now the focus of scientific investigation to discover novel natural products which could be used as medicines (Bordbar, Anwar, & Saari, 2011).

Members of the class Echinodermata such as the sea-stars (or starfish) have become virtually a symbol of sea life. They were used in ancient oriental medicine as a source of bioactive compounds and are now used for extraction and purification of cytotoxic, hemolytic, antiviral, antifungal, antifouling, antimicrobial, and even anti-tumoral compounds (Bordbar et al., 2011; Cardoso et al., 2016; Lee, Hsieh, Hsieh, & Hwang, 2014; Sharmin, 2017; Thao, Luyen, Kim, Kang, Kim, et al., 2014).

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Some part of this work has been presented by MB in the 22nd meeting of the French Society of Toxinology, Toxins: New Targets and New Functions held in Pasteur institute, Paris, France in December 2014. The abstract was published in special issue of Toxicon titled "Hemotoxic Activity of Sea-star Coelomic Fluid", Toxicon 116 (2016) 72-86
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processes induced by *Androctonus australis hector* (Aah) scorpion venom, using a selective antagonist of this receptor as pretreatment (JNJ-7777120). Inflammation in hepatic and renal tissues was evaluated by histological analysis, vascular permeability changes, and cellular peroxidase activities (eosinophil peroxidase and myeloperoxidase) and also, by measuring some serum metabolic enzyme activities. The Aah scorpion venom induced a marked tissue disorganization of the liver parenchyma, with appearance of necrotic zones and centrolobular vein congestions. The renal cortex revealed a reduction of light tubules, hemorrhages and edema formation with inflammatory cell infiltration. The migration of inflammatory cells into these tissues was indicated by the high myeloperoxidase and eosinophil peroxidase activities, preceded by the elevation of the hepatic and the renal vessel permeability associated with elevated serum enzyme activities. The inhibition of histamine access to the H4-receptor before the experimental envenomation resulted in a prevention of many histological alterations including centrolobular vein congestions in the liver, hemorrhages, edema and inflammatory cell infiltration in the renal tissue. Moreover, this antagonist was able to decrease both hepatic and renal vascular permeability and to reduce neutrophil influx into these tissues. Eosinophil infiltration was markedly reduced in the liver but seems to be slightly decreased in the renal tissue. In addition, this pretreatment reduced the serum metabolic enzyme activities. Obtained results point to the H4 receptor as a new target for inflammatory signal modulation and offer a perspective for the therapeutic exploitation of this pharmacological target in the scorpion venom-induced excretory system inflammatory disorders.

HEMOTOXIC ACTIVITY OF SEA-STAR COELOMIC FLUID

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The marine venomous animals collectively represent the largest group of venomous species. To date approximately 16,000 marine natural products have been isolated from marine organisms. Members of the class Echinodermata such as the sea-star or starfish have become virtually a symbol of sea life. They were used in ancient oriental medicine as a source of bioactive compounds and are now used for extraction and purification of cytotoxic, hemolytic, antiviral, antifungal, antifouling, antimicrobial and even anti-tumoral compounds.
Anticoagulants and platelet aggregation inhibitors are in high demand due to their ever increasing demand as therapeutic molecules against thrombosis, which is one of the major killer diseases of modern times. Biochemical and pharmacological profiles of sea-star coelomic fluid (SCF) are not well studied in India. Very little effort has been made till now to discover potential drugs from sea-stars. Sea-star *Astropecten indicus* was collected from the coast of Goa, India during low tides. Coelomic fluid was obtained by injecting minimal volume of phosphate buffer saline through the aboral pore of starfish and immediately withdrawing some coelomic fluid. Care was taken to not extract too much SCF from one starfish so that they are not sacrificed. The starfish were released back into the sea after extraction of SCF. The SCF after extraction was dialysed, concentrated and assayed for various hemotoxic activities such as fibrinogenolysis, fibrinolysis and hemolysis. It was found to possess fibrinogen and fibrin digesting ability at a dose of 4 μg/ml within 30 minutes of incubation with the substrate. Additionally, SCF could hemolyse chicken RBC by 98.4%, fish RBC (*Pangasius sp.*) by 30.58% and human RBC by 5.32%. Histopathological studies revealed no change in fish injected with SCF. SDS-PAGE analysis showed that SCF is a cocktail of various proteins and peptides. Three protein bands yielded strong fibrinolytic, fibrinogenolytic, caseinolytic and gelatinase activities as revealed by zymography. Fractionation of SCF by DEAE Sephadex ion exchange resin and other chromatography techniques yielded pure protein(s) showing proteolytic activity at very low concentrations. Further purification of these proteins to yield pure toxin(s) and characterization of these toxin(s) is presently being pursued.

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IONIC MECHANISMS IMPLICATED IN CIGUATOXIN-INDUCED MEMBRANE HYPEREXCITABILITY AND CELL SWELLING

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Ciguatoxins (CTXs) are a family of lipid-soluble, highly oxygenated, heat stable, cyclic polyether compounds responsible for ciguatera, a human seafood intoxication linked mainly to benthic dinoflagellates (*Gambierdiscus sp.*) and acquired by eating contaminated fish. The aim of the present study was to determine the ionic mechanisms implicated in Pacific ciguatoxin-1B (P-CTX-1B)-induced membrane hyperexcitability and cell swelling of NG108-15 neuroblastoma cells and frog myelinated axons, using electrophysiology and confocal microscopy. No marked variation in the three-dimensional projected area of NG108-15 neuroblastoma cells stained with FM1-43, measured as an index value of cell volume, was detected during the action of P-CTX-1B, although the toxin induced a significant and tetrodotoxin (TTX)-sensitive increase in the relative fluorescence intensity of Sodium Green-loaded cells. Taking into account that P-CTX-1B produced transient repetitive action potentials only after cell stimulation but not spontaneously, these results strongly suggest that Na^+ ions flowing through toxin-modified voltage-gated sodium channels are not sufficient to produce cell swelling, and that those flowing through unmodified sodium channels activated during spontaneous and repetitive action potentials are necessary. In frog myelinated axons, substituting external NaCl by NaMeSO4 did not affect P-CTX-1B-induced spontaneous and repetitive action potentials and axonal swelling. This indicates that the toxin action was not dependent on external Cl^- ions. In contrast, substituting external NaCl by LiCl suppressed the spontaneous action potentials and prevented the axonal swelling induced by the toxin. This strongly suggests that the selectivity of toxin-modified voltage-gated sodium channels is less for Li than for Na^+ ions, and further supports that Na^+ entry through channels opened during spontaneous action potentials is required to produce cell swelling. Finally, blocking voltage-gated potassium channels with tetraethylammonium (TEA) or 3,4-diaminopyridine did not prevent P-CTX-1B-induced spontaneous and repetitive action potentials but markedly reduced axonal swelling. Interestingly, blocking potassium channels with TEA and allowing K^+ efflux with valinomycin restored a marked toxin-induced axonal swelling that was reversed by hyperosmolar D-mannitol and prevented by TTX. In conclusion, water movements responsible for cell swelling are dependent on both Na^+ influx and K^+ efflux during the action of P-CTX-1B. This may help to understand the human neurological symptoms induced by ciguatera fish poisoning, especially a decreased nerve conduction, and paves the way for further studies regarding treatment of this poisoning.
This work was partially funded by grant Aquaneurotox ANR-12-ASTR-0037-01 from the Agence Nationale de la Recherche (France).

BIOPHYSICAL INVESTIGATIONS OF THE ADENYLATE CYCLASE (CyaA) TOXIN FROM *BORDETELLA PERTUSSIS*

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Adenylate cyclase (CyaA) is one of the major virulence factors produced by *B. pertussis*, the causative agent of whooping cough (Ladant and Ullmann, 1999). The toxin is a large and complex multi-domain protein (1706 aa) synthesized as an inactive precursor, pro CyaA, that is converted into the active form upon a specific acylation of two residues of lysine (Lys 860 and Lys 983). This is performed by a specific acyltransferase coexpressed in the bacteria. Once secreted across the bacterial cell envelope through a type I secretion machinery, the toxin invades eukaryotic

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Goa-based scholar discovers thrombolytic and wound healing properties of starfish

RAMANDEEP KAUR | NT KURIOCITY

When one thinks of starfish, the first thing that usually comes to mind is how pretty they look. Yet besides this, these sea creatures are also model organisms for regenerations studies owing to their immense regenerative potential. And Mansi Baveja, a PhD scholar at BITS Pilani K K Birla Goa Campus set out to research further on the starfish found in Goa.

Based in Goa, Mansi together with her PhD guide professor Dibakar Chakrabarty and co-guide Angshuman Sarkar wanted to design a study based out of local natural resources available here.

"With hundreds of sea stars being washed ashore especially during the months of September-November, we wanted to explore them for their biomedical potential," says Mansi. However, with starfish being a relatively unexplored species, they faced a lot of challenges in designing the work and standardising protocols.

In fact it took them nearly two years just to figure out that the best time to catch them and was during September-November. They then designed their work plan such that the samples for research were collected during these two months, processed and then stored for use for the rest of the year.

During the course of their research they discovered that the body fluid of these starfish had therapeutic potential and the findings was published through a publication house called Springer Nature. "The coelomic fluid (or body fluid) of Astropecten indicus (the common sea star found along the coast of Goa and Maharashtra) contains proteolytic, thrombolytic and wound healing compounds. Thrombosis is one of the most popular cardio vascular disorders that causes thousands of deaths each year," says Mansi.

Explaining how she stumbled upon these findings, Mansi discloses that she was in fact exploring many bio-activities in this particular sea star species. One of her research objectives was to explore cytotoxic agents present in them which could further be used to target cancer cells. "On the contrary, we observed that cells proliferation was increasing post-exposure to this coelomic fluid.

Continued on page 8

PIC BY VIPUL REGE | NT KURIOCITY

Mansi Baveja, a PhD scholar at BITS Pilani K K Birla Goa Campus who has been researching starfish found in Goa has discovered that the body fluid of these sea creatures has therapeutic potential. **NT KURIOCITY** learns more

How will the properties of starfish benefit man?

Deep vein thrombosis leading to pulmonary embolism is one of the leading causes of death worldwide. Together, DVT and PE are referred to as venous thromboembolism (VTE), the third largest cardiovascular killer worldwide after heart attack and stroke. This can be treated with administration of thrombolytic drugs like heparin and warfarin, etc. which are associated with side effects such as uncontrolled bleeding, haemorrhage. Therefore the search for ideal thrombolytic drugs from many sources is on. The coelomic fluid of star fish being non-cytotoxic could be a useful lead in developing novel anticoagulant drugs with lesser side effects.

Goa-based scholar discovers thrombolytic and wound healing properties of starfish

Continued from page 1

We repeated our experiments with different kinds of cells and each time we observed an increase in cell viability upon exposure to the coelomic fluid of this sea star. This led us to change the course of our work and study its wound healing potential instead," she says.

Mansi states that theirs is the first report on the bio-active potential of the starfish Asropecten indicus to be published internationally, adding that there is an extreme death of available literature on the bioactive potential of this species.

Apart from this there is only one national level report on this species which denotes its possible anti-microbial potential and another report on Saponins extracted from these species.

"Most reputed scientific journals aim to publish a novel, characterised and purified bio-compound from natural sources. It is extremely difficult to make an international level publication reporting the bioactive potential of a crude resource." In fact, Mansi and her guide faced a lot of rejection over a year before they could finally substantiate the work with repeated experimentation making it eligible for acceptance for publication.

Originally from Delhi, Mansi further says that researchers normally lose motivation if they are not given a platform to showcase their original findings just because it is not groundbreaking enough. "Every result is important, even negative ones. It would be great if those could be given as much importance as positive results," she says.

Starfish hold key to treating blood clots

Scientists: Fluid In Organism Can Beat Thrombosis

Newton.Sequeira
@timesgroup.com

NATURE'S CURE

- Starfish are predominantly **found between Sept & Dec** along Goa's coast
- Marine biologists have discovered the **potential of the body fluid of these species to treat thrombosis**
- Research shows that starfish **coelomic fluid contains molecules that can dissolve blood clots and heal wounds**

Incidence of **venous thromboembolism in Asia and India is comparable** to that in Western countries

Panaji: Just as the monsoons begin to subside, thousands of starfish begin to flourish along the Konkan coast, often washing up on the shore with the ebb and flow of the tide. These marine invertebrates, the Astropecten indicus species, are predominantly found between September and December along Goa's coast.

Marine biologists have now discovered that these delicate starfish could hold the key to treat deep vein thrombosis and pulmonary embolism, a common and potentially life threatening condition that affects thousands of patients in India and globally.

"We observed the potential of the body fluid of these species (known as the coelomic fluid) to treat thrombosis, a cardiovascular disease which is a leading cause of death world-wide," says Mansi Baveja, a PhD scholar with the BITS Pilani biological sciences department.

Thrombosis is a cardiovascular condition wherein blood clots appear within the blood vessels and travel via the blood circulation to other parts of the body. It can completely stop the blood flow through arteries or veins and eventually cause death.

This potentially life-threatening condition, often remains under diagnosed, and awareness among Indians regarding this potentially life-threatening disease remains woefully low. Professor Dibakar Chakrabarty, who is pursuing research in anticoagulant toxins in jellyfish and starfish venoms, found that the regenerative ability of a starfish offered a potential treatment for blood clots.

"Starfish has a body cavity or coelom, which is bathed by a fluid. This fluid contains a great variety of substances ranging from nutrients, factors responsible to maintain immune system, enzymes and many other molecules required for survival of starfish," says Chakrabarty.

The professor, who teaches at BITS Pilani's Goa Campus, says that the starfish has a legendary capacity to grow its arms when they are severed accidentally. "In our search for the molecules responsible for this great healing ability, a molecule was found that not only can heal wounds created on a layer of cells in a petri-dish, but could also effectively dissolve artificially made blood clots," he says.

The incidence of venous thromboembolism in Asia and India is comparable to that in western countries. It is a common medical complication associated with hospitalization and preventable death. While no definite statistics are available for India, research data shows that it takes as many as 30,000 lives every year in the United States alone.

"There is a need for thrombolytic drugs of natural origin having lesser side effects. The coelomic fluid of sea star Astropecten indicus was found to be non-toxic and hence offered potential as a useful drug," Baveja says.

The young researcher says that despite the starfish's natural ability to heal itself, no wound healing agents from been reported from sea stars till date. The invertebrate species exhibit remarkable potential in the pharmaceutical as well as cosmetic industries, say researchers at the BITS Pilani, K K Birla Goa Campus.

Chakrabarty is quick to exercise caution, and says that further scientific tests are required to establish and develop a marketable treatment for mankind.

"Our results show that starfish coelomic fluid contains molecules with very good ability to dissolve blood clots and heal wounds. The results, however, need to be replicated in animals before making a comment that 'we have found the ultimate medicine to heal all wounds'," he said.

prevent any inconvenience to traders and the public that could be caused by dust pollution if carried out after the rains. He added that the demolition will be carried out by a contractor who has offered to execute the demolition of the building at no cost to the municipality.

Though the CCMC had evicted all occupants of the building, many have returned to their old premises to conduct business as usual, with the municipality turning a blind eye to the development.

Phadte, while admitting that some traders have refused to shift, stressed that the demolition work would go ahead as scheduled. "It would take nearly one to one-and-a-half months to complete the demolition work," he said.

Following an order of the high court of Bombay at Goa, the CCMC had ordered all 42 traders occupying the building to vacate the premises. While most of the occupants of the old municipal building have been accommodated in the Goa State Urban Development Agency's (GSUDA) commercial complex near the KTC bus stand, some have been allowed to erect temporary structures in the nearby plot owned by the municipality "at their own cost and risk".