STUDIES ON THE EFFECTS OF RUSSELL'S VIPER VENOM ON HUMAN ALVEOLAR CANCER CELLS AND THE INVOLVEMENT OF SMALL GTPASES

THESIS

Submitted in partial fulfillment
of the requirements for the degree of
DOCTOR OF PHILOSOPHY

by

JIGNI PATHAN

Under the supervision of

Dr. Dibakar Chakrabarty
&
Dr. Angshuman Sarkar

BITS Pilani
K K Birla Goa Campus

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI
K. K. BIRLA GOA CAMPUS
2017

Department of Biological Sciences
Birla Institute of Technology and Science
Pilani K K Birla Goa Campus
Zuarinagar - 403 726, Goa, INDIA.

CERTIFICATE

This is to certify that the thesis entitled ***"Studies on the effects of Russell's viper venom on human alveolar cancer cells and the involvement of small GTPases"*** and submitted by Jigni Pathan ID NO 2011PHXF0006G, under our joint guidance for the award of Ph.D of the Institute embodies original work done by her under our supervision.

(Dr. Dibakar Chakrabarty)
(Supervisor)
Associate Professor

(Dr. Angshuman Sarkar)
(Co-supervisor)
Associate Professor

Date: 22/01/2018

This Thesis is dedicated to .....

..... My beloved Father

ACKNOWLEDGMENTS

I would like to acknowledge that the work presented in my thesis is a result of concerted efforts by many people to whom I express my heartfelt thanks for contributing to various phases of my work.

Prior to everything, I would like to express my deep sense of gratitude towards my principal supervisor Dr. Dibakar Chakrabarty, Associate Professor in Biological Sciences, BITS-Pilani K K Birla Goa campus, for granting me the opportunity to work in his research group. Thank you sir, for believing in me since day one. And i thank for his invaluable guidance, wonderful support, unstinted efforts and timely help in planning and executing the work in time. The thoughts he has offered have enriched my thesis. Without his encouragement, guidance and support, completion of this thesis would not have been possible. I learned a lot of things from him for over the last five years of research, you have reinforced in me the values of independence and perseverance and I am confident that it will stand me in good stead in the future.

I would like to express my sincere gratitude to my co-supervisor Dr. Angshuman Sarkar, Associate Professor in Biological Sciences, BITS-Pilani K K Birla Goa campus, for giving me an opportunity to work on my favourite subject under his guidance, for the continuous support during my study, for his patience and motivation. He has always been approachable, open to new ideas and given me intellectual freedom while providing valuable insights. My appreciation to Dr. Dibakar Chakrabarty and Dr. Angshuman Sarkar cannot be described in words. I am blessed to have them as my mentor.

I take this opportunity to express my special thanks to Prof. S.Bhattacharya, Vice-Chancellor, Prof. S.K. Verma, Dean, ARD, BITS-Pilani, Prof. Raghuraman G, Director, Prof. Sunil Bhand, Dean, SRCD, Dr. Prasanta Kumar Das Associate Dean, ARD, Dr. Judith Maria Braganaca, Head of the Department, Biological Sciences, BITS-Pilani K K Birla Goa Campus, for all the support.

I wish to thank my DAC members Dr. Utpal Roy and Dr.Indrani Talukdar who evaluated my research proposal and guided the progress of my research work during the DAC presentations

I owe a great debt of gratitude towards Birla Institute of Technology and Science, Pilani K K Birla Goa campus for supporting me with Institute fellowship. I also express my gratitude towards University Grant Commission (UGC), for the Maulana Azad National Fellowship (MANF), India, for supporting me to carry out my research. I would like to thank Department of Biotechnology (DBT), India for travel grant for presenting a poster at 22nd Meeting of the French Society of Toxinology (SFET) 2014, Paris, France.

I gratefully acknowledge Prof. Sunil Bhand for sharing microscopy facilities. I extend my graditude towards School Of Life Sciences, Manipal for providing the facility of MALDI-TOF-MS for characterization of protein sample. I also appreciate the hard work of the service staff, especially Veena, Pratap, Kamna, Mahadev and Mahalinga.

It has indeed been my very good fortune to work with members of the venom group Chandrashekar Chanda, Akriti Rastogi, Mansi Baveja, Chitra Nair and Clarice Periera, the departmental research scholars especially Ramya Ramachandran, Ajay Ghosh Chalasani, Om Prakash and Mohammad Monsoor Shaik whose experiences and expertise I have benefited from. The friendly atmosphere they created has been and always be unforgettable.

Thanks to all the friends I have made from all walks of life, from high school and through my undergraduate and postgraduate studies. They have accepted me as I am and helped to add a lighter side to life.

My love goes to my parents, brother, Sisters and brother-in-laws and their kids, for showing an interest in my work and for their constant unfailing encouragement, love, prayers and wholehearted encouragement rendered to me with all kinds of support and countless blessings by strengthening my soul.

I am grateful to my husband, Mr. Akbar Pathan, for being the source of inspiration and my spirital support when I face difficulties in my life and who supported me emotionally throughout my study.

My great love and thanks to my in laws for understanding how much my studies meant to me, a big thank you for taking care of my smallest needs, and always having faith in my abilities. Their patient understanding and encouragement have finally paid off.

Anam my little angel, goal of my life, who patiently waited for me, you were Mummy's mental break, keeping me entertained all the way through, while watching you grow along with my work.

I also bow my head before the Almighty for guiding, supporting and answering to my prayers throughout this journey, the help He has sent my way from so many (and sometimes unexpected) quarters. I am greatly indebted to him for showering me with the most wonderful surprise that one could ever wish for.

JIGNI

ABSTRACT

Russell's viper (*Daboia russelii*), an important member of the Viperideae family of snakes is widely distributed over large areas of Asia. Russell's viper is one of the most frequently encountered venomous snakes responsible for majority of snakebite morbidity and mortality in many Southeast-Asian countries. Viper venom is considered to be one of the most potent toxins and is known for its cytotoxicity. In this thesis, we have demonstrated the cytotoxic effects of Russell's viper venom (RVV) and Daboialectin on human alveolar adenocarcinoma (A549) cells growing *in vitro*. For the ease of understanding, this thesis is divided into following chapters:

Chapter 1: This covers a brief introduction about snakes, and specifically Russell's viper, their classification, structure, function and evolution and a review of relevant literature. This chapter also focuses on the pathophysiological significance of the isolated protein(s)/peptide(s) from snake venoms and provides a brief detail on the biomedical application of such venom-derived protein(s)/peptide(s). An insight in to the story of Rho GTPases and a brief note on mode of cell death is included. This chapter also resents the aim and objectives of the study.

Chapter 2: In this chapter the cytotoxicity of RVV on A549 cells and its effects on regulation of small GTPases and apoptotic related proteins is explained. The results of this section concludes that Russell's viper venom-exhibits cytotoxic effects against A549 cells in a dose and time dependent manner with decreased expression of Hsp70, anti-apoptotic proteins and small GTPases (RhoA and Rac1) with up-regulation of Cdc42, pro-apoptotic proteins and p53 levels.

Chapter 3: This chapter describes the isolation, purification and characterization of a C- type lectin from Russell's viper venom. The observations from this chapter concludes that the low

molecular weight C-type lectin (18.5 kDa) isolated from Russell's viper venom named 'Daboialectin' showed cytotoxic effects on human broncho-alveolar carcinoma derived (A549) cell lines.

Chapter 4: This chapter includes the results of the activity of the purified protein Daboialectin on A549 cells and elucidates the possible mechanism involved in changes in morphology of the cells and also describes the mechanism of cell death caused by Daboialectin.

Summary and conclusion includes the major findings and visualizes the future prospects of the research findings of this study.

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LIST OF ABBREVIATIONS

A549 cells	broncho-alveolar carcinoma-derived cell line
AO	acridine orange
BSA	bovine Serum Albumin
CLPs	C-type lectin-related proteins
DDW	double distilled water
DMSO	dimethyl sufloxide
EDTA	ethylene diamine tetra acetic acid
EtBr	ethidium bromide
FBS	fetal Bovine Serum
h	hour
HPLC	high performance liquid chromatography
IEC	Ion-exchange chromatography
LD	lethal dose
MALDI	matrix assisted laser desorption/ionization
MEM	minimum Essential Medium
mg	milligram
ml	milliliter
MS	mass spectrophotometry
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
MW	molecular weight
PBS	phosphate buffered saline
PCR	polymerase chain reaction
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
RVV	russell's viper venom
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard Error of the Mean
µg	microgram

LIST OF MATERIALS

Sources of various chemicals employed in the study are as follows:

Chemicals	Company
2- Mercaptoethanol	Sigma, USA
Acridine orange	Sigma, USA
Acrylamid/bis-Acrylamid	Sigma, India
Agarose for gel electrophoresis	Invitrogen, USA
Ammonium persulfate (APS)	Merck, India
Ampicillin (100mg/ml in dH2O, stored at -20°C)	Sigma, USA
Bovine Serum Albumin	Himedia, India
Bromphenol blue	Sigma, USA
CM Sephadex C-50	Sigma, USA
Chloroform	Merck, India
DTT (DL-Dithiothreitol)	Sigma, USA
EDTA	Sigma, USA
Ethanol	Merck, India
Ethidium Bromide	Sigma USA
Formaldehyde	Sigma, USA
Glycerol	Merck, India
Glycine	Sigma, USA
Glycogen	Sigma, USA
Hydrochloric acid	Merck, India
Isoamylalcohol (3-methyl-1-butanol)	Merck, India
Isopropanol (2-propanol)	Merck, India
Methanol	Merck, India
Sodium hydroxide	Sigma, USA
Potassium di-hydrogen ortho-phosphate	Fisher Scientific,India
Di-Potassium hydrogen ortho-phosphate	Fisher Scientific,India
Proteinase- K	Sigma, USA
Phalloidine	Sigma, USA
Resazurin	Sigma, USA

RNAase	Sigma, USA
Russell viper venom	Calcutta Snake Park, Kolkata, India
Sodium chloride	Sigma, USA
Sodium dodecyl sulfate (SDS)	Sigma, USA
TEMED	Sigma, USA
Tris (Tri(hydroxymethyl)aminoethane)	Sigma, USA
TRIzol Reagent	Invitrogen,USA
Trypsin	Himedia, India
Trypan Blue dye	Himedia, India

Following kits and antibodies were employed in the study:

Kits /antibodies	**Company**
Reactive oxygen species kit	Abcam, USA
cDNA synthesis kit	Bioline, USA
Caspase 3 activity kit	Biovision, India
Small GTPases mAB kit	Cell signaling, USA
(Rac1, RhoA, Cdc42, phosphor Rac1/RhoA)	
Hsp70	Sigma, USA
p53	Abcam,, USA
Beta actin	Sigma, USA

Sources of Plastic and Glasswares are the following:

Plastic and Glass wares	**Company**
0.2mL & 0.5mL PCR tubes	Axygen, USA
1.5mL micro centrifuge tubes	Tarson, India
10cm petri plate (tissue culture), T 25 and T 75 tissue culture Flasks	Corning, USA
15mL & 50mL sterile centrifuge tubes	Tarson, India
1mL, 0.5mL, 0.1mL pipette tips	Tarson, India
Cryo vials	Corning, USA
Glass beaker - 50mL, 100mL, 500mL, 1000mL	Borosil, India
Test tubes - 5mL, 10ML	Borosil, India

Details of the Instruments employed for the study:

Instruments	Company
Agarose Gel Electrophoresis System	Balaji Scientific, Chennai
Vertical Gel Electrophoresis System	Balaji Scientific, Chennai
Centrifuge	Thermo Scientific, USA
Micro-Filt Laminar hood	Pune, India
Gradient MilliQ	Merk, Millipore, USA
HPLC	Shimadzu, Germany
Spectrophotometer (UV-1800)	Shimadzu
Multiskan Go	Thermo Scientific, USA
Microwave oven (Samsung)	Japan
Nanodrop spectrophotometer (ND-1000)	Thermo Scientific, USA
Veriti Thermal Cycler	Applied Biosystem, USA
Spin win	Tarson, India
BioSep-SEC-s2000(300×7.8mm)	Phenomenex ,USA
UV-Gel documentation system	Applied Biosystems, USA
Water bath	Chromous, India

Various Cell Culture media, chemicals and cell lines employed for the study

Cell Culture	Company
DMSO	Sigma, USA
Dulbecco's Modified Eagle Medium (DMEM)	Sigma, USA
Fetal Bovine Serum (FBS)	Invitrogen, USA
A549 cells	National Centre for Cell Science, Pune
Penicillin	Sigma, USA
Trypsin	Sigma, USA

Different Buffers and Reagents employed for the study:

Buffer - Complete medium	Composition : 90% DMEM, 10% FBS, 0.1 X Antibiotic-antimycotic solution
Freezing medium	90% FBS, 10% DMSO
TBE (10×)	0.9 M Tris base, 0.09 M boric acid, 0.02 M EDTA pH 8.0
TE solution (10×)	10 mM Tris-HCl, 1 mM EDTA disodium salt pH 7.5
TBS (1×)	10 mM Tris-HCl pH 7.5, 100 mM sodium chloride.
Protein Extraction Buffer (PEB)	20mM TrisCl (pH 7.4), 1mM EDTA (pH 8) 1mM PMSF, 0.1% Triton × 100 Protease cocktail: 1 tablet per 100 ml
DNA Extraction Buffer	10mM Tris, pH (8.0), 0.1 M EDTA (pH 8.0), 0.5% SDS. Add RNAse (10mg/ml) just before use
Phosphate Buffer Saline [pH- 7.4] (1000mL)	3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl, pH 7.4.
5× TBE [pH-8.3] (1000mL)	0.45 M Tris-Borate, 0.01 M EDTA, pH 8.3
Gel Running Buffer 1× (Tris-glycine electrophoresis buffer)	25 mM Tris base, 190 mM Glycine, 0.1% SDS
Western Blot,Transfer Buffer1×	25 mM Tris base,190 mM Glycine,20% Methanol
Ponceau Stain (50 ml 0.1%):	0.05 g ponceau stain powder,500 µl Glacial Acetic Acid, 49.5 ml distilled water

Different PCR Primer list employed for the study:

Gene Name (annealing temperature)	**Primer Sequence**
Cdc42 (50^0C)	Forward primer 5' AGA GGA AAT ACG AGG GGT GGT 3'
	Reverse primer 5' CCT GAC TGG TCC CCA TGT TG 3'
Rac1 (60^0C)	Forward primer 5'GCC AAT GTT ATG GTA GAT GGA 3'
	Reverse primer 5'TTA AGA ACA CAT CTG TTT GCG 3'
RhoA (55^0C)	Forward primer 5'CCT TGT CTT GTG AAA CCT TG3'
	Reverse primer5' ACT GGT AGC AAG ATG ACT TC3'
Hsp70 (50^0C)	Forward primer 5' ACA CGA ATC CCT GCG GTA AA3'
	Reverse primer 5' AGC AGG CGA TAA GAT GGC AC3'
p53 (50^0C)	Forward primer 5'TGC TCA AGA CTG GCG CTA AA3'
	Reverse primer 5'CAA TCC AGG GAA GCG TGT CA3'
Bcl2 (60^0C)	Forward primer 5'GAA CTG GGG GAG GAT TGT GG 3'
	Reverse primer 5'GGC AGG CAT GTT GAC TTCAC3'
Bax (70^0C)	Forward primer 5'GGC CCT TTT GCT TCA GGG TTT C3'
	Reverse primer 5'CAG TCG CTT CAG TGA CTC GG 3'
Caspase 8 (65^0C)	Forward primer 5'GAG TGA GTC ATC TCT GTT CTG CTT3'
	Reverse primer 5'TCA CTT CCT GTT GAG TTG ACT AGC3'
Caspase 9 (60^0C)	Forward primer 5'CTG TTC AGG CCC CAT ATG ATC G3'
	Reverse primer 5'AGA GCA CCG ACA TCA CCA AA3'
Caspase 3 (65^0C)	Forward primer 5'GCT CTG GTT TTC GGT GGG TG3'
	Reverse primer 5'CTG AGG TTT GCT GCA TCG AC3',

Chapter 1

Introduction
&
Review of Literature

1. Introduction

Snakes form an integral part of' this immense universe. They have been the objects that fascinate many people while frightening others across all lands. Among all worshipful animals the serpent predominated as good and evil. The Greeks and Egyptians also had various representations of snakes' appearances in their religion. In Hindu religion, markings on the hood of cobra snakes are believed to be from lord Vishnu's stepping (Wake, 1873). Whereas, native Americans consider rattlesnake as "grandfather" and were rarely killed (Klauber, 1956).

Fear about snakes is due to the presence of venom. The oldest record of snake poison is given in an egyptian papyrus dating around 1600 B.C (Hearst Medical Papyrus, 1600 B.C). Snake is known as a powerful symbol in Indian mythology (Figure 1.1).

Figure 1.1

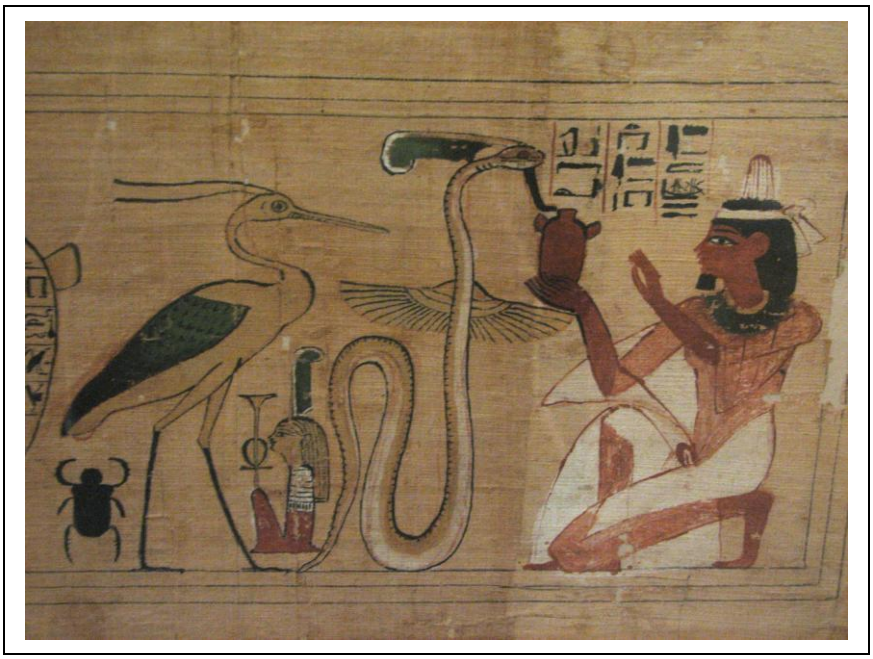

Figure 1.1: Ancient Egyptian papyrus of Death kneeling before a snake.

(Source: Photo by Art Media/Print Collector/Getty Images)

Snakes are regularly seen as resurrected ancestors or the source of mankind. In India, Hindus refuse to kill snakes since they believe that there may be reincarnation (Wake, 1873). Mayans and Aztecs believe serpent as their sun god and originator of mankind (Figure 1.2).

Figure 1.2

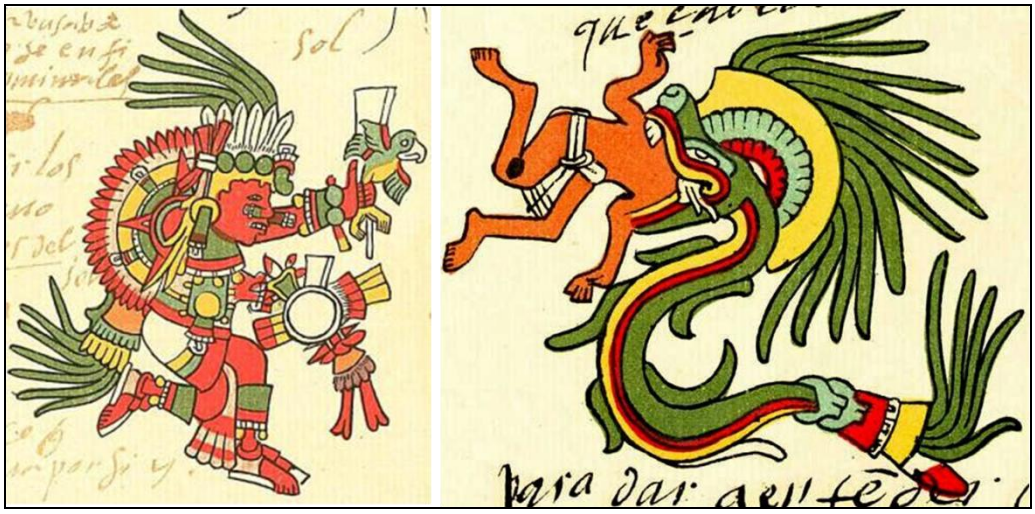

Figure 1.2. Portrayals of Aztec god showing Quetzalcoatl as a serpent eating a man. (Source: ancient-origins.net/myths-legends)

The fear of snakes has provoked a large number of the myths about snakes. Japanese worship snakes as a mysterious being, often as the embodiment of a spirit (MacCulloch, 1951). The Chinese also worship it in temples and believe that the dragon (or naga) still protects their empire. In Korea, people feed and worship serpents as the guardian genei of their households. The Romans also kept snakes in large numbers in temples and houses and worshipped them as embodiments of the genius (Wake, 1873).

The cult of the serpent in our country is typical, more so because it is believed to exist all through the life of man right from the prenatal stage. Although the serpent

is frequently worshipped, certain races and people of both Burma and Borneo hold the opinion that the dangerous snakes are embodiments of demonic powers or evil spirits. Whereas, some Indonesians believe that the spirits appear as snakes. The serpent is generally associated with evil in Babylonia. The Ophites, too, consider the serpent as a symbol of evil (Bauchot, 1995).

Apart from the above facts, snakes were also believed to be associated with rain and wind. For example, In Hinduism, Vritra, a dragon with three heads who hides in clouds and was slain by Indra, the giver of rain. Snakes are also associated with life and health and are consider as “bringer of health and good fortune” (Wake, 1873).

Interestingly, snakes were evolved 100 million years ago from lizards and have inhabitated all major ecosystems outside the Polar Regions (they are not found in Arctic, New Zealand and Ireland). Till date, around the world more than 3000 species of snakes are identified and out of this only 400 species of snakes are known to be venomous (O'Shea, 2008). Snakes have figured conspicuously in the historical backdrop of humanity and still are imperative right now. Apart from their role in several religions, snakes have been associated in the treatment of various diseases. Venomous snakes are considered to be most powerful and alarming animals with a lethal weapon called as "venom". Snake venom is generally a thick exocrine secretion from a specialized gland called a Jacobson gland which was proved to have several pharmacological properties.

1.1. Taxonomy and distribution of snakes

The suborder Serpentes consists of around 3000 types of snakes (Kasturiratne *et al.*, 2008). All venomous snakes purportedly belong to super family Colubroidea and subdivided into Colubridae, Atractaspididae, Elapidae and Viperidae (Pough *et al.*,

2004). The family Viperidae consists of 310 species with two main sub-families; the Viperinae (pit-less Vipers) and the Crotalinae (pit Vipers) (Lawson *et al.*, 2005). Russell's viper which belongs to Viperids (old-world vipers) is known to produce highly potent toxic venom which is responsible for number of human mortality and morbidity globally (O'Shea, 2008).

In India over 250 species and subspecies of snakes has been identified, among those about 50 species and subspecies were found to be highly venomous (Whitaker and Captain, 2004). Among venomous snakes, the "Big Four" including Indian cobra (*Naja naja*), Russell's viper (*Daboia russelii*), saw-scaled viper (*Echis carinatus*) and common krait (*Bungarus caeruleus*) are the most venomous snake species responsible for causing threat to human beings. The distribution of all these four species is widely through the nation like North-east region, the Himalayan region and the Andaman and Nicobar Islands (Table 1) (Whitaker and Whitaker, 2012).

Table 1.1: Distribution of Big Four snakes in India

Common name	**Scientific Name**	**Family**	**Distribution**
Indian Spectacled Cobra	*Naja naja*	Elapidae	Inhabits throughout India, sea level up to 2,000 m (6,600 ft)
Common krait	*Bungarus caeruleus*	Elapidae	Throughout India, sea level up to 1700 m.
Russell's viper	*Vipera russelii*	Viperidae	Inhabits throughout India, sea level up to 2300–3000 m (7,500-9,800 ft)
Saw-scaled viper	*Echis carinatus*	Viperidae	India (in rocky regions of Rajasthan, Uttar Pradesh, Maharashtra, and Punjab)

1.2. Snake venom composition: A brief overview

The first detailed account of the venom glands of a true viper was published by Wolter (1924) for *Vipera berus.* The viper's venom gland contains four distinct regions: the posterior two-third is occupied by main gland, whereas, fang sheath contains primary and secondary duct and accessory glands (Stocker, 1998) (Figure 1.3).

Figure 1.3

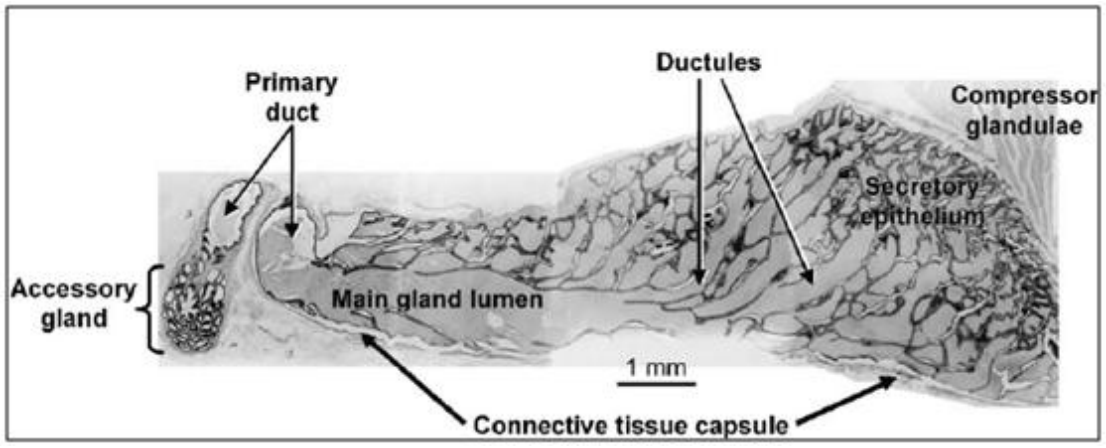

Figure 1.3: Longitudinal section of venom gland (Mackessy & Baxter, 2006)

Chemists were among the first to consider the venoms with interest, and they endeavoured to define their composition and attempted to extract the toxic constituents. Jung (1964) explained the molecular structure of benzene by Kekule who, in the 19th century, influenced by the memory of ancient symbols seen in dream that a snake holding its tail in its mouth and related the circular shape of the snake to the cyclic structure of benzene (Figure 1.4).

Figure 1.4

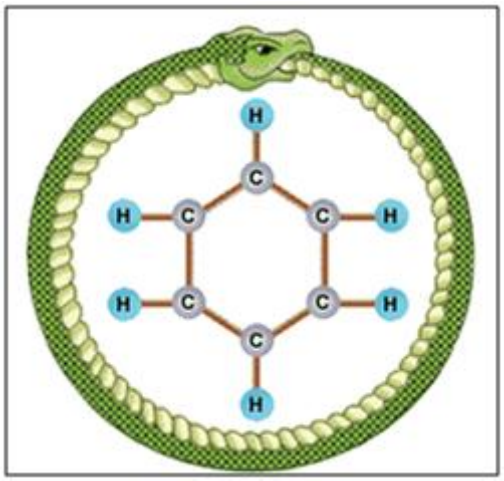

Figure 1.4: Kekule's cyclic structure of benzene

(Source: Read J. From alchemy to chemistry. Courier Corporation; 1957.)

Venoms target vital physiological processes causing deleterious effects in the target animal. Rapid urbanization in developing countries has increased snake-human conflicts and cases of snake-bite. According to WHO (2017), an estimated 5 million people are bitten each year with up to 2.5 million envenoming and around three times as many amputations and other permanent disabilities are caused by snakebites annually.

Among all the naturally producing venoms and toxins, snake venoms are the most complex and complicated containing at least 26 different types of enzymes (Aird *et al.*, 2015). Based on the patho-physiological effects they exhibit, snake venoms are classified into several fundamental groups.

The three functions of snake venom include, primarily weakening and immobilizing the prey. Secondly, it acts as defence tool against predators and thirdly, digestion of prey (Kang *et al.,* 2011). Snake venom typically comprises of solids as well as water (60-80%). It is a complex mixture of both organic and inorganic

components. Proteins and peptides comprise about 90-95% of the dry weight of the venom. Every individual component of the venom has a special role to play with characteristic properties of high affinity, selectivity and potency towards its substrate which can be explored to serve as potential drugs and scaffolds of drug design (Stocker, 1998). The strength of venom is measured by its lethal potency, which is abbreviated as LD_{50} (LD_{50} represents the optimum dose required to kill 50 percent of test animals).

The non-protein components which constitute the rest of the venom (5%) comprises of salts and organic components, such as nucleic acids, neurotransmitters and amino acids, whereas, the protein components (usually the abundant and active part of venom) constitutes enzymatic and non-enzymatic proteins (Sarkar and Devi, 1968).

1.3. Enzymatic snake venom proteins

Snake venoms are rich in enzymes. There are about 20 enzyme families detected in snake venoms and 12 are known to be found in most of the snake venoms (Kang *et al.*, 2011). Some of the common snake venom enzymes are metalloproteinases, hyaluronidase, phospholipase A2 (PLA2), nucleotidases (5′-nucleotidase, ATPase, phosphodiesterase and DNAse), acetylcholinesterase (AChE), and L-amino acid oxidase (LAAO) (Figure 1.5).

1.4. Non-enzymatic proteins

Non-enzymatic proteins of snake venom facilitate immobilization of prey by binding to specific receptors, ion channels and enzymes to influence their normal functions. They are usually rich in di-sulphide bridges which make them stable molecules (McCleary & Kini, 2013). Some of the well identified non-enzymatic venom proteins

are **Three-finger toxins (3FTx)** targeting various receptors (nicotinic acetylcholine receptors, biogenic amine receptors and integrin receptors), enzymes (acetylcholine esterase and blood coagulation factors) and ion-channels (calcium ion-channels and proton gated acid sensing ion channels (ASICs), **Serine protease inhibitors (SPIs) or Kunitz-type protease inhibitors**; **C-type lectin-related proteins (CLPs)**, possess biological properties such as anti-coagulant, pro-coagulant and agonists/antagonist effects on platelet activation (Morita, 2004).

Figure 1.5

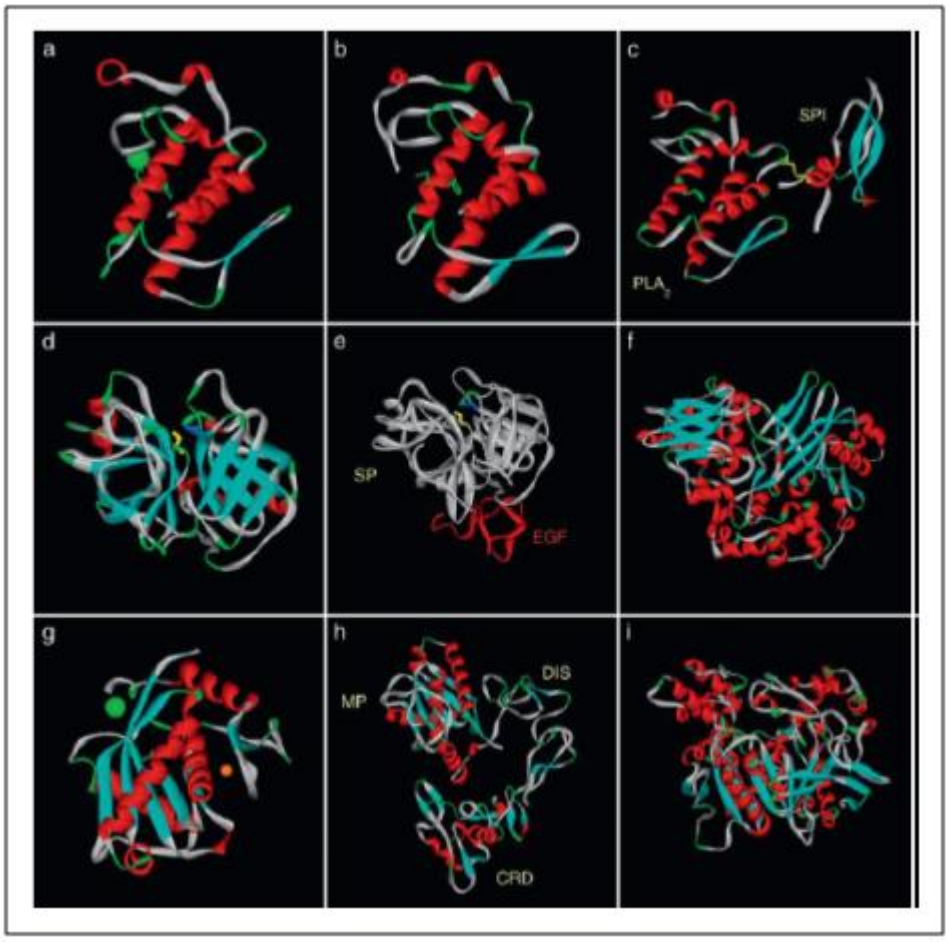

Figure 1.5: Common Enzymes found in snake venom. (a) Class I PLA2. (b) Class II PLA2. (c) β-Bungarotoxin, a presynaptic toxin with PLA2 linked to Kunitz-type serine proteinase inhibitor. (d) Serine proteinase (TSVPA). (e) Group D prothrombin

activator. (f) l-Amino acid oxidase. (g) PI metalloproteinase. (h) PIII metalloproteinase VAP2 (vascular apoptosis-inducing protein 2). (i) AChE.

The structures of hyaluronidase and nucleotidases are not yet known. Abbreviations: CRD – cysteine–rich domain; DIS – disintegrin domain; EGF – epidermal growth factor domain; MP – metalloproteinase domain; PLA2 – phospholipase A2 subunit; SP – serine proteinase domain; SPI – serine proteinase inhibitor subunit (**Source -** Kini, 1997**).**

A brief description of C-type lectins is provided below, as the work described in this thesis involves one C-type lectin.

1.5. Lectins

Boyd (1954) has coined the term "Lectin" which is derived from the Latin word "legere", which means "to select". Initially Lectins were described in plants, but in later years multiple lectins were identified from micro-organisms and animals (Ghazarian *et al.,* 2011). Lectins are non-immune and non-enzymatic proteins possessing the ability to bind carbohydrates or to a carbohydrate moiety. Divalency or polyvalency in Lectins permits lectin interaction with sugar moieties on adjacent cell surfaces, resulting in cell agglutination. Some lectins are specific for simple sugars while others interact with complex carbohydrates (Rabia *et al.,* 2013).

Mainly two types of calcium dependent (C-type) lectins have been identified in snake venoms.

A) **True C-type glycan-binding lectins** are homo-dimeric proteins, composed of two identical disulfide-linked polypeptide monomers with approximately molecular mass of 15 kDa, with a functional carbohydrate-recognition domain which binds to carbohydrates. (Sartim and Sampaio, 2015).

B) **C-type lectin-like proteins** (also named **"snaclecs"**) are hetero-dimeric proteins with carbohydrate-recognition domain (McGreal *et al.,* 2007).

1.6. Applications:

In the recent past, several types of Lectins are identified and elucidated, due to unique properties, lectins play an important role in therapeutic areas of research and medical applications. (Hamid *et al.,* 2013; Rabia *et al.,* 2013).

1.6. C-type lectins

In 1902, Flexner and Noguchi have reported the studies on snake venom lectins. However, almost 80 years later, Gartner and co-workers (1980) reported characterisation and isolation of galactoside-binding lectin from *Bothrops atrox*. Since then, several studies on structural and functional properties of various lectins from *Crotalidae, Elapidae,* and *Viperidae* families were reported (Sarray *et al.,* 2004; Momic *et al.,* 2011). The first lectin classified as "lectin-like" was isolated from *Lachesis muta stenophrys* (Ogilvie *et al.,* 1985*)*. Till to date, most of the lections identified from snake venom are glycan-binding lectins due to their ability to interact with terminal galactoside residues in a calcium-dependent manner (Sartim *et al.,* 2015). Further, C-type lectins like proteins (calcium-dependent), from venoms of various snake species were also isolated, characterized and sequenced. CTL proteins are known to have several properties like platelet aggregation, inhibition and activation of specific platelet membrane receptors that can influence hemostasis and thrombosis. CTL proteins are also studied for the properties like inhibition of cell proliferation, *in vitro* agglutination, cytotoxicity and mitogenic activity of erythrocytes (Sharon, 2004). Owing to their fine specificity; lectins have various

applications including cancer research, Sarray and co-workers (2004) have shown the anti-tumor effects of lebecetin from *M. lebetina* venom (Sarray *et al.*, 2004).

Crude venom consists of low concentration of CTL's; purification of such lectins requires large amounts of crude venom with time consuming and multiple purification techniques. Therefore, cloning of these molecules is necessary. In the present study, unique CTLs (accession no. Q4PRC6) was identified from the previously constructed cDNA library of the *Daboia siamensis* (Zhong *et al.*, 2006).

1.7. Biomedical applications of snake venom proteins: A brief overview

Several studies have reported the pharmacological and biological properties of snake venoms (Figure 1.6). However, snake venoms exhibit discrepancy in their potency of toxicity, and this variation is considered at several tiers which include: Interfamily, Intergenus, Interspecies and Intraspecies (Fry, *et al.*, 2012; Fatima and Fatah, 2014). This discrepancy may be due to geographical distribution, age, diet etc. The effects of snake venom toxins vary and these are divided into following types:

- Hemorrhage - due to presence of Hemorrhagic toxins by preventing the clotting compounds from functioning correctly, leading to uncontrollable bleeding through gaps arising on the endothelial layer of blood vessels caused by proteolytic toxins or disintegrins.
- Myonecrosis – due to presence of myotoxins
- Hemostatic alterations - due to presence of coagulant and anti-coagulant enzymes
- Cytotoxic effects - target specific cellular sites
- Neurotoxic effects – due to neurotoxins.

Figure: 1.6

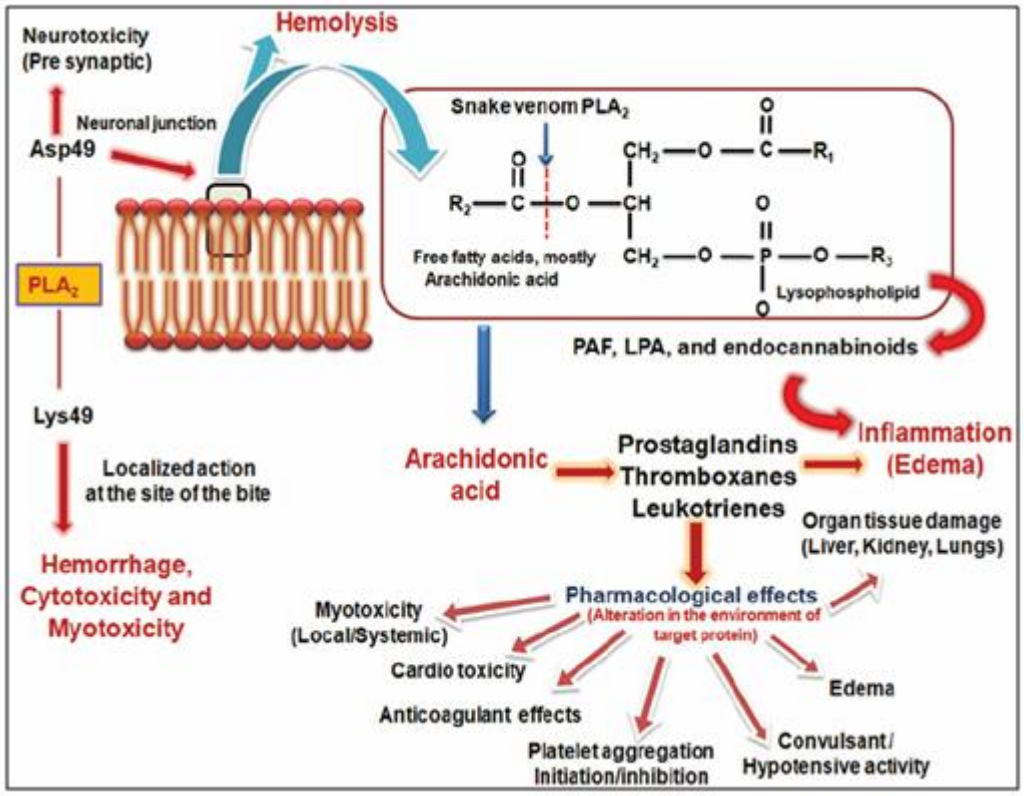

Figure 1.6: Diagrammatic representation of various properties of snake venom proteins (Chan et al., 2016).

Among the snake venom hemorrhagic proteins the most intensively studied are disintegrins and hemorrhagins.

1.8. Disintegrins

Disintegrins isolated from the venom of snakes are low molecular weight cysteine-rich peptides with high affinity to integrins on the surface of platelets and other cells (Kele *et al.*, 2015). They are known potent inhibitors of integrin function and therefore named "disintegrins".

1.9. Hemorrhagins

The presence of snake venom components that damage the blood vessel wall has been known for about a century. The term hemorrhagin or hemorrhagic principle

designates a venom agent causing bleeding by a direct action on the blood vessel wall, as distinct from venom agents that affect hemostasis through an action on blood coagulation (Baldo *et al.,* 2010). Based on the molecular size, hemorrhagins are of two types; small hemorrhagins (25 kDa) and large hemorrhagins (50-90 kDa). HR1 and HR2 were the first hemorrhagins isolated from *Tnmeresurus flavovindis (*Kishimoto and Takahashi 2002*).*

Hemorrhage is one of the most pronounced effects observed in Rattle snake, viperid and crotalid envenomation. Several studies have conducted on snake venom components especially those influencing the process of hemostasis, for a novel and more helpful therapeutic agents to manage various diseases like thrombosis, hypertension and cancer (Panfoli *et al.,* 2010).

High specificity of anti-coagulant or pro-coagulant proteins from snake venom has been used for practical applications for the advancement of a few routine laboratory tests like quantification of plasma factor V levels and defects in the protein C- pathway by venom factor V activators, and factor RVV-X used in the diagnosis of quantitative conversion of the zymogen factor X into factor Xa (Takeya, 1992). Pro-coagulant proteins isolated from venom are used for lupus anticoagulant assay and also in the diagnosis of coagulation factor VII and X deficiencies (Marsh. 2002). Further, prothrombins from snake venom are also used to detect lupus anticoagulant (Thiagarajan *et al.,* 1986), factor Va deficiency and fully carboxylated prothrombin in human plasma (Quick, 1971).

Cardiovascular diseases are a leading cause of death in developed countries and are one of the main contributors to the disease burden in developing countries (WHO, 2010). Unfortunately, many natural anticoagulants such as heparin and

warfarin used as anti-thrombotic drugs are associated with adverse effects limited their usage (Frenkel *et al.,* 2005; Holbrook *et al.,* 2012). As such, snake venom derived potent anti-coagulants such as inhibitors of thrombin, factor Xa and have been considered as an alternative for the development of better anti-thrombotic (Huang *et al.,* 2016).

Several non-enzymatic anti-coagulant proteins, peptides and enzymes from snake venom have been investigated for properties like defibrinogenation and digestion of fibrin clots (Kini, 2006). Several studies have suggested the potential application of such enzymes for the treatment of thrombotic ailments such as strokes, heart attacks and other diseases associated with the formation of thrombus. Snake venom fibrinolytic proteases such as fibrolase have been explored for their anti-thrombotic and thrombolytic potential (Koh and Kini, 2012).

1.10. Snake venom proteins as potential anticancer agents

Modern life style has exposed human beings to vagaries of environmental pollution, chemicals, radiation, therapeutic agents and biological agents like viruses that play a major role in the induction of cancer. Cancer unlike other diseases evokes a widespread fear among the people. In fact, cancer is the second largest killer disease in the modern world. The number of global cancer deaths is expected to increase 45% from 2007 to 2030 (from 7.9 to 11.5 million deaths). It is estimated that there will be 15.5 million new cases every year by 2030 (WHO, 2010). Despite making great strides in cancer research and availability of large paraphernalia of state of the art treatment strategies, the complete cure of cancer remains elusive. Thus, there is a need for more effective anti-cancer agents which could be clinically more useful for the cancer patients (Calvete *et al.,* 2007; Calderon *et al.,* 2014).

Several *in vivo* and/or *in vitro* studies have been reported on the anti-cancer potential of snake venom derived proteins and toxins by various mechanisms listed below

1. Alterations in the organisation of actin filaments thus causing damage to cell membrane.
2. Alterations in the expression levels of cell cycle regulatory proteins and inhibition of cell proliferation
3. Increase in ROS generation
4. Alteration in the expression levels of pro-apoptotic and anti-apoptotic proteins.

It is surprising that substances that have been so well designed for killing could also be useful in saving life. In 1933, Calmette and his colleagues reported the first medically active substance isolated from a snake's venom as anticancer agent. Since then, various snake venom components were screened for their anti-cancer potential (Jain and Kumar, 2012; Sarray *et al.,* 2013).

Snake venom derived proteins like RGD disintegrins are well studied for their anticancer properties. Triflavin, RGD derived disintegrins isolated from *Trimerisurus flavoviridis*, was first protein was shown to inhibit the cancer cell migration and adhesion (Huang *et al.*, 1991).

Rhodostomin, antiplatelet disintegrin isolated *Crotalus atrox* was found to be inhibit thrombin associated metastasis and growth of breast cancer cells (Yeh *et al.*, 2001). Similarly, several types of disintegrins were isolated from various snake venom like Contortrostatin from *Agkistrodon contortrix contortrix (*Zhou *et al.*, 1999*),* Viperistatin from *Vipera palaestinae (*Momic *et al.,* 2011*),* Lebestatin from *Macrovipera lebetina (*Olfa *et al.,* 2005*)* and Salmosin from *Agkistrodon halys*

brevicaudus have shown anti-cancer potential on various cancer cells (Kang *et al.*, 2000).

Snake venom components like C-type lectins were also extensively studied for their anti-cancer potential, Lebestatin a CTL's isolated from *Macrovipera lebetina (*Sarray *et al.*, 2004*)* and Vixapatin from *Vipera palastinae* (Momic, *et al.*, 2012) exhibited anti-cancer properties by inhibiting tumour cells growth adhesion and metastasis.

Snake venom derived L- amino oxidases (LAAO's) were also studied for their cytotoxicity on various cell lines. The cytotoxicity exhibited by LAAO's may be due to increase in ROS generated but the exact mechanism of cytotoxcity still remains unclear (Costa *et al.*, 2014).

Toxins isolated from snake venom have been found to possess anti-cancer potential. Taipoxin, a neurotoxin derived from *Oxyuranus scutellatus scutellatus* has shown anti-cancer effect on small cell lung cancer cells (Poulsen *et al.*, 2005). Several other toxins like INN toxin, Cardiotoxin III, Cardiotoxin NK-CT1 were found to possess anticancer potential on various cancer cell lines (Yang *et al.*, 2007; Ponnappa *et al.*, 2008; Debnath *et al.*, 2010).

1.11. Indian Russell's viper (Daboia russelii russelii): A brief overview

Russell's viper is also called as Indian Viper (or) Daboia was named in the honour of Dr. Patrick Russell (Daniels, 2002), a Scottish surgeon who described this species and named the genus "Dabioa" means "lurker" (Figure 1.7).

1.12. Systemic classification

Kingdom: Animalia

Phylum: Chordata

Group: Vertebrata

Class: Reptilia

Subclass: Diapsida

Order: Squamata

Suborder: Serpentes

Family: Viperidae

Subfamily: Viperinae

Genus: *Daboia*

Species: *russelii*

Figure 1.7

Figure 1.7: ***Indian Russell's viper (Daboia russelii russelii)***

(Courtesy: Dr. Dibakar Chakrabarty)

1.13. Habitat & Distribution

Russell's viper is distributed throughout the Indian subcontinent. It is predominantly seen in countries like India, Pakistan, Bangladesh, Sri Lanka and Nepal, but less commonly in places like Sudan, Southern China and Taiwan (McDiarmid *et al.*, 1999). Russell's viper habitats mainly in open field areas and forest plantations

Daboia russelii has been categorized based on geographical distribution. *Daboia russelii russelii* is commonly seen in the places like India, Bangladesh and Pakistan. The subspecies *Daboia russelii mordicus* is found in Northern Indian region. Whereas, countries like Taiwan and Indonesia *Daboia russelii formosensis* and *Daboia russelii limits* are abundantly seen (Mallow *et al.*, 2003). Newer classification was based on morphological and mitochondrial DNA, Russell's viper are two types *Daboia russelii russelii* and *Doboia russelii siamensis* (Lenk *et al.*, 2001*)* (Figure 7)

Figure 1.8

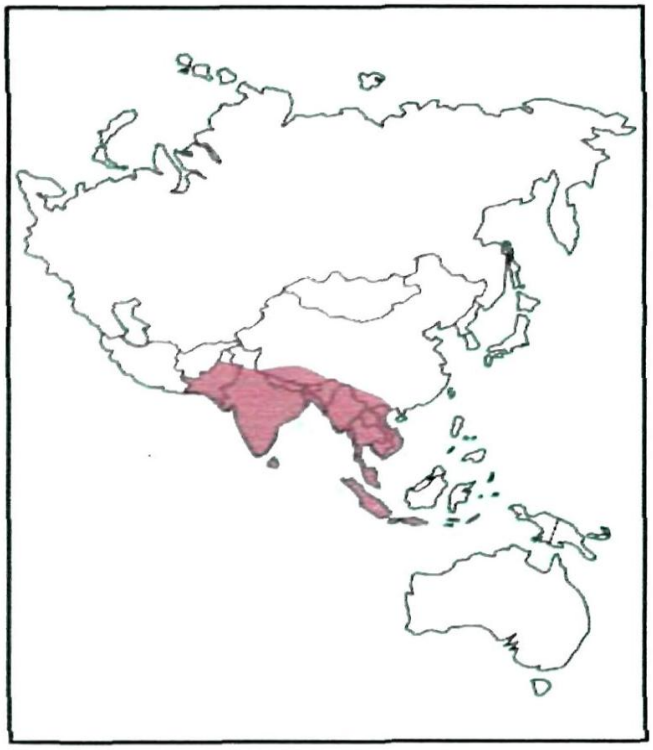

Figure 1.8: Russell's viper habitat in south-east Asia including India. (source:http://danger.mongabay.com/survival/afm/e.html)

1.14. Russell's viper envenomation, pathophysiology and clinical manifestations

Russell's viper is one of the deadliest snakes which can inject 225-250 mg of venom. Several studies have shown the lethal dose of RVV for humans is 40-70 mg (Mukherjee *et al.,* 2000). Bite of RV exhibits regional lymphadenitis, vomiting, epigastric pain and drowsiness (Warrell, 1989).

1.15. Biological activities of RVV

Several PLA2 enzymes from RVV were screened for the anti-cancer potential and cytotoxicity on various cell lines (Rosing and Tans, 1988; Carlisle *et al.,* 1990). The drCT-I peptide has shown to be effective against U937, K562 cell lines and Ehrlich ascites carcinoma cells (Furie and Furie, 1988). The anti-cancer potential of drCT-II may be due to arrest of the cell cycle at G1 phase by inducing apoptosis (Gomes *et al.,* 2015).

The anti-tumor activity of RVV-7 was studied against B16F10 cells. RVV-7 peptide suppressed cell proliferation and metastasis in B16F10 cells (Maity *et al.,* 2007). Drs-PLA2 peptide isolated from *Daboia russelii siamensis* venom has shown the cytotoxic potential both *in vitro* and *in vivo* conditions (Khunsap *et al.,* 2011). The cytoskeleton is a vital network of filamentous proteins that transverse through the crowded cytoplasmic environment, giving shape to cells and framework in order to accomplish vital cellular processes via the signals emanating from outside the cell to the cell interior.

Rho GTPases link extracellular cues to changes in cell function. One of their targets is the actin cytoskeleton, and as a result they regulate a large variety of cellular processes including cell morphology, cell motility and cell adhesion. The following is an overview of the biology of Rho GTPases, emphasizing how GTPases and Rho proteins control membrane integrity in response to extracellular stimuli is discussed.

1.16. Actin cytoskeleton dynamics in cell migration

The actin cytoskeleton consists of a network of actin filaments (F-actin) which are polymerised from actin monomers (G-actin). Each filament has a 'barbed' or 'plus' end where new monomers are constantly being added and a 'pointed' or 'minus' end that undergoes depolymerisation. Only adenosine triphosphate (ATP) loaded G-actin is added to the 'barbed' end and over time the ATP is hydrolysed to adenosine diphosphate (ADP) and inorganic phosphate (Pi). The Pi is then released leaving ADP-loaded F-actin which is depolymerised from the 'pointed' end. The ADP on the released actin monomer is exchanged for ATP and the ATP-G-actin is ready to be reincorporated into F-actin. This process is constantly occurring and is known as actin treads milling. In addition to these continual changes to each filament, the actin cytoskeleton as a whole undergoes constant dynamic changes and this is particularly important during cell migration (Lee *et al.*, 2010). There are a number of proteins that mediate these changes in the actin cytoskeleton. These mediators of actin cytoskeleton dynamics are tightly controlled in cells by a range of regulatory mechanisms. In particular, the Rho family of GTPases including Rho, Rac and Cdc42 are well known to be key regulators of the actin cytoskeleton (Hall, 1998). Signalling through these and other pathways ensure that protrusions are generated and cells migrate only when necessary. Indeed, in invasive cancer cells, many of these proteins and components of

their upstream signalling pathways are up-regulated, contributing to the migration, invasion, and metastasis of cancer cells (Ridley, 2001).

Reorganization of the actin cytoskeleton is regulated by complex signalling pathways that link extracellular cues to actin-binding proteins. Members of the Rho family of GTPases have recently emerged as major regulators of actin organization and dynamics in cells (Lee and Dominguez, 2011).

1.17. Small GTPases

These are the family of proteins that regulate several cytoplasmic functions by alternating between a GDP-bound forms (inactive), to a GTP-bound form (active). This group includes the Ras; Rab; Rho; Ran, Arf, and Arf-like subfamilies. All these proteins contain in one of their domains a conserved threonine that recognizes Mg^{2+} and the phosphate of GTP. The Cdc42, Rac1, and RhoA are the best known, 'classical' Rho GTPases (Mishra and Lambright, 2016).

1.18. Regulators of small GTPases

There are three regulators which are involved in this mechanism those are guanine nucleotide exchange factor (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). GEF are the proteins that stimulate the release of GDP and allow the binding of GTP thus activating the GTPases. There are around 80 Rho-GEFs in humans. They are further divided into two families, the DbI family, and the DOCK. DbI was first isolated in mammals in the year 1985 as an oncogene in NIH3T3 cells. Later it was found to have a potential to catalyze nucleotide exchange on Cdc42. This potential was due to the conserved domain which was present between DbI and Cdc42 and this domain was named as DH (DbI

homology) domain (Aghazadeh *et al.,* 1998). Adjacent to DH domain of DH protein there is also a tandem pleckstrin homology (PH), PH domain is involved in targeting GEFs to their correct intracellular location. RHO GEFs may be specific or nonspecific for GTPases

GEFs bind to the GDP bound form GTPases and destabilize the GDP–GTPase complex while stabilizing a nucleotide-free reaction intermediate. Leading to increase the intracellular concentration of GTP thus, favouring the released of GDP and replacing it with GTP thus activating the small GTPases (Cherfils and Chardin, 1999). DOCK gene was first cloned in 1996 encoding 180 kDa protein named as DOCK 180. (Hasegawa *et al.,* 1996)

The RhoGAP's Bcr (breakpoint cluster region) was the first RhoGAP discovered in 1989. Also known as accelerating proteins which interact with the GTP-loaded conformation of GTPases. The RhoGAP domains are formed of 9 alpha helices and the hallmark is a conserved arginine amino acid residue present in a loop region, the so-called "Arginine finger" (Bourne, 1997). There is considerable evidence to suggest that the activity of RhoGAP within a cell is controlled by diverse means for instance protein-protein interaction, lipid binding, post-translation modification such as phosphorylation (Fauré and Dagher, 2001). Crystallographic studies have revealed that the amino acids close to the arginine form a catalytic site involved in direct hydrolyzing GTP reaction to GDP a release of an inorganic phosphate (Pi) (Bourne *et al.,* 1991).

1.19. Rho-GTPases

Rho-GTPases are expressed in all eukaryotic organisms and regulate many aspects of cell behaviour, such as cell polarity and motility, through their effects on the

cytoskeleton, membrane trafficking and cell adhesion (Symons, 1996). Rho-GTPases belong to the Ras family of small GTPases and, to date, 22 human members of the Rho family have been identified. The most studied members of the family are RhoA, Rac1, and Cdc42 (Kaibuchi *et al.*, 1999). In the early 1990s, the group of Alan Hall showed that RhoA, Rac1, and Cdc42 displayed different roles in Swiss 3T3 cells (Hall, 1994). Using this cellular model system, they demonstrated that RhoA regulates the formation of contractile actin-myosin filaments and focal adhesions in response to a variety of extracellular stimuli (Machacek *et al.*, 2009). In contrast, Rac1 was demonstrated to induce actin polymerisation at the cell front to produce lamellipodia, and Cdc42 was found to promote actin filament assembly and filopodia formation (Ridley and Hall, 1992). The members of rho subfamily shares with ras family in having a highly conserved G domain which is about 150 amino acids long. This provides an essential site for nucleotide exchange. Additionally, rho family members contain an alpha helical structure made of 12 amino acid residues. Rho-GTPases do not only regulate pathways linked to the actin cytoskeleton. They also participate in the regulation of cell polarity, gene transcription, G1 cell cycle progression, microtubule dynamics, and vesicular transport pathways, to name a few (Etienne and Hall, 2002).

Rho-GTPases cycle between an active state when they are bound to GTP and an inactive state when they are bound to GDP (Figure 6). The cycling between GDP and GTP-bound states is primarily controlled by two classes of regulatory proteins, i.e. GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs). GAPs catalyse GTP hydrolysis, converting the Rho proteins to the GDP-bound inactive conformation whereas GEFs catalyse the exchange of GDP for GTP.

The third set of regulatory proteins i.e. the guanine nucleotide dissociation inhibitors (GDIs), sequester Rho-GTPases in the cytosol in a GDP-bound state (Etienne and Hall, 2002) (Figure 1.9).

Figure 1.9

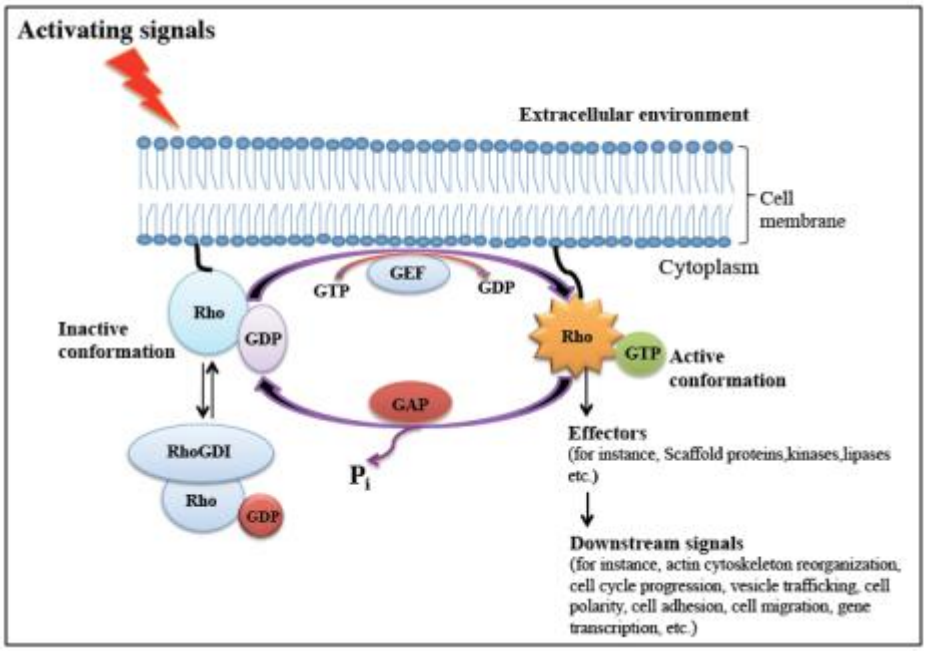

Figure 1.9: Regulators and targets of Rho GTPases

(Source: Mackay and Hall, 1998)

In recent years, studies on snake venoms and their components have demonstrated the cytotoxic effects of different toxins (Montecucco *et al.*, 2008). It is known that the cytotoxicity induced by venoms is mainly related to changes in the cellular metabolism of cells either by apoptosis or necrosis, however, the mechanisms of action of cell death by venom are still to be clarified, which makes them target of many studies with interest in their therapeutic potential (Jain and Kumar, 2012).

1.20. Rho GTPases and Reactive Oxygen Species:

Reactive oxygen species (ROS) and reactive nitrogen species (RNS), are key regulators in a variety of signal transduction pathways, including integrin signaling, extracellular matrix adhesion, and inflammation (Yan and Smith, 2000; Chiarugi *et al.*, 2003). ROS/RNS and Rho GTPases operate through a wide array of regulatory mechanisms, crosstalk between ROS/RNS and Rho GTPases is thought to play a pivotal role in many of their physiological functions and cancer (Myant *et al.*, 2013) (Figure 1.10).

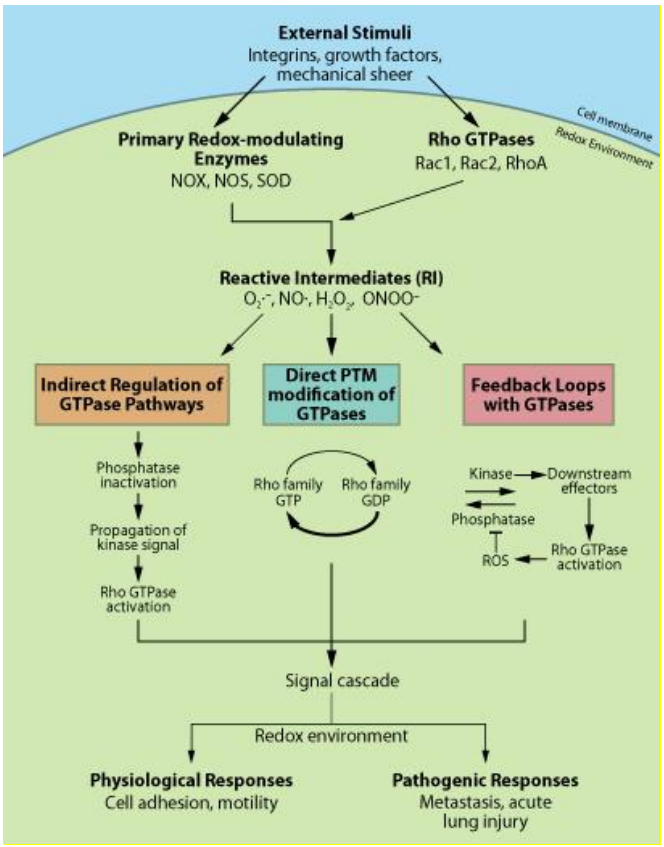

Figure 1.10: Schematic representation of crosstalk between Rho GTPases and ROS in Signal Transduction. (Source: CYTOSKELETON NEWS, 2014)

1.21. Cell death

Cell death may be classified based on "cellular morphology, enzymological criteria, functional aspects or immunological characteristics". According to Leist and Jaattela (2005) cell death can be categorised into four groups; apoptosis, apoptosis-like programmed cell death, necrosis-like programmed cell death and accidental necrosis. Other forms of cell death have also been described and include autophagy (self-digestion), paraptosis, necroptosis, coagulation necrosis and oncosis (Sperandio *et al.*, 2000; Mizushima, 2007). In general, cell death is divided into two main categories: apoptosis, which is an active, programmed cell death and accidental necrosis, a passive, unregulated form of cell death.

1.21.1. Necrosis

The term "necrosis" is derived from the Greek "nekros", meaning corpse. Necrosis is a pathological or accidental mode of cell death, which typically occurs when cells suffer a trauma. Necrosis is typically characterized by a noticeable gain in cell volume (oncosis), swelling and distortion of cytoplasmic organelles, irreversible changes in the nucleus (karyolysis, pyknosis and karyorhexis), bioenergetic failure, and loss of membrane integrity, cell rupture and the subsequent release of cellular contents (Figure 1.11).

Figure 1.11

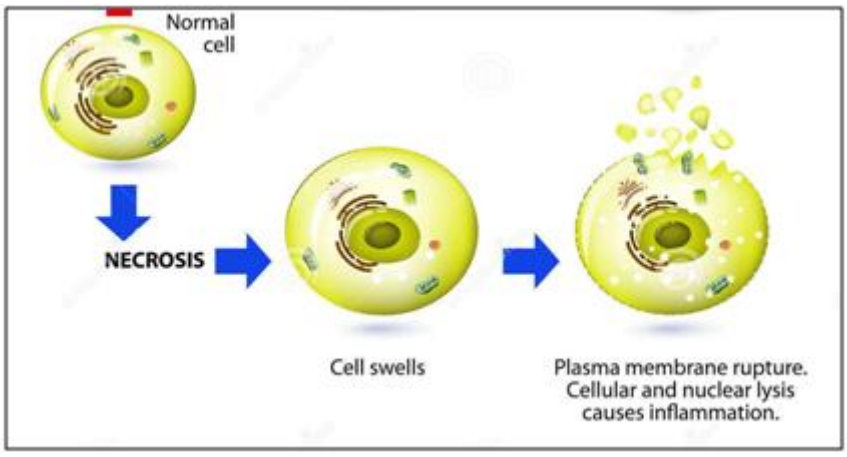

Figure 1.11: Schematic representation of the process of necrotic cell death

(Source: https://thumb1.shutterstock.com)

Necrosis can be triggered by a number of molecular events, including increased mitochondrial reactive oxygen species (ROS) production, channel-mediated calcium uptake, activation of non-apoptotic proteases, and/or enzymatic destruction of cofactors required for adenosine triphosphate (ATP) production (Majno, 1995).

1.21.2. Apoptosis

Apoptosis is a form of controlled or programmed cell death. It is a process that is involved in the development of organs and digits during embryology and later plays a role in hormone-dependent involution, immune system development, and selective immune cell deletion in adults. Apoptosis is important in the regulation of cell populations of tissues under both physiological and pathological conditions (Elmore, 2007).

Stages of apoptosis

Apoptosis occurs in three stages. The first stage is the initiation stage and involves stimulation of the apoptotic machinery. During the second or effector stage, the

apoptotic machinery becomes fully activated. In the final, or degradation, stage the morphological and biochemical features typical of apoptosis appear (Elmore, 2007).

Morphological and biochemical features of apoptosis

Apoptotic cells are typically characterised by morphological features that include cell shrinkage, chromatin condensation, blebbing of the cell membrane and the formation of apoptotic bodies (Figure 1.12). The biochemical features of apoptosis are considered to be the fragmentation of the cells' DNA.

Figure 1.12

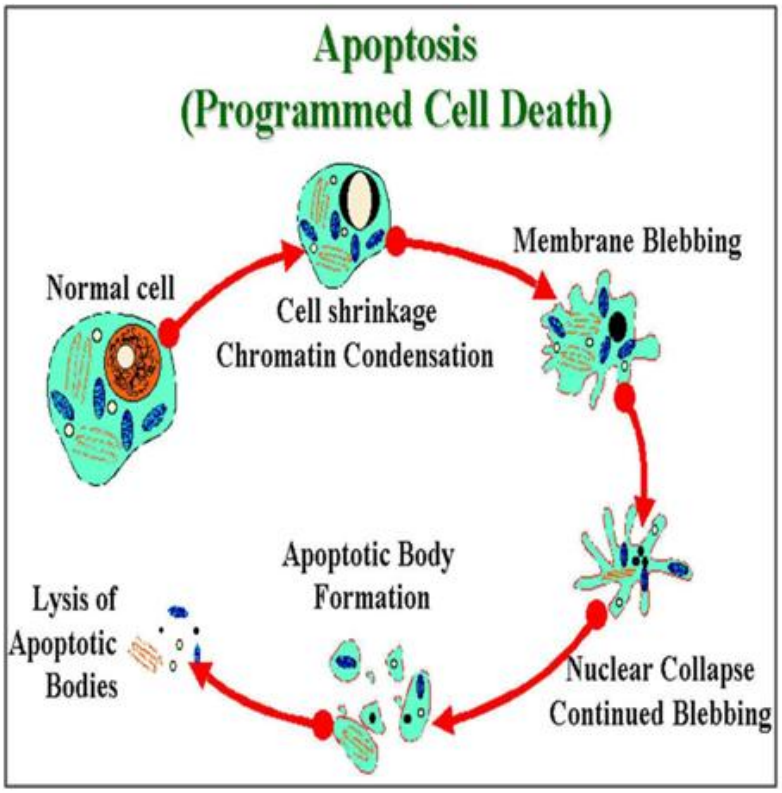

Figure 1.12: Typical morphological features of apoptotic cells, including cellular shrinkage, chromatin condensation, blebbing and the formation of apoptotic bodies. Also shown is the phagocytosis of apoptotic bodies, *in vivo*, or secondary necrosis, *in vitro*, following apoptosis.

(**Source:** http://biologyfora2.blogspot.in/2011/09/apoptosis.html)

Cells undergoing apoptosis show distinctive and specific morphological characteristics. Cell shrinkage is the earliest apoptotic feature that occurs due to the net outward movement of fluid from the cell. The nucleus also condenses during apoptosis. Following cytoplasmic and nuclear condensation, there is a formation of blebbing or “bubbling”. Blebbing is thought to be due to the disruption of the fodrin network in the cortical cytoskeleton (Martin *et al.,* 1995). In the final stages of apoptosis, the cell breaks up into apoptotic bodies, defined as "membrane-bound compact but otherwise well-preserved cell remnants" (Kerr *et al.,* 1972). Inter-nucleosomal cleavage of DNA has been considered a characteristic of apoptosis (Cohen, 1997). This DNA cleavage can be recognised as a DNA ladder on agarose gel electrophoresis.

The caspases and inhibitor of apoptosis proteins

The caspases are a group of aspartate-specific cysteine proteases. They are expressed as pro-enzymes, which become activated through two cleavage reactions. The caspases may auto-activate themselves or may be activated by another caspase as part of an amplification cascade.

There are 11 human enzymes in the caspases gene family. Seven family members are involved in the apoptotic pathway, while three others (caspases 1, 4, and 5) are involved the activation of pro-inflammatory cytokines. Those caspases that are involved in apoptosis can be divided into the initiator (caspases 2, 8, 9 and 10) and executioner caspases (caspases 3, 6 and 7).

Initiator caspases are responsible for converting apoptotic signalling, in response to death receptor ligation or cellular stress, to proteolytic activity. They

cleave and thus activate the executioner caspases. The execution caspases then cleave a number of cellular proteins, which results in cell death.

The intrinsic apoptotic pathway

The intrinsic apoptotic pathway (Figure 1.13) can be triggered by a number of death stimuli from within the cell. Such stimuli include cytotoxic agents, the expression of oncogenes and DNA damage, caused, for example, by drugs (Riedl & Shi, 2004; Saraste & Pulkki, 2000). Following DNA damage, the p53 transcription factor is often activated, which then induces the expression of pro-apoptotic Bcl2 members while suppressing the anti-apoptotic Bcl2 members.

The extrinsic apoptotic pathway

The extrinsic pathway (Figure 1.13) is induced by externally supplied signals and is initiated by ligation of the death receptors. An extracellular death ligand, such as FasL, binds to its appropriate cell-surface death receptor, Fas. The death ligands are homotrimeric, causing the cytoplasmic death domains (DDs) of the death receptor (Fas) to trimerize. The trimerized Fas then recruits adaptor proteins called FADD (Fas-Associating Death Domain-containing protein). The FADD molecules bind via their death domains to the DDs of Fas. The adaptors recruit initiator pro-caspases (pro-caspases 8) to form the death-inducing signalling complex (DISC). Formation of this complex causes the activation of caspase 8, which will in turn cleave and activate the effector caspase, caspase 3 (Hengartner, 2000; Saraste and Pulkki, 2000; Zimmermann *et al.*, 2001; Riedl and Shi, 2004;).

The intrinsic and extrinsic apoptotic pathways converge at the level of caspase 3 activation. Downstream of this, the pathway diverges and the end result is the ordered dismantling and removal of the cell (Hengartner, 2000).

Figure 1.13

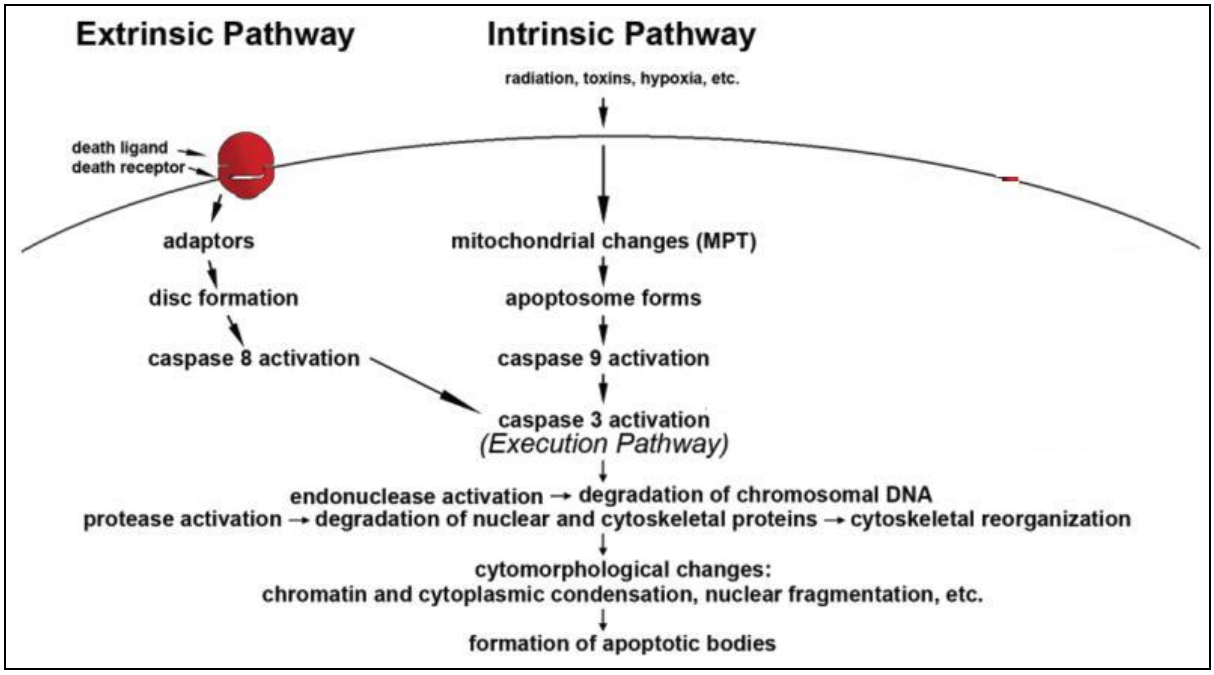

Figure 1.13: Schematic representation of intrinsic and extrinsic pathways of apoptosis.

Gaps in existing research

Several active secretions produced by animals have been utilized in the advancement of new drugs to treat various diseases. Toxins and enzymes from snake's venom contributed significantly for the treatment of many medical conditions (Chaitanya *et al.*, 2016). Snake venom is a complex mixture of peptides, often with diverse and selective pharmacologies plays an important role in addressing the crucial issues of potency and stability. It is this evolved biodiversity that makes venom peptides a unique source of leads and structural templates from which new therapeutic agents might be developed. Snake venoms are also the richest sources of anti-adhesion proteins. These proteins have amazing ability to inhibit metastasis and control angiogenesis. Search for these peptides which can control cancer has become a very

challenging field for the researchers. The rationale for this project is that if there is a significant effect of purified venom proteins on the cancer cells, then the proteins could be used for treatment of cancer. These toxins can also be helpful in the field of cancer research for better understanding of the underlying mechanism involved in tumor metastasis and help in designing/discovering new classes of drugs.

It is well known that, crude preparations as well as purified components of venoms isolated from Asian, American and European snakes (*Naja naja, Naja nigricollis, Naja nigricollis nigricollis, Trimeresurus flavoviridis* and *Agkistrodon rhodostoma*) have been shown to inhibit the growth of various cancer cells (Oron *et al.*, 1992; Sheu *et al.*, 1994). It is of interest to note that some investigators claim snake venom to be less toxic to normal cells than to cancer cells. The toxic effect on cancer cells may be due to specifically binding of proteins or peptides to cell membranes, affecting the migration and proliferation and limiting metastasis. Earlier studies have also shown that cytotoxins purified from the venoms of a few cobra species penetrate smoothly into the A549 cells and also accumulate in the lysosomes. Cytotoxin have a high propensity for internalizing exogenous ligands in cancer cell to produce pathology and also causes disruption of the phospholipid membrane structure indicating the higher affinity of cytotoxins towards some tumor cells compared to normal human cells. The intracellular molecular mechanisms underlying the cytotoxicity are still unclear but it is known that this process requires remodelling of the actin cytoskeleton. There is now compelling evidence that Rho GTPases, in particular RhoA, Racl and Cdc42, are important signalling elements within the cell growth and they have been shown to play a role downstream of almost all guidance cell receptors. However, there has been no study addressing the question of whether

or not Russell's viper venom (RVV) possesses any anti-cancer activity on A549 cells. The detailed *in vitro* studies on RVV induced cellular morphology and regulation of small GTPases are lacking. The present study was to investigate the anti-cancer activity of the RVV *in vitro* with following aim and objectives:

Aims and scopes of this study:

1. To study the effects of Russell's viper venom (RVV) on the morphology of human A549 cells and its effects on expression and regulation of small GTPases.

2. To study the effects of Russell's viper venom (RVV) induced regulation and expression of stress protein (Hsp70) and apoptosis- related genes.

3. Purification and partial characterization of toxin(s) responsible for above-mentioned effects from Russell's viper venom (RVV).

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Chapter 2

Regulation of Small GTPases and cytotoxic effects by Russells's viper venom (RVV) In A549 cell lines

2.1. Introduction:

Russell's viper is an old world snake falling under the elite viperine snake family. Its single species with five sub-species, namely, *russelli, pulchella* (sometimes considered synonymous with *russelli), formosensis Maki, limitis* and *nordicus* are distributed throughout South-East Asia. Envenomation by Russell's viper is known for its diverse patho-physiological consequences, e.g., myotoxicity, edema, pituitary insufficiency, 'dramatic hemorrhage' and renal failure apart from death. The amazing specificity of snake venom toxins to target physiological systems also made them potential molecules of drug development. A number of toxins with anticancer potentials have been purified in recent times from different snake venoms, including Russell's viper venom (Vyas *et al.,* 2013). The anticancer potential of Russell's viper venom lies in its cytotoxic effects. Most contemporary research in the development of anticancer therapeutics from venoms has focused on investigating the molecular mechanism by which an agent induces cytotoxicity and apoptosis in cancer cells (Yang *et al.*, 2005; Son *et al.,* 2007).

It is well documented that Rho family of small GTPases play a vital role in the control of cell movement, morphology and adhesion by regulating the actin cytoskeleton (Bishop and Hall, 2000). Tumour invasion and metastasis involves various intracellular molecules, which includes formation of membrane protrusions like filopodia, lamellipodia, and pseudopodia (Abraham *et al.,* 2001). In the recent past, studies have shown that Rho proteins are most likely involved in the cancer cell migration, tissue invasion and metastasis (Aznar and Lacal, 2001). Although, the cytotoxic effects of Russell's viper venom have been observed, the true mechanism of the same is not known. Therefore, the present study was undertaken to evaluate the morphological changes in cultured lung adenocarcinoma cells following exposure to whole Russell's

viper (*Vipera russelli russelli*) venom and to elucidate the mechanism of cytotoxicity. One of the major pathways for cellular morphological change is through the cytoskeleton. Expression of Rac, Rho and Cdc42 genes are known to be involved in cytoskeletal changes. In this study, an attempt was made to understand the involvement of small GTPases in the RVV mediated cytotoxicity.

Since GTPase expression is important for cancer progression and metastasis, understanding regulation of GTPase is of clinical importance. The present investigations address for the first time the cytotoxic effects of RVV on human *in vitro* cultured broncho-alveolar carcinoma-derived cell line (A549). A549 cells exhibit similarity to the type-II alveolar cell phenotype and share many characteristics with the human primary epithelial cells (Fuchs *et al.,* 2003). Therefore, this cell culture based study was considered as an appropriate *in vitro* model system to evaluate the cytotoxicity and the events involved in the cell death caused by RVV. In the present work, the effect of RVV on A549 cell line was analysed in order to elucidate a putative direct cytotoxic action, cytotoxicity with respect to the expression and regulation pattern of small GTPases. Further, the involvement of caspase-3, Bcl2, Bax in relation to apopototic effects of RVV on A549 cells was also studied. We have studied for the first time the expression pattern of Hsp70 at mRNA as well as in protein level. In this study, extent of nuclear damage and invasive property of A459 cells on exposure to RVV was noted.

2.2. Materials and Methods:

2.2.1. Maintenance of Cell Line: All cell culture works were performed in a laminar flow hood under appropriate aseptic conditions. Human broncho-alveolar carcinoma derived (A549) cells were purchased from National Centre for Cell Science, Pune,

India NCCS, Pune India) were used in all experiments. These cell lines were maintained in Dulbecco's Modified Eagle's Medium (GIBCO, HIMEDIA) containing 10% Fetal Bovine Serum (FBS). FBS supplemented with 0.1% antibiotic anti-mycotic solution. The supplemented essential cell culture medium is henceforth referred to as "growth medium". A549 cells were cultured in growth medium and incubated at 37°C with 5% CO_2 in a humidified air incubator prior to use. All described experiments in the present study were performed using A549 cells between cell passage no. 9 and 22. Cell passage is defined as the number of times cells divide to enable cell growth *in vitro* (MacDonald, 1994). For example, passage 9 refers to the nine serial passages of the cells.

2.2.2. Freezing of A549 cells

Prior to long-term usage, cells were kept at low temperature at - 80°C. The A549 cells were generated from a confluent cell layer grown in cell culture flask and detached from the flask following trypsinisation and re-suspended in growth medium. The cells were counted using a hemocytometer and cell suspensions of 1 million cells in 150 µl of 10% (v/v) dimethyl sulfoxide (DMSO) was prepared in FBS. DMSO is used as a cryoprotective agent to protect cells from damage caused by low temperature. The suspensions were kept in cryovials, marked with date and passage number and stored overnight at - 80°C.

2.2.3. Thawing of A549 cells

Thawing of A549 cells involved retrieving the cells previously stored at low temperature -80°C. Prior to thawing, cryovials containing A549 cells were moved to a water bath heated to 37°C. In a cell culture hood, cells were then transferred from the cryovials into an appropriate volume of growth medium pre-warmed at 37°C to allow

dilution of the cryoprotectant DMSO. The suspension containing cells and media was then centrifuged at 1650 revolutions per minute (rpm) for 5 min. The supernatant was removed and the cell pellet was resuspended in fresh growth medium. Cells were then transferred to a T-25 cm^2 cell culture flask and an appropriate volume of growth medium was added to allow cell growth.

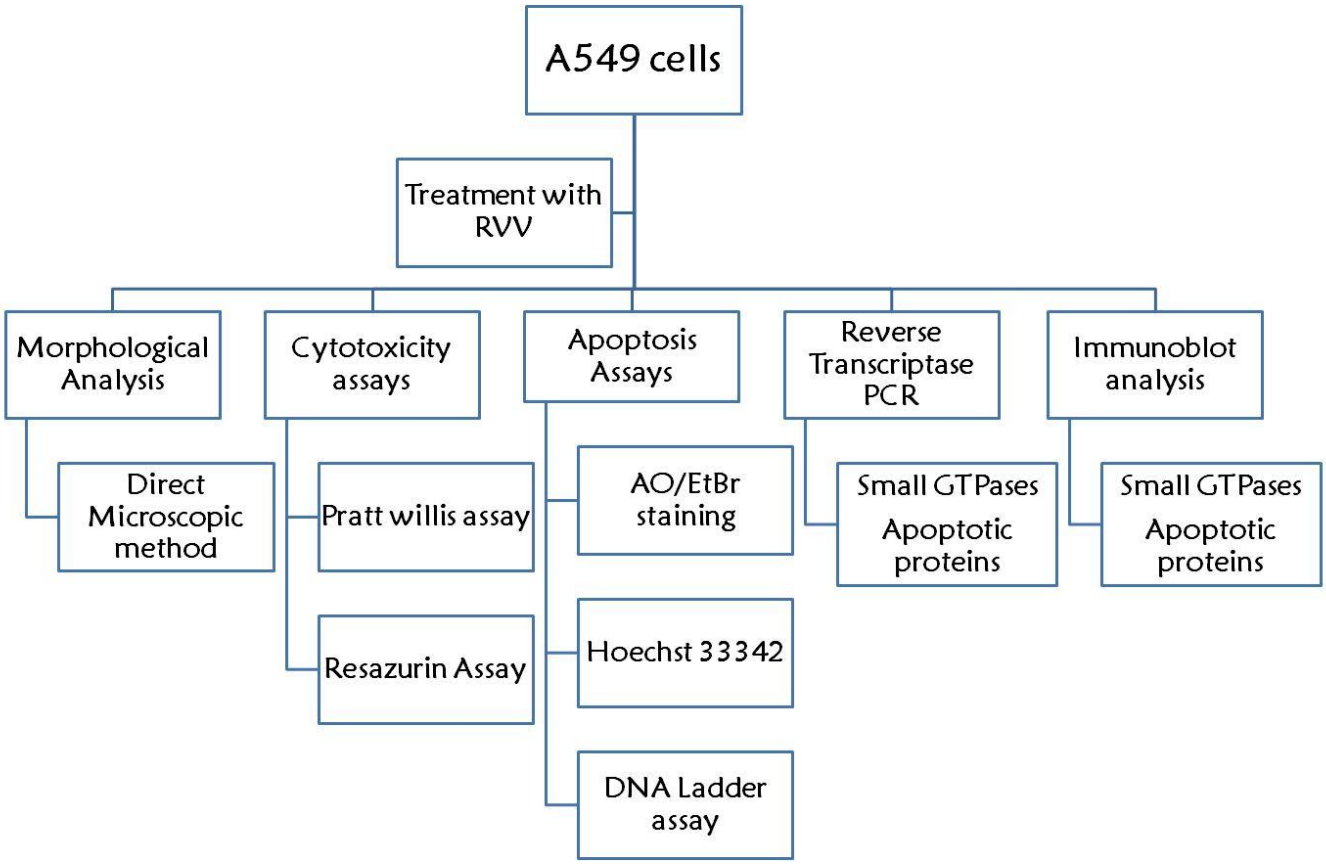
2.2.4. Growth of A549 cells

The cells were grown in a T-25 cm^2 cell culture flask. Cell passage and splitting was performed when cells reached 80-100% confluency. Cell confluency was assessed by a light optical microscope (Nikon Eclipse TS100, Tokyo, Japan). Prior to cell passage, the growth medium in the flask was discarded and the cells were washed twice with sterile phosphate buffered saline (PBS) to remove any FBS particles. Cells were then exposed to trypsin (0.1%) solution and incubated at 37°C for 5 min until the cell monolayer had dislodged from the flask. Trypsin was deactivated by adding of growth medium containing 10% (v/v) FBS into the flask. A single cell suspension was generated and centrifuged at 3000 rpm for 5 min. The supernatant was removed and the pellet containing the cells was re-suspended in 10 ml of growth medium. Cells were then counted using a haemocytometer. The average of four different counts was used to calculate cell numbers in a 10 ml suspension.

After counting, cell suspensions were either split in a ratio of 1 to 10 into a fresh T-25 cm^2 cell culture flask or seeded into multi-well cell culture plates (coated with fibronectin to allow cells to adhere to the plates) to be used for subsequent experiments. Cell incubation was maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.2.5. Preparation of RVV solutions: Dry RVV was reconstituted in 20 mM phosphate buffer (pH 7.4) over night at 4^{o}C. The solution was then centrifuged at 5000 rpm for 5 min at 4^{o}C to discard the cells debris and other particulate matters as described by Chakrabarty and co-workers (2000). This was further diluted with DMEM to obtain the desired concentration. RVV was freshly prepared from the original stock just before the treatment.

EXPERIMENTAL DESIGN



2.3. *Cytotoxicity assays*

2.3.1. *Experimental design*

A fixed number (5×10^5) of exponentially growing A549 cells were seeded into several individual T-25cm^2 flasks and were treated as follows:

1. ***Control:*** Cells not treated with RVV
2. ***RVV alone*****:** The cultures of these groups were treated with various concentrations of RVV (2.5µg/ml and 5µg/ml) alone for 24 and 48 h.

After the treatment with RVV, media was removed and cells were trypsinized for 1 min to dislodge the cells to get single cell suspension. The cytotoxic effects of RVV were analyzed by Pratt and Willis test as explained below.

2.3.2. Total viable cell count

The cytotoxic effect of RVV was evaluated by Pratt and Willis test (1985). Briefly, after treatment with various concentrations of RVV, the RVV containing medium was removed from the culture flasks and washed with 2 ml of sterile phosphate-buffered saline (PBS). The cells were then dislodged by a brief exposure (1 min) to trypsin solution following inactivation with addition of few drops of serum. Trypsinized cells were collected for staining with 0.5% trypan blue with 1:1 ratio of the dye and cell suspension. Mixed suspension (20µl) was applied to haemocytometer and a minimum of two thousand cells including both viable and dead cells were counted at 40X magnification using an inverted light microscope (Nikon Eclipse TS 100). Dead cells appeared blue in colour, while viable cells appeared colourless. Three replicate counts were taken per sample. The percentage of cell viability was calculated as follows:

$$\text{Viability (\%)} = \frac{\text{Number of non-stained cells}}{\text{Total cell number cells}} \times 100$$

2.3.3. Resazurin cytotoxicity Assay

The assay was carried out according to the protocol described by O'Brien and co-workers (2000). Cell viability through metabolic activity was assayed using Resazurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one). Live and healthy cells reduce Resazurin (blue) to resorufin (pink) and dihydroresorufin intracellularly (Figure 2.1).

Figure 2.1

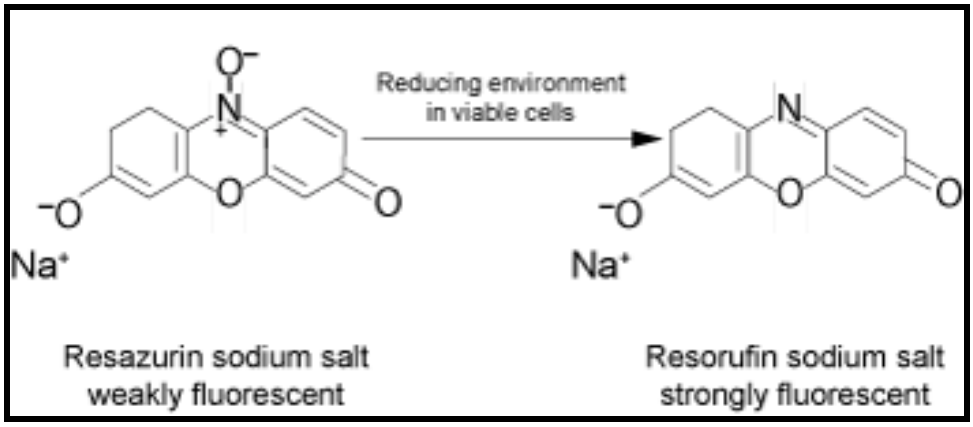


Figure 2.1: Conversion of resazurin to resorufin by viable cells results in a fluorescent product. (**Source:** https://www.promega.in)

Exponentially growing A549 cells (1,00,000) were seeded into 96 well plate and incubated for 24 h at 37°C in CO_2. Eight wells each served as control and blank. Rests of the wells were used for different toxin treatment groups. The culture medium was removed and substituted with fresh medium with different concentrations of RVV (0 - 10µg/ml) for 24 and 48 h. After the treatment, the medium containing RVV was removed and 10 µl of resazurin solution was added to each well. After four hours of incubation at 37^{o}C in a 5% CO_2 incubator positive difference in absorbance at

wavelength of 600 nm and 690 nm of each well culture against control was monitored and the percentage reduction was calculated and reported as a measure of toxicity as described by Anoopkumar and co-workers (2005). The limiting value corresponding to 0% reduction was obtained by measuring A_{600}–A_{690} of negative Control.

2.4. Morphological analysis of cells

The effects of RVV induced morphological changes in A549 cell was assessed by simple microscopic method. After the cells were treated with different concentrations of RVV for 24 and 48 h, the cells from all the treated groups were photographed using inverted microscope using an attached digital camera (Nikon-Eclipse, TS 100F, USA) along with an unexposed sample as a control.

2.5. Fluorescent staining with Phalloidin FITC

The cytoskeletal changes rendered by RVV in A549 cells were assessed by Phalloidin FITC. Phalloidin binds specifically to F-actin. When labeled with fluorescein molecules, such as FITC, phalloidin enables the visualization of the actin cytoskeleton within cells. Phalloidin staining is a useful tool to clearly visualize the cytoskeleton of the cell.

Phalloidin staining was performed directly in the multi-well dishes, in which the cell monolayers were grown, cells were treated with various concentrations of RVV for 24 and 48 h. After treatment, cells in each group were fixed with 3.7% formaldehyde for 30 min at room temperature, rinsed three times with 1 × PBS, then treated with 0.1% Triton X-100 for 10 min at room temperature, and rinsed thrice with PBS. The cells were further incubated with (1:100) dilution FITC-phalloidin for 2 h in the dark at room temperature. To label the nuclei, Hoechst 33342 (10 μg/ml) was added for 15

min. in the dark at room temperature. The cells were then washed in 1× PBS to remove the unbound FITC-phalloidin and Hoechst 33342. Finally, the cytoskeletal and nuclear morphology was imaged by a fluorescence microscope (Nikon-Eclipse, TS 100F, USA).

2.6. Measurement of apoptosis

To evaluate the apoptotic effects of RVV, fixed number (10^6) of A549 cells were inoculated into several individual 25 cm^2 culture flasks and were allowed to grow for 24 h. Cultures were then divided into following groups:

1. ***Control group:*** The cells of this group were not treated with RVV
2. ***RVV alone group:*** The cells of this group were treated with 2.5 and 5 µg/ml for 24 and 48 h.

After various treatments, cells were trypsinized and further processed for microscopy by Hoechst 33342, EtBr/AO and DNA fragmentation pattern assays.

2.6.1. Analysis of apoptotic cells by fluorescence microscopy

The morphological changes during apoptosis were analyzed by the differential uptake of fluorescent DNA binding EtBr/AO stains as described by Renvoize and co-workers (1998). Acridine orange is taken up by both viable and nonviable cells and emits green fluorescence if intercalated into double stranded nucleic acid (DNA) or red fluorescence if bound to single stranded nucleic acid (RNA). Ethidium bromide is taken up only by nonviable cells and emits red fluorescence by intercalation into DNA. Based on this assay four types of cells were distinguished according to fluorescence emission and the morphological aspects of chromatin condensation in the stained nuclei. The following points were considered for characterization: **(1)** Viable cells have uniform bright green nuclei with organized structure (PMNCs also have orange cytoplasm).

(2) Early apoptotic cells (which still have intact membranes but have started to undergo DNA cleavage) have green nuclei, but peri-nuclear chromatin condensation is visible as bright green patches or fragments.

(3) Late apoptotic cells have orange to red nuclei with condensed or fragmented chromatin. **(4)** Necrotic cells have a uniformly orange to red nuclei (Figure 2.2).

Figure 2.2

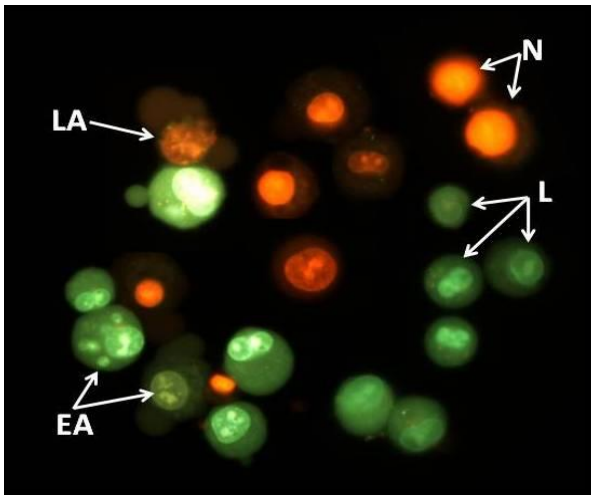


Figure 2.2: Typical representative images of A549 cells treated with or without RVV showing morphological analysis of apoptosis and necrosis using AO/EtBr staining. Magnification (40×), (**L**-live cell, **EA**-early apoptotic, **LA**- late apoptotic and **N**-necrotic cell).

After treatment, cells were dislodged by a brief (1 min) exposure to trypsin solution following inactivation with addition of few drops of serum. Trypsinized cells were stained with mixture of AO (5 µg/ml) and EtBr (5 µg/ml) and observed immediately under fluorescence microscope (Nikon-Eclipse, TS 100F, USA). At least 100 target cells were counted. The experiment was conducted in triplicates. The percentages of apoptotic cells were determined according to the following formula.

$$\text{Apoptotic index} = \frac{\text{No. of apoptotic cells}}{\text{Total number of cells}} \times 100$$

2.6.2. Fluorescent staining with Hoechst 33342

Cell staining with Hoechst 33342 stain ($C_{27}H_{37}C_{l3}N_6O_4$) is a dye having ability to penetrate both intact and lysed cell membranes and bind strongly to specific grooves in DNA (Adenine-thymine). When bound to DNA, the stain emits blue light, which allows counting of all cells (live and dead) when imaged using a fluorescence microscope (Parrilla *et al.,* 2004). Hoechst 33342 dye dilution was prepared from a stock of 10 mg/ml of Hoechst 33342 trihydrochloride. A dilution of 1 mg/ml of Hoechst 33342 dye in sterile water was used for cell counting. The stain dilution was found necessary to minimize experimental error that may result from using small volume of the reagent. The monolayer was washed twice with 1x PBS. Cells were fixed in 10% formaldehyde and dehydrated using 90% methanol. After fixing and dehydrating, the monolayer was washed once and maintained in 1x PBS. Hoechst 33342 (final concentration 1µg/ml) was directly added to it by gently shaking at 4°C for 5 min. Stained nuclei were visualized under Nikon eclipse TS 100 fluorescence microscope at 20 × magnification with an excitation wavelength of 355-366 nm and an emission wavelength of 465-480 nm. The apoptotic cells were stained bright blue because of their chromatin condensation, while normal cells were stained pale blue (Figure 2.3).

Figure 2.3

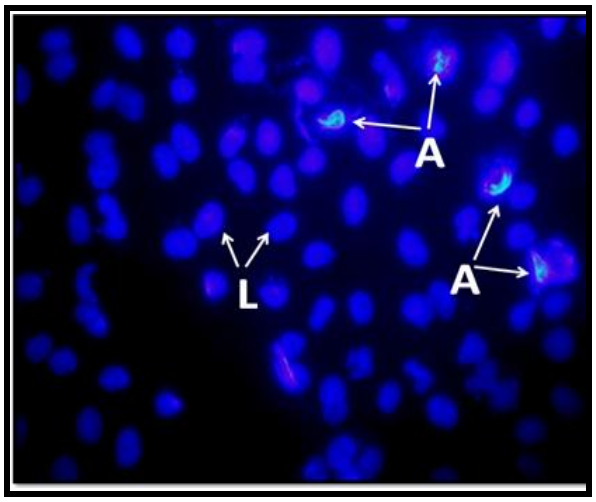


Figure 2.3: Typical representative images of A549 cells treated with or without RVV showing morphological analysis of apoptosis using Hoechst 33342 staining. Magnification (40×), (**L**-live cell, **A**-apoptotic cell)

2.6.3. Detection of DNA degradation by gel electrophoresis:

The formation of ladder pattern from the DNA fragmentation a biochemical hallmark of apoptosis, this assay was performed by using agarose gel electrophoresis according to the protocol described by Giri and co-workers (2003). During the process of apoptosis, chromatin DNA is digested by Ca^{2+}/Mg^{2+} endonucleases into internucleosomal fragments of 180-200 base pairs, which can be visualized by agarose gel electrophoresis analysis, a method for qualitative determination of DNA fragmentations. After the treatments, the floating and adherent cells from above groups were pooled and incubated overnight at 37°C in a lysis buffer (0.02M EDTA, 0.05M Tris HCl, 1%NP40). After centrifugation at 800 rpm for 10 min, the supernatant was collected and incubated with 10µl of RNAse (10 mg/ml) and 20 µl of 20% SDS at 56°C for 2 h. This was followed by treatment with 5 µl of proteinase

K (20mg/ml) at 56°C for 8 h. DNA was precipitated overnight with ammonium acetate (10 M) and ethanol and the DNA pellet was dissolved in 20 µl of TE buffer. Later, 10ng of DNA was separated on a 1.5% agarose gel followed by ethidium bromide staining. The results were analyzed using a gel documentation system (Bio-Rad, UK).

2.7. Reverse Transcriptase PCR

To investigate the role of RVV on small GTPases and apoptosis, we examined the expression levels of some GTPases and apoptosis-related proteins in A549 cells. A fixed number of cells were inoculated into several individual culture flasks and were allowed to grow for 24 h. Cultures were then divided into following groups.

1. **Control group:** The cells of this group were not treated with RVV
2. **RVV group:** The cultures of this group were treated with RVV.

After various treatments, cells were processed for RT-PCR by the following method.

2.7.1. Total Ribonucleic Acid (RNA) Extraction

Total RNA was extracted from the control and experimental cells as per the manufacturer's (Invitrogen, USA) protocol using TRIzol® LS Reagent. 1ml of the TRIzol® LS reagent was added directly to the monolayer after removing the media and the cells were homogenized by repeated pipeting. 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 spectrophotometrically at 260 nm and then stored at -80^{0}C for future applications.

2.7.2. RNA quantification

Quantification of both RNA and cDNA was done using a Nanodrop (NanoDrop technologies, USA). Reading was taken at 260 and 280 nm as described by the manufacturer. A260/A280 ratio of more than 2.0 were regarded as pure quality.

2.7.3. cDNA Synthesis: First strand cDNA was synthesized from RNA samples (2 µg) using the cDNA synthesis kit for RT-PCR (Tetro cDNA synthesis kit) from Bioline, USA, as per specifications provided in the kit.

2.7.4. Primer designing and Synthesis: The cDNA sequences for each gene were obtained from The Reference Sequence (Ref Seq) database NCBI. The primers specific for each template was designed using NCBI primer blast soft ware. The list of primers used along with their respective gene name is given in the material list section.

2.7.5. Reverse Transcriptase PCR: Polymerase chain reaction (PCR) is a technique that allows logarithmic amplification of short DNA sequences of between 100 to 600 BP within a longer double stranded DNA molecule. The cDNA obtained through reverse transcription was used as a DNA template.

The PCR was performed using a master mix from chromous biotech, India. The reactions were subjected to approximately 30 cycles of the three PCR steps (denaturation, annealing and extension) after the initial denaturation step and followed by a final extension step. The steps were as follow with some variation in annealing temperature and extension time depending on the nucleotide sequence of the gene of interest. (97^0 C for 2 min, 94^0 C for 1 min, 50^0 C for 2 min, 70^0 C for 1 min). The products were either stored at 4ºC for further use or electrophoresed on 1.2 % agarose gel**.**

2.7.6. Agarose gel electrophoresis of DNA

The principle of agarose gel electrophoresis involves separation of nucleic acids according to their size and conformation. During this technique the DNA fragments can be visualized by staining the gel with ethidium bromide which intercalates between bases of DNA and illuminating the gel under UV light on a transluminator.

Agarose gel electrophoresis is a technique used for separation of nucleic acids according to size and charge. The DNA fragments were visualized by staining the gel with ethidium bromide (1 μg/ml), which intercalates between bases of DNA and illuminating the gel under UV light using a transluminator. Agarose gel was prepared in 1× TBE. The DNA samples were electrophoresed on agarose gel. A 100 base pair ladder from Genei Pvt Ltd., India was used DNA molecular weight marker. Gels were then subjected to electrophoresis with 80 volts for 90 min in 1× TBE buffer.

2.8. Immunoblot analysis

To further investigate the role of RVV, we examined the expression levels of some apoptosis-related proteins in A549 cells. A fixed number (10^6) of cells were inoculated into several individual culture flasks and were allowed to grow for 24 h. Cultures were then divided into following groups.

1. ***Control group:*** The cells of this group were not treated with RVV
2. ***RVV alone group:*** The cultures of this group were treated with optimal concentrations of RVV.

After various treatments, cells were processed for Western Blot Analysis by using anti-Hsp70, anti-Rac1, anti-RhoA, anti-Cdc42 and anti-phosphor Rac1/Cdc42 monoclonal antibodies by the method described below.

2.8.1. Total protein extractions

Total cellular protein was extracted from controls and tested cells, using lysis buffer. The monolayer was washed with 1× PBS and the cells were precipitated after trypsinisation. The pellet was then homogenized in lysis buffer using a tissue homogenizer. The extract was centrifuged at 10,000 rpm at 4°C for 10 min. The supernatant containing the soluble protein was transferred to a new sterile vial and was stored at -80^{0}C for further use.

2.8.2. Bradford Assay

Samples of the protein extractions were analysed using the Bradford Assay (1976) to determine the concentration of protein in each sample. This is important to load equal amount of each sample. Bovine serum albumin (BSA) protein standards were prepared in water at 0.25-1 mg/ml concentrations in order to construct a standard curve. Ten microlitres of each sample was added, in duplicate, to individual wells of a 96 well plate. Bradford reagent (250 µl) was then added to each well and the plate was left at room temperature for 15 min. The plate was then shaken for 30 seconds and then optical density was measured at 595 nm using an ELISA reader.

2.8.3. Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were separated on a 10% separating PAGE. The samples were thawed and boiled for 3 min at 100^{0}C and 30 µg of each sample was loaded onto a 10% SDS-PAGE. The voltage was set at 50V in stacking gel and 70V in resolving gel.

2.8.4. Preparation of 10% SDS-PAGE

To prepare two plates of SDS-PAGE, 10 ml of the gel was prepared as follows, 3.75 ml of bis/acrylamide (40%-37:5:1), 2.5 ml of 1.5M Tris-Cl (pH 8.8); 100 µl of 10% SDS; 100 µl of 10% freshly prepared ammonium persulphate (APS) and 3.15 ml of water were added to a 15ml tube of TEMED (5 µl) was then added to the mixture to start polymerization. The mixture was then poured into gel casting apparatus (BioRad, USA). After polymerization, stacking gel (prepared as follows 3.7 ml of water; 0.625 ml of 0.5 M Tris, pH 6.8, 0.5 ml of acrylamide/bis-acrylamide 30%; 50 µl of 10% SDS; 50 µl of 10% APS, 5 µl TEMED) was prepared and poured on top of the resolving gel.

2.8.5. Electrophoretic Transfer

Upon completion of protein separation by SDS-PAGE, gel was transferred onto the polyvinylidene fluoride (PVDF) membrane in transfer buffer and incubated for 2 h. The electro-blotting cassette (BioRAD, USA) was assembled according to the manufacturer's instructions. Briefly, the PVDF membrane and the gels were placed between the two filter papers and electrophoresis was performed at 25 V at 4°C for 2 h.

2.8.6. Immunobloting

Upon completion of the protein transfer on to a charged PVDF membrane, the membrane was stained with Ponceou Stain. After washing off the excess stain the membrane was incubated in 1×PBS for 5 min before being transferred to blocking buffer followed by overnight incubation at 4^0C. After blocking, the membrane was incubated in primary antibody solution for 4-6 h at room temperature. After incubation the blot was washed for 5 min in 1× TBST, followed by two washes in 1× TBS. The blot was then incubated in HRP-conjugated secondary antibody for 3 h.

2.8.7. Detection and exposure

Membrane was immune-blotted with the corresponding primary antibodies like Anti-Bcl2, anti-Bax, anti-caspase 3, anti-Rho GTPases and anti-actin antibodies at $4^{o}C$ overnight. Protein expression levels were detected by incubating with Chemiluminescent Substrate (Thermo scientific, Rockford, USA) for 5 min. The blot was then exposed to X-ray film (Thermo Scientific, USA) for an appropriate time. The X-ray film was then developed in the dark.

2.9. Caspase 3 activity Assay

The Caspase 3 Activity Assay is a fluorescent assay that detects the activity of caspase 3 in cell lysates. During the assay, activated caspase 3 cleaves this substrate DEVD-pNA, generating highly fluorescent p-NA (p - nitroaniline). Cleavage of the substrate only occurs in lysates of apoptotic cells; therefore, the amount of p-NA produced is proportional to the number of apoptotic cells in the sample.

Caspase 3 activity was determined using CPP32 colorimetric protease assay kit (Invitrogen, Frederick, MD, USA). The cells were seeded at a density of 1×10^{6} in a 60 mm dish and maintained at 5% CO_2 at 37°C in an incubator. After the treatment the cells were harvested by cell lysis buffer and 200µg of protein lysate were loaded in a 96 well plate. Caspase 3 activity was determined by adding DEVD-pNA substrate and incubating for 2 h in the dark at 37°C. The samples were read at 405 nm using an enzyme-linked immunosorbent assay (ELISA) microplate reader.

2.10. Transwell invasion assay

Transwell inserts allow the examination of chemotaxis, each transwell insert has an anchored microporous membrane, which is placed in a multi-well dish, thus providing upper and lower compartments. The upper compartment is inoculated with the cell

suspension, while the lower compartment contains the chemo attractants or media to be tested. Usually, media composition in the upper and lower compartments differ, with cells suspended in serum-free (SF) media in the upper chamber, so that a chemoattractant gradient is established across the microporous membrane. This allows the cells in the upper compartment to migrate through the pores towards the lower compartment containing chemoattractants. Once cells have migrated through the pores, they adhere on the under-side of the membrane. Non-migrating cells on the upper side of the microporous membrane can be removed by gentle swabbing (thus not damaging the membrane or dislodging cells on the lower side), enabling the fixing, staining and counting of the migrated cells.

Transwell chambers were uniformly coated with 50 µl Matrigel diluted with DMEM to a 30 µg/ml final concentration as per manufacturers protocol (Sigma, USA) followed by incubation at 37^0C for 24 h. A549 cells along with treatment were suspended in 100 µl DMEM and seeded in the upper chambers, and 600 µl DMEM containing 10% FBS was added to the lower chamber. After incubation at 37^0C for 24 and 48 h respectively, the cells were fixed and stained with 0.5% methylene blue for 1 h. After being washing twice with 1× PBS (pH 7.4), cells that remained on the top of the filter were removed using wet cotton swabs and whole filters were photographed and the invaded cells were counted under inverted microscope in 3 random fields, and the average value was calculated.

Statistical evaluation: The repeated measures ANOVA test was performed to compare the results of different groups, while Student's t-test (unpaired) was performed for two group comparisons. All the data were expressed as Mean ± SD (Standard Deviation) and the difference of $P < 0.05$ or more was considered significant.

2.11. RESULTS

2.11.1. Dose and time dependent effects of RVV on viability of A549 cells

A549 cell lines were assayed for cell viability with 24 and 48 h exposure for different doses RVV. Cells treated for 24 and 48 h with 2.5 µg/ml and 5 µg/ml RVV showed significant reduction in cell count when compared with respective control, indicating the cytotoxic effects of RVV (Figure 2.4).

Figure 2.4

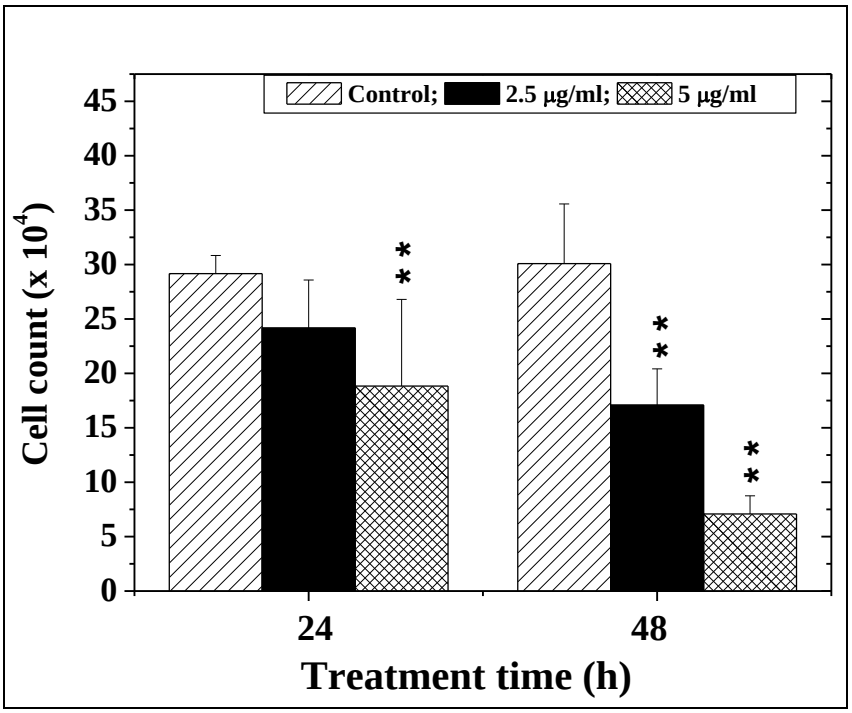


Figure 2.4: Evaluation of cytotoxicity by Pratt Willis test in A549 cells following exposure to RVV. All results are shown as Mean ± SEM from the data of a minimum of three separate experiments. **P< 0.01; No symbol- Non significant (when compared with respective control group).

2.11.2. Cytotoxic effects of RVV on A549 cells

The cytotoxic effects of RVV on A549 cells cultured *in vitro* were measured by Resazurin assay. The assay was performed using 2.5 and 5 μg/ml of RVV for 24 and48 h. The results showed that the growth of RVV exposed A549 cells was inhibited (% reduction) at 48 h as compared to 24 h. RVV dose and time dependent increase was indicated by increase in absorbance at 660 nm. Cytotoxicity of RVV was more evident after 48 h incubation than at 24 h for all concentrations of RVV tested. The half maximal inhibitory concentration (IC_{50}) value of RVV for A549 cells at studied times was 6 μg/ml for 24 h and 9 μg/ml for 48 h, respectively (Figure 2.5).

Figure 2.5

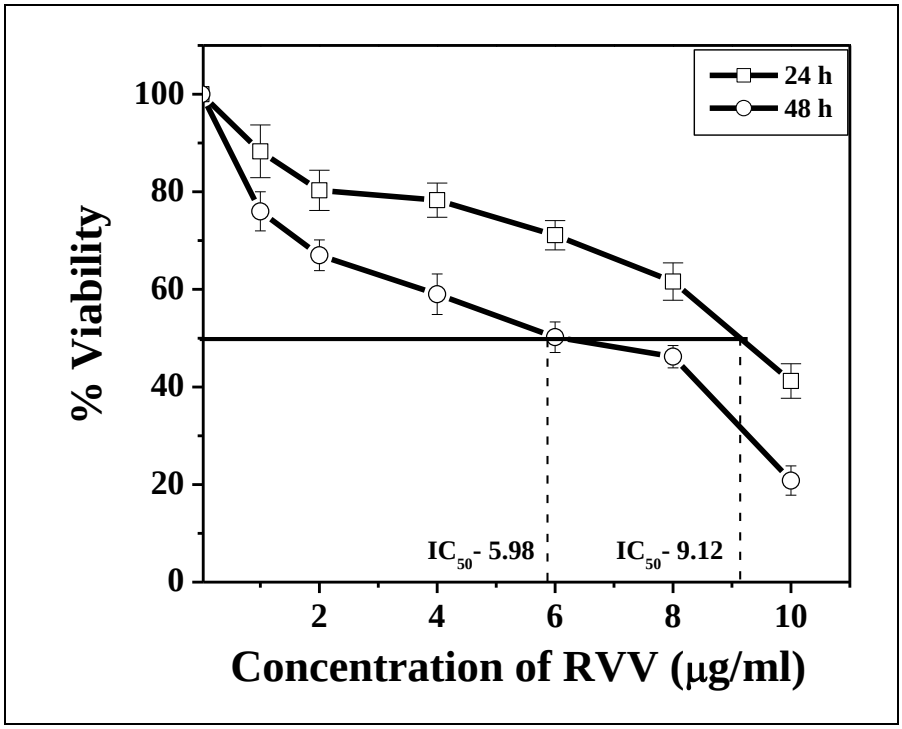


Figure 2.5: Evaluation of cytotoxicity by Resazurin assay in human lung adenocarcinoma cell line (A549): Cell viability was assayed after RVV exposure for 24 and 48 h using spectrophotometer. IC_{50} for 24 and 48 h is 9.12 and 5.98 μg/ml respectively.

2.11.3 RVV changes cell morphology in human A549 cells in a dose and time dependent manner

The results of the present study showed that, control A549 cells remained adherent and were homogeneously distributed on cultured field and presented a normal epithelial shape (Figure 2.6 A & D). However, A549 cells showed a time and concentration dependent detectable morphological changes after incubation with different concentrations of RVV at different time intervals (24 and 48 h). RVV (2.5 and 5 μg/ml) treated for 24 h showed change of shape from normal epithelial to fusiform shape. Stress granules and punctuate structures were also noticed (Figure 2.6 B & C). Increase in treatment of cells with 5 μg/ml RVV for 48 h resulted in further elongation of the cells, with increase in stress granules and punctuate structures (Figure 2.6 E). Rounding of cells with increase in extracellular space was noticed. Few areas devoid of cells were also observed in the culture plates (Figure 2.6 F).

Figure 2.6

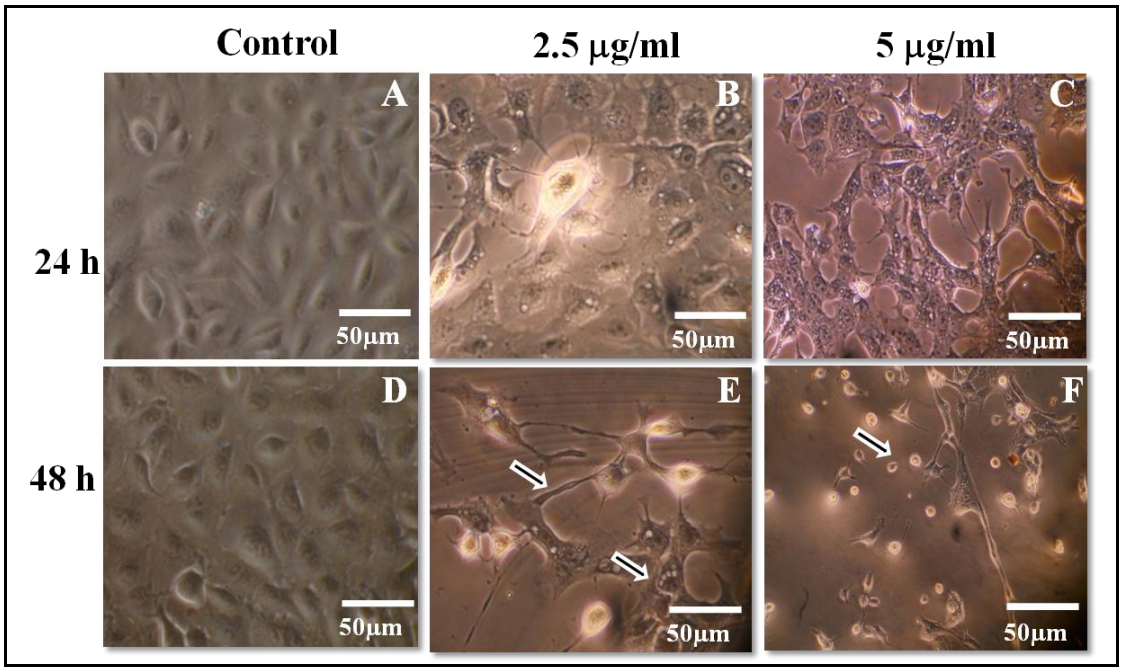


Figure 2.6: RVV induces Morphological changes in human lung adenocarcinoma cell line (A549): Representative microscopic images (20×) from both untreated and treated A549 cells for 24 and 48 h showing concentration dependent morphological changes such as formation of spindle like structures, stress granules and punctuate structures, in A549 cells exposed to RVV for various incubation periods. (A) Control (B) 2.5 μg/ml (C) 5 μg/ml (24 h); (D) Control (E) 2.5 μg/ml (F) 5 μg/ml (48 h), with 48 h exposure time cells.

2.11.4. RVV induced changes on F-actin by FITC-phalloidin staining

Effects of RVV on cytoskeletal changes were further studied by FITC phalloidin staining. The results indicated that the cells in the control group were rich in regular and parallelly organized actin (Figure 2.7A). Cells treated with RVV for two different time points showed changes in F-actin distribution leading to decreased number of actin fibers resulting in cell shrinkage (Figure 2.7 C and E). As shown in Figure 2.6

cells treated with RVV showed dose and time dependent increase in actin condensation, resulting in rounding of cells (Figure 2.7 F).

Figure 2.7

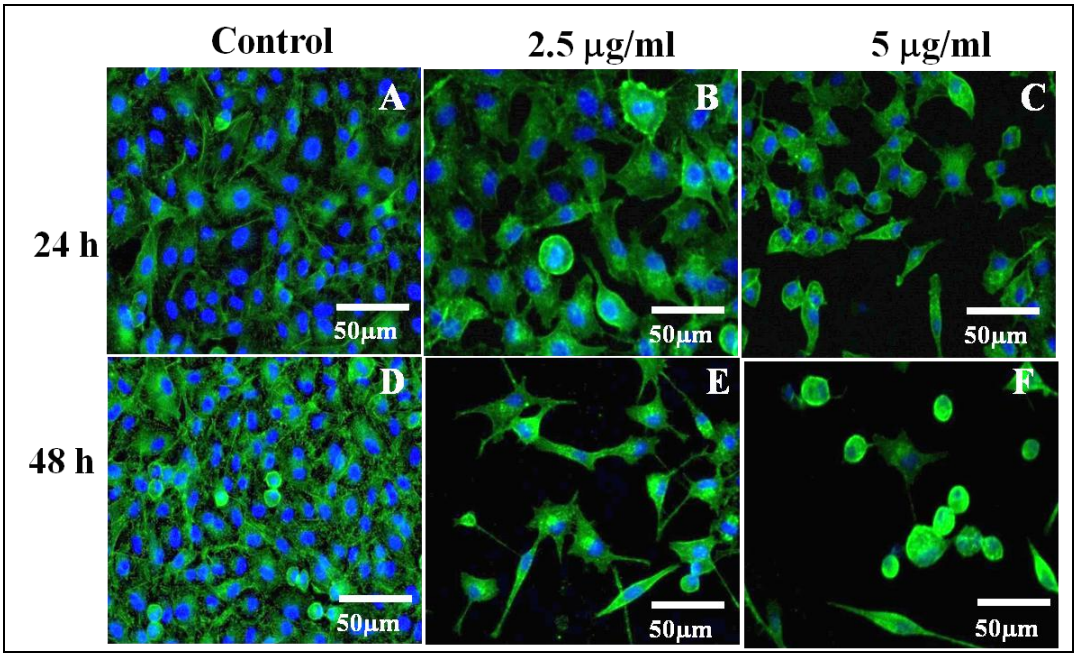


Figure 2.7: Morphology of the cytoskeleton and nuclei in A549 cells before and after treatment with RVV: The blue colour indicated the location of nuclei stained with Hoechst 33342, the green colour indicated the location of F-actin stained with FITC-phalloidin. (A) Control; (B) 2.5 µg/ml; (C) 5 µg/ml- 24 h; (D) Control; (E) 2.5 µg/ml; (F) 5 µg/ml- 48 h.

2.11.5. Assessment of RVV induced apoptosis by Hoechst 33342 staining

Apoptosis can be differentiated from necrosis by their characteristic nuclear changes. Hoechst 33342 is a nuclear stain which is observed as blue fluorescence when excited under fluorescence microscope. In our present study, Hoechst 33342 staining revealed the changes associated with apoptosis in A549 cells treated with the RVV (Figures 2.8). The morphological changes associated with apoptosis such as chromatin condensation,

nuclear fragmentation, and condensation of nucleus margin (marked by arrows in Figure 2.7 are evident in A549 cells upon treatment. The RVV treated group showed a dose and time dependent significant increase in the apoptotic index.

Figure 2.8

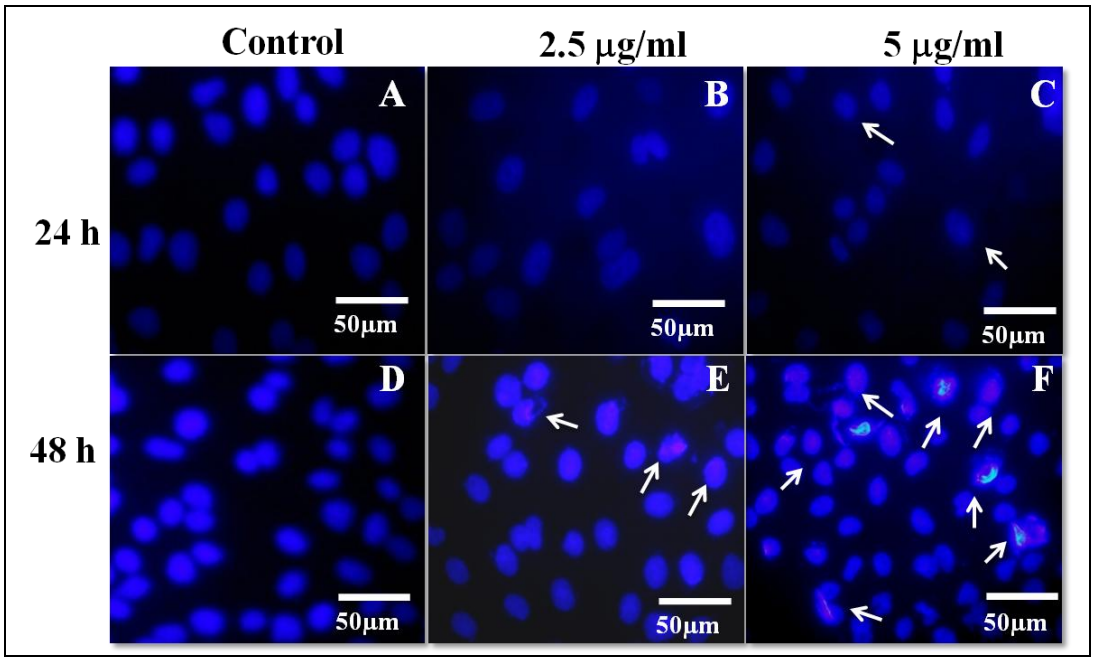


Figure 2.8: Nuclear staining of human lung cancer cells A549 using Hoechst 33342. Arrows indicate cell shrinkage, nuclear fragmentation, and margination of the nucleus. All associated with the apoptotic mode of cell death A) Control; (B) 2.5 µg/ml (C) 5µg/ml- 24 h (D) Control (E) 2.5 µg/ml (F) 5 µg/ml- 48 h. Quantitative results for the number of apoptotic cells per 100 cells in total. (Magnification 20×).

Table 2.1

Concentration of RVV (μg/ml)	Apoptotic index ± SEM	
	24 h	48 h
Control	3.15 ± 0.41	3.51 ± 0.63
2.5	16.37 ± 1.28	23.92 ± 2.41
5	24.82 ± 2.16	31.29 ± 2.36

Table 2.1: Results of apoptotic index by acridine orange / EtBrstaining of A549 cells treated with different concentrations of RVV for 24 and 48 h.

2.11.6. Study of Nuclear morphology by AO/EtBr staining

Cellular morphology can be used as a parameter for measuring the effect of a compound on cytotoxicity. Since the nuclear condensation occurs in this stage of apoptosis, the apoptotic morphology of the nucleus will be evident upon staining. The staining with acridine orange/ethidium bromide shows that the cells in control groups are uniformly green and well distributed (Figure 2.9A). Whereas, typical morphological changes, including cytoskeletal collapse, the formation of apoptotic bodies and nuclear fragmentation and loss of membrane integrity are observed in the RVV treated cells (shown with arrows in Figure 2.9). Nuclei of cells treated with RVV showed damage including swelling, membrane dissolution and DNA condensation (Figure 2.9 E & F), which are known manifestations of apoptosis. After the exposure of cells to RVV there was a significant increase in apoptotic index in the cells with increase in dose (2.5 μg/ml and 5 μg/ml) and time at 24 h and 48 h, respectively (Table 2.1).

Figure 2.9

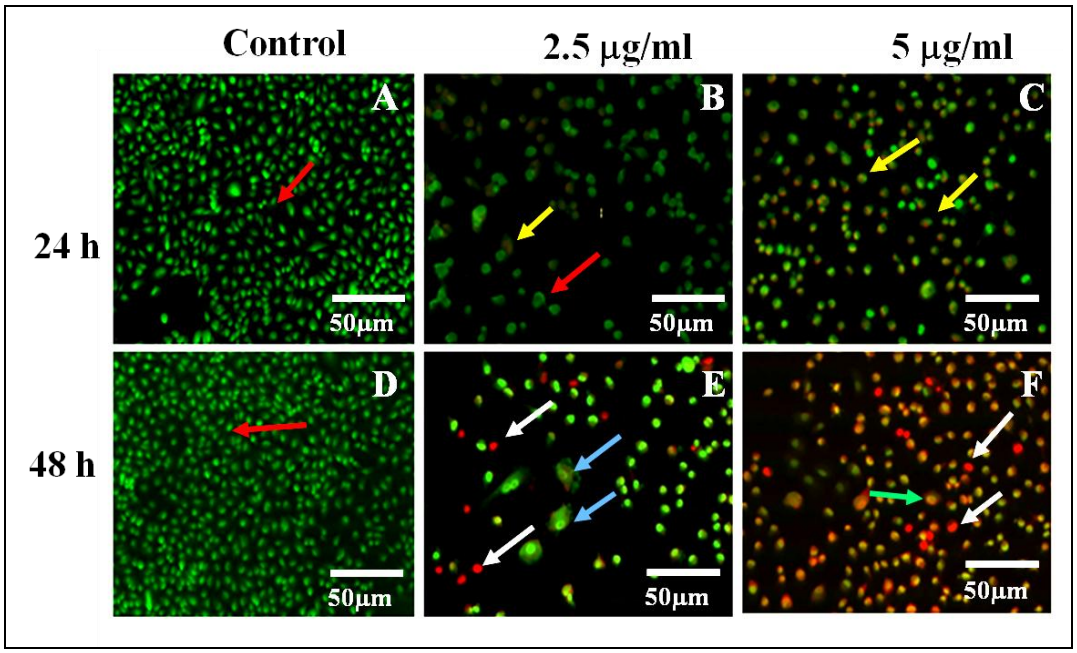


Figure 2.9: Cellular staining using acridine orange/ethidium bromide of human lung cancer cells A549. Untreated viable cells are uniformly green (red arrow) (A & D). Apoptotic cells show characteristic loss of membrane integrity (blue arrow) and chromatin condensation, stained yellow-orange (yellow arrow) (B & C); dead cells showed bright orange-red in appearance (white arrow) (E & F). (A) Control; (B) 2.5 µg/ml; (C) 5 µg/ml- 24 h; (D) Control; (E) 2.5 µg/ml; (F) 5 µg/ml- 48 h. Cells were photographed at (20×).

2.11.7. DNA Ladder assay

To gain further insights into the mode of cell death caused by RVV, its effect on the DNA fragmentation was determined. A549 cells were exposed to 2.5 and 5 µg/ml of RVV for 24 and 48 h. RVV exposed cells showed typical oligonucleosomal ladder pattern as compared as an indicator for early apoptosis (Figure 2.10).

Figure 2.10

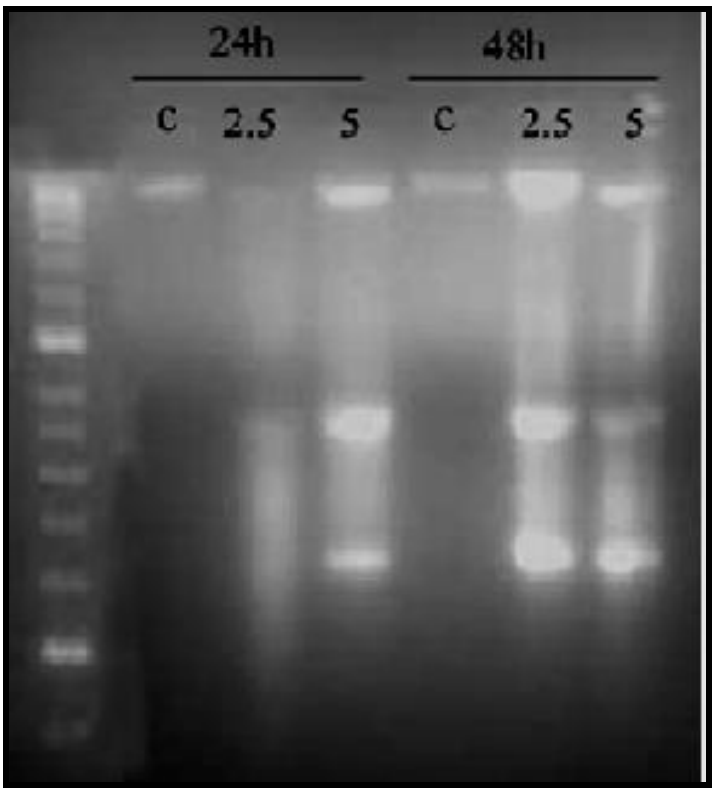


Figure 2.10: Gel electrophoresis image obtained after DNA fragmentation assay for apoptosis detection. Analysis of genomic DNA fragmentation in A549 cells after the treatment at 24 and 48 h with various concentrations of RVV. Supernatants of control and treated cells were subjected to lysis and total DNA from replicate cultures was prepared using the standard protocol and fragmentation was assessed by 1.2% agarose gel electrophoresis and ethidium bromide staining.

2.11.8. Effects of RVV on caspases, Bcl-2 and Bax mRNA expression

To elucidate the possible mechanisms of apoptosis by caspase family in A549 cells, present study was aimed to investigate the effects of RVV with respect to mRNA expression by RT-PCR. The result indicated that the expression levels of caspase 3, 8,

and 9 increased in the treated group compared to that of untreated group. Bcl2 (pro-apoptotic) and Bax (anti-apoptotic) are apoptosis-related protein and play an important role in keeping mitochondrial membrane integrity and permeability. To further investigate the functional role of RVV, the expression levels of Bax and Bcl2 were determined. Different concentrations of RVV were added to A549 cells and treated for different time 24 and 48 h. RT-PCR results show that, cells pre-treated with RVV promoted the mRNA expression of Bax and down-regulated Bcl-2 expressions in a dose and time dependent manner (Figure 2.11 (A)). Expression profile is also accompanied with densitometric scanning profile of protein (Figure 2.11 (B)). Collectively, these results suggest that RVV induce apoptosis by activating expression of Bax and inhibiting Bcl2.

Figure 2.11

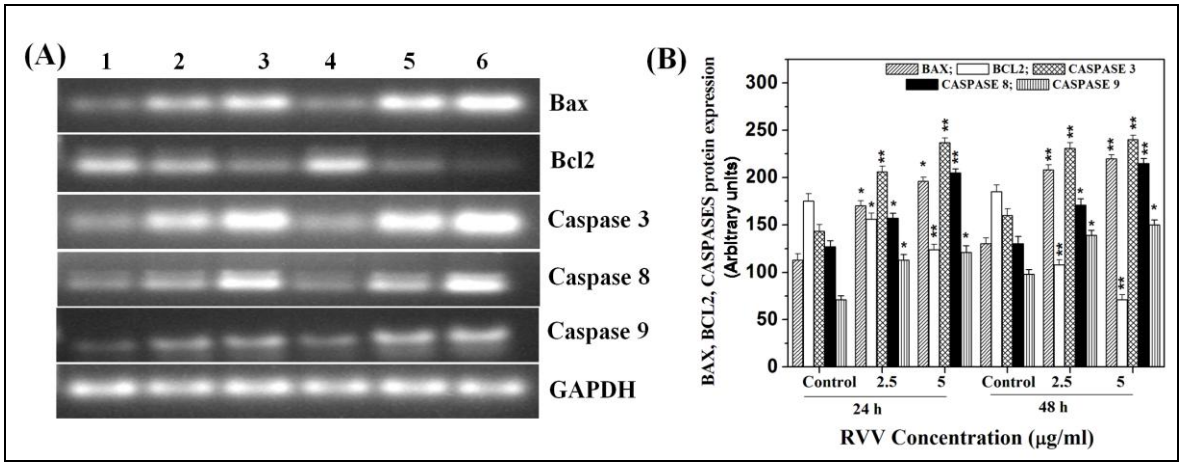


Figure 2.11: (A) Reverse transcriptase PCR (RT-PCR) for the gene expression of apoptosis related genes: Total RNA was extracted and RT-PCR was carried out. The amplified product was analyzed by agarose gel electrophoresis. GAPDH was used as a control to ensure equal loading. The results showed dose and time dependent up-regulation at mRNA level compared to control. Lane-(1) Control; (2) 2.5 µg/ml; (3) 5

µg/ml -24 h, (4) Control; (5) 2.5 µg/ml; (6) 5 µg/ml, 48 h **(B)** Quantification of RT-PCR results by quantity one analysis that reveals the underlying expression pattern. The bars represent Mean±SEM of at least two independent experiments each performed in triplicate, $^{**}p<0.01$, $^{*}p<0.05$ represents significant differences as compared to that in control group.

2.11.9. Effects of RVV on small GTPases (Rac1, RhoA and Cdc42) mRNA

The results of the present study showed no alterations in the mRNA expression of small GTPases in untreated group, whereas, RhoA protein which maintains the cell adhesions, and Rac1 proteins which plays an important role in cell membrane integrity and polarity, on exposure of A549 cells to RVV (2.5 and 5 µg/ml) for 24 and 48 h caused down-regulation of mRNA levels. Whereas, Cdc42 involved in filopodia formation was up-regulated on exposure of cells to RVV (2.5 and 5 µg/ml) for 24 and 48 h (Figure 2.12 (A)). GAPDH and β-actin served as internal controls, respectively. Expression profile is also accompanied with densitometric scanning profile of mRNA (Figure 2.12 (B)).

Figure 2.12

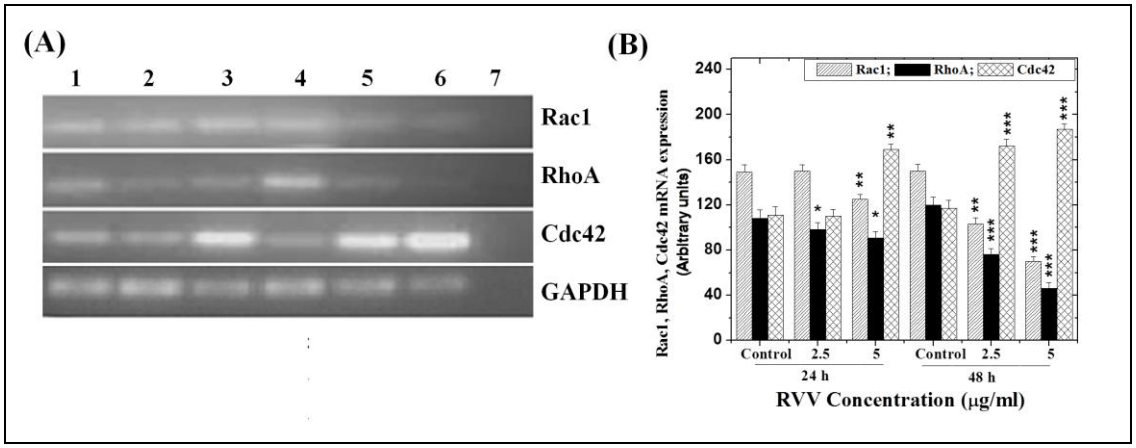


Figure 2.12: (A) Reverse transcriptase PCR (RT-PCR) for the gene expression of small GTPases: A549 cells were subjected to RVV treatment. Total RNA was extracted and RT-PCR was carried out. The amplified products (Rac1, RhoA and Cdc42) were analyzed by agarose gel electrophoresis. GAPDH was used as an internal control. The results showed that there was down regulation in the expression of Rac1 and RhoA at mRNA level compared to control. Whereas, dose and time dependent up-regulation of Cdc42 was seen. Lanes-**1**: Control; **2**: 2.5 µg/ml; **3**: 5 µg/ml;-24 h, **4**: Control; **5:** 2.5 µg/ml; **6:** 5 µg/ml and **7:** Negative Control-48 h. **(B)** Quantification of RT-PCR results of small GTPases (Rac1, RhoA and Cdc42) reveals the underlying expression pattern. The bars represent Mean±SEM of at least two independent experiments, ***p<0.001, **p<0.01, *p<0.05 represents significant differences as compared to that in control group.

2.11.10. Effects of RVV on small GTPases (Rac1, RhoA and Cdc42) protein

Rho-GTPase plays an important role in the actin cytoskeleton rearrangements. The effect of RVV on the protein expression of Rac1, RhoA, and Cdc42 was investigated. As showed in Figure 2.13, Rac1 and RhoA expression decreased after RVV treatment,

whereas, Cdc42 increased when treated with RVV (2.5 µg/ml and 5 µg/ml) for 24 and 48 h (Figure 2.13 (A) and (B)).

Figure 2.13

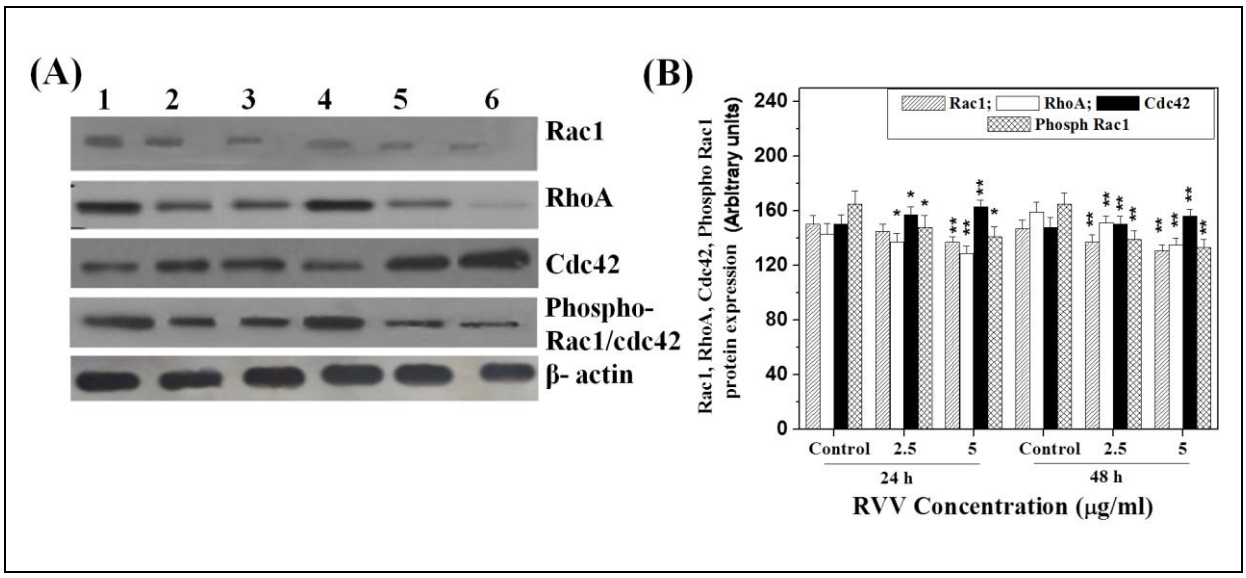


Figure 2.13: (A) RVV regulates the expression of Rho-GTPases in A549 cells Protein expressions of small GTPases were analysed by western blot analysis: After the treatment with RVV the protein was extracted and western blot was carried out with different monoclonal antibodies. The results at specific protein level showed that there was down regulation in the expression pattern of Rac1 and RhoA compared to controls of 24 and 48h. Conversely Cdc42 exhibited up-regulation. The expression pattern of small GTPases at protein level showed similar expression pattern of mRNA levels. **Lanes-1**: Control; **2:** 2.5 µg/ml; **3:** 5 µg/ml -24 h, **4:** Control; **5:** 2.5 µg/ml; **6:** 5 µg/ml-48 h. **(B)** Quantification analysis of western blot results. The bars represent Mean±SEM of at least two independent experiments, ***p<0.001, **p<0.01, *p<0.05 represents significant differences as compared to that in control group.

2.11.11. Treatment with RVV causes p53 regulation

To assess the possible involvement of tumor suppressor protein p53, which plays an important role in apoptosis, RT PCR analysis was carried out. RVV treated cells resulted in significant concentration and time dependent elevation of tumor suppressor p53 (Figure 2.14 (i)) at mRNA level. Expression profile is also accompanied with densitometric scanning profile (Figure 2.14 (ii)).

Figure 2.14

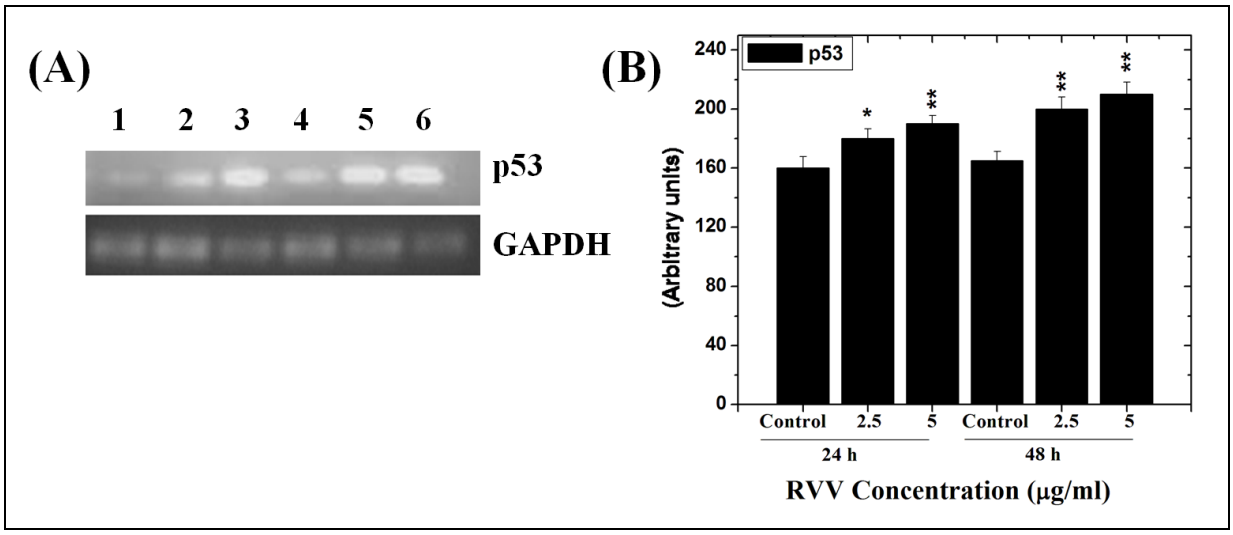


Figure 2.14: (A) Expression profile of p53 mRNA up on RVV exposure: The results showed that there was up regulation at mRNA level compared to control with respect to dose and time. **Lanes-1:** Control; **2:** 2.5 µg/ml; **3:** 5 µg/ml -24 h, **4:** Control; **5:** 2.5 µg/ml; **6:** 5 µg/ml-48 h. **(B)** Quantification of RT-PCR results of expression profile of p53 mRNA. The bars represent Mean±SEM of at least two independent experiments, **p<0.01, *p<0.05 represents significant differences as compared to that in control group.

2.11.12. RVV exposure affects Hsp70 expression pattern at mRNA level

To further examine the role of Hsp70 upon treatment with RVV in A549 cells. RT-PCR was performed in cells exposed to RVV for 24 and 48h. Exposure to RVV resulted in

decreased expression of Hsp70 in dose and time dependent (Figure 2.15 (A)). GAPDH was served as an internal control for each sample. Expression profile is also accompanied with densitometric scanning profile (Figure 2.15 (B)).

Figure 2.15

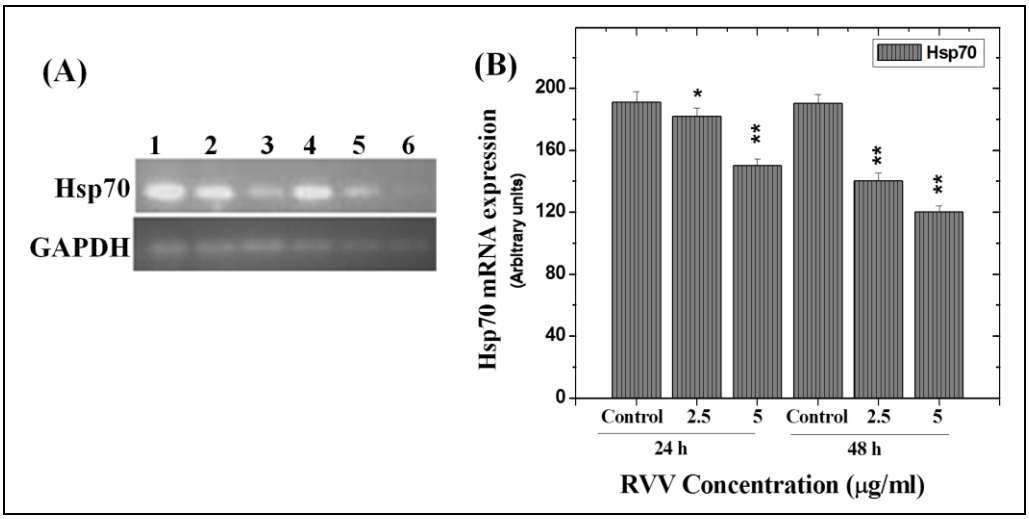


Figure 2.15: (A) Expression of heat shock protein (Hsp70) at mRNA level: The A549 cells were exposed to RVV. Total RNA was extracted and RT-PCR was carried out. The amplified product was analyzed by agarose gel electrophoresis. GAPDH was used as an internal control. The results showed that there was down regulation at mRNA level compared to that in control with respect to dose and time. **Lanes-1:** Control; **2:** 2.5 µg/ml; **3:** 5 µg/ml -24h, **4:** Control; **5:** 2.5 µg/ml; **6:** 5 µg/ml -48h. **(B)** Quantification of RT-PCR results of heat shock protein (Hsp70) by quantity one analysis that reveals the underlying expression pattern. The bars represent Mean±SEM of at least two independent experiments, $^{**}p<0.01$, $^{*}p<0.05$ represents significant differences as compared to that in control group.

2.11.13. RVV exposure affects Hsp70 expression pattern at protein level

To evaluate Hsp70 expression level by RVV treatment, Western blot analysis was performed in cells exposed to RVV for 24 and 48 h (Figure 2.15 (i)). Hsp70 levels were

determined after the exposure to 2.5 and 5µg/ml for 24 and 48 h. Exposure to RVV at above concentrations showed decrease in expression of Hsp70 protein levels when compared with that in control group (Figure 2.16 (A)). β actin served as an internal control for each sample. Expression profile is also accompanied with densitometric scanning profile (Figure 2.16 (B)).

Figure 2.16

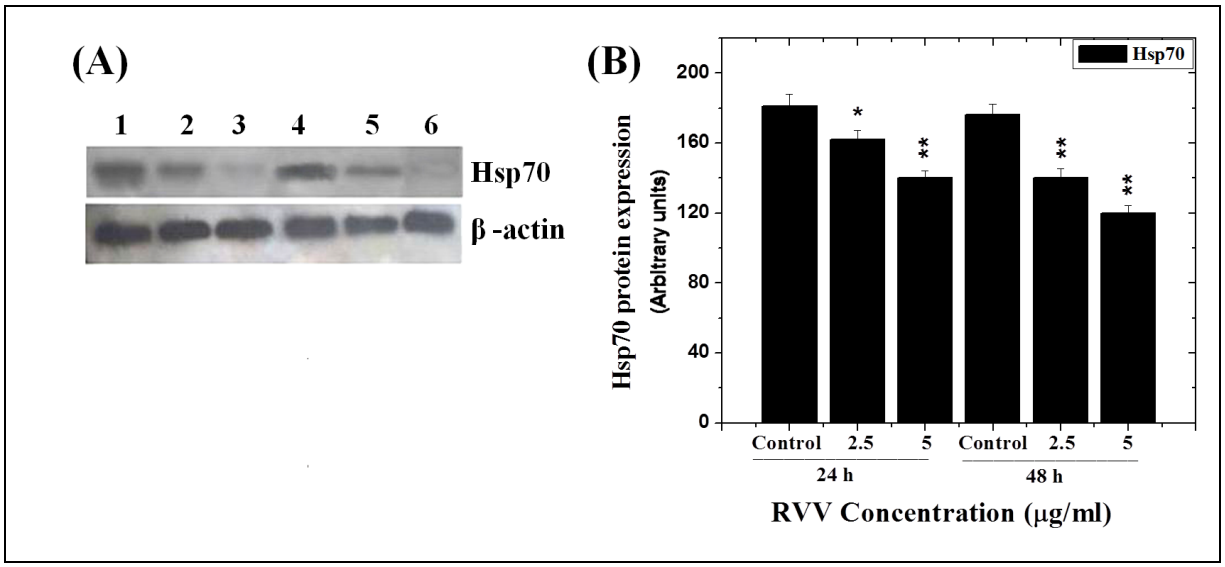


Figure 2.16: (A) Expression of heat shock protein (Hsp70) at protein level: The western blot results of the same experiment using Hsp70 monoclonal antibody revealed down regulation in the expression of Hsp70. The expression pattern of Hsp70 at protein level showed similar to the expression of mRNA levels.Lanes-**1:** Control; **2:** 2.5 µg/ml; **3:** 5 µg/ml-24 h, **4:** Control; **5:** 2.5 µg/ml; **6:** 5 µg/ml-48 h. **(B)** Quantification of western blot results of Heat shock protein Hsp70.The bars represent Mean±SEM of at least two independent experiments, $^{**}p<0.01$, $^{*}p<0.05$ represents significant differences as compared to that in control group.

2.11.14. RVV induced caspase 3 activation

The role of caspases in the execution phase of apoptosis is well established. EtBr staining resulted in increase number of apoptotic cells on treatment with RVV. Thus, led us to examine the effects of RVV on caspase activation. After the treatment with different concentration of RVV for 48 h resulted in significantly increase in 2.5 and 5 µg/ml RVV treated cells respectively for 48 h as compared to that of control (Figure 2.17).

Figure 2.17

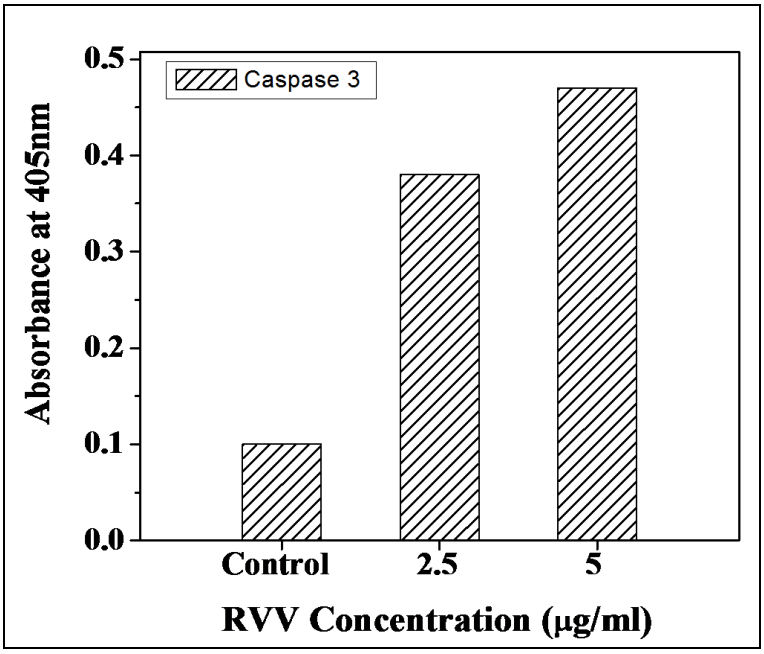


Figure 2.17: Measurement of caspase 3 activity in RVV treated A549 cells: After treatments for 48 h, cell lysates were prepared, and enzymatic activities of caspase-3 were measured by colorimetric assay. Each bar graph represents the mean ± SD of at least three independent assays. The caspase 3 activities in untreated cells are taken as 1-fold, and the figures on the chart display the relative changes of these activities in the treated cells. *Statistical significance ($p < 0.05$) versus control at equal incubation periods.

2.11.15. Transwell invasion assay

The transwell assay with matrigel showed a significant decrease in the invasive potential of A549 cells after the treatment with RVV at different doses (2.5 and 5 µg/ml) for 24 and 48 h, respectively (Figure 2.18 (A) & (B)).

Figure 2.18

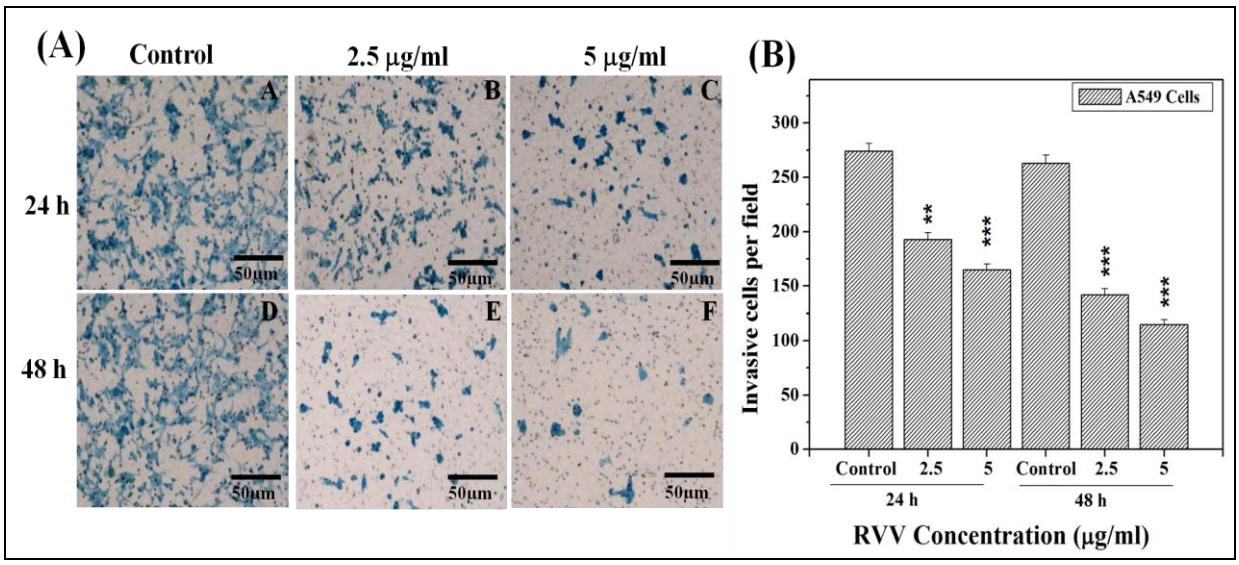


Figure 2.18: Representative images and quantification (A & B) of the transwell invasion assay: The number of invaded cells A549 were treated with RVV was significantly decreased with increase in concentration and time. The bars represent Mean±SEM of at least two independent experiments, ***p<0.001, **p<0.01 represents significant differences as compared to that in control group.

2.12. Discussion

Russell's Vipers is considered one of the most dangerous snakes in the world (Maheshwari and Mittal, 2004; Lang Balija *et al.*, 2005) and its venom is known for cytotoxicity among other pathophysiological manifestations in bite victims. Currently, researchers suggest that cell-based assay of venom induced cytotoxicity is an alternative to animal testing (Bustillo *et al.,* 2009; Kalam *et al.,* 2011). Studies employing a variety of cell lines in culture showed that snake venoms display a relatively broad cytotoxic spectrum (Omran *et al.,* 2004; Damico *et al.,* 2007). Similar observations were made with several other toxins, mostly from viperid snake venoms (Bonfim *et al.,* 2006).

The results of microscopy assays clearly demonstrated that, treatment of cells with RVV showed alterations in morphological changes with an increase in the extracellular spaces. Further, additional studies by FITC-phalloidin assay also confirmed that cells treated with RVV for various time intervals showed changes in F-actin distribution leading to decreased number of actin fibers resulting in cell shrinkage in a time and dose dependent manner. Therefore, the present study shows the involvement of RVV causing a change in the cell morphology through regulation of small GTPases. Through cell invasion assay using RVV on A549 cells; it was observed that RVV also hindered the cell invasion ability.

In this study, RVV mediated cytotoxic effects on A549 cells were confirmed by cell viability assay with trypan blue dye exclusion and resazurin assays. The IC_{50} values obtained from the resazurin assay for RVV at 24 and 48 h was 9.12 and 5.98, respectively. The decrease in cell viability observed following exposure to RVV, suggest a cytotoxic activity of the venom *in vitro*. Though it was shown by Maung-

Maung-Thwin, and co-workers in 1995 that daboiatoxin is cytotoxic to HeLa cells causing cytolysis of the cells 24 h post-exposure to toxin, there is no report on the cytotoxic effects of RVV on human A549 cells.

Apoptosis is a form of programmed cell death in which cell undergoes loss of mitochondrial membrane potential, loss of plasma membrane symmetry, chromatin condensation and the fragmentation of the cellular DNA (Elmore, 2007). We carried out experiments to understand the mechanism of cytotoxicity that may lead to apoptosis. In the present study a significant increase in the DNA fragmentation in cells treated with RVV was observed. There was significant increase in percentage of apoptotic index when RVV treated cells were stained with Hoechst 33342. These observations are consistent with the earlier reports on ability of scorpion venom to induce morphological alterations, early apoptosis with nuclear margination and chromatin condensation in A549 cells (Diaz-Garcia *et al.,* 2013).

In order to further elucidate the molecular mechanism responsible for the RVV induced apoptosis the levels of some critical genes associated with apoptosis such as caspases, Bcl2 and Bax using RT-PCR. Bcl2 family of proteins regulate the release of cytochrome *c* and other inter-membrane space proteins were investigated. While, Bcl2 is one of the most important anti-apoptotic members in this family, it interacts with Bax, a pro-apoptotic member, to prevent the release of cytochrome c and cause subsequent apoptosis. Increased expression of Bax can induce apoptosis, while Bcl2 protects cells from apoptosis (Cory and Adams, 2002). In the present study, RVV effectively elevated programmed cell death by increasing apoptotic features including caspase activation, decreasing anti-apoptotic molecules (Bcl2), and increasing pro-apoptotic molecules (Bax).

Heat shock proteins are the molecular chaperones and identified as important stress induced proteins which are abundantly expressed in cancer cells. Elevated HSPs have a wide variety of roles which may contribute to prolonged survival of tumour cell via several mechanisms by regulating protein folding and synthesis, cellular stress defence and many other functions against oxidant-induced DNA damage and apoptosis. Such inhibition of cell death by Hsp70 may be due to involvement of various anti-apoptotic proteins (Rashmi *et al.,* 2004; Dudeja *et al.,* 2009; Jiang *et al.,* 2009). Present study clearly demonstrated that RVV exposure caused down-regulation of Hsp70. In fact, the data on Hsp70 expression at mRNA level contrasted the impact of Hsp70 expression during cell cytotoxicity. This was further confirmed at protein expression level by western blot analysis.

p53 is one of the most abundant and important proteins for regulating gene expression responses during cellular stress. p53 activity along with many other proteins is important for various regulatory functions like cell cycle arrest, cellular senescence and apoptotic cell death (Vogelstein *et al.,* 2000). Results of the present study showed that treatment of RVV resulted in significant concentration and time dependent elevation of tumor suppressor p53 at mRNA level indicating the induction of apoptosis.

The Rho family of proteins belong to Ras superfamily of low molecular weight proteins. Rho, Rac and Cdc42 are well characterized members of Rho family (Hall, 1998). RhoA induces the assembly of actin stress fibers and focal adhesion complexes, whereas activated Rac1 causes the formation of lamellipodia (Kaibuchi *et al.,* 1999) and membrane ruffles and Cdc42 causes the production of actin filaments termed filopodia. The fate of the migrated tumor cell depends upon the activation of Rac1 and RhoA proteins. Rho GTPases are regarded as best regulators of the actin cytoskeleton

(Sahai and Marshall, 2003; Sanz-Moreno and Marshall, 2009). Observations from the RT-PCR study showed no alterations in the expression of small GTPases in untreated group. Treatment with RVV showed down-regulation of RhoA and Rac1 with up-regulation of Cdc42 protein levels. These findings are consistent with cell morphology results where increase in the percentage of non-adherent cells, loss of cell morphology and the rounding of the cells in RVV treated group indicating the regulatory role of RVV on small GTPases. Similar expression pattern was also observed in the present study by western blot. In this study, it was observed that elevation of p53 expression pattern at mRNA level activates the transition in cell morphology accompanied by differential expression pattern of small GTPases as reported earlier (Lassus *et al.,* 2000; Muller *et al.,* 2011).

Taken together, the present study demonstrated that Russell's viper venom induces cytotoxic effects against A549 cells in a dose and time dependent manner with decreased expression of Hsp70 and small GTPases, Present findings also showed down-regulation of RhoA, Rac1, capases and Bcl2 with up-regulation of Cdc42 protein Bax and p53 levels. This is probably the first scientific evidence showing the cytotoxic potential of RVV on A549 cell line.

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Chapter 3

Purification and Characterisation of biologically active protein from Russell's viper venom

3.1. Introduction:

Humanity has always been fearfully interested about serpents. The long shiny rope of a creature with fatal bites gifted the serpents a peculiar position in the minds of humans. Since the dawn of civilization snakes have been considered as a creature of mystery. Early Egyptians and then early Christians believed that snakes great creatures of knowledge. The greek physician, Aesculapius got the ultimate medicine from a snake!! Similarly, in Hindu mythology also serpents are treated with respect and fear. The core reason of these myths about snakes probably lies in the snake venom. Although venom has become almost synonymous with snakes, only a few snakes are venomous. In venomous snakes, venom is produced by specialized glands which are evolutionarily related to salivary glands (Kochva, 1987). Venoms contain a wide mixture of proteins and peptides which make up 90-95% of their venom. About 5% of the venom constitution is amino acids, nucleotides, free lipids, carbohydrates and metallic elements bound to proteins (Russell, 1980; Tu, 1988). Venoms are of biological interest due to their diverse and selective pharmacological and physiological effects. Many studies have demonstrated the significance of snake venom proteins as valuable tools for basic research, disease diagnosis and drug development (Marsh, 2001; Nair *et al.*, 2007).

The Russell's Viper (***Daboia russelii***) is a member of the big four snakes of India. Crude RVV contains several toxins including phospholipase A2 (PLA2), Russell's viper venom-factor X activator (RVV-X), Russell's viper venom-factor V activator (RVV-V), proteinases, and other proteins as yet unidentified. Russell's viper venom is typically hemotoxic (blood toxins), necrotic and anticoagulant (preventing the blood

from clotting). Neurotoxic components are also present in the venom of some species (Tans and Rosing, 2001).

Snake venom also contains important molecules like Disintegrins and Lectin proteins (McLane *et al.,* 2004). Disintegrins have the potential use in medical applications such as the treatment of strokes, heart attacks, diabetes, cancers, and osteoporosis (Arruda Macêdo *et al.,* 2015; McLane *et al.,* 2004). Snake venom lectins have been isolated from the Crotalidae, Elapidae, and Viperidae families (Hirabayashi *et al.,* 1991). They are known as a modulator of platelet aggregation (Sarray *et al.,* 2004). CTL proteins have been shown to induce the agglutination of erythrocytes in vitro, possess mitogenic activity on lymphocytes, cytotoxicity, and cell proliferation inhibition (Kassab *et al.,* 2004). Recently, Jebali and co-workers (2009) demonstrated that lebecetin isolated from *M. lebetina* venom inhibited integrin-mediated adhesion, migration, and invasion of human tumor cells.

In this study, one unique CTL like 7 (accession no. Q4PRC6) was identified from the protein sequences deposited in the NCBI database created from the venom of the *Daboia siamensis* with 61% sequence homology. The amino acid sequence of C-type lectin 7 was compared with those of other C-type lectins to study the structure-function relationships. This chapter describes purification and characterization of one of the responsible toxins of RVV.

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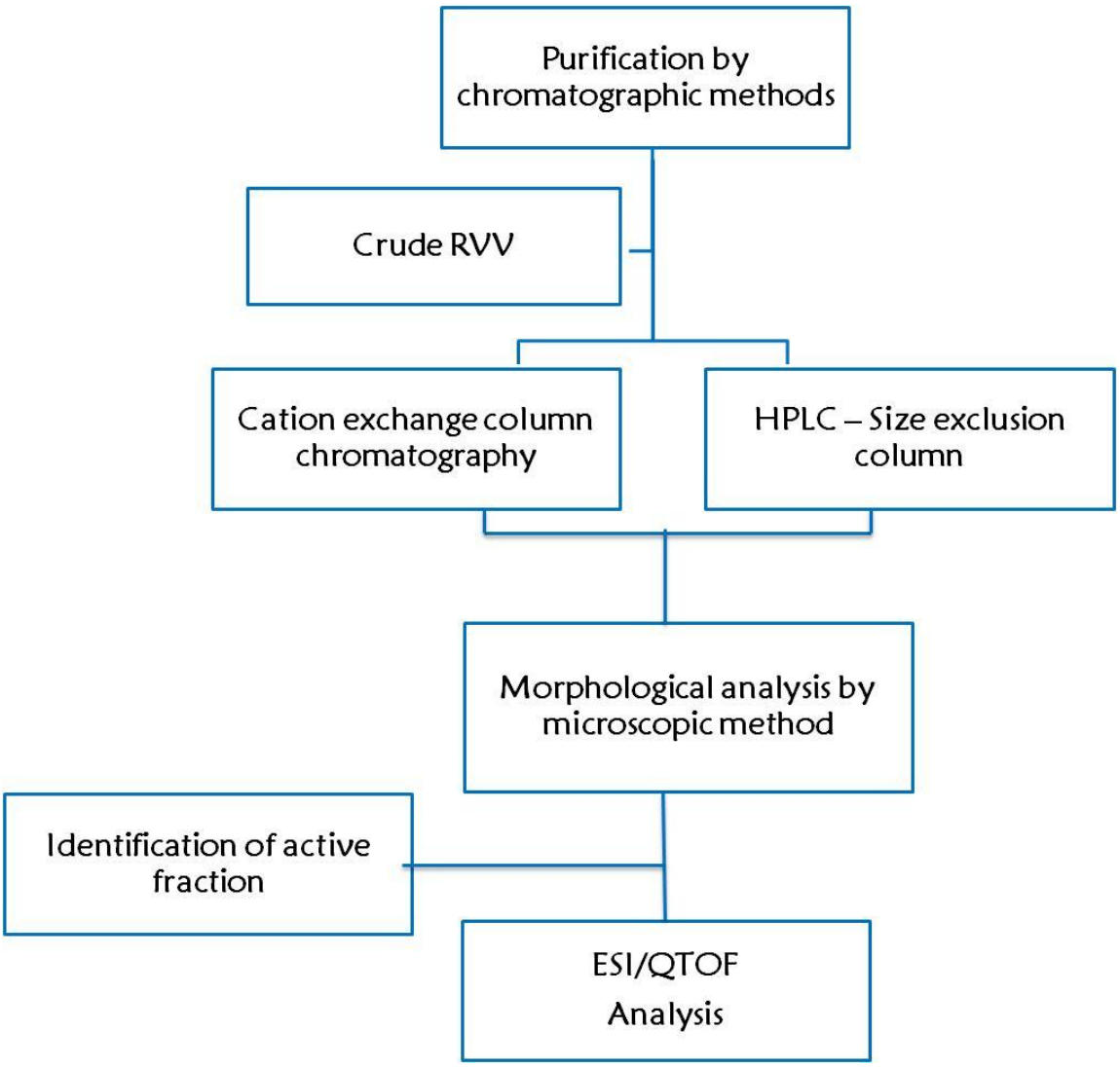
EXPERIMENTAL DESIGN



3.2. METHODOLOGY

3.2.1. Ion-exchange chromatography (IEC)

The purification of cytotoxin was done by using ion-exchange chromatography, which allows the separation of charged molecules based on their affinity to the ion exchanger resin. This technique enables the separation of similar types of molecules that would be difficult to separate by other techniques because the charge carried by the molecule of interest can be readily manipulated by changing buffer pH. Ion-exchange chromatography involves two primary steps, first the binding of a protein to a charged resin and second the elution or displacement of the protein from the resin. Critical to the former are the pH of the buffer used to equilibrate and load the proteins onto the column. Factors that control the elution are pH or ionic strength.

3.2.2. Preparation of IE columns

Column matrix was mixed with several volumes of double distilled (DD) water and fines were discarded by decantation. The mixture was then degassed by a vacuum pump and allowed to swell by soaking in DD water over night at 4°C. Column of dimension (1 cm × 60 cm) was fixed to a stand using a clamp at cold chamber (4°C). Matrix solution was then poured into the column by streaming over a glass rod to avoid formation of air bubbles. Equilibration of the column was done by passing several bed volumes of 20 mM potassium phosphate buffer, pH 7.4 into the column so as to obtain constant pH throughout the column matrix.

3.2.3. Fractionation of venom on IE columns

Fifty milligrams of lyophilized crude RVV was suspended in 20mM phosphate buffer, pH 7.4 (running buffer) overnight at 4°C and the solution was centrifuged to remove cell debris and clear solution was loaded on to the column. The column was washed

with two column volumes of running buffer to elute the unabsorbed fractions. The adsorbed fractions were then eluted with a linear gradient of NaCl (0 - 0.5 M) in 3 column volumes of running buffer. The fraction size was restricted to 1ml. Protein concentration of fractions was measured at 280 nm. Each fraction was screened for cell morphological assay and cytotoxicity activity. Fractions that showed cytotoxicity were pooled, concentrated and further fractionated by size-exclusion high performance liquid chromatography using BioSep-SEC-s2000 column (300 mm × 7.8 mm). Molecular weight and purity of fractions were determined by SDS-PAGE and verified by mass spectrometry.

3.2.4. Dialysis and sucrose concentration

Dialysis of protein sample to eliminate salts and unwanted low molecular weight peptides was performed using semi-permeable dialysis membranes of different molecular weight cut-off. Dialysis membrane was pre-treated according to the manufacturer's protocols. Protein sample was then packed inside the membrane using dialysis clips. Dialysis was performed against three to four changes of 20 mM potassium phosphate buffer, pH 7.4 overnight at 4°C on a magnetic stirrer with continuous stirring. The protein sample thus obtained was concentrated by placing the dialysis bags in a petridish and covered with dry sucrose. The petridish was further covered with aluminium foil at 4°C for 30 minutes.

3.2.5. Purity by SDS-PAGE

Purity of isolated protein was verified by SDS-PAGE (5% stacking gel/15% separating gels) The Protein samples were treated with sample buffer (100 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, and 0.01% Bromophenol blue) at 100°C for 3 min in a boiling water bath. Electrophoresis was carried out at room temperature on a mini gel

electrophoresis system. The voltage was set at 50V in stacking gel and 70V in resolving gel. The gel was stained overnight with 1% coomassie brilliant blue R-250. Coomassie stained gels were destained by using 30% methanol and 10% acetic acid in distilled water.

3.2.6. Molecular mass determination and LC- MS/MS analysis

The molecular weight analyses were performed by ESI/QTOF analysis (Agilent 65200). The active fractions eluted from GFC-HPLC column were subjected to in-solution trypsin digestion, after reduction and alkylation, for overnight at 37°C. The digested peptides were dried and reconstituted in 15.0 µl of the 0.1% formic acid and were subjected to standard 70 min MS/MS analysis with collision induced dissociation as the fragmentation method coupled to a mass spectrometer (LTQ Orbitrap Discovery, Thermoscientific, USA). The generated data was searched following standard approach for the identity using MASCOT 2.4 as search engine on Proteome Discoverer 1.4. The data was searched against Uniprot/Swiss-Prot database (non redundant database with reviewed proteins) from NCBI. The data was also searched against Daboia database downloaded from NCBI using PEAKS 7.0 software.

3.3. Results

Purification and characterization of protein from RVV

3.3.1. Fractionation of Russell's viper venom (RVV)

Fractionation of Russell's viper venom (RVV) was performed on CM-sephadex, C-50 cation exchange column. The adsorbed fractions were eluted with a 0.5 M NaCl gradient. The whole venom fractionated into six major protein peaks. Three of which were in the unadsorbed region and three in the adsorbed region. All peaks were numbered with roman numerals I, II, III, IV, V and VI (Figure 3.1)

Figure 3.1

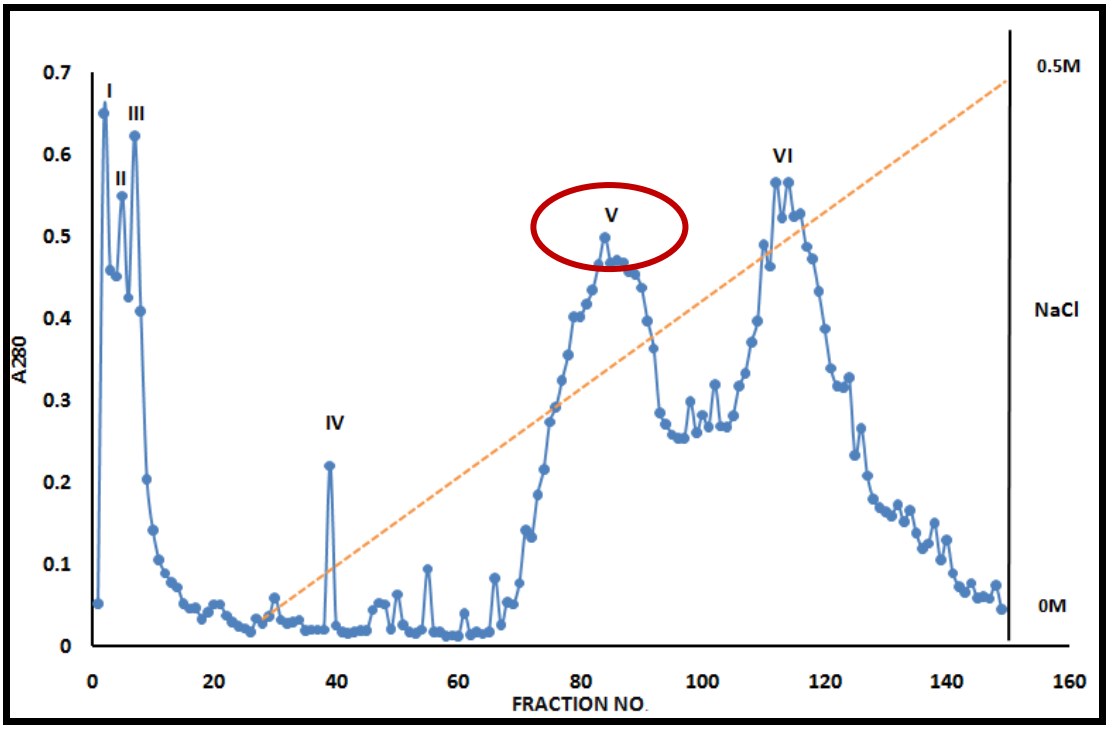


Figure 3.1: Cation-exchange chromatography of RVV on CM-Sephadex C-50. Fractions were eluted with a linear gradient of NaCl (0.0 to 0.5 M). The dotted line represents linear gradient of NaCl.

3.3.2. Screening for cytotoxicity

The peaks eluted at unadsorbed region were subjected to assay for cytotoxicity on A549 cells. The V and VI peaks showed cytotoxic activity on A549 cells. But the cytotoxic activity exhibited by peak V was similar to the activity seen by crude venom earlier. Therefore, peak V was chosen for further studies (Figure 3.2).

Figure 3.2

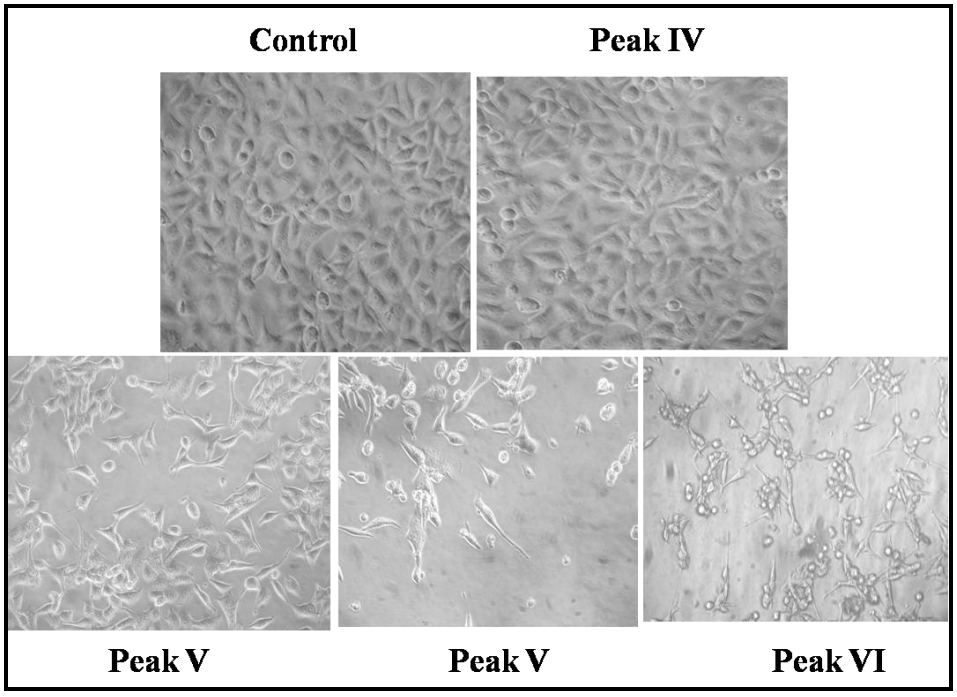


Figure 3.2: Assessment of cytotoxicity by the various fractions of RVV

The fractions under the peak V were pooled and dialysed using dialysis membrane of molecular weight 3 kDa cut-off against 20 mM potassium phosphate buffer pH 7.4 overnight at 4°C. Most of the lower molecular weight proteins were moved out of the dialysis membrane during this process. The sample obtained was then concentrated on sucrose and gel permeation chromatography was performed for further purification.

The chromatogram obtained after second purification using GFC column showed one major peak at retention time 5.8 min. and several minor peaks followed. The major peak obtained after chromatography of Peak V showed a single band of approximately 18.5 kDa (Figure 3.3).

Figure 3.3

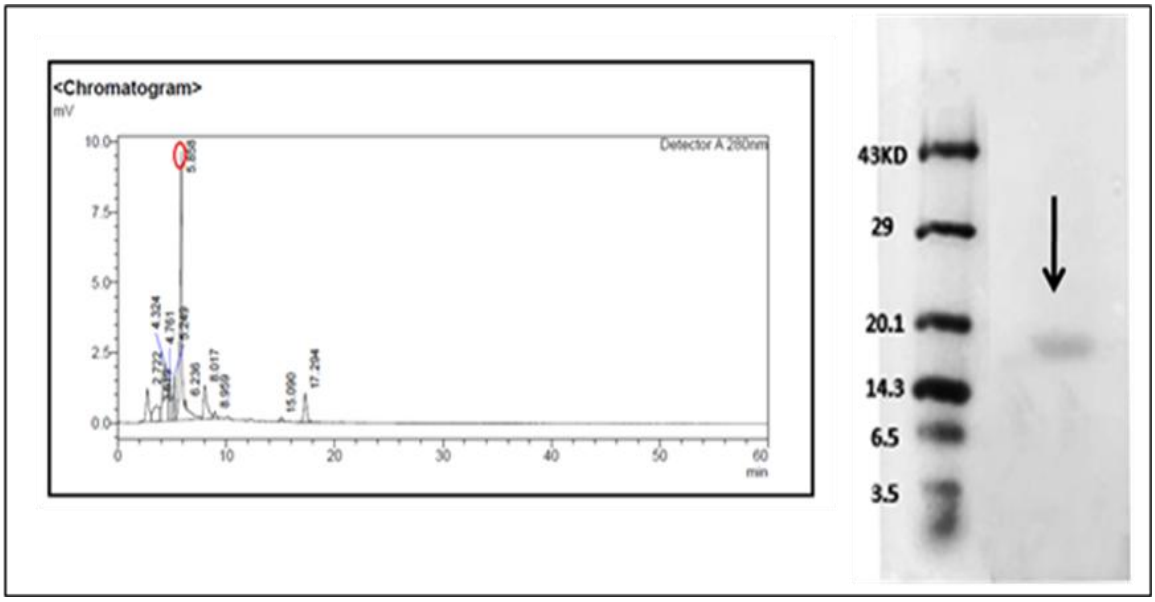


Figure 3.3: Protein profile of major peak obtain after second round of purification, sample (20 µl) was denatured and ran on 15% SDS-PAGE.

3.3.3. Molecular mass determination and ESI/QTOF analysis

The protein purity was assessed by mass spec ESI/QTOF analysis showing the actual mass of Daboialectin as 18.512 kDa. The LC-MS/MS analysis of Daboialectin showed highest identity with a 18.5 kDa C-type lectin-like 7 isolated from *Daboia siamensis* (accession no. Q4PRC6) against snake venom protein sequences deposited in the NCBI database and also showed 61% sequence homology (Figure 3.4 & Table 3.1).

Figure 3.4

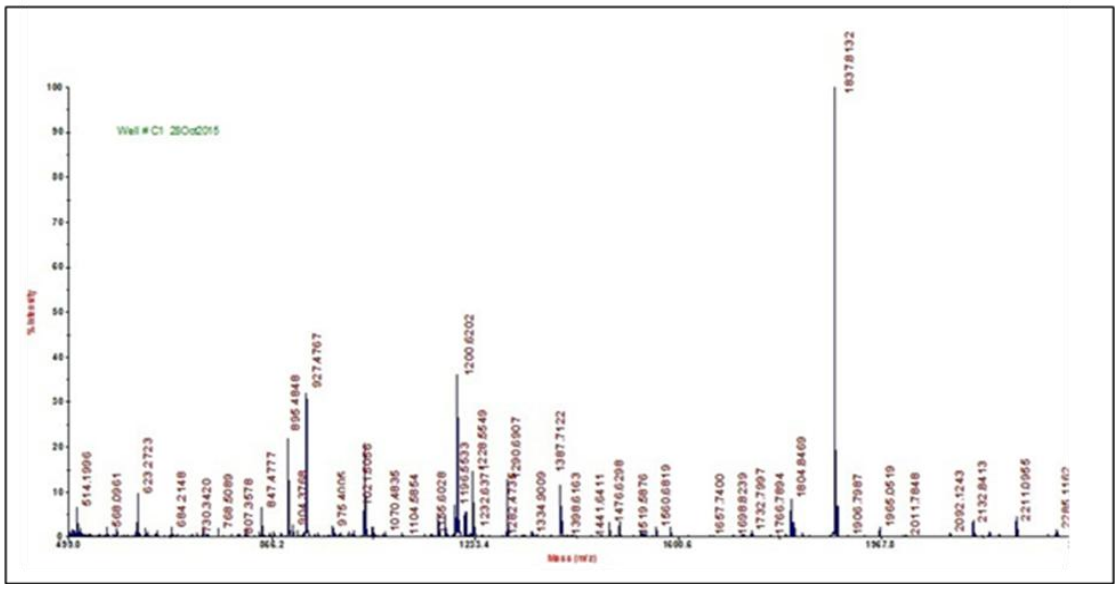


Figure 3.4: Mass spec ESI/QTOF analysis of Daboialectin

```
          **   **      **         *  **  * *      * *     *  *                  * *
Q4PRC6   KQDCLSDWSFYEGYCYKVFNEKKTWEDAEKFCNEQVNGGYLVSFRSSEEMDFVIRMTFPIFR--FDFFWIGLRDFW  97
Q8AV97   DFDCIPGWSAYDRYCYQAFSKPKNWEDAESFCEEGVKTSHLVSIESSGEGDFVAQLVAEKIKTSFQYVWIGLRIQN  99
Q4PRC8   KQDCLSDWSFYEGYCYKVFNEKKTWEDAEKFCTEQHKGSHLLSLHNIAEADFVLKKTLAMLK--DGVIWMGLNDVW  97
B5U6Y6   --DCLPDWSPYQGHCYRVFNQKMTWADAEKFCTEQANGGHLASFHSSKEVDFMVSLAFPMLK--VDFVWIGMSDFW  95
Q6X5S0   DQDCLPDWSSHERHCYKVINEYKTWEEAEQYCTEEANGGHLVSFHNRQEVAFVVKLGYTILK--ADVVWIGLRDFW  97
                  eee  eeeeee     hhhhhhhhhhh            hhhhhhhhhhhhh       eeeeeeee


            *   *       *          *          *    *       **
Q4PRC6   --RDCYWRWSDGVNLDYKAWSR--EPNCFVSKTTD--NQWLRWNCNDPRYFVCKSRVSC  150
Q8AV97   KEQQCRSEWSDASSVNYENLVKQFSKKCYALKKGTELRTWFNVYCGTENPFVCKYTPEC  158
Q4PRC8   --NECNWGWTDGAKLDYKAWNE--GTNCFVFKIAK--NHWSHMDCSSTHNFVCKFRV--  148
B5U6Y6   --RDCEWKWSDGAKLDYKAWNN--ELNCFVSKTTD--NQWLRWDCSRTNNVACKYPL--  146
Q6X5S0   --RECQWEWSNGAKLNYKGWSD--EPNCFIAYTVG--NRWVRRKCSSTHPFVCKSPA--  148
             eeee     eeee          eeeeee      eeee     eeeeeee
```

Table 3.1: Multiple sequence alignment of *Daboialectin* with several *Daboia* toxins

Multiple sequence alignment (MSA) of snake C-type lectin-like proteins. MSA generated using PROMALS3D (Pei *et al.*, 2008), where '*' represents highly conserved residue and consensus secondary structure prediction symbols (bottom): alpha-helix as 'h' and beta-strand as 'e'. Underlined fragments are exact match of trypsin digested Daboialectin peptides. Cys involved in intra- and inter-chain disulfide bonds are shaded using grey and black, respectively. Q4PRC6: Snaclec 7 from *Daboia*

siamensis (*Eastern Russell's Viper*); Q8AV97: Snaclec flavocetin-A from Protobothrops flavoviridis (Habu); Q4PRC8: Snaclec 5 from Daboia siamensis (Eastern Russell's viper); B5U6Y6: Snaclec CTL-Eoc124 from Echis ocellatus (Ocellated saw-scaled viper); Q6X5S0: Snaclec 7 from Echis pyramidum leakeyi (Leakey's carpet viper).

3.4. Conclusion:

Snake venom from *Russell's Viper* (***Daboia russelii russelli***) was analyzed using Ion-exchange chromatography followed by size-exclusion HPLC. Different fractions were collected and analysed for cell morphological studies and cytotoxic activity on A549 cells (Figure 3.2). The molecular weight and purity of fractions were determined by SDS-PAGE and mass spectrometry. MALDI-ESI/QTOF analysis spectra were interpreted were matched with proteins to the references and identified. MS raw data were analyzed by depositing the venom protein sequences NCBI database, resulting in identification of highest identity with an 18.5 kDa C-type lectin-like 7 isolated from *Daboia siamensis* (accession no.Q4PRC6) with 61% sequence homology. The isolated protein was named as Daboialectin. Further, studies with this purified toxin were carried out to understand the mode of cytotoxicity and mechanism of cell death involved on A549 cells.

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Chapter 4

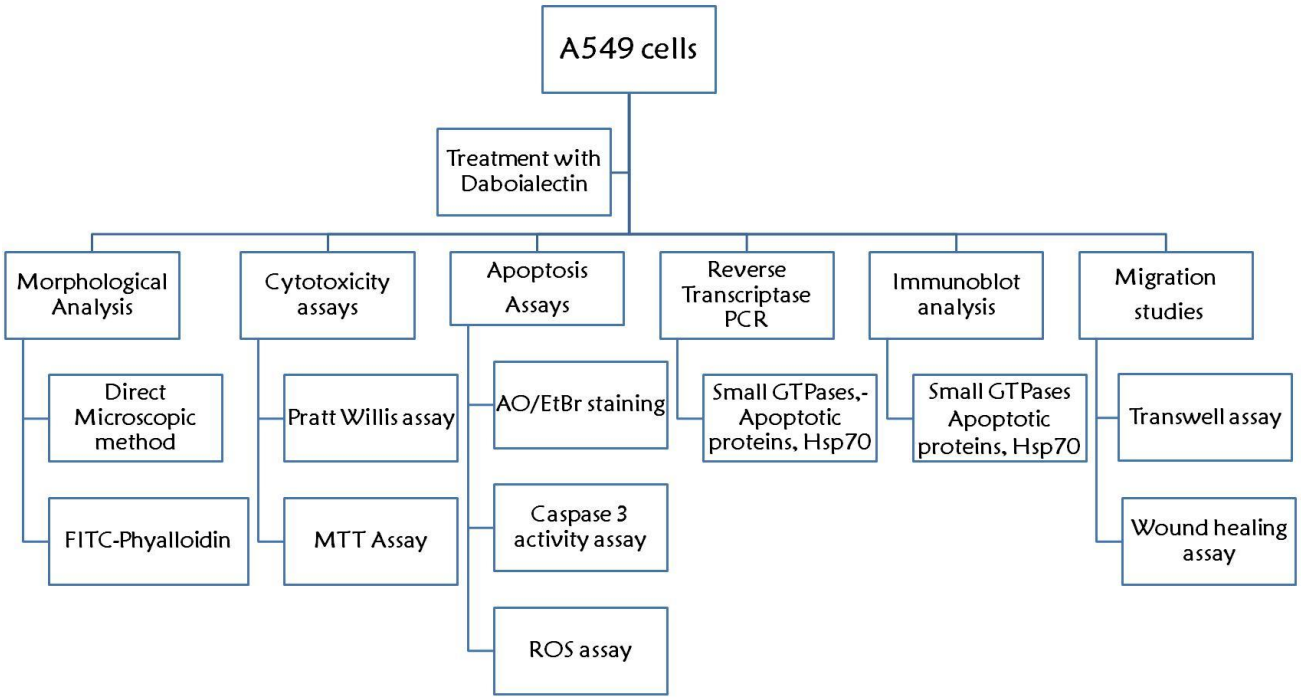
Daboialectin, a C-type lectin from Russell's viper venom induces cytoskeletal damage and apoptosis in human lung cancer cells in vitro

4.1. Introduction:

Venoms of snakes are the most studied among all natural toxins. Snake venoms are made up of hundreds of different types of peptides, enzymes, and toxins. Each venom component has its own specific function, specifically acting on various critical points in the physiological systems of target animals causing mortality and morbidity. The pharmacological specificity of snake venom toxins are attractive molecules for new drug development. However, till date no snake venom component has been marketed as medicine. Identification and characterization of toxic compounds present in snake venoms are the main steps not only to understand the pathophysiological changes observed after bites, but also to find novel drugs in the toxins.

Russell's viper (*Daboia russelii*) is one of the dominating venomous snakes distributed over the tropical nations of South-East Asia including India. Venoms from the family Viperidae contain a wide mixture of proteins and peptides making up to 90-95% of their dry weight (Russell, 1980). About 5% of the venom composition is amino acids, nucleotides, free lipids, carbohydrates and metallic elements bound to proteins (Heise *et al.,* 1995). Russell's viper venom (RVV) is known to develop a complex pharmacological scenario in the victim's body. Earlier studies have already shown the effect of RVV on regulation of small GTPases in A549 cell line (chapter 2). It was found that whole RVV effectively induced apoptosis through nuclear breakdown and activating caspases and pro-apoptotic genes. Anti-apoptotic genes were also affected significantly when challenged with RVV. Significant change in F-actin distribution, leading to severe morphological changes was noticed. This chapter describes "Daboialectin" one of the responsible toxins of RVV for the said cytotoxic changes in A549 cells.

EXPERIMENTAL DESIGN



4.2. Material and Methods

4.2.1. *Maintenance of cell line and in vitro culture of human A549 Cells*

The details regarding cell maintenance and culture procedures are explained in Chapter 2 under section 2.2.

4.3. *Cytotoxicity assays*

4.3.1. *Experimental design*

A fixed number (5×10^5) of exponentially growing A549 cells were seeded into several individual T-25cm^2 flasks and were treated as follows:

1. ***Control group:*** Cells not treated with Daboialectin
2. ***Daboialectin group*****:** The cultures of these groups were treated with various concentrations of Daboialectin (50 and 100 nM) alone for 24 and 48 h.

After the various treatments, media was removed and cells were dislodged by trypsin EDTA (0.1%) treatment and the cells were processed for following assays.

4.3.2. Total viable Cell count

Exponentially growing cells were sub-cultured into 25 cm^2 culture flasks and were allowed to grow for 24 h. Cultures were then treated with various concentrations of Daboialectin for 24 and 48 h. briefly, after the treatment; the medium containing Daboialectin was removed from the culture flasks and washed with 2 ml of sterile phosphate-buffered saline (PBS). The cells were then dislodged by a brief exposure (1 min) with trypsin solution and collected for staining with 0.5% of trypan blue with 1:1 ratio of the dye and cell suspension as described by Pratt and Willis in 1985 and Pathan and co-workers in 2015.

4.3.3. Assessment of cytotoxicity by MTT Assay

The viability of A549 cells under the influence of Daboialectin was measured by MTT assay as described by Mossman (1983). MTT assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT to form dark blue formazan crystals (Figure 4.1). These crystals are largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. A solubilization solution (usually DMSO or a solution of the detergent sodium dodecyl sulfate in dilute hydrochloric acid) is added to dissolve the insoluble formazan product into a purple coloured solution. The number of surviving cells is directly proportional to the colour intensity of the dissolved formazan and can be quantified using a multi-well spectrophotometer at 540 nm wavelength.

Figure 4.1

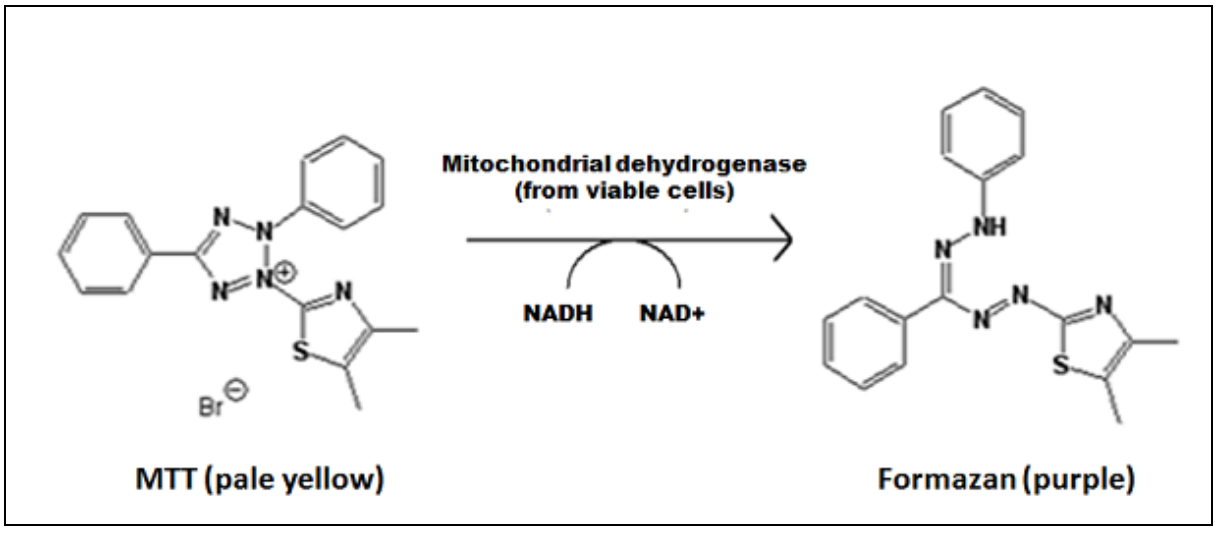


Figure 4.1: Reduction of tetrazolium rings of MTT by mitochondrial dehydrogenase in viable cells to purple formazan crystals. (Source: https://upload.wikimedia.org/wikipedia/commons/thumb/d/de/MTT_reaction.png/500px-MTT_reaction.png).

A fixed number (5,00,000) of exponentially growing cells were seeded at a density of 2-4 × 10^4 cells/well in a 96-well-plate in 100 µl of culture medium and incubated at 37°C. After 24 h., the cells were treated with different concentrations of Daboialectin and incubated for 24 and 48 h respectively. After the incubation time, MTT solution (1mg/ml, Himedia) was added to each well and incubated for 3 h at 37°C, 200 ul of DMSO was added following incubation. Absorbance of the dye was measured spectrophotometrically at a 595 nm wavelength using an automated microplate reader (Bio-Rad). The percentage of inhibition was determined by comparing the absorbance values of treated cells with that of untreated controls: [(absorbance of treated cells / absorbance of untreated cells) × 100].

4.4. Morphological analysis of cells

Briefly, cells were grown on tissue culture dishes and treated with or without Daboialectin at concentrations of 50 and 100 nM. After 24 and 48 h, the cells from all the treated groups were photographed using inverted microscope using an attached digital camera (Nikon-Eclipse, TS 100F, USA) along with an unexposed sample as a control.

4.5. Fluorescent staining with Phalloidin FITC

A549 cells were seeded at a density of 1 × 10^6 cells/ml in six-well plates, and treated with different concentrations of Daboialectin (50 and 100 nM) for 24 and 48 h respectively. The actin filaments in A549 cells were visualized by staining with FITC-phalloidin. The methodologies for this entire assay have been described in Chapter 2 under section 2.5

4.6. Cell death by apoptosis

To evaluate the apoptotic effect of Daboialectin, a fixed number of A549 cells (10^6) were inoculated into several individual 25 cm^2 culture flasks and were allowed to grow

for 24 h. Cultures were then divided into following groups:

1. ***Control group:*** The cells of this group were not treated with Daboialectin
2. ***Daboialectin group:*** The cultures of this group were treated with 50 and 100 nM for 24 and 48 h.

After post-incubation, media and cells were dislodged by trypsin treatment and the cells were processed for microscopy by, EtBr/AO and ROS production assay.

4.6.1. Analysis of apoptotic cells by fluorescence microscopy

Microscopic EtBr/AO staining was carried out according to the protocol described by Renvoize and co-workers (1998) to detect morphological evidence of apoptosis on the Daboialectin treated cells. The methodologies for this entire assay have been described in Chapter 2 under section 2.6.1

4.7. Measurement of ROS using DCFH-DA

ROS are essential intermediates in oxidative metabolism. ROS can damage cells by oxidizing lipids and disrupting structural proteins, enzymes and nucleic acids. The generation processes of ROS can be monitored using the luminescence analysis or fluorescence methods.

The intracellular ROS generation of cells can be investigated using the DCFH-DA as a well-established compound to detect and quantify ROS produced intra-cellularly. The conversion of the non-fluorescent DCFH to the highly fluoresecent compound 2',7'-dichlorfluorescein (DCF). DCFH-DA is a cell permeable flurogenic compound which diffuse into cells and is de-acetylated by cellular esterases to non-fluorescent 2', 7'-dichlorodihydrofluorescin (DCFH), which is rapidly oxidized to highly fluorescent 2', 7'-dichlorodihydrofluorescein (DCF) by ROS (Figure 4.2). The fluorescence intensity is proportional to the ROS levels in the cytosol.

Figure 4.2

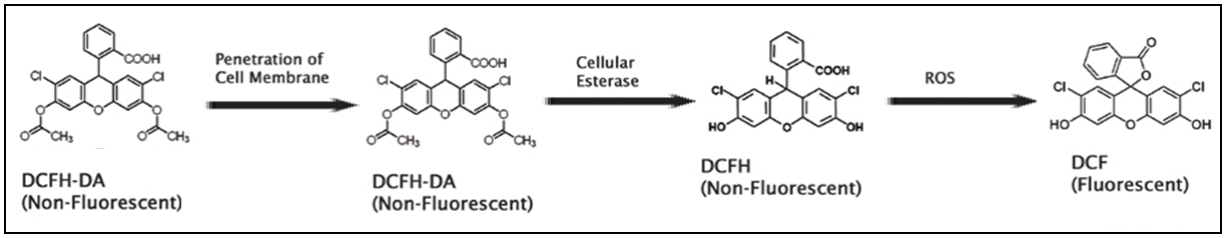


Figure 4.2: Formation of fluorescent compound DCF by ROS. (Source: http://pubs.rsc.org/services/images/RSCpubs.ePlatform.Service.FreeContent.ImageService.svc/ImageService/Articleimage/2015/RA/c5ra05889a/c5ra05889a-f10_hi-res.gif)

Briefly, cells seeded in 96 well plates (2×10^5 cells/well) treated with or without various concentrations of Daboialectin (50 and 100 nM) were incubated for 24 and 48 h respectively. Further cells were washed with 1× buffer and stained with 10 μM DCFH-DA in 1× buffer for 45 min and incubated at 37°C. The fluorescence resulting from the rate of oxidation of the dye in the cells was measured using a plate reader with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

4.8. RT-PCR analysis

To investigate the effect of Daboialectin on expression of Rho-GTPases and apoptosis related proteins in A549 cells. A fixed number of cells were inoculated into several individual culture flasks and were allowed to grow for 24 h. Cultures were then divided into following groups.

1. ***Control group:*** Cells of this group were not treated with Daboialectin
2. ***Daboialectin group:*** Cells of this group were treated with 50 and 100 nM Daboialectin.

After various treatments, cells were processed for RT-PCR. The methodologies for this entire assay have been described in Chapter 2 under section 2.5

4.9. *Immunoblot analysis*

To further investigate the role of Daboialectin, the expression levels of some apoptosis-related proteins in A549 cells were examined. A fixed number of cells (10^6) were inoculated into several individual culture flasks and were allowed to grow for 24h. Cultures were then divided into following groups.

1. ***Control group:*** Cells of this group were not treated with Daboialectin
2. ***Daboialectin group:*** Cells of this group were treated with optimal concentrations of Daboialectin.

After various treatments, cells were processed for Western Blot analysis by using anti-Hsp70, anti-Rac1, anti-RhoA, anti-Cdc42 and anti- phospho Rac1/Cdc42 monoclonal antibodies by the method described in Chapter 2 under section 2.7.

4.10 Caspase 3 activity Assay:

Caspases are key mediators of cell death. Caspase-3 activity assay was performed using CPP32 colorimetric protease assay kit (Bio vision, Frederick, MD, USA). Cells were treated with 50 and 100 nM of Daboialectin for 24 and 48 h. The methodologies for this assay have been described in Chapter 2 under section *2.9*

4.11. Transwell invasion assay

Transwell invasion assay is an in vitro cell invasion assay developed to determine the invasive property of malignant cells, most commonly called as Boyden chamber assay using a basement membrane matrix preparation, Matrigel, as the matrix barrier and the conditioned media as the chemo-attractant. The entire methodologies for this assay have been described in Chapter 2 under section 2.10

4.12. In vitro wound healing

The *in vitro* wound-healing assay is one of the earliest developed and easy to perform methods to study directional cell migration. This method mimics cell migration during wound healing *in vivo*.

Briefly, A549 cells were grown in 6 well plates at a density of 3×10^6/ml, and a small linear scratch was created in the confluent monolayer by 200 µl auto-pippette tip. Cells were extensively rinsed with medium to remove cellular debris before treating with different concentrations (50 and 100 nM) of Daboialectin in FBS deprived condition for 24 h and 48 h. Controls were used separately to judge the rate of cell migration. After treatment, images of the migrated cells were taken using a digital camera connected to the inverted microscope (Nikon-Eclipse, TS 100F, USA), and analyzed by Image J image analysis software. Extent of wound healing was determined by the distance traversed by cells migrating into the denuded area.

4.13. Platelet aggregation assay

Platelet aggregation assays were performed in whole human blood as follows. Nine parts of whole human blood was collected in 1 part of 3.8% (w/v) sodium citrate. A Chrono-Log Whole Blood aggregometer was used to monitor platelet aggregation. Five hundred microliters of 0.85% saline was incubated at 37°C for 5 min. and mixed with equal volumes of citrated whole blood for each assay. Blood samples were then treated with different concentrations of Daboialectin for 2 min. Agonist ADP (10 µM) was added immediately to the above treated blood sample and impedance patterns were monitored. Blood samples treated with platelet aggregation agonist alone were

considered as positive control. Blood samples without any treatment (i.e., agonists or Daboialectin) were considered as negative controls.

Statistical analysis

Data are presented as the Mean ± SEM from at least three independent experiments. Student's t-test was used for comparison of two independent groups. For all tests, $p < 0.05$ was considered statistically significant.

4.14. RESULTS

4.14.1. Daboialectin induced morphological changes in A549 cells

Cellular morphology can be used as a parameter for measuring the effects of a bioactive protein on cytotoxicity. In the present work, the cells A549 were treated with different concentration of Daboialectin, incubated for 24 and 48 h, and images were taken using phase contrast microscope. At the end of the incubation period the number of cells was significantly reduced in treated wells when compared to their vehicle-treated counterparts (Figure 4.3). The growth inhibitory effects of Daboialectin were confirmed in microscopic images showing fewer cells present in Daboialectin treated cultures (Figure 4.3). Phase contrast views reveal that Daboialectin treated cells acquired a long, thin and spindle shape with boundaries resembling those of loosely adhered cells. The filopodia-like extensions induced by Daboialectin appear to reach out to other cells in the field (Figure 4.3). The morphological changes induced by Daboialectin started to appear within 24 h of treatment. Further details of thinning of the cytoplasm can be clearly appreciated upon 48 h. This confirmed the intra-cellular effects of Daboialectin on cell shape.

Figure 4.3

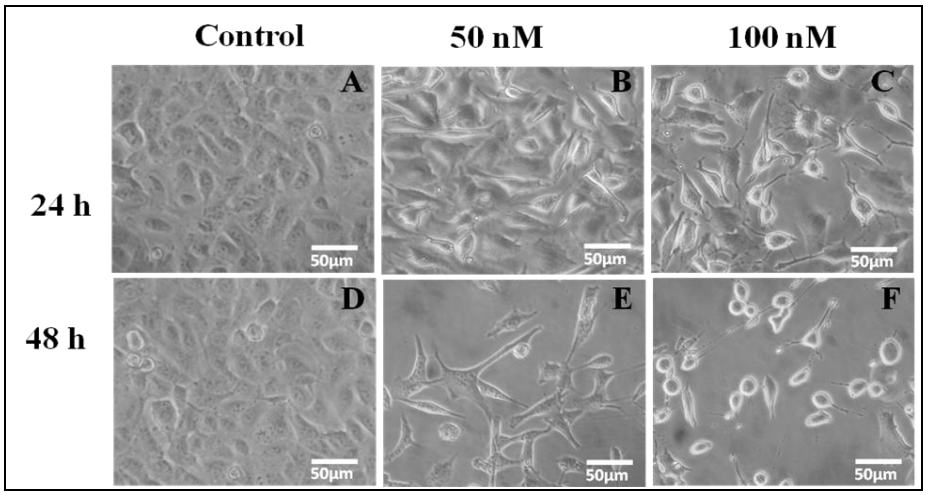


Figure 4.3: Morphological assessment of Daboialectin treated A549 cells. A549 cells display distinct morphological changes in response to Daboialectin treatment. Equal number of cells were plated and allowed to attach for 24 h. Cells were then exposed to different doses of Daboialectin and incubated for a period of 24 and 48 h. At the end of the experiment phase contrast microscope images were captured.

4.14.2. Daboialectin affects reorganization of the actin cytoskeleton

Actin filaments play a crucial role in cellular mobility and cancer cells are no exception. Therefore, changes in filamentous actin (F-actin) in A549 cells was assessed with FITC-phalloidin stain following treatment with Daboialectin at different doses. In sub-confluent A549 controls, elongated cell morphology was observed with a high number of F-actin filaments (Fig. 4.4). However, treatment with Daboialectin at 50 and 100 nM concentrations for 24 h led to significant decrease in F-actin content and at 48 h of incubation resulted in almost complete disappearance of F-actin leading to rounding of cells (Fig. 4.4).

Figure 4.4

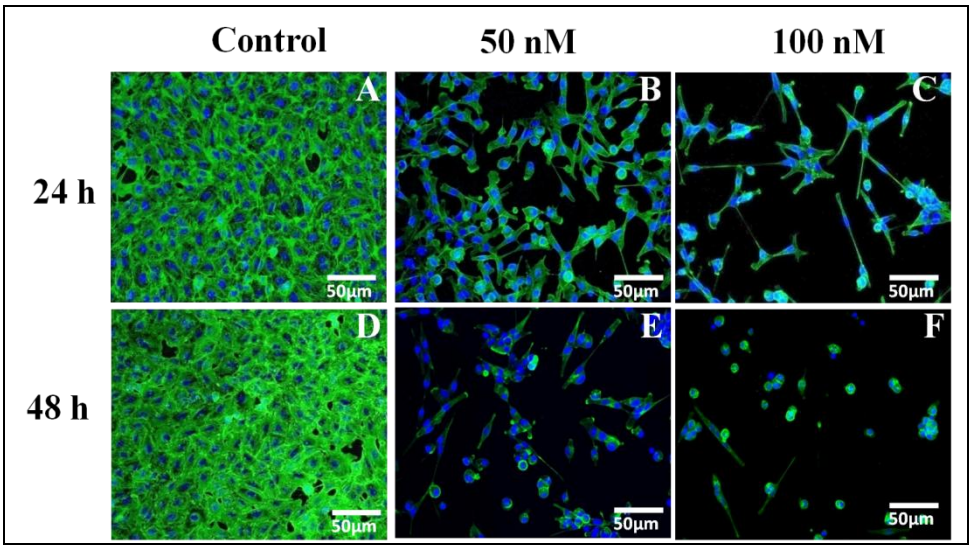


Figure 4.4: Morphological assessment of Daboialectin treated A549 cells. Effect of Daboialectin on cytoskeletal actin filaments. A549 cells were cultured and then exposed to different concentration of Daboialectin for 24 and 48 h, following which immune-fluorescence staining was used to visualize the cytoskeletal proteins. Phalloidin was utilized to visualize F-actin and Hoechst 3384 to label cell nuclei.

4.14.3. Cell viability assay

Cell viability was reduced to approximately 46% and 64 % in Daboialectin (1 and 2 µg/ml) treated groups after 24 h treatment. The viability of A549 cell lines after 48 h treatment with 50 and 100 nM of Daboialectin was 60% and 84%, which were significantly lower ($p<0.001$) than control (Fig. 4.5).

Figure 4.5

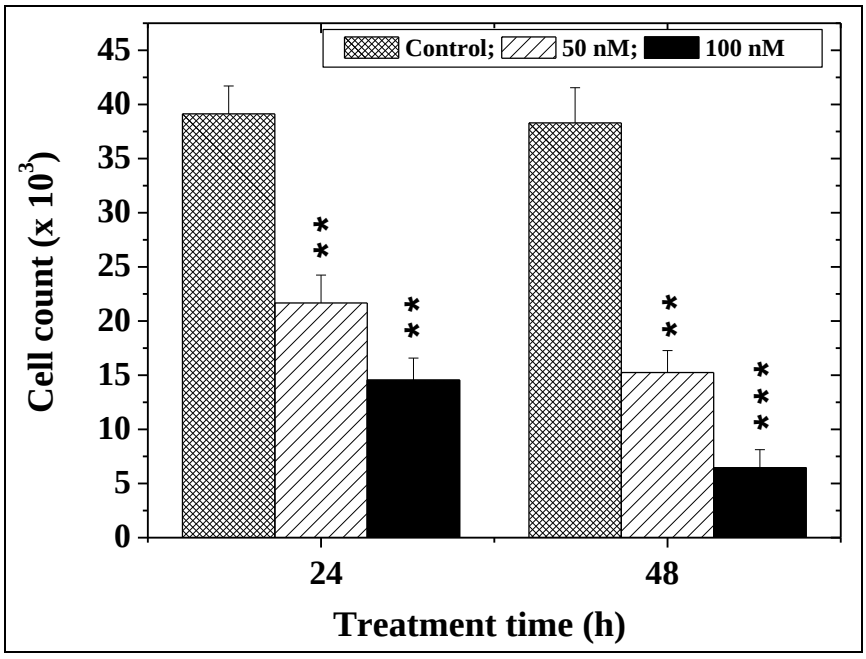


Figure 4.5: Cytotoxic effects of Daboialectin in A549 cells. Cells were treated with Daboialectin in dose-dependent manner for 24 and 48 h. The ratio of cell viability was measured by trypan blue. Data are presented as Mean ± SEM from three independent experiments. ***p<0.01, ***p<0.001* compared to control.

4.14.4. Daboialectin inhibits the proliferation of A549 cells

MTT-based cytotoxicity assay was performed in the present study, complete dose-response curves were generated and IC_{50} values were calculated. The treatment of cells with various concentrations of Daboialectin (25 – 150 nM) showed good inhibitory effect against A549 cell lines with an IC_{50} of 56.47 nM at 24 h and IC_{50} of 83.68 nM at 48 h indicating that Daboialectin is deleterious to A549 cell lines (Fig. 4.6).

Figure 4.6

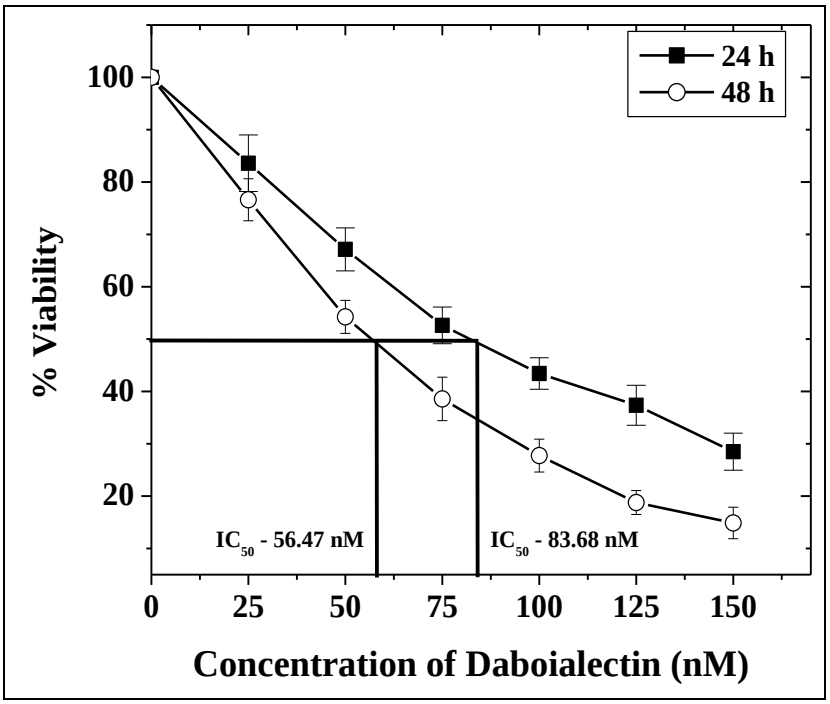


Figure 4.6: Cytotoxic effects of Daboialectin in A549 cells. Cells were treated with Daboialectin at various doses for 24 and 48 h. The ratio of cell viability was measured by MTT assay. Data are presented as mean ± SEM from three independent assays.

Figure 4.7

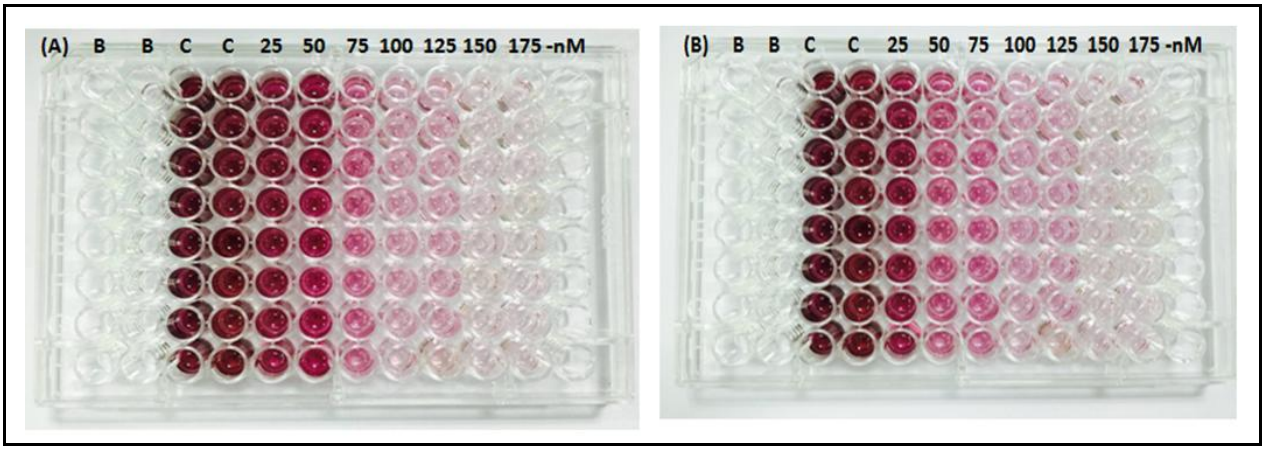


Figure 4.7: A microtiter plate after an MTT assay. (A) 24 h treated plate (B) 48 h treated plate.

4.14.5. Dabiolectin induced apoptosis

Quantitative analysis of apoptotic indices by AO/EB staining in Figure 4.8 and Table 4.1 illustrates the in vitro apoptotic indices (%) induced by Daboialectin in cultured cells. A549 cells after treatment with Daboialectin (50 and 100 nM) for 24 and 48 h showed bright green early apoptotic cells with nuclear margination and chromatin coagulation, indicating the increase in the percentage of apoptotic cells (Table 4.1). Whereas, green live A549 cells with a normal morphology were seen in the untreated group (Figure 4.8). Apoptotic cells were quantified by counting the number of apoptotic nuclei per number of total nuclei in the same microscopic field. The apoptotic index was averaged for 5 fields showing the total of about 500 cells per treatment. The results suggested that Daboialectin is able to induce apoptosis in A549 cells in a concentration-dependent manner.

Table 4.1: Results of Apoptotic index by Acridine orange/Ethidium bromide (AO/EtBr). All the results are shown as mean ± SEM from the data of three independent experiments.

Concentration of Daboialectin (nM)	**Apoptotic index ± SEM**	
	24 h	**48 h**
Control	3.024 ± 0.271	3.154 ± 0.314
50	21.281 ± 1.523	36.852 ± 2.053
100	34.153 ± 2.751	43.023 ± 2.581

Figure 4.8

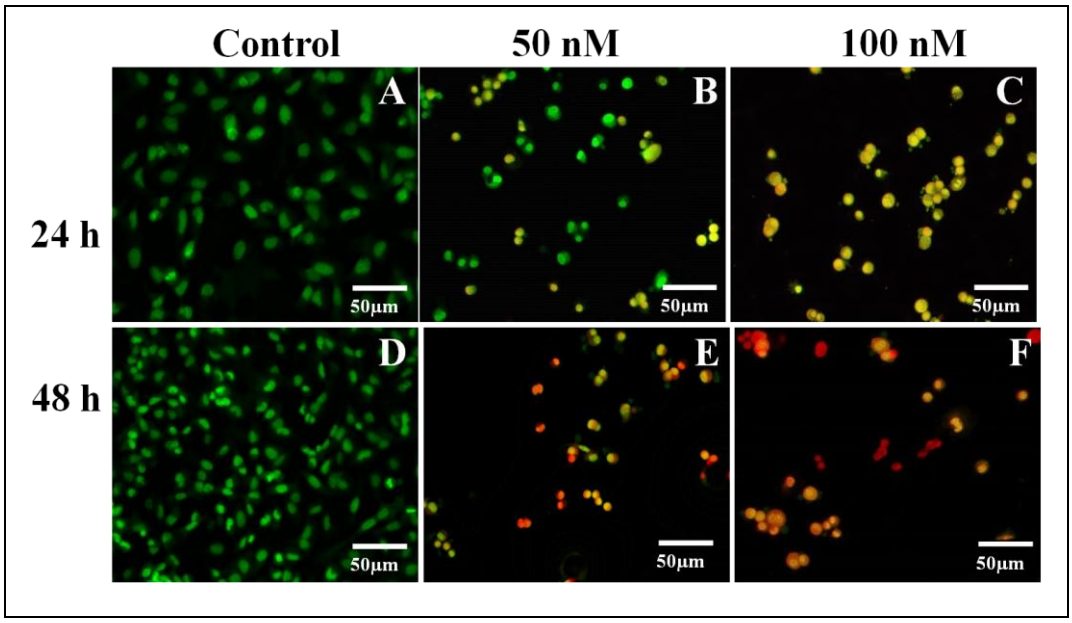


Figure 4.8: Morphological observation following acridine orange (AO) and ethidium bromide (EtBr) staining (magnification 20×). A549 cells were treated with and without Daboialectin at 50 and 100 nM for 24 and 48 h. After 24 h the early apoptotic cells could be observed: the cells were stained with AO, their membranes were in integrity, and the nuclei exhibited bright condensed chromatic or fragmented chromatin. Some cells appeared with typical apoptotic bleb phenomenon; (C) After being treated for 48 h, the late apoptotic cells could be observed: their cell membrane lost integrity; and stained red with EtBr.

4.14.6. Daboialectin induced cellular ROS production

Daboialectin induced ROS in A549 cells was determined by measuring fluorescence after loading with DCFDA, a dye that is oxidized into a highly fluorescent form in the presence of peroxides. A DCF fluorescence level, which is an indicative of intracellular ROS, increased in Daboialectin treated cells ($p<0.01$) in a significant time-dependent manner as compared to that in control group (Fig. 4.9).

Figure 4.9

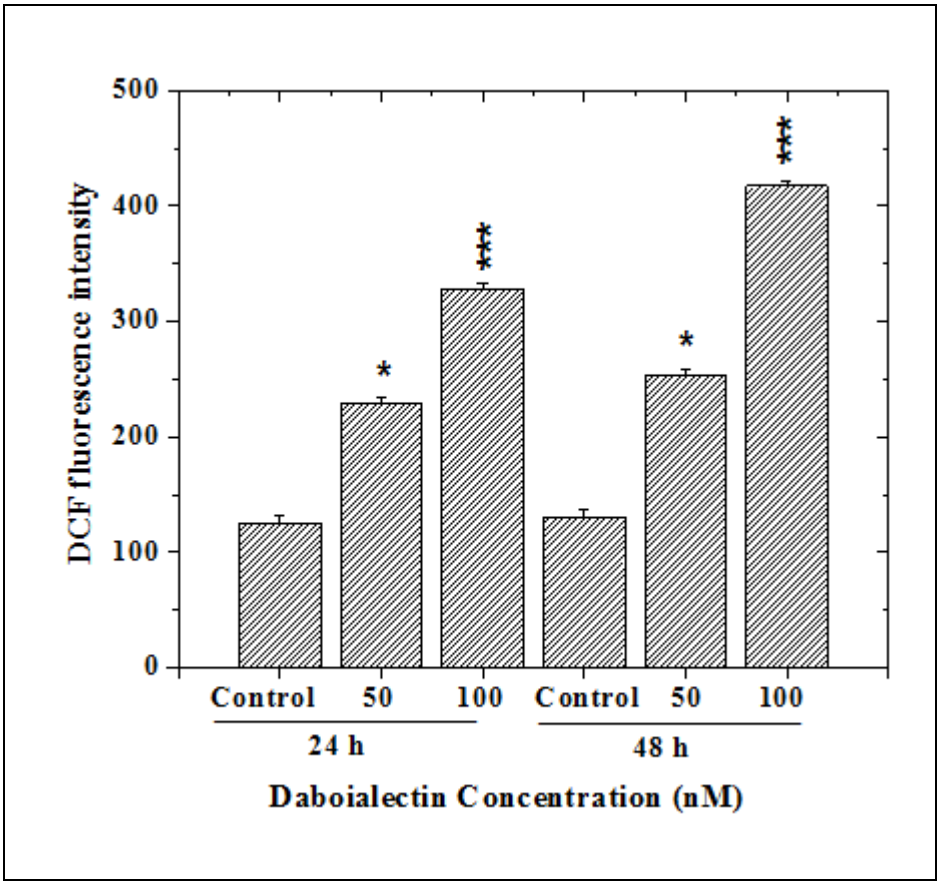


Figure 4.9: Daboialectin generated ROS in A549 cells. Cells were treated with different concentrations of Daboialectin, 10 μM of DCFH-DA for 24 and 48 h, ROS productions were determined by fluorescence spectrophotometic analysis. Data are presented as Mean ± SEM from three independent experiments. *$*p<0.05$, ***$***p<0.001$* compared to control.

4.14.7. Daboialectin affects the expression of small GTPases at mRNA and protein level

It is well known that Rho family GTPases which include RhoA, Rac1, and Cdc42 participate in regulation of the actin cytoskeleton and cell adhesion events. To understand the role of Daboialectin on regulation of the actin cytoskeleton, mRNA and protein from treated cells were analyzed to evaluate the regulation of small GTPases.

Rho which plays a role in the formation of stress fibers and focal adhesions showed down regulation at both mRNA and protein levels at 24 h by 1.1 and 1.2 folds, whereas, Daboialectin (50 and 100 nM) at 48 h showed 1.3 and 1.4 folds of RhoA inhibition. Similarly, Rac1 which is involved in membrane ruffling, cell motility showed down regulation at both mRNA and protein levels at 24 h (1.1 and 1.2 folds) and 48 h (1.1 and 1.3 folds). However, Cdc42 which participates in filopodia formation and cell-cell adhesions, showed up-regulation at both mRNA and protein at 24 h by 1.0 and 1.1 folds, whereas at 48 h by 1.2 and 1.2 folds in the presence of Daboialectin when compared with control groups (Fig. 4.10 and 4.11).

Figure 4.10

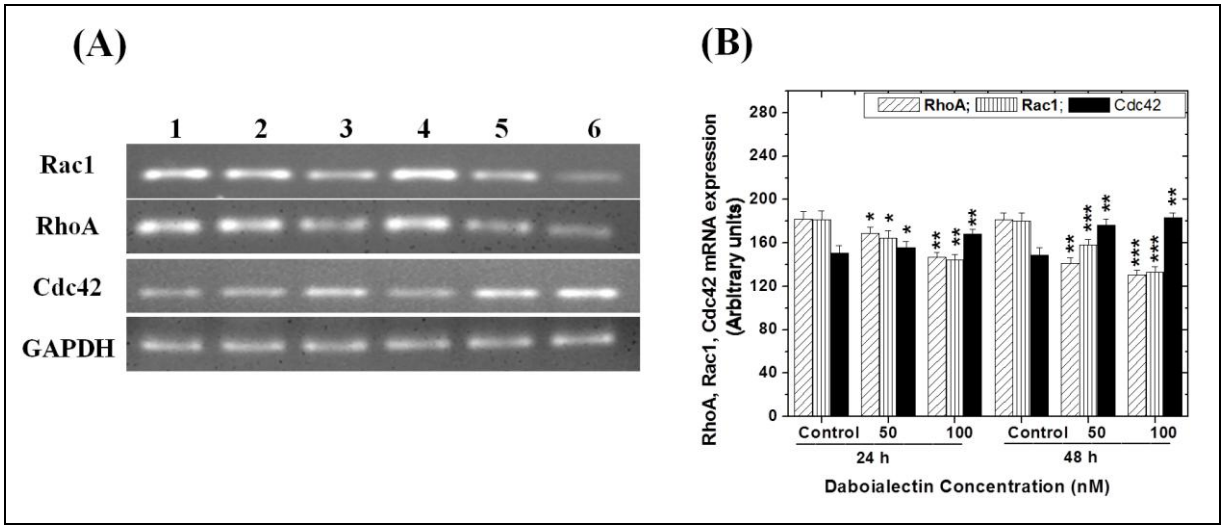


Figure 4.10: (A) Daboialectin regulates the expression of small GTPases (Rac1, Rho and Cdc42) in A549 cells. Following treatment with Daboialectin for 24 and 48 h. The A549 cells were lysed and expression of Rac1, RhoA, Cdc42 and phospho Rac1/RhoA were detected by RT-PCR, where GAPDH was used as internal control. **(B)** Quantification of small GTPase expression as modulated by Daboialectin has been

depicted in bar diagram. Data are presented as Mean ± SEM from three independent experiments. ***p<0.01, ***p<0.001* compared to control.

Figure 4.11

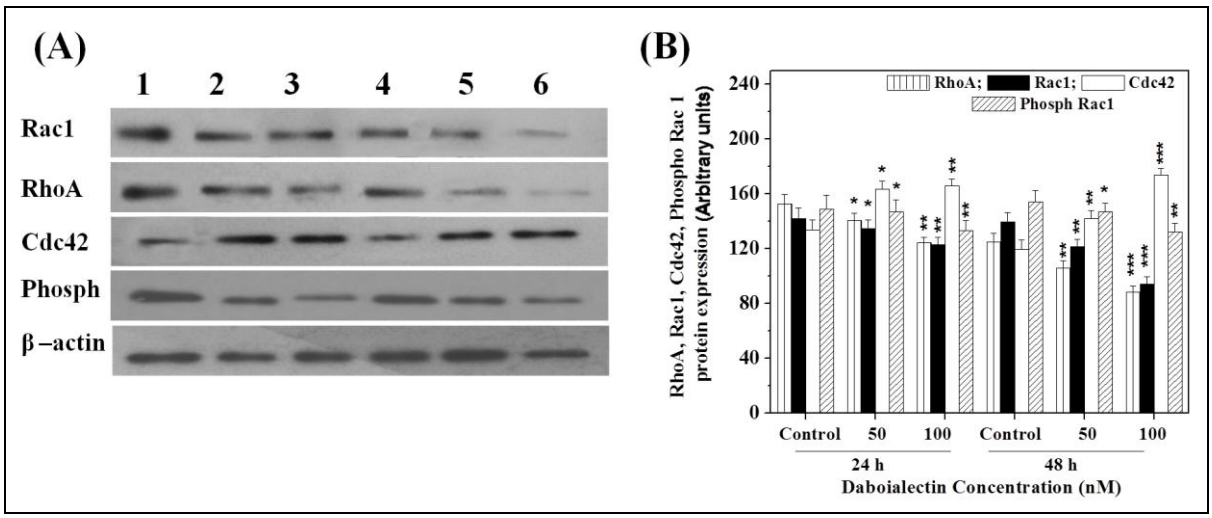


Figure 4.11: (A) Daboialectin regulates the expression of small GTPases (Rac1, RhoA and Cdc42) in A549 cells. Following treatment with Daboialectin for 24 and 48 h. The A549 cells were lysed and expression of Rac1, RhoA, Cdc42 and phospho Rac1/RhoA were detected by western blot analysis, where β- actin was used as internal control. **(B)** Quantification of small GTPase expression as modulated by Daboialectin has been depicted in bar diagram. Data are presented as Mean ± SEM from three independent experiments. **p<0.05, **p<0.01, ***p<0.001* compared to control.

4.14.8. Effects of Daboialectin on expressions of Bax, Bcl2 and caspase 3, 8, 9 in A549 cells

An early event in the cell that sensitizes it to apoptosis is the expression of the apoptotic proteins. In the present study, reduced cell viability and increased levels of apoptotic cells were observed on exposure to Daboialectin. A semi-quantitative RT-PCR assay was used to examine whether these changes were also evident at the mRNA level.

Treatment of A549 cells with Daboialectin for 24 and 48 h treatment showed significant increase in the Bax expression levels when compared with untreated cells. Since we observed elevated expression of Bax, further counter-gene Bcl2 expression was also observed. Following treatment with Daboialectin for 24 and 48 h, a decrease in Bcl2 expression was detected in the Daboialectin treated cells.

Since Daboialectin down regulated Bcl2 gene expression, it was examined whether these induced downstream processes involve caspase activation. Following secretion of cytochrome c from the mitochondria, it binds to pro-caspase 9, resulting in the activation of caspase 9 and subsequently, caspase 3. A549 cells treated with Daboialectin for 24 and 48 h showed increased expression of caspase 3, caspase 8, and caspase 9 proenzyme levels in treated cells. (Fig. 4.12).

Figure 4.12

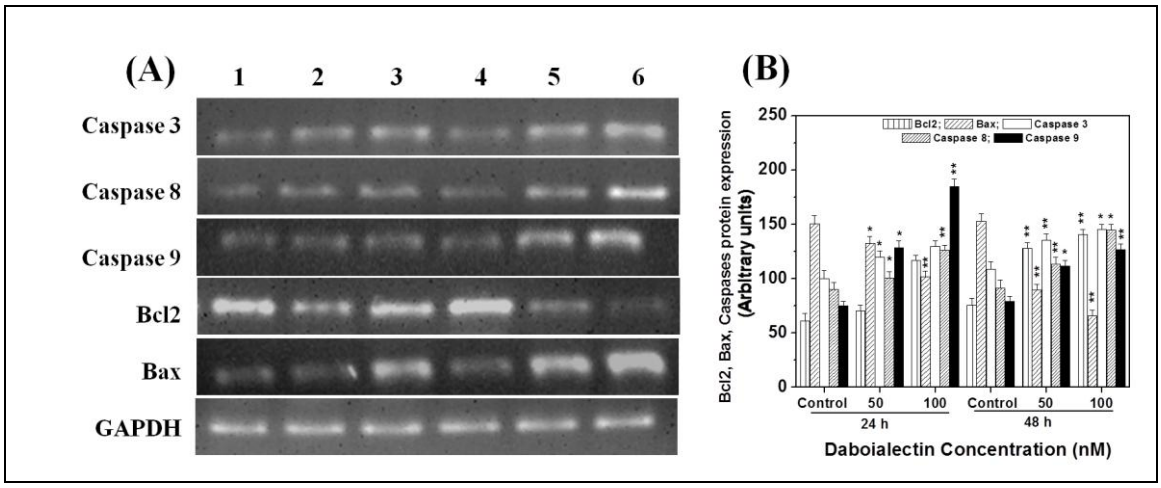


Figure 4.12: (A) Expression of apoptotic genes (Bax, Bcl2, caspase 3, 8 and 9) at mRNA level was monitored by RT-PCR technique. RT-PCR was performed using specific primers mentioned earlier in the text. The amplified PCR products were run in a 1.2% agarose gel and visualized by EtBr staining. Bcl2 had been found to be reduced whereas Bax and other caspases were seen to have enhanced expression after treatment

with different concentrations of Daboialectin. GAPDH was used as a house-keeping control gene. **(B)** Quantification of apoptotic protein expression as modulated by Daboialectin has been depicted in bar diagram. Data are presented as Mean ± SEM from three independent experiments. *$p<0.05$, **$p<0.01$ compared to control.

4.14.9. Daboialectin inhibits invasion of A549 cells

The effect of Daboialectin on the migration ability of A549 cells was assayed by using Matrigel invasion assay. Results show that Daboialectin at two different doses (50 and 100 nM) for 24 and 48 h significantly inhibited A549 cells penetrating through Matrigel. It inhibited the invasive ability of A549 cells in a dose and time dependent manner. Present observations suggest that Daboialectin negatively affects on growth and migration ability of A549 cells (Fig. 4.13).

Figure 4.13

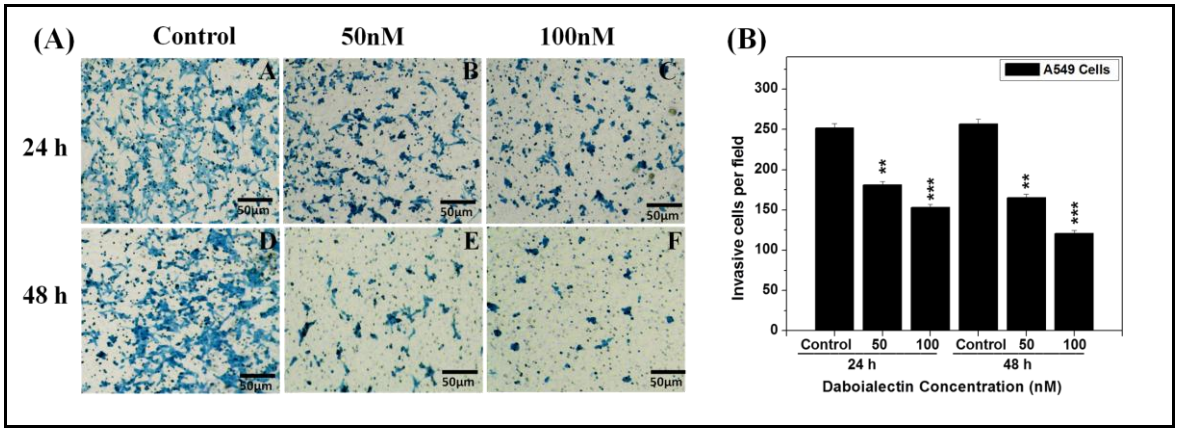


Figure 4.13: For migration assay, A549 cells were seeded into the upper chamber of the transwell system. Cells were treated with different doses of Daboialectin for 24 and 48 h and were allowed to migrate. **(A)** Migrated cells were fixed, stained with methylene blue and photographed. It was found that Daboialectin had an inhibitory effect on A549 cell migration. **(B)** Bar diagrams represent percentage of invaded cells. Results are expressed as mean ± SEM of three independent experiments. Data are

presented as mean ± SEM from three independent experiments. ***p<0.01, ***p<0.001* compared to control.

4.14.10. Daboialectin induced caspase 3 activation

As caspase 3 is well established as the major executioner caspase and its activation ultimately leads to cell death, it is thus suited as a read-out in an apoptosis assay. We evaluated the Daboialectin induced apoptosis in association with changes in caspase 3 activity. A549 cells treated with 50 and 100 nM for 24 and 48 h were analyzed for such activity using *in vitro* colorimetric substrate DEVD-pNA (Fig. 4.14).

Figure 4.14

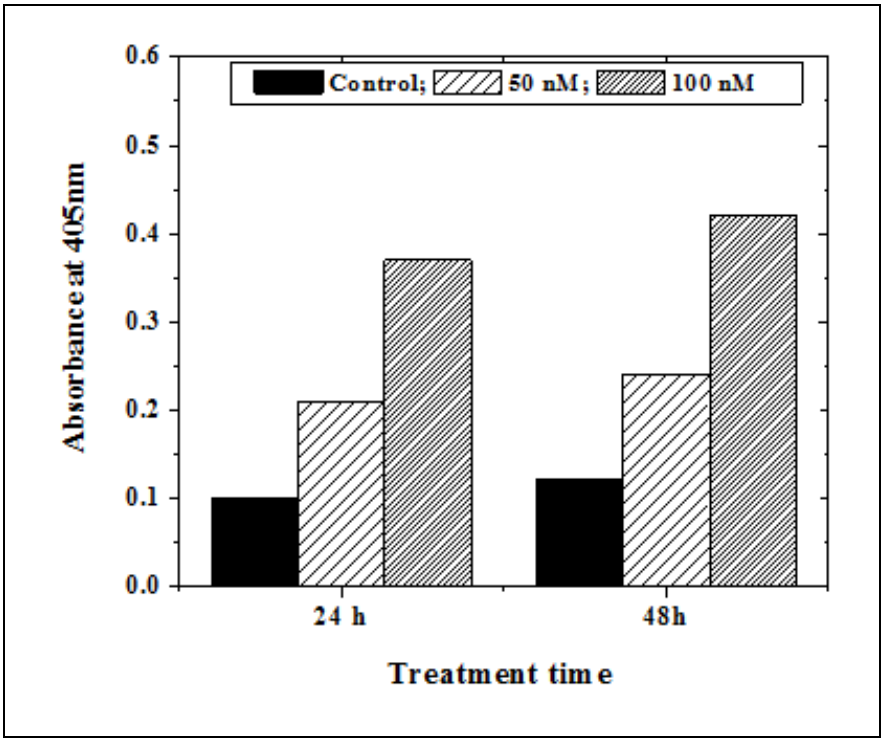


Figure 4.14: Daboialectin activated caspase 3 in A549 cells. Cells were treated with different concentrations of Daboialectin for 24 and 48 h and caspase activity was assayed using DEVD-pNA.

4.14.11. Effects of Daboialectin on Hsp70 expression pattern at mRNA and protein levels

To evaluate Hsp70 regulation by Daboialectin treatment, RT-PCR and Western blot analysis were performed in cells exposed to Daboialectin for 24 and 48 h (Fig.4.14). Hsp70 levels were determined after the exposure to 50 and 100 nM of Daboialectin for 24 and 48 h. Exposure to Daboialectin at concentrations showed down-regulation of Hsp70 protein levels. Further, estimation of mRNA levels relatively decreased the expression levels in Daboialectin treated group at both 24 and 48 h respectively. GAPDH and β-actin served as internal controls for each sample. The expression profile of Hsp70 was similar to that of densitometric scanning profile of both mRNA and protein levels (Fig. 4.15 and 4.16).

Figure 4.15

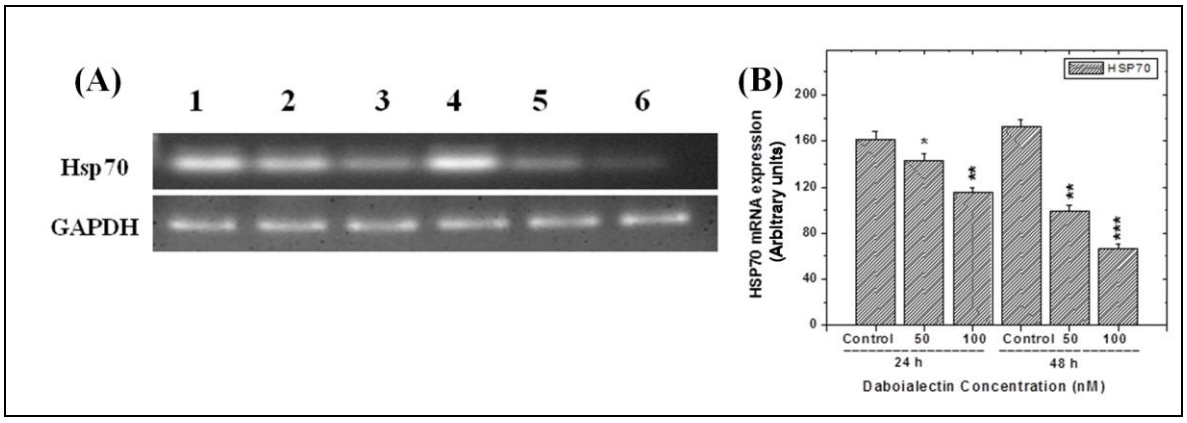


Figure 4.15: Effect of Daboialectin on Hsp70 expression. **(A)** Exponentially growing cells were exposed to different concentrations of Daboialectin for 24 and 48 h cells were processed for RT-PCR. Results showed relatively decreased levels of mRNA with increasing concentrations of Daboialectin. GAPDH was used was used as a house-keeping control gene. **(B)** Quantification of Hsp70 expression as modulated by

Daboialectin has been depicted in bar diagram. Data are presented as mean ± SEM from three independent assays. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to control.

Figure 4.16

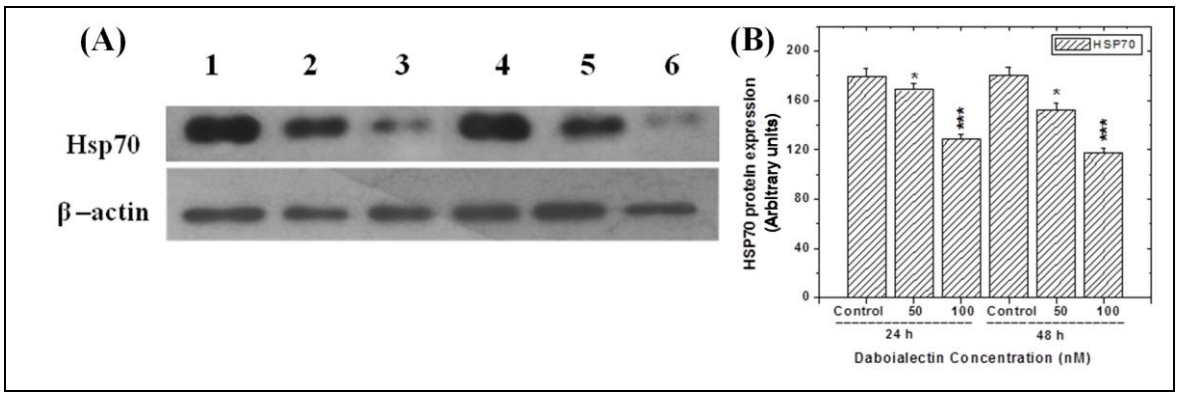


Figure 4.16: Effect of Daboialectin on Hsp70 expression. **(A)** Exponentially growing cells were exposed to different concentrations of Daboialectin for 24 and 48 h. Cells were harvested, total proteins were extracted and 40 µg of protein was subjected to western blotting using antibodies against Hsp70. Results showed repression of Hsp70 with increasing concentrations of Daboialectin. β-actin was used as control to ensure equal loading of protein. **(B)** Quantification of Hsp70 expression as modulated by Daboialectin has been depicted in bar diagram. Data are presented as mean ± SEM from three independent experiments. *$p<0.05$, ***$p<0.001$ compared to control.

4.14.12. Daboialectin reduces the migratory capacity of A549 cells

Present study was conducted to investigate the effects of Daboialectin on the migration ability of A549 cell lines. The untreated group showed complete migration ability. However, treatments with 50 and 100 nM of Daboialectin for 24 and 48 h blocked the migrating ability of A549 cells (Fig. 4.17A). The Daboialectin inhibition of migration activity was 44% (50 nM) and 72% (100 nM) at 24 h, whereas, at 48 h 56% and 86% reduction were observed with same doses, respectively (Fig. 4.17B).

Figure 4.17

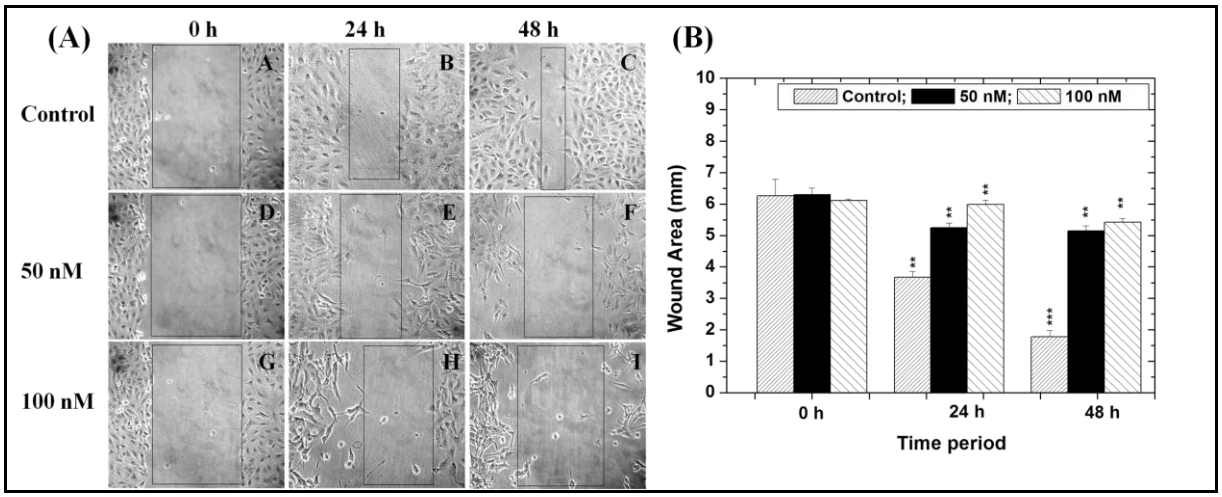


Figure 4.17: Effect of Daboialectin on migratory capacity of A549 cells - A wound healing assay was utilized to study modulation on cell migration of the Daboialectin, in A549 cells. **(A)** Wound healing assay was initiated by a uniform scratch in a Pertidish containing A549 cells grown to confluence. Scratch was monitored under a microscope. The width of the scratch was measured and the percentage of closure was estimated. **(B)** Results expressed as mean ± SEM (n=3) shows a significant inhibition of wound closure ($p<0.001$) by Daboialectin.

4.14.13. Dose dependent inhibition of ADP induced platelet aggregation

Treatment of whole blood to different concentrations of Daboialectin inhibited ADP induced platelet aggregation in a concentration dependent manner. 52.1% inhibition of platelet aggregation was observed with 50 nM of Daboialectin and about 73% inhibition with 100 nM of Daboialectin (Fig. 4.18).

Figure 4.18

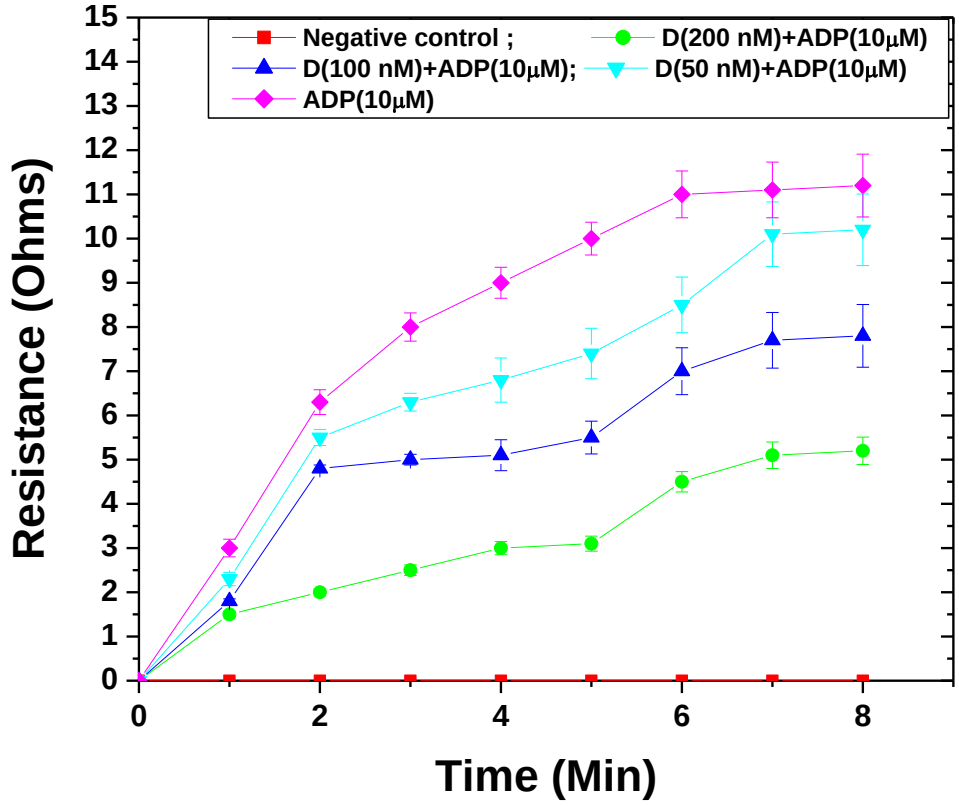


Figure 4.18: Effects of Daboialectin on ADP induced platelet aggregation. Blood was treated with Daboialectin at different concentrations and the platelet aggregation process was induced by ADP (n =3). Aggregation kinetics were observed in terms of resistance (Ohms) measured by the platelet aggregometer.

4.15. Discussion

Snake venoms are the most versatile and complicated forms of all naturally produced venoms containing hundreds of proteins, peptides and non-protein molecules. Several studies have shown the potential of bioactive compounds from snake venoms as cytotoxic, anti-tumor and apoptosis inducing agents in different cancer cell lines as well as in some *in vivo* models (Chan *et al.*, 2016). It is obviously important to identify and isolate the responsible agents from the whole venom for better understanding. The present study was attempted to isolate at least one of the toxins from RVV responsible for morphological changes observed following treatment with whole RVV. The protein toxin of 18.5 kDa purified from RVV by cation-exchange chromatography and size-exclusion HPLC, was found to cause significant morphological changes in A549 cells *in vitro*. MALDI-TOF analyses showed that a protein obtained by trypsin digestion of the purified 18.5 kDa toxin has significant homology with C-type lectins isolated from snake venoms. Hence, the pure toxin was named Daboialectin.

It is well documented that C-type lectins (snaclec) are abundant components of viperine snake venoms and are structurally homologous to the carbohydrate recognition domain of animal C-type lectins (Sartim and Sampaio, 2015). CLPs are characterized by various biological activities, and are considered as modulators of platelet aggregation by targeting vWF, GPIb-IX-V, GPVI and possibly other platelet receptors. Recently, some CLPs were highlighted for their potential anti-tumor effects by blocking adhesion, migration, proliferation and invasion of different cancer cell lines (Morita, 2005).

In the present study, exposure of A549 cells with different concentrations of Daboialectin for different time periods resulted in long, thin, spindle-like shape with

boundaries resembling those of loosely adhered cells, indicating that effects of Daboialectin on cell morphology are in accordance with our earlier findings with whole RVV. Further, FITC-phalloidin assay on Daboialectin treated cells showed an alteration of the actin cytoskeleton and cell morphology like those observed with whole RVV.

Investigations on the mechanism of such changes were made by assessing the status of molecules known to be responsible for maintaining the shapes of cells, namely the cytoskeletal proteins. Dynamic interactions between the cell, the extracellular matrix (ECM) and the cytoskeleton play important roles in the process of cell migration (Vicente-Manzanares, *et al.,* 2009). Some snake venoms have been shown earlier to contain toxins that inhibit cell adhesion, proliferation, migration and can induce cell death (Chan *et al.,* 2016).

Our earlier observation on RVV induced dose and time dependent cytotoxic effects against A549 cells were accompanied by decreased expression of Hsp70 and small GTPases. Whole RVV also down-regulated RhoA, Rac1 caspases and Bcl-2, while up-regulating Cdc42 protein Bax and p53 levels (Pathan *et al.,* 2015).

Assessment of toxicity is important in development of a drug from natural or synthetic sources. Our data clearly showed marked cytotoxic effects of Daboialectin on A549 cells which was evident from the cell viability assays. These results were further confirmed by MTT-based cytotoxicity assay, where treatment of cells with various concentrations of Daboialectin showed strong inhibitory effects against A549 cell lines with an IC_{50} of 56.47 nM at 24 h and IC_{50} of 83.68 nM at 48 h, indicating that Daboialectin is deleterious to A549 cell lines. Our observations are in accordance with other studies on cytotoxic effect of venoms on cancer cells (Balasubashini *et al.,* 2006, Zare *et al.,* 2008, Chen *et al.,* 2009).

Snake venoms are known to inhibit cell growth through chromatin condensation as well as cytoplasmic and mitochondrial alteration, thus pushing the cells towards programmed cell death (Karthikeyan *et al.,* 2007, Abu-Sinna et al. 2003, Tolleson *et al.,* 1996,). In the present study, cells treated with Daboialectin showed a significant increase in the percentage of apoptotic cells (apoptotic index). Bcl2, Bax and caspase proteins, contribute to the regulation of apoptosis (Yang *et al.,* 2007, Salakou *et al.,* 2007, Mbazima *et al.,* 2008). Daboialectin induced apoptosis was accompanied by up-regulation of Bax genes and down regulation of Bcl2 gene in treated A549 cells. Snake venoms have earlier been reported to arrest cancer cell growth by induction of apoptosis (Yang 2005; Yang *et al.,* 2006; Son *et al.,* 2007). During the induction of apoptosis, the level of reactive oxygen species (ROS) is expected to increase and the mitochondrial membrane released cytochrome c into the cytosol results in activation of caspase 3 and 9 (Shivapurkar *et al.,* 2003). Similar observations were also made in the present study, where Daboialectin treated cells showed increase in the intracellular ROS levels with up regulation of caspase proteins at mRNA level.

Among several small GTPases, Rho proteins are essential for focal adhesion formation when cells are plated on a matrix. Furthermore, integrins regulate signaling pathways to members of Rho GTPases, RhoA, Rac1 and Cdc42, which are molecular switches that control the dynamics and structure of actin-based processes, such as filopodia, lamellipodia, and stress fiber formation (Hall and Nobes, 2000). The molecular mechanisms underlying the above processes have been largely unknown, until recently. Some of the molecular pathways that connect the Rho family GTPases to the control of cytoskeleton and cell adhesion have been established. To understand the role of Daboialectin on regulation of the actin cytoskeleton, mRNA and protein from

treated cells were analysed to evaluate the regulation of small GTPases. Data from the present study indicates that RhoA and Rac1 proteins showed down regulation at both mRNA and protein levels, Cdc42 showed up-regulation at both mRNA and protein levels. These observations are in agreement with those made with RVV.

The above mentioned observations prompted investigation of the average migration ability of Daboialectin treated A549 cells by using *in vitro* wound healing and matrigel invasion assays. Treatment of A549 cells with Daboialectin significantly inhibited penetration ability of the cells through Matrigel in a concentration and time dependent manner. Similarly, *in vitro* wound-healing assay showed significant blockage of migration ability of A549 cells in the presence of Daboialectin. Similar events were also observed in earlier studies (Tseliou *et al.*, 2016)

Russell's viper venom is a mixture of many proteins and peptides. A biological function observed with whole venom may or may not be attributed to a single toxin. However, the purified toxin, Daboialectin could reproduce all cytotoxic effects related to change in cell shape observed with RVV. This indicates that Daboialectin is at least one of the C-type lectins in the venom of Russell's viper venom capable of pushing A549 cells to apoptotic pathway. The strong anti-migration activity of Daboialectin adds value of this toxin as a potential therapeutic candidate against malignance or invasive tumor formation.

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Summary & Conclusion

SUMMARY AND CONCLUSIONS

Snakes have held a unique place in the history of Indian culture and civilization. This unique status of snakes is richly articulated in Indian art, science and also in religious rituals. Snakes are considered the most alarming of all animals that co-inhabit our planet. Most of the fear about snakes prevails due to the forbidding pathological effects observed after envenomation.

Venoms of snakes are the most studied among all venoms. Snake venom components are of biological interest because of their ability to cause mortality and morbidity in many parts of the world. In India about 250 species and subspecies of snakes have been reported of which 50 are poisonous (Deoras, 1965). Venomous snakes possess one of the most sophisticated integrated weapon systems in the natural world. Snake venom toxins are studied with great interest for their therapeutic potentials against cancer, thrombosis, chronic pain, etc. (Pal *et al.*, 2002). These venoms are also of intense academic interest because of pharmacological specificity of component toxins. The pharmacological specificity also may be exploited for development of better therapeutic tools.

Russell's snake (*Daboia russelii*) commonly known as Daboia, Chain Viper or Indian Russell's Viper, is one of the dominating venomous snakes dispersed over the tropical nations of South-East Asia including India and is frequently considered a serious and major health hazard. Russell’s viper is an old world snake falling under the elite viperine snake family. Venom from Russell's viper is a mixture of proteins and peptides making up to 90-95% of their dry weight (Russell, 1980). About 5% of the venom composition is amino acids, nucleotides, free lipids, carbohydrates and metallic elements bound to proteins (Heise *et al.*, 1995). Many enzymatic and non-enzymatic

components from snake venom are known to induce multiple toxic effects. Most of the toxic effects of the venom are attributed to the proteinaceous components of the venom. These proteins bring about lethal and debilitating effects as a consequence of their neurotoxic, cardiotoxic and cytotoxic nature. Peptides such as *dr*CT-1 (7.2 kDa), RVV-7 (7.2 kDa) and Drs-PLA2 (13.6 kDa) with anticancer potential has also been reported from RVV [132-134]. All of these peptides are characterized as Phospholipase A_2 (PLA2) enzymes with cytotoxic and anticancer potential. Maung-Maung and co-workers (1995) reported that purified lethal toxin from Burmese Russell's viper (*Daboia russelli siamensis*) showed cytotoxicity on HeLa cells. However, there has been no study addressing the question of whether or not Russell's viper venom (RVV) possesses any anticancer activity on A549 cells. The detailed *in vitro* studies on RVV induced change in cellular morphology and regulation of small GTPases are lacking. The present study was to investigate the anticancer activity of the RVV *in vitro* with following aim and objectives:

Aims and scopes of this study:

The present study was aimed to evaluate the effects of Russell's viper venom (RVV) on the morphology of human A549 cells and its role on expression and regulation of small GTPases, stress protein (Hsp70) and apoptosis related genes. The study was also aimed for the purification and partial characterization of toxin(s) responsible for above mentioned effects from Russell's viper venom (RVV).

In the present work, RVV induced cyto-morphological changes were evaluated by treating the human A549 cells with different doses of RVV for 24 and 48 h. A significant alteration in cellular morphology was observed and an increase in the dose with time had shown the presence of stress granules, appearance of axo-dendrionic

extension, rounding-off of the cells, formation of punctate structures and loss in cell to cell contacts. The cytotoxic effects of RVV were evaluated by Resazurin assay. The findings of Resazurin assay confirmed that cytotoxicity was more evident at a lower concentration at 48 h group as compared to that of 24 h at all tested concentrations. The half maximal inhibitory concentration (IC_{50}) value of RVV for A549 cells was 6μg/ml for 24 h and 9 μg/ml for 48 h respectively.

Small GTPases regulate cellular morphology, polarity, movement and overall signalling cascade. Therefore, further studies were made to understand the involvement of smGTPases at mRNA and protein level through RT-PCR and western blot. The results indicated a difference in expression pattern of Rac1, RhoA and Cdc42 genes. Similarly, a significant difference in the expression pattern of Hsp70 and p53 at the mRNA as well as at the protein level were noted when cells were treated with different concentrations of RVV. These findings were consistent with the changes in cellular morphology results. All experiments were compared with RVV-unexposed cells as control. Present study, clearly indicates that RVV causes morphological changes in human A549 cells through modulation of small GTPases at their mRNA and protein level for the first time. RVV also affects the cellular nuclear architecture affecting migration and proliferation of these cells.

RVV, like all snake venoms is a mixture of hundreds of proteins, peptides and non-protein molecules. It is obviously important to identify and isolate the responsible agents from the whole venom for better understanding. In the present study, identification and characterization of proteins from RVV was analyzed using ion-exchange chromatography. Different fractions were collected and analysed for cyto-morphological studies and cytotoxic activity on A549 cells. Molecular size of the active

fraction was further interpreted by HPLC using Size exclusion column (SEC). The molecular weight and purity of fractions were determined by SDS-PAGE and verified by mass spectrometry. MALDI- ESI/QTOF analysis spectra provided clue to the identity of the purified toxin. MS raw data were analyzed by depositing the venom protein sequences in NCBI database, resulting in identification of highest identity with an 18.5 kDa C-type lectin-like 7 isolated from *Daboia siamensis* (accession no. Q4PRC6) with 61% sequence homology. The isolated protein was named as Daboialectin. Further, the molecular characterization of this protein was performed to understand the mode of cytotoxicity and mechanism of cell death involved observed in A549 cells.

The cytotoxic effects of 'Daboialectin', a C-type lectin (18.5 kDa) isolated from Russell's viper venom was evaluated on A549 cell lines. Daboialectin-induced inhibition of A549 cell growth was time and dose dependent with severe morphological changes caused by alteration of the functions of small GTPases such as Rac1, RhoA and Cdc42. Our results indicate that Daboialectin alters morphology of A549 cells via regulation of cytoskeleton through Rho-GTPases. In the present study, apoptosis of A549 cells induced by Daboialectin was confirmed using fluorescence microscopic analysis. Cells treated with Daboialectin showed a significant increase in the percentage of apoptotic cells (apoptotic index). Bcl2, Bax and caspase proteins are known to contribute in the regulation of apoptosis. Daboialectin was found to induce apoptosis by up-regulation of Bax genes and down regulation of Bcl2 gene in treated A549 cells. On other hand, Daboialectin treated cells showed an increase in the intracellular ROS levels with up-regulation of caspase proteins at mRNA level. Daboialectin was also found to be inhibitory to anti-adhesive and anti-invasive to A549 cells *in vitro*. Daboialectin is

the first Snaclec reported to induce cytoskeletal changes through regulation of Rho-GTPases and blocking the anti-apoptotic pathway for a cancer cell line.

From the present investigation it can be concluded that the cytotoxic effects of RVV and Daboialectin in A549 cells can be attributed to the following:

- RVV/Daboialectin significantly altered the cellular morphology and changes in F-actin distribution leading to decreased number of actin fibers, thus effecting the migration and invasive ability of the cells
- RVV/Daboialectin showed significant reduction in the percentage cell survival.
- RVV/Daboialectin showed alterations in the expression of Rho-GTPases at mRNA and protein levels.
- RVV/Daboialectin showed alterations in the expression of Hsp70 at mRNA and protein levels.
- RVV/Daboialectin exhibited apoptotic effect and resulted in significant concentration and time dependent elevation of tumour suppressor p53 protein. It also exhibited an increase in expression of apoptotic proteins thus favouring the cells to undergo apoptosis

Taken together, the present thesis demonstrated that Russell's viper venom induced cytotoxic effects against A549 cells in a dose and time dependent manner with decreased expression of Hsp70 and small GTPases showed down-regulation of RhoA and Rac1 with up-regulation of Cdc42 and p53 levels (Figure 1). The purified toxin Daboialectin was also found to be inhibitory to anti-adhesive and anti-invasive to A549 cells *in vitro*. It is evident that Daboialectin is at least one of the major components of RVV capable of the cytotoxic activities observed with RVV. Daboialectin is the first

Snaclec reported to induce cytoskeletal changes through regulation of Rho-GTPases and blocking anti-apoptotic pathway for a cancer cell line (Figure 2 & 3).

FIGURE 1: PROPOSED MODEL OF RVV INDUCED CYTOTOXICITY

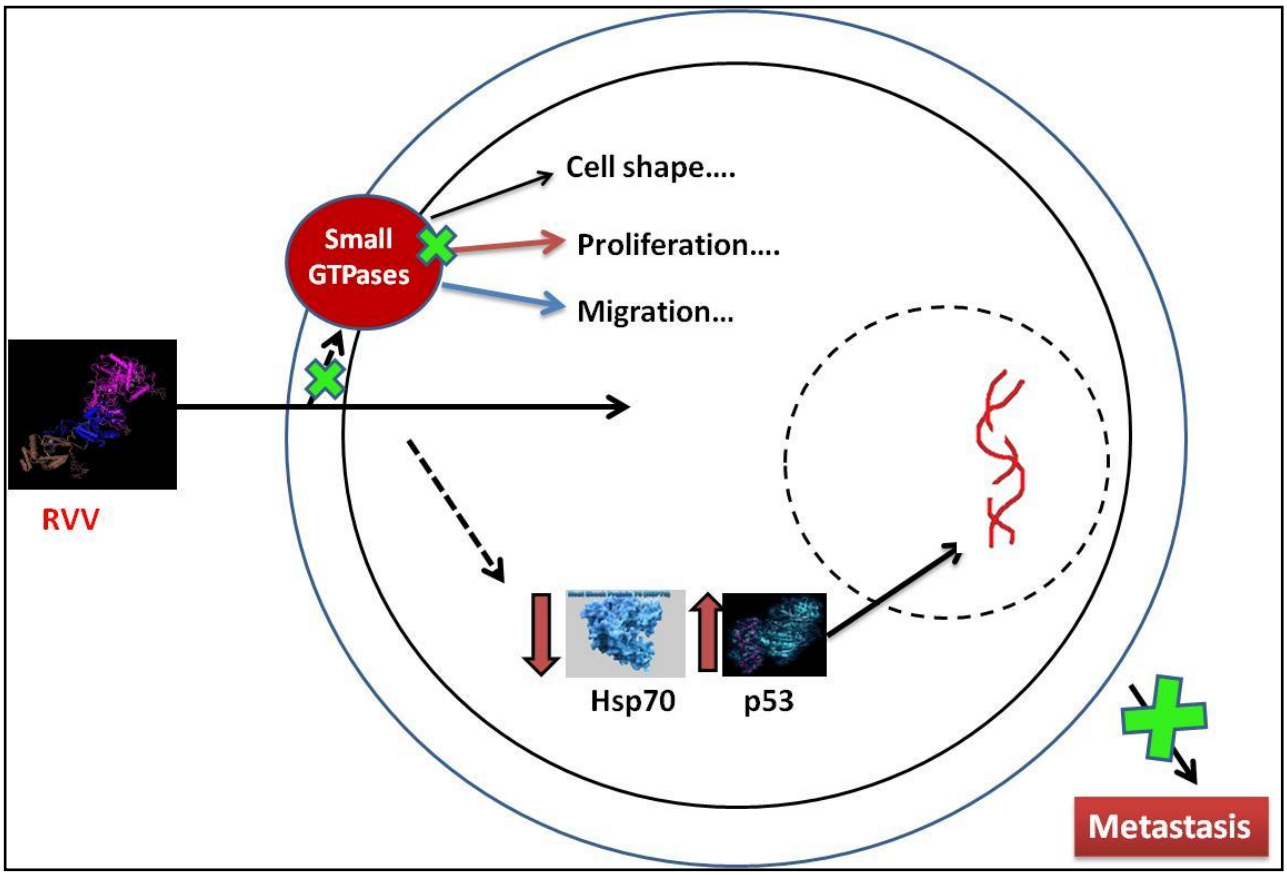


FIGURE 2: PROPOSED MODEL OF DABOIALECTIN INDUCED ALTERATIONS OF SMALL GTPASES

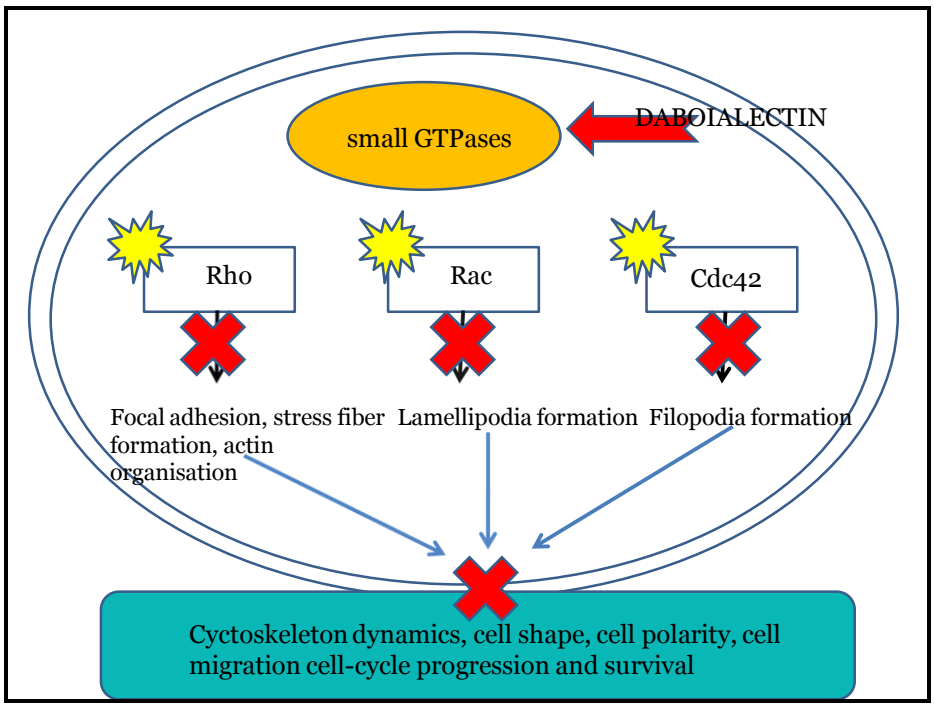
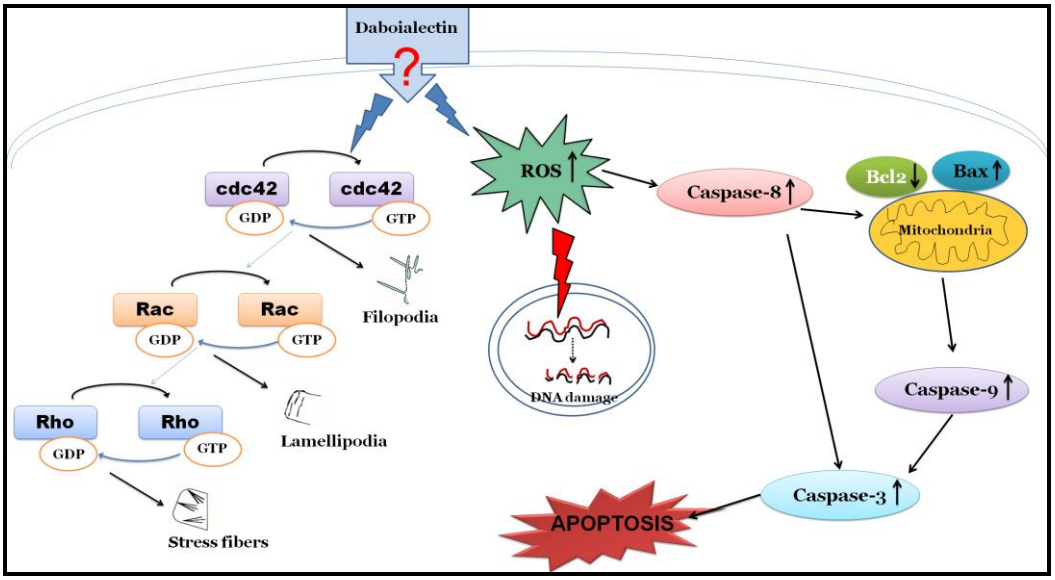


FIGURE 3: PROPOSED MODEL OF DABOIALECTIN INDUCED APOPTOTIC PATHWAY



FUTURE SCOPE

Future investigation of snake venomics can not only help researchers understand the evolution and the geographical effects of the venoms, but also benefit researchers to develop new drugs to treat diseases. The work described in this thesis has addressed;

1. The effects of Russell's viper venom (RVV) on the morphology of human A549 cells and its effects on expression and regulation of small GTPases, stress protein (Hsp70) and apoptosis- related genes.
2. Purification and partial characterization of toxin(s) responsible for above-mentioned effects from Russell's viper venom (RVV).

However, there still remain some imperative questions in this research, answers for which will open up new avenues of investigation in the future.

Some of the future prospective is discussed below.

Elucidating the structure-activity relationship of C-type Lectin

C-type lectins are known to be involved in immune response, cell proliferation, and apoptosis. The involvement of Daboialectin for its cytotoxicity on A549 cells is described in detail in this thesis. It is important to conduct further research on lectins, to determine its specificity and cytotoxicity against other malignant cell lines. Further detailed studies on the structure-activity relationship of Daboialectin will help us in discovering the various molecular mechanisms involved in the physiological processes. In addition, these studies might help in the development of various new therapeutic agents for the treatment of cancer.

Further *in vivo* studies with C-type Lectin

Lectins can induce apoptosis through different pathways, some being more effective than others in specific cell lines. In the present study, by Western blot techniques the expression of Rho-GTPases and apoptotic pathways was evaluated. It is well known that, Lectins can induce apoptosis through different pathways, some being more effective than others in specific cell lines. It is important to understand the process and mechanisms by which lectins affect cancerous cells. Investigations using *in vivo* experiments may provide a better indication on how a specific lectin may react in a clinical setting and what cytotoxic effects the lectin may hold. The basis for cancer therapy using lectins stems from the ability of these proteins to target multiple cellular components in a wide range of cancer cells.

List of publications

Original articles:

- ✓ Pathan J, Martin A, Chowdhury R, Chakrabarty D, Sarkar A. Russell's viper venom affects regulation of small GTPases and causes nuclear damage. Toxicon. 2015 Dec 15;108:216-25.

- ✓ Pathan J, Mondal S, Sarkar A, Chakrabarty D. Daboialectin, a C-type lectin from Russell's viper venom induces cytoskeletal damage and apoptosis in human lung cancer cells in vitro. Toxicon. 2017 Mar 1;127:11-21.

Conference proceedings:

- ✓ Pathan JS, Martin A, Chakrabarty D, Sarkar A. Cytotoxic effect of Russell's viper venom (RVV) in human alveolar adenocarcinoma cells (A549) cultured in vitro. Toxicon. 2016 Jun 15;116:84.

- **PATENTS**

  Filed a patent with the Indian Patent Office in April 2013 (Ref. no. 1370/MUM/2013)

- **AWARDS**

  - Best **Oral Presentation Award** at "National Conference of Young Researchers - 2017 on New Frontiers in Life Sciences and Environment" conference at GOA University, organized by Faculty of Life sciences & Environment, GOA on March 16-17, 2017.

  - Awarded Research Fellowship by Maulana Azad National Fellowship for Minority students (MANF-2012-13-MUS-GOA-14344). University Grants Commission, New Delhi.

List of Conferences Attended

- Jigni Pathan, *Sukanta Mondal*, Dibakar Chakrabarty and Angshuman Sarkar "Daboialectin, A Snaclec from Russell's Viper Venom induces cytoskeletal damage and Apoptosis in Human Lung Cancer Cells In Vitro'' presented oral presentation at the national conference on **"National Conference of Young Researchers - 2017 on New Frontiers in Life Sciences and Environment"** conference at GOA University, organized by Faculty of Life sciences & Environment, GOA on March 16-17, 2017.

- Jigni Pathan, Dibakar Chakrabarty and Angshuman Sarkar: Regulation of Small GTPases and Nuclear Damage Caused by Russell's Viper Venom (RVV), presented at the International conference on **"Trends in Cell and Molecular Biology-TCMB-2015"** conference at BITS PILANI, K K BIRLA GOA CAMPUS

- Jigni Pathan, Ansie Martin, Dibakar Chakrabarty and Angshuman Sarkar: Cytotoxic effects of Russell's Viper Venom (RVV) In Human Alveolar Adenocarcinoma cells cultured *In-vitro* presented poster at a **22nd meeting of the French Society of Toxinology (SFET)**, held in Pasteur Institute, Paris, France, 2014.

- Jigni Pathan, Dibakar Chakrabarty and Angshuman Sarkar: Russell's Viper Venom (RVV) causes alteration in cellular morphology through regulation of small GTPases in a time and dose dependent manner, presented at the international conference on **"3rd Annual Toxinological Society of India-TSICON-2013"** conference at BITS PILANI, K K BIRLA GOA CAMPUS.

- Attended the "**National Stem Cell Update-2009"** conference held at Manipal University, Manipal- April, 2009

- Attended the international conference on ***"Indo-US workshop on mitochondrial research & medicine"*** held at Manipal University, Manipal- November, 2008.

- Attended the *"**Genetically Modified Foods: A Third World Perspective"*** conference held at St.Xavier's College, Mapusa, Goa- September, 2005.

Toxicon 108 (2015) 216–225

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Russell's viper venom affects regulation of small GTPases and causes nuclear damage

Jigni Pathan [a], Ansie Martin [a], Rajdeep Chowdhury [b], Dibakar Chakrabarty [a, *], Angshuman Sarkar [a, **]

[a] Department of Biological Sciences, BITS Pilani, K K Birla Goa Campus, Zuarinagar, Goa 403726, India
[b] Department of Biological Sciences, BITS Pilani, Pilani Campus, Rajasthan 333031, India

ARTICLE INFO

Article history:
Received 12 August 2015
Received in revised form
15 October 2015
Accepted 20 October 2015
Available online 28 October 2015

Keywords:
Cell damage (nuclear damage)
Cytotoxicity
Cytoskeleton
Russell's viper venom
smGTPase
Apoptosis
Snake venom

ABSTRACT

Russell's viper with its five sub-species is found throughout the Indian subcontinent. Its venom is primarily hemotoxic. However, its envenomation causes damage to several physiological systems. The present work was aimed to study the dose and time dependent cytotoxic effects of Russell's viper venom (RVV) on human A549 cells grown *in vitro*. Time dependent changes have been observed in cellular morphology following exposure to RVV. Presence of stress granules, rounding-off of the cells, and formation of punctate structure and loss of cell–cell contact characterized the cellular effects. Fluorescence microscopic studies revealed that apoptotic cell population increased on exposure to RVV. Further to understand the mechanism of these effects, status of small GTPase (smGTPases) expression were studied by Western blot and RT-PCR; as smGTPases play pivotal roles in deciding the cellular morphology, polarity, cell movement and overall signaling cascade. It was shown for the first time that expression patterns of Rac, Rho and CDC42 genes are altered on exposure to RVV. Similarly, significant difference in the expression pattern of HSP70 and p53 at the mRNA levels were noted. Our results confirmed that RVV induces apoptosis in A549 cells; this was further confirmed by AO/EtBr staining as well as caspase-3 assay. All experiments were compared using RVV unexposed cells. We propose for the first time that RVV induces morphological changes in human A549 cells through modulation of smGTPase expression and affects the cellular-nuclear architecture which in turn interferes in proliferation and migration of these cells along with apoptosis.

1. Introduction

Russell's viper is an old world snake falling under the elite viperine snake family. Its single species with five sub-species, namely, *russelli*, *pulchella* (sometimes considered synonymous with *russelli*), *formosensis Maki*, *limitis* and *nordicus* are distributed throughout south-East Asia. Envenomation by Russell's viper is known for its diverse patho-physiological consequences, e.g., myotoxicity, edema, pituitary insufficiency, 'dramatic hemorrhage' and renal failure apart from death. The amazing specificity of snake venom toxins to target physiological systems also made them potential molecules of drug development. A number of toxins with anti-cancer potentials have been purified in recent times from different snake venoms, including Russell's viper venom. The anti-cancer potential of Russell's viper venom lies in its cytotoxic effects. Most contemporary research in the development of anticancer therapeutics from venoms have focused on investigating the molecular mechanism by which an agent induces cytotoxicity and apoptosis in cancer cells (Son et al., 2007; Yang et al., 2005).

It is well documented that Rho family of small GTPases play a vital role in the control of cell movement, morphology and adhesion by regulating the actin cytoskeleton (Bishop and Hall, 2000). Tumor invasion and metastasis involves various intracellular molecules, which includes formation of membrane protrusions like filopodia, lamellipodia, and pseudopodia (Abraham et al., 2001). In the recent past, studies have shown that Rho proteins are most likely involved in the cancer cell migration, tissue invasion and metastasis (Aznar and Lacal, 2001). Although, the cytotoxic effects Russell's viper venom has been observed, the true mechanism of

* Corresponding author.
** Corresponding author.
E-mail addresses: dibakarchakrabarty@goa.bits-pilani.ac.in (D. Chakrabarty), asarkar@goa.bits-pilani.ac.in (A. Sarkar).

http://dx.doi.org/10.1016/j.toxicon.2015.10.011
0041-0101/

Toxicon 127 (2017) 11–21

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Daboialectin, a C-type lectin from Russell's viper venom induces cytoskeletal damage and apoptosis in human lung cancer cells *in vitro*

Jigni Pathan, Sukanta Mondal, Angshuman Sarkar, Dibakar Chakrabarty*

Department of Biological Sciences, Birla Institute of Technology and Science, Pilani, K K Birla Goa Campus, Zuarinagar, Goa, India

ARTICLE INFO

Article history:
Received 17 November 2016
Received in revised form
26 December 2016
Accepted 31 December 2016
Available online 3 January 2017

Keywords:
A549 cells
Apoptosis
Cytotoxicity
Daboialectin
RHO-GTPases
Russell's viper venom

ABSTRACT

'Daboialectin', a low molecular weight C-type lectin (18.5 kDa) isolated from Russell's viper venom showed cytotoxic effects on human broncho-alveolar carcinoma derived (A549) cell lines. Daboialectin induced inhibition of A549 cell growth was time and concentration dependent with severe morphological changes by altering the functions of small GTPases such as Rac, Rho and cdc42. ROS induced DNA damage may result in apoptosis by inducing disruption of membrane integrity, blebbing and nuclear disintegration by activating caspases. Our results indicate that Daboialectin, a snake c type lectin (Snaclec) isolated from RVV alters morphology of A549 cells via regulation of cytoskeleton through RHO-GTPases. On other hand, the HSP70 and some other anti-apoptotic proteins required for the survival of cancer cells was found to be down-regulated in presence of Daboialectin. Daboialectin was also found to be inhibitory to anti-adhesive and anti-invasive to A549 cells *in vitro*. Daboialectin is the first Snaclec reported to induce cytoskeletal changes through regulation of RHO-GTPases and blocking anti-apoptotic pathway for a cancer cell line.

1. Introduction

Russell's viper (*Daboia russelii*) with its five sub-species is found in a wide geographical area in South-Asia. Venoms from the family Viperidae contain a wide variety of proteins and peptides making up to 90–95% of their dry weight (Russell, 1980). Many of these are known to have potent hemorrhagic, fibrinolytic (Chakrabarty et al., 2000) and cytotoxic activities (Maung-Maung-Thwin et al., 1995). A few peptides such as drCT-1, RVV-7 and Drs-PLA2 with anticancer potential have also been reported from RVV (Mukherjee, 2014). Russell's viper venom induced cytoskeletal changes though small GTPases was reported earlier from this laboratory. RVV also was found to down regulate the anti-apoptotic genes in cultured A549 lung carcinoma cells. Significant change in F- actin distribution, leading to severe morphological changes was noticed in A549 cells exposed to whole Russell's viper venom (Pathan et al., 2015). In the present study, we characterized a protein toxin purified from RVV that could mimic almost all the cytotoxic activities of Russell's viper venom reported earlier by our group. The present article describes one of the major apoptosis inducing toxins purified and named Daboialectin from Indian Russell's viper venom.

2. Material and methods

2.1. Chemicals

Dry pooled venom of Russell's viper (RVV) was purchased from Calcutta Snake Park, Kolkata, India. All the general laboratory chemicals were purchased either from Sigma-Aldrich (USA), Bioline (India) or from Invitrogen (USA). CM Sephadex C-50 was purchased from Sigma-Aldrich, USA. A Chrono-Log Whole Blood aggregometer was purchased from purchased from Chronolog Corporation, USA. Molecular weight markers were purchased from Bangalore Genei Pvt. Ltd., India. Cell culture media, fetal bovine serum (FBS) and Antibiotics were bought from Cell One, USA or from Invitrogen, USA. Human broncho-alveolar carcinoma derived (A549) cell lines were obtained from National Center for Cell Science (NCCS), Pune India. PCR primers for reverse transcriptase PCR experiments were synthesized by Integrated DNA Technologies, USA. cDNA synthesis kit was purchased from Thermo Fisher, USA. Hsp70 mAB and Hoechst 33342 was purchased from Sigma-Aldrich (USA). Small GTPase (Rac1, RhoA and Cdc42) mAB were purchased from Cell Signalling Technology, Inc. USA. For cell culture all the

* Corresponding author. Department of Biological Sciences, BITS Pilani, K K Birla Goa Campus, Zuarinagar, Goa 403726, India.
E-mail addresses: dibakarchakrabarty@goa.bits-pilani.ac.in, diba27@yahoo.com (D. Chakrabarty).

http://dx.doi.org/10.1016/j.toxicon.2016.12.013
0041-0101/

CURRICULUM VITAE OF JIGNI PATHAN

Jigni Pathan

Research Scholar
Department of Biological Sciences,
Birla Institute of Technology and Science Pilani,
K K Birla Goa Campus. N.H. 17B,
Zuarinagar-403726, Goa, India.

ACADEMIC QUALIFICATIONS

M.Sc. (2010) (Medical Biotechnology)	**:**	Manipal Life Sciences Centre, Manipal University, Manipal, Karnataka. **Dissertation**: ***2-Deoxy-D-Glucose induced modulation of genetic damage in irradiated cell lines***
B.Sc. (2007) (Zoology-Biotechnology)	**:**	Carmel college, Nuvem, Margao, Goa.

PRESENT RESEARCH

Currently working as Senior Research Fellow at Department of Biological Sciences, BITS Pilani, K K Birla Goa Campus, Zuarinagar, Goa, under the supervision of Dr. Dibakar Chakrabarty and co-supervisor Dr. Angshuman Sarkar, project entitled ***"Studies on the effects of Russell's viper venom on human alveolar cancer cell lines and the involvement of small GTPases"***

RESEARCH EXPERIENCE

- Worked and presented a project during Masters Degree entitled ***"2-Deoxy-D-glucose induced modulation of genetic damage in irradiated cell lines in cultures"*** under the supervision of Dr. P.M. Gopinath, Department of Biotechnology, Manipal Life Sciences Centre, Manipal University, Manipal - 2010.
- Worked and presented a project during Bachelors degree entitled ***"Preliminary study of skin mycoflora in gardeners of Goa"*** under the supervision of Dr. Manoj R. Borkar, Biodiversity Research Cell, Carmel College For Women, Nuvem Goa -2007.

CURRICULUM VITAE OF DIBAKAR CHAKRABARTY

Name: **Dibakar Chakrabarty**

Associate Professor

Current Affiliation: Department of Biological Sciences, Birla Institute of Technology and Science Pilani, K K Birla Goa Campus. N.H. 17B, Zuarinagar-403726, Goa.

Academic Records:

M.Sc. in Physiology (1985), University of Calcutta, India

Ph.D., Jadavpur University (1993) for thesis entitled "Studies on Haemorrhagic Principle of Russell's Viper venom"

Currently engaged in teaching Animal Physiology, Developmental Biology and Reproductive Physiology as theoretical courses. Teaching Experimental techniques as laboratory based course for post-graduate biotechnology students.

Major research interest: Animal toxins as drug leads

Publications

Book Chapters

- Dibakar Chakrabarty and Chandrasekhar Chanda (2015) **Snake Venom Disintegrins in the Snake Venoms** (DOI 10.1007/978-94-007-6648-8_14-1).
- Dibakar Chakrabarty and Akriti Rastogi. (2015) **Hemotoxic Activity of Jellyfish Venom in Handbook of Clinical Toxinology in the Asia-Pacific and Africa,** Chapter 26, pp. 539-552 (Springer Handbooks of Toxinology, ed. P. Gopalakrishnakone).
- Dibakar Chakrabarty and Angshuman Sarkar (2016). **Cytotoxic Effects of Snake Venoms.in the Snake Venoms** (Springer Handbooks of Toxinology, ed. P. Gopalakrishnakone).DOI : 10.1007/978-94-007-6648-8_34-1

Research Articles

1. Jigni Pathan, Sukanta Mondal, Angshuman Sarkar and **Dibakar Chakrabarty**. **Daboialectin, a C-type lectin from Russell's viper venom induces cytoskeletal damage and apoptosis in human lung cancer cells in vitro**.**Toxicon,** 2017. 127 11-21.
2. Geetanjali Ravindran, **Dibakar Chakrabarty** and Angshuman Sarkar . **Cell Specific Regulation of p53 mRNA Expression Induced by Cadmium. Animal Cells and Systems** (Taylor & Francis).DOI: 10.1080/19768354.2016.1267041.
3. Chandrasekhar Chanda, Angshuman Sarkar and **Dibakar Chakrabarty**. **Novel thrombolytic protein from Cobra venom with Anti-Adhesive Properties.** Archives of Biochemistry and Biophysics, 2016. 590,20-26.
4. Jigni Pathan, Ansie Martin, Rajdeep Chowdhury, **Dibakar Chakrabarty*** and Angshuman Sarkar**. Russell's Viper Venom Affects Regulation Of Small Gtpases and Causes Nuclear Damage. Toxicon** doi.10.1016/j.toxicon.2015.10.011.
5. Geethanjali Ravindran, **Dibakar Chakrabarty** and Angshuman Sarkar. **Cadmium toxicity alters mRNA expression of Rho-like genes in A549 cell line.** Journal of Cell and Molecular Biology 2013. 11(1&2):13-20.
6. Chanda Chandrasekhar, Sarkar Angshuman, Sistla Srinivas, **Chakrabarty Dibakar** (2013)**Anti-Platelet Activity of A Three-Finger Toxin (3ftx) From Indian Monocled Cobra (Naja kaouthia) Venom.** Biochem Biophys Res Commun. 2013 Nov 22; 441 (3):550-4. Doi: 10.1016/J.Bbrc.2013.10.125.
7. Akriti Rastogi, Sumit Biswas, Angshuman Sarkar, and **Dibakar Chakrabarty Anticoagulant activity of Moon jellyfish (Aurelia aurita) tentacle extract**. **Toxicon.** 2012. 60(5):719-23.
8. C. Chandra Sekhar and **Dibakar Chakrabarty**. **Fibrinogenolytic Toxin from Indian Monocled Cobra (Naja kaouthia) Venom.** J. Biosci. 2011. 36, 355–361.
9. **Dibakar Chakrabarty**, Kausiki Dutta, Antony Gomes and Debasish Bhattacharyya. **Haemorrhagic protein of Russell's viper venom with fibrinolytic and esterolytic activities**. Toxicon, 2000. 38, 1475-1490.
10. Labanyamoy Kole, **Dibakar Chakrabarty**, Kaushiki Dutta and Debasish Bhattacharyya. **Purification and partial characterisation of an organ specific**

haemorrhagic toxin from Vipera russelli russelli venom. Indian J. Biochem. Biophys. 2000. 37, 114-120.

11. **Dibakar Chakrabarty**, Debasish Bhattacharyya, H.S. Sarkar and Sites C.Lahiri. **Purification and partial characterisation of a haemorrhagin (VRH-1) from Vipera russelli russelli venom**. Toxicon, 1993. 31, 1601-1614.
12. **Dibakar Chakrabarty**, Antony Gomes, Sites C.Lahiri and Asish K.Nag Chaudhuri. **Lethal and Haemorrhagic activity of Russell's viper venom—Neutralization by polyvalent, monovalent and toxoid antiserum**. Indian J. Exp. Biol., 1991. 29, 456-459.

CURRICULUM VITAE OF ANGSHUMAN SARKAR

Name: **Angshuman Sarkar**

Associate Professor

Current Affiliation: Department of Biological Sciences, Birla Institute of Technology and Science Pilani, K K Birla Goa Campus. N.H. 17B, Zuarinagar-403726, Goa,

Academic Records:

Doctoral Research (1999-2004)

Subject: Biotechnology.

Place: Department of Biotechnology, University of Pune, Pune, India.

Supervisor: Prof. Jayanta K Pal, Ph. D., FNASc., FMASc.

Thesis Title: The heme-regulated eukaryotic initiation factor 2alpha kinase: Expression and regulation of protein synthesis in human cells *in vitro* during heat-shock and lead toxicity.

Post Doctoral Research Experience (From 2004 till 2009 in USA)

- Cold Spring Harbor Laboratory, New York, USA, Molecular Developmental Biology.
- Case Western Reserve University; School Micro Biology and Molecular Biology, Cleveland, OH, USA.
- Department of Cancer Biology, Cleveland Clinic Foundation, Cleveland, OH, USA; 2008.

Carrier Highlights:

- ✓ Awarded fellow from All India Society of Cell Biology, 2016.
- ✓ Awarded 'Young Investigator' Pilot Project Grant by DBT 'Cancer Biology Mission' in 2015.
- ✓ Awarded "Appreciation Certificate" by Memorial Sloan Sloan-Kettering Cancer Center, USA, in 2008 for outstanding leadership and commitment in the Cancer Research in 2009.

- ✓ Awarded Research Associateship by Department of Cell and Molecular Biology [CWRU, Cleveland, USA in 2007.
- ✓ Offered a Fellowship by Cold Spring Harbor Laboratory (CSHL), New York, USA for Post Doctoral Training in 2004.
- ✓ Published a paper in one of the high ranking Cell Press Journal about the role of some proteins in maintaining germ line stem cells niche in 2007, this paper was subsequently selected as top 20 cited papers in USA for the year 2007.
- ✓ Received CSIR, SRF by Govt. of India, New Delhi in 2002.

Publications:

Research Articles

1. Jigni Pathan, Sukanta Mondal, **Angshuman Sarkar**, Dibakar Chakrabarty, **Daboialectin, a C-type lectin from Russell's viper venom induces cytoskeletal damage and apoptosis in human lung cancer cells in vitro** Toxicon 127 11-21 2017.
2. G. Ravindran, D. Chakrabarty and **A. Sarkar**. **Cell specific regulation of p53 mRNA expression induced by cadmium.** *Animal Cells and Systems* (TACS) (Accepted in Dec. 2016).
3. C. Chanda, **A. Sarkar**, D. Chakrabarty, Thrombolytic protein from cobra venom with anti-adhesive properties. ***Arch. Biochem Biophys***. **2016 Jan 10; 590:20-26**.
4. J. Pathan, A Martin, R. Chowdhury, D. Chakrabarty **A. Sarkar, Russell's viper venom affects regulation of small GTPases and causes nuclear damage.** *Toxicon*. 2015 Dec 15;108:216-25.
5. G. Ravindran, D. Chakrabarty and **A. Sarkar**; **Cadmium toxicity alters mRNA expression of Rho-like genes in A549 cell line.** *Journal of Cell and Molecular Biology*, Haliç University Press Publication, Vol. 11 (1 & 2):13-20, 2013.
6. C. Chanda, A**. Sarkar**, S. Sistla, and D. Chakrabarty; **Anti-platelet activity of a three-finger toxin (3FTx) from Indian monocled cobra (*Naja kaouthia*) venom.** *Biochem Biophys Res Commun*. 2013 Nov 22; 441 (3):550-4. doi: 10.1016/j.bbrc.2013.10.125. Epub. 2013 Oct 30.

7. **Sarkar A**., Ravindran G. and Krishnamurthy V., **A brief review on the effect of cadmium toxicity: from cellular to organ level.** International Journal of Bio-Technology and Research (IJBTR) ISSN 2249-6858 Vol. 3, Issue 1, 2013, 17-36.
8. Santimano M.C., Martin A., Kowshik M. and **Sarkar A**. **Zinc Oxide Nanoparticles Cause Morphological Changes in Human A549 Cell Line Through Alteration in the Expression Pattern of Small GTPases at mRNA Level.** *J. Bionanosci*. 7, 300-306, 2013.
9. A. Rastogi, S. Biswas, **A. Sarkar** and D. Chakrabarty. **Anticoagulant activity of Moon jellyfish (Aurelia aurita) tentacle extract.** *Toxicon. 2012* Oct; 60 (5):719-23.
10. R. K. Sinha, K.P.Krishnan and **A Sarkar**, **Heat shock response as a cue for phenotypic variability: A study from the psychrotrophic and mesophilic strains of Cellulosimicrobium cellulans;** *Annals of Microbiology*, (DOI 10.1007/s13213-011-0411-6) 2012.
11. Benjamin B Parrott, Yuting Chiang, **A. Sarkar**, A. Hudson, A. Guichet and C. Schulz, **The Nucleoporin98-96 function is required for transit amplification divisions in the germ line of Drosophila melanogaster.** *PLoS One*. 2011;6(9).
12. **A. Sarkar**, N, Parikh, S. Hearn, M. T. Fuller, S. Tazuke and C. Schulz, **Antagonistic roles of Rac and Rho in organizing the germ cell micro-environment.** *Current Biology*, Vol. 17, 1253-1258, July 17, 2007* **['Top 20 paper in USA' selected in the year 2007]**.
13. **A. Sarkar** and C. Schulz. **An approach for Immufluoroscence of Drosophila S2 cells.** *CSHL Protocols*, Vol. 2; Issue. 6; 2007.
14. S. Salokhe, **A. Sarkar**, A. Kulkarni, S. Mukherjee and J. K. Pal, **Flufenoxuron, an acylura insect growth regulator, alters development of Tribolium castaneum (Herbst) (coleoptera: tenebrionidae) by moulding levels of chitin, soluble protein content, and HSP70 and p34cdc2 in the larval tissue.** *Pesticide Biochem & Physiology*, **Vol. 85**, **84-90, 2006**.
15. **A. Sarkar**, A. Kulkarni, S. Chattopadhyay, D. Mogare, K. K. Sharma, K. Singh, J. K. Pal, **Lead-induced upregulation of the heme-regulated eukaryotic initiation factor 2α kinase is compromised by hemin in human K562 cells.** *BBA-Gene structure and expression*, 1732, 15-22, 2005.

16. **A. Sarkar**, S. Chattopadhyay, R. Kaul and J. K. Pal, **Lead exposure and heat shock inhibit cell proliferation in human HeLa and K562 cells by inducing expression and activity of the heme-regulated eIF-2α kinase.** *Journal of Biochemistry, Molecular biology and Biophysics*, Vol. 6 (6), 391-396, December 2002.
17. J. K. Pal., **A. Sarkar**., and B. Katoch, **Detergent mediated destaining of Coomassie Brilliant Blue –stained SDS polyacrylamide gels.** *Indian Journal of Experimental Biology*, Vol. 39, January 2001, 95-97, 2001.

Book chapter

1. Dibakar Chakrabarty and **Angshuman Sarkar, Cytotoxic Effects of Snake Venoms,** *Springer Science*, Business Media Dordrecht P. Gopalakrishnakone et al. (eds.), Snake Venoms Toxinology, 2016, DOI 10.1007/978-94-007-6648-834-1.

EXAMINER'S COMMENTS

Reviewer 1: Professor Amal Kanti Bera

Comment: There is serious lack of addressing the under lying mechanisms of RVV induced cell death. Following the exposure of RVV, there could be changes in the expression of N no genes. The claim that the alteration of small GTPases or some other proteins is the reason for RVV-induced cytoskeleton remodelling/cell death have not been supported by any experiment.

Response: In the present study, the proposed mechanism of cell death by RVV was explained in the conclusion section with figures. However to support our hypothesis on cell death, various parameters have been monitored in the present study. It is well known that small GTPases and regulatory proteins are the signature proteins for the cytoskeleton remodelling (Porter AP, Papaioannou A, Malliri A. Deregulation of Rho GTPases in cancer. Small GTPases. 2016 Jul 2;7(3):123-38.). Effects of RVV on the cytoskeleton remodelling in A549 cells was assessed by Phalloidin-FITC. Whereas, to understand the cell death by RVV on A549 cells, apoptosis was assessed by using three different methods as each of these assays have their own merits - like fluorescence microscopy using ethidium bromide and acridine orange dual staining technique is highly useful to differentiate necrotic cells from apoptotic cells after various treatments. Whereas, Hoechst 33342 assay determines the apoptotic cells. This gives a quantitative measure of total apoptotic cells. However, a qualitative analysis such as DNA fragmentation pattern ladder assay which is also considered as a gold standard gives a much more confirmatory picture. The details of the all the procedures were explained in the chapter no 2 under section 2.5 and 2.6. Similarly, these end points for detection of apoptosis in various cell lines were also used earlier and also the potential of these assays were convincingly described in the following paper as well.

1. Peng L, Liu JJ. A novel method for quantitative analysis of apoptosis. Lab Invest. 1997 Dec;77(6):547-55. Review.

Comment: Why the effect of RVV was tested only one cell line? It would have been interesting to check IC50 of RVV on cancerous vs. non –cancerous cell lines.

Response: As indicated by the reviewer, information on the IC50 evaluation on normal cell lines was not performed in the present study. But the cytotoxic effects on normal cell lines was monitored on HEK cell lines by MTT assay at the tested concentrations (2.5µg and 5µg/ml for 24 and 48 hrs). It showed no significance in the cell kill when compared with untreated cells indicating no cytotoxic effect on normal cell lines at above tested concentration (data not shown). Snake venoms were found to be less toxic to normal cells than to cancer cells. The toxic effect on cancer cells may be due to specific binding of proteins or peptides to cell membranes receptors and can readily penetrate into cancer cells by disruption of the phospholipid membrane structure and accumulate in lysosomes and internalized through a non-canonical pathway to produce pathology. The internalization of cytotoxins likely explains the higher sensitivity of some tumor cells to cytotoxin-induced toxicity compared to normal human cells. However, the mechanism of their entry into the cytosol is still unclear. Although it is thought that the disruptive action of these toxins on membrane phospholipids is the probable mechanism. On the other hand, cytotoxins can completely disturb ATP-dependent metabolic processes in the cell. They have also been observed to enter nucleus and produce apoptotic effects through binding with DNA. (Gasanov SE, Dagda RK, Rael ED. Snake venom cytotoxins, phospholipase A2s, and Zn2+-dependent metalloproteinases: mechanisms of action and pharmacological relevance. Journal of clinical toxicology. 2014 Jan 25;4 (1):1000181)

Comment: Most results are presented without proper explanation. Some results are not presented in proper scientific format.

Response: As advised by the reviewer, necessary corrections are incorporated in the revised chapters.

Comment: Semi- quantative PCR is not a right way of quantifying mRNA level. One needs to do real time PCR for that one would expect more detail and extensive work in a PhD thesis.

Response: With the earlier advent of RT-PCR, the sensitivity for mRNA determination has been increased dramatically, and this technique is becoming widely used. In the present study, due to unavailability of Real Time PCR in our institution (during the course of this

work) and also based on earlier studies, semi-quantitative PCR was used in quantifying mRNA levels.

- Zamorano PL, Mahesh VB, Brann DW. Quantitative RT-PCR for neuroendocrine studies. A minireview. Neuroendocrinology. 1996 May;63(5):397-407. Review.
- Marone M, Mozzetti S, De Ritis D, Pierelli L, Scambia G. Semiquantitative RT-PCR analysis to assess the expression levels of multiple transcripts from the same sample. Biol Proced Online. 2001 Nov 16;3:19-25.

Comment: Following RVV exposure there was a change in expression of Rho, Rac and Cdc42. There was a change in cell morphology and cytoskeleton. As Rho, Rac and Cdc42 are associated with the maintenance of cytoskeleton integrity, it was concluded that therefore it is the first report that confirms the involvement of RVV causing a change in cell morphology through regulation of small GTPases (page no 85). This is not acceptable unless it is substantiated with proper experiment. For example, IC50 of RVV can be determined on A549 cells after over- expression and knocking down these GTPases. If there is a shift of IC50 of RVV that could confirm if indeed small GTPases are involved or not. IC50 values mentioned in the discussion (3rd line from the bottom page 85) should be other way round.

Response: We agree with reviewer's opinion on the over-expression and knocking down the GTPases before evaluating IC50. But based on earlier studies, Small GTPases normally show over expression in the cancer cells (Porter AP, Papaioannou A, Malliri A. Deregulation of Rho GTPases in cancer. Small GTPases. 2016 Jul 2;7(3):123-38.), based on these evidences IC50 was calculated with further expression of Small GTPases.
As suggested by the reviewer the following points were included in the revised manuscript.
The sentence on page no 85 is now modified accordingly as suggested.
The IC50 values mentioned in the discussion (3rd line from the bottom page 85) is now corrected.

Comment: Fig. 2.11B, 2.12B, 2.13B and in many figures like this, I failed to understand how the normalization is done. It is expressed as fold control. If it is so, the value should be 2,3,4 etc. meaning 2,3,or 4 times more than control. However, the values are like 160, 170, etc. Which essentially means 160 or 170 times more than control, which is definitely not the case. I assume the control value is normalized to 100. In that case fold control is completely misleading. Ideally, if the control value is normalized to 100, there should not be any error

bar on control. However, in every fig control value is more or less than 100 with an error bar. How?

Response: In the present study, densitometric analysis from agarose gel was used to measure expression levels and intensities of specific bands, using Image J software. The analysis was performed as explained by the user guide lines and manufactures protocol. The observations from the figures 2.11B, 2.12B, 2.13B, etc., explains the amount of expression of mRNA present in the test and control group.

The values obtained from the above Software Image analysis were further statistically analysed to find out any significant change between the controls and treated group. The analysis was performed three different times independently and the mean and SEM was calculated.

Similarly, densitometry analyses from agarose gel were also used earlier in the following paper as well.

- Gassmann, M., Grenacher, B., Rohde, B. and Vogel, J. (2009). Quantifying western blots: pitfalls of densitometry. Electrophoresis 30, 1845-1855. doi: 10.1002/elps.200800720
- Tan, H. Y. and Ng, T. W. (2008). Accurate step wedge calibration for densitometry of electrophoresis gels. Optics Communications 281, 3013-3017.

Comment: A 18.5 KD protein, purified from the RVV was found to possess the cytotoxicity effect exhibited by the whole RVV. As it is a big protein, it is not likely to cross the lipid bilayer. How is it altering the gene expression of small GTPases and other proteins? Obiviously, it is triggering some signalling cascade by interacting with some membrane bound receptor/protein, which has not been discussed. Although this point has been touched in Fig.3 (page no 147), it depicts the functional activation- cascade but not the alteration of gene expression, is there any cross-talk between ROS generation and Rho activation?

Response: I agree with the reviewer's concern about the permeability of 18.5 KD protein purified from the RVV. Several mechanisms have been proposed about the various ways in which proteins associate with the lipid bilayer (Molecular Biology of the Cell. 4th edition.). In the present study, the exact mechanism of association and transportation of the Daboialectin protein was not assessed.

The issue raised by the reviewer about the involvement of ROS and GTPases is well taken and has been incorporated in the Review of literature section chapter 1 page no 25.

Comment: In the Fig.3 (page 147), one should be Rho-GTP and another Rho-GTP and another Rho-GDP. Both are written as GDP.

Response: Comments are well taken; the correction in the above figure was made accordingly in the revised portion in the thesis.

Comment: It is surprising that one purified protein mimicking the whole crude RVV in terms of its toxic effects. How is it possible? The changes in the cytoskeleton, alteration of gene expression, induction of apoptotic gene expression, all parameters studied are almost same in both whole RVV-treated as well as the purified protein treated cells. It gives an impression that this is the only toxic protein present in crude RVV, which is obviously not. One would expect that some properties of crude RVV will be carried by the purified protein but not all. The cell death caused by crude RVV is a cumulative effect of many proteins and peptides. What about MMPs? They can also do the similar changes in cell morphology.

Response: In the present study, the preliminary findings of using RVV as whole extract on A549 cell lines have shown the anti-cancer efficacy of RVV. The further isolation and identification of the active components in the various fractions and the purity of the each active principle were analyzed.

As explained in the chapter 3 under section 3.3.2, screening for cytotoxicity, all the tested fractions have shown little or no toxicity against A549 cells. Among all tested fractions Daboialectin proved to possess significant cytotoxic potential and therefore, all further assays in the present study were performed by using Daboialectin only.

The cytotoxic effect rendered by RVV was at concentration of 2.5 to 5 µg/mL, whereas, the purified compound from whole RVV extract has shown similar observations like RVV at concentration of 50 and 100 nM, indicating that the anticancer potential of RVV on A549 cells is may be due to Daboialectin.

Comment: In fig 3.1, the peaks are not numbered, Page 134, the IC50 values are written wrongly. 164ug/ml should be for 24 hrs. In fig. 4.6 it is expressed in nM. Why in two different ways in two different places? What kind of statistical test has been performed for comparison? Only P values are written.

Response: We regret for the typographical error, the correction of IC50 has been incorporated. Student's t-test was used in the present study for comparison of two independent groups. For all tests, $p < 0.05$ was considered statistically significant, the details about the statistics used in the present study was given in the page no 114.

Reviewer 2: Professor S.B Deshpande

Chapter1: In this chapter introduction and review of literature are presented. This is the weak part of the thesis. The literature survey is not focused to the methods or the topics under investigation. First 2-3 pages are reductant and figures 1.1 and 1.2 are not referenced. Candidate has to present the source for these figures to the research committee. In page 6, last para the % value of the composition of snake venom is not clear. Is it the % of solids or total % weight of the venom? Figure 1.12 is not made by the candidate and has been taken from some source hence source has to be identified.

The introduction part is not clear, why this work is under taken? What are lacunae in the research topic of work?

Response: As advised by the reviewer necessary changes were made in the introduction section. Regarding the work undertaken details are explained in the Aims and objectives section Chapter 1 page no 33.

About the lacunae in the research topic of work was explained in details under the section "Gaps in existing research" Chapter 1 page no 31.

As suggested by the reviewer, source for the figures 1.1 and 1.2 are now included in the revised manuscript. Details about the snake venom composition are also now modified accordingly, as suggested. All the other corrections suggested were incorporated accordingly in the respective lines of revised manuscript.

Chapter 2: In this chapter the cytotoxicity effects of RVV are evaluated on A549 cell lines using trypan blue staining method and resazuring fluorescent staining techniques. RVV produced dose and time dependent toxicity on these cell lines. Caspases activity and DNA fragmentation were assayed. The results indicated increased pro-apoptotic cdc42 and decreased anti apoptotic Hsp70 pathways. Further, expression of mRNA for Rac1 and RhoA(bindicators of small GTPase activity) was decreased after RVV exposure.

In this section, two important points require to be clarified by the candidate. Firstly, the toxic effects of RVV was restricted to cancer cells but what are the toxic effects RVV on normal (non- cancerous epithelial) cells? Secondly, the A549 cell line are mentioned as human alveolar adenocarcinoma cells in the thesis but these are human pulmonary epithelial cell lines in literature which is correct?

Response: As suggested by the reviewer the study of cytotoxic effect on normal cell lines was done by MTT assay on HEK cell lines at the tested concentrations (2.5µg and 5µg/ml for 24 and 48 hrs each). No significant cell kill was observed when compared with untreated cells indicating no cytotoxic effect on normal cell lines at above tested concentration.

A549 cell line was first developed in 1972 by D.J. Giard, *et al*., through the removal and culturing of cancerous lung tissue in the explanted tumor of a 58-year-old Caucasian male. The cells produced were adenocarcinomic alveolar basal epithelial cells with a modal chromosome number of 66.

Chapter 3: Purification of RVV is dealt with in this chapter. Purification was done using ion exchange chromatography. The peaks shown in Fig 3.1 are not clear. Fig 3.2 is not clear. Only one control was available in this series and toxicity on cells is not clearly visible. Other methods are fine for the molecular weight determination and sequencing of the toxin are fine.

Response: As suggested by the reviewer the figures 3.1 and 3.2 is now modified accordingly in the revised manuscript.

Chapter 4: In this toxicity of Daboialectin activity in reference apoptosis and small GTPases activity was assessed. Also the effect on ADP-induced platelet aggregation and wound healing was assessed. However, the effects of crude venom on the platelet aggregation and wound healing are not available. Why these parameters are included in this chapter and their interpretations are not clear.

Response: In the present study wound healing was performed to understand the migration ability of A549 cells after the treatment with Daboialectin. However, many such toxins also act on the various known and unknown receptors present on the platelet membranes. Binding of such toxins with these membranes may cause inhibition or activation of platelet aggregation. Cancer cell entry into the blood stream triggers platelet-mediated recognition and is amplified by cell surface receptors, cellular products, extracellular factors, and immune cells. In some cases, these interactions suppress immune recognition and elimination of cancer cells or promote arrest at the endothelium, or entrapment in the microvasculature, and survival. Therefore, Daboialectin was also assayed for platelet aggregation ability**.** (Platelets and cancer: a casual or causal relationship: revisited. David G. Menter, Stephanie C. Tucker, Scott Kopetz, Anil K. Sood, John D. Crissman, and Kenneth V. Honn. Cancer Metastasis Rev. 2014 Mar; 33(1): 231–269).

Summary and conclusions: this has summarized the observation further, the candidate has included the mechanisms underlying the effects are mentioned in figures 1-3. Are these original figures prepared by the candidate? At the end candidate has provided the future scope of the study.

Response: The figures shown in the summary and conclusion were prepared by self and were not taken from any source.

Overall this is a well planned study, performed with appropriate methods and the presentation is satisfactory. I recommend this thesis work for the award of Ph.D degree of the BITS, Pilani K.K Birla, Goa campus.

Reviewer 3: Dr. Dibakar Chakrabarty

This work has shown for the first time thrown light on the Russell's viper venom induced apoptosis in lung carcinoma cells.

The candidate has successfully purified a c-type lectin from RVV and could show that it is a very important apoptotic protein

The thesis also describes the mechanism involved in the apoptotic effects of c- type lectin

The text is well written, albeit some minor mistakes given below

Comment: Fig. 1.8 is to be bigger in scale

Response: As suggested figure 1.8 is now modified accordingly

Comment: RNA Quantification in pg.61 should be A260/A280

Response: As suggested RNA Quantification is now corrected accordingly

Comment: Fig on pg.79 Margin to be corrected

Pg 82, 84 figures margin to be corrected

Pg.142 should be anticancer potential

Response: As suggested by the reviewer all the above changes were made accordingly in the respective lines of the revised manuscript.

Reviewer 4: Dr. Angshuman Sarkar

No comments