University of Strathclyde

Strathclyde Institute of Pharmacy & Biomedical Sciences

Effect of *Crotalus ruber ruber* and *Agkistrodon piscivorus leucostoma* venoms on the dissemination of A2780 ovarian cancer cells *in vitro*

by

Ibtisam Kaziri

A thesis presented in fulfilment of the requirements for

the degree of

Doctor of Philosophy

Supervisors

Dr. Valerie Ferro

Dr. Edward Rowan

Mrs. Louise Young

2017

Copyright Statement

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed: Ibtisam Kaziri Date:

ACKNOWLEDGEMENTS

I am deeply grateful to my supervisor Dr. Valerie Ferro, for her endless support and encouragement. This study would never have been completed successfully without her invaluable guidance and fruitful ideas. I was always impressed by her patience. She was truly outstanding in many respects.

My sincere gratitude and special thanks go to Professor David Flint, for his patient answers to my queries and generous assistance during this research.

Special thanks to Dr. Edward Rowan and Mrs. Louise Young for their cooperation and patience. I also wish to thank all of my colleagues (Sajjad, Sanad, and Lougin) for their help.

I would like to thank my parents, Gheith Kaziri, Fatma Mohammed, my husband, Ali Rafea, my brothers, Kald, Towfig and Wessam, my sisters Hoda and Euman and my daughters Ghadi, Gheida and my son Saleh. To them, I dedicate this thesis.

Table of Contents

Copyright Statement.....i

Acknowledgements.....ii

Table of Contents iii

List of Figures xii

List of Tables.....xviii

List of Abbreviations.....xix

Abstract.....xx

Introduction.....1

1.1 Ovarian cancer.....1

1.2 Metastasis of ovarian cancer.....2

1.3 Current treatment for ovarian cancer.....5

1.4 Integrins.....5

1.4.1 Classification of integrins.....6

1.4.2 Integrins expression in ovarian cancer.....7

1.5 Snake venom.....8

1.5.1Classification of snakes and their venoms.....8

1.5.2 Components of snake venoms.....9

1.5.3 Anticancer activity of snake venom.....9

1.5.4 Snake venom metalloproteinases (SVMPs).....10

1.5.4.1 SVMPs and cancer treatment.....11

1.5.5 Phospholipases (PLA2).....13

1.5.5.1 svPLA2 and cancer treatment.....14

1.5.6 L-Amino acid oxidase (LAAO) 16

1.5.6.1 SV-LAAO and cancer treatment.....16

1.5.7 Snake venom serine proteinases (SVSPs).....18

1.5.7.1SVSPs and cancer treatment.....18

1.5.8 Disintegrins.....18

1.5.8.1 Disintegrins and cancer treatment.....20

1.5.9 Snake C-type lectins proteins (Snaclec)..... 24

1.5.9.1 CLPs and cancer treatment.....24

1.5.10 Three finger toxins (3FTxs).....25

1.5.10.1 3FTxs and cancer treatment.....26

1.5.11 Snake Venom Cysteine-Rich Secretory Protein (CRISP).....27

1.6 Snake venom studies.....27

1.6.1 *Crotalus ruber ruber* (Red diamond rattlesnake) snake venom.....26

1.6.2 *Agkistrodon piscivorus leucostoma* (Western Cottonmouth Moccasin) snake venom.....29

1.7 Project aims.....31

2.0 Materials and methods.....32

2.1 Materials.....32

2.2 Equipment.....34

2.3 Methods.....35

2.3.1 Tissue culture.....35

2.3.2Cell lines and growth conditions.....35

2.3.3 Cell culture.....36

2.3.4 Venom preparation.....36

2.3.5 Growth curve of A2780 ovarian cell line.....37

2.3.6 Effect of snake venom on the morphology of A2780 cells after 4h.....37

2.3.7 SYTOX®Green assay.....37

2.3.8 AlamarBlue® cytotoxicity assay. 38

2.3.9 Spreading of A2780 cells on plastic and different ECM proteins.....38

2.3.10 Alpha/beta integrin-mediated cell adhesion array.....39

2.3.11 Effect of function blocking integrin antibodies on A2780 cell adhesion.....40

2.3.12 Effect of snake venom on adhesion of A2870 cells to ECM and poly- L-lysine (an integrin-independent substratum).....40

2.3.13 Effect of snake venom on migration of A2870 cells.....41

2.3.14 Migration assay using a Cytoselect™ 24-well Cell Migration Assay.....41

2.3.15 Invasion assay using a Cytoselect™ 24-well Cell Invasion Assay.....42

2.3.16 Western blotting.....43

2.3.16.1 Preparation of whole cell extracts.....43

2.3.16.2 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).....44

2.3.16.3 Electrophoretic transfer of proteins to nitrocellulose membrane.....44

2.3.16.4 Immunological detection of protein.....44

2.3.17 Size exclusion chromatography.....45

2.3.17.1 Column packing and preparations.....45

2.3.17.2 Venom preparation.....45

2.3.17.3 Lyophilisation.....46

2.3.18 Fraction identification.....46

2.3.18.1 SDS-PAGE.....46

2.3.18.2 Nanoflow HPLC Electrospray Tandem Mass Spectrometry (nLC-ESI-MS/MS) 47

2.3.18.3 Liquid chromatography mass spectrometry (LC-MS).....47

2.3.18.4 Assessment of the metalloproteinase inhibitor ethylenediaminetetraacetic acid (EDTA) on activity of F3 from *Crotalus ruber ruber* venom.....48

2.3.18.5 Assessment of metalloproteinase inhibitor (1, 10-phenanthroline) on the activity of F6 of *Agkistrodon piscivorus leucostoma* venom.....48

3.0 Results.....49

3.1 Effects of *Crotalus ruber ruber* venom on cell adhesion, migration and invasion of A2780 cells.....49

3.1.1 Growth characteristics of the A2780 ovarian cancer cell line.....51

3.1.2 Growth curve for A2780 cells.....52

3.1.3 Effect of *Crotalus ruber ruber* venom on the morphology of A2780 cells after 4h.....53

3.1.4 Effect of *Crotalus ruber ruber* venom on membrane integrity of A2780 cells after 4h.....54

3.1.5 Effect of *Crotalus ruber ruber* venom on A2780 cell viability after 24, 48 and 72h.....54

3.1.6 Spreading of A2780 cells on plastic and various substrata.....57

3.1.7 Integrin expression by A2780 cells.....59

3.1.8 Effect of function blocking integrin antibodies on A2780 cell adhesion to fibronectin and vitronectin.....59

3.1.9 Effect of function blocking integrin antibodies on A2780 cell adhesion to poly-L-lysine.....63

3.1.10 Effect of *Crotalus ruber ruber* venom on adhesion of A2780 cells to vitronectin and fibronectin.....64

3.1.11 Effect of *Crotalus ruber ruber* on adhesion of A2780 cells to an integrin-independent substratum (poly-L-lysine).....64

3.1.12 Effect of *Crotalus ruber ruber* venom on migration of A2780 cells…….....68

3.1.13 Migration assay using a Cytoselect™ 24-well Cell Migration Assay.....68

3.1.14 Effect of *Crotalus ruber ruber* venom on invasion of A2780 cells across the basement membrane using a Cytoselect™ 24-well Cell Invasion Assay.....68

3.1.15 Effect of *Crotalus ruber ruber* venom on level of α5, β1, and αv integrins expressed by A2780 cells.....74

3.1.16 Fractionation of *Crotalus ruber ruber* venom by a size exclusion chromatography.....77

3.1.17 Gel electrophoresis.....77

3.1.18 Effect of fractions from *Crotalus ruber ruber* venom on the morphology of A2780 cells after 4h.....77

3.1.19 Effect of F3 on morphology of A2780 cells after 4h.....80

3.1.20 Effect of F3 on membrane integrity of A2780 cells after 4h.....81

3.1.21 Effect of F3 on A2780 cell viability after 24, and 48h.....81

3.1.22 Effect of F3 on adhesion of A2780 cells to vitronectin and fibronectin.....81

3.1.23 Effect of F3 on adhesion of A2780 cells to an integrin-independent substratum (poly-L-lysine).....86

3.1.24 Effect of F3 on migration of A2780 cells by using Cytoselect™ 24-well Cell Migration Assay.....87

3.1.25 Effect of F3 on invasion of A2780 cells across the basement membrane using Cytoselect™ 24-well Cell Invasion Assay.....87

3.1.26 Effect of F3 on level of α5, β1, and αv integrins expressed by A2780 cells.....87

3.1.27 Identification of the active peptide in fraction 3 from *Crotalus ruber ruber* venom.....94

3.1.27.1 SDS-PAGE for F3 of *Crotalus ruber ruber* venom.....94

3.1.27.2 Nanoflow HPLC Electrospray Tandem Mass Spectrometry.....94

3.1.27.3 Liquid chromatography mass spectrometry (LC-MS).....96

3.1.27.4 Assessment of the effect of metalloproteinase inhibitor (ethylenediaminetetraacetic acid) (EDTA) on activity of F3 of *Crotalus ruber ruber* venom……..97

3.2 Effects of *Agkistrodon piscivorus leucostoma* snake venom on cell adhesion, migration and invasion of A2780 cells.....100

3.2.1 Effect of *Agikistrodon piscivorus leucostoma* venom on the morphology of A2780 cells after 4h.....101

3.2.2 Effect of *Agkistrodon piscivorus leucostoma* snake venom on membrane integrity of A2780 cells after 4h.....102

3.2.3 Effect of *Agkistrodon piscivorus leucostoma* venom on A2780 cell viability after 24, 48 and 72h.....102

3.2.4 Effect of *Agkistrodon piscivorus leucostoma* venom on adhesion of A2780 cells to fibronectin and vitronectin.....105

3.2.5 Effect of *Agkistrodon piscivorus leucostoma* venom on adhesion of A2780 cells to an integrin-independent substratum (poly-L-lysine).....108

3.2.6 Effect of *Agkistrodon piscivorus leucostoma* venom on migration of A2780 cells.....110

3.2.7 Migration assay using Cytoselect™ 24-well Cell Migration Assay.....110

3.2.8 Effect of *Agkistrodon piscivorus leucostoma* venom on invasion of A2780 cells across the basement membrane using Cytoselect™ 24-well Cell Invasion Assay.....110

3.2.9 Effect of *Agkistrodon piscivorus leucostoma* venom on the level of α5, β1, and αv integrins expressed by A2780 cells.....116

3.2.10 Fractionation of *Agkistrodon piscivorus leucostoma* venom by size exclusion chromatography.....119

3.2.11 Gel electrophoresis.....119

3.2.12 Effect of F6 from *Agkistrodon piscivorus leucostoma* venom on the morphology of A2780 cells after 4h.....121

3.2.13 Effect of F6 of *Agkistrodon piscivorus leucostoma* venom on membrane integrity of A2780 cells after 4h.....122

3.2.14 Effect of F6 of *Agkistrodon piscivorus leucostoma* venom on A2780 cell viability after 24, and 48h.....122

3.2.15 Effect of F6 on adhesion of A2780 cells to vitronectin and fibronectin.....125

3.2.16 Effect of F6 on adhesion of A2780 cells to an integrin-independent substratum (poly-L-lysine).....125

3.2.17 Effect of F6 on migration of A2780 cells.....130

3.2.18 Effect of F6 on invasion of A2780 cells across the basement membrane.....132

3.2.19 Effect of F6 on level of expression of α5, β1, and αv integrins of A2780 cells.....134

3.2.20 Identification of active peptide(s) in F6 from *Agkistrodon piscivorus leucostoma* venom.....137

3.2.20.1 SDS-PAGE for F6 from *Agkistrodon piscivorus leucostoma* venom.....137

3.2.20.2 Nanoflow HPLC Electrospray Tandem Mass Spectrometry.....137

3.2.20.3 Liquid chromatography mass spectrometry (LC-MS).....139

3.2.20.4 Assessment of the effect of metalloproteinase inhibitor (1,10-phenanthroline) on activity of F6 of *Agkistrodon piscivorus leucostoma* venom.....140

4.0 Discussion.....143

4.1 *Crotalus ruber ruber* and *Agkistrodon piscivorus leucostoma* venom affect morphology of A2780 cells to prevent adhesion in different ways.....143

4.2 *Crotalus ruber ruber* and *Agkistrodon piscivorus leucostoma* venoms had no effect on viability of A2780 cells at specific concentrations that had an effect on adhesion of A2780 cells 145

4.3 A2780 cells showed a good spread on fibronectin using α5β1 integrins.....148

4.4 *Crotalus ruber ruber* venom inhibited the adhesion of A2780 cell to fibronectin, but not vitronectin and poly-L-lysine.....149

4.5 Both venoms had an inhibitory effect on migration and invasion of A2780 cells.....153

4.6 Five fractions from *Crotalus ruber ruber* venom and nine fractions from *Agkistrodon piscivorus leucostoma* venom by using a size exclusion chromatography.....155

4.7 The active peptide in F3 and F6 is rubelysin and leucostoma peptidase A respectively.....156

4.7.1 SDS-PAGE, Tandem Mass Spectrometry and LCMS.....156

4.7.2 The activities of F3 and F6 were abolished by EDTA and 1, 10- phenanthroline respectively.....158

5.0 Conclusion and future work.....159

References.....163

6.0 List of Figures:

Figure 1: A proposed model for intraperitoneal dissemination of ovarian cancer.....4

Figure 2: Integrin signalling during tumour progression……..6

Figure 3: Structure of three finger toxins.....26

Figure 4: *Crotalus ruber ruber* snake.....28

Figure 5: *Agkistrodon piscivorus leukostoma* snake.....30

Figure 6: Cell morphology of A2780 cells.....51

Figure 7: Growth curve of A2780 cells.....52

Figure 8: Effect of *Crotalus ruber ruber* venom on the morphology of A2780 cells after 4h.....53

Figure 9: Effect of *Crotalus ruber ruber* venom on A2780 cell membrane integrity after 4h.....55

Figure 10: Effect of *Crotalus ruber ruber* venom on viability of A2780 cells after 24, 48 and 72h.....56

Figure 11: Spreading of A2780 cells on plastic and ECM (collagen, fibronectin, and vitronectin)….....57

Figure 12: Spreading of A2780 cells on plastic and different extracellular matrix proteins.....58

Figure 13: α and β integrins expressed by A2780 cells.....60

Figure 14: Effect of function blocking integrin antibodies on A2780 cell adhesion to (A) fibronectin and (B) vitronectin.....61

Figure 15: Effect of function blocking integrin antibodies on A2780 cell adhesion to (A) fibronectin and (B) vitronectin. 62

Figure 16: Effect of function blocking integrin antibodies on A2780 cell adhesion to poly-L-lysine.....63

Figure 17: Effect of *Crotalus ruber ruber* venom on adhesion of A2780 cells in the presence of (A) fibronectin and (B) vitronectin…….....65

Figure 18: Effect of *Crotalus ruber ruber* venom on adhesion of A2780 cells to (A) fibronectin and (B) vitronectin.....66

Figure 19: Effect of *Crotalus ruber ruber* venom on adhesion of A2780 cells to poly-L-lysine.....67

Figure 20: Effect of *Crotalus ruber ruber* venom on migration of A2780 cells.....69

Figure 21: Effect of *Crotalus ruber ruber* venom on migration of A2780 cells.....70

Figure 22: Effect of *Crotalus ruber ruber* venom on migration of A2780 cells.....71

Figure 23: Effect of *Crotalus ruber ruber* venom on invasion of A2780 cells across the basement membrane.....72

Figure 24: Effect of *Crotalus ruber ruber* venom on invasion of A2780 cells across the basement membrane.....73

Figure 25: Effect of *Crotalus ruber ruber* venom on level of α5 (A) and β1 (B) integrins expressed by A2780 cells 75

Figure 26: Effect of *Crotalus ruber ruber* venom on level of αv integrins expressed by A2780 cells.....76

Figure 27: Separation of *Crotalus ruber ruber* venom using (A) size exclusion chromatography and (B1) SDS-PAGE of the crude venom (B2) SDS-PAGE of fractions.....78

Figure 28: Effect of *Crotalus ruber ruber* fractions on the morphology of A2780 cells after 4h.....79

Figure 29: Effect of F3 on the morphology of A2780 cells.....80

Figure 30: Effect of F3 of *Crotalus ruber ruber* venom on A2780 cell membrane integrity after 4h.....82

Figure 31: Effect of F3 on viability of A2780 cells after 24h (A) and 48h (B).....83

Figure 32: Effect of F3 of *Crotalus ruber ruber* venom on adhesion of A2780 cells to (A) fibronectin and (B) vitronectin.....84

Figure 33: Effect of F3 on adhesion of A2780 cells to (A) fibronectin and (B) vitronectin.....85

Figure 34: Effect of F3 on adhesion of A2780 cells in the presence of poly-L-lysine.....86

Figure 35: Effect of F3 of *Crotalus ruber ruber* venom on migration of A2780 cells.....88

Figure 36: Effect of F3 on migration of A2780 cells.....89

Figure 37: Effect of F3 on invasion of A2780 cells across the basement membrane.....90

Figure 38: Effect of F3 on invasion of A2780 cells across the basement membrane.....91

Figure 39: Effect of F3 on level of expression of (A) α5 and (B) β1 integrins of A2780 cells.....92

Figure 40: Effect of F3 on level of expression of αv integrins of A2780 cells.....93

Figure 41: SDS-PAGE for F3 of *Crotalus ruber ruber* venom.....95

Figure 42: Analysis of F3 by Liquid chromatography mass spectrometry (LC-MS) 96

Figure 43: Effect EDTA (metalloproteinase inhibitor) on the activity of F3 of *Crotalus ruber ruber* venom.....98

Figure 44: Effect of EDTA on activity of F3 of *Crotalus ruber ruber* venom after 24h.....99

Figure 45: Effect of *Agkistrodon piscivorus leucostoma* venom on the morphology of A2780 cells after 4h.....101

Figure 46: Effect of *Agkistrodon piscivorus leucostoma* venom on A2780 cell membrane integrity after 4h.....103

Figure 47: Effect of *Agkistrodon piscivorus leucostoma* venom on viability of A2780 cells after (A) 24, (B) 48 and (C) 72h.....104

Figure 48: Effect of *Agkistrodon piscivorus leucostoma* venom on adhesion of A2780 cells to (A) fibronectin and (B) vitronectin.....106

Figure 49: Effect of *Agkistrodon piscivorus leucostoma* venom on adhesion of A2780 cells to (A) fibronectin and (B) vitronectin.....107

Figure 50: Effect of *Agkistrodon piscivorus leucostoma* venom on adhesion of A2780 cells to poly-L-lysine.....108

Figure 51: Effect of *Agkistrodon piscivorus leucostoma* venom on adhesion of A2780 cells to poly-L-lysine.....109

Figure 52: Effect of *Agkistrodon piscivorus leucostoma* venom on migration of A2780 cells.....112

Figure 53: Effect of *Agkistrodon piscivorus leucostoma* venom on migration of A2780 cells.....112

Figure 54: Effect of *Agkistrodon piscivorus leucostoma* venom on migration of A2780 cells.....113

Figure 55: Effect of *Agkistrodon piscivorus leucostoma* venom on invasion of A2780 cells across the basement membrane.....114

Figure 56: Effect of *Agkistrodon piscivorus leucostoma* venom on invasion of A2780 cells.....115

Figure 57: Effect of *Agkistrodon piscivorus leucostoma* venom on level of (A) α5 and (B) β1 integrins expressed by A2780 cells.....117

Figure 58: Effect of *Agkistrodon piscivorus leukostoma* venom on level of αv integrins expressed by A2780 cells.....118

Figure 59: Separation of *Agkistrodon piscivorus leucostoma* venom using (A) size-exclusion chromatography and (B1) SDS PAGE of the crude venom and (B2) SDS-PAGE of the fractions.....120

Figure 60: Effect of F6 on the morphology of A2780 cells.....121

Figure 61: Effect of Fraction F6 on A2780 cell membrane integrity after 4h.....123

Figure 62: Effect of F6 on viability of A2780 cells after (A) 24 and (B) 48h.....124

Figure 63: Effect of F6 of *Agkistrodon piscivorus leucostoma* venom on adhesion of A2780 cells to (A) fibronectin and (B) vitronectin.....126

Figure 64: Effect of F6 on adhesion of A2780 cells to (A) fibronectin and (B) vitronectin.....127

Figure 65: Effect of F6 on adhesion of A2780 cells to poly-L-lysine.....128

Figure 66: Effect of F6 on adhesion of A2780 cells to poly-L-lysine.....129

Figure 67: Effect of F6 of *Agkistrodon piscivorus leucostoma* venom on migration of A2780 cells.....130

Figure 68: F6 of *Agkistrodon piscivorus leucostoma* venom inhibited the migration of A2780 cells.....131

Figure 69: Effect of F6 on invasion of A2780 cells across the basement membrane.....132

Figure 70: Effect of F6 on invasion of A2780 cells across the basement membrane.....133

Figure 71: Effect of F6 of *Agkistrodon piscivorus leucostoma* venom on level of expression of (A) α5 and (B) β1 integrins of A2780 cells.....135

Figure 72: Effect of F6 of *Agkistrodon piscivorus leucostoma* venom on level of expression of αv integrins of A2780 cells.....136

Figure73:SDS-PAGE for F6 of *Agkistrodon piscivorus leucostoma* venom.....138

Figure74: Analysis of F6 by Liquid chromatography mass spectrometry (LC-MS) 139

Figure 75: Effect of phenanthroline (metalloproteinase inhibitor) on activity of F6 of *Agkistrodon piscivorus leucostoma* venom after 24h.....141

Figure 76: Effect of phenanthroline (metalloproteinase inhibitor) on activity of F6 of *Agkistrodon piscivorus leucostoma* venom after 24h.....142

List of Tables

Table 1: PLA2 and anti-cancer activity.....15

Table 2: LAAO and anti-cancer activity.....17

Table 3: Disintegrins and anti-cancer effect.....23

Table 4: Scientific classification of *Crotalus ruber ruber* snake.....28

Table 5: Scientific classification of *Agkistrodon piscivorus leucostoma* snake.....30

List of Abbreviations

Angiotensin converted enzyme (ACE)

C-type lectins protein (CLPs)

Cysteine-Rich Secretory Protein (CRISP)

Dithiothreitol (DTT)

Epithelial mesenchymal transition (EMT)

Ethylenediaminetetraacetic acid (EDTA)

Extracellular matrix (ECM)

Foetal calf serum (FCS)

Fluorescence-activated cell sorting (FACS)

L-Amino acid oxidase (LAAO)

Liquid chromatography mass spectrometry (LC-MS)

Mg^{2+}-Ca^{2+} -free Hank's balanced salt solution (HBSS)

Mesenchymal epithelial transition (MET)

Phospholipases (PLA2)

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Snake venom metalloproteinases (SVMPs)

Snake venom serine proteinases (SVSPs)

Three finger toxins (3FTxs)

Abstract

Ovarian cancers are the second most common female cancer in the UK. In the majority of patients there are no observable symptoms of ovarian cancer at early stage and no effective screening strategy to detect cancer before metastasis. Moreover, chemo-resistance is another problem which hinders the successful treatment of ovarian cancer. Therefore, discovery of novel anti-ovarian cancer drugs remains critically important. Snake venoms contain different proteins, peptides, enzymes and low molecular weight components that have been investigated for development of new therapies to treat many diseases including cancer. The present study was carried out to evaluate the effect of *Crotalus ruber ruber* and *Agkistrodon piscivorus leucostoma* snake venoms both from the Viperidae family on dissemination and processes of metastasis of an ovarian cancer cell line (A2870). The first part of the project involved examination of changes to cell morphology, cytotoxicity, migration, invasion and the effect of the crude venom on levels of the main integrins involved in cell adhesion. Cell morphology differences were observed with both venoms; *Crotalus ruber ruber* crude venom caused the cells to round up so that they were poorly spread at 1.5 and 3.1µg/ml, while, *Agkistrodon piscivorus leucostoma* venom caused the cells to detach from the growing surface at 0.7, 1.5, and 3.1µg/ml. An AlamarBlue® Cell Viability Assay and SYTOX®Green (nucleic acid stain) were used to test the viability of the cells following treatment and to demonstrate that the morphological alterations were not due to a cytotoxic effect, but to anti-adhesive activity. Moreover, an integrin-independent substratum (poly-L-lysine) was used to determine if the venom is specific for the integrin family of adhesion molecules or not. As a result, the effect of *Crotalus ruber ruber* venom on

adhesion was found to be specific for the integrin family of adhesion receptors, while the effect of *Agkistrodon piscivorus leucostoma* venom on adhesion was not specific for the integrin family. Both venoms had an inhibitory effect on migration and invasion of the cells by inhibiting α5, β1integrins (fibronectin receptor) for *Crotalus ruber ru*ber venom and α5, β1integrins and αv integrins (fibronectin and vitronectin receptor) for *Agkistrodon piscivorus leucostoma* venom. The next part of the project was to semi-purify both venoms. This was achieved using gel chromatography to obtain faction 3 (F3) from *Crotalus ruber ruber* venom and F6 from *Agkistrodon piscivorus leucostoma* venom. The assays carried out with the crude venoms were repeated using the fractions instead. F3 of *Crotalus ruber ruber* venom and F6 of *Agkistrodon piscivorus leucostoma* venom were found to be the fractions that contained components responsible for the activity of the venoms. F3 had an inhibitory effect on the level of expression of α5, β1 integrins while F6 had an inhibitory effect on α5, β1 and on αv integrins. Moreover, it was proposed that F6 had hydrolytic activity against the extracellular matrix. Finally preliminary analysis was carried out on F3 and F6 using SDS-PAGE, Nanoflow HPLC Electrospray Tandem Mass Spectrometry, liquid chromatography mass spectrometry and metalloproteinase inhibitor studies to elucidate the peptides as rubelysin for F3 and leucostoma peptidase A for F6. These results suggest that rubelysin and leucostoma peptidase A have antimetastatic activity against ovarian cancer.

1. Introduction

Cancer

One of the major causes of death in the world (Jemal *et al*., 2011). Cancer is a malignant neoplasm and it develops due to uncontrolled growth and division of cells. In 2012, there were a total of 1.638.910 new cancer cases diagnosed in United States and approximately 577.190 deaths (Siegel *et al*., 2012). The 5 year survival rate has increased in the United States from 50% in 1976 to 68% in 2006 (American Cancer Society, 2012). In addition, in the USA the rate of death is 1 in 4 of cancer cases (Deepika and Sudhir, 2010). Cancer accounts for the second most common cause of death after heart disease in United States (Jemal *et al*., 2007).

1.1 Ovarian cancer

Ovarian cancer is one of the major causes of death amongst gynaecological cancers, because it is often discovered when cancer metastasis has occurred and that has a poor prognosis (Sabatier *et al*., 2011). It is the fifth leading cause of cancer death among USA women, with nearly 225,000 new cases and 125,000 deaths worldwide every year and is the second most common gynaecologic malignancy in the UK (Asher *et al*., 2011). Ovarian cancers classified as epithelial tumours account for more than 80% of cases, while other types are defined as germ and stromal cell tumours (Muccioli *et al*., 2012). In the majority of patients there are no observable symptoms of ovarian cancer at early stage and no effective screening strategy is available to date. As a result, ovarian cancer is identified at late stage with peritoneal metastasis and results in a low survival rate (Muccioli *et al*., 2012) and only 25% of patients are diagnosed at an early stage (Badgwell & Bast, 2007). There is a high degree of variation geographically; highest incidence of the disease is found in the

USA and Northern Europe, while lower incidence is found in Africa and Asia (Asher *et al*., 2011). Causes of ovarian cancer are unknown; however, the risk factors include older women who have never given birth, a family history of the disease, and women with endometriosis. It has been demonstrated that use of oral contraceptive pills, first pregnancy at early age, and last pregnancy at older age have a protective effect (Bandera, 2005). Genetic predisposition of ovarian cancer accounts for only 10% and mutations in BRCA1 and BRCA2 genes have been reported as a cause (Muccioli *et al*., 2012). The incidence of ovarian cancer also increases with age. Signs and symptoms include abdominal or pelvic pain, abdominal mass, abnormal vaginal bleeding, and weight loss (Smith *et al*., 2005). Patients with early stages of ovarian cancer have a good prognosis (5 year survival rate > 80%), while patients with metastatic disease have a significantly poor prognosis (20% of patients surviving 5 years). The overall survival rate is 30% (Breedlove & Busenhart, 2005).

1.2 Metastasis of ovarian cancer

The majority of patients (80%) are diagnosed with metastatic disease and only 20% are diagnosed at early stage (stage I); at this stage about 90% of the patients can be treated using cytoreductive therapy and chemotherapy (Badgwell and Bast, 2007). Ovarian cancer is different from other cancers in the way dissemination occurs because it is mainly spread through the abdominal cavity by seeding of the tumour cells within the peritoneal cavity (Figure 1) and rarely using the vascular system. Also it can spread by direct extension to adjacent organs such as the bladder or colon. The omentum and the peritoneum are the most common secondary sites of metastasis (reviewed by Lengyel, 2010). Ovarian carcinoma cells undergo a process of epithelial mesenchymal transition (EMT) before detaching from the primary site

and in this process the cells lose their E-cadherin, which is an important adhesion molecule between epithelial cells. This allows the cancer cells to become more aggressive and acquire a more invasive behaviour (Huber *et al*., 2006). Ovarian cancer cells at the metastatic site show low E-cadherin expression in comparison to cells at the primary site and this is associated with poor prognosis (Huber *et al*., 2006). Poor patient survival rate of ovarian cancer is also associated with absence of E-cadherin expression (Darai *et al.*, 1997). After detachment from the original site, the cancer cells are transported through the abdominal cavity and form spheroids to overcome anoikis. Anoikis is a form of programmed cell death that occurs when cells detach from the ECM. Cancer cells involve integrins to attach to the secondary site (mesothelium). After implantation, cancer cells undergo mesenchymal epithelial transition (MET) and migration, then invasion across the mesothelium into the underlying extracellular matrix (ECM) (Masoumi *et al*., 2012). It has been demonstrated that CD44 which is a cell receptor for hyaluronic acid also plays a role in adhesion of ovarian cancer cells to mesothelial cells (Cannistra *et al*., 1993). The matrix metalloproteinases (MMP) are family of enzymes whose activity has been implicated in a number of physiological and pathologic processes. Pathological condition includes tumour progression and metastasis. As a result, these proteases have come to represent important therapeutic and diagnostic targets for the treatment and detection of human cancers. Tumor cells overexpress proteases in order to degrade the basement membrane and invade the surrounding tissue (Curran and Murray, 1999). In ovarian cancer MMP-2, -9, and -14 are the most studied MMPs as biomarkers for ovarian cancer. MMP-9 activity was significantly increased in advanced ovarian cancers compared with benign tumors (Al-Alem, & Curry 2015).

After adhere ovarian cancer cells to mesothelial cells, the cancer cells up-regulate MMP-2, which then cleaves the fibronectin and vitronectin into fragments (Kenny *et al.*, 2008).

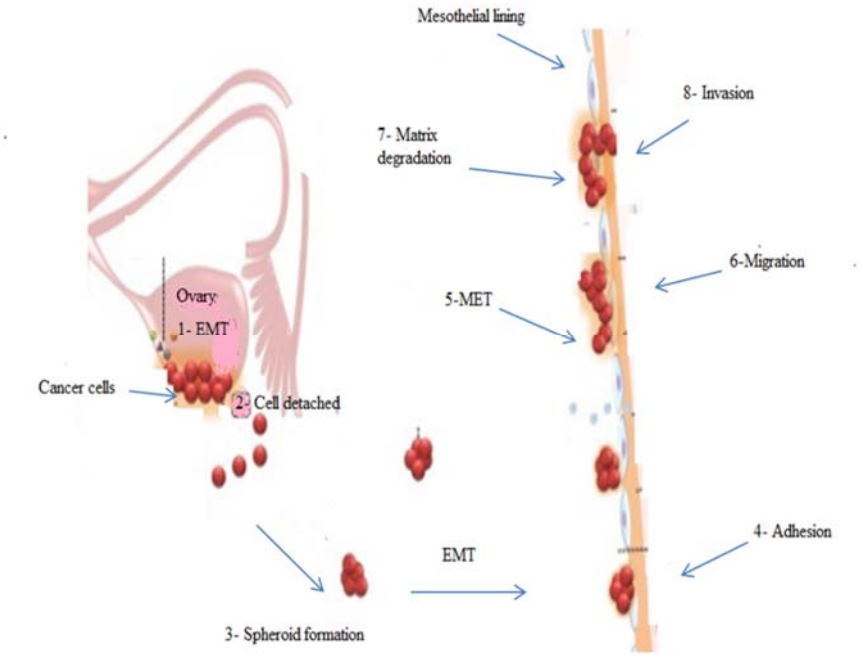


Figure 1: A proposed model for intraperitoneal dissemination of ovarian cancer (adapted from Masoumi *et al.*, 2012). Steps 1 to 8 indicate the process of intraperitoneal dissemination of ovarian cancer.

Abbreviations: EMT (epithelial mesenchymal transition); MET (mesenchymal epithelial transition).

1.3 Current treatment for ovarian cancer

There are no effective screening methods to detect ovarian cancer before metastasis occurs so there is an urgent need to prevent this process. The current treatment options include cytoreductive therapy and combination of platinum and taxane-based chemotherapy. In addition, outcomes are improved when no residual tumour is left during surgical cytoreduction. However, the current treatment fails to achieve complete relief (Bristow *et al*., 2002). Integrins are an important mediator between ovarian cancer and mesothelial cells that line the peritoneum (Casey *et al.,* 2001), so integrin inhibitors are believed to be a promising approach for treatment of ovarian cancer.

1.4 Integrins

Integrins are transmembrane receptors, which maintain cell-cell and cell-ECM interaction. They represent the major class of adhesion molecules. Integrins are heterodimers which are composed of alpha and beta subunits. In mammals, there are 18 alpha and 8 beta subunits, from which one alpha subunit combines with one beta subunit by noncovalent interactions to make up 24 different integrin combinations (Schaffner *et al*., 2013). Each integrin subunit has a large extracellular domain, a transmembrane domain, and a short cytoplasmic domain. The main functions of integrins are their role as adhesion receptors for extracellular ligands and activation of intracellular signalling, which is involved in cell adhesion, migration, growth, and progression of tumours (Figure 2) (Aoudjit & Vuori, 2012).

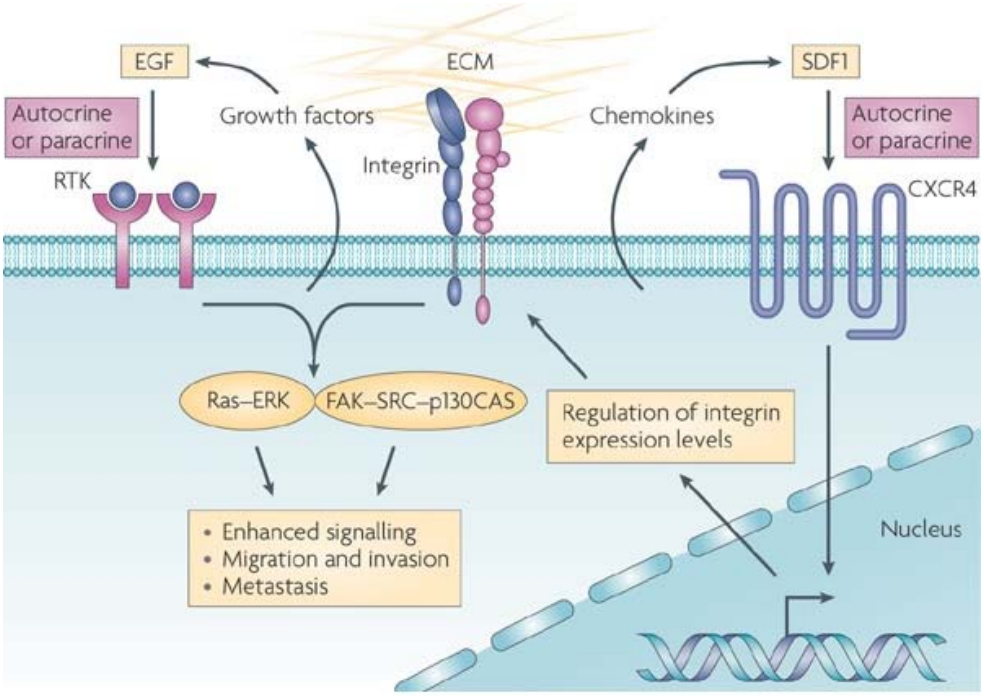


Figure 2:Integrin signalling during tumour progression (Desgrosellier and Cheresh 2010).

1.4.1 Classification of integrins

The binding of integrins to the ECM depends on the extracellular domain of the integrins. Integrins α1β1, α2β1, α10β1, and α11β1 are collagen receptor (Hynes, 2002). The major fibronectin receptors are α5β1, α8β1, αIIbβ3 and the αvβ1 integrins. In addition, the major laminin receptors are α3β1, α6β1, α6β4 and α7β1(van der Flier & Sonnenberg, 2001).

1.4.2 Integrins expression in ovarian cancer

Integrins are an important mediator between ovarian cancer cells and mesothelial cells that line the peritoneal cavity (Casey *et al.,* 2001). It has been shown that α5 integrin has an important role in peritoneal dissemination of ovarian cancer (Ohyagi-Hara *et al*., 2013). After detachment from the original site, the ovarian cancer cells float in ascites as single or multicellular spheroids and involve the integrins to attach to the secondary site. It has been demonstrated that α5β1integrin is responsible for spheroid formation of ovarian cancer cells and their adhesion at the secondary sites (Casey *et al*., 2001). These spheroids express α5β1integrin at a high level. Inhibition of α5β1integrin results in increased survival in a mouse model of ovarian cancer (Strobel & Cannistra, 1999). The most abundant proteins in the ECM of the peritoneum are fibronectin and collagen (Kenny *et al*., 2009). The α5β1integrin recognises the arginine-glycine-aspartic acid (RGD) motif of fibronectin. Use of antibodies against α5β1 suppresses peritoneal spread of ovarian cancer in a xenograft model of ovarian cancer (Schaffner *et al*., 2013). Blocking of α5β1integrin inhibits ovarian cancer binding to the mesothelium and that proves the importance of α5β1 receptors in mediating the binding of ovarian cancer cells to the mesothelium (Strobel & Cannistra, 1999). Integrin signalling pathways regulate invasion, migration, and proliferation in cancer cells. Furthermore, because the α5β1integrin plays a crucial role in the attachment of ovarian cancer cells to the mesothelium, so α5β1integrin inhibitors could provide therapeutic agents to prevent metastasis (Ohyagi-Hara *et al*., 2013). Moreover, recent studies have shown that use of natural products such as peptides, proteins and enzyme from snake venom can prevent cancer metastasis (Sarray *et al.,* 2013). It has been demonstrated that snake venom

contains peptides with anti-integrin activity. There are changes in integrin expression during tumour migration and growth (Schaffner *et al*., 2013). Researchers have shown that the expression of integrins differ between cancer and normal cells; in normal cells integrins such as αvβ3, α5β1 and αvβ6 are undetectable, but are expressed at high levels in cancer cells. Research has shown that use of integrin inhibitors can effect adhesion, migration, invasion, survival, and the microenvironments of the tumour (Ganguly *et al*., 2013). The major integrins expressed by A2780 ovarian cells used in this project are α5β1, αv and α2β1 integrins (Markland *et al*., 2011).

1.5 Snake venom

Snake venom is produced by the mandibular gland and is composed of a complex mixture of components, which includes enzymes, peptides, proteins and carbohydrates. It provides a source of molecules with different pharmacological activities (Calvete *et al*., 2007). It has been estimated that snakebite envenomation affects 2,500,000 people per year and results in 125,000 deaths in the world. The highest incidence of snakebite deaths occur in Africa, Asia, and Latin America (Albuquerque *et al*., 2013). According to the World Health Organization (WHO), envenomation due to snakebites is considered a major public health problem.

1.5.1 Classification of snakes and their venoms

There are approximately 2700 species of snake, of which the venomous types compromise just one fifth. Venomous snakes are divided into four families: Viperidae, Atractaspididae, Elapidae and Colubridae. In addition, snakes under each family have venoms of a common protein complex. For example, venoms of the Viperidae family have enzymes that disturb coagulation and produce minor

neurotoxicity. Furthermore, extreme neurotoxic activity has been observed in the venoms of Elapidae. In the Colubridae species, venom activities are analogous to those in the Viperidae and Elapidae families. Whereas, cardiotoxicity is most likely to be observed in venoms of the Atractaspididae family (Fox & Serrano, 2008).

1.5.2 Components of snake venoms

Snake venoms are comprised of a highly complex mixture of components with different natures and pharmacological activities. These components act as toxins against an attacked victim and destroy important physiological functions and lead to death. Snake venom components vary from species to species and even within a single species depending on season, age, and temperature (Chippaux *et al*., 1991). Components of snake venom are mainly proteins and peptides (up to 90-95%) while the remaining portion are amino acids, nucleotides, lipids and carbohydrates (De Lima *et al*., 2005). The proteins and polypeptides are categorised into either enzymatic or non-enzymatic groups (Doley & Kini, 2009). Enzymes in snake venom include acetylcholinesterase (AChE), L-amino acid oxidases (SV-LAAOs), serine proteases (SVSPs), metalloproteases (SVMPs), and phospholipases A2 (svPLA2). Non-enzymatic groups include disintegrins, three finger toxins (3FTxs), C-type lectin proteins (CLPs) and cysteine-rich secretory protein (CRISP) (Calvete *et al*., 2005).

1.5.3 Anticancer activity of snake venom

Snake venom has been studied for its therapeutic potential to fight cancer, despite having toxic effects (Shaikh and Jokhio, 2005). It has been reported that using components of snake venom can stop tumour metastasis by blocking adhesion, migration, and invasion of cancer cells (Trikha *et al.,* 1994).

1.5.4 Snake venom metalloproteinases (SVMPs)

SVMPs are the major components of Viperidae and Crotalid venoms. Metalloproteinases are zinc dependent enzymes with a molecular weight ranging from 20,000 to 100,000Da. Synthesis of SVMPs occurs in the venom gland as zymogens that are then processed to active forms (Cominetti *et al*., 2003). SVMPs are classified into different classes according to their molecular weight and organisation of their domains. P-I SVMPs are the simplest ones with a molecular weight of 20,000-30,000Da and contain only a metalloproteinase domain. P-II SVMPs include metalloproteinase and disintegrin domains with a molecular weight between 30,000-60,000Da. P-III SVMPs contain metalloproteinase, disintegrin, and cysteine rich domains and their molecular weight range is 60,000-100,000Da. P-IV SVMPs contain a C-type lectin-like domain in addition to metalloproteinase, disintegrin, and cysteine rich domains with a molecular weight greater than 90,000Da and is now included as a subclass of P-III SVMPs (Markland & Swenson, 2013). The disintegrin-like domain of metalloproteinase is different from true disintegrin in the number of disulphide bonds and the presence of RGD that is why it is called a disintegrin-like domain. SVMPs require zinc (Zn^{2+}) for catalytic activity and calcium (Ca^{2+}) for stabilisation of their structure (Hamza *et al*., 2010). SVMPs are responsible for haemorrhage, necrosis, oedema, and blood coagulation disorders following snakebite. Some SVMPs have the ability to cause proteolysis of proteins of the ECM, such as laminin, fibronectin, and type IV collagen and cell surface integrins. In addition, some SVMPs destroy blood clotting factors and affect platelet function. It has been reported that some P-III SVMPs inhibit platelet aggregation and enhance haemorrhage (Boukhalfa *et al*., 2009). Platelet collagen receptor α2β1

integrin is degraded by jararhagin (P-III SVMPs) which was isolated from *Bothrops jararaca* and is associated with platelet aggregation inhibition (Correa *et al*., 2002). SVMPs are Zn^{2+}dependent metalloproteinase and their activity is inhibited by ethylenediaminetetraacetic acid (EDTA) and 1,10-phenanthroline (Boukhalfa *et al*., 2009).

1.5.4.1 SVMPs and cancer treatment

There are various SVMPs that have anticancer activity by their inhibitory effect on integrin mediated adhesion of cancer cells and antiangiogenic activity. Tumour growth and progression is dependent on angiogenesis. Endothelial cell proliferation, migration, invasion and differentiation are important steps of angiogenesis (Bateman *et al*., 2013). It has been demonstrated that P-III SVMPs (BaG) from *Bothrops alternatus* snake venom inhibited fibronectin cell adhesion of leukemic cells by its inhibitory effect on α5β1 integrin (Cominetti *et al*., 2003). HV1 (P-III SVMPs) from *Trimeruserus flavoviridis* snake venom, has been shown to inhibit adhesion of vascular endothelial cells and induce apoptosis. Vascular endothelial cells are important for cancer progression because of their role in angiogenesis (Masuda *et al*., 2001). In addition, VLAIPs are SVMPs, which have been purified from *Vipera lebetina* snake venom, that have antitumour activity by inhibiting proliferation and inducing of apoptosis of vascular endothelial cells (reviewed by Saray *et al*., 2013). VLAIPs inhibit the adhesion of vascular endothelial cells to fibronectin, fibrinogen, collagen, and vitronectin (Trummal *et al*., 2005). P-III SVMP jararhagin, a hemorrhagic metalloproteinase from *Bothrops jararaca* venom inhibits the adhesion of human melanoma cells (SKmel-28) to ECM protein coated plates and the cells become rounded. Furthermore, migration and invasion of human malignant

melanoma cells were significantly inhibited after treatment with jararhagin (Correa *et al*., 2002). Marked reduction in the number of metastatic cells in the lungs of AIRmin mice was observed after jararhagin administration (Correa *et al*., 2002). Jararhagin has been reported to induce change in morphology of endothelial cells with decreased spreading and detachment of the cells and leads to cell death (Tanjoni *et al*., 2005). Halysase, a novel hemorrhagic metalloproteinase from *Gloydius halys*, has been shown to be a proliferation inhibitor and apoptosis inducer of human umbilical vein endothelial cells (HUVECs). In addition, it has been demonstrated that halysase inhibited the adhesion of HUVECs to ECM by acting on α1β1 and α5β1, but not αvβ3 and αvβ5 integrins. It has been suggested that the disintegrin domain of halysase modifies the proteolytic activity of the enzyme in hydrolysing ECM proteins and integrins (You *et al.,* 2003). Graminelysin I has only a metalloproteinase domain and has been purified from *Trimeresurus gramineus* venom. It has been reported that graminelysin I inhibits the adhesion of HUVECs to fibrinogen and induces apoptosis, but their adhesion to collagen is not affected. The activity of graminelysin I against HUVECs has been inhibited and abolished after adding EDTA, suggesting that the catalytic activity of graminelysin I on ECM proteins is responsible for its apoptotic activity against HUVECs (Wu *et al*., 2001). TSV-DM (P-III SVMP), purified from *Trimeresurus stejnegeri* venom, has an inhibitory effect on the proliferation of a human umbilical vein endothelial cell derived cell line (ECV304). It has been demonstrated that TSV-DM induces morphological changes of ECV304, but has no apoptotic effect. The activity of TSV-DM against ECV304 is not affected by adding EDTA, suggesting there is no role for a catalytic domain in this activity (Wan *et al*., 2006).

1.5.5 Phospholipases (PLA2)

According to their primary structure, phospholipases (PLA2) are classified into different classes and the major distinct classes include cytosolic PLA2, secreted PLA2, platelet activating factor acetyl-hydrolases (PAF-AH), Ca^{2+}-independent PLA2, and lysosomal PLA2 (Burke & Dennis, 2009). The major source of secretory PLA2 is snake venom (Perumal Samy *et al*., 2010) and the Colubridae, Elapidae, and Viperidae families are the main providers. They consist of 120-135 amino acids with low molecular masses ranging between 14,000-18,000Da and they have 5-8 disulphide bonds. Snake venom PLA2 (svPLA2) needs the presence of Ca^{+2} ions for their activity (reviewed by Saray *et al*., 2013). PLA2 are enzymes that are responsible for catalysing the hydrolysis of ester bonds of phospholipids and result in generating free fatty acids and lysophospholipids (Zouari-Kessentini *et al*., 2009). svPLA2s are divided into two groups (I and II) according to their primary structure and pattern of disulphide bonds. Group I svPLA2s are found in the Elapid family, while group II have been isolated from the Viperid and Crotalid families (Zouari-Kessentini *et al*., 2009). Group II are divided into two subgroups according to the amino acid residue at position 49 in the primary structure. The subgroups are Asp-49 PLA2s and Lys-49 PLA2s. Asp-49PLA2s contain aspartic acid at position 49 with high catalytic activity, while Lys-49 PLA2s have lysine instead of aspartic acid with low catalytic activity (Perumal Samy *et al*., 2010). svPLA2s are responsible for numerous pathophysiological effects such as, cardiotoxicity, neurotoxicity, hypotension, myotoxicity, antiplatelet activities, oedema and anticoagulation (Bazaa *et al*., 2009). Aggregation inhibition is due to release of lysophospholipids. It has

been demonstrated that svPLA2s inhibit tumour cell adhesion and migration and have antiangiogenic activity (reviewed by Sarray *et al*., 2013).

1.5.5.1 svPLA2 and cancer treatment

Some svPLA2s have been reported to have antitumour and antiangiogenic activities. There is a novel PLA2 from *Macrovipera lebtina* venom (MVL-PLA2) that has been shown to have anticancer activity by inhibiting adhesion and migration of various cancer cells such as melanoma and fibrosarcoma. The anticancer activity of MVL-PLA2 occurred by inhibiting α5β1 and αv integrins. Antitumour activity of MVL-PLA2 is independent from its catalytic activity because it has been shown that by inhibiting the enzymatic activity there was no effect on anticancer activity (Bazaa *et al*., 2009). Furthermore, an acidic PLA2 protein (Bth-A-I- PLA2) was isolated from *Bothrops jararacussu* venom that had antitumour activity on breast adrenocarcinoma, human leukemia T and Erlich ascites tumour. The cytotoxicity and antitumour activity was linked to the catalytic activity (Roberto *et al*., 2004). Myotoxins are basic PLA2 which have been purified from *Bothrops jararacussu* venom and have shown antitumour activity on promyelocytic leukemia, hepatocellular carcinoma, and murine pheochromocytoma by inducing apoptosis (Prinholato da Silva *et al*., 2015). Two PLA2 from *Cerastes cerastes* snake venom (CC-PLA2-1 and CC-PLA2-2) have an inhibitory effect on adhesion of melanoma and fibrosarcoma to fibronectin and fibrinogen. Markedly both CC-PLA2-1 and CC-PLA2-2 inhibit the migration of fibrosarcoma. It has been suggested that the effect of CC-PLA2-1 and CC-PLA2-2 is integrin-dependent because there is no effect on adhesion of melanoma and fibrosarcoma to poly-L-lysine (an integrin–independent substrate). The antitumour activity of CC-PLA2-1 and CC-PLA2-2 is independent

from its catalytic activity (Zouari-Kessentini *et al*., 2009). The inhibitory activity of both these PLA2 on migration of fibrosarcoma have the same IC_{50}, suggesting that the differences in amino acid sequence of both CC-PLA2-1 and CC-PLA2-2 have no effect on their anti-integrin activity (Zouari-Kessentini *et al*., 2009). It has been reported that CC-PLA2-1 and CC-PLA2-2 have antiangiogenic activity by inhibiting migration and adhesion of human brain microvascular endothelial cell through blocking of α5β1 and αv integrins (Kessentini-Zouari *et al*., 2010). Different PLA2 with their cytotoxic effect on different cancer cell lines has been shown in (Table 1).

Table 1: PLA2 and anti-cancer activity

PLA2	**Snake**	**Effects**	**References**
phospholipase B	*Australian elapid*	Cytotoxic effect on rhabdomyosarcoma	(Bernheimer *et al*., 1987)
PLA2s	*Bothrops newweidii*	Cytotoxic effect on melanoma cells	(Gomes *et al*., 2010)
PLA2s	*Naja naja*	Cytotoxic effect on Ehrlich ascites tumour	(Gomes *et al*., 2010)
PLA2s	*Prothobotrops flavoviridis*	Apoptotic activity against leukemic cells	(Murakami e*t al*., 2011)

PLA2s have been isolated from different snake venoms with cytotoxic and apoptotic activity against different cancer cell lines.

1.5.6 L-Amino acid oxidase (LAAO)

Snake venom L-amino acid oxidases (SV-LAAOs) are flavoenzymes. They are the most common enzymatic constituents of venoms that have been isolated from Viperidae, Crotalidae and Elapidae snake families. The yellow colour of many snake venoms is due to flavins of SV-LAAOs (Costa *et al*., 2014). These enzymes usually constitute about 1-9% of the total protein in the venom. Oxidative deamination of L-amino acids is catalysed by SV-LAAOs and associated with production of α-keto acids, ammonia, and hydrogen peroxidase (H_2O_2) (Burin *et al*., 2013). SV-LAAOs have a molecular weight of about 110,000-150,000Da and are dimeric flavoproteins. Each subunit binds non-covalently to flavin adenine dinucleotide as a co-factor. SV-LAAO has various uses in medicine because of its antimicrobial, anticoagulant, antiviral, and anticancer activity (Abdelkafi-koubaa *et al*., 2014). It has been demonstrated that all the biological activity of SV-LAAO such as apoptosis, bactericidal, cytotoxicity, and platelet aggregation induction or inhibition could be due to the release of H_2O_2 from the oxidative deamination reaction (Ali *et al*., 2000).

1.5.6.1 SV-LAAO and cancer treatment

Researchers have reported that SV-LAAOs have a cytotoxic effect on different cancer cell lines including, breast adenosarcoma, leukaemia, murine melanoma and pheochromocytoma (Suhr & Kim, 1996). However, SV-LAAOs are capable of affecting normal cells as well, but it is a less significant effect when compared to cancer cells. This is thought to be due to the higher lipid content of the cell membranes of tumour cells compared to normal cells and lipids are usually damaged by reactive oxygen species and so cancer cells are more susceptible (Costa *et al*., 2014). LAAO from *Bothrops jararaca* snake venom was shown to inhibit the growth

of Ehrlich ascites tumours *in vivo* (De Vieira Santos *et al*., 2008). BpirLAAO-I, LAAO purified from *Bothrops pirajai* snake venom has also been shown to have cytotoxic activity against acute T cell leukaemia and human breast cancer (Burin *et al*., 2013). It has been reported that, SV-LAAOs are apoptotic inducers for different cancer cell lines derived from pro-myelocytic leukaemia, ovarian, stomach, melanoma, fibrosarcoma and colorectal cancers (Costa *et al*., 2014). LAAOs from haemorrhagic snake venom cause apoptosis in endothelial cells (Suhr & Kim, 1996), while Bl-LAAO from *Bothrops leucurus* venom has a cytotoxic effect on stomach and colorectal cancers (Naumann *et al*., 2011). Different SV-LAAOs with anticancer activity has been shown in (Table 2).

Table 2: LAAO and anti-cancer activity

LAAO	**Snake**	**Antitumour effect**	**References**
ACTX-6	*Agkistrodon acutus*	Antitumour activity by inducing apoptosis in HeLa cells	(Zhang&Wu, 2008)
LAAO	*Ophiophagus Hannah*	Cytotoxic activity on fibrosarcoma, stomach and colorectal cancer	(Ahn *et al*.,1997)
Apoxin I	*Crotalus atrox*	Induced apoptosis in HUEVC and A2780 ovarian cells	(Torii *et al*., 2000)

SV-LAAOs have been isolated from different snake venoms with cytotoxic and apoptotic activity against different cancer cell lines.

1.5.7 Snake venom serine proteinases (SVSPs)

Snake venom serine proteinases (SVSPs) have been purified from Viperidae, Crotalidae, Elapidae and Colubridae snakes and it is a subfamily of trypsin-like proteinases. SVSPs usually constitute about 20% of the total venom proteins. SVSPs display several physiological functions such as fibrinogenolytic and fibrinolytic activity, and affect the haemostatic system by acting on the coagulation cascade, which is responsible for platelet aggregation (Serrano & Maroun, 2005). *Trimeresurus stejnejeri* venom plasminogen activator is a serine proteinase which activates plasminogen. It has been reported that, SVSPs have been used for therapy and diagnosis of coagulant disorders (Parry *et al*., 1998).

1.5.7.1 SVSPs and cancer treatment

The antitumour activity of SVSPs is still not well understood. However, there are a few studies to show their activities. For example, Crotalase, a serine protease, isolated from *Crotalus adamanteus* snake venom, inhibits the growth of B16 melanoma cells *in vitro*. In addition, batroxobin, a thrombin-like serine protease from *Bothrops moojeni* venom has anti-metastatic activity (reviewed by Calderon *et al*., 2014).

1.5.8 Disintegrins

Disintegrins are one of the most important non-enzymatic complexes found in snake venoms. They are low molecular weight proteins and cysteine rich. Disintegrins and disintegrin-like proteins are mainly derived from the venom of four families of snakes*:* Atractaspididae, Elapidae, Viperidae, and Colubridae. Their molecular mass ranges from 4,000-15,000Da (Galan *et al*., 2008). Disintegrins are potent inhibitors of platelet aggregation and have anti-integrin activity. Disintegrins bind to integrin

receptor αIIbβ3 on platelets and are associated with inhibition of platelet aggregation. Snake venom disintegrins also have antiangiogenic activity (Marcinkiewicz *et al.*, 2003). It has been demonstrated that disintegrins have been released into venom from proteolytic processing of metalloproteinase II. Studies have suggested that disintegrins can be divided into five groups according to the number of disulphide bridges and length of their polypeptides (Olfa *et al*., 2005). Short disintegrins are included in the first group, which are composed of 49-51 amino acids with four disulphide bonds; examples include echistatin, eristocophin, eristostatin and ocellatusin. The second group include medium disintegrins that consist of about 70 amino acids and six disulphide bonds; examples include trigramin, kistrin, flavoridin, albolabrin and barbourin. Long disintegrins are comprised of 83 amino acids and seven disulphide bonds an example of which is salmosin (Calvete *et al*., 2005). It has been reported that the fourth group of disintegrins consist of disintegrin-like domains, which have been derived from P-III SVMPs and have about 100 amino acids and 8 disulphide bonds (Sarray *et al*., 2013). Short, medium, and long disintegrins are single chain molecules. The fifth category includes homo and heterodimers (Calvete *et al*., 2005). Anti-integrin activities of disintegrins depend on the presences of appropriate cysteine residues, which determines the inhibitory loop. Determination of the binding of disintegrins to the integrin receptor is dependent on the binding loop conformation that contains a tripeptide motif (Juarez *et al*., 2008). Most single chain disintegrins have an RGD tripeptide motif in its binding loop; however, small disintegrins such as eristocophin II have a MGD motif (Met-Gly-Asp). On the other hand, atrolysin has a MVD motif (Met-Val-Asp) as does rubistatin (Calvete *et al*., 2005). Disintegrins bind to integrins and prevent the

binding of integrins to natural ligands (Tian *et al*., 2007). It has been shown that disintegrins bind to different integrin receptors according to the tripeptide motifs, for example, KTS (Lys-Thr-Ser) disintegrin binds to the α1β1 integrin, whereas MLD disintegrins have the ability to bind to integrin receptors such as α4β1, α4β7, and α9β1. Moreover, RGD disintegrins bind to α5β1, αvβ3, and αIIbβ3 integrin receptors and VGD and MGD disintegrins bind and inhibit the function of α5β1 integrin receptors (Juarez *et al*., 2008). In addition, disintegrins have anti-tumour activity by interfering with integrin-mediated cell functions, such as cell adhesion and migration. Disintegrins act as competitive inhibitors and prevent the binding of integrins to their preferred ligand and that results in alteration of cell-ECM interactions and cell-cell interactions, thus affecting cellular function (Galan *et al*., 2008). The process of angiogenesis results in the formation of new blood vessels and it is an important step for tumour progression. Integrins are an important regulator for angiogenesis, so inhibition of integrins affects angiogenesis and inhibits tumour progression (Marcinkiewicz *et al*., 2003).

1.5.8.1 Disintegrins and cancer treatment

Disintegrins from snake venom have an important role in inhibiting platelet aggregation and preventing tumour migration by blocking the integrin signalling pathway. Platelet aggregation plays an important role in facilitating tumour metastasis. The disintegrin peptides found in Crotalid and Viperid venoms are different from the disintegrin-like domains of metalloproteinases by nature of their disulphide bonds. Disintegrins and metalloproteinases have the same affinity and ability of inhibiting integrin binding. Tumour growth is dependent on continuous neovascularisation, so antiangiogenic activities of some disintegrins play a

significant role in hindering tumour progression (Zhou *et al*., 2000). Contortrostatin is a homodimer disintegrin isolated from the venom of *Agkistrodon contortrix contortrix* snakes with a molecular weight of 13.500Da (Swenson *et al*., 2005). It has been shown to inhibit the progression of breast cancer in a xenographic model(Zhou *et al*., 2000). Furthermore, the adhesion of HUVECs to vitronectin was significantly inhibited by contortrostatin and showed antiangiogenic activity. Adhesion of breast cancer cells to fibronectin and vitronectin was inhibited by contortrostatin, but had no effect on binding of the cells to laminin (Zhou *et al*., 2000). The anti-integrin activity of contortrostatin occurs through binding to αvβ3, α5β1, αvβ5 integrins and interference of adhesion, migration and invasion of different cancer cells such as human glioblastoma, ovarian cancer and breast cancer cells (Swenson *et al*., 2005). It has been reported that contortrostatin inhibited ovarian cancer dissemination *in vitro* and *in vivo* (Markland *et al*., 2001). Eristostatin, a RGD-disintegrin purified from *Eristicophis macmahoni* venom, inhibited migration of five melanoma cell lines (MV3, WM164, M24met, 1205Lu, and C8161) (Tian *et al.*, 2007). The inhibition activity of eristostatin on migration occurred mainly by affecting the fibronectin receptor, but not laminin or collagen receptors, suggesting that eristostatin is specific for fibronectin binding integrins (Tian *et al.*, 2007). It has been reported that eristostatin has an inhibitory effect on platelet aggregation, but has no effect on proliferation of melanoma cell or angiogenesis (Tian *et al*., 2007). Moreover, it has been suggested that eristostatin has an inhibitory effect on α4β1integrin (Sarray *et al*., 2013). Obtustatin, a disintegrin isolated from *Vipera lebetina obtusa* snake venom, contains a KTS motif instead of the RGD motif. Obtustatin is a selective inhibitor for α1β1 integrin and that makes it a potent inhibitor of angiogenesis,

because a recent study has shown that α1β1 is an important integrin in blood vessel formation. It has been reported that obtustatin reduces tumour growth in a Lewis lung syngeneic mouse model (Marcinkiewicz *et al*., 2003). Rhodostomin, a RGD disintegrin, was purified from the venom of *Calloselasma rhodostoma* and has antiangiogenic and antitumour activity by inhibiting αvβ3, αvβ5 integrin receptors (reviewed by Calderon *et al*., 2014). It has been demonstrated that rhodostomin inhibited angiogenesis by inhibiting proliferation, migration and invasion of HUVECs (Yeh *et al*., 2001). Lebestatin, a KTS disintegrin purified from *Macrovipera lebetina* venom, inhibits α1β1 integrins and it displays an inhibitory effect on adhesion of rat pheochromocytoma to collagen type I and IV. Furthermore, lebestatin inhibits migration and adhesion of endothelial cells and has antiangiogenic activity (Olfa *et al*., 2005). Rubistatin, a disintegrin isolated from *Crotalus ruber ruber* snake venom, has an inhibitory effect against proliferation and migration of human melanoma cells. It is a MVD disintegrin and the receptor targeted is still unknown (Carey *et al*., 2012). It has been demonstrated that saxatilin (a RGD disintegrin purified from *Gloydius saxatilis* venom) inhibited platelet aggregation and ovarian cancer cell proliferation and invasion by inhibiting αIIb β3integrin (Kim *et al*., 2007). Different disintegrins with anticancer activity has been shown in (Table 3).

Table 3: Disintegrins and anti-cancer activity

Disintegrins	Snake	Integrins	Effects	References
Accutin	*Agkistrodon acutus*	αvβ3	Inhibited angiogenesis by blocking αvβ3 integrin on endothelial cells and induction of apoptosis	(Yeh *et al*., 1998)
DisBa-01	*Bothrops alternatus*	αvβ3	Anti-metastatic activity against melanoma cells, antiangiogenic activity	(Ramos *et al*., 2008)
Salmosin	*Agkistrodon halys brevicaudus*	αvβ3	Inhibited proliferation of malignant melanoma cell lines (SK-Mel-2 and B16)	(Chung *et al*., 2003)
Triflavin	*Trimeresurusflavoviridis*	α5β1, αvβ3, and α3β1	Inhibits adhesion of HUVEC to ECM proteins (fibronectin, vitronectin, laminin and collagen type IV)	(Sheu *et al*., 1997)
Atrolysin E/D(MVD disintegrin)	*Crotalus atrox*	α1β1 α2β1 αIIbβ1	Inhibitsplatelet aggregation	(Carey *et al*., 2012)

Disintegrins and anti-cancer effect on different cancer cell lines

1.5.9 Snake C-type lectin proteins (Snaclec)

The CLPs are one of the most abundant components in snake venom. The CLPs are a non-enzymatic protein complex. They also consist of heterodimers consisting of two subunits, α and β, linked covalently by a disulphide bond (Sarray *et al*., 2004). The molecular weight of CLPs is 30,000Da and the heterodimer structure is multimerised to form a larger structure. Some CLPs are part of snake venom metalloproteinase IV (Eble *et al*., 2009). They have several biological activities such as, modulatar of platelet aggregation by targeting of platelet receptors. Some CLPs have anticoagulant activity by binding to von Willbrand factor receptor (vWF) and others induce platelet aggregation by activating collagen receptors (Sarray *et al*., 2004).

1.5.9.1 CLPs and cancer treatment

CLPs have been highlighted for their anticancer activity. Recently, it has been demonstrated that CLPs have anti-integrin and antitumour activities by preventing adhesion, migration, invasion, and proliferation of cancer cells (Marcinkiewicz *et al.*, 2000). EMS16, a CLP purified from *Echis multisquamatus* venom, has been shown to inhibit the adhesion and migration of HUVECs by blocking α2β1 integrin. It has been shown that α2β1integrin of endothelial cells plays a vital role in angiogenesis, so blocking α2β1 by EMS16 is an important step to block angiogenesis and neovascularisation (Marcinkiewicz *et al*., 2000). Lebectin, another CLP isolated from *Macrovipera lebetina* venom, has anti-platelet aggregation and antitumour activity. It has been shown that lebectin inhibits adhesion of different cancer cell lines such as melanoma, fibrosarcoma, adenosarcoma, and leukaemia to ECM proteins (Sarray *et al*., 2004). The inhibitory effect of lebectin on adhesion has been demonstrated using fibronectin, but not the collagen receptor (Sarray *et al*., 2004). Furthermore, the

antiangiogenic activity of lebectin has been reported *in vitro* and *in vivo.* The anti-adhesive and antiangiogenic activity of lebectin occurs by blocking α5β1 and αv integrins (Pilorget *et al*., 2007). BJcuL, a CLP purified from the venom of *Bothrops jararacussu* snake, displays anti proliferative activity against renal, pancreatic, melanoma, and prostatic cancer cells. Moreover, BJcuL is considered an apoptotic inducer in human gastric carcinoma by inhibition of cell adhesion, but the involved integrin has not yet been established (Nolte *et al*., 2012).

1.5.10 Three finger toxins (3FTxs)

3FTxs are non-enzymatic snake venom proteins and mostly present in the Elapids, Hydrophiid, and Colubrid snakes. They are composed of 60-74 amino acids and four or five disulphide bridges with a molecular weight ranging between 6,000 to 8,000Da (Fry *et al*., 2003). 3FTxs share a similar structure, but different functional properties and include neurotoxins, cardiotoxins, calciseptine and dendroaspins. It has been reported that cardiotoxins interact with cell membranes, while neurotoxins target the cholinergic system (Roy *et al*., 2010). This family of proteins is named because of their appearance (Figure 3). In addition, calciseptin and droaspins are another type of 3FTxs which block L-type Ca^{2+} channel and antagonists of cell adhesion receptors, respectively (Roy *et al*., 2010).

1.5.10.1 3FTxs and cancer treatment

A 3FTx purified from *Daboia russelli russelli* venom drCT-II, has anti-proliferative and apoptotic activity against HepG2 (human liver cancer cells) (Gomes *et al*., 2010). Cytotoxin, a 3FTx from *Naja naja atra* venom, has cytotoxic activity against different cancer cell lines such as breast cancer and leukemia cells (Gasanov *et al*., 2014).

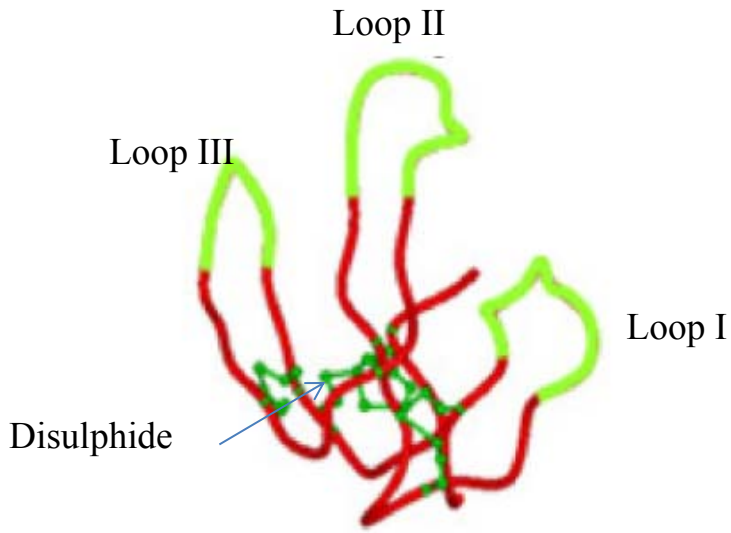


Figure 3: Molecular structure of a three finger toxin (Naimuddin *et al*., 2011)

1.5.11 Snake Venom Cysteine-Rich Secretory Protein (CRISP)

CRISPs are non-enzymatic snake protein and the majority of snake venoms contain them. They are single polypeptides with molecular masses ranging from 20,000-30,000 Da. They belong to the nontoxic components of snake venom. It has been reported that CRISPs have anti-protozoa activity (Adade *et al*., 2014) and have been shown to selectively block a number of ion channels (L type calcium channel and cyclic nucleotide gated ion channels). ES-CRISP, purified from *Echis carinatus sochureki* venom has antiangiogenic activity (Lecht *et al*., 2015).

1.5.12 Snake venom studies

In this project the effect of two kinds of snake venoms (from *Crotalus ruber ruber* and *Agkistrodon piscivorus leucostoma*) on dissemination of ovarian cancer was studied.

1.5.12.1 *Crotalus ruber ruber* (Red diamond rattlesnake) snake venom

Crotalus ruber ruber is a viper species found in south-western California in the United States (Figure 4, Table 4). On envenomation, the venom is known to cause severe tissue damage and is associated with local and systemic haemorrhage and this is due to action of proteases (Mackessy, 1985). The snake venom is known as a haemotoxic venom because it disrupts red blood cell homeostasis, which leads to systemic bleeding. The venom consists of various proteases, phosphodiesterases, LAAO and disintegrins. The proteolytic activity of adult venom is higher than the proteolytic activity of juvenile venom (Mackessy, 1985). *Crotalus ruber ruber* venom has a significant quantity of the disintegrin rubistatin, which acts as an inhibitor of platelet aggregation and cell migration of melanoma cells (Carey *et al*., 2012)

Table 4: Scientific classification of *Crotalus ruber ruber* snake

Kingdom:	Animalia
Phylum	Chordata
Subphylum	Vertebrata
Class	Reptilia
Order	Squamata
Suborder	Serpentes
Family	Viperidae
Subfamily	Crotalinae
Genus	*Crotalus*
Species	*C. ruber*

Figure 4: *Crotalus ruber ruber* snake

It has been reported that, rubistatin is a MVD disintegrin and its biological activity is not understood well. In addition, rubistatin inhibits proliferation and induces apoptosis of SK-Mel-28 (melanoma cancer cells), but not HeLa or T24 (transitional-cell human bladder carcinoma) cell lines and the receptor which is targeted by rubistatin is still unknown (Carey *et al*., 2012). The molecular mass of rubistatin is 6,558Da and consists of 61 amino acids. Researchers have shown that the crude venom contains many potent proteases (Mackessy, 1985). Moreover, there are several different proteases of the venom with proteolytic activity. Rubelase is a non haemorrhagic metalloprotease with hydrolytic activity on collagen, fibrinogen and

elastase substrates (Komori *et al.*, 2011). The molecular weight of rubelase is 23,266 Da. Rubelysin (haemorrhagic metalloproteinase HT-2) is another component isolated from the venom and its' amino acid residues are extremely homologous to rubelase (Takeya *et al*., 1990). The differences between ruberlysin and rubelase are the amino acid residues of Trp, Leu, Asn, Glu at positions 81, 87, 114 and 151, respectively in ruberlysin, while in rubelase these are Val, Lys, Lys, and Lys. Rubelysin is composed of 202 amino acids and has a molecular mass of 23,321Da (Takeya *et al*., 1990). It has been reported that rubelysin has a toxic effect on HUVECs and the effect of rubelase on the cells was weaker in comparison to that of rubelysin. C. *ruber* venom lectin (CRL) consists of 135 amino acid residues with 9 Cys residues. In addition, CRL is a disulphide-linked homodimer of a 15.000Da subunit. It has been reported that CRL has no effect on platelet aggregation induction (Hamako *et al*., 2007).

1.5.12.2 *Agkistrodon piscivorus leucostoma* (*Western Cottonmouth Moccasin*)

Agkistrodon piscivorus leucostoma belongs to the Viperidae family, found in South-Eastern United States (Figure 5, Table 5).

Table 5: Scientific classification of *Agkistrodon piscivorus leucostoma* snake

Kingdom	Animal
Phylum	Chordata
Subphylum	Vertebrata
Class	Reptilia
Order	Squamata
Suborder	Serpentes
Family	Viperidae
Subfamily	Crotalinae
Genus	Agkistrodon
Species	A.piscivorus

Figure 5:*Agkistrodon piscivorus leukostoma* snake

The venom from this snake is rich in enzymes with high proteolytic activity. Leucostoma peptidase A has been found to be the largest portion of the venom and has a MW of 22,500Da (Wagner *et al.*, 1986). Furthermore, two different classes of metalloproteinase have been purified from the venom (AplVMP1, AplVMP2) with molecular weights of 46,610Da and 51,160Da, respectively (Jia *et al*., 2009). AplVMP1 belongs to the metalloproteinase I family, while, AplVMP2 belongs to the metalloproteinase II family. AplVMP2 contains a metalloproteinase domain and a disintegrin domain, which has a KGD motif (Jia *et al*., 2009). The proteolytic and fibrinogenolytic activity of both metalloproteinases is abolished after treating with EDTA or 1,10-phenanthroline. Leucostoma peptidase A from *Agkistrodon piscivorus leucostoma* has the same properties as neutral protease which is a non-specific metalloprotease (Spiekerman *et al*., 1973).

1.5.13 Project aims:

Ovarian cancer is associated with poor prognosis and a poor survival rates because of late diagnosis after metastasis has occurred. The major steps in ovarian cancer metastasis are adhesion, migration and invasion. The initial aim of this project was to study the effect of *Crotalus ruber ruber* and *Agkistrodon piscivorus leucostoma* snake venoms on dissemination of ovarian cancer by studying the effect of both venoms on cell morphology, viability, adhesion, migration and invasion of an ovarian cancer cell line (A2870). A2780 cells were used in this study because it is one of the most commonly used cell line models for ovarian cancer. Since integrins play a major role in all of these steps, the subsequent aims were to demonstrate the effect of both venoms on the level of integrins expressed. Both venoms were semi-purified in order to obtain component-enriched fractions, which were then re-examined for their effect on cell morphology, viability, adhesion, migration and invasion. In this way, it was anticipated that identification of the active components against the process of metastasis could be made. Preliminary component structure elucidation was also attempted by SDS-PAGE, Nanoflow HPLC Electrospray Tandem Mass Spectrometry, liquid chromatography mass spectrometry (LC-MS) and assessment of the effect of metalloproteinase inhibitors.

2.0 Materials and methods

2.1 Materials

- 1% Virkon® (Antec International, Sudbury, UK)
- 25cm^2 and 75 cm^2 sterile flask (Thermo Fisher Scientific Inc, Renfrew, UK)
- A2780 cells (American Type Culture Collection) (ATCC, Middlesex, UK)
- Alamar Blue® Cell Viability Assay (Thermo Fisher Scientific Inc, Renfrew, UK)
- Alpha- and beta-integrin binding plates (AvantiCell Science, Ayr, UK)
- Antibodies: human anti-integrin α5, human anti-integrin β1, human anti-integrin αv (Abcam, Cambridge, UK)
- Bio- Safe-Coomassie, G-250 stain (Bio-Rad Laboratories, Hemel Hempstead, UK)
- Bromophenol blue (Sigma-Aldrich Ltd, Dorset, UK)
- Collagen I from human plasma (Sigma-Aldrich Ltd, Dorset, UK)
- Crystal violet (Sigma-Aldrich Ltd, Dorset, UK)
- Dithiothreitol (DTT) (Sigma-Aldrich Ltd, Dorset, UK)
- Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich Ltd, Dorset, UK)
- Fibronectin from human plasma (Sigma-Aldrich Ltd, Dorset, UK)
- Foetal calf serum (FCS) (Thermo Fisher Scientific Inc, Renfrew, UK)
- Function-blocking integrin antibodies: MAB1956 (anti-α5), MAB2253Z (anti-β1), ab24697 (anti-α2β1), MAB1980 (anti-αV) (Millipore, Watford, UK)
- Glycerol (Sigma-Aldrich Ltd, Dorset, UK)
- Glycine (Sigma-Aldrich Ltd, Dorset, UK)

- Haemocytometer (Hawksley, Sussex, UK)
- Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories Inc, West Grove, USA)
- Ibidi 2-chamber inserts (Thistle Scientific Ltd, Glasgow, UK)
- Invasion assay kits (Cambridge Bioscience Ltd, Cambridge, UK)
- Kanamycin (Thermo Fisher Scientific Inc, Renfrew, UK)
- LDS sample buffer 4X (Thermo Fisher Scientific Inc, Renfrew, UK)
- L-glutamine (Thermo Fisher Scientific Inc, Renfrew, UK)
- Mg^{2+}-Ca^{2+} -free Hank's balanced salt solution (HBSS) (Thermo Fisher Scientific Inc, Renfrew, UK)
- Migration assay kits (Cambridge Bioscience Ltd, Cambridge, UK)
- Ninety six well plates (Greiner Bio-one, Stonehouse, UK)
- Non-essential amino acids (Thermo Fisher Scientific Inc, Renfrew, UK)
- Phosphate buffered saline tablets (Sigma-Aldrich Ltd, Dorset, UK)
- Polyacrylamide gels 10% - 15% (Bio-Rad Laboratories, Hemel Hempstead, UK)
- Poly-L-lysine (Sigma-Aldrich Ltd, Dorset, UK)
- Pre-stained SDS-Page molecular weight markers (Bio-Rad Laboratories, Hemel Hempstead, UK)
- Rabbit polyclonal NFκB p65 (C-20)(Santa Cruz Biotechnology Inc, California, USA)
- RPMI 1640 (Sigma-Aldrich Ltd, Dorset, UK)
- Sephadex G75 (Sigma-Aldrich Ltd, Dorset, UK)

- Snake venoms (*Crotalus ruber ruber* and *Agkistrodon piscivorus leucostoma)* (National Natural Toxins Research Centre of Texas, Texas, USA)
- Sodium chloride (NaCl) (Sigma-Aldrich Ltd, Dorset, UK)
- Sodium dodecyl sulphate (SDS)(Sigma-Aldrich Ltd, Dorset, UK)
- Sodium pyrophosphate tetrabasic (Sigma-Aldrich Ltd, Dorset, UK)
- Sodium pyruvate (Thermo Fisher Scientific Inc, Renfrew, UK)
- Tris base (Sigma-Aldrich Ltd, Dorset, UK)
- Tris HCl (Sigma-Aldrich Ltd, Dorset, UK)
- Triton X100 (Sigma-Aldrich Ltd, Dorset, UK)
- TrypLE Express (Thermo Fisher Scientific Inc, Renfrew, UK)
- Tween (Sigma-Aldrich Ltd, Dorset, UK)
- Vitronectin from human plasma (Sigma-Aldrich Ltd, Dorset, UK)
- Western Blot Chemiluminescent (Bio-Rad Laboratories, Hemel Hempstead, UK)
- X-ray film (Thermo Fisher Scientific Inc, Renfrew, UK)

2.2 Equipment

- Bio-Rad Mini-PROTEAN 3TM electrophoresis tank (Bio-Rad Laboratories, Hemel Hempstead, UK)
- Edward freeze dryer modulyo (Edwards, Crawley, UK)
- KODAK M35-M X-OMAT processor (Kodak, New York, USA)
- Liquid Chromatography Mass Spectrometry (LC-MS)
    - ThermoFinnigan LTQ-Orbitrap (ThermoFisher Scientific, Bremen, Germany).

- Microscope**:** a Nikon TMS inverted phase contrast microscope (Motic, Wetzlar, Germany)
- Nanoflow HPLC Electrospray Tandem Mass Spectrometry (nLC-ESI-MS/MS)
    - Electrospray ionisation (ESI) mass spectrometry (Bruker Daltonics, Billerica, USA)
    - Nano flow uHPLC system (Thermo Scientific, Massachusetts, USA)
- SpectraMax M5 micro plate reader (Molecular Devices, California, USA)
- Size exclusion chromatography (GE healthcare, Hatfield, UK)
    - A Shimadzu LC-6A liquid chromatography pump controller
    - Desktop computer running Microsoft XP operating system for fraction detection and generation of chromatogram
    - Pharmacia LKB.FRAC.100 fraction collector
    - Rheodyne model 7725 injector with a 1ml loading loop
    - Shimadzu SCL-6B system controller
    - Shimadzu SPD-6A UV spectrophotometric detector

2.3 Methods

2.3.1 Tissue culture

All the experiments were carried out in a tissue culture laminar flow hood and all the equipment was cleaned with 70% ethanol before and after the experiments.

2.3.2 Cell lines and growth conditions

The cell line used in this study was the A2780 human ovarian cancer cell line, which was established from tumour tissue. It is epithelial in nature and cisplatin sensitive. The medium for growing the cells was RPMI 1640 supplemented with 10% (v/v)

foetal calf serum, 1% (v/v/) kanamycin, 1% (v/v) non-essential amino acids, 1% (v/v) L-glutamine, 1% (v/v) sodium pyruvate, and named complete medium. The cells were grown at 37°C in a humidified atmosphere with 5% CO_2.

2.3.3 Cell culture

Medium was discarded into 1% Virkon®, from a 75 cm^2 flask containing cells growing in log phase. The cells were washed with 5ml Mg^{2+}-Ca^{2+} -free Hank's balanced salt solution (HBSS) and 5ml of TrypLE Express was incubated with the cells at 37°C for 5min to detach the cells from the flask. The cells were viewed under a light microscope to make sure all the cells had detached and the action of TrypLE Express was stopped by adding 10ml of complete medium. Then the cells were centrifuged at 1000g for 2min. The medium was removed and the pellet resuspended in 10ml of complete medium. The number of the cells was determined using a haemocytometer by counting the cells in the four large corner squares of the haemocytometer under a light microscope. The mean from the four corners $X10^4$ gave the number of cells/ml. A2780 cells were maintained in complete medium and the media was refreshed every 48h and passaged when 80-90% confluence was achieved. Preparations of frozen stocks of cells were made by suspending the pellet in 10% (v/v) DMSO and 90% (v/v) FCS and aliquoted into 1ml cryovials and kept at -80°C overnight before being transferred to liquid nitrogen.

2.3.4 Venom preparation

Snake venom was prepared as a 5mg/ml solution in complete medium and aliquoted into 20µl volumes to avoid repeated freeze-thaw cycles and stored at -20°C.

2.3.5 Growth curve of A2780 ovarian cell line

Cells were seeded in 12 flasks (T25) at $65x10^4$ cells/flask. Every 24h the cells were counted using a haemocytometer (two flasks per day). A growth curve was constructed using the cell number plotted against time.

2.3.6 Effect of snake venom on the morphology of A2780 cells after 4h

Cells were seeded at $1x10^6$ cells per well (500µl) in 12 well plates. Venom was prepared at 6.25µg/ml (crude) and 3.125µg/ml (fractions) in 500µl complete medium (section 2.3.18), and added to 500µl of cell suspension so the final concentration of venom was 3.1µg/ml (crude) and 1.5µg/ml (fractions) and incubated with cells at 37°C in a humidified atmosphere with 5% CO_2 for 4h. A Nikon TMS inverted phase contrast microscope was used to take images. More than 90% of the cells had affected.

2.3.7 SYTOX®Green assay

SYTOX®Green is a high-affinity nucleic acid stain that easily penetrates cells with compromised plasma membranes, but will not penetrate intact cell membranes. Cells were seeded at $1x10^4$ cells per well in complete medium in a 96 well plate (75µl) and incubated for 24h. Venom was prepared in complete medium and 25µl added to the wells and incubated for 4h (venom prepared at concentrations of 0.7, 1.5, 3.1, 6.25, 12.5, 25 and 50µg/ml in 25µl). SYTOX®Green was used at a final concentration of 5µM in each well and incubated for 15min at 37°C in a humidified atmosphere with 5% CO_2. A SpectraMax M5 micro plate reader was used to measure the fluorescence intensity at 485-535nm. The results were calculated as a % viability of the untreated control (100% viable).

2.3.8 AlamarBlue® cytotoxicity assay

The AlamarBlue® assay, based on resazurin, was used to detect the effect of venom on cell viability. Resazurin is a non-toxic, cell permeable compound that is blue in colour and virtually non-fluorescent. Upon entering metabolically active cells, resazurin is reduced to resorufin, a compound that is red in colour and highly fluorescent. Cells were prepared at $1x10^4$ cells per well in complete medium (75µl), incubated for 24h at 37°C. Venom was prepared in complete medium and added at a final concentration between 0.1-12.5µg/ml (25µl), incubated for 24, 48, 72h. Ten µl of AlamarBlue® was added to each well for 6h before the end of the incubation period. The plates were incubated at 37°C in a humidified atmosphere with 5% CO_2 and a SpectraMax M5 micro plate reader was used to measure the fluorescence intensity at 560-590nm. The results were calculated as a % viability of the untreated control, which was considered 100% viable.

2.3.9 Spreading of A2780 cells on plastic and different ECM proteins

Collagen I was prepared at 10µg/ml in 1x phosphate buffered saline (PBS) (one tablet was dissolved in 200ml of deionized water and made upto pH 7.4). Fibronectin and vitronectin were prepared at 10 and 5µg/ml, respectively, in distilled water. Then 100µl of each protein was added per well, incubated for 1h at 37°C, removed and washed with PBS. Cells were prepared at $4x10^5$ cells per ml in 0.2% (w/v) bovine serum albumin (BSA) in RPMI 1640 medium (100µl/well in a 96 well plate). Cells were added per well and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 4h. The plate was then washed once with PBS, fixed with 4% (w/v) paraformaldehyde (PFA) in PBS (100µl) for 10min. The PFA was discarded and 100µl of 0.01 % (w/v) crystal violet in distilled water was added per well for at least

10min at room temperature to penetrate the cell membrane and stain the nucleus. The stain was removed and each well was washed 3 times with 300µl of distilled water. A SpectraMax M5 micro plate reader was used to measure the absorbance at 540nm. Before solubilisation (addition of 0.1%, v/v, Triton X-100) readings were used to calculate cell spreading, since the increased area of the nucleus gave a higher absorbance and this was a crude measurement. The absorbance was plotted against the different ECM proteins. However, the post-solubilisation readings were used in other experiments because it gave a reading representing the number of cells since all of the dye was eluted from the nucleus independent of whether cells were spread or not.

2.3.10 Alpha/beta integrin-mediated cell adhesion array

This experiment was carried out using α and β integrin-mediated cell adhesion array kits in order to identify the principle integrins expressed by A2780 cells. The manufacturer's instructions were followed. Briefly, the kits contained plates coated with mouse monoclonal antibodies raised against α and β human integrins. The wells were rinsed 3x with 300µl/well of wash buffer I and a suspension (100µl) of A2780 cells in serum free medium, adjusted to $4x10^5$ cells/ml was added per well. The plate was incubated for 4h at 37°C in a humidified atmosphere with 5% CO_2. The medium and any unbound cells were discarded from the wells and the plates washed 3x with 200µl/well wash buffer II. Adherent cells were stained with 100µl of stain solution for 10min at room temperature. The stain was removed and the wells were washed 4x with 200µl of wash buffer II. The final wash was discarded and 100µl of extraction solution was added to each well to elute the stain from the cells at room temperature for 10min. A SpectraMax M5 micro plate reader was used to measure

the absorbance at 540nm (after the solubilisation reading). A graph was plotted of cell adhesion (absorbance) against the different integrins.

2.3.11 Effect of function blocking integrin antibodies on A2780 cell adhesion

The function blocking antibodies used were, MAB1956 (anti-α5), MAB2253Z (anti-β1), ab24697 (anti-α2β1), and MAB1980 (anti-αV). Cells were adjusted to $4x10^5$ cells/ml in 0.2% (w/v) BSA in RPMI 1640 and added (90µl/well) to 96 well plates coated with fibronectin, vitronectin (see section 2.3.9) and 50µg/ml of poly-L-lysine (positively charged amino acid polymer to determine, if there was nonspecific electrostatic interaction between negatively charged ions of the cell membrane with the lysine residues) together with 10µl/well of antibodies (final concentration 10µg/ml). The cells were cultured for 4h at 37°C in a humidified atmosphere with 5% CO_2. The plate was then washed once with PBS, fixed with PFA and stained with crystal violet (see section 2.3.9). Subsequently, 300µl of 0.1% (v/v) Triton X-100 in PBS was used to extract the dye from the cells. A SpectraMax M5 micro plate reader was used to measure the absorbance at 540nm. Images were recorded on a Nikon TMS inverted phase contrast microscope. The results were calculated as % adhesion, where the untreated control was considered 100% adhesion.

2.3.12 Effect of snake venom on adhesion of A2870 cells to vitronectin, fibronectin and poly-L-lysine (an integrin-independent substratum)

Ninety six well plates were coated with 5µg/ml of vitronectin, 10µg/ml of fibronectin, or 50µg/ml of poly-L-lysine (100µl/well) for 1h. The plates were then washed with PBS (see 2.3.9), after which, 75µl of $4x10^5$cells/ml in 0.2% (w/v) BSA in RPMI 1640 and 25µl of venom at a final concentration between 0.1-6.25µg/ml were added to the coated plate and incubated at 37ºC in a humidified atmosphere

with 5% CO_2 for 4h. The non-adherent cells were then washed away and the adherent cells were fixed, stained with crystal violet and lysed with 0.1% (v/v) triton X-100 (section 2.3.11). A SpectraMax M5 micro plate reader was used to measure the absorbance at 540nm. The results were calculated as % adhesion, where the untreated control was considered 100% adhesion.

2.3.13 Effect of snake venom on migration of A2870 cells

The cell number was adjusted to $5x10^5$cells/ml in complete medium. Seventy µl of cell suspension was added into both sides of an ibidi migration chamber in a 24-well plate. After 24h the ibidi chamber was carefully removed, the medium discarded and 500µl of fresh complete medium with different concentrations of *Crotalus ruber ruber* snake venom (between 0.7-12.5µg/ml) was added, while for *Agkistrodon piscivorus leucostoma* snake venom the concentrations were 0.08, 0.1, 0.3, 0.7 and 1.5µg/ml. These concentrations of *Agkistrodon piscivorus leucostoma* snake venom were chosen as they did not cause cell detachment. After 48h, the medium was removed and cells were fixed for 30min with 200µl PFA, stained with crystal violet (as in section 2.3.9) and images taken. The migration was determined using Image J software. A graph was drawn by plotting the % migration against treatment. The absorbance value of the wound area was calculated using a template for the original wound (by photographing an empty insert). The absorbance of a confluent monolayer was similarly determined and the % migration was the absorbance in the wound divided by the absorbance of a confluent monolayer x100.

2.3.14 Migration assay using a Cytoselect™ 24-well Cell Migration Assay

Following the manufacturer's instructions, 300µl of a cell suspension containing $1x10^6$ cells/ml in serum free medium with the venom at final concentrations of 1.5 or

3.1µg/ml were added to the inside of each polycarbonate membrane inserts (8µm pore size to assay the migratory properties of cells) in a 24 well plate (200µl of cell suspension and 100µl of venom at 4.5 and 9.375µg/ml). Culture medium (500µL) containing 20% (v/v) foetal calf serum (chemo attractant) was added to the lower compartment of the migration plate and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 24h. The medium was aspirated and the cells on the upper surface of the membrane were wiped using a cotton swab. The cells that migrated into the lower surface of the membrane were stained, photographed, and then the stain was extracted with extraction solution and quantified by measuring the absorbance at 560nm using a SpectraMax M5 micro plate reader. The results were calculated as % migration of the untreated control, which was considered 100% migration.

2.3.15 Invasion assay using a Cytoselect™ 24-well Cell Invasion Assay

Following the manufacturer's instructions, the protocol was as above, except the basement membrane layer of the cell culture inserts was rehydrated by adding 300µl of warm serum free media to the inner compartment and incubated at room temperature for 1h. The difference between the invasion assay kit and migration assay kit is the inserts are coated with a uniform layer of dried basement membrane matrix solution in the invasion assay inserts. The basement membrane layer in the invasion assay kit serves as a barrier to discriminate invading from non-invading cells. The results were calculated as a % invasion of the untreated control, which was considered 100% invasion.

2.3.16 Western blotting

2.3.16.1 Preparation of whole cell extracts

Cells were seeded at $1x10^6$ cells per well in 12 well plates. Venom was added at a final concentration of 3.1µg/ml for crude venom and 1.5µg/ml for fractions (see section 2.3.17), incubated at 37°C in a humidified atmosphere with 5% CO_2 for 4h. The plate was placed on ice to stop any protein degradation/dephosphorylation reactions and the medium was discarded. Two hundred µl of Laemmli's sample buffer (63mM Tris-HCl (pH6.8), 2mM $Na_4P_2O_7$, 5mM ethylenediaminetetraacetic acid (EDTA), 10% (v/v) glycerol, 2% (w/v) sodium dodecyl sulphate (SDS), 50mM dithiothreitol (DTT), 0.007% (w/v) bromophenol blue) was added. The cells were then scraped with a cell scraper, transferred to 1.5ml microfuge tubes and boiled for 5min to denature the proteins in the samples, before storing at -20°C until further use.

2.3.16.2 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Polyacrylamide gels were assembled in a Bio-Rad Mini-PROTEAN 3TM electrophoresis tank, with both reservoirs filled with electrophoresis buffer (25mM Tris base, 129mM glycine, 0.1%, w/v, SDS). A micro syringe was used to load the previously prepared samples into the wells (30µl/well). Pre-stained SDS-PAGE molecular weight markers of known molecular weights ranging from 10,000 to 250,000Da were run concurrently in order to aid identification of the separated protein bands of interest. Samples were electrophoresed at a constant voltage of 130V, until the bromophoenol dye had reached the bottom of the gel.

2.3.16.3 Electrophoretic transfer of proteins to nitrocellulose membrane

The proteins separated by SDS-PAGE were transferred to nitrocellulose membranes using electrophoretic blotting following a standard protocol (Towbin *et al.*, 1979). The gel was pressed firmly against a nitrocellulose sheet and assembled in a transfer cassette sandwiched between 2 filter papers and two sponge pads. The cassette was immersed in transfer buffer (25M Tris base, 19mM glycine, 20%, v/v, methanol in water) in a Bio-Rad Mini-PROTEAN 3TM electrophoresis tank and a constant current of 300mA was applied for 1h, whilst the tank was cooled by inclusion of an ice reservoir.

2.3.16.4 Immunological detection of protein

Following transfer of the proteins from the gel to the nitrocellulose membrane, the membrane was removed and any remaining protein blocked by incubation in a solution of 3% (w/v) BSA in NaTT buffer (150mM NaCl, 20mM Tris, pH 7.4, 0.2%, v/v, Tween -20) for 90min with gentle agitation on a platform shaker. The blocking buffer was then removed and membranes incubated overnight with antiserum specific to the target protein diluted 1:1000 in NaTT buffer containing 1% (w/v) BSA . On the following day, membranes were washed in NaTT at 15min intervals for 90min with gentle agitation. The membranes were then incubated for a further 90min at room temperature with secondary horseradish peroxidase conjugated IgG directed against the first immunoglobulin diluted 1:1000 in NaTT buffer containing 1% (w/v) BSA. After 6 washes in NaTT for 90 min as described before, immunnoreactive protein bands were detected by incubation in enhanced chemiluminescence (ECL) reagent for 3min with agitation. The membranes were then mounted onto an exposure cassette, covered with cling film, then exposed to

X-ray film (B Plus – Full Blue) until correct development had occurred in a darkroom and developed using a KODAK M35-M X-OMAT processor. P65 was used as loading control to normalize the levels of protein detected by confirming that protein loading is the same across the gel. The protein levels of loading controls remain constant under the tested conditions.

2.3.17 Size exclusion chromatography

Size exclusion chromatography is a separation technique based on the molecular size of the components. Gel filtration chromatography is mainly used for the fractionation of proteins and this technique is good for separation of large molecules from small molecules. The column used is filled with material (such as Sephadex) containing many pores. Small molecules diffuse into the pores so they flow more slowly through the column, while large molecules flow quickly through the column because they do not pass into the pores.

2.3.17.1 Column packing and preparations

Sephadex G-75 slurry was prepared with ammonium acetate buffer (0.1 M, pH 6.8) and autoclaved for 121˚C for 20min. A column (1.5 x 100 cm) was secured onto a clamp stand. Running buffer was added gently and the Sephadex slurry was added to the column from which air bubbles had been removed under vacuum. The Sephadex was allowed to settle under gravity and the column was opened and the buffer allowed to flow through. The column was washed with ~2-3 volumes of running buffer.

2.3.17.2 Venom preparation

Snake venom (100mg) was dissolved in 700µl of ammonium acetate buffer (0.1 M, pH 6.8), followed by centrifugation at 5000g for 10min to remove any insoluble

material. The supernatant was applied to the Sephadex column. Venom fractionation was carried out under isocratic conditions, using a constant flow rate 0.3mL/min of ammonium acetate buffer and the chromatographic run was monitored by UV absorbance at 290nm. SDS-PAGE was used for further analysis of the fractions to make sure the separation was efficient.

2.3.17.3 Lyophilisation

The fractions obtained from the columns were shown as peaks on the screen of a desktop computer attached to the chromatography set up. All fractions obtained were kept at -80°C overnight, then all the fractions were lyophilised in a freeze dryer for 72h and stored at −20°C for further experiments.

2.3.18 Fraction identification

2.3.18.1 SDS-PAGE

SDS-PAGE was carried out by preparing a stock solution of venom (3mg venom in 1ml distilled water). Stain buffer (5µl) and venom (15µl) were added to a 1.5ml microfuge tube, then heated at 70ºC for 15min. Polyacrylamide gels were assembled in a Bio-Rad Mini-PROTEAN 3TM electrophoresis tank, with both reservoirs filled with electrophoresis buffer and 20µl of each sample was added to each well. The leads were attached according to the colour code. Then 130V of current was passed through the electrophoresis tank. When the first band reached the lower edge of the gel the current was stopped. The gel was removed and placed in a plastic container. Fresh distilled water was added to the gel with gentle agitation for 5min and this was repeated thrice. Then, the water was removed and replaced with G250 stain with gentle agitation on a platform shaker for 1h. Then the stain was removed and the gel was placed in distilled water for 30min and the water replaced with fresh distilled

water. Images were taken by mobile phone camera and the bands of interest were excised and sent to the Proteomics Laboratory, Glasgow Polyomics Wolfson Wohl Cancer Research Centre performed by Dr. Richard Burchmore for identification.

2.3.18.2 Nanoflow HPLC Electrospray Tandem Mass Spectrometry (nLC-ESI-MS/MS)

MS/MS analysis was used for protein identification. Peptides were solubilised in 2% (v/v) acetonitrile containing 0.1% (v/v) trifluroacetic acid and fractionated on a nanoflow uHPLC system before online analysis by electrospray ionisation (ESI) mass spectrometry on an Amazon Speed ion trap MS/MS. MS analysis was performed using a continuous duty cycle of survey MS scan followed by up to ten MS/MS analyses of the most abundant peptides, choosing the most intense multiply charged ions with dynamic exclusion for 120s. MS data was processed using Data Analysis software (Bruker) and the automated Matrix Science Mascot Daemon server (v2.4.1). Peptide identifications were assigned using the Mascot search engine to interrogate protein sequences in the NCBI Genbank database, allowing a mass tolerance of 0.4Da for both MS and MS/MS analyses.

2.3.18.3 Liquid chromatography mass spectrometry (LC-MS)

LC-MS was used to determine the mass of proteins in fractions, and was performed by Dr. Tong Zong (SIPBS Department at University of Strathclyde). One mg of fraction was dissolved in 1ml distilled water and 10µl of the solution was injected along with a gradient from 90% water and 10% (v/v) acetonitrile containing 0.1% (v/v) formic acid to 90% (v/v) acetonitrile and 10% water containing 0.1% (v/v) formic acid at a flow rate of 0.3ml/min. High resolution electron impact mass spectra

were recorded. Positive ion and negative ion mode ESI experiments were carried out on a ThermoFinnigan LTQ-Orbitrap.

2.3.18.4 Assessment of the metalloproteinase inhibitor ethylenediaminetetraacetic acid (EDTA) on activity of F3 from *Crotalus ruber ruber* venom

Ethylenediaminetetraacetic acid (EDTA) is an inhibitor of metalloproteases and acts as a chelator of the Zn^{2+} in the active site of metalloproteases. Cells were seeded at $3x10^5$ cells per ml in a 24 well plate. The third fraction (F3) was prepared and added to the cells at a final concentration of 1.5µg/ml and incubated with a serial dilution of EDTA from 25µg/ml to 100µg/ml at 37^{o}C in a humidified atmosphere with 5% CO_2 for 24h. A Nikon TMS inverted phase contrast microscope was used to record images. After which, the medium was removed and cells washed with PBS, fixed with PFA, stained with crystal violet and lysed with 0.1% (v/v) triton X-100. Absorbance readings at 540nm was taken using a SpectraMax M5 micro plate reader. The graph for this experiment was plotted of the absorbance (cell adhesion) against different concentrations of EDTA with 1.5µg/ml of F3.

2.3.18.5 Assessment of metalloproteinase inhibitor (1,10-phenanthroline) on the activity of F6 of *Agkistrodon piscivorus leucostoma* venom

Phenanthroline is an inhibitor of metalloproteinase and mainly targets zinc metalloproteinases with a much lower affinity for calcium. The protocol was as above, except the concentration of 1, 10-phenanthroline was 25µg/ml because a high concentration has a toxic effect on A2780 cells.

3.0 Result

A preliminary study was carried out using different snake venoms and *Crotalus ruber ruber* and *Agkistrodon piscivorus leucostoma* venoms were chosen because of their effect on the morphology of A2780 cells. About 100 ovarian cancer cell lines are available. Genomic differences between cancer cell lines and tissue samples have been pointed out in several studies. The most commonly used cell line models for ovarian cancer are SK-OV-3, A2780, OVCAR-3, CAOV3 and IGROV1. The two most frequently used ovarian cancer cell lines SK-OV-3 and A2780. In the current work A2780 cell line was used because its commonly used and also this cell line express α5β1 integrin at high level.

3.1 Effects of *Crotalus ruber ruber* venom on adhesion, migration and invasion of A2780 cells

The overall aim of this section was to examine the effect of *Crotalus ruber ruber* snake venom and its active fraction on morphology, viability, adhesion, migration and invasion of A2780 cells. This was achieved by:

- Examining the effect of the crude venom and its fractions on the morphology of cells under light microscopy. A preliminary study was carried out using a range of concentrations of the venom at different time points and observing the effect on the cells. Based on this, an appropriate non-toxic concentration and time point was chosen for further experiments.
- Examining the viability of the cells treated with venom using SYTOX®Green which is a high-affinity nucleic acid stain that easily penetrates cells with compromised plasma membranes, but will not penetrate intact cell membranes.

- Carrying out cell viability assays at additional time points to rule out the probability that the activity of the venom against ovarian cancer cell migration, adhesion, and invasion is due to cytotoxicity; the cell viability assay was carried out at 24, 48 and 72h.
- Examining the main integrins expressed by A2780 cells and the integrins that are used for their adhesion on fibronectin and vitronectin.
- Examining the effect of the venom on adhesion of A2780 cells on fibronectin, vitronectin, and poly-L-lysine in order to determine if the venom is specific for the integrin family of adhesion receptors.
- Investigating the effect of the venom on migration of A2780 cells using a wound closure assay and to confirm the inhibitory effect of the venom on migration of A2780 cells using a Cytoselect^{TM} 24-well Cell Migration Assay.
- Investigating the effect of the venom on invasion of A2780 cells across the basement membrane.
- Examining the effect of the venom on the levels of α5, β1, and αv integrins expressed by A2780 cells.
- Fractionating of venom to obtain the component responsible for the activity of venom against dissemination of ovarian cancer cells.
- Demonstrating the effect of the active fraction of venom on morphology, viability, adhesion, migration and invasion of A2780 cells.
- Identifying the active component in the fraction by carrying out Nanoflow HPLC Electrospray Tandem Mass Spectrometry, liquid chromatography mass spectrometry (LC-MS) and assessment of the effects of metalloproteinase inhibitors.

3.1.1 Growth characteristics of the A2780 ovarian cancer cell line

A2780 cells have a typical epithelial, round morphology and grow in tight clusters (Figure 6).

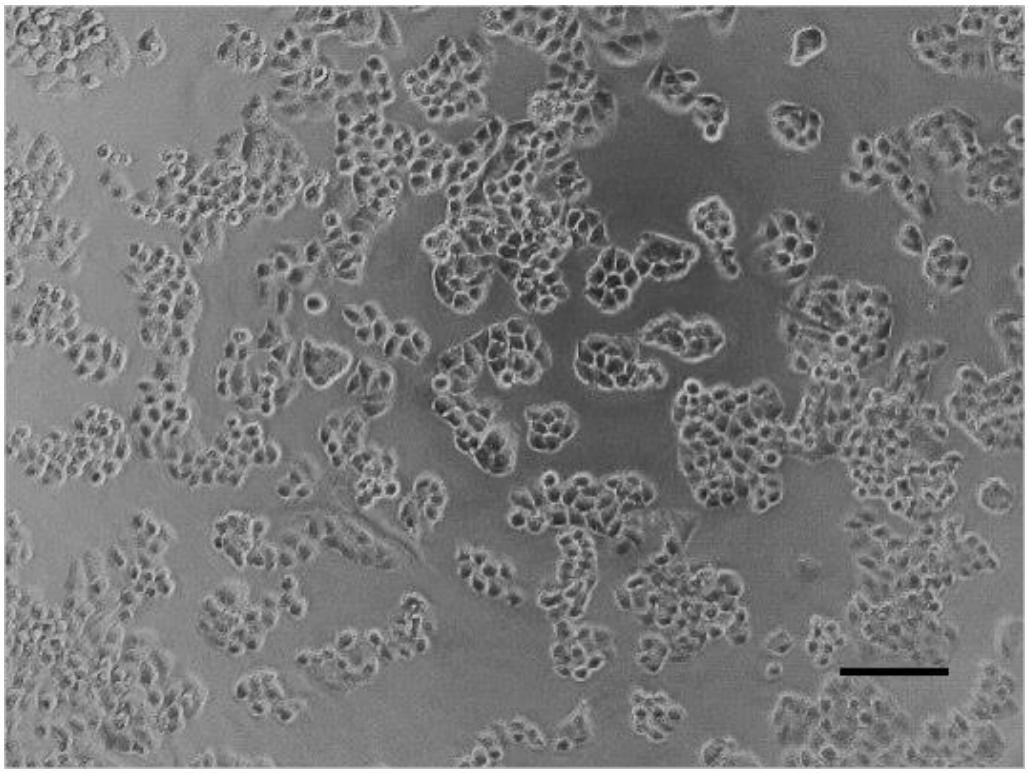


Figure 6: Cell morphology of A2780 cells. Cells were seeded in 96 well plates at $1x10^4$ cells per well in complete medium and grown overnight at 37°C in a humidified atmosphere with 5% CO_2. Images were taken using a Nikon TMS inverted phase contrast microscope. The magnification bar represents 100µm. Objective lens x10.

3.1.2 Growth curve for A2780 cells

The doubling time for A2780 cells was determined by seeding the cells in 12 flasks (T25). Figure 7 shows the lag, log, stationary, and decline phase for A2780 cells and that the cells started to double after 24h.

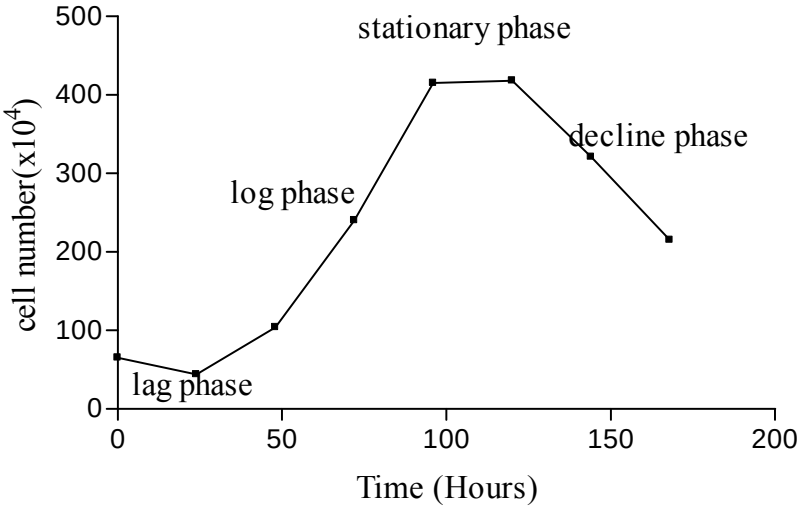
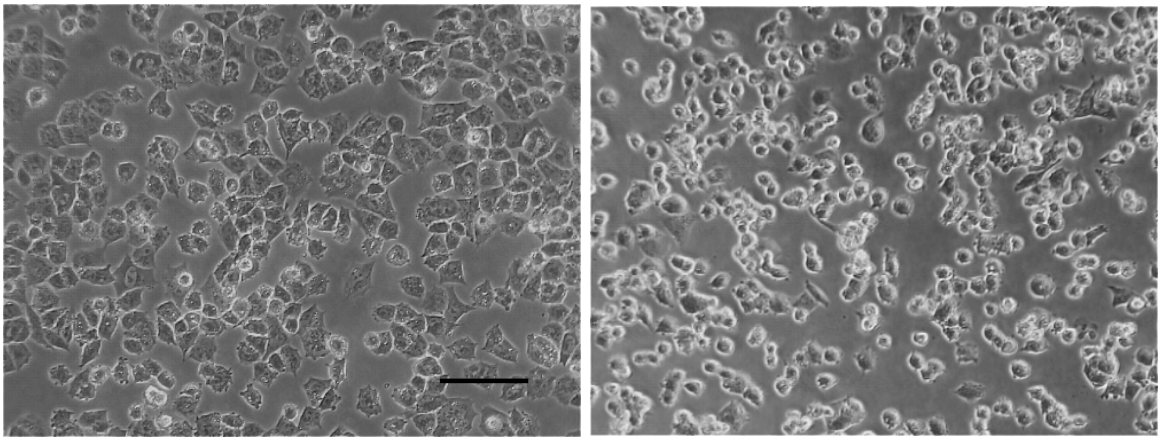


Figure 7: Growth curve of A2780 cells. The cell number was adjusted to $65x10^4$ cells/flask in complete medium and 12 flasks were prepared. The cell numbers were counted for 6 days (two flasks per day).

3.1.3 Effect of *Crotalus ruber ruber* venom on the morphology of A2780 cells after 4h

This experiment was carried out to determine the effect of venom at 3.1µg/ml on the morphology of A2780 cells after 4h. The control well shows some degree of cell attachment and spreading. Wells that had *Crotalus ruber ruber* venom showed the cells were clustered together, rounded up and poorly spread, suggesting there is an effect on adhesion (Figure 8).



Control 3.1µg/ml of venom

Figure 8: **Effect of *Crotalus ruber ruber* venom on the morphology of A2780 cells after 4h.** Cells were seeded at $1x10^6$ cells per well in a 12 well plate and incubated with venom (3.1µg/ml) for 4h. Images were taken using a Nikon TMS inverted phase contrast microscope. The magnification bar represents 50µm. Objective lens x20.

3.1.4 Effect of *Crotalus ruber ruber* venom on membrane integrity of A2780 cells after 4h

This experiment was carried out using SYTOX®Green in order to prove the morphological alterations of A2780 cells after adding *Crotalus ruber ruber* venom was not due to cytotoxicity. There was no effect on membrane integrity at concentrations between 0.1-3.1µg/ml, but with 1.5 and 3.1µg/ml, changes in morphology were observed. There was an effect on membrane integrity at high concentrations 6.25 and 12.5µg/ml (Figure 9).

3.1.5 Effect of *Crotalus ruber ruber* venom on A2780 cells viability after 24, 48 and 72h

This experiment was carried out using an AlamarBlue® assay. The effect of different concentrations of venom on the viability of A2780 cells after 24, 48 and 72h was examined. *Crotalus ruber ruber* venom was found to have a significant (P value < 0.01) effect on the viability of the A2780 cells at concentrations 6.25 and 12.5µg/ml at all the time periods, but the viability of the cells was not affected at low concentrations of venom at 24 and 48h (Figures 10A and B). The viability was affected at 3.1µg/ml and above after 72h (Figure 10C).

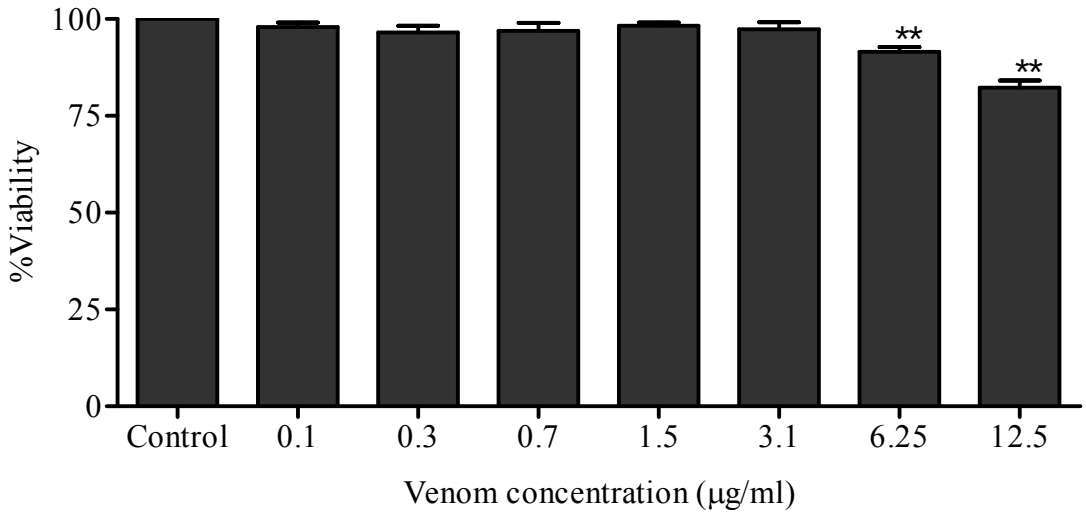
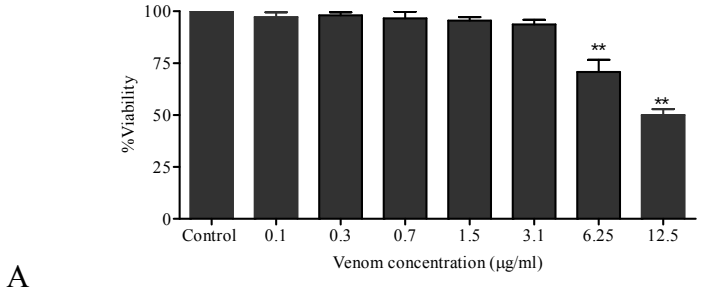
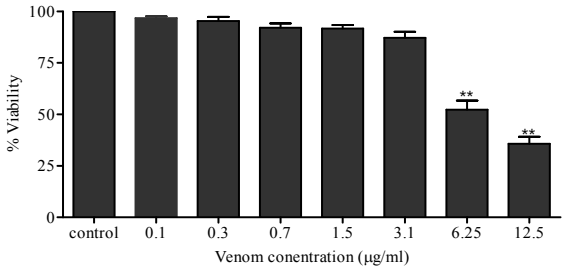


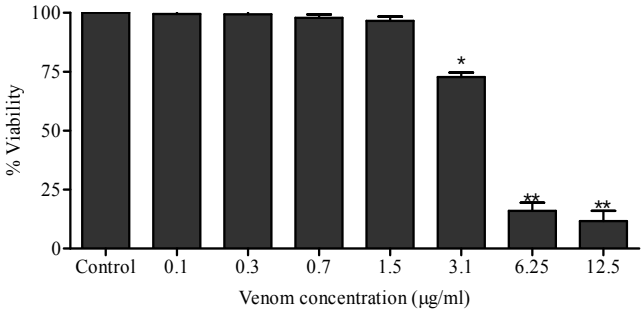
Figure 9: Effect of *Crotalus ruber ruber* venom on A2780 cells membrane integrity after 4h. Cells were seeded at $1x10^4$cells per well in complete medium in a 96 well plate (75µl) and incubated for 24h. Venom prepared in complete medium was added to the wells (25µl) at a final concentration between 0.1-12.5µg/ml and incubated for 4h. SYTOX®Green at a final concentration of 5µM/well was incubated for 15min at 37°C. The fluorescence intensity at 485-535nm was measured. Values represent the mean ± SEM of 3 values. Statistical analysis was performed using one way ANOVA with Dunnett's post-hoc test. ** indicates significantly ($p < 0.01$) lower values compared with the untreated control.



A



B



C

Figure 10: Effect of *Crotalus ruber ruber* venom on viability of A2780 cells after 24(A), 48 (B) and 72(C)h. Cells were seeded in a 96 well plate at $1x10^4$ cells per well in complete medium (75µl) and incubated for 24h. Following which, venom was prepared and added to the cells at final concentrations between 0.1-12.5µg/ml, incubated for 24, 48 and 72h. Ten µl AlamarBlue® was added to each well for 6h before the end of the incubation period. Fluorescence intensity at 560-590 nm was read. Statistical analysis was performed using one way ANOVA with Dunnett's post-hoc test. * and ** indicates significantly ($p < 0.05$, $p < 0.01$) lower values compared with the untreated control, respectively.

3.1.6 Spreading of A2780 cells on plastic and various substrata.

The purpose of this experiment was to assess the spreading of A2780 cells on plastic, fibronectin, vitronectin and collagen. A2780 cells on fibronectin showed a high degree of cell attachment and spreading, while on vitronectin the cells showed a lower degree of attachment. However, cells were not spread or adherent on collagen and plastic (Figures 11 and 12).

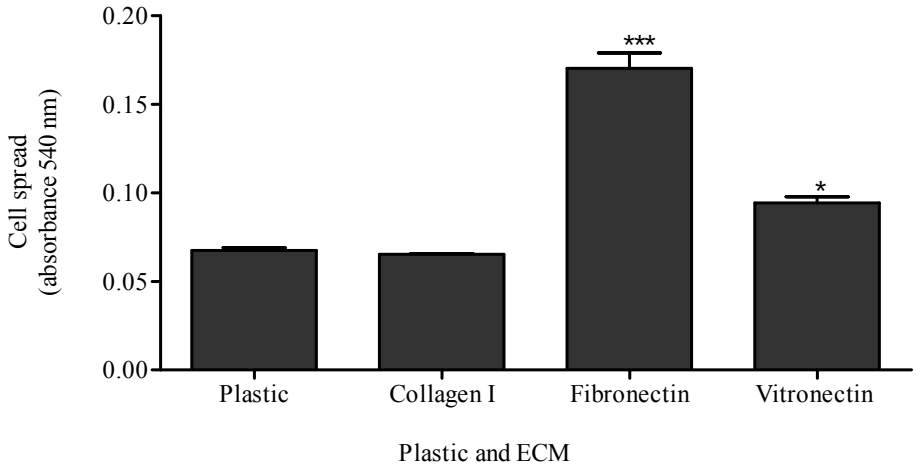


Figure 11: Spreading of A2780 cells on plastic and ECM (collagen, fibronectin and vitronectin). A2780 cells were adjusted to $4x10^5$cells/ml in 0.2% (w/v) BSA in RPMI 1640 and seeded onto plates coated with collagen (10µg/ml), fibronectin (10µg/ml), or vitronectin (5µg/ml) for 4h then fixed and stained with crystal violet. The absorbance was measured at 540nm. The values are means ± SEM of 3 values. Statistical analysis was performed using one way ANOVA with Bonferrori's multiple comparison tests. * and *** indicates significantly ($p < 0.05$, $p < 0.001$) higher values compared with cells seeded on plastic, respectively.

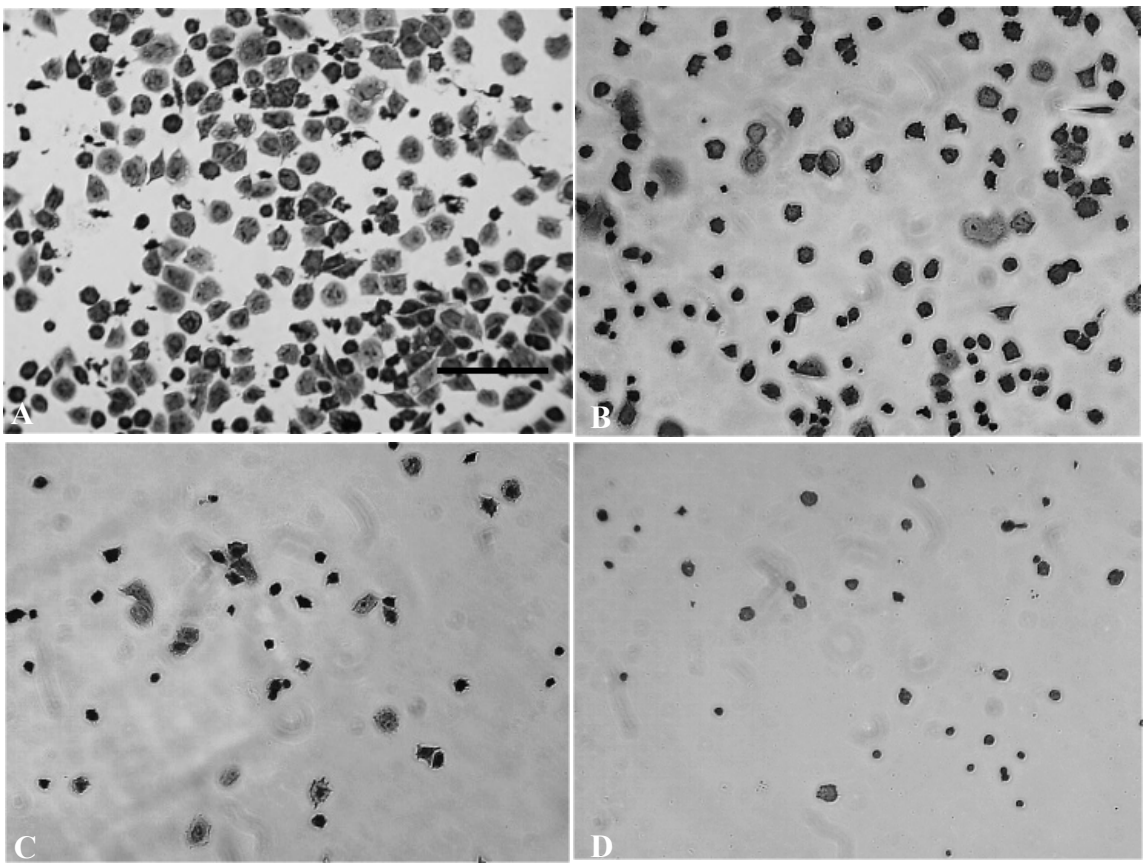


Figure 12: Spreading of A2780 cells on plastic and different extracellular matrix proteins. Ninety six well plates were coated with (A) fibronectin (10µg/ml), (B) vitronectin (5µg/ml), and (C) collagen I (10µg/ml) or left untreated (D). A2780 cells were adjusted to $4x10^5$ cells/ml in 0.2% (w/v) BSA in RPMI 1640 and added to the coated plates and incubated for 4h. After which, the cells were washed, fixed and stained with crystal violet, Images were taken. The magnification bar represents 50µm. Objective lens x20.

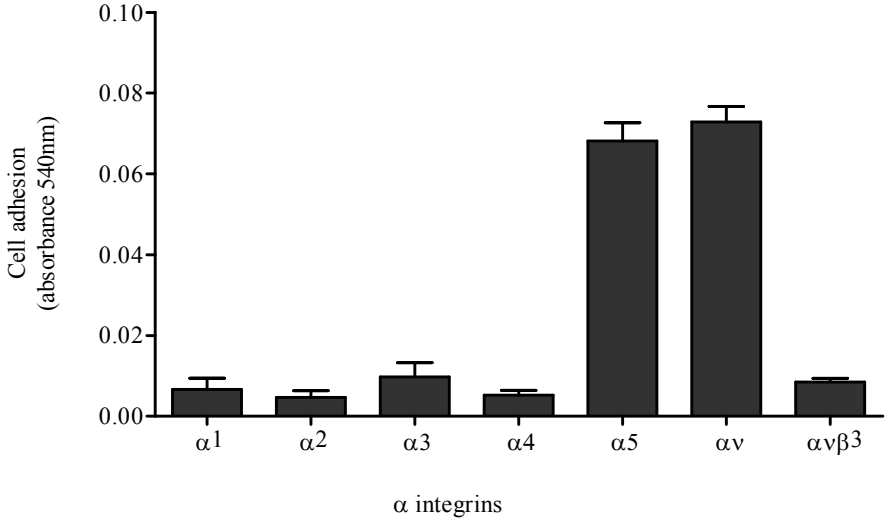
3.1.7 Integrin expression by A2780 cells

The purpose of this experiment was to determine the major integrins expressed by A2780 cells. The result showed that the major α integrins expressed by A2780 cells are α5, αv (Figure 13A) and the major β integrins are β1 and α5β1 (Figure 13B).

3.1.8 Effect of function blocking integrin antibodies on A2780 cells adhesion to fibronectin and vitronectin

After determining the principle integrins expressed by A2780 cells from the previous experiment, this experiment was carried out to determine which integrins were involved in the interaction between A2780 cells and ECM (fibronectin and vitronectin). Function blocking integrin antibodies to β1, α5, and αv inhibited adhesion of A2780 cells to fibronectin, although the effect of function blocking antibodies to β1, α5 had a greater effect than the function blocking antibodies to αv on A2780 cells adhesion (Figures 14A and 15A). Function blocking integrin antibodies to αv have an effect on adhesion of A2780 cells to vitronectin (Figures 14B and 15B).

A



B

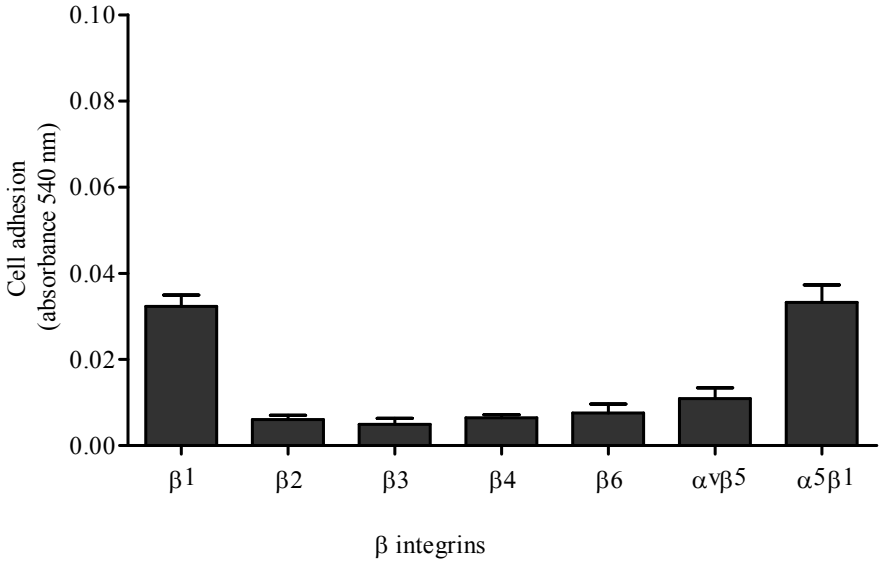
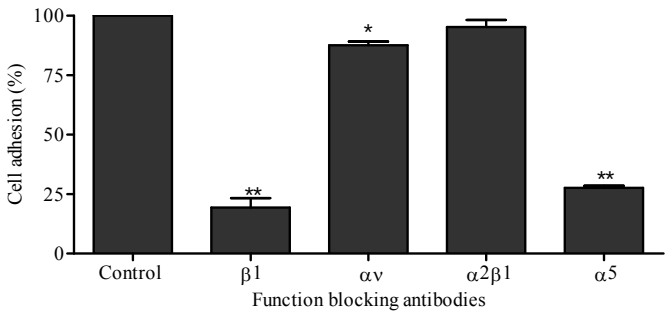
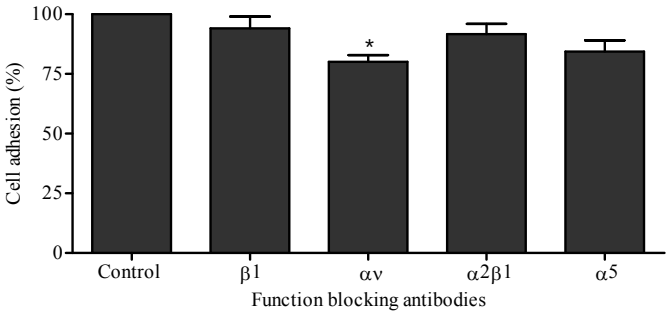


Figure 13: α and β integrins expressed by A2780 cells. A2780 cells were adjusted to $4x10^5$ cells /ml in serum free RPMI 1640 medium and seeded onto (A) alpha and (B) beta integrin binding plates for 4h at 37°C. Then the plates were fixed, stained and extraction solution was added to each well to elute the stain. The absorbance at 540nm was measured and the values represent means ± SEM of 3 values.



A



B

Figure 14: Effect of function blocking integrin antibodies on A2780 cells adhesion to (A) fibronectin and (B) vitronectin. Cells were adjusted to $4x10^5$ cells/ml in 0.2% (w/v) BSA in RPMI 1640 (90µl/well) and plated onto pre-coated (with fibronectin or vitronectin) 96 well plates. After which, 10µl of each antibodies (final concentration 10µg/ml) was added per well and cultured the plates for 4h at 37°C. The plate was then washed, fixed, stained with crystal violet, and lysed with 0.1% (v/v) Triton X-100. The absorbance was measured at 540nm and the values represent means ± SEM of 3 values. Statistical analysis was performed using one way ANOVA with Dunnett's post-hoc test. * and ** indicates significantly ($p < 0.05$, $p < 0.01$) lower values compared with the untreated control, respectively.

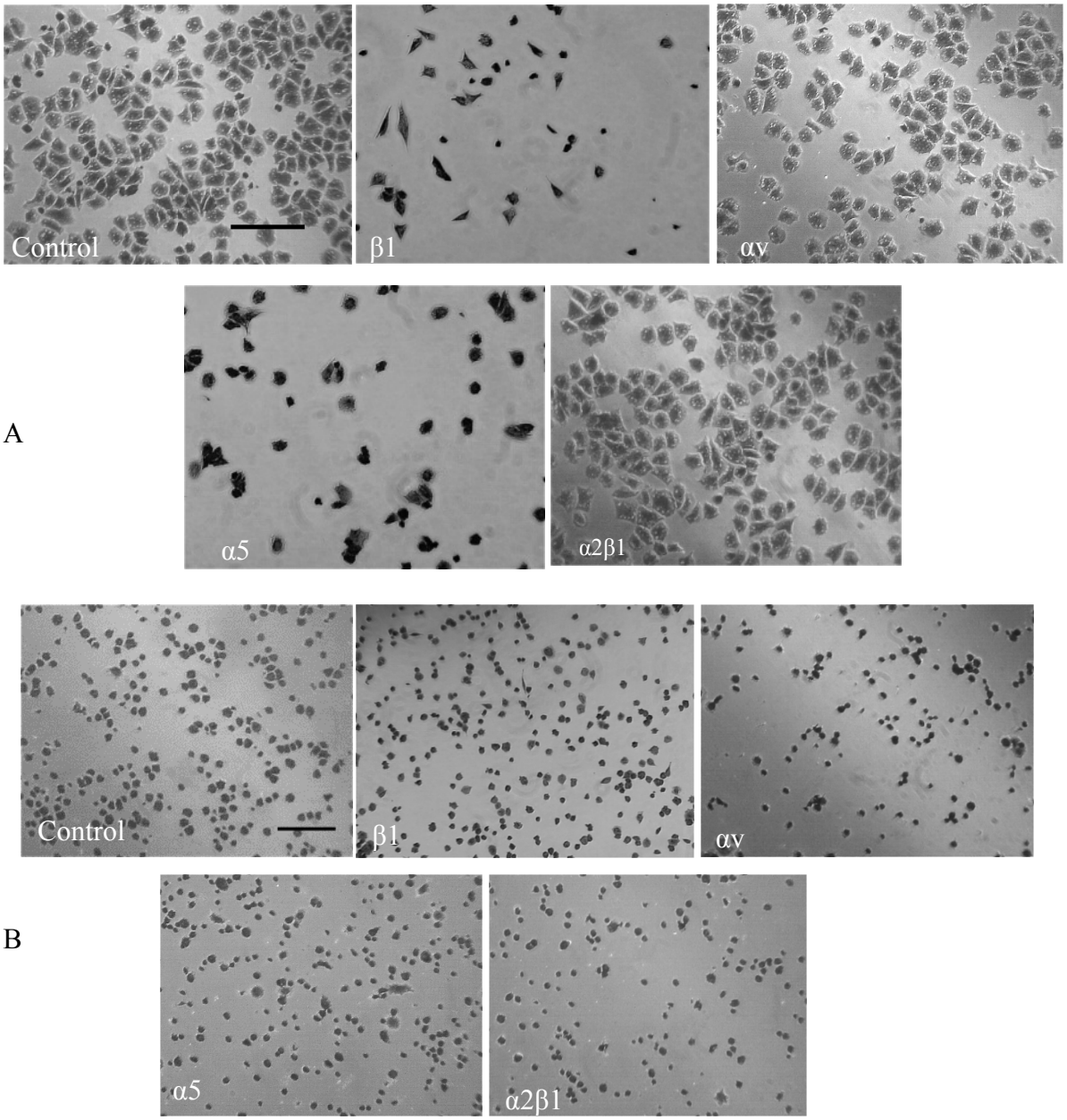


Figure 15: Effect of function blocking integrin antibodies on A2780 cells adhesion to (A) fibronectin and (B) vitronectin. Cells were adjusted to $4x10^5$ cells/ml then plated in 96 well plates coated with fibronectin (10µg/ml) or vitronectin (5µg/ml) with 10µl of antibodies (10µg/ml) for 4h. After washing, adherent cells were fixed, stained with crystal violet and images were taken. The magnification bar represents 50µm. Objective lens x20.

3.1.9 Effect of function blocking integrin antibodies on A2780 cells adhesion to poly-L-lysine

The purpose of this experiment was to examine whether the adhesion of the cells to poly-L-lysine involve integrins or not. There was no effect of function blocking antibodies on adhesion of A2780 to poly-L-lysine (Figure 16).

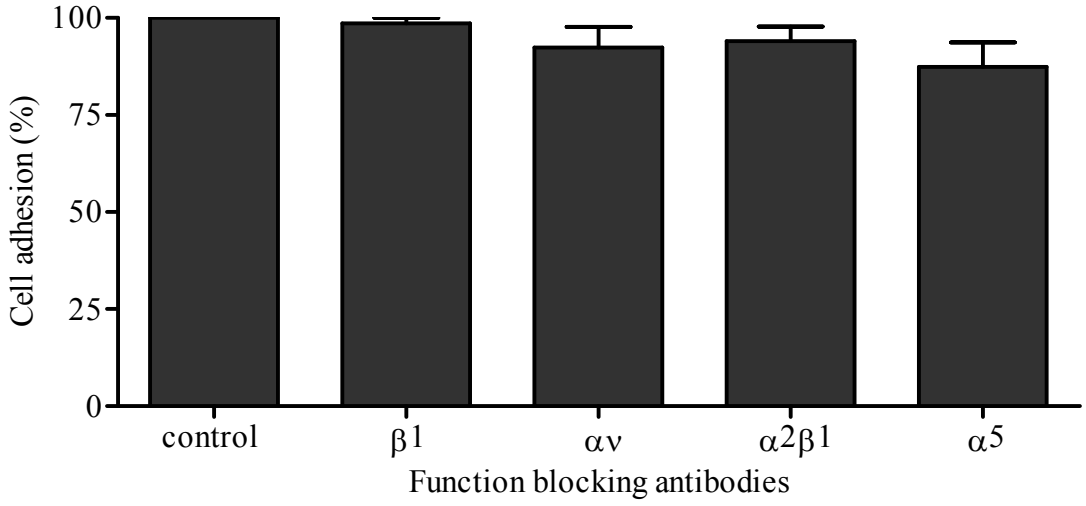


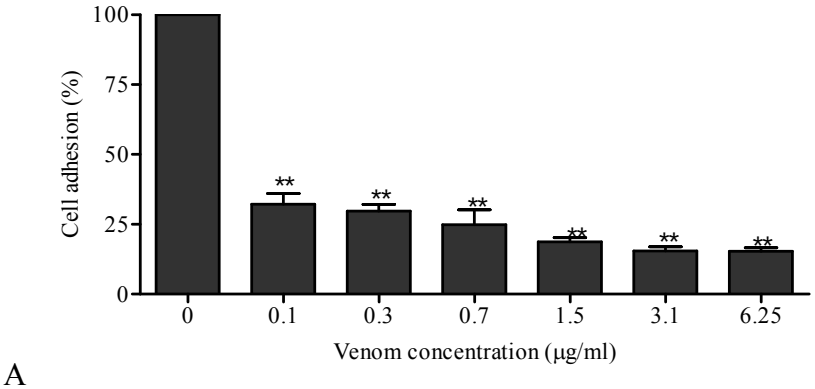
Figure 16: Effect of function blocking integrin antibodies on A2780 cells adhesion to poly-L-lysine. Cells were adjusted to $4x10^5$ cells/ml in 0.2% (w/v) BSA in RPMI 1640 (90µl/well) and plated onto pre-coated 96 well plates (with poly-L-lysine 50µg/ml) together with 10µl/well antibodies (final concentration 10µg/ml). The cells were cultured for 4h at 37°C. The cells were washed, fixed, stained with crystal violet, and lysed with 0.1% (v/v) Triton X-100. Absorbance at 540nm was read and the values represent means ± SEM of 3 values. Statistical analysis was performed using one way ANOVA with Dunnett's post-hoc test.

3.1.10 Effect of *Crotalus ruber ruber* venom on adhesion of A2780 cells to vitronectin and fibronectin

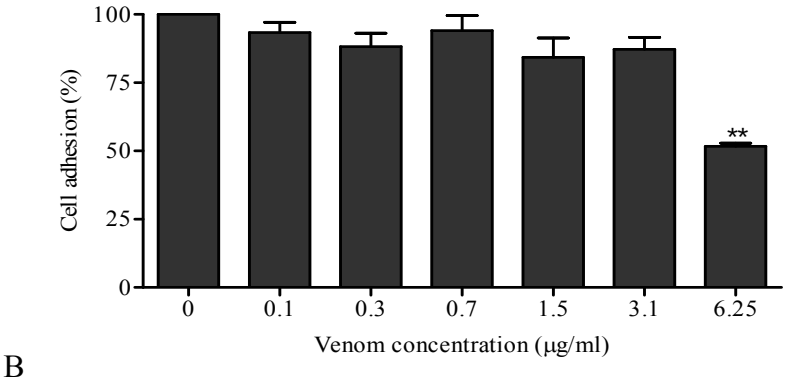
The effect of venom on adhesion of A2780 cells in the presence of fibronectin and vitronectin was examined. A2780 cells adhered and spread well on fibronectin in untreated wells. After adding the venom, cells became rounded up and lost their adherence to fibronectin (Figures 18A). After washing, most of the cells were lost. The effect of *Crotalus ruber ruber* venom on adhesion of A2780 cells to fibronectin was dose dependent (Figure 17A). A2780 cells did not spread well on vitronectin and there was no effect of the venom on the adhesion of the cells to vitronectin (Figures17B and 18B).

3.1.11 Effect of *Crotalus ruber ruber* venom on adhesion of A2780 cells to an integrin-independent substratum (poly-L-lysine)

This experiment was carried out to assess the effect of *Crotalus ruber ruber* venom on adhesion of A2780 cells to poly-L-lysine. There was no effect of venom on adhesion of A2780 cells to poly-L-lysine (Figure 19) at concentrations that were not toxic to the cells (concentrations between 0.1-3.1μg/ml), suggesting that the effect of the venom is specific for the integrin family of adhesion receptors.

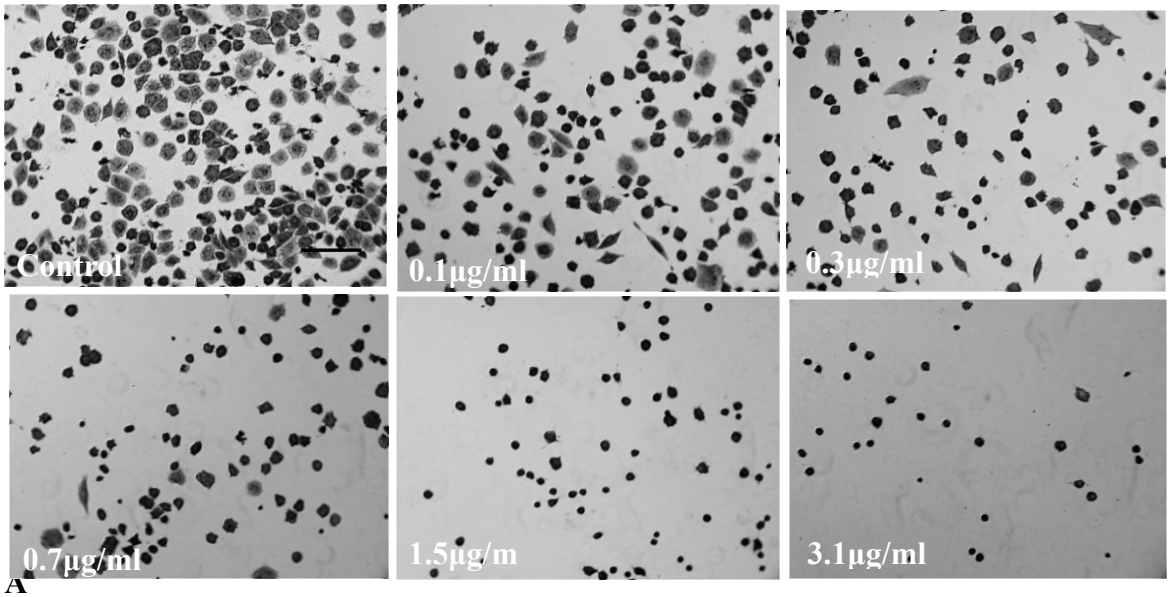


A

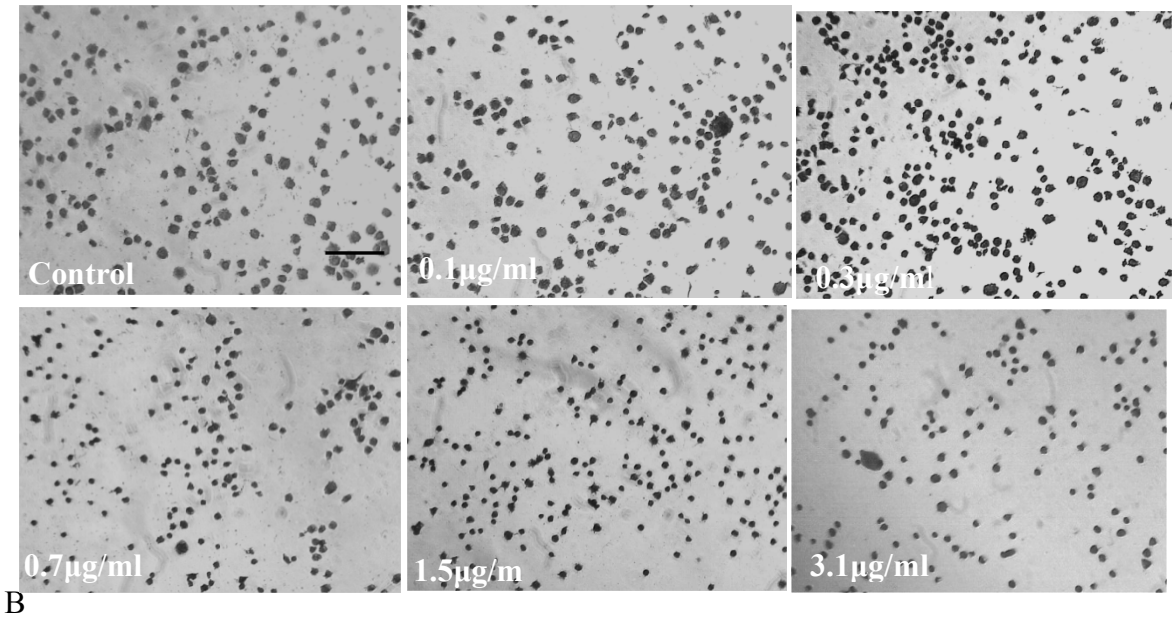


B

Figure 17: Effect of *Crotalus ruber ruber* venom on adhesion of A2780 cells in presence of (A) fibronectin and (B) vitronectin. Tissue culture wells of 96 well plates were coated with fibronectin or vitronectin, incubated for 1h, removed and washed. After which, 75µl of $4x10^5$cells/ml in 0.2% (w/v) BSA in RPMI 1640 with 25µl of venom at a final concentration between 0.1-6.25µg/ml was added to precoated plates and incubated at 37ºC. After 4h, the plate was washed, fixed, stained with crystal violet and lysed with 0.1% (v/v) Triton X-100. The absorbance was measured at 540nm. The values are means ± SEM of 3 values. Statistical analysis was performed using one way ANOVA with Dunnett's post-hoc test. ** indicates significantly ($p < 0.01$) lower values compared with the untreated control.



A



B

Figure 18: Effect of *Crotalus ruber ruber* venom on adhesion of A2780 cells to (A) fibronectin and (B) vitronectin. Tissue culture wells of 96 well plates were coated with fibronectin (A) or vitronectin (B). After which, cells with venom were added to the precoated plate and incubated at 37ºC. After 4h, the non-adherent cells were washed away and the adherent cells were fixed, stained, and images were taken. The magnification bar represents 50µm. Objective lens x20.

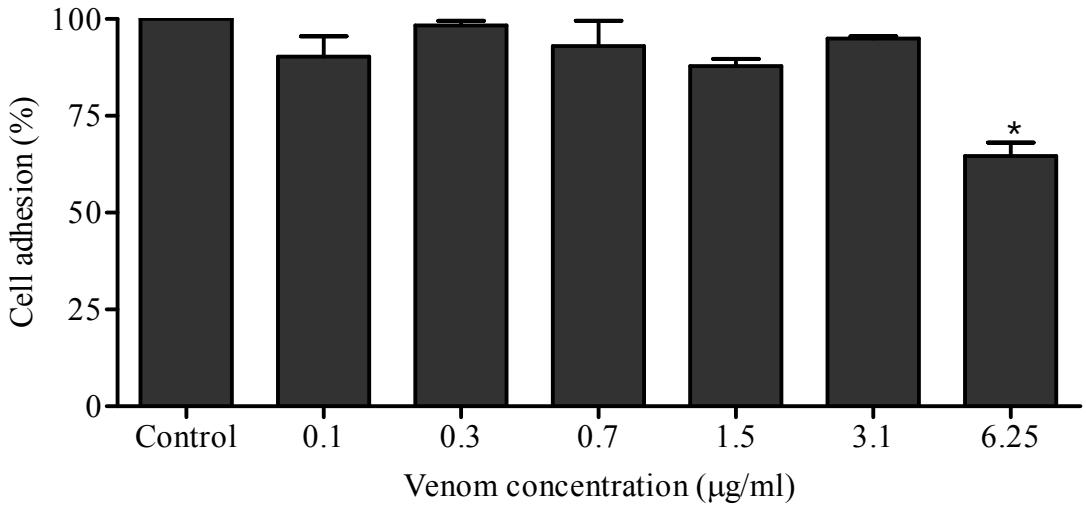


Figure 19: Effect of *Crotalus ruber ruber* venom on adhesion of A2780 cells to poly-L-lysine. Tissue culture wells of 96 well plate were coated with 50µg/ml of poly-L-lysine, incubated for 1h, removed and washed with PBS. After which, cells and venom at different concentrations were added and incubated. After 4h, the plate was washed, fixed, stained with crystal, and lysed with 0.1% (v/v) Triton X-100. The absorbance was measured at 540nm. The values are means ± SEM of 3 values. Statistical analysis was achieved using one way ANOVA with Dunnett's post-hoc test. * indicates significantly ($p < 0.05$) lower values compared with the untreated control.

3.1.12 Effect of *Crotalus ruber ruber* venom on migration of A2780 cells

The aim of this experiment was to determine the effect of *Crotalus ruber ruber* venom on migration of A2780 cells using a wound closure assay. The venom inhibited migration of cells in a dose dependent manner**;** the venom evidently inhibited migration at 1.5 and 3.1µg/ml (Figures 20 and 21). Both venom concentrations of 6.2 and 12.5µg/ml had a toxic effect on A2780 cells after 48h therefore these doses were not considered.

3.1.13 Migration assay using a Cytoselect™ 24-well Cell Migration Assay

The inhibitory effect of *Crotalus ruber ruber* venom on migration of A2780 cells was confirmed using a Cytoselect™ 24-well Cell Migration Assay. Both venom concentrations 1.5 and 3.1µg/ml had a significant ($p < 0.01$) inhibitory effect on migration (Figure 22). The % migratory inhibition of A2780 cells treated with 1.5 and 3.1µg/ml of the venom was 50 and 60% respectively.

3.1.14 Effect of *Crotalus ruber ruber* venom on invasion of A2780 cells across the basement membrane using a Cytoselect™ 24-well Cell Invasion Assay

The effect of *Crotalus ruber ruber* venom on invasion of A2780 cells across the basement membrane was assessed using a Cytoselect™ 24-well Cell Invasion Assay. The result of this experiment showed that *Crotalus ruber ruber* venom at 1.5 and 3.1µg/ml significantly ($p < 0.01$) inhibited the invasion of A2780 cells across the basement membrane (Figures 23 and 24). The results showed that the venom at a concentration of 3.1µg/ml had an inhibitory effect on invasion of A2780 cells of more than 45%.

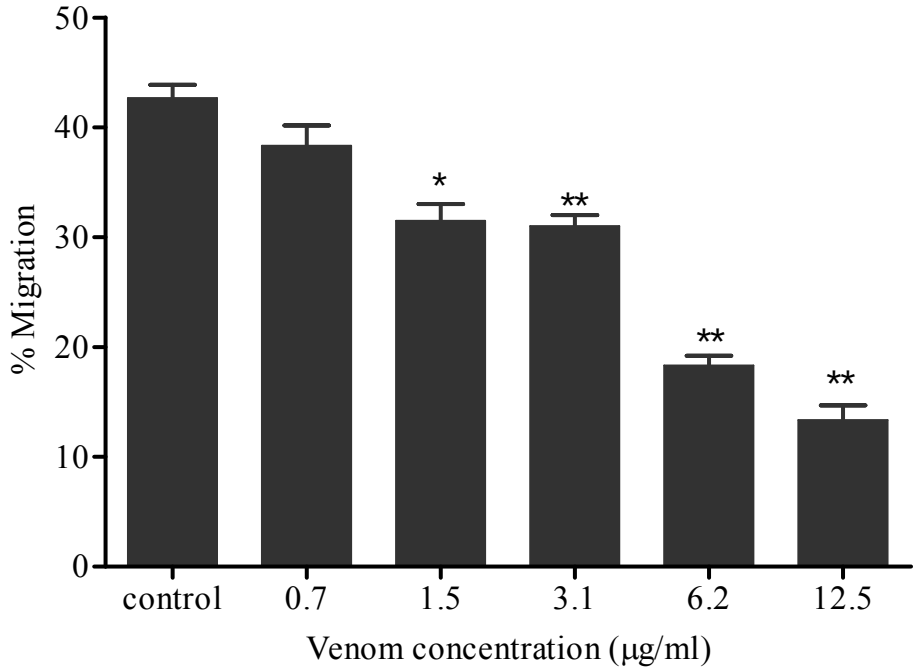


Figure 20: Effect of *Crotalus ruber ruber* venom on migration of A2780 cells using wound closure assay. The cell number was adjusted to $5x10^5$ cells/ml in complete medium and added into both sides of an ibidi migration chamber in a 24 well plate. After 24h the ibidi chamber was carefully removed, the medium discarded and 500µl of complete medium with different concentrations of venom between 0.7-12.5µg/ml were added and incubated for 48h at 37°C. After which, cells were fixed, stained with crystal violet, images were taken. Image J analysis was used to determine wound closure. Values are means ± SEM of 3 values. Statistical analysis was carried out using one way ANOVA with Dunnett's post-hoc test. * and ** indicates significantly ($p < 0.05$, $p < 0.01$) lower values compared with the untreated control, respectively.

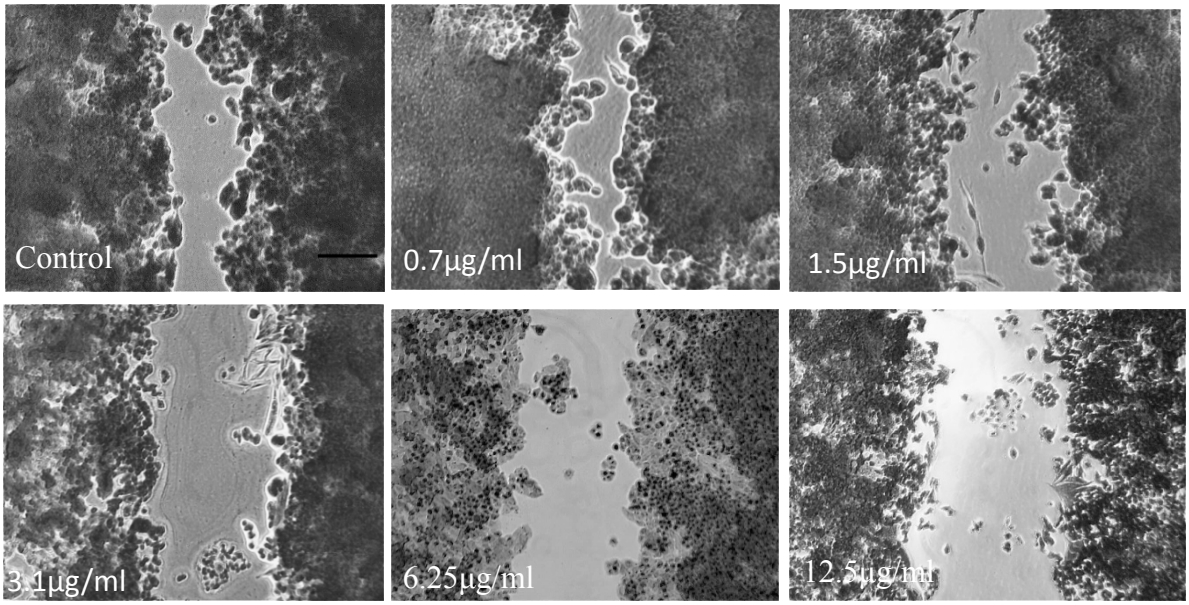


Figure 21: Effect of *Crotalus ruber ruber* venom on migration of A2780 cells using wound closure assay. The cells were prepared in complete medium and added into both sides of ibidi migration chambers in a 24 well plate. After 24h, the ibidi chambers were removed and fresh medium with serial dilution of venom was added and incubated for 48h. After which, the cells fixed, stained, and images were taken. The magnification bar represents 100µm. Objective lens x10.

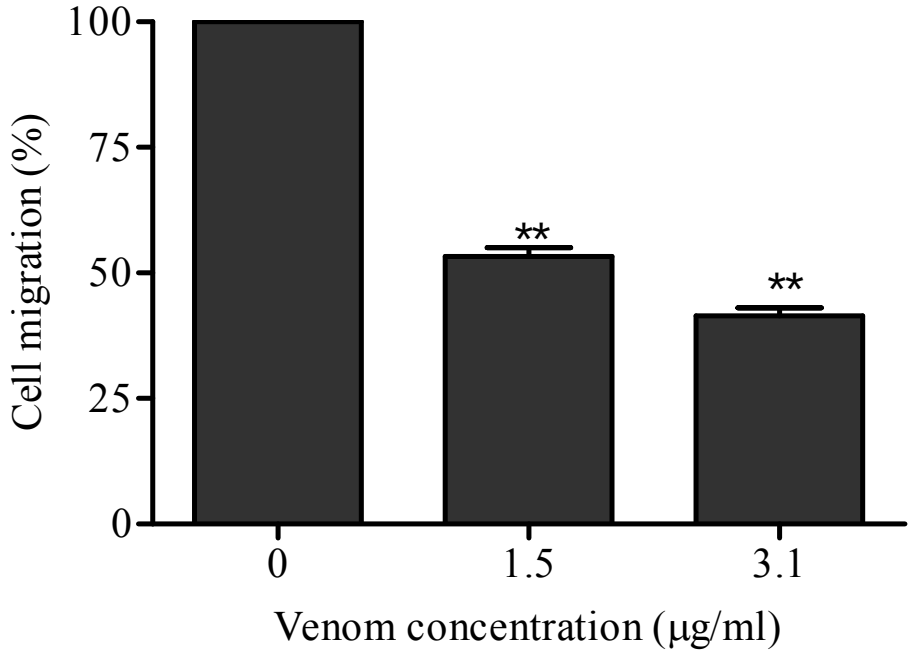


Figure 22: Effect of *Crotalus ruber ruber* venom on migration of A2780 cells using a cell migration assay. Three hundred µl of a cell suspension containing $1x10^6$ cells/ml in serum free medium with the venom at a final concentration of 1.5 or 3.1µg/ml was added to the inside of each insert in a 24 well plate. Then cells were incubated for 24h. The cells that migrated were stained, photographed, and quantified. The absorbance was measured at 560nm. The values are means ± SEM of 3 values. Statistical analysis was performed using one way ANOVA with Dunnett's post-hoc test. ** indicates significantly ($p < 0.01$) lower values compared with the untreated control.

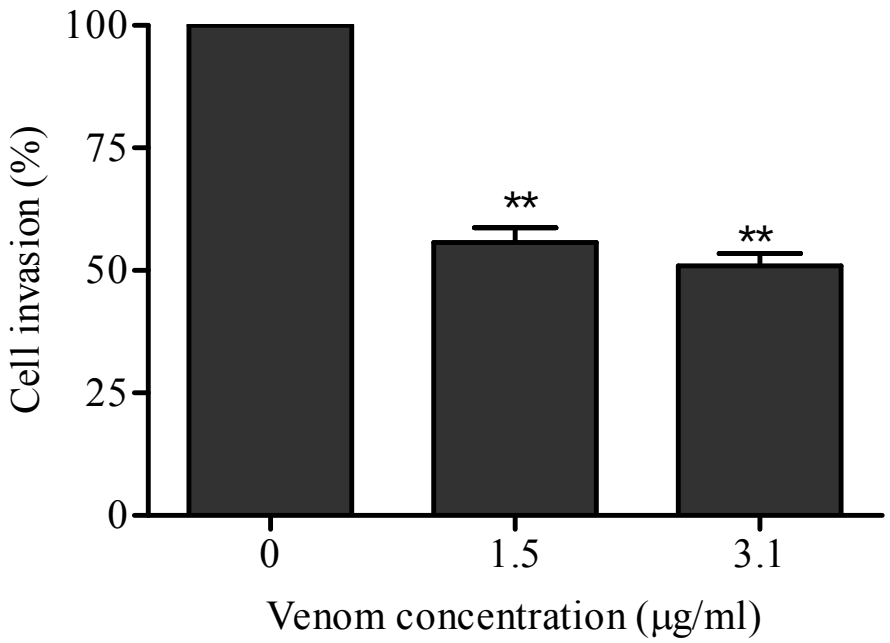


Figure 23: Effect of *Crotalus ruber ruber* venom on invasion of A2780 cells across the basement membrane. Cells were adjusted to $1x10^6$ cells /ml in serum free medium and 300 µl of cell suspension was added to each insert in a 24 well plate with venom at a final concentration 1.5 or 3.1µg/ml. The cells were incubated for 48h and those that invaded into the lower surface of the membrane were stained, photographed, and quantified. The absorbance was measured at 560nm. The values are means ± SEM of 3 values. Statistical analysis was performed using one way ANOVA with Dunnett's post-hoc test. ** indicates significantly ($p < 0.01$) lower values compared with the untreated control.

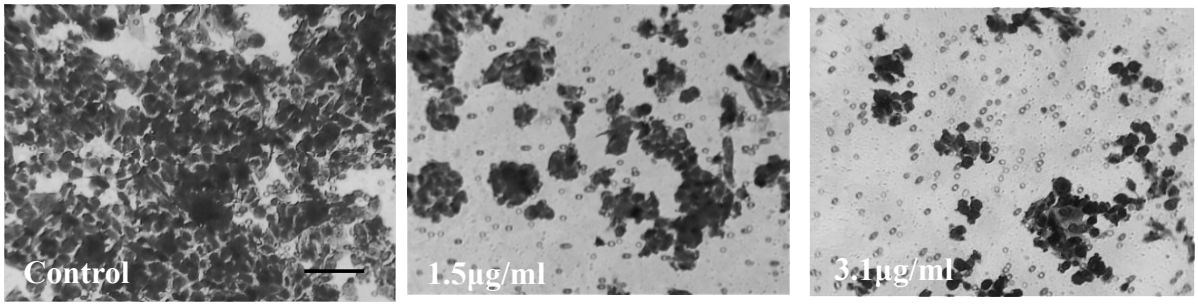


Figure 24: Effect of *Crotalus ruber ruber* venom on invasion of A2780 cells across the basement membrane. Cells were adjusted to $1x10^6$ cells /ml in serum free medium and added with 1.5 or 3.1µg/ml final concentration of venom to each insert in a 24 well plate. The cells were incubated for 48h and the cells that had invaded were stained and images taken. The magnification bar represents 50µm. Objective lens x20.

3.1.15 Effect of *Crotalus ruber ruber* venom on level of α5, β1, and αv integrins expressed by A2780 cells

From observations of the previous experiments of the effect of *Crotalus ruber ruber* venom on adhesion of the cells to fibronectin and vitronectin this experiment was carried out in order to determine which fibronectin or vitronectin receptor had been affected. The venom at 3.1μg/ml had an inhibitory effect on the level of α5 and β1 integrins expressed by A2780 cells (Figures 25A and B). However, there was no effect on the level of αv integrins (Figure 26). The results showed that the inhibitory effect of the venom on the level of expression of α5 and β1 is about 50%.

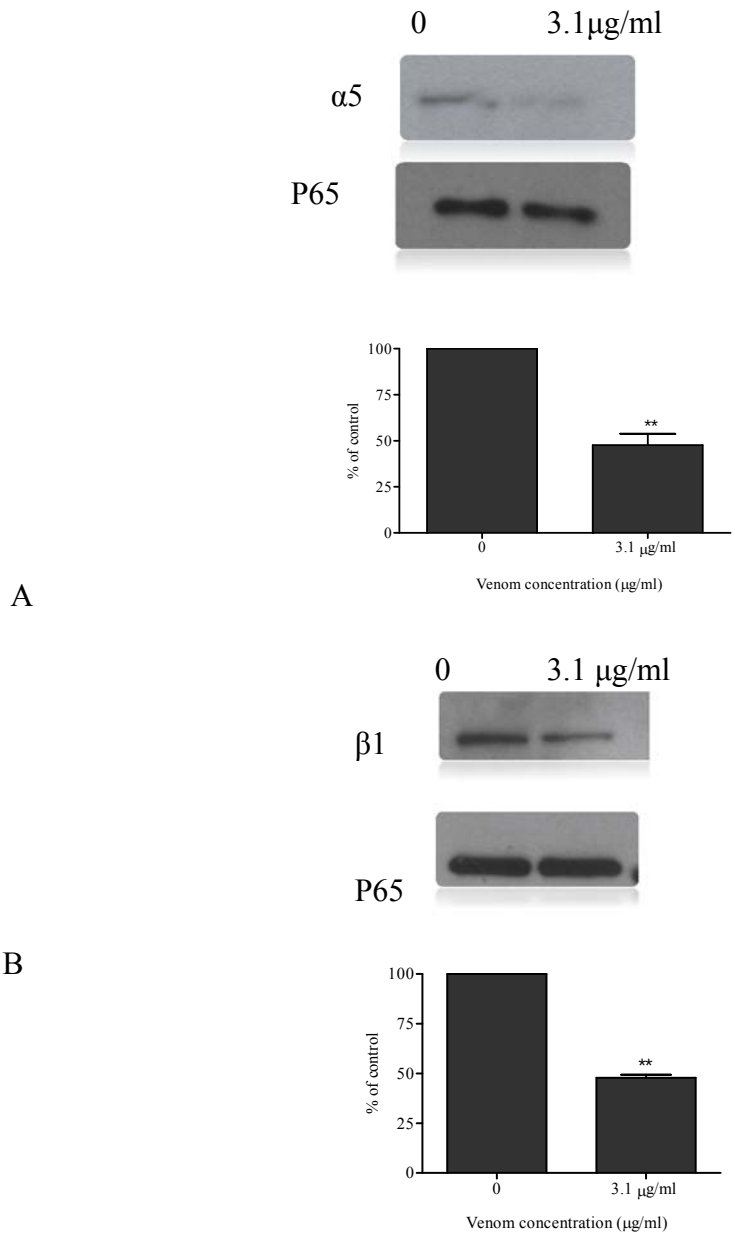


Figure 25: **Effect of *Crotalus ruber ruber* venom on the level of α5 (A) and β1 (B) integrins expressed by A2780 cells.** Cell lysates from A2780 cells treated with 3.1µg/ml of *Crotalus ruber ruber* venom were separated by SDS-PAGE and immunoblotted with antibodies against α5 and β1 integrins. P65 was used as a loading control. Dilution of primary and secondary antibodies was 1:1000. Statistical analysis was carried out using Student's paired t-test. ** indicates significantly ($p < 0.01$) lower values compared with the untreated control.

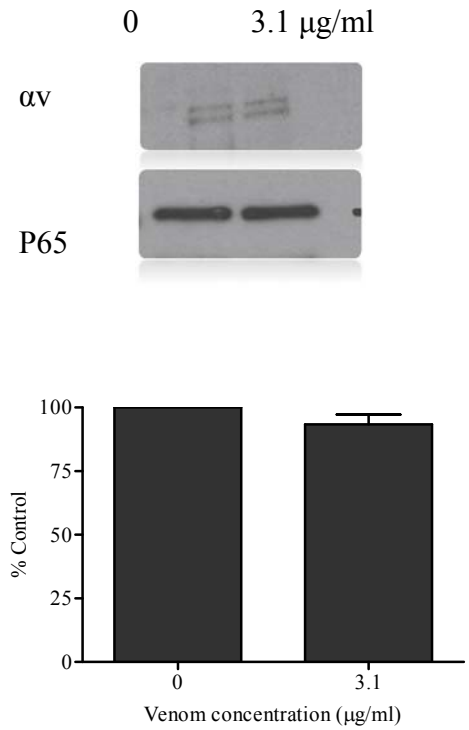


Figure 26: **Effect of *Crotalus ruber ruber* venom on the level of αv integrins expressed by A2780 cells.** Cell lysates from A2780 cells treated with 3.1µg/ml of *Crotalus ruber ruber* venom were separated by SDS-PAGE and immunoblotted with antibodies against αv integrins. P65 was used as a loading control. Dilution of primary and secondary antibodies was 1:1000. Statistical analysis was carried out using a Student's paired t-test.

3.1.16 Fractionation of *Crotalus ruber ruber* venom by a size exclusion chromatography

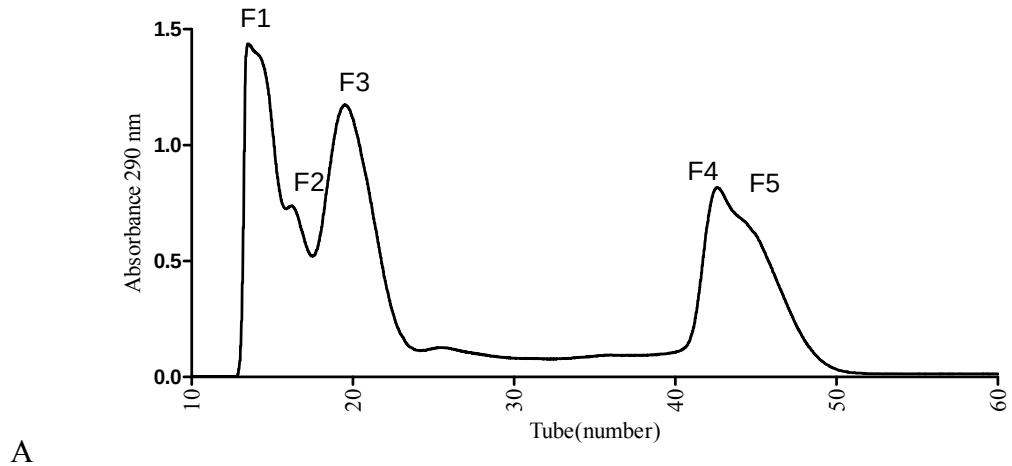
Separation of *Crotalus ruber ruber* venom was achieved by a size exclusion chromatography (Sephadex G75). The fractions obtained were pooled into five groups (Figure 27A) and lyophilised.

3.1.17 Gel electrophoresis

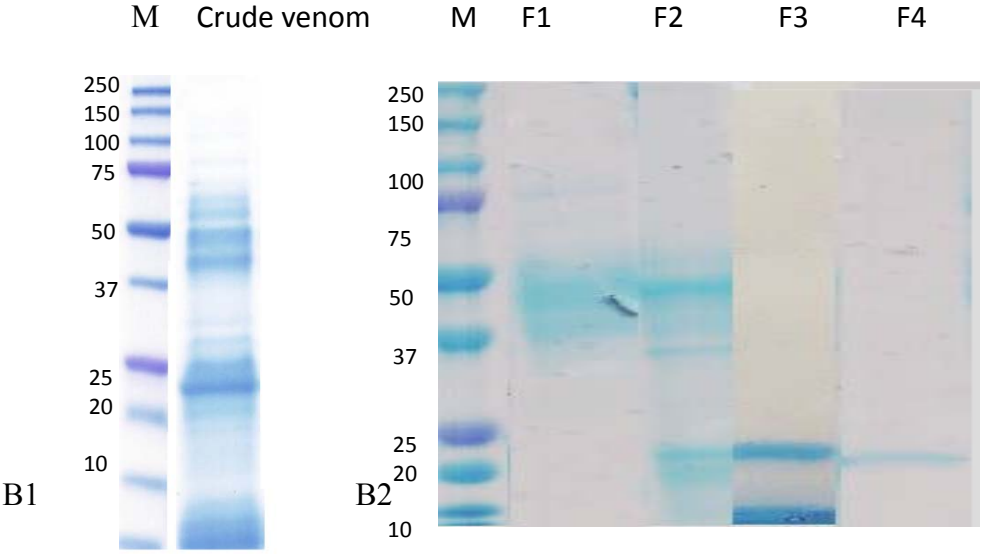
SDS-PAGE was used for further analysis of the crude venom and fractions to make sure the separation was efficienct. At least 10 protein bands ranging in molecular weights from less than 10,000 to 150,000Da were observed in the crude venom. Fraction 1 (F1) contained proteins with molecular weights ranging from 40,000-50,000Da, F2 contained proteins with molecular weights from 20,000-50,000 Da and F3 and F4 contained proteins less than 25,000Da (Figures 27B1 and B2).

3.1.18 Effect of fractions from *Crotalus ruber ruber* venom on the morphology of A2780 cells after 4h

This experiment was carried out in order to determine if fractions obtained from *Crotalus ruber ruber* venom would induce any visible morphological changes after 4h. The control well showed some degree of cell attachment and spreading. Fractions 1, 2, and 4 had no effect on the morphology of the cells. While F3 caused the cells to cluster together, round up, and lose their spreading on the surface (Figure 28), suggesting that F3 contains components that are responsible for morphological alterations and anti-adhesive properties of *Crotalus ruber ruber* venom. There was no powder observable for F5, so its activity was not tested.



A



B1 B2

Figure 27: Separation of *Crotalus ruber ruber* venom using (A) size exclusion chromatography and (B1) SDS-PAGE of the crude venom (B2) SDS-PAGE of the fractions. The crude venom (100 mg) was dissolved in 700µl of ammonium acetate buffer applied to a chromatographic column (1.5x100cm). The absorbance was monitored at 290nm. Fractions were eluted at a flow rate of 0.3ml/min and then pooled and lyophilised. Crude venom and fractions (15µl of stock at 3mg/ml) were mixed with 5µl of stain buffer. The mixture was centrifuged, heated and then loaded on 10% gels.

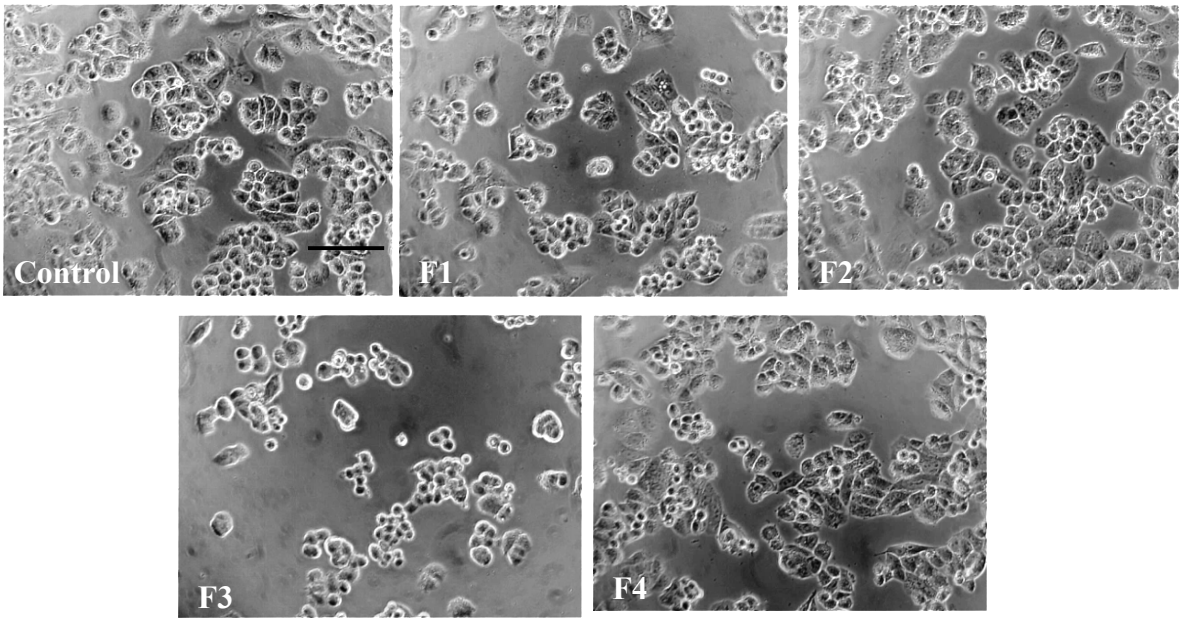


Figure 28: Effect of *Crotalus ruber ruber* fractions on the morphology of A2780 cells after 4h. Cells were seeded at $1x10^4$ cells per well in a 96 well plate and incubated with 1.5µg/ml of fractions for 4h. Images were taken. The magnification bar represents 100µm. Objective lens x10.

3.1.19 Effect of F3 on the morphology of A2780 cells after 4h

This experiment was carried out in order to demonstrate the effect of F3 of *Crotalus ruber ruber* venom on the morphology of A2780 cells. After adding F3, cells became rounded up, lost their spreading ability, clustered together, had less contact with the surface, and there was some cell detachment compared to the untreated control (Figure 29). Among these results, F3 appeared to be the most interesting fraction, exhibiting morphological changes and anti-adhesive activity on A2780 cells.

Control 1.5µg/ml of F3

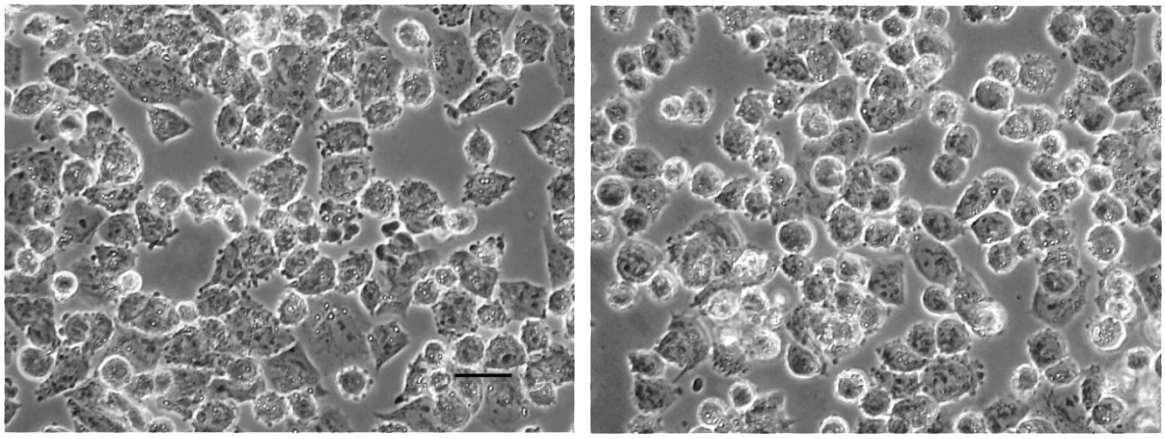


Figure 29: Effect of F3 on the morphology of A2780 cells. A2780 cells were seeded at a density of $1x10^6$ cells per well in a 12 well plate and incubated with 1.5µg/ml of F3 for 4h. Images were taken. The magnification bar represents 25µm. Objective lens x40.

3.1.20 Effect of F3 on membrane integrity of A2780 cells after 4h

The effect of F3 on viability of A2780 cells after 4h has examined using SYTOX®Green. There was no effect on membrane integrity at concentrations from 0.1 to 6.25µg/ml of F3, but there were changes in morphology at concentrations between 1.5 - 6.25µg/ml. However, 12.5 µg/ml had an effect on membrane integrity after 4h and the effect on viability was significant ($p < 0.01$) (Figure 30).

3.1.21 Effect of F3 on A2780 cells viability after 24, and 48h

The effect of different concentrations of F3 on the viability of A2780 cells after 24 and 48h was assessed using AlamarBlue® assay. F3 was found to have a significant effect ($p < 0.01$) on the viability of the A2780 cells at concentrations of 6.25 and 12.5µg/ml at 24 and 48h. Low concentrations of F3 did not affect cell viability (Figure 31A), while 3.1µg/ml had a toxic effect after 48h (Figure 31B) which was significant ($p < 0.01$).

3.1.22 Effect of F3 on adhesion of A2780 cells to vitronectin and fibronectin

This experiment was carried out to assess the effect of F3 on adhesion of A2780 cells in the presence of fibronectin and vitronectin. Cells were considered adherent if they became spread and flattened. F3 decreased adhesion of A2780 cells to fibronectin in a dose dependent manner (Figure 32A). The untreated control showed a high degree of spreading and adherence to fibronectin. Cells became rounded up and less flattened on the substrate after adding different concentrations of F3. After the washing procedure most of the cells were lost (Figure 33A). However, F3 did not significantly affect adhesion of A2780 cells to vitronectin (Figures 32B and 33 B).

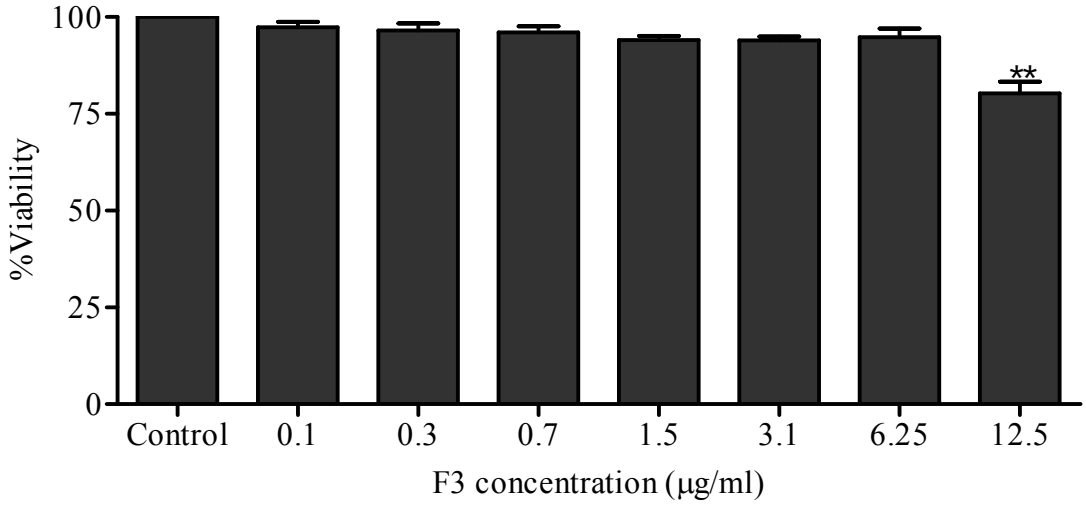
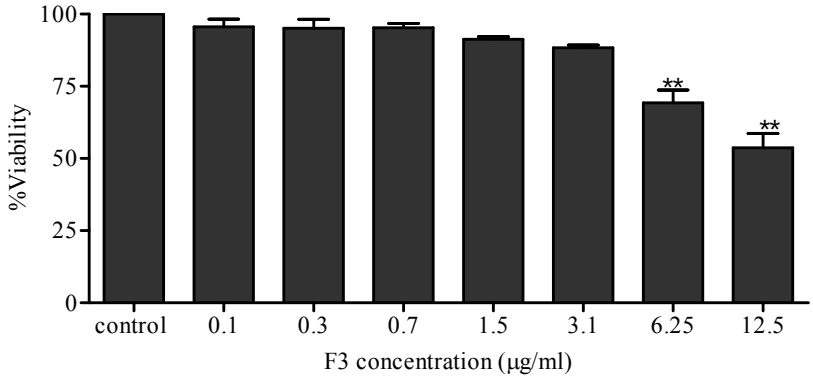
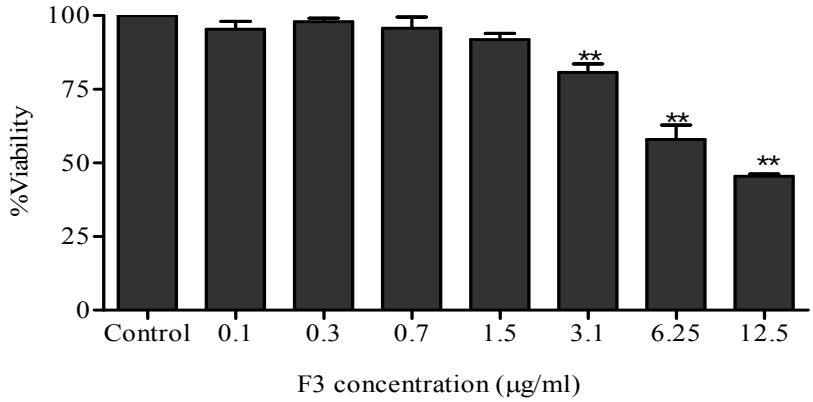


Figure 30: Effect of F3 of *Crotalus ruber ruber* venom on A2780 cells membrane integrity after 4h. Cells were seeded in a 96 well plate at a density of $1x10^4$ cells per well in complete medium (75µl) and incubated overnight. After which, venom was prepared in complete medium and added to the wells (25µl) for 4h. SYTOX®Green was used at a final concentration of 5µM in each well and incubated for 15min at 37°C. The fluorescence intensity was measured at 485-535nm. Values represent the mean ± SEM of 3 values. Statistical analysis was carried out using one way ANOVA with Dunnett's post-hoc test. ** indicates significantly ($p < 0.01$) lower values compared with the untreated control.

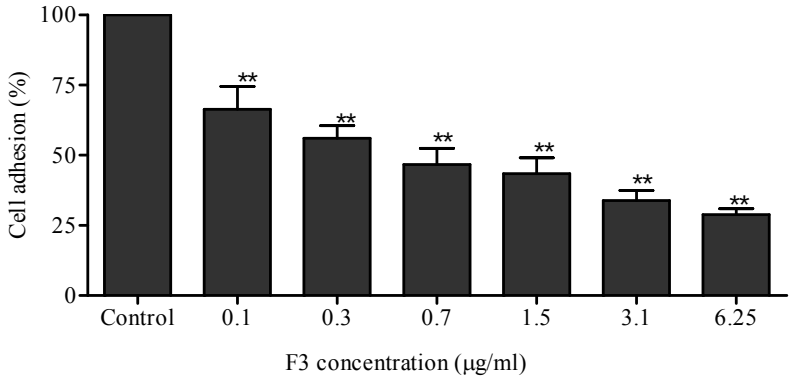


A

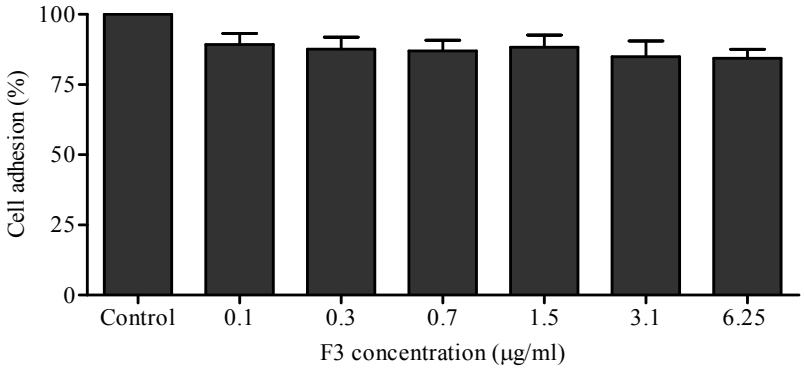


B

Figure 31: Effect of F3 on viability of A2780 cells after 24h (A) and 48h (B). Cells were cultured in a 96 well plate at a density of $1x10^4$ cells per well and incubated overnight. Following which, cells were exposed to different concentrations between 0.1-12.5µg/ml of F3. Following 24 and 48h incubation, 10µl of AlamarBlue® was added to the cells in each well 6h before the end of the incubation period and incubated at 37°C. The fluorescence intensity at 560-590nm was read. Values represent the mean ± SEM of 3 values. Statistical analysis was performed using one way ANOVA with Dunnett's post-hoc test. ** indicates significantly ($p < 0.01$) lower values compared with the untreated control.

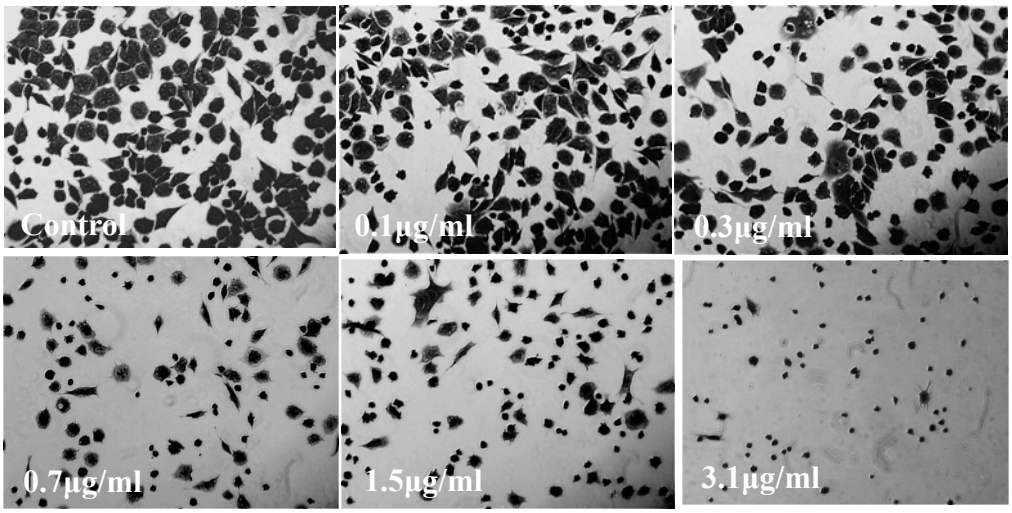


A

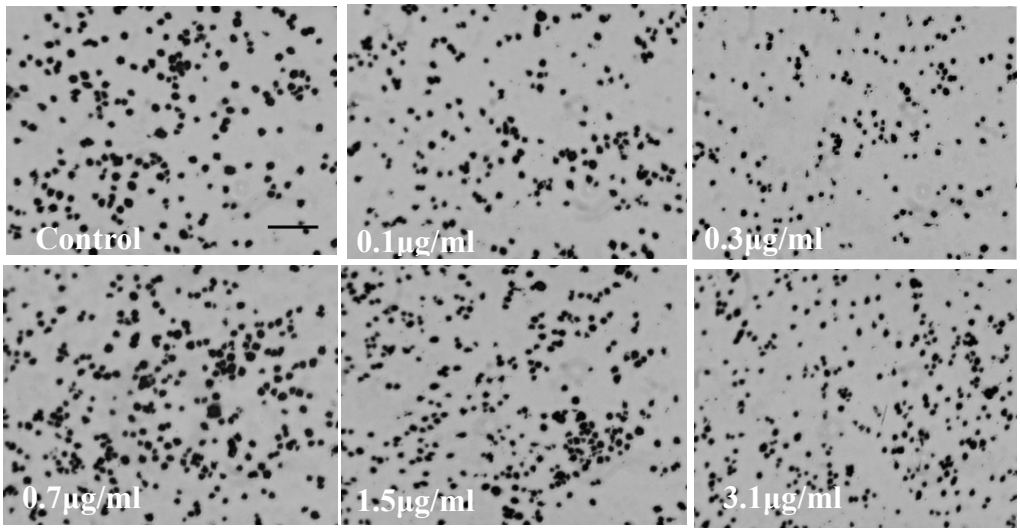


B

Figure 32: Effect of F3 of *Crotalus ruber ruber* venom on adhesion of A2780 cells to (A) fibronectin and (B) vitronectin. After coating a 96 well plate with 10µg/ml of fibronectin or 5µg/ml of vitronectin, cells ($4x10^5$cells/ml in 0.2% (w/v) BSA in RPMI 1640) with F3 (at final concentrations between 0.1-6.25µg/ml) were added and incubated. After 4h, the plate was washed, fixed, stained, and lysed. The absorbance was read at 540nm. The values are means ± SEM of 3 values. Statistical analysis was performed using one way ANOVA with Dunnett's post-hoc test. ** indicate significantly ($p < 0.01$) lower values compared with the untreated control.



A



B

Figure 33: Effect of F3 on adhesion of A2780 cells to (A) fibronectin and (B) vitronectin. Tissue culture wells of 96 well plates were coated with fibronectin (A) or vitronectin (B). After which, cells with venom were added to the precoated plate and incubated at 37ºC. After 4h, the plate was washed, fixed, stained, and images were taken. The magnification bar represents 50µm. Objective lens x20.

3.1.23 Effect of F3 on adhesion of A2780 cells to an integrin-independent substratum (poly-L-lysine)

The effect of F3 on adhesion of A2780 cells to an integrin-independent substratum was assessed using a poly-L-lysine coated plate. There was no effect of F3 on adhesion of A2780 cells to poly-L-lysine (Figure 34), suggesting that F3 is specific for the integrin family of adhesion receptors.

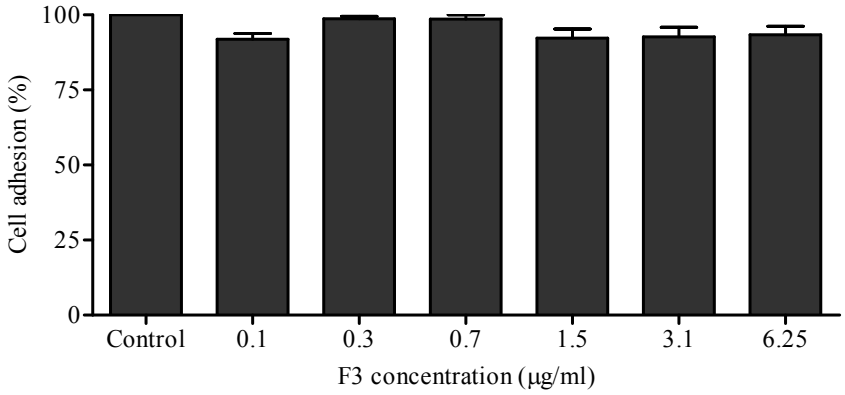


Figure 34: Effect of F3 on adhesion of A2780 cells in the presence of poly-L-lysine. Tissue culture wells were coated with poly-L-lysine, incubated for 1h, removed and washed with PBS. After which, cells and F3 at different concentrations were added and incubated. After 4h, the plate was washed, fixed, stained, and lysed with 0.1% (v/v) triton X-100. The absorbance was measured at 540nm. The values are means ± SEM of 3 values. Statistical analysis was achieved using one way ANOVA with Dunnett's post-hoc test.

3.1.24 Effect of F3 on migration of A2780 cells by using Cytoselect™ 24-well Cell Migration Assay

This experiment was carried out based on the results of the previous experiment of the effect of F3 on adhesion of A2780 cells. The effect of F3 on migration of A2780 cells was assessed using Cytoselect™ 24-well Cell Migration Assay. F3 inhibited A2780 cell migration at 1.5 and 3.1µg/ml (Figures 35 and 36). The result showed that F3 at 1.5 and 3.1µg/ml inhibited cell migration by 30% and 50%, respectively.

3.1.25 Effect of F3 on invasion of A2780 cells across the basement membrane using a Cytoselect™ 24-well Cell Invasion Assay

The result of this experiment showed that F3 inhibited the invasion of A2780 cells across the basement membrane at 1.5 and 3.1µg/ml (Figures 37 and 38). F3 at 1.5 and 3.1µg/ml inhibited the invasion of cells by 40% and 60%, respectively.

3.1.26 Effect of F3 on level of α5, β1, and αv integrins expressed by A2780 cells

Effect of F3 of *Crotalus ruber ruber* venom on α5, β1, and αv integrins expressed by A2780 cells was assessed. F3 at 1.5µg/ml had an inhibitory effect on the expression of α5 and β1 (Figures 39A and B). However, there was no effect on the level of expression of αv (Figure 40).

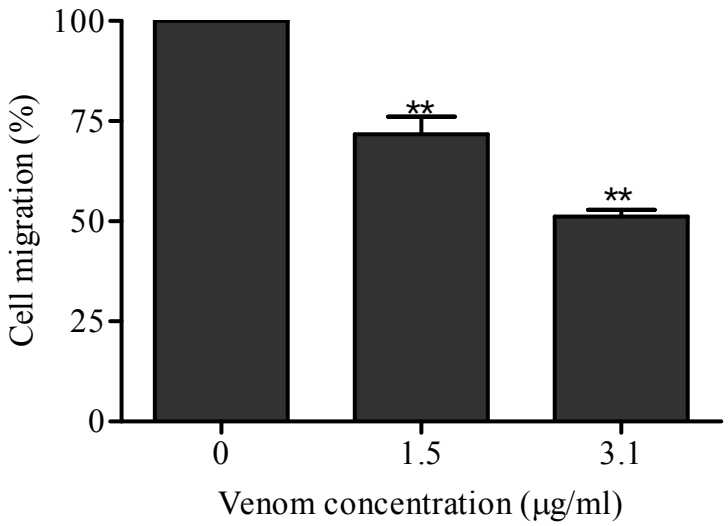
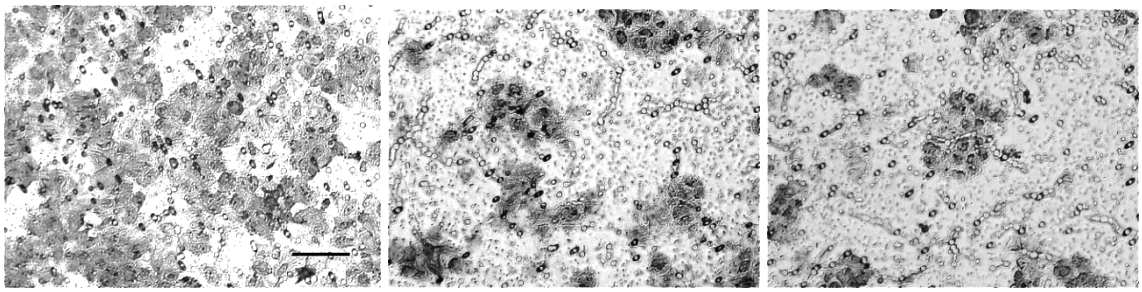


Figure 35: Effect of F3 of *Crotalus ruber ruber* venom on migration of A2780 cells using cell migration assay. Cells were adjusted to $1x10^6$ cells /ml in serum free medium and added to inserts in a 24 well plate with F3 at a final concentration of 1.5 or 3.1µg/ml, and then incubated for 24h. The cells that migrated were stained, photographed, and quantified. The absorbance was determined at 560 nm. The values are means ± SEM of 3 values. Statistical analysis was achieved using one way ANOVA with Dunnett's post-hoc test. ** indicates significantly ($p < 0.01$) lower values compared with the untreated control.



Control 1.5µg/ml of F3 3.1µg/ml of F3

Figure 36: Effect of F3 on migration of A2780 cells. Cells were adjusted to 1x10 ^{6} cells /ml in serum free medium and added to the inserts in a 24 well plate with 1.5 or 3.1µg/ml of venoms, then incubated for 24h. The cells that migrated into the lower surface of the membrane were stained and images were taken. The magnification bar represents 50µm. Objective lens x20.

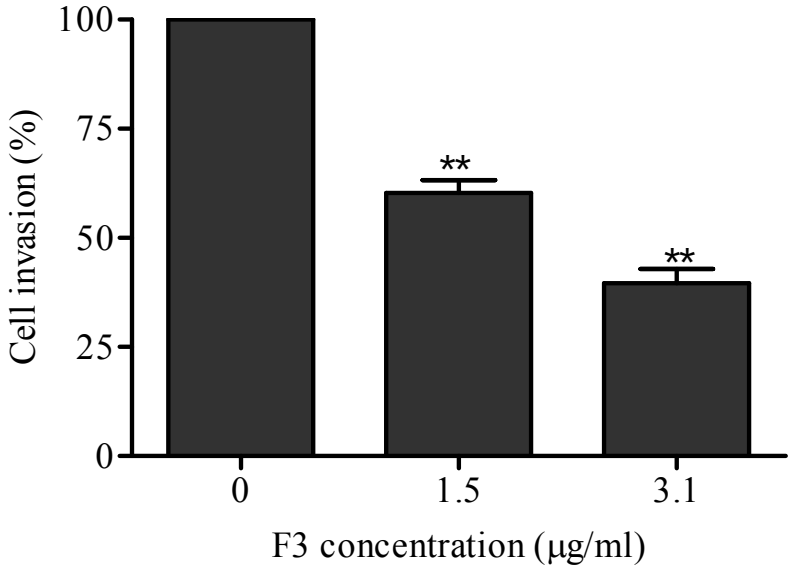
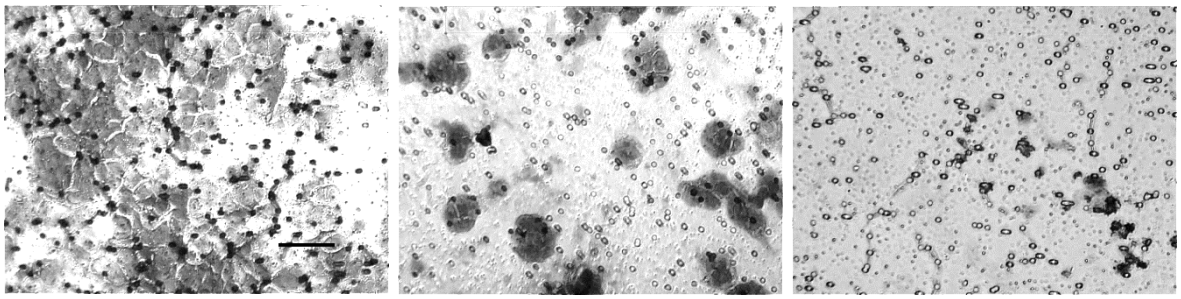
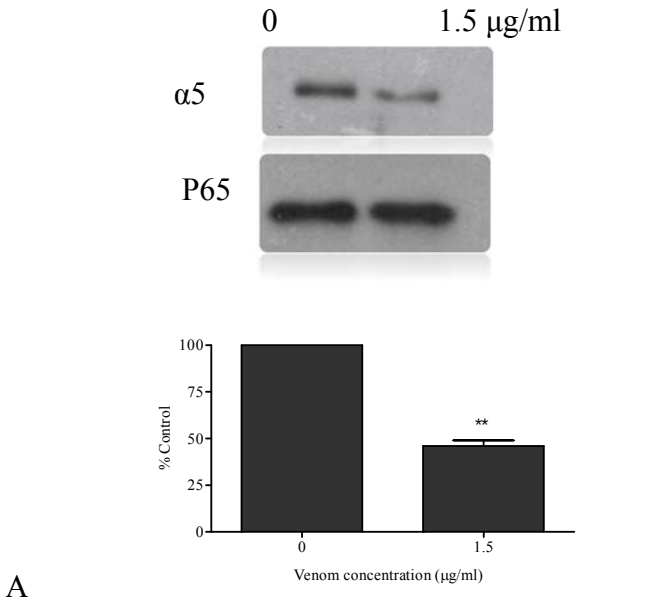


Figure 37: Effect of F3 on invasion of A2780 cells across the basement membrane. Cells were adjusted to $1x10^6$ cells /ml in serum free medium and 300µl of cell suspension was added to each insert in a 24 well plate with F3 at a final concentration of 1.5 or 3.1µg/ml and incubated for 48h. The cells that invaded into the lower surface of the membrane were stained, photographed, and quantified. The absorbance was determined at 560nm. The values are means ± SEM of 3 values. Statistical analysis was achieved using one way ANOVA with Dunnett's post-hoc test. ** indicates significantly ($p < 0.01$) lower values compared with the untreated control.

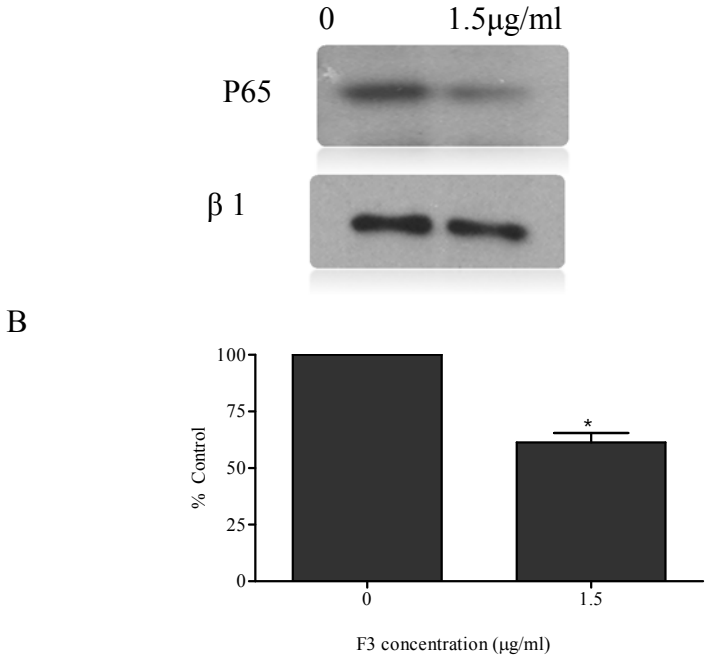


Control 1.5µg/ml 3.1µg/ml

Figure 38: Effect of F3 on invasion of A2780 cells across the basement membrane. Cells were adjusted to $1x10^6$ cells /ml in serum free medium and added with 1.5 or 3.1µg/ml final concentration of F3 to each insert in a 24 well plate and incubated for 48h. The cells that invaded were stained and images were taken. The magnification bar represents 50µm. Objective lens x20.



A



B

Figure 39: Effect of F3 on the level of expression of (A) α5 and (B) β1 integrins of A2780 cells. Cell lysates from A2780 cells treated with 1.5µg/ml of F3 were prepared, separated by SDS-PAGE and immunoblotted with antibodies against α5 and β1integrins. P65 was used as a loading control. Dilution of primary and secondary antibodies was 1:1000. Statistical analysis was carried out using a Student's paired t-test. * and ** indicates significantly ($p < 0.05$, $p < 0.01$) lower values compared with the untreated control, respectively.

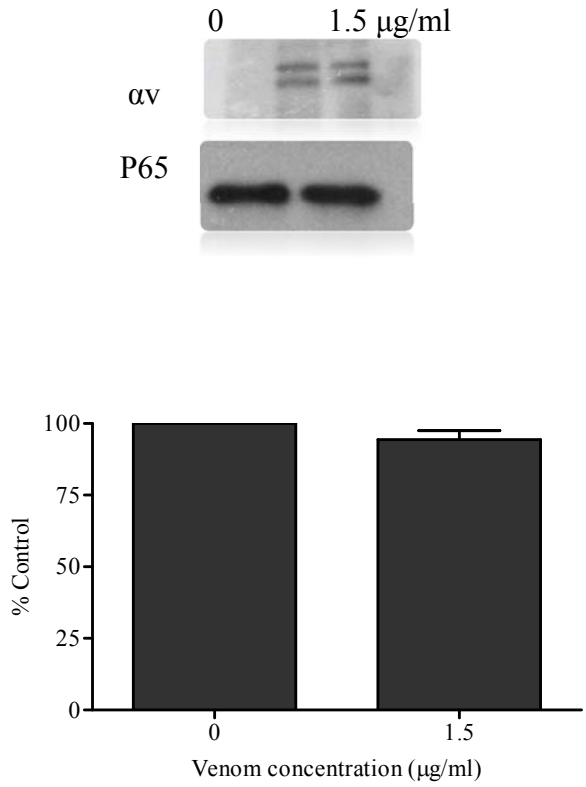


Figure 40: Effect of F3 on the level of expression of αv integrins of A2780 cells. Cell lysates from A2780 cells treated with 1.5µg/ml of F3 were prepared, separated by SDS-PAGE and immunoblotted with antibody against αv integrins. P65 was used as a loading control. Dilution of primary and secondary antibodies was 1:1000. Statistical analysis was carried out using a Student's paired t-test.

3.1.27 Identification of the active component in F3 from *Crotalus ruber ruber* venom

The objective of these investigations was to identify the active component in F3 responsible for effects on adhesion, migration, invasion and expression of α5 and β1 integrins.

3.1.27.1 SDS-PAGE for F3 of *Crotalus ruber ruber* venom

In order to identify the molecular weight of each peptides in F3 SDS-PAGE was used. Firstly, F3 was analysed by SDS-PAGE on 15% (w/v) polyacrylamide gels and a single peptide was observed with a molecular weight between 23,000 and 24,000Da. The band on the gel was excised and sent for Tandem Mass Spectrometry (Figure 41). In this experiment a 15% polyacrylamide gel was used instead of 10% gels used in previous experiments (section 3.1.17) to make sure low molecular weight proteins were not missed.

3.1.27.2 Nanoflow HPLC Electrospray Tandem Mass Spectrometry

In order to identify the active peptides in F3 Nanoflow HPLC Electrospray Tandem Mass Spectrometry was used. Tandem Mass Spectrometry was used because F3 is not pure and mixture so the best way to identify the component of the interest in a mixture is Tandem Mass Spectrometry. The result showed that the highest score was for ruberlysin (1041), while the other peptides were less than the ruberlysin with scores rang 67, 63 and 54. So the main peptide in F3 according to Nanoflow HPLC Electrospray Tandem Mass Spectrometry is most probably Hemorrhagic toxin II (rubelysin). Rubelysin is a *Crotalus ruber* metalloendopeptidase II, 202 amino acid and its molecular weight is 23,321Da.

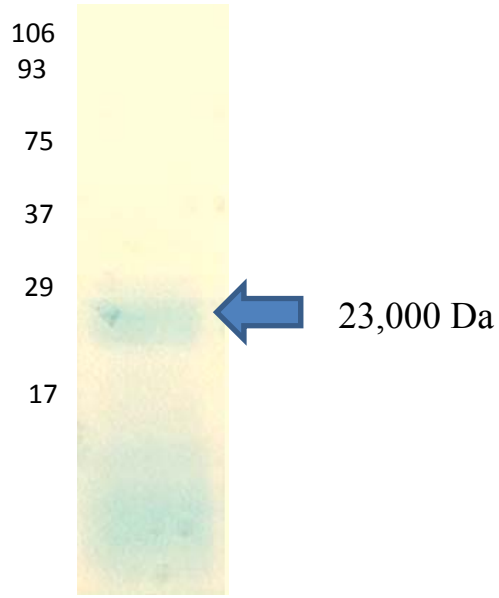


Figure 41: **SDS-PAGE for F3 of *Crotalus ruber ruber* venom.** Three mg of the F3 dissolved in distilled water, and then 5µl stain buffer and 15µl of venom were mixed together, centrifuged, heated, and then the sample loaded (20µl) onto a 15% polyacrylamide gel. When the first band reached the lower edge of the gel the current was stopped and the gel removed, washed and stained.

3.1.27.3 Liquid chromatography mass spectrometry (LC-MS)

This technique was performed to identify the molecular mass and the percentage of peptides in F3. F3 was found to have 4 peptides of varying percentage. The percentage of the major peptide was 73 % and its molecular weight was 23,300Da and the percentage of the other 3 peptides was 2 %, 3 %, and 14 % (Figure 42).

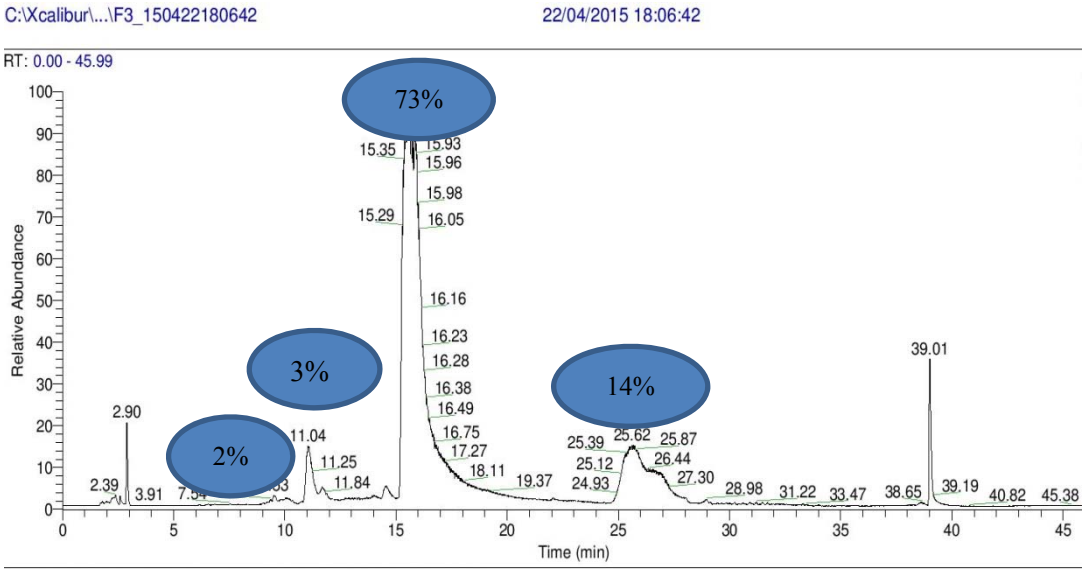


Figure 42: Analysis of F3 by liquid chromatography mass spectrometry (LC-MS). One mg of the fraction was dissolved in 1ml of water and 10µl of the solution was injected along with gradient from 90% water and 10% acetonitrile (0.1% (v/v) formic acid) to 90% acetonitrile and 10% water (0.1% (v/v) formic acid) at a flow rate of 0.3 ml/min for 30min. The separation was performed on an ACE-C_{18} column (150×3mm, 3µm).

3.1.27.4 Assessment of the effect of metalloproteinase inhibitor (ethylenediaminetetraacetic acid) (EDTA) on activity of F3 of *Crotalus ruber ruber* venom

This final experiment was carried out to determine the effect of different concentrations of EDTA on the activity of F3 against A2780 cells in order to prove that the active peptide is metalloproteinase. The activity of F3 was inhibited in the presence of EDTA. The effect of F3 on morphology and adhesion of A2780 cells was abolished after adding 100µg/ml of EDTA. The effect of EDTA on the activity of F3 was in a dose dependent manner (Figure 43). The morphological changes of A2780 cells that observed after adding 1.5µg/ml of F3 tended to disappear after adding EDTA (Figure 44). Results obtained from this study indicated that EDTA at 100µg/ml inhibited the activity of metalloproteinase II and stopped the effect of F3 on the adhesion of A2780 cells. The inhibition of the activity of F3 by EDTA, suggested that the active peptide in this fraction could be the zinc-metalloproteinase II (rubelysin).

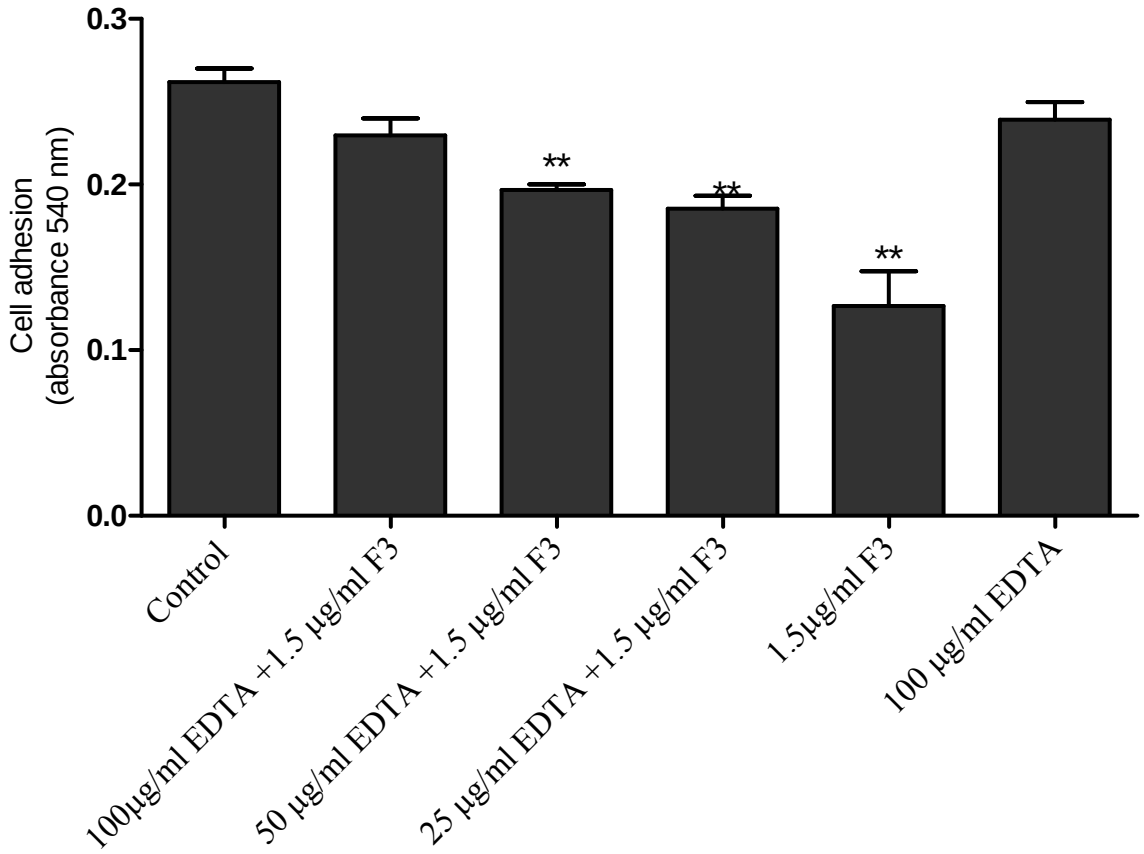


Figure 43: Effect EDTA (metalloproteinase inhibitor) on the activity of F3 of *Crotalus ruber ruber* venom. A2780 cells were prepared at $3x10^5$ cells / ml in complete medium and added to a 24 well plate with F3 (1.5µg/ml) and incubated with a serial dilution of EDTA from 25 to 100µg/ml at 37°C for 24h. After which, the medium was removed and cells washed, fixed, stained, and lysed with 0.1% (v/v) triton X-100. The absorbance was determined at 540nm. The values are means ± SEM of 3 values. Statistical analysis was performed using one way ANOVA with Dunnett's post-hoc test. ** indicates significantly ($p < 0.01$) lower values compared with the untreated control.

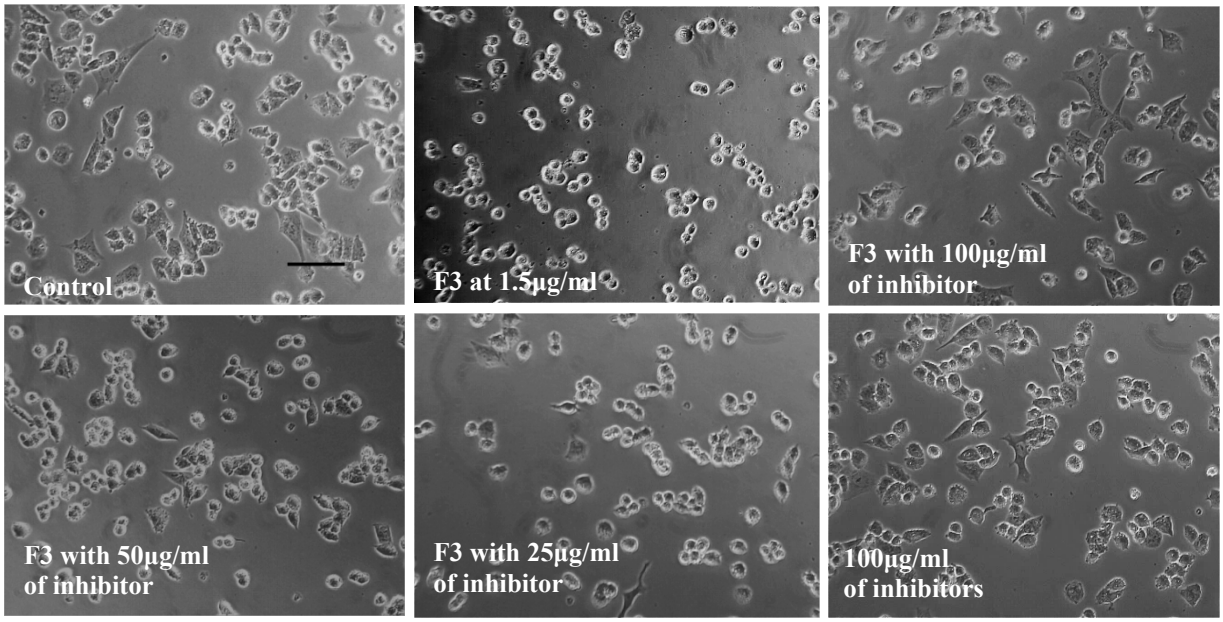


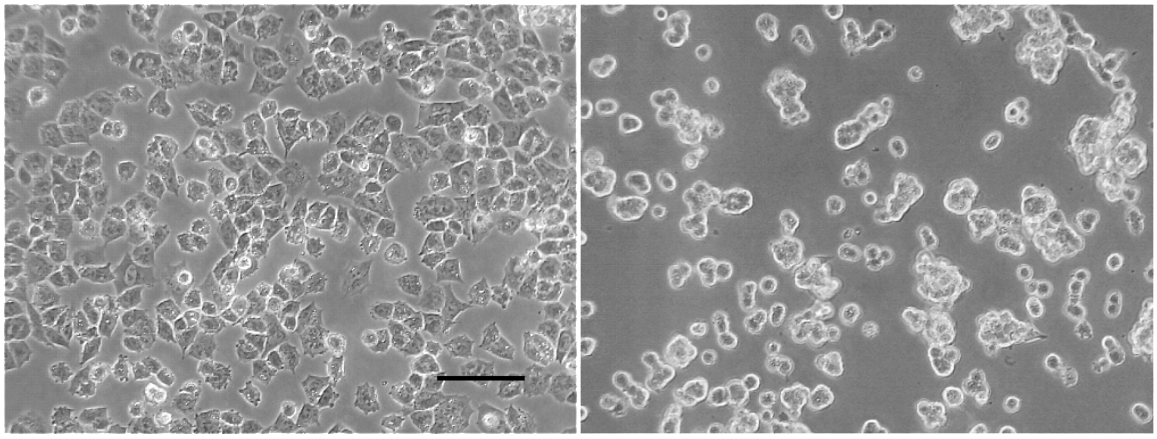
Figure 44: **Effect of EDTA on activity of F3 of *Crotalus ruber ruber* venom after 24h.** Cells were seeded at $3x10^5$ cells per ml in a 24 well plate. Venom was prepared at a final concentration of 1.5µg/ml then different concentrations of EDTA were added to the plate and incubated for 24h. After which, images were taken. The magnification bar represents 50µm. Objective lens x20.

3.2 Effects of *Agkistrodon piscivorus leucostoma* snake venom on cell adhesion, migration and invasion of A2780 cells

The major aims of this section were to demonstrate the effect of *Agkistrodon piscivorus leucostoma* snake venom and its active fraction on morphology, viability, adhesion, migration and invasion of A2780 cells. The same experiments were carried out as in section 3.1.

3.2.1 Effect of *Agkistrodon piscivorus leucostoma* venom on the morphology of A2780 cells after 4h

The aim of this experiment was to exhibit the morphological changes of A2780 cells induced by venom. The control well showed some degree of cell attachment and spreading, while the wells with *Agkistrodon piscivorus leucostoma* venom showed cells floating in the wells, detached from the surface and clustered together (Figure 45).



Control 3.1μg/ml of venom

Figure 45: Effect of *Agkistrodon piscivorus leucostoma* venom on the morphology of A2780 cells after 4h. Cells were seeded at $1x10^6$ cells per well in a 12 well plate and incubated with venom (3.1μg/ml) for 4h. Images were taken using a Nikon TMS inverted phase contrast microscope. The magnification bar represents 50μm. Objective lens x 20.

3.2.2 Effect of *Agkistrodon piscivorus leucostoma* snake venom on membrane integrity of A2780 cells after 4h

This was achieved by carrying out cell viability assays using SYTOX®Green in order to prove the morphological changes after adding venom is not due to cytotoxic effects of the venom. Depending on the venom concentrations, there was no effect on membrane integrity at concentrations between 0.1-6.25µg/ml, although drastic alterations in morphology were observed for concentrations between 1.5 – 6.25µg/ml. There was an effect on the membrane integrity (viability) at high concentrations 12.5µg/ml (Figure 46).

3.2.3 Effect of *Agkistrodon piscivorus leucostoma* venom on A2780 cells viability after 24, 48 and 72h

This experiment was conducted to check the effect of different concentrations of venom on cell viability after 24, 48 and 72h. The effect on the viability was evaluated using an AlamarBlue® assay. *Agikistrodon piscivorus leucostoma* venom was found to have a significant ($p<0.01$) effect on the viability of A2780 cells at concentrations of 6.25 and 12.5µg/ml at all the time periods, but the viability of the cells was not affected at low concentrations of venom for 24 and 48h (Figures 47A and B). The viability was affected at 1.5 and 3.1µg/ml after 72h (Figure 47C).

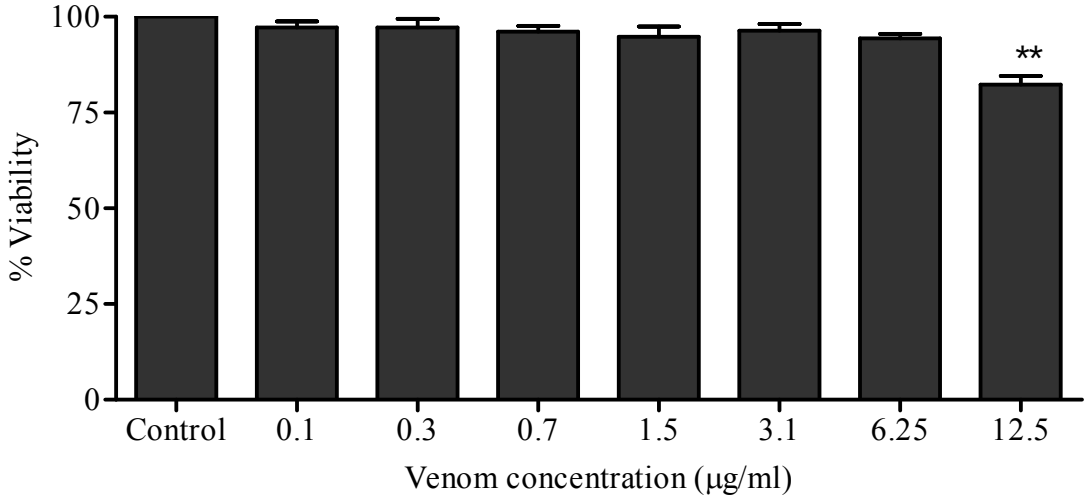
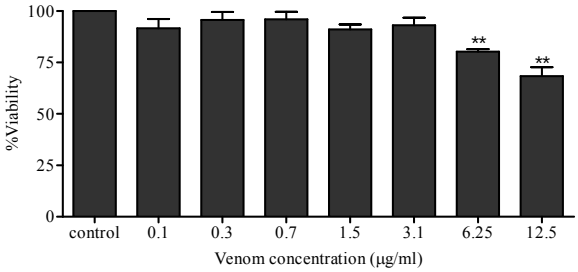
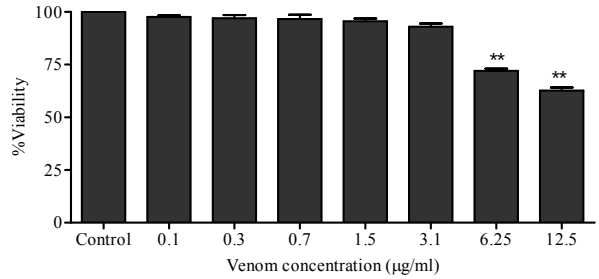


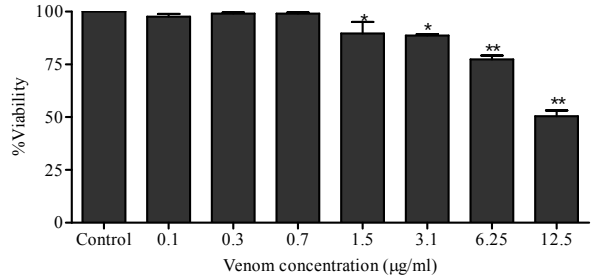
Figure 46: Effect of *Agkistrodon piscivorus leucostoma* venom on A2780 cells membrane integrity after 4h. Cells were adjusted to $1x10^4$ cells per well in 75µl complete medium and incubated for 24h. Venom (25µl) was added and incubated for 4h. SYTOX®Green was used at a final concentration of 5µM in each well and incubated for 15min in a 37°C incubator. The fluorescence intensity at 485-535nm was measured. Values represent the mean ± SEM of 3 values. Statistical analysis was performed using one way ANOVA with Dunnett's post-hoc test. ** indicates significantly ($p < 0.01$) lower values compared with the untreated control.



A



B

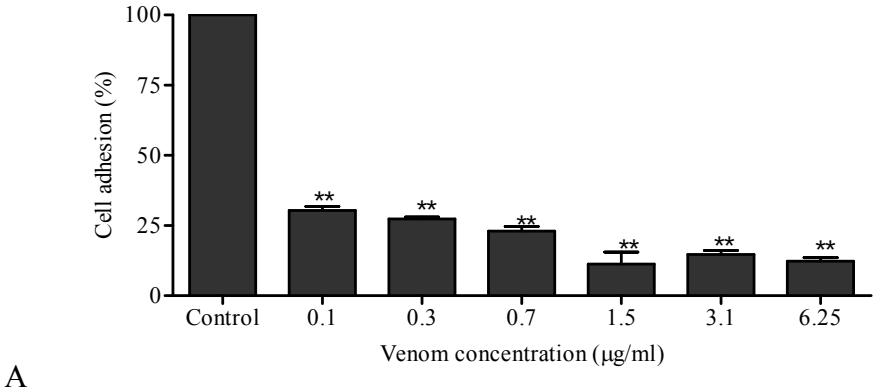


C

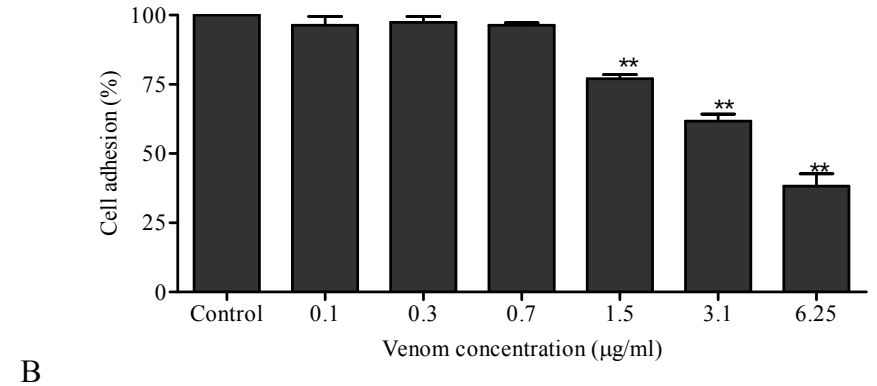
Figure 47: Effect of *Agkistrodon piscivorus leucostoma* venom on viability of A2780 cells after (A) 24, (B) 48 and (C) 72h. Cells were prepared at $1x10^4$ cells per well in 75µl complete medium. After 24h, venom (25µl) was added to the cells at final concentrations between 0.1 - 12.5µg/ml. After 24, 48 and 72h, 10µl of AlamarBlue® was added to each well 6h before the end of the incubation period. The fluorescence intensity at 560-590nm was read. Statistical analysis was performed using one way ANOVA with Dunnett's post-hoc test. * and ** indicates significantly ($p < 0.05$, $p < 0.01$) lower values compared with the untreated control, respectively.

3.2.4 Effect of *Agkistrodon piscivorus leucostoma* venom on adhesion of A2780 cells to fibronectin and vitronectin

The effect of *Agkistrodon piscivorus leucostoma* venom on adhesion of A2780 cells in the presence of fibronectin and vitronectin was assessed. A2780 cells spread well on fibronectin and after adding venom the cells lost their adherence to fibronectin. After washing, most of the cells were lost. The effect of *Agkistrodon piscivorus leucostoma* venom on adhesion of A2780 cells to fibronectin was in a dose-dependent manner (Figures 48A and 49A). A2780 cells did not spread well on vitronectin and there was an effect of *Agkistrodon piscivorus leucostoma* venom on the adhesion of the cells on vitronectin, which was significant ($p < 0.01$) at concentrations between 1.5 - 6.25µg/ml (Figures 48B and 49B).

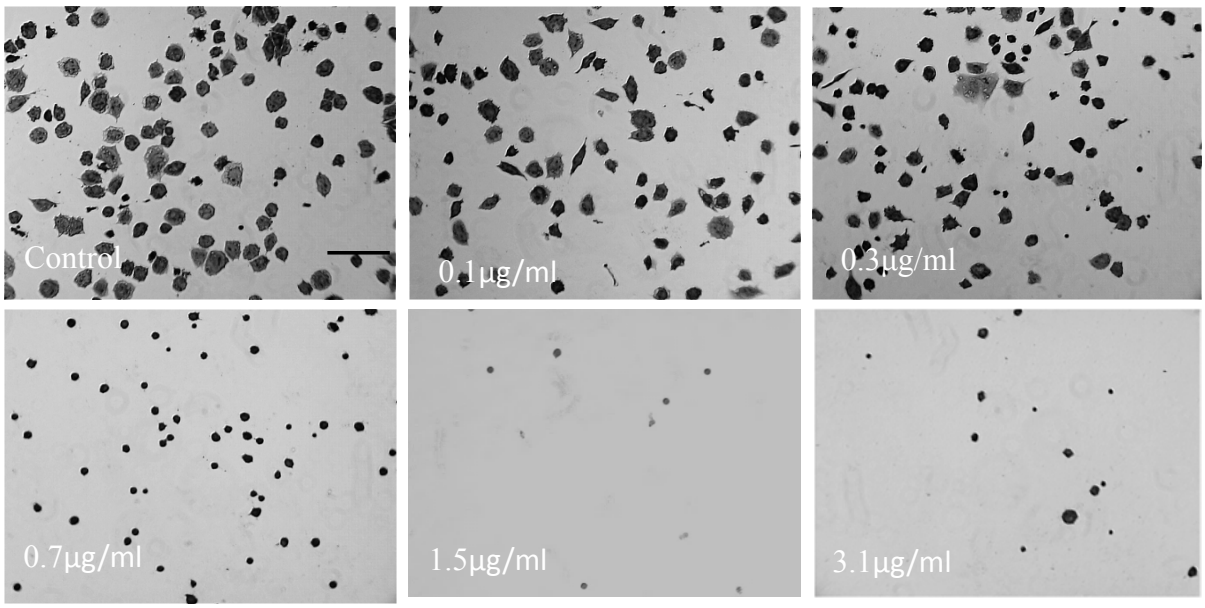


A

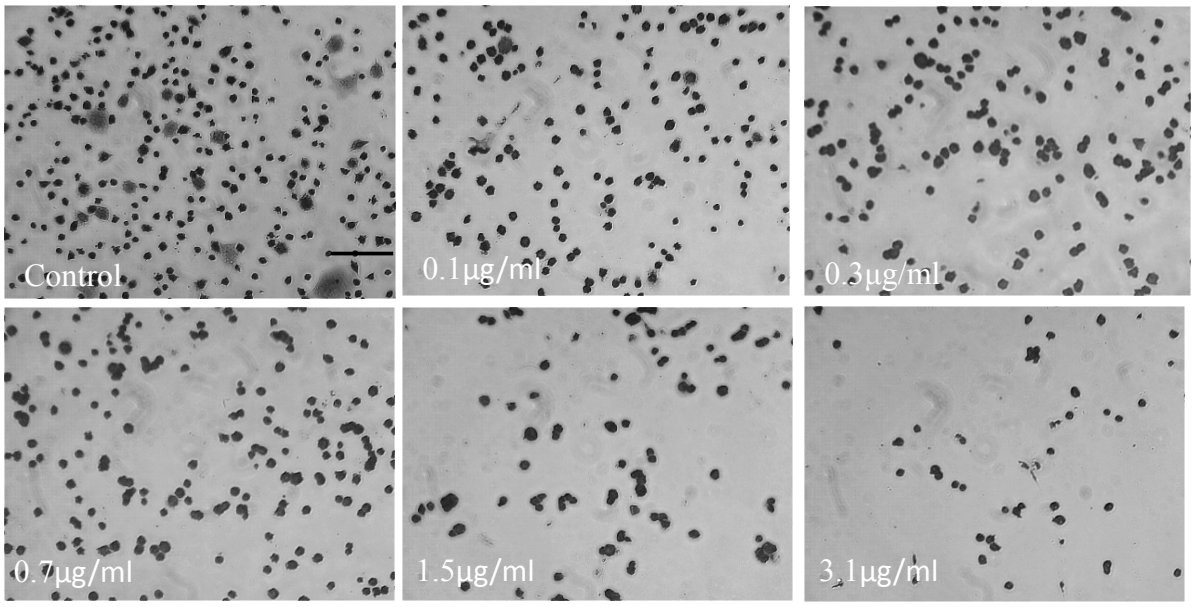


B

Figure 48: Effect of *Agkistrodon piscivorus leucostoma* venom on adhesion of A2780 cells to (A) fibronectin and (B) vitronectin. Ninety six well plates were coated with either fibronectin or vitronectin and incubated for 1h. Cells were adjusted to $4x10^5$cells/ml in 0.2% (w/v) BSA in 75µl RPMI 1640 medium and added to the coated plates with 25µl of venom at a final concentration between 0.1-6.25µg/ml. After incubation at 37ºC for 4h, unattached cells were removed by gently washing and residual attached cells were fixed with 4% (v/v) PFA. After staining with crystal violet, cells were lysed with 0.1% (v/v) Triton X-100. The absorbance at 540nm was measured. The values are means ± SEM of 3 values. Statistical analysis was performed using one way ANOVA with Dunnett's post-hoc test. ** indicate significantly ($p < 0.01$) lower values compared with the untreated control.



A



B

Figure 49: Effect of *Agkistrodon piscivorus leucostoma* venom on adhesion of A2780 cells to (A) fibronectin and (B) vitronectin. Cells with different concentrations of venom were added to fibronectin or vitronectin coated plates and incubated for 4h. After 4h, the plates were washed, fixed, stained with crystal violet, and images were taken. The magnificiation bar represents 50µm. Objective lens x20.

3.2.5 Effect of *Agkistrodon piscivorus leucostoma* venom on adhesion of A2780 cells to an integrin-independent substratum (poly-L-lysine)

This experiment was carried out in order to determine if *Agkistrodon piscivorus leucostoma* venom is specific for the integrin family of adhesion molecules or if it would affect any adhesion molecules. *Agkistrodon piscivorus leucostoma* venom had an inhibitory effect on adhesion of A2780 cells to poly-L-lysine at the concentration between 0.1-6.25µg/ml (Figures 50 and 51). As a result, the effect of the venom on adhesion is believed to be non-specific.

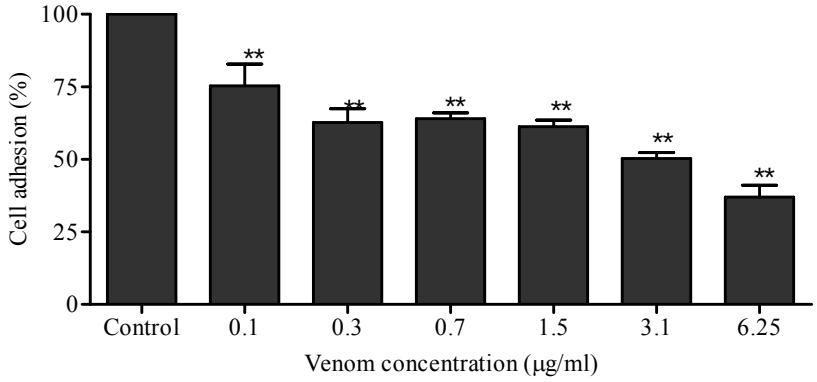


Figure 50: Effect of *Agkistrodon piscivorus leucostoma* venom on adhesion of A2780 cells to poly-L-lysine. Ninety six well plates were coated with poly-L-lysine (50µg/ml) and incubated at 37ºC for 1h. Following washes, cells ($4x10^5$cells/ml in 0.2% (w/v) BSA in RPMI 1640) with venom at a final concentration between 0.1-6.25µg/ml were added to the coated plate and incubated for 4h. After which, the plate was washed, fixed, stained, and lysed with 0.1% (v/v) triton X-100. The absorbance at 540nm was read. The values are means ± SEM of 3 values. Statistical analysis was performed using one way ANOVA with Dunnett's post-hoc test. ** indicate significantly ($p < 0.01$) lower values compared with the untreated control.

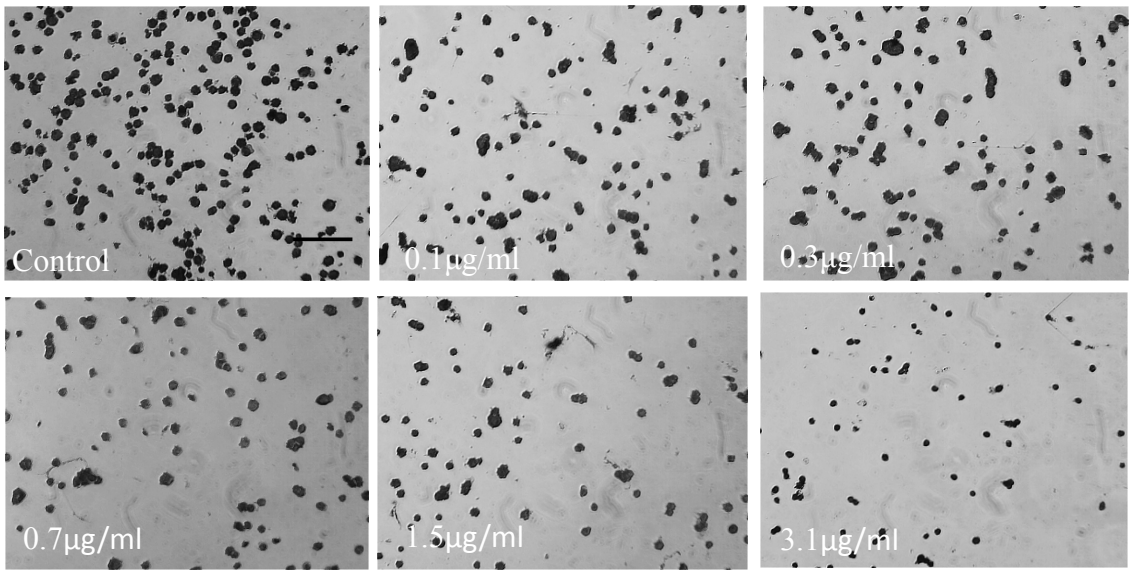


Figure 51: Effect of *Agkistrodon piscivorus leucostoma* venom on adhesion of A2780 cells to poly-L-lysine. Cells with different concentrations of venom were added to a poly-L-lysine coated plate and incubated. After 4h, the non-adherent cells were washed away and the adherent cells were fixed, stained, and images taken. The magnificiation bar represents 50µm. Objective lens x20.

3.2.6 Effect of *Agkistrodon piscivorus leucostoma* venom on migration of A2780 cells

To assess migration of A2780 cells in response to *Agkistrodon piscivorus leucostoma* venom, an *in vitro* wound closure assay was used. The assay revealed a delayed closure of the wound comparable to the untreated control. In the presence of different concentrations of venom, the migration of A2780 cells was affected in a concentration-dependent manner (Figures 52 and 53).

3.2.7 Migration assay using a Cytoselect™ 24-well Cell Migration Assay

This experiment was carried out to confirm the inhibitory effect of *Agkistrodon piscivorus leucostoma* venom on migration of A2780 cells. Both venom concentrations 1.5 and 3.1µg/ml had a significant ($p < 0.01$) inhibitory effect on cell migration (Figure 54).

3.2.8 Effect of *Agkistrodon piscivorus leucostoma* venom on invasion of A2780 cells across the basement membrane using a Cytoselect™ 24-well Cell Invasion Assay

The effect of *Agkistrodon piscivorus leucostoma* venom on invasion of A2780 cells across a basement membrane was assessed using a Cytoselect™ 24-well Cell Invasion Assay. The result of this experiment showed that the venom at 1.5 and 3.1µg/ml significantly ($p < 0.01$) inhibited the invasion of cells across the basement membrane (Figure 55). As shown in Figure 56, the number of A2780 cells which invaded after exposure to venom was markedly less than the untreated control.

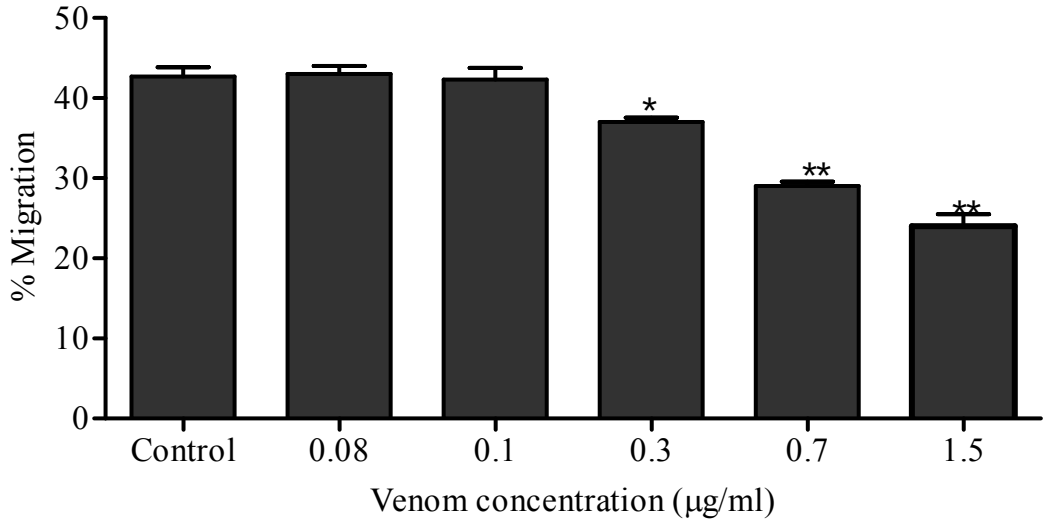


Figure 52: Effect of *Agkistrodon piscivorus leucostoma* venom on migration of A2780 cells using wound closure assay. The cells ($5x10^5$cells/ml in complete medium) were added into both sides of an ibidi migration chamber. After 24h, the ibidi chamber was removed, the medium discarded and fresh complete medium with different concentrations of venom (between 0.08-1.5µg/ml) was added. After 48h of incubation at 37^{o}C, the cells were fixed, stained with crystal violet, images were taken. Image J analysis was used to determine wound closure. Values are means ± SEM of 3 values. Statistical analysis was carried out using one way ANOVA with Dunnett's post-hoc test. * and** indicates significantly ($p < 0.05$, $p < 0.01$, respectively) lower values compared with the untreated control.

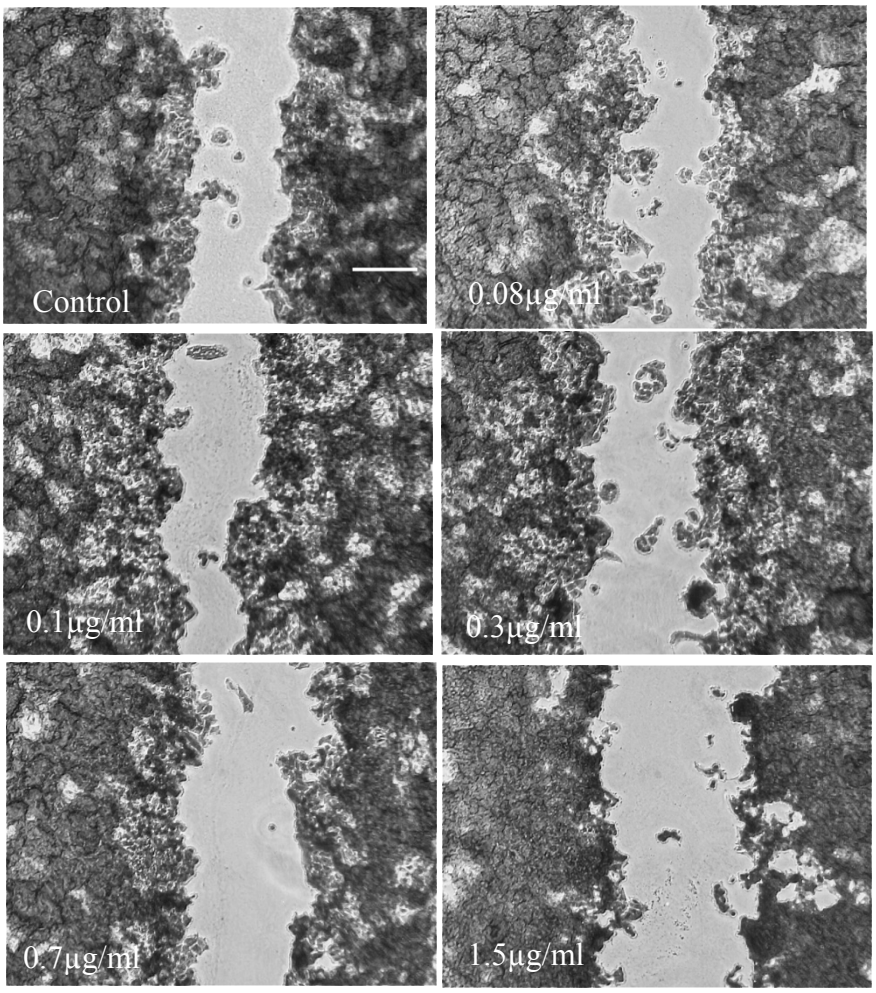


Figure 53: Effect of *Agkistrodon piscivorus leucostoma* venom on migration of A2780 cells wound closure assay. The cell number was adjusted to $5x10^5$ cells /ml in complete medium and added into both sides of ibidi migration chambers in a 24 well plate. After 24h, the ibidi chambers were removed and the medium was discarded and 500µl of medium with a serial dilution of the venom was added and incubated for 48h. After which, the cells fixed, stained with crystal violet and images taken. magnificiation bar represents 100µm. Objective lens x10.

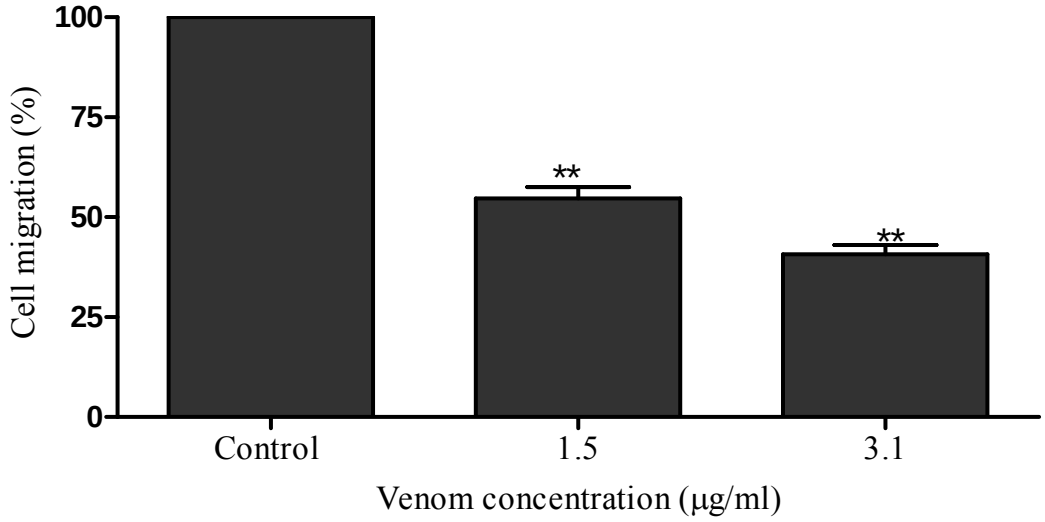


Figure 54: Effect of *Agkistrodon piscivorus leucostoma* venom on migration of A2780 cells using a cell migration assay. Cells were adjusted to $1x10^6$ cells /ml in serum free medium and added to each insert in a 24 well plate with 1.5 or 3.1µg/ml of venom, and then incubated for 24h. The cells that migrated into the lower surface of the membrane were stained, photographed and quantified. The absorbance at 560nm was measured. The values are means ± SEM of 3 values. Statistical analysis was performed using one way ANOVA with Dunnett's post-hoc test. ** indicates significantly ($p < 0.01$) lower values compared with the untreated control.

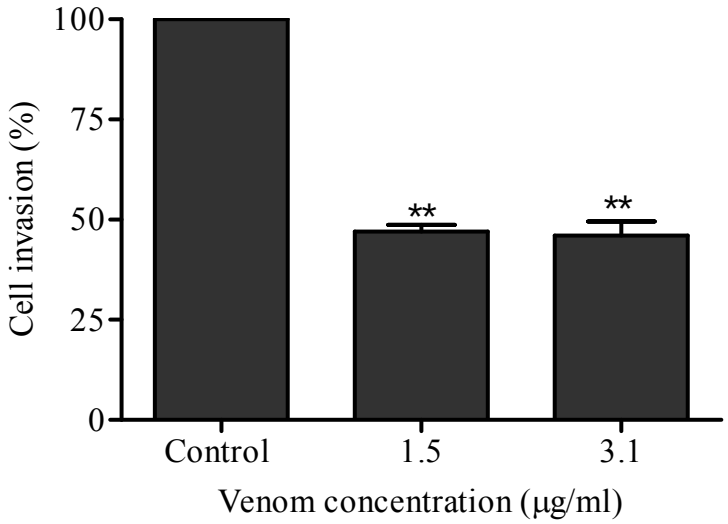
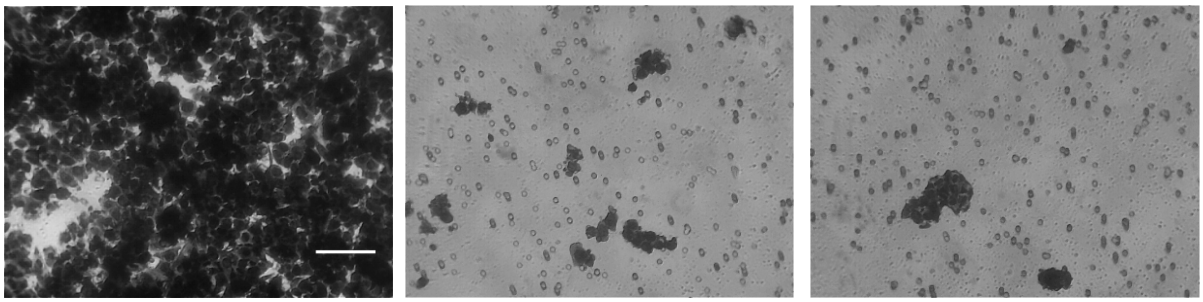


Figure 55: Effect of *Agkistrodon piscivorus leucostoma* venom on invasion of A2780 cells across the basement membrane. Cells were adjusted to $1x10^6$ cells /ml in serum free medium and 300µl of cell suspension was added to each insert in a 24 well plate with a final concentration of venom at 1.5 or 3.1µg/ml and incubated for 48h. The cells that invaded were stained, photographed and quantified. The absorbance at 560nm was measured. The values are means ± SEM of 3 values. Statistical analysis was performed using one way ANOVA with Dunnett's post-hoc test. ** indicates significantly ($p < 0.01$) lower values compared with the untreated control.

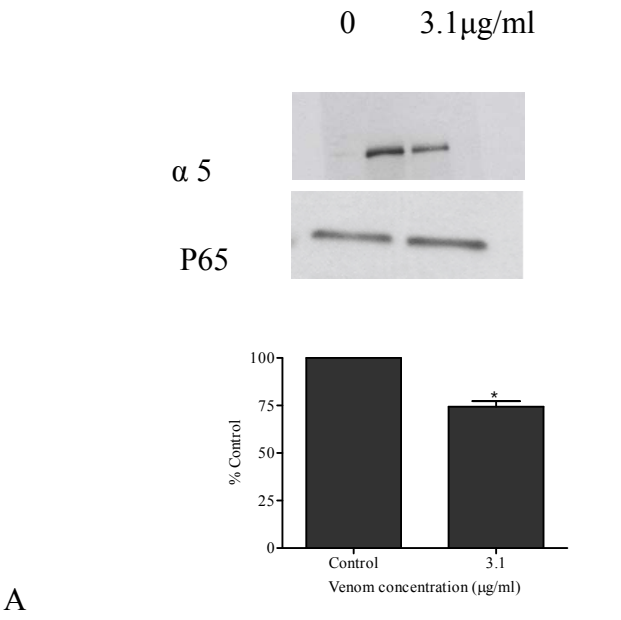


Control 1.5µg/ml 3.1µg/ml

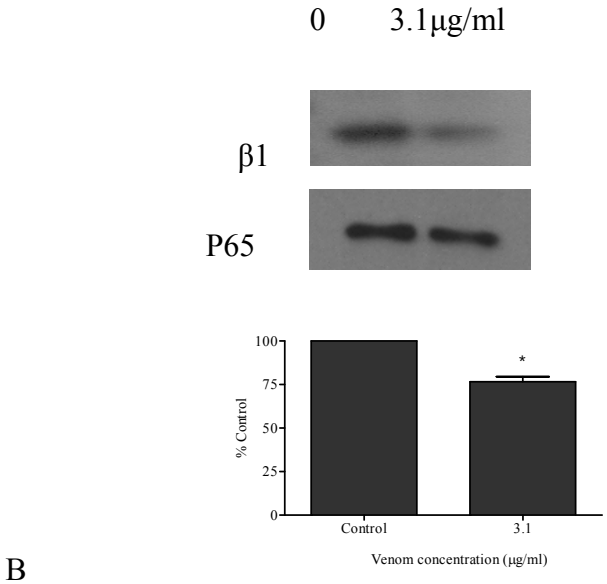
Figure 56: Effect of *Agkistrodon piscivorus leucostoma* venom on invasion of A2780 cells. A2780 cells were plated in an insert in a 24 well plate in the presence of 1.5 or 3.1µg/ml of venom and allowed to invade for 48h. The invaded cells were stained and images were taken under a Nikon TMS inverted phase contrast microscope. The magnificiation bar represents 50µm. Objective lens x20.

3.2.9 Effect of *Agkistrodon piscivorus leucostoma* venom on the level of α5, β1, and αv integrins expressed by A2780 cells

This experiment was carried out to determine the effect of *Agkistrodon piscivorus leucostoma* venom on expression of α5, β1, and αv integrins. The venom at 3.1μg/ml had an inhibitory effect on the level of α5, β1 and αv integrins, expressed by A2780 cells Figures 57A, B and Figure 58.



A



B

Figure 57: Effect of *Agkistrodon piscivorus leucostoma* venom on the level of (A) α5 and (B) β1 integrins expressed by A2780 cells. Cell lysates from A2780 cells treated with 3.1µg/ml of *Agkistrodon piscivorus leucostoma* venom were prepared, separated by SDS-PAGE and immunoblotted with antibodies against α5 and β1 integrins. P65 was used as a loading control. Dilution of primary and secondary antibodies was 1:1000. The values are means ± SEM of 3 values. Statistical analysis

was carried out using a Student's paired t-test. * indicates significantly ($p < 0.05$) lower values compared with the untreated control.

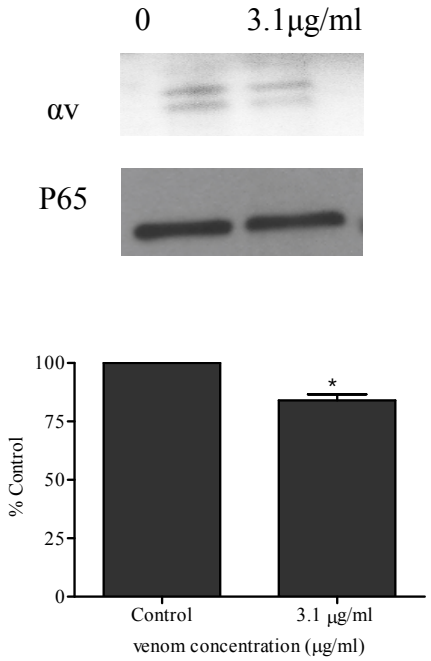


Figure 58: Effect of *Agkistrodon piscivorus leucostoma* venom on the level of αv integrins expressed by A2780 cells. Cell lysates from A2780 cells treated with 3.1μg/ml of venom were prepared, separated by SDS-PAGE and immunoblotted with antibodies against αv integrins. P65 was used as a loading control. Dilution of primary and secondary antibodies was 1:1000. The values are means ± SEM of 3 values. Statistical analysis was carried out using a Student's paired t-test. * indicates significantly ($p < 0.05$) lower values compared with the untreated control.

3.2.10 Fractionation of *Agkistrodon piscivorus leucostoma* venom by size exclusion chromatography

Separation of venom was achieved by a size exclusion chromatography (Sephadex G75). The fractions obtained were pooled into 9 groups (Figure 59A) and lyophilised.

3.2.11 Gel electrophoresis

Separation of venom by SDS-PAGE showed that there were more than 10 protein bands ranging in molecular weight from less than 10,000 to 150,000Da (Figure 59B1). Nine fractions (F1-F9) were obtained from the crude venom and lyophilised. Only F3 to F9 were separated by SDS-PAGE, as there was no powder observed for F1 and F2. Fractions F3 and F4 contained proteins with molecular weights ranging from 50,000 to 80,000Da and from 20,000 to 40,000Da respectively. The other fractions had proteins with molecular weights less than 25,000Da (Figure 59B2).

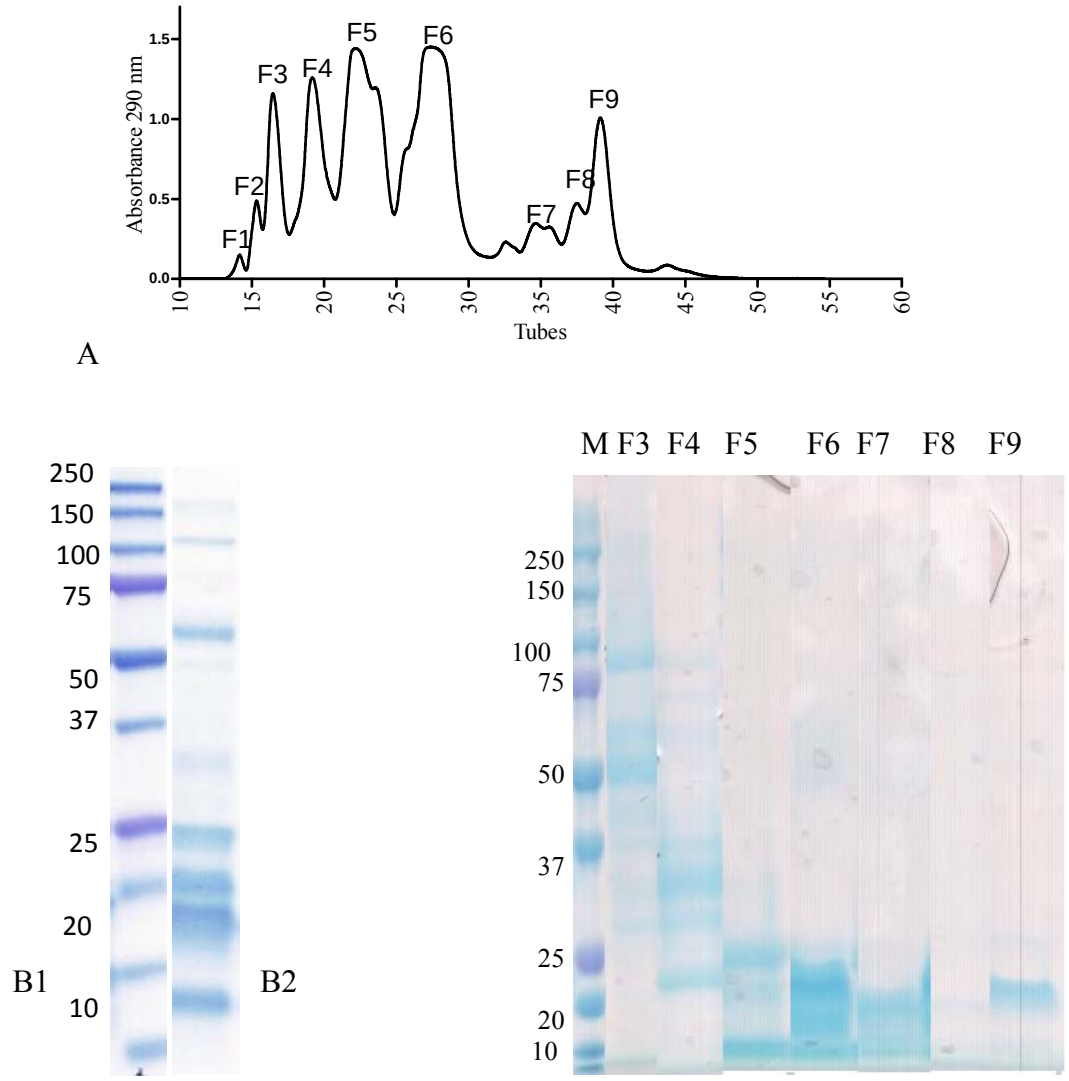
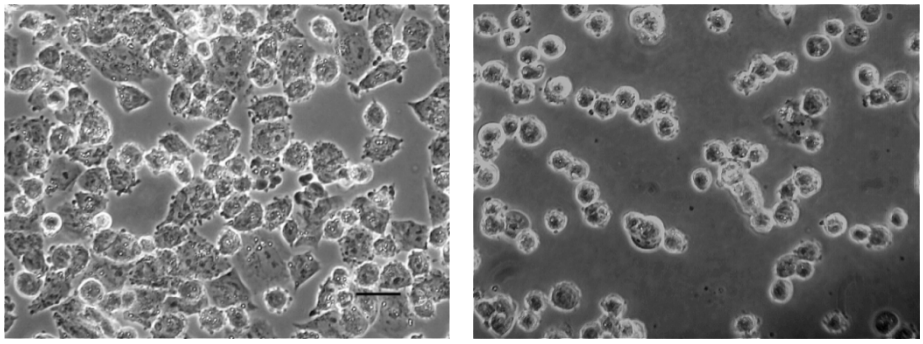


Figure 59: Separation of *Agkistrodon piscivorus leucostoma* venom using (A) size exclusion chromatography and (B1) SDS-PAGE of the crude venom (B2) SDS-PAGE of fractions. The crude venom (100 mg) was dissolved in 700µl of ammonium acetate buffer applied to a chromatographic column (1.5x100cm). The absorbance was monitored at 290nm. Fractions were eluted at a flow rate of 0.3ml/min and then pooled and lyophilised. Crude venom and fractions (15µl of stock at 3mg/ml) was mixed with 5µl of stain buffer. The mixture was centrifuged, heated and then loaded on 10% gel.

3.2.12 Effect of F6 from *Agkistrodon piscivorus leucostoma* venom on the morphology of A2780 cells after 4h

A preliminary experiment was carried out with fractions from *Agkistrodon piscivorus leucostoma* venom to determine if the fractions would induce any visible morphological changes (data not shown). Among these results, F6 was chosen as the most interesting fraction with anti-adhesive activity**.** At 1.5µg/ml F6 induced visible morphological changes in the cells after 4h incubation. The control well showed cell attachment and spreading compared with the F6 treated wells that showed the cells clustered together, less flattened, and more round, suggesting there was an effect on adhesion (Figure 60).



Control 1.5µg/ml

Figure 60: Effect of F6 on the morphology of A2780 cells. A2780 cells were seeded at a density of $1x10^6$ cells per well in a 12 well plate and incubated with 1.5µg/ml of F6 for 4h. After which, images were taken using a Nikon TMS inverted phase contrast microscope. The magnification bar represents 25µm. Objective lens x40.

3.2.13 Effect of F6 of *Agkistrodon piscivorus leucostoma* venom on membrane integrity of A2780 cells after 4h

The effect of F6 on viability of A2780 cells after 4h has examined using SYTOX®Green. This experiment was conducted to demonstrate the effect of different concentrations of F6 on membrane integrity (viability) of A2780 cells after 4h in order to prove the morphological alterations of the cells after adding F6 was not due to cytotoxicity. No effects on membrane integrity were observed at concentrations between 0.1-6.25µg/ml, unlike at higher concentrations (> 6.25µg/ml) (Figure 61).

3.2.14 Effect of F6 of *Agkistrodon piscivorus leucostoma* venom on A2780 cells viability after 24, and 48h

The effect of F6 on viability of A2780 cells after 24, 48, and 72h has examined using AlamarBlue® assay. F6 was found to have an inhibitory effect on the viability of A2780 cells at concentrations between 6.25 and 12.5µg/ml at 24 and 48h, while the concentrations between 0.1-1.5µg/ml did not affect the cell viability at theses times (Figures 62A and B). The concentration 3.1µg/ml had an inhibitory effect on cell viability after 48h.

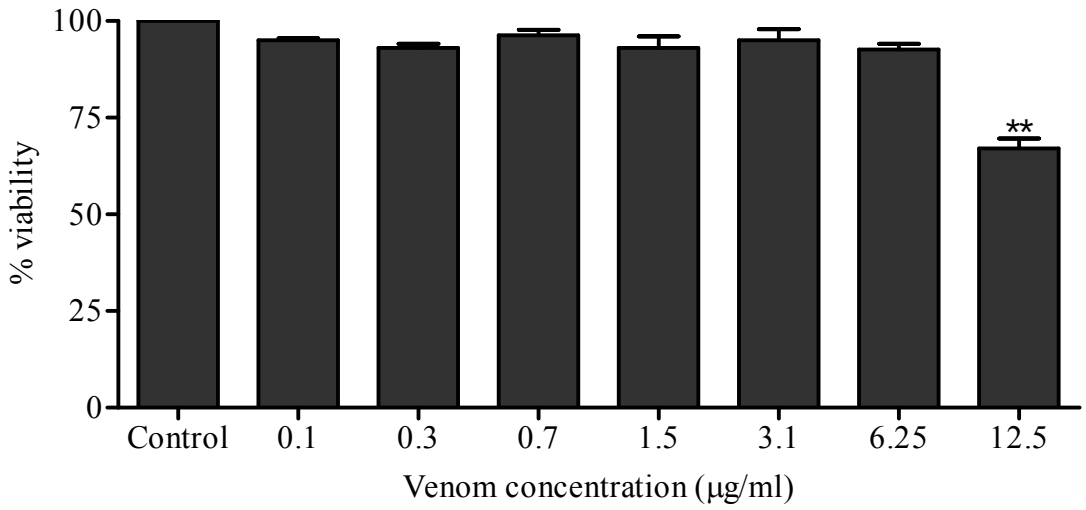
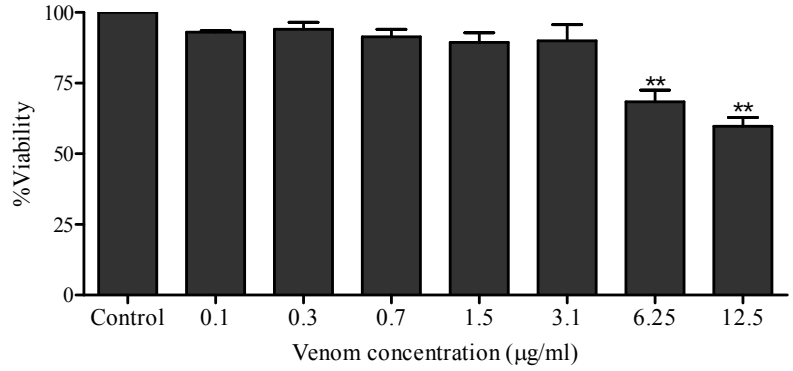
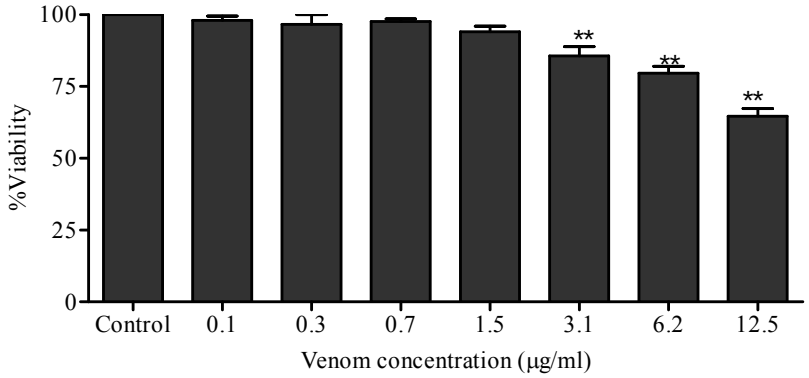


Figure 61: **Effect of F6 on A2780 cells membrane integrity after 4h.** Briefly, $1x10^4$ cells per well in complete medium (75µl) was added and incubated overnight. After which, venom (25µl) was added to the wells at a final concentration between 0.1-12.5µg/ml, incubated for 4h. After which, SYTOX®Green was used at a final concentration of 5µM in each well and incubated for 15min at 37°C. The fluorescence intensity was measured at 485-535nm. Values represent the mean ± SEM of 3 values. Statistical analysis was performed using one way ANOVA with Dunnett's post-hoc test. ** indicates significantly ($p < 0.01$) lower values compared with the untreated control.



A



B

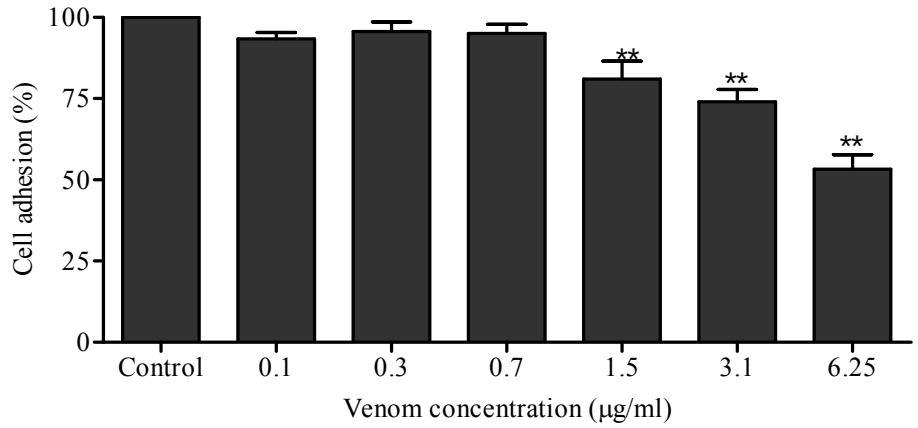
Figure 62: Effect of F6 on viability of A2780 cells after (A) 24 and (B) 48h. Cells were cultured in a 96 well plate at a density of $1x10^4$ cells per well and incubated overnight. Following which, cells were exposed to different concentrations of F6 between 0.1-12.5µg/ml. Six hours prior to the end of the 24 and 48h incubations, 10 µl of AlamarBlue® was added to the cells in each well and incubated at 37°C. The fluorescence intensity was read at 560-590nm. Statistical analysis was performed using one way ANOVA with Dunnett's post-hoc test. ** indicates significantly ($p < 0.01$) lower values compared with the untreated control.

3.2.15 Effect of F6 on adhesion of A2780 cells to vitronectin and fibronectin

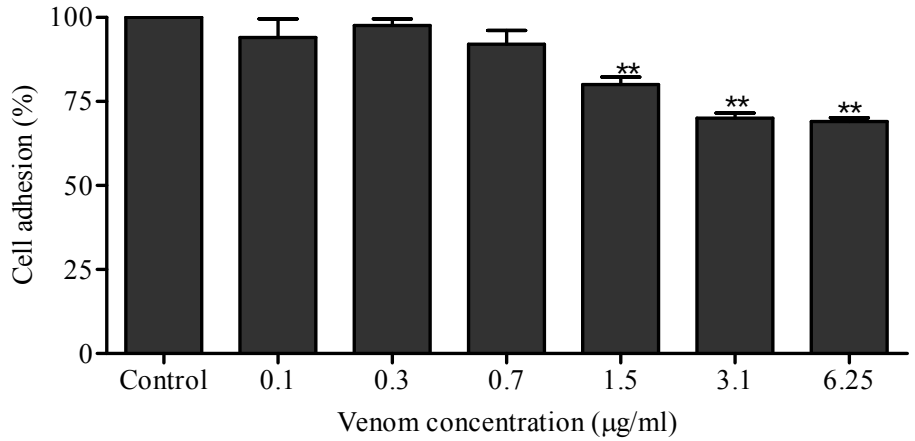
This was carried out to examine whether the morphological alterations induced after adding F6 was due to an effect on the adhesion of the cells. A2780 cells spread and adhered well to fibronectin (observed as flattened cells), while F6 decreased adhesion (Figures 63A and 64A). Adhesion of A2780 cells to vitronectin was significantly affected ($p < 0.01$) with concentrations greater than 1.5μg/ml (Figures 63B and 64B). No venom concentration below 1.5μg/ml affected the adhesion of A2780 cells to vitronectin nor to fibronectin.

3.2.16 Effect of F6 on adhesion of A2780 cells to an integrin-independent substratum (poly-L-lysine)

This experiment was carried out to determine if F6 affects the adhesion of A2780 cells to an integrin-independent substratum. There was an effect observed (Figures 65 and 66), suggesting that F6 is not specific for the integrin family of adhesion receptors.



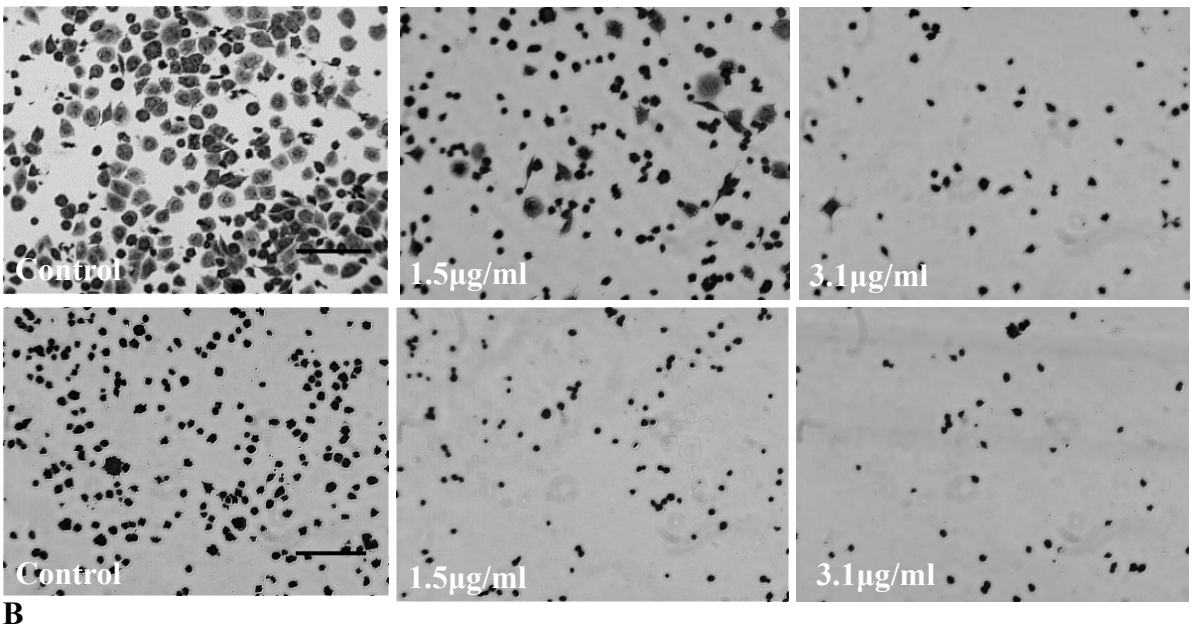
A



B

Figure 63: Effect of F6 of *Agkistrodon piscivorus leucostoma* venom on adhesion of A2780 cells to (A) fibronectin and (B) vitronectin. After coating a 96 well plate with fibronectin or vitronectin for 1h, the proteins were removed and the plate washed with PBS. Cells ($4x10^5$cells/ml) were added with F6 (at final concentrations between 0.1-6.25µg/ml). After 4h, the plate was washed, fixed, stained with crystal violet, and lysed with 0.1% (v/v) Triton X-100. The absorbance was measured at 540nm. The values are means ± SEM of 3 values. Statistical analysis was performed using one way ANOVA with Dunnett's post-hoc test. ** indicate significantly ($p < 0.01$) lower values compared with the untreated control.

A



B

Figure 64: **Effect of F6 on adhesion of A2780 cells to (A) fibronectin and (B) vitronectin.** After coating a 96 well plate with fibronectin or vitronectin, A2780 cells ($4x10^5$cells/ml) and F6 (at final concentrations of 1.5 or 3.1µg/ml) were added to the coated plate and incubated. After 4h, the plate was washed, fixed, stained, and images were taken. The magnification bar represents 50µm. Objective lens x20.

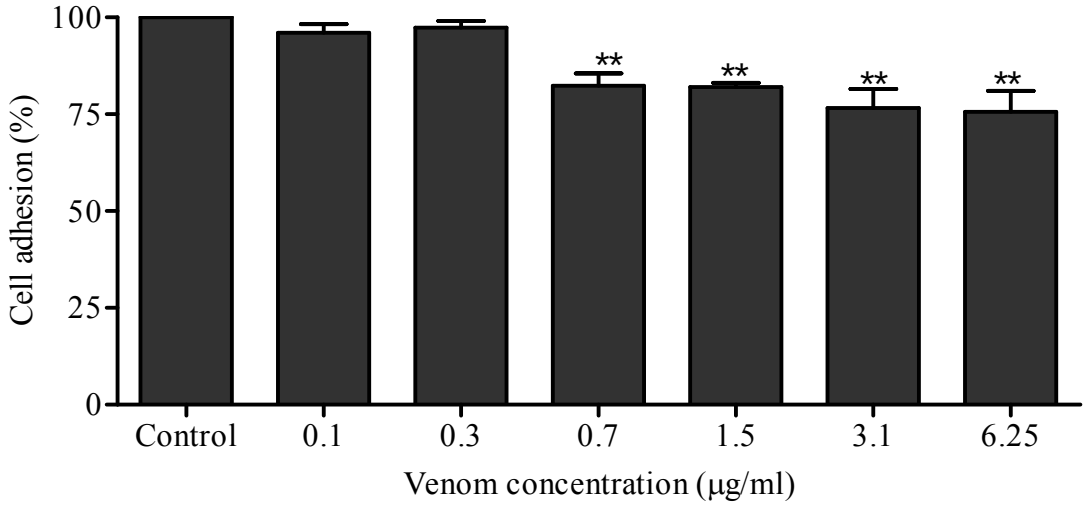


Figure 65: Effect of F6 on adhesion of A2780 cells to poly-L-lysine. Tissue culture wells were coated with poly-L-lysine (50µg/ml), incubated for 1h, removed and washed with PBS. A2780 cells were seeded in the coated plate and incubated in the presence of different concentrations of F6 (0.1-6.25µg/ml) for 4h. The plate was washed, fixed, stained with crystal violet, and lysed with 0.1% (v/v) Triton X-100. The absorbance was read at 540nm. The values are means ± SEM of 3 values. Statistical analysis was performed using one way ANOVA with Dunnett's post-hoc test. ** indicates significantly ($p < 0.01$) lower values compared with the untreated control.

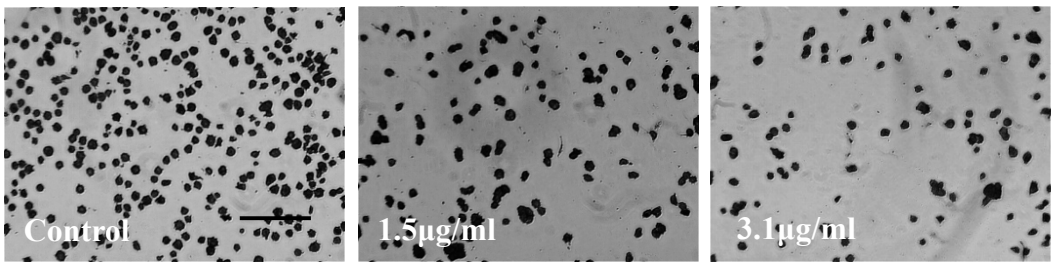


Figure 66: **Effect of F6 on adhesion of A2780 cells to poly-L-lysine.** A2780 cells were adjusted to $4x10^5$ cells/ ml and added to a poly-L-lysine coated plate (50µg/ml). F6 was added to the cells at final concentrations of 1.5 or 3.1µg/ml and incubated for 4h. Then the cells were fixed, stained, and images were taken. The magnification bar represents 50µm. Objective lense x20.

3.2.17 Effect of F6 on migration of A2780 cells

This experiment was conducted to examine the effect of F6 on migration of A2780 cells using cell migration assay. The result of this experiment showed F6 at 1.5 and 3.1μg/ml significantly ($p < 0.01$) inhibited the migration of A2780 cells (Figures 67 and 68).

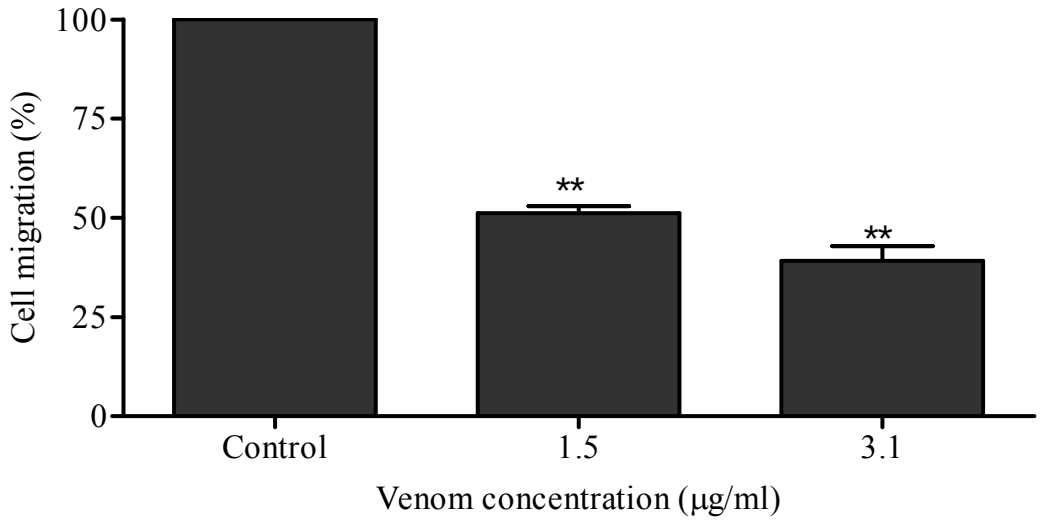


Figure 67: Effect of F6 of *Agkistrodon piscivorus leucostoma* venom on migration of A2780 cells. Cells were adjusted to $1x10^6$ cells /ml in serum free medium with F6 at final concentrations of 1.5 and 3.1μg/ml, and then incubated for 24h. The cells that migrated into the lower surface of the membrane were stained, photographed, and quantified. The absorbance was determined at 560nm. The values are means ± SEM of 3 values. Statistical analysis was achieved using one way ANOVA with Dunnett's post-hoc test. ** indicates significantly ($p < 0.01$) lower values compared with the untreated control.

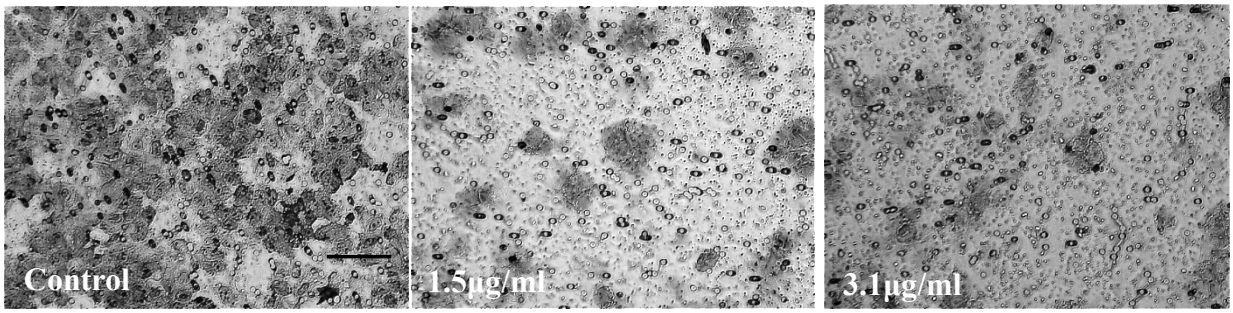


Figure 68: F6 of *Agkistrodon piscivorus leucostoma* venom inhibited the migration of A2780 cells. In a volume of 300µl serum free medium, $1x10^6$cells /ml with F6 at a final concentration of 1.5 or 3.1µg/ml was added per insert in a 24 well plate. Cells were allowed to migrate for 24h at 37°C. The migrated cells were stained and images were taken. The magnification bar represents 50µm. Objective lens x20.

3.2.18 Effect of F6 on invasion of A2780 cells across the basement membrane

The effect of F6 on invasion of A2780 cells across a basement membrane was assessed using a Cytoselect^{TM} 24-well Cell Invasion Assay F6 had an inhibitory effect on invasion of A2780 cells across the basement membrane at 1.5 (30%) and 3.1µg/ml (45%) (Figures 69 and 70).

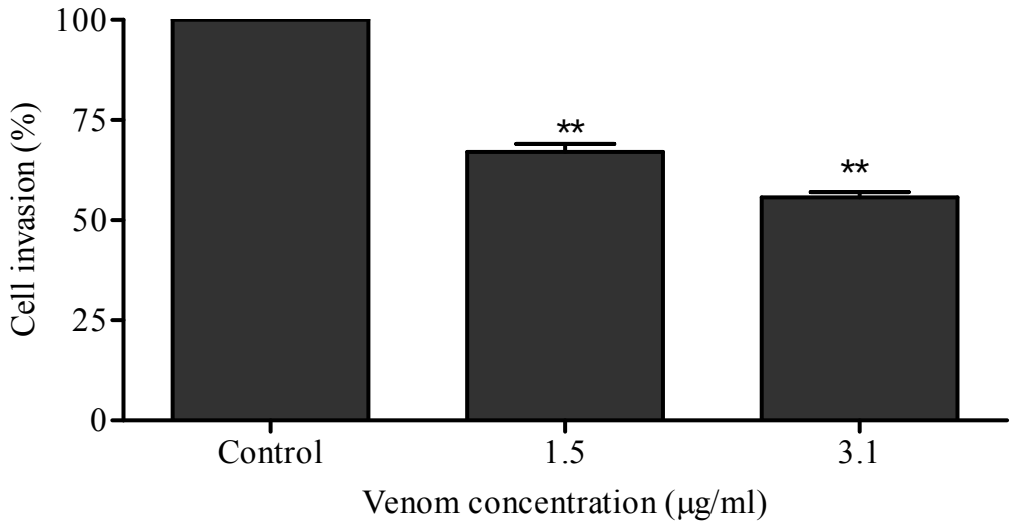


Figure 69: **Effect of F6 on invasion of A2780 cells across the basement membrane.** Cells were adjusted to $1x10^6$ cells /ml in serum free medium and 300µl of cell suspension was added to each insert in a 24 well plate with F6 at a final concentration of 1.5 or 3.1µg/ml. Then cells were incubated for 48h. The cells that invaded into the lower surface of the membrane were stained, photographed, and quantified. The absorbance was determined at 560nm. The values are means ± SEM of 3 values. Statistical analysis was achieved using one way ANOVA with Dunnett's post-hoc test. ** indicates significantly ($p < 0.01$) lower values compared with the untreated control.

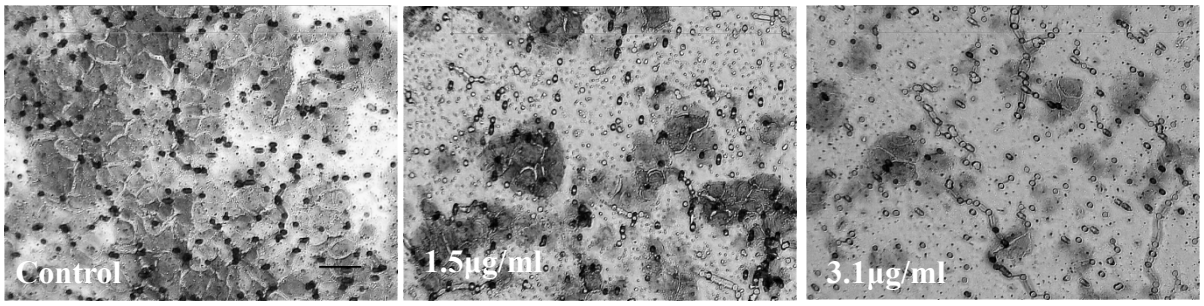
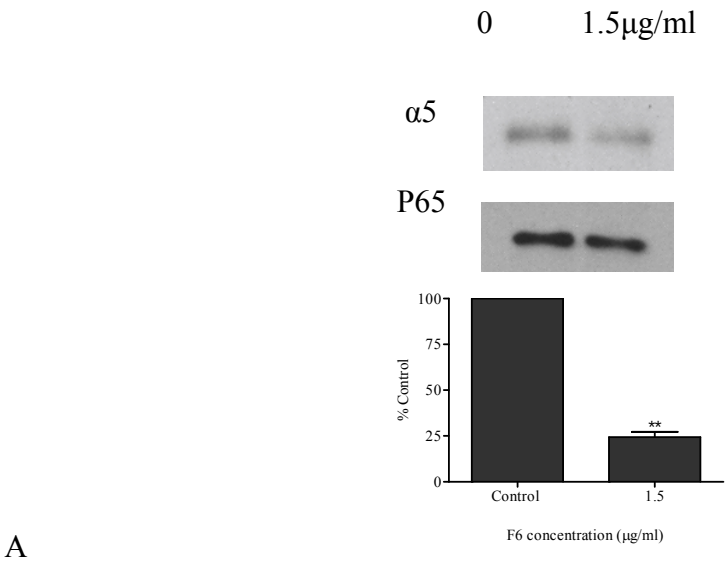


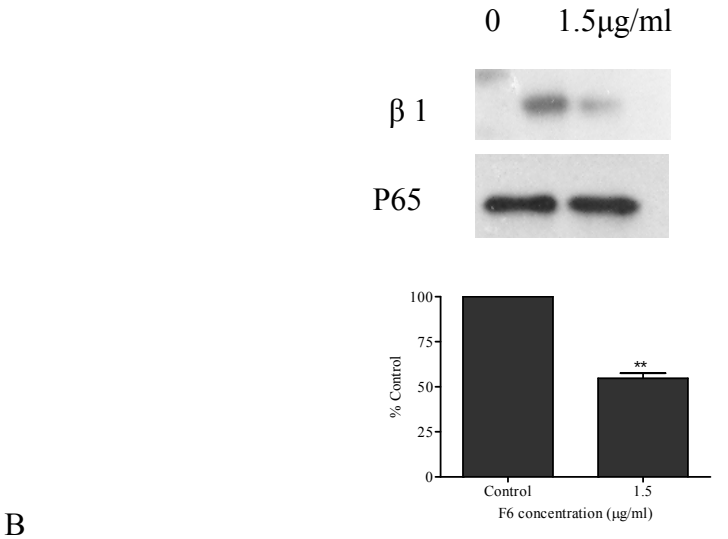
Figure 70: **Effect of F6 on invasion of A2780 cells across the basement membrane.** Cells ($1x10^6$ cells /ml) were incubated in the presence of F6 (1.5 or 3.1µg/ml) for 48h. The invaded cells were stained and images were taken. The magnification bar represents 50µm. Objective lens x20.

3.2.19 Effect of F6 on the level of expression of α5, β1, and αv integrins of A2780 cells

This experiment was carried out to assess the effect of F6 of *Agkistrodon piscivorus leucostoma* venom on expression of α5, β1 and αv of A2780 cells. F6 at 1.5µg/ml had an inhibitory effect on expression of α5, β1 (Figures 71 A and B), and on αv integrins (Figure 72). The percentage of inhibition was 75%, 50% and 70% for α5, β1 and αv integrins, respectively.



A



B

Figure 71: Effect of F6 on the level of expression of (A) α5 and (B) β1 integrins of A2780 cells. Cell lysates from A2780 cells treated with 1.5µg/ml of F6 were separated by SDS-PAGE and immunoblotted with antibodies against α5 and β1 integrins. P65 was used as a loading control. Dilution of primary and secondary antibodies was 1:1000. The values are means ± SEM of 3 values. Statistical analysis was carried out using Student's paired t-test. ** indicates significantly ($p < 0.01$) lower values compared with the untreated control.

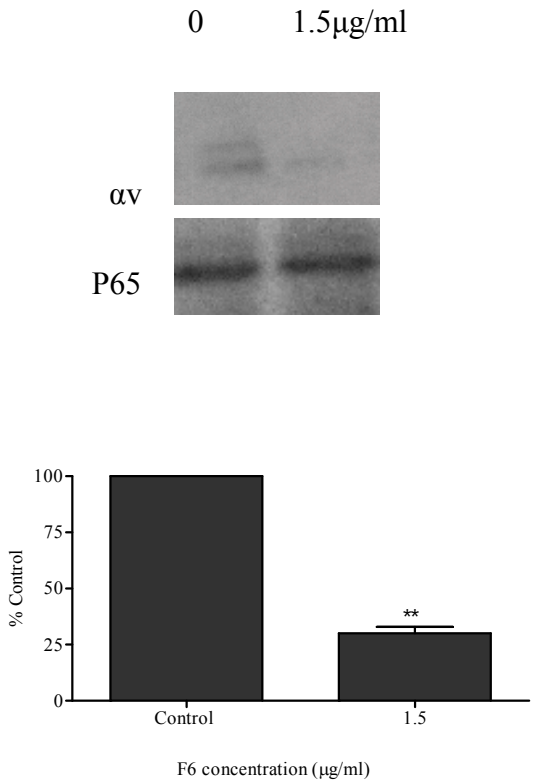


Figure 72: Effect of F6 of *Agkistrodon piscivorus leucostoma* venom on the level of expression of αv integrins of A2780 cells. Cell lysates from A2780 cells which were treated with 1.5µg/ml of F6 were separated by SDS-PAGE and immunoblotted with antibodies against αv integrins. P65 was used as a loading control. Dilution of primary and secondary antibodies was 1:1000. The values are means ± SEM of 3 values. Statistical analysis was carried out using Student's paired t-test. ** indicates significantly ($p < 0.01$) lower values compared with the untreated control.

3.2.20 Identification of active compnent(s) in F6 from *Agkistrodon piscivorus leucostoma* venom

The major aim of these investigations was to identify the active peptides in F6 responsible for inhibiting adhesion, migration and invasion of A2780 cells through inhibition of the expression of α5, β1 and αv integrins.

3.2.20.1 SDS-PAGE for F6 from *Agkistrodon piscivorus leucostoma* venom

In order to identify the molecular weight of each peptides in F6 SDS-PAGE was used. F6 was separated on SDS-PAGE (15% polyacrylamide gels) and revealed the presence of 2 peptides with molecular weights between 23,000-25,000Da (denoted F6.1) and 13,000-17,000Da (denoted F6.2) (Figure 73). The two bands were excised and sent for proteomic analysis.

3.2.20.2 Nanoflow HPLC Electrospray Tandem Mass Spectrometry

In order to identify the active peptides in F6 Nanoflow HPLC Electrospray Tandem Mass Spectrometry was used. Tandem Mass Spectrometry was used because F6 is not pure and mixture.The first band F6.1 was identified as snake venom metalloproteinase ACLH with molecular weights of 46,109Da (highest score) which was contradictory to the SDS-PAGE analysis. The F6.2 band according to Nanoflow HPLC Electrospray Tandem Mass Spectrometry was identified as phospholipase A2 (highest score) with a molecular weights of 13,961Da.

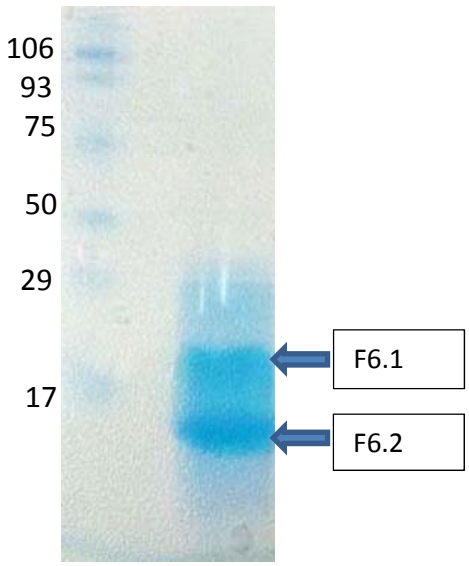


Figure 73: **SDS-PAGE for F6 of *Agkistrodon piscivorus leucostoma* venom.** Five µl stain buffer and 15µl of venom (3mg/ml) were mixed together, centrifuged, heated, and loaded on a 15% polyacrylamide gel. When the first band reached the lower edge of the gel, the current was stopped and the gel removed, washed, and stained. Bands indicated by the arrows were excised and sent for identification by Tandem Mass Spectrometry.

3.2.20.3 Liquid chromatography mass spectrometry (LC-MS)

This technique was performed to identify the molecular mass and the percentage of peptides in F6. F6 was found to have 3 peptides with molecular weights of 13,947, 13,988, and 23,000Da. The percentage quantity of these peptides was 28.88%, 39.30% and 12.08%, respectively (Figure 74). The first two peaks are thought to be the same molecule, but appear as two peaks because of the separation procedure using ammonium acetate for purification of the venom (personal communication Dr JonansTusiimire, SIPBS).

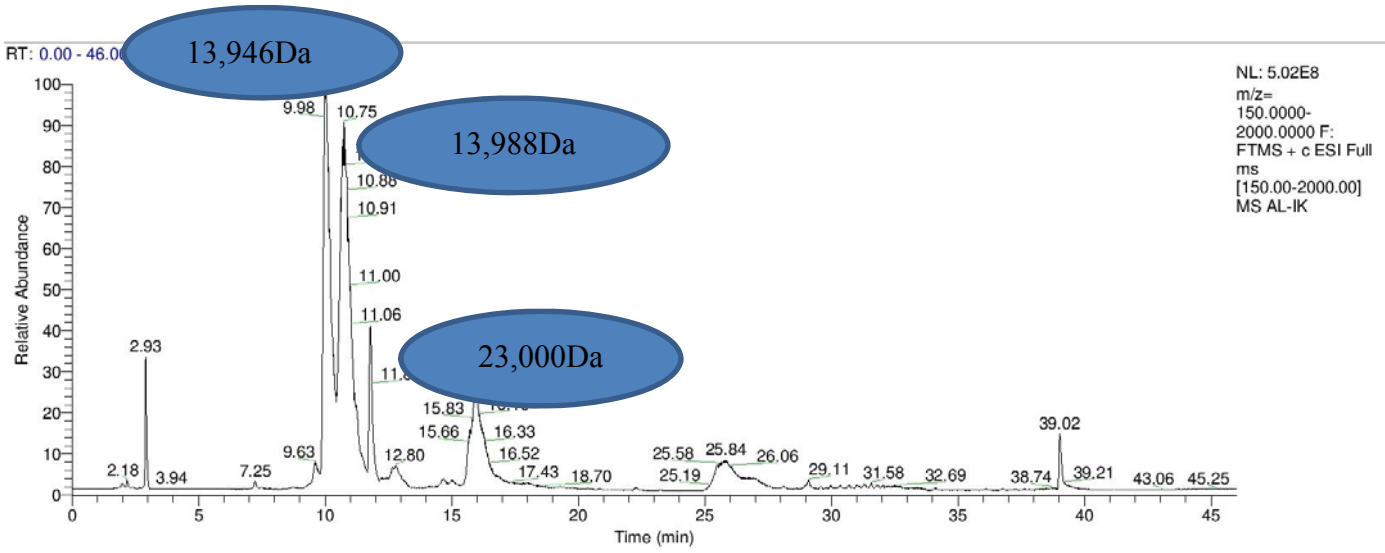


Figure 74: Analysis of F6 by liquid chromatography mass spectrometry (LC-MS). One mg of the fraction was dissolved in 1ml water and 10µl of the solution was injected along with a gradient from 90% water and 10% acetonitrile with 0.1% (v/v) formic acid to 90% acetonitrile and 10% water and 0.1% (v/v) formic acid at a flow rate of 0.3 ml/min for 30 min.

3.2.20.4 Assessment of the effect of metalloproteinase inhibitor (1,10-phenanthroline) on activity of F6 of *Agkistrodon piscivorus leucostoma* venom

In order to identify which component of F6 is responsible for morphological changes and anti-adhesion activities, this final experiment was carried out in the presence of enzyme inhibitors. Metalloproteinase activity is Zn^{2+}-dependent, while phospholipase A2 is Ca^{+2} dependent. EDTA was not suitable to assess which one was the active peptide (metalloproteinase or phospholipase) because it is a chelating agent for both Zn^{2+} and Ca^{+2}. Phenanthroline is an inhibitor of metalloproteinase and mainly targets Zn^{2+} with a much lower affinity for Ca^{+2}, so phenanthroline was chosen in this experiment. The result of this experiment showed that the morphological alterations and anti-adhesion activity of F6 were abolished after adding 25µg/ml of phenanthroline (Figures 75 and 76). Inhibition of the activity of F6 by phenanthroline, suggests that the active peptide in this fraction is snake venom metalloproteinase. A serial dilution of phenanthroline was tested from 12.5 to 100µg/ml, but 25µg/ml was chosen as 100 and 50µg/ml had a toxic effect on the cells.

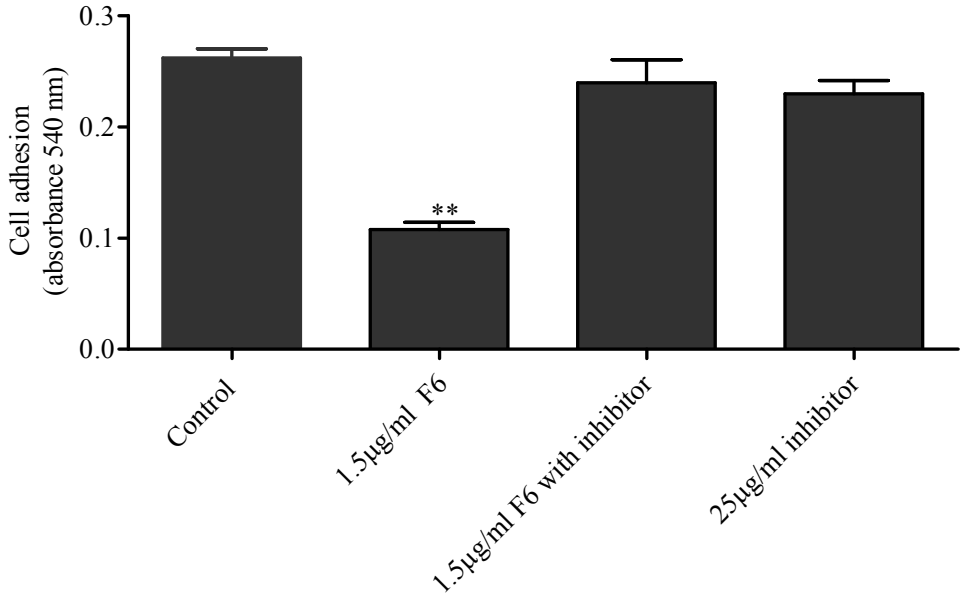


Figure 75: Effect of 1,10-phenanthroline (metalloproteinase inhibitor) on activity of F6 after 24h. A2780 cells were prepared at $3x10^5$ cells/ ml in complete medium and added to a 24 well plate with F6 (1.5µg/ml) plus 25µg/ml of phenanthroline at 37°C. After 24h, the medium was removed and cells washed with PBS, fixed with PFA, stained with crystal violet, and lysed with 0.1% (v/v) triton X-100. The absorbance was determined at 540 nm. The values are means ± SEM of 3 values. Statistical analysis was performed using one way ANOVA with Dunnett's post-hoc test. ** indicates significantly ($p < 0.01$) lower values compared with the untreated control.

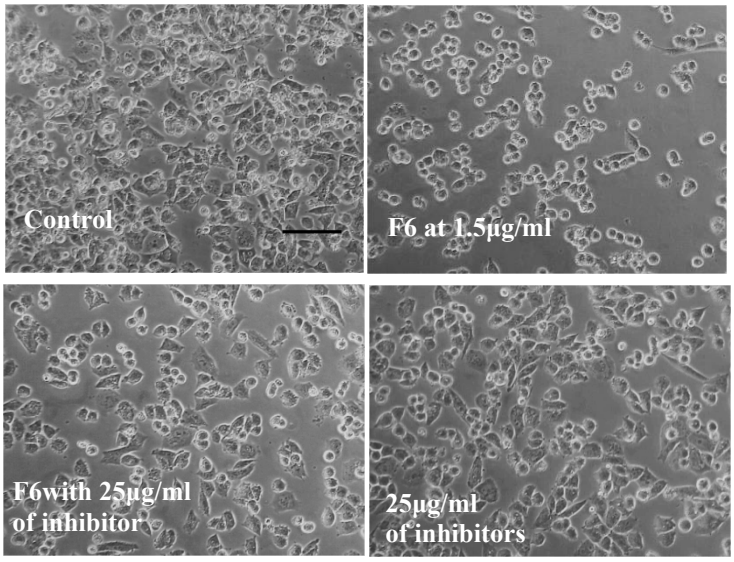


Figure 76: Effect of 1,10-phenanthroline (metalloproteinase inhibitor) on activity of F6 after 24h. Cells were seeded at $3x10^5$ cells per ml in a 24 well plate. Venom was prepared and added at a final concentration of 1.5µg/ml then 25µg/ml of inhibitor was added to the plate and incubated for 24h and images were taken. The magnification bar represents 50µm. Objective lens x20.

4.0 Discussion

Recently, numerous studies have suggested that peptides from snake venom can be used to provide therapeutics against different types of cancer and prevent metastasis by their anti-integrin activities (reviewed by Calderon *et al*., 2014). Several peptides and proteins from venom have been used in many pharmacological applications (Lewis & Garcia, 2003), so this work examined the effect of two kinds of snake venom on dissemination of ovarian cancer cells. Metastasis of ovarian cancer occurs through detachment of cancer cells from the original site into the peritoneal cavity as single cells or multicellular spheroids. It has been demonstrated that, α5β1integrin is responsible for spheroid formation and attachment of ovarian cancer cells at metastatic sites (Casey *et al*., 2001), while the work of Ohyagi-Hara *et al.* (2013) reported that α5 integrin plays a vital role in peritoneal spreading of ovarian cancer (Ohyagi-Hara *et al.*, 2013). In order to metastasise to new sites, cell adhesion, migration and invasion is required. So this project focussed on the effect of *Crotalus ruber ruber* and *Agkistrodon piscivorus leucostoma* venom and their active fractions on viability, adhesion to different ECM proteins, migration and invasion of A2780 cells. Integrins have been known as an important factor for tumour cell adhesion, migration and invasion (reviewed by Sawada *et al*., 2012), so the effect of venom on α5, β1 and αv integrins of A2780 cells was examined.

4.1 *Crotalus ruber ruber* and *Agkistrodon piscivorus leucostoma* venoms affect morphology of A2780 cells to prevent adhesion in different ways

Both venoms and their active fractions had an effect on the morphology of the cells as follows: after adding *Crotalus ruber ruber* venom or F3, cells became rounded up and lost their ability to spread on the culture support plastic surface, suggesting there

was an effect on adhesion. However, after adding *Agkistrodon piscivorus leucostoma* venom or F6, the cells lost their spreading ability, became less flattened and more aggregated. The effect of both venoms was quite different because the effect of *Agkistrodon piscivorus leucostoma* led to the cells becoming abnormal in shape and aggregated, while this was not the case with the *Crotalus ruber ruber* venom. Nevertheless, overall, the effect of both venoms indicated anti-adhesive properties. To date, there has been no study of the effect of these particular venoms on the morphology of A2780 cells. However, there is a study on the effect of rubelysin and rubelase (metalloproteinases purified from *Crotalus ruber ruber* venom) on toxicity and viability of HUVECs (Komori *et al*., 2011). In addition, rubistatin (a disintegrin from *Crotalus ruber ruber* venom) has been shown to have an inhibitory effect on migration and proliferation of melanoma cells, but it has not been studied for its effect on morphology and adhesion of these cells (Carey *et al*., 2012). The result of the current work is similar to the result of another study that demonstrated jararhagin (a metalloproteinase from *Bothrops jararaca* snake venom) induced morphological alterations of melanoma cells in terms of the cells becoming rounded and clustered together (Correa *et al*., 2002). A similar effect was observed in normal endothelial cells, but there was no effect on morphology of murine peritoneal adherent cells, which was attributed to the specificity of jararhagin to specific cell receptors expressed by certain types of cells (Tanjoni *et al*., 2005). It was demonstrated that those morphological changes were associated with the anti-adhesive properties of the venoms and that correlates with the result of the current work. Similarly, other studies have indicated that BaH-1 (hemorrhagic toxin from venom of *Bothrops asper*) enhanced morphological alterations of endothelial cells (Borkow *et al*., 1995),

while rounding up and granulation of HeLa cells was observed after exposure to ICD-85 (a peptide from *Agkistrodon halys* venom)(Sarzaeem *et al*., 2012).

4.2 *Crotalus ruber ruber* and *Agkistrodon piscivorus leucostoma* venoms had no effect on viability of A2780 cells at specific concentrations that had an effect on adhesion of A2780 cells

To clarify that the actions of the venoms on morphology were due to their effect on adhesion and not cytotoxicity, the effect on viability after 4, 24, 48 and 72h was tested. This work demonstrated that there was no significant effect on the viability of the cells at concentrations that had a profound effect on morphology (1.5 and 3.1μg/ml). So only these two concentrations were considered in subsequent experiments. It has been proposed that morphological alterations of endothelial cells observed in the presence of *Loxosceles intermedia* venom also did not correlate to cytotoxicity of the venom (Paludo *et al*., 2006). Similarly, jararhagin was shown to affect morphology and adhesion of melanoma cells without affecting viability within 3h (Correa *et al*., 2002). Moreover, BaH-1 affected morphology of endothelial cells at concentrations not associated with cytotoxicity (Borkow *et al*., 1995). Thus, the finding of the current study was not unexpected and indicated potential therapeutic activity and justification to use this approach. The effect of time was significant because there was no effect on cell viability for concentrations from 0.1 to 3.1μg/ml for 4, 24 and 48h for both crude venoms. However, after 72h *Agkistrodon piscivorus leucostoma* venom had an effect on viability at concentrations of 1.5 and 3.1μg/ml. Similarly *Crotalus ruber ruber* venom at 3.1μg/ml had an effect on viability after 72h. Both of active fractions at 3.1μg/ml had a significant effect on viability after 48h. Treatment with both concentrations resulted in morphological changes and a

decrease in adhesion after 4, 24 and 48h. After 72h of treatment with 3.1µg/ml of the crude venom, the viability of the cells was significantly affected, so morphology and adhesion could not be determined for this time-point. Similarly, 3.1µg/ml of the fractions had a marked effect on cell viability after 48h. There was a strong correlation between detachment of cells and loss of viability after 72h. Cell death after detachment from ECM is known as anoikis (Paoli *et al*., 2013). It has been reported that jararhagin induces apoptosis (anoikis) in endothelial cells because it disrupts cell adhesion and leads to loss of cell anchorage and anoikis after 48h (Tanjoni *et al*., 2005). Integrins are the important mediator between the cells and ECM, and that is the likely mechanism of action of the venoms used in this study. The work of Nowatzki *et al.* (2012), demonstrated that *Loxosceles intermediate* venom induced detachment of the endothelial cells from their substrate and caused them to cluster together. In addition, there was no effect on viability of the cells after 3h, while the cell viability reduced after 48h of incubation with the venom and resulted in anoikis (Nowatzki *et al* 2012). It has been reported that endothelial cells need 48h to execute anoikis after detachment (Aoudjit and Vuori, 2001). This correlates with the results of the current work, which demonstrated that it is not possible to detect the reduction in cell viability at early time points. The correlation between cell detachment and anoikis was confirmed using jararhagin on cells in suspension. The results showed that jararhagin did not change the number of apoptotic cells in non-adherent conditions compared to the no treatment controls (Tanjoni *et al.,* 2005). Anoikis is an important process to prevent the attachment of detached cells to incorrect places and to prevent dysplastic growth (Paoli *et al*., 2013). There are different pathways involved in anoikis that lead to activation of

caspase, DNA fragmentation and cell death. The two apoptotic pathways involved in execution of anoikis are intrinsic (mitochondria) or extrinsic (death receptors) pathways (Paoli *et al*., 2013). A pro-apoptotic protein in the intrinsic pathway leads to permeabilisation of the outer mitochondria membrane and release of cytochrome C into cytosol, which is associated with the activation of caspase. Stimulation of the death receptor is associated with the extrinsic pathway and leads to activation of caspase 8. Recently, it has been reported that detachment of the cells from ECM leads to release of some mitochondrial proteins to the cytosol and activates caspase-independent anoikis (Guadamillas *et al*., 2011). Some tumor cells have developed mechanisms to resist anoikis and thereby survive after detachment from their original site. Resistance to anoikis in tumor cells could be due to defects in the death receptor pathway of caspase activation or a defect in mitochondrial pathway. As a result, failure to perform the anoikis could result in cells adhere and proliferate at ectopic site. Targeting defects in these pathways can restore sensitivity to anoikis and work as the basic for therapy against tumor metastasis (Paoli *et al*., 2013). In case of ovarian cancer, after detachment from the original site, the cancer cells are transported through the abdominal cavity and form spheroids to overcome anoikis, however, single cells undergo the process of anoikis (Masoumi *et al*., 2012). The reason for ovarian cancer cells escape anoikis still is not clear, however, there is a study has showed that the upregulation of miR-141 and the subsequent suppression of KLF12 are essential for anoikis resistance in ovarian cancer cells. The upregulation of miR-141 is associated with inhibition of intrinsic apoptotic pathway (Mak *et al*., 2017). Future work could focus on the anoikis pathways stimulated by

the venoms studied in the current project. In this project, the time was not sufficient to study the anoikis pathways.

4.3 A2780 cells showed a good spread on fibronectin using α5β1 integrins

In light of the possible role of anoikis from the previous section, the major aim of these experiments was to examine integrin expression patterns at the cell surface and to demonstrate on which substrata the cells spread and adhered to well. Functional blocking integrin antibodies were used to determine the integrins used by the cells to adhere to different ECM proteins (fibronectin, vitronectin). Integrins are well-known adhesion molecules, which mediate attachment of cells to other cells and the ECM (Schaffner *et al*., 2013). The main functions of integrins are their role as adhesion receptors for extracellular ligands and activation of intracellular signalling, which is involved in cell adhesion, migration, and progression of tumours. Although the involvement of integrins in ovarian, and in particular A2780 cells has been established before (reviewed by Sawada *et al*., 2012), these experiments were repeated in this project to make sure the cells were still expressing particular integrins as cancer cells undergo changes all time and there are changes in integrin expression during tumour migration and growth (Schaffner *et al*., 2013). The results showed that the cells adhered and spread well on fibronectin, but showed a lower degree of attachment on vitronectin. Under the conditions used in these experiments, A2780 cells did not spread or adhere well on collagen and plastic. These observations support the findings of the current work that the major integrins expressed by A2780 cells were α5, β1 (fibronectin receptor) and αv (fibronectin and vitronectin receptor), but not α2β1 (collagen receptor). Using functional blocking integrin antibodies in this study confirmed that the integrins required for adhesion of

A2780 cells were α5, β1 and αv. The work of Markland *et al.* (2008), showed the major integrins expressed by A2780 cells are αv, α5β1, β1 and α2β1. This result supported the findings of the current work except for their observation of the expression of α2β1 (collagen receptor). The reason for this could be because the integrins expressed by these cancer cells have changed or maybe because they used a different technique to identify the integrins expressed. In the current work an alpha/beta integrin-mediated cell adhesion array was used, while in the Markland *et al.* study, fluorescence-activated cell sorting (FACS) was used (Markland *et al.,* 2011). In the current work, three pieces of evidence were used to back up that there was no collagen receptor in these A2780 cells: (1) the cells did not spread on collagen I, (2) the cells did not express α2β1integrin and (3) using functional blocking antibodies the cells did not use α2β1 integrin for adherence. Another study showed that epithelial ovarian cancer cells adhere well to collagen I using α2β1 integrin and immunoprecipitation analyses showed that the cells expressed α2β1 integrin (Fishman *et al*., 1998). In a review by Lengyel (2010), α2β1 integrin plays a role in spheroid formation of ovarian cancer cells and is vital in their adhesion to collagen I (reviewed by Lengyel, 2010). In contrast, α5β1-integrin is responsible for spheroid development of ovarian cancer cells and their adhesion at metastatic sites (Casey *et al.,* 2001).

4.4 *Crotalus ruber ruber* venom inhibited the adhesion of A2780 cells to fibronectin, but not vitronectin and poly-L-lysine

In the current work, *Crotalus ruber ruber* venom and F3 had an inhibitory effect on adhesion of A2780 cells to fibronectin, but there was no effect on their adhesion to vitronectin and poly-L-lysine. As a result, it can be concluded that the venom is

specific at least for the fibronectin receptor. The result clearly showed that the *Crotalus ruber ruber* venom and F3 had an inhibitory effect on the level of α5 and β1 integrins (fibronectin receptor), while, there was no effect on the level of αv integrins (vitronectin and fibronectin receptor) of A2780 cells. *Agkistrodon piscivorus leucostoma* venom and F6 inhibited the adhesion of the cells to fibronectin, vitronectin and poly-L-lysine. The inhibitory activity of *Agkistrodon piscivorus leucostoma* venom and F6 could be due to hydrolysis of ECM, α5, β1 and αv integrins. In a study carried out by You *et al.* (2003), they demonstrated that degradation of the ECM by metalloproteinases led to disturbance of ECM function and caused cell detachment (You *et al*., 2003).The present study has shown that there was no effect of *Crotalus ruber ruber* venom on adhesion of A2780 to poly-L-lysine, unlike *Agkistrodon piscivorus leucostoma* venom which had an inhibitory effect. Thus, *Crotalus ruber ruber* venom is more specific for the integrin family of adhesion receptors, while *Agkistrodon piscivorus leucostoma* venom is not. The result of the effect of *Crotalus ruber ruber* venom and F3 was similar to the results reported by Cominetti *et al.* (2003), where BaG (metalloproteinase III from *Bothrops alternatus* snake venom) inhibited the adhesion of human erythroleukemia to fibronectin, but not to collagen I. The mechanism of action was through inhibition of the binding of α5β1 integrin to fibronectin. BaG has disintegrin domains which are thought to be responsible for its anti-integrin activity (Cominetti *et al*., 2003). This work on BaG correlates to the result of the current work since *Crotalus ruber ruber* venom and F3 inhibited the adhesion of the cells to fibronectin by inhibiting α5 and β1 integrins, but had no effect on αv integrin. Similarly EC3 (a disintegrin from *Echis carinatus* venom) inhibited the adhesion of leukemic cell (K562 cells) to

fibronectin via α5β1integrin and also had an inhibitory effect on α4β1 and α4β7 integrins (fibronectin) (Brando *et al*., 2000). *Echis carinatus* snake venom and the venoms which were used in this study were from the Viperidae family. Moreover, contortrostatin, a disintegrin from *Agkistrodon contortrix contortrix* venom, has been shown to bind to α5β1integrins and prevent cell adhesion to fibronectin (Cominetti *et al*., 2003), as well as adhesion of HUVEC to vitronectin (Zhou *et al*., 2000). Contortrostatin interacts with different integrins αIIbβ3, $\alpha5\beta1$, $\alpha v\beta3$, $\alpha v\beta5$, which explains how adhesion to fibronectin and vitronectin are affected. CC-PLA2-1 and CC-PLA2-2 (phospholipase A2 isolated from *Cerastes cerastes* venom) inhibited the adhesion of human fibrosarcoma and melanoma cells to fibronectin and fibrinogen (Zouari-Kessentini *et al*., 2009), but not to poly-L-lysine. The mechanism of inhibitory activity on cell adhesion occurred by blocking α5β1 and αv integrins and that correlates with the current work of the inhibitory effect of *Agkistrodon piscivorus leucostoma* venom on α5β1 and αv integrins and the adhesion assay used was the same as in the present study (Zouari-Kessentini *et al*., 2009). MVL-PLA2 (PLA2 from *Macrovipera lebetina transmediterranea* venom) inhibited the adhesion of different tumour cells such as melanoma and fibrosarcoma to fibrinogen and fibronectin through inhibition of α5β1 and αv integrins, however, their effect on adhesion of cells to vitronectin was slight and there was no effect on adhesion to poly-L-lysine or collagen I (Bazaa *et al*., 2009). The adhesion assay used was the same as in the current study and the results were similar to the result of the present work since *Crotalus ruber ruber* venom and F3 had no effect on adhesion of A2780 cells to poly-lysine and had an inhibitory effect on α5β1integrins. Halysase, a metalloproteinase III from *Gloydius halys*, induced apoptosis of HUVECs by

degradation of α5β1 and α1β1 integrins, but had no effect on αvβ3 and αvβ5 integrins (You *et al.,* 2003). Halysase is thought to hydrolyse fibronectin, vitronectin and IV collagen, but has no effect on types I, II and V collagen (You *et al.,* 2003). Both the metalloproteinase and disintegrin domains are responsible for the activity of halysase. The effect of halysase is similar to the result of the current work since the *Agkistrodon piscivorus leukostoma* venom and F6 have anti-metastatic activity by inhibiting α5, β1 and αv integrins, but could also have hydrolysis effect on ECM proteins such as fibronectin and vitronectin. Studying the effect of F6 on ECM could be done by incubating F6 with ECM for certain time, then the proteolytic activity can be analysed using SDS-PAGE. In this project the proteolytic activity of F6 has not been studied because of the time. Another study demonstrated that a CLP isolated from *Macrovipera lebetina* venom (lebectin), was shown to have anti-adhesive activity against various cell lines such as melanoma, fibrosarcoma, adenocarcinoma and leukemia cells (Sarray *et al*., 2004). Lebectin is a potent inhibitor of adhesion to fibronectin, but not collagen by blocking α5β1 and αv integrins and that correlates with the observations of the current work since *Agkistrodon piscivorus leucostoma* venom had an inhibitory effect on α5β1 and αv integrins (Pilorget *et al*., 2007). Eristostatin is a RGD-disintegrin which mainly inhibited the adhesion of five melanoma cell lines (MV3, WM164, M24met, 1205Lu, and C8161) by blocking the fibronectin receptor, but not laminin or collagen IV receptors (Tian *et al*., 2007) and that is similar to the response by *Crotalus ruber ruber* venom and F3. It has been reported that CC5 and CC8 (disintegrins from *Cerastes cerastes* venom) also have an inhibitory effect on α5β1 and αvβ3 integrins (Ben-Mabrouk *et al*., 2016). All the above mentioned peptides that affect the α5β1 integrin belong to the Viperidae

family which is the same family of the venoms used in this study. From the results of the current work it is thought that the mechanism of action of *Crotalus ruber ruber* venom and F3 occurs by decreasing the level of α5 and β1 integrins, possibly through the catalytic activity of rubelysin. Although rubelysin belongs to P-I SVMP which has only a metalloproteinase domain, it has anti-integrin activity. It is thought P-I SVMP was post-translationally processed from P-II or P-II SVMP (Wu *et al*., 2001). However, the mechanism of action of *Agkistrodon piscivorus leucostoma* venom is thought to be due to hydrolysis of the ECM and integrins and result in loss of cell adhesion and to stop tumour dissemination. Both of the venoms in this study belong to the Viperidae family and the difference in responses observed is thought to be because of the percentage of each component that is responsible for their activities in the venoms. For *Agkistrodon piscivorus leucostoma* venom leucostoma peptidase A is the most abundant protease (neutral metalloproteinase) and accounts for 17% of the total content (Wagner *et al.,* 1986), while *Crotalus ruber ruber* venom has a high activity level of proteases (metalloproteinase).

4.5 Both venoms had an inhibitory effect on migration and invasion of A2780 cells

To further clarify the actions of venoms on A2780 cells, cell migration and invasion were examined. It has been reported that integrins are important in cell migration (Hood & Cheresh, 2002). The current work demonstrated that both *Crotalus ruber ruber* venom and *Agkistrodon piscivorus leucostoma* venom had an inhibitory effect on migration and invasion of A2780 cells. This result was similar to a study carried out by Sarray *et al.* (2004), which demonstrated that lebectin prevented migration and invasion of melanoma, fibrosarcoma, adenosarcoma and leukaemia cells by

inhibiting the α5β1 and αv integrins using the same migration assay but a different invasion assay (tumour cell invasion of fibrin gel as opposed to a Cytoselect^{TM} 24-well Cell Invasion Assay) (Sarray *et al*., 2004). The current study demonstrated that *Crotalus ruber ruber* venom and F3 inhibited cell migration and invasion by causing a significant decrease in protein levels of α5 and β1 integrins as determined by Western blot analysis. *Agkistrodon piscivorus leucostoma* venom had an inhibitory effect on tumour migration and invasion through inhibiting α5, β1 and αv integrins in a similar result to a study with MVL-PLA2 blocking the migration of fibrosarcoma to fibronectin and fibrinogen through inhibiting α5β1and αv integrins (Bazaa *et al*., 2009). However, in this case, the migration assay used was quite different from the one used in the current work where the bottom side of the insert was coated with fibronectin or collagen which act as chemoattractant toward which the cells migrate. Eristostatin, a RGD-disintegrin purified from *Eristicophis macmahoni* venom, inhibited migration of five melanoma cell lines by blocking fibronectin receptor from binding to fibronectin (Tian *et al*., 2007) and that correlates with the result of the effect of *Crotalus ruber ruber* venom and F3 on inhibiting the α5β1 integrin (fibronectin receptor). The work of Tian *et al.* (2007), used an *in vitro* wound closure assay as was used in the current work to assess the effect of venom on migration of A2780 cells with the exception of coating the plate with fibronectin. CC-PLA2-1 and CC-PLA2-2 inhibited the migration of fibrosarcoma to fibronectin and fibrinogen through inhibiting α5β1 and αv integrins (Zouari-Kessentini *et al*., 2009). Previous work pointed out that rubistatin (r-rub) (a disintegrin purified from *Crotalus ruber ruber* venom) inhibited migration of human melanoma cells (SK-Mel-28), although the integrin receptor involved is still unknown (Carey *et al.,* 2011). The wound

healing assay was used in both studies. In the current work the activity of *Crotalus ruber ruber* venom and F3 is not related to rubistatin because its activity was inhibited after adding a metalloproteinase inhibitor. It has also been reported that lebestatin (a disintegrin isolated from *Macrovipera lebetina* venom) has an inhibitory effect on migration of human microvascular endothelial cells and rat pheochromocytoma by inhibiting α1β1 integrin (Olfa *et al*., 2005). Triflavin inhibits the migration of HUVECs by inhibiting α5β1, αvβ3, and α3β1 (Sheu *et al*., 1997), affecting the fibronectin receptor as well as vitronectin and laminin receptors and it correlates more with the effect of the active fraction of *Agkistrodon piscivorus leucostoma* venom which was not specific for certain integrins and ECM. It has been reported that targeting integrins affects tumour cell adhesion, migration and invasion and that makes integrin inhibitor therapies a promising target to treat ovarian cancer (reviewed by Sawada *et al*., 2012).

4.6 Five fractions from *Crotalus ruber ruber* venom and nine fractions from *Agkistrodon piscivorus leucostoma* venom were obtained by using size exclusion chromatography

Separation of the venom was achieved by size exclusion chromatography, which is applied to fractionate large molecules such as proteins. Snake venom contains proteins and peptides that is why size exclusion chromatography was chosen as a good technique to fractionate the venoms. The fractions obtained from *Crotalus ruber ruber* venom (Sephadex G75) were pooled into five groups, while *Agkistrodon piscivorus leucostoma* venom (separated on Sephadex G75) produced 9 fractions. A study in the literature reported fractionation of *Crotalus ruber ruber* venom by size exclusion chromatography (separated on Sephadex G75) resulted in 6 peaks

(Mackessy, 1985). In the current work F1 contained proteins with approximate molecular weights ranging from 40,000-50,000Da, F2 contained proteins with a molecular weight range from 20,000-50,000Da and F3 and F4 contained proteins below 25,000Da. The work of Mackessy (1985), showed that Peak I contained peptides with molecular weight of 64,500 to 67,000 and peak III had peptides with molecular weights of 19,000 to 20,500, but they did not show the other peaks (Mackessy, 1985). A study carried out previously showed *Agkistrodon piscivorus leucostoma* venom was separated using anion exchange and then gel filtration chromatography (Sephadex 75) to obtain pure leucostoma peptidase A (Wagner *et al*., 1968) which seems a good approach to obain pure leucostoma peptidase A. The present work showed that SDS-PAGE analysis for F3 of *Agkistrodon piscivorus leucostoma* venom contained proteins with molecular weights ranging from 50,000 to 80,000Da, F4 contained proteins with molecular weights from 20,000 to 40,000Da, and the other fractions had proteins with a molecular weight less than 25,000Da. In the literature, there is very little characterisation of *Agkistrodon piscivorus leucostoma* venom and not much information about this venom. In the current work there was a problem with availability of the venom to carry out purification on a large-scale in order for more in-depth characterisation to be carried out, that is why other methods were carried out to identify the active component in each fraction.

4.7 The active peptide in F3 and F6 is identified as rubelysin and leucostoma peptidase A, respectively

4.7.1 SDS-PAGE, Tandem Mass Spectrometry and LCMS

The analysis of F3 on SDS-PAGE revealed a single band with a molecular weight between 23,000 and 24,000Da. According to the Nanoflow HPLC Electrospray Tandem Mass Spectrometry the peptide which appears as a single band on SDS-PAGE was thought to be hemorrhagic toxin II (rubelysin). It consists of 202 amino acid and belongs to P-I SVMP. However, the LCMS data showed F3 consisted of 4 components in different quantities. The highest percentage was 73% for a component with a MW of 23,300Da, suggesting that this component is rubelysin. The percentage of the other components was 2, 3, and 14%. Rubelysin is a hemorrhagic metalloproteinase-II (MW 23,321Da). The previous work of Takeya *et al.* (1990), showed that ruberlysin of *Crotalus ruber ruber* venom appeared from SDS-PAGE to have a MW between 23,000-24,000Da (Takeya *et al*., 1990). So that correlates to the result of the present work where the most prominent band on SDS-PAGE was rubelysin. This is the first work to demonstrate the effect of rubelysin on the levels of integrins in A2780 cells and associated with inhibiting A2780 cells adhesion, migration and invasion. The analysis of F6 of *Agkistrodon piscivorus leucostoma* venom on SDS-PAGE showed there were two bands. One band had a molecular weight between 22,000-25,000Da, while the other one ranged between 13,000 and 15,000Da. The result of the Nanoflow HPLC Electrospray Tandem Mass Spectrometry showed that one band with a MW of 13,989Da was most likely phospholipase A2, while the other band was snake venom metalloproteinase ACLH with a MW of 46,109Da. This result for the second band did not correlate with the SDS-PAGE analysis (22,000-25,000Da). Previous work of Spiekerman *et al.* (1973), pointed out that *Agkistrodon piscivorus leucostoma* venom is a rich source for leucostoma peptidase A enzyme with a MW of 22,500Da (Spiekerman *et al*., 1973).

Since leucostoma peptidase A is not present in the Tandem Mass Spectrometry database, it could not be identified. This allows the possibility that the second band observed on SDS-PAGE could be leucostoma peptidase A. Leucostoma peptidase A is a non-specific metalloprotease, belonging to the general class known as neutral proteases (Spiekerman *et al*., 1973). It cleaves fibronectin, collagen IV, and to a lesser extent collagen I and that fits in with the result of the current work since F6 expect to have an inhibitory effect on fibronectin, but the effect on collagen was not examined because the A2780 cells did not adhere to collagen. The result of LCMS showed that F6 consisted of 2 peptides in different quantities and with different molecular weights (23,000 Da and the other one consisted of two peaks with MW 13,946 and 13,988 Da). The result of LCMS supports the SDS-PAGE data and therefore this fraction is more likely to consist of leucostoma peptidase A and PLA2. From the literature leucostoma peptidase A and rubelysin are metalloproteinases and they could be inhibited by metalloproteinase inhibitors. In addition, PLA2 is Ca^{+2}dependent enzyme and its activity could be inhibited using a chelating agent.

4.7.2 The activities of F3 and F6 were abolished by EDTA and 1,10-phenanthroline respectively

Since it was proposed that the active component in F3 of *Crotalus ruber ruber* venom is rubelysin, further studies were carried out to try and demonstrate this. Rubelysin is a haemorrhagic metalloprotease inhibited by EDTA; therefore experiments were performed in the presence of this metalloproteinase inhibitor. One hundred μg/ml of EDTA inhibited the activity of F3 which further strongly suggests that the active component in this fraction is rubelysin. This is also an indication that the catalytic domain is important in activity of rubelysin against dissemination of

ovarian cancer cells. For the F6 component, proposed to consist of PLA2 and leucostoma peptidase A, a different approach had to be taken since PLA2 is a Ca^{+2} dependent enzyme, while leucostoma peptidase A is a Zn^{2+} dependent enzyme. An alternative to EDTA had to be used since EDTA is a chelating agent for both Zn^{2+} and Ca^{+2}. Phenanthroline is a metalloproteinase inhibitor that mainly targets Zn^{2+} with a much lower affinity for Ca^{+2} (Salvesen *et al*., 2001), so was considered a good choice to elucidate which peptide in F6 was responsible for the activity observed. As a result leucostoma peptidase A (a non-specific metalloproteinase) is believed to be responsible for F6 activity. The previous work of Spiekerman *et al*. (1973), demonstrated that the activity of leucostoma peptidase A was inhibited by phenanthroline and the activity could be restored by the addition of Zn^{2+}. In the current work, the morphological alterations and anti-adhesive activity of F6 were abolished after adding 25µg/ml of phenanthroline. This type of study has been carried out with other metalloproteinases eg jararhagin activity and graminelysin I were abolished in the presence of EDTA (Tanjoni *et al*., 2005; Wu *et al*., 2001). According to these results it is believed that the active components of both fractions F3 and F6 are snake venom metalloproteinases. This work demonstrates for the first time the effect of these venoms on dissemination of A2780 cells and in particular identification of the role of rubelysin and leucostoma peptidase A in disrupting some of the process of metastasis.

5.0 Conclusion and future work

The aim of this study was to demonstrate the effect of two kinds of snake venom on ovarian cancer dissemination *in vitro* and to carry out preliminary identification of the peptides responsible for any activity. Both crude venoms showed anti-metastatic

properties and were further fractionated using size exclusion chromatography. The most significant findings in this project are that both venoms have anti-disseminating activity against A2780 cells using two different mechanisms of action. The anti-disseminating effect of *Crotalus ruber ruber* venom and its active fraction was through inhibition of α5 and β1 integrins, while the activity of *Agkistrodon piscivorus leucostoma* was achieved by inhibiting α5, β1 and αv integrins and possibly through hydrolysis of the ECM. Both venoms were functional at concentrations that were not cytotoxic. *Crotalus ruber ruber* venom inhibited adhesion of the cells to fibronectin, but not vitronectin, while *Agkistrodon piscivorus leucostoma* venom inhibited the adhesion on fibronectin and vitronectin and that correlates with the results of the inhibitory effect of *Agkistrodon piscivorus leucostoma* venom on fibronectin and vitronectin receptor. This work also demonstrated that the effect of *Crotalus ruber ruber* venom and its active fraction is specific for the integrin family of adhesion molecule, whereas, *Agkistrodon piscivorus leucostoma* and its active fraction were non-specific and not integrin-dependent. This study reported that both venoms had an inhibitory effect on migration and invasion of A2780 cells. From preliminary proteomic work, it can be concluded that the active component in *Crotalus ruber ruber* venom could be rubelysin, and for *Agkistrodon piscivorus leucostoma* venom is possibly leucostoma peptidase A.

Future work would focus on isolation of pure rubelysin from *Crotalus ruber ruber* venom by using gel filtration on a Sephadex G-75 column, followed by ion exchange chromatography on a CM-Sephadex C-50 column (Takeya *et al*., 1990). In addition, pure leucostoma peptidase A could be isolated from the venom using ion exchange

chromatography (DEAE-Sephadex A50), followed by gel filteration chromatography (Sephadex 75) (Wagner *et al*., 1968). Researchers have used different techniques to fractionate venoms and get pure metalloproteinase, some used gel filtration followed by anion exchange, affinity chromatograph and reverse-phase HPLC and others started with anion exchange.

The pure compounds may have anti-metastatic effect on ovarian cancer and could be used for production of safer, natural and active anti-metastatic lead candidate drugs. It has been explored that the spheroids of ovarian cancer cells are resistance to chemotherapy more than single cells (reviewed by Lengyel, 2010), so future work could focus on producing 3D structures of ovarian cancer cells to demonstrate the effect of venoms on spheroids. Three dimensional cancer models are known to provide a more accurate picture of the metastatic process and drug susceptibility in order to develop a better cancer treatment (Xu *et al*., 2011). Furthermore, future work would need to focus on *in vivo* assessment, intially using a suitable rodent experimental model to improve ovarian cancer patient outcomes. It has been reported that contortrostatin (disintegrin isolated from Southern copperhead venom), showed significant decrease in the number and size of ovarian tumours in female athymic nude mice compared to untreated controls. Female athymic nude mice received an intraperitoneal injection of ovarian cancer cells. After four days of allowing the cells to implant, the mice received daily 30μg/ml intraperitoneal injection of native contortrostatin for 28 days (Swenson *et al*., 2005). Since most of the ovarian cancer cells are confined within the peritoneal cavity it may be that intraperitoneal treatment is the most efficient way of peptide delivery. It has been reported that using intraperitoneal administration with intravenous chemotherapy improves the outcome

of patients with ovarian cancer compared with intravenous chemotherapy alone (Armstrong *et al*., 2006).

Further work could focus on the mode of delivery of the pure active peptide to target the cancer cells without causing haemorrhage because both active peptides belong to metalloproteinase family, which is known to play an important role in haemorrhage after envenomation. It has been shown using nanoparticles to deliver drugs to cancer cells offer a great opportunities to fight cancer. A venom-loaded silica nanoparticle had an inhibitory effect on prostatic cancer proliferation and with an IC_{50} less than venom alone (Bader *et al.,* 2013). It has been shown that venom-loaded silica nanoparticle induce apoptosis of breast cancer cells without affecting normal cells (Al-Sadoon *et al*., 2012), so future work could focus on loaded rubelysin and leucostoma peptidase A with silica nanoparticles.

There are a number of venom-derived medicines now used clinically such as captopril (designed based on the structure of peptide which is isolated from *Bothrops jaracusa* venom), the first line of successful angiotensin converted enzyme (ACE) inhibitor. Integrilin, designed on peptide derived from *Sisturus miliarus barbourin* snake venom is used as a platelet aggregation inhibitor and in treatment of acute coronary disease. In addition, reptilase (isolated from *Bothrops jaraca* venom) has been used for diagnosis of blood coagulation disorders (reviewed by Sarray *et al*., 2013). In this study we investigated the actions of two venoms on dissemination of A2780 cells. This provides promise of an alternative therapeutic solution. In terms of future progression to a commercially viable product it seems rubelysin from *Crotalus ruber ruber* venom through its specific action on α5β1 integrins is a better proposition and future work should continue with this venom.

References

Abdelkafi-Koubaa, Z., Morjen, M., Srairi-Abid, N., El Ayeb, M., & Marrakchi, N. (2014). [Snake Venom L-Amino Acid Oxidases potential biomedical applications]. *Arch Inst Pasteur Tunis, 91*(1-4), 15-32.

Adade, C. M., Carvalho, A. L., Tomaz, M. A., Costa, T. F., Godinho, J. L., Melo, P. A., Souto-Padron, T. (2014). Crovirin, a snake venom cysteine-rich secretory protein (CRISP) with promising activity against Trypanosomes and Leishmania.*PLoS Negl Trop Dis, 8*(10), e3252.doi: 10.1371/journal.pntd.0003252

Ahn, M. Y., Lee, B. M., & Kim, Y. S. (1997). Characterization and cytotoxicity of L-amino acid oxidase from the venom of king cobra (Ophiophagus hannah). *Int J Biochem Cell Biol, 29*(6), 911-919.

Al-Alem, L., & Curry, T. E. (2015). Ovarian cancer: involvement of the matrix metalloproteinases. *Reproduction (Cambridge, England)*, *150*(2), R55–R64. http://doi.org/10.1530/REP-14-0546.

Albuquerque, P. L., Jacinto, C. N., Silva Junior, G. B., Lima, J. B., Veras Mdo, S., & Daher, E. F. (2013). Acute kidney injury caused by Crotalus and Bothrops snake venom: a review of epidemiology, clinical manifestations and treatment. *Rev Inst Med Trop Sao Paulo, 55*(5), 295-301. doi: 10.1590/s0036-46652013000500001

Ali, S. A., Stoeva, S., Abbasi, A., Alam, J. M., Kayed, R., Faigle, M., Voelter, W. (2000). Isolation, structural, and functional characterization of an apoptosis-inducing L-amino acid oxidase from leaf-nosed viper (Eristocophis macmahoni) snake venom. *Arch Biochem Biophys, 384*(2), 216-226. doi: 10.1006/abbi.2000.2130

Al-Sadoon, M. K., Abdel-Maksoud, M. A., Rabah, D. M., & Badr, G. (2012).

Induction of apoptosis and growth arrest in human breast carcinoma cells by a snake (*Walterinnesia aegyptia*) venom combined with silica nanoparticles: crosstalk between Bcl2 and caspase 3. *Cellular Physiology and Biochemistry*, *30*(3), 653-665.

Aoudjit, F., & Vuori, K. (2001). Matrix Attachment Regulates FAS-Induced Apoptosis in Endothelial Cells: A Role for C-Flip and Implications for Anoikis. *J Cell Biol, 152*(3), 633-644.

Aoudjit, F., & Vuori, K. (2012). Integrin Signaling in Cancer Cell Survival and Chemoresistance. *Chemotherapy Research and Practice, 2012*, 16. doi: 10.1155/2012/283181

Armstrong, D. K., Bundy, B., Wenzel, L., Huang, H. Q., Baergen, R., Lele, S., Burger, R. A. (2006). Intraperitoneal cisplatin and paclitaxel in ovarian cancer. *N Engl J Med, 354*(1), 34-43. doi: 10.1056/NEJMoa052985

Asher, V., Warren, A., Shaw, R., Sowter, H., Bali, A., & Khan, R. (2011). The role of Eag and HERG channels in cell proliferation and apoptotic cell death in SK-OV-3 ovarian cancer cell line. *Cancer Cell Int, 11*, 6. doi: 10.1186/1475-2867-11-6.

Badr, G., Al-Sadoon, M. K., Rabah, D. M., & Sayed, D. (2013). Snake (Walterinnesia aegyptia) venom-loaded silica nanoparticles induce apoptosis and growth arrest in human prostate cancer cells. *Apoptosis*, *18*(3), 300-314.

Badgwell, D., & Bast, R. C., Jr. (2007). Early detection of ovarian cancer. *Dis Markers, 23*(5-6), 397-410.

Bandera, C. A. (2005). Advances in the understanding of risk factors for ovarian cancer. *J Reprod Med, 50*(6), 399-406.

Bateman, E., Venning, M., Mirtschin, P., & Woods, A. (2013). The effects of selected Australian snake venoms on tumour-associated microvascular endothelial cells (TAMECs) in vitro. *J Venom Res, 4*, 21-30.

Bazaa, A., Luis, J., Srairi-Abid, N., Kallech-Ziri, O., Kessentini-Zouari, R., Defilles, C., Marrakchi, N. (2009). MVL-PLA2, a phospholipase A2 from Macrovipera lebetina transmediterranea venom, inhibits tumor cells adhesion and migration. *Matrix Biol, 28*(4), 188-193. doi: 10.1016/j.matbio.2009.03.007

Ben-Mabrouk, H., Zouari-Kessentini, R., Montassar, F., Koubaa, Z. A., Messaadi, E., Guillonneau, X., Marrakchi, N. (2016). CC5 and CC8, two homologous disintegrins from Cerastes cerastes venom, inhibit in vitro and ex vivo angiogenesis. *Int J Biol Macromol, 86*, 670-680. doi: 10.1016/j.ijbiomac.2016.02.008

Bernheimer, A. W., Linder, R., Weinstein, S. A., & Kim, K. S. (1987). Isolation and characterization of a phospholipase B from venom of Collett's snake, Pseudechis colletti. *Toxicon, 25*(5), 547-554.

Borkow, G., Gutierrez, J. M., & Ovadia, M. (1995). In vitro activity of BaH1, the main hemorrhagic toxin of Bothrops asper snake venom on bovine endothelial cells. *Toxicon, 33*(10), 1387-1391.

Boukhalfa-Abib, H., Meksem, A., & Laraba-Djebari, F. (2009). Purification and biochemical characterization of a novel hemorrhagic metalloproteinase from horned viper (Cerastes cerastes) venom. *Comp Biochem Physiol C Toxicol Pharmacol, 150*(2), 285-290. doi: 10.1016/j.cbpc.2009.05.008

Brando, C., Marcinkiewicz, C., Goldman, B., McLane, M. A., & Niewiarowski, S. (2000). EC3, a heterodimeric disintegrin from Echis carinatus, inhibits human and

murine alpha4 integrin and attenuates lymphocyte infiltration of Langerhans islets in pancreas and salivary glands in nonobese diabetic mice. *Biochem Biophys Res Commun, 267*(1), 413-417. doi: 10.1006/bbrc.1999.1897

Breedlove, G., & Busenhart, C. (2005). Screening and detection of ovarian cancer. *J Midwifery Womens Health, 50*(1), 51-54. doi: 10.1016/j.jmwh.2004.10.002

Bristow, R. S., Tomacruz, D. K., Armstrong, E. L., Trimble, F. J. (2002). Survival effect of maximal cytoreductive surgery for advanced ovarian carcinoma during the platinum era: a meta-analysis. *Journal of Clinical Oncology,* 20 (5), 1248–1259.

Burin, S. M., Ayres, L. R., Neves, R. P., Ambrosio, L., de Morais, F. R., Dias-Baruffi, M., de Castro, F. A. (2013). L-amino acid oxidase isolated from Bothrops pirajai induces apoptosis in BCR-ABL-positive cells and potentiates imatinib mesylate effect. *Basic Clin Pharmacol Toxicol, 113*(2), 103-112. doi: 10.1111/bcpt.12073

Burke, J. E., & Dennis, E. A. (2009). Phospholipase A2 structure/function, mechanism, and signaling. *J Lipid Res, 50 Suppl*, S237-242. doi: 10.1194/jlr.R800033-JLR200

Calderon, L. A., Sobrinho, J. C., Zaqueo, K. D., de Moura, A. A., Grabner, A. N., Mazzi, M. V., Soares, A. M. (2014). Antitumoral activity of snake venom proteins: new trends in cancer therapy. *Biomed Res Int, 2014*, 203639. doi: 10.1155/2014/203639

Calvete, J. J., Marcinkiewicz, C., Monleon, D., Esteve, V., Celda, B., Juarez, P., & Sanz, L. (2005). Snake venom disintegrins: evolution of structure and function. *Toxicon, 45*(8), 1063-1074. doi: 10.1016/j.toxicon.2005.02.024

Calvete, J.J., Juárez, P., Sanz, L.(2007). Snake venomics. Strategy and applications. *Journal of Mass Spectrometry,*42(11),1405–1414.

Cannistra, S. A. (2004). Cancer of the Ovary. *New England Journal of Medicine, 351*(24), 2519-2529. doi: doi:10.1056/NEJMra041842

Cannistra, S.A., Kansas, G.S., Niloff, J., DeFranzo, B., Kim, Y., Ottensmeier, C. (1993). Binding of ovarian cancer cells to peritoneal mesothelium in vitro is partly mediated by CD44H. *Cancer Res*, 53:3830–3838.

Carey, C. M., Bueno, R., Gutierrez, D. A., Petro, C., Lucena, S. E., Sanchez, E. E., & Soto, J. G. (2012). Recombinant rubistatin (r-Rub), an MVD disintegrin, inhibits cell migration and proliferation, and is a strong apoptotic inducer of the human melanoma cell line SK-Mel-28. *Toxicon, 59*(2), 241-248. doi: 10.1016/j.toxicon.2011.12.002

Casey, R. C., Burleson, K. M., Skubitz, K. M., Pambuccian, S. E., Oegema, T. R., Jr., Ruff, L. E., & Skubitz, A. P. (2001). Beta 1-integrins regulate the formation and adhesion of ovarian carcinoma multicellular spheroids. *Am J Pathol, 159*(6), 2071-2080.

Chippaux, J. P., Williams, V., & White, J. (1991). Snake venom variability: methods of study, results and interpretation. *Toxicon, 29*(11), 1279-1303.

Chung, K. H., Kim, S. H., Han, K. Y., Sohn, Y. D., Chang, S. I., Baek, K. H., Kang, I. C. (2003). Inhibitory effect of salmosin, a Korean snake venom-derived disintegrin, on the integrin alphav-mediated proliferation of SK-Mel-2 human

melanoma cells. *J Pharm Pharmacol, 55*(11), 1577-1582. doi: 10.1211/0022357022160

Cominetti, M. R., Ribeiro, J. U., Fox, J. W., & Selistre-de-Araujo, H. S. (2003). BaG, a new dimeric metalloproteinase/disintegrin from the Bothrops alternatus snake venom that interacts with alpha5beta1 integrin. *Arch Biochem Biophys, 416*(2), 171-179.

Correa, M. C., Jr., Maria, D. A., Moura-da-Silva, A. M., Pizzocaro, K. F., & Ruiz, I. R. (2002). Inhibition of melanoma cells tumorigenicity by the snake venom toxin jararhagin. *Toxicon, 40*(6), 739-748.

Costa Tá, R., Burin, S. M., Menaldo, D. L., de Castro Fí, A., & Sampaio, S. V. (2014). Snake venom L-amino acid oxidases: an overview on their antitumor effects. *J Venom Anim Toxins Incl Trop Dis, 20*, 23.

Curran, S., & Murray, G.I. (1999). Matrix metalloproteinases in tumor invasion and metastasis. *J Pathol* 189*(3):*300–308.

Darai, E., Scoazec, J. Y., Walker-Combrouze, F., Mlika-Cabanne, N., Feldmann, G., Madelenat, P., & Potet, F. (1997). Expression of cadherins in benign, borderline, and malignant ovarian epithelial tumors: a clinicopathologic study of 60 cases. *Hum Pathol, 28*(8), 922-928.

De Lima, D. C., Alvarez Abreu, P., de Freitas Cí, C., Santos, D. O., Borges, R. O., dos Santos, T. C., Castro, H. C. (2005). Snake Venom: Any Clue for Antibiotics and CAM? *Evid Based Complement Alternat Med, 2*(1), 39-47. doi: 10.1093/ecam/neh063

Desgrosellier, J., and Cheresh, D. (2010). Integrins in cancer: biological implications

and therapeutic opportunities. *Nature Reviews Cancer*, 10: 9-22.

De Vieira Santos, M. M., Sant'Ana, C. D., Giglio, J. R., da Silva, R. J., Sampaio, S. V., Soares, A. M., & Fecchio, D. (2008). Antitumoural effect of an L-amino acid oxidase isolated from Bothrops jararaca snake venom. *Basic Clin Pharmacol Toxicol, 102*(6), 533-542. doi: 10.1111/j.1742-7843.2008.00229.x

Doley, R., & Kini, R. M. (2009). Protein complexes in snake venom. *Cell Mol Life Sci, 66*(17), 2851-2871. doi: 10.1007/s00018-009-0050-2

Eble, J. A., Niland, S., Bracht, T., Mormann, M., Peter-Katalinic, J., Pohlentz, G., & Stetefeld, J. (2009). The alpha2beta1 integrin-specific antagonist rhodocetin is a cruciform, heterotetrameric molecule. *FASEB J, 23*(9), 2917-2927. doi: 10.1096/fj.08-126763.

Fishman, D. A., Kearns, A., Chilukuri, K., Bafetti, L. M., O'Toole, E. A., Georgacopoulos, J., Stack, M. S. (1998). Metastatic dissemination of human ovarian epithelial carcinoma is promoted by alpha2beta1-integrin-mediated interaction with type I collagen. *Invasion Metastasis, 18*(1), 15-26. doi: 24495

Fox, J. W., & Serrano, S. M. (2008). Exploring snake venom proteomes: multifaceted analyses for complex toxin mixtures. *Proteomics, 8*(4), 909-920. doi: 10.1002/pmic.200700777

Fry, B. G., Wuster, W., Kini, R. M., Brusic, V., Khan, A., Venkataraman, D., & Rooney, A. P. (2003). Molecular evolution and phylogeny of elapid snake venom three-finger toxins. *J Mol Evol, 57*(1), 110-129. doi: 10.1007/s00239-003-2461-2

Galan, J. A., Sanchez, E. E., Rodriguez-Acosta, A., Soto, J. G., Bashir, S., McLane, M. A., Perez, J. C. (2008). Inhibition of lung tumor colonization and cell

migration with the disintegrin crotatroxin 2 isolated from the venom of Crotalus atrox. *Toxicon, 51*(7), 1186-1196. doi: 10.1016/j.toxicon.2008.02.004

Ganguly, K. K., Pal, S., Moulik, S., & Chatterjee, A. (2013). Integrins and metastasis. *Cell Adh Migr, 7*(3), 251-261. doi: 10.4161/cam.23840

Gasanov, S. E., Dagda, R. K., & Rael, E. D. Snake Venom Cytotoxins, Phospholipase A(2)s, and Zn(2+)-dependent Metalloproteinases: Mechanisms of Action and Pharmacological Relevance. *J Clin Toxicol, 4*(1), 1000181-.

Gomes, A., Bhattacharjee, P., Mishra, R., Biswas, A. K., Dasgupta, S. C., & Giri, B. (2010). Anticancer potential of animal venoms and toxins. *Indian J Exp Biol, 48*(2), 93-103.

Guadamillas, M. C., Cerezo, A., & del Pozo, M. A. (2011). Overcoming anoikis – pathways to anchorage-independent growth in cancer. *Journal of Cell Science, 124*(19), 3189-3197. doi: 10.1242/jcs.072165

Hamako, J., Suzuki, Y., Hayashi, N., Kimura, M., Ozeki, Y., Hashimoto, K., & Matsui, T. (2007). Amino acid sequence and characterization of C-type lectin purified from the snake venom of Crotalus ruber. *Comp Biochem Physiol B Biochem Mol Biol, 146*(3), 299-306. doi: 10.1016/j.cbpb.2006.11.022

Hamza, L., Gargioli, C., Castelli, S., Rufini, S., & Laraba-Djebari, F. (2010). Purification and characterization of a fibrinogenolytic and hemorrhagic metalloproteinase isolated from Vipera lebetina venom. *Biochimie, 92*(7), 797-805. doi: http://dx.doi.org/10.1016/j.biochi.2010.02.025

Huber, M.A., Kraut, N., Beug H.(2006). Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr Opin Cell Biol*, 17:548–558.

Hood, J. D., & Cheresh, D. A. (2002). Role of integrins in cell invasion and migration. *Nat Rev Cancer, 2*(2), 91-100. doi: 10.1038/nrc727

Hynes, R. O. (2002). Integrins: bidirectional, allosteric signaling machines. *Cell, 110*(6), 673-687.

Jia, Y., Lucena, S., Cantu, E., Jr., Sanchez, E. E., & Perez, J. C. (2009). cDNA cloning, expression and fibrin(ogen)olytic activity of two low-molecular weight snake venom metalloproteinases. *Toxicon, 54*(3), 233-243. doi: 10.1016/j.toxicon.2009.04.008

Juarez, P., Comas, I., Gonzalez-Candelas, F., & Calvete, J. J. (2008). Evolution of snake venom disintegrins by positive Darwinian selection. *Mol Biol Evol, 25*(11), 2391-2407. doi: 10.1093/molbev/msn179

Kenny, H. A., Dogan, S., Zillhardt, M., A, K. M., Yamada, S. D., Krausz, T., & Lengyel, E. (2009). Organotypic models of metastasis: A three-dimensional culture mimicking the human peritoneum and omentum for the study of the early steps of ovarian cancer metastasis. *Cancer Treat Res, 149*, 335-351. doi: 10.1007/978-0-387-98094-2_16

Kenny, H.A., Kaur, S., Coussens, L., Lengyel, E. (2008). The initial steps of ovarian cancer cell metastasis are mediated by MMP-2 cleavage of vitronectin and fibronectin. *J Clin Invest*, 118:1367–1379.

Kessentini-Zouari, R., Jebali, J., Taboubi, S., Srairi-Abid, N., Morjen, M., Kallech-Ziri, O., Luis, J. (2010). CC-PLA2-1 and CC-PLA2-2, two Cerastes cerastes venom-derived phospholipases A2, inhibit angiogenesis both in vitro and in vivo. *Lab Invest, 90*(4), 510-519. doi: 10.1038/labinvest.2009.137

Kim, D. S., Jang, Y. J., Jeon, O. H., & Kim, D. S. (2007). Saxatilin, a snake venom disintegrin, suppresses TNF-alpha-induced ovarian cancer cell invasion. *J Biochem Mol Biol, 40*(2), 290-294.

Komori, Y., Sakai, K., Masuda, K., & Nikai, T. (2011). Isolation and Biochemical Characterization of Rubelase, a Non-Hemorrhagic Elastase from Crotalus ruber ruber (Red Rattlesnake) Venom. *Toxins (Basel), 3*(7), 900-910. doi: 10.3390/toxins3070900

Lecht, S., Chiaverelli, R. A., Gerstenhaber, J., Calvete, J. J., Lazarovici, P., Casewell, N. R., Marcinkiewicz, C. (2015). Anti-angiogenic activities of snake venom CRISP isolated from Echis carinatus sochureki. *Biochim Biophys Acta, 1850*(6), 1169-1179. doi: 10.1016/j.bbagen.2015.02.002

Lengyel, E. (2010). Ovarian Cancer Development and Metastasis. *Am J Pathol, 177*(3), 1053-1064.

Lewis, R. J., & Garcia, M. L. (2003). Therapeutic potential of venom peptides. *Nat Rev Drug Discov, 2*(10), 790-802.

Mackessy, S. P. (1985). Fractionation of red diamond rattlesnake (Crotalus ruber ruber) venom: protease, phosphodiesterase, L-amino acid oxidase activities and effects of metal ions and inhibitors on protease activity. *Toxicon, 23*(2), 337-340.

Mak, C. S. L., Yung, M. M. H., Hui, L. M. N., Leung, L. L., Liang, R., Chen, K., Chan, D. W. (2017). MicroRNA-141 enhances anoikis resistance in metastatic progression of ovarian cancer through targeting KLF12/Sp1/survivin axis. *Molecular Cancer*, *16*, 11. http://doi.org/10.1186/s12943-017-0582-2

Marcinkiewicz, C., Lobb, R. R., Marcinkiewicz, M. M., Daniel, J. L., Smith, J. B., Dangelmaier, C., Niewiarowski, S. (2000). Isolation and characterization of

EMS16, a C-lectin type protein from Echis multisquamatus venom, a potent and selective inhibitor of the alpha2beta1 integrin. *Biochemistry, 39*(32), 9859-9867.

Marcinkiewicz, C., Weinreb, P. H., Calvete, J. J., Kisiel, D. G., Mousa, S. A., Tuszynski, G. P., & Lobb, R. R. (2003). Obtustatin: a potent selective inhibitor of alpha1beta1 integrin in vitro and angiogenesis in vivo. *Cancer Res, 63*(9), 2020-2023.

Markland, F.S., Shieh, Jr. K., Zhou,Q.(2001). A novel snake venom disintegrin that inhibits human ovarian cancer dissemination and angiogenesis in an orthotopic nude mouse model. *Haemostasis*, 31(3-6),183-191.

Markland, F. S., & Swenson, S. (2013). Snake venom metalloproteinases. *Toxicon, 62*, 3-18. doi: 10.1016/j.toxicon.2012.09.004

Markland, F. S. (2008). *Development of a Multifaceted Ovarian Cancer Therapeutic and Imaging Agent*. University of Southern California Los angeles.

Masoumi Moghaddam, S., Amini, A., Morris, D. L., & Pourgholami, M. H. (2012). Significance of vascular endothelial growth factor in growth and peritoneal dissemination of ovarian cancer. *Cancer Metastasis Rev, 31*(1-2), 143-162. doi: 10.1007/s10555-011-9337-5

Masuda, S., Hayashi, H., Atoda, H., Morita, T., & Araki, S. (2001). Purification, cDNA cloning and characterization of the vascular apoptosis-inducing protein, HV1, from Trimeresurus flavoviridis. *Eur J Biochem, 268*(11), 3339-3345.

Muccioli, M., Sprague, L., Nandigam, H., Pate, M., & Benencia, F. (2012). Toll-like receptors as novel therapeutic targets for ovarian cancer. *ISRN Oncol, 2012*, 642141. doi: 10.5402/2012/642141

Murakami, T., Kamikado, N., Fujimoto, R., Hamaguchi, K., Nakamura, H., Chijiwa, T., Oda-Ueda, N. (2011). A [Lys(4)(9)]phospholipase A(2) from Protobothrops flavoviridis venom induces caspase-independent apoptotic cell death accompanied by rapid plasma-membrane rupture in human leukemia cells. *Biosci Biotechnol Biochem, 75*(5), 864-870.

Naimuddin, M., Kobayashi, S., Tsutsui, C., Machida, M., Nemoto, N., Sakai, T., & Kubo, T. (2011). Directed evolution of a three-finger neurotoxin by using cDNA display yields antagonists as well as agonists of interleukin-6 receptor signaling. *Molecular Brain, 4*(1), 1-16. doi: 10.1186/1756-6606-4-2

Naumann, G. B., Silva, L. F., Silva, L., Faria, G., Richardson, M., Evangelista, K., Sanchez, E. F. (2011). Cytotoxicity and inhibition of platelet aggregation caused by an l-amino acid oxidase from Bothrops leucurus venom. *Biochim Biophys Acta, 1810*(7), 683-694. doi: 10.1016/j.bbagen.2011.04.003

Nolte, S., de Castro Damasio, D., Barea, A. C., Gomes, J., Magalhaes, A., Mello Zischler, L. F., Moreno-Amaral, A. N. (2012). BJcuL, a lectin purified from Bothrops jararacussu venom, induces apoptosis in human gastric carcinoma cells accompanied by inhibition of cell adhesion and actin cytoskeleton disassembly. *Toxicon, 59*(1), 81-85. doi: 10.1016/j.toxicon.2011.10.012

Nowatzki, J., Sene, R. V., Paludo, K. S., Rizzo, L. E., Souza-Fonseca-Guimaraes, F., Veiga, S. S., Trindade, E. S. (2012). Brown spider (Loxosceles intermedia) venom triggers endothelial cells death by anoikis. *Toxicon, 60*(3), 396-405. doi: 10.1016/j.toxicon.2012.04.333

Ohyagi-Hara, C., Sawada, K., Kamiura, S., Tomita, Y., Isobe, A., Hashimoto, K., Kimura, T. (2013). miR-92a inhibits peritoneal dissemination of ovarian cancer

cells by inhibiting integrin alpha5 expression. *Am J Pathol, 182*(5), 1876-1889. doi: 10.1016/j.ajpath.2013.01.039

Olfa, K. Z., Jose, L., Salma, D., Amine, B., Najet, S. A., Nicolas, A., Naziha, M. (2005). Lebestatin, a disintegrin from Macrovipera venom, inhibits integrin-mediated cell adhesion, migration and angiogenesis. *Lab Invest, 85*(12), 1507-1516. doi: 10.1038/labinvest.3700350

Paludo, K. S., Gremski, L. H., Veiga, S. S., Chaim, O. M., Gremski, W., de Freitas Buchi, D., Franco, C. R. (2006). The effect of brown spider venom on endothelial cell morphology and adhesive structures. *Toxicon, 47*(8), 844-853. doi: 10.1016/j.toxicon.2006.02.006

Paoli, P., Giannoni, E., & Chiarugi, P. (2013). Anoikis molecular pathways and its role in cancer progression. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1833*(12), 3481-3498.

Parry, M. A., Jacob, U., Huber, R., Wisner, A., Bon, C., & Bode, W. (1998). The crystal structure of the novel snake venom plasminogen activator TSV-PA: a prototype structure for snake venom serine proteinases. *Structure, 6*(9), 1195-1206.

Perumal Samy, R., Gopalakrishnakone, P., Bow, H., Puspharaj, P. N., & Chow, V. T. (2010). Identification and characterization of a phospholipase A2 from the venom of the Saw-scaled viper: Novel bactericidal and membrane damaging activities. *Biochimie, 92*(12), 1854-1866. doi: 10.1016/j.biochi.2010.07.012

Pilorget, A., Conesa, M., Sarray, S., Michaud-Levesque, J., Daoud, S., Kim, K. S., Luis, J. (2007). Lebectin, a Macrovipera lebetina venom-derived C-type lectin,

inhibits angiogenesis both in vitro and in vivo. *J Cell Physiol, 211*(2), 307-315. doi: 10.1002/jcp.20935

Prinholato da Silva, C., Costa, T. R., Paiva, R. M. A., Cintra Aé, C. O., Menaldo, D. L., Antunes Lâ, M. G., & Sampaio, S. V. (2015). Antitumor potential of the myotoxin BthTX-I from Bothrops jararacussu snake venom: evaluation of cell cycle alterations and death mechanisms induced in tumor cell lines. *J Venom Anim Toxins Incl Trop Dis, 21*. doi: 10.1186/s40409-015-0044-5

Ramos, O. H., Kauskot, A., Cominetti, M. R., Bechyne, I., Salla Pontes, C. L., Chareyre, F., Bonnefoy, A. (2008). A novel alpha(v)beta (3)-blocking disintegrin containing the RGD motive, DisBa-01, inhibits bFGF-induced angiogenesis and melanoma metastasis. *Clin Exp Metastasis, 25*(1), 53-64. doi: 10.1007/s10585-007-9101-y

Roberto, P. G., Kashima, S., Marcussi, S., Pereira, J. O., Astolfi-Filho, S., Nomizo, A., Franca, S. C. (2004). Cloning and identification of a complete cDNA coding for a bactericidal and antitumoral acidic phospholipase A2 from Bothrops jararacussu venom. *Protein J, 23*(4), 273-285.

Roy, A., Zhou, X., Chong, M. Z., D'Hoedt, D., Foo, C. S., Rajagopalan, N., Kini, R. M. (2010). Structural and functional characterization of a novel homodimeric three-finger neurotoxin from the venom of Ophiophagus hannah (king cobra). *J Biol Chem, 285*(11), 8302-8315. doi: 10.1074/jbc.M109.074161

Sabatier, R., Finetti, P., Bonensea, J., Jacquemier, J., Adelaide, J., & Lambaudie, E. (2011). A seven-gene prognostic model for platinum-treated ovarian carcinomas. *Br J Cancer, 105*. doi: 10.1038/bjc.2011.219

Sarray, S., Berthet, V., Calvete, J. J., Secchi, J., Marvaldi, J., El-Ayeb, M., . . . Luis, J. (2004). Lebectin, a novel C-type lectin from Macrovipera lebetina venom, inhibits integrin-mediated adhesion, migration and invasion of human tumour cells. *Lab Invest, 84*(5), 573-581. doi: 10.1038/labinvest.3700088

Sarray, S., Luis, J., El Ayeb, M., and Marrakchi, N. "Snake venom peptides: promising molecules with anti-tumor effects," in Bioactive Food Peptides in Health and Disease, B. Hernandez- ´ Ledesma and C. C. Hsieh, Eds., InTech, 2013. www.intechopen.com/books/bioactive-food-peptides-in-health-and-disease/snake-venom-peptides-promising-molecules-with-anti-tumor-effects.

Sarzaeem, A., Zare Mirakabadi, A., Moradhaseli, S., Morovvati, H., & Lotfi, M. (2012). Cytotoxic effect of ICD-85 (venom-derived peptides) on HeLa cancer cell line and normal LK cells using MTT Assay. *Arch Iran Med, 15*(11), 696-701. doi: 0121511/aim.0010

Sawada, K., Ohyagi-Hara, C., Kimura, T., & Morishige, K. (2012). Integrin inhibitors as a therapeutic agent for ovarian cancer. *J Oncol, 2012*, 915140. doi: 10.1155/2012/915140

Schaffner, F., Ray, A. M., & Dontenwill, M. (2013). Integrin α5β1, the Fibronectin Receptor, as a Pertinent Therapeutic Target in Solid Tumors. *Cancers (Basel), 5*(1), 27-47.

Serrano, S. M., & Maroun, R. C. (2005). Snake venom serine proteinases: sequence homology vs. substrate specificity, a paradox to be solved. *Toxicon, 45*(8), 1115-1132. doi: 10.1016/j.toxicon.2005.02.020

Shaikh, D., & Jokhio, R. (2005). Effect of snake venom on nucleic acids and total Proteins in various normal and cancerous animal tissues. *Pak J Physio, 1,* 1-2.

Sheu, J. R., Yen, M. H., Kan, Y. C., Hung, W. C., Chang, P. T., & Luk, H. N. (1997). Inhibition of angiogenesis in vitro and in vivo: comparison of the relative activities of triflavin, an Arg-Gly-Asp-containing peptide and anti-alpha(v)beta3 integrin monoclonal antibody. *Biochim Biophys Acta, 1336*(3), 445-454.

Smith, L. H., Morris, C. R., Yasmeen, S., Parikh-Patel, A., Cress, R. D., & Romano, P. S. (2005). Ovarian cancer: can we make the clinical diagnosis earlier? *Cancer, 104*(7), 1398-1407. doi: 10.1002/cncr.21310

Spiekerman, A. M., Fredericks, K. K., Wagner, F. W., & Prescott, J. M. (1973). Leucostoma peptidase A: a metalloprotease from snake venom. *Biochim Biophys Acta, 293*(2), 464-475.

Strobel, T., & Cannistra, S. A. (1999). Beta1-integrins partly mediate binding of ovarian cancer cells to peritoneal mesothelium in vitro. *Gynecol Oncol, 73*(3), 362-367. doi: 10.1006/gyno.1999.5388

Suhr, S. M., & Kim, D. S. (1996). Identification of the snake venom substance that induces apoptosis. *Biochem Biophys Res Commun, 224*(1), 134-139. doi: 10.1006/bbrc.1996.0996

Swenson, S., Costa, F., Ernst, W., Fujii, G., & Markland, F. S. (2005). Contortrostatin, a snake venom disintegrin with anti-angiogenic and anti-tumor activity. *Pathophysiol Haemost Thromb, 34*(4-5), 169-176. doi: 10.1159/000092418

Takeya, H., Onikura, A., Nikai, T., Sugihara, H., & Iwanaga, S. (1990). Primary structure of a hemorrhagic metalloproteinase, HT-2, isolated from the venom of Crotalus ruber ruber. *J Biochem, 108*(5), 711-719.

Tanjoni, I., Weinlich, R., Della-Casa, M. S., Clissa, P. B., Saldanha-Gama, R. F., de Freitas, M. S., Moura-da-Silva, A. M. (2005). Jararhagin, a snake venom metalloproteinase, induces a specialized form of apoptosis (anoikis) selective to endothelial cells. *Apoptosis, 10*(4), 851-861. doi: 10.1007/s10495-005-2945-1

Tian, J., Paquette-Straub, C., Sage, E. H., Funk, S. E., Patel, V., Galileo, D., & McLane, M. A. (2007). Inhibition of melanoma cell motility by the snake venom disintegrin eristostatin. *Toxicon, 49*(7), 899-908. doi: 10.1016/j.toxicon.2006.12.013

Torii, S., Yamane, K., Mashima, T., Haga, N., Yamamoto, K., Fox, J. W., Tsuruo, T. (2000). Molecular cloning and functional analysis of apoxin I, a snake venom-derived apoptosis-inducing factor with L-amino acid oxidase activity. *Biochemistry, 39*(12), 3197-3205.

Towbin, H., Staehelin, T., Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A. 76*(9):4350-4.

Trikha, M., De Clerck, Y. A., & Markland, F. S. (1994). Contortrostatin, a snake venom disintegrin, inhibits beta 1 integrin-mediated human metastatic melanoma cell adhesion and blocks experimental metastasis. *Cancer Res, 54*(18), 4993-4998.

Trummal, K., Tonismagi, K., Siigur, E., Aaspollu, A., Lopp, A., Sillat, T., Siigur, J. (2005). A novel metalloprotease from Vipera lebetina venom induces human

endothelial cell apoptosis. *Toxicon, 46*(1), 46-61. doi: 10.1016/j.toxicon.2005.03.008

van der Flier, A., & Sonnenberg, A. (2001). Function and interactions of integrins. *Cell Tissue Res, 305*(3), 285-298.

Wagner, F. W., Spiekerman, A. M., & Prescott, J. M. (1968). Leucostoma peptidase A. Isolation and physical properties. *J Biol Chem, 243*(17), 4486-4493.

Wan, S. G., Jin, Y., Lee, W. H., & Zhang, Y. (2006). A snake venom metalloproteinase that inhibited cell proliferation and induced morphological changes of ECV304 cells. *Toxicon, 47*(4), 480-489. doi: 10.1016/j.toxicon.2006.01.006

Wu, W. B., Chang, S. C., Liau, M. Y., & Huang, T. F. (2001). Purification, molecular cloning and mechanism of action of graminelysin I, a snake-venom-derived metalloproteinase that induces apoptosis of human endothelial cells. *Biochem J, 357*(Pt 3), 719-728.

Xu, F., Celli, J., Rizvi, I., Moon, S., Hasan, T., & Demirci, U. (2011). A three-dimensional in vitro ovarian cancer coculture model using a high-throughput cell patterning platform. *Biotechnol J, 6*(2), 204-212. doi: 10.1002/biot.201000340

Yeh, C. H., Peng, H. C., & Huang, T. F. (1998). Accutin, a new disintegrin, inhibits angiogenesis in vitro and in vivo by acting as integrin alphavbeta3 antagonist and inducing apoptosis. *Blood, 92*(9), 3268-3276.

Yeh, C. H., Peng, H. C., Yang, R. S., & Huang, T. F. (2001). Rhodostomin, a snake venom disintegrin, inhibits angiogenesis elicited by basic fibroblast growth factor and suppresses tumor growth by a selective alpha(v)beta(3) blockade of endothelial cells. *Mol Pharmacol, 59*(5), 1333-1342.

You, W. K., Seo, H. J., Chung, K. H., & Kim, D. S. (2003). A novel metalloprotease from Gloydius halys venom induces endothelial cell apoptosis through its protease and disintegrin-like domains. *J Biochem, 134*(5), 739-749.

Zhang, L., & Wu, W. T. (2008). Isolation and characterization of ACTX-6: a cytotoxic L-amino acid oxidase from Agkistrodon acutus snake venom. *Nat Prod Res, 22*(6), 554-563. doi: 10.1080/14786410701592679

Zhou, Q., Sherwin, R. P., Parrish, C., Richters, V., Groshen, S. G., Tsao-Wei, D., & Markland, F. S. (2000). Contortrostatin, a dimeric disintegrin from Agkistrodon contortrix contortrix, inhibits breast cancer progression. *Breast Cancer Res Treat, 61*(3), 249-260.

Zouari-Kessentini, R., Luis, J., Karray, A., Kallech-Ziri, O., Srairi-Abid, N., Bazaa, A., Marrakchi, N. (2009). Two purified and characterized phospholipases A2 from Cerastes cerastes venom, that inhibit cancerous cell adhesion and migration. *Toxicon, 53*(4), 444-453. doi: 10.1016/j.toxicon.2009.01.003