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Immunological Effects of *Haliotis Rubra* Hemolymph and Hemolymph Components

A dissertation submitted to The University of Sydney in full fulfillment of the requirements for the degree of Master of Philosophy

By

Humaira Noor

School of Chemical and Biomolecular Engineering

The University of Sydney

March 2017
DECLARATION

I declare the work presented in this thesis is, to the best of my knowledge original, except as acknowledged in the text, and has not been submitted for a degree at any other university. Some parts of this research work have been published and a list of publications arising from this research is included in the thesis.

Humaira Noor

March 2017
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ABSTRACT

Marine derived drugs are emerging with promises to positively intervene in a range of diseases including viral, bacterial, fungal, and to even some forms of cancers. It is due to their reduced cost and negligible side effect on patients in contrast to synthetic drugs available today. Hemolymph of several molluscs and arthropods are of particular interest for their immunomodulatory properties that enhance their anti-microbial and anti-cancerous mechanisms. One such potential active compound is hemocyanin, and hemocyanin derived proteins in the hemolymph of molluscs.

Our research aims to uncover the immunological effects of *Haliotis rubra* (black lipped abalone) hemolymph, purified hemocyanin and hemolymph permeate. Their immunomodulatory properties can be key to treating Herpes Simplex Virus -1, HSV-1, infected patients and prostate cancer patients. In this study, we cultured HaCat (cultured human keratinocyte) cells and Human oral primary keratinocytes (as *in vitro* model for oral administration of abalone sera) and prostate cancer cell line (PC-3) to observe immunomodulatory effects of the extracts on such form of cancer. Treatment of these cells with variable concentrations of abalone sera, purified hemocyanin and the sera/hemolymph permeate for optimization and determination of dose-dependent and time dependent immunomodulatory effects were carried out. It was found that a 16 hour treatment period was optimum for greatest immunomodulatory properties, while the permeate proved to be the most potent immunomodulator in the case of all three models. Six different cytokine level changes in the cell culture supernatant post treatment were determined using BD Cytometric Bead Array Kit and analyzing with Flow Cytometer.

Results show a 50% inhibition in Interleukin-6 (IL-6) expression, 21% inhibition in Interleukin-8 (IL-8) expression and an increase in Tumor Necrosis Factor (TNF) concentration were observed after 16 hr incubation with HaCat Cells treated with 20% v/v sera concentration. Other cytokine levels were unaltered. On a separate 6 day treatment of HaCat cells, results showed that the hemolymph successfully
primed the HaCat cell for the expression of an additional proinflammatory cytokine Interleukin-1β, which was not expressed previously on the 16 h treatment model. This shows varying immunomodulatory effects of the sera/hemolymph that rely on treatment frequency and length.

There is an observed inhibition of IL-6 expression in permeate treated PC-3 cells by over 50%, the value followed closely by sera treatment, while purified hemocyanin yielded an inhibition of IL-6 expression by 27%. Additionally, the effects also extend to IL-8 as a 29% inhibition in IL-8 results from treatment with permeate for 5 h, followed by a 25% reduction from treatment with AH (purified abalone hemocyanin) and lastly a 16% reduction from treatment with the abalone sera.

Further, the effect of abalone sera, AH and permeate 5 h treatment on the prostate cancer cell morphology was observed and results suggest that the permeate had a size reduction effect on the morphology on all of the cells compared to untreated control.

The most significant results were obtained for primary oral keratinocytes treatment with the abalone sera yielding a sharp 35 fold increase in IL-8 concentration and 23 fold increase in IL-6 concentration as compared to the untreated control. Further, dose-dependent effects of all extracts were subsequently confirmed on the primary oral keratinocytes. It has also been shown that Human Oral Keratinocytes are much more sensitive to the sera in terms of IL-8 modulation than IL-6 modulation.

Hence, immunomodulatory potential of the three extracts have been studied on the three cell lines, and immunomodulation has been confirmed at varying degrees for all cases. Additionally, targeted modulation of proinflammatory cytokines IL-6 and IL-8 has also been observed. Particular potency in immunomodulation has been observed in the sera permeate component of the hemolymph.

Further, identification of the nature of the potent hemolymph permeate was carried out using Western Blot technique. It was shown that the fragments of proteins
present in the permeate originate from the hemocyanin. According to the results, they are highly likely to be proteins/sub-units/clusters of functional units dissociated from the hemocyanin.

These findings not only support the immunomodulatory potential of abalone sera, sera permeate and purified hemocyanin but also support its potential to be effective on the immunomodulatory treatments of HSV-1 patients and prostate cancer patients since literature suggests, particularly IL-6, IL-8 and TNF modulation are key to these diseases.
PUBLICATIONS

H. Noor, P. Valtchev, V. G. Gomes, F. Dehghani “Marine Derived Immunomodulatory Drugs- A review paper” (manuscript in preparation)

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GLOSSARY

AH Abalone Hemocyanin
AIDs Acquired Immune deficiency syndrome
CBA Cytokine Beads Array
CCH Concholepas Concholepas Hemocyanin
CCK-8 Cell Counting Kit -8
CMV Cytomegalovirus
DC cells Dendritic Cells
ELISA Enzyme Linked Immuno Sorbent Assay
FU Functional Unit
GFP Green fluorescent protein
HaCat Human Cultured Keratinocytes
HIV Human Immunodeficiency Virus
HSV Herpes Simplex Virus
IFN Interferon
IgA Immunoglobulin A
IgG Immunoglobulin G
IgM Immunoglobulin M
IL-10 Interleukin -10
IL-11 Interleukin -11
IL-12 Interleukin -12
IL-13 Interleukin -13
IL-1β Interleukin -1β
IL-2 Interleukin -2
IL-6 Interleukin -6
IL-8 Interleukin -8
IRF-5 Interferon Regulatory Factor 5
KLH Keyhole Limpet Hemocyanin
MAPK Mitogen Activated Protein Kinase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>MERS</td>
<td>Middle East Respiratory Syndrome</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear Factor Kappa Beta</td>
</tr>
<tr>
<td>NK Cells</td>
<td>Natural Killer Cells</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Pattern</td>
</tr>
<tr>
<td>PC-3</td>
<td>Prostate Cancer Cell</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern Recognition Receptor</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RTH</td>
<td><em>Rapana Thamasania</em> Hemocyanin</td>
</tr>
<tr>
<td>SARS</td>
<td>Severe Acute Respiratory Syndrome</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper cell 1</td>
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<tr>
<td>Th2</td>
<td>T helper cell 2</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll Like Receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
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<tr>
<td>Treg</td>
<td>T regulatory Cells</td>
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<tr>
<td>TT</td>
<td>Tetanus Toxin</td>
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<tr>
<td>WSSV</td>
<td>White spot syndrome virus</td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS

Chapter 1: Introduction........................................................................................................... 2

1.1 Objectives.......................................................................................................................... 5
1.2 Hypothesis........................................................................................................................... 6
1.3 Significance......................................................................................................................... 7

Chapter 2: Review of Literature............................................................................................. 10

2.0 Introduction...................................................................................................................................... 11
2.1 Hemocyanin..................................................................................................................................... 12
   2.1.1 Structure of arthropod and molluscan hemocyanin............................................................ 12
   2.1.2 Properties and functions of hemocyanin................................................................................ 13
   2.1.3 Immune system modulating potential of hemocyanin.......................................................... 14
   2.1.4 Anti-fungal, anti-viral and anti-cancer properties of hemocyanin, and use of hemocyanin as a conjugate vaccine................................................................. 15
2.2 Innate immunity......................................................................................................................... 17
   2.2.1 Use of PAMPs, PRRs and TLRs............................................................................................ 18
   2.2.2 Other aspects of innate immunity......................................................................................... 19
2.3 Cytokines of the innate immune system..................................................................................... 20
   2.3.1 Proinflammatory and anti-inflammatory cytokines............................................................... 21
   2.3.2 InterLeukin-6, Interleukin-8 and Interleukin-10................................................................... 21
2.4 Marine derived immune system modulating compounds......................................................... 25
   2.4.1 Immune system modulating marine drugs......................................................................... 26
2.5 Viruses and human herpes simplex virus.................................................................................. 28
   2.5.1 What are viruses?.................................................................................................................. 29
   2.5.2 Properties of viruses.............................................................................................................. 29
   2.5.3 Mechanisms of viral entry................................................................................................... 30
2.5.4 Effector immune responses against viral infections.................................31
2.5.5 Role of immune system in chronic HSV infections.................................32
  2.5.5.1 Importance of IL-6, IL-8 and related cytokines in HSV infections...33

2.6 Methods for determination of cytokine concentration...............................35

Chapter 3: Hypotheses, Materials and Methodology.........................................48

  3.0 Introduction..................................................................................................49
  3.1 Materials .....................................................................................................50
  3.2 Methodology.................................................................................................52
    3.2.1 Cell culture protocol.............................................................................52
    3.2.2 Coating of cell culture plates with Poly-l-lysine..................................53
    3.2.3 Determination of required seeding cell density of HaCat cells per well
        in 6 well plates.........................................................................................53
    3.2.4 Cytotoxicity test standard curve ..........................................................53
    3.2.5 Determination of the cytotoxicity of abalone sera, purified
        hemocyanin and permeate on HaCat cell line and PC-3 cell line.............55
    3.2.6 Extraction of fresh sera (hemolymph) from the abalone.....................55
    3.2.7 Filtration and sterilization of the fresh sera (hemolymph)....................55
    3.2.8 Purification of abalone hemocyanin using ultrafiltration....................56
    3.2.9 Determination of protein concentration using Qubit..........................58
    3.2.10 Incubation with the test extracts.........................................................59
      3.2.10.1 Incubation of test extracts with HaCat cells.................................63
      3.2.10.2 Incubation of test extracts with Prostate Cancer cells...............63
      3.2.10.3 Incubation of test extracts with Human Primary Oral Keratinocytes........................................................................................................64
    3.2.11 Human Inflammatory Cytokine Beads Array....................................65
    3.2.12 Materials for CBA Human Inflammatory Cytokine Kit......................66
    3.2.13 Preparation of standards.....................................................................67
    3.2.14 Preparation of the assay......................................................................70
    3.2.15 Flow Cytometer setup using the setup beads and sample
        acquisition..................................................................................................70
    3.2.16 FCAP ARRAY software....................................................................71
    3.2.17 Statistical analysis...............................................................................71

Chapter 4: Cytotoxicity Analysis: HACAT Cell line and PC-3 Cell line..............72
  4.0 Introduction.................................................................................................73
  4.1 Results...........................................................................................................74
4.1.1 The effect of abalone sera and purified AH on the viability of HaCat cells and PC-3 cells

Chapter 5: Immunomodulatory effects on Human Keratinocytes
5.0 Introduction
5.1 Results
  5.1.2 Effect of short time treatment with abalone hemolymph
    5.1.2.1 Immunogenic effects with 5% abalone sera
    5.1.2.2 Immunogenic effects with 2% abalone sera
  5.1.3 Effects of extended treatment time with abalone hemolymph and its components
    5.1.3.1 Immunogenic effects of 16 h treatment with variable concentrations of abalone hemolymph extracts
    5.1.3.2 6 day dose dependent study on priming of HaCat cells
  5.1.4 Dose and time dependent modulation of IL-6 and IL-8

5.2 Immunomodulatory effects on Human Oral Primary Keratinocytes
  5.2.2 Immunogenic effects of 16 h treatment with 20% abalone sera concentration
  5.2.3 Dose dependent effect of abalone sera on Human Oral Primary Keratinocytes
  5.2.4 Dose dependent effect of abalone sera permeate

Chapter 6: Immunomodulatory effects on prostate cancer cells and identification of the permeate proteins
  6.2 Immunogenic effects of variable extracts with 5 h treatment
  6.3 Effect of extracts on cell morphology
  6.4 Identification of the permeate proteins using Western Blot

Chapter 7: Discussions, Conclusions and Future Directions
  7.1 Conclusions
  7.2 Future Directions

References
APPENDIX
LIST OF FIGURES

Figure 2.0: Structure of Interleukin-6.

Figure 2.1: Three-dimensional structure of interleukin 8 in solution.

Figure 3.1: Experimental setup of cytotoxicity determination: bird's eye view of the 96 well plate.

Figure 3.2: Partial purification and dilution of hemocyanin by ultrafiltration using filters with Molecular weight cut off of 100 kDa and Tris buffer. Impurities were eluted with ultrapure water.

Figure 3.3: Experimental procedure for incubation for the 6 day treatment of HaCat cells.

Figure 3.4: Fluorescence peaks of six different cytokines measured in all of the cytometric beads array human inflammatory cytokines assay conducted.

Figure 3.5: Cytokine Standard serial dilution procedure with assay diluent.

Figure 3.6: Shift of fluorescence peaks on six cytokine standards in accordance with concentration of the standard A. 0 pg/ml B. 20 pg/ml C. 40 pg/ml D. 80 pg/ml E. 156 pg/ml F. 312 pg/ml G. 625 pg/ml H. 1250 pg/ml I. 2500 pg/ml J. 5000 pg/ml

Figure 4.1: Effect of variable concentrations (0.1%, 0.5%, 1%, 2%, 5% and 10% v/v) of abalone sera on the viability of HaCat cells, which were treated for 30 min, 3 hour, 4 hour and 6 hour. Absorbance detected at 450 nm.

Figure 4.2: Effect of variable concentrations (0.1%, 0.5%, 1%, 2%, 5% and 10% v/v) of abalone sera on the viability of HaCat cells which were treated for 24 hour and 48 hour. Absorbance detected at 450 nm.

Figure 4.3: Effect of variable concentrations (10, 20, 40, 60, 80 and 100% v/v) of abalone sera on the viability of HaCat cells which were treated for 30 min, 2 hour, 4 hour and 6 hour. Absorbance detected at 450 nm.
**Figure 4.4:** Effect of variable concentrations (10, 20, 40, 60, 80 and 100% v/v) of abalone sera on the viability of HaCat cells which were treated for 30 min, 1 hour, 4 hour and 24 hour and 48 hour. Absorbance detected at 450 nm.

**Figure 4.5:** Effect of variable concentrations of purified hemocyanin (1%, 2%, 4%, 6%, 8%, 10% as percentages of 400 microgram/mL solution) on the viability of HaCat cells which were treated for 30 min 24 h and 48 h. Absorbance detected at 450 nm.

**Figure 4.6:** Effect of variable concentrations of abalone sera and purified abalone hemocyanin ((1%, 2%, 4%, 6%, 8%, 10% as percentages of 400 microgram/mL) on PC-3 cells, treated for 24 h. Absorbance detected at 450 nm.

**Figure 5.1:** Immunogenic effects of 15 min treatment with 5% v/v abalone sera concentration on HaCat cells and determination of cytokine concentration (IL-6 and IL-8). Data obtained with flow cytometry.

**Figure 5.2:** Immunogenic effects of 30 min treatment with 5% v/v abalone sera concentration on HaCat cells and determination of cytokine concentration (IL-6 and IL-8). Data obtained with flow cytometry.

**Figure 5.3:** Immunogenic effects of 45 min treatment with 5% v/v abalone sera concentration on HaCat cells and determination of cytokine concentration (IL-6 and IL-8). Data obtained with flow cytometry.

**Figure 5.4:** Immunogenic effects of 30 min treatment with 2% v/v abalone sera concentration on HaCat cells and determination of cytokine concentration (IL-6 and IL-8). Data obtained with flow cytometry.

**Figure 5.5:** Immunogenic effects of 1.5 h treatment with 2% v/v abalone sera concentration on HaCat cells and determination of cytokine concentration (IL-6 and IL-8). Data obtained with flow cytometry.

**Figure 5.6:** Immunogenic effects of 2.5 h treatment with 2% v/v abalone sera concentration on HaCat cells and determination of cytokine concentration (IL-6 and IL-8). Data obtained with flow cytometry.
sera concentration on HaCat cells and determination of cytokine concentration (IL-6 and IL-8). Data obtained with flow cytometry.

**Figure 5.7:** Immunogenic effects of 16 h treatment with 10% and 20% abalone sera, 20% purified AH and 20% v/v sera permeate concentrations on HaCat cells and determination of IL-6 concentration. Data obtained with flow cytometry.

**Figure 5.8:** Immunogenic effects of 16 h treatment with 10% and 20% v/v abalone sera, 20% purified AH and 20% sera permeate concentrations on HaCat cells and determination of IL-8 concentration. Data obtained with flow cytometry.

**Figure 5.9:** Immunogenic effects of 16 h treatment with 10% and 20% v/v abalone sera, 20% purified AH and 20% sera permeate concentrations on HaCat cells and determination of TNF-alpha concentration. Data obtained with flow cytometry.

**Figure 5.10:** Immunogenic effects of 6 day dosage treatment (1.5 h treatment per day) of HaCat cells with 10% and 20% v/v abalone sera and determination of IL-6 concentration. Data obtained with flow cytometry.

**Figure 5.11:** Immunogenic effects of 6 day dosage treatment (1.5 h treatment per day) of HaCat cells with 10% and 20% v/v abalone sera and determination of IL-8 concentration. Data obtained with flow cytometry.

**Figure 5.12:** Immunogenic effects of 6 day dosage treatment (1.5 h treatment per day) of HaCat cells with 10% and 20% v/v abalone sera and determination of IL-1β concentration. Data obtained with flow cytometry.

**Figure 5.13:** Immunogenic effects of 6 day dosage treatment (1.5 h treatment per day) of HaCat cells with 10% and 20% v/v abalone sera and determination TNF-alpha concentration. Data obtained with flow cytometry.
**Figure 5.14:** Quantification of IL-6 in HaCat cell supernatant post treatment with 1%, 5%, 10% and 20% v/v Sera for 1 h, 2 h and 5 h. Data obtained with Flow Cytometry. Indicating the release of IL-6 increases with higher treatment time. Dose dependent effect for the concentration range tested (1% to 20% v/v) is not observed. 5 h treatment time clearly indicates a significantly higher IL-6 production compared to 1h, 2 h or the control (0%).

**Figure 5.15:** Quantification of IL-8 in HaCat cell supernatant post incubation with 1%, 5%, 10% and 20% v/v Sera for 1 h, 2 h and 5 h. Data obtained with Flow Cytometry. Indicating the release of IL-8 increases with higher treatment time. Dose dependent effect for the concentration range tested (1% to 20% v/v) is not observed. 5 h treatment time clearly indicates a significantly higher IL-8 production compared to 1h, 2 h or the control (0%).

**Figure 5.16:** Immunogenic effects of 16 h treatment with 20% v/v abalone sera concentration on Human Primary Oral Keratinocytes and determination of cytokine concentration (IL-6 and IL-8). Data obtained with flow cytometry.

**Figure 5.17:** Dose-dependent immunogenic effects of 16 h treatment with 5%, 10%, 20% v/v abalone sera concentration on Human Primary Oral Keratinocytes and determination of IL-6 concentration. Data obtained with flow cytometry.

**Figure 5.18:** Dose-dependent immunogenic effects of 16 h treatment with 5%, 10%, 20% v/v abalone sera concentration on Human Primary Oral Keratinocytes and determination of IL-8 concentration. Data obtained with flow cytometry.

**Figure 5.19:** Dose-dependent immunogenic effects of 16 h treatment with variable concentrations of purified AH on Human Primary Oral Keratinocytes and determination of IL-6 concentration. Data obtained with flow cytometry.

**Figure 5.20:** Dose-dependent immunogenic effects of 16 h treatment with
variable concentrations of purified AH on Human Primary Oral Keratinocytes and determination of IL-8 concentration. Data obtained with flow cytometry.

**Figure 5.21:** Dose-dependent immunogenic effects of 16 h treatment with variable concentrations of abalone sera permeate on Human Primary Oral Keratinocytes and determination of IL-6 concentration. Data obtained with flow cytometry.

**Figure 5.22:** Dose-dependent immunogenic effects of 16 h treatment with variable concentrations of abalone sera permeate on Human Primary Oral Keratinocytes and determination of IL-8 concentration. Data obtained with flow cytometry.

**Figure 6.1:** Immunogenic effects of 5 h treatment with 20% v/v abalone sera, 20% purified AH and 20% sera permeate concentrations on PC-3 cells and determination of IL-6 concentration. Data obtained with flow cytometry.

**Figure 6.2:** Immunogenic effects of 5 h treatment with 20% v/v abalone sera, 20% purified AH and 20% sera permeate concentrations on PC-3 cells and determination of IL-8 concentration. Data obtained with flow cytometry.

**Figure 6.3:** Effect of abalone sera, purified AH and permeate 5 h treatment on the morphology of PC-3 cells. (A) Control (B) 20% v/v Sera (C) 20% v/v hemocyanin (D) 20% v/v permeate.

**Figure 6.4:** Identify the protein in the permeates by Coomassie blue stain (A) and western blot with the anti-hemocyanin antibody (B). Marker units: kDa.

**Figure 8.1:** Flow cytometer Gating strategy for the analysis of cytokine concentration results observed.

**Figure 8.2:** Gating of IL-6, IL-8 and IL-1β.

**Figure 8.3:** Mean values of cytokines detected in each gated sub-population.
LIST OF TABLES

Table 2.1: Marine derived therapeutic compounds.

Table 2.2: Immunomodulatory compounds from marine species.

Table 3.1: Cell culture protocol followed for HaCat cells, Human Oral Keratinocytes and PC-3 cells in propagation, passage and freezing.

Table 3.2: Dilutions of the purified hemocyanin and permeate following 20 minute centrifugation at 4000 x g, 4°C.

Table 3.3: Dilutions carried out on variable concentration samples of purified AH and permeate to assess the concentration using Qubit 2.0 Fluorometer.

Table 6.1: Total Protein concentration of three permeates.
CHAPTER 1:

Introduction
1.0 Introduction

In this era of increasing numbers of emerging diseases: SARS (2003), Chikungunya (2005), swine flu (2009), MERS (2012), Ebola (2014) and Zika (2015), it is essential to have a stack of potential compounds and their mechanisms in preparation to control the deadly diseases from having a pandemic fate. Seoul virus outbreak struck the U.S as recently as February 2017, and such emerging diseases are to be expected with time. Another deadly word in the medical field is cancer. To date, people continue to die of several forms of cancer despite the exhaustive research carried out to combat the disease throughout the world. An estimated 130,466 new cancer cases have been diagnosed in Australia during 2016 (sourced from https://canceraustralia.gov.au). This deadly disease infests and cripples every organ of human body.

Immunomodulation – controlling the human immune response – can be a potential weapon to control such diseases. In fact, immunotherapy is one of the most prevalent emerging fields of treatment to combat a variety of diseases such as ischemia reperfusion injury, acute lung injury and sepsis, Alzheimer's disease, Diabetes, Parkinson’s disease, Multiple sclerosis, Myeloma, arthritis, autoimmune diseases, leukemia, immune deficiency syndrome, systemic sclerosis, and viral diseases such as Herpes simplex infections, HIV/AIDS, etc.

The benefit of immunomodulation is primarily due to the concept of healing oneself with the fighter cells present in their own body. This organic method of disease treatment can be triggered by certain compounds, a great number of which can be derived from nature. A significant number of the synthetic immunomodulatory drugs available today, and on Clinical Trials, are comprised of molecules identical to certain naturally derived immunomodulatory compounds. A thorough list of these is presented in Chapter 2: Review of the Literature.
If we differentiate between terrestrial and marine species, a significant 700,000 different species reside in the marine world. Marine world is plagued with different viruses and all forms of diseases, and the fact that these species continue to survive and exist over millions of years give a shining hint that they are equipped with components of exceptionally strong immune systems. These immunomodulatory compounds of marine species can be used today to prolong human lives and better human health. This is the key factor behind increasing numbers of research carried out on marine-derived compounds in the medical field.

One of the well known compounds present in the hemolymph of gastropods and arthropods is the blue coloured copper-based respiratory protein - hemocyanin. Respiration is not the only function of hemocyanin; in fact it adds greatly to the innate immunity of these species, as most of these species are not equipped with forms of immune cells. While several hemocyanins have been identified, characterized and the properties researched extensively, research of hemocyanin from *Haliotis rubra* (Australian black-lipped abalone) hemolymph is still at dawn. The immunomodulatory properties of this hemocyanin on human immune system have not been studied yet.

An unpublished clinical study was conducted in Marine Biotechnology Australia’s initiative, to test the effect of *Haliotis rubra* hemolymph on HSV-1 patients: ‘A Randomised, Double-Blind, Placebo-Controlled Proof-of-Concept Study to Evaluate Safety and Efficacy of Topical Abalone Haemocyanin Once Daily for the Treatment of Herpes Labialis in Immunocompetent Patients’. This clinical study implemented two modes of tests, one where purified abalone hemocyanin (AH) was applied topically on the cold sores of patients (50mg applied topically once daily for 5 days within 6 hours of profromal symptom), and another where oral administration of 25 ml whole hemolymph was offered to HSV-1 patients for 10 consecutive days. There were patients with identical symptoms who were orally administered with placebos. Peripheral blood was collected from the subjects prior to the start of the study and immediately
following the end of the study period for natural killer cell (NK) and viral induced proliferation assays.

All of the subjects in this study reported a substantial improvement following treatment with the extracts with less cold sore episodes and reduced intensity of cold sores. Of particular interest was the cytokine profiling before and after the treatment, which demonstrated a drastic modulation of interleukin-10 (IL-10) in all of the HSV-1 patients that were orally administered with Haliotis rubra hemolymph (Dr. Valtchev, 2015, pers. comm). This was the first sign of immunomodulation indicating a hypothesis that several other cytokines related to the control of HSV-1, and potentially cancer, can also be modulated on oral administration of abalone hemolymph.

At this point, it was important to perform in vitro studies to identify the levels of immunomodulation, the site of immunomodulation, the optimum treatment time, the optimum treatment concentration, any cytotoxicity to host cells, the component of the hemolymph responsible for immunomodulation and to reach the broad view of the significance of this finding.

A review of literature is presented throughout Chapter (2) of this thesis. The hypotheses, materials and methodology of all the experiment procedures conducted are outlined in detail in Chapter (3).

Cytotoxicity analysis of abalone sera, purified AH and permeate are explained in Chapter (4), while Chapter (5) of this thesis incorporate the in vitro studies conducted with Human Primary Oral Keratinocytes and HaCat cell lines. Immunomodulation was determined by the quantification of proinflammatory cytokine concentration changes in cell supernatants pre- and post treatment with extracts.

Varying concentrations of three different extracts (purified hemocyanin, whole sera/hemolymph and sera permeate), on several incubation time periods were used as treatment on in vitro cultured cells. Dose-dependent effects of the extracts and cytotoxicity to host cells were also evaluated.
The quantification of cytokine concentration changes was performed with the Cytometric Beads Array Human Inflammatory Cytokine Kit (BD Biosciences) and analysed on Flow Cytometer. The six inflammatory cytokines that were determined included IL-10, IL-6, IL-8, IL-1 β, IL-12 and TNF-alpha.

In Chapter (6), the immunomodulatory prospect of *Haliotis rubra* hemolymph extracts were assessed on prostate cancer cell line PC-3 cells. A study on the effect of various extract treatment on the cell morphology is also outlined in this chapter. Additionally, the origin and nature of the hemolymph permeate was studied using SDS-PAGE and Western Blot Analysis. Various concentrations of the permeate was used to rule out anomaly and obtain a confirmed result.

Finally, Chapter (7) incorporates a thorough discussion of all the findings of this study, leading to directions for future research and a concluding remark.

1.1 Objectives:

- Establish and validate a suitable *in vitro* model for cytokine modulation by abalone sera.
- Investigate the effect of abalone sera on cytokine expression *in vitro*.
- Identify the specific cytokines modulated by abalone sera
- Identify the component of the hemolymph which exert immunological activity
- Determine the optimum delivery system of the drug onto patients.
1.2 Hypothesis:

(a) Proteins derived from the hemolymph of *Haliotis rubra* have immunomodulatory properties towards the human innate immune system.

(b) The effect on immune response occurs through the modulation of certain proinflammatory cytokines.

(c) Based on previous studies we hypothesize that hemocyanin is the main protein involved in immunomodulation in abalones.

In order to assess the hypotheses, *in vitro* studies were designed to treat keratinocytes (HaCat cells and Human Oral Primary Keratinocytes) and cancer cells (PC-3 prostate cancer cell line) with variable concentrations of *Haliotis rubra* hemolymph and the consequent modulation of expressed proinflammatory cytokine levels in the cell supernatant was assessed using flow cytometry.

In order to identify the component of the hemolymph that is responsible for the immunomodulatory behavior, hemocyanin was purified from the hemolymph. Purified hemocyanin (retentate) and the permeate, along with unprocessed hemolymph was then used as treatment extracts. This was also done to determine the most potent immunomodulator, and ascertain if all the three extracts were effective in immunomodulation of the cells.

Dose dependent immunomodulatory effects of the extracts were then assessed, with variable concentrations of the treatment extracts on the cells. Time-dependent immunomodulatory effects of the extracts were also assessed, that involved a variation of treatment duration and the corresponding modulation of proinflammatory cytokines.
1.3 Significance

Targeting the immune system to alleviate diseases is a crucial aspect in medicine because most chronic diseases have an immune component. The critical role of the immune system with respect to HSV-1 recurrence has been well studied and demonstrated (citation needed) but this promising therapeutic route has not been exploited. There are no drugs on the market which target this interaction. Current anti-viral drugs available against HSV-1, such as acyclovir, are not effective in immune-compromised patients. Moreover, these drugs do not have any effect on the recurrence of the disease, which is a major issue in HSV infected patients. Investigating the immunomodulatory potential of abalone sera and its components, identifying which component from the abalone sera is the active ingredient, what it does and what is the mechanism of action, can have implications beyond the treatment of HSV and may help treat other viral infections and forms of cancer. Since the number of immunomodulatory drugs available is limited, new immunomodulatory candidates are needed. There are added benefits of this candidate originating from a naturally occurring marine derived extract.

The novel aspects of this research include:

- Immunomodulatory properties of whole abalone hemolymph and hemolymph permeate on human primary oral keratinocytes have not been studied previously.
- Immunomodulatory properties of Haliotis rubra abalone hemocyanin (and other hemolymph components) have not been reported previously.
- The origin of the abalone hemolymph permeate proteins is a novel finding.
- The effect of Haliotis rubra hemolymph on the morphology of Prostate cancer cells has not been observed previously.
- Anti-cancer effects and immunomodulation of prostate cancer cells with treatment of *Haliotis rubra* whole hemolymph has not been investigated previously.
- The component of abalone hemolymph that induces the greatest immunomodulation among other abalone hemolymph components has not been identified before.
CHAPTER 2:

Review of the Literature
2.0 Introduction

Marine derived drugs are emerging at a rate higher than ever as research is being carried out on selective species constituents from the pool of a staggering 700,000 different marine species. This promising variety and their successful existence over millions of years despite inhabitancy with harmful viruses and bacteria in the marine world, is indeed a strong indication that marine derived drugs can help human health.

Abalone hemocyanin is such a marine derived extract that have shown to be active in multiple instances, and in particular helps patients with HSV-1 when orally consumed before and during outbreaks. A report, with substantial evidence from the blood of patients who dose dependently ingested abalone sera for ten days, showed that the level of cytokines, in particular IL-10, were reduced. This indicates an immune response triggered by hemocyanin that helps alleviate and reduce the frequency of HSV-1 outbreaks. The hypothesis that immune system modulating effect due to abalone sera takes place in contact with epithelial and neighbouring cells in the mouth and gut prior to digestion is to be tested in this project, while the aim remains to map out the immune response pathway and ultimately devising an abalone hemocyanin derived anti-viral drug.

While reviewing the literature for the titled research project, it was important to look closely at the existing findings and on going research in the field of immunology and marine derived drugs. In particular, there is emphasis placed on the mechanisms and importance of innate immunity, branching out to cytokines. Both marine derived drugs available commercially and those undergoing clinical trials are discussed. Other immune system modulating drugs and their mechanism are evaluated. However, the first point of discussion is the suspected active ingredient within the abalone sera, hemocyanin itself. The structure, properties and functions of both molluscan and arthropod hemocyanin are thoroughly researched and put together that advocates the potency of this multifarious molecule based on findings so far.
2.1 Hemocyanin

2.1.1 Structure of hemocyanin from arthropods and molluscs

The transport of oxygen is an essential feature for aerobic metabolism, thus life, in most animals. The widely known haemoglobin has two other lesser counterparts hemerythrins and hemocyanins (Kensal et. al, 2001). In 1878, Léon Fredericq, discovered and named hemocyanin, which are respiratory glycoproteins present in the hemolymph of arthropods and molluscs (Holde et. al, 1992); oxygen binding, transport and release is the primary function of the protein. In the molluscan hemocyanins, each functional unit (FU) within the protein subunit contains two copper binding sites that can bind to one molecule of oxygen (O₂). As the copper is in cuprous state, oxygen binding generates a particular light absorbance at 340 nm, causing the blue colour of oxygenated hemocyanins (Harris et. al, 1999).

There are some inherent difference in the structure of hemocyanin from arthropods and molluscs. Arthropod hemocyanins consist of multiple hexamers, each of which is made up of monomers weighing approximately 75 kDa (Durstewitz et. al, 1997; Feldmaier et. al, 2000). Variations of the common monomer sequence occupy a certain position on the whole molecule. The hemocyanin subunit is divided into three domains, and the highly helical domain contains the active site copper pair. Each of the copper is ligated with three histidine residues (Volbeda et. al, 1989).

Molluscan hemocyanins on the other hand, have structures built from large polypeptide chains of 350-450 kDa, each divided into 7 or 8 functional units weaved through linker peptide chains (Lieb et. al, 2000). Within the various molluscan hemolymph, hemocyanin molecules may exist as subunits of dimers, decamers, or oligomers of the decamers,
causing hollow cylindrical arrays of very large sizes resulting in as many as 160 active sites per molecule (Hazes et. al, 1993). The two domains of such hemocyanins are of N terminus (highly helical) and C terminus (β-sheet). The two copper sites are positioned such that they are well separated; one near the N-terminus and the other near the C-terminus. Each copper site is ligated by histidine residues, similar to the arthropod hemocyanin (Magnus et. al, 1994).

2.1.2 Properties and functions of hemocyanin

Hemocyanin typically encompasses up to 95% of the total protein in the hemolymph of molluscs and arthropods, and has proven to be involved in a varied range of functions in addition to the respiratory objective. These include osmoregulation, energy storage, molt cycle, exoskeleton formation and immune defense (Zheng et. al, 2016; Coates et. al, 2014; Decker et. al, 2007).

Hemocyanin from various species has been widely studied for its anti-viral, anti-bacterial, anti-fungal, anti-cancer and immunogenic activities over the past three decades. The substantial findings have placed hemocyanin as a key interest to researchers in the field, and its potency is still unraveling in the recent years.

2.1.3 Immune system modulating potential of hemocyanin

The immunomodulatory effects of hemocyanin, particularly *keyhole* limpet hemocyanin (KLH), have been established in a number of different studies both in vivo and in vitro. Murai et. al (2015) found a direct relation between the reduction of IL-10 gene expression in orally immunized chicken with KLH in response to anti-biotics. IL-10, which is mainly produced by T-regulatory cells (Treg), suppresses immune responses and contributes to immune tolerance in the intestine.
(Veenbergen et. al, 2012). This is a promising result since recent study by Shanmugasundaram et. al. found the close similarity between the regulatory immune system of chickens to that of mammalian Treg cells, deducing that CD4+ CD25+ cells in chickens are analogous to mammalian Treg cells that produce considerable amounts of IL-10 and TGF-β (Shanmugasundatam et. al, 2011). Similar reduction in IL-10, along with IL-4 and IL-5 was observed by Kamachia et. al in BALB/c mice when they were injected with antigen KLH induced OX40L cDCs (conventional dendritic cells). This indicated a reduction in Th2 production in the lymph node of these mice (Kamachia et. al, 2014). In a study of the effect of Strontium 90 ingestion in mice that were previously vaccinated with antigens of KLH and TT (tetanus toxin), there was no reduction in the IgG specific to KLH (a strong Th2 inducer antigen). In contrast there was an increase in immunoglobulins specific for the KLH antigen (Nicholas et. al, 2016). Effect of hemocyanin on the level of cytokine and antibody production provides a degree of clarity to its immunomodulatory potential.

2.1.4 Anti-fungal, anti-viral and anti-cancer properties of hemocyanin, and use of hemocyanin as a conjugate vaccine

In vitro studies on fungal spores demonstrated an interesting finding where a particular mechanism was adopted by Penaeid shrimp hemocyanin to combat fungal cells. This mechanism involved the peptide PvHCT, released from the C-terminus of hemocyanin, adopting an alpha helical structure in membrane-mimicking media and displaying a random conformation in aqueous environment. In contact with F. oxysporum, PvHCT bound to the surface of fungal hyphae but there was no import into the cytoplasm. Notably there was a gradual degeneration of the cytoplasm resulting in cell lysis in fungal spores and hyphae (Petita et. al, 2016). This is further supported by Destoumieux-Garzón et. al. in the study of infected penaeid shrimp plasma, which appeared to contain an elevated amount of peptides derived from the C-terminus of its hemocyanin, revealing that
microbial infections trigger the increase of these peptides in shrimp plasma (Garzon et al., 2001).

Hemocyanin acts as an anti-viral agent in the hemolymph of Red Swamp Crayfish when the fish is exposed to White Spot Syndrome Virus (WSSV). Two hemocyanin subunit genes of Crayfish challenged with WSSV showed the same regulation pattern in terms of their mRNA levels, which indicated that both of these genes may participate in the anti-viral immune response of crayfish (Zeng et al., 2008). This is an example of how hemocyanin is responsible in immune modulation in species that carries it. In other words, it serves as an immune defense component in the crayfish (and abalone). This has been extended to study the anti-viral effect of hemocyanin on mammalian viral infections, including the very common Herpes Simplex Type 1 Virus (HSV-1). Vero cells infected with HSV 1 were treated with abalone hemocyanin on a dose dependent manner where inhibitory effects through a receptor mimicking mechanism were observed. Viral attachment and entry to Vero cells was inhibited through specific binding with the viral surface glycoproteins, and this was also achieved by using a small dimer subunit of the hemocyanin indicating that only a small hemocyanin analogue could be used for its antiviral activities (Zanjani et al., 2016). Peptides based on the overlapping fragments of the haliotisin region of abalone hemocyanin (located within the linking sequence between the α-helical domain and β-sheet domain of abalone hemocyanin functional unit E), which is considered as the putative anti-microbial region, were tested for their bactericidal potential through incubating Gram-positive and Gram-negative bacteria in the presence of certain haliotisin peptides. There was significant reduction in microbial growth and additional damages to the microbial cell wall (Zhuang et al., 2014). A novel approach that is increasingly being adopted and tested by researchers is the use of hemocyanin as a bio-adjuvant. The adjuvants are substances that have the ability to enhance the magnitude and duration of the immune response and to increase vaccine efficacy. Gesheva et al. tested hemocyanin from
gastropod *Helix pomatia* as a bio-adjuvant for bacterial antigens, where hemocyanin was combined with tetanus toxoid and the complex was used for immunization on mice. This resulted in an increased number of anti-TT IgG producing plasmocytes and induced a significant increase of B and T cell proliferation (Gesheva et al., 2006). Bio-adjuvant hemocyanin which acts as a natural protein carrier for vaccine, further illustrates its ability to orchestrate the immune response in an effective manner for bacterial attacks. Immunogenicity of similar conjugates were tested with Trinitrophenyl-KLH on rabbits, that induced an average of 375 µg of anti-TNP antibody and up to 2.5 mg of anti-KLH antibody per ml serum within a week after second injection (Marvin et al., 1966) This further validates the potency of KLH as an adjuvant in inducing immune response.

The use of hemocyanin as an adjuvant is being implemented for treatments of various types of cancers due to its cancer cell specific inhibitory effects. *Rapana thomasiana* hemocyanin adjuvants with choline amino acid salts has specific anti-proliferative effects on breast cancer cells *in vitro*, and in contrast stimulatory effects on fibroblasts (Guncheva et al., 2015). An *in vivo* study of KLH conjugate vaccines for melanoma showed that mice immunized with these conjugates had a stronger antibody response to G(D3) than mice immunized with unconjugated G(D3). The strongest response was observed in mice immunized with the KLH conjugate of the G(D3) aldehyde derivative and the adjuvant QS-21. These mice showed a long-lasting high-titer IgM and IgG response indicating recruitment of T-cell help (Helling et al., 1994). Later the same year, this study went to the clinical trials phase – I to be tested as vaccine on patients with malignant melanoma identifying 100 µg dose of QS-21 as the optimal well tolerated dose for induction of antibodies against both the melanoma ganglioside/GM2 and the protein KLH in melanoma patients (Livingston et al, 1994). The rapid advancement accredits itself to the promise of hemocyanin as a potential anti-cancer drug.
Bladder cancer cells responded to Helix lucorum and Rapana venosa hemocyanin in vitro by showing anti-tumor activity of both the hemocyanins through induction of apoptosis. Besides the antiproliferative effect, there was downregulation of the genes with metastatic potential (Antenova et. al, 2015). Hemocyanin from shrimp showed similar antiproliferative effect on cervical cancer cells, Hela Cells, through apoptosis in HeLa cells characterized by the formation of apoptotic body-like vesicles, chromatin condensation and margination. In addition, the reactive oxygen species (ROS) and caspase-9/3 activities significantly altered in hemocyanin-treated HeLa cells (Zheng et. al, 2016).

The potential of hemocyanin further extends to its application in contraception. GnRH-hemocyanin conjugate immunocontraceptive vaccine formulation was used and it successfully prevented reproduction and inhibited production of sex hormones in various mammalian species, particularly feline species, for an extended period of time (Sung et. al, 2015).

2.2 Innate immunity

Specific bacteria with the doubling time of one hour can reproduce into as many as 20 million a day to infect the host (Alberts et. al, 2002). At this rate, immediate immune response to combat the infection is vital. Innate immunity takes action within hours of the bacterial infection, while otherwise relying on the adaptive immune response would takes effect in as much as few days that could potentially cost a life. Hence, the importance of innate immunity is evident.

Innate immunity is a primordial immune defense system, which every individual is gifted from birth. (Wiley et. al, 2009) As first line of defense, a number of physical and chemical barriers are present to prevent further entry of foreign bodies. Majority of foreign bodies cannot penetrate skin, while some may enter through sebaceous glands they are immediately fought with fatty acids and
hydrolytic enzymes. Skin also has an additional supply of lymphocytes and plasma cells beneath the epithelium. (Edwin et. al, 1982) Innate immunity mechanism also includes mucus in respiratory and gastrointestinal tracts which entraps most microorganisms and the cilia lining in epithelial cells facilitates their quick exit. Macrophages in the alveoli deal with any substances that remain in the respiratory tract by phagocytosis. Neutrophils are the most abundant leukocyte in humans and essential to the innate immune response against invading pathogens. The ability of neutrophils to kill microorganisms is immediate, non-specific, and not dependent on previous exposure to microorganisms. Microglial cells in the central nervous system phagocytose those who find their way to that area while acidity of the stomach, bile in small intestine and enzymes in saliva contribute further to innate immunity. (Wiley et. al, 2009) The thymus, spleen, tonsils, lymph nodes, appendix and bone marrow are the major organs participating in our immune system, from being the originators of cells of immunity to the mediators of our immune system (Edwin et. al, 1982).

2.2.1 Use of PAMPs, PRRs and TLRs

The fundamental mechanism by which innate immunity functions is by distinguishing self from non-self. This prevents the immune response from destructing own cells, absence of which would prove to be fatal. Infectious agents possess invariant molecular constituents that are essential for the microbe’s survival. Hence, these special components are not subject to mutations or variability, moreover these structures are common across infectious microorganisms. Innate immunity has evolved over the years to combat this common series of Pathogen-Associated-Molecular-Patterns (PAMPs) present in every invading pathogens. Lipopolysaccharides, techoic acids and double stranded RNAs are some examples of PAMPs (Ruslan et. al, 1987). The media used by host cells to recognize these structures are germline-encoded Pattern Recognition Receptors (PRRs) which may be present extracellularly, intracellularly or secreted by host defense cells.
One such example is the transmembrane protein Toll like receptors (TLRs). TLRs have a cytosolic region identical to IL-1 receptors and thus this domain is termed TIR. The ectodomain contains leucine-rich repeats. Each of these domains serve a purpose with the extracellular domain focusing on the recognition of PAMPs, while the TIR domain mediating signal transduction (Taro et. al, 2010) Eleven different proteins have been identified in mammalian TLRs, and each are fine-tuned to recognize specific PAMPs and induce varied necessary signaling pathways. NFkB (Nuclear Factor Kappa Beta), MAPK (Mitogen Activated Protein Kinase) and IRF5 (Interferon Regulatory Factor 5) are common signaling pathways amongst many induced by different TLRs, resulting in the gene encoding and transcription of immunomodulation messenger proteins such as cytokines, TNFs and interferons (Taro et. al, 2005).

TLRs recognize and respond to infectious agents both on the extracellular and intracellular region of cells. Intracellular microorganisms are also recognized by nucleotide binding oligomerization proteins (NOD 1 and NOD 2), that may induce the according signal transduction (Takeda et. al, 2005).

### 2.2.2 Other aspects of innate immunity

Opsonization (binding the pathogen for aided engulfing) and phagocytosis (ingestion and elimination of pathogens) are the other arms of innate immune response, which are highly well integrated with selective “division of labor” between cells and targets for effective elimination of the foreign invader. Phagocytic immune cells such as macrophages have ancillary receptors to identify specific types of microorganisms to orchestrate the optimum means of phagocytosis (Spencer et. al, 2014).

A crucial finding in immunology relates to innate immunity’s direct influence on the functions of adaptive immunity, which was formerly
considered as mutually exclusive. Innate immune response not only initiates but also subsequently orchestrates the pathway taken by adaptive immunity. Additionally, innate immunity takes considerable responsibility in dealing with the infectious agents already targeted by adaptive immune response (Janeway et. al, 2001; Akiko et. al, 2015).

2.3 Cytokines of the innate immune system

During an immune response, small protein molecules ranging from 8 - 40,000 Daltons (Watkins et. al, 2013) are secreted by immune cells (e.g T helper cells or macrophages) or non-immune cells (e.g endothelial cells or fibroblasts), which help cell to cell communication and interaction (Jun-Ming et al, 2007). Cytokine is the generalized name of a collection of such molecules including lymphokine (cytokines made by lymphocytes), monokine (cytokines made by monocytes), chemokine (cytokines with chemotactic activities), and interleukin (cytokines made by one leukocyte and acting on other leukocytes) (Richard et. al, 1997). Secreted cytokines may act in three different manners: autocrine where the cytokines act on the cells that they have been secreted from, paracrine where they act on nearby cells, and endocrine where they act on cells positioned at a distance (Constantin et. al, 2007). In an immune response, cytokines may either have pro-inflammatory effects or anti-inflammatory effects, and all cytokines are attributed to each specific category of effects (Angus et. al, 1997).

Different cell types secrete the same cytokine while a single cytokine can act on several different cell types (i.e pleiotropy). Additionally, similar immune response can be stimulated by different cytokines. Generally, cytokines are produced in a cascade where one cytokine stimulates the target cells to produce additional cytokines (Robin et. al, 1996).

2.3.1 Proinflammatory and anti-inflammatory cytokines

Proinflammatory cytokines have been extensively studied in conditions such as periodontal disease (Sledziewski et. al, 2015), coronary heart disease (Provotorov et. al, 2015), non-alcoholic fatty liver disease (Stojisavljevic et. al, 2014), colon cancer (Hummel et. al, 2014) and so on.
Interestingly, increased levels of proinflammatory cytokines like TNF-alpha and IL-6 are associated with aging (Brunsgaard et al., 2001). Increased proinflammatory cytokines IL-1 and TNF-alpha is responsible for the defect in Erythropoietin production in severe systemic and renal inflammatory diseases (Jelkmann et al., 1998). Hence, reduction in the biological activities of proinflammatory cytokines is necessary to prevent harm to host and promote healing. Biological activity of IL-1 and TNF can be achieved by several different strategies, including neutralizing antibodies, inhibitors of proteases receptor antagonist, and soluble receptors (Dinarello et al., 2000).

Anti-inflammatory cytokines on the other hand control the proinflammatory cytokine response. This concert of cytokines with specific cytokine inhibitors and soluble cytokine receptors regulate the human immune response. Some of the major anti-inflammatory cytokines are IL-10 and IL-13 (Opal et al., 2000).

### 2.3.2 Interleukin-6, Interleukin-8 and Interleukin-10

Interleukin-6, IL-6, was initially recognized for its ability to promote the population expansion and activation of T cells, the differentiation of B cells, and regulation of the acute-phase response (Yasukawa, K. et al. 1987; Hirano, T. et al. 1986). Hormone-like attributes of IL-6 affecting vascular disease, insulin resistance, the neuroendocrine system and neuropsychological behavior was later recognized (Bethin et al. 2000; Kraakman et al. 2015). The structure of IL-6 is illustrated in Fig. 2.0. Majority of stromal cells and cells of the immune system secrete IL-6. It is produced mainly by certain pathways involving Toll-like receptors, prostaglandins, adipokines, stress responses and other cytokines. IL-1β and tumor-necrosis factor are major activators of IL-6 expression as well. Concentrations of IL-6 in normal human serum are as low as 1–5 pg/ml, but these are rapidly increased in disease conditions (Wagee et al., 1989). IL-6 is, in fact, a better predictor of disease activity as infection,
autoimmunity and cancer have an associated elevated IL-6 (Fraunberger, P. et al. 2006; Mroczko et. al 2010; Panichi et. al, 2004).

On the other hand, as IL-6 is a lymphocyte-stimulating factor, its deficiency causes impaired innate and adaptive immunity to viral, parasitic and bacterial infection (Dienz, et. al 2012; Garbers, et. al 2011; Hoge, et. al 2013; Smith et. al, 2014).

Figure. 2.0: Structure of Interleukin- 6. Source: Xu et. al (1997)

IL-6 modulates almost every aspect of the innate immune system, such as hematopoiesis and the migration of neutrophils at sites of infection through the control of granulopoiesis (Chou et. al 2012; Liu et. al 1997). The driving force behind this is the regulation of neutrophil-activating chemokines and neutrophil apoptosis by IL-6. IL-6 reduces influenza virus–induced inflammation and protects against fatal lung pathology (Launder et. al, 2013). Competent host defense, excessive tissue damage prevention and the transition from the recruitment of neutrophils to the recruitment of mononuclear cells are further effects controlled by IL-6 (Lally et. al, 2005). IL-6 augments macrophage differentiation and restricts the formation of dendritic cells in vitro (Chomarat et. al, 2000), and controls the macrophage colony-stimulating factor receptor (encoded by Csf1r) (Jenkins et. al 2004). IL-6 also inhibits activation of the transcription factor NF-κB.
In the adaptive immune system, IL-6 is identified as a lymphokine that triggers the maturation of B cells into antibody-secreting cells. It is also involved in the survival and maintenance of long-lived plasma cells. This was supported by reports that IL6−/− mice immunized with a T cell-dependent antigen showed lower immunoglobulin G production than wild-type mice. It was also demonstrated that IL-6 deficiency correlates with diminished antibody responses and susceptibility to infection (Kopf et. al, 1994). Castleman’s disease is one such clinical example of IL-6 and B cell association. Furthermore, the ability of IL-6 to promote humoral immunity is due to its effects on follicular helper T cells (TFH cells), a specialized subset of CD4+ T cells that express the chemokine receptor CXCR5 and localize to B cell follicles. It is there that they promote B cell proliferation and immunoglobulin class switching (Ma et. al, 2012). IL-6 serves as a central link between T cell responses and B cell responses (Nurieva et. al, 2008; Dienz et. al, 2009; Harker et. al, 2011).

Interleukin-8 (IL-8) belongs to a family of small, structurally related cytokines similar to platelet factor 4. IL-8 is a chemotactic factor that attracts neutrophils, basophils, and T-cells, but not monocytes. It is also involved in neutrophil activation. It is released from several cell types, including macrophages, epithelial cells, endothelial cells and other cell types (Hedges et. al, 2000), in response to an inflammatory stimulus. The structure of IL-8 is illustrated in Fig. 2.1. The primary function is not only inducing chemotaxis of neutrophils and other granulocytes in target cells, and causing them to migrate toward the site of infection but also inducing phagocytosis once they have migrated to the site. IL-8 increases Ca2+, exocytosis and the respiratory burst (Modi et. al, 1990).

Interleukin-8 secretion is increased by oxidant stress, cyclically causing the recruitment of inflammatory cells and inducing an increase in oxidant stress mediators itself, making it a key parameter in localized inflammation (Vlahopoulos et. al, 1999).
IL-8 has demonstrated significance in a number of clinical situations; it has been cited as a pro-inflammatory mediator in gingivitis (Haake et. al, 2002) and psoriasis, autocrine growth factor for colon carcinoma cell lines (Brew et. al, 2000), elevated IL-8 has been linked to schizophrenia (Zhang et. al, 2004), and IL-8 has also been implicated in the pathology of cystic fibrosis (Reeves et. al, 2011).

![Image of IL-8 structure](image)

Figure 2.1: Three-dimensional structure of interleukin 8 in solution. 
*Source: Clore et. al, (1990)*

IL-10 is particularly of interest since it is produced by a variety of immune and non immune cells, and its level in blood or cell supernatant from various treatment of infections can be a key indicator of the immunological effects of the treatments. It primarily inhibits the action of Th1 cells, NK cells and macrophages during infection. This helps prevent tissue damage. As a consequence IL-10 impedes pathogen clearance and ameliorates immunopathology. IL-10 is mainly produced in different tissues during acute or chronic infections (Coupar et. al, 2008). In addition to the anti inflammatory action of IL-10 however, there is a paradoxical degree of pro-inflammatory effect demonstrated by IL-10 as many reports suggest that IL-10 enhances the function of NK cells, causing an increased antigen availability. Also, IL-10 preserves the ability of antigen presenting cells for antigen uptake. This also hampers their migration to draining lymph nodes (Mocellin et. al, 2003).
2.4 Marine derived immune response modulating compounds

A number of marine derived compounds have shown to regulate cytokines in vivo and in vitro in the recent years. Orally administered Oligosaccharides of kappa/beta-carrageenan from the red alga Tichocarpus crinitus, induced IL-10 production in human blood cells. Compared to the source polysachcharide, the low molecular weight counterpart induced more IL-10. Polysachcharides from lichens have shown to have immunostimulatory effect in some studies as well (Sreshtha et. al, 2015; Hirabayashi et. al, 1989; Schepetkin et. al, 2006). A comprehensive list of marine derived immunomodulatory compounds is outlined in Table 2.2.

Other compounds such as seaweed lectins, or Pseudopterane Diterpene isolated from the octocoral Pseudopterogorgia acerosa, particularly the latter have shown to have anti-inflammatory effects through the inhibition of inflammatory mediators. It also inhibited the macrophage activation and initiation of adaptive immune response (Gonzalez et. al, 2013; Rivanor et. al, 2014). Marine sponge-derived reagent alpha-galactosylceramide (alpha-GalCer), induces a rapid production of cytokines and activation of other immune cell types (Parekh et. al, 2013). 11-episinulariolide acetate from soft coral reduced COX-2 and IL-8, and blocked intracellular Ca2+ increase and Ca2+ dependent transcription factor activation in human epidermoid carcinoma cells, which can be used for targeting Ca2+ signaling-dependent inflammatory diseases (Hsu et. al, 2013).
2.4.1 Immune system modulating marine drugs

A number of marine derived drugs are on shelves today, continuing to alleviate human diseases. Acyclovir, a derivative of Ara-A from marine sponge Cryptotethya Cryta, is the primary drug for Herpes Simplex Virus type 1 and 2 (HSV).

Acyclovir, an acrylic purine nucleoside analog, which although is an inhibitor of HSV, has very low toxicity for the normal host cells. The reason for this selectivity is that these viruses code for a viral thymidine kinase that phosphorylates acyclovir to a monophosphate; uninfected cells are incapable of this (Elion et. al, 1982). Cellular enzymes later convert this acyclovir monophosphate (acyclo-GMP) to acyclovir triphosphate (acyclo-GTP). Acyclo-GTP persists in HSV-infected cells for many hours after acyclovir is removed from the medium. Acyclo-GTP acts as a more potent inhibitor of the viral DNA polymerases than of the cellular polymerases (Brigden et. al, 1983). Pachnio et. al. found the immune response modulating potential of Acyclovir showing that low dose acyclovir treatment has the potential to modulate components of the T cell response to Cytomegalovirus (CMV) antigen proteins (Pachnio et. al, 2015).

A well known immune modulatory drug is Cyclosporine, a cyclic undecapeptide from the marine fungus Microdochium nivale, which has immunosuppressive effects because they block T-cell activation or proliferation (Stuart et. al, 1992). Activation is the G0-G1 cell-cycle transition, which takes place due to the antigen binding to the T-cell receptor (TCR). Genes such as those encoding the interleukin-2 (IL-2) and its receptors are switched on by the consequent Ca2+ dependent TCR signal transduction pathway. On the other hand, proliferation occurs at G1-S transition that recruits from autocrine signaling by ILh-2 (Jeannette et. al, 1993). Cyclosporine was initially used as an immunosuppressant drug to prevent rejection in organ transplant patients, but have now
being applied to diseases such as rheumatoid arthritis and severe psoriasis (Lowe et al., 1996).

Various marine derived drugs have entered Clinical trials including IPL-576 (aka HMR-4011A) from marine sponge *Petrosia contignata*, which inhibits the release of histamine from mast cells and lung tissue enabling itself to be a treatment as an anti-allergen for asthma. It has passed Clinical Trial phase II in 2002 (Singh et al., 2008). KRN7000, a glycolipid that has a novel α-galactosylceramide (α-GalCer) structure, is no longer extracted from the native source marine sponge, but is chemically synthesized, which exhibits potent anti-tumor activity through stimulation of a strong immune response in Natural Killer Cells (NKT cells). (Alline et al., 2011; Chang et al., 2005).

The most common mechanism of action of marine derived drugs to date is, however, through inhibition of tumor cell proliferation in association with G(2)-M arrest and tubulin polymerization and microtubule stabilization (Iwasaki et al., 2015).

Besides drugs, application of commercially available marine bioproducts extends to molecular probes, such as manoalide and green fluorescent protein (GFP), enzymes such as vent and deep vent DNA polymerase, nutritional supplements such as formulaid from marine algae, and pigments like phycoerythrin (Roger et al., 2002).

2.5 Viruses and the Human herpes simplex virus (HSV)

Virology, the study of viruses, originated during the 1890s when infectious agents targeted tobacco plants. However, the evidence of viral infections dates back to 1157 BC with the discovery of small pox lesions present in the mummified body of the young pharaoh Ramses V. The viral pestilence timeline incorporates the prevalence of small pox throughout the early 15th century, followed by outbreak of measles, dengue fever, influenza and polio until early
18th century. AIDS epidemic spread through early 1980s and the culprit was finally discovered as the human immunodeficiency virus (HIV) by Luc Montagnier. During the past two centuries, there have been considerable accounts of new viral outbreaks including Zika (first isolated from humans in Nigeria in 1954), Asian influenza, Rubella, Marburg hemorrhagic fever originated from infected African green monkeys from Uganda, Germany and Yugoslavia. Ebola, SARS, Avian influenza Chikungunya, Porcine reproductive and respiratory syndrome, Schmallenberg, Swine Flu, Hantavirus, MERs, Zika and Powassan virus have been most prevalent and damaging in the last two decades (Woolhouse et. al, 2012). HSV type 1 and type 2 viruses are very common to date, with the former, affecting 80% of the Australian population.

With emerging viral diseases there has been numerous advents of virus discovery techniques. To briefly outline in chronological order of virus discovery technique implementation Filtration technique is the most classical method developed in 1890s, followed by Complement fixation technique (1929), Mice, embryonated eggs and electron microscopy in 1930s, Tissue culture as host in late 1940s, Monoclonal antibodies and Sanger Sequencing in 1970s, Polymerase chain reaction (PCR) in mid 1980s, Bioinformatics in 1990s followed by most recent techniques of high throughput sequencing, Panmicrobial arrays, Unbiased next generation sequencing (NGS) and Metagenomics during the 2000s (Woolhouse et. al, 2012).

2.5.1 What are Viruses?

Unable to reproduce by itself, virus is a small parasite that infects a susceptible cell inducing the cell to act as a machine to generate more viruses of the kind. Viruses can range in its complexity with the simplest form only being able to encode four proteins and the most complicated ones up to 200 proteins. Their genetic material is either RNA or DNA (Krulik et. al, 1999)

The active and infectious component of the virus is termed virion and it consists of the nucleic acid with an outer coat of protein. These proteins, or
capsid, can be in the form of replicates of one protein or an amalgamation of various types of proteins. The entire particle containing the nucleic acid and the capsid is termed nucleocapsid. In some cases, the nucleocapsid is further protected by an external envelope mainly formed by a phospholipid bilayer containing types of glycoproteins encoded by virus. This essential feature of viruses mimicks the phospholipids in the infected host cell’s plasma membrane and is formed by the budding act with the host cell’s plasma membrane (Maramosh et. al).

2.5.2 Properties of Viruses

The nucleocapsid adds to one of the key properties of viruses which is its protection from outside environment that would otherwise quickly degrade the viral genome. The unprotected viral genome is susceptible to physical, chemical and enzymatic damages. It is sensitive to the extent that the breakage of a single phosphodiester bond may inactivate a virus.

Recognition and interaction with the host cell is another key property and may be performed through either the protein capsid or virally encoded glycoprotein coat acting as spikes to enter the host cell.

Viruses with small viral genomes such as poliovirus can self assemble. The process of self assembly of viruses require minimal energy and, in addition, is reversible resulting in a stable structure capable of assembling and disintegrating itself according to prompts.

In order to avoid errors caused by DNA/RNA replication, transcription and protein synthesis stages, a viral genome is restricted to code for only a particular number of proteins depending on the size of the viral genome. Hence the accuracy of protein coding performed by the viral genome represents high fidelity.
Economy in genome-coding capacity is controlled by the existence of capsomers composing a spherical structure for most volume and least surface area (Maramosh et. al).

2.5.3 Mechanisms of viral entry

Different viruses have varying preferred routes of entry to the host. They enter the host through the skin or mucous membranes found in the respiratory tract, gastrointestinal tract, genital tract or conjunctiva.

Following entry to host, the virus replication is either localized at the site of entry or they spread systemically to the neighboring lymph nodes. Examples of localized infections may be papillomavirus, adenovirus and norovirus while HSV-1, HSV-2 and Enterovirus cause systemic infections.

2.5.4 Effector immune responses against viral infections

Non-specific defense mechanism is the first mode of encounter when a primary infection enters in a non-immune, susceptible host. It occurs within few hours and the defense consists of interferon production, inflammation, fever, phagocytosis, and natural killer cell activity. The primary infection may then be prevented or aborted. Failure of this non-specific defense mechanism leads to the spread of the virus through local spread, viremia, or nerve spread, to a number of target organs, producing a generalized infection (Baron et. al, 1984).

Production of antiviral antibody through specific immune response, and cell-mediated immunity takes place. The dispersed antibody response in serum is predominantly IgG (followed by IgM) while the local antibody response in secretions is predominantly secretory IgA (with some IgM). Antibodies either neutralize virus or destroy virus-infected cells via antibody dependent cell-mediated cytotoxicity or complement pathway (Biron et. al, 1994). In cases where virus spreads throughout the body through blood stream, for instance measles and polio, the serum anti-body counters the generalized infection.
In localized infections of mucosal surfaces such as respiratory tract infection and infection of the gastrointestinal tract, IgA antibody is much more important. Serum antibody may not protect against recurrence of latent infections, such as herpes zoster (shingles) and herpes simplex, both because the virus may be shielded by its intracellular location and because cell-mediated immunity may be the more important defense (Brenner et. al 1991). In persistent infections, serum antibody may be responsible for certain long-term sequelae.

In situations where antibody is unable to reach the virus, cell-mediated immunity is essential for the control of viral infections. Examples are infections involving oncogenic viruses or viruses that spread directly from cell to contiguous cell. Cell mediated immunity specializes in recognizing the virally induced antigens on the surface of the infected cell, to tackle the infection (Hirsh et. al 1980). Cytotoxic T cells are a type of effector cells that fulfill this task.

When the virus reaches target organs, it becomes much more complex. Then the host defense plays an important role causing inflammation, fever, and interferon production and cell-mediated immunity (Rouse et. al, 1988).

### 2.5.5 Role of immune system in chronic HSV infections

Herpes simplex virus (HSV) is a human pathogen belonging to the family Herpesviridae. There are two serotypes of the alpha herpes viruses: HSV-1 and HSV-2. They are the causative agents of the highly prevalent oral and genital herpes. Not only the immune response to these pathogens is complex and multifactorial but also, these viruses have multiple viral evasion mechanisms. To understand antiviral immunity and viral pathogenesis, it is thus vital to the study of anti-HSV immune responses.
Both innate and adaptive immune mechanisms are involved in the immune response against HSV. However, the innate antiviral response plays a pivotal role in consequent the outcome of the HSV infection. Production of type I interferon (IFN - IFNα and IFNβ) has shown to protect against HSV in both mouse models and in human studies (Ellermann-Eriksen et. al, 1986; Shupack et. al, 1992; Vollstedt et. al, 2004).

In vivo, multiple cell types have shown to contribute to the innate immune responses to HSV, such as natural killer (NK) cells and plasmacytoid dendritic cells (pDCs). NK cells are involved in cytokine production, recognition, and killing of virally infected cells. PDCs cells are involved type I IFN production in vivo (Biron et. al, 2001; Cerwenka et. al, 2001)

Disease progression, latency and control of virus spread are controlled through the adaptive immune response. Neutralizing antibody levels have been negatively correlated with disease severity (Milligan et. al, 1995; Seppanen et. al, 2006) On the other hand, cellular response plays a vital role in the process. For instance, CD8+ T cells through the production of IFNγ is highly involved in antiviral defense, with (Dobbs et. al, 2005; Iijima et. al, 2008). In the absence of other immune effectors, CD4+ T cells provide some degree of protection against HSV (Johnson et. al, 2008).

### 2.5.5.1 Importance of IL-6, IL-8 and related cytokines in HSV infection

Cytokines produced during the innate inflammation not only provides antiviral effects themselves, but also induces further adaptive immune response. Innate immune response acts through signaling induced by pattern recognition receptors including Toll-like receptors. Viral pathogens are perceived as hazardous by these receptors that initiates a cascade of signaling and resulting inflammatory response. Such signals are mediated by inflammatory cytokines, particularly interferons (IFN-α/β), IL-12 and TNF (Orange et. al., 1996). These proinflammatory molecules attract other
inflammatory cells into the infection area and activate antigen presenting
cells to induce adaptive immunity.

Moreover, human and animal herpes virus models have demonstrated
signaling occurring number of TLRs including TLR2, which recognizes
virion components including surface glycoproteins (Boehme et. al 2006;
Compton et. al 2003), which pinpoints dsRNA (Iwakiri et. al 2009) as well
as TLR9 which is responsible for the detection of genomic DNA (Lund et. al
2003).

Nason et. al. (2016) found a significant association between Herpes
Simplex Virus shedding and high levels of IL-6. The increase in IL-6 in
primary oral keratinocytes may induce shedding of HSV-1, and according to
Nason et. al (2016) the increased IL-6 may also prevent subsequent
reactivation of the virus as long as the immune-environment remained
optimal for this cause.

Interestingly, Donnarumma et. al (2010) found an increase in IL-1 B,
IL-6, IL-8, TNF-alpha, (i.e proinflammatory cytokines) in epithelial cells and
concluded that their method of HSV-1 treatment indeed facilitates and
triggers the proinflammatory immune response in HSV-1 infection. Additionally, the importance of targeted immune responses in different
stages of the infection is also stressed.

As previously mentioned about the ability of IL-6 to further modulate
IFNs, a cytokine highly equipped with antiviral effects, McLoughlin et. al
(2003) has found an interplay between levels of IL-6 and IFN-gamma
during acute inflammation, which consequently has an immediate effect in
innate immune response through recruitment of neutrophils to the site. IL-
6 has shown to attenuate neutrophil influx due to IFN-gamma, while IFN-
gamma has also shown to modulate IL-6 signaling through its soluble
receptor (sIL-6R) to promote their apoptosis and clearance. This cyclic
interplay dominates immune response against HSV infections.
2.6 Methods for Determination of Cytokine Concentration

An emerging demand for rapid, precise and cost-effective quantification of soluble cytokines and other analytes in serum, is evolving in research laboratories as it is an essential factor in the management of diseases (Elshall et al 2007)

Quantification of cytokines as an indication of immune response can be performed in a number of methods. These include Enzyme Linked Immunosorbant Assay (ELISA), Western Blot and Multiplex Bead-based Immunoassay. While the most classic and standard method of cytokine quantification from cell supernatant is ELISA followed by Western Blot, they are not suitable for high throughput multiplex assays Multiplex Bead-based immunoassay is becoming more common due to its advantages over the former methods.

In brief, the working mechanism of ELISA involves coating microtitre plates with antibodies to capture the protein of interest. Then the protein sample is added followed by the addition of a secondary antibody to the same antigen and probing for that secondary antibody.

Western Blot involves resolving the proteins by size on a protein gel, transferring them to a membrane and probing with an antibody to the protein of interest.

Multiplex Bead-based immunoassays work on a capture-detection sandwich-type assay using fluorescent microspheres analyzable by Luminex instruments or flow cytometers (Moncunill et. al 2014).

Up to 30 different proteins can be quantified with a 50 microlitre sample with bead-based immunoassay without losing sensitivity, accuracy, or reproducibility (Castillo et. al 2012), while only 1 protein can be quantified with 100 to 200 microlitre sample with ELISA/Western Blot. Thus, the sample volume required is
tentatively one sixth the quantity necessary for ELISA assays due to the detection of six proteins in one sample. Furthermore, in preparation of the standard, a single set of diluted standards is used to generate a standard curve for each analyte. A bead-based experiment takes less time than a single ELISA while providing results that would normally require six ELISA experiments.

In terms of the comparison of results obtained from these methods, there is a need to know how comparable these two methods are for quantitative analyses. Specific aspects of these assays, such as the clones of monoclonal antibodies used for detection and reporting, are crucial in producing comparable results from both assays. Two commercially produced bead array kits were compared with ELISA (DuPont et al. 2005) demonstrating considerable correlations between ELISA and Luminex for seven cytokines (IL-1β, IL-4, IL-5, IL-6, IL-10, IFN-gamma and TNF-alpha), fair correlations for IL-13, and a poor correlation in the case of IL-12.

A larger study of more than 2000 serum specimens, compared multiplex bead based assays with ELISA to determine its potential as a replacement for ELISA techniques, finding that the multiplex results were, on average, 2.36-fold higher than ELISA values (Ray et. al 2005).

As a solution, using identical capture and reporter antibodies, similar diluents and serum blockers will minimize the deviations between the two methods. As these factors also vary between different vendors of multiplex bead assay kits, multiplex bead assay kits should not be considered interchangeable (Elshal et. al 2007)

By thorough consideration of the above variables, it is possible to use multiplex bead array assays in place of ELISAs particularly for research demanding high throughput analysis of numerous analytes.

Throughout our research BD Cytometric Bead Array (CBA) Human inflammatory assay was used to quantify the cytokine concentrations.
<table>
<thead>
<tr>
<th>Name of Drug</th>
<th>Marine source</th>
<th>Target Disease</th>
<th>Nature of compound</th>
<th>Molecular weight (g/mol)</th>
<th>Molecular formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manoalide</td>
<td>South Pacific sponge, Luffariella variabilis</td>
<td>Anti-inflammatory and analgesic properties in addition to its antileukemic ...</td>
<td>Sesterterpenoid</td>
<td>416.55034</td>
<td>C25H36O5</td>
</tr>
<tr>
<td>IPL-576092</td>
<td>Marine sponge Petrosia contignata</td>
<td>Antiasthma</td>
<td>Contignasterol (polyhydroxylated sterols)</td>
<td>1157.40804</td>
<td>C48H56N10O12S6</td>
</tr>
<tr>
<td>CGX-1007dictyodendrins</td>
<td>Fish-hunting snail, Conus geographus</td>
<td>Ischemic and excitotoxic brain injury, neuronal apoptosis, pain, epilepsy, ...</td>
<td>17 amino acid peptide Conantokin</td>
<td>2264.21</td>
<td>-</td>
</tr>
<tr>
<td>GTS-21 (aka DMBX)</td>
<td>Amphiporous lactiflores</td>
<td>Alzheimer’s disease, nicotine dependence, and, most significantly, for ...</td>
<td>3-(2,4-dimethoxy-benzylidene) anabaseine</td>
<td>308.374</td>
<td>C19H20N2O2</td>
</tr>
<tr>
<td>CGX-1160</td>
<td>Conus Geographus</td>
<td>Pain and epilepsy</td>
<td>-</td>
<td>2038.20926</td>
<td>C89H144N20O34</td>
</tr>
<tr>
<td>Ascididemnin</td>
<td>Didemnum sp. tunicate</td>
<td>Colon cancer, breast cancer and leukemia</td>
<td>Pyridoacridine alkaloid</td>
<td>283.28356</td>
<td>C18H9N3O</td>
</tr>
<tr>
<td>Salicylihalimides A</td>
<td>Marine sponge Haliclona</td>
<td>Melanoma</td>
<td>Benzo lactone enamide</td>
<td>439.544</td>
<td>C26H33NO5</td>
</tr>
<tr>
<td>Thiocoraline</td>
<td>Micromonospora sp from marine soft coral in indian ocean from ...</td>
<td>Anti tumor</td>
<td>Cyclic thiodepsipeptide and 2-fold symmetric or pseudosymmetric bicy cyclic octade ...</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Compound</strong></td>
<td><strong>Origin</strong></td>
<td><strong>Cytotoxicity</strong></td>
<td><strong>Molecular Formula</strong></td>
<td><strong>Molecular Weight</strong></td>
<td></td>
</tr>
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<td>----------------</td>
<td>----------------------------------------------------------------------------</td>
<td>------------------------------------------------------------</td>
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<td></td>
</tr>
<tr>
<td>Sarcodictyin</td>
<td><em>Sarcodictyon Roseum</em>, <em>Eleutherobia aurea</em>, <em>Bellonella albiflora</em></td>
<td>Ovarian cancer, leukemia</td>
<td>2,11-cyclized</td>
<td>C28H36N2O6</td>
<td></td>
</tr>
<tr>
<td>Eleutherobin</td>
<td><em>Eleutherobia species</em> (possibly <em>E. albiflora</em>, <em>Alcyonacea</em>, <em>Alcyoniidea</em>)</td>
<td>Various types of cancer</td>
<td>Diterpene glycoside</td>
<td>656.76302</td>
<td></td>
</tr>
<tr>
<td>Peloruside A</td>
<td><em>Mycale hentscheli</em></td>
<td>Mainly ovarian cancer</td>
<td>Macrolide (organic heterobicyclic)</td>
<td>548.66342</td>
<td></td>
</tr>
<tr>
<td>Diazonamide</td>
<td><em>Colonial ascidian</em> <em>Diazza angulata</em></td>
<td>Cancer</td>
<td>Single atropisomer</td>
<td>765.64056</td>
<td></td>
</tr>
<tr>
<td>Curacin A</td>
<td><em>Lyngya Majuscula</em></td>
<td>Renal colon and breast cancer</td>
<td>Jamaicamide,</td>
<td>373.5951</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>mupriocin</em>, and</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>pederin</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lauimalide</td>
<td><em>Cacospongia mycofijiensis</em></td>
<td>Cancer</td>
<td>Macrolide</td>
<td>514.65028</td>
<td></td>
</tr>
<tr>
<td>Vitilevuamide</td>
<td><em>Didemnum cuculiferum</em> and <em>Polysyncranton lithostrotum</em></td>
<td>Anti tumour and anti cancer</td>
<td>Bicyclic 13 amino acid peptide</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>KRN-7000</td>
<td>Okinawan marine sponge <em>Agelas mauritianus</em>. KRN7000 is no longer extracted from the native source, but</td>
<td>Anti tumor activity infectious diseases, autoimmune disease and graft versus host disease</td>
<td>Agelasphins (glycosphingolipids),α-galactosylceramide</td>
<td>858.322</td>
<td></td>
</tr>
<tr>
<td><strong>Squalamine</strong></td>
<td>Squalus aquanthius</td>
<td>Eye disorders, ovarian cancer continue</td>
<td>Aminosterol (steroidal Alkaloid)</td>
<td>627.962</td>
<td>C34H65N3O5S</td>
</tr>
<tr>
<td><strong>HTI-286 (hemiasterlin derivative)</strong></td>
<td>Hemiasterella minor</td>
<td>Prostate cancer</td>
<td>Tripeptide</td>
<td>473.64802</td>
<td>C27H43N3O4</td>
</tr>
<tr>
<td><strong>ES-285 (spisulosine)</strong></td>
<td>Spisula polynyma or polynoma</td>
<td>Antitumour activity variety of malignancies, ranging from hematological malignancies to solid tumors</td>
<td>Phospholipid sphingosine</td>
<td>285.50836</td>
<td>C18H39NO</td>
</tr>
<tr>
<td><strong>Discodermolide</strong></td>
<td>Discodermia dissoluta</td>
<td>Breast, ovarian and colon cancer</td>
<td>Polyketide</td>
<td>593.7917</td>
<td>C33H55NO8</td>
</tr>
<tr>
<td><strong>E7389 (erybulin mesylate)</strong></td>
<td>In the Halichondria genus of sponges</td>
<td>Breast cancer, osteosarcoma</td>
<td>Macrocyclic ketone (synthetic) halichondrin B which is a polyether macroalide(natural)</td>
<td>826.00222</td>
<td>C41H63NO14S</td>
</tr>
<tr>
<td><strong>Kahalalide F</strong></td>
<td>Elysia Rufescens (but might get it from the feed algae)</td>
<td>Cancer and psoriasis</td>
<td>Cyclic depsipeptide</td>
<td>1477.87126</td>
<td>C75H124N14O16</td>
</tr>
<tr>
<td><strong>ILX 651 (Tasidotin Hydrochloride)</strong></td>
<td>an orally active synthetic microtubule-targeted derivative of the marine depsipeptide dolastatin-15</td>
<td>Breast cancer</td>
<td>Pentapeptide</td>
<td>643.30106</td>
<td>C32H59ClN6O5</td>
</tr>
<tr>
<td><strong>Bryostatin 1</strong></td>
<td>Bryozoan</td>
<td>Anti-cancer and Anti-AIDS/HIV</td>
<td>Macroyclic lactone</td>
<td>905.03262</td>
<td>C47H68O17</td>
</tr>
<tr>
<td><strong>Cryptophycins (also arenastatin A)</strong></td>
<td>Cyanobacteria Nostoc sp.</td>
<td>-</td>
<td>Macrolide</td>
<td>606.70588</td>
<td>C34H42N2O8</td>
</tr>
<tr>
<td>--------------------------------------</td>
<td>-------------------------</td>
<td>---</td>
<td>------------</td>
<td>-------------</td>
<td>-----------</td>
</tr>
<tr>
<td><strong>Aplidine</strong></td>
<td>Aplidium albinicans</td>
<td>Pancreatic, stomach, bladder, and prostate cancers-Antitumor Anti leukemic</td>
<td>Peptide (cyclic depsipeptide)</td>
<td>1110.33858</td>
<td>C57H87N7O15</td>
</tr>
<tr>
<td><strong>Girolline</strong></td>
<td>Cymbastela cantharella</td>
<td>Anti tumour</td>
<td>2-aminoimidazole derivative</td>
<td>190.63074</td>
<td>C6H11CIN4O</td>
</tr>
<tr>
<td><strong>Ecteinascidin 743</strong></td>
<td>Ecteinascidia turbinata</td>
<td>Ovarian cancer</td>
<td>Tetrahydroisoquinoline alkaloid</td>
<td>761.83722</td>
<td>C39H43N3O11S</td>
</tr>
<tr>
<td><strong>Soblidotin</strong></td>
<td>Dollabella Auricularia</td>
<td>Anti tumour and vascular</td>
<td>Peptide</td>
<td>701.97918</td>
<td>C39H67N5O6</td>
</tr>
<tr>
<td><strong>Dolastatin-10</strong></td>
<td>Sea hare Dolabella auricularia, and we now report its isolation from the marine cyanobacterium Symploca sp. VP642 from Palau</td>
<td>Anti cancer</td>
<td>Pentapeptide</td>
<td>785.09092</td>
<td>C42H68N6O6S</td>
</tr>
</tbody>
</table>
### Table 2.2: Immunomodulatory compounds from marine species

<table>
<thead>
<tr>
<th>Marine Source</th>
<th>Immunomodulatory Compound(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Shark</strong></td>
<td>Shark Liver Oil, Shark Cartilage, Squalamine</td>
</tr>
<tr>
<td><strong>Red Seaweed</strong></td>
<td>peptides and polysaccharides</td>
</tr>
<tr>
<td><strong>Marine Fungus: Phoma Herbarum</strong></td>
<td>YCP Alpha-D-glucan (polysaccharides of D glucose monomer)</td>
</tr>
<tr>
<td><strong>Kuruma Shrimp</strong></td>
<td>Lactic Acid Bacteria from shrimp intestine</td>
</tr>
<tr>
<td><strong>Fermented marine Alga</strong></td>
<td>Bacillus subtilis</td>
</tr>
<tr>
<td><strong>Brown Algae</strong></td>
<td>Fucoidan</td>
</tr>
<tr>
<td><strong>Hydrothermal Vent bacteria</strong></td>
<td>Over-sulphated low molecular exopolysaccharide</td>
</tr>
<tr>
<td><strong>Oyster (Crassostrea hongkongensis)</strong></td>
<td>Peptides OPENF</td>
</tr>
<tr>
<td><strong>Marine Bacteria: Pseudoalteromonas strains</strong></td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td><strong>Marine microalgae: Sarcinochrysis marina Geitler</strong></td>
<td>Degraded polysaccharide (SMP)</td>
</tr>
<tr>
<td><strong>Marine algae Solieria filiformis (SfL), Pterocladiella capillacea (PcL) and Caulerpa cupressoides (CcL)</strong></td>
<td>Lectins</td>
</tr>
<tr>
<td><strong>fish oil</strong></td>
<td>n-3 polyunsaturated fatty acids</td>
</tr>
<tr>
<td><strong>Jellyfish: Chironex fleckeri</strong></td>
<td>Cnidarians</td>
</tr>
<tr>
<td><strong>Red Algae: Halopithys incurva and Hypnea spinella</strong></td>
<td>Polysaccharides</td>
</tr>
<tr>
<td><strong>Brown Algae</strong></td>
<td>Hyposulfated (hypoS), deacetylated (deAc), and both hyposulfated and deacetylated (hypoSdeAc) derivatives of native Fucus evanescens fucoidan</td>
</tr>
<tr>
<td><strong>Marine sponges</strong></td>
<td>Callyspongidiol and 14,15-dihydrorhodisponodiol are Polyacetylenediols</td>
</tr>
<tr>
<td>--------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>Marine snail: Telescopium telescopium</strong></td>
<td>Cytosol fraction of spermatheca and/or the ovotestis</td>
</tr>
<tr>
<td><strong>red alga: Mastocarpus stellatus</strong></td>
<td>Floridoside</td>
</tr>
<tr>
<td><strong>Marine algae</strong></td>
<td>Sulfated polymannuroguluronate (SPMG)</td>
</tr>
<tr>
<td><strong>Fresh water blood cyanobacterium</strong></td>
<td>Microcystins (MCs)</td>
</tr>
<tr>
<td><strong>Marine fish oil</strong></td>
<td>Polyunsaturated fatty acid n-3</td>
</tr>
<tr>
<td><strong>Bugula neritina</strong></td>
<td>Bryostatin 1</td>
</tr>
<tr>
<td><strong>Tunicates: Didemnidae</strong></td>
<td>Didemnins</td>
</tr>
<tr>
<td><strong>Lichen</strong></td>
<td>Polysachcharides</td>
</tr>
<tr>
<td><strong>Starfish</strong></td>
<td>Five new steroid glycosides, luzonicosides B-E</td>
</tr>
<tr>
<td><strong>Limpet: Fissurella latimarginata</strong></td>
<td>Hemocyanin</td>
</tr>
<tr>
<td><strong>Gastropod: Rapana thomasiana</strong></td>
<td>Hemocyanin</td>
</tr>
<tr>
<td><strong>Styela Clava</strong></td>
<td>(1) Cholesteric-7-en-3 beta-ol, (2) cholesteric-4-en-3 beta,6 beta-diol, (3) cholesterol, (4) batilol, and (5) ceramide</td>
</tr>
<tr>
<td><strong>Soft coral: Lobophytum crassum</strong></td>
<td>Immunomodulatory effects of marine emembrane compounds, (9E,13E)-5-acetoxy-6-hydroxy-9,13-dimethyl-3-methylene-3,3a,4,5,6,7,8,11,12,14a-decahydro-2H-cyclotrideca[b]furan-2-one, (9E,13E)-5-acetoxy-6-acetyl-9,13-dimethyl-3-methylene-3,3a,4,5,6,7,8,11,12,14a-decahydro-2H-cyclotrideca[b]furan-2-one, lobocrassin B, (--) 14-deoxycrassin, cembranolide B and 13-acetoxyxsaroccrassolide</td>
</tr>
</tbody>
</table>
CHAPTER 3:

Materials and Methodology
3.0 Introduction

Naturally derived compounds as potential drug candidates have been growing increasingly in research interest throughout the world. Particular interest lies in marine derived compounds, since the pool of a staggering 700,000 species rests in the marine world and varied spectrum of potential compounds promises healing and disease prevention, largely due to their successful existence over millions of years despite inhabitancy with harmful viruses and bacteria present in the marine environment.

A valuable nature of certain marine-derived compounds is their immunomodulatory properties. This is mainly not only due to the lack of abundance in immunomodulatory synthetic drugs in the market, but also due to the near negligible side effects of marine-derived drugs (Nair et. al, 2015). Additionally, the cost of such compounds is much lower than synthetic compounds. Immunomodulatory drugs can be used to treat a broad range of major chronic diseases such as: Alzheimers disease, diabetes, Parkinson’s disease, multiple sclerosis, herpes simplex infections, HIV/AIDs, etc. Thus, the vast need for immunomodulatory drugs in the medical field, combined with much lessened cases of side effects from marine derived compounds and their low cost attributes, the benefits are extraordinary.

In this study, we focus on the immunomodulatory properties of the hemolymph from the abalone species *Haliotis rubra*. Immunomodulatory effects are assessed on human immune response *in vitro* in three different cell lines. The level of cytokine modulation acts as the indication of immunomodulation effectiveness.
3.1 Materials

HaCat cells (spontaneously immortalized human keratinocytes) were kindly provided by Junlae Cho (Department of Pharmacy, University of Sydney) and they were propagated in Gibco Dulbecco’s Modified Eagle’s Medium (DMEM Glutamax) supplemented with 10% Fetal Bovine Serum (FBS). PC-3 cells were kindly provided by my colleague Mr. Balakrishna Shammugaswami and they were propagated in Gibco RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS). Both the media along with DPBS Buffer solution were sourced from ThermoFisher while the FBS was supplied by Sigma-Aldrich. Human Oral Primary Keratinocytes (HOK Cells) were purchased from ABMGood (local agent: Resolving images Australia) and they were propagated in the PriGro X cocktail medium on poly-l-lysine coated T75 flasks sourced from the same source. Cytotoxicity was assessed with Cell Counting Kit (CCK-8 reagent) supplied by Sigma Aldrich. Cytokine level changes were determined with the use of BD Human inflammatory Cytokine Cytometry Beads Array (CBA) kit, Calibrite 3 BEADS for flowcytometer and FCAP Array software that were all purchased from BD Biosciences Australia. For propagating the primary keratinocytes Trypsin Neutralizing Solution (TNS) was purchased from Lonza, 0.1 M pH 7.4 Tris Buffer solution was sourced from Astral Scientific. Poly-l-lysine solution bioreagent 0.01% (mol wt 70,000-150,000 Da) were used for coating to aid cell adhesion and was supplied from Sigma Aldrich. Trypsin and DMSO required for cell propagation were also purchased from Sigma Aldrich. Trypan Blue (Gibco) was used during cell counting along Qbit assays from Invitrogen were used for protein concentration quantification. Fresh abalone sera was kindly provided by Marine Biotechnologies Australia which was then centrifuged in Biofuge Stratos sterilized with Millipore 0.22μm vacuum driven filtration. Purification of the hemocyanin was achieved with Ultra-4 Centrifugal Filter Units (100 KDa, Millipore).
Table 3.1: Cell culture protocol followed for HaCat cells, Human Oral Keratinocytes and PC-3 cells in propagation, passage and freezing.

<table>
<thead>
<tr>
<th></th>
<th>HaCat Cells</th>
<th>Human Oral Keratinocytes</th>
<th>PC-3 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Propagation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Media Used</td>
<td>DMEM</td>
<td>PriGroX</td>
<td>RPMI 1640</td>
</tr>
<tr>
<td>% FBS in Media</td>
<td>10</td>
<td>Serum-free</td>
<td>10</td>
</tr>
<tr>
<td>Type of flask used</td>
<td>T75</td>
<td>2 μg/cm2 Poly-l-lysine coated T75</td>
<td>T75</td>
</tr>
<tr>
<td>% Anti-biotic</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vol of media per</td>
<td>14</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>flask</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seeding cell density</td>
<td>1 x 10⁶</td>
<td>1 x 10⁶</td>
<td>1 x 10⁶</td>
</tr>
<tr>
<td>Cell adhesion to flask</td>
<td>12 hours</td>
<td>10-12 days</td>
<td>12 hours</td>
</tr>
<tr>
<td>Cell confluence</td>
<td>48 hrs</td>
<td>14 days</td>
<td>48 hrs</td>
</tr>
<tr>
<td><strong>Passage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Confluence at</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>passage</td>
<td>70%</td>
<td>90%</td>
<td>70%</td>
</tr>
<tr>
<td>Passage frequency</td>
<td>2.5 days</td>
<td>14 days</td>
<td>2.5 days</td>
</tr>
<tr>
<td>Wash Buffer</td>
<td>DPBS</td>
<td>DPBS</td>
<td>DPBS</td>
</tr>
<tr>
<td>Trypsin Volume</td>
<td>2 ml</td>
<td>2 ml</td>
<td>2 ml</td>
</tr>
<tr>
<td>Trypsin Dilution?</td>
<td>no</td>
<td>diluted in 8 ml DPBS</td>
<td>no</td>
</tr>
<tr>
<td>Trypsin Neutralizer?</td>
<td>only media</td>
<td>Trypsin Neutralizing Solution (TNS)</td>
<td>only media</td>
</tr>
<tr>
<td>Passage ratio</td>
<td>1:3</td>
<td>1:2</td>
<td>1:3</td>
</tr>
<tr>
<td><strong>Freezing</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell density</td>
<td>1 x 10⁶</td>
<td>1 x 10⁶</td>
<td>1 x 10⁶</td>
</tr>
<tr>
<td>Freezing Media</td>
<td>20% FBS &amp; 10%DMSO</td>
<td>70% Media, 20% FBS and 10% DMSO</td>
<td>20% FBS &amp; 10%DMSO</td>
</tr>
</tbody>
</table>
3.2 Methodology

3.2.1 Cell Culture Protocol

Cell culture protocols of HaCat and PC-3 cells were similar and followed the regular procedure for propagation, passage and freezing. Human Oral Keratinocytes, however required a more sensitive procedure. Poly-l-sine coated T75 flasks were required for propagating these cells, Trypsin was required to be diluted with DPBS2:8 ratio, and trypsin neutralizing solution was required to deactivate the trypsin following 1 to 2 minute incubation. The deactivated cell suspension solution in the passage was transferred to 5ml FBS prior to centrifuge and final transfer to PriGroX Media.

3.2.2 Coating of Cell culture plates with Poly-l-lysine

While Poly-l-lysine coated T75 flasks were purchased (ABM Good), cell culture 6 well plates and 96 well plates needed to be manually coated for the Human Primary Oral Keratinocytes plate attachment. The required concentration of Poly-l-lysine was 2 μg / cm². This was done 24 h prior to cell seeding.

For 6 well plates:

384 μL of poly-l-lysine solution was added to each well and gently rocked to ensure entire surface area was covered per well.

For 96 well plates:

14 μL of poly-l-lysine solution was added to each well and gently rocked to ensure entire surface area was covered per well.
The plates were then incubated for 5 minutes followed by complete aspiration. Each well was thoroughly rinsed with sterile tissue culture grade water and allowed to dry for at least 2 hours before use.

3.2.3 Determination of required seeding cell density of HaCat cells per well in 6 well plates:

200 µl pipette tips were autoclaved for sterilization and used to seed passaged HaCat cells in triplicates of varying concentrations in the range 10,000, 20,000, 30,000, 40,000, 50,000, 100,000, 150,000 200,000, 250,000 and 300,000 cells per well in four 6 well plates. The cell confluence was observed and reported for 72 hours to determine the appropriate seeding cell density with 90% confluence at 72 hour mark. This was required according to the timing of subsequent experiments.

3.2.4 Cytotoxicity Test standard curve

Cells were recovered from T75 culture flask after trypsinization, counted with a hemocytometer and the concentration adjusted to 5 x 10^4 cells per ml. 100 µl of media was added to each well of 96 well plates and triplicate serial dilutions of variable cell concentrations were performed (2.5x10^4, 1.25x10^4 and 6.25x10^3). The 96 well plate was then incubated for a period of 24 and 48 hours in a CO2 incubator. 10 µl of Cell Counting Kit- 8 (CCK-8) was added to each well. The microplates were incubated for 1 h, 2 h, 3 h and 4 h time slots. Using spectrophotometer, colorimetric reading was taken using a filter for 450 nm. The optimum seeding cell density for each cell type was determined. The optimum CCK-8 incubation time was also obtained from the standard curve generated (absorbance vs number of cells).

3.2.5 Determination of the cytotoxicity of Abalone sera, purified hemocyanin and permeate on HaCat cell line and PC-3 cell line

The effect of AH, sera and sera permeate on cell viability was assessed using CCK-8
assay to determine the highest concentration, which could be tolerated by the HaCat cells and PC-3 cells in the subsequent assay. The experimental set up of the plates is illustrated in Fig. 3.1.

Cells were recovered from T75 culture flask after trypsinization, counted with a hemacytometer and the concentration adjusted according to findings from the standard curve. 100 µl of media was added to each well of 96 well plates containing triplicates of 2000 cells per well. The 96 well plate was then incubated for a period of 24 hrs to allow the cells to attach to the plate. The media was then discarded, and 100 µl fresh media was added to each well, including triplicate of negative control wells. 10 µl of test extract (sera, purified hemocyanin and permeate) were added in variable concentrations, and incubated for set periods (depending on the experiment performed ranging from 30 mins to 72 hrs). 10 µl of Cell Counting Kit-8 (CCK-8) was added to each well and incubated for 1 h, followed by absorbance reading in the spectrophotometer at 450 nm.

![Figure 3.1: Experimental setup of cytotoxicity determination: bird’s eye view of the 96 well plate.](image)
3.2.6 Extraction of Fresh Sera (hemolymph) from the Abalone

*Haliotis rubra* (black lipped abalone) was meticulously selected and those without scars and signs of disease were gathered from Tasmanian coastline bays by Marine Biotechnologies Australia Pty Ltd, followed by 5 days of conditioning in their factory, in recirculating salt water system (in 14 °C and 33 parts per thousand salinity). Prior to extraction of sera, prevention of bacterial contamination was ensured through spraying of the abalone with 0.2 μm filtered salt water. The animal was then rested at an angle of 30 ° to allow for drainage of excess water.

The abalone was then positioned shell side down at a 45° angle, 400 mm height above the extraction surface. The extraction was performed with a Terumo Surflo LV catheter with dimensions 14 g x 1.73 id x 51 mm, which was inserted at a long axis angle of 40° behind the abalone’s neck. The soft outer catheter plastic was left behind as the internal needle of the catheter was removed immediately. The outer catheter was then gradually withdrawn until the cephalic arterial sinus was intersected. Around 10 mL hemolymph was drained to waste, and a sterile silicon tube was attached to the catheter’s end to act as the transport vessel to a sterile container. Approximately 150 mL of hemolymph can be safely collected without causing mortality to the animal (Dr. Valtchev, 2015, priv. comm.).

3.2.7 Filtration and Sterilization of the Fresh Sera (hemolymph)

Upon reaching Professor Dehghani’s lab at Sydney University, the hemolymph was then centrifuged (Biofuge stratos) at 4000×g for 15 min to remove hemocytes and debris. The hemolymph was sterilised using vacuum driven filtration (0.22μm, Millipore) and stored at 4°C for experimental use. Sterilization was required to prevent any bacterial contamination that may degrade hemocyanin.
### 3.2.8 Purification of Abalone Hemocyanin using ultrafiltration

Ultrafiltration was performed using Ultra-4 Centrifugal Filter Units (100 KDa, 4 mL, Millipore) (Illustrated in Fig. 3.2).

4 mL sterile grade water was centrifuged at 4000 x g for 3 minutes at 4°C to wash the filter and to let the centrifuge reach the low temperature setting. The water was then discarded and 4 mL sera was centrifuged at 4000 x g for 20 minutes at 4°C. The permeate was then collected and protein concentration determined using Qubit assay. The retentate (purified hemocyanin) was topped up with Tris Buffer (0.1 M 7.4 pH) to match the initial total volume of 4 mL. Tris buffer was used because of its useful buffer range of pH 7-9, which mimics the physiological pH typical of most living organisms.

The protein concentration was determined using Qbit assay. Table 3.2 notes the dilution measurements for each concentration of extracts. Subsequent dilutions of the purified hemocyanin and permeate (containing smaller peptides) were performed using Tris buffer (either 4 mL or 2 mL) to achieve various concentrations of the purified hemocyanin and permeate. All samples were incubated on ice to prevent protein disintegration prior to experiments.

Table 3.2: Dilutions of the purified hemocyanin and permeate following 20 minute centrifugation at 4000 x g, 4°C

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Vol. of Tris buffer added for dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Purified hemocyanin</strong></td>
<td></td>
</tr>
<tr>
<td>2.3 x 10^3</td>
<td>4 mL</td>
</tr>
<tr>
<td>1.3 x 10^3</td>
<td>8 mL</td>
</tr>
<tr>
<td><strong>Permeate</strong></td>
<td></td>
</tr>
<tr>
<td>556</td>
<td>0 mL</td>
</tr>
<tr>
<td>398</td>
<td>4 mL</td>
</tr>
<tr>
<td>63</td>
<td>6 mL</td>
</tr>
</tbody>
</table>
Tris Buffer added to dilute permeate

Abalone Hemocyanin (AH)

Hemolymph permeate containing small peptides

Tris Buffer added to achieve initial volume

Tris Buffer added to dilute hemocyanin
Figure 3.2: Partial purification and dilution of hemocyanin by ultrafiltration using filters with Molecular weight cut off of 100 kDa (used to separate the hemocyanin molecules which are much larger in size than this cut off) and Tris buffer. Impurities were eluted with ultrapure water.

3.2.9 Determination of Protein concentration using Qubit

Qubit assay (Invitrogen) was found to be the most appropriate (reproducible and accurate) method of protein quantification compared to Bradford assay and BCA, according to Dr. Zanjani (Thesis, 2014). Therefore protein concentration quantification was performed using Qubit, which yields a fluorometric quantitation of protein content. Measurements are based on the signals emitted when the fluorescent dye binds to the protein. It is a highly sensitive assay.

Three assay tubes were set up for standards, and one tube for each sample i.e three for permeate samples and two for hemocyanin samples. Qubit reagent was diluted 200 times in Qubit buffer solution to prepare the working solution. Three pre-diluted BSA standards (Invitrogen) were used for the calibration of instrument (Qubit® 2.0 Fluorometer, Invitrogen). The dilution measurements are outlined in Table 3.3 below.

Various dilutions of protein samples according to (Table: Dilution of the purified hemocyanin and permeate following 20 minute centrifugation at 4000 x g, 4°C) were further diluted in 0.5-mL optical-grade real-time PCR tubes, with the working solution according to the following table and incubated in dark at room temperature for 15 minutes after vortexing all tubes for 2 to 3 seconds.
Table 3.3: Dilutions carried out on variable concentration samples of purified AH and permeate to assess the concentration using Qubit 2.0 Fluorometer.

<table>
<thead>
<tr>
<th></th>
<th>Hemocyanin 1</th>
<th>Hemocyanin 2</th>
<th>Permeate 1</th>
<th>Permeate 2</th>
<th>Permeate 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vol. of working solution</strong></td>
<td>198 μL</td>
<td>198 μL</td>
<td>190 μL</td>
<td>190 μL</td>
<td>195 μL</td>
</tr>
<tr>
<td><strong>Vol. of sample</strong></td>
<td>2 μL</td>
<td>2 μL</td>
<td>10 μL</td>
<td>10 μL</td>
<td>5 μL</td>
</tr>
<tr>
<td><strong>Total Vol.</strong></td>
<td>200 μL</td>
<td>200 μL</td>
<td>200 μL</td>
<td>200 μL</td>
<td>200 μL</td>
</tr>
</tbody>
</table>

Using the Qubit fluorometer, the absorbance values of standards and samples were measured. The concentrations of the protein samples containing hemocyanin and permeates were then calculated using the following equation:

\[
\text{Concentration of Protein (μg/mL)} = \text{Absorbance Value} \times 20 \times 100
\]

**3.2.10 Incubation with the test extracts**

Immunological effects of the abalone sera, purified hemocyanin and small peptide containing permeate were to be observed on various cell types including Human oral primary keratinocytes, HaCat Cells and PC-3 Prostate cancer cells. This required various experimental designs to find the optimum incubation time, type of extracts and concentration. We tested for various parameters in the initial experiments to optimize the experimental design for highest practically obtainable cytokine yield from treatment with extracts. Varying the dosage gave us an ideal dosage % of sera, high cell density helped us obtain the appropriate in vitro model and the different time points indicated the optimum treatment time in vitro. In the results section of these treatment experiments the error bars are ± SD. Some of the treatments are
conducted in n=3, while others are n=2 and n=1. This depended on the importance of the treatment and dose dependent effects noted.

3.2.10.1 Incubation of test extracts with HaCat Cells

HaCat cell line was used to develop the protocol for test extract incubation and cytokine detection.

(i) Short time span incubation of low cell density wells with low concentration range sera:

Passage 15 cells (passaged from 70% confluent T75 cell culture flasks) were counted using a hemocytometer and seeded at 3200 cells per well of 12 well plate dishes and allowed to adhere to the plate for 24 hours. Sera concentrations of 1%, 5%, 10% and 20% v/v were injected and incubated for 1 h, 2 h and 5 h. Serum free media was used as triplicate controls.

(ii) Instantaneous immune response with low cell density and fixed concentration of sera

Passage 17 cells (passaged from 70% confluent T75 cell culture flasks) were counted using a hemocytometer and seeded at 3200 cells per well of 12 well plate dishes and allowed to adhere to the plate for 24 hours. Sera concentration of 5% v/v were injected in triplicate wells and incubated for 15 min, 30 min and 45 min. Undisturbed control was used to prevent immune reaction of cells caused by turbulence of the surrounding. Two independent experiments were conducted in triplicates.
(iii) High cell density, low sera concentration and short time span study of immune response

Passage 15 cells (passaged from 70% confluent T75 cell culture flasks) were counted using a hemocytometer and seeded at 135,000 cells per well of 6 well plate dishes and allowed to adhere to the plate for 24 hours. Sera concentration of 2% v/v were injected in triplicate wells and incubated for 30 min, 1.5 h and 2.5 h. Serum free media was used as control. Additional undisturbed controls were also used. Two independent experiments were conducted in triplicates.

(iv) High cell density, high sera concentrations and long incubation time study of immune response

Passage 15 cells (passaged from 70% confluent T75 cell culture flasks) were counted using a hemocytometer and seeded at 135,000 cells per well of 6 well plate dishes and allowed to adhere to the plate for 24 hours. Sera concentrations of 10% and 20% v/v were injected in triplicate wells and incubated for 16 h. Serum free media was used as control. Two independent experiments were conducted in triplicates.

(v) High cell density, purified hemocyanin test extract and long incubation time study of immune response

Passage 15 cells (passaged from 70% confluent T75 cell culture flasks) were counted using a hemocytometer and seeded at 135,000 cells per well of 6 well plate dishes and allowed to adhere to the plate for 24 hours. Purified hemocyanin (using ultrafiltration according to the protocol stated previously) at concentration of 20% v/v was
injected in triplicate wells and incubated for 16 h. Serum free media was used as control. Two independent experiments were conducted in triplicates.

(vi) High cell density, ultrafiltered sera permeate test extract and long incubation time study of immune response

Passage 15 cells (passaged from 70% confluent T75 cell culture flasks) were counted using a hemocytometer and seeded at 135,000 cells per well of 6 well plate dishes and allowed to adhere to the plate for 24 hours. Ultrafiltered hemolymph permeate at concentration of 20% v/v was injected in triplicate wells and incubated for 16 h. Serum free media was used as control. Two independent experiments were conducted in triplicates.

(vii) 6 day dosage basis incubation with high sera concentrations – study of immune response

Passage 15 cells (passaged from 70% confluent T75 cell culture flasks) were counted using a hemocytometer and seeded at 35,000 cells per well of 6 well plate dishes and allowed to adhere to the plate for 24 hours. 10% and 20% v/v sera concentration were injected each day, incubated for 1 to 2 hrs. The media (including the test extract) was then removed completely and replaced with fresh media. This was repeated for 6 days, while the 6th day incubation time was 5 h. Experimental procedure for this experiment is illustrated in Fig. 3.3 below.
3.2.10.2 Incubation of test extracts with Prostate Cancer Cells (PC-3)

Only 1 condition for PC-3 cells as PC-3 cells are very sensitive to the extracts and only low treatment time of 5 hrs was sufficient for cytokine yield.

(i) High cell density, ultrafiltered sera permeate test extract and short incubation time study of immune response

Passage 13 cells (passaged from 70% confluent T75 cell culture flasks) were counted using a hemocytometer and seeded at 240,000 cells per well of 6 well plate dishes and allowed to adhere to the plate for 24 hours. Ultrafiltered hemolymph permeate at concentration of 20% v/v, purified hemocyanin at concentration of 20% v/v and abalone hemolymph at concentration of 20% v/v were injected in triplicate wells and incubated for 5 h. Serum free media was used as control. Two independent experiments were conducted in triplicates.
3.2.10.3 Incubation of test extracts with Human Primary Oral Keratinocytes

In this case, we have already optimized the experimental conditions for 1 type of keratinocytes (HaCat cells), hence we used the optimized condition of high cell density, 16 hr treatment time and maximum 20% sera conc.

(i) High cell density, high sera concentrations and long incubation time study of immune response

Passage 3 cells (passaged from 90% confluent Poly-l-lysine coated T75 cell culture flasks) were counted using a hemocytometer and seeded at 125,000 cells per well of poly-l-lysine coated 6 well plate dishes and allowed to adhere to the plate for 48 hours. Sera concentration 20% v/v were injected in triplicate wells and incubated for 16 h. Tris buffer was used as control. Two independent experiments were conducted in triplicates.

(ii) Dose dependent immune response effect of abalone sera

Passage 4 cells (passaged from 90% confluent Poly-l-lysine coated T75 cell culture flasks) were counted using a hemocytometer and seeded at 102,000 cells per well of poly-l-lysine coated 6 well plate dishes and allowed to adhere to the plate for 48 hours. Sera concentration 5%, 10%, 20% and 30% v/v were injected in triplicate wells and incubated for 16 h. Tris buffer was used as control.

(iii) Dose dependent immune response effect of purified hemocyanin

Passage 4 cells (passaged from 90% confluent Poly-l-lysine coated T75 cell culture flasks) were counted using a hemocytometer and
seeded at 102,000 cells per well of poly-l-lysine coated 6 well plate dishes and allowed to adhere to the plate for 48 hours. Purified hemocyanin concentrations $2.3 \times 10^3 \mu g/mL$ and $1.3 \times 10^3 \mu g/mL$ were injected in triplicate wells and incubated for 16 h. Tris buffer was used as control.

(iv) Dose dependent immune response effect of ultrafiltered hemolymph permeate

Passage 4 cells (passaged from 90% confluent Poly-l-lysine coated T75 cell culture flasks) were counted using a hemocytometer and seeded at 102,000 cells per well of poly-l-lysine coated 6 well plate dishes and allowed to adhere to the plate for 48 hours. Permeate concentrations 556 $\mu g/mL$, 398 $\mu g/mL$ and 63 $\mu g/mL$ were injected in triplicate wells and incubated for 16 h. Tris buffer was used as control.

3.2.11 Human Inflammatory Cytokine Beads Array

Immunological effects of the abalone sera, purified hemocyanin and small peptide containing permeate were to be observed on various cell types including Human oral primary keratinocytes, HaCat Cells and PC-3 Prostate cancer cells.

In order to quantify the immune response, Human Inflammatory Cytokine Beads Array was used to observe cytokine level changes pre and post incubation with the test extract. The CBA Kit quantitatively measures IL-8, IL 1 B, IL -6, IL -10, TNF and IL 12 protein levels. It provides a method of capturing a set of analytes with beads of known sizes and fluorescence, enabling it to detect analytes with flow cytometry.

A single capture bead is conjugated with specific antibody that forms a sandwich complex with the analyte and phycoerythrin detection reagent that provides a
fluorescent signal proportional to the amount of bound analyte. The six bead population has distinct fluorescence intensities and are coated with capture antibodies for IL-8, IL 1 B, IL -6, IL -10, TNF and IL 12 proteins. Fig 3.4 identifies each of the peaks of these cytokines.

![Fluorescence peaks of six different cytokines measured in all of the cytometric beads array human inflammatory cytokines assay conducted.](image)

**3.2.12 Materials for CBA Human Inflammatory Cytokine Kit**

The following materials were procured:

6 vials of capture beads for each detectable cytokine (0.8 mL each)
Human Inflammatory Cytokine PE Detection Reagent (4 mL)
Human Inflammatory Cytokine Standards (2 lyophilized vials)
Cytometer setup beads (0.5 mL)
PE Positive control detector (0.5 mL)
FITC Positive control detector (0.5 mL)
Wash Buffer (260 mL)
Assay Diluent (30 mL)
Serum Enhancement buffer (10 mL)
3.2.13 Preparation of Standards

A vial of lyophilized Human Inflammatory Cytokine standard was transferred to a 15 mL polypropylene tube, reconstituted with 2 mL Assay diluent and marked as Top Standard. This was equilibrated for 15 minutes and gently mixed with pipette.

12 x 75 mm tubes were labeled in descending order of standard concentrations: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256. The flow cytometric results of each of these standards are captured in Fig 3.6, where the increasing concentration is depicted by shifting of the peaks to the right.

300 μL of Assay Diluent was pipetted to each of the tubes and serial dilution performed by transferring 300 μL from the Top Standard to the 1:2 dilution tube. This was mixed thoroughly with pipette and 300 μL was transferred to the 1:4 tube and so on until 1:256 tube was serially diluted. One tube contained only the assay diluent and acted as negative control. Serial dilution procedure is illustrated in Fig. 3.5.

![Figure 3.5: Cytokine Standard serial dilution procedure with assay diluent.](image)

"300 μL 300 μL 300 μL 300 μL 300 μL 300 μL 300 μL 300 μL"

Top 1:2 1:4 1:8 1:16 1:32 1:64 1:128 1:256"
Figure 3.6: Shift of fluorescence peaks on six cytokine standards in accordance with concentration of the standard A. 0 pg/ml B. 20 pg/ml C. 40 pg/ml D. 80 pg/ml E. 156 pg/ml F. 312 pg/ml G. 625 pg/ml H. 1250 pg/ml I. 2500 pg/ml J. 5000 pg/ml
3.2.14 Preparation of the Assay

- Number of required assay tubes was determined according to the number of samples, controls and standards allocating 10 μL aliquot of each capture bead for each assay tube after vigorously vortexing. For instance, 24 samples meant 240 μL of each capture bead of the six. The tube was labeled “Mixed capture beads”, and was thoroughly vortexed.
- 50 μL of this mixed capture beads were added to all assay tubes including the standard tubes.
- 50 μL of each unknown sample (cell culture supernatant from test extract incubated cell and media) was added to appropriately labeled tube (e.g. 20% v/v sera incubation)
- 50 μL of Human inflammatory PE detection reagent was added to all assay tubes.
- The assay tubes were incubated in dark for 3 hours, followed by pipetting of 1 mL wash buffer in each tube and centrifugation at 200 x g for 5 minutes.
- It was then carefully aspirated and resuspended with 300 μL of Wash Buffer.

3.2.15 Flow Cytometer Setup using the Setup beads and Sample Acquisition

During the incubation, the flow cytometer setup was conducted following the procedure below:

100 μL of cytometer setup beads were added to a 12 x 75 mm sample acquisition tube and gently vortexed with 400 μL of wash buffer. The tube was loaded onto the cytometer under appropriate file and was acquired. The FSC and SSC voltages were adjusted until the singlet bead population fit the P1 gate in the template. The APC voltage was adjusted until the mean of P2 was 70,000. The PE voltage was adjusted until the mean of P3 was approximately 75. The FITC voltage was adjusted until the mean of P4 was approximately 75. The settings were then saved to catalog under
the cytometer settings option. The Assay tubes were then acquired in FACS Cantos II flow cytometer, after being thoroughly vortexed. The analysis was conducted using FCAP Array software and graphs generated using Microsoft Excel.

### 3.2.16 FCAP ARRAY Software

Analysis of BD CBA assay acquired on the FACS Canto II flow cytometer was performed using this software which was specifically designed for this assay. Upon creating a new experiment and loading the .fcs files from acquired samples from the flow cytometer, “standard” “sample” and “control” labels were assigned. Bead model was selected and Debris filtering was performed. Beads were assigned to each peak and manual clustering was performed if necessary. Quality Control was monitored and the standard graphs were generated. Raw data of “Fitted concentration of each analyte or sample” were reported. The raw data achieved was then processed in Excel to generate graphs showcasing the results of each experiment.

### 3.2.17 Statistical Analysis

The results of the experiments were expressed as means±S.D. Comparisons between groups were made using one-way ANOVA. Statistical significance was defined at a p-value of < 0.05.
CHAPTER 4:

Cytotoxicity Analysis on HaCat Cells & Prostate Cancer Cells
4.0 Introduction

Cytotoxicity and cell viability analysis was carried out to negate the effects of the sera, purified hemocyanin and sera permeate on cell survival rate. In numerous cases the cytotoxic nature of an extract triggers several immune responses, particularly in keratinocytes. It is important to prove that the concentration of extracts used during immune studies experiments is significantly lower than the CC50 of the extracts. (CC50 - cytotoxic concentration of the extracts at which 50% of the viable cells are dead). At CC50, there would be triggered immune responses that are not related to the anti-viral nature of the extracts. Without cytotoxicity test, these immune responses may be mistaken for cytotoxic effects of the extracts.

A more common reasoning of performing cytotoxicity test is to analyse the safe concentrations of use of the extracts: a concentration at which it targets the virus without causing harm to host cells.

Various incubation time periods and concentration of extract combinations were used in the cytotoxicity tests as outlined below. Cytotoxicity of abalone sera was measured with CCK-8 Assay on HaCat Cells, PC-3 Cells and Human oral primary keratinocytes.

To the best of our knowledge there are no available literature data on the cytotoxicity of abalone hemocyanin, abalone sera and abalone sera permeate on the particular cell lines of HaCat cells, Prostate cancer cell line PC-3 cells and Human Oral Keratinocytes. However, studies show the cytotoxic effects of few of the above extracts on related cell lines. Cytotoxicity of marine snail hemocyanin (Rapana thomasiana hemocyanin) with series of imidazolium-based amino acid ionic liquids [emim][AA] was tested on 3T3 murine fibroblasts (non-cancerous cells) and breast cancer cell line MCF-7 (Guncheva et. al 2016). It was found that the extract produced weak (10–15%) to moderate (15–20%) anti-proliferative effects toward MCF-7 and 3T3 cells. Additionally, HTB68 melanoma cell line proliferation was inhibited by 28% by KLH (McFadden et. al 2007) and the cell
viability of the bladder cancer cell lines 647 v, CAL 29 and T 24 after treatment with *Helix lucorum* hemocyanin (HlH) for 72 hours were 20.8 %, 59.2 % and 46.36 % respectively (Antonova et. al 2015). Dendritic cells pulsed with KLH showed a viability of 87.4 % (Teitz-Tennenbaum et. al 2008).

The cytotoxic effect of abalone hemocyanin, abalone sera and abalone sera permeate on keratinocytes and prostate cancer cell line will thus be a novel study.

4.1 Results

The cytotoxicity results are presented as absorbance values vs concentration of abalone sera. Absorbance is a direct indication of viability of cells (higher absorbance corresponds to higher cellular activity, hence higher cell viability). Hence viability vs concentration graph is directly comparable to the graph presented. In our case the graphs presented here are a better representation to avoid negative % and % above 100 that resulted from varying cellular proliferation between wells. Additionally there was no significant effect on cell viability in any case that would have resulted in a sensible viability graph by overcoming the varying proliferation issue.

4.1.1 The effect of abalone sera and purified AH on the viability of HaCat cells and PC-3 cells

Cytotoxicity of a range of low abalone sera concentrations (0.1 %, 0.5 %, 1 %, 2 %, 5 % and 10% v/v) was tested upon incubation of HaCat cells with the extract for 30 min, 3 h, 4 h and 6 h time frames. Cytotoxicity at low time period was of particular interest to mimic the short exposure time during the process of abalone sera oral ingestion.
Figure 4.1: Effect of variable concentrations (0.1%, 0.5%, 1%, 2%, 5% and 10% v/v) of abalone sera on the viability of HaCat cells, which were treated for 30 min, 3 hour, 4 hour and 6 hour. Absorbance detected at 450 nm.

The results in figure 4.1 show that no cytotoxicity is observed for this low extract concentration range and corresponding incubation time periods. As expected there was observation of the direct correlation between incubation time and absorbance readings due to cell proliferation and hence, increased cell density at each subsequent checkpoint. Interestingly, at 10% v/v sera concentration absorbance readings (which is a direct indication of cell viability) start to reduce at all incubation time periods. This might indicate a beginning of cytotoxic nature of the extract towards HaCat cells. Hence, much higher incubation time frames of 24 h was chosen to observe the effects of prolonged exposure to the extract on the HaCat cells. The low concentration parameters were kept constant, and in figure 4.2 no cytotoxicity was observed.
In contrast, a stark increase in cell proliferation is indicated at extract concentrations from 0.1 % to 5 % v/v, particularly at 48 h with 0.1% and 0.5 % v/v abalone sera concentrations. This led to testing the cytotoxicity at higher concentrations of 10 %, 20 %, 40 %, 60 %, 80 % and 100 % for shorter incubation time frames of 30 min, 2 h, 4 h and 6 h. As depicted in figure 4.3 at this higher concentration range cytotoxicity is observed, however it is not significant and CC50 is not reached.
Figure 4.3: Effect of variable concentrations (10, 20, 40, 60, 80 and 100% v/v) of abalone sera on the viability of HaCat cells which were treated for 30 min, 2 hour, 4 hour and 6 hour. Absorbance detected at 450 nm.

Approximately there is a 30% decrease in cell viability for all incubation time frames. A maximum abalone sera concentration of 20% is thus chosen for subsequent cytokine assays for the results to be unaffected by the cytotoxicity of abalone sera. As expected there was again observation of the direct correlation between incubation time and absorbance readings due to cell proliferation and hence, increased cell density at each subsequent checkpoint. The number of cells per well for the cytotoxicity assay was reduced for the absorbance readings to fall below 1 and the experiment was repeated with the higher concentration range of 10%, 20%, 40%, 60%, 80% and 100% under diverse incubation time frames of 30 min, 1 h, 4 h, 24 h and 48 h. The results at figure 4.4 shows no observed cytotoxicity at 30 min, 1 h, 4 h and 24 h.
Figure 4.4: Effect of variable concentrations (10, 20, 40, 60, 80 and 100% v/v) of abalone sera on the viability of HaCat cells which were treated for 30 min, 1 hour, 4 hour and 24 hour and 48 hour. Absorbance detected at 450 nm.

However, at 48 h there was an anomalous 50% reduction of cell viability at 10% v/v sera concentration, low concentration proving to be more cytotoxic than the higher concentrations. This may be due to the structure of the hemocyanin and coagulation or agglomeration properties. This does not cause concern as for subsequent cytokine assays, incubation time of extract chosen was much shorter.

The cytotoxic effect of variable concentrations of purified hemocyanin (as percentage of 400 microgram/mL solution) on the viability of HaCat cells which were treated for 30 min 24 h and 48 h were tested and no observed cytotoxicity surfaced (Figure 4.5) in the concentration range 1 %, 2 %, 4 %, 6 %, 8 % and 10 % v/v.
Figure 4.5: Effect of variable concentrations of purified hemocyanin (1%, 2%, 4%, 6%, 8%, 10% as percentages of 400 microgram/mL solution) on the viability of HaCat cells which were treated for 30 min 24 h and 48 h. Absorbance detected at 450 nm.
Furthermore, effect of variable concentrations (1 \%, 2 \%, 4 \%, 6 \%, 8 \% and 10 \% as percentages of 400 microgram/mL) of abalone sera and purified AH were tested on **PC-3 cells**, incubated for 24 h. Again, no observed cytotoxicity surfaced for this concentration range as depicted in Figure 4.6.

![Figure 4.6: Effect of variable concentrations of abalone sera and purified abalone hemocyanin (1\%, 2\%, 4\%, 6\%, 8\%, 10\% as percentages of 400 microgram/mL) on PC-3 cells, treated for 24 h. Absorbance detected at 450 nm.](image-url)
CHAPTER 5:

Immunomodulatory Effects on HaCat Cells & Human Primary Oral Keratinocytes
5.1 Results

Variable duration of treatment and concentration of test extracts were investigated to obtain an abalone hemolymph treatment model on human keratinocytes. BD Cytometric Bead Array (CBA) Human inflammatory assay was used to quantify the six cytokine concentrations (IL-1β, IL-12, IL-6, IL-8, IL-10 and TNF-alpha). Amongst these, the biggest impact was observed particularly on the concentrations of TNF-alpha, IL-6 and IL-8.

5.1.2 Effect of short duration treatment with abalone hemolymph

HaCat cells were treated with low concentrations of abalone hemolymph (2 % v/v and 5% v/v) with a short treatment time spanning from 15 min to 2.5 h. Short treatment time was investigated to observe the instantaneous cytokine modulation upon contact with low concentration abalone hemolymph. This would give an indication of the sensitivity and effectiveness of the treatment on these cell types.

5.1.2.1 Immunogenic effects with 5% v/v abalone sera

In the process of experimental design optimization, a study of immediate (15 min, 30 min and 45 min) immune response on HaCat cell triggered by treatment with 5% v/v abalone sera was conducted. A slight increase in the IL-8 and IL-6 concentrations compared to the control was observed at 15 min and 30 min incubation mark (figure 5.1 and figure 5.2) followed by a reduction in the same cytokines at 45 min mark (figure 5.3) This was, however, not a significant difference and further optimization of the experiment was required.
Figure 5.1: Immunogenic effects of 15 min treatment with 5% v/v abalone sera concentration on HaCat cells and determination of cytokine concentration (IL-6 and IL-8). (p<0.05) Data obtained with flow cytometry.

Figure 5.2: Immunogenic effects of 30 min treatment with 5% v/v abalone sera concentration on HaCat cells and determination of cytokine concentration (IL-6 and IL-8). (p<0.05) Data obtained with flow cytometry.
Immune response on HaCat cell triggered by treatment with 5% v/v abalone sera (15 min, 30 min and 45 min treatments) was conducted. A slight increase in the IL-8 and IL-6 concentrations compared to the control was observed at 15 min and 30 min incubation mark followed by a reduction in the same cytokines at 45 min mark. This was, however, not a significant difference and further optimization of the experiment was required.

5.1.2.2 Immunogenic effects with 2% abalone sera

To increase the method's sensitivity, a higher seeding cell density was required for more reliable results. With much higher cell seeding density and short treatment time of 30 min, 1.5 h and 2.5 h no significant immune response was observed in terms of IL-8 and IL-6 concentration changes as depicted in figure 5.4, figure 5.5 and figure 5.6, however, the increased cell seeding density corresponded to heightened cytokine levels which was the purpose of this experiment to optimize the experimental design in terms of sensitivity. It is worth noting that basal IL-8 concentration in HaCat cells is 7 fold greater than their basal IL-6 concentration.
Figure 5.4: Immunogenic effects of 30 min treatment with 2% v/v abalone sera concentration on HaCat cells and determination of cytokine concentration (IL-6 and IL-8). (p<0.05) Data obtained with flow cytometry.

Figure 5.5: Immunogenic effects of 1.5 h treatment with 2% v/v abalone sera concentration on HaCat cells and determination of cytokine concentration (IL-6 and IL-8). (p<0.05) Data obtained with flow cytometry.
Figure 5.6: Immunogenic effects of 2.5 h treatment with 2% v/v abalone sera concentration on HaCat cells and determination of cytokine concentration (IL6 and IL-8). (p<0.05) Data obtained with flow cytometry.

Short treatment time of 30 min, 1.5 h and 2.5 h no significant immune response was observed in terms of IL-8 and IL-6 concentration changes as depicted. However, the increased cell seeding density corresponded to heightened cytokine levels, which was the purpose of this experiment to optimize the experimental design to produce higher yield of cytokines. It is worth noting that basal IL-8 concentration in HaCat cells is 7 fold greater than their basal IL-6 concentration.

5.1.3 Effect of extended treatment with abalone hemolymph and its components

5.1.3.1 Immunogenic effects of 16 h treatment with variable concentrations of different extracts

A study with longer incubation time period of 16 h with increased seeding cell density aimed at increased cytokine response was then conducted. Abalone sera was ultrafiltered according to the protocol outlined previously, and purified hemocyanin and the permeate were collected to be used as treatment extracts. More than 2 fold reduction in IL-6 concentration was observed on all tested
extracts (20% sera, 10% sera and 20% v/v permeate), while 1.5 fold reduction in IL-6 was observed with treatment with purified AH as shown in figure 5.7. The abalone sera permeate was the most potent extract with the most immunomodulating property amongst the three extracts tested.

![Bar chart showing immunogenic effects of 16 h treatment with 10% and 20% abalone sera, 20% purified AH and 20% v/v sera permeate concentrations on HaCat cells and determination of IL-6 concentration.](image)

Figure 5.7: Immunogenic effects of 16 h treatment with 10% and 20% abalone sera, 20% purified AH and 20% v/v sera permeate concentrations on HaCat cells and determination of IL-6 concentration. (p<0.05) Data obtained with flow cytometry.

IL-8 concentrations were also reduced by approximately 25 % compared to the control with treatment of 10% and 20% v/v abalone sera, while the permeate and purified AH were less effective in modulating IL-8 as shown in figure 5.8.
Figure 5.8: Immunogenic effects of 16 h treatment with 10% and 20% v/v abalone sera, 20% purified AH and 20% sera permeate concentrations on HaCat cells and determination of IL-8 concentration. (p<0.05) Data obtained with flow cytometry.

There was a significant 9-fold increase in TNF-alpha concentrations in the cell supernatant particularly on treatment with 20% v/v sera, while no significant modulation of this cytokine was observed upon treatment with purified hemocyanin and the permeate. This is demonstrated in figure 5.10.

Figure 5.10: Immunogenic effects of 16 h treatment with 10% and 20% v/v abalone sera, 20% purified AH and 20% sera permeate concentrations on HaCat
cells and determination of TNF-alpha concentration. (p<0.05) Data obtained with flow cytometry.

5.1.3.2 6 day dose dependent study on priming of HaCat cell

The effect of a 6 day treatment with sera was tested to observe any priming effect on the cells that may modulate the cytokines differently. HaCat cells were supplemented with 10% and 20% v/v sera each day, and media changed after 1.5 hours. Same was repeated each day for 6 consecutive days. This was done to mimic a daily dose model. According to the results in figure 5.10 compared to the control, different concentration of sera indicated different effects. 10% v/v sera concentration indicated a 22% inhibition in IL-6 after 6 days. While 20% v/v sera concentration indicated a stark 40% increase in IL-6 concentration after 6 days. This indicates that on a dosage basis model, concentration of sera plays a crucial role in steering the immune response.

Figure 5.10: Immunogenic effects of 6 day dosage treatment (1.5 h treatment per day) of HaCat cells with 10% and 20% v/v abalone sera and determination of IL-6 concentration. (p<0.05) Data obtained with flow cytometry.

Further, in figure 5.11, the results are very similar to the results from IL-6, where 10% v/v sera causes a 21% reduction in IL-8. However, unlike the increase in IL
6 with 20% v/v sera, here the effects of 20% v/v sera concentration does not differ much from the control. This again stresses the importance of sera concentration on a dosage basis model.

Figure 5.11: Immunogenic effects of 6 day dosage treatment (1.5 h treatment per day) of HaCat cells with 10% and 20% v/v abalone sera and determination of IL-8 concentration. (p<0.05) Data obtained with flow cytometry.

An additional stimulating effect on IL-1β was noted as shown in figure 5.12, which was solely attributed to the 6 day treatment as the effect on IL-1β was not previously observed on the 16 h incubation model.

Figure 5.12: Immunogenic effects of 6 day dosage treatment (1.5 h treatment per day) of HaCat cells with 10% and 20% v/v abalone sera and determination of IL-1β concentration. (p<0.05) Data obtained with flow cytometry.
TNF-alpha concentration was also modulated to result in a 12-fold increase compared to untreated control, during the 6 day treatment, specifically by 20% v/v sera concentration. Surprisingly, 10% v/v sera concentration did not have any such modulatory effect, which allows us to conclude that the sensitivity of HaCat cell of TNF-alpha production drastically increases between sera concentrations of 10% and 20%, reflecting almost an exponential increase, as shown in figure 5.13.

![Figure 5.13](image)

**Figure 5.13**: Immunogenic effects of 6 day dosage treatment (1.5 h treatment per day) of HaCat cells with 10% and 20% v/v abalone sera and determination TNF-alpha concentration. (p<0.05) Data obtained with flow cytometry.

### 5.1.4 Dose and Time-dependent modulation of IL-6 and IL-8

Another study was conducted for the quantification of IL-6 and IL-8 in HaCat cell supernatant post incubation with 1%, 5%, 10% and 20% v/v sera for 1 h, 2 h and 5 h. Result indicates (figure 5.14 and figure 5.15) the release of IL-6 and IL-8 increase with higher treatment time. Dose dependent effect for the concentration range tested (1% to 20%) was not observed. 5 h treatment time clearly indicates a significantly higher IL-6 and IL-8 production compared to 1h, 2 h or the
control. As expected there was an increase of cytokine production with time. Including two other controls at different time points would have helped to see the effect of the treatments.

**Figure 5.14:** Quantification of IL-6 in HaCat cell supernatant post treatment with 1%, 5%, 10% and 20% v/v sera for 1 h, 2 h and 5 h. Data obtained with Flow Cytometry. Indicating the release of IL-6 increases with higher treatment time. Dose dependent effect for the concentration range tested (1% to 20% v/v) is not observed. 5 h treatment time clearly indicates a significantly higher IL-6 production compared to 1h, 2 h or the control (0%).
Figure 5.15: Quantification of IL-8 in HaCat cell supernatant post incubation with 1%, 5%, 10% and 20% v/v sera for 1 h, 2 h and 5 h. Data obtained with Flow Cytometry. Indicating the release of IL-8 increases with higher treatment time. Dose dependent effect for the concentration range tested (1% to 20% v/v) is not observed. 5 h treatment time clearly indicates a significantly higher IL-8 production compared to 1h, 2 h or the control (0%).

Dose dependent effect for the concentration range tested (1% to 20% v/v) is not observed. 5 h treatment time clearly indicates a significantly higher IL-6 and IL-8 production compared to 1h, 2 h or the control. As expected there was an increase of cytokine production with time. Including two other controls at different time points would have helped to see the effect of the treatments.
5.2 Immunomodulatory effects on Human Oral Primary Keratinocytes

5.2.1 Results

The abalone hemolymph ingestion study mentioned in Chapter 1 had shown modulation in cytokine concentration in the serum of patients treated with oral dosage of the whole hemolymph. The contact barrier between the extract and the recipient were oral keratinocytes. Immunomodulatory effect of the abalone hemolymph, and its components, on the human primary oral keratinocytes was, thus, crucial in our investigation. Determination of cytokine concentration modulation at this site was an important aspect in our research to validate our hypothesis.

Maximum concentration of 20% v/v sera was used as a treatment for 16 h incubation with the Human Primary Oral Keratinocytes to test the effect on cytokine concentration.

In summary, immunomodulatory properties of the three extracts at varying concentrations (dose-dependent) on Human Oral Primary Keratinocytes has been demonstrated. Permeate was found to be the most potent of all extracts in cytokine modulation for both the cytokines of interest.

5.2.2 Immunogenic effects of 16 h treatment with 20% abalone sera concentration

A sharp 35 fold increase in IL-8 concentration and 23 fold increase in IL-6 concentration was observed as shown in figure 5.16. In terms of basal cytokine expressions of Human Oral Keratinocytes, concentrations of IL-8 are twice as much as IL-6 concentration. This can be visualized in figure 5.16 that shows the shifting means of IL-6 and IL-8 peaks as compared to the control.
Figure 5.16: Immunogenic effects of 16 h treatment with 20% v/v abalone sera concentration on Human Primary Oral Keratinocytes and determination of cytokine concentration (IL-6 and IL-8). (p<0.05) Data obtained with flow cytometry.

5.2.3 Dose Dependent effect of abalone sera on Human Oral Primary Keratinocytes

Confirming dose-dependent effect of abalone sera on human oral keratinocytes was important to demonstrate the immunomodulatory properties of the sera and it is, thus, validated as shown in figure 5.17. 5% v/v sera concentration yields 4 fold increase, 10% v/v sera concentration yields 6 fold increase and 20% v/v sera concentration yields 10 fold increase in IL-6 concentration in Human Oral Keratinocytes cell supernatant after treatment.
Figure 5.17: Dose-dependent immunogenic effects of 16 h treatment with 5%, 10%, 20% v/v abalone sera concentration on Human Primary Oral Keratinocytes and determination of IL-6 concentration. (p<0.05) Data obtained with flow cytometry.

The effect on IL-8 concentration seemed to reach a plateau at approximately 8 fold increase at 5%, 10% and 20% v/v sera concentration treatment of the Human Oral Keratinocytes as shown in figure 5.18. This suggests that the Human Oral Keratinocytes are much more sensitive to the sera in terms of IL-8 modulation than IL-6 modulation. Further experiment with sera concentrations much lower than 5% v/v is required for dose-dependent effect to show.
Figure 5.18: Dose-dependent immunogenic effects of 16 h treatment with 5%, 10%, 20% v/v abalone sera concentration on Human Primary Oral Keratinocytes and determination of IL-8 concentration. (p<0.05) Data obtained with flow cytometry.

Figure 5.19 shows the dose-dependent effect of purified AH treatment on IL-6 where 1300 microgram/ml yields a two fold increase and 2300 microgram/ml yields 2.5 fold increase in concentration. As with the sera, sensitivity of Human Oral Keratinocytes to purified AH in terms of IL-8 modulation is higher than IL-6 modulation; as shown in figure 5.20, 1300 microgram/ml yields a 5 fold increase in IL-8 concentration after treatment and 2300 microgram/ml yields 8 fold increase in concentration after treatment. Hence, the dose dependent effect of purified AH was demonstrated.

Figure 5.19: Dose-dependent immunogenic effects of 16 h treatment with variable concentrations of purified AH on Human Primary Oral Keratinocytes and determination of IL-6 concentration. (p<0.05) Data obtained with flow cytometry.
Dose-dependent immunogenic effects of 16 h treatment with variable concentrations of purified AH on Human Primary Oral Keratinocytes and determination of IL-8 concentration. (p<0.05) Data obtained with flow cytometry.

5.2.4 Dose dependent effect of Abalone Sera Permeate

Abalone sera permeate was tested as a treatment on the Human Oral Keratinocytes and the dose dependent effect was observed as shown in Figure 5.21. The aim was to obtain high, medium and low concentration of the permeate. These were obtained by dilution, and it was out of our control to obtain exact or rounded concentrations. These concentrations of 63 and 398 microgram/ml were measured using Qubit after dilution. The difference between them was large enough for a valid dose-dependent study.

63 microgram/ml permeate yielded a 2.4 fold increase in IL-6 concentrations, followed by 3.5 fold increase with 398 microgram/ml and 4.5 fold increase with 556 microgram/ml of sera permeate. Again, sensitivity of Human Oral Keratinocytes to abalone sera permeate (similar to the sera and purified AH) in terms of IL-8 modulation is higher than IL-6 modulation; as shown in figure 5.22, 63 microgram/ml yields a 6 fold increase in IL-8 concentration after treatment, 398 microgram/ml yields an anomalous 13 fold increase and 556 microgram/ml yields an 8 fold increase in IL-8 concentration after treatment.

The drastic increase may be due to a large pipetting error in either cell seeding or treatment dose. The “decrease” in 556 microgram/ml treatment is still higher.
than the yield obtained from 63 microgram/ml. Hence, it makes sense to call the drastic increase an anomaly/experimental error.

Hence, the dose dependent effects of sera permeate was demonstrated.

Figure 5.21: Dose-dependent immunogenic effects of 16 h treatment with variable concentrations of abalone sera permeate on Human Primary Oral Keratinocytes and determination of IL-6 concentration. (p<0.05) Data obtained with flow cytometry.

Figure 5.22: Dose-dependent immunogenic effects of 16 h treatment with variable concentrations of abalone sera permeate on Human Primary Oral Keratinocytes and determination of IL-8 concentration. (p<0.05) Data obtained with flow cytometry.
CHAPTER 6:

Immunomodulatory Effects On Prostate Cancer Cells & Identification Of The Permeate Proteins
6.0 Introduction

The role of hemocyanin as an immunotherapeutic for various forms of cancer has been studied extensively with some promising results. The most prevalent KLH and now CCH (Concholepas concholepas hemocyanin), has been widely used as alternatives in superficial bladder cancer (SBC) immunotherapy. Hemocyanins are particularly preferable as a treatment for superficial urinary bladder cancer (SBC), which is most commonly treated with Bacillus Calmette-Guerin (BCG). Unlike BCG that has the risk of anaphylactic reaction or formation of large abscess, the fact that hemocyanin does not cause side effects, renders them preferable for long-term repetitive treatments (Campo et. al, 2011). Thus, the search for natural immunostimulants is a promising avenue to explore (Lamm et. al, 2003). Olsson et al treated superficial bladder cancer with immunization of patients with 5 mg of KLH and observed a significant reduction in the recurrence of superficial bladder cancer. Reduction in tumor growth in mice was reported by Lamm et al. KLH has also shown bladder tumor recurrence prevention with negligible side effects (Winkner et. al, 2000; Jurincic et. al, 1996).

Due to the similarity between the structures of KLH and AH, the effect of AH on treating cancer cells is expected to be in line with the effects of KLH and thus, we treated prostate cancer cell line (PC-3) with purified AH, sera and sera permeate we expected immunomodulatory effects as well as cell proliferation inhibition. Effects of KLH on prostate cancer cell lines have been studied mainly as carrier protein, including 10 clinical trials in various stages of development mainly using MUC-2 antigen with KLH. Monovalent and poly-valent vaccines are under debate since, although, both have immunostimulatory effects on prostate cancer, monovalent vaccines yield higher titres of antibodies while poly-valent vaccines reach a greater range of antibodies (Slovin et. al, 2007). A more recent study conducted on prostate cancer patients, with the use of an adjuvant of another form of hemocyanin, CCH, showed that it is safe and is able to generate memory T cell responses in patients and is thus a potential substitute for KLH in immunotherapies.
The effect of KLH (50 μg/well), IL-2 (10,000 IU/well), IFN-a (1500 IU/well) and their combination has demonstrated an astounding reduction of HTB68 melanoma cell line where KLH and IFN-a combined yielded a 67% reduction in cell growth and increased apoptotic activity by 14% (McFadden et. al, 2006). KLH has thus shown to enhance the effect of IFN-a, which is a powerful immunotherapeutic against melanoma (Rizvi et. al., 2007). Cancer cell lines estrogen-dependent breast (MCF-7), estrogen-independent breast (ZR75-1), and pancreas (PANC-1, MIA-PaCa) were treated with KLH and significant inhibitions of cell growth was observed, while a 6% inhibition of prostate cancer cell growth was also observed upon treatment with KLH (Riggs et. al, 2002).

Other forms of cancer such as epithelial derived Barrett’s esophageal adenocarcinoma in an in vitro model was tested by McFadden et. al, (2003) where a reduced viability of cancer cells in a dose and time-dependent manner were observed upon treatment with KLH. Several other in vivo and in vitro hemocyanin treatment models exist for various forms of cancer such as breast, oesophageal, melanoma, pancreatic and prostatic.

6.1 Results

In summary, immunomodulating properties of the three extracts at varying concentrations on PC-3 cell have been demonstrated. Permeate continues to be the extract with strongest immunomodulating potency on IL-6. A cell morphology change and reduction in cell size on prostate cancer cells is observed when treated with the permeate.

6.2 Immunogenic effects of variable extracts with 5 h treatment

Prostate cancer cell line, PC-3 cells, were treated with variable concentrations of the abalone sera for 5 h and the resulting cytokine concentration changes were determined. Figure 6.1 shows that all three extracts (sera, AH and permeate)
have a modulatory effect inhibiting production of IL-6 in the PC-3 cells. Permeate, again, is proven to be the most potent treatment extract, inhibiting IL-6 in PC-3 cells by over 50%, followed closely by 20% v/v sera. Purified AH, although has the inhibiting IL-6 production effect, is the least potent among the three, inhibiting the IL-6 concentration by 27%.

Figure 6.1: Immunogenic effects of 5 h treatment with 20% v/v abalone sera, 20% purified AH and 20% sera permeate concentrations on PC-3 cells and determination of IL-6 concentration. (p<0.05) Data obtained with flow cytometry.

A 29% reduction in IL-8 results from treatment with permeate for 5 h, followed by a 25% reduction from treatment with AH, and lastly a 16% reduction from treatment with the abalone sera. This is portrayed in Figure 6.2. Basal IL-8 concentration is much more abundant in PC-3 cells than IL-6 as can be seen in Figure 6.2.
Figure 6.2: Immunogenic effects of 5 h treatment with 20% v/v abalone sera, 20% purified AH and 20% sera permeate concentrations on PC-3 cells and determination of IL-8 concentration. (p<0.05) Data obtained with flow cytometry

The most potent treatment extract is the permeate, inhibiting IL-6 in PC-3 cells by over 50%, followed closely by 20% v/v sera. Purified AH, although has the inhibiting IL-6 production effect, is the least potent among the three, lowering the IL-6 concentration by 27%. 29% reduction in IL-8 results from treatment with permeate for 5 h, followed by a 25% reduction from treatment with AH, and lastly a 16% reduction from treatment with the abalone sera.
6.3 Effect of extracts on Cell morphology

The effect of abalone sera, AH and permeate 5 h incubation on the prostate cancer cell morphology was observed and findings in Figure 6.3 suggest that the permeate had a direct effect on cancer cell morphology resulting in size and aspect reduction of the cells compared to untreated control. No such effect was observed with abalone sera and AH treatment. This may be a crucial finding to support the permeate’s capacity in reducing the size and aspect of cancer cells on direct contact.

Figure 6.3: Effect of abalone sera, purified AH and permeate 5 h treatment on the morphology of PC-3 cells. The PC-3 cells are at 100 x magnification. (A) Control (B) 20% v/v sera (C) 20% v/v hemocyanin (D) 20% v/v permeate.
6.4 Identification of the permeate proteins using Western Blot

Due to the continued high potency of the hemolymph permeate in immunomodulation across all tested cell lines, it was important to have an insight on the nature and origin of the permeate proteins. To detect if the permeate proteins originate from the hemocyanin (dissociated fragments) or if they are entirely unrelated to the hemocyanin, Western Blot was performed. Three different concentrations of permeate samples were tested. Due to the low concentration of even the undiluted permeate, Coomassie blue stain could not detect the permeate proteins. However, the highly sensitive Western Blot experiment showed protein bands at at least 11 molecular weight markers as shown in Fig 6.4. All of which are detected by anti hemocyanin antibody. This suggests that these small proteins are fragments/sub-units-functional units of the hemocyanin dissociated from it. Clearly, the 1st permeate, which is undiluted, shows the strongest stain, followed by the 2nd and 3rd permeate. Concentrations of these permeates are shown in Table 6.1.

Figure 6.4: Identify the protein in the permeates by Coomassie blue stain (A) and western blot with the anti-hemocyanin antibody (B). Marker units: kDa. Samples were processed by me, while the Coomassie Blue Stain and Western Blot experiment was performed by Jiadai Wu at Westmead Millenium Institute.
Protein cannot be detected by Coomassie blue stain due to the little amount (Figure 6.4-A). Same amount of sample was loaded for Western blot with the polyclonal antibody raised against native hemocyanin.

<table>
<thead>
<tr>
<th>Total protein concentrations of three permeates</th>
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<tbody>
<tr>
<td><strong>Absorbance</strong></td>
</tr>
<tr>
<td>1st permeate</td>
</tr>
<tr>
<td>2nd permeate</td>
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<tr>
<td>3rd permeate</td>
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</tbody>
</table>

The result indicated protein bands at at least 9 molecular weight markers ranging from 28 kDa to 250 kDa (and a faint band above the 250 kDa marker) in the 1st permeate. A very dense protein band was identified at 55 kDa marker for this sample.

The 2nd permeate consisted of the highest number of protein bands (at 11 molecular weight markers, 9 of them at identical molecular weight to 1st permeate). The additional bands were identified between 100 kDa and 250 kDa.

The 3rd permeate, being the most diluted amongst the three samples, showed a series of faint protein bands at identical molecular weight markers as 2nd permeate from 55 kDa to 250 kDa.

The protein bands detected are the proteolytic molecules derived from native hemocyanin (Figure 6.4-B).

Permeate with the highest concentration of proteins showed the most significant modulation of IL-6. In contrast, permeate with medium concentration of proteins showed the most significant modulation of IL-8. The modulation of IL-8 may be caused by the additional proteins detected exclusively in the 2nd permeate (Fig. 5.21 and Fig. 5.22).
CHAPTER 7:

Discussions, Conclusions
& Future Directions
7.0 Discussions

Immunomodulatory properties of *Haliotis rubra* (abalone) hemocyanin (AH) along with whole sera and sera permeate, had been investigated on three different cell types, two of which are human keratinocytes and one of which is a human prostate cancer cell line. Immunomodulatory and dose-dependent effects on all three cell types through the modulation of cytokines is observed for all three extracts, while the sera permeate (consisting of hemocyanin derived proteins, according to Western Blot Analysis) consistently proved to be the most potent immunomodulator in all cases. Among the tested six cytokines (IL-10, IL-8, IL-6, IL-12, TNF-alpha and IL-1β), significant modulation of IL-6 and IL-8 is observed on all cell types and extracts used. Modulation of IL-1β and TNF-alpha were also observed under certain parameters, such as daily dose model on HaCat cells.

The significance of this finding branches out to and opens a vast field of potential anti-viral, anti-bacterial and anti-cancerous effects of this marine-derived extract. Some immunomodulatory behavior of certain hemocyanins have been previously reported; studies by Zhong et.al (2016) have found the ability of three different hemocyanins (Concholepas concholepas, Fissurella latimarginata, and Megathura crenulata (*keyhole limpet*)) to induce a potent Th1 dominant immune response following upregulation of IL-6, TNF-alpha and IL-1β in macrophages, which is coherent with the findings in this study. Downregulation effects of these hemocyanins were also observed by Zhong et. al on different cytokine gene expressions including IL-4 and IL-5. Additionally, Donnarumma et. al (2010) found an increase in IL-1β, IL-6, IL-8, TNF-alpha, in epithelial cells upon treatment with KLH, all of which fall common with our results with Human Primary Oral Keratinocytes (a form of epithelial cells). Findings of Zhong et. al (2016) and Donnarumma et. al (2010) and the ability of hemocyanin to upregulate and downregulate certain cytokines is, thus, in accordance with our findings. The in vitro production of cytokines by mucosal lymphocytes immunized by oral administration of KLH that used cholera toxin as an adjuvant showed heightened levels of IL-4, IL5 and IL-6 with lower levels of IL-2 and
interferon-gamma (IFN-gamma) in the lamina propria (mucosa). This finding is in line with our research outcome of human oral keratinocytes with significant increase in IL-6 levels. However, HaCat cells were not a suitable model for the primary keratinocytes as they responded differently to treatment (a response similar to PC-3 cells) due to their immortalized status, and hence its effect on their receptors and various signaling pathways.

The effect of immunomodulation with whole sera and permeate has not been studied previously. While there are fragments of the hemocyanin present in the sera permeate, it is not clear if any non-hemocyanin derived proteins are present in the permeate that facilitates the augmented immunomodulatory effect of the permeate compared to purified hemocyanin and whole sera. Zanjani et. al (2014) found 20kDa size proteins in the permeate, which along with other fragmented proteins, are derived from hemocyanin according to our analysis.

An interplay between the innate and humoral immune response is observed in a number of cases. Immunogenicity of Rapana thomasiana hemocyanin (RTH) has been found by Tichorbanov et. al (2008), where humoral immune response is observed in vivo through secretions of anti-hemocyanin antibodies against RTH, two sub units of the hemocyanin RTH1 and RHT2 upon immunization with the extracts. Similar humoral immune response has been found with KLH as a carrier with saponin adjuvant, where high titres of IgM and IgG were induced (Slovin et.al 2005). In addition, humoral response of heightened IgA levels were observed in the mucosa. It was thus assumed that reaction to cholera toxin/keyhole limpet hemocyanin is due to by Th2-type cytokines (Wilson et. al, 1991).

As the immunomodulatory effects of hemocyanin is evident on both innate and humoral immune response, it is important to decode the mechanisms that are involved in the process. There are quite a few hypothesis: while the mechanism of action initiated by macropinocytosis and clathrin-mediated endocytosis is reported by Arancibia et. al in the case of macrophage activation following hemocyanin inoculation, the mechanism of action on the keratinocyte level is yet
to be studied. The mechanism of action of Litopenaeus vannamei hemocyanin on human cervical cancer cells (Zheng et al) follows apoptosis through mitochondria triggered pathway. This particular mechanism sheds light into a potential interaction of the hemocyanin and hemocyanin derived proteins with mitochondria to modulate the cytokines through receptors on the keratinocytes and cancer cell line in our study.

Another possible mechanism could involve enhanced humoral immune response due to the association of modified fecal Lactobacillus content (Murai et al 2016). This can be related to the study conducted by Murai et al, 2016, where orally administered chickens (with KLH) have shown enhanced humoral immune response due to the above modification. This may be a mechanism of action in humans orally administered with AH followed by the modulation of IL-6 and IL-8, bridging the innate immune response with humoral immune response in combating diseases such as HSV-1 and cancer.

Alternatively, activation of NF-κB is a potential mechanism of immune response of the hemocyanin and hemocyanin derived proteins, as proven by Yasuda et al (2016) in their study with KLH where human monocytic THP-1 cells demonstrated activation of NF-κB through partial mediation by Syk and Erk pathways. The transcription factor, NF-κB, plays crucial roles in the immune system (Ghosh et al., 1998; Karin et al, 2004) and the NF-κB activation results in the production of pro-inflammatory cytokines (Kingeter and Lin, 2012). The similarity between the structures of AH and KLH (constituting of high levels of sugar residues, unpublished data) supports the notion that this NF-κB activation may also be induced by AH as it is initiated through mannose or C-lectin receptors since, like KLH (Mansour et al. 2006), AH and AH derived proteins may also be rich in mannose. Additionally, Ines et al (2013) reported that secretion of cytokines was dependent on the presence of oligosaccharides on FLH, which demonstrated a role of lectin/mannose receptors in the mechanism of hemocyanin action.

There are observations that oppose this hypothesis; Endocytosis inhibition experiments performed on human DCs with an anti-mannose receptor antibody
and KLH, demonstrated the partial inhibition of KLH incorporation by DCs. Additionally, it continued the promotion of activation and maturation of DCs reflected by the up regulation of MHC class II molecules (Presicce et al., 2008). However, murine DCs pulsed with KLH did not undergo DC maturation (Teitze-Tennenbaum et al., 2008) coherent with the in vivo results (Teitz-Tennenbaum et al., 2008; Moltedo et al., 2009).

The mechanism of action by the hemocyanin is also shown to involve the adaptive arm of immune response. As carbohydrates are natural adjuvant themselves, triggering the adaptive arm of immunity without any other adjuvant is quite possible by hemocyanins. A mechanism of CD4+ T cell reacting to preserved xenogenic peptidic sequences stimulate the T cells to secrete Th1 cytokines. This results in a breakage of tumor tolerance and enhancement of immune response against the tumor. Arancibia et al. (2012) have found evidence that supports this mechanism of action, showing that high secretions of IFN-Y and IL-2, was found in lymph nodes after exposure to hemocyanin. Additionally, cytokine environment tending towards a Th1 response was observed when mice primed with CCH or KLH were exposed to bladder cancer cells. Furthermore, there was a significant increase of CD4+ T cells in the submucosa and urothelial cells, as opposed to only a slight increase in CD8+ T cells after intravesical KLH therapy in patients with bladder cancer (Jurincic et al. 1995). Lastly, tumor antibodies (IgG1 and IgG3 isotypes) resulted from KLH conjugated vaccines against cancer (Musselli et al, 2001).

Hemocyanin has been studied extensively for cancer treatment. Research on the effect of KLH on bladder cancer has led to multicenter clinical trials confirming the efficacy of intravesically treated KLH on patients during varying stages of bladder cancer (Lamm et al., 2000). However, it is worth noting that at the phase III clinical trial stage, when the anti-cancerous effects of KLH treatment on non-muscle invasive bladder cancer was tested and compared to the effects of mitomycin (Lammers et al, 2012), experiences of fever, flu-like symptoms, and fatigue occurred significantly more after KLH treatment but KLH tended to be more effective than mitomycin in preventing progression of cancer. Becker et. al
(2009) and Moltedo et al. (2006) have studied the effect of CCH on bladder cancer finding a reduction in tumor growth and prolonged survival. A further study conducted by Becker et al. subsequently identified the more potent subunit CCH-A that has greater anti tumor effect compared to CCH-B. This shows anti-tumor, and hence immunomodulatory, activity may vary between subunits of hemocyanin and it is, thus, worth studying different subunits of AH.

The direct effect of IL-8 on prostate cancer cells have been reported by Waugh et al. (2008) where it is shown that IL-8 signaling increases proliferation and survival prostate cancer cells, and potentiates the migration of cancer cells. He suggests that inhibiting the effects of IL-8 signaling may be a significant therapeutic intervention in targeting the tumor microenvironment. Additionally, Neveu et al. (2014) reported a direct correlation between IL-8 secretion and primary prostate cancer cell aggressiveness. The suppression of IL-8 secretion, post treatment with the abalone hemolymph, is thus an indication of anti-cancerous effect through immunomodulation.

On the other hand, the role of IL-6 in prostate cancer is discussed by Nguyen et al. (2014) stating that IL-6 is able to promote prostate cancer cell proliferation and inhibit apoptosis in vitro and in vivo. IL-6 is also associated with aggressive prostate cancer phenotype. Lou et al. (2000) found that IL-6 induces prostate cancer cell growth by activation of stat3 signaling pathway. However, Mallinowska et al. (2009) reported the activation of androgen receptor in prostate cancer cell by IL-6. Either way, inhibition of IL-6 secretion have an anti-tumor effect due to these reasons, which supports the finding of IL-6 suppression by abalone hemolymph treatment.

Immune response is intricately involved in herpes simplex virus 1 (HSV 1), and in particular innate immune response plays a critical role in early control of the disease (Paludan et al. 2011). Human and animal herpes virus models have demonstrated signaling occurring number of TLRs including TLR2, which recognizes virion components including surface glycoproteins (Boehme et al. 2006; Compton et al. 2003), which pinpoints dsRNA (Iwakiri et al. 2009) as well
as TLR9 which is responsible for the detection of genomic DNA (Lund et. al 2003). As already found (Zanjani et. al 2014), abalone hemocyanin and derived proteins are glycoproteins that prevent viral entry by binding to viral glycoproteins, and thus, according to the above it may act on the immune system through the TLR signaling pathway.

Nason et. al. (2016) found a significant association between Herpes Simplex Virus shedding and high levels of IL-6. The increase in IL-6 in primary oral keratinocytes may induce shedding of HSV-1, and according to Nason et. al (2016) the increased IL-6 may also prevent subsequent reactivation of the virus as long as the immune-environment remained optimal for this cause. Interestingly, Donnarumma et. al (2010) found an increase in IL-1β, IL-6, IL-8, TNF-alpha, (i.e proinflammatory cytokines) in epithelial cells and concluded that their method of HSV-1 treatment indeed facilitates and triggers the proinflammatory immune response in HSV-1 infection. Additionally, the importance of targeted immune responses in different stages of the infection is also stressed. This corroborates the idea that abalone sera, purified AH and permeate can, indeed, be potential extracts that combat HSV-1 through immunomodulation, since there was observed augmentation in the levels of all of the above cytokines (IL-1B, IL-6, IL-8, TNF-alpha).

Augmented levels of proinflammatory cytokines in the human primary oral keratinocyte cell supernatant, post treatment with abalone hemolymph extracts, fits well with reports of Tumpey et. al (1996) that shows both immunocompetent and immunodeficient mice, neutrophils play a significant role in controlling the replication and spread of HSV-1. Milligan et. al (1999) suggested that neutrophils helped control HSV-2 in murine model of the infection. Both IL-6 and IL-8, the target cytokines modulated by the abalone hemolymph extracts, attract and activate neutrophils during infection and inflammation (Borish et. al, 1989; Hammond et. al, 1995). Hence, the IL-6 and IL-8 modulatory effects of abalone hemolymph may have a direct effect on the attraction and activation of neutrophils in HSV infections.
The finding of permeate proving to be the most potent immunomodulator can be explained by the finding of Zhang et. Al (2004), which demonstrated that individual proteins were slightly more active than native hemocyanin. Additionally, the mixture proteins were much more active than native hemocyanin. This may correspond to the relative abundance of *Haliotis rubra* hemocyanin fragmented proteins in the permeate as compared to purified hemocyanin, and the higher immunomodulatory effect corresponding to the higher anti viral activity. It is also highly likely that the functional units of the hemocyanin fragmented in the permeate are responsible for the boosted immunomodulatory properties as compared to the whole sera or purified hemocyanin, however there is a lack of literature that studied this aspect. According to our findings, all 11 differently sized proteins present in the permeate are derived from hemocyanin. However, it may be possible that there are proteins present in the permeate, which were not detected in SDS-PAGE, that are foreign to hemocyanin. Nevertheless, an explanation may be found in the findings of Dang et al. (2011) that proposed antiviral activity of hemolymph from *Haliotis laevigata*, a closely related species to *Haliotis rubra*, was due to heat-resistant peptides and not hemocyanin.

**7.1 Conclusion**

Only a few immunomodulatory drugs are on the market today. Examples of these drugs include prostaglandins, cyclosporine A, levamisole, niridazole and penicillamine. As most of these have severe side effects new drugs from natural sources may offer new mechanisms of activity and may underpin new therapeutic approaches.

The impact of naturally derived immunomodulators are potentially greater due to the preferable observation that there is minimal side effects associated with these compounds, in contrast to the synthetic immunomodulators commercially available in the market today (Natanranjan et. al 2016).
Immunomodulatory compounds particularly derived from shellfish have been widely studied in recent years, and proteins, peptides, lipopolysaccharides, glycoproteins and lipid derivatives are found to have the most immunomodulatory effects (Tzianabos et. al 2000). Proteinaceous molecules with much lower molecular weights have also demonstrated potent immunomodulation e.g lectins, glycoproteins, agglutinins, etc.

The need for immunomodulatory drugs derived from natural sources is of great importance, as it minimizes the problems associated with cost and severe side effects in patients with viral diseases and cancer, both of which are intricately dictated by the state and responses of the immune system.

The research questions in this case were:

- Whether abalone sera, hemocyanin or the permeate have immunomodulatory properties.
- What is the nature and degree of immunomodulation observed?
- Which component of the sera is a more potent immunomodulator and what is the nature of the immunomodulatory extract?
- What kind of immune response is triggered by the extract?
- Is there any cytotoxicity to the host cells?
- Is there an effect on the cancer cells?

Answering these research questions would facilitate studies of hemocyanin as a cancer therapeutic and as a model for developing new class of drugs for against diseases such as HSV-1.

In this study, I demonstrated that *Haliotis rubra* sera, purified hemocyanin and permeate from the same, have no significant cytotoxicity to the host cells and yet, have a direct and powerful effect on the innate immune response by stimulating expression of inflammatory cytokines.
Immunomodulatory effect of abalone sera and its components has been demonstrated for the first time in an in vitro system consisting of human oral primary keratinocytes. IL-6 and IL-8 were the target cytokines in all tested cell lines that responded to stimulation with abalone sera and its components.

There was a sharp 35-fold increase in IL-8 concentration and 23-fold increase in IL-6 concentration with treatment of 20% v/v abalone sera, as compared to the untreated control. Further, dose-dependent effects of all extracts (whole sera, purified hemocyanin and permeate) were subsequently confirmed on the primary oral keratinocytes. It has also been shown that human oral keratinocytes are much more sensitive to the sera in terms of IL-8 modulation than IL-6 modulation. Additionally, it has been demonstrated that the basal expression of IL-8 concentration in Human Oral Keratinocyte supernatant is twice as much as IL-6 concentration.

Receptor saturation and agonistic affect was demonstrated at high concentrations of the sera and components. Long term stimulation is necessary to achieve sustained modulation of IL-6 and IL-8 necessitating the development of sustained release system for mucosal delivery. Although IL-10 was decreased in patients who consumed abalone sera previously (25 ml oral dose daily), no such reduction was demonstrated by cultured primary keratinocytes. This suggests that other cells beside keratinocytes were the potential targets for the components of abalone sera.

The two cytokines are relevant for treatment of HSV and other viral infections and demonstrate potential for subsequent modulation of the innate immune system and engagement of the cellular immune system.

Treatment of HaCat cells with the abalone sera showed a 50% inhibition in IL-6 expression, 21% inhibition in IL-8 expression and an increase in TNF-alpha concentration in the HaCat cell supernatant after 16 h treatment. Other cytokine levels were unaltered.
A 50% inhibition of IL-6 expression and 29% inhibition of IL-8 expression was observed in prostate cancer cell line treated with abalone hemolymph permeate. Other extracts from the abalone hemolymph showed similar results. Abalone hemolymph also showed selectivity towards the cancer cells as their morphology was affected by the extracts, while the morphology of keratinocytes were not. Hence, it has been successfully demonstrated that all three extracts are capable of varying degrees of immunomodulation, targeting IL-6 and IL-8. Under some specific parameters mentioned above, effects on TNF-alpha and IL-1β (Other proinflammatory cytokines) have also been significant.

Small proteins and peptides from the low molecular weight fraction of abalone sera (hemolymph permeate) have demonstrated higher immunomodulatory activity than native hemocyanin. The nature of the hemolymph permeate was studied to answer whether the proteins present in the permeate originate from hemocyanin or they are foreign to hemocyanin. Western Blot results showed that the proteins in the permeate are indeed fragments of the hemocyanin, which are more effective than the much larger parent molecule. This finding promises, upon isolation of the most immunomodulatory peptide fragmented in the permeate, that a synthetic hemocyanin fragment derived immunomodulatory drug in a form of peptide will be much simpler to formulate and produce on large scale and formulate.

Overall, these results are very promising as they demonstrate the immunomodulatory potential of abalone hemolymph extract on three different cell lines. The results justify further studies on the immunological activity of these peptides and identifying the precise mechanism of action.

7.2 Future Directions

Functional studies are needed to demonstrate modulation of the immune system beyond cytokine release. Innate immune response modulation resulting from the
treatment with abalone sera, purified AH and the permeate should be further explored with studies involving cells of the innate immunity such as macrophages, T cells, NK cells and antigen presenting cells such as dendritic cells. Cytokine modulation in these cells as well as effects on their maturation should be studied. This will help mapping the effector cells involved and identify the receptors responsible for the triggered immune response. Signaling pathways and precise mechanism of action may be decoded.

Modulation of the cellular immune response, and potential interplay between innate and adaptive immune system should also be investigated.

Furthermore, studies should be extended to observe immunomodulatory effects of the abalone hemolymph on other human epithelial cells, such as fibroblasts. Screening of other cancer cells is also justified to understand the mechanism of hemolymph extracts’ direct anti-cancer effect. The promising observations presented in this work justify further, more detailed studies in this field.
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Appendix

A.1 Effect of Abalone Hemolymph Treatment on IL-6 and IL-8

Below are the effects of 20% v/v abalone hemolymph treatment observed on FACSCanto II scatter plots. The peaks shifting to the right post-treatment are that of IL-6 and IL-8.

![Control](image1)

![20% Sera](image2)

Figure 8.1: FACSCanto II images of effects of 16 h 20% v/v sera treatment on the peaks of IL-6 and IL-8.
A.2 Effects of Abalone hemolymph extracts

The modulation of IL-6 and IL-8 can be visualized in the scatter plots below (from FACSCanto II flow cytometer). Different extracts have variable degrees of immunomodulation as can be observed by the shifting peaks of IL-6 and IL-8.

Figure 8.2: FACSCanto II images of effects of 16 h treatment of Human Primary Oral Keratinocytes with (A) Tris control, (B) 20% v/v Sera, (C) low concentration permeate and (D) high concentration purified AH on the peaks of IL-6 and IL-8.
A.3 Flow Cytometer Analysis Gating Strategy

The gating strategy followed for the analysis of flow cytometric observations are demonstrated below. The beads enclosed by the gate are stained beads containing captured cytokines. The rest are cellular debris, which were not included in the analysis. This gating strategy was followed for observations under all experimental parameters.

Figure 8.31: Flow cytometer Gating strategy for the analysis of cytokine concentration results observed.

These beads were further sub-divided into 6 peaks of the 6 cytokines detected. Three (in some cases two) sub-populations: P2, P3 and P4 were further gated as they showed the most modulation from the basal cytokine concentration. They are IL-8, IL-1β and IL-6 respectively.

Figure 8.32: Gating of IL-6, IL-8 and IL-1β.
The figure below indicates the quantification of the detected cytokines in each gate P2, P3 and P4. Relatively, it can be concluded that IL-6 (P2) has experienced the highest immunomodulation followed by IL-6 and IL-1β. The cytokine concentration was quantified in pg/ml using FCAPArray software.

Figure 8.33: Mean values of cytokines detected in each gated sub-population.