ETHANOLIC AND PARTIALLY PURIFIED AQUEOUS EXTRACTS FROM THE SEA CUCUMBER *HOLOTHURIA POLII* INDUCE A TUMOR SUPPRESSIVE PHENOTYPE IN MDA-MB-231 BREAST CANCER CELLS

by

MIKE IBRAHIM EL KAREH

A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Biology
of the Faculty of Arts and Sciences
at the American University of Beirut

Beirut, Lebanon
June 2014
ETHANOLIC AND PARTIALLY PURIFIED AQUEOUS EXTRACTS FROM THE SEA CUCUMBER HOLOTHURIA POLII INDUCE A TUMOR SUPPRESSIVE PHENOTYPE IN MDA-MB-231 BREAST CANCER CELLS

by

MIKE IBRAHIM EL KAREH

Approved by:

Dr. Rabih Talhouk, Professor
Department of Biology

Dr. Marwan El-Sabban, Professor
Department of Anatomy, Cell Biology and Physiological Sciences

Dr. Diana Jaalouk, Assistant Professor
Department of Biology

Dr. Najat Saliba, Professor
Department of Chemistry

Date of thesis defense: June 31, 2014
AMERICAN UNIVERSITY OF BEIRUT

THESIS, DISSERTATION, PROJECT RELEASE FORM

Student Name: _________________________________________________________________

Last    First    Middle

☐ Master’s Thesis      ☐ Master’s Project      ☐ Doctoral Dissertation

I authorize the American University of Beirut to: (a) reproduce hard or electronic copies of my thesis, dissertation, or project; (b) include such copies in the archives and digital repositories of the University; and (c) make freely available such copies to third parties for research or educational purposes.

I authorize the American University of Beirut, three years after the date of submitting my thesis, dissertation, or project, to: (a) reproduce hard or electronic copies of it; (b) include such copies in the archives and digital repositories of the University; and (c) make freely available such copies to third parties for research or educational purposes.

__________________________________________________
Signature       Date

This form is signed when submitting the thesis, dissertation, or project to the University Libraries
ACKNOWLEDGMENTS

My greatest gratitude goes to Dr. Rabih Talhouk. I’m indebted to all the support you provided me with and the things you taught me, not only science related but on the personal level as well. You have trained me well, you have changed a lot in me in ways I can’t explain … I cherish every single thing you did and didn’t do … I can’t find the exact words to describe my experience, simply, the time I spent in your laboratory was enough to make a better man out of me … I will never forget this, I truly won’t … Your attitude was influential in so many ways that made me recognize things from different perspectives … Your decisiveness and excellence as a scientist and as a person … I totally respect you on all levels.

My committee members, I’m honored you served on my committee. In no particular order, Dr Marwan El Sabban, Dr Diana Jaalouk and Dr Najat Salib, you have always been the driving force throughout my research, each in his/her specific way … I wouldn’t have enjoyed my work at AUB if it wasn’t for your presence. Dr Nadine Darwiche, I will never forget all the support and the care … I’m really sorry that you were not a member of my thesis committee … You have encouraged me in so many ways … Thank you for everything.

RST members, you are my second family and I wouldn’t have done it without you… Rana, a special thanks for all your help throughout my work.

Gilbert Rahme, I will never forget everything you did to me … you treated me as a brother … Wish you all the best in your life … Thank you for introducing me to Ms. Rima Chedid, the amazing person who trained me from all her heart … Ms. Rima, I thank you, you are an inspiring person.

I thank all my friends for supporting me throughout my research, a special thanks to all of those whom tolerated me when I was so hard to deal with … You made my stay more valuable, enjoyable, fun and unforgettable.

I owe everything to my family, Sandy, my father Ibrahim, my mother Maha and my sister Carla.

Mike El Kareh
Beirut
June 2014
AN ABSTRACT OF THE THESIS OF

Mike Ibrahim El Kareh for Master of Science
Major: Biology

Title: Ethanolic and Partially Purified Aqueous Extracts from the Sea Cucumber *Holothuria polii* Induce a Tumor Suppressive Phenotype in MDA-MB-231 Breast Cancer Cells

The concept of functional food has always been implemented in traditional remedies against various chronic diseases. Sea cucumbers are considered as one of the most important functional foods for their effectiveness against various human disorders. We sought to investigate the presence of anti-cancer bioactivities in *Holothuria polii*, the most abundant sea cucumbers inhabiting the Lebanese coast in the Mediterranean. Sea cucumber ethanolic extract (SCE) inhibited the proliferation of highly invasive breast cancer MDA-MB-231 cells by 60% in both, 2D (plastic) and 3D (matrigel) cultures compared to untreated cells. Cell cycle analysis showed a 140% S-phase prolongation in SCE treated cells compared to untreated cells in 2D conditions. Further, SCE treatment favored spherical aggregate morphology over stellate outgrowths in 3D cultures of MDA-MB-231 cells, coupled by a 70% decrease in their invasive ability as shown by trans-well matrigel invasion assays. RT-qPCR data showed that SCE treatment decreased the mRNA levels of Vimentin in 2D and 3D cultures of MDA-MB-231 cells by 20% and 40% respectively. Twist expression was decreased by 60% in 3D but not in 2D cultures, in contrast to Zeb1 expression, which showed a 25% decrease in 2D cultures but not in 3D conditions. Western blot analysis demonstrated a decrease in Vimentin protein expression in 2D and 3D cultures of SCE treated MDA-MB-231 and a downregulation in N-Cadherin expression in 3D conditions. Bio-guided fractionation showed that the aqueous (Aq) fraction among all the collected fractions retained the activity detected in SCE, whereby Aq fraction treatment decreased proliferation and invasion, prolonged S-phase of the cell cycle, favored spherical clusters over stellate outgrowths and decreased the expression of EMT markers in MDA-MB-231 cells. In conclusion, the tumor suppressive phenotype induced by SCE and the purified Aq fraction is not solely attributed to an inhibition in cellular proliferation but also in conjunction with a partial reestablishment of epithelial integrity as part of mesenchymal-epithelial transition (MET). Currently, studies are underway to further characterize and further purify the aqueous fraction and determine its active component(s).
# CONTENTS

ACKNOWLEDGMENTS .................................................................................. v

ABSTRACT .................................................................................................. vi

ILLUSTRATIONS ......................................................................................... xi

TABLES ........................................................................................................ xii

ABBREVIATIONS ........................................................................................ xiii

Chapter

I. LITTERATURE REVIEW .......................................................................... 1

A. Overview of Sea Cucumber ....................................................................... 2
   1. Distribution and Life Cycle .................................................................... 2
   2. Market and Nutritional Value ................................................................. 3
      a. Market value and commercial consumption ...................................... 3
      b. Cultivation .......................................................................................... 4
      c. Nutritional value ................................................................................. 5

B. Bioactivities from Sea Cucumber .............................................................. 7
   1. Bioactivities from Holothuria Polii ......................................................... 7
      a. Anti-schistosomal/Anti-parasitic activity ........................................... 8
      b. Anti-microbial activity ..................................................................... 9
      c. Anti-cancer activity ......................................................................... 10
   2. Bioactivities from sea cucumbers other than Holothuria Polii ............ 11
      a. Anti-cancer ...................................................................................... 11
      b. Anti-microbial ................................................................................ 22
      c. Anti-viral .......................................................................................... 25
      d. Anti-inflammatory .......................................................................... 26
      e. Anti-coagulant ................................................................................ 26
      f. Anti-hypertension ............................................................................ 28
      g. Metabolic bioactivities ................................................................... 28
      h. Neural regenerative activities ......................................................... 30
C. Bioactivities from Marine Natural Products .................................................. 30
  1. Aplidine ........................................................................................................ 31
  2. Trabectidin (ET-743) ................................................................................... 33
  3. Eribulin mesylate ......................................................................................... 35
  4. Kahalalide F ................................................................................................. 37

II. MATERIALS AND METHODS ................................................................. 39

A. Cell Culture .................................................................................................. 39

B. Drug Preparation and Solvent Control ....................................................... 40
  1. Ethanol Extraction ....................................................................................... 40
  2. Initial Ethanolic Extract Preparation and Control Solvents ..................... 40
  3. Sequential Solvent Fractionation and Controls Solvents ......................... 41

C. Preparation of Cell Pellet from 3D Cultures .............................................. 42

D. Cell Counting Using Trypan Blue Exclusion Assay ................................. 42

E. Protein Extraction and Immunoblotting ..................................................... 43
  1. Total Cellular Protein Extraction from cells plated in 2D cultures .......... 43
  2. Total Cellular Protein Extraction from Cells Plated in 3D Cultures ......... 43
  3. Western Blot Analysis of Proteins .............................................................. 44

F. 3D Morphogenesis Assay ........................................................................... 44

G. RNA Extraction and qPCR ........................................................................ 45
  1. RNA Extraction .......................................................................................... 45
  2. Primers and qPCR Reaction ...................................................................... 45

H. Invasion Assay ............................................................................................. 47

I. Cell Cycle Analysis ....................................................................................... 47

J. Immunoassay of Interleukin-6 (IL-6) ........................................................... 48

K. Griess Reaction Assay for Nitric Oxide (NO) ............................................. 48

L. SDS-Substrate Gel Electrophoresis (Zymography) ................................... 48

III. RESULTS .................................................................................................. 50
A. SCE Decreases MDA-MB-231 Cell Count in 2D and 3D Cultures, Alters Cellular Morphology in 3D Cultures of MDA-MB-231 and Impede Cells in the S-Phase of the Cell Cycle ........................................... 50

B. Partially Purified Aq Fraction Decreases MDA-MB-231 Cell Count in 2D and 3D Cultures, Alters the 3D Morphology and Restrain Cells in the S-Phase of the Cell Cycle .................................................. 56

C. SCE and Aq Treatment Inhibit Trans-well Invasion of MDA-MB-231 ...... 62

D. SCE and Aq Treatment Decrease mRNA Expression Levels of Vimentin But Differentially Regulate Twist1 and Zeb1 ......................... 65

E. SCE and Aq Treatment Rescue the Expression of E-Cad in 3D Cultures and Decrease the Expression of N-Cad and Vimentin in 2D and 3D Cultures of MDA-MB-231........................................... 68

IV. DISCUSSION .................................................................................. 72

V. APPENDIX ....................................................................................... 89

1. Anatomy and Physiology................................................................. 89
   a. Body Plan ................................................................................. 89
   b. Nervous System ..................................................................... 90
   c. Respiratory System ................................................................. 91
   d. Muscular System .................................................................. 94
   e. Skeletal System ................................................................... 94
   f. Reproductive System .............................................................. 94
   g. Circulatory system ................................................................. 95
   h. Digestive System .................................................................. 97
      i. Evisceration: types and mechanism ...................................... 98
      ii. Visceral Regeneration: morphological and cellular mechanisms ......................................................... 99
      iii. Visceral plasticity during aestivation ............................... 102
   i. Body Wall ............................................................................. 103
2. Defense Mechanism ....................................................................... 105
   a. Cuvierian Tubules .................................................................. 105
   b. Regeneration ......................................................................... 106

REFERENCES .................................................................................... 109
# ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Effect of SCE on the proliferation of human breast cancer cell line, MDA-MB-231 in 2D and 3D cultures</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Effect of SCE on the proliferation of normal mouse mammary epithelial cells SCp2</td>
<td>51</td>
</tr>
<tr>
<td>2</td>
<td>Effect of SCE on growth morphology of MDA-MB-231</td>
<td>52</td>
</tr>
<tr>
<td>3</td>
<td>Effect of SCE on cell cycle progression of MDA-MB-231</td>
<td>53</td>
</tr>
<tr>
<td>4</td>
<td>Effect of all isolated organic extracts on the proliferation of MDA-MB-231</td>
<td>54</td>
</tr>
<tr>
<td>5</td>
<td>Effect of Aq fraction on the proliferation of human breast cancer cell line, MDA-MB-231 in 2D and 3D cultures</td>
<td>57</td>
</tr>
<tr>
<td>6</td>
<td>Effect of Aq fraction on growth morphology of MDA-MB-231</td>
<td>58</td>
</tr>
<tr>
<td>7</td>
<td>Effect of Aq fraction on cell cycle progression of MDA-MB-231</td>
<td>60</td>
</tr>
<tr>
<td>8</td>
<td>Effect of SCE and Aq fraction on trans-well invasion potential of MDA-MB-231</td>
<td>61</td>
</tr>
<tr>
<td>9</td>
<td>Effect of SCE and Aq fraction on RNA expression of EMT markers in 2D and 3D cultures of MDA-MB-231</td>
<td>63</td>
</tr>
<tr>
<td>10</td>
<td>Effect of SEC and Aq treatment on the protein levels of several EMT markers in 2D and 3D cultures of MDA-MB-231</td>
<td>66</td>
</tr>
<tr>
<td>11</td>
<td>Effect of SCE and Aq treatment on the protein levels of several EMT markers in 2D and 3D cultures of MDA-MB-231</td>
<td>69</td>
</tr>
</tbody>
</table>
TABLES

Table | Page
-----|-----
1. List of primers used | 46
2. Percentage of SCE treated cells | 55
3. Percentage of SCE treated cells normalized to control | 55
4. Percentage of Aq treated cells | 62
5. Percentage of Aq treated cells normalized to control | 62
6. Expected and observed mRNA expression from cells treated with SCE and Aq under both 2D and 3D conditions | 68
7. Expected and observed protein expression from total cell lysates treated with SCE and Aq under both 2D and 3D conditions | 71
ABBREVIATIONS

°C  Degrees Celsius

%  Percent

/  Per

®  Registered trademark

$  Dollar

µg  Microgram

µl  Microliter

µM  Micromolar

µm  Micrometer

12-MTA  12-methyltetradecanoic acid

13C-NMR  Carbon-nuclear magnetic resonance

1H-NMR  Proton-nuclear magnetic resonance

2D  Two Dimensional

3D  Three Dimensional

5-HETE  5-hydroxyeicosatetraenoic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin Converting Enzyme</td>
</tr>
<tr>
<td>AFP</td>
<td>Alpha-fetoprotein</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis Of Variance</td>
</tr>
<tr>
<td>Aq</td>
<td>Aqueous</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>BAX</td>
<td>Bcl-2–associated X protein</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic Fibroblast Growth Factor</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>BuOH</td>
<td>Butanol</td>
</tr>
<tr>
<td>CAM</td>
<td>Chick Chorioallantoic Membrane</td>
</tr>
<tr>
<td>c-Bid</td>
<td>BH3-interacting domain death agonist</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>Cdc25c</td>
<td>Cell division cycle 25 C</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary DeoxyriboNucleic Acid</td>
</tr>
<tr>
<td>CHCl₃</td>
<td>Chloroform</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
</tr>
<tr>
<td>cntrl</td>
<td>Control</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>DEN</td>
<td>DiethylNitrosamine</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic Acid</td>
</tr>
<tr>
<td>DHEA</td>
<td>24-dehydroechinoside A</td>
</tr>
<tr>
<td>DMEM-F12</td>
<td>Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>DeoxyriboNucleic Acid</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s Phosphate Buffered Saline</td>
</tr>
<tr>
<td>DSEA</td>
<td>Desulfated-Echinoside A</td>
</tr>
<tr>
<td>EA</td>
<td>Echinoside A</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Epithelial Cadherin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene Diamine Tetraactic Acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epithelial Growth Factor</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epithelial Growth Factor Receptor</td>
</tr>
<tr>
<td>EHS</td>
<td>Engelbreth-Holm Swarm</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked ImmunoSorbent Assay</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial Mesenchymal Transition</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic Acid</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal-related Kinase</td>
</tr>
<tr>
<td>ET</td>
<td>Endotoxin</td>
</tr>
<tr>
<td>et.al</td>
<td>et allii</td>
</tr>
<tr>
<td>ET-743</td>
<td>Ecteinascidin-743</td>
</tr>
<tr>
<td>EtAc</td>
<td>Ethyl-acetate</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal Adhesion Kinase</td>
</tr>
<tr>
<td>Fas</td>
<td>Fas cell surface death receptor</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FDA</td>
<td>U.S Food and Drug Administration</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>FuCS</td>
<td>Fucosylated Chondroitin Sulfate</td>
</tr>
<tr>
<td>G0</td>
<td>Gap 0</td>
</tr>
<tr>
<td>G1</td>
<td>Gap 1</td>
</tr>
<tr>
<td>G2</td>
<td>Gap 2</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>GGT</td>
<td>γ-glutamyltranspeptidase</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter type 4</td>
</tr>
<tr>
<td>gp-120</td>
<td>glycoprotein-120</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>H1</td>
<td>Holothuroidin 1</td>
</tr>
<tr>
<td>H2</td>
<td>Holothuroidin 2</td>
</tr>
<tr>
<td>HA1</td>
<td>Holothurin A1</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular Carcinoma</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>HeLa</td>
<td>Henrietta Lacks cells</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IC&lt;sub&gt;30&lt;/sub&gt;</td>
<td>half maximal inhibitory concentration</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible Nitric Oxide Synthase</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>IRS-1</td>
<td>Insulin Receptor Substrate 1</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>KDa</td>
<td>KiloDalton</td>
</tr>
<tr>
<td>KDR</td>
<td>kinase insert domain receptor</td>
</tr>
<tr>
<td>KF</td>
<td>Kahalalide F</td>
</tr>
<tr>
<td>Kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LOX</td>
<td>Lipoxygenases</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>M</td>
<td>Mitosis</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinases</td>
</tr>
<tr>
<td>MCF-10A</td>
<td>Michigan Cancer Foundation cells number 10 A</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Michigan Cancer Foundation cells number 7</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>induced myeloid leukemia cell differentiation protein</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Monroe Dunaway Anderson Metastatic Breast cancer cells number 231</td>
</tr>
<tr>
<td>MDR1</td>
<td>Multidrug Resistance protein 1</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal Epithelial Transition</td>
</tr>
<tr>
<td>Mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>MilliMolar</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinases</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NAFLD</td>
<td>Nonalcoholic Fatty acid Liver Disease</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>Neuronal Cadherin</td>
</tr>
<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide Excision Repair</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>nM</td>
<td>NanoMolar</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>N-terminus</td>
<td>Amino terminus</td>
</tr>
<tr>
<td>O/N</td>
<td>OverNight</td>
</tr>
<tr>
<td>OA</td>
<td>Orotic Acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-Derived Growth Factor</td>
</tr>
<tr>
<td>PE</td>
<td>Petroleum ether</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>PPARα</td>
<td>Peroxisome Proliferator-Activated Receptor α</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene Diflouride</td>
</tr>
<tr>
<td>qPCR</td>
<td>Real-Time PCR</td>
</tr>
<tr>
<td>Rac</td>
<td>Ras-related C3 botulinum toxin substrate</td>
</tr>
<tr>
<td>RNA</td>
<td>RiboNucleic Acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Rose Park Memorial Institute</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>RTKs</td>
<td>Receptor Tyrosine Kinase</td>
</tr>
<tr>
<td>S</td>
<td>Synthesis</td>
</tr>
<tr>
<td>SCE</td>
<td>Sea Cucumber Extract</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SCp2</td>
<td>SubClonal petit 2</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecylsulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>SJAMP</td>
<td>Stichopus Japonicus acid mucopolysaccharide</td>
</tr>
<tr>
<td>SMase</td>
<td>sphingomyelase</td>
</tr>
<tr>
<td>SP</td>
<td>Side Population</td>
</tr>
<tr>
<td>Src</td>
<td>Sarcoma</td>
</tr>
<tr>
<td>STC</td>
<td>Stichoposide C</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue Inhibitor of MMP</td>
</tr>
<tr>
<td>Tm</td>
<td>melting Temperature</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>Topo-2α</td>
<td>Topoisomerase 2-alpha</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet–Visible</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular Endothelial Growth Factor Receptor</td>
</tr>
<tr>
<td>Vol/vol or v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless-related integration site</td>
</tr>
<tr>
<td>-----------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>ZEB</td>
<td>Zinc finger E-box-binding</td>
</tr>
</tbody>
</table>
The oldest undisputed fossils of isolated spicules from sea cucumbers were identified in the Silurian era, 400 million years ago (Gilliland, 1993). Sea cucumbers are an abundant group of marine invertebrates classified as echinoderms (phylum Echinodermata) belonging to the class Holothuroidea (Arndt et al., 1996). A total of 1250 extant species of sea cucumbers have been identified and divided into six orders, Apodida, Molpadiida, Aspidochirotida, Elasipodida, Dactylochirotida, and Dendrochirotida (Paulay, 2014). The most conventional way to separate orders of sea cucumbers is by their oral tentacles (Roberts, 1982), and sometimes, by either reduced or absent tube-feet, but still, this morphology-based phylogenesis is not radically congruent. Much of this uncertainty regarding the evolution and classification of sea cucumbers originates from the fact that this group of echinoderms, unlike others, lack an integrated skeleton, which is considered a vital feature in providing informative characteristics and fossil records (Lafay et al., 1995; Smith et al., 1995). Recently, biologists are working on understanding the evolution of this group based on molecular and morphological phylogenesis in order to remodel the current fundamental classifications at the ordinal and even at the subclass level (Kerr et al., 1999).

Taxonomically, echinoderms are closer to vertebrates than any other invertebrate phyla (Kitto et al., 1998). Their important phylogenetic position makes them an indispensable model system to understand vertebrate evolution in general.
A- Overview of Sea Cucumber

1. Distribution and Life Cycle

Sea cucumbers inhabit benthic (on, in or near the seabed) zones from the intertidal to the deepest trenches, where they are thought to encompass 90% of the seabed biomass (Conand, 2004). Their ability to control the buoyancy of their body and tube-feet, by means of the water vascular system, makes it possible for them to either stick to the bottom of the ocean or actively swim above it relocating to new sites (Li et al., 2013). They tend to live in dense populations populating almost all marine environments with the greatest numbers being found on the sea floor of the Asia Pacific region (Conand, 1990).

Sea cucumbers are known as filters of the oceans, they serve a general purpose whereby they aid in recycling and breaking down debris or detritus organic matter for other animals to feed on (Uthicke, 1999). Basically, sea cucumbers ingest large amounts of sand or sediments and absorb whatever nutrients exist in them, such as algae, molluscan shell fragments, crustaceans, pelagic and benthic foraminifera and diatoms (Moriarty, 1982; Zhang et al., 1995). In sedentary states, sea cucumbers extend their tentacles out in the water in favor of catching food.

Sea cucumbers spawn once a year, usually from June to August, the cues for spawning is believed be environmental (Battaglene, 1999; Hagen, 1996). After spawning, fertilization occurs in the water; for some species of sea cucumbers, fertilization occurs in a basin-like arrangement on the back of the animal where the eggs had been laid (Conand, 1993). The developing egg grows in sea water and hatches giving rise to a planktonic larvae. The developing larvae stays floating in water for up to
70 days finishing its embryonic development, eventually settling at the bottom of the sea as a juvenile sea cucumber (Kelly, 2005). At this stage, the sea cucumber is the most vulnerable to predators, as such, they tend to hide in the sand, sea weed and stones (Kelly, 2005). The development or metamorphosis of the larvae to an adult is generally a long process requiring around four years to culminate (Kelly, 2005).

2. Market and Nutritional Value:

a. Market value and commercial consumption

Among all classes of echinoderms, sea cucumbers are the most extensively commercialized and fished due to their high pharmacological and nutritious value (Kiew et al., 2012). Sea cucumbers, commonly called “bêche-de-mer”, have long been used in folk medicine in South-East Asia and the Middle East against hypertension, asthma, rheumatism, burns, and constipation amongst other conditions (Bordbar et al., 2011; Fredalina et al., 1999). The dehydrated sea cucumber or bêche-de-mer is commercially produced and sold in Asian markets mainly in China, Korea, Indonesia and Japan and trivially exported to parts of North America and Australia (Conand, 2004). In accordance with the latest update of the trade and catch data, Asia and the Pacific are the two most sea cucumber producing regions in the world with around 20,000 to 40,000 tons of dry weight per year (Wen et al, 2010).

The market value of a sea cucumber is usually recognized based on the species being sold, its appearance, the dried animal size, the extent of dryness, the thickness of the body wall and the marketing season (Toral-Granda, 2006). For example, Japan implements the best processing techniques and produce the best quality products reaching 400$ per kg whereas products from china, Indonesia and the Philippines are
considered of lower quality (Wen et al., 2010). It is worth mentioning that certain but not all species of sea cucumbers are edible or of commercial value, mainly the species that are selected for commercial consumption belong to two families and seven genera of Aspidochirotids and one family and one genus of Dendrochirotids (Bruckner et al., 2003).

Considering the immense market demand and the multi-million dollar industry of sea cucumbers, many fisheries, especially in Indonesia and the Philippines, either show evidence of overexploitation or have already collapsed due to the absence of management or restrictions (Toral-Granda et al., 2008). This lack of specific regulations in some areas has endangered certain species of sea cucumbers and depleted others due to poor management, conservation strategies and unguided activities (Kiew et al., 2012). It is reported that populations with densities under a certain threshold may take as long as 50 years to recover (Toral-Granda, 2006).

b. Cultivation

Taking into account the above mentioned risks, cultivation is becoming a must in order to meet market demand and protect endangered species by recovering the wild stocks. Sea cucumber cultivation started in Japan in the 1930’s and is now well established and practiced there, in China, India, Maldives, and Australia (Kelly, 2005). In the past decade or so, enormous improvements in cultivation methods have been implemented and China is now in the post-research stage with large scale reseeding techniques being applied and 50% of the country’s annual needs are sustained from cultivation (Battaglene, 1999). Same goes for Japan, whereby it is estimated that its annual production increased to around 2.5 million juveniles (Battaglene, 1999).
In brief, cultivation usually starts by collecting mature adult sea cucumbers from the wild and culture them in tanks with sea water. Thermal stimulation by increasing the water temperature by 3 to 5°C for 1 hour stimulates spawning (the release of male and female gametes). Once the gametes mix in sea water, fertilization occurs spontaneously and the eggs tend to develop rapidly at a temperature of 28°C. The resulting larvae is fed a gradually increasing amount of certain microalgae species over the larvae lifecycle until it becomes a sea cucumber juvenile (Kelly, 2005). The most critical step of culturing sea cucumbers is the settlement of the juvenile sea cucumber where the suitable settlement cues usually differ between species. In general sea grass and bacteria are used as plates to which the juvenile sea cucumbers adhere and settle (Kelly, 2005). When the juveniles reach a length of 10 to 20 mm they are passaged to sandy substrata and fed algal extracts. This phase usually takes around 6 months and the juveniles reach a length of approximately 2 to 8 cm. The last phase is the growth phase whereby the juveniles are released into copep areas of the sea floor (Kelly, 2005). It is worth mentioning that one of the most vital and demanding aspect of sea cucumber cultivation is the establishment of systems that are adjusted to be species-specific as this would have great impacts on the genetic diversity of the animal.

c. Nutritional value

The remarkable nutritious profile of sea cucumbers comprise an ideal combination of micro and macro-nutrients offering a beneficial dietary source with various physiological functions. Sea cucumbers contain low fat levels, high protein content and are rich in vitamin A, vitamin B1 (thiamine), vitamin B2 (riboflavin), vitamin B3 (niacin) and vitamin C and various essential minerals such as Calcium,
Magnesium, Iron and Zinc along with other minerals namely, Chromium, Manganese, Nickel, Cobalt and Cupper (Wen et al., 2010). Of note, Chromium is considered a toxic heavy metal however, sea cucumbers contain traces of Chromium which have been reported to be essential for many physiological functions mainly involved in the regulation of insulin levels (Anderson, 1997; Chen et al., 2009).

Among the important nutritious benefits of almost all species of sea cucumbers is their amino-acid composition that is rich in essential amino-acids such as threonine, and phenylalanine and other conditionally essential amino-acids like glycine, glutamic acid, aspartic acid, tyrosine, alanine and arginine (Wen et al., 2010). Glycine, glutamic acid and arginine are well known as immune-modulators, glycine being an enhancer of interleukin-2 (IL-2) secretion and phagocytosis, glycine and glutamic acid act as precursors for glutathione production and arginine stimulates T-cell activation and proliferation (Bordbar et al., 2011). In addition, the low lysine to arginine ratio is an added value for its important effect on lowering blood cholesterol levels in patients having hyperlipidemia (Wen et al., 2010; Bordbar et al., 2011). The essential amino-acid composition to total amino-acid content and to non-essential amino-acids in almost all species is close to the ideal pattern recommended by the World Health Organization (WHO) indicating that sea cucumbers are of a great nutritional value (Yuan et al., 2010).

In non-processed sea cucumbers, the protein content is as high as 55% of total animal weight while processed or dried sea cucumbers hold a protein content of about 80 to 90% of total weight (Chen, 2003). An added characteristic to the high protein content of dried sea cucumbers is the presence of insoluble collagen in the body wall.
which is vitally important for hematogenesis and the presence of tryptophan rich gelatin which makes it more beneficial than other gelatins known to contain only non-essential amino-acids (Saito et al., 2002; Ahmed et al., 2004).

Although sea cucumbers contain low fat levels, certain omega acids such as C20:5 (n-3), Eicosapentaenoic Acid (EPA) and C22:6 (n-3), Docosahexaenoic Acid (DHA) had been extracted from *S. chloronotus* in considerable amounts (Fredalina et al., 1999). EPA and DHA are both known to be involved in numerous physiological processes mainly related to immunity, cardiovascular diseases and type II diabetes (Mori et al., 2006). Besides the main macro-nutrients available in sea cucumbers, other exotic molecules such as glycosaminoglycans and chondroitin sulfates, extracted from the gelatinous body wall, are known to be effective against arthritis (Imanari et al., 1999). In addition, saponins or triterpene glycosides from sea cucumbers are heavily studied and show anti-cancer and anti-inflammatory activities (Kim et al., 2012).

Please refer to Chapter V, the appendix, for information about the anatomy and physiology of sea cucumbers.

B- Bioactivities from Sea Cucumber

1. Bioactivities from *Holothuria Polii*

The sea cucumber *Holothuria Polii* belongs to the order Aspidochirotida (Vergara-Chen et al., 2010; Shoukr et al., 1984). This species is the most abundant among sea cucumbers in Lebanon and prominently inhabits the Mediterranean
(Vergara-Chen et al., 2010), yet, very few studies have been conducted to identify bioactivities from this species.

a. Anti-schistosomal/Anti-parasitic activity

Schistosomiasis is a chronic human parasitic infection caused by flat worms known as blood flukes (trematode worms) belonging to the genus Schistosoma (Adam et al., 2005). This disease is considered as the second most devastating parasitic disease after malaria according to WHO. Nearly 243 million people required treatment in 2011 according to WHO’s fact sheet N0115 (updated March 2013) whereby the treatment should be repeated over years. Praziquantel is the recommended treatment for schistosomiasis over the past 30 years however, drug resistance of certain strains against Praziquantel has been reported (Adam et al., 2005; Botros et al., 2005; Ismail et al., 1999).

Ethanol extract prepared from the tegument (skin and its appendages) of *Holothuria Polii* showed an anti-schistosomal activity in mice infected with *Schistosoma mansori*, when administered orally (Mona et al., 2012). A 60% and 90% reduction in worm burden was observed in male and female mice respectively, in 30 days infected mice, along with a reduction in egg count by 56% in 45 days infected mice (Mona et al., 2012). Moreover, scanning electron microscopy (SEM) of recovered worms from treated mice showed pores, blebs, rupturing of tubercles and other alterations that would impair adhesion aiding in the dislodgment of the worm (Mona et al., 2012). These comparative results to Praziquantel suggest that the holothurin (triterpene glycoside) rich ethanol extract from *Holothuria Polii* should be considered for new studies as a source for an anti-parasitic drug or an adjuvant alongside with conventional therapy.
Another study aiming to identify anti-schistosomal activity from methanol extracts of 79 marine organisms, against *Schistosomamansori*, *in vitro*, showed that the strongest activities were found in the extracts of the sea cucumbers *A. echinites* and *Holothuria polii* (Melek et al., 2012). Bio-guided chromatographic separation of these methanolic extracts and structural analysis specified that the active anti-schistosomal activity is attributed to the two triterpene glycosides, echinosides A and B, with IC$_{50}$ of 0.19 μg/ml and 0.27 μg/ml respectively (Melek et al., 2012).

b. Anti-microbial activity:

Being benthic organisms, sea cucumbers are highly exposed to microbial contaminants such as bacteria, fungi and viruses which are considered as a vital source of food, which they rely on for their survival and development. As such, should there exist an anti-microbial mechanism to protect against infections. Studies on *Holothuria Polii*, collected from the coast of Egypt, confirmed the presence of five general Gram-negative bacteria and one genus of Gram-positive bacterium in addition to fungal contamination in their body wall, intestines and gonads (Omran et al., 2013). Histopathological studies of the aforementioned tissues showed no signs of infection in these sea cucumbers (*Holothuria Polii*) (Omran et al., 2013). Two ethanolic extracts from the body wall of *Holothuria Polii* were tested for their anti-microbial activities against a wide range of human pathogenic bacteria and fungi including those identified within *Holothuria Polii* (Omran et al., 2013). The two extracts differ in the mode of preparation whereby extract A is dried using a rotary evaporator at 50°C while extract B was lyophilized using a freeze dryer. Extract B showed anti-fungal activity against all tested fungi while extract A showed anti-fungal activity only against four of the tested...
fungi (Aspergillus niger, Scloretium sp., Aspergillus Flavus, Candida Albicans). Only extract B showed activity against yeast (Malassezia furfur), and against two gram-negative bacteria (A. hydrophila and S. choleraesuis) (Omran et al., 2013). According to the authors, this study on Holothuria Polii comes in agreement with many identified anti-fungal activities among other tested species of sea cucumbers, however, very few studies have shown anti-bacterial activities. The fact that very few anti-bacterial activities from sea cucumbers have been reported in the literature, this doesn’t rule out the validity of this study. The authors related this paradoxical variability to different modes of extract preparations which is highlighted in their own work. Even though extracts A and B were prepared in parallel from the same tissue, different drying methods resulted in only extract B having anti-bacterial activity (Omran et al., 2013).

c. Anti-cancer activity:

The same group that was trying to identify anti-microbial activities in body wall extracts of Holothuria Polii aimed in another study to elucidate the structure of the bioactive compound(s) and test for anti-cancer activities against HCT116 (colon adenocarcinoma) and MCF7 (breast adenocarcinoma) cell lines (Omran et al., 2012). The ethanolic extract was protein-free as tested on sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) electrophoresis and the active compound was identified to be a non-sulfated triterpene glycoside known as bivittoside by means of infrared (IR), proton-nuclear magnetic resonance (1H NMR), ultraviolet–visible (UV–Vis) and mass spectra (Omran et al., 2012). Cytotoxicity analysis showed that the ethanolic extract inhibited the viability of MCF-7 and HCT116 cells in a dose
dependent manner with an IC$_{50}$ of 17.4 μg/ml and 18 μg/ml respectively (Omran et al., 2012).

2. Bioactivities from sea cucumbers other than Holothuria Polii

Besides their nourishing value, sea cucumbers offer a multitude of therapeutic and medically valuable metabolites including fatty acids, sulfated and non-sulfated polysaccharides, cerebrosides and various secondary metabolites with anti-cancer, anti-coagulant, anti-microbial, anti-inflammatory, anti-oxidant and anti-viral bioactivities amongst many other.

a. Anti-cancer

12-methyltetradecanoic acid (12-MTA) is a branched chain fatty acid derived from Fronadol, a patented product of the sea cucumber Cucumaria frondosa (Yang et al., 2003). Fronadol has shown anti-inflammatory activities in mammals by inhibiting lipoxygenases 5 and 12 (5-LOX and 12-LOX) (Collin, 2001). 5-LOX and 12-LOX are known to be involved in the eicosanoid metabolism which requires the oxidation of arachidonic acid (AA) to form eicosanoid products such as 5-hydroxyeicosatetraenoic acid (5-HETE) (Sala et al., 2001). Evidence from prostate cancer cell lines and human prostate tumor tissues have shown a positive correlation between 5-LOX, 5-HETE and carcinogenesis in both models (Anderson et al., 1998; Gupta et al., 2001). Moreover, inhibition of 5-LOX was correlated with suppression of proliferation and apoptosis (Hong et al., 1999). Based on the relationship of eicosanoid metabolism and prostate carcinogenesis, 12-MTA was tested for its apoptotic effect and anti-proliferative activity on five human cancer cell lines (H1299, PC3, A549, MCF-7, and DU145) (Yang et al.,
The highest cytotoxic effect, as measured by MTT, was against PC3 cells with an IC$_{50}$ of 20.45 µg/ml. Using scanning electron microscopy (SEM), morphological changes were detected as early as 1 hour post treatment (25 µg/ml of 12-MTA), whereby cells have shown reduced cell-cell contact (Yang et al., 2003). Over a time period of 24 hours, cells have exhibited profound morphological changes distinctive of apoptotic cell death such as, cell roundness, loss of cell surface cilia, DNA fragmentation and the formation of apoptotic bodies (Yang et al., 2003). This was further confirmed by showing an increase in the percentage of apoptotic cell using propidium iodide (PI) staining and increased caspase-3 activity (Yang et al., 2003). Moreover, 12-MTA selectively reduced formation of the 5-LOX metabolite, 5-HETE, from PC3 cells by 66% (Yang et al., 2003).

Glycosaminoglycans or acidic mucopolysaccharides are among the most important polysaccharides isolated from sea cucumbers. They are water soluble and consist of a long unbranched chain of repeating disaccharide units (Esko et al., 2009). Chondroitin sulfates as the most prevalent (Kusche-Gullberg et al., 2003) and precious metabolite of this family.

**HS**, an anti-thrombotic Heparin-like acidic mucopolysaccharide, isolated from *Holothuria leucospilota*, was shown to also act as an anti-metastatic agent by inhibiting angiogenesis and tumor cell invasion (Zhang et al., 2009). *In vitro*, HS inhibited proliferation, migration, transwell invasion, tube formation ability of VEGF-induced HUVECs (Zhang et al., 2009). Moreover, HS inhibited angiogenesis *in vivo* as assayed in both, formation of bFGF-induced neo-vessels in mouse matrigel plug assay and neo-vascularization in chick chorioallantoic membrane (CAM) assay (Zhang et al., 2009).
Furthermore, HS inhibited the growth and invasion of B16F10 cells in vitro and western blot analysis on B16F10 cells showed a reduced expression of VEGF, MMP-2 and MMP-9 (Zhang et al., 2009). In vivo, HS inhibited metastatic lung foci with no significant effect on the primary tumors of C57BL/6 mice subcutaneously injected with B16F10 cells into their footpads (Zhang et al., 2009). Immunohistochemical analysis of primary tumors also showed reduced levels of VEGF, MMP-2 and MMP-9 (Zhang et al., 2009).

In another study, acidic mucopolysaccharides isolated from the body wall of Stichopus Japonicus have shown anti-cancer effects on rats with diethylnitrosamine (DEN)-induced hepatocellular carcinoma (HCC) (Song et al., 2013). Rats treated with Stichopus Japonicus acid mucopolysaccharide (SJAMP) were all rescued at the end of the experiment (16 weeks) and showed a higher mean body weight with fewer, yet smaller, nodule volumes and neatly arranged hepatic lobules unlike the tumor control group (Song et al., 2013). Observation of hepatic injury revealed significant recovery of SJAMP treated rats as measured by lower serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and γ-glutamyltranspeptidase (GGT) compared to untreated animals, in addition to lower levels of the tumor marker Alpha-fetoprotein (AFP) (Song et al., 2013). Furthermore, immunohistochemical analysis on hepatic tissues from treated animals showed a significant decrease in the levels of the PCNA (a marker for cell proliferation) and increase in the levels of p21 (cell cycle inhibitor), indicating that SJAMP anti-cancer effect could be in part a result of an anti-proliferative effect (Song et al., 2013).
A fucosylated chondroitin sulfate (FucCS) isolated from the sea cucumber *Ludwigothurea grisea*, showed resemblance to motifs of sialyl-Lewis glycans’ present on cancer and immune cells (Borsig et al., 2007). Usually the cell surface of cancer cells display a transformed pattern of glycosylation which commonly correlates with bad prognosis (Hakamori et al., 1996). The presence of highly branched and sialylated oligosaccharides such as sialyl-Lewis is a characteristic of metastatic tumor cells (Kim et al., 1999). This altered glycosylation aid in the binding of carcinoma cells to adhesion molecules such as P and L-selectins found on endothelial cells thus favoring intravasation of circulating tumor cells (Koenig et al., 1998). Moreover, these surface oligosaccharides offer another level of protection to circulating cancer cells by acting as a docking sites for platelets, thus shielding cancer cells from immune detection (Fuster et al., 2005). Several studies on heparin, an anti-coagulant drug, have reported an anti-metastatic and anti-inflammatory activity *in vitro* and in animal models independent of its anti-coagulant activity (Borsig et al., 2001). Basically, heparins’ effect is executed by blocking P and L-selectin on activated endothelial cells thus preventing the binding of cancer and immune cells to adhesion molecules (Borsig et al., 2001). Owing to the fact that FucCS shares structural similarity with glycans on cancer cells, Borsig et al showed that when tested *in vitro*, FucCS impaired the binding of P and L-selectin-Ig chimeras and LS180 adenocarcinoma cells to immobilized sialyl-Lewis at concentrations much lower than those of heparin. When tested *in vivo*, FucCS inhibited tumor cell-platelet association in lung sections of mice injected with MC-38 adenocarcinoma cells coupled with a pronounced decrease in metastatic lung foci (Borsig et al., 2001). Accordingly, the sea cucumber FucCS is much more potent than heparin with no undesirable effects as those observed after heparin treatment (hemorrhage).
Triterpene glycosides or saponins are the most abundant and the most studied class of secondary metabolites isolated from sea cucumbers. They are best known for their cytotoxic effects in tumor cell lines and in pre-clinical animal models. Triterpene glycosides are amphipathic molecules, composed of a glycosidic and triterpenoid moieties, the latter could be associated with either one, two or three sulfate groups (Osbourn et al., 2011). The glycosidic part is extremely variable and consist of one or more sugar units, this variability in structure comes in agreement with the promiscuous bioactivities conveyed by triterpene glycosides.

A wide range of isolated triterpene glycosides from sea cucumbers have shown anti-cancer activities against several cancer models with Frondoside A being the most promising and most studied amongst all. Frondoside A, isolated from Cucumaria frondosa (Jin et al., 2009), has been tested in various cancer cell lines and in various animal models of breast, pancreatic, prostatic and lung cancer, among many others, showing potent anti-cancer properties in almost all reported models.

In relation to pancreatic cancer, frondoside A was able to inhibit the proliferation of AsPC-1 pancreatic cancer cells in vitro by inducing apoptotic cell death as demonstrated by morphological changes, enrichment of subG0/G1 cell population, positive annexin-V and tunnel staining, increase in pro-apoptotic proteins levels (Bax and caspases3/7/9) and the cell cycle inhibitor p21 and a decrease in anti-apoptotic proteins (Bcl-2 and Mcl-1) (Li et al., 2008). In vivo, low concentrations of frondoside A (10 μg/kg/day) were able to inhibit the growth of subcutaneously transplanted AsPC-1 cells in athymic mice without any undesirable effect (Li et al., 2008).
When tested on breast cancer cell lines, frondoside A decreased the viability of MDA-MB-231 cells by 90%, 48 hours after treatment with lower toxicity on normal mammary epithelial cells, MCF-10A (Marzouqi et al., 2011). Up-regulated P53 and caspases 3, 7 and 9 indicates that frondoside A induced apoptotic cell death in MDA-MB-231 cells. Migration and invasion of MDA-MB-231 cells were totally inhibited when treated with non-cytotoxic concentrations of frondoside A (Marzouqi et al., 2011). The growth of MDA-MB-231 tumor xenografts were reduced by 87% after i.p. injections of 100 μg/kg/day of frondoside A for 24 days with no signs of toxicity or undesirable effects either on the animal behavior and body weight or on the levels of white blood cells, red blood cells, platelets, hemoglobin, on serum levels of creatinine, urea nitrogen, lactate dehydrogenase and activities of alanine aminotransferase and aspartate aminotransferase (Marzouqi et al., 2011). Moreover, it was found that frondoside A when added in combination with paclitaxel, it enhances the cytotoxic effect of the latter on MDA-MB-231 cells (Marzouqi et al., 2011).

Adding to the growing body of evidence, that frondoside A may be a promising anti-cancer compound, is a study showing anti-metastatic effects of frondoside A on breast cancer via a novel mechanism whereby frondoside A inhibits lung metastasis by antagonizing prostaglandin E2 (PGE2) signaling through prostaglandin E receptors 2 and 4 (EP2 and EP4) in vitro and in vivo (Ma et al., 2012).

With regard to lung cancer, frondoside A induced apoptotic cell death in LNM35 human lung cancer cells in vitro and reduced the volume of LNM35-xenographs by 44% and mean lymph node weigh of LNM35-bearing mice to half without any sign of toxicity (Attoub et al., 2013). This anti-metastatic effect was
accompanied with partial and complete inhibition of respectively basal and bFGF-induced angiogenesis as monitored by CAM assay. Furthermore, a synergistic effect between frondoside A and the chemotherapeutic agent, cisplatin, was noted. Combined treatment resulted in an increased in the regression of LNM35-xenographs volume from 44%, in case of single treatments, up to 68%, in combination, compared to control (Attoub et al., 2013).

Another triterpene glycoside, **Ds-echinoside A (DSEA)**, is a non-sulfated triterpene glycoside isolated from the sea cucumber *Pearsonothuria graeffei* (Zhao, et al., 2011). DSEA treatment had a toxic effect on Hep-G2 cells in 2D cultures, decreased average cell adhesion rate in 3D cultures and inhibited cell migration and trans-well invasion by more than 80% (Zhao, et al., 2011). Moreover, DSEA induced an anti-angiogenic effect by inhibiting tube formation ability of EVC-304 cells *in vitro* and CAM neovascularization *in vivo* (Zhao, et al., 2011). At the molecular level, DSEA treated Hep-G2 cells showed a decrease in the expression levels of MMP9, an ECM endopeptidase, together with an increase in the expression of TIMP-1 (Zhao, et al., 2011), an endogenous inhibitor of MMP9 (Hornebeck et al., 2005). Furthermore, western blot analysis showed that DSEA inhibited VEGF, a pro-angiogenic factor, and P65 expression in Hep-G2 cells (Zhao, et al., 2011), the latter being known to regulate the expression of both MMP9 and VEGF (Alferez et al., 2008).

**Echinoside A (EA)** is the sulfated counterpart of DSEA, isolated from the sea cucumber *Holothuria nobilis* Selenka and is the first isolated marine derived compound with topoisomerase-2α inhibitory activity (Li et al., 2010). Unlike classical topoisomerase inhibitors used in clinic, EA is a non-intercalative top2α inhibitor, it
induces permanent DNA double strand breaks by competing with DNA for the enzyme thus abrogating the pre-strand passage cleavage/ligation instead of post-strand passage (Li et al., 2010). When tested in vitro, EA was able to induce cell death in 26 human tumor cell lines from diverse tissue origins with an average IC$_{50}$ of 2.3µM (Li et al., 2010). Noticeably, EA was able to induce cytotoxicity in 6 cancer cell lines known to display high degree of drug resistance against referenced compounds such as Adriamycin, vincristine and etoposide (Higgins et al., 2007). In vivo, i.v. injections of 1.5 mg/kg/day of EA inhibited tumor growth of S-180 mouse model and mouse H$_22$ hepatoma model by 88% and 66% respectively, and PC-3 xenograph volume in nude mice by 54% (Li et al., 2010). Due to its potent anticancer activity in preclinical testing and its unique mode of action, EA offers a new and promising category of anticancer agents that could be clinically tested or chemically-optimized for clinical use.

A comparative study of two other triterpene glycosides, Holothurin A1 (HA$_1$) and 24-dehydroechinoside A (DHEA), isolated from Pearsonothuria graeffei, shows that both compounds were cytotoxic against Hep-G2 and ECV-304 cells in vitro, with DHEA being more potent than HA$_1$ on both cell lines (Zhao et al., 2010). HA$_1$ and DHEA showed anti-metastatic effects when tested at non-cytotoxic concentrations, both compounds inhibited cell adhesion to matrigel and to endothelial cells, inhibited migration and trans-well invasion of Hep-G2 cells, again with DHEA showing more potency compared to HA$_1$ (Zhao et al., 2010). In accordance with their anti-metastatic effects, HA$_1$ and DHEA both inhibited the protein levels of MMP9 and increased the expression of TIMP-1 in Hep-G2 cells. Furthermore, HA$_1$ and DHEA disrupted tube formation ability of ECV-304 in vitro and inhibited CAM neovascularization in vivo (Zhao et al., 2010). This anti-angiogenic effect was coupled with a decrease in VEGF
protein levels of HA$_1$ and DHEA treated Hep-G2 cells (Zhao et al., 2010). Contrary to the observed trend, only HA$_1$ was able to inhibit NF-κB expression in Hep-G2 cells (Zhao et al., 2010), indicating that HA$_1$ and DHEA could be instigating their anti-metastatic effects via two different mechanisms of action.

Philinopside E is a sulfated triterpene glycoside isolated from the sea cucumber Pentacta quadrangularis (Tian et al., 2005). PE treatment induced apoptotic cell death in HMECs and HUVECs and 10 tumor cell lines in vitro (Tian et al., 2005). At non-cytotoxic concentrations, PE was able to inhibit directional cell migration, adhesion on fibronectin and tube formation ability of HMECs and HUVECs (Tian et al., 2005). In vivo, PE was capable of suppressing neovascularization as detected by CAM assay (Tian et al., 2005). Testing of PE against two mouse models of sarcoma 180 and hepatoma 22, showed tumor growth suppression by 61% and 59% respectively for each model (Tian et al., 2005). Double immunostaining for endothelial cells (CD31) and apoptosis (TUNEL) revealed a marked PE anti-angiogenic/tumor effects noted by decreased micro-vessel density and branching and an increase in apoptotic cell death of endothelial and tumor cells (Tian et al., 2005). Moreover, PE treatment inhibited protein levels of phosphorylated-VEGF receptor 2 (KDR/Flk-1) and downstream kinase activity of FAK, paxillin, Akt and ERK in VEGF-induced HMEC cells (Tian et al., 2005). Kinetic studies showed that PE treatment was able to inhibit the phosphorylation of KDR induced by VEGF without inhibiting the kinase domain, even at very high concentration, instead it abrogated the interaction of VEGF to the extracellular domain.
of receptor (Tian et al., 2007). And since αvβ3 integrins are known to associate with KDR at the extracellular level upon VEGF activation (Borges et al., 2000), PE treatment was able to hinder this binding and prevent internalization of the complex as seen in coimmunoprecipitation and immunofluorescent assays (Tian et al., 2007). Consequently, downstream integrin cues, communicated intracellularly upon physical interaction with KDR, have been significantly inhibited by PE, as monitored by the phosphorylation levels of downstream targets ERK, FAK, paxillin and Akt in HMECs (Tian et al., 2007). With paxillin playing a major role in providing the infrastructure for the formation of focal adhesion and actin cytoskeleton (Mitra et al., 2005), its alteration by the use of PE was also accompanied by loss of focal adhesions and stress fiber formation (Tian et al., 2007), as such, endothelial cell adhesion has been suppressed due to the effect of PE on adhesion formation and cytoskeletal reorganization and their downstream cues (Tian et al., 2007). Consequently, these studies indicate that PE is a promising anti-cancer compound showing no undesired side effects due to its specific mode of action in contrast to many naturally identified compounds which are either general kinase or cell cycle inhibitors.

**Philinopside A**, another member of the philinopside triterpene glycoside family isolated from the sea cucumber *Pentacta quadrangularis*, shown to have a nonspecific inhibitory activity across a broad spectrum of RTKs including VEGF receptor, FGF receptor-1, PDGF receptor-β and EGF receptor (Tong et al., 2005). As per its inhibitory activity on the aforementioned RTKs, Philinopside A possess anti-cancer and anti-angiogenic effects *in vitro* and *in vivo* (Tong et al., 2005).
Stichoposide C (STC), a triterpene glycoside, isolated from Thelenota anax, induces apoptotic cell death in leukemic (primary leukemic cells, HL-60, THP-1, K562, and NB4) and colorectal cancer cells (CT-26, HT-29, and SNU-C4) with no cytotoxic effects on normal human hematopoietic progenitor cells (Yun et al., 2012). In vivo, STC suppressed tumor growth in mouse CT-26 tumors and HL-60 leukemia xenographs with no evident toxicity (Yun et al., 2012). It was shown that STC signals through both, the intrinsic and extrinsic apoptotic pathways and partly through caspase-dependent pathways (Yun et al., 2012). Little is known about the mechanism of action of STC nevertheless, what is known so far is that STC initiates apoptosis by activating FAS and caspase 8 followed by activation of sphingomyelinease (SMase) (Yun et al., 2012), which is responsible for the release of ceramide from the membrane (Hannun et al., 2008), the latter plays the role of a pro-apoptotic secondary messenger (Hannun et al., 1996). It is known that the activation of SMase correlates with the depletion of GSH and the production of ROS (Liu et al., 1998; Van den Dobbelsteen et al., 1996) which are evidently seen in bothleukemic and colorectal cancer models, along with an increase in ceramide levels, in vitro and in vivo (Yun et al., 2012).

Other sub-families of triterpene glycosides have been isolated from different species of sea cucumbers and have shown pre-clinical anti-cancer activities, to name a few, Argusides A, B, C, D and E isolated from Bohadschia argus JAEGER, have preferential anti-cancer effects against HCT-116 colon adenocarcinoma cell line (Liu et al., 2007; Liu et al., 2008(A); Liu et al., 2008(B)). Hillasides A, B and C isolated from Holothuria hilla LESSON, show cytotoxic effects against several tumor cell lines (Wu et al., 2007; Wu et al., 2006)
Collectively, all these sea cucumber triterpene glycosides are very potent cytotoxic agents towards a wide array of cancer types owing to their structural diversity, particularly in the combination and length of their sugar moieties. Despite the fact that a big a number of saponins have been isolated and identified so far, few have been studied comprehensively such as Frondoside A, Echinoside A and Philinopside E due to their extreme toxicity.

b. Anti-microbial

Antifungal activity is the second most studied biological activity of sea cucumber triterpene glycosides. Among the multiplex of triterpene glycosides isolated from sea cucumbers with antimicrobial activities, methanolic extract from *Bohadschia vitiensis* showed antifungal activity *in vitro* against *Candida albicans*, *Cryptococcus neoformans*, *Sporothrix schenckii*, *Trichophyton mentagrophytes*, *Aspergillus fumigatus* and *Candida parapsilosis* (Lakshmi et al., 2012). Bio-guided fractionation of the methanolic extract indicated that the aqueous extract retained the activity among the four fractions prepared, against *C. albicans*, *C. neoformans*, *S. schenckii*, *T. mentagrophytes* and *A. fumigatus*, and that the activity was endowed to the presence of bivittoside-D in the aqueous fraction (Lakshmi et al., 2012).

Patagonicosides A, B and C, isolated from the ethanolic extract of the sea cucumber *Psolus patagonicus*, and their de-sulfated analogs, showed antifungal activities against *Cladosporium cladosporoides* (Careaga et al., 2011).

Arguside F, impatienside B, and pervicoside D isolated from the sea cucumber *Holothuria (Microthele) axiloga*, and marmoratoside A, 17α-hydroxy impatienside A,
marmoratoside B, 25-acetoxy bivittoside isolated from the sea cucumber Bohadschia marmorata Jaeger showed antifungal activity against 6 fungal strains, Candida albicans, Cryptococcus neoformans, Aspergillus fumigatus, Trichophyton rubrum, Candida tropicalis and Candida krusei (Yuan et al., 2009A; Yuan et al., 2009B).

Bioguided fractionation of the methanolic extract of the sea cucumber Actinopyga lecanora lead to the identification of three triterpene glycosides (Kumar et al., 2007). Holothurin B, a previously known glycoside was among the identified saponins from Actinopyga lecanora, when tested in vitro, holothurin B showed antifungal activity against twenty fungal strains (Kumar et al., 2007).

Besides triterpene glycosides, other molecules isolated from sea cucumbers have been shown to possess antimicrobial activities. Two peptides among the nine identified from protein extracts of Holothuria tubulosa immune cells (coelomocytes) have been chemically synthesized and tested for anti-bacterial and anti-biofilm formation (Schillaci et al., 2013). Based on blasting and in silico comparative analysis of peptides to antimicrobial data bases (Wang et al., 2004), Holothuroidin 1 and 2 (H1 and H2) were selected for biological testing due to their physiochemical properties which mimics previously defined antimicrobial peptides (Schillaci et al., 2013). Both peptides showed activity against planktonic Gram-positive (staphylococcal reference strains and Enterococcus faecalis ATCC 29212) and Pseudomonas aeruginosa ATCC 15442 (strain type) (Schillaci et al., 2013). Also, H1 and H2 obstructed the formation of Pseudomonas aeruginosa ATCC 15442 biofilm by 69.9% and 64.3% respectively (Schillaci et al., 2013). When tested for hemolytic activity, which usually goes along
with most antimicrobial treatments, H1 and H2 showed no detectable toxicity against human RBCs (Schillaci et al., 2013).

Due to the lack of a prominent malaria vaccine and to the development of refractory strains against commonly used anti-malarial drugs, focus have been shifted towards genetic manipulation of anopheles mosquitoes, which act as carrier vectors of this parasite. Several transgenic anopheles mosquitoes have been hypothetically designed in a way that would resist the transmission of malarial parasite and few have been put to practice. Among the tested mosquitoes, it was shown that they either can’t resist the human malaria parasite, *P. falciparum*, or they were less fit than the wild type mosquitoes (Moreira et al., 2004). CEL-III is a calcium dependent hemolytic lectin, isolated from the sea cucumber *Cucumaria echinata*, known to transcend its hemolytic effect by binding to, and inducing pore formation of cell membranes via its interaction with specific cell surface polysaccharides (Hatakeyama et al., 1995). *In vitro* testing shows that CELL-III was able to induce hemolytic effects on human and rat erythrocytes, which acts as a host for the development of the parasite inside the mosquitoes’ midgut (Yoshida et al., 2007). Furthermore, CEL-III was able to bind and induce pores in ookinete, the mature fertilized parasitic zygote, and when incubated with male and female gametocytes, CEL-III inhibited the formation of ookinete by 95% with an IC$_{50}$ of 15nM (Yoshida et al., 2007). Transgenic *A. stephensi*, expressing CEL-III conditionally in the midgut, was shown to be reproductively fit as the non-transgenic mosquitoes (Yoshida et al., 2007). When tested for their effect on uninfected human erythrocytes, blood fed transgenic mosquitoes showed excessive hemolytic activity compared to non-transgenic mosquitoes after histopathological examination of the gut (Yoshida et al., 2007). Subsequently, transgenic mosquitoes impaired the formation of
P. berghei oocyst, from infected rats, and absolutely inhibited the transmission ability to uninfected animals. Most notably, similar results were obtained when CELL-III transgenic mosquitoes were tested for their effect on human Plasmodium development (P. falciparum) (Yoshida et al., 2007).

c. Anti-viral

Several drugs against human immunodeficiency virus (HIV) have been approved by FDA, impeding different stages of the virus lifecycle at the level of fusion or entry (fusion inhibitors (FIs)), integration (integrase inhibitors (INIs)), replication and expression (reverse transcriptase inhibitors (RTIs)) and splicing (protease inhibitors (PIs)) (De Clercq et al., 2002; Ran et al., 2010). However, toxicity is still the main hurdle constraining the usage of these drugs in addition, advent mutant strains of the virus eventually becomes resistant to the treatment (Kagone et al., 2011; Parczewski et al., 2011; El Annaz et al., 2011). Therefore, the search for new effective yet less toxic compounds is still ongoing. A 12.5 KDa depolymerized glycosaminoglycan (FuCS-1), from the sea cucumber Thelenota ananas have shown intriguing results in vitro by suppressing the replication of several resistant strains (T-20-resistant strains) of HIV with no detectable toxicity (Huang et al., 2013). FuCS-1 inhibited the replication of HIV laboratory strains (HIV-1IIIB and HIV-1RF), clinical strains (HIV-1KM018, HIV-1TC-2), RTIs and PIs resistant strains and most notably all T-20 (Enfuvirtide) resistant strains in C8166 and PBMCs with no detectable cytotoxicity (Huang et al., 2013). Most of the tested polysaccharides showing anti-HIV activity were reported to inhibit RT activity however, FuCS-1 appears to mediate its activity via a different mechanism than those of sulfated polysaccharides (Vives et al., 2005). Results show that FuCS-1
inhibited 99% of viral attachment and fusion through binding to viral gp120 glycoprotein thus preventing it from docking to CD4 and co-receptors on target cells which aid in viral attachment and eventually fusion (Huang et al., 2013).

d. Anti-inflammatory

No compound has been comprehensively studied and reported to show promising anti-inflammatory activity besides the patented anti-inflammatory fronadol extracted from sea cucumber Cucumaria frondosa and discussed above. The ethyl acetate fraction, rich in polyphenolics, from the sea cucumber Stichopus japonicas, inhibited the secretion of NO and PGE₂ from LPS induced-RAW264.7 murine macrophages by inhibiting protein and mRNA levels of iNOS and COX-2 respectively (Himaya et al., 2010). Furthermore, the ethyl acetate fraction was able to inhibit the secretion and mRNA levels of TNF-α and IL-1β. The inhibitory activity of the ethyl acetate fraction was attributed to its ability to inhibit MAPK pathway by reverting the LPS induced-phosphorylation of ERK, p38 and JNK in RAW264.7 cells (Himaya et al., 2010).

e. Anti-coagulant

The best known and utilized anticoagulant drug is heparin; its activity is mediated through inhibition of both thrombin and factor Xa, the endopeptidase which converts prothrombin into thrombin (Luo et al., 2013). However, heparin usage is usually restricted in certain clinical conditions due to it hemorrhagic side effects, as a result, the search for an alternative anticoagulant compound with lower risk factors is still on the seek. Several chondroitin polysaccharides isolated from sea cucumbers have shown
promising anticoagulant activity when compared to heparin with minimal hemorrhagic effects. Two polysaccharides, fucosylated chondroitin sulfate (FuCS) and a sulfated fucan isolated from the sea cucumber *Ludwigothurea grisea*, have a similar 2,4-disulfated fucose units that differ in their attachment point (Fonseca et al., 2009); it’s important to mention that the presence of this structural motif and its spatial-organization are well known to favor the anticoagulant activity of the native compound they are linked to (Pereira et al., 1999). FuCS displays a branching pattern of the 2,4-disulfated fucose repeat while the fucan expresses them as components of its linear structure (Fonseca et al., 2009). *In vitro* and *in vivo* results reveal anticoagulant activities, comparable to heparin, with FuCS inhibiting heparin cofactor II and fucan showing anti-thrombin and heparin cofactor II inhibitory effects (Fonseca et al., 2009). When tested for their respective bleeding effects, only fucan was able to induce an anticoagulant activity without any undesirable side effects (Fonseca et al., 2009). For that matter, the localization of the 2,4 disulfated fucose units are essential to the interaction with coagulation cofactors and proteases which eventually controls the bioactivity and side effects.

Another fucosylated chondroitin sulfate (FuCS) isolated from the sea cucumber *Ludwigothurea grisea* induced anticoagulant activity *in vitro* by inhibiting both thrombin activity and heparin cofactor-II, the latter is a serine proteinase inhibitor which rapidly inhibits thrombin (Mourao et al., 1996). Mild acid hydrolysis of the FuCS which releases sulfated fucose branches reduced the anticoagulant activity of the innate FuCS molecule thus indicating a positive correlation between sulfation pattern, its correct spatial organization and the corresponding anticoagulant activity (Mourao et al., 1996). The results were replicated *in vivo* using stasis thrombosis model in rabbits whereby
1.5mg/kg of FuCS completely prohibited thrombosis from occurring 10 min after stasis and the removal of the sulfated branches lowered the efficacy (Mourao et al., 1998; Pacheco et al., 2000).

f. Anti-hypertension:

Angiotensin Converting Enzyme (ACE) increases blood pressure by simply converting angiotensin-I into angiotensin-II, the latter is a forceful vasoconstrictor which has the ability to tighten the vessels. Anti-ACE strategies have been implemented since the discovery of ACEs in plasma by Skeggs in 1956. A novel peptide “MEGAQEAQG”, isolated through a bio-guided study, from protein lysates of the sea cucumber Acaudina molpadioidea have shown promising anti-ACE effects in vitro and anti-hypertension effects in vivo, using a spontaneously hypertensive rat (SHR) model (Zhao et al., 2009).

g. Metabolic bioactivities

The notion that sea cucumbers comprise a remarkable nutritional profile offering various health benefits have been linked to the existence of different families of primary and secondary metabolites with bioactivities directly linked to metabolic processes, particularly those revolving around lipid metabolism. Seven groups of rats’ assigned different feeding regimes namely, control, whole extracts of the sea cucumber Cucumaria frondosa, saponins, polysaccharides, collagen peptides, dregs and non-saponin residues for 28 days.Screenings for serum cholesterol levels, HDL, triglycerides and hepatic lipid concentrations revealed that dietary saponin supplementation from Cucumaria frondosa is responsible for lowering hepatic lipid accumulation and serum
lipids (Hu et al., 2012). The mode by which saponins were able to induce their lipid-lowering effects is via inhibiting pancreatic lipase thus stalling cholesterol and triglyceride absorption (Hu et al., 2012). Another study on dietary saponins isolated from the sea cucumber *Pearsonothuria graeffei*, showed that saponin treatment eased the orotic acid (OA)-induced nonalcoholic fatty acid liver disease (NAFLD) in rats’ and lowered serum cholesterol and triglycerides concentrations (Hu et al., 2010). Screenings have shown that the activity and RNA expression of hepatic lipogenic enzymes such as fatty acid synthase and glucose-6 phosphate dehydrogenase have diminished after saponin treatment along with an enhancement in β-oxidation as measured by the activity of peroxisome proliferator-activated receptor α (PPARα) (Hu et al., 2010). Accordingly, saponin treatment was able to ease fatty liver in rats by inhibiting hepatic lipogenesis and enhancing β-oxidation. Using the same rat model of OA induced-NAFLD, cerebroside isolated from the sea cucumber *Acaudina molpadioides* showed similar easing results as those of saponins on the fatty livers accompanied by an inhibition at the level of hepatic lipogenesis (Zhang et al., 2012). However, cerebroside was unable to enhance β-oxidation as seen after saponin treatment, instead, treatment was accompanied by an increase in serum triglycerides indicating that cerebrosides utilizes the release of haptic triglycerides as a mode of ameliorating fatty livers (Zhang et al., 2012).

Eicosapentaenoic acid-enriched phosphatidylcholine, an exotic lipid fraction rich in EPA, isolated from the sea cucumber *Cucumaria frondosa* (*Cucumaria*-PC) lowered the levels of blood glucose and enhanced insulin secretion and glycogen synthesis in STZ-induced hyperglycemic rats’ (Hu et al., 2013). This anti-hyperglycemic effect is partly mediated by promoting cues correlating with increased insulin secretion as
measured by an increase in the abundance and phosphorylation of phosphoinositide 3-kinase (PI3K) and protein kinase B (PKB) and increase in the expression of insulin receptor substrate-1 (IRS-1) and glucose transporter-4 (GLUT4) (Hu et al., 2013).

h. Neural regenerative activities

Ever since the identification of neuronal progenitor/stem cells by Reynolds and Weiss in 1992 interest in the basic biology of these cells and their tremendous potential for clinical applications to treat neurodegenerative diseases have been exponentially increasing.

A bio-guided fractionation study aimed at identifying compounds that induce neurosphere migration has revealed a new sulfated polysaccharide (SJP) isolated from the sea cucumber *Stichopus japonicas* (Sheng et al., 2012). SJP treated neurospheres, unlike the control group, were able to attach to the bottom of the plate, migrate and form neurite outgrowths (Sheng et al., 2012). Under mitogen deprivation conditions, SJP treatment prohibited apoptotic cell death which was observed in untreated neurospheres. Differentiation markers as for neurons (TUJI) and astrocytes (GFAP) were assessed after SJP treatment and the majority (90%) of the cells were positively stained (Sheng et al., 2012). Furthermore, SJP treated neurospheres increased the RNA and protein expression of N-Cadherin which is known to promote cell-matrix interactions and neurite outgrowths (Sheng et al., 2012; Utton et al. 2001).

C- Bioactivities from Marine Natural Products
It is not a coincidence that most of the drugs utilized in clinic today are either directly isolated from nature or echoes back to a definite natural source. Biodiversity and complexity of metabolites acknowledged from smart natural systems cannot be easily reproduced in a lab. The resulting natural biodiversity stems from a history of interactions in-between organisms themselves and their environments, these metabolites are articulated in a manner that best suit each and every organism in its corresponding environment ultimately favoring its fitness within its habitat. Many areas of research, particularly medical research, have benefitted from this natural biodiversity and examined compounds for their respective therapeutic bioactivities across different systems and diseases. For the next section to rhyme with the purpose of this study, anticancer derived natural products from marine organisms in clinic or undergoing clinical trials will be the primary focus of the coming section.

1. Aplidine:

Aplidine is the generic name and Aplidin® is the trade name, the first marine compound with anticancer properties to enter clinical trials (Chun et al, 1986), it is a cyclic depsipeptide isolated from a Mediterranean tunicate, *Aplidium albicans*. Early studies with this compound heads back to the early 1990’s where Faircloth and colleagues, showed that aplidine anticancer activity is selective for non-small-cell lung cancer, melanoma, ovarian and colorectal cancer across the national cancer institute panel of human cancer cell lines (Faircloth et al., 1997). Early studies against primary tumor cells showed a dose-response on short term and long term exposures of aplidine against colon cancer, non-small-cell lung cancer, melanoma, breast cancer, ovarian cancer and lymphomas (Depenbrock et al., 1998). Also in opening studies, aplidine
showed activity against a variety of xenograft models of human tumors (Urdiales et al., 1996). Early mechanistic studies have shown that aplidine is able to induce apoptotic cell death in Hela cells by inducing an oxidative stress concomitant with an increase in the expression and activity of JNK, p38 MAPK and ERK, ultimately leading to the release of mitochondrial cytochrome c and activation of intrinsic caspases (García-Fernández et al., 2002). In another study in MDA-MB-231, JNK, p38 and ERK were also found to be constitutively active upon aplidine treatment. However, the oxidative stress stimulus eliciting the activation of the aforementioned kinases was found to be glutathione (GSH) depletion whereby aplidine favors oxidized (GSSG) form over reduced (GSH) form of glutathione, the later known to inhibit Rac1 GTPase, an activator of JNK (González-Santiago et al., 2006). Upon GSH depletion, Src, EGFR and JNK become activated leading to the constitutive activation of downstream p38 MAPK and ERK that in turn activate intrinsic effector caspases thus transducing aplidine pro-apoptotic activity in MDA-MB-231 (Cuadrado et al., 2003). In leukemic cell lines and primary leukemic cultures, aplidine was able to activate both, intrinsic (mitochondrial pathway) and extrinsic (Fas/CD95) apoptotic pathways together with the cleavage of c-Bid, an apoptotic mediator known to link the extrinsic and intrinsic apoptotic pathways. When tested on normal primary hepatocytes and resting peripheral blood lymphocytes, aplidine had no cytotoxic effects indicating a selectivity towards tumor cells (Gajate et al., 2003).

Besides the canonical pro-apoptotic activities, aplidine was able to induce apoptotic cell death via an anti-angiogenic mechanism. Aplidine inhibited the secretion of VEGF and the expression of VEGF and VEGFR-1, thus abrogating the autocrine loop essentially needed for the growth of human leukemia cell line MOLT-4 (Broggini
et al., 2003). When tested in vivo using CAM assay, aplidine revealed an anti-angiogenic effect whereby it inhibited spontaneous vascularization, VEGF and FGF-2 induced vascularization and tumor-made vascularization with no noticeable effect on epithelia and fibroblastic components of the CAM (Taraboletti et al., 2004). In vitro, aplidine inhibited VEGF- and FGF-2-induced proliferation, motility and invasion, it also disrupted tube formation of HUVECs when plated on matrigel and inhibited the levels of total and activated MMP-2 and MMP-9 from PMA-activated HUVECs (Taraboletti et al., 2004).

Phase I clinical trials were successfully completed for solid tumors and non-Hodgkin’s lymphoma with aplidine dosage of 7mg/m$^2$ as non-toxic concentrations (Jimeno et al., 2002) administered as an intravenous infusion for 3 hours every 2 weeks (Indumathya et al., 2013). Aplidine is currently in phase II/III clinical trials in a variety of solid tumors and hematologic malignancies. In 2004 it got the orphan drug designation by the FDA and European Medicines Agency for the treatment of multiple myeloma (Indumathya et al., 2013). For a detailed timeline of clinical and physiological assessment of aplidine refer to this reference (Le Tourneau et al., 2013). Moreover, clinical studies of aplidine in combination with other compounds are ongoing because it generally lacks cross-resistance with other known cytotoxic drugs suggesting that aplidine could be a potential therapeutic drug in refractory cancers (Schwartsmann et al., 2003).

2. Trabectidin (ET-743):

Yondelis® is the trade name, ecteinascidin-743 (ET-743) is a marine alkaloid isolated from Caribbean tunicate, Ecteinascidia turbinate, the first marine derived
anticancer compound to reach the market. Initial DNA binding studies showed that ET-743 could specifically bind in a reversible manner to the exocyclic amino group of guanine in the minor groove of DNA with preferred GC triplets flanking the binding site; this interaction is reversed upon DNA double strand separation (Pommier et al., 1996). The interaction between DNA and ET-743 takes place through 5 hydrogen bonds revolving around the alkylated guanine at the level of the DNA minor groove (Pommier et al., 1996). ET-743 is composed of 3 tetrahydroisoquinolone subunits (A, B and C) whereby rings A and B are in direct contact with DNA, subunit C flanks outside of the minor groove without any contact with the DNA, however this subunit seems to be essential for the compound’s potency, any modification to subunit C abrogates the anticancer activities of ET-743 (Zewail-Foote et al., 1999).

A study back in 1999 shows that DNA lesions occurring after DNA alkylation via ET-743 are a result of topoisomerase I recruitment to ET-743-DNA complex (Takebayashi et al., 1999). Initial in vitro studies of ET-743 across the NCI human cell line panel showed cell cycle arrest and apoptosis within the nanomolar range against a variety of tumor types in addition to activities against primary tumor cell cultures freshly explanted from cancer patients (Valoti et al., 1998). The mechanism by which ET-743 renders its anti-proliferative and pro-apoptotic activities is still vague, one investigation revealed the requirement of DNA nucleotide excision repair (NER) mechanism for manifestation of ET-743 activity. Cell lines deficit of the genes responsible for NER (XPG, XPA and XPD) showed resistance to ET-743 however, complementation with wild type genes rescued drug sensitivity, as such, it is confirmed that ET-743 requires NER machinery to induce lethal DNA strand breaks (Takebayashi et al., 2001). Subsequent, in vivo studies confirmed ET-743 activity against murine
leukemia, melanoma, human mammary carcinoma, ovarian and human non-small cell cancer xenografts (Valoti et al., 1998). Nevertheless, the exact mechanism through which ET-743 exerts its anticancer effect is still unknown, several reports proposed different modes of action including DNA bending, interaction with transcription factors, disorganization of microtubules, cell cycle arrest, and the interference of DNA repair mechanisms and topoisomerase I however, most of the proposed mechanisms do occur at high concentrations beyond effective concentration of ET-743. An interesting study done to investigate the effect of ET-743 interference with minor groove binding transcriptional proteins revealed an intriguing finding whereby physiological concentrations of ET-743 interferes with the binding of NF-Y known to bind the gene promoter elements of MDR1 leading to its transcriptional activation and drug resistance (Jin et al., 2000).

Trabectedin is approved as an anticancer drug for soft tissue sarcoma and ovarian cancer, additionally it is currently in phase II/III trials against breast, ovarian and prostate cancers and other solid tumors (Tohme et al., 2011), most importantly, almost all clinical studies so far reports that trabectidin has remarkable antitumor activity and offers a safety profile suitable for chronic administration (Jimeno et al., 2004; Molinski et al., 2009).

3. Eribulin mesylate:

Halichondrin B a polyether macrolide isolated initially from the sponge Halichondria okadai (Molinski et al., 2009) and later from several marine sponges (Wang et al., 2000) with very potent anticancer activities in vitro and in vivo targeting tubulin polymerization and microtubule assembly (Wang et al., 2000). Main hurdles
stumbled the way for this compound to progress towards high scale and clinical studies are mainly the scarcity of sampling and the complexity of synthesizing (requiring 90 steps to be synthesized if started from natural raw material) (Wang et al., 2000). Total synthesis of simple fragments of the compound was made and each tested for its corresponding anticancer activities in favor of identifying a smaller analogue of the natural compound with α and β tubulin binding (Wang et al., 2000). Two fragments identified, ER-076349 and ER-086526, with a mimetic activity to the parent compound Halichondrin B, supported by in vitro and in vivo evidence for G2-M cell cycle arrest and disruption of tubulin based structures (Towle et al., 2001). The process continued with another synthetic macrolytic ketone fragment E7389 (later known as Eribulin Mesylate), showed identical tubulin based antimitotic activity as early as 2 hours similar to Halichondrin B in U937 and LNCap cells which eventually lead to apoptotic cell death starting 12 hours post-treatment (Kuznetsov et al., 2004). The novel mechanism by which E7389 targets microtubules is through inhibiting microtubule end-growth and inducing aggregates without interfering with the shortening process of microtubules, resulting in disorganized mitotic spindle unable to bypass M-phase of the cell cycle, in contrast to regular antimitotic drugs, such as paclitaxel, which affects both the growth and shortening processes (Jordan et al., 2005).

Clinical trials for eribulin mesylate were approved in 2002 and it’s currently in phases II/III as monotherapy against various solid tumors (Molinski et al., 2009) and it was approved to by the FDA in 2010 to treat metastatic breast cancer in patients already received two prior chemotherapies (Indumathya et al., 2013). Preliminary data from several clinical studies reveals an increase in survival rate and manageable tolerability of doses ranging between 1 and 2 mg/m² depending on the treatment cycle and rate of
infusion (Goel et al 2009; Tan et al., 2009; Vahdat et al., 2009; Cortes et al., 2010). In a phase III clinical study, registered under then number NCT00388726, 762 women with recurrence of metastatic breast cancer (treated previously with 2 to 5 chemotherapeutic compounds) were randomized in 2 groups, either receiving eribulin mesylate or a treatment of physician’s choice (TPC). Results showed that eribulin mesylate treated cases had a 33% higher overall survival rate compared to all TPC’s signifying that an increase in overall survival of heavily treated cases within a refractory settings is possible (Cortes et al., 2011).

4. Kahalalide F:

Kahalalides A-F are cyclic depsipeptides isolated from a Hawaiian marine sacoglossan mollusk, *Elysia rufescens* and the green alga *Bryopsis* on which it feeds (Hamann et al., 1996). Among the six peptides, the largest, kahalalide F (KF), contains 13 amino acids and 5-methylhexanoic acid at the N-terminus, recently exhibited very interesting antitumor activity (López-Macià et al., 2001). Primary *in vitro* screenings reveal potent cytotoxicity against a panel of human breast and prostate cancer cells while non-cancerous human endothelial, kidney, breast and prostatic cells amongst others, showed resistance to the treatment by a magnitude of 40 folds than did their cancerous counterparts (Suárez et al., 2003). Also *in vitro* testing showed cytotoxic effects of KF on colon and lung cancer models (Rawat et al., 2006). The mechanism by which KF induces its cytotoxic action didn’t seem to be inhibited neither by overexpressed MDR1 or general caspase inhibitor (Z-VAD-fmk) nor with transcription or translation inhibitors (actiomycin D, cycloheximide) (Suárez et al., 2003). KF didn’t induce cell cycle arrest nor apoptotic cell death, what was observed after KF treatment.
was massive vacuolization of the cytoplasm accompanied by mitochondrial, lysosomal and plasma membrane rupturing and surprisingly a somehow intact nucleus, indicating that cells were dying due to oncosis, a phenomenon which usually culminates in necrotic cell death (Suárez et al., 2003). Of note, it is believed that KF tends to target lysosomes with a propensity towards cells having active lysosomal activity, this best explains its activity against prostate cancer cells which are known to secret a lot of lysosomal proteins, however evidence supporting this hypothesis is still absent.

Kahalalide F is currently in phase II clinical trials against prostate and refractory cancers (Newman et al., 2004; Rademaker-Lakhai et al., 2005) in addition to other solid tumors such as melanoma, NSCLC and hepatocellular carcinoma (Molinski et al., 2009).
CHAPTER TWO
MATERIALS AND METHODS

A. Cell Culture

MDA-MB-231 human mammary adenocarcinoma cell line was kindly provided by Dr. Mina Bissell (LBNL, CA). MDA-MB-231 cells were grown in humidified incubator (95% air, 5% CO2) at 37°C, in RPMI 1640 media (Lonza, Belgium) supplemented with 1% penicillin-streptomycin as well as 10% Fetal Bovine Serum (FBS) (Sigma, St. Louis). This media was changed once every two to three days. When reaching 80% confluency, cells were washed with 1x Dulbecco’s Phosphate Buffered Saline (DPBS) (Lonza, Belgium) then incubated with 2x trypsin-EDTA (containing 5.0g porcine trypsin, 2.0g EDTA, 4NA per liter of 0.9% NaCl; Sigma, St. Louis) at 37°C for 2 minutes (mins). The cells were washed with complete media, and centrifuged at 208x g for 5 mins, and the pellet was re-suspended in the appropriate amount of media and transferred into new culture plates. Low passage number (15 to 25) SCp2 cells were used throughout. Cells were maintained in growth medium (5% FBS) of DMEM-F12 containing 5% fetal bovine serum (FBS), insulin (5 μg/ml) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere (95% Air; 5% CO2).

For three-dimensional (3D) cultures, we used Growth Factor Reduced Matrigel obtained from BD Biosciences (BD Biosciences, Discovery Labwork, Two Oak Park, Bedford, MA, BD No. 354230). 12 well-plates or 6 well-plates were coated with 2-3 mm thick layer, corresponding to 250 μl and 500 μl respectively, of growth factor-
reduced Matrigel. The plates were incubated at 37°C for at least 30 mins for the matrix to solidify. Cells were then counted and diluted in complete media with 2% Matrigel, to achieve a final concentration of 4 x 10⁴ cells for 12-well plate and 8 x 10⁴ cells for 6-well plates, which were then plated on top of the solidified matrix. Clusters start to form by day 2, and cells were treated as specified later and kept in culture up to 5 days post treatment.

B. Drug Preparation and Solvent Control

1. Ethanol Extraction

The Sea Cucumbers were dissected into small pieces, around 2 cm³, washed with distilled water and snap frozen in liquid nitrogen. The small pieces were freeze dried for 2 days and then pulverized using a blender and the resulting powder is stored at -20°C. The powdered material is reconstituted in 80% ethanol, for each 1 gram of tissue 10 ml of 80% ethanol was added, then the solution was homogenized well for five times using a homogenizer, left for 10mins at room temperature and centrifuged at 700x g for 10mins. The supernatant was collected, filtered through 100 µm nylon mesh and lyophilized.

2. Initial Ethanolic Extract Preparation and Control Solvents

The lyophilized material was reconstituted in PBS and dimethyl sulfoxide (DMSO) (10:1-v/v), referred to hereafter as “10% DMSO”. The 10% DMSO solution was vortexed for 1 min and centrifuged at 17,000x g for 10 mins. The supernatant was filtered through 0.2 µm nylon mesh and the resulting ethanolic extract referred to here after as Sea Cucumber Extract (SCE) is added on the cells as specified in each
condition; the stock was aliquoted in 1 ml tubes and stored at -20°C. Serial dilutions were made so that all treated conditions contain the same % of PBS/DMSO as the highest concentration, in addition, an SCE free medium referred to as “sham” containing also equal amounts of PBS/DMSO as the highest concentration was tested in parallel in all experiments.

3. Sequential Solvent Fractionation and Controls Solvents

The lyophilized material from the ethanol extraction was re-suspended in water and methanol (MeOH) (10:1-v/v), referred to here after as “10% MeOH”; for each 1g of lyophilized material 5ml of 10% MeOH was added. The 10% MeOH solution was vortexed for 1 min and partitioned first against petroleum ether (PE) solvent in a ratio of 1:2 v/v (10%MeOH:PE). The mixture of 10% MeOH and petroleum ether was mixed in a separatory funnel which was kept for 15 mins at room temperature resulting in an observable separation of 2 phases, one being organic (PE) and the other an aqueous (Aq) layer. The Aq phase was re-partitioned for a second run with PE also in a ratio of 1:2 and the resulting PE layers from the two extractions were pooled. The remaining Aq phase is now partitioned with chloroform, the same steps were followed as per the extraction with PE and the resulting two fractions of chloroform were pooled and the Aq phase was passaged to be partitioned in ethyl acetate and butanol respectively. The organic layers of the four solvents (PE, chloroform, ethyl-acetate and butanol) were evaporated by nitrogen evaporator while the Aq layer was lyophilized. The yield was 13.5%, 3%, 3.5%, 26% and 54.2% for PE, chloroform, ethyl-acetate, butanol and Aq respectively, out of the initial mass. The resulting mass from every fraction was dissolved in 10% DMSO centrifuged at 17,000x g and the supernatant filtered through
0.2 µm nylon mesh and added to the cells as indicated in each condition. In parallel to this sequential fractionation of SCE, a control of 10% MeOH free of SCE was partitioned similarly and sequentially in all the aforementioned solvents and evaporated in the same way resulting in five control fractions. Cells were treated with the same volume either from the control fractions or the fractions containing SCE.

C. Preparation of Cell Pellet from 3D Cultures

Cells were plated in 6 well plates, as described previously, and kept for 5 days post treatment in culture unless specified otherwise. At day 5, old media was removed and the well was washed with 1x PBS. Then, the culture dish was placed at 4 °C on a shaker and each well was supplemented with 2 mL of 2.5 milliMolar (mM) PBS-EDTA. After a 60 mins incubation, the volume in each well was collected into 15 ml conicals and left to settle on ice for 10mins. The mixture was centrifuged at 208x g for 5 mins, supernatant removed and pellet washed with 1x PBS. The mixture is subsequently re-centrifuged at 208x g for 5 mins and the pellet is recovered for cell counting, protein and RNA extraction.

D. Cell Counting Using Trypan Blue Exclusion Assay

MDA-MB-231 cells were plated in 12 well plates at a density of 4 x 10^4 cells in each well. The cells were counted from triplicates after 24, 48, and 72 hours post treatment. First, media was removed, and the cells subsequently trypsinized and collected in the initial media of each plate that contains dead cells. Cells were then diluted in Trypan Blue (1:1) ratio (v/v) and counted using a hematocytometer. Experiments were repeated at least three times.
For 3D proliferation assessment, MDA-MB-231 cells were plated in triplicates in 12 well plates at a density of $4 \times 10^4$ cells in each well. The cells were maintained for 5 days post treatment and counted on a daily basis. On each time point, cell pellet was collected as described previously and trypan blue counting was pursued similar to counting from 2D cultures. Statistical significance was determined using one-way ANOVA and Post-Hoc.

**E. Protein Extraction and Immunoblotting**

1. *Total Cellular Protein Extraction from cells plated in 2D cultures.*

   MDA-MB-231 cells are collected at 80% confluency, washed twice with 1X PBS and frozen at -80 °C. Cells were scraped through 300 μl of lysis buffer (50mM Tris-Cl, pH 7.5, 150mM NaCl, 1% Nonidet P40, 0.5% Sodium deoxycholate) to which 40 μl/ml Protease inhibitors (CompleteTM) were added. The mixture was sheared by passage through 27-gauge needle, and then centrifuged at 14,000x g for 30min at 4°C. The supernatant was stored at -20°C. DC Protein Assay (Bio-Rad, Hercules, CA) was used to quantify proteins using different concentrations of bovine serum albumin (BSA, Sigma Chemical Co.) as standards. Protein extracts were then mixed with 2x sample buffer with 10% β- mercapto-ethanol in a 1:1 ratio (v/v) at the time of gel electrophoresis.

2. *Total Cellular Protein Extraction from Cells Plated in 3D Cultures.*

   Using the 3D culture cell pellet obtained as previously described, total protein extraction was performed by adding 150μl lysis buffer and proceeding similarly to protein extraction from two-dimensional cultures.
3. Western Blot Analysis of Proteins

On basis of equal protein loading, protein extracts were resolved on polyacrylamide gels under denaturing conditions of SDSpage electrophoresis running buffer (39mM glycine, 48mM Tris base and 0.037% SDS). After electrophoresis, proteins were transferred on PVDF membrane (BioRad, Hercules, CA) using wet blot apparatus in transfer buffer (39mM glycine, 48mM Tris base, 0.037% SDS and 20% methanol). Blocking of the membranes was carried out for 1.5-2 hrs in wash buffer (100mM Tris-Cl, pH 8, 150mM NaCl, 0.1% Tween-20) with 5% skimmed milk, then incubated O/N at 4°C with 1% milk in wash buffer with the primary antibody of interest (dilutions were as specified by company’s datasheet). The bound antibody was detected by addition of horse raddish peroxidase- conjugated anti-rabbit IgG or anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) followed by enhanced chemiluminescence (ECL, Santa Cruz). All incubations were performed at room temperature (RT). Equal loading was determined by probing total extracts for mouse anti-GlycerAldehyde 3-Phosphate DeHydrogenase (GAPDH) (1:5000 – v/v) and mouse anti-actin (1:10000 – v/v), while Lamin A/C (1:1000) was used to determine equal nuclear extract loading.

F.3D Morphogenesis Assay

MDA-MB-231 cells were plated in 12 well plates as described previously. A minimum of ten fields of each well were imaged at low power. Colony morphology was quantified by counting the number of spherical versus stellate colonies within each image. Equal numbers of colonies were counted and the % of stellate and spherical colonies were calculated. A colony was considered stellate if it had two or more
extensions from the central colony of cells. Statistical significance was determined using one-way ANOVA.

G. RNA Extraction and qPCR

1. RNA Extraction

Total RNA was extracted from cells using RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions and stored at -20 °C overnight. Cells plated in 2D and 3D cultures were freshly subjected to RNA extraction. In the case of cells plated on 3D, the cell pellet was obtained as previously described. Reverse-transcription PCR was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The program included a reverse transcription step at 47°C for 30 mins, followed by enzyme deactivation at 94°C for 2 mins. This was followed by a cycle of 94°C for DNA denaturation, 55°C for primer annealing, 72°C for extension, and the cycle was repeated between 25 and 35 times, depending on the transcript abundance. At the end of the cycles, the PCR was concluded with a final elongation stage at 72°C for 5 mins, and the samples were then stored at 4°C till used. We then checked for DNA and protein contamination of our samples using the Nanodrop 2000c UV-VIS Spectrophotometer (Thermo scientific).

2. Primers and qPCR Reaction

The primers (TIM MOLBIOL, Berlin, Germany) were selected based on verified sequences (http://pga.mgh.harvard.edu/primerbank/). The primer sequence and amplicon lengths, end specificity, G/C-contents, absence of secondary structures and melting temperature (Tm) differences were checked in silico. Non-specific amplicons
were not specifically found, when performing the BLAST-search (www.ncbi.nih.gov/BLAST) for each primer pair. We used iQ SYBR Green Supermix (Biorad Laboratories, California) to conduct our quantitative Polymerase Chain Reaction (qPCR) reactions in triplicates in a CFX96 Real-Time PCR Detection System (Bio-Rad), using conditions suggested by the manufacturer and tested with each primer pair for optimal temperature and concentration. Reactions were adjusted to a final volume of 20µl/well using 4µl of cDNA, 2µl of each primer (150nM), 2µl of nuclease free water and 10µl of Master Mix.

The qPCR program was as follows: Hot start at 95°C for 3 mins, followed by 39 two-step cycles (10 seconds at 95°C, 30 seconds at annealing temperature). A melt analysis (ramping from 65°C to 95°C, rising the temperature by 0.5°C at every step with 5-seconds interval), exhibited only one peak characterizing a homologous amplicon in respective runs. Raw data were analyzed using Bio-Rad CFX Manager software v 62.1 (Qiagen, Vienna, Austria). The run validity can be estimated by $R^2$-value and reaction efficiency (E).

Table 1: List of primers used.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Annealing temperature (°C)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>AAGGTGAAGGTCGGAGTCAAC</td>
<td>58</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGGGTCAATGGGCAACAATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vimentin</td>
<td>Forward</td>
<td>AGTCCACTGAGTACCAGGAGAC</td>
<td>58</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGTTCCTTTAAGGGCATCCAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zeb1</td>
<td>Forward</td>
<td>TTACACCTTTACATACAGAACC</td>
<td>58</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TTTACGATTACACCCAGACTGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Twist1</td>
<td>Forward</td>
<td>GGGCGTGGGGGGCGCACTTTTA</td>
<td>66</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CGCTGCCCCTCGTGGAATCA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**H. Invasion Assay:**

Six well plates were fitted with inserts (8 μm pore size). The inserts were coated with 400μl of EHS-Media (0% FBS) solution in a ratio of 1:3 or 1:20 (v/v) and incubated at 37°C for 2 hrs. eGFP transfected MDA-MB-231 (transfected with an empty 4.7Kilobases (Kb) pEGFP-N1 vector plasmid) were plated at a density of 8 x 10⁴ cells per inserts, and left overnight (8 hours at most) to adhere. The next day, the cells were treated, as indicated later, and left for 24 hours to invade through the Matrigel. 24 hours later, the cells were fixed using 4% formaldehyde in PBS for 20 minutes at room temperature (or kept at 4°C for a maximum of 2 weeks). The cells towards the inside of the insert were removed by using a cotton swab. The insert is then cut and mounted on a microscopic slide using Vector labs hardmount fluorescence media and examined by fluorescence microscopy. Fluorescent cells that successfully invaded through the matrix and the 8μm pores were counted (10 random fields at low magnification) and plotted on a histogram as percentage of invaded cells relative to the control (untreated) cells.

**I. Cell Cycle Analysis:**

Cell pellets from 2D cultures were prepared on day 1, 2 and 3 post treatment, while cell pellets from 3D cultures were prepared on day 3, 4 and 5 post treatment. On the given time point’s, cells were trypsinised and centrifuged at 208g’s for 5min at 4 °C. The pellet was resuspended in ice cold 70% ethanol and stored at -20°C (a minimum of O/N and a maximum of two weeks). The fixed cells were then centrifuged (208 g’s, 5 min, 4°C) to remove the ethanol, and the cellular pellet was then washed with 1XDPBS.
DNAse free RNase A was added at a concentration of 0.2mg/ml (150μl) over the cellular pellet and the cells were kept at 37°C in a dry bath for an hour and thirty minutes to allow full digestion of RNA. The pellet was washed twice again in 1XDPBS before final re-suspension of cells with 420 μl of 1XDPBS into flow tubes (BD falcon, USA). 30 μl of 2mg/ml propidium iodide were then added to each flow tube and the cells were analysed on FACScan (Becton Dickinson, San Jose, CA).

J. Immunoassay of Interleukin-6 (IL-6)

IL-6 secretion in response to ET treatment in SCp2 cells was measured by enzyme-linked immunosorbent assay (ELISA). The collected medium secreted by control and treated cells, at the time points indicated post-ET treatment, was assayed using ELISA against IL-6 (DuoSet kit; R&D Systems Inc, Minneapolis, MN) according to the manufacturer’s protocol. Samples were assayed in duplicates and data presented as the average of IL-6 (pg/ml) of three experiments ± standard deviation (SD) based on a standard known standard of IL-6.

K. Griess Reaction Assay for Nitric Oxide (NO)

NO was measured by the Griess reaction assay that measures nitrite (the stable spontaneous oxidation product of NO) using a Griess Reagent Kit (Molecular Probes, Eugene, OR) according to the manufacturer’s protocol. Samples were assayed in duplicates and data is represented as an average concentration of NO₂ from three independent experiments ± SD (μM ± SD).

L. SDS-Substrate Gel Electrophoresis (Zymography)
Gelatinase activity in the medium of control and treated samples was analyzed on the basis of equal loading volumes of samples on a 10% polyacrylamide gel impregnated with gelatin (3mg/ml). The gels were run in electrophoresis running buffer (25 mM TRIS-base, 192mM glycine; 0.1% SDS, water) followed by two washes at room temperature, 30 mins each, in 2.5% Triton X-100 solution. After washing the gels were incubated for 16-20 hrs in development (substrate) buffer (50mM Tris HCl; 5mM CaCl$_2$; 0.02% NaN$_3$; 0.5% Triton X; Water). The gels were then stained for at least 2 hours, at room temperature, in 0.1% comassie blue R-250, in 50% methanol, and 10% acetic acid. The gels were then destained for 15 min in destaining solution (MeOH: acetic acid: water 4.5:1:4.5). The gelatinase activity was visualized as clear white bands on darkly stained blue gels and analyzed by Gel documentation (Bio RAD) using the software Quantity 1.
CHAPTER THREE

RESULTS

A. SCE Decreases MDA-MB-231 Cell Count in 2D and 3D Cultures, Alters Cellular Morphology in 3D Cultures of MDA-MB-231 and Impede Cells in the S-Phase of the Cell Cycle

To test if Holothuria polii holds any compound(s) with anticancer bioactivity, whole body tissues were extracted with 80% ethanol, lyophilized and dissolved in PBS/10%DMSO; this ethanolic extract will be referred to hereafter as SCE (Sea Cucumber whole Extract).

To assess whether SCE holds any anticancer activity, MDA-MB-231 breast cancer cells were treated with different concentrations of SCE, then cellular proliferation, cell cycle analysis and morphology were assessed in 2D and 3D cultures. To monitor cellular proliferation of MDA-MB-231, cell counts using trypan blue exclusion assay was done across a period of 3 days in 2D cultures and 5 days in 3D cultures. 2D-growth analysis showed that SCE treatment at concentrations of 1, 2 and 3mg/ml decreased cellular proliferation at day 3 by 25%, 55% and 70% respectively, compared to control and sham treated cells (Figure 1A). In 3D cultures, SCE treatment at 2mg/ml showed no effect while a 30% and 60% decrease in proliferation was observed by day 5 at concentrations of 4 and 6mg/ml respectively (Figure 1B). The percentage of dead cells, in both 2D and 3D cultures, stained by trypan blue was not significant and did not exceed 5% across all conditions and time points.
Figure 1: Effect of SCE on the proliferation of human breast cancer cell line, MDA-MB-231 in 2D and 3D cultures. SCE significantly reduced growth in both 2D and 3D cultures of MDA-MB-231 with no significant cell death (≤10%) across all conditions. (A) 2D-Growth curve analysis by trypan blue counting showed 50% and 60% decrease in cell counts by day 3 at SCE concentrations of 2mg/ml and 3mg/ml respectively. (B) 3D-Growth curve analysis by trypan blue counting showed no effect, 30% and 60% growth inhibition by day 5 at concentrations of 2mg/ml, 4mg/ml and 6mg/ml respectively. Statistical analysis from three independent experiments revealed significant differences represented by, (***) asterisks for p<0.001, (**) asterisks for p<0.01, and (*) asterisk for p<0.05.
In a parallel study to check SCE for anti-inflammatory activities in a normal mouse mammary epithelial cell model, SCp2 cells were less sensitive to SCE treatment and showed no significant decrease in proliferation at 2.5 mg/ml and a 35% decrease at 10 mg/ml by day 4 (Figure 2). Moreover, proliferation of SCp2 cells treated with SCE totally recovered and achieved growth rates comparable to untreated cells 24 hours after treatment wash-out (Figure 2), indicating that the anti-proliferative effect of SCE is reversible and induces no permanent damage. This recovery in proliferation after treatment wash-out is yet to be tested on MDA-MB-231 cells.

![SCp2 Recovery After SCE Treatment](image)

**Figure 2:** Effect of SCE on the proliferation of normal mouse mammary epithelial cells SCp2. Growth curve analysis of SCp2 treated cells and their subsequent recovery after treatment wash-out by trypan blue counting.

No morphological changes could be observed by day 3 between control and SCE treated MDA-MB-231 when grown in 2D conditions (Figure 3A left panel), whereas 3D growth morphology analysis showed that SCE treatment increased spherical formation (white arrow heads) and reduced stellate outgrowths (white arrows) by more than 60% when compared to control at a concentration of 6 mg/ml by day 5 (Figure 3A right panel and 3B).
(A) [Images of cell cultures with different conditions: ctrl, SCE 3 mg/ml, and SCE 6 mg/ml.]

(B) [Bar graphs showing the percentage of stellate and spherical clusters for different days and concentrations of SCE mg/ml.]

- DAY 3:
  - Stellate %: 29, 27, 38, 56, 80
  - Spherical %: 71, 73, 62, 44, 20

- DAY 4:
  - Stellate %: 21, 25, 38, 49, 62
  - Spherical %: 79, 75, 62, 51, 38

- DAY 5:
  - Stellate %: 18, 30, 40, 53
  - Spherical %: 84, 70, 60, 47

- CNTRL: [Data for control conditions]
Figure 3: Effect of SCE on growth morphology of MDA-MB-231 in 3D cultures.

(A) Representative phase contrast images of cntrl and treated (SCE 3mg/ml) cells at day 3 in 2D cultures (Left panel) and of cntrl and treated (SCE 6mg/ml) in 3D cultures (Right panel). White arrows indicate stellate aggregates and white arrow heads represent spherical colonies. (B) Histogram analysis of the percentages of stellate versus spherical clusters on days 3, 4 and 5 post SCE treatment showed that SCE increased spherical formation and reduced stellate outgrowths by more than 60% at 6mg/ml up to day 5 in culture. Scale bars (in white) = 100µm. Statistical analysis from three independent experiments revealed significant differences represented by, (***) asterisks for p<0.001, (**) asterisks for p<0.01, and (*) asterisk for p<0.05.

The noted inhibition in proliferation of MDA-MB-231 cells after SCE treatment was accompanied by a delay in the S-phase of the cell cycle. A 140% increase in the amount of cells trapped in the S-phase was observed compared to the control at an SCE concentration of 3mg/ml by day 3 (Figure 4D). As expected, cells impeded in the S-phase was complemented by a decrease in the percentage of cells in G0/G1 and G2/M phases of the cell cycle (Figure 4A, 4B and 4C).
Table 2: Percentage of SCE treated Cells

<table>
<thead>
<tr>
<th>Time</th>
<th>Pre Go</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
<th>Sum</th>
</tr>
</thead>
<tbody>
<tr>
<td>cntrl</td>
<td>0.68</td>
<td>59.72</td>
<td>18.17</td>
<td>17.41</td>
<td>95.98</td>
</tr>
<tr>
<td>SCE 1mg/ml</td>
<td>0.66</td>
<td>59.65</td>
<td>24.59</td>
<td>12.45</td>
<td>97.35</td>
</tr>
<tr>
<td>SCE 2mg/ml</td>
<td>0.95</td>
<td>67.69</td>
<td>19.78</td>
<td>10.03</td>
<td>98.45</td>
</tr>
<tr>
<td>SCE 3mg/ml</td>
<td>1.05</td>
<td>62.03</td>
<td>19.67</td>
<td>14.82</td>
<td>97.57</td>
</tr>
</tbody>
</table>

Table 3: Percentage of SCE treated Cells normalized to control

<table>
<thead>
<tr>
<th>Time</th>
<th>Pre Go</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
<th>Sum</th>
</tr>
</thead>
<tbody>
<tr>
<td>cntrl</td>
<td>0.72</td>
<td>57.67</td>
<td>16.66</td>
<td>21.92</td>
<td>96.97</td>
</tr>
<tr>
<td>SCE 1mg/ml</td>
<td>9.26</td>
<td>50.57</td>
<td>18.83</td>
<td>16.97</td>
<td>95.63</td>
</tr>
<tr>
<td>SCE 2mg/ml</td>
<td>15.14</td>
<td>54.87</td>
<td>16.36</td>
<td>11.71</td>
<td>98.08</td>
</tr>
<tr>
<td>SCE 3mg/ml</td>
<td>9.22</td>
<td>47.52</td>
<td>26.11</td>
<td>15.1</td>
<td>97.95</td>
</tr>
</tbody>
</table>

Figure 4: Effect of SCE on cell cycle progression of MDA-MB-231 in 2D cultures. SCE treatment delayed the S-phase of the cell cycle in 2D cultures of MDA-MB-231. (A) Fluorescence histogram plots showing cell counts (Y-axis) versus fluorescence intensity (X-axis) with green circles demonstrating the amount of cells in the S-phase in both cntrl and treated conditions. (B) Table showing percentages of cells in pre-G0, G0/G1, S, G2/M.
G0/G1, S and G2/M phases of the cell cycle over 24, 48 and 72 hours post-treatment. (C) Table showing percentages of cells in pre-G0, G0/G1, S and G2/M phases of the cell cycle normalized to the cntrl over 24, 48 and 72 hours post-treatment. (D) Quantitation showing the percentage of S-phase cells, normalized to cntrl, on 24, 48 and 72 hours post-treatment. SCE delayed the cell cycle by inducing a 140% increase in the amount of cells trapped in the S-phase compared to the cntrl at a concentration of 3mg/ml by 72 hours post treatment. Statistical analysis from three independent experiments revealed significant differences represented by, (***) asterisks for p<0.001, (**) asterisks for p<0.01, and (*) asterisk for p<0.05.

B. Partially Purified Aq Fraction Decreases MDA-MB-231 Cell Count in 2D and 3D Cultures, Alters the 3D Morphology and Restrain Cells in the S-Phase of the Cell Cycle

After an initial insight into the prospective anticancer activity of SCE, a bio-guided fractionation was conducted for identifying a purified fraction retaining the anticancer activity against MDA-MB-231 recognized in SCE. The lyophilized ethanol extracted powder was dissolved in deionized water/10% methanol and successively partitioned in 4 organic solvents of increasing polarity, petroleum ether (PE), chloroform (CHCl₃), ethyl-acetate (EtAc) and n-butanol (BuOH), at a ratio of 1:2 v/v (SCE:solvent). The solvent fractionation procedure generated 5 fractions, four extracts for each of the organic solvents and a remaining aqueous (Aq) fraction. In parallel to this sequential fractionation of SCE, a control of deionized water/10% methanol free of SCE was partitioned similarly and sequentially in all the aforementioned solvents resulting in five control fractions.

To test whether any of the five isolated extracts retain the anticancer activity identified in SCE, MDA-MB-231 cells were treated with comparable concentrations to SCE from each fraction and counted across 3 days using trypan blue exclusion assay. The trend shows no significant decrease in proliferation as measured by cell counts in
CHCl₃ and BuOH treated cells at concentrations up to 2mg/ml and 1.5mg/ml respectively (Figure 5B and 5D). PE and EtAc treatments were either ineffective at concentrations up to 1.5mg/ml and 0.5mg/ml or cytotoxic at 2mg/ml and 1.5mg/ml respectively (Figure 5A and 5C), reflecting a very small margin between the minimal active and cytotoxic concentrations which does not correlate with what was noted in SCE.
Figure 5: Effect of all isolated organic extracts on the proliferation of MDA-MB-231 in 2D cultures. Growth analysis by trypan blue showed that (A) PE extract did not affect cell proliferation at 1 and 1.5 mg/ml and was cytotoxic at 2mg/ml. (B) CHCl₃ did not inhibit proliferation up to 2mg/ml. (C) EtAc extract is cytotoxic on cells at 0.5 and 1.5 mg/ml. (D) n-BuOH extract showed no effect on proliferation up to 1.5mg/ml.

In contrast with the four organic fractions tested, the Aq fraction significantly reduced the proliferation of MDA-MB-231 over a period of 3 days in culture without inducing any cytotoxic effect, comparable to what was confirmed in SCE. A decrease by 50%, 60% and 80% in cellular proliferation was detected at 2, 4 and 6mg/ml of Aq treatment by day 3 (Figure 6A), with no significant cell death (≤10%) across all conditions. To confirm whether this decrease in proliferation observed in 2D could translate to 3D cultures as noted in SCE, cells were grown on matrigel (3D), treated with different concentrations of Aq fraction for a period of 5 days and cell counts were monitored. 3D-Growth analysis showed a 40% and 60% growth inhibition by day 5 at Aq concentrations of 6mg/ml and 9mg/ml respectively (Figure 6B).
Figure 6: Effect of Aq fraction on the proliferation of human breast cancer cell line, MDA-MB-231 in 2D and 3D cultures. The Aq fraction significantly reduced growth in both 2D and 3D cultures of MDA-MB-231 with no significant cell death (≤10%) across all conditions. (A) 2D-Growth curve analysis by trypan blue counting showed 50%, 60% and 80% decrease in cell counts by day 3 at Aq concentrations of 2mg/ml, 4mg/ml and 6mg/ml respectively. (B) 3D-Growth curve analysis by trypan blue counting showed 40% and 60% growth inhibition by day 5 at concentrations of 6mg/ml and 9mg/ml respectively. Statistical analysis from three independent experiments revealed significant differences represented by, (***) asterisks for p<0.001, (**) asterisks for p<0.01, and (*) asterisk for p<0.05.

Similar to SCE treatment, no morphological changes were detected comparing control and Aq treated MDA-MB-231 when grown in 2D conditions by day 3 (Figure 7A left panel), whereas 3D growth morphology analysis was in line with what was observed after SCE treatment. A shift in morphology was observed whereby the percentage of spherical colonies (white arrow heads) increased and that of stellate outgrowths (white arrows) decreased by more than 60% at 9mg/ml when compared to control by day 5 (Figure 7A right panel and 7B).
Figure 7: Effect of Aq fraction on growth morphology of MDA-MB-231 in 3D cultures. (A) Representative phase contrast images of cntrl and treated (Aq 6mg/ml) cells at day 3 in 2D cultures (Left panel) and of cntrl and treated (Aq 9mg/ml) in 3D cultures (Right panel). (B) Histogram analysis of the percentages of stellate versus spherical clusters on days 3, 4 and 5 post treatment showed that Aq fraction increased spherical formation and reduces stellate outgrowths by more than 60% at 9mg/ml up to day 5 in culture. Scale bars (in white) = 100µm. Statistical analysis from three independent experiments revealed significant differences represented by (****) asterisks for p<0.001.

As previously indicated that the decrease in cellular proliferation upon SCE treatment is concomitant with a delay in the S-phase of the cell cycle, analysis of the cell cycle progression of Aq treated MDA-MB-231 cells in 2D cultures was performed. Similar, yet inferior to what was observed after SCE treatment, Aq treated cells with 4 and 6mg/ml delayed the S-phase of the cell cycle by 30% and 40% respectively by day 3 when compared to the control (Figure 8D). Noteworthy to state is that the delay in S-phase was accompanied only by a decrease in G2/M phase of the cell cycle, distinct from the S-phase delay observed after SEC treatment, which was accompanied by a decrease in both G0/G1 and G2/M phases (Figure 8A, 8B and 8C).
(B) Table 4: Percentage of Aq treated Cells

<table>
<thead>
<tr>
<th></th>
<th>Pre Go</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
<th>Sum</th>
</tr>
</thead>
<tbody>
<tr>
<td>cntrl</td>
<td>3.68</td>
<td>67.85</td>
<td>19.61</td>
<td>8.39</td>
<td>99.53</td>
</tr>
<tr>
<td>Aq 4mg/ml</td>
<td>1.95</td>
<td>66.12</td>
<td>24.72</td>
<td>6.64</td>
<td>99.43</td>
</tr>
<tr>
<td>Aq 6mg/ml</td>
<td>1.29</td>
<td>67.77</td>
<td>25.76</td>
<td>4.24</td>
<td>99.06</td>
</tr>
</tbody>
</table>

(C) Table 5: Percentage of Aq treated Cells normalized to cntrl

<table>
<thead>
<tr>
<th></th>
<th>Pre Go</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>cntrl</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Aq 4mg/ml</td>
<td>52.99</td>
<td>97.45</td>
<td>126.06</td>
<td>79.14</td>
</tr>
<tr>
<td>Aq 6mg/ml</td>
<td>35.05</td>
<td>99.88</td>
<td>131.36</td>
<td>50.54</td>
</tr>
</tbody>
</table>

(D) Cell Cycle Analysis-Aq Treatment

Figure 8: Effect of Aq fraction on cell cycle progression of MDA-MB-231 in 2D cultures. Aq treatment delayed the S-phase of the cell cycle in 2D cultures of MDA-MB-231. (A) Fluorescence histogram plots showing cell counts (Y-axis) versus fluorescence intensity (X-axis) with green circles demonstrating the amount of cells in the S-phase in both cntrl and treated conditions. (B) Table showing percentages of cells in pre-G0, G0/G1, S and G2/M phases of the cell cycle, 72 hours post-treatment. (C) Table showing percentages of cells in pre-G0, G0/G1, S and G2/M phases normalized to the cntrl, 72 hours post-treatment. (D) Quantitation of G0/G1, S and G2/M phases of cells normalized to cntrl, 72 hours post-treatment. Scale bars (in white) = 200µm. Statistical analysis from three independent experiments revealed significance represented by, (***) asterisks for p<0.001 and (*) asterisk for p<0.05.

C. SCE and Aq Treatment Inhibit Trans-well Invasion of MDA-MB-231
As SCE and Aq treatments favored spherical aggregates over stellates, a notion that correlates implicitly but not exclusively with cellular phenotypes undergoing EMT versus MET respectively, and considering invasion as an indispensable element of cells undergoing EMT, the invasive ability of SCE and Aq treated cells was assessed using matrigel trans-well invasion assays. Stable transfectants of MDA-MB-231 expressing e-GFP (enhanced Green Fluorescent Protein) were used to monitor cellular invasion. Of note, a previous study from our lab (Talhouk et al. 2013) showed that these eGFP-transfected MDA-MB-231 cells act identically to untransfected cells be it at the level of proliferation, morphology and invasion amongst others, hence e-GFP transfected cells are eligible to be used throughout this assay. Figure 9A shows phase contrast and fluorescent eGFP images of transfected MDA-MB-231 and their merge indicating more than 80% transfection efficiency. Cell scoring for e-GFP labeled cells that invaded through the matrigel barrier (diluted 1:3) showed that SCE and Aq treatments inhibited the invasion potential of these cells by 30% across all tested conditions (Figure 9B). However, Aq pre-treated MDA-MB-231 cells on plastic for 24 hours followed by treatment wash-out and platting on top of the matrigel (diluted 1:20) showed a 30% increase in invasion, while cells treated with Aq after adhering to the matrigel (diluted 1:20) showed a 70% decrease in invasion compared to untreated cells (Figure 9C).
Figure 9: Effect of SCE and Aq fraction on trans-well invasion potential of MDA-MB-231. (A) Phase contrast and fluorescent GFP images of eGFP transfected MDA-MB-231 and their merge showing more than 80% transfection efficiency. (B) Fluorescent images of cntrl, SCE and Aq treated eGFP transfected MDA-MB-231 cells that invaded through the matrigel (diluted 1:3). Histogram analysis of cntrl and treated cells showing a 30% decrease in invasiveness of SCE and Aq treated cells at all tested concentrations compared to cntrl. (C) Histogram analysis of pre-treated (left) and post-treated (right) MDA-MB-231 cells that invaded across the matrigel (diluted 1:20). Scale bars (in white) = 200µm. Statistical analysis from three independent experiments revealed significant differences represented by (*) asterisks for p<0.05.
D. SCE and Aq Treatment Decrease mRNA Expression Levels of Vimentin But Differentially Regulate Twist1 and Zeb1

To be able to determine the effect of SCE and Aq treatment on EMT, particularly at the molecular level, real time q-PCR was used to quantitate for mRNA levels of key EMT markers known to be deregulated in cells undergoing EMT, in concurrence with the noted tumor suppressive effects of both treatments at the proliferative, morphological and functional levels. RNA samples from control, SCE and Aq treated cells were isolated at day 3 in 2D cultures and at day 5 in 3D cultures. GAPDH was used as a reference gene as it showed no significant expression fluctuations when tested in real time following treatment across all samples. SCE and Aq treated cells showed a 20% decrease in Vimentin expression under 2D conditions at concentrations of 3 and 6mg/ml respectively (Figure 10A and 10B, first lane), while in 3D cultures, a 30% and 40% decrease was observed with SCE at 3 and 6mg/ml respectively, and a 75% decrease was detected with 9mg/ml of Aq treatment (Figure 10C and 10D, first lane). Data analysis from Zeb1 expression showed that SCE but not Aq treatment was able to decrease Zeb1 expression by around 30% at 3mg/ml under 2D (Figure 10A and 10B, last lane) but not 3D conditions (Figure 10C, last lane), whereas Zeb1 expression exhibited around 2-fold increase in 3D conditions at 9mg/ml of Aq treatment (Figure 10D, last lane). Twist expression profile was rather more peculiar than Vimentin and Zeb1 profiles as noted by a more pronounced decrease in its expression at low concentration of SCE but not at higher ones under 3D conditions, and only at low concentration in 2D cultures. SCE treatment at 2mg/ml decreased Twist1 mRNA levels by 20% whereas 3mg/ml showed no alteration in expression under 2D conditions (Figure 10A, middle lane). A similar trend, yet more pronounced, was observed after
SCE treatment in 3D cultures, low concentrations of SCE (3mg/ml) decreased Twist1 expression by 80% whereas higher concentrations of SCE (6mg/ml) decreased Twist1 expression only by 40% (Figure 10C, middle lane). Unexpectedly, Twist1 expression levels were increased after Aq treatment by 10-folds in 2D (Figure 10B, middle lane) and 3.7-folds in 3D cultures (Figure 10D, middle lane). Table 6 shows the expected mRNA expression of each of the tested markers and summarizes the observed results for both SCE and Aq treatments under 2D and 3D conditions.

2D

(A) Vimentin

(B) Twist1

Table 6 shows the expected mRNA expression of each of the tested markers and summarizes the observed results for both SCE and Aq treatments under 2D and 3D conditions.
Figure 10: Effect of SEC and Aq treatment on the RNA expression of EMT markers in 2D and 3D cultures of MDA-MB-231. (A) (B) Bar Diagrams showing the fold change normalized to GAPDH of Vimentin, Twist1 and Zeb1 of SCE and Aq treated MDA-MB-231 cells in 2D cultures, respectively. (C) (D) Bar Diagrams showing the fold change normalized to GAPDH of Vimentin, Twist1 and Zeb1 of SCE and Aq treated MDA-MB-231 cells in 3D cultures, respectively. Statistical analysis from at least three independent experiments revealed significant differences represented by (*) asterisks for p<0.05 and (****) for p<0.001.
Table 6: Expected and observed mRNA expression from cells treated with SCE and Aq under both 2D and 3D conditions

<table>
<thead>
<tr>
<th>Marker</th>
<th>Expected expression</th>
<th>Expression relative to control and normalized to GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SCE (mg/ml)</td>
<td>Aq (mg/ml)</td>
</tr>
<tr>
<td></td>
<td>2D</td>
<td>3D</td>
</tr>
<tr>
<td>Vimentin</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td>Zeb1</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td>Twist1</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

E. SCE and Aq Treatment Rescue the Expression of E-Cad in 3D Cultures and Decrease the Expression of N-Cad and Vimentin in 2D and 3D Cultures of MDA-MB-231

Since the morphological changes observed were supported by changes at the level of RNA expression of key EMT markers, it became more evident that SCE and Aq treatments are inducing their tumor suppressive effect by partially reverting/delaying EMT. To better understand the mechanism by which this is occurring, protein expression levels of major epithelial and mesenchymal markers were analyzed between control and treated cells in both culture substratum’s, at day 3 in 2D and day 5 in 3D. Western blot analysis of total cellular extracts showed that the levels of the epithelial marker E-cadherin were significantly rescued, only under 3D conditions and after Aq but not SCE treatment (Figure 11A and 11D). Moreover, both SCE and Aq treatments significantly repressed the expression of the both mesenchymal markers, N-cadherin and vimentin, in 2D and 3D cultures (Figure 11B, 11C and 11E, 11F). Table 7 shows the expected protein expression of each of the tested markers and summarizes the observed results for both SCE and Aq treatments under 2D and 3D conditions.
2D

(A)  
<table>
<thead>
<tr>
<th></th>
<th>SCE mg/ml</th>
<th>Aq mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>cntrl</td>
<td>2  3</td>
<td>4  6</td>
</tr>
</tbody>
</table>

E-Cad

GAPDH

(B)  
<table>
<thead>
<tr>
<th></th>
<th>SCE mg/ml</th>
<th>Aq mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>cntrl</td>
<td>2  3</td>
<td>4  6</td>
</tr>
</tbody>
</table>

N-Cad

GAPDH

(C)  
<table>
<thead>
<tr>
<th></th>
<th>SCE mg/ml</th>
<th>Aq mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>cntrl</td>
<td>2  3</td>
<td>4  6</td>
</tr>
</tbody>
</table>

Vimentin

GAPDH
Figure 11: Effect of SCE and Aq treatment on the protein levels of several EMT markers in 2D and 3D cultures of MDA-MB-231. Western blot showing the expression of (A) E-Cadherin, (B) N-Cadherin and (C) Vimentin in cntrl and treated total cell lysates of MDA-MB-231 in 2D cultures. Western blot showing the expression of (D) E-Cadherin, (E) N-Cadherin and (F) Vimentin in cntrl and treated total cell lysates of MDA-MB-231 in 3D cultures. Besides each western blot analysis its corresponding densitometric quantitation represented in bar diagrams for each protein. GAPDH western blot demonstrates equal loading in the blots for all the proteins.
tested. Statistical analysis revealed significant differences represented by (*) asterisk for p<0.05, (**) for p<0.01 and (***) for p<0.001 with the exception of E-cadherin (n=1).

Table 7: Expected and observed protein expression from total cell lysates treated with SCE and Aq under both 2D and 3D conditions.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Expected expression</th>
<th>Expression relative to control and normalized to GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SCE (mg/ml)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2D</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>↑</td>
<td>UND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UND</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Vimentin</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

UND: undetected
CHAPTER FOUR

DISCUSSION

Extensive studies on sea cucumbers aimed at identifying bioactive molecules against several human disorders, chiefly against cancer, have unveiled a library of bioactive compounds against a wide variety of acute and chronic diseases. Surprisingly, no report has been issued regarding isolation of bioactive molecules from *Holothuria polii*, a sea cucumber heavily inhabiting the Mediterranean and the most abundant species in Lebanon (personal communication with Dr. Michel Bariche). Given that sea cucumbers offer a large repertoire of bioactive compounds (Reviewed by Li et al., 2013; Kim et al., 2012; Choi et al., 2012; Kiew et al., 2012; Bordbar et al., 2011; Osbourn et al., 2011), this study is intended at identifying and characterizing anticancer molecules extracted from *Holothuria polii*.

To investigate whether *Holothuria polii* harbors any compound(s) with anticancer effects, sea cucumbers were chopped into small pieces, frozen dried and pulverized into fine powder which was then extracted with ethanol and subsequently lyophilized. The corresponding lyophilized material was dissolved in PBS/10%DMSO and referred to henceforth as SCE. This general methodology implemented here as an initial step in extracting molecules from sea cucumbers is in line with the majority of studies addressing biomass extraction with solvent systems using sea cucumbers (Zhang et al., 2006; Girard et al., 1990; Tian et al., 2005; Tong et al., 2005; Tian et al., 2007; Zhang et al., 2006). Likewise, our methodology is similar to what was described by Riguera
regarding the isolation of bioactive compounds from marine resources (Riguera., 1997 updated 2008).

Treatment with SCE decreased cellular proliferation of MDA-MB-231 cells by 25%, 55% and 70% at concentrations of 1, 2 and 3mg/ml respectively, on day 3 in 2D cultures. Recently, accumulated evidence defies therapeutic strategies performed on flat 2D contexts as giving misleading results compared to 3D frameworks, which more faithfully mimic cellular responses in vivo and provide more accurate predictions of drug efficacies (Horning et al., 2008; Li et al., 2010). This discrepancy between predictions from 2D cultures and in vivo efficacy is exemplified by using Sunitinib, an inhibitor of VEGFR and PDGFR, which was shown to induce apoptotic cell death in glioblastoma cells grown on flat 2D cultures in vitro without any survival benefit in vivo (Fernandez-Fuente et al., 2014). This lack in correlation between 2D cultures and in vivo work has been attributed to several aspects of cell culture techniques such as elasticity, dimensionality and matrix composition in which major signaling pathways are differentially regulated (Pedron et al., 2013; Chen et al., 2012; Gurski et al., 2009; Xu et al., 2012; Loessner et al., 2010; Dangi-Garimella et al., 2013; Weaver et al., 2002). For instance, thirty seven different tumor cell lines treated with nine chemotherapeutic compounds were shown to be more responsive to almost all treatments when cultured on flat 2D matrices compared to 3D cultures (Fernandez-Fuente et al., 2014). In particular, glioblastoma stem-like cells showed a complete chemoresistance to kinase inhibitors, such as Sunitinib, in 3D compared to 2D cultures, this context-depended resistance was mediated partly by MEK-ERK and PI3K-Akt pathways (Fernandez-Fuente et al., 2014).
Given that the 3D microenvironment provides not only a physical simulation of the multicellular tumor architecture but govern specific functions by transmitting contextual information, to that matter, we pursued to study the effect of SCE on the proliferation of MDA-MB-231 cells in this context. SCE treatment decreased cellular proliferation by 30% and 60% at 4 and 6 mg/ml respectively, on day 5 in 3D cultures. The percentage of dead cells, in both 2D and 3D cultures, was not significant and did not exceed 5% across all conditions and time points, confirming that the decrease in proliferation is attributable to an inhibition or a delay in cell cycle and not a product of cytotoxicity. In an attempt to further assess the growth arrest endowed by SCE, we noticed a 140% increase in the amount of cells arrested in the S-phase of the cell cycle at an SCE concentration of 3 mg/ml by day 3 in 2D cultures of MDA-MB-231. As expected, the increase in the percentage of cells impeded in the S-phase was accompanied by a decrease in the percentage of cells in G0/G1 and G2/M phases of the cell cycle. Given that triterpene glycosides are by far the most abundant class of isolated metabolites from holothurians, the majority of these compounds are identified as potent cytotoxic agents able to induce cell death at very low concentrations with a perceptible increase in sub-G0/G1 cell population, as is the case with Frondoside A (Li et al., 2008; Marzouqi et al., 2011; Attoub et al., 2013), DSEA (Zhao, et al., 2011), Philinopside E and A (Tian et al., 2005; Tong et al., 2005) and Stichoposide C (Yun et al., 2012). Our cellular proliferation data in the initial ethanolic extract and the purified Aq fractions along with the cell cycle data comes in contrast with anticancer activities facilitated by these triterpene glycosides. Very few extracts or pure compounds from sea cucumbers have shown anticancer activities at non-cytotoxic concentrations nonetheless, one of the very few extracts, frondanol A5 isolated from the sea cucumber
*Cucumaria frondosa*, induced S-phase and G2/M arrest by increasing the protein levels of p21 and decreasing those of Cdc25c, a phosphatase responsible for M-Phase entry (Janakiram et al., 2010; Dong et al., 2004). As such, future investigations to better characterize SCE-induced cell cycle arrest will include monitoring of p21 expression and localization in addition to other potential candidates involved in regulating S-phase such as cyclinA and p27, the former is essential for S-phase progression (Caffarelli et al., 2013; Vidal-Laliena et al., 2013; Tane et al., 2009; Wheeler et al., 2008) and the latter is shown to induce an S-phase arrest (Yadav et al., 2012; Uehara et al., 2012; Hsu et al., 2011; Chassot et al., 2008; Shibahara et al., 2005).

MDA-MB-231 cells grown in 3D cultures exhibit stellate cluster morphology with protruding outgrowths, a typical phenotype of cells undergoing EMT. These phenotypic features that correlate with EMT involves the loss of various epithelial characteristics such as, intercellular junctions and apico-basal polarity and their replacement with mesenchymal features such as front-rear polarity and enhanced cellular motility (Roxanis., 2013; Drasin et al., 2011; Wang et al., 2011). SCE treatment shifted cluster morphology into a predominance of spherical clusters over stellate; 3D growth morphology analysis showed that 6mg/ml of SCE increased spherical formation by more than 60% when compared to untreated control at day 5. This morphological switching has been observed in the poorly differentiated Hs578T breast cancer cell line treated with geodiamolide H, a depsipeptide isolated from the marine sponge *Geodia corticostylifera* (Rangel et al., 2006). It was shown the geodiamolide H was able to revert the stellate clustering of Hs578T cells plated on top of matrigel into spherical-like aggregates that resemble normal polarized acini of MCF-10A cells. Moreover, this reversion into normal-like spheroids was accompanied by an
inhibition in cellular motility and invasion and was shown by confocal images to be coupled with a partial acquisition of a polarized phenotype as monitored by the apical orientation of Golgi apparatus similar to what was observed in MCF-10A (Freitas et al., 2008). Essentially, our data indicates that the morphogenic reversion from stellate into spherical clustering suggests that SCE holds potential compound(s) able to partially revert the mesenchymal phenotype in MDA-MB-231 cells.

Together, the aforementioned results warranted a bio-guided fractionation of the whole extract to better characterize and isolate the compound(s) responsible for the ensued biological activity. A sequential gradient partitioning of SCE in four organic solvents (petroleum ether, chloroform, ethyl-acetate and n-butanol) of increasing polarity gave four organic fractions and one aqueous layer containing compounds distributed according to their respective polarities. The five obtained fractions were guided for their anti-proliferative effects on MDA-MB-231 to identify the active fraction(s). Based on previous literature discussing extracts from marine resources, petroleum ether and chloroform fractions and rich in low polarity metabolites including hydrocarbons, fatty acids, steroids and terpenes while compounds from ethyl-acetate and n-butanol fractions are abundantly of medium and high polarity respectively (Riguera, 1997). Medium polarity compounds include peptides and depsipeptides (a peptide in which one or more of the amide bonds are replaced by ester bonds) and those of high polarity comprise alkaloid salts, amino acids and triterpene glycosides (Ebada et al., 2008). The remaining Aq layer is less described in terms of its composition compared to other organic fractions however, it is known to hold for compounds that are extremely water soluble and immiscible in any of the organic solvents. The Aq fraction of sea cucumber extracts in particular is less comprehensively studied
compared to the organic fractions due to the limited number of bioactivities and the diverse properties of the compounds it holds in addition to the expense and complexity of purifying hydrophilic compounds. While the organic fractions from extracts of sea cucumbers have been reported to convey biological activities, the more polar fractions, especially butanol, are among the most representative in terms of anticancer activities due to the fact that triterpene glycosides such as frondoside A (Jin et al., 2009), Holothurin A1 (Zhao et al., 2010) and Philinopsides A and E (Tian et al., 2005) preferably dissolve in these highly polar solvents. In our study, fractions extracted with chloroform and n-butanol showed no anti-proliferative activity when tested on MDA-MB-231 at respective concentrations, 2mg/ml and 1.5mg/ml. Petroleum ether and ethyl-acetate fractions were both ineffective at concentrations up to 1.5mg/ml and 0.5mg/ml and cytotoxic at 2mg/ml and 1.5mg/ml respectively, reflecting a very small margin between the minimal active and cytotoxic concentrations. These results do not rule out the possibility of the latter two fractions containing a biological activity per se. However, the observed cytotoxic effects do not seem to correlate with the cell cycle data noted in SCE, in which the inhibitory activity encountered was not accounted for by cytotoxicity, instead inhibition was mediated dose dependently along with a broad range between minimal active and cytotoxic concentrations. Still, it would be interesting to further investigate and characterize these two fractions using a scaled down margin of concentrations.

The Aq fraction recapitulated the activity highlighted initially in SCE regarding growth inhibition, reversion in morphology and cell cycle arrest with minute differences in concentrations required to attain comparable activities. A decrease of 50%, 60% and 80% in cellular proliferation of MDA-MB-231 was detected at 2, 4 and 6mg/ml of Aq
treatment by day 3 in 2D cultures. Moreover, 3D-growth analysis showed a 40% and 60% growth inhibition by day 5 in culture at Aq concentrations of 6mg/ml and 9mg/ml respectively with no significant counts of dead cells (<10%) across all conditions and time points in both, 2D and 3D cultures. Growth morphology analysis in 3D cultures was in line with what was observed following SCE treatment, a shift in morphology was observed whereby the percentage of spherical colonies increased and that of stellate outgrowths decreased by more than 60% at 9mg/ml by day 5 in culture. The only discrepancy encountered so far between SCE and the partially purified Aq fraction is assent to cell cycle analysis. Aq testing showed a similar, yet diminished S-phase prolongation compared to what was observed after SCE treatment; Aq treated cells with 4 and 6mg/ml delayed the S-phase of the cell cycle by 30% and 40% respectively by day 3 compared to a 140% delay in the S-phase after SCE treatment. Noteworthy to state that the delay in S-phase provoked by Aq was accompanied only by a decrease in the percentage of cells in G2/M phase of the cell cycle, in contrast to the S-phase delay observed after SEC treatment, which was escorted by a decrease in both G0/G1 and G2/M phases. This might hint towards a more refined activity in the Aq fraction compared to the initial extract as a result of purification. This has also been observed in the polar fraction of Frondanol A (Frondanol A5P); the polar fraction, in contrast to Frondanol A, induced only a G2/M arrest compared to a dual S and G2/M arrest noted after Fronadol A treatment in human pancreatic cancer cells AsPC-1 and S2013(Roginsky et al., 2010). Nevertheless, the mechanism by which SCE and Aq impedes the exit of cells from S-phase is yet to be determined, possibly by probing for essential cell cycle regulators of this particular phase. Since both treatments are affecting the DNA synthesis phase of the cell cycle and both facilitate G2/M exit,
despite differences in their respective efficacies, one could speculate that the mechanism involved does not require alterations in microtubule dynamics, as seen in microtubule targeting agents such as plant derived paclitaxel (Drago-Ferrante et al., 2008) and Vinca Alkaloids (Jordan et al., 2004) or marine derived Dictyostatin-1 (Isbrucker et al., 2003) and Eribulin mesylate (Jordan et al., 2005), but could partly include either a regulation of cell cycle mediators and their respective inhibitors or a DNA binding compound(s) as potential predictions for the noted cell cycle arrest.

As SCE and Aq treatments favored spherical aggregates over stellates, a notion that correlates implicitly but not exclusively with cellular phenotypes undergoing EMT versus MET respectively (Debnath et al., 2005), this indicates that the tumor suppressive activity along with the corresponding morphological switching observed hints towards a partial revert or a delay in EMT. That being said, we hypothesized that the inhibition in tumorigenicity of MDA-MB-231 breast cancer cells is not solely attributed to an inhibition in cellular proliferation but also in conjunction with a partial reestablishment of epithelial integrity as part of MET. To that matter, we assessed the effects of SCE and Aq treatment on trans-well invasion capacity of MDA-MB-231, an important characteristic of cells undergoing EMT. Moreover, we monitored RNA and protein expression levels of key epithelial and mesenchymal markers known to be deregulated in cells undergoing EMT, in concurrence with the noted reversion in cellular morphology.

SCE and Aq treatment inhibited the invasive potential of MDA-MB-231 by 30% across a 1 mm thick matrigel (diluted 1:3). In an alternate approach (El-Sabban and Talhouk; unpublished data) we noted increased invasion of Aq treated MDA-MB-231
cells when cells were pre-treated for 24 hours on plastic prior to testing for their respective invasion potential across a matrigel coat (diluted 1:20). We thus carried out a comparative study to monitor the effect of Aq on the invasive potential of MDA-MB-231 whereby cells were either pretreated for a period of 24 hours on plastic, followed by washing and plating on top of a matrigel coat (diluted 1:3 and 1:20) or post-treated after being plated on top of the matrigel coat (diluted 1:3 and 1:20). More cells were able to pass-through the more diluted (1:20) matrigel as compared to the less diluted (1:3) one. This was in line with previous studies quantitating invasive potential of tumor cells across different matrigel dilutions where the percentage of Hs242/3T3, T24/3T3, 3T3 and MRC-5 cells that invaded the matrigel was inversely proportional to the matrix amount being used (Albini et al., 1987). Interestingly, the data obtained in the comparative study showed that pre-treatment enhanced invasiveness compared to the untreated control only across a 1:20 diluted matrigel. Notably, Aq post-treatment procedure which showed a 30% decrease in invasion when tested across a thick matrigel barrier (1:3) showed 70% decrease in invasion when tested across the more diluted matrigel coat (1:20).

It is evident that post-treatment retards invasion whether the matrigel dilution is 1:3 or 1:20 whereas, pre-treatment enhances invasion only across the more diluted matrigel (1:20). One possible explanation could be that pre-treatment might cause the cells to arrest in the S phase of the cell cycle when cultured on plastic, but when the treatment is washed out and the cells are tested for invasion, they might recover and go through the cell cycle again, replicate and invade in a synchronized manner and hence reflect the noted increase in their invasive ability. Supporting evidence of the latter possibility is that treating mouse mammary epithelial cells SCp2 with SCE for a period
of 4 days at 7.5 and 10mg/ml decreased cellular proliferation by around 25% and 35% respectively. However, treatment wash-out and 24 hours of recovery were accompanied by a rescue in cellular proliferation of cells treated with 7.5 mg/ml and partial recovery in cells treated with 10mg/ml. This rescue noted in the proliferation rate of pre-treated cells achieving similar rates to untreated cells indicates that washing cells post-treatment permits the cells to re-enter the cell cycle (no DNA damage) in a more synchronized manner that could partly be responsible to the noted increase in the invasive ability of pre-treated cells.

Another prospect for the discrepancy noted between the two techniques could be due to the heterogeneity of MDA-MB-231 cell population. MDA-MB-231 are known to contain a subset or a subpopulation of stem-like/progenitor cells (CD44<sub>high</sub>/CD24<sub>low</sub>) known as side population (SP) with an elevated metastatic potential and drug resistance compared to other subpopulations (Cordenonsi et al., 2011). A similar event in pancreatic cancer cell lines BxPC3 and HPAC was observed after recovery from treatment with the nucleoside analogue, gemcitabine (Lee et al., 2014). Resistant cells to gemcitabine revealed an enriched population with higher invasive and migratory abilities, expressing high levels of the surface marker CD44 in addition to an elevated notch signaling all of which correlate with the acquisition of a stem-like state. Treatment of the resistant cells with DAPT, a notch signaling inhibitor, decreased the percentage of CD44<sup>+</sup> subpopulation and reversed the invasive behavior of the cells (Lee et al., 2014). Based on the above, one guess would be that pre-treatment with Aq might be enriching a cell population that is resistant to the treatment, supposedly with a more invasive potential than the heterogeneous untreated cells hence, the outcome illustrates
an increase in invasion as a result of a possible aggregate of an aggressive/resistant subpopulation.

Considering our data and the above, we believe that our data demonstrates that SCE and Aq treatments are able to inhibit the invasion of MDA-MB-231 cells however, it remains important to assess the molecular signature of pre-treated cells after their recovery, in agreement with the two prospects discussed above. This is essential to decipher the mechanism regulating the enhanced invasion noted in pre-treated cells in order to understand the increase observed in the invasion of pre-treated (recovered) cells.

Minding that several mechanisms are involved in regulating the intricate invasion ability of metastatic cells, such as cellular motility, cytoskeletal protein expression/dynamics and secreted soluble factors amongst others, it remains unclear exactly how SCE and Aq treatments are able to mediate this effect. Vimentin expression correlates with poorly differentiated cancers and with highly invasive phenotype of breast cancer cells (Korsching et al., 2005). Over expression of Vimentin in the low-invasive MCF-7 cells increased cellular motility and invasiveness whereas knocking down Vimentin in MDA-MB-231 cells, which constitutively express Vimentin, reverted these mesenchymal characteristics and abrogated invasion (Satelli et al., 2011). Given the well-established role of Vimentin as an important contributor to cellular invasion, particularly in cells undergoing EMT such as MDA-MB-231, we sought to investigate the effect of SCE and Aq treatments on Vimentin expression levels. A decrease in the mRNA and proteins levels of Vimentin was noted after SCE and Aq treatments in both 2D and 3D substrata; Vimentin mRNA levels from SCE and Aq treated cells were decreased by 20% in 2D cultures and by 40% and 75% in 3D.
cultures respectively. Protein levels of Vimentin were decreased by more than 80% in both 2D and 3D cultures of SCE and Aq treated cells compared to untreated cells on both substrata. In fact, several studies show evidence that Vimentin regulates the expression and modulates E-cadherin complexes (Wei et al., 2008), yet the mechanism is still unclear however, evidence from thyroid carcinoma cells ATC shows that Vimentin and E-cadherin are reciprocally regulated via a common pool of microRNAs (Braun et al., 2010), in addition, a study in prostate cancer cells PC-3M-1E8 showed that his reciprocal effect is attributed to src regulation (Wei et al., 2008). In accordance with the decreased mRNA and protein levels of Vimentin after SCE and Aq treatment and considering that the loss of cell-cell adhesion receptor E-Cadherin, which conveys its tumor-suppressive effect primarily via regulation of β-catenin signaling, cell-cell adhesion and possibly through molecular links with regulators of epithelial polarity, we sought to determine the effect of SCE and Aq treatment on the protein expression of E-cadherin. In our study, SCE and Aq treatments rescued the expression of E-cadherin protein levels in 3D cultures of MDA-MB-231 compared to extracts from untreated cells. The possibility that the re-expression of E-Cadherin after treatment is accompanied by membrane tethering and formation of signaling complexes with its associated cytoskeletal partners is yet to be determined. This could be assayed for using immunocytochemistry and co-immunoprecipitation studies.

While the effect of SCE and Aq treatment on the invasive ability of MDA-MB-231 needs to be further characterized, the presented data suggest that the decrease in Vimentin and the re-expression of E-cadherin levels are strong indicators of the anti-invasive potential of those fractions. Moreover, studies on anti-inflammatory activities of SCE and Aq have revealed that the Aq fraction but not SCE is able to decrease the
secretion of endotoxin-induced MMP9 by 30% from SCp2 cells (data not shown). In case the ability of the partially purified Aq fraction to decrease invasion of MDA-MB-231 is sustained by a decrease in secreted matrix metalloproteinases is a matter of future investigation.

In an attempt to better characterize the SCE- and Aq-induced partial MET, other markers that play central roles in mediating EMT besides Vimentin and E-cadherin were assessed for their respective mRNA or protein expressions using real time qPCR and western blot analysis. Ectopic expression of Twist or Snail transcription factors in normal human mammary epithelial cells (HMLEs) was associated with an acquisition of a fibroblast-like, mesenchymal morphology, a down-regulation in E-cadherin and an upregulated expression of mesenchymal markers including N-cadherin and Vimentin all of which connect to EMT (Mani et al., 2008). On the other hand, knockdown of Zeb1 in moderately aggressive breast cancer cells, MCF-7 and PMC42-LA and in highly aggressive cells, PMC42-ET and MDA-MB-231 caused a dominant compact-spherical clustering, re-expression of E-cadherin and reduced levels of Vimentin and Snail1 all of which connect to epithelialization or MET (Hugo et al., 2013). Therefore, EMT and MET effectors are vital players in determining cellular morphology, integrity and ultimately cell state. In our study, data analysis of Zeb1 expression showed that SCE was able to decrease Zeb1 levels by around 30% under 2D but not 3D conditions. This indicates that the 3D context of MDA-MB-231 is in a sense antagonizing the suppressive effect on Zeb1 noted in 2D cultures after SCE treatment. Likewise, after noticing no change in Zeb1 expression in Aq treated cells in 2D cultures, the levels of Zeb1 were increased by 2-fold in 3D cultures, capitalizing on the contextual effects altering Zeb1 expression. Since Zeb1 is known to directly repress the expression of E-
We infer that cues regulating the induction of E-cadherin after SCE and Aq treatment in 3D conditions are mediated through alternative pathways independent of Zeb1 suppression. Other non-examined key suppressors known to inhibit E-cadherin expression are Zeb2, and Snail1 and 2 also known to enhance the expression of N-cadherin (Sanchez-Tillo et al., 2012). N-cadherin which positively correlates with EMT showed decreased protein expression levels by more than 90% in cells treated with SCE and Aq in both 2D and 3D cultures. N-cadherin was shown to enhance the metastatic phenotype of cancer cells by promoting cellular motility and invasion through cooperation with FGF receptor, leading to an increase in the expression of MMP9 (Suyama et al., 2002; Hazan et al., 2004). When transfected in the weakly metastatic MCF-7 breast cancer cells, N-cadherin expressing cells exhibited increased migratory and invasive abilities, alleviated MMP9 secretion and in vivo metastasis widely to the liver, pancreas, salivary gland, omentum, lung, lymph nodes, and lumbar spinal muscle (Hazan et al., 2000). In addition, N-cadherin caused FGF receptor upmodulation, resulting in EMT and stem/progenitor like properties, involving Snail and Slug upregulation and mammosphere formation in MMTV-Neu mouse (Qian et al., 2013). This suggests that the decrease in N-cadherin protein expression, as a result of SCE and Aq treatment, is accounted for with respect to the anti-invasive effect observed.

Lastly, we monitored the expression of Twist1 in SCE and Aq treated MDA-MB-231. Previous studies provide evidence that the expression of Twist1 is fundamental for inducing EMT, increasing cellular invasion and promote anchorage-independent growth in breast cancer by regulating the expression of several downstream mediators (Yang et al., 2004; Cheng et al., 2008; Li et al., 2009; Cheng et al., 2007)
such as epigenetically repressing E-cadherin (Qin et al., 2012) and enhancing N-cadherin expression (Alexander et al., 2006). Recent studies also positively correlate the expression of Twist1 with the acquisition of stem cell traits observed in disseminated tumor cells (Vesuna et al., 2009). In line with the above, our data shows a decrease in Twist1 mRNA levels in SCE treated MDA-MB-231 cells. Unexpectedly, a more pronounced decrease in Twist1 expression was observed at low concentrations of SCE (80% decrease) but not at higher ones (40% decrease) under 3D conditions, and only at low concentration (20% decrease) in 2D cultures.

Possible explanations, under our experimental conditions, include the occurrence of a negative-feedback loop associated with the increase in SCE treatment concentration. This however remains highly speculative and requires further investigation. Additionally, the noted increase in Twist1 expression following Aq treatment in 2D and 3D cultures comes in contrast with data showing a decrease of Twist1 after SCE treatment. This variation observed in Twist1 expression between SCE and Aq is similar to that observed in Zeb1 expression, implying that SCE and Aq may prompt a similar tumor suppressive phenotype but the mechanism by which this is occurring does not seem to completely overlap for both fractions. A feasible explanation to this could be related to the diverse constituents of both fractions as a result of purification. It is also worth mentioning that qRT-PCR is a highly sensitive assay and that despite the lack of fluctuations in GAPDH mRNA expression levels across all tested conditions and replicates, not normalizing GAPDH to other reference genes leave a small window of suspicion that might explain some of the fluctuations observed in the regulation of Zeb1 and Twist1 whereby we might have missed, underestimated or overestimated some of the data.
Collectively, the results elucidate the potential tumor suppressive effects of SCE and Aq fractions in breast cancer cells by reverting EMT and partially re-establishing an epithelial-like phenotype. Current studies are underway to characterize and further purify the Aq fraction. Several spectroscopic and structural analysis was done on the Aq including UV visible spectra analysis, $^1$H-NMR, $^{13}$C-NMR, and IR spectroscopy. Preliminary data analysis indicates that the Aq fraction contains few organic compounds along with other impurities such as salts. Coomassie staining of different concentrations of the Aq fraction on SDS-PAGE showed a single broad band around 10KDa which might indicate the existence of a single glycosylated peptide. Meanwhile, we are attempting to desalt the Aq fraction using centicon membrane filter tubes and sephadex LH-20. So far, preliminary data indicates that out of the 10 sub-fractions collected from sephadex LH-20 column, the third and fourth fractions when pooled together seem to retain the activity observed in the Aq fraction. Moreover, as part of the anti-inflammatory studies conducted with SCE and Aq, we have noted no effect of SCE on the secretion of IL-6, NO and MMP9 from ET-induced SCp2 cells while Aq treatment significantly decreased the secretion of IL-6, NO and MMP9. In addition and similar to the results obtained with SCp2 cells, SCE had no effect on the secretion of ET-induced IL-1β from PMA activated THP-1 cells, while Aq treatment was able to significantly decrease its secretion (Talhouk and Nahas unpublished data). These findings capitalize on the refined chemical profile and in particular the anti-inflammatory component of the Aq fraction as compared to SCE.

In summary, the findings highlight the potential of SCE and Aq treatment in inducing a tumor suppressive phenotype by inhibiting cellular proliferation and inducing a partial MET in MDA-MB-231 breast cancer cells. The data revealed a
decrease in cellular proliferation accompanied by cell cycle arrest in S-phase and a 3D-phenotypic switching from stellate into spherical clusters supported with an inhibition of cellular invasion. Moreover, the mechanism by which SCE and Aq treatments convey this partial MET was through reducing the expression of several pivotal EMT players such as Vimentin and N-cadherin and re-expressing the epithelial marker, E-cadherin. Furthermore, the chemical purification process has shown to be of equal importance to the biological assays conducted. In that context, structural analysis revealed the existence of small water soluble compounds. Despite the technical challenges to purify compounds of this nature, assessing such features actually provides advantages over water insoluble compounds at the level of administration route, bioavailability and efficacy. This study provides robust evidence that *Holothuria polii* holds potential anticancer compound(s) that are able to suppress tumorigenicity in a highly aggressive breast cancer cell model by partly re-establishing an epithelial-like phenotype.
CHAPTER FIVE
APPENDIX

1. Anatomy and Physiology of sea cucumbers

a. Body Plan

The definitive cylindrical shape of a sea cucumber is sometimes deviated in certain species by which the body-frame resembles a sphere (Mashanov et al., 2011). A minute number of fossil records indicates that sea cucumbers once were U-shaped, with a skeleton of overlapping plates (Parsley, 1994). This body form evolved to become cylindrical and the skeleton reduced to microscopic ossicles which are currently used as a one of the main identification features of sea cucumbers (Parsley, 1994). Commonly, sea cucumbers live for about 5 to 10 years and are soft-bodied having a leathery skin and a gelatinous body with a mouth, an anus and ventrally positioned tube-feet. Being echinoderms, sea cucumbers are classified as bilaterians, that is, they are bilaterally symmetrical at the larvae stage but achieve the typical five-radial or fivefold symmetry at the adult stage, the latter is usually evident only internally or around the mouth (Kerr et al., 1999; David et al., 1996). On average, the length of a sea cucumber ranges between 10 and 30 cm, however, some small species of just 3 mm in length and others as long as 5 meters (Synapta maculate) have also been reported (Lane, 1992; Kerr et al., 1999). The mouth at the anterior end, is ringed with digitate (simple), peltate (branched) or dendritic (tree-like) feeding tentacles which are usually retractable into the mouth, while the anus lies at the posterior end (David et al., 1996; Mashanov et al., 2005). Found posterior to the tentacles is the calcareous ring which is unique to holothurians
and consist of a circumpharyngeal calcitic ossicles of usually 10 plates (discussed below).

**b. Nervous System**

The central nervous system (CNS) of echinoderms in general, and sea cucumbers in particular, is capable of fast and complete regeneration after any damage (San Miguel-Ruiz et al., 2009). It is arguably considered as one of the most taxonomically relevant model systems to provide insight into how regeneration could be reactivated in higher phyla. As mentioned above, sea cucumbers achieve a fivefold or pentaradial body plan, the five sections are individually provided by five separated radial nerve cords (RNC) (San Miguel-Ruiz et al., 2009). The five RNCs merge together at the oral side of the body to form a single anatomical unit, sometimes called the nerve ring (San Miguel-Ruiz et al., 2009). Individually, each RNC is made up of two parallel nerve tissues, a thick outer ectoneural and a thin inner hyponeural neuroepithelium, separated by a layer of connective tissue but connected by small neural bridges (Mashanov et al., 2009). The ectoneural system makes up the nerve ring and most of the RNC and contain both sensory and motor neurons, while the hyponeural is found in the RNC and associates with the skeletal muscle system and is thought to be responsible for motion (Mashanov et al., 2009). Two epinueral and hypneural canals overlay the two ectoneural and hypneural tissues respectively, covering the entire longitudinal stretch of the nerve cord (Mashanov et al., 2009). The linings of these canals is made up of simple flattened glial cells with no neuronal elements, while the ectoneural and hypneural tissues contain a meshwork of tall and radial glial cells scattered between neuronal cell bodies and glial protrusions (Mashanov et al., 2013). These radial glial
cells have been shown to dedifferentiate into precursor cells in response to a lesion in the CNS of the sea cucumber, Holothuria glaberrima (Mashanov et al., 2013). Initially, and at the vicinity of the lesioned CNS, both glial cells and neuronal cells undergo cell death. However, the dedifferentiated glial subpopulation provides an infrastructure by forming a “tubular scaffold” that grows at the tip of the lesion, and elongates, for new neuronal elements to populate again (Mashanov et al., 2013). Furthermore, it is also shown that these dedifferentiated glial cells are themselves the source of the new neurons which intriguingly survive for more than 4 months and show typical differentiation markers (Mashanov et al., 2013).

On another note, some of the studied components of the nervous system in sea cucumbers do not correlate with the two ectoneural and hyponeural systems described above, noticeably, those innervating the viscera (García-Arrarás et al., 1999; García-Arrarás et al., 2001). The enteric nervous system in cucumbers is divided into four plexuses, visceral plexus, basiepithelial plexus, mucosal neuroendocrine plexus and the connective tissue plexus, each associated with a certain type of tissue in the gut, and has no resemblance to any of the two described systems above (García-Arrarás et al., 1999; García-Arrarás et al., 2001).

Considering the significant taxonomic position of echinoderms and their enormous ability to regenerate their CNS in particular, such studies could be employed in the context of understanding the limited regenerative potential of the mammalian CNS and how to translate this understanding into therapeutic strategies.

c. Respiratory System
The last organ to start developing after fertilization is the respiratory tree, in some species, such as *Cucumaria japonica*, the respiratory trees are still unobserved even 6 months post-fertilization (Dolmatov et al., 1995). Very little is known about the detailed structure of the respiratory trees, which are unique organs restricted to holothurians among all echinoderms. Only three species of sea cucumbers (*Cucumaria frondosa, Apostichopus japonicas* and *Cucumaria japonica*) have been thoroughly studied (Dolmatov et al., 2011). The left and the right respiratory tissues like all other internal organs are made up of coelomic epithelium, a muscle layer, separated from the luminal epithelia by a connective tissue flanked by basal lamina on both sides (Dolmatov et al., 2011). The coelomic epithelial layer is composed of two cell types, the myoepithelial cells and the peritoneocytes, whereas the luminal epithelial layer consists of basal and secretory cells with the innermost apical epithelium expressing microvilli to increase the surface area with sea water (Dolmatov et al., 2011). Both the left and right respiratory trees are attached to the body wall by thin threads of connective tissues overlaying the coelomic epithelium (Dolmatov et al., 2011).

At the functional level, respiratory trees are thought to operate in a manner similar to lungs than to gills (Dolmatov et al., 2011). The sea water is inhaled or pumped into the cloaca near the anus where it is transported inside as a result of the relaxation of the muscular layer lining the walls of the respiratory tree, and expelled due to muscular contraction (Dolmatov et al., 2011). Due to the apical microvilli and the well-developed system of zonula adherens and septate junctions on the luminal cells, selectively, oxygen from sea water is diffused inside and metabolic wastes and carbon dioxide amongst others are expelled out through the respiratory trees (Dolmatov et al., 2011).
In general, the two months post fertilization (2 mm long animal) phase is ascribed by an outgrowth of the respiratory trees from the wall of the cloaca (Spirina et al., 2001; Spirina et al., 2003). During this phase, a separate outgrowth of the left and right respiratory trees is observed but the common base can only be observed 3 months after fertilization (4 mm long animal) (Dolmatov et al., 2011). Lateral branching starts to develop at this stage and continue to grow until both the left and right trees are fully developed (Dolmatov et al., 2011). In the adult stage, respiratory trees become highly branched organs with the left having twice the size of the right respiratory tree (Dolmatov et al., 2011).

Sea cucumbers belonging to the order Aspidochirotida which are known to be able to eviscerate are also capable of regenerating the whole respiratory tree after evisceration (discussed later) (Dolmatov et al., 2009). It is evident that after evisceration of the respiratory trees along with the gut, the histogenesis phase of the regeneration process is initiated by stem cells and dedifferentiated cells at the cloaca (Dolmatov et al., 2009). Myoepithelial cells and peritoneocytes migrate from the cloaca to reconstruct the coelomic epithelial layer, while dedifferentiated luminal cells lining the cloaca migrate to form the luminal epithelial layer (Dolmatov et al., 2009). It was observed that dedifferentiated luminal cells and myoepithelial cells migrate individually whereas peritoneocytes retain their junctional integrity and collectively migrate from the cloaca (Dolmatov et al., 2009). The muscular tissue within the respiratory tree is regenerated from myoepithelial cells of the cloaca’s coelomic epithelium which are known to rapidly dedifferentiate, migrate and proliferate (Dolmatov et al., 2009). Consequently, the end product of the regeneration process is a functional organ, nonetheless, with a much smaller frame-size than the original one (Dolmatov et al., 2009).
d. Muscular System

Unlike vertebrates, where a clear difference between visceral smooth and skeletal striated muscles takes place, be it at the structural or functional level, echinoderms majorly exhibit non-striated muscle fibers albeit associated with the body wall, appendages and visceral organs (Elphick et al., 2001).

e. Skeletal System

Sea cucumbers have a mesodermally derived endoskeleton beneath their skin. The lack of a definitive skeleton is compensated for by a calcareous ring and microscopic spicules (ossicles) embedded in the connective tissue layer of the dermis (Barnes., 1982; Kerr et al., 1999). These are calcified structures that are joined by connective tissue, in some species they are enlarged and in others they are absent (Barnes, 1982).

f. Reproductive System

Sea cucumbers are usually dioecious, having separate male and female individuals each with a single branched gonad made up of tubules converging into a single duct near the tentacles (Sui et al., 1985; Pang et al., 2006). Despite being dioecious, they show no anatomical sexual dimorphism making the gonads hard to differentiate except microscopically (Kato et al., 2009). Some species are protandric, meaning that the organism is born as a male or female and at some point in their lifespan they tend to switch sex (Sui et al., 1985). Most species start reproducing at an age of 2 years and cease at an age of 6 years (Kato et al., 2009). A number of species
are reported to reproduce asexually by fission while the majority reproduce by spawning, accordingly, sperms and eggs are released through the mouth into the water where fertilization occurs (Thornea et al., 2013).

g. Circulatory system

Sea cucumbers retain two circulatory systems, the water vascular system and the hemal system.

The water vascular system is connected to the outside, whereas the coelomic system is not, and is thus filled with coelomic fluid instead of sea water (Nichols, 1972). It consists of a ring canal just below the calcareous ring near the mouth, a stone canal protruding from the anterior dorsal wall of the ring canal and a peripheral system (Li et al., 2013). The stone canal protrudes just a small distance from the ring canal where it ends at a button-like structure called madreporite, which in turn is attached to the dorsal mesentery and not to the outside as is observed in sea stars (Li et al., 2013). Another protrusion from the ring canal is the polian vesicle, a thin hollow bag-like structure with no known function besides its role as a reservoir holding water fluid under slight pressure (Li et al., 2013). The peripheral system consists of five radial canals ascending anteriorly from the ring canal, subsequently, each duct reverts direction and extends posteriorly along the length of the body wall. Across the body wall, the running ducts branch to give rise to various lateral canals which are enclosed under a layer of muscle tissue and each leading to the ampulla of a tube feet called podia (Li et al., 2013).

In terms of function, the water vascular system provides hydraulic pressure for the tentacles and tube feet to move (Li et al., 2013). In addition, it is important in
regulating the body buoyancy which aids in locomotion. It also assist in gas exchange to
the internal organs of the body and in the removal of the wastes through the respiratory
trees (Li et al., 2013).

The hemal system in sea cucumbers is somehow more developed than other
echinoderms, it consist of a hemal ring just beneath the water ring but of a small
diameter (Kitto et al., 1998). From the ring core, five radial hemal canals grow and run
parallel to the radial water canals along the body length (Kitto et al., 1998). Two of the
canals are considered intestinal vessels since they run across the sides of the visceral
tube. These two canals branch into the ascending and descending intestines and connect
with each other within the coelomic space (Kitto et al., 1998). The system of capillaries
shaping the intestines is called “rete mirable” meaning miraculous net (Dolmatov et al.,
2011). This net like structure of capillaries is best observed in sea cucumbers belonging
to the order Aspidochirotida (Dolmatov et al., 2011). The hemal vessels consist of
coelemic ciliated epithelium, a muscular layer and nerve cells, and an inner most layer
of luminal endothelium. There exist two functional regions for circulation, the first, at
the upper part of the small intestine, supplied by small single chambered muscular
structures acting as miniature hearts capable of pumping blood across the hemal system,
and the second vasculature exists in the vicinity of the left respiratory tree (Barnes,
1982).

One of the cellular components of the blood is the phagocytic coelomocyte, this
type of cells is formed inside the hemal canals and has an analogous function to white
blood cells of vertebrates (Barnes, 1982). The red blood cells of sea cucumbers are of
three types, yet all are nucleated, each having a different morphology and express certain types of globins with unique binding features (Kitto et al., 1998).

h. Digestive System

The digestive tube is the main visceral organ within the coelomic space, it is long and looped and show clear variances between difference species reflecting differences in the feeding mode and food consumption (García-Arrarás et al., 2001). Despite species specific differences, it is generally divided into seven functional zones, the pharynx, esophagus, stomach, ascending and descending intestines, and the cloaca, all of which are attached to the body wall by a continuous mesentery sheets (Mashanov et al., 2010). Morphologically, the different zones throughout the digestive tract show morphofunctional similarities with the variability limited to the thickness of certain tissue layers (García-Arrarás et al., 2001). The general pattern from the outside-in consist of an outer connective tissue, coelomic epithelial bilayer, longitudinal and circular muscle layer, internal connective tissue layer flanked by basal lamina from both sides, and an inner most pseudostratified luminal epithelial layer (enterocytes) (García-Arrarás et al., 2001). Although the architecture of the above mentioned layers seems straightforward, some controversies regarding the nomenclature of certain elements do exist. For example, some investigators consider the outer connective tissue, the coelomic epithelial layer and the muscular layer as a single entity termed the “perivisceral mesothelium” (García-Arrarás et al., 2001). Regardless of these hitches, the mesothelial layer plays an important role in the regeneration processes following evisceration (García-Arrarás et al., 2001). In addition, the mesenteries have also been shown to play a pivotal role in regenerating the digestive tract, as it is believed that the
histological organization of the mesenteries mimics that of the digestive tract (García-Arrarás et al., 2001).

### i. Evisceration: types and mechanism

Autotomy or amputation occurs by one of two means, either through evisceration of internal organs or by fission, a mode of asexual reproduction (García-Arrarás et al., 2001). Following autotomy, irrespective of the prompting stimulus, the lost organs are replaced by a regeneration process that reestablishes the whole framework back in position (García-Arrarás et al., 2001). The phenomena of evisceration and regeneration largely differ in the mode, the type of organs expelled and the order of regeneration between different orders of sea cucumbers (García-Arrarás et al., 2001). However, even after eviscerating the digestive tube, the hemal system and the respiratory trees, a general outline of regeneration exists, thus the lost organs are regenerated and the animal survives pretty well.

Although the cues and the significance of such a phenomenon is obscure, the complex and highly regulated mechanism of evisceration is well studied. In simple terms, the composite mechanism of evisceration starts by weakening the attachments of the visceral ligaments and tendons to the cloaca, mesenteries and the body wall (García-Arrarás et al., 2001). Sequentially, the contraction of the longitudinal muscles in the vicinity of the visceral organs along with the fragility of their attachments, leads to the rupture and exclusion of the disconnected viscera (García-Arrarás et al., 2001).

Sea cucumber species belonging to the order Dendrochirote eviscerate through the mouth (García-Arrarás et al., 2001). The jaws or the lantern retractors disintegrates
and the muscles of the body wall around the tentacles contract forcing the tentacles, the nerve ring and the water vascular system to be expelled through the mouth opening, trailed by the attached stomach, intestines, hemal system and part of the gonad. The remaining body wall with its muscles and neural innervations and the cloaca regenerates all the eviscerated organs (García-Arrarás et al., 2001).

Species belonging to the order Aspidochirota, in contrast to Dendrochirota members, eviscerate through an opening in the cloaca with the exception of some species that eviscerate through openings in the body wall, such as, H. surinamensis and H. parvula (García-Arrarás et al., 2001). Moreover, differences in the nature of eviscerated organs even among species which eviscerate through the same route do occur. In general, contractions of the body wall muscles force the visceral organs to be expelled, however the oral organs remain intact (García-Arrarás et al., 2001). Some species for instance, Stichopus mollis and S. regalis loose both of their respiratory trees along with the digestive tube and the hemal system. In other species, parts of the gonads are lost depending on the reproductive maturity of the animal (García-Arrarás et al., 2001).

It is worth mentioning that evisceration has never been reported in any order except the two aforementioned ones.

ii. Visceral Regeneration: morphological and cellular mechanisms

Visceral regeneration in sea cucumbers has been documented as early as the 19th century. However, it was not comprehensively and accurately reported until the 1930’s by the work of Fausta Bertolini, whom was the first to study the cellular and histological
events accompanying visceral regeneration and her studies was considered instrumental, and still are, for all consequent studies over more than 70 years (García-Arrarás et al., 2001).

As previously mentioned, after the viscera is gradually disconnected from the body wall and the mesenteries, it eventually expels out, leaving the anterior and posterior lines of attachment blindly flanking in the coelomic space (Ortiz-Pineda et al., 2009). In all species of sea cucumbers capable of evisceration and regeneration, the flanking ends of the mesenteries play a vital role in reinitiating the regeneration process. The tips of the mesenteries start thickening and as the regeneration process proceeds, the thickenings extend homogenously from both sides, ultimately meeting and reestablishing a straight connection from the esophageal region to the cloaca (Ortiz-Pineda et al., 2009).

At the cellular level, investigations in Holothuria glaberrima showed that the origin of stem/dedifferentiated cells forming the thickenings at the tip of the mesenteries are supplied from the composite mesothelial layer, more specifically, from the peritoneocytes of the coelomic epithelial layer (García-Arrarás et al., 2011). The mesothelial layer starts remodeling its architecture with the peritoneocytes and myoepithelial cells forming simple epithelial sheets of irregular shapes, this remodeling of the tissue is paralleled by intracellular changes indicative of dedifferentiation/transdifferentiation at the level of both cell types (García-Arrarás et al., 2011). The peritoneocytes cleave their long bundles of intermediate filaments, and the myoepithelial cells condense their myofilaments into spindle-like structures. These dedifferentiated cells pass through the basal lamina and invade into the underlying
connective tissue towards the tip of the mesenteries. Furthermore, they adopt new roles favoring organogenesis, namely, proliferative, self-renewing, phagocytic and extracellular matrix remodeling roles; and due to their histoplasticity, they are subject to differentiation into other types of specialized cells thus forming the visceral primordial tube (Mashanov et al., 2011). As the luminal epithelial layer starts taking position, the lumen opens up and the complete lining of the intestinal tube forms (Mashanov et al., 2011). For a detailed understanding of the formation of all the tissue layers and the patterns of spatiotemporal changes at the histological and cellular levels, the following references are advised (García-Arrarás et al., 2011; García-Arrarás et al., 2001; García-Arrarás et al 1998; Mashanov et al., 2011; Mashanov et al., 2005).

It is not before the formation of this semi-mature digestive tube that regeneration of other eviscerated organs starts to take place (4 weeks post-evisceration) (García-Arrarás et al., 2001). In both species of the order Aspidochirotes, Stichopus mollis and Holothuria scabra, it was shown that the ventral and dorsal hemal vessels originate at the junction between the mesentery and the intestinal tube; and the cells initiating the hemal regeneration are derived from the coelomie epithelial layer of the intestinal primordial mesothelium (García-Arrarás et al., 2001). No studies have been done regarding hemal regeneration on species in the order Dendrochirotides.

Sea cucumbers that eviscerate through the cloaca (Aspidochirotes), only part of the reproductive organ is eviscerated, the gonad duct is lost while the base containing the germinal cells remains (García-Arrarás et al 1998). This mimics what occurs seasonally during breeding, as such, the lost tissues are regenerated from the remaining ones (García-Arrarás et al., 2001). In case the basal tissue of the gonad eviscerates, no
studies until now have detected gonad regeneration, even after complete regeneration of the viscera.

In brief, at the molecular level, several in situ hybridization, qRT-PCR, one-to-one gene studies and microarray analysis have revealed an analogy between oncogenesis and regeneration at the level of gene expression and the association of epithelial mesenchymal transition (EMT) (García-Arrarás et al., 2011). Oncogenes that are commonly expressed in malignant human tissues where concomitantly expressed in the regenerating organs of holothurians, to name a few, matrix metalloproteinase (MMP’s) (Ortiz-Pineda et al., 2009), survivin, mortalin, translationally controlled tumor protein (TCTP), wnt9, and bone morphogenic protein1/tolloid (BMP1/Tll) (Mashanov et al., 2012; Mashanov et al., 2010).

iii. Visceral plasticity during aestivation

Since sea cucumbers feed on low-quality food, they tend to continuously consume high amounts of matter during their active metabolic phases to supply their demands (Mayer et al., 1997). Yet, certain environmental changes increase the demand of food consumption even beyond the capacity of the gut volume. Therefore, the life cycle of nearly all species includes phases of low metabolic rates through which the animal ceases from consuming food and enter an inactive phase (Gao et al., 2008). The environmental cues prompting inactive periods are mostly attributed to an increase in water temperature during the summer (above 18 °C) and shortage in supplies, the former being the predominant (Yuan et al., 2007; Gao et al., 2008). This physiological safe mode lasts on average, for three months, and is sometimes called “aestivation”, regarded by a lack of movement, and appetite, a decrease in intestinal size (1mm in
diameter and half of its original length), body mass (by 50%) and metabolic rate (Wang et al., 2006; Gao et al., 2008). Moreover, studies in A. japonicus indicate that the levels and activities of digestive enzymes do fluctuate during this inactive period, accordingly, few enzymes were incapable of recovering after this period (Gao et al., 2008). For example, the levels of pepsin increased during this phase, whereas the levels of carbohydrases, amylase, cellulase, alginate, lipase, and trypsin decreased significantly (Gao et al., 2008). During the recovery phase, the mass and the length of the intestines recovered along with the levels and activities of the digestive enzymes with the exception of amylase and cellulase, but why inability to recover actually happens, is still unclear (Gao et al., 2008). On another note, studies in the same species have shown that at even higher temperatures (25 – 30 °C) the animal decreased oxygen and waste exchange by 30% and 25% respectively (Yang et al., 2006).

i. Body Wall

One of the remarkable features of sea cucumbers is their body wall or the dermis, consisting primarily of large amounts of viscoelastic extracellular matrix, mainly collagen fibrils, proteoglycans and microfibrils (Trotter et al., 1989; Trotter et al., 1994; Szulgit, 2007). It is a catch connective tissue or mutable collagenous tissue that shows large fluctuations in stiffness providing the dermis with enough plasticity to adopt three different yet reversible mechanical statuses (Yamada et al., 2010). These are the stiff, standard and soft states. The nervous system regulates this swift and extensive change in the viscosity and elasticity of the tissue within seconds to minutes, depending on the species in question, after certain physical or chemical stimulation (Wilkie, 2002). Several proteins have been identified as regulators of this dermal plasticity, tensilin, a
33 KDa protein isolated from sea cucumbers (Cucumaria frondosa and Holothuria leucospilota) converts the state of the dermis from soft to standard but is incapable to converting standard into stiff (Yamada et al., 2010). However, a new yet partially purified protein from the dermis of Holothuria leucospilota, having a molecular weight of 2.4 KDa, is able to convert the dermal standard state to stiff, but was unable to stiffen the soft dermis, suggesting that the mechanisms involved in the switch from soft to standard are different from those implicated in the switch from standard to stiff (Yamada et al., 2010). It is believed that this dynamic and unique feature of the dermis is due to the absence of permanent associations between the collagen fibrils; unlike collagenous tissues of adult vertebrates (Yamada et al., 2010). In vitro studies have shown that tensilin binds collagen fibrils and increases their crosslinking, thus stiffening the tissue, however, this stiffness regressed when a tensilin specific protease was added (Yamada et al., 2010).

Recently, the identification of relaxant neuropeptides (SALMFamide) in the starfish Asterias rubens and in several sea cucumbers introduced a new aspect to the concept of the sea cucumber body wall malleability (Elphick et al., 2001). Neuropeptides are small signaling byproducts of large cleaved precursor proteins having an N-terminal signaling peptide targeting them to the endoplasmic reticulum as an initial step in their secretory route (Elphick et al., 2001). Immunocytochemistry analysis have shown that neuropeptides could be released from the terminals of motor neurons (Inoue et al., 1999), however, the mechanisms by which they exert their function are still unclear. One possibility is that these peptides might exert there relaxing effect by stimulating the release of Nitric Oxide from the neurons that innervate the muscle tissues (Boeckxstaens et al., 1997). Another possible scenario is that neuropeptides act
directly on muscle cells via G-protein coupled receptors, which are known to bind neuropeptides, thus inducing intracellular changes in the levels of certain second messenger molecules, such as c-AMP (Gudermann et al., 1996; Elphick et al., 2001). Holothurin 1, a neuropeptide isolated from stichopus japonicas was shown to reduce muscle contraction and soften the body wall dermis (Elphick et al., 2001). Furthermore, GFSKLYFamide, another neuropeptide, induced relaxation in both visceral and longitudinal body wall muscles of holothuria galberrima and reverted the contracting action of the major excitatory neurotransmitter known in echinoderms, acetylcholine (Elphick et al., 2001). Additionally, immunocytochemistry analysis confirmed the presence of a “stiffening” peptide, NGIWYamide, in Stichopus japonicas nerve fibers across the body wall dermis signifying the involvement of neuropeptides in the dermal architecture (Elphick et al., 2001).

This suggests that the concept of “mutable connective tissue” is to be reassessed in order to incorporate the neuromuscular system as a major contributor, in addition to the collagenous component, to this dermal plasticity.

2. Defense Mechanism

a. Cuvierian Tubules

Sea cucumbers belonging to the order Aspidochirotida, exhibit another peculiar system for self-defense characterized by secreting renewable Cuvierian tubules to catch any neighboring object (Flammang, 1996). These tubules are attached to the base of the left respiratory tree and their terminals blindly float in the coelomic cavity (VandenSpiegel et al., 2000). Mechanical stress or heat stress leads to the spur of numerous white tubules that turn sticky upon contact with any physical body and clip it.
Around 10 to 20 filaments have been reported to be secreted each time, in response to a gentle stimulus, and up to 300 upon harsh stimuli, and the length of each filament tend to elongate more than 20 times its initial length after being secreted. Eventually, after being secreted outside, the attachment between the tubules and the base of the respiratory tree is lost providing the animal with a chance to escape (VandenSpiegel et al., 2000).

Studies have shown that the mesothelial layer of these tubules is the predominant source of adhesive proteins responsible for this glueiness (Baranowska et al., 2011). Morphologically, quiescent tubules are hollow organs with a thin lumen surrounded by an inner epithelial layer, a thick connective tissue with muscle fibers and nerve processes, and an outer mesothelial layer (VandenSpiegel et al., 1987). The mesothelium is composed of two layers, a peritoneocytes layer and a granular cells layer. It is observed that upon the sprouting of the tubules, the threads are not initially sticky, however, the outer cell layer of the tubules is shed away making it possible to the granular cells of the mesothelium to secrete granules of insoluble proteins that would stick together and to any object they come in contact with (VandenSpiegel et al., 2000). Biochemical analysis have shown that the adhesive material from the tubules is made up of 60% glycine-rich proteins and 40% carbohydrates (DeMoor et al., 2003).

b. Regeneration

After discharge of the tubules, a swift regeneration takes place, however when only few tubules are expelled in response to a mild stimulus, a lag period exist before regeneration starts (VandenSpiegel et al., 2000). Regeneration of the tubules proceeds in stepwise manner with each 10-30 tubules at a time, with an interval of 10 days between
successive regeneration steps (VandenSpiegel et al., 2000). This mode of regeneration has supposedly evolved to skillfully consume energy in an intermittent manner. Each tubule requires five weeks to fully regenerate and the regeneration process is divided into three phases, a repair phase, a regeneration phase and a growth phase taking 48 hours, 4 weeks and 7 days respectively (VandenSpiegel et al., 2000).

The repair phase is recognized by the phago-lysis of the damaged tissue via phagocytic cells derived from the cells of the coelomic fluid which tend to migrate to the site of injury. This wound healing step is followed by reestablishing the epithelial integrity of the left respiratory tree which has been compromised during autotomy (VandenSpiegel et al., 2000). After this 48 hours of wound healing, the regeneration process starts and takes about 4 weeks to finalize (VandenSpiegel et al., 2000). Regeneration starts by thickening the mesothelial layer and renewing the luminal epithelial layer thus reforming the lumen. Next, this primordial tubule starts to regenerate itself and the connective tissue in between the epithelial layers start to thicken, eventually becoming the thickest among the three layers. Finally, the last step is when the newly formed tubule becomes functional (VandenSpiegel et al., 2000). However, after regeneration, the tubule is still half the size of a mature Cuvierian tubule, and this is where the growth phase starts eventually giving rise to a full size tubules in a period of 1 week (VandenSpiegel et al., 2000).

At the cellular level, immunohistochemistry analysis have shown that perotoneocytes of the mesothelium, and their dedifferentiated population, are the primary source of almost all the cells involved in regenerating the tubule (VandenSpiegel et al., 2000). The dedifferentiation step starts at the level of the
mesothelial layer and later extends to the other two layers, nevertheless, the percentage of these dedifferentiated cells starts to regress as proliferation, migration and differentiation takes over (Candia Carnevali et al., 1997).

“In conclusion, holothuroids are as unusual echinoderms as echinoderms are unusual animals”. Their ability to regenerate almost every adult tissue, should be complemented with very potent tumor suppressor mechanisms that might expand our understanding of the relation between regeneration and cancer favoring better treatment regimes.
REFERENCES


Mani, SA., Guo, W., Liao, MJ., Eaton, EN., Ayyanan, A., Zhou, AY., Brooks, M., Reinhard, F., Zhang, CC., Shipitsin, M., Campbell, LL., Polyak, K., Brisken, C.,


Michelangelo Cordenonsi,1,* Francesca Zanconato,1 Luca Azzolin,1 Mattia Forcato,2 Rosato, A., Frasson, C., Inui, M., Montagner, M., Parenti, A., Poletti, A.,


Tian, F., Zhu, C., Zhang, X., Xie, X., Xin, X., Yi, Y., Lin, L., Geng, M., Ding, J. (2007). Philinopside E, a New Sulfated Saponin from Sea Cucumber, Blocks the Interaction between Kinase Insert Domain- Containing Receptor (KDR) and
αvβ3 Integrin via Binding to the Extracellular Domain of KDR. *Mol Pharmacol.* 72, 545-552.


leucospilota has antitumor effect by inhibiting angiogenesis and tumor cell invasion in vivo and in vitro. *Cancer Biol Ther.* 8, 1489-1499


