ANTICANCER POTENTIAL OF NEW ZEALAND SURF CLAM EXTRACTS

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By

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ABSTRACT

The field of nutraceuticals, functional foods, and chemo-preventive compounds in the treatment/control of cancer is experiencing an increased awareness in both scientific and public domains. This study stems from my earlier work on the antioxidative properties associated with New Zealand surf clam extracts.

In this thesis, I have evaluated and presented the cytotoxic activities of extracts from three New Zealand surf clam species: Diamond Shell (*Crassula aequilatera*), Storm shell (*Mactra murchisoni*), and Tua Tua (*Paphies donacina*). These were tested against seven cancer cell lines: A549, Hep G2, MIA PaCa-2, MCF-7, PC-3, SiHa, and WiDr. Clams were either heat processed (blanched and oven dried) or cold processed (frozen and freeze-dried) prior to extraction. These were extracted in distilled water and absolute ethanol in series. A portion of the ethanolic extracts was further fractioned into two parts using petroleum ether (pe) or ethyl acetate (ea). A cell viability study using the MTT assay was then performed to screen for the most potent fractions and concentrations. Cell viability was deterred in all cell lines at the three-day time point, to varying degrees. Then, cells underwent an apoptosis assay, which revealed that apoptosis was indeed induced in NZ surf clam extract-treated cancer cells. The apoptosis observed was confirmed by measuring the caspase-3 and -7 activities in the treated cells. High caspase activities were observed in all treated cells. Furthermore, a cell cycle analysis was carried out, to investigate cell cycle arrest. Cell cycle arrest was observed in the G2/M- and S- phases in most cases.

The differences in biochemical components and cytotoxic effects of extracts from the two methods of preparation - heat processing and cold processing, indicate that heat treatments of extracts are associated with lower cytotoxic activities, mainly cell proliferation inhibition, induction of apoptosis, and concomitant caspase-3/7 activities.

This study is the first comprehensive report on the cytotoxic properties of the three tested New Zealand surf clams' extracts. My results build on my previous work on the clam's antioxidant properties, and propose its role in health-related applications, such as a bioactive food constituent. The promising compounds found in this study can also now be isolated and employed in possible synergetic combination with traditional chemotherapy against cancer.

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ATTESTATION OF AUTHORSHIP

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Tinu Odeleye

April 2019

CANDIDATE CONTRIBUTION TO CO-AUTHORED WORKS

Chapters two, three, four, and five in this thesis represent papers that have either been published or are under review in peer-reviewed journals. All co-authors on the chapters/papers indicated in the following table have approved the inclusion of these papers in this doctoral thesis.

Chapter publication reference	**Author %**
Chapter 2: Odeleye, T., White, W.L., & Lu, J. (2019). Extraction techniques and potential health benefits of bioactive compounds from marine molluscs: A review. *Food & Function*.	TO = 80%; LW = 10%; JL = 10%
Chapter 3: Odeleye, T., White, W.L., & Lu, J. (2019). Cytotoxicity of New Zealand surf clams against hormone sensitive cancer cell lines. *Foods* (under review).	TO = 85%; LW = 5%; JL = 10%
Chapter 4: Odeleye, T., White, W.L., & Lu, J. (2019). Cytotoxicity of extracts from New Zealand surf clams against organ cancer cell lines. *Biomedicines*.	TO = 85%; LW = 5%; JL = 10%
Chapter 5: Odeleye, T., Zeng, Z., White, W.L., Wang, K., Li, H., Xu, X., Xu, H., & Lu, J. (2019). Effects of preparation method on the anticancer activity and biochemical characterization of New Zealand surf clam extracts. *Food Chemistry* (under review).	TO = 80%; ZZ = 5%; LW = 5%; JL = 5%, Others (5%)

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Appendix B

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Jude 24-25

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Godiya da sujada na ba Ka, ya Yesu, maicetona. Ni nagode!

DEDICATION

To these men I highly respect, and for whom I am eternally grateful, answers to prayers in every way. When I thought I did not have an answer, I was blessed with these people. When I needed company, at 5am or 11pm- they took shifts. When I ran samples too many for one person, they jumped in to help.

"Whatever it takes, my dear. Whatever it takes."

"Anything you need, Tinu. What time do you want me there?"

"Tinu... I will help you, don't worry."

I will look back and remember those words for a very long time!

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Legends.

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Piyush Budge, BSc, MSc, PhD.

Kelvin Wang, BSc, MSc, PhD.

CHAPTER 1

INTRODUCTION AND RATIONALISATION

1.1 FOREWORD

For several decades now, natural products have played a very important role as established cancer chemotherapeutic agents (Kinghorn et al., 2009). Natural products are a rich source of bioactive compounds for the treatment of cancer. Since they are metabolites of living organisms, they are likely to easily bind or interact with biomolecules in human living cells such as proteins, enzymes, DNA, RNA, and receptors, some of which happen to be drug targets (Kittakoop, 2015).

There is growing interest in naturally occurring compounds with anticancer potential because they are relatively non-toxic, inexpensive and available in ingestive forms (Gali-Muhtasib, Roessner, & Schneider-Stock, 2006).

More recently, interest is growing in marine natural products. So far, more than 22,000 marine natural products have been reported in the literature (Chen et al., 2015). Marine organisms are rich in functional compounds including polyunsaturated fatty acids (PUFA), polysaccharides, essential minerals and vitamins, antioxidants, enzymes (Kim & Wijesekara, 2010) and bioactive peptides (Kim et al., 2008). The oceans provide an ample scope for the extraction of chemicals for therapeutic purposes (Chakraborty & Ghosh, 2010) and medical research (Ruggieri, 1975) and remains the largest remaining reservoir of natural molecules to be evaluated for drug activity (Gerwick, 1987).

There are very many compelling arguments as to why there is a shift to marine organisms as sources of anticancer agents. This is due to the fact that they have demonstrated

tremendous abilities in producing anti-cancer compounds, and secondary metabolites which act against infectious diseases and inflammation (Bhatnagar & Kim, 2010). Marine metabolites have got such unique physical structures, with biological properties which can be exquisitely potent against cellular targets. Marine metabolites also exert their pharmacological activities through interactions at novel drug sites, such as enzymes or receptors not targeted by any current pharmacological agent (Simmons, & Gerwick, 2007).

Among the first marine derived bioactive compounds is spongouridine, which was isolated in the early 1950s from the Caribbean sponge (*Cryptotheca crypta*) (Jimeno et al., 2004). It was approved as an anticancer drug 15 years after it was isolated (Hussain et al., 2012).

One of the marine natural products currently in clinical trial phase that is a potential new anticancer agent is the marine ecteinascidin-743, isolated from the tunicate *Ecteinascidia turbinate* (Hussain et al., 2012).

From the mollusk phylum, a compound called kahalalide F has been isolated. It is reported to have high cytotoxic activity against cell lines and tumor specimens derived from various human solid tumors, including prostate, breast, lung, ovarian, and colon carcinomas (Janmaat et al., 2005). Moreover, kahalalide F has shown antitumor activity against human prostate cancer xenografts in mouse models (Faircloth et al., 2000).

1.2 SURF CLAMS

Surf clam is the collective term for molluscan bivalves that filter feed and live in the sandy substrate on high-energy surf type beaches through out the world. They tend to be medium-sized, growing up to 5cm long on the average, with some (e.g. the Atlantic surf clam) as long as 22.6cm (Cargnelli et al., 1999).

1.2.1 SURF CLAMS IN NEW ZEALAND

Surf clams are found in the surf zone of exposed sandy beaches throughout New Zealand (Cranfield, & Michael, 2001). According to the Ministry for Primary Industries (MPI) (2012), three families of sub-tidal surf clams occur in New Zealand: Veneridae, Mactridae, and Mesodesmatidae. There are seven species of surf clams in New Zealand: *Paphies donacina*, *Crassula aequilatera*, *Mactra discors*, *Mactra murchisoni*, *Dosinia anus*, *Dosinia subrosea*, and *Bassina yatei* (MPI, 2012).

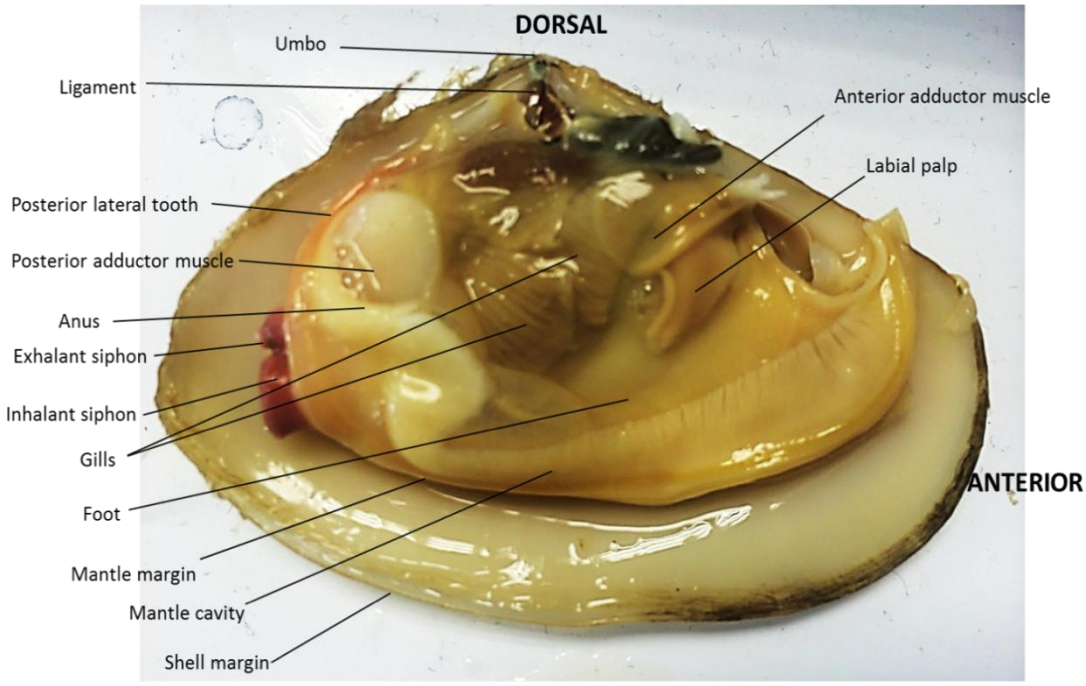


Figure 1.1: Internal anatomy of Deepwater Tua tua (Paphies donacina)

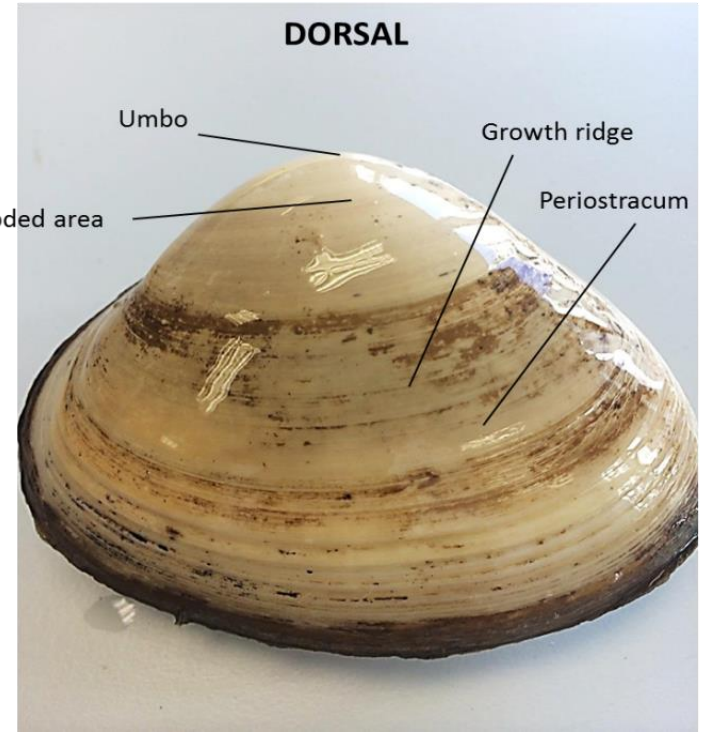


Figure 1.2: External structure of Storm clam (Mactra murchisoni)

New Zealand surf clams grow rapidly in their first 3 years but considerably slower thereafter. Individuals grow quickly in summer but hardly at all in winter. South Island clams grow faster and to a larger size as compared to the same species from the North Island. It is also reported that at a growth size of 7cm, the deeper water species, such as the moon shell (*Dosinia anus*), and the diamond shell (*Crassula aequilatera*), a species most abundant in shallower waters, had a life expectancy of up to 25 years and about 5 years respectively (Cranfield & Michael, 2001).

Surf clams are found in and immediately beyond, the surf zone of exposed sandy beaches. Surf clam species are distributed subtidally to depths of 10 m, each species generally within a distinct depth zone. The various surf clam species follow the same order of depth succession throughout New Zealand, but the depth distribution of each species may vary between locations. The zonation of species with depth allows a degree of species targeting during harvest (Cranfield et al., 1994).

Landings of surf clam in New Zealand have increased over the years. Within the space of ten fishstock years (2006/7 to 2015/6), SAE, PDO, and MMI have gone up from 46.6 tons to 375.1 tons (Ministry for Primary Industries, 2017a), 21.2 tons to 207.4 tons (Ministry for Primary Industries, 2017b), and from 61.6 tons to 71.8 tons (Ministry for Primary Industries, 2017c) respectively.

1.2.2 BIOLOGY OF SURF CLAMS

1.2.2.1 Classification of surf clams

Following Linnaeus' system of organism classification, Fay, Neves, & Pardue, (1983) classified surf clams as shown in Figure 1.3 below.

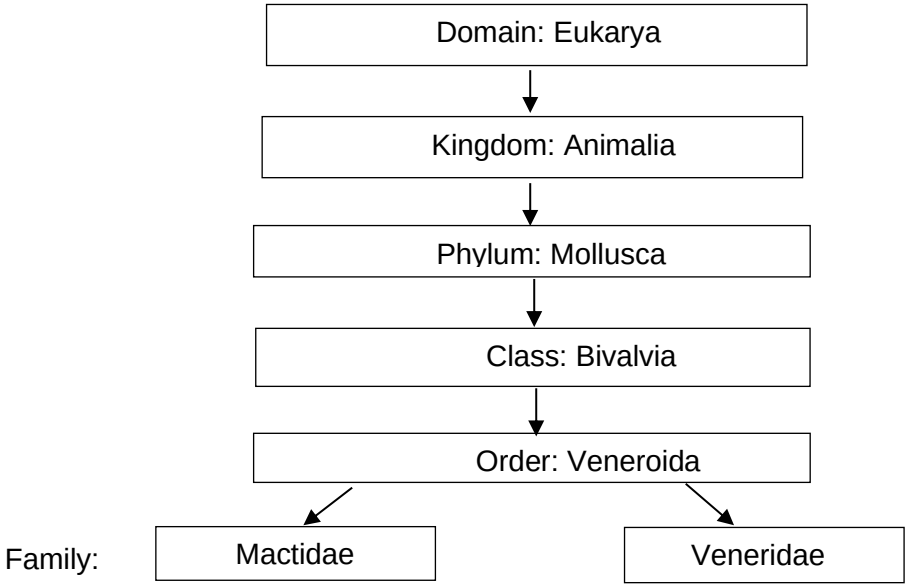


Figure 1.3: Classification of surf clam (Redrawn from Fay, Neves, & Pardue, 1983).

1.2.2.2 Feeding

Surf clams are described as filter feeders (McLachlan et al., 1996). Research suggests that they ingest particulate organic matter from the overlying water just above the bottom. Surf clams have a funnel-like siphon into which water and food can flow, and use this siphon in reproduction and locomotion (Cargnelli, Griesbach, Packer, & Weissberger, 1999). The water is then exhaled through the exhalent siphon after respiration and ingestion (Sasaki et al 2004). Even though 'food' to a clam is the

suspended particles in the water of their habitat, most of their food is obtained from microalgae (Sasaki et al 2004).

1.2.2.3 Reproduction

Clams reproduce sexually. There are male, female or hermaphroditic, and it is nearly impossible to determine their sex by external appearance (Aljadani, 2013). As male and female surf clams are identical externally, the sexes are differentiated through the histological examination of gonads (Joaquim et al., 2008; Aljadani, 2013). Surf clams spawn from late spring through early fall, shedding their eggs and sperm directly into the water column (Fay et al., 1983; Ropes, 1968). Larvae spend about three weeks in the water column as plankton before settling to the bottom to live. The larval life is thought to be about two to three weeks, and there is evidence of significant recruitment variation between years (Fay et al., 1983; Ropes, 1968)

1.2.2.4 Growth

The larvae develop into juveniles, which sink to the sandy bottoms to start their lives on the ocean's floor. They take up minerals from their surrounding. These minerals are used to form the clam's two hinged shells, which is composed of calcium carbonate. Clams reach maturity as soon as they have reached harvestable size, and this is different from clam to clam. If left unharvested, clams in the wild may live for several decades. Surf clams can live for up to 35 years. On average, those living in open water live longer than those living inshore (Fishwatch U.S. Seafood Facts, 2014). The maximum longevity, however, is suggested to be 37 years (Cargnelii et al., 1999).

A number of environmental variables influence the growth and development of surf clams (Douglas et al., 1983). This includes water temperature (Ansell, 1968; Mann, 1979; Robert & Debra, 1992; Orban et al., 2004; Karakoltsidis, Zotos, & Constantinides, 1995), water depth (Jones, 1980; Ambrose et al., 1980) and population density (Cargnelli et al.,

1999), the availability of food (Robert & Debra, 1992), salinity of the water (Orban et al., 2002; Gardner and Thompson, 2001; Islay, 2004; Orban et al., 2006), turbidity of the waters and/or quantity of sediments present in the water body (Aldridge, Payne, & Miller, 1987) and dissolved oxygen in ocean bottom waters (Fay, Neves, & Pardue, 1983).

1.2.2.5 Food chain

Surf clams are very important in the surf zone food webs (McLachlan et al., 1996; Menn, 2002). They feed on phytoplankton and detritus, and are consumed by birds, fish, and crabs (Laudien, Brey, & Arntz, 2003). They also play an important ecological role, as they can be exploited for bait and harvested for human consumption (Laudien, Brey, & Arntz, 2003).

1.2.2.6 Respiration

As in other bivalves, oxygen uptake in surf clams is dependent on body size of the organism, and to a lesser extent, temperature. As such, surf clams have similar oxygen uptake to low-tide green mussel (Marsden, 1999). Surf clams respirate through the movement of water across their gills. Water is drawn through the incurrent siphon to the gills (AlJadani, 2013). Some oxygen intake takes place along their body mantle, however. Water is then propelled across the gills and the body by means of cilia, (AlJadani, 2013).

1.2.3 THERAPEUTIC EFFECTS OF CLAM EXTRACTS

Research on surf clams have been carried out in different parts of the world, as it is known to exhibit a variety of biological activities. It is common practice in China that the mollusc *Mactra veneriformis* is not only utilised as a delicious food but also as a traditional Chinese medicine with unique functions, such as antihyperglycemic, anticancer, anticoagulant, protecting vascular system and so on (Liu et al., 2012; Ji et al.,

2013). Clam extracts have been associated with a huge range of therapeutic activities. Surf clams, such as *Cyclina sinensis* are rich in protein, amino acid, lipid and polysaccharides that may contribute to the biological functions, such as anti-tumor, anti-inflammation and immune-regulation (Abdulkadir & Tsuchiya, 2008; Gu, Yu, & Cai, 2006; Liu, Zhang, Dou, Lu, & Guo, 1997).

Therapeutic activities are summarised in Table 1.1 below.

Table 1.1: Therapeutic activities of surf clam extracts

Therapeutic activity	Clam species	Type of study	Reference
Anticoagulant	Mactra *spissula*	*In vitro and In vivo*	Frommhagen, Fahrenbach, Brockman Jr., & Stokstad, 1953
	Artica islandica		
Antiviral	*Meretrix casta, Villorita cyprinoides* and	*In vitro*	Chatterji et al., 2002;
	Polymesoda erosa		
	Meretrix petechialis	*In vitro*	Amornrut et al., 1999
Antineoplastic	*Mercenaria mercenaria*	*In vivo*	Schmeer, 1964;
	Mercenaria mercenaria	*In vivo*	Li, Prescott, Eddy, Chu & Martino, 1968
Antibacterial	*Anadara granosa*	*In vitro*	Ramasamy & Murugan, 2005;
	Meretrix Casta	*In vitro*	Mariappan & Balasubramanian, 2012
	Tridacna Maxima		
Reducing cholesterol level and hepatic lipids	*Corbicula fluminea*	*In vivo*	Chijimatsu, Tatsuguchi, Oda, & Mochizuki, 2009
Antioxidant	*Mactra veneriformis*	*In vitro*	Luan, Wang, Wu, Jin, & Ji, 2011;
	Mactra chinensis	*In vitro*	Chang, Li, Sun, Yang, & Sun, 2012;

	Meretrix casta	*In vitro*	Nazeer, Prabha, Kumar, & Ganesh, 2013
Hepatoprotective	*Corbicula fluminea*	*In vivo*	Hsu, Hsu, & Yen, 2010;
	Corbicula fluminea	*In vivo and In vitro*	Chen, Lin, Hiao, & Pan, 2008
Chronic hepatitis treatment	*Corbicula fluminea*	*In vivo*	Peng et al., 2008
Skin wound healing	*Rapana venosa*	*In vivo*	Badiu, Luque, Dumitrescu, Craciun, & Dinca, 2010
Antihypertensive	*Corbicula fluminea*	*In vitro*	Tsai, Lin, Chen, & Pan, 2006
Hypocholesterolemic	*Corbicula fluminea*	*In vitro*	Lin, Tsai, Hung, & Pan, 2010
Leukaemia therapy	*Mercenaria mercenaria*	*In vivo*	Li, Prescott, Liu, & Martino, 1968
Anti-hyperglycaemia	*Mactra veneriformis*	*In vivo*	Wang, Wu, Chang, & Zhang, 2011
Antiulcer	*Villorita cyprinoides*	*In vivo*	Ajithkumar, 2012

1.3 RESEARCH AIMS AND OBJECTIVES

Chapter Two provides a review into the background of specific extraction techniques, and how the extraction process directly influences the bioactivity of a compound. This was specifically investigated in terms of (1) the extraction techniques surrounding proteins, lipids, and polysaccharides from marine molluscs, (2) how the extraction process affects the yield (type and function) of an extract, and (3) the health benefits of resultant extract(s). This chapter is now published in the journal ***Food and Function (*****doi: 10.1039/c9fo00172g*****).***

In Chapters Three and Four, a series of complementary assays were conducted to answer the broad question, 'Do NZ clam extracts possess anticancer properties'? Chapters Three and Four also investigate (1) cell viability after treatment with NZ surf clam extracts (2) whether cell death was due to apoptosis (3) the influence of caspases 3 and 7, and (4) cell cycle arrest. Chapter Three focused on three cancer cell lines associated with hormonal sensitivity- PC-3, MCF-7, and SiHa. Chapter Four focused on four other organ cancer cell lines- A549, Hep G2, MIA PaCa-2, and WiDr. Chapter Three is under review in the journal ***Food Bioscience***, and Chapter Four is now published in the journal ***Biomedicines (doi:10.3390/biomedicines7020025).***

Chapter Five provides a comparison between two different preparation techniques prior to extraction. In the first, clams were blanched (as undertaken in commercial processes) and then oven dried, in the second, clams were frozen and then freeze-dried. Chapter Five answers the following specific questions (1) does heat have adverse effect on the cytotoxic properties of NZ surf clam extracts? (2) Is the biochemical characterisation of

NZ surf clam extracts affected by preparations involving heat? This chapter is now under review in the journal ***Food Chemistry***.

Finally, Chapter Six discusses the findings from the above data chapters and ties all of the major results obtained and described in each of the preceding chapters. It harmonizes the central findings, and describes in details how these findings achieve the aim of this research, and how the information may influence further studies in identifying bioactive substances within NZ surf clam extracts, and related fields moving forward.

1.4 ORIGINALITY AND SIGNIFICANCE OF THESIS

The current research makes significant contributions to cancer research, food chemistry, and endeavours to connect readily available food sources with preventive healthcare. This study is in response to the call for new anticancer drugs with little to no side effects, given that current chemotherapy drugs possess short- and long-term consequences.

This thesis describes a relationship between potent bioactive compounds in surf clam extracts and the pro death of cancer cells, showing approximately when, and in what part of the cell cycle apoptosis is induced. It employs the use of three different surf clam species, and compares the activities of four extracts from each clam species. It provides a wide range of analyses across seven cancer cell lines. This research highlights the effect of heat on drying the clam flesh before extraction.

1.5 STRUCTURE OF THESIS

Under Auckland University of Technology's Doctoral Thesis Pathway 2, this thesis is structured and presented in six chapters, four of which are comprised of peer-reviewed journal publication formats.

The review article (Chapter Two) is entitled: **Extraction techniques and potential health benefits of bioactive compounds from marine molluscs: A review.**

The third, fourth, and fifth chapters comprise of original research articles that have either been published or are currently under review.

The structure of each chapter includes:

- A short foreword describing the rational and summary of the research presented in the article, author list, affilation, and contribution of the PhD candidate.
- The manuscript following publication. Each article comprises of an abstract, introduction, detailed materials and methods, and results and discussion sections.
- Each manuscript has been reformatted according to AUT's guideline for thesis presentation.

The title of each original research article in Chapters 3, 4, and 5 is listed below:

Chapter Three: **Cytotoxicity of New Zealand surf clams against hormone sensitive cancer cell lines**

Chapter Four: **Cytotoxicity of extracts from NZ surf clams against organ cancer cell lines**

Chapter Five: **Effects of preparation method on the anticancer activity and biochemical characterization of New Zealand surf clam extracts**

The sixth Chapter is the concluding chapter.

The Appendix section contains a conference (poster) presentation, and other data that helped to improve the thesis content.

CHAPTER 2

EXTRACTION TECHNIQUES AND POTENTIAL HEALTH BENEFITS OF BIOACTIVE COMPOUNDS FROM MARINE MOLLUSCS: A REVIEW

2.1 ABSTRACT

Marine molluscs and their bioactive compounds are of particular relevance to the growing pool of nutraceutical resources under global investigation. Several extraction techniques have been developed to isolate bioactive compounds according to their chemical characterization, such as protein, carbohydrate and lipid. We briefly reviewed those methods in general. Bioactive molecules are 'concealed' in the primary structure of molluscs' tissue samples as amino acid, lipids or carbohydrate which are released by mechanical and chemical processes. The major health benefits of molluscs' extracts include antioxidant, anticancer, anti-infectious diseases and cardiovascular protection, which have been reviewed in detail. This review provides a novel view into the efficacy of isolation techniques and subsequent bioactivity analysis of compounds under investigation. Future development in extraction-bioactivity has also been discussed.

Keywords: Bioactive compounds; mollusc; extract; isolate; marine metabolite

2.2 INTRODUCTION

Functional foods are edible products which have been augmented either with new beneficial ingredients or increased levels of known beneficial ingredients. The augmentation therefore is adding to the functionality of the food beyond taste and nutritional effects. In terms of beneficially affecting one or more target functions in the body, functional foods provide healthful resources for the body to utilize, improve the general condition of the body, decrease risk of disease, prevent nutrition-related diseases, cure illness and improve mental and physical wellness (Stanton et al., 2005; Hamed et al., 2015). The term 'functional food' was first described in Japan in the 1980s, encompassing foods or food ingredients containing nutritive value as well as possessing substances capable of having a positive impact on a person's health and physical performance (Hardy, 2000). Foods can be 'made' functional by the addition, removal, or modification of particular components to improve their bioactivity, provided that component possesses functional effects (Roberfroid, 1999).

Bioactive compounds are constituents of foods in the human diet that are capable of supplying support and control of regulatory activities beyond simple nutritional analysis. In fact, some food-derived bioactive compounds, which possess non-nutrient characteristics, are still considered as essential aids in maintaining good health. The ability to reduce the risks of certain diseases, and/or improve certain physiological functions upon consumption is a characteristic that is independent of nutritional valuation (Meisel, 2004). Bioactive compounds are essentially ingredients or components that make food functional. Dietary fibres, essential fatty acids, oligosacchraides, and vitamins, examples of such bioactive compounds (Lobo et al.,

2010), have been extracted from diverse sources (Tanaka et al., 2012; Fisher et al., 1956; Luque-Garcia et al., 2001). While extraction and isolation of these compounds is prominent in literature, the purpose of this review surrounds proteins, lipids, and polysaccharides.

In today's world, consumers are demanding natural and safe food products (Pinto et al., 2009), as well as drugs with low amounts of additives (Gómez-Guillén et al., 2017). These positive consumer attitudes are reinforced by continued successful developments of healthy food and supplement options, particularly those arising from marine-derived sources. There are several compelling arguments as to why there is a shift towards focusing on marine organisms as sources of dietary bioactive molecules, compared with producing synthetic supplemental compounds (Zhang et al., 2010). The marine environment is the largest reservoir of natural molecules to be evaluated for beneficial drug activities (Gerwick, 1987). Marine organisms are rich in bioactive compounds, with polyunsaturated fatty acids (PUFA), polysaccharides, essential minerals and vitamins, antioxidants, enzymes (Kim & Wijesekara, 2010) and bioactive peptides (Kim et al., 2008) identified by researchers as areas of interest. Marine organism-derived anti-cancer compounds and secondary metabolite bioactives, which act against infectious diseases and inflammation, further strengthens public perception about, and subsequent demand for, beneficial products from the sea (Bhatnagar & Kim, 2010). In addition, marine organisms possess structural and chemical features which are not found in their terrestrial counterparts. The physical and chemical conditions in the marine environment have shaped marine organisms to possess a vast range of bioactive compounds possessing distinct potential bioactivity (Kijjoa & Sawangwong, 2004). It is now widely accepted that when functional foods are combined with a healthy lifestyle,

there is a beneficial health effect which goes beyond mere nutrition (Grienke, Silke, & Tasdemir, 2014), and can contribute significantly to overall health and wellbeing (Harnedy & FitzGerald, 2012). Marine bioactive compounds have such unique physical structures, with concomitant biological properties, that they can be exquisitely potent against cellular targets. These substances are known to exert their pharmacological activities through interactions at novel drug sites, in association with enzymes or receptors not targeted by any other current pharmacological agent (Simmons & Gerwick, 2007). Moreover, with the advent of high-tech machines and instruments for isolation, characterization and analyses of natural products, there is an increasing consideration given to the importance and effectiveness of marine life as a continual source of biologically-active elements (Yang et al., 2009).

The first marine derived bioactive compound is spongouridine, which was isolated in the early 1950s from the Caribbean sponge (*Cryptotheca crypta*) (Jimeno et al., 2004). It was approved as an anticancer drug 15 years after it was isolated (Hussain et al., 2012). A wide range of health benefits is well described in many marine invertebrates, including shrimp (Gómez-Guillén et al., 2018; Saelao et al., 2017; Poonsin et al., 2017; Latorres at al., 2018; Li et al., 2017; Gómez-Estaca et al., 2016; Montero et al., 2016; Mezzomo et al., 2015), crab (Jiang, Hu, Li, & Liu, 2017), salmon (Girgih et al., 2013; Harnedy et al., 2018; Neves et al., 2017; Yang et al., 2009), sole (Giménez et al., 2009; Rajapakse et al., 2005), cuttlefish (Jridi et al., 2017; Hamzeh et al., 2017), sea cucumber (Zhao et al., 2009), tuna (Lee et al., 2010), zebra blenny (Ktari et al., 2017), lobster (Battison et al., 2008), hoki (Kim, Je, & Kim, 2007), Alaska Pollack (Byun & Kim, 2001), and cod (Picot et al., 2006).

Many of these bioactive compounds have passed the research phase and are currently being used in a range of applications from ready-to-eat food, to food additives/ingredients e.g. natural antioxidants such as polyphenol and tocopherols; natural colorants such as carotenoids (Carocho et al., 2015), medicine e.g. cryotin enzyme (cancer inhibition) from shrimp (Kannan et al., 2011), Neutrase (antioxidant) from sea urchin (Qin et al., 2011) and Protamex (cancer inhibition) from snow crab (Doyen et al., 2011), neutraceutical supplements e.g. *Undaria* macroalgal supplement (iodine supplement), which lessens effects of herpes infection (Fitton et al., 2007), therapeutic applications (Chakraborty & Ghosh, 2010) and cosmetics e.g. pseudopterosins, which prevent skin irritation from the Caribbean sea whip (gorgonian) (Kijjoa & Sawangwong, 2004); glutathione (skin whitening agent) from macroalgae (Fitton et al., 2007); and scytonemin (sunscreen) from cyanobacteria (Rastogi, Sonani, & Madamwar, 2015)).

Mollusca is the second largest animal phylum on earth, constituting about 7% of living animals, 52,000 of which are named (Benkendorff, 2010). Molluscs have provided a source of nutritional and traditional medicine for centuries in many cultures worldwide, including those in ancient Crete, Rome, China, South Africa, and India (Benkendorff, 2010). A clam from coast of Iceland has been discovered to be the longest-lived (at 507 years) non-colonial animal known to science (Butler et al, 2013), which sparked health and longevity interest in those animals. At present, a number of compounds from molluscs are in clinical or preclinical phases for their use in the pharmaceutical industry. Kahalalide F, a prostate cancer treatment currently in different phases of clinical trials, ziconotide (still under development as an analgesic), and dolastatin (an anticancer agent currently in the second phase of clinical trials), (Cruz et al., 2009; Proksch et al., 2012)

are each recognised as key discoveries from molluscan sources. The potential bioactive compounds of mollusc extracts and by-products are theoretically endless, ranging from alkaloids and polysaccharides, to proteins such as the ubiquitous MSP (molluscan shell proteins), and lipids (Avila, 2006).

Bioactive compounds require a range of extraction techniques in order to successfully isolate them. The removal of non-functional elements in attempt to purify an isolate suitable for analysis is common practise. Selection of correct solvents, buffers, pH ranges, temperatures, etc. will result in optimised conditions for the extraction of desired compounds. The extraction processes of bioactive compounds, crude or purified isolates, are essentially similar, with minor modifications. Homogenization is an efficient initial step preferred for a more efficient extraction. Centrifugation and filtration, among other separation techniques, further isolate compounds from mixtures. Drying is utilised to concentrate these isolates. The solvents and conditions of the extraction are the major determinants in the isolation of a targeted compound and its innate bioactivities.

Malacological reviews on this topic reveal that the focus has largely been on either individual molluscs, rather than the generalised phylum (e.g. a particular family such as Muricidae), on a single isolated component (e.g. peptides) or a part of the organism (e.g. mantle, shells, flesh). Furthermore, reviews to date have centred on either the observed activity from an entire extract (e.g. ethyl acetate fraction), or on the biological and chemical diversity of the phylum and/or marine animals as a whole (Benkendorff, 2010; Harnedy & FitzGerald, 2012; Marin et al., 2007; Di Cosmo & Polese, 2013). This review is the first to focus on the effects of extraction techniques on the structure (if reported), composition, and beneficial properties of mollusc derived extracts (or specifically named compounds). Representative molluscan classes in this review include gastropoda,

bivalvia, and cephalopoda. The extraction techniques and testing methods associated with each of the exemplar organisms are also highlighted in order to best illustrate the impact of methods on known bioactive substance functions.

2.3 EFFECT OF EXTRACTION TECHNIQUES ON ISOLATE

Numerous molecules/compounds possessing health benefits have been isolated from marine organisms. The mechanism of action of a molecule may be greatly dependent on the extraction technique and solvent(s) used. Certain compounds have consistently featured in scientific literature and will provide the basis for discussions relative to extraction techniques, identification of solvent fractions and components within the identified fractions. This review's efforts to identify correlations between these noted elements of isolation techniques and the bioactivities of proteins, lipids, and polysaccharides, is intent on presenting developing trends in nutritional supplementation and healthful dietary practices.

It is worth noting that the extraction method itself is as important as the end product and its functionality. There are multiple pathways to discovering, identifying, and developing supplemental bioactives. The relationship between extraction techniques and bioactivities necessarily involves a method which maintains molecular function, and assesses all viable candidates.

2.3.1 PROTEINS, PEPTIDES, AND PROTEIN HYDROLYSATES

A vast range of extraction techniques have been employed in isolating these peptides and hydrolysates. Extreme pH adjustments, either acidic or basic, temperature, and

enzymatic manipulations highlight the extraction techniques (Lye, Asenjo, & Pyle, 1994; Deak & Johnson, 2007, Gillett et al., 1977). Observed hydrolysate activities also tend to correspond to further specific data relating to protein or peptide effectors. In the case of peptide isolates, anti- (oxidant, metastatic, microbial, fungal, Deoxyribonucleic (DNA) fragmentation, hypertensive, and enzymatic) roles are noted as characterized bioactivities (Badiu et al., 2010; Shavandi et al., 2017; Sivaraman et al., 2016; Tsai et al., 2006; Salem et al., 2018).

Proteins with specific bioactivities can be isolated from various parts of a given mollusc of interest. The method of extraction must match the desired outcome for a recovered isolate. For instance, shells require the use of acetic acid or a similar solvent to 'free' the sequestered compounds. Demineralization is also expedient in preparations involving shells (Shavandi et al., 2015). With mollusc flesh, on the other hand, several chemicals have been employed in the extraction of proteins, e.g. water (Tsai et al., 2006), NaOH (Shavandi et al., 2017), physiological buffered systems (Zhang et al., 2016), and K_2SO_4 : $CuSO_4$ mixtures coordinated with concentrated H_2SO_4 washes (Badiu et al., 2010).

It is a common practise to prepare hydrolysates as either boiled then filtered milieus, or merely as distilled solutions. These solutions are not necessarily buffered to a specific pH. Hydrolysate preparations contain a broad spectrum of compounds which require further analysis in order to identify specific bioactive elements. This is also indicative that species-to-species differences exist when isolating and characterizing protein metabolites. The efficacy of individual isolates is expected to vary between not only preparations, but also species from which they are extracted.

Hydrolysate mixtures may contain non-proteinaceous components or inactive protein macromolecules, and may need to be purified in order to obtain the particular peptide

class(es), as per their molecular weight. This purification can be carried out by centrifugation or ultrafiltration. Further separation procedures may include gel and ion exchange chromatography techniques and RP-HPLC (Grienke et al., 2014). It is important to note however, that separation and/or purification techniques are not always advantageous regarding bioactivity. For example, mixtures of peptides, amino acids and sugars have shown higher bioactivity than a single purified peptide (Sarmadi & Ismail, 2010).

Proteins isolated from molluscan samples may act as hormones in their targeted cascades, initiating signal transductions with their associated messengers to elicit cellular physiological responses. Furthermore, oxidative reactions and stabilization of compounds during the extraction processes significantly affect final therapeutic effectiveness (Benkendorff et al., 2015). As such, it is essential that the isolation process does not render the protein inactive, denatured or otherwise modified, whereby its native function is lost. In this case, the cascade is likely associated with pathways which differ from the lipid-soluble extracts, given that a peptide and transport across cell membranes will differ from the relatively free passage of a soluble molecule. It is therefore of interest to elucidate the mechanisms of identified molecules or compounds that arise from the hydrolysates isolated from molluscs.

2.3.2 LIPIDS AND STEROLS

More often than not, lipid extraction is carried out following Folch et al. (1956) or Bligh & Dyer (1959), with the latter, a slight modification of the former, proving to be a lot less time-consuming and requiring smaller and convenient volumes of solvents. Another critical advantage is that extraction and purification can be performed in a single

operation. Optimum lipid extraction results when the tissue of interest is homogenized using a mixture of chloroform and methanol, which when mixed with water yields a monophasic solution. The chloroform layer contains the lipids, and the methanol-water layer the non-lipids. Generally, the Folch method is employed in the extraction of lipids from solid tissues, while the Bligh method is preferred when carrying out extraction of biological fluids (Schiller et al., 2004). Lipids from molluscan shells are also extracted in the same way (Farre & Dauphin, 2009).

There are strong interactions between lipids and other cell biopolymers, such as polysaccharides and proteins. Extraction solvents should therefore be selected to disrupt these molecular associations, with a balance of polar and nonpolar characteristics. Sterols and triacyglycerols, nonpolar lipids lacking hydrophilic groups, for instance, are best extracted with alkane-based solvents. Polar lipids (e.g. glycerophospholipids) on the other hand, possess the ability to bind to cell biopolymers through ionic interactions that cannot be easily disrupted by polar organic solvents. In this instance, pH adjustments and acidification, which interrupts the ionic interactions and increase lipid hydrophobicity, can be beneficial for achieving lipid extraction efficiency (Pati et al., 2016). One should note however, that esters, as part of the molecular framework of lipids, are vulnerable to acids and long exposures to concentrated acids may result in the loss of lipids. Furthermore, prolonged extraction under acidic conditions results in the degradative conversion of phosphoglycolipids to glycolipids. This problem has been overcome by replacing hydrochloric acid in extraction protocols with 5% trichloroacetic acid (TCA) (Nishihara & Yosuke, 1987). TCA is easily removed by washing with methanol-water (Nishihara & Yosuke, 1987) and proves less damaging to lipid isolations.

A further method for extracting lipids from tissues employs the use of hexane:isopropanol (HIP) (usually in the ratio 2:1). This is followed by washing the extract with aqueous Na_2SO_4 (Hara & Radin, 1978). The advantages the HIP methods has over the Folch and Bligh methods are numerous. First, relatively 'mild' solvents are used. Chloroform and methanol are both known for their carcinogenicity and damage to the visual system respectively. Hexane, on the other hand, is seemingly relatively toxic in laboratory usage and is probably not a hazard after sewage processing. Chloroform decomposition yields phosgene and HCL acid, both of which are capable of modifying lipid species (Schmid et al., 1973). Secondly, lipids extracted using the HIP method are void of proteins and/or proteolipids, while those extracted with other methods (namely, Folch's) contain a significant amount of protein. In addition, HIP extracted lipids contain less of other nonlipid tissues, yielding close to pure lipids than any other method (Hara & Radin, 1978). Furthermore, the use of milder solvents allow for usage of plastic laboratory materials, such as pipettes, tips, and eppendorf tubes, whereas this is impossible with solvent mixtures containing small amounts of chloroform (Schiller et al., 2004).

The methyl tert-butyl ether (MTBE) method was developed especially for complex lipidomics from samples with excessive amounts of biological matrices. The recovery of lipids of almost all major classes is reported to be the same or better than can be achieved by the Folch method (Matayash et al., 2008). MTBE has the advantage of replacing chloroform with MTBE, circumventing the instability of the chrolofrom-methanol analytical pipeline (Matayash et al., 2008). It does this be accumulating the non-extractable pellet at the bottom, providing an easier access to the upper non-polar

and lower polar fractions. Lastly, the MTBE extraction involves a single extraction from small amounts of tissue (Matayash et al., 2008; Chen et al., 2013).

The chloroform-methanol method, albeit the practical foundation of lipid extraction, and the basis for many other subsequent protocols, has its imperfections. Some of shortcomings of the method include time-consuming and labor-intensive procedures, inconsistent efficiencies, and the production of emulsions, which may result in the partial or complete loss of complex glycolipids and polar prostaglandins (Pati et al., 2016). Taken as a whole, literature provides indications that lipid levels and fatty acid composition vary with species, sex, age, season of the year, food availability, salinity, and water temperature (Wlieg & Body, 1988). These information are critical especially when drawing up nutrition sheets or calculating populational intake of nutrients (Sorihuer et al., 1997).

Of note, very few lipid extracts have been characterised in molluscs. Within the scope of this review, two lipid extracts are reported. The lipid extract of a clam, *M. lusoria* has been shown to be a rich source of steroids that belongs to the 5α, 8α-epidioxyl sterol family, and possesses apoptosis-inducing activity (Pan et al., 2007). A sterol extract from black clam possessing anti-inflammatory and antioxidant activities has also been described (Joy & Chakraborty, 2018). The sterol and lipid isolates from these samples are associated with several activities. Given that the solubility of the active compound is appropriate for effective cell-membrane absorption or transport, research in this area will offer intriguing findings.

Lipids are responsible for facilitating a range of structural and functional properties. They reside in cell membranes as principal constituents of the bilayer. Phospholipids are one of the most abundant constituents of biological membranes (Pati et al., 2016).

2.3.3 POLYSACCHARIDES

The extraction of polysaccharides involves defatting the soft body tissue, usually prior to homogenizing. The organic solvent, its concentration, and the duration differ from publication to publication, but the practice remains the same. Ethanol (95% - Zhu et al., 2011; 90% - Jiang et al., 2011; Jiang et al., 2013; 75% - Qiao et al., 2009) and acetone (Volpi & Maccari, 2003; Liao et al., 2013; Mohan et al., 2016; Aldairi et al., 2018), or a combination of both (Qui et al., 2010) are the common solvents used in this process. Defatting maximises the purity of the polysaccharide samples as the presence of fats can lead to the formation of an emulsion which may trap the polysaccharides from being extracted from the matrix. Additionally, removal of lipids from samples can involve either carbon tetrachloride or chloroform:methanol solvent mixtures (Jung & Kim, 2009; Li et al., 2011).

Proteins interact with polysaccharides and interfere with their stability so deproteination is necessary. Much of the proteins can be extracted from the crude sample with mild solvents (e.g. buffers) at low temperatures (Vincent, 2002). The most popular extraction temperature for this technique is 4^{o}C (Volpi & Maccari, 2003; Aldairi et al., 2018; Mohan et al., 2016). Alcohols are also used in the deproteinization process. The use of an alcohol to deproteinate removes extraneous proteins from the liquid phase by centrifugation. Deproteinization inarguably removes potential proteins and peptides, but it frees the targeted polysaccharides. Many a publication follow the

method by Sevag et al. (1938), and employ the use of absolute ethanol (Volpi & Maccari, 2003). Shells are deproteinized and demineralized as well, in order to isolate a polysaccharide as close to being pure as possible (Qurashi et al., 1992; Rasti et al., 2017).

Sulfated polysaccharides, of which GAGs are a type, are widely distributed in animals, including molluscs. Most physiological properties of these polysaccharides are largely hinged upon their sulfate groups. Since the degree of sulfation (DS) dominate the effects of a polysaccharide in biological systems, it has been proven that the higher the DS, the better its biological potency (Ghosh et al., 2009). These sulfate groups can be removed (desulfation) or introduced in order to elucidate the molecular origins of the functions demonstrated, thereby creating novel biopolymer-based pharmaceutical ingredients (Ryo, 2002). Desulfation can be acid-catalyzed, alkali-catalyzed, solvolytic, or silylating reagent mediated. Sulfated GAGs are crucial in interacting with bioactives, especially proteins, which 'produces' the aforementioned bioactivities. Therefore, since the sulfation pattern and location determine specificity in protein-polysaccharide interractions, desulfation at particular positions is important in striving to understand the observed bioactivities at molecular levels. Furthermore, combining this chemical modification (desulfation) with methylated techniques is expedient in the analysis of the linkage position of each individual sugar in order to effectively harness the structure of a polysaccharide (Ryo, 2002).

Molluscan GAGAs are found to be similar in structure to the standard heparin, but may fall short in their activity in the presence of impurities. The activity, the degree of activity, or the absence thereof, of polysaccharides depend greatly on the amount of impurities carried over during the extraction and isolation processes (Periyasamy et al., 2013). This may be the reason behind differences in the effectiveness of isolates from

molluscan origins. Thus, the isolation process must needs be optimised in order to maximise extraction.

The production and use of polysaccharides as therapeutic agents are increasingly important fields of research, especially as polysaccharides display several biological activities ranging from anticoagulant and antithrombotic capacities to antimetastatic and anti-inflammatory roles in biological systems (Wang et al., 2018). In fact, marine polysaccharides may possess advantages over their mammalian counterparts, as they are considerably less likely to be contaminated with viruses and/or prions. Good examples of GAGs are the Heparin (Hep) and Heparan sulfate (HS) types of polysaccharides, which are widely distributed in the soft tissues of molluscs and have been posited to be substitute sources of heparinoids. Hep fractions, possessing similar chemical structures to the standard commercial Hep, have been isolated from clams (e.g. *Katelysia opima*, *Anomalocardia brasiliana*, *Donnax striatus*) and scallops (e.g. *Argopecten irradians*) (Vijayabaskar, Balasubramanian, & Somasundaram, 2008). In addition, several other polysaccharides, such as hyaluronic acid (HA) and dermatan sulfate (DS) have been isolated from molluscs with reported bioactivities and health benefits, including anticoagulant (Periyasamy, Murugan, & Bharadhirajan, 2013), antitumour (Li, Yuan, and Hou 2008; Zhang et al. 2013), antioxidant (Chang et al., 2012), immunomodulatory (Dai et al., 2009; Li et al., 2014), and antiviral (Yu, Liu, and Han 2008) activities. Molluscan wastes from scallops (*Chlamys hastate*), cockles (*Cerastoderma edule*, *Clinocardium nuttalli*), whelks (*Buccinum undatum*), clams and mussels (*Mercenaria mercenaria*, *Mytilus galloprovincialis*, *Mytilus edulis*), and oysters (*Crassostrea gryphoides*, *Crassostrea gigas*) have been redirected from industrial disposal practices towards the more-effective extraction of molecules of nutraceutical

interest, which in turn can be used as nutritional substances and animal feed (Ruocco et al., 2016).

2.4 BIOACTIVE COMPOUNDS FROM MARINE MOLLUSCS

Numerous health benefits have been reported from marine animal extracts, ranging from anti-HIV, anti-inflammatory, and antioxidant mechanisms to anti-bacterial and anti-enzymatic functions. Table 2.1 outlines standard bioactives reported over the last two decades, which also serve as the basis of discussion for the correlations presented in this review. Mollusc derived bioactives are known to promote human health in addition to assisting in the prevention and/or treatment of chronic diseases (Harnedy & FitzGerald, 2012). Table 2.1 gives a clear insight into some biological activities of marine animal extracts, with the following subsections detailing these activities and attempting to bring them into current scientific perspective. This section reviews bioactives with properties applicable to some of the leading causes of death globally.

Table 2.1: Summary of mollusc-derived bioactives. Exemplar species along with identified compounds are summarized.

Health benefit	Organism	Isolate	Reference	Health benefits
Anti-inflammatory	Peru squid	Squid cartilage type II collagen (SCII)	Dai, Liu, Wang, & Sun, 2018	No clear health risk has been identified in the literature.
	Clam (*Paphia malabarica*)*		Joy & Chakraborty, 2017	
	Black clam (*Villorita cyprinoides*)*	Aryl polyketides	Joy & Chakraborty, 2018	
	Squid (*Todarodes pacificus*)*	Sterols	Zhang et al., 2016	
		Liquid extract (containing peptides proteins minerals)		
	Clams (*Paphia malabarica*, *Villorita cyprinoides*) αβ	Clam extract	Joy, et al., 2016	
Anti-microbial	Squid (*Illex argentinus*)	Chitosan	Huang et al., 2018	No clear health risk has been identified in the literature, but chitosan supplements may interfere with blood thinners and some antiviral agents (WebMD, 2019).
	Marine clam (*Anadara granosa*)	Broad range of bioactive compounds	Ramasamy & Baluasubramanian, 2012	
	Clam (*Anadara granosa*)	Crude extract	Kumar et al., 2017	
	Bivalve shellfish (*Arca inflata*)	N-terminal peptide of hemoglobin, AI-hemocidin 2	Li et al., 2017	
	Blue mussel (*Mytilus edulis*)	Peptides	Charlet et al., 1996	
	Mussel (*Mytilus galloprovincialis*)	Mytillin	Mitta, et al., 1999	
Anti-oxidant	Arrow squid (*Nototodarus sloanii*)	Protein hydrolysates	Shavandi et al., 2017	No clear health risk has been identified in the literature.
	North Pacific squid (*Ommastrephes bartrami*)	Squid viscera autolysates (SVAs)	Song, Zhang, & Wei, 2016	
	Indian squid (*Loligo duvauceli*)	Squid protein hydrolysates (SPH)	Sivaraman et al., 2016	
	Scallop (*Patinopecten yessoensis*)		Wu et al., 2016	

	Jumbo squid The Pacific oyster (*Crassostrea gigas*) Clam (*Mactra veneriformis*)	Scallop female gonad hydrolysate (SFGH) Jumbo Squid Fin (JSF) insoluble collagen 3,5-dihydroxy-4-methoxybenzyl alcohol Crude extracts and fractions of alcohol extracts	Cuevas-Acuña et al., 2016 Watanabe et al., 2012 Luan et al., 2011	
Anti-enzymatic	Octopus (*Octopus vulgaris*)[*α] Jumbo flying squid (*Dosidicus gigas*)[*#]	Octopus protein hydrolysates (OPHs), Squid hydrolates and peptide fraction	Salem at al., 2018 Mosquera et al., 2016	No clear health risk has been identified in the literature.
Anti-hypertensive	Oyster (*Crassostrea gigas*)* Freshwater clam (*Corbicula fluminea*) Clams (*Paphia malabarica* & *Villorita cyprinoides*)	Hydrolysate Protein hydrolysates and hot water extract Clam extract	Lee at al., 2018 Tsai, Lin, Chne, & Pan, 2006 Joy, Chakraborty, & Pananghat, 2016	No clear health risk has been identified in the literature.
Anti-proliferative	Abalone (*Haliotis discus hannai* Ino) Abalone (*Haliotis discus hannai*) Bay scallop Indian green mussel (*Perna viridis*)	N/a Peptide (A2) Scallop polysaccharide Hydrolysates	Lee et al., 2010 Kim et al., 2016 Li et al., 2017 Mitra & Chatterji, 2004	No clear health risk has been identified in the literature.

Proliferative	Mediterranean mussel (*Mytilus galloprovincialis*)	Proteic extract	Badiu et al., 2010	No clear health risk has been identified in the literature, but chitosan supplements may interfere with blood thinners and some antiviral agents (WebMD, 2019).
	Mussel (*Mytilus edulis*) and Oyster (*Crassostrea gigas*)	N/a	Latire et al., 2017	
	Squid (*Sepioteuthis lessoniana*)	ß-Chitosan	Subhapradha et al., 2017	
	The Pacific oyster (*Crassostrea gigas*)	3,5-dihydroxy-4-methoxybenzyl alcohol	Fuda et al., 2015 Huang, et al., 2017	
	Clam (*Meretrix meretrix*)*	Oligopeptides	Ajith et.al., 2012	
	Black water clam (*Villorita cyprinoides*)	Villorita cyprinoides extract (VCE)	Hasnat et al., 2015	
	Abalone (*Haliotis discus hannai* Ino)*	Water extract and fermented hydrolysate Water soluble polysaccharide MP-I	Xu et al., 2008 Sudhakar & Nazeer, 2017	
	Mussel (*Mytilus coruscus*)	Tri-peptide		
	Octopus (*Octopus aegina*)			
Sanguineous	Abalone (*Haliotis rubra*)	Hydrolysate enriched with sulphated polysaccharides	Suleria et al., 2017	No clear health risk has been identified in the literature.
	Abalone (*Haliotis discus hannai* Ino)	water-soluble neutral polysaccharide	Zhu et al., 2009 Lin et al., 2010	
	Freshwater clam (*Corbicula fluminea*, Muller)	Hot water extract and hydrolysate	Wang et al., 2011	
	Clam (*Mactra veneriformis*)	Crude polysaccharide extract	Ninan et al., 2007	
	Blue mussel (*Mytilus edulis*)	Mussel extract (MAPS)		

Anti-coagulant	Blue mussel (*Mytilus edulis*)	*M. edulis* anticoagulant peptide (MEAP) (oligopeptide)	Jung & Kim, 2009	No clear health risk has been identified in the literature.
	Abalone (*Haliotis discus hannai* Ino)	Sulfated polusaccharide	Li et al., 2011	
		GAGs	Volpi & Maccari, 2005	
	Mussel (*Anodonta anodonta*)	Molluscan GAG	Vijayabaskar et al., 2008	
	Clam (*Katelysia opima*)	Heparan sulfate	Gomes et al., 2010	
	Paw scallop (*Nodipecten nodosus*)	Heparan sulfate-like GAGs	Mohan et al., 2016	
	Snail (*Harpa conoidalis*)			

Some organisms possess more than one bioactivity. Additional health benefits have been assigned symbols defined below.

* = antioxidant

= antimicrobial

α = anti-diabetic

β = anti-inflammatory

N/a = not applicable

The role of proteins and peptides as physiologically functional elements in the diet is increasingly recognized (Korhonen & Pihlanto, 2006). Bioactive peptides are 'concealed' in the primary structure of plant and animal proteins as dormant amino acid sequences which are released by food processing, fermentation, and proteolysis catalysed by enzymes in the digestive tract following consumption (Udenigwe & Aluko, 2011). Bioactive peptides often contain between 3 and 20 amino acid residues per molecule, and possess activities based on their amino acid composition and sequence (Pihlanto-Leppala, 2001). Based on their amino acid sequence, they may be involved in various biological activities (Kim & Wijesekara, 2010). The changes in functional properties and/or the multifunctionality of peptides are based on their low molecular mass and intrinsic factors such as hydrophobicity (Cho et al., 2008). Marine organisms possess a variable range of bioactive peptides, ranging in size from di-, tri, and oligopeptides. Additionally, high molecular weight polypeptides, derived from proteins or isolated from protein hydrolysates, have shown numerous health benefits, including immunomodulatory, antihypertensive, and antioxidative (Kannan et al., 2011). Peptides derived from food proteins are also easily absorbed (Yike et al., 2006). The mechanisms of action of a few marine protein-derived peptides have been described. The mechanism of ACE inhibition is by competitive inhibition, characterised by peptides competing with ACE substrate for the enzyme catalytic sites (Sato et al., 2002). Meanwhile, antioxidant activities of peptides include metal chelating, scavenging of free radicals and/or reactive oxygen species (ROS), and ferric reducing activities (Luan et al., 2011). Hydrolysates containing anti-inflammatory peptides are reported to protect against gamma radiation induced immunosuppression by enhancing spleen production of IL-12, as well as

augmenting $CD4^+$ Th cells (Udenigwe & Aluko, 2011). Other physiological functions of bioactive peptides include immunomodulatory, antithrombic and antihypertensive activities (Murray & FitzGerald, 2007).

Lipids are an expansive group of naturally occurring molecules, including fats, waxes, sterols, fat-soluble vitamins (for example vitamins A, D, E and K), mono-, di- and triacylglycerols, diglycerides, and phospholipids (Holdt & Kraan, 2011). Glycerolipids, sphingolipids, prenol lipids, and polyketides also fall within the category of these isolated compounds. (Pati et al., 2016). Lipids' chemical properties render them soluble in organic solvents, and therefore are capable of relatively easy-to-replicate isolation techniques in order to identify bioactive components. Lipid extracts of similar nature are known to be rich in PUFAs (Takeungwongtrakul et al., 2012) and tocopherols (Özogul, Özogul, & Kuley, 2011). PUFAs are lipids that have been shown to demonstrate biological functions in the human body (Rein et al., 2012). α-tocopherol is usually the only tocopherol present in oils from marine fish, and is notably only present at low concentrations. Nonetheless, other forms of tocopherol, such as β-, γ- and δ-tocopherols, have been identified in Shrimp (*Parapenaus longirostris*) and reported to be of biological importance (Özogul, Özogul, & Kuley, 2011).

Sterols, bioactive lipid metabolites, have been identified in marine organisms as components of complex chemical blends, and are usually identified by GC-MS. Some of the organisms containing sterols include invertebrates such as molluscs, corals, and bryozoans (Sarma et al., 2005). Significantly, marine derived steroids and sterols are found to exhibit therapeutic properties such as anti-inflammatory, antimicrobial, and cytotoxic activities (Joy et al., 2017, Yang et al., 2018).

The polysaccharides are a complex group of macromolecules with incredible structural heterogeneity, comprised of long chains of monosaccharide subunits (Cruz et al., 2010). Furthermore, marine polysaccharides have regular and well-defined structures responsible for their potentially useful biological activities (Suleria et al., 2017). Among the functional components of sea animals, polysaccharides may be the molecules of greatest interest to researchers and neutraceutical companies in their efforts to develop candidate supplements and treatments (Wang et al., 2017).

Glycosaminoglycans (GAGs) (acidic, highly sulfated complex polysaccharides, such as acidic mucopolysaccharides) were first discovered in certain invertebrates in the 70s (Cássaro & Dietrich, 1977), and have since been extracted from several molluscs such as the scallop and pearl oysters (Suleria et al., 2017). Glycosaminoglycans are comprised of repeating disaccharides and are chiefly found in the connective tissues of molluscs. These molecules have shown multiple activities such as antithrombotic, antiviral, antioxidant and antitumor (Cui, Wang & Yuan, 2014), anti-inflammatory, neutrite growth, and anticoagulant capacities (Valcarcel et al., 2017), to mention a few.

2.4.1 OXIDATIVE STRESS

Reactive oxygen species (ROS) cause extensive damage to biological macromolecules such as nucleic acids (DNA and Ribonucleic acid- RNA), proteins and lipids. Besides well-known dietary antioxidants such as vitamin C, vitamin E, polyphenols and carotenoids, other dietary compounds demonstrate protective mechanisms against oxidative damage (Erdmann, Cheung, & Schröder, 2008). To deal with such negative effects living organisms utilize self-antioxidant defense systems. However, some of the important antioxidants need to be supplemented through external diet (Pawar et al., 2013).

Research shows that protein hydrolysates as well as peptides are able to act as metal chelators, free radical scavengers, and possess lipid peroxidation activities, like in the arrow squid, for example (Shavandi et al., 2017). Furthermore, a thin layer chromatographic separation of the ethanolic extract of *Perna viridis* revealed the presence of alkaloids, polyphenols and sterols. The said extract showed a dose dependent activity in scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, and its postulated mode of action is its hydrogen donating ability (Sreejamole et al., 2013).

Many antioxidants have been found/ identified in marine molluscs, including taurine, carotenoids, α-tocopherol and *n*-3 polyunsaturated fatty acids (Luan et al., 2011). The biological pathways of these natural antioxidants is either unknown or not well understood, however, *n*-3 polyunsaturated fatty acids used as a dietary supplement, have been known to mitigate oxidative stress (Gao et al., 2007) through the induction of cellular antioxidant responses.

2.4.2 CANCER

Cancer is a diverse group of at least 100 diseases characterized by the uncontrolled proliferation of anaplastic cells which tend to invade surrounding tissues and metastasize to other tissues and organs. Cancer results from a mutation in the chromosomal DNA of a normal cell, which can be triggered by both external factors (tobacco, alcohol, chemicals, infectious agents and radiation) and internal factors (hormones, immune conditions, inherited mutations, and mutations occurring in metabolism).

Clinical interest in the pharmacology of medicinals from marine organisms has heightened due to anticancer effects indicated for *Mercenaria* marine clam components (Schmeer, 1979). Evaluations into the apoptotic effects of the hard clam extract have shown it to possess inhibitory effects on human cancer cell proliferation (Pan, Huang, Chan, Ho, & Pan, 2007). The liver extract of this clam is a general inhibitor of cancer growth, as it has been reported to have a therapeutic effect upon Leukaemia 1210 in mice and a prophylactic effect against adenovirus 12-induced-tumor formation in hamsters (Ruggieri, 1975). The hot water extract of *Corbicula fluminea* and the aqueous, ethanol, and ethyl acetate extracts of *Meretrix lusoria* have been reported to have significant antitumor activities and significant inhibitory effects on growth of human gastric cancer cells (SGC7901) and human ovarian carcinoma cells (SKOV3 and A2780) respectively (Liao, Chen, & Xingqian, 2013; Kong, Chiang, Fang, Shinohara, & Pan, 1997; Pan, Huang, Chang, Ho, & Pan, 2006; Pan, Huang, Chan, Ho, & Pan, 2008). Furthermore, extracts derived from *Cyclina sinensis* have been found to induce apoptosis in human gastric cancer cells (Jiang, Wang, Liu, Gan, & Zeng, 2011).

2.4.3 INFECTIOUS DISEASES

Studies have been carried out on the effects of isolated substances from marine animals as potential antiviral and antibacterial agents. The results have indicated that certain compounds present in the abalone (*Holiotis rufescens*), oyster (*Crassosfrea virginica*), common clam (*Mercenaria mercenaria*), and queen conch (*Strombus gigas*), possess significant antiviral activity in vitro and in vivo (Li et al., 1965). More than 100 species of marine organisms have also been shown to exhibit antimicrobial activity (Ramasamy & Murugan, 2005). A range of extracts from shellfish has yielded differential activities

against viral and bacterial targets based on the solvents used for isolation of the resulting treatment fractions.

Investigations conducted by Chatterji et al. in 2002 showed that extracts prepared from economically important Indian marine bivalves, including the estuarine clam (*Meretrix casta*), black clam (*Villorita cyprinoides*) and mud clam (*Polymesoda erosa*), were found to possess high antiviral activity when tested against influenza virus strains type-A (A/Mississippi 1/85/H3N2) and type-B (B/Harbin 7/94). Li and Traxler (1972) also reported that the aqueous extract of *Mya arenaria* had antiviral activity in vivo. Moreover, a new type of D-galactan sulfate was isolated from Meretrix *petechialis*. Its structure was characterised and its antiviral activity was examined (Amornrut et al., 1999). It has been reported that these polysulfates exert their anti-HIV activity by either interfering with CD4 binding to gp120 (thus inhibiting syncytial formation (Uzair, Mahmood, & Tabassum, 2011)) or by binding to the V3 loop, preventing infectivity (Hayashi, Hayashi, & Kojima, 1996). Most of the sulphated polysaccharides that have been found to inhibit HIV replication appear to inhibit syncytia formation (Amornrut et al., 1999).

Two clam species, *Meretrix casta* and *Tridacna maximawere*, were screened for antibacterial activity using whole body tissue extracts obtained from different solvents. The ethanol and methanol extracts were able to inhibit all of the tested pathogens, exhibiting broad spectral antibiotic activity. Ethanol extracts of *M. casta* showed highest activity against *E. coli* and *Staphylococcus aureus*, while aqueous extracts showed highest activity against *E. coli*, *Salmonella typhi* and *Staphylococcus aureus*. Other extracts showed the lower activity against *Klebsiella pneumoniae* and *Lactobacillus vulgaris*. Similarly, the ethanol extract of *Tridacna maxima* exhibited highest activity

against *Klebsiella oxytoca*, *Proteus mirabilis* and *Staphylococcus aureus* (Mariappan & Balasubramanian, 2012)

2.4.4 CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) is the sole leading cause of death in the United States of America and other first world countries (Erdmann, Cheung, & Schröder, 2008). CVD includes stroke, heart failure, atherosclerosis and coronary heart disease (Harnedy & FitzGerald, 2012).

Diverse marine derived protein hydrolysates have been shown to display ACE inhibition *in vitro*. These include proteases and peptides from clam (e.g. *Corbicula fluminea)*, and cephalopods (e.g. Jumbo flying squid) (see Table 2.1).

In the last 40 years, a growing body of evidence has accumulated on the beneficial effects of n-3 PUFAs on CVD in reducing triglyceride levels, controlling inflammation processes, improving endothelial function, regulating platelet aggregation and decreasing the incidence of arrhythmias. n-3 PUFAs are among the drugs recommended for managing dyslipidemia and preventing CVD. In addition, higher circulating n-3 PUFA levels have very recently been reported to be associated with lower total mortality, especially deaths due to coronary heart disease (CHD) in older adults (Bilato, 2013).

2.5 FUTURE DIRECTIONS

In order to elucidate the mechanisms of action of the vast array of potential bioactives, there is a dire need for more targeted research in areas pertaining to mollusc-derived

compounds. Based on the current knowledge set available from published literature, most of the biological effects of mollusc derived bioactivities are *in vitro*, with only a few of these tested in animal models. Although significant improvement has been made in identifying and/or isolating potential bioactives and novel drugs from the ocean, tremendous efforts are still required to further these substances to clinical applications.

Today, there are many newer extraction techniques, such as fluidphase partitioning methods (e.g. single-drop and liquid microextraction, supercritical and pressurized liquid extraction, microwave-assisted extraction), and sorptive and membrane-based extraction methods (e.g. solid-phase microextraction, sorptivephase developments, and hollow-fiber membrane extractions) (Raynie, 2006). These newer techniques possess several advantages over the older conventional methods, such as significant reduction in sample degradation and organic solvent consumption, improved extract selectivity, removal of additional steps e.g. sample clean-up, efficient extraction, etc. (Huie, 2002), and these may improve the bioactivity of the extract/isolate, as fewer functional groups are damaged and/or eliminated from their native forms. Even though the development and implementation of extraction techniques have 'evolved' over the years, extant extraction procedures are still common practice in many laboratories today190. The Bligh-Dyer method of lipid extraction, for example, is still preferred over other newer lipid extraction methods, as it provides the best yields (Tanamati et al., 2005). Furthermore, even though these modern techniques are quicker, more user friendly, and require less organic solvent, they possess very similar basic principles (e.g. the use of liquid solvents) to those of classic extraction techniques (Huie, 2002).

Furthermore, should naturopathic preferences for healthy food options remain on their current ever increasing trend, efforts should be taken to study the identified natural

reservoirs of in-demand bioactive substances (such as marine populations of targeted gastropods) and ascertain appropriate levels of harvest and replacement practices. Maintenance of the food and supplement sources is paramount in order to ensure that utility of the populations occurs without depleting or destroying them, seeing that our own well-being is intimately tied to the sea.

It is interesting that in all of the efforts to isolate polysaccharides of leading interest, lipids (defatting) and proteins (deproteinization) are often removed and discarded from analysis of any bioactivity, unless they were the targeted isolates. Further studies would benefit from analysis of lipids and/or proteins, that are otherwise lost, merely because polysaccharides are the isolate of interest. Evidence supports that lipids and proteins carry beneficial activities in physiological systems (Table 2.1). As such, investigations are warranted to further elucidate their roles.

2.6 CONCLUSION

Finding functional food products and drugs with low amounts of additives is a great challenge in medical and pharmacological research. In recent years, research has moved away from synthetic production of potentially bioactive compounds to identifying the bioactive compounds already present in marine organisms. Driven by consumer demand for foods with greater health benefits, as well as academic and industrial interests, a great number of bioactive compounds have been isolated and identified from marine animals and their by-products. The isolation and extraction of bioactive compounds is critical preparatory to understanding the health benefits of marine organism food sources. Extraction techniques are paramount in diagnosis and elucidation of isolates and their putative health benefits.

A majority of identified bioactive compounds exhibit *in vitro* activities. Further research is required for *in vivo* studies/testing in order to effectively apply findings to promote human health. Furthermore, studies to determine whether the ingestion of these organisms can improve health by acting on cellular pharmacological targets are essential.

CHAPTER 3

CYTOTOXICITY OF NEW ZEALAND SURF CLAMS AGAINST HORMONE SENSITIVE CANCER CELL LINES

3.1 ABSTRACT

The cytotoxicity of three species of New Zealand (NZ) surf clam extracts were investigated in three hormonal cancer cell lines. Four extracts from each clam species were used: water (cd), ethanol (et), petroleum ether (pe), and ethyl acetate (ea). The last three of these significantly induced apoptosis via caspase cascades and cell cycle arrest. This was observed in the percentages of early apoptotic cells in SS-treated PC-3, MCF-7, and SiHa, with 30, 30.97, and 34.55% respectively at 400 µg/ml. Cell cycle arrest at 72 hours is highlighted by the induction of arrest in the S- and G2-M phases. Previous analysis reveal that pe and ea extracts from NZ clams are rich in lipids, and current indications based on fractional efficacy, point to a lipid soluble bioactive compound. This study provides a basis for further development of NZ clam extracts for treatment, or as a supplement, for prostate, breast, and cervical cancers.

Keywords: cytotoxic activity; surf clam; cancer; functional foods; marine compounds; extract, New Zealand

3.2 INTRODUCTION

Marine organisms have developed defensive and protective mechanisms (such as the production of bioactive metabolites) to survive in the competitive and aggressive environment in which they live (Beaulieu et al., 2013). These marine bioactive metabolites have unique physical structures and biological properties that can be potent against cellular targets. They could exercise their pharmacological activities through interactions at novel drug sites that are not targeted by any current pharmacological agent (Simmons and Gerwick, 2007). Marine animals possess a wide range of bioactive properties. Mussel (*Mytilus edulis*) extracts, for instance, promote catabolic pathways of human dermal fibroblasts (Latire et al., 2017). Protective effects on hepatic cells are reported from Squid (*Sepioteuthis lessoniana*) (Subhapradha et al., 2017). Wound healing (cuttlefish), and antibacterial properties (Squid (*Illex argentinus*)) have been reported as well (Jridi et al., 2017; Huang et al., 2018). Some of these bioactives are drug candidates in clinical development at different phases of clinical trials. The range of these bioactives includes anticancer drug candidates (e.g. APL (dehydrodidemnin B, Aplidin™) a cyclic depsipeptide derived from *Aplidium albicans*), Kahalalide F derived from the sea slug (*Elysia rufescens*), and asthma treatment drugs (e.g. IPL512602 isolated from the sponge *Petrosia contignata* (Haefner, 2003).

Surf clams (or beach clams) is the collective term for filter feeding bivalve molluscs that live in the sand on high energy sandy beaches (McLachlan et al., 1996). Clams are not only a source of food, but also a source of medicine (Ludien et al., 2003), particularly in traditional Chinese medicine (Childs et al., 1990; Luan et al., 2011). The bioactivities of clam extracts have been extensively researched, including anti-inflammatory (Joy and

Chakraborty, 2017), anti-diabetic and anti-hypertensive (Joy et al., 2016), antibacterial (Ramasamy and Balusubramanian, 2012; Kumar et al., 2017), antioxidant (Luan et al., 2011), ACE inhibitory (Tsai et al., 2006), antiulcerogenic (Ajithkumar et al., 2012), and anti-hyperglycemia (Wang et al., 2011). In New Zealand, there are seven species of subtidal surf clam: *Paphies donacina*, *Crassula aequilatera*, *Mactra discors*, *Mactra murchisoni*, *Dosinia anus*, *Dosinia subrosea*, and *Bassina yatei* (Ministry for Primary Industries, 2016). Previously, we described the antioxidant activities of three NZ surf clam extracts (Odeleye et al., 2016).

Reproductive hormones play a major part in the aetiology of certain cancers. Hormones such as oestrogen, preogestreon, ovarian and adrenal androgens, estrone and estradiol are associated with breast cancer (Endogenous Hormones and Breast Cancer Collaborative Group, 2011; Althuis et al., 2004; Bernstein and Ross, 1993). Androgens are also implicated in the aetiology of prostate cancer (Shaneyfelt et al., 2000; Gann et al., 1996). So are estrogens and prolactin (Henderson et al., 1982). Hormones are also risk factors in the aetiology of cervical carninomas (Krishnanurthi et al., 2000), as the cervix epithelium is a hormone-dependent epithelium (Allen and Gardner, 1941; Rinaldi et al., 2011). Previous studies report that sex steroid hormones, particularly estrogens, are required for the inception of atypical metaplasia in invasive cervical carcinoma (Brake and Lambert, 2005). Other culprit hormones are estrone and estriol (Zheng, 1990). Of the six most common cancers in NZ, three (breast, cervical, and prostate) are hormone sensitive (Ministry of Health, 2019). Three cancer cell lines (MCF7, SiHa, and PC-3) were therefore selected to represent those cancers. The aim of this study is to investigate the *in vitro* cytotoxic effect of extracts from three NZ surf clam species on three hormonal cancer cell lines, and explore their possible mechanism of action.

3.3 MATERIALS AND METHODS

3.3.1 CHEMICALS AND MATERIALS

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) formazan powder was purchased from Sigma-Aldrich (St. Louis, MO, USA). Petroleum ether and Ethyl acetate were purchased from Global Science (Auckland, NZ), ethanol was purchased from ThermoFisher (Auckland, NZ). Foetal Bovine Serum (FBS) was purchased from Medica Pacifica (Auckland, NZ). All organic solvents used were of analytical grade. Roswell Park Memorial Institute (RPMI) 1640 medium, no phenol red, L- Glutamine (200 mM), Penicillin- Streptomycin (10,000 U/mL), TrypLE™ Express, no phenol red, Trypan blue stain (0.4%), and Dulbecco's Phosphate Buffered Saline (D-PBS) were all purchased from Life Technologies (Auckland, NZ). Dimethyl sulfoxide (DMSO) was purchased from Merck Chemicals (city, country); Apo-ONE® Homogeneous Caspase-3/7 Assay kit was purchased from In Vitro Technologies (Auckland, NZ). Alexa Fluor® 488 annexin V/ Dead Cell Apoptosis kit was purchased from Thermo-Fisher Scientific (Auckland, NZ).

3.3.2 CLAM COLLECTION

Clams were obtained from Cloudy Bay Clams Ltd (Blenheim, NZ). Wild clams were caught in sand 3-5 meters under the water surface. Clams were cleaned with United States Food and Drug Administration (USFDA)-approved water, and monitored to ensure it maintained the highest possible quality. Already blanched clams arrived at Auckland University of Technology (AUT) packed and labelled by their species, in polystyrene boxes that regulate the temperature at around 4 ^{o}C (Odeleye et al., 2016).

3.3.3 EXTRACTION AND FRACTIONATION OF CLAM SAMPLES

Three New Zealand surf clam species provided all samples characterised in this study, including Storm shell (SS) (*Mactra murchisoni*), Diamond shell (DS) (*Crassula aequilatera*), and Tuatua (TT) (*Paphies donacina*). Blanched clam meat was deshelled and drained of excess fluids. After coming to room temperature, the flesh was oven dried at 60ºC to constant weight, pulverised, milled and stored at -20ºC until use. We employed a modified extraction method from Luan et al. (2011). All clam extraction and measurements were carried out in dim light to reduce any possibility of oxidation. Initial extraction fractions were generated in parallel using water (cd) and ethanol (et) as solvents. Clam powder was solubilized in distilled water and stirred constantly at room temperature using a magnetic stir-bar for one hour. The supernatant was removed, replaced with fresh solvent and stirred for another hour. This process was repeated until the solvent was colourless. The supernatant was collected by centrifugation. Ethanol extraction was carried out in the same way. The clear solution was collected and evaporated under reduced pressure using a Rota evaporator (Buchi Rotavapor R-215, Global Science, Auckland, NZ) until complete dryness. The water (cd) and ethanol extracts (et) were collected after evaporation and stored at -20ºC. The ethanol extraction (et) of each clam species was further fractionated by liquid-liquid extraction steps according to the polarities of petroleum ether (pe) and ethyl acetate (ea). The ethanol extracts were dissolved in 100ml distilled water and fractionated with petroleum ether. Extracts (‘pe’) were collected and concentrated under reduced pressure. Further step-by-step fractionation was done using ethyl acetate, which resulted in the fractions of ‘ea’. Each fractionation process was repeated until the

solvent was colourless. Fractions were evaporated to dryness and stored at -20ºC until ready for use.

Table 3.1: Extracts obtained from NZ surf clams. DS, SS, and TT extracts were subdivided according to solvent polarities. Nomenclatures for each of these divisions are as listed.

Table 3.1: Details of extracts

	Water extract	**Ethanol extract**	**Pet ether extract**	**Ethyl acetate extract**
Clam species				
Diamond shell- DS	DScd	DSet	DSpe	DSea
Storm shell- SS	SScd	SSet	SSpe	SSea
Tua tua- TT	TTcd	TTet	TTpe	TTea

3.3.4 CELL LINES AND DETERMINATION OF CYTOTOXICITY

Cell lines were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were maintained in RPMI supplemented with 10% (v/v) FBS and penicillin-streptomycin under an atmosphere of 5% CO_2 – 95% air in an incubator at 37º. Three cancer cell lines were employed in this study. Details of each cell line are listed in Table 3.2 below.

Table 3.2: Details of cell lines employed

Cell line	**Tissue**	**Morphology**	**Species**	**Disease**	**ATCC Cat No**
PC-3	Prostate	Epithelial	Human	Adenocarcinoma	CRL-1435™
SiHa	Cervix	Epithelial	Human	Squamous cell carcinoma	HTB-35™
MCF7	Breast	Epithelial	Human	Adenocarcinoma	HTB-22™

The cytotoxic effect of NZ surf clam extracts on three cancer cell lines was measured employing the MTT assay. The cells were seeded in a 96-well plate at a concentration of 1×10^5 cells ml^{-1} using the RPMI medium. After incubation in a humidified 37 °C, 5% Carbon dioxide ($CO_{2)}$ incubator (Series II Water Jacket supplied by Thermo Scientific) overnight, the cells were treated by NZ surf clam extracts at a concentration range from 25 to 1000 µg/mL. The cells were further incubated for an additional 24, 48, and 72 hours independently at 37 °C. After incubation, MTT stock solution was then added to each well and incubated for a further 4 hours. The formazan crystals in each well were dissolved in 100 µl of DMSO. The amount of purple formazan was determined by measuring the absorbance at 540 nm.

Data interpretation

Treated cells with absorbance values lower than the control cells indicate a reduction in the rate of cell proliferation. On the other hand, higher absorbance values indicate an increase in cell proliferation.

%cell viability= {(At-Ab)/ (Ac-Ab)} x100

Where,
At= Absorbance value of test compound
Ab= Absorbance value of blank
Ac=Absorbance value of control
% cell inhibition= 100 - cell viability

3.3.5 FLOW CYTOMETRY ANALYSIS OF APOPTOSIS

The apoptotic effect of NZ clam extracts was determined by the Alexa Fluor® 488 annexin V staining method and measured by flow cytometer. Cells were seeded onto 6-well plates, at a density of 4 x 10^5 cells per well and allowed to incubate overnight. The

cells were then treated with NZ clam extracts for 7 hours at 400 and 600µg/ml. After treatment, the cells were washed with PBS and harvested using trypsin. Careful measures were taken in order to prevent the loss of detached dead or apoptotic cells. The harvested cells were washed twice with cold PBS, then resuspended in 1 x binding buffer. 4µl of Alexa Fluor® 488 annexin V and 1µl of 100 µg/ml Propidium iodide (PI) (Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit) were added to each 100 µl of cell suspension, with a slight modification to the manufacturer's protocols. After the incubation period, 400 µl 1X annexin-binding buffer was added to each sample and analysed. Fluorescence-activated cell samples were recorded at 10,000 events using a flow cytometer (MoFlo™ XDP, Beckman Coulter, CA, USA). The fluorescence emission was measured at 530 nm, and 575 nm.

3.3.6 CASPASE-3/7 ASSAY

The Apo-ONE Homogeneous Caspase-3/7 Assay Kit was used to evaluate the activities of apoptosis by evaluating the activities of caspase-3/7 in the clam treated cells. Cells were seeded onto 96-well plates at a density of 5 x 10^3 cells per well, and allowed to incubate overnight. The cells were treated with NZ clam extracts for 24 hours at 400 and 600µg/ml. After incubation, an equal volume of Apo-ONE caspase-3/7 reagent was added to each well, and incubated while shaking for 1 hour at room temperature. The fluorescence of each well was read at 495 ± 10 (excitation) and 520 ± 10 (emission).

3.3.7 FLOW CYTOMETRY ANALYSIS OF CELL CYCLE

Cell lines were seeded onto 6-well plates, at a density of 3 x 10^5 cells per well and allowed to incubate overnight. The cells were then treated with NZ surf clam extracts for 72 hours. After treatment, the supernatant was collected, cells were washed with PBS and treated with trypsin. Careful measures were taken in order to prevent the loss

of detached dead or apoptotic cells. The harvested cells were washed twice with PBS at 4ºC, and fixed with ice cold 80% ethanol for no longer than 7 days. The cells were then gently centrifuged at 1200RM for 2 minutes, after which permeabilizing solution was added to each sample, and incubated for 30 minutes at 37ºC. After permeabilization, PI was added and incubated for a further 5 minutes. Fluorescence-activated cells in each sample was recorded at 10,000 events using a flow cytometer (Beckman Coulter's MoFlo^{TM} XDP).

3.3.8 STATISTICS

MTT and caspase data were collected from duplicate experiments of triplicate samples. Apoptosis and cell cycle assays were carried out twice, in duplicates. Results are presented as mean ± standard error of the mean and $p<0.05$ was considered statistically significant. MTT and caspase data were analysed using Microsoft Excel. Analysis of Flow cytometry data was performed using Kaluza Analysis 1.3 (Beckman Coulter, Miami, FL, USA).

3.4 RESULTS AND DISCUSSION

We first carried out cell viability studies on PC-3, MCF-7, and SiHa, after treatment with NZ surf clam extracts. We then conducted both apoptosis and cell cycle arrest tests and supported our results with the caspase-3/7 activity data, to better understand the cascades employed in the observed apoptosis. Previous studies show that the side-by-side occurrence of apoptosis and disturbances to the cell cycle effectively contributes

and/or monitors cell proliferation and induces cell death (Xu et al., 2011). This is the first report to demonstrate that cancer cell viability (*in vitro)* is inhibited by NZ surf clam extracts.

3.4.1 ANTI-PROLIFERATIVE ACTIVITIES OF NZ SURF CLAM EXTRACTS

Initial examinations of the effect of the surf clam extracts on the survival and proliferation of the cancer cells were carried out using the MTT assay. Three cell lines were challenged with extracts from the clam species, and compared to their untreated control counterparts (denoted by 'C' throughout this article). Each individual species was processed to obtain the extracts of the study (Table 3.1). Most extracts displayed a dose and time- dependent inhibition on the proliferation of all cancer cells (Figs 3.1- 3.3). Results are expressed as percentage of viable cells compared to the control.

Prior to the addition of the MTT reagent, the treatment media was removed and replaced with fresh media, thereby limiting interaction between treatment and MTT. The cell lines were further treated with the extracting solvents to rule out possibilities of solvent interference (data not shown). In addition, NZ surf clam extracts have been shown to possess various antioxidant activities (Odeleye et al., 2016), and may be capable of reducing MTT irrespective of cellular mechanism. To yield data with the correct estimations of percentage dead cells, an MTT assay was performed on the extracts only, in the absence of cells. In the former case, no apoptosis was induced by all solvents employed in the extraction process, and in the latter, MTT was not reduced by the extracts (data not shown).

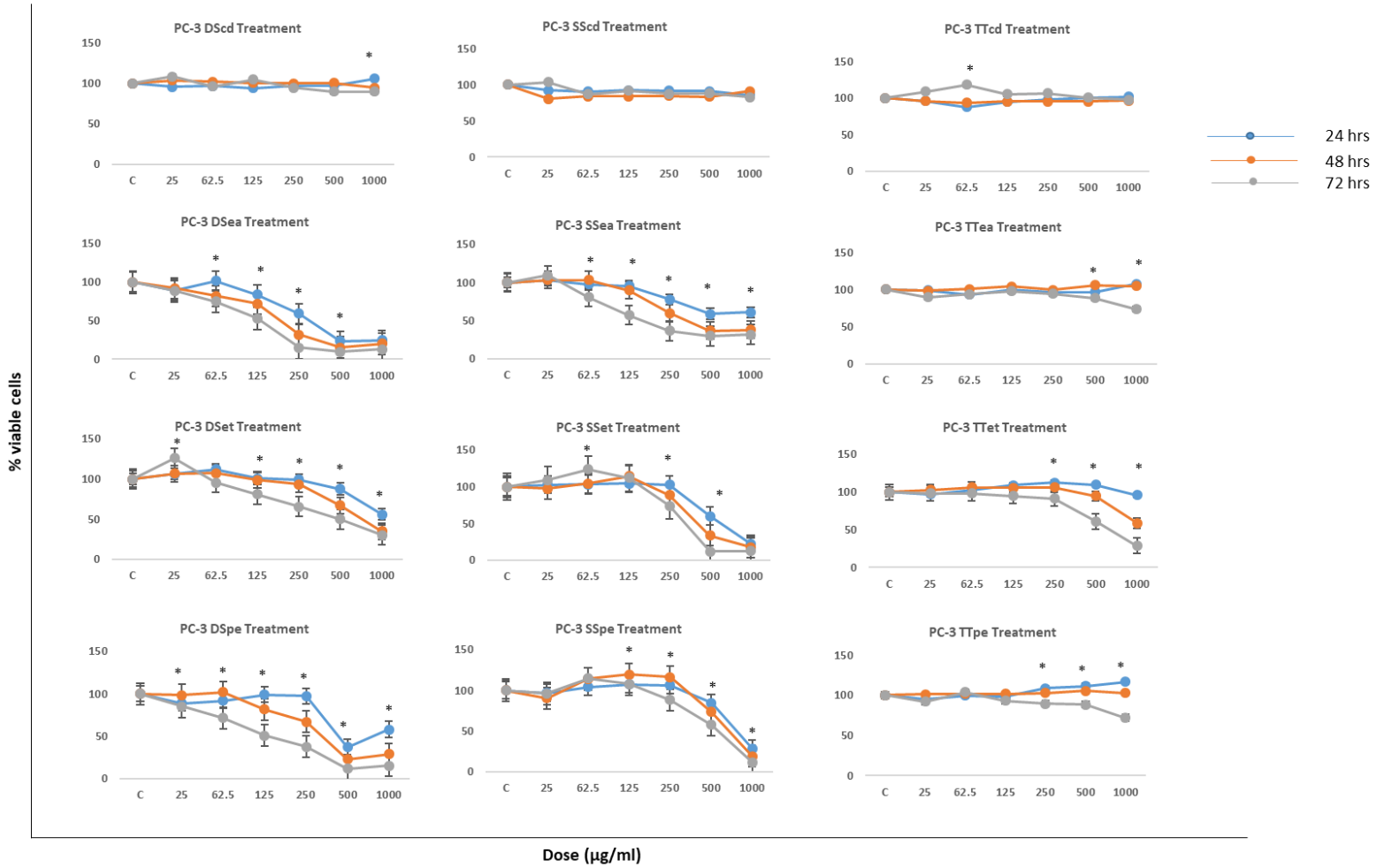


*Figure 3.1: Analysis of cell viability of NZ surf clam extract-treated PC-3 cells as determined by MTT assay. Cells were treated with different concentrations for 24, 48, and 72 hours, as indicated in each legend. Data are represented as means ± SE (n = 3). * indicates significant difference, p<0.05.*

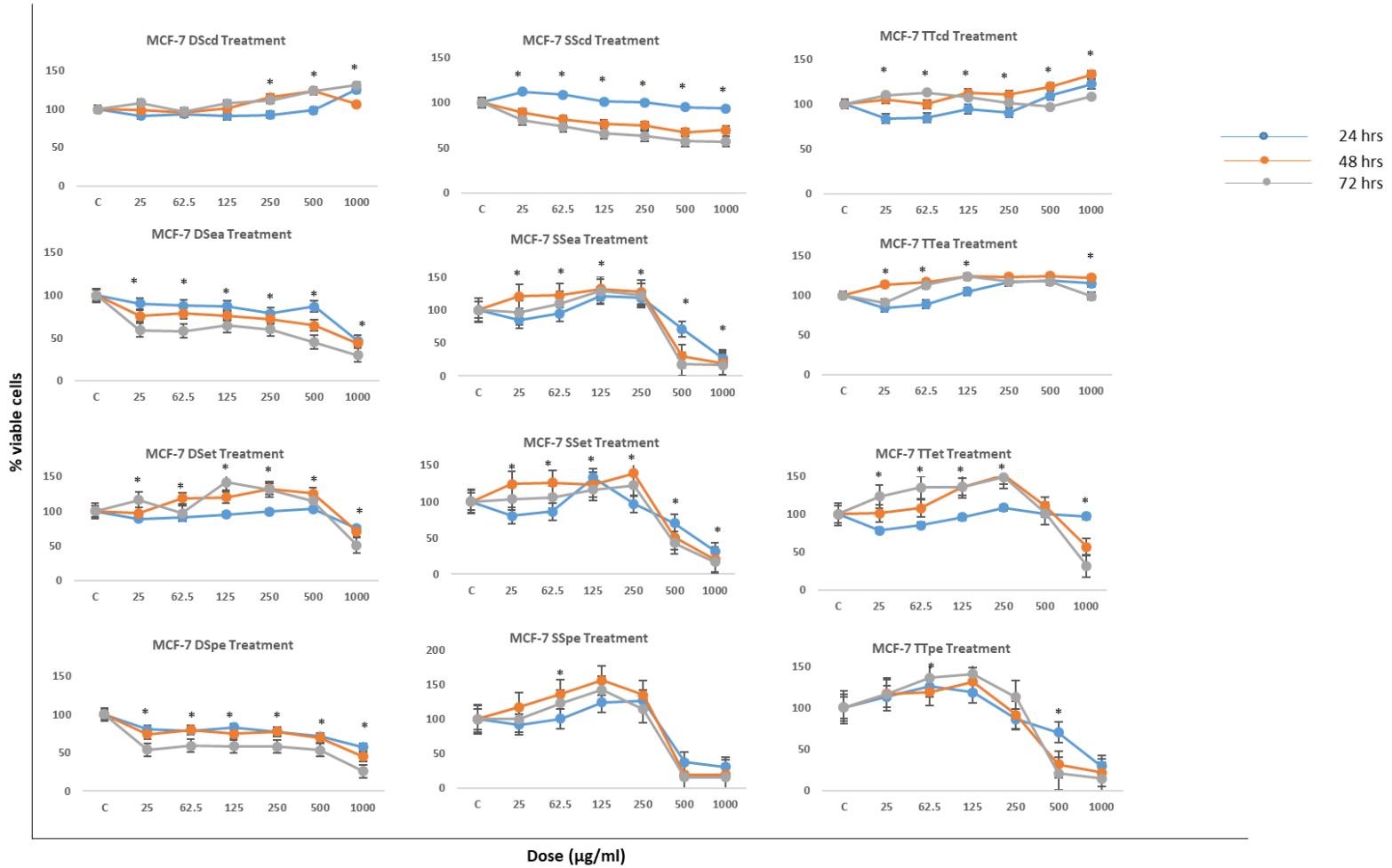


*Figure 3.2: Analysis of cell viability of NZ surf clam extract-treated MCF-7 cells as determined by MTT assay. Cells were treated with different concentrations for 24, 48, and 72 hours. Data are represented as means ± SE (n = 3). * indicates significant difference, p<0.05.*

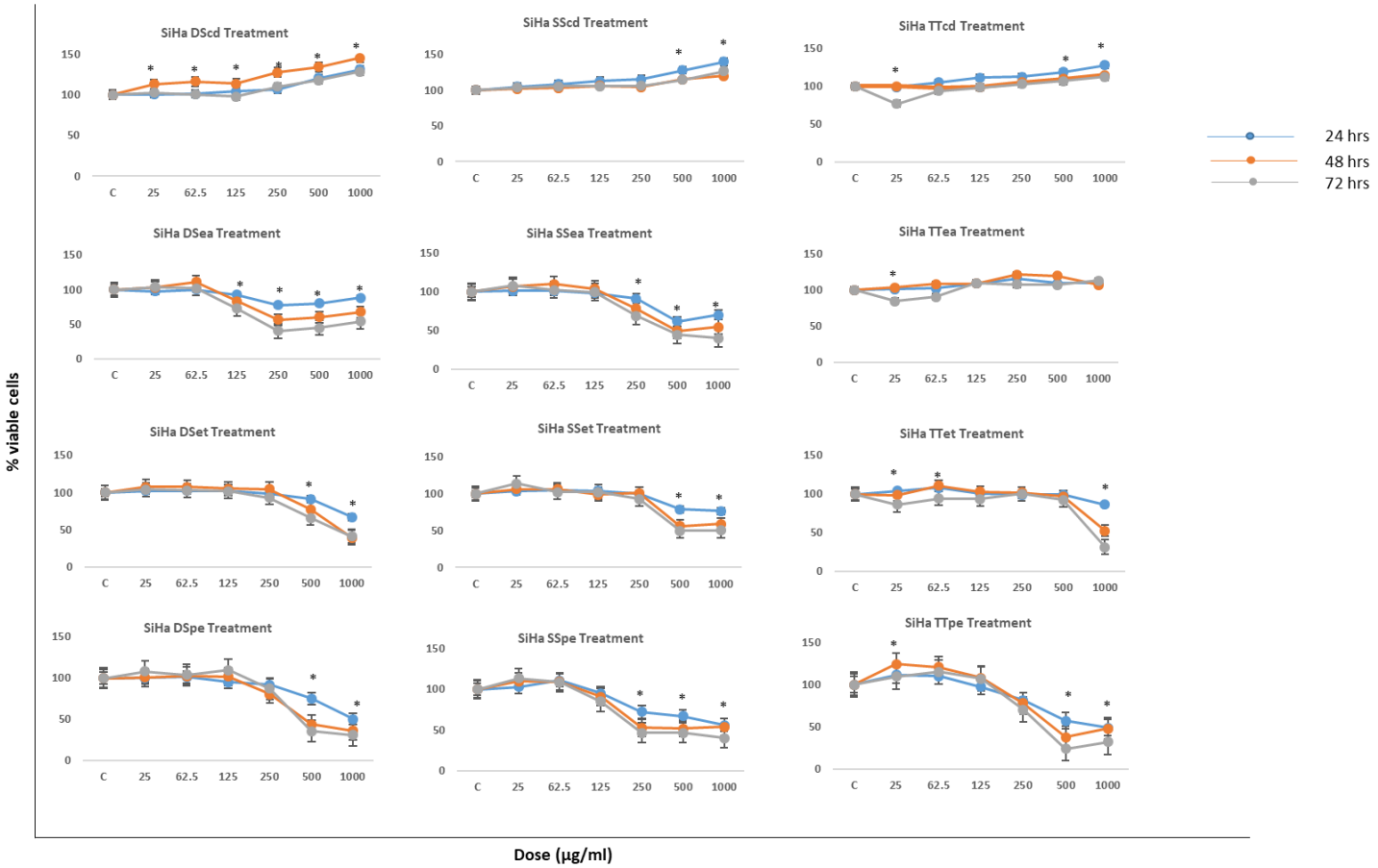


*Figure 3.3: Analysis of cell viability of NZ surf clam extract-treated SiHa cells as determined by MTT assay. Cells were treated with different concentrations for 24, 48, and 72 hours. Data are represented as means ± SE (n = 3). * indicates significant difference, p<0.05.*

Effects of NZ surf clam extract treatments suggest significant inhibition on cancer cell growth. To examine the inhibitory effects of NZ surf clam extracts on hormonal cancer cell viability, cells were treated with a series of extracts at concentrations between 25 and 1000µg/ml for 0, 24, 48, and 72 hours employing the MTT assay. The MTT assay is a well established and widely used assay to test the cytotoxicity against cancer cell lines. Viable cells are able to convert the soluble yellow tetrazolium salt, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl- tetrazolium bromide, to an insoluble purple coloured formazan precipitate by the mitochondrial enzyme succinate dehydrogenase. Dead cells, on the other hand, are unable to carry out this reaction (Sreejamole and Radhakrishnan, 2013).

Although each cell line was subject to 12 treatments each (Figs 3.1 – 3.3), only the best three (one from each clam species) are fully described (Table 3.1). This is not in any way due to poor activity of the extract (albeit this is the case for most cd extracts). For example, a 72-hour DSpe treatment of PC-3 displayed an inhibition of 62%, 88%, and 84% at 250, 500, and 1000 µg/ml respectively (data not shown). However, DSea treated PC-3, under the same conditions, displayed inhibition values of 85%, 90%, and 87%. In addition, for brevity, only the inhibition values of treatments, and their IC_{50} values, at 250, 500, and 1000 µg/ml are discussed.

The percent growth inhibition increased in most cases, with an increase in treatment concentration (Figures 3.1-3.3). The IC_{50} values of the extracts (DS, SS and TT) at 72 hours were 120.4, 378.5, 568.5 µg/ml (PC-3), 300, 334.7, 320.5 µg/ml (MCF-7), and 431, 390, 377.5 µg/ml (SiHa). There were a few cases where the viability plateaued at 500 µg/ml. A possible explanation for this may be that the extract reached saturation at 500 µg/ml

and thus was unable to cause any further or significant cell viability inhibition. Remarkably, in all cell lines, cell viability at 1000 µg/ml, and at 72 hours did not exceed 40%.

The MTT differential sensitivities, based on a 72-hour time point, were greatest to least as follows: PC-3 > MCF-7 > SiHa. Inhibition values of DSea and SSet treated PC-3 (500 µg/ml), at 48 hours were 85 and 67% respectively, and both showed greater than 80% inhibition at 72 hours. Furthermore, MCF-7 and PC-3 showed comparable sensitivities to treatments. The percentage inhibition of DSpe, SSpe, and TTpe treated SiHa cells at 72 hours (1000 µg/ml), on the other hand, were 69, 60, and 68% respectively.

Cell viability was largely unaffected at lower concentrations up to 125 µg/ml. As a matter of fact, in some cases, concentrations between 25 and 125 µg/ml promoted the growth of cancer cell lines (IC50 not provided, but see Fig 3.1). Our results are consistent with Kong et al. (1997), where extracts of the clams, *Meretrix lusoria, Anadara granosa*, and *Sinonovacula constricta,* increased the relative viability of HB4C5 cells at concentrations as low as 1.25, 2.5, 5, 10, and 20 µg/ml. Liao et al (2016) also reported that MCF-7 and MDA-MB-231 cells were not affected by freshwater clam (*Corbicula fluminea*) extracts at $\leq$250 µg/ml. This may be indicate that the cells undergo a short-term recovery or healing afore death, resulting in a temporary increase in the survival of the cells.

In comparison with hard clam (*Meretri*x spp.) extract treatment on AGS cells, the NZ surf clam extracts performed significantly better especially at concentrations as low as 250, 500, and 1000 µg/ml, producing similar and better results, compared to the range (0.3125 – 2.5 mg/ml) employed in said study (Song et al., 2017). The IC_{50} values in MCF-7 treated cells obtained in this study were 300, 334.7, and 320.5 µg/ml for DSea, SSpe, and TTpe treatments respectively. Compared to *C. fluminea* extract- treated MDA-MB-

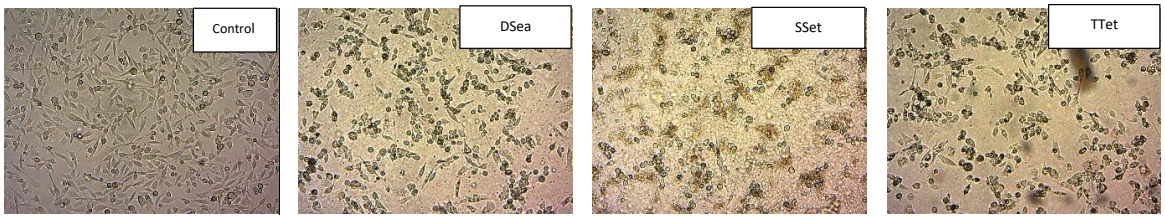
231 cells at similar concentrations, the NZ surf clam was almost four-fold more effective at reducing cell viability (Liao et al., 2016).

3.4.2 MORHPOLOGICAL CHANGES IN CULTURED CELLS INDUCED BY NZ SURF CLAM EXTRACTS

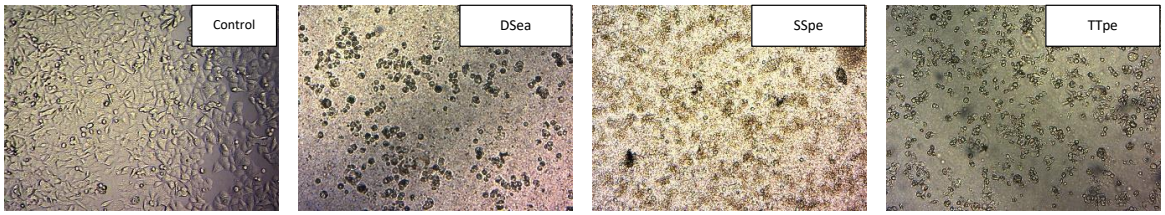
Apoptosis is characterized by biological and cytological features leading to morphological modifications, which mainly occur in the final stages of apoptosis (Abbro & Dini, 2003). Morphological characteristics of apoptosis include chromatin condensation, nuclear fragmentation in membrane bound vesicles, formation of blebs, loss of adhesion and rounding (in adherent cells), cell shrinkage and growth decline, and rapid phagocytosis by nearby cells (Saraste & Pulkki, 2000; Zimmermann et al., 2001).

In this study, morphological bioactivity testing of treated cells lines were taken. The morphological changes indicated that cell death occurred in all three cell lines (Fig 3.4). In untreated controls, the cells appeared morphologically normal, while treated cells showed signs of apoptosis. This experiment suggests that NZ surf clam extracts possess active constituents responsible for the morphological changes and cell death. In this study, cell shrinkage, cell disintegration, reduction in cell number, and loss of adhesion indicated cell death.

a. PC-3



b. MCF-7



c. SiHa

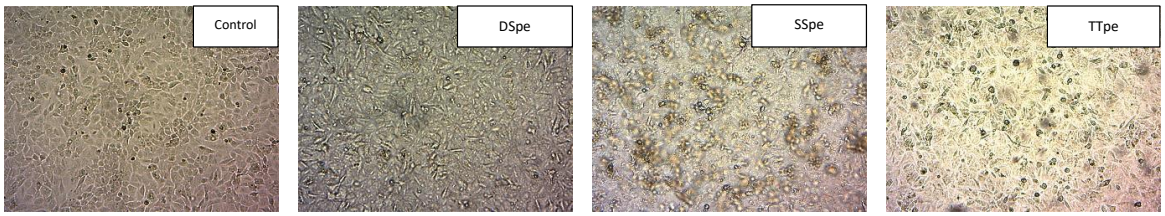


Figure 3.4: Morphological observation of NZ surf clam extracts on PC-3 (a), MCF-7 (b), and SiHa (c) cells. Cells were cultured in RPMI media and treated for 72 hours, at 600 µg/ml. The cell morphology was observed using an inverted microscope at 40x magnification (Zeiss).

3.4.3 APOPTOSIS INDUCING ACTIVITY OF NZ SURF CLAM EXTRACTS

A reduction in cancer cell growth and an induction of cancer cell death are two major mechanisms of inhibiting tumor growth. Apoptosis is a specific type of cell death that brings balance between cell division and cell death, of which when induced, is the most common anticancer mechanism employed by many a cancer therapy (Huang et al., 2003). Externalization of PS is one of the many biological changes of cells associated with apoptosis (Johnson et al., 2000). This can be analysed to detect the binding of annexin V to PS using a flow cytometer (Cheng et al., 2004). To confirm the morphological cell

death observed in Fig 3.4 was due to apoptosis, an apoptosis assay was utilized. In this study, the optimal incubation time was 7 hours. At this time point, the extracts exerted significant inhibitory effects at the concentrations employed (400 and 600 μg/ml). This study demonstrates that NZ surf clam extracts can change the cell distribution among the four analytical quadrants of the apoptosis analysis. Such changes indicate significant cancer cell growth inhibition in the cell lines. Apoptosis was confirmed by fluorescence analysis of apoptosis using the Alexa Fluor® 488 annexin V/ Dead Cell Apoptosis kit. Of note, in this experimental condition, little necrotic cell death (<12% in all cases) was observed across all three cell lines (Fig 3.5a, 3.5b, and 3.5c).

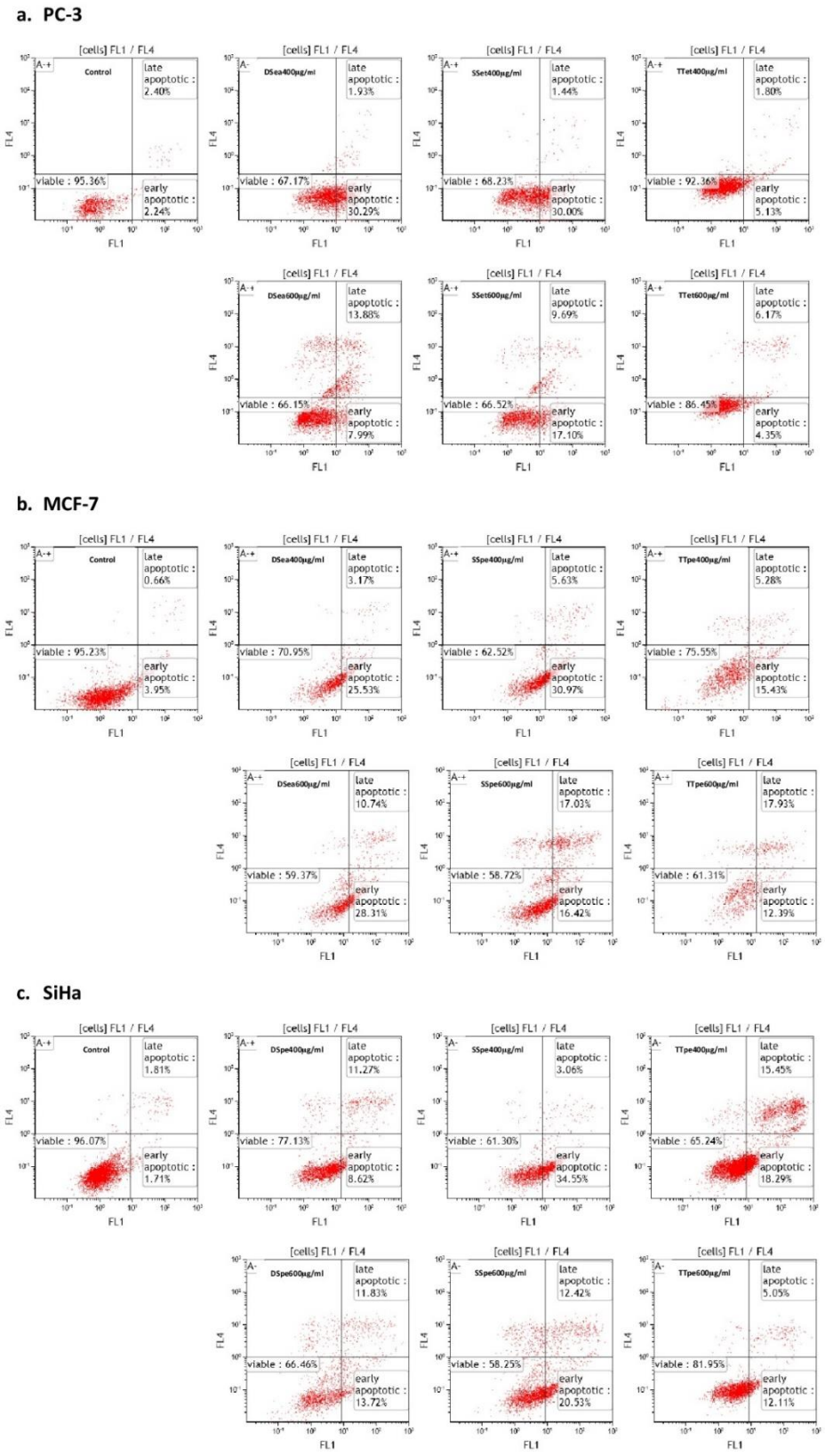


Figure 3.5: Induction of apoptosis on PC-3, MCF-7, and SiHa cells by NZ surf clam extracts in a, b, and c respectively. Annexin V/Dead Cell Apoptosis Kit with Aleza® Fluor 488 annexin V and PI determined the percentage of apoptotic cells, examined by flow cytometry.

The toxic effect displayed by the extracts after a short treatment incubation was possibly caused by disruption of cell membrane integrity or mitochondrial function in the cells (Saleem et al., 2002). In PC-3 cells, cell viability decreased in DSet and SSet treated cells (400 µg/ml) to 67% and 68% respectively. In TTet treated PC-3 however, cell viability was 92% at 400 µg/ml, which dropped to 87% at 600 µg/ml. This tally along with the MTT data, where TTet PC-3 treated cells were the least responsive of the three (with a percentage cell viability of 113%, 110%, and 96%, at 250, 500, and 1000 µg/ml). There was an insignificant decrease in cell viability between both concentrations (400 and 600 µg/ml) when treated with DSet and SSet, however, there was a remarkable increase in the late apoptotic population, from 1.9 to 13.88%, 1.44 to 9.7%, and from 1.8 to 6.2% in DSet, SSet and TTet treated PC-3 cells respectively. The same is true for MCF-7 and SiHa (Figs 3.5b and c). In all cases, there was a considerable decrease in the percentage of viable cells, and an increase in the percentage of late apoptotic cells at 600 µg/ml, compared to the percentage of early apoptotic cells. The only exception to this trend was observed in TTpe treated SiHa.

The induction of apoptosis occurred within hours, consistent with the 6 hour *Corbicula fluminea* (fresh water clam) treated HL-60 cells, suggesting that NZ surf clam extracts induced apoptosis by activating pre-existing apoptosis machinery (Huang et al., 2006).

3.4.4 CASPASE-3/7 ACTIVITY

Caspases are the major executioners of the apoptosis process, through the activation of the mitochondrial death cascade. Caspase -3, and -7 are two of the three effector caspases. The activation of caspase -3 leads to the final stages of cellular death, and subsequent activation of pro-apoptotic factors (Yacobi et al., 2004). Caspase -7 is similar

to Caspase -3 in terms of substrate specificity, and is suggested to play a supportive or synergistic role with Caspase -3 in apoptosis (Yin et al., 2004). To further confirm apoptotic processes, we assayed the Caspase-3/7 activity using the Apo-ONE Homogeneous Caspase-3/7 Assay Kit. The level of caspase-3/7 activity was estimated after 24 hours of treatment for each cell line. The results suggest that NZ surf clam extracts induced apoptosis via high expressions of caspase 3/7 cascades.

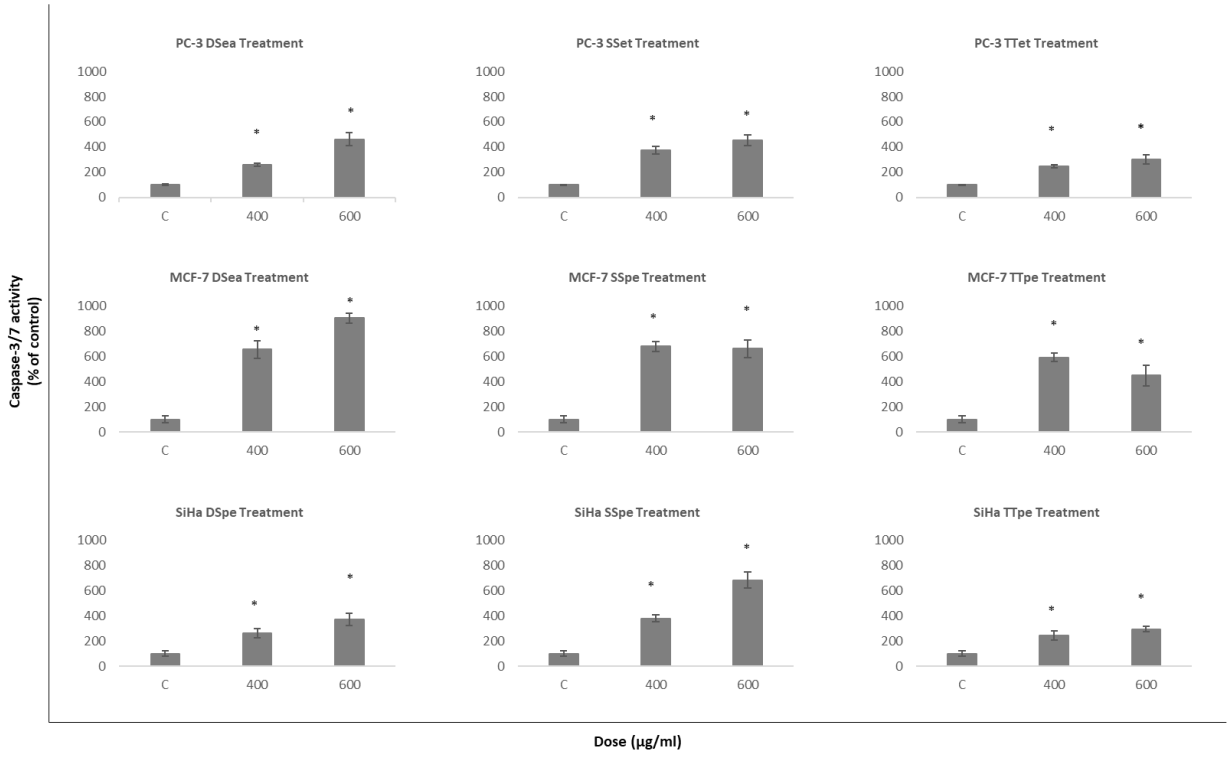


*Figure 3.6: The caspase 3/7 activities in PC-3, MCF-7, and SiHa after treatment of NZ surf clam extracts. Cell lines were treated with two concentrations for 24 hours. The caspase 3/7 activities were evaluated by Apo-ONE Homogeneous Caspase-3/7 Assay kit. The Caspase-3/7 activity of each group was indicated by their rate fluorescence (RFU). Data are presented as mean ± SE (n = 3). * indicates significant difference, p<0.05.*

As shown in Fig 3.6, 24hr treatment with NZ surf clam extracts at concentrations 400 and 600 µg/ml revealed an increase in levels of Caspase 3/7 activities in all three cell lines, corresponding closely with the apoptosis results. MCF-7 showed the greatest

caspase activity, with about a 6-fold increase in DSea, SSpe, and TTpe treated cells at 400 µg/ml. Although SiHa and PC-3 cells possessed high caspase activity, MCF-7 cells showed almost twice their activities. These results indicate that besides exerting cytotoxic effects, NZ surf clam extracts also induce the activation of caspase-dependent cellular apoptosis in a population of hormonal cancer cells. There is notable difference in highest caspase activity rankings compared to data from MTT results. Where PC-3 exhibited highest susceptibility to treatments in the MTT assay, its caspase activity was lower than MCF-7 values. This difference may indicate that subtle differences exist in the role of caspase cascades eliciting apoptotic responses in these cell lines.

3.4.5 CELL CYCLE ALTERATIONS

Interference to the normal cell cycle may be due to modifications to the distribution of cells across different stages of the cell cycle and regulation of the genes associated with the distribution, culminating in the inhibition of cell growth and proliferation (Song et al., 2017). These cell cycle-associated genes seemed to directly or indirectly induce cell death, mediated by the surf clam extracts. In the present study, the cell distribution of cancer cells were analysed after 72 hours of treatment with NZ surf clam extract. The percentage of cells in their different phases after treatment showed there was a shift in the distribution of identifiable populations. The sub-G1population was used as an indication of DNA fragmentation.

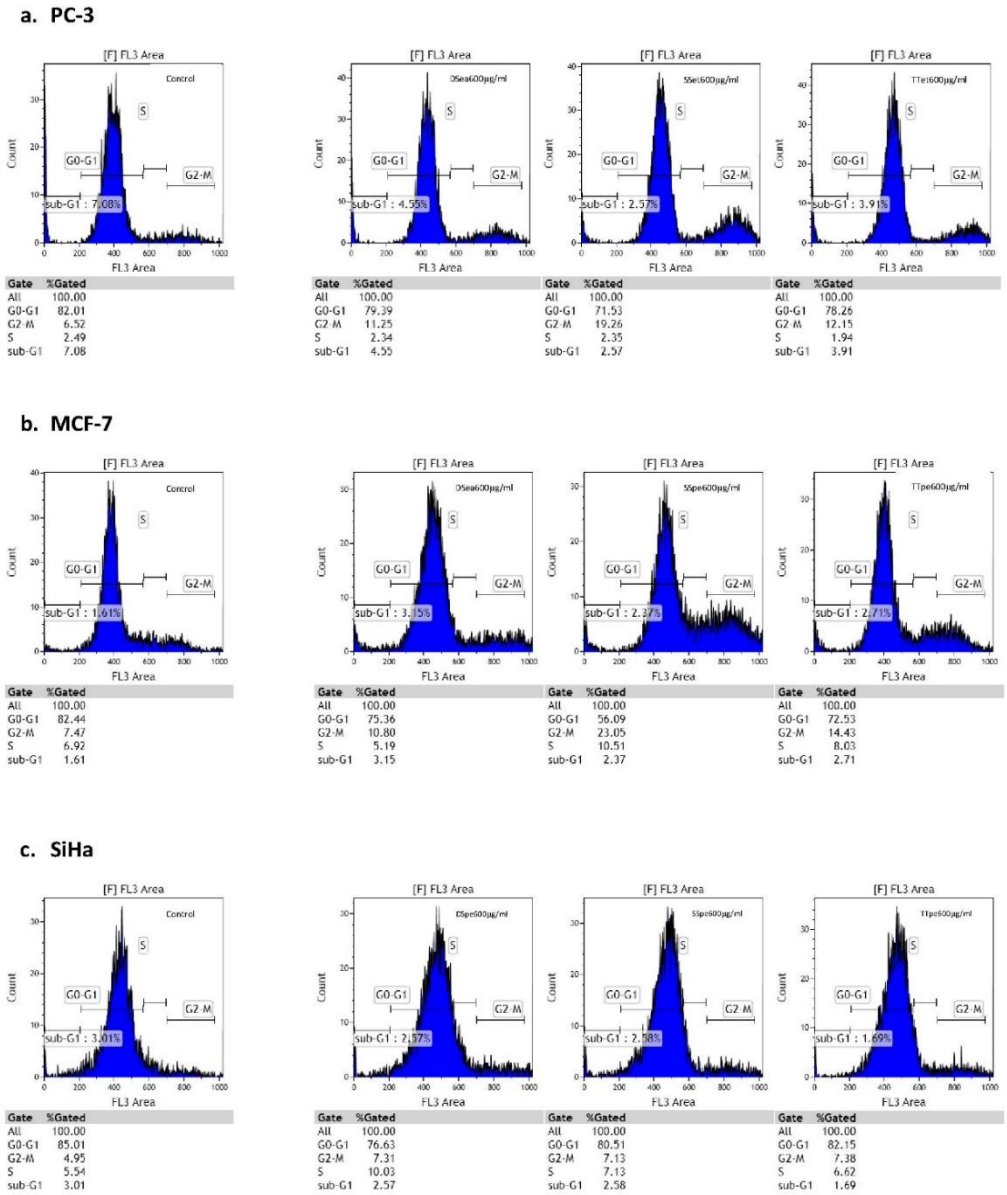


Figure 3.7: The induction of cell cycle arrest on PC-3, MCF-7, and SiHa by NZ surf clam extracts. Cell lines were treated with 600 µg/ml of various extracts. The percentages of cells in the different phases of cell cycle was determined by PI staining and examined by flow cytometry.

NZ surf clam extracts did not result in an increase in the G0-G1 phase, implicating inhibition of cell proliferation (Fig 3.7). Furthermore, the accumulation of extract-exposed cells in the G2-M phase suggest that the cells are not re-entering the G1-GO phase of the cell cycle. This was particularly observed in PC-3 and MCF-7, where,

compared to the control, there was a 72.5, 195.4, 68.3% and a 44.6, 208.6, 93.2% increase of cells in the G2-M phase in PC-3 and MCF-7 respectively. These results also show that NZ surf clam treatment induced the accumulation of S phase cells especially in MCF-7 and SiHa cells, insinuating a cell cycle arrest in the S phase. The induction of S-phase arrest are in agreement with treatment of MCF-7 cells with *Corbicula fluminea* extracts (Liao et al., 2016). The induction of arrest in the S- and G2-M phases may be the major mechanism by which the growth of these cancer cells is inhibited. It was indeed clear that cell cycle progression was obstructed. In addition, the cells shrunk during the 72 hour treatment, further confirming the occurrence of cell death after treatment (Fig 3.4).

3.4.6 CHEMICAL COMPOSITION OF FRACTIONS

The ethyl acetate and petroleum ether fractions exhibited the most significant growth inhibition across all cell lines, as reported in our previous study. The cytotoxicity described in this study is in line with characterised antioxidant activity of the isolated extracts (Odeleye et al., 2016). The other extract showing high cytotoxicity was the et extract, occurring twice in PC-3 treatments (SSet and TTet). The use of ethanol is employed in the extraction of polysaccharides to defat soft body tissue (Zhu et al., 2011). Analysis of et extracts revealed the presence of proteins (Zhu et al., 2011) and amino acids (Luan et al., 2011), amongst others, which serve as drug targets in cancer therapy, and can be conjugated to a cytotoxic drug to deliver to a cancer cell expressing the

corresponding peptide receptor (Thundimadathil, 2012). The ea fraction of all clam species is a protein/lipid mixture, slightly favouring greater presence of lipids over proteins. They contained negligible amounts of carbohydrates (Odeleye et al., 2016). The ea fraction of *Mactra veneriformis* possess high amounts of nucleosides and oligosaccharides (Luan et al., 2011). Nucleotide analogues display a wide range of other biological activities, such as antiviral and antitumor activities (Spanou et al., 2007). In studies of an ethyl acetate fraction from herbal extracts, Zheng et al. (2016) surveyed anti-caspase activity in HT-29 cells indicated high levels of activity against the colon cells. The same is true for the ea extracts of the stem bark of Cudrang (*Cudrania Tricuspidata*) (Seo et al., 2001) and Danewort (*Sambucus ebulus*) fruits (Shokrzadeh et al., 2009). In this study, within DS, the ea fraction appears to be the most effective fraction, with the greatest cytotoxicity evidenced in two of the three cell lines tested. . Even though similar ea fractions are discussed here, the methods of extraction have slightly different preceding steps. As a result, we expect compositional differences among the range of ea fractions. The pe fraction is predominantly comprised of lipids. The abundance of lipids in NZ surf clams is greatest in DS (84%), followed by TT (72%), and SS (64%), correlating to findings by Luan et al. (2011) on the clam, *Mactra veneriformis*. Moreover, the activity observed in this study is also likely, in addition to others, hinged on the unsaturated fatty acid content of the extracts, if there is a similarity in the components. The lipid extract of the hard clam *Meretrix lusoria* is a rich

source of a broad range of steroids belonging to the 5α, 8α-epidioxy sterol family (Pan et al., 2007). Lipid-rich mollusc extracts possess fat fractions, sterols, sterol esters, sphingolipids, etc, responsible for the bioactive property observed in their respective tests (Chijimatsu et al., 2013; Whitehouse et al., 1997; Chijimatsu et al., 2011; Gauvin et al., 2000; Chang and Duh, 2000). Interestingly, the components from Luan et al. (2011) are associated with inflammatory responses. The pe fractions are also characterised by negligible amounts of both proteins and carbohydrates (Odeleye et al., 2016). Surprisingly, all three SiHa treatment extracts in this study were pe fractions, showing at least 44% cytotoxicity at 100 µg/ml on Day 1 as observed in MTT studies.

The limitation of this study is that this is an early investigation of clam species of New Zealand without in depth chemical characterization. Since there is little information about bioactivities of New Zealand clams, early investigation with large-scale screening like the current study is essential, which will provide foundation for further and future clam bioactive study.

3.5 CONCLUSION

In summary, this study describes the cytotoxic activities of NZ surf clam extracts *in vitro*. The anti-proliferative effects of the surf clam extracts increase, in most cases, with increasing time and concentrations in the cell lines tested. These results demonstrate that the extracts have remarkable potent medicinal properties that show effective anti-

cancer activities in three hormonal cancer cells, and cause attenuation of the proliferation of these cancer cell lines. These results also show that at least two major factors are involved in the cytotoxic effects in PC-3, MCF-7, and SiHa- disruption of proliferation and cell cycle progression, and apoptosis. These findings may provide a theoretical basis for the prevention and/or treatment of several hormonal cancers with NZ surf clam extracts. However, the detailed mechanism of action and functional components responsible for the cytotoxic effect of NZ surf clam extracts needs to be further investigated. Furthermore, the data obtained in this study may not completely represent the *in vivo* situation. The administration of the extracts into an animal model, the subsequent metabolic processing into metabolites, metabolites' identification of cancer cells and resultant signal transduction cascades, are currently outside the scope of this study, but warrant further investigation. A future animal study will indeed answer these questions and more.

CHAPTER 4

CYTOTOXICITY OF EXTRACTS FROM NZ SURF CLAMS AGAINST ORGAN CANCER CELL LINES

4.1 ABSTRACT

In this study, we examined the cytotoxic effects of four fractions from three species of New Zealand (NZ) surf clam on four common organ cancer cells. In most cases, a dose and time-dependent inhibition on the proliferation of the cancer cells was observed. This was most significant in WiDr (colon) cells where the percentages of viability reduced to as low as 6%, 5%, and 17% (at 1000 µg/ml; 72 hours) by extracts from Diamond shell, Storm shell, and Tua tua species respectively. A549 (lung) cells were the least susceptible to the treatment, with viability percentages at 82%, 15%, and 45%, under the same conditions. Induction of caspase-dependent apoptosis and alterations to the cell cycle further supported the observed morphological analysis. The ethanol, petroleum ether, and ethyl acetate fractions of NZ surf clam, rich in lipids and proteins, were more potent than their water based counterpart. This is the first demonstration that extracts from NZ surf clams show the ability to inhibit the growth and proliferation of cancer cell lines. We suggest that NZ surf clam extracts have the potential to be further studied and developed as candidates for cancer supplementary management/treatment.

Keywords: extract; bioactive; surf clam; organ cancer cells; nutraceutical; New Zealand

4.2 INTRODUCTION

Marine animals possess bioactive substances (such as oligosaccharides, vitamins, and fatty acids) capable of reducing the risks of certain diseases (Lobo et al., 2010). These bioactives are diverse in structure, chemical and biological compositions, and possess extremely potent bioactive capabilities. Immunomodulatory, anti-viral (Singh et al., 2008), anticoagulant (Jung & Kim, 2009; Mohan et al., 2016), antithrombotic (Gomes et al., 2010), antihypertensive (Lee et al., 2018), antibacterial (Kumar et al., 2017), anti-inflammatory (Zhang et al., 2016), antidiabetic (Salem et al 2018), and antioxidant (Odeleye et al., 2016) activities have been reported.

The extraction (primitive or otherwise) and/or use of bioactive substances from marine organisms is a practice dating back to ancient Egyptian (Petit et al., 1970), Crete (Benkendorff, 2010), Indian (Benkendorff, 2010), and Chinese times (Hua et al., 2012). Molluscs rank high on the list of marine organisms explored for their bioactive metabolites. This may be because molluscs are more likely to produce metabolites to protect themselves from predation and pathogens (Beaulieu et al., 2013). Clams, among other molluscs, have been exploited for their bioactive properties (Joy & Chakraborty, 2017; Joy & Chakraborty, 2018; Ramasamy & Balasubramanian, 2012; Tsai et al., 2006; Ajithkumar et al., 2012; Lin et al., 2010). There are seven main species of surf clams in New Zealand (NZ), and the NZ surf clam market is a growing globally (Odeleye et al., 2016). NZ's most harvested and exported surf clams, the Diamond shell (*Crassula aequilatera*), Storm shell (*Mactra murchisoni*) and Tua tua (*Paphies donacina*), are used in this research.

Gastrointestinal (GI) cancer is a term describing a group of cancers that affect the digestive system. This includes the gall bladder, oesophagus, liver, pancreas, stomach, small intestine, colon and rectum (GI Cancer Institute, 2017). Cancers of the GI tract are the most common form of cancer in men and women alike (GI Cancer Institute, 2017), and are a significant cause of mortality and morbidity globally (Song & Wilson, 2005). GI cancers account for roughly 15% of diagnosed cancer in men and women (British Columbia Cancer, 2018). Lung cancer includes small cell lung cancer and non-small cell lung cancer. The estimated deaths of lung cancer in 2018 are 154,050, constituting 25.3% of all cancer deaths (National Institutes of Health- NIH, 2018). Surf clam extracts' anticancer activities have been described in literature (Liao et al., 2013; Liao et al., 2016; Pan et al., 2007). Our previous studies indicate that NZ surf clam extracts possess antioxidant activities (Odeleye et al., 2016). This study aims to, for the first time, determine and evaluate the effects of extracts from three NZ surf clam species on four types of cancer cell lines, including three GI/ digestive cancer cell lines (Liver- Hep G2, Pancreatic- MIA PaCa-2, and Colon- WiDr) and one respiratory cancer cell line (Lung- A549) *in vitro*.

4.3 MATERIALS AND METHODS

4.3.1 MATERIALS

A549, human lung cancer cells (Cat No. CRL-185); WiDr, human colon cancer cells, (Cat No. CCL-218); Hep G2, human liver cancer cells (HB-8065); and MIA PaCa-2, human pancreatic cancer cells (CRL-1420) were purchased from American type culture collection (ATCC) (Manassas, VA, USA). Ribonuclease A from bovine pancreas (Cat No.

R4875- 100mg) and Triton™ X-100 for molecular biology (Cat No. T8787-250ML) were purchased from Sigma-Aldrich (St Louis, MO, USA). MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) Formazan powder (Sigma Aldrich, St. Louis, USA); Roswell Park Memorial Institute (RPMI) 1640 medium, no phenol red (Life technologies, Auckland, NZ); Petroleum ether and Ethyl acetate (Global Science, Auckland, NZ), ethanol (ThermoFisher, Auckland, NZ). Foetal Bovine Serum (FBS) (Medica Pacifica, Auckland, NZ), L- Glutamine (200 mM; 100 ml) (Life technologies, Auckland, NZ), Penicillin- Streptomycin (10,000 U/mL; 100 mL) (Life technologies, Auckland, NZ); TrypLE™ Express, no phenol red (Life technologies, Auckland, NZ), Dimethyl sulfoxide (DMSO) (Merck chemicals); Trypan blue stain (0.4%) (Life technologies, Auckland, NZ); Dulbecco's Phosphate Buffered Saline (D-PBS) (Life technologies), Apo-ONE® Homogeneous Caspase-3/7 Assay kit (In vitro technologies, Auckland, NZ), Alexa Fluor® 488 annexin V/ Dead Cell Apoptosis kit (Thermo-Fisher Scientific, Auckland, NZ).

4.3.2 EXTRACTION AND FRACTIONATION OF CLAM SAMPLES

Three New Zealand surf clam species provided all samples characterised in this study, including Storm shell (SS) (*Mactra murchisoni*), Diamond shell (DS) (*Crassula aequilatera*), and Tuatua (TT) (*Paphies donacina*). Blanched clam meat was deshelled and drained of excess fluids. After allowed to come to room temperature, the flesh was oven dried in a hot air oven at 60ºC to a constant weight. Flesh was pulverised. Milled clam powder was stored at -20ºC until use. All clam extraction and measurements were carried out in dim light to reduce any possibility of oxidation. The extraction method from a previous study was adopted with slight modifications (Gauvin et al., 2000). Initial extraction fractions were generated in parallel using water (cd) and ethanol (et) as solvents. Clam powder solubilized in distilled water was stirred constantly at room

temperature using a magnetic stir-bar for one hour. The supernatant was removed, replaced with fresh solvent and stirred for another hour. This process was repeated until the solvent was colourless. The supernatant was collected by centrifugation. Ethanol extraction was carried out in the same way. The clear solution was collected and evaporated under reduced pressure using a Rota evaporator (Buchi Rotavapor R-215, Global Science, Auckland, NZ) until complete dryness. The water (cd) and ethanol extracts (et) were collected after evaporation and stored at -20^{o}C. The ethanol extraction (et) of each clam species were further fractionated by liquid-liquid extraction steps. The ethanol extract was further fractioned according to the polarities of petroleum ether (pe) and ethyl acetate (ea). The extracts were dissolved in 100ml distilled water and fractionated with petroleum ether. Extracts ('pe') were collected and concentrated under reduced pressure. Further step-by-step fractionation was done using ethyl acetate, which resulted in the fractions of 'ea'. Each fractionation process was repeated until the solvent was colourless. Fractions were evaporated to dryness and stored at -20ºC until ready for use.

Extracts were named thusly, for example, DScd is the cd extract of DS, SSet is the et fraction of SS, TTea is the ea fraction of TT, and so on.

4.3. CELL PROLIFERATION ASSAY

The cytotoxic effect of NZ surf clam extracts on three cancer cell lines was measured employing the MTT assay. The cells were seeded in a 96-well plate at a concentration of 1×10^5 cells ml^{-1} using the RPMI medium supplemented with 1 % Penicillin-Streptomycin, 1 % L-glutamine and 10 % fetal bovine serum. After incubation in a humidified 37 °C, 5% CO_2 incubator (Series II Water Jacket supplied by Thermo Scientific) overnight, the cells were treated by NZ surf clam extracts at a concentration range from

25 to 1000 µg/mL. The cells were further incubated for an additional 24, 48, and 72 hours independently at 37 °C. After incubation, MTT stock solution was then added to each well and incubated for a further 4 hours. The formazan crystals in each well were dissolved in 100 µl of DMSO. The amount of purple formazan was determined by measuring the absorbance at 540 nm.

Data interpretation

Treated cells with absorbance values lower than the control cells indicate a reduction in the rate of cell proliferation. On the other hand, higher absorbance values indicate an increase in cell proliferation.

%cell viability= {(At-Ab)/ (Ac-Ab)} × 100

Where, At= Absorbance value of test compound; Ab= Absorbance value of blank; Ac=Absorbance value of control; % cell inhibition= 100 - cell viability

4.4. CELL APOPTOSIS ASSAY

The apoptotic effect of NZ clam extracts was determined by the Alexa Fluor® 488 annexin V staining method and measured by flow cytometer. Cells were seeded onto 6-well plates, at a density of 4 x 10^5 cells per well and allowed to incubate overnight. The cells were then treated with NZ clam extracts for 7 hours at 400 and 600 µg/ml. After treatment, the cells were washed with PBS and harvested using trypsin. Careful measures were taken in order to prevent the loss of detached dead or apoptotic cells. The harvested cells were washed twice with cold PBS, then resuspended in 1 x binding buffer. 4µl of Alexa Fluor® 488 annexin V and 1µl of PI (Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit) were added to each 100 µl of cell suspension, with a slight modification to the manufacturer's protocols. After the incubation period, 400 µl 1X

annexin-binding buffer was added to each sample and analysed. Fluorescence-activated cell samples were recorded at 10,000 events using a flow cytometer (Beckman Coulter's MoFloTM XDP).

4.5. CELL CYCLE ASSAY

Cell lines were seeded onto 6-well plates, at a density of 3 x 10^5 cells per well and allowed to incubate overnight to ensure maximum cell attachment. The cells were then treated with NZ surf clam extracts for 72 hours. After treatment, the supernatant was collected, cells were washed with PBS and treated with trypsin. Careful measures were taken in order to prevent the loss of detached dead or apoptotic cells. The harvested cells were washed twice with PBS at 4^{o}C, and fixed with ice cold 80% ethanol at -20^{o}C for no longer than 7 days. The cells were then gently centrifuged for 2 minutes, after which permeabilizing solution was added to each sample, and incubated for 30 minutes at 37ºC. After permeabilization, PI was added and incubated for a further 5 minutes. Fluorescence-activated cells in each sample was recorded at 10,000 events using a flow cytometer (Beckman Coulter's MoFlo^{TM} XDP).

4.6. THE APO-ONE® HOMOGENEOUS CASPASE-3/7 ASSAY

The Apo-ONE Homogeneous Caspase-3/7 reagents was prepared according to the manufacturer's recommendations. Cells were seeded onto 96-well plates at a density of 5 x 10^3 cells per well, and allowed to incubate overnight. The cells were treated with NZ clam extracts for 24 hours at 400 and 600µg/ml. After incubation, an equal volume of Apo-ONE caspase-3/7 reagent was added to each well, and incubated while shaking for 1 hour at room temperature. The fluorescence of each well was read at 495 ± 10 (excitation) and 520 ± 10 (emission).

4.7. STATISTICAL ANALYSIS

MTT and caspase data were collected from duplicate experiments of triplicate samples. Apoptosis and cell cycle assays were carried out twice, in duplicates. Results are presented as mean ± standard error of the mean and $p<0.05$ was considered statistically significant. The use of t-test, non-parametric comparison, and 1- and 2- way ANOVA applications were employed. Also, post-analysis Dunnett testing was used to identify differences in data from this study. MTT and caspase data were analysed using Microsoft Excel. Analysis of Flow cytometry data was performed using Kaluza Analysis 1.3 (Beckman Coulter, Miami, FL, USA).

4.4 RESULTS AND DISCUSSION

4.4.1 ANTI-PROLIFERATIVE ACTIVITIES OF NZ SURF CLAM EXTRACTS

Initial examinations of the effects of NZ surf clam extracts on the proliferation of the selected cancer cells were carried out employing the MTT assay. The cell lines were treated with 12 extracts each (Supplementary Figs. 4.1a - d), but only the best three extracts are discussed for brevity. Most extracts displayed a dose- and time-dependent inhibition on the proliferation of the cancer cells used in this study (Fig. 4.1). Results are expressed as percentages of viable cells compared to the control.

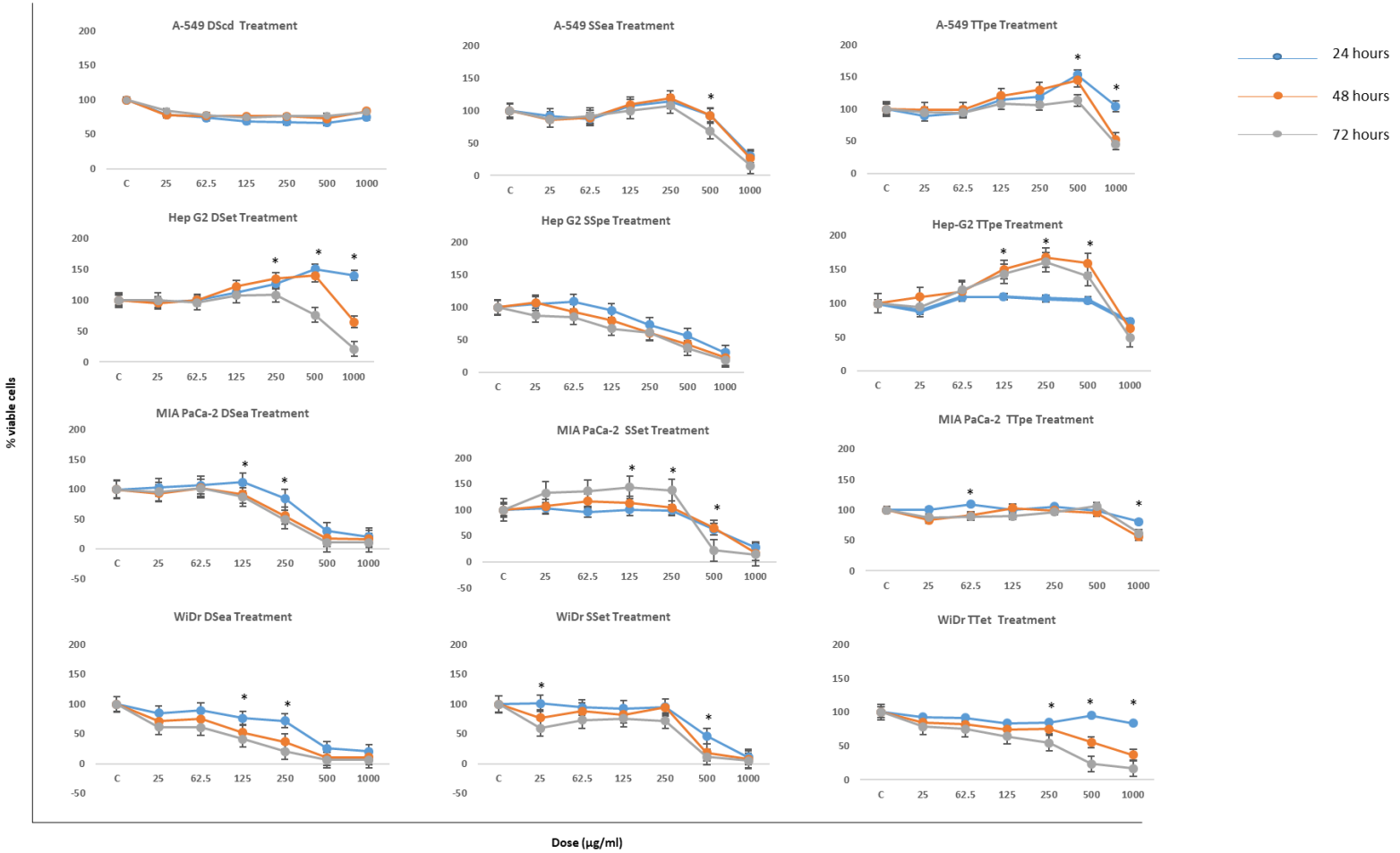


*Figure 4.1: The inhibitory effect of NZ surf clam extracts on the growth of A-549, Hep G2, MIA PaCa-2, and WiDr after an incubation time of 24, 48, and 72 hours. Cells were incubated in the presence of various extract concentrations. A relative cell viability of 100% was designated as the total number of cells that grew after each time point. Each experiment was carried out twice, in triplicates. Data is presented as means ± SD. * indicates statistical significance, $p < 0.05$.*

In order to ensure an accurate context for cellular viability in this study, control treatments of cells were carried out using only the extracting solvents. MTT assays were performed, and determined that solvent-treated cells did not induce apoptosis (results not shown). Additionally, extract only (no cells present) did not chemically reduce MTT (results not shown). This is to ensure that the observed activities are a function of their constituent compounds.

Effects of NZ surf clam extract treatment on A-549, Hep G2, MIA PaCa-2, and WiDr suggest significant inhibition. Cell lines were treated with a series of concentrations between 25 and 1000 μg/ml, and assessed employing the MTT assay after 24, 48, and 72 hours of treatment.

The MTT differential sensitivities, based on a 72-hour time point, were least to greatest as follows: A549 > Hep G2 > MIA PaCa-2 > WiDr. MIA PaCa-2 and WiDr cell lines were the most susceptible to treatment with NZ surf clam extracts, showing dose-dependent inhibitions after only 24 hours of treatment. Inhibition values of DSea-treated MIA PaCa-2 and WiDr at 250, 500, 1000 μg/ml (72 hours) were 51, 89, 89%, and 80, 94, 94%. SSet-treated MIA PaCa-2 and WiDr cells also showed incredible inhibition values of 73, 83, 86%, and 89, 93, 95% respectively at 1000 μg/ml (24, 48, and 72 hours). After only 48 hours of treatment at 250 μg/ml, TTet- treated WiDr cell proliferation was inhibited by 63%. The best inhibition rates at 24, 48, and 72 hours (1000 μg/ml) was observed in SSet-treated WiDr in all cases. In MIA PaCa-2, the inhibition rate of DSea-treated cells, was marginally better than its SSet counterpart. Similar results of high cell viability inhibition were obtained by hard clam (*Meretrix lusoria*) extract-treated AGS and HL-60 cells at similar concentrations (Pan et al., 2007).

A549 and Hep G2 were the least susceptible cell lines to treatment. At 1000 μg/ml (72 hours), of their respective DS, SS, and TT treatments, both cell lines displayed

percent viabilities of 82, 15, 45, and 31, 19, and 49. These inhibition values were already achieved by MIAPaCa-2 and WiDr treated cells at 24 hours. SSpe-treated Hep G2, on the other hand, showed remarkable cell viability inhibition throughout the course of study, showing percentage inhibition values of 73, 57, 31% (24 hours), 61, 44, 23% (48 hours), and 61, 38, 19% (72 hours) at 250, 500, and 1000 μg/ml respectively. A549 was even less sensitive to treatments than Hep G2, with the its best inhibitory effect at 71, 74, and 85% after 24, 48, and 72 hours of treatment, respectively.

Clam extract-treated MCF-7 and MBA-MB-231 at 1500 μg/ml, cell proliferation inhibition values were 78.8% and 64.3% respectively (72 hour treatment) (Liao et al., 2016). Such values were obtained in this study at 1000 μg/ml, after only 24 hours of treatment in SSea-treated A549, SSpe-treated Hep G2, DSea-treated MIA PaCa-2 and WiDR, and SSet-treated WiDr cells. Moreover, compared to BCP-A (Blood clam-*Tegillarca granosa*) -treated (5 mg/ml) H1299, HeLa, and DS-145 cells, which showed inhibition rates of 77.28, 90.58, and 87.54% respectively (Chi et al., 2015), the NZ surf clam extracts performed significantly better, showing similar inhibition values at 500 and 1000 μg/ml (in MIA Paca-2 and WiDr cells) after 24 hours of treatment in both cases.

In most cases, no obvious dose-dependent inhibition was observed at concentrations up to 250 μg/ml (Fig. 4.1). In all cases, the inhibitory effect was most significant in concentrations above 125 μg/ml. Overall, concentrations between 25 and 125 μg/ml promoted the growth of cancer cell lines. Similar observations were made by Liao et al (2016), where breast cancer cells were unaffected by *Corbicula fluminea* extract treatments at concentrations $\leq$ 250 μg/ml. This temporary increase in cells' survival may indicate that the cells go through a short-term healing or recovery before death (2011), or that lower concentrations are simply ineffective, thereby promoting

cell growth, and resulting in treatment resistance in the cells. Another possible reason might be that at lower concentrations, the cells activate pro-survival pathways, which are shut down at higher concentrations.

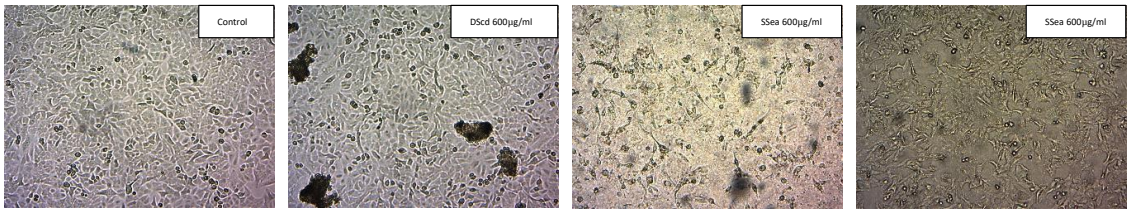
The cytotoxicity of *Cratoxy formosum* extract against Hep G2 cells at 500 µg/ml, after 48 hours of treatment is similar to those observed in this study (SSpe-treated Hep G2) at same concentration and time (Buranrat et al., 2017). Similar cytotoxicity was observed at 250 µg/ml in DSea-treated WiDr cells at 48 hours.

The results indicate that DSea and SSet fractions exhibited great suppression on cell viability especially in the MIA PaCa-2 and WiDr cell lines at 250, 500, and especially 1000 µg/ml (at all time points tested) (Fig. 4.1). The results also show that NZ surf clam extracts exhibited a broad spectrum of cytotoxicity against different cancer cells under identical conditions.

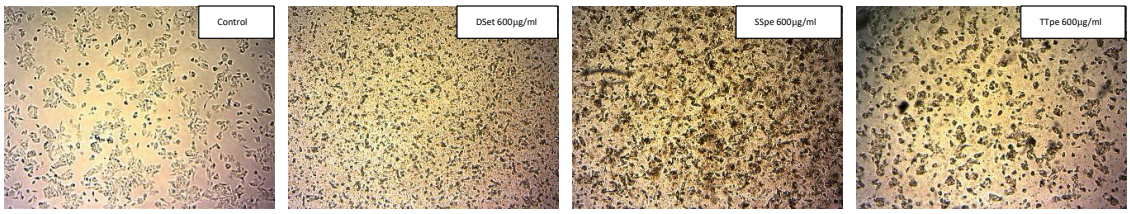
4.4.2 MORPHOLOGICAL ANALYSIS

Chromatin condensation, nuclear compaction and fragmentation, formation of blebs, shrinkage of cytoplasm, and growth decline are all morphological hallmarks of apoptosis (Huang et al., 2017). The morphological examination of NZ surf clam-treated cancer cell lines was carried out to observe signs of apoptosis. Cells were treated with respective extracts and incubated for 72 hours. The cell morphology was observed using an inverted microscope at 40x magnification (Zeiss). It was observed that untreated controls appeared morphologically normal, while treated cells showed signs of apoptosis, such as reduction in cell number, cell shrinkage and disintegration, and severe loss of adhesion (Fig. 4.2).

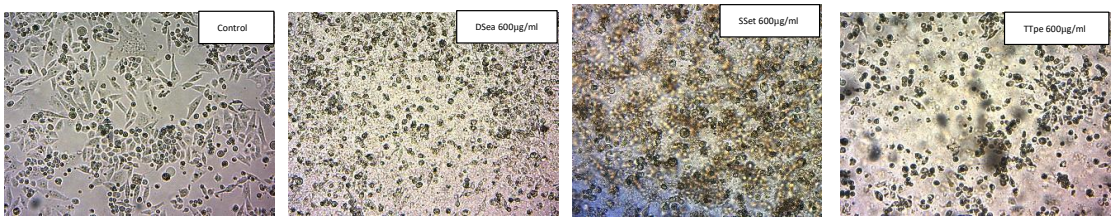
a. A549



b. Hep G2



c. MIA PaCa-2



d. WiDr

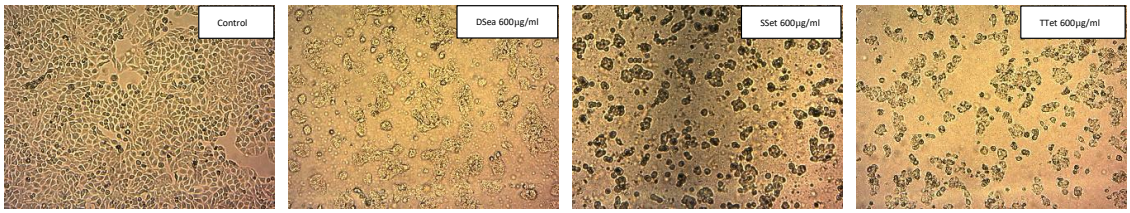


Figure 4.2: Morphological examination of extract-treated cancer cell lines, A549 (a), Hep G2 (b), MIA PaCa-2 (c), and WiDr cells (d).

4.4.3 APOPTOSIS INDUCING ACTIVITY OF NZ SURF CLAM EXTRACTS

Apoptosis is the most common anticancer mechanism in many cancer therapies (Zimmermann et al., 2001). To confirm that exposure to NZ surf clam extracts induced the death of A549, Hep G2, MIA PaCa-2, and WiDr cells by apoptosis *in vitro*, an apoptosis assay was performed. Cells were treated with two concentrations of their respective DS, SS, and TT extracts (400 and 600 μg/ml), and then stained with Annexin V and PI. Annexin V identifies apoptotic cells by binding to phosphatidyl serine exposed on the outer leaflet of the plasma membrane. PI stains dead cells by binding to the nucleic acids in the cell (Huang et al., 2003).

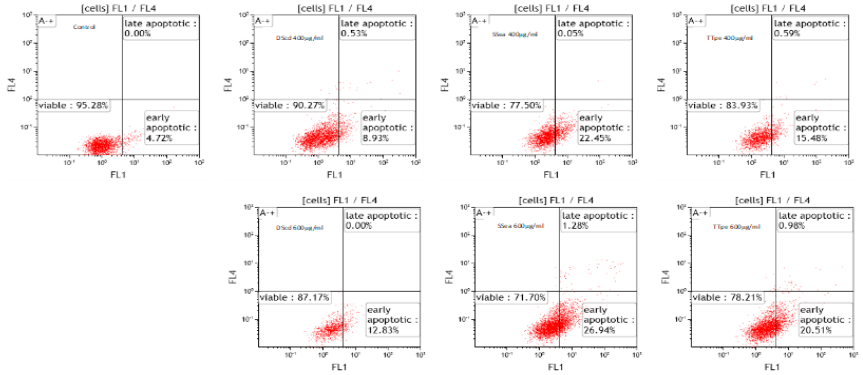
The percentage of apoptosis was examined after 7 hours of treatment, based on earlier optimization studies (data not shown). As shown in Fig. 4.3, cell distribution was altered, showing a decrease in viability. Notably, there was a very low percentage of late apoptotic cells in all cell lines tested. This may be due to a relatively short treatment time of 7 hours, correlating the early stages of apoptosis in the cell lines tested. Given the nature of this assay, it is plausible that a longer treatment time would reflect higher populations of late apoptotic cells (Fig. 4.3). Therefore, only two populations are discussed in this study: viable and early apoptotic cells. Furthermore, cells in this study did not undergo preliminary serum starvation; therefore, the apoptosis observed was initiated by the treatments, rather than in association with cell culture conditions.

A549 cells were the least responsive to treatments, corresponding with the MTT data. The viability of A549 cells did not indicate apoptosis to the degree in which was seen in the other cell lines. The cell viability percentages in A549 cells after a 400 μg/ml treatment were 90.27 (DScd), 77.50 (SSea), and 83.93% (TTpe). WiDr, the most susceptible cell line, on the other hand, showed percentage viability of 60.26 (DSea),

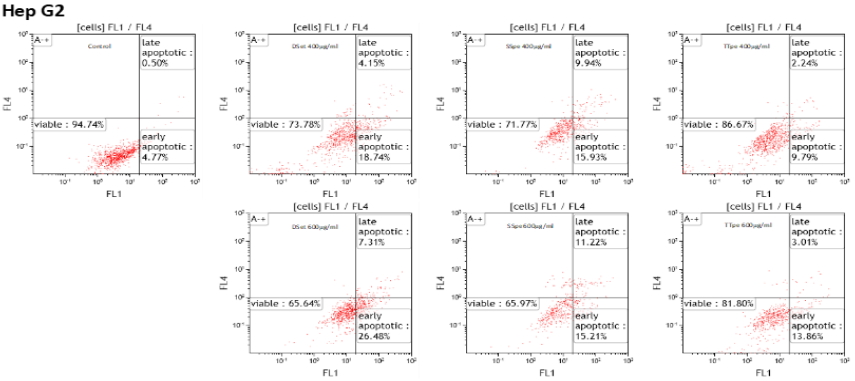
65.19 (SSet), and 69.33% (TTet) under the same conditions. The apoptosis observed in DS, SS, and TT-treated MIA PaCa-2 cells at 600 μg/ml (30.38, 38.32, and 26.19%) was similar to WiDr treated cells (42.49, 31.33, and 30.88%). In like manner, Hep G2 demonstrated high sensitivities to extracts with viable cells of 65.64 (DSet), 65.97 (SSpe), and 81.80% (TTpe) at 600 μg/ml. This may suggest that slightly different mechanisms are associated with different cell lines. Notably, A549 is a lung cancer cell line, while the others are GI-associated, and likely share more commonality in response cascades. These differences appear to be associated with caspase-3/7 cascade activation in the different cell lines (as is discussed in section 2.4).

NZ surf clam extracts produced great apoptosis results after the optimized 7 hours of treatment, compared to other studies. Protein hydrolysates from the blood clam (*Tegillarca granosa*), for example, achieved 14.29, 17.71, and 20.28% early apoptotic cells at 1.5, 2, and 2.5 mg/ml, after 20 hours of treatment (Chi et al., 2015). Moreover, the percentages of early apoptotic cells in Sepia ink oligopeptide-treated DU-145, PC-3, and LNCaP at 15 mg/ml were 38.26, 39.96, and 16.11% respectively (Huang et al., 2012). Similar and greater percentages of early apoptotic cells were achieved in this study at 400 and 600 μg/ml. The induction of apoptosis after only several hours suggests that apoptosis was induced by pre-existing apoptosis machinery (Huang et al., 2006).

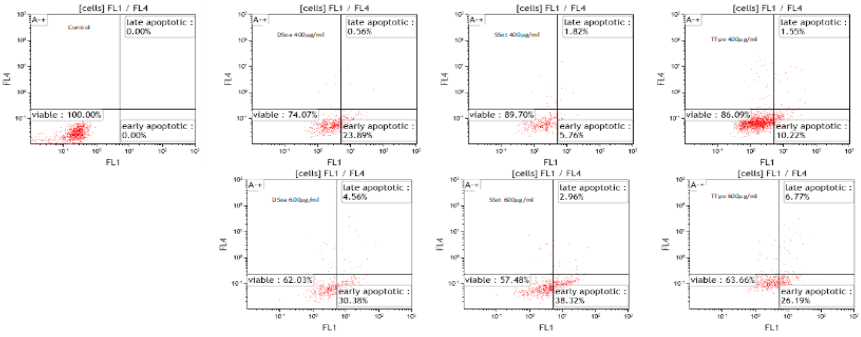
a. A549



b. Hep G2



c. MIA PaCa-2



d. WIDr

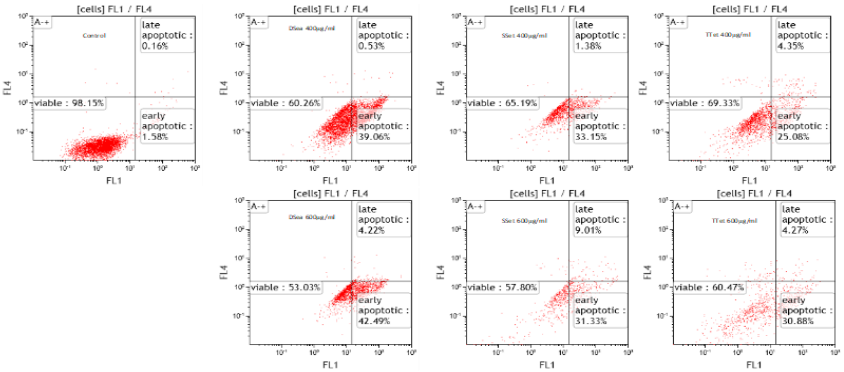


Figure 4.3: Induction of early apoptosis in A549, Hep G2, MIA PaCa-2, and WiDr cells by NZ surf clam extracts. Annexin V/Dead Cell Apoptosis Kit with Aleza® Fluor 488 annexin V and PI determined the percentage of early apoptotic cells, examined by flow cytometry. Each experiment was carried out twice, in duplicates. Data are represented as mean ± SE.

4.4.4. CASPASE-3/7 ACTIVITY OF NZ SURF CLAM EXTRACTS

To further confirm apoptotic activities by NZ surf clam extracts, we assayed the Caspase-3/7 activity employing the Apo-ONE Homogeneous Caspase-3/7 Assay kit. The level of caspase-3/7 activity was estimated after 24 hours of treatment for each cell line. The results suggest that NZ surf clam extracts induced apoptosis in a caspase-dependent manner.

In this study, the caspase-3/7 activities in all four cell lines correspond closely with the apoptosis results. The major difference in caspase-3/7 activities is seen between A549 and MIA PaCa-2 cells. TTpe- treated A549 at 400 and 600 µg/ml, showed higher caspase activities (344.7 and 375.3%) compared to TTpe-treated MIA PaCa-2 treated cells (216.1 and 147.9%). The caspase activities of DS- and SS- treated MIA PaCa-2, however, exceeded those of the A549 cells. As expected, WiDr cells possessed the highest caspase-3/7 activities in this study.

The most obvious increase in caspase activity was observed in DSea (864.5 and 1268.3%) and SSet (1435.8 and 973.2%)-treated WiDr cells, at both 400 and 600 µg/ml, compared to the control (100%). The extract-induced apoptosis in the cell lines studied herein seem to be related to the mitochondria-mediated pathway, based on the activation of caspases-3 and -7.

In this study, A549 cells showed lower caspase-3/7 activities (Fig. 4.4a). However, these data would be indicative of the activity, and not the expression of the caspase-3/7 proteins, isolating and defining A549 cells, relative to the expressions defining the GI cancers tested. Albeit A549 displayed relatively lower inhibitions and apoptotic activities (Figs. 4.1 and 4.3), its apoptotic activity was caspase-dependent and concentration dependent. These findings suggest greater activation of caspase-3/7 cascades in GI

associated cancer cell lines by the extracts, in preference to lower activations in lung cancer cells.

Compared to the Caspase-3/7 activities of *Angelica dahurica* extracts on colon cancer cells (HT-29) (48 hour treatment), the NZ surf clam extracts performed significantly better, showing approximately 3, 5, and 1-fold increases in Caspase-3/7 activities at the same concentration (400 μg/ml) in DSea, SSet, and TTet- treated WiDr (colon cancer) cells (24 hour treatment) (Zheng et al., 2016). Also, comparison of *Cratoxy formosum*-treated Hep G2 to NZ surf clam-treated Hep G2, reveals interesting results. Buranrat et al (2017) demonstrate a 2.9-fold increase in caspase activity over their control at 100 μg/ml treatment. Our studies highlight 400 μg/ml treatments of Hep G2 cells result in an approximate 4-fold increase in caspase activities, across DSet, SSpe, and TTpe extracts. Although our observed activities occur at a higher concentration, there is indication of potential similarities or improvement over the *Cratoxy formosum* extract.

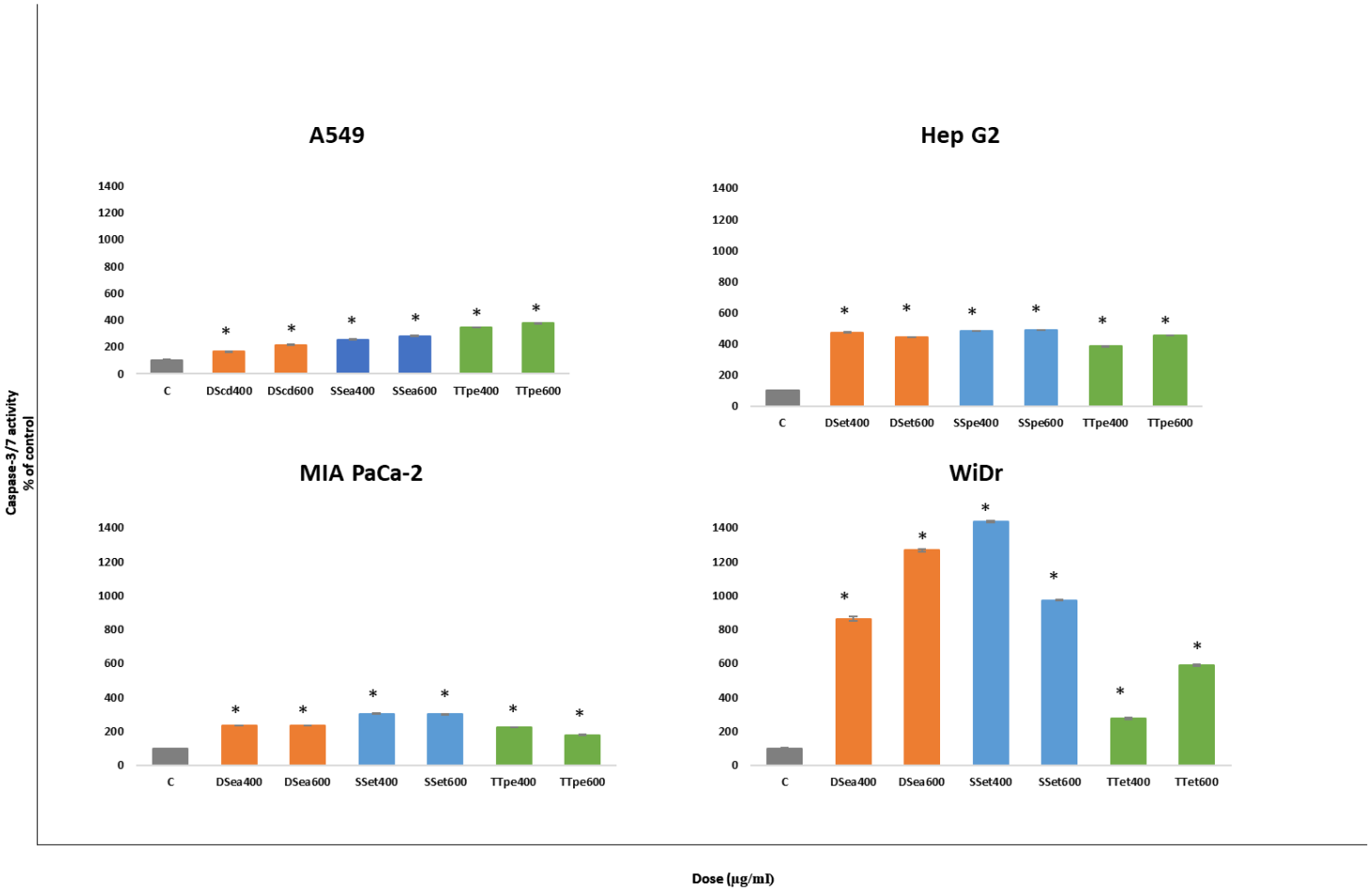


*Figure 4.4: Caspase 3/7 activities in A549, Hep G2, MIA PaCa-2, and WiDr cells after treatment with NZ surf clam extracts. Cell lines were treated with two concentrations (400 and 600 µg/ml) for 24 hours. Caspase 3/7 activities were evaluated by Apo-ONE Homogeneous Caspase-3/7 Assay kit. The Caspase-3/7 activity of each group was indicated by their rate fluorescence (RFU). Each experiment was carried out twice, in triplicates. Data is presented as mean ± SE. * indicates significant difference to control groups, p<0.05.*

4.4.5. CELL CYCLE ALTERATIONS BY NZ SURF CLAM EXTRACTS

The inhibition of cell growth and proliferation is significantly characterized by interference to the normal cell cycle (Huang et al., 2006). In this study, the cell distribution was analyzed after a 72 hour treatment. The percentage of cells in their different phases indicates an alteration to normal cell population distribution (Fig. 4.5).

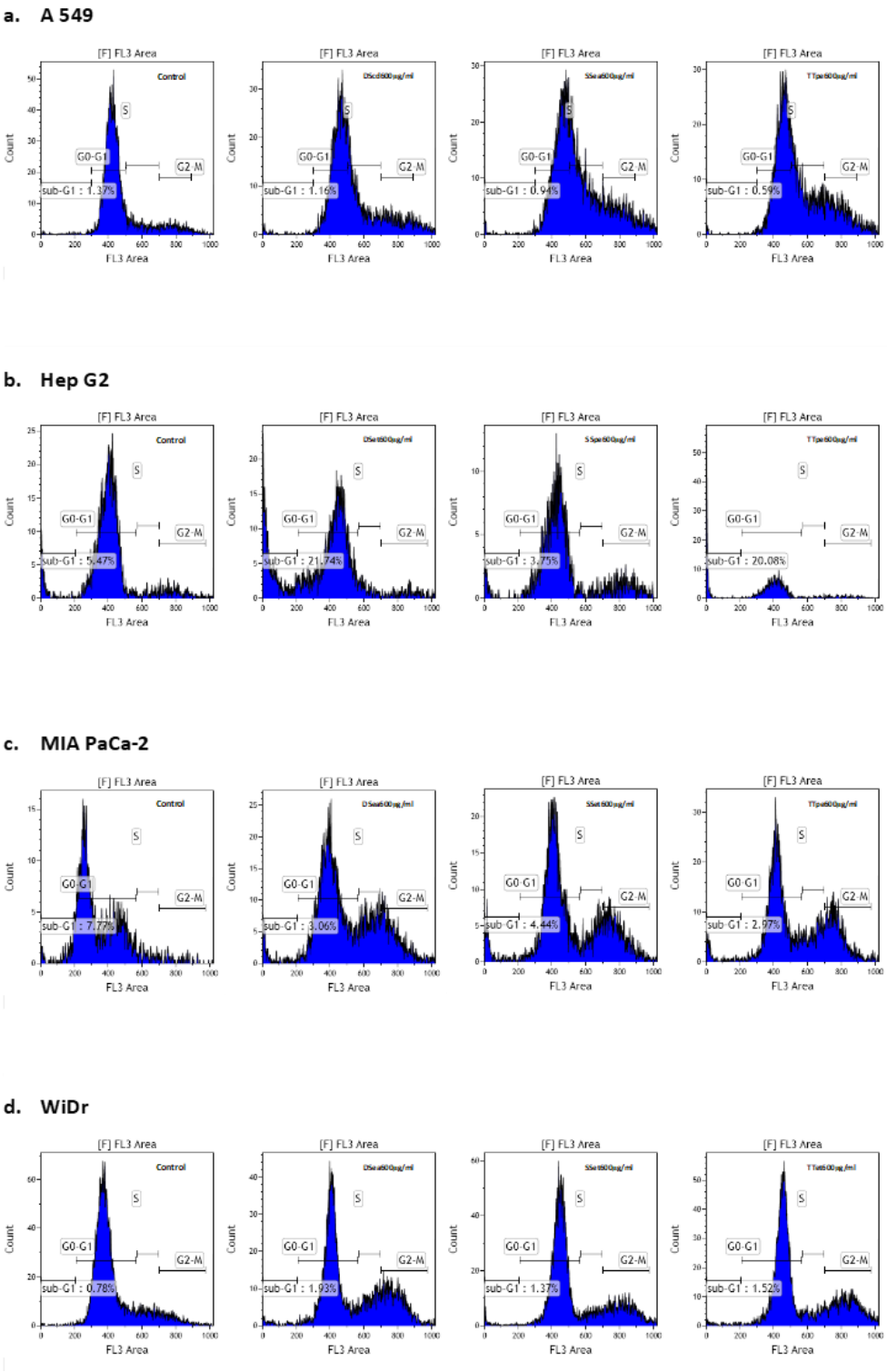


Figure 4.5: Induction of cell cycle arrest on (a) A549 cells (DScd, SSea, and TTpe treatments), (b) Hep G2 cells (DSet, SSpe, and TTpe treatments), (c) MIA PaCa-2 cells (DSea, SSea, and TTpe treatments), and (d) WiDr cells (DSea, SSet, and TTet treatments) respectively by NZ surf clam extracts. Cell lines were treated with 600 µg/ml of various extracts. The percentages of cells in the different phases of cell cycle was determined by PI staining and examined by flow cytometry. Each experiment was carried out twice, in duplicates. Data are represented as mean ± SE.

The cell cycle assay helps in the analysis of cell cycle parameters of surviving cells, the capacity to inhibit the progression of cell cycle, and the proportions of sub-G1 (indicative of cell death) cells. The sub-G1 population is generally used as an index of apoptotic DNA fragmentation (Liao et al., 2016). There was little to no increase in the sub-G1 cells in all cases, but DSet- and TTpe-treated Hep G2 cells (600 µg/ml) (Fig 4.5b). The sub-G1 cell populations in both cases were 21.7 and 20% respectively. In all cases, including Hep G2 treated cells, there was a significant decrease of cells in the G0-G1 population, indicating cell proliferation inhibition. In MIA PaCa-2 cells, for example, there was a 1.4 (DSea treatment), 1.4 (SSet treatment), and a 1.5 (TTpe treatment) fold decrease in the G0-G1 population, compared to the control. These results also reveal that NZ surf clam treatment induced the accumulation of cells in the G2-M and S-phases, except in DSet- and TTpe-treated Hep G2 cells. In the SS treatment of all cell lines, there was a 163.29 (A549), 207.99 (Hep G2), 544.50 (MIA PaCa-2), and 285.81 (WiDr) percentage increase of cells in the G2-M phase compared to their respective controls. The accumulation of cells in the G2-M phase suggests that the extract-exposed cells are not re-entering the G1-G0 phase of the cell cycle. The accumulation of cells in the G2-M- and S- phases, and ergo the strong G2/M and S- phases cell cycle arrest, may be the major mechanism through which the proliferation of A549, MIA PaCa-2, and WiDr cancer cells is inhibited. This accumulation of cells in the G2-M and S-phases correlate well with findings by Huang et al (2012) (Sepia ink-treated DU-145 and LNCaP), and Liao et al. (2016) (*Corbicula fluminea* extracts -treated MCF-7).

4.4.6. CHEMICAL COMPOSITION OF EXTRACTS

The fractions obtained from the entire extraction process were previously characterized (Odeleye et al., 2016). The crude extract obtained from DS, SS, and TT, contained comparable amounts of carbohydrates (15.48, 15.80 and 17.10%) and proteins (16.25, 15.48, 16.48%). Similar protein content was found in the DSea, SSea, and TTea fractions (12.49, 15.58, and 17.89%). The major content of the et fraction is lipid, with most of it extracted in the pe fraction (84.19%- DSpe, 63.50%- SSpe, and 72.01%- TTpe). A significant amount of lipids was also found in the ea fractions (21%- DSea, 19.50%- SSea, and 56.49%- TTea).

In this study, the et, pe, and ea fractions exhibited the most significant growth inhibitions across all four cell lines. The ea fraction is particularly comprised of proteins and lipids, suggesting that either the protein- or the lipid-, or both components are responsible for the cytotoxicity observed. Other studies show that the lipid extracts of molluscan extracts possess fat, sterols, sterol esters, and steroids, responsible for the observed bioactivity (Chijimatsu et al., 2011; Chijimatsu et al., 2013; Whitehouse et al., 1997; Gauvin et al., 2000). The pe fraction clearly contains the most lipids than any other fraction. It is highly plausible that lipids are responsible for some bioactive component which elicits the observed responses. Notably, the TTpe fraction was the most effective TT fraction in A549, Hep G2, and MIA PaCa-2. TTpe-treated WiDr showed higher susceptibility at 1000 µg/ml than TTet-treated WiDr at 72 hours (results not shown). However, cumulatively, TTet-treated WiDr was chosen because of its remarkable effects over a range of concentrations rather than effectiveness over a single higher concentration. This is more desirable as we have activities at lower concentrations in TTet-treated WiDr over its TTpe-treated WiDr counterpart.

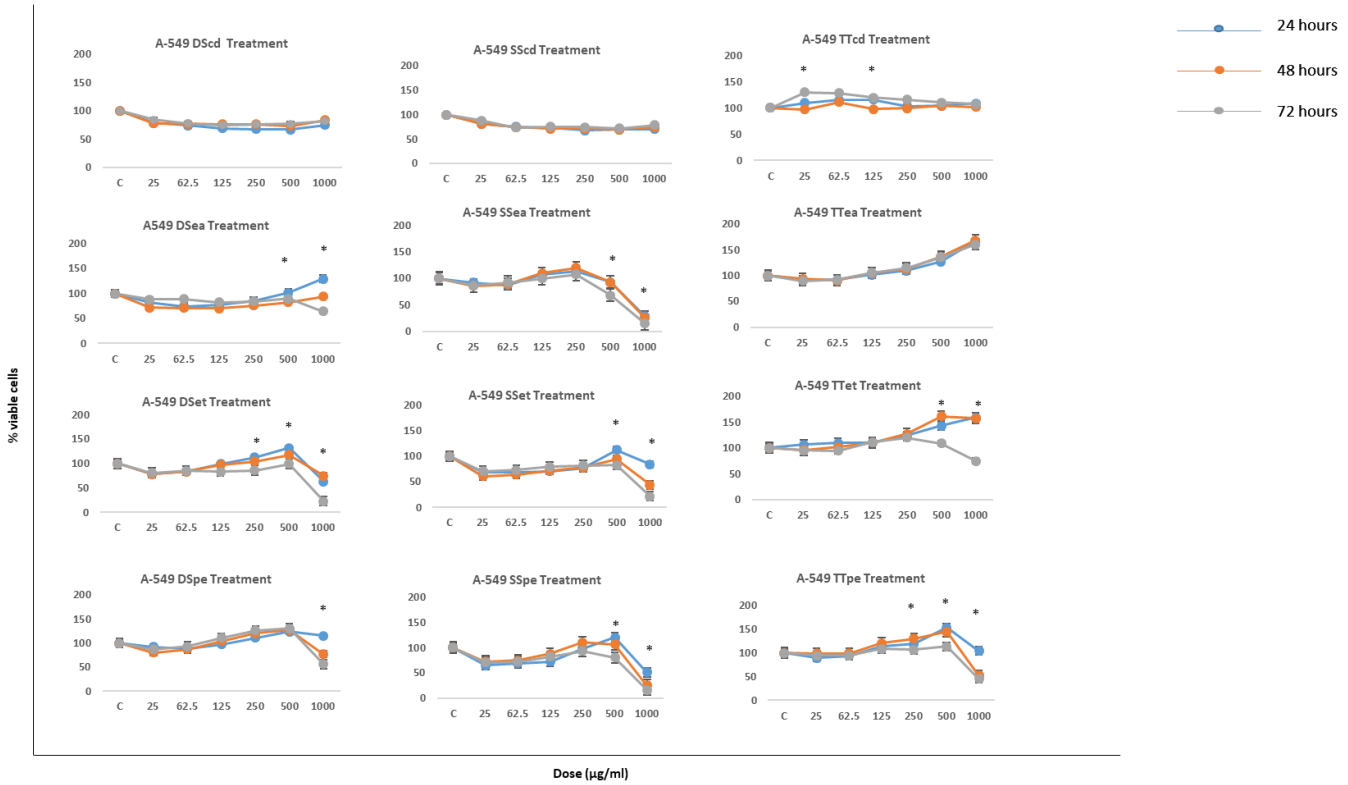
4.5 CONCLUSION

This is the first study to describe the preparation of NZ surf clam extracts and their anticancer activities. Results show that cell proliferation was time and concentration dependent, decreasing as both variables increased. The results demonstrate that NZ surf clam extracts possess remarkable bioactivity, responsible for the observed *in vitro* caspase-dependent apoptosis and disruption of cell cycle progression in the four cancer cell lines. These results may provide a basis for the further investigation and development of NZ surf clam extracts in cancer treatment or treatment supplement.

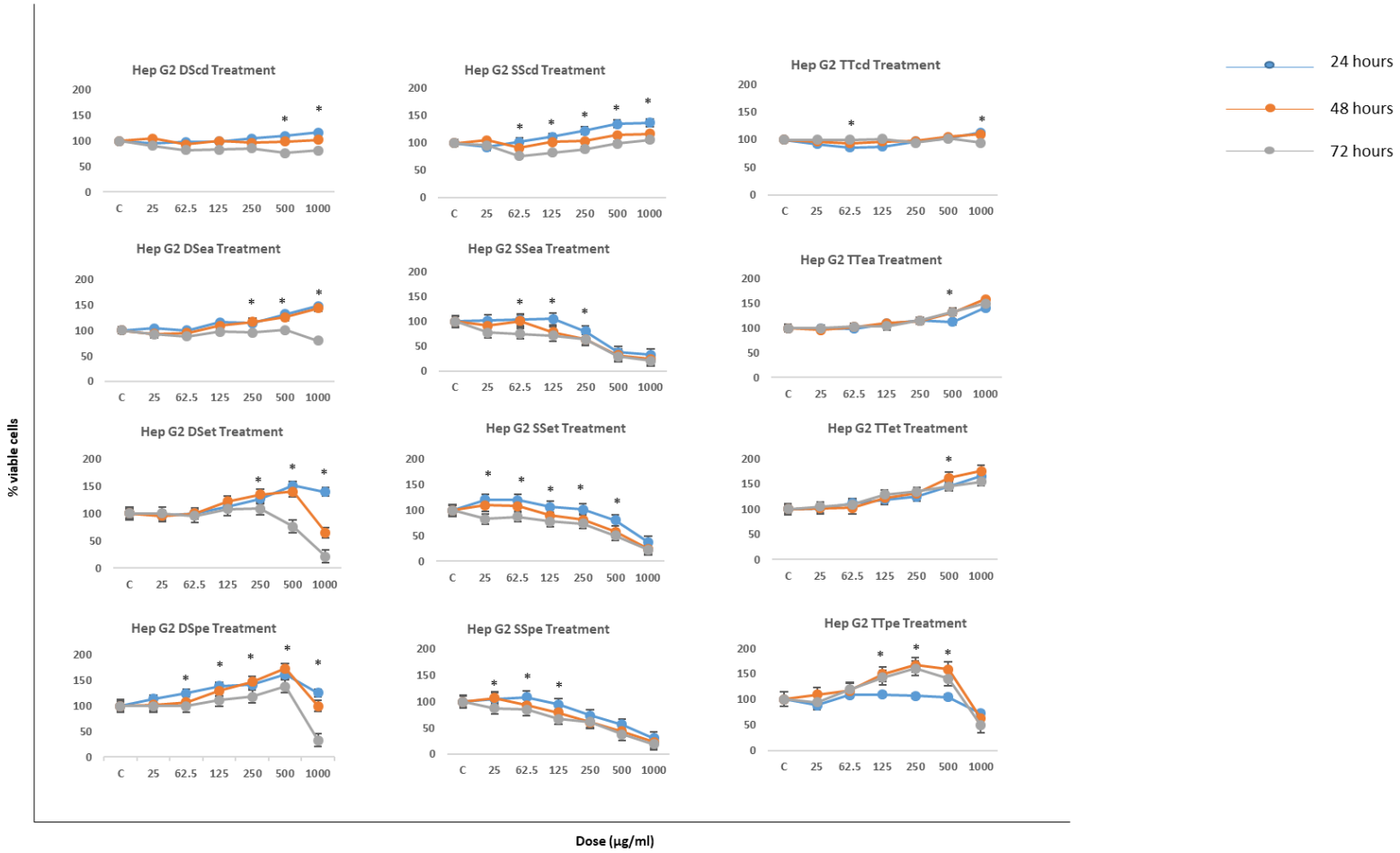
4.6 FUTURE RESEARCH

This study confirms the anticancer potential of NZ surf clam extracts. Additional data are needed to better understand their bioactivity. The functional components in the extracts require further investigation (isolation, identification, and chemical analysis). Furthermore, an *in vivo* study will answer the question – whether the extracts' bioactivities still exist and to what extent in whole organism. Lastly, it is well known that heat may denature certain proteins and/or other metabolites. Therefore, a study on an alternative clam flesh drying method (versus oven-drying in this study) will be of tremendous importance.

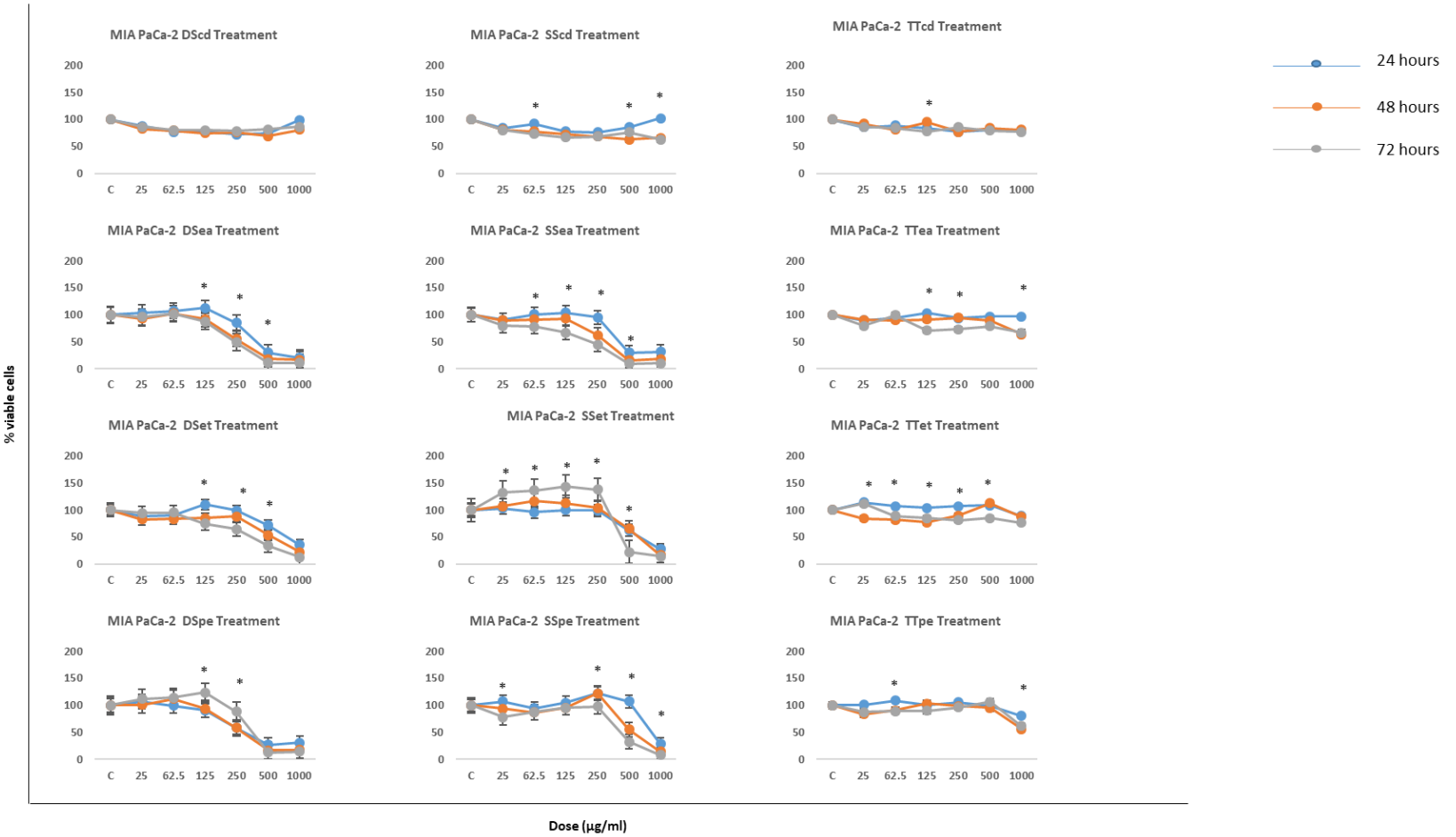
4.8 SUPPLEMENTARY DATA



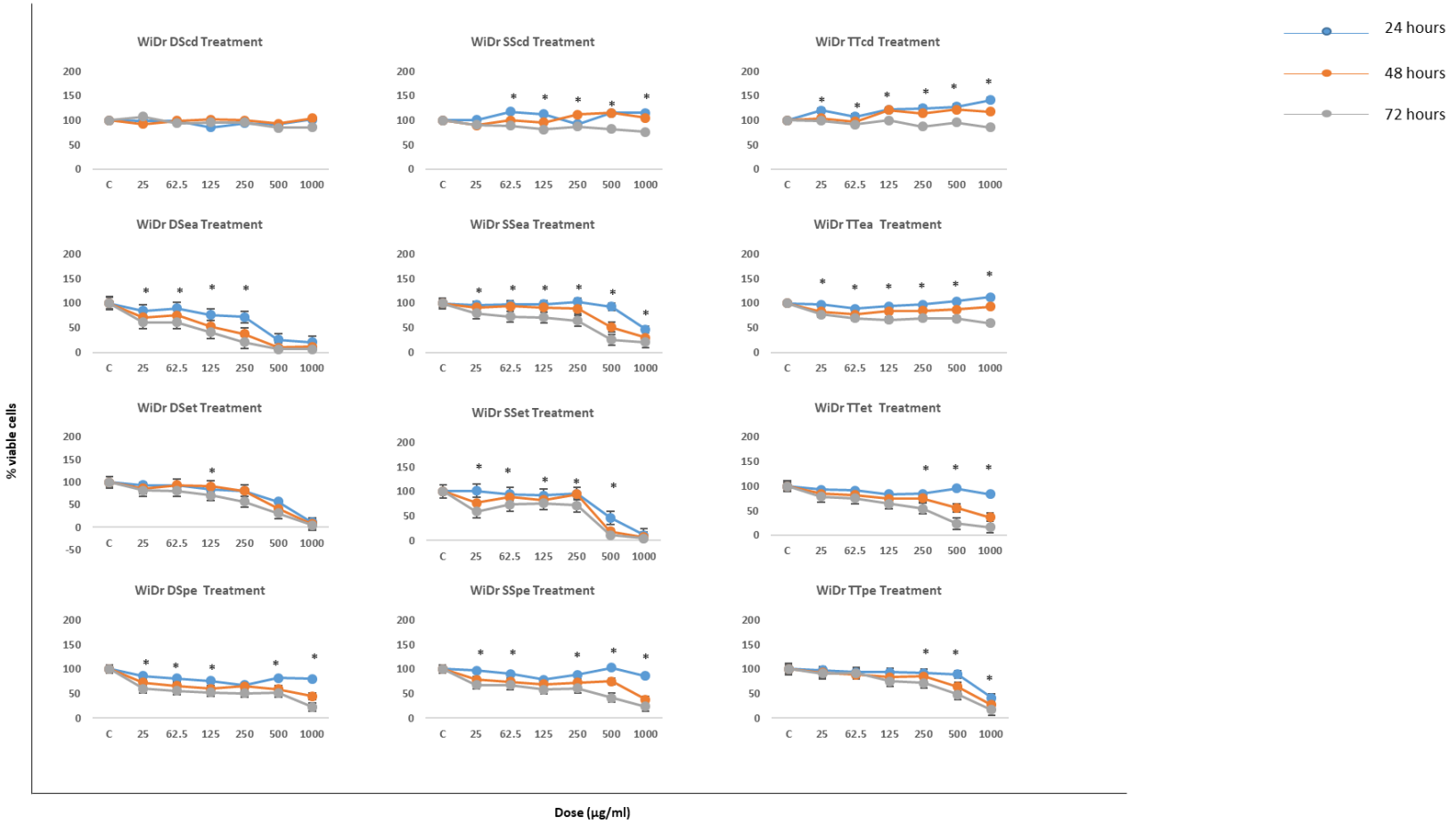
Supplementary Fig. 4.1a: The inhibitory effect of NZ surf clam extracts on the growth of A549 after an incubation time of 24, 48, and 72 hours. Cells were incubated in the presence of various extract concentrations. A relative cell viability of 100% was designated as the total number of cells that grew after each time point. Each experiment was carried out twice, in triplicates. Data is presented as means ± SE. * indicates statistical significance, $p < 0.05$.



Supplementary Fig. 4.1b: The inhibitory effect of NZ surf clam extracts on the growth of Hep G2 after an incubation time of 24, 48, and 72 hours. Cells were incubated in the presence of various extract concentrations. A relative cell viability of 100% was designated as the total number of cells that grew after each time point. Each experiment was carried out twice, in triplicates. Data is presented as means ± SE. * indicates statistical significance, $p < 0.05$.



Supplementary Fig. 4.1c: The inhibitory effect of NZ surf clam extracts on the growth of MIA PaCa-2 after an incubation time of 24, 48, and 72 hours. Cells were incubated in the presence of various extract concentrations. A relative cell viability of 100% was designated as the total number of cells that grew after each time point. Each experiment was carried out twice, in triplicates. Data is presented as means ± SE. * indicates statistical significance, $p < 0.05$.



Supplementary Fig. 4.1d: The inhibitory effect of NZ surf clam extracts on the growth of WiDr after an incubation time of 24, 48, and 72 hours. Cells were incubated in the presence of various extract concentrations. A relative cell viability of 100% was designated as the total number of cells that grew after each time point. Each experiment was carried out twice, in triplicates. Data is presented as means ± SE. * indicates statistical significance, $p < 0.05$.

CHAPTER 5

EFFECTS OF PREPARATION METHOD ON THE CYTOTOXIC ACTIVITY AND BIOCHEMICAL CHARACTERIZATION OF NEW ZEALAND SURF CLAM EXTRACTS

5.1 ABSTRACT

Molluscan extracts confer a wide range of health promoting properties, e.g. cytotoxic properties. Extraction and processing can affect the efficacy and properties of bioactive molecules. Cold processing and heat processing have differential effects on the biochemical composition and cytotoxic activities of New Zealand surf clam extracts across seven cancer cell lines. Apoptosis was induced through all cell lines, as further evidenced in Caspase-3/7 activities. Cell cycle arrest was notably focused on G2/M- and S- phases. Petroleum ether and ethyl acetate fractions, known for greatest activity in this study, are both rich in lipids and proteins, indicating likely fractional localization of relevant bioactives. Cold processing of clam preparations were responsible for lowest cell viability in MTT assays, and induced greater apoptosis, confirmed by caspase-3/7 activities. This difference in cytotoxicity indicates that heat processing affects the relevant bioactive molecules/components or their efficacy. Overall, cold processed extracts performed better than heat-processed preparations.

Keywords: surf clam, drying, biochemical components, cytotoxic activity, biological activity

5.2 INTRODUCTION

Active components in food, particularly those possessing apoptosis-inducing activity against cancer cells, could prove important in the deterrence and/or treatment of cancer (Pan et al., 2007). Some molluscan extracts have bioactive properties both *in vitro* and *in vivo*. These bioactivities include decreasing inhibiting inflammatory responses in LPS-activated macrophages, ameliorating hypercholesterolaemia hepatoprotective properties, as well as anticoagulant, hypocholesterolaemic, antioxidant, and antimicrobial activities (chapters 3 and 4). The three most harvested species of surf clams in New Zealand (NZ), the Diamond shell (*Crassula aequilatera*), Storm shell (*Mactra murchisoni*) and Deepwater Tua tua (*Paphies donacina*), are used in this research.

The first step in the extraction of natural products, for characterization or to test bioactivity, more often than not involves drying the tissues. Molluscan flesh is prepared either by steaming prior to extraction (Chijimatsu et al., 2013), oven drying (Liu et al., 2012, Odeleye et al., 2016), freezing (Murphy et al., 2003), or freeze drying (Joy & Chakraborty, 2017), all prior to extraction. Raw, unprocessed flesh studies have also been carried out (Leng et al., 2005, Orban et al., 2007, Wang et al., 2013).

The effect of various drying processes on the bioactivity of molluscs has been reported in literature. The clam lipid content (oven drying at different temperatures) (Liu et al., 2018), as well as NZ green lipped mussel fat content (freezing versus freeze-drying) have been assessed for bioactivity as a function of preparatory methods (Murphy et al., 2003). Furthermore, the effect of drying procedures (freeze-drying, spray-drying, and rotary

evaporation-drying) on the physicochemical properties and antioxidant activities of polysaccharides from *Crassostrea gigas* has been described, revealing that spray-dried polysaccharides exhibited stronger antioxidant activities than the activities observed in other preparation methods (Hu et al., 2017).

On a much wider scale, preparations of plant-sourced polysaccharides revealed that freeze-dried finger citron fruits' extracts were the extracts of choice, possessing higher antioxidant activities, over extracts from two other methods of drying (hot air drying and vacuum drying) (Wu, 2015). The preferred drying method usually possesses significantly higher, though comparable, activities as per the assays investigated. This indicates that different methods of drying do not completely eliminate bioactivities. However, there are some methods of drying which tend to maintain notably higher levels of bioactivity.

This study adds important information to a very specific area of knowledge, as it is the first study to compare the cytotoxic activity of cold processed (FD) and heat processed (OD) NZ surf clam extracts. Previous literature reveals the importance in considering preparatory methods of food sources as a means of maintaining bioactivities. The aim of this study is to assess the effects of heat (in the forms of blanching and oven drying) versus cold processing (in the forms of freezing and freeze-drying) on the subsequent biochemical composition and cytotoxic activity of NZ surf clam extracts.

5.3 MATERIALS AND METHODS

5.3.1 CHEMICALS AND REAGENTS

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) formazan powder was purchased from Sigma-Aldrich (St. Louis, MO, USA). Petroleum ether and Ethyl

acetate were purchased from Global Science (Auckland, NZ), ethanol was purchased from ThermoFisher (Auckland, NZ). Foetal Bovine Serum (FBS) was purchased from Medica Pacifica (Auckland, NZ). Roswell Park Memorial Institute (RPMI) 1640 medium, no phenol red, L- Glutamine (200 mM), Penicillin- Streptomycin (10,000 U/mL), TrypLE™ Express, no phenol red, Trypan blue stain (0.4%), and Dulbecco's Phosphate Buffered Saline (D-PBS) were all purchased from Life Technologies (Auckland, NZ). Dimethyl sulfoxide (DMSO) was purchased from Thermo-Fisher Scientific, (Auckland, NZ); Apo-ONE® Homogeneous Caspase-3/7 Assay kit was purchased from In Vitro Technologies (Auckland, NZ). Alexa Fluor® 488 annexin V/ Dead Cell Apoptosis kit was purchased from Thermo-Fisher Scientific (Auckland, NZ).

5.3.2 SURF CLAM PREPARATION AND EXTRACTION

Blanched clams were prepared as previously described (Odeleye et al., 2016; Odeleye et al., 2019). Raw clams were received in ice packed polysterene boxes. Clams were shucked and drained of any water. Clams were then frozen in a -80ºC freezer, and then freeze-dried commercially according to the company's protocol. Freeze-dried raw clams were then pulverised in a laboratory blender (model: 800 watts Sunbeam Multiblender-Pro blender). Initial extraction fractions were generated in parallel using water (cd) and ethanol (et) as solvents. Clam powder was solubilized in distilled water and stirred constantly at room temperature using a magnetic stir-bar for one hour. The supernatant was removed, replaced with fresh solvent and stirred for another hour. This process was repeated until the solvent was colorless. The supernatant was collected by centrifugation. Ethanol extraction was carried out in the same way. The clear solution was collected and evaporated under reduced pressure using a Rota evaporator (Buchi

Rotavapor R-215, Global Science, Auckland, NZ) until complete dryness. The water (cd) and ethanol extracts (et) were collected after evaporation and stored at -20ºC. The ethanol extraction (et) of each clam species was further fractionated by liquid-liquid extraction steps according to the polarities of petroleum ether (pe) and ethyl acetate (ea). They were dissolved in 100ml distilled water and fractionated with petroleum ether. Extracts ('pe') were collected and concentrated under reduced pressure. Further step-by-step fractionation was done using ethyl acetate, which resulted in the fractions of 'ea'. Each fractionation process was repeated until the solvent was colorless. Fractions were evaporated to dryness and stored at -20ºC until ready for use Water, ethanol, petroleum ether, and ethyl acetate fractions were abbreviated as cd, et, pe, and ea fractions respectively.

5.3.3 BIOCHEMICAL ANALYSIS OF EXTRACTS

Biochemical composition analysis was performed according to the method previously described (Odeleye et al., 2016). Each fraction was tested for carbohydrate, protein and lipid content. Protein amount in each sample was measured by an Enhanced BCA Protein Assay kit (Beyotime Biotechnology, Shanghai, China) according to manufacturer's instruction, using bovine serum albumin as the standard. Total carbohydrate content in each sample, after simple filtration to filter out insolubles, was measured by phenol-sulphuric acid method using glucose as the standard. Lipid was measured by total lipid extraction method. In brief, sample was extracted by chloroform–methanol (2:1 v/v) for 3 h, and then 1% NaCl solution was added to the sample. The chloroform layer was removed and chloroform was evaporated under nitrogen at 75 °C. The sample was then dried at 105°C to constant weight. The resulting lipid is then weighed.

5.3.4 CELL CULTURE AND DETERMINATION OF CYTOTOXICITY

A total of seven cancer cell lines were utilised in this study. Cell lines (Table 5.1) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The cells were cultured in the Roswell Park Memorial Institute tissue 1640 medium, supplemented with 10% (v/v) FBS, L-glutamine, and penicillin-streptomycin under an atmosphere of 5% CO_2, 95% air in an incubator at 37ºC.

Table 5.1: Details of cell lines employed

CELL LINE	TISSUE	MORPHOLOGY	DISEASE	ATCC CATALOG
PC-3	Prostate	Epithelial	Adenocarcinoma	CRL-1435™
WiDr	Colon	Epithelial	Colorectal adenocarcinoma	CCL-218™
A549	Lung	Epithelial	Carcinoma	CRL-185™
Hep G2	Liver	Epithelial	Hepatocellular carcinoma	HB-8065™
SiHa	Cervix	Epithelial	Squamous cell carcinoma	HTB-35™
MCF-7	Breast	Epithelial	Adenocarcinoma	HTB-22™
Mia PaCa-2	Pancreas	Epithelial	Carcinoma	CRL-1420™

The inhibitory rate of extracts was assessed by the way of MTT. Briefly, the cells were seeded in 96-well plates at a concentration of 1×10^5 cells ml^{-1} using the RPMI medium. After incubation in a humidified 37 °C, 5% CO_2 incubator (Series II Water Jacket supplied by Thermo Scientific) overnight, the cells were treated by NZ surf clam extracts at a concentration range from 25 to 1000 µg/mL. The cells were further incubated for an additional 24, 48, and 72 hours independently at 37 °C. After incubation, MTT stock solution was then added to each well and incubated for a further 4 hours. The formazan crystals in each well were dissolved in 100 µl of DMSO. The amount of purple formazan

was determined by measuring the absorbance at 540 nm (Multiskan microplate reader by Thermo Fisher Scientific).

5.3.5 ANNEXIN V FLOW CYTOMETRIC ASSAY

The apoptotic effect of NZ clam extracts was determined by the Alexa Fluor® 488 annexin V staining method and measured by flow cytometer (Beckman Coulter's MoFlo^{TM} XDP). Cells were placed in 6-well plates at a density of 4 x 10^5 cells per well and incubated overnight. Cells were then treated with different concentrations (400 and 600 µg/ml) of NZ surf clam extracts for 7 hours. After treatment, the cells were harvested, washed twice with PBS, and resuspended in 1X binding buffer. Alexa Fluor® 488 annexin (4 µl) and PI (1µl) (Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit) were added to each 100 µl of cell suspension. After incubation, 400 µl 1X annexin-binding buffer was added to all samples prior to analysis.

5.3.6 CELL CYCLE ANALYSIS

Cells were seeded in 6-well flat-bottom plates at a density of 3 x 10^5 cells/well, and cultured for 24 hours. They were then treated with NZ surf clam extracts (600 µg/ml) for 72 hours. Supernatant was collected, cells were washed with PBS, and treated with trypsin. Cells were washed twice with PBS at 4ºC, and then fixed with ice cold 80% ethanol, and stored at -20ºC for no longer than 7 days. Upon use, cells were gently centrifuged (1200 RPM, 2 minutes), decanted, resuspended in permeabilizing solution for 30 minutes at 37ºC, and incubated with PI for 5 minutes. The mixture was then analysed with flow cytometer (Beckman Coulter's MoFlo^{TM} XDP).

5.3.7 DETERMINATION OF CASPASE-3/7 ACTIVITY

The Apo-ONE Homogeneous Caspase-3/7 Assay Kit was used to evaluate the activities of apoptosis by measuring the activities of caspase-3/7 in the clam extract-treated cells. Cells were seeded in 96 well plates at a density of 5 x 10^3 cells/ well, and incubated overnight. cells were then treated with NZ surf clam extracts for 24 hours (400 and 600 µg/ml). After treatment, an equal volume of Apo-ONE caspase-3/7 reagent was added to each well, and incubated while shaking for 1 hour at room temperature. The fluorescence of each well was read at 495 ± 10 (excitation) and 520 ± 10 (emission) (Spark 10M multimode microplate reader by Tecan, Switzerland).

5.3.8 STATISTICAL ANALYSIS

MTT and caspase data were collected from duplicate experiments of triplicate samples. Apoptosis and cell cycle assays were carried out twice, in duplicate. Results are presented as mean ± standard error of the mean and $p<0.05$ was considered statistically significant. MTT and caspase data were analysed using Microsoft Excel. Analysis of Flow cytometry data was performed using Kaluza Analysis 1.3 (Beckman Coulter, Miami, FL, USA). The use of t-test, non-parametric comparison, and 1- and 2- way ANOVA applications were employed. Also, post-analysis Dunnett testing was used to identify differences in data from this study.

5.4 RESULTS AND DISCUSSION

5.4.1. COMPOSITION OF EXTRACTS

The biochemical constituents of each fraction (cd, et, pe, and ea) of Diamond shell (*C. aequilatera*), Storm shell (*M. murchisoni*) and Tua tua (*P. donacina*) were studied. Table

5.2 shows a summary of the carbohydrate, protein and lipid components of each OD and FD fraction.

Table 5.2: Biochemical composition of extracts

Species	Fraction	Carbohydrate (%)		Protein (%)		Lipid (%)	
		Freeze dried	Oven dried	Freeze dried	Oven dried	Freeze dried	Oven dried
Diamond shell	cd	3.24 ± 0.06	9.86 ± 1.17	15.46 ± 0.14	12.22 ± 0.26	4.00 ± 2.04	2.83 ± 0.24
	pe	8.89 ± 0.22	9.29 ± 0.22	6.91 ± 0.05	7.80 ± 0.12	70.67 ± 3.70	79.67 ± 2.32
	ea	10.17 ± 0.09	10.27 ± 0.70	8.98 ± 0.18	7.49 ± 0.02	57.67 ± 1.84	83.17 ± 1.43
	et	8.37 ± 0.21	5.89 ± 0.24	5.49 ± 0.31	6.05 ± 0.10	54.67 ± 3.17	66.00 ± 1.78
Storm shell	cd	1.60 ± 0.32	7.30 ± 1.40	14.25 ± 0.35	11.53 ± 0.22	2.00 ± 1.78	4.33 ± 1.31
	pe	10.29 ± 0.09	7.74 ± 0.03	7.92 ± 0.20	4.94 ± 0.05	78.33 ± 7.42	78.33 ± 9.00
	ea	8.69 ± 0.30	9.72 ± 0.12	7.10 ± 0.16	8.30 ± 0.15	58.67 ± 5.54	76.83 ± 4.48
	et	8.21 ± 0.16	6.36 ± 0.13	6.04 ± 0.17	4.65 ± 0.11	67.17 ± 6.51	60.17 ± 5.07
Tua tua	cd	14.83 ± 0.95	10.81 ± 1.60	15.83 ± 0.03	11.51 ± 0.13	8.83 ± 4.92	3.33 ± 2.05
	pe	10.48 ± 0.09	6.37 ± 0.08	7.89 ± 0.13	8.49 ± 0.10	65.00 ± 3.27	70.17 ± 1.65
	ea	10.57 ± 0.16	5.69 ± 0.07	7.11 ± 0.06	18.59 ± 0.19	74.17 ± 9.26	53.83 ± 2.36
	et	5.21± 0.14	5.62 ± 0.12	3.76 ± 0.08	6.35 ± 0.06	12.33 ± 3.57	46.17 ± 0.62

Carbohydrates range from 1.60 to 14.83% in freeze-dried (FD) extracts, with an average of approximately 8.4%. The average carbohydrate content in the oven dried (OD) samples is 7.91%, from a range of 5.62% to 10.81%. The pe and ea fractions, especially in the FD samples possess the highest carbohydrate content. The low carbohydrate content in all fractions does not suggest that the observed bioactivity is devoid of carbohydrate influence. Among the functional ingredients of molluscs, polysaccharides, a class of carbohydrates, may be the most studied, as they have very diverse and well defined types and structures (Cruz et al., 2010; Suleria et al., 2017). Marine polysaccharides have been reported to display diverse biological activities, e.g. cytotoxic (Wang et al., 2018). The carbohydrate content is consistent with that of *Meretrix meretrix* (Xie et al., 2012), and *Meretrix lusoria* foot (wet weight), mantle, and viscera samples (Karnjanapratum et al., 2013).

NZ surf clam extracts contain more proteins in the cd fraction than any other fraction, with the exception of OD TTea, which had a protein content of 18.59%. The FD cd extracts possess more protein content than their OD counterparts do. Marine derived proteins and peptides have shown several bioactivities such as antihypertensive and immunomodulatory (Kannan et al., 2011). Certain peptides are the major components of the extracts responsible for observed anticancer activity (Leng et al., 2005). This may be true for the NZ surf clam extracts. Other peptides inducing antioxidant (blue mussel (*Mytilus edulis*) (Wang et al., 2013)) and antiproliferative (bivalve- *Arca subcrenata* (Chen et al., 2013)) activities have been described. The mean crude protein (11.8% in males; 11.9% in female) in the surf clam, *Mactra violacea*, (Laxmilatha, 2009) is similar to those obtained in the OD DScd (12.22%), SScd (11.53%), and TTcd (11.51%) extracts in this study. The FD cd extracts displayed higher protein content.

Lipid was the major component of the et, pe, and ea fractions. The pe and ea fractions contain the most lipids, suggesting that the bulk of lipid content was extracted from the et fraction into the pe and ea fractions. Lipids from the ethyl acetate fraction of plant (Hu et al., 2010) and animal (Pan et al., 2007) extracts possess cytotoxic activities. Other lipid components from the freshwater clam (*Corbicula fluminea*) have been reported to ameliorate hypercholesterolaemia *in vivo* (Chijimatsu et al., 2013). The chemical composition of the ea fraction of the Indian green mussel, *Perna viridis*, revealed the presence of sterols, polyphenols, alkaloids, and terpenes. These components have been attributed to biological activities including lipid peroxidation (polyphenols) and cytotoxicity (alkaloids) (Sreejamole & Radhakrishnan, 2013). Seafood products, including clams, are the only significant source of *n*-3 PUFA in the human diet. *n*-3 PUFA is a class of essential nutrients important in the prevention of diseases (Orban et al., 2007).

5.4.2 EFFECT OF NZ SURF CLAM EXTRACTS ON CELL VIABILITY

To determine whether NZ surf clam FD-extracts have a growth inhibition effect on seven human cancer cells, a cell viability assay was performed. The extracts significantly decreased cell viability in a dose dependent manner, especially at concentrations above 125 µg/ml (Supplementary Fig. 5.1). Results (Table 5.3) are expressed as percentages of viable cells in treated cells compared to the control, after 72 hours of treatment. Each cell line was challenged with 12 FD and 12 OD extracts, however, only the best three extracts are shown and discussed.

*Table 5.2: The inhibitory effect of NZ surf clam extracts (Oven dried- OD, and Freeze dried- FD) after an incubation time of 72 hours. Experiments were carried out twice, in triplicates. Data is presented as means ± SE. *p < 0.05; **p < 0.01; ***p < 0.005; ****p < 0.0005; NS = not significant (Student's t-test).*

Cell line	% Cell viability: Oven dried (OD) treatment				% Cell viability: Freeze-dried (FD) treatment			
A549		**250 µg/ml**	**500 µg/ml**	**1000 µg/ml**		**250 µg/ml**	**500 µg/ml**	**1000 µg/ml**
	DScd	76	76	82	DSea	64*	44**	8****
	SSea	107	68	15	SSea	80**	59*	19^{NS}
	TTpe	106	113	45	TTea	87 **	35***	11***
Hep G2	DSet	92	69	31	DSea	87 ^{NS}	65 ^{NS}	9***
	SSpe	61***	38***	19	SSea	108	84	15 ^{NS}
	TTpe	161	140	49	TTea	115***	24****	18***
MCF-7	DSea	57*	20***	24	DSpe	68	44	15*
	SSpe	114	15	15	SScd	59***	16 ^{NS}	8*
	TTpe	112	20	14	TTpe	64***	14 ^{NS}	14 ^{NS}
MIA PaCa-2	DSea	49	11	11	DSea	21***	11 ^{NS}	11 ^{NS}
	SSea	138	22**	14	SSea	84***	40	12 ^{NS}
	TTpe	97	106	62	TTpe	40*	12****	14***
PC-3	DSea	15***	10***	13*	DSpe	72	62	23
	SSet	74	12**	12*	SSpe	53***	30	20
	TTet	91	61	28	TTpe	79*	54*	16*
SiHa	DSpe	87**	36	31	DSea	107	21**	21*
	SSpe	46***	46	40	SSea	85	38*	18***
	TTpe	70	24	32	TTpe	69 ^{NS}	23 ^{NS}	18*

WiDr	DSea	20***	6***	6*	DSpe	53	27	14
	SSet	72	11*	5*	SSpe	34***	23	13
	TTet	54	24	17	TTpe	55 ^{NS}	16*	15 ^{NS}

WiDr cell line was the most susceptible to treatment on all three days, with percentage inhibition values of 47% (DSpe), 66% (SSpe), and 45% (TTpe) at 250 µg/ml, after 72 hours of treatment (Table 5.3). The OD data of 28% (SSet), and 46% (TTet) (Odeleye et al., 2019) correlates closely with the FD treated WiDr cells, with the exception of DS (DSea), which was statistically different ($p = 0.0010$). It is interesting that another colon cancer cell line (LoVo) showed almost identical susceptibility to treatments (data not shown). At 250 µg/ml (72 hours), LoVo cell growth was inhibited by 61% (DSpe), 72% (SSpe), and 58% (TTpe) (data not shown). Colon cancer cells may have one or more molecular receptor sites with which the extracts react effectively and efficiently, to yield such cytotoxic responses. Furthermore, there was no significant difference in the proliferative responses of OD and FD extract-treated WiDr and LoVo cell lines. Of note, FD-treated WiDr cells were more susceptible to extract treatment at 250 and 500 µg/ml on Day 1, while OD-treated (DS and SS treatments) cells were more susceptible on Day 3 (Odeleye et al., 2019).

Contrary to colon cancer cell line data, the results indicate lung (A549) and liver (Hep G2) cancer cell lines were the least susceptible to treatment, consistent with the OD data from previous results (Odeleye et al., 2019). After 72 hours of treatment, A549 was more susceptible to FD treatments than their OD counterpart, with percentage viable cells of 64%, 80%, and 87% at 250 µg/ml, ($p \leq 0.0136$). This contradicts a study by Sahayanathan et al. (2017), where A549 cells were the most susceptible to treatments of *Donax variabilis* (marine clam) extracts. A likely explanation for this disparity may be found in the extreme specificity of proteins isolated from the clam mantle, opposed to

total extracts from an entire clam employed in this study. The absence of 'impurities' may ameliorate the bioactivity of a refined natural extract.

Notably, there was a better 24-hour response in FD-treated MIA PaCa-2 and PC-3 cells versus OD-treated cells The same is true in both FD and OD SSea- and TTea-treated MIA Paca-2 (Table 5.3). Conversely, in DSea-treated MIA PaCa-2, OD- and FD-treated cells both exhibited inhibition values of 89% at 1000 µg/ml ($p = 1.0000$).

As expected, 72 hour-treatments were the most effective, and cell viability decreased with an increase in concentration in most cases. This shows that NZ surf clam extracts induced cytotoxicity to cancer cells in a time dependent manner. In a few cases such as TTpe-treated MCF-7, DSea-treated MIA PaCa-2, and DSea-treated SiHa, however, cell viability seemingly plateaued at 500 µg/ml. Saturation effects are likely the cause in these observed results.

At a concentration of 400 µg/ml, the inhibitory effects of polysaccharides (CSPS-3) from *Cyclina sinensis*, on BGC-823 cells, was 79.89% (Jiang et al., 2011). Such an outcome was achieved at 500 µg/ml in certain LoVo, MCF-7, MIA PaCa-2 and WiDr-treated cells in this study. Furthermore, Leng et al. (2005) reported that a *Mercenaria (Meretrix meretrix* Linnaeus) peptide on BGC-823 cells at only 4 µg/ml reached an inhibition of 60%. Such inhibitory rates were achieved at concentrations of 250 µg/ml and above in this study, from crude extracts. The similarity in cytotoxicity, despite disparate clam extract concentrations, may be due to two reasons- first; a peptide (or polysaccharide in the case of CSPS-3) was purified and employed in treatment, suggesting that a purified extract is potentially more effective in its targeting and elimination of cancer cells, rather than an un-purified extract. Second, the whole flesh of the clam (*M. meretrix* Linnaeus) was extracted without prior drying. Although the cytotoxicity of OD and FD- NZ clam

extracts demonstrated similar results, the effect of an initial drying before extraction, may in turn determine the observed bioactivity.

Certain molecular characteristics of cancer cell lines direct their growth inhibition, death, and drug sensitivity (Shoemaker, 2006). This is very likely one explanation for the 'strong' variation in the cytotoxicity of NZ surf clam extracts in different cell lines. Furthermore, cell culture media contains growth factors, metabolites, and antibiotics in abundance, all of these being provided in the supplements contained within a small environment. This artificial atmosphere differs from normal *in vivo* environments, resulting in less competition for survival (Ertel et al., 2006). This may affect a cell line's responsiveness, or the lack thereof, to specific extract treatments. The regulation of certain cell survival pathways may be rendered inactive, due to a more senescent existence. Hence the difference in cytotoxicity of NZ clam extracts in different cell lines.

The use of heat in the drying process of OD extracts did not correlate to a decrease in bioactivity efficacy in OD extracts. However, there were notable improvements in treatment outcomes from the FD extracts: 32 out of 49 significant differences identified in this study favoured FD extracts (Table 5.3). Corollaries are notably seen between both methods of drying and effective inhibition in all noted cancer cell lines.

5.4.3 EFFECT OF NZ SURF CLAM EXTRACTS ON CELL DEATH

The ability of NZ surf clam extracts to induce cell death was estimated by analysing their effect on cell morphology. We examined the morphological modifications on treated cells, compared to untreated cells. This was performed as previously described (Odeleye et al., 2019).

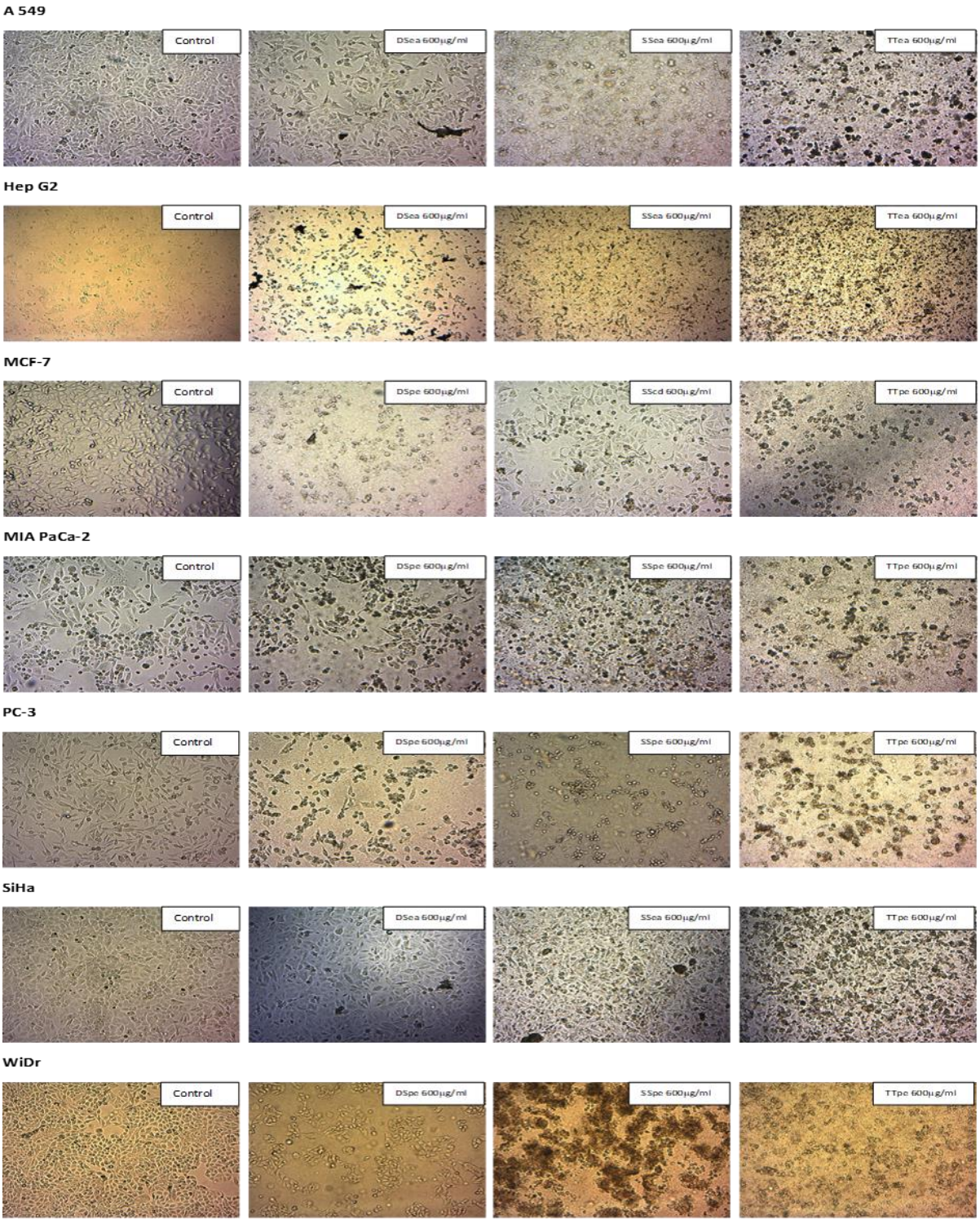


Figure 5.1: Photographs of seven cancer cells cultivated with or without FD treatment under an inverted microscope at 40x magnification (Zeiss).

Treatment of cells with NZ surf clam extracts (at 600 µg/ml, for 72 hours) caused morphological changes compared to closely packed control cells, and this may be indicative of apoptosis. The control cells displayed a typical spindle shape, while in the

treated cells, there were very few adherent cells left after treatment, and those attached were poorly attached. Most cells were distorted, and looked dead, shrunken and/or disintegrated. Growth decline and reduction in cell number were also observed (Figure 5.1), and these were identical to those of the OD extract-treated cells (Odeleye et al., 2019). This concurs with observations reported by Sahayanathan et al. (2017). Chromosomal condensation was also observed. Our results suggest that the FD-extracts possess active ingredients responsible for the observed morphological changes, thereby confirming the MTT data.

5.4.4 APOPTOSIS INDUCING ACTIVITY

The effect of NZ surf clam extract on cell death and degree of apoptosis was also analysed by flow cytometry. The assay was carried out to further investigate the mechanism of cell death induced by the extracts. This method differentiates the fraction of viable cells from apoptotic (non disrupted) cells and necrotic (disrupted) cells. The flow cytometric analysis showed that after treating cells at 400 µg/ml and 600 µg/ml, significant inhibitory effects were observed. FD- and OD-treated cells are displayed side by side in Table 5.4.

Table 5.3: Induction of apoptosis by NZ surf clam extracts. Annexin V/Dead Cell Apoptosis Kit with Aleza® Fluor 488 annexin V and PI determined the percentages of viable and apoptotic cells, examined by flow cytometry. Each experiment was carried out twice, in duplicate.

Cell line/ Extracts		**Oven dried (OD) treatment**			**Freeze-dried (FD) treatment**		
			Early Apoptosis (%)	**Late Apoptosis (%)**		**Early Apoptosis (%)**	**Late Apoptosis (%)**
A549		C	4.72	0	C	4.72	0
	DS	DScd 400µg/ml	8.93	0.53	DSea 400µg/ml	25.03	0.17
		DScd 600µg/ml	12.83	0	DSea 600µg/ml	33.66	0.58
	SS	SSea 400µg/ml	22.45	0.05	SSea 400µg/ml	27.12	4.45
		SSea 600µg/ml	26.94	1.28	SSea 600µg/ml	37.86	7.62
	TT	TTpe 400µg/ml	15.48	0.59	TTea 400µg/ml	35.26	4.24
		TTpe 600µg/ml	20.51	0.98	TTea 600µg/ml	42.67	5.14
Hep G2		C	4.77	0.50	C	4.77	0.50
	DS	DSet 400µg/ml	18.87	4.15	DSea 400µg/ml	13.74	16.56
		DSet 600µg/ml	26.48	7.31	DSea 600µg/ml	23.38	28.72
	SS	SSpe 400µg/ml	15.93	9.94	SSea 400µg/ml	17.60	7.12
		SSpe 600µg/ml	11.22	15.21	SSea 600µg/ml	21.27	9.89
	TT	TTpe 400µg/ml	9.97	2.24	TTea 400µg/ml	23.53	27.76
		TTpe 600µg/ml	13.86	3.01	TTea 600µg/ml	0.92	35.49

MCF-7		C	3.95	0.66	C	3.95	0.66
	DS	DSea 400µg/ml	25.53	3.17	DSpe 400µg/ml	14.80	27.06
		DSea 600µg/ml	28.31	10.74	DSpe 600µg/ml	24.16	21.96
	SS	SSpe 400µg/ml	30.97	5.63	SScd 400µg/ml	25.85	23.92
		SSpe 600µg/ml	16.42	17.03	SScd 600µg/ml	9.43	50.60
	TT	TTpe 400µg/ml	15.43	5.28	TTpe 400µg/ml	8.51	14.25
		TTpe 600µg/ml	12.39	17.93	TTpe 600µg/ml	7.91	27.68
MIA PaCa-2		C	0	0	C	0	0
	DS	DSea 400µg/ml	23.89	0.56	DSpe 400µg/ml	20.26	12.72
		DSea 600µg/ml	30.38	4.56	DSpe 600µg/ml	11.53	17.85
	SS	SSet 400µg/ml	5.76	1.82	SSpe 400µg/ml	34.13	11.23
		SSet 600µg/ml	38.32	2.96	SSpe 600µg/ml	38.23	24.41
	TT	TTpe 400µg/ml	10.22	1.55	TTpe 400µg/ml	25.97	28.57
		TTpe 600µg/ml	26.19	6.77	TTpe 600µg/ml	66.79	16.02
PC-3		C	2.24	2.40	C	2.24	2.40

	DS	DSea 400µg/ml	30.29	1.93	DSpe 400µg/ml	5.75	18.40
		DSea 600µg/ml	7.99	13.88	DSpe 600µg/ml	8.49	37.58
	SS	SSet 400µg/ml	30	1.44	SSpe 400µg/ml	9.72	30.56
		SSet 600µg/ml	17.10	9.69	SSpe 600µg/ml	5.14	35.96
	TT	TTet 400µg/ml	5.13	1.80	TTpe 400µg/ml	11.66	23.32
		TTet 600µg/ml	4.35	6.17	TTpe 600µg/ml	15.70	31.15
SiHa		C	1.71	1.81	C	2.61	0.24
	DS	DSpe 400µg/ml	8.62	11.27	DSea 400µg/ml	3.43	17.09
		DSpe 600µg/ml	13.72	11.83	DSea 600µg/ml	5.02	27.55
	SS	SSpe 400µg/ml	34.55	3.06	SSea 400µg/ml	13.28	20.73
		SSpe 600µg/ml	20.53	12.42	SSea 600µg/ml	1.72	38.82
	TT	TTpe 400µg/ml	18.29	15.45	TTpe 400µg/ml	1.30	30.73
		TTpe 600µg/ml	12.11	5.05	TTpe 600µg/ml	1.74	44.53
WiDr		C	1.58	0.16	C	1.08	0
	DS	DSea 400µg/ml	39.06	0.53	DSpe 400µg/ml	46.47	8.96
		DSea 600µg/ml	42.49	4.22	DSpe 600µg/ml	64.83	13.87

SS	SSet 400µg/ml	33.15	1.38	SSpe 400µg/ml	10.86	1.35
	SSet 600µg/ml	31.33	9.01	SSpe 600µg/ml	42.98	3.56
TT	TTet 400µg/ml	25.08	4.35	TTpe 400µg/ml	34.58	4.61
	TTet 600µg/ml	30.88	4.27	TTpe 600µg/ml	23.93	4.27

The cell lines differed in their susceptibility to the bioactivities of the extracts. The highest proportion of early apoptotic cells were observed in FD DSpe-treated WiDr cells (64.83%).

The late apoptosis percentage of PC-3 cells induced by the protein hydrolysate of Blood clam (*Tegillarca granosa*) muscle was 21.77% at 3 mg/ml (Chi et al., 2015). This outcome was lower than the early and late apoptotic percentages of PC-3-treated cells in the FD-treated cells in this study. FD DSpe-, SSpe-, and TTpe- treated PC-3 cells' late apoptotic populations numbered 37.58%, 35.96%, and 31.15% respectively, at 600 µg/ml. Furthermore, the early apoptotic percentage of a peptide isolated from the shell mollusc, *Bullacta exarata*, on PC-3 cells was 17.51% at 5 mg/ml (Ma et al., 2013). A similar level of apoptosis was attainted by TTpe-treated PC-3 (15.70%) at 600 µg/ml.

In FD-treated A549 cells, there was an observed increase in the early apoptotic cell percentages, and fewer late apoptotic cell percentages, compared to untreated cells (Supplementary Fig. 5.3a). Observed early apoptotic percentage in DSea-, SSea-, and TTea-treated A549 cells were 0.58%, 7.62%, and 5.14% (Table 5.4). This increase is based on a 7 hour, 600 µg/ml treatment. Similar values were observed in DSpe-, SSpe-, and TTpe-treated WiDr cells, with 13.87%, 3.56%, and 4.27% respectively (Supplementary Fig. 5.3g). The magnitude of the early apoptosis difference between treated A549 and WiDr cells is consistent with previous OD data. However, the observed low percentages of early apoptosis in treated A549 cells is unique when compared against the remaining FD-treated cells.

OD-treated MIA PaCa-2 displayed low percentages of late apoptotic cells (DSea- 4.56%, SSet- 2.96%, and TTpe- 6.77%) (Table 5.4), while in FD-treated MIA PaCa-2 cells, late

apoptotic cell percentages were markedly higher (DSpe- 17.85%, SSpe- 24.41%, and TTpe- 16.02%). The same is true for Hep G2 cells. The only exception was found in the SS-treatments, where FD SSea-treated Hep G2 cells displayed a late apoptotic cell percentage of 9.89%, versus 11.22% observed in the OD SSpe-treated cells.

In MCF-7 cells, FD extracts elicited greater than approximately two-fold higher late apoptotic cell populations, when compared to OD extract-treatments. OD DSea, SSpe, and TTpe treatments (10.74%, 17.03%, and 17.93%) were all significantly different from FD DSpe, SScd, and TTpe treatments (21.76%, 50.60%, and 27.68%). This observed higher late apoptosis is intensified in treated PC-3 cells, with notable 2.7-, 3.7-, and 5-fold increases in DS, SS- and TT- treated cells, from OD to FD. SiHa treated cells also demonstrated a similar trend, with 2.3, 3.1, and 8.8-fold increase.

In Hep G2, MCF-7, MIA PaCa-2, PC-3, and SiHa cells, the FD late apoptotic cells were significantly higher than the OD counterpart was. Despite OD and FD- extract similarities of viability, as indicated by the MTT assay results, FD-extract treated cells showed greater percentages of apoptosis. Furthermore, caspase-3/7 activity from FD extracts was elevated above OD extracts (Table 5.5), indicating differential responses between the extracts obtained from the two methods of drying. It is likely that some active components- structure and/or composition- which might have been otherwise altered, denatured, disintegrated, etc. during the prolonged oven drying processes, have been retained in their original/ active form in the FD extracts.

5.4.5 CASPASE-3/7 ACTIVITY

As Caspases-3 and -7 are the most important caspases in the control of apoptosis, the activation of these caspases leads to potent apoptosis. In order to quantify the activities of effector caspases like caspases-3 and -7, the initiator caspases (-2, -8, -9, and -10)

must needs be activated to initiate the caspase cascade. It is therefore safe to infer that no events in the activation of caspases-3 and -7 were disturbed or altered in the process, as the high caspase-3/7 activities displayed are consistent in the major apoptosis markers observed (Li et al., 2007). To further confirm the observed apoptotic processes, we assayed the Caspase-3/7 activity using the Apo-ONE Homogeneous Caspase-3/7 Assay Kit. The level of caspase-3/7 activity was estimated after 24 hours of treatment for each cell line, at 400- and 600 µg/ml.

In most treatment cases, a dose dependent caspase-3/7 activity was observed. The exceptions were TTpe-treated MCF-7, PC-3, and WiDr cells, where the caspase-3/7 activities dropped slightly at 600 µg/ml, but remained higher than the control values. Our results demonstrate that FD SSea-treated A549, Hep G2, and SiHa cells showed incredibly high caspase-3/7 activities, compared to their respective controls (Supplementary Fig. 5.4). As expected, WiDr cells displayed the highest caspase activity, with a 4.8, 10, and 11-fold increase in DSpe-, SSpe-, and TTpe-treated cells at 400 µg/ml.

*Table 5.4: Caspase-3/7 activities after treatment with NZ surf clam extracts. Caspase-3/7 activities were evaluated by Apo-ONE Homogeneous Caspase-3/7 Assay kit. The Caspase-3/7 activity of each group was indicated by their rate fluorescence (RFU). Experiments were carried out twice, in triplicate. Data is presented as mean ± SE. *p < 0.05; **p < 0.01; ***p < 0.005; ****p < 0.0005; NS = not significant (Student's t-test).*

Cell line/ Extracts	Oven dried (OD) treatment		Freeze-dried (FD) treatment	
		Caspase-3/7 activity (%)		Caspase-3/7 activity (%)
A549	C	100	C	100^{NS}
DS	DScd 400µg/ml	165	DSea 400µg/ml	228***
	DScd 600µg/ml	216	DSea 600µg/ml	301****
SS	SSea 400µg/ml	257	SSea 400µg/ml	494****
	SSea 600µg/ml	282	SSea 600µg/ml	591****
TT	TTpe 400µg/ml	345*	TTea 400µg/ml	338
	TTpe 600µg/ml	378	TTea 600µg/ml	401***
Hep G2	C	100	C	100^{NS}
DS	DSet 400µg/ml	474	DSea 400µg/ml	750****
	DSet 600µg/ml	445	DSea 600µg/ml	757****
SS	SSpe 400µg/ml	485	SSea 400µg/ml	977****
	SSpe 600µg/ml	490	SSea 600µg/ml	979****
TT	TTpe 400µg/ml	384	TTea 400µg/ml	551****
	TTpe 600µg/ml	455	TTea 600µg/ml	685****
MCF-7	C	100	C	100^{NS}
DS	DSea 400µg/ml	653****	DSpe 400µg/ml	472
	DSea 600µg/ml	903****	DSpe 600µg/ml	801
SS	SSpe 400µg/ml	679	SScd 400µg/ml	758****
	SSpe 600µg/ml	659	SScd 600µg/ml	895****

TT	TTpe 400µg/ml	590	TTpe 400µg/ml	913****
	TTpe 600µg/ml	447	TTpe 600µg/ml	789****
MIA PaCa-2	C	100	C	100 NS
DS	DSea 400µg/ml	236	DSpe 400µg/ml	395****
	DSea 600µg/ml	236	DSpe 600µg/ml	520****
SS	SSet 400µg/ml	305	SSpe 400µg/ml	474****
	SSet 600µg/ml	302	SSpe 600µg/ml	725****
TT	TTpe 400µg/ml	225	TTpe 400µg/ml	276***
	TTpe 600µg/ml	180	TTpe 600µg/ml	284****
PC-3	C	100	C	100 NS
DS	DSea 400µg/ml	280***	DSpe 400µg/ml	246
	DSea 600µg/ml	530****	DSpe 600µg/ml	338
SS	SSet 400µg/ml	374***	SSpe 400µg/ml	351
	SSet 600µg/ml	454	SSpe 600µg/ml	667****
TT	TTet 400µg/ml	248	TTpe 400µg/ml	475****
	TTet 600µg/ml	301	TTpe 600µg/ml	395****
SiHa	C	100	C	100 NS
DS	DSpe 400µg/ml	261	DSea 400µg/ml	439****
	DSpe 600µg/ml	373	DSea 600µg/ml	539****
SS	SSpe 400µg/ml	379	SSea 400µg/ml	550****
	SSpe 600µg/ml	683	SSea 600µg/ml	1066****
TT	TTpe 400µg/ml	244	TTpe 400µg/ml	434****
	TTpe 600µg/ml	294	TTpe 600µg/ml	575****

WiDr	C	100	C	100 ^{NS}
DS	DSea 400µg/ml	865****	DSpe 400µg/ml	480
	DSea 600µg/ml	1268	DSpe 600µg/ml	1377****
SS	SSet 400µg/ml	1436****	SSpe 400µg/ml	1014
	SSet 600µg/ml	973	SSpe 600µg/ml	1896****
TT	TTet 400µg/ml	280	TTpe 400µg/ml	1056****
	TTet 600µg/ml	589	TTpe 600µg/ml	671****

In A549 cells, FD extracts were responsible for highest caspase-3/7 activity in the SSea-treated cells. Both FD TTea and OD TTpe extracts showed comparable activities ($p < 0.05$ at 400 µg/ml), along with lowest activity demonstrated by FD DSea and OD DScd (Odeleye et al., 2019). In all FD- and OD treated- A549 cells, a dose dependent Caspase-3/7 activity was observed, with 600 µg/ml eliciting the highest activities (Table 5.5).

Notably, In Hep G2 cells, all FD extracts were responsible for higher activities compared to the OD-treated cells ($p < 0.0005$ in all cases). FD SSea extracts yielded the highest activities (2 fold increase over any OD activity). The highest activity observed in OD-treated Hep G2 cells was in all treatments of DSet and SSpe extracts, with the later having 490% caspase activity. Whereas in the FD- treated cells, the lowest activity was observed in TTea-treated cells at 400 µg/ml (551%).

Compared to the OD-treated results, individual reactions to extract treatments were slightly different, but overall, all cell lines (OD- and FD-treated) showed high caspase-3/7 activities (Table 5.5). In almost all cases, the highest Caspase-3/7 values were generated from FD extract treatments.

5.4.6 CELL CYCLE ALTERATION

To understand the mechanism of inhibition of NZ surf clam extracts, the effect of the extracts on cell cycle progression was analysed by flow cytometry. In this study, cells were treated with 600 µg/ml of extracts. As shown in Supplementary Fig. 5.5, the cell cycle analysis of FD-treated cell lines revealed that in most cases, there was a decrease in the percentage of the G0/G1 phase, and an increase in the S- and G2/M phases respectively. The G2/M (pre-mitosis) and S (pre-replication) phases are the two main

checkpoints for DNA damage (Li et al., 2007). This is consistent with the OD-treated cancer cell lines (Table 5.6).

Table 5.5: Induction of cell cycle arrest in the S- and G2/M phases, examined by flow cytometry. Induction of cell cycle arrest in the S- and G2/M phases, examined by flow cytometry. Cells were treated with FD and OD clam extracts, at 600 µg/ml. Each experiment was carried out twice, in duplicate.

Cell lines	**G2-M Phase**				**S Phase**			
	Oven dried (%)		**Freeze-dried (%)**		**Oven dried (%)**		**Freeze-dried (%)**	
A549	C	8.58	C	8.58	C	11.01	C	11.01
	DScd	11.49	DSea	5.73	DScd	25.03	DSea	20.53
	SSea	14.01	SSea	5.81	SSea	32.03	SSea	33.25
	TTpe	15.47	TTea	2.77	TTpe	28.39	TTea	16.39
Hep G2	C	6.88	C	6.88	C	2.92	C	2.92
	DSet	4.91	DSea	5.81	DSet	4.6	DSea	4.62
	SSpe	14.31	SSea	8.17	SSpe	3.34	SSea	8.00
	TTpe	6.96	TTea	4.84	TTpe	2.43	TTea	3.69
MCF-7	C	7.47	C	7.47	C	6.92	C	6.92
	DSea	10.80	DSpe	6.88	DSea	5.19	DSpe	15.74
	SSpe	23.05	SScd	12.87	SSpe	10.51	SScd	8.04
	TTpe	14.43	TTpe	20.39	TTpe	8.03	TTpe	5.67
MIA PaCa-2	C	3.91	C	3.91	C	4.69	C	4.69
	DSea	17.22	DSpe	17.54	DSea	16.48	DSpe	12.74
	SSet	21.29	SSea	19.04	SSet	11.60	SSea	13.67
	TTpe	23.62	TTpe	25.09	TTpe	14.14	TTpe	8.65

PC-3	C	6.52	C	6.52	C	2.49	C	6.52
	DSea	11.25	DSpe	18.99	DSea	2.34	DSpe	18.99
	SSet	19.26	SSpe	20.79	SSet	2.35	SSpe	20.79
	TTet	12.15	TTpe	24.89	TTet	1.94	TTpe	24.89
SiHa	C	4.95	C	4.95	C	5.54	C	5.54
	DSpe	7.31	DSea	11.09	DSpe	10.03	DSea	6.79
	SSpe	7.13	SSea	11.26	SSpe	7.13	SSea	10.53
	TTpe	7.38	TTpe	13.57	TTpe	6.62	TTpe	16.05
WiDr	C	7.61	C	7.61	C	8.81	C	8.81
	DSea	24.20	DSpe	22.50	DSea	13.84	DSpe	10.14
	SSet	21.75	SSpe	26.99	SSet	7.75	SSpe	14.18
	TTet	26.03	TTpe	15.99	TTet	7.42	TTpe	10.86

In FD-treated Hep G2 cells, there was a notable accumulation of cells in the sub-G1 phase, which is considered to represent apoptotic cells. Table 5.6 reveals DSea, SSea, and TTea- treated cell data of 2-, 2.3-, and 5.8- fold increases in the sub-G1 cell fraction, compared to the control. This increase in the sub-G1 cells was also observed in OD DSet, and OD TTpe- treated Hep G2, where the sub-G1 populations for both treatments were 21.7% and 20% respectively (Odeleye et al., 2019).

In A549, in particular, treatments increased the population of cells in the S-phase, to 20.53% (DSea), 33.25% (SSea), and 16.39% (TTea) (Table 5.6). The accumulation of cells in the S phase suggests a cell cycle arrest in this phase. Similar results were obtained in the OD- treated A549 cells (Table 5.6). All other cell lines induced the accumulation of cells in the G2-M phase.

Across PC-3 and SiHa cells, FD extracts induced greater arrests in the G2-M and S- phases (with the exception of DS-treated SiHa) (Table 5.6).

In this study, NZ surf clam extracts blocked the cell cycle across multiple phases in different cell lines. The mechanism of action of the extracts likely affects regulatory pathways, involving protein kinases, which inhibit cell growth at different checkpoints of the cell cycle (Flatt & Pietenpol, 2000). Consistent with variable cell lines, diverse mechanisms are expected to be involved in these processes. This is in line with a study by Hseih et al. (2006), where regulation occurred at different phases of the cell cycle in each of the cell lines employed for this analysis.

Microtubules are the targets of most natural product anticancer drugs (Jordan, 2002). Marine organism extracts have been reported to block mitosis in the G2/M phase, and

induce cell death, by deterring tubulin polymerization, either directly or via receptor-mediated signal transduction processes (Ning et al., 2009). Dolastatin 10, a peptide isolated from the shell-less mollusc, *Dolabela auricularia*, has been shown to bind to tubulin and block cancer cell cycle in the G2/M phase (Turner et al., 1998), and this may be the major cause of reduced viability and cellular growth inhibition of the cancer cell lines studied herein. The cell cycle arrest in the G2/M- and S- phases of other molluscs have also been reported (Ning et al., 2009; Huang et al., 2012; Liao et al., 2016).

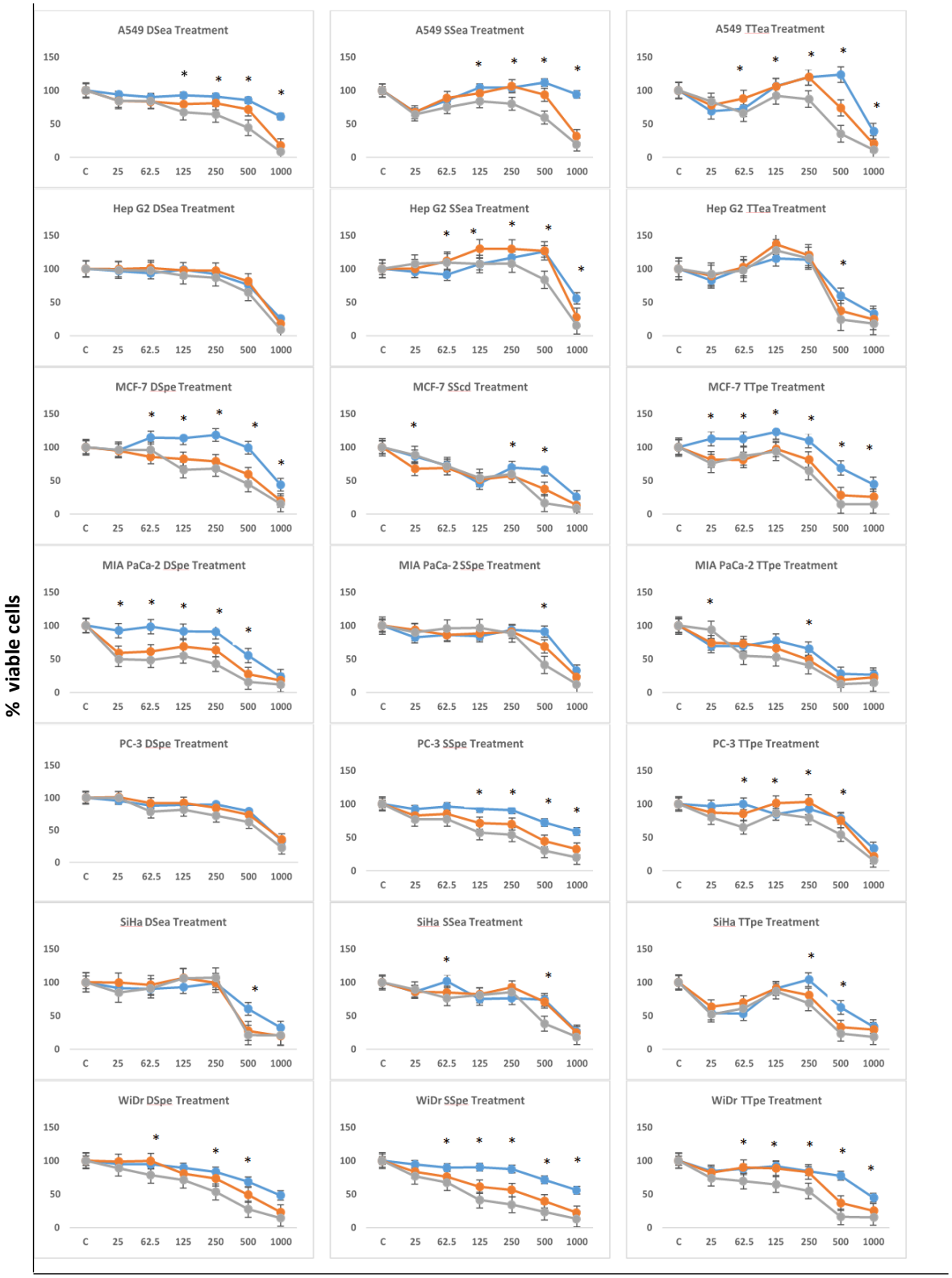
The FD- associated reduction of cells in the GO/G1 phase, and accumulation of cells in the G2/M- and S-phases are consistent throughout the OD-treated results (Table 5.6). These similar modes of cell cycle alteration, the induction of apoptosis via the activation of caspases-3 and -7 indicate that the different processing techniques (freeze-drying and oven drying) prior to extraction result in similar trends. However, the ability of the extract to elicit greater beneficial responses is higher in FD extracts over OD extracts.

5.5 CONCLUSION

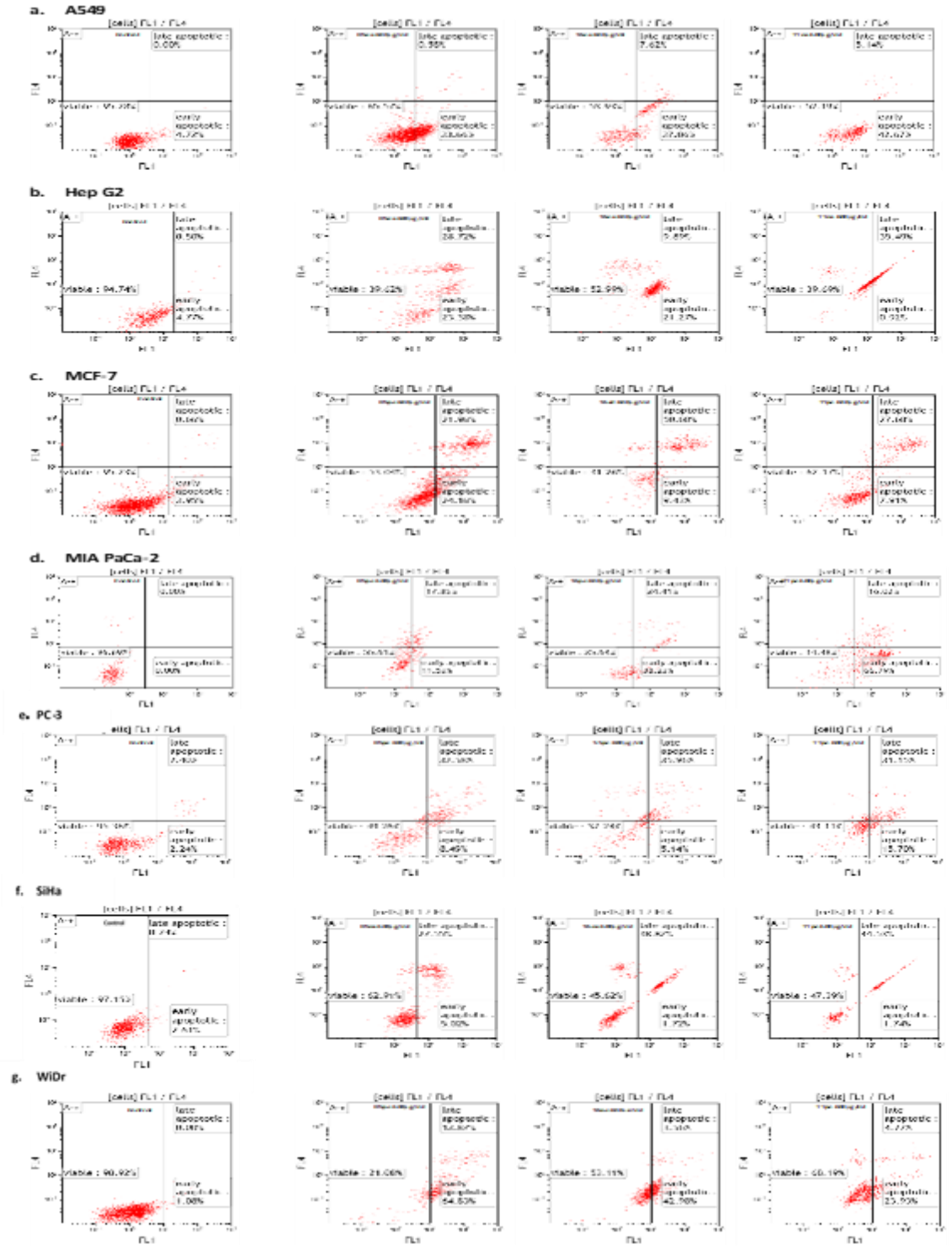
In conclusion, we found that NZ surf clam extracts reduced cell viability by inducing apoptosis and altering the normal cell cycle progression in seven cancer cell lines. The results of our study show that NZ surf clam extracts have a cytotoxic effect on certain cancer cells, in a concentration-dependent manner. Our observations of altered cell morphology are consistent with previous reports of cells undergoing apoptosis. The apoptosis and Caspase-3/7 assays further confirmed this. In line with the above, a cell cycle assay also confirmed that NZ surf clam extracts induced apoptosis. Indications from compositional studies revealed highest levels of lipids and proteins across all fractions from both heat- and cold-processed preparations. In line with these findings, the apoptotic effects of NZ surf clam extracts may be due to molecules, cofactors or

additional aspects associated with the components of the protein- and lipid-rich fractions. Despite similarities in components, FD preparations were responsible for lowest cell viability in MTT assays, and induced greater percentages of apoptosis, confirmed by the highest caspase-3/7 activities. This may indicate that the application of heat in the processing (drying and extraction) protocol affects the relevant bioactive molecules/components or their efficacy in the prepared extracts of this study.

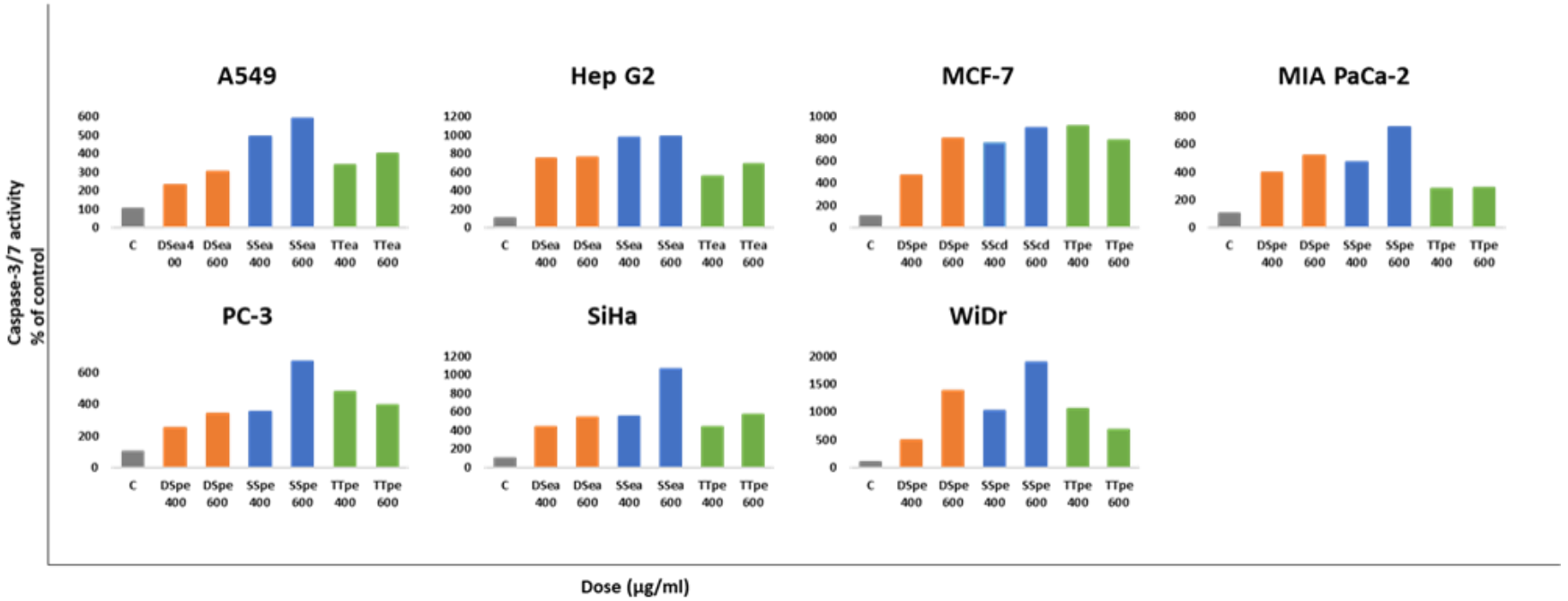
5.7 SUPPLEMENTARY DATA



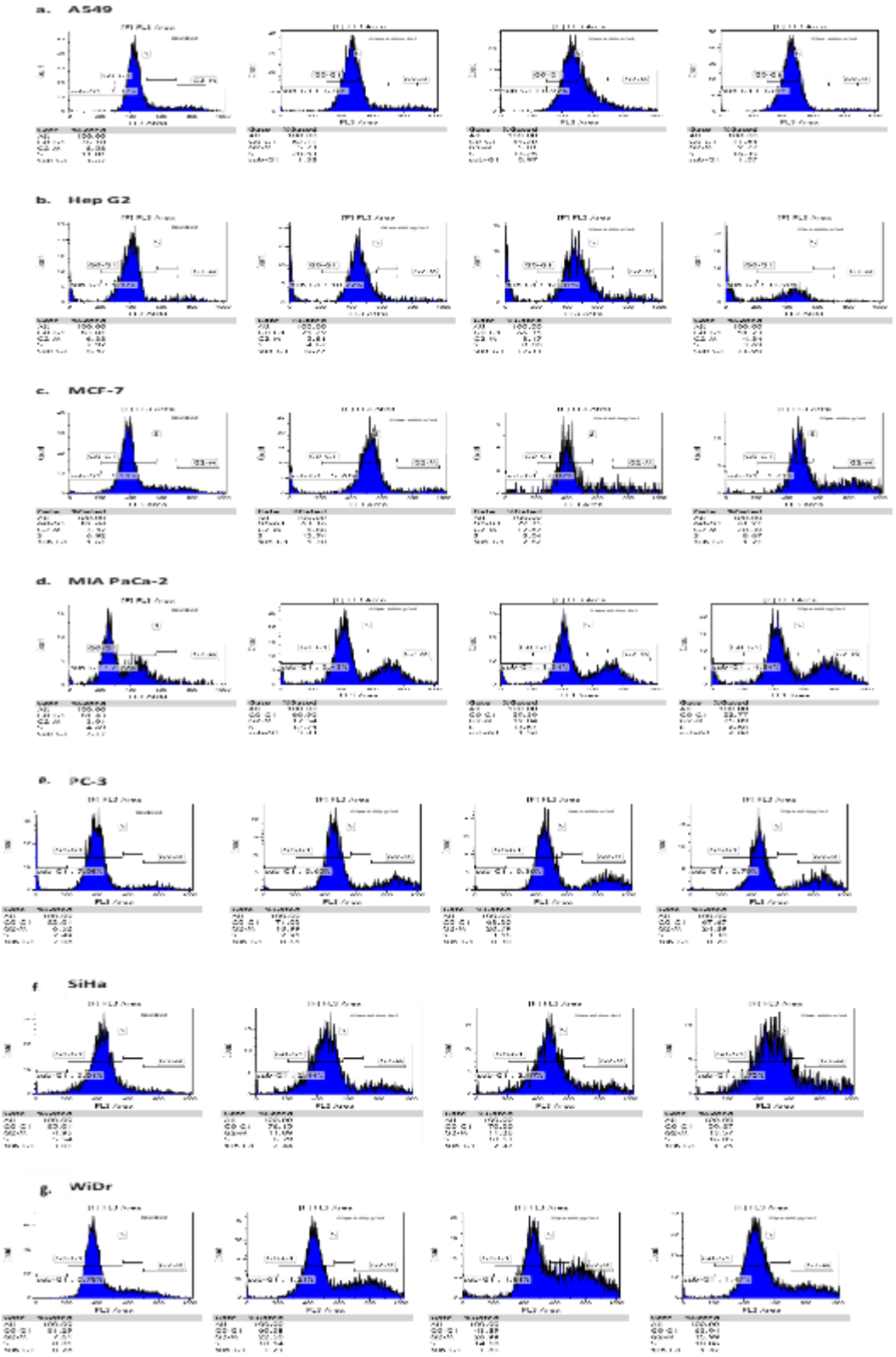
*Supplementary Figure 5.1. The inhibitory effect of FD New Zealand (NZ) surf clam extracts on cell growth after an incubation time of 24, 48, and 72 h (24hrs- blue; 48hrs- orange; 72hrs- grey). Cells were incubated in the presence of various extract concentrations. A relative cell viability of 100% was designated as the total number of cells that grew after each time point. Each experiment was carried out twice, in triplicate. Data is presented as means ± SE. * indicates statistical significance, $p < 0.05$*



Supplementary Figure 5.2. Induction of apoptosis by FD NZ surf clam extracts. Annexin V/Dead Cell Apoptosis Kit with Aleza® Fluor 488 annexin V and PI determined the percentages of viable and apoptotic cells, examined by flow cytometry.



Supplementary Figure 5.3. Caspase-3/7 activities after treatment with FD NZ surf clam extracts. Caspase-3/7 activities were evaluated by Apo-ONE Homogeneous Caspase-3/7 Assay kit. The Caspase-3/7 activity of each group was indicated by their rate fluorescence (RFU). Experiments were carried out twice, in triplicate. Data is presented as mean ± SE.



Supplementary Figure 5.4. Induction of cell cycle arrest, examined by flow cytometry.

CHAPTER 6

CONCLUSION

6.1 OVERALL CONCLUSION

Molluscs have provided worldwide nutritional and medicinal utility for centuries. Marine molluscs are rich in bioactive compounds, with polyunsaturated fatty acids (PUFA), polysaccharides, essential minerals and vitamins, antioxidants, enzymes and bioactive peptides that are readily available for human digestion. A wealth of essentially-hidden individual mechanistic compounds still remained hidden and unidentified within their tissues. Isolation and descriptive details regarding mollusc-derived bioactive compounds are at the forefront of scientific investigations and require accurate methods for a successful combination of extraction and subsequently maintained molecular functions.

6.1.1 BIOACTIVES FROM NATURAL SOURCES

Today's consumers are demanding natural, safe and healthy food products, as well as drugs with low adverse reactions. These positive consumer attitudes are reinforced by continued successful developments of healthy food and food supplement, particularly those arising from marine-derived sources. It is now widely accepted that when functional foods are combined with a healthy lifestyle, there is a beneficial health effect, which goes beyond mere nutrition, and can contribute significantly to overall health and wellbeing.

Many ready-to-eat foods and food additives/ingredients resulting from molluscan studies are currently in human consumption. With a pool of theoretically endless bioactive compounds within their tissues, the removal of established non-functional elements is a common practise. However, in order to characterize these bioactive compounds from this vast spectrum of potential bioactives, tremendous efforts are still required to isolate, screen and promote these substances to clinical applications.

The three most harvested species of surf clams in New Zealand (NZ), the Diamond shell (*Crassula aequilatera*), Storm shell (*Mactra murchisoni*) and Deepwater Tua tua (*Paphies donacina*), are used in this research. Four extracts from each clam species were used: water (cd), ethanol (et), petroleum ether (pe), and ethyl acetate (ea).

6.1.2 PROCESSING INFLUENCE ON BIOACTIVITIES OF NZ SURF CLAMS

The cytotoxicity of the heat processed (OD) extracts were tested in three hormonal cancer cell lines (PC-3, MCF-7, and SiHa), and four other organ cancer cell lines (A549, Hep G2, MIA PaCa-2, and WiDr). In each cell line, cell viability was assessed via the MTT assay, over a three day time point, and over 6 concentrations (25, 62.5, 125, 250, 500, and 1000 µg/ml). Cell viability was deterred in all cell lines over time, especially at concentrations above 125 µg/ml. Beyond initial viability studies, an investigation of apoptotic markers was carried out by surveying caspase-3 and -7 activities in order to assess the quality of cellular death observed as a result of extract treatments. Cells (400 and 600 µg/ml) were stained with Annexin V and PI to identify apoptotic cells after treatment with OD clam extracts. After treatment for 7 hours, results revealed great apoptosis in all treated cells. This was further confirmed by assaying the caspase-3/7 activities. The results revealed an increase in levels of caspase-3 and -7 activities in all cell lines, corresponding with the apoptosis results. The interference to normal cell cycle

progression was also determined by cell cycle assay. This revealed that NZ surf clam-treated cells did not result in an increase the cells in the G0-G1 phase. Furthermore, cell cycle arrest was notably focused on G2/M- and S- phases, and in the sub G1 phase in DSet- and TTpe-treated Hep G2 cells.

Extraction and processing influence the efficacy and properties of bioactive molecules obtained from our food sources. Processes such as cold processing (FD) and heat processing (OD) are both utilized in preparation of mollusks, as preparatory methods for shipping and/or consumption. It is expected that differential effects on the harvested tissues will manifest themselves in kind due to potential denaturation or inactivation. Therefore, the biochemical compositions and preferential activities of three New Zealand surf clam extracts were assessed, focusing on their method of preparation (cold versus heat processing) as a means for identifying notable inhibitory activities against seven cancer cell lines. The results from these studies show that at least two major factors (apoptosis and cell cycle alteration) are involved in the extracts' effects on the cell lines tested. Cell viability studies, followed by general apoptosis and caspase-specific activities, with concluding cell cycle arrest identifications, were used as hallmarks for characterizing the selected clam extracts. Unequivocally, FD extracts yielded lower cell viability, induced greater apoptosis and higher caspase-3/7 activity. This suggests that heat processing affects the bioactivity of OD extracts.

NZ surf clam-treated cells were significantly influenced by the petroleum ether and ethyl acetate fractions of the tested extracts. The ethanol, petroleum ether, and ethyl acetate fractions of NZ surf clam, rich in lipids and proteins across both preparatory methods, were more potent than their water based counterpart, indicating likely fractional localization of relevant bioactives.

6.2 SIGNIFICANCE OF STUDY

This is the first study to describe the preparation of NZ surf clam extracts and their cytotoxic activities. Results show that cell proliferation was time and concentration dependent, decreasing as both variables increased. The results demonstrate that NZ surf clam extracts possess remarkable cytotoxicity, responsible for the observed *in vitro* caspase-dependent apoptosis and disruption of cell cycle progression in the seven cancer cell lines tested. This study provides a basis for further development of NZ clam extracts for cancer treatment, or as a supplementary treatment.

Moreover, natural compounds, such as the NZ surf clam extracts, are active at reasonable concentrations (as displayed in this thesis), and mild enough to allow safe synergetic combinations with each other, and with other natural compounds (Boik, 2001). These attributes make natural compounds suitable for supplemental cancer chemotherapy.

Furthermore, natural compounds from food are not viewed as 'medicine'. This may mean that natural compounds may find widespread long term use (Pan et al., 2007). There is therefore room for the development and 'acceptance' of naturally derived drugs, for preventive and curative purposes.

6.3 FUTURE DIRECTIONS

This study confirms the cytotoxic potential of extracts from three species of NZ surf clams. Additional data, to include extracts' mechanism of action, are however needed to better understand their bioactivity. The functional components in the extracts require

further investigation (ergo isolation, identification, and chemical analysis to include the structures and functional mechanism).

6.3.1 POSSIBLE COMBINATION STUDY

When used alone, the inhibitory effects of natural compounds are moderate at best. Their true inherent bioactivity is best observed when they are used in synergy with other natural compounds *in vivo* (Boik, 2001). Synergetic combinations between extracts, and with other natural compounds, may yield significantly 'better' results. A combination of extracts/compounds may target different pro-cancer events simultaneously and redundantly (where one compound serves as a backup for another). Furthermore, synergetic combinations with current chemotherapy drugs will also provide a basis for improved outcomes.

With a rise in the advent and use of new technologies, natural-product-based drug discovery is continually gaining popularity. These drugs are in the form of anticancer agents, chemopreventive compounds, nutraceuticals and functional foods. There is an extensive number of anticancer agents from diverse microorganisms, plants, and animals (Nobili et al., 2009). Marine compounds in clinical investigation, including trabectedin, bryostatin, kahalalide F, and squalamine, have been reported (Nobili et al., 2009). Despite the natural-product drugs being a robust field today, preclinical and clinical data are lacking (Nobili et al., 2009). An *in vivo* study will be of great benefit in understanding the extracts' bioactivities (or lack thereof), and potency, uptake, and metabolism/ absorption in a whole organism.

6.3.2 FURTHER PRE-CLINICAL AND MECHANISM STUDIES

Furthermore, the effects of NZ surf clam extracts on the growth and proliferation of human normal cell lines must needs be explored. Determining what extract is cytotoxic to cancer cells, with little to no cytotoxic effects in normal cells, is crucial in the furtherance of this study.

Lastly, other experiments, such as western blot, TUNNEL and immunoblotting assays, to further characterize apoptosis-related protein expression, along with cytotoxic activity of NZ surf clam extracts will add value to the current findings.

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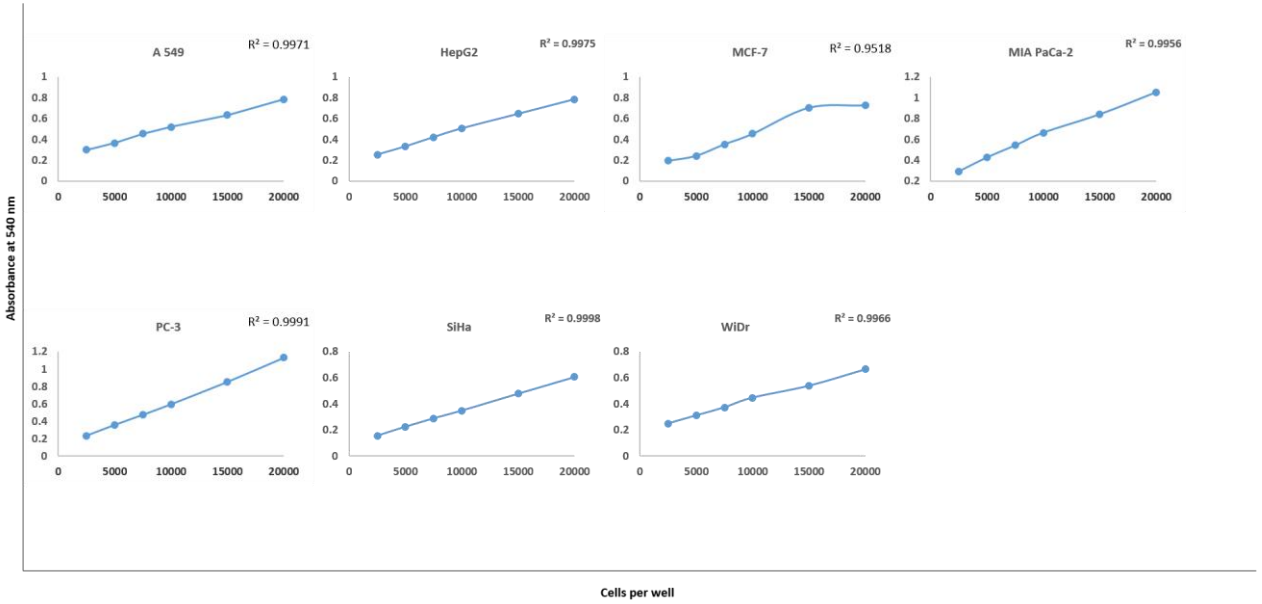
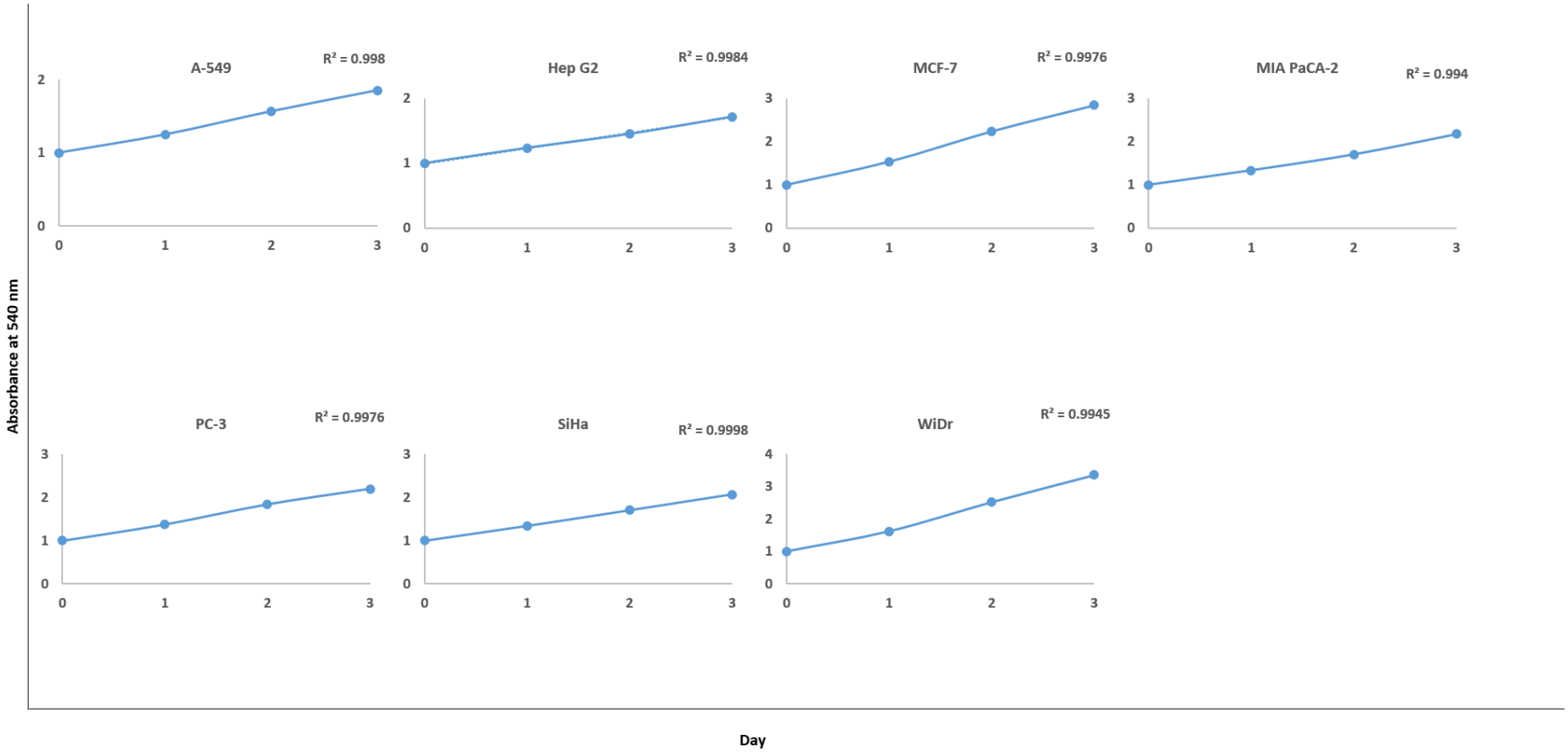
APPENDIX

Appendix A. Growth curves of cell lines used in the study. Initial curves were calculated for variable seeding concentrations. Secondary studies detailed the growth of cells over 1, 2, and 3 days post-incubation. Control studies for cellular responses to the extraction process is demonstrated with solvent-only treatments of applicable cell lines.

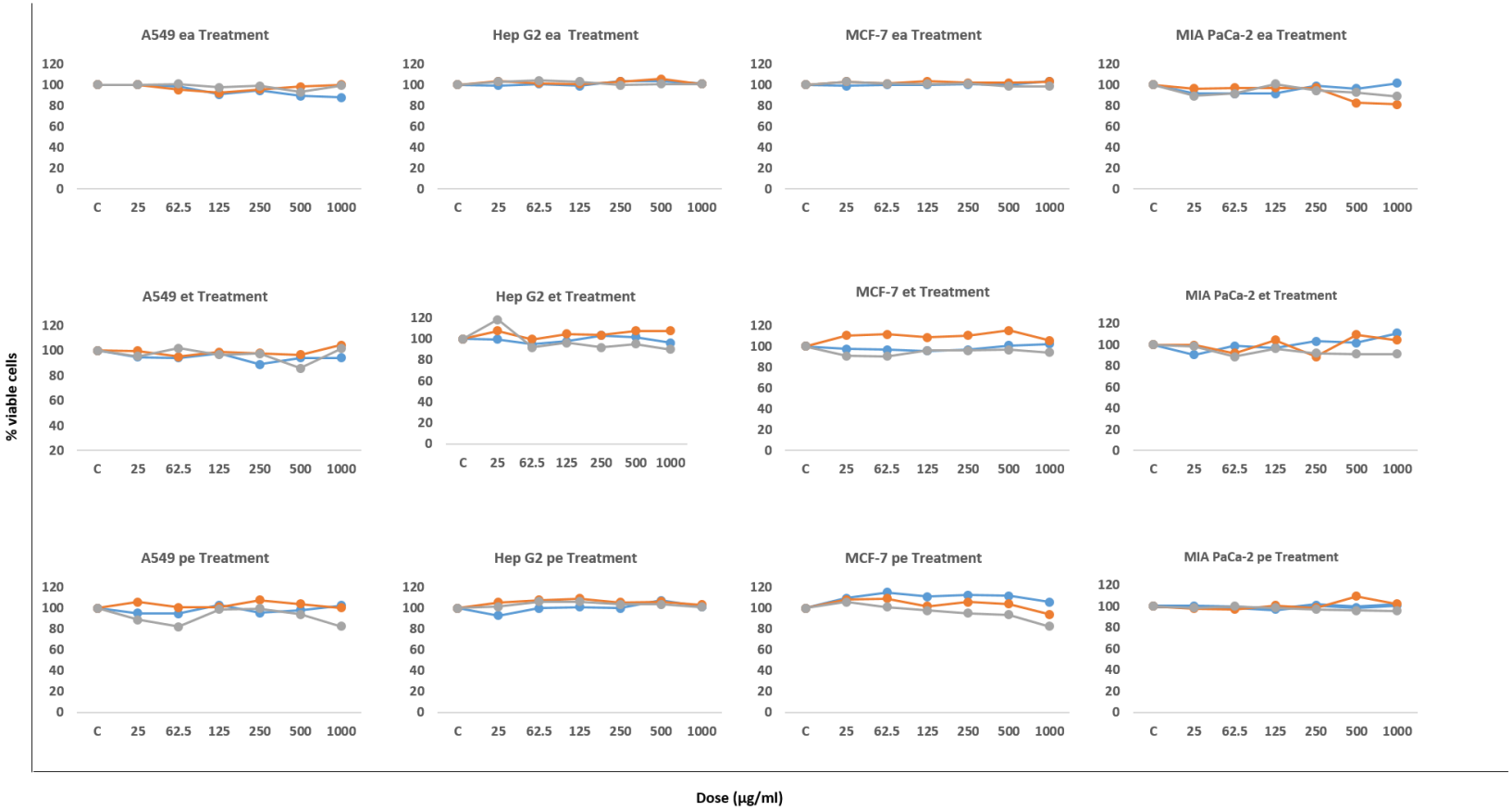
A.1. Growth curves of all cell lines across the range of cell seeding concentrations after 24 hours of incubation. Absorbance measurements were taken at 540 nm and are indicative of cell-specific growth patterns for the cell lines investigated.



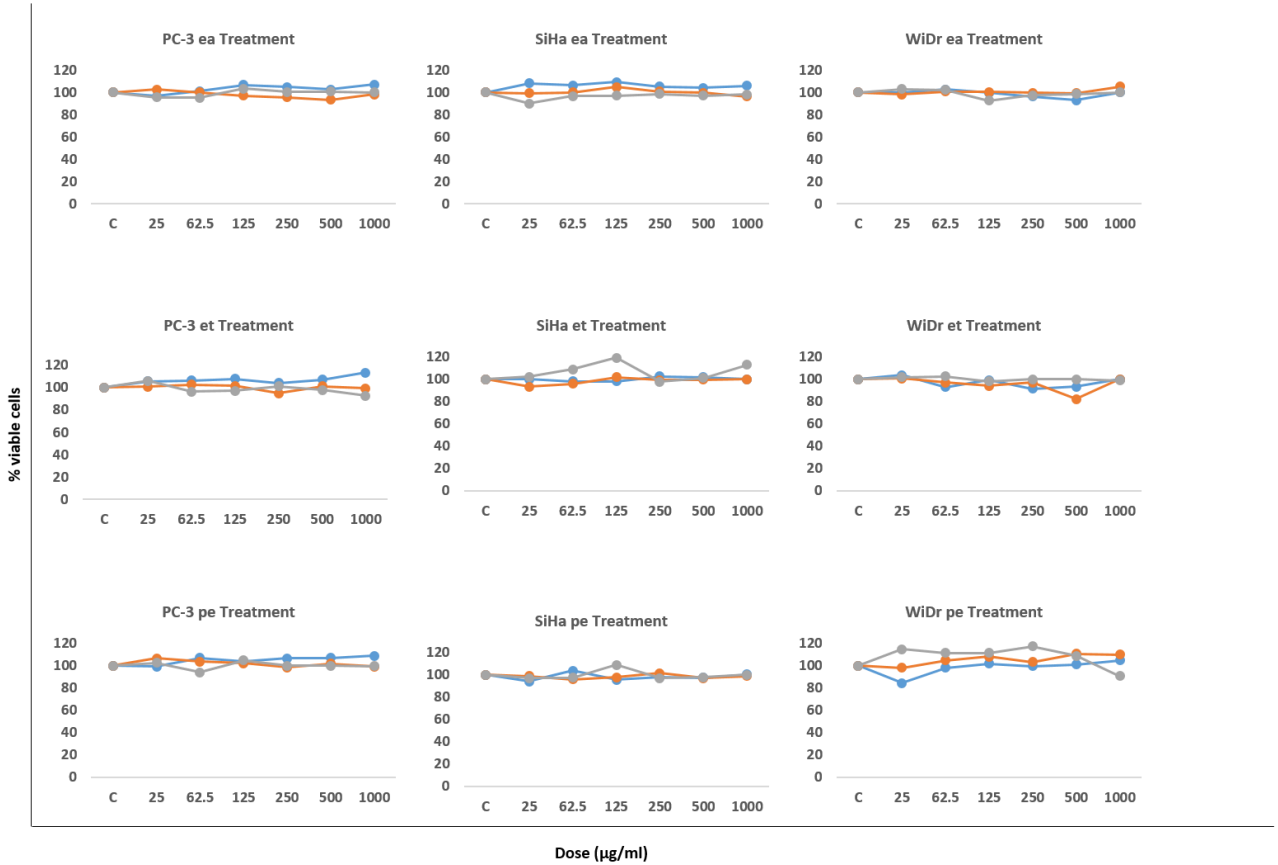
A.2. Growth curves of all cell lines. Growth of 5,000 cells per well wash meaasured after 1, 2, and 3 days (24, 48, and 72 hours) of incubation. Absorbance measurements were taken at 540 nm and are indicative of cell-specific growth patterns for the cell lines investigated.



A.3. Cell viability after treatment with extraction protocols solvents. Ethyl acetate, ethanol, and petroleum ether were administered to cell lines, and viability as determined by MTT assay is indicated. Cells (A549, Hep G2, MCF-7, and MIA PaCa-2) were treated with different concentrations for 24, 48, and 72 hours. Data are represented as mean ± SE (n = 3).



A.4. Cell viability after treatment with extraction protocol solvents. Ethyl acetate, ethanol, and petroleum ether were administered to cell lines, and viability as determined by MTT assay is indicated. Cells (PC-3, SiHa, and WiDr) were treated with different concentrations for 24, 48, and 72 hours. Data are represented as mean ± SE (n = 3).



Appendix B. Poster presentation at Queenstown Research Week, 2017. Research summarizing the successful identification of antioxidant activities associated with extracts from three NZ surf clam species. This initial antioxidant work provided the impetus for current studies which are detailed in this thesis.

Anticancer Activity of New Zealand Surf Clams

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Most cancer chemotherapy drugs possess serious side effects. Some non-toxic bioactive peptides, particularly from marine organisms, either possess anticancer activities or can increase the efficacy of conventional chemotherapy drugs in a synergistic manner. As a result, natural anticancer compounds from marine sources, which may elicit few or no side effects, have become a focus for the development of effective therapeutic compounds to treat various cancers. Our previous work has demonstrated that extracts from NZ surf clams have high antioxidant activity, leading us to extend our studies to investigate potential anticancer activity.

Introduction

In New Zealand, "surf clam" is the collective term for seven species of bivalve molluscs that burrow in the sand, below the low tide mark, on our high energy sandy beaches. These shellfish are abundant in New Zealand and there is a growing fishery being developed. It is estimated that this fishery, when mature, could be NZ's largest by dollar value, generating in excess of NZ$300m per annum.

Clam extracts are known to exhibit a variety of biological activities such as anticancer, antihyperglycemic, anticoagulant, protection of the vascular system, anti-inflammation, and immune-regulation. Clams are rich in proteins, amino acids, lipids and polysaccharides that may contribute to the observed biological functions. Natural products play an important role as cancer chemotherapeutic agents. There is growing interest in naturally occurring compounds with anticancer potentials, as they are relatively non-toxic, inexpensive and available in ingestive forms. There has also been a shift to using marine organisms as sources of anticancer agents due to their demonstrated abilities to produce anticancer compounds and secondary metabolites which act against infectious diseases and inflammation.

Objectives

- To determine the anticancer properties of extracts from three NZ surf clam species in a range of cancer cells *in vitro*.
- To compare and identify which species and/or fraction of surf clam has the highest anticancer activity.
- To identify the compound in the clam responsible for the anticancer activity, and its mode of action.

Methods

Extraction & Fractionation

Freeze dried raw samples of three clam species (Fig. 1) were pulverised with a blender. Extraction and measurements were carried out using distilled water, ethanol, petroleum ether, ethyl acetate, and n-butanol using methods adapted from Luan et al. (2011).

Cell lines

A total of 11 cell lines were studied in this research; eight cancer cell lines (breast, prostate, colon, pancreatic, liver, lung, cervix), and three normal cell lines (umbilical vein, dermal fibroblast, and embryonic kidney). This poster presents results from three of the cancer cell lines used in our study; colon, prostate, and breast cancer cell lines treated with petroleum ether extract of three surf clam species.

MTT Assay

MTT assays were used to determine cancer cell viability after treatment with various doses of clam extracts across different incubation times (24, 48 and 72 hours).

The novelty of this study requires initial selection of treatments which will accurately ascertain the efficacy of extract fractions. As a result, a range of concentrations were selected to define study parameters, and to guide future experimental doses. From low to high, concentrations of 25, 62.5, 125, 250, 500, and 1000µg/mL were prepared for use in protocols.

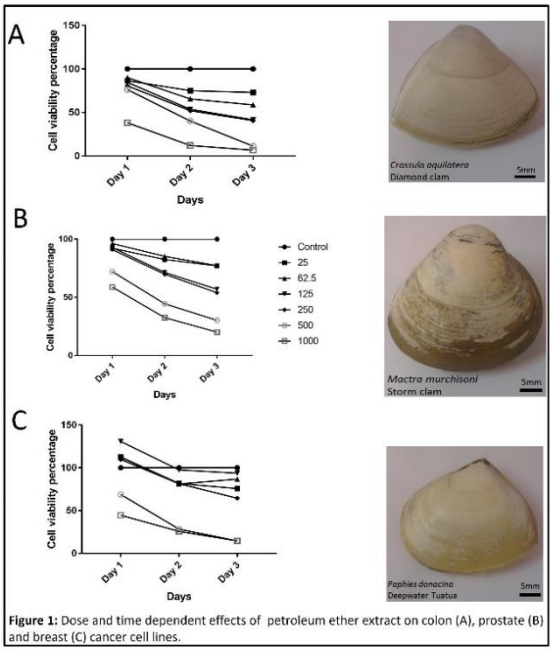

Figure 1: Dose and time dependent effects of petroleum ether extract on colon (A), prostate (B) and breast (C) cancer cell lines.

Results and Discussion

We evaluated the activity of NZ surf clam extracts on eight cancer cell lines and found out that all cell lines were sensitive to three or more clam extracts. All surf clam extracts showed cancer cell growth inhibition in a dose dependent manner, albeit they all had distinctive IC50 values.

The fraction (notably from all three clam species presented) indicating highest levels of inhibition in prostate, colon and breast cancer cell lines was the petroleum ether extract. In fact, the two highest concentrations of this extract exhibited the highest inhibition of MTT activity across all cell lines. This was also true in the ethyl acetate and ethanol (data not shown).
It was also observed that in most cell lines tested, longer treatment incubation periods produced greater inhibition, and vice versa.

These results show that extracts from NZ surf clams possess antineoplastic effects, however, further study is required to identify the active component(s) and elucidate the mechanism of action.

IC50 values were calculated using Graphpad Prism software.

Acknowledgements

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References

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Appendix C. Flow cytometer cell cycle FL3 voltage settings. FL3 settings of the flow cytometer were controlled across the cell cycle data analyses in this study for the purpose of most effective signal compensations. Treatments and cell lines are presented with their given settings, ranging from 490 – 680 nm, as indicated.

Treatment	Cell line
	A549
C	580
DSeaR	570
SSeaR	570
TTeaR	555
DScdOD	580
SSeaOD	570
TTpeOD	570
	HepG2
C	570
DSeaR	580
SSeaR	580
TTeaR	585
DSetOD	580
SSpeOD	580
TTpeOD	580

Treatment	Cell line
	PC-3
C	680
DSpeR	650
SSpeR	660
TTpeR	665
DSeaOD	670
SSeaOD	655
TTetOD	660
	SiHa
C	545
DSeaR	540
SSeaR	545
TTpeR	535
DSpeOD	545
SSpeOD	535
TTpeOD	540

	MCF-7
C	670
DSpeR	655
SScdR	650
TTpeR	635
DSeaOD	650
SSpeOD	630
TTpeOD	635
	MIA PaCa-2
C	490
DSpeR	520
SSpeR	525
TTpeR	530
DSeaOD	525
SSetOD	525
TTpeOD	530

	WiDr
C	590
DSpeR	580
SSpeR	570
TTpeR	570
DSeaOD	590
SSetOD	575
TTetOD	570