Heliyon 6 (2020) e04357

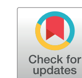
Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Effects of preparation method on the biochemical characterization and cytotoxic activity of New Zealand surf clam extracts



Tinu Odeleye [a], Zhiyong Zeng [b], William Lindsey White [a], Kelvin Sheng Wang [a], Hui Li [b], Xu Xu [b], Hong Xu [b], Jinyao Li [c], Tianlei Ying [d], Baohong Zhang [e], Tao Feng [f], Jun Lu [a,b,f,g,h,i,j,*]

[a] School of Science, Faculty of Health and Environmental Sciences, Auckland University of Technology, Auckland 1010, New Zealand
[b] College of Life Sciences and Oceanography, Shenzhen University, Shenzhen 518071, Guangdong Province, China
[c] Xinjiang Key Laboratory of Biological Resources and Genetic Engineering, College of Life Science and Technology, Xinjiang University, Urumqi 830046, China
[d] Key Laboratory of Medical Molecular Virology of MOE/MOH, Shanghai Medical College, Fudan University, Shanghai 200032, China
[e] Engineering Research Center of Cell and Therapeutic Antibody, Ministry of Education; School of Pharmacy, Shanghai Jiao Tong University, Shanghai 200240, China
[f] School of Perfume and Aroma Technology, Shanghai Institute of Technology, Shanghai 201418, China
[g] School of Public Health and Interdisciplinary Studies, Faculty of Health and Environmental Sciences, Auckland University of Technology, Auckland, New Zealand
[h] Institute of Biomedical Technology, Auckland University of Technology, Auckland 1010, New Zealand
[i] Maurice Wilkins Centre for Molecular Biodiscovery, Auckland, New Zealand
[j] College of Food Engineering and Nutritional Science, Shaanxi Normal University, Xi'an 710119, Shaanxi, China

ARTICLE INFO

Keywords:
Food science
Food technology
Surf clam
Drying
Cytotoxicity
Storm shell
Tua tua
Dimond shell
Crassula aequilatera
Mactra murchisoni
Paphies donacina
New Zealand

ABSTRACT

Molluscan extracts confer a wide range of health promoting properties, one of them is cytotoxicity. Extraction and processing can affect the efficacy and properties of bioactive molecules. New Zealand (NZ) surf clams have never been thoroughly studied for bioactives until recently. However, the effect of cold and heat extraction procedure on biochemical composition and cytotoxic activities of NZ surf clam remains unanswered. The objective is to compare the effects on cytotoxicity of three NZ surf clams (Diamond shell, *Crassula aequilatera*; Storm shell, *Mactra murchisoni*; and Deepwater Tua tua, *Paphies donacina*) extracts via cold or heat process across cancer cell lines to find out which process can preserve bioactivity better. Fractions of extracts prepared via cold or heat procedures were tested for cell growth inhibition, apoptosis induction and cell cycle arrest in seven cancer cell lines. Apoptosis was induced through all cell lines, as further evidenced in Caspase-3/7 activities. Cell cycle arrest was focused on G2/M- and S- phases. Petroleum ether and ethyl acetate fractions, with the greatest bioactivity in this study, are rich in lipids and proteins, indicating likely bioactive sources. Cold preparation was responsible for the lowest cancer cell viability and induced greater apoptosis. Cold process retained better bioactivity/cytotoxicity than that of heat-processed extracts. This information may guide future health/nutraceutical clam product development.

1. 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 [1]. 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 [2].

The first step in the extraction of natural products, for characterization or to test bioactivity, often involves drying the tissues. Molluscan tissues are normally prepared either by steaming prior to extraction [3], oven drying [4, 5], freezing [6], or freeze drying [7], all prior to extraction. Raw, unprocessed flesh studies have also been carried out [8, 9, 10].

The effect of various drying processes on the bioactivity of molluscs has been reported in literature [3, 4, 5, 6, 7]. The clam lipid content (oven drying at different temperatures) [11], as well as NZ green lipped mussel fat content (freezing versus freeze-drying) have been assessed for bioactivity as a function of preparatory methods [6]. Furthermore, the

* Corresponding author.
E-mail address: jun.lu@aut.ac.nz (J. Lu).

https://doi.org/10.1016/j.heliyon.2020.e04357
Received 31 July 2019; Received in revised form 15 November 2019; Accepted 26 June 2020
2405-8440/© 2020 Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

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 [12]. 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) [13]. 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 freeze-dried (FD) and blanched-oven dried (OD) NZ surf clam extracts. Previous literature reveals the importance in considering preparatory methods of food sources as a means of maintaining bioactivities. This research provides a comparison between two different preparation techniques prior to extraction. In the first technique, clams were blanched and then oven dried. In the second, clams were frozen and then freeze-dried. Therefore, the aim of this study is to assess the effects of heat preparations and cold preparations on the subsequent biochemical composition and cytotoxic activity of NZ surf clam extracts, and to compare between both preparations to ascertain which technique had the least effect on the biochemical composition of its extracts. 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.

2. Materials and methods

2.1. 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 Thermo-Fisher (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).

2.2. Surf clam preparation and extraction

Three 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. Two sets of clams (obtained from Cloudy Bay Clams Ltd, Blenheim, New Zealand) were employed in this study. In each case, 6kg (clam-in-shell weight) was used. First, blanched clams were prepared as previously described [2, 5]. Briefly, blanched clams were shucked, and oven dried (Heratherm 50125590 Model IMC18, Thermo Scientific, Auckland, NZ) at 60 °C to constant weight. Blanched-oven dried clams were pulverised in a laboratory blender (model: 800 W Sunbeam Multiblender-Pro blender). Blanched-oven dried (OD) clam powder was stored away from sunlight.

Second, raw live clams were received in ice packed polystyrene boxes. Live clams were shucked and drained of any water. Clams were then frozen in a -80 °C freezer, and then freeze-dried commercially according to the company's (Riddet Institute, Massey University, NZ) protocol. Frozen-freeze dried raw clams were then pulverised in a laboratory blender (model: 800 W Sunbeam Multiblender-Pro blender). Frozen-freeze dried (FD) clam powder was stored away from sunlight.

Initial extraction fractions were generated in parallel using distilled water (cd) and ethanol absolute (et) as solvents. For water extraction, clam powder (OD and FD separately) was solubilized in distilled water and stirred constantly at room temperature using a magnetic stir-bar for 1 h. The supernatant was removed, replaced with fresh distilled water, and stirred for another hour. This process was repeated until the solvent was colourless. The supernatant was collected by centrifugation, then evaporated under reduced pressure using a Rota evaporator (Buchi Rotavapor R-215, Global Science, Auckland, NZ). Ethanol extraction was carried out in the same way, using ethanol absolute. The resulting solution was collected and evaporated under reduced pressure using a Rota evaporator (Buchi Rotavapor R-215, Global Science, Auckland, NZ) until complete dryness.

The resultant water (cd) and ethanol extracts (et) were collected after evaporation and stored at -20 °C. 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'. After fractionation, the water layer was evaporated and remaining dry material was stored at -20 °C, but was not used in this study. Each fractionation process was repeated until the solvent was colourless. Fractions were evaporated to dryness and stored at -20 °C until ready for use. Water, ethanol, petroleum ether, and ethyl acetate fractions were abbreviated as cd, et, pe, and ea fractions respectively. The extract amounts for the cd, et, pe, and ea fractions of Diamond shell, Storm shell, and Tua tua were 4.45g, 3.55g, 4.49, 0.09g; 5.92g, 2.68g, 0.81g, 0.18g; and 6.36g, 5.48g, 1.64g, 2.17g respectively. Each extract was resuspended in RPMI media to a stock concentration of 5 mg/ml for use in further cell cycle methodologies.

2.3. Biochemical analysis of extracts

Biochemical composition analysis was performed according to the method as previously described by Odeleye et al. [5]. 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.

2.4. Morphological analysis

We examined the morphological modifications on treated cells, compared to untreated control cells. Cells were seeded in 6 well plates using the RPMI medium. After incubation in a humidified 37 °C, 5% CO_2 incubator (Series II Water Jacket supplied by Thermo Scientific) overnight, cells were then treated with respective clam extracts (600 μg/ml)

and incubated for 72 h. Cell morphology was observed under an inverted microscope (Zeiss Primotech microscope), at 40 x magnification.

2.5. Cell culture and determination of cytotoxicity

A total of seven cancer cell lines were utilised in this study. Cell lines (Table 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 (Series II Water Jacket supplied by Thermo Scientific) at 37 °C.

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 of 25, 62.5, 125, 250, 500, and 1000 μg/mL. The cells were further incubated for an additional 24, 48, and 72 h independently at 37 °C. After incubation, MTT stock solution was then added to each well and incubated for a further 4 h. 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).

2.6. 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™ 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 h. After treatment, the cells were harvested, washed twice with PBS, and resuspended in 1X binding buffer. Alexa Fluor® 488 annexin (4 μl) and PI (1 μl) (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.

2.7. 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 h. They were then treated with NZ surf clam extracts (600 μg/ml) for 72 h. Supernatant was collected, cells were washed with PBS, and treated with trypsin. Cells were washed twice with PBS at 4 °C, and then fixed with ice cold 80% ethanol, and stored at -80 °C for no longer than 7 days. Upon use, cells were gently centrifuged (1200 xg, 2 min), decanted, resuspended in permeabilizing solution for 30 min at 37 °C, and incubated with PI for 5 min. The mixture was then analysed with flow cytometer (Beckman Coulter's MoFlo™ XDP).

2.8. 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 h (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 h 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).

2.9. 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.

3. Results and discussion

3.1. 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 2 shows a summary of the carbohydrate, protein and lipid components of each OD and FD fraction.

Carbohydrates range from 1.60 to 14.83% in 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%–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 [14, 15]. Marine polysaccharides have been reported to display diverse biological activities, e.g. cytotoxic [16]. The carbohydrate content is consistent with that of *Meretrix meretrix* [17], and *Meretrix lusoria* foot (wet weight), mantle, and viscera samples [18].

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 [19]. Certain peptides are the major components of the extracts

Table 1. Details of cell lines employed.

Cell line	Tissue	Morphology	Disease
PC-3	Prostate	Epithelial	Adenocarcinoma
WiDr	Colon	Epithelial	Colorectal adenocarcinoma
A549	Lung	Epithelial	Carcinoma
Hep G2	Liver	Epithelial	Hepatocellular carcinoma
SiHa	Cervix	Epithelial	Squamous cell carcinoma
MCF-7	Breast	Epithelial	Adenocarcinoma
Mia PaCa-2	Pancreas	Epithelial	Carcinoma

All cell lines were purchased from ATCC.

Table 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

Fractions: cd, water; pe, petroleum ether; ea, ethyl acetate; et, ethanol.

responsible for observed anticancer activity [8]. This may be true for the NZ surf clam extracts. Other peptides inducing antioxidant (blue mussel (*Mytilus edulis*)) [20] and antiproliferative (bivalve- *Arca subcrenata* [21]) activities have been described. The mean crude protein (11.8% in males; 11.9% in female) in the surf clam, *Mactra violacea* [22], 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 [23] and animal [1] extracts possess cytotoxic activities. Other lipid components from the freshwater clam (*Corbicula fluminea*) have been reported to ameliorate hypercholesterolaemia *in vivo* [3]. 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) [24]. 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 [9].

3.2. 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 Figure 1). Results (Table 3) are expressed as percentages of

Table 3. The inhibitory effect of NZ surf clam extracts after an incubation time of 72 h.

Cell line	% Cell viability: Oven dried (OD) treatment				% Cell viability: Freeze-dried (FD) treatment			
		250 μg/ml	500 μg/ml	1000 μg/ml		250 μg/ml	500 μg/ml	1000 μg/ml
A549	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}

OD, Oven dried; FD, Freeze dried; 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).

viable cells in treated cells compared to the control, after 72 h of treatment. Each cell line was challenged with 12 FD and 12 OD extracts, however, only the best three extracts are shown and discussed.

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 h of treatment (Table 3). The OD data of 28% (SSet), and 46% (TTet) [2] 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 h), 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[2].

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 [2]. After 72 h 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. [25], 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 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 h-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% [26]. 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. [8] 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 [27]. 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 [28]. 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 3). Corollaries are notably seen between both methods of drying and effective inhibition in all noted cancer cell lines.

3.3. 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 [2].

Treatment of cells with NZ surf clam extracts (at 600 μg/ml, for 72 h) 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 (Supplementary Figure 2), and these were identical to those of the OD extract-treated cells. This concurs with observations reported by Sahayanathan et al. [25]. 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.

3.4. 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 4.

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 [29]. 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 [30]. 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. 3a). Observed early apoptotic percentage in DSea-, SSea-, and TTea-treated A549 cells were 0.58%, 7.62%, and 5.14% (Table 4). This increase is based on a 7 h, 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. 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

Table 4. Induction of apoptosis by NZ surf clam extracts.

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 400 μg/ml	8.93	0.53	DSea 400 μg/ml	25.03	0.17
	DScd 600 μg/ml	12.83	0	DSea 600 μg/ml	33.66	0.58
SS	SSea 400 μg/ml	22.45	0.05	SSea 400 μg/ml	27.12	4.45
	SSea 600 μg/ml	26.94	1.28	SSea 600 μg/ml	37.86	7.62
TT	TTpe 400 μg/ml	15.48	0.59	TTea 400 μg/ml	35.26	4.24
	TTpe 600 μg/ml	20.51	0.98	TTea 600 μg/ml	42.67	5.14
Hep G2	C	4.77	0.50	C	4.77	0.50
DS	DSet 400 μg/ml	18.87	4.15	DSea 400 μg/ml	13.74	16.56
	DSet 600 μg/ml	26.48	7.31	DSea 600 μg/ml	23.38	28.72
SS	SSpe 400 μg/ml	15.93	9.94	SSea 400 μg/ml	17.60	7.12
	SSpe 600 μg/ml	11.22	15.21	SSea 600 μg/ml	21.27	9.89
TT	TTpe 400 μg/ml	9.97	2.24	TTea 400 μg/ml	23.53	27.76
	TTpe 600 μg/ml	13.86	3.01	TTea 600 μg/ml	0.92	35.49
MCF-7	C	3.95	0.66	C	3.95	0.66
DS	DSea 400 μg/ml	25.53	3.17	DSpe 400 μg/ml	14.80	27.06
	DSea 600 μg/ml	28.31	10.74	DSpe 600 μg/ml	24.16	21.96
SS	SSpe 400 μg/ml	30.97	5.63	SScd 400 μg/ml	25.85	23.92
	SSpe 600 μg/ml	16.42	17.03	SScd 600 μg/ml	9.43	50.60
TT	TTpe 400 μg/ml	15.43	5.28	TTpe 400 μg/ml	8.51	14.25
	TTpe 600 μg/ml	12.39	17.93	TTpe 600 μg/ml	7.91	27.68
MIA PaCa-2	C	0	0	C	0	0
DS	DSea 400 μg/ml	23.89	0.56	DSpe 400 μg/ml	20.26	12.72
	DSea 600 μg/ml	30.38	4.56	DSpe 600 μg/ml	11.53	17.85
SS	SSet 400 μg/ml	5.76	1.82	SSpe 400 μg/ml	34.13	11.23
	SSet 600 μg/ml	38.32	2.96	SSpe 600 μg/ml	38.23	24.41
TT	TTpe 400 μg/ml	10.22	1.55	TTpe 400 μg/ml	25.97	28.57
	TTpe 600 μg/ml	26.19	6.77	TTpe 600 μg/ml	66.79	16.02
PC-3	C	2.24	2.40	C	2.24	2.40
DS	DSea 400 μg/ml	30.29	1.93	DSpe 400 μg/ml	5.75	18.40
	DSea 600 μg/ml	7.99	13.88	DSpe 600 μg/ml	8.49	37.58
SS	SSet 400 μg/ml	30	1.44	SSpe 400 μg/ml	9.72	30.56
	SSet 600 μg/ml	17.10	9.69	SSpe 600 μg/ml	5.14	35.96
TT	TTet 400 μg/ml	5.13	1.80	TTpe 400 μg/ml	11.66	23.32
	TTet 600 μg/ml	4.35	6.17	TTpe 600 μg/ml	15.70	31.15
SiHa	C	1.71	1.81	C	2.61	0.24
DS	DSpe 400 μg/ml	8.62	11.27	DSea 400 μg/ml	3.43	17.09
	DSpe 600 μg/ml	13.72	11.83	DSea 600 μg/ml	5.02	27.55
SS	SSpe 400 μg/ml	34.55	3.06	SSea 400 μg/ml	13.28	20.73
	SSpe 600 μg/ml	20.53	12.42	SSea 600 μg/ml	1.72	38.82
TT	TTpe 400 μg/ml	18.29	15.45	TTpe 400 μg/ml	1.30	30.73
	TTpe 600 μg/ml	12.11	5.05	TTpe 600 μg/ml	1.74	44.53
WiDr	C	1.58	0.16	C	1.08	0
DS	DSea 400 μg/ml	39.06	0.53	DSpe 400 μg/ml	46.47	8.96
	DSea 600 μg/ml	42.49	4.22	DSpe 600 μg/ml	64.83	13.87
SS	SSet 400 μg/ml	33.15	1.38	SSpe 400 μg/ml	10.86	1.35
	SSet 600 μg/ml	31.33	9.01	SSpe 600 μg/ml	42.98	3.56
TT	TTet 400 μg/ml	25.08	4.35	TTpe 400 μg/ml	34.58	4.61
	TTet 600 μg/ml	30.88	4.27	TTpe 600 μg/ml	23.93	4.27

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. C: control.

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 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

Table 5. Caspase-3/7 activities after treatment with NZ surf clam extracts.

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 400 μg/ml	165	DSea 400 μg/ml	228***
	DScd 600 μg/ml	216	DSea 600 μg/ml	301****
SS	SSea 400 μg/ml	257	SSea 400 μg/ml	494****
	SSea 600 μg/ml	282	SSea 600 μg/ml	591****
TT	TTpe 400 μg/ml	345*	TTea 400 μg/ml	338
	TTpe 600 μg/ml	378	TTea 600 μg/ml	401***
Hep G2	C	100	C	100 ^{NS}
DS	DSet 400 μg/ml	474	DSea 400 μg/ml	750****
	DSet 600 μg/ml	445	DSea 600 μg/ml	757****
SS	SSpe 400 μg/ml	485	SSea 400 μg/ml	977****
	SSpe 600 μg/ml	490	SSea 600 μg/ml	979****
TT	TTpe 400 μg/ml	384	TTea 400 μg/ml	551****
	TTpe 600 μg/ml	455	TTea 600 μg/ml	685****
MCF-7	C	100	C	100 ^{NS}
DS	DSea 400 μg/ml	653****	DSpe 400 μg/ml	472
	DSea 600 μg/ml	903****	DSpe 600 μg/ml	801
SS	SSpe 400 μg/ml	679	SScd 400 μg/ml	758****
	SSpe 600 μg/ml	659	SScd 600 μg/ml	895****
TT	TTpe 400 μg/ml	590	TTpe 400 μg/ml	913****
	TTpe 600 μg/ml	447	TTpe 600 μg/ml	789****
MIA PaCa-2	C	100	C	100 ^{NS}
DS	DSea 400 μg/ml	236	DSpe 400 μg/ml	395****
	DSea 600 μg/ml	236	DSpe 600 μg/ml	520****
SS	SSet 400 μg/ml	305	SSpe 400 μg/ml	474****
	SSet 600 μg/ml	302	SSpe 600 μg/ml	725****
TT	TTpe 400 μg/ml	225	TTpe 400 μg/ml	276***
	TTpe 600 μg/ml	180	TTpe 600 μg/ml	284****
PC-3	C	100	C	100 ^{NS}
DS	DSea 400 μg/ml	280***	DSpe 400 μg/ml	246
	DSea 600 μg/ml	530****	DSpe 600 μg/ml	338
SS	SSet 400 μg/ml	374***	SSpe 400 μg/ml	351
	SSet 600 μg/ml	454	SSpe 600 μg/ml	667****
TT	TTet 400 μg/ml	248	TTpe 400 μg/ml	475****
	TTet 600 μg/ml	301	TTpe 600 μg/ml	395****
SiHa	C	100	C	100 ^{NS}
DS	DSpe 400 μg/ml	261	DSea 400 μg/ml	439****
	DSpe 600 μg/ml	373	DSea 600 μg/ml	539****
SS	SSpe 400 μg/ml	379	SSea 400 μg/ml	550****
	SSpe 600 μg/ml	683	SSea 600 μg/ml	1066****
TT	TTpe 400μg/ml	244	TTpe 400μg/ml	434****
	TTpe 600 μg/ml	294	TTpe 600 μg/ml	575****
WiDr	C	100	C	100 ^{NS}
DS	DSea 400 μg/ml	865****	DSpe 400 μg/ml	480
	DSea 600 μg/ml	1268	DSpe 600 μg/ml	1377****
SS	SSet 400 μg/ml	1436****	SSpe 400 μg/ml	1014
	SSet 600 μg/ml	973	SSpe 600 μg/ml	1896****
TT	TTet 400 μg/ml	280	TTpe 400 μg/ml	1056****
	TTet 600 μg/ml	589	TTpe 600 μg/ml	671****

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). C: control.

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), 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.

3.5. 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 [31]. 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 h 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 Figure 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.

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 [2]. 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).

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). In almost all cases, the highest Caspase-3/7 values were generated from FD extract treatments.

3.6. 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 Figure 5, the cell cycle analysis of FD-treated cell lines revealed that in most cases, there was a decrease in the

Table 6. Induction of cell cycle arrest in the S- and G2/M phases, examined by flow cytometry.

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

Cells were treated with FD and OD clam extracts, at 600 μg/ml. Each experiment was carried out twice, in duplicate. C: control.

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 [31]. This is consistent with the OD-treated cancer cell lines (Table 6).

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 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 [2].

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 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 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 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 [32]. 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. [33], 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 [34]. 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 [35]. 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 [36], 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 [35, 37, 38].

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 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 (cold and heat) 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.

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 FD and OD 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 extraction protocol affects the relevant bioactive molecules/components or their efficacy in the prepared extracts of this study. This study calls for further research to identify and elucidate the compounds in NZ surf clam extracts that are responsible for the observed cytotoxic effects.

Declarations

Author contribution statement

T. Odeleye: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Z. Zeng, K. S. Wang: Performed the experiments; Contributed reagents, materials, analysis tools or data.

J. Lu: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

W. L. White: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

H. Li, X. Xu, H. Xu, T. Feng, T. Ying, J. Li, B. Zhang: Contributed reagents, materials, analysis tools or data.

Funding statement

This research received funding from Cloudy Bay Clams Ltd., New Zealand and T.O. received scholarship from the AUT Vice-Chancellor Doctoral Scholarship Fund.

Competing interest statement

The authors have received samples free of charge from the Cloudy Bay Clam company

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2020.e04357.

References

[1] M.H. Pan, Y.T. Huang, C.I. Chang, C.T. Ho, B.S. Pan, Apoptotic-inducing epidioxysterols identified in hard clam (*Meretrix lusoria*), Food Chem. 102 (2007) 788–795.

[2] T. Odeleye, W.L. White, J. Lu, Cytotoxicity of extracts from New Zealand surf clams against organ cancer cell lines, Biomedicines 7 (2019).

[3] T. Chijimatsu, M. Umeki, Y. Kataoka, S. Kobayashi, K. Yamada, H. Oda, S. Mochizuki, Lipid components prepared from a freshwater Clam (*Corbicula fluminea*) extract ameliorate hypercholesterolaemia in rats fed high-cholesterol diet, Food Chem. 136 (2013) 328–334.

[4] R. Liu, J. Ji, L. Wang, S. Chen, S. Guo, H. Wu, Characterisation of nucleosides and nucleobases in *Mactra veneriformis* by high performance liquid chromatography coupled with diode array detector-mass spectrometry (HPLC–DAD–MS), Food Chem. 135 (2012) 548–554.

[5] T. Odeleye, Y. Li, W.L. White, N. Shaoping, S. Chen, J. Wang, J. Lu, The antioxidant potential of the New Zealand surf clams, Food Chem. 204 (2016) 141–149.

[6] K. Murphy, N. Mann, A. Sinclair, Fatty acid and sterol composition of frozen and freeze-dried New Zealand Green Lipped Mussel (*Perna canaliculus*) from three sites in New Zealand, Asia Pac. J. Clin. Nutr. 12 (2003) 50–60.

[7] M. Joy, K. Chakraborty, First report of two new antioxidative meroterpeno 2H-pyranoids from short-necked yellow-foot clam *Paphia malabarica* (family: Veneridae) with bioactivity against pro-inflammatory cyclooxygenases and lipoxygenase, *Natural Product Research* Formerly *Natural Product Letters* 31 (2017) 615–625.

[8] B. Leng, X.D. Liu, Q.X. Chen, Inhibitory effects of anticancer peptide from *Mercenaria* on the BGC-823 cells and several enzymes, FEBS Lett. 579 (2005) 1187–1190.

[9] E. Orban, G. Di Lena, T. Nevigato, I. Casini, R. Caproni, G. Santaroni, G. Giulini, Nutritional and commercial quality of the striped venus clam, *Chamelea gallina*, from the Adriatic sea, Food Chem. 101 (2007) 1063–1070.

[10] L.C. Wang, L.Q. Di, R. Liu, H. Wu, Characterizations and microsphere formulation of polysaccharide from the marine clam (*Mactra veneriformis*), Carbohydr. Polym. 92 (2013) 106–113.

[11] Z.Y. Liu, D.Y. Zhou, X. Zhou, F.W. Yin, Q. Zhao, H.K. Xie, D.Y. Li, B.W. Zhu, T. Wang, F. Shahidi, Effect of various hot-air drying processes on clam *Ruditapes philippinarum* lipids: composition changes and Oxidation development, J. Food Sci. 83 (2018) 2976–2982.

[12] S. Hu, G. Zhao, Y. Zheng, M. Qu, Q. Jin, C. Tong, W. Li, Effect of drying procedures on the physicochemical properties and antioxidant activities of polysaccharides from *Crassostrea gigas*, PloS One 12 (2017), e0188536.

[13] Z. Wu, Effect of different drying methods on chemical composition and bioactivity of finger citron polysaccharides, Int. J. Biol. Macromol. 76 (2015) 218–223.
[14] A.K.M. Cruz, G.P.V. Andrade, S.F. Chavante, C.L. de Vasconcelos, R.B. Garcia, E.L. Leite, A.P. Valente, M.P. Sales, F.W. Oliveira, Structural elucidation of an acidic galactan from the eggs of mollusc *Pomacea lineata*, Carbohydr. Polym. 79 (2010) 975–980.
[15] H. Suleria, P. Masci, K.N. Zhao, R. Addepalli, W. Chen, S. Osborne, G. Gobe, Anti-coagulant and anti-thrombotic properties of blacklip abalone (*Haliotis rubra*): *In vitro* and animal studies, Mar. Drugs 15 (2017) 240.
[16] L. Wang, L. Di, H. Wu, Abundance of saccharides and scarcity of glycosaminoglycans in the soft tissue of clam, *Meretrix meretrix* (Linnaeus), Acta Histochem. 120 (2018) 551–557.
[17] W. Xie, C. Chen, X. Liu, B. Wang, Y. Sun, M. Yan, X. Zhang, *Meretrix meretrix*: active components and their bioactivities, Life Sci. J. 9 (2012) 756–762.
[18] S. Karnjanapratum, S. Benjakul, H. Kishimura, Y.H. Tsai, Chemical compositions and nutritional value of Asian hard clam (*Meretrix lusoria*) from the coast of Andaman Sea, Food Chem. 141 (2013) 4138–4145.
[19] A. Kannan, N.S. Hettiarachchy, M. Marshall, S. Raghavan, H. Kristinsson, Shrimp shell peptide hydrolysates inhibit human cancer cell proliferation, J. Sci. Food Agric. 91 (2011) 1920–1924.
[20] B. Wang, L. Li, C.F. Chi, J.H. Ma, H.Y. Luo, Y.F. Xu, Purification and characterisation of a novel antioxidant peptide derived from blue mussel (*Mytilus edulis*) protein hydrolysate, Food Chem. 138 (2013) 1713–1719.
[21] L. Chen, L. Song, T. Li, J. Zhu, J. Xu, Q. Zheng, R. Yu, A new antiproliferative and antioxidant peptide isolated from *Arca subcrenata*, Mar. Drugs 11 (2013) 1800–1814.
[22] P. Laxmilatha, Proximate composition of the surf clam *Mactra violacea* (Gmelin 1791), Indian J. Fish. 56 (2009) 147–150.
[23] W. Hu, S.K. Lee, M.J. Jung, S.I. Heo, J.H. Hur, M.H. Wang, Induction of cell cycle arrest and apoptosis by the ethyl acetate fraction of *Kalopanax pictus* leaves in human colon cancer cells, Bioresour. Technol. 101 (2010) 9366–9372.
[24] K.L. Sreejamole, C.K. Radhakrishnan, Antioxidant and cytotoxic activities of ethyl acetate extract of the Indian green mussel *Perna viridis*, Asian J. Pharmaceut. Clin. Res. 6 (2013) 197–201.
[25] G.J. Sahayanathan, S. Guha, A. Chinnasamy, Antiproliferative effect of crude proteins extracted from marine clam *Donax variabilis* on human cancer cell lines, Int. J. Pharmaceut. Sci. Res. 9 (2018) 3180–3188.
[26] C. Jiang, M. Wang, J. Liu, D. Gan, X. Zeng, Extraction, preliminary characterization, antioxidant and anticancer activities in vitro of polysaccharides from *Cyclina sinensis*, Carbohydr. Polym. 84 (2011) 851–857.
[27] R.H. Shoemaker, The NCI60 human tumour cell line anticancer drug screen, Nat. Rev. Canc. 6 (2006) 813.
[28] A. Ertel, A. Verghese, S.W. Byers, M. Ochs, A. Tozeren, Pathway-specific differences between tumor cell lines and normal and tumor tissue cells, Mol. Canc. 5 (2006) 55.
[29] C.F. Chi, F.Y. Hu, B. Wang, T. Li, G.F. Ding, Antioxidant and anticancer peptides from the protein hydrolysate of blood clam (*Tegillarca granosa*) muscle, J. Funct. Foods 15 (2015) 301–313.
[30] J. Ma, F. Huang, H. Lin, X. Wang, Isolation and purification of a peptide from *Bullacta exarata* and its impaction of apoptosis on prostate cancer cell, Mar. Drugs 11 (2013) 266–273.
[31] Y.L. Li, G.P. Gan, H.Z. Zhang, H.Z. Wu, C.L. Li, Y.P. Huang, Y.W. Liu, J.W. Liu, A flavonoid glycoside isolated from *Smilax china* L. rhizome *in vitro* anticancer effects on human cancer cell lines, J. Ethnopharmacol. 113 (2007) 115–124.
[32] P.M. Flatt, J.A. Pietenpol, Mechanisms of cell-cycle checkpoints: at the crossroads of carcinogenesis and drug discovery, Drug Metab. Rev. 32 (2000) 283–305.
[33] T.C. Hsieh, P. Wu, S. Park, J.M. Wu, Induction of cell cycle changes and modulation of apoptogenic/anti-apoptotic and extracellular signaling regulatory protein expression by water extracts of I'm-Yunity™(PSP), BMC Compl. Alternative Med. 6 (2006) 30.
[34] M.A. Jordan, Mechanism of action of antitumor drugs that interact with microtubules and tubulin, Curr. Med. Chem. Anti Canc. Agents 2 (2002) 1–17.
[35] X. Ning, J. Zhao, Y. Zhang, S. Cao, M. Liu, P. Ling, X. Lin, A novel anti-tumor protein extracted from *Meretrix meretrix* Linnaeus induces cell death by increasing cell permeability and inhibiting tubulin polymerization, Int. J. Oncol. 35 (2009) 805–812.
[36] T. Turner, W.H. Jackson, G.R. Pettit, A. Wells, A.S. Kraft, Treatment of human prostate cancer cells with dolastatin 10, a peptide isolated from a marine shell-less mollusc, Prostate 34 (1998) 175–181.
[37] F. Huang, Z. Yang, D. Yu, J. Wang, R. Li, G. Ding, Sepia ink oligopeptide induces apoptosis in prostate cancer cell lines via caspase-3 activation and elevation of Bax/Bcl-2 ratio, Mar. Drugs 10 (2012) 2153–2165.
[38] N. Liao, J. Zhong, R. Zhang, X. Ye, Y. Zhang, W. Wang, Y. Wang, S. Chen, D. Liu, R. Liu, Protein-bound polysaccharide from *Corbicula fluminea* inhibits cell growth in MCF-7 and MDA-MB-231 human breast cancer cells, PloS One 11 (2016), e0167889.