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

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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 antiocoagulant, hypcholesterolaemic, 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
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-sulfoxide (DMSO) was purchased from Thermo-Fisher Scientific. TropLE™ 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 then oven dried (Heratherm 50125590 Model IMC18, Thermo Scientific, Auckland, NZ) at 60 °C to constant weight. Blanched-oven dried clams were then 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 Odelaye 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% CO2 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% CO2, 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 × 10⁵ cells ml⁻¹ using the RPMI medium. After incubation in a humidified 37 °C, 5% CO2 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® MoFlo™ XDP). Cells were placed in 6-well plates at a density of 3 x 10⁵ 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 10⁵ 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 10⁴ 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. aequalata), 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 isoria foot (well 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.
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, 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 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 |
| Storm shell | cd | 5.21 ± 0.14 | 5.62 ± 0.12 | 3.76 ± 0.08 | 3.75 ± 0.06 | 12.33 ± 3.57 | 46.17 ± 0.62 |
| 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 | 8.99 ± 0.22 | 9.29 ± 0.22 | 6.91 ± 0.05 | 7.80 ± 0.12 | 7.06 ± 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 ± 0.33 | 6.05 ± 0.10 | 54.67 ± 3.17 | 66.00 ± 1.78 |

| Table 3. The inhibitory effect of NZ surf clam extracts after an incubation time of 72 h. |
|-----------------------------------------------|
| Cell line | % Cell viability: Freeze-dried (FD) treatment |
|          | 250 μg/ml | 500 μg/ml | 1000 μg/ml |
|          | 8.***<0.0005; ****<0.0001; ***<0.005; **<0.01; *<0.05 | |
|          |          |          |          |
| A549     | DSdc 76 | 76 | 82 | DSdc 44*<0.05 | DSdc 64*<0.0005 |
|          | DSsey 107 | 68 | 15 | DSdc 59*<0.0005 | DSdc 39*<0.0001 |
|          | DTpe 106 | 113 | 45 | DSdc 35*<0.0005 |
| Hep G2   | DSdc 92 | 69 | 31 | DSdc 80*<0.0005 | DSdc 90*<0.0001 |
|          | SSey 61*** | 38*** | 19 | SSdc 108 | SSdc 113*<0.0005 |
|          | DTpe 161 | 140 | 49 | DTpe 115*** | DTpe 24***<0.0001 |
| MCF-7    | DSey 57* | 20*** | 24 | DSey 68 | DSey 15*<0.007 |
|          | SSey 114 | 15 | 15 | SSdc 16*<0.007 | SSdc 8*<0.007 |
|          | DTpe 112 | 20 | 11 | DTpe 21*** | DTpe 11***<0.0005 |
| MIA PaCa-2 | DSey 49 | 11 | 11 | DSey 21*** | DSey 11***<0.0005 |
|          | SSey 138 | 22*** | 14 | SSdc 40 | SSdc 12***<0.0005 |
|          | DTpe 97 | 106 | 62 | DTpe 40* | DTpe 14***<0.0005 |
| PC-3     | DSey 15*** | 10*** | 13* | DSey 72 | DSey 23 | DSey 23 |
|          | SSey 74 | 12** | 12* | SSdc 53*** | SSdc 30 | SSdc 20 |
|          | DTet 91 | 61 | 28 | DTpe 79* | DTpe 16*<0.007 |
| SiHa     | DSey 87*** | 36 | 31 | DSey 107 | DSey 21** | DSey 21**<0.01 |
|          | SSey 46*** | 46 | 40 | SSdc 85 | SSdc 38***<0.0005 |
|          | DTpe 70 | 24 | 32 | DTpe 69 NS | DTpe 18***<0.0005 |
| WiDr     | DSey 26*** | 6*** | 6* | DSey 53 | DSey 27 | DSey 14 |
|          | SSey 72 | 11* | 5* | SSdc 34*** | SSdc 23 | SSdc 13 |
|          | DTet 54 | 24 | 17 | DTpe 55 NS | DTpe 15 NS | DTpe 15 NS |
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% (TTTe) [2] correlates closely with the FD treated WiDr cells, with the exception of DS (DSsea), 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 2.

Contrary to colon cancer cell line data, the results indicatelung (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 < 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 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 SiHAs, 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 DSea-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 DSea-, 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 attained 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-, SSpe-, and TTea-treated A549 cells were 2.59%, 7.62%, and 5.14% (Table 4). This increase is based on a 7 h, 600 μg/ml treatment. Similar values were observed in DSea-, 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                        | DSea 400 μg/ml      | 25.03              | 0.17              |
|                   | DScd 600 μg/ml             | 12.83                       | DSea 600 μg/ml      | 33.66              | 0.58              |
| SS                 | SSea 400 μg/ml             | 22.45                       | SSea 400 μg/ml      | 27.12              | 4.45              |
|                   | SSea 600 μg/ml             | 26.94                       | SSea 600 μg/ml      | 37.86              | 7.62              |
| TT                 | TTpe 400 μg/ml             | 15.48                       | TTena 400 μg/ml     | 35.26              | 4.24              |
|                   | TTpe 600 μg/ml             | 20.51                       | TTena 600 μg/ml     | 42.67              | 5.14              |
| Hepl G2            | C 4.77                    | 0.50                        | C 4.77             | 0.50              |
| DS                 | DSea 400 μg/ml             | 18.87                       | DSea 400 μg/ml      | 13.74              | 16.56             |
|                   | DSea 600 μg/ml             | 26.48                       | DSea 600 μg/ml      | 23.38              | 28.72             |
| SS                 | SSpe 400 μg/ml             | 30.97                       | SSpe 400 μg/ml      | 25.85              | 23.92             |
|                   | SSpe 600 μg/ml             | 16.42                       | SSpe 600 μg/ml      | 9.43               | 50.60             |
| TT                 | TTpe 400 μg/ml             | 15.43                       | TTpe 400 μg/ml      | 8.51               | 14.25             |
|                   | TTpe 600 μg/ml             | 12.39                       | TTpe 600 μg/ml      | 7.91               | 27.68             |
| MCF-7              | C 3.95                    | 0.66                        | C 3.95             | 0.66              |
| DS                 | DSea 400 μg/ml             | 25.53                       | DSpe 400 μg/ml      | 14.80              | 27.06             |
|                   | DSea 600 μg/ml             | 28.31                       | DSpe 600 μg/ml      | 24.16              | 21.96             |
| SS                 | SSpe 400 μg/ml             | 30.97                       | SSpe 400 μg/ml      | 34.13              | 11.23             |
|                   | SSpe 600 μg/ml             | 16.42                       | SSpe 600 μg/ml      | 38.23              | 24.41             |
| TT                 | TTpe 400 μg/ml             | 10.22                       | TTpe 400 μg/ml      | 25.97              | 28.57             |
|                   | TTpe 600 μg/ml             | 26.19                       | 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                       | DSpe 400 μg/ml      | 20.26              | 12.72             |
|                   | DSea 600 μg/ml             | 30.38                       | DSpe 600 μg/ml      | 11.53              | 17.85             |
| SS                 | SSpe 400 μg/ml             | 38.52                       | SSpe 400 μg/ml      | 34.13              | 11.23             |
|                   | SSpe 600 μg/ml             | 17.10                       | SSpe 600 μg/ml      | 38.23              | 24.41             |
| TT                 | TTpe 400 μg/ml             | 5.13                        | TTpe 400 μg/ml      | 11.66              | 23.32             |
|                   | TTpe 600 μg/ml             | 4.35                        | 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                        | DSea 400 μg/ml      | 11.27              | 17.09             |
|                   | DSpe 600 μg/ml             | 13.72                       | DSea 600 μg/ml      | 11.83              | 27.55             |
| SS                 | SSpe 400 μg/ml             | 34.55                       | SSea 400 μg/ml      | 13.28              | 20.73             |
|                   | SSpe 600 μg/ml             | 20.53                       | SSea 600 μg/ml      | 1.72               | 38.82             |
| TT                 | TTpe 400 μg/ml             | 18.29                       | TTpe 400 μg/ml      | 1.30               | 30.73             |
|                   | TTpe 600 μg/ml             | 12.11                       | TTpe 600 μg/ml      | 1.74               | 44.53             |
| WiDr               | C 1.58                    | 0.16                        | C 1.08             | 0.00              |
| DS                 | DSea 400 μg/ml             | 39.06                       | DSpe 400 μg/ml      | 46.47              | 1.08              |
|                   | DSea 600 μg/ml             | 42.49                       | DSpe 600 μg/ml      | 64.83              | 13.87             |
| SS                 | SSpe 400 μg/ml             | 33.15                       | SSpe 400 μg/ml      | 10.86              | 1.35              |
|                   | SSpe 600 μg/ml             | 31.33                       | SSpe 600 μg/ml      | 42.98              | 3.56              |
| TT                 | TTpe 400 μg/ml             | 25.08                       | TTpe 400 μg/ml      | 34.58              | 4.61              |
|                   | TTpe 600 μg/ml             | 30.88                       | TTpe 600 μg/ml      | 23.93              | 4.27              |

Annexin V/Dead Cell Apoptosis Kit with Alexa® 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%, SSpe- 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 twofold 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               |                           |                            |
| DS                 | DScd 400 µg/ml            | DSea 400 µg/ml             |
|                    | 165                       | 228***                     |
|                    | DScd 600 µg/ml            | DSea 600 µg/ml             |
|                    | 216                       | 301***                     |
| SS                 | SSea 400 µg/ml            | SSea 400 µg/ml             |
|                    | 257                       | 494****                    |
|                    | SSea 600 µg/ml            | SSea 600 µg/ml             |
|                    | 282                       | 591****                    |
| TT                 | TTpe 400 µg/ml            | TTpe 400 µg/ml             |
|                    | 345*                      | 338                        |
|                    | TTpe 600 µg/ml            | TTpe 600 µg/ml             |
|                    | 378                       | 401***                     |
| Hep G2             |                           |                            |
| DS                 | DSet 400 µg/ml            | DSea 400 µg/ml             |
|                    | 474                       | 750***                     |
|                    | DSet 600 µg/ml            | DSea 600 µg/ml             |
|                    | 445                       | 759****                    |
| SS                 | SSpe 400 µg/ml            | SScd 400 µg/ml             |
|                    | 485                       | 977****                    |
|                    | SSpe 600 µg/ml            | SScd 600 µg/ml             |
|                    | 490                       | 979****                    |
| TT                 | TTpe 400 µg/ml            | TTpe 400 µg/ml             |
|                    | 384                       | 551****                    |
|                    | TTpe 600 µg/ml            | TTpe 600 µg/ml             |
|                    | 455                       | 685****                    |
| MCF-7              |                           |                            |
| DS                 | DSea 400 µg/ml            | DSpe 400 µg/ml             |
|                    | 653****                   | 472                        |
|                    | DSea 600 µg/ml            | DSpe 600 µg/ml             |
|                    | 903****                   | 801                        |
| SS                 | SSpe 400 µg/ml            | SSpe 400 µg/ml             |
|                    | 679                       | 758****                    |
|                    | SSpe 600 µg/ml            | SSpe 600 µg/ml             |
|                    | 659                       | 895****                    |
| TT                 | TTpe 400 µg/ml            | TTpe 400 µg/ml             |
|                    | 590                       | 913****                    |
|                    | TTpe 600 µg/ml            | TTpe 600 µg/ml             |
|                    | 447                       | 789****                    |
| MIA PaCa-2         |                           |                            |
| DS                 | DSea 400 µg/ml            | DSpe 400 µg/ml             |
|                    | 236                       | 395****                    |
|                    | DSea 600 µg/ml            | DSpe 600 µg/ml             |
|                    | 236                       | 520****                    |
| SS                 | SSet 400 µg/ml            | SSpe 400 µg/ml             |
|                    | 305                       | 474****                    |
|                    | SSet 600 µg/ml            | SSet 600 µg/ml             |
|                    | 302                       | 725****                    |
| TT                 | TTet 400 µg/ml            | TTet 400 µg/ml             |
|                    | 225                       | 276****                    |
|                    | TTet 600 µg/ml            | TTet 600 µg/ml             |
|                    | 180                       | 284****                    |
| PC-3               |                           |                            |
| DS                 | DSea 400 µg/ml            | DSpe 400 µg/ml             |
|                    | 280****                   | 246                        |
|                    | DSea 600 µg/ml            | DSpe 600 µg/ml             |
|                    | 530****                   | 338                        |
| SS                 | SSet 400 µg/ml            | SSet 400 µg/ml             |
|                    | 374****                   | 351                        |
|                    | SSet 600 µg/ml            | SSet 600 µg/ml             |
|                    | 454                       | 667****                    |
| TT                 | TTet 400 µg/ml            | TTet 400 µg/ml             |
|                    | 248                       | 475****                    |
|                    | TTet 600 µg/ml            | TTet 600 µg/ml             |
|                    | 301                       | 395****                    |
| SiHa               |                           |                            |
| DS                 | DSpe 400 µg/ml            | DSea 400 µg/ml             |
|                    | 261                       | 439****                    |
|                    | DSpe 600 µg/ml            | DSea 600 µg/ml             |
|                    | 373                       | 539****                    |
| SS                 | SSe 400 µg/ml             | SSe 400 µg/ml              |
|                    | 379                       | 550****                    |
|                    | SSe 600 µg/ml             | SSe 600 µg/ml              |
|                    | 683                       | 1066****                   |
| TT                 | TTet 400 µg/ml            | TTet 400 µg/ml             |
|                    | 244                       | 434****                    |
|                    | TTet 600 µg/ml            | TTet 600 µg/ml             |
|                    | 294                       | 575****                    |
| WiDr               |                           |                            |
| DS                 | DSea 400 µg/ml            | DSpe 400 µg/ml             |
|                    | 865****                   | 480                        |
|                    | DSea 600 µg/ml            | DSpe 600 µg/ml             |
|                    | 1268                      | 1377****                   |
| SS                 | SSe 400 µg/ml             | SSe 400 µg/ml              |
|                    | 1436****                  | 1014                       |
|                    | SSe 600 µg/ml             | SSe 600 µg/ml              |
|                    | 973                       | 1896****                   |
| TT                 | TTet 400 µg/ml            | TTet 400 µg/ml             |
|                    | 280                       | 1056****                   |
|                    | TTet 600 µg/ml            | TTet 600 µg/ml             |
|                    | 589                       | 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 caspase-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 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 assessed 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

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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       | DSea       | 11.49 | 5.73 | DSea     | 14.01 | SSea      | 8.81 | TTea     | 15.47 | TTea     | 2.77 | TTpe     | 15.47 |
|            |            |      |         |          |          |          |       |          |       |          |       |          |       |
| Hep G2     | C          | 6.88 | C        | 6.88 | C          | 2.92 | C        | 2.92 |
| DSet       | DSea       | 4.91 | 5.81 | DSea     | 14.31 | SSea      | 8.17 | TTpe     | 6.96 | TTea     | 4.84 | TTpe     | 4.84 |
| SSpe       |            |      |         |          |          |          |       |          |       |          |       |          |       |
|            |            |      |         |          |          |          |       |          |       |          |       |          |       |
| MCF-7      | C          | 7.47 | C        | 7.47 | C          | 6.92 | C        | 6.92 |
| DSea       | DSea       | 10.80 | 6.88 | DSea     | 23.05 | SScd      | 12.87 | TTpe     | 14.43 | TTpe     | 20.39 | TTpe     | 20.39 |
| SSpe       |            |      |         |          |          |          |       |          |       |          |       |          |       |
|            |            |      |         |          |          |          |       |          |       |          |       |          |       |
| MIA PaCa-2 | C          | 3.91 | C        | 3.91 | C          | 4.69 | C        | 4.69 |
| DSea       | DSea       | 17.22 | 17.54 | DSea    | 21.29 | SSea      | 19.04 | TTpe     | 23.62 | TTpe     | 25.09 | TTpe     | 14.14 |
| SSset      |            |      |         |          |          |          |       |          |       |          |       |          |       |
|            |            |      |         |          |          |          |       |          |       |          |       |          |       |
| PC-3       | C          | 6.52 | C        | 6.52 | C          | 2.49 | C        | 2.49 |
| DSea       | DSea       | 11.25 | 18.99 | DSea    | 19.26 | SSsp      | 20.79 | TTet     | 12.15 | TTet     | 24.89 | TTet     | 24.89 |
| SSset      |            |      |         |          |          |          |       |          |       |          |       |          |       |
|            |            |      |         |          |          |          |       |          |       |          |       |          |       |
| SiHa       | C          | 4.95 | C        | 4.95 | C          | 5.54 | C        | 5.54 |
| DSppe      | DSppe      | 7.31 | 11.09 | DSppe   | 7.13 | SSea      | 11.26 | TTea     | 7.38 | TTpe     | 13.57 | TTpe     | 6.62 |
|            |            |      |         |          |          |          |       |          |       |          |       |          |       |
|            |            |      |         |          |          |          |       |          |       |          |       |          |       |
| WiDr       | C          | 7.61 | C        | 7.61 | C          | 8.81 | C        | 8.81 |
| DSea       | DSea       | 24.20 | 22.50 | DSea    | 21.75 | SSsp      | 26.99 | TTet     | 26.03 | TTpe     | 15.99 | TTet     | 7.42 |

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 Hsieh 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, Dolabola 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.

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Competing interest statement

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

Additional information

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