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The cytotoxic activity of extracts of the brown alga *Cystoseira tamariscifolia* (Hudson) Papenfuss, 1950 against cancer cell lines changes seasonally

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Abstract

Brown seaweeds have been highlighted for their abundant production of bioactive substances that may provide novel drugs or drug scaffolds for a range of diseases, particularly cancer. Indeed, a number of promising compounds that can modulate growth arrest or apoptosis have already been isolated. As previous work has highlighted seasonal differences in concentrations of secondary metabolites, this study aimed to evaluate seasonal variation in the cytotoxic anticancer activity of *Cystoseira tamariscifolia* extracts. Primary and secondary metabolites were measured using colourimetric techniques and extracts were exposed to human leukaemia, HL60 and THP-1, and human prostate cancer PC3 cell lines *in vitro* and cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The results confirm a large but inconsistent seasonal variation in the concentrations of biomolecules, although, generally, levels were higher in spring and summer. IC_{50} values for cytotoxicity also showed variability both seasonally and with extraction protocol, but again, this was inconsistent. These data suggest that, when collecting brown algae in order to isolate novel bioactive compounds, repeated sampling across annual or biotic cycles of the seaweed and the use of multiple solvent extraction methods could give a greater possibility of discovering bioactive-lead compounds.

Keywords: Anticancer; Chemical metabolites; Seasonal variation; Seaweed.

Introduction

Cancer has become one of the major causes of premature death in an ageing population. With the risk of developing cancer approaching 50% by the age of 80, combined with current life expectancy figures that are increasing in developing countries, cancer is likely to remain a major affliction to patients and a challenge to healthcare services. Prostate cancer remains one of the leading causes of cancer-related death, while 6.8 million cancer deaths reported globally in 2018, 1.8 million of which were caused by prostate cancer only. Not far from that, leukaemia of various types, also claiming many lives around the world every year, especially children (Siegel et al. 2019; Bray et al. 2018), which makes the search for a cure for these fatal diseases a noble goal of our research. The most common treatments for cancer are still surgery, radiotherapy and chemotherapy, including direct and indirect hormonal and immune therapies (Coffelt and de-Visser 2015; Senthebane et al. 2017). Currently, most of the chemotherapeutic strategies used to treat different cancers are not completely effective and are often associated with severe side effects that can significantly reduce the quality of life of the patient. Thus, much research is being dedicated to finding novel compounds that show clinical efficacy with reduced side effects, with much of this research concentrated on natural products including those from marine algae (Anand et al. 2016). Bioactive compounds from brown seaweeds have been highlighted for their importance as anticancer agents (Ashwini et al. 2016) and most of these compounds are generally safe and are abundant in brown seaweeds, which make them a promising potential source of therapeutic compounds (Zubia et al. 2009). A wide range of these bioactive ingredients has been reported to have anti-tumour activity. Polyphenols have been linked with anticancer activities of seaweed crude extracts, for instance, phloroglucinol and dioxinodehydroeckol from brown algae display an antiproliferative activity against colon and breast tumours (Lopes-Costa et al. 2017; Murphy et al. 2014). Similarly, phloroglucinol from an *Ecklonia cava* extract showed apoptosis enhancing effects on the MCF-7 human breast cancer cell line (Kong et al. 2009). Furthermore, crude extracts from brown algae *Palmaria palmata*, *Laminaria setchellii* and *Macrocystis integrifolia* exhibited cytotoxicity against the cervical cancer HeLa cell line (Yuan and Walsh 2006). The anticancer or cytotoxic mechanism of polyphenols is still unclear; however, it has been

suggested that polyphenols may inhibit the formation of mitotic spindles by preventing normal microtubule formation, decreasing angiogenesis and cell adhesion and invasion (Olivares-Bañuelos et al. 2019; Zenthoefer et al. 2017; Wells et al. 2016).

Seaweed flavonoids such as flavones, catechins, chalcones, flavanols and isoflavonoids are well recognised to exhibit a potent anticancer activity. For example, flavonoids extracted in methanol, chloroform and ethyl acetate from the brown algae *Padina gymnospora*, and *Sargassum wightii* reduced the cell viability of the human cancer cell lines A549, HCT-15 and PC-3 (Murugan and Iyer 2013). One possible mechanism of flavonoids may be exerted by altering steroid hormone production through inhibition of aromatase, preventing the development of cancer cells (Sithranga et al. 2010).

Polysaccharides from seaweeds, such as sulphated laminarin, porphyran and fucoidan are naturally active as anticancer agents against human breast, gastric, liver, prostate, colon, lung and urinary bladder cancers and melanoma (Yamasaki-Miyamoto et al. 2009; Alekseyenko et al. 2007; Lowenthal and Fitton JH 2015). The suggested mechanism is that polysaccharides cause cell cycle arrest at the G0/G1 phase (Senthilkumar et al. 2013). A number of proteins have been isolated from seaweed such as lectins, glycoproteins and phycobiliproteins which are the most studied as bioactive anticancer compounds against human lymphoma MCL, colon cancer Colo201 cells and cervix cancer HeLa cell lines (Pangestuti and Kim 2015; Harnedy and FitzGerald 2011). They may be able to induce cell cycle arrest and inhibit the IGF-IR signalling pathway (Park et al. 2013).

Given the wide range of compounds that they contain and initial evidence on the activities of those compounds, brown algae are a promising source of novel pharmaceutical compounds. Among the brown seaweeds, the genus *Cystoseira* contains about 40 species distributed widely in the Atlantic Ocean and Mediterranean Sea (De Sousa et al. 2017). Crude extracts of different species have shown anti-tumour activity against a wide variety of cancer cell lines. Cystoseira spp. provide a good source of polyphenolic compounds (De Sousa et al. 2017), which are well known to show cytotoxic activities against tumour cells (Gutiérrez-Rodríguez et al. 2017). Polyphenols from these algae include those with unique structural features and exhibit

effective anticancer activities (Yong-Xin et al. 2011), for example, hexane fractions extracted from *Cystoseira crinita* and *C. stricta* showed 87% and 50% reductions in proliferation respectively in colorectal carcinoma (Caco2) cells after 72 h exposure with 200 μgmL^{-1} extracts and the antiproliferative effect correlated with their polyphenol and flavonoid contents (Alghazeer et al. 2016).

Several studies have demonstrated that the bioactivity properties of brown seaweeds vary seasonally (e.g. Stengel et al. 2011). These studies have indicated that changes in biotic and abiotic factors between seasons and locations affect the production of their bioactive compounds (Celis-Plá et al. 2016; Zatelli et al. 2018). However, as far as we are aware, while concentrations of secondary metabolites have been measured, no assessment of seasonal variations in cytotoxicity against cancer cell lines has been undertaken. Here, we investigate the seasonal variation of the chemical composition and the cytotoxic activity of extracts from *C. tamariscifolia* collected from south-west England. We show that extracts from *C. tamariscifolia* harvested in the winter and spring contained higher levels of secondary metabolites in accordance with previous work (Celis-Plá et al. 2016). Furthermore, we tested cytotoxicity of our extracts against leukaemia and prostate cancer cell lines and we show seasonal variation in the cytotoxic activity of four different solvent extracts against these cell lines. These cytotoxic activities can range from complete cell death to undetectable depending on the season and thus, we suggest that when considering collecting marine algae from temperate seas in order to search for bioactive compounds, a consideration of seasonality is made and samples are collected during different seasons to maximise the possibility of finding bioactive compounds.

Material and methods

Collection of *Cystoseira tamariscifolia*:

Cystoseira tamariscifolia was collected by hand from the shallow subtidal at Hannafore Point, Cornwall, UK (DD N 50.342234, DD W -4.453528） in mid-June (summer), mid-October (autumn), and early March (winter) and early May (spring). The collected samples were transferred to the laboratory in polythene bags kept in an icebox. On arrival, the samples were rinsed with distilled water, cleaned of adhering debris and epiphytes and then freeze-dried at −20^{o}C using Edwards super Modulyo freeze dryer.

Solvent extraction

Extracts were made in chloroform, methanol (100% and 70%) and water. Fifty grams of freeze-dried samples were mixed with solvents (1:10, w/v), and then homogenised for 2 min by using an IKA T10B Ultra-Turrax disperser at 24^{o}C. The extract was then stirred for 3 min, centrifuged (5,000 g, 10 min, room temperature [RT] and the supernatants recovered. The extracts were dried at 40^{o}C under vacuum. Solvents were used to re-suspend all extracts, and then the extracts were stored at −20^{o}C for biological activity screening (Vizetto-Duarte et al*.* 2016).

Total polyphenol content (TPC)

The Folin-Ciocalteu (F-C) colourimetric technique (Velioglu et al*.* 1998) was used to determine total phenolic concentrations (TPC). Briefly, 5 µL of the extracts at 0.1, 1 and 10 mg/mL were mixed with 100 µL of 10-fold diluted F–C reagent, incubated at RT for 5 min and mixed with 100 µL of sodium carbonate (75 g L^{-1}, w/v). Following a 90 min incubation period at RT, absorbance was measured at 725 nm on a microplate reader (Omega, BMG Labtech). The concentration of TPC was calculated as phloroglucinol equivalents (PGE) utilising a calibration curve prepared with phloroglucinol standard solutions and is expressed as mg PGE g^{-1} dry weight.

Total flavonoid content (TFC)

Flavonoid concentrations were quantified according to the method described by Ahn et al. (2007), with modifications. Briefly, 50 µL of 2% (w/v) aluminium chloride–ethanol solution was added to 50 µL of the extracts at 0.1, 1, 10 mg mL^{-1}. After one hour at RT,

the absorbance was measured at 420 nm on a microplate reader (Omega, BMG Labtech). Quercetin was used as a standard, and results are expressed as mg of quercetin equivalents (QE) g^{-1} dry weight.

Total polysaccharide content

Total polysaccharide concentrations were measured by the phenol-sulfuric acid method (Masuko et al. 2005). 50 µL of samples at the concentrations 0.1, 1 and 10 mg/mL was added to 150 µL of sulfuric acid (96% reagent grade). The mixture was incubated in a 96 well plate floating on a water bath at 90ºC for 5 min. 30 µL of 5% phenol was added to the mixture for another 5 min in the water bath. The plate was then floated on cold water for additional 5 min to cool and the absorbance was measured at 490 nm on a microplate reader (Omega, BMG Labtech). Glucose was used as a standard and results are expressed as mg of glucose equivalents (G) g^{-1} dry weight.

Total protein

The BCA assay was carried out to determine total protein concentrations using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) in a 96-well-plate format. The assay mixture contained 100 µL of the reagent and 100 µL of the sample; crude extracts at concentrations 0.1, 1 and 10 mg mL^{-1}. A standard curve with serial bovine serum Albumin BSA solutions (ranging from 0.1 µg mL^{-1} to 100 µg mL^{-1}) was used for calibration. The mixture was incubated at 37°C and absorbance was measured at 560 nm on a microplate reader (Omega, BMG Labtech).

Cell line maintenance and culture

The culturing of the human leukaemia cancer cell lines THP-1 and HL60 was as a suspension and the human prostate cancer cell line (PC3) was as a monolayer. Cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC), and divided every 3-4 d and were used regularly between passages 10-35. The cells were cultured in RPMI 1640 complete growth medium supplemented with 10% (v/v) fetal bovine serum and 2 mM L-glutamine and maintained at 37ºC, under 5% CO_2 in a humidified incubator.

Cell vitality assay

The cells were incubated at a density of 2 x10^5 cells/well in a 96-well microplate for 72 h at 37ºC with an equal volume of assay medium containing thiazolyl blue tetrazolium (2 mg mL^{-1} in PBS). The resulting formazan crystals were solubilised in 150 µL DMSO. The absorbance was read at 540 nm using a microplate reader (Omega, BMG Labtech). The IC_{50} values were calculated from a sigmoidal dose-response curve of the data generated in SigmaPlot v. 13.0.

Fluorescent staining for HL60 and THP-1 cells

THP-1 and HL60 were seeded at 10^6 cells/ml in 24-well culture plates and treated with 100% methanol extracts of *C. tamariscifolia* for 48 h. Cells were centrifuged and suspended in PBS. Propidium iodide (5 µg mL^{-1}in PBS; Sigma Aldrich) was used for staining cells for 5 min at room temperature in the dark and images were captured using a Nikon fluorescence microscope.

Flow Cytometry Analysis

Flow cytometry was performed to determine the apoptotic effect of *C. tamariscifolia* methanol extracts on human leukaemia cell lines HL 60 and THP-1. Cells were seeded at a density of 10^6 mL^{-1} and exposed to extracts at a concentration of 150 µg mL^{-1} before being incubated at 37°C in a 5% CO_2 incubator for 24h. Cells were washed in PBS and fixed in cold 70% ethanol for 1h. Cells were incubated in 50 µg mL^{-1} RNase and 50 µg mL^{-1} fluorescein diacetate (FITC; Sigma Aldrich) solution for 30 min before cells were analysed by Becton Dickinson FACSCalibur Flow Cytometer. All experiments were performed in three replicates.

Results

Effects of extraction method and season on the biochemical composition.

To assess the cytotoxic bioactivity of *Cystoseira tamariscifolia* extracts from the south-western coast of England, various extraction solvents were applied to material collected in all four seasons, and the concentrations of primary and secondary metabolites were measured (Table 1).

100% methanol extracts of C. *tamariscifolia* contained the highest yields of primary and secondary metabolites. Polyphenol content was found to be higher in 100% methanol extracts with a concentration of approximately 100 mg g^{-1} DW, while chloroform extracts showed the highest content of flavonoids with up to 45 mg g^{-1} DW. Polysaccharide content ranged from 10 and 45 mg g^{-1} DW in the water and 100% methanol extracts, respectively (Table 1).

The spring and summer extracts generally had higher concentrations of metabolites while in autumn we recorded the lowest concentration of total polyphenols and flavonoids. *C. tamariscifolia* crude extracts had low protein content compared with the previous bioactive compounds. The greatest concentration of protein was in the autumn in both 100% methanol and water extracts with concentrations of roughly 19 and 6 mg g^{-1} DW respectively, while the chloroform extract could not be used due to interference with the assay.

Extraction method and seasonality impact the anticancer activity of *C. tamariscifolia* extracts.

We were interested in seeing the effect of the extraction method and season on any potential cytotoxic activity as *Cystoseira* spp. extracts have been shown to contain various bioactive compounds, particularly polyphenols that show cytotoxicity against cancer cell lines. Three different cell lines, HL-60, THP-1 and PC3 cells, representing two leukaemia-derived lines and a prostate cancer line respectively, were treated with increasing concentrations of *C. tamariscifolia* extracts and cell vitality was assessed via the MTT assay. Cells were treated with extracts from all four seasons extracted via all four extraction regimes separately (Figures 1, 2 and 3) and IC_{50} values for cell vitality were calculated (Table 2).

The solvent used to make the extracts influenced the cytotoxic activity of the extracts. Generally, 100% methanol and chloroform extracts had the most cytotoxic effect on the three cell lines tested with some differences between them (Table 2). The 100% MeOH extract was approximately 17 times more cytotoxic in HL-60 compared to water extracts, while both 100% methanol and chloroform extracts had almost identical activity in HL60 and THP-1. Extracts made in water were the most cytotoxic in PC3 cells (Figure 2) with IC_{50} values approximately 2-5 fold higher than for the other solvents whereas water extracts were much less cytotoxic than the other solvents for HL-60 and THP-1 cells, being between approximately 2-130 fold less cytotoxic (Figures 1, 3).

In addition to the extraction method effect, there was a clear seasonality in the cytotoxic effects on the three different cell lines. Summer and spring extracts showed the most potent cytotoxic effect on THP-1 and HL-60 cells with approximately 8 times higher cytotoxicity compared to the autumn and winter extracts. There was, however, an exception in that winter extracts were also highly cytotoxic against the prostate cancer cells PC3.

HL-60 cells showed the greatest sensitivity to *C. tamariscifolia* extracts with a mean IC_{50} over all seasons and extracts of 80.61 ± 21.74 µg mL^{-1} while the similar THP-1 model showed a mean IC_{50} over all seasons and extracts of 199.78 ± 37.23 µg mL^{-1} and PC3, 162.15 ± 36.11 µg mL^{-1}. The highest cytotoxic activity was seen in the summer extracts in 100% and 70% MeOH and 100% chloroform on HL-60 cells (IC_{50} values; 2.32 ± 0.21, 7.34 ± 0.30 and 7.92 ± 0.12 µg mL^{-1} respectively; Figure 1). These values show that the cytotoxic activity of these crude extracts is very high against HL-60 cells and is between approximately 17 and 62 fold higher than the corresponding effects on THP-1 and PC3 cells, respectively.

Methanol extracts of *C. tamariscifolia* induce apoptosis in HL60 and THP-1 Cell lines

To investigate whether the cytotoxicity activity of *C. tamariscifolia* extracts is related to the induction of apoptosis, HL60 and THP-1 cells were exposed to a concentration of 150 mgmL^{-1} of methanol extracts of *C. tamariscifolia* for 24 h and nuclear morphological changes of HL60 and THP-1 cells were observed using propidium

iodide staining (Fig. 4). Compared with the normal nuclear morphology of the control cells, the cells treated with *C. tamariscifolia* extracts presented typical morphological characteristics of apoptosis, including nuclear fragmentation. Additional confirmation of apoptosis induced by methanol extracts of *C. tamariscifolia* was performed by flow cytometry-based on fluorescent diacetate (FITC) staining as shown in figure 5. Control cells that had not been treated with extract displayed a largely homogeneous population with >99% of cells being vital. Upon treatment with the extract, this dropped to 36.28 and 17.54% in HL-60 and THP-1 cells respectively and an increase in cells in both necrosis and late apoptosis was observed with c. 50% of cells in late apoptosis in both lines confirming the data from the cell vitality assay.

Discussion

In temperate seas, one would expect that seasonal differences would have a large influence on the concentrations of likely bioactive compounds and thus bioactivity of those extracts. Surprisingly, little work has been performed on these links. Here we have investigated the effects of season and extraction method on primary/secondary metabolite concentrations in extracts and their cytotoxic activity against three cancer cell lines.

Methanol (100%) was generally the most effective solvent, extracting the highest levels of the four metabolite classes, although all four solvents gave detectable levels of all the investigated metabolites, the exception being chloroform that interfered with the BCA assay for proteins. Concentrations of polyphenols and flavonoids were high, a result in accordance with previous research showing brown algae to be good sources of these two metabolite classes (Thomas et al. 2011; Alghazeer et al. 2016). A similar pattern of results was obtained by Mhadhebi et al. (2011) and Yegdaneh et al. (2016) who also concluded that methanol and chloroform extracts contain high concentrations of metabolites, especially polyphenols. This result can be explained by the difference in secondary metabolite polarity. For example, the high variation in the structures and both hydrophilic and hydrophobic parts of polyphenols (Li et al. 2011) allow them to be extracted typically in polar solvents including methanol and water but some can also be extracted in low polarity solvents such as chloroform (Airanthi et al. 2011; Vizetto-Duarte et al. 2016), which may explain the high levels of polyphenols in chloroform extracts of *Cystoseira*. Protein represented the least common of the four classes of metabolite as might be expected in brown algae.

As might be expected, there was a clear seasonality to the levels of the metabolites although this varied between compound and extraction method (Celis-Plá et al. 2016; Rickert et al. 2016; Cikoš et al. 2018). *C. tamariscifolia* begins to grow in late winter with most growth in the spring and summer before stopping in autumn. Polyphenols were highest in each extraction method in spring and summer except for chloroform extracts from winter. This is consistent with observations by Abdala-Díaz et al. (2006), who showed that polyphenol concentration in the tissue of *C. tamariscifolia* (in Spain from June 1988 to July 2000) ranged from 2% in the winter to 8% in summer. Polyphenols from species of the brown algal order Dictyotales, Fucales and

Laminariales, have been correlated with the tissue age and stage of the life cycle and there were significantly lower levels in new branches in late winter than older, bigger branches in summer (Denton et al. 1990; Mannino et al. 2014). For flavonoids, polysaccharides and protein, the picture was more complex with winter and autumn often offering at least one higher value than in spring/autumn. The complexity of the variation of flavonoid, polysaccharide and protein levels extracted from *C. tamariscifolia* has been highlighted in previous studies. For instance, a study by Abdala-Díaz et al. (2006) found that spring and winter extracts of *C. tamariscifolia* showed the highest concentration of flavonoids, however, contradictory results were reported by Ramah et al. (2014) and Mannino et al. (2014) who showed that the flavonoid peak occurred in summer. During the year, the highest levels of polysaccharides in *C. tamariscifolia* were observed in summer, supporting the observations of Teas et al. (2013) and Hurtado and Critchley (2018). These authors noted that *Undaria pinnatifida*, *Ascophyllum nodosum* and *Fucus vesiculosus* showed the maximum levels of polysaccharides in summer. However, other results by Skriptsova (2016) revealed that *Saccharina japonica* and *Sargassum pallidum* showed maximum polysaccharide content in autumn. The protein content in *C. tamariscifolia* varied from 3.28 ± 0.36 to 19.57±1.42 mg g^{-1} dry weight, and it differs from season to season and according to the extraction method. In general, the highest protein values in *C. tamariscifolia* were found in autumn and spring, while the lowest were found winter and summer. Similar results were reported by Kim et al. (2011) and Pangestuti and Kim (2015), who suggested that minimum protein concentration in summer could be linked with protein destruction.

Given the temperate nature of the collection site, autumn and winter offer lower water temperatures, fewer hours of sunlight and increased wave action that will lead to decreased growth and investment into primary and secondary metabolites (Fleurence and Levine 2016). This may also coincide with decreased pressures of herbivory during these seasons and less requirement for secondary metabolites (Duffy and Hay 1990). We found that new growth of *C. tamariscifolia* was already clearly apparent in early March (winter) with many fresh new phosphorescent fronds present presumably ready for the increased sunlight hours of spring and summer. Conversely, in October (autumn) most of the thalli were showing early signs of senescence where the main

axis and the primary laterals had elongated while the branches were relatively short and often covered in epiphytes. Despite this, autumn algae still contained high levels of some metabolites although polyphenol levels were generally lowest in autumn, but this depended on the extraction method. One explanation of this result could be attributed to the increase in the need for the secondary metabolites as a protection mechanism against increased herbivory in summer (Duffy and Hay 1990; Jormalainen and Honkanen 2008). Another reason for the increase in secondary metabolites in summer may be due to the photoprotective role against the high radiation dosages in summer daylight (Connan et al. 2004). They also contribute to protection against oxidative stress, bacterial infection and epiphytes and perform a role in algal reproduction which all increase in summer (Plouguerné et al. 2006; Ferreres et al. 2012; Jennings and Steinberg 1997; Thomas et al. 2011).

In line with Duarte (2016), who demonstrated that a hexane extract of *C. tamariscifolia* had a high level of cytotoxicity against AGS, HCT-15 and HepG2 cell lines with IC_{50} values of 32.36, 23.59 and 13.15 μg mL^{-1} respectively, we showed that our extracts possess cytotoxic activity against cancer cell lines. In particular, our extracts were considerably more cytotoxic against HL-60 cells and our evidence suggests that cell death is induced via apoptosis. There is a large seasonal variation not only in the levels of primary and secondary metabolites but also a significant seasonal variation in their cytotoxic effects. In particular, our results clearly underline the need to test extracts from different seasons and using different solvent extraction protocols. Of particular interest, our data suggest that generalisations cannot be drawn with respect to season or extraction method and that there is no best season or extraction method when results are compared between cell types. For example, the difference between the effect of the summer extracts in organic solvents for HL-60 cells and the aqueous winter extracts on PC3 cells highlights this difference. Indeed, we carried out statistical analyses to examine any potential interactions between metabolite composition and the cytotoxicity of the extracts with season and extraction method (data not shown). There was no clear interaction that could link the cytotoxic effects to any metabolite group which might also suggest that various bioactive compounds may be present in the extracts and that their relative amounts may vary with both season and extraction method. It would be tempting to assume that summer (or an early or midpoint of the

growing season) might be the best time to collect material, but our results show that this is not necessarily the case in all cell lines or extracts. We have not fully fractionated our extracts to isolate the specific compound(s), but it is likely that different compounds are responsible for the observed effects and that these change levels with season. Equally, we cannot discount that there may be synergistic interactions between compounds that only become apparent in different seasons. We believe that it is possible that many promising extracts potentially containing novel cytotoxic/chemotherapeutic agents may have been discarded in the past where samples were taken in a single season, extracted using a single solvent or tested using a single cell line.

We suggest that, where possible, samples are taken at multiple times of the year that represent different phases in the growth or annual life cycle of the plant/species in question. Given that many of the bioactive compounds with potential medical use are probably produced in response to some type of biotic or abiotic stress, it may also be good practice to include the presence of any obvious stressors (seasonal temperature, irradiance, herbivores, for example) into any consideration of sampling times so as to maximize the possibility of finding novel compounds.

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Table 1: Metabolite concentrations in *Cystoseira tamariscifolia* as a function of season and extraction solvent. DW: dry weight. Total polyphenol Content: milligram phloroglucinol acid equivalents per gram dry weight; Total flavonoid Content: milligram Quercetin equivalents per gram dry weight. Total polysaccharide: milligram Glucose equivalents per gram dry weight; Total protein: milligram Bovine Serum Albumin equivalents per gram dry weight. Values are presented as mean ± SD (n = 3).

Metabolite (mg g^{-1} DW)	Season	Extraction solvent			
		100%MeOH	70%MeOH	Water	Chloroform
Total polyphenol content	Summer	102.23±1.85	57.70±2.06	83.24±1.03	41.99±0.90
	Autumn	71.27±4.16	9.67±1.27	20.61±3.07	7.81±0.31
	Winter	71.16±4.40	31.20±1.19	14.62±0.91	48.89±1.91
	Spring	85.46±2.7	62.35±1.86	46.29±0.28	68.75±2.79
Total flavonoid content	Summer	22.87±0.80	5.55±0.46	8.56±0.71	22.27±0.66
	Autumn	27.86±1.20	3.26±0.27	5.99±0.99	16.69±0.52
	Winter	35.23±1.03	5.66±0.91	4.89±0.28	45.19±2.12
	Spring	25.54±0.5	6.69±0.97	4.80±0.12	49.21±4.83
Total polysaccharide content	Summer	48.84±3.66	8.16±0.40	14.95±1.85	42.84±3.84
	Autumn	31.10±5.80	11.19±0.96	10.25±1.58	27.81±0.71
	Winter	18.04±2.96	3.92±0.33	3.12±069	25.78±8.74
	Spring	39.11±1.46	19.30±2.26	16.02±040	26.75±0.29
Total protein content	Summer	9.28±0.28	8.10±0.59	7.18±0.63	-
	Autumn	19.57±1.42	3.31±0.81	13.51±1.08	-
	Winter	9.95±0.36	5.95±0.31	3.28±0.36	-
	Spring	11.17±0.12	5.36±0.43	3.7±0.07	-

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Table 2: IC_{50} values (µg mL^{-1}) for extracts on HL60, PC3 and THP-1 cell lines. Cells were exposed for 72 h to crude extracts of the brown alga *Cystoseira tamariscifolia* collected in all seasons. IC_{50} values were calculated by sigmoidal dose-response of the data using SigmaPlot v. 13.0.

Season	cells	70%MeOH	100%MeOH	Chloroform	Water
Summer	HL60	7.33 ± 0.30	2.32 ± 0.21	7.9170 ± 0.12	293.54 ± 25
	PC3	452.01 ± 20	40.09 ± 3.40	112.28 ± 11	24.88 ± 0.21
	THP-1	116.48 ± 15	134.68 ± 24	60.35 ± 0.98	64.72 ± 6.2
Autumn	HL60	110.17 ± 9.6	110.43 ± 9.8	46.25 ± 39	221.51 ± 19
	PC3	469.76 ± 31	64.72 ± 5.3	263.59 ± 24	50.42 ± 5.6
	THP-1	236.74 ± 24	262.62 ± 21	396.04 ± 54	24.62 ± 4.1
Winter	HL60	39.37 ± 3.2	74.86 ± 0.51	24.63 ± 0.13	149.93 ± 0.20
	PC3	168.27 ± 15	105.60 ± 12	149.88 ± 0.41	38.69 ± 2.7
	THP-1	196.66 ± 22	342.39 ± 22	467.11 ± 27	444.29 ± 45
Spring	HL60	24.29 ± 0.27	24.28 ± 0.20	24.46 ± 0.19	128.38 ± 17
	PC3	190.75 ± 19	110.83 ± 15	99.35 ± 8.7	228.39 ± 22
	THP-1	74.98 ± 0.45	24.80 ± 0.31	74.84 ± 0.30	169.13 ± 16

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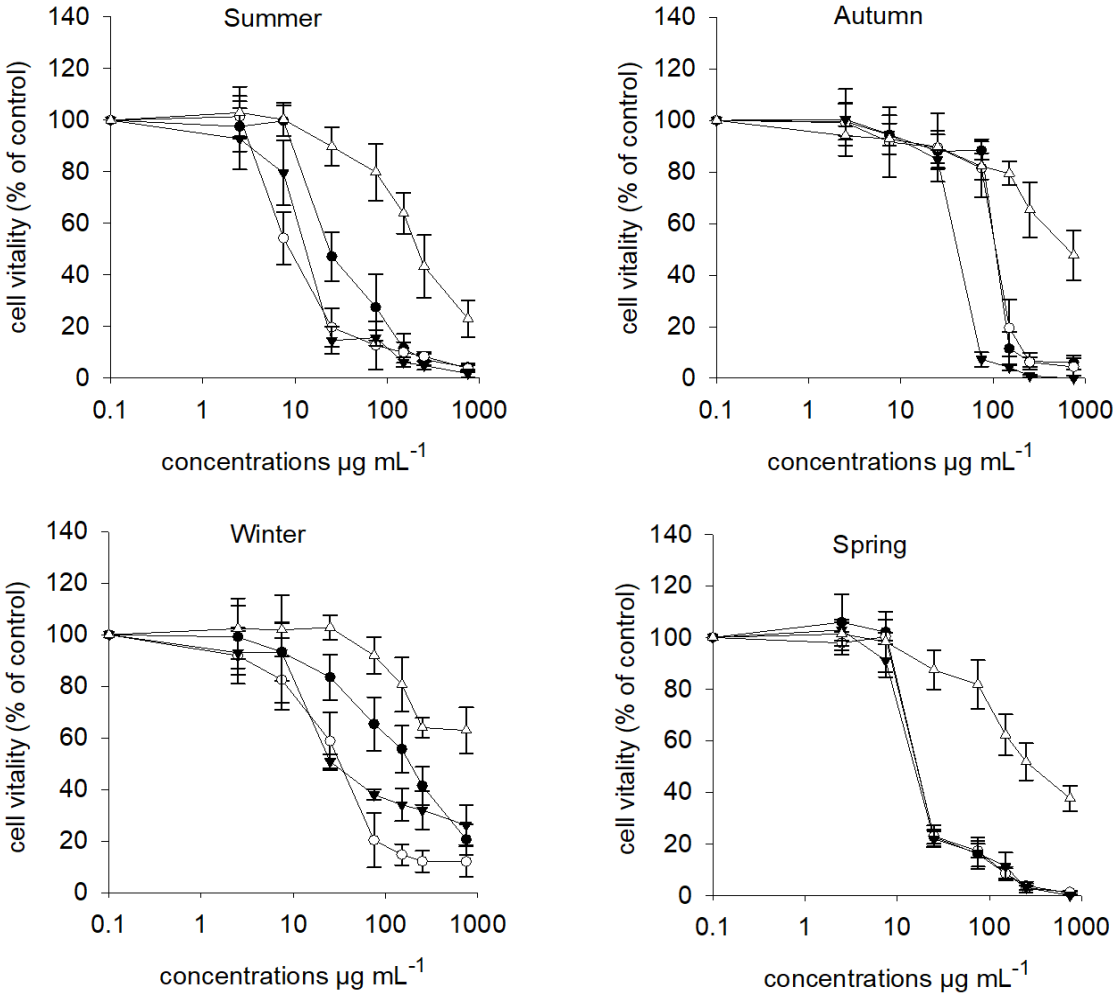


Figure 1: Dose-response curves for cell vitality in HL-60 cells. HL60 cells were exposed for 72 h to crude extracts of the brown alga *Cystoseira tamariscifolia* collected in all seasons. Black circles - 70% MeOH, white circles - 100% MeOH, chloroform - black triangles, white triangles - water. (mean ± SD; n = 9).

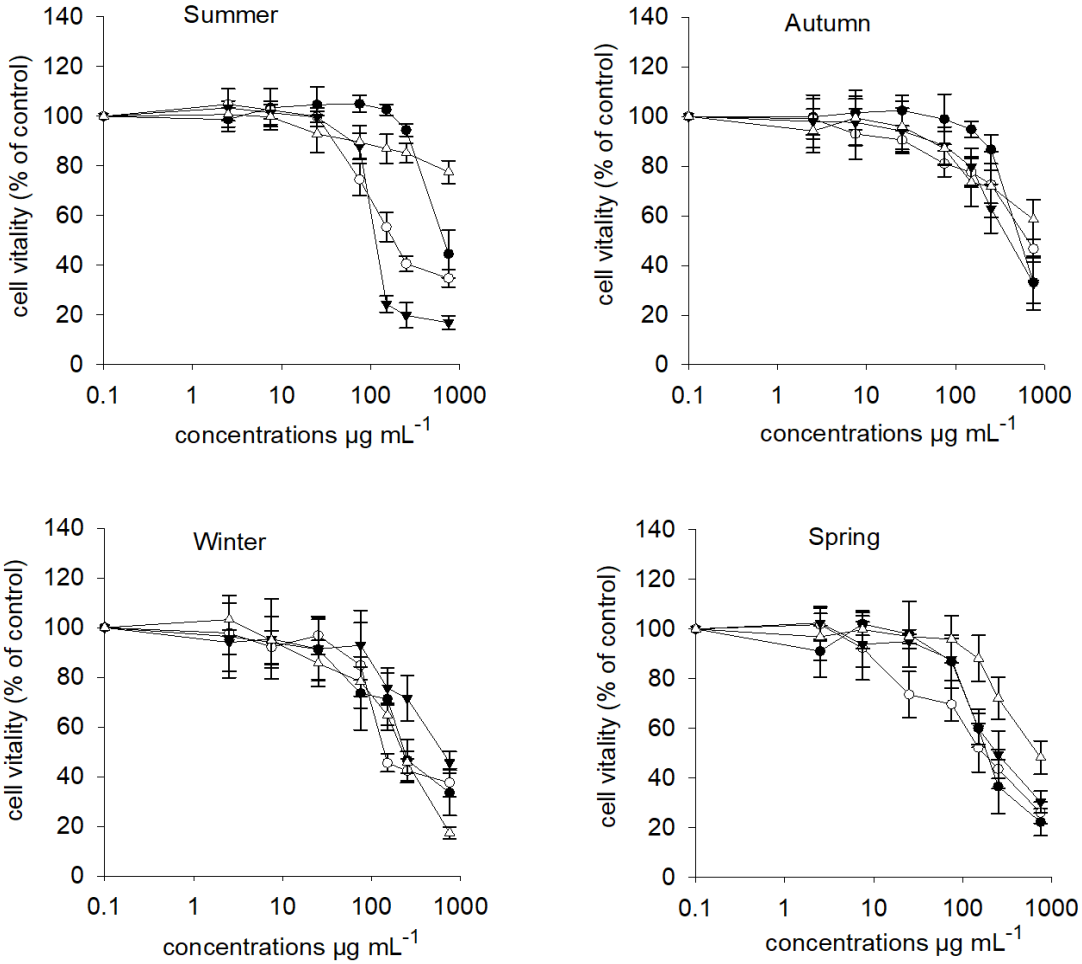


Figure 2: Dose-response curves for cell vitality in PC3 cells. PC3 cells were exposed for 72 h to crude extracts of the brown alga *Cystoseira tamariscifolia* collected in all seasons. Black circles - 70% MeOH, white circles - 100% MeOH, chloroform - black triangles, white triangles - water. (mean ± SD; n = 9).

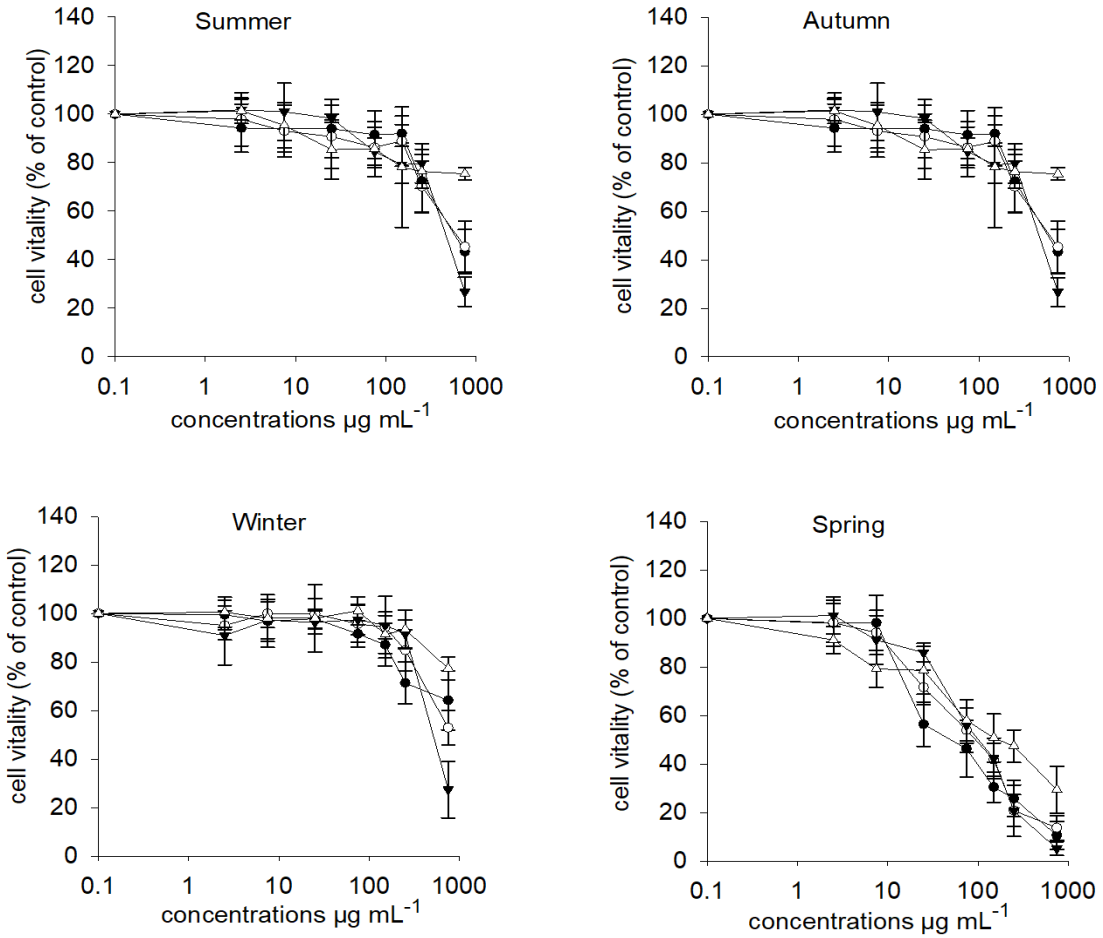
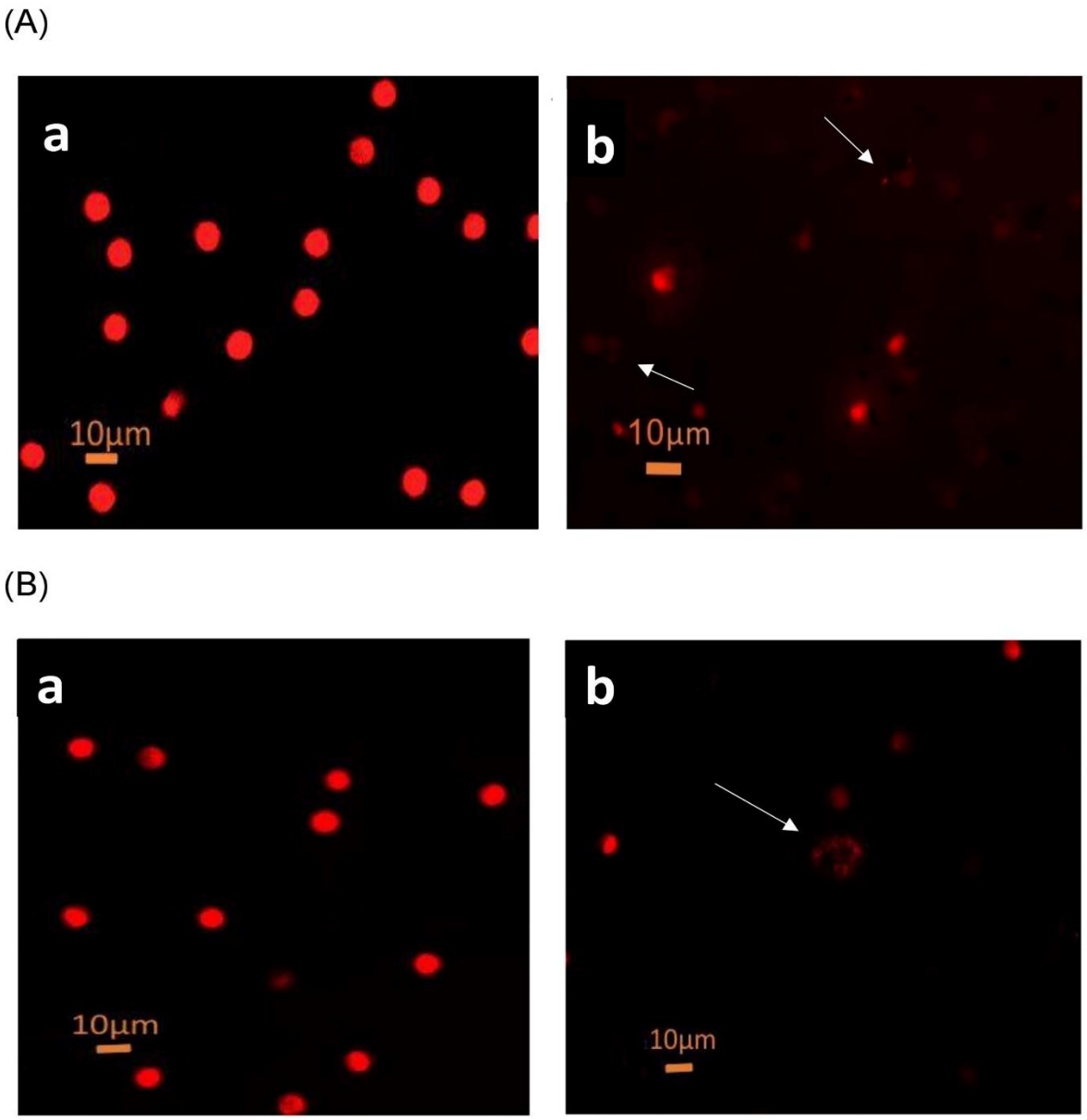


Figure 3: Dose-response curves for cell vitality in THP-1 cells. THP-1 cells were exposed for 72 h to crude extracts of the brown alga *Cystoseira tamariscifolia* collected in all seasons. Black circles, 70% MeOH, white circles, 100% MeOH, chloroform black triangles, white triangles water. (mean ± SD; n = 9).



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563 Figure 4: Fluorescent staining of cancer cell nuclei with propidium iodide. (A) HL60
564 and (B) THP-1 were treated with 100% methanol summer extracts at 150 µg mL^{-1} for
565 36h. Fragmented nuclei and apoptotic bodies were seen in the *C tamariscifolia* extract-
566 treated cells (b), but not in the control treatment (a). Magnification 200×, images
567 representative of three independent experiments.
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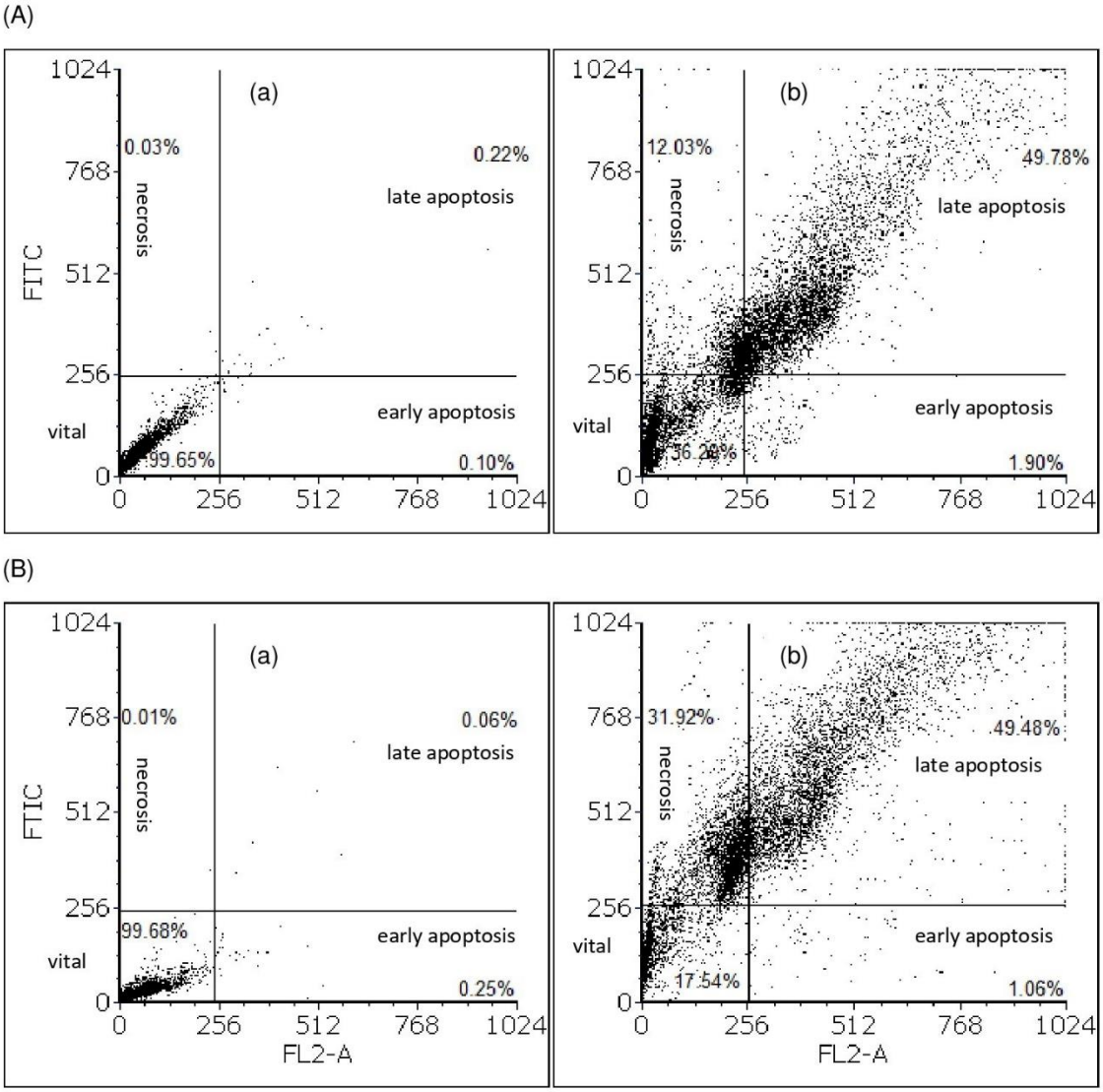

Figure 5: The effect of *C. tamariscifolia* methanol extracts on the human leukaemia cell lines HL 60 (A) and THP-1 (B). HL 60 and THP-1 cells were resuspended in PBS and then RNase, and fluorescein diacetate (FITC) were added for 30 minutes, and cells were analysed by flow cytometry. Control cells (a) received no drug treatments. (b) Extract-treated HL60 and THP-1 cells. Early, late apoptosis and necrosis were found in treated cells but not in control. Experiments were carried out in three replicates.