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An Unfractionated Fucoidan from *Ascophyllum nodosum*: Extraction, Characterization, and Apoptotic Effects in Vitro

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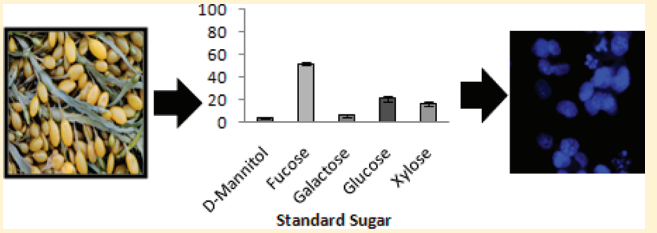
Supporting Information

ABSTRACT: An unfractionated fucoidan was extracted from the brown alga *Ascophyllum nodosum*. Extraction of fucoidan from seaweed was carried out using an innovative low-chemical process. A combinational approach involving compositional analysis, HPAEC, IR analysis, GPC, and NMR was employed to elucidate the composition and structure of an unfractionated fucoidan from *A. nodosum*. This fucoidan is composed mainly of fucose (52.1%), and also galactose (6.1%), glucose (21.3%), and xylose (16.5%). Sulfate content was determined to be 19%. GPC data indicated a polydisperse fucoidan containing two main size fractions (47 and 420 kDa). NMR analyses revealed a fucoidan displaying broad, complex signals as expected for such a high molecular weight and heterogeneous polymer with resonances consistent with a fucoidan isolated previously from *A. nodosum*. The effects of fucoidan on the apoptosis of human colon carcinoma cells and fucoidan-mediated signaling pathways were also investigated. Fucoidan decreased cell viability and induced apoptosis of HCT116 colon carcinoma cells. Fucoidan treatment of HCT116 cells induced activation of caspases-9 and -3 and the cleavage of PARP, led to apoptotic morphological changes, and altered mitochondrial membrane permeability. These results detail the structure and biological activity of an unfractionated fucoidan from *A. nodosum*.



Seaweed polysaccharides are highly active natural substances having valuable applications,[1] and brown algae represent a rich and renewable source of key polysaccharides of structural and biological interest.[2] One such seaweed polysaccharide, fucoidan, has been the subject of much interest in recent years. These polysaccharides have been extracted from several species of marine algae and also from some marine echinoderms such as the sea urchin and sea cucumber.[3] Since its initial extraction from seaweed almost 100 years ago,[4] fucoidan has ignited much interest as a polysaccharide with assorted biological activities. Besides their well-demonstrated anticoagulant and antithrombotic activities, fucoidans have anti-inflammatory effects and antiproliferative and antiadhesive effect on cells, can protect cells from viral infection, and can interfere with mechanisms involved in marine echinoderm fertilization.[5,6] Marine algal fucoidans are typically heterogeneous in structure. In contrast to the "fucan"-type polysaccharides identified in marine invertebrates, which are largely sulfated L-fucose polymers, marine algal fucoidans generally contain 20−60% L-fucose and various amounts of other hexose and pentose sugars, as well as acidic sugars.[7,8] What has emerged from research to date is the knowledge that a single algal species can contain more than one type of "fucoidan" polymer in terms of molecular size and composition.[7,9−12]

Despite being known for the last century, the precise structures of marine algal fucoidans remain somewhat of a mystery, and to date, there appears to be little defined structural regularity. Many factors such as geographic location, environmental conditions, and harvest season can influence the polysaccharide content of seaweed,[13,14] and this hampers efforts to elucidate definitive structures for fucoidans. In addition, the method of extraction can affect the monosaccharide composition, sulfation patterns, fine structure, and size.[13] Despite this, fucoidans can be broadly classified as a family of sulfated homo- and heteropolysaccharides composed mainly of α-(1→2)- and/or α-(1→3)-L-fucose residues.[15,16] Structural studies on fucoidans from selected species of the Fucales have revealed a potential repeating unit of alternating α-(1→3)-L-fucose-2-sulfate and α-(1→4)-L-fucose-2,3-disulfate, which can be partially sulfated at C4 of α-(1→3)-L-fucose units (fucoidans from some species of Fucales may be partially acetylated at C4).[17−19] Other than fucose and sulfate, fucoidans also contain various monosaccharides such as mannose, galactose, glucose, and xylose and may even contain uronic acids, acetyl groups, and some protein.[20]

In the context of potential pharmacological applications, studies have indicated that size, composition, sulfation, and structure can all have an impact on functional properties and bioactivity. In healthy cells, there is a delicate balance between normal and unregulated growth. However, cancer cells possess defects in the regulatory circuits that govern cell proliferation and homeostasis.[21] Two of these specific alterations are the ability of cancer cells to evade programmed cell death (apoptosis) while being able to proliferate infinitely. Several marine algal polysaccharides, fucoidans in particular, have been found to induce

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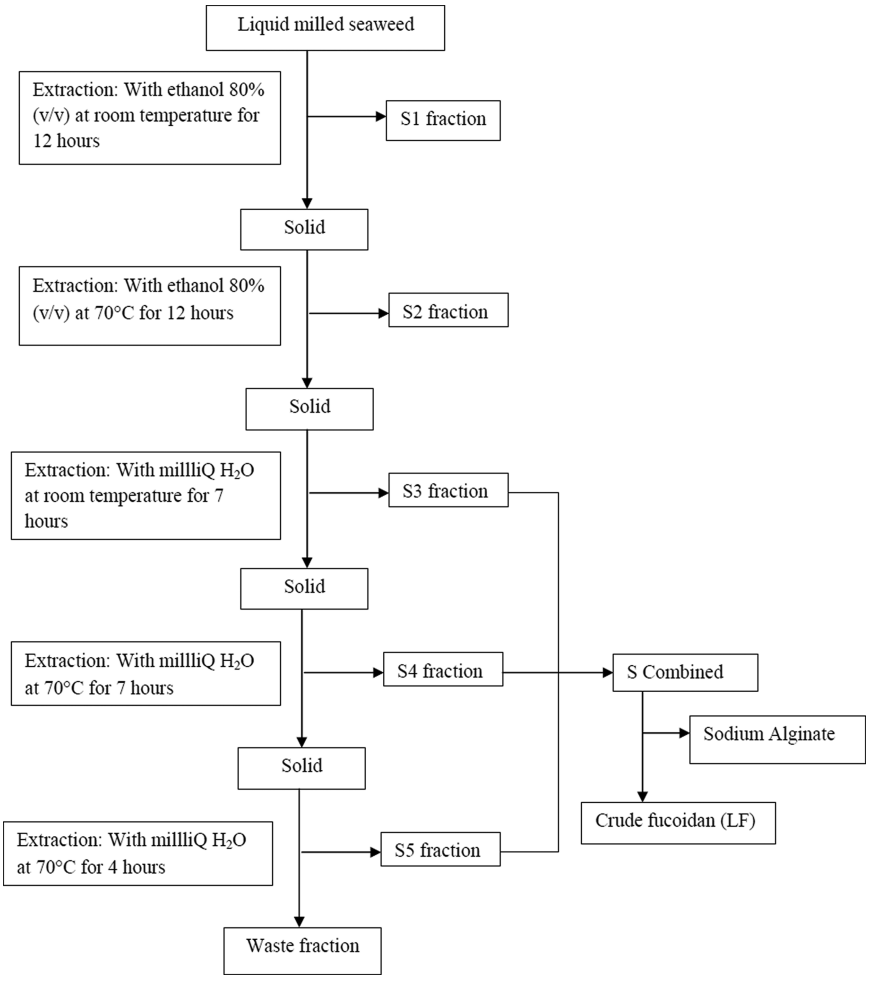

Figure 1. Extraction of polysaccharides with selective solvents (EtOH and millliQ H_2O).

apoptosis in cancer cells. The transformation of colorectal epithelium to carcinoma, in particular, is associated with a progressive inhibition of apoptosis.[22] The inhibition of apoptosis in colorectal cancer contributes to tumor growth, promotes neoplastic progression, and confers resistance to cytotoxic anticancer agents.[23] Apoptosis occurs via two principal pathways termed the mitochondrial-mediated (intrinsic) and death receptor-mediated (extrinsic) pathways. The extrinsic pathway mediates apoptosis in response to Fas ligand (FasL) and Apo2L/TRAIL (Apo2 ligand or tumor necrosis factor (TNF)-related apoptosis-inducing ligand), which signal, respectively, through the death domain (DD)-containing receptors Fas or death receptor (DR) 4 and DR5. These receptors use the adaptor molecule Fas-associated DD (FADD) to recruit the initiator proteases caspase-8 and -10 into a death-inducing signaling complex (DISC), where they are activated.[24] The intrinsic pathway can be activated by various intracellular stresses such as oxidative damage, drug treatment, or DNA damage, leading to the release of cytochrome *c* from the mitochondria to the cytoplasm. The intrinsic pathway is centered on the balance of activity between pro- and antiapoptotic members of the BCL-2 superfamily of proteins, which act to regulate the permeability of the mitochondrial membrane.[25] The two main apoptotic pathways converge on caspase-3 and subsequently on other proteases and nucleases that drive the terminal events of apoptosis.

In this study, we report on a stepwise, low-chemical approach to isolate a pure, native fucoidan from *Ascophyllum nodosum* and present a detailed analysis of the chemical composition and characteristics of this fucoidan. We also report on the ability of *A. nodosum* fucoidan to inhibit the growth of colon cancer cells and determine if apoptosis induction is a mechanism relevant to this effect.

RESULTS AND DISCUSSION

A native fucoidan was isolated from the marine alga *A. nodosum,* harvested in September 2006 from the west coast of Ireland, using a low-chemical approach (Figure 1), at a yield of 1.75% dry weight [3.3% (w/w) from wet seaweed]. As shown in Table 1, this yield is similar to values reported for other species of Fucales and for other varieties of brown seaweeds.[12,26] Selection of this harvesting time-point was based on information from previous studies[27,28] in which fucoidan levels in brown algae (selected Fucales and Laminariales) harvested in the Northern hemisphere were highest toward the end of the main photosynthetic period (i.e., end of summer). The fucoidan preparation was analyzed in detail to determine the total carbohydrate content as well as the presence of residual mannitol, polyphenols (or

Table 1. Yields of Fucoidan (% dry weight of algal biomass) Reported for Marine Brown Macroalgae Belonging to the Order Fucales

algal species	.	reference
Ascophyllum nodosum	1.75[a]	this work
	6.0−8.0	64
	4.0−10.0	65
	11.0	66
	1.25	11
Fucus distichus	0.26−7.05	19
F. evanescens	4.8−12.0	28, 67
F. serratus L.	0.42−7.16	10
F. spiralis	9.0−11.0	64
F. vesiculosus	3.4	26
Hizikia fusiformis	2.7	34
Pelvetia canaliculata	20.0	64
Sargassum horneri	5.17	68
S. polycystum	2.74	69
S. stenophyllum	4.9	70
S. tenerrimum	2.19	71

[a] 3.3% wet weight.

phlorotannins), and protein. The results obtained showed that the fucoidan contained 65.4% carbohydrate, 3.5% free mannitol, 13.5% residual polyphenol, and 18.5% protein by weight. The total carbohydrate levels agree well with, and in some cases are higher than, values reported to date for this parameter including values reported for commercial fucoidan. Few reports exist on the residual protein or polyphenol levels in fucoidans isolated to date. However, protein has been reported in fucoidan preparations isolated from other brown algae, as well as in commercial fucoidan.[22,26,29,30] Values reported for this *A. nodosum* fucoidan preparation are similar to those reported previously by Zvyaginsteva and co-workers[28] and Duarte and colleagues[31] for species of *Fucus* and *Sargassum*, respectively.

Carbohydrate analysis of the fucoidan, by high-performance anion exchange chromatography (HPAEC), showed it to be composed of significant proportions of L-fucose (52.1%) and smaller percentages of glucose (21.3%), galactose (6.1%), and xylose (16.5%). The HPAEC profile and relative abundance of the monosaccharides present in fucoidan from *A. nodosum* are shown in Figure 2. The results concur with fucoidan being composed mainly of L-fucose. Commercial fucoidan (Sigma Aldrich), from *F. vesiculosus*, has been reported to contain fucose, galactose, glucose, mannose, xylose, uronic acid, glucosamine, and sulfate.[7] However, the presence of rhamnose, mannose, arabinose, or uronic acids was not detected in the *A. nodosum* fucoidan reported in this study, in contrast to fucoidans reported from *A. nodosum* and other algal sources (Tables 2 and 3). In addition, total sulfate estimation by the $BaCl_2$-gelatin method[32] was found to be 19% for this fucoidan. The percentage sulfate is also in keeping with other published data (Tables 2 and 3). Early work by Percival and Ross showed 31.7% sulfation for a *Fucus vesiculosus* fucoidan.[33] A report in 1990 analyzed the chemical composition of a number of fucoidans from various sources and found the *F. vesiculosus* fucoidan to contain 14−39% sulfate.[29] The molar ratio of fucose to sulfate (Fuc:SO_4^{2-}) for the *A. nodosum* fucoidan isolated in this study was 1.0:0.466 (Table 3). Comparison of the total sugar and sulfate contents of the *A. nodosum* fucoidan with other fucoidans from this algal species is presented in Table 2. Altogether, the structural analysis of the fucoidan purified from the brown alga *A. nodosum* shows that it is chemically heterogeneous with respect to monosaccharide composition. The molar ratio of Fuc:Gal:Glc:Xyl:Sulfate (SO_4^{2-}) in this polysaccharide is 1.0:0.107:0.373:0.346:0.466. The sugar and sulfate compositions of high molecular weight fucoidans isolated to date from brown algae, including the fucoidan isolated from *A. nodosum* in this work, are compared in Table 3.

The monosaccharide composition indicated by HPAEC revealed a relatively high glucose content. From a preliminary investigation by thin layer chromatography (TLC, data not shown), glucose was found to be a component of this fucoidan, and while this was confirmed by the HPAEC analysis, the relative concentration present was considered significant. Glucose was reported to be present in fucoidans previously,[7,34] and while glucose has been reported in a fucoidan from *A. nodosum* previously,[11] reported levels were lower than observed here.

Gel permeation chromatography (GPC) confirmed the extraction of a high molecular weight fucoidan from *A. nodosum*. The molecular weight profiles (data not shown) determined by GPC suggest that *A. nodosum* fucoidan may consist of two molecular size fractions, one very large, approximately 420 kDa, and one smaller, with a peak molecular weight of around 47 kDa. The polydispersity of the fucoidan was 4.6, reflecting the broad range of different size species in this sample. The higher molecular weight must be regarded as a rough guide only, as no sulfated polysaccharide calibrants were available to cover such high molecular weights, and the value given is based on an extrapolation of the calibration curve.[35] Molecular weight determination of fucoidans can be difficult due to their heterogeneous compositions.[36] In previous reports, the average molecular weight of fucoidan from *F. vesiculosus* (Sigma) was found to vary between 100 and 217 kDa by GPC.[37,38]

Further analytical data on the isolated *A. nodosum* fucoidan were provided by infrared analysis (Figure S1). The IR spectrum showed typical absorption bands of fucoidans. The fucoidan contained a hydroxy group band at 3371 cm^{-1} as well as a carbonyl band at 1627 cm^{-1}.[16,39] No distinct bands were seen in the fucoidan sample at 1720 cm^{-1}, which would have indicated the presence of *O*-acetyl groups.[40] Absorption at 840 cm^{-1} was reported in a previous study and was suggested to be due to sulfate groups at the axial C-4 position.[41] Sulfate groups at the equatorial C-2 and C-3 positions were reported to give a small shoulder of absorption at 820 cm^{-1}, and there is such a shoulder in the fucoidan extract at 818 cm^{-1}. Small disparities in the IR spectra obtained in different reports can be due to a number of factors including sample handling and the extraction processes used. While IR spectroscopy confirmed key signature features of fucoidans in the *A. nodosum* isolated, comparative analysis of IR spectra obtained in this work for commercial fucoidan and the fucoidan extract clearly supports the identification of the *A. nosodum* polysaccharide as a fucoidan (Figure S1).[17] Since the fucoidan material was harvested at a different location and possibly in a different season from other fucoidans from *A. nodosum*, and a low-chemical extraction approach was used here, in comparison to more chemically intensive approaches used in other reports, the chemical composition is also likely to vary for each particular fucoidan isolated. Fucoidans from *A. nodosum* are characterized by their heterogeneous and branched structures.[17,42]

Structural analysis of the high molecular weight fucoidan extract was conducted using 1D NMR. The 500 MHz ^{1}H NMR spectrum exhibited broad, complex signals as expected for such a high molecular weight and heterogeneous polymer (Figure 2C). For this reason the structure could not be deduced from a 2D NMR study, but the spectrum was similar to that of an *A. nodosum* fucoidan reported some time ago.[43] Spectra of the algal fucoidans are usually complex, and the resonances broadened, indicating heterogeneity and possible branching.[44] An NMR study of a low molecular weight fragment of *A. nodosum* fucoidan revealed that its structure was that of a repeating disaccharide [→3)-α-L-Fuc(2SO_3-)-(1→4)-α-L-Fuc(2,3diSO_3-)-(1→],[45] and resonances in the spectrum of our sample (and in a 2D TOCSY spectrum, not shown) are fully consistent with the presence of this structure (Figure 2C). In addition, strong signals and TOCSY cross-peaks linking anomeric (H-1) signals between 4.5 and 5.0 ppm with ring proton signals between 4.0 and 3.0 ppm indicate the presence of unsulfated sugars; the same group of signals is consistent with the presence of glucose and/or galactose in the *A. nodosum* fucoidan isolated in this work.[44] The well-resolved H-6 methyl signal at 1.2 ppm indicates the presence of a high proportion of fucose in a different environment from the disaccharide shown above. This work reports the extraction of a polydisperse, high molecular weight fucoidan composed mainly of L-fucose, but also containing significant proportions of glucose. This fucoidan was difficult to characterize by GPC and NMR, but

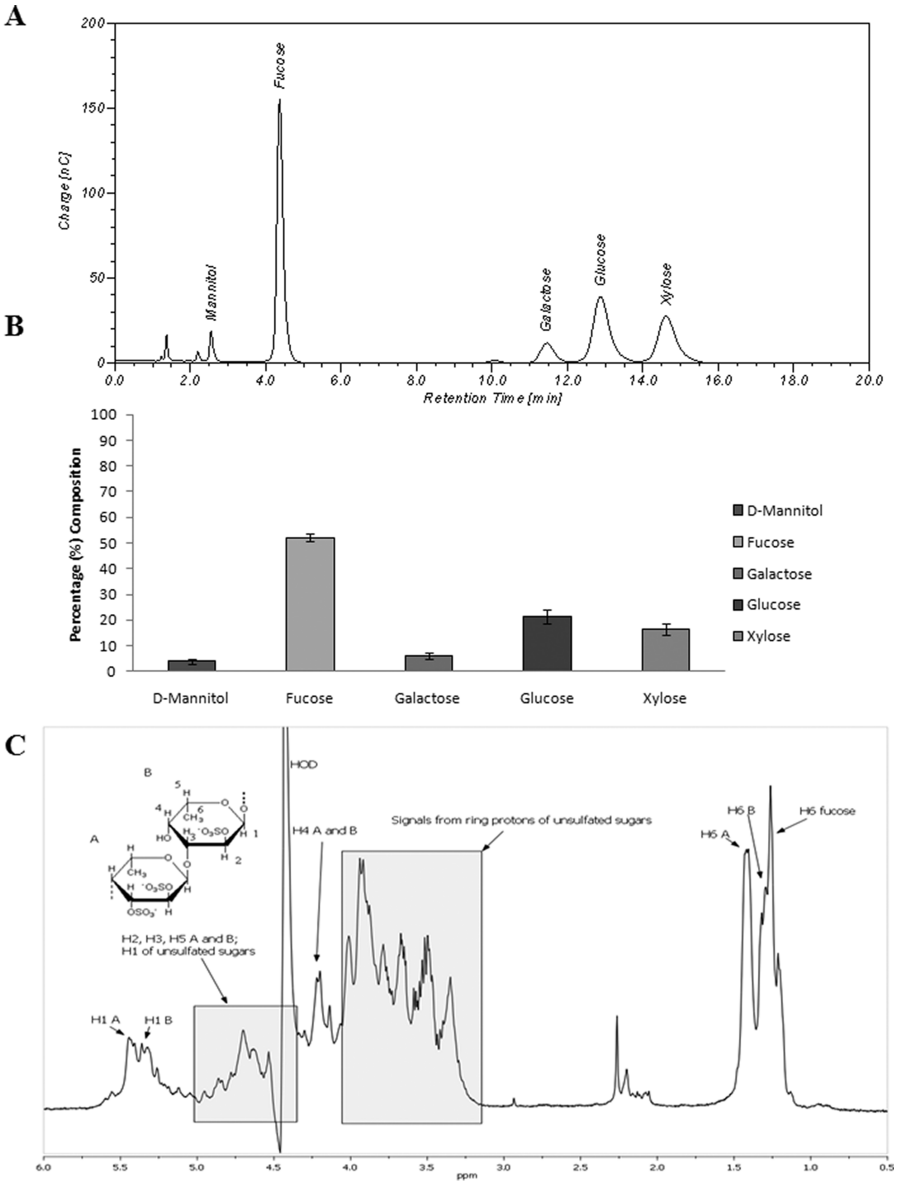

Figure 2. Composition of fucoidan from *A. nodosum*. (A) HPAEC chromatogram of unfractionated fucoidan following TFA hydrolysis. Fucose is the main monosaccharide present with smaller amounts of galactose, glucose, and xylose with some minor proportions of mannitol noted in addition. (B) Monosaccharide composition of fucoidan by HPAEC analysis. The values presented are expressed as percentages from the mean ± SD of three independent experiments. (C) ^{1}H NMR spectrum at 500 MHz, 60 °C, of the high molecular weight fucoidan extracted from *A. nodosum* in D_2O. Signals from the disaccharide structure illustrated from H-6 of fucose in other environments and from unsulfated sugar components (in the gray boxes) can be identified, as labeled. The signal marked HOD arises from traces of residual H_2O in the sample. The group of unlabeled signals between 2.0 and 2.3 ppm arises from traces of solvent and possibly from a small proportion of acetylated polysaccharide.

Table 2. Comparison of Fucose, Sulfate, and Uronic Acid Contents of Fucoidans Isolated from *Ascophyllum nodosum*

source	Size (kDa)	% fucose	% sulfate	% uronic acid	fucose:sulfate
this work	420 (and 47)	52.1	19.0	nd[a]	1.0:0.365
72	nr[b]	38.7	33.7	nr[b]	1.0:0.871
73	556	31.3	26.1	5.7	1.0:0.834
	516	35.8	18.4	11.6	1.0:0.514
	600	43.2	35.3	1.7	1.0:0.817
74	25	47.0	30.0	6.0	1.0:0.638
75	18.6	39.7	27.0	4.1	1.0:0.680
	5.5[c]	42.5	29.4	2.6	1.0:0.692
	19.0[c]	41.9	34.3	1.4	1.0:0.819
76, 77	13	43.0	34.0	5.0	1.0:0.791
78	13	42.0	31.0	5.0	1.0:0.738
12	417	45.4	22.1	9.9	1.0:0.488
	1,323	46.5	22.3	9.3	1.0:0.480
11	nr[b]	28.4	19.4	5.8	1.0:0.683

[a] nd, not detected. [b] nr, not reported. [c] Both fractions were derived from the 18.6 kDa fucoidan preparation following ion-exchange chromatography.

partial analysis revealed structures consistent with a disaccharide unit reported previously.[17]

Subsequent to characterization studies, fucoidan was used as a treatment on human cancer cell lines to determine effects on proliferation and viability. Fucoidan decreased viability in all cell types screened compared to untreated cells, and results are shown in Figure 3. In all cases, the observed effects of fucoidan treatment may be due to induction of death in cells that are otherwise normal or a reduction in the rate of cell division. The effects on proliferation of MCF-7 and HCT-15 cells correspond to data published previously.[46,47] As both normal growth and apoptotic mechanisms are not functional in cancers, the apoptotic pathway was chosen as a hypothesis for the effect of fucoidan on viability and proliferation. It is possible that fucoidan may induce apoptosis in cancer cells, thus leading to a loss of viability. The HCT116 cell line was chosen for this work, as fucoidan caused a loss of cell viability in this cell type.

The induction of apoptosis by fucoidan was determined by Annexin-V/PI flow cytometry. By flow cytometry it can be seen that untreated and cells treated with a 300 μg/mL solution of fucoidan show less than 10% apoptotic cells (9.8% and 8.25%, respectively). These results correlate well with the XTT assay results for HCT 116 cells (Figure 3C). Treatment with 300 μg/mL fucoidan had little to no effect on viability and also does not induce significant amounts of apoptosis in HCT 116 cells. Treatment with 1000 μg/mL fucoidan induces a significant amount of apoptosis (57.15%, 48 h) in HCT 116, which compares well to the XTT viability results seen previously. These results are shown in Figure 4A.

The loss of mitochondrial membrane potential ($\Delta\Psi$m) is a hallmark for apoptosis and is directly linked to the induction of apoptosis.[48] To quantify loss of $\Delta\Psi$m, tetramethylrhodamine ethyl ester (TMRE), a potentiometric fluorescent dye that is incorporated into mitochondria in a $\Delta\Psi$m-dependent manner, was used. A positive control, 2-[(3-chlorophenyl)hydrazinylidene]propanedinitrile, was used to uncouple the mitochondrial electron transport chain and thus cause loss of membrane potential. HCT116 cells were cultured in the presence or absence of 1000 μg/mL fucoidan. The cells were then incubated with TMRE for 30 min and analyzed by flow cytometry. Fucoidan (1000 μg/mL) caused significant membrane depolarization after 24 h treatment (42%), as can be seen in Figure 4B. This represents 80% of the TMRE negative cells seen for the positive control (52%). The percentage TMRE negative cell number in the untreated sample is 12%. The function of fucoidan in mitochondrial membrane depolarization has been documented previously. A recent study[22] found that fucoidan increased the number of cells with depolarized mitochondrial membranes in a concentration-dependent manner. This work demonstrates the capacity of fucoidan to induce mitochondrial membrane depolarization, which may be regulated by Bax and Bax in this instance. The formation of pores in the mitochondrial membrane allows the release of pro-apoptotic cytochrome *c*, which can lead to caspase activation and other pro-apoptotic events.

Nuclear morphological changes during apoptosis are distinct, and effector caspases have been implicated to play a central role in these processes.[49] Fucoidan (1000 μg/mL) was found to induce changes in nuclear morphology after 24 h treatment, as shown in Figure 4C. The imaging of apoptotic nuclear morphology in HCT 116 cells was found to be quite difficult. The retention of early stage apoptotic cells on glass coverslips was particularly difficult, and therefore, collecting enough image data to infer statistical significance was not possible. However, it was possible to identify chromatin condensation and nuclear fragmentation in the images captured. Various fucoidans from different sources have been shown previously to induce morphological changes in a range of cancer cell types.[46,47,50,51]

The cleavage of poly-ADP-ribose polymerase (PARP) by caspases, at a single site, at an early stage of apoptosis, has been shown by Kaufmann and colleagues.[52] PARP is a nuclear zinc-finger DNA-binding protein that detects DNA strand breaks.[53] If fucoidan can induce cleavage of PARP, this would indicate the involvement of caspases in fucoidan-mediated apoptosis. Several caspases can cleave PARP after the apoptotic cascade has been triggered,[54] but caspase-3 has been found to be primarily responsible for the cleavage of PARP during cell death.[55] Fucoidan (both concentrations) was found to induce cleavage of PARP to the 89 kDa polypeptide (Figure 5C) after 48 h treatment. From this, it may be assumed that caspases are involved in fucoidan-mediated apoptosis, although caspase-independent PARP cleavage has been reported previously in transforming growth factor β1-induced apoptosis in murine hepatocytes.[56] Induction of PARP cleavage by fucoidans from various sources has been previously reported.[22,46,47,51]

Apoptotic events are orchestrated primarily, but not exclusively, by members of a family of cysteine aspartate-specific proteases known as caspases.[54,57] Caspases have been assigned into different subfamilies based on their substrate preferences, extent of sequence identity, and structural similarities.[58] Initiator caspases are the first to be activated in a particular death pathway, and they constitute the first step in a minimal two-step cascade by activating the executioner caspases.[59] Caspase-9 is an initiator caspase central to the intrinsic, or mitochondrial, apoptotic pathway. Therefore caspase-9 activation was chosen as a marker for possible involvement of this pathway in fucoidan-mediated apoptosis in HCT 116 cells. Total protein was harvested, and caspase-9 cleavage was examined by Western blotting. Caspase-9 activation to the 37, 35, and possibly the 17 kDa polypeptides is shown in Figure 5A. Activation of caspase-9 is most prominently seen after 48 h treatment with 300 and 1000 μg/mL fucoidan.

Table 3. Sugar Composition of Large Molecular Weight Fucoidans Isolated from Brown Macroalgae[a]

species	extraction method	size (kDa)	fucose	galactose	mannose	glucose	rhamnose	xylose	arabinose	uronic acid	% Fuc or Fuc:SO_4^{2-}
			52.1	6.1	nd	23.1	nd	16.5	nd	nd	19.0
			23.5[a]	2.52[a]	nd	8.76[a]	nd	8.14	nd	nd	10.95[a]
	aqueous/ethanolic extraction;		1.0[b]	0.107[b]	0.0	0.373[b]	0.0	0.346[b]	0.0	0.0	0.466[b]
Ascophyllum nodosum	$CaCl_2$ to recover alginates	420 and 47									1.0:0.445
A. nodosum	nr	F1: >500	0.8[b]	0.1[b]	0.1[b]	nr	nr	nr	nr	nr	nr
		F2: ~500	1.0[b]	<0.02[b]	<0.02[b]	nr	nr	nr	nr	nr	nr
A. nodosum	alkali extraction	F1: 556	31.3	nr	nr	nr	nr	nr	nr	5.7	nr
		F2: 516	35.8	nr	nr	nr	nr	nr	nr	11.6	nr
		F21: 156	23.1	nr	nr	nr	nr	nr	nr	23.5	nr
		F22: 600	43.2	nr	nr	nr	nr	nr	nr	1.7	nr
Cladosiphon okamuranus	aqueous extraction; ultrafiltration; acid and alkali	200	4.0[b]	nr	nr	nr	nr	nr	nr	1.0[b]	2.0[b]
		380	40.2	nr	nr	nr	nr	nr	nr	3.7	18.6
Fucus evanescens	solvent defatting, acid, ethanol, Cetavlon	FeF1: 150−500	90.0	1.2	0.0	6.0	0.0	2.8	nr	nr	1:0.8[b]
		FeF2: 200−500	90.5	0.0	0.0	1.5	0.0	7.0	nr	nr	1.0:1.2[b]
F. vesiculosus	aqueous acid/alkali	F3: 1,600									33.5−39.1
1.9−2.2	nr	25.3	nr	2.3−2.8	nr	nr	11.5				
Hisikia fusiforme	$CaCl_2$, ethanol and chromatography	F1: 959	29.6	22.7	29.3	8.8	0.9	7.9	0.8	17.4	19.3
		F2: 507	38.2	18.2	30.3	3.5	1.4	7.6	0.8	29.3	11.6
		F31: 776	44.5	33.0	17.3	1.0	1.1	3.1	nd	13.4	23.9
Laminaria augustata var. *longissima*	nr	640	1.0 [b]	0.6 [b]	nr	0.08 [b]	nr	0.08 [b]	nr	0.64 [b]	1.0:0.87
L. japoninca	nr	F-A: >200	52.8	27.4	3.4	6.1	3.3	1.9	3.3		
			1.0[b]	0.5[b]	0.11[b]	0.07[b]	0.06[b]	0.04[b]	0.06[b]	18.4	28.3
Saccharina longicruris	$CaCl_2$, NaCl, ethanol and chromatography	2379	21.5								
		2179	18.5								
		2188	20.9								
		2051	12.8								
	ethanol, $CaCl_2$, acid and chromatography	A: 455	nr	nr	nr	nr	nr	nr	nr	22.0	12.0
		B: 576	nr	nr	nr	nr	nr	nr	nr	8.2	14.2
Undaria pinnatifida	acid, ethanol, and $CaCl_2$	2100	70.0	14.8	10.9	nr	nr	1.3	nr	26.2	7.4

[a] Results presented as % compound weight, mol %,[a] and/or molar ratios[b]. nd, not detected; nr, not reported.

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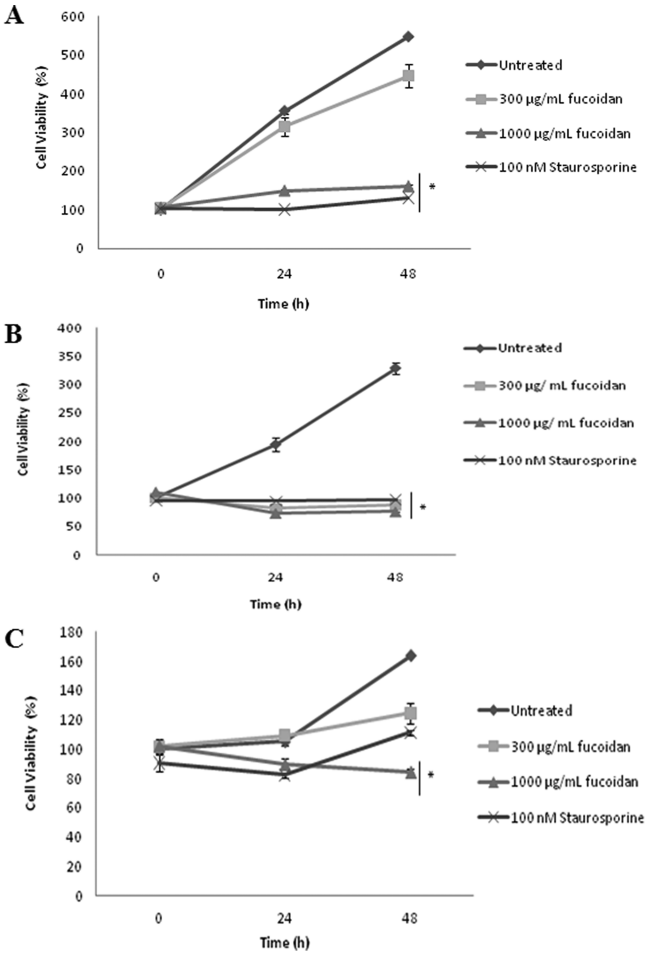

Figure 3. Fucoidan from *A. nodosum* decreases proliferation and viability of various cancer cell lines. (A) MCF-7 cells, (B) HCT-15, and (C) HCT116. A total of 10 000 cells were seeded in 96-well plates and treated with 0, 300, and 1000 μg/mL fucoidan and 100 nM staurosporine for up to 48 h to determine effects on proliferation. The signal for the zero time untreated control represents 100%, and each result is calculated as a percentage of the control. Each bar represents the mean ± SD (n = 3) *p < 0.05.

Treatment with 1 μM staurosporine (STS) resulted in caspase-9 cleavage after 24 h. Activation of caspase-9 leads to eventual cleavage of the central effector caspase, caspase-3. Cleavage of caspase-3 following fucoidan treatment can be seen in Figure 5B. Fucoidan induces caspase-3 cleavage to the 19 and 17 kDa fragments after 48 h treatment. No cleavage can be seen at 24 h for either concentration of fucoidan.

It is clear from this work that fucoidan decreases viability and has pro-apoptotic activity in HCT116 cancer cells. This apoptotic effect is triggered via a pathway dependent upon caspase-9 activation and subsequent activation of caspase-3, leading to PARP cleavage. Fucoidan also causes loss of mitochondrial membrane potential and alterations in nuclear morphology. These apoptotic hallmarks lead us to believe fucoidan is eliciting apoptotic effects through the mitochondrial, or intrinsic, pathway. We suggest that *A. nodosum* fucoidan is a potential therapeutic agent for colon cancer treatment. Further work will be necessary to unravel the precise mechanism of fucoidan-mediated apoptosis and the proteins critical to this process, and the role of fucoidan structure with regard to biological activity. It is hoped that elucidation of a chemical structure for fucoidan may facilitate in determining the role of particular structures in the biological activity of fucoidans of algal origin.

EXPERIMENTAL SECTION

Infrared Spectroscopy (IR). Spectra were recorded on a Spectrum One FT-IR spectrometer (Perkin-Elmer Ireland Ltd., Ballymount, Dublin, Ireland) over the range 650 to 4000 cm^{-1}. The entire sample chamber was cleaned with acetone and lint free paper prior to use. Freeze-dried fucoidan (~10 mg) was placed between two plates in the IR spectrometer, and the press applied to the specimen. The sample was analyzed, and the plates and surrounding area were thoroughly cleaned before analyzing another specimen.

NMR Spectroscopy of Fucoidan. NMR spectroscopy was carried out according to the methods of Pereira and Mulloy.[43] Briefly, ^{1}H NMR spectra were recorded at 500 MHz using a Varian Unity 500 spectrometer (Varian Inc., Santa Clara, CA, USA). The fucoidan sample (~10 mg) was dissolved in approximately 0.7 mL of 99.8% deuterated H_2O (D_2O) and lyophilized (~2 h). Subsequently, 500 μL of D_2O was added, and the fucoidan was lyophilized again. A 500 μL volume of D_2O was added to the fucoidan sample and mixed using a vortex for 5−10 min. Finally, it was placed on a hot plate in a vial of H_2O and allowed to solubilize overnight prior to analysis. Spectra were recorded at 60 °C with suppression of the HOD signal by presaturation. A TOCSY spectrum was recorded using the pulse sequence provided by the spectrometer manufacturer.

Algal Materials. *Ascophyllum nodosum* was harvested from the Donegal seashore (Kilcar, Co. Donegal, Ireland) in September 2006. The seaweed was milled on site in Donegal by Oilean Glas Teoranta (OGT), Kilcar, Co. Donegal, Ireland, using a commercial blender, and the resulting homogenate was stored at −20 °C (long-term) or 4 °C (immediate use) until required.

Extraction Procedures. All polysaccharides were extracted as described below and summarized in Figure 1. Seaweeds were milled to produce a homogeneous paste. Polysaccharides were extracted from the milled seaweeds (800 mL) using selective solvents with constant mechanical stirring.[60] Temperature was controlled using a hot plate and thermometer. First, EtOH (80% v/v) at room temperature (~23 °C, RT) for 12 h and at 70 °C for 12 h was used to extract mannitol and some salts (S1 and S2). Subsequent extraction involved millliQ H_2O at RT for 7 h, at 70 °C for 7 h, and at 70 °C for 4 h (S3, S4, and S5). The resultant fraction (Sc, combination of S3, S4, and S5) was treated with 2 M $CaCl_2$ at RT for 5 h in order to precipitate alginates. The alginate-rich precipitate was removed by centrifugation at 10 000 rpm for 30 min. After alginate removal, the resulting extract was enriched in fucoidan. Dialysis was carried out (cut-off 1000 Da) at 4 °C over a 48 h period to decrease salinity, and Milli-Q H_2O was changed every 12 h. Fucoidan was freeze-dried and stored in aliquots at −20 °C until required.

Chemical Composition. All chemicals, unless otherwise stated, were from Sigma-Aldrich. Total sugars were quantified for each polysaccharide fraction using the phenol−H_2SO_4 method,[61] and the total carbohydrate content was calculated by reference to the sugar standard (0−200 μg/mL fucose) at 490 nm. Total polyphenol content was measured by the Folin-Ciocalteau (FCR) method, as FCR is the preferred method for routine measurement of total phenols.[62] Tests and controls were analyzed in triplicate. All absorbance measurements were carried out at λ = 765 nm using phloroglucinol as a standard (0−80 μg/mL). Sulfate content was estimated quantitatively using the $BaCl_2$-gelatin turbidimetric method.[32] Again, test and control samples were assayed in triplicate, and absorbance (turbidity) measurements were made at λ = 550 nm. The concentration of sulfate present was determined by reference to a sulfate standard curve using K_2SO_4 (0−300 μg/mL). The concentration of soluble protein was determined

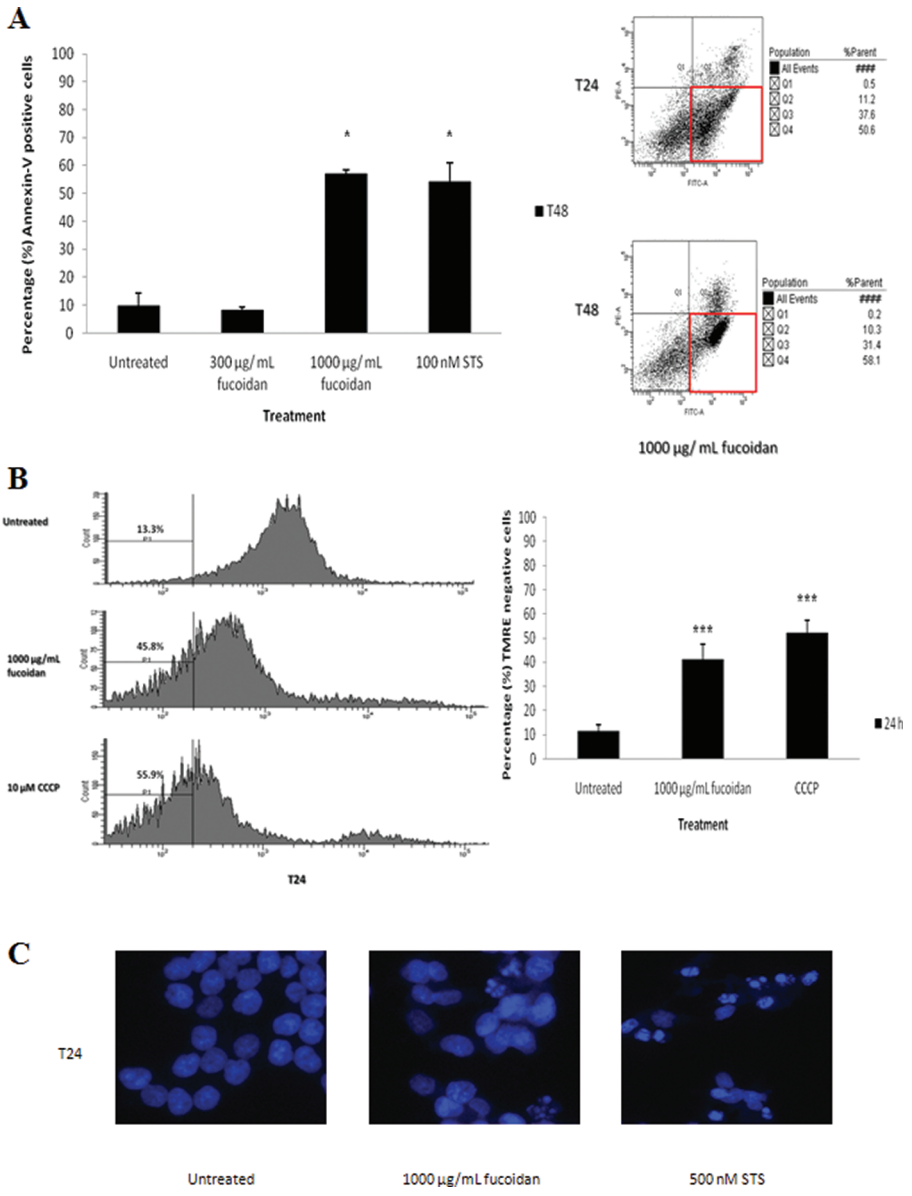

Figure 4. Fucoidan induces apoptosis in HCT116 cells. (A) Fucoidan induces apoptosis as shown by Annexin-V/PI double staining in the graph on the left and dot plot on the right. (B) Alterations in mitochondrial membrane potential (Ψm) after fucoidan treatment, as assessed by TMRE staining and flow cytometry. (C) Apoptotic morphology in HCT 116 cells after fucoidan treatment. Condensed, intensely stained apoptotic nuclei can be seen after fucoidan treatment (24 h) and STS treatment (12 and 24 h). Each bar represents the mean ± SD (n = 3) $^{*}p < 0.05$, $^{***}p < 0.001$.

using the Bradford method with bovine serum albumin (BSA, fraction V) as a standard,[63] and measurements were made at λ = 595 nm with reference to a BSA standard curve (0−30 μg/mL). Finally, all samples were analyzed for D-mannitol content using the D-mannitol/L-arabinatol kit according to the manufacturer's instructions (Megazyme International Ireland Ltd., Bray Business Park, Bray, Co.Wicklow, Ireland).

Trifluoroacetic Acid (TFA) Hydrolysis of Carbohydrates. Samples were boiled in TFA/MeOH solution for 4 h and dried using a Hetovac VR-1 concentrator (Heto Lab Equipment A/S Birkerod, Denmark). The sugar hydrolysate was subsequently washed with 100% EtOH several times to remove residual acid. In between washes the samples were dried to remove acid and ethanol using the Hetovac VR-1 concentrator. Specifically, for high-performance anion exchange chromatography (HPAEC), purified fucoidan (200 μL) was combined with a mixture of 4 M TFA/MeOH and H_2O (4:2:1) and heated to 100 °C for 4 h to break glycosidic bonds. After hydrolysis the products were co-evaporated as described above with EtOH four to five times to remove any residual acid or methanol using a Hetovac VR-1 concentrator.

High-Performance Anion Exchange Chromatography. The monosaccharide compositions of commercial and extracted fucoidans and commercial laminaran were determined quantitatively by high-performance ion exchange chromatography on an ICS-3000 ion chromatography system (Dionex Corporation, Sunnyvale, CA, USA). The sugars mannitol, fucose, rhamnose, glucose, galactose, and xylose were used as standards for HPAEC as a mix of 200 mg of each sugar in a 1 L final volume (i.e., 0.2 mg/mL). A 5 mg quantity of commercial fucoidan (Sigma-Aldrich), commercial laminaran (Sigma-Aldrich), and the fucoidan extract (200 μL) were hydrolyzed as described above. The hydrolysis products were separated on a CarbopacPA-100 column (Dionex Corp., Sunnyvale, CA, USA), with the appropriate guard column, using a decreasing 200−18 mM NaOH gradient at a flow rate of 1 mL/min at RT. An ED40 electrochemical detector (Dionex Corp., Sunnyvale, CA,

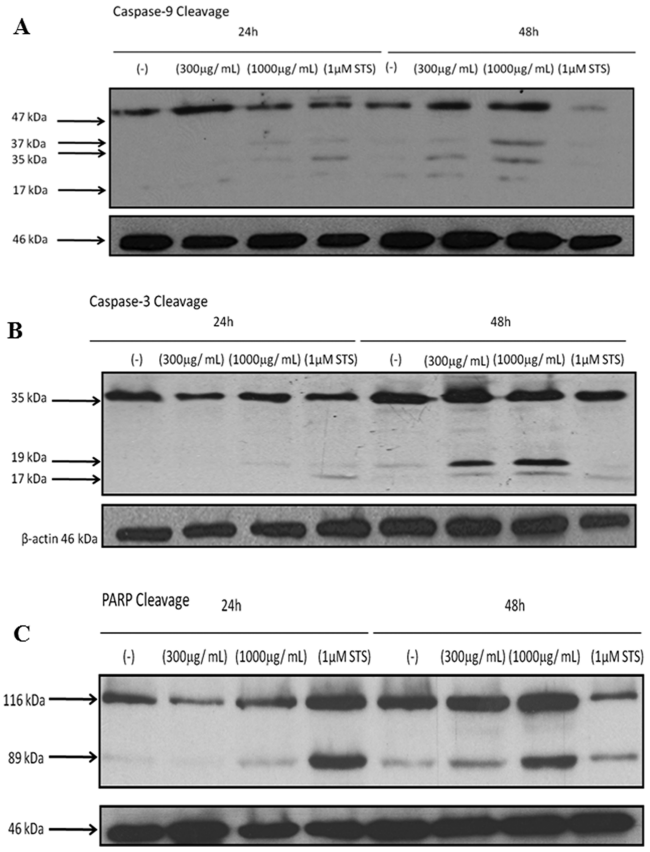

Figure 5. Fucoidan activates caspases and can cleave caspase substrates. (A) Fucoidan activates caspase-9 after 24 and 48 h treatments (1000 μg/mL). (B) Caspase-3 clevage in response to fucoidan treatment at 48 h. (C) Fucoidan causes cleavage of PARP, a caspase-3 substrate, after 48 h.

USA), in the integrated amperometry mode, facilitated detection of eluting product peaks. Data were collected and processed using Chromeleon Version 6.70 software (Dionex Corp., Sunnyvale, CA, USA). Aliquots of TFA-hydrolyzed standard sugars and samples were separated under identical conditions.

Molecular Weight Determination. The molecular size profile of the extracted fucoidan was determined by preparative gel permeation chromatography as previously described.[35] The gel permeation chromatography system consisted of TSK G4000 SW-XL (300 × 7.8 mm) and TSK G3000 SW-XL (300 × 7.8 mm) columns (Tosoh Corporation, Tokyo, Japan) in series, a 0.1 M NH_4OAc acetate eluant used to irrigate the columns at 0.5 mL/min, and a refractive index detector (Tosoh Corporation, Tokyo, Japan). Lyophilized fucoidan (9.9 mg) was resuspended in 800 μL of 0.1 M NH_4OAc eluant buffer. This mixture was allowed to solubilize briefly and then filtered through a sterile 0.2 μm filter before being manually injected into the GPC unit. The gel permeation columns were calibrated using narrow heparin standards as previously described.[44] Peak molecular weights and polydispersities were calculated using the Cirrus software package (Polymer Laboratories/Agilent).

Cell Culture. The HCT 116 colorectal carcinoma cell line (ref. no. 91091005) was obtained from American Type Culture Collection (ATCC) through a European distributor (LGC Standards, Queens Road, Teddington, Middlesex, UK) and was routinely cultured in McCoy's 5A medium and incubated at 37 °C with 5% CO_2. Medium was supplemented with 2 mM L-Glutamine (L-Glut), 10% (v/v) fetal bovine serum (Gibco-Invitrogen Life Technologies, Paisley, UK) heat inactivated at 56 °C for 1 h, and 1% (v/v) penicillin−streptomycin solution (10 000 units of penicillin and 10 mg of streptomycin/mL).

Cell Viability Assay (XTT Assay). Exponentially growing cells were seeded (1×10^3/well in 100 μL) into 96-well plates. Two concentrations of fucoidan and a positive control, staurosporine (100 nM to 1 μM), were chosen for this investigation. A time-course of 48 h allows growth to be monitored, and samples were harvested every 24 h (0, 24, and 48 h). For fucoidan dosage, a lower (300 μg/mL) and higher (1000 μg/mL) concentration were chosen to determine effects of different concentrations of fucoidan on viability and proliferation. Treatments were added to the plates 24 h later as outlined above. Following the incubation period indicated in figure legends, cell viability was quantified using an XTT cell proliferation kit assay (Cell Proliferation Kit II, Roche Diagnostics, Mannheim, Germany). After drug exposure, plates were incubated for 4 h with XTT before reading them at 490 nm with a reference wavelength at 650 nm using a WALLAC Victor Multilabel microtiter plate reader. Cell viability was expressed as a percentage in relation to controls. All data were averaged from at least three independent experiments ± SD.

DAPI Staining. To stain DNA molecules, cells were incubated with 5 μL of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) solution for 5 min in the dark at RT. A 1:10 000 dilution of stock DAPI (2.5 mg/mL) solution (in 1× PBS) was used. After incubation, coverslips were rinsed four times with 1× PBS. Coverslips were then inverted and mounted on SuperFrost glass slides (Menzel Gläser, Thermo Fisher Scientific, Braunschweig, Germany) with VectaShield Mounting Medium (Vector Laboratories, Inc., Burlingame, CA, USA). The edges of the coverslips were sealed with clear nail varnish and stored at 4 °C in the dark. Slides were visualized at 40× and 100× magnifications, using an Olympus 1X51 fluorescent microscope (Olympus, Essex, UK). Images were analyzed using CellR Imaging software (Olympus, Essex, UK).

Apoptosis Assay (Annexin-V and Propidium Iodide (PI) Double Staining). Cells were seeded at 1×10^5 cells per mL of complete medium in a 24-well plate and allowed to attach for 24 h. Cells were then treated as described previously with fucoidan and staurosporine for 24 and 48 h. At harvesting, medium (containing floating cells) was removed from wells and placed in sterile 1.5 mL microcentrifuge tubes. Wells were washed with 300 μL of prewarmed HBSS; then cells were trypsinised by adding 200 μL of 1× Trypsin-EDTA and were transferred to the tubes, which were incubated at 37 °C with intermittent shaking to allow recovery from minor damage to the cell membrane mediated by trypsinization. Cells were collected by centrifugation at 2000 rpm for 5 min. The supernatant was removed, and cells were resuspended in 50 μL of calcium buffer (10 mM HEPES pH 7.5, 140 mM NaCl, 2.5 mM $CaCl_2$) containing 1 μL of Annexin V dye and incubated on ice in the dark for 15 min. Subsequently, a 4 μL aliquot of propidium iodide (50 μg/mL) in 300 μL of calcium buffer was added to the tubes, and the cells were sorted and analyzed by flow cytometry using channels for FITC/PE in a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA).

Mitochondrial Membrane Potential ($\Delta\Psi$m) Assay. Cells were seeded at 1×10^5 cells per mL in complete medium in a 24-well plate and allowed to attach for 24 h. Cells were then treated as described previously with fucoidan and staurosporine for 24 and 48 h. At harvesting, medium was removed from wells and placed in sterile 1.5 mL microcentrifuge tubes. Cells were trypsinized with 140 μL of 2× Trypsin-EDTA and incubated at 37 °C for 5 min. Once detached, the trypsinised cells were combined with the medium in the 1.5 mL microcentrifuge tubes. To generate the positive control, 0.6 μL of 10 mM 2-[(3-chlorophenyl)hydrazinylidene]propanedinitrile was added to a designated tube and incubated at 37 °C for 30 min. A 1.5 μL aliquot of 40 μM TMRE was added to each tube to give a final concentration of 100 nM. Cells were then incubated for 30 min at RT in the dark with occasional mixing of settling cells. Mitochondrial

membrane potential was monitored using flow cytometry by measuring the fluorescence intensity on a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA).

Western Blotting. Cells were lysed with whole cell lysis buffer (20 mM HEPES pH 7.5, 350 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1% (v/v) Ipegal-630, 0.5 mM DTT, 1.0 μM phenylmethanesulfonyl fluoride, 1.0 μg/mL pepstatin, 10 μM leupeptin, 2.5 μg/mL aprotinin, and 250 μM ALLN.). The supernatant extracts were centrifuged at 12000*g* for 10 min at 4 °C to remove debris, and the resulting supernatant was used for Western blot analysis. Equal amounts of protein were resolved using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (BioRad, Alpha Technologies, Blessington, Co. Wicklow, Ireland) and electrophoretically transferred to nitrocellulose membranes. The membranes were subsequently blocked with 5% (w/v) milk in PBS-Tween (PBS-T, 0.05% Tween) and incubated with the indicated antibodies. Western blotting was performed using anti-human caspase-3,-9 and PARP rabbit antibody as primary antibody (Cell Signaling Technologies, 1:1000 dilution in 5% milk in PBS/0.05% Tween-20) and goat anti-rabbit IgG-horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, 1:5000 dilution in PBS-T in 5% milk) as secondary antibody. Proteins were visualized by enhanced chemiluminescence.

Statistical Analysis. All values are expressed as the mean ± standard deviation. Statistical significance was compared between each treatment group and control by a one-way analysis of variance (ANOVA) followed by Dunnett's test using GraphPad Prism version 5.04 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com.

■ ASSOCIATED CONTENT

Supporting Information. Infrared spectra for commercial (Sigma) fucoidan and our fucoidan extract. This material is available free of charge via the Internet at http://pubs.acs.org.

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