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ORIGINAL ARTICLE

Fucus spiralis extract and fractions: anticancer and pharmacological potentials

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Summary

Purpose: *Sea macroalgae are an important source of biologically highly valuable compounds. The main aim of this study was to investigate the in vitro anticancer properties and chemical composition of the dichloromethane-methanol extract and three fractions of the Fucus spiralis from coastline of Morocco.*

Methods: *Fractions were made from dichloromethane: methanol (1:1) extract of Fucus spiralis: petroleum-ether, ethyl-acetate and n-butanol. Extract and fractions were screened for in vitro cytotoxicity by MTT assay against human cervical adenocarcinoma (HeLa), colorectal adenocarcinoma (LS-174T), lung carcinoma (A549), and normal human lung fibroblasts (MRC-5). Cell cycle distribution of the HeLa cells was evaluated using flow cytometry. Acridine orange (AO)-ethidium bromide (EB) staining was used to assess morphological changes of HeLa cells under fluorescence microscope. Anti-migration and anti-angiogenic properties were investigated using scratch and tube formation assays against human endothelium-derived permanent EA.hy926 cell line. Antidiabetic activity was tested using anti-a-glucosidase assay. Antimicrobial effect was tested using micro- dilution method.*

Results: *Petroleum-ether fraction of Fucus spiralis rich in fatty acids exerted the highest cytotoxicity against HeLa cells. Ethyl-acetate and petroleum-ether fractions induced the highest accumulation of the HeLa cells in sub-G1 and G2/M phases. Extract and fractions showed proapoptotic effect on HeLa cells under fluorescent microscope. They exhibited antimigratory and antiangiogenic effects in vitro. IC_{50} value for a-glucosidase inhibitory activity was much stronger than standard acarbose. n-Butanol fraction exerted the highest antibacterial and antifungal activity.*

Conclusions: *The investigation of various biological activities of the extract and fractions obtained from Fucus spiralis may suggest a promising anticancer and pharmacological potential of this edible macroalga.*

Key words: *cancer, cytotoxic, fatty acids, Fucus spiralis, cancer cells, in vitro*

Introduction

Macroalgae have been explored and used not only as food but also as medicinal and pharmaceutical natural agents. It has been reported that macroalgae have anticancer properties including cytotoxic and antimitogenic [1]. One of the active substances isolated from macroalgae *Bryopsis sp.*, kahalalide F, was investigated in phase 2 clinical trials for malignant melanoma, liver, prostate, and

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breast cancer [1-3]. The trial was discontinued in patients with malignant melanoma due to lack of objective response [2]. It was also found that this substance exhibited antitumor effect against lung, colon and prostate cancer [1].

Macroalgae produce bioactive components in order to endure harsh environmental conditions: fatty acids, vitamins, proteins, essential amino acids, dietary fibers, polysaccharides, phlorotannins (unique phenolic compounds) and secondary metabolites as chemical defense and cell wall structure as defense from herbivores [4]. These metabolites might be medicinally useful for the treatment or prevention of different diseases, including cancer [5].

Bioactive components that have been isolated from the brown algae are polysaccharides, alginic acid, laminarians and fucoidans (sulfated polysaccharides with antiviral, immunomodulating and antitumor activity) [5,6]. Fucoidan isolated from brown algae induced apoptosis of human colon cancer cells *in vitro* [7].

Edible brown macroalga *Fucus spiralis* Linnaeus (*Phaeophyta*) inhabits littoral shores of Europe and North America. It is found along the Atlantic coast of France, Spain, Morocco and Azores. Specific microclimate on the Sidi Bouzid location in Morocco (strong and freezing cold ocean current) makes this brown alga rich in unique secondary metabolites [8].

Fucus spiralis was found to have a high content of biologically active compounds: phloroglucinol, mannitol, oleic acid, fucosterol, arachidonic and eicosapentaenoic acids [5]. There is an increasing interest for using brown macroalgae in nutrition, due to their potent antioxidative activities. It is well-known that *Fucus spiralis* has an important place in the food chain of Mediterranean countries [5]. Polyphenolic compounds have been shown to exert numerous health-beneficial effects. Their antioxidative and antidiabetic properties had been reported [9]. The polyphenols may have a promising anticancer potential, since they can induce cell cycle arrest and apoptosis, inhibit angiogenesis and reduce metastatic potential of cancer cells [10]. Fatty acids, which may exert inhibitory effects on the development and progression of cancer, may be also helpful in the prevention and treatment of diabetes type 2 and cardiovascular diseases and also they may be used as potential antimicrobial and anti-inflammatory agents [11,12].

There is a link between diabetes and cancer. People with diabetes have increased risk of several types of cancer (female reproductive organs, colorectal, breast, liver, urinary tract and pancreas) and their mortality is also increased. Patients with diabetes are often troubled with hyperglycemia, obesity and increased oxidative stress, which are factors that can also lead to increased cancer risks [13]. Furthermore, commercial antidiabetic medications, such as acarbose, have toxic side effects, which bring up the search for natural alternatives.

People with cancer are more prone to microbial infections which can be multidrug resistant [14]. Also, chronic infection can lead to chronic inflammation which could lead to cancer [15]. So it is very important to find novel antimicrobial agents that can alone or with combination of known antibiotics and antimycotics contribute to eradication of multidrug resistance that can be fatal.

The main aim of this research was to investigate the anticancer effects of the dichlormethane-methanol extract and fractions obtained from *Fucus spiralis* collected at the Sidi Bouzid on the Atlantic coast of Morocco. To further explore their biological effects, the antioxidative, antidiabetic, antibacterial and antifungal activities were evaluated. In addition, the fatty acid composition and total phenolic content of the extract and fractions were determined.

Methods

Algae collection and preparation of extract and fractions

Investigated algae were collected at the Atlantic coast of Morocco (Sidi Bouzid coast) between March-April 2014. The examined brown alga was identified as *Fucus spiralis* Linnaeus, at the Laboratoire de Cryptogamie, MNHN Paris, France. The specimens were air-dried in the dark and powdered. The powder was extracted in dichloromethane/methanol (50:50) as described by Caccamese and Azzolina [16]. In brief, the powder was extracted in a solvent overnight at room temperature. The resulting extract was centrifuged and the supernatant was concentrated to dryness in a rotary evaporator (Heidolph Instruments GmbH & Co., KG, Germany) under reduced pressure (at 45°C), until crude extract was obtained and was conserved at 4°C. In order to obtain fractions, the extract was then suspended in 100 mL of distilled water and successively extracted with petroleum ether (Pet-Et, 3x100 mL), ethyl-acetate-(EtOAc, 3x100 mL) and n-butanol (n-BuOH, 3x100 mL). The obtained extracts and fractions were stored at -20°C until further analysis.

Fatty acid assay

Fatty acids from algae dry petroleum-ether fractions were trans-esterified with hydrochloric acid in methanol, according to the method described by Ichihara and Fukubayashi, 2010 [17], and fatty acid methyl esters (FAMEs) were obtained. Fatty acid methyl esters were further analyzed using an Agilent Technologies 7890A Gas Chromatograph with a flame ionization detector. Separation of the FAMEs was performed on a CP-Sil 88 capillary column (100 m×0.25 mm×0.2 μm) using helium as a carrier gas at a flow rate of 1 mL/min. The samples were injected at the starting oven temperature of 80°C, injector temperature was 250°C, and detector temperature

was 270°C. The oven temperature was programmed to increase from 80°C, 4°C/min to 220°C, 5 min, 4°C/min to 240°C, 10 min. Fatty acids were identified by their retention time in comparison with the reference fatty acid standards (Supelco FAME Mix, Bellefonte, PA). The results were expressed as a percentage of individual fatty acid in the total dry petroleum-ether fractions.

Total phenolic content

The content of total phenolics in investigating extracts was analyzed using a modified Folin-Ciocalteu method [18]. Results were expressed as milligrams of Gallic acid equivalents (GAE) per gram of the investigating extracts based on the standard calibration curve obtained with a series of Gallic acid standard solutions. Data were presented as mean ± standard deviation (SD) of three independent experiments.

DPPH free radical scavenging assay

The antioxidant activity of the extracts was analyzed based on their free radical scavenging activity (RSA) on the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical, carried out according to the procedure described previously [19] with slight modifications. All test experiments were run in three independent experiments and data were presented as mean ± SD.

Cytotoxic activity

The tested cell lines human cervical adenocarcinoma (HeLa), colorectal adenocarcinoma (LS-174T), lung carcinoma (A549), and normal human lung fibroblasts (MRC-5) were grown in RPMI-1640 medium. Somatic human umbilical vein endothelial (EA.hy926) cell line was grown in Dulbeccos's modified Eagle's medium (DMEM). The tested cell lines were grown in the complete nutrient medium according to previously described procedure for treatment of cancer cell lines [20]. Stock solutions (100 mg/mL) of the extract and fractions, made in dimethylsulfoxide (DMSO), were dissolved in a corresponding medium to the required working concentrations. The final concentrations applied to the cells were 200, 100, 50, 25 and 12.5 μg/mL. To the control cells only the nutrient medium was added. The procedure has been already described [20]. The effects of the extract and fractions on the cell survival were determined by the microculture tetrazolium test (MTT) according to Mosmann [21] with modification by Ohno and Abe [22]. All experiments were done in triplicate.

Cell cycle analysis

HeLa cells were seeded into 6-well plates (200000 cells per well) and after 24 h they were treated with IC_{50} and $2\times IC_{50}$ concentrations of the investigated extract and fractions for 24 h. After incubation, the cells were collected by trypsinization, fixed in 70% ethanol on ice and stored at -20°C for one week [20]. The cells were washed with PBS and incubated with RNaseA at 37°C for 30 min. After this, propidium iodide (PI) was added. Cell cycle phase distribution was analyzed by FACSCalibur flow cytometer (BD Biosciences Franklin Lakes, NJ, USA) using CELLQuest software.

Fluorescence microscopy

HeLa cells (50000 cells per well) were seeded into 6-well plates. The next day, the tested extract and fractions were added to cells at concentrations $2\times IC_{50}$. After 24 h of treatment, the cells were stained with a mixture of acridine orange (AO)/ethidium bromide (EB) (3 μg/mL AO and 10 μg/mL EB) in phosphate buffered saline (PBS). Photomicrogaphs were taken under a fluorescence microscope - Carl Zeiss PALM MicroBeam with AxioObserver.Z1 using AxioCamMRm (filters Alexa 488 and 568), as previously described [20].

In vitro scratch assay

EA.hy926 cells were seeded into 24-well plates. Confluent cell monolayers were formed after 24 h and scratched with a p200 sterile pipette tip to create a straight central scratch line, as described earlier [23]. After washing with nutrient medium, cells were treated with subtoxic concentrations (IC_{20}) of extracts and fractions for 24 h. Photomicrographs were captured immediately after making the wound and then 24 h later under inverted phase-contrast microscope. The experiments were carried out in triplicate and the results are shown as mean ± SD.

Tube formation assay

EA.hy926 cells were plated on the surface of the 24-well plates, coated with 200 μL of Corning®Matrigel® basement membrane matrix. The assay was described previously elsewhere [23]. Afterwards, in control wells with cells, DMEM was added, while solutions of subtoxic concentrations (IC_{20}) of extract and fractions were added to other wells. After 20-h incubation, photomicrographs of cells were taken under inverted phase-contrast microscope.

α-Glucosidase inhibitory activity

The α-glucosidase inhibitory activity was estimated by the modification of the procedure described by Matsui et al [24]. The α-glucosidase (Sigma-Aldrich St. Louis, MO, USA) solution was set at 400 mU/mL in a 0.1 M phosphate buffered saline (PBS) (pH=6.7). The tested extracts were diluted in the same phosphate buffer, so that the highest concentrations were 166.67 μg/mL, from which serial double dilutions were made. Fifty μL of each extract in DMSO was diluted in 50 μL of enzyme at 37°C for 15 min. The reaction was started by adding 50 μL of substrate solution (1.5mg/mL PNP-G (p-nitrophenyl α-D-glucopyranoside, Sigma-Aldrich, St. Louis, MO, USA) in the buffer. After measuring absorbance A1 at 405 nm, the solution was incubated at 37°C for 15 min. Second absorbance A2 was measured at 405 nm. Acarbose (Sigma-Aldrich St. Louis, MO, USA) was used as positive control. The percentage of the enzyme inhibition was calculated according to the equation 100×(A2S–A1S)/(A2B-A1B), where A1B, A2B and A1S, A2S represent the absorbance of the blank (phosphate buffer, DMSO, enzyme dilution, and PNP-G dilution) and the sample, respectively. All experiments were done in triplicate.

Antimicrobial activity

Bacterial inoculi and suspensions of fungal spores were prepared as described by Kosanic et al [25] and according to the procedure recommended by NCCLS [26]. The minimal inhibitory concentration (MIC) was determined by broth microdilution method with using 96-well micro-titer plates [27]. A series of dilutions with concentrations ranging from 25 to 0.012 mg/mL for test samples were used in the experiment against every microorganism tested. The starting solutions of the test samples were obtained by measuring certain quantity of extract and dissolving it in DMSO. As a positive control of growth inhibition, streptomycin and ketoconazole were used. A DMSO solution was used as a negative control for the influence of the solvents. All experiments were performed in triplicate.

Statistics

The results are expressed as mean±standard deviation of three independent experiments. Statistical analyses were performed using one-way ANOVA with Dunnett's comparison test. P value <0.05 showed statistical significance.

Results

Fatty acid assay

Fatty acid content was determined in the petroleum-ether fraction of the *Fucus spiralis* since this solvent is known as the most effective for isolation of fatty acids. The obtained results are presented in Table 1. Saturated fatty acids (SFA) with the highest content were palmitic and myristic acids. Among the examined monounsaturated acids the oleic acid was the most abundant. The following polyunsaturated fatty acids (PUFA) were detected in the fraction: eicosadienoic acid, arachidonic acid (AA), eicosapentaenoic acid (EPA), alfa linolenic acid (ALA), and linoleic acid (LA). PUFA to SFA ratio was 0.50.

Total phenolic content and DPPH radical scavenging activity

The total phenolic contents and DPPH radical scavenging activities of *Fucus spiralis* extract and fractions are shown in Figure 1. The n-butanol fraction had the highest total phenolic content (15.16±0.04 mg GAE/g), followed by *Fucus spiralis* whole extract (7.11±0.06 mg GAE/g), ethyl-acetate (6.14±0.10 mg GAE/g) and petroleum-ether fraction (3.62±0.03 mg GAE/g) (Figure 1). The ethyl-acetate fraction showed the highest DPPH free radical scavenging activity (9.48±2.17) mg/mL, followed by *Fucus spiralis* whole extract (9.78±0.2 mg/mL), petroleum-ether (15.67±0.11 mg/mL) and n-butanol fraction (21.16±2.77 mg/mL).

In vitro cytotoxicity

Results of the examination of the cytotoxic activities of the extract and fractions are presented in Table 2. HeLa cells were the most sensitive to

Table 1. Fatty acid content of the Fucus spiralis (petroleum-ether fraction)

	Fatty acids	*F. spiralis %*
Myristic acid	C 14:0	16.24
pentadecanoic acid	C 15:0	0.7
palmitic acid	C 16:0	27.77
stearic acid	C 18:0	1.87
ΣSFA	ΣSFA	46.58
palmitoleic acid	C 16:1	1.08
oleic acid	C 18:1 n-9	24.15
ΣMUFA	ΣMUFA	25.23
linoleic acid (LA)	C 18:2 n-6	3.27
alfa linolenic acid (ALA)	C 18:3 n-3	3.57
eicosadienoic acid	C 20:2 n-6	6.32
arachidonic acid (AA)	C 20:4 n-6	6.15
eicosapentaenoic acid (EPA)	C 20:5 n-3	3.95
ΣPUFA	ΣPUFA	23.26
PUFA/SFA	PUFA/SFA	0.50
n-9	n-9	24.15
n-6	n-6	15.74
n-3	n-3	7.52
n-6/n-3	n-6/n-3	2.09

SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids

the cytotoxic effects of the tested extract and fractions. The strongest cytotoxic activity on HeLa cells was exerted by the *Fucus spiralis* petroleum-ether fraction with IC_{50} value of 43.74±7.85 μg/mL, followed by the ethyl-acetate fraction (47.25±1.32 μg/mL) and dichloromethane-methanol extract with (52.18±3.21μg/mL) and n-butanol (105±6.77 μg/mL). All tested samples exerted lower intensity of the cytotoxic activity on LS174T and A549 cells when compared with their activities on HeLa cells. The ethyl-acetate fraction was the most active on LS174 cells with IC_{50} value of 72.58±0.55 μg/mL, followed by dichloromethane-methanol extract (81.75±4.60 μg/mL), petroleum-ether (85.12±3.49 μg/mL) and n-butanol (159.00±1.45 μg/mL) fractions. A549 cells were the most susceptible to the cytotoxic action of the ethyl-acetate fraction (132.68±3.93 μg/mL), then to the petroleum-ether (142.99±0.37 μg/mL), dichloromethane-methanol extract (147.41±1.25 μg/mL) and n-butanol (>200 μg/mL) fraction. Each of the tested extract and fractions exhibited notably lower intensity of the cytotoxic activity on normal MRC5 cells in comparison with their activity on the cancer cell lines.

Cell cycle analysis

Analysis of the cell cycle of HeLa cells treated with IC_{50} of the extract and fractions after 24 h (Figure 2) showed that ethyl-acetate fraction induced the highest increase of HeLa cells in the subG1 phase when compared with this percentage in the control untreated cells. This fraction also led to accumulation of cells in the G2/M cell cycle phase, compared to control cells. HeLa cells treated with petroleum-ether fraction showed the highest accumulation in the G2/M phase and induced increase of treated cells in the subG1 phase. The extract and n-butanol fraction also induced increase of HeLa cells within the subG1 phase. When treated with $2\times IC_{50}$ concentrations, the highest increase of HeLa cells within the subG1 phase was shown in cells treated with ethyl-acetate and petroleum-ether

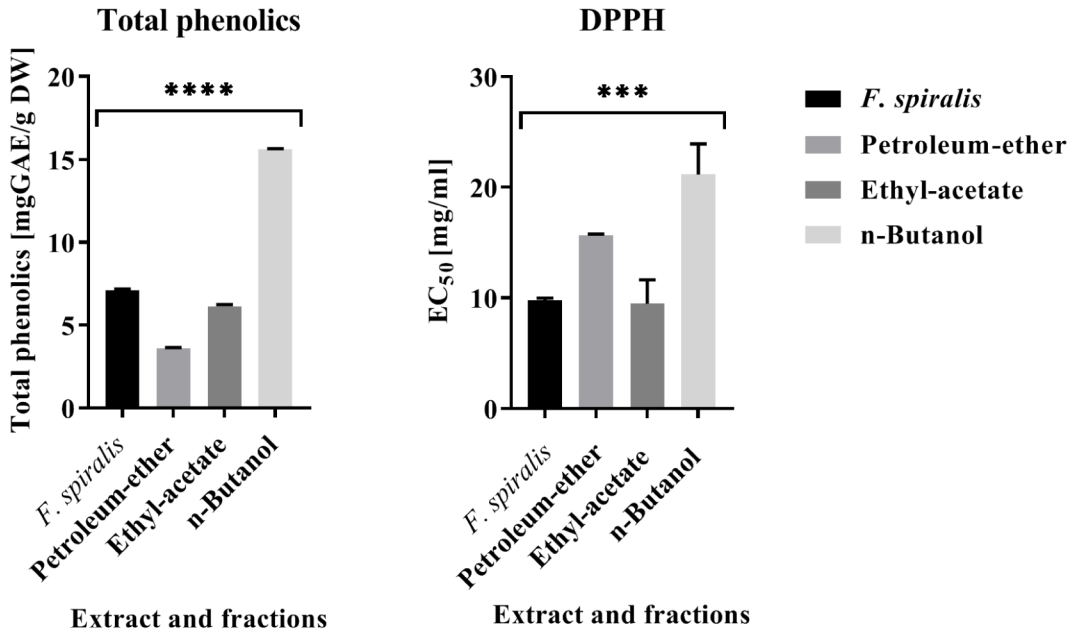

Figure 1. Total phenolic content and DPPH radical scavenging activity of algal extract and fractions (****p< 0.0001, ***p< 0.001).

Table 2. IC50 values for the investigated extract and fractions

*F. spiralis / IC_{50}, μg/mL**	*HeLa*	*LS174T*	*A549*	*MRC5*
extract	52.18±3.21	81.75±4.60	147.41±1.25	>200
petroleum-ether	43.74±7.85	85.12±3.49	142.99±0.37	>200
ethyl-acetate	47.25±1.32	72.58±0.55	132.68±3.393	>200
n-butanol	105.25 ±6.77	159.89±1.45	>200	>200

*Concentrations of the examined extract and fractions inducing 50% decrease in cell survival rate (expressed as IC_{50} value). Extracts were incubated with cells for 72 h, followed by cytotoxic activity determination in vitro by the MTT assay. Results are presented as the mean value ± SD of three independent experiments.

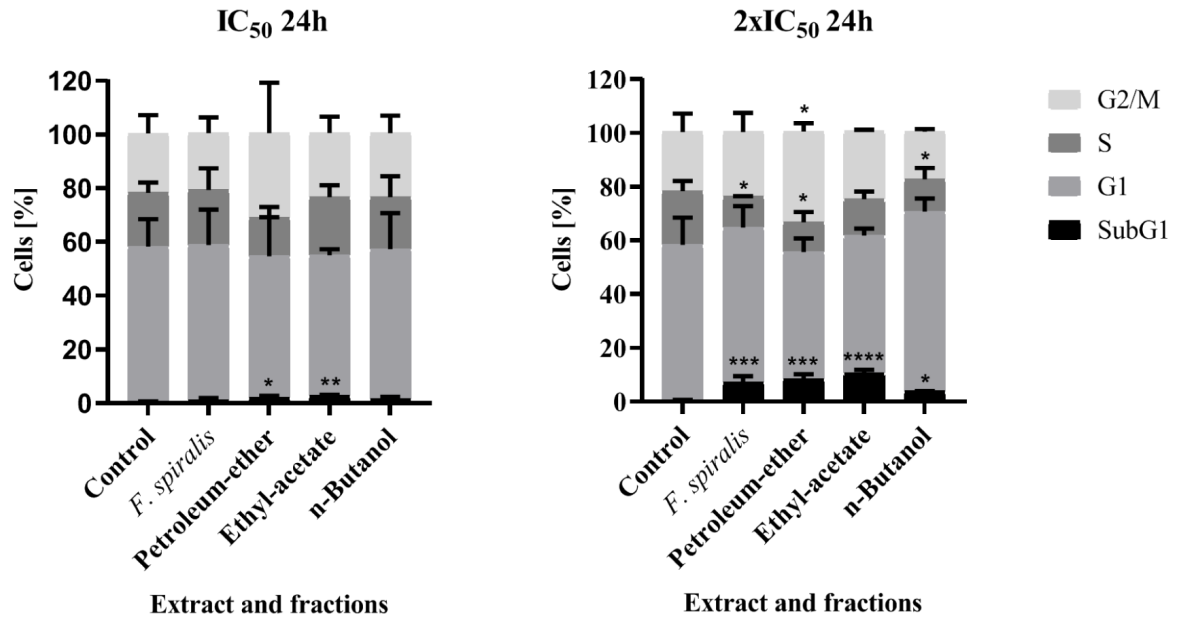

Figure 2. Changes in cell cycle phase distribution of HeLa cells induced by the Fucus spiralis extract and fractions after 24-h treatment (treated cell samples were compared with control cell sample (****p<0.0001, ***p<0.001, **p<0.01, *p<0.05).

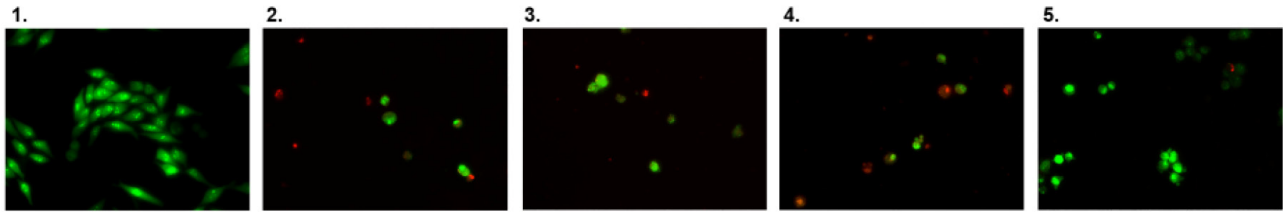

Figure 3. Fluorescence photomicrographs of acridine orange-ethidium bromide stained HeLa cells 24 h after treatment with 2×IC_{50} concentration of F.spiralis extracts and fractions; **1:** control, **2:** extract (supernatant), **3:** petroleum-ether fraction (supernatant), **4:** ethyl-acetate fraction (supernatant), **5:** n-butanol fraction (supernatant). Control cells are attached to the cover slips and have normal morphology. The majority of HeLa cells after treatment are detached from cover slips. Cells treated with 2×IC_{50} concentration of F.spiralis extract and fractions showed typical signs of apoptosis: cells are rounded, with blebbing membrane and condensed or fragmented nuclei. Orange-red stained cells present late apoptosis (apoptotic bodies) and secondary necrosis.

fractions. The extract and n-butanol fraction also induced increase in the percentage of cells in the subG1 cell cycle phase. Furthermore, accumulation of cells in the G2/M phase was observed in HeLa cells incubated in the presence of petroleum-ether and ethyl-acetate fractions, and whole extract.

Fluorescence microscopy

Morphological analysis by fluorescence microscopy showed that the majority of HeLa cells after treatment with extract and fractions were detached from cover slips, and therefore the supernatant samples were analyzed (Figure 3). Control untreated cells were still attached to the cover slips and had normal morphology. HeLa cells treated with 2×IC_{50} concentrations of *Fucus spiralis* extract and fractions showed typical signs of apoptosis: cells had round shape, their membrane was blebbing, and their nuclei were condensed or fragmented. In addition, orange-red stained cells were observed showing the signs of late apoptosis (apoptotic bodies) and secondary necrosis. The ethyl acetate fraction exhibited the strongest proapoptotic effect.

In vitro scratch assay

The influence of the *Fucus spiralis* extract and fractions on the migration of endothelial EA.hy926 cells was examined by *in vitro* scratch assay. The ethyl-acetate fraction showed the best antimigratory activity (percentage of gap reduction was 2.34±2.11), followed by the dichloromethane-methanol extract (6.85±0.64%), and petroleum-ether fraction (8.00±5.07%), while n-butanol frac-

tion exerted the poorest effect (46.20±3.56%); the percentage of gap reduction in control cells was 57.60±1.83 (Figure 4).

Tube formation assay

Endothelial cell tube formation assay was used to examine the *in vitro* antiangiogenic properties of *Fucus spiralis* extract and fractions. As can be seen in Figure 5, the *Fucus spiralis* whole extract, petroleum-ether, and ethyl-acetate fractions inhibited elongation and connections of endothelial EA.hy926 cells grown on the surface of matrigel matrix, and also prevented their organization into tubular structures. Control cells and cells treated with n-butanol fraction showed formation of large vessel structures and complex meshes.

α-glucosidase inhibitory activity

α-glucosidase inhibitory activity of the tested extract and fractions is shown in Table 3. The *Fucus spiralis* extract and fractions showed strong α-glucosidase inhibitory activities. The best activity was observed for ethyl-acetate fraction, followed by petroleum-ether fraction, whole *Fucus spiralis* extract and n-butanol fraction. These results were much better than standard drug acarbose. The tested algal samples demonstrated significantly better α-glucosidase inhibitory activity when compared with the activity of standard drug acarbose.

Microbiology

Results of microbiology analysis are shown in Tables 4 and 5. Best antibacterial activity exhibited n-butanol fraction (from 0.04 mg/mL for *B.cereus* and *B.subtilis* to 0.14 for *P.mirabilis* and *E.coli*), followed by ethyl-acetate (from 0.06 mg/mL for *B.cereus* to 0.47 mg/mL for *E.coli*), petroleum-ether (from 0.17 mg/mL for B. cereus and *B.subtilis* to 1.33 mg/mL for *E.coli*) and whole *Fucus spiralis* extract (from 0.12 mg/mL for *B.cereus* to 0.95 mg/mL for *E.coli*). N-butanol fraction was also most effective against the tested fungal strains (from 0.14 mg/mL for *T. mentagrophytes* and *C.albicans* to 0.56 for *M. mucedo* and *A. niger*), followed by ethyl acetate (from 0.19 mg/mL for *C. albicans* to 1.53 for *M. mucedo*), petroleum-ether (from 0.33 mg/mL for *C. albicans* to 2.67 for *M. mucedo* and *A. niger*) and whole *Fucus spiralis* extract (from 0.95 mg/mL for *T.mentagrophytes* to 3.78 for *M. mucedo* and *A. niger*).

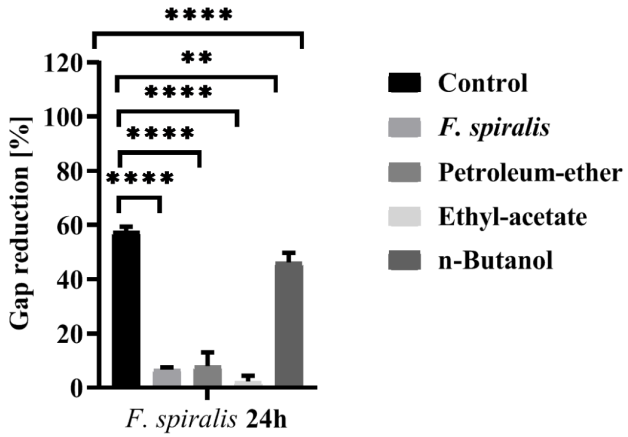

Figure 4. Scratch assay result chart showing percentages of gap reduction with or without treatments of F. spiralis dichloromethane-methanol extract and fractions (****p<0.0001, **p<0.001).

Discussion

Marine macroalgae have shown excellent cytotoxic activities and their dietary consumption is believed to be chemopreventive against several

Table 3. The IC_{50} values of the α-glucosidase inhibitory activity of the extracts and fractions

F. spiralis	*IC_{50} (μg/mL)*
extract	14.18±0.25
petroleum-ether	12.05±0.01
ethyl-acetate	10.37±0.27
n-butanol	16.42±0.45
acarbose	229.35±3.24

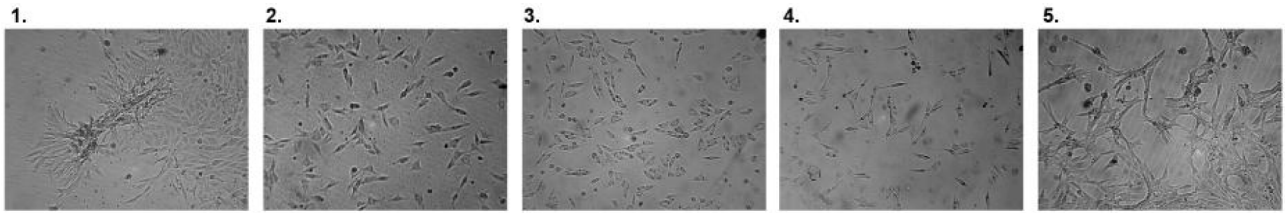

Figure 5. Light microscopy micrographs of EA.hy926 cells incubated in the presence of F.spiralis dichloromethane-methanol extract and their fractions after 24 h. **1:** control, **2:** extract, **3:** petroleum-ether fraction, **4:** ethyl-acetate fraction, **5:** n-butanol fraction. Control ea.hy926 cells and cells treated with n-butanol fraction showed formation of large vessel structures and complex meshes. F.spiralis extract, petroleum-ether, and ethyl-acetate fractions inhibited elongation and connections of endothelial cells.

Table 4. The antibacterial activity of the extract and fractions

Microorganisms	*Staphylococcus aureus*	*Bacillus subtilis*	*Bacillus cereus*	*Escherichia coli*	*Proteus mirabilis*
F. spiralis			*MIC*		
extract	0.47	0.24	0.12	0.95	0.47
petroleum-ether	0.33	0.17	0.17	1.33	0.67
ethyl-acetate	0.23	0.12	0.06	0.47	0.23
n-butanol	0.07	0.04	0.04	0.14	0.14
streptomycin	0.03	0.02	0.02	0.06	0.06

Minimum inhibitory concentration (MIC); values given as mg/mL for extracts and antibiotic

Table 5. The antifungal activity of the extract and fractions

Microorganisms	*Mucor mucedo*	*Trichophyton mentagrophytes*	*Aspergilus niger*	*Candida albicans*	*Penicillium italicum*
F. spiralis			*MIC*		
extract	3.78	0.95	3.78	0.47	1.89
petroleum-ether	2.67	0.67	2.67	0.33	1.33
ethyl-acetate	1.53	0.38	0.76	0.19	1.53
n-butanol	0.56	0.14	0.56	0.14	0.28
ketokonazole	0.16	0.08	0.08	0.04	0.16

Minimum inhibitory concentration (MIC); values given as mg/mL for extracts and antimycotic

cancer types (breast cancer, gastrointestinal, and skin cancers) [28,29].

Our results showed that *Fucus spiralis* is rich in fatty acids and has fair amount on PUFAs. Other researchers have found that PUFA to SFA ratio in *Fucus spiralis* extract vary due to the season and place they were harvested [30]. It is observed that algae found in warm waters have more SFA than the ones grown in cold waters, and that season influence contributes to different fatty acids profile [31]. It has been shown that n-3 PUFAs may contribute to reduced cancer and cardiovascular risks. Consumption of fatty acids can lower the occurrence of chronic diseases like cancer, diabetes, obesity and heart diseases [32].

Fucales and *Fucus spiralis* are known for their high phenolic content and antioxidative activities [4,33]. It is found that phlorotannins, the unique polyphenols found in brown algae have cytotoxic, antioxidative, antidiabetic, and antimicrobial properties [34,35]. Ethyl acetate is used to elute phlorotannins [36]. In this research, the ethyl-acetate fraction showed the highest DPPH free radical scavenging activity despite the fact that the n-butanol fraction had the highest total phenolic content. Although many studies have shown direct correlation between total phenolic content and DPPH activity, many others didn't find direct link [4, 37]. Beside polyphenols, other bioactive compounds can contribute to the antioxidative action of algal extracts and fractions [33]. In the same algal extract there are other components that exert synergistic or antagonistic effects on the radical scavenging activities [4]. Therefore, the type and content of different polyphenols in the extracts and their synergistic or antagonistic actions should be taken into account when considering the bioactivity of the algal extracts and fractions. In 2010, Luo et al reported that ethyl acetate and petroleum ether fractions had the highest DPPH effects, suggesting that phenolic compounds with medium polarity might be major contributors to the antioxidative activity of brown seaweeds [38].

Our results demonstrated that the algal extract and some fractions had pronounced and highly selective cytotoxic activity in HeLa cells, and less in LS174T and A549 cells. The best cytotoxic activities were exerted in petroleum-ether and ethyl acetate fractions that have plenty of fatty acids and polyphenols. To the best of our knowledge, these are the first findings about anticancer activity of the extract and fractions obtained from *Fucus spiralis* from this region and their mechanism of action. Earlier studies have shown that also the fucoidan in the whole extract could be responsible for cytotoxic effects, since it was found that fucoidan from *Sargasum* and *Fucus vesiculosus* reduced the viability of lung carcinoma cells and melanoma cells [39]. Also, Alves et al [40] showed cytotoxic activity of *Fucus spiralis* extract from Portugal shoreline on human hepatocellular carcinoma cells. It has been suggested that different classes of primary and sec-

ondary metabolites such as terpenes, phenols, fatty acids, hydrocarbons, and fucoxanthin are responsible for the cytotoxic effects of *Fucus spiralis* extract on MCF-7 cells [41].

The investigation of the effects on changes in cell cycle phases revealed that *Fucus spiralis* extract and fractions exerted pro-apoptotic effects in HeLa cells in a dose-dependent manner. Highest accumulation of cells in the subG1 and G2/M phases were shown by ethyl-acetate and petroleum-ether fractions, rich in polyphenols and fatty acids. Both polyphenols and fatty acids are found to have proapoptotic effects and influence on cell cycle arrest [10,42,43]. Beside polyphenols and fatty acid influence on anticancer activities, fucoidan could be one of the active components in *Fucus spiralis* extract behind these activities. Fucoidan was found to induce cell cycle arrest and apoptosis [7]. Furthermore, Kim et al [7] showed that fucoidan inhibited the proliferation of human colon cancer cells HT-29 and HTC116 and induced apoptosis, while at the same time had no toxic effects on normal human colon FHC cells. Fucoidan could also have synergistic effects with current anticancer agents thus preventing toxicity [44].

Results of the *in vitro* scratch assay showed that ethyl-acetate fraction, followed by *Fucus spiralis* extract and petroleum-ether fraction exerted the best antimigratory activity. These results may suggest that polyphenol and fatty acids contributed to the inhibition of migration of endothelial cells as found in the literature [10,43]. In 2012, Ferres et al showed that phlorotannins prevent cancer cell migrations [45]. Furthermore, fucoidan decreased metastasis in rats and in MDA-MB-231 cells [7,44].

Antiangiogenic effects in our experiments were demonstrated by *Fucus spiralis* extract, ethyl-acetate and petroleum-ether fractions. This may suggest the role of polyphenols and fatty acids as potential antiangiogenic agents, as confirmed by the literature data [10,46]. Also, we can't exclude the role of fucoidan in the extract, as literature data report that it may suppress angiogenesis via inhibition of binding vascular endothelial growth factor (VEGF) to the cell membrane receptor [7,46].

The tested extract and fractions showed similar effects and much better anti-α-glucosidase action than standard acarbose. Studies have shown that polyphenols, beside their ability to reduce oxidative stress, can also inhibit the polysaccharide hydrolyzing enzymes, thus preventing hyperglycemia [47,48]. It has been shown that fatty acids and phlorotannins can inhibit α-glucosidase [49]. Our results also indicate that other extract constituents, beside polyphenols, and fatty acids all together contributed to the high rate of α-glucosidase inhibition. This result is in accordance with study of *Fucus spiralis* ethanol-based extract which showed α-glucosidase and amylase inhibitory activities [50].

Microbiology results correlate with total phenolic and fatty acids content of extract and fraction as n-butanol fraction had the highest phenolic content, and petroleum-ether fraction was rich in fatty acids. Literature data showed that polyphenols and fatty acids have antimicrobial activities [51,52]. Researchers also found that phlorotannin rich extract and terpenes could be responsible for antimicrobial activities [53-55]. Earlier studies, using less sensitive disk-diffusion method, also showed the *Fucus spiralis* antimicrobial action [8,56-58]. Etahiri et al in 2003 [59] recorded that seasonal variation had influence on antibacterial activity and that algae collected in March to May had better antibacterial activity than algae collected in December and January.

Conclusion

The extract and fractions obtained from *Fucus spiralis* showed cytotoxic effects against cancer cell lines, while at the same time they were less toxic against normal MRC-5 fibroblasts. The strongest cytotoxic activities was exerted in petroleum-ether fraction, ethyl-acetate fraction, and dichloromethane-methanol extract against HeLa cell line. These two fractions induced increase of HeLa cells within subG1 and G2/M cell cycle phases. Furthermore, they demonstrated antimigratory and antiangiogenic effects *in vitro*. These results, in addition to other biological properties of tested extract and fractions, may suggest a promising anticancer and pharmacological potential of *Fucus spiralis*.

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Conflict of interests

The authors declare no conflict of interests.

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