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SEASONAL VARIATION IN BIOPHARMACEUTICAL ACTIVITY AND FATTY ACID CONTENT OF ENDEMIC *FUCUS VIRSOIDES* ALGAE FROM ADRIATIC SEA

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Abstract: Macroalgae from *Fucus* genus are a valuable source of bioactive components as they are abundant in complex polysaccharides, fatty acids and polyphenols. In this work, the biological activity and chemical composition of extracts and fractions obtained from endemic *Fucus virsoides* J. Agardh species collected in the summer and the fall were investigated. From dichloromethane:methanol (1 : 1) extract three fractions were made: petroleum-ether, ethyl-acetate and n-butanol. The aim of the study was to examine the influence of the seasonal variations on algal composition and activity. The significant seasonal variation in content and biological activity of *Fucus virsoides* samples was found. Fall extract and fractions exerted higher cytotoxic effects on cancer cell lines in comparison with summer extract and fractions. The examined extracts and fractions showed higher cytotoxic activity towards cancer cells compared to normal fibroblast MRC-5 cells. Morphological evaluation and cell cycle distribution analysis demonstrated their proapoptotic activity in human cervical adenocarcinoma HeLa cells. Fall extract and fractions better suppressed the migration and tube formation of EA.hy926 cells in comparison with summer extract and fractions. Fall extract and fractions were more potent in inhibition of α-glucosidase enzymatic activity. Ethyl-acetate fractions, from both seasons, exhibited the best antibacterial and antifungal activity on all tested bacteria and fungi. In conclusion, the two fall fractions ethyl-acetate and petroleum-ether rich in polyphenols and polyunsaturated fatty acids were the most active and exhibited prominent anticancer and anti-α-glucosidase activities.

Keywords: Cytotoxic, *Fucus virsoides*, fatty acid, α-glucosidase

Fucus virsoides J. Agardh is an endemic brown alga found only in the Adriatic Sea. Other members of the *Fucus* genus have shown anticancer potential, namely *F. vesiculosus* L. (1) and its most active component fucoidan. Algae from the *Fucus* genus are rich in active biological compounds such as polysaccharides, polyphenols, fatty acids and vitamins (2). Fucoidans have been documented for numerous biological activities such as antiviral, anti-inflammatory, anticoagulant, anti-angiogenic, immunomodulatory, and anti-adhesive activities (3, 4). The biological effects of these compounds can vary due to chemical structure differences that depend on the species from which they have been isolated (3). The anticancer or antidiabetic properties of *Fucus virsoides* have not been investigated in detail.

Polyphenols, secondary metabolites, have been studied for their role in the prevention of cancer, diabetes, cardiovascular diseases, osteoporosis, and neurodegenerative diseases. Polyphenols exert antioxidant, free radical scavenging activities; they

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can affect enzyme activity, cell receptor function and various intracellular signal transduction pathways. Many studies have been focused on polyphenol antimicrobial activities, given that they can inhibit the formation of biofilms, reduce host ligand adhesion, neutralize bacterial toxins and show synergism with antibiotics. Given the increase in the microbial resistance against standard antibiotic and antimycotic therapies, polyphenols have potential as novel treatments for drug-resistant bacterial and fungal infections (5). Fatty acids from macroalgae extracts may also be used as potential antimicrobial agents (6). In addition, they may have therapeutical benefit in several major diseases, such as cancer, type 2 diabetes, coronary heart disease, arrhythmias, atherosclerosis, and inflammation (7). The balanced ratio of n-3 to n-6 fatty acids found in macroalgal extracts may have health beneficial effects. The seasonal variations of environmental conditions play an important role in the life cycle of the algae and the composition of its secondary metabolites (8).

Cancer and diabetes type 2 have a biological connection. Diabetes can impact the malignant transformation when the organism is in a prolonged state of hyperinsulinemia, hyperglycemia or chronic inflammation (9). The α-glucosidase is an enzyme found at the membrane of the small intestine enterocytes (10, 11). It plays an important role in the digestion of polysaccharides, as it catalyzes their breakdown to the monosaccharides that can then be absorbed (12). Thus, inhibition of α-glucosidase could be important for the prevention and the treatment of diabetes type 2.

Standard drugs used for cancer treatment and α-glucosidase inhibitors have many side effects. Many contemporary cancer drugs are derived from natural products (e.g. vincristine, taxol), and given their bioactive properties algae extracts have great potential for both diabetes and cancer drug development.

To the best of our knowledge, *F. virsoides* species has not been studied in detail for its anticancer properties, especially for anti-angiogenesis and antimigration activities, or for antidiabetic effects.

The aim of this study was to investigate biological activity and chemical composition of extracts and fractions prepared from endemic *F. vir-*

Table 1. Fatty acid content of *F. virsoides* (petroleum-ether fraction).

	Fatty acids	*F. virsoides* fall %	*F. virsoides* summer %
Lauric acid	C 12 : 0	0.52	/
Myrisitc acid	C 14 : 0	11.22	13.62
Pentadecanoic acid	C 15 : 0	1.18	0.57
Palmitic acid	C 16 : 0	16.50	20.51
Stearic acid	C 18 : 0	1.84	1.49
ΣSFA	ΣSFA	31.26	36.19
Palmitoleic acid	C 16 : 1	3.05	1.72
Oleic acid	C 18 : 1 n-9	22.59	26.01
ΣMUFA	ΣMUFA	25.64	27.73
Linoleic acid (LA)	C 18 : 2 n-6	5.38	5.45
Alfa linolenic acid (ALA)	C 18 : 3 n-3	8.38	8.51
Eicosadienoic acid	C 20 : 2 n-6	3.30	/
Arachidonic acid (AA)	C 20 : 4 n-6	12.61	8.38
Dihomo-gamma-linolenic acid (DGLA)	C 20 : 3 n-6	0.71	/
Eicosapentaenoic acid (EPA)	C 20 : 5 n-3	6.86	3.66
ΣPUFA	ΣPUFA	37.24	26.00
PUFA/SFA	PUFA/SFA	1.19	0.72
n-9	n-9	22.59	26.01
n-6	n-6	22.00	13.83
n-3	n-3	15.24	12.17

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

soides species, collected in the summer and the fall, as well as to explore the influence of the seasonal variations on their composition and activity.

EXPERIMENTAL

Algae collection and preparation of extracts and fractions

Fucus virsoides J. Agardh was collected at Boka Kotorska bay, Montenegro, in late September 2014 (fall sample), and July 2015 (summer sample). Vouchers were deposited in the Natural History Museum in Podgorica, Montenegro, collection numbers 162 (a, b), inventory number 6527-600podalg. Prior to extraction, algae material was washed with fresh water and dried at room temperature. Ground algae were extracted with a mixture of dichloromethane:methanol (1 : 1) by stirring at room temperature. Extraction was repeated until the colorless filtrate was obtained. All filtrates were combined and the solvent was evaporated under vacuum at the temperature below 40°C. The extract was then suspended in 100 mL distilled water and successively extracted with petroleum ether (Pet-Et; 3 × 100 mL), ethyl-acetate (EtOAc; 3 × 100 mL) and n-butanol (n-BuOH; 3 × 100 mL). Obtained extracts and fractions were stored at -20°C until further analysis.

Fatty acid assay

Fatty acids from marine plants dry petroleum-ether fractions were trans-esterified with hydrochloric acid in methanol, according to the method described by Ichihara and Fukubayashi, (13), 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 reference fatty acid standards (Supelco FAME Mix, Bellefonte, PA). The results were expressed as a percentage of individual fatty acid in total dry petroleum-ether fractions.

Total phenolic content

Content of total phenolics in extracts and fractions was analyzed using a modified Folin-Ciocalteu method (14). Briefly, stock solutions of the extracts (100 mg/mL in DMSO) were diluted in deionized water (50% vol) and filtered through 0.45 μm filters. A 0.125 mL of the obtained solutions were added to 0.5 mL of deionized water and 0.125 mL of Folin–Ciocalteu reagent mixed thoroughly and incubated for 6 min at room temperature (RT). Afterward, a 1.25 mL of 7% sodium-carbonate water solution and 0.75 mL of deionized water were added, mixed and after 90 min of incubation at RT, the absorbencies of the solutions were measured at 760 nm. Results were expressed as milligram of Gallic acid equivalents (GAE) per gram of investigating extracts based on the standard calibration curve obtained with a series of Gallic acid standard solutions within the concentration range of 5-600 μg/mL. Data are presented as mean ± SD for three replications. Statistical analysis was performed using One-way ANOVA with Tukey's multiple comparison test.

DPPH free radical scavenging assay

The antioxidant activity of investigated extracts and fractions 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 (15) with slight modifications. The antiradical activity was evaluated using a dilution series, in order to obtain a large spectrum of sample concentrations. Algae extracts were mixed with a methanolic solution of DPPH. Absorbance at 517 nm was measured after 30 min of incubation on RT in the dark. The percentage of inhibition was calculated using the following equation: RSA (%) = [(A0-Ai)/A0] × 100, where A0 is absorbance of the control and Ai is absorbance of the samples. All test analyses were run in triplicate. Statistical analysis was performed using One-way ANOVA with Tukey's multiple comparison test.

In vitro cytotoxicity

The tested cell lines: human cervical adenocarcinoma (HeLa), colorectal adenocarcinoma (LS-174T), lung epithelial carcinoma (A549) and lung fetal fibroblasts (MRC-5) were maintained in RPMI-1640 medium. Somatic human umbilical vein endothelial (EA.hy926) cell line was grown in DMEM. The cell lines were obtained from the American Type Culture Collection (USA). Stock solutions of extracts and fractions (50 mg/mL) were dissolved in dimethylsulfoxide (DMSO). HeLa, LS-174T, A549, MRC-5, and EA.hy926 cells were seeded into 96-well microtiter plates. Seeding densi-

ties for each cell line were: 2000, 7000, 5000, 5000 and 5000 cells per well, respectively. Cells were allowed to adhere overnight and treated with serial dilutions of extracts and fractions ranging from 12.5 to 200 µg/mL, according to the protocol described previously (16). The final concentrations of DMSO to which the cells were exposed were non-toxic, lower than 0.5%. Experiments were repeated three times.

The effects of the extracts and fractions on cell survival after 72 h of treatment (for HeLa, LS-174T, A549, and MRC-5), and 24 h treatment (for EA.hy 926 cells), were determined by MTT cell survival assay, described in detail previously (16). IC_{50} was defined as the concentration of an agent that inhibited cell survival by 50% compared to control.

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 × IC_{50} concentrations of investigated extracts 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 (16). The cells were washed and incubated with RNaseA (100 µg/mL) at 37°C for 30 min. Next, cells were incubated with propidium iodide (40 µg/mL) and cell cycle phase distribution was analyzed by FACSCalibur flow cytometer (BD Biosciences Franklin Lakes, NJ, USA) using CELLQuest software. The results were obtained from two independent experiments and presented with standard deviations.

Fluorescence microscopy

HeLa cells were seeded (50000 cells per well) into 6-well plates on coverslips. The next day, tested extracts and fractions were added to cells at concentrations 2 × IC_{50}. After 24 h of treatment, the cells were stained with a mixture of acridine orange and ethidium bromide dyes (3 µg/mL acridine orange and 10 µg/mL ethidium bromide in phosphate-buffered saline (PBS). Photo-micrographs were taken under a fluorescence microscope – Carl Zeiss PALM MicroBeam with AxioObserver.Z1 using AxioCamMRm (filters Alexa 488 and 568), as previously described (16).

***In vitro* scratch assay**

EA.hy926 cells were seeded into 24-well plate. Confluent cell monolayers were formed after 24 h and scarped with a p200 pipette tip to create a straight central scratch line, as described earlier (16). After washing with nutrient medium cells were treated with subtoxic concentrations (IC_{20}) of extracts and fractions for 24 h. IC_{20} concentration is the concentration that inhibits 20% of cell survival, and values for fall samples were 88.0 µg/mL, 94.3 µg/mL, 72.9 µg/mL, 122.1 µg/mL and for summer 147.2 µg/mL, 156.6 µg/mL, 160.9 µg/mL, 161.8. µg/mL for dichloromethane-methanol, petroleum-ether, ethyl-acetate, and n-butanol extracts and fractions respectively. Photomicrographs were captured immediately after making the wound and 24 h later under the inverted phase-contrast microscope.

Tube formation assay

EA.hy926 cells were plated on the surface of the wells coated with 200 µL of Corning® Matrigel® basement membrane matrix. The assay was described previously elsewhere (16). Afterward, in control wells, the nutrient medium was added, while solutions of subtoxic concentrations (IC_{20}) of extracts and fractions were added to other wells. After 20 h of incubation, photomicrographs of cells

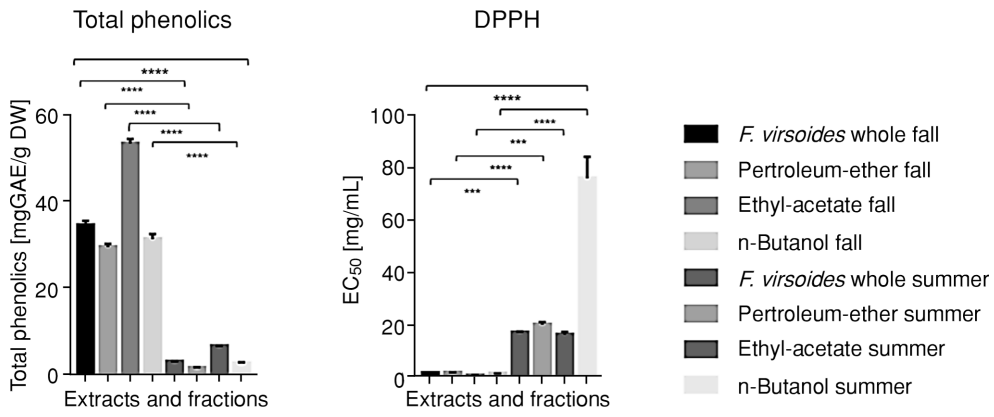

Figure 1. Total phenolic content and DPPH of algal extracts and fractions (**** $p < 0.0001$, *** $p < 0.001$)

Table 2. Cytotoxic activity of the investigated extracts and fractions.

Algae extracts /IC_{50}, µg/mL	HeLa	LS174	A549	MRC-5
F. virsoides fall	53.91 ± 1.48	138.68 ± 4.99	183.40 ± 2.86	> 200
Petroleum-ether	44.38 ± 2.86	146.90 ± 8.72	147.50 ± 0.59	> 200
Ethyl-acetate	55.85 ± 9.91	120.33 ± 4.55	143.40 ± 0.98	> 200
n-butanol	112.67 ± 35.26	174.96 ± 11.60	> 200	> 200
F. virsoides summer	175.15 ± 1.39	195.81 ± 2.66	196.91 ± 2.01	> 200
Petroleum-ether	161.98 ± 1.42	193.44 ± 4.42	195.39 ± 1.58	> 200
Ethyl-acetate	157.67 ± 0.41	185.21 ± 0.76	166.49 ± 4.66	> 200
n-butanol	> 200	> 200	> 200	> 200

Concentrations of examined extracts inducing 50% decrease in cells survival rate (expressed as IC_{50} value). Results are presented as the mean value ± SD of three independent experiments

were taken under the inverted phase-contrast microscope.

α-glucosidase inhibitory activity

Extracts and fractions were tested for α-glucosidase inhibitory activity using the method described by McCue et al. (17) with some modifications. Briefly, 400 mU/mL of α-glucosidase (*from Saccharomyces cerevisiae*, was dissolved in 0.1 M phosphate buffer (pH = 6.8). Extracts dissolved in DMSO were diluted in 0.1 M phosphate buffer (pH = 6.8) so that the final concentrations of the extracts in each well were 20.83, 10.42 and 5.21 µg/mL. The extract dilutions or 10% DMSO for blank were preincubated with enzyme solution at 37°C for 15 min. Next, substrate solution *p*-nitrophenyl α-D-glucopyranoside (1.5 mg/mL in phosphate buffer) was added into each well. After measuring absorbance A1 at 405 nm the solution was incubated at 37°C for 5 min. Second absorbance A2 was measured at 405 nm. Acarbose was used as a positive control. Experiments were conducted in duplicate and IC_{50} value (estimated concentration of extracts that caused 50% inhibition of α-glucosidase activity) was determined using linear regression analysis.

Antimicrobial activity

All the bacteria and fungi used were obtained from the American Type Culture Collection (ATCC). Bacterial and fungal cultures were maintained and prepared as previously described by Kosanic et al (18). The minimal inhibitory concentration (MIC) was determined by the broth microdilution method with using 96-well microtiter plates (19). 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. Further steps have already been described in detail (18). As a positive control of growth inhibition, streptomycin was used in the case of bacteria, ketoconazole in the case of fungi. DMSO solution was used as a negative control for the influence of the solvents.

RESULTS

Fatty acid assay

Analysis of fatty acid content revealed seasonal differences between the examined extracts and fractions. Fatty acids contents of summer and fall petroleum-ether fractions of *F. virsoides* are shown in Table 1. Both fractions were rich in fatty acids. Total percentages of saturated fatty acids (SFA) were 31.26% for fall fraction and 36.19% for summer fraction; for monounsaturated fatty acids (MUFA) were 25.64% for fall fraction and 27.73% for summer fraction, and for polyunsaturated acids (PUFA) were 37.24% for fall sample and 26.00% for summer sample. Among detected SFAs in both fractions, the most abundant was palmitic acid (16.5% fall fraction, 20.51 % summer fraction), followed by myristic acid, stearic acid, and pentadecanoic acid, while lauric acid was only detected in the fall fraction. Oleic acid was dominant MUFA in both fractions; 22.59% in the fall fraction, 26.01% summer fraction, while the content of palmitoleic acid was significantly lower. Arachidonic acid (12.61% in fall fraction, 8.38% in summer fraction), alpha-linolenic acid (8.38% in fall, 8.51% summer fraction) and linoleic acid (5.38% in fall, 5.45% in summer fraction) were the most abundant among PUFAs in both tested fractions, while eicosadienoic acid and dihomo-gamma-linolenic acid were detected only in fall fraction. Fall fraction of *F. virsoides* had better polyunsaturated fatty acids (PUFA) to

saturated fatty acids (SFA) ratio and higher content of n-6 and n-3 fatty acids when compared with summer sample.

Total phenolic content and DPPH radical scavenging activity

The radical scavenging activities and total phenolic contents of algal extracts and fractions are shown in Figure 1. *F. virsoides* fall extract and fractions exerted stronger radical scavenging activity in comparison with summer extract and fractions. The highest phenolic content and lowest IC_{50} values for DPPH scavenging activity were found in fall ethyl-acetate fraction (53.57 ± 1.06 mgGAE/g and 1.38 ± 0.05 mg/mL respectively), followed by *F. virsoides* fall extract (34.78 ± 0.96 mgGAE/g, 2.28 ± 0.03 mg/mL), n-butanol (31.59 ± 1.17 mgGAE/g, 2.07 ± 0.04 mg/mL) and petroleum-ether (29.73 ± 0.78 mgGAE/g, 2.48 ± 0.04 mg/mL) fractions. Summer fractions had lower polyphenol content when compared with fractions, ranging from 7.00 ± 0.06 mgGAE/g for ethyl-acetate fraction to 1.99 ± 0.12 mgGAE/g for petroleum-ether fraction. DPPH scavenging activity for summer fractions varied from

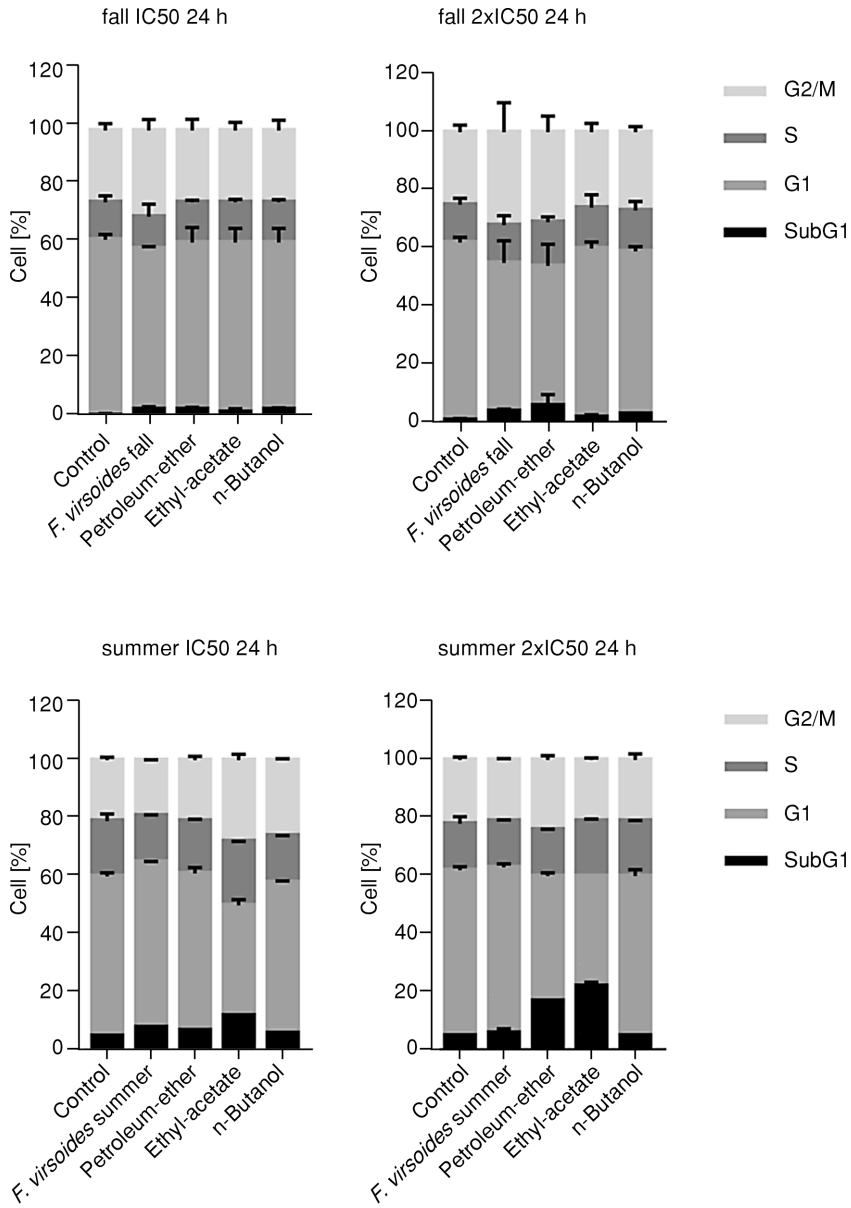

Figure 2. Cell cycle changes in phase distribution of HeLa cells induced by *F. virsoides* extracts and fractions after 24 h of treatment

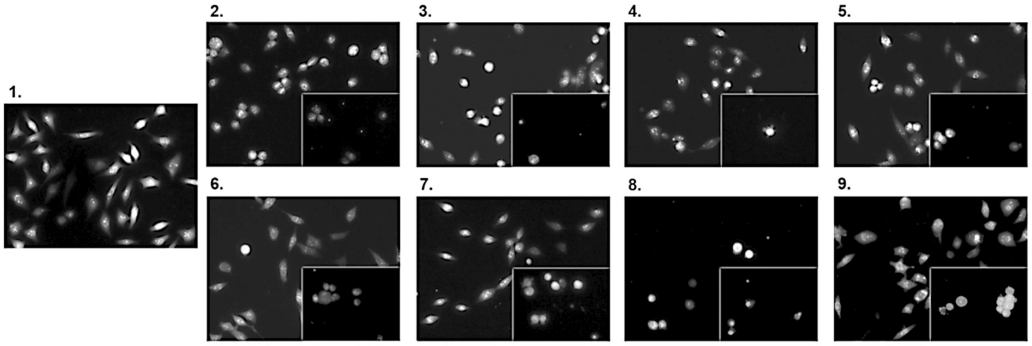

Figure 3. Fluorescence photomicrographs of acridine orange-ethidium bromide-stained HeLa cells 24 h after treatment with *F. virsoides* extracts and fractions; matching supernatant micrographs are displayed in bottom right corners. 1) control, 2) whole fall extract, 3) petroleum-ether fall fraction, 4) ethyl-acetate fall fraction, 5) n-butanol fall fraction, 6) whole summer extract, 7) petroleum-ether summer fraction, 8) ethyl-acetate summer fraction, 9) n-butanol summer fraction

16.86 ± 1.08 mg/mL for ethyl-acetate to 76.28 ± 8.23 mg/mL for n-butanol fraction.

In vitro cytotoxicity

The cytotoxic activities of the extracts and fractions are shown in Table 2. Human cervical adenocarcinoma HeLa cells were the most sensitive to the cytotoxic effects of the tested extracts and fractions. The strongest cytotoxic activity on HeLa cells was exerted by the *F. virsoides* fall petroleum-ether fraction with an IC_{50} value of 44.38 ± 2.86 µg/mL, followed by the fall dichloromethane-methanol extract with an IC_{50} value of 53.91 ± 1.48 µg/mL and ethyl-acetate fall fraction with an IC_{50} value of 55.85 ± 9.91 µg/mL. Summer extract and fractions showed the lower intensity of cytotoxic activity on HeLa cells when compared with the activity of fall extract and fractions, with IC_{50} values ranging from 157.67 ± 0.41 µg/mL for ethyl-acetate to more than 200 µg/mL for n-butanol fraction. All tested extracts and fractions exerted lower intensities of cytotoxic activity on human colon adenocarcinoma LS174 and lung carcinoma A549 cells when compared with their activities on HeLa cells. The ethyl-acetate fall fraction was the most active on LS174 cells with IC_{50} value of 120.33 ± 0.4.55 µg/mL, while the summer extract and summer petroleum-ether and n-butanol fractions showed the lowest cytotoxic effects on LS174 cells with IC_{50} values of 195.81 ± 2.66, 193.44 ± 4.42, and higher than 200 µg/mL, respectively. A549 cells were the most sensitive to the cytotoxic action of the fall petroleum-ether and ethyl-acetate fractions (IC_{50} values of 147.50 ± 0.59 and 143.40 ± 0.98 µg/mL). The lowest cytotoxicity against A549 cells showed summer extract, summer petroleum-ether fraction and n-butanol fraction (IC_{50} values of 196.91 ± 2.01, 195.39 ± 1.58, and higher than 200 µg/mL, respectively). Each of the tested extracts and fractions showed the notably lower intensity of the cytotoxic activity on normal lung fibroblasts MRC-5 in comparison with their activity on cancer cell lines. The fall extract and fractions exerted stronger cytotoxic activities on all the tested cancer cell lines when compared with the summer extract and corresponding fractions.

Cell cycle analysis

Cell cycle distribution analysis revealed that fall *F. virosides* extract applied at IC_{50} concentration caused increase in the percentage of HeLa cells within G2/M phase (29.95 ± 3.82%, compared to control 24.73 ± 2.38%) after 24 h treatment The fall extract (31.82 ± 10.14%) and petroleum-ether fraction (31.23 ± 5.48%) applied at $2 \times IC_{50}$ concentration showed the highest accumulation of HeLa cells within G2/M phase compared to control (Fig. 2). The summer extract and fractions induced a higher overall increase of cells in subG1 cell cycle phase when compared with fall extract and fractions, applied at $2 \times IC_{50}$ concentrations. Among the tested extracts and fractions, ethyl-acetate and petroleum-ether summer fractions exerted the highest increase in the percentage of cells in subG1 phase of the cell cycle (21.56 ± 1.44% and 17.25 ± 0.12%, respectively; applied at $2 \times IC_{50}$ concentrations) (Fig. 2).

Fluorescence microscopy

HeLa cells morphology was observed under a fluorescence microscope after 24 h incubation with $2 \times IC_{50}$ concentrations of *F. virosides* extracts and fractions. HeLa cells exposed to the fall extract and fractions showed typical signs of apoptosis: still attached cells had round shape, their membrane was blebbing, and their nuclei were condensed or frag-

mented, while orange-red stained cells in the supernatant were showing the signs of late apoptosis (apoptotic bodies) and secondary necrosis (Fig. 3). The fall extract and petroleum-ether fraction exerted the strongest pro-apoptotic activities. HeLa cells treated with summer extract and fractions also showed signs of early and late apoptosis. The ethyl acetate fraction exhibited the strongest effect (Fig. 3).

In vitro scratch assay

In vitro scratch assay was used to explore the influence of the *F. virsoides* extracts and fractions on the migration of endothelial EA.hy926 cells. The fall extract and its ethyl-acetate fraction showed the best antimigratory activity (percentage of gap reduction was 9.80 ± 0.49), followed by the petroleum-ether fraction (15.17 ± 2.16%) and dichloromethane-methanol fraction (19.55 ± 3.93%), while n-butanol fraction exerted the poorest effect (45.65 ± 8.89%) (Fig. 4). The summer extract and fractions exhibited weaker antimigratory effects compared to the fall extract and fractions. Among summer samples, the dichloromethane-methanol fraction was the most effective in suppressing EAhy.926 cell migration (the percentage of gap reduction was 38.68 ± 2.55). Slightly lower antimigratory effect was exerted by summer petroleum-ether fraction (41.36 ± 9.56%). The effect of the ethyl-acetate fraction was lower when compared with the effect of the previous two fractions (49.84 ± 8.78%). The n-butanol fraction was the least effective in inhibiting cell migration (85.37 ± 7.65%).

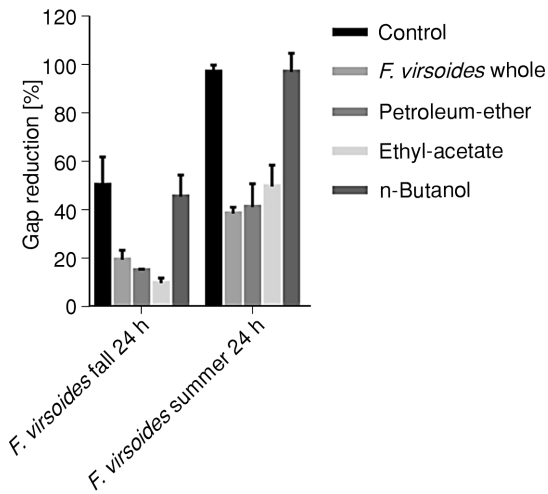

Figure 4. Scratch assay result chart showing percentages of gap reduction with or without treatments of two *F. virsoides* dichloromethane-methanol extracts and their fractions

Tube formation assay

The angiogenic assessment showed that fall and summer ethyl-acetate fraction exerted *in vitro* anti-angiogenic effects and prevented any tube formation or cells interaction. The fall extract and petroleum-ether fraction were the less effective in inhibiting angiogenesis of EA.hy926 cells. Summer extract and other tested fractions did not show any anti-angiogenic effects. The treated cells were similar to control EA.hy926 cells in which polygonal structures and complex meshes were observed, as shown in Figure 5.

α-glucosidase inhibitory activity

The IC_{50} values for the α-glucosidase inhibitory activity of the extracts and fractions are given in

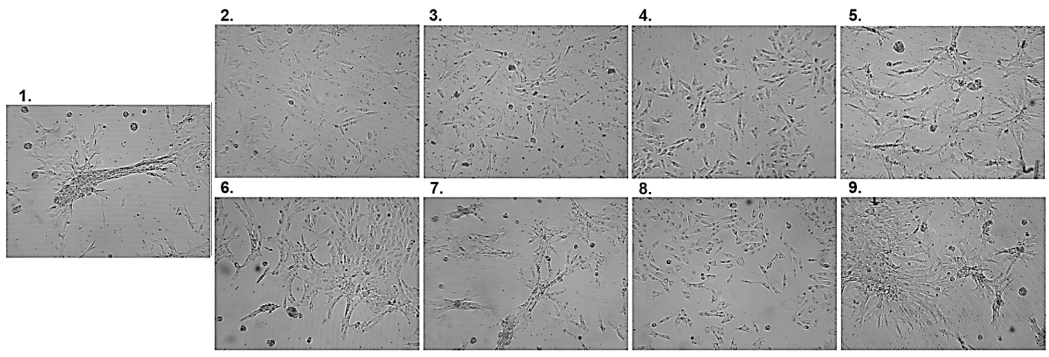

Figure 5. Light microscopy micrographs of EA.hy926 cells incubated in the presence of two *F. virsoides* dichloromethane-methanol extracts and their fractions after 24 h. 1) control, 2) whole fall extract, 3) petroleum-ether fall fraction, 4) ethyl-acetate fall fraction, 5) n-butanol fall fraction, 6) whole summer extract, 7) petroleum-ether summer fraction, 8) ethyl-acetate summer fraction, 9) n-butanol summer fraction

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

Algae	IC_{50} (µg/mL)
F. virsoides fall	20.05 ± 0.33
Petroleum-ether	15.90 ± 0.12
Ethyl-acetate	14.98 ± 0.07
n-butanol	24.42 ± 0.78
F. virsoides summer	90.75 ± 0.81
Petroleum-ether	45.85 ± 1.28
Ethyl-acetate	72.57 ± 3.40
n-butanol	132.60 ± 19.20
Acarbose	229.35 ± 3.24

Table 3. Fall extract and fractions showed better α-glucosidase inhibitory effects than the summer ones. Ethyl-acetate fall extract showed the most potent inhibitory activity with an IC_{50} value of 14.98 ± 0.07 µg/mL, closely followed by petroleum-ether fraction (IC_{50} 15.90 ± 0.12 µg/mL). All algal extracts and fractions showed significantly better α-glucosidase inhibitory activity when compared with the activity of standard drug acarbose (IC_{50} 229.35 ± 3.24 µg/mL).

Microbiology

The antibacterial and antifungal activities of the extracts and fractions are shown in Tables 4 and 5.

Table 4. The antibacterial activity of the extract and fractions.

Microorganisms	*Staphylococcus aureus*	*Bacillus subtilis*	*Bacillus cereus*	*Escherichia coli*	*Proteus mirabilis*
Extracts	MIC				
F. virsoides fall	5.86	5.86	2.93	11.72	11.72
Petroleum-ether	3.50	3.50	1.75	7.00	3.50
Ethyl-acetate	1.25	0.63	0.31	1.25	1.25
n-butanol	2.50	1.25	1.25	2.50	2.50
F. virsoides summer	5.31	2.65	2.65	10.62	10.62
Petroleum-ether	11.30	5.65	5.65	11.30	11.30
Ethyl-acetate	0.62	0.31	0.31	1.25	0.62
n-butanol	6.90	3.45	3.45	/	6.90
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*
Extracts	MIC				
F. virsoides fall	11.72	5.86	11.72	2.93	11.72
Petroleum-ether	7.00	7.00	/	3.50	/
Ethyl-acetate	1.25	1.25	2.50	0.31	2.50
n-butanol	2.50	2.50	2.50	1.25	2.50
F. virsoides summer	10.62	5.31	10.62	2.65	10.62
Petroleum-ether	11.30	11.30	/	5.65	/
Ethyl-acetate	1.25	0.62	1.25	0.31	1.25
n-butanol	/	6.90	6.90	3.45	/
Ketokonazole	0.16	0.08	0.08	0.04	0.16

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

The fall and summer ethyl-acetate fractions showed the best antibacterial and antifungal activities on all tested bacteria and fungi. The highest MIC values among fall extract and fractions exhibited ethyl-acetate fraction against *Bacillus cereus* (0.31 mg/mL) and *Bacillus subtilis* (0.62 mg/mL) and up to 1.25 mg/mL for *Staphylococcus aureus*, *Escherichia coli* and *Proteus mirabilis*. The MIC values for the summer fraction were also high and ranged from 0.31 mg/mL for both *B. cereus and B. subtilis*, 0.62 mg/mL for *S. aureus* and *P. mirabilis* to 1.25 mg/mL for *E. coli*. Ethyl-acetate fall fractions MIC for fungi were the highest towards *Candida albicans* (0.31 mg/mL) and up to 2.50 mg/mL for *Aspergillus niger* and *Penicillium italicum*; Summer ethyl-acetate fraction had the same MIC for 0.31 mg/mL for *C. albicans* as the fall one, followed by 0.62 mg/mL for *Trichophyton mentagrophytes* and ranged to 1.25 mg/mL for *Mucor mucedo*, *A. niger* and *P. italicum*. Ranges for n-butanol fraction (fall sample) on bacteria were second best and varied from 1.25 mg/mL (*B. cereus* and *B. subtilis*), to 2.50 mg/mL (*S. aureus, E. coli and P. mirabilis*). Second by antibacterial activity was *F. virsoides* dichloromethane-methanol summer sample with ranges from 2.65 mg/mL (*B. cereus* and *B. subtilis*) to 10.62 mg/mL (*E. coli and P. mirabilis*). All other samples showed slightly better activity toward bacteria compared to fungi.

DISCUSSION

Fucus spp. is an important food source with high nutritional value due to its complex polysaccharide, fatty acid and polyphenol contents. They are also a valuable source of bioactive compounds that may possess therapeutic potential. Literature data show that macroalgae from *Fucus* genus exhibit antioxidant, anticancer (proapoptotic, antimetastatic and anti-angiogenic properties) anti-inflammatory, antidiabetic, anticoagulant, and antithrombotic effects (20).

In our study, fatty acids were isolated from petroleum-ether fraction, because it was expected that they would be the most abundant in this fraction. Obtained data showed that fall fraction was richer in polyunsaturated fatty acids and that it had better PUFA to SFA ratio. In addition, the fall sample had more n-3 fatty acids than the summer sample. This finding is in concordance with the previous studies that have shown that there was a seasonal change in the production of fatty acids in macroalgae (21). Macroalgae are adapting to the colder water temperature by increasing the amount of PUFAs, MUFAs and other bioactive molecules (8). It has been shown that PUFA, especially n-3, exert cytotoxic effects on breast cancer cell lines, prostate and colorectal cancer cells (8, 22, 23). In particular, n-3 PUFAs were shown to decrease tumor growth and ability of tumors to metastasize. It is perceived that the observed effects may be attributed to their influence on gene expression, transcription factors, and suppression of neoplastic stimulations, and to their ability to influence the production of ROS, cell metabolism and induce apoptosis (24, 25, 26). Fatty acids were also shown to enhance the effectiveness of standard chemo- or radiotherapy (27, 28). On the contrary, SFA and MUFA can stimulate tumorigenesis. Therefore, the ratio of PUFAs to SFA and MUFA seems to be very important (8).

Our study showed that the total amount of polyphenols in the extract and the fractions were in direct correlation with their free radical scavenging activity. The ethyl-acetate fall fraction, which showed the highest total phenolic content, also had the highest capacity to remove DPPH free radicals. Based on the literature data and solvent properties it was expected that the ethyl-acetate fraction should have the highest total phenolic content (29). In addition, the amount of total polyphones was much higher in fall than in summer extract and fractions indicating seasonal variations. Polyphenols are known for their anticancer properties. They exert pro-apoptotic, anti-angiogenetic, DNA-damaging, antioxidative, and immunostimulatory activities. Polyphenols are also found to have the ability to target protein kinases, involved in malignant transformation and cell cycle regulation. They have also been identified as potential anticancer agents that might suppress aggressive metastatic cancers (30).

The examination of the cytotoxic activities of the summer and fall extracts and fractions showed selectivity in their cytotoxicity on cancer cells when compared with their effects on normal MRC-5 cells. Furthermore, the influence of seasonal variations on the cytotoxic activity of the extracts and fractions was observed with the fall extract and fractions having stronger cytotoxic activities on cancer cells. The strongest cytotoxic effects of *F. virsoides* fall petroleum-ether fraction, dichloromethane-methanol fraction and ethyl-acetate fraction were in correlation with a high content of polyphenols and fatty acids (PUFAs) in these fractions and their possible synergistic action in the whole extract. These results are in accordance with literature data that showed that polyphenols and fatty acid have cytotoxic properties (8, 30).

Cell cycle distribution analysis and fluorescence microscopy showed pro-apoptotic effects of

the summer and fall extracts, which could be explained by a high content of polyphenols and fatty acids in these fractions as well as their possible synergistic effect in the whole extract. Confirmation of the influence of polyphenols and fatty acids on cell cycle arrest and apoptosis is found in the literature (8, 30, 31).

Results of the *in vitro* scratch assay showed that fall extract and its ethyl-acetate fraction, followed by petroleum-ether fraction and extract exerted the best antimigratory activity. These results may suggest the connection between polyphenol-rich fraction, and fatty acid-rich fraction and their ability to inhibit migration of endothelial cells. Furthermore, the influence of the seasonal composition of extracts and fractions on inhibition of cell migration was found, where the summer extract and fractions had a weaker effect on suppression of cell migration. The summer extract and fractions also had a lower amount of polyphenols and n-3 fatty acids, the two main components that exert antimigratory effects (8, 31).

Anti-angiogenic effects of the ethyl-acetate summer and falls fractions may suggest the promising role of polyphenols as potential anti-angiogenic agents, as confirmed by the literature data (31). In addition, the anti-angiogenic effects exhibited by the fall extract and petroleum-ether fraction may indicate that fatty acids could contribute to the anti-angiogenic properties (8).

The fall extract and fractions also showed better α-glucosidase inhibitory activity when compared to the summer extract and fractions. IC_{50} values for α-glucosidase inhibitory activity of ethyl-acetate and petroleum-ether fraction were the lowest, which may indicate that polyphenols and fatty acids play an important role in this activity. Of interest, all of the samples were much more effective in α-glucosidase inhibition than standard drug acarbose, suggesting their possible use in the prevention of postprandial hypoglycemia or even as a complementary natural product against type 2 diabetes. To the best of our knowledge, there were no previous literature data about the α-glucosidase inhibitory activity of *F. virsoides*. However, Husni et al. (32), found a significant correlation between total phenolic content and phlorotannins and α-glucosidase inhibition in brown alga *Padina pavonica*. Furthermore, Kellog et al. (33), showed that the ethyl acetate fraction of Alaskan *Fucus distichus*, that also had the highest total phenolic content, had the highest α-glucosidase inhibitory effect. It was previously reported that fucoidan from *F. vesiculosus* and *F. vesiculosus* extract alone inhibited this enzyme (34, 35). Su et al. (36) have also shown that both saturated and unsaturated pure fatty acids inhibited α-glucosidase stronger than acarbose. The best effect was shown by unsaturated oleic, followed by linoleic fatty acids, but also α- linoleic, arachidonic, palmitoleic, and SFAs (palmitic mystiric, stearic, lauric fatty acids) had inhibitory effects.

In this study, ethyl-acetate fall fractions of both samples, which exhibited the best antimicrobial action, were the richest in polyphenols. It is well known that the most active components should be concentrated in this fraction. It has already been shown that polyphenols have antimicrobial properties (5). The petroleum-ether fraction, rich in fatty acids, showed good antimicrobial activity as well. Saritha et al. (37) reported the antibacterial effects of the fatty acid fractions from three different macroalgae. Results of this research are in accordance with the study of Rizzo et al. (38) that showed the antibacterial activity of ethanol *F. virsoides* crude extract and isolated polysaccharides.

CONCLUSIONS

Results of this research showed seasonal variations in content and biological activity of the *F. virsoides* extracts and fractions. The petroleum-ether and ethyl-acetate fall fractions rich in polyphenols and fatty acids were the most active and exhibited prominent anticancer as well as anti-α-glucosidase and antimicrobial activities.

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Conflict of interest

The authors declare no conflicts of interest.

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