

Neuroprotective and antibacterial effects of phlorotannins isolated from the cell walls of brown algae *Fucus vesiculosus* and *Pelvetia canaliculata*

Darya Meshalkina,^{1,2†} Elena Tsvetkova,^{1,3†} Anastasia Orlova,⁴ Renata Islamova,¹ Elana Kysil,⁵ Maria Grashina,¹ Daria Gorbach,^{1,5} Vladimir Babakov,⁶ Antonio Francioso,⁷ Luciana Mosca,⁷ Elena Tarakhovskaya,^{1,8*} Andrej Frolov^{5*}

¹St. Petersburg State University, St. Petersburg, Russia, ²Sechenov Institute of Evolutional Physiology and Biochemistry, ³Institute of Experimental Medicine, Department of General Pathology and Pathological Physiology, St. Petersburg, Russia, ⁴K.A. Timiryazev Institute of Plant Physiology RAS, Moscow, Russia, ⁵Leibniz Institute of Plant Biochemistry, Department of Bioorganic Chemistry, Halle/Saale, Germany, ⁶Research Institute of Hygiene, Occupational Pathology, and Human Ecology, Federal Medicobiological Agency, St. Petersburg, Russia, ⁷Sapienza University, Department of Biochemical Sciences, Roma, Italy, ⁸Vavilov Institute of General Genetics RAS, St. Petersburg Branch, St. Petersburg Russia.

[†]These authors contributed equally to the manuscript

*Corresponding authors:

Dr. Andrej Frolov

Leibniz Institute of Plant Biochemistry

Department of Bioorganic Chemistry

Weinberg 3,

06120, Halle (Saale), Germany

Tel. +4934555821350

Fax. +4934555821309

Email: afrolov@ipb-halle.de

Dr. Elena Tarakhovskaya

St. Petersburg State University

Department of Plant Physiology and Biochemistry

Universitetskaya nab. 7-9,

199034, St. Petersburg, Russia

Tel. +78123289695/Fax. +78123289703

Email: elena.tarakhovskaya@gmail.com

Abstract

The Phaeophyceae (brown algae) essentially contribute to biotopes of cold and temperate seas. Their thalli are rich in biologically active natural products which are strongly and universally dominated with phlorotannins – polyphenols of complex and diverse structure based on multiple differently arranged phloroglucinol units. These electron-rich compounds are strong antioxidants with antimicrobial, anti-inflammatory and neuroprotective activities. In the algal cells phlorotannins can either accumulate in cytoplasm or can be secreted into the cell wall (CW) with subsequent covalent binding to the alginate network. The biological activities of easily extractable intracellular phlorotannins were comprehensively characterized, whereas the properties of the CW-bound polyphenol fraction are still mostly unknown. Recently, we identified dibenzodioxin bonding as the principal structural feature of the CW-bound phlorotannins of furoid algae, whereas soluble intracellular phlorotannins relied on aryl- and ether bonds. However, profiles of biological activity associated with these structural differences are still unknown. Therefore, here, to the best of our knowledge, for the first time, we address the antioxidant, cytotoxic, neuroprotective, and antibacterial properties of the CW-bound phlorotannin fractions isolated from two representatives of the order Fucales - *Fucus vesiculosus* and *Pelvetia canaliculata*. The CW-bound phlorotannins appeared to be softer antioxidants, stronger antibacterial agents and were featured with essentially less cytotoxicity in comparison to the intracellular fraction. However, the neuroprotective effects of both sub-cellular phlorotannin fractions of *F. vesiculosus* and *P. canaliculata* were essentially similar. Thus, due to their lower cytotoxicity, CW-bound phlorotannins can be considered as promising antioxidants and neuroprotectors.

Keywords: Alzheimer disease, antibacterial, antioxidant, anti-neurodegenerative, brown algae, cell culture, cell wall-bound phlorotannins, neuroprotective, Parkinson disease, Phaeophyceae, phlorotannins

1. Introduction

The brown algae are represented with approximately 256 genera with 2040 species, 95% of which inhabit cold and temperate waters [1]. About 90 species of brown algae, rich in biologically active metabolites, can be found in the global coastline area which makes them commercially important organisms [2]. Phlorotannins represent the most abundant group of valuable secondary metabolites (depending on species, season and habitat accounting 0.5 to 25% of dry weight [3]), which are unique for brown algae and are present at detectable concentrations across almost all orders of Phaeophyceae [4]. These polyphenols act as integral components of cell walls (CW), antioxidants, metal chelators and herbivore protectants [4,5].

Natural phlorotannins represent complex mixtures of water-soluble oligo- and polymerization products of phloroglucinol (1,3,5-trihydroxybenzene, Figure 1) with molecular size ranging from 126 Da (monomer molecule) to 650 kDa [6]. Based on their structure (i.e. the types of inter-monomer chemical bonds), these compounds can be splitted in four classes: (i) fuhalols and phlorethols with ether linkages (Aryl-O-Aryl), (ii) phenyl-linked fucols (Aryl-Aryl), (iii) fucophlorethols, which possess both ether and phenyl linkages and can have branched structure, and (iv) dibenzodioxin-linked eckols and carmalols [7]. The high isomerization potential of phlorotannins underlies impressing structural diversity of these structurally similar compounds [8]. Thus, the distinct molecular size fractions (<1, 1–10, 10–100, and >100 kDa) of phlorotannins demonstrated high variability depending on the species, geographical region, and thallus zone [9]. This fact and the absence of commercially available standards make characterization of phlorotannins a challenging problem [10], which in the most efficient way can be addressed by ultra-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) and nuclear magnetic resonance (NMR) techniques [11,12].

Algal cells usually contain a pool of intracellular phlorotannins in the specialized organelles, physodes, which are supposed to be formed in the endoplasmic reticulum (ER) and Golgi

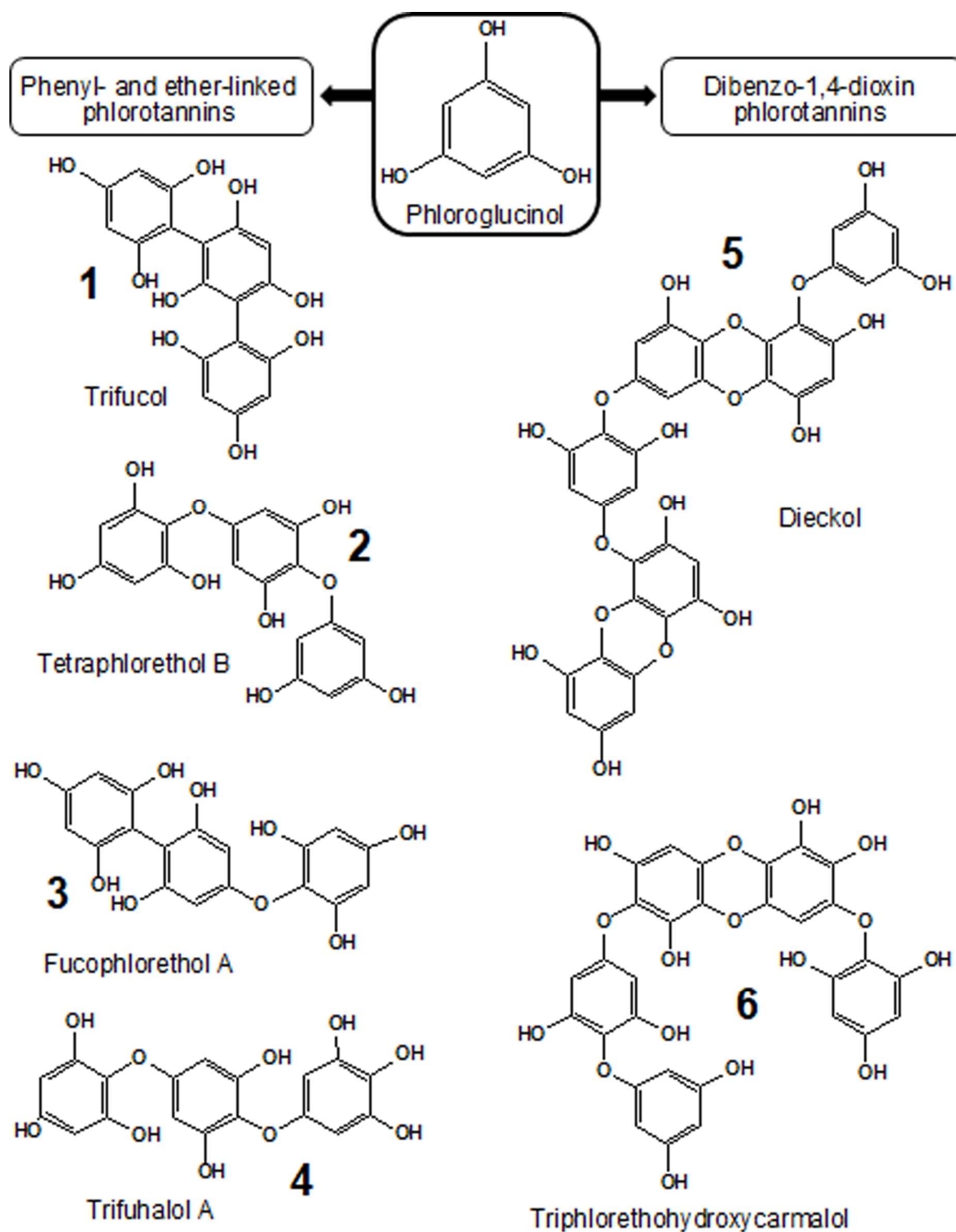


Figure 1 Structures of phloroglucinol and representatives of major phlorotannin classes: fucols (1), phlorethols (2), fucophlorethols (3), fuhalols (4), eckols (5), and carmalols (6) [9].

apparatus. Phlorotannin precursors might be synthesized in the ER and then transferred to the Golgi for further processing [13]. Histochemical studies distinguish at least two types of

physodes: the vesicles accumulating around the nucleus and those moving to the periphery of the cytoplasm and then secreting their contents into the apoplast, where phlorotannins form complexes with alginic acid [13]. The content of CW-bound phlorotannins in the algal tissue is an order of magnitude lower than the concentration of soluble ones [14]. At present, the fraction of phlorotannins associated with the cell wall is almost unstudied.

Recently, due to their biological activity, phlorotannins attracted a special attention of food chemists, cosmetics producers and pharmacists [15]. Similar to the polyphenols of vascular plants, phlorotannins exhibit pronounced antioxidant properties [16], which are clearly manifested with high antioxidant capacity and reactive oxygen species (ROS) scavenging activity comparable to those of ascorbic acid, butylated hydroxytoluene (BHT) and propyl gallate [17,18]. Moreover, phlorotannin-rich algal extracts were shown to suppress lipid peroxidation and formation of hydroperoxides with the efficiency comparable to that of propyl gallate [19].

Moreover, phlorotannins are featured with pronounced antimicrobial effects towards both Gram-positive and negative microorganisms, which were comprehensively reviewed by Catarino et al [15]. Besides this, phlorotannins were proved to be anti-inflammatory [20-22], cytotoxic and antiproliferative compounds [23-25]. Phlorotannin preparations effectively inhibited α -amylase and α -glucosidase activities, i.e. exerted anti-diabetic effect [26,27]. Both phloroglucinol and low-molecular-weight phlorotannins reduced formation of advanced glycation end-products (AGEs), which are responsible for such diabetic complications as retinopathy, neuropathy and cardiomyopathy [28].

Unfortunately, although the biological activity of soluble phlorotannins is well characterized, the properties of the cell wall polyphenolic fraction are still insufficiently addressed and remain mostly unknown. Recently, in a comprehensive high resolution-mass spectrometry (HR-MS) survey [29], we unambiguously showed that the CW-bound phlorotannin fraction is clearly

different by its structure (i.e. monomer unit/bonding type) from the soluble one (aryl/ether vs dibenzodioxin-type units). As this structural diversity might underlie differential biological activity, here we address the antioxidant, antimicrobial and neuroprotective (antineurodegenerative) effects of the cell wall fractions isolated from *Fucus vesiculosus* and *Pelvetia canaliculata* thalli and compare these properties with their soluble counterparts.

2. Materials and methods

2.1 Algal material

Samples of two species of Fucales (*F. vesiculosus* L. and *Pelvetia canaliculata* (L.) Dcne and Thur.) were collected in the Keret Archipelago (Kandalaksha Bay, White Sea; 66° 17' 28.76" N, 33° 40' 03.46" E) in August 2020. Mature thalli from the typical habitats of each species (low-intertidal and high-intertidal zones, respectively) were frozen and transported to the laboratory for subsequent homogenization and phlorotannin extraction.

2.2 Materials

Unless stated otherwise, materials were obtained from the following manufacturers: Duchefa Biochemie (Haarlem, The Netherlands): dimethylsulfoxide (>99.9 atom % D); Ecos-1 (Moscow, Russia): *n*-hexane (analytical grade), ethyl acetate (analytical grade), methylene chloride (analytical grade); Honeywell (Seelze, Germany): acetonitrile (>99.9%, LC-MS grade). All other chemicals were purchased from Merck KGaA (Darmstadt, Germany). Water was purified in house with a water conditioning and purification system GenPure Pro UV-TOC system (resistance 18 mΩ/cm, Thermo Fisher Scientific, Langenselbold, Germany).

2.3 Extraction of the intracellular and CW-bound phlorotannins from the algal material

Extraction of intracellular and CW-bound phlorotannins was performed according to [14] with modifications. Briefly, samples of 1-2 g frozen algal material were homogenized using a cryogenic laboratory mill Freezer/Mill 6870 (SPEX SamplePrep, Germany), transferred to the 15 mL falcon tubes, poured with 10 mL of acetone:water (70:30, v/v) and left soaking for one hour.

Afterwards, each extract was centrifuged ($5000 \times g$, 10 min), the supernatant was transferred to a fresh tube, and the pellet was re-extracted with another 10 mL of aqueous acetone. The supernatants of five extraction rounds were combined, and acetone was evaporated under reduced pressure in a centrifugal evaporator (CentriVap vacuum concentrator system, Labconco, USA). The CW-bound phlorotannin fraction was extracted from the precipitate of the remaining algal material after the extraction of intracellular phlorotannins. First, the precipitate was washed with 3 more 10 mL portions of aqueous acetone to remove the possible remnants of the intracellular phenolics. Then the precipitate was resuspended in 5 mL of 1 mol/L aqueous NaOH solution (80°C) and then incubated for 2.5 h at room temperature with continuous shaking (750 rpm). After centrifugation ($5000 \times g$, 10 min) the supernatant was transferred to another tube. The alkaline extraction was repeated three times. The combined supernatants were neutralized with concentrated HCl to pH 6.8-7.0. At the next step, both intracellular and CW-bound phlorotannin extracts were defatted three times partitioning against dichloromethane (1:1, v/v), and phlorotannins were extracted by five successive portions of ethyl acetate (1:1, v/v). Ethyl acetate extracts were dried and resuspended in 1 mL water.

A modification of the Folin–Ciocalteu micro-method was used to measure the phlorotannin content in the intracellular and CW-bound phlorotannin extracts [30]. Phloroglucinol (Sigma-Aldrich 79330) was used as the standard. The reaction mixture containing 0.3 mL of sample (diluted as necessary), 0.3 mL of Folin reagent and 2.4 mL of 5% (w/v) aqueous Na_2CO_3 , was incubated for 20 min at 45°C , and then absorbance was measured at 750 nm using a SPEKOL 1300 spectrophotometer (Analytik Jena AG, Jena, Germany).

2.3 Antioxidant assays

The antioxidant effects of the extracts enriched in intracellular and CW-bound phlorotannins isolated from the thalli of *F. vesiculosus* and *P. canaliculata* were addressed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging, Trolox equivalent antioxidant capacity (TEAC)

and nitroblue tetrazolium (NBT) assays. The analyses were accomplished according to Masci et al [31] with minor modifications as follows.

2.3.1 DPPH free radical scavenging effect

The phlorotannin-rich extracts were diluted with distilled water to achieve the concentration of 1 mg/mL. The 10 μ L aliquots of these solutions (10 μ g in total) were supplemented to 1 mL portions of 40 μ mol/L methanolic solution of stable nitrogen centered free radical DPPH \bullet . The absorbance was monitored spectrophotometrically at 517 nm after 1h of incubation at room temperature (RT). The capacity of the phlorotannin-rich preparations for scavenging of the DPPH \bullet radical was estimated from the difference in the absorbance acquired in presence and in absence of the algal isolates. The corresponding values were expressed as the percentage of DPPH \bullet consumption as a function of the sample concentration.

2.3.2 Trolox equivalent antioxidant capacity (TEAC) assay

The 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was dissolved in water to obtain a 7 mmol/L solution which was further oxidized to corresponding radical cation (ABTS $^{+\bullet}$) in presence of 2.45 mmol/L potassium persulfate (16 h at RT in dark). The radical cation reagent (ABTS $^{+\bullet}$) was diluted with ethanol to obtain the absorbance of 0.70 (± 0.02) at 734 nm. Aliquots of the phlorotannin samples, diluted as described in the previous section, were supplemented with 1 mL of the ABTS $^{+\bullet}$ solution. Absorbance was measured at 734 nm after six minutes of incubation in the dark at room temperature. Antioxidant capacities of the phlorotannin-rich extracts were reported as equivalents of Trolox.

2.3.3 Assessment of extract capacity to scavenge superoxide anion radicals (NBT assay)

The stock solutions (1 mmol/L) of phenazine methosulfate (PM) in ethanol, NBT in water, and β -NADH in 0.05 mol/L phosphate buffer (pH 7.4) were freshly prepared daily. The reaction mixtures contained 73 μ mol/L β -NADH, 15 μ mol/L PM, 50 μ mol/L NBT, and 10 μ g of samples

in 1 mL of 0.02 mol/L Tris-HCl buffer, pH 8.0. The absorbance was determined at 560 nm immediately after mixing the reagents and after 15 sec of reaction. The change of absorbance in time ($\Delta\text{Abs}/\text{min}$) and absorption coefficient of 1 $\mu\text{mol/L}$ formazan solution 0.03 were used to calculate the rate of production of superoxide anion radicals.

2.4 Antimicrobial assays

The analysis of the antimicrobial activity was accomplished as described by Orlova et al [32] with changes. Thereby, the minimum inhibitory concentrations (MIC) of the intracellular and CW-bound phlorotannin extracts were determined by broth microdilution method, as recommended by Clinical Laboratory Standards Institute, USA [33]. The following bacteria strains were cultured under aerobic conditions according to the approved standard protocol: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Listeria monocytogenes* EGD, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* SG-511, *MRSA* ATCC 33591, *Micrococcus luteus* CIP A270. Strains *MRSA* ATCC 33591 and *Listeria monocytogenes* EGD were provided by Prof. R. Lehrer (University of California Los Angeles, USA); *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Micrococcus luteus* CIP A270, *Staphylococcus aureus* ATCC 25923 - Department of Molecular Microbiology, IEM; strain *Staphylococcus aureus* SG 511 - by Professor H.G. Sahl (University of Bonn, Germany). The microorganisms from an agar plate culture were incubated for 2-6 hours in 2.1% (w/v) Mueller-Hinton broth (MHB, HiMedia, India) at 37° C on an orbital shaker at an agitation rate 150 rpm. After adjusting the turbidity to 0.5 McFarland ($1.5 \times 10^8 \text{ CFU/mL}$), suspensions were diluted in sterile 2.1% (w/v) MHB till the final bacterial concentration of $1.0 \times 10^6 \text{ CFU/mL}$. The intracellular and CW-bound phlorotannin extracts from *F. vesiculosus* and *P. canaliculata* were serially two-fold diluted (the initial concentration was 500 $\mu\text{g/mL}$ for intracellular phlorotannins and 125 $\mu\text{g/mL}$ for CW-bound phlorotannins) with sterile 2.1% (w/v) MHB and 50 μL aliquots were added to the wells of a 96-well sterile U-shaped plate (GreinerBio-one,

Austria). Afterwards, 50 μ L of bacterial suspension was added in each well. The controls of bacterial growth and viability and medium sterility were included. As a quality control MIC of gentamicin for *Staphylococcus aureus* ATCC 25923 was determined, and the results were within recommended limits. The microtiter plates were incubated aerobically without shaking at 37°C for 18 h. MICs were defined as the lowest extract concentrations that inhibited the visual growth of microorganisms. The experiments were performed in triplicates, the final results were calculated as the medians based on the data from three independent experiments, each accompanied with the complete set of the controls.

2.5 Assessment of antineurodegenerative effects

Evaluation of the anti-neurodegenerative effects relied on the cell models of Alzheimer and Parkinson diseases established with SH-SY5Y human neuroblastoma cells and amyloid peptide A β ₂₅₋₃₅.

2.5.1 Cell culture

Human neuroblastoma SH-SY5Y cells were obtained from ICLC (Genova, Italy). Cells were cultured in T25 cell culture flasks (Eppendorf, Germany) in DMEM/F12 with 10% fetal bovine serum (FBS), 2 mmol/L *L*-glutamine in the atmosphere of 95% air and 5% CO₂ at 37°C and humidity \geq 95%. Cells were passed twice per week by detaching with accutase (2 – 3 min followed with dilution with full medium). Before the accutase treatment, the cells were washed two times with 1 mL of PBS, pH 7.4. The cells were not split in ratios lower than 1:3 – 1:5. Cell counting relied on an automatic cell counter (TC20, Bio Rad, CA, USA) or manual calculation with Neubauer chamber.

2.5.2 Synthesis and aggregation of A β ₂₅₋₃₅ amyloid peptide

A β ₂₅₋₃₅ was obtained by solid phase peptide synthesis as described elsewhere [24] and stored at -20°C. The peptide was resuspended in 1,1,1,3,3,3-hexafluoro-2-propanol, incubated for 1 hour with gentle shaking at 4°C, then aliquoted and the solvent was completely evacuated under

reduced pressure by centrifugal evaporation in SpeedVac during 20 minutes. The aliquots were stored under a vacuum glass bell. The day before being used, aliquots were re-suspended in phosphate buffered saline (PBS) at a final concentration of 1 mmol/L, incubated in an ultrasonic bath on ice for 30 min to induce aggregation, followed by gentle shaking at 4°C overnight. Directly prior to cell treatment A β ₂₅₋₃₅ was diluted in culture medium at the final concentration of 30 μ mol/L.

2.5.3 Anti-Alzheimer assay

Cells were seeded at the density of 4×10^4 cells per each well of 96-well cell culture treated microtiter plates (Eppendorf, Germany). Next morning, the medium was discarded and 50 μ L of fresh medium supplemented with serially diluted phlorotannin-rich extracts (1 - 10 μ g/mL, n = 4) were added to each well. Half an hour later, 50 μ L of aggregated A β ₂₅₋₃₅ peptide was added to each well to yield the concentration of 25 μ mol/L. The control cells were supplemented with 50 μ L of fresh medium. After the incubation for 24 hours at 37°C, the methylthiazolyldiphenyl-tetrazolium bromide (MTT) test was accomplished in quadruplicates. For this, 5 mg/mL MTT solution in PBS (pH 7.4) was added to each well to obtain the final concentration in culture medium of 0.5 mg/mL. After a 2 h incubation at 37°C in a CO₂ incubator (Sanyo, Kyoto, Japan), the medium was removed and formazan crystals were dissolved in 100 μ L/well of DMSO. Absorbance was measured by DU-50 (Beckman Coulter, Indianapolis, USA) at 570 nm with a reference at 630 nm. Cell viability was calculated for each well as a ratio of specific absorbance to mean absorbance obtained for untreated cells (with blanks subtracted).

2.5.4 Anti-Parkinson assay

For the assessment of the anti-parkinsonian activity of the intracellular phlorotannin extracts we used paraquat model of neurodegeneration established on differentiated SH-SY5Y cells. The day before the differentiation procedure, the cells were seeded into the wells of 96-well plate at the concentration 50000 cells per well. SH-SY5Y cell differentiation was induced by culture growth

in DMEM/F-12 medium with 3% FBS containing 10 $\mu\text{mol/L}$ retinoic acid and 10 $\mu\text{mol/L}$ phorbol myristate acetate. Differentiation proceeded for 10 days with medium exchange every two days and was routinely controlled visually by phase contrast microscopy. At the day of experiment the algal extracts were added to the cells for 30 min in 50 μL of culture medium (1-10 $\mu\text{g/mL}$). Neurodegeneration was modeled by the toxicity of 800 $\mu\text{mol/L}$ paraquat incubated during 24 hours with differentiated cells that resulting in 50% viability of the culture. Afterwards MTT solution was applied and the viability values were retrieved as described in 2.5.3 for anti-Alzheimer assay.

3. Results

For all analyses the extract concentrations were normalized to the total phenolics contents. Phlorotannins highly dominate in the phenolic profiles of brown algae (especially, fucoids) and it was shown that non-phenolic compounds contribute less than 5% of Folin-Ciocalteu reactive substances [34], so total phenolic content is an adequate proxy for the phlorotannin content.

3.1 Antioxidant effects

The extracts enriched with intracellular and CW-bound phlorotannins isolated from *F. vesiculosus* and *P. canaliculata* differed in their patterns of antioxidant activity. Thus, as can be seen from Table 1, the highest normalized free radical-scavenging activity, discovered in the DPPH test, was observed for the intracellular phlorotannin fraction of *F. vesiculosus* (93.44%). The CW-bound phlorotannins of *F. vesiculosus* and intracellular phlorotannin-rich fraction of *P. canaliculata* yielded similar values (85.06% and 83.58%, respectively), while the extract from the cell walls of *P. canaliculata* showed a much lower activity (57.49%). On the other hand, all the tested extracts demonstrated similar electron-donating behavior, that was manifested with practically the same free cation-radical scavenging activity in the TEAC tests (about 0.65 $\mu\text{mol/L}$ Trolox equivalents/ μg).

Finally, the activity of the extracts in neutralizing of the superoxide anion radicals was addressed in the NBT tests. Thereby, the highest activity was detected in the extracts of CW-bound phlorotannins of the both algae (49.89 nmol of $O_2^{\bullet-}$ /min for *F. vesiculosus* and 37.14 nmol of $O_2^{\bullet-}$ /min for *P. canaliculata*), whereas the efficiency of the intracellular phlorotannin-rich fractions was much lower (51.32 nmol of $O_2^{\bullet-}$ /min for *F. vesiculosus* and 52.04 nmol of $O_2^{\bullet-}$ /min for *P. canaliculata*, Table 1).

Table 1 The antioxidant activities assessed by DPPH, TEAC and NBT assays for the extracts enriched with intracellular and CW-bound phlorotannins of *F. vesiculosus* and *P. canaliculata*

Extract	DPPH normalized activity, %	TEAC, μ mol/L Trolox eq./ μ g	NBT assay, nmol of $O_2^{\bullet-}$ /min
<i>Fucus vesiculosus</i> CW	85.06 \pm 0.04	0.65 \pm 0.01	49.89 \pm 0.37
<i>Fucus vesiculosus</i> intracellular	93.44 \pm 0.13	0.65 \pm 0.06	51.32 \pm 0.39
<i>Pelvetia canaliculata</i> CW	57.49 \pm 0.10	0.65 \pm 0.03	37.14 \pm 0.28
<i>Pelvetia canaliculata</i> intracellular	83.58 \pm 0.06	0.65 \pm 0.03	52.04 \pm 0.39
Blank	-	-	65.89 \pm 0.49

DPPH – 2,2-diphenyl-1-picrylhydrazyl free radical scavenging assay; TEAC – Trolox equivalent antioxidant capacity; NBT (nitroblue tetrazolium) assays – assessment of capacity to scavenge superoxide anion radicals

3.2 Antimicrobial activity

The antibacterial activity of all four phlorotannin-enriched extracts was investigated *in vitro* by broth microdilution assay. Extracts were probed against several gram-positive and gram-negative microorganisms and MICs were determined (Table 2). The assays revealed low or moderate antimicrobial activity for all extracts. The MIC comparison of intracellular and CW-bound

phlorotannin isolates of both algae showed that, on the whole, they were quantitatively and qualitatively similar. However, the antibacterial activity of the CW-extracts was generally higher in comparison to the intracellular phlorotannin-rich extracts. The highest antibacterial activities were observed against the gram-negative strain *Escherichia coli* ATCC 25922 and the gram-positive strain *Staphylococcus aureus* ATCC 25923 (MIC 62.5 µg/mL for intracellular phlorotannins vs 31.2 µg/mL for CW-bound phlorotannins). The lowest activities were revealed against the gram-positive strain *MRSA* ATCC 33591 (MIC 500 µg/mL for intracellular phlorotannins vs 125 µg/mL for CW-bound phlorotannins).

Table 2 Antimicrobial activities of the extracts of intracellular and CW-bound phlorotannins of *F. vesiculosus* and *P. canaliculata*

Microorganism strain	Activity (MICs, µg/mL)			
	intracellular fraction		cell wall fraction	
	<i>Fucus vesiculosus</i>	<i>Pelvetia canaliculata</i>	<i>Fucus vesiculosus</i>	<i>Pelvetia canaliculata</i>
<i>Escherichia coli</i> ATCC 25922	62.5	62.5	31.2	31.2
<i>Pseudomonas aeruginosa</i> ATCC 27853	250.0	125.0	125.0	62.5
<i>Staphylococcus aureus</i> SG-511	250.0	125.0	62.5	62.5
<i>Staphylococcus aureus</i> ATCC 25923	62.5	62.5	31.2	31.2
<i>MRSA</i> ATCC 33591	500.0	500.0	125.0	125.0
<i>Micrococcus luteus</i> CIP A270	62.5	62.5	62.5	62.5
<i>Listeria monocytogenes</i> EGD	250.0	250.0	125.0	125.0

Antimicrobial activities were expressed as minimal inhibitory concentrations, MICs

3.3 Cytotoxic effects of phlorotannin-enriched extracts observed with the SH-SY5Y cell culture

To obtain the range of non-toxic and low-toxic concentrations of the tested algal extracts, their cytotoxicity was addressed with the differentiated SH-SY5Y cell culture prior to the assessment of the neuroprotective antineurodegenerative effects. For this, a broad concentration range (from 1 to 100 $\mu\text{g/mL}$) of the extracts was applied to the cells and to the cell-free wells as a control (to subtract the effect of extract dark color from the resulting MTT signal). For all the extracts tested, the dose-effect curves were obtained and the LD50 values could be assessed (Figure 2). In our experiments, the cell wall extracts showed slightly lower toxicity (LD50 of 24 and 54 $\mu\text{g/mL}$ for *F. vesiculosus* and *P. canaliculata*, respectively), than the extracts of intracellular phlorotannins (LD50 of 25 and 16 $\mu\text{g/mL}$ for *F. vesiculosus* and *P. canaliculata*, respectively). Based on these results, we selected the extract concentrations lower than 10 $\mu\text{g/mL}$ for application in the further antineurodegenerative activity assay.

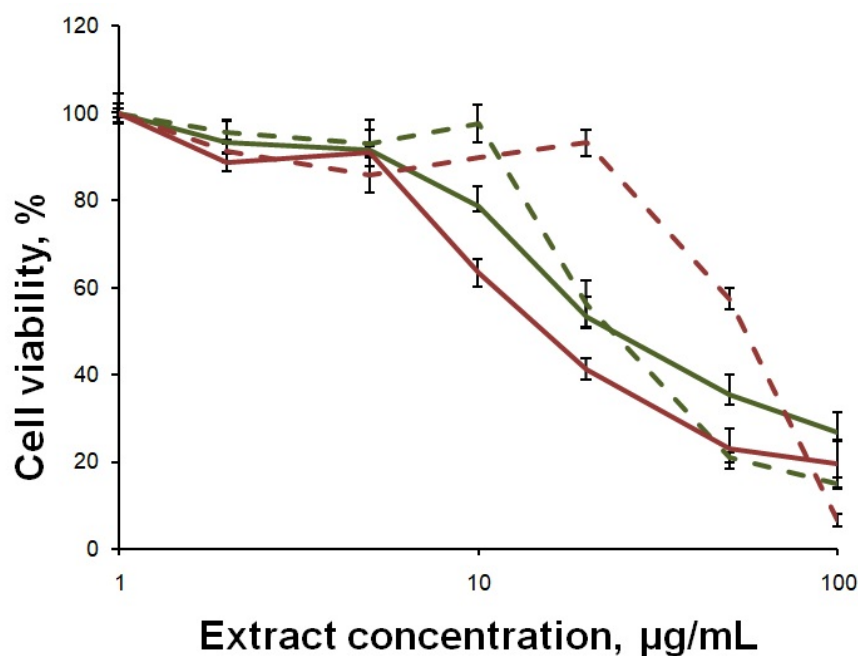


Figure 2 Dose-response curves ($n = 4$) built for cytotoxicity of the extracts of intracellular (solid lines) and CW-bound (dashed lines) phlorotannin-rich isolates obtained from *F. vesiculosus* (green lines) and *P. canaliculata* (red lines) against the differentiated culture of SH-SY5Y cells. Error bars are standard deviations, the concentration axis is scaled logarithmically.

3.5 Neuroprotective (anti-neurodegenerative) activity

Anti-neurodegenerative activity was assessed in two models of toxicity established for the differentiated SH-SY5Y cells. Thereby, paraquat toxicity was employed as the cellular model of Parkinson cell death, while the cellular model of Alzheimer disease relied on the A β (25-35) toxicity.

The extracts of both brown algae showed modest but significant protective effects in the paraquat cell model of Parkinson disease (Figure 3). Specifically, the intracellular phlorotannins of *F. vesiculosus* significantly improved viability of paraquat-treated cells only at the highest concentration tested (10 μ g/mL), which was toxic to the control cells not treated with paraquat (Figure 3A), whereas the extract of *P. canaliculata* demonstrated protective effect at all the concentrations tested (Figure 3B).

Surprisingly, although the extracts containing CW-bound phlorotannins were less toxic for the model cells, they did not demonstrate more pronounced protective effects. Thus, only the two lowest concentrations of the *F. vesiculosus* extract (1 μ g/mL and 2 μ g/mL) resulted in significant increase of cell viability, whereas the cell wall extract of *P. canaliculata* thalli did not produce any protective effect (Figure 3C,D).

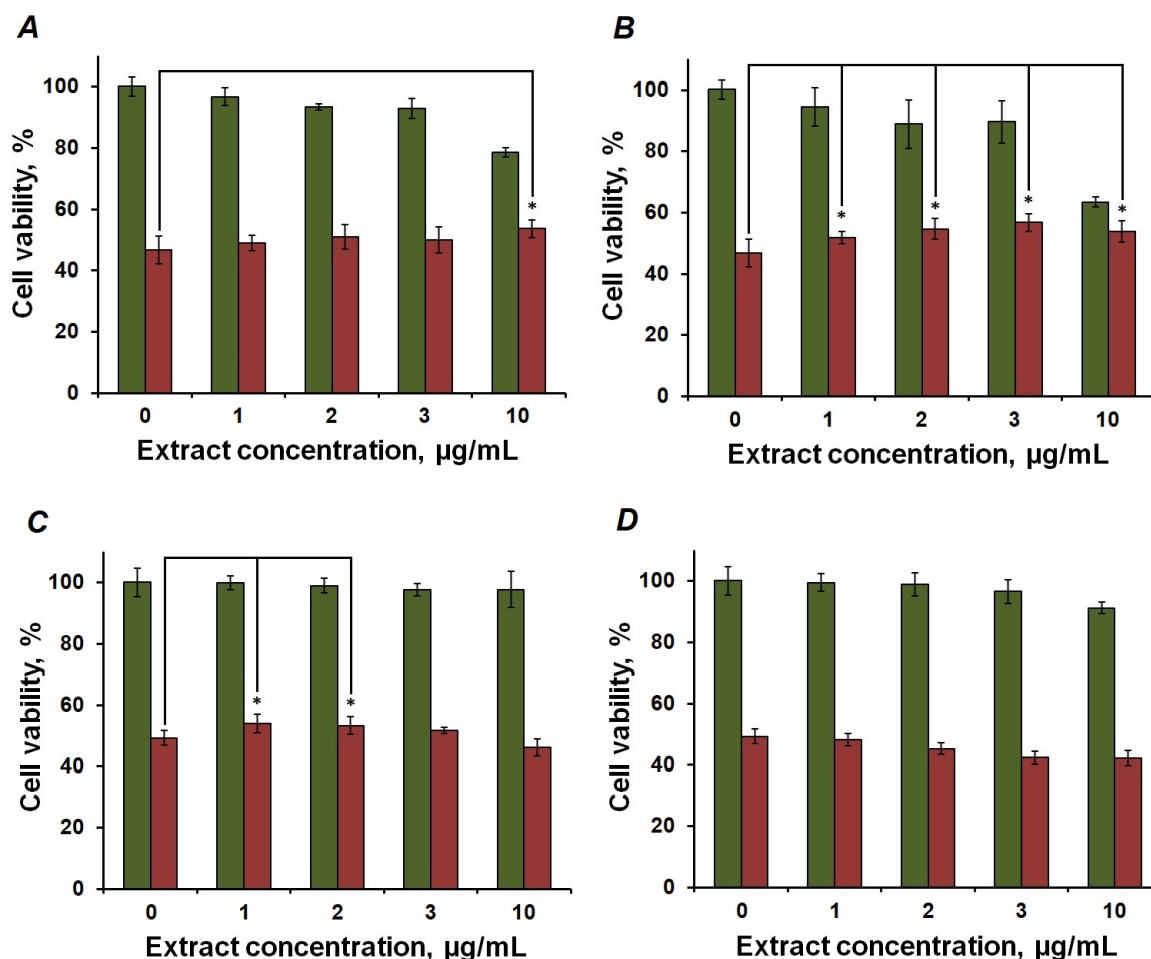


Figure 3 Protective effects of brown algal extracts against 800 µmol/L paraquat (PQ) toxicity registered in the MTT test. SH-SY5Y cells (pre-differentiated with retinoic acid and PMA) were pre-treated with intracellular phlorotannin-rich extracts of *F. vesiculosus* (A) and *P. canaliculata* (B) or extracts enriched with CW-bound phlorotannins of *F. vesiculosus* (C) and *P. canaliculata* (D) for 0.5 h and subsequently paraquat was applied for 24 h before the MTT application. * indicates statistically significant (t-test, $p \leq 0.05$) effects of the treatment with the algae extracts in comparison to paraquat only.

In the A β (25-35) cell model of Alzheimer disease tested extracts of brown algae demonstrated well-pronounced protective activity, i.e. cell viability could be restored to almost the control

levels (Figure 4). The solutions containing the extracts were applied to the cells for 0.5 h prior to the solution of A β (25-35) and the resulting signal was compared with the control of A β (25-35) only-wells. In these experiments, the extracts of *P. canaliculata* demonstrated more pronounced protective activities (practically all the concentrations tested produced observable effect) than the extracts of *F. vesiculosus* (only the highest concentrations of 5 μ g/mL and 10 μ g/mL were protective). The highest concentrations in the tested range produced the strongest effect, implying therapeutic doses of the extracts may be rather close to the toxic doses.

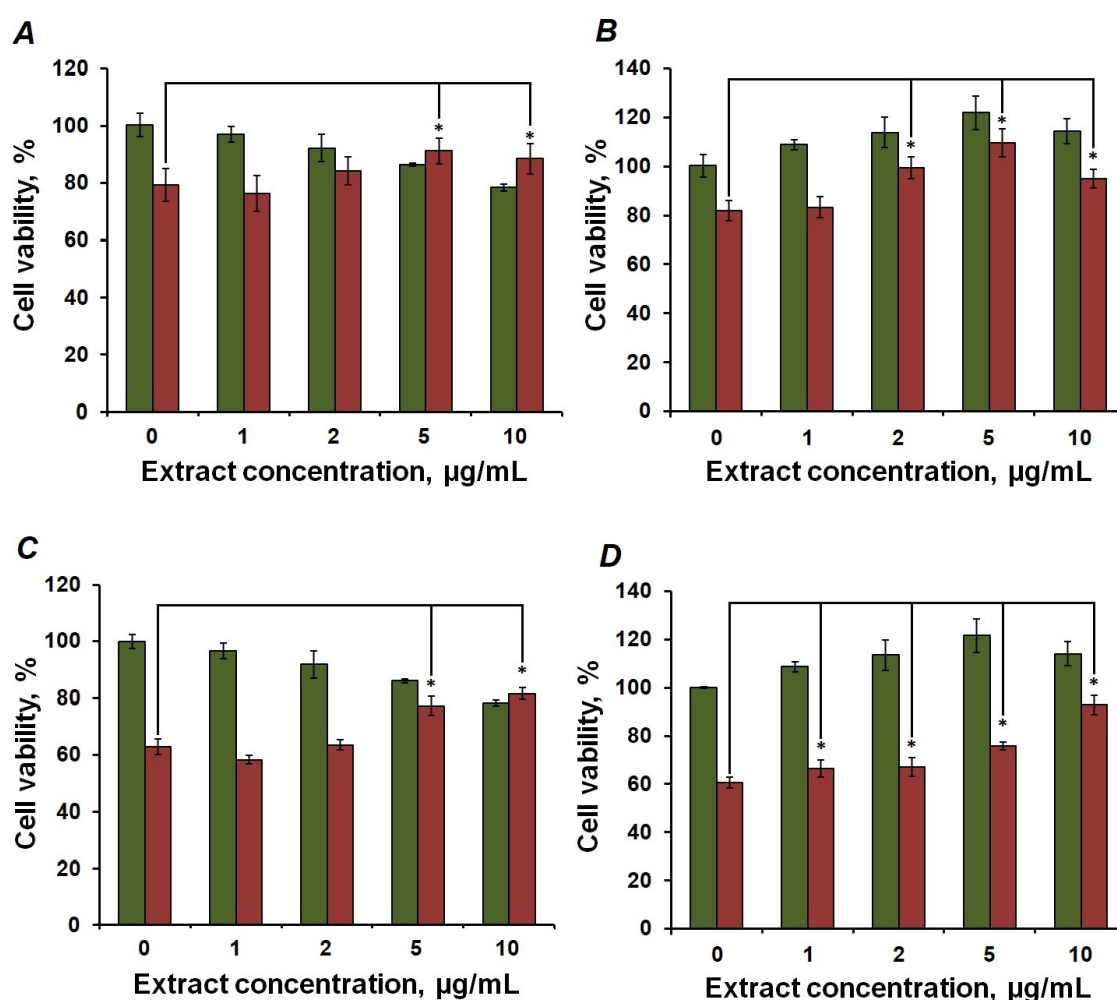


Figure 4 Protective effects of brown algal extracts against 25 μ mol/L A β (25-35) toxicity registered in the MTT test. SH-SY5Y cells (pre-differentiated with retinoic acid and PMA) were pre-treated with the extracts rich in intracellular phlorotannins of *F. vesiculosus* (A) and *P.*

canaliculata (B) or CW-bound phlorotannin extracts of *F. vesiculosus* (C) and *P. canaliculata* (D) for 0.5 h and subsequently A β (25-35) was applied for 24 h before the MTT application. * - represents a statistically significant effect of extract treatment of cells with A β (25-35) application in comparison to A β (25-35) only.

Discussion

High antioxidant potential is characteristic for the most of studied brown algae, including *F. vesiculosus* and *P. canaliculata* [35,36]. The observed ROS-protective activities of phenol-rich preparations obtained from the thalli of brown algae clearly indicate association of their pronounced antioxidant properties with the high contents of phlorotannins [37-39]. This observation is further supported by the high antioxidant activities of phlorotannins isolated in individual form, which were comparable or even more pronounced in comparison to raw phenol-rich isolates from the thalli of brown algae [40,41]. Thus, it was logical to assume that pronounced antioxidant activities of *F. vesiculosus* and *P. canaliculata* extracts are promising in therapy of human diseases characterized with high impact of oxidative stress on their pathogenesis [42-45].

As antioxidant properties can be underlied by different mechanisms (i.e. unspecific radical scavenging or direct transfer of electron to hydroxyl or superoxide anion radical [46-48]), all these aspects need to be addressed. This information can be acquired by simultaneous application of several antioxidant tests - DPPH, TEAC, and NBT assays giving access to the total scavenging activity, direct electron transfer in redox reactions and reduction of superoxide anion [31], that was also done here. Similar to the polyphenols of terrestrial plants, the phlorotannins represent electron-rich compounds and are readily involved in electron donation reactions. In the presence of oxidants these reactions are accompanied by formation of resonance-stabilized phenoxyl radicals as reaction intermediates [4,49]. Thus, the antioxidant capacity of phlorotannins strongly depends on their potential for formation of such phenoxyl radicals. This potential, in turn,

depends of the number of phenolic moieties per molecule and their spatial orientation. It is in agreement with the well-proved fact that oligomeric compounds, especially those forming branched structures, exhibit higher antioxidant activity in comparison to low-molecular-weight representatives of the class [4,50]. Addressing several sides of the phlorotannin antioxidant activity allowed understanding the observed effects.

Generally, the high antioxidant potential of the algal extracts, revealed in this study, did not conflict with the literature data. However, based on the DPPH radical scavenging assay, our results clearly indicate that intracellular phlorotannin-rich extracts of *F. vesiculosus* demonstrated significantly higher activity in comparison to the isolates of *P. canaliculata* (93% vs. 84%), although the TEAC and NBT tests failed to reveal any significant differences in electron-donating properties of these preparations (Table 1). This fact might indicate higher radical scavenging potential of phlorotannins of *F. vesiculosus* in comparison to those of *P. canaliculata* with the similar electron-donating properties[34], i.e. formation of phenoxyl radicals dominated over direct reduction of ROS.

In agreement with the report of Liu et al [18], our experiments revealed a lower antioxidant activity of the CW-bound phlorotannins in comparison to the intracellular phlorotannin fraction (both in the DPPH and NBT assays, Table 1). Thereby, for *F. vesiculosus* the difference in activity of intracellular and CW-bound phlorotannin fractions was much less pronounced than for *P. canaliculata*. For the latter organism the concerted difference in electron-donating and radical-scavenging properties was observed, that might indicate uneven distribution of protective phlorotannins between cell wall and cytoplasm. Most likely, these biochemical inter-species differences reflect ecological specialization of the algae. Indeed, in contrast to *F. vesiculosus*, which is characteristic for low-intertidal zone, *P. canaliculata* is a high-intertidal organism which is regularly exposed to very harsh conditions, i.e. subjected to prolonged desiccation, light and temperature stress [29,51]. This definitely requires higher plasticity in metabolic adaptation,

and functional differentiation of two subcellular fractions of phlorotannins may contribute to survival of the organism.

Certainly, the observed differences in the antioxidant activity of phlorotannins need to be addressed in the context of the available information on their structure and contents. Thus, although both *F. vesiculosus* and *P. canaliculata* have a comparable distribution of oligomers by the chain length (3-49 and 7-48 phloroglucinol units, respectively), according to earlier studies, the total content of polyphenolic compounds, including phlorotannins and fucoxanthins, in *F. vesiculosus* significantly exceeds that in *P. canaliculata* [52-54]. Given the well-known fact that the polyphenolic compounds are one of the most promising algae constituents with a pronounced antioxidant potential [34], the weaker antioxidant activity of *P. canaliculata* extracts might get its logical explanation.

Thus, in our earlier work [29] we showed that the fundamental skeleton of the intracellular phlorotannins relies mostly on aryl- and ether bonds, whereas the dibenzodioxin-based structures represent the only class of the CW-bound phlorotannins. It can be assumed that, along with occupation of multiple hydroxyl groups for conjugation with cell wall polymers, this lower structural diversity of the CW-bound phlorotannins underlies their less pronounced antioxidant properties. On the other hand, this fact can be explained by the approximately 10-fold lower content of phlorotannins in cell walls as compared to the cytoplasm [29]. Thus, due to the lower contents, the cell wall-bound phlorotannins fraction in both studied species has significantly lower activity than the total extracts (Table 1).

Thus, our data indicate, that the CW-bound phlorotannins represent a milder antioxidant in comparison to the intra-cellular fraction. Moreover, despite the known toxicity of the CW-bound phenolics fraction [53,54], cytotoxicity of the extracts correlated with their antioxidant activity, i.e. the CW-bound phlorotannin fractions were less toxic in comparison to the intracellular ones with the toxicity of the *P. canaliculata* the least pronounced (Figure 1). At least partly it can be

explained by the lower representation of toxic phlorotannins. Taking it into account the oxidative nature of neurodegenerative processes, the CW-bound phlorotannin fractions can be assumed to be efficient neuroprotective agents. Therefore, here we addressed their anti-neurodegenerative (neuroprotective) properties in comparison to the intracellular phlorotannin-rich isolates. Thereby, two neurodegenerative amyloidogenic pathologies, namely Alzheimer (AD) and Parkinson (PD) diseases, were addressed.

Paraquat is considered as a substance inducing Parkinson-like pathology in animals and therefore is also used for modeling this pathology in monoaminergic cell culture (such as neuroblastoma SH-SY5Y or pheochromocytoma PC-12,[55-57]. Monoamine metabolism is very sensitive to oxidative stress produced by paraquat through dopamine quinones production, affecting mitochondrial function and protein folding [58-60]. In our experiments we adhered to paraquat concentration 800 $\mu\text{mol/L}$, stably resulting in 50% differentiated SH-SY5Y cell culture viability. Activity of brown algal extracts was not tested before in paraquat model of neurodegeneration, but their antioxidant properties suggested the ability to prevent paraquat toxicity. The extracts demonstrated modest, but significant protective effects. For *F. vesiculosus* extracts, protective activities were revealed at the highest concentration for intracellular phlorotannins and at the lowest concentrations for the fraction rich with CW-bound phlorotannins. For *P. canaliculata*, only the intracellular phlorotannin fraction demonstrated protective activity for all concentrations tested.

Currently, using of A β (full 1-40, 1-42 or truncated, e.g. 25-35 fragment) is considered to be a gold standard for induction of neurotoxicity in AD research [61-63]. The models based on the application of A β appear to be easier in implementation, although they suffer from several limitations. Thus, they are not able to address the progressive nature of neurodegeneration, and the concentrations of A β typically used for modeling are three orders of magnitude higher in comparison to the contents naturally occurring in tissues [64,65]. Among all the variants of A β

used, the A β_{25-35} peptide has the shortest sequence (and, hence, the most easy in synthesis and handling), but it still retains cytotoxic activity and aggregation properties of A β_{1-42} [62,63,66]. Also, indeed, this peptide can be treated as naturally relevant, as it is present in the brains of patients with AD [66]. These characteristics make this truncated variant of A β to be one of the most widely applied models of AD-associated cytotoxicity.

Extracts of phlorotannins isolated from *F. vesiculosus* and *P. canaliculata* already demonstrated some effectiveness in this model of AD based on the toxicity of aggregated A β_{1-42} [67,68]. Such effect can be attributed to the antioxidant properties which are showed for phlorotannins from many brown algal species (refer to introduction section). Phlorotannins are also prone to bind proteins, therefore that can cause binding to A β and disaggregation, protection of cell membranes and surface receptors from interaction with toxic oligomers or enzymatic inhibition [69]. In our experiments algal extracts demonstrated prominent protective effect against A β_{25-35} toxicity. For *F. vesiculosus* only high concentrations tested produced the effect (5 and 10 $\mu\text{g/mL}$). Extracts of *P. canaliculata* revealed the activity practically at all concentrations tested, while viabilities at the highest concentrations approached the values of control untreated cells. This may be indicative to promising neuroprotective and antineurodegenerative activity of *P. canaliculata* metabolites in the subsequent animal studies.

Antimicrobial effects represent another aspect of biological activity of phlorotannins, closely related to their characteristic structure and related antioxidant properties. Indeed, in earlier studies, it was shown that the antibacterial properties of phlorotannins are related to the ability of phenolic aromatic rings and hydroxyl groups to bind to bacterial proteins by hydrophobic and hydrophilic bonds. This process in turn causes inhibition of oxidative phosphorylation, changes in microbial cell permeability, loss of internal macromolecules, which ultimately leads to the death of the microbial cell [4,70-72]. Taking this into account, we addressed here also the antibacterial properties of CW-bound phlorotannins. Though the MIC values, observed for the

intracellular phlorotannin fractions of *F. vesiculosus* and *P. canaliculata* (Table 2) were higher than those known for some other brown algae (30-60 µg/mL for phlorotannins extracted from *Ecklonia stolonifera* from the order Laminariales), they were considerably less than the values reported for *Fucus spiralis* from the order Fucales (2-15 mg/mL) [73,74]. Thus, although CW-bound phlorotannin extracts showed higher antibacterial efficiency in comparison to the intracellular phenolics (the lowest MIC values 31.2 vs 62.5 µg/mL), their antimicrobial potential is still too low to consider them as prospective prototype for antibacterial preparations.

Conclusions

The intracellular and CW-bound phlorotannins represent clearly distinguished fractions of brown algal polyphenols featured with different bonding of phloroglucinol units in the polymeric network. Recently we identified dibenzodioxin bonding as the principal structural feature of the CW-bound phlorotannins that differed from the intracellular fraction based mainly on the aryl- and ether bonds. As the next step we addressed the biological activities associated with the revealed structural features of the two sub-cellular phlorotannin fractions. Thereby, we compared the characteristic activity profiles of the extracts enriched in the intracellular and CW-bound phlorotannins, isolated from two fucallean algae, *F. vesiculosus* and *P. canaliculata*. This allowed efficient comparison with the available literature data that increased the validity of the results. Our study revealed several favorable biological properties of the CW-bound phlorotannin fractions of the both organisms. Indeed, their less cytotoxicity and milder antioxidant activity (in comparison to the intracellular phlorotannin-rich isolates), in combination with the neuroprotective properties, makes them promising compounds for pharmaceutical applications and design of functional foods. However, the whole spectrum of activities needs to be addressed before the phlorotannin-based preparations can be implemented in the every-day diet. Obviously, it will be the next nearest step in the study of CW-bound phlorotannins.

Abbreviations: ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid; AGE, advanced glycation end-product; AD, Alzheimer disease; BHT, butylated hydroxytoluene; CW, cell wall; DMSO, dimethyl sulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FBS, fetal bovine serum; ER, endoplasmic reticulum; HR-MS, high resolution mass spectrometry; MHB, Mueller-Hinton broth; MIC, minimum inhibitory concentration; MTT, methylthiazolyldiphenyl-tetrazolium bromide; NADH, nicotinamide adenine dinucleotide reduced form; NBT, nitroblue tetrazolium; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; PD, Parkinson disease; PM, phenazine methosulfate; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; RT, room temperature; TEAC, Trolox equivalent antioxidant capacity; UPHLC-MS/MS, Ultra-performance liquid chromatography-tandem mass spectrometry

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Conflict of interest:

The authors state no conflict of interest

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