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Unravelling the potential of seaweeds from the Black Sea coast of Romania as bioactive compounds sources. Part I: *Cystoseira barbata* (Stackhouse) C. Agardh

Adriana Trifan[a], Alexandru Vasincu[b,*], Simon Vlad Luca[a,c,**], Christiana Neophytou[d], Evelyn Wolfram[e], Sebastian E.W. Opitz[f], Daciana Sava[g], Laura Bucur[h], Bogdan I. Cioroiu[i], Anca Miron[a], Ana Clara Aprotosoaie[a], Oana Cioanca[a], Monica Hancianu[a], Alexandra Jitareanu[j], Andreas I. Constantinou[d]

[a] Department of Pharmacognosy, Faculty of Pharmacy, Grigore T. Popa University of Medicine and Pharmacy Iasi, Iasi, 700115, Romania
[b] Department of Pharmacodynamics and Clinical Pharmacy, Faculty of Pharmacy, Grigore T. Popa University of Medicine and Pharmacy Iasi, Iasi, 700115, Romania
[c] Biothermodynamics, TUM School of Life and Food Sciences Weihenstephan, Technical University of Munich, Freising, 85354, Germany
[d] Department of Biological Sciences, University of Cyprus, Nicosia, 1678, Cyprus
[e] Centre for Biochemistry and Bioanalytics, Institute of Chemistry and Biotechnology, Zurich University of Applied Sciences, Wädenswil, 8820, Switzerland
[f] Phytopharmacy and Natural Products Research Group, Institute of Chemistry and Biotechnology, Zurich University of Applied Sciences, Wädenswil, 8820, Switzerland
[g] Department of Natural Sciences, Faculty of Natural and Agricultural Sciences, Ovidius University, Constanta, 900470, Romania
[h] Department of Pharmacognosy, Faculty of Pharmacy, Ovidius University, Constanta, 900470, Romania
[i] Fiterman Pharma LLC, Iasi, 700544, Romania
[j] Department of Toxicology, Faculty of Pharmacy, Grigore T. Popa University of Medicine and Pharmacy Iasi, Iasi, 700115, Romania

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ABSTRACT

The Romanian coastlines of the Black Sea have abundant seaweed resources, but little effort has been done to investigate their biological potential. The aim of the present study was to assess the *in vitro* antioxidant and anti-proliferative effects of *Cystoseira barbata* (Stackhouse) C. Agardh (Sargassaceae), a brown alga inhabiting the Black Sea coast of Romania. The 70% acetone, methanol and water extracts of *C. barbata* were evaluated for their total phenolic content, antioxidant activity and anti-proliferative potential against human tumor cell lines (pulmonary A549, colon HT-29, mammary MCF-7) and the non-tumor mammary epithelial MCF-10A cell line. *C. barbata* 70% acetone extract (CBAE) displayed the highest antioxidant and cytotoxic activities. The mechanism of CBAE anti-proliferative activity involved initially increased intracellular ROS accumulation, followed by increased DNA content in the subG1 phase and DNA fragmentation leading to excessive apoptosis. Thus, our study provides a theoretical basis for the use of CBAE as a tumor preventive agent. Furthermore, UHPLC-DAD-QTOF-MS analysis of CBAE tentatively identified 18 phlorotannins as fucophlorethol and eckol derivatives, containing three up to seven phloroglucinol units. In conclusion, *C. barbata* represents a valuable source for the development of macroalgal-based products with putative use as nutraceuticals and pharmaceuticals.

1. Introduction

Over the past decades, marine macroalgae have attracted an emerging interest mainly due to their bioactive constituents endowed with a broad spectrum of activities such as antioxidant, antimicrobial, anti-inflammatory and anti-tumor (Rocha et al., 2018; Michalak and Chojnacka, 2015; Brown et al., 2014). Among macroalgae, brown seaweeds (Phaeophyta) have been intensively studied, as they accumulate a variety of compounds with biological activity, such as polysaccharides, terpenoids, phlorotannins, sterols, lipids, proteins and vitamins (Balboa et al., 2013; Jiménez-Escrig et al., 2012; Wijesinghe and You-Jin, 2011).

Phlorotannins, phenolic compounds found only in Phaeophyceae, are formed *via* acetate-malonate pathway by the polymerization of phloroglucinol units (1,3,5-trihydroxybenzene). According to the type of linkage between monomer units, phlorotannins are divided into four

* Corresponding author. Faculty of Pharmacy, Grigore T. Popa University of Medicine and Pharmacy Iasi, Universitatii Str. 16, Iasi, 700115, Romania.
** Corresponding author. Faculty of Pharmacy, Grigore T. Popa University of Medicine and Pharmacy Iasi, Universitatii Str. 16, Iasi, 700115, Romania.
E-mail addresses: alexandru.vasincu@umfiasi.ro (A. Vasincu), simon-vlad.v.luca@d.umfiasi.ro (S.V. Luca).

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classes: fucols (aryl-aryl bonds), fuhalols and phloroethols (ether bonds), fucophloroethols (both aryl-aryl and ether bonds) and eckols (dibenzodioxin bonds) (Thomas and Kim, 2011). Several phlorotannins have been identified and isolated from *Ecklonia*, *Eisenia*, *Fucus* and *Cystoseira* species; their bioactivities, including antioxidant, anti-inflammatory, anti-diabetic, anti-allergic, anti-hyaluronidase, anti-tyrosinase and anti-tumor activities, have also been assessed (Bruno de Sousa et al., 2017; Kwon et al., 2013; Ferreres et al., 2012; Li et al., 2011). Among terpenoids, the xantophyll pigment fucoxantin, responsible for the brown-olive color of brown seaweeds, has been found to exhibit antioxidant, anti-inflammatory and anti-tumor effects (Molina et al., 2014; Das et al., 2008). With regard to polysaccharides, brown algae are known to produce fucoidan, laminarin, alginic acid and its derivatives, widely used in food, cosmetic and pharmaceutical industries as thickening and gelling agents. Data on their composition, structural characteristics and therapeutic properties have been reported. Fucoidans and alginic acid exhibit mainly anticoagulant and antithrombotic, antitumor, anti-inflammatory, antioxidant and immunomodulatory effects (Wijesinghe and You-Jin, 2011; Zhang et al., 2010; Das et al., 2008).

Research on bioactive compounds from Phaeophyceae has attracted an increased interest (Kamiya et al., 2010; Zubia et al., 2009; Nahas et al., 2007), as these secondary metabolites might counteract the deleterious effects of the oxidative stress in the human body. It is known that increased reactive oxygen species (ROS) generation leads to DNA damage, lipid peroxidation and protein denaturation, playing a key role in the etiology and pathogenesis of a wide range of diseases and age-related disorders such as cardiovascular diseases, diabetes mellitus, atherosclerosis, inflammatory conditions, cancer, cataract, Alzheimer's and Parkinson's diseases (Halliwell, 2011; Sies, 1997).

Regarding Phaeophyta phylum, the chemistry of Sargassaceae has been widely studied. The genus *Cystoseira* C. Agardh, one of the most representative of the sargassacean family, comprises 46 species that have been flagged as currently accepted taxonomically, alongside other 129 species in the Algaebase (Guiry and Guiry, 2019). In terms of phytochemical and bioactivity screenings, mostly *Cystoseira* species from Mediterranean and Atlantic Ocean coastal lines (such as *C. tamariscifolia*, *C. nodicaulis*, *C. usneoides*, *C. abies-marina, C. crinita, C. sedoides* and *C. compressa*) have been the focus of various research groups (Mhadhebi et al., 2014; Montero et al., 2014; Ferreres et al., 2012; Zubia et al., 2009). *Cystoseira* genus was shown to possess significant biomedical properties, as it contains molecules endowed with antioxidant effects and anti-proliferative activity in cancer cell lines (Bruno de Sousa et al., 2017; Vizetto-Duarte et al., 2016; Mhadhebi et al., 2014; Heffernan et al., 2015). Considering the bioactive potential of *Cystoseira* species, the aim of the present study was to assess the *in vitro* antioxidant and anti-proliferative activities of *Cystoseira barbata* (Stackhouse) C. Agardh (Sargassaceae), a brown alga inhabiting the Romanian Black Sea coastlines. We evaluated the total phenolic content and antioxidant activities of the 70% acetone, methanol and water extracts of *C. barbata*. The anti-proliferative potential was assessed in human tumor pulmonary adenocarcinoma A549, colorectal adenocarcinoma HT-29, mammary adenocarcinoma MCF-7 and non-tumor mammary epithelial MCF-10A cell lines. The most bioactive extract of *C. barbata* (70% acetone extract) was further evaluated in terms of its ability to induce DNA fragmentation and apoptosis in cancer cells. The extract was then subjected to UHPLC-DAD-QTOF-MS analysis in order to identify the compounds responsible for the antioxidant and anti-proliferative effects.

2. Material and methods

2.1. Chemicals

2,2′-Diphenyl-1-picrylhydrazyl radical (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 2′,7′-dichlorodihydrofluorescein diacetate, (R)-(+)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), caffeic acid, disodium phosphate dodecahydrate, Folin-Ciocalteu's phenol reagent, gallic acid, hydrogen peroxide (H_2O_2), phosphate-buffered saline (PBS), potassium ferricyanide, iron (III) chloride, phloroglucinol, sodium carbonate, were purchased from Sigma-Aldrich (Steinheim, Germany). Dimethyl sulfoxide (DMSO) and monopotassium phosphate were purchased from Merck (Darmstadt, Germany). Potassium persulfate and trichloroacetic acid were purchased from Riedel-de Haën (Seelze, Germany). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), streptomycin and penicillin were purchased from Gibco Invitrogen (Carlsbad, CA, USA). All other solvents and reagents were of analytical grade.

2.2. Algal material

Cystoseira barbata was collected from the Black Sea coastal areas of Romania (location: Mangalia - 43°48′0″ N, 28°35′0″ E). Fresh seaweed material was washed thoroughly with distilled water to remove salt, sand and epiphytes; the air dried sample (40 °C) was powdered and used for further experiments. The species was authenticated by Associate Professor Daciana Sava (Ovidius University, Constanta, Romania). A voucher specimen (no. CB120617) was deposited in the Department of Pharmacognosy, Faculty of Pharmacy, Grigore T. Popa University of Medicine and Pharmacy Iasi, Iasi, Romania.

2.3. Preparation of algal extracts

Acetone (70%), absolute methanol and water were used as extraction solvents. Dried algal powders (5 g) were extracted with 150 mL of each of the above mentioned solvents for 2 h at room temperature in the dark (Lopez et al., 2011). After filtration, the extracts were dried under reduced pressure at 40 °C and lyophilized. Significant variations in the extraction yield were observed, as follows: 70% acetone (24.31%) > water (11.59%) > methanol (3.80%). The extracts were stored at −20 °C until use.

2.4. Total phenolic content

Total phenolic contents were determined using a method previously described (Trifan et al., 2018). Phloroglucinol was used as standard. Total phenolic content was expressed as mg of phloroglucinol equivalents (PE)/g of dry extract.

2.5. Antioxidant activity assays

Antioxidant activity was evaluated by three *in vitro* assays (DPPH and ABTS radical scavenging assays, and reducing power assay) based on methods previously described (Trifan et al., 2018). In all antioxidant tests gallic and caffeic acids were used as positive controls. The EC_{50} values were calculated by linear interpolation between values above and below 50% activity, except for reducing power assay, where the EC_{50} values are the effective concentrations at which the absorbance is 0.5 (Ferreira et al., 2007).

2.6. In vitro cell-based assays

2.6.1. Extracts

Stock solutions of *C. barbata* extracts were prepared in order to be used for further experimental studies. Thus, 70% acetone and methanol extracts were dissolved at concentration of 200 mg/mL in DMSO and were then diluted in phosphate buffered saline (PBS, pH 7.4). Water extract was dissolved in PBS at concentration of 200 mg/mL. Controls contained 0.05% DMSO/PBS or PBS only.

2.6.2. Cell lines

Human tumor pulmonary adenocarcinoma A549 (ATCC® CCL 185), colorectal adenocarcinoma HT-29 (ATCC® HTB 38), mammary adenocarcinoma MCF-7 (ATCC® HTB 22) and non-tumor "immortalized" mammary epithelial MCF-10A (ATCC® CRL 10317) cells were cultured as follows. Thus, MCF-7 cells were maintained in DMEM, supplemented with FBS (10%) and antibiotic-antimycotic (1%), HT-29 cells in McCoy's 5A (modified) medium, supplemented with FBS (10%) and antibiotic-antimycotic (1%), A549 cells in RPMI 1640 + GlutaMax medium, supplemented with FBS (10%) and antibiotic-antimycotic (1%). MCF-10A cells were maintained in DMEM/F-12 medium, supplemented with 5% horse serum, 1% antibiotic-antimycotic, insulin, hydrocortisone, cholera toxin and EGF.

2.6.3. MTT assay

Cell viability was measured using the MTT colorimetric assay, as described by Mosmann (1983), with slight modifications. Briefly, cells were seeded in 96-well plates (5×10^3 cells/well) and treated with different concentrations of *C. barbata* extracts for 48 h. DMSO was used as solvent vehicle in case of 70% acetone and methanol extracts, and PBS was used as solvent vehicle in case of water extract. MTT (10 μL) was next added to each well and cells were further incubated for 3 h. The medium was then removed and DMSO (150 μL/well) was added for 20 min. The absorbance was recorded at 570 nm with a Wallac Victor^{3}™ 1420-012 microplate reader (PerkinElmer Inc., Waltham, USA). Results were expressed as *cell viability (%)* calculated as follows: % cell viability = absorbance of sample (cells treated with extract)/absorbance of untreated cells × 100. Results were expressed in terms of cell viability (%) and as half maximal inhibitory concentration (IC_{50}, μg/mL). The *selectivity index* (SI) of the extracts was determined using the following equation: $SI = C_T/C_{NT}$, where C_T and C_{NT} indicate the extract concentration inducing cytotoxicity on tumor (e.g., MCF-7) and non-tumor cells (e.g., MCF-10A), respectively (Oh et al., 2011).

2.6.4. Cell cycle analysis

MCF-7 cells were seeded in 6-well plates (2×10^5 cells/well) and incubated in the presence or absence of CBAE (50 and 100 μg/mL) for 48 h. Then, cells were fixed with 70% ethanol and treated with propidium iodide staining solution (containing 1 mg/mL propidium iodide and 100 μg/mL ribonuclease) for 30 min. Cells were analyzed using Guava EasyCyte™ flow cytometer (Millipore, Watford, UK). Results were expressed as *percentage of cells/cell cycle phase* (sub-G1, G0/G1, S, G2/M), calculated using the GuavaSoft analysis software.

2.6.5. Annexin V/Propidium iodide staining assay

MCF-7 cells were seeded in 6-well plates (1×10^5 cells/well) and incubated in the presence or absence of CBAE (50 and 100 μg/mL). After 48 h, cells were stained using annexin V Alexa Fluor® 488/propidium iodide (Annexin V AF/PI) staining, as described by Alexa Fluor™ 488 Annexin V/Dead Cell Apoptosis kit (Life Technologies). Cell viability, death and apoptosis were evaluated using the Guava EasyCyte™ flow cytometer. The percentages of viable (Annexin V AF^-/PI^-), early apoptotic (Annexin V AF^+/PI^-), late apoptotic (Annexin V AF^+/PI^+) and dead (Annexin V AF^-/PI^+) cells were extracted by the GuavaSoft analysis software (Neophytou et al., 2014).

2.6.6. Cell death detection ELISA (enzyme-linked immunosorbent assay)

MCF-7 cells were seeded in 96-well plates (1×10^4 cells/well) and incubated in the presence or absence of CBAE (50 and 100 μg/mL) for 48 h. The quantification of mono- and oligonucleosomes present in the cytoplasm of apoptotic cells was performed using the cell death detection ELISA^{PLUS} kit (La Roche, Indianapolis, IN, USA), following the manufacturer's instructions (Neophytou et al., 2014). The results were expressed as the specific *enrichment factor* (arbitrary units, AU) of mono- and oligo-nucleosomes released into the cytoplasm, calculated using the following formula: enrichment factor (AU) = absorbance of sample (cells treated with extract)/absorbance of untreated cells.

2.6.7. 2′,7′-Dichlorodihydrofluorescein diacetate assay

The intracellular pro-oxidant effects of CBAE (100 μg/mL) were evaluated according to the method described by Girard-Lalancette et al. (2009), with slight modifications (Vasincu et al., 2014). MCF-7 cells were seeded in 12-well plates (4×10^4 cells/well) and treated with 100 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) for 30 min. Next, the cells were incubated in the presence or absence of CBAE extract for 1, 2, 3 and 24 h or H_2O_2 (100 μM) as positive control. The fluorescence intensity was measured with an Infinite F200 automated plate reader (Tecan, Männedorf, Switzerland) at an excitation wavelength of 485 nm and emission wavelength of 535 nm. Data were expressed as *pro-oxidant activity (%)* in comparison to the positive control group (H_2O_2 treated cells).

2.7. UHPLC-DAD-QTOF-MS analysis

Chromatographic analysis of CBAE using ultra high performance liquid chromatography (UHPLC) was carried out on a system consisting of an Agilent 1290 Infinity II system with a diode array detector (DAD) coupled with an Agilent 6540 Ultra High Definition (UHD) Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) mass spectrometer (Agilent Technologies, USA). Chromatographic separations by UHPLC were carried out at 30 °C on a Zorbax Rapid Resolution High Definition Eclipse Plus C18 column for UHPLC (2.1 × 50 mm, 1.8 μm particles; Agilent Technologies, USA). Injection volume was 0.5 μL. The mobile phases were 0.1% formic acid in water (solvent A) and acetonitrile (solvent B), with a flow of 0.3 mL/min and the following gradient: 0–1 min, 0.5% B; 1–7 min, 0.5–30% B; 7–8 min, 30–95% B; 8–10 min, 95% B; then back to initial conditions in 5 min. Dual Agilent Jet Stream Electrospray Ionization (Dual AJS ESI) source was operated in negative ion mode. The mass spectrometer was used upon following settings: capillary voltage 2.5 kV; sheath gas flow rate 10 L/min; temperature of drying gas and sheath gas 300 °C; pressure of nebulizer 35 psi; skimmer 65 V; fragmentor voltage 120 V. The spectra were scanned in the range of 100–2000 *m/z*. Collision energy for the MS/MS experiments was 20 eV. The UV spectra were recorded between 200 and 600 nm, and the wavelength of 280 nm was chosen for determination. Data were analyzed with Agilent MassHunter software (Version B.08.00, Agilent Technologies, USA, 2016). All compounds had eluted within the first 6.5 min and therefore the chromatograms are of this duration. The individual compounds were tentatively identified by comparison of the exact molecular masses, UV spectra, mass spectra and fragmentation patterns to those from online available databases and literature data (Ferreres et al., 2012; Martínez and Castañeda, 2013; Montero et al., 2014; Li et al., 2017; Vissers et al., 2017; Hermund et al., 2018; Lopes et al., 2018).

2.8. Statistical analysis

All experiments were performed in triplicates and results were expressed as means ± standard error of the means (SEM). Statistical analyses were carried out using the one-way analysis of variance, Duncan's multiple range tests and paired Student's *t*-test. A difference was considered significant at the 5% level ($p < 0.05$).

3. Results and discussion

3.1. Total phenolic content

The total phenolic content of *C. barbata* extracts varied significantly. The 70% acetone extract showed the highest phenolic content (236.03 ± 1.79 mg PE/g), followed by methanol and water extracts (Table 1). Phenolic levels within the range found in our study were reported for the methanol extract of *C. abies-marina* collected from the

Table 1
Total phenolic content and antioxidant activity of *C. barbata* extracts.

Extract/Positive control	Total phenolic content*	DPPH radical scavenging assay EC_{50}**	ABTS cation radical scavenging assay		Reducing power assay EC_{50}**
			EC_{50}**	TEAC***	
70% Acetone[a]	236.03 ± 1.79[b,c]	88.5 ± 0.3[b,c,d,e]	13.9 ± 0.2[b,c,d,e]	0.54 ± 0.00[b,c,d,e]	16.07 ± 0.02[b,c,d,e]
Methanol[b]	140.60 ± 0.80[a,c]	155.1 ± 2.6[a,c,d,e]	20.5 ± 0.2[a,c,d,e]	0.49 ± 0.00[a,c,d,e]	25.56 ± 0.30[a,c,d,e]
Water[c]	126.45 ± 1.20[a,b]	211.3 ± 2.9[a,b,d,e]	22.1 ± 0.3[a,b,d,e]	0.45 ± 0.00[a,b,d,e]	35.41 ± 0.33[a,b,d,e]
Gallic acid[d]	–	1.6 ± 0.0[a,b,e]	0.5 ± 0.0[a,b,c,e]	19.28 ± 0.0[a,b,c,e]	1.58 ± 0.00[a,b,c,e]
Caffeic acid[e]	–	3.6 ± 0.0[a,b,d]	1.6 ± 0.0[a,b,c,d]	6.11 ± 0.00[a,b,c,d]	2.01 ± 0.00[a,b,c,d]

*mg PE/g extract; **μg/mL; *** μM Trolox equivalent to 1 μg/mL extract/positive control; a. $p < 0.001$ differences to 70% acetone extract; b. $p < 0.001$ differences to methanol extract; c. $p < 0.001$ differences to aqueous extract; d. $p < 0.001$ differences to gallic acid; e. a < 0.001 differences to caffeic acid.

Azores Islands (135.08 mg/g) (Barreto et al., 2012) and for the water extract of Tunisian *C. barbata* (207.95 mg/g) (Haddar et al., 2012). However, Mhadhebi et al. (2014) reported slightly higher phenolic content for the methanol extract of *C. crinita,* native to the Tunisian Mediterranean Sea (261.53 mg/g). This variation in phenolic amount between *Cystoseira* species might be attributed not only to differences in extraction methods of algal material, but also to spatial and temporal environmental variability (sunlight, temperature, salinity, nutrients, climate, season) and reproductive development of seaweed (Abdala-Diaz et al., 2006; Stengel et al., 2011).

3.2. Antioxidant activity

Antioxidant activities are attributed to the multifunctional properties of bioactive compounds which may act as radical scavenging, reducing and metal ions chelators (Prior et al., 2005). The antioxidant activity of *C. barbata* extracts was assessed by three *in vitro* antioxidant assays and compared with the activity of commonly used positive controls, such as gallic and caffeic acids (Table 1).

In all assays, the highest antioxidant effect was obtained with the 70% acetone extract, followed by methanol and water extracts, as indicated by the EC_{50} and the Trolox equivalent antioxidant capacity (TEAC) values (Table 1). However, the positive controls showed significantly higher antioxidant activities than *C. barbata* extracts. A direct comparison of our results with those reported by other studies for macroalgae is not possible due to the fact that different methods and different ways of expressing antioxidant results are used. However, it is noteworthy that under similar experimental conditions, *C. barbata* extracts showed higher ABTS radical scavenging activity (EC_{50} values range: 13.9 ± 0.2–22.1 ± 0.3 μg/mL) than phlorotannins dieckol and phlorofucofuroeckol-A isolated from *Eisenia bicyclis* (EC_{50} values of 41.85 ± 2.3 and 36.84 ± 0.9 μg/mL, respectively) (Kwon et al., 2013). Regarding the DPPH scavenging potential, EC_{50} values within the range found in our study were reported for different extracts from *Cystoseira* species, such as acetone extract of *C. amentacea* endemic in the Adriatic Sea (150.2 μg/mL) (Stanojković et al., 2014) and methanol extract of Tunisian *C. crinita* (107 μg/mL) (Mhadhebi et al., 2014).

The antioxidant activities of *C. barbata* extracts increased in a concentration-dependent manner in all tests (data not shown). Dose-dependent antioxidant activities were reported for extracts derived from brown algae species by several *in vitro* studies (Chandini Kumar et al., 2008; Kumar et al., 2008; Ye et al., 2009; Vinayak et al., 2011; Chakraborty et al., 2013). More, among *C. barbata* extracts, high and significant correlations between total phenolic content (TPC) and antioxidant assays (EC_{50} values) were established by the Pearson correlation analysis (TPC-DPPH radical scavenging activity: $R = -0.9374$; TPC-ABTS radical cation scavenging activity: $R = -0.9978$; TPC-reducing power: $R = -0.9150$). Andrade et al. (2013) suggested that the antioxidant activity of *Cystoseira* sp. is mainly due to their high content in phlorotannins and phenolic compounds, but other constituents (e.g. proline, mannitol, palmitic acid, oleic acid, fucosterol) might also contribute to the overall antioxidant effects.

3.3. In vitro effects in human cell lines

3.3.1. Cell viability

MTT assay was used to investigate the impact of *C. barbata* extracts on cell viability in three human tumor cell lines (pulmonary adenocarcinoma A549, colorectal adenocarcinoma HT-29, mammary adenocarcinoma MCF-7 cells) and one non-tumor mammary epithelial cell line (MCF-10A). *C. barbata* extracts presented a significant inhibitory activity on viabilities of MCF-7 cells in a dose-dependent manner (Fig. 1). Among tested *C. barbata* extracts, 70% acetone extract had the highest cytotoxic activity in all tumor cell lines tested, except for the non-tumor MCF-10A cells. This effect was more pronounced in MCF-7 cells (IC_{50} = 72.12 ± 1.53 μg/mL) compared to A549 and HT-29 cell lines (Table 2). The comparison of our results with those reported in the literature for brown seaweeds-derived extracts is not straightforward due to limited information available about chemical phlorotannin composition and differences in the *in vitro* testing. However, the cytotoxic effects on the A549 cell line observed for Romanian-sourced *C. barbata* 70% acetone extract (IC_{50} = 196.12 ± 3.43 μg/mL) were similar to those reported by Kosanić et al. (2015) for an acetone extract of *C. barbata* collected from the Adriatic Sea (IC_{50} = 190.34 ± 0.56 μg/mL).

Importantly, the viability of the "normal immortalized" non-tumor MCF-10A cells was practically unaffected when treated with up to 500 μg/mL of 70% acetone extract (IC_{50} of 562.39 ± 4.19 μg/mL). Thus, *C. barbata* 70% acetone extract showed a selective cytotoxic potential for tumor cells when comparing MCF-7 and MCF-10A cells (SI = 7.8), as samples with SI values higher than 3 are considered as highly selective (Mahavorasirikul et al., 2010). Based on these results, *C. barbata* 70% acetone extract (CBAE) was further used to study the mechanisms of action associated with the cytotoxicity observed in MCF-7 cells.

3.3.2. Cell cycle

Cell cycle has a significant impact on regulation of cell physiology, such as cell proliferation and apoptosis. Cell cycle arrest is a common cause of growth inhibition (Alberts et al., 2002). To determine whether the inhibitory effects of CBAE on MCF-7 cell proliferation involve alterations in cell cycle progression, cell cycle distribution analysis by flow cytometry using propidium iodide as a probe was undertaken. The binding of water soluble DNA intercalating propidium iodide correlates with the amount of DNA within a given cell and the relative content of DNA indicates the distribution of a population of cells throughout the cell cycle (Chao et al., 2019; Wang et al., 2019).

In order to determine the effect of CBAE on the cell cycle phase distribution, MCF-7 cells were incubated with the extract (either 50 μg/mL or 100 μg/mL) for 24 h. CBAE produced a substantial accumulation in subG1 phase, while the amounts of cells in G0/G1, S and G2/M phases were correspondingly decreased (Fig. 2Aa,b). In comparison to untreated control, CBAE in doses of 50 μg/mL and 100 μg/mL exhibited a 23.7% and 44.1%, respectively, increase in the percentage of MCF-7 cells in subG1 phase (Fig. 2Ac). SubG1 cells are apoptotic cells with

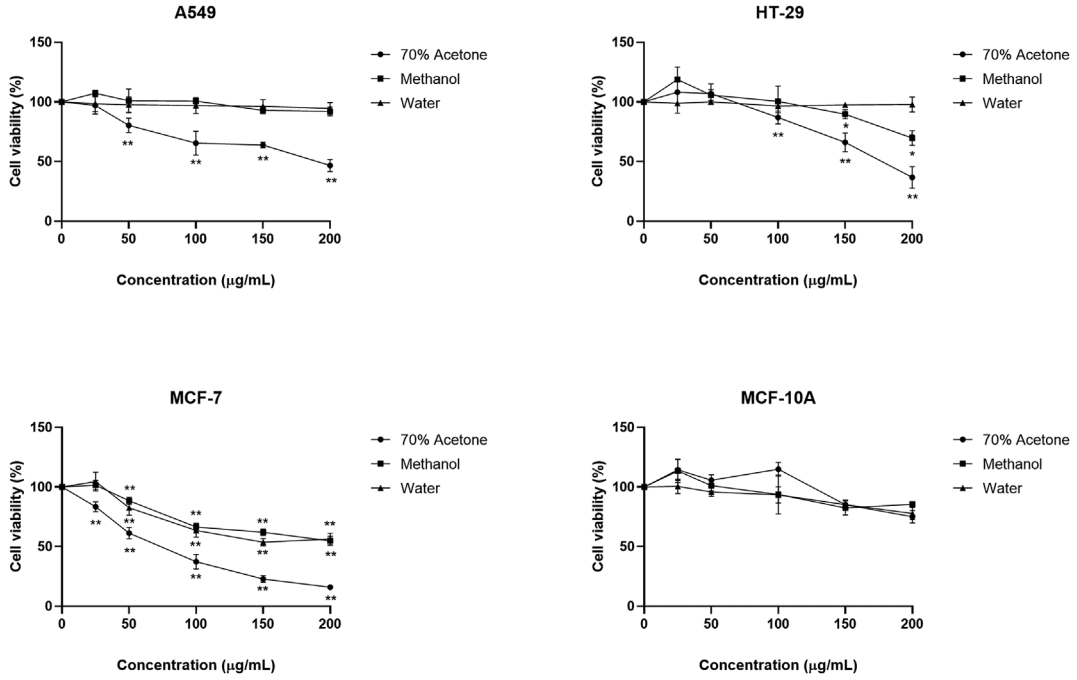

Fig. 1. The effects of *C. barbata* extracts on the viabilities of A549, HT-29, MCF-7 and MCF-10A cell lines. Cells were seeded in 96-well plates (5×10^3/well) and treated with different concentrations of *C. barbata* extracts (25–200 μg/mL). After 48 h, cell viability was evaluated using the MTT assay. Each experimental group was repeated in triplicate and data are representative of three individual experiments. Bars correspond to the standard error of mean (SEM); *$p < 0.05$ *vs.* solvent vehicle; **$p < 0.01$ *vs.* solvent vehicle.

fragmented DNA (Matson and Cook, 2017), indicating that the CBAE-induced cytotoxicity in MCF-7 cells was not related to cell cycle arrest and is likely due to mechanisms associated with apoptosis.

3.3.3. Apoptosis

Apoptosis is a physiological process defined as the programmed cell death and characterized by certain morphological and biochemical features that distinguish it from other forms of cell death (Brady, 2004). Induction of apoptosis is the major pathway to inhibit tumor cell proliferation by various agents (Martin and Green, 1995). To investigate the mechanisms involved in reducing MCF-7 cell viability, the ability of CBAE to induce apoptosis was further examined by flow cytometry with annexin V AF/PI staining. Analysis of the externalization of phosphatidylserine using flow cytometry is a well-established method for the detection of early and late stage apoptotic cells. During apoptosis, the loss of membrane phospholipid asymmetry with the translocation of phosphatidylserine from the inner to the outer leaflet of the cell membrane occurs even before membrane damage and nuclear breakdown. Since Annexin V strongly binds to phosphatidylserine, the degree of apoptosis can be quantitatively assessed by using a cell analyzer able to distinguish between intact, early apoptotic, late apoptotic, or necrotic cells (Koopman et al., 1994). Treatment of MCF-7 cells with CBAE for 24 h resulted in the appearance of early apoptotic cells (Fig. 2Ba,b,c). At 100 μg/mL, CBAE induced early apoptosis in MCF-7 cells, which was evident with 5.8 ± 0.2% of cells staining with Annexin V AF/PI (Fig. 2Bc). However, CBAE treatment determined a significant increase of MCF-7 cells in late apoptosis (3-fold increase) (Fig. 2Bd).

Fragmentation of nuclear DNA into nucleosomal units through activation of endonucleases can be also hallmark of apoptosis (Matassov et al., 2004). Therefore, the pro-apoptotic effects of CBAE were additionally analyzed by the ELISA cell death assay, which enabled the detection of mono- and oligonucleosomes released in the cytoplasm. CBAE (at a concentration of 100 μg/mL) produced a 3.5-fold increase in DNA fragmentation compared to vehicle-treated MCF-7 cells (Fig. 2C). Overall, these results indicate that apoptosis contributed significantly to the reduction in MCF-7 cell viability when exposed to CBAE.

3.3.4. Intracellular ROS level

ROS play an important role in living organisms, as growth factors and intracellular signal molecules. At low doses, ROS are involved in regulation of physiological processes such as cell cycle progression and proliferation, differentiation, migration and cellular death. In certain conditions, excess cellular levels of ROS cause protein, nucleic acid and lipid damage that can lead to activation of apoptosis. The anti-proliferative effect is often associated with induction of oxidative stress in cancer cells. Therefore, oxidative stress might be considered as the main cause of cell apoptosis or necrosis (Halliwell, 2011; Redza-Dutordoir and Averill-Bates, 2016).

The ability of CBAE to induce pro-oxidant effects in tumor cells was evaluated by DCFH-DA assay at 1, 2, 3 and 24 h of incubation. Fig. 3 shows the ROS content in MCF-7 cells treated with 100 μg/mL of CBAE.

Table 2
In vitro cytotoxic activity, expressed as IC_{50} values (μg/mL), of *C. barbata* extracts on A549, HT-29, MCF-7 and MCF-10A cell lines.

Extract	IC_{50} (μg/mL)			
	A549	HT-29	MCF-7	MCF-10A
70% Acetone	196.12 ± 3.43	174.3 ± 2.13	72.12 ± 1.53	562.39 ± 4.19
Methanol	247.09 ± 5.64	305.56 ± 4.37	261.82 ± 2.54	664.12 ± 5.21
Water	210.85 ± 4.52	280.26 ± 5.19	223.79 ± 4.78	613.20 ± 7.11

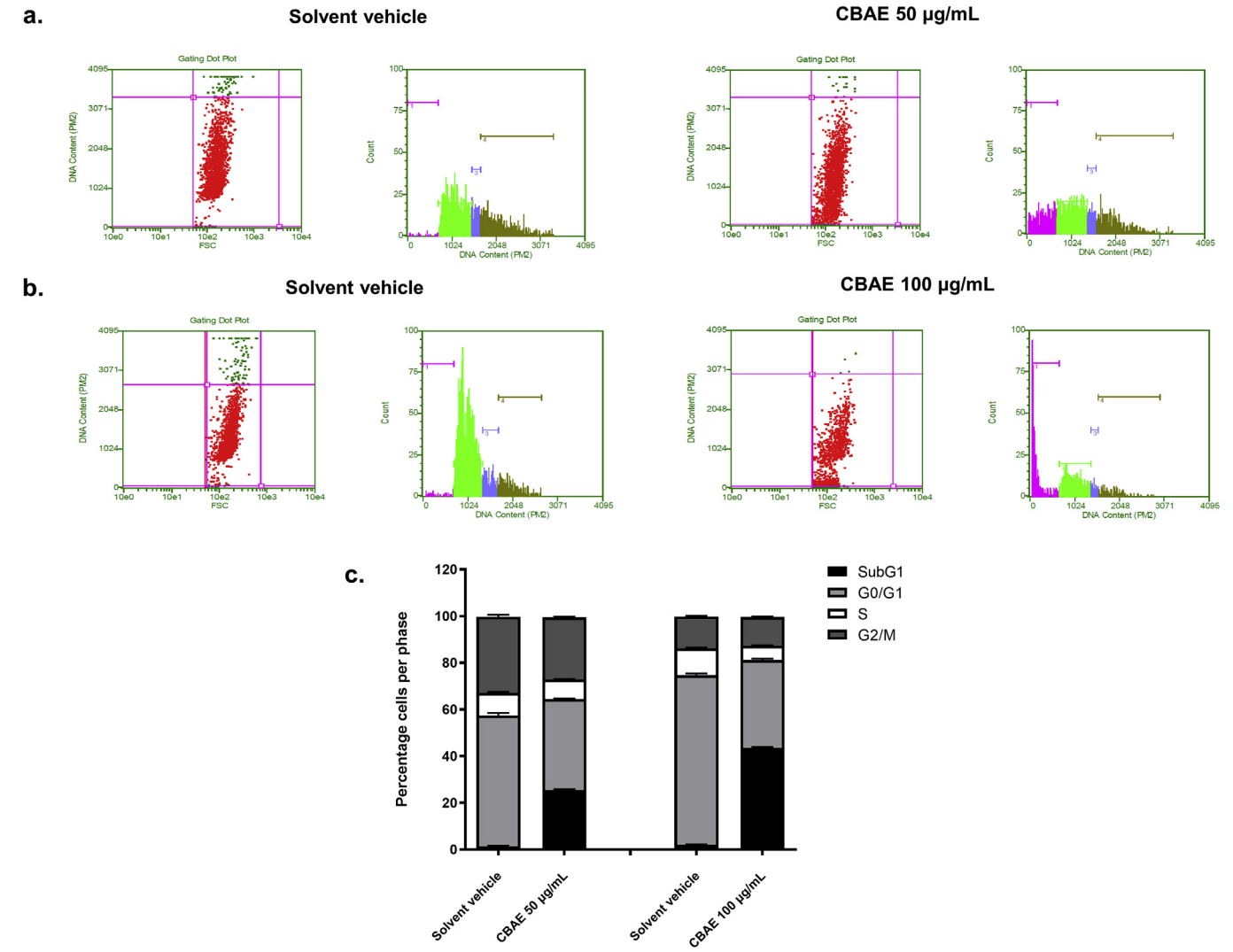

Fig. 2. Effects of CBAE on the cell cycle progression, cell apoptosis and DNA integrity of MCF-7 cells. Cells were incubated with extract at concentrations of 50 µg/mL and 100 µg/mL or solvent vehicle for 48 h. **(A)** Effects of CBAE on the cell cycle progression of MCF-7 cells. Cell cycle distribution was evaluated using propidium iodide staining. Cytograms and histograms results of MCF-7 cells after being treated for 48 h with CBAE 50 µg/mL *vs.* solvent vehicle (**a**), and CBAE 100 µg/mL *vs.* solvent vehicle (**b**). *Left side* (cytograms): propidium iodide fluorescence *vs*. forward scatter; green-colored cells indicate viable cells; red-colored cells indicate dead cells. *Right side* (histograms): cell counts *vs*. propidium iodide fluorescence; purple (1) – cells in subG1 phase; light green (2) – cells in G0/G1 phase; blue (3) – cells in S phase; dark green (4) – cells in G2/M phase. Percentage (%) of cells per phase of cell cycle (**c**).
(B) Incubation of MCF-7 cells with CBAE promotes apoptosis. Flow cytometric results of MCF-7 cells after being treated for 48 h with either solvent vehicle (**a**), CBAE 50 µg/mL (**b**), and CBAE 100 µg/mL (**c**). Scatter plots showing distribution of Annexin-V/PI staining: upper left quadrant – dead cells; upper right quadrant – late apoptotic cells; lower left quadrant - live cells; lower right quadrant - early apoptotic cells. Percentage (%) of early apoptosis and late apoptotic cells *vs*. concentration of CBAE (**d**).
(C) Effect of CBAE on DNA integrity of MCF-7 cells. Cells were treated for 48 h with CBAE 50 µg/mL and 100 µg/mL *vs.* solvent vehicle. The presence of nucleosomes in the cytoplasm was determined with the ELISA cell death detection kit and is expressed as Enrichment factor.
Each experimental group was repeated in triplicate; bars correspond to the SEM; ****$p < 0.001$ *vs*. solvent vehicle. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

As compared to the negative control, CBAE increased the production of ROS in MCF-7 cells in a time-dependent manner. After 24 h incubation time, the ROS content in CBAE treated cells was significantly higher as compared to non-treated cells (2.8-fold increase). The above results suggest that CBAE induced excessive ROS accumulation in MCF-7 cells that could initiate DNA damage and lead to apoptosis.

Taken together, our data suggest that the reduced viability of MCF-7 cells after CBAE treatment is mainly due to the induction of apoptosis. The core mechanism for this induction might consist of intracellular ROS accumulation as the initiator of apoptosis, followed by the accumulation of cells in subG1 phase, DNA fragmentation, ultimately leading to apoptosis. Our results are in agreement with recent studies that evidenced the *in vitro* anti-proliferative, pro-apoptotic and growth-inhibiting effects of brown seaweeds extracts in various types of cell lines, such as melanoma, lymphoma, glioblastoma, hepatic, colon, gastric, ovarian and lung cancer cell lines (Bruno de Sousa et al., 2017; Rocha et al., 2018).

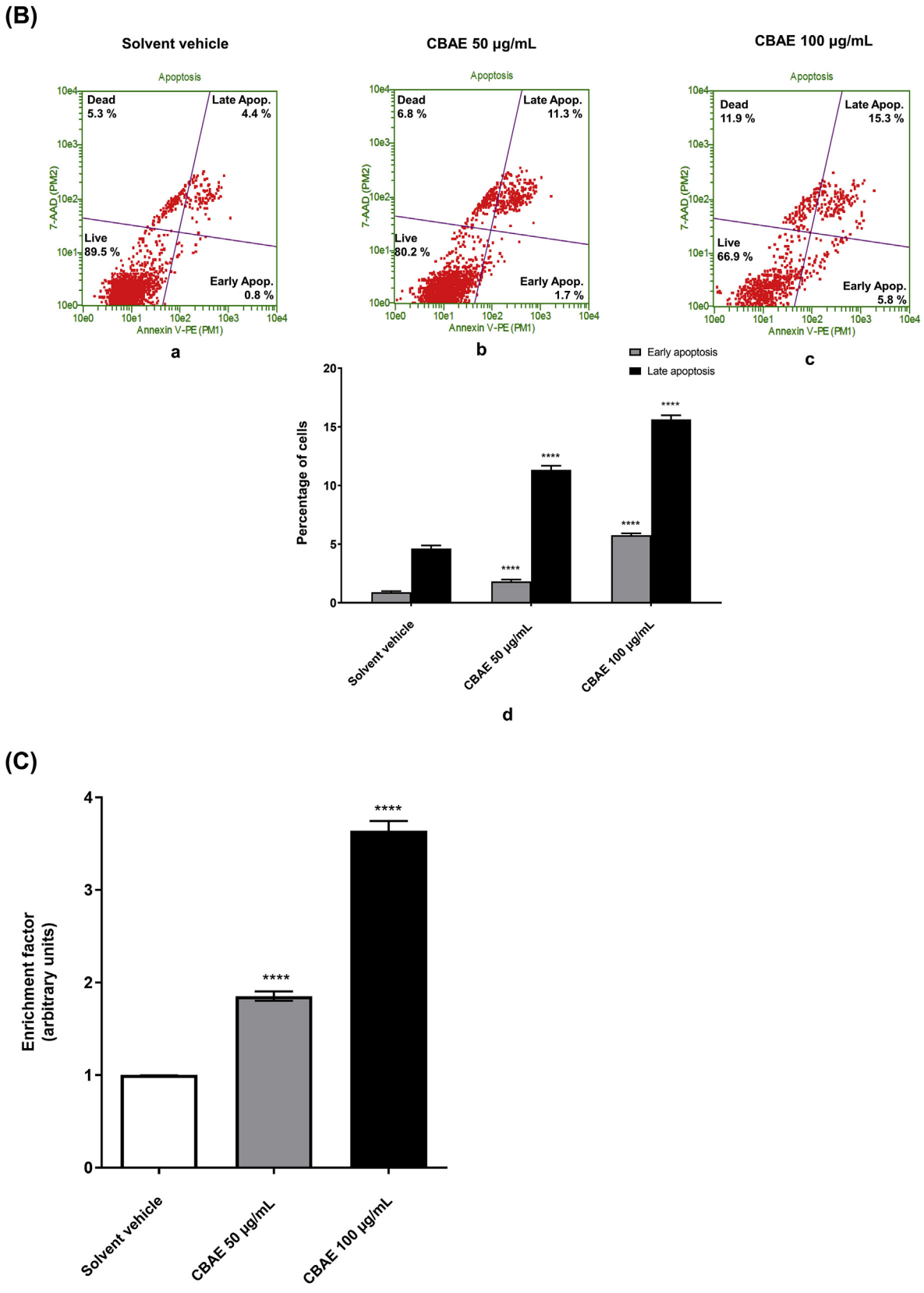

Fig. 2. (*continued*)

3.4. Phlorotannin profile

Literature survey revealed that only a small number of phlorotannins have been isolated and chemically characterized from *Cystoseira* species (Ferreres et al., 2012; Montero et al., 2014). Related to *C. barbata*, Sellimi et al. (2017) investigated the chemical profile of Tunisian-sourced brown seaweed by HPLC-MS analysis, with the tentative identification of phenolic compounds based on their mass

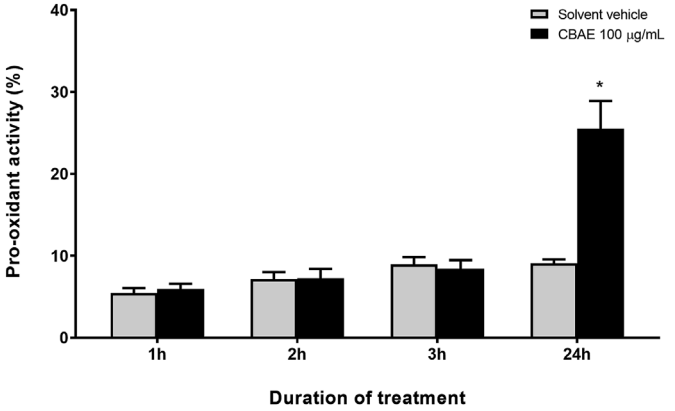

Fig. 3. Effect of CBAE on intracellular ROS levels of MCF-7 cells after incubation with 100 µg/mL of extract for 1, 2, 3 and 24 h; bars correspond to the SEM of three repeated experiments; *p < 0.05 *vs.* solvent vehicle.

spectra. To the best of our knowledge, mass spectra confirming the presence and polymerization degree of phlorotannins in Romanian-sourced *C. barbata* were not investigated.

Since CBAE exhibited the highest total phenolic content and both the highest antioxidant and anti-proliferative activities, it was further analyzed using UHPLC-DAD-QTOF-MS, in order to characterize the main phlorotannins found in *C. barbata* for better understanding of its bioactivity. The study of phlorotannin derivatives in CBAE was conducted using the known isotopic masses of the most common phlorotannins previously identified in brown seaweeds and, notably, in *Cystoseira* species. Thus, the extracted ion chromatograms (EICs) of the deprotonated molecular ions ($[M-H]^-$) corresponding to eckol (*m/z* 371.0409), fucophloroethol (*m/z* 373.0565), 7-phloroeckol (*m/z* 495.0569), fucodiphloroethol (*m/z* 497.0725), phlorofucofuroeckol (*m/z* 601.0624), fucotriphloroethol (*m/z* 621.0886), dieckol (*m/z* 741.0733), and fucophloroethols with six (*m/z* 745.1046) and seven phloroglucinol units (*m/z* 869.1207) were depicted in Fig. 4, together with the UV chromatogram at 280 nm.

On the basis of these data, a number of 18 compounds were tentatively annotated as phlorotannins. Furthermore, the diagnostic fragment ions with *m/z* 125, 247/249, 371, 495/497 or 619/621 observed in their MS/MS spectra (Table 3) confirmed the presence of one or more phloroglucinol units. Nevertheless, it can be noticed that the identified phlorotannins exhibited multiple ion peaks corresponding to the same $[M-H]^-$ ion but with different retention times (Fig. 4). Phlorotannins are known to exist in various conformations that differ in the branching position of the phenyl and ether linkages of the subsequent phloroglucinol units (Ferreres et al., 2012; Martínez and Castañeda, 2013). This might give rise to complex interactions with the stationary phase of the UHPLC column and could explain the variable elution times observed in our study. However, due to the limitations of UHPLC-DAD-QTOF-MS technique, the full structures of these conformers were not proposed.

Compounds 1–3 had the deprotonated molecular ions $[M-H]^-$ at *m/z* 373 showing similar fragmentation patterns, with several fragment ions that are suggestive to phlorotannin-based structures. Thus, these compounds exhibited the neutral losses of one water molecule [−18 Da (*m/z* 355)], one phloroglucinol unit [−126 Da (*m/z* 247)], one phloroglucinol unit and one oxygen atom [−126 Da, −16 Da (*m/z* 233)] or one phloroglucinol unit and water [−126 Da, −18 Da (*m/z* 229)]. These observations led to the conclusion that compounds 1–3 with $[M-H]^-$ at *m/z* 373 are phlorotannin trimers that contain both phenyl and ether couplings, being tentatively identified as isomers of fucophloroethol (Li et al., 2017; Martínez and Castañeda, 2013; Wang et al., 2012). The structure of a fucophloroethol isomer together with its proposed fragmentation patterns are given in Fig. 5. Phlorotannins with similar precursor and fragment ions were identified in *C. abies-marina* and other brown seaweeds such as *Fucus* and *Laminaria* species (Hermund et al., 2018; Lopes et al., 2018; Vissers et al., 2017).

Compound 4 with the deprotonated molecular ion $[M-H]^-$ at *m/z* 495 was identified as a phlorotannin tetramer, corresponding to a phloroekol isomer. Compounds 5–7 presented similar deprotonated molecular ions at *m/z* 497 which were assigned to phlorotannin tetramers (Table 3). The main MS/MS fragment (*m/z* 235) observed for

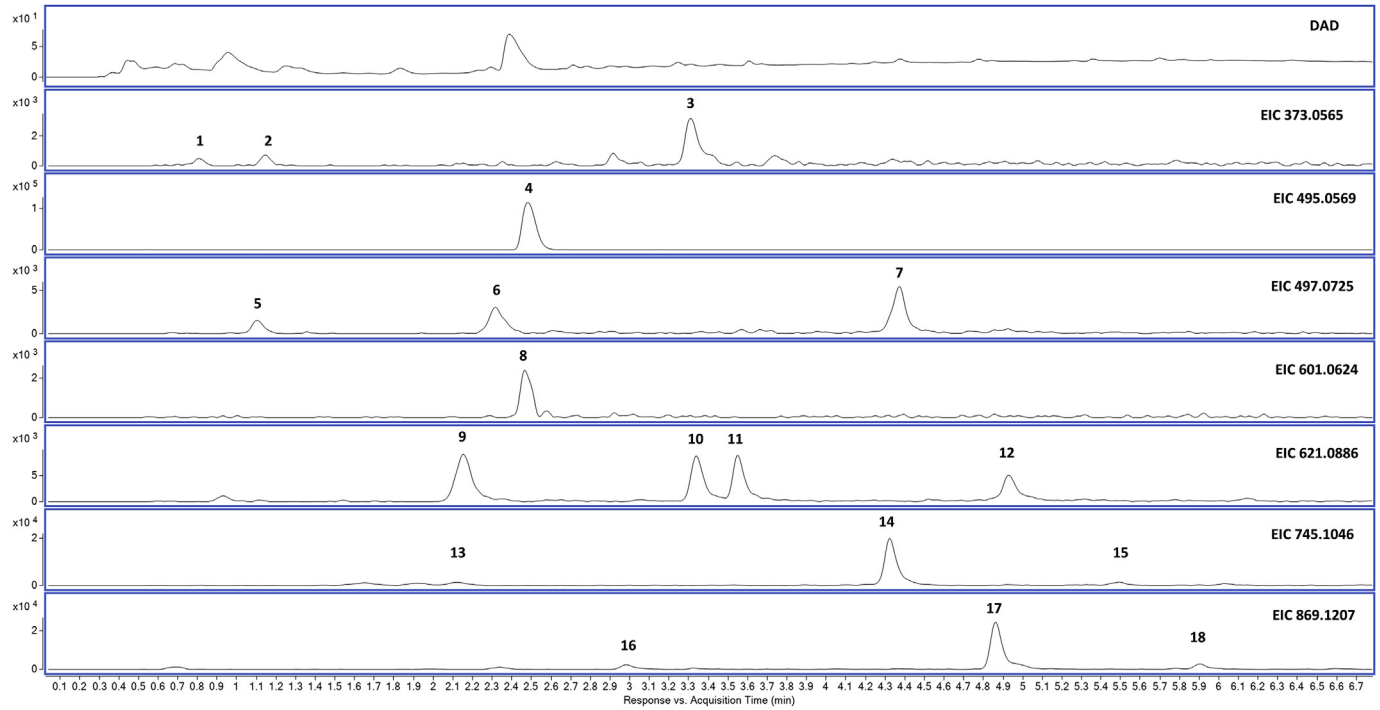

Fig. 4. UV (280 nm) and Extracted Ion Chromatograms (EIC) obtained from UHPLC-DAD-QTOF-MS of CBAE $[M-H]^-$ (*m/z*): **1–3**, 373.0565; **4**, 495.0569; **5–7**, 497.0725; **8**, 601.0624; **9–12**, 621.0886; **13–15**, 745.1046; **16–18**, 869.120.

Table 3
Molecular formula, retention time, UV (nm) and mass spectrometric data of phlorotannins from *C. barbata* 70% acetone extract determined by UHPLC-DAD-QTOF-MS.

Compound[a]	Phlorotannin oligomer	Molecular formula	RT (min)	UV (nm)	$[M-H]^-$, *m/z*	MS/MS, *m/z*
1	Trimer	$C_{18}H_{14}O_9$	0.802	208, 276sh	373.0552	355.0461, 293.0465, 275.0311, 249.0416, 233.0465, 231.0311, 207.0622, 205.0596, 191.0712, 189.0557, 181.0544, 165.0567, 163.0482, 139.0388, 137.0311, 111.0466
2	Trimer	$C_{18}H_{14}O_9$	1.149	207, 270sh	373.0560	311.0589, 285.0392, 271.0611, 259.0442, 241.0295, 227.0155, 187.0411, 183.0175, 141.0193, 129.0133, 127.0408
3	Trimer	$C_{18}H_{14}O_9$	3.307	207, 274sh	373.0547	355.0488, 311.0511, 293.0487, 285.0411, 283.0619, 269.0451, 267.0301, 257.0441, 243.0664, 241.0511, 225.0522, 215.0588, 203.0311, 201.0112, 177.0551, 169.0314, 151.0544, 107.0133
4	Tetramer	$C_{24}H_{16}O_{12}$	2.485	208, 276sh	495.0569	389.0432, 373.0411, 371.0211, 247.0289, 203.0342, 167.0311, 121.0112
5	Tetramer	$C_{24}H_{18}O_{12}$	1.105	208, 272sh	497.0727	235.0267
6	Tetramer	$C_{24}H_{18}O_{12}$	2.325	211, 271sh	497.0740	373.0531, 371.0387, 355.0482, 353.0271, 335.0194, 291.0155, 249.0405, 247.0274, 245.0213, 233.0462, 231.0391, 229.0518, 219.0312, 205.0545, 177.0589, 161.0322, 139.0475, 125.0274, 111.0411
7	Tetramer	$C_{24}H_{18}O_{12}$	4.372	209, 270sh	497.0718	479.0571, 373.0539, 355.0451, 289.0296, 263.0422, 247.0306, 245.0036, 229.0572, 219.0348, 205.0563, 203.0322, 177.0558, 161.0384, 135.0115, 125.0225, 111.0435
8	Pentamer	$C_{30}H_{18}O_{14}$	2.463	208, 273sh	601. 0623	583.0455, 493.0413, 477.0511, 371.0315, 369.0122, 355.0481, 353.0293, 247.0211, 125.0239
9	Pentamer	$C_{30}H_{22}O_{15}$	2.154	212, 275sh	621.0864	603.0733, 585.0689, 413.0356, 373.0511, 371.0328, 355.0482, 353.0295, 343.0325, 327.0491, 309.0344, 289.0358, 283.0685, 247.0211, 245.0051, 243.0611, 231.0348, 229.0580, 207.0612, 205.0544, 203.0388, 177.0521, 165.0595, 163.0436, 161.0240, 139.0188, 125.0247
10	Pentamer	$C_{30}H_{22}O_{15}$	3.340	209, 277sh	621.0879	603.0711, 479.0619, 373.0511, 355.0439, 289.0360, 263.0478, 247.0312, 245.0043, 229.0580, 219.0344, 205.0535, 203.0299, 177.0542, 161.0211, 135.0186, 125.0264
11	Pentamer	$C_{30}H_{22}O_{15}$	3.550	208, 275sh	621.0878	603.0755, 585.0661, 495.0582, 479.0654, 477.0462 461.0479, 373.0590, 371.0301, 355.0388, 353.0410, 337.0482, 309.0382, 283.0656, 247.0222, 245.0155, 231.0334, 229.0599, 207.0616, 201.0128, 177.0544, 163.0424, 139.0112, 125.0293
12	Pentamer	$C_{30}H_{22}O_{15}$	4.930	209, 276sh	621.0879	577.0882, 495.0534, 371.0325, 265.0511, 263.0421, 247.0256, 245.0012, 231.0299, 229.0550, 201.0156, 177.0555, 161.0244, 139.0112, 125.0285
13	Hexamer	$C_{36}H_{26}O_{18}$	4.323	209, 274sh	745.1059	497.0681, 479.0541, 353.0422, 247.0299, 245.0182, 229.0518, 219.0385, 205.0545, 177.0215, 175.0348, 159.0312, 125.0199
14	Hexamer	$C_{36}H_{26}O_{18}$	5.498	208, 278sh	745.1051	389.0572, 369.0144, 355.0582, 353.0411, 325.0422, 263.0441, 247.0289, 245.0133, 231.0375, 229.0544, 219.0388, 201.0188, 139.0152, 125.0277
15	Hexamer	$C_{36}H_{26}O_{18}$	6.028	208, 278sh	745.1058	341.0433, 321.0152, 271.0642, 247.0214, 245.0149, 229.0534, 219.0322, 203.0344, 177.0515
16	Heptamer	$C_{42}H_{30}O_{21}$	2.976	209, 277sh	869.1218	621.0851, 537.0622, 497.0611, 479.0522, 461.0348, 387.0622, 371.0411, 283.0615, 247.0388, 245.0199, 229.0544, 219.0345, 203.0341, 163.0455, 135.0146
17	Heptamer	$C_{42}H_{30}O_{21}$	4.869	207, 276sh	869.1203	851.1062, 619.0712, 495.0523, 479.0585, 371.0452, 353.0411, 309.0378, 249.0416, 247.0322, 245.0155, 229.0582, 219.0380, 205.0582, 203.0318, 177.0551, 161.0244, 133.0254
18	Heptamer	$C_{42}H_{30}O_{21}$	5.906	209, 274sh	869.1211	601.0598, 497.0688, 387.0611, 371.0422, 353.0488, 351.0322, 323.0388, 285.0444, 263.0485, 249.0398, 245.0201, 231.0344, 229.0558, 201.0142, 159.0284

[a] Peak identity as in Fig. 4.

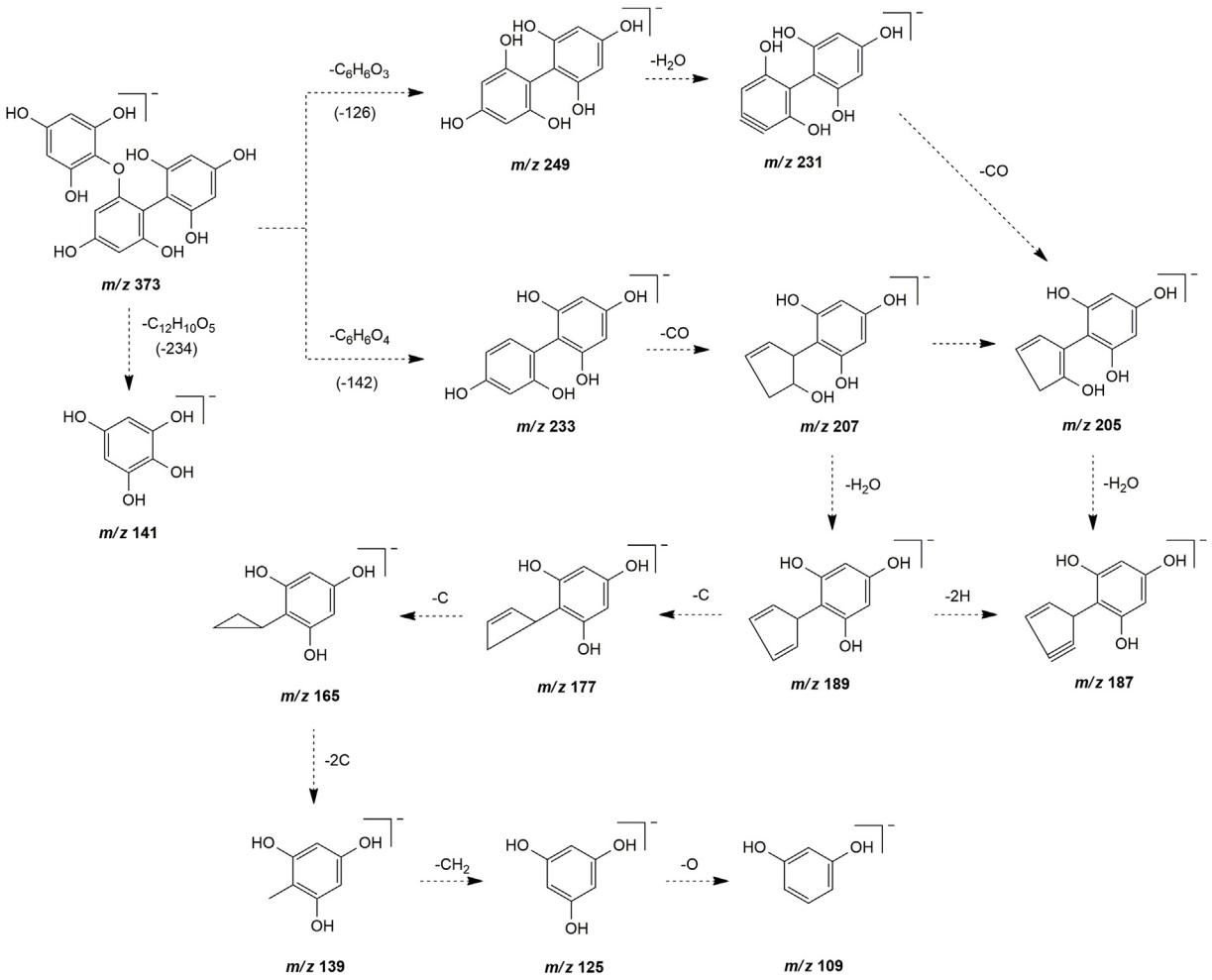

Fig. 5. Proposed fragmentation patterns of phlorotannin trimers (*m/z* 373) identified in 70% acetone extract of *C. barbata*.

compound 5 resulted from the loss of two phloroglucinol units and a methyl group (−124 Da, −124 Da, −14 Da), indicating the presence of two fucol moieties linked by an aryl-ether bond. Thus, compound 5 was tentatively identified as bisfucophlorethol (Lopes et al., 2018). Compounds 6 and 7 showed similar fragmentation patterns, with the loss of two phloroglucinol units that indicated the presence of two aryl-ether bonds, likely corresponding to isomers of fucodiphlorethol. Compound 8 with the deprotonated molecular ion ($[M-H]^-$) at *m/z* 601 was identified as a phlorotannin pentamer, corresponding to a phlorofucofuroeckol isomer. Compounds 9–12 with the same deprotonated molecular ion ($[M-H]^-$) at *m/z* 621 showed similar fragmentation patterns and were suggested to be phlorotannins composed of five phloroglucinol units, possibly isomers of fucotriphlorethol (Hermund et al., 2018; Lopes et al., 2018). Furthermore, isomers with $[M-H]^-$ at *m/z* 745 (compounds 13–15) and *m/z* 869 (compounds 16–18) were tentatively identified as fucophlorethols with six and seven phloroglucinol units, respectively. The fragmentation patterns of the identified isomers showed several structural differences, thus indicating a high phytochemical diversity of phlorotannins found in *C. barbata*. Nevertheless, conclusive structural data of the isomers found in *C. barbata* could be provided by the means of nuclear magnetic resonance (NMR) analysis, with previous purification of the algal extract. Findings in the present study are in agreement with other reports stating the occurrence of phlorotannins belonging to fucophlorethol and eckol groups in *Cystoseira* species (Ferreres et al., 2012; Montero et al., 2014). More, Ferreres et al. (2012) showed the presence of phlorotannins with a similar degree of polymerization for *Cystoseira* species (*C. nodicaulis*, *C. tamariscifolia*, *C. usneoides*) native to Atlantic Ocean.

As regards the link between the *in vitro* anti-proliferative activity and the phytochemical profile of *C. barbata*, fucophlorethol- and eckol-type phlorotannins have been previously reported to possess anti-proliferative effects, acting in different hallmarks of cancer. Thus, fucodiphloroethol G, dieckol, 1-(3′,5′-dihydroxyphenoxy)-7-(2″,4″,6″-trihydroxyphenoxy)-2,4,9-trihydroxy-dibenzo-1,4-dioxin, eckol and phlorofucofurofuroeckol A isolated from *Ecklonia cava* were found able to exert specific cytotoxicity towards several human tumor cell lines (HeLa, HT1080, A549 and HT-29) with IC_{50} values between 180.3 and 362.5 μM (Li et al., 2011). More, dieckol (200–400 μg/mL) was shown to suppresses the cell proliferation of MCF-7 and SK-BR-3 human breast cancer cells. Dieckol induced cell cycle arrest at the G2/M phase and apoptosis by activating the intrinsic apoptotic pathway as evaluated by an increased Bax/Bcl-2 ratio (You et al., 2018). Dioxinodehydroeckol (100 μM) was also found to exert inhibitory growth effects against MCF-7 cells with apoptosis inducing properties (Kong et al., 2009). Moreover, phlorotannins might act as chemopreventive agents due to their antioxidant potential, since oxidative stress is recognized to play a key role in cancer initiation, promotion and progression (Rocha et al., 2018). In conclusion, our study provided evidence of antioxidant and anti-proliferative activities of Romanian-sourced *C. barbata* as assessed by *in vitro* tests. The 70% acetone extract of *C. barbata* displayed a high content of phenolic compounds as well as a selective cytotoxic effect against mammary carcinoma MCF-7 cells. More, it reduced cell proliferation and inhibited cell growth through apoptosis induction. We reported herein for the first time mass spectra confirming the presence and degree of polymerization of phlorotannins in *C. barbata*, compounds that might play a role as natural protective agents against

mammary carcinoma. However, the present research possessed certain limitations due to the fact that the experiments were performed *in vitro*. The human body is an extremely sophisticated physiological system, therefore the anti-proliferative activity of *C. barbata* needs to be further investigated *in vivo*.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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