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ANTIOXIDANT, ANTIPLATELET AND CYTOTOXIC ACTIVITY OF EXTRACT OF *CYSTOSEIRA AMENTACEA* FROM THE COAST OF MONTENEGRO (SOUTH-EAST ADRIATIC SEA)

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Seaweeds are an excellent source of compounds with biological activity. Since algae have been used in traditional medicine for a long time and also some algal substances have shown cytotoxic, anticoagulant and antiplatelet activity, they have been extensively studied. We examined the cytotoxicity and cell cycle distribution of acetone extract of Brown alga *Cystoseira amentacea,* on different human malignant cell lines. The antioxidant and antiplatelet activity of the extract has been evaluated. The results indicated that extract of *Cystoseira amentacea* significantly decreased cell survival in all tested cell lines. The IC50 values in the MTT assay in human cervix carcinoma (HeLa), two human breast cancer cell lines (MDA-MB-361 and MDA-MB-453) and a human colon carcinoma (LS174) cells were ranged from 22.87±1.26 to 64.34±2.78μg/ml. In vitro antitumor activites was accompained by an important sub-G1 accumulation of all cells after treatment of tested cell lines with extract. Antioxidant activity of *Cystoseira amentacea* extract in vitro was shown using chemically and cell-based assays, in erythrocytes and neutrophils. Finally, extract of *Cystoseira amentacea* showed significant in vitro inhibitory effects on both platelet activation and aggregation with other blood cells in response to agonist in healthy donors.

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1. Introduction

The discovery of drugs from marine natural products has enjoyed a renaissance in the past few years [1,2]. Broad range of bioactivities of marine natural products is reviewed in many articles, including marine pharmacology, drugs and cosmetics from the sea, unconventional natural sources for future drug discovery, new structures and bioactivities for small-molecule natural products, and marine natural products and related compounds in clinical and advanced preclinical trials [1].

Marine algae have long been used as food and medicine in Asian countries such as Japan, China and Korea. Consumption of the brown marine algae is thought to ameliorate some inflammatory disorders and high cholesterol level [3,4]. Also, brown marine algae have been used in the traditional medicine for treating some types of cancers and for providing many health benefits.

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Marine algae have yielded a variety of secondary metabolites that possess novel chemical structures and interesting pharmacological activities [5,6]. Recently, researchers have described a wide range of biological activities for algal compounds including anti-HIV, anticoagulant, anticonvulsant, anti-inflammatory, bacteriostatic, antineoplastic, and cytotoxic activities [7,8]. *Cystoseira* (Cystoseiraceae) is a widely distributed genus of brown algae with antibacterial, antifungal, and cytotoxic activities [9,10,11]. *Cystoseira spp*. possesses, among their more significant compounds, different types of terpenes. Terpenes containing aryl groups have been attracting more and more attention because they present broad spectra of pharmacological activities, and combine valuable curative properties with practically no harmful side effects. A number of diterpenes and sterols have been isolated from the brown algae belonging to the genus *Cystoseira* [10-14].

Brown alga, *C. amentacea* var. *spicata* is found in abundance at the coastal area of the Boka Kotorska, Montenegro. This study was designed to determine biological properties of these marine algae extract. The extract were tested for their cytotoxic activity *in vitro* against the human cancer cell lines: HeLa (cervix adenocarcinoma cell line), LS174 (human colon carcinoma), MDA-MB-361 and MDA-MB-453 (breast cancer cell lines). The changes in cell cycle progression were evaluated using FACS analysis. Also, the antioxidant activity, using both chemically-based and cell-based assays and antiplatelet activity of the extract has been evaluated. *C.amantacea* has never been examined for anticancer, antioxidant or antiplatelet activity.

2. Experimental Section

2.1. Algal sampling and classification

Samples of brown alga, *C. amentacea* var. *spicata,* were collected from the Boka Kotorska, Montenegro on the peninsula Luštica in June 2009. The alga was identified by V. Mačić at the Institute of Marine Biology Kotor, Montenegro according to Ercegović (1952), [15] Giaccone & Bruni (1973), [16] and Gomez-Garreta et al. (2001) [17]. The voucher specimens were deposited at repository of the Institute. The collected samples were washed thoroughly with fresh water to remove salts, sand, and epiphytes, and stored at –20°C until further use.

2.2. Preparation of algal extract

Frozen samples of the investigated algae (50 g) were extracted using acetone in a Soxchlet extractor. The extract was filtered and then concentrated under reduced pressure in a rotary evaporator. The dry extract was stored at -18°C until it was used in the tests. The extract was dissolved in DMSO for the experiments with the concentration of DMSO in final reaction mixtures less than 0.2%. DMSO solution corresponding to the concentration in the extract solution was used as a control.

2.3. Cytotoxic activity

2.3.1. Cell culture

Human cervix adenocarcinoma HeLa cells, human breast cancer MDA-MB-361 and MDA-MB-453 cells and human colon carcinoma LS174 cells (American Type Culture Collection, USA) were cultured as a monolayer in the RPMI 1640 nutrient medium, with 10% (inactivated at 56ºC) FBS, 3 mM of L-glutamine, and antibiotics, at 37ºC in humidified air atmosphere with 5% CO_2. For the growth of MDA-MB-361 and MDA-MB-453 cells complete medium was enriched with 1.11 gL^{-1} glucose.

2.3.2. In vitro cytotoxic assay

In vitro assay for cytotoxic activity of investigated extract was performed when the cells reached 70–80% of confluence. Stock solution (50 mg mL^{-1}) of extract was dissolved in corresponding medium to the required working concentrations. Neoplastic HeLa cells (2000 cells per well), MDA-MB-361 cells (7000 cells per well), MDA-MB-453 cells (3000 cells per well) and LS174 cells (7000 cells per well) were seeded into 96-well microtiter plates, and 24 h later, after cell adherence, 5 different, double diluted concentrations of investigated extract were added to the

wells. Final concentrations of the extract were 100, 50, 25, 12.5, and 6.25μg mL^{-1} except for the control wells, where only nutrient medium was added. The cultures were incubated for the next 72 h. The effect on cancer cell survival was determined 72 h after the addition of extract, by the MTT test [18]. Briefly, 20 μL of MTT solution (5 mg mL^{-1} PBS) was added to each well and incubated for a further 4 h at 37°C in 5% CO_2 and humidified air. Subsequently, 100 μL of 10% SDS was added to solubilise the formazan crystals formed from MTT after the conversion by mitochondrial dehydrogenases of viable cells. Absorbencies proportional to the number of viable cells were measured using a microplate reader (Multiskan EX, Thermo Scientific, Finland) at 570 nm. Each experiment was performed in triplicate and independently repeated at least four times.

2.4. Flow cytometry analysis

Cellular DNA content and cell distribution were quantified by flow cytometry using propidium iodide (PI). Cells (3×10^5 cells/well) were seeded in 6-well plates and incubated with or without IC50 concentration of investigated extract for 24 h. After treatment, the cells were collected by trypsinization, and fixed in ice-cold 70% ethanol at −20 ◦C overnight. After fixation, the cells were washed in PBS and pellets obtained by centrifugation was treated with RNase (100 μg m^{-1}) at 37 ◦C temperature for 30 min and then incubated with propidium iodide (PI) (40 μg mL^{-1}) for at least 30 min. DNA content and cell cycle distribution were analyzed using a Becton Dickinson FAC-Scan flow cytometer. Flow cytometry analysis was performed using a CellQuestR (Becton Dickinson, San Jose, CA, USA), on a minimum of 10,000 cells per sample [19].

2.5. Antioxidant activity

2.5.1. DPPH radical scavenging assay

Free radical scavenging activity (RSA) of the extract on the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical was carried out according to the procedure described previously with slight modifications [20]. The antiradical activity was evaluated using a dilutions series, in order to obtain a large spectrum of sample concentrations. Plant extracts (100 μl) were mixed with 1400 μl of 80 μM methanolic solution of DPPH. Absorbance at 517 nm was measured after 20 min. The percentage of inhibition was calculated using following equation:

RSA (%) = $[(A_0\text{-}A_i)/A_0] \times 100$, where A_0 is absorbance of the control and A_i is absorbance of the samples. IC_{50} values were estimated using a nonlinear regression algorithm. All test analyses were run in triplicate. Trolox was used as a positive control.

2.5.2. Blood samples

Peripheral venous blood for *in vitro* testing of cellular antioxidative and antiplatelet effects of investigated extract was obtained from 4 healthy human volunteers, aged between 30 and 50 years. All participants provided written informed consent. All blood samples were taken in the morning after overnight fasting by venipuncture according to the guidelines for blood sampling in platelet analysis.

2.5.3. Determination of antioxidant protection of erythrocytes and polymorphonuclear granulocytes

Cellular antioxidant activity of investigated algal sample was based on the antioxidant protection of erythrocytes (red blood cells, RBC's) and polymorphonuclear leucocytes (PMNs) exposed to reactive oxygen species (ROS). The cellular antioxidant protection assay, based on the staining of both cell types with two different intracellular dyes: 2′,7′-dichlorofluorescein-diacetate (DCF-DA) and dihydrorhodamine (DHR), was performed as previously described with modifications regarding lower level of extracellular ROS applied [21,22]. Packed RBC's were isolated from the whole blood of donors by three subsequent washings with PBS. PMNs were isolated from the whole blood of same donors by a double gradient of Histopaque 1077 and 1119, according to the protocol recommended by the manufacturer followed by the lysis of RBC's using FACSLysing solution. Obtained RBC's and PMN's, re-suspended in PBS, were treated with serial dilutions of investigated sample (1h, 37°C). After the incubation cells were washed twice with PBS to remove extracellular antioxidants, and incubated with intracellular dyes, DCF-DA (50μM)

or DHR (1μM), washed again and treated with hydrogen peroxide (1 mM) for 30 min. Intracellular ROS levels were analysed by flow cytometry (FACSCalibur, BD, USA) based on fluorescence of dichlorofluorescein (DCF) or rhodamin, fluorescent product of DCF-DA and DHR reaction with intracellular H_2O_2 . Mean fluorescence intensity (MFI) of total number of analysed RBC's and PMN's were used for calculation of and presented as mean ± SD of data obtained in three subjects. All analyses were performed in duplicates.

2.6. Determination of platelet activation - in vitro

Platelet activation marker, P-selectin, was measured by the whole-blood flow cytometry according to the previously published protocol with slight modifications for *in vitro* testing [23]. In brief, after the venepunction aliquots of dissolved (1:10 in Hepes-Tyrode Buffer, pH 7,4) anti-coagulated blood (3.2% citrate) were incubated with serial (2x) dilutions of investigated extracts (30 min, 37°C) and subsequently incubated with CD61-PerCP (panplatelet marker), CD62P-PE (anti-P-selectin) monoclonal antibodies with suboptimal concentration of platelet agonists (0,5 mM ADP) for 20 minutes in dark at room temperature. After the incubation with antibodies, samples were fixed with para-formaldehyde solution (0.5%) for 15 minutes and analysed. Sample analysis was performed on FACSCalibur flow cytometer with CellQuest software (Becton Dickinson, USA). Results were presented as antigen positive platelets (%) in the platelet pool (20000 events).

2.6.1. Determination of platelet-monocyte and platelet-neutrophil aggregation - in vitro

Determination of platelet-monocyte and platelet-neutrophil aggregation - in vitro was performed according to the previously published protocol with slight modification regarding *in vitro* assessment [24]. Briefly, after the venepunction aliquots of anti-coagulated blood (3.2% citrate) were incubated with serial (2x) dilutions of investigated extracts (30 min, 37°C) and subsequently incubated with CD61-FITC (panplatelet marker), CD11b-PE and CD14-PerCP monoclonal antibodies with suboptimal concentration of platelet agonists (0,5 mM ADP) for 20 minutes in dark at room temperature. After the incubation with antibodies, samples were lysed using FACSLysing solution and subsequently fixed with para-formaldehyde solution (0.5%) for 15 minutes and analysed. Sample analysis was performed on FACSCalibur flow cytometer with CellQuest software (Becton Dickinson, USA).

Results were presented as percentage of CD14+CD61+ events, representing platelet-monocyte aggregates in the pool of CD14 positive cells (1000 events) and CD11b+CD61+ events, representing platelet-neutrophil aggregates in the pool of CD11b positive cells (≥ 20000 events).

2.7. Statistical analysis

All results are presented as mean ± standard deviations (SD). Data were analysed by one sample t-test and paired-samples t-test. Pearson's correlation coefficients were calculated to examine the relationships between obtained data. $p < 0.05$ was considered statistically significant. SPSS program, version 19 (SPSS Inc., Chicago, IL) was used for the analysis.

3. Results and discussion

3.1. Antitumor activity of Cystoseira amentacea extract (CAE)

3.1.1. In vitro cytotoxic effect of CAE on malignant cells

To examine potential cytotoxic effects of *CAE*, several human malignant cell lines were cultured for 72 hours at various concentrations of algae extract and analyzed by MTT test.

The *in vitro* assay showed that *CAE* exhibited significant cytotoxic effects in all tested cell lines (Table 1.). The order of sensitivity of various human cell lines to extract's cytotoxic action was: human breast cancer MDA-MB-453 (IC_{50}=22.87±0.98 μg mL^{-1}), > human cervix adenocarcinoma HeLa (IC_{50}=35.15±0.53 μg mL^{-1}), > human colon carcinoma LS174 (IC_{50}=55.43±1.56 μg mL^{-1}), > human breast cancer MDA-MB-361 (IC_{50}=64.34±1.21 μg mL^{-1}). The sensitivity of the human MDA-MB-453 and HeLa cell lines to the algal extract were generally stronger than LS174 and MDA-MB-361 cell lines. Our obtained data indicated that the extract can

be a source of bioactive compounds responsible for cytotoxic activity. As shown in some recent works, *Cystoseira spp.* possess, among their more significant compounds, different types of terpenes [25]. Terpenes containing aryl groups have been attracting more and more attention because they present broad spectra of pharmacological activities, and combine valuable curative properties with practically no harmful side effects. Various diterpenes have been identified by Zubia et al. [26] as the bioactive compounds in several species of the genus *Cystoseira*. These diterpene compounds could be responsible for the antitumoral activities of the *Cystoseira* extracts.

Table 1 shows IC_{50} values of CAE against cell lines.

Extract	*IC50(μg mL-1)*			
	HeLa	MDA-MB-361	MDA-MB-453	LS174
Cystoseira amentacea	35.15±0.53	64.34±1.21	22.87±0.98	55.43±1.56

IC_{50} values (μg mL^{-1}) of acetone extract of *Cystoseira amentacea* against tumor cell lines. Results are expressed as mean ± SD.

3.1.2. Effects of CAE on malignant Cell-Cycle Arrest

In order to obtain more information regarding events related to cytotoxic activity of investigated extract, changes in cell cycle progression were evaluated using FACS analysis. For that purpose, HeLa, MDA-MB-453, MDA-MB-361 cells as well as LS174 cells were treated for 24 hours with *CAE* at concentration corresponding to IC_{50} value. Investigated extract of *Cystoseira amentacea*, after 24 hours of continual incubation with HeLa cells induced increase of the percent of apoptotic cells (sub-G1 peak), along with a slight decrease of the percent of cells in G0/G1 phase (Fig.1). Also slight accumulation of cells in S phase of cell division was found after 24 hours of treatment of HeLa cells with algae extract. The greatest percent (23%) of apoptotic cells was found in MDA-MB-453 cell lines upon 24 hours of continual treatment with algae extract. This accumulation of MDA-MB-453 cells in sub-G1 phase was followed by the strong decrease of the cells in G0/G1 phase of cell cycle. Treatment of LS174 cells with algae extract induced accumulation of cells in sub-G1 peak (20%), and slight decrease of the cells in G0/G1, as well as G2/M phase of cell cycle. Extract of *Cystoseira amentacea*, after 24 hours treatment of MDA-MB-361 cells had no effect on cell-cycle progression in MDA-MB-361 cell lines. In this study, we demonstrated that extract of *Cystoseira amentacea* is capable to induced perturbation of cell cycle phase distribution in tested cell lines.

3.2. Antioxidant activity of Cystoseira amentacea extract (CAE)

3.2.1. DPPH assay

Reactive oxygen species (ROS) could be formed by several metabolic pathways as well as by various exogenous factors. Balance between production of ROS and activity of antioxidant defence system is of crucial importance for normal cell function. Because of possible disintegration of cell membranes and damage of essential bio-molecules such as proteins, DNA and lipids which can lead to many diseases it is very important that antioxidant system is functioning properly. Beside enzymes such as superoxide dismutases, catalases, gluthatione peroxidase and some other molecules that are first line of defence, natural antioxidants from vegetables, fruits and other foods represent are significant especially because many synthetic antioxidants have shown toxic and mutagenic effects [27,28,29]. It has been shown that compounds present in seaweeds such as polysaccharides, dietary fibres, proteins, amino acids, polyphenols and carotenoids possess antioxidant activity [30]. Stable synthetic free radical 1,1-dyphenyl-2-picrylhydrazil (DPPH) has been widely used for rapid evaluation of potential radical scavenging activity *in vitro* based on possibility of DPPH reduction in presence of a hydrogen donating antioxidant [31,32,33]. Radical scavenging activity of the CAE was determined by decrease in absorbance of DPPH radical measured at 517 nm. Tested extract showed significant antiradical activity EC_{50}=150.2 ± 2.9 μg mL^{-1} even if it was less pronounced than the activity of Trolox (EC_{50}=4.2 ± 0.2μg mL^{-1}). Compared to DPPH radical-scavenging activity of other brown algae from genus *Cystoseira*, activity of CAE was stronger than the activity of

dichlormethane:methanol (1:1) extract of *C. tamariscifolia* (EC_{50}=0.49 ± 0.01 mg mL^{-1}) [26]. Airanthi *et al.* [34] showed that methanol extract of *C. hakodatensis*, one of the five species of brown algae analyzed, was the best source of antioxidants demonstrating the highest antioxidant activity probably due to the high content of phenolics and fucoxanthin.

3.2.2. Antioxidant Protection of Erythrocytes –in vitro

Red blood cells are used in the study as a simple cellular model for the evaluation of antioxidants interactions with biological systems regarding their bioavailability and effective intracellular protection against reactive oxidant species (ROS). Antioxidant activity of investigated extract was evaluated based on the reduction of intracellular ROS, detected after cell-staining with two intracellular dyes: 2′,7′-dichlorofluorescein diacetate (DCF-DA) and dihydrorhodamine 123 (DHR). In the presence of ROS these agents were oxidized into fluorescent products, dichlorofluorescein (DCF) and rhodamine123, respectively, and their fluorescence was measured by flow cytometry.

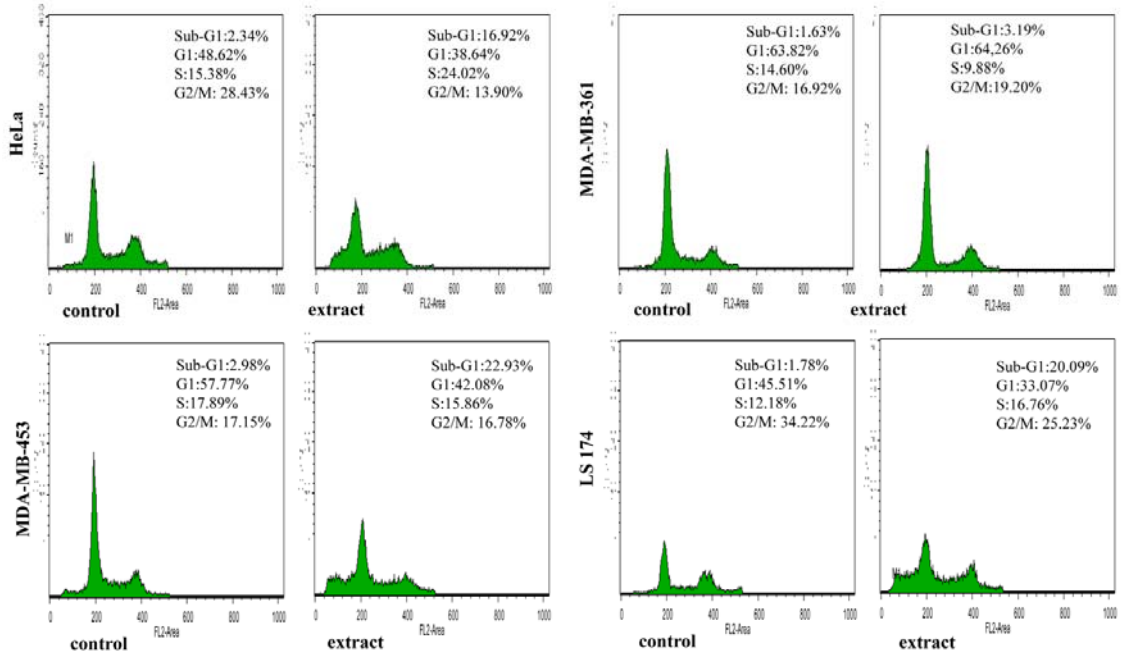

Fig. 1. Cell cycle analysis of HeLa, MDA-MB-361, MDA-MB-453 and LS174 cells untreated (control) and treated with the IC_{50} concentration of Cystoseira amentacea extract for 24 h.

Treatment of freshly isolated erythrocytes with H2O2 (1mM) induced the increase in both DCF and rhodamine fluorescence. Pre-treatment with CAE (50µg mL^{-1}) caused the reduction of H_2O_2 induced fluorescence, indicating the decrease in H_2O_2 levels within the cell. The obtained reduction rates were 39.31 ± 15.9% ($p = 0.054$) and 47.9 ± 12.8 % ($p < 0.05$), measured using DCF-DA and DHR123 respectively. Representative graphs of DCF and rhodamine fluorescence decrease in CAE pre-treated erytrocytes are shown in Fig. 2.

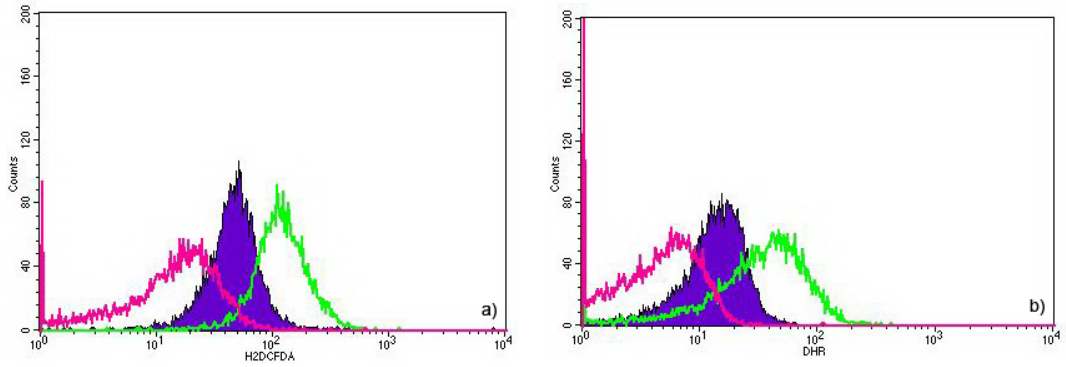

Fig. 2. Representative histogram showing (a) DCF and (b) rhodamin fluorescence decrease in erythrocytes pre-treated with CAE and subsequently exposed to H_2O_2 (full area), compared to the fluorescence of non-pretreated cells, exposed to H_2O_2 (green borderline) and cells without H_2O_2 exposure (purple borderline).

Obtained results demonstrate the potential of investigated extract to pass through the biological membrane of erythrocytes and exhibit antioxidant potential within the cell. Antioxidant activity of algal extracts and isolated compounds in a cell-based assay have been previously reported. Takamatsu et al. compared antioxidant activity using chemical and cell-based assays for a great number of marine natural products, including secondary metabolites of brown algae (*Pheophyta*) [35]. Based on the results of this study several bioactives from marine organisms showed antioxidant activity in DPPH assay but without effects within promyelocytic HL60 cellular model, while others showed antioxidant activity in both in vitro models. Strong antioxidant activity within the cell without radical scavenging activity against DPPH radical was also observed for some algal secondary metabolites, suggesting that it could be a result of interaction between bioactive compound and biological system regarding activation. Kang et al. [36] investigated DPPH scavenging activity and antioxidant activity in rat kidney homogenates for 17 different algal extracts. Three algal extracts, including extract of brown alga *Ecklonia stolonifera* inhibited ROS generation upon stimuli, measured by using DCF-DA. The data obtained in our study showed similar results, showing marked antioxidant activity of CAE in a cellular model at concentrations that provided moderate radical scavenging activity in DPPH assay. At the same time red blood cells are considered to be more convenient model for cellular antioxidant activity screening, compared to transformed or malignant cells, which are often characterized by disturbed metabolism and redox regulation [22].

3.2.3. Inhibition of ROS induced ROS formation by PMNs - in vitro

PMN cells are highly reactive immune cells characterized by the rapid production of ROS, as their functional feature, in response to both oxidative damage and pro-inflammatory stimuli. In our experimental conditions, in freshly isolated PMNs treated with lower concentrations H_2O_2 (100μM) compared to those used on erythrocytes, a marked increase in intracellular ROS is observed, based on both DCF and rhodamine 123 fluorescence. Pre-treatment of PMNs with investigated CAE (50μg mL^{-1}) did not influence the raise in intracellular ROS after H_2O_2 stimulation, based on DCF fluorescence, but it inhibited rhodamine 123 fluorescence increase for about 42.7 ± 15.34 % ($p < 0.05$). Representative graphs of DCF and rhodamine fluorescence change in basal conditions and after hydrogen-peroxide treatment, with and without CAE pre-treatment are shown in Figure 3. The results obtained with DHR staining indicate that investigated extract could be effective in protection of PMN cells from oxidative damage, induced by extracellular H_2O_2. At the same time the difference in the effects observed with DHR and DCFDA in parallel could be used in evaluation of the precise mechanisms of action of algal bioactives as inhibitors and oxidant scavengers in immune response, exploiting differential reactivities of DCDHF and DHR [37].

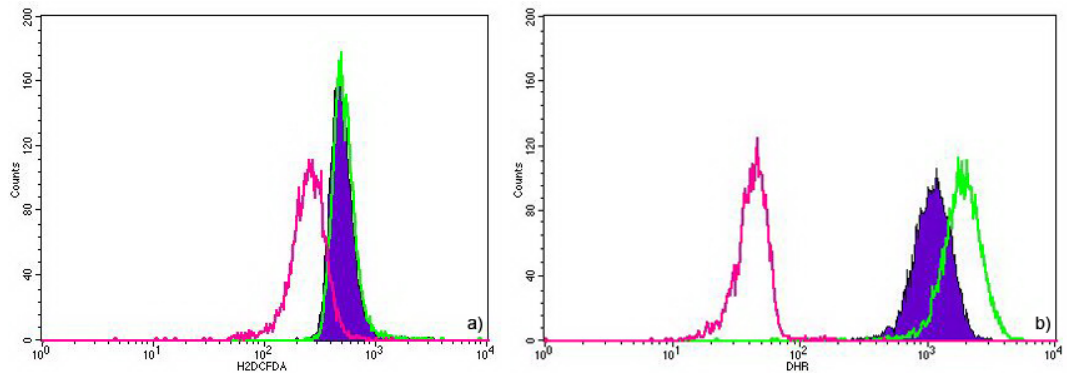

Fig. 3. Representative histogram showing (a) DCF and (b) rhodamin fluorescence decrease in PMNs pre-treated with CAE and subsequently exposed to H_2O_2 (full area), compared to the fluorescence of non-pretreated cells, exposed to H_2O_2 (green borderline) and cells without H_2O_2 exposure (purple borderline).

3.3. Antiplatelet activity of Cystoseira amentacea extract (CAE) -in vitro

3.3.1. Inhibition of platelet activation

We investigated the effects of CAE on platelet activation based on the expression of P-selectin on platelet surface after stimulation with suboptimal concentration (0.5 μM) of adenosine-diphospate (ADP), by using the whole blood flow-cytometry. Pre-incubation with CAE (50μg mL^{-1}) induced the significant decrease of P-selectin positive platelets (%) in the platelet pool compared to non-pretreated samples. The percent of reduction in P-selectin expression was 30.08 ± 11.17 % ($p < 0.05$). Mean fluorescence intensity (MFI) values as a relative measure of the average P-selectin density in P-selectin positive platelets, was not influenced by the treatment with CAE, suggesting the specific effect on hyper-reactive subpopulation of platelets, which could be hypothetically linked to the antioxidant potential of investigated extract.

P-selectin is a protein constitutively expressed in the α-granules of platelets and following platelet stimulation it becomes expressed on the platelet surface. Active role of P-selectin in thrombosis, coagulation and crucial role in pathogenesis of atherosclerosis implies mostly its role in leucocyte recruitment (notably monocytes) as an inflammation process [38]. Enhanced number of activated platelets in basal conditions and platelet hyper-reactivity, defined as enhanced response to low agonist concentration ex vivo, are shown in patients with traditional risk factors for chronic diseases rationalizing the investigation of anti-platelet effects of bioactives in health promotion and the prevention of chronic diseases [39.40,41]. Anti-platelet effects of secondary metabolites of brown algae were previously reported by other authors. Cumashi *et al*. [42] showed the significant inhibitory effects of fucoidans, polysaccharides from brown algae, on P-selectin-dependent adhesion of PMNs to adherent platelets under flow, and also on adhesion of malignant cells to immobilized platelets, a process involving P-selectin. Concentration-dependent inhibition of tumor cell adhesion to P-selectin was also reported for sulfated polysaccharide fraction isolated from red alga *Delesseria sanguine* [43].

3.3.2. Inhibition of platelet-leucocyte aggregation

The aggregation of platelets with both monocytes and neutrophils, induced by the ex vivo action of suboptimal concentration of agonist (ADP), was significantly influenced by in vitro CAE pre-treatment. Percentage of platelet-monocyte aggregates (PMA) in the population of monocytes after ex vivo action of suboptimal ADP was 73.79 ± 15.02 %. Pre -treatment of the whole blood with CAE (50μg mL^{-1}) significantly inhibited platelet-monocyte aggregation and the observed change was 46.44 ± 14.75 % ($p < 0.01$) compared to the percentage of PMA in the samples that were not pretreated. Percentage of platelet-neutrophil aggregates (PNA) in the population of neutrophils was 42.6 ± 15.82 %, and also showed marked significant decrease if the whole blood samples were pre-treated with CAE, and subsequently treated with ADP as agonist. The decrease

of PNA, compared to the non-pretreated samples was 52.02 ± 16.78 % ($p < 0.01$). Representative dot-plots of the platelet activation and platelet-leucocyte aggregation analysis by flow cytometry are shown in the Fig. 4.

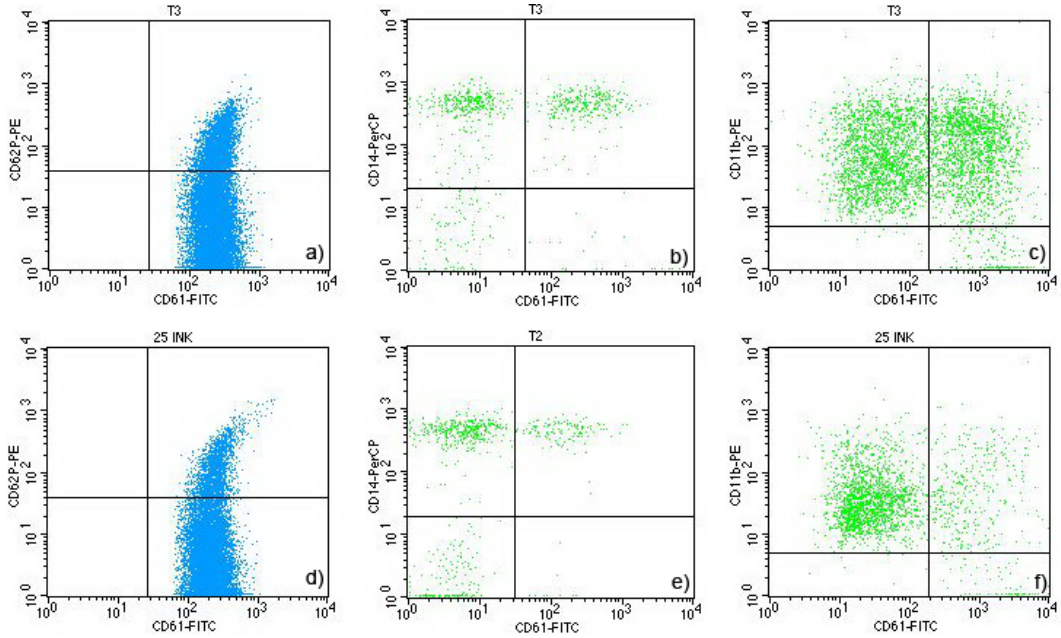

Fig. 4. Representative dot-plots showing (in upper-right quadrants) P-selectin positive platelets (a, d) platelet-monocyte aggregates (PMA) (b, e) and platelet-neutrophil aggregates (PNA) (c, f) after ADP agonist action, in non-pretreated samples and CAE pretreated samples, respectively.

Obtained data for P-selectin expression (%), platelet-monocyte aggregates (%) and platelet-neutrophil aggregates (%), after ex vivo ADP action, without and with CAE pre-treatment are shown in the Fig. 5. Data are presented as Tukey box-plots. Boxes represent the 25^{th}-75^{th} percentile, horizontal line represents median and whiskers represent the highest and lowest value, obtained in six subjects.

Regardless the observed inter-individual variation of the data, it could be concluded that there is a marked trend of decrease of all investigated parameters of platelet function after CAE treatment. There is also strong positive correlation ($r = 0.964$, $p = 0.036$) between the effects of CAE on PMA(%) and PNA(%).

Based on our knowledge this is the first investigation of the effects of brown algae bioactives on platelet function, regarding the expression of their activation markers and heterotypic aggregation with leucocytes. Based on these data, *Cystoceira amantacea* has the potential to reduce some of the biomarkers linked to the chronic diseases risk. Future studies should be targeted to the evaluation of the major compound present in the extract responsible for the observed activities, and exploitation of these effects in health promotion and the prevention of diseases.

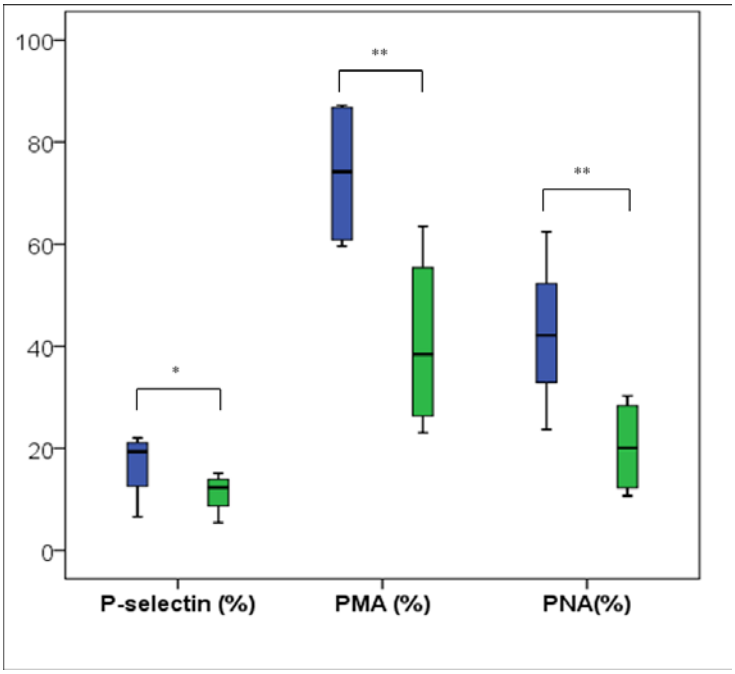

*Fig. 5. P-selectin positive platelets (%) and platelet-monocyte (PMA) and platelet-neutrophil (PNA) aggregates (%) in population of monocytes and neutrophils, respectively, after CAE pre-treatment and subsequent ADP agonist action (blue boxes), compared to the control (non-pre-treated) ADP activated samples (green boxes), n = 4. Significantly different from non-pretreated samples (paired-samples t-test), * $p < 0.05$ and ** $p < 0.01$.*

An increased or disturbed activation and aggregation of platelets plays a major role in the pathophysiology of thrombosis and atherogenesis and is related to etiology and pathogenesis of peripheral, coronary, cerebrovascular and other vascular diseases [44, 45]. Very recent study showed that direct contact with activated platelets primes tumor cells for metastasis, and induces an epithelial-mesenchymal-like transition via synergistic activation of both the TGFb and NF-kB pathways. Specific ablation of platelet-derived TGFb or NF-kB signalling in cancer cells prevents metastasis [46]. Platelet-tumor cell interactions and the signalling pathways that they trigger are therefore crucial determinants of cancer metastasis and potential targets for anti-metastatic therapies [47]. According to these findings antiplatelet activity of different agents, including brown algae bioactives, could be evaluated as an effect complement to other mechanisms of antitumor action.

4. Conclusions

In conclusion, the results showed significant cytotoxic activity, against all cell lines tested. In vitro antitumor activity was accompanied by an important apoptotic fraction of tested cell lines after treatment with extract, except for MDA-MD-361 cells. Antioxidant activity of *C.amentacea* extract was shown in both chemical and cell based assays. Antiplatelet effects show in this study could also contribute to the putative antitumor action of extract. Finally, it can be stated that the tested extract have a good antioxidant, antiplatelet and anticancer activity *in vitro*. Based on these results, further studies should be carried out to search for new compounds from brown algae that exhibit strong biological activities.

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