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Full Length Research Paper

In vitro antitumoral activities of *Padina pavonia* on human cervix and breast cancer cell lines

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Marine algae represent a potent source of anticancer agents. Brown alga, *Padina pavonia* L. was collected from Adriatic Sea, Montenegro. Cytotoxic and apoptosis effects were tested against human cervix (HeLa) and breast cancer (MDA-MB-453) cell lines. *P. pavonia* methanol extract possesses cytotoxic activity with IC_{50} values 86.45 µg/ml related to HeLa cell and 74.59 µg/ml related to MDA-MB-453 cell. The extracts did not exert any significant cytotoxicity toward normal human fetal lung fibroblast cells (MRC-5). Reduction in the number of cells with distorted shapes and condensation of cytoplasm was noticed. An increase in cells containing sub-G1 amounts of DNA was observed, indicating that the tested extract induced apoptosis. Nuclear DNA laddering was noted 24 h after the HeLa cells were exposed to 2×IC_{50} concentration of *P. pavonia* methanol extract. As brown algae represent a rich source of polysaccharides and glycosides, this activity could be connected with these compounds, but also with phenolics detected in examined extract (12.2 mg gallic acid equivalents/g dry weight (GAE/g dry weight).

Key words: Brown alga, HeLa, MDA-MB-453, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test.

INTRODUCTION

Seaweeds have been of great interest in Asian culture as marine food sources and they are largely employed in the human nutrition (Rioux et al., 2009), while in Serbian and Montenegro, there is no long tradition of the consumption of algae. Marine organisms are also prominent sources of new compounds with diverse biological activities, but when compared with plants; their use is much more restricted in traditional medicine. On the other hand, seaweeds and their extracts have attracted great interest in the pharmaceutical industry as a source of bioactive compounds (Pereira and Costa-Lotufo, 2012).

Seaweeds are rich in antioxidants such as carotenoids, pigments, polyphenols, enzymes and diverse functional polysaccharides (Morgan et al., 1980; Ye et al., 2008). Marine algae are also rich sources of unsaturated fatty acids (Kamenarska et al., 2002). It has been shown that fatty acids block growth and systemic spread of human breast cancer via mechanisms independent of the host immune system, probably by peroxidation of intracellular lipids (Zubia et al., 2009). Polysaccharides and terpenoids from brown algae are considered as promising bioactive molecules with anticancer activity (Taskin et al., 2010; Devery et al., 2001).

The characteristic brown algal polysaccharides (alginates and laminarans) were found in *Padina pavonia* (Khafaji, 1986) and *Padina tetrastromatica* (Rao et al., 1984). Numerous macroalgae have shown potent cytotoxic activities and certain authors have suggested the consumption of algae as a chemo-preventive agent against several cancers. Dehydro-thrsiferol and halomon extracted from *Laurencia viridis* Gil-Rodriguez et Haroun

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and *Portieria horemanii* (Lyngbye) P.C. Silva, respectively have been tested in the preclinical phase (Taskin et al., 2010; Devery et al., 2001).

P. pavonia (L.) Gaill. is a representative of brown algae (Phaeophyceae), widely distributed in unpolluted environments including North Carolina to Florida in the United States, the Gulf of Mexico, throughout the Caribbean and tropical Atlantic and the Eastern Atlantic, Mediterranean and Adriatic Seas (Orlando-Bonaca et al., 2008).

It is usually located on rocky substrates, various shell bottoms and coral fragments in shallow waters. Peres and Picard (1964) indicated that *P. pavonia* usually occur in shallow waters due to its tolerance to variations in edaphic factors (Peres and Picard, 1964). In a previous study, Kamenarska et al. (2002) examined in detail the chemical composition of the brown alga, *P. pavonia* (L.) Gaill. from the Southern Adriatic Sea. Based on the data obtained in this work, we assume that the active compounds are responsible for the biological activity of *P. pavonia.*

Twelve sterols were identified in the sterol fraction, the main ones being cholesterol and fucosterol. The main fatty acids in the lipids were also identified. By gas chromatography/mass spectrometry (GC/MS) analysis of the volatile and polar fractions, 40 compounds were identified. In the volatile fraction, free fatty acids, aromatic esters, benzyl alcohol and benzaldehyde predominated. Low concentrations of terpenoids, phenols and sulfur containing compounds were also identified (Kamenarska et al., 2002).

The aim of this work was to investigate cytotoxic and apoptosis effects of methanol extract of *P. pavonia* collected in Adriatic Sea, Montenegro. In this study, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay were carried on two cancer cell lines, human cervix (HeLa) and MDA-MB-453 as well as normal human fetal lung fibroblast cells (MRC-5). The cell cycle distribution was estimated by cytofluorometric analysis. DNA fragmentation analysis was performed by agarose gel electrophoresis. The amount of total phenolics was also recorded.

MATERIALS AND METHODS

Plant

Brown alga, *P. pavonia* (L.) Gaill. (Pheophycaea) was collected from Mediterranean Sea in Boka Kotorska, Montenegro during May 2011. The sample was washed thoroughly with fresh water to remove salt, sand and epiphytes, and was dried at room temperature.

Preparation of extracts

Dried *P. pavonia* was ground into fine powder and extracted with 150 ml methanol in a Soxhlet apparatus for 24 h. The extract was filtered and evaporated in vacuum at 40°C to yield residue (1.6 g).

Determination of total phenolics

The total phenolic content was estimated by the Folin-Ciocalteu method (Waterman and Mole, 1994), with slight modifications. Briefly, 200 µl of the extract (5 mg/ml) was added to 1 ml of 10 fold diluted Folin-Ciocalteu reagent. After 4 min, 800 µl of sodium carbonate (75 g/L) was added. After 2 h of incubation at room temperature, the absorbance at 765 nm was measured. Gallic acid (0 to 100 mg/L) was used for calibration of a standard curve. The results were expressed as mg of gallic acid equivalents/g dry weight of plant extract (mg GAE/g DW). Triplicate measurements were taken and mean values were calculated.

Cytotoxic activity

Cell lines

The human cervix carcinoma HeLa and human breast cancer MDA-MB-453 cell lines and normal human fetal lung fibroblast MRC-5 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Both cancer cell lines and normal human fetal lung fibroblast cells were maintained in the recommended RPMI-1640 medium supplemented with 10% heat-inactivated (56°C) fetal bovine serum, l-glutamine (3 mM), streptomycin (100 mg/ml), penicillin (100 IU/ml), and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and adjusted to pH 7.2 by bicarbonate solution. Cells were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Treatment of cell lines

Stock solutions (100 mg/ml) of extract, made in dimethylsulfoxide (DMSO), were dissolved in corresponding medium to the required working concentrations. Neoplastic HeLa cells (2000 cells per well) and neoplastic MDA-MB-453 cells (3000 cells per well) and normal human fetal lung fibroblast MRC-5 cells (5000 cells per well) were plated in 96-well flat-bottomed microplates and five different, double diluted, concentrations of investigated extract were added 24 h later. The final concentrations applied to target cells were 200, 100, 50, 25 and 12.5 µg/ml except in control wells, where only the nutrient medium was added to the cells. Cells were exposed to various concentrations of the extract for 72 h.

MTT test

Cell survival was determined by MTT test (Mosmann, 1983; Ohno and Abe, 1991) 72 h upon addition of the extract. Briefly, 20 µl of MTT solution (5 mg/ml in phosphate buffered saline (PBS)) was added to each well. Samples were incubated for further 4 h. Then, 100 µl of 10% sodium dodecyl sulfate (SDS) was added to extract the insoluble product formazan, resulting from the conversion of the MTT dye by viable cells. The number of viable cells in each well was proportional to the intensity of the absorbance of light, which was read in an enzyme-linked immunosorbent assay (ELISA) plate reader at 570 nm.

The absorbance (A) at 570 nm was measured 24 h later. To determine cell survival (%), the A of a sample with cells grown in the presence of various concentrations of the investigated extracts was divided by the control optical density (the A of control cells grown only in nutrient medium) and multiplied by 100. It was implied that the A of the blank was always subtracted from the A of the corresponding sample with target cells. IC_{50} was defined as the concentration of an agent inhibiting cell survival by 50% when compared with a vehicle-treated control. All experiments were performed in triplicate.

Morphological changes

Morphological changes in HeLa and MDA-MB-453 cell treated with the various concentrations of extract were monitored under an inverted Carl Zeiss light microscope.

Flow cytometry

Aliquots of 3×10^5 control or cells were treated with investigated extract for 24 h (concentrations corresponded to $2\times IC_{50}$ values) were fixed in 70% ethanol on ice for at least 1 week and centrifuged. The pellet was treated with RNase (100 µg/ml) at 37°C temperature for 30 min and then incubated with propidium iodide (PI) (40 µg/ml) 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 (Zeng et al., 1996).

DNA fragmentation

The presence of a 'ladder-like' DNA fragmentation pattern typical of apoptotic cells was evaluated after 24 h of exposure to medium containing $2\times IC_{50}$ extract. There were also control cells with no extract. Both floating and attached cells in medium were collected, washed in PBS and resuspended in 200 µl PBS. DNA was extracted using QIAamp DNA Blood Mini Kit (Qiagen, Germany) according to the protocol for cultured cells. Electrophoresis of the extracted DNA, to which a 6X loading dye solution (Fermentas UAB, Lithuania) had been added, was carried out on 1.0% agarose gels; Lambda DNA/HindIII Marker 2 and Mass Ruler DNA Ladder High Range (Fermentas UAB, Lithuania) were used to compare the size of the DNA fragments. DNA bands were visualized after ethidium bromide staining (Serva Electrophoresis, Germany).

RESULTS AND DISCUSSION

Total phenolic content

The amount of total phenolics in *P. pavonia* methanol extract was 12.2±0.2 mg GAE/g DW. Phenolic compounds are commonly found in the edible brown, green and red seaweeds (Devi et al., 2008). Methanol extracts of seven brown seaweeds were analyzed for total phenolics by Vinayak et al. (2011), and the content varied from 13.19±0.32 to 25.29±0.44 mg GAE/g being the highest in *P. tetrastromatica*. Amornlerdpison et al. (2007) detected significantly greater amount of total phenolics (217.40±11.28 mg GAE/g) in aqueous extract of *P. minor* collected in Thailand. In general, aqueous extracts of different seaweed showed higher contents of total phenolics then methanol or ethanol extracts (Boonchum et al., 2011).

Morphological changes in HeLa and MDA-MB-453 cells after treatment with the extract

Obvious morphological changes in HeLa and MDA-MB-453 cells were observed after 72 h of treatment with the extract. Results of microscopic examination of investigated cancer cells after 72 h treatment with *P. pavonia* are shown in Figure 1. Reduction in the number of cells with distorted shapes and condensation of cytoplasm were noticed, which resulted in significantly smaller cells as compared to the control cells.

Cytotoxic activity of the *P. pavonia* methanol extract

The cytotoxic activity of the studied *P. pavonia* methanol extract related to tested cell lines is shown in the Table 1. Results showed that the extract demonstrated significant cytotoxic activity. More pronounced activity of the extract was obtained against MDA-MB-453 cell. In contrast, *P. pavonia* methanol extract was found to be a weak cytotoxic agent against the normal cells MRC-5 *in vitro* with an IC_{50}>200 µg/ml.

As brown algae represent a rich source of polysaccharides and glycosides, this activity could be connected with these compounds, but also with phenolics detected in examined extract. Based on the published work (Kamenarska et al., 2002), some sterols fatty acids, aromatic esters, terpenoids, benzyl alcohol and benzaldehyde may be responsible for the biological activity of *P. pavonia.* Also, several sulfated polysaccharides separated from algae have shown antitumor, anticancer, antimetastatic activities in mice (Combe et al., 1987; Khanavi et al., 2010). In addition, as reported (Awad et al., 2009) neither cold nor hot aqueous polysaccharide extracted from *P. pavonia* exhibited cytotoxic effect against cultured U251, while hot water extract showed cytotoxic activity against cultured HepG2 *in vitro.* Also, fractions isolated from polysaccharide extracts were cytotoxic against HepG2. Ktari and Guyot (1999) demonstrated that *P. pavonia* showed a cytotoxic activity against KB cells. Oxysterol, hydroperoxy-24 vinyl-24 cholesterol was identified as responsible compound for this activity (Ktari and Guyot, 1999; Sheu and Sung, 1991).

Cell cycle analysis

After treatment of HeLa and MDA-MB-453 cells with *P. pavonia* extract ($2\times IC_{50}$), the cells were harvested and analyzed with a FACS calibur flow cytometer. Figure 2 shows a representative cell-cycle distribution of HeLa and MDA-MB-453 cells incubated in the absence or presence of *P. pavonia* extract for 24 h. We observed that *in vitro* antiproliferative activity was accompanied by an important sub-G1 fraction of HeLa and MDA-MB-453 cell lines after treatment with the extract. An increase in cells containing sub-G1 amounts of DNA was observed, indicating that the tested extract induced apoptosis. In addition, when treated with *P. pavonia* ($2\times IC_{50}$), increase in the percentage of cells in G1 phase and concomitant decrease in S and G2/M were observed, supporting a G1 phase arrest.

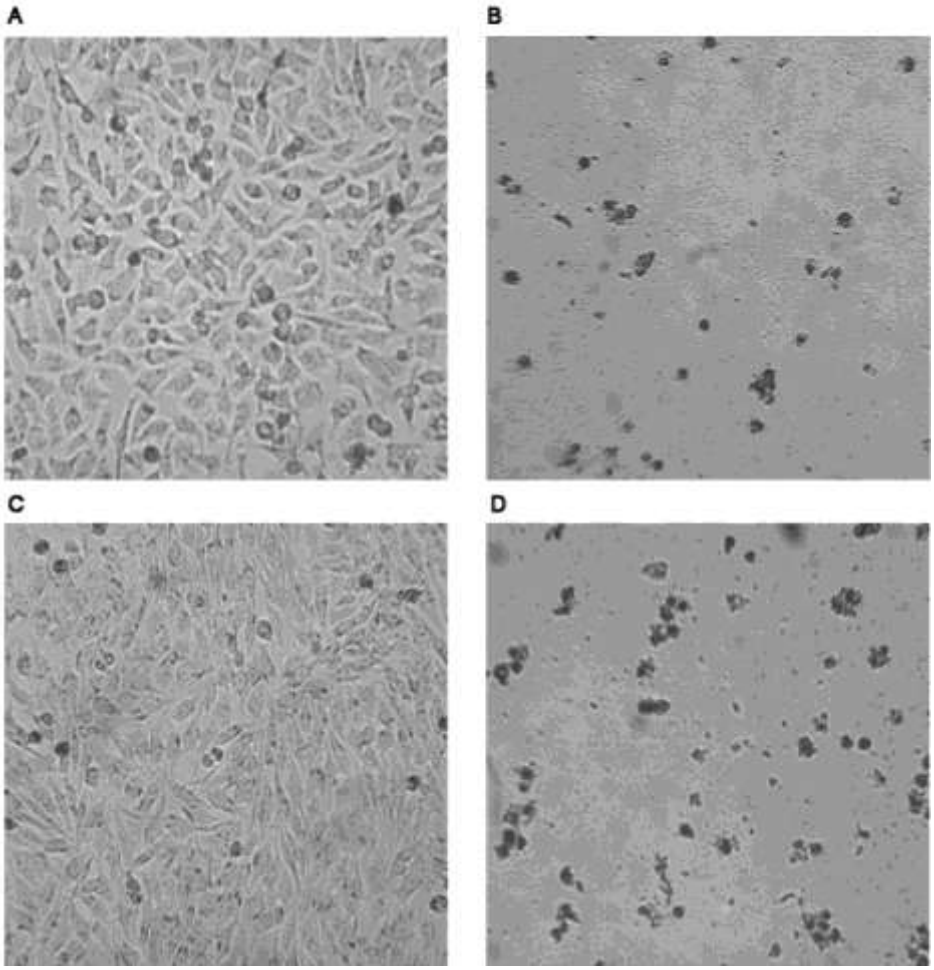

Figure 1. Morphological changes in HeLa and MDA-MB-453 cells by *P. pavonia* methanol extract. Control cells: HeLa (A) and MDA-MB-453 (C). HeLa (B) and MDA-MB-453 (D) cells treated with the 200 µg/ml *P. pavonia* extract for 72 h.

Table 1. Cytotoxic effects of *P. pavonia* methanol extract on HeLa, MDA-MB-453 and MRC-5 cells.

Extract	HeLa	MDA-MB-453	MRC-5
			IC50 (µg/ml)
Padina pavonia	86.45±0.21	74.59±0.79	>200

*Concentrations of examined extract that induced a 50% decrease in HeLa, MDA-MB-453 and MRC-5 cell survival (expressed as IC50, µg/ml±SD). *Padina pavonia* extract were incubated with cells for 72 h. At the end of this incubation period, antiproliferative activity *in vitro* was determined by the MTT assay. Results are presented as the mean value±SD of three independent experiments.

DNA ladder induced by *P. pavonia* methanol extract in HeLa cells

Nuclear DNA fragmentation was examined using agarose gel electrophoresis. As shown in Figure 3, DNA laddering was noted 24 h after the HeLa cells were exposed to $2\times IC_{50}$ concentration of *P. pavonia* methanol extract.

Our results are consistent with already published data on the bioactivity of some brown algae. In fact, after HCT-15, HCT-116, HT-29 and MCF-7 cells were treated with some polysaccharides isolated from brown algae, several apoptotic events such as DNA fragmentation, chromatin condensation and increase of the population of sub-G1 hypodiploid cells were observed (Hyun et al. 2009).

Conclusion

In our experiments, *P. pavonia* methanol extract showed relatively strong cytotoxic effect on the HeLa and MDA-MB-453 cancer cell lines, but did not exert a significant cytotoxic activity toward human MRC-5 cell lines. The extract also demonstrated high DNA damage. Cell growth inhibition was mainly due to apoptosis, proved by DNA fragmentation analysis. In the present study, the results clearly demonstrate that methanol extract of the studied

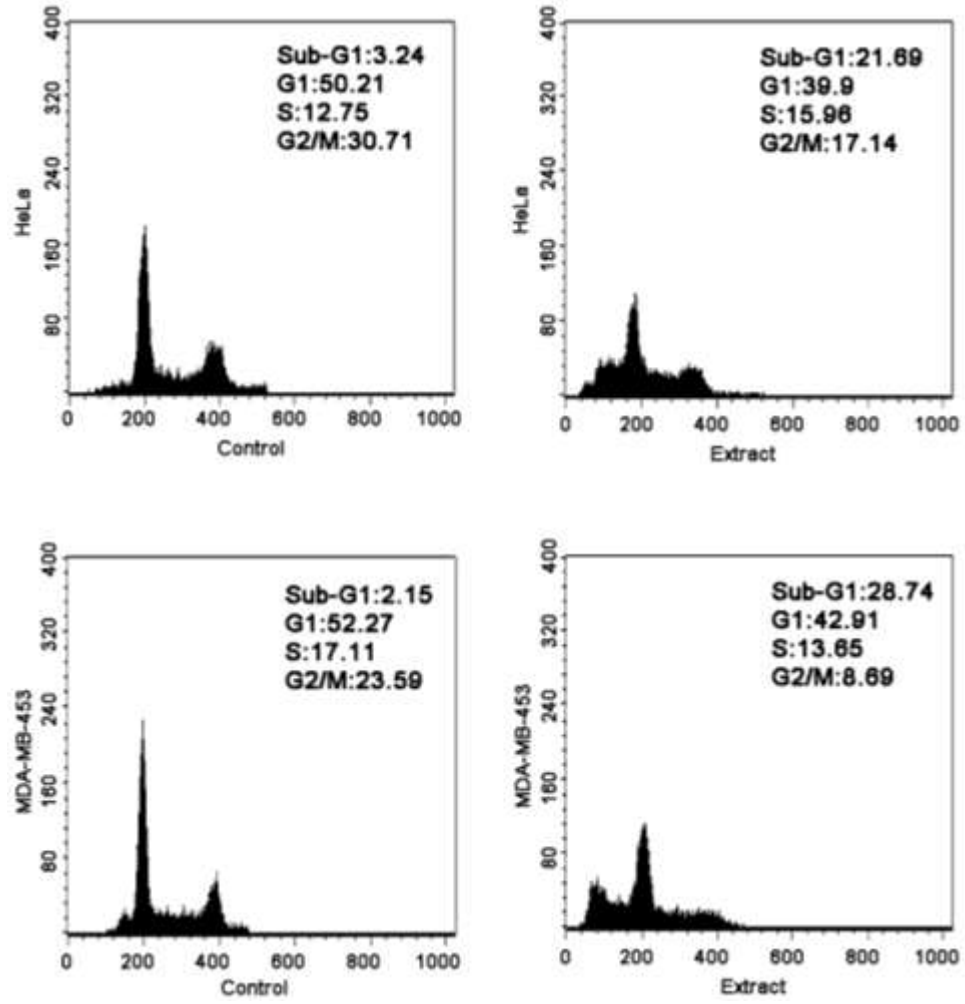

Figure 2. Cell cycle distribution after 24 h of continuous action of ($2\times IC_{50}$) *P. pavonia* methanol extract in HeLa and MDA-MB-453 cell lines.

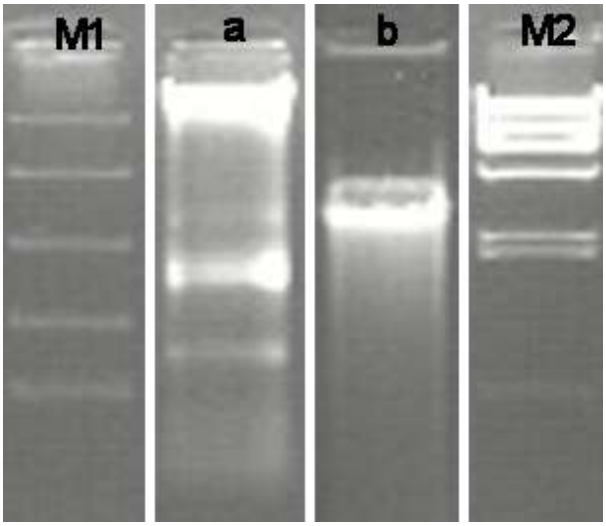

Figure 3. Agarose gel electrophoresis of DNA from HeLa cells treated with the *P. pavonia* methanol extract (M1, M2 - DNA marker; lane **a** - treated with the *P. pavonia* extract; lane **b** -control).

algae induced strongest increase in the percentage of the sub-G1 population in the HeLa and MDA-MB-453 cell lines. A decrease of cells in the G2/M phase led to an increase in apoptosis, for both cell lines. According to our results, methanol extract of brown algae *P. pavonia* could be of especial interest in searching for new marine drugs.

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