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An insight into cytotoxic and antimicrobial effects of *Cystoseira humilis* crude extract

Nadja GROZDANIĆ[1], Marijana KOSANIĆ[2], Samira ETAHIRI[3], Omar ASSOBHEI[3], Zoran KLJAJIĆ[4], Tatjana P. STANOJKOVIĆ[1*]

[1] Institute for Oncology and Radiology of Serbia, Experimental Oncology, Pasterova 14,11000 Belgrade, Serbia,

[2] Department of Biology, Faculty of Science, University of Kragujevac, Radoja Domanovića 12, 34000 Kragujevac, Serbia.

[3] Université Chouaib Doukkali, Faculté des Sciences, BP 20 El Jadida, Morocco.

[4] Institute of Marine Biology, University of Montenegro, Laboratory of Chemistry, Biochemistry and Molecular Biology, P.O. Box 69, 85330 Kotor, Montenegro.

*E-mail: tanjast@lycos.com

ABSTRACT

Sea algae are a part of human diet and they are widely used in traditional medicine. We have investigated dichloromethane-methanol (50/50) Cystoseira humilis (Schousboe ex Kützing) crude extract from Atlantic coastline of Morocco for cytotoxic and antimicrobial activity. Extract cytotoxic action was investigated on HeLa and K562 cell lines using MTT test. HeLa cells were treated with two different concentrations and observed under fluorescence microscope in order to determine type of cell death. A quantitative investigation was conducted on the antimicrobial effect of the extract against various microorganisms: bacteria (Gram positive: Bacillus cereus, Bacillus subtilis Staphylococcus aureus, and Gram negative: Escherichia coli and Klebsiella pneumoniae) and micromycetes (Aspergillus flavus, Aspergillus fumigatus, Candida albicans, Penicillium purpurescens and Penicillium verrucosum). Crude extract from Cystoseira humilis species exhibited pronounced cytotoxic and antimicrobial activities for natural product.

Keywords: *Cystoseira humilis,* cytotoxic, antifungal, antibacterial, algae

INTRODUCTION

Modern medicine is faced with numerous challenges such as search for more effective anticancer and antimicrobial agents. Cancer is the second leading cause of death in developed countries ("WHO|Cancer," n.d.). Antimicrobial resistance is one of the biggest concerns lately according to the WHO ("WHO|Antimicrobial resistance," n.d.). Macroalgae could be the source of potential anticancer and antimicrobial agents. Macroalgae are important part of human diet and the source of soluble dietary fibers, proteins, minerals,

vitamins, antioxidants, phytochemicals and polyunsaturated fatty acids (Mendis et al., 2011). They are in the scope of medical research against various diseases such as allergy, cancer, diabetes, hypertension, oxidative stress, inflammation, thrombosis, obesity and other degenerative disorders (El Gamal, 2010, Mendis et al., 2011). Some of the phytochemicals produced by algae are valuable biomolecules such as highly unsaturated fatty acids, tannins, carotenoids and sulfated polysaccharides (Rajapakse and Kim, 2011). Biodiversity of the oceans is enormous. Oceans cover more than 70% of the Earth's surface and contain more than 300,000 described species of plants and animals. See algae are one of its treasures as they produce unique secondary metabolites with various biological activities (Sasidharan et al., 2010).

Macroalgae produce chemically active metabolites in order to protect themselves against predators. Those compounds with wade range of biological activities include alkaloids, polyketides, cyclic peptide, polysaccharide, phlorotannins, diterpenoids, sterols, quinones, lipids and glycerols (Al-Saif et al., 2014, Cabrita et al., 2010).

Algae from Cystoseira genus have shown anticancer, antimicrobial and antioxidative effects (Vizetto-Duarte et al., 2016, Oumaskour et al., 2012). *Cystoseira humilis* distribution includes: Adriatic sea, Balearic Islands, Britain, Corsica, France, Greece, Italy, Malta, Portugal, Sicily, Spain, Turkey, Azores, Canary Islands, Cape Verde Islands, Madeira, Salvage Islands, Algeria Mauritania, Morocco, Tunisia, Cyprus and Syria (Cormaci et al., 2012, De Toni 1895, Engelen et al., 2008, Guiry and Guiry 2016, Pardi et al., 2000. Taskin et al., 2012).

Cystoseira humilis from the Sidi Bouzid location in Morocco grows in the specific microclimate, exposed to freezing cold strong ocean current (Oumaskour et al., 2012).

The aim of this study was to investigate and quantify dichloromethane/methanol (50/50) *Cystoseira humilis* (Schousboe ex Kützing) crude extract for cytotoxic and antimicrobial activity.

MATERIAL AND METHODS

Plant material and extraction

Cystoseira humilis was collected in the Atlantic coastline of Morocco (Sidi Bouzid coast) in a period of March-April 2011. Algae investigated were identified as *Cystoseira humilis ,* at the Laboratoire de Cryptogamie, MNHN Paris, France. The specimens were air-dried in the shade and powdered. The powder was extracted in dichloromethane/methanol (50:50) as described by Caccamese and Azzolina (1979). In breef powder was extracted in solvent for a night at room temperature.The resulting extract was centrifuged and the supernatant was concentrated to dryness in a rotary evaporator (Heidolph instruments GmbH&Co., KG, Germany) under reduced pressure (at 45°C), until crude extract was obtained and was conserved at 4°C.

Cell culture

Human cervical adenocarcinoma HeLa cells and human chronic myelogenous leukemia K562 cells were grown in the complete nutrient medium, at 37°C in humidified air atmosphere with 5% CO_2. Nutrient medium RPMI 1640 was supplemented with 100 g/l heat-inactivated (56°C) fetal bovine serum (FBS), 3 mmol/l L-glutamine, 100 mg/l streptomycin, 100 I/ ml penicillin and 25 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and adjusted to pH 7.2 with bicarbonate solution. Cells were maintained in humidified atmosphere (95% air/5% CO_2 (v/v)) at 37°C.

Treatment of cells

Stock solutions (100 mg/ml) of the extract, made in DMSO, were dissolved in a corresponding medium to the required working concentrations. HeLa cells were seeded 2000 cells per well and incubated for 24 hours to achieve cell adherence. K562 cells were seeded 5000 cells per well into 96-well microtiter plates, and 2 hr after seeding, five different doubly diluted concentrations of the investigated extracts were added to the wells. The final concentrations applied to the cells were 200, 100, 50, 25 and 12.5 μg/ml. In the control wells only the nutrient medium was added. Nutrient medium with corresponding concentrations of compounds, without the cells, was used as blank.

Determination of cell survival

The effect of extracts on the cancer cell survival was determined by the microculture tetrazolium test (MTT) according to Mosmann (1983) with modification by Ohno and Abe (1991). Briefly, 10 μl of MTT solution (5 mg/ml phosphate-buffered saline) was added to each well. After 4 hours of incubation at 37°C in a humidified atmosphere (95% air/5% CO_2 (v/v)), 100 μl of 100 g/l sodium dodecyl sulfate was added in order to dissolve formasan crystals. The absorbance (A) was measured at 570 nm 24 h later in the enzyme-linked immunosorbent assay (ELISA) plate reader (Thermo Scientific Multiskan EX Microplate reader). The number of the viable cells in each well was proportional to the intensity of the absorbance (A). To determine the cell survival percentage, the A of a sample of cells grown in the presence of various concentrations of extracts was divided by the control optical density (the A of control cells grown only in nutrient medium) and multiplied by 100. The A of the blank was subtracted from the A of each sample. IC_{50} was defined as concentration of the extract inhibiting cell survival by 50% compared to vehicle-treated control. The final concentration of DMSO solvent never exceeded 0.5%, which was non-toxic to the cells. All experiments were done in triplicate.

Morphological evaluation of cell death:

HeLa cells were seaded 80 000 cells per well. After 24 h cells were treated with two diferent concentrations of the extract IC_{50} and 2 times IC_{50}. In control wells only nutrient medium was added. Then after another 48 h of incubation cell were stained with acridine orange AO and ethidium bromide EB (3 μg/ml AO +10 μg/ml EB in PBS) then visualized under a fluorescence microscope (Carl Zeiss PALM MicroBeam with Axio Observer.Z1 using AxioCam MRm (filters Alexa 488 and 568), as described in literature (Matic et al., 2013).

Antimicrobial activity

As test organisms in this study were used Gram-positive bacteria *Bacillus cereus* (ATCC 10987), *Bacillus subtilis* (ATCC 6633) and *Staphylococcus aureus* (ATCC 25923), and Gram-negative bacteria *Escherichia coli* (ATCC 25922) and *Klebsiella pneumoniae* (ATCC 13883). The bacteria used were obtained from the American Type Culture Collection (ATCC). The fungi used as test organisms were: *Aspergillus flavus* (ATCC 9170), *Aspergillus fumigatus* (DBFS 310), *Candida albicans* (ATCC 10231), *Penicillium purpurescens* (DBFS 418) and *Penicillium verrucosum* (DBFS 262). They were from the American Type Culture Collection (ATCC) and the Mycological collection maintained by the Mycological Laboratory within the Department of Biology of Kragujevac University's Faculty of Science (DBFS). Bacterial cultures were maintained on Müller-Hinton agar substrates (Torlak, Belgrade). Fungal cultures were maintained on potato dextrose (PD) agar and Sabourad dextrose (SD) agar (Torlak, Belgrade). All cultures were stored at 4°C and subcultured every 15 days.

The sensitivity to extracts of the investigated species of microorganisms was tested by determining the minimal inhibitory concentration (MIC).

Bacterial inoculi were obtained from bacterial cultures incubated for 24 h at 37°C on Müller-Hinton agar substrate. Fungal inoculi were prepared from fresh mature (3- to 7-day-old) cultures that grew at 25 to 37°C (***depending*** on ***species)*** on a SD agar substrate. Bacterial and yeast suspensions were prepared by direct colony method. The turbidity of initial suspension was adjusted by comparing with 0.5 McFarland's standard to approximately 10^8 CFU/ml for bacteria and 10^6 CFU/ml for yeast. Fungal spores were rinsed with sterile distilled water, used to determine turbidity spectrophotometrically at 530 nm, and then further diluted to approximately 10^6 CFU/ml according to the procedure recommended by NCCLS (1998).

Minimal inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) was determined by the broth microdilution method with using 96-well micro-titer plates (Sarker et al., 2007). A series of dilutions were used in the experiment against every microorganism tested. The starting solutions of the test samples were obtained by measuring certain quantity of extract and dissolving it in DMSO. Twofold dilutions of the test samples were prepared in Müller-Hinton

broth for bacterial cultures and SD broth for fungal cultures. The MIC was determined with resazurin. Resazurin is an oxidation-reduction indicator that is used for the evaluation of microbial growth. It is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. The boundary dilution without any changing colour of resazurin was defined as the minimal inhibitory concentration (MIC) for the tested microorganism at the given concentration. As a positive control of growth inhibition, streptomycin and ketoconazole were used. A DMSO solution was used as a negative control for the influence of the solvents. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

Cytotoxic activity

Investigated extract from *Cystoseira humilis* exerted dose-dependent cytotoxic activity on two types of tested malignant cells. The decrease in survival of target cancer cells induced by the extract is shown in Table 1 and Figure 1 and 2. Generally this activity is considered to be moderate, keeping in mind that this is natural product. Extract showed slightly better action against HeLa cells compared to K562 cells.

Table 1: IC50 values for the investigated extracts.

IC_{50}, µg/ml*	HeLa	K562
Cystoseira humilis	88.16±2.43	92.33±0.94

*Concentrations of examined extract inducing 50% decrease in HeLa and K562 cell survival rate (expressed as IC50 value).

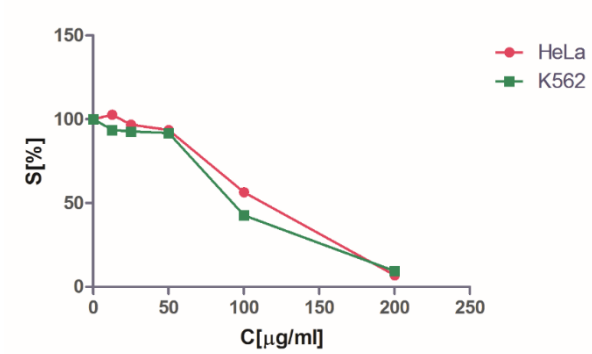

Figure 1. Survival of HeLa and K562 cells grown for 72 h in the presence of increasing concentrations of *Cystoseira humilis* extract, determined by MTT test. Representative graphs are shown.

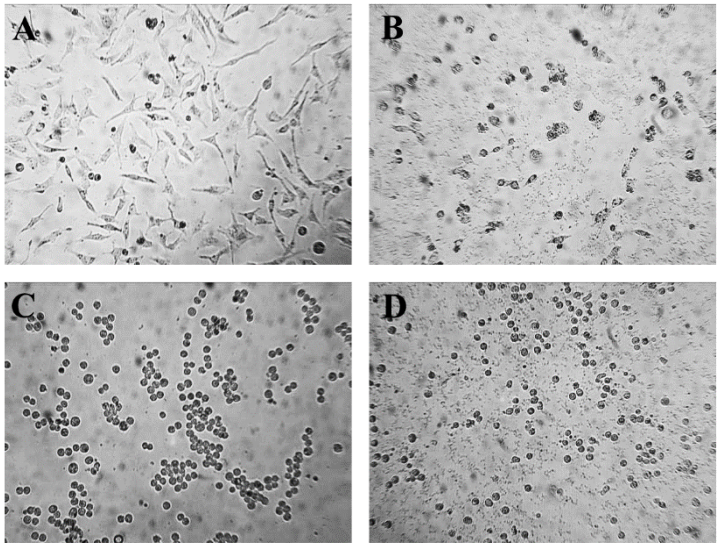

Figure 2. Morphological changes under the light microscope of HeLa and K562 cells after 72 h incubation with 200μg/ml dichlormethane-methanol *Cystoseira humilis* extract . Control cells HeLa (A), K562 (C), HeLa cells after treatment (B) K562 cells after treatment (D).

Extracts were incubated with cells for 72 hr, followed by cytotoxic activity determination *in vitro* by the MTT assay. Results are presented as the mean value ± SD of three independent experiments.

Other research of *Cystoseira humilis* dichloromethane/methanol extract showed high cytotoxic activity using test of brine shrimp lethality for larvae and on KB cells (human buccal epidermal carcinoma) cells (IC_{50} was 10 μg/ml). After purification and characterization of cytotoxic compounds, hydroperoxy-24 vinyl-24 cholesterol was isolated and identified as responsible for this activity as the IC_{50} for KB cells was about 6.5 μg/ml. (Boujaber et al., 2013)

Morphological changes

In order to reveal the pro-apoptotic effect of the extract morphological analysis of cell death mode was performed using fluorescent microscopy and acridine orange/ethidium bromide staining of the HeLa cells. Acridine orange dye can penetrate into the living cells emitting green fluorescence, while ethidium-bromide enters the cells after cell membrane damage and emits red fluorescence. The changes of the cells morphology after 48 hours of treatment of IC_{50} and $2xIC_{50}$ of extract and untreated control are shown on the Figure 3.

Observed control cells (A) are with normal morphology, stained green. Cells treated with IC_{50} dilution of the extract (B) had changed morphology, and in those cells apoptotic signs can be observed: shrinkage of the cells, condensation of nuclear chromatin. On the right side of the image (C) we can observe late apoptotic and necrotic changes to the HeLa cells after treatment of the $2xIC_{50}$ concentrations of extract. Morphological evaluation of the cell death indicated that *Cystoseira humilis* extract had pro-apoptotic effect on the HeLa cells and active components of the crude extract should be further examined.

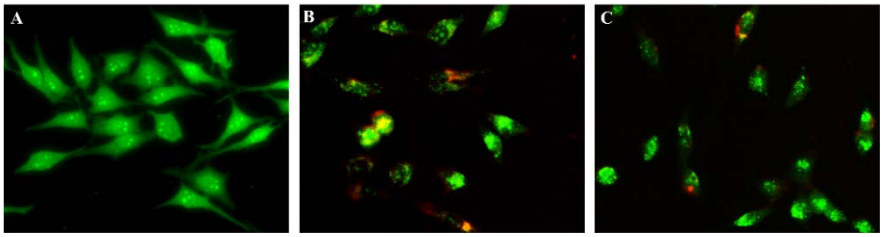

Figure 3. Morphological changes observed on HeLa cells after 24 hours treatment with IC_{50} and $2xIC_{50}$ concentrations of extract. A) control; B) IC_{50}; C) $2xIC_{50}$, Fluorescent microscope (PALM Micro Beamsystems, Carl Zeiss, 20x)

Antimicrobial activity

Antibacterial and antifungal activities of the *Cystoseira humilis* extract are shown in Tables 2 and 3. The extract exerted pronounced antimicrobial activity for natural product. However, commercial antibiotic and antimycotic were more effective then the extract. Extract was most effective against following bacterial species: Gram positive *Bacillus cereus,* followed by *Bacillus subtilis, Staphylococcus aureus*, and Gram negative *Escherichia coli* and *Klebsiella pneumoniae*. Antifungal activity of the extract was most pronounced against *Candida albicans*, followed by *Aspergillus fumigatus* and *Penicillium verrucosum* and least effective against *Aspergillus flavus* and *Penicillium purpurescens*.

In general, Gram positive strains are more susceptible to antibacterial agents than Gram negative, due to differences is cell wall (Yang and Anderson 1999, Kosanić et al. 2012). Cell walls of Gram-positive bacteria have peptidoglycan and teichoic or teichuronic acid and can be surrounded by a protein or polysaccharide envelope. Cell walls of Gram-negative bacteria are made of peptidoglycan, lipopolysaccharide, lipoprotein, phospholipid and protein. The most important point of action of anti-cell-wall agents is the peptidoglycan layer as it is crucial for the bacterial survival in hypotonic environment. Loss or damage of this layer results in bacteria death. Outer wall of Gram-positive bacteria permits easy diffusion of antibacterial agents, but outer wall of Gram-negative species have narrow channels so antibacterial agents have trouble to pass trough . (Neu and Gootz 1996). Cell wall of micromycetes is poorly permeable and consists of polysaccharides such as chitin and glucan

(Farakas, 2003). Therefore micromycetes are less sensitive to antifungal agents. However this screening showed pronounced extract effect on tested micromycetes, especially on *Candida albicans*.

Table 2. The antibacterial activity of the extracts

Algae extracts	*Bacillus cereus*	*Bacillus subtilis*	*Staphylococcus aureus*	*Klebsiella pneumoniae*	*Escherichia coli*
			MIC		
Cystoseira humilis	1.56	3.12	6.25	12.5	6.25
*Streptomycin**	78.1	78.1	156.2	312.5	312.5

*Minimum inhibitory concentration (MIC); values given as mg/ml for extract and as μg/ml for antibiotic.

Table 3. The antifungal activity of the extracts

Algae extract	*Aspergillus flavus*	*Aspergillus fumigatus*	*Candida albicans*	*Penicillium purpurescens*	*Penicillium verrucosum*
			MIC		
Cystoseira humilis	12.5	6.25	3.12	12.5	6.25
*Ketoconazole**	39	39	19.5	78.1	78.1

*Minimum inhibitory concentration (MIC); values given as mg/ml for extract and as μg/ml for antimycotic.

Earlier report using disc diffusion methods showed strong activity of *Cystoseira humilis* dicholrmethane-methanol extract on *Bacillus cereus and Staphylococcus aureus* > 15 mm; and moderate activity on *Bacillus thuringiensis and Bacillus subtillus* < 15 mm. The same study didn't reveal antifungal activity of *Cystoseira humilis* extract (Oumaskour et al., 2012).

CONCLUSIONS

Crude extract from *Cystoseira humilis* species exhibited pronounced cytotoxic and antimicrobial activities for natural product. Further analyses and research are nessesary to identify active components of this crude extract that could exhibit better cytotoxic as well as antimicrobial activities.

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To the memory of dr Zoran Kljajić.

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