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BIOLOGICAL ACTIVITY OF PRODUCTS FROM *COELASTRELLA* SP.

RESUME

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The public defense of the Dissertation will take place on ….. December 2020 from ….. in the Conference Hall of IPPG-BAS (21 Acad. Georgi Bonchev Str., 2^{-nd} floor, Sofia).

Members of the scientific jury:

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Prof.dn. Dr. Elena Nikolova - IEMPAM, BAS
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The materials on the defense are available in the Library of the Institute of Plant Physiology and Genetics - BAS (21 Acad. Georgi Bonchev Str., 2^{-nd} floor, room 225, Sofia) and on the website of the Institute http://www.bio21.bas.bg.ippg/

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TABLE OF CONTENTS:

ABBREVIATIONS USED

BALB/c 3T3	Normal cell line - murine fibroblasts
CV	Cell viability
DAPI	4 ', 6-diamino-2-phenylindole (fluorescent dye)
DMEM	Dulbecco Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
Dox	Doxorubicin
EtBr	Ethidium bromide
FBS	Fetal bovine serum
GC-MS	Gas chromatography mass spectrometry
HeLa	Human cancer cell line from cervical cancer
MUFA	Monounsaturated fatty acids
OD	Optical density
PBS	Phosphate buffered saline
PUFA	Polyunsaturated fatty acids
SDA	Agar (Sabouraud's DextroseAgar)
SFA	Saturated fatty acids
EE	Ethanol extract
AO	Acridine orange
ADS	Absolute dry substance
HT	High temperature aqueous extract
EPSH	Exopolysaccharide
IR-FT	Infrared spectroscopy with Fourier transform

CM	Culture medium
OE	Oil extract
MIC	Minimum inhibitory concentration
FAs	Fatty acids
US	Unsaponifiable substances
LT	Low temperature aqueous extract
TAA	Total antioxidant activity

I. INTRODUCTION

In recent decades, interest in microalgae as a promising and inexhaustible source of a number of useful products with applications in the food, pharmaceutical, cosmetic and textile industries, in ecology, agriculture, biofuel production, etc. has not abated. The increase in the incidence of oncological diseases and mortality among people, the insufficiently effective antitumor therapy, as well as the appearance of bacterial strains resistant to the widely used antibiotics, necessitate the search for new natural products with antitumor and antibacterial action, combining high specificity, wide spectrum of action and harmlessness to the organism. Microalgae are sources of such products, which have diverse biosynthetic and biotechnological potential and provide an opportunity for controlled cultivation and targeted production of metabolites desired for practice.

Among them, green microalgae (Chlorophyta division) *Chlorella vulgaris*, *Haematococcus pluvalis*, *Dunaliella salina*, *Scenedesmus obliquus* and cyanoprokaryotic microalgae *Arthrospira* (*Spirulina*) *platensis* (Cyanophyta division) have wide practical applications. They are producers of valuable biomass and biologically active substances - pigments, polysaccharides, sterols, fatty acids, vitamins and etc. with significant biomedical importance. The carotenoids, astaxanthin, β-carotene, lutein, zeaxanthin, lycopene are of particular interest. Carotenoids have various health benefits such as antioxidant, antitumor, antidiabetic, neuroprotective and anti-obesity, anti-inflammatory, cardioprotective and osteoprotective activity, leading to their widespread use in the prevention and treatment of neurodegenerative, diabetic and cardiac diseases, cancer, cataracts, etc. They are also used in the production of food supplements, cosmetics and pharmaceuticals. *Haematococcus pluvilais* is one of the best sources of natural astaxanthin, and *Muriellopsis* sp. of lutein. *Dunaliella salina* is used for mass production of β-carotene. Polysaccharides from cyanoprokaryotes, green and red microalgae have an immunostimulating effect and powerful anticoagulant, anti-inflammatory, antitumor, antiviral and antioxidant properties. A number of studies have shown the great potential of intracellular and extracellular products from microalgae in the fight against pathogens such as bacteria, viruses, fungi and protozoa.

Despite the growing achievements in the field of experimental and applied algology, only a part of the huge wealth of microalgae species has been studied, and the potential of this useful bioresource remains unknown and unutilized.

The present thesis aims to reveal the possibilities of the Bulgarian strain of green microalgae to produce substances with antibacterial, antifungal and cytotoxic action and to assess its potential for practical application.

II. AIM AND TASKS

The aim of the present work was to study the biological activity of products from a selected strain of green microalga *Coelastrella* sp.

To achieve this goal we set the following **tasks:**

1. Comparison of growth characteristics and biochemical composition of strains of the genus *Coelastrella* and selection of an experimental object.

2. Preparation of products from the selected strain *Coelastrella* sp. - low temperature and high temperature aqueous extracts, culture medium, ethanol extract, oil extract, fatty acids, unsaponifiable substances and exopolysaccharide.

3. Characterization of the oil extract, fatty acids and exopolysaccharide from the selected strain of *Coelastrella* sp.

4. Study of the biological activity of the products obtained from the selected strain of *Coelastrella* sp., including:

4.1. *In vitro* study of antitumor activity in a experimental model system - a human tumor cell line from cervical adenocarcinoma (HeLa);

4.2. Attempts to elucidate the mechanism of antitumor action in HeLa tumor cells by applying fluorescent assay methods (dual intravital staining with fluorochromes and staining with DAPI);

4.3. Study of antibacterial and antifungal activities;

4.4. Determination of the antioxidant potential of an ethanol) extract.

5. Assessment of the biological activity and biotechnological potential of the selected strain of *Coelastrella* sp. for practice.

III. MATERIALS AND METHODS

III. 1. Algae material. The studies were performed with 4 strains microalgae of the genus *Coelastrella* - *Coelastrella* sp. BGV (strain from the collection of the Laboratory "Experimental Algology" at IPPG - BAS), *Coelastrella multistriata* var. *corcontica* 308, *Coelastrella multistriata* 309 and *Coelastrella vacuolata* 356 (provided by the collection of the Institute of Botany, Trebon (CCALA), Czech Academy of Sciences (ASCR, Třeboň).

III. 2. Cultivation of microalgae, measurements and analyzes. The cultivation of microalgae was carried out under sterile conditions, in facilities constructed in IPPG-BAS.

Luministat - used to maintain the experimental strains in a nutrient medium under extensive conditions - constant temperature 20° C and illumination 80 μmol photons / m^2 / s;

Intensive cultivation unit - used for culturing microalgae in glass containers with a working volume of 200 mL under intensive conditions - preset temperature, one-sided and / or two-sided lighting (8000/16000 Lx), blowing with air enriched with 2-3% carbon dioxide.

Two culture media were used to cultivate the microalgae - Blue – Green (BG11) and Setlik's culture medium (1967), modified by Georgiev et al., (1978). In the experiments to obtain and test the biological activity of algae products, *Coelastrella* sp. BGV was cultured in Setlik nutrient medium modified by Georgiev et al., (1978) based on the data of Draganova (2018).

The biological condition of the culture was monitored daily. The microscopic control was performed under a light microscope NU 2 - Karl Zeiss-Jenna and Olympus BX51 with an Olympus DP71 camera at a magnification of up to 780 ×.

III. 2.1. The amount of absolute dry substance (ADS) of the algae was determined by weight after removal salts and drying of the biomass to constant weight at 105°C.

III. 2.2. The growth rate [μ] was calculated by the formula (Levasseur et al., 1993):

$$\mu = \frac{Ln(m_{t2}/m_{t1})}{t_2 - t_1}$$

, where m_{t1} is ADS at the beginning of the cultivation period (t_1) - (t_1 = 0) and mt_2 is ADC on the first and fourth day of cultivation (t_2 = 1 and t_2 = 4).

III. 2.3. Biomass productivity (BP) (dry biomass, grams / liter per day) is calculated by the formula:

$$BP = (ADS\ t_2 - ADS\ t_1)/t_2 - t_1,$$

where ADS t_2 and ADS t_1 represent the dry weight of the biomass at time t (days) - ($t_2 = 5$ and $t_1 = 0$).

III. 2.3.1. The content of chlorophyll a, b and carotenoids in algal biomass was determined spectrophotometrically (λ- 665 nm for chlorophyll *a;* λ-650 nm for chlorophyll *b*; and λ-460 nm for carotenoids), after extraction with boiling methanol and calculated by the formulas of Mackinney (1941).

III. 2.3.2. The content of total proteins was determined by the method of Lowry (1951).

III. 2.3.3. The carbohydrate content was determined by the phenol-sulfur method of Dubois et al. (1956).

III. 2.3.4. The content of total lipids was determined by a method described by Petkov (1990).

III. 3. Gas chromatography - mass spectrometry analysis of lipid extract. The chemical composition of the obtained lipid sample was determined by gas chromatography - mass spectral analysis, after the conversion of fatty acids into their corresponding methyl esters.

The gas chromatography analysis was performed on a gas chromatograph with a mass-selective detector PE Clarus680/SQ8S, equipped with a capillary injector with electronic pressure control and two gas sampling valves.; GC column: PE-5MS (30 m × 0.25 mm × 0.25 μm). Stationary phase - 5% Phenyl, 95% Methyl Siloxane.

Chromatography conditions: Mobile phase - Helium 5.0 (helium); Mobile phase mode - constant flow - 0.9 ml / min.; Injector temperature - 290^{o}C; Injection mode - Split 15: 1, Injection volume - manual - 1μL; The elution of the compounds was performed with the following temperature program: Initial temperature - 80oC - holding 1 min .; Temperature gradient 1 - 8^o /min. up to 140^{o}C - holding 0 min.; Temperature gradient 2 - 4^o / min. up to 210^{o}C - holding 0 min.; Temperature gradient 3 - 6^o /min. up to 270^{o}C - holding 4.0 min.; Total execution time - 40 minutes. Temperature of the MSD transfer line - 270^{o}C; MSD ion source temperature - 220^{o}C; MSD solvent delay - 4.0 min.; MSD scanning - m/z - 45 - 450E$^+$ - from 4.0 to 40.0 min.

Instrument Control, Data Acquisition and Data Processing were performed by GCMSD software TurboMass v.6.1.0.1963.

III. 4. Chemical characteristics and composition of extracellular polysaccharide

III.4.1. Determination of total uronic acid content. The anhydrourone content in the polysaccharide fraction was determined colorimetrically with 3-hydroxybiphenyl by the method of Blumenkrantz and Asboe-Hansen (1973), using D-GalA as a standard (5.0-100.0 μg/mL).

III.4.2. Determination of the degree of esterification. The polysaccharide sample was saponified (0.5 M NaOH, 1 h) and after neutralization (1M HCl), the amount of methanol released was determined by a combined enzyme-colorimetric method of Klavons and Bennett (1986). The assay was performed according to the procedure described by Anthon and Barrett (2008), using 4-amino-5-hydrazino-1,2,4-triazole-3-thiol (Purpald®) as a chromogen.

III.4.3. Determination of acetyl content. The acetyl content was determined photometrically by the hydroxam method of McComb and McCready (1957), using *β*-D-glucose-pentaacetate (24.0-120.0 μg / mL) as standard.

III.4.4. Determination of protein content. The protein content of the polysaccharide sample was determined by the dye-binding method of Bradford (1976).

III.4.5. Infrared spectroscopy with Fourier transform (IR-FT spectroscopy). The IR spectra of the tested EPSH were recorded by a Bruker IR-FT spectrophotometer in a KBr tablet.

III.4.6. Determination of molecular weight of polysaccharides. Molecular weight was determined by Agilent 1220 HPSEC-RID chromatography system, using pullulan standards (0.59×10^4 - 78.8×10^4), mobile phase 150 mM NaH_2PO_4 (pH 7.0) and Agilent Bio SEC-3 column (300 A, 4.6 × 300 mm, 3 μm).

III.4.7. Determination of monosaccharide composition. The monosaccharide composition of the isolated polysaccharides was determined by HPLC-UV chromatographic system Agilent 1220 by the method of Honda et al. (1989), with a modification by Yang et al. (2005). For this purpose, the polysaccharides were hydrolyzed with 4M trifluoroacetic acid (TFA), for 8 h at 110°C. The released monosaccharides were derivatized with 1-phenyl-3-methyl-5-pyrazolone (PMP) to UV-absorbent products. Separation was performed on an Agilent TC-C18 column (5 μm, 4.6 × 250 mm) with mobile phase 50 mM phosphate buffer (Na_2HPO_4-NaH_2PO_4, pH 6.9), with added acetonitrile, in gradient elution mode (Yang et al., 2005).

III.5. Preparation of samples from *Coelastrella* sp. BGV for biological activity evaluation.

III.5.1. Culture medium with extracellular secretions (CM). A culture in the stationary phase of growth was used. The cell mass was removed after centrifugation at 5000 rpm, and the supernatant was lyophilized. The resulting dry substance was suspended in sterile distilled water at the required concentration.

III.5.2. Low temperature aqueous extract (LT). Freeze-dried biomass from *Coelastrella* sp. BGV was flooded with boiling distilled water in a ratio of 1: 8. After cooling to 40°C, the mixture was placed in a refrigerator (4°C) for 48 hours with periodic stirring. The cell mass was removed after centrifugation at 4000 rpm and the aqueous extract was freeze-dried. The resulting dry low temperature aqueous extract was suspended in sterile distilled water at the required concentration.

III.5.3. High temperature aqueous extract (HT). Freeze-dried biomass from *Coelastrella* sp. BGV was suspended in distilled water in a ratio of 1: 8 and heated on a water bath at a mixture temperature of 80-90°C and stirring constantly for 90 minutes. After separating the cell mass, the aqueous extract was lyophilized. The resulting dry high temperature aqueous extract was suspended in sterile distilled water at the required concentration.

III.5.4. Ethanol extract (EE). Fresh biomass from *Coelastrella* sp. The HBV was flooded with 96% ethanol in a ratio of 1:10 and the resulting mixture was heated on a water bath at 50°C for 4 hours. The extract was filtered and evaporated to dryness on a rotary evaporator at 60°C. The dry substance was suspended in DMSO (a suitable solvent for lipophilic substances used in biological experiments with cultured cells) at the desired concentration.

III.5.5. Fatty acids (FA) and unsaponifiable substances (US). A 5% solution of potassium hydroxide in ethanol was added to 25-30% ethanol extract of *Coelastrella* sp. in a ratio of 1: 1. The hydrolysis was carried out at reflux for 4 hours at the boiling point of the mixture. Upon completion of the saponification, the reaction mixture was cooled to 20-25°C and diluted with 3-4 times the amount of water and hexane with vigorous stirring. After layering, the two layers are separated. The upper layer with orange-red color contains unsaponifiable matter - hydrocarbons, phytol, sterols, carotenoids, fat-soluble vitamins. The extract was washed with water to remove ethanol and evaporated to dryness. The dry substance was suspended in DMSO at the desired concentration. The lower dark green layer contains fatty acids in the form of potassium salts. After acidification to pH~2 with 10-15% H_2SO_4, the potassium salts of the fatty acids were converted into free fatty acids, which were extracted with hexane. The hexane was evaporated and replaced with ethanol (for a sample

concentration of 20 mg/mL). To convert the fatty acids into water-soluble sodium salts, saturated $NaHCO_3$ solution was added to the sample.

III.5.6. Oil extract (OE). For direct extraction of carotenoids (astaxanthin), the algae suspension was mixed with sunflower oil in a ratio of 1:1, with continuous stirring for 48 hours at room temperature. The contents were poured into a separatory funnel and after layering the lower layer was separated. The top layer was centrifuged at 4000 rpm for 10 minutes and the amount of pigment was measured spectrophotometrically at a wavelength of 480 nm.

III.5.7. Isolation of extracellular polysaccharides (exopolysaccharides - EPSH). The polysaccharides released in the culture medium were precipitated with cooled 96% ethanol in a ratio of 1:2 for 24 hours. The precipitate was washed three times with 65% ethanol, freeze-dried and dissolved in sterile distilled water. The concentration of carbohydrates was determined by the method of Dubois et al., (1956).

III.6. Study of biological activity of metabolites and extracts of *Coelastrella* sp. BGV

III.6.1. Study of antitumor activity

HeLa tumor cells. A permanent human HeLa cell line from cervical cancer was used in the experiments. Cells were cultured in DMEM medium enriched with 10% fetal bovine serum and antibiotics (Penicillin and Streptomycin) at 37°C, 95% humidity and 5% CO_2. For *in vitro* experiments, cells were dissociated from the culture flask by trypsinization (Trypsin-EDTA), washed with phosphate buffered saline (PBS), by centrifugation (1200 rpm), adjusted to the desired concentration and seeded at 96- or 24-well plates depending on the test method. After 24 hours of cultivation in a CO_2 incubator to obtain a monolayer, the cells were treated (according to a scheme) with algae products and cell viability was determined by MTT test.

BALB/c 3T3 mouse fibroblasts. Normal BALB/c 3T3 murine fibroblasts (permanent, non-tumorigenic mouse embryonic fibroblast cell line) were also used as a test system in the *in vitro* experiments. Cultivation of BALB/c 3T3 cells and treatment with algae samples were performed at conditions and concentrations as described for HeLa tumor cells.

III.6.1.1. Antiproliferative effect of algae products - MTT test. The MTT test described by Mossmann (1983) was used. The method is based on the reduction of the yellow tetrazolium salt MTT (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide) to insoluble formazan crystals (violet in color) by the mitochondrial enzymes of the viable cells. The formation of formazan crystals is proportional to the activity of mitochondrial enzymes and, accordingly, to the viability of the cells.

HeLa tumor cells (1×10^5 cells / mL) in DMEM medium with 10% FBS were seeded in 96-well tissue culture plates in a volume of 100 μL/well. After 24 hours of incubation in a CO_2 thermostat for good cell adhesion and spreading, the medium was replaced with 100 μL of fresh medium containing products of *Coelastrella* sp. BGV, at various concentrations, and the cells were incubated for additional 24 and 48 hours. Tumor cells cultured in medium alone (negative control) and tumor cells cultured in the presence of the antitumor antibiotic Doxorubicin (positive control) were used as controls. At the indicated time (24th and 48th hour) of treatment, the culture medium was removed and 100 μL of MTT solution (0.5 mg/mL) was added to each well. The cells were incubated for an additional 3 hours in a CO_2-thermostat. Formazan crystals were dissolved in 100 μL/well lysis solution (DMSO: Ethanol = 1: 1). The optical density (OD) of the dissolved formazan was measured at 570 nm and 620 nm (as reference length) by an ELISA spectrophotometer (TECAN, SunriseTM, Grödig/Salzburg, Austria). Cell viability was calculated by the formula:

$$CV\ (\%) = OD570\ (experiment)/OD570\ (negative\ control) \times 100.$$

III.6.1.2. Cytomorphological studies of HeLa tumor cells performed using fluorescent methods.

III.6.1.2.1. Staining with acridine orange (AO) and ethidium bromide (EtBr) by the method of Wahab et al., (2009).

HeLa tumor cells (1×10^5 cells/mL) in DMEM medium with 10% FBS and antibiotics were seeded on sterile glass lamellae placed on the bottom of 24-well plates and cultured for 24 hours in a CO_2 incubator to form a cell monolayer. The next day, the cells were treated with algae products and cultured in a CO_2 incubator under standard conditions for another 24 hours. HeLa tumor cells cultured on glass lamellae in medium alone (negative control) and tumor cells cultured in the presence of Doxorubicin (5 μg/mL) (positive control) were used as controls. At the end of the incubation, the glass lamellae were washed twice with PBS to remove unadhered tumor cells and stained with a mixture of equal volumes of fluorescent dyes (10 μg/mL AO and 10 μg/mL EtBr). Freshly stained tumor cells were observed and photographed within 5-10 minutes after staining with a fluorescence microscope (Leica DM 5000B, Wetzlar, Germany) before the fluorescent color began to fade.

III.6.1.2.2. Evaluation of the nuclear morphology of HeLa tumor cells treated with algae products from *Coelastrella* sp. BGV - Staining with DAPI (4',6-diamino-2-phenylindole) according to the manufacturer's protocol and instructions.

The cultivation of HeLa tumor cells and their treatment with algae samples were performed under conditions and concentrations as described in III.6.1.2.1. DAPI cell staining was performed after fixation with 3% paraformaldehyde. Samples of treated and untreated (control) HeLa cells were mounted with Mowiol (a water-soluble hydrocolloid mucoadhesive based on polyvinyl alcohol) on microscope slides. They were stored in the dark until examination with a fluorescence microscope (Leica DM 5000B, Wetzlar, Germany).

III.6.2. Study of antibacterial and antifungal activities

III.6.2.1. Agar diffusion method of Perez et al., (1990) (Agar well method). The test bacteria *Staphylococcus aureus* (Gram +) and *Ecsherichia coli* (Gram -) and the fungal pathogen *Candida albicans* were used. As a positive control, standard antibiotics were used - Gentamicin (0.1 mg/mL) for bacterial strains and Amphotericin B (0.1 mg/mL) for *Candida albicans*. Bacterial strains were seeded in MHA (Müller-Hinton Agar) and *Candida albicans* in Sabouraud (Sabouraud Dextrose Agar - SDA). 0.1 mL of microbial suspension (1.0×10^5 CFU/mL) corresponding to a concentration of 2×10^5 cells/mL was seeded on MHA agar previously placed into 100 mm diameter petri dishes. The fungal strain (1.0×10^5 CFU/mL) was seeded superficially on Sabouraud Dextrose Agar in the same concentration (2×10^5 cells/mL). A medium (MHA or Sabouraud) containing 10% DMSO was used as a negative control.

Six wells with a diameter of 8 mm were made in each petri dish with a template. The algal sample stock solutions were diluted to a concentration of 5 mg/mL. Each algae sample (metabolites, extracts) was added dropwise into the wells in a volume of 0.1 mL (500 μg/ well) in three replicates. The petri dishes were left for 2 hours at 4°C for pre-diffusion of the samples and then placed in a thermostat at a temperature suitable for the growth of pathogens.

The antibacterial and antifungal activity of the algal products was assessed by the diameter (mm) of the inhibitory zones in the agar after 24 hours of incubation at 37°C for bacterial strains and after 48 hours of incubation at 30°C for *Candida albicans*.

III.6.2.2. Disc-diffusion method of Essawi and Srour (2000). Ten conditionally pathogenic test microorganisms represented by nine bacterial strains and one fungal pathogenic strain (*Candida albicans* NBIMCC 74) were used. Two of the bacterial strains, were Gram-positive: *Bacillus cereus* NBIMCC 1085, *Staphylococcus aureus* ATCC 25923, and seven were Gram-negative - *Escherichia coli* ATCC 25922, *Escherichia coli* NBIMCC 8954 (UPEC), *Escherichia coli* (EPEC)-clinical isolate, NBIMCC 3700, *Salmonella*

typhimurium NBIMCC 3669, *Proteus mirabilis* NBIMCC 8747 and *Klebsiella pneumoniae* NBIMCC 3670.

The test microorganisms were seeded in appropriate culture media (described in III.6.2.1.). Algal samples sterilized through a bacterial filter (45 μm) were applied to sterile paper discs (in a volume of 25 μL), which afterthat were placed on the surface of the agar media preliminary inoculated with test microorganisms. Tetracycline-soaked discs (30 μL) for bacterial strains and Nystatin for fungal pathogenic strains were used as positive controls. The experiment was performed in triplicate for each algae sample.

After 24 hours of culturing the bacterial strains at 37°C and 48 hours of culturing *C. albicans* at 30°C, the antibacterial and antifungal activity were assesed by measuring of the diameter (mm) of the resulting inhibitory zones in the agar.

III.6.2.3. Determination of minimum inhibitory concentration (MIC) by the method of micro-dilutions in a liquid medium described by Andrews (2001).

Pre-cultured pathogens (*Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*) were equated to the McFarland standard, equivalent to 1.0×10^5 CFU/mL for bacteria and 2.0×10^5 CFU/mL for *C. albicans*. 0.1 mL of the appropriate algal sample (5 mg/mL) was added dropwise to the first well of a 96-well plate, followed by serial dilution of the sample 1:1 with the appropriate liquid culture medium for bacteria or candida. Bacterial or fungal culture (0.1 mL/well) was added to each well of one series. After culturing for 24 hours, under appropriate conditions, the optical density (OD650) was measured by an ELISA spectrophotometer. MIC was determined - the lowest concentration (corresponding to the highest dilution) of the tested algae product that inhibits visible microbial growth (no turbidity) after 24 hours of incubation at 37°C (for bacteria) and after 48 hours at 30°C (for the fungi). Each experiment was performed in 3 replicates.

III.6.3. Antioxidant activity (AO) of an ethanol extract of *Coelastrella* sp. BGV

III.6.3.1. Determination of total phenol content (Singleton et al., 1999). The reaction mixture contained 100 μL of ethanol extract; 1500 μL Folin-Ciocalteu reagent (1:10 in H_2O) and 1400 μL 7.5% Na_2CO_3. The control sample contained 100 μL of ethanol; 1500 μL Folin-Ciocalteu reagent (1:10 in H_2O) and 1400 μL 7.5% Na_2CO_3. After vortexing and incubating in the dark for 30 minutes at room temperature, the staining intensity was measured at $\lambda = 765$ nm. The total phenol content was calculated from a standard curve as gallic acid equivalents (GAE).

III.6.3.2. Determination of total flavonoid content (Chang et al., 2002). Reaction mixture containing 125 μL of ethanol extract; 875 μL ethanol; 50 μL 10% $AlCl_3$; 50 μL 1M CH_3COOH was prepared. The contontrol sample contained 1000 μL of ethanol, 50 μL 10% $AlCl_3$ and 50 μL 1M CH_3COOH. The samples were incubated for 30 minutes at room temperature in the light. The color intensity was measured at $\lambda = 415$ nm. The total flavonoid content was calculated as quercetin equivalents (QE) according to a pre-prepared standard curve.

III.6.3.3. Determination of total antioxidant activity (TAA) (Prieto et al., 1999). The method is based on the reduction of Mo^{+6} to Mo^{+5} and the subsequent formation of a green phosphomolybdate complex. A reaction mixture of 250 μL ethanol extract and 2500 μL reagent (0.6 M H_2SO_4, 28 mM NaH_2PO_4, 4 mM $(NH_4)_6Mo_7O_4$) was used. The control contained 250 μL ethanol and 2500 μL reagent. After incubating of the samples for 90 minutes at 90°C, the reaction was stopped in an ice bath. The color intensity is measured at $\lambda = 695$ nm. Total antioxidant activity (TAA) is calculated by the formula:

$$TAA\ [mM/g\ extract] = \frac{E_{695} \times V}{\varepsilon \times g}$$

V - mL ethanol in which the extract is dissolved
ε - molar absorption coefficient of tocopherol (ε = 4)
g - weight of the dry ethanol extract

III.7. Statistical analysis. All experiments were performed in triplicate. Results were presented as means and standard deviations (± SD). A statistically significant difference between treatments was found by one-way analysis of variance (ANOVA), followed by Bonferroni's test using GraphPAD PRISM software, Version 5 (GraphPad Software Inc., San Diego, USA). P * <0.05 values are considered significant.

IV. RESULTS AND DISCUSSION

IV.1. Cultivation of green microalgae of the genus *Coelastrella* sp. BGV.

The cultivation was performed on an intensive cultivation unit at 8000 Lx illumination of the surface of the vessels, temperature 28°C, aeration with air enriched with 2-3% carbon dioxide and nutrient medium BG11 for 168 hours (Fig. 1).

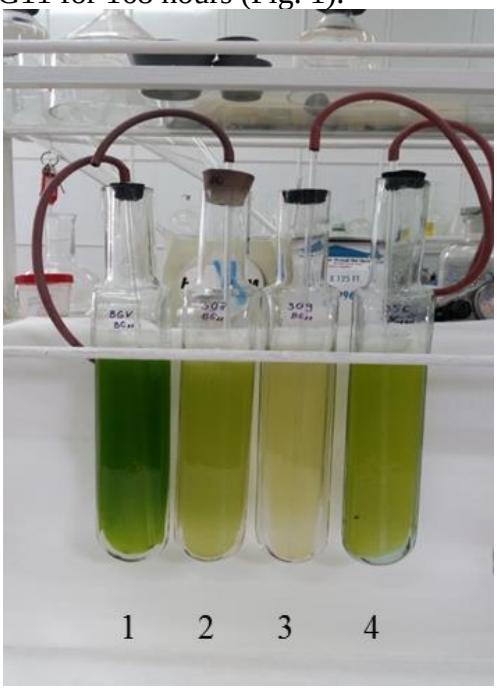


Figure 1. Cultures of: 1 - *Coelastrella* sp. BGV, 2 - *Coelastrella multistriata* var. *corcontica* (CCALA 308), 3 - *Coelastrella multistriata* var. *multistriata* (CCALA 309) and 4 - *Coelastrella vacuolata* (CCALA 356).

The growth and biochemical composition (proteins, carbohydrates, lipids and pigments) of the biomass in exponential and stationary growth phase of four strains of the genus *Coelastrella* - *Coelastrella* sp. BGV (Bulgarian strain), *Coelastrella multistriata* var. *corcontica* (CCALA 308), *Coelastrella multistriata* (CCALA 309) and *Coelastrella vacuolata* 356 (CCALA 356) were determined. For brevity, in the following text, the strains from the Czech collection are presented as *Coelastrella* 308, *Coelastrella* 309 and *Coelastrella* 356, respectively.

IV.1.1. Biomass productivity and specific growth rate. The highest density of the algal suspension (4.97 g/L) and biomass productivity (0.814 g/L/d) was achieved by *Coelastrella* sp. BGV, at growth rates [μ] 0.745 and 0.413 (t_2 = 1 and t_2 = 4, respectively) (Table 1). In this culture, the linear growth was maintained up to the 120th hour (Fig. 2). The maximum density achieved was 2.5 g/L, 1.84 g/L and 1.67 g/L for *Coelastrella* 308, *Coelastrella* 309 and *Coelastrella* 356, respectively. In the last 3 cultures, a growth

retardation from 48 to 96 hours was registered, followed by a new linear growth up to 120 hours.

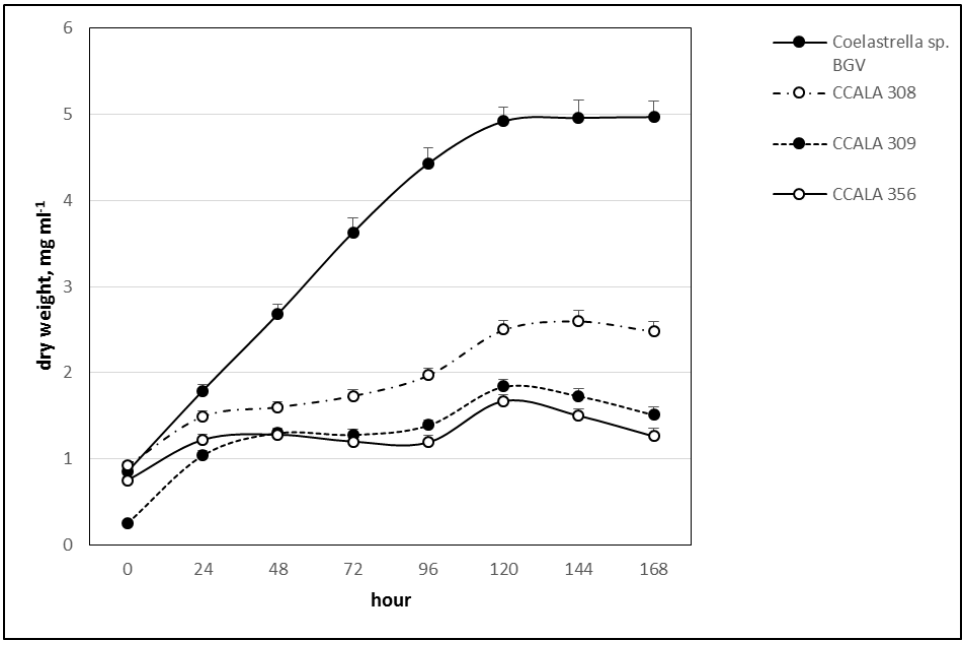


Figure 2. Growth curves of *Coelastrella* sp. BGV, *Coelastrella multistriata* var. *corcontica* (CCALA 308), *Coelastrella multistriata* (CCALA 309) and *Coelastrella vacuolata* 356 (CCALA 356

Table 1. Change in the amount of biomass (ADS, g/L) with the time of cultivation (days), growth rate [μ] and biomass productivity (g/L/d) of *Coelastrella* sp. BGV, *Coelastrella* 308, *Coelastrella* 309 and *Coelastrella* 356.

Day	***Coelastrella* sp. BGV**	***Coelastrella* 308**	***Coelastrella* 309**	***Coelastrella* 356**
	ADS, g/L			
0	0.85	0.92	0.25	0.75
1	1.79	1.49	1.04	1.22
4	4.43	1.97	1.39	1.19
5	4.92	2.50	1.84	1.67
7	4.97	2.48	1.51	1.26
	[μ]			
μ t_2=1	0.745	0.482	1.425	0.486
μ t_2=4	0.413	0.190	0.429	0.122
	Biomass productivity g/L/d			
	0.814	0.316	0.318	0.184

In optimized biphasic cultivation of Indian isolate of *Coelastrella* sp. [μ] 0.56 and biomass productivity of 302.0 mg/L/d were achieved (Minhas et al., 2020). In two other Indian isolates, *Coelastrella oocsytiformis* grown in BG11 medium at 25°C, 5000 Lx and 3%

CO_2 and *Coelastrella* sp. M-60 grown in medium BG11, at 25°C, 1500 Lx and a photoperiod of 12:12 hours, biomass productivity was 89 mg/L/d and 56.3 ± 0.9 mg/L/d, respectively (Iyer et al., 2015; Karpagam et al., 2015).

IV.1.2. Biochemical composition of biomass. The content of the main components of the biomass of the studied 4 strains of *Coelastrella*, in exponential and stationary phase of algae growth is presented in Figure 3.

In the exponential phase (Fig. 3A), the protein content was approximately the same (about 35% of ADS) in three of the studied strains, and only in *Coelastrella* 309 it was lower - 26.8%. The content of carbohydrates was different in individual strains - the highest in *Coelastrella* 309 and *Coelastrella* 308, respectively 45.2% and 44.6% of ADS and the lowest in *Coelastrella* 356 - 27.6% of ADS. Studies by Draganova (2018) showed that depending on the cultivation conditions, the amount of carbohydrates of *Coelastrella* sp. BGV varied from 35.8 to 68.2% of ADS. Iyer et al. (2015) found 44% carbohydrates in *Coelastrella oocystiformis*.

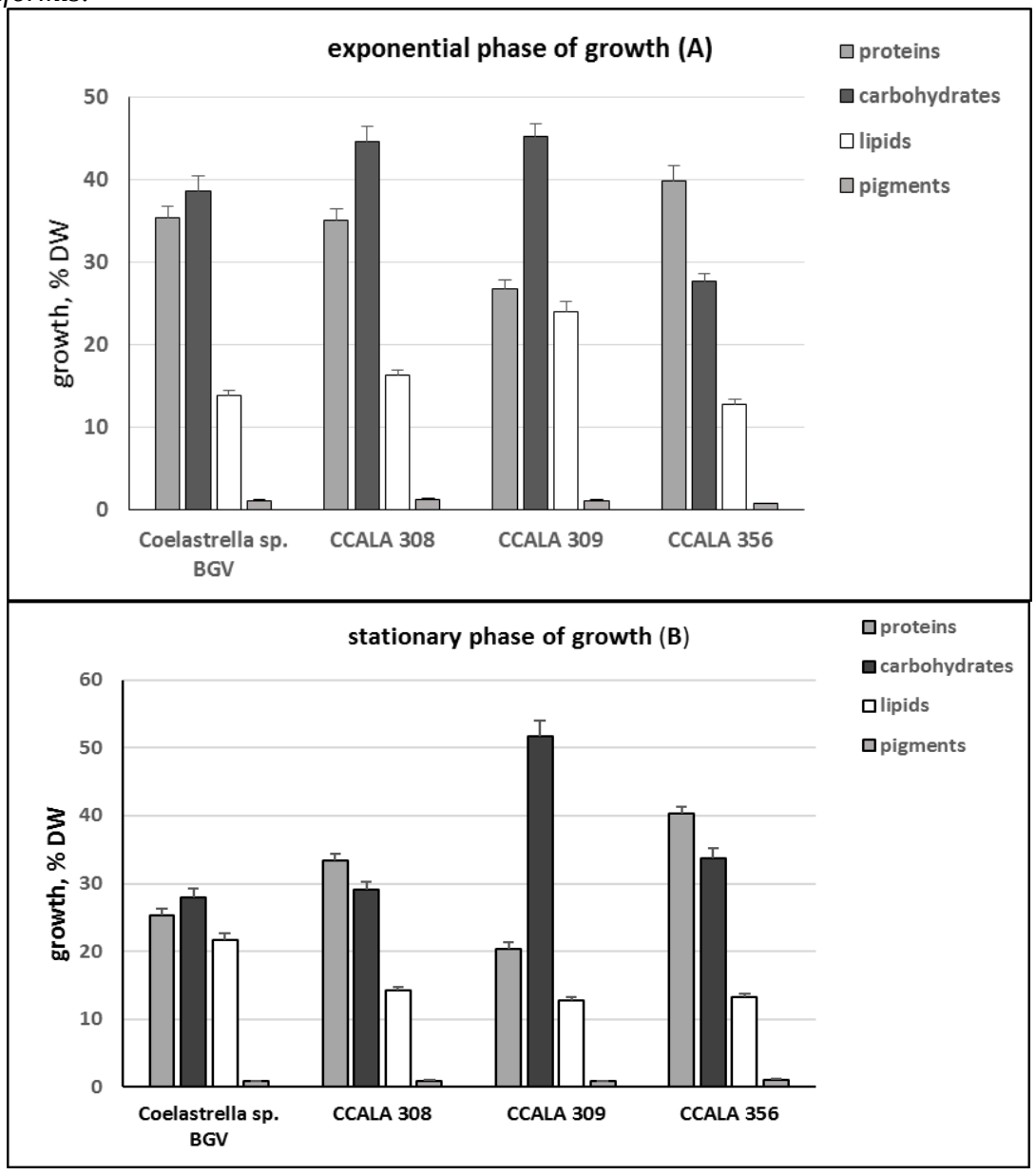


Figure 3. Biochemical composition of *Coelastrella* sp. BGV, *Coelastrella multistriata* var. *corcontica* (CCALA 308), *Coelastrella multistriata* (CCALA 309) and *Coelastrella vacuolata* (CCALA 356) in exponential (A) and stationary (B) growth phase

Regarding lipids, the values in *Coelastrella* sp. BGV, *Coelastrella* 356 and *Coelastrella* 308 varied within narrow limits - from 12.8 to 16.2% of ADS, and in *Coelastrella* 309 the value is higher - 24.0%. The lipid content in the biomass of *Coelastrella* sp. BGV in exponential growth phase (13.9%) was close to that of the heat-resistant strain *Coelastrella* sp. F50 and *Coelastrella* sp. M-60 (16% of ADS) in the early stationary phase reported by Hu et al., (2013) and Karpagam et al., (2015). The total amount of pigments (chlorophyll *a* + chlorophyll *b* + carotenoids) ranged from 0.7% (*Coelastrella* 356) to 1.3% of ADS (*Coelastrella* 308). In the Bulgarian strain the pigments were 1.14% of ADS. For comparison, Iyer et al., (2015) found 24.6%, 44%, 27% and 2%, content of proteins, carbohydrates, lipids and carotenoids in the biomass of *Coelastrella oocystiformis*, respectively.

At the stationary phase of growth (Fig. 3B), the protein content was highest in *Coelastrella* 356 - 40.3%, followed by that in *Coelastrella* 308 - 33.4%, as for *Coelastrella* sp. BGV the reported quantity was 25.3% of the ADS. The protein content (27%) of *Coelastrella oocystiformis* (Iyer et al., 2015) cultured in BG11 medium and 3% CO_2 is comparable to that found for *Coelastrella* sp. BGV. Two of the studied strains of *Coelastrella* sp. BGV and *Coelastrella* 308 have an equal carbohydrate content of 28.0% and 29.1%, respectively. Higher values were reported for strain *Coelastrella* 356 - 33.8% and highest for *Coelastrella* 309 - 51.7% of ADS. In this phase, the lipid content was the highest in the biomass of the Bulgarian strain (21.6%). In contrast to the exponential, in the stationary phase of growth, the three strains from the Czech collection have an equal content of lipids - 13-14% of ADS. In all four studied strains, the content of pigments in the stationary phase was about 1% of ADS.

Draganova (2018) found a change in the lipid content from 17.7% to 36.5% of ADS in the early stationary phase in *Coelastrella* sp. BGV, depending on the concentration of the nutrient medium. The percentage of lipids was highest in the cultivation of algae in 1/8 medium of Setlik (1967), modified by Georgiev et al., (1978), with the lowest concentration of nutrients. The lipid content of *Coelastrella* sp. F50 grown in modified Bold 3N medium and double stress (high salt concentration and illumination), increased from 16 ± 1.0 % to 22 ± 1.7% of dry weight, but biomass productivity decreased from 200 ± 4.7 to 134 ± 9.0 mg/L/d (Hu et al., 2013). The lipid content of *Coelastrella* sp. QY01 grown in BG11 medium with added wastewater in different proportions, however, was approximately the same in all crops, and ranged from 22.4% to 25.5% (Luo et al., 2016). Change in the biochemical composition (lipids, proteins, carbohydrates) of the biomass of the green microalga *Coelastrella oocystiformis* depending on the amount of CO_2 was reported by Iyer et al. (2015). The study by Razooki et al. (2020) also showed that the type and amount of carbon source were ~~a~~ key factors in the productivity of lipids, proteins and carbohydrates from *Coelastrella* MH923012. The dosed and increased supply of CO_2 solution in the nutrient medium increased the content of lipids and proteins (20.3% and 41.5%, respectively) and reduced the content of carbohydrates (30.1%) compared to the control (lipids 14.2%, proteins 27.7% and carbohydrates 50.7 %). In addition, it affected the composition and increased the amount of saturated and unsaturated fatty acids.

The established changes in the chemical composition of the biomass of the four studied strains in the two phases of growth can be explained by the physiological features and the different adaptive capabilities of the individual strains to the reduction of food resources during longer cultivation.

The results obtained showed that *Coelastrella* sp. BGV has high growth potential and balanced composition of biomass (exponential/stationary growth phase: proteins - 35.3/25.3%, carbohydrates - 38.7/28%, lipids - 13.9/22% and pigments - 1.14/1.00% of ADS). Under the same cultivation conditions, the Bulgarian strain accumulated 2 to 3 times

more biomass, and therefore a higher amount of valuable metabolites, which makes it more promising in biotechnological terms than the others *Coelastrella* strains studied. This fact determined the choice of *Coelastrella* sp. BGV as a subject of the subsequent studies.

IV.2. Characterization of oil extract, fatty acids and exopolysaccharide from *Coelastrella* sp. BGV

IV.2.1. Absorption spectrum of oil extract (OE) from *Coelastrella* sp. BGV.

In our experiments, an oil extract from the green microalga *Coelastrella* sp. BGV was obtained by a method for direct extraction with vegetable oil (sunflower oil). The absorption spectrum of OE (Fig. 4) showed absorption in the range of 400–500 nm with a maximum at 460 nm in length, which is an indication for the presence of carotenoids and especially astaxanthin.

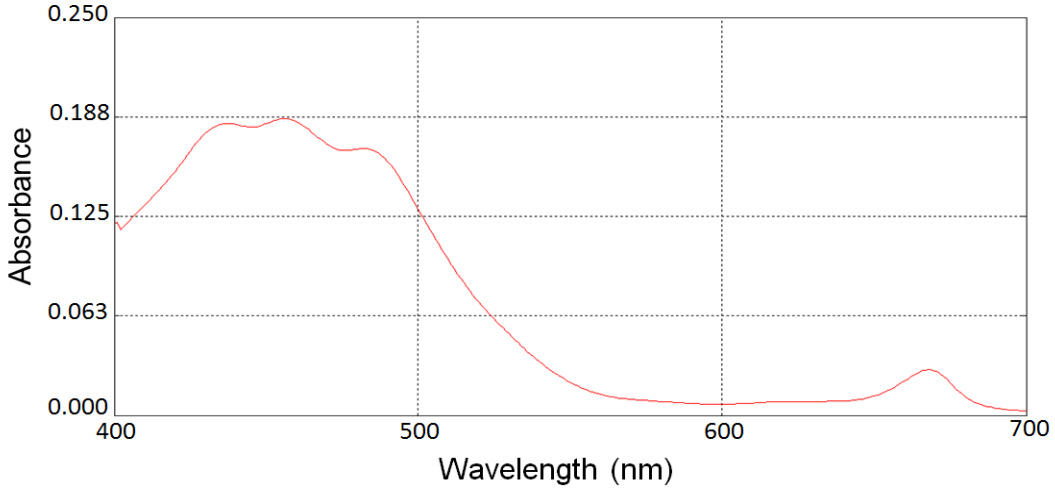


Figure 4. Absorption spectrum of oil extract of *Coelastrella* sp. BGV obtained by direct extraction with sunflower oil.

Our results are consistent with other data in the literature. Kang and Sim (2008), using vegetable oils, identified astaxanthin in *Haematococcus* culture extract with an absorption maximum at 474 nm. The presence of a small peak in the region of 700 nm, the authors associated with the presence of another type of oil-soluble pigments in the oil extract, for example chlorophyll. The advantages of vegetable oils used to obtain extracts enriched with carotenoids is that they are a good barrier against ongoing oxidative processes, do not require separation of carotenoids from the biosolvent and can be used directly in various commercial products (Rao et al., 2007). Abe et al., (2007) showed that algae extract of *Coelastrella striolata* var. *multistriata* containing the carotenoids cantaxanthin, astaxanthin and *β*-carotene has a strong antioxidant potential when used in food products.

IV.2.2. The fatty acid composition in a lipid extract obtained from the green microalga *Coelastrella* sp. BGV was determined using a gas chromatograph with a mass-selective detector (GC-MS PE Clarus680/SQ8S) under the chromatography conditions of analysis described in the section “Material and Methods”.

The resulting GC-MS chromatogram was analyzed using an internal standard and database. The qualitative and quantitative composition (%, m/m) of the fatty acids in the algae sample was determined. The results are presented in Figure 5 and Table 2.

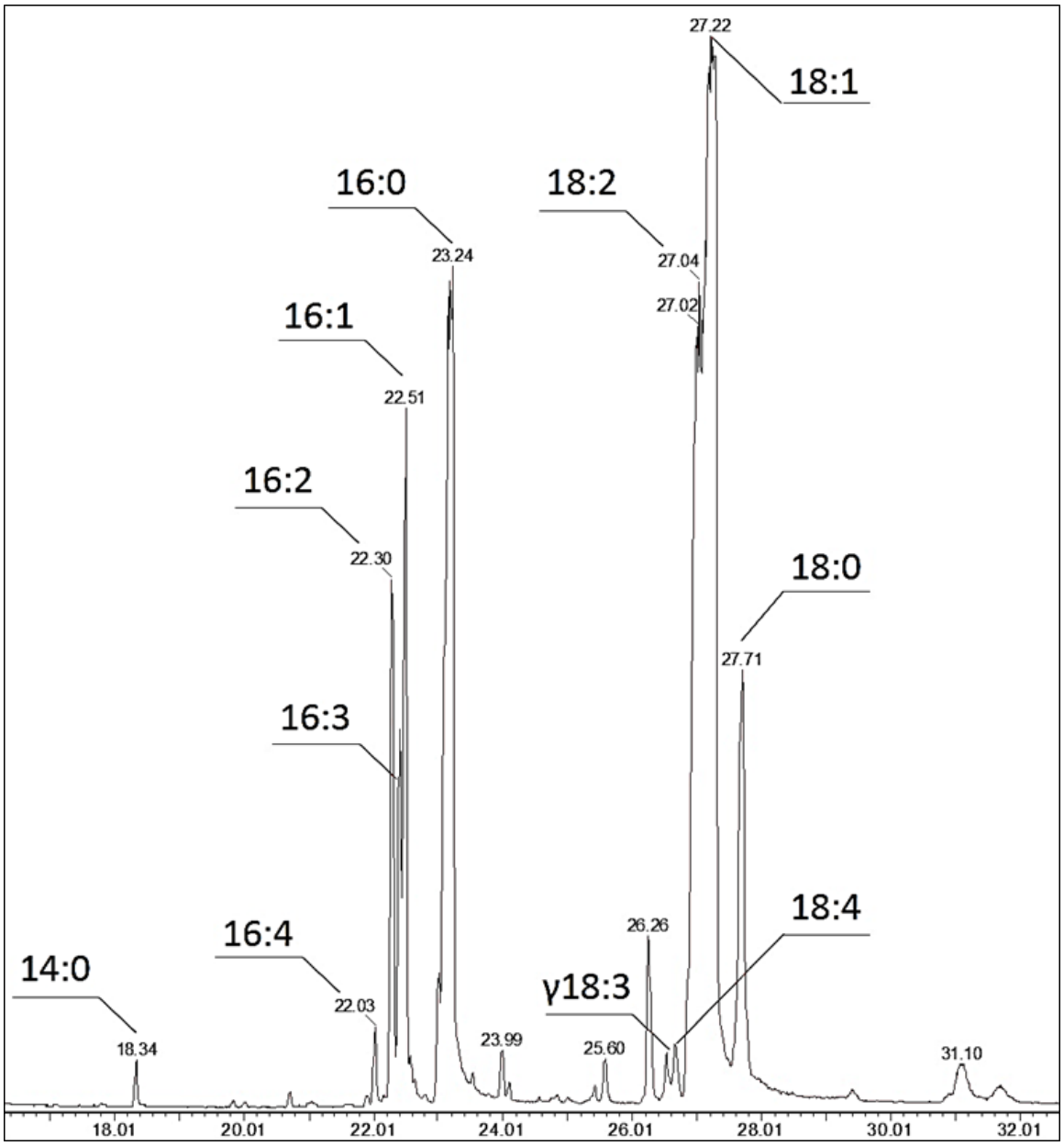


Figure 5. Gas chromatogram of methyl esters of fatty acids of *Coelastrella* sp. BGV.

The identified fatty acids were classified into saturated fatty acids (SFA) (myristic, palmitic and stearic acids), monounsaturated fatty acids (MUFA) (palmitoleic, oleic acid) and polyunsaturated fatty acids (PUFA) (linoleic, hexadecadienoic, hexadecatrienoic, hexadecatetranoic acid, γ-linolenic and octadecatetraenoic). Dimitrova et al., (2018) found six essential fatty acids in *Coelastrella* sp. BGV determined after gas chromatography analysis: myristic (14: 0), palmitic (16: 0), stearic (18: 0), oleic (18: 1), linoleic (18: 2), α -linolenic (α-18: 3). An unidentified fatty acid was also found. The present investigation is a more extensive study of the fatty acid composition of *Coelastrella* sp. BGV.

Table 2. Qualitative and quantitative composition of fatty acids from *Coelastrella* sp. BGV.

Fatty acid		Formula	Type	Positions of double bonds	RT	%, m/m
Saturated fatty acids (SFA)						
14:0	**Tetradecanoic acid Myristic acid**	$C_{14}H_{28}O_2$	**-**	**-**	**18.34**	**0,4**
16:0	**Hexadecanoic acid Palmitic acid**	$C_{16}H_{32}O_2$	**-**	**-**	**23.24**	**19,2**
18:0	**Octadecanoic acid Stearic acid**	$C_{18}H_{36}O_2$	**-**	**-**	**27.71**	**6,8**
Quantity of Saturated fatty acids						**26,4**
Monounsaturated fatty acids (MUFA)						
16:1	**Hexadecenoic acid Palmitoleic acid**	$C_{16}H_{30}O_2$	**ω-7**	Δ^9	**22.51**	**6,1**
18:1	**Octadecenoic acid Oleic acid**	$C_{18}H_{34}O_2$	**ω-9**	Δ^9	**27.22**	**31,7**
Quantity of Monounsaturated fatty acids						**37,8**
Polyunsaturated fatty acids (PUFA)						
16:2	**Hexadecadienoic acid**	$C_{16}H_{28}O_2$	**ω-6**	$\Delta^{7,10}$	**22.3**	**4,7**
16:3	**Hexadecatrienoic acid**	$C_{16}H_{26}O_2$	**ω-3**	$\Delta^{7,10,13}$	**22.42**	**3,2**
16:4	**Hexadecatetranoic acid**	$C_{16}H_{24}O_2$	**ω-3**	$\Delta^{4,7,10,13}$	**22.03**	**0,6**
18:2	**Octadecadienoic acid Linoleic acid**	$C_{18}H_{32}O_2$	**ω-6**	$\Delta^{9,12}$	**27.04**	**25,9**
γ-18:3	**γ-Octadecatrienoic acid γ-linolenic acid**	$C_{18}H_{30}O_2$	**ω-6**	$\Delta^{6,9,12}$	**26.54**	**0,6**
18:4	**Octadecatetraenoic acid**	$C_{18}H_{28}O_2$	**ω-3**	$\Delta^{6,9,12,15}$	**26.67**	**0,7**
Quantity of Polyunsaturated fatty acids						**35,7**
Total amount of Fatty acids						**99,9**

* The IUPAC (International Union of Pure and Applied Chemistry) name is written first, and the trivial name is given in parentheses.

From Table 2 and Figure 5 it can be seen that eleven fatty acids have been identified in the lipid extract of *Coelastrella* sp. BGV. Oleic (C18: 1), linoleic (C18: 2) and palmitic (C16: 0) fatty acids are present in the largest amount and in descending order - 31.7, 25.9 and 19.2 (%, m/m), respectively. Octadecanoic (stearic acid) (18: 0), hexadecenoic (palmitoleic acid) (16: 1), hexadecadienoic (16: 2) and hexadecatrienoic acid (16: 2) were 6.8, 6.1, 4.7 and 3.2 (%, m/m), respectively, and the remaining tetradecanoic (myristic acid), hexadecatetranoic, γ-octadecatrienoic (γ-linolenic acid) and octadecatetraenoic fatty acids were in amounts below 1%.

The total saturated fatty acids represented 26.4% of all fatty acids in the studied samples, with the highest percentage of hexadecanoic (palmitic acid) FA - 19.2%. Total monounsaturated fatty acids (MUFAs) were 37.8% and polyunsaturated fatty acids (PUFAs) were 35.7% of all fatty acids. We can draw the conclusion that the lipid extract of

Coelastrella sp. BGV contained over 70% of unsaturated FAs and the ratio of mono- and polyunsaturated FAs is 1:1. The amount of monounsaturated - oleic (C18: 1) and polyunsaturated - linoleic (C18: 2) was over 50% of all fatty acids in the tested samples. Our results are similar to those reported by other authors, in which unsaturated oleic and linoleic acids and saturated palmitic acid are present in algae of the genus *Coelastrella*. However, qualitative and quantitative differences in the chemical composition of fatty acids are observed in different strains, as well as in the same strain depending on the cultivation conditions, nutrient medium, temperature, light, CO_2, pH, seasonality and others (Abe et al., 2007; Li et al., 2011; Zhu et al., 2013; Iyer et al., 2015; Dimitrova et al., 2018). In a study by Dimitrova et al. (2018), for example, the content of an unidentified fatty acid, for which the authors hypothesized that it was a fatty acid with 16 carbon atoms and more than one double bond (probably 16: 2 or 16: 3), varied widely depending on the conditions of cultivation. The amount of palmitic acid ranged from 15.2% at 30°C and 8,000 Lx to 53.5% at 35°C and 8,000 Lx. The oleic acid content was significantly reduced at 30°C and one-sided lighting. The amount of linoleic acid was doubled at 25°C and double-sided lighting and decreased at 20°C. The most significant change in the content of palmitic, oleic and α-linolenic acids in a narrow temperature (30-35°C) range was established (Draganova, 2018).

Iyer et al., (2015) analyzed the fatty acid profile of *Coelastrella oocystiformis* (Mumbai, India) also by GC-MS. The authors reported that the main fatty acids present in green cells were Undecanoic acid; Tricosanoic acid; Hexadecanoic acid; 9,12-Hexadecadienoic acid; 7,10,13-Hexadecatrienoic acid; 4,7,10,13 - Hexadecatretranoic acid; 9-Octadecenoic acid; 9,12-Octadecadienoic acid; γ-linolenic acid; 9,12,15-Octadecatrienoic acid; cis-6,9,12,15-Octadecatetraenoic acid.

Abe et al., (2007) found that the microalga *Coelastrella striolata* var. *multistriata* contained eight fatty acids with 16 and 18 carbon atoms. Both saturated and unsaturated FAs were presented in both types of algae cells (green and red-orange). Of the saturated FAs, palmitic acid predominated (16: 0), and of the unsaturated ones - polyunsaturated linoleic (18: 2) and monounsaturated oleic acid (18:1). Red-orange algae cells produced more fatty acids (total fatty acid content: 319 mg/g ADS) and mostly oleic acid (113.4 mg/g ADS).

The profile of *Coelastrella* sp. QY01 (newly isolated freshwater lake strain) grown in BG11 medium (in the presence of different wastewater concentrations) was mainly represented by palmitic acid (C16: 0), linoleic acid (C18: 2) and linolenic acid (C18: 3), in the amount of 23.8–30.8%, 11.9–20.8% and 40.5–53.8% of the total fatty acid content, ie unsaturated FAs predominated (Luo et al., 2016).

IV.2.3. Chemical characteristics and composition of extracellular polysaccharide

IV.2.3.1. Determination of the molecular weight of exopolysaccharide obtained from *Coelastrella* sp. BGV using the Agilent 1220 HPSEC-RID chromatography system. The obtained elution profile of the polysaccharide is presented in Fig. 6

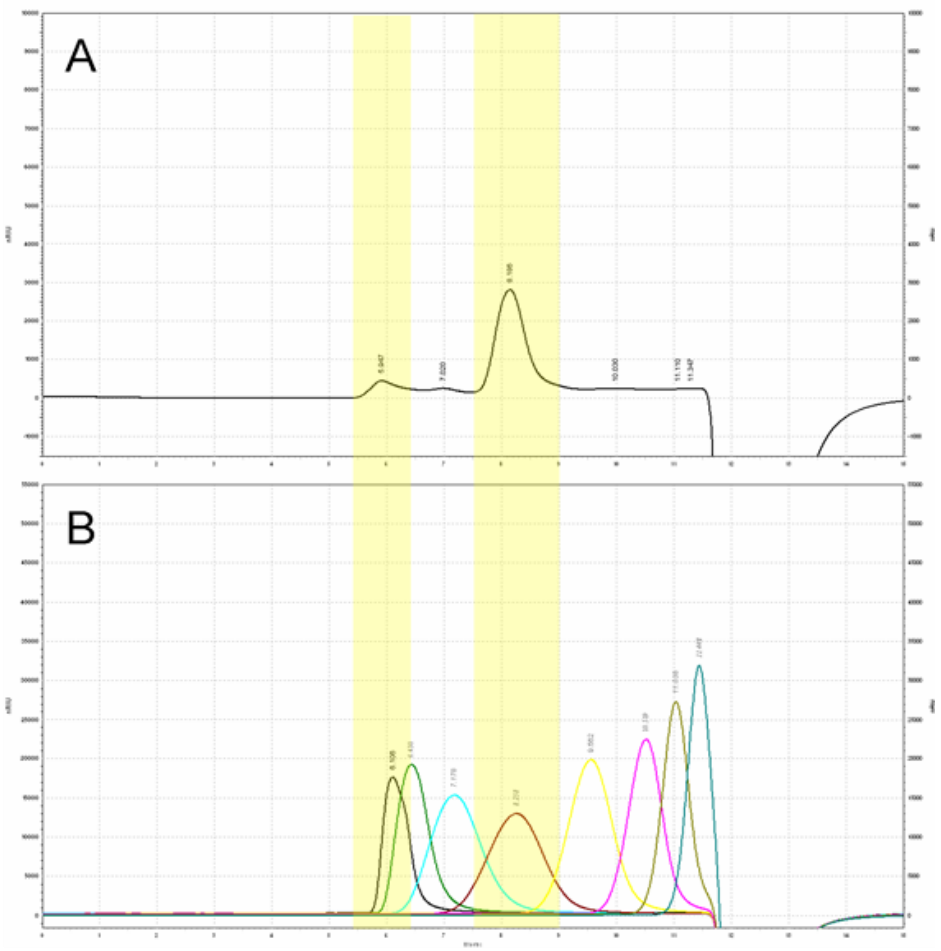


Figure 6. HPSEC elution profile of exopolysaccharide from *Coelastrella* sp. BGV. A - exopolysaccharide; C - pullulan standards. Molecular weights of the standards used - from left to right: 78.8 × 10^4, 40.4 × 10^4, 21.2 × 10^4, 11.2 × 10^4, 4.73 × 10^4, 2.28 × 10^4, 1.18 × 10^4, 0.59 × 10^4 Da.

The molecular weight distribution of EPSH showed the presence of three polysaccharide fractions (Fig. 6A). The fraction with a molecular weight of 11.5 × 10^4 Da (marked in the yellow zone of a pronounced peak) was in the largest amount (72.5%). In front of it, two fractions (two small peaks) with higher molecular weight, but in smaller quantities in the sample - 72.4 × 10^4 (11.8%) and 30.7 × 10^4 (5.7%), respectively were eluted. The data obtained show that the isolated EPSH from *Coelastrella* sp. BGV is low molecular weight.

IV.2.3.2. The IR-FT spectrum of the EPSH on the IR-FT spectrophotometer in a KBr tablet was taken (Fig. 7).

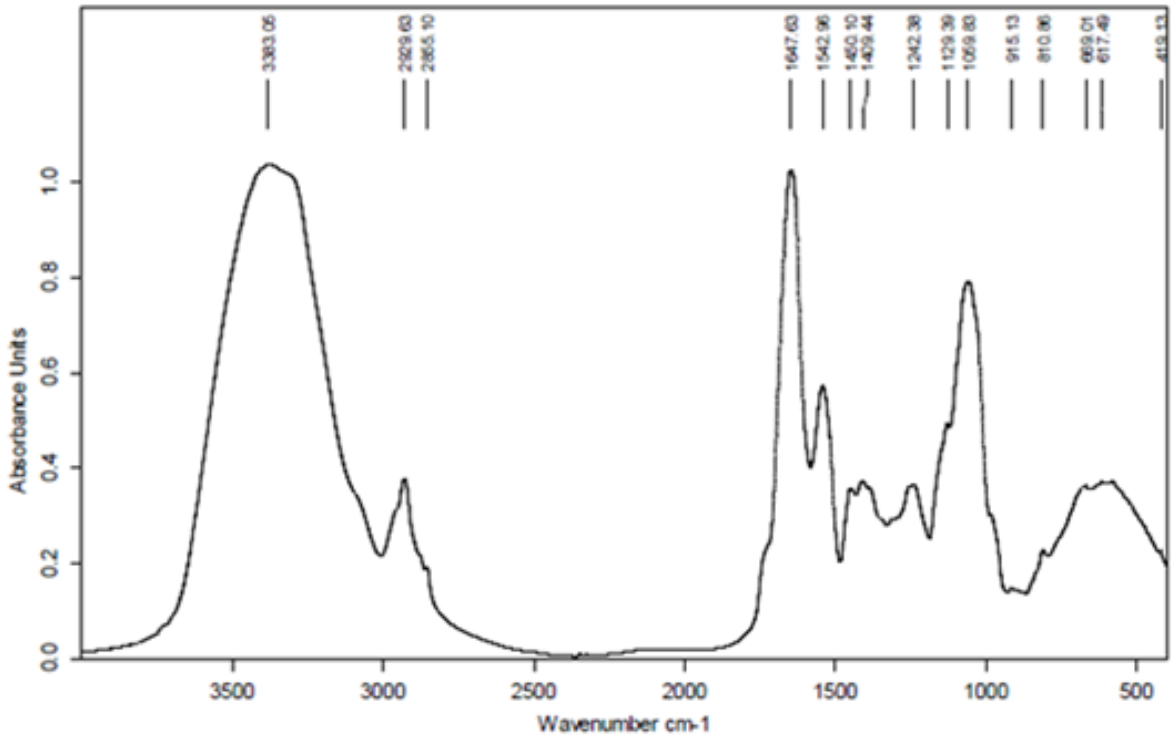


Figure 7. IR-FT spectrum of EPSH from *Coelastrella* sp. BGV.

The resulting spectrum was analyzed according to an available database. The individual absorption bands were compared with the presence of well-defined functional groups. The spectrum showed bands for the presence of: free hydroxyl groups (O-H and N-H valence oscillations in the range of 3400-3225 cm^{-1}); of $NH3^{+}$ (amine group) at 3383 cm^{-1}; of the CH_2 (methylene) group at 2929 cm^{-1} (valence oscillations of the C–H bond of the CH_2 group); for -NH_2 group of primary amine and N-H group of amide at 1650-1550 cm^{-1}, presence of amino sugars and/or protein component (two bands at 1647 cm^{-1} and 1542 cm^{-1}); signals (1409 cm^{-1}) for the C-N bond of amines and amides (in the range 1400-1418 cm^{-1}). Bands characteristic of polysaccharide sulfate esters were not detected in the spectrum. The obtained results showed that the IR-FT spectrum of EPSH from *Coelastrella* sp. BGV is typical of a polysaccharide (or proteoglycan) composed predominantly of neutral sugars.

IV.2.3.3. Determination of the monosaccharide composition of the isolated exopolysaccharide from *Coelastrella* sp. BGV. The monosaccharide composition was determined on an Agilent 1220 HPLC-UV chromatography system after hydrolysis of the EPSH and derivatization of the released monosaccharides to UV-absorbing products (Fig. 8). The conditions are detailed in the Material and Methods section.

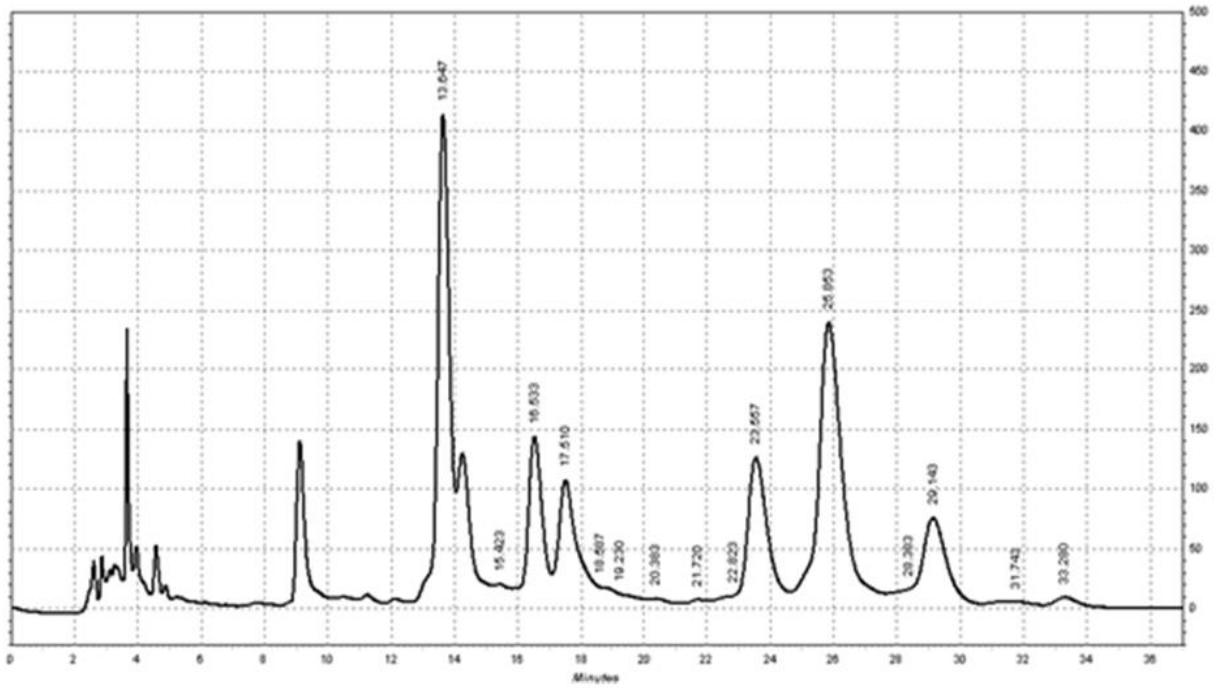


Figure 8. HPLC-UV chromatogram of PMP (1-phenyl-3-methyl-5-pyrazolone) - monosaccharide derivatives obtained from EPSH from *Coelastrella* sp. BGV.

Two monosaccharides were identified by the chromatography analysis - galactose and fucose, and five other unidentified monosaccharides were present in the sample (Table 3). Based on the analyzes performed and the origin of the sample, it can be assumed that some of the unidentified monosaccharides are unacetylated galactosamine and/or glucosamine, or that the sample is a proteoglycan. The presence of anhydrous sugars (eg 3,6-anhydro-D/L-galactose) cannot be ruled out. Additional studies are needed including additional standards (amino sugars, ribose, deoxysaccharides).

Table 3. Chemical characteristics of exopolysaccharide isolated from *Coelastrella* sp. BGV (w/w%).

Protein	7.14
Monosaccharides	
Rhamnose (Rha)	-
Arabinose (Ara)	-
Galactose (Gal)	1.7
Glucose (Glc)	-
Mannose (Man)	-
Xylose (Xyl)	-
Fucose (Fuc)	0.4
Galacturonic acid (GalA)	-
Glucuronic acid (GlcA)	-
Total content of uronic acids	4.5
Degree of methoxylation, mol%	0.74 µg/mL
Content of acetyl groups	not found

Bernaerts et al. (2018) compared ten species of microalgae that are of commercial interest as a functional food or nutritional supplement, in terms of biomass composition, cell-associated polysaccharides and exopolysaccharides, and their chemical composition (monosaccharide profile, uronic acid content and sulfates). They found EPSH in only four species of microalgae (*Porphyridium cruentum*, *Odontella aurita*, *Arthrospira platensis* and *Chlorella vulgaris*). The highest content of EPSH was found in *P. cruentum*, a red microalga known for its high content of sulphated extracellular polysaccharides (Arad and Levy-Ontman, 2010). Other authors have also reported an EPSH of *Chlorella* sp. (Morineau-Thomas et al., 2002; Xiao and Zheng, 2016).

From *Chlorella pyrenoidosa*, Sheng et al., (2007) isolated a polysaccharide (PZH) consisting of two PZH fractions (designated CPPS I and CPPS II) with a molecular weight of 69658 Da and 109406 Da, respectively. The monosaccharide profile of the two fractions, determined by gas chromatography, showed the same qualitative and different quantitative composition. Both PZH fractions contained rhamnose, mannose, glucose and galactose and up to 10% unknown monosaccharide(s). The dominant monosaccharide in the CPRS I fraction was Galactose (46.5%), and rhamnose (37.8%) in the CPRS II fraction. Xue et al., (2003) and Xie et al., (2005) obtained three polysaccharide fractions from *Dunaliella salina* by hot water extraction, ion exchange purification (DEAE-32 ion exchange column) and gel filtration (Sephadex G-100). The three fractions were defined as glucan, sulphate proteoglycan and sulphate heteropolysaccharide, containing mainly glucose. From the extraction residue of *D. salina*, Dai et al., (2010) obtained a crude polysaccharide extract (PD) containing four fractions - PD1, PD2, PD3, PD4 and two subfractions PD4a and PD4b. The results of the monosaccharide analysis showed that PD1 and PD4a are acidic heteropolysaccharides containing glucose and galactose, respectively, and PD4a contains sulfated groups. PD2 and PD3 are glucans, while PD4b is a polysaccharide complex linked to nucleic acids by covalent bonds. The composition of PD4a (Mw = 424.2 kDa) supports the claim of Fabregas et al., (1999) that aqueous extracts of *D. salina* exhibiting antiviral activity contain high molecular weight sulfated polysaccharides.

IV.3. Biological activity of products derived from *Coelastrella* sp. BGV

Exopolysaccharide and extracellular secretions (lyophilized culture medium) were isolated from culture fluid of *Coelastrella* sp. BGV during cultivation. Aqueous extracts (low temperature and high temperature), ethanol extract, oil extract, fatty acids and unsaponifiable substances were prepared from algal biomass. The antitumor, antibacterial and antifungal activities of these algae products were studied in *in vitro* experiments.

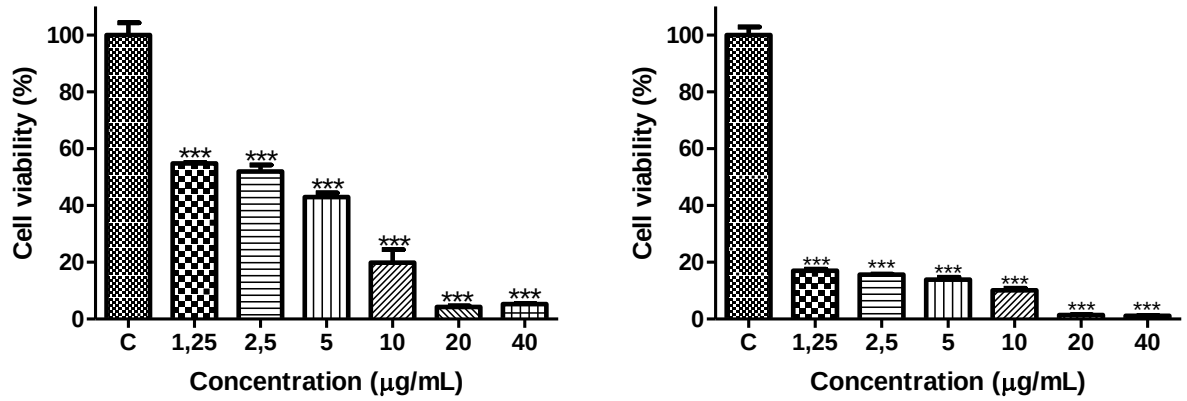
IV.3.1. Antitumor activity

IV.3.1.1. Antiproliferative effect of algae products from *Coelastrella* sp. BGV.

The antitumor activity of the algae products was studied against human tumor cell line HeLa by MTT assay. Tumor cells HeLa were treated with different concentrations of the algae products for 24 and 48 hours. In all experiments, HeLa cells cultured in DMEM medium with 10% FBS were used for the negative control (C), in which the value of cell viability was accepted to be 100%. The concentration at which 50% inhibition of tumor cell growth (IC_{50}) was achieved was calculated.

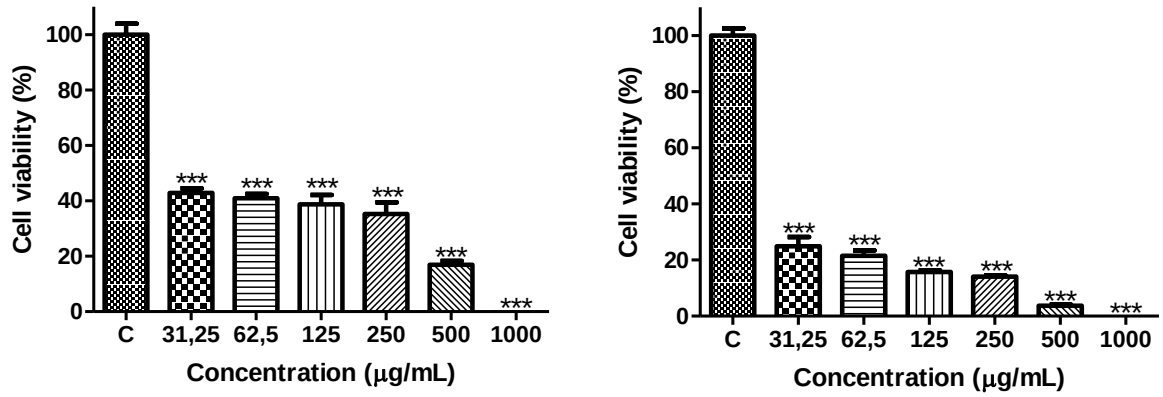
The antitumor antibiotic Doxorubicin (Dox), widely used in clinical practice for the treatment of human malignancies, was used as a positive control in the experiments. HeLa tumor cells were cultured for 24 and 48 hours in the presence of Dox at concentrations ranging from 40 to 1.25 μg/mL. It was established that, the Dox significantly reduced cell viability/proliferation, with values between 5 and 54% at the 24^{th} hour and between 1.0% and 17% at the 24^{th} hour of treatment. The observed effect was concentration- and time-

dependent. The IC_{50} values determined at the 24th and 48th hour were 2.254 μg/mL and 0.07573 μg / mL, respectively (Fig. 9A, B).



A IC_{50}=2.254 μg/mL **B** IC_{50}=0.07573 μg/mL

Figure 9. Effect of Doxorubicin on HeLa tumor cell proliferation recorded at the 24th hour (A) and the 48th hour (B) by MTT assay. *** ($p < 0.001$)



A IC_{50} =30.77 μg/mL **B** IC_{50} =6.019μg/mL

Figure 10. Effect of ethanol extract (EE) from *Coelastrella* sp. BGV, on the proliferation of HeLa tumor cells, recorded at the 24th hour (A) and the 48th hour (B) by MTT test. *** ($p < 0.001$)

The ethanol extract (EE) of *Coelastrella* sp. BGV was tested in concentrations from 1000 to 31.25 μg/mL. As early as the 24th hour, at all tested concentrations, a statistically significant decrease of the cell viability ($p < 0.001$) compared to the control - untreated tumor cells (C) was found. The reported values were between 0 and 42.9 ± 3.3% (Fig. 10A). At the 48th hour of treatment, the reported values were lower - between 0 and 24.9 ± 6.5% (Fig. 10B). The calculated IC_{50} concentrations were 30.77 μg/mL at the 24th hour and 6,019 μg/mL at the 48th hour of treatment.

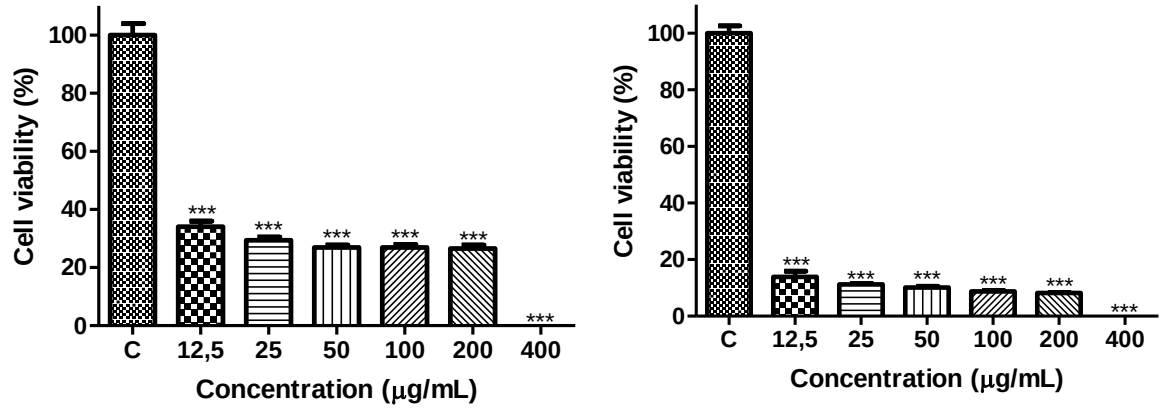


Figure 11. Effect of fatty acids (FAs) from *Coelastrella* sp. BGV on proliferation of HeLa tumor cells, recorded at the 24th hour (A) and the 48th hour (B) by MTT assay. *** (p <0.001)

Fatty acids (FAs) were tested at concentrations from 400 to 12.5 µg/mL. Statistically significant inhibition of cell proliferation was observed at all concentrations tested, and this effect was more pronounced at the 48th hour. The cell viability values ranged between 100% and 65.98 ± 3.993% at the 24th hour and between 100% and 86.09 ± 3.9% at the 48th hour. (Fig. 11A, B). IC_{50} values 3.046 µg/mL and 0.1387 µg/mL were calculated at the 24th and 48th hour, respectively.

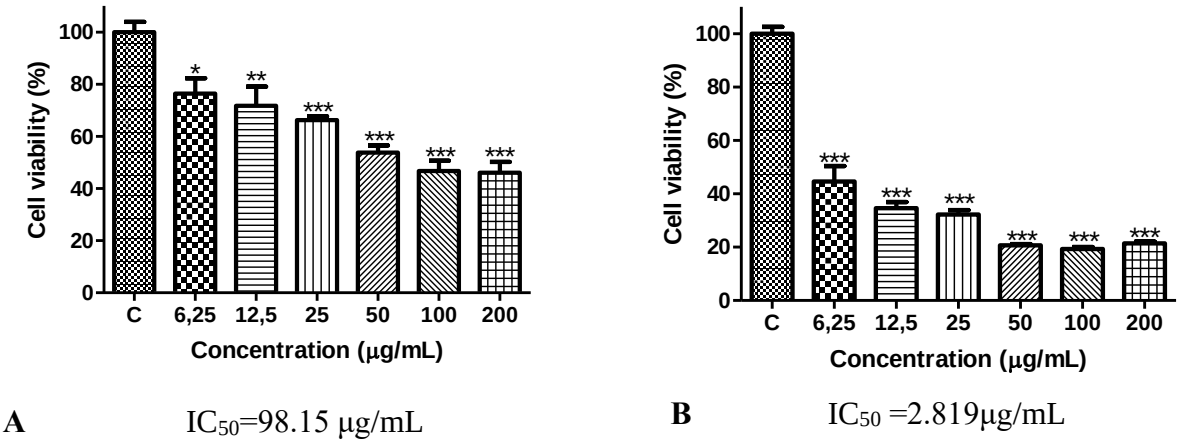


Figure 12. Effect of unsaponifiable substances (US) from *Coelastrella* sp. BGV, on the proliferation of HeLa tumor cells, recorded at the 24th hour (A) and the 48th hour (B) by MTT test. * p <0.05, ** p <0.01, *** (p <0.001)

Unsaponifiable substances (US) were administered in concentrations from 200 to 6.25 µg/mL. Concentration-dependent inhibition of HeLa tumor cell proliferation was observed at the 24th hour in the range between 53.91 ± 7.077% and 23.56 ± 11.72% (Fig. 12A), which increased at the 48th hour and reached 78.64 ± 1.340% and 55.41 ± 8.181% for the highest (200 µg/mL) and lowest (6.25 µg/mL) concentrations, respectively (Fig. 12B). The calculated IC_{50} values were 98.15 µg/mL and 2.819 µg/mL for the 24th hour and 48th hour of treatment.

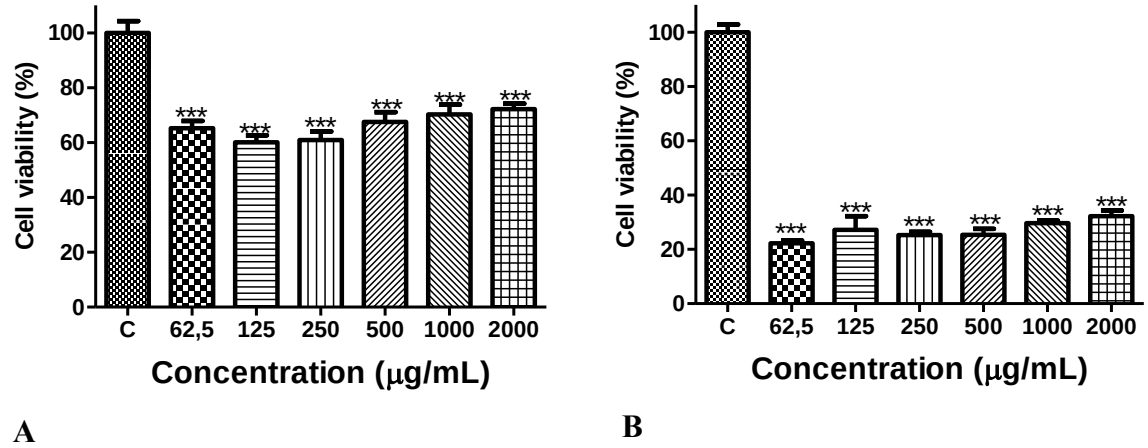


Figure 13. Effect of extracellular secretions in culture medium (CM) from *Coelastrella* sp. BGV, on the proliferation of HeLa tumor cells, recorded at the 24th hour (A) and the 48th hour (B) by MTT test. *** ($p < 0.001$)

Extracellular secretions in culture medium (CM) administered at six different concentrations (from 2000 to 62.5 μg/mL) induced a statistically significant inhibition of HeLa cell proliferation ($p < 0.001$) with values of 60-72% at the 24th hour (Fig. 13A), and up to about 2.5 times lower values (22-32%) at the 48th hour (Fig. 13B). The effect was time-dependent.

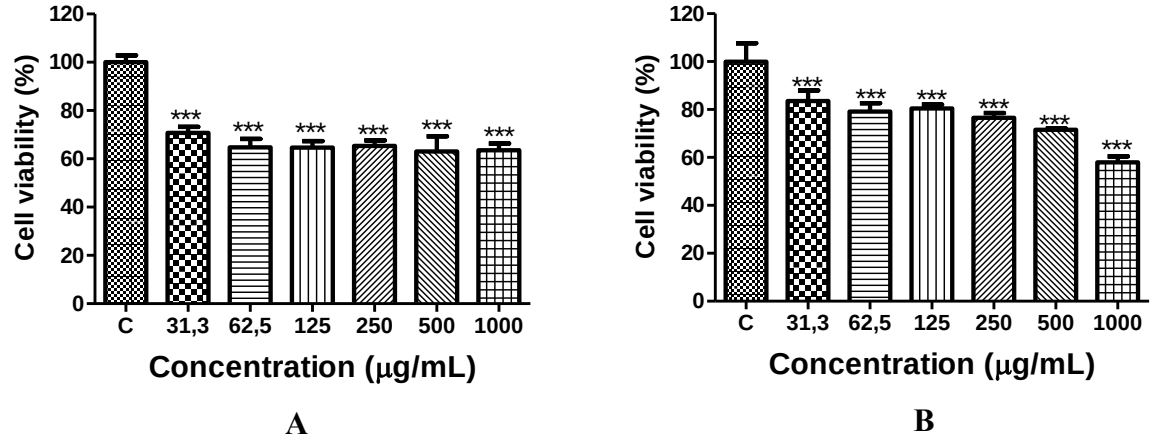
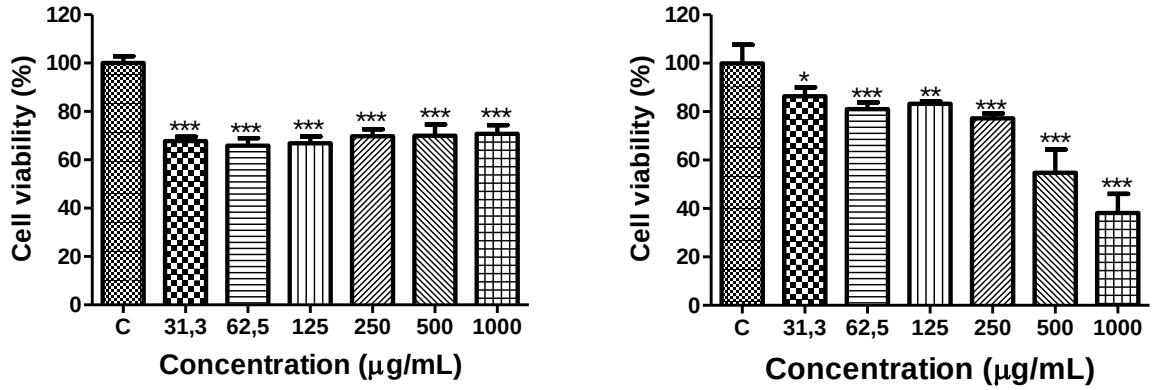


Figure 14. Effect of low temperature aqueous extract (LT) of *Coelastrella* sp. BGV, on the proliferation of HeLa tumor cells, recorded at the 24th hour (A) and the 48th hour (B) by MTT test. *** ($p < 0.001$)

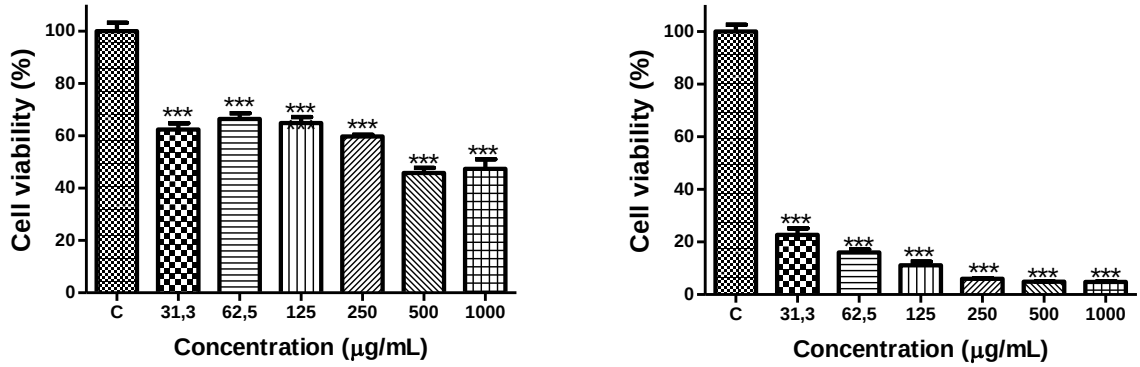
Treatment of HeLa tumor cells with low temperature aqueous extract (LT) significantly reduced the cell viability at both time intervals of examination (Fig. 14A, B). The cell viability values recorded at the 24th hour were lower than those at the 48th hour for all concentrations tested (from 1000 to 31.3 μg/mL), except for the highest one. The lowest viability of HeLa cells was observed at a concentration of LT of 1000 μg/mL - 63.6 ± 2.8% and 58 ± 2.4% at the 24th hour and 48th hour, respectively.



A **B** IC_{50}=486.9μg/mL

Figure 15. Effect of high temperature aqueous extract (HT) of *Coelastrella* sp. BGV, on the proliferation of HeLa tumor cells, recorded at the 24th hour (A) and the 48th hour (B) by MTT test. * p <0.05, ** p <0.01, *** (p <0.001)

The high temperature aqueous extract (HT) was tested at concentrations from-1000 to 31.3 μg/mL. A statistically significant antiproliferative effect was observed at all concentrations. At the 24th hour of treatment, the viability of HeLa cells varied between 65.8 and 70.7% (Fig. 15A). At the 48th hour, at the lower concentrations, the percentage of viable cells was relatively high. The treatment with concentrations higher than 250 μg/mL resulted in a decrease of cell proliferation to 54.7 ± 9.6% and 38.1 ± 8.0% at 500 μg/mL and 1000 μg/mL, respectively (Fig. 15 B). The IC_{50} value at the 48th hour was 486.9 μg/mL.

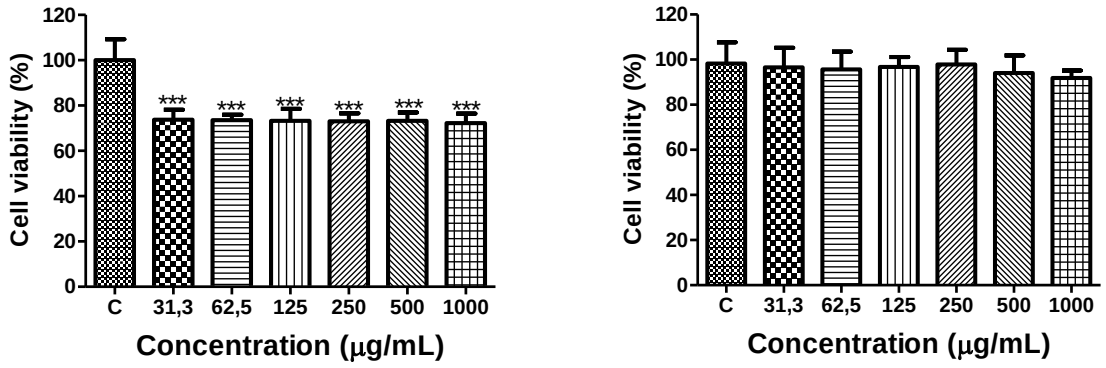


A IC_{50}=643.110 μg/mL **B** IC_{50}=4.310 μg/mL

Figure 16. Effect of exopolysaccharide (EPSH) from *Coelastrella* sp. BGV on the proliferation of HeLa tumor cells, recorded at the 24th hour (A) and 48th hour (B) by MTT test. *** (p <0.001)

The viability of HeLa tumor cells cultured in the presence of exopolysaccharide (EPSH) from *Coelastrella* sp. BGV reported at the 24th hour was between 62.3 ± 4.8% and 45.8 ± 3.9% compared with that of untreated tumor cells (Fig. 16A). At 48th hour the values were 3 to 10 times lower than those at the 24th hour and were 22.7 ± 4.3%, 16.0 ± 2.2%, 11.1 ± 2.7%, 6.0 ± 0.24%, 4.9 ± 0.22% and 4.8 ± 0.44% for EPSH concentrations of 31.3, 62.5, 125, 250, 500, and 1000 μg/mL, respectively (Fig. 16B). The viability of tumor cells was lowest at the highest concentrations - 500 μg/mL and 1000 μg/mL. The observed inhibitory

effect of EPSH was time- and concentration-dependent at the 48th hour of treatment. The IC_{50} values calculated for the 24th hour and 48th hour were 643.110 μg/mL and 4.310 μg/mL, respectively.



A **B**

Figure 17. Effect of oil extract (OE) of *Coelastrella* sp. BGV, on the proliferation of HeLa tumor cells, recorded at the 24th hour (A) and the 48th hour (B) by MTT test. *** ($p < 0.001$)

The oil extract of the green microalga *Coelastrella* sp. BGV decreased significantly the proliferation of HeLa tumor cells only at the 24th hour of the study, and the cell viability values were 72-73% at all concentrations administered (Fig. 17A).

Table 4 summarizes the IC_{50} (μg/mL) values of *Coelastrella* sp. BGV on HeLa tumor cells. The fatty acids (Fas) showed the highest antitumor activity after 24 hours of exposure followed by EE, US and EPSH. After 48 hours of exposure, again the effect of FAs was the strongest, followed by US, EPSH, EE, CM and HT.

Table 4. IC_{50} (μg/mL) of products from *Coelastrella* sp. BGV

Algae product	IC_{50} (μg/mL) - 24th hour	IC_{50} (μg/mL)- 48th hour
Ethanol extract (EE)	30.77	6.019
Fatty acids (FAs)	3.046	0.1387
Unsaponifiable substances (US)	98.15	2.819
Extracellular secretions in culture medium **(**CM**)**	-	<62.5
Low temperature aqueous extract (LT)	-	-
High temperature aqueous extract (HT)	-	486.9
Exopolysaccharide (EPSH)	643.110	4.310
Oil extract (OE)	-	-
Doxorubicin (Dox)	2.254	0.07573

The analysis of the obtained results showed that HeLa tumor cells are sensitive to the algal products isolated from *Coelastrella* sp. BGV. The *in vitro* experiments with the studied extracts and metabolites of the Bulgarian strain showed a statistically significant reduction of the tumor cells viability established by MTT test after treatment for 24 and 48 hours. A dose-

and time-dependent effect was found for EE, US and FAs, and the effects of CM and EPSH were time-dependent. With an extension of the treatment time to 48 hours, the reported IC_{50} values were many times lower than those of the 24th hour. For EPSH, for example, the reduction of IC_{50} was 150 times, and for FAs it was 22 times. After 48 hours of treatment, the EPSH exhibited dose-dependent antiproliferative activity. The highest cytotoxicity against HeLa tumor cells at the 24th hour was induced by FAs, followed by EE, US and EPSH. At the 48th hour, the FAs again showed the highest effect, followed by US, EPSH, EE, CM and HT. It is noteworthy that the fatty acids (FAs) isolated from *Coelastrella* sp. BGV showed an antiproliferative effect at the 24th hour with an IC_{50} value close to that of the classical cytostatic drug Doxorubicin. A similar, comparable to cytostatic effect was observed for US at 48th hour of treatment. The high activity of the FAs of *Coelastrella* sp. BGV may be due to the content of palmitic, oleic, linoleic, and γ-linolenic fatty acids, which have previously been shown to inhibit tumor cell growth (Bergé et al., 2002; Ramesh and Das, 1998).

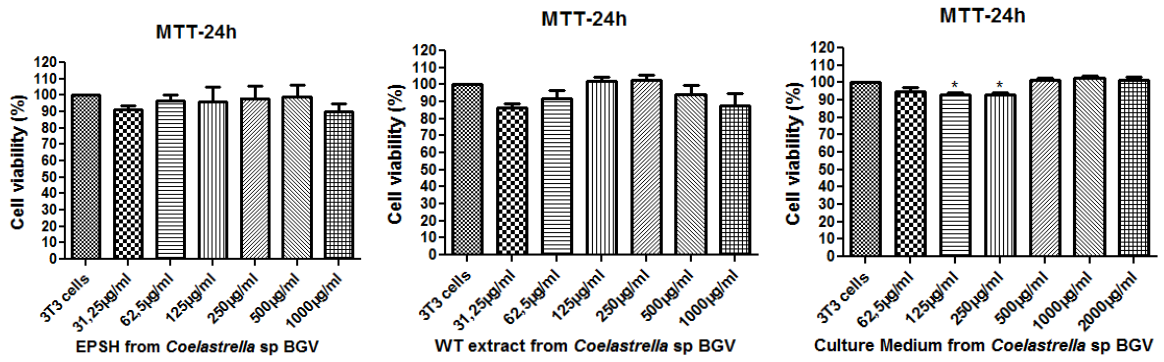
The studied LT (low temperature) and HT (high temperature) aqueous extracts, and oil extract (OE) from the biomass of *Coelastrella* sp. BGV significantly reduced the viability of HeLa tumor cells for both time intervals (24th and 48th hour), with the exception of OE, which had no effect at the 48th hour. After 24 hours of exposure, the three extracts administered in the same concentrations had a similar antiproliferative effect. However, after 48 hours of exposure, the high concentrations (500 μg/mL and 1000 μg/mL) of HT aqueous extract induced a significantly higher inhibition of the proliferation of the HeLa cells compared to the same concentrations of the LT aqueous extract.

Gacheva (2012) and Gigova et al., (2011) investigated ethanolic (lipophilic) and aqueous cell extracts and culture fluid from green microalgae for cytotoxicity against HeLa tumor cells. The culture fluid from *Coelastrella* sp., *Scenedesmus incrassatulus* and 3B1, and aqueous extracts from *Chlorella* sp., *Scenedesmus obliquus*, 3B1 and *Coelastrella* sp. inhibited the proliferation of HeLa cells, and the effect of aqueous extracts were concentration dependent. Aqueous extracts showed a stronger cytotoxic effect, compared to the culture fluids. Lipophilic extract of *Scenedesmus incrassatulus* showed low activity, while the lipophilic substances from *Chlorella* sp. were the most toxic to HeLa cells of all tested extracts (80% inhibition of cell growth) even at the lowest applied concentrations (100 μg/mL). Fatty acids from *Scenedesmus incrassatulus* and *Scenedesmus obliquus* showed high activity, but the authors found the highest activity for fatty acids from *Coelastrella* sp. and *Chlorella* sp. with an IC_{50} at the 24th hour lower than 15 μg/mL (Gacheva, 2012; Gigova et al., 2011). Silambarasan et al., (2014) found that the methanolic extract of *Scenedesmus obliquus* RDS01 showed high cytotoxic activity against HeLa (with an IC_{50} of 50 μg/mL). *Scenedesmus obliquus* diethyl ether extract was reported to inhibit the proliferation of three human tumor cell lines - HepG2 (human hepatocellular carcinoma), HCT116 (colon cancer) and MCF-7 (mammary adenocarcinoma) with IC_{50} concentrations of 24.6 μg/mL for HepG2, 42.77 μg/mL for HCT116 and 93.8 μg/mL for MCF-7 (Marrez et al., 2019). Lipids from *Scenedesmus obliquus* extracted with chloroform:methanol (2:1 v/v) showed high antitumor activity against MCF7, HepG2 and HCT116 with IC_{50} values of 11.62 μg/mL, 14.5 μg/mL and 15.22 μg/mL, respectively (Abd El Baky et al., 2014). In a study by Iyer et al., (2015), an extract of *Coelastrella oocystiformis* containing mainly carotenoids and administered at concentrations of 40 μg/mL and 80 μg/mL, inhibited the growth of only 5% of DU-145 cancer cells (human prostate cancer cell line). Of the two low molecular weight polysaccharide fractions isolated from *Chlorella pyrenoidosa*, the galactose-containing fraction I, had a higher activity against A549 human tumor cells (lung cancer) than the second PZH fraction containing mainly rhamnose. Administered at a concentration of 1000 μg/mL, after 48 hours, fraction I inhibited 68.7% of tumor cells and fraction II inhibited 49.5% (Sheng et al., 2007). Compared to *Chlorella pyrenoidosa* polysaccharide fraction I,

Coelastrella sp. BGV exopolysaccharide, which also contains mainly galactose, showed higher antitumor activity (with an IC_{50} of 4.310 μg/mL). The difference in antitumor efficacy may be due to different molecular weights, monosaccharide composition, chain conformation, charge of different polysaccharides (Sheng et al., 2007; Zhang et al., 2007), as well as tested tumor cell lines.

Antiproliferative activity of EPSH, HT and CM against normal mouse fibroblasts (3T3)

The effect of EPSH, high temperature aqueous extract (HT) and culture medium (CM) from *Coelastrella* sp. BGV on the viability/proliferation of 3T3 murine fibroblasts, widely used for control in experimental practice as a normal cell line was examined.



A **B** **C**

Figure 18. Effect of EPSH (A), HT (B) and CM (C) obtained from the green microalga *Coelastrella* sp. BGV on the viability/proliferation of 3T3 murine fibroblasts, recorded at the 24th hour by MTT test.

The results of the MTT test showed a statistically insignificant decrease of the viability of 3T3 murine fibroblasts after 24 hours of treatment with algae samples. The EPSH, administered in the same concentrations as in HeLa cells, reduced the viability of 3T3 fibroblasts from 98.11 ± 15.03% to 89.84 ± 10.36%. The HT induced ~~a~~ decrease of the viability of normal 3T3 cells up to 13% and the values showed 86.38 ± 5.0%. The CM inhibited the prolifetation of 3T3 fibroblasts with about 8% and reaches 92.86 ± 2.74%. (Fig. 18A, B, C).

The obtained results give grounds to assume specific activity of algal products against HeLa human tumor cells.

IV.3.1.2. Cytomorphological studies of HeLa tumor cells cultured in the presence of algal products from *Coelastrella* sp. BGV, by fluorescence methods

The aim of these studies was to determine the type of cell death (apoptosis or necrosis) in the treated tumor cells by analyzing the morphological changes in the cells at the cell and nucleus level after double staining with fluorochromes - AO/EtBr and staining with DAPI. HeLa tumor cells were cultured in the presence of algae products administered in concentrations equal to the IC_{50} for the respective product, recorded after 24 hours of treatment (Table 4, FAs - 3 μg/mL; EE - 30 μg/mL; US - 100 μg/mL; EPSH - 600 μg/mL), or at the maximum initial dose (LT - 1000 μg/mL; HT - 1000 μg/mL; OE - 1000 μg/mL; CM - 1000 μg/mL).

IV.3.1.2.1. Cytomorphological changes of HeLa tumor cells cultured in the presence of algal products from *Coelastrella* sp. BGV evaluated after intravital staining with acridine orange (AO) and ethidium bromide (EtBr).

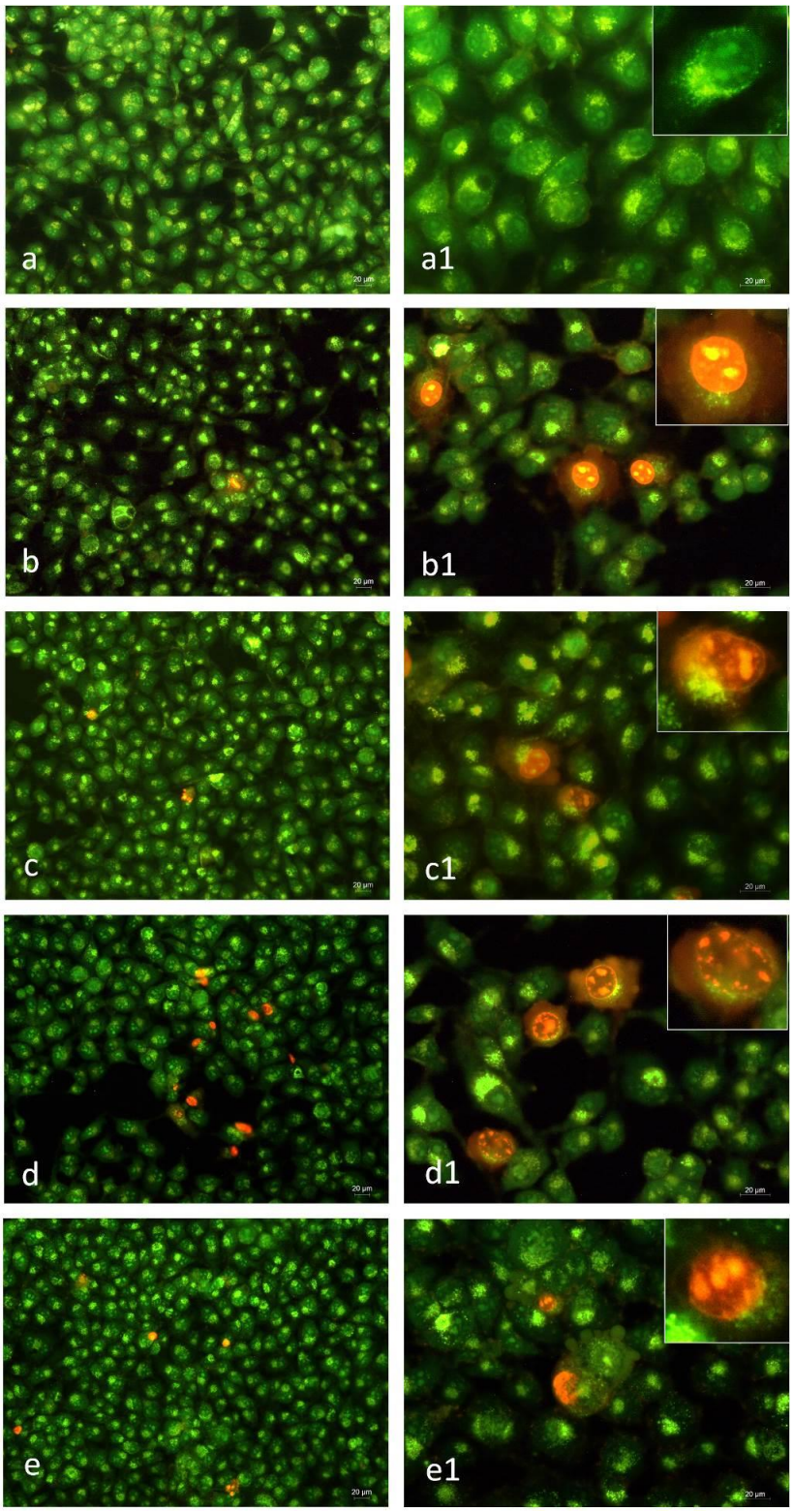


Figure 19. Fluorescence microscopy of HeLa tumor cells cultured in the presence of algal products from *Coelastrella* sp. BGV. a, a1 - in culture medium (control); b, b1 - ethanol extract (EE); c, c1 - fatty acids (FAs); d, d1 - unsaponifiable matter (US); e, e1 - culture medium (CM); magnification 200 × (a-e) and 400 × (a1-e1); bar 20 µm; AO/EtBr staining.

AO stains viable and dead cells, emitting strong green fluorescence as a result of its intercalation in double-stranded DNA, as well as red-orange fluorescence when bound to

single-stranded RNA. Unlike AO, EtBr is a fluorochrome that does not stain viable cells and those with an intact membrane (early apoptotic). It stains dead and late apoptotic cells that have increased membrane permeability.

Untreated HeLa cells (negative control) showed normal (typical) for the respective cell type uniform monolayer growth and normal morphology - slightly elongated in shape, green-stained cells with pale green nuclei containing 3-4 yellow-green nuclei and clusters of yellow-orange granules located around the nucleus (Fig. 19 a, a1).

Similar morphological changes with different degrees of variation were observed in HeLa tumor cells after treatment with each of the tested algae samples from the green microalga *Coelastrella* sp. BGV.

Impaired monolayer growth and cells with different morphological changes - the presence of cells with almost normal morphology; cells with vacuolation of the cytoplasm; cells with signs of early apoptosis (rounded, with membrane blebing); single cells with signs of late apoptosis (orange nucleus with bright yellow nucleoli and chromatin condensation) were observed in HeLa tumor cells cultured in the presence of EE (Fig. 19 b, b1).

HeLa tumor cells cultured in the presence of FAs showed impaired monolayer growth and cells with more severe morphological changes. Rounded cells with signs of early apoptosis with unevenly distributed chromatin in the nucleus in the form of dense green areas, and cells with signs of late apoptosis with swelling of the cell membrane, bright orange nucleus with bright yellow nucleoli and chromatin condensation were observed (Fig.19 c, c1).

The morphological changes of HeLa cells after the treatment with US were similar to that one described for FAs (Fig. 19 d, d1). Significant cytotoxic effect, uneven monolayer growth, cells with cytoplasmic swellings and bright orange nuclei with condensation, margination and fragmentation of chromatin were observed.

After treatment of HeLa tumor cells with CM, monolayer growth was also impaired. Cells in stages of early apoptosis predominated - light green in color with a light green nucleus and unevenly distributed chromatin in the nucleus in the form of dense green areas and cytoplasmic swellings. Late apoptotic cells had a bright orange stained nucleus with condensation and fragmentation of chromatin (Fig.19 e, e1.

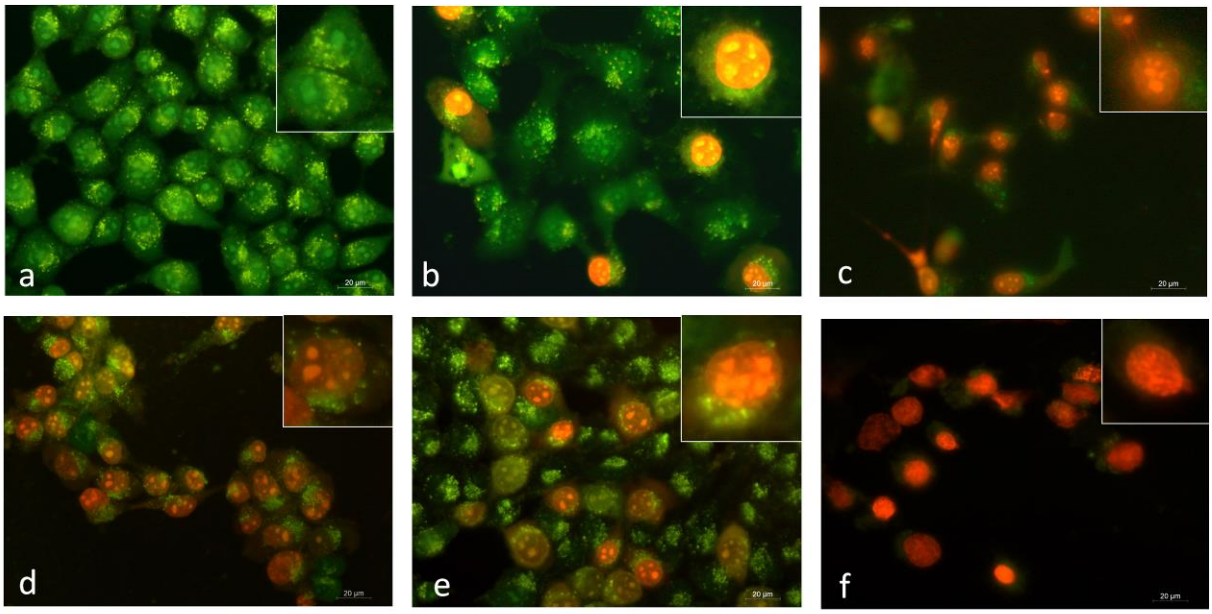


Figure 20. Fluorescence microscopy of HeLa tumor cells cultured in the presence of algal products derived from *Coelastrella* sp. BGV. a - in culture medium (negative control); b - low temperature aqueous extract (LT); c - in high temperature aqueous extract (HT); d -

exopolysaccharide (EPSH); e - oil extract (OE); f - Doxorubicin (10 µg/mL) (positive control); magnification 400 ×; bar 20 µm; AO/EtBr staining.

The HeLa tumor cells treated with LT, HT and OE (1000 µg/mL) and EPSH (600 µg/mL) from *Coelastrella* sp. BGV for 24 hours showed typical morphological characteristics of early and late apoptotic cells. Most of the cells were with late apoptotic changes – saturated orange-red stained nucleus with chromatin condensation (Fig. 20).

HeLa tumor cells cultured in the presence of LT (Fig. 20b): Cells in the stages of early apoptosis predominated. Cells and nuclei were stained green and redistribution of nuclear chromatin (condensation) was observed. The unclear cell contour and nuclear membrane, as well as the appearance of small red-colored granules in the cytoplasm were impressive.

HeLa tumor cells cultured in the presence of HT (Fig. 20c): Severely impaired monolayer growth and severely decreased cell numbers were observed. Strongly altered cells with morphology similar to Doxorubicin-treated positive control were seen. Single orange-red nuclei with condensation of chromatin were visible. Reduction of cell and nuclear volume (picnosis) and fragmentation of the nucleus (karyorrhexis) were also observed.

HeLa tumor cells cultured in the presence of EPSH (Fig. 20d) showed: impaired monolayer growth, decreased cell volume, presence of cells in the late stages of apoptosis (saturated orange-red stained nucleus with chromatin condensation and fragmentation).

HeLa tumor cells cultured in the presence of ME (Fig. 20e) showed morphological changes characteristic of late apoptosis. The nuclei were from orange-brown to deep orange-red in color with condensation, chromatin margination and fragmentation.

HeLa tumor cells cultured in the presence of Dox (Fig. 20f): A strong reduction of the cell number and single bright reddish-orange nuclei and cytoplasmic shadows were observed. The nuclei were polymorphic in size with condensation of chromatin and fragmentation of the nucleus.

The obtained results showed the presence of cells with impaired morphology, which leads to a decrease in their viability and proliferation. Data on cytomorphological changes of the algae-treated tumor cells were in accordance with the results obtained by the MTT test.

IV.3.1.2.2. Evaluation of the nuclear morphology of HeLa tumor cells treated with algae products from *Coelastrella* sp. BGV - DAPI staining

The aim of the research was to analyze the alterations in the nuclear morphology and to obtain information about the potenial of the algae products to induce apoptotic cell death in the tumor cells. The DAPI molecule can cross an intact cytoplasmic membrane, making it a suitable agent for studying the nuclear morphology of both living and fixed cells.

The control, untreated HeLa tumor cells (Fig. 21 a, a1) were with intact nuclei, round to slightly oval in shape and almost uniform in size, with smooth outlines and evenly distributed chromatin. Cell nuclei in different phases of mitosis were observed.

HeLa tumor cells cultured in the presence of EE, FAs, US and CM showed atypical nuclear morphology - nuclear polymorphism (different in shape and size nuclei), uneven outline of the nuclei, unevenly distributed chromatin (pronounced margination and condensation of chromatin), nucleus fragmentation and apoptotic bodies (Fig. 21 b, b1; c, c1; d, d1; e, e1).

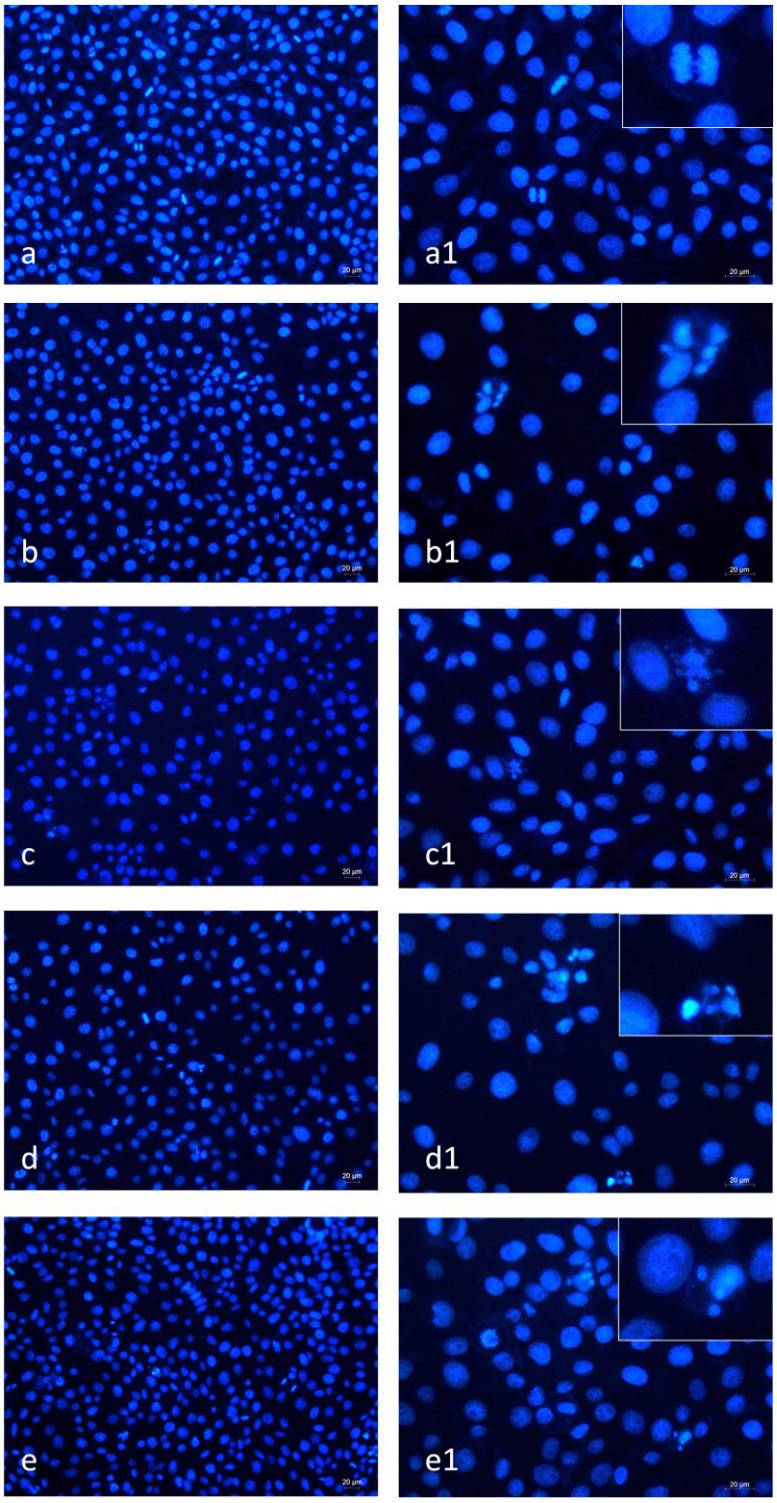


Figure 21. Morphological changes in the nuclei of HeLa tumor cells cultured for 24 hours in the presence of algal products from *Coelastrella* sp. BGV. a, a1 - culture medium (negative control); b, b1 - ethanol extract (EE); c, c1 - fatty acids (FAs); d, d1 - unsaponifiable matter (US); e, e1 - lyophilized culture medium (CM); magnification 200 × (a-e) and 400 × (a1-e1); bar 20 µm; DAPI.staining.

Morphological changes in the nuclei of HeLa tumor cells treated for 24 hours with LT, HT, EPSH and OE from *Coelastrella* sp. BGV, and visualized by DAPI staining are shown in Figure 22. Untreated control HeLa cells had the nuclear morphology described in the previous figure (Fig. 21 a). In contrast to controls, LT-, HT-, EPSH- and OE-treated cells showed

significant morphological changes in the nuclei typical of apoptosis: nuclear polymorphism, severely impaired chromatin pattern (chromatin condensation and margination), nucleus fragmentation and formation of apoptotic bodies (Fig. 22 b, c, d, e). HeLa tumor cells treated with the anthracycline antitumor antibiotic Doxorubicin were used as a positive control in the experiments (Fig. 22 f). Doxorubicin-treated cells showed greatly reduced number of nuclei with uneven outlines, condensed chromatin, and fragmented nuclei with multiple apoptotic bodies. The lower number of nuclei in the samples treated with algal metabolites compared to the untreated control and the similar morphological features of HT- and Doxorubicin-treated tumor cells were impressive (Fig. 22 c, f).

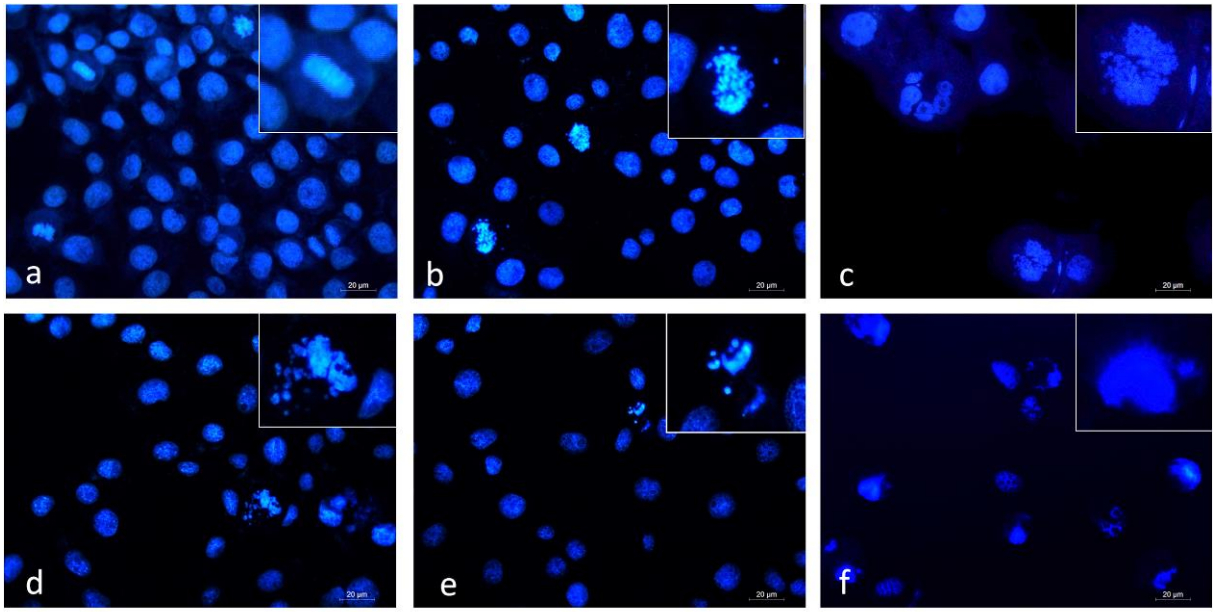


Figure 22. Morphological changes in the nuclei of HeLa tumor cells cultured for 24 hours in the presence of algal products derived from *Coelastrella* sp. BGV. a - cell culture medium (negative control); b - low temperature aqueous extract (LT); c - high temperature aqueous extract (HT); d - exopolysaccharide (EPSH); e - oil extract (OE); f - Doxorubicin (10 µg/mL) (positive control); magnification 400 ×; bar 20 µm; DAPI staining.

The results obtained by applying the MTT test and fluorescent test methods showed that the tested products of *Coelastrella* sp. BGV significantly reduced the viablity of HeLa tumor cells, which is associated with the induction of programmed cell death.

According to literature data, the antitumor action of a number of agents of microalgal origin (carotenoids, sulfated polysaccharides, phenolic derivatives, sterols, lipopeptides, depsipeptides, polyketides, alkaloids, etc.) is due to the induction of programmed cell death (Martins et al., 2008 ; Abd El-Hack et al., 2019; Martínez Andrade et al., 2018). Microalgae-derived metabolites have been shown to interact with molecular targets in tumor cells such as DNA, receptor protein kinases, microtubules, and proteins at cell cycle checkpoints, leading to cell cycle arrest, mitochondrial dysfunction, oxidative damage, changes in the membranes, activation of a caspase cascade and eventually to cell death. Therefore, apoptosis-inducing metabolites have high pharmacological value for antitumor therapy. Carotenoids from *Chlorella ellipsoidea* (mainly violaxanthin) and *Chlorella vulgaris* (mainly lutein), for example, induce apoptosis in HCT116 cells (human colon carcinoma cell line), as evidenced by translocation of phosphatidylserine on the outer surface of plasmatic membrane, visualized by annexin V-FITC fluorescent analysis (Kwang et al., 2008). Zhang et al., (2008) reported an antiproliferative effect of fucoxanthin on EJ-1 cells (bladder cancer) and the induction of

apoptosis characterized by condensed chromatin, nuclear fragmentation, and apoptotic bodies in addition to the DNA ladder. Dolastatins 10 and 15 isolated from cyanoprokaryotes stop the G2/M phase cell cycle and induce apoptosis by altering gene expression, including activation of caspase 3 in lung cancer cells A549 (Catassi et al., 2006) or by inhibition of microtubule assembly in various human myeloma cell lines (RPMI8226, U266 and IM9) (Sato et al., 2007). The lipopeptide somocystinamide A, isolated from the blue-green alga *Lyngbya majuscula*, is a potent inhibitor of the proliferation (in μM concentrations) of various cancer cell lines such as leukemia, lung and breast carcinomas, melanoma and neuroblastoma. Antiproliferative activity is largely due to the activation of the caspase 8-dependent apoptotic pathway (Wrasidlo et al., 2008). Lin et al., (2017) investigated the effect of an aqueous extract of *Chlorella sorokiniana* on two human lung adenocarcinoma cell lines (A549 and CL1-5). The authors found the presence of early apoptotic cells (by annexin V+/PI−staining), activated forms of caspase-9 and caspase-3, and inactivation of the nuclear enzyme poly (ADP-ribose) polymerase (which signals the presence of of DNA damage and facilitates its recovery). Based on these results, they concluded that the extract induced the death of A549 and CL1-5 cells by the mitochondria-mediated apoptotic pathway.

Data on the antitumor effect of algae products from green microalgae of the genus *Coelastrella* are scarce (Iyer et al., 2015). In the present work, the effect of extracts and metabolites isolated from *Coelastrella* sp. BGV, on the proliferation of human tumor cells HeLa, as well as the mechanism of their antitumor action were examined for the first time.

IV.3.2. Antibacterial and antifungal activities of algal products derived from the green microalgae *Coelastrella* sp. BGV

IV.3.2.1. Agar - diffusion method of Perez et al. (1990). The antibacterial and antifungal activities of algae products from *Coelastrella* sp. BGV – ethanol extract (EE), fatty acids (FAs), unsaponifiable substances (US) and culture medium (CM), administered at a concentration of 5 mg/mL, was determined by agar - diffusion method, against the test bacteria *Staphylococcus aureus* (Gram+) and *Ecsherichia coli* (Gram-) compared to the standard antibiotic Gentamicin (0.1 mg/mL) and against *Candida albicans* compared to the standard antibiotic Amphotericin B. Antibacterial and antifungal activity was assessed by the diameter (mm) of the inhibitory zones in the agar after 24 hours incubation at 37°C for bacteria and 48 hours at 30°C for the fungus. The results of the experiments are presented in Figure 23 and Table 5.

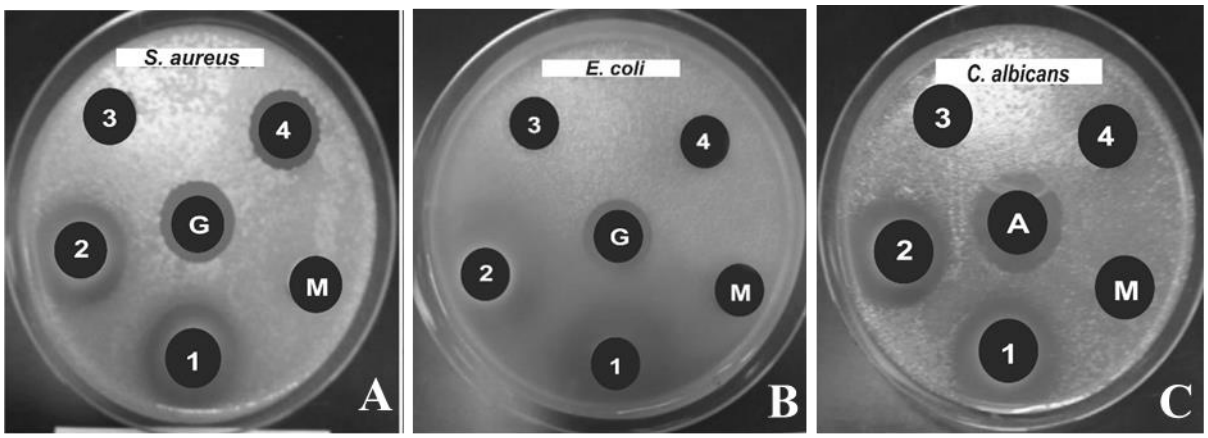


Figure 23. Antibacterial and antifungal effects of algal products derived from *Coelastrella* sp. BGV against *Staphylococcus aureus* (A); *Ecsherichia coli* (B) and *Candida albicans* (C). 1 - ethanol extract; 2 - fatty acids; 3 - unsaponifiable substances; 4 – culture medium; M - culture

medium containing 10% DMSO (negative control); G and A - positive controls Gentamicin and Amphotericin B, respectively.

Table 5. Zones of inhibition (diameter in mm) of the pathogens *Staphylococcus aureus*; *Ecsherichia coli* and *Candida albicans*, induced by the algae products. Gentamicin and Amphotericin B - positive controls.

Test samples from *Coelastrella* sp./Concentrations	**INHIBITORY ZONE (mm)**		
	TEST-MICROORGANISMS		
	***Ecsherichia coli* Gram (-) bacteria**	***Staphylococcus aureus* Gram (+) bacteria**	***Candida albicans***
Gentamycin /0.1 mg/mL/	**5.0**	**5.0**	**-**
Amphotericin B /0.1 mg/mL/	**-**	**-**	**7.0**
Ethanol extract (EE) /5 mg/mL/	**12.0**	**10.0**	**10.0**
Fatty acids (FAs) /5 mg/mL/	**14.0**	**8.0**	**8.0**
Unsaponifiable substances (US) /5 mg/mL/	**4.0**	**0**	**0**
Culture medium (CM) /5 mg/mL/	**0**	**4.0**	**0**

The study showed that two of the four microalgae products inhibited the growth of *S. aureus* with an inhibitory zone - 10 mm for EE and 8 mm for FAs. For comparison, the inhibitory zone for the Gentamicin (positive control administered at a concentration of 0.1 mg/mL) was - 5 mm. The extracellular secretions of *Coelastrella* sp. BGV (KM) were less active (the zone of inhibition was 4 mm), and US were inactive against this Gram-positive pathogenic bacteria.

Clearly defined inhibitory zones for EE - 12 mm, for FAs - 14 mm and a smaller zone for US - 4 mm, against *Ecsherichia coli* (Gram-negative bacteria) were observed, with a control of - 5 mm zone for Gentamicin.

The inhibitory zones observed for *Candida albicans* were 10 mm in diameter for EE, 8 mm for FAs, and 7 mm for the positive control Amphotericin B, used at a concentration of 0.1 mg/mL.

EE and FAs of *Coelastrella* sp. BGV showed the highest activity (largest diameter of the inhibition zone) and the widest antipathogenic effect (inhibition of growth of the three tested pathogens). The culture medium containing 10% DMSO used as a negative control showed no antimicrobial and antifungal activity.

IV.3.2.2. Method of micro-dilutions in a liquid medium (Andrews, 2001). The antibacterial and antifungal activities of the four samples from *Coelastrella* sp. BGV were

also tested by the method of micro-dilutions in a liquid medium. This method makes it possible to determine the minimum inhibitory concentration (MIC), a quantitative expression of antimicrobial activity. The MIC of the studied algae extracts and metabolites was determined spectrophotometrically, after serial double dilutions of the samples in 96-well microplates. The obtained results are presented in Table 6.

Table 6. Minimum inhibitory concentrations (MIC) (μg/mL) of algae products from *Coelastrella* sp. BGV against *Staphylococcus aureus*, *Ecsherichia coli* and *Candida albicans*.

Test sample / Control	***S. aureus***	***E. coli***	***C. albicans***
Gentamycin	**20±0.05**	**20±0.04**	
Amphotericin B			**10±0.05**
Ethanol extract (EE)	**600±0.2**	**400±0. 2**	**500±0.1**
Fatty acids (FAs)	**500±0.1**	**300±0.2**	**600±0.2**
Unsaponifiable substances (US)	**0.0**	**>2500±0.3**	**0.0**
Culture medim (CM)	**>3000±0.2**	**0.0**	**0.0**

All algal samples tested, with the exception of CM, showed the highest activity (lowest MIC values) against the Gram-negative bacterium *E. coli*. The MIC of FAs was 300 μg/mL and that of EE was 400 μg/mL. Unsaponifable substances inhibited the growth of *E. coli* at much higher concentrations (MIC>2500 μg/mL), but are inactive against the other two pathogens. Fatty acids were also most active against Gram-positive *Staphylococcus aureus* (the value of MIC was 20% lower than that of EE and more than six times lower than the value of CM). Only EE and FAs showed an effect against *Candida albicans*, and the activity of EE was 20% higher.

Gacheva (2012) has found that culture media from *Coelastrella* sp., *Chlorella* sp. and 3B1 showed activity against *Candida albicans* with a MIC of 12.5 mg/mL. Aqueous extract of *Scenedesmus quadricauda* inhibited the growth of *Trichophyton rubrum*, *Aspergillus flavus* and *Microsporium canis* with a MIC value for all pathogenic fungi of 0.4 mg/mL (Al-Rekabi, 2011). Marrez et al., (2019) investigated the activity of diethyl ether extract of *Scenedesmus obliquus* against two Gram-positive, four Gram-negative pathogenic bacteria and nine species of fungi and found the highest activity of the extract against the Gram-negative *Salmonella typhi* and the fungus *Aspergillus steynii* with MIC values 0.5 and 0.6 mg/mL, respectively. Ethanol extracts from ten strains of green microalgae of three genera (*Desmococcus*, *Chlorella* and *Scenedesmus*) showed high antibacterial activity against three Gram-positive and four Gram-negative bacteria, with MIC values ranging from 0.001 to> 0.125 mg/mL (Ördög et al., 2004). Mendiola et al., (2008) compared the activity of carbon dioxide extracts from the green microalga *Dunaliella salina* against *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus niger*. The authors found that the composition of the extracts, and their antimicrobial activity, differ depending on the extraction conditions. The activity of the algae extract was highest at 314 bar and 9.8°C, with values of the minimum bactericidal/fungicidal concentration (MBC/MFC, in mg/mL) of 3.1 ± 0.4 (*E. coli*), 3.9 ± 0.3

(S. aureus), 8.3 ± 0.4 (*C. albicans*) and 30.0 ± 0.5 (*A. niger*). In a similar study performed with *Haematococcus pluvialis*, the highest antimicrobial activity was reported for ethanol extract obtained from red hematocysts under pressure and 100°C, with MBC/MFC values of 1.9 ± 0.1 mg/mL (*E. coli*), 2.5 ± 0.2 mg/mL (*S. aureus*), 3.9 ± 0.3 mg/mL (*C. albicans*) and 12.8 ± 0.7 mg/mL (*A. niger*) (Santoyo et al., 2009).

The comparative analysis showed that the results for antibacterial and antifungal activity of the samples from *Coelastrella* sp. BGV obtained by applying the precise quantitative method of micro-dilutions in a liquid medium confirm the data from the semi-quantitative agar-diffusion method. This makes the use of easier and faster agar/disk diffusion methods suitable for a larger study of the antipathogenic activity of algal samples (when the number of samples and test microorganisms is higher).

IV.3.2.3. Disc-diffusion method of Essawi and Srour, (2000). The antibacterial and antifungal activities of fatty acids (FAs), ethanol extract (EE) and culture medium (CM), low temperature aqueous extract (LT) and exopolysaccharide (EPSH) from *Coelastrella* sp. BGV have also been studied by the disk-diffusion method, expanding the spectrum of test pathogens. Nine bacterial strains were used, of which two Gram-positive (*Bacillus cereus*, *Staphylococcus aureus*) and seven Gram-negative (*Escherichia coli* ATCC, *Escherichia coli* UPEC, *Escherichia coli* EPEC, *Salmonella typhimurium*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Klebsiela pneumoniae*) and one fungal strain - *Candida albicans*. Antibacterial and antifungal activity was assessed by the diameter (mm) of the resulting inhibitory zones in the agar, after 24 hours of incubation at 37°C for bacterial strains and after 48 hours of cultivation for the fungal strain at 30°C. The results are presented in Table 7.

The table 7 shows that three extracts and two metabolites of *Coelastrella* sp. BGV displayed varying degrees of antimicrobial activity against the pathogens tested, inhibiting the growth of a large number of them.

The ethanol extract of *Coelastrella* sp. BGV was active against six bacterial strains – five Gram (-) (*E. coli* ATCC, *E. coli* UPEC, *Proteus mirabilis*, *P. aeruginosa*, *K. Pneumoniae*), one Gram (+) (*B. cereus*), and against *C. albicans*. (Table 7).

The FAs were active against three Gram (-) bacterial strains (*E. coli* UPEC, *P. aeruginosa*, *K. pneumoniae*), one Gram (+) (*B. cereus*), and against *C. albicans* (Table 7). The CM was active against Gram-negative *E. coli* UPEC, *Proteus mirabilis* and *P. aeruginosa* and against *C. albicans*. The EPSH showed activity against two Gram (-) bacterial strains (*Proteus mirabilis* and *P. aeruginosa*) and one Gram (+) (*B. cereus*), as well as against *C. albicans*. Antimicrobial activity against Gram-negative *Proteus mirabilis* and *P. aeruginosa*, and against *C. albicans* was found for LT aqueous extract. (Table 7).

The ethanol extract (EE), which was active against six of the nine bacterial strains tested showed the widest antibacterial spectrum, followed by fatty acids (Fas) which inhibited the development of four bacterial strains. The EPSH and CM inhibited the growth of three and LT extract of two of the bacterial strains studied.

Table. 7. Antimicrobial activity of products of *Coelastrella* sp. BGV

Samples from *Coelastrella* sp.BGV/Concentration	**INHIBITORY ZONE (mm)**
	TEST MICROORGANISMS

	Gram (-) bacteria							Gram (+) bacteria		Fungal strain
	E. coli ATCC	***E. coli UPEC***	***E. coli EP EC***	***S. typhi muri um***	***P. mirabilis***	***P. aerugino sa***	***K. pneum oniae***	***B. cereus***	***S. aur eus***	***C. albicans***
Low temperatur e aqueous extract (LT) /35 mg/mL/	**0**	**0**	**0**	**0**	**10/17***	**8**	**0**	**0**	**0**	**8**
Exopolysacc haride (EPSH) /20 mg/mL/	**0**	**0**	**0**	**0**	**9**	**10/18***	**0**	**10/15***	**0**	**7**
Culture medium (CM) /20 mg/mL/	**0**	**9/12***	**0**	**0**	**12/30***	**8**	**0**	**0**	**0**	**7**
Ethanol extract (EE) /25 mg/mL/	**9/14***	**8**	**0**	**0**	**13**	**7**	**9**	**7**	**0**	**9**
Fatty acids (FAs) /8 mg/mL/	**0**	**11/25***	**0**	**0**	**0**	**11/22***	**15**	**10**	**0**	**9**

*Bacteriostatic action of the extract (diameter of the bacteriostatic zone).

Although the ethanol extract inhibited the growth of a larger number of tested microorganisms (6 strains), the fatty acid sample (4 strains) demonstrated more pronounced antibacterial activity against the tested microorganisms, in terms of the size of the inhibitory zones measured. The diameter of the inhibition zones ranged from 10 to 15 mm for the fatty acids, and from 8 to 13 mm for the ethanol extract. The fatty acids sample showed the highest antibacterial activity of all *Coelastrella* sp. BGV products tested with a 15 mm zone of inhibiton of *K. pneumoniae* (Table 7).

As we found, the oil extract of *Coelastrella* sp. BGV contained over 70% unsaturated FAs. Earlier studies demonstrated antibacterial activity of unsaturated FAs from other green microalgae, such as *Haematococcus pluvialis* (Rodriguez-Meizoso et al., 2010) and *Chlorococcum* sp. HS-101 (Bhadury and Wright, 2004).

Pseudomonas aeruginosa and *Proteus mirabilis* were the most sensitive of the tested bacterial strains, as their growth was inhibited by five and four algae samples, respectively.

The highest activity against *Pseudomonas aeruginosa* was again recorded in the fatty acid sample (11 mm inhibition zone) and against *Proteus mirabilis* in the ethanol extract (13 mm inhibition zone). *Escherichia coli* strain EPEC, *Salmonella typhimurium* and *Staphylococcus aureus* were resistant, while *C. albicans* was sensitive to the action of the five algal samples. The registered zones of inhibition of *C. albicans* had the largest diameter in the two most active samples - fatty acids and ethanol extract (9 mm each) (Table 7).

The algal extracts and metabolites studied by both methods (agar-diffusion and disk-diffusion) showed different degrees of antibacterial and antifungal activity against Gram-positive, Gram-negative pathogens and *C. albicans*, inhibiting the growth of a large number of them (Table. 5 and Table 7). The high therapeutic potential of fatty acids and ethanol extract of *Coelastrella* sp. BGV against one of the most significant Gram-negative pathogens, such as *Escherichia coli*, against *Pseudomonas aeruginosa* and *Proteus mirabilis*, and against fungal pathogen *Candida albicans* was noteworthy.

FAs isolated from other green microalgae (*Chlorella* sp., *Scenedesmus obliquus*, *Scenedesmus incrassatulus*, *3B1*, *Coelastrella* sp.), tested by the agar well method, at a concentration of 20 mg/mL were active only against Gram-positive bacteria (*S. aureus* and *Streptococcus pyogenes*) with a diameter of the inhibition zones from 6 to 15 mm (Gacheva, 2012; Najdenski et al., 2013). Inhibition zones with a diameter ranging from 3 to 9 mm were measured after tretment with *Chlorella vulgaris* extracts, depending on the solvent (ethanol, methanol, chloroform and diethyl ether) as well as on the test pathogens (ten Gram-positive and Gram -negative bacteria) (Dineshkumar et al., 2017). *Heterochlorella luteoviridis* culture fluid inhibited the growth of *C. albicans*, forming an area less than 10 mm in diameter (Mudimu et al., 2014). Bhagavathy et al., (2011) found that extracts with eight different organic solvents and pigments *β*-carotene, chlorophyll *a* and chlorophyll *b* from the green microalga *Chlorococcum humicola* inhibited the growth of *C. albicans*, *Aspergillus flavus* and *Aspergillus niger*, with the diameter of the inhibition zones varying from 20 to 20 mm for extracts and from 7 to 11 mm for pigments. Diethyl ether extract of *Scenedesmus obliquus*, tested by the disk diffusion method showed activity against two Gram-positive, four Gram-negative pathogenic bacteria and nine species of fungi with a diameter of the zones of inhibition for bacterial pathogens between 12.5 mm and 19.5 mm and for fungi between 8.7 mm and 18.3 mm (Marrez et al., 2019).

V.3.3. Antioxidant activity (AO) of an ethanol extract (EE) of *Coelastrella* sp. BGV.

The ethanol extract of *Coelastrella* sp. BGV showed high antitumor and antibacterial activity, which gave us reason to study its antioxidant potential.

Antioxidant activity was determined by indicators characterizing the state of the antioxidant defense system of cells - the total amount of phenols and flavonoids, and total antioxidant activity (TAA). The results are presented in Figure 24.

The results showed a high content of total phenols and flavonoids and a high level of total antioxidant activity for an ethanol extract obtained from the biomass of *Coelastrella* sp. BGV. Phenolic content and carotenoid content correlate with high antioxidant capacity, which is an indication that these compounds contribute significantly to the overall antioxidant activity of the microalgae.

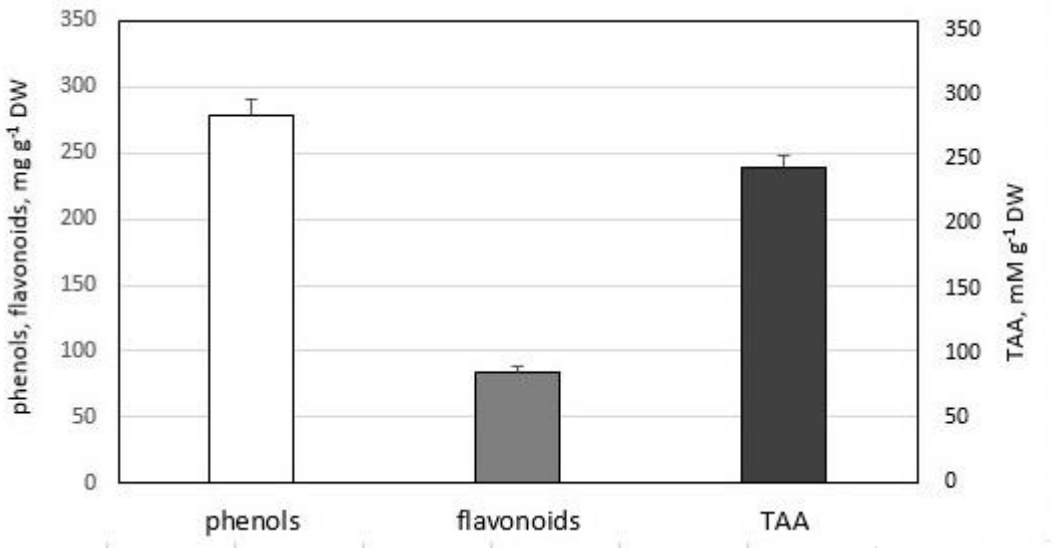


Figure 24. Total phenols (mg/g^{-1} DW), flavonoids (mg/g^{-1} DW) and total antioxidant activity (mM/g^{-1} DW) in an alcoholic extract obtained from the biomass of *Coelastrella* sp. BGV

Maadane et al., (2015) investigated the antioxidant activity of extracts with three different solvents obtained from nine strains of microalgae using DPPH analysis. The nine ethanol extracts showed significantly higher activity than the extracts with ethanol/water and water. The extacts of *Tetraselmis* sp., *Dunaliella salina* and *Navicula* sp. showed the highest antioxidant activity. The ethanol extract of *Nannochloropsis gaditana* had the highest phenolic content (32.0 ± 0.5 mg GAE/g extract), followed by *Tetraselmis* sp. with 25.5 ± 1.5 mg GAE/g. The ethanol extracts of *Dunaliella* sp., *Phaoedactilum tricornutum* and *Navicula* sp. had a total phenolic content of more than 15 mg GAE/g, while the lowest phenolic content was found in *Chlorella* sp. (8.1 ± 0.1 mg GAE/g). The amount of total phenols in the ethanol extract of *Coelastrella* sp. BGV significantly exceeded the values measured in extracts of cyanoprokaryotes *Nostoc commune* and *Arthronema africanum* (Petrova et al., 2020). In a study by Sawant and Mane (2018), it was found that of the seven different extracts obtained from *Chlorella emersonii* KJ725233, the methanol extract had the highest antimicrobial potential against the tested Gram-positive, Gram-negative bacterial strains and fungal pathogens. This extract showed the highest antioxidant potential as well as the highest content of phenols (5.19 ± 0.10 mg GAE/g DW) and flavonoids (24.60 ± 1.18 mg QE/g DW). Ethyl acetate extracts of *Nannochloropsis oculata* and *Gracilaria gracilis* were rich in flavonoid compounds (71.79 ± 2.32 and 66.48 ± 1.87 mg QE/g extract), while their methanol extracts contained smaller amounts (42.08 ± 1.09 and 26.47 ± 1.203 mg QE/g extract, respectively). The maximum and minimum amounts of total phenols were found in the ethyl acetate extract of *N. oculata* and to the methanolic extract of *G. gracilis* (41.45 ± 1.42 and 29.39 ± 2.01 mg GAE/g extract) (Ebrahimzadeh et al., 2018). Compared to other microalgae, *Coelastrella* sp. BGV appeared as a potentially rich source of natural antioxidants.

The ethanol extract obtained from the biomass of *Coelastrella* sp. BGV exhibited high antioxidant, antitumor, antibacterial and antifungal activities and the determination of its chemical composition and the identification of the compounds responsible for its biological activity are suitable tasks for future research.

The results of the research conducted and described in the dissertation on the possibilities of *Coelastrella* sp. BGV to grow rapidly, to accumulate biomass, to produce substances with antitumor, antibacterial, antifungal and antioxidant action and their comparison with literature data from similar studies of other, mainly green microalgae show

that this Bulgarian strain has a high potential for biomass and products for future practical application.

The results obtained support the idea that the study of newly isolated microalgae, as well as the expansion of research on those showing potential such as *Coelastrella* sp. BGV will help to make fuller use of this rich bioresource to obtain effective and beneficial to health and practice products.

V. CONCLUSIONS

1. From the studied four strains of green microalgae of the genus *Coelastrella* - *Coelastrella* sp. BGV, *Coelastrella multistriata* var. *corcontica* (CCALA 308), *Coelastrella multistriata* var. *multistriata* (CCALA 309) and *Coelastrella vacuolata* (CCALA 356), the Bulgarian strain *Coelastrella* sp. BGV showed the highest growth potential in laboratory conditions.

2. The qualitative composition of the biomass of *Coelastrella* sp. BGV in exponential and stationary growth phase is quantitatively balanced, which together with high productivity makes this strain promising for biotechnological use.

3. Eleven fatty acids have been identified in the lipid extract of *Coelastrella* sp. BGV, of which oleic (C18:1), linoleic (C18:2) and palmitic (C16:0) were in the largest amount. The ratio of unsaturated to saturated fatty acids was 3:1, and that of monounsaturated to polyunsaturated fatty acids was 1:1.

4. The exopolysaccharide from *Coelastrella* sp. BGV is low molecular weight and contains predominantly neutral sugars. It is composed of seven monosaccharides, two of which have been identified - galactose and fucose

5. Extracellular secretions and components of the biomass of *Coelastrella* sp. BGV showed high biological activity in *in vitro* experiments.

6. The test products obtained from *Coelastrella* sp. BGV, showed varying degrees of antitumor/antiproliferative activity against HeLa human tumor cells (cervical adenocarcinoma) as detected by the MTT assay.

6.1. The highest statistically significant antitumor effect was found for the fatty acids (FAs), followed by the ethanol extract (EE), unsaponifiable substances (US) and exopolysaccharides (EPSH) at both time intervals sutudied and the effect of FAs (at 24 hours and 48 hours) and US (at 48 hours) was comparable to that of the classic cytostatic Doxorubicin.

6.2. The extracellular secretions in culture medium (CM), low temperature (LT) and high temperature (HT) aqueous extracts induced a statistically significant inhibition of the viability of HeLa tumor cells at both time intervals. The effect of the LT, HT and oil extract (OE) was stronger at the 24^{th} hour, while the effect CM was 2-3 times more pronounced at the 48^{th} hour.

7. Treatment of HeLa tumor cells with extracts and metabolites of *Coelastrella* sp. BGV induced varying degrees of morphological changes in the cell and nucleus, characteristic of early and/or late apoptosis, established by the application of fluorescent test methods (double staining with AO and EtBr and staining with DAPI).

8. The results of the fluorescent test methods are in accordance with the data obtained from the MTT test and showed that the products isolated from the Bulgarian strain *Coelastrella* sp. BGVs exert antitumor activity against HeLa tumor cells by induction of apoptosis.

9. Encouraging results have been obtained regarding the therapeutic potential of extracts and metabolites of *Coelastrella* sp. BGV (ethanol extract, fatty acids,

exopolysaccharide and culture medium) against a wide range of Gram-negative and Gram-positive bacteria, as well as the fungal pathogen *Candida albicans*. The highest activity was found for the FAs and EE.

10. The ethanol extract from the biomass of *Coelastrella* sp. BGV has a high content of total phenols, flavonoids and a high level of total antioxidant activity.

11. The obtained results for antitumor, antibacterial and antifungal activities of the extracts and metabolites of *Coelastrella* sp. BGV show perspectives for future application of this Bulgarian strain in practice.

VI. CONTRIBUTIONS

1. The biochemical characteristics of the Bulgarian strain of green microalgae *Coelastrella* sp. BGV were enriched and expanded. The chemical composition of the biomass in two phases of growth - exponential and stationary was cheracterized. An exopolysaccharide was isolated from *Coelastrella* sp. BGV for the first time.

2. The fatty acid profile of the oil extract and the chemical composition of the EPSH from *Coelastrella* sp. BGV were cheracterized.

3. Original data on biological activity of extracts and metabolites of *Coelastrella* sp. BGV was obtained.

4. Antitumor activity of extracts and metabolites of *Coelastrella* sp. BGV against HeLa human tumor cells was established for the first time in *in vitro* experiments.

5. High antitumor activity of fatty acids, unsaponifiable substances, ethanol extract and exopolysaccharides from *Coelastrella* sp. BGV was found. The activity of fatty acids and unsaponifiable substances against HeLa tumor cells was comparable to that of the antitumor antibiotic Doxorubicin, widely used in clinical practice.

6. Antibacterial activity of fatty acids and ethanol extract of a strain of *Coelastrella* sp. BGV, against Gram-negative pathogens (*E. coli* ATCC, *E. coli* UPEC, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*) was found for the first time.

7. It has been found that *Coelastrella* sp. BGV is a rich source of natural antioxidants.

8. The Bulgarian strain of the green microalga *Coelastrella* sp. BGV is a potential source of biomass and products derived from it, for application in the development of new natural drugs for the treatment of tumors and bacterial infections in experimental and clinical conditions.

VII. PUBLICATIONS

VII.1. Published in scientific journals, referenced and indexed in a world-famous database of scientific information

1. Toshkova-Yotova, T., A. Georgieva, P. Pilarski, R. Toshkova. Aqueous extracts of green microalga *Coelastrella* sp. BGV display antiproliferative and proapoptotic activity in vitro against HeLa tumor cells. *Compt. Rend. Acad.Bulg. Sci. 2019,* **in press,** ISSN (print) 1310-1331; ISSN (online) 2367-5535; Impact Factor IF- 0.321/2018, SJR- 0.21/2018; Q2

2. Toshkova-Yotova, T., S. Alexandrov, P. Pilarski, L. Yocheva, D. Petrova, G. Chaneva. Screening of antimicrobial and antioxidant properties of green microalga *Coelastrella* sp. BGV. *Oxidation Communications*, 2020, 43, No 2, 265-279, ISSN 02094541; SJR- 0.21/2018; Q3

VII.2. Participation in national and international forums

1. **Toshkova-Yotova T.**, A. Georgieva, P. Pilarski, R. Toshkova. Antiproliferative and proapoptotic activity of water extracts obtained from green microalga *Coelastrella* sp. BGV against HeLa tumor cells**.** Proceedings of the Thirteenth workshop with international electronic participation: Biological activity of metals, synthetic compounds and natural products, 19-21 November, 2018, ISSN: 2367-5683. (report)

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