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Freshwater algae *Cladophora glomerata* and *Vaucheria* sp. from Serbia as sources of bioactive compounds: chemical analysis and biological activities

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Abstract: We examined potential biological activities of two taxa of freshwater algae, *Cladophora glomerata* and *Vaucheria* sp., from Serbia. The total phenolic and pigment contents, antioxidant potential, antimicrobial, antibiofilm activities, and cytotoxicity of the ethanol and acetone extracts were evaluated. The extracts were also subjected to Fourier transform infrared spectroscopy (FTIR) analysis. The levels of total phenolic compounds, chlorophylls *a* and *b*, and carotenoids varied based on both the algal taxa and the type of extracts. FTIR analysis showed the presence of lipids, unsaturated fatty acids, protein, carbohydrates, and phenols in the algal extracts. The extracts had moderate DPPH radical scavenging activity and lower reducing power compared with ascorbic acid. The antimicrobial activity expressed as minimum inhibitory concentrations ranged from 0.31 mg/mL to 10 mg/mL. The strains of *Staphylococcus aureus* and *Bacillus cereus* isolated from food samples, as well as *S. aureus* ATCC 25923, were the most sensitive. For the first time, the antibiofilm activity test revealed 98.7% inhibition of *S. aureus* biofilm formation. The extracts exhibited cytotoxic effects on choriocarcinoma JAR cells but without selectivity on normal fetal lung fibroblast MRC-5 cells. This is the first report on the biological activities of freshwater macroalgae from Serbia.

Keywords: freshwater algae; algal extracts; antioxidant activity; antimicrobial activity; antibiofilm activity; cytotoxicity

INTRODUCTION

Algae are a diverse group of photosynthetic organisms, representing the most prevalent primary producers [1]. Although algae are widespread in various ecological habitats, including air, soil, or extreme habitats, they usually inhabit freshwater or marine aquatic biotopes [1]. Algae synthesize and accumulate various compounds in response to abiotic and biotic stress conditions. These compounds exhibit a wide range of biological functions, including antioxidant, anti-inflammatory, cardioprotective, antibacterial, antiviral, and anticancer properties, among others [2-5]. The composition and content of these biologically active compounds are changeable and depend on many factors, including species, habitat, climatic conditions, stress factors, time of biomass collection, and the method of extraction [6,7]. Biologically active compounds can be categorized into several groups, including polysaccharides, lipids, saturated and unsaturated fatty acids, phenolic compounds, pigments (phycobilins, carotenoids, chlorophylls), peptides, and proteins, as well as vitamins and minerals [8-11].

Research on the biological activities and the detection of biologically active compounds in freshwater macroalgae have intensified in recent years. In Serbia, the biological activities of

algae are rarely investigated. The existing information concerns terrestrial filamentous strains of Cyanobacteria [12,13] and an aerophyte green algae, *Trentepohlia umbrina* [14]. Based on the database BAES *ex situ* [15], a total of 127 taxa of benthic macroalgae (*Cyanobacteria*, *Rhodophyta*, *Xanthophyceae*, *Chrysophyceae*, *Phaeophyceae*, *Chlorophyta*, *Charophyta*) were identified at 209 locations in 80 Serbian ecosystems. The most frequently occurring were species of the genus *Cladophora*, accounting for 26.31% of the investigated locations. *Cladophora glomerata* (family Cladophoraceae, Chlorophyta) is a freshwater green filamentous alga. The filaments are abundantly branched and can be quite long. The thalli grow attached to stony substrata or artificial stones in riverbeds but can also be found floating in water [16]. Globally, *Cladophora* species are the most widespread freshwater macroalgae, commonly found in eutrophic waters [16]. *Cladophora glomerata* possesses valuable nutritional characteristics, containing significant levels of carbohydrates, minerals, and proteins [10]. Moreover, due to its diverse chemical composition, including saturated and unsaturated fatty acids, sterols, terpenoids, and phenolic compounds, *C. glomerata* represents a potential raw material for pharmaceutical and cosmetic applications [17-20].

The coenocytic yellow-green alga *Vaucheria* (family Vaucheriaceae, Xanthophyceae) frequently occurs in freshwater, marine, and terrestrial habitats, forming greenish mats resembling velvety, felt-like coatings on various substrates. The genus consists of about 70 species distributed worldwide. Around 40 of them have been found in freshwater and terrestrial habitats of Europe. In Serbian rivers, *Vaucheria* was found in 11% of the 209 investigated localities [15]. It has a remarkable growth mode. Owing to cell elongation occurring in the cross-wall, large multinucleate cells are located within a tube of indefinite length, known as a siphon. The ecology and distribution of *Vaucheria* have been largely overlooked, mainly due to the reliance on sporadically occurring sexual organs for species identification in nature. [21]. One of the unique characteristics of the genus is its ability to tolerate desiccation, making it ideal for habitats with fluctuating water levels. Because of this, *Vaucheria* species are capable of enduring dry conditions and promptly resuming growth once moisture becomes available again. [22]. Little is known about the potential biological applications of *Vaucheria* species. El-Tablawy et al. [22] reported the phenolic-flavonoid content and antioxidant potential of the soil species *Vaucheria geminata*. Iqbal et al. [23] analyzed the biochemical profile of marine *Vaucheria karachiensis* and evaluated its nutritional, antioxidant, antimicrobial, and hypoglycemic potentials. As far as freshwater *Vaucheria* species go, there has been little investigation of their biological activities. There is a growing interest in novel and rapidly renewable sources of biologically active compounds. In this regard, freshwater macroalgae could be the best candidates for a wide range of compounds with biological properties. Thus, the present study aimed to perform chemical analysis and evaluate the biological activities of extracts of the freshwater macroalgae, *Cladophora glomerata*, and *Vaucheria* sp., collected from Serbia, with a focus on biomolecules of potential therapeutic interest.

MATERIALS AND METHODS

Collection and preparation of algal material

C. glomerata thalli were collected from the Drina River (N 44° 08' 15", E 19° 28' 54"), and those of *Vaucheria* sp. from the Ibar River (N 42° 93' 31", E 20° 42' 15") (central and western region of Serbia) in July-September 2019, when the algal biomass was at its annual maximum. The algae samples were collected manually from rocky substrata. The thalli were put into a plastic container with water from the same habitat at a ratio of 3:1 and transported to the laboratory, where they were repeatedly rinsed with fresh water to eliminate impurities. The samples were dried at 40°C under ventilation in an oven, and dry material was blended using a commercial blender.

Preparation of the extracts

Dry, ground algal material was extracted by maceration. Ten g of dry material was extracted with ethanol or acetone in a 1:30 dry material:solvent ratio. The extraction was repeated with fresh amounts of solvents three times (every 24 h), with both ethanol and acetone. After extraction, the filtrates were concentrated to dryness using a rotary evaporator (IKA, Germany) at 40°C, and the dry extracts were stored at -20°C until use. The percentage yield of extracts was calculated using the following equation (1):

$$\text{Percentage yield} = \frac{\text{weight (g) of crude extract}}{\text{weight (g) of starting dried algal material}} \times 100 \quad (1)$$

Chemical analysis

Fourier transform infrared spectroscopy (FTIR)

FTIR spectroscopy was used to identify the chemical bond types (functional groups) of the compounds extracted based on the peak values in the infrared radiation region. The wavelength of the absorbed light is indicative of the attraction, observable across the entire spectrum. Small amounts of dry powders of the extracts were individually encapsulated in a KBr pellet to prepare a translucent sample disk preparation. The samples were examined using an FTIR spectrometer (Perkin Elmer spectrum 2, IR, Waltham, USA) with a scanning range from 450 to 4000 cm⁻¹ and a resolution of 4 cm⁻¹ [24].

Determination of chlorophyll *a*, *b*, and carotenoid contents

The levels of chlorophylls and carotenoids were evaluated spectrophotometrically as previously described [25]. The absorbances of the samples were measured at wavelengths of 470 nm, 645 nm, 649 nm, 662 nm, and 665 nm using a Jenway 6405 spectrophotometer. The concentrations of chlorophyll *a*, *b*, and carotenoids were measured as µg/mL of the extract solution according to the following equations:

For ethanol extracts

$$\text{Chl } a \text{ (}\mu\text{g/mL)} = 13.36_{A665} - 5.19_{A649}$$

$$\text{Chl } b \text{ (}\mu\text{g/mL)} = 27.43_{A649} - 8.12_{A665}$$

$$C \text{ (}\mu\text{g/mL)} = 1000_{A470} - 2.13 \text{ Chl } a - 97.64 \text{ Chl } b / 209$$

For acetone extracts

$$\text{Chl } a \text{ (}\mu\text{g/mL)} = 11.24_{A662} - 2.04_{A645}$$

$$\text{Chl } b \text{ (}\mu\text{g/mL)} = 20.13_{A645} - 4.19_{A662}$$

$$C \text{ (}\mu\text{g/mL)} = 1000_{A470} - 1.90 \text{ Chl } a - 63.14 \text{ Chl } b / 214,$$

where Chl *a* is the concentration of chlorophyll *a*, Chl *b* is the concentration of chlorophyll *b*, and C is the concentration of carotenoids. All measurements were performed in triplicate, and results are expressed as the mean ± standard deviation.

Determination of total phenolic content

The total phenolic content (TPC) was quantified according to the Folin-Ciocalteu method, as previously described [26]. Briefly, a ten-fold diluted Folin-Ciocalteu reagent (1.5 mL, Sigma Aldrich, St. Louis, USA) was added in 0.2 mL of tested extract (1 mg/mL, in methanol) followed by 1.5 mL of NaHCO₃ (6%, v/w). The absorbance of samples was measured after 90 min of incubation at 725 nm. The TPC was expressed as mg gallic acid (Sigma Aldrich, St. Louis, USA) equivalents (GAE) per g of extract (mg GAE/g of extract), using the standard curve of gallic acid ($y = 0.008x + 0.0077$, $R^2 = 0.996$). All measurements were performed in triplicate, and the results are expressed as the mean ± standard deviation.

Determination of antioxidant activity

DPPH radical scavenging capacity assay

The ability of algal extracts to scavenge DPPH free radicals was assessed using the method described previously [27]. A methanol solution of the extract (1 mL) was mixed with 3 mL of DPPH solution (40 µg/mL, in methanol, Sigma Aldrich, St. Louis, USA). Tested

concentrations of plant extracts were 0.625 mg/mL, 0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 1.0 mg/mL, and 2.0 mg/mL. The absorbance of samples was measured at 517 nm after 60 min of incubation. Ascorbic acid (Sigma Aldrich, St. Louis, USA) was used as a reference compound. The percentage of DPPH radical scavenging activity was calculated using the following equation (2):

$$\% = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100 \quad (2)$$

where $A_{control}$ is the absorbance of the DPPH solution, and A_{sample} is the absorbance of the tested extract. All measurements were performed in triplicate, and the results are expressed as the mean values \pm standard deviation.

Reducing power assay

The reducing power of the algal extracts was tested at the following concentrations: 0.625 mg/mL, 0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 1.0 mg/mL, and 2.0 mg/mL. The assay was performed according to the procedure described previously [28]. The absorbance of the reaction mixture was measured at 700 nm. Ascorbic acid was used as a reference compound. All measurements were performed in triplicate, and the results are expressed as mean values \pm standard deviation.

Determination of antimicrobial activity

Tested microorganisms and inoculum preparation

Antimicrobial activity was evaluated against 17 bacterial strains and 2 yeast strains. The strains were kept in a 20% glycerol/medium stock at 80°C. The bacterial strains were subcultured twice on Nutrient agar (Torlak, Serbia) and yeast strains on Sabouraud dextrose agar (Torlak, Serbia) before the experiments. The inoculum was prepared in sterile 0.85% physiological saline by the direct colony suspension method [29,30]. For bacterial and yeast inoculum standardization, 0.5 McFarland turbidity standard was used, which indicates the density of a bacterial suspension of $1-2 \times 10^8$ colony forming units (CFU)/mL and yeast suspension of $1-5 \times 10^6$ cells/mL. The density of the inoculum was determined using a McFarland densitometer (DEM-1, BioSan, Latvia).

Microdilution method

The antimicrobial activity of algal extracts was tested using the broth microdilution method [29,30]. In 96-well microtiter plates (Sarstedt, Germany), a series of double dilutions of the stock solutions of extracts ranging from 0.156 mg/mL to 10 mg/mL were prepared in Mueller-Hinton broth (Torlak, Serbia) for bacteria and Sabouraud dextrose broth (Torlak, Serbia) for fungi. The total volume in the wells was 100 μ L. To give a bacterial count of 5×10^5 CFU/mL and 1×10^4 cells/mL of yeast, 10 μ L of each diluted inoculum were added to the appropriate wells. The microplates were incubated at 37°C for 20 h for bacteria and 25°C for 48 h for yeast. The growth was detected using resazurin, an indicator of microbial growth. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of tested extracts that inhibited the change in color of resazurin from blue to pink. The minimum microbiocidal concentration (MMC) was defined as the lowest extract concentration at which no bacterial/yeast growth was observed after subculturing the content of the wells with no color change. Each test included growth control (broth+bacteria) and sterility control (broth+extract). Stock solutions of the extracts were obtained by dissolving them in 10% dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, USA), which served as a negative control. The antibiotic amoxicillin (Sigma-Aldrich Co., St. Louis, USA) and the antimycotic itraconazole (Sigma-Aldrich, St. Louis, USA) were used as positive controls.

Determination of antibiofilm activity

The inhibitory effect of algal extracts on bacterial biofilm formation was evaluated in 96-well tissue culture microtiter plates (Sarstedt, Germany) with a modified crystal violet staining assay

[31]. Tryptic soy broth (TSB) (Torlak, Belgrade, Serbia), supplemented with 1% glucose, was used as a nutrient broth, dispensed at 100 μ L per well. Two-fold serial dilutions of the extracts were made, and the tested concentration range was from 1.25 mg/mL to 10 mg/mL. The samples were inoculated with 10 μ L bacterial suspensions, adjusted to 0.5 McFarland turbidity, containing the previously confirmed biofilm-producing strains, *Staphylococcus aureus* ATCC 25923, *S. aureus* LM2, and *Proteus mirabilis* LM1. Finally, the microtiter plates were incubated at 37°C for 20 h. Following incubation, the contents of each well were gently pipetted out, and the wells were washed with sterile deionized water to eliminate free-floating bacteria. Subsequently, the biofilm was fixed by adding 96% ethanol. The ethanol was then removed, and the plate was air-dried. The biofilms were stained by adding 100 μ L of 0.1% aqueous solution of crystal violet (Fisher Scientific, Belgium) for 15 min. The excess stain was rinsed off with deionized water, and 100 μ L of 10% acetic acid was added to solubilize the dye from the biofilms. The absorbances of the resulting samples were measured at 550 nm using an ELISA plate reader (RT-2100C, Rayto, Shenzhen, China). Stock solutions of the extracts were obtained by dissolving them in 10% DMSO (5% DMSO and lower, final). Each experiment included a growth control (broth + bacteria), extract control (broth + extract), and broth-only control. The absorbance values of the controls (broth only and extract control) were subtracted from the absorbance values of the growth control and tested samples, respectively. The percentage of biofilm inhibition was calculated using formula (2), where $A_{control}$ is the absorbance of the growth control, and A_{sample} is the absorbance of the tested extract.

Determination of cytotoxic activity

The human choriocarcinoma cells (JAR) and normal fetal lung fibroblasts (MRC-5), obtained from the American Type Culture Collection, USA, were cultured in DMEM (Dulbecco's Modified Eagle Medium, Capricorn, Germany) under previously described laboratory conditions [32]. The experiment was conducted when the cells reached 80% confluency. The cytotoxic effects of algal extracts were determined using an MTT assay [33]. The JAR and MRC-5 cells were seeded in a 96-well plate (10^4 cells/well). After 24 h, ethanol and acetone extracts of *C. glomerata* and *Vaucheria* sp., in concentrations of 2, 20, 100, 200, 500, and 1000 μ g/mL, were used for the treatment (24 h and 72 h). Untreated cells were used as the control. All method and result-processing procedures were described previously [34]. The IC_{50} values were calculated from the dose curves using the software program CalcuSyn. Results are presented as the mean values \pm standard error of three independent experiments.

Statistical analysis

The results obtained from phytochemical and biological assays were analyzed using SPSS software (version 21, IBM SPSS Statistics, Chicago, USA). The normality of data distribution was assessed using the Shapiro-Wilk test. An independent T-test was used to analyze antibiofilm activity, and the Mann-Whitney test was used to assess antimicrobial activity and chlorophyll *b* content. One-way analysis of variance (ANOVA) was used to determine the significant difference between groups for cytotoxicity, antioxidant activity, TPC, and chlorophyll *a* and carotenoid contents. $P < 0.05$ was considered significant.

RESULTS

Chemical analysis

FTIR spectroscopy

FTIR spectroscopy was used to identify the functional groups of the components based on the peak values in the region of IR radiation. IR spectra of both algal taxa (Figs. 1 and 2) were comparable, and 13 of the most intense bands were analyzed (Table 1). The peaks were detected in the single bond area ($2500-4000\text{ cm}^{-1}$), the double bond area ($1500-2000\text{ cm}^{-1}$), and the fingerprint region ($600-1500\text{ cm}^{-1}$), whereas no peaks were detected in the triple bond region ($2000-2500\text{ cm}^{-1}$). The literature confirmed the average positions of protein (amides),

lipid, and carbohydrate absorption bands. The peak recorded around 3400 cm^{-1} indicates the stretching vibrations of the hydroxyl groups that make up carbohydrates, H-bonded alcohols, and phenols [35]. The bands detected in the 3050 to 2800 cm^{-1} region correspond to the asymmetric and symmetric stretching vibrations of (CH₃) and (CH₂) groups, respectively, present in hydrocarbons from lipids. [36]. The extracts were rich in lipids with unsaturated fatty acids, as indicated by the intensity of the bands due to the unsaturated functional group (CH=CH) around 3010 cm^{-1} [37]. In addition, both ethanol and acetone extracted neutral lipids in *Vaucheria* sp. Extracts, as evidenced by the intensity of the ester band at $1738/1741\text{ cm}^{-1}$ [37]. The polar lipids, including sugars/carbohydrates and starch, were extracted in the algal extracts, as indicated by the bands around 1000 cm^{-1} (COC, COH) and around 3400 cm^{-1} (OH) [37]. Prominent peaks ranging from 1628 to 1428 cm^{-1} indicated asymmetric and symmetric stretching vibrations related to carboxylate anions (COO⁻) [35]. The characteristic band of the amide groups of protein, amide I, was observed around 1653 cm^{-1} [38]. The region between 1250 cm^{-1} and 1000 cm^{-1} corresponded to the stretching vibrations of C-O of polysaccharides from carbohydrates and stretching vibrations of P=O of phospholipids and nucleic acids [36,37].

Extract yield, total phenolic content (TPC), total carotenoid content and chlorophyll content

The extract yield, TPC, and the pigment contents (chlorophylls *a*, *b*, and carotenoids) in the algal extracts are presented in Table 2. A higher extract yield was obtained from *C. glomerata* dry material than from *Vaucheria* sp. In terms of extraction efficiency, ethanol was shown to be a better extractant than acetone. The TPC indicated that the differences depended on algal taxa and solvent polarities (Table 2). *Vaucheria* sp. extracts contained a higher level of phenolic compounds than *C. glomerata* extracts; in the case of ethanol extracts, the difference was statistically significant ($P=0.001$). The ethanol and acetone extracts of *Vaucheria* sp. contained 20.39 and 17.69 mg GAE/g , respectively, while those of *C. glomerata* contained 11.35 and 16.37 mg GAE/g , respectively. *Vaucheria* sp. contained a significantly higher content of both chlorophyll *a* and carotenoids. *Vaucheria* sp., as a species of Xanthophyceae, does not contain chlorophyll *b*.

Antioxidant activity

The DPPH radical scavenging activity demonstrated the antioxidant effect of *C. glomerata* and *Vaucheria* sp. extracts through their hydrogen donating ability, which reduced the stable violet DPPH radical to the yellow DPPH-H. A high percentage of radical scavenging activity indicated a strong antioxidant activity in the samples. The algal extracts showed moderate and concentration-dependent antioxidant activity compared with the control. The extracts exhibited similar antioxidant activity in the range of 28.68 - 42.44% for *C. glomerata* extracts and 27.37 - 41.03% for *Vaucheria* sp. extracts ($P=0.171$) (Fig. 2). At the tested concentrations, vitamin C exhibited 83.0 - 96.7% of the activity of the reference compound. The reducing power of algal extracts is related to their electron transfer ability and may serve as a significant indicator of potential antioxidant activity. The increased absorbances of the sample indicated a rise in reducing power. As shown in Fig. 3, all the extracts demonstrated concentration-dependent reducing power. Higher activity was exhibited by *Vaucheria* sp. extracts at concentrations of 0.5 mg/mL , 1 mg/mL , and 2 mg/mL , as compared to the *C. glomerata* extracts. However, there was no statistically significant difference between *C. glomerata* and *Vaucheria* sp. ($P=0.610$). *Vaucheria* sp. extracts showed activity with absorbance values ranging from 0.04 to 0.86 . In comparison, the absorbance values of vitamin C, the reference compound, were from 1.19 to 2.96 . It can be inferred that the tested extracts showed lower reducing power compared to the control.

Antimicrobial activity

In vitro antimicrobial activity of acetone and ethanol extracts of *C. glomerata* and *Vaucheria* sp. was tested against various microorganisms, including reference strains, human pathogenic bacteria, foodborne pathogenic bacteria, and yeasts, to gauge the broad-spectrum antimicrobial efficacy. The MIC and MMC values are shown in Table 3. It was observed that 10% DMSO did not inhibit the growth of microorganisms.

The antimicrobial activity of *C. glomerata* and *Vaucheria* sp. was similar ($P=0.196$). Additionally, among the tested extracts as well as among the groups of isolates classified by origin, no statistically significant difference in activity was noted ($P>0.05$). The activity interval of the extracts was from 0.31 mg/mL to 10 mg/mL. The most sensitive were *S. aureus* and *Bacillus cereus* isolated from food samples and the *S. aureus* ATCC 25923 reference strain (with MIC/MMC 0.31/2.5 mg/mL). The *S. aureus* strains isolated from human materials, *Klebsiella pneumonia* and *P. mirabilis* strains isolated from food samples, along with the tested reference ATCC strains, were moderately sensitive to the acetone extract of *C. glomerata* (MIC/MMC 2.5/5 mg/mL). Most tested strains, especially those isolated from human material, showed sensitivity at the highest tested concentration (MIC/MMC 10 mg/mL). In addition, the tested extracts exhibited varied degrees of antifungal activity against *Candida albicans* strains. The MIC/MMC values ranged from 1.25 mg/mL to 10 mg/mL. *C. albicans* and reference ATCC strains were more sensitive than the clinical isolate of *C. albicans*.

Antibiofilm activity

The antibiofilm activity of *C. glomerata* and *Vaucheria* sp. extracts was evaluated by measuring the inhibition of biofilm formation in three biofilm-positive strains (Fig. 5). There was no statistically significant difference in antibiofilm activity between *C. glomerata* and *Vaucheria* sp. ($P=0.118$). The activity was concentration- and strain-dependent. The percentage of biofilm inhibition ranged from 0% to 98.07%. The strongest activity was observed at a concentration of 10 mg/mL (36.48-87.91% in *Vaucheria* sp. extracts and 12.92-98.07% in *C. glomerata* extracts). The most effective inhibition of biofilm formation among the tested strains was observed in the following descending order: *S. aureus*, *S. aureus* ATCC 25923, *P. mirabilis*.

Cytotoxic effect

The cytotoxic effect of *C. glomerata* and *Vaucheria* sp. extracts were evaluated on human choriocarcinoma (JAR) cells and normal fetal lung fibroblast (MRC-5) cells. The results show a significant decrease in JAR cell viability at higher concentrations (Fig. 6). However, on MRC-5 cells, results did not indicate a selective effect (Fig. 7). IC_{50} values, as a measure of cytotoxicity, show the presence of a time-dependence effect (Table 4). The most significant effect was obtained with the *C. glomerata* acetone extract, which had the lowest IC_{50} values.

DISCUSSION

Freshwater macroalgae are a significant source of bioactive compounds with diverse beneficial biological activities [2-5]. However, they are still insufficiently researched. The biological activities of species from the *Vaucheria* genus are almost unexplored, and for *C. glomerata* from Serbia, these are the first results. Studies have demonstrated that the phenolic compounds, pigments, terpenes, fatty acids, and polysaccharides of algae significantly contribute to their bioactive capacity [3,19,20,39,40]. In this study, total phenolic compound and pigment contents and FTIR analysis confirm the presence of active compounds. The FTIR analysis of *C. glomerata* and *Vaucheria* sp. extracts shows that lipids, unsaturated fatty acids, protein, carbohydrates, phenols, and P- and S-containing compounds are present. The total phenolic and total pigment contents significantly depend on solvent polarity. The results indicated that compounds with different polarities were present in the algal material and could be successfully extracted using solvents of different polarities. Ethanol and acetone proved to be good

extractants of these compounds. In the study by Korzeniowska et al. [20], different extraction techniques (ultrasound-assisted extraction, solvent extraction, microwave-assisted extraction) and different solvents were used. The authors reported that the best technique for extracting phenolic compounds from *C. glomerata* was ultrasound-assisted extraction, providing a TPC content of 0.09 ± 0.01 mg GAE/g dry weight (DW) in the ethanol extract [20]. In the study by Messyasz et al. [19], ultrasound-assisted extraction of *C. glomerata* with methanol, a solvent with similar polarity, yielded a TPC value of 21.50 ± 1.71 mg GAE/g, chlorophyll *a* 0.30 ± 0.01 mg/g, and carotenoids 0.08 ± 0.01 mg/g. The TPC was determined in 70% methanol extracts of *C. glomerata* collected from four Lithuanian rivers, yielding concentrations of 0.95, 1.10, 1.22, and 1.32 mg GAE/g [41]. In the mentioned study, chlorophyll *a* was from 0.57 to 0.74 mg/g DM. The content of carotenoids was from 0.17 to 0.23 mg/g in the study by Nauautaitė et al. [41] and 0.89 mg/g in the study by Khuantrairong and Traichaiyaporn [42]. A similar concentration of carotenoids was detected in our study. HPLC analysis of *C. glomerata* extract confirmed the presence of several phenolic compounds [20,39,41]. Phenolic acids (gallic, chlorogenic, syringic, *p*-coumaric, 3,4-dihydroxybenzoic, vanillic, 4-hydroxybenzoic, tannic acids) and flavonoids (rutin, quercetin, isoquercetin, kaempferol, myricetin, catechin) were identified [20,39,41]. Gas chromatography-mass spectrometry (GC-MS) analysis of fatty acids and volatile compounds confirmed the presence of long-chain hydrocarbons, methyl esters of complex fatty acids, and alkenes [43].

Vaucheria spp. is an even less researched alga. The genus produces active compounds and can be an interesting source of bioactive compounds. In this study, *Vaucheria* sp. extracts were found to contain a higher number of tested compounds than *C. glomerata* extracts. For comparison, the soil alga *V. geminata* possesses 13.68 ± 1.11 mg/g TPC, 0.29 ± 0.003 mg/g chlorophyll *a*, and 0.10 ± 0.000 mg/g carotenoids [22]. HPLC chromatography confirmed the presence of phenolic acids (gallic, chlorogenic, caffeic, coumaric, ferulic, and syringic acids) [22]. Conversely, in the methanol extract of *Vaucheria* sp. collected in India, phenolic compounds were not detected, while chlorophyll *a* was 3.7 mg/g and carotenoids 2.8 mg/g [44]. Therefore, the chemical composition of algal biomass, total phenolic content, and pigment content depend on numerous factors such as taxa, habitat, climatic conditions, environmental stressors, biomass collection, time, and extraction techniques.

Antioxidants are crucial in regulating oxidative stress-related diseases in humans and serve as preservatives in foods and pharmaceuticals [45,46]. Natural antioxidants are efficient inhibitors of the oxidation process without any side effects [47]. In this instance, the antioxidant activity of *C. glomerata* and *Vaucheria* sp. extracts was investigated. Antioxidant activity was assessed in terms of the radical scavenging ability and reducing power. The tested extracts manifested noticeable DPPH radical-scavenging activity in a dose-dependent manner and reached 40% activity at a concentration of 2 mg/mL. Therefore, *Vaucheria* sp. extracts could be an effective electron donor that can react with free radicals to convert them to more stable products. This is the first report on the reducing power of *Vaucheria* sp. extracts.

Other studies have confirmed the antioxidant properties of *C. glomerata*. A methanol extract of *C. glomerata* at a concentration of 10 mg/mL exhibited 65.23% of DPPH scavenging activity [19]. The ethyl acetate extract of *C. glomerata* revealed the free radical DPPH neutralization potential of 49.8% at 100 mg/mL [48]. Amornlerdpison et al. [49] showed that *C. glomerata* aqueous extract, at a concentration of 20 mg/mL, also elicited antioxidant activity in DPPH and superoxide and lipid peroxidation assays, with values of 71%, 73%, and 66%, respectively. *C. glomerata* methanol extract could reduce oxidative stress in mitochondria [50]. Moreover, Yarnpakdee et al. [39] confirmed the use of *C. glomerata* ethanol extract as an alternative natural antioxidant to prevent lipid oxidation of fish meat. The DPPH activity of *V. geminata* extract at a dose of 10 mg/g was 36.89% [22]. In comparison, the DPPH activity of *Vaucheria* sp. extracts in the present study was 30–35% at a concentration of 2.0 mg/mL. The

higher antioxidant capacity of *C. glomerata* and *Vaucheria* sp. extracts in this study may be attributed to the amounts and synergistic effects of phenolic compounds, chlorophyll *a*, and carotenoids; the presence of other antioxidant compounds could also contribute to this effect.

Regarding the antimicrobial potential of algal extracts, they have been successfully analyzed against various pathogens of great concern to human health. In general, the extracts showed moderate activity, and the activity was strain-dependent. Some food contaminants, such as *B. cereus*, *S. aureus*, and *P. mirabilis*, were successfully inhibited by *C. glomerata* and *Vaucheria* sp. extracts, suggesting their potential use as food preservatives. Literature reports describe the antibacterial activities of *C. glomerata*, while *Vaucheria* sp. extracts are unexplored. The methanol extract of *C. glomerata* was tested for antibacterial activity using the disk diffusion method [42] against Gram-positive bacteria, *S. aureus* and *B. subtilis*, and Gram-negative bacteria *Xanthomonas campestris* and *Ralstonia solanacearum* at different doses (500; 1000, and 1500 ppm). The extract showed moderate antibacterial action against *B. subtilis* and *R. solanacearum*, and weak antibacterial activity against *S. aureus* and *X. campestris*. In the study [51], the aqueous extract of *C. glomerata* exhibited no activity, whereas the hot acetone extract exhibited varying zones of growth inhibition at different concentrations (12.5-100 mg/mL), with *S. aureus* being the most sensitive. Significant antibacterial effects of *C. glomerata* extracts have been reported against human pathogens, such as the multidrug-resistant *Acinetobacter baumannii*, and against bacterial pathogens of fish [52]. The hexane extract of *C. glomerata* exhibited a different level of activity against tested bacterial and fungal strains (MIC=1 mg/mL, 10 mg/mL) [53]. The authors proposed that the fatty acids present in the extract could serve as potential antimicrobial compounds. Besides fatty acids, other compounds such as alkanes, phenols, and long-chain hydrocarbons were recognized as active compounds [20,53,54]. Moderate antibacterial activities of the methanol and ethanol extracts of *Vaucheria* sp. against *Bacillus* species and low antibacterial activities against Gram-negative *Salmonella typhimurium*, *E. coli*, and *Pseudomonas aeruginosa* were observed [44]. The results are comparable with our results.

The antimicrobial mechanisms of action attributed to compounds extracted from algae are diverse. These encompass alterations in membrane permeability due to interactions with proteins and lipids, as well as enzyme inhibition. Different compounds might be incorporated into the membrane of microbial cells, leading to a loss of selective permeability and leakage of solutes, distorting their shape, size, and constituents [2,3].

Many pathogenic bacteria can form biofilms, making them even more harmful and difficult to control. A bacterial biofilm is a three-dimensional and sessile community of cells that are irreversibly attached to the substratum and embedded in a self-producing matrix of hydrated extracellular polymeric substances (EPS) [55]. This study evaluated the antibiofilm activities of *C. glomerata* and *Vaucheria* sp. extracts. The acetone and ethanol extracts of *Vaucheria* sp. exhibited a biofilm formation inhibitory effect on bacterial strains. The most significant inhibitory effect was observed at a concentration of 10 mg/mL. As the concentration of the extract decreased, the inhibitory effect on the bacterial strains also decreased. *C. glomerata* extracts showed weaker activity. The algal extracts probably prevented the initial cell attachment and thus interrupted the formation of biofilm. While these findings represent the first results, there is considerable potential for further research, especially against *S. aureus* biofilms.

The cytotoxic impact of ethanol and acetone extracts from *C. glomerata* and *Vaucheria* sp. on choriocarcinoma JAR cells was investigated. Additionally, the effects of these treatments on normal fetal lung fibroblast MRC-5 cells were assessed. Our results suggest that the algal extracts decrease choriocarcinoma cell viability only at higher doses. However, a notable disparity was already noticeable at a concentration of 20 µg/mL, with the acetone extract of *C. glomerata* demonstrating a dose- and time-dependent impact. The acetone extract of *C.*

glomerata exhibited the most significant anticancer effect. Based on other literature data, *C. glomerata* showed a cytotoxic effect on other cell lines as well, such as the breast cancer cell line MCF-7 [56] and colon cancer cells HT29 [57]. According to Bézivin et al. [58], an extract has a favorable selective effect if the IC₅₀ value on healthy cells is at least 3-fold greater than on cancer cells. The observed effects on normal fetal lung fibroblast MRC-5 cells indicated that there was no selectivity of the examined extracts, regardless of the type of extract. Our study reports a cytotoxic effect against both cancer and healthy cells for the first time, providing novel insight into the potential activity of *Vaucheria* species. Given their weak individual cytotoxic activity, it is necessary to thoroughly investigate the mechanism of action of these algal extracts in conjunction with cytostatic agents.

CONCLUSIONS

Extracts of freshwater macroalgae *C. glomerata* and *Vaucheria* sp. from Serbia are valuable sources of biologically active compounds. Phytochemical analysis confirmed the presence of lipids, unsaturated fatty acids, proteins, carbohydrates, phenolic compounds, and pigments. The extracts exhibited diverse biological activities, including DPPH radical-scavenging and antimicrobial activities; moreover, they inhibit the growth of common foodborne bacterial contaminants and prevent *S. aureus* biofilm formation. They also exhibit a cytotoxic effect on choriocarcinoma JAR cells without selective cytotoxicity toward normal fetal lung fibroblast MRC-5 cells. These extracts can be further explored for use in the cosmetic, pharmaceutical, and food industries.

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Data availability: Data underlying the reported findings have been provided as a raw dataset, which is available here:

https://www.serbiosoc.org.rs/NewUploads/Uploads/Stefanovic%20et%20al_Raw%20Dataset.xlsx

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Table 1. FTIR detected functional groups of compounds in *Cladophora glomerata* and *Vaucheria* sp. extracts.

No	<i>C. glomerata</i>		<i>Vaucheria</i> sp.		Functional group/assignment
	Ethanol extract	Acetone extract	Ethanol extract	Acetone extract	
	wavenumber (cm ⁻¹)				
1	3433.21	3432.68	3432.13	3433.95	H-bonded, OH stretching vibration
2	3011.19	3010.25	3006.94	3009.01	C-H stretching
3	2924.49	2925.45	2925.88	2926.43	Asymmetric stretching vibrations of CH ₂
4	2853.67	2854.34	2854.54	2854.95	Symmetrical stretching vibrations of CH ₂
5	/	/	1738.23	1741.40	Ester bond
6	1713.61	1715.40	1714.54	1712.04	Stretching vibrations of C=O, C=C
7	1631.43	1632.08	1631.40	1623.55	Stretching vibrations of C=O, C=C, vibrations of NH
8	1463.65	1462.95	1463.00	1463.26	Stretching vibrations of C=O, C=C, vibrations of NH
9	1383.98	1382.65	1378.30	1378.64	Symmetric bending vibrations of CH ₃
10	/	1269.12	1245.67	1242.89	Stretching vibration of carbonyl C-O, P=O stretching
11	1173.33	1172.16	1169.59	1166.41	Stretching vibration of carbonyl C-O
12	1056.45	1102.39	1080.32	1116.85	Stretching vibrations of C-C; C-O-P stretching vibrations; S-O stretching vibrations
13	722.57	730.83	722.70	722.87	Aromatic ring

Table 2. Extract yield, total phenolic content (TPC), and pigment contents in tested algal extracts.

Parameters	<i>C. glomerata</i>		<i>Vaucheria</i> sp.	
	Ethanol extract	Acetone extract	Ethanol extract	Acetone extract
Extract yield (%)	3.40	3.10	2.30	2.0
TPC (mg GAE/g)	11.35 ± 0.28 ^{aA}	16.37 ± 0.39 ^{bC}	20.39 ± 0.75 ^{cB}	17.60 ± 0.35 ^{dC}
Chlorophyll <i>a</i> (µg/ml)	7.18 ± 0.01 ^{aA}	10.53 ± 0.02 ^{bC}	13.25 ± 0.07 ^{cB}	12.82 ± 0.04 ^{dD}
Chlorophyll <i>b</i> (µg/ml)	3.77 ± 0.01 ^a	1.71 ± 0.03 ^b	NC	NC
Carotenoids (µg/ml)	ND	0.80 ± 0.01 ^A	1.60 ± 0.02 ^a	2.0 ± 0.1 ^{bB}

ND – not detected; NC – not contained. Different small letters as superscripts mean a statistically significant difference between extracts of the same algal species ($P < 0.05$); different uppercases as superscripts mean a statistically significant difference between extracts of different algal species ($P < 0.05$).

Table 3. Antimicrobial activity (MIC and MMC values) of tested algal extracts and positive controls.

Bacteria	<i>C. glomerata</i>				<i>Vaucheria</i> sp.				Amoxicillin/ Itraconazole ($\mu\text{g/mL}$)	
	Ethanol extract (mg/mL)		Acetone extract (mg/mL)		Ethanol extract (mg/mL)		Acetone extract (mg/mL)		MIC	MMC
	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC		
Referent strains										
<i>Escherichia coli</i> ATCC 25922	10	10	5	10	10	10	10	10	6.37	25.5
<i>Klebsiella pneumoniae</i> ATCC 70063	10	10	5	10	10	10	10	10	> 25.5	> 25.5
<i>Pseudomonas aeruginosa</i> ATCC 10145	5	10	5	5	10	10	10	10	> 25.5	> 25.5
<i>Staphylococcus aureus</i> ATCC 25923	0.62	0.62	0.31	0.31	2.5	2.5	1.25	1.25	< 0.40	1.60
Isolates from human material										
<i>E. coli</i> LM1	> 10	> 10	> 10	> 10	> 10	> 10	> 10	> 10	6.25	12.5
<i>K. pneumoniae</i>	10	10	5	5	10	10	10	10	> 25.5	> 25.5
<i>P. mirabilis</i> LM1	> 10	> 10	10	10	> 10	> 10	> 10	> 10	> 25.5	> 25.5
<i>Proteus</i> sp. LM2	> 10	> 10	10	10	> 10	> 10	> 10	> 10	3.12	6.25
<i>P. aeruginosa</i>	10	> 10	10	10	> 10	> 10	> 10	> 10	> 25.5	> 25.5
<i>S. aureus</i> LM1	10	> 10	5	5	>10	>10	10	10	6.25	6.25
<i>S. aureus</i> LM2	10	10	5	5	10	10	10	10	< 0.40	< 0.40
Isolates from food samples										
<i>E. coli</i> LM2	10	10	10	10	10	10	10	10	> 25.5	> 25.5
<i>K. oxytoca</i>	10	10	> 10	> 10	> 10	> 10	> 10	> 10	6.25	25.5
<i>Salmonella enterica</i>	10	10	10	10	10	> 10	10	10	1.60	3.10
<i>P. mirabilis</i> LM3	5	5	2.5	5	10	10	10	10	> 25.5	> 25.5
<i>S. aureus</i> LM3	1.25	1.25	1.25	1.25	2.5	2.5	0.62	1.25	25.5	> 25.5
<i>Bacillus cereus</i>	1.25	2.5	0.62	0.62	0.62	0.62	0.31	1.25	25.5	> 25.5
Yeast										
<i>Candida albicans</i> ATCC 10231	5	10	1.25	2.5	2.5	2.5	2.5	2.5	1.95	1.95
<i>C. albicans</i>	10	> 10	5	10	10	> 10	10	> 10	1.95	1.95

MIC – minimum inhibitory concentration; MMC – minimum microbiocidal concentration

Table 4. Cytotoxic effect (IC₅₀ values, µg/mL) of tested algal extracts on JAR choriocarcinoma cells and MRC-5 fetal lung fibroblasts.

Extract	JAR		MRC-5	
	24 h	72 h	24 h	72 h
<i>C. glomerata</i> ethanol extract	661.41±4.39	303.62±1.18	582.75±1.02	441.38±1.02
<i>C. glomerata</i> acetone extract	499.19±1.55	191.95±4.82	314.51±1.20	417.09±0.98
<i>Vaucheria</i> sp. ethanol extract	640.30±2.66	532.36±2.1	587.93±3.50	321.51±1.14
<i>Vaucheria</i> sp. acetone extract	571.03±4.22	410.76±3.46	555.33±2.13	718.38±3.41

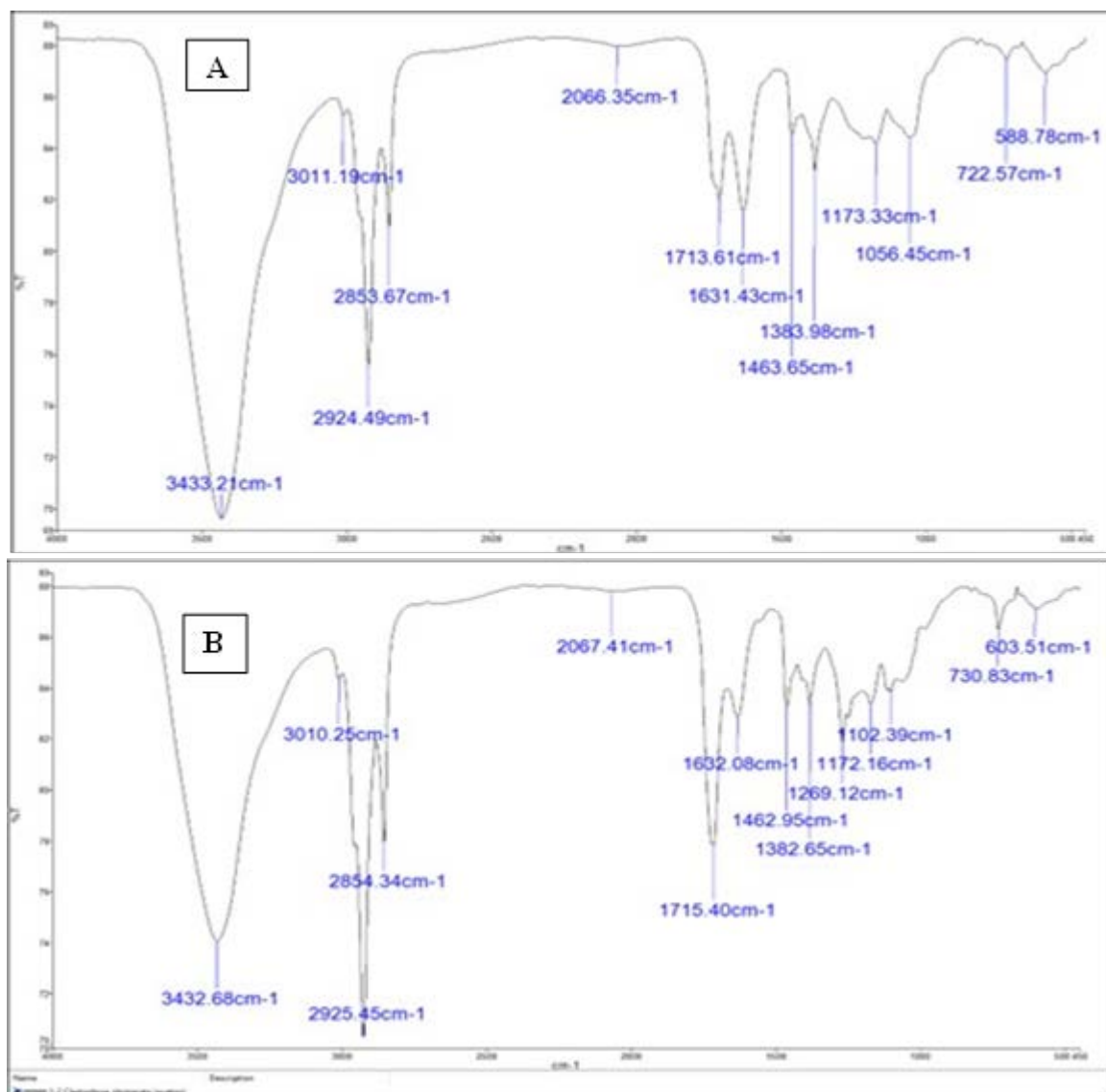


Fig. 1. FTIR spectra of *Cladophora glomerata* ethanol extract (A) and acetone extract (B).

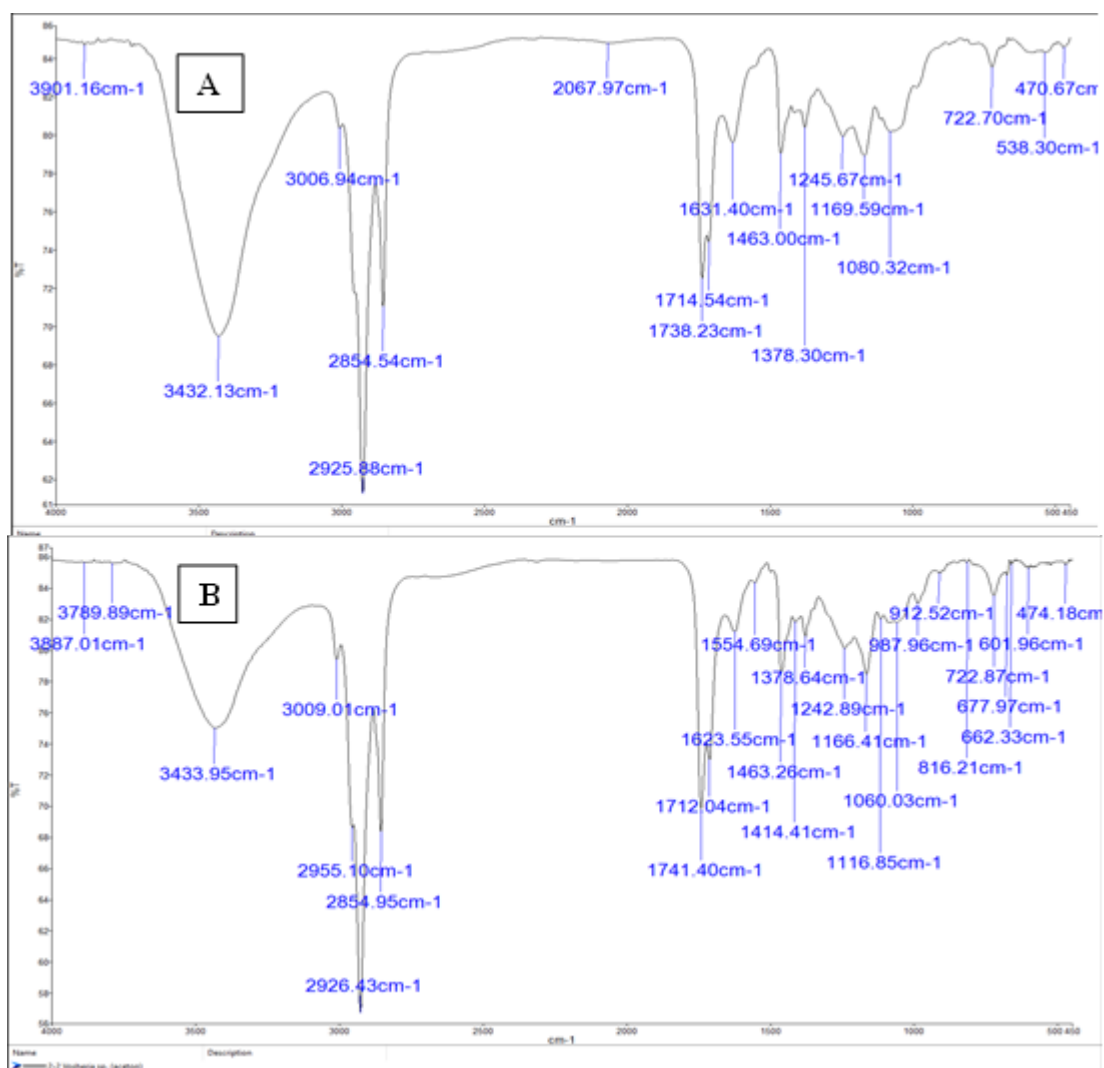


Fig. 2. FTIR spectra of *Vaucheria* sp. ethanol (A) and acetone extracts (B).

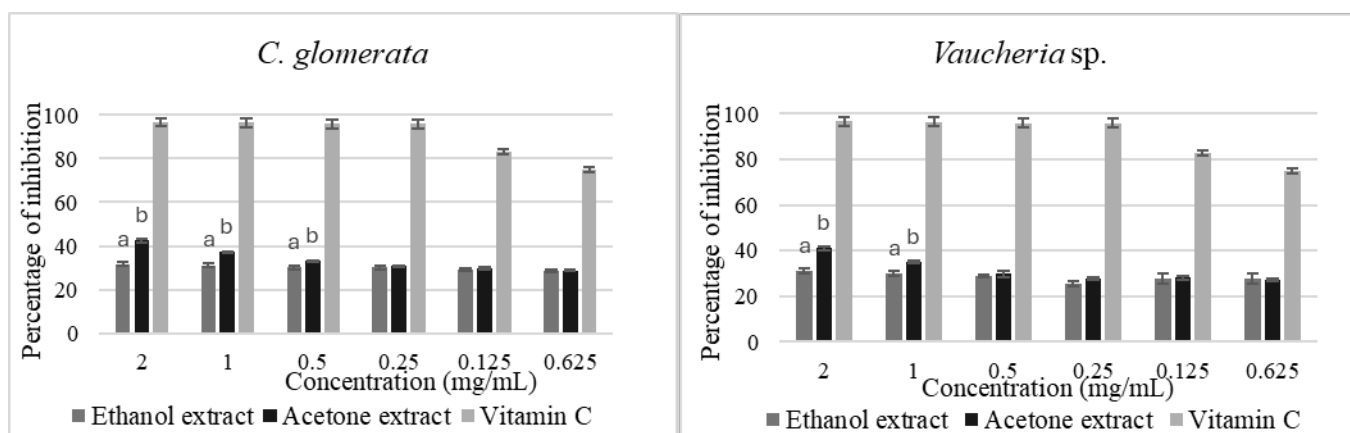


Fig. 3. DPPH radical scavenging activity of tested algal extracts and positive control. ^{a,b} – Statistically significant difference ($P < 0.05$).

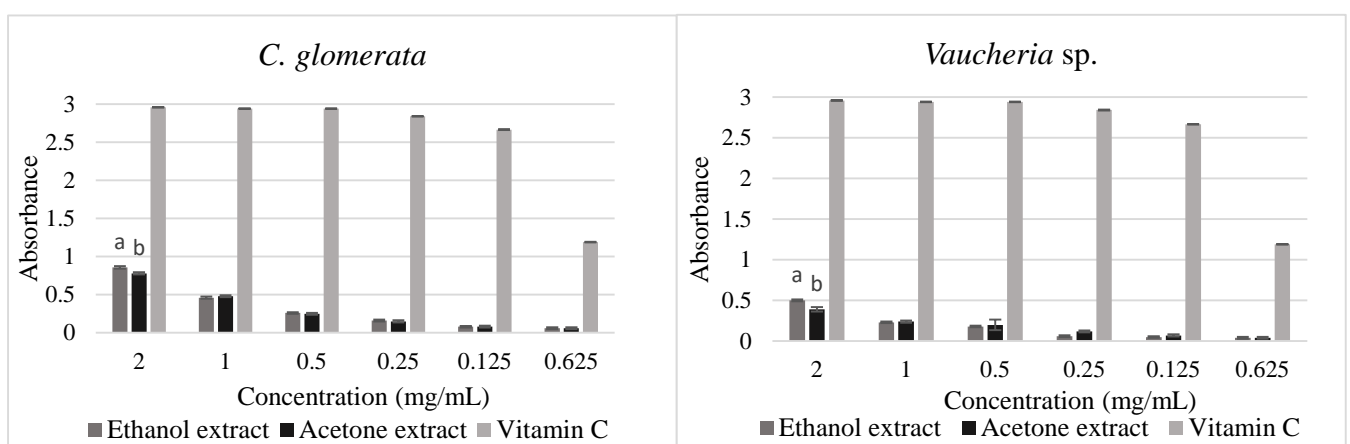


Fig. 4. Reducing power of tested algal extracts and positive control. ^{a,b} – Statistically significant difference ($P < 0.05$).

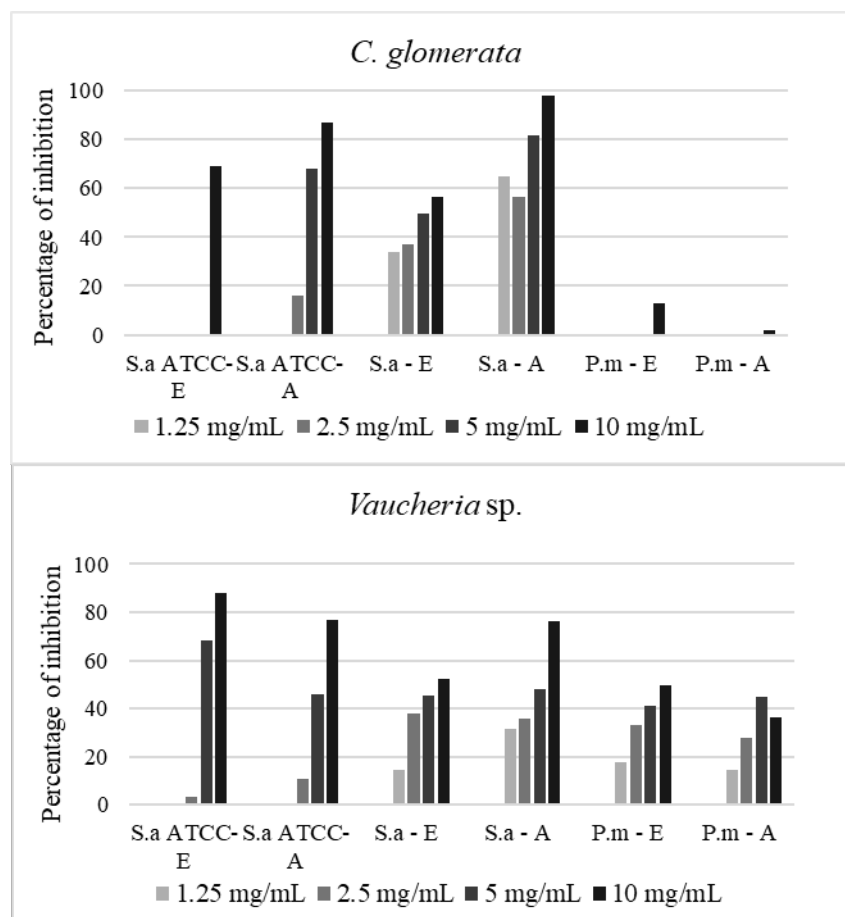


Fig. 5. Antibiofilm activity of tested algal extracts. S.a ATCC – *Staphylococcus aureus* ATCC 25923; S.a. – *Staphylococcus aureus* LM2; P.m. – *Proteus mirabilis* LM1; E – ethanol extract; A – acetone extract.

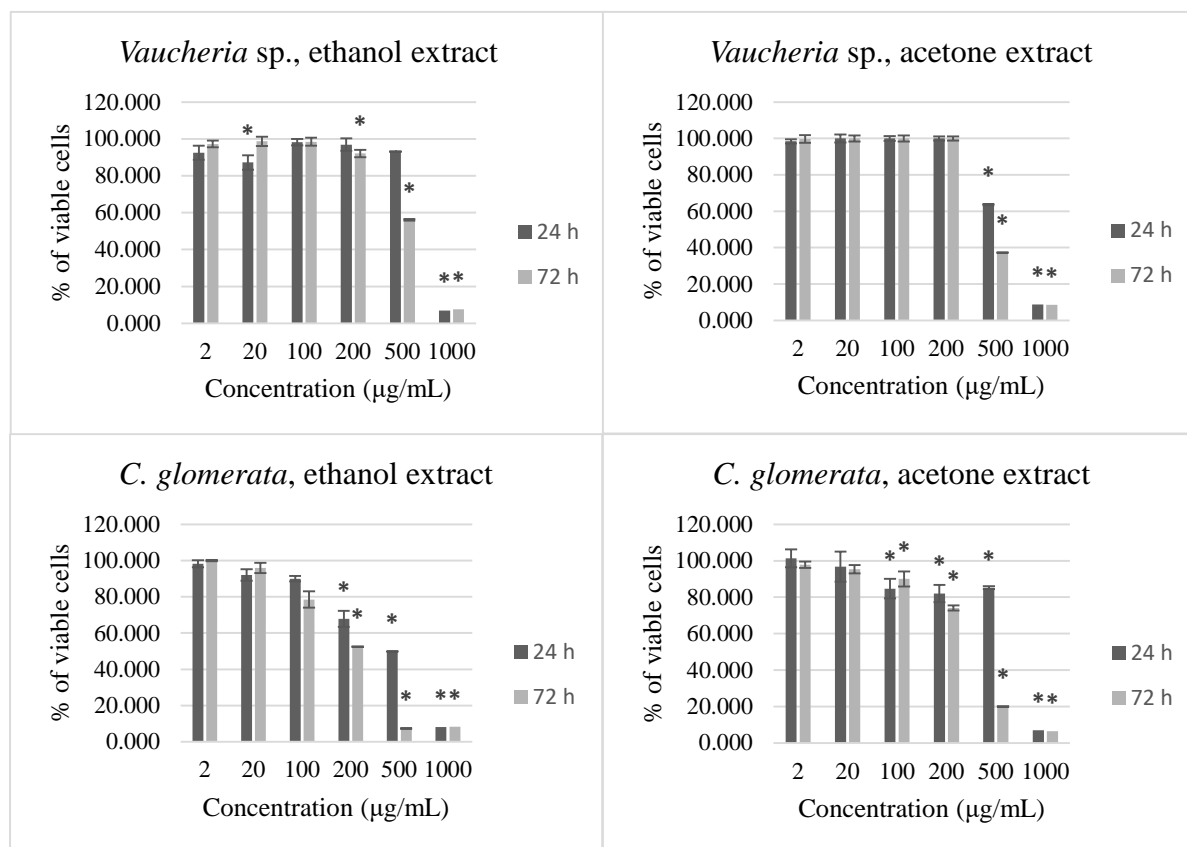


Fig. 6. Cytotoxic effect of *Cladophora glomerata* and *Vaucheria sp.* extracts on JAR choriocarcinoma cells 24 h and 72 h after treatment. * – Statistically significant difference ($P < 0.05$) relative to the control (100%).

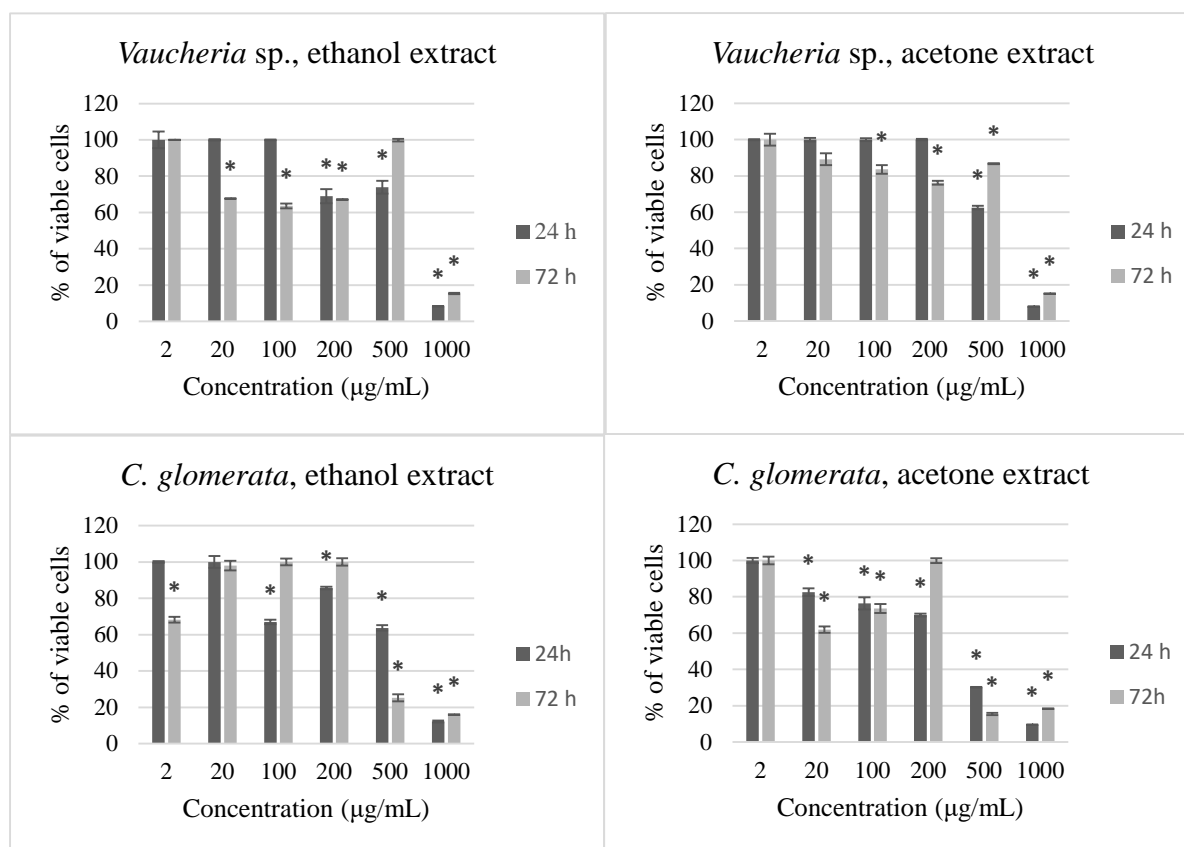


Fig. 7. Cytotoxic effect of *Cladophora glomerata* and *Vaucheria sp.* extracts on MRC-5 fetal lung fibroblasts 24 and 72 h after treatment. * – Statistically significant difference ($P < 0.05$) relative to the control (100%).