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Ecological benefits and antioxidant activity of the green macroalga *Cladophora glomerata*

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This study evaluated the ecological and industrial potential of *Cladophora glomerata* from the Aytoska River. The species was analyzed for bioactive compounds, including phenolic acids and flavonoids, and for antioxidant activity using five complementary methods. Additionally, the adsorption properties of *C. glomerata* were assessed for potential use in bioremediation of water pollutants. The content of phenolic acids and flavonoids in extracts obtained with different solvents did not exceed 2.8 mg GAE/g and 2.2 mg RE/g, respectively. These findings highlight the dual potential of *C. glomerata* as a natural source of antioxidants and as a sustainable material for water purification applications. Low antioxidant activity was determined by DPPH, ABTS, H_2O_2, FRAP and CUPRAC analysis. *C. glomerata* demonstrates potential for the removal of nitrates and nitrites from water.

Key words: Aquatic ecosystem, *Cladophora glomerata*, total phenolic content, total flavonoid content, antioxidant activity

INTRODUCTION

Macroalgae have attracted the attention of many researchers as a source of compounds with different biological activities, including both complex organic compounds and primary and secondary metabolites. These include lipids, peptides, enzymes, carbohydrates, tannins, phenolic compounds, terpenoids, polyunsaturated fatty acids (PUFAs), phytopigments, and others. Thus, algae are a viable and economical biomass source of valuable compounds with potential applications in nutraceutical, pharmaceutical, chemical, food and cosmetic industries due to their biologically active and regenerative properties [1-4]. In general, macroalgae are classified as red (*Rhodophyta*), brown (*Phaeophyta*) and green (*Chlorophyta*) species according to their chemical composition and mainly due to the presence of specific pigments (phycobilins, fucoxanthin and chlorophyll, respectively) [5]. The *Chlorophyta* make up the majority of green algae and together with the *Streptophyta* belong to the *Viridiplantae* [6]. They are the most diverse group of algae worldwide today in terms of number of species (at least 7000), organization of the plant body (unicellular to multicellular) and habitat (from the snow surface to various symbiotic relationships) [7]. *Chlorophyta* can be effective phyco-mediators. Their presence in water can reduce the contents of dyes [8], heavy metals [9, 10], and nutrients nitrogen and phosphorus from different types of waste water [11-13] with the possibility of using the residual biomass as biofuel [14, 15], as biofertilizer by recovering nutrients from wastewater and converting them to biochar [16], and producing bioplastics and additives as plasticizers to enhance the quality of the final product [17]. The genus *Cladophora* belongs to *Chlorophyta* and has different forms and many species that are widely distributed in brackish and freshwater environments. It adapts well to a wide range of environmental conditions, such as different temperature, salinity and nutrient concentrations [18]. The most common in freshwater ecosystems are the species of the genus *Cladophora* Kützing [19]. This genus is rich in phytochemical compounds that can be used to maintain the health of both humans and animals. Due to the variety of secondary metabolites, species of this group possess antioxidant, antidiabetic, antihypertensive, antiparasitic, antimicrobial, anticancer, and cytotoxic properties [20]. *Cladophora glomerata* is a representative of the green filamentous algae belonging to the family *Chlorophyceae*, and is one of the most common algae, both in freshwater and marine environments [21]. The identification of filamentous algae is often a complex task. Several taxonomic characters are used in the genus *Cladophora:* thallus color, types of branching, cell design and size, general structure of the plant, basal cells, and shape of zoospores [22]. Populations of *C. glomerata* occurring in water bodies in Bulgaria

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are described as forming dense green cotton-like tufts or long "tails" up to 50 cm long. The thallus is attached to stones, concrete, or wood by a disc-shaped holdfast of short-branched filaments. The filaments are single-row, cylindrical with cells most often 50-90 µm wide and up to four times their width long. The lateral branches are formed just below the apical cell at an angle of about 40-70 °. The branches are thinner than the main axis and often intertwine, forming a dense mat. Each cell contains a large reticulate chloroplast with several clearly distinguishable pyrenoids. The cells are multinucleated and have a relatively thick, sometimes layered cell wall. Reproduction is most often asexual by tetraflagellate zoospores, and sexual stages are rare. The mass development of the algae takes place in spring and early summer in nutrient-rich rivers and reservoirs. During the summer, the tuffs break up into fragments overwinter attached to the substrate [23]. The species is reported to be a rich source of saturated and unsaturated fatty acids which have antibacterial activity [24]. A study evaluating the biochemical profile of freshwater *C. glomerata* reported that the algae contain high levels of quercetin, rutin, and lutein, along with tyrosinase inhibition corresponding to an SPF of approximately 12, suggesting that the algae are a viable natural UV-protector and antioxidant for the production of creams and lotions in skin care [25]. With improved extraction methods, *C. glomerata* proves to be an important source of phenols, tocopherols, carotenoids and chlorophyll pigments, which makes it suitable for use as a functional food and dermal antioxidant [26]. In a study evaluating the biosorption properties of *C. glomerata*, the algae achieved 57% Pb removal, 49% Cd, 39% chemical oxygen demand (COD), and 31% total nitrogen removal from industrial wastewater [27]. Another study on a pilot wetland dominated by *C. glomerata* found 81% elimination of total nitrogen, with the species reported to have a preference for NH_4^+ [28].

The aim of this article is to evaluate the benefits of *C. glomerata* investigating the content of bioactive compounds such as phenolic acids and flavonoids with potential applications in the food, cosmetic and pharmaceutical industries, and to evaluate the antioxidant activity of the algae. Additionally, preliminary experiments were conducted to test *C. glomerata* as a biosorbent for the removal of nitrogen compounds (nitrate, nitrite and ammonium) in polluted waters.

MATERIALS AND METHODS

Sampling of waters and C. glomerata

Water sampling was carried out from 5 points: 1 - Aytoska River - estuary N 42.5086 E 27.3372; 2 - Aytoska River - after Kameno N 42.5712 E 27.3088; 3-Burgas Lake-west N 42.4931 E 27.3442,4-Burgas Lake - center N 42.5105 E 27.3676; 5- Chakarliyska River - estuary N 42.4933 E 27.3439. Algae samples were taken from May to October 2024, from the mouth of the Aytoska river (point 1), just before the flow into the Burgas lake - the largest natural lake in Bulgaria (Figure 1).

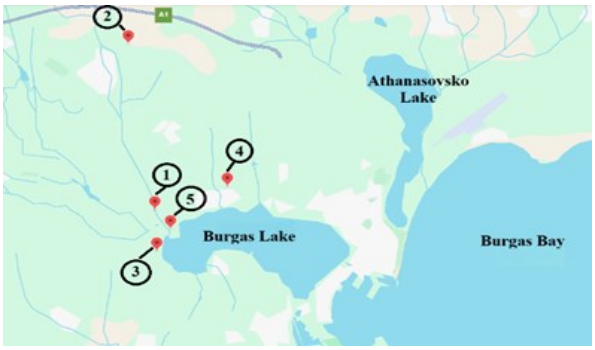

Figure 1. Location of monitoring points.

Sample preparation

Fresh *C. glomerata* biomass was collected from the sampling site, thoroughly washed with tap water to remove sand particles, epiphytes, shells, and other debris, and left on filter paper to reduce excess surface water. For further experiments, the algal biomass was dried under sunlight for 5 days (ambient temperature 25–30 °C, relative humidity 50–60%) or in an oven at 40–45 °C in order to preserve thermolabile compounds [29].

For the experiments on the antioxidant activity of macrophytes, the plant material was ground in a grinder to obtain a uniform powder, which was sieved through a 2-mm sieve [20, 21, 30, 31]. A portion of the dried algae was chopped and separated into particles with an average size of 1-5 mm, for the adsorption experiments [32].

Reagents and instruments

All chemicals were of analytical grade quality and were purchased from Honeywell and Sigma Aldrich (USA). The main physicochemical parameters (pH, dissolved oxygen, temperature, salinity, total dissolved solids (TDS), resistivity, and conductivity were measured with a WTW Multi 3630 IDS portable multimeter. The indicators N-NO_2 - nitrite nitrogen, NO_2 - nitrite, were measured with ready-to-use Hach® LCK cuvette tests with DR

3900 spectrophotometer (Hach, Loveland, CO, USA).

Assays for total phenolic content (TPC), total flavonoid content (TFC), antioxidant activity (by DPPH, ABTS, HPSA, FRAP, CUPRAC assay) were performed with Spectroquant Pharo 300, UV/Vis (Merck, USA).

Determination of the biosorption capacity, total phenolic content, total flavonoid content and antioxidant activity

- *Biosorption capacity.* Water samples collected from five monitoring points were pre-filtered using 0.45 µm syringe filters to remove suspended solids. A series of batch adsorption experiments was then prepared, consisting of 125 mL aliquots of filtered water and 0.25 mg of the adsorbent material. The adsorbent (*C. glomerata* biomass) was placed in permeable mesh bags (dimensions: 4.5×6 cm; mesh thickness: 20–40 µm) to allow free exchange with the water phase. The bags were immersed in the prepared water samples, and the optimal contact time was set at 120 min based on previously reported studies [33, 34].
- *Extraction method.* To determine the total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity, the same extraction was performed. Dry leaves and stems of *C. glomerata* were powdered and extracted with 60% methanol, 40% ethanol and distilled water using a mechanical shaker. The extracts were filtered and used for further analyses.
- *Total phenolic content (TPC) measured by the Folin-Ciocalteu (FC) assay.* The TPC of the extracts was assessed according to the method described by Kirkova *et al.* [35] using the FC assay: 0.1 mL of plant extracts (60% methanolic, 40% ethanolic or aqueous extract), 6 mL of water and 0.5 mL of 0.2 M FC reagent were placed into the test tube. After 4 min 3.4 mL of 7.5% Na_2CO_3 was added. Samples and blank were stored in the dark for 2 h and then were measured at 765 nm against the blank sample using a spectrophotometer (Spectroquant Pharo 300, Merck, USA). The concentration of the phenolic compounds in the extracts was calculated using gallic acid as standard, and the results were expressed as milligrams gallic acid equivalents per gram of extract (mg GAE/g) [35].
- *Determination of total flavonoid content (TFC).* The TFC of extracts was determined as follows: 0.8 mL of the extract was mixed with 1.6 mL of 1% $AlCl_3$ and 1.2 ml of 1 M CH_3COOK. Samples and blank were stored in the dark for 2.5 h after which the absorbance was measured at 440 nm against the blank sample on a spectrophotometer (Spectroquant Pharo 300, Merck, USA) [36].
- *Determination of antioxidant activity. DPPH assay.* The 1,1-diphenyl-2-picryl-hydrazyl (DPPH) scavenging activity of the 60% methanolic and 40 ethanolic extracts was measured following the method described by Kirkova *et al.* [35]. This method assesses the reaction of the antioxidants with stable DPPH free radicals. The absorbance was measured at 515 nm against a methanol blank using a spectrophotometer (Spectroquant Pharo 300, Merck, USA) [35].
- *ABTS assay.* The 2,2′- azino-bis (3-ethylbenzothiazoline-6-sulfonate) radical cation ($ABTS^{\bullet+}$) scavenging activity of the extracts was evaluated according to the original method of Re *et al*. [37] with slight modifications from our previously study [35]. The $ABTS^{\bullet+}$ was generated by reacting ABTS (7 mM) in H_2O and $K_2S_2O_8$ (2.45 mM) at room temperature (25 °C) in the dark for 14-16 h. The absorbance at 734 nm was measured against a methanol blank using a spectrophotometer (Spectroquant Pharo 300, Merck, USA) [35].
- *H_2O_2 assay.* Hydrogen peroxide scavenging activity (HPSA) of the extracts was measured using 0.2 M phosphate buffer (PB, pH = 7.4) and H_2O_2 (2 mM dissolved in PB). A mixture of 0.1 mL plant extract, 0.6 mL of H_2O_2 and 3.3 mL of PB was incubated in dark for 10 min. The absorbance was measured at 230 nm using a spectrophotometer Spectroquant Pharo 300 (Merck, USA) [35].
- *FRAP assay.* The ferric reducing antioxidant power (FRAP) of the extracts was determined using the method of Benzie [19], with modifications as described by Docheva *et al.* [38]. This method is based on the reduction of Fe^{3+} to Fe^{2+}. Absorbance was measured at 593 nm using the same spectrophotometer.
- *CUPRAC assay*. The cupric ion reducing antioxidant capacity (CUPRAC) was assessed according to Apak *et al.* [39], with slight modifications [35]. The reaction mixture consists of 1 mL of $CuCl_2$ solution (10 mM), 1 mL of neocuproine ethanolic solution (7.5 mM) and 1 mL of CH_3COONH_4 buffer solution (1 M, pH=7). Then 0.1 mL of extract and 1 mL of deionized H_2O were added to a final volume of 4.1 mL. The absorbance was measured at 450 nm after 30-min incubation in dark using the same spectrophotometer [35].

For all antioxidant assays Trolox was used as a standard and results were expressed as millimoles of Trolox equivalents per gram of extract (mM TE/g).

Statistical analysis

All experimental measurements were carried out in triplicate. Data represent mean ± standard deviation (SD) of three independent experiments. Statistical analysis was conducted using Microsoft Excel 2020. The percentage removal of nitrate (N-NO_3, NO_3), ammonium (N-NH_4, NH_4) and nitrite (N-NO_2, NO_2) from water samples was calculated in agreement with the formula given in [40].

RESULTS AND DISCUSSION

In this study *C. glomerata* showed potential for water purification, as a reduction of N-NO_3 from 4.54 mg/L to 3.3 mg/L, and NO_3 from 20.1 mg/L to 14.6 mg/L was observed in samples from sampling point 2. The percent removal was 27.31% for N-NO_3, and 27.36% for NO_3. The results of water samples from sampling points 3, 4, and 5 showed reduction of N-NO_2 and removal of 16.22%, 66.67%, and 73.81%, respectively.

A reduction in NO_2 was observed in samples from sampling points 4 and 5 of 67.44% and 73.19%, respectively, and results are presented in Figure 2.

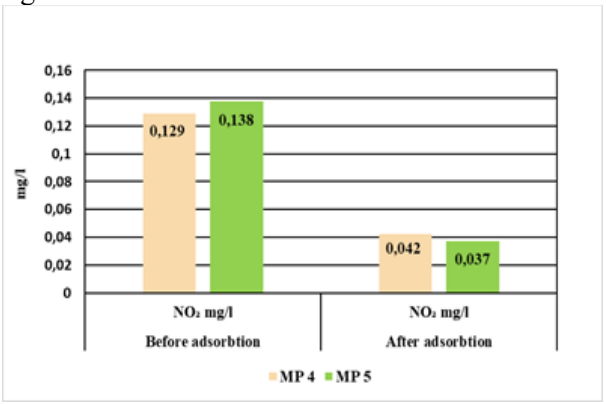

Figure 2. NO_2 before and after adsorption

Only in samples from sampling point 4 a decrease of N-NH_4 and NH_4 was observed and the percent removal was 58.23% and 58.03%, respectively. Basic physicochemical parameters were measured before and after adsorption in all samples from the 5 sampling points. The results showed pH values varying from 8.09 to 8.54 before adsorption and from 7.9 to 8.9 after adsorption. This suggests that the parameter does not change drastically and remains in the alkaline zone. pH is an important parameter in adsorption processes as it creates a suitable environment for retaining certain ions on the surface of the biosorbent [41]. For example, Cu^{2+} and Pb^{2+} are adsorbed significantly better by *C. glomerata* at pH 4-5 (and below) due to the increased availability of negatively charged functional groups [42]. For dissolved oxygen (O_2 mg/L), the initial concentration was between 8.44 mg/L to 8.63 mg/L, and after adsorption it was 8.02 – 8.26 mg/L. A slight decrease in dissolved oxygen values may indicate possible metabolic activity in the processes of oxidation, reduction and active ion transport, which may further assist the purification process [43]. The temperature conditions remained almost unchanged: before adsorption - an average of 22.7 °C, and after adsorption 22.4 °C. This might suggest a stable process without need for parameter adjustment. Temperatures in the order of 25 °C are used as a standard for optimal conditions that increase the adsorption capacity of algae [44].

Total phenolic and total flavonoid content

Numerous factors determine the chemical composition of algal biomass and the level of biologically active compounds, including taxa, habitat, climate conditions, environmental stressors, biomass collection time, and techniques. In general, algae are a well-known natural raw material that contains phenolic compounds. One of the most significant and most discussed features of phenolic and flavonoid compounds is their antioxidant activity. Its primary function is to bind, stabilize and inactivate free radicals [45]. The total phenolic content (TPC) of various *C. glomerata* extracts is presented in Table 1.

Table 1. Total phenolic and total flavonoid content of *C. glomerata* extracts

Extract Solvent	Total phenolic content, (mg GAE/g) *	Total flavonoid content, (mg RE/g) **
60% Methanol	0.9 ± 0.04	0.6 ± 0.03
40% Ethanol	2.8 ± 0.07	0.5 ± 0.02
Distilled water	1.2 ± 0.05	2.2 ± 0.03

* GAE: Gallic acid equivalent ** RE: Rutin equivalent

The highest TPC content was measured in the extract obtained with 40% ethanol (2.8±0.07 mg GAE/g). The content of phenolic acids detected in the aqueous extracts was approximately two times lower (1.2±0.05 mg GAE/g), while in the 60% methanolic extracts three times lower (0.9±0.04 mg GAE/g) compared to the 40% ethanolic extract. The relatively low TPC in *C. glomerata* may be attributed to the presence of only three phenolic acids – gallic acid, *p*-hydroxybenzoic acid, and *p*-coumaric acid. In previous research it was found that the variation in phenolic acids content among different algal biomasses is relatively low [44]. Our results are consistent with those reported by

Nutautaitė *et al.* [45] who investigated the TPC in *C. glomerata* using acidified aqueous-methanol extraction and reported values ranging from 0.22 mg GAE/g DM to 1.32 mg GAE/g DM. In a study conducted by Ruiz-Medina *et al.* [1] *C. glomerata* samples collected from the Canary Islands and extracted with 80% methanol yielded a significantly higher TPC: 5.90±0.83 mg GAE/g DM. These values are notably higher than those observed in our study, in which samples collected from the Aytoska River were used. The highest flavonoid content in our study was obtained using distilled water (2.2±0.03 mg RE/g). No significant differences were found between the extracts obtained with 60% methanol (0.6±0.03 mg RE/g) and 40% ethanol (0.5±0.02 mg RE/g). The results are summarized in Table 1. Ruiz-Medina *et al.* [1] reported a TFC of 3.04±0.24 mg CAE/g DM in *C. glomerata* extracts from the Canary Islands, obtained by extraction with 80% methanol. This value is similar to that found in the aqueous extracts of *C. glomerata* collected from the Aytoska River.

J. Fabrowska *et al.* suggested that low or decreasing levels of phenolic acids or flavonoids may be due to aging algal populations [46].

Antioxidant activity

Algae are a natural material with a diverse and complicated chemical composition. The methods for assessing antioxidant activity have proven that it is not only due to the existence of phenolic compounds, but also to the presence of additional antioxidants [45]. In this regard the antioxidant activity of *C. glomerata* was evaluated using five different methods: DPPH, ABTS, HPSA, FRAP, and CUPRAC. Higher values indicate stronger antioxidant activity of the extracts (Figure 3).

The antioxidant activity of *C. glomerata* determined in this study is in line with previous reports, although some variations are observed depending on the extraction method and drying conditions. For instance, Lithuanian samples showed relatively low DPPH radical scavenging activity (8.22–11.09%), while other authors reported much higher values, up to 65.2% for methanolic extracts from Thailand [47].

The highest antioxidant activity of *C. glomerata* was reported by the HPSA method - 38.4 mM TE/g in the aqueous extracts, 30.5 mM TE/g - in 60% methanol extracts, and 27.9 mM TE/g - in 40% ethanol extracts.

The antioxidant activity reported by the CUPRAC method was weaker than by the HPSA method, varying from 22.4 mM TE/g (40% ethanolic extracts) to 14.9 mM TE/g (60% methanolic extracts). The extracts from *C. glomerata* exhibited the lowest activity, determined by the ABTS method, with no difference observed between the extracts (from 5.5 mM TE/g in 40% ethanol extracts to 5.0 mM TE/g in 60% methanol extracts).

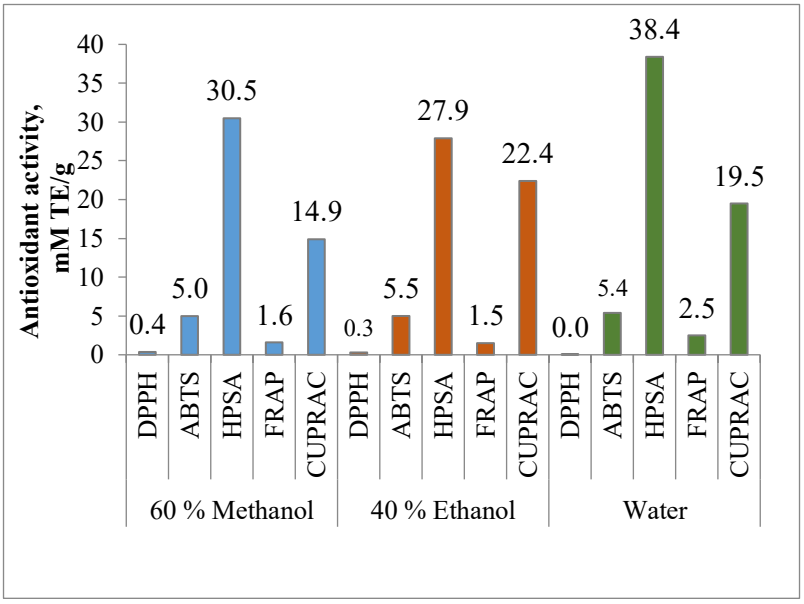

Figure 3. Antioxidant activity of 60% methanolic, 40% ethanolic and aqueous extracts

Our data are in accordance with the studies of Sáez-González *et al.* [48] who investigated *C. glomerata* extracts obtained with different solvents and found that the antioxidant activity of the extracts, determined by ABTS, varies on average about 30 mg T/g for aqueous and ethanolic extracts

(equivalent to 5.2 mM TE/g). Our studies indicate a higher activity of the extracts, determined by the FRAP method, and a lower activity by the DPPH method compared to the studies of Sáez-González *et al.* [48].

CONCLUSION

This study is among the first ones to simultaneously evaluate the bioremediation capacity and antioxidant profile of *C. glomerata*, highlighting its dual functionality. While previous studies have focused primarily on its ecological role or on its bioactive compounds, the present work demonstrates its integrated potential both as a sustainable biosorbent for water quality improvement and as a valuable natural source of phenolic and flavonoid antioxidants. Among the tested samples, the aqueous extracts showed the highest activity measured by five different methods (HPSA, CUPRAC, ABTS, FRAP, and DPPH), which can be attributed to their higher content of water-soluble flavonoids. This combined perspective provides a new framework for the future use of *C. glomerata* in circular bioeconomy strategies, bridging environmental and industrial applications.

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