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Chemical Characterization of Selected Algae and Cyanobacteria from Bulgaria as Sources of Compounds with Antioxidant Activity

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Abstract: The current research focused on algae from the waters of the Black Sea—*Chaetomorpha linum*, *Ulva intestinalis*, *Ericaria crinita*, and bioreactors—*Chlorella* spp. and *Arthrospira platensis/cyanobacterium/*. Pigment content, total phenolic content, and antioxidant capacity were investigated for their use as pharmaceutical, food, and cosmetic ingredients. *E. crinita* exhibited the highest antioxidant activity by ORAC and HORAC (463.3 µmol TE/g and 463.3 µmol GAE/g) and the highest total content of polyphenols and rutin of the investigated algae. Lower protein content was found in saltwater algae than in freshwater algae. For the first time, biologically active substances from the Bulgarian *A. platensis* and *Chlorella* spp., produced in a bioreactor, have been quantitatively identified. *A. platensis* contained rutin (141.25 mg/100 g), naringenin (42.17 mg/100 g), quercetin (26.74 mg/100 g), kaempferol, and quercetin-3-β-glycoside. Phenolic acids were isolated: neochlorogenic (172.27 mg/100 g) for lyophilized and 5783 mg/100 g for convection-dried *A. platensis*. It has been found that the protein content in *A. platensis* (convection dried) and *Chlorella* spp. (lyophilized) was nearly 54% higher than that of green algae *U. intestinalis* from Varna. The lyophilization process reduced the protein content of *A. platensis* samples by almost 20%. The high protein content of convection-dried *A. platensis* (43.4%) and lyophilized *Chlorella* spp. (43.7%) identified them as suitable emulsifiers in colloidal and emulsion systems.

Keywords: *Chaetomorpha linum*; *Ulva intestinalis*; *Ericaria crinita*; *Arthrospira platensis*; *Chlorella* spp.; antioxidant activity; flavonoids; phenolic acids; protein; chlorophyll

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1. Introduction

In recent years, algae have attracted the attention of researchers as a natural source of antioxidants. Biomass of cyanobacteria and seaweed has been employed as food since the Middle Ages [1,2]. Around 420 companies in Europe produce algae and cyanobacterial biomass for use as ingredients in ready-to-eat foods, as well as fresh and fermented products. They can be added to biscuits, pasta, bread, and drinks [3]. Algae are a source of extremely valuable natural bioactive compounds with the potential to provide new nutrients for the human body. They can produce three main organic pigments: chlorophylls, carotenoids or phycobilins [4]. Macroalgae that are rich in chlorophyll *a* or *b* appear green, while algae appear greenish-brown due to a combination of different types of pigments.

Marine algae contain various inorganic and organic compounds, which have a beneficial effect on human health. Seaweed extracts exhibit various pharmacological effects,

among which antioxidant, antidiabetic, anti-acetylcholinesterase, antimicrobial [5], anticancer [6], antitumor [7], anti-inflammatory [8], photoprotective [9], and antiviral [10] properties. The main groups of antioxidants in macroalgae, along with specific examples and potential algal sources for application, are presented in Tables 1 and 2.

Table 1. Antioxidant components and health benefits for compounds, derived from marine algae.

Antioxidant	Health Benefits	References
β-carotene, lutein	Protection against breast cancer	[11]
Bromophenol Carrageenan Oligosaccharides Fucoidan	Inhibition of α-glucosidase Antitumor effect Anti-HIV-effect Improves hyperoxaluria Protection against neurodegenerative disorder	[12] [13] [14] [15] [16]
Fucoflorethols	Chemopreventive effect	[17]
Fucoxanthin	Antiangiogenic effect Protective effect against retinol deficiency	[18] [19]
Galactan sulfate	Antiviral effect	[20]
Phenolic functional groups and mycosporine (as amino acids)	Anticancer effect	[21]
Phlorotannins	Anti-inflammatory and bactericidal effect Inhibition of H_2O_2—mediator of DNA damage Photochemopreventive effect	[21] [22] [22]
Phycoerythrin	Improvement of diabetic complications	[23]
Polyphenols	Vascular chemoprotection Antimicrobial effect Inhibition of α-glucosidase	[24]
Porphyran, shinorine	Anti-aging effect	[25]

Table 2. Main groups antioxidants in macroalgae.

Components	Algal Source	References
β-carotene	*Chondrus crispus* *Mastocarpus stellatus*	[26]
Fucoxanthin	Brown algae	[27]
Antheraxanthin Lutein Violaxanthin Xanthophylls Zeaxanthin	Red algae	[28]
Stypodiol Isoepitaondiol Taondiol	*Taonia atomaria*	[29]
Terpenoids	*Ericaria crinita*	[30]
Phycoerythrin Phycocyanin	Red algae	[31] [32] [33] [34]
Catechin Epicatechin gallate	*Halimeda* spp.	[35]

Table 2. *Cont.*

Components	Algal Source	References
Flavonoids Phlorotannins	*Palmaria palmata* *Sargassum pallidum* *Fucus vesiculos*	[20] [36] [37]
Ascorbate	*Chondrus crispus* *Mastocarpus stellatus* *Sargassum* spp.	[25] [37]
Vitamin A	*Kappaphycus alvarezii*	[6]
Fucoidan Alginic acid Laminaran	*Turbinaria conoides*	[38]
Fucoidan Sulfated galactans (Lambda-carrageenan)	*Saccharina japonica* (formerly *Laminaria japonica*) Some red seaweeds	[16] [39,40]
Galactans Sulfated glycosaminoglycan	Most of the red algae *Sargassum wightii*	[41] [42]
Porphyran	*Porphyra* spp.	[43]

Marine algae (red, brown, and green) are an excellent source of sulfated polysaccharides with an anticoagulant activity of non-mammalian origin. Some of the structural similarities between algal polysaccharides and heparin have prompted groups of scientists to explore their antithrombotic [44], antiadhesive [39,45], antiparasitic [46], and other properties.

Scientific interest is directed not only at the antioxidant effect of marine algae, but also at the antioxidant activity of freshwater algae cultivated in bioreactors such as *Arthrospira/Spirulina* (Cyanobacteria). According to various studies, *Arthrospira/Spirulina* (Table 3) has the potential to improve heart health by influencing the blood lipid composition, blood pressure [47,48], and cholesterol profile, by lowering LDL (bad) cholesterol and total triglycerides and increasing good (HDL) cholesterol [49,50].

Chlorella spp. has high levels of essential fatty acids, antioxidants, and vitamins, and this makes it a superior choice for applications in culinary technology and food industry. Merchant and Andre summarize the data from several clinical trials regarding the effect of *Auxenochlorella pyrenoidosa* (formerly *Chlorella pyrenoidosa*) in the treatment of fibromyalgia, hypertension, and ulcerative colitis [51].

Green algae (*U. intestinalis, C. linum*) are widespread in the Bulgarian Black Sea region, relatively easy to harvest, and have a rich chemical composition. On the other hand, the brown alga (*E. crinita*) is more difficult to obtain (their habitat is the rocky seabed close to the seashore at a depth of 2 to 3 m). Freshwater algae (*Arthrospira platensis, Chlorella* spp.) grown in a bioreactor are characterized by high yield, controlled chemical composition, ecological purity, and safety. The current research aims to compare the chemical composition of bioreactor freshwater algae with the green and brown algae described above. Knowledge of this composition is a prerequisite for their differentiated application. Parameters such as antioxidant activity, total phenolic content, protein content, rutin, chlorophyll, flavonoids, and phenolic acids are essential for evaluating the quality of raw materials in the production of dietary supplements, using them in foods, etc.

Table 3. Effects of the use of *Arthrospira/Spirulina* against various diseases.

Disease, Associated with	Model	Treatment	Results	References
Obesity	Rats	1000 mg/kg/day for 30 days	Improvement of the measured parameters	[52]
	Human	2.8 g of *Arthrospira/Spirulina* thrice a day over a period of 4 weeks	Statistically significant reduction of body weight in obese outpatients	[53]
High cholesterol	Human	*Arthrospira/Spirulina* 2 g daily for 2 months	Reduces total cholesterol and triglycerides, free fatty acid levels	[54]
Cerebral ischemia injury	Rats	*Arthrospira/Spirulina* at a dose of 180 mg/kg once a day, for 7 days.	Improvement of neurological deficit score, reduction of oxidative stress biomarkers	[55]
Diabetes	Human	2 g water soluble fraction of *Arthrospira/Spirulina* for 21 days	Reduce blood glucose	[56]
Oral leukoplakia	Human	1 g/day for 1 year	Complete regression of lesions in 45% of the intervention group	[57]

There is no in-depth research on the Bulgarian Black Sea region's rutin, flavonoids, and phenolic acid content of *U. intestinalis*, *C. linum*, and *E. crinita*. There is scarce data available on the antioxidant activity and total phenolic content of such algae of the Turkish Black Sea region [58], as well as on the protein, chlorophyll *a* and *b* [59], rutin, and flavonoids [60] of algae from the Romanian Black Sea region. Research has been carried out on Bulgarian Black Sea region algae regarding the content of α—tocopherols and fatty acid composition [61,62], as well as heavy metal content [63] and mineral content [64].

The present study aimed to fill these gaps by providing the necessary data on some macroalgae from the Bulgarian Black Sea and the microalgae *A. platensis* (Cyanobacteria) and *Chlorella* spp. (*Chlorophyta*) from bioreactors.

2. Materials and Methods

2.1. Samples for Research

Three biological species of seaweed were collected, namely (*E. crinita*, *U. intestinalis*, *C. linum*). Freshwater algae (*A. platensis* and *Chlorella* spp.), cultivated in a bioreactor in Bulgaria (near Varvara), were also studied.

The seaweed were extracted from a depth between 1 and 3 m in June 2021. Taxonomic analysis of the samples was performed at the Institute of Oceanology—[BAS]—Varna (Table 4).

Table 4. Taxonomy of investigated marine macroalgae.

	U. intestinalis	***C. linum***	***E. crinita* (Formerly *Cystoseira crinita*)**
Phylum	Chlorophyta	Chlorophyta	Phaeophyta
Class	Ulvophyceae	Cladophorophyceae	Phaeophyceae
Order	Ulvales	Cladophorales	Fucales
Family	Ulvaceae	Cladophoraceae	Cystoseiraceae
Genus	*Ulva*	*Chaetomorpha*	*E. crinita*
Locality	Asparuhovo, Varna	Asparuhovo, Varna	Pomorie
Geographical Coordinates	43.173645, 27.916596	43.189680, 27.884336	42.560546, 27.633244
Number of samples	3	3	3

2.1.1. Conditions for Production of Biomass into a Bioreactor

The investigated freshwater samples were grown in a bioreactor in Varvara, Bulgaria, and purchased from a producer for the investigation. Photos from the bioreactor are presented in Figure 1a,b.

(**a**) (**b**)

Figure 1. Bioreactor in Varvara, Bulgaria (**a**) condions into the reactor; (**b**) production of biomass.

The steps for the production of biomass were the following:

- Creating sowing;
- Changing the habitat of the samples from the laboratory into the production conditions;
- Growing the samples into a large volume;

2.1.2. Creating Sowing

Sowing was created in a facility for laboratory accumulation of biomass: glass baths with a volume of up to 50 L, illumination from 8000–10,000 lx. $T_{/water/}$ = 33 °C ÷ 35 °C. 100 L of gas mixture (air and carbon dioxide up to 1% per liter) were passed through 1 L of algae suspension in an 1 h.

2.1.3. Changing the Habitat of the Samples from the Laboratory into the Production Conditions

For production cultivation, it was necessary to gradually increase the volumes by transferring the crops to pools of different sizes. In this way, after 25–30 days, a suspension of algae with the required density was obtained.

2.1.4. Growing the Samples into a Large Volume

The aqueous suspension was stirred by means of water wheel blades. Carbon dioxide was added after each fin turn in the direction of flow. Evaporated water during cultivation was compensated automatically. At night, light was administered both from above and below.

2.2. Methods

2.2.1. Methods for Conservation, Storage, Preparation, Analysis, and Observation of Algal Samples

Freezing and Refrigerator Storage

The samples were stored in a refrigerator at −18 °C and packed in plastic bags.

Convective Drying

A. platensis were dried in a thin layer with transversely oriented airflow towards the product layer at 45 ± 2 °C and relative humidity of the circulating air, on average, 10%. Reaching the sample's constant mass indicates the end of the drying process. The cooled pieces of dried algae were packed in paper bags and stored at 20 °C without light.

Lyophilization

The lyophilization of algae was performed at a freezing temperature of −28 to −30 °C and a drying temperature of 32 °C using a laboratory lyophilizer BK-FD10S, Biobase, Chandong, China. The layer thickness of *A. platensis* and *Chlorella* spp. was 8–9 mm.

Microscopic Studies

Photographs of the Black Sea algae samples were taken with a Levenhuk MED D30T digital microscope (PRC, Levenhuk, Inc. (Tampa, FL, USA)). The magnification of the microscope was of the order of 20×.

2.2.2. Methods for Evaluating the Antioxidant Activity, Total, and Individual Polyphenolic Compounds

Samples Preparation

A sample of 1 g of lyophilized algae was extracted twice with 80% acetone (Merck KGaA, Darmstadt, Germany) containing 0.5% of formic acid (Merck KGaA, Darmstadt, Germany) [65] in solid to the solvent ratio (1:10 *w*/*v*) for 60 min with a constant stirring on a magnetic stirrer Ika RCT Basic (IKA®-Werke GmbH & Co. KG, Staufen (Staufen im Breisgau) Germany). The mixture was centrifuged for 20 min at 6000× *g* by centrifuge Nahita, model 2640/12 (Nahita, Beriain, Spain). The supernatants were collected and used for subsequent analysis of the antioxidant activity and total and individual polyphenolic compounds content.

Oxygen Radical Absorbance Capacity (ORAC) Method

An ORAC method developed by Ou et al. [66] was used in this study with slight modifications. The capacity of particular antioxidants to neutralize peroxyl radicals was measured. AAPH (2,2′-azo-bis (2-amidinopropane) dihydrochloride) (Sigma-Aldrich Chemie GmbH, Darmstadt, Germany) was used as a free radical generator. A sample of 10 µL of extract and 170 µL of fluorescein (70 nmol/L) were tempered for 20 min at 37 °C in the apparatus. Twenty microliters of AAPH (51.5 mM final concentration) was added to the reaction mixture. The reaction volume was 200 µL, and all solutions were prepared in phosphate buffer (75 mM, pH 7.4). The mixture was shaken, and the fluorescence was evaluated every minute until a zero value was reached. To express the antioxidant activity, the results obtained for standard solutions of Trolox (6.25, 12.5, 25, 50, and 100 µM, Sigma-Aldrich Chemie GmbH, Darmstadt, Germany) were used, based on which a standard curve was generated. Antioxidant concentration was directly proportional to the area under the decaying fluorescence curve, and the obtained data were presented in µmol Trolox equivalents. The measurements were performed on a Fluostar OPTIMA fluorometer (BMG LABTECH, Offenburg, Germany). The excitation wavelength was 485 nm, and the emission wavelength was 520 nm.

Hydroxyl Radical Averting Capacity (HORAC) Method

The HORAC method developed by Ou et al. [67], was used to measure the complexing ability of an antioxidant under Fenton reaction conditions caused by an interaction between Co (II) and H_2O_2. The Co (II) solution was prepared in the following way: 15.7 mg $CoF_2 \times 4H_2O$ and 20 mg picolinic acid (Sigma-Aldrich Chemie GmbH, Darmstadt, Germany) were dissolved in distilled water. A sample of 10 µL of extract and 170 µL of fluorescein (final concentration 60 nM) prepared in phosphate buffer (75 mM, pH = 7.4) (Merck KGaA, Darmstadt, Germany) was heated at 37 °C for 20 min directly in the apparatus. Then, 10 µL of H_2O_2 (27.5 mM, final concentration) and 10 µL of cobalt solution (Co (II), 230 µM final concentration, (Merck KgaA, Darmstadt, Germany) were added to the reaction mixture. After shaking, the initial fluorescence was measured every minute until zero fluorescence was reached. Gallic acid solutions (100, 200, 400, 500, and 600 µM, Sigma-Aldrich Chemie GmbH, Darmstadt, Germany) were prepared in phosphate buffer (75 mM, pH = 7.4, Merck KgaA, Darmstadt, Germany) and were used to construct the standard curve. The area

under the decaying fluorescence curve of a gallic acid solution with a concentration of 1 µmol is taken as one HORAC unit. The results were expressed in µmol equivalents of gallic acid. The measurements were performed on a FLUOstar OPTIMA fluorimeter (BMG LABTECH, Offenburg, Germany) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

Determination of Total Polyphenolic Content

The determination was performed by the Singleton & Rossi's method [68], which is based on the reducing effect of phenolic compounds on a mixture of phosphotungstic ($H_3PW_{12}O_{40}$) and phosphomolybdic ($H_3PMo_{12}O_{40}$) acids (Sigma-Aldrich Chemie GmbH, Darmstadt, Germany), converted into a mix of oxides: wolfram blue (W_8O_{23}) and molybdenum oxide (Mo_8O_{23}) (Sigma-Aldrich Chemie GmbH, Darmstadt, Germany). The resulting blue color had a maximum absorption at λ = 765 nm and it was proportional to the amount of phenolic substances. The results were calculated according to a standard calibration curve constructed with gallic acid solutions and were expressed as mg gallic acid equivalents (GAE) in 100 g of fresh raw material. The determinations were performed on a VIS spectrophotometer Camspec M107 (Spectronic-Camspec, Ltd., Leeds, United Kingdom).

Determination of Pigment Content

For the analysis of chlorophyll *a* (C_a), and chlorophyll *b* (C_b), each sample was extracted with 100% acetone (Merck KgaA, Darmstadt, Germany) in a sample to a solvent ratio (1:50 *w*/*v*). The extraction was performed in an ultrasonic bath VWR USC100T (Singapore, Malaysia) with a frequency of 45 kHz, power 30 W at 40 °C for 20 min. The extraction procedure was repeated twice, and the acetone extracts were filtered through filter paper. The absorbance (A) of the combined final extracts was measured at three wavelengths: 662 nm, 645 nm, and 470 nm. The pigment amount was calculated according to Equations (1) and (2) reported by Lichtenthaler and Wellburn [69].

$$C_a = 11.76 \cdot A_{662} - 2.35 \cdot A_{645} \quad (1)$$

$$C_b = 18.61 \cdot A_{645} - 3.96 \cdot A_{662} \quad (2)$$

HPLC Determination of Phenolic Acids and Flavonoids

Gallic acid (3,4,5-trihydroxybenzoic acid), neochlorogenic acid, protocatehuic acid (3,4-dihydroxybenzoic acid), chlorogenic acid, catechin, vanillic acid (4-hydroxy-3-methoxyben zoic acid), caffeic acid (3,4-dihydroxycinnamic acid), epicatechin, *p*-coumaric acid, ferulic acid, rutin, ellagic acid, naringin, myricetin, quercetin, cinnamic acid, naringenin, and kaempferol were quantified on a Nexera-I LC2040C Plus UHPLC system (Shimadzu Corporation, Kyoto, Japan) with a UV detector and a binary pump. A wavelength of 280 nm was used to determine most of the analytes, whereas chlorogenic acid, caffeic acid, ferulic acid, rutin, quercetin, and kaempferol were detected at 360 nm. The separation of phenols and flavonoids was performed on an Agilent TC-C18 column (5 µm, 4.6 mm × 250 mm) at 25 °C. The mobile phases consisted of 0.5% acetic acid (A) and 100% acetonitrile (B) at a flow rate of 0.8 mL/min. The gradient condition started with 14% B, between 6 min and 30 min linearly increased to 25% B, then to 50% B at 40 min. The sample injection volume was 20 µL. The results were calculated with standard curves for each analytical standard and expressed as mg/100 g sample.

Protein Analysis

The crude protein content was analyzed by the micro-Kjeldahl method (N × 6.25). The determination of nitrogen expressed as ammonia content of the digested sample was performed by the acetylacetone–formaldehyde colorimetric method using ammonium sulfate as a standard [70].

2.3. Data Analysis

Three samples of the examined algae were collected in the area of the indicated geographical point. Three parallel measurements were made for each investigated parameter. The obtained data were processed, and the mean and standard deviation of the mean (SD) were obtained. Analysis of variance was used to compare means with a significance level of $p < 0.05$. One-way analysis of variance and Duncan's post hoc test for multiple comparisons based on the parameters studied were performed for all samples studied. The least square method was used to determine linear regression modes between antioxidant activity and total phenolic content.

3. Results and Discussion

3.1. Characteristics of Wild Seaweed and Freshwater Algae

3.1.1. Identification of Plant Material

Two species of dominant green algae and one brown alga (Black Sea) were selected. Results from the taxonomic analysis of the samples are presented in Table 4. Microscopic photos are shown in Figure 2.

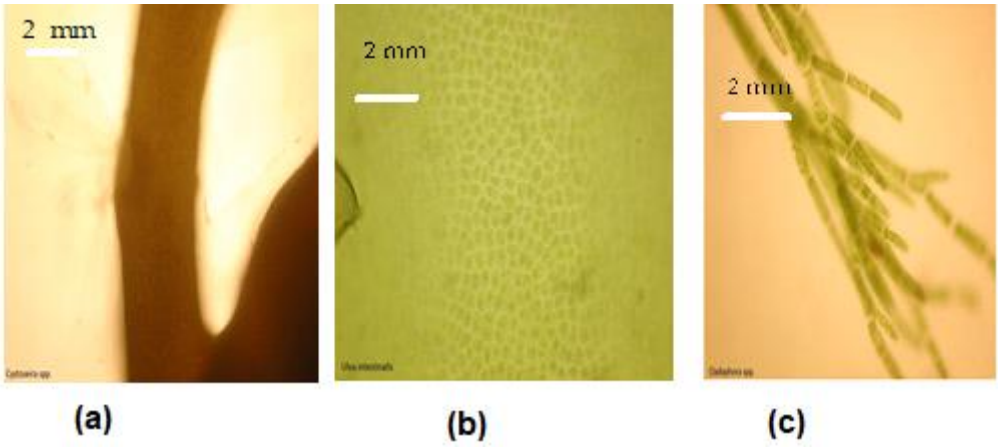

Figure 2. Microscopic structures of marine algae from Bulgarian Black Sea (magnification 20×) (**a**) *E. crinita*; (**b**) *U. intestinalis*; (**c**) *C. linum*.

3.1.2. Marine Algae

✓ Brown algae (Phaeophyceae),
- ○ *E. crinita*

E. crinita samples (Figure 2a) had brown to dark brown thallus with fleshy tissue consisting of a central axis, from which secondary axes branch off, up to 40 cm long with a thickness of the central axis at the base up to 1 cm.

✓ Green algae (Chlorophyta),
- ○ *U. intestinalis*
- ○ *C. linum*

U. intestinalis (Chlorophyta) samples (Figure 2b) had green to dark green thallus with fleshy tissue reaching between 10–30 cm in length and 0.7 cm in thickness. *C. linum* samples (Figure 2c) had green thallus, characterized by reticulate tissue, 10–40 cm long.

3.1.3. Freshwater Algae and Cyanobacteria, Cultivated in a Bioreactor

- ○ *A. platensis* (Cyanobacteria)
- ○ *Chlorella* spp. (Chlorophyta)

3.2. Some Chemical and Phytochemical Components

The total phenolic content (TPC) (mg GAE/100 g) and the antioxidant activity according to ORAC (μmol TE/g) and HORAC (μmol GAE/g) of algae *A. platensis* (convection dried and lyophilized), *U. intestinalis*, *C. linum*, *E. crinita*, and *Chlorella* spp. (lyophilized samples) were determined (Table 5).

Table 5. Total phenolic content (mg GAE/100 g) and antioxidant activity of algae according to ORAC (μmol TE/g) and HORAC (μmol GAE/g).

Algae	TPC, mg GAE/100 g	ORAC, μmol TE/g	HORAC, μmol GAE/g
	Mean ± SD	Mean ± SD	Mean ± SD
A. platensis (*convection dried*)	410.7 ± 16.5 ^{c}	87.2 ± 4.3 ^{b}	14.2 ± 1.2 ^{d}
A. platensis (*lyophilized*)	419.7 ± 12.2 ^{c}	86.0 ± 5.1 ^{b}	15.4 ± 0.4 $^{c, d}$
U. intestinalis (*lyophilized*)	512.8 ± 23.5 ^{b}	101.0 ± 5.3 ^{b}	17.5 ± 0.5 ^{c}
C. linum (*lyophilized*)	403.9 ± 16.4 ^{c}	129.6 ± 8.6 ^{b}	30.6 ± 2.3 ^{b}
E. crinita (*lyophilized*)	2662.4 ± 54.2 ^{a}	463.3 ± 25.6 ^{a}	113.4 ± 2.7 ^{a}
Chlorella spp. (*lyophilized*)	267.7 ± 8.3 ^{d}	51.2 ± 4.1 ^{b}	10.9 ± 1.1 ^{e}

Means in a column with a common superscript letter (a–e) differ ($p < 0.05$) as analyzed by one-way ANOVA.

The total phenol content in the tested samples was found to be between 2662.4 mg/100 g and 267.7 mg/100 g (Table 5). Brown algae (*E. crinita*) had the highest values for antioxidant activity and polyphenolic content. With *Chlorella* spp., the values of the studied indicators were almost ten times lower. Despite their relatively high polyphenol content, *U. intestinalis* showed lower antioxidant activity compared to *C. linum* according to HORAC.

Lyophilization of *A platensis* gave statistically indistinguishable values of polyphenol content and antioxidant activity compared to conventional drying. The data for polyphenol content and antioxidant activity obtained through lyophilization of *A. platentis* were statistically insignificant compared to conventional drying techniques. The values for the antioxidant activity by the ORAC method were 4 to 5 times higher than those specified by HORAC. The antioxidant activity of *A. platensis* by the ORAC method was 42% higher than *Chlorella* spp. extract. In both cases, the values of the antioxidant activity were highest in brown macroalgae *E. crinita*.

A correlation was found between the antioxidant capacity and the phenolic content of the studied algae species (marine and freshwater). The coefficient of correlation is high, and the results are presented in Table 6. A similar correlation between antioxidant activity and phenolic content of macroalgae was published by Jimenez-Escrig, although there are few studies on the relationship between these two algae parameters [71]. Similar results were obtained by Massoumeh Farasat et al. [72] in a study of antioxidant activity, total phenolic and flavonoid contents of methanolic extracts of four species of green macroalgae: *Ulva clathrata*, *Ulva linza*, *Ulva flexuosa* and *U. intestinalis* from different parts of the northern shores of the Persian Gulf and south of Iran. The species *Ulva clathrata* showed the highest activity, as well as the highest total phenolic and flavonoid content.

Table 6. Correlation between total phenolic content and antioxidant activity.

Regression Model	Correlation Coefficient
ORAC = 0.1651.TPC + 24.357	0.993
HORAC = 0.0422.TPC + 0.7389	0.990

Rutin is a phytochemical with multiple pharmacological actions—analgesic and antiarthritic [73,74], aggregate antiplatelet effect [75], neuroprotective activity [76], antiviral activity [77], and many others [78]. In the scientific literature, there is not much information regarding the study of rutin content in algae. In *Ulva reticulata*, *Caulerpa chemnitzia* (formerly *Caulerpa occidentalis*), *Cladophora socialis* (Chlorophyta), *Dictyota ciliolata* (Phaeophyceae), and *Gracilaria dendroides* (Rhodophyta), from the Jeddah coast of the Red Sea, Saudi Arabia, authors reported rutin values from 0.04 to 10.5 mg/kg [79]. Of 27 different species (6 green, 11 brown, and 10 red) of Japanese seaweeds, rutin was found in 13 of them (in 7 of the red and 6 of 17 green and brown algae samples), varying over a wide range of 457 μg/g up to 30,000 μg/g dry weight [80].

The results for the rutin and protein content of the studied samples are presented in Table 7. The highest levels were found in *E. crinitea* (284.17 mg/100 g) followed by *C. linum* 150.79 mg/100 g and *U. intestinalis* 148.14 mg/100 g.

Table 7. Rutin content of algae, determined by HPLC method (mg/100 g), and protein contents (%).

Algae	Rutin Content, mg/100 g	Protein Content, %
	Mean ± SD	Mean ± SD
A. platensis (*convection dried*)	141.25 ± 2.00 ^{c}	43.4 ± 0.3 ^{a}
A. platensis (*lyophilized*)	116.97 ± 2.00 ^{e}	34.4 ± 0.2 ^{b}
U. intestinalis (*lyophilized*)	148.14 ± 1.00 ^{b}	28.2 ± 0.4 ^{d}
C. linum (*lyophilized*)	150.79 ± 2.00 ^{b}	29.4 ± 0.5 ^{c}
E.crinitea (*lyophilized*)	284.17 ± 4.00 ^{a}	29.7 ± 0.3 ^{c}
Chlorella spp. (*lyophilized*)	127.99 ± 4.00 ^{d}	43.7 ± 0.1 ^{a}

Means in a column with a common superscript letter (a–e) differ ($p < 0.05$) as analyzed by one-way ANOVA.

The protein content in *A. platensis* and *Chlorella* spp. (lyophilized) was nearly 54% higher than that of green seaweed. *U. intestinalis* had the lowest protein content (12.1%). As can be seen, in *A. platensis*, dried by the lyophilization process, the protein content is nearly 20% lower. Therefore, the lyophilization process did not positively affect the preservation of the protein content.

The amount of proteins in the studied samples of *Chlorella* spp. (lyophilized) was lower than that described from Bito et al. [81] by about 59%. Similar protein content results were reported by Chisti, for *C. pyrenoidosa* (57%) [82] and by Becker for *C. vulgaris* 51–58% [53]. Green macroalgae (*Ulva rigida*) and brown macroalgae (*Gongolaria barbata*) freshly harvested from the coastal waters of the Dardanelles had a lower protein content of 8% and 16%, respectively, than the seaweeds studied [83].

Algal proteins are known to be of high quality compared to other plant materials, such as wheat, beans, and rice, but inferior to animal proteins [84]. For example, *A. platensis* is a superfood because it contains 670% more protein than tofu, more antioxidant activity in 3 g of cyanobacteria than in five servings of fruits and vegetables, and 3100% more beta carotene than carrots [85]. From the research conducted, we can conclude that *A. platensis* (convection-dried) and *Chlorella* spp. (lyophilized) are suitable for use as emulsifiers in colloidal and emulsion systems due to their high protein content (43.4 and 43.7%, respectively).

The content of flavonoids determined by HPLC method is presented in Figure 3.

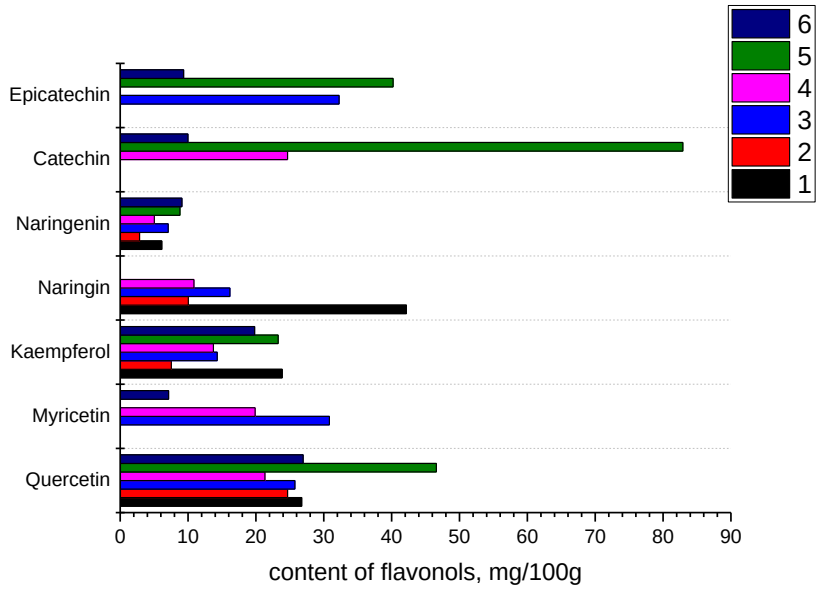

Figure 3. Content of flavonoids determined by HPLC method, mg/100 g. 1. *A. platensis* (convection dried); 2. *A. platensis* (lyophilized); 3. *U. intestinalis*; 4. *C. linum*; 5. *E. crinita*; 6. *Chlorella* spp.

E. crinita had the highest content of quercetin, catechin, and epicatechin (Figure 3). Quercetin-3-β-glycoside and catechin were not detected in *U. intestinalis*, but it possessed the highest levels of myricetin (30.82 mg/100 g) compared to other species examined. *E. crinita* did not contain myricetin. Catechin and epicatechin were not detected in both *A. platensis* samples. The lyophilization process of *A. platensis* did not affect the content of quercetin but led to a decrease in the values of quercetin-3-β-glycoside, kaempferol, narginine, and naringenin of nearly three times (Figure 3).

Flavonols (rutin, quercetin, naringin, myricetin, and kaempferol) had the highest antioxidant activity among flavonoids. Quercetin has the highest antioxidant capacity against water-soluble and fat-soluble free radicals. Krachanova M. [86], shows that it has even higher antioxidant activity than vitamins C and E.

Another class of biologically active flavonoids are catechins. In addition to their high antioxidant activity, they are characterized by various therapeutic effects: they reduce the accumulation of atherosclerotic plaques, have anti-cancer effects, and reduce the risk of heart attack and stroke. The brown algae *E. crinita* were rich in quercetin and catechin. *Chlorella* spp. had a higher content of naringin and kaempferol compared to *U. intestinalis* and *C. linum*, and *A. platensis* (grown in a bioreactor). Some authors report that the least diversity in flavonoid components found in the cyanobacterium *A. platensis*, in which only the flavone apigenin is present [87].

Polyphenols (especially flavonoids) prevent oxidative damage caused by free radicals through ingestion activity, play a crucial role in preventing degenerative neuropathies or diabetes, and have anti-inflammatory and antiviral functions. Phenolic acids are also responsible for antimicrobial, anti-inflammatory, antiviral, and anti-cancer effects. They act as antioxidants by preventing radical formation [88].

The composition and the amount of phenolic acids in the studied algae are presented in Figure 4.

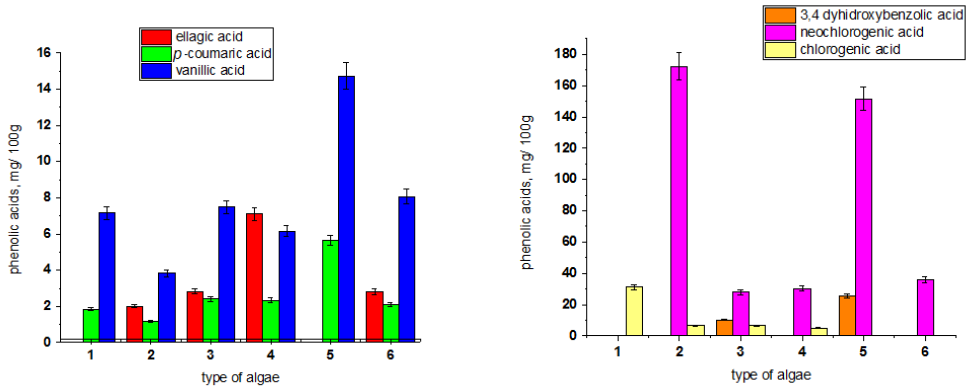

Figure 4. The phenolic acids content of algae (mg/100 g) determined by HPLC. 1. *A. platensis* (convection dried); 2. *A. platensis* (lyophilized); 3. *U. intestinalis;* 4. *C. linum*; 5. *E. crinita*; 6. *Chlorella* spp.

The amount of chlorogenic acid was highest in *A. platensis* grown in a bioreactor 31.13 mg/ 100 g and lowest in *Cladophora* ssp. 4.94 mg/100 g. In the brown seaweed *E. crinita* and freshwater algae *Chlorella* spp., chlorogenic acid was not detected. The presence of 3,4, dihydroxybenzoic acid was found in *U. intestinalis* and in *E. crinita*, where it is about 2.5 times higher than in *U. intestinalis*.

Other macroalgae for which the presence of this acid has been reported are *Neopyropia tenera* (formerly *Porphyra tenera*) (Rhodophyta) and *Undaria pinnatifida* (Phaeophyceae)—690 and 211 ng·g^{-1}, respectively [89]. *E. crinita* had the highest values for vanillic acid (14.73 mg/100 g), and in the other samples, it was almost twice lower. All tested samples contained neochlorogenic acid (Figure 4), excluding conventionally dried *A. platensis*, but the lyophilized one demonstrated high values; therefore, the lyophilization process allowed the preservation of the neochlorogenic acid. The case of chlorogenic acid was just the opposite, lyophilization reduced its values, in *E. crinita* and *Chlorella* spp. chlorogenic acid was not detected. Of the acids studied, Koen Goiris et al. reported a *p*-coumaric acid content of 920 ng/g in *Arthrospira* (Cyanobacteria), 770 ng/g for *Porphyridium* (Rhodophyta) and 7000 ng/g for *Tetraselmiss* (Chlorophyta) [87].

In addition to phenolic acids and rutin, algae contain pigments such as chlorophyll and carotenoids. Chlorophyll is a major photosynthetic pigment and natural dye. The values for the content of chlorophyll *a* and *b* in freshwater algae *Chlorella* spp., *A. platensis* (lyophilized and conventionally dried) as well as *U. intestinalis* and *E. crinita* (lyophilized feedstock) are presented in Table 8. Chlorophyll *b* dominated in all samples compared to chlorophyll *a*, with one exception—lyophilized *U. intestinalis*, where chlorophyll *b* values were many times lower than those of the other samples. The highest values of chlorophyll *b* were found in *A. platensis*—4843 µg/g dw. Chlorophyll values were between 589 and 4237 µg/g dw (Table 8).

Table 8. Chlorophyll content in algae samples.

Algae	Chlorophyll *a*, µg/g	Chlorophyll *b*, µg/g
	Mean ± SD	Mean ± SD
Chlorella spp. (lyophilized)	1659.1 ± 1.2 ^{b}	3533.7 ± 2.1 ^{b}
E. crinita (lyophilized)	163.6 ± 0.78 ^{e}	251.1 ± 0.78 ^{d}
U. intestinalis (lyophilized)	193.1 ± 0.83 ^{d}	9.9 ± 0.1 ^{e}
A. platensis (lyophilized)	588.41 ± 1.0 ^{c}	1337.02 ± 2.0 ^{c}
A. platensis (convection dried)	4237.29 ± 4.1 ^{a}	4843.27 ± 5.1 ^{a}

Means in a column with a common superscript letter (a–e) differ ($p < 0.05$) as analyzed by one-way ANOVA.

The chlorophyll content obtained by ultrasonic extraction at 45 kHz from dry *A. platensis* samples is similar to the one reported by Park et al. for ultrasonic acetone extraction between (2.6–4.7) mg/g) [90]. According to Choi, W.Y. [91], the optimal conditions for ultrasonic extraction of chlorophyll are with ethanol at 20.52 kHz, 32.59 °C, for 4.91 h and yield 17.98 mg/g. Increasing temperature and extraction time can cause the breakdown of chlorophylls in *A. platensis*, as chlorophylls are sensitive to heat [91].

Our chlorophyll content data are close to those presented by Panzella, L. et al. [92] for chlorophyll *b* content obtained by CO_2 supercritical extraction from dry *A. platensis*. Freeze-dried seaweeds *E. crinita* and *U. intestinalis* have lower chlorophyll content than marine green macroalga *Cladophora glomerata* (0.30 mg/g) [93].

The chlorophyll content, especially chlorophyll *b* detected in *E. crinita*, that we detected was higher than the one found in *Gongolaria barbata* (formerly *Cystoseira barbata*) (Phaeophyceae) from Rusalka Cape, Bulgaria [94] and comparable with the data reported for the same age from the Black sea coast in Sinop, Turkey [95]. Of course, we should not overlook the fact that the chlorophyll content depends on algae nutrient media and environmental factors [96–98]

Algae are a fascinating natural source of novel components with biological activity used as functional ingredients. Some types of algae are organisms that live in complex habitats and are subject to extreme conditions (such as changes in water salinity, temperature, nutrients, and ultraviolet radiation); therefore, in order to survive, they must quickly adapt to new environmental conditions to survive, producing a wide variety of secondary (biologically active) metabolites that cannot be found in other organisms [99]. A key component in the nucleus of the *Chlorella* spp. cell is the so-called growth factor (*Chlorella Growth Factor—C.G.F*). This nucleotide-peptide complex of substances is found only in *Chlorella* spp. It is called a growth factor because it is produced during intensive photosynthesis and this allows these algae to reproduce very quickly. C.G.F. contains nucleic acids and amino acids, peptides, polysaccharides, vitamins and beta-glucans. This complex of substances is not found in other minerals, herbs or foods and can help improve the body's natural defenses and contribute to its detoxification [100].

4. Conclusions

Macro- and microalgae, and *A. platensis* have biologically active metabolites of natural origin, which allows them to be used according to the needs of different industrial areas. We were successful in quantifying for the first time the content of some biologically active substances such as rutin, naringin, kaempferol, quercetin, and naringenin in the Bulgarian *A. platensis* produced in a bioreactor and selected algae from the Bulgarian waters of the Black Sea. There are differences between algae collected from the wild and algae from bioreactors. Brown algae (*E. crinita*) had the highest total phenolic content and antioxidant activity according to ORAC and HORAC and rutin, while *A. platensis* (convection dried) had the highest concentrations of chlorophyll *a* and *b*, and *Chlorella* spp of protein. The content of flavonoids and phenolic acids was different in the studied samples. The obtained results can serve as a good basis for developing new applications in the pharmaceutical and food industries.

Author Contributions: K.N.—constructed and conceived the project. I.M.-T. and D.B.—designed the study. I.P. and V.G.—performed the study. G.G., N.P., and V.A.—methodology, I.M.-T. and K.N.—analyzed the data and interpreted the results. V.G. and E.P.—visualization. G.G., K.N., and N.P.—wrote the article. All authors have read and agreed to the published version of the manuscript.

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