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Biocompounds from Green Algae of Romanian Black Sea Coast as Potential Nutraceuticals

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Abstract: Three green algae, collected from the Romanian Black Sea coast, are studied: *Ulva lactuca*, *Enteromorpha intestinalis*, and *Cladophora vagabunda*, which were collected from six different coastal areas. This paper aims to identify the bioactive compounds of these green algae and their beneficial properties, in order to use them as potential nutraceuticals using different analytical methods. Pharmacognostic, microbiological, and physico-chemical methods used for the analysis of algal materials revealed a rich and diverse array of biocompounds with nutritional value. In the case of green algae, a high percentage of sulphates, carbohydrates and dietary fibers were identified. Moisture, ash, total nitrogen, protein and lipid contents were determined. The contents of pigments, vitamins, and metals determined complement the nutritional qualities of the three seaweed species studied as future nutraceuticals. The content of flavonoid and phenolic compounds identified in the composition of seaweeds justifies their antioxidant and antimicrobial properties. The antioxidant capacity was tested by means of DPPH, reducing power, and TEAC methods, and the algae studied exhibit important antioxidant properties that can be used to enhance their potential as nutraceuticals. The studied algae show good antibacterial activity on both Gram (+) and Gram (−) bacteria, with slightly better activity on Gram (−) bacteria. Biocompounds from green algae from the Black Sea coast may represent an important source of marine nutraceuticals with medical and nutritional potential.

Keywords: nutraceuticals; algae; nutritional constituents; antioxidant activity; antibacterial activity; *Ulva lactuca*; *Enteromorpha intestinalis*; *Cladophora vagabonda*

Citation: Cadar, E.; Negreanu-Pirjol, T.; Sirbu, R.; Dragan, A.-M.L.; Negreanu-Pirjol, B.-S.; Axente, E.R.; Ionescu, A.-M. Biocompounds from Green Algae of Romanian Black Sea Coast as Potential Nutraceuticals. *Processes* **2023**, *11*, 1750. https://doi.org/10.3390/pr11061750

Academic Editor: Carla Silva

Received: 1 May 2023
Revised: 24 May 2023
Accepted: 31 May 2023
Published: 8 June 2023

1. Introduction

Nutraceuticals are attracting increased consumer interest. Studies show the interest of researchers in biocompounds derived from biocompatible natural sources with high physiological benefits [1]. In this context, particular attention should be paid to bioactive compounds found in seaweed. These marine plants, although living in most seas and oceans, have not been studied extensively enough as possible raw materials for obtaining nutraceuticals of interest, which can be used as alternative treatments for maintaining human health [2–4]. Since prehistoric times in the Chinese, Japanese, and Korean diet, seaweed has remained an essential nutrient [5]. The marine biomass of green algae continues

to serve as food for humans, and can be used in environmental pollutant removal [6–10]. In the literature, the antimicrobial properties of *Cladophora* species have been reported [11,12]. For *Ulva* species, studies on antimicrobial and antioxidant activities have also been reported [13,14]. Antibacterial activity was also reported for green algae from the Saudi Arabian Red Sea, Arabian Gulf, and algae from the territorial waters of Taiwan [15–17].

The antioxidant activity of green algae collected from the territorial waters of Korea has been investigated for use in healthcare products, such as nutraceuticals and supplements [18]. Antioxidant activities have also been reported for green algae in the Persian Gulf area [19,20]. In the marine environment of the Black Sea three species of algae, *Ulva lactuca* (L), *Cladophora vagabunda* (L. Hoek), and *Enteromorpha intestinalis* (L. Nees), are found in abundance [21–23]. This natural resource provided by algal biomass is currently untapped—seaweed not being utilized in food or in other biomedical applications. Because of this, the Romanian marine ecosystem has been studied in order to identify habitats where green seaweeds can be found [24,25]. There have been researchers who have found that algal biomass is vulnerable to eutrophication [26,27].

Seaweeds from the Black Sea have been studied as potential new players in drug delivery systems, in combination with other bioactive compounds, but also for their rich polysaccharide (MAPs) and monosaccharide (MAO) composition, due to which other seaweeds are used by Asian populations as food, nutraceuticals, and pharmaceuticals [28–30]. Seaweeds, due to their marine algal oligosaccharide (MAO) content, can be considered functional foods, as nutraceuticals that demonstrate a beneficial health effect beyond the traditional nutritional benefit [31]. In this sense, seaweeds that have a high marine algae oligosaccharide content can be consumed as dry biomass, functional dietary ingredients, dietary supplements, or nutraceuticals [32]. Bioactive algal compounds collected from Australian territorial waters have been reported as prebiotics and nutraceuticals [33]. Researchers in China have also reported potential prebiotic activities of oligosaccharide-rich algae [34].

The objective of this study is the biochemical composition research of green algae from the Romanian Black Sea coast with the aim of using this natural marine bioresource to obtain nutraceuticals, prebiotics, and functional foods.

For this purpose, we have developed a project design for the methods and analyses needed to identify the biocomponents of interest. Antioxidant and antimicrobial properties were also studied for each type of algae tested. Classes of bioactive compounds and biological actions that have concrete beneficial effects on human health were monitored.

2. Materials and Methods

2.1. Process Design for Green Algal Analyses and Preparatory Activities

The algal material was collected from six areas, namely S1—Midia, S2—Constanta, S3—Eforie Nord, S4—Eforie Sud, S5—Neptun, and S6—Mangalia. The green algae analyzed were monitored from May to October 2022. The biomass processing was performed on average samples derived from three determinations for each type of algae, according to specific plant product methodologies [35,36]. The investigated species of green algae developed appreciable biomass in the prevernal, vernal and autumnal seasons, and are presented in Figure 1 [37].

Seaweeds were processed by means of washing, drying, and grinding to obtain a uniform powder [38]. Seaweed powder samples were kept in the refrigerator at 4 ± 0.5 °C. The dried samples were then pulverized in a grinder and sieved through a 40-mesh sieve.

Figure 1. Green algae species investigated [3].

2.2. Reagents

The reagents used in this study to determine the phenolic compounds—acetonitrile, acetic acid, 1,1-diphenyl-2-picryl hydrazyl (DPPH), catechin, tannic acid, sodium carbonate, and aluminum chloride—were purchased from Fluka, Buchs, Switzerland. Methanol, diethyl ether, ethanol, gallic acid, and Folin–Ciocalteu reagent were purchased from Merck Co., Darmstadt, Germany. Acetone, L-ascorbic acid, sodium carbonate, sodium nitrite, beta carotene standard, line catalog no. C 4582, $AlCl_3$ $6H_2O$ and NaOH were provided by Sigma-Aldrich, Albuch, Germany. The ACW (water-soluble antioxidative capacity) kit was a part of the Photochem device, Analytik Jena AG, Berlin, Germany. Pyrogallol acid as standard was supplied by Extrasynthese, Genay Cedex, Paris, France. Other solvents and chemicals were of analytical grade. Bacterial strains and culture media were donated by the Veterinary and Food Safety Directorate in Constanta, Romania.

2.3. Algal Extracts Preliminary Pharmacognostic Analysis

Three extracts were obtained from dried samples of algae with different solvents: ether extract solution (A); alcohol extract solution (B); and water extract solution (C) [39,40]. Pharmacognostic analysis was performed for each extract via specific physicochemical methods as shown in the design process (see Figure 2). Each extract was then analyzed to identify the active principles. The process design for the analysis of biocompounds of interest (secondary metabolites) in green algae from the littoral zone of the Romanian Black Sea is shown in Figure 2.

2.4. Seaweed Extract Preparation

- Analysis of the ether extract solution

Twenty grams of the freshly powdered algal product of the studied species was weighed and extracted initially with diethyl ether (2 × 100 mL) by means of refluxing for 15 min. After refluxing, the refluxed solutions were filtered and then concentrated in a water bath to 50 mL. The solution thus obtained was further used to identify lipophilic compounds.

- Analysis of the alcoholic extract solution (in ethanol)

The algal plant product, depleted in ether, was placed in a water bath to remove traces of ether and then extracted with ethanol by means of refluxing for half an hour. The combined ethanolic solution was concentrated via solvent distillation and divided into 2 parts. One part was used for the identification of active principles in the non-hydrolyzed

solution and the other part was subjected to hydrolysis in a 10% HCl medium for 30 min. The ethanol-depleted plant product was stored for further analysis.

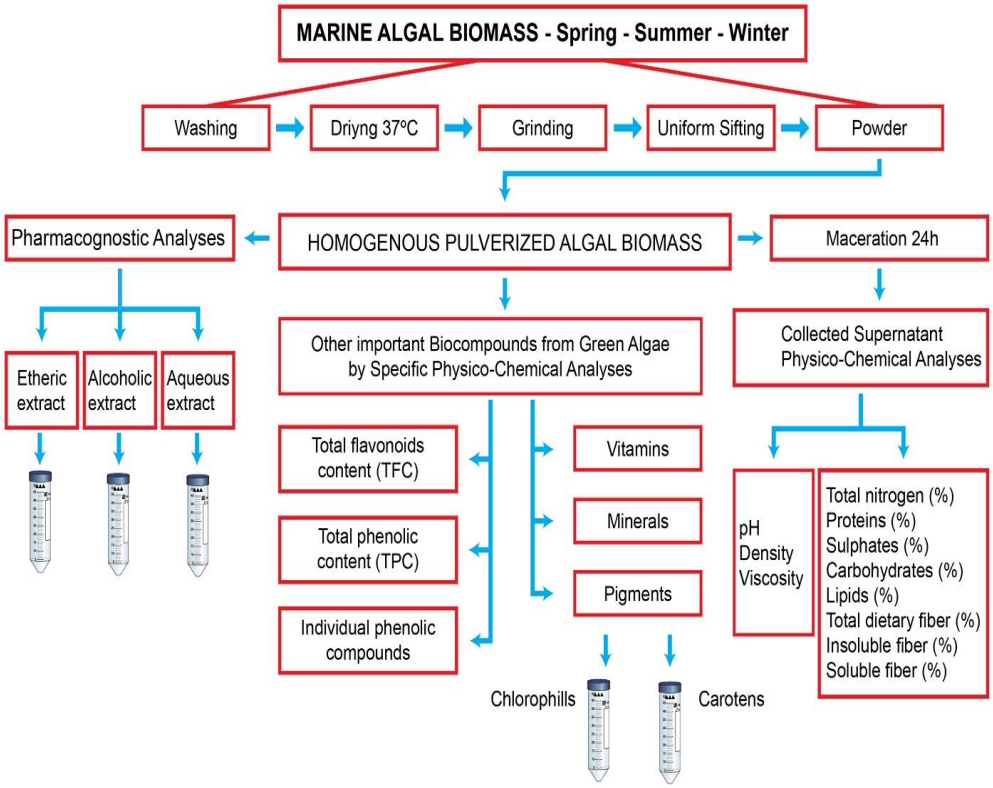

Figure 2. Process design to identify and obtain bioactive compounds from Black Sea green algae.

- Analysis of the water extract solution

The remaining plant product after alcohol extraction was dried and then extracted with 100 mL of distilled water in a water bath at 90 °C for 30 min. The extraction process was carried out by soaking the algal powder in alcohol solvent (90% methanol), (1:10 w/v), for 24 h. The received materials were concentrated in a rotary evaporator, BUCHI, Switzerland, at 40 °C to reduce the volume, filtered through Whatman No. 1 paper three times until clear extracts were obtained and were collected using a borosilicate glass container.

2.5. Green Algae Physicochemical Compositions

The moisture content (%) of seaweed was determined by drying 2 g samples in a temperature-controlled incubator at 105 °C to a constant weight [36,41]. Ash content was determined by heating the samples for 4 h in an electric oven at 500 °C [36,41,42]. The determination of sulphate content was carried out by means of quantitative chemical analysis [35].

For the determination of carbohydrates in aqueous solution, we turned to the most commonly used method, which is based on the DuBois et al. 1956 method, adapted by Albalasmeh et al., 2013. This is known as the phenol-sulphuric acid method [43]. In this analysis, a solution of 5% phenol in water (w/w) was prepared immediately before measurement and the acidic solution containing carbohydrates was added. Total carbohydrates were estimated spectrophotometrically at a maximum of 490 nm wavelength using the VWR UV-6300 PC double beam spectrophotometer. There is a strong linear correlation between carbohydrate concentrations and light absorption measured by this method. The results were calculated based on a standard glucose calibration curve with the following Equation (1):

$$y = 0.1009x - 0.0024 \quad (1)$$

where y is the carbohydrate concentration and x is the absorbance. The correlation coefficient is $R^2 = 0.992$ [41,43].

Total proteins were determined using the total nitrogen method (Nx6.25 method) [36]. Total nitrogen content was obtained by means of the Kjeldahl method. The UdK DK6 digester equipped with distillation unit 127 and Velp software was used. First, mineralization with sulfuric acid in the presence of mercury and selenium catalyst was perfomed. After alkalinization, ammonia was steam-entrained and trapped in boric acid solution, then titrated with hydrochloric acid. The results were reported as the amount of total algal powder analyzed and expressed as a percentage.

Lipids were extracted using a modified Rohani-Ghadikolalalel et al. method [44]. Lipids from the algal powder samples were extracted with dichloroethane in a Soxhlet apparatus for 5 h. After solvent evaporation, lipids were determined gravimetrically. Total lipids were determined gravimetrically on two aliquots of each lipid extract. The results were expressed as percentages, depending on the amount of algal powder used [41,45].

Total dietary fiber and soluble/insoluble fractions were determined by means of the methods of Yaich et al. [46]. The fiber content of green algae was quantified and compared using two different methods. Yaich et al. showed that there was a small difference in the result obtained for total dietary fiber content using the two procedures: the Englyst procedure (enzymatic-chemical method) and the Prosky method (gravimetric method) [46]. Differences occur, however, when the aim is to differentiate into soluble and insoluble fibers. The gravimetric method is easier to use.

2.6. Green Algae Vitamin Content

For the vitamin content, algae alcoholic and water extracts were used, according to specific methods [41].

Vitamin C content was determined by means of an analytical method with iodine using redox titration in algae water extract [35]. As iodine was added during titration, ascorbic acid was oxidized into dehydroascorbic acid and iodine was reduced into iodide ions. The reaction occurred as long as ascorbic acid was present in the reaction environment.

Vitamins A (*Retinol*) and E (*Tocopherol*) can be identified and quantified via the HPLC method. The equipment used was Agilent 1100 Series HPLC, Column Zorbax Eclipse XDB-C18, 150 × 4.6 mm, 5 µm; DAD detector. The analyte detector wavelength for vitamin A was 325 nm the retention time was 4 min, and for vitamin E the wavelength was 293 nm and the retention time was 20 min [47].

Vitamin B_1 (*Thiamine*) and vitamin B_2 (*Riboflavin*) were analyzed by means of a simple, selective, and reproducible UV-Vis spectrophotometric method.

A method of simultaneous determination of the two vitamins in the same analytical sample was used. The concentrations of each vitamin were obtained by means of isotope regression. [48]. The apparatus used for absorption spectra was a Shimadzu UV-1601 spectrophotometer. The vitamin content was determined at wavelength of 450 nm for thiamine and 410.5 nm for riboflavin, respectively [48].

Vitamin B_3 (*Niacin*) and vitamin B_6 (*Pyridoxine*) were determined by means of the UV spectrophotometric method using a Jasco V-530 UV-VIS spectrophotometer. This method was based on the chemical action of the mixture of potassium iodide and potassium iodate in an aqueous medium on the vitamin to form yellow tri-iodide ions [49]. Absorbance measurements were performed at 290 nm and 335 nm for vitamin B_6, and 288 nm and 350 nm for vitamin B_3, respectively.

2.7. Green Algae Mineral Content

The mineral content analysis was performed through flame atomic absorption spectroscopy. The equipment used was a HR-CS AAS ContrAA700 spectrophotometer, Analytik Jena AG, Germany. The method briefly comprised: sample mineralization with nitric acid ACS reagent 70% concentration (Sigma-Aldrich), establishment of calibration curves for each mineral, exposure of mineralized solutions to flame ionization, and measurement of

specific absorbance, calculation of heavy metal content [44,50,51]. To express the metal concentrations in mg metal/kg sample, the following equation was applied (2):

$$Metal\ concentration(\text{mg/Kg}\ sample) = \frac{V_b \times c}{m_{sample}} \times 100 \quad (2)$$

where: V_b = volume of the volumetric flask in which the sample solution was prepared; c = read *metal concentration* (mg/L); and m_{sample} is the sample mass in g.

2.8. Green Algae Pigments Determination

The content of total carotenoids was spectrophotometrically analyzed using a WPA S106 spectrophotometer, according to AOAC (Association of Official Analytical Chemists) method [41]. The total carotenoid content was obtained using absorbance readings at 470 nm, [52,53] with a reference solution of 2.5 mg β-carotene in 100 mL of benzene. The β-carotene concentration was obtained using a graphical method with a standard curve following Equation (3):

$$Y = A \times X;\ A = 0.0860326 \quad (3)$$

with correlation coefficient $R^2 = 0.992366$ [43].

Beta carotene standard used line catalog no. C 4582, from Sigma-Aldrich Company, Purified $\geq$ 95% HPLC.

For the chlorophyll pigment content, the 80% acetone extract of each algal species was used and determined spectrophotometrically using a WPA S106 UV-Vis spectrometer, at wavelengths of 647 nm for *Chl a* [37,53–55] and 664 nm for *Chl b* [37,53,54].

2.9. Determination of TFC and TPC

2.9.1. Total Flavonoid Content (TFC)

For the total flavonoid content, samples were analyzed with aluminum chloride. The method was adapted from Ling et al. [56]. A calibration curve based on catechin solutions at different concentrations ranging from 10 to 100 µg/mL in methanol was used. The absorbance was at a wavelength of 415 nm using a JASCO-550 UV-Vis spectrophotometer. The flavonoid content of the hydroalcoholic extracts in 80% menthol was expressed in terms of catechin equivalents in mg CE/100 g d.w. [56]. The standard curve follows Equation (4):

$$y = 0.0067x + 0.0132 \quad (4)$$

where x represents different concentrations of catechin solutions and y represents the absorbances. The correlation coefficient was $R^2 = 0.999$.

2.9.2. Determination of Total Phenol Content (TPC)

The method described by Farvin et al. was used [57]. Gallic acid solutions of various concentrations with Folin–Ciocalteu reagent (1:10 v/v) in sodium carbonate medium were used for the calibration curve [58]. After 30 min, the absorbance at a wavelength of 765 nm was read using a VWR UV-6300 PC double beam spectrophotometer [59]. The total phenol content was calculated, and the results were expressed in terms of gallic acid equivalent in mg GAE/100 g d.w. of the algal sample [60]. The calibration curve characteristics are given by Equation (5):

$$y = 0.0078x + 0.1861 \quad (5)$$

where x represents different concentrations of gallic acid solution in mg/L and y represents the absorbances. The correlation coefficient was $R^2 = 0.9959$.

2.10. Antioxidant Properties

2.10.1. DPPH Test

The free radical scavenging effect of α-diphenyl-β-picrylhydrazyl (DPPH) was measured using the method described by Farasata et al. and Ghareeba et al. [20,40]. Green

algae powder and 20 mg/L DPPH solution were dissolved in methanol [61]. At 517 nm, absorbances were noted after 30 min. The analytical equipment used was a VWR UV-6300 PC double beam spectrophotometer. DPPH activity was determined with relation (6) [62]:

$$\%Inhibition = \left(A_{control} - \frac{A_{sample} - A_{blank}}{A_{control}} \right) \times 100 \quad (6)$$

where A_{sample} is the sample absorbance after 30 min for the algae extract, and DPPH absorbance without algae is represented by $A_{control}$. The positive control was ascorbic acid, and the absorbance of the blank sample is indicated as A_{blank}.

The IC50 inhibition was calculated from graphs using non-linear regression [63].

2.10.2. Reducing Power

The spectrophotometric AOAC method was used on methanol 80% hydroalcoholic extract with absorbance measurements at 700 nm wavelength [41,59]. Readings were made with a VWR UV-6300 PC double beam spectrophotometer. The L-ascorbic acid solution was chosen as a reference [64]. The results were expressed as average ± SD of three replicates.

2.10.3. Determination of Total Antioxidant Activity (TEAC)

The determination of the antioxidant activity by means if the TEAC method was based on the photochemiluminescence method, an ACL (Antioxidant Capacity of Lipid Soluble substances)-specific procedure of Analytik Jena, performed on 10% hydroalcoholic algae extract in a 70% ethanol concentration [65]. The Photochem Analytik Jena AG, Germany, with luminol as a photosensitizer for UV light, at a wavelength of λ = 351 nm, was used [36,37]. The work was completed with Trolox as a standard substance. Calibration curves for Trolox solutions were plotted using Equation (7):

$$1/Y\ (X) = 0.15553(1/X\hat{}2) + (1/X) + 0.76378 \quad (7)$$

where on the y axis, 1/Inhibition is represented, and on the x axis, 1/Quantity is represented.

The statistical parameters were Syo = 0.0222 and R^2 = 0.9998.

The results were obtained in Trolox equivalent units (nmol/volume of sample)

2.11. Antibacterial Activity

For the antibacterial activity, four Gram-negative species were used: *Klebsiella pneumonia* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus mirabilis* (ATCC 25933), and *Escherichia coli* (ATCC 25322). Two species of Gram-positive bacteria were also tested: *Staphylococcus aureus* (ATCC 25923), and *Streptococcus epidermidis* (ATCC 12228). Bacterial strains were grown in a nutrient agar medium at 37 °C. Nutrient agar medium consisted of 5 g of peptone, 3 g of beef extract, 5 g of NaCl, and 20 g of agar in distilled water. Bacterial suspensions were spread to achieve a uniform turf culture [66–68]. The antimicrobial activities of green algal hydroalcoholic extracts obtained in ethanol 70% were evaluated by means of the agar well diffusion assay with some modifications [67]. Antimicrobial activity was assessed by measuring the diameter of the circular inhibition zones in the well using the Kirby–Bauer diffusimetric method, and the values represent the means of three replicates [68]. The minimum inhibitory concentration (MIC) was studied with the measurement of the diameters of the inhibition zone made by the seaweed ethanolic extracts [38,68].

2.12. Statistical Methods

The results were studied using SPSS 16.0 parametric tests. One-way ANOVA was utilized in order to correlate the chemical composition data of the marine algae groups. When differences were found, the Duncan multiple comparison test was used, and differences were considered significant at $p < 0.05$.

3. Results

Following the analyses proposed by the scheme in Figure 2, the following results are presented for the biocomponents identified in the marine algae collected from the coastal waters of the Romanian Black Sea coast. The identified biocompounds support the biological activities analyzed.

3.1. Chemical Composition

3.1.1. Preliminary Pharmacognostic Composition

Based on physicochemical methods used in phytochemical studies, we conducted a pharmacognostic study in order to identify the classes of bioactive compounds that can be found in three representative green seaweeds species from the Black Sea coast, *Cladophora vagabunda*, *Enteromorpha intestinalis*, and *Ulva lactuca*. The importance of these intuitive analyses represents the possibility of focusing on the secondary metabolites of algae. The chemical methods used for each extract on the three solvent classes—ether, alcohol, and water—are emphasized in the scheme in Figure 2. The specific physicochemical reactions leading to the identification of the important active principles are emphasized in Figure 3 [27,35].

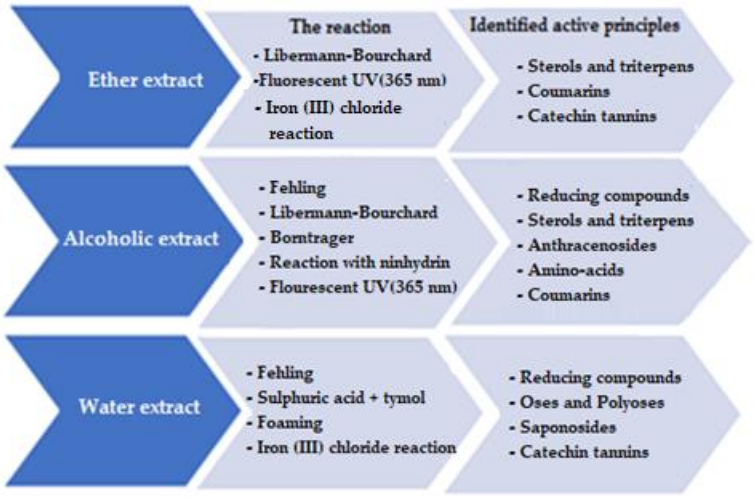

Figure 3. The selective reactions used in the chemical analysis and the active principles identified from seaweeds.

Lipophilic compounds, namely sterols and triterpenes, coumarins, and catechin tannins, were identified in the ether extract. The largest number of classes of compounds with hydrophilic structures was identified in the alcoholic extract, namely reducing compounds, sterols, and triterpenes, anthracenosides, amino acids, and coumarins. The next highest number of classes of compounds with hydrophilic structures was identified in the water extract: reducing compounds, oses and polyoses, saponosides, and catechin tannins.

3.1.2. Bioactive Components with Nutritional Potential

The algal biochemical composition was determined by highlighting moisture and ash, establishing the sulphate, protein, lipid, carbohydrate, oligosaccharides, polysaccharides, and dietary fiber content for each type of algae.

In terms of moisture and ash, *Enteromorpha intestinalis* had the highest moisture (11.98 ± 0.84%) and ash content (25.65 ± 0.98%), and *Cladophora vagabunda* had the lowest moisture (9.78 ± 0.56%) and ash content (24.63 ± 0.84%). These results are similar to the results obtained by Rohani-Ghadikolalel et al., Metin et al., and Turan et al. [44,69,70]. From the pharmacognostic study (Figure 3) and the biochemical composition analysis of the studied algal material, the high carbohydrate content consists of sulphated and non-sulphated polysaccharides, and various oligosaccharides are noted. In the literature, studies have

confirmed the existence of polysaccharides in green seaweeds, such as those reported by Hernández-Garibay et al. and Hentati et al. [71,72]. Sulfate-oligosaccharides from green algae have been reported by Liu et al. [73]. Figure 4 contains the results obtained from the elemental analysis.

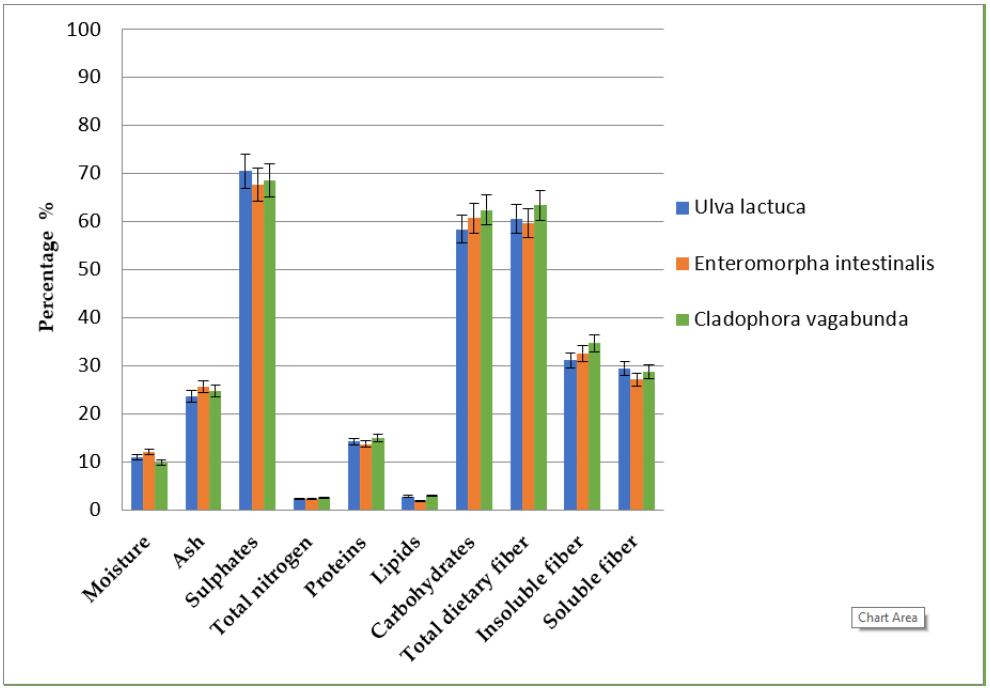

Figure 4. Physico-chemical composition of green algae from the Romanian Black Sea Coast. All values represent the mean of three replicates ± SD. There were no significant differences in the statistical analysis at $p < 0.05$.

3.2. Green Algae Vitamin Content

Fat-soluble vitamins A and E and water-soluble vitamins C, B_1, B_2, B_3, and B_6 were identified by means of specific analyses. Vitamin C was identified at contents in the following order: *Cladophora vagabunda*, *Ulva lactuca*, and *Enteromorpha intestinalis*, with ascorbic acid being a water-soluble vitamin with remarkable antioxidant potential.

The results are presented in descending order for the vitamin content obtained in Table 1.

Table 1. Vitamin content of green algae from the littoral zone of the Romanian Black Sea. No statistical differences can be observed at $p < 0.05$ between seasons (spring–summer–autumn) in vitamin content.

Sample	*Ulva lactuca* mg/100 g d.w.	*Enteromorpha intestinalis* mg/100 g d.w.	*Cladophora vagabunda* mg/100 g d.w.
Vitamin C (Ascorbic acid)	146.63 ± 0.95	136.16 ± 0.85	149.66 ± 0.58
Vitamin E (*Tocopherol*)	8.22 ± 0.11	9.93 ± 0.83	8.54 ± 0.63
Vitamin B_1 (*Thiamine*)	3.72 ± 0.25	3.95 ± 0.52	4.16 ± 0.25
Vitamin B_3 (*Niacin*)	2.97 ± 0.28	1.84 ± 0.45	2.59 ± 0.32
Vitamin B_2 (*Riboflavin*)	0.99 ± 0.07	0.97 ± 0.02	0.89 ± 0.06
Vitamin B_6 (*Pyridoxine*)	0.58 ± 0.12	0.53 ± 0.66	0.69 ± 0.06
Vitamin A (*Retinol*)	0.57 ± 0.06	0.49 ± 0.05	0.58 ± 0.03

3.3. Green Algae Mineral Content

Analysis of the mineral content shows that green algae have a wide range of minerals in their composition. In Figure 5, it is interesting to analyze their distribution in each type of algae sample collected.

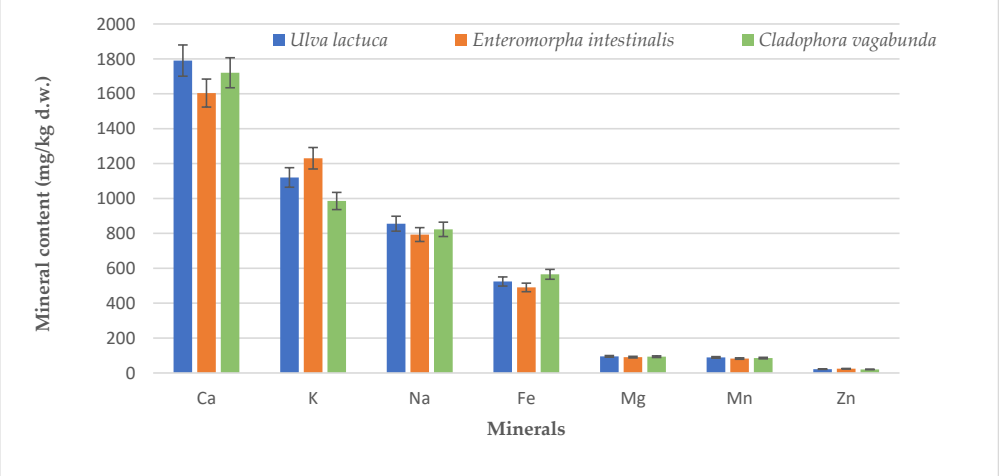

Figure 5. Minerals contained in the studied algae from the Romanian Black Sea. Values are given as the average ± SD of three replicates, d.w. (dry weight). No statistical differences can be observed at $p < 0.05$ for all mineral data.

The contents of the minerals found are in the following order: Ca > K > Na > Fe > Mg > Mn > Zn.

3.4. Green Algae Pigment Content

In Table 2, the basic pigments identified in green seaweeds, namely total carotenoids, total chlorophylls (Total *Chl*), chlorophylls *a* (*Chl* a), and chlorophylls *b (Chl b)*, are presented. The results were statistically analysed by the ANOVA test, and there are no significant differences.

Table 2. Pigment content of algae from the Romanian Black Sea. Values are given as the average ± SD of three replicates and were expressed in mg/g d.w. (dry weight).

Sample	Total Carotenoids	Total *Chl*	*Chl a*	*Chl b*
Ulva lactuca	16.25 ± 1.3	35.37 ± 1.7	26.95 ± 1.5	8.42 ± 1.56
Enteromorpha intestinalis	15.98 ± 1.98	30.51 ± 1.82	23.56 ± 1.88	6.95 ± 1.6
Cladophora vagabunda	17.66 ± 1.56	41.64 ± 1.52	29.25 ± 1.56	12.39 ± 1.35

3.5. Total Flavonoid Content (TFC) and Total Phenol Content (TPC)

Phenolic compounds are considered one of the most important classes of natural antioxidants. According to their chemical structure, flavonoids and phenolics acids, isoflavonoids, lignans, and phenolic polymers are known. [56,57]. Results were obtained by means of specific methods and are presented in Table 3.

Table 3. The content of flavonoid and phenolic compounds of green algae. Total flavonoid content is expressed as mg catechin equivalents in 100 g of dried sample (mg CE/100 g d.w.). Total phenolic content is expressed as mg gallic acid equivalents in 100 g of dried sample (mg GAE/100 g d.w.). The results are the mean ± SD for three replicates. d.w. (dry weight).

Sample	TFC	TPC
Ulva lactuca	16.6 ± 1.65	417.6 ± 1.56
Enteromorpha intestinalis	14.1 ± 1.68	413.5 ± 1.26
Cladophora vagabunda	12.3 ± 1.78	409.8 ± 1.68

3.6. Green Algae Biological Properties

3.6.1. Antioxidant Properties

- DPPH assay

This type of analysis determination is based on radical scavenging by antioxidants [38,40]. The radical compound is stable and does not need to be generated [62]. The percentage of DPPH inhibition was quantified for different concentrations of methanol extracts from the green algae species studied. In Figure 6, the results are presented.

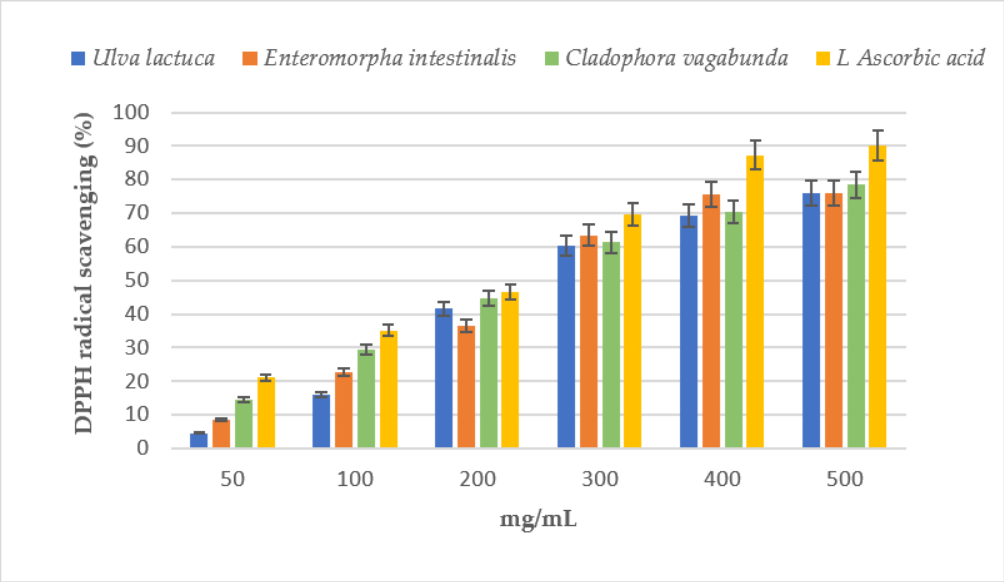

Figure 6. DPPH radical scavenging (%) for the green algae studied. Values are given as the average ± SD of three replicates. L-ascorbic acid was used as a control. No statistical differences can be observed at $p < 0.05$ compared to the control.

The IC50 value was determined in order to quantify the scavenging effects of DPPH radicals. The lowest IC50 value indicates the highest capacity of the extract to absorb DPPH radicals. The results are shown in Figure 7.

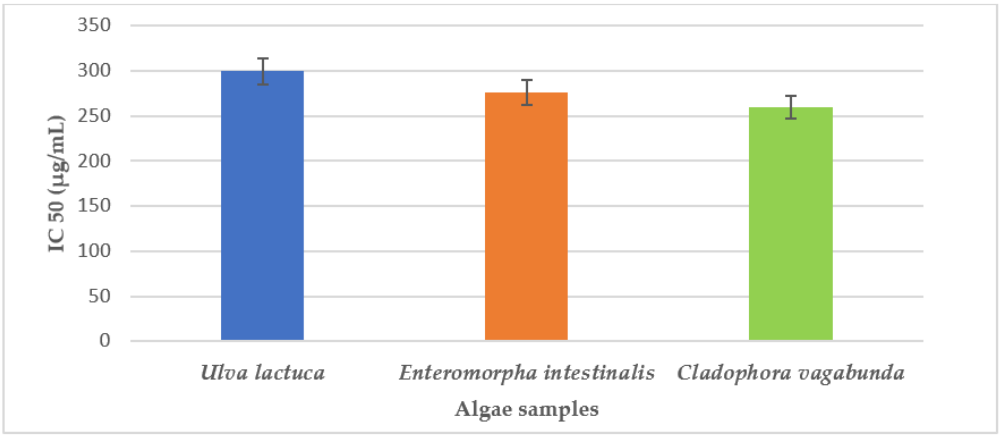

Figure 7. IC50 values for green algae from the Romanian Black Sea coast. Values were given as the average ± SD of three replicates.

- Reducing power assay

The reducing power attests that antioxidants are electron donors and can intervene in the lipid peroxidation process. Thus, they can become primary and secondary antioxidants.

The reducing power is dependent on increasing concentration. It is also affected by free radical chain reactions, protein degradation, lipid peroxidation, and transition metal ion. The reducing potential of green algae ethanolic extracts for *Cladophora vagabunda* showed significantly higher uptake compared to the assimilation of *Ulva lactuca* and *Enteromorpha intestinalis* at 700 nm (see Figure 8).

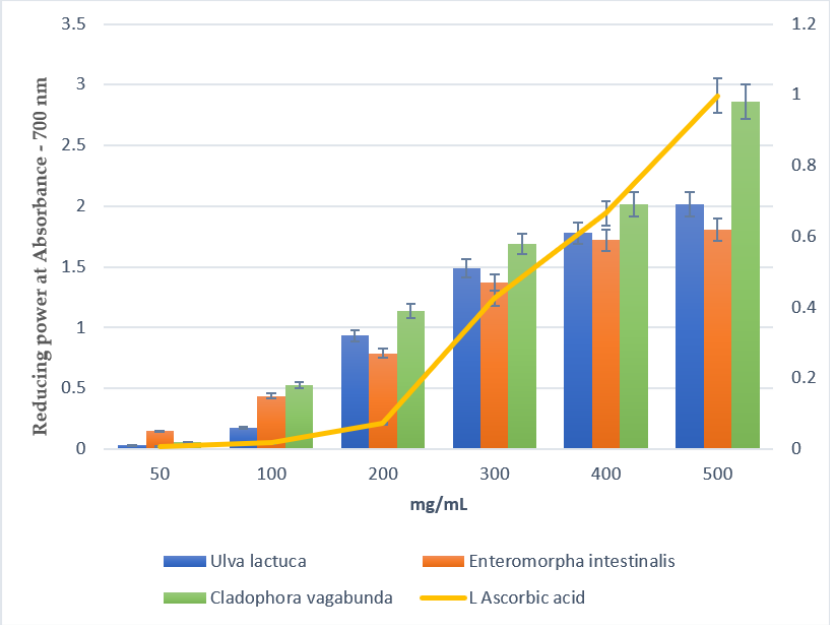

Figure 8. Reducing power for green algae from the Romanian Black Sea shore. Values are given as the average ± SD of three replicates. L-ascorbic acid was used as the control. No statistical differences can be observed at $p < 0.05$ compared to the control.

- Antioxidant capacity (TEAC)

Total antioxidant capacity was obtained by means of chemiluminescence measurements. The values are presented in Table 4 and show Trolox unit equivalents (mmol/g dry sample); the calculated results are given in nmol Trolox/g sample for each type of green algae.

Table 4. Values of TEAC for green algae from the Romanian Black Sea. The results are expressed as the mean ± SD ($n = 3$).

TEAC Trolox units Equivalent		***Ulva lactuca***	***Enteromorpha intestinalis***	***Cladophora vagabunda***
	nmol/vol sample	1.31 ± 0.1	1.18 ± 0.1	1.11 ± 0.2
	nmol/g dry sample	65.32 ± 0.1	59.05 ± 0.2	55.21 ± 0.4

3.6.2. Antimicrobial Activity

The results of the antibacterial activities in the inhibition zones are expressed as the diameter in mm of the discs filled with ethanolic extracts of green algae. Gram-negative strains, namely *K. pneumoniae* and *E. coli*, were the most sensitive. The rest of the tested pathogens reacted differently to the action of green algal ethanol extracts. The *P. mirabilis* strain showed low sensitivity to all green algal extracts, comparable to the inhibition zone of chloramphenicol.

The concentration of the seaweed extract solution used was 10 mg/mL, and that of the chloramphenicol solution was 5 mg/mL. Chloramphenicol served as a control. No statistical differences were observed at $p < 0.05$ compared to the control. In Figure 9, we present pictures of the plates used to calculate the inhibition zone.

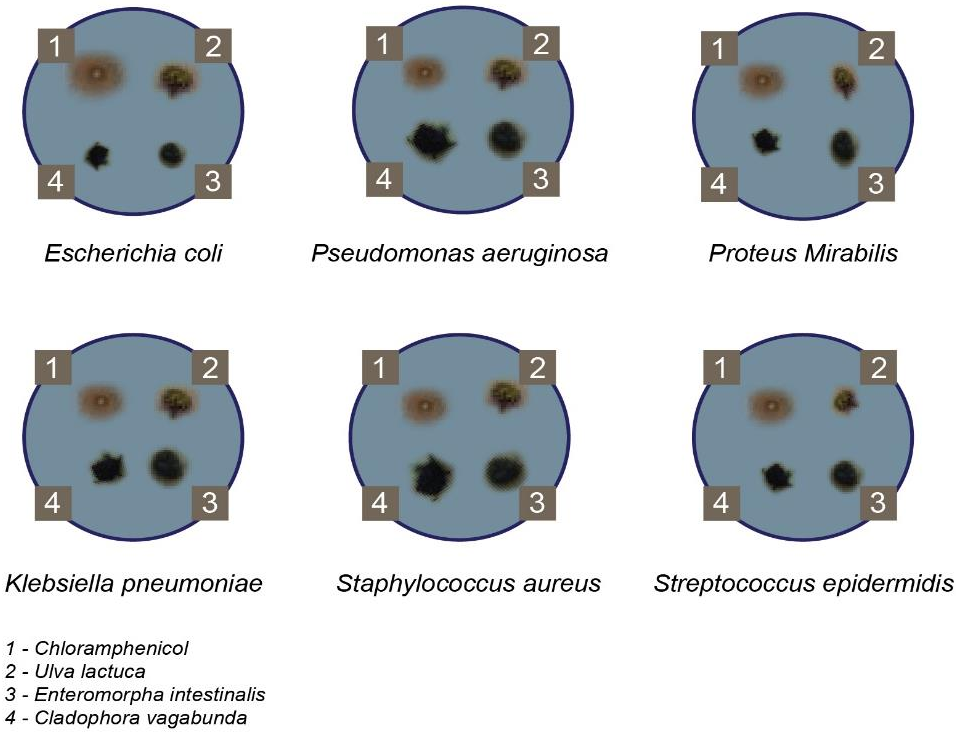

Figure 9. Pictures of the plates used to calculate the inhibition zone in the antibacterial study of the three green algae extracts.

The results for the zones of inhibition achieved by each algal extract against Gram-negative and Gram-positive bacteria are shown in Figure 10.

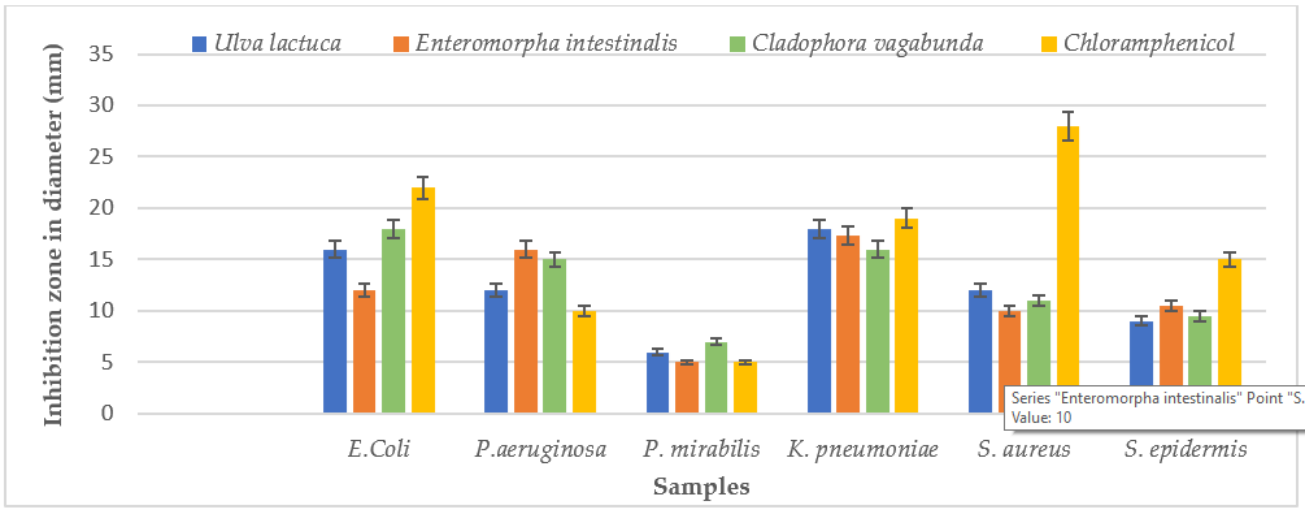

Figure 10. The antibacterial activities of green algae extracts from the Romanian Black Sea coast. The results are expressed as the mean ± SD (n = 3). Chloramphenicol served as a control. No statistical differences can be observed at $p < 0.05$ compared to the control.

The critical micellar concentration (MIC) values obtained from the antimicrobial tests ranged from 10 µg/mL to >100 µg/mL, as shown in Table 5.

Table 5. Minimal inhibitory concentration (MIC) of green algae extracts from the Romanian Black Sea coast. The results are expressed as the mean ± SD (n = 3).

Bacterial Strains	MCI (µg/mL)		
	Ulva lactuca	*Enteromorpha intestinalis*	*Cladophora vagabunda*
Escherichia coli	25	25	25
Pseudomonas aeruginosa	50	50	50
Proteus mirabilis	>100	>100	>100
Klebsiella pneumonia	25	25	25
Staphylococcus aureus	50	50	50
Streptococcus epidermidis	75	75	75

4. Discussion

4.1. Nutritional Potential

The nutritional potential of green algae, which are natural products derived from marine sources, is highlighted by the content of bioactive compounds such as proteins, lipids, carbohydrates, and dietary fibers (see Figure 4). In Table 6, systematized data obtained for green algae harvested from the Romanian Black Sea coast both for the determination of their nutritional potential and their biocomponents that support their biological activities such as antioxidant and antimicrobial activity are presented. Following the results, we found a good correlation with the literature data, leading us consider the potential nutritional and beneficial effects of these algae for human health (see Table 6).

Table 6. Data obtained in the present study and literature data for the characterization of the physicochemical compositions of the three green seaweeds studied.

	Ulva Lacutca			*Enteomorpha intestinalis*			*Cladophora vagabunda*		
	Data from the Present Study	Data from the Literature	Reference	Data from the Present Study	Data from the Literature	Reference	Data from the Present Study	Data from the Literature	Reference
Moisture (%)	10.85 ± 0.26	11.2	[3]	11.98 ± 0.84	12.15 ± 0.18%	[69]	11.98 ± 0.84	5.71 ± 0.92	[35]
Ash (%)	23.62 ± 0.59	16.9	[70]	25.65 ± 0.98	22.4 ± 1.46	[44]	24.63 ± 0.84	26.38 ± 0.31	[35]
Sulphates (%)	70.46 ± 1.87	68.86 ± 1.99	[35]	67.68 ± 1.63	60.57 ± 6.12	[35]	68.65 ± 1.78	67.92 ± 0.53	[35]
Protein (%)	14.13 ± 0.85	13.6	[3]	13.63 ± 0.96	14.67-13.42	[69]	14.94 ± 0.92	15.43 ± 0.36	[35]
Lipid (%)	2.78 ± 0.69	3.6 ± 0.42	[44]	1.72 ± 0.56	1,69 ± 0,47	[35]	2.86 ± 0.75	3.85 ± 0.47	[35]
Carbohydrate (%)	58.36 ± 1.64	59.1 ± 0.37	[44]	60.68 ± 1.36	58.0-70.64	[69]	62.37 ± 1.74	48.45 ± 0.5	[35]
Total dietary fiber (%)	60.56 ± 1.1	54.9	[72]	59.66 ± 1.95	52.36 ± 3.26	[35]	63.35 ± 1.24	61.56 ± 1.5	[35]
Insoluble fiber	31.13 ± 0.85	34.37	[46]	32.57 ± 0.95	31.54 ± 1.58	[35]	34.68 ± 0.82	38.64 ± 2.68	[35]
Soluble fiber %	29.45 ± 1.23	31.5	[46]	27.09 ± 1.86	20.82 ± .0.6	[35]	28.67 ± 1.75	22.92 ± 1.6	[35]
Vitamin C mg/ 100 g d.w.	146.63 ± 0.95	143.461 ± 0.35	[35]	136.16 ± 0.85	147 ± 2.00	[69]	149.661 ± 0.58	89.665 ± 2.58	[35]
Vitamin E mg/ 100 g d.w.	8.221 ± 0.11	7.919 ± 0.11	[35]	9.935 ± 0.83	5.13 ± 1.0	[69]	8.541 ± 0.63	8.132 ± 1.03	[35]
Vitamin B1 mg/ 100 g d.w.	3.725 ± 0.25	4.87	[3]	3.955 ± 0.52	0.125 ± 0.25	[35]	4.162 ± 0.25	0.153 ± 0.02	[35]
Vitamin B3 mg/ 100 g d.w.	2.971 ± 0.28	4.7	[6]	1.841 ± 0.45	2.42 ± 0.09a	[69]	2.591 ± 0.32	2.495 ± 0.19	[35]
Ca mg/kg d.w.	1790.35 ± 2.55	1620.35 ± 2.55	[35]	1604.15 ± 2.96	15977	[69]	1720.64 ± 2.87	1495.64 ± 2.87	[35]
K mg/kg d.w.	1120.54 ± 1.03	515.6 ± 35.68	[44]	1230.56 ± 1.65	1052.70	[69]	985.64 ± 2.03	920.56 ± 1.67	[35]
Fe mg/kg d.w.	524.25 ± 0.64	464.1 ± 1.53	[44]	490.36 ± 1.56	338.70	[69]	565.35 ± 1.05	330.56 ± 1.95	[35]
Chlorophyll-*a* mg/ g d.w.	26.95 ± 1.5	28.16 ± 2.69	[53]	23.56 ± 1.88	16.74 ± 1.65	[36]	29.25 ± 1.56	24.13 ± 2.57	[35]
Chlorophyll-*b* mg/ g d.w.	8.42 ± 1.56	5.97 ± 0.85	[53]	6.95 ± 1.6	4.23 ± 0.45	[35]	12.39 ± 1.35	9.54 ± 1.55	[35]
TFC mg/g d.w.	16.25 ± 1.3	23.91 ± 1.55	[56]	15.98 ± 1.98	12.73 ± 1.32	[35]	17.66 ± 1.56	13.90 ± 0.42	[35]
TPC mg. GAE/ 100 g d.w.	417.6 ± 1.56	285.5 ± 0.6	[38]	413.5 ± 1.26	268.8 ± 0.7	[38]	409.8 ± 1.68	356.8 ± 0.3	[38]

The protein content established based on the nitrogen content was highest in *Cladophora vagabunda*, followed by *Ulva lactuca* and *Enteromorpha intestinalis* (see Table 6). These results are similar to those obtained in other research works on algae from other seas around the world, such as that of Rasyid, who studied *Ulva sp.* from Pameungpeuk waters, Indonesia, and Turan et al., who studied green seaweeds from Iskenderun Bay, on the Northeastern Mediterranean coast of Turkey [3,70]. In the case of green algae, the carbohydrate content is high. The highest percentage of carbohydrate content is presented by *Cladophora vagabunda*, followed by *Enteromorpha intestinalis* and *Ulva lactuca* (58.36 ± 1.64 %). These results are in agreement with the green algae contents in other marine waters, such as those reported

by Hernández-Garibay et al. in their study on green algae from the Pacific Ocean coast of Mexico, Ensenada, Baja, and California, by Hentati et al. in their study on green algae from the Mediterranean Sea off Tunisia's coast and by Liu at al. in their study on green algae from the marine waters of China [71–73]. For sulphates, the highest percentage is presented by *Ulva lactuca*, followed by *Cladophora vagabunda* and then by *Enteromorpha intestinalis.* The data are comparable with the green algae results from the Turkish Mediterranean coast, reported by Metin et al. [69]. Sulphated polysaccharide and oligosaccharide biocompounds generate important biological activities such as anti-inflammatory, antidiabetic, antiobesity, immunomodulatory, antioxidant, antitumoral, antimicrobial activities, as reported by Liu et al. for green algae from marine water from China and by Kang et al. for green algae from the coastal waters of Korea. [73,74]. In this study, as shown in Figure 4 and Table 6, the lipid content in algae was in the following ascending order: the lowest in *Enteromorpha intestinalis,* followed by *Ulva lactuca*, and highest in *Cladophora vagabunda*. Lipids in seaweed have been studied for use in nutrient and nutraceutical applications by various algae researchers from other parts of the world, such as Rohani-Ghadikolaei et al., who studied green seaweeds from the Persian Gulf of Iran, Ansari et al., who studied green seaweeds from marine waters of South Africa, and Kumari et al., who studied green algae from the North Sea coast of Aberdeen, the United Kingdom [44,45,75]. The upper percentage of total dietary fibers was identified for *Cladophora vagabunda*, followed by *Ulva lactuca* and *Enteromorpha intestinalis* (see Table 6). The percentage content of insoluble dietary fibers was the highest for *Cladophora vagabunda*, followed by *Ulva lactuca*, and the lowest was observed for *Enteromorpha intestinalis* (see Table 6). These results are in good accordance with the results of other researchers regarding the content of total dietary fibers, such as those reported by Yaich et al., who studied algae from the Mediterranean Sea off the Tunisian coast [46]. The highest content of soluble dietary fibers was shown by *Ulva lactuca*, followed by *Cladophora vagabunda* and *Enteromorpha intestinalis* (see Figure 4 and Table 6). The high-level content of soluble fibers suggests a favorable nutritional effect for people who need it for medical reasons. Carvalho et al. also suggest the necessity of developing seaweed products that are more attractive to consumers [76].

Regarding the vitamin content results, vitamin C was found in considerable amounts in all algae analyzed: the highest content was observed in *Cladophora vagabunda*, followed by *Ulva lactuca* and *Enteromorpha intestinalis* (see Tables 1 and 6). Among the fat-soluble vitamins, the highest vitamin E (*Tocopherol*) content was identified in *Enteromorpha intestinalis*, followed by *Cladophora vagabunda* and *Ulva lactuca* (see Table 6). Among the B-complex vitamins, the highest content of vitamin B1 (thiamine) was found in in *Cladophora vagabunda*, followed by *Enteromorpha intestinalis* and *Ulva lactuca* (see Tables 1 and 6). These results are also confirmed by vitamin values identified in green algae from other seas, such as the values reported by Rasyid, who studied algae from Pameungpeuk waters, Indonesia, by Abirami et al., who reported data on seaweeds from Ramanathapuram district, India, by Sirbu et al., who studied green algae from the Romanian Black Sea coast in 2019, and by Metin et al., who studied algae from the northeastern Mediterranean coast of Turkey [3,6,35,69]. The lowest content was shown for vitamin A (*Retinol*), which was identified in *Enteromorpha intestinalis* (0.61 ± 0.05 11 mg/100 g d.w.), followed by *Ulva lactuca* (0.67±0.06 mg/100 g d.w.) and *Cladophora vagabunda* (0.69 ± 0.03 mg/100 g d.w.), as also confirmed by Sirbu et al. in 2019 [35]. The most significant green algae vitamin contents reported in our study suggest that they may represent a real source of natural compounds, particularly due to their ascorbic acid content.

From the analysis of the results on green algae mineral content, the highest amounts of minerals were recorded for Ca and K compared to other minerals (see Figure 6). For the quantity of Ca, identified in the studied green algae, significant values were obtained for *Ulva lactuca*, followed by *Cladophora vagabunda* and *Enteromorpha intestinalis* (see Figure 5 and Table 6). The highest K content was obtained in *Enteromorpha intestinalis*, followed by *Ulva lactuca* and *Cladophora vagabunda.* For Na, the highest content was obtained in *Cladophora vagabunda* (853.15 ± 0.89 mg/kg d.w), followed by *Ulva lactuca* (825.50 ± 0.56 mg/kg d.w)

and *Enteromorpha intestinalis* (793.31 ± 1.20 mg/kg d.w). For Mg, the highest content was obtained in *Ulva lactuca* (95.26 ± 1.05 mg/kg d.w), followed by *Cladophora vagabunda* (93.45 ± 0.91 mg/kg d.w), and *Enteromorpha intestinalis* (90.87 ± 0.96 mg/kg d.w). For Mn, the highest content was obtained in *Ulva lactuca* (89.25 ± 0.95 mg/kg d.w), followed by *Cladophora vagabunda* (85.45 ±1.85 mg/kg d.w) and *Enteromorpha intestinalis* (82.64 ± 0.57 mg/kg d.w). A low level was obtained for Zn concentrations. The highest Zn content was determined in *Enteromorpha intestinalis* (24.74 ± 0.86 mg/kg d.w.), followed by *Ulva lactuca* (21.62 ± 0.65 mg/kg d.w.) and *Cladophora vagabunda* (20.26 ± 0.85 mg/kg d.w.) The mineral content in green algae was in agreement with data reported in the literature by Rohani-Ghadikolaei et al. on green seaweeds from the Persian Gulf of Iran and Negreanu-Pirjol et al. on green algae from Romanian Black Sea coast [44,51]. The order of values for mineral content is comparable to our data. Thus, Rasyid et al. reported the mineral content for *Ulva reticulata* from Pameungpeuk Waters, Indonesia, in the following order, Ca > K > Na > Fe > P, and Metin et al. reported the mineral content for *Enteromorpha intestinalis* from the Turkish Mediterranean coast in the following order: Ca > K > Fe > Mn > Cu > Zn [3,69].

Concerning the pigment content results, two types of *chlorophylls*, *a* and *b,* in green seaweed were identified and were reported in mg/g (see Tables 2 and 6). Regarding the total chlorophyll content, the maximum chlorophyll content was detected in *Cladophora vagabunda* (41.64 ± 1.52 mg/g d.w), followed by *Ulva lactuca* (35.37±1.7 mg/g d.w), and the lowest content was found in *Enteromorpha intestinalis* (30.51±1.82 mg/g d.w). For *chlorophyll a*, the highest level was observed in *Cladophora vagabunda*, followed by *Ulva lactuca* and *Enteromorpha intestinalis* (see Table 6). In the case of chlorophyll *b*, the values obtained were attributed to the algae in ascending order as follows: *Cladophora vagabunda, Ulva lactuca*, and *Enteromorpha intestinalis* (see Table 6). The results are in agreement with data from the literature reported by Sirbu et al. and by Negreanu-Pirjol T. et al., who studied green algae from the Romanian Black Sea coast, and by Abd El-Baky et al., who studied Mediterranean green algae [35,37,53]. In the literature, we found out that these derivates show antimutagenic effects and may play a significant role in cancer prevention, as reported by Lee et al., who studied seaweeds from the marine waters of Taiwan [17]. Abd El-Baky et al. showed that, among natural pigments, chlorophyll *b* derivatives exhibited stronger antioxidant activity than chlorophyll *a* derivatives [53].

Relating to the total carotenoid content, the maximum amount was found in *Cladophora vagabunda* (17.66 ± 1.56 mg/g d.w.), followed by *Ulva lactuca* (16.25 ± 1.3 mg/g d.w.), and the lowest content was found in *Enteromorpha intestinalis* (15.98 ± 1.98 mg/g d.w.). The data in the present study are consistent with those of Christaki et al., who studied Aegean Sea algae, and of Abd El-Baky et al. (total carotenoid content for *Ulva lactuca* was 12.73 ± 1.32 mg/g d.w.), who studied Mediterranean green algae [52,53]. The results are comparable with data on chlorophyll *a* and total carotenes reported by Turan et al. for *Ulva lactuca* regarding green algae from the waters of the Mediterranean Sea [70].

The total flavonoid content (TFC) was obtained in terms of catechin equivalents (mg CE/100 g), with the upper level recorded in *Ulva lactuca* followed by *Enteromorpha intestinalis*. The lowest level was recorded in *Cladophora vagabunda* (see Tables 3 and 6). The presence of flavonoids has been considered responsible for the antioxidant and antimicrobial activity of algae. The obtained results are in accordance with the literature data reported by Sirbu et al., who analyzed Black Sea green algae in 2019, and by Ling et al., who studied seaweeds in the waters of Sabah, Malaysia [38,56].

The total phenol content (TPC) is expressed in terms of mg GAE/100 g d.w, and the results reveal the increasing order of values obtained: *Cladophora vagabunda*, followed by *Enteromorpha intestinalis* and *Ulva lactuca* (see Tables 3 and 6). The results are comparable with data obtained by Sirbu et al. for Black Sea green algae in 2019 and by Wekre et al., who studied algae from the western coast of Norway [38,60].

4.2. Biological Activities

The total phenol and flavonoid contents determine the antioxidant capacity, and the antioxidant activity was tested by means of three different specific methods, DPPH, reducing power, and TEAC. The type of extractant had a great impact on both the phenol and flavonoid content, and thus on the antioxidant capacity and antimicrobial activity of seaweed extracts. Ethanol was the most effective for phenolic extraction. In order to better exploit the potential of seaweed, new and improved extraction technologies need to be developed without using large amounts of toxic organic solvents.

In the DPPH test, from Figure 6, the mean values of DPPH (%) range between 4.85 ± 0.2% and 76.0 ± 0.6% for *Ulva lactuca*, between 8.66 ± 0.5% and 76 ± 0.6% for *Enteromorpha intestinalis*, and between 14.6 ± 0.3% and 78.578.5 ± 0.3% for *Cladophora vagabunda*. The antioxidant activity of green algae extract is comparable to that of the positive control, ascorbic acid. The highest values for 50% IC were found in *Ulva lactuca* (299.34 ± 1.31 µg/mL), followed by *Enteromorpha intestinalis* (276.33 ± 1.58 µg/mL), and the lowest value was observed in *Cladophora vagabunda* (259.66 ± 1.49 µg/mL) (see Figure 7). The results obtained are confirmed by many researchers who have shown the correlation between the total phenol content and the high antioxidant activity in seaweed, such as Zaatout et al., who studied seaweeds collected from the northern coasts of the Persian Gulf, Iran, and Alagan et al., who studied seaweeds from the marine coast of India [40,61]. Consistent results for the antioxidant activity of seaweeds have been reported by Kedare et al., collected from the marine waters of India, and by Chew et al., collected from the marine waters of Malaysia [62,63].

Reducing power has been established for concentrations in range of 50–500 mg/mL. The control solutions were L-ascorbic acid solutions with the same concentration range. Absorbances were read at 700 nm wavelength (see Figure 8). The highest values were recorded in *Cladophora vagabunda*, with values ranging from 0.02 ± 0.18 to 0.98 ± 0.45, followed by *Ulva lactuca*, with values ranging from 0.01 ± 0.01 to 0.69 ± 0.03, and the lowest value was observed in *Enteromorpha intestinalis*, ranging from 0.05 ± 0.21 to 0.62 ± 0.18. In the TEAC assay (Table 4), no negative values were recorded for the inhibition area or Trolox unit equivalents, indicating that all extracts used in this study exhibit remarkable antioxidant activity. Analysis of green algae extracts shows the best inhibition for *Ulva lactuca* extract (65.32 ± 0.1 nmol/g dry sample), followed by *Enteromorpha intestinalis* extract (59.05 ± 0.2 nmol/g dry sample), and the lowest inhibition was observed for *Cladophora vagabunda* extract (55.21 ± 0.4 nmol/g dry sample). TEAC values are expressed as nmol/g of dry samples. The results for the antioxidant activity of seaweeds are in agreement with those of other researchers, such as those reported by Farasat et al. for seaweeds collected from the northern coasts of the Persian Gulf, by Farvin et al. for seaweeds collected from the Danish coast, and by Alagan et al., who analyzed seaweeds collected from the marine coast of India [20,57,61]. Consistent results for reducing power have been reported by Karawita et al. for algae collected from the marine waters of South Korea [64]. The results obtained for antioxidant activity are also confirmed by Negreanu–Pirjol T. et al. in their study on seaweeds collected from the Romanian Black Sea coast and by Cho et al. in their study on seaweeds collected from the marine waters of South Korea [65,77].

The antibacterial activity of green algae was more efficient against Gram-negative bacteria in comparison with Gram-positive bacteria, as shown in Figure 9. From Figure 10, we can observe that *Cladophora vagabunda* ethanolic extracts showed good inhibition against Gram-negative bacteria: *E. coli* (18 ± 0.1 mm), *K. pneumoniae* (16 ± 0.1 mm), and *P. aeruginosa* (15 ± 0.1 mm); and Gram-positive bacteria: *S. aureus* (11 ± 0.2 mm) and *S. epidermis* (9.5 ± 0.2 mm). *Ulva lactuca* ethanolic extracts showed high inhibition against Gram-positive bacteria: *S. aureus* (12 ± 0.1 mm); and Gram-negative bacteria: *K. pneumoniae* (18 ± 0.1 mm), *P. aeruginosa* (12 ± 0.1 mm), *E. coli* (16 ± 0.1 mm) and *S. epidermis* (9.5 ± 0.1 mm). *Enteromorpha intestinalis* extracts demonstrated good inhibition against Gram-positive bacteria: *S. aureus* (10 ± 0.1 mm) and *S. epidermidis* (10.5±0.1 mm); and Gram-negative bacteria: *K. pneumoniae* (17.3 ± 0.1 mm), *E. coli* (12 ± 0.1 mm), and *P.*

aeruginosa (16 ± 0.1 mm). Alcoholic extracts of all three algal species tested showed low inhibition against *P. mirabilis*, with the highest observed for *Cladophora vagabunda* extracts (7 ± 0.2 mm), followed by *Ulva lactuca* ethanolic extracts (6 ± 0.2 mm) and *Enteromorpha intestinalis* extracts (7 ± 0.2 mm). In Table 5, the minimal inhibitory concentrations (MICs) of green algae extracts are presented. The most sensitive strains were *E. coli* and *K. pneumonia*, which showed the lowest MIC values (25 µg/mL). The bacterial strains *S. aureus* and *P. aeruginosa* showed MIC values of 50 µg/mL. The bacterial strain *S. epidermidis* was responsive to green algal extracts with an MIC value of 75 µg/mL. The bacterial strain *P. mirabilis* was the least sensitive strain to all green algal extracts, with MIC values of up to 100 µg/mL. Ethanol extraction showed higher antibacterial activity than methanolic extract extraction, which was also confirmed by Devi et al. and by Kolanjinathan et. al., who studied seaweeds collected from Tamil Nadu, India [78,79]. There are studies in the literature confirming that marine macroalgae can be considered sources of antimicrobial agents through their antibacterial activity, such as that reported by Al-Saif et al. in 2014 for algae from the Jeddah coast of the Red Sea, Saudi Arabia, [80]. Other research on the antibacterial activity of algae has been conducted by Spanish researchers such as Pérez et al. in 2016 and researchers in Indonesia, such as Ardita et al., who reported the antibacterial activity of *Ulva* species against *S. aureus* in 2021 [81,82] Figure 11 schematizes the direct links between biochemical compositions and biological actions, which are based on certain classes of compounds that have biomedical action, adapted from El-Beltagi et al. [83].

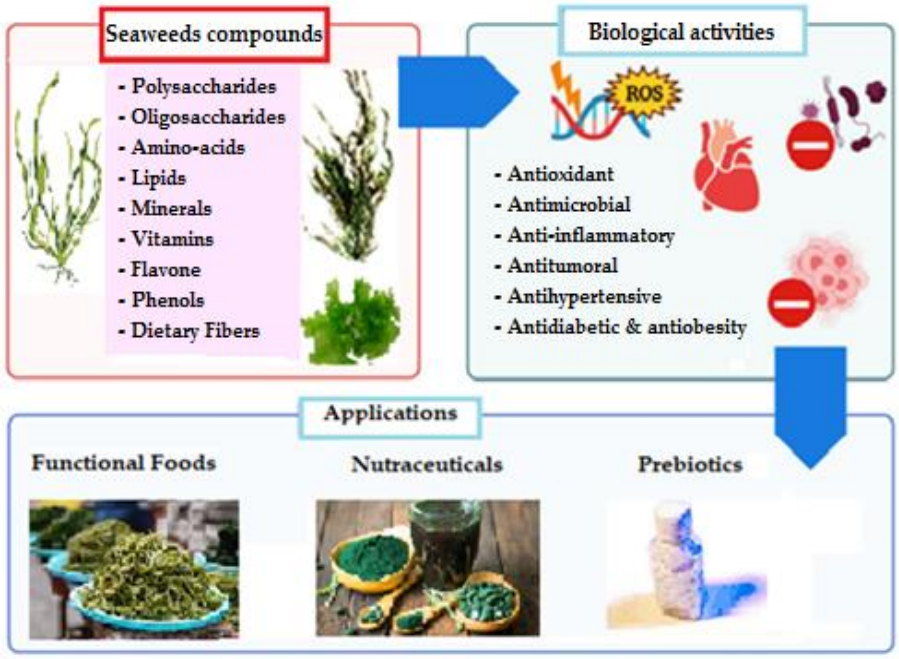

Figure 11. Qualitative value of seaweed nutraceuticals based on biochemical composition and biological actions.

5. Conclusions

In the present study, the biochemical compositions of green algae from the Romanian Black Sea coast were studied in order to highlight the rich resource that the bioactive compounds of these marine products represent, meaning that they can be considered valuable potential nutraceuticals.

As a conclusion of this phytochemical study carried out on three different algal extracts (alcoholic, etheric and aqueous), many important active principles were identified, such as sterols and triterpenes, coumarins, anthracenoids, amino acids, catechins, tannins, oses, polyoses, saponosides, and reducing compounds.

The conclusion of our study to determine the biochemical composition of marine algae from the Romanian coast was that the studied algal material presented a rich content

of compounds with nutritional value, such as sulphate compounds, polysaccharides, lipids, proteins, dietary fibers, pigments, chlorophylls and carotenoids, vitamins, phenolic compounds and flavonoids, which support their use in medical applications. In particular, the following important ideas can be highlighted.

- *Cladophora vagabunda* species have a higher carbohydrate (62.37 ± 1.74 %) and protein content than *Ulva sp*. (*Ulva lactuca* and *Enteromorpha intestinalis*). However, sulphate compounds are found in higher amounts in *Ulva lacuca* (70.46 ± 1.87%) compared to *Cladophora vagabunda*. The highest lipid content was identified in *Cladophora vagabunda* (2.86 ± 0.75 %) compared to *Ulva lactuca* and *Enteromorpha intestinalis*. The highest soluble dietary fiber content was identified in *Ulva lactuca* (29.45 ± 1.23%), followed by *Cladophora vagabunda* and *Enteromorpha intestinalis*.
- From the vitamins studied, the vitamin with the highest identified content was vitamin C. The marine algae *Cladophora vagabunda* (149.661 ± 0.58 mg/100 g d.w.) showed the highest vitamin C content compared with the other studied seaweeds.
- Algae from the Romanian Black Sea coast can be considered natural marine sources of calcium and potassium. *Ulva lactuca* has the highest Ca content (1790.35 ± 2.55 mg/100 g d.w.) and *Enteromorpha intestinalis* has the highest potassium content. Seaweeds from the Romanian coast are found in uncontaminated habitats and have a very low content of heavy metals. The green algae studied showed values well below the limits for toxic metals (such as Cd and Pb).
- From the content analysis of chlorophyll pigments, the content of both *chlorophyll a* and *chlorophyll b* was higher in *Cladophora vagabunda* (29.25 ± 1.56 mg/g d.w. for *chlorophyll a* and 12.39 ± 1.35 mg/g d.w. for *chlorophyll b*). The same result was obtained in the case of total carotenoid pigments: *Cladophora vagabunda* presented a higher content (17.66 ± 1.56 mg/g d.w.) compared to the other studied species. Laboratory studies showed that the highest content of flavonoid and phenolic compounds was recorded for *Ulva lactuca* compared to *Enteromorpha* and *Cladophora vagabunda.*
- Antioxidant and antibacterial activities were tested, and were supported by the rich and varied composition of the three seaweeds studied. The antioxidant activity was tested by means of three methods (the DPPH test, reducing power and TEAC analyses). In the whole range of concentrations tested, *Ulva sp*. showed higher values compared to *Cladophora vagabunda* in all three methods. In addition, *Ulva lacuca* showed higher values compared to *Enteromorpha intestinalis* for IC50, reducing power and TEAC tests.

Antibacterial activity was recorded for all green algae, but the most effective antibacterial activity was recorded against Gram-negative bacteria.

Cladophora vagabunda showed the best inhibition against *Escherichia coli*, while *Ulva lactuca* demonstrated the best inhibition against *Klebsiella pneumoniae.* These algae can be considered as a source of natural antibacterial agents.

As a general conclusion, future research directions can be explored for the industrial production of nutraceuticals of marine origin that are effective due to their multiple activities, including antioxidant, antibacterial, anti-inflammatory, anti-tumor, anti-diabetic and nutritive. Marine algae from the Black Sea represent an untapped natural source of potential nutraceuticals that can be used for new foods with health benefits.

Author Contributions: Conceptualization, E.C., T.N.-P. and R.S.; methodology, T.N.-P., R.S. and B.-S.N.-P.; validation, R.S. and T.N.-P.; investigation, R.S., E.C., T.N.-P., B.-S.N.-P. and A.-M.L.D.; software, E.C.; formal analysis, R.S., T.N.-P., E.C., A.-M.L.D., B.-S.N.-P. and E.R.A.; writing—original draft preparation, E.C., R.S. and A.-M.L.D.; writing—review and editing E.C., R.S., A.-M.I. and B.-S.N.-P.; resources, R.S., A.-M.I., B.-S.N.-P. and T.N.-P.; project administration, T.N.-P. and R.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Data are contained within in the article.

Acknowledgments: The authors thank the management of the National Marine Development Research Institute "Grigore Antipa" of Constanta, Romania and the Veterinary and Food Safety Directorate for the resources of green seaweed and for the help given in completing this study.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ACW: water-soluble antioxidative capacity; TFC: total flavonoid content; TPC: total phenol content; DPPH: α-diphenyl -β-picrylhydrazyl; TEAC: Trolox equivalent antioxidant capacity; T-Chl: total chlorophylls; *Chl a* and *Chl b*: chlorophylls *a* and *b*; AOAC: official statistical methods for analysis of variance data; SD: standard deviation; MIC: minimal inhibitory concentration; ATCC: American-type culture collection.

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