

# Evaluation of Antioxidant and Antimicrobial Activity in Relation to Total Phenolic Content of Green Algae from Black Sea

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*Worldwide, marine algae are known to produce a wide variety of bioactive secondary metabolites and several compounds have been derived from them for the prospective development of novel drugs by the pharmaceutical industry. In this context, Ulva lactuca, Enteromorpha intestinales, and Cladophora vagabunda, isolated from Black Sea coastal waters of Romania, were evaluated for their potential for bioactivity. Extracts of the algae selected for the study were prepared using ethanol. The study presents original results concerning the total phenolic content (TPC), determined by the spectrometric method (Folin-Ciocalteu method) and the individual phenols from each species of seaweed studied were identified by HPLC-DAD techniques. Total antioxidant activity (ACL) was studied by photochemiluminescence method, and antibacterial activity was studied on two strains of bacteria, Escherichia coli ATCC 10536 - gram-negative and Staphylococcus aureus ATCC 6538P - gram-positive. Results indicated that the highest total phenolic content was obtained for Cladophora vagabunda (325.8 mg/100 g f.w.), followed by Ulva lactuca (285.5 mg/100 g f.w.) and the lowest rate was registered by Enteromorpha intestinalis (268.8 mg/100 g f.w.). Through HPLC-DAD techniques, 6 individual phenolic compounds were identified in all analyzed seaweeds. In addition, two other phenolic compounds were identified in lower concentrations in Ulva lactuca, but only one phenolic compound in Enteromorpha intestinalis. The results of this study show that there is a strong correlation between antioxidant activity (ACL), antibacterial activity and total phenolic content (TPC). Antioxidant activity shows the same order as total phenolic content (TPC): Cladophora vagabunda (64.15 nmol/g sample) followed by Ulva lactuca (22.8 nmol/g sample) and Enteromorpha intestinalis (10.4 nmol/g sample). The antibacterial activity recorded in all green seaweeds shows that the inhibition sites are higher on Escherichia coli compared to Staphylococcus aureus.*

**Keywords:** green seaweeds, antioxidant activity, antibacterial activity, total phenolic content, HPLC-DAD

The effects of light and the presence of oxygen and active free radicals ( $O_2$ ,  $OH \cdot$ ,  $LOO \cdot$ ) on living systems has become a frequently researched topic [1]. Oxidative degradation of lipids from raw or processed food products is not responsible only for the loss of nutritional value, but it also contributes to carcinogenesis, atherosclerosis and the ageing process in humans [2]. Phenols are extremely efficient antioxidants for polyunsaturated fatty acids; in fact, they can easily transfer a hydrogen atom to the peroxy lipid radical (LOO) thus forming the aryloxy radical (ArO), which cannot act as chain carrier. This couples itself with another radical, thus stopping the chain process [3]. Amongst marine organisms, algae represent one of the richest sources of antioxidants [4]. Phenolic compounds are present in all plants [5-7]. They can be found in significant quantities in algae, alongside other important active biologic compounds, such as polysaccharides, fatty acids, proteins, terpenes, and so on, which suggest antitumor, antibacterial, antiviral and antioxidant activity [8, 9]. Marine algae are distributed globally in seas and oceans and can be found attached to rocks or other hard substrates from coastal regions, with an 0.01% availability of photosynthetic light [10]. These can be found in every climate area, from warm tropical waters to polar, freezing regions [11]. Green algae can be found worldwide, growing in intertidal zones, attached to a hard substrate or free [12]. These are considered opportunistic algae and they proliferate in eutrophic coastal waters [13]. There are numerous studies regarding green algae [14, 15]. Thus, phenols and active biologic compounds have been found in several green algae species which can be observed in

various seas and oceans [16]. Marine algae have been studied in the past years as a potential source of bioactive compounds, due to the fact that they have the ability of producing a series of secondary metabolites with a variety of interesting biological effects, such as antimicrobial, antiviral and antioxidant activity [17, 18]. There has been a particular interest in researching new antimicrobial substances originating from green marine algae from various seas and oceans [19-21]. Algae contain a high number of antibacterial compounds, such as fatty acids [22], phenols [23], steroids [24, 25] and halogenated compounds [26, 27]. In the past years, algae extracts have been widely used in the prevention and treatment of viral and bacterial disease in aquatic animals [28]. Algae extracts have shown a powerful antibacterial activity against aquatic pathogenic bacteria [29]. From the green algae, *Ulva lactuca* has been studied by numerous researchers and it has become a model for morphogenetic studies [30]. Studies regarding green algae composition were also performed by other researchers who have taken into account the seasonal changes in compositions for *Ulva lactuca* [31]. Other studies have determined the chemical composition of ulvan [32, 33] and have isolated extracts with antioxidant and antibacterial activity [34-36]. The Black Sea, like other seas and oceans, offers a rich algae mass which can be harvested, thus representing an important resource for the medical and pharmaceutical fields [37]. Until now, studies regarding the characterization of marine algae, which have been used in various purposes, have been performed [38]. Regarding Black Sea algae, marine habitats in which algae develop have been studied,

especially in the Romanian shore area [39]. However, the antioxidant and antimicrobial activity of the seaweed remains unexplored. The main objective of this paper is to evaluate the total phenol content and its correlation with antioxidant and antimicrobial activity of *Clorophyta* green algae. The following species have been selected: *Ulva Lactuca* (Linnaeus, 1753) [40] and *Enteromorpha intestinales* (Linnaeus-Nees, 1820) from the *Ulvophyceae* class, *Ulvales* order, *Ulvaceae* family and *Cladophora vagabunda* (Linnaeus-van den Hoek, 1963) [41], from the *Chlorophyceae* class, *Cladophorales* order, *Cladophoraceae* family. Phenolic content in alcoholic extracts from algae harvested from six different regions of the Romanian shore was determined, and the antioxidant (ACL) and antimicrobial activity of the algae was evaluated. The total phenolic content was determined through the Folin-Ciocalteu methods. Phenolic compounds from green algae extracts were determined through HPLC-DAD techniques. Antioxidant capacity (ACL) was evaluated through the chemiluminescence method, using trolox as the standard substance. Green algae antibacterial activity was evaluated on two strains of bacteria: gram negative *Escherichia coli* and gram positive *Staphylococcus aureus*.

## Experimental part

### Plant materials

The samples of green seaweed were collected during July-October 2017, when algae reach maturity in different harvesting sites. Samples were harvested from 6 areas, and named as: S1 through S6: S1- Mamaia North, S2 - Constanta Casino, S3 -Constanta harbor, S4 -Eforie South, S5 -Costinesti, S6- Mangalia. Each collected sample was thoroughly washed with seawater to remove all the extraneous matter such as epiphytes, sand particles, pebbles and shells. In the laboratory the sample was cleaned with fresh water and distilled water, then dried and powdered using mixture grinder. The powdered seaweeds were passed through a 0.5 mm sieve in order to obtain a uniform powder which was stored in the freezer until extraction.

### Chemicals

All used reagents were of analytical reagent grade. Gallic acid was purchased from Fluka and Folin - Ciocalteu reagent from Merck. Gallic acid (standard phenol compound)  $1 \times 10^{-2} \text{ mol} \times \text{L}^{-1}$  solution was prepared by dissolving 376 mg of gallic acid in 100 mL of ethanol. Folin - Ciocalteu reagent was diluted with distilled water 1:10 (v:v). The gram positive and gram negative bacteria strains were grown on culture media.

### Apparatus

Spectrometric measurements were carried out using a UV-Vis *Cintra 10e* scanning spectrophotometer. The identification and quantitative determination of phenolic compounds were performed using HPLC system (Agilent 1200) with quaternary pump, DAD, auto sampler. The antioxidant capacity of lipid soluble substances determination was performed with a Photochem instrument with ACL kit (Analytic Jena  $\Delta$ G).

### Preparation of algal extract

Ethanol was used as an extraction solvent. For the preparation of the ethanol extract, 5 g of powdered seaweed was extracted overnight with 50 mL of 70 % ethanol (v:v) at room temperature and centrifuged at 2800 rpm for 10 min. The supernatant was collected in a separate bottle and the residue was re-extracted three times under

the same conditions as mentioned above. The combined filtrate was evaporated in a rotary evaporator (BUCHI) below 40 °C. The extract obtained after evaporation of the organic solvent was weighed for calculation of extraction yield and was used as a natural antioxidant.

### Determination of total phenolic content (TPC)

Total phenolic contents in extracts were determined according to the Folin - Ciocalteu method [42, 43] using a UV spectrophotometer (*Cintra Xe*) according to a colorimetric reduction of a phosphowolframate - phosphomolibdate complex to blue products in sodium carbonate media. The absorbance relative to a gallic acid standard curve was measured and results are expressed as gallic acid equivalents (mgGAE/L). All samples were performed in triplicate and the mean value was reported.

### Calibration curve

The calibration curve (fig. 1) was graphically represented by mixing 1 mL aliquots of 50, 100, 150, 200, 250, 300, 350, 400 and 450 mg/L gallic acid solutions with 5.0 mL of Folin-Ciocalteu reagent (1:10) and 4.0 mL of sodium carbonate solution (75g/L). Absorbance was measured after 30 min at 765 nm. In order to determine total phenolic content from ethanol extracts, 1 mL of sample was mixed with the same reagents as the ones used for the calibration curve.

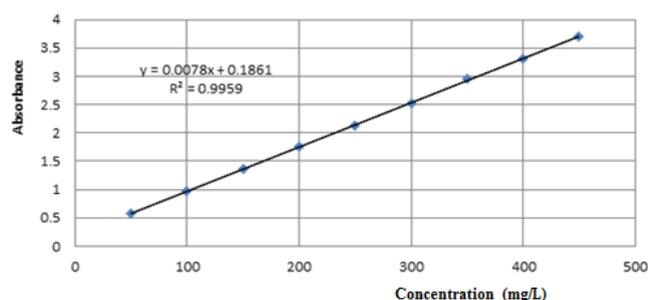


Fig.1. Calibration curve of gallic acid standard

The amount of total phenol contents was calculated as Gallic acid equivalents in mg/100 g of dried seaweed (mg GAE/100 g f.w.).

### Identification of phenolic acids by HPLC

Identification of phenolic acid was performed through HPLC-DAD method. The extract was passed through a 0.45  $\mu\text{m}$  filter (Millipore) before being injected into the HPLC. Reverse phase HPLC was performed with an Agilent 1100 series HPLC (Agilent Technologies), equipped with a DAD detector (Agilent G13158). The column used was a ZORBAX Eclipse XDB C8 analytical column (150 x 4.6 mm) with a 5 $\mu\text{m}$  packing material. The elution was performed with an isocratic mixture of methanol and 10 mM ammonium acetate buffer, pH 5.4 (12:88 v/v) at a flow of 1 mL/min. Detection was done using a diode array detector (DAD) with reference wavelength of 280 nm. Retention times and peak areas were monitored and computed automatically by Chem32 integrator (Agilent). The standard reference substances chosen were gallic acid, protocatechuic acid, gentisic acid, p-hydroxybenzoic acid, vanillic acid, caffeic acid, salicylic acid, benzoic acid, ferulic acid, elagic acid and p-coumaric acid, for which a 0.05 mg/mL concentration was established. Standards were provided by Sigma Aldrich. Individual phenolic acids were identified by comparing the retention time of sample chromatographic peaks with those of authentic standards using the same HPLC operating conditions. Table 1 presents the determined retention time of standards.

**Table 1**  
THE RETENTION TIME OF STANDARDS

| No. | Standards             | Retention time $\pm$ SD |
|-----|-----------------------|-------------------------|
|     | Gallic acid           | 0.990 $\pm$ 0.025       |
|     | Protocatechuic acid   | 3.630 $\pm$ 0.008       |
|     | Gentisic acid         | 4.272 $\pm$ 0.009       |
|     | p-Hydroxybenzoic acid | 5.933 $\pm$ 0.006       |
|     | Vanillic acid         | 7.752 $\pm$ 0.003       |
|     | Caffeic acid          | 8.981 $\pm$ 0.061       |
|     | Salicylic acid        | 9.177 $\pm$ 0.051       |
|     | Benzoic acid          | 9.468 $\pm$ 0.098       |
|     | Ferulic acid          | 10.488 $\pm$ 0.009      |
|     | Elagic acid           | 15.630 $\pm$ 0.031      |
|     | p-Coumaric acid       | 15.971 $\pm$ 0.063      |

#### Statistical analysis

All experiments were conducted in triplicate and ANOVA (using SPSS 11.5 statistics) software was used to compare the mean values of each treatment. Significant differences between the means values of parameters were determined by using the Duncan test ( $P < 0.05$ ).

#### Determination of total antioxidant activity (ACL)

The ACL assay was performed using Photochem instrument (Analytic Jena) against the superoxide anion radicals from luminol as photosensitizer, upon the UV light [44]. In order to determine total antioxidant capacity (ACL) of biological samples, the well dried algae were triturated to a fine powder, after which a cold extract was obtained from each alga species, using 20 mg of dried vegetal product dissolved in 1 mL ethylic alcohol p.a. [45]. Extraction was performed for 24 h at room temperature in yellow glass sealed bottles. Mixtures were stirred regularly and extraction separation was performed through decantation, without filtration. Antioxidant capacity was determined using the standardized ACL method (antioxidant capacity of lipid soluble substances, Analytik Jena 10) in comparison to the standard substance trolox (brand name Hoffman-La Roche-6-hydroxy-2, 5, 7, 8-tetramethylchromen-2-carboxylic). For the calibration curve, an Analytik Jena standard reagent kits was used: R1 (dilution solvent), R2 (buffer reagent), R3 (photosensitive reagent), R4 (reagent dimension).

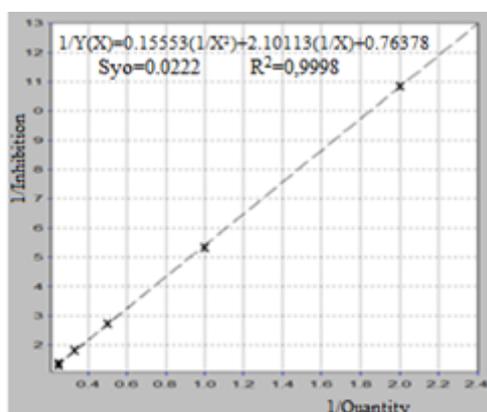


Fig. 2. Calibration curve for trolox in the calculation of trolox equivalents for ACL measurements (generated by PCLsoft)

The calibration curve was designed through the measurement of a series of standard solutions containing 0.5, 1.0, 2.0, 3.0 and 4.0 nmol trolox and is presented in Fig. 2. Photochemical reaction was generated by exposure to external radiation from a Hg lamp which yields maximum energy at  $\lambda = 351$  nm. Total antioxidant capacity of the sample was measured by comparison to the trolox standard, in standard equivalent units [45].

#### Antibacterial activity

In order to test antimicrobial activity of the green seaweed, we used several culture media. We worked on two types of bacteria: gram-positive *Staphylococcus aureus* ATCC 6538P and gram-negative *Escherichia coli* ATCC 10536. The analysis method used was the nutrient agar well diffusion method. The methodology involves the preparation of nonspecific nutrient agar plates, filled with a known concentration of bacterial medium and measuring the inhibition halos formed by the ethanol extract solutions from green seaweed. Algae species were washed, dried and brought to a corresponding grinding degree. Dried algae were subjected to ethanol extraction (5 g powder per 100 mL ethanol) through continuous stirring at 37 °C, for 24 h. The samples were subjected to centrifugation for 30 minutes and the supernatant was evaporated under vacuum. The extracts were re-dissolved in ethanol.

#### Preparation of nutrient agar plates

Culture media was prepared for each strain, *Staphylococcus aureus* and *Escherichia coli* (fig.3). Petri dishes in which 1 mL reference solution (Inoculum) and 80 mL of nonspecific nutrient agar were added, sterilized and homogenized (fig.3). The plates were left to cool at room temperature until the agar became solid. Two plates with positive witness samples were also prepared in order to compare results.

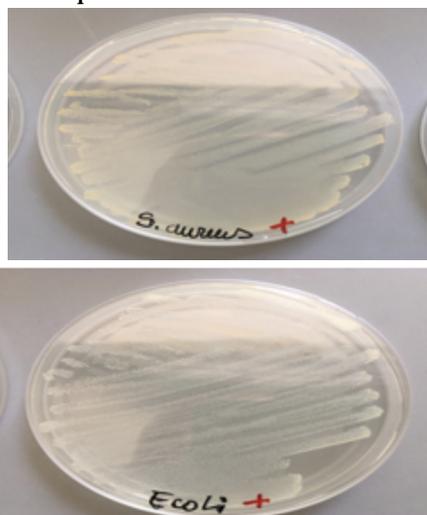


Fig.3 Culture media for *Staphylococcus aureus* and *Escherichia coli*

For each of the strain, a standardized reference solution with a known concentration ( $3 \times 10^{-8}$  g/mL) was prepared. After solidification of the agar from the Petri dishes the wells were made, using a sterilized matrix with a known diameter, corresponding to each solution volume. Thus, for a 0.25 mL volume, was a matrix with a diameter of 10 mm was used, whereas, for a 0.5 mL volume, we used a matrix with a diameter of 15 mm, and for a 1 mL volume, the a diameter was of 17 mm. Interpretation of the results of this antimicrobial analysis was performed through the comparison of the obtained samples with the positive witness plate for each studies pathogen culture. The diffusion radius of the green algae extract solution in the agar contaminated with the study strain was measured.

The halo formed around the wells represents growth inhibition and the halt of pathogen spread; the radii differ according to the type of alga, as well as the concentration and the solution volume from the algae extracts.

#### Statistical analysis

A statistical approach was designed and the experimental data were evaluated using one-way analysis of variance (ANOVA), with  $p < 0.05$  as threshold for statistical significance. The statistical results confirm the hypothesis that the differences between the results are either not significant ( $p > 0.05$ ). The average of multiple measurements (triplicates or more) was listed in the tables together with the standard deviations. Statistical analysis was performed using Excel software package.

### Results and discussions

#### Total phenol content (TPC)

Three different green seaweed from the *Chlorophyta* class were studied: *Ulva lactuca*, *Enteromorpha intestinalis* and *Cladophora vagabunda*. The results of total phenolic content (TPC) determined for ethanol extracts of these green algae are presented in table 2.

The total phenolic content ranged from 268.8 to 325.8 mg/100 g dried seaweed in ethanol. The lowest, but still rich, total phenolic content was obtained for *Enteromorpha intestinalis* (268.8 mg/100 g f.w.). The highest total phenolic content was obtained for *Cladophora vagabunda* (325.8 mg/100 g f.w.) followed by *Ulva lactuca* (285.5 mg/100 g f.w.).

There are studies in literature which outline the fact that the type of solvent influences selectivity of phenol compounds [42]. The study performed by Wang et al. [23], shows that water as solvent is inferior to polar organic solvents in the extraction of polyphenolic compounds, which confirms our use of ethanol as a solvent.

| No. | Sample                                     | Taxonomy  | Concentration (mg GAE/100 g f.w.) |
|-----|--|---|-----------------------------------|
| 1   | <i>Ulva lactuca</i> (L.)                   | <i>Chlorophyta, Ulvales, Ulvaceae</i>             | 285.5 ± 0.6                       |
| 2   | <i>Enteromorpha intestinalis</i> (L.-Ness) | <i>Chlorophyta, Ulvales, Ulvaceae</i>             | 268.8 ± 0.7                       |
| 3   | <i>Cladophora vagabunda</i> (L.-C Hoek)    | <i>Chlorophyta, Cladophorales, Cladophoraceae</i> | 356.8 ± 0.3                       |

Values are mean ± SD (n = 3)

**Table 2**  
TOTAL PHENOL CONTENT (TPC) OF ETHANOLIC EXTRACTS OF GREEN SEAWEEDS FROM THE ROMANIAN BLACK SEA COAST

| No. | Phenolic compound     | <i>Ulva lactuca</i> (L.)<br>mg/100 g f.w. | <i>Enteromorpha intestinalis</i> (L.-Ness)<br>mg/100 g f.w. | <i>Cladophora vagabunda</i> (L.-C Hoek)<br>mg/100 g f.w. |
|-----|-----------------------|---|---|--|
| 1   | Gallic acid           | 5.3±0.1                                   | 8.5±0.05  | 7.9±0.1  |
| 2   | Protocatechuic acid   | 72.8±0.2                                  | 55.6±0.1  | 75.9±0.03  |
| 3   | Gentisic acid         | 130.5±0.1                                 | 98.6±0.1  | 155.3±0.15   |
| 4   | p-Hydroxybenzoic acid | 2.3±0.1                                   | 1.5±0.05  | 2.6±0.01   |
| 5   | Vanillic acid         | 1.6±0.02                                  | 1.8±0.02  | 12.8±0.01  |
| 6   | Caffeic acid          | 13.0±0.13                                 | 15.7±0.11   | 11.7±0.09  |
| 7   | Salicylic acid        | 1.3±0.02                                  | 0.9±0.01  | 0  |
| 8   | Benzoic acid          | 1.5±0.03                                  | 0   | 0  |
| 9   | Ferulic acid          | 0   | 0   | 0  |
| 10  | Elagic acid           | 0   | 0   | 0  |
| 11  | p-Coumaric acid       | 0   | 0   | 0  |
|     | Total                 | 228.3                                     | 182.6   | 266.2  |

Values are mean ± SD (n = 3)

**Table 3**  
INDIVIDUAL PHENOLIC CONCENTRATIONS DETERMINED BY HPLC -DAD IN GREEN SEAWEED FROM THE ROMANIAN BLACK SEA COAST

#### HPLC analysis of phenol compounds

Table 3 presents the individual phenolic concentrations determined by HPLC -DAD expressed in mg/100 g f. w. By correlating the total phenolic content (TPC) and individual phenols the majority phenolic content can also be expressed as percentage (table 4). It can be observed that the total phenols identified and quantified were in different percentages for the ethanol extracts from the three algae. The highest percentage of individual phenols was identified in *Ulva lactuca* (79.96%), followed by *Cladophora vagabunda* (74.6%), while the lowest percentage was obtained for *Enteromorpha intestinalis* (67.93%). Table 3 shows that all three *Chlorophytele* green algae contain gentisic acid; the highest quantity was identified in *Cladophora vagabunda* (155.3 mg/100 g f.w.), followed by *Ulva lactuca* (130.5 mg/100 g f.w.) and *Enteromorpha intestinalis* (98.6 mg/100 g f.w.). If we correlate the total phenol content (TPC) identified and quantified in the analyzed seaweed with the gentisic acid content found (see Table 4), it can be observed that this acid has the highest percentage of total existing phenols in *Ulva lactuca* (46.0%), followed by 43.52% in *Cladophora vagabunda* and 36.68% in *Enteromorpha intestinalis*.

Protocatechuic acid is another phenol compound found in considerable amounts in green seaweed ethanolic extracts. This acid was found in *Cladophora vagabunda* (75.9 mg/100 g f.w.), in *Ulva lactuca* (72.8 mg/100 g f.w.) and the least in *Enteromorpha intestinalis* (55.6 mg/100 g f.w.).

Table 4 outlines the following percentage order in which protocatechuic acid was found: 25% in *Ulva lactuca*, 21.27% in *Cladophora vagabunda* and 20.68% in *Enteromorpha intestinalis* from the total content of polyphenols identified in each alga.

**Table 4**

THE PERCENTAGES (%) OF TOTAL PHENOLS AND PERCENTAGES (%) OF MAJOR PHENOL COMPOUND IDENTIFIED IN TESTED ALCOHOLIC BERRIES EXTRACTS DETERMINED BY HPLC-DAD REPORTED TO TPC VALUES

| No. | Sample                           | Total phenols identified and quantified % | The major phenolic compound identified and quantified |       |                     |       |                   |      |
|-----|----------------------------------|---|---|-------|---------------------|-------|-------------------|------|
|     |                                  |   | phenolic compound                                     | %     | phenolic compound   | %     | phenolic compound | %    |
| 1   | <i>Ulva lactuca</i>              | 79.96                                     | Gentisic acid   | 46.0  | Protocatechuic acid | 25.5  | Caffeic acid      | 4.55 |
| 2   | <i>Enteromorpha intestinalis</i> | 67.93                                     | Gentisic acid   | 36.68 | Protocatechuic acid | 20.68 | Caffeic acid      | 5.84 |
| 3   | <i>Cladophora vagabunda</i>      | 74.60                                     | Gentisic acid   | 43.52 | Protocatechuic acid | 21.27 | Caffeic acid      | 3.28 |

Another acid found in significant amounts is represented by caffeic acid: in *Enteromorpha intestinalis* (15.7 mg/100 g f.w. with a percentage of 5.84%), in *Ulva lactuca* (13.0 mg/100 g f.w. with a percentage of 4.55%) and in *Cladophora vagabunda* (11.7 mg/100 g f.w. with a percentage of 3.28%). It has been noted that in *Cladophora vagabunda*, the vanillic acid (12.8 mg/100 g f.w.) was found in comparable amounts with caffeic acid (11.7 mg/100 g f.w.). The remainder phenolic acids which were identified and quantified through HPLC-DAD, were found in smaller concentrations. Due to different extraction methods and different species studied, as well as the measurements used and the lack of information regarding phenolic composition of Black Sea algae in literature on the matter, a direct comparison between our results and studies performed in other seas is not feasible.

#### Antioxidant activity (ACL)

It is very important to examine the correlation between the total phenol content and the total antioxidant activity (ACL) in the studied algae. The extraction method used to obtain fluid extracts was cold macerated in 70% concentration ethylic alcohol, for 24 h in the dark. A good total antioxidant capacity was generated, as it can be seen from the curves obtained using the Photochem instrument with ACL kit (fig.4). Table 5 presents the results for ACL expressed as Trolox units equivalents (nmol/g of dry sample) for the three algae.

The highest ACL values were registered by *Cladophora vagabunda* (64.15 nmol/g dry alga), followed by *Ulva lactuca* (22.8 nmol/g dry alga) and *Enteromorpha intestinalis* (10.4 nmol/g dry alga). This order is in accordance with the total polyphenol content (TPC) in the analyzed algae: *Cladophora vagabunda* (325.8 mg/100 g f.w.), followed by *Ulva lactuca* (285.5 mg/100 g f.w.) and *Enteromorpha intestinalis* (268.8 mg/100 g f.w.).

The results of this study confirm the fact that there is a strong correlation between total antioxidant activity (ACL) and total polyphenol content (TCL).

It is believed that the antioxidant properties of polyphenols are the result of their ability to act as reducing agents, hydrogen donors and free radicals and of caffeol's function as metal chelator, which impede the catalytic functioning of metal in the radical initiation process [16, 46].

#### Antibacterial activity

The antibacterial activity of the three green algae is the result of their high phenolic compound concentrations. Figure 5 and figure 6 present the inhibition halos formed as a result of the own antimicrobial action exercised by each alga extract (*Cladophora vagabunda*, *Ulva lactuca*, *Enteromorpha intestinalis*), on bacterial strains of *Escherichia coli* and *Staphylococcus aureus*.

It can be observed that the analyzed alga extracts exhibit different radii for bacterial growth inhibition for each

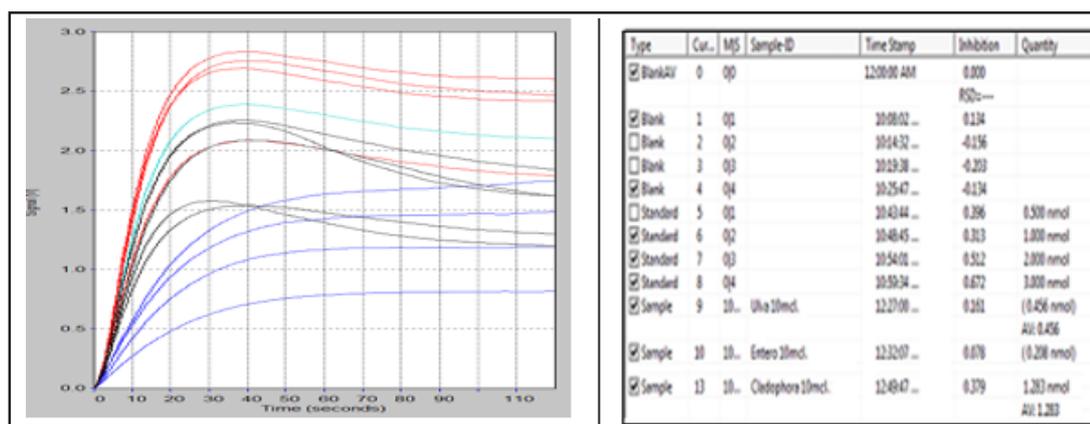


Fig. 4 Antioxidant activity (nmol trolox / mg fresh weight) for undiluted sample and diluted samples 1:25 measured using PHOTOCHEM equipment

| Sample (type of green alga)      | Volume of used sample (µL) | Analysis time (sec.) | Trolox units equivalent (nmol/sample volume) | Trolox units equivalent (nmol/ g dry sample) |
|----------------------------------|----------------------------|----------------------|--|--|
| <i>Ulva lactuca</i>              | 20                         | 120                  | 0.456  | 22.8   |
| <i>Enteromorpha intestinalis</i> | 20                         | 120                  | 0.208  | 10.4   |
| <i>Cladophora vagabunda</i>      | 20                         | 120                  | 1.283  | 64.15  |

**Table 5** RESULTS OBTAINED FOR THE ACT DETERMINATION ON GREEN ALGAE EXTRACTS

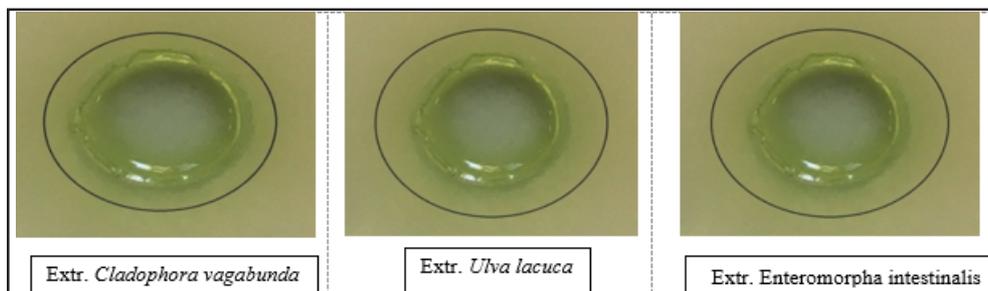


Fig.5 The antimicrobial effect of alcoholic alga extracts on *Escherichia coli*



Fig.6 The antimicrobial effect of alcoholic alga extracts on *Staphylococcus aureus*

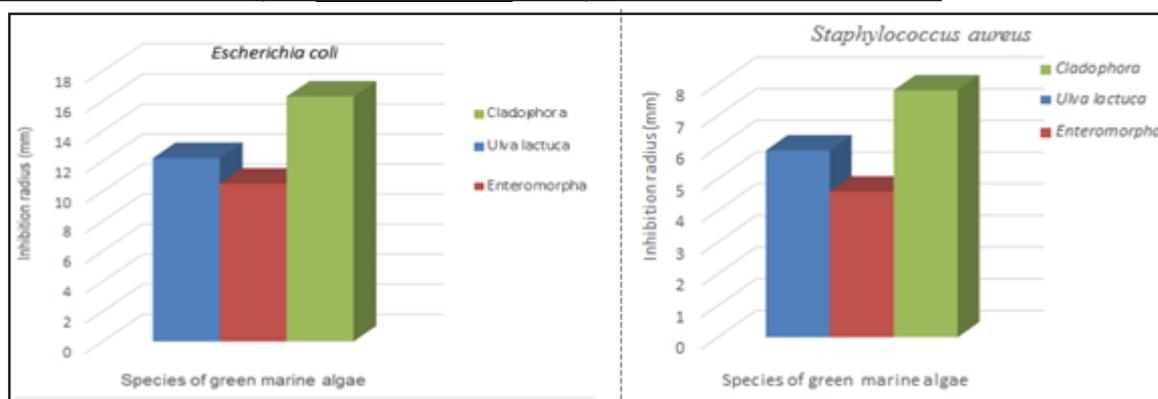


Fig.7. Antibacterial Activity of ethanolic extracts from green seaweeds in the Romanian Black Sea coast on strains of bacteria (*Escherichia coli* and *Staphylococcus aureus*)

of the three algae. This antimicrobial effect, evidenced through the size of the inhibition areas is an important property of seaweed, of great use in the pharmaceutical field. Figure 7 shows the graphical representation of antimicrobial activity of seaweed extracts on the analyzed pathogen germs. Inhibition radii obtained for the studied algae extracts are similar in value, with a different antimicrobial effect on the bacterial strains that can be observed. In our study the highest activities of ethanol extracts (10.5 mm, 12.2 mm and 16.3 mm) were recorded for *Escherichia coli* and the lowest (4.6 mm, 5.9 mm and 7.8 mm) for *Staphylococcus aureus* at 100 µg/mL. Although literature on the matter holds few data regarding antibacterial activity of green alga in the Black Sea, our results are in accordance with the antibacterial activity of other green seaweed species (*Ulva reticulata* and *Cladophora socialis*) in ethanolic extracts that can be found in the Red Sea [47]. In literature, the maximum activity was recorded in ethanol and ethyl acetate extracts from seaweeds and both of these extracts exhibited reasonable activity against all the tested pathogens [48, 49], which confirms our choice of solvent. Studies performed on different types of seaweed originating from the territorial South African waters have been reported, in which the antibacterial activity manifests itself all throughout the year at different intensities [50].

Other researchers claim that the activity of algae originating from India's territorial waters can be due to the variation in cellular chemical and biological composition according to season, habitat, and life stage of the respective alga [51]. Recent studies performed by Chinese researchers

[52] have shown that there is an evident seasonal variation in antibacterial activity of green algae harvested from China's territorial waters [52]. Also, Chinese researchers have observed that *Ulva lactuca* has the highest antibacterial activity from the beginning of winter (December) until mid-spring (April) [52].

### Conclusions

This is the detailed study of in vitro antioxidant and antibacterial activity of seaweeds from the Romanian Black Sea coast in which phenolic compounds were identified and quantified from a range of green seaweeds. The results of this screening experiment demonstrated that extracts of the different seaweed species contain different levels of total and individual phenolics and possess diverse antioxidant and antibacterial properties.

Three green algae species from the *Clorophyta* class were analyzed: *Ulva lactuca* (L.), *Enteromorpha intestinalis* (L-Nees) and *Cladophora vagabunda* (L-Hoek). The total phenolic content (TPC) as determined by spectrophotometry (the Folin-Ciocalteu method) was found in all studied algae, in varying concentrations. The highest total phenolic content was obtained for *Cladophora vagabunda* (325.8 mg/100g f.w.), followed by *Ulva lactuca* (285.5 mg/100g f.w.) and *Enteromorpha intestinalis* (268.8 mg/100g f.w.).

Using HPLC-DAD techniques 6 individual phenol compounds were found, in varying quantities, in all the analyzed algae. High contents were obtained for: gentisic acid (98.6-155.3 mg/100 g f.w.), protocatechuic acid 55.6-75.9 mg/100 g f.w.), caffeic acid (11.7-15.7 mg/100 g f.w.),

gallic acid (5.3-8.5 mg/100 g f.w.), and in lower contents for p-vanillic acid (1.6-12.8 mg/100 g f.w.) and p-hydroxybenzoic acid (1.5-2.6 mg/100 g f.w.). Moreover, salicylic acid was found in *Ulva lactuca* (1.3 mg/100 g f.w.) and in *Enteromorpha intestinalis* (0.9 mg/100 g f.w.), while benzoic acid was found in *Ulva lactuca* (1.5 mg/100 g f.w.). The results of this study show that there is a strong correlation between the total antioxidant activity (ACL), the antibacterial activity, and the total polyphenol content (TCL). Antioxidant activity, as determined through chemiluminescence, registers the same order as the total phenolic content: *Cladophora vagabunda* (64.15 nmol/g sample) followed by *Ulva lactuca* (22.8 nmol/g sample) and *Enteromorpha intestinalis* (10.4 nmol/sample). The antibacterial activity for the two strains of bacteria analyzed in this study also registers the same order as that of the total phenolic content. What is more, ethanolic extracts from the green algae show larger areas of inhibition for *Escherichia coli* compared to *Staphylococcus aureus*. It must be noted that the Black Sea has an important potential of yielding pharmaceutically useful algae, which can be harnessed for the development of drugs for use in management of human pathogens, cancer, tumor, AIDS and many human degenerative diseases.

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