# Physico-Chemical Methods Applied To Bioactive Compounds Determination From Ceramium Rubrum On The Romanian Coast Of The Black Sea

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## Abstract

The red seaweed species Ceramium rubrum found in abundance along the Romanian Black Sea coast. Phylum Rhodophyta, Class Florideophycidae, Family Ceramiaceae, Genus Ceramium to which the species Ceramium rubrum belongs, is recognized due to two compounds: agar-agar and carrageenan which are widely used in the medical, pharmaceutical and food industry for their properties, agar-agar and carrageenan. The study aims to determine and quantify the active principles in the red seaweed Ceramium rubrum using IR and UV-VIS spectrometry and chromatographic methods. For IR spectrometry, dry algal powder and standard β-carotene used. For simultaneous evaluation of β-carotene, chlorophyll a and chlorophyll b, a chromatographic method (high performance liquid chromatography) applied. The antioxidant capacity analyzed by chemiluminescence method, showing remarkable results.

**Keywords:** Ceramium rubrum, red algae, bioactive compounds, UV-VIS, IR, HPLC analysis

## Introduction

The species Ceramium rubrum is part of the red algae group: Phylum Rhodophyta, Class Florideophycidae, Family Ceramiaceae, Genus Ceramium (Huds. C. Ag.), which represented by multicellular algae. This is also present in the Black Sea, throughout the year, but especially in spring and summer, at depths from 0.5m to 4.5m [1]. Ceramium rubrum colonizes the rocky substrata on the infralittoral and medium, being an annual species. Algae are well known for their biotechnological significance,

especially red algae, but they are also appreciated as a natural food source [2]. Nowadays, we want to discover new biologically active compounds from red seaweeds, these being the least studied, with pharmaceutical, cosmetic and nutraceutical applicability [3]. Polysaccharides are also important compounds found in all types of algae. A study determined a yield of 4.27% polysaccharides obtained from the species Ceramium rubrum, the galactan type being in the highest proportion of 78%. In Figure 1 is shown Ceramium rubrum algae.

**Figure 1. Ceramium rubrum algae**

Galactans (agar and carrageenan) used in the food industry as a food additive to thicken or stabilize food, but also for technical purposes [4]. Another study carried out on Ceramium rubrum in Turkey, concluded that this species has antioxidant properties due to the high content of phenolic compounds, flavonoids and carotenoids [5]. In a recent study where 3 species of algae from the Sea of Marmara were examined, including the species Ceramium rubrum, it revealed an increased content in total proteins and lipids for this species. Fatty acids were also determined, and among them the most abundant were palmitic and oleic acids. Linoleic acid and α-linolenic acid, which cannot be synthesized by humans and are important for nutrition, have been detected in Ceramium rubrum [6]. Red algae are also important due to their antimicrobial capacity, which has been proven in various studies, including one that was conducted on 4 species of red algae, including the species Ceramium rubrum, collected from the Red Sea in Egypt. These were tested against ten multidrug resistant clinical isolates of Gram + and Gram - bacteria and different concentrations of methanol extract of C. rubrum show activity against S. flexneri, E. coli, P. aeruginosa and K. pneumoniae. The bioactive compounds that have been identified in red algae and due to which the antioxidant capacity is present are phenols, terpenes, acetogenins, indoles, fatty acids and volatile halogenated hydrocarbons [7]. Chlorophylls are greenish pigments, playing a key role in capturing energy from the light source, transferring it and separating charges during photosynthesis. They are non-polar, and they contain a hydroporphyrin ring or a porphyrin centrally linked to a magnesium ion. Cancer research has shown that chlorophyll is a very powerful therapeutic remedy for this condition,

chemoprevention and chemotherapy [8]. Carotenoids have an important role in the protection of all plants against photooxidative processes, because they have an effect of trapping peroxyl radicals and singlet oxygen, due to their antioxidant capacity α-carotene, β-carotene and their derivatives are the main carotenoins found in red algae. Figure 2 shows the chemical structures of the 3 pigments chlorophyll a, chlorophyll b and β-carotene.

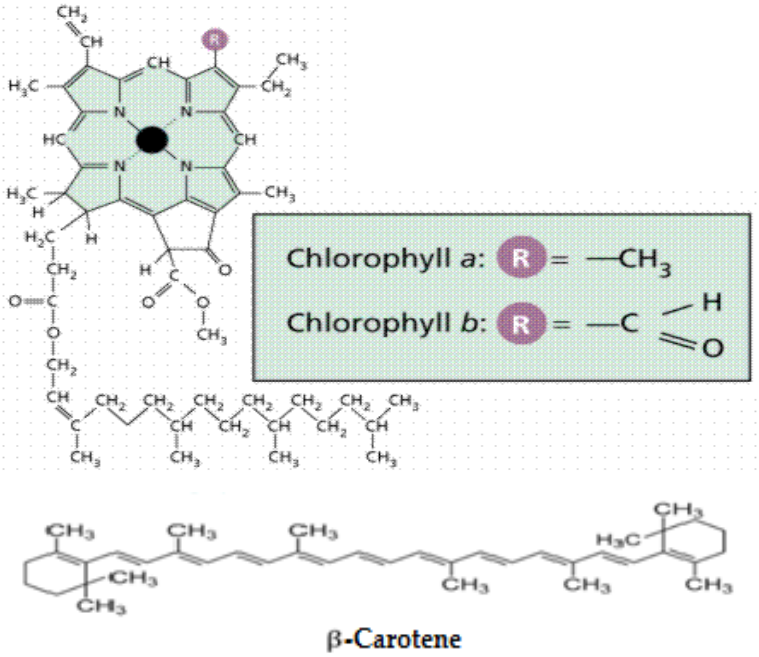



**Figure 2. The chemical structures of chlorophyll a, chlorophyll b and β-carotene**

Containing a conjugated polyene in its structure, beta-carotene is an unusual type of oxygen-free lipid antioxidant. β-carotene may reduce the occurrence of epithelial cell cancers, which account for more than 90% of cancer deaths, and a diet rich in it leads to a reduced risk of cancer occurrence, according to observational studies [9].

In this study, a chromatographic method used to separate, identify and quantify chlorophyll a, chlorophyll b and β carotene in a sample. This method applied to the analysis of Ceramium rubrum extract with good results compared to external standards. Identification and determination of each component was done after correlating the spectrophotometric and chromatographic spectral profile. The antioxidant capacity of this species was also determined using the photochemiluminescence method. Firstly, phytochemical studies were carried out on 3 types of extracts of Ceramium Rubrum to determine the active principles found in this type of algae. From the three extracts obtained, etheric, alcoholic and aqueous, a series of particularly important bioactive compounds, from a pharmaceutical point of

view were highlighted, such as reducing compounds, catehic tannin, cumarines, flavonoic aglicoles, ozes and poliozes [10].

## Materials and Methods

Macroalgal material – the main step to perform detailed laboratory analysis is the harvesting and preservation of algae. In order to collect the intact material, it is usually done manually with the help of a knife. In order to be able to differentiate them when harvesting, they are placed in a bag with water, on which must be specified the place of harvest, the depth and the date. The extraction was carried out with 90% acetone for these Ceramium rubrum red algae, after they had previously been dried in the dark at room temperature and finely ground. After filtering the extract, it introduced into a volumetric flask containing the same solvent. To analyze by spectrophotometer and chromatographic analyses, the stock solution diluted with different solvents.

Standards – The analyzes are carried out on the red algae extract against the standards of β-carotene, chlorophyll a and chlorophyll b, reporting the correlated results of IR, UV-VIS spectrometry and HPLC. In a volumetric flask, the 3 standards of chlorophyll a, chlorophyll b and β-carotene were dissolved in diethyl ether. To prepare standard solutions by dilution with the appropriate solvents, the stock solution used to perform these chromatographic and spectrophotometric analyses.

## Equipment

- The Fourier Transform Infrared Spectrometer (FT/IR 4200) by Jasco is a highly advanced instrument for infrared spectroscopic analysis. Below is an overview of its key features and specifications: wavenumber Range: 7800 to 350 $cm^{-1}$; accuracy: ±0.01 $cm^{-1}$; high-intensity ceramic source; detector: DLATGS; ATR correction.
- The **CINTRA 10e Spectrometer** is a UV-Visible spectrophotometer designed for high-speed, accurate spectral analysis. Here are the key specifications and features: w**avelength Range: 190–1200 nm; Monochromator: Czerny-Turner mounting with holographic grating; automatic lamp Peaking; detector: Silicon photodiode.**
- The High-Performance Liquid Chromatography (HPLC) system by GBC. Below is a detailed overview of its configuration and key features: LC 1150 quaternary solvent delivery system; LC 1150 Column oven; LC 1460 vacuum on-line degasser; detector: LC 5000 photodiode array detector (DAD); WinChrom chromatography data management system.
- Photochemiluminometer PHOTOCHEM Analytik Jena, Germany, 2008 - is an advanced analytical device used primarily to measure the antioxidant capacity of various substances. It operates based on

photochemiluminescence (PCL). This light emission is directly proportional to the antioxidant capacity of the sample being tested [11].

## Methods

The results of the HPLC method, which was carried out on sample and standard solutions, were illustrated by the superimposed chromatogram, maximum spectrum plot and its graph. A chromatogram generated by the maximum spectrum plot, showing the maximum absorbance at each point, can show how the chromatogram will appear when the wavelength for each sample, are optimized. In order to have the spectra for each peak detected in real time, with the diode-array detector. The expression of the ability of the column to retain the compound is rendered by the capacitance factor and ranges from 1 to 15. To analyze the sample solutions and standards, used in spectrophotometric method were compared. By the spectral plot and superimposed spectra, the results were illustrated [12].

Antioxidant capacity - The plant product (20 mg) was dissolved in 1 mL of ethyl alcohol, after first drying, grinding and finely pulverizing to a fine powder, and a cold extract was obtained. Extraction was carried out for 24 and 72 hours respectively in an amber beaker at room temperature. Separation was carried out by decantation, without filtration, after the mixtures had been shaken regularly. By comparison with the standard substance Trolox the antioxidant capacity for each algal mass extract was determined using the standardized method ACL – (Antioxidant capacity of lipid soluble substances). In order to obtain a photochemical reaction, the samples were exposed to external radiation from a phosphorus-lined Hg lamp, which provides maximum energy at 351 nm and produces free radicals in the sample to be analyzed. Antioxidants present in the sample annihilate the free radicals after they have been released, but some of them remain in the sample and mix with the photosensitive reagent without the need for an external excitation source. The photomultiplier detects the photon that has been emitted and amplified as a result of the chemical reaction. The total antioxidant capacity of the device electrical signal will be converted into concentration values and measured [13].

## Results and Discussion

HPLC analysis - The chromatographic conditions used for the analysis of the standard solution of 25mg/L of each component: β-carotene, chlorophyll a and chlorophyll b, are presented in Table 1. The Table 2, lists the results for the 3 solutions that were analyzed at the same time and the same chromatographic conditions.

By comparing the retention time of the standard with the sample, they are usually identified. By superimposed normalized spectra of the sample and the standard, the diode array detector achieves a better identification. The identity of each separate component was confirmed using the similarity index. The results reveal very well separated components in Figure 2.

**Table 1. The parameters of HPLC**

| Tehniques | Features HPLC |
|---|---|
| Mobile Phase | methanol/water/ethyl acetate=18/1/1<br>A: 5% water<br>C: 90% methanol<br>D: 5% ethyl acetate |
| Detector | PDA (200–700 nm); units: mAU |
| Column | RP-18 Nucleosil® 5 µ 150 ×4.6 mm; Guard column RP-18 Nucleosil® 5 µ; 50 × 4.6 mm |
| Injection | Rheodine valve |
| Temperature | 30 °C |
| Elution | Isocratic |
| Flow rate | 1mL/min |
| Sample size | 100 µL |
| Sample concentration | 0,5 mg/mL |
| Standard concentration | 12,5 mg/L (dilution with elution solvent) |

**Table 2. The chromatographic analysis results**

| Peak No | Peak I | Peak II | Peak III | Peak IV |
|---|---|---|---|---|
| Component | Mixture | Chlorophyll a | Chlorophyll b | β-carotene |
| Resolution | 0 | 2.67 | 3.818 | 13.99 |
| Area (%) | 50.56 | 13.89 | 22.94 | 12.624 |
| λ max (nm) | 271 | 391 | 422 | 409 |
| $t_R$ (min) | 1.95 | 5.90 | 4.17 | 16.86 |
| Capacity factor | 18.4 | 56 | 40.7 | 167 |

The standard substances chlorophyll a, chlorophyll b and β-carotene were prepared by dissolution in ethyl ether, the optimal solvent for all three compounds. After chromatographic optimization of the concentration and separation method, standard solutions of 25 ppm concentration were prepared by dilution with the elution solvent. These were analyzed chromatographically. In Figure 3 are presented the chromatogram for standard solution of chlorophyll a, chlorophyll b and β-carotene 12,5 mg/L each. To make the working solution by diluting with elution solvent.

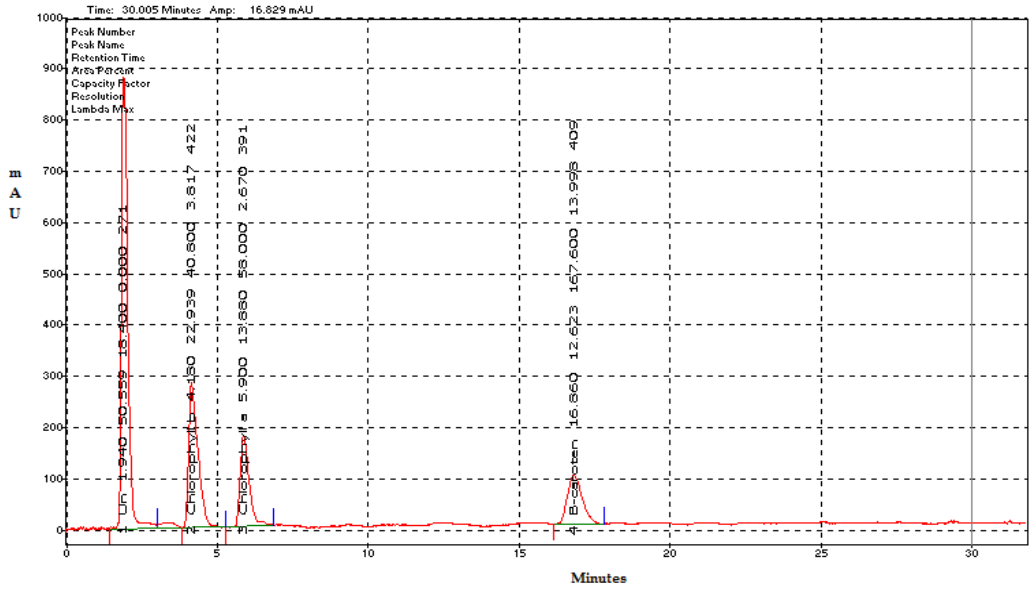



**Figure 3. The chromatogram for standard solution of chlorophyll a, chlorophyll b and β-carotene 12,5 mg/L each. Peaks 2, 3 and 4 show the presence of chlorophyll b, chlorophyll a and β-carotene respectively.**

Ceramium rubrum extract in 90% acetone was used. Dried and shredded algae were macerated in cold and dark for 12 hours in a cloth cartridge with 90% acetone: 1g plant product weighed to analytical precision with 40mL 90% acetone. After 12 hours, the extract was quantitatively filtered, the cartridge was washed with 90% acetone and brought to a 50 mL volumetric flask with the same solvent. The concentration of the extract obtained is 20 mg plant product/mL 90% acetone. In order to discover the optimal chromatographic concentration, several diluted solutions were tested. To reach a better reproducibility, more than 5 chromatographic analyzes were used at this concentration level (0.5 mg/mL). Table 3 and Figure 4 show the results of the chromatographic analysis of the Ceramium rubrum standard solution.

**Table 3. The results of chromatographic analysis for the Standard solution of Ceramium rubrum 0,5 mg/mL**

| Peak No | Peak I | Peak II | Peak III | Peak IV | Peak V | Peak VI |
|---|---|---|---|---|---|---|
| Resolution | 0 | 7.172 | 1.847 | 2.325 | 22.144 | 10.692 |
| Area (%) | 90.779 | 0.984 | 1.669 | 1.942 | 1.505 | 0.609 |
| λ max (nm) | 273 | 241 | 241 | 241 | 241 | 241 |
| $t_R$ (min) | 1.99 | 5.65 | 7.5 | 9.55 | 21.36 | 25.16 |
| Capacity factor | 18.8 | 55.4 | 75 | 94.7 | 212.9 | 250.8 |

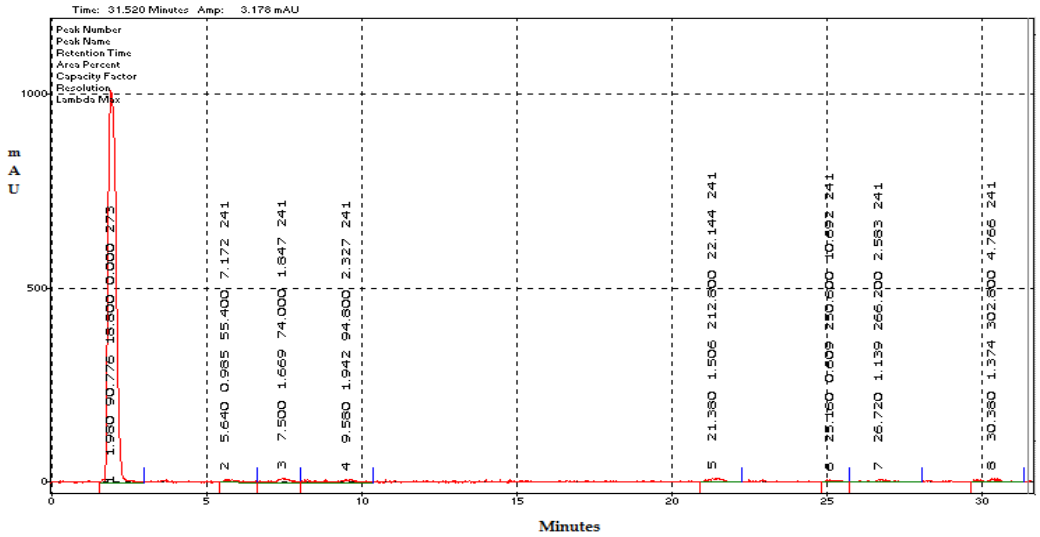
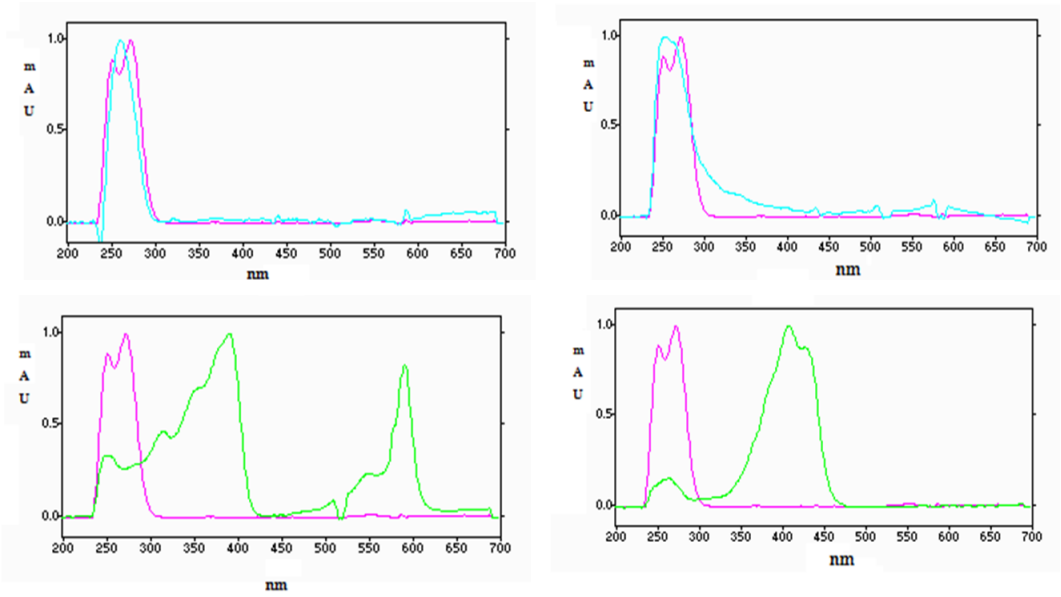



**Figure 4. The chromatogram for Ceramium rubrum 0,5 mg/mL**




In the entire chromatogram for Ceramium rubrum from Figure 4, there are 7 peaks that represent compounds from chlorophyll and carotenoid pigments. These peaks are highlighted in Table 3, where we especially note the area of the peak (%). The size of the peak area is conditioned by the amount of pigment found in the composition of the algae. We also find from Table 3 that the level of the areas of peaks 2-7 is below approximately 2%. The most important peak is peak I, which has an area of 90.779%.

In Figure 5 are shown in detail the peak I superimposed spectra of the solutions of the red alga Ceramium rubrum and the standard solution of chlorophyll a. In Figure 6 are shown the peak I superimposed spectra of the solution of the red alga Ceramium rubrum and the standard solution of β-carotene.

Using the peak area under the curve, the sample was quantified. The calculation of the concentration takes into account the mass of the sample, the volume of the flask for the extract, the dilution factors (F) and the concentration determined by the chromatographic method:

$$C = [\frac{V_{S,flask}\ (mL)\ X\ 10^{-3}}{m_{sample}\ (g)}\ X\ F\ X\ C_{det.}\ \left(\frac{mg}{L}\right) X\ 10^{-3}]\ X\ 100\ g\ active\ subst/100g\ sample \quad (1)$$

$$C = [\frac{50\ (mL) X\ 10^{-3}}{1.009\ (g)}\ X\ 40\ X\ 18.115\ \left(\frac{mg}{L}\right) X\ 10^{-3}]\ X\ 100 = 3.59\ g\ chlorophylls/100g\ sample \quad (2)$$

For the optimal separation of the chlorophyll a, chlorophyll b, and β-carotene standards, a chromatographic method was used, the HPLC method, which offers an improvement in precision, versatility and reproducibility. Also, through these methods the presence of the three types of pigments, chlorophyll a, chlorophyll b and β-carotene in the algal mass used by Ceramium rubrum was demonstrated and was calculated with the help of the above equation and the amount of total chlorophylls found in it studied species, 3.59 g chlorophylls/100g sample.

Although there are few studies on Ceramium rubrum that highlight the levels of total chlorophyll and β-carotene, we can still compare the existence of these pigments with studies done on other species of red algae. Thus, Rosemary et al. (2019) highlighted the chlorophyll pigments: chlorophyll a content (8.96 ± 0.39 mg/g d.w.) and chlorophyll b content (7.74 ± 0.33 mg/g d.w.) and total carotenoid content (1.28 ± 0.5 mg/g d.w.) on the red alga Gracilaria corticata originating from the southeast coast of India, and Choudhary et al (2023) studied the red alga Halymenia porphyriformis originating from the Arabian Sea and reported the existence of chlorophyll biocompounds through total chlorophyll content (7.0 ± 0.04 mg/g d.w.) and total carotenoid content (0.20 ± 0.01 mg/g d.w.) [14,15].

Spectrophotometric analysis in UV-VIS for Ceramium rubrum

UV-VIS spectrophotometric analysis was performed on extracts of the seaweed Ceramium rubrum obtained by maceration with acetone. The absorption spectrum obtained in the range 300-1000 nm is presented in Figure 7. The presentation of the whole spectrum of the alga Ceramium rubrum allows to highlight the component constituents.

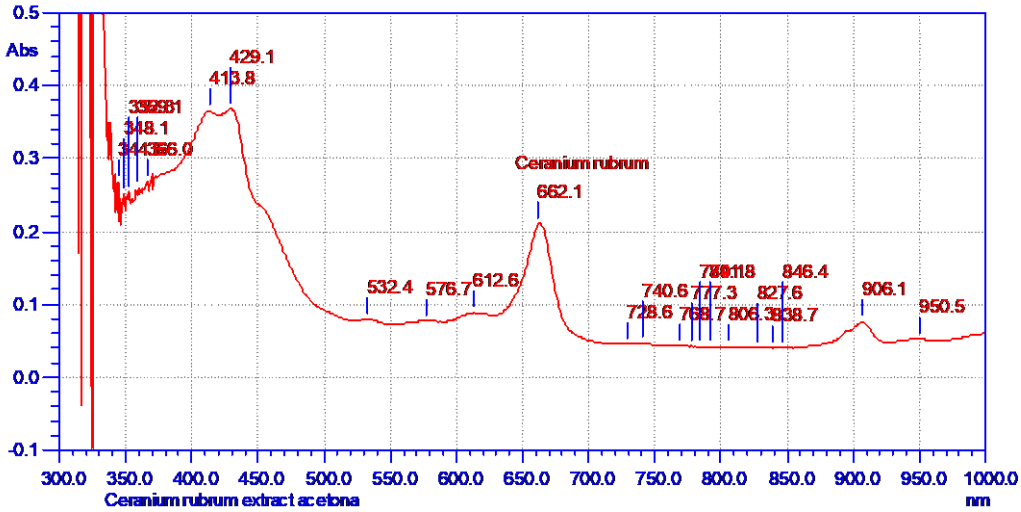



**Figure 7. The absorption spectrum of the Ceramium rubrum extracts 35 mg/mL in acetone 90%, in the range of 300–1000 nm**

**Spectrophotometric analysis in IR**

The IR spectrophotometric study was performed on dried powders of the seaweed Ceramium rubrum and standard substances β-carotene, cholecalciferol, retinol and ergocalciferol.

In order to highlight the structures of the different compounds of interest in the composition of the red algae Ceramium rubrum, we used the method of superimposing the IR spectra of the algae spectrum with the spectra of the studied compounds.

A specific IR profile for the dry powder of Ceramium rubrum is 4.000–630 $cm^{-1}$ and can be seen in Figure 8. Next, this IR spectrum is overlapped with other spectra of interest taken in the study.

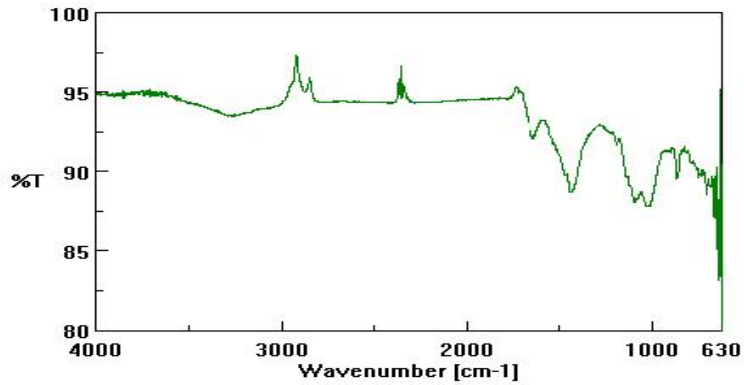


**Figure 8. The spectrum of Ceramium rubrum dried powder, in the range of 4.000–630 $cm^{-1}$**

Figure 9 shows the superimposed IR spectra for Ceramium rubrum seaweed powder and β-carotene solid substance. Figure 10 shows the superimposed IR spectra of the seaweed Ceramium rubrum dry powder and cholecalciferol solid substance.

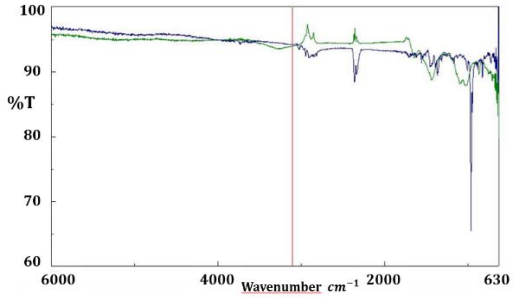



**Figure 9.** The Overlaid IR spectra of Ceramium rubrum dried powder (upper curve) and β-carotene (lower curve), in the range of 4000–630 $cm^{-1}$

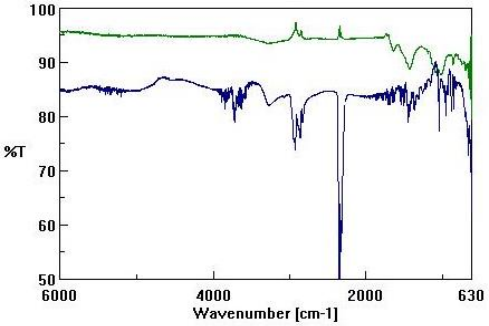



**Figure 10**. The Overlaid IR spectra of Ceramium rubrum dried powder (green) and cholecalciferol solid substance (blue), in the range of 4000–630 $cm^{-1}$

Figure 11 shows the superimposed IR spectra for Ceramium rubrum seaweed powder and retinol solid substance. Figure 12 shows the superimposed IR spectra of the seaweed Ceramium rubrum dry powder and ergocalciferol solid substance.

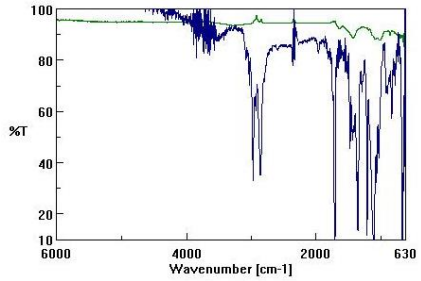



**Figure 11.** The Overlaid IR spectra of Ceramium rubrum dried powder (green) and retinol solid substance (blue), in the range of 4000–630 $cm^{-1}$

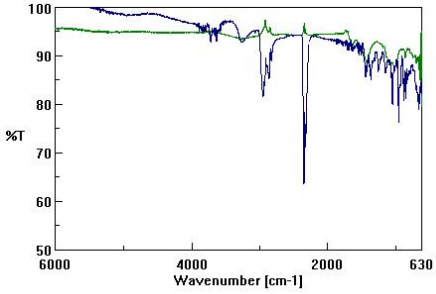



**Figure 12.** The Overlaid IR spectra of Ceramium rubrum dried powder (green) and ergocalciferol solid substance (blue), in the range of 4000–630 $cm^{-1}$

Using the IR spectrophotometry method, the presence of β- carotene and some vitamins, such as cholecalciferol (Vitamin D3), ergocalciferol (Vitamin D2) and retinol

(Vitamin A), were determined in Ceramium rubrum algae, which are important sources in nutrition and also contributes to the antioxidant capacity.

**Antioxidant capacity**

Analytik Jena Germany reagent kits used the calibration curve to determine the antioxidant capacity, these being: R1 (dilution solvent), R2 (buffer reagent), R3 (photosensitive reagent), R4 (reagent size). By measuring a series of standard solutions containing 0.5, 1.0, 2.0, 3.0 nmol Trolox (suitable for 5-30 µL R4), as can be seen in Figure 13, this is how the curve was constructed calibration. Table 4 show the results in nmol/sample, Trolox equivalent units. According to the ACL method quantified by comparison with the Trolox standard substance, the antioxidant capacity of the algal mass was expressed at the time of extraction and the volume of the sample used.

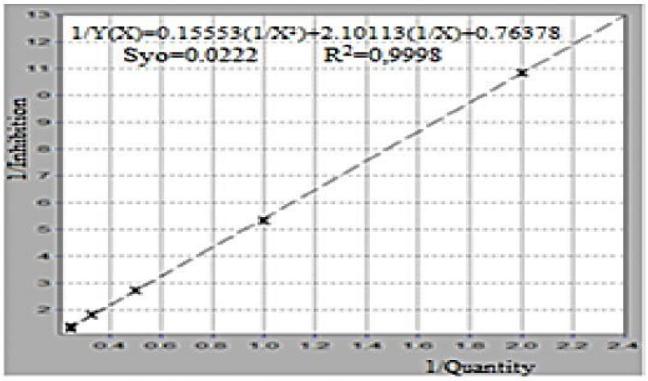



**Figura 13. Calibration curve for standard Trolox (ACL method, Analytik Jena, Germany)**

**Tabel 4. The antioxidant capacity of Trolox (ACL method)**

| Algae species | Volume sample (µL) | Analysis time (s) | Extraction time (Hours) | Equivalent units Trolox (nmol/g dry sample) | Equivalent units Trolox (nmol/volume sample) |
|---|---|---|---|---|---|
| Ceramium rubrum | 20 | 120 | 24 | 52.3 | 1.043 |
| | 20 | 120 | 72 | 141.5 | 2.84 |

By means of the chemiluminescent method, it was demonstrated that this species of red algae Ceramium rubrum has antioxidant properties, being able to be used in the pharmaceutical and medical fields in the treatment of many ailments. The highest antioxidant capacity is identified in the case of the Ceramium rubrum sample at 72 hours, which proves that it has a high antioxidant capacity.

Compared to other studies that were carried out to determine the antioxidant capacity of the Ceramium rubrum species, the values that were obtained in this study are close. The antioxidant properties of the biocompounds of Ceramium rubrum species have also been proven by other studies. Biris-Dorhoi et al (2018), in the study carried out on five species of green, brown and red algae, including the species Ceramium rubrum, the following values were obtained for Ceramium rubrum 0.1%- 0.530 nmol/volume sample, for Ceramium rubrum 1%- 1.623 nmol/ volume sample, and for Ceramium rubrum 5% - 1,271 nmol/volume sample [16].

## Conclusions

The rich composition of the red alga Ceramium rubrum was investigated by modern methods and techniques. By the physicochemical analysis presented in this study, (HPLC), the existence of chlorophyll pigments (chlorophyll a and chlorophyll b) and β-carotene is highlighted as the main conclusion. Other bioactive compounds of pharmacognostic interest are also highlighted from UV- VIS spectral analysis.

From the study of the antioxidant activity by chemiluminescence method, the antioxidant capacity due to the biochemical composition of Ceramium rubrum algae was evidenced

By IR spectrophotometric analysis it was demonstrated the existence of β-carotene pigments and vitamins A, D2 and D3 in the biochemical composition of the red algae Ceramium rubrum.

Red algae, especially this Ceramium rubrum species, are very little studied for their potential, although it has been demonstrated that they constitute an important source of valuable compounds and can be further exploited for its antioxidant capacity and it can be used in the pharmaceutical and nutraceutical fields.

The present study can be considered a start for new researches that highlight the bioavailability of biocompounds from Ceramium rubrum. It also deserves extended future research to expand the possibilities of using this species of algae in other industries such as the food industry or the cosmetics industry, along with applications in the pharmaceutical industry.

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