Valuable Bioactive Compounds Extracted from *Ceramium rubrum* on the Romanian Seaside with Medical Interest

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Abstract

Recently in science, seaweeds are widely used because of their relevant compounds and potential origin of antimicrobial, antiviral and antioxidant activity. Seaweeds are rich in the trace elements and essential minerals that are hard to find in terrestrial plants. *Ceramium rubrum* is one of the red seaweeds that can be found in the Black Sea on the Romanian coast that has not been enough studied for their bioactive compounds. Identification and quantification of the biomolecules is carried out by specific methods. The compounds that have been found are cumarines, sterols, flavonoic aglicoles, triterpenes and polysaccharides. Is well known that this kind of biomolecules are very important in the pharmaceutical industry. Ozes and poliozes, reducing compounds and catehic tanin we're also identified from the red algae.

Keywords: *Ceramium rubrum*, red seaweed, antioxidant, flavonoids, polysaccharides, bioactive compounds.

Introduction

Nowadays, worldwide a remarkable interest of medical and pharmaceutical professionals is focused on the superior use of natural resources for therapeutic purposes. Marine ecosystems have productivity and diversity that are very important in maintaining the health of the marine and terrestrial environment and provide important sources for the cosmetics, food and pharmaceutical industries. They represent about 70% of the Earth's surface. Although the literature presents a wide range of medicines from marine resources, it is modest when it comes to the pharmaceutical use of marine resources in the Black Sea. Recently, a real field of investigation has been developed for biological substances in marine organisms, as they have been found to be a rich source of valuable compounds. The importance of algae and phanerogams in the general bioproductivity of the marine environment, especially in shallow waters, is becoming increasingly evident both economically and ecologically. The algal macroflora of the Black Sea totals a number of 277 species of algae, of which the most common are green and red algae. Although green algae have been extensively studied for their properties, red algae have not received much attention and I believe that they should also be fully explored. The research developed in this paper treats the red seaweed existing in the territorial waters of the Romanian coast. The capitalization of seaweed is of particular importance, being a valuable resource for the medical-pharmaceutical field [1].

Ceramium rubrum belongs the red algae group: Div. *Rhodophyta*, Subcl. *Florideophycidae*, Fam. *Ceramiaceae*. This group includes pluricellular algae that can be found in an aquatic environment and it grows spontaneously and abundantly in the Black Sea [2]. The red algae (*Rhodophyta*) make a distinct photosynthetic eukaryotic lineage which consists of around 6,000 species that includes unicellular to large multicellular taxa. Red algae differ from other eukaryotes by the lack of flagella and centrioles during their entire life cycle. Because of their particular life cycles (e.g., triphasic life cycle: gametophyte, tetrasporophye and carposporophyte phases), *Florideophyceae* is one of the most important algal groups in marine environments [3]. Due to the environmental conditions, the species of *Ceramium* are easily differentiated. It is probable to provide different names, if the examinations of the samples are not done prudently [4]. The genus *Ceramium* is found in the northern sector of the Black Sea coast, this being an opportunistic species that prefers most types of substrates from the extended platform of sarmatic limestone to live or dead mussel shells. This is a constant presence on our coast and develops appreciable biomass even in waters with a high load of nutrients. All year long, generally in spring and summer, red alga richly develops along the entire Romanian coastal area, on rocks, at depths of 0,5-4,5 m [5][6][7].

Polyphenols, vitamins, polysaccharides and polyunsaturated fatty acids are the most valuable and researched bioactive compounds from marine algae. Due to the presence of non-digestible polysaccharides in the algal cell wall, algae can be considered a very good source of dietary fibers. Polyphenols are a class of compounds commonly found in plant foods, such as, vegetables, fruits, spices, herbs, tea, etc. They can neutralize harmful free radicals that would damage the human cells and increase the risk of condition like cancer, heart diseases and diabetes, they can act as antioxidants. Polyphenols are also thought to reduce the origin cause of the most chronic ilnesses, the inflammation. They can be further categorized into 4 main groups: polyphenolic acids, polyphenolic amides, flavonoids and other polyphenols. Flavonoids are an extensively distributed group of structurally associated compounds with a 2-phenylchromane skeleton that have, in the C2 or C3 position, a phenyl substituent. The subclasses that they are divided depends on the degree of oxidation of the central pyran ring. Flavonoids play an imperative role in plants as resistance and warning compounds in pathogenesis, symbiosis and reproduction, they are of physiological and biological importance. In some recent studies, phenolic acids and specifically flavonoids have been described to exhibit various effects including antiinflamatory, antioxidant, anti-cancer, antimicrobial, anti-allergic, anti-thrombotic, anti-atherogenic and estrogenic, vasodilatory and capillary fragility and permeability lowering actions [8]. Polysaccharides are omnipresent biopolymers that can be found widely in nature. These are polymers formed with glycosidic linkages of simple sugars, which are monosaccharides. Polysaccharides can be found in different types. Distinct chemical and physical properties exist because of the structural differences. These are natural biodegradable biopolymers, nontoxic. Algae are an important source of polysaccharides, especially the red macro algae. They are largely used as stabilizers, gelling agents, emulsifiers and as thickeners in food products. Another important group are sulfated polysaccharides. These can be valuable ingredients in pharmaceutical, nutraceutical and food industry because they can act as defensive barrier against phatogen. Agar and carrageenan are the most relevant sulfated polysaccharides and both are encountered in red algae [9]. Polyunsaturated fatty acids (PUFA) play an important role in metabolism, as a regulatory molecule and as a fundamental component of all the organs membranes. These have more than one double bond in their backbone. In recent studies it has been demonstrated that PUFA have anti-cancer and antiinflammatory effects and they are regulators of lipid metabolism, notably through epigenetic mechanisms [10]. Potential sources of polyunsaturated fatty acids are the marine algae [9].

Materials and Methods

Algal Material

The marine biomass was selected manually and exposed to a pretreatment that consisted of repeated washings with potable water and finally with distilled water. Macroscopic and microscopic examinations were one of the investigations that were performed on the fresh product. Other examinations that the algal biomass was selected for are chemical, physico-chemical and microbiological analysis. For this matter, the algal material was dried at temperatures between 25-35 degrees. The dry product, by grinding, was ground to a powder and was passed through a 0.5 mm sieve to obtain a uniform powder [11], see Figure 1.

The algal flora was harvested from the Black Sea coastline in may-november period, from water at a distance of 5-25 m from the shore, from the areas of Eforie Nord, Eforie Sud, Costinesti, 2 Mai, Mangalia, Vama Veche, Năvodari and Constanța Casino.

Figure 1. Red algae in the algal colony

Macroscopic Examination

The first phase in the study of algal products is the macroscopic examination. This is done by examinating the whole algae (cauloid, phylloid and rhizoid) both with help from a magnifying glass and with the eyes to detect its color, size, appearance, taste and smell [1][5][12]. From this macroscopic examination it was found that *Ceramium rubrum* is a red alga with a 10-15 cm height and with a philament like thalle, fixed on the substratum through the rhizoid, having a bushy appearence, with ramifications. It has an articulate aspect because of the filaments that have dichotomic and are formed of a single row of connecting cells. The entire surface of the thallus is covered by cortical cells that are formed by the continuous division of periaxial cells produced and located in the nodal area. Tow short twisted branches are situated at the end of each filament [5-7][13].

Microscopic Examination

Whole, fresh algae was used for microscopic examination and kept in seawater jars throughout the analysis. The algae were placed in a bowl of clean water after being rinsed, very well, with distilled water to remove impurities. The materials that are used in this examination are fragments of thallus from the red seaweed, a Microscope and Micros photomicroscope (10/0.25), forceps, blades, spatulate needles and Petri dishes. The microscop analysis was performed directly on fresh thallus fragments obtained by slicing it with a slice and brought into a Petri dish with distilled water, because the macrophytic algae species have single-cell or two-layer thallus.

There, in the transversal section of the thalle, in the center, is a large cell, surrounded by eight pericentral cells, which are also surrounded by another layer of cortical cells. There are large strip-shaped plastides in the central cell.

Physico-chemical Methods

Qualitative chemical analysis requires the successive and selective extraction of plant products with solvents of diverse polarities and separation using chemical methods, followed by specific reactions with which to classify different groups of active compounds. The active principles are extracted first in order to perform the global chemical analysis. From the algae product sprayed with a non-polar solvent (chloroform, ethyl ether, petroleum ether, benzene, hexane, etc.), then with a medium polarity solvent (ethanol, methanol) and finally the following are obtained: a sol. etheric extractive, a sol. alcoholic extract and a sol. aqueous extract. In the alcoholic and aqueous extract, the hydrophilic compounds are determined, and in the etheric one, the lipophilic compounds [14-17]. The three solutions are analyzed separately, using methods corresponding to the physicochemical properties of each group of active principles [18].

Analysis of the alcoholic extractive solution

The ether-depleted algal product was used and brought to the water bath to remove traces of ether. The plant product was extracted with methanol (2 x 100 mL) by refluxing for half an hour. The combined methanolic solution was concentrated to 50 mL by distillation of the solvent and partitioned into 2 parts. One part is subjected to hydrolysis with 15 mL 10% HCl on the electric nest at 3 grade for 30 minutes and the other part is used to identify the active ingredients on the non-hydrolyzed solution (25 mL). For further extraction, the methanol depleted plant product is stored [19].

Analysis of the ether extractive solution

Weighed 20 g of freshly sprayed vegetable product from the red algae, which was initially

extracted with ethyl ether (2 x 100 mL) by refluxing for 15 minutes. The resulting solutions were filtered and collected in a fluted cube flask, after each reflux. Then the combined ether solutions have to be concentrated on a water bath to 50 ml and the first solution is obtained. It is used to perform reactions characteristic of lipophilic compounds [20].

Analysis of the aqueous extractive solution

Was used the remaining plant product from the alcohol extraction which was dried and then extracted with 100 mL of water in a water bath at 900 ° C for 30 minutes.

Results and discussions

Following the identification reactions discussed above, and the following results were obtained and they can be seen in Table 1.

Table 1. The reactions used to identify bioactive compounds [1]

Analyzed solution	Reactions	Identified active compounds
Alcoholic extract	Iron Chlorure reaction	Catehic Tanin
	Liebermann-Bouchard	Triterpenic heterozides
	Fehling	Reducing compounds
	Borntrager	Antracenozide
	Fluorescent UV(λ=365nm)	Cumarines
Etheric Extract	Fluorescent UV(λ=365nm)	Cumarines
	Liebermann-Bouchard	Sterols, Triterpenes
Aqueous Extract	Foaming	Soapozides
	Fehling	Reducing compounds
	$FeCl_3$	Catehic Tanin
	H2SO4 conc.+tymol	Ozes and poliozes

In the non-hydrolyzed alcoholic solution

The reaction with ferric chloride is blackish green, so positive for catehic tannin in this species *Ceramium rubrum*. The reaction with Styassny's reagent is negative. The analyzed species does not contain alkaloids because the residue obtained after evaporation of the non-hydrolyzed alcoholic solution was taken up with a 2% aqueous HCl solution then basified with ammonium hydroxide and extracted with ether; after evaporation of the ether solution and resumption of the residue with 2% HCl, the Mayer and Bertrand reactions were carried out and they were negative. Reducing compounds are present because by performing the Fehling reaction a reddish-red precipitate was obtained. In *Ceramium rubrum* the reaction with ninhydrine of the aqueous solution obtained from the residue of the alcoholic solution was negative.

In the hydrolyzed alcoholic solution

The Liebermann - Bourchard reaction performed on the residue of the hydrolyzed alcoholic solution did not result in a green - purple coloration, so triterpene heterosides are not present. The residue obtained after evaporation of the hydrolyzed alcoholic solution is treated with 50% methyl alcohol; the Shibata reaction on the alcoholic solution was positive, which reported the presence of flavonoids in this species. Antracenozides are not detected in this red alga, a result of the negative Borntrager reaction. The solution shows fluorescence under the incidence of UV radiation, so cumarines are identified.

In the etheric solution

From the Lieberman-Burchard reaction performed on the ether extract, which came out positive, it appears that sterols and triterpenes are present in the analyzed algae. Carotenoids are identified in this species by the Carr-Price reaction which was positive. The residue obtained by evaporation of the ether extract was taken up with methyl alcohol; the alcohol solution was reacted with Shibata; the reaction was positive, so they contain flavonoic aglicoles. Upon evaporation of the ether extract, a residue was obtained which was subsequently taken up with aluminum hydroxide. Borntrager reagent did not stain the solution orange, which shows that the alga does not contain endemoles. The same residue taken up with ammonium hydroxide show intense fluorescence under UV radiation, so *Ceramium rubrum* contain cumarines.

In the aqueous extractive solution

The aqueous extractive solution is evaporated to the residue, then a few drops of concentrated sulfuric acid and an alcoholic solution of thymol are added - a red coloration is detected which attests the presence of oozes and polioozes. The identification reaction for starch (with Lugol's reagent) was positive for this red alga. The reducing compounds are found in *Ceramium rubrum* due to the Fehling's reaction which was positive. The foaming reaction of the soapozides was negative, that means soapozides are missing from the analyzed species. The basic alkaloids were not identified, because the reactions of Mayer and Bertrand were negative. The aqueous extractive solution reacts with dilute FeCl2 and a blackish green color appears which confirms the presence of catehic tanin.

In Table 2 are presented the results of all identifications.

Table 2. The compounds detected in *Ceramium Rubrum*

The seaweed	Catehic tanin	Oozes and polioozes	Reducing compounds	Soapozides	Flavonoic aglicoles	Cumarines
Ceramium rubrum	++	++	+	-	+	+

Polysaccharides are usually the major component of red algae. The various polysaccharides are the main composition of the cell walls of algae. The most important polysaccharides in red algae include carrageenan and agar along with other identified polysaccharide compounds, see Table 3.

Table 3. Polysaccharides identified in red algae

Polysaccharides	**Red algae**
Agar	X
Carrageenan	X
Cellulose	X
Starch	X
Manan	X
Parphyran	X
Galactan sulphates	X
Xylenes	X

Carrageenans are the essential components of red algae cell walls and are linear polysaccharide chains with half sulfate esters attached to the carbohydrate unit. Thay are divided into three forms: lambda, iota and kappa, dipending on the degree of molecular sulfation [21], see Figure 2.

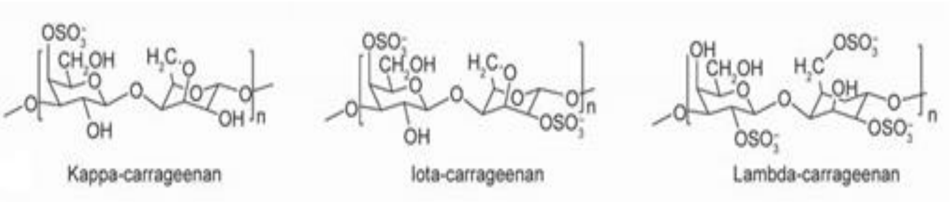

Figure 2. The structures of the three forms of carrageenan

Carrageenan is a sulfated anionic polysaccharide with a straight vertical column structure formed by bonds that alternate 3 β D-galactopyranose with residues of 4-α-galactopyranose. Many of the α-galactose residues may be in the form of 3,6-anhydrous derivatives. Sulfated ester, methyl and acetal groups of pyruvic acid and sometimes monosaccharides can substitute these derivates.

The biological activities of carrageenan

They have been tested for the treatment of hepatitis A, respiratory diseases, the H1N1 flu strain and the African swine fever virus.

The superoxidase dismutase biosensor, which is modulated by the k-carrageenan gel membrane, has been developed and has been reported to test the cleansing properties of commercial drugs. It has been used in the manufacture of the microbial temperature-time-environment indicator [22].

Agar-agar is obtained from a series of red seaweeds (aragophytes) and it is an organic product. It is formed of galactoside residues esterified at C6 with a sulfonic group. See Figure 3. Agars are a mixture of linear polysaccharide agarose, with a heterogeneous mixture of smaller molecules called agaropetin [23], see Figure 3. It has a very high gelling power.

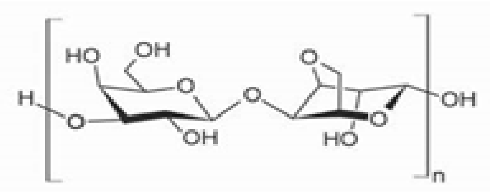

Figure 3. The sructure of the agar

The biomedical activity of the agar

Agar has been extensively used as a gelling agent in the food industry due to its hydrophilic colloidal properties, which is obtained from a mixture of agarose and pectin agar and it has the ability to form reversible gels easily by cooling the hot aqueous solution. It is frequently used as a microbiological medium to ensure the firmness of the gel. The resulting gels are transparent, relatively brittle, clear and they melt on heating. Despite its degradability and excellent gelling power, agar has not been widely used due to its poor aging properties.

Photodegradation and fluctuations in ambient temperature and humidity can modify the crystallinity of the agar, leading to the production of microfractures and the fragility of the polymers [24-26].

Conclusions

From this study, the following conclusions can be drawn: 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 catehic tannin, reducing compounds, cumarines, flavonoic aglicoles, ozes and poliozes. Variations in the marine ecosystem along the Romanian Black Sea coast, as well as temperature variations can lead to different results for algae species compositions, so carefull monitoring is needed each year to track changes in seaweed. These are categories of compounds of great pharmaceutical interest that can be used, with good results in the future in topical pharmaceuticals.

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