Antioxidant and antibacterial potential of *Ulva lactuca* species from Romanian Black Sea Coast

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Abstract

Green algae biomass represents an important natural resource found in marine environments which could offer new applications in the pharmaceutical and cosmeceutical industries. The paper aim was to characterize some fluid extracts of *Ulva lactuca* L. green macroalgae species, in the Ulvaceae family, from Romanian Black Sea Coast, through physical, chemical and biological methods. For biomedical applications it is important to study how the difference between the marine environment specific conditions, from which they are harvested, can influence the chemical composition of macroalgae. Another important aspect which could affect the bioactive green macroalgae extracts composition, is the influence of applied extraction method. In this way, the less distructive cold maceration method in different solvent concentrations was chosen, according with literature data. The extracts have been studied in order to determine total polyphenols content, total antioxidant capacity by photochemiluminescence method and antibacterial activity. The results emphasized an antioxidant and antibacterial activity of the studied macroalgae extracts, which can be improved in mixture with other bioactive natural substances, in order to obtain new pharmaceutical preparations for topical applications. The valuable obtained results offer the possibility to propose this natural resource for next studies as biomaterial in tissue engineering, wound dressing and drug delivery systems. We consider that *Ulva lactuca* L. green macroalgae species of Romanian Black Sea Coast, could be an important source of marine bioactive compounds with various uses in the biomedical field.

Keywords: *Ulva lactuca* L., bioactive compounds, antioxidant capacity, antibacterial activity

Introduction

Marine algae serve as a large part of the phytoplankton found in various marine environments of the world. Macroalgae represent, macroscopic algae with increased growth rate, which can be found in various areas and have relatively low use of fresh water [1]. Interest in algal biotechnology has been on the rise in recent times, especially on the production of various macroalgae species, used in the food, medical, cosmetic and pharmaceutical industries. Of significant importance is the increased global production of seaweeds in the last few decades [2]. In data literature, there are multiple studies conducted on *Ulva lactuca* L., which grows in the waters of seas and oceans [3], [4].

(a)

(b)

(b)

Fig. 1 (a, b). *Ulva lactuca* L. green macroalgae species

Ulva lactuca L., popular known as *sea lettuce*, see Fig. 1, represents a green macroalgae which belongs to the Chlorophyta Phylum, as described by Linnaeus in the 17th century [5]. It can be found connected, sessile or floating, in various marine environments and it is widespread on the Romanian Black Sea shore. *Ulva lactuca* L. presents polymorphism which led to the assumption, that different species exist depending on the environment. Linnaeus was the first one to discover that *Ulva lactuca* L. presents different phenotypes with a tubular form tail. Taxonomists from the 19th century believed that green algae with tubular tail represent a completely different genus called *Enteromorpha*. In time though, different studies of genetic analysis showed that diverse phenotypes were not linked to genetic variations, so there is no real evidence to support the existence of species other than *Ulva lactuca* L. [6]. Literature still describes distinct *Ulva* species, but reproduction among these species has been demonstrated and therefore they can only be described from now on as *Ulva lactuca* L. variants.

An increased number of studies enphasize marine habitats and other factors such as water temperature or marine pollutants can influence the chemical composition of marine algae. The study of macroalgae increased in interest due to their role of bioindicators [7], [8]. They represent markers that can highlight negative changes in marine ecosystems due to the accumulation of different pollutant agents in their thallus [9]. *Ulva lactuca* L. predominantly invades and blooms near beaches, where it can affect tourism and damage marine ecosystems. The acidic vapors and biodegradation can induce animal deaths and possibly human deaths, due to *Ulva lactuca* L. large quantities degradation [10]. *Ulva lactuca* L. blooms have been reported all over the world, but the biggest event in the world so far was the green tide from the Yellow Sea for ten consecutive years which covers approximately 10% of the whole Yellow Sea surface [11]. In Romania, the South-east coast of the Black Sea, near Constanta and Mangalia cities reported the biggest *Ulva lactuca* L. blooms.

The Romanian Black Sea coast offers great possibilities for harvesting *Ulva lactuca* L., which can be found in significant numbers near the shore and at low depths. *Ulva lactuca* L. is an edible green seaweed with no threats to human health, traditionally consumed in various Asian countries but also approved in Europe [12].

Ulva lactuca L. consists of various natural compounds that can be utilized in many areas like pharmaceutical, cosmetical, chemical, energy or food sector. Seaweed composition includes soluble and insoluble dietary fibers together with minerals, polysaccharides, vitamins, chlorophylls, proteins. The integral use of the plant can represent a more profitable approach in order to utilize all the different constituents that *Ulva lactuca* L. possesses [13], [14].

In the last thirty years, medicinal plants practice has become a major trend worldwide, as the analysis of algae, plant extracts and natural products has shown that green algae represent a potential source of antibacterial activity. Traditional compounds face crises due to the rapidly growing resistance of bacteria, e.g. *Staphylococcus aureus*. Unlike chemicals, herbs are considered non-toxic due to their natural origin and long-term use as folk medicine. In this paper we present the results obtained regarding the antioxidant and antibacterial activity evaluation of this common green macroalgae species present along Romanian Black Sea Coast.

Materials and methods

The analyzed samples consist of green algae species *Ulva lactuca* L., class Ulvophyceae, order Ulvales, family Ulvaceae. The vegetal material was obtained from Romanian Black Sea shore, from four different zones: Vadu, Constanta (the highest macrophyte seaweed area), Mamaia and Navodari, Constanta County, Romania, in the August - October 2021 period. The marine algae collected samples were processed by washing thoroughly with tap water and drying the harvested vegetal material, at room temperature, on metal sieves, 25 ± 2 ˚C, for 24 hours, followed by drying at 40 ˚C in oven with natural convection and grinded in an electric grinder to a fine powder.

Analysis methods were used in accordance to phytochemical, physico-chemical and pharmacognostical studies in literature data. Studies and experiments were made in the laboratories of Ovidius University of Constanta, Faculty of Pharmacy.

The chemical composition was explored in order to identify and classify the classes of compounds that support the physical-chemical and biological characteristics of the samples in comparison with the literature data [3].

Three types of extracts were obtained on using distinct solvents and different reactions for identifying the presence of important active principles of pharmaceutical interest, as Table 1.

For chemical compounds identification of the three extracts, they were analysed separately, using methods fit for the physical and chemical properties for each group of active principles [3].

Table 1. Type of methods and specific reactions used in farmacognostic study [3]

Type of extractive solution	Reaction/Method utilised
Etheric extractive solution (A)	Fluorescent UV (λ=365nm) and Lieberman-Burchard
Alcoholic extractive solution (B)	Fehling reaction, Borntrager reaction and UV method (λ = 365 nm), Iron Chlorure reaction, Liebermann Bourchard reaction
Water extractive solution (C)	Fehling reaction, reaction with H_2SO_4 conc. and tymol, Foaming reaction and reaction with $FeCl_3$

Total phenolic content determination and pigments chemical composition

Total phenolic content determination (TPC): Total phenols present in *Ulva lactuca* L.

samples were determined UV-Vis spectrophotometrically, applying Folin-Ciocalteu reagent as stated by Singleton et al. [15]. Absorbance was determined at 760 nm in comparison to the calibration curve of gallic acid.

T*otal chlorophylls determination* (T-Ch): We homogenized three grams of *Ulva lactuca* samples in acetone (30 mL, 80%) and incubated over the night, to protect it from sunlight, at 4 °C in order to obtain a complete extraction followed by centrifugation at 10,000 xg for 5 min. Total chlorophyll (T-Chl), chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) found inside the supernatant were measured UV-Vis spectrophotometrically at 664 nm wavelength, in accordance to Lichtenthaler and Buschmann method [16].

Total carotenoids determination (TCAR): Total carotenoids were determined UV-Vis spectrophotometrically at 450 nm wavelength, in accordance to AOAC [17]. *β*-carotene was utilised as a standard.

Antioxidant activity

Algae fluid extracts were obtained using two different methods, cold maceration and Soxhlet method, see Fig. 2.

Sample preparation - The vegetal product has been made into fluid extracts using as solvents, ethyl alcohol 50% and 70% concentration, using two common extraction methods:

Cold maceration for variable concentrations of vegetal product, 2.5 g, 5.0 g, respectively 10 g in 100 mL, both ethyl alcohol 50% and 70% concentration, for 12 days at darkness, room temperature, periodic stirring.

Soxhlet extraction for three hours refluxing for variable concentrations of vegetal product, 2.5 g, 5.0 g, respectively 10 g in 150 mL ethyl alcohol 70% concentration.

All the hydroalcoholic extracts obtained were filtered at normal pressure through quantitative Whatman filter paper and present different colors, from pale green to green-brown and dark green.

For determining *total antioxidant capacity (TEAC),* 10 μL volume of algae extracts were taken for analysis. Each determination lasted 120s. Analyses employed the photo-chemiluminescence method by ACL (Antioxidative Capacity in Lipid Soluble Substances) procedure Analytik Jena, using Photochem apparatus Analytik Jena AG, Germany, see Fig. 2. Triplicate samples of hydroalcoholic extracts were quantified by comparison with the standard substance Trolox®, Hoffman-LaRoche's trade name (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). For calibration, the standard kit of reagents, Analytik Jena, Germany, was used: R1 (dilution solvent), R2 (buffer reagent), R3 (photosensitive reagent), R4 (reagent sized). For the calibration curve standard solutions containing 0.5, 1.0, 2.0, 3.0 nmol Trolox were measured (suitable for 10 μL R4), as Trolox calibration curve, Fig. 3.

Fig. 2. *Ulva lactuca* L. hydroalcoholic extracts samples obtained by cold maceration and Soxhlet extraction and Photochem apparatus Analytik Jena AG, Germany

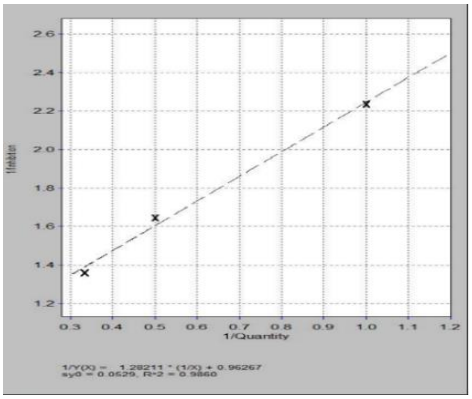

Fig. 3. Trolox standard calibration curve

Antibacterial activity

In order to demonstrate the antibacterial activities, we measured areas of inhibition for the different microorganisms that were classified under standardized conditions: from the gram positive bacteria we had *Staphylococcus aureus* (MTCC 737) and *Staphylococcus epidermidis* (MTCC 3615). We also had the gram negative bacteria - *Escherichia coli* (MTCC 1687) and *Pseudomonas aeruginosa* (MTCC 424). Ampicillin was used as reference substance, the standard drug.

Solvents chloroform, *n*-hexane and ethyl alcohol – water (1:1) were used to extract the material previously powdered (300 g), using the maceration process for 72 hours, ocassionally stirring from time to time.

Each of the three extracts were concentrated after it was brought together with the filtrate and we kept them in the desiccator for future experiments. The *n*-hexane extract, chloroform extract and ethanol: water (1:1) extract were concentrated after combining the filtrate and kept in desiccator for further investigation.

Results and Discussions

The marine algae samples were first treated with a non-polar solvent such as benzen or dyethil ether, then with a medium polarity solvent such as methyl alcohol or ethyl alcohol and finally with water. Each extract has been afterwards analysed in order to identify the presence of active principles of pharmaceutical interest. The extracts were analysed individually using the specific methods for each group of active principles.

Table 2. Active principles identified in green algae *Ulva lactuca* L.

Table 2

Type of extractive solution	Class of active principles
Etheric extractive solution (A)	steroles
	triterpenes
	cumarines
Alcoholic extractive solution (B)	catehic tannins
	reducing compounds
	antracenozides
	cumarines
Water extractive solution (C)	reducing compounds
	saponosides
	catehic tannins

highlights the active principles identified in green algae *Ulva lactuca* L. It is interesting that in all three extractive solutions studied different classes of bioactive compounds have been identified that are responsible for the antioxidant and antimicrobial activity that we studied in the paper.

Total phenolic content determination and pigments chemical composition

The antioxidant capacity and antibacterial potential of the marine algae samples were highlighted by identifying and determining different natural compounds and their active principles, recognized in literature data for having the potential to give antioxidant and antibacterial activity. In the above table we showed our results for the determination of total phenolic contents, total chlorophylls (*a +b*) and total carotenoids. Table 3 presents the results obtained for the compositions of the studied marine algae. There is a good consistency with the literature data of other researchers who have worked on green seaweed. Phenolic compounds can be defined by their aromatic rings and their hydroxyl groups. A phenolic compound which contains an aromatic ring with one or two groups of hydroxyls are named simple phenols, for example benzoic acid or hydroxycinnamic acid which can be found in marine algae structure. Polyphenols represent a large family comprised by multiple phenol units [18]. From this method we can conclude that the most stable extracts and the highest inhibition are the *Ulva lactuca* L. extracts made with the Soxhlet extraction, from which it can highlight *Ulva lactuca* L. extract 10 g/100 mL ethanol 70%, with an increased antioxidant activity. From the cold maceration, the best results were

obtained from *Ulva lactuca* L. extract 5 g/100 mL ethanol 50% and *Ulva lactuca* L. extract 2.5 g/100 mL ethanol 70%.

Table 3. Total phenolic content, total chlorophylls and total carotenoids contents from extracts of green macroalgae *Ulva lactuca* L. from the Romanian Black Sea shore

Sample	Total phenolic TPC (mg. GAE/100g d.w.)	Total chlorophylls T-Chl (a) mg/g d.w	Total chlorophylls T-Chl (b) mg/g d.w	Total carotenoids (TCAR):
Ulva lactuca L.	286.1 ± 0.6	21.08 ± 2.69	3.77±0.36	10.57±0.85

All values show mean of three replicates, ± standard deviation

The unique chemical, physical and biological structure of polyphenols can divide them into different classed like phloroglucinols and phlorotannins. In recent times, various studies showed multiple beneficial therapeutical actions for phenolic compounds found in marine algae, such as antioxidant, antibacterial, anticancer and anti-inflammatory activities.

Marine algae organisms can synthesize different pigments such as carotenoids, phycobiliproteins and chlorophylls [19]. The green pigment for *Ulva lactuca* L. is given by the presence of *clorophylls a* and *b* [19].

Antioxidant activity

Samples were prepared and the results were expressed as nmol Trolox equivalents/sample volume, according to Table 4.

Table 4. Maximum inhibition of free radicals and total antioxidant capacity of *Ulva lactuca* L. extracts

Extract samples	Max. Inhibition of free radicals	Total antioxidant capacity (nmol equiv. Trolox/sample volume)
Ulva lactuca extract 2.5g/100mL ethanol 50%, cold maceration	0.213	0.109
Ulva lactuca extract 5g/100mL ethanol 50%, cold maceration	0.257	0.161
Ulva lactuca extract 10g/100mL ethanol 50%, cold maceration	0.178	0.023
Ulva lactuca extract 2.5g/100mL ethanol 70%, cold maceration	0.381	0.285

Ulva lactuca extract 5g/100mL ethanol 70%, cold maceration	0.245	0.138
Ulva lactuca extract 10g/100mL ethanol 70%, cold maceration	0.024	0.022
Ulva lactuca extract 2.5g/100mL ethanol 70%, Soxhlet extraction	0.140	0.035
Ulva lactuca extract 5g/100mL ethanol 70%, Soxhlet extraction	0.231	0.111
Ulva lactuca extract 10g/100mL ethanol 70%, Soxhlet extraction	0.311	0.272

In the hydroalcoholic extracts of *Ulva lactuca*, obtained by cold maceration, a significant total antioxidant capacity was recorded for *Ulva lactuca* extract 5 g/100 mL ethanol 50% with 0.161 nmol equiv. Trolox/10 µL sample and for *Ulva lactuca* extract 2.5 g/100 mL ethanol 70% with 0.285 nmol equiv. Trolox/10 µL sample.

In the hydroalcoholic extracts of *Ulva lactuca*, obtained by reflux, Soxhlet extraction, a significant total antioxidant capacity, compared to extracts obtained by cold maceration, was recorded for *Ulva lactuca* extract 10 g/100 mL ethanol 70%, with 0.272 nmol equiv. Trolox/10 µL sample.

The highest antioxidant activity was obtained through cold maceration for *Ulva lactuca* extract 2.5 g/100 mL ethanol 70% sample, with a value of 0.285 nmol equiv. Trolox/10 µL sample.

The results obtained emphasize that extracts obtained in ethanol 70% induce the highest values of total antioxidant activities for different concentrations of the samples.

Antioxidants are very important compounds that can stop the production of free radicals and the chain reactions that may affect important cells in the organism through oxidation. They are also used in food and pharmaceutical preservation, with increasing percentage in recent times because of the toxicity and danger to human health caused by the use of synthetic antioxidants [20]. Seaweeds represent an important natural source for antioxidants with very little side effects. In human organism antioxidants are significant in their activity of regulating the oxidative stress related diseases [20, 21].

Ulva lactuca L. contain a varied compounds array with antioxidant activit such as, tocopherols, carotenoids and polyphenols. An important compound with antioxidant potential in green seaweeds is represented by ulvan, a sulfated heteropoly-saccharide obtained from *Ulva lactuca* [22].

Antioxidant potential was measured by scavenging activity of hydroxyl and superoxide radicals, metal chelating potential and superoxide radicals. Sulfate contents in ulvan has been altered by using trioxide/*N,N*-dimethyl formamide. After the modification all the parameters studied gave better results, higher scavenging activity, better reducing power and stronger chelating ability [22].

From this study we draw the conclusion that even if sulfates are already in the composition of ulvan, by increasing the number of sulfate radicals we can enhance the potential of its antioxidant activity [22].

Antibacterial activity

Many species of marine algae have been studied in order to examine their antibacterial and antiviral properties. A lot of polysaccharides have been found in the composition of seaweeds, from which we highlight some that could potentially exhibit antibacterial and antiviral activity: alginic acid, agar, laminarin, fucoidan, galactans, carrageenan, and mannans [23]. In this present study we explore the potential antibacterial activity of green seaweed *Ulva lactuca* L. Review of literature has highlighted possible antibacterial activity in different compounds present in the green algae. In the view of this, an effort was made to verify some of the isolated compounds from *Ulva lactuca* L. for their antibacterial activity. The area of inhibition of microorganisms under standardized conditions was used in order to demonstrate antibacterial action for the various compounds. For present work, efficacy of eight compounds were detected against *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa* and *Escherichia coli.*, see Fig. 4. The concentration used for the test compound was 1mg/mL. and we took Ampicillin as the standard drug.

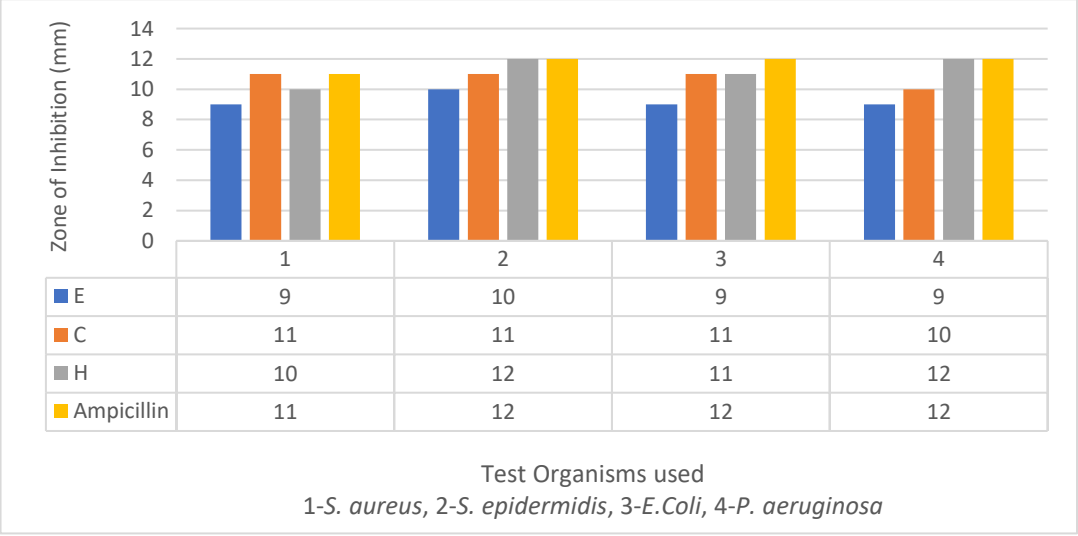

Fig. 4. Antibacterial activity of *Ulva lactuca* L. species

Against *Staphylococcus aureus* the chloroform extract showed the highest inhibition zone (11 mm) comparable to that of the standard ampicillin. In the case of *Staphylococcus epidermides* and *Pseudomonas aeruginosa,* the extract in *n*-hexane achieved the largest inhibition zone (12 mm) comparable to that of th*e* ampicillin standard. In the case of *Escherichia coli*, the extracts in *n*-hexane and in chloroform achieved the largest areas of inhibition (11 mm) but are below the values presented by the ampicillin standard. From the experiments we concluded that *n*-hexane and chloroform extracts had antibacterial activity comparable to the standard drug, ampicilin.

Conclusions

Green algae *Ulva lactuca* L. shows promising potential for both antioxidant and antimicrobial activity, that are due to the existence of known bioactive chemical compounds that support these characteristics. *Ulva lactuca* L. is also a source for many essential bioactive compounds ranging from antioxidants and essential minerals to the highly signified and complete profiles of amino acids and fatty acids. In the present study we determined the total phenolic content, total chlorophylls T-Chl (*a + b*) and total carotenoids. We managed to demonstrate that various compounds from *Ulva lactuca* L. exhibit antioxidant activity which is given by various compounds such as phenolic constituents, pigments and other active principles and we showed that the *n*-hexane and chloroform extracts display good antibacterial activity on various gram positive and negative bacteria.

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