Chemical Analysis of Selected Seaweeds and Seagrass from the Adriatic Coast of Montenegro

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Three seaweeds (Halimeda tuna, Codium bursa and Cystoseira barbata) and one seagrass (Cymodocea nodosa) were collected from the Coast of Montenegro, Gulf of Boka Kotorska and their chemical analysis was performed. In seagrass C. nodosa, three phenolic compounds were identified (diosmetin 7-sulfate, caftaric and coutaric acid). The content of β-glucan, fatty acids, sterols and micro- and macro-elements were investigated among all samples. The highest content of β-glucan was detected in C. nodosa seagrass (13.04 ± 0.42 g/100 g). The highest polyunsaturated fatty acids (PUFAs) level was reported in C. barbata, the brown alga (7.157 mg/g), which also had the significant sterol content (fucosterol, 21.76 ± 0.1 μg/g). Green algae, C. bursa and H. tuna, showed the highest level of sterols (β-sitosterol, 95.21 ± 0.16 μg/g and 73.90 ± 0.08 μg/g, respectively). H. tuna had the highest content of calcium (Ca) in amount of 55125 μg/g. In C. bursa, C. barbata and C. nodosa, the Na/K ratio was low (0.43, 0.46 and 0.69, respectively).

Keywords: Halimeda tuna, Codium bursa, Cystoseira barbata, Cymodocea nodosa, seaweeds.

Introduction

Seaweeds present an important source of biologically active compounds in industry of pharmaceutics, food and agronomy.[1] Many of these active principles are used to treat diseases like cancer, pain, inflammation, arthritis, as well as fungal, bacterial and viral infections. There is an increasing focus on seaweeds in food and nutrition because of high amount of carbohydrates, proteins and minerals.[2] Seaweeds have low fat content and they are rich in low-digestible carbohydrates, so they can be supported as low calorie foodstuffs.[3,4]

High content of minerals in seaweeds is well established (8–40%).[5] Seaweeds are rich in the trace elements and essential minerals that are hard to find in terrestrial plants.[6] Environment, season, physiology, as well as the geographical origin of seaweeds may induce the variations in mineral content, which is generally higher than that in terrestrial plants.[7,8] Ash content in vegetables, according to statistic of United States Department of Agriculture (USDA), ranges between 5–10 g/100 g dry weight, while in seaweeds it might be above 30 g/100 g dry weight.[9]

β-Glucans, the long-chain polysaccharides, are found in the higher plants, cell wall of fungi and in yeasts, and they are concerned as naturally occurring non-specific immunostimulants.[10] Soluble β-1,3/1,4-glucan from barley[11] and soluble β-1,3/1,6-glucan from fungi[12] have been investigated for use in
medicinal products and food. An interest in finding new sources of β-glucan increase, and algae, certainly, might be one of them. Bobadilla et al.\textsuperscript{[13]} detected a water-soluble form of β-1,3/1,6-glucan from brown algae and described its immunostimulatory effect.

Algae and seagrass are rich in phytosterols and polyunsaturated fatty acids (PUFAs). Phytosterols and PUFAs are biologically active lipids identified in many terrestrial plants and marine organisms.\textsuperscript{[14]} There has been increased scientific interest in biological and nutritive properties of phytosterols and PUFAs in human health.\textsuperscript{[15]} Numerous clinical trials confirmed the link between the consumption of phytosterols and decreased cholesterol level in sera.\textsuperscript{[16]} Other important properties of plant sterols include antitumor, anti-inflammatory, antifungal, antioxidant, antibacterial and antilulcer activities.\textsuperscript{[17,18]} In brown seaweeds, the main sterols were fucosterol and its derivatives.\textsuperscript{[19]}

There has been a lot of research in the field of algae and seagrass from various geographic regions, but there are only few studies on health benefits, chemical composition and nutritive potential of seaweeds from the Adriatic Sea. Seaweeds, widely distributed in the Adriatic Sea whose potential application in food or pharmaceutical industry have not been sufficiently tested, were selected for this research. Biological activities, health protective and nutritive potential of selected seaweeds from the Montenegro Coast Halimeda tuna (J.Ellis & Solander) J.V.Lamouroux, Halimedaceae; Codium bursa (Oliv) C.Agardh, Codiaceae; Cystoseira barbata (Stackhouse) C.Agardh, Sargassaceae; Cymodocea nodosa (Urchia) Ascherson, Cymodoceaceae) were presented in our previous work.\textsuperscript{[20]} The green alga, H. tuna was found in Mediterranean Sea, Atlantic and Indian Ocean.\textsuperscript{[21]} The green macroalga C. bursa, the brown alga C. barbata and seagrass C. nodosa are distributed in North Atlantic and Mediterranean Sea.\textsuperscript{[21]} In our previously published study, H. tuna had the best cytotoxic activity against human colon carcinoma cell lines, LS174 (IC\textsubscript{50} = 17.92 ± 1.54 μg/mL). C. nodosa demonstrated strong cytotoxicity against human adenocarcinoma cell lines, HeLa (IC\textsubscript{50} = 13.28 ± 0.39 μg/mL) and human chronic myelogenous leukemia cell lines, K562 (IC\textsubscript{50} = 19.64 ± 1.55 μg/mL). C. barbata had the best anti α-glucosidase (IC\textsubscript{50} = 9.98 ± 3.34 μg/mL) and antimicrobial activity (minimal inhibitory concentration of 100 μg/mL) for Staphylococcus aureus and Bacillus subtilis. C. bursa showed the highest nutritional value (490.4 kcal).\textsuperscript{[20]}

In continuation of our research, health protective activity and chemical analysis of selected biomaterial were further studied.

Results and Discussion

Content of β-Glucan

The highest level of β-glucan was found in C. nodosa (13.04 ± 0.42 g/100 g), seagrass, and the lowest was in C. bursa (2.43 ± 0.23 g/100 g), brown seaweed (Table 1). In comparison to results of Bobadilla et al.\textsuperscript{[13]}

| Sample            | α-Glucan | β-Glucan | Total glucan |
|-------------------|----------|----------|--------------|
| Codium bursa      | 4.89 ± 0.14 | 2.43 ± 0.23 | 7.32 ± 0.37 |
| Cystoseira barbata| 0.16 ± 0.21 | 5.80 ± 0.31 | 5.96 ± 0.52 |
| Halimeda tuna     | 5.81 ± 0.01 | 2.74 ± 0.14 | 8.55 ± 0.15 |
| Cymodocea nodosa  | 0.66 ± 0.17 | 13.04 ± 0.42 | 13.07 ± 0.59 |

The number of independent experiments n = 2. The results were expressed as mean ± SD.

that have found for Durvillaea antarctica (Chamisso) Hariot (DA) (significant immunomodulatory activity because of high content of 1,3;1,6-β-d-glucan (4–15 g/100 g)), we can assume that C. nodosa and C. barbata have potentially high immunostimulatory activity. Besides immunomodulatory function, β-glucans can decrease the blood cholesterol level.\textsuperscript{[22]} Laminarin (1,3-β-d-glucan) is the most studied polysaccharide isolated from brown seaweed, which acts as a reserve of energy, stored in the macroalgal cell vacuoles.\textsuperscript{[23]} Data shows that use of brown seaweeds (Laminaria) in nutrition can lower the breast cancer risk.\textsuperscript{[24]} Also, it is known that 1,3-β-d-glucan can alter the fecal flora enzymatic activity, and it can stimulate the immune response.\textsuperscript{[22]}

Fatty Acids Content

Seaweeds are not generally rich in lipids. However, many researchers have reported high levels of polyunsaturated fatty acids (PUFAs) which have many health benefits.\textsuperscript{[26]} In our research, we report the highest level of palmitic acid (C16:0) which was found in all tested extracts as a dominant fatty acid (Table 2). Myristic acid (C14:0) and stearic acid (C18:0) were found in all tested seaweed cyclohexane extracts as well. Among monounsaturated fatty acids (MUFA), oleic acid (C18:1 n-9 cis) was abundant in all seaweeds, and the highest amount of 2.170 mg/g was found in C. barbata. The highest level of PUFAs was in C. barbata, the brown alga (7.157 mg/g). All tested seaweeds contain significant amount of omega-3 fatty acids, and there were further studies.

Table 1. The content of α-glucan and β-glucan in selected seaweeds [g/100 g].
acid such as \(\alpha\)-linoleic acid (ALA) in a wide range between 0.029 – 3.291 mg/g. Fatty acids such as \(\alpha\)-linolenic, eicosapentanoic, docosahexanoic acid protect against coronary heart disease.\[27\] C. barbata, C. nodosa and C. bursa contain odd chain fatty acids like: heptadecanoic (C17 : 0) and pentadecanoic (C15 : 0) acid. Some researchers find correlation of odd chain fatty acids with the decreasing risk of cardiovascular and metabolic disease.\[28\] The most abundant among n-6 fatty acids, in investigated seaweed extracts, was linoleic acid (C18:2 n-6) as major fatty acid that regulates low-density lipoproteins (LDL) metabolism.\[27\]

**Content of Sterols**

Sterols present important components of all cell membranes of eukaryotic organisms. They have role in membrane fluidity control and membrane permeability.\[29\] In brown alga C. barbata, fucosterol was detected in amount of 21.76 \(\pm\) 0.1 mg/g (Table 3). Presence of fucosterol in brown algae was previously reported by Patterson.\[30\] The small amount of fucosterol might be due to season of sample collection (July) which is in accordance to Boulom et al.\[31\] who reported that fucosterol and 24-methylenecholesterol show significantly higher results during winter. Significant decrease of 24-methylenecholesterol and fucosterol was found in summer.\[31\] Investigation of Honya et al.\[32\] supports that the concentration of fucosterol in Laminaria japonica, brown seaweed, gets its maximum during winter period, which decreases in autumn. It is well-known that fucosterol exhibits various biological activities including anticancer, antioxidant, antihyperlipidemic, blood cholesterol reducing and blood vessel thrombosis preventive.\[33\] It was reported that the sterol composition in green algae, is complex and differs than in other groups of algae.\[30\] In both examined green alga samples (H. tuna and C. bursa), significant amount of \(\beta\)-sitosterol was found.

| Fatty acid | Halimeda tuna | Cystoseira barbata | Cymodocea nodosa | Codium bursa |
|------------|---------------|---------------------|------------------|--------------|
| C12:0      | 0             | 0                   | 0.137            | 0            |
| C14:0      | 0.171         | 0.741               | 0.466            | 0.316        |
| C15:0      | 0             | 0.068               | 0.148            | 0.037        |
| C16:0      | 0.717         | 6.376               | 11.580           | 3.638        |
| C17:0      | 0             | 0.050               | 0.193            | 0            |
| C18:0      | 0.146         | 0.471               | 1.071            | 0.736        |
| C 20:0     | 0             | 0.064               | 0                | 0            |
| C 22:0     | 0             | 0.089               | 0.326            | 0.187        |
| C 23:0     | 0             | 0                   | 0.209            | 0            |
| C 24:0     | 0.019         | 0                   | 0.306            | 0            |
| \(\Sigma\) SFA | 1.053     | 7.859              | 14.436           | 4.914        |
| C16:1      | 0.056         | 0.452               | 0                | 0.227        |
| C18:1 n-9 cis | 0.070   | 2.170              | 12.768           | 0.895        |
| C18:1 n-9 trans | 0.085 | 0                  | 0                | 0            |
| \(\Sigma\) MUFA | 0.211       | 2.622              | 12.768           | 1.122        |
| C18:2 n-6   | 0.138         | 0.355               | 3.866            | 0            |
| C18:2 n-9 trans | 0           | 0                  | 0.250            | 0            |
| C18:3 n-3   | 0.029         | 0.272               | 3.291            | 0.240        |
| \(\Sigma\) PUFA | 0.167    | 0.627              | 7.157            | 0.490        |
| PUFA/SFA    | 0.159         | 0.080               | 0.496            | 0.100        |

| Sterol      | Halimeda tuna | Cystoseira barbata | Cymodocea nodosa | Codium bursa |
|-------------|---------------|---------------------|------------------|--------------|
| \(\beta\)-Sitosterol | 73.90 \(\pm\) 0.08 | 0                   | 5.70 \(\pm\) 0.03 | 95.21 \(\pm\) 0.16 |
| \(\beta\)-Stigmasterol | 0           | 0                   | 6.04 \(\pm\) 0.08 | 0            |
| Fucosterol  | 0             | 21.76 \(\pm\) 0.1   | 0                | 0            |
| Campesterol | 3.78 \(\pm\) 0.12 | 0                   | 0                | 0            |

The number of independent experiments \(n=2\). The results were expressed as mean \(\pm\) SD.

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**Table 2.** Fatty acid composition of selected seaweeds [mg/g].

**Table 3.** Sterols content in selected seaweeds [\(\mu\)g/g].
Data shows the effect of β-sitosterol in benign prostate hyperplasia risk reduction, in prevention of colorectal cancer, heart disease, modulating the immune system, in treatment of tuberculosis, hypercholesterolemia, rheumatoid arthritis and cervical cancer. Campesterol was determined in very small amounts was: Ca > Na > K > Mg > P > Si > B > Zn > Bi > Ba > Cr > Cu > Li. C. bursa, C. nodosa and C. bursa have the highest level of potassium (K). The order of minerals in C. nodosa and C. bursa was: K > Na > Mg > Ca > P > Si > Zn > Bi > Ba > Cr > Cu > Li. Mineral content in C. barbata, the brown alga, differs, in comparison to other tested samples: K > Na > P > Si > Mg > Bi > In > Zn > Ba > Ag, while Ca was below the limit of detection. According to American Heart Association, the level of Na/K in food is very important in treatment of hypertension. In C. bursa, C. barbata and C. nodosa, the Na/K ratio was low (Table 4) which supports the inclusion of these seaweeds in the antihypertensive diet.

Very high level of strontium (Sr), as well as high level of mercury (Hg) and lead (Pb) in all tested samples might be due to environmental contamination [20]. In seaweed H. tuna, we noticed very high level of Sr (approximately 30 times higher than ones in C. nodosa and approximately 48 times higher than ones in C. bursa). Very high accumulation of Sr in H. tuna might be in correlation with very high level of Ca in comparison to all investigated seaweeds, keeping in mind the fact, that Sr is analog of Ca in living organisms, and that Sr has high affinity to plasma membrane transporters for calcium and potassium [40–42].

**LC/MS Data Analysis**

Among investigated samples, C. nodosa had the best cytotoxic activity in our previous research (IC₅₀ = 13.28 ± 0.39 μg/mL, human adenocarcinoma cell lines (HeLa)). C. nodosa also has shown the highest antioxidative activity in DPPH test and the highest total phenolic content. Due to those data, further

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**Table 4.** Mineral content in selected seaweeds [μg/g].

| Minerals | Halimeda tuna | Cystoseira barbata | Cymodocea nodosa | Codium bursa |
|----------|--------------|-------------------|-----------------|-------------|
| Ag       | 0            | 60                | 0               | 0           |
| As       | 0            | 0                 | 0               | 0           |
| B        | 110          | 0                 | 560             | 166         |
| Ba       | 26           | 250               | 27              | 31          |
| Bi       | 32           | 360               | 33              | 34          |
| Ca       | 55125        | 0                 | 2476            | 2456        |
| Cd       | 0            | 0                 | 0               | 0           |
| Co       | 0            | 0                 | 0               | 0           |
| Cu       | 0            | 180               | 25              | 36          |
| Fe       | 168          | 0                 | 319             | 134         |
| K        | 2871         | 4242              | 17515           | 39346       |
| Li       | 7            | 40                | 5               | 6           |
| Mg       | 1393         | 2800              | 3392            | 2471        |
| Mn       | 0            | 0                 | 51              | 0           |
| Na       | 9823         | 19470             | 12051           | 17025       |
| Ni       | 0            | 0                 | 0               | 0           |
| P        | 1104         | 15990             | 2380            | 735         |
| Pb       | 11           | 50                | 22              | 11          |
| Si       | 420          | 5340              | 407             | 558         |
| Sr       | 18492        | 6880              | 619             | 387         |
| Ti       | 0            | 0                 | 0               | 0           |
| Zn       | 58           | 290               | 69              | 59          |
| Hg       | 47           | 380               | 37              | 34          |
| Na/K     | 3.42         | 0.46              | 0.69            | 0.43        |
investigation led to chemical composition analysis of *C. nodosa* seagrass. In seaweed *C. nodosa*, three compounds were identified (diosmetin 7-sulfate (1); caftaric acid (2); coutaric acid (3)) according to their UV, mass and NMR spectra, library and literature. Peaks were identified in negative mode of ESI spectra, and for compound 1 using HR-MS and 1H-NMR data. Caftaric (2) and coutaric (3) acids were identified comparing their UV spectra with standard and literature data, while diosmetin 7-sulfate (1) was identified according UV, mass and 1H-NMR spectra.

In negative mode, at fragmentor voltage of 100 V, compound 1 had molecular ion weight 379 (M⁻). We increased ionization energy at 170 V and the compound 1 ion had molecular weight 299 (M⁻). Neutral loss of 80 Da, according to literature, indicates separation of the sulfate ion. At higher ionization energy of 250 V, we got the dominant ion with molecular weight of 285 (M⁻). Loss of 15 Da, according to literature, indicates separation of methyl group.

Compounds 1 had HPLC-DAD λₘₐₓ (band I) 347 nm (band II) 252 nm, 268 nm (shoulder, in acetonitrile as solvent) which additionally confirms flavonoid aglycon diosmetin.

To get additional data, compound 1 was isolated by semipreparative chromatography, and additional HR-MS were recorded using Q-TOF-MS/MS detector. The molecular formula was C₁₆H₁₁O₅S, and it was obtained from the deprotonated molecule at m/z 379.0155 [M−H]⁻ (calc. 379.0167 for [M−H]⁻ C₁₆H₁₁O₅S, Δppm = −3.69). The main fragment ion of Q-TOF-MS/MS spectrum was m/z 299.0577 (C₁₆H₁₁O₅), yielded by loss of SO₂ (−80 Da). The minor fragment ion at m/z 284.0355 (C₁₅H₇O₆) was determined by additional demethylation from the deprotonated molecule. After the hydrolysis of the compound 1, using sulfatase, the aglycone was identified as diosmetin compared to commercial authentic standard (Figure 1).

The position of sulfate group was assumed using UV spectral analysis and 1H-NMR data. According to published data of Enerstvedt et al., UV absorption spectra of luteolin 7-sulfate and luteolin are relative similar, whilst the significant hypsochromic shift in the UVₘₐₓ of luteolin 7,3'-disulfate, is strongly indicating the presence of a sulfate group in the 3'- or 4'-position on the B-ring. Introducing a sulfate group to the flavonoid A-ring does not influence the UV absorption significantly, but sulfation in the 3'- or 4'-position on the B-ring will cause a large hypsochromic shift in band I. Also, flavonoid sulfates seem to have analogous UV/VIS spectral characteristics as their corresponding flavonoid glycosides, however, introducing a sulfate group in 3'- or 4'-position on the B-ring will cause a large hypsochromic shift in band I, and band II appears as a single peak.'

In our results, UV spectra of compound 1 is relative similar to diosmetin UV spectra and band II appears as a double peak which is characteristic to a diosmetin aglycon (Figure 2) without hypsochromic shift in UVₘₐₓ (HPLC-DAD λₘₐₓ (band I) 347 nm (band II) 252 nm, 268 nm (shoulder, in acetonitrile as solvent)). According to this data, we can assume that our isolated compound 1 is the most probably diosmetin 7-sulfate (Figure 3).

1H-NMR data for compound 1 are in correlation with the results of Grignon-Dubois and Rezzonico for diosmetin 7-sulfate.

Specific data of identified compounds in the seagrass *C. nodosa* are listed below:

**Diosmetin 7-sulfate (1):** HPLC-DAD λₘₐₓ (nm): (band I) 347, (band II) 252, 268 nm (shoulder, in acetonitrile as solvent). LC/MS-ESI m/z: 379 (M⁻-1), 299 (M⁻-1-80, 100 %). 1H-NMR (400 MHz, DMSO): 3.94 (s, MeO); 6.67 (d, J₁/₄ = 1.67, H-6); 6.69 (s, H-3); 7.08 (d, J₁/₄ = 1.43, H-8); 7.10 (d, J₁/₄ = 8.58, H-5); 7.43 (d, J₁/₄ = 2.13, H-2); 7.58 (dd, J₁/₄ = 8.59, 2.15, H-6). HR-MS m/z: 379.0155 [M−H]⁻ (calc. for [M−H]⁻ C₁₆H₁₁O₅S, 379.0167). qTOF-MS/MS (15 eV) m/z (rel. int.): 379.0155 (40), 299.0577 (100), 284.0355 (7).

**Coutaric acid (2):** HPLC-DAD λₘₐₓ (nm): 314. LC/MS-ESI m/z: 295 (M⁻-1).

**Caftaric acid (3):** HPLC-DAD λₘₐₓ (nm): 328. LC/MS-ESI m/z: 311 (M⁻-1).

**Biodiversity of Selected Seaweeds and Seagrass**

*H. tuna* is a calcareous green seaweed, attached to the seabed by a holdfast. This species is found in the tropical and subtropical Indo-Pacific region, the Mediterranean Sea and the western Atlantic Ocean. It grows on rocky bottom from the shallow subtidal zone down to depths of about 70 m. *C. bursa* thallus looks, youth, almost perfectly spherical, then flattens out and hollowed in the middle. It is distributed in southwest Pacific, Northeast Atlantic and the Mediterranean. *C. barbata* (STACKHOUSE) C.AGARDH is a brown macroalga endemic to the Mediterranean. Populate upper sublittoral zone, 0.2 m depth, on open and sheltered rocky shores, in semi-closed areas and coastal lagoons. *Codium* species populate Mediterranean Sea, Black Sea, North-East Atlantic from Portugal to the Canary Islands; Indian Ocean (India and Pakistan). The seagrass, *C. nodosa* is found in shallow parts of the Mediterranean Sea and the adjoining parts of the
Atlantic Ocean, the coasts of Portugal, Mauritania and Senegal and round the Canary Islands, Madeira and the island of Cape Verde. It grows at depths of down to ten meters in sandy sediments in sheltered locations and needs clear waters for photosynthesis.\textsuperscript{[46]}

Flavonoids (diosmetin 7-sulfate) and phenolic acids (coutaric and caftaric acid), detected in \textit{C. nodosa}, are known as phenolic compounds, defensive secondary plant metabolites. They have antiherbivory properties, antimicrobial activity, helping in the nutrient storage by protecting oxidation of fatty acids, and also protecting marine macrophytes and seaweeds against pathogen attacks and harmful ultraviolet radiation.\textsuperscript{[47]}

In process of adaptation to environmental conditions, such as marine water salinity, temperature, UV light exposure, presence of other marine organisms, seagrass and seaweeds produce different biologically active compounds. There is an increasing evidence that flavonoid sulfates increase the physiological survival of seagrasses in marine environment.\textsuperscript{[45]}

\textbf{Figure 1.} HR-MS spectra of compound 1 and diosmetin.
Sulfated flavonoids are reported to be involved in plant growth regulation, and their presence in marine plants represents an ecological adaptation to the saline environment.

Conclusions

*H. tuna*, as a promising source of calcium, should be further included in supplementation and nutritional research. With its high level of β-sitosterol, *H. tuna*, together with *C. bursa*, should be further investigated regarding to benign prostate hyperplasia risk reduction and treatment of hypercholesterolemia. *C. nodosa* and *C. barbata* have shown the significant level of β-glucan, and further research should lead to its immunostimulatory effect investigation. Also keeping in mind, the lack of the information about biological and pharmacological activities of diosmetin 7-sulfate, our further scientific interest should be directed to that way. Results, presented in this work, show that *C. nodosa*, *H. tuna*, *C. bursa* and *C. barbata* present a great potential of Adriatic Sea for future novel food and potential source for new drug investigations. Further research should lead to new natural products discovery and the potential application of selected seaweeds in medicine, pharmacy and supplementation.

Experimental Section

Chemicals

K-YBGL kit was from Megazyme International (Ireland). Sulfatase, diosmetin, caftaric acid and coutaric acid were purchased from Fluka (Munich, Germany). Organic solvents used in this work were of HPLC grade, purchased from J.T. Baker (Deventer, Netherlands). Other used chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Derivatization reagent for sterol determination was bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (99% BSTFA + 1% TMCS), purchased from Sigma-Aldrich (Steinheim, Germany). The standard for fatty acid methyl esters (FAMES) was Supelco TM 37 Component FAME Mix (Sigma–Aldrich, Steinheim, Germany).

Algae and Seagrass

The raw samples of *H. tuna*, *C. bursa*, *C. barbata* and *C. nodosa* were identified and collected by Slavica Petović from the Adriatic Sea, Gulf of Boka Kotorska in July 2016. A Voucher specimen (registration number 9739; 9740; 9741; 9742) are kept with the Natural History Museum of Montenegro, Podgorica, Montenegro. Collected material was washed with seawater to remove rocks and sand and packed in plastic bags. Each sample of seaweed was later washed thoroughly with distilled water to remove salt and epiphytes, and dried in shade.

The dry sample yield was about 10% of weight of the raw seaweed. Dried samples were powdered using an electric grinder.

Phenolic compounds were analyzed in dichloromethane/methanol (1:1) dry seaweed extracts (DME). Powdered samples were extracted by cold maceration using dichloromethane/methanol (1:1) solvent for 48 h, with periodical shaking. The sample/solvent ratio was 1:5. The extracts were filtered before vacuum evaporation at 40°C to yield residue (3.75 ± 0.5%).

Determination of β-Glucans

The determination of β-glucans was performed in accordance to the method published in the work of Kolundžić et al.[48]
Fatty Acids Composition

The determination of fatty acids and sterols from cyclohexane seaweed extracts was performed in accordance to work of Muszyńska et al. Cyclohexane extracts were obtained from 1 g of pulverized seaweeds. The equipment contained Gas Chromatograph Agilent 6890 N type, with a split/splitless injector (260 °C). Capillary column was Agilent J&W HP-88, 100 m × 0.25 mm, 0.20 μm film thickness. Detection was performed with FID detector and with the Agilent 5975 C MS Detector, operating in the EI mode at 70 eV. The injected volume was 2 μL. Experiments were done in duplicate.

The identification of the FAMEs was done on the comparison of their retention times (tR) and mass spectra of the representative standards ran under the same chromatographic conditions and to those from the NIST/NBS 05 and Wiley (8th edition) libraries. Relative percentages of the compounds were calculated based on the peak areas from the FID data.

Sterols Determination

The previously obtained unsaponifiable fraction was used for the analysis of sterols. Dry petroleum ether extract was dissolved in dichloromethane to achieve the approximately 10 mg/mL concentration. 500 μL of this solvent were mixed with 200 μL of derivatization reagent for sterols (bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (99 % BSTFA + 1 % TMCS)), heated at 70 – 75 °C in water bath for 90 min, filtrated and further used for GC and GC/MS analysis.

Further GC analyses were performed in accordance to method described in work of Kundaković et al.

The compounds were identified based on the mass spectra from data bases and literature (NIST/NBS 05; Wiley, 8th edition).

Determination of Micro- and Macro-Elements

Preparation of the sample for determining heavy metal concentration carried out by wet digestion with nitric acid was in accordance to method published in a work of Kolundžić et al. Because dry sample of C. barbata was hard to digest by standard procedure, the digestion was performed on the same way as other samples, but with the addition of concentrated hydrochloric acid, and allowed to stand 48 h for complete digestion.

Measurements were done in triplicate on ICP-OES (Inductively Coupled Plasma-Optical Emission Spectrometry, ARCOS FHE12, SPECTRO, Germany) according to the manufacturer instruction.

LC/MS (Liquid Chromatography-Mass Spectrometry) Analysis of Phenolic Compounds

Dry DME were dissolved in acetonitrile to rich the approximately 10 mg/mL concentration of each seaweed extract. The analysis of phenolic compounds was performed in accordance to method described in a work of Petrović et al. with some modifications.

Before injection, samples went through 0.45 μm membrane filter. The equipment consisted of Agilent LC/MS System 1260/6130 (Agilent Technologies, Germany), software: ChemStation, Revision (B.04.03-SP1), quaternary pump (G1311B/1260), degasser (model G1311B), fraction collector (G1364C), autosampler (G1329B), single quadrupole API-ESI mass selective detector (MSD) (6130), diode array detector (DAD) (G4212B), analytical column (Zorbax SB-C18, 250 × 4.6 mm; 5 μm).

Mobile phase consisted of 0.1 % formic acid (solvent A) and acetonitrile (solvent B). A gradient elution was: in 0 min-10 % B, in 60 min-100 % B, 65 min-100 % B. Injection volume was 10 μL and the flow rate was 0.8 mL/min. The temperature of column was set at 25 °C. DAD detector was operating at 210–380 nm. Negative ionization mode was used for recording the electrospray mass spectra, with nebulization with nitrogen at flow rate of 10 L/min, temperature of 350 °C and pressure of 40 psi. Deprotonated molecules signals were registered by fragmentor voltage of 100 V, whereas additional fragment ions were recorded at 170 and 250 V. Capillary voltage was set at 3500 V. The mass range, for recording the mass spectra was set from 100 to 1200 m/z.

The identification of compounds was performed in accordance to data from NIST/NBS 05 and Wiley (8th edition) libraries, spectral analysis (UV, NMR) after extraction and literature. The number of identified compounds was presented as area percent of total compounds in extract.
Semipreparative Extraction of Selected Compound

Semipreparative extraction was used for isolation of dominant compound, found in the seagrass C. nodosa. Extracted compound 1 was further identified with spectral analysis.

For semipreparative extraction, the next equipment was used: Agilent LC/MS System 1260/6130 (Agilent Technologies, Germany) with characteristics mentioned above. We used the Zorbax SB C18 column (9.4 × 250 nm, 5 μm) and DAD was used for detection. Binary mobile phase consisted of water (solvent A) and acetonitrile (solvent B). Applied gradient elution was: in 0 min-35% B, in 20 min-100% B, in 25 min-100% B, with post time of 5 min for returning the system to initial conditions. Injection volume was 70 μL and the flow rate was 3.3 mL/min. Column temperature was set at 30°C. DAD detector was operating at 210–380 nm. Fraction collector was adjusted to collect one dominant fraction. Fraction weighted 2 mg after mobile phase evaporation.

LC/MS Analysis of Isolated Compound

High resolution mass spectra (HR-MS) data of isolated compound was performed in accordance to method published in a work of Milutinović et al.[53] using LC system (Waters Alliance 2695) coupled to a Q-TOF (quadrupole/time-of-flight) mass spectrometer (Waters SYNAPT). Column, YMC AQ C18 (2 × 100 mm, 3 μm), was used for separation at 30°C. Mobile phase A (0.1% HCO3/H2O) and mobile phase B (acetonitrile) was used for elution, with flow rate of 0.2 mL/min. Gradient characteristics were: 6–65% B over 30 min and 65–100% B for 10 min. Resolving power for the high resolution mass measurements were 10,000, and Leu-enkephalin was used as the lock mass. Argon was used as collision gas, at a collision energy of 15 eV.

NMR Analysis of Isolated Compound

Bruker (Germany) NMR spectrometer (Avance AVIII 400, equipped with a 5 mm PABBO BBO probe, operating at 400 MHz for 1H and 101 MHz for 13C nuclei) was used for recording the NMR spectra. Compound 1 was dissolved in dimethyl sulfoxide (DMSO). Coupling constants (J) were expressed in Hz and chemical shifts (δ) in ppm.

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Author Contribution Statement

Sanja Milović performed the experiments regarding to LC MS analysis, compound extraction, analyzed data and wrote the article. Ivan Stanković participated in the data processing and design of experiments. Dejan Nikolić performed the HR-MS analysis. Jelena Radović performed the experiments regarding to analysis of sterols and fatty acids. Marina Kolundžić performed the experiment for determination of β-glucans. Vesna Nikolić performed the analysis of micro- and macro-elements. Tatjana Stanojković participated in the data processing and design of experiments. Slavica Petović collected and identified the samples of tested seaweeds and seagrass. Tatjana Kundaković-Vasović corresponding author, participated in the data processing and design of experiments.

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