ORIGINAL ARTICLE

ANTI α-GLUCOSIDASE, ANTITUMOUR, ANTIOXIDATIVE, ANTIMICROBIAL ACTIVITY, NUTRITIVE AND HEALTH PROTECTIVE POTENTIAL OF SOME SEAWEEDS FROM THE ADRIATIC COAST OF MONTENEGRO

SANJA MILOVIĆ[1], TATJANA KUNDAKOVIĆ[1], VESNA MAČIĆ[2], JELENA ANTIĆ STANKOVIĆ[1], NAĐA GROZDANIĆ[3], IVANA ĐURIČIĆ[1], IVAN STANKOVIĆ[1], TATJANA STANOJKOVIĆ[3]*

[1]*Faculty of Pharmacy, University of Belgrade, Vojvode Stepe 450, 11221 Belgrade, Serbia*
[2]*Institute of Marine Biology, P.O. Box 69, 85330 Kotor, Montenegro*
[3]*Institute of Oncology and Radiology of Serbia, Pasterova 14, 11000 Belgrade, Serbia*

**corresponding author: tanjast@lycos.com*

Manuscript received: April 2016

Abstract

The aim of the present study was to reveal the potential biological activities of the dichlorometane:methanol (1:1) dry extract (DME) and the nutritional value of selected seaweeds: *Cymodocea nodosa*, *Halimeda tuna, Cystoseira barbata* and *Codium bursa*, collected from the Adriatic coast, Montenegro. We assessed the chemical composition and several biological activities such as: anti- α-glucosidase, antitumour, antimicrobial and antioxidative activity. *H. tuna* had the best cytotoxic activity against human colon carcinoma cell line, LS174 (IC_{50} = 17.92 ± 1.54 µg/mL). *C. nodosa* demonstrated strong cytotoxicity against human adenocarcinoma cell line, HeLa (IC_{50} = 13.28 ± 0.39 µg/mL) and human chronic myelogenous leukaemia cell line, K562 (IC_{50} = 19.64 ± 1.55 µg/mL). *C. barbata* had the best anti α-glucosidase (IC_{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).

Rezumat

Scopul studiului a fost de a investiga activitățile biologice potențiale ale extractului uscat și valoarea nutritivă a algelor marine selectate: *Cymodocea nodosa*, *Halimeda ton*, *Cystoseira barbata* și *Codium bursa*, provenite de pe coasta Mării Adriatice, Muntenegru. Au fost evaluate compoziția chimică, activitatea anti-α-glucozidază, antitumorală, antimicrobiană și antioxidantă. *Tonul H.* a avut cea mai bună activitate citotoxică asupra liniei celulare de carcinom de colon uman, LS174 (IC_{50} = 17,92 ± 1,54 pg/mL). *C. nodosa* a demonstrat o citotoxicitate puternică asupra liniei celulare de adenocarcinom uman, HeLa (IC_{50} = 13,28 ± 0,39 µg/mL) și a liniei celulare de leucemie mielogenă cronică umană, K562 (IC_{50} = 19,64 ± 1,55 µg/mL). *C. barbata* a avut cea mai bună activitate anti- α-glucozidază (IC_{50} = 9,98 ± 3,34 µg/mL) și activitate antimicrobiană (concentrație minimă inhibitoare de 100 µg/mL) pentru *Staphylococcus aureus* și *Bacillus subtilis*. *C. bursa* a arătat cea mai mare valoare nutritivă (490,4 kcal).

Keywords: biological activities, seaweed, nutrients, anti α-glucosidase, antitumour, antimicrobial, antioxidant

Introduction

There are over 10,000 metabolites isolated from seaweeds, many of which showed pharmacological properties. A broad spectrum of biological activities has been detected, such as antibiotic, antifungal, hypoglycaemic, cytotoxic, neurotoxic, hypolipemic, antimitotic, antiviral and antineoplastic and in recently they were used against AIDS, as immunosuppressant, antiinflammatory, against Alzheimer’s disease, ageing processes and some tropical diseases [8, 29, 44]. Although there has been a lot of research in the field of seaweeds from various geographic regions, there are only few studies on the health benefits of marine algae from the Adriatic Sea [16, 39].
Halimeda tuna (J. Ellis & Solander) J. V. Lamouroux, *Halimedaceae* family was found in Atlantic and Indian oceans, as well as in the Mediterranean Sea [2]. *Codium bursa* (Olivi) C. Agardh, *Codiaceae* family the green macroalga, the brown alga *Cystoseira barbata* (Stackhouse) C. Agardh, *Sargassaceae* family and one seagrass *Cymodocea nodosa* (Urcia) Acherson, *Cymodoceaceae* family are distributed in North Atlantic and Mediterranean Sea [2]. The green alga (*Chlorophyta*) has a significant amount of essential amino acids, essential fatty acids, chlorophyll, vitamins, carotenoids, minerals, pigments and polysaccharides [1]. The brown alga (*Phaeophyta*) is an excellent source of iodine, complex polysaccharides (soluble fibres) and vitamins [1].
Previous studies have shown the presence of several primary and secondary metabolites in selected marine organisms [11, 12].

Seaweeds are important and promising resources in cancer research and a number of compounds from these organisms have undergone clinical trials as antitumour agents [41]. Several authors have found seventeen seaweed species with *in vitro* cytotoxic potential against L1210 leukemic cells [13]. Also, the algae are known to biosynthesize a number of bioactive secondary metabolites which stave off many potential invaders either in a constitutive or an inducible way [5], with known antioxidant activity [25, 43].

A recent study of Kolsi *et al.* has shown significant α-amylase inhibitory activity of sulphated polysaccharides from *C. nodosa* and protection of pancreatic β-cells in rats with experimentally induced diabetes [18]. Also, the lipid peroxidation and toxic damage was decreased in the pancreas, suggesting the protective effects of *C. nodosa* polysaccharides in liver, pancreas and kidney. The same authors have shown the anti-obesity and lipid lowering activity of polysaccharide fraction of *C. nodosa* in rats fed with a high cholesterol diet (the decrease of total cholesterol, triglycerides and low density lipoprotein cholesterol (LDL-C) and an increase in high density lipoprotein cholesterol (HDL-C) levels) [18]. Also, the polysaccharides administration lead to the antioxidant activity shown *in vivo*. Different extracts of *C. nodosa* (hexane, ethylacetate and methanol) have shown antibacterial and antifungal activity [18]. The chemical studies are rare. Kontiza *et al.* have shown antibacterial and cytotoxic activity of diarylheptanoids isolated from *C. nodosa* [19].

The data about brown alga *C. barbata* are also scarce. Hexane extract of *Cystoseira barbata* from the Coast of Izmir have shown significant antibacterial activity [27]. Also, antioxidative, lipolytic and hypoglycaemic activities have been shown, fatty and amino acids composition, trace metals, and fucans were identified [20].

Halitunal, an antiviral diterpenic compound was isolated from *H. tuna* [17]. For this green alga, several biological activities have been shown (antibacterial, hypolipidemic, cytotoxic and antioxidant) [22, 36].

The biological activities of *C. bursa* were studied (antibacterial, antiprotozoal, anti-mycobacterial and cytotoxic), but only one compound was isolated from this species [42].

The recent research of the organic extracts from some macroalgae such as: *Rhodomela confervoides* (Huds.) Silva, *Gracilaria textorii* (Suringar) De Toni, *Plocamium telfairiae* Harv, *Dictyopteris divaricata* (Okam.) Okam, have shown inhibitory activity of α-glucosidase [7]. α-Glucosidase represents the key enzyme in carbohydrate digestion catalysing the hydrolysis of 1,4-α-glucosidal bond, present in carbohydrates, promoting the increase of glucose blood level after meals. Acarbose and voglibose, acting as α-glucosidase inhibitors, are clinically used oral antihyperglycaemics which delay intestinal carbohydrate absorption, slowing down the rising in blood sugar level [3]. However, these α-glucosidase inhibitors often cause strong gastrointestinal side effects leading towards the investigation of natural products for antidiabetic activity for the treatment of postprandial hyperglycaemia.

In many cultures, there has been a long tradition in using seaweeds in human nutrition as vegetables. In Western countries, seaweeds have been used as sources of phycocolloids, gelling and thickening agents for different applications in industry and food. In 1990, the French government published the report of authorizing the seaweeds as vegetables and condiments [3] and expended the exploitation of seaweeds.

Seaweeds contain large amounts of polysaccharides that are extracted by the hydrocolloid industry: alginate from brown seaweeds, carrageen and agar from red seaweeds. Other minor polysaccharides are also found in the cell wall: polysaccharides containing sulphated fucose (from brown seaweeds), xylans (from certain red and green seaweeds) and cellulose (which occur in all genera, but at lower levels than found in higher plants) [26]. Marine algae are also rich in polyunsaturated fatty acids (PUFAs) [48] and have potential value as sources of essential fatty acids, important for human nutrition [6, 31].

Keeping in mind the complex composition of seaweeds and their potential biological activities, we assessed the antimicrobial, antidiabetic, cytotoxic and antioxidant activities of selected seaweeds. Besides the biological activities, we examined the nutritional value, fatty acid composition, dietary fibre and polyphenolic content of two green algae *H. tuna*, *C. bursa*, one brown alga *C. barbata* and one seagrass *C. nodosa* from the Adriatic Sea, Coast of Montenegro.

Materials and Methods

Chemicals. Dichlorometane, methanol, dimethylsulfoxide (DMSO), 1,1-diphenyl-2-picryl hydrazyl (DPPH), Folin-Ciocalteu reagent, gallic acid standard, p-nitrophenyl α-D-glucopyranoside (PNP-G), α-glucosidase and acarbose standard were obtained from Sigma-Aldrich (USA). Fatty acid standards (FAME Mix) were obtained from Supelco (USA). 2,3,5-triphenyl-tetrazolium chloride solution (TTC), cis-diaminedichloroplatinum (cis-DDP), sodium dodecyl sulphate, formazan and RPMI-1640 medium were obtained from Sigma-Aldrich (USA). 10% foetal bovine serum, L-glutamine, and penicillin-streptomycin were obtained also from Sigma-Aldrich, (USA). Nitric acid, trichloroacetic acid, chloroform, acetic acid and concentrated hydrochloric acid were obtained from Merck (USA). Müller-Hinton broth and Sabouraud agar were obtained from Torlak (Serbia). All other chemicals were of analytical grade.

Plant material. The raw seaweed samples (*Halimeda tuna* (J. Ellis & Solander) J. V. Lamouroux, *Halimedaceae*, *Codium bursa* (Olivi) C. Agardh, *Codiaceae*, *Cystoseira barbata* (Stackhouse) C. Agardh, *Sargassaceae* and *Cymodocea nodosa* (Urcia) Acherson, *Cymodoceaceae*) were collected from the Adriatic Sea, Gulf of Boka Kotorska in November 2013. Collected species were washed with marine water to remove sand and rocks, packed in plastic bags and brought to the laboratory. Each species was washed thoroughly with distilled water to remove salt and epiphytes, and dried in shade. Dried samples were powdered using an electric grinder and divided into two parts. One part was immediately used for extraction and the other part was used for energy value and dietary fibre determination.

Nutritional value determination

Humidity content. Humidity of powdered seaweeds was determined using the gravimetric technique. Weighing the mass before and after sample drying in an appropriate dish, in the oven temperature at 105ºC, the amount of evaporated water was determined. Humidity was expressed as percent of evaporated water using the formula:

$$\text{Water (\%)} = (a * 100)/p$$

where: a – the difference of the mass of seaweed sample before and after drying in the appropriate dish, p – the mass of seaweed sample, before drying.

Dry matter content (Ash). The dry matter determination was performed by calculation using the percent of evaporated water in the following formula:

$$\text{Ash (\%)} = 100 - \text{water (\%)}.$$

Total fat content. The total fat determination was performed by the Weibull-Stoldt method using a Soxhlet device for the extraction procedure. 20 g of sample were weighed in an appropriate glass, and covered with 100 mL of cold water and 60 mL of concentrated hydrochloric acid (Merck). The sample was heated on a hotplate for 20 min at 100ºC. After heating, the sample was filtered over filter paper. The filter paper was dried in the oven, and after drying it was used for the extraction. Filter paper with dried sample was transferred in an appropriate capsule, closed with cotton wool and transferred in the Soxhlet device for extraction. The extraction procedure was performed using chloroform as organic extraction solvent. The solvent was collected in appropriate tarred dish and removed using a rotary evaporator at 50ºC. The results were expressed as a percentage of lipids in the dry seaweed powder.

Total protein content. The total protein was determined by Kjeldahl method. The protein was calculated using a nitrogen content factor of 6.25 [33]. Results were expressed as percent of dry weight.

Cellulose content. Cellulose content was estimated using Scharrer-Kürshner method. Cellulose reagent contains 75 mL of 70% acetic acid, 5 mL of concentrated nitric acid and 2 g of trichloroacetic acid. 1 g of dry seaweed powder was quantitatively transferred into a volumetric flask, covered with cellulose reagent and heated for 30 minutes. The hot solution was quantitatively transferred on a tarred sintered glass funnel (G3). The sintered glass funnel with sample was dried in the oven at 105ºC for 1 hour and after cooling the mass was measured. Cellulose content was calculated using the formula:

$$\text{Cellulose (\%)} = [(a - b) * 100]/p$$

where: a – mass of sintered glass funnel with cellulose, b – mass of empty sintered glass funnel, p – mass of dry seaweed powder.

Carbohydrates content. The content of carbohydrates was calculated from the difference of determined values for fat, protein, cellulose, ash and water [38], using the formula:

$$\text{Carbohydrates (\%)} = 100 - \text{water (\%)} - \text{ash (\%)} - \text{fat (\%)} - \text{protein (\%)} - \text{cellulose (\%)}.$$

Dietary fibre content. Insoluble and soluble dietary fibres were determined according to the AOAC (Association of Official Analytical Chemists) enzymatic-gravimetric method [34]. The total dietary fibre was determined by summing the insoluble dietary fibres and the soluble dietary fibres. Tests were performed in triplicate and presented as a percent of dietary fibre of dry seaweed mass.

Fatty acid assay. Fatty acids from seaweeds dry dichlorometane:methanol (1:1) dry extracts (DMEs) were trans-esterified with hydrochloric acid in methanol, according to the method described by Ichihara and Fukubayashi [14], and fatty acid methyl esters (FAMEs) were obtained. Fatty acid methyl esters were further analysed using an Agilent Technologies 7890A Gas Chromatograph with a flame ionization detector. Separation of the FAMEs was performed on a CP-Sil 88 capillary column (100 m × 0.25 mm × 0.2 µm) using helium as a carrier gas at a flow rate of 1 mL/min. The samples were injected at the starting oven temperature of 80°C, the injector temperature was 250°C, and the detector temperature was 270°C. The oven temperature was programmed to increase from 80°C, 4°C/min to 220°C, 5 min, 4°C/min to 240°C, 10 min. Fatty acids were identified by their retention time in comparison with reference fatty acid standards (FAME Mix, Supelco (USA)). The results were expressed as a percentage of individual fatty acid in total DME.

Energy value determination. Energy value (EV) was calculated for 100 g of seaweed powder based on the recommendations of Association of Official Analytical Chemists (AOAC):

Energy, kcal/100 g of seaweed powder = 9 (crude fat content, g) + 4 (protein content, g + carbohydrate content, g) +2 (fibre content, g).

Extraction procedure. Powdered samples were extracted by cold maceration using dichlorometane: methanol (1:1) solvent for 48 hours, with periodical shaking. The sample:solvent ratio was 1:5. The extracts were filtered and evaporated in vacuum at 40°C to yield residue.

Evaluation of antioxidative activity. The antioxidative activity of DMEs was determined by DPPH free-radical scavenging assay. All extracts were dissolved in dichlormethane:methanol (1:1). An aliquot of this solution was mixed with 0.5 mL of 0.5 mM DPPH in methanol, and the final volume was adjusted to 2.5 mL. Mixtures were vigorously shaken and left 30 min in the dark at room temperature. According to the work of Marxen *et al.* [28], in order to eliminate intensive pigment interferences, sample absorbance was measured at 550 nm using the methanol solution of investigated extracts with corresponding concentration, as blank. The IC_{50} value, which represents the estimated dry extract concentration providing 50% of the inhibition of DPPH radical, was calculated using the probit analysis [35, 46]. Experiments were conducted in triplicate.

Total phenolic content (TPC). The total phenolic content was determined using Folin-Ciocalteu method [45] with slight modifications. Gallic acid (GA) was used as reference standard. 100 μL of marine plant extract (10 mg/mL) dissolved in methanol were mixed with 750 μL of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) and allowed to stand at 22°C for 5 min; 750 μL of Na_2CO_3 (60 g/L) solution was added to the mixture. The absorbance was measured at 725 nm, after 90 minutes. Results were expressed as μg of GA/mg of dry extract. Experiments were conducted in triplicate.

Antimicrobial activity. Microorganisms included in this study were: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212**,** *Bacillus subtillis* ATCC 6633 and *Candida albicans* ATCC 24433. Antimicrobial activity test and determination of the minimal inhibitory concentration (MIC) were performed by the microdilution assay using 2,3,5-triphenyl-tetrazolium chloride solution (TTC) for the microbial growth detection [4]. The MIC is defined as the lowest concentration of the extract at which the microorganism does not demonstrate visible growth. The experiment was performed in triplicate in five serial dilutions within 50 - 800 μg/mL extract concentration range (50, 100, 200, 400 and 800 μg/mL), using Müller-Hinton broth for antibacterial activity testing and Sabouraud agar for antifungal activity testing.

Antitumour activity

Treatment of cell lines. Target cells HeLa, cervix adenocarcinoma cell line (2000 cells per well), LS174, human colon carcinoma cell line (7000 cells per well), K562, human chronic myelogenous leukaemia cell line (5000 cells per well), and non-cancerous MRC-5 (5000 cells per well) were seeded into wells of a 96-well flat-bottomed microtiter plate. Twenty-four hours later, after the cell adherence, different concentrations of the investigated extracts were added to the wells, except for the control cells to which only a nutrient medium was added. The final concentrations range chosen was 1 - 200 μg/mL (12.50, 25, 50, 100, and 200 μg/mL). The final concentration of DMSO solvent never exceeded 0.5%, which was non-toxic to the cells. Especially, extracts were applied to the suspension of K562 cells 2 h after the cell seeding. All concentrations were assessed in triplicate. Nutrient medium with corresponding concentrations of investigated compounds, but without cells, was used as a blank, also in triplicate. The cultures were incubated for 72 h.

Determination of cell survival. The effect of the prepared DMEs on cancer cell survival was determined by the microculture tetrazolium test (MTT) according to Mosmann with modification by Ohno and Abe, 72 h after the addition of the compounds, as described earlier [30, 32]. Briefly, 20 mL of MTT solution (5 mg/mL phosphate-buffered saline) was added to each well. Samples were incubated for 4 h at 37ºC in a humidified atmosphere of 95% air/5% CO_2 (v/v). Then, 100 mL of 100 g/L sodium dodecyl sulphate was added to extract the insoluble product formazan resulting from the conversion of the MTT dye by viable cells. The number of viable cells in each well was proportional to the intensity of the absorbance, which was read in an enzyme-linked immunosorbent assay (ELISA) plate reader at 570 nm. The absorbance (A) at 570 nm was measured 24 h later. To determine cell survival (%), the A of a sample with cells grown in the presence of various concentrations of the investigated compounds was divided by the control optical density (the A of control cells grown only in nutrient medium) and multiplied by 100. It was implied that the A of the blank was always subtracted from the A of the corresponding sample with target cells. IC_{50} was defined as the concentration of an agent inhibiting cell survival by 50% compared with a vehicle-treated control. As positive control, cis-diaminedichloroplatinum (cis-DDP) was used. The extracts were screened against 4 cell lines including HeLa, LS174, K562 and non-cancerous MRC5 by MTT assay. The evaluation was conducted in accordance with the Protocol of the American Cancer Institute (NCI), which recommends that IC_{50} values $\leq$ 30 μg/mL should be considered significant for crude

extracts of plant origin as well as IC_{50} values ≤ 4 μg/mL for pure substances [10]. All experiments were performed in triplicate.

Anti α-glucosidase activity. Anti α-glucosidase activity of dry DMEs was determined using α-glucosidase inhibitory activity test. The enzyme solution was set at 400 mU/mL of α-glucosidase in a 0.1M phosphate buffer (pH = 6.7). For each well we used 50 μL of the tested extract in DMSO, diluted in a 0.1 M phosphate buffer (pH = 6.7) so that the final concentrations of the extracts in each well were 166.67, 83.33, 41.67, 20.83, 10.42, 5.21 μg/mL. In 96 well plates, we pre-incubated 50 μL of extract dilutions with 50 μL of enzyme solution for each well at 37°C for 15 min. The reaction was started by adding 50 μL of substrate solution, p-nitrophenyl α-D-glucopyranoside (1.5 mg/mL PNP-G in phosphate buffer), and after measuring absorbance A1 at 405 nm the solution was incubated at 37°C for 15 min. Then the second absorbance A2 was measured at 405 nm. Acarbose was used as a positive control. The percent of the enzyme inhibition was calculated as $100 \times (A_{2S} - A_{1S})/(A_{2B} - A_{1B})$, where A_{1B}, A_{2B} and A_{1S}, A_{2S} represent the absorbance of the blank (phosphate buffer, DMSO, enzyme dilution, and PNP-G dilution) and sample, respectively. Experiments were conducted in duplicate and IC_{50} value (estimated concentration of DME marine plant extract that caused 50% inhibition of α-glucosidase activity) was determined using the linear regression analysis.

Results and Discussion

Seaweeds from the Adriatic Sea, Coast of Montenegro, have been investigated only in a few researches, which mainly included biological activity assessment [16, 20, 39]. In this research, we investigated the nutritive and health protective effects of *H. tuna*, *C. bursa*, *C. nodosa* and *C. barbata* seaweeds.

H. tuna had the lowest nutritional value (234.01 kcal), which in combination with its high cellulose content (30 ± 0.08%) indicates a low digestibility and low nutritional value. Despite that, the percent of dry, mineral material was high (30 ± 0.13%) which was expected for calcareous algae, rich in calcium carbonate [11].

The highest level of docosahexaenoic acid (DHA, C 22:6, n-3) and eicosapentaenoic acid (EPA, C 20:5, n-3) in *H. tuna* dry extract (2.83% and 4.97% respectively) rates this seaweed as good source of essential fatty acids. The World Health Organization (WHO) published that EPA and DHA in combination decrease colorectal cancer risk and decrease the risk for breast cancer. *H. tuna* DME in our research have shown the best cytotoxic activity against LS174, colorectal carcinoma cells, and high cytotoxic activity against HeLa, human cervical adenocarcinoma (Table I).

Table I

Concentrations of seaweeds DMEs that induced a 50 % decrease in HeLa, LS174 and K562, malignant cells and MRC5 normal cell survival (expressed as IC_{50} (μg/mL))

Sample	IC_{50} [a] (μg/mL)			
	HeLa	LS174	K562	MRC5
Cymodocea nodosa	13.28 ± 0.39	62.09 ± 1.24	19.64 ± 1.55	> 200
Cystoseira barbata	22.85 ± 1.36	66.51 ± 0.92	36.27 ± 2.41	> 200
Halimeda tuna	17.92 ± 1.54	25.34 ± 2.37	29.53 ± 1.29	> 200
Codium bursa	57.42 ± 1.73	88.52 ± 1.43	75.29 ± 1.42	> 200
cis-DDP	0.83 ± 0.19	2.58 ± 0.16	3.21 ± 0.41	13.21 ± 0.37

[a]IC_{50} values are expressed as the mean ± SD determined from the results of MTT assay in three independent experiments.

Due to its n-3 fatty acid composition (DHA and EPA) and its cytotoxic activity, *H. tuna* can have the potential to reduce colorectal carcinoma risk and risk for breast cancer (WHO). Kurt *et al.* have also demonstrated the cytotoxic activity of *H. tuna* extract on MCF-7 breast cancer cell line [22]. Moderate antioxidative activity of *H. tuna* (IC_{50} = 839.44 ± 5.482 μg/mL) and its phenolic compounds content (9.03 ± 0.4 μg GA/mg dry extract) indicates its health protective potential against oxidative stress (Table II), which can cause damage of cell function, but also induce tumourigenesis [50].

Table II

In vitro antioxidative activity (DPPH free-radical scavenging activity) and TPC of selected seaweeds DMEs

Sample	Antioxidative activity (DPPH test) IC_{50} [a], μg/mL	TPC[b] (μg GA/mg dry extract)
Cymodocea nodosa	112.73 ± 2.051	37.82 ± 1.23
Cystoseira barbata	367.21 ± 3.621	14.94 ± 0.6
Halimeda tuna	839.44 ± 5.482	9.03 ± 0.4
Codium bursa	1369.72 ± 7.351	7.43 ± 0.0.2

[a]IC_{50} values are expressed as the mean ± SD determined from the results of DPPH test in three independent measurements; [b]TPC values are expressed as the mean ± SD determined from the results of Folin-Ciocalteu reagent method test in three independent measurements

H. tuna and *C. barbata* had the highest antimicrobial activity against *B. subtilis* (MIC = 100 µg/mL). *C. albicans* and *S. aureus* were also susceptible to *H. tuna* DME (Table III). The discovery of halitunal, a diterpene aldehyde, with antiviral activity against murine coronavirus, together with many researches in antibacterial activity of *H. tuna* promises the use of this seaweed as a source of antimicrobial compounds [13]. Antimicrobial activity of other tested seaweed species had not been studied before, so these are the first results which have shown promising antifungal activity of the tested extracts against *C. albicans*.

The amount of total dietary fibre was the lowest in comparison to other examined species. *H. tuna* had a 10 times higher amount of insoluble than soluble fibres (Table IV).

Table III

Antimicrobial activity of selected seaweed DMEs expressed as minimal inhibitory concentrations (MIC)

Sample	Minimal inhibitory concentration (MIC), µg/mL				
	Escherichia coli	***Staphylococcus aureus***	***Bacillus subtillis***	***Enterococcus faecalis***	***Candida albicans***
Cymodocea nodosa	[a]nd	200	[a]nd	[a]nd	400
Cystoseira barbata	[a]nd	100	100	[a]nd	200
Halimeda tuna	[a]nd	800	100	[a]nd	400
Codium bursa	[a]nd	400	200	[a]nd	400

[a]nd - Not demonstrated antimicrobial activity

Insoluble fibres are metabolically inert and provide bulking, or they can be prebiotic and metabolically ferment in the large intestine. Bulking fibres absorb water as they move through the digestive system, easing defecation. Soluble fibres (pectins, guar gum etc.), are readily fermented in the colon into gases and physiologically active byproducts, and can be prebiotic and viscous. Soluble fibres have been correlated to hypocholesterolemic and hypoglycaemic effects [26]. *H. tuna* DME has shown significant anti α-glucosidase activity (IC_{50} = 19.16 ± 0.65 µg/mL) in our research, that indicates the potential use of this seaweed in the management of diabetes.

C. barbata seaweed has shown the highest content of total dietary fibres (70.05 ± 0.21%) among all investigated species, and the highest insoluble dietary fibres content (58.95 ± 0.06%) in our research. Many researchers have shown the health benefits of dietary fibres in the management of diabetes, by improving the insulin sensitivity [24]. Dietary fibres intake has been shown to improve the risk factors associated with cardiovascular disease, such as obesity, atherogenic dyslipidaemia, hyper-tension and inflammation in diabetes and metabolic syndrome [9]. In our research, *C. barbata* DME had the best anti α-glucosidase activity (IC_{50} = 9.98 ± 3.34 µg/mL) in comparison to other examined species. Brown algae are a rich source of poly-phenolic compounds called phlorotannins which have exhibited antidiabetic activity through various mechanisms such as α-glucosidase and α-amylase inhibitory effect, which can indicate the potential use of these seaweeds as beneficial functional food in the management of diabetes including *C. barbata* from our research [23, 40]. According to data presented in Table III, *C. barbata* dry extract has shown the highest antibacterial activity (MIC = 100 µg/mL) against *S. aureus* and *B. subtilis* (MIC = 100 µg/mL).

Table IV

Chemical composition and energy value of selected seaweeds

Components	*Cymodocea nodosa*	*Halimeda tuna*	*Codium bursa*	*Cystoseira barbata*
Humidity (%)	10 ± 0.59	4.8 ± 0.1	7.8 ± 0.8	10.5 ± 1.33
Ash (%)	11 ± 0,71	30 ± 0.13	9 ± 0.2	10 ± 0.1
Total fat (%)	5 ± 0.37	3.75 ± 0.07	7.1 ± 0.17	4.95 ± 0.06
Total protein (%)	11.5 ± 0.14	7.5 ± 0.07	4.0 ± 0.07	8.5 ± 0.07
Cellulose (%)	45 ± 0.14	30 ± 0.08	18 ± 0.08	22 ± 0.11
Carbohydrates (%)	17.5 ± 0.21	23.95 ± 0.3	69. 7 ± 0.15	44.05 ± 0.15
Energy value (kcal, for 100 g of seaweed powder)	293.16	234.01	490.4	394.85
Soluble dietary fibre (%)	10.5 ± 0.11	3.53 ± 0.2	35.85 ± 0.08	11.10 ± 0.15
Insoluble dietary fibre (%)	55.58 ± 0.11	33.7 ± 0.04	30 ± 0.04	58.95 ± 0.06
Total dietary fibre (%)	66.08 ± 0.22	37.23 ± 0.24	65.85 ± 0.12	70.05 ± 0.21

C. barbata has also shown high antioxidative activity (IC_{50} = 367.21 ± 3.621 µg/mL) and medium TPC (14.94 ± 0.6 µg GA/mg dry extract). *C. barbata*, had a prominent cytotoxic activity, particularly against HeLa and K562 cells, in MTT assay. According to these biological activity results *C. barbata* can be important in pharmaceutical industry and novel drugs research. Reviewing the fatty acid profile of *C. barbata* we can see the high level of SFA and MUFA (Table V), and some odd chain fatty acids such as C 15:0 and C 15:1. Some researchers found a correlation

of odd chain fatty acids with the lower risk of cardio-metabolic disease [15].

C. nodosa seagrass had the highest content of cellulose (45 ± 0.14%) which in combination to its low energy value (293.16 kcal) limits its use in nutrition. Despite of its low nutrient value, the amount of non-nutritive health protective agents, such as dietary fibres and polyphenol compounds, was high compared to other investigated species.

Table V

Fatty acid composition of selected seaweeds DMEs (expressed as % of total extract)

Fatty acid, % of total fatty acids	***Halimeda tuna***	***Cystoseira barbata***	***Cymodocea nodosa***	***Codium bursa***
C 14:0	7.72	7.63	2.38	1.87
C 15:0	[a]nd	0.3	[a]nd	[a]nd
C 16:0	32.6	29.41	33.11	32.07
C 17:0	7.24	[a]nd	[a]nd	[a]nd
C 18:0	0.7	1.2	2.85	0.98
C 20:0	[a]nd	[a]nd	[a]nd	[a]nd
C 22:0	0.79	7.06	[a]nd	[a]nd
∑ SFA	***49.05***	***45.6***	***38.34***	***34.92***
C 14:1	0.4	0.57	[a]nd	0.79
C 15:1	[a]nd	0.87	[a]nd	[a]nd
C 16:1	3.31	3.66	5.66	1.14
C 17:1	1.53	[a]nd	[a]nd	3.9
C 18:1 n-9 cis	10.97	15.37	6.04	21.23
C 18:1 n-9 trans	[a]nd	0.84	[a]nd	[a]nd
C 20:1	[a]nd	[a]nd	[a]nd	[a]nd
C 22:1 n-9	1.67	1.26	1.79	1.98
C 24:1	2.38	0.77	1.58	0.38
∑ MUFA	***20.26***	***23.34***	***15.07***	***29.42***
C 18:2 n-6 cis	13.63	5.28	17.6	15.32
C 18:2 n-6 trans	0.4	[a]nd	[a]nd	[a]nd
C 18:3 n-6	0.75	0.8	[a]nd	1.92
C 18:3 n-3	3.15	6.55	27.11	9.72
C 20:2	0.6	[a]nd	[a]nd	[a]nd
C 20:4 n-6	3.76	12.44	[a]nd	6.77
C 20:5 n-3	4.97	3.24	[a]nd	[a]nd
C 22:2	0.61	0.75	[a]nd	[a]nd
C 22:6 n-3	2.83	0.32	[a]nd	[a]nd
∑ PUFA	***32.3***	***29.38***	***44.71***	***33.73***
PUFA/SFA	***0.66***	***0.64***	***1.16***	***0.97***
n-6	18.54	18.52	17.6	24.1
n-3	7.8	10.11	27.11	9.72

[a]nd – Not detected, SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids.

The highest TPC of *C. nodosa* DME, found to be 37.82 ± 1.23 µg GA/mg dry extract, correlated to the highest *in vitro* antioxidative activity (IC_{50} = 112.73 ± 2.051 µg/mL). *C. nodosa* has been recognized as an important source of phenol-carboxylic acids, possessing high antioxidative activity [12], which could probably explain the results [21]. High dietary fibre content in combination with high anti α-glucosidase activity (Table VI), indicates the use of *C. nodosa* in the management of diabetes and metabolic syndrome.

Table VI

Anti α-glucosidase activity of selected seaweeds DMEs (expressed as IC_{50} (µg/mL)

Sample	**Anti α-glucosidase activity; IC_{50}[a], µg/mL**
Cymodocea nodosa	11.48 ± 3.57
Cystoseira barbata	9.98 ± 3.34
Halimeda tuna	19.16 ± 0.65
Codium bursa	13.85 ± 1.41
Acarbose (standard)	59.8 ± 12.3

[a]IC_{50} values are expressed as the mean ± SD determined from the results of anti α-glucosidase inhibitory activity test in two independent measurements

C. nodosa DME has shown significant antimicrobial activity against *S. aureus* (MIC = 200 µg/mL) [41]. The highest cytotoxic activity against HeLa (IC_{50} = 13.28 ± 0.39 µg/mL) and K562 (IC_{50} = 19.64 ± 1.55 µg/mL) cells was found for *C. nodosa* DME. Kontiza *et al.* have reported the presence of diaryl-

heptanoids in *C. nodosa*, namely: cymodienol and cymodienal, which exhibited cytotoxic activity against two lung cancer cell lines [41]. *C. nodosa* had the highest content of n-3 fatty acids with the highest level of α-linolenic acid (27.11%) among all investigated samples. Polyunsaturated fatty acids (α-linolenic, eicosapentanoic, docosahexanoic) collectively protect against coronary heart disease.

The most abundant n-6 fatty acid in all investigated seaweeds was linoleic acid (C18:2, n-6) as a major fatty acid that regulates low-density lipoproteins (LDL-c) metabolism by down regulating LDL-c production and enhancing its clearance [47]. Linoleic acid was detected in all investigated samples in a range from 5.28% to 17.6%. Linoleic acid was also shown to improve insulin sensibility of tissues and also possess antimicrobial activity and cytotoxic activity against HeLa cell types [37]. Also, the highest content of linoleic acid (17.6 %) in *C. nodosa* DME might explain of the highest cytotoxicity against HeLa tumour cell line.

C. bursa, the green seaweed, had the highest energy value among all investigated species (490.4 kcal) which is in correlation to the highest fat (7.1 ± 0.17%) and carbohydrate (69.7 ± 0.15%) content. The high content of soluble dietary fibres in *C. bursa* (Table IV) can induce satiety and help in weigh control [49]. Fatty acid profile shows a high level of cardioprotective PUFAs, and a low level of SFA. In comparison to other investigated seaweeds, the biological activities of *C. bursa* were reduced. Anti α-glucosidase activity of *C. bursa* DME was significantly higher than acarbose standard (IC_{50} = 59.8 ± 12.3 μg/mL) which has been shown in Table I.

Conclusions

Seaweeds have been proposed as an interesting, almost unlimited, natural source in the search for novel natural functional ingredients, and several works have shown the possibility to find bioactive compounds in these organisms. Results of this study showed that *H. tuna*, *C. barbata*, *C. nodosa* and *C. bursa,* present a great potential in future novel food and medicine investigations, because they represent a renewable and huge source of biologically active compounds. Further investigation should lead to novel substances investigation and potential implementation of these seaweeds in nutrition and supplementation.

Acknowledgement

This work was supported from Ministry of Education, Science and Technological Development of the Republic of Serbia (Grant Nos. 175011; 173021), Ministry of Science of the Republic of Montenegro; project "KOTOR" - Institute of Marine Biology, Department of Chemistry Biochemistry and Molecular Biology, Kotor.

References

1. Cooksley V.G., Seaweed: Nature's Secret to Balancing Your Metabolism, Fighting Disease, and Revitalizing Body and Soul. 2nd Ed. New York: Stewart, Talory and Chang, 2007.
2. Costello M.J., European register of marine species: a check-list of the marine species in Europe and a bibliography of guides to their identification. Paris: Muséum national d'histoire naturelle, 2001.
3. De La Santé D.G., Avis du Conseil Supérieur d'Hygiène Publique de France, section des maladies transmissibles, relatif à la conduite à tenir devant un sujet atteint de pédiculose du cuir chevelu (séance du 27 juin 2003). *Ann. Dermatol. Venereol.*, 2004; 131: 1122-1124.
4. Eloff J.N., A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Med.*, 1998; 64(8): 711-713.
5. Ely R., Supriya T., Naik C.G., Antimicrobial activity of marine organisms collected off the coast of South East India. *J. Exp. Ma.r Biol. Ecol.*, 2004; 309(1): 121-127.
6. Floreto E.A., Teshima S.I., Koshio S., The effects of seaweed diets on the lipid and fatty acids of the Japanese disc abalone *Haliotis discus hannai*. *Fisheries Sci.*, 1996; 62(4): 582-588.
7. Frantz S., Calvillo L., Tillmanns J., Elbing I., Dienesch C., Bischoff H., Ertl G., Bauersachs J., Repetitive postprandial hyperglycemia increases cardiac ischemia/reperfusion injury: prevention by the α-glucosidase inhibitor acarbose. *The FASEB J.*, 2005; 19(6): 591-593.
8. Freeman B.C., Beattie G.A., An overview of plant defenses against pathogens and herbivores. *The Plant Health Instructor*, 2008: 226(1): 1-12.
9. Galisteo M., Duarte J., Zarzuelo A., Effects of dietary fibers on disturbances clustered in the metabolic syndrome. *J. Nutr. Biochem.*, 2008; 19: 71-84.
10. Geran R.I., Greenberg N.H., Macdonald M.M., Modified protocol for the testing of new synthetics in the Ll210 lymphoid leukaemia mini model in the program of DR and DP, DCT, NCI. *Cancer Chemother. Rep.*, 1975; 5: 15-19.
11. Goreau T.F., Calcium carbonate deposition by coralline algae and corals in relation to their role as reef-builders. *Ann. NY Acad. Sci.*, 1963; 109(1): 127-167.
12. Grignon-Dubois M., Rezzonico B., The economic potential of beach-cast seagrass–*Cymodocea nodosa*: a promising renewable source of chicoric acid. *Bot. Mar.*, 2013; 56(4): 303-311.
13. Harada H., Kamei Y., Selective cytotoxicity of marine algae extracts to several human leukemic cell lines. *Cytotechnology*, 1997; 25(1-3): 213-219.
14. Ichihara K.I., Fukubayashi Y., Preparation of fatty acid methyl esters for gas-liquid chromatography. *J. Lipid Res.*, 2010; 51(3): 635-640.

15. Jenkins B., West J.A., Koulman A., A review of odd-chain fatty acid metabolism and the role of pentadecanoic acid (C15: 0) and heptadecanoic acid (C17: 0) in health and disease. *Molecules*, 2015; 20(2): 2425-2444.
16. Kamenarska Z., Gasic M.J., Zlatovic M., Rasovic A., Sladic D., Kljajic Z., Stefanov K., Seizova K., Najdenski H., Kujumgiev A., Tsvetkova I., Chemical composition of the brown alga *Padina pavonia* (L.) Gaill. from the Adriatic Sea. *Bot. Mar.*, 2002; 45(4): 339-345.
17. Koehn F.E., Sarath G.P., Neil D.N., Cross S.S., Halitunal, an unusual diterpene aldehyde from the marine alga *Halimeda tuna*. *Tetrahedron Let.*, 1991; 32(2): 169-172.
18. Kolsi R.B., Frikha D., Jribi I., Hamza A., Fekih L., Belgith K., Screening of antibacterial and antifungical activity in marine macroalgae and magnoliophytea from the coast of Tunisia. *Int. J. Pharm. Pharmaceut. Sci.*, 2015; 7(3): 47-51.
19. Kontiza I., Stavri M., Zloh M., Vagias C., Gibbons S., Roussis V., New metabolites with antibacterial activity from the marine angiosperm *Cymodocea nodosa*. *Tetrahedron*, 2008; 64(8): 1696-1702.
20. Kosanić M., Ranković B., Stanojković T., Biological potential of marine macroalgae of the genus *Cystoseira*. *Acta Biol. Hung.*, 2015; 66(4): 374-384.
21. Kuda T., Tsunekawa M., Hishi T., Araki Y., Antioxidant properties of driedkayamo-nori', a brown alga *Scytosiphon lomentaria* (*Scytosiphonales*, *Phaeophyceae*). *Food Chem.*, 2005; 89(4): 617-622.
22. Kurt O., Özdal-Kurt F., Tuğlu M.I., Akçora C.M., The cytotoxic, neurotoxic, apoptotic and antiproliferative activities of extracts of some marine algae on the MCF-7 cell line. *Biotech. Histochem.*, 2014; 89(8): 568-576.
23. Lee S.H., Jeon Y.J., Anti-diabetic effects of brown algae derived phlorotannins, marine polyphenols through diverse mechanisms. *Fitoterapia*, 2013; 86: 129-136.
24. Li C., Uppal M., Canadian diabetes association national nutrition committee clinical update on dietary fibre in diabetes: Food sources to physiological effects. *Can. J. Diabetes.*, 2010; 34(4): 355-361.
25. Lim S.N., Cheung P.C., Ooi V.E., Ang P.O., Evaluation of antioxidative activity of extracts from a brown seaweed, *Sargassum siliquastrum*. *J. Agr. Food. Chem.*, 2002; 50(13): 3862-3866.
26. Mabeau S., Fleurence J., Seaweed in food products: biochemical and nutritional aspects. *Trends Food Sci. Tech.*, 1993; 4(4): 103-107.
27. Manilal A., Sujith S., Selvin J., Shakir C., Seghal Kiran G., Antibacterial activity of *Falkenbergia hillebrandii* (Born.) from the Indian coast against human pathogens. *Phyton (Buenos Aires)*, 2009; 1: 161.
28. Marxen K., Vanselow K.H., Lippemeier S., Hintze R., Ruser A., Hansen U.P., Determination of DPPH radical oxidation caused by methanolic extracts of some microalgal species by linear regression analysis of spectrophotometric measurements. *Sensors*, 2007; 7(10): 2080-2095.
29. Mohamed S., Hashim S.N., Rahman H.A., Seaweeds: a sustainable functional food for complementary and alternative therapy. *Trends Food Sci. Tech.*, 2012; 23(2): 83-96.
30. Mosmann T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Met.*, 1983; 65(1-2): 55-63.
31. Newton I.S., Long chain fatty acids in health and nutrition. *J. Food Lipids*, 1996; 3(4): 233-249.
32. Ohno M., Abe T., Rapid colorimetric assay for the quantification of leukemia inhibitory factor (LIF) and interleukin-6 (IL-6). *J. Immunol. Met.*, 1991; 145(1-2): 199-203.
33. Ortiz J., Romero N., Robert P., Araya J., Lopez-Hernández J., Bozzo C., Navarrete E., Osorio A., Rios A., Dietary fiber, amino acid, fatty acid and tocopherol contents of the edible seaweeds *Ulva lactuca* and *Durvillaea antarctica*. *Food Chem.*, 2006; 99(1): 98-104.
34. Prosky L., Asp N.G., Schweizer T.F., DeVries J.W., Furda I., Determination of insoluble, soluble, and total dietary fiber in foods and food products: interlaboratory study. *J. Assoc. Anal. Chem.*, 1988; 71(5): 1017-1023.
35. Popescu M.L., Costea T., Nencu I., Duțu L.E., Gîrd C.E., Polyphenols contents and antioxidant activity of some romanian wild edible mushrooms. *Farmacia*, 2016; 64(2): 231-236.
36. Ruqqia K., Sultana V., Ara J., Ehteshamul-Haque S., Athar M., Hypolipidaemic potential of seaweeds in normal, triton-induced and high-fat diet-induced hyperlipidaemic rats. *J. Appl. Phycol.*, 2015; 27(1): 571-579.
37. Sagar P.S., Das U.N., Koratkar R., Ramesh G., Padma M., Kumar G.S., Cytotoxic action of cis-unsaturated fatty acids on human cervical carcinoma (HeLa) cells: relationship to free radicals and lipid peroxidation and its modulation by calmodulin antagonists. *Cancer Lett.*, 1992; 63(3): 189-198.
38. Southgate D.A., Determination of carbohydrates in foods. I. Available carbohydrate. *J. Sci. Food. Agr.*, 1969; 20(6): 326-330.
39. Stanojković T.P., Scaron K., Zdunić G., Kljajić Z., Grozdanić N., Antić J., *In vitro* antitumoral activities of *Padina pavonia* on human cervix and breast cancer cell lines. *J. Med. Plants Res.*, 2013; 7(8): 419-424.
40. Sun Z., Chen F., Evaluation of the Green Alga *Chlorella pyrenoidosa* for Management of Diabetes. *J. Food Drug Anal.*, 2012; 20: 246-249.
41. Synytsya A., Kim W.J., Kim S.M., Pohl R., Synytsya A., Kvasnička F., Čopíková J., Park Y.I., Structure and antitumor activity of fucoidan isolated from sporophyll of Korean brown seaweed *Undaria pinnatifida*. *Carbohyd. Polym.*, 2010; 81(1): 41-48.
42. Süzgeç-Selçuk S., Meriçli A.H., Güven K.C., Kaiser M., Casey R., Hingley-Wilson S., Lalvani A., Tasdemir D., Evaluation of Turkish seaweeds for antiprotozoal, antimycobacterial and cytotoxic activities. *Phytother. Res.*, 2011; 25(5): 778-783.
43. Takamatsu S., Hodges T.W., Rajbhandari I., Gerwick W.H., Hamann M.T., Nagle D.G., Marine natural products as novel antioxidant prototypes. *J. Nat. Prod.*, 2003; 66(5): 605-608.

44. Tierney M.S., Croft A.K., Hayes M., A review of antihypertensive and antioxidant activities in macroalgae. *Bot. Mar.*, 2010; 53(5): 387-408.
45. Velioglu Y.S., Mazza G., Gao L., Oomah B.D., Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. *J. Agr. Food Chem.*, 1998; 46(10): 4113-4117.
46. Viturro C., Molina A., Schmeda-Hirschmann G., Free radical scavengers from *Mutisia friesiana* (*Asteraceae*) and *Sanicula graveolens* (*Apiaceae*). *Phytother. Res.*, 1999; 13(5): 422-424.
47. Wijendran V., Hayes K.C., Dietary n-6 and n-3 fatty acid balance and cardiovascular health. *Annu. Rev. Nutr.*, 2004; 24: 597-615.
48. Wood B.J., Lipids of algae and protozoa. *Microb. Lipids*, 1988; 1: 807-867.
49. Ye Z., Arumugam V., Haugabrooks E., Williamson P., Hendrich S., Soluble dietary fiber (Fibersol-2) decreased hunger and increased satiety hormones in humans when ingested with a meal. *Nutr. Res.*, 2015; 35(5): 393-340.
50. Zubia M., Robledo D., Freile-Pelegrin Y., Antioxidant activities in tropical marine macroalgae from the Yucatan Peninsula, Mexico. *J. Appl. Phycol.*, 2007; 19(5): 449-458.