Antioxidant, antimicrobial and cytotoxic activity of selected macroalgae from the Adriatic Sea (Boka Kotorska Bay, Montenegro)

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Received: October 28, 2024
Accepted: November 8, 2024
Published on-line: December 23, 2024
Published: December 25, 2024

In this study, the antioxidant, antimicrobial, and cytotoxic activities of four marine macroalgae, *Codium bursa, Codium effusum, Laurencia obtusa* and *Padina pavonica* from the Adriatic Sea were investigated. The antioxidant activities of the extracts were characterized by the DPPH (1,1-diphenyl-2-picrylhydrazyl) method and their total phenolic and flavonoid contents were quantified. The antimicrobial activity of four species against 8 pathogenic bacteria: *Staphylococcus aureus, Bacillus cereus, Micrococcus luteus, Listeria monocytogenes, Pseudomonas aeruginosa, Salmonella Typhimurium, Escherichia coli, Enterobacter cloacae* and 8 fungi: *Aspergillus fumigatus, Aspergillus versicolor, Aspergillus ochraceus, Aspergillus niger, Trichoderma viride, Penicillium funiculosum, Penicillium ochrochloron* and *Penicillium verrucosum* var. *cyclopium*) were assessed. The antitumor activities were determined using two different tumor cell lines (HeLa and K562). The results have shown potential antioxidant activity of *Padina pavonica*, antibacterial and antifungal activity of *Codium effusum*, and strong cytotoxic activity of *Laurencia obtusa*. The results of this study show that marine macroalgae *Codium effusum, Padina pavonica* and *Laurencia obtusa* can be a good choice in the search for new compounds with antioxidant, antimicrobial and antitumor effects.

Keywords: macroalgae, extracts, antioxidant activity, antimicrobial activity, cytotoxic activity

https://doi.org/10.61652/leksir2444010S

1. INTRODUCTION

Marine algae, traditionally classified as green algae (Chlorophyta), brown algae (Phaeophyceae), and red algae (Rhodophyta), are rich sources of structurally novel and biologically active metabolites. They are also a potential renewable resource in the marine environment (Barbosa et al., 2014). Research over the past 50 years has led to the isolation of over 15 thousand new compounds. Many of the isolates have been shown to possess biological activity. Some of the bioactive compounds are carotenoids, dietary fiber, proteins, essential fatty acids, vitamins, and minerals (Kelman et al., 2012; Negreanu-Pirjol et al., 2022; Stanojković et al., 2013).

The potential of macroalgae for functional foods or pharmaceutical applications is enormous due to their exceptional richness in bioactive compounds that can exert antioxidant, antimicrobial, anti-inflammatory, and antitumor effects (Negreanu-Pirjol et al., 2022). Reactive oxygen species (ROS) are generated in living organisms during metabolism and cause extensive oxidative damage, which in turn leads to geriatric degenerative conditions, cancer, and a variety of other human diseases (Yangthong et al., 2009). As photosynthetic

organisms, algae are exposed to a combination of light and high oxygen concentration that leads to the formation of free radicals and other oxidative reagents. The finding of no structural damage to algal organs has led the scientific community to believe that their protection from oxidation is due to their natural content of antioxidant substances or their production under stress (Kelman et al., 2012; Negreanu-Pirjol et al., 2022). In recent years, molecules from marine algae have led to promising results in studies of various cancers (Salehi et al., 2019). Numerous macroalgae have shown potent cytotoxic activities, and some authors have suggested consumption of algae as a chemopreventive agent against various cancers (Stanojković et al., 2013).

Bacteria are common pathogens in humans, as demonstrated by the wide clinical use of antibiotics. The use of antibiotics to develop resistance in pathogenic bacteria has increased at an alarming rate. Alternative prevention and treatment methods are needed, and natural sources such as plants and algae are increasingly being used (Rajauria et al., 2013). The antimicrobial activity of algal extracts has been reported by almost all groups and in different geographical areas (Pane et al., 2015). The aim of this work was to investigate the antioxidant, antimicrobial, and cytotoxic effects of methanol extracts from four marine algae: *Codium bursa*, *C. effusum*, *Laurencia obtusa* and *Padina pavonica* collected in the Boka Kotorska Bay the Adriatic Sea, Montenegro.

2. MATERIALS AND METHODS

2.1. Seaweed material

Samples of *Codium bursa, C. effusum, Laurencia obtusa* and *Padina pavonica* were collected in the Adriatic Sea, in the Bay of Kotor (Montenegro). Upon delivery to the laboratory, they were placed on ice. The seaweed samples were thoroughly washed with fresh water to remove salt, sand and epiphytes. Part of the cleaned algae was freeze-dried at -35 °C and then ground into fine powder using a grinder. The other portion of the cleaned algae was air-dried at 50 °C with forced convection for 48 hours and then ground to fine powder with a mill. The samples, both lyophilized (L) and freeze-dried (D), were stored at -20 °C until further use.

2.2. Preparation of seaweed material for *in vitro* antibiological susceptibility testing

Briefly, 30 g of sample powder (lyophilized (L) or freeze-dried (D)) was mixed with
ethanol/methanol in a 10:1 ratio (v/w). The mixture was kept at room temperature in an orbital shaking incubator for 72 hours. Each mixture was clarified by centrifugation at 3000 x for 10 minutes (Knežević et al., 2017). The supernatant was filtered with Whatman No. 4 filter paper and concentrated under reduced pressure in a rotary evaporator (BUCHI R-114, Switzerland). Prior to analyses, extracts were dissolved in appropriate solvent.

2.3. Antioxidative activity (DPPH• radical scavenging assay)

The radical scavenging activity of the extracts was determined spectrophotometrically based on the reduction of a methanol solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH•) (Blois 1958). 1800.0 µL of a 4% methanol solution of DPPH- and 200.0 µL of an extract of defined concentration (series of double dilutions from 20.0 mg mL^{-1} to 0.312 mg mL^{-1}) were mixed and shaken vigorously. After incubation for 30 min in the dark, the absorbances of the reactive mixtures were measured at 517 nm against methanol as a blank using a spectrophotometer (CECIL CE2501, UK). The negative control contained all reaction reagents except the extract. The scavenging effect was calculated according to the following equation:

$$DPPH^{\bullet} scavenging\ effect\ (\%) = \frac{A_0 - A_{sample}}{A_0} \times 100$$

A_0 - the absorbance of the negative control; A_{sample} - the absorbance of the reaction mixture.

The extract concentration (mg extract mL^{-1}) giving 50% of DPPH reduction (EC 50) was determined by interpolation from linear regression analysis. The commercial antioxidant butylated hydroxyanisole (BHA) at a concentration range of 20.0-0.312 mg/mL was used as a positive control.

2.4. Total phenolic content

Total phenolic compounds in the methanol extracts of the algae were determined with the Folin-Ciocalteu reagent according to the method described by Singleton and Rossi (1965) using gallic acid as a standard. 1000.0 µL of the 10% Folin-Ciocalteu reagent and 200.0 µL of the extract were reacted in the dark for 6 min before 800.0 µL of 7.5% Na_2CO_3 was added. The reaction mixture was shaken vigorously and incubated for 2 h in the dark at room temperature on a rotary shaker (100 rpm). The absorbance was measured spectrophotometrically at 740 nm against the blank (mixture without extract). The total concentration of phenolic compounds in the tested extracts was determined as µg gallic acid equivalents (GAE) per mg dry extract, using an equation obtained from the standard gallic acid diagram as follows:

$$Absorbance = 1.966 \times total\ phenols\ (\mu g\ of\ gallic\ acid) + 5.346\ \ (R^2 = 0.991)$$

2.5. Total flavonoid content

Total flavonoid content was determined according to the method of Park et al. (1997) using quercetin as the standard. 1000.0 µL of the extract was diluted with 4300.0 µL of mixture containing 4100.0 µL of 80% ethanol, 100.0 µL of 10% aluminium nitrate, and 100.0 µL of 1 M aqueous potassium acetate. The reaction mixture was incubated at room temperature for 40 minutes and the absorbance was measured spectrophotometrically at 415 nm. The mixture with the ethanol extract served as a blank. The amount of total flavonoids was expressed as µg of quercetin equivalents (QE) per mg of dry extract, using an equation obtained from the diagram of standard quercetin hydrate as follows:

$$Absorbance = 0.457 \times total\ flavonoid\ (quercetin\ hydrate) - 0.989\ R^2 = (0.957)$$

A total of 16 microbial pathogens (eight bacterial and eight fungal strains), were tested in this study. The Gram-positive bacteria *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (food isolate), *Micrococcus luteus* (ATCC 10240), and *Listeria monocytogenes* (NCTC 7973), and the Gram-negative bacteria *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella Typhimurium* (ATCC 13311), *Escherichia coli* (ATCC 35210), and *Enterobacter cloacae* (human isolate) were used. The following strains of microfungi were evaluated: *Aspergillus fumigatus* (human isolate), *A. versicolor* (ATCC 11730), *A. ochraceus* (ATCC 12066), *A. niger* (ATCC 6275), *Trichoderma viride* (IAM 5061), *Penicillium funiculosum* (ATCC 36839), *P. ochrochloron* (ATCC 9112)

and *P. verrucosum* var. *cyclopium* (food isolate). All the tested microorganisms are deposited in the Mycological Laboratory, Department of Plant Physiology, Institute of Biological Research "Siniša Stanković" - National Institute of the Republic of Serbia, University of Belgrade, Serbia.

2.6. *In vitro* antimicrobial assays

2.6.1. Bacterial and fungal strains tested

A total of 16 microbial pathogens (eight bacterial and eight fungal strains), were tested in this study. The Gram-positive bacteria *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (food isolate), *Micrococcus luteus* (ATCC 10240), and *Listeria monocytogenes* (NCTC 7973), and the Gram-negative bacteria *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella Typhimurium* (ATCC 13311), *Escherichia coli* (ATCC 35210), and *Enterobacter cloacae* (human isolate) were used. The following strains of microfungi were evaluated: *Aspergillus fumigatus* (human isolate), *A. versicolor* (ATCC 11730), *A. ochraceus* (ATCC 12066), *A. niger* (ATCC 6275), *Trichoderma viride* (IAM 5061), *Penicillium funiculosum* (ATCC 36839), *P. ochrochloron* (ATCC 9112) and *P. verrucosum* var. *cyclopium* (food isolate). All the tested microorganisms are deposited in the Mycological Laboratory, Department of Plant Physiology, Institute of Biological Research "Siniša Stanković" - National Institute of the Republic of Serbia, University of Belgrade, Serbia.

2.6.2. Microdilution method

The *in vitro* antimicrobial test was performed using the broth microdilution method (CLSI, 2015) to determine the minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC), and minimum fungicidal concentrations (MFC) of the tested agents. The tested samples were dissolved in 5% DMSO or physiological solution (antimicrobial drugs). The bacterial suspensions were adjusted to a concentration of 1.0×10^5 CFU/mL using sterile saline. Fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v), and the spore suspension was adjusted to a concentration of 1.0×10^5 with sterile saline. Inocula were prepared daily and stored at +4 °C until use. Dilutions of the inocula were cultured on solid medium to verify the absence of contamination and to check the validity of the inoculum. The MIC values obtained in the susceptibility testing of various bacteria to the extracts tested were also determined using a colorimetric assay to determine microbial viability based on the reduction of *p*-iodonitrotetrazolium violet (INT) [2-(4-iodophe nyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride; Sigma Aldrich, St. Louis, MO, USA] color and compared with the positive control for the strains of the individual microorganisms. The lowest concentrations without visible growth (under the binocular microscope) were defined as MICs, for testing the various fungi. The minimum bactericidal (MBC) and fungicidal concentrations (MFC) concentrations were determined by serial sub-cultivation of 2 µL into microtiter plates with 100 µL of broth per well and further incubation at 37 °C for 24 hours for bacteria, and at 28 °C for 72 hours for fungal strains. All experiments were performed in duplicate and repeated three times. The final tested concentration of methanol extracts was in a range of 0.0625–16.0 mg/mL for all tested strains. The following synthetic commercial antibiotics, Streptomycin (Sigma- Aldrich S6501) and Ampicillin (Sigma-Aldrich A9393) and the commercial fungicides, Bifonazole (Srbolek, Belgrade, Serbia) and Ketoconazole (Zorkapharma, Šabac, Serbia) were used as positive controls (1 mg/mL in 0.01 M PBS), in final tested concentration ranges of 0.00015–0.03 mg/mL (antibiotics) and 0.1–3.50 mg/mL (antimycotics), respectively. Five percent DMSO and 0.01 M PBS was used as a negative control.

2.7. Cytotoxic activity

2.7.1. Cell lines

The cervical adenocarcinoma cell line (HeLa) and human chronic myeloid leukemia cells (K562) were grown in RPMI-1640 medium (Sigma). The media were supplemented with 10% fetal bovine serum, L-glutamine and penicillin/streptomycin (Sigma).

2.7.2. Treatment of cell lines

Target HeLa cells (2000 cells per well) and K562 (5000 cells per well) were seeded in the wells of a 96-well flat-bottomed microtiter plate. Twenty-four hours later, after attachment of the cells, different concentrations of the extracts studied were added to the wells, except for the control cells, to which only nutrient medium was added. Stock solutions (100 mg/mL) of the extracts prepared in dimethyl sulfoxide (DMSO) were dissolved in the appropriate medium to the required working concentrations. The final concentration range chosen was 1-100 µg/mL (1, 8.25, 16.5, 33 and 100 µg/mL). The final solvent DMSO concentration never exceeded 0.5%, which was not toxic to the cells. Notably, the compounds were applied to the suspension of K562 cells 2 hours after cell seeding. All concentrations were prepared in triplicate. Culture medium containing the appropriate concentrations of the compounds studied, but without cells, was also used in triplicate as a blank. The cultures were incubated for 72 hours.

2.7.3. Treatment of cell lines

The effect of the prepared compounds on cancer cell survival was determined by the microculture tetrazolium test (MTT) of Mosmann (1983) with modification by Ohno and Abe (1991) 72 hours after addition of the compounds as previously described. Briefly, 20 mL of MTT solution (5 mg/mL phosphate-buffered saline) was added to each well. Samples were incubated for an additional 4 hours at 37 °C in a humidified atmosphere of 95% air/5% CO_2 (v/v). Then, 100 mL of 100 g/L sodium dodecyl sulfate was added to extract the insoluble product formazan resulting from the conversion of MTT dye by viable cells. The number of viable cells in each well was proportional to the intensity of light absorbance measured in an ELISA plate reader at 570 nm. The absorbance (A) at 570 nm was measured 24 hours later. To determine cell survival (%), the A of a sample containing cells grown in the presence of different concentrations of the compounds studied was divided by the optical density of the control (the A of control cells grown only in culture medium) and multiplied by 100. It was assumed that the A of the blank sample was always subtracted from the A of the corresponding sample with target cells. IC_{50} was defined as the concentration of an agent that inhibited cell survival by 50% compared with a vehicle-treated control. Positive controls were cis-diamine dichloroplatin (cis-DDP). All experiments were performed in triplicate.

2.8. Statistical analysis

For each species, three samples were used and all the assays were carried in triplicate. The results expressed a mean values and standard errors, and analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HDS Test with α=0.05. This analysis was carried out using SPSS v. 18.0 program.

3. RESULTS

The extraction yields of the methanolic extracts on a lyophilizates and dry weight basis has shown in Table 1. The extracts

based on freeze-dried weight had higher yields than the extracts based on dry weight for all algal samples. The species with the highest extraction yield was *C. effusum* (lyophilized), 21.07%.

Table 1. Extraction yield of seaweeds methanolic extracts on lyophilized (L) and dry (D) weight basis

Extracts	Yield (%) w/w
Codium bursa (L)	11.83
Codium bursa (D)	10.10
Codium effusum (L)	21.07
Codium effusum (D)	13.83
Laurencia obtusa (L)	14.87
Laurectia obtusa (D)	2.73
Padina pavonica (L)	13.40
Padina pavonica (D)	5.20

3.1. Total phenolic (TP) and flavonoid (TF) content

The results in Table 2 show that flavonoids content was significantly higher in all tested extracts comparing to phenolic content. It has been demonstrated that drying method used during material preparation does not affect the amount of extracted phenolic compounds. In contrast, the amount of extracted flavonoids was significantly higher in extracts obtained from oven-dried material compared to lyophilized samples (Table 2).

Table 2. Total phenolic (TP) and flavonoid (TF) contents of seaweeds lyophilized (L) and dry (D) extracts

Extracts	Total phenols (μg GAE/mg extract)	Total flavonoids (μg QE/mg extract)
Codium bursa (L)	2.89 ± 0.35	10.83 ± 0.10
Codium bursa (D)	3.19 ± 0.14	11.94 ± 0.80
Codium effusum (L)	0.54 ± 0.45	10.08 ± 0.86
Codium effusum (D)	2.81 ± 0.28	22.12 ± 0.74
Laurencia obtusa (L)	ND	7.91 ± 0.21
Laurencia obtusa (D)	0.95 ± 0.49	25.89 ± 3.25
Padina pavonica (L)	10.25 ± 0.94	11.70 ± 1.45
Padina pavonica (D)	10.44 ± 0.94	73.30 ± 2.62

Values are expressed as mean ± standard error
ND, no detected total phenolic and flavonoid contents

3.2. Antioxidative activity

The antioxidant activity of methanolic extracts, lyophilized and dry, determined by DPPH assays, is shown in Table 3. Antioxidative activity expressed in EC_{50} values ranged from 5.88 to 84.05 mg/mL. The extracts *of P. pavonica*, lyophilized, showed the strongest radical scavenging activity (EC50=5.88 mg/mL). The antioxidant activity of the methanolic extract of *C. bursa* (dried) was not detected. The activity of the commercial antioxidant butylated hydroxyanisole (BHA) was 0.06 ± 0.03 mg/mL.

Table 3. Antioxidant activity of seaweeds lyophilized (L) and dry (D) extracts determined by DPPH• assays

Extracts	DPPH• EC_{50} (mg/mL)
Codium bursa (L)	71.41± 4.85
Codium bursa (D)	ND
Codium effusum (L)	63.83 ± 3.22
Codium effusum (D)	36.96 ± 0.40
Laurencia obtusa (L)	60.20 ± 0.97
Laurencia obtusa (D)	32.06 ± 0.30
Padina pavonica (L)	5.88 ± 0.13
Padina pavonica (D)	84.05 ± 10.03

Values are expressed as mean ± standard error
ND, no detected antioxidant activity

3.3. Antibacterial and antifungal activity

The antibacterial activities of all tested extracts of four seaweeds are shown in Table 4. The seaweed extracts showed antibacterial activity against all bacterial species, but at different levels. The minimum inhibitory concentration (MIC) ranged from 0.0625-4.000 mg/mL, while the minimum bactericidal concentration (MBC) was 0.125-8.000 mg/mL. Their antibacterial potential can be presented as follows extracts: *C. effusum* (dried) > *P. pavonica* (dried) > *L. obtusa* (dried) > *P. pavonica* (lyophilized) > *L. obtusa* (lyophilized) > *C. effusum* (lyophilized) > *C. bursa* (dried) > *C. bursa* (lyophilized). The best antibacterial activity was obtained for the dry extract of *C. effusum*, with MIC ranging from 0.0625-2.000 mg/mL and MBC from 0.125-4.000 mg/mL. The lowest antibacterial activity among all extracts tested here was determined for freeze-dried extracts of *C. bursa*. The most sensitive bacterial species was *B. cereus* with an MIC of 0.0625-3.000 mg/mL and an MBC of 0.125-4.000 mg/mL. *L. monocytogenes* was the most resistant species with inhibitory activity between 1.500-4.000 mg/mL and bactericidal activity of 3.000-8.000 mg/mL. Streptomycin possessed inhibitory activity of 0.0003-0.015 mg/mL and bactericidal activity of 0.0006-0.030 mg/mL. The MIC range for ampicillin was 0.00015-0.00045 mg/mL and the MBC was 0.0003-0.0009 mg/mL. Comparing the biological activity of the extracts with the commercially available antibiotics, we found that the tested samples had lower antibacterial potential (Table 4). The results of the antifungal activity of the different types of four algae are shown in Table 5. All the tested extracts showed antifungal activities in the MIC range of 1.000-8.000 mg/mL and in the MFC range of 2.000-18.000 mg/mL. The best antifungal activity was obtained for the extract of *C. effusum* (lyophilized) with inhibitory activity of 1.000-4.000 mg/mL and fungicidal activity of 4.000-8.000 mg/mL. *A. niger*, was the most sensitive fungus tested, while *P. verrucosum* was the most resistant. Bifonazole showed inhibitory activity of 0.1000.200 mg/mL and fungicidal activity of 0.200-0.2500 mg/mL, while ketoconazole showed inhibitory activity of 0.200-2.500 mg/mL and fungicidal activity of 0.500-3.500 mg/mL. The synthetic fungicides showed better antifungal activity than the tested extracts.

The tested methanolic extracts of algae possessed better antibacterial activity than antifungal potential.

3.4. Cytotoxic activity

Screening of methanolic extracts of the studied algae was performed with two human cancer cell lines: the cervical adenocarcinoma cell line (HeLa) and human chronic myeloid leukemia (K562). The cytotoxic effects of the tested extracts estimated by the MTT assay are shown as IC_{50} values in Table 6. First, all tested extracts showed excellent to moderate activity against malignant cells. Very important information that emerged from our results is that all methanolic dry extracts (D) of the algae showed significant or several times better cytotoxic activity compared to the freeze-dried samples (L). Moreover, the HeLa cells were found to be more sensitive than the K562 cell lines. The dry extracts (D) from *L. obtusa* and *P. pavonica* algae showed pronounced cytotoxic activity in both cell lines tested (Table 6). In contrast, the lyophilized (L) extract of *P. pavonica* showed the weakest activity against HeLa cells and moderate activity against K562 when comparing the lyophilized (L) extracts. For all other algal samples, good to moderate cytotoxicity is reported against the malignant cells studied.

Table 4. Minimum inhibitory (MIC) and bacterial (MBC) concentrations of tested methanol extracts of macroalgae (mg/mL)

Extracts	*Codium effusum* L	*Codium effusum* D	*Codium bursa* L	*Codium bursa* D	*Padina pavonica* L	*Padina pavonica* D	*Laurencia obtusa* L	*Laurencia obtusa* D	Streptomycin	Ampicillin
Bacteria	MIC MBC	MIC MBC	MIC MBC	MIC MBC	MIC MBC	MIC MBC	MIC MBC	MIC MBC	MIC MBC	MIC MBC
Staphylococcus aureus	1.00±0.07^{a} 2.00±0.07^{d}	0.125±0.30^{b} 0.25±0.01^{ab}	3.00±0.05^{f} 4.00±0.10^{e}	3.00±0.000^{f} 4.00±0.20^{e}	0.50±0.07^{d} 1.00±0.10^{c}	0.25±0.02^{c} 0.35±0.02^{b}	0.50±0.007^{d} 1.00±0.02^{c}	0.50±0.02^{d} 1.00±0.05^{c}	0.0004±0.00005^{a} 0.0008± 0.00007^{a}	0.0002± 0.00007^{a} 0.0004± 0.0001^{a}
Bacillus cereus	1.00±0.10^{d} 2.00±0.000^{f}	0.0625±0.04^{a} 0.125±0.008^{ab}	3.00±0.05^{e} 4.00±0.000^{g}	3.00±0.08^{e} 4.00±0.10^{g}	0.125±0.000^{ab} 0.25±0.02^{b}	1.00±0.000^{d} 1.50±0.10^{e}	0.25±0.02^{b} 0.50±0.08^{c}	0.75±0.08^{c} 1.00±0.07^{d}	0.0003±0.00007^{a} 0.0006±0.00003^{a}	0.00015±0.00002^{a} 0.0003±0.00005^{a}
Micrococcus luteus	2.00±0.000^{d} 4.00±0.20^{d}	1.00±0.80^{b} 2.00±0.10^{c}	4.00±0.10^{f} 6.00±0.20^{e}	3.00±0.10^{e} 4.00±0.000^{d}	2.00±0.000^{d} 4.00±0.07^{d}	0.75±0.08^{b} 1.50±0.10^{b}	3.00±0.10^{e} 4.00±0.10^{d}	1.50±0.07^{c} 2.00±0.10^{c}	0.00035±0.00005^{a} 0.0007±0.0001^{a}	0.0004±0.00007^{a} 0.0008±0.00007^{a}
Listeria monocytogenes	4.00±0.07^{d} 8.00±0.20^{d}	2.00±0.20^{c} 4.00±0.05^{c}	4.00±0.20^{d} 8.00±0.000^{d}	2.00±0.07^{c} 4.00±0.20^{c}	2.00±0.10^{c} 4.00±0.07^{c}	1.50±0.10^{b} 3.00±0.10^{b}	4.00±0.000^{d} 8.00±0.20^{d}	2.00±0.15^{c} 4.00±0.000^{c}	0.015±0.002^{a} 0.030±0.00007^{a}	0.00015±0.000^{d} 0.0003±0.00007^{a}
Pseudomonas aeruginosa	1.00±0.10^{d} 2.00±0.000^{e}	0.25±0.05^{b} 0.50±0.07^{b}	4.00±0.20^{f} 8.00±0.000^{g}	3.00±0.07^{e} 4.00±0.000^{f}	0.50±0.10^{c} 1.00±0.10^{d}	0.35±0.05^{bc} 0.75±0.05^{c}	0.35±0.02^{b} 0.50±0.10^{b}	0.25±0.02^{b} 0.50±0.07^{b}	0.0004±0.000^{a} 0.0008±0.00007^{a}	0.00015±0.00002^{a} 0.0003±0.00007^{a}
Escherichia coli	2.0±0.20^{e} 4.00±0.05^{e}	1.00±0.10^{c} 2.00±0.10^{d}	4.00±0.08^{g} 6.00±0.20^{f}	3.00±0.07^{f} 4.00±0.10^{e}	1.50±0.000^{d} 2.00±0.10^{c}	0.75±0.02^{b} 1.50±0.08^{b}	3.00±0.10^{f} 4.00±0.000^{e}	1.50±0.10^{d} 2.00±0.10^{d}	0.0004±0.0001^{a} 0.0008±0.00007^{a}	0.00015±0.00002^{a} 0.0003±0.000^{a}
Enterobacter cloacae	2.00±0.07^{c} 8.00±0.10^{d}	2.00±0.000^{c} 4.00±0.10^{c}	4.00±0.20^{e} 8.00±0.000^{d}	2.00±0.10^{c} 4.00±0.20^{e}	3.00±0.10^{d} 4.00±0.07^{c}	0.75±0.08^{b} 1.50±0.20^{b}	4.00±0.08^{e} 8.00±0.20^{d}	1.00±0.000^{b} 4.00±0.10^{c}	0.015±0.002^{a} 0.03±0.007^{a}	0.0002±0.0001^{a} 0.0004±0.00007^{a}
Salmonella typhimurium	2.00±0.000^{e} 4.00±0.08^{f}	1.00±0.15^{c} 2.00±0.15^{e}	3.00±0.10^{f} 4.00±0.10^{f}	4.00±0.20^{g} 8.00±0.20^{g}	1.50±0.10^{d} 2.00±0.000^{e}	0.75±0.08^{b} 1.50±0.20^{d}	0.75±0.000^{b} 1.00±0.20^{c}	0.15±0.02^{a} 0.35±0.02^{b}	0.00045±0.00005^{a} 0.0009±0.0001^{a}	0.00045±0.00002^{a} 0.0009±0.0001^{a}

[MIC/MBC (mg/mL); L lyophilized; D dry]

Table 5. Minimum inhibitory (MIC) and fungicidal (MFC) concentrations of tested methanol extracts of macroalgae (mg/mL)

Extracts	*Codium effusum* L	*Codium effusum* D	*Codium bursa* L	*Codium bursa* D	*Padina pavonica* L	*Padina pavonica* D	*Laurencia obtusa* L	*Laurencia obtusa* D	Bifonazole	Ketoconazole
Fungi	MIC MFC	MIC MFC	MIC MFC	MIC MFC	MIC MFC	MIC MFC	MIC MFC	MIC MFC	MIC MFC	MIC MFC
Aspergillus fumigatus	4.00±0.000^{cd} 8.00±0.20^{c}	4.00±0.10^{c} 8.00±0.000^{c}	4.00±0.07^{cd} 8.00±0.10^{e}	4.00±0.20^{d} 18.0±1.00^{e}	4.00±0.08^{cd} 8.00±0.10^{c}	4.00±0.000^{cd} 16.0±0.30^{d}	2.00±0.10^{b} 4.00±0.10^{b}	4.00±0.07^{cd} 8.00±0.70^{c}	0.15±0.02^{a} 0.20±0.10^{a}	0.20±0.02^{a} 0.50±0.000^{a}
Aspergillus versicolor	2.00±0.10^{d} 4.00±0.10^{c}	2.00±0.20^{d} 4.00±0.05^{c}	4.00±0.10^{e} 16.0±0.70^{f}	2.00±0.10^{d} 4.00±0.000^{c}	1.00±0.000^{c} 2.00±0.10^{b}	2.00±0.10^{d} 4.00±0.20^{c}	8.00±0.10^{f} 16.0±0.30^{e}	4.00±0.07^{e} 8.00±0.10^{d}	0.10±0.03^{a} 0.20±0.10^{a}	0.20±0.07^{b} 0.50±0.07^{a}
Aspergillus ochraceus	4.00±0.20^{c} 8.00±0.000^{d}	2.00±0.000^{b} 4.00±0.20^{c}	4.00±0.07^{c} 18.0±1.00^{f}	4.00±0.07^{c} 16.0±0.70^{e}	4.00±0.20^{c} 8.00±0.20^{d}	4.00±0.000^{c} 8.00±0.10^{d}	4.00±0.08^{c} 8.00±0.000^{d}	4.00±0.03^{c} 16.0±0.30^{e}	0.15±0.03^{a} 0.20±0.000^{a}	1.50±0.05^{a} 2.00±0.20^{b}
Aspergillus niger	1.00±0.10^{b} 4.00±0.000^{c}	1.00±0.20^{b} 2.00±0.20^{b}	1.00±0.000^{b} 2.00±0.08^{b}	2.00±0.10^{c} 4.00±0.10^{c}	2.00±0.07^{c} 8.00±0.10^{d}	4.00±0.10^{d} 16.0±0.07^{e}	2.00±0.10^{c} 8.00±0.10^{d}	4.00±0.02^{d} 16.0±0.30^{e}	0.15±0.02^{a} 0.20±0.10^{a}	0.20±0.02^{a} 0.50±0.07^{a}
Trichoderma viride	2.00±0.07^{c} 4.00±0.20^{d}	2.00±0.000^{c} 4.00±0.10^{de}	2.00±0.20^{c} 4.00±0.20^{e}	2.00±0.000^{c} 4.00±0.07^{de}	2.00±0.10^{c} 4.00±0.10^{de}	4.00±0.20^{2} 8.00±0.10^{f}	1.00±0.005^{b} 2.00±0.10^{c}	2.00±0.10^{c} 8.00±0.07^{f}	0.15±0.02^{a} 0.20±0.02^{a}	1.0±0.10^{b} 1.0±0.07^{b}
Penicillium funiculosum	1.00±0.20^{b} 4.00±0.08^{b}	2.0±0.07^{c} 4.00±0.000^{b}	2.00±0.20^{c} 4.00±0.07^{b}	2.00±0.000^{c} 4.00±0.10^{b}	4.00±0.08^{d} 16.0±0.30^{d}	2.00±0.08^{c} 4.00±0.10^{b}	2.00±0.10^{c} 4.00±0.10^{c}	4.00±0.03^{d} 8.00±0.20^{c}	0.20±0.10^{a} 0.25±0.02^{a}	0.20±0.07^{a} 0.50±0.000^{a}
Penicillium ochrochloron	4.00±0.07^{c} 8.00±0.10^{c}	4.00±0.10^{c} 8.00±0.10^{c}	4.00±0.20^{c} 16.0±0.000^{de}	4.00±0.05^{c} 16.0±0.70^{d}	4.00±0.000^{c} 16.0±0.70^{e}	2.00±0.20^{b} 4.00±0.20^{b}	2.00±0.10^{b} 4.00±0.08^{b}	4.00±0.03^{c} 16.0±0.70^{e}	0.20±0.000^{a} 0.25±0.02^{a}	2.50±0.20^{b} 3.50±0.20^{b}
Penicillium verrucosum var. cyclopium	4.00±0.08^{d} 8.00±0.000^{c}	4.00±0.07^{d} 16.0±0.70^{d}	4.00±0.20^{d} 16.0±0.20^{de}	8.00±0.08^{e} 16.0±0.30^{e}	8.00±0.000^{e} 16.0±0.70^{d}	2.00±0.10^{c} 4.00±0.20^{b}	1.00±0.10^{b} 8.00±0.10^{c}	4.00±0.07^{d} 16.0±0.000^{de}	0.10±0.007^{a} 0.20±0.03^{a}	0.20±0.03^{a} 0.30±0.02^{a}

[MIC/MFC (mg/mL); L lyophilized; D dry]

Table 6. Cytotoxic activity of seaweeds lyophilized (L) and dry (D) extracts on HeLa and K562 cells.

Extracts	HeLa	K562
	IC50 (μg/mL)	
Padina pavonica (D)	21.27 ± 0.89	15.17 ± 2.34
Padina pavonica (L)	87.27 ± 2.05	68.15 ± 1.02
Codium effusum (D)	37.43 ± 0.87	44.39 ± 0.32
Codium effusum (L)	54.68 ± 2.23	47.91 ± 0.69
Codium bursa (D)	59.03 ± 2.52	77.04 ± 2.65
Codium bursa (L)	67.93 ± 1.35	80.31 ± 1.47
Laurencia obtusa (D)	6.51 ± 0.35	16.95 ± 2.37
Laurencia obtusa (L)	42.07 ± 1.96	20.46 ± 0.75

4. DISCUSION

Previously have been shown that polar solvents are usually the solvent of choice for the extraction of various compounds from macroalgal species. The differences in the yields of the various extracts have been attributed to the polarity of the different compounds present in the plants (Ye et al., 2009). In the present study, methanol was used to extract antioxidant, antimicrobial and antitumor compounds from four species of algae. According to Kelman et al. (2012), methanol was found to have the highest extraction efficiency. The phenolic content of these algae was determined by the Folin-Ciocalteu method. Phenolic compounds have various biological activities, including antioxidant activity. The variation in phenolic content was quite wide, ranging from 0.54 to 10.44 μg GAE/mg of extract (Table 2). The brown algae *P. pavonica* (lyophilized and dried) had significantly higher phenolic content than red algae *L. obtusa* and green algae *C. bursa* and *C. effusum*. It has been reported that brown algae generally contain higher amounts of polyphenols than red and green algae (Heffernan et al., 2015). The range of variation in flavonoid content was also quite wide, ranging from 7.91 to 73.30 μg QE/mg of extract (Table 2). Flavonoids are the largest class of polyphenols and contribute most to the antioxidant capacity of plants. They act by either blocking the formation of hypervalent metal forms, scavenging free radicals, or interrupting chain reactions of lipid peroxidation (Zaragozá et al., 2008). The significantly higher flavonoid content was found in brown algae *P. pavonica* (dried). Zaragoza et al. (2008) and Rajauria et al. (2013) observed flavonoid content in methanolic extracts of brown algae, *Fucus vesiculosus* and *Himanthalia elongate*.

In this study, the antioxidative activities of algal extracts were tested using the DPPH assay. DPPH is a useful reagent to study the radical scavenging activities of compounds (Duan et al., 2006). The antioxidant activity of the methanolic extracts was significantly different among the four tested algae. Among them, *P. pavonica* showed the strongest radical scavenging activity and the highest TP among. Praba and Sumaya (2022) also reported similar results in other species of the genus *Padina* (*P. antillarum*, *P. tetrastomatica*, *P. gymnospora*). *Codium fragile* are known to have relatively low antioxidant activity (KeskiNkaya et al., 2022). We found that *C. bursa* and *C. effusum* have low antioxidant activity. The methanolic extracts of *L. obtusa* showed low antioxidant activity. According to Kelman et al. (2012) and Al-Enazi et al. (2018), *Laurencia obtusa* is a good source of biologically active secondary metabolites but does not exhibit high antioxidant activity.

Results clearly showed that antioxidative activities of *C. effusum* and *L. obtusa* were strongly correlated to flavonoids content. The linear correlation between radical scavenging activity and TP and TF content suggests that algae may also contain other antioxidants such as ascorbic acid, carotenoids, folic acid, and thiamine (Yangthong et al., 2009).

The best antibacterial activity was obtained for the methanolic extract of *C. effusum* (dry) and the lowest antibacterial activity of all extracts tested here was obtained for extracts of *C. bursa* (freeze-dried). According to Albayati et al. (2020) extracts of *C. bursa* showed antimicrobial activity. The tested methanolic extracts of algae possessed better antibacterial activity than antifungal potential. According to Khaled et al. (2012), *P. pavonica* exhibited antifungal activity against four *Candida* species.

Dry extracts (D) from *L.obtusa* and *P. pavonica* algae showed pronounced cytotoxic activity in both cell lines tested (HeLa and K562). Stanojković et al. (2013) reported strong cytotoxic activity of methanolic extract of *P. pavonica* on HeLa cancer cell line. Awad et al. (2008) reported the cytotoxic activity of *P. pavonica* against human lung carcinoma (H460) and liver carcinoma (HepG2) cell lines. According to Ktari and Guyot (1999), dichloromethane extract of *P. pavonica* showed cytotoxic activity against KB cells.

5. CONCLUSION

In the pursuit of new natural compounds with potential antimicrobial and antitumor properties, algae have demonstrated significant promise as a valuable source. This study highlights the potential of algae, particularly *Padina pavonica*, as a rich source of bioactive compounds with antioxidant, antimicrobial, and cytotoxic properties. Methanol was effective in extracting phenolic and flavonoid compounds, with *P. pavonica* exhibiting the highest antioxidant activity among tested species. Antimicrobial effects were more pronounced in antibacterial assays, notably in *C. effusum*, while *L. obtusa* and *P. pavonica* showed strong cytotoxicity against cancer cell lines. These findings suggest that specific algae may serve as promising natural sources for developing therapeutic agents.

ACKNOWLEDGMENTS

This work was financially supported by Ministri of Science, Technological Development and Innovation of the Republic of Serbia (Grants No., 451-03-65/2024-03/200178, 451-03-66/2024-03/200178, 451-03-66/2024-03/ 200007, 451-03-66/2024-03/200026 and 451-03-66/2024-03/200043).

CONFLICT OF INTEREST

The authors declare that they have no financial conflicts of interest.

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