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BIOLOGICAL POTENTIAL OF MARINE MACROALGAE OF THE GENUS *CYSTOSEIRA*

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In the present investigation the acetone extracts of three macroalgae, *Cystoseira amentacea, Cystoseira barbata* and *Cystoseira compressa,* were tested for antioxidant, antimicrobial and cytotoxic potential. As a result of the study, *C. amentacea* extract had more potent free radical scavenging activity (IC_{50} = 409.81 µg/mL) than *C. barbata* and *C. compressa* extracts. For reducing power, measured values of absorbance varied from 0.0352 to 0.8873. The IC_{50} values for superoxide anion scavenging activity for different extracts were 521.45–976.62 µg/mL. Total phenol and flavonoid contents in extracts were 39.96–81.28 µg PE/mg and 20.85–64.58 µg RE/mg respectively. Further, all three *Cystoseira* species exhibited a similar antimicrobial activity. The lowest MIC value (0.312 mg/mL) was shown in the extract obtained from *C. compressa* against *Bacillus subtilis.* Finally, extract of *C. amentacea* expressed the strongest cytotoxic activity toward tested cell lines with IC_{50} values ranging from 94.72 to 186.55 µg/mL. Based on these results, it can be stated that the tested macroalgae can be used as potential natural antioxidants and antimicrobial and cytotoxic agents.

Keywords: Acetone extracts – cytotoxic activity – antimicrobial activity – antioxidant activity – algae

INTRODUCTION

Marine organisms are source material for structurally unique natural products with pharmacological and biological activities. Among the marine organisms, the macroalgae (seaweeds) occupy an important place as a source of biomedical compounds [27]. Marine macroalgae are the most interesting algae group because of their broad spectrum of biological activities such as antimicrobial [9], anti-allergic [17], anticoagulant [4], cytotoxic [27], antiviral [3] and antioxidant activities [27]. As an aid to protect themselves against other organisms in their surrounding, macroalgae produce a wide variety of chemically active metabolites that have a broad range of biological activities. Some of these metabolites have been used in the pharmaceutical industry.

The *Cystoseira* genus includes approximately 294 species and is one of the most representative member of the *Sargassaceae* family (composed by the recent merging of the two former *Sargassaceae* and *Cystoseiraceae* families) [6]. *Cystoseira* are

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among the most dominant and ecologically important species in the Mediterranean and the Adriatic Sea [21].

Previous investigation of members of the genus *Cystoseira* resulted in the discovery of broad range of biological activities such as: anti-inflammatory, antibiotics, antiprotozoal, antioxidant and cytotoxic activities [14, 27, 28]. Despite the diversity in quality and quantity of the Adriatic coast flora, with its rich content of marine organisms and seaweeds, most of them have not yet been investigated for pharmacological and biological activities [13]. Therefore, the objective of this research was to evaluate antioxidant, antimicrobial and cytotoxic activities in *C. amentacea*, *C. barbata* and *C. compressa* acetone extracts.

MATERIAL AND METHODS

Algal samples

Algal samples of *C. amentacea* (C. Agardh) Bory de Saint-Vincent, *C. barbata* (Stackhouse) C. Agardh and *C. compressa* (Esper) Gerloff & Nizamuddin, were collected from the Adriatic Sea, in June of 2013. The voucher specimen of the algae (Voucher Nos 44, 45 and 46) was deposited at the Department of Biology, Faculty of Science, University of Kragujevac. To determinate tested algae were used several standard keys [5].

Preparation of the algal extracts

Finely dry ground thalli of the examined algae (100 g) were extracted using acetone (500 mL) in a Soxhlet extractor. The extracts were filtered and then concentrated under reduced pressure in a rotary evaporator. The dry extracts were stored at −18 °C until they were used in the tests. The extracts were dissolved in 5% dimethyl sulphoxide (DMSO) for the experiments. DMSO was dissolved in sterile distilled water to the desired concentration.

Antioxidant activity

Antioxidant activity was evaluated by free radical scavenging, superoxide anion radical scavenging and reducing power. The free radical scavenging activity of lichen extracts was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH) according to the Simić et al. method [25]. Oyaizu method [20] was used to determine the reducing power. The superoxide anion radical scavenging activity was detected according to the described method by Nishimiki et al. [19].

Determination of total phenolic and flavonoid compounds

The amount of total phenols in the lichen extracts was determined as pyrocatechol equivalent using Folin-Ciocalteu reagent according to Slinkard and Singleton's method [26]. Dowd method [12] was used for the quantification of total flavonoid in the extracts with rutin as standard.

Antimicrobial activity

The bacteria used in this study: *Bacillus mycoides* (ATCC 6462), *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Klebsiella pneumoniae* (ATCC 13883) were obtained from the American Type Culture Collection (ATCC). The fungi used as test organisms were: *Aspergillus flavus* (ATCC 9170), *Aspergillus fumigatus* (ATCC 1022), *Candida albicans* (ATCC 10231), *Penicillium purpurescens* (DBFS 418) and *Penicillium verrucosum* (DBFS 262). They were from the ATCC and the mycological collection maintained by the Mycological Laboratory in the Department of Biology of the Faculty of Science of the Kragujevac University. Bacterial cultures were maintained on Müller-Hinton agar substrates (Torlak, Belgrade). Fungal cultures were maintained on potato dextrose (PD) agar and Sabourad dextrose (SD) agar (Torlak, Belgrade). All cultures were stored at 4 °C and subcultured every 15 days.

Bacterial inoculi were obtained from bacterial cultures incubated for 24 h at 37 °C on Müller-Hinton agar substrate and brought up by dilution according to the 0.5 McFarland standard to approximately 10^8 CFU/mL. Suspensions of fungal spores were prepared from fresh mature (3- to 7-day-old) cultures that grew at 30 °C on a PD agar substrate. Spores were rinsed with sterile distilled water, used to determine turbidity spectrophotometrically at 530 nm, and then further diluted to approximately 10^6 CFU/mL according to the procedure recommended by NCCLS [18].

The minimal inhibitory concentration (MIC) was determined by the broth micro-dilution method with using 96-well micro-titer plates [23]. A series of dilutions with concentrations ranging from 40 to 0.0047 mg/mL for extracts was used in the experiment against every microorganism tested. The starting solutions of test samples were obtained by measuring off a certain quantity of samples and dissolving it in DMSO. Twofold dilutions of test samples were prepared in Müller-Hinton broth for bacterial cultures and SD broth for fungal cultures. The MIC was determined with resazurin. Resazurin is an oxidation-reduction indicator used for the evaluation of microbial growth. It is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. The boundary dilution without any changing color of resazurin was defined as the MIC for the tested microorganism at the given concentration. Streptomycin (for bacteria) and ketoconazole (for fungi) were used as a positive control. A solvent control test was performed to study an effect of 5% DMSO on the growth of a microorganism.

Cytotoxic activity

Human colon carcinoma LS174 cells, human lung carcinoma A549 cells, malignant melanoma Fem-x cells and chronic myelogenous leukaemia K562 cells (American Type Culture Collection, USA) were cultured as a monolayer in the RPMI 1640 nutrient medium, with 10% (inactivated at 56 °C) FBS, 3 mM of L-glutamine, and antibiotics, at 37 °C in humidified air atmosphere with 5% CO_2.

In vitro assay for cytotoxic activity of investigated extract was performed when the cells reached 70–80% of confluence. Stock solution (50 mg/mL) of extract was dissolved in corresponding medium to the required working concentrations. Neoplastic LS174 cells (7000 cells per well), A549 cells (5000 cells per well), Fem-x cells (5000 cells per well) and K562 cells (5000 cells per well) were seeded into 96-well microtiter plates, and 24 h later, after cell adherence, 5 different, double-diluted concentrations of investigated extract were added to the wells. Final concentrations of the extract were 200, 100, 50, 25, and 12.5 μg/mL except for the control wells, where only nutrient medium was added. The cultures were incubated for the next 72 h. The effect on cancer cell survival was determined 72 h after the addition of extract, by the MTT test [15]. Briefly, 20 μL of MTT solution (5 mg/mL PBS) was added to each well and incubated for a further 4 h at 37 °C in 5% CO_2 and humidified air. Subsequently, 100 μL of 10% SDS was added to solubilise the formazan crystals formed from MTT after the conversion by mitochondrial dehydrogenases of viable cells. Absorbencies proportional to the number of viable cells were measured using a microplate reader (Multiskan EX, Thermo Scientific, Finland) at 570 nm. Each experiment was performed in triplicate and independently repeated at least four times.

Data analyses

Data analyses were performed with the EXCEL and SPSS softwares package. To determine the statistical significance of antioxidant activity, Student's *t*-test was used. All values are expressed as mean ± SD of three parallel measurements.

RESULTS

The scavenging DPPH radicals and superoxide anion radical scavenging of the studied extracts are represented in Table 1. As seen in the table, the extract from *C. amentacea* showed larger antioxidant activities than *C. barbata* and *C. compressa*. The IC_{50} values of all extracts ranged from 409.81 to 812.22 μg/mL for the DPPH scavenging effect and 521.45–976.62 μg/mL for the superoxide anion scavenging activity. The results of the reducing power assay of tested species are summarized in Table 2. High absorbance indicates high reducing power. Measured values of absorbance varied from 0.0352 to 0.8873. The reducing power in the tested extracts decreased in the following order: *C. amentacea* > *C. barbata* > *C. compressa*. In various antioxidant

Table 1
DPPH radical scavenging activity, superoxide anion scavenging activity, total phenolics and flavonoid content of acetone extracts of *Cystoseira amentacea*, *Cystoseira barbata* and *Cystoseira compressa*

Algal species	DPPH radical scavenging IC_{50} (µg/mL)	Superoxide anion scavenging IC_{50} (µg/mL)	Phenolics content (µg PE/mg of extract)	Flavonoid content (µg RE/mg of extract)
C. amentacea	409.81 ± 1.36	521.45 ± 2.12	81.28 ± 1.065	64.58 ± 1.099
C. barbata	564.78 ± 2.35	675.93 ± 2.02	61.74 ± 1.013	55.14 ± 1.078
C. compressa	812.22 ± 2.93	976.62 ± 2.73	39.96 ± 1.002	20.85 ± 0.991
Ascorbic acid	6.42 ± 0.18	115.61 ± 1.16		

Values are expressed as mean ± SD of three parallel measurements.
PE – pyrocatechol equivalents.
RE – rutin equivalents.

activities, there was a statistically significant difference between extracts and control ($P < 0.05$). Various antioxidant activities were compared to ascorbic acid. The results showed that standard antioxidant had stronger activity than the tested samples.

The total phenolic and flavonoid contents of the algal extracts are given in Table 1. Phenolic content for tested extracts is obtained from the regression equation of calibration curve of pyrocatechol (y = 0.0057 × total phenols [µg PE/mg of dry extracts] –0.1646, R2 = 0.9203). The concentrations of the flavonoids were calculated according to the equation that was obtained from the standard rutin graph (y = 0.0296 × total flavonoid [µg RE/mg of dry extracts] + 0.0204, R2 = 0.9595). The total phenolics content in extracts of *C. amentacea*, *C. barbata* and *C. compressa* were 81.28, 61.74 and 39.96 µg PE/mg, while concentration of flavonoids were 64.58, 55.14 and 20.85 µg RE/mg, respectively.

The antimicrobial activity of the algal extracts against the test microorganisms is shown in Table 3. Extract from *C. amentacea*, *C. barbata* and *C. compressa* exhibited a similar antimicrobial activity. They inhibited all the tested bacteria and fungi. The MIC for bacteria ranged from 0.312 mg/mL to 5 mg/mL and MIC for fungi ranged from 2.5 mg/mL to 10 mg/mL. The strongest antimicrobial activity was found in *C. compressa* extract against *B. subtilis*. The most sensitive among the bacteria were *B. mycoides* and *B. subtilis* and the highest resistance was shown in *E. coli.* Among the fungi, the most sensitive appeared to be *C. albicans.* The antimicrobial

Table 2
Reducing power of acetone extracts of *Cystoseira amentacea*, *Cystoseira barbata* and *Cystoseira compressa*

Algal species	Absorbance (700 nm)				
	1000 µg/mL	500 µg/mL	250 µg/mL	125 µg/mL	62.5 µg/mL
C. amentacea	0.8873 ± .031	0.6741 ± .025	0.1870 ± .008	0.0622 ± .004	0.0425 ± .002
C. barbata	0.6823 ± .028	0.5568 ± .024	0.3692 ± .009	0.1949 ± .006	0.0901 ± .003
C. compressa	0.1309 ± .015	0.0983 ± .011	0.0781 ± .008	0.0522 ± .004	0.0352 ± .003
Ascorbic acid	2.113 ± .032	1.654 ± .021	0.0957 ± .008	0.0478 ± .008	0.0297 ± .004

Values are expressed as mean ± SD of three parallel measurements.

Table 3
Minimum inhibitory concentration (MIC) of acetone extracts of *Cystoseira amentacea*, *Cystoseira barbata* and *Cystoseira compressa*

Algal species	*C. amentacea*	*C. barbata*	*C. compressa*	S – K	
Bacillus mycoides	0.625	0.625	0.625	7.81	–
Bacillus subtilis	0.625	0.625	0.312	7.81	–
Escherichia coli	5	5	5	31.25	–
Klebsiella pneumoniae	1.25	1.25	1.25	1.95	–
Staphylococcus aureus	2.5	1.25	1.25	31.25	–
Aspergillus flavus	5	5	5	–	3.9
Aspergillus fumigatus	5	5	5	–	3.9
Candida albicans	2.5	2.5	2.5	–	1.95
Penicillium purpurescens	5	10	5	–	3.9
Penicillium verrucosum	5	10	5	–	3.9

Values given as mg/mL for tested samples and as μg/mL for antibiotics. Values are the mean of three replicate. Antibiotics: K – ketoconazole, S – streptomycin.

activity was compared with the standard antibiotics, streptomycin (for bacteria) and ketoconazole (for fungi). The results showed that standard antibiotics had stronger activity than tested samples as seen in Table 3. In a negative control, DMSO had no inhibitory effect on the tested organisms.

The data obtained for the cytotoxic effect of *C. amentacea*, *C. barbata* and *C. compressa* extracts are shown in Table 4. The best cytotoxic effect had the extract from *C. amentacea* (IC_{50} = 162.88, 186.55, 94.72 and 150.69 μg/mL, respectively). *C. barbata* extract shows activity against LS174, A569 and K562 cells while cytotoxic activity against Fem-x cells was not detected (IC_{50} > 200 μg/mL). *C. compressa* extract showed a weakest cytotoxic activity. It inhibited only K562 cells (IC_{50} = 172.93) whereas in LS174, A569 and Fem-x cells cytotoxic activity was not detected (IC_{50} > 200 μg/mL). Furthermore, all extracts showed significantly less activity compared to *cis*-DDP as a positive control.

Table 4
Growth inhibitory effects of acetone extracts of *Cystoseira amentacea*, *Cystoseira barbata* and *Cystoseira compressa* on LS174, A549, Fem-x and K562 cell lines

Algal species	Cell lines			
	LS174	A549	Fem-x	K562
	IC_{50} (μg/mL)			
Cystoseira amentacea	162.88±3.53	186.55±2.26	94.72±0.51	150.69±2.36
Cystoseira barbata	176.02±0.87	190.34±0.56	>200	172.93±1.69
Cystoseira compressa	>200	>200	>200	165.61±2.34
Cis-DDP	0.86±0.33	4.91±0.42	3.18±0.29	2.22±0.08

DISCUSSION

The tested algal extracts have moderate antioxidant activity when using various antioxidant bioassays. The recorded activity was shown to correlate with the total phenolic content in the algal extracts. In most algae, phenols are important antioxidants because of their ability to scavenge free radicals such as singlet oxygen, superoxide and hydroxyl radicals [24]. Like our results, numerous studies have found a high correlation between antioxidative activities of algae and phenolic content [14, 25]. On the other hand, less studies such as for example that of Heo and Cha [8] reported that the antioxidant activity does not always correlate with the total phenolic content in algal extract. These results may indicate the possible participation of other active substances which exhibit antioxidant activity such as pigments (chlorophyll, carotenoids), essential oils, and low molecular weight polysaccharides [16].

Genus *Cystoseira* was previously tested on antioxidant activity. For example, the lipid extracts of eight marine algae belonging to the *Cystoseira* genus were evaluated for their antioxidant activity in a micellar model system by Ruberto et al. [22]. They found that all the lipid extracts showed antioxidant activity and among them the *C. amentacea* var. *stricta* extract was the most active. This is because this alga species is one of the richest in tetraprenyltoluquinols with an intrinsic capability of transferring a hydrogen atom to the peroxyl radical. Stanojković et al. [27] also found relatively strong antiradical activity for acetone extract of *C. amentacea*. In accordance with their results, in our study among the tested species *C. amentacea* also exhibited the strongest antioxidant activity, probably due to the higher content of antioxidant active compounds in extracts. Similar metabolites were present in the other extracts but their antioxidant activity was weaker. This behaviour is to be ascribed to a lower concentration of these metabolites that of the previous alga. Mhadhebi et al. [14] found very strong antioxidant activities for aqueous extracts of *C. crinita*, *C. sedoides* and *C. compressa* species. The *C. compressa* and *C. crinita* extracts showed excellent DPPH radical scavenging activity, with an IC_{50} value of 12 μg/mL and 20 μg/mL, respectively, whereas the *C. sedoides* extracts exhibited less DPPH radical scavenging activity with an IC_{50} value of 75 μg/mL, respectively. Compared with these results, the results of our study demonstrated that the acetone extracts of *C. amentacea, C. barbata* and *C. compressa* have a weaker antioxidant activity. This difference probably depends on the algae species, variability in the environmental conditions, methods used for testing, solvents used for extraction and etc. Previous antioxidant activity for different *Cystoseira* species, using other extraction solvents; the antioxidant activity studies have demonstrated was confirmed by acetone algal extracts. Different extraction solvents, according to the their polarity, may extract various compounds including pigments (chlorophylls a, b, carotenoids), alkaloids, and phenolic compounds, as well as essential oil which can participate in the great antioxidant activity [24]. This means that synergistic effects may occur between these constituents leading to the pronounced antioxidant activity of algal extracts (containing the antioxidant active components). On the contrary, extracts that have a lower pig-

ment contents but also lower content of phenolic compounds have reduced antioxidant activity.

Numerous algae have been screened for antimicrobial activity in search of new antimicrobial agents [9, 28]. In our experiments, the tested algal species show relatively strong antimicrobial activity. The intensity of the antimicrobial effect depended on the species of algae, its concentration and the tested organism. Differences in antimicrobial activity of different species of algae are probably a consequence of the presence of different components with antimicrobial activity [10]. Similar to our results, numerous researchers found strong antimicrobial activity for several *Cystoseira* species. For example, *C. trinodis* harvested from the Persian Gulf, was active against numerous gram-positive and gram-negative species bacteria including *S. aureus* and *E. coli* [28]. The MIC of extract was 1.031 mg/ml for *S. aureus*, 0.687 mg/ml for *S. epidermidis*, 4.125 mg/ml for *E. coli*, and 6.6 mg/ml for *P. aeruginosa*. Therefore, they found that the MIC for gram-positive bacteria was lower than the MIC for gram-negative bacteria which also agrees with our results. Antibacterial activity of methanolic extract of *C. barbata* from the Aegean Sea (Turkey) was investigated in another study [29]. In accordance with our results, *C. barbata* exhibited antibacterial effect against all test bacteria including *S. aureus* and *E. coli*. Ibtissam et al. [9] investigated the antibacterial activities of methanol extract from several species of *Cystoseira* which were collected from the coast of Morocco. Similar to our results, extract of *C. tamariscifolia* showed activity against *S. aureus, E. coli,* and other test bacteria. Also, extracts of *C. mediterranea, C. humilis* and *C. usneoides* had antibacterial effect on *S. aureus* and *E. coli*. Whereas, in contrast to our work, extract of *C. crinita* was not effective against *E. coli* and the extract from *C. compressa* did not show antibacterial effect on any of the test bacteria. It is obvious that there are both similarities and dissimilarities between the results of mentioned articles and our results. The reasons for these dissimilarities could be the differences in various algal species or strains for production of substances, geographical zones and habitats, seasonal variations, life phase, as well as solvents and protocols used for extraction [28].

The extracts used in this study, had stronger antibacterial than antifungal activity. The probable reason for this is difference in the composition and permeability of their cell walls. The cell walls of gram-positive bacteria are made of peptidoglycans and teichoic acids, while the cell walls of gram-negative bacteria are made of peptidoglycans, lipopolysaccharides, and lipoproteins. The lipid portion of the outer membrane of gram-negative bacteria is poorly permeable to antimicrobials, and it is the reason for their greater resistance. The cell walls of fungi are the least permeable and consist of polysaccharides such as hitchin and glucan. This observation is in accordance to many other studies focused on the antimicrobial activity which has demonstrated that structure and permeability of the cell wall are the reason for different sensitivity gram-positive bacteria, gram-negative bacteria and fungi [1, 7, 25].

In our study, the best cytotoxic effect was shown by the extract obtained from *C. amentacea* which inhibited all cell lines tested. *C. barbata* extract showed activity against LS174, A569 and K562 cells while *C. compressa* inhibited only K562 cells.

Similar to us, Stanojković et al. [27] demonstrated that *C. amentacea* extract had cytotoxic effects in various human cell lines (human breast cancer, human cervix adenocarcinoma, human colon carcinoma). As shown in a recent work Zubia et al. [30], *Cystoseira* species possess, among their more significant compounds, different types of terpenes as the bioactive compounds. These terpene compounds could be responsible for the cytotoxic activities of the *Cystoseira* extracts. In our experiment, among the tested species *C. amentacea* probably contains the most metabolites responsible for the noticed cytotoxic effect. *Cystoseira* species extracts were also screened for their cytotoxic activity by other researchers against different cell lines. For instance, *C. tamariscifolia* extract exhibited strong cytotoxic activity against Daudi, Jurkat and K562 tumor cell lines [30]. The hexane fraction of the methanolic extract from *C. myrica* (S.G.Gmelin) C. Agardh showed an interesting activity against T47D (breast carcinoma) (IC_{50} = 99.9 µg/mL) and mainly against MDA-MB468 (breast carcinoma estrogen receptor independent (IC_{50} = 56.5 µg/mL) [11]. Also, the dichloromethane extract of fresh *C. abiesmarina* displayed selective and antiproliferative activity against HeLa cell line, induced by apoptosis (IC_{50} = 8.8 µg/mL) [2]. Compared with their results, the findings of our research suggest that the tested *Cystoseira* species showed moderate anticancer activity.

In conclusion, it can be stated that the algal extracts tested have a certain degree of antioxidant, antimicrobial and cytotoxic activities *in vitro*. Based on the results, the macroalgae tested appear to be good natural antioxidant, antimicrobial and cytotoxic agents. Identification of the active compounds of these algal species will lead to their evaluation in considerable commercial potential in medicine, food production and cosmetic industry.

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