

Bioprospecting of *Turbinaria* Macroalgae as a Potential Source of Health Protective Compounds

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The present study aims to focus on the bioprospecting of marine macroalgae of *Turbinaria* species, plenteous biomass of the world ocean. Three types of solvents, i.e., H₂O, MeOH/H₂O (80:20, v/v) and hexane/i-PrOH (50:50, v/v), were used for extraction. Both the biological activity and the pattern of present chemicals were characterized. For the cell proliferation assay, the human embryonic kidney 293 cells, cervix/breast/pancreatic adenocarcinoma, and osteosarcoma cells were used. For the antioxidant activity determination, both intracellular assay with human embryonic kidney and cervix adenocarcinoma cells, as well as the biochemical DPPH test, were employed. To complete the information about macroalgae composition, organic compounds were characterized by the liquid chromatography coupled with high resolution tandem mass spectrometry. Attention was concentrated mainly on the lipidomic profile characterization. In spite the fact that any significant antiproliferative effect was not observed for cancer cells, both the *Turbinaria* species were shown to be good protectors against the oxidative stress of the non-cancer cells. Most of the antioxidants were determined in the hexane/i-PrOH extract. As regards the lipids identified, most of them belonged to the triacylglycerols followed by sphingomyelins, diacylglycerols, and polar (lyso) phospholipids. Additionally to fatty acids with 14, 16 and 18 carbons, also those with odd carbon numbers were frequently present.

Keywords: Algae, *Turbinaria*, Bioprospecting, Antiproliferation assay, Antioxidant activities, Liquid chromatography, Mass spectrometry.

Introduction

Recently, marine macroalgae or other seaweeds have been used as a potential source of highly valuable nutrients or pharmaceutically attractive compounds, which predetermines their usage as components of foods, dietary supplements for human or animal nutrition, or cosmetics.^[1–4] Although the oceans cover ca. 70% of the planet's surface, only minority of inhabiting organisms have been explored and characterized so far. Characterization of macroalgae in terms of the chemical composition of major/minor components (secondary metabolites), in combination with the biological effects, is the main task of bioprospecting.

Algae bioprospecting is a very complex process, comprising sample collection, determination of biological activity, identification of active compounds (if

possible), and their effective isolation. To be applicable, the isolation process should be easily transformable from the laboratory to the industrial scale, especially in terms of the availability of technologies and economical profitability. Sometimes, the large-scale production of the rare marine organisms in bioreactors is necessary to assure a high quantity of desired biomass. Nowadays, despite the fact that several dozens of compounds exhibiting a broad spectrum of health positive biological activities have been identified, only few large-scale sustainable production technologies have been developed to supply these chemicals in the economically rentable amounts.

According to the available literature, a wide range of pronounced bioactivities of marine macroalgae have been provided by small-molecular secondary metabolites. For example, halimedatrial and

caulerpenin produced by green algae, lanosol, vidalol or elatol generated by red seaweeds, or dictyopterene C, pachydictyol A, fucodiphlorethol and epitaondiol produced by brown algae have attracted the attention of scientists for decades.^[1] Except these, also the healthy lipids, *i.e.*, mainly those containing the 'omega-3' long-chain polyunsaturated fatty acids, polar lipids as phospholipids/glycolipids with the positive health impact or healthy sterols can be very promising in terms of further use in food/feed industry. Recently, several papers focusing on the lipidomic characterization of marine resources have discussed this topic. *Melo et al.*^[5] characterized the polar lipids and fatty acid composition of macroalgae *Chondrus crispus*, *Santos et al.*^[6] published a detailed study about lipophilic composition of *Codium tomentosum*, *Ulva lactuca*, *Gracilaria vermiculophylla* and *C. crispus*, and *Kendel et al.*^[7] focused on the lipidomic characterization of *Ulva armoricana*, and *Solieria chordalis* seaweeds. Another very perspective group of chemicals from this point of view can be the sulfated polysaccharides (fucoidans, galactans, and ulvans) which have been revealed to possess many beneficial biological and therapeutic properties such as anticoagulant, antiviral, antioxidative, antitumor, immunomodulating, anti-hyperlipidemic, and antihepatotoxic.^[8–10] Also peptide compounds have been described as highly promising health promoters. For example, *Fitzgerald et al.*^[2] clearly summarized the information about hypotensive peptides originating from macroalgae which can be incorporated into functional foods' such as beverages and soups.

Many of the bioprospecting studies also discussed the overall biological effects and pharmacological activities of the macroalgae and seaweed, without the specific assignment to the responsible compound. For example, *Yende et al.*^[11] reviewed the therapeutic potential and health benefits of *Sargassum* species (*i.e.*, the analgesic, antiinflammatory, antioxidant, neuroprotective, antimicrobial, antitumor, antiviral and others). *Almeida et al.*^[12] published a similar overview for the *Gracilaria* genus, and *Mariya et al.*^[13] focused on the collecting the information about the algae of genus *Rhodophyta*, *Phaeophyta* and *Chlorophyta*.

In the present study, we aimed to characterize the *Turbinaria* macroalgae species, an abundant biomass of the Indian and Pacific oceans. The biological activity, as well as the chemical pattern of possibly responsible compounds, was assessed. Both *in vitro* cell-based tests, and the biochemical assays for characterization of antiproliferation and antioxidant capacity, were applied here. For completing the information about macroalgae composition, the compounds

(mainly secondary metabolites) were characterized by using ultra-performance liquid chromatography coupled with ultra-high resolution tandem mass spectrometry (U-HPLC/HR-MS/MS). The detailed composition of *Turbinaria* species lipids was described for the first time.

Results and Discussion

In recent years, an interest in research of free radicals, and their role in the oxidative stress in living organisms, has been continuously increasing. Although the reactive oxygen species (ROS) such as hydroxyl radical (OH[•]), H₂O₂, or superoxide anions (O₂^{•-}) play important roles in many biological processes,^[5] the overproduction of these species often contributes to the vast number of diseases such as inflammatory, cancer, atherosclerosis, diabetes mellitus, hypertension and others.^{[14][15]} Beside the ROS, also the other compounds of natural or synthetic origin can cause modifications of structures of important biopolymers (lipids, proteins, and nucleic acids), and thereby influence their functions, and cause various health troubles.

On the other hand, many antioxidants (as tocopherols, carotenoids, polyphenols, flavonoids, catechins) or other health beneficial compounds present in natural products can counteract this trend. The mutual interactions of all these compounds cause various synergistic or inhibitory effects influencing the overall metabolic processes in the living organisms. That is why the modern biological activity studies aim to characterize the overall biological effect of the sample (or sample extract), better than characterize the presence of individual compounds.

The *in vitro* cell-based or biochemical methods can be very descriptive in terms of unknown natural material characterization. In our study, we concentrated on the characterization of the antiproliferation and antioxidant activity of brown macroalgae as a potential source of interesting health beneficial compounds. Both the cell-based and *in vitro* biochemical tests were performed here. It should be noticed that the physicochemical properties as polarity or solubility, as well as the types of chemical bonds, influence their extractability from the natural material, and have to be taken into the account. That is why in our study, three extracts of different polarity, thus different chemical composition, were investigated.

The Effects of Algae Extracts on Cell Proliferation

The antiproliferative (cytotoxic) effect of extracts from *Turbinaria ornata* and *Turbinaria decurrens* was

investigated on one non-cancer, and four cancer cell lines. As depicted in Fig. 1, any significant antiproliferative effect on all cancer cell lines tested was not observed. On the contrary, the induction of proliferation was observed in some cases; *i.e.*, for MCF7 and U2OS cells in case of some concentrations of H₂O and MeOH extracts both from algae. As regards the non-cancer cells, the proliferation of HEK 293T was significantly suppressed by application of MeOH extracts from both species at the highest doses used (Fig. 1).

Antioxidant Capacity of Algae Extracts Measured in vitro by Human Cells Systems

One non-cancer cell line (HEK 293T) and one cancer cell line (HeLa) were used for *in vitro* ROS scavenging activity assay. H₂O₂ was used as the activator of oxidative stress. All the extracts from both algae species significantly protected the non-cancer HEK 293T cells against the ROS formation (Fig. 2a). On the contrary, the protective effect for HeLa cells was significantly less persuasive; only the aqueous-MeOH extracts from both algae were effective in this respect (Fig. 2b). Some degree of HeLa cells protection was observed also in the case of hexane/*i*-PrOH extract from *T. decurrens*.

Antioxidant Capacity of Extracts Measured by in vitro Biochemical Tests

For the determination of the antioxidant activity of the extracts of brown Indonesian macroalgae, the DPPH method of radical scavenging activity testing was used. As seen in Fig. 3, extracts of different polarity showed significantly different results in terms of the overall capacity to scavenge the free DPPH radicals. Regarding the scavenging activity of aqueous extract, it was 9.6 and 9.8% for *T. decurrens* and *T. ornata*, respectively. When comparing these values with scavenging activity of ascorbic acid (antioxidant often used as a reference compound in this assay), we obtained the equiv. of 316 and 325 mg/l of extract, referring to 38 and 39 mg/g of dry weight of original algae, respectively. The lower scavenging activity was determined for the aqueous-MeOH extracts of macroalgae species. When re-calculating this scavenging activity (*i.e.*, 1.7 and 1.4% for *T. decurrens* and *T. ornata*, respectively) for the activity of the ascorbic acid, we obtained 7 and 5.6 mg/g of algae, respectively. Taking into account the successive extraction, the polar and semi-polar antioxidants were probably extracted during the first extraction step, *i.e.*, extraction with H₂O.

Surprisingly, the highest scavenging activity was observed for the non-polar hexane/*i*-PrOH extract, *i.e.*, 43 and 47% for *T. decurrens* and *T. ornata*, respectively. When expressing these values as the ascorbic acid equiv., we obtained 172 and 187 mg/g of algae, respectively. Unfortunately, in spite of a high potential of U-HPLC/HR-MS/MS instrumentation (described below) which was used for screening of compounds present in the respective extracts, we were not able to unambiguously determine the compounds responsible for this bioactivity. The reasons could be either the ionization suppression caused by other co-eluting compounds, or too low (non-detectable) concentrations of antioxidants, which probably act in a mixture as synergists to develop such strong scavenging effect.

Additionally to the DPPH method, we also screened the antioxidant capacity based on the overall polyphenols and flavonoids content. As shown in Fig. 4, similar results as in the case of DPPH method were obtained. The highest amount of both flavonoids, as well as overall polyphenols, expressed as the equiv. of quercetin and phloroglucinol, was found in the less polar hexane/*i*-PrOH extract.

U-HPLC/HR-MS/MS Non-target Screening and Tentative Compounds Identification

With the aim to characterize the chemical composition of the brown algae, all extracts differing in the polarity, thus extraction ability, were injected into the U-HPLC/HR-MS/MS system, and the non-target screening and tentative identification of major low-molecular sample components (up to 1200 Da) was performed (Table 1). The laborious and time demanding data minding was realized by searching the chromatograms and identification of the most intensive ions in the mass spectra. The accurate mass, conformity of theoretical and experimental isotopic profile, as well as presence of the fragment ions in MS/MS spectra were assessed as the criteria for tentative compounds identification (see the example for fucoxanthin shown in Fig. 5). The true is that for the unambiguous identification, pure analytical standards would be necessary, to verify the retention times and the conformity of MS/MS spectra. Because the standards were not available, the identity of presented compounds was validated by their occurrence in similar matrices published in relevant scientific literature.

Unfortunately, it should be noticed that in spite of the high effort devoted to this task, a lot of ions acquired still remained unidentified, because of extreme complexity of algae matrices, and limited

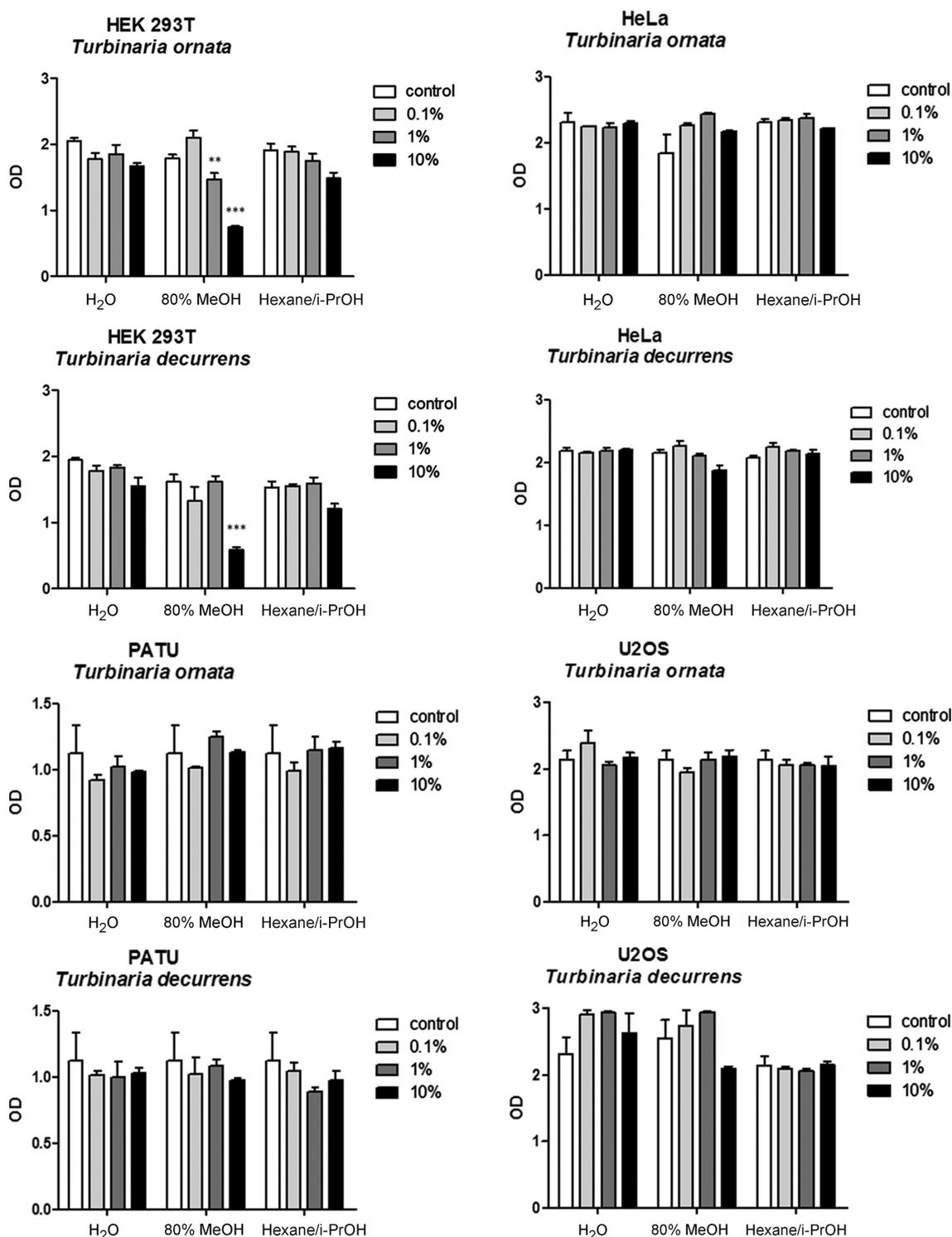


Figure 1. The antiproliferative effects of extracts. OD – optical density. White columns – negative control, gray and black columns – represent extracts added at indicated concentrations. The results represent data from triplicate measurements. Statistical analysis of the results is based on Fisher's exact test and was performed using GrahPadPrism5 software. *P* values, * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001, were considered statistically significant.

information contained in present compounds databases.

In general, both environmental contaminants, and natural biologically active compounds were identified

in the samples. As regards the contaminants, the surface active agents with polar head and non-polar chain as alkylamio oxides, alkylbenzenesulfonates, alkylsulfates, and betaine derivatives were identified.

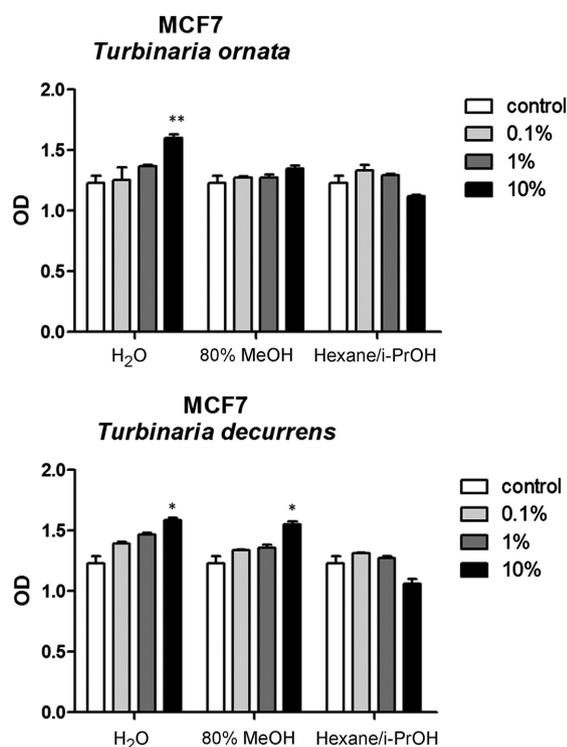


Figure 1. (cont.)

These compounds are widely used in laundries, cosmetics, and industrial application, unintentionally polluting the aquatic environment. In particular, dodecyl (dimethyl)amine oxide, dodecyl hydrogen sulfate, dodecylbenzenesulfonic acid, tridecylbenzenesulfonic acid, (oxyethylene)myristoyl sulfate, di(oxyethylene)lauryl sulfate, tri(oxyethylene)lauryl sulfate, dihydroxy-octadecyl hydrogen sulfate, dodecyl hydrogen sulfate, 2-dodecylethyl hydrogen sulfate, tridecyl hydrogen sulfate, sodium tetradecyl sulfate and 3,6,9,12-tetraoxatetracos-1-yl hydrogen sulfate, or cocamidopropyl betaine were confirmed, mainly in the MeOH and hexane/i-PrOH extracts. Among others environmental contaminants, chloralkylphosphates and alkyl phthalates (bis(2-ethylhexyl) phthalate and dibutyl phthalate) used as industrial plasticizers, and pesticide tetradecadienyl acetate were characterized. Also these compounds were identified in the semi-polar and non-polar extracts. Intensities of ions of all of these contaminants were very similar between the analyzed samples, giving the evidence that the samples were collected in one geographical area burdened by industry.

As concerns the natural compounds originating from the biomass, we detected a large number of relatively common organic compounds like free fatty acids, monosaccharides, sugar alcohols, purine/pyrimidine bases or amino acids. Nevertheless, only the

potentially interesting chemicals found in the algae samples are mentioned in paragraphs below.

The major compound found in polar H₂O extract was ectoin with systematic name 1,4,5,6-tetrahydro-2-methylpyrimidine-4-carboxylic acid. Ectoin is a natural chemical substance serving as a protective agent by acting as an osmolyte, thus helping the organisms to survive extreme osmotic and temperature stress. Usually it is found in higher concentrations in halophilic microorganisms, where it confers the resistance towards salts. This compound was also described as an effective protectant against UVA-induced premature photoaging.^[16] Primarily, it was identified in the *Ectothiorhodospira halochloris*, but since that it had been found in a wide range of *Gram*-negative and *Gram*-positive bacteria. In algae, its presence has not been published yet.

All of the samples were also positive on fucoxanthin, which was found in both semi-polar, and non-polar extracts. It is a xanthophyll compound found as an accessory pigment in the chloroplasts of brown algae, giving them a brown or olive-green color. The all-*trans*-fucoxanthin exhibits also the strong antioxidant properties.^{[17][18]}

In the non-polar *T. decurrens* extract, sesquiterpene tanacetol A, previously identified in higher plants of *T. vulgare*^[19] was tentatively identified. However, because of rather low intensity of the primary ion, thus lack of fragment ions in the MS/MS spectra, identity of this compound could not be confirmed, and, according to the literature, two other proposals for the compound identity exist. The first alternative is isochroman pseudoanguillosporin B, showing the strong antimicrobial activity.^[20] The second option is embelin, undecylcyclohexadiene substituted by hydroxy and keto groups, showing *in vivo* antitumor, anti-inflammatory^[21] and antioxidant activity.^{[22][23]} In the same algae strain, bengazole C with antitumor, antibiotic and anthelmintic properties^[24] was found in the semi-polar extract. It is worth to notice that in samples of *T. ornata*, the presence of these compounds was not confirmed.

U-HPLC/HR-MS/MS Lipidomic Profiling

Composition of lipids present in macroalgae samples was assessed in semi-polar and non-polar sample extracts analyzed on the reversed-phase U-HPLC coupled with HR-MS/MS. Contrary to the gas chromatography-based methods, the direct analysis of lipid species in their native forms is possible by this liquid chromatography approach (when analyzing by gas chromatography, lipids have to be firstly

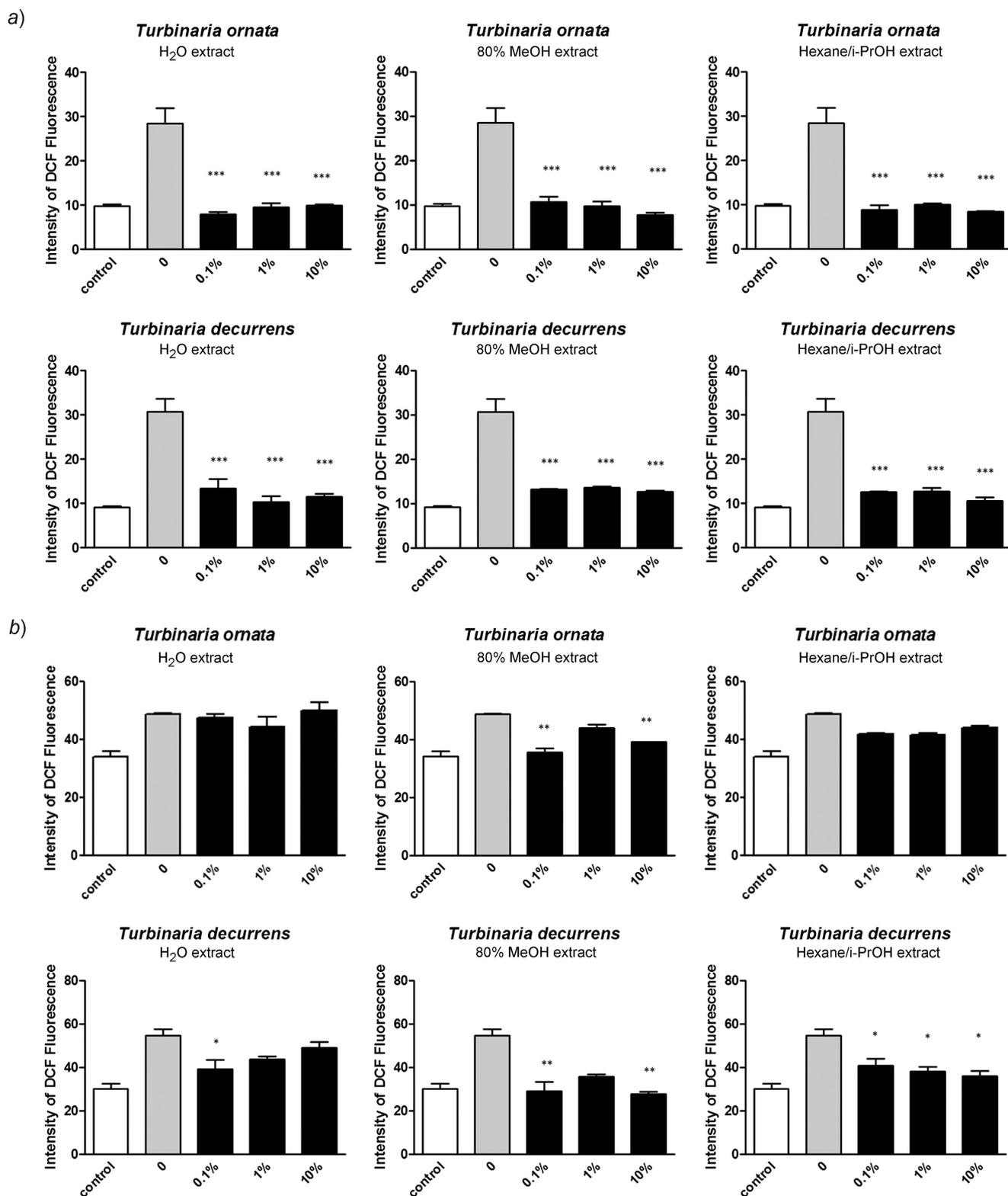


Figure 2. Effect of algae extracts on intracellular ROS concentration a) HEK 293T cells; b) HeLa cells. Fluorescence corresponds to the concentration of ROS generated by using H₂O₂. The results represent data from triplicate measurements * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. White column – negative control in the absence of H₂O₂, gray and black columns – represent extracts in indicated concentrations in the presence of 100 μM H₂O₂. ROS – reactive oxygen species, TO – *Turbinaria ornata*, TD – *Turbinaria decurrens*, DCF – 7'-dichlorodihydrofluorescein diacetate.

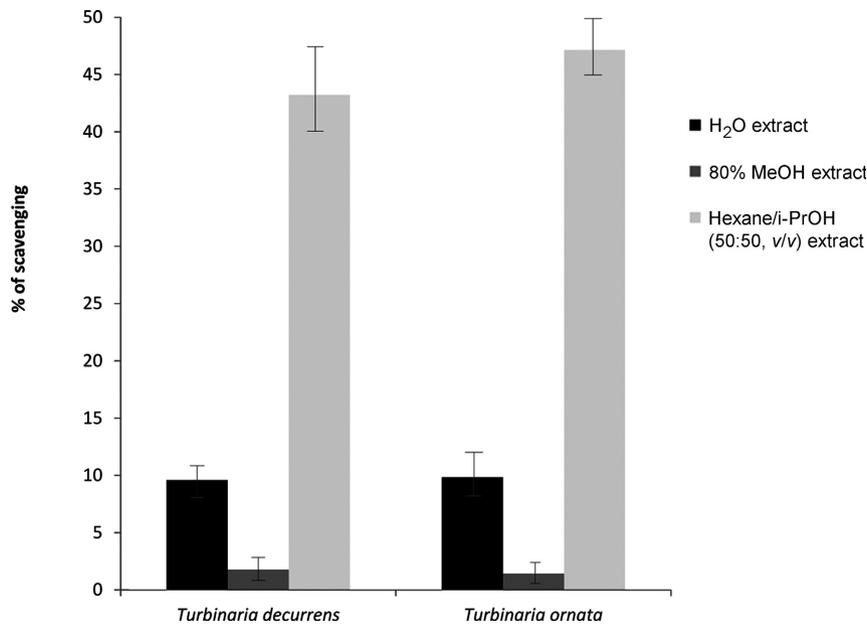


Figure 3. Antioxidant activity of algae extracts (equiv. of 8.33 mg/kg of the original algae biomass) measured by the DPPH method. The error bars indicate the standard deviation between the results obtained from triplicate analysis.

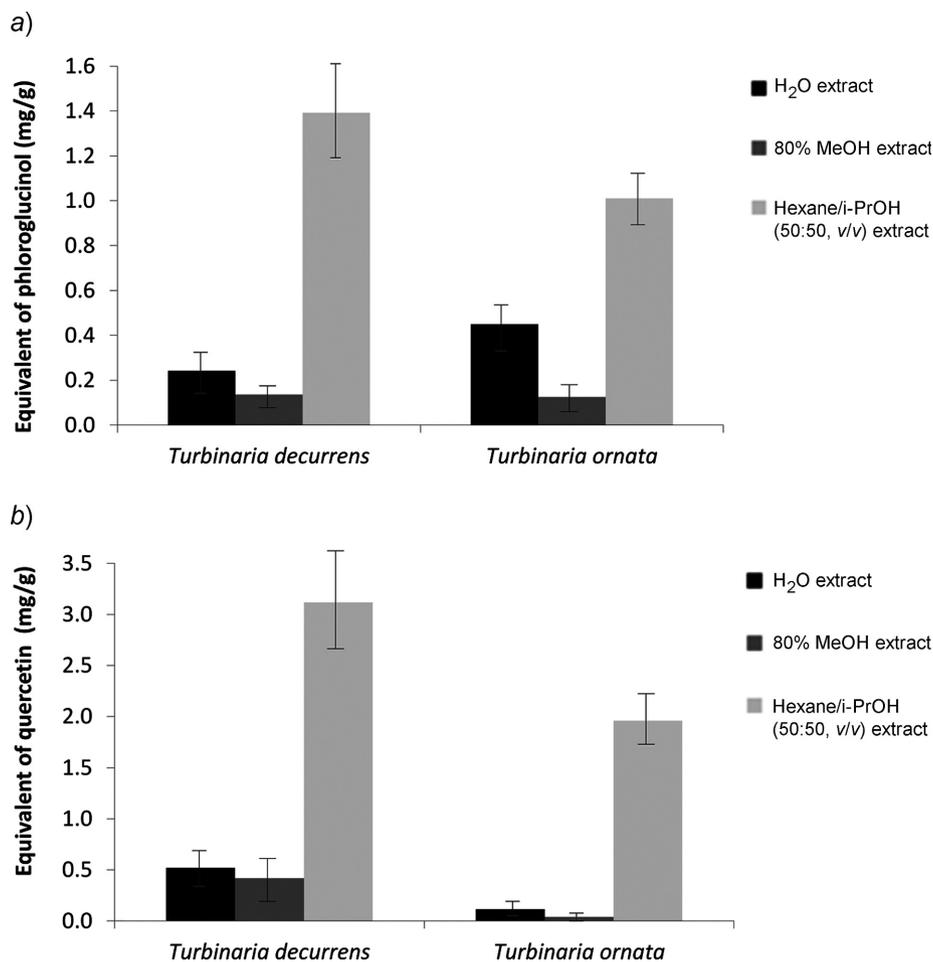


Figure 4. Total a) polyphenols and b) flavonoids content in the brown algae extracts determined as equiv. of phloroglucinol/quercetin in mg/kg; determined in the extract with the equiv. of 8.33 mg/kg of the original algae biomass. The error bars indicate the standard deviation between the results obtained from triplicate analysis.

Table 1. Compounds identified in the algae extracts during the non-target screening

	Summary formula of the neutral compound	Exact mass of the ion (m/z)/ionization mode	Measured mass of the ion (m/z)/mass detection error [ppm]	Intensity of the ion	t _R [min]	Compliance of the isotopic profile	Presence of fragment ions in the MS/MS spectra	Compound identified
H ₂ O extract								
<i>Turbinaria decurrens</i>	C ₆ H ₁₀ N ₂ O ₂	143.0815/ESI ⁺	143.0811/2.8	1.66E+05	1.86	Yes	Yes	Ectoine
<i>Turbinaria ornata</i>	C ₆ H ₁₀ N ₂ O ₂	143.0815/ESI ⁺	143.0816/-0.7	1.70E+05	1.85	Yes	Yes	Ectoine
80% MeOH Extract								
<i>T. decurrens</i>	C ₄₂ H ₅₈ O ₆	659.4306/ESI ⁺	659.4287/2.9	1.82E+05	8.08	Yes	Yes	Fucoxanthin
<i>T. ornata</i>	C ₄₂ H ₅₈ O ₆	659.4306/ESI ⁺	659.4291/2.3	1.68E+05	8.09	Yes	Yes	Fucoxanthin
<i>T. decurrens</i>	C ₂₆ H ₄₂ N ₂ O ₈	509.2868/ESI ⁻	509.2868/0.1	1.20E+05	6.55	Yes	Yes	Benzazole C
<i>T. ornata</i>	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Hexane/i-PrOH (50:50, v/v) extract								
<i>T. decurrens</i>	C ₄₂ H ₅₈ O ₆	659.4306/ESI ⁺	659.4286/3.1	3.10E+05	8.10	Yes	Yes	Fucoxanthin
<i>T. ornata</i>	C ₄₂ H ₅₈ O ₆	659.4306/ESI ⁺	659.4293/2.0	5.90E+05	8.09	Yes	Yes	Fucoxanthin
<i>T. decurrens</i>	C ₁₇ H ₂₆ O ₄	293.1758/ESI ⁻	293.1756/0.8	3.12E+04	7.27	Yes	No	Tanacetol A ^a
<i>T. ornata</i>	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
<i>T. decurrens</i>	C ₁₇ H ₂₆ O ₄	293.1758/ESI ⁻	293.1756/0.8	3.12E+04	7.27	Yes	No	Pseudoanguillosporin B ^a
<i>T. ornata</i>	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
<i>T. decurrens</i>	C ₁₇ H ₂₆ O ₄	293.1758/ESI ⁻	293.1756/0.8	3.12E+04	7.27	Yes	No	Embelin ^a
<i>T. ornata</i>	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

^a Compounds of the same summary formula (i.e., of the same mass); fragment ions were not identified because of low intensity of primary ion, thus more precise determination of compound's identity was not possible.

hydrolyzed to release the fatty acids, which are to be further derivatized and then analyzed). It should be noticed that the U-HPLC/HR-MS/MS method employed in this study does not allow the direct quantification of lipids because of absence of suitable respective analytical standards, but the profiling and between-samples comparison is easily possible. This can be very promising for selection of algae strains with interesting lipid profile.

Altogether, the LipidView software automatically identified more than 1000 lipid species in the investigated macroalgae samples. As can be seen from the Table 2, the vast majority of identified lipids belonged to the triacylglycerol (TG) class (790 hits in average). The second mostly frequented lipid class were ceramides (CER) followed by sphingomyelins (SM)^[14] and diacylglycerols (DG) with the average number of species 103, 86, and 84, respectively. From the group of phospholipids, also lysophosphatidylcholines, phosphatidylcholines, and lysophosphatidylglycerols were quite frequent with 18, 8, and 10 identified species. The remaining less frequent lipids belonged to monoacylglycerols, phosphatidylethanolamines, lysophosphatidylserines, phosphatidylserines, phosphatidylglycerols and lysophosphatidylethanolamines.

As depicted in Fig. 6 demonstrating the mutual between-samples comparison of present lipid classes, higher amount of TG was determined in *T. ornata* sample (TG contribution in *T. decurrens* was ca. 70%, when the response in *T. ornata* is considered as 100%). Higher responses of lipids in *T. ornata* were observed also for DG and sphingomyelins, but the difference from the *T. decurrens* was not such significant. Both these lipid classes dominated in *T. ornata* and their contribution in *T. decurrens* was 89 and 93%, respectively. As concerns CER, phospholipids, and lysophospholipids, the trend was the opposite; higher amount was found in *T. decurrens*, whereas in *T. ornata*, only 32, 20, and 79%, respectively, was assessed.

Within the assessment of overall lipidomic profile of the macroalgae, also composition of fatty acids which are bound in lipid molecules is very important (mainly with respect to their possible use as food or feed supplements). It is worth to notice that the profiles of particular fatty acids between the samples differed rather significantly. As shown in Table 3, the most frequent fatty acids represented in lipid molecules were C14:0, C14:1, C16:0, C16:1, C18:1, and C18:2 for both algae species. Interestingly, also the fatty acids with the odd number of carbons occurred in the

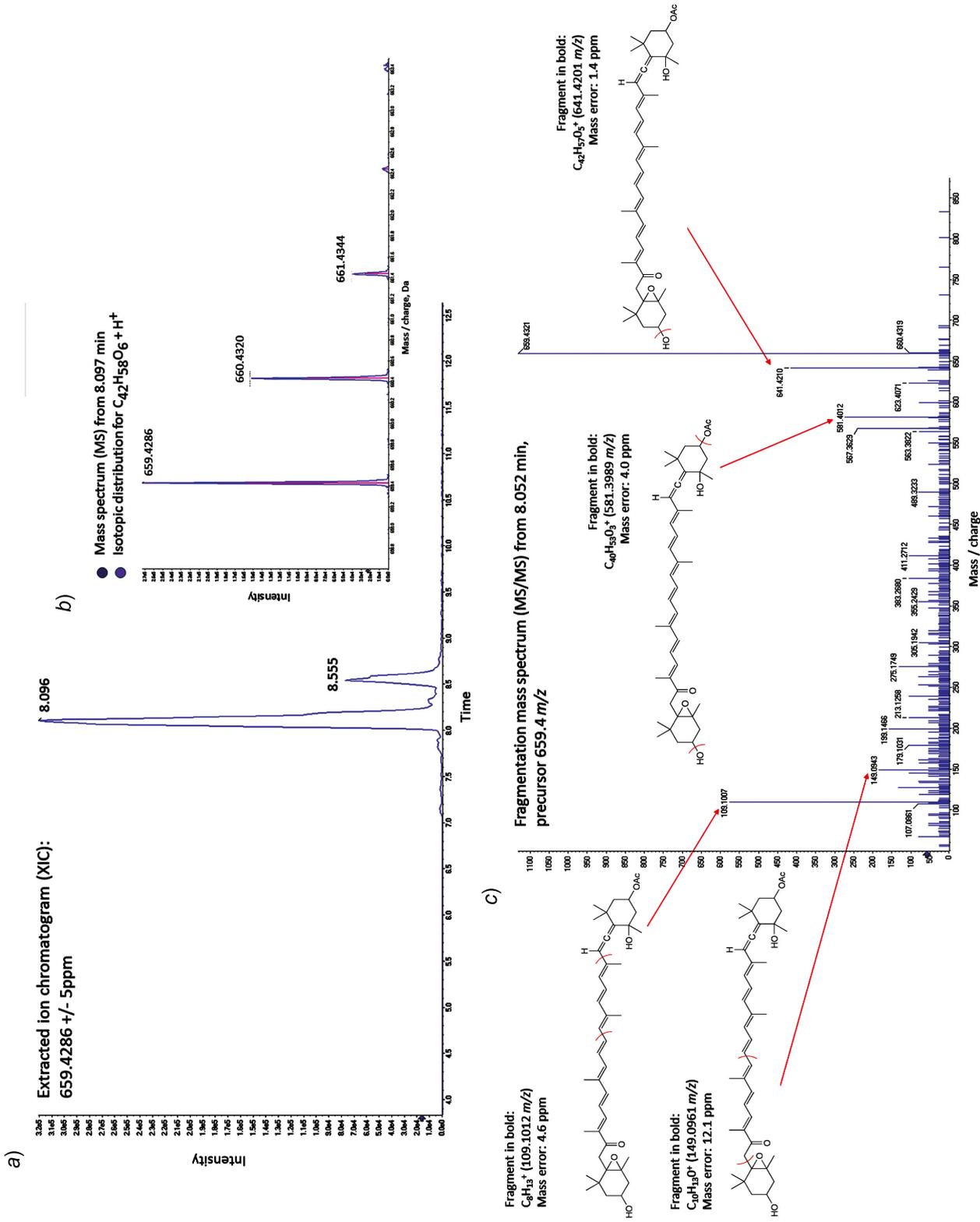


Figure 5. Example of tentative identification of fucoxanthin (8.096 min) in hexane/*i*-PrOH (50:50, v/v) extract of *Turbinaria decurrens* algae; **a)** Extracted ion chromatogram of m/z 659.4306 \pm 5 ppm; **b)** Mass isotopic profile of fucoxanthin in 8.097 min, and its compliance with the theoretical one; fragmentation mass spectrum in 8.052 min, precursor m/z 659.4. Note: the identity of peak in 8.555 min was not revealed, fragmentation spectra were not acquired because of low intensity of the primary ion.

Table 2. Number of lipids species identified in the particular extracts of different polarities

Lipid class	<i>Turbinaria decurrens</i>			<i>Turbinaria ornata</i>			Arithmetical mean of number of lipid species identified in both extracts
	Lipid species identified in extract 2	Lipid species identified in extract 3	Lipid species identified in both extracts	Lipid species identified in extract 2	Lipid species identified in extract 3	Lipid species identified in both extracts	
TG	535	487	725	568	547	855	790
CER	56	57	92	69	75	113	103
SM	61	34	84	57	44	87	86
DG	37	54	78	49	62	90	84
LPC	12	7	16	18	5	20	18
PS	4	6	7	6	6	9	8
LPG	10	1	11	7	4	9	10
PG	5	2	7	5	1	6	6.5
MG	4	6	10	6	4	8	9
PE	5	4	9	4	3	6	7.5
LPS	6	5	7	5	6	6	6.5
PC	10	7	14	8	11	16	15
LPE	4	1	5	1	0	1	3

TG, triacylglycerol; CER, ceramides; SM, sphingomyelins; DG, diacylglycerols; LPC, lysophosphatidylcholines; PC, phosphatidylcholines; LPG, lysophosphatidylglycerols; MG, monoacylglycerols; PE, phosphatidylethanolamines; LPS, lysophosphatidylserines; PS, phosphatidylserines; PG, phosphatidylglycerols; LPE, lysophosphatidylethanolamines.

samples, mainly the C15:0, C15:1, C17:0 and C17:1 (in spite of the fact that their presence is rather unusual for mammals, those are quite common for microorganisms because of different biochemical pathways of lipids biochemical genesis).^{[25][26]}

Conclusions

The research described in this article was focused on bio-prospecting of *Turbinaria* macroalgae sampled on the Indonesian coast. The investigation of biological activity was combined with analyses of chemical composition. The main results obtained are summarized in following paragraphs:

- The results of the cell based *in vitro* tests showed significant differences between the protection against oxidative stress (ROS formation) of the cancer and non-cancer cell lines, the latter were more protected. Both *Turbinaria* species tested in this study well protected the non-cancer cells against the ROS.
- No significant antiproliferative effect was observed for the cancer cell lines. On the contrary, slight induction of proliferation was observed in some cases.
- As regards the biochemical tests characterizing the antioxidant capacity of the algae extracts, the highest amount of antioxidants was determined for the non-polar hexane/*i*-PrOH one. This less polar

extract was also the richest in terms of antioxidative compounds, at least fucoxanthin, embelin, and non-specified flavonoids and polyphenols.

- Majority of lipids identified in *Turbinaria* algae belonged to the TGs followed by sphingomyelins, DG, and polar (lyso)phospholipids. Additionally to the relatively common fatty acids with 14, 16 and 18 carbons, also the fatty acids with odd carbons number, with so far not fully characterized biological functions, were identified.

Experimental Section

Samples

Two types of brown algae were selected for the analysis: *T. ornata* and *T. decurrens*. The samples were collected in September 2013 from Pameungpeug Beach, Garut, West Java. Immediately after collection, they were washed in running fresh H₂O to remove salt and other adhering matters, and were freeze-dried. The species identification was done by the Research Centre of Oceanography – Indonesian Institute of Sciences. Details about the species description and taxonomical classification are summarized in Table 4.

Other Materials and Chemicals

The deionized H₂O (18 MΩ) was produced by a Milli-Q system (Millipore; Bedford, MA, USA). MeOH (HPLC

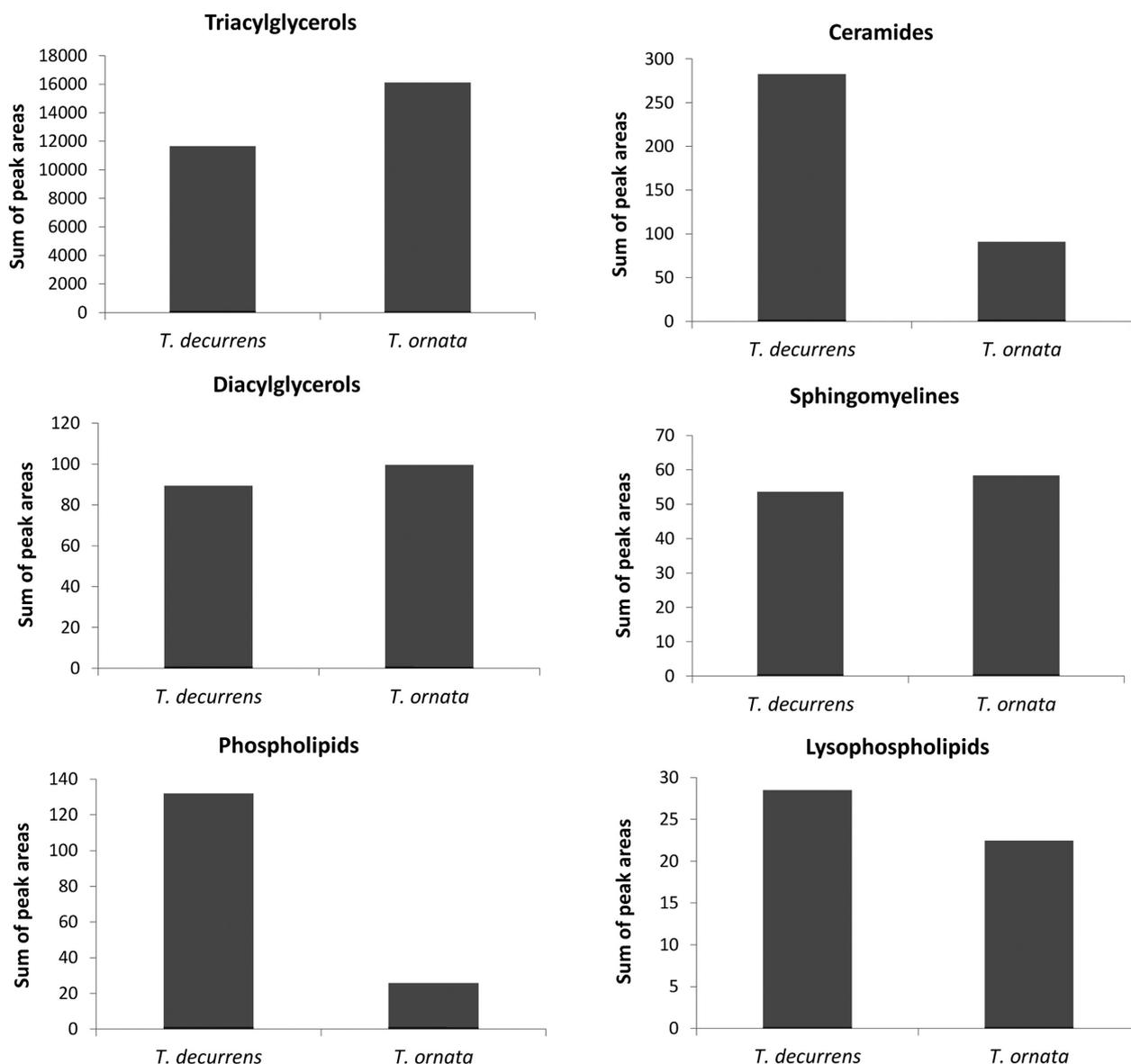


Figure 6. The between-samples comparison of present lipid classes; the summary areas of all peaks belonging to the same class are considered.

grade), EtOH (96%), hexane (HPLC grade), i-PrOH (HPLC grade), CH_2Cl_2 (HPLC grade), and CHCl_3 (HPLC grade) were obtained from Merck (Darmstadt, Germany). DMSO, ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl, Folin–Ciocalteu's phenol reagent, AlCl_3 , Na_2CO_3 , phloroglucinol, potassium phosphate dibasic trihydrate β -nicotinamide adenine dinucleotide, nitrotetrazolium blue chloride, phenazine methosulfate, quercetin, L-tyrosine, tyrosinase, α -glucosidase, and 4-nitrophenyl- α -D-glucopyranoside were obtained from Sigma–Aldrich (USA). NaOH was provided by Lach-Ner (Czech Republic) and HCl by Penta (Czech Republic). Glass beads (glass micro beads – ballotina TF8, i.d. 400 – 600 μm) for sample disintegration were purchased from Ginzler Ltd. (Czech Republic).

Sample Preparation

Firstly, the freeze-dried algae samples were homogenized under liquid N_2 to get the consistent powder. Then the subsequent extraction of algae biomass was performed by using of solvents with different polarities (according to the procedure of Stranska-Zacharišova *et al.*)^[27] to get extracts containing large scope of compounds of different physico-chemical properties. Firstly, to 500 mg of freeze-dried algae, the ballotina (inert glass beads used for supporting the mechanical distortion of algae cells) and 0.5 ml of hot H_2O was added. The mixture was vortexed for 3 min, and then, the rest of the extraction H_2O (5.5 ml) was

Table 3. Composition of fatty acids bound in triacylglycerols

Fatty acid	<i>Turbinaria decurrens</i>	<i>Turbinaria ornata</i>
16:01	4442	2708
18:02	1813	2158
16:00	1304	2249
18:01	1204	2079
14:01	627	824
14:00	506	2000
15:00	355	916
18:00	272	265
18:03	216	345
17:00	193	162
17:01	181	284
19:00	123	12
15:01	83	488
20:00	70	44
13:00	44	541
12:00	41	785
20:01	33	20
17:03	30	31
22:01	30	26
14:02	18	7
20:02	13	19
16:02	11	10
17:02	11	15
20:04	10	11
19:03	9	18
20:03	9	8
19:01	8	10
15:02	2	33

pipetted. The suspension was vortexed again for another 2 min. Then the mixture was centrifuged (5 min, 10,000 *g*), and the whole aqueous supernatant was transferred into a container, and stored in $-18\text{ }^{\circ}\text{C}$ before the follow-up U-HPLC/HR-MS/MS and bioactivity analyses.

Then the algae biomass residue remaining after the extraction with H_2O was repeatedly extracted with

6 ml of 80% MeOH (to ca. 0.25 ml of H_2O remaining in the algae biomass, 1 ml of additional H_2O and 4.75 ml of MeOH were added). The mixture was vortexed for 2 min, centrifuged, and the supernatant was transferred into a container and stored at $-18\text{ }^{\circ}\text{C}$ before the follow-up UHPLC/HR-MS/MS and bioactivity analyses.

Further, the algae biomass residue resting after the 80% MeOH extraction was repeatedly extracted with 6 ml of hexane/*i*-PrOH (50:50, v/v). Suspension was vortexed again for 2 min, centrifuged, and the supernatant was transferred into a container and stored at $-18\text{ }^{\circ}\text{C}$ before the follow-up U-HPLC/HR-MS/MS analyses. For the testing of biological activity of this non-polar hexane/*i*-PrOH extract, the solvent was removed by the gentle stream of N_2 , and the residuum was re-dissolved in 10% DMSO solution, to assure the better compatibility with polar solvents, and inorganic salts used in the particular assay.

Biological Activity Determined by in vitro Models on Human Cells

HEK 293T (human embryonic kidney 293 cells – non-cancerous cells), HeLa (human cervix adenocarcinoma), PaTu (human pancreatic adenocarcinoma), U2OS (human osteosarcoma) and MCF7 (human breast adenocarcinoma) cell lines were maintained and grown in a humidified atmosphere containing 5% CO_2 at $37\text{ }^{\circ}\text{C}$ in DMEM (*Dulbecco's Modified Eagle's Medium*, Sigma, pH = 7.4) supplemented with 10% fetal bovine serum.

Cell Proliferation Assay. HeLa, HEK 293T, MCF7, PaTu and U2Os cells were plated into 96 wells in concentration of 2000 cells per well, and extracts were added 24 h after cell seeding. Three extracts (H_2O ,

Table 4. Description of the brown algae samples analyzed

Sample	Species description	Taxonomical classification:
Sample 1: <i>Turbinaria decurrens</i>	Brown algae with stiff and erect thallus, 2 – 30 cm tall, with yellowish brown to dark brown color. Leaves characteristically triangular, often with a concave surface.	Division: <i>Phaeophyta</i> Class: <i>Phaeophyceae</i> Order: <i>Fucales</i> Family: <i>Sargassaceae</i> Genus: <i>Turbinaria</i> Species: <i>Turbinaria decurrens</i>
Sample 2: <i>Turbinaria ornata</i>	Brown algae with stiff and erect thallus, 2 – 30 cm tall with yellowish brown to dark brown color. Blades are conical, hard and thick with stiff spines around the margin of the blade.	Division: <i>Phaeophyta</i> Class: <i>Phaeophyceae</i> Order: <i>Fucales</i> Family: <i>Sargassaceae</i> Genus: <i>Turbinaria</i> Species: <i>Turbinaria ornata</i>

80% aqueous MeOH, and hexane/*i*-PrOH (50:50, *v/v*) from two the *algae* species (*T. ornata*, *T. decurrens*) were prepared and tested at the concentration of 0.1, 1 and 10% (corresponding to 0.0833, 0.833 and 8.33 mg/ml) for 48 h. All the samples were analyzed in triplicates. The amount of the cells was determined by *WST-1* colorimetric assay (cell proliferation reagent, Roche) according to the manufacturers' instruction, measured with the plate reader (*xMark™*, BioRad) at 490 and 650 nm. Data were evaluated by using the GraphPad Prism software.

Intracellular ROS Assay. HEK 293T and HeLa cells were seeded into 96 wells in the concentration of 5000 cells per well. After 24 h, the cells were exposed to all three extracts (H₂O, 80% aqueous MeOH, and hexane/*i*-PrOH (50:50, *v/v*) from two *algae* species at the concentrations of 0.1, 1; 10% (corresponding to 0.0833, 0.833 and 8.33 mg/ml) together with 100 μM H₂O₂, that was used as activator of oxidative stress. Cells without the H₂O₂ treatment are reported as a negative control. After the exposure to H₂O₂ (alone (positive control) or in combination with the tested extracts) for 60 min, the media were removed and the cells were incubated 15 min at 37 °C with 10 μM DCF-DA (7'-dichlorodihydrofluorescein diacetate) in PBS. The results obtained by fluorimetric measurement using the microplate fluorescence reader (*Fluoroskan Ascent FI*, Thermo Labsystem), excitation at 488 nm, emission at 530 nm are reported as intensity of DCF fluorescence. All the samples were analyzed in triplicates. Differences were considered statistically significant when *P* values were less than 0.05.

Biological Activity Determined by Screening by the Biochemical Tests

DPPH Method. The DPPH antiradical activity was determined spectrophotometrically in a 96 plate reader (*Epoch Microplate Spectrophotometer*, BioTech), by monitoring the decrease of DPPH' concentration at 517 nm, according to a described procedure with some modifications.^[28] The mixtures in the sample wells consisted of 100 μl of sample extract (*i.e.*, 8.33 mg of the original matrix per 1 ml of solvent) and 200 μl of 132 μM DPPH' dissolved in MeOH. The plate was incubated for 15 min in the dark after the addition of DPPH'. Experiments were performed in triplicates. The absorbance was read at 517 nm, and scavenging activity was calculated according to eqn (1), where 'positive control' is the DPPH' solution with highest absorbance, 'sample' is DPPH' with the sample extract, and 'sample blank' is the sample

extract with MeOH. Ascorbic acid was used as standard control. The standard curve was prepared by 0, 2.5, 5, 10, 15, and 20 mg/l solutions of ascorbic acid in H₂O, 80% MeOH and 10% DMSO solvent (specific solvents in which sample extracts are prepared).

$$\% \text{ of scavenging} = A_{\text{positive control}} - \frac{(A_{\text{sample}} - A_{\text{sample blank}})}{A_{\text{positive control}}} \times 100. \quad (1)$$

Total Polyphenols Content. To determine the total polyphenol content in the *algae* extracts, the modified method with *Folin–Ciocalteu* reagent was used. To the sample wells were added 165 μl of distilled H₂O/*Folin–Ciocalteu* solution (9:1, *v/v*) and 15 μl of sample extract. After 3 min, 60 μl of 2M Na₂CO₃ and 80 μl of distilled H₂O were added. After 1 h standing at laboratory temp., absorbance was measured at 725 nm. The total polyphenol content was calculated as a phloroglucinol equiv. from the calibration curve of phloroglucinol standard solutions (concentrations: 0, 2, 10, 50, 100, 250, 500, and 1000) in H₂O, 80% MeOH and 10% DMSO solvent (specific solvents in which sample extracts are prepared). All measurements were conducted in triplicates.

Total Flavonoids Content. Colorimetric AlCl₃ method was used for flavonoid determination.^[29] To the sample wells were added 50 μl of sample extract, 10 μl of 10% AlCl₃, 10 μl of 1M AcOK, and 240 μl of distilled H₂O, and left at r.t. for 30 min. The absorbance of the reaction mixture was measured at 415 nm. Total flavonoid contents were determined as quercetin concentrations assessed from a calibration curve. The calibration curve was prepared by colorimetric measurements of quercetin solutions at concentrations 0, 2.5, 5, 10, 15, 25, and 50 mg/l in H₂O, 80% MeOH and 10% DMSO solvent (specific solvents in which sample extracts were prepared).

U-HPLC/HR-MS/MS Analysis

Ultra-high performance liquid chromatography coupled with a high resolution tandem mass spectrometry (U-HPLC/HR-MS/MS) was used for the non-target screening of *algae* extracts.

The chromatographic separation was performed by *Dionex UltiMate 3000 RS UHPLC* system (*Thermo Scientific*, Waltham, USA). For analysis of semi-polar (80% aqueous MeOH) and non-polar (hexane/*i*-PrOH) extracts, the reversed-phase chromatography was

used. The *Acquity UPLC*[®] *BEH-C18* analytical column (100 × 2.1 mm, 1.7 μm i.d.; Waters, Milford, MA, USA) was held at 60 °C was used for separation of sample components. As the mobile phases, H₂O/MeOH (95:5, v/v), 5 mM HCOONH₄, 0.1% HCOOH in H₂O (A) and i-PrOH/MeOH/H₂O (60:30:5, v/v/v), 0.1% HCOOH (B) were used. The gradient was as follows: start with 10% B, linear increase to 50% B in 4 min, for next 2 min another linear increase to 100% B, keeping up to 11 min, switching to 10% B in 11.1 min, and column equilibration for 3 min before the next injection start. Samples injection volume was 5 μl, the flow rate was 300 μl min⁻¹. As a HILIC-phase system for separation of polar extracts, *Atlantis*[®] *HILIC* silica analytical column (100 × 2.1 mm, 3 μm i.d.; Waters, Milford, MS, USA) held at 40 °C was used for separation of sample components. As mobile phases, 50 mM HCOONH₄, 0.2% HCOOH in H₂O (A) and MeCN (B) were used. The gradient was as follows: start with 20% A, linear increase to 40% A in 6 min, keeping up to 8 min, switching to 20% A in 8.1 min, and column equilibration for 4 min before the next injection start. Injection volume was 5 μl, the flow rate was 300 μl min⁻¹.

As the mass spectrometric detection system, *TripleTOF*[®] 5600 quadrupole–time-of-flight mass spectrometer (SCIEX, Concord, ON, Canada) was used. The ion source was a *Duo Spray*[™] with combined electrospray (ESI) and atmospheric-pressure chemical ionization (APCI). While the APCI was used for exact mass calibration of the *TripleTOF* instrument, the ESI was employed for the sample extract analysis. In the positive ESI mode the source parameters, metabolic fingerprinting, were as follows: capillary voltage: +4500 V; nebulizing gas pressure: 60 psi; drying gas pressure: 50 psi; temp.: 550 °C; and declustering potential: 80 V. The capillary voltage in negative ESI was –4000 V, other source settings were the same.

For the data collection, the 'information dependent acquisition'^[12] was used to record the MS and MS/MS spectra. Within the MS experiments, ions of *m/z* 100 – 1200 were acquired. For the acquisition of MS/MS spectra, product ions ranging from *m/z* 50 to 1200 originating after fragmentation of precursor ions selected by the quadrupole with extraction window of 1 Da, were monitored. The collision energy applied was 35 V and collision energy spread was ±15 V. The achieved resolving power was > 31,000 (*m/z* 321.0192) full width at half maximum in both polarities. The PI spectra were measured in a high sensitivity mode, which provides half resolving power.

The data acquisition was carried out with the *Analyst 1.6 TF* software (SCIEX), and the qualitative analysis was performed using *PeakView 2.0* (SCIEX)

equipped with the *MasterView*, *Formula Finder* (SCIEX) and directly linked to *ChemSpider* database. For the evaluation of lipids, the specialized software *LipidView* (SCIEX) was employed.

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