Advanced polysaccharides extraction techniques of Adriatic Sea algae

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DOCTORAL DISSERTATION

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Prehrambeno – biotehnološki fakultet

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Napredni postupci izolacije polisaharida iz algi Jadranskoga mora

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Mentor:

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Advanced polysaccharides extraction techniques of Adriatic Sea algae

Ana Dobrinčić, 348/PT

Short abstract: Fucoidans, sulfated polysaccharides from the brown algae *Fucus virsoides* and *Cystoseira barbata*, show a wide range of biological activities (antioxidant, antibacterial, and antifungal) which depend on their chemical structure (sulfate group, fucose and uronic acid content, monosaccharide composition, molecular weight) and are related to the algal species, extraction technique and parameters, as well as pre-treatment used to remove interfering compounds. In addition to a higher yield compared to the conventional technique, the application of advanced extraction techniques (microwave-assisted extraction - MAE and pressurised liquid extraction - PLE) significantly reduced the extraction time.

Keywords: *polysaccharides, fucoidan, algae, Fucus virsoides, Cystoseira barbata, advanced extraction techniques*

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Napredni postupci izolacije polisaharida iz algi Jadranskoga mora

Ana Dobrinčić, 348/PT

Sažetak: Fukoidani, sulfatirani polisaharidi iz smeđih algi *Fucus virsoides* i *Cystoseira barbata* pokazuju široki raspon bioloških aktivnosti (antioksidacijsko, antibakterijsko i antifungalno djelovanje) koje ovise o njihovoj kemijskoj strukturi (udio sulfatnih grupa, fukoze, uronskih kiselina, sastav monosaharida, molekulska masa) i povezani su s vrstom alge, tehnikom i parametrima ekstrakcije te korištenim pred-tretmanom za uklanjanje interferirajućih spojeva. Uz veće prinose u odnosu na konvencionalnu tehniku, primjenom naprednih tehnika ekstrakcije (ekstrakcija potpomognuta mikrovalovima – MAE i ubrzana ekstrakcija otapalima pri povišenom tlaku – PLE) vrijeme ekstrakcije je značajno skraćeno.

Ključne riječi: *polisaharidi, fucoidan, alge, Fucus virsoides, Cystoseira barbata, napredne tehnike ekstrakcije*
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The dissertation topic was accepted at the 1st regular session of the Faculty Council of the Faculty of Food Technology and Biotechnology, the University of Zagreb in the academic year 2019/2020 held on October 29th 2019., and the University of Zagreb Senate approved the initiation of the procedure for obtaining a doctorate of science within the doctoral study on April 28th, 2020 at the 8th regular session in the 351st academic year (2019/2020).

Extended abstract

Brown algae cell walls are a good source of sulfated polysaccharide fucoidan which shows a wide range of biological activities (antioxidant, antimicrobial, antiviral, antitumor). These biological activities depend on the chemical structure of the fucoidan, which is closely related to the applied technique and extraction parameters, but also to the applied pre-treatment by which the interfering compounds are removed. The conventional polysaccharide extraction technique is time-consuming and requires the use of high temperatures, so the application of advanced extraction techniques is a promising alternative. The aim of this study was to define optimal conditions and compare the efficiency (yield, structural parameters, and antioxidant capacity) of polysaccharide extraction from the brown algae *Fucus virsoides* and *Cystoseira barbata* using conventional extraction (CE), microwave-assisted extraction (MAE), pressurised liquid extraction (PLE), ultrasound-assisted extraction (UAE), and non-thermal plasma extraction (NTP). In addition, the influence of pre-treatments with various solvents and pre-treatments that promote cell wall damage (UAE and NTP), on the yield and chemical structure of fucoidan, was investigated. The highest polysaccharide yield for *F. virsoides* was obtained with PLE (0.1 M H_2SO_4, two cycles of 15 min, 140 °C), while for *C. barbata* a similar yield was obtained with PLE and CE, and the lowest yields for both algae were obtained with UAE and NTP. Along with higher or similar yields compared to CE, the extraction time was reduced from 3 h to 30 min and 10 min with PLE and MAE, respectively. CE resulted in polysaccharides with the lowest sulfate groups content and the highest uronic acids content, while UAE and NTP resulted in significantly higher sulfate group content and lower fucose and uronic acids content. The use of 0.1M H_2SO_4 as extraction solvent improved the yield and purity of the obtained extracts, and the combination of acetone and 96% ethanol proved to be the best for the removal of interfering compounds in the pre-treatment. UAE and NTP as pre-treatments did not result in greater cell wall breakage, giving more or less comparable yields as CE. Polysaccharides from the algae *F. virsoides* and *C. barbata* did not show embryotoxic and cardiotoxic effects and did not cause behavioral changes in zebrafish but they showed antimicrobial activity against *Bacillus subtilis*. Stronger *in vitro* and *in vivo* antioxidant activity was observed with polysaccharides from the alga *C. barbata*, which also showed antifungal activity against *Candida albicans*.

Keywords: *polysaccharides, fucoidan, algae, Fucus virsoides, Cystoseira barbata, advanced extraction techniques*

Proširenisažetak

Stanične stjenke smeđih algi dobar su izvor sulfatiranih polisaharida fukoidana koji pokazuju široki raspon bioloških aktivnosti (antioksidacijska, antimikrobna, antivirusna, antitumorska). Navedene biološke aktivnosti ovise o kemijskoj strukturi fukoidana koja je usko povezana s primijenjenom tehnikom i parametrima ekstrakcije, ali i primijenjenim pred-tretmanom kojim se uklanjanje interferirajući spojevi. Konvencionalna metoda ekstrakcije polisaharida je dugotrajna te zahtjeva upotrebu visoke temperature zbog čega primjena naprednih metoda ekstrakcije predstavlja obećavajuću alternativu. Cilj ovog istraživanja bio je definirati optimalne uvjete i usporediti učinkovitost (prinos, parametri strukture i antioksidacijski kapacitet) ekstrakcije polisaharide iz smeđih algi *Fucus virsoides* i *Cystoseira barbata* primjenom konvencionalne ekstrakcije (CE), ekstrakcije potpomognute mikrovalovima (MAE), ubrzane ekstrakcije otapalima pri povišenom tlaku (PLE), ekstrakcije potpomognute ultrazvukom (UAE) i ekstrakcije hladnom atmosferskom plazmom (NTP). Nadalje, istražen je utjecaj pred-tretmana različitim otapalima te pred-tretmana koji pospješuju oštećenje stanične stjenke (ultrazvuk i hladna atmosferska plazma) na prinos i kemijsku strukturu fukoidana. Najviši prinos polisaharida za *F. virsoides* postignut je primjenom PLE (0.1 M H_2SO_4, dva ciklusa od 15 min, 140°C), dok je kod *C. barbata* sličan prinos postignut primjenom PLE i CE, a najniži prinosi za obje alge postignuti su primjenom UAE i NTP. Uz veće ili slične prinose u odnosu na CE, primjenom PLE i MAE vrijeme ekstrakcije je skraćeno s 3 sata na 30 min odnosno 10 min. CE je rezultirala polisaharidima s najmanjim udjelom sulfatnih grupa i najvećim udjelom uronskih kiselina dok su UAE i NTP rezultirale značajno većim udjelom sulfatnih grupa te nižim udjelom fukoze i uronskih kiselina. Upotreba 0.1M H_2SO_4 kao ekstrakcijskog otapala poboljšala je prinos i čistoću dobivenog ekstrakta, a kombinacija acetona i 96% etanola pokazala se kao najbolja za uklanjanje interferirajućih spojeva u pred-tretmanu. UAE i NTP kao pred-tretmani nisu rezultirali većim lomljenjem stanične stjenke, dajući više ili manje usporediv prinos kao CE. Polisaharidi iz algi *F. virsoides* i *C. barbata* nisu pokazali embriotoksične i kardiotoksične učinke, niti su izazvali promjene u ponašanju zebrica, ali su pokazali antimikrobnu aktivnost protiv *Bacillus subtilis*. Jača *in vitro* i *in vivo* antioksidativna aktivnost dobivena je u polisaharidima iz alge *C. barbata* koji su pokazali i antifungalno djelovanje protiv *Candida albicans*.

Ključne riječi: *polisaharidi, fucoidan, alge, Fucus virsoides, Cystoseira barbata, napredne tehnike ekstrakcije*

Želim se od sveg srca zahvaliti svojoj mentorici prof. dr. sc. Verici Dragović-Uzelac na ukazanom povjerenju da budem dio njezinog tima. Hvala mentorici koja je na cijelom ovom putu bila uz mene, koja mi je uvijek nastojala prenijeti svoje znanje i iskustvo, čiji su mi savjeti bili od velike pomoći, koja me je znala usmjeriti na pravi put i pokazati kako da samostalno i odvažno pronađem rješenje za sve probleme. Hvala mentorici koja me naučila puno više od same znanosti, naučila me da nemoguće ne postoji, da se vrijedi izboriti za ono u što vjeruješ, i da su čast i poštenje iznad svega. Hvala mentorici što mi je svojim zaraznim osmijehom, pjesmom i plesom uljepšavala i najtmurnije dane, i čije su riječi podrške uvijek stizale baš kad su mi trebale. Bila mi je iznimna čast i zadovoljstvo učiti i raditi uz osobu koja me svojim entuzijazmom, predanošću radu i životnom energijom svakodnevno inspirira. Hvala.

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Information about the supervisor - Verica Dragović-Uzelac, Ph.D. Full Professor

VERICA DRAGOVIĆ-UZELAC has been working at the Faculty of Food Technology and Biotechnology at the University of Zagreb since 1993 and was appointed full professor in January 2013. She is actively involved in scientific research and has published over 128 scientific papers, 80 of which are high-ranking articles (a1) and have been cited 3628 times. She has also published a number of papers in journals indexed in secondary databases (a2), in congress books with international peer review (a3), and she has participated in a number of national and international congresses. Since 2013 she has been the head of the Laboratory for drying technologies and monitoring of biologically active compounds. She actively participates in teaching at undergraduate, graduate, and doctoral studies, and has successfully supervised 8 doctoral dissertations, 3 professional theses, and over 150 undergraduate and graduate theses. She has particularly excelled in leading national and EU-funded projects in the field of food technology and food chemistry: "Sour cherry Marasca (*Prunus cerasus* var. Marasca) as an ingredient for functional food", "The application of innovative technologies in bioactive compounds isolation from organic waste in the wine production", "Application of innovative technologies for the production of plant extracts as ingredients for functional food", "Isolation and encapsulation of bioactive molecules of wild and cultivated nettle and fennel and effects on organism physiology" and "Medicinal plants' bioactive molecules as natural antioxidants, microbicides and preservatives". She participated as a collaborator in the project "Processing raw materials into excellent and sustainable end products while remaining fresh", "Equipping the semi-industrial practicum for the development of new food technologies", "Center of Excellence for Marine Bioprospecting BioProCro" and the project "Bioprospecting of the Adriatic Sea". She has received numerous awards including: annual state award for significant scientific achievement (2015), RegioStars recognition for the most successfully implemented project from the Operational Program: Regional Competitiveness 2007-2013 (Brussels, 2016), award from the Faculty of Food Technology and Biotechnology for published papers in recognized international journals and exceptional achievements in scientific research and teaching (2014), annual award for popularization and promotion of science (2012), certificate for the most successful local EU project in the Republic of Croatia for the period from 2010 to 2013 (IPA project - Cherry Marasca (*Prunus cerasus* var. Marasca) as an ingredient in functional food), ARCA Gold Plaque 2015 - for the same IPA project. She was President of the Organizing and Executive Committee of the "7th, 8th and 9th International Congress of Food Technologists, Biotechnologists and Nutritionists" (2011, 2014 and 2018).

Authors publications included in the doctoral dissertation:

Publication No. 1

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Publication No. 2

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Publication No.3

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Table of contents

List of abbreviations

%PS – polysaccharide yield

ABTS – 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)

CE – conventional extraction

DPPH – 2,2-diphenyl-1-picrylhydrazyl

EAE – enzyme-assisted extraction

EtOH – ethanol

FRAP – ferric reducing antioxidant power

H2O – water

H_2O_2 – hydrogen peroxide

H_2SO_4 – sulfuric acid

HCl – hydrochloric acid

HPLC – high performance liquid chromatography

MAE – microwave-assisted extraction

MANOVA – multivariate analysis of variance

MIC – minimum inhibitory concentration

M_n – number average molecular weight

M_w – weight average molecular weight

NTP – non-thermal plasma

ORAC – oxygen radical absorbance capacity

PDI – polydispersity index

PLE – pressurised liquid extraction

RNS – reactive nitrogen species

ROS – reactive oxygen species

UAE – ultrasound-assisted extraction

ZET – zebrafish embryotoxicity test

General introduction

Seaweeds or marine macroalgae are an excellent source of nutrients and bioactive molecules with a broad range of applications in the food, pharmaceutical, cosmetics and textile industry. According to the latest worldwide statistics on aquaculture compiled by FAO, in 2018 total production of aquatic algae was 32.4 million tons with a total sale value of USD 13.3 billion (FAO, 2020). Among the nutrients and bioactive molecules present in macroalgae (polysaccharides, proteins, vitamins, minerals, pigments, polyphenols, amino acids, peptides etc.), polysaccharides are the most prominent with respect to possible commercial application. Seaweed polysaccharides do not have analogues among plant polysaccharides, as they could be methylated, acetylated or sulfated. They are specific for each of the algal groups, so fucoidan, laminarin and alginate are found in brown, carrageenan and agar in red and ulvan in green algae (Samaraweera et al., 2011). Fucoidan is composed of mainly fucose interconnected by β (1,3) glycoside bonds, alternating β (1,3) and β (1,4) bonds and rarely β (1,2) bonds (Lim & Wan Aida, 2017). Apart from fucose, it also contains other monosaccharides, including galactose, glucose, mannose, xylose, rhamnose, and uronic acids, and its sulfate content varies between 5% and 38% (Lim & Wan Aida, 2017). The chemical structure of fucoidan may significantly determine its physical, chemical and biochemical properties (De Jesus Raposo et al., 2015). Moreover, biological activities of fucoidan are strongly associated with their chemical structure (Garcia-Vaquero et al., 2017) however the correlation between structure and biological activity is still not sufficiently clarified (Dobrinčić et al., 2020). The structure and composition of fucoidan can be influenced by algae species, location and harvesting season (Jiao et al., 2011) as well as extraction techniques and different extraction conditions (e.g. pH, time, temperature, pressure, particle size, solvent, sample to solvent ratio, agitation speed) (Ale et al., 2011b; Garcia-Vaquero et al., 2017; Praveen et al., 2019).

The extraction of algal polysaccharides can be carried out by conventional and advanced techniques, and the extraction efficiency and yield can be significantly improved by applying various pre-treatments prior to the algae polysaccharide extraction. It is beneficial to apply pre-treatment in order to remove proteins, lipids, phenols as well as mannitol and chlorophyll i.e. compounds which are highly bound to the polysaccharides. For that purpose, various solvents and solvent mixtures of different polarity have been used e.g. mixture of methanol, chloroform and water at 4:2:1 (v/v/v), acetone, ethanol and methanol (Guerra Dore et al., 2013; Hadj Ammar et al., 2016; Hentati et al., 2018). The second type of pre-treatment is carried out with the aim to disrupt cell wall material and enhance the mass transfer of the target compounds to the extraction solvent, resulting in improved extraction yield (Hahn et al., 2012). Several

mechanical and physical methods for cell disruption have been described in the literature including milling, high pressure extrusion, ultrasonication and microwave pre-treatment (Mzibra et al., 2019).

Conventional polysaccharide extraction (CE) is generally performed with water, dilute acid or dilute alkali for a long time, at high temperature and by using large solvent volume, thus not being economically and environmentally friendly. To overcome these limitations, advanced technologies such as microwave assisted extraction (MAE), ultrasound assisted extraction (UAE), pressurized liquid extraction (PLE) and enzyme-assisted extractions (EAE) have been applied to extract brown algae polysaccharides. These techniques, with different "modus operandi" target the breakdown of the brown algae cell walls, where most of the bioactive molecules, including fucoidan, are stored (Okolie et al., 2019).

The marine environment offers a wide variety of organisms as the different sea zones provide a diverse environment. Out of all marine species, 98% of them live on or in the ocean floor, in ecological zone known as benthic zone. Benthic fauna of the Croatian part of the Adriatic Sea includes 2597 species of algae, among which 170 species are brown algae from 11 taxonomic orders (Antolić et al., 2011). One of the endemic brown algae species is *Fucus virsoides*, the only representative of Fucus genus in the Mediterranean, growing mainly in the northern Adriatic, from the Venice Lagoon to Dalmatia (Guiry, 2021). *Cystoseira barbata* belongs to genus Cystoseira whose representatives have an important role in the structure and functioning of the rocky habitats of the Mediterranean and the Black Sea, providing shelter, food and nursery grounds for a variety of organisms (Berov et al., 2015).

Within the Scientific Center of Excellence for Marine Bioprospecting (BioProCro) and the project Bioprospecting of the Adriatic Sea, brown algae were selected as one of the marine organisms with the highest biological potential, mainly due to their abundance in sulfated polysaccharides. This dissertation in form of published papers and a final comprehensive review aims to evaluate the influence of various pre-treatment solvents on impurities removal, polysaccharide yield and polysaccharide chemical composition. Moreover, the aim was to compare CE, MAE and PLE, and to evaluate the influence of their parameters (solvent, temperature and time) on polysaccharide yield, chemical composition (total sugar, fucose, sulfate group and uronic acid content, monosaccharide composition, molecular weight) and antioxidant activity, of the fucoidan from *F. virsoides* and *C. barbata*. Application of UAE and NTP as pre-treatment and polysaccharide extraction method were also examined. Additionally,

in vitro and *in vivo* antioxidant activity, antimicrobial activity, embryotoxicity, genotoxicity, cardiotoxicity and behavioural changes of *F. virsoides* and *C. barbata* polysaccharides were evaluated.

Chapter 1

Theoretical background

- algae - general information
- *Fucus virsoides* and *Cystoseira barbata*
- ***Publication No. 1*:**
  "Advanced technologies for the extraction of marine brown algal polysaccharides"
- most recent studies on brown algae polysaccharide extraction
- hypothesis, research objectives, and expected scientific contributions

1. Algae

The ocean is one of the world's most important repositories of biodiversity. Within marine habitats, water column communities include pelagic microbes, phyto- and zooplankton, while seafloor communities include benthic micro-, macro-, and megafauna, as well as primary producers such as seaweeds and macroalgae (Cochrane et al., 2016). The term *algae* is not a taxonomic category, but a term used to describe heterogeneous assembly of organisms, including both prokaryote and eukaryote species, with a profound diversity in size, cellular structure, levels of organization and morphology, colonized habitats, pigments for photosynthesis, reserve and structural polysaccharides (Barsanti & Gualtieri, 2014). Algae are biochemically and physiologically very similar to plants: they produce the same storage compounds, have the same metabolic pathways, possess chlorophyll (Gallardo, 2014), and use similar defence strategies against predators and parasites (Barsanti & Gualtieri, 2014). What distinguishes algae from plants is their lack of embryo and multicellular envelope around the sporangia and gametangia (Gallardo, 2014), lower degree of differentiation (plants have roots, leaves, stems and xylem/phloem vascular network), and their monogenetic and digenetic life cycles (all plants have a digenetic life cycle) (Barsanti & Gualtieri, 2014). Algal phycologists believe that there are from 30 000 to more than one million species, most of which are marine (Gallardo, 2014). There is no easily definable classification system because taxonomy is under constant and rapid revision (Barsanti & Gualtieri, 2014). Based on cellular structure, pigment composition, and molecular sequence or genome architecture information, the algae have been classified into several different classification schemes (Graham & Wilcox, 2000). One of the classification schemes divides algal species among eleven divisions: two prokaryotic divisions (Cyanophyta, Prochlorophyta) and nine eukaryotic divisions (Glaucophyta, Dinophyta, Euglenophyta, Haptophyta, Heterokontophyta, Cryptophyta, Chlorarachniophyta, Rhodophyta, Chlorophyta) and the number of genera and species significantly varies among different divisions (Graham & Wilcox, 2000).

Algae are unicellular or multicellular organisms which, with the exception of the Cyanophyta, have cellular organelles surrounded by membranes (Gallardo, 2014). Their size ranges from picoplankton only 0.2 to 2.0 mm in diameter, to giant seaweeds up to 60 m long (Barsanti & Gualtieri, 2014), for what reason they are divided as microalgae and macroalgae. Algae can live exposed to the atmosphere (subaerial), or completely submerged in water (aquatic) (Barsanti & Gualtieri, 2014). Due to their tolerance for a wide range of pH,

temperature, turbidity, O_2 and CO_2 concentration, aquatic algae are found almost anywhere, from freshwater spring to salt lakes (Barsanti & Gualtieri, 2014). Most unicellular and colonial species usually live suspended in the water as part of plankton while other algae grow attached to a substrate as part of the benthos. Benthic algae are attached on stones (epilithic), mud or sand (epipelic), other algae or plants (epiphytic), or on animals (epizoic) (Barsanti & Gualtieri, 2014). Marine algae can grow above the high-tide level within the reach of waves (supralittoral), on shores exposed to tidal cycles (intertidal), or in the benthic environment from the extreme low-water level to around 200 m deep (sublittoral) (Barsanti & Gualtieri, 2014).

Algae are well known for their rich nutrient composition including vitamins, lipids, proteins, carbohydrates and minerals, what makes them a good and healthy food source (Carvalho & Pereira, 2014). Some of the seaweed species most often used for food are *Ulva* (Chlorophyta), *Porphyra* (Rhodophyta), *Undaria*, *Laminaria*, *Himanthalia* and *Saccharina* (Phaeophyceae) (Pereira, 2011). Alginic acid (or its mineral salt, alginate), carrageenan and agar are gelling agents produced from certain brown and red seaweeds, often used stabilizers and emulsifiers in food, in the pharmaceutical and textile industries, as well as in the manufacture of paints, paper, and other industrial uses (Gallardo, 2014). Besides their application as human food, microalgae are used as feeding stocks for aquaculture (Carvalho & Pereira, 2014) and they are used in a wide variety of technological applications. Due to their small size, fast growth rate, short generation times, and easy cultivation under laboratory conditions algae are useful laboratory organisms (Graham & Wilcox, 2000). Algae are used as environmental monitors, to assess high levels of nutrients in water and as a very profitable tool for bioremediation (Graham & Wilcox, 2000). Macroalgae extracts are widely accepted as crop fertilizers to improve yield, increase uptake of soil nutrients, increase resistance to some pests, increase resistance to frost and improve seed germination (Barsanti & Gualtieri, 2014). Due to their considerable hydrating skills and cosmetic effects algae extracts have been used in cosmetic industry (Carvalho & Pereira, 2014) for wrinkle reduction, tissue regeneration, cell proliferation, prevention of striae formation and regulation of skin metabolism (Barsanti & Gualtieri, 2014). Microalgae abundance in fatty acids, which can be converted into biodiesel, and seaweeds abundance in sugars, which can be converted into bioethanol, makes them so suitable for biofuel production (Carvalho & Pereira, 2014).

Macroalgae or seaweed are multicellular photoautotrophic organisms which, besides being primary producers, play an important role in the structuring and maintenance of the

marine ecosystems, introducing important nursery spots for a variety of marine species (Carvalho & Pereira, 2014). There are three major groups of macroalgae based on pigment composition: Chlorophyta (green algae), Rhodophyta (red algae) and Phaeophyceae (brown algae) (Carvalho & Pereira, 2014).

1.1. Green algae

Green algae, members of the division Chlorophyta, are a large and ecologically important group of photosynthetic eukaryotes (Leliaert, 2019). They are the most diverse group of algae with more than 15000 species in 500 genera (Leliaert, 2019). Although green algae are predominant in fresh water, there are also many marine representatives globally distributed from tropical to arctic regions, occurring in a wide range of aquatic habitats (Leliaert, 2019). Approximately 214 species were identified in the Mediterranean Sea, and Croatian part of the Adriatic Sea contains 118 species in orders Bryspidophyceae, Chlorophyceae and Ulvophyceae (Antolić et al., 2011).

Thallus size varies from microscopic to macroscopic forms. Furthermore, thallus structure varies from swimming and non-motile unicells, to filaments, colonies and different levels of tissue organization (pseudoparenchymatous, parenchymatous, thalloid) and branching morphologies. Because green algae have the same type of pigments and produce the same kind of carbohydrates as land plants, it is most likely they have a common ancestor. Chlorophylls a and b are dominant photosynthetic pigments responsible for their green colour since they are not masked by carotenoids and xanthophylls. Starch, located within the chloroplast, is the major storage product of green algae.

Green algae could be very interesting natural source of new biologically active compounds important for functional food development. Protein content in some green seaweeds, such as the species belonging to the genus *Ulva*, can represent between 10 and 26% algae dry weight (Fleurence, 1999). Green algae are source of sulfated polysaccharide called ulvan whose major monosaccharide unit is α-L-rhamnose. Ulvan exhibited various health-improving properties and biological activities including anti-inflammatory, antivirus, antitumor, immunomodulatory, and anticoagulant activities (Rahimi et al., 2016). Compared to brown algae species, green and red algae have lower concentrations of phenols (Holdt & Kraan, 2011) as well as iodine (Rajapakse & Kim, 2011). The most dominant phospholipid in green alga is phosphatidylglycerol (Pérez et al., 2016) while predominant sterols in green algae

are 28-isofucocholesterol, cholesterol, 24-methylene-cholesterol and β-sitosterol (Hakim & Patel, 2020). Due to their ability to absorb nutrients from industrial, domestic and agriculture wastewater, some *Ulva*, *Cladophora* and *Chaetomorpha* species, are considered to be related to seawater pollution (Bonanno & Orlando-Bonaca, 2018).

1.2. Red algae

Red algae, or Rhodophyta, are phylogenetically the oldest division of lower plants (Usov, 2011) and they contain between 5000 and 5500 species, which are distributed in 500 to 600 genera (Antolić et al., 2011). Only about 150 species, form 20 genera, are known in fresh water while the rest are marine (Antolić et al., 2011). They are found in all the regions of the world, growing attached to the bottom or other hard surfaces. In Mediterranean Sea approximately 650 red algae species were identified, of which 340 species, belonging to Bangiophyceae, Compsopogonophyceae and Florideophyceae orders, were identified in Croatian part of the Adriatic Sea (Antolić et al., 2011).

Red algae are mostly multicellular and their thallus structure varies from filamentous, branched, feathered, and sheet like forms. The coralline algae are very important in the formation of tropical coral reefs, as they secrete hard shell of calcium carbonate on their surface what prevents them from being eaten and gives them strength and support. The red colour is a result of the presence of the phycoerythrin and phycocyanin which masks other pigments such as chlorophyll a (there is no chlorophyll b), β-carotene and xanthophylls. This pigments reflect red light and absorb blue light that can penetrate water to a greater depth, allowing red algae to photosynthesize and thus live at greater depths than majority of other algae (Tschudy, 1934). Red algae store sugars as floridean starch, the type of starch that consists of highly branched amylopectin without amylose (Usov, 2011) while classical starch is absent.

Red algae contain a large variety of structurally diverse compounds including proteins, polysaccharides, dietary fibres, PUFAs, carotenoids, polyphenols, vitamins (especially vitamin C, B1, B2 and B2), and minerals (iodine), all of which can be attractive for use in functional food and nutraceutical products (Alves et al., 2018). With 10 to 47% dry weight, they have a higher protein content than green or brown algae, and they are a source of protein–pigment complexes called phycobiliproteins (Alves et al., 2018). Red algae polysaccharides, agar and carrageenan, have been widely used as stabilizers, emulsifiers and gelling agents in food, pharmaceutical and cosmetic industry (Alves et al., 2018). Although algal lipid content is

generally low, their proportion in PUFAs is high (8 to 63%), particularly eicosapentaenoic acid (EPA) and α-linolenic acid as ω-3 fatty acids, and arachidonic acid (AA) and linoleic acid as ω-6 fatty acids (Alves et al., 2018). The most dominant phospholipid in red alga is phosphatidylcholine (Pérez et al., 2016) while predominant sterols are desmosterol, cholesterol, sitosterol, fucosterol and chalinasterol (Hakim & Patel, 2020).

1.3. Brown algae

The brown algae comprise the class Phaeophyceae, large group of multicellular golden-brown algae that range from small filamentous forms to large, complex seaweeds. Of the estimated 1500 to 2000 species, in approximately 265 genera, less than 1% are found in freshwater habitats. Most brown algae live in marine environments, in moderately cold water within the Northern Hemisphere. They are the most numerous group of algae in the Mediterranean Sea with approximately 265 identified species, of which 170 species are identified in Croatian part of the Adriatic Sea (Antolić et al., 2011). Those 170 species are included in 11 taxonomic orders: Cutleriales, Desmarestiales, Dictyotales, Discosporangiales, Ectocarpales, Fucales, Laminariales, Ralfsiales, Scytosiphonales, Sphacelariales and Sporochnales (Antolić et al., 2011).

All brown algae are multicellular as there are no known unicellular or colonial representatives. They grow in a wide range of sizes and forms, from tiny, feathery tufts of threadlike cells just few centimetres long, to the giant, 50 m long, kelp *Macrocystis pyrifera*, the largest algae of them all. Dominant pigment in brown algae is fucoxanthin which is responsible for their distinct greenish-brown color and masks other pigments like chlorophyll a and c (there is no chlorophyll b) as well as other carotenoids and xanthophylls (β-carotene, lutein, violaxanthin, astaxanthin, neoxanthin, zeoxantine).

Brown seaweed represent a good source of various bioactive compounds with specific biological activities, including unique secondary metabolites such as phlorotannins (algal polyphenols) and phytosterols (fucosterol, cholesterol and brassicasterol) (Hakim & Patel, 2020). Alginic acid (alginate), fucoidan and laminarin are polysaccharides specific for brown algae cell walls, and they are considered dietary fibres. Alginate is extracted commercially and used as an industrial thickening agent in food while fucoidan is sulfated polysaccharide that possesses a wide range of positive effects such as antioxidant, anti-inflammatory and antitumor (Ale et al., 2011b; Li et al., 2008; Lim & Wan Aida, 2017). Brown algae are recognized for

their superior ability to accumulate minerals such as calcium, magnesium, phosphorus, potassium, sodium and iron. Standout aspects of brown algae minerals, compared to plants, are low Na/K ratio and high iodine level what is an important for good maintenance of cardiovascular health.

1.3.1. Fucus virsoides

Fucus is a genus of brown multicellular algae in the order Fucales, family Fucaceae, found in the intertidal zones of rocky seashores all over the world. Among 716 Fucus species identified worldwide (Guiry, 2021), the most common are *Fucus vesiculosus*, *Fucus distichus*, *Fucus serratus* and *Fucus spiralis*. Members of the Fucus genus have a high nutritional value due to the abundance of dietary fiber and minerals, as well as high biological potential due to the bioactive compounds such as fucoidan, fucoxanthin and phlorotannins (Catarino et al., 2018).

F. virsoides J. Agardh it very likely the only species of the Fucus genus to occur in the Adriatic Sea (Guiry, 2021). It is endemic to the Adriatic Sea and it is considered to be a glacial relict. Due to the influence of fresh water, which is suitable for their growth, it is predominant in the northern and central Adriatic, from the Venice Lagoon to Dalmatia (Guiry, 2021), with its southern range limit situated along the Albanian coast (Mačić, 2006). *F. virsoides* is psychrophilic organism meaning its optimal growth temperature is below 15°C explaining its prevalence in northern Adriatic as air and sea temperatures south of Boka Kotorska are too high for its survival (Linardić, 1949). It prefers clean environment and habitats with specific trophic condition, therefore it can be considered as an indicator for water quality.

F. virsoides (Figure 1) is light to dark brown in color, sometimes with a tinge of green, and its size varies from 2 - 4 cm to the maximum of 20 - 22 cm (Linardić, 1949). It has perennial flat dark brown thallus which is dichotomous branched, flattened and with a distinct midrib (Linardić, 1949). The base of the thallus is attached to the rock by a basal plate and around the midrib gas-filled aerocysts (air-vesicles) are located. Thallus is covered with mucus that allows it to survive outside of water where it spends half of its life as it inhibits intertidal zones (Linardić, 1949).

Figure 1. *Fucus virsoides* (www.specieaspim.it, accessed 8th September, 2021)

1.3.2. Cystoseira barbata

Cystoseira is a genus of brown algae in the order Fucales and one of the most significant representative of the Sargassaceae family, composed of recently merged two former families, Sargassaceae and Cystoseiraceae (Kosanić et al., 2015). Algae of this genus are found mainly on the shores of the Mediterranean and Black Seas, as well as on the east coast of the Atlantic Ocean (Trica et al., 2019). More than 294 species of this genus are known worldwide (Kosanić et al., 2015) and about 56 of them are found in the Adriatic Sea (Rožić et al., 2012), including *Cystoseira barbata*, *Cystoseira compressa*, *Cystoseira corniculata*, *Cystoseira crinita* and *Cystoseira spinosa* (Antolić et al., 2010). Numerous studies conducted on species belonging to *Cystoseira* genus identified a wide range of secondary metabolites, such as polysaccharides, florotanins and terpenoids, with various biological activities (Trica et al., 2019).

C. barbata (Stackhouse) is an endemic species of the Mediterranean Sea, growing mostly in the coastal area of the northern Adriatic most abundantly during April and May (Nita et al., 2014). Morphological differences could be observed between different habitats and environmental conditions (Falace & Bressan, 2006). The natural habitat of this species are open and sheltered rocky shores, semi-closed areas and coastal lagoons (Berov et al., 2015). It usually grows in the upper sublittoral zone at 0-2 m depth (Berov et al., 2015), but also at depth of 20 to 30 m, depending on the permeability of seawater to light (Antolić et al., 2011).

C. barbata (Figure 2) is a perennial, large, strongly branched, flexible, brown alga that can grow up to 2 m. It has a thallus which consists of a perennial cauloid and a dendroid frond

(Falace & Bressan, 2006). It is attached to the seabed by a strong basal disc, from which multiple cylindrical branches emerge, with various round or oval-shaped, lined-up or isolated, aerocysts, acting as floating devices to ease maintaining the vertical position in the water (Nita et al., 2014).

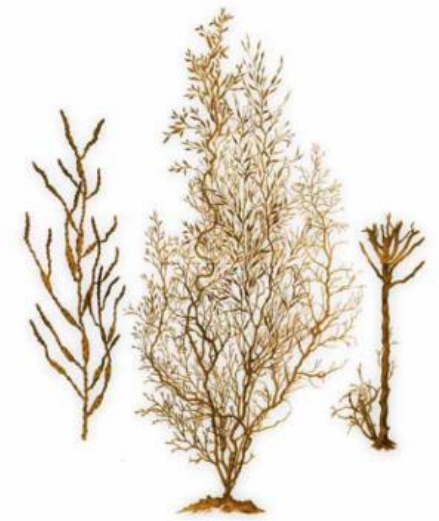

Figure 2. *Cystoseira barbata* (www.specieaspim.it, accessed 8th September, 2021

2. Application of advanced technologies for the extraction of marine brown algal polysaccharides

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Sandra Pedisić: writing—review and editing

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Ivona Elez Garofulić: conceptualization, methodology, investigation, writing— original draft preparation, review and editing

Verica Dragović-Uzelac: project administration, funding acquisition

marine drugs

MDPI

Review

Advanced Technologies for the Extraction of Marine Brown Algal Polysaccharides

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Abstract: Over the years, brown algae bioactive polysaccharides laminarin, alginate and fucoidan have been isolated and used in functional foods, cosmeceutical and pharmaceutical industries. The extraction process of these polysaccharides includes several complex and time-consuming steps and the correct adjustment of extraction parameters (e.g., time, temperature, power, pressure, solvent and sample to solvent ratio) greatly influences the yield, physical, chemical and biochemical properties as well as their biological activities. This review includes the most recent conventional procedures for brown algae polysaccharides extraction along with advanced extraction techniques (microwave-assisted extraction, ultrasound assisted extraction, pressurized liquid extraction and enzymes assisted extraction) which can effectively improve extraction process. The influence of these extraction techniques and their individual parameters on yield, chemical structure and biological activities from the most current literature is discussed, along with their potential for commercial applications as bioactive compounds and drug delivery systems.

Keywords: polysaccharides; marine algae; extraction; fucoidan; laminarin; alginate

1. Introduction

The term macroalgae refers to aquatic photosynthetic organisms which are included in the *Eukaryota* domain as well as *Plantae* and *Chromista* kingdoms [1]. They differ according to several characteristics such as cell wall composition, presence or absence of flagella and ultrastructure of mitosis [2]. Their distribution, diversity and chemical composition are mainly limited by the environmental conditions, e.g., sunlight availability (chromatic adaptation) and water temperature. Based on their pigmentation and chemical composition, macroalgae can be classified into three groups: brown (*Phaeophyceae*), red (*Rhodophyceae*) and green (*Chlorophyceae*) [3,4].

Brown algae are a rich source of bioactive molecules such as proteins, amino acids, polysaccharides, fatty acids, vitamins, minerals, dietary fibre, sterols, pigments, polyphenols etc. which possess a broad spectrum of biological activities (anticoagulant, antithrombotic, anti-viral, anti-cancer, anti-inflammatory and antibacterial) [5]. These compounds therefore provide high potential for the application of brown algae extracts in the treatment of arteriosclerosis, rheumatic processes, hypertension, goitre, asthma, ulcers, menstrual disorders, syphilis, skin diseases etc. [4,6,7]. The biological potential of brown algae is significantly contributed by polysaccharides as one of the most common and most important groups of bioactive compounds. Over the last years, considerable interest has been raised about different types of polysaccharides in brown algae cell walls, including laminarins, alginates and fucoidans which have high potential for biological applications in functional foods, cosmeceutical and pharmaceutical products [8]. The structure and composition of algal polysaccharides

(APS) is determined by algae species however it is also influenced by other factors causing inter-species variation, e.g., growth location and harvesting season [8]. Vast structural variation between the APS therefore presents a challenge in terms of pre-treatments application, extraction techniques and optimization, characterization of isolated fractions and determination of their biological properties.

Chemical structure and yield of APS isolated from marine macroalgae by conventional extraction (CE) techniques can be affected by various experimental conditions (pH, time, temperature, pressure, particle size, solvent, sample to solvent ratio, agitation speed etc.). In addition, different advanced techniques such as microwave assisted extraction (MAE), ultrasound assisted extraction (UAE), pressurized liquid extraction (PLE), enzyme-assisted extractions (EAE) are assessed and applied for APS extraction [9–11].

In general, the chemical structure of polysaccharides determines its physical, chemical and biochemical properties as well as its biological activities [12]. Several studies have reported that their biological activity is strongly associated with their chemical structure [9]. Due to very complex mechanisms that are affected by many factors, the correlation between polysaccharide structure and biological activity is still not sufficiently clarified.

In order to improve isolation of APS, pre-treatments are usually applied to the algal biomass prior to the extraction process with the two aims: (i) to prevent co-extraction of interfering bioactive compounds with similar solubility; and (ii) to disrupt cell walls and improve mass transfer of APS into extraction solvent. The first type of pre-treatments is therefore used to remove compounds which are highly bound to the APS such as proteins, phenols and lipids, as well as mannitol and chlorophyll [13]. For that purpose, the application of various pre-treatment solvents at different temperatures has been studied. A mixture of methanol, chloroform and water at 4:2:1 (v/v/v) has been successfully used for defatting, and acetone [14] as well as mixture of acetone and ethanol [15] or methanol [16] were used to remove lipids and pigments. The second type of pre-treatments which is carried out in order to disrupt cell wall material and enhance the mass transfer of the target compounds to the extraction solvent result in an improved extraction yield [13]. In addition, several mechanical and physical methods for cell disruption which include milling, high pressure extrusion, ultrasonication and microwave pre-treatment have been described in the literature [17].

Advanced technologies may overcome some limitations inherent to CE procedures (water, acid, salt solutions) such as relatively low yields, long time, and high energy consumption and costs. The application of advanced extraction techniques such as MAE, UAE and EAE [9], as well as purification techniques (membrane separation, affinity chromatography, ion-exchange chromatography and size-exclusion chromatography) [9] has shown the potential for the recovery of APS and other marine bioactive compounds. Hence, this review presents an overview of conventional and advanced extraction techniques of marine brown APS in the latest researches done in this field. Compared to some other review articles [3,11,13] that covered similar topics, more focus is given on extraction techniques and parameters, and their influence on structural properties and biological activity of the extracted polysaccharides. Furthermore, commercial application of APS from the most current literature is also discussed.

2. The Chemical Structure and Bioactivity of Polysaccharides from Marine Brown Algae

Polysaccharides from marine macroalgae differ greatly from the ones present in terrestrial plants such as cellulose and starch [18]. Brown seaweed cell walls contain sulfated polysaccharides i.e., laminarin and alginate along with fucoidan, which is not present in any other type of seaweeds. These three types of APS have their own unique physical and chemical characteristics which are influenced by species, geographic location, season and population age [19].

2.1. Laminarin

Laminarin is a water-soluble linear polysaccharide that consists of β (1→3) and β (1→6) glucan in a 3:1 ratio [20] (Figure 1). Molecular weight (MW) of laminarin is around 5 kDa depending on the

degree of polymerization [21]. In addition, in dependence on the type of sugar at the reducing end, there are M chains with terminal 1-O-substituted D-mannitol, and G chains with glucose. Laminarin is mainly isolated from the brown algae species *Laminaria* and *Alaria*. Based on the type of algae and harvest season [22] as well as the environmental conditions such as sea temperature, salinity, sea currents, depth and availability of nutrients [23], laminarin represents around 22–49% of algal dry matter. Apart from contributing to dietary fibre intake, studies have shown that laminarin and products of its enzymatic hydrolysis inhibit the production of melanoma cells and colon cancer [24] and also show anti-metastatic effects which makes them potentially useful in cancer treatment [25].

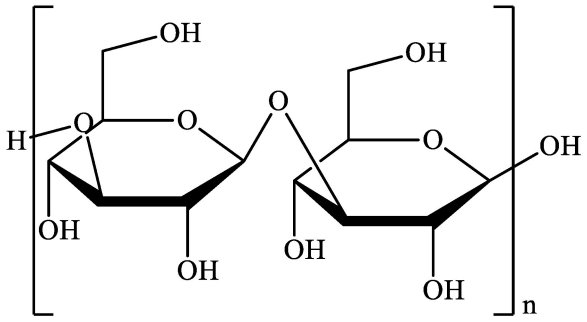

Figure 1. Structure of laminarin [26].

2.2. Alginates

Alginates are linear hetero-polysaccharides composed of β-D-manuronic acid (M) and α-L-guluronic acid (G) (Figure 2). These two monomers are linked in a 1→4 configuration and arranged as homogeneous MM, GG or alternatively MG blocks. The proportion of these three block types is responsible for the physical properties of alginates whereas alginates with high M blocks share have higher viscosity while alginates with high G blocks share have better gelling properties [27]. Alginates are obtained from cell walls of various brown algae that grow in colder seas such as *Microcystis*, *Laminaria* and *Ascophyllum* sp. [28]. In addition, alginates can be present in alginic acid form as well as in the form of its salt, which make about 40% dry matter of the algae biomass [29].

Studies have shown that alginic acid has a positive effect on preventing the absorption of heavy metals in the body, reducing blood pressure and cholesterol as well as assisting in the absorption of cholesterol [30,31]. In addition, alginates with molecular mass larger than 50 kDa showed a positive effect in prevention of diabetes and adiposity [32]. Furthermore, they have anticarcinogenic properties [31] and inhibitory effect on microorganisms such as *Staphylococcus*, *Listeria*, *Salmonella* and *Escherichia coli* [33]. Due to their properties, alginates are widely used by the food industry as stabilizers, emulsifying agent or thickeners, as well as by the cosmetic and pharmaceutical industries [34].

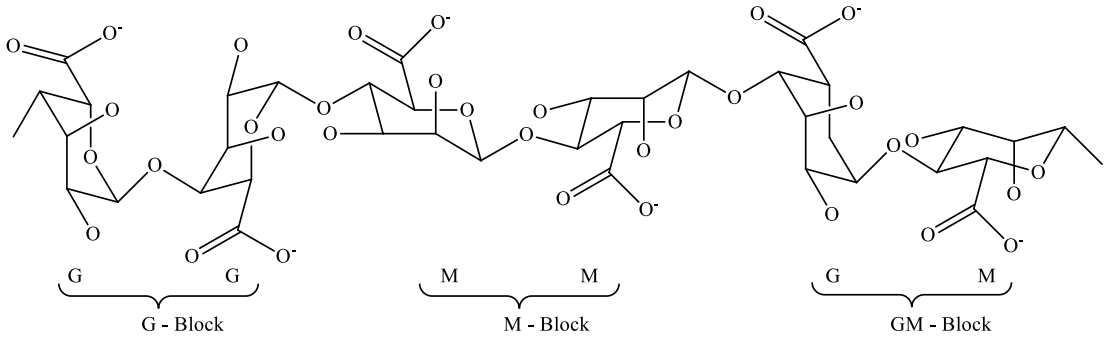

Figure 2. Structure of alginates [35].

2.3. Fucoidan

Fucoidan is sulfated polysaccharide found in fibrillar tissue of the cell wall and intercellular space of brown algae. It consists mainly of fucose interconnected by α−(1,3) glycoside bonds, alternating α−(1,3) and α−(1,4) bonds and rarely α−(1,2) bonds (Figure 3). Apart from fucose, it also contains other monosaccharides, including galactose, glucose, mannose, xylose, rhamnose, and uronic acids which contents vary depending on algal species and season [19,36]. The average relative MW of fucoidan varies from 7 kDa [37] to 2300 kDa [38]. Fucoidan is the most abundant in orders *Laminariales* and *Fucales* and, depending on the algae type, it represents approximately 5% to 10% of algae dry matter while its sulfate content varies between 5% [19] and 38% [39].

Fucoidan is one of the most researched algae molecules and studies have found that it shows a wide range of positive effects such as antioxidant, anti-inflammatory and antitumor [10,19,40]. It has been increasingly studied not only for its potential applications as the heparin-like anticoagulant and antithrombotic agent but also, due to its non-toxicity and biodegradability, as a functional additive for developing novel drug delivery systems and functional foods. Biological activities of fucoidan are closely correlated to its physicochemical properties such as MW, types and ratios of constituent monosaccharides, features of glycosidic linkages, sulfation degree and the position of sulfate groups.

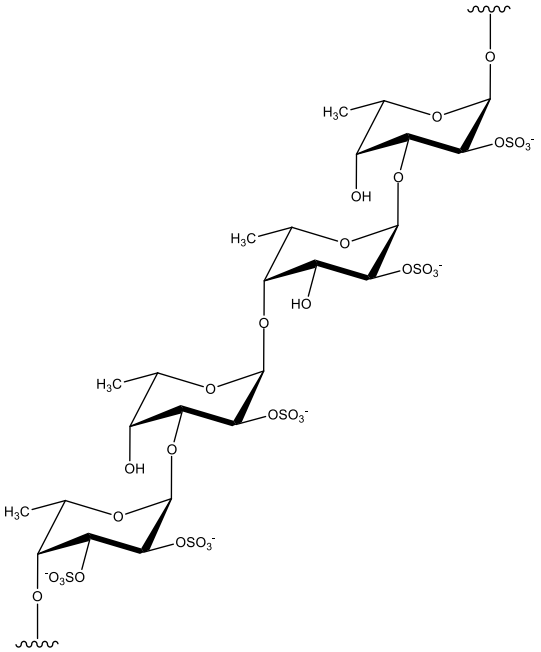

Figure 3. Structure of fucoidan from *Fucus vesiculosus*, with a backbone of alternating (1→3)-linked α-L-fucopyranose and (1→4)-linked α-L-fucopyranose residues and the presence of sulfate groups on both *O*-2 and *O*-3 [41].

3. The Perspective of Advanced Technologies for Polysaccharide Extraction from Marine Brown Algae

In general, APS isolation process (Figure 4) includes several complex and time consuming steps such as seaweed preparation, pre-treatment, extraction (conventional or advanced) and purification. These procedures are usually followed by biological activity assays which enable the determination of APS potential industrial use.

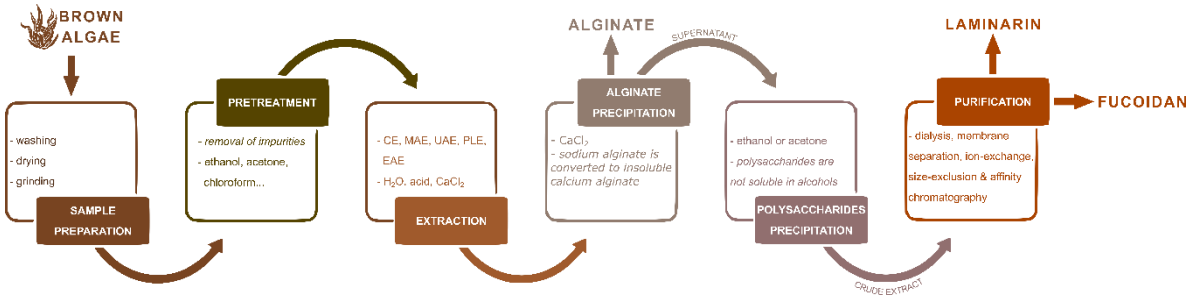

Figure 4. Schematic overview of essential steps for extraction of brown algae polysaccharides.

Preparation of the seaweed includes washing the seaweed, preferably with distilled water to remove salt and impurities, as well as drying or freeze drying and milling in order to achieve the powder with higher surface-to-volume ratio. Removal of interfering algal compounds prior to polysaccharide extraction is useful for prevention of contamination of the target polysaccharide. Conventional polysaccharide extraction is usually performed by hot aqueous or acidic solutions at high temperatures for several hours. On the other hand, advanced technologies (e.g., MAE, UAE, PLE and EAE) have already demonstrated numerous advantages over CE especially in the terms of increased extraction efficiency, reduction of extraction time, energy and solvent usage as well as preservation of sensitive and unstable bioactive molecules (e.g., polyphenols). Regardless of which extraction technique is performed, the extraction parameters should be optimized as they may influence APS chemical structure, bioactivity and potential industrial use (Figure 5). Moreover, preservation of the APS structural integrity is crucial for obtaining the relevant structural characteristics required for their specific biological activities [10].

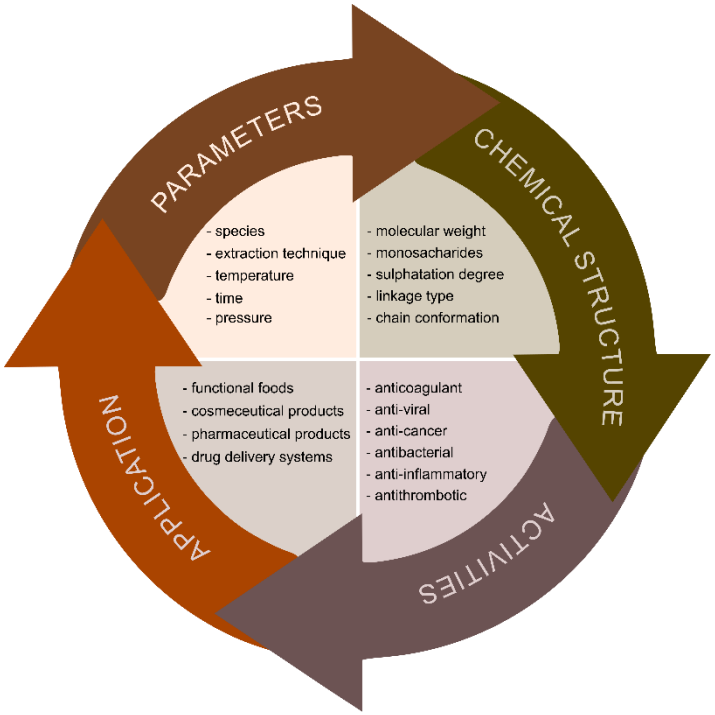

Figure 5. Schematic diagram of process parameters, chemical structure properties, biological activity and potential industrial uses of brown algae polysaccharides.

3.1. Pre-treatment of Marine Brown Algae

Prior to the APS extraction, it is beneficial to apply pre-treatment in order to remove lipids, pigments and low molecular weight compounds from the seaweed material. For that purpose, various

solvents and solvent mixtures with different polarity, that do not cause any APS structural changes, have been used [13]. Lipids are traditionally removed by lower polarity solvents, such as chloroform, petroleum ether or dichloromethane; pigments with semipolar solvents, such as acetone, methanol or ethanol; while other high polarity molecules, such as monosaccharides, proteins and minerals are extracted in water [20]. Prior to laminarin extraction from *Cystoseira barbata*, Sellimi et al. [42] seaweed powder was sequentially treated twice with acetone-methanol (7:3) and twice with chloroform for 24 h at 30 °C with constant stirring (250 rpm). Similarly, January et al. [43] used the mixture of methanol-chloroform-water (4:2:1) as a pre-treatment prior to fucoidan extraction. However, as chloroform is toxic and classified as an extremely hazardous substance in the United States as it is defined in Section 302 of the U.S. Emergency Planning and Community Right-to-Know Act (42 U.S.C. 11002), new chloroform-free pre-treatment alternatives are being proposed. As an alternative solvent, relatively low cost and non-toxic petroleum ether has been used by Sahera et al. [44]. Furthermore, *Cystoseira myrica* powder was treated with petroleum ether and acetone in Soxhlet apparatus prior to polysaccharides extraction. The same procedure was applied by Abid et al. [45] for sodium alginate extraction from *Dictyopteris membranaceae* and *Padina pavonica*. However, in their work, algae powder was previously macerated three times by methanol and dichloromethane (1:1, v/v) for 48 h. In addition to acetone, 95% ethanol (v/v), 80% ethanol (v/v) and methanol were also used [15,16,46]. Compared to previously described procedures, where higher temperatures were not used because they might promote the extraction of fucoidan, with 80% ethanol (v/v) as a solvent, higher temperature is acceptable. Since fucoidan is not soluble in ethanol, even if it was unintentionally extracted, it would be precipitated and thus only impurities like pigments and lipids would be removed by filtration [19].

Prior to APS extraction, pre-treated seaweed is further exposed to the conventional, vacuum or freeze drying methods. In addition, as seaweed rigid cell walls are hard to break [47], a cell disruption pre-treatment is generally required to remove or weaken them, making the intracellular molecules more accessible to solvent in further extraction steps. For that purpose, various cell disruption pre-treatment methods such as mechanical, chemical, thermal, enzymatic or advanced techniques, like ultrasound and microwave, could be applied. Ultrasound and microwave pre-treatments are based on the energy waves that have an effect on the cell wall material causing its lysis and release of intracellular molecules. However, this approach is more often used in bioethanol production from algae where pre-treatment is intended for cell disruption and complex carbohydrates release. Complex carbohydrates are then broken down into their monosaccharide components (simple sugars) which are fermented into bioethanol and carbon dioxide [48]. To the best of our knowledge, the only research where cell disruption pre-treatment was applied prior to polysaccharide extraction from brown seaweed was made by Kadam et al. [49]. In their work, ultrasound pre-treatment for 10 min (20 Hz, amplitude 20–100%, 13 mm diameter probe) was applied followed by simple extraction in orbital shaker for 1 to 22 hours to extract fucose and uronic acid from *Ascophyllum nodosum*. Compared to control extraction, ultrasound pre-treatment increased fucose and uronic acid extraction yield, whereas ultrasound amplitude was the most significant factor contributing to the pre-treatment efficiency [49].

3.2. Extraction Techniques

Following pre-treatment, seaweed samples are subjected to various extraction techniques. General principal of these procedures is to extract target compounds with minimum co-extraction of other polysaccharide constituents, e.g., isolate fucoidan from alginate. If alginate is co-extracted, further steps are needed to remove alginate from fucoidan, thus increasing the purity of the extracted fucoidan [20].

3.2.1. Conventional Extraction Technique (CE)

Conventional extraction of APS is typically performed by treating the algal material with various solvents such as hot water, acidic or salt solutions at high temperatures for several hours. Table 1 summarizes the most frequently used parameters of CE for APS recovery from brown algae.

CE with hot water (80–100 °C) is often used to extract water-soluble sulfated APS from *Sargassum henslowianum* and *Dictyopteris divaricate* [46,50]. However, this method is not sufficiently selective as all types of APS (fucoidan, alginate and laminarin) and other water-soluble compounds from the seaweed samples could be also extracted. Consequently, more isolation steps are needed to increase the purity of fraction with target polysaccharide.

Furthermore, for the improvement of the extraction yield, the use of 0.1 M HCl solution has been shown to be effective since it enables cell walls hydrolysis and facilitates fucoidan and laminarin extraction from the matrix [42,51]. In addition, the acid converts alginate into water-insoluble alginic acid, which is removed, together with solid seaweeds residues, resulting in relatively pure fucoidan fraction [20]. For alginate extraction, solid residue remaining after water and acid extraction could be treated with sodium carbonate (Na_2CO_3) to convert alginic acid into sodium alginate, which is water-soluble but not alcohol-soluble. Therefore, by the addition of ethanol, formed sodium alginate can be precipitated, separated from the rest of the mixture and dried [16,52].

To effectively remove alginate which is present in brown algae cell walls in the form of calcium, magnesium and sodium salts of alginic acid, 2% calcium chloride ($CaCl_2$) solution is often used [15,45,53]. Since only sodium salt is water soluble, aqueous solution of $CaCl_2$ enables fucoidan and sodium alginate extraction and dissolution, while high temperature and mechanical agitation additionally enhance the extraction process. However, when sodium alginate gets in contact with calcium ions, they replace the sodium ions in the polymer and solid calcium alginate is formed. It is not soluble in water and can be easily separated, leaving relatively pure fucoidan in the extract.

January et al. [43] used all three previously described solvents (salt - $CaCl_2$, acid and water) to extract fucoidan from *Ecklonia maxima*, *Laminaria pallida* and *Splachnidium rugosum*. Their results showed that conventional hot water extraction (HWE) resulted in the highest concentration of L-fucose while acid extraction resulted in the highest sulfate and uronic acid content. On the contrary, while investigating fucoidan extraction from *Sargassum fusiforme*, Liu et al. [54] achieved the lowest sulfate and uronic acid content by applying acid as a solvent. Furthermore, they achieved the highest fucoidan yield with acid extraction (11.24%) and the lowest with $CaCl_2$ method (3.94%). MW of fucoidan extracted with acid was significantly lower while acid and salt extraction removed almost all protein indicating higher purity of the extract. DPPH and hydroxyl radical scavenging activities were much higher for fucoidan extracted with water and salt compared to acid extracted fucoidan which was positively correlated with the uronic acid content, MW and monosaccharide composition (glucose + galactose).

3.2.2. Advanced Extraction Techniques

Recently, advanced extraction techniques used for polysaccharide extraction from algae are microwave-assisted extraction (MAE), ultrasound assisted extraction (UAE), pressurized liquid extraction (PLE) and enzymatic assisted extraction (EAE). However, due to various extraction conditions, APS degradation could occur which may affect the extract viscosity, sulfate content, monosaccharide composition, MW and bioactivity. Therefore, extraction parameters such as temperature, time, power and sample to solvent ratio should be optimized.

Table 1. The most frequent conventional extraction parameters used for the recovery of brown algae polysaccharides.

Algae	Polysaccharide	PRETREATMENT	EXTRACTION	Purification	Yield	References
		Solvent; Time; Temperature	Solvent; Time; Temperature			
S. henslowianum	fucoidan	95% EtOH; 2 × 12 h	H_2O; 3 × 2 h; reflux	EtOH precipitation; dialysis (12000 Da)	5.1%	[46]
S. fusiforme	fucoidan	95% EtOH; 24 h; 30 °C	H_2O; 3 h; 80 °C 1.0M HCl; 6 h; 25 °C 2% $CaCl_2$; 3 h; 50 °C	EtOH precipitation; dialysis (3,5 kDa)	3.94–11.24%	[54]
E. maxima *L. pallida* *S. rugosum*	fucoidan	/	H_2O; 24 h; 70 °C 0.15M HCl; 2 h; 65 °C	EtOH precipitation	/	[43]
		methanol-chloroform-H_2O (4:2:1); overnight; room temp.	2% $CaCl_2$; 5 h; 85 °C	10% CTAB		
C. barbata	laminarin	acetone-methanol (7:3); 2 × 24 h; 30 °C chloroform; 2 × 24 h; 30 °C	0.1M HCl; 2 × 2 h; 60 °C	EtOH precipitation; ultrafiltration (50, 10 & 1 kDa)	7.27%	[42]
Cystoseira compressa	sodium alginate	acetone; 2 × 24 h; 25 °C methanol; 2 × 24 h; 25 °C	0.1M HCl; 2 h; 60 °C 3% Na_2CO_3; 2 h; 60 °C	EtOH precipitation; dialysis (3,5 kDa)	fucoidan—5.2%	[16]
Dictyopteris divaricata	polysaccharides	/	H_2O; 5–7 h; 80–100 °C; water to solid ratio 90–110 mL/g	EtOH precipitation	3.05%	[50]
Sargassum latifolium	sodium alginate	/	2% citric acid; 2 h; room temperature 3% Na_2CO_3; 1–3 h; 25–45 °C	EtOH precipitation	18.89–40.43%	[52]
Fucus serratus *F. vesiculosus* *A. nodosum*	fucoidan	85% EtOH; overnight; room temp.	0.1M HCl; 4 h; 80 °C 1% $CaCl_2$; overnight; 4 °C	EtOH precipitation	*F. serratus*—4.2–7.5% *F. vesiculosus*—8.1–12.2% *A. nodosum*—6.5–8.9%	[51]
D. Membranaceae *P. Pavonica*	sodium alginate	methanol-dichloromethane (1:1); 3x48h; room temp. petroleum ether; soxhlet acetone; soxhlet	2% $CaCl_2$; 3 × 3h 1M Na_2CO_3; 2 h	EtOH precipitation	*D. Membranaceae* - 18.93% *P. Pavonica*—66.72%	[45]

Table 1. *Cont.*

Algae	Polysaccharide	PRETREATMENT	EXTRACTION	Purification	Yield	References
		Solvent; Time; Temperature	Solvent; Time; Temperature			
Cystoseira sedoides	fucoidan sodium alginate	acetone; 24 h; 25 °C 80% EtOH; 24 h; 25 °C 80% EtOH; 24 h; 78 °C	2% $CaCl_2$; 7 h; 70°C 2% Na_2CO_3; 70°C	dialysis (7 kDa)	fucoidan—4.2% alginate—11%	[15]
C. myrica	polysaccharides	petroleum ether acetone	H_2O; 8 h; 80°C	EtOH precipitation; 10% CTAB; dialysis	5.3%	[44]
Cystoseira crinite *C. compressa* *C. sedoides*	fucoidan	methanol-dichloromethane (1:1); 3 × 48 h; room temp.	2% $CaCl_2$; 3 × 3h	dialysis (30 kDa)	2.8–3.7%	[55]

Microwave Assisted Extraction (MAE)

MAE is considered one of the most efficient extraction techniques that can overcome drawbacks of conventional procedures. During microwave treatment, heat is generated directly within the material (volumetrically distributed heating) by ionic conduction of dissolved ions and/or dipole rotation of polar solvent. Non-polar compounds are hence not heated when exposed to microwaves. Rapid internal heating during MAE causes an effective cell wall rupture and release of the intracellular compounds into the extraction solvent [55]. Microwave radiation can also stimulate cuticular layer destruction which was observed as very rough algae surface with many cavities after high pressure (120 psi) MAE application [56]. MAE has been successfully used for isolation of various bioactive compound from seaweeds [57–60] as well as polysaccharides from other plants [61–63]. Application of MAE for brown APS extraction is summarized in Table 2 along with its effect on chemical structure and bioactivities of target polysaccharides.

Alboofetileh et al. [64] confirmed that MAE is more efficient than conventional HWE of polysaccharides from *Nizamuddinia zanardinii* with extraction yields being 6,17% vs. 5,2%, respectively. Higher crude extract yield can be correlated to higher amount of bioactive compounds, but there is a possibility that it is a result of higher amount of impurities [65] which can explain higher yields obtained by CE compared to MAE in other research [55,65]. These contradictory results may be attributed to differences in extraction protocols, algae species, origin, harvest time and extraction conditions [65]. Another point that should be taken into consideration when considering method efficiency is much shorter extraction time of MAE as well as 3 times lower solvent volume [55].

Polysaccharides obtained by MAE have higher concentration of sulfate groups and lower MW [65,66]. Alboofetileh et al. [64] reported higher sulfate content and higher MW while Yuan and Macquarrie [55] reported lower sulfate content and lower MW of the polysaccharides extracted by MAE compared to HWE. MAE had no significant effect on type of glycosidic bond and monosaccharide compositions in fucoidan from *Sargassum thunbergii* [66] while higher fucose content was reported for *A. nodosum* and *N. zanardinii* fucoidan [55,64,65] as well as lower uronic acid content [55,64,65]. On the contrary, higher uronic acid, galactose and neutral sugars contents was reported by Okolie et al. [65]. Moreover, polysaccharide extracts obtained by MAE showed higher antioxidant and hydroxyl radical scavenging activity as well as potential hypoglycemic activity [66] due to the lower MW and higher sulfate groups content in comparison to CE. They also improved the growth rate of *Lactobacillus delbruecki* ssp *bulgaricus*, while no apparent effect was found on the growth rates of *Lactobacillus casei* [65]. Fucoidan extracted by MAE at 90 °C had similar DPPH scavenging effect and even higher reducing power than fucoidan extracted by CE [55]. At 2 mg/mL MAE polysaccharides inhibited the growth of *Escherichia coli* although showed lower inhibitory activity against HSV-2 strain [64].

Besides extraction technique, extraction parameters such as microwave power, irradiation time, temperature and pressure also have an impact on polysaccharide yield and thus should be optimized. Polysaccharides extraction yield from *Sargassum pallidum* and *S. thunbergii* increased significantly with increased time, temperature and power, while optimal MAE conditions were set at 10 min, 90 °C, 800 W [67] and 20 min, 70 °C, 600 W [66]. Similar trend for time and temperature influence was observed by Yuan & Macquarrie [55] on *A. nodosum* polysaccharides, where the highest yield was achieved at 120 °C for 15 min. It is expected that an increase in temperature should decrease the viscosity and surface tension therefore improving compound solubility, diffusion rate and mass transfer in the solvent [68]. In the initial extraction stage higher temperature, longer time and higher power accelerated the mass transfer of intracellular substances. However, excessive extraction time, temperature and microwave power can lead to the degradation of some APS and reduced yield. As shown by Rodriguez-Jasso et al. [56] interaction between pressure and extraction time in MAE was highly significant ($p < 0.01$) for fucoidan yield from *Fucus vesiculosus* and maximum yield (18.22%) was achieved when the highest pressure (120 psi) and the lowest extraction time (1 min) were applied.

Table 2. Reported parameters of microwave assisted extraction for extraction of brown algae polysaccharides.

Algae	Polysaccharide	PRETREATMENT	EXTRACTION	Purification	Yield	References
		Solvent; Time; Temperature	Solvent; Time; Temperature			
F. vesiculosus	fucoidan	/	H_2O; 1–31 min; 30, 75, 120 psi 1% $CaCl_2$; overnight; 4 °C	EtOH precipitation	1.06–18.22%	[56]
S. thunbergii	polysaccharides	/	H_2O; 15-25 min; 60–80 °C; 500–700 W; water to sample ratio 25:1, 30:1, 35:1 mL/g	EtOH precipitation	2.41–2.75%	[66]
N. zanardinii	fucoidan	85% EtOH; overnight; room temp.	H_2O; 2 × 20 min; 90 °C; 700 W 1% $CaCl_2$; 14 h; 4 °C	EtOH precipitation	6.17%	[64]
A. nodosum	fucoidan	80% EtOH; 20 h; room temp. 80% EtOH; 5 h; 70°C	0.01M HCl; 15 min; 90 °C 2% $CaCl_2$; overnight; 4 °C	EtOH precipitation	5.71%	[65]
A. nodosum	fucoidan	80% EtOH; 18 h; room temp. 80% EtOH; 4 h; 70°C	0.01M HCl; 5–30 min; 90–150 °C 2% $CaCl_2$; overnight; 4°C	EtOH precipitation	6.48–16.08%	[55]
S. pallidum	polysaccharides	/	EtOH (19–27%) and ammonium sulfate (20–24%); 5–25 min; 70–110°C; 600–100 W	dialysis (3000Da); EtOH precipitation	5.6–8.26%	[67]

MAE parameters also had a strong influence on fucoidan monosaccharide composition, sulfatation degree, MW and biological activities. The monosaccharide composition of fucoidan from *A. nodosum* indicated that fucose is the major constituent of fucoidan extracted at 90 °C while glucuronic acid is the main component of fucoidan isolated at 150 °C [55]. Similarly, by increasing the extraction pressure from 30 psi to 120 psi fucose content, in fucoidan from *F. vesiculosus*, decreased from 100% to 27% and galactose content increased from 0% to 57% [56]. In addition to sulfatation degree of fucoidan that decreased with the increase of extraction temperature during MAE [55,56], scavenging effect on DPPH free radicals and reducing power also decreased with the increase in temperature and time [55]. Sulfate groups could contribute to the hydrogen-donating ability of the polysaccharides by activating the hydrogen atom of the anomeric carbon [69]. Therefore, increased sulfatation degree observed during these MAE experiments could potentially increase antioxidant, anticoagulant and anti-HIV activity of extracted APS [55].

Ultrasound Assisted Extraction (UAE)

Among the novel techniques, UAE is the most practical for the industrial level because of its simplicity, faster extraction rate, increased yield as well as reduced cost and processing time [70]. UAE can also be combined with other non-conventional technologies, such as enzymatic processing [71] or MAE [64]. The acoustic cavitation in UAE generates physical forces such as shear, shockwaves, micro jets and acoustic streaming [72], causing cell walls disruption, particle size reduction and better contact between solvent and target compounds. Furthermore, ultrasound causes a rapid formation and collapse of cavitation bubbles in treated liquid medium, leading to intense stress and irreversible chain splitting [73]. Ultrasound treatment could cause structural (MW, monosaccharide compositions, sulfate content) and microstructural modifications of the sulfated APS. Alboofetileh et al. [71,74] examined surface microstructure of fucoidan with scanning electron microscope (SEM) and micrographs showed fucoidan as distributed fluffy powder under 200 fold magnification and an irregular semi-spherical shape with no uniform size and plenty of pores at 500 and 1000 fold magnification. UAE efficiency is dependent on various factors, such as ultrasound power, temperature, time, solvents to solids ratio and characteristics of the compounds to be extracted, hence optimization of the extraction conditions is important.

Improved yield achieved by UAE in comparison to CE is attributed to the bubble cavitation phenomenon generated by ultrasonic waves [75], that was previously observed in fucoidan extraction from *Sargassum witghtii* [70], *Undaria pinnatifida* [76] and laminarin extraction from *A. nodosum* and *Laminaria hyperborean* [75]. No statistical difference between UAE and CE was reported in fucoidan extraction from *N. zanardinii* [64] and *Fucus evanescens* [77], while Okolie et al. [65] reported significantly higher yield by CE (11.9%) than by UAE (4.56%) and also no statistical differences between UAE, MAE and EAE. Alboofetileh et al. [74] compared UAE, EAE and combined UAE-EAE for isolation of polysaccharides from *N. zanardinii*. Extraction technique had a strong impact on fucoidan yield - the lowest yield was obtained by UAE (3.6%) while alcalase enzyme improved disintegration of cell wall, hence UAE-EAE exhibited the highest yield (7.87%).

Temperature controlled UAE equipment enables the study of temperature impact on polysaccharide yield. The effect of temperature in range of 30–90 °C and 70–90 °C was studied by Youssouf et al. [78] and Alboofetileh et al. [71], respectively. They noted that extraction yield increased linearly with temperature increase and reached maximum at 90 °C. By increasing the temperature, surface tension and solvent viscosity are reduced and vapour pressure is increased. Therefore, it is easy to form cavitation bubbles which intensify cellular damage, promote intracellular polysaccharides extraction and improve extraction yield [79]. By increasing the ultrasound power from 75 W to 150 W, extraction yield of alginates from *Sargassum binderi* and *Turbinaria ornata* also improves [78]. Similarly, fucoidan yield from *N. zanardinii* increased by increasing the power from 50 W to 200 W but slightly decreased above 200 W [71]. It was previously established that ultrasonic power facilitates cell walls breakdown and APS diffusion into the solution. However, greater ultrasound

power could lead to chemical decomposition caused by hydroxyl radicals generated by acoustic cavitation [79]. Fucoidan and alginate yield increased linearly with increased extraction time until they reached plateau at 40 and 30 min respectively [71,78]. Youssouf et al. [78] noted positive correlation between pH and alginate yield since high pH leads to the formation of water soluble sodium alginate.

Regarding sulfate content there was no unique trend among different authors, obtained by UAE in comparison with CE, since some reported higher sulfate concentration obtained by UAE [71], some similar [70] and some even lower [65]. Fucoidan extracted by UAE (22.97%) had lower sulfate content than the one extracted by EAE (29.6%) but higher than UAE-EAE (21.78%) [74]. In majority of extracted fucoidans main monosaccharides were fucose, mannose, galactose, xylose and glucose, with no noted impact of the extraction technique used. By comparing UAE and CE, Okolie et al. [65] reported that there was no significant difference for fucose and galactose concentration. Alboofetileh et al. [64] found no significant difference in fucose and uronic acid concentration but galactose content was slightly increased by UAE. However, destructive effect of ultrasound on fucose structure caused slight reduction of fucose concentration in UAE (23.72%) compared to HWE (26.21%) [70]. UAE demonstrated to be more efficient in extraction of higher MW fucoidan [64,65,71] and laminarin [75] then CE, MAE, PLE, EAE and UAE-EAE. Nevertheless, UAE reduced average MW of the *U. pinnatifida* polysaccharides [77].

Considering antioxidant activity of APS extracts, Hanjabam et al. [70] reported lower DPPH radical scavenging activity and reducing power along with higher metal chelating activity of fucoidan extracted by UAE in comparison to CE. Laminarin extracted from *L. hyperborea* and *A. nodosum* by UAE using acid as a solvent had the highest DPPH activity, 87.58% and 93.24% respectively [75]. Furthermore, anticancer activity of fucoidan extracted by UAE was lower than the one extracted by EAE and UAE-EAE [74]. Even though sulfated polysaccharides extracted from *N. zanardinii* by UAE didn't show antibacterial activity they exhibited potential anti-HSV-2 activity, with EC50 value of 0.082 μg/mL compared to 0.031 μg/mL in HWE [64]. Table 3 summarizes the most frequent used parameters of UAE for APS recovery from brown algae.

Pressurized Liquid Extraction (PLE)

PLE is novel extraction technique based on using elevated temperatures and pressures to extract compounds from samples in oxygen and light-free environment, in a short period of time and using less solvent. Elevated temperature allows the sample to become more soluble and achieves a higher diffusion rate, while elevated pressure keeps the solvent below its boiling point [80]. Depending on the solvent used for the extraction and its diverse working conditions, PLE is often called pressurized fluid extraction (PFE), pressurized solvent extraction (PSE), accelerated solvent extraction (ASE), subcritical water extraction (SWE) or hot water extraction (HWE) [80]. For polysaccharide extraction from brown algae different type of static [81] or dynamic [82–85] PLE equipment has been used. Commercial laboratory-scale equipment commonly used for PLE was developed by the Dionex Corporation in 1995 and it can only be operated in static (batch) mode while some in-house equipment can be used in dynamic mode (continuous flow) [86].

High temperature (>100 °C) and pressure (> 10 MPa) modify physical properties of solvent that improves its penetration, capillary effects and cell destruction, resulting in increased fucoidan yield of water PLE (13.15%) compared to CE (5.2%) from *N. zanardinii* [64]. Along with improved yield, extraction time was reduced from 6 h (two cycles of 3 h) to 20 min (two cycles of 10 min). Likewise, fucoidan yield from *N. zanardinii* obtained by PLE under optimized conditions (29 min, 150 °C, water to sample ratio of 21 mL/g and pressure of 7.5 bar) was 25.98% which was considerably higher than 5.2% obtained by CE [87]. Saravana et al. [85] achieved almost 2 times higher fucoidan yield from *Saccharina japonica* by PLE under optimized conditions (11.98 min, 127.01 °C, 80 bar and 0.04 g/mL) than by CE, while *S. japonica* extraction yield was more than 4-times higher by PLE (140 °C, 50 bar) with 0.1% NaOH as a solvent compared to 0.05M HCl CE [82].

Table 3. Reported parameters of ultrasound assisted extraction for extraction of brown algae polysaccharides.

Algae	Polysaccharide	PRETREATMENT	EXTRACTION	Purification	Yield	References
		Solvent; Time; Temperature	Solvent; Time; Temperature			
L. hyperborean *A. nodosum*	laminarin	/	H_2O and 0.03M HCl; 15 min; 60% amplitude; 20 Hz	EtOH precipitation	5.29–6.24%	[75]
U. pinnatifida	polysaccharides	/	0.01N HCl; 3-24 h; 80% amplitude	dialysis	25%	[76]
F. evanescens	fucoidan	70% EtOH; 10 days; 23 °C	H_2O; 23 °C; 5–30 min	ion-exchange chromatography	3.99–4.75%	[77]
S. witghtii	fucoidan	/	H_2O; 30 min; 50% amplitude	EtOH precipitation	14.61%	[70]
A. nodosum	fucoidan	80% EtOH; 20 h; room temp. 80% EtOH; 5 h; 70 °C	0.01M HCl; 35 min; 40% amplitude; 20 kHz 2% $CaCl_2$; overnight; 4 °C	EtOH precipitation	4.56%	[65]
N. zanardinii	fucoidan	85% EtOH; overnight; room temp.	H_2O; 2 × 20 min; 55 °C; 200 W; 20 kHz 1% $CaCl_2$; 14 h; 4 °C	EtOH precipitation	3.6%	[64]
N. zanardinii	fucoidan	85% EtOH; 24h; room temp.	H_2O; 59 min; 70 °C; 196 W; 20 kHz $CaCl_2$; overnight; 4°C	EtOH precipitation	3.6%	[74]
N. zanardinii	fucoidan	85% EtOH; 24h; room temp.	H_2O; 40–60 min; 70–90 °C; 100–200 W; 20 kHz 1% $CaCl_2$; overnight; 4 °C	EtOH precipitation	3.51%	[71]
S. binderi *T. ornata*	alginate	80% EtOH; overnight; room temp.	H_2O; 30 min; 30–90 °C; 75–150 W; 20 kHz	EtOH precipitation; 5% $CaCl_2$	27%	[78]

Even though PLE caused lower APS sulfate content in comparison to HWE, MAE and EAE [64], sulfate content achieved by using PLE with 0.1M NaOH as solvent was almost 2-folds higher than in CE [85]. In both of these studies uronic acid and fucose content were higher, while galactose and glucose content obtained by PLE were lower. Additionally, Saravana et al. [85] obtained higher total sugar, protein and phenolic content by PLE, meaning that besides improved yield impurities were also increased. Polysaccharides obtained by PLE had lower MW and higher polydispersity [64,82,85]. In contrast to polysaccharides obtained by CE, PLE extracted polysaccharides showed antibacterial activity and antiviral activity against HSV-2 infection [64]. Moreover, fucoidan obtained by PLE also showed good antioxidant, modest anti-mitotic and moderate anti-proliferative activities in cell lines [85]. There was no significant difference in DPPH radical scavenging activity between CE and PLE with water, 0.1% NaOH and 0.1% formic acid as solvents while $ABTS^+$ radical scavenging activity was significantly higher for PLE extract obtained with 0.1% formic acid and water then for CE extract [82]. Hydrogen atoms from different monosaccharide components and their side-chain linkages may be the reason for scavenging capacity of polysaccharides extracted with water and NaOH [82].

Temperature plays a key role with respect to fucoidan yield. In general, elevated temperature results in improved extraction yield [83,87] up to a certain point after which yield stagnates or decreases [82,85]. Similar effect occurs with UAE and has been previously explained. A group of authors [83,85] determined polysaccharides yields from *Saccharina japonica* hydrolysates and found them to be enhanced by increasing the pressure from 20 to 80 bar [85] and from 13 to 520 bar [83]. Similarly, polysaccharide yield increased when extraction time was increased from 10 to 30 min in the research of Alboofetileh et al. [87]. Sample to solvent ratio was the main factor affecting the fucoidan yield in the research by Saravana et al. [85]. In a tested range from 0.04 to 0.09 g/mL yield was enhanced up to 0.05 g/mL and decreased with further sample to solvent ratio increase. RSM and Box–Behnken design (BBD) were used to determine optimal conditions for fucoidan extraction from *N. zanardinii* [87] which were determined at the temperature of 150 °C, time of 29 min and sample to solvent ratio of 21 g/mL. Temperature of 127.01 °C, pressure of 80 bar and sample to solvent ratio of 0.04 g/mL were optimal conditions for fucoidan extraction from *S. japonica* [85].

By using 0.1% NaOH, 0.1% formic acid and water as extraction solvent, higher temperature positively influenced the sulfate content, although temperatures higher than 170 °C under the pressure of 75 bar did not contribute to further yield increase [82]. Fucoidan extracted with 0.1% formic acid had the lowest sulfate content followed by water extract and 0.1% NaOH extract. Ester bonds between polysaccharide chain and sulfate groups are not easily broken by NaOH while water at high temperature breaks down ester bonds more effectively [82]. The highest uronic acid content in extracted fucoidan was obtained by using formic acid at 110 °C and 25 bar, while the highest total sugar concentration was obtained at 140 °C and 50 bar [82] and at 180 °C and 13 bar [83]. As temperature and pressure further increased, concentration of uronic acid and sugars gradually decreased indicating that monosaccharides are not stable at higher temperature and pressure [83]. Fucose was the main monosaccharide of the fucoidan extracted by PLE, while mannose, galactose, xylose and glucose were also present in the majority of samples [81,82,87]. MW of fucoidan extracted with formic acid was significantly lower than that of fucoidan extracted with 0.1% NaOH, water or ethanol, indicating that acid extraction may have caused APS chains decomposition [82]. Saravana et al. [84] used subcritical water treatment to depolymerize fucoidan powder extracted from *U. pinnatifida* and to study the influence of different parameters on the antioxidant activity (DPPH and $ABTS^+$). Results showed that antioxidant activity is increased as temperature and pressure are increased for both DPPH and $ABTS^+$, however after 250 °C the activity was reduced. Table 4 summarizes the most frequently used parameters of PLE for APS recovery from brown algae.

Table 4. Reported parameters of pressurized liquid extraction for extraction of brown algae polysaccharides.

Algae	Polysaccharide	PRETREATMENT	EXTRACTION	Purification	Yield	References
		Solvent; Time; Temperature	Solvent; Time; Temperature			
N. zanardinii	fucoidan	85% EtOH; overnight; room temp.	H_2O; 2 × 10 min; 150 °C; 1500 W 1% $CaCl_2$; 14 h; 4°C	EtOH precipitation	13.5%	[64]
N. zanardinii	fucoidan	85% EtOH; 24 h; room temp.	H_2O; 10-30 min; 90–150 °C; 1500 W; 7.5 bar; 20–40 mL/g; 1% $CaCl_2$; overnight; 4 °C	EtOH precipitation	4.99–23.77%	[87]
S. japonica	fucoidan	/	H_2O; 0.1% NaOH; 0.1% formic acid; 70% EtOH; 50% EtOH; 25% EtOH; 5 min; 80–200 °C; 5–100 bar; 200 rpm 1% $CaCl_2$; overnight; 4°C	EtOH precipitation	8.23%	[82]
S. japonica	fucoidan	supercritical CO_2; 4 h; 50°C; 300 bar	0.1% NaOH; 5–15 min; 100–180 °C; 20–80 bar; 100–300 rpm 0.04–0.09 mg/mL	EtOH precipitation	0.1–12.89%	[85]
Himanthalia elongata	polysaccharides	/	H_2O; 20 min; 100°C	EtOH precipitation; dialysis	15.1%	[81]

Enzymes Assisted Extraction (EAE)

Enzymes assisted degradation of cell wall polysaccharides is a useful technique widely used to improve extraction efficiency of bioactive compounds from terrestrial plants but not that often from seaweed. EAE showed higher extraction yield, faster extraction rate, lower energy consumption and simpler recovery with reduced solvent usage in comparison to CE [88]. EAE of polysaccharides could be performed with enzymes capable of cell wall degradation or enzymes that cause partial degradation of desirable polysaccharides into smaller fragments with the aim of facilitating the extraction. Various types of commercially available carbohydrate hydrolytic enzymes and proteases are used for APS extraction. Their use is practical in commercial applications and cost effective for industry, whereas seaweed polysaccharide-specific hydrolytic enzymes such as fucoidanases and alginases are still difficult to access [89]. Since fucoidans are closely associated with cellulose and proteins, which limit their extractability by chemicals, their hydrolysis by commercially available carbohydrate hydrolytic enzymes and proteases can facilitate weakening of the cell wall complex and release of the target APS (fucoidan and alginate) without significant degradation [89]. Some of the commercially available enzymes are: (i) Alcalase—protease which hydrolyse peptide bonds; (ii) Viscozyme—a mixture of carbohydrases (cellulase, β-glucanase, hemicellulase and xylanase) that catalyzes the breakdown of pectin-like substances in the algal cells; (iii) Celluclast—able to break down cellulose in algal cells into glucose, cellobiose and longer glucose polymers; (iv) AMG—a type of amyloglucosidase which breaks down starches consisting of 1,4 and 1,6 linkages; (v) Termamyl—a type of heat-stable α-amylase; and (vi) Ultraflo—a type of multiactive β-glucanase [18]. Apart from the enzyme type other process parameters such as temperature, time, pH and enzyme concentration or enzyme to sample ratio, are crucial for the extraction process and should be optimized.

Results demonstrated by Alboofetileh et al. [90] showed better cell wall disruption by Alcalase. Higher extraction efficiency of fucodian (5.58%) was therefore obtained with enzymatic assistance compared to conventional HWE (5.2%). Furthermore, fucoidan yield obtained by the use of Celluclast (4.8%) and Viscozyme (4.28%) was lower than the one obtained by CE. The reason for that could be that polysaccharides were probably partially hydrolysed after prolonged extraction time (24 h) in the presence of the enzymes. The lowest extraction yield of alginate (3.30%) was achieved with water extraction while slight increase was observed after alcalase (3.5%) and cellulase (3.47%) treatments [91]. Similarly, the yield of sulfated polysaccharides from *Turbinaria turbinate* obtained from cellulose, amyloglucosidase and vicozyme assisted extraction were higher than those obtained without EAE processes [92]. Alginate yield increased up to 6.60% after Cellulase treatment while Alcalase did not improve alginate yield in comparison with conventional water extraction without enzyme assistance (3.8%) [93]. Total sugar yield and composition was differently affected by carbohydrate hydrolases (Viscozyme, Celluclast and Ultraflo) and proteases (Alacalase, Neutrase and Flavourzyme) [89]. Viscozyme and Celluclast produced around twice as much total sugars than proteases and only Neutrase, Celluclast and Viscozyme and Celluclast mixture slightly improved total sugar yield in comparison to the corresponding controls. In another research by Alboofetileh et al. [74] EAE with alcalase had higher fucoidan yield (5.58%) than UAE (3.6%) but combining EAE with UAE resulted in even higher yield (7.87%). Cell wall weakening that occurs during the enzymes hydrolysis treatments could potentially increase the effect of the following extraction process [94]. Extraction yields obtained with 2h alcalase treatment coupled with PLE and PLE alone were not statistically different ($p > 0.05$) while viscozyme treatments coupled with PLE produced lower yields [94].

Research by Hammed et al. [92] revealed that hydrolysis time, extraction stages and enzyme concentration had significant positive effects on sulfated polysaccharides yield. Accordingly, the highest fucoidan yield (25.13%) was achieved under optimum conditions determined at hydrolysis time of 19.5 h, 2 extraction stages and enzyme concentration of 1.5 µl/mL. Regarding the pH effect on total sugar yield, significantly higher concentration was achieved at pH 4.5 compared to pH 6–8 while MWs were significantly lower at pH 4.5 [89].

Alcalase extracted fucoidans had the highest sulfate content and MW, the lowest uronic acid and protein content, while monosaccharide composition remained the same for EAE and CE extracted fucoidans [90]. Protease and carbohydrase enzymes used before extraction, reduced the amount of proteins in alginates below 0.4% [91]. Likewise, Alcalase and Cellulase treatment produced alginates with the lowest chemical contamination with proteins and polyphenols as well as with the lowest MW [93]. EAE reduced MW of the extracted polysaccharides from *Ecklonia radiata* by 20–50% compared to control CE what indicates that enzymes have the ability to hydrolyse certain bonds within fucoidan and alginate molecules [89]. Celluclast led to the highest polysaccharide yield, the highest sulfate content and the lowest protein content compared to the AMG, Viscozyme and Alcalase in sulfated polysaccharide extraction from *Sargassum horneri* [95]. Anticancer and immunomodulatory activities of fucoidan are influenced by higher sulfate content and higher MW which explains why Alcalase treated fucoidan exhibited higher anticancer and immunomodulatory activity [90]. Alginate produced by Alcalase and Cellulase treatment displayed higher DPPH radical scavenging activity and higher reducing power [93] while Celluclast assisted extracts of sulfated polysaccharide from *S. thunbergii* demonstrated the strongest DPPH radical and hydrogen peroxide scavenging activity [96]. Table 5 summarizes the most frequent used parameters of EAE for APS recovery from brown algae.

3.3. Purification Procedure

In addition to having variable MWs, monosaccharides composition and sulfate content, extracted APS are usually contaminated with proteins and low molecular weight compounds which were also dissolved in water during extraction [3]. Therefore, they can be additionally purified using different purification methods such as ethanol precipitation, membrane separation, ion-exchange, size-exclusion and affinity chromatographic methods. As it depends on the purity requirements and further function, there are no standard protocols set for purification. In functional food industry ethanol precipitation is the most frequently used method while in scale-up testing membrane separation can be used [3].

Ethanol precipitation is often used as the first step in APS purification, which removes low molecular weight impurities from the polysaccharides. Numerous researchers conducted purification step by adding two or three volumes of ethanol to the extract and allowing the mixture to precipitate overnight at 4 °C [16,42,46,52,54]. Due to shielded oppositely charged groups APS are soluble in solvents with higher dielectric constants such as water. [13]. However, by using solvent with lower dielectric constant, like ethanol, polysaccharides sulfate groups and positive ions will form ionic bonds, which will result in precipitation. Precipitated polysaccharides can therefore be separated from the supernatant by centrifugation. Supernatant with other non-polysaccharide impurities is discarded while precipitate is then dissolved in water and sometimes treated with $CaCl_2$ to precipitate alginates. Alginates are then removed by centrifugation, while fucoidan is left in supernatant. Since fucoidan is a macromolecule, supernatant goes through dialysis against water to remove other low molecular weight impurities (laminarin, oligosaccharides and inorganic salts) prior to drying. To isolate fucoidan from the extract January et al. [43] and Sahera et al. [44] used cationic detergent hexadecyltrimethylammonium bromide (CETAVLON or CTAB). In addition, asfucoidan is a sulfated (SO_3^{2-}) polysaccharide, it is negatively charged (polyanion) and may form salts with cationic detergents. These salts are highly insoluble in water and hence they will precipitate. Laminarin and alginate are neutral APS and therefore do not react with cationic detergents and remain water soluble [97].

After isolation fucoidan needs to be dried with one of the various drying methods such as oven drying, vacuum drying, spray-drying and lyophilisation. Selection of drying method depends on the analysis requirements along with potential use of the extracted fucoidan [19]. Although it is quite slow and has relatively low capacity, lyophilisation is usually the preferred method due to its low effect on the fucoidan structure and bioactivity [19]. Furthermore, various combinations of chromatographic techniques can be used to achieve high throughput screening and percent purification. For validation purposes, numerous analytical equipment and instrumental techniques can be used to identify and quantify the active fractions of extracted compounds [98].

Table 5. Reported parameters of enzymes assisted extraction for extraction of brown algae polysaccharides.

Algae	Polysaccharide	EXTRACTION	Purification	Yield	Reference
		Enzyme; Concentration; PH; Temperature; Time			
N. zanardinii	fucoidan	Alcalase (2.5 mL/g; pH7; 50°C; 24 h)	1% $CaCl_2$; overnight; 4°C EtOH precipitation	5.58%	[74]
S. thunbergii	sulfated polysaccharide	24h Viscozyme, Celluclast, AMG, Termamyl, Ultraflo, Protamex, Kojijyme, Neutrase, Flabourzyme, Alcalase	EtOH precipitation	18.4–28.3%	[96]
N. zanardinii	fucoidan	Alcalase (5% v/v; pH8; 50 °C; 24 h) Celluclast (5% w/v; pH4.5; 50 °C, 24 h) Viscozyme (5% v/v; pH4.5; 50 °C; 24 h) Flavourzyme (5% v/v; pH7; 50 °C; 24 h)	$CaCl_2$ - alginates removal EtOH precipitation	Alcalase—5.58% Celluclast—4.80% Viscozyme—4.28% Flavourzyme—4.36%	[90]
Colpomenia peregrina	alginates	Alcalase (5% w/w; pH8; 50 °C; 24 h) Cellulase (5% w/w; pH4.5; 50 °C; 24 h)	3% Na_2CO_3; pH11; 65°C; 3 h EtOH precipitation	Alcalase—3.8% Cellulase—6.6%	[93]
Sargassum angustifolium	alginates	Alcalase (5% w/w; pH8; 50 °C; 24 h) Cellulase (5% w/w; pH4.5; 50 °C; 24 h)	3% Na_2CO_3; pH11; 65°C; 3 h EtOH precipitation	Alcalase—3.5% Cellulase—3.47%	[91]
Turbinaria turbinata	polysaccharides	cellulase, amyloglucosidase and vicozyme	EtOH precipitation	14–21%	[92]
S. horneri	sulfated polysaccharide	24h AMG, Celluclast, Viscozyme, Alcalase	EtOH precipitation	AMG—71.63 Celluclast—88.7% Viscozyme—84.68% Alcalase—81.25%	[95]
Sargassum muticum	bioactive compounds	Viscozyme (pH4.5; 50 °C; 2 and 4 h) Alcalase (pH7; 50 °C; 2 and 4 h)	/	13.6–23.5%	[94]
E. radiata	carbohydrates	10% v/w; 50 °C; 24 h Viscozyme (pH 4.5); Celluclast (pH 4.5); Ultraflo (pH 7); Alcalase (pH 8); Neutrase (pH 6); Flavourzyme (pH 7)	EtOH precipitation	/	[89]

4. Brown Algae Sulfated Polysaccharides as Drug Delivery Systems and Their Safety

Common commercial applications of APS are mainly related to their colloid properties such as high water-solubility, hydrophilicity and chain aggregation [99]. These properties enable the APS to act as emulsifiers, stabilizers, flocculants as well as gelling, hydrating and thickening agents [100]. APS such as alginate, extracted from brown seaweed, as well as agar and carrageenan from red seaweed, are used by the food industry in numerous food products and beverages adding up to a market value of $10.000 per ton of dry seaweed [101]. As it was shown, alginate and other brown algae sulfated polysaccharides such as fucoidan and laminarin are also being researched for their anti-tumor [102], anti-microbial [103], immunostimulatory [104] and anti-inflamatory [105] activity. In addition, new advances are being made in the research of their drug delivery, tissue engineering and skin rejuvenating properties.

Newly developed active substances are lately calling for innovative, tailor-made drug delivery systems that can secure controlled and targeted drug release. Application of brown APS, due to their excellent gel forming properties, biodegradability and non-toxicity offers many possibilities for the creation of various controlled release matrices, i.e., beads, microparticles, nanoparticles, films etc. [106]. Such polysaccharide matrices are loaded with active ingredients which are, by diffusion and pH related erosion, released in targeted tissues [107]. Release mechanisms of active compounds from different grades of alginate matrices at different acidity were investigated by Sriamornsak et al. [108]. They found all tested alginate salts, except sodium/calcium alginate in acidic and ammonium/calcium alginate in both neutral and acidic medium, can extend drug release period to 8–10 h. In addition, added sodium bicarbonate had influence on acidic environment, which means that it acted successfully as a local pH modifier. On the other hand, calcium acetate can be added to enhance cross-linked gel formation through ionic interactions of alginate G-blocks and calcium cations. These mechanisms thereafter enable brown APS to target the release of active compounds to specific parts of the digestive system, e.g., for gastric or intestinal delivery [109]. Incorporation of compounds such as citric acid, sodium, calcium and zinc salts can also modify the drug release rates of brown APS delivery systems [99,110,111]. Furthermore, drug release targeting can be made more accurate by innovative modulations with chitosan, gelatine and pectin [112–117]. These modulations often have superior pH stability and bioadhesive properties and can, e.g., enable the release of active compounds in the colon by biodegrading [118]. Gastroretention can be achieved by the formation of porous systems that are able to keep buoyant in the stomach for up to 24 hours [119–121]. In addition, brown algae sulfated polysaccharides drug delivery systems are also being developed for the ophthalmic drugs application and offer enhanced ocular bioavailability as well as adequate hydrogel strength and optical clarity [122,123]. Innovative techniques such as nanoencapsulation are also used to further improve active compounds bioavailability and decrease systemic toxicity [124–126]. The variety of drugs for which the delivery can be targeted by brown algae sulfated polysaccharides include antitumor drugs [127], anti-tubercular drugs [128], peptide and protein drugs [129,130], antibiotics [131] etc.

Brown algae sulfated polysaccharides also play very important role in tissue engineering research and development of wound specific healing dressings. Their hydrogels can offer optimal moist environment and oxygen flow while also act as efficient bacterial barriers. They are also highly biocompatible materials and can be easily removed, e.g., via saline irrigation [132]. Lee et al. [133] investigated the wound healing properties of alginate in the treatment of full-thickness skin defects in rats and found significant decrease of wound sizes in alginate treated groups. Fucoidan alone or in combination with chitosan also showed the best reepithelization and fastest wound closure results in rabbit dermal burns [134]. On the other hand, Custódio et al. [135] have shown that laminarin can be used for the formation of biocompatible, mechanically stable and injectable hydrogels that can be used for soft tissue repair. Similarly, alginate and its composites are being researched in the field of bone tissue engineering due to their superior adhesion to cells, biocompatibility and regeneration properties [136]. Besides the physical effects of wound protection, brown APS also possess important pharmacological function in the wound healing process. This role is often attributed to the bioactivity

of calcium ions, deriving from calcium alginate, for example [137,138]. In addition, more complex wound healing mechanisms based on the coordinated anti-inflamatory, antioxidative and growth-factor dependant properties of APS are proposed by other authors [133,134]. Additionally, various studies are combining their wound healing activity and drug delivery properties therefore creating active tissue healing accelerators. For this purpose, a variety of pharmacologically active compounds e.g., vitamins, antibiotics, growth factors, etc., are being evaluated [139].

Following the global clean labelling trends, cosmetic industry is recently reducing the use of synthetic chemicals and aiming towards the formulations based on natural sources. Due to remarkable biological activity which includes antioxidative, anti-inflammatory and anti-aging activity as well as depigmenting and UV-shielding effects, brown APS are recently in the focus of numerous studies which deal with their application in skin care products [140]. Wang et al. [141] researched the moisture absorption and retention ability of polysaccharides extracted from various seaweed and found that brown APS had the best results even when compared to hyaluronic acid. Sulfated groups were determined as the main active sites for this ability. Fernando et al. [142] concluded that brown algae sulfated polysaccharides have notable antioxidative and anti-inflammatory properties and are able to inhibit collagenase, elastase and tyrosinase therefore causing anti-wrinkling and skin-whitening effects. Brown algae extracts evaluated in the research of Fitton et al. [143] positively affected skin protection, soothing and spot reduction.

However, much more research is needed to characterize the bioactive content from natural sources such as of brown algae to fully understand their benefits and possible concerns. As yet no reports about toxic effects of marine APS are available and no undesirable effects of overdose and sensitivity to polysaccharides are reported [3]. One of the studies which reported that consumption of fucoidan at the recommended dose (2,000 mg/kg bw/day) did not cause any toxicological indications was performed by Hwang et al. [144]. Neither toxicity nor mutagenicity of fucoidan from *L. japonica* was observed at concentration of 5,000 μg/mL [144] and no toxicity was measured in any blood samples when consuming *F. vesiculosus* extract with 85% fucoidan (300 mg) for 12 weeks [145]. Acute toxicity results by Lim et al. [146] classified *S. binderi* extract as category 5 (unclassified) according to the OECD Guideline since no mortality in rats was observed at the highest dosage (2,000 mg/kg).

5. Future Challenges and Potential Industry Application of Brown Algae

The market demand for functional foods enriched with ingredients derived from natural sources is rapidly increasing. Due to the various biological activities, marine APS have a great potential to be used in a wide range of functional foods, cosmeceutical and pharmaceutical products. Development of the standard extraction procedure, including pre-treatment and purification step, appears to be crucial for preservation of the polysaccharides structural integrity and consequently their biological properties. Even though advanced extraction techniques, such as MAE, UAE, PLE and EAE, may display higher extraction efficiencies along with reduced time, cost and energy consumption, their application in marine APS extraction is so far limited to the laboratory research since no research on an industrial scale has been reported. Furthermore, it is highly desirable to develop a simple and reliable method for marine APS structural characterization as it could contribute to the better understanding of their structure-bioactivity relationships which is, regardless of intensive research, not completely clarified.

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3. The overview of the most recent studies on brown algae polysaccharide extraction

The number of published articles dealing with the extraction and potentially beneficial biological activities of brown algae polysaccharides increased considerably in recent years. An introduction to this broad topic and the latest, most important findings on the application of pre-treatments and various techniques for the extraction of brown algal polysaccharides were summarized and presented in *Publication No. 1*. Overview of publications published after March 2020 (when *Publication No. 1* was published) where conventional (CE) and advanced extraction techniques, such as microwave-assisted extraction (MAE), ultrasound assisted extraction (UAE), and enzymatic assisted extraction (EAE), were used for brown algae polysaccharide extraction is given in Tables 1 and 2. Various combinations of pre-treatments and CE parameters (solvent, time and temperature) have been applied. Pre-treatment is usually performed with ethanol (EtOH) as one step process, with either combination of room temperature and longer time (12-24 h) (Manikandan et al., 2020; Narayani et al., 2020; Zou et al., 2021) or combination of higher temperature (60-70°C) and shorter time (4-5 h) (Shan et al., 2020), or as two steps process combining these two approaches (Mohd Fauziee et al., 2021; Sharma & Baskaran, 2021). Most frequently used solvents for fucoidan extraction are H_20 (Manikandan et al., 2020; Shan et al., 2020), and mild acidic conditions, 0.05M (Zou et al., 2021) and 0.1M HCl (Benslima et al., 2021; Sharma & Baskaran, 2021), while Na_2CO_3 is frequently used for alginate extraction (Sharma & Baskaran, 2021; Zhang et al., 2020). Applied extraction temperature ranged from 60 to 110°C and time from 1 to 9 h.

Conventional techniques for polysaccharide extraction require long extraction time and high extraction temperature, but the extraction efficiency is usually low. Therefore, advanced techniques which can overcome those disadvantages, have been widely employed for brown algae polysaccharide extraction. A detail overview of studies in which UAE, MAE and EAE have been used for brown algal polysaccharide extraction is given in our review article (Dobrinčić et al., 2020). Overview of most recent studies with similar subject, from 2020 and 2021, is shown in Table 2. Wang et al. (2021) applied CE, MAE and UAE for extraction of *Sargassum siliquosum* fucoidan and noted the highest extraction efficiency with MAE, followed by CE and UAE (S. H. Wang et al., 2021). What makes MAE especially efficient for fucoidan extraction is significant time reduction, from 60 min to 10 min (S. H. Wang et al., 2021). On the other hand, the application of higher ultrasonic power resulted in polysaccharides degradation (S. H. Wang et al., 2021). Higher yield of *Ascophyllum nodosum* sodium alginate

was achieved with CE than MAE and even higher with combination of EAE and CE (Okolie et al., 2020). Likewise, higher *Fucus evanescens* fucoidan yield was obtained with EAE, using commercial cellulase preparation (Cellic®CTec2) and alginate lyase, than CE (Nguyen et al., 2020).

Table 1. Most recently reported conventional extraction parameters used for the recovery of brown algae polysaccharides

Algae	Polysaccharide	PRETREATMENT	EXTRACTION	Purification	Yield	Reference
		solvent; time; temperature	solvent; time; temperature			
Cystoseira schiffneri	fucoidan	acetone-methanol (7:3); 2x24h; 30°C chloroform; 2x24h; 30°C	0.1M HCl; 2 h; 60°C	dialysis (14 kDa); EtOH precipitation	1-2.4%	(Benslima et al., 2021)
M. pyrifera	fucoidan	80% EtOH; 24 h; room temp.	0.05M HCl; 3 h; 60°C	dialysis (3.5 kDa); EtOH precipitation	3.3%	(Zou et al., 2021)
A. nodosum *Laminaria japonica* *Kjellmaniella crassifolia*	fucoidan	95% EtOH; 4 h; 60°C	H_2O; 2×3 h; 80°C	dialysis (10 kDa); EtOH precipitation	/	(Shan et al., 2020)
Turbinaria decurrens	fucoidan	85% EtOH; 12 h; room temp.	H_2O; 1 h; 65°C	EtOH precipitation	5.32%	(Manikandan et al., 2020)
Sargassum polycystum *Turbinaria ornate* *Padina boryana*	fucoidan; laminarin	85% EtOH; 16 h; 23°C 85% EtOH; 5 h; 70°C	2% $CaCl_2$; 3 h; 70°C	EtOH precipitation; dialysis (10 kDa i 2 kDa)	1.16%; 0.76% 0.65%; 0.39% 1.59%; 0.53%	(Mohd Fauziee et al., 2021)
Sargassum cinereum	fucoidan	EtOH; 12 h; room temp.	pH 2-9; 3-5 h; 80-110°C	EtOH precipitation	5.651%	(Narayani et al., 2020)
S. cinereum	fucoidan	/	H_2O; 4-5 h; 80 °C	EtOH precipitation	2.78%	(Nurhidayati et al., 2020)

Sargassum sp.	fucoidan	/	0.1 N HCl; 4 h; 80°C	EtOH precipitation	7.5%	(Sari et al., 2020)
F. vesiculosus *Laminari thalli*	fucoidan	/	H_2O; 2.5 h; 80°C 6% HCl; 3 h; room temp.	/	/	(Sopelkina et al., 2020)
Padina tetrastromatica	laminaran+fucoidan	85% EtOH; 2*12 h; 23°C	2% $CaCl_2$; 3 h; 70°C	EtOH precipitation; dialysis (1000 Da)	12.4%	(Sharma & Baskaran, 2021)
	fucoidan		0.01 M HCl; 3×3 h; 70°C		9.4%	
	alginate	85% EtOH; 2*5 h; 70°C	3% Na_2CO_3 (w/v); 3×3 h; 70°C		71.3%	
Carpophyllum flexuosum *Carpophyllum plumosum* *Ecklonia radiata* *Undaria pinnatifida*	sodium alginate	Soxhlet; EtOH; 2 h	1.5% Na_2CO_3; 1 h; 60°C	EtOH precipitation	5.2-15.5%	(Zhang et al., 2020)

Table 2. Most recently reported advanced extraction techniques (MAE - microwave-assisted extraction; UAE – ultrasound-assisted extraction; EAE – enzymatic-assisted extraction) used for the recovery of brown algae polysaccharides

Algae	**Polysaccharide**	**PRETREATMENT**	**EXTRACTION**	**Purification**	**Yield**	**Reference**
		solvent; time; temperature	**solvent; time; temperature**			
S. siliquosum	fucoidan	95% EtOH; 4 h; room temp.	CE – H_2O; 1h; 100°C UAE - H_2O; 10-20 min; 50-200 W MAE - H_2O; 10-20 min; 750 W	dialysis (14 kDa); EtOH precipitation	5.08% 4.78% 6.94%	(S. H. Wang et al., 2021)
Sargassum filipendula	fucoidan	chloroform:methanol (2:1); 20 min; room temp.	UAE - 350 W; 40 kHz; 70°C; 0.01M, 0.03M, 0.05M HCl; 10, 15, 20 min	EtOH precipitation	4.54-6.07%	(Laeliocattleya et al., 2020)

Sargassum mcclurei	fucoidan	/	UAE – EtOH; $CaCl_2$; 20-60 min; 40-60°C; 200-800 W	/	7.1203 mg g^{-1}	(Thao My et al., 2020)
Ecklonia cava	fucoidan	/	McIlvaine buffer (0.1 M, pH 4.5) CE – 3 h; 90°C UAE - 3 h; 50°C EAE – 2% (v/v) amyloglucosidase from *Aspergillus niger*; 3 h; 50°C	EtOH precipitation	/	(J. J. Park & Lee, 2021)
A. nodosum	sodium alginate	80% EtOH; 20 h; room temp. 80% EtOH; 5h; 70°C	CE – 0.01M HCl - 3 h - 70°C; 3% Na_2CO_3 - 3 h MAE – 0.01M HCl - 15 min - 90°C; 3% Na_2CO_3 - 10 min - 100°C UAE – 0.01M HCl - 750 W - 20 kHz - 35 min - 40% amplitude - 13 mm probe; 3% Na_2CO_3 EAE+CE – 0.1 M sodium acetate buffer (pH 4.5) - 50°C - Cellulase (pH 4.5, 50°C) - 24 h; 3% Na_2CO_3 - 3 h - 70°C	EtOH precipitation	72% 56% 70% 90%	(Okolie et al., 2020)
F. evanescens *Saccharina latissima*	fucoidan	/	CE – 0.1 MHCl; 3 h; 70°C EAE - 55 mM phosphate-38 mM citrate buffer (pH 6); 24 h; 40°C; 5% Cellic®CTec2; 0.35% alginate lyase	EtOH precipitation	40%; 43% 29%; 29%	(Nguyen et al., 2020)

4. Hypothesis, research objectives, and expected scientific contributions

The research hypothesizes that:

(i) the use of advanced extraction techniques will improve brown algae polysaccharide extraction in terms of yield and time reduction;
(ii) pre-treatment, species, extraction techniques and extraction conditions will differently affect polysaccharides yield, chemical composition and antioxidant activity.

In order to accept or decline established hypotheses, the following objectives are defined:

(i) to examine the effect of different pre-treatment solvents and advanced pre-treatments (ultrasound and non-thermal plasma) on the yield and chemical structure of *F. virsoides* and *C. barbatta* polysaccharides;
(ii) to compare the effectiveness of conventional, microwave-assisted and pressurised-liquid extraction and to define their optimal conditions for *F. virsoides* and *C. barbata* polysaccharide extraction;
(iii) to characterize isolated polysaccharides and determine their biological activity.

Due to its complexity and scope, this research is divided into 3 parts, which are systematically interconnected:

In the first part of the research, the influence of various pre-treatment solvents on polyphenols and pigment removal, polysaccharide yield and polysaccharide chemical composition were examined (***Publication No.2***).

The second part included optimization (solvent, time and temperature) of conventional, microwave-assisted and pressurised-liquid extraction, of *F. virsoides* and *C. barbata* polysaccharides, and influence of extraction method on polysaccharides chemical composition and antioxidant activity (***Publication No. 2, Publication No. 3***).

The third part of the research examined the application of ultrasound-assisted extraction and non-thermal plasma for *F. virsoides* and *C. barbata* polysaccharide pre-treatment and extraction, and their influence on polysaccharides chemical composition and antioxidant activity (***Publication No. 4***).

Throughout this dissertation the following questions were examined:

1) Which combination of solvents used in pre-treatment, prior to polysaccharide extraction, will remove the highest amount of interfering compounds (polyphenols and pigments), give the highest polysaccharide yield and how it will effect polysaccharide chemical composition? (***Publication No. 2***)
2) What are the optimal solvent, time and temperature for conventional polysaccharide extraction? How different extraction parameters affect polysaccharide chemical composition? (***Publication No. 2***)
3) What are the optimal solvent, time and temperature for microwave-assisted and pressurised-liquid polysaccharide extraction? (***Publication No. 3***)
4) Can advanced extraction techniques, microwave-assisted extraction and pressurised-liquid extraction, improve polysaccharide extraction and how will they affect polysaccharide chemical composition and antioxidant activity? (***Publication No. 3***)
5) Can application of high power ultrasound and non-thermal plasma improve polysaccharide extraction if used prior to conventional method? Can these techniques be used alone for polysaccharide extraction and how will they affect polysaccharide chemical composition and antioxidant activity (***Publication No. 4***).
6) Do *F. virsoides* and *C. barbata* polysaccharides have embriotoxic, cardiotoxic or genotoxic effect or cause behavioural changes in zebrafish embryos? Do they have antioxidant, antibacterial or antifungal activity? (***Unpublished data***)

Throughout this dissertation next was achieved:

1) the knowledge of the most efficient pre-treatment solvent for brown algae polysaccharide extraction
2) the knowledge of the optimal parameters for conventional, microwave-assisted and pressurised-liquid extraction, for brown algae polysaccharides
3) a better understanding of the influence of extraction technique on brown algae polysaccharide yield, chemical composition and antioxidant activity
4) a better understanding of the influence of ultrasound and non-thermal plasma on brown algae polysaccharides
5) characterization of the *F. virsoides* and *C. barbata* polysaccharide chemical structure
6) insight into the toxicity of *F. virsoides* and *C. barbata* polysaccharides and their antioxidant and antimicrobial activities

Chapter 2

Publication No. 2: The effectiveness of the *Fucus virsoides* and *Cystoseira barbata* fucoidan isolation as a function of applied pre-treatment and extraction conditions

Algal Research

Publication No. 2

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Sandra Pedisić: Investigation.

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The effectiveness of the *Fucus virsoides* and *Cystoseira barbata* fucoidan isolation as a function of applied pre-treatment and extraction conditions

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ABSTRACT

Fucus virsoides and *Cystoseira barbata* are important sources of sulfated polysaccharide fucoidan which shows a wide range of biological activities. These activities are significantly dependent on chemical composition which is influenced by species, anatomical part of the seaweed, growing location and conditions, extraction procedures and analytical methods. The objective of this study was to evaluate influence of various pre-treatment solvents and conventional extraction parameters (solvent, temperature and time) on polysaccharide yield and chemical composition (total sugar, fucose, sulfate group and uronic acid content) of the fucoidan from *F. virsoides* and *C. barbata*. Combination of acetone and ethanol was chosen for pre-treatment since it removed the most interfering compounds and resulted with the highest polysaccharide yield. Applying acid (0.1 M HCl and 0.1 M H_2SO_4) instead of water for polysaccharide extraction improved yield and resulted with fucoidan with higher sulfate group content, lower uronic acid content but lower fucose content. Extraction at higher temperatures and longer time resulted with higher polysaccharide yield, uronic acid and total sugars content. However, they had the opposite effect on fucose and sulfate group content between *F. virsoides* and *C. barbata*.

1. Introduction

There are between 1500 and 2000 brown algae (*Phaeophyceae*) species known worldwide [1] among which order *Fucales* includes some of the most common littoral seaweeds genera - *Fucus* and *Cystoseira*. *Fucus virsoides* is an endemic species of brown algae from the Adriatic Sea and it is the only species of *Fucus* genus occurring in the Adriatic Sea, mainly in the northern Adriatic, from the Venice Lagoon to Dalmatia [2]. *Cystoseira barbata* is representative of the Cystoseira genus, one of the most dominant and ecologically most important genera in the Mediterranean and Adriatic Sea [3].

These algae species have been proven to contain polysaccharides with various biological activities, including laminarin, alginate and fucoidan. Fucoidan is sulfated polysaccharide (PS) composed of mainly fucose interconnected by β (1,3) glycoside bonds, alternating β (1,3) and β (1,4) bonds and rarely β (1,2) bonds [4]. Apart from fucose, it also contains other monosaccharides, including galactose, glucose, mannose, xylose, rhamnose, and uronic acids, and its sulfate content varies between 5% and 38% [4].

Fucoidan is one of the most researched algae molecules and studies have found that it shows a wide range of biological activities such as antioxidant, anti-inflammatory and antitumor [4–6] thus having a great potential in the production of functional foods, cosmeceutical and pharmaceutical products [7]. Each of these properties is associated with a specific fucoidan, since their chemical composition, and consequently their biological activity, is significantly dependent on species, anatomical part of the seaweed, growing location and conditions, extraction procedures and analytical methods [8]. Prior to the algae PS extraction, it is beneficial to apply pre-treatment in order to remove proteins, lipids, phenols as well as mannitol and chlorophyll i.e. compounds which are highly bound to the PS. For that purpose, various solvents and solvent mixtures with different polarity have been used e.g. mixture of methanol, chloroform and water at 4:2:1 (v/v/v), acetone, ethanol and methanol [9–11].

From the industrial point of view, it is extremely important to be able to produce a sufficient quantity of an identical product with the desired properties. However, the ability to replicate the extracted fucoidan is significantly reduced without clear knowledge of the environmental and

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extraction parameters influence on chemical composition and biological activities [12]. The characteristics of "good quality fucoidan" are high levels of L-fucose, high degree of sulfation and low levels of contaminants such as uronic acid and protein [13]. Furthermore, it is also important to emphasize the importance of retaining the native structure of fucoidan as these structural moieties often have bioactive properties in higher biological systems [13].

Seeing how versatile fucoidan molecule can be, in this research we aimed to investigate fucoidan from endemic *F. virsoides* which up until now has not been characterized and *C. barbata*. Literature search revealed that novel extraction techniques such as microwave-assisted extraction, ultrasound-assisted extraction, enzyme-assisted extraction and autohydrolysis, have been applied and optimized for PS extraction from brown seaweed [8,14–16]. Despite all the advantages of these novel techniques, conventional extraction technique is still widely used for polysaccharide extraction but it has rarely been optimized to such an extent. Only Ale et al. and Zhu et al. [5,18] conducted such research but with fewer variables examined (polysaccharide yield and fucose content) [17,18]. Furthermore, to the best of our knowledge influence of pre-treatment solvent on polysaccharide yield (%PS) and chemical composition of the extracted brown algae polysaccharide has not been examined before. Therefore, the objective of our study was to evaluate influence of various pre-treatment solvents and extraction parameters (solvent, temperature and time) on polysaccharide yield (%PS) and chemical composition (total sugar, fucose, sulfate group and uronic acid content) of the fucoidan from *F. virsoides* and *C. barbata*.

2. Materials and methods

All chemicals and reagents used in this study were of analytical grade. Ethanol, acetone, sodium carbonate (Na_2CO_3), D(+)-glucose and potassium sulfate (K_2SO_4) were purchased from Gram-mol doo (Croatia), L-cystein gelatin, sodium tetraborate, sulfamic acid and potassium hydroxide from Acros Organics (Belgium), Folin-Ciocalteu reagent and trichloroacetic acid (TCA) from Fisher Scientific (UK), barium chlorid ($BaCl_2$) from abcr GmbH (Germany), phenol, fucoidan from *Fucus vesiculosus*, D-galacturonic acid and *m*-hydroxydiphenyl from Sigma-Aldrich (USA), hexane, absolute ethanol and ethyl acetate from Carlo Erba Reagents (Italy), sulfuric acid (H_2SO_4) from Scharlab S.L. (Spain), sodium hydroxide from Lach-Ner (Croatia) and hydrochloric acid (HCl) from TKI Hrastnik (Slovenia).

2.1. Algal material and preliminary treatments

Fucus virsoides was harvested from southwest coast of the Novigrad Sea, Croatia (44°12′02″ N; 15°28′51″ E) and *Cystoseira barbata* from coastal region of Zadar, Croatia (44°12′42″ N; 15°09′23″ E) in December 2018. Algae were identified by marine biologist Donat Petricioli, initially washed in seawater and then rinsed with distilled water. They were frozen at −60 °C in a ScanCool SCL210P freezer (Labogene ApS, Denmark) and freeze drying was performed on a CoolSafe lyophilizer, Model: 55-9 PRO, (Labogene, Denmark) for 24 h. The freeze dried algae were milled with an electric mill and the powder was stored at −20 °C until extraction was carried out. Schematic diagram of the experiment is

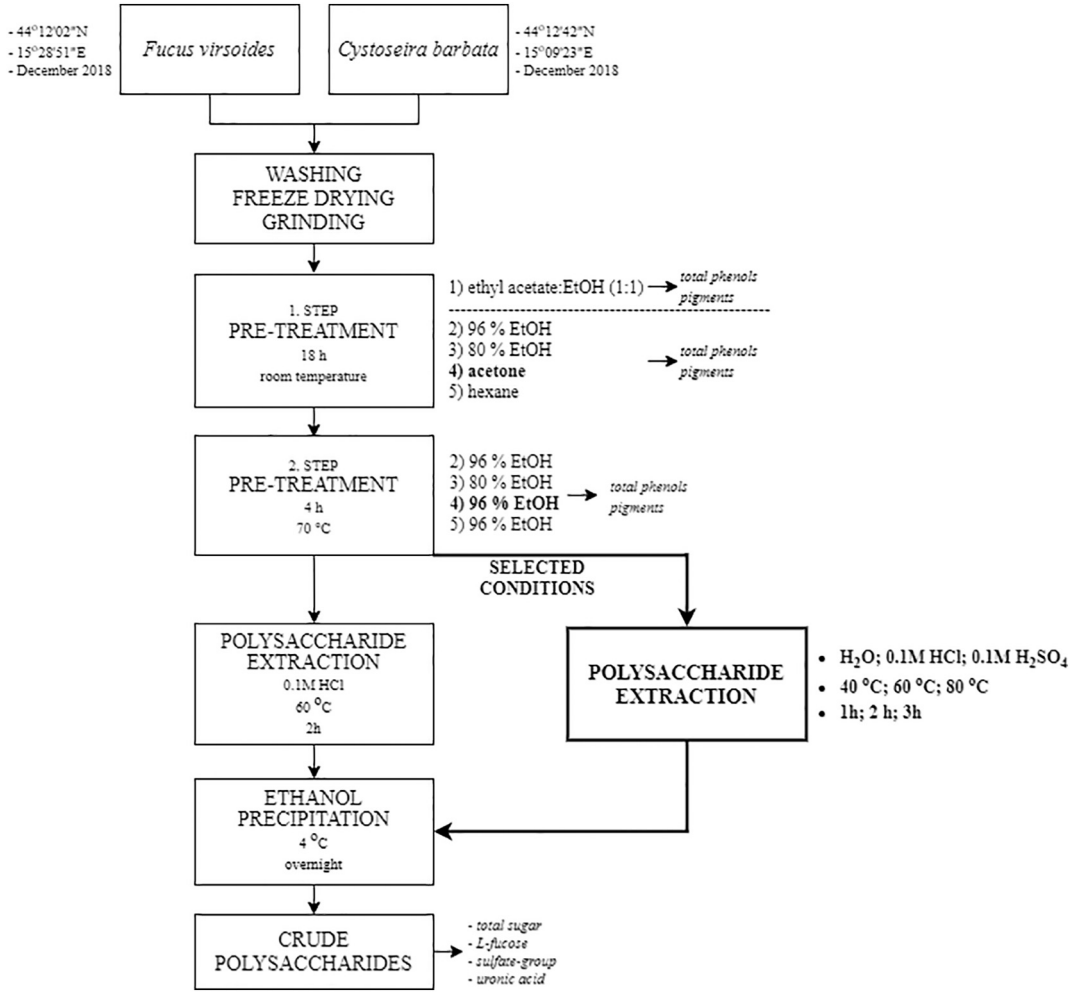

Fig. 1. Schematic diagram of the experiment.

given on Fig. 1.

2.2. Pre-treatment

Pre-treatment process of milled algae samples with selected solvents was carried out with constant stirring in two steps: first 18 h at room temperature and then 4 h at 70 °C. Five different solvents or solvents mixtures were used: (1) mixture of ethyl acetate and 96% ethanol (1:1) only in first step; (2) 96% ethanol in both steps; (3) 80% ethanol in both steps; (4) acetone in first and 96% ethanol in second step; (5) hexane in first and 96% ethanol in second step. After centrifugation and filtration, residual algae were dried and subjected to conventional PS extraction with 0.1 M HCl, for 2 h at 60 °C as described in Section 2.3. This process was performed in duplicate. Extracts obtained after pre-treatment were analyzed for phenols and pigments content according to the methods described in Section 2.4. The solvent that proved to be the most effective in removing the interfering compounds and with which the highest %PS were reached was used for further PS extraction optimization (Section 2.3).

2.3. Extraction of polysaccharides

After pre-treatment with selected solvents, depigmented and dried seaweed (1 g) was extracted with H_2O, 0.1 M HCl or 0.1 M H_2SO_4 (30 mL) for 1, 2 or 3 h at 40, 60 or 80 °C under constant stirring (400 rpm). After filtration, PS were precipitated from supernatant by the addition of 2 volumes absolute ethanol at 4 °C, overnight. PS were recovered by centrifugation at 5500 ×*g* for 30 min, dried for 48 h at room temperature, milled in a mortar and pestle to a fine powder and stored at −20 °C.

Extraction was performed in duplicate and PS extraction yield (%PS) was calculated according to Eq. (1) where WP is the weight obtained after ethanol precipitation and WA is the algae weight used in each experiment.

$$\%PS = \frac{WP}{WA} * 100 \quad (1)$$

2.4. Chemical analyses of the extract

2.4.1. Total phenols

Total phenols (TP) were determined by Folin-Ciocalteu method, according to the previously described procedure with some modifications [19]. In a test tube, 100 μL of each extract, 200 μL of Folin-Ciocalteu reagent and 2 mL of distilled water were mixed, after 3 min 1 mL of saturated sodium carbonate was added, and the mixture was shaken on a vortex. The absorbance at 765 nm was measured on spectrophotometer (UV–1600PC, VWR International, USA) after tempering for 25 min in water bath at 50 °C. All determinations were carried out in duplicate. The TP content was calculated according to the gallic acid standard calibration curve and expressed as mg of gallic acid equivalents (GAE) per g of powder ± standard deviation (SD).

2.4.2. Pigments

Extracts were analyzed for chlorophyll-a (C_a), chlorophyll-b (C_b) and carotenoids ($C_{(x+c)}$) content using spectrophotometric method. Extracts obtained with 80% ethanol, hexane and ethyl acetate-ethanol mixture were evaporated to dryness in vacuum concentrator (SpeedVac SPD2010P1, Thermo Fisher Scientific, USA) and then dissolved in 96% ethanol. The absorption spectrum was measured at 470, 644.8, 649, 661.6 and 664 nm depending on the solvent used for extraction or dissolution. The following equations were used for the quantification of C_a, C_b and $C_{(x+c)}$ [20]:

Acetone:

$$C_a\ (\mu g\ mL^{-1}) = 11.21\ A661.6 - 2.04\ A644.8 \quad (2)$$

$$C_b\ (\mu g\ mL^{-1}) = 20.13\ A644.8 - 4.19\ A661.6 \quad (3)$$

$$C_{(x+c)}\ (\mu g\ mL^{-1}) = (1000\ A470 - 1.9\ C_a - 63.14\ C_b)/214 \quad (4)$$

Ethanol:

$$C_a\ (\mu g\ mL^{-1}) = 13.36\ A664 - 5.19\ A649 \quad (5)$$

$$C_b\ (\mu g\ mL^{-1}) = 27.43\ A649 - 8.12\ A664 \quad (6)$$

$$C_{(x+c)}\ (\mu g\ mL^{-1}) = (1000\ A470 - 2.13\ C_a - 97.63\ C_b)/209 \quad (7)$$

All determinations were carried out in duplicate. Total pigments content was calculated as sum of C_a, C_b and $C_{(x+c)}$ and expressed as mg of pigments per g of dry algae powder ± standard deviation (SD).

2.5. Chemical composition of polysaccharides

2.5.1. Total sugars

Total sugars concentration in dried PS samples was determined by the colorimetric phenol-sulfuric acid method [21]. The 5 mg of dried PS was dissolved in 5 mL of water and then 400 μL of dissolved sample and 400 μL of 5% phenol solutions (*w/v*) were mixed with 2 mL of 95% H_2SO_4, strongly vortexed and left on room temperature for 20 min. The absorbance was measured at 490 nm and all measurements were done in duplicate. D-(+)-Glucose was used as a standard and a calibration curve, ranging from 12.5–100 mg/L, was plotted and results were expressed as percentage of total sugars in dry PS extract.

2.5.2. Analysis of L-fucose content

The content of L-fucose units in PS was determined by colorimetric assay with L-cysteine using L-fucose as standard [22]. Aliquots of 1 mL of PS sample, concentration 1 mg/mL, and 4.5 mL of diluted sulfuric acid (1:6, H_2O:H_2SO_4) were mixed, boiled at 100 °C for 10 min, cooled on ice for 5 min, 100 μL 3% (w/v) L-cysteine HCl solution was added and the solutions were left to stand for 30 min. A sample blank without L-cysteine HCl was used to calibrate the spectrophotometer prior to absorbance readings at 396 nm and 427 nm. To correct for the presence of hexoses the following equation was used: A396 nm − A427 nm. Calibration curve ranging from 0.05–0.175 mg/mL of L-fucose was used to determine the content of fucose in the sample extracts. All measurements were done in duplicate and results were expressed as percentage of fucose in dry PS extract.

2.5.3. Analysis of sulfate-group content

The sulfate-group (-SO_3H) content of PSs was determined by the $BaCl_2$-gelatin turbidity method [23] with slight modifications. Firstly, 0.3% gelatin solution was prepared in hot water (70 °C) and stored at 4 °C overnight. Two grams of $BaCl_2$ was dissolved in gelatin solution and left to stand for 2–3 h at 25 °C. Aliquots of 200 μL of PS solution (8 mg of extracted PS was hydrolysed in 3 mL 1 mol/L HCl, for 5 h at 105 °C, in a sealed glass tube), 3.8 mL of TCA and 1 mL of $BaCl_2$-gelatin reagent were mixed and left to stand on room temperature 15 min. The same protocol was applied for standard K_2SO_4 solutions (K_2SO_4 was dried at 105 °C, and then 181.4 mg was accurately weighed and dissolved in 100 mL 1 mol/L HCl). A blank was prepared with 200 μL of water instead of sample. Absorbance was measured at 360 nm, all measurements were done in duplicate and results were expressed as percentage of sulfate groups in dry PS extract. Calibration curve ranging from 0.125–1 mg/mL of —SO_3H groups was plotted.

2.5.4. Analysis of uronic acid content

Quantitative measurement of total uronic acid is commonly done with colorimetric methods after hydrolysing the PS in sulfuric acid [24]. Uronic acid content is estimated using the method in which sulfamate suppresses the formation of brown pigments from the neutral sugars and

tetraborate increases the sensitivity of the reaction with uronic acids [25]. According to the protocol [26], 5 mg of dried PS and 1 mL of concentrated sulfuric acid were added into borosilicate glass tubes and caped. Tubes were placed in an ice bath on a magnetic stirrer and stirred for 5 min. This process was repeated one more time with acid and two times with water. Reagent control was set up containing only 1 mL of concentrated sulfuric acid. Content of each tube was diluted with water to 10 mL, and centrifuged for 10 min at 2000 ×*g* at room temperature. Aliquots of 400 μL from each hydrolysate sample and reagent control were mixed with 40 μL of 4 M sulfamic acid/potassium sulfamate solution (pH 1.6) and 2.4 mL of 75 mM sodium tetraborate in sulfuric acid solution, vortexed vigorously, capped and placed in a 100 °C water bath for 20 min. After cooling in an ice bath for 10 min, 80 μL *m*-hydroxydiphenyl solution was added to 2 tubes of each sample and the 2 reagent control tubes while 80 μL of 0.5% NaOH was added to the third tube of each sample (sample control). Contents of the tubes were mixed on vortex three times and after 15 min absorbance was read at 525 nm against the reagent control. From sample absorbance, corresponding values for the sample control were subtracted. Calibration curve was created with D-galacturonic acid in a range from 5 to 40 μg/400 μL. All measurements were done in duplicate and results were expressed as percentage of uronic acids in dry PS extract.

2.6. Statistical analysis

Statistical analysis was done using STATISTICA v. 8 software (StatSoft Inc., Tulsa, OK, USA). For PS extraction, dependent variables were: %PS, total sugar (g/g), sulfate group (g/g), fucose (g/g) and uronic acid (g/g) content while independent variables were: (a) solvent (H_2O, 0.1 M HCl; 0.1 M H_2SO_4), (b) temperature (40, 60 and 80 °C), (c) time (1, 2 and 3 h). Continuous variables were analyzed by multivariate analysis of variance (MANOVA). Marginal means were compared with Tukey's HSD multiple comparison tests. The significance levels for all tests were $\alpha \leq 0.01$. Descriptive statistics was used to assess grand mean. For pre-treatment experiment, one-way ANOVA and Tukey's HSD multiple comparison tests were performed with total phenols (mg/g), total pigments (mg/g), %PS, total sugar (g/g), sulfate group (g/g), fucose (g/g) and uronic acid (g/g) content as dependent variables.

3. Results and discussion

3.1. Pre-treatment

Pre-treatment is an important step in the isolation of brown algae PS and it is usually applied prior to the extraction process with the aim of preventing coextraction of interfering bioactive compounds (polyphenols, pigments, lipids) with similar solubility [27]. In this research, pre-treatment was performed in two extraction steps (except with ethyl acetate-EtOH mixture) in order to successively remove structurally different interfering compounds. First extraction step was performed with ethyl acetate-EtOH mixture, 96% EtOH, 80% EtOH, acetone and hexane at room temperature while second extraction step in all pre-treatments was performed with EtOH at 70 °C to enhance impurities extraction. Any accidental PS extraction that could occur at higher temperature would result in their precipitation since they are not soluble in EtOH. Results of *F. virsoides* and *C. barbata* pre-treatment using different combination of solvents are shown in Tables 1 and 2. As it can be observed from Table 1, total polyphenols content removed from *F. virsoides* was significantly ($p \leq .01$) higher with second and third combination of solvents (96% and 80% EtOH) while higher total pigments content was removed with combination of acetone and EtOH. These results were somehow expected since polyphenols have better solubility in solvents with higher polarity [28], in this case 80% EtOH and 96% EtOH, while acetone gives very sharp chlorophyll absorption peaks [29] and it is widely used as the solvent for pigment extraction. In *C. barbata* significantly ($p \leq .01$) higher total polyphenols and pigments content was removed with combination of acetone and EtOH. Furthermore, the highest %PS for both seaweeds was achieved with combination of acetone and EtOH. Even though there was a statistically significant ($p \leq .01$) difference in total sugar, sulfate group, fucose and uronic acid content between PS obtain in various pre-treatments none of them showed to be distinguishably more effective than the others. Taking in to consideration all the results mentioned above, combination of acetone and EtOH was used for further PS extraction optimization.

3.2. Influence of extraction parameters on crude polysaccharide yield

First, it should be noted that all extracts analyzed in this study are crude extracts that could contain other co-extracts such as alginic acid. Extract are not purified, and therefore authors consider it is more accurate to report them as polysaccharide yield (%PS) rather than fucoidan yield. Average crude %PS from *F. virsoides* and *C. barbata* (Tables 3 and 4) obtained in this research were 15.07% and 7.80% respectively, what is mostly higher than those obtained by other authors on seaweeds from *Fucus* genus [12,30–33] and *Cystoseira* genus [11,34–37] as seen in Table 5. Only by applying advanced extraction techniques, microwave assisted extraction (MAE) and autohydrolysis, *F. vesiculosus* %PS was higher [8,16]. However, it should be taken in to consideration that only some of these results are expressed as crude polysaccharides while others are referring to the purified fucoidan fractions.

As confirmed in this research, PS content highly depends on the species of seaweed and according to recent study [38] it ranges from 0.4 to 21% in various seaweed species. Furthermore, within the same algal genus %PS is also influenced by several other factors including harvest season, geographic location and the maturity of the plant [38] as well as the extraction procedure and extraction solvent [39].

The choice of extraction solvent was found to have a significant ($p \leq .01$) influence on %PS from both seaweeds. The lowest %PS was obtained with H_2O while both acids resulted with significantly higher %PS, even more than three times higher with 0.1 M H_2SO_4. This can be explained by the facilitated PS extraction due to the cell wall hydrolysis that occurs with the use of acids [40]. Liu et al. [40] also obtained more than two times higher %PS by 1 M HCl (11.24%) than with H_2O (4.63%) from brown seaweed *Sargassum fusiforme* [40] and Saleem Ahmad [41]

Table 1
Influence of pre-treatment solvents on total polyphenols (mg/g) and total pigments (mg/g) removed in the pre-treatment, polysaccharide yield (%PS) and chemical structure of the extracted polysaccharides (total sugars (%), fucose (%), sulfate group (%) and uronic acid (%) content) from *Fucus virsoides*.

Solvent		Total polyphenols (mg/g)	Total pigments (mg/g)	%PS	Total sugar (%)	Fucose (%)	Sulfate group (%)	Uronic acid (%)
1 step	2 step	$p \leq .01$*	$p \leq .01$	$p \leq .01$	$p \leq .01$	$p \leq .01$	$p \leq .01$	$p \leq .01$
Etil acetate-EtOH (1:1)		10.69 ± 0.26^{a}	$1.73 \pm 0.02^{a,b}$	15.06 ± 0.33^{a}	$22.75 \pm 1.00^{b,c}$	$9.67 \pm 0.52^{b,c}$	79.13 ± 3.48^{b}	9.45 ± 0.37^{a}
96% EtOH	96% EtOH	12.22 ± 0.26^{b}	2.26 ± 0.02^{c}	14.54 ± 0.33^{a}	10.63 ± 0.44^{c}	14.74 ± 0.79^{a}	62.85 ± 0.15^{a}	15.05 ± 0.55^{b}
80% EtOH	80% EtOH	12.73 ± 0.26^{b}	1.71 ± 0.02^{a}	$16.11 \pm 0.33^{a,b}$	8.16 ± 0.02^{a}	20.13 ± 0.81^{b}	54.70 ± 4.69^{a}	13.06 ± 0.35^{b}
Acetone	96% EtOH	$10,01 \pm 0.26^{a}$	3.24 ± 0.02^{d}	17.45 ± 0.33^{b}	$8.69 \pm 0.04^{a,b}$	$22.67 \pm 0.30^{b,c}$	$68.00 \pm 4.63^{a,b}$	18.06 ± 0.31^{c}
Hexane	96% EtOH	9.35 ± 0.26^{a}	1.83 ± 0.02^{b}	14.58 ± 0.33^{a}	10.10 ± 0.01^{c}	24.46 ± 0.89^{c}	79.15 ± 0.46^{b}	9.77 ± 0.86^{a}

Values with different letters within column are statistically different at $p \leq .01$.
* $p \leq .01$. Results are expressed as mean ± SD.

Table 2
Influence of pre-treatment solvents on total polyphenols (mg/g) and total pigments (mg/g) removed in the pre-treatment, polysaccharide yield (%PS) and chemical structure of the extracted polysaccharides (total sugars (%), fucose (%), sulfate group (%) and uronic acid (%) content) from *Cystoseira barbata*.

Solvent		Total polyphenols (mg/g)	Total pigments (mg/g)	%PS	Total sugar (%)	Fucose (%)	Sulfate group (%)	Uronic acid (%)
1 step	2 step	$p \leq .01$*	$p \leq .01$	$p \leq .01$	$p \leq .01$	$p \leq .01$	$p \leq .01$	$p \leq .01$
Etil acetate-EtOH (1:1)		25.59 ± 0.96^{a}	0.62 ± 0.01^{a}	$8.48 \pm 0.26^{a,b}$	15.07 ± 0.87^{c}	11.65 ± 0.10^{c}	$22.51 \pm 0.00.80^{a}$	$38.50 \pm 0.26^{b,c}$
96% EtOH	96% EtOH	29.12 ± 0.96^{a}	1.43 ± 0.01^{c}	$8.45 \pm 0.26^{a,b}$	10.75 ± 0.70^{b}	8.28 ± 0.22^{a}	$32.00 \pm 0.02.40^{c}$	$29.54 \pm 1.81^{a,b,c}$
80% EtOH	80% EtOH	36.28 ± 0.96^{b}	1.52 ± 0.01^{d}	7.07 ± 0.26^{a}	8.15 ± 0.06^{a}	7.91 ± 0.26^{a}	$31.29 \pm 0.00.20^{c}$	39.40 ± 4.99^{c}
Acetone	96% EtOH	39.52 ± 0.96^{b}	1.60 ± 0.01^{e}	9.66 ± 0.26^{b}	$10.64 \pm 0.27^{a,b}$	10.25 ± 0.18^{b}	$25.49 \pm 0.00.60^{a,b}$	26.44 ± 2.16^{a}
Hexane	96% EtOH	28.54 ± 0.96^{a}	1.26 ± 0.01^{b}	9.39 ± 0.26^{b}	$9.06 \pm 0.81^{a,b}$	9.82 ± 0.65^{b}	$29.66 \pm 0.00.50^{b,c}$	$28.53 \pm 0.58^{a,b}$

Values with different letters within column are statistically different at $p \leq .01$.
* $p \leq .01$. Results are expressed as mean ± SD.

reported that *L. hyperborea* extraction with water gave very low amount of crude fucoidan compared to the dilute acid [41]. It appears that lowering the pH increased the PS yield [33] since 0.1 M H_2SO_4 with pH 0.7 is much more effective for PS extraction than 0.1 M HCl with pH 1. Similar finding was reported by Ptak et al. [33] who achieved marginally better fucoidan and laminarin yield with 100 mM HCl (pH 2) then with 10 mM H_2SO_4 (pH 4) for seaweed harvested in France [33].

Another observation regarding the use of different solvents that can be seen on Fig. 2, is that PSs obtained by acids are much lighter in color compared to water extract. Lighter color of PS extract indicates it has higher purity, thus higher quality [38]. Brown color of PSs extracted by H_2O indicates the presence of seaweed pigments (fucoxantin, β - carotene, violaxanthin, chlorophyll a and c) bound to PS during the extraction process [42]. In the presence of acid, pigments undergo chemical changes and ultimately become oxidized to colourless compounds [43] what explains lighter color of those extracts. In addition to pigments, brown color of the extracts correlates with their TP content [44].

Except extraction solvent, %PS was closely correlated with extraction temperature and time. By increasing the temperature from 40 to 80 °C, marginal mean of %PS increased from 14.65% to only 15.37% for *F. virsoides*, and from 6.19% to 9.65% for *C. barbata*. Furthermore, by prolonging the extraction from 1 h to 3 h, *F. virsoides* %PS significantly ($p \leq .01$) increased, while for *C. barbata* there was no statistical difference ($p \geq .01$) between 2 and 3 h. At higher temperature, kinetic of chemical reactions becomes higher and faster [38], viscosity and surface tension are reduced so extraction rate increases [45]. Similarly, by increasing the temperature from 80 to 120 °C, in fucoidan and laminarin MAE from *F. vesiculosus*, *Fucus serratus* and *Fucus evanescens*, Ptak et al. [33] noted the highest yield increase [33] while Baba et al. [38] obtained higher yield at 65 °C than on 45 or 85 °C [38]. Lower temperatures are more likely less efficient in providing higher PS% but at higher temperatures and longer extraction times PS may be subjected to thermal degradation [38] so a combined effect of temperature and time should be taken in to consideration. For *C. barbata* it can be observed (Table 5) that at lower temperatures (40 and 60 °C) PS% increased by prolonging the extraction time while at high temperature (80 °C) there was no significant difference between 2 and 3 h indicating thermal degradation. Adversely, for *F. virsoides* there was a continuous increase in %PS with simultaneous elevation of temperature and prolongation of extraction time. Similar to our observations on *C. barbata*, in research by Baba et al. [38] there was no difference in PS% between 3 h and 5 h at lower temperatures (45 and 65 °C), while at high temperature (85 °C) yield decreased when time increased from 1 to 5 h [38].

3.3. Influence of extraction parameters on total sugar content

Total sugar content in PS extracted from *F. virsoides* range between 8.72 and 54.36% with the average of 25.67%. This is in accordance with values reported for various seaweed species such as *F. vesiculosus*, *A. nodosum*, *Saccharina longicruris*, *C. barbatta*, *C. sedoides*, *C. compressa*, *C. crinite* and *Sargassum filipendula* in which total sugar content range between 8.9% and 66.7% [11,31,34–36,39,46,47]. Average total sugar content in PS extracted from *C. barbata* was 5.81% what is below 13 to 51.3% range reported by other authors for *Cystoseira* genus [11,34–36].

Because glycosidic bonds in the PS chain could be hydrolysed by acids more readily than by water it was somehow expected to obtain higher sugar content with acids as solvents [48]. However, both acidic extractions resulted with significantly ($p \leq .01$) lower concentration of total sugars than with water extraction. These results are reverse from the results for %PS what suggests that total sugars obtained with water are mainly monosaccharides and/or short chain PS, explaining the low %PS. On the contrary, sugars obtained with acids are probably PS of long chain able to agglomerate and precipitate as sulfated PS [8], causing an elevated %PS. Similarly, Liu et al. [40] achieved much higher %PS with acid while total sugar content was not significantly different between extracts obtained with water and acid [40].

For both seaweeds, fewer sugars were extracted at 40 °C and 1 h of extraction. Increasing the temperature to 60 °C for *F. virsoides* and 80 °C for *C. barbata* improved total sugar extraction. After 3 h of extraction total sugar content in *F. virsoides* PSs was slightly higher than after 1 and 2 h while in *C. barbata* PSs the highest total sugar content was achieved after 2 h. Time and temperature also increased sugar content in MAE of fucoidan from *A. nodosum* [49]. Rodriguez-Jasso et al. [8] reported that total sugar concentration increased by increasing the pressure in MAE from 30 to 120 Psi, what corresponded to temperature increase from 122 to 172 °C [8], while time had positive effect on total sugar concentration on lower temperature while on higher temperature longer extraction did not improved sugar concentration.

3.4. Influence of extraction parameters on fucose content

The colorimetric method used for analysis of L-fucose content is based on reaction that occurs between fucose and mixture of L-cysteine and sulfuric acid. The change in absorbance between 396 nm and 427 nm differentiates the presence of fucose content from that of other hexoses because the absorbance of other hexoses remains the same at both wavelengths [50]. Fucose content of PS extracted from *F. virsoides* obtained in this research ranged between 7.48 and 59.83% with the average of 29.58% while *C. barbata* fucose content ranged between 4.55% and 45.51% with the average of 16.5%. In comparison, the reported fucose content in *F. serratus*, ranged between 18 and 28% [12], in *F. vesiculosus* between 26 and 39% [12], in *Sargassum* sp. fucose content was 3.80% [38], in *Cystoseira sedoides* 17.6% [36] and 54.5% [35], in *C. crinite* 43.4% [35], in *C. compressa* 61.5% [35]. Therefore, it can be observed that the fucose content in brown seaweed *F. virsoides* and *C. barbata* from Adriatic Sea, is within the range. According to the IUPAC standards of nomenclature and terminology, a sulfate PS containing 20–60% of L-fucose can be classified as fucoidan [51].

There are many internal and external factors which can influence the fucose content in brown seaweeds such as species [12], structure [52] and maturity of the seaweed [53], harvest time [12], extraction technique and extraction parameters [38]. Extraction solvent, temperature and time showed statistically significant effect ($p \leq .01$) on fucose content. Significantly higher ($p \leq .01$) fucose content for both seaweeds

Table 3
Influence of extraction parameters on polysaccharide yield (%PS), total sugars (%), fucose (%), sulfate group (%) and uronic acid (%) content from *Fucus virsoides*.

	N	%PS	Total sugars (%)	Fucose (%)	Sulfate group (%)	Uronic acid (%)
Solvent		$p \leq .01$*	$p \leq .01$*	$p \leq .01$*	$p \leq .01$*	$p \leq .01$*
H_2O	18	6.32 ± 0.00^{a}	36.37 ± 0.31^{c}	27.95 ± 0.26^{b}	24.28 ± 0.24^{a}	8.97 ± 0.14^{b}
0.1 M HCl	18	15.39 ± 0.00^{b}	26.90 ± 0.31^{b}	41.50 ± 0.26^{c}	32.84 ± 0.24^{b}	9.31 ± 0.14^{b}
0.1 M H_2SO_4	18	23.51 ± 0.00^{c}	13.73 ± 0.31^{a}	19.29 ± 0.26^{a}	32.92 ± 0.24^{b}	7.61 ± 0.14^{a}
Temperature (°C)		$p \leq .01$*	$p \leq .01$*	$p \leq .01$*	$p \leq .01$*	$p \leq .01$*
40	18	14.65 ± 0.00^{a}	24.24 ± 0.31^{a}	31.97 ± 0.26^{c}	27.63 ± 0.24^{a}	5.76 ± 0.14^{a}
60	18	15.20 ± 0.00^{b}	26.91 ± 0.31^{b}	30.14 ± 0.26^{b}	31.78 ± 0.24^{c}	7.53 ± 0.14^{b}
80	18	15.37 ± 0.00^{c}	25.85 ± 0.31^{b}	26.64 ± 0.26^{a}	30.61 ± 0.24^{b}	12.60 ± 0.14^{c}
Time (h)		$p \leq .01$*	$p \leq .01$*	$p \leq .01$*	$p \leq .01$*	$p \leq .01$*
1	18	11.60 ± 0.00^{a}	25.21 ± 0.31^{a}	33.11 ± 0.26^{c}	25.72 ± 0.24^{a}	7.36 ± 0.14^{a}
2	18	16.49 ± 0.00^{b}	25.07 ± 0.31^{a}	31.50 ± 0.26^{b}	31.63 ± 0.24^{b}	8.03 ± 0.14^{b}
3	18	17.12 ± 0.00^{c}	26.72 ± 0.31^{b}	24.13 ± 0.26^{a}	32.68 ± 0.24^{c}	10.50 ± 0.14^{c}
Solvent; temperature (°C)		$p \leq .01$*	$p \leq .01$*	$p \leq .01$*	$p \leq .01$*	$p \leq .01$*
H_2O; 40	6	4.30 ± 0.00^{a}	36.06 ± 0.0053^{d}	25.14 ± 0.45^{b}	23.91 ± 0.41^{a}	7.25 ± 0.24^{c}
H_2O; 60	6	6.32 ± 0.00^{b}	41.92 ± 0.0053^{e}	29.93 ± 0.45^{c}	24.92 ± 0.41^{a,b}	9.02 ± 0.24^{d}
H_2O; 80	6	8.34 ± 0.00^{d}	31.13 ± 0.0053^{c}	28.79 ± 0.45^{c}	24.00 ± 0.41^{a}	10.65 ± 0.24^{e}
0.1 M HCl; 40	6	7.45 ± 0.00^{c}	24.44 ± 0.0053^{b}	51.11 ± 0.45^{f}	26.71 ± 0.41^{b}	7.27 ± 0.24^{c}
0.1 M HCl; 60	6	17.25 ± 0.00^{e}	24.40 ± 0.0053^{b}	40.27 ± 0.45^{e}	38.77 ± 0.41^{e}	7.85 ± 0.24^{c}
0.1 M HCl; 80	6	21.46 ± 0.00^{f}	31.85 ± 0.0053^{c}	33.12 ± 0.45^{d}	33.03 ± 0.41^{c,d}	12.80 ± 0.24^{f}
0.1 M H_2SO_4; 40	6	23.05 ± 0.00^{g}	12.21 ± 0.0053^{a}	23.09 ± 0.45^{b}	32.28 ± 0.41^{c}	2.76 ± 0.24^{a}
0.1 M H_2SO_4; 60	6	25.90 ± 0.00^{h}	14.41 ± 0.0053^{a}	24.31 ± 0.45^{b}	31.67 ± 0.41^{c}	5.73 ± 0.24^{b}
0.1 M H_2SO_4; 80	6	21.58 ± 0.00^{f}	14.57 ± 0.0053^{a}	10.47 ± 0.45^{a}	34.80 ± 0.41^{d}	14.35 ± 0.24^{g}
Solvent; time (h)		$p \leq .01$*	$p \leq .01$*	$p \leq .01$*	$p \leq .01$*	$p \leq .01$*
H_2O; 1	6	5.63 ± 0.00^{a}	38.45 ± 0.53^{h}	28.88 ± 0.45^{c}	24.21 ± 0.41^{b}	8.56 ± 0.24^{c,d}
H_2O; 2	6	5.87 ± 0.00^{b}	37.51 ± 0.53^{h}	27.63 ± 0.45^{c}	23.43 ± 0.41^{b}	8.32 ± 0.24^{c,d}
H_2O; 3	6	7.45 ± 0.00^{c}	33.15 ± 0.53^{g}	27.35 ± 0.45^{c}	25.18 ± 0.41^{b}	10.04 ± 0.24^{e}
0.1 M HCl; 1	6	13.74 ± 0.00^{d}	26.77 ± 0.53^{e}	50.59 ± 0.45^{e}	19.68 ± 0.41^{a}	7.55 ± 0.24^{b,c}
0.1 M HCl; 2	6	16.31 ± 0.00^{f}	23.64 ± 0.53^{d}	45.96 ± 0.45^{d}	36.81 ± 0.41^{e}	9.02 ± 0.24^{d,e}
0.1 M HCl; 3	6	16.12 ± 0.00^{e}	30.29 ± 0.53^{f}	27.95 ± 0.45^{c}	42.00 ± 0.41^{f}	11.36 ± 0.24^{f}
0.1 M H_2SO_4; 1	6	24.58 ± 0.00^{i}	10.41 ± 0.53^{a}	16.43 ± 0.45^{a}	33.27 ± 0.41^{d}	5.98 ± 0.24^{a}
0.1 M H_2SO_4; 2	6	23.41 ± 0.00^{h}	14.06 ± 0.53^{b}	16.83 ± 0.45^{a}	34.61 ± 0.41^{d}	6.76 ± 0.24^{a,b}
0.1 M H_2SO_4; 3	6	22.53 ± 0.00^{g}	16.72 ± 0.53^{c}	24.61 ± 0.45^{b}	30.86 ± 0.41^{c}	10.11 ± 0.24^{e}
		$p \leq .01$*	$p \leq .01$*	$p \leq .01$*	$p \leq .01$*	$p \leq .01$*

Table 3 (*continued*)

	N	%PS	Total sugars (%)	Fucose (%)	Sulfate group (%)	Uronic acid (%)
Time (h); temperature (°C)						
1; 40	6	11.52 ± 0.00^{a}	23.33 ± 0.53^{a,b,c}	34.58 ± 0.45^{f}	22.96 ± 0.41^{a}	5.78 ± 0.24^{a,b}
1; 60	6	15.40 ± 0.00^{c}	29.89 ± 0.53^{f}	30.56 ± 0.45^{c,d}	28.67 ± 0.41^{c}	7.20 ± 0.24^{c}
1; 80	6	17.03 ± 0.00^{e}	22.40 ± 0.53^{a}	30.76 ± 0.45^{c,d}	25.53 ± 0.41^{b}	9.10 ± 0.24^{d}
2; 40	6	11.57 ± 0.00^{a,b}	22.82 ± 0.53^{a,b}	32.62 ± 0.45^{d,e,f}	26.52 ± 0.41^{b}	5.24 ± 0.24^{a}
2; 60	6	17.32 ± 0.00^{f}	25.63 ± 0.53^{c,d}	30.08 ± 0.45^{c}	32.85 ± 0.41^{e}	6.52 ± 0.24^{b,c}
2; 80	6	16.70 ± 0.00^{d}	26.76 ± 0.53^{d,e}	27.72 ± 0.45^{b}	35.51 ± 0.41^{f}	12.33 ± 0.24^{e}
3; 40	6	11.71 ± 0.00^{b}	26.56 ± 0.53^{d,e}	32.14 ± 0.45^{c,d,e}	33.41 ± 0.41^{e}	6.26 ± 0.24^{a,b,c}
3; 60	6	16.75 ± 0.00^{d}	25.21 ± 0.53^{b,c,d}	33.87 ± 0.45^{e,f}	33.83 ± 0.41^{e,f}	8.88 ± 0.24^{d}
3; 80	6	17.64 ± 0.00^{g}	28.39 ± 0.53^{e,f}	13.89 ± 0.45^{a}	30.79 ± 0.41^{d}	16.37 ± 0.24^{f}
Grand mean	54	15.07	25.67	29.58	30.01	8.63

Values with different letters within column are statistically different at $p \leq .01$.
* $p \leq .01$. Results are expressed as mean ± SE.

was achieved with 0.1 M HCl. Conversely, Baba et al. [38] reported that acidic properties of HCl might cause the breakage of chemical bonds between the structures of fucose what resulted in the failure of fucose detection in fucoidan from *Sargassum* sp. [38]. Furthermore, Mak [63] reported higher fucose content in fucoidan from *Undaria pinnatifida* extracted with water (9.52%) than with acid (4.26%). Temperature and time showed opposite influence on fucose content between two seaweeds. While their increase led to lower fucose content in *F. virsoides*, in *C. barbata* it resulted with higher fucose content. Ale et al. [17] and Balboa et al. [54] noted that fucose content dropped steadily as the duration of the extraction time increased [17,54]. The reason is degradation of fucose chain that can occur at higher temperatures and longer extraction time [38].

3.5. Influence of extraction parameters on sulfate group content

It has been reported that the increase of sulfate content plays an important role in biological activities such as anti-HIV [55], anticoagulant [56] and antioxidant [14]. It was suggested that sulfate group contributes to hydrogen donating ability of the PS by activating the hydrogen atom of the anomeric carbon [14]. It can be observed that sulfate content is higher than 20% in all samples what is highly advantageous since it has been reported that sulfate content lower than 20% leads to a complete loss of anti-proliferative and anticoagulant activity [12]. The average sulfate content of *F. virsoides* obtained in this research was 30%, which is in upper spectrum of the 9 to 40.3% range of those reported for *Fucus* genus [8,12,16,31,57,58]. At 64.10%, the average sulfate content of *C. barbata* is higher than any previously reported value for *Cystoseira* genus [11,34–37]. Except sulfate content alone, some authors emphasize that sulfate/fucose ratio is an effective indicator of antioxidant activity as it appears to relate to metal chelating, free radical and hydroxyl radical scavenging activities of fucoidan [14,51,59,60]. Furthermore, Nishino et al. [61] found that anticoagulation activity of fucoidans was positively correlated with sulfate content and that only fucoidans with a sulfate/total sugar ratio higher than one possessed significant activity [61]. High average sulfate/fucose ratios of *F. virsoides* and *C. barbata* fucoidans obtained in this

Table 4
Influence of extraction parameters on polysaccharide yield (%PS), total sugars (%), fucose (%), sulfate group (%) and uronic acid (%) content from *Cystoseira barbata*.

	N	%PS	Total sugars (%)	Fucose (%)	Sulfate group (%)	Uronic acid (%)
Solvent		$p \leq .01$*	$p \leq .01$*	$p \leq .01$*	$p \leq .01$*	$p \leq .01$*
H_2O	18	4.22 ± 0.04^{a}	12.57 ± 0.07^{c}	11.69 ± 0.38^{a}	36.75 ± 0.73^{a}	8.87 ± 0.10^{b}
0.1 M HCl	18	4.68 ± 0.04^{b}	3.67 ± 0.07^{b}	20.98 ± 0.38^{c}	72.27 ± 0.73^{b}	7.39 ± 0.10^{a}
0.1 M H_2SO_4	18	14.51 ± 0.04^{c}	1.19 ± 0.07^{a}	16.84 ± 0.38^{b}	83.27 ± 0.73^{c}	8.71 ± 0.10^{b}
Temperature (°C)		$p \leq .01$*	$p \leq .01$*	$p \leq .01$*	$p \leq .01$*	$p \leq .01$*
40	18	6.19 ± 0.04^{a}	5.35 ± 0.07^{a}	12.55 ± 0.38^{a}	74.32 ± 0.73^{c}	5.78 ± 0.10^{a}
60	18	7.57 ± 0.04^{b}	5.27 ± 0.07^{a}	15.53 ± 0.38^{b}	66.49 ± 0.73^{b}	6.35 ± 0.10^{b}
80	18	9.65 ± 0.04^{c}	6.82 ± 0.07^{b}	21.42 ± 0.38^{c}	51.48 ± 0.73^{a}	12.84 ± 0.10^{c}
Time (h)		$p \leq .01$*	$p \leq .01$*	$p \leq .01$*	$p \leq .01$*	$p \leq .01$*
1	18	7.00 ± 0.04^{a}	4.07 ± 0.07^{a}	13.58 ± 0.38^{a}	69.06 ± 0.73^{c}	7.43 ± 0.10^{a}
2	18	8.14 ± 0.04^{b}	6.86 ± 0.07^{c}	17.14 ± 0.38^{b}	65.47 ± 0.73^{b}	10.37 ± 0.10^{b}
3	18	8.26 ± 0.04^{b}	6.50 ± 0.07^{b}	18.78 ± 0.38^{c}	57.77 ± 0.73^{a}	7.18 ± 0.10^{a}
Solvent; temperature (°C)		$p \leq .01$*	$p \leq .01$*	$p \leq .01$*	$p \leq .01$*	$p \leq .01$*
H_2O; 40	6	3.03 ± 0.07^{a}	12.36 ± 0.12^{g}	8.79 ± 0.66^{a}	43.08 ± 1.26^{b}	8.03 ± 0.17^{d}
H_2O; 60	6	4.09 ± 0.07^{c}	11.25 ± 0.12^{f}	11.94 ± 0.66^{b}	35.63 ± 1.26^{a}	7.43 ± 0.17^{c,d}
H_2O; 80	6	5.54 ± 0.07^{d}	14.12 ± 0.12^{h}	14.33 ± 0.66^{b,c}	31.55 ± 1.26^{a}	11.15 ± 0.17^{e}
0.1 M HCl; 40	6	2.88 ± 0.07^{a}	2.60 ± 0.12^{c}	12.70 ± 0.66^{b}	89.59 ± 1.26^{e,f}	4.02 ± 0.17^{a}
0.1 M HCl; 60	6	3.70 ± 0.07^{b}	3.63 ± 0.12^{d}	17.88 ± 0.66^{d}	80.18 ± 1.26^{c,d}	4.42 ± 0.17^{a}
0.1 M HCl; 80	6	7.44 ± 0.07^{e}	4.80 ± 0.12^{e}	32.34 ± 0.66^{e}	47.04 ± 1.26^{b}	13.73 ± 0.17^{f}
0.1 M H_2SO_4; 40	6	12.64 ± 0.07^{f}	1.10 ± 0.12^{a,b}	16.17 ± 0.66^{c,d}	90.30 ± 1.26^{f}	5.30 ± 0.17^{b}
0.1 M H_2SO_4; 60	6	14.92 ± 0.07^{g}	0.92 ± 0.12^{a}	16.78 ± 0.66^{c,d}	83.67 ± 1.26^{d,e}	7.19 ± 0.17^{c}
0.1 M H_2SO_4; 80	6	15.95 ± 0.07^{h}	1.54 ± 0.12^{b}	17.58 ± 0.66^{d}	75.84 ± 1.26^{c}	13.65 ± 0.17^{f}
Solvent; time (h)		$p \leq .01$*	$p \leq .01$*	$p \leq .01$*	$p \leq .01$*	$p \leq .01$*
H_2O; 1	6	3.38 ± 0.07^{a}	9.27 ± 0.12^{f}	10.07 ± 0.66^{a}	37.66 ± 1.26^{a}	9.14 ± 0.17^{d,e}
H_2O; 2	6	4.84 ± 0.07^{c}	15.0 ± 0.12^{h}	8.61 ± 0.66^{a}	38.79 ± 1.26^{a}	11.26 ± 0.17^{f}
H_2O; 3	6	4.44 ± 0.07^{b}	13.41 ± 0.12^{g}	16.38 ± 0.66^{b,c}	33.81 ± 1.26^{a}	6.22 ± 0.17^{b}
0.1 M HCl; 1	6	3.61 ± 0.07^{a}	2.14 ± 0.12^{c}	15.59 ± 0.66^{b}	83.41 ± 1.26^{d}	5.47 ± 0.17^{a,b}
0.1 M HCl; 2	6	5.09 ± 0.07^{c,d}	3.93 ± 0.12^{d}	26.17 ± 0.66^{e}	74.66 ± 1.26^{c}	11.28 ± 0.17^{f}
0.1 M HCl; 3	6	5.33 ± 0.07^{d}	4.95 ± 0.12^{e}	21.17 ± 0.66^{d}	58.74 ± 1.26^{b}	5.42 ± 0.17^{a}
0.1 M H_2SO_4; 1	6	14.00 ± 0.07^{e}	0.80 ± 0.12^{a}	15.10 ± 0.66^{b}	86.10 ± 1.26^{d}	7.67 ± 0.17^{c}
0.1 M H_2SO_4; 2	6	14.50 ± 0.07^{f}	1.60 ± 0.12^{b,c}	16.65 ± 0.66^{b,c}	82.96 ± 1.26^{d}	8.57 ± 0.17^{d}
0.1 M H_2SO_4; 3	6	15.02 ± 0.07^{g}	1.16 ± 0.12^{a,b}	18.79 ± 0.66^{c,d}	80.75 ± 1.26^{d}	9.90 ± 0.17^{e}
Time (h); temperature (°C)		$p \leq .01$*	$p \leq .01$*	$p \leq .01$*	$p \leq .01$*	$p \leq .01$*
1; 40	6	5.90 ± 0.07^{a}	3.57 ± 0.12^{a}	8.22 ± 0.66^{a}	78.51 ± 1.26^{d}	6.31 ± 0.17^{b,c}
1; 60	6	7.12 ± 0.07^{c}	4.79 ± 0.12^{c}	16.60 ± 0.66^{b}	75.96 ± 1.26^{d}	6.50 ± 0.17^{b,c}
1; 80	6	7.94 ± 0.07^{e}	3.84 ± 0.12^{a,b}	15.94 ± 0.66^{b}	52.70 ± 1.26^{a,b}	9.46 ± 0.17^{d}
2; 40	6	6.19 ± 0.07^{a,b}	8.22 ± 0.12^{e}	15.06 ± 0.66^{b}	78.06 ± 1.26^{d}	6.11 ± 0.17^{b,c}
2; 60	6	7.61 ± 0.07^{d}	6.43 ± 0.12^{d}	14.04 ± 0.66^{b}	66.05 ± 1.26^{c}	6.73 ± 0.17^{c}
2; 80	6	10.63 ± 0.07^{f}	5.93 ± 0.12d	22.33 ± 0.66^{c}	52.30 ± 1.26^{a,b}	18.27 ± 0.17^{f}
3; 40	6	6.44 ± 0.07^{b}	4.26 ± 0.12^{b,c}	14.38 ± 0.66^{b}	66.41 ± 1.26^{c}	4.93 ± 0.17^{a}
3; 60	6	7.98 ± 0.07^{e}	4.57 ± 0.12^{c}	15.97 ± 0.66^{b}	57.47 ± 1.26^{b}	5.81 ± 0.17^{b}
3; 80	6	10.37 ± 0.07^{f}	10.68 ± 0.12^{f}	25.99 ± 0.66^{d}	49.43 ± 1.26^{a}	10.80 ± 0.17^{e}
Grand mean	54	7.80	5.81	16.50	64.10	8.32

Values with different letters within column are statistically different at $p \leq .01$.
* $p \leq .01$. Results are expressed as mean ± SE.

Table 5
Review of analysis of extracted fucoidan found in the literature.

Seaweed	%PS	Total sugar (%)	Fucose (%)	Sulfate group (%)	Uronic acid (%)	Reference
F. virsoides	15.07	25.67	29.58	30.01	8.63	Current research
C. barbata	7.80	5.81	16.50	64.10	8.32	Current research
F. vesiculosus	9.80	/	35	19	/	[12]
	1.40	42.1	/	22.4	8.6	[31]
	11.10	/	/	/	/	[33]
	18.22	/	/	35.55	/	[8]
	18.14	/	/	30.78	/	[16]
F. serratus	6.00	/	24	34	/	[12]
	9.52	/	/	/	/	[33]
F. evanescens	9.00	33	/	9.00	26.12	[58]
F. distichus	21.50	/	/	/	/	[57]
C. barbata	5.45	50.79	/	22.51	7.13	[34]
C. compressa	5.20	50.67	/	14.65	8.65	[11]
	3.70	13	61.5	16.6	9.3	[35]
C. sedoides	4.20	51.3	17.6	15.5	7.6	[36]
	3.30	21.3	54.5	16.3	5.9	[35]
C. crinita	2.80	44.5	43.4	15.7	13.8	[35]
C. myrica	5.30	/	/	22.3	/	[37]
Sargassum sp.	2.7	64.5	/	4.71	25.19	[47]
S. longicruris	1.3	50.5	/	12	22	[31]
A. nodosum	1.1	45.4	/	22.1	9.9	[31]
	8.9	/	40	15	/	[12]
L. japonica	/	66.7	/	25.9	8.45	[46]
Padina sp.	2.6	62.9	/	8.82	12.91	[47]

research, 1.01 and 3.88 respectively, indicate possible antioxidant and anticoagulation activity that should be further investigated.

Higher sulfate content in both seaweeds was obtained with both acid extractions compared to water extraction since acid induces the sulfate ester cleavage so more sulfate groups can be liberated [13,62]. However, for *F. virsoides* there was no statistical difference ($p \geq .01$) between 0.1 M HCl and 0.1 M H_2SO_4 while for *C. barbata* higher content was achieved with 0.1 M H_2SO_4. Opposite results were reported by Liu et al. [40] for *S. fusiforme*, Saravana et al. [48] for *Saccharina japonica* and Mak [63] for *U. pinnatifida* [40,48,63]. Acetic acid, HCl and H_2SO_4, in dilute, mild and highly acidic conditions, have been used for fucoidan extraction and generally HCl is preferred since H_2SO_4 may interfere with sulfate analysis [13]. According to the literature, dilute HCl tends to yield low

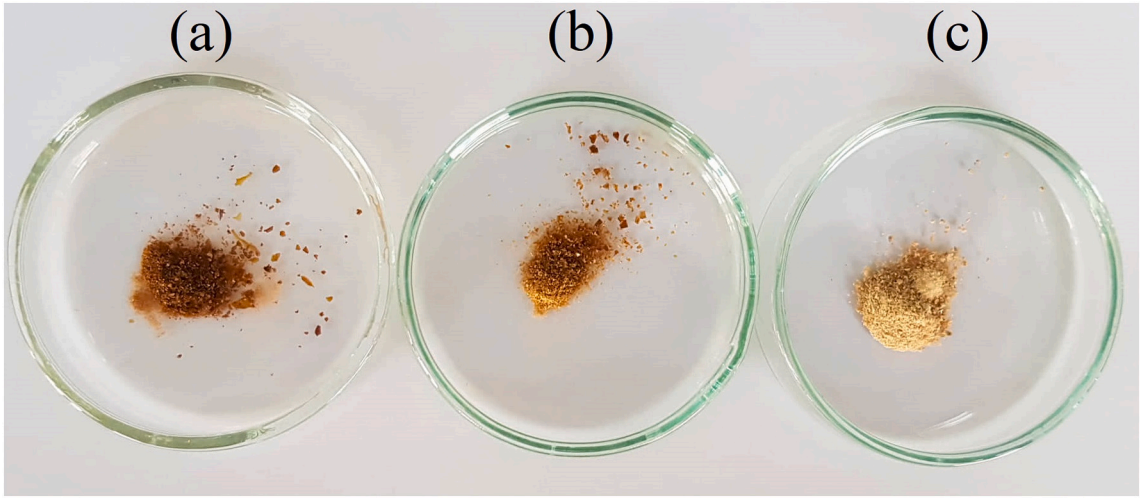

Fig. 2. *Fucus virsoides* polysaccharides extracts obtained with (a) H_2O, (b) 0.1 M HCl, (c) 0.1 M $H2SO_4$. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

sulfate fucoidan while dilute H_2SO_4 tends to yield high sulfate fucoidan due to the sulfate group in the sulfuric acid [62]. While in *C. barbata* temperature increase led to decrease in sulfate group content, in *F. virsoides* sulfate content of fucoidan increased as temperature increased from 40 to 60 °C but with further temperature increase on 80 °C sulfate content decreased. Similar trend was reported by Rodríguez-Jasso et al. [16] in autohydrolysis of *F. vesiculosus* [16] and Saravana et al. [48] in pressurized liquid extraction from *Saccharina japonica* [48] while Yuan and Macquarrie [49] reported decrease of sulfate content with temperature increase in MAE from *Ascophyllum nodosum* [49]. Longer extraction time led to higher sulfate content in *F. virsoides* but lower sulfate content in *C. barbata*. These opposite results are not a surprise since there are studies in which time had a positive effect [8,16] on sulfate content as well as negative [17,64]. However, by looking in to combined effect of temperature and time it can be observed that at higher temperature longer extraction time led to sulfate content decrease.

3.6. Influence of extraction parameters on uronic acid content

Uronic acids are found in fucoidan in different concentrations depending on various factors such as species and extraction parameters. Opposite to the sulfate group and fucose content, fucoidan with higher uronic acids content shows lower anticoagulant [65] and anti-complement activity [66] so its lower content is more desirable property. Uronic acid content in PS extracted from *F. virsoides* obtained in this research ranged between 2.48 and 20.30% with the average of 8.63% while *C. barbata* uronic acid content ranged between 3.08 and 22.90% with the average of 8.32%. Other research articles reported uronic acid content as low as 0.59% in *A. nodosum* [67] and 1.2% in *Nizamuddinia zanardinii* [68] and *S. filipendula* [39] through 5.9% [35] and 7.6% [36] in *C. sedoides*, 8.45% in *Laminaria japonica* [46], 8.65% [11] and 9.3% [35] in *C. compressa*, 13.8% in *C. crinite* [35] and as high as 25.19% in *Sargassum* sp. [47] and 26.12% in *F. evanescens* [58].

Extraction solvent, temperature and time showed statistically significant effect ($p \leq .01$) on uronic acid content. The lowest uronic acid content in *F. virsoides*, was achieved with 0.1 M H_2SO_4 while in *C. barbata*, 0.1 M HCl resulted with the lowest uronic acid content indicating that acid extraction leads to lower alginate contamination since uronic acid is a major component of alginate (alginic acid) which is abundant in brown seaweed [13]. Ponce et al. [64], January et al. [13] and Mak [63] obtained lower total uronic acid content with hot water then acid extraction while Liu et al. [40] obtained 8.3% total uronic acid by water and 4.5% by 1 M HCl in *S. fusiforme* fucoidans. These findings show that there is no exact correlation between solvent pH and uronic acid content. By looking at the interaction between solvent and temperature it can be observed that at 80 °C the lowest uronic acid content was obtained with water and it increased as the pH decreased what is exactly the same tendency as reported by Vriesmann et al. [69] for uronic acid content of pectins from cacao pod husks [69]. By increasing the temperature from 40 to 80 °C uronic acid content in both seaweeds fucoidan increased. Likewise, in extraction from *Adenocystis utricularis* and *A. nodosum*, Yuan & Macquarrie [49] and Ponce et al. [64] demonstrated that higher temperatures lead to higher proportion of uronic acid [49,64] while Saravana et al. [48] found that uronic acid decreases when there is an increase in temperature [48]. By prolonging the extraction time from 1 to 3 h uronic acid content in *F. virsoides* increased while in *C. barbata* it initially increased and then decreased as time prolonged further. Similar correlation between extraction time and uronic acids was observed by Ale et al. [17] and Balboa et al. [54] for *Sargassum* sp. and *Sargassum muticum* respectively [17,54].

4. Conclusion

The results of this study indicate that Adriatic Sea endemic brown seaweed species *F. virsoides* as well as *C. barbata* may be a good source of fucoidan. The data also confirmed the long known facts that brown algae cell wall PS are complex, and that their yield and chemical composition are significantly influenced by the algae species and the conditions used to extract them. Pre-treatment solvent had an impact on %PS, removal of interfering substances and chemical composition of the extracted fucoidan and combination of acetone and 96% ethanol showed the most promising results. Compared to *C. barbata*, *F. virsoides* contained almost twice as much PS with higher fucose but lower sulfate group content. The highest *F. virsoides* %PS was achieved with 0.1 M H_2SO_4 as solvent for 3 h at 80 °C while for *C. barbata* parameters that gave the highest % PS are 0.1 M H_2SO_4, 2 h and 80 °C. Applying acid instead of water not only improved %PS but resulted with PS with higher sulfate group content but lower uronic acid, fucose and total sugar content. Extraction at higher temperatures and longer time resulted with lower fucose and higher sulfate group content in *F. virsoides* and oppositely in *C. barbata*. It is important to emphasize the importance of these finding for application in any future study in which fucoidan will be evaluated for biological activity.

CRediT authorship contribution statement

Ana Dobrinčić: Conceptualization, Investigation, Formal analysis,

Writing - Original Draft. Erika Dobroslavić & Sandra Pedisić: Investigation. Sandra Balbino & Ivona Elez-Garofulić: Writing - Review & Editing. Rozelindra Čož-Rakovac: Funding acquisition. Verica Dragović-Uzelac: Conceptualization, Supervision, Writing - Review & Editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter 3

Publication No. 3: Microwave assisted extraction and pressurized liquid extraction of sulfated polysaccharides from *Fucus virsoides* and *Cystoseira barbata*

Foods

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foods

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Article

Microwave Assisted Extraction and Pressurized Liquid Extraction of Sulfated Polysaccharides from *Fucus virsoides* and *Cystoseira barbata*

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Abstract: Sulfated polysaccharide fucoidan isolated from brown algae shows a wide range of biological activities that are significantly dependent on its chemical composition, which is closely related to the applied technique and extraction parameters. Therefore, the objective of this study was to evaluate the influence of microwave assisted extraction (MAE) and pressurized liquid extraction (PLE) parameters (solvent, temperature, time, and number of cycles) on the *Fucus virsoides* and *Cystoseira barbata* polysaccharide yield (%PS) and chemical composition (total sugar, fucose, and sulfate group). The optimal MAE parameters that resulted in the highest polysaccharide extraction from *F. virsoides* and *C. barbata* were 0.1 M H_2SO_4 for 10 min at 80 °C, while the optimal PLE parameters were 0.1 M H_2SO_4, for two cycles of 15 min at 140 °C. Furthermore, the %PS, chemical structure, molecular properties, and antioxidant activity of the *F. virsoides* and *C. barbata* polysaccharide extracts obtained with MAE, PLE, and conventional extraction (CE) performed under previously determinate optimal conditions were compared. PLE resulted in a significantly higher %PS from *F. virsoides*, while for *C. barbata*, a similar yield was achieved with CE and PLE, as well as CE and MAE, for both algae. Furthermore, the polysaccharides obtained using PLE had the highest polydispersity index, fucose, and sulfate group content, and the lowest uronic acid content; however their antioxidant activity was lower.

Keywords: *Fucus virsoides*; *Cystoseira barbata*; polysaccharides; fucoidan; microwave assisted extraction; pressurized liquid extraction

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1. Introduction

Because of the increased consumer awareness of functional food ingredients, seaweeds are increasingly being considered as a potential source of bioactive compounds. With about 2000 species, brown seaweeds are the second most abundant group of marine algae [1]. Their various biological properties, such as their anticoagulant, antithrombotic, anti-viral, anti-cancer, anti-inflammatory, and antibacterial effects [2], have been attributed to the sulfated polysaccharide fucoidan. Fucoidan is composed of mainly fucose interconnected by β (1, 3) glycoside bonds; alternating β (1, 3) and β (1, 4) bonds; and, rarely, β (1, 2) bonds [3]. Apart from fucose, it also contains other monosaccharides, including galactose, glucose, mannose, xylose, rhamnose, and uronic acids, and its sulphate content varies between 5% and 38% [3]. The chemical structure of fucoidan may significantly determine its physical, chemical, and biochemical properties [4]. Moreover, the biological activities of fucoidan are strongly associated with their chemical structure [5]; however, the correlation between the structure and biological activity has still not been sufficiently clarified [2]. The structure and composition of fucoidan can be influenced by the algae species, location, and

harvesting season [6], as well as extraction techniques and different extraction conditions (e.g., pH, time, temperature, pressure, particle size, solvent, sample to solvent ratio, and agitation speed) [5,7,8].

Conventional polysaccharide extraction (CE) is generally performed with water, dilute acid, or dilute alkali for a long time; at a high temperature; and using a large solvent volume, and is thus not economically and environmentally friendly. To overcome these limitations, advanced technologies such as microwave assisted extraction (MAE), ultrasound assisted extraction (UAE), pressurized liquid extraction (PLE), and enzyme-assisted extractions (EAE) have been applied to extract brown algae polysaccharides (PS). MAE utilizes microwave energy for heating, and thus increases the mass transfer rate of the solutes from the sample matrix into the solvent [9]. The MAE open vessels system under atmospheric pressure can be operated at a maximum temperature determined by the boiling point of the solvents [10]. In PLE, elevated temperatures and pressures are used to extract compounds from samples in an oxygen and light-free environment, in a short period of time and using less solvent [11]. Elevated pressure keeps the solvent below its boiling point so the application of temperatures above a solvent boiling point (at atmospheric pressure) is possible.

The benthic fauna of the Adriatic Sea includes 2597 species of algae, 152 of which are endemic. One of the endemic brown algae species is *Fucus virsoides*, the only representative of Fucus genus in the Mediterranean, growing mainly in the northern Adriatic, from the Venice Lagoon to Dalmatia [12]. *Cystoseira barbata* belongs to the genus Cystoseira, whose representatives have an important role in the structure and functioning of the rocky habitats of the Mediterranean and the Black Sea, providing shelter, food, and nursery grounds for a variety of organisms [13].

Even though MAE and PLE have been successfully applied for the extraction of numerous biologically active compounds from a wide variety of plants, their application in brown algae PS extraction is sparsely reported [1,14,15], especially their comparison in terms of the yield and chemical composition of the extracted PS. As conventional PS extraction is long and complex, a significant time reduction and lower energy consumption makes these novel techniques really interesting for application in processed and functional food, pharmaceutical, and chemical industries. In this research, MAE and PLE were applied to extract polysaccharides from brown algae *F. virsoides* and *C. barbata,* and one of the goals was to investigate the influence of extraction parameters (solvent, time, temperature, and number of cycles) on the yield and chemical structure (total sugar, fucose, and sulfate group content) of the extracted PS. Furthermore, the yield, chemical structure (total sugar, fucose, sulfate group and uronic acid content, and monosaccharide composition), molecular weight, and antioxidant activity of the PS extracts obtained with MAE, PLE, and CE, performed under previously determined optimal conditions [16], were compared.

2. Materials and Methods

All of the chemicals and reagents used in this study were of analytical grade. Ethanol, acetone, sodium carbonate (Na_2CO_3), and potassium sulfate (K_2SO_4) were purchased from Gram-mol doo (Zagreb, Croatia); sodium tetraborate, L-cystein, sulfamic acid, gelatin, potassium hydroxide, chloroform, 1-phenyl-3-methyl-5-pyrazolone (PMP), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) from Acros Organics (Geel, Belgium); acetonitrile, trichloroacetic acid (TCA), silicone oil, and Folin–Ciocalteu reagent from Fisher Scientific (Leicestershire, UK); barium chlorid ($BaCl_2$) from abcr GmbH (Karlsruhe, Germany), fucoidan from *Fucus vesiculosus*, D-galacturonic acid, phenol, *m*-hydroxydiphenyl, 2,2-diphenyl-1-picrylhydrazyl (DPPH), arabinose, D-(+)-glucose, L-rhamnose, D-(−)-fructose, trimethylamine, 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), D-(+)-mannose, L-(−)-fucose, and ammonium acetate from Sigma-Aldrich (St. Louis, Missouri, USA); ethyl acetate, hexane, and absolute ethanol from Carlo Erba Reagents (Cornaredo, Italy); hydrochloric acid (HCl) from TKI Hrastnik (Hrastnik, Slovenia); sodium hydroxide from Lach-Ner (Zagreb, Croatia); sulfuric acid (H_2SO_4)

from Scharlab S.L. (Barcelona, Spain); D-(−)-ribose from TCI (Portland, OR, USA); and fluorescein sodium salt from Honeywell Riedel-de-Haën (Bucharest, Romania).

2.1. Algal Material and Preliminary Treatments

Cystoseira barbata was harvested from the coastal region of Zadar, Croatia (44°12′42″ N; 15°09′23″ E), and *Fucus virsoides* was harvested from the southwest coast of the Novigrad Sea, Croatia (44°12′02″ N; 15°28′51″ E), in December 2018, and the identification was performed by marine biologist Donat Petricioli. Freshly collected algae were firstly washed in seawater and then distilled water, and were then frozen at −60 °C in a ScanCool SCL210P freezer (Labogene ApS, Hillerød, Denmark); lyophilisation was done on a Cool-Safe lyophilizer, Model: 55-9 PRO, (company, Labogene, Denmark) for 24 h. The lyophilised algae were ground in an electric grinder and were stored at −20 °C until extraction.

2.2. Pre-Treatment

The pre-treatment process of the algae samples was performed with continuous stirring in two phases: 18 h at room temperature with acetone, followed by 4 h at 70 °C with 96% ethanol [16]. Afterwards, the residual algae were dried and subjected to conventional, MAE, or PLE PS extraction.

2.3. Extraction of Polysaccharides

2.3.1. Conventional Extraction

The pre-treated dried seaweed (1 g) was extracted under constant stirring (400 rpm) at previously determined optimal conditions [16], as follows: 0.1 M H_2SO_4 (30 mL) as a solvent, for 3 h at 80 °C.

2.3.2. Microwave Assisted Extraction

The pre-treated dried seaweed (1 g), 30 mL of extraction solvent (H_2O, 0.1 M HCl, or 0.1 M H_2SO_4), and the magnetic stirrer were put into an extraction cell and placed in a microwave reactor, Ethos Easy (Milestone, Italy). The time required to achieve the extraction temperature was set at 2 min; ventilation after extraction at 5 min; extraction temperatures of 60, 80, or 100 °C; and extraction times of 10, 20, or 30 min.

2.3.3. Pressurized Liquid Extraction

PLE was performed using an accelerated solvent extractor (ASE 350, Dionex, Sunnyvale, CA, USA), equipped with a solvent controller. The 22-mL stainless steel extraction cells were filled in consecutive layers with two glass filters on the bottom, with about a 2 cm layer of diatomaceous earth, 1 g of pre-treated dry algae powder mixed with 2 g of diatomaceous earth, and diatomaceous earth again layered on top to fill the extraction cells. Distilled water and 0.1 M H_2SO_4 were used as the extracting solvent and a pressure of 1500 psi was retained for all of the analyses. Extractions were performed at different extraction temperatures (60, 100, and 140 °C) in one or two extraction cycles for 5, 10, and 15 min. The warming-up time changed depending on the extraction temperature (2 min if the extraction temperature was 60 °C, 5 min if the extraction temperature was 100 °C, and 6 min if the extraction temperature was 140 °C). The solvent was purged from the cell with nitrogen for 120 s and the system was depressurized. The extracts were collected in glass collection vials and afterwards were transferred to an Erlenmeyer flask.

2.3.4. Processes after Extraction

After extraction, the extracts were filtrated and PS precipitation from the supernatant was done overnight, at 4 °C, by adding double the volume of absolute ethanol. After centrifugation at 5500 RPM for 30 min, the PS were dried at room temperature for 48 h, crushed to a fine powder in a mortar and pestle, and stored at −20 °C.

Extractions were performed in duplicate and the PS extraction yield (% PS) was calculated according to Equation (1), where WP is the weight obtained after ethanol precipitation and WA is the algae weight used in each experiment.

$$\% \text{ PS} = \frac{WP}{WA} \times 100 \quad (1)$$

2.4. Chemical Composition of Polysaccharides

The total sugar concentration in the PS was determined using the colorimetric phenol-sulfuric acid method, using glucose as the standard (Dubois et al., [17]). The content of L-fucose units in the PS was determined using a colorimetric assay with L-cysteine, as described by Dische and Shettles [18], using L-fucose as the standard. The sulfate group content was quantified after a hydrolysis of PS in 1 M HCl at 105 °C for 5 h, according to the turbidimetric $BaCl_2$-gelatin method, with K_2SO_4 as the standard, as described by Dodgson and Price [19]. The uronic acid content was measured with the modified sulfamate/m-hydroxydiphenyl colorimetric method using D-galacturonic acid as the standard, in which sulfamate suppressed the formation of brown pigments from the neutral sugars and tetraborate increased the sensitivity of the reaction with uronic acids (Filisetti-Cozzi and Carpita, [20]). The results were expressed as the percentage of total sugar, fucose, sulfate group, or uronic acids in the dry PS extract.

2.5. Monosaccharide Analysis of Polysaccharides by High Performance Liquid Chromatography (HPLC)

The monosaccharide composition was analyzed according to a previously reported method [21], with minor modifications. A sample of PS (100.0 mg) was dissolved in 1 mol/L H_2SO_4 (2.0 mL) and was incubated for 4 h at 110 °C. After cooling, the reaction mixture was neutralized to pH 7 with 2 mol/L sodium hydroxide, and the internal standard solution (2 mL) was added. The mixture was shaken well, diluted to 10 mL, and filtered.

The mixture of filtered hydrolyzed PS solution (or monosaccharide standards; 100 µL), 0.5 mol/L methanolic solution of PMP (100 µL), and 0.3 mol/L aqueous sodium hydroxide (100 µL) was incubated at 70 °C for 30 min. The reaction mixture was then cooled and neutralized with 0.3 mol/L hydrochloric acid. Chloroform (1 mL) was added to the solution, shaken well on a vortex and centrifuged at 5000 RPM for 10 min. The chloroform layer was discarded and the aqueous layer was extracted twice with chloroform. The final aqueous layer was analyzed directly by HPLC.

An accurate amount of ribose (~1 mmol) was dissolved in water, diluted to 50 mL, and used as the internal standard solution. Five known concentrations of the following standards (mixed with internal standard) were prepared by consecutive dilutions from stock solutions and were injected into the instrument: glucose (0.25 to 2 mg/mL), fucose (0.1875 to 1.5 mg/mL), galacturonic acid (0.25 to 1.75 mg/mL), arabinose (0.25 to 1.75 mg/mL), mannose (0.25 to 1.75 mg/mL), fructose (0.25 to 1.75 mg/mL), and rhamnose (0.25 to 1.75 mg/mL). Calibration curves (Table 1) were plotted as the ratio of the peak areas of the standard monosaccharide and the internal ribose standard versus concentration.

Table 1. Calibration curves for the 1-phenyl-3-methyl-5-pyrazolone (PMP) sugars.

PMP Sugar	Standard Curve	R^2
arabinose	y = 1.2784x − 0.2645	0.9948
glucose	y = 2.8327x − 0.6781	0.9925
fucose	y = 0.3549x − 0.0712	0.9940
galacturonic acid	y = 2.3517x − 0.6414	0.9951
rhamnose	y = 0.0706x + 0.0064	0.9961
fructose	y = 0.1633x − 0.0453	0.9925

An HPLC Agilent Infinity 1260 system (Agilent Technologies, Santa Clara, CA, USA) equipped with UV/VIS and DAD, an automatic injector, and ChemStation software on a Zorbax Eclipse XDB-C18 column (4.5 × 250 mm, 5 μm; Agilent Technologies, USA) was used for the analysis. Solvent A was a mixture of 0.4% trimethylamine in 20 mmol/L ammonium acetate buffer solution (pH 6.30 with acetic acid) and acetonitrile in a 9 to 1 ratio. Solvent B was a mixture of 0.4% triethylamine in a 20 mmol/L ammonium acetate buffer solution (pH 6.30 with acetic acid) and acetonitrile in a 4 to 6 ratio. Chromatographic separation was achieved with the following gradient: 0–9 min, 10% to 14% B; 9–30 min, 64% B; 30–35 min, 64% B; and 35–37 min, 10% B. The mobile phase was delivered at a flow rate of 1 mL/min and the column temperature was set at 25 °C. The chromatograms were monitored at 245 nm and the sample injection volume was 10 μL. All of the experiments were carried out in duplicate.

2.6. Determination of Molecular Properties

The molecular properties (average molecular weight (M_w), the number for average molecular weight (M_n), and the polydispersity index (PDI)) of *F. virsoides* and *C. barbata* PS extracted with CE, MAE, and PLE at optimal conditions were detected and measured using high-performance size exclusion chromatography with a refraction index detector (HPSEC-RID). The 1260 Infinity II LC system (Agilent Technologies) consisted of a quaternary gradient pump G7111B, an autosampler G4767A, a multicolumn thermostat G7116A, and a refraction index detector G7162A. HPSEC analysis was performed using PL aquagel-OH guard column (8 μm, 50 × 7.5 mm; Agilent Technologies) and PL aquagel-OH MIXED-M column (8 μm, 300 × 7.5 mm; Agilent Technologies). The mobile phase was H_2O, and the temperatures of the column and RID, the flow rate, and the injection volume were set to 30 °C, 40 °C, 0.5 mL/min, and 50 μL, respectively. Data were collected and processed using OpenLAB CDS ChemStation Edition (Agilent Technologies). A molecular weight calibration curve was constructed with known pullulan standards (Agilent Technologies) with M_w in a range from 180 to 700 kDa.

2.7. DPPH Radical Scavenging Assay

The ability of the extracts to scavenge the DPPH radical was assessed spectrophotometrically. In a test tube, 1.5 mL of polysaccharide solution (1 mg/mL) was mixed with 1.5 mL of DPPH solution (0.2 mM in 70% ethanol), and it was vortexed and kept in the dark at room temperature for 30 min. The decrease in absorbance was measured at 517 nm in duplicate. The free radical scavenging activity was calculated as follows:

$$\text{Scavenging effect (\%)} = (A - C)/A \times 100 \quad (2)$$

where A is the absorbance of the control (DPPH without sample) and C is the absorbance of the sample.

2.8. Oxygen Radical Absorbance Capacity (ORAC) Assay

The antioxidant capacity was evaluated by the oxygen radical absorbance capacity (ORAC) assay according to the research of Elez Garofulić et al. (2020) [22]. The ORAC procedure used an automated plate reader (BMG LABTECH, Offenburg, Germany) with 96-well plates, and the data were analyzed using MARS 2.0 software. Dry PS extracts were dissolved in distilled water (4 mg/mL) and were filtered. AAPH, fluorescein solution, and different dilutions of Trolox were prepared in a 75 μM phosphate buffer (pH 7.4). Dissolved samples were added in a 96-well black plate containing a fluorescein solution (70.3 nM). The plate was incubated for 30 min at 37 °C and after the first three cycles (representing the baseline signal), AAPH (240 mM) was injected into each well to initiate the peroxyl radical generation. On each plate, different dilutions of Trolox (3.12–103.99 μM) were used as the reference standard. The fluorescence intensity (excitation at 485 nm and emission at 528 nm) was monitored every 90 s over a total measurement period of 120 min. The measurements were performed in duplicate, and the results are expressed as μmol of the

Trolox equivalents (TE) per g of dry PS sample, as the mean value ± standard deviation (N = 4 replicates).

2.9. Statistical Analysis

Statistical analysis was done using STATISTICA v. 8 software (StatSoft Inc., Tulsa, OK, USA). The dependent variables were %PS, total sugar content, fucose content, and sulfate group content, while the independent variables were as follows: (a) solvent (MAE—H_2O, 0.1 M HCl, and 0.1 M H_2SO_4; PLE—H_2O and 0.1 M H_2SO_4), (b) temperature (MAE—60, 80, and 100 °C; PLE—60, 100, and 140 °C), (c) time (MAE—10, 20, and 30 min; PLE—5, 10, and 15 min), and (d) number of cycles in PLE (1 and 2). For a comparison of the different extraction techniques (CE, MAE, and PLE; independent variable), the dependent variables were %PS, total sugar, fucose, sulfate group, uronic acid, and monosaccharide composition. The continuous variables were analyzed using a multivariate analysis of variance (ANOVA). Marginal means were compared with Tukey's HSD (honestly significant difference) multiple comparison tests. The significance levels for all of the tests were $\alpha \leq 0.05$.

3. Results and Discussion

3.1. Microwave Assisted Extraction

First, it is important to emphasize that all of the extracts analyzed in this study were crude extracts that could contained other co-extracted compounds such as alginic acid [23]. Because the extracts were not purified, we considered it to be more accurate to report their polysaccharide yield (%PS) rather than their fucoidan yield. MAE was applied to extract the PS from *F. virsoides* and *C. barbata,* and the influence of the solvent (H_2O, 0.1 M HCl, or 0.1 M H_2SO_4), temperature (60, 80, or 100 °C), and time (10, 20, or 30 min) on the yield and chemical composition are shown in Table 2. The average crude %PS from *F. virsoides* and *C. barbata* obtained by MAE were 13.19% and 6.43%, respectively. In general, the algae from the *Fucus* genus have reported a PS content in the range of 1.40% to 21.50% [15,24–27], and the only commercially available source of fucoidan is from *F. vesiculosus*. On the contrary, the reported fucoidan content in the algae from the *Cystoseira* genus ranges from 2.80% to 5.45% [28–32]. Extracts from *F. vesiculosus* obtained by MAE, at a pressure of 120 psi for 1 min and with water as a solvent (solvent-to-sample ratio 25:1 *w*/*v*), had 18.22% sulfated PS [1], while MAE at 120 °C for 30 min with 10 mM sulfuric acid resulted in 11.1% PS from *F. vesiculosus* and 9.52% from *F. serratus* [15]. MAE at 120 °C for 15 min was previously used to extract 16.08% sulfated PS from *Ascophyllum nodosum* [14], while 1000 W of microwave power for 5 min extracted 1699.80 ± 83.80 mg fucose/100 g dw from *A. nodosum* [33]. The optimized MAE conditions of 547 W and 80 °C for 23 min were used to extract 2.84% of PS from brown macroalgae *Sargassum thunbergii* [12]. Furthermore, MAE was likewise used to extract PS from green macroalgae, e.g., at 140 °C for 10 min, the ulvan yield from *Ulva meridionalis* and *Ulva ohnoi* was 40.4 ± 3.2% and 36.5 ± 3.1%, respectively.

The extraction solvent showed a significant ($p \leq 0.01$) influence on %PS, as both of the applied acids resulted in a significantly higher %PS than water, even three-fold higher with 0.1 M H_2SO_4. Cell wall hydrolysis that occurs with the use of acids facilitates PS extraction [34], resulting in a higher %PS with dilute acids compared with the water from brown seaweed *Sargassum fusiforme* [34] and *L. hyperborean* [35]. Additionally, the PS yield increased by lowering the pH [15], as 0.1 M HCl with pH 1 was less effective for PS extraction than 0.1 M H_2SO_4 with pH 0.7. Similarly, a better fucoidan and laminarin yield was achieved with 100 mM HCl (pH 2) than with 10 mM H_2SO_4 (pH 4) [15].

Table 2. Influence of algae species (*F. virsoides* and *C. barbata*), extraction solvent (water, 0.1 M HCl, and 0.1 M H_2SO_4), time (10, 20, and 30 min), and temperature (60, 80, 100 °C) in microwave assisted extraction on the yield (%PS) and chemical composition of the extracted polysaccharides.

	N	% PS	Total Sugar (%)	Fucose (%)	Sulfate Group (%)
Algae		$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †
F. virsoides	54	13.19 ± 0.02 ^{b}	15.40 ± 0.12 ^{b}	58.55 ± 0.44 ^{b}	25.60 ± 0.25 ^{a}
C. barbata	54	6.43 ± 0.02 ^{a}	6.37 ± 0.12 ^{a}	26.13 ± 0.44 ^{a}	34.80 ± 0.25 ^{b}
Solvent		$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †
H_2O	36	5.87 ± 0.03 ^{a}	9.07 ± 0.14 ^{a}	33.49 ± 0.54 ^{a}	19.26 ± 0.31 ^{a}
0.1 M HCl	36	8.14 ± 0.03 ^{b}	13.20 ± 0.14 ^{b}	50.67 ± 0.54 ^{c}	25.11 ± 0.31 ^{b}
0.1 M H_2SO_4	36	15.43 ± 0.03 ^{c}	10.39 ± 0.14 ^{a}	42.86 ± 0.54 ^{b}	46.22 ± 0.31 ^{c}
Time (min)		$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.05$ †
10	36	9.91 ± 0.03 ^{b}	10.35 ± 0.14 ^{a}	43.67 ± 0.53 ^{b}	30.02 ± 0.31 ^{a,b}
20	36	9.79 ± 0.03 ^{a}	10.58 ± 0.14 ^{a}	42.16 ± 0.53 ^{a,b}	30.89 ± 0.31 ^{b}
30	36	9.74 ± 0.03 ^{a}	11.72 ± 0.14 ^{b}	41.19 ± 0.53 ^{a}	29.68 ± 0.31 ^{a}
Temperature (°C)		$p \leq 0.01$ †	$p \leq 0.01$ †	$p = 0.34$ ‡	$p \leq 0.05$ †
60	36	8.54 ± 0.03 ^{a}	9.39 ± 0.14 ^{a}	42.43 ± 0.54 ^{a}	31.59 ± 0.31 ^{b}
80	36	10.30 ± 0.03 ^{b}	11.69 ± 0.14 ^{b}	42.85 ± 0.54 ^{a}	31.26 ± 0.31 ^{b}
100	36	10.60 ± 0.03 ^{b}	11.58 ± 0.14 ^{b}	41.74 ± 0.54 ^{a}	27.75 ± 0.31 ^{a}
Algae; solvent		$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †
F. virsoides; H_2O	18	8.30 ± 0.04 ^{b}	14.22 ± 0.20 ^{c}	47.30 ± 0.76 ^{c}	18.77 ± 0.43 ^{a}
F. virsoides; 0.1 M HCl	18	12.93 ± 0.04 ^{c}	19.95 ± 0.20 ^{d}	72.60 ± 0.76 ^{e}	21.71 ± 0.43 ^{a}
F. virsoides; 0.1 M H_2SO_4	18	18.35 ± 0.04 ^{d}	12.04 ± 0.20 ^{c}	55.76 ± 0.76 ^{d}	36.30 ± 0.43 ^{c}
C. barbata; H_2O	18	3.43 ± 0.04 ^{a}	3.92 ± 0.20 ^{a}	19.69 ± 0.76 ^{a}	19.76 ± 0.43 ^{a}
C. barbata; 0.1 M HCl	18	3.35 ± 0.04 ^{a}	6.45 ± 0.20 ^{b}	28.73 ± 0.76 ^{b}	28.51 ± 0.43 ^{b}
C. barbata; 0.1 M H_2SO_4	18	12.51 ± 0.04 ^{c}	8.74 ± 0.20 ^{b}	29.96 ± 0.76 ^{b}	56.13 ± 0.43 ^{d}
Algae; time (min)		$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †	$p = 16$ ‡
F. virsoides; 10	18	13.85 ± 0.04 ^{d}	15.24 ± 0.20 ^{c}	62.23 ± 0.76 ^{d}	26.17 ± 0.43 ^{a}
F. virsoides; 20	18	12.67 ± 0.04 ^{c}	14.68 ± 0.20 ^{c}	55.86 ± 0.76 ^{c}	25.11 ± 0.43 ^{a}
F. virsoides; 30	18	13.06 ± 0.04 ^{c,d}	16.29 ± 0.20 ^{d}	57.57 ± 0.76 ^{c}	25.51 ± 0.43 ^{a}
C. barbata; 10	18	5.96 ± 0.04 ^{a}	5.48 ± 0.20 ^{a}	25.11 ± 0.76 ^{a}	33.88 ± 0.43 ^{b}
C. barbata; 20	18	6.91 ± 0.04 ^{b}	6.48 ± 0.20 ^{b}	28.45 ± 0.76 ^{b}	36.67 ± 0.43 ^{c}
C. barbata; 30	18	6.42 ± 0.04 ^{a,b}	7.16 ± 0.20 ^{b}	24.82 ± 0.76 ^{a}	33.85 ± 0.43 ^{b}
Algae; temperature (°C)		$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †
F. virsoides; 60	18	11.72 ± 0.04 ^{d}	13.78 ± 0.20 ^{d}	61.51 ± 0.76 ^{c}	26.46 ± 0.43 ^{b}
F. virsoides; 80	18	14.36 ± 0.04 ^{f}	16.85 ± 0.20 ^{f}	60.51 ± 0.76 ^{c}	27.41 ± 0.43 ^{b}
F. virsoides; 100	18	13.50 ± 0.04 ^{e}	15.58 ± 0.20 ^{e}	53.64 ± 0.76 ^{b}	22.92 ± 0.43 ^{a}
C. barbata; 60	18	5.37 ± 0.04 ^{a}	5.00 ± 0.20 ^{a}	23.34 ± 0.76 ^{a}	36.72 ± 0.43 ^{d}
C. barbata; 80	18	6.24 ± 0.04 ^{b}	6.53 ± 0.20 ^{b}	25.20 ± 0.76 ^{a}	35.10 ± 0.43 ^{d}
C. barbata; 100	18	6.69 ± 0.04 ^{c}	7.59 ± 0.20 ^{c}	29.84 ± 0.76 ^{a}	32.58 ± 0.43 ^{c}

Values with different letters are statistically different at $p \leq 0.05$. † Statistically significant variables at $p \leq 0.05$. ‡ Statistically insignificant variables at $p \leq 0.05$.

A shorter extraction time and slightly higher temperature led to an increased %PS. However, an additional increase from 80 to 100 °C did not further improve the extraction yield. Considering these results, the optimal parameters that would result in the highest PS extraction from *F. virsoides* and *C. barbata* were 0.1 M H_2SO_4 for 10 min at 80 °C.

This research confirmed that the chemical composition of the extracted PS was influenced by the algal species, extraction solvent, temperature, and time. *F. virsoides* has a higher total sugar and fucose content, but a significantly lower sulfate group content. The average total sugar contents in the PS extracted from *F. virsoides* and *C. barbata* were 15.40% and 6.37%, respectively, which is in accordance with values reported for numerous seaweed species like *C. barbatta*, *F. vesiculosus*, *C. compressa*, *C. sedoides*, *C. crinite A. nodosum*, *Sargassum filipendul*, and *Saccharina longicruris*, in which the total sugar content ranged between 8.9% and 66.7% [26,28–31,36–38]. With 58.55% fucose obtained in this research, *F.*

virsoides was above the 24–35% range reported for algae from the *Fucus* genus [24], while *C. barbata*, with 26.13% fucose, was within the 16.5–61.5% range for algae from the *Cystoseira* genus [16,30,31]. The *F. virsoides* sulfate group content (25.6%) was within the 9–40.3% range reported for *Fucus* genus [1,24,26,39–41], while the 34.8% sulfate groups in *C. barbata* were slightly above the 14.65–22.51% range reported for *Cystoseira* genus [28–32].

The PS obtained with water had a significantly lower ($p \leq 0.01$) total sugar, fucose, and sulfate group content. The highest total sugar and fucose contents were achieved with 0.1 M HCl, while the sulfate group content was the highest with 0.1 M H_2SO_4. Acid promotes sulfate ester breakage, so more sulfate groups can be liberated [23,42], thus explaining the higher sulfate group content obtained by the acids. However, opposite results were reported for *S. fusiforme* [34], *Saccharina japonica* [13], and *Undaria pinnatifida* [43]. Because of the sulfate group in H_2SO_4, which might interfere with the sulfate analysis, diluted H_2SO_4 tended to yield a high sulfate fucoidan, while diluted HCl tends to yield low sulfate fucoidan [42]; nevertheless, it is generally preferred [23].

A longer time improved the total sugar extraction, while the fucose and sulfate group contents were reduced after 30 min of extraction. A higher temperature resulted in a higher total sugar and lower sulfate group content, but it did not have a significant ($p \leq 0.05$) influence on the fucose content. The same trend was reported for *A. nodosum* [14] and, similarly, by increasing the pressure from 30 to 120 Psi, which corresponded to a temperature increase from 122 to 172 °C, the *F. vesiculosus* total sugar concentration increased [1].

3.2. Pressurised Liquid Extraction

PLE was used to extract the polysaccharides from *F. virsoides* and *C. barbata*, and the influence of the extraction solvent (water and 0.1 M H_2SO_4), temperature (60, 100, and 140 °C), time (5, 10, and 15 min), and number of cycles (1 and 2) on the yield and chemical composition of the extracted polysaccharides is shown in Table 3. The average *F. virsoides* %PS obtained by PLE was 10.22%, which is significantly lower than the 11.7% obtained for *C. barbata*. PLE was used to extract fucoidan from *Nizamuddinia zanardinii*, and the obtained yield ranged from 4.99 to 23.77% [44] and 13.15% [45]. Likewise, *S. japonica* crude fucoidan yield obtained with PLE ranged from 0.1 to 12.89% [46] and 8.23% [13].

The type of solvent had the same effect as in MAE on %PS, as the acid lead to a significantly higher %PS for both algae. By increasing the time, temperature, and number of cycles, the *F. virsoides* and *C. barbata* %PS increased. The increased yield obtained at elevated temperatures is explained by the increased mass transfer, lower surface tension, and higher solubility of numerous compounds [47]. The extraction yield of *S. japonica* increased with an increase in temperature from 180 °C to 420 °C [47], while the *N. zanardinii* extraction yield increased when the extraction time was increased from 10 to 30 min [44]. Similarly, the %PS from *S. japonica* was enhanced by increasing the pressure from 20 to 80 bar [46] and from 13 to 520 bar [47]. Regarding the results obtained in this research, the optimal PLE parameters that will result in the highest PS extraction from *F. virsoides* and *C. barbata* are 0.1 M H_2SO_4, for two cycles of 15 min at 140 °C. A temperature of 150 °C, time of 29 min, and solvent to sample ratio of 21 mL g^{-1} were determined to be the optimal conditions for fucoidan extraction from *N. zanardinii* [44], while the optimal conditions for PS extraction from *S. japonica* were a temperature of 127.01 °C, pressure of 80 bar, and sample to solvent ratio of 0.04 g mL^{-1} [46].

Table 3. Influence of algae species (*F. virsoides* and *C. barbata*), extraction solvent (water and 0.1 M H_2SO_4), temperature (60, 100, and 140 °C), time (5, 10, and 15 min), and number of cycles (1 and 2) in pressurized liquid extraction on the yield (%PS) and chemical composition of the extracted polysaccharides.

	N	% PS	Total Sugar (%)	Fucose (%)	Sulfate Group (%)
Algae		$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †
F. virsoides	72	10.22 ± 0.03 ^{a}	14.50 ± 0.09 ^{b}	42.03 ± 0.19 ^{b}	65.70 ± 0.42 ^{b}
C. barbata	72	11.77 ± 0.03 ^{b}	7.83 ± 0.09 ^{a}	13.57 ± 0.19 ^{a}	60.45 ± 0.42 ^{a}
Solvent		$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †
H_2O	72	5.07 ± 0.03 ^{a}	17.19 ± 0.09 ^{b}	33.18 ± 0.19 ^{b}	58.32 ± 0.42 ^{a}
0.1 M H_2SO_4	72	16.93 ± 0.03 ^{b}	5.14 ± 0.09 ^{a}	22.41 ± 0.19 ^{a}	67.83 ± 0.42 ^{b}
Temperature (°C)		$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.05$ †	$p \leq 0.01$ †
60	48	8.39 ± 0.04 ^{a}	11.86 ± 0.11 ^{c}	27.24 ± 0.24 ^{a}	75.42 ± 0.52 ^{b}
100	48	10.96 ± 0.04 ^{b}	11.17 ± 0.11 ^{b}	28.02 ± 0.24 ^{a,b}	57.26 ± 0.52 ^{a}
140	48	13.64 ± 0.04 ^{c}	10.48 ± 0.11 ^{a}	28.13 ± 0.24 ^{b}	56.55 ± 0.52 ^{a}
No. of cycles		$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †
1	72	10.67 ± 0.03 ^{a}	11.70 ± 0.09 ^{b}	28.54 ± 0.19 ^{b}	64.86 ± 0.42 ^{b}
2	72	11.33 ± 0.03 ^{b}	10.64 ± 0.09 ^{a}	27.05 ± 0.19 ^{a}	61.29 ± 0.42 ^{a}
Time (min)		$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †
5	48	9.94 ± 0.04 ^{a}	12.37 ± 0.11 ^{b}	28.48 ± 0.24 ^{b}	62.29 ± 0.52 ^{a}
10	48	11.21 ± 0.04 ^{b}	10.49 ± 0.11 ^{a}	26.22 ± 0.24 ^{a}	60.64 ± 0.52 ^{a}
15	48	11.84 ± 0.04 ^{c}	10.65 ± 0.11 ^{a}	28.69 ± 0.24 ^{b}	66.30 ± 0.52 ^{b}
Algae; solvent		$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †
F. virsoides; H_2O	36	6.40 ± 0.05 ^{b}	22.03 ± 0.13 ^{d}	54.85 ± 0.27 ^{d}	63.48 ± 0.60 ^{b}
F. virsoides; 0.1 M H_2SO_4	36	14.04 ± 0.05 ^{c}	6.98 ± 0.13 ^{b}	29.20 ± 0.27 ^{c}	67.93 ± 0.60 ^{c}
C. barbata; H_2O	36	3.73 ± 0.05 ^{a}	12.36 ± 0.13 ^{c}	11.51 ± 0.27 ^{a}	53.17 ± 0.60 ^{a}
C. barbata; 0.1 M H_2SO_4	36	19.81 ± 0.05 ^{d}	3.31 ± 0.13 ^{a}	15.62 ± 0.27 ^{b}	67.73 ± 0.60 ^{c}
Algae; temperature (°C)		$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †
F. virsoides; 60	24	7.10 ± 0.06 ^{a}	15.49 ± 0.16 ^{d}	45.17 ± 0.33 ^{d}	80.52 ± 0.73 ^{d}
F. virsoides; 100	24	9.17 ± 0.06 ^{b}	14.21 ± 0.16 ^{c}	39.99 ± 0.33 ^{c}	58.12 ± 0.73 ^{b}
F. virsoides; 140	24	14.40 ± 0.06 ^{e}	13.82 ± 0.16 ^{c}	40.92 ± 0.33 ^{c}	58.46 ± 0.73 ^{b}
C. barbata; 60	24	9.68 ± 0.06 ^{c}	8.22 ± 0.16 ^{b}	9.31 ± 0.33 ^{a}	70.32 ± 0.73 ^{c}
C. barbata; 100	24	12.75 ± 0.06 ^{d}	8.14 ± 0.16 ^{b}	16.05 ± 0.33 ^{b}	56.40 ± 0.73 ^{a,b}
C. barbata; 140	24	12.89 ± 0.06 ^{d}	7.13 ± 0.16 ^{a}	15.34 ± 0.33 ^{b}	54.63 ± 0.73 ^{a}
Algae; no. of cycle		$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †
F. virsoides; 1	36	10.02 ± 0.05 ^{a}	15.49 ± 0.13 ^{a}	44.32 ± 0.27 ^{d}	65.13 ± 0.60 ^{b}
F. virsoides; 2	36	10.43 ± 0.05 ^{b}	13.52 ± 0.13 ^{b}	39.74 ± 0.27 ^{c}	66.27 ± 0.60 ^{b}
C. barbata; 1	36	11.31 ± 0.05 ^{c}	7.91 ± 0.13 ^{a}	12.76 ± 0.27 ^{a}	64.59 ± 0.60 ^{b}
C. barbata; 2	36	12.23 ± 0.05 ^{d}	7.76 ± 0.13 ^{a}	14.37 ± 0.27 ^{b}	56.31 ± 0.60 ^{a}
Algae; time (min)		$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †
F. virsoides; 5	24	8.47 ± 0.06 ^{a}	16.04 ± 0.16 ^{e}	44.24 ± 0.33 ^{e}	63.54 ± 0.73 ^{b,c}
F. virsoides; 10	24	10.33 ± 0.06 ^{b}	13.92 ± 0.16 ^{d}	39.22 ± 0.33 ^{c}	67.90 ± 0.73 ^{d}
F. virsoides; 15	24	11.87 ± 0.06 ^{d,e}	13.55 ± 0.16 ^{d}	42.63 ± 0.33 ^{d}	65.66 ± 0.73 ^{c,d}
C. barbata; 5	24	11.41 ± 0.06 ^{c}	8.69 ± 0.16 ^{c}	12.71 ± 0.33 ^{a}	61.03 ± 0.73 ^{b}
C. barbata; 10	24	12.10 ± 0.06 ^{e}	7.06 ± 0.16 ^{a}	13.23 ± 0.33 ^{a}	53.38 ± 0.73 ^{a}
C. barbata; 15	24	11.80 ± 0.06 ^{d}	7.74 ± 0.16 ^{b}	14.75 ± 0.33 ^{b}	66.95 ± 0.73 ^{d}

Values with different letters are statistically different at $p \leq 0.05$. † Statistically significant variables at $p \leq 0.05$.

F. virsoides had a higher total sugar and fucose content than *C. barbata*, which is aligned with the MAE results, while the sulfate group content in PLE was higher in *F.virsoides*. The sulfate group content was higher with acid, which is in accordance with the results for MAE, while a higher fucose and total sugar content were obtained with water, which is reversed from the experiment with MAE. However, the same trend for the fucose content was observed in the fucoidan from *U. pinnatifida* [48] and *Sargassum* sp. [43] because of to the possible breakage of chemical bonds between the fucose structures caused by acid [48].

3.3. Comparison of Different Extraction Methods

Table 4 shows the yields and chemical composition of the polysaccharides extracted from *F. virsoides* and *C. barbata*, by conventional and advanced extraction techniques performed under determined optimal conditions. The extraction technique had a significant influence ($p \leq 0.05$) on the %PS for both algae. Along with a reduced extraction time, from 3 h to 30 min (2 cycles of 15 min), the PLE resulted in a significantly higher %PS from *F. virsoides*, while for *C. barbata*, there was no statistical difference between CE and PLE. Even though MAE was only 10 min, there was no statistical difference ($p \leq 0.05$) in %PS between the CE and MAE for both algae, meaning that a similar yield was achieved in a much shorter time. Compared with the CE techniques, applying PLE resulted in a significantly higher PS% from *N. zanardinii* [44] and *S. japonica* [13,46], while CE was more efficient than MAE from *A. nodosum* [14,49]. The *N. zanardinii* %PS obtained by PLE (water; 1500 W; 150 °C; two cycles of 10 min) was 13.15 ± 1.05%, which is significantly higher than the 6.17 ± 0.62% and 5.2 ± 0.5% obtained by MAE and conventional hot water extraction, respectively [45]. Under a high temperature and pressure, the physical properties of a solvent are modified, resulting in improved cell destruction, capillary effects, mass transfer, and solvent penetration, and consequentially in increased extraction yields [50].

Table 4. Yield (% PS) and chemical composition of CE (conventional), MAE (microwave) and PLE (pressurised liquid) extracted polysaccharides from *Fucus virsoides* and *Cystoseira barbata*.

		% PS	Total Sugar (%)	Fucose (%)	Sulfate Group (%)	Uronic Acid (%)
F. virsoides		$p \leq 0.05$ †	$p \leq 0.05$ †	$p \leq 0.05$ †	$p \leq 0.05$ †	$p \leq 0.05$ †
	CE	18.53 ± 0.00 ^{a}	20.17 ± 0.00 ^{c}	41.54 ± 0.01 ^{a}	28.46 ± 0.01 ^{a}	20.06 ± 0.00 ^{c}
	MAE	20.42 ± 0.28 ^{a}	15.65 ± 0.09 ^{a}	48.48 ± 1.36 ^{b}	37.13 ± 0.26 ^{b}	15.93 ± 0.77 ^{b}
	PLE	24.22 ± 0.94 ^{b}	18.24 ± 0.13 ^{b}	60.08 ± 0.22 ^{c}	51.82 ± 1.72 ^{c}	5.32 ± 0.51 ^{a}
C. barbata		$p \leq 0.05$ †	$p \leq 0.05$ †	$p = 0.11$ ‡	$p \leq 0.05$ †	$p \leq 0.05$ †
	CE	16.47 ± 0.18 ^{a,b}	6.34 ± 0.18 ^{a,b}	22.53 ± 0.14 ^{a}	35.53 ± 0.80 ^{a}	15.72 ± 0.34 ^{c}
	MAE	15.27 ± 0.17 ^{a}	7.14 ± 0,53 ^{b}	26.61 ± 2.15 ^{a}	45.56 ± 0.34 ^{b}	12.52 ± 0.08 ^{b}
	PLE	18.77 ± 0.82 ^{b}	4.40 ± 0.19 ^{a}	28.06 ± 0.44 ^{a}	57.58 ± 2.19 ^{c}	7.15 ± 0.36 ^{a}

Values with different letters are statistically different at $p \leq 0.05$. † Statistically significant variable at $p \leq 0.05$. ‡ Statistically insignificant variable at $p \leq 0.05$.

While the highest total sugar content in *F. virsoides* was obtained by CE and the lowest by MAE, in *C. barbata*, the total sugar content obtained by PLE and MAE was not statistically different from that of CE. Similar to our results, Alboofetileh et al. [45] achieved the lowest total sugar content with MAE, followed by PLE and CE. On the contrary, a lower total sugar content was obtained for CE than for MAE [49] and PLE [44,46]. The *F. virsoides* fucose content was higher with both of the applied advanced techniques, while the *C. barbata* fucose content was not significantly influenced ($p \geq 0.05$) by the extraction technique. Comparable results were reported for *N. zanardinii* polysaccharides, where the extract obtained by PLE had a higher fucose content than for MAE and CE. Likewise, a higher fucose content was obtained by MAE [49] and PLE in comparison with CE [46]. The sulfate group content for both algae was the highest with PLE, followed by MAE and CE. In the research by Alboofetileh et al. [45], the higher sulfate group content was achieved with MAE and the lowest with PLE. However, it has been reported that MAE resulted in a lower sulfate group content than CE [14,49], while the sulfate group content was lower for PLE in the research by Alboofetileh et al. [44], but higher for Sivagnanam Saravana et al. [46]. A higher PS sulfate group content is an advantageous property, as it has been reported that PS with a higher sulfate content display a higher biological activity. Therefore, the increased sulfate group content observed during these advanced extraction techniques could potentially increase the antioxidant, anticoagulant and anti-HIV (human immunodeficiency virus) activity of extracted PS [14]. For both algae, CE resulted in the highest uronic acid content, while the application of PLE led to a lower uronic acid content, which is opposite to the results obtained for *N. zanardinii*, where the uronic acid content

was the highest in the extract obtained by PLE, while there was no statistical difference between MAE and CE [45]. In comparison with CE, a higher uronic acid content was reported by PLE for fucoidan from *S. japonica* [46], as well as MAE for *A. nodosum* [14,49]. On the other hand, *N. zanardinii* fucoidan contained 2.07% uronic acid for PLE and 3.9% for CE [44].

The monosaccharide compositions of the CE, MAE, and PLE extracted fucoidans from *C. barbata* and *F.virsoides* are shown in Table 5. In all of the extracted fucoidans, L-fucose was the predominant monosaccharide, which was expected and in accordance with most of the previously published studies [45,51–54]. Other detected monosaccharides were glucose, galacturonic acid (oxidized form of D-galactose), and arabinose, while mannose, rhamnose, and fructose were not detected. Glucose and galactose were detected in the majority of similar studies, but in different ratios, e.g., Foley et al. [53] reported 21.3 mol% glucose and 6.1 mol% galactose in *A. nodosum* fucoidan, while Wang et al. [52] reported 1.93 mol% glucose and 24.33 mol% galactose in *Laminaria japonica*. This study also confirmed that the monosaccharides ratio varies according to the extraction method used, which was previously observed by Alboofetileh et al. [45].

Table 5. Monosaccharide composition and molecular properties (weight average molecular weight—M_w; number average molecular weight—M_n; polydispersity index—PDI) of CE (conventional), MAE (microwave), and PLE (pressurized liquid) extracted polysaccharides from *Fucus virsoides* and *Cystoseira barbata*.

		Monosacharide Composition (%)				Molecular Properties		
		Glucose	Fucose	Galacturonic Acid	Arabinose	M_w (kDa)	M_n (kDa)	Polydispersity (M_w/M_n)
F. virsoides		$p \leq 0.05$ †	$p \leq 0.05$ †	$p \leq 0.05$ †	$p \leq 0.05$ †			
	CE	18.65 ± 0.24 ^{b}	44.83 ± 0.45 ^{b}	19.48 ± 0.26 ^{b}	17.04 ± 0.25 ^{c}	693.43	264.42	2.62
	MAE	13.26 ± 0.36 ^{a}	78.35 ± 0.21 ^{c}	n.d. ^{a}	8.39 ± 0.19 ^{a}	891.25	332.14	2.68
	PLE	19.04 ± 0.41 ^{b}	41.90 ± 0.33 ^{a}	26.52 ± 0.31 ^{c}	12.55 ± 0.35 ^{b}	521.72	149.64	3.49
C. barbata		$p \leq 0.05$ †	$p \leq 0.05$ †	$p \leq 0.05$ †	$p \leq 0.05$ †			
	CE	n.d.^{a}	100 ± 0.00 ^{c}	n.d. ^{a}	n.d. ^{a}	766.00	322.87	2.37
	MAE	27.22 ± 0.16 ^{c}	61.27 ± 0.51 ^{b}	n.d. ^{a}	11.52 ± 0.32 ^{b}	1252.19	681.34	1.84
	PLE	16.95 ± 0.38 ^{b}	49.5 ± 0.28 ^{a}	19.75 ± 0.38 ^{b}	13.79 ± 0.48 ^{c}	1031.94	415.75	2.48

Values with different letters are statistically different at $p \leq 0.05$. † Statistically significant variables at $p \leq 0.05$.

The molecular properties of the PS extracts (Table 5) were analyzed using HPSEC, which provided information on the weight average molecular weight (M_w), the number average molecular weight (M_n), and the polydispersity index (PDI). M_n is the statistical average molecular weight of all of the polymer chains within a sample, whereas M_w represents the molecular size of the sample [55]. M_w is more influenced by high molecular weight chains, while M_n is more influenced by lower molecular weight chains [55]. PDI is the ratio between M_w and M_n and it measures the heterogeneity of the molecular weight distributions of polymers, where a larger difference between M_w and M_n (larger PDI) indicates a more heterogeneous molecular weight distribution [55].

The M_w of CE, MAE, and PLE extracted PS ranged between 521.72 to 891.25 kDa and 766 to 1252.19 kDa for *F.virsoides* and *C. barbata*, respectively, and fell within the range of reported fucoidan M_w values, namely 1.4–1323 kDa [14,26]. The differences in the molecular weight, when compared with the literature values, could be attributed to the algal species and growth conditions, and it is highly dependent on the extraction methodology used [55]. According to their molecular weight, fucoidans can be classified as low-molecular-weight fucoidans (<10 kDa), medium-molecular-weight fucoidans (10–10,000 kDa), and high-molecular-weight fucoidans (>10,000 kDa) [56]. For both algae, the highest M_w was achieved in the samples obtained by MAE, while the lowest M_w for *F. virsoides* was in the PLE samples and for *C. barbata* it was in the CE samples. Likewise, the *N. zanardinii* M_w obtained by MAE was the highest (1184 kDa), followed by CE (823 kDa), while a significantly lower M*w* was in the extract obtained by PLE (670 kDa) [45]. On the contrary, the M_w of the *A. nodosum* fucoidan extracted by MAE (30.8 kDa) was significantly

lower compared with the samples obtained by CE (40.2 kDa), ultrasound-assisted extraction (121.1 kDa), and enzyme-assisted extraction (100.1 kDa) [49], as microwave heating contributed to the cell wall degradation and splitting of the poly-/oligo-saccharides in the extraction medium [49].

Natural polymers such as proteins are usually monodisperse with a PDI of approximately 1, while polysaccharides are polydisperse with PDIs higher than 1. The PDI of the PS extracted in this study ranged from 1.84 to 3.49, and it fell within the 1 to 6.2 [31,45] range of reported fucoidan PDI values. For both algae, the PDI was the highest in the samples obtained by PLE, indicating a larger degradation during the extraction process. Likewise, PDI was lower in the *N. zanardinii* fucoidan extracted with CE (1.56) compared with MAE (1.84) and PLE (1.78) [45].

In order to determine the antioxidant capacity of the PS extracts obtained at optimized conditions for each method, ORAC and DPPH assays were employed and the results are shown in Figure 1. The PS from *F. virsoides* and *C. barbata* obtained with MAE had the highest ORAC value of 42.22 ± 0.12 and 38.62 ± 0.12 µmol TE g^{-1}, respectively. Furthermore, the extracts obtained with PLE had the lowest ORAC and DPPH values for both algae, which is possibly linked with their chemical structure (higher sulfate content, lower uronic acid content, and lower M_w). Even though the antioxidant activity of fucoidan has been previously confirmed, the relationship between chemical structure and antioxidant activity, up until now, has not been established. It is known that the antioxidant activity is not a function of a single factor, but a combination of several related physicochemical characteristics, such as the uronic acid content, sulfate group content, protein content, and molecular weight [34]. Therefore, we checked if there was a correlation between the structural characteristics (uronic acid, sulfate group, M_w, and PDI) and antioxidant measurements (ORAC and DPPH values). Sulfate group content had a significant ($p \leq 0.05$) strong positive correlation ($r > 0.8$) with the ORAC and DPPH values, while the uronic acid content, M_w, and PDI did not correlate with the ORAC and DPPH values. The ORAC values were not well correlated with the total sulfate content and molecular weight of red algae *Gigartina skottsbergii* and *Schizymenia binderi* and brown algae *Lessonia vadosa* [57].

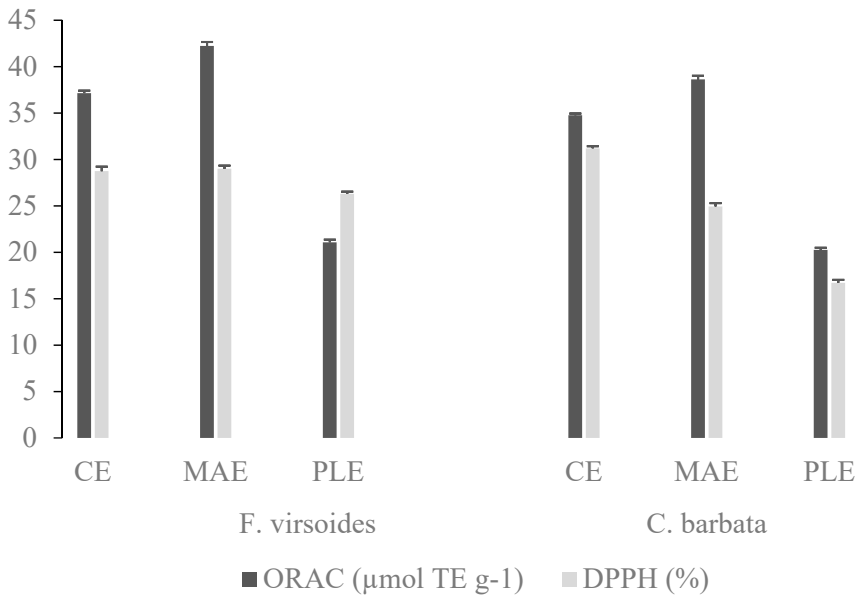

Figure 1. Antioxidant capacity of *F. virsoides* and *C. barbata* polysaccharides, obtained by conventional extraction (CE), microwave assisted extraction (MAE), and pressurized liquid extraction (PLE), determined by oxygen radical absorbance capacity (ORAC) and DPPH assays.

4. Conclusions

Advanced extraction techniques, namely, MAE and PLE, were successfully applied and optimized to extract sulfated polysaccharides from brown algae *F. virsoides* and *C. barbata*. PLE under optimal extraction parameters (0.1 M H_2SO_4, for two cycles of 15 min at 140 °C) resulted in a significantly higher %PS from *F. virsoides*, while for *C. barbata*, a similar yield was achieved with CE and PLE. Likewise, a similar yield was achieved with MAE (0.1 M H_2SO_4 for 10 min at 80 °C) and CE for both algae. Although advanced extraction techniques did not excessively improve the polysaccharide yield, the extraction time was reduced from 3 h to 30 min (PLE) or 10 min (MAE), which contributed to significant energy saving. Furthermore, the polysaccharides obtained by PLE had the highest PDI, fucose, and sulfate group content, and the lowest uronic acid content; however, the antioxidant activity was lower. The correlation between the chemical structure and biological activity, as one of the emerging questions in this field, still remains unclear. These findings indicate that PLE and MAE could be effectively used as a potential method for polysaccharide extraction from brown seaweed, while their apparent antioxidant activity makes these polysaccharides interesting for use in processed and functional food, and in the pharmaceutical and chemical industries.

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Chapter 4

Publication No. 4: Application of ultrasound-assisted extraction and non-thermal plasma for *Fucus virsoides* and *Cystoseira barbata* polysaccharides pre-treatment and extraction

Processes

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processes

MDPI

Article

Application of Ultrasound-Assisted Extraction and Non-Thermal Plasma for *Fucus virsoides* and *Cystoseira barbata* Polysaccharides Pre-Treatment and Extraction

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Abstract: Brown algae *Fucus virsoides* and *Cystoseira barbata* are an abundant source of sulfated polysaccharide fucoidan, which has shown a wide range of biological activities. These activities are significantly dependent on the fucoidan chemical composition, which is closely linked with the applied extraction technique and process parameters. In order to overcome the drawbacks of lengthy conventional extraction (CE), advanced extraction techniques, such as ultrasound-assisted extraction (UAE) and non-thermal plasma (NTP), were applied. Furthermore, this study also investigated the efficiency of different solvents as well as UAE and NTP as 5 min pre-treatments prior to CE as a more effective course of cell wall breakage and, consequently, a higher polysaccharide yield (%PS). Apart from %PS, the effect of this procedure on the chemical composition and antioxidant capacity of the extracted polysaccharides was also monitored. When comparing the extraction solvent, the application of 0.1 M H_2SO_4, instead of H_2O, resulted in a three-fold higher %PS, a higher sulfate group, and a lower fucose content. Application of CE resulted in higher %PS, uronic acids, and fucose content as well as oxygen radical absorbance capacity (ORAC) and DPPH values, while the average molecular weight (M_w), sulfate group, and glucose content were lower during CE when compared to 30 min of UAE and NTP treatment. Application of UAE and NTP as 5 min pre-treatments decreased fucose content, while %PS and sulfate content were similar to values obtained when using CE.

Keywords: ultrasound-assisted extraction; non-thermal plasma; polysaccharides; extraction; brown algae; advanced extraction techniques; fucoidan; antioxidant capacity

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1. Introduction

Seaweeds or marine macroalgae are an excellent source of nutrients and bioactive molecules with a broad range of applications in the food, pharmaceutical, cosmetics, and textile industries. Among the nutrients and bioactive molecules present in macroalgae (polysaccharides, proteins, peptides, amino acids, polyphenols, pigments, vitamins, minerals, etc.), polysaccharides (PS) are the most prominent with respect to possible commercial application. According to the latest worldwide statistics on aquaculture compiled by the FAO in 2018, total production of aquatic algae was 32.4 million tons with a total sale value of USD 13.3 billion [1].

Fucus virsoides is an endemic species in the Adriatic Sea, and it is the only species of the Fucus genus to occur in the Adriatic Sea [2]. It has a perennial flat dark brown thallus that is dichotomously branched, flattened, and with a distinct midrib [3]. *Cystoseira barbata* is an endemic species of the Mediterranean Sea, growing mostly in the coastal area of the

northern Adriatic and most abundantly during April and May [4]. It is a perennial, large, strongly branched, flexible, brown alga that can grow up to 2 m.

F. virsoides and *C. barbata* are potentially a good source of PS—fucoidan, laminarin, and alginate. Fucoidan is sulfated PS with high fucose content interconnected by α-(1,3) glycoside bonds or by alternating α-(1,3) and α-(1,4) bonds (and very rarely α-(1,2) bonds). Apart from fucose, it contains lower amounts of other monosaccharides including glucose, galactose, mannose, xylose, rhamnose, and uronic acids [2]. It is found in the fibrillar tissue of the cell wall and the intercellular space of brown algae, and it has been established that it possesses a wide range of positive effects such as antioxidant, anti-inflammatory, and antitumor [5–7], which give it great potential for use in a wide range of functional food, cosmeceutical, and pharmaceutical products [8]. Biological functions of fucoidan are closely correlated with its physicochemical properties [9], namely, sulfate group content, sulfate group position, molecular weight, types and ratios of constituent monosaccharides, and features of glycosidic bonds. However, these physicochemical properties are influenced by algal species, location, harvesting season [10], extraction techniques, and different extraction conditions (e.g., time, temperature, solvent, pH, particle size, pressure, agitation speed, and sample-to-solvent ratio) [6,9,11].

Compared to the currently employed conventional extraction (CE) techniques, advanced techniques have advantages of shorter extraction time; lower temperature; reduced energy, cost, and organic solvents consumption [12]. As it was previously summarized [8], microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), pressurized liquid extraction (PLE), and enzyme-assisted extraction (EAE) have been successfully used for brown algae PS extraction. Although these extraction techniques have different principles of operation, their common goal is breakdown of brown algae cell walls, where most of the bioactive molecules, including fucoidan, are stored [13].

UAE is recognized as an effective extraction technique based on the application of high-frequency sound waves (>20 kHz), and it has been studied for the extraction of bioactive compounds from various plant and algal matrices [14–17]. The ultrasonication technique applies physical forces in the extraction of molecules generated by acoustic cavitation, such as shear, shockwaves, microjets, and acoustic streaming [18], which results in rapid formation and collapse of cavitation bubbles within irradiated liquid medium, leading to intense stress and irreversible chain splitting [19].

Recently, the potential of cold or non-thermal plasma (NTP) in isolation of bioactive molecules has also been explored. NTP is generated by the application of an electric or electromagnetic field to a gas. This accelerates the free electrons and ionizes the gas atoms and molecules releasing more free electrons, provoking new ionizations and producing molecular dissociations [20]. Therefore, the plasma is constituted of molecules and atoms in an excited state, positive and negative ions, free radicals, electrons, UV radiation, and reactive oxygen and nitrogen species such as ozone, hydroxyl radicals, superoxide, atomic oxygen, singlet oxygen, nitric oxide, or nitrogen dioxide [20]. These reactive species could damage cell structure, rapidly and easily diffuse into the cells, and provoke damage of lipids, proteins, nucleic acids, and carbohydrates [20]. In the food industry, NTP is mostly used for food preservation and maintenance of food safety due to the fact of its effectiveness in microbial inactivation. However, some studies also reported its application in phenolics extraction from tomato pomace [21], essential oil extraction [22–25], lipid extraction from microalgae *Nannochloropsis gaditana* [26], and as a pre-extraction procedure prior to Soxhlet extraction from coffee grounds [27]. To the best of our knowledge, there are no reported studies on the use of NTP for algal PS extraction.

Since physicochemical properties of algal PS are influenced by various parameters, and the aim of this research was to study the effect of different solvents (i.e., H_2O, 0.1 M HCl, and 0.1 M H_2SO_4) and extraction techniques (i.e., CE, UAE, and NTP) for their extraction from brown algae *F. virsoides* and *C. barbata* and to compare them in terms of yield, chemical composition, and antioxidant capacity. Furthermore, the research goal was to investigate if application of UAE and NTP as a pre-treatment prior to CE would

cause more cell wall breakage and, consequently, higher yield as well as how it would affect chemical composition of the extracted PS. Overall, this study aimed to determine if the application of advanced extraction techniques could successfully extract algal PS but with a reduced time and lower temperature, which is economically and ecologically more beneficial.

2. Materials and Methods

2.1. Chemicals and Reagents

All chemicals and reagents used in this research were of analytical grade. Gelatin, L-cysteine, sodium tetraborate, sulfamic acid, chloroform, potassium hydroxide, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and 1-phenyl-3-methyl-5-pyrazolone (PMP) were purchased from Acros Organics (Geel, Belgium). Fucoidan from *Fucus vesiculosus*, phenol, *m*-hydroxydiphenyl, D-(+)-glucose, L-(−)-fucose, D-(+)-mannose, D-(−)-fructose, D-galacturonic acid, L-rhamnose, arabinose, trimethylamine, ammonium acetate, and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ethanol, acetone, potassium sulfate, and sodium carbonate were obtained from Gram-mol Ltd. (Zagreb, Croatia); Folin–Ciocalteu reagent, acetonitrile, trichloroacetic acid (TCA), and silicone oil from Fisher Scientific (Leicestershire, UK); absolute ethanol, hexane, and ethyl acetate from Carlo Erba Reagents (Cornaredo, Italy). Fluorescein sodium salt was purchased from Honeywell Riedel-de-Haën (Bucharest, Romania); barium chloride from abcr GmbH (Karlsruhe, Germany); sodium hydroxide from Lach-Ner (Zagreb, Croatia); D-(−)-ribose from TCI (Portland, OR, USA); sulfuric acid from Scharlab S.L. (Barcelona, Spain); hydrochloric acid (HCl) from TKI Hrastnik (Hrastnik, Slovenia).

2.2. Algal Material and Preliminary Treatments

In February 2020, from the coastal region of Zadar (Croatia), marine biologist Donat Petricioli harvested and identified brown algae *Fucus virsoides* (44°12′02″ N; 15°28′51″ E) and *Cystoseira barbata* (44°12′42″ N; 15°09′23″ E) (Figure 1). Freshly harvested algae were washed, frozen at −60 °C (ScanCool SCL210P, Labogene ApS, Lillerød, Denmark), and freeze-dried (CoolSafe lyophilizer, 55-9 PRO, Labogene, Lillerød, Denmark) for 24 h. The freeze-dried algae were milled with an electric grinder WSG30E/K (Waring Commercial, Stamford, CT, USA), and the powder was stored at −4 °C for one week before the extraction process.

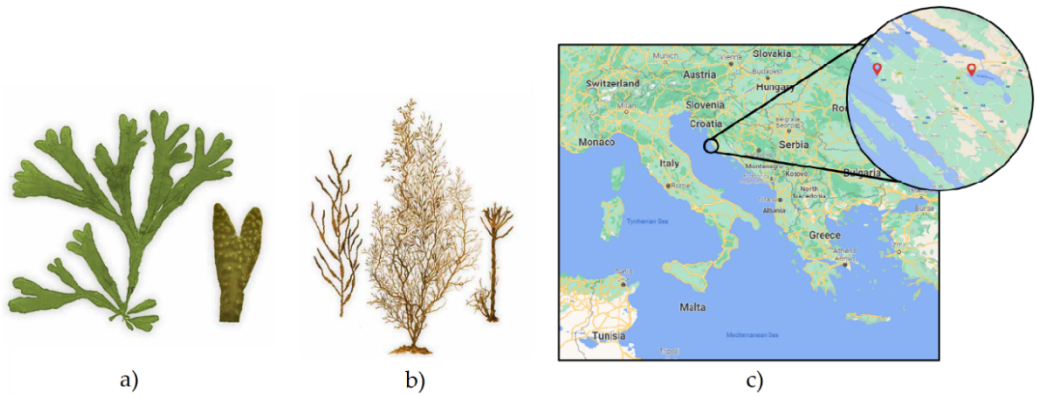

Figure 1. (**a**) Fucus virsoides; (**b**) Cystoseira barbata (**c**); location of seaweed collection.

2.3. Extraction of PS

Prior to extraction, a pre-treatment process was performed with continuous stirring in two steps under previously established conditions [28]: 18 h at room temperature with acetone followed by 4 h at 70 °C with 96% ethanol. After centrifugation, filtration, and

air-drying, pre-treated dried algae (1 g) were extracted with H_2O, 0.1 M HCl, or 0.1 M H_2SO_4 (30 mL) by the following procedures (Figure 2):

(a) CE for 3 h at 80 °C under constant stirring (400 rpm) [28];
(b) UAE with an ultrasonic homogenizer UP200HT (200 W, 26 kHz) (Hielscher, Teltow, Germany), titanium sonotrode Ø14 mm (154 mm^2) for 30 min at 100% amplitude, and a cold water bath was used to keep the temperature at approximately 25 °C;
(c) Five minutes of UAE treatment followed by CE for 3 h at 80 °C under constant stirring (400 rpm);
(d) Hybrid NTP with a pulse high-voltage generator HVG60/1 PL (Impel, Croatia) for 30 min; electrical voltage—30 kV; frequency—60 Hz; electric current—10 mA; distance between electrodes—1 cm; gas—argon at 1 L h^{-1};
(e) Five minutes NTP treatment followed by CE for 3 h at 80 °C under constant stirring (400 rpm).

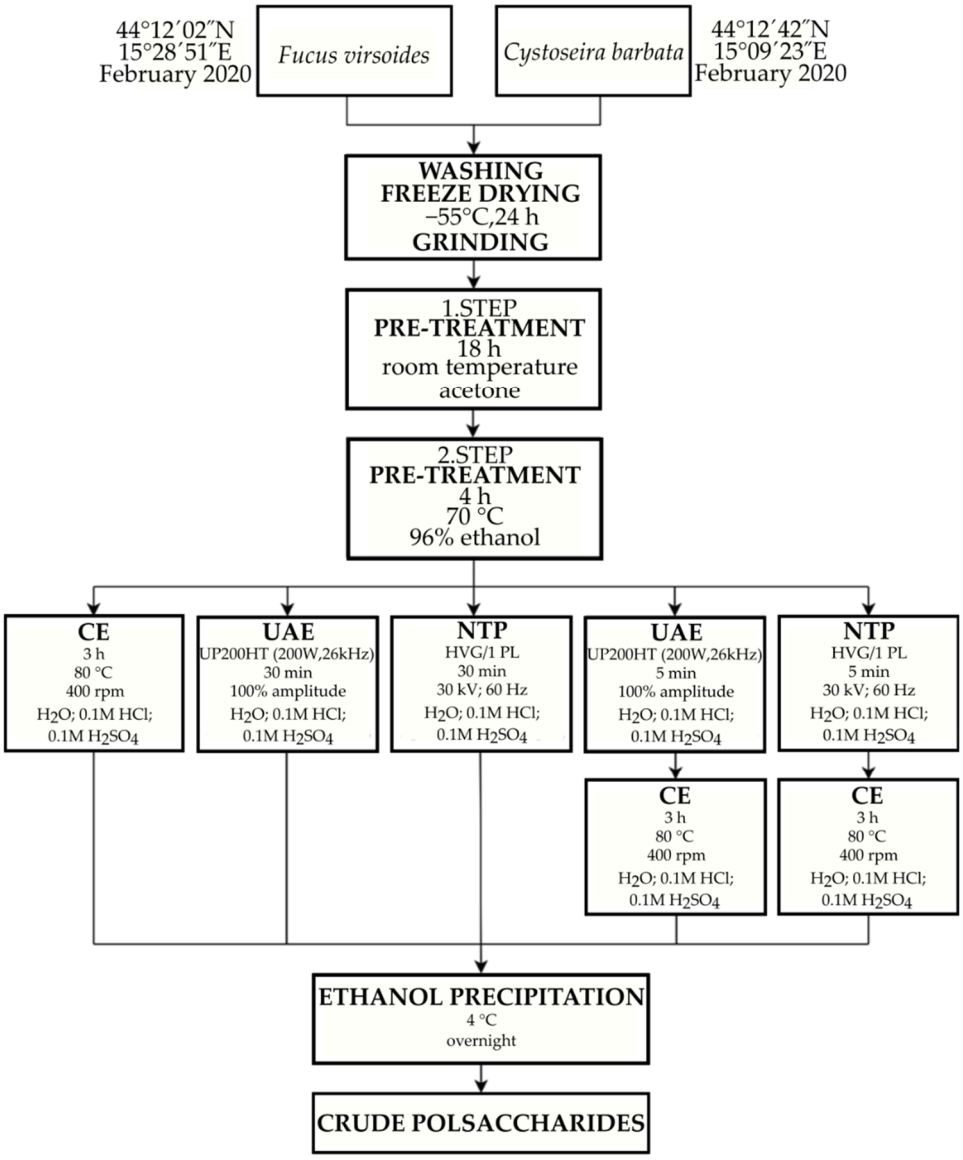

Figure 2. Schematic diagram of the experiment.

Afterwards, a double volume of absolute ethanol (60 mL) was added to the filtrates in order to precipitate PS. The mixture was kept overnight at +4 °C after which it was centrifuged (5500 rpm, 30 min), dried for 48 h at room temperature, and milled in a mortar

and pestle to a fine powder. Dried samples were stored at −4 °C. PS extraction yield (%PS) was calculated according to Equation (1), where WP is the weight obtained after ethanol precipitation, and WA is the algae weight used in each experiment.

$$\% \text{ PS } = \frac{WP}{WA} \times 100 \quad (1)$$

2.4. Chemical Composition of PS

The concentration of L-fucose units in algal PS was determined according to the colorimetric assay with L-cysteine, using L-fucose as the standard [29]. Sulfate group content was determined by the turbidimetric barium chloride–gelatin method, with potassium sulfate as the standard, after PS hydrolysis (1 M HCl, 105 °C, 5 h) [30]. Total sugar content in PS was measured by the colorimetric phenol–sulfuric acid method with glucose as the standard [31]. Uronic acid concentration was determined through modified sulfamate/m-hydroxydiphenyl colorimetric method with D-galacturonic acid as the standard [32].

2.5. HPLC Analysis of PS Monosaccharide Composition

HPLC analysis of PS monosaccharide composition was performed according to the previously described method [33] with some modifications. The PS sample (100.0 mg) was hydrolyzed in 1 M H_2SO_4 (2.0 mL) and incubated in an oil bath at 110 °C for 4 h. After cooling to a room temperature, 2 M sodium hydroxide was used to neutralize (pH 7) the reaction mixture. An Internal standard solution (2 mL) was added, shaken well, diluted to 10 mL, and filtered.

A mixture of filtered hydrolyzed PS solution (or monosaccharide standards) (100 µL), 0.5 M methanolic solution of PMP (100 µL), and 0.3 M aqueous sodium hydroxide (100 µL) was incubated in water bath for 30 min at 70 °C. Afterwards, the reaction mixture was cooled to a room temperature and neutralized (pH 7) with 0.3 M hydrochloric acid. Chloroform (1 mL) was added to the solution, shaken well on vortex mixer and centrifuged (5000 rpm, 10 min). The chloroform layer was discarded, while the aqueous layer was extracted two more times with chloroform, and the final aqueous layer was analyzed by HPLC.

The internal standard solution was ribose (~1 mmol) (dissolved in water and diluted to 50 mL). Five known concentrations of the following monosaccharides standards (mixed with internal standard) were prepared by successive dilutions from stock solutions and injected into the instrument: arabinose (0.25–1.75 mg mL^{-1}), glucose (0.25–2 mg mL^{-1}), fucose (0.1875–1.5 mg mL^{-1}), galacturonic acid (0.25–1.75 mg mL^{-1}), rhamnose (0.25–1.75 mg mL^{-1}), fructose (0.25–1.75 mg mL^{-1}), and mannose (0.25–1.75 mg mL^{-1}). Calibration curves were plotted (Table 1) as the ratio of the peak areas of the standard monosaccharide and the internal ribose standard vs. concentration.

Table 1. Calibration curves for PMP sugars.

PMP Sugar	Standard Curve	R^2
fucose	y = 0.3549x − 0.0712	0.9940
glucose	y = 2.8327x − 0.6781	0.9925
arabinose	y = 1.2784x − 0.2645	0.9948
galacturonic acid	y = 2.3517x − 0.6414	0.9951
fructose	y = 0.1633x − 0.0453	0.9925
rhamnose	y = 0.0706x + 0.0064	0.9961

An HPLC Agilent Infinity 1260 system (Agilent Technologies, Santa Clara, CA, USA) equipped with UV/Vis and DAD, an automatic injector, ChemStation software, and a Zorbax Eclipse XDB-C18 column (4.5 × 250 mm, 5 um) (Agilent Technologies, Santa Clara, CA, USA) were used for monosaccharide analysis. A mixture of 0.4% trimethylamine in 20 mmol L^{-1} ammonium acetate buffer solution (pH 6.30 with acetic acid) and acetonitrile

(9:1) was used as solvent A, while a mixture of 0.4% triethylamine in 20 mmol L^{-1} ammonium acetate buffer solution (pH 6.30 with acetic acid) and acetonitrile (4:6) was used as solvent B. Chromatographic separation was achieved with the following gradient: 0–9 min, 10–14% B; 9–30 min, 64% B; 30–35 min, 64% B; and 35–37 min, 10% B. The mobile phase flow rate was set at 1 mL min^{-1}, column temperature at 25 °C, injection volume at 10 µL, and the chromatograms were monitored at 245 nm. All experiments were carried out in duplicate.

2.6. Determination of Molecular Properties

High-performance size exclusion chromatography with refraction index detector (HPSEC-RID) was used to assess molecular properties (average molecular weight (M_w), the number average molecular weight (M_n), and the polydispersity index (PDI)) of *F. virsoides* and *C. barbata* PS extracted with CE, UAE, and NTP with H_2SO_4. The 1260 Infinity II LC system (Agilent Technologies) consisted of a quaternary gradient pump G7111B, an autosampler G4767A, a multicolumn thermostat G7116A, and a refraction index detector G7162A. HPSEC analysis was performed using PL aqua gel–OH guard column (8 µm, 50 × 7.5 mm; Agilent Technologies) and PL aqua gel–OH MIXED-M column (8 µm, 300 × 7.5 mm; Agilent Technologies). Column temperature was 30 °C, RID temperature was 40 °C, mobile phase was H_2O, flow rate was 0.5 mL min^{-1}, and injection volume was 50 µL. Pullulan standards (Agilent Technologies), with M_w in a range from 180 to 700 kDa, were used to construct a molecular weight calibration curve and OpenLAB CDS ChemStation Edition (Agilent Technologies) was used for data collection and processing.

2.7. Oxygen Radical Absorbance Capacity (ORAC) Assay

The ORAC assay was used to measure the antioxidant capacity in *F. virsoides* and *C. barbata* PS extracts obtained with CE, UAE, and NTP with H_2SO_4 [34]. An automated plate reader (BMG LABTECH, Offenburg, Germany) with 96-well plates was used, and data were analyzed by MARS 2.0 software. Fluorescein solution, AAPH, and different Trolox dilutions were prepared in 75 µM phosphate buffer (pH 7.4). After dissolving dry PS in ddH_2O (4 mg mL^{-1}) and filtration, samples were added in a 96-well black plate containing a fluorescein solution (70.3 nM). The plate was incubated at 37 °C for 30 min, and after the first three cycles (representing the baseline signal) to initiate the peroxyl radical generation, AAPH (240 mM) was injected into each well. Different Trolox dilutions (3.12–103.99 µM) were used as the reference standard. During a total measurement time of 120 min, fluorescence intensity (emission at 528 nm and excitation at 485 nm) was observed every 90 s.

2.8. DPPH Radical Scavenging Assay

Spectrophotometric assay was used to measure the ability of the extracts to scavenge DPPH radical. Polysaccharide solution (1 mg mL^{-1}) (1.5 mL) was mixed with DPPH solution (0.2 mM in 70% ethanol) (1.5 mL), shaken on a vortex mixer, and kept at room temperature, in dark, for 30 min. Absorbance decrease was measured at 517 nm in duplicate. The free radical scavenging activity was calculated according to Equation (2):

$$\text{Scavenging effect } (\%) = \frac{\text{A–C}}{\text{A}} \times 100 \quad (2)$$

where A is the control absorbance (DPPH without sample), and C is the sample absorbance.

2.9. Statistical Analysis

All extractions and measurements, apart from molecular properties determination, were performed in duplicate. The results were analyzed for statistical significance at $p \leq 0.05$, using the STATISTICA 8.0 software (StatSoft Inc., Tulsa, OK, USA). Continuous variables were analyzed by multifactor analysis of variance (ANOVA) and marginal means were compared with Tukey's HSD multiple comparison tests. Dependent variables were:

%PS, sulfate group content (%), and fucose content (%), while independent variables were: (a) seaweed species (*C. barbata* and *F. virsoides*), (b) solvent (H_2O, 0.1M HCl and 0.1 M H_2SO_4), and (c) treatment (CE, 30 min UAE, 5 min UAE + CE, 30 min NTP and 5 min NTP + CE). For comparison of different extraction techniques (CE, UAE, and NTP—independent variable) one-way ANOVA with Tukey's HSD multiple comparison tests were used, where the dependent variables were %PS, total sugar, fucose, sulfate group, uronic acid content, monosaccharide composition, M_w, M_n, and PDI. The design matrix for the experiment and the regression model for each response were calculated as follows [35]:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (3)$$

where Y is the predicted response; β_0 is the fixed response; β_i, β_{ii}, and β_{ij} are the linear, quadratic, and interaction coefficients; X_i and X_j are independent factors.

3. Results and Discussion

3.1. Influence of Algal Species, Solvent, and Extraction Technique on PS Yield

PS from *F. virsoides* and *C. barbata* were extracted using CE as well as advanced techniques: UAE and NTP. Furthermore, UAE and NTP were also applied as 5 min pre-treatments prior to CE. The use of three different extraction solvents (i.e., H_2O, 0.1 M HCl, and 0.1 M H_2SO_4) was also examined on yield and chemical composition of the extracted PS. It is necessary to emphasize that all extracts analyzed in this study were crude (not purified) extracts; thus, it was more accurate to report them as %PS rather than fucoidan yield.

Influence of algae species, extraction solvent, and extraction treatment on polysaccharide yield (%PS), sulfate group, and fucose content of the extracted polysaccharides is shown in Table 2 and regression model equations in Table 3. All studied parameters had a significant ($p \leq 0.05$) influence on %PS. Average crude %PS from *F. virsoides* was 9.75% and from *C. barbata* 8.86%. Although both of these seaweeds are order *Fucales*, PS% highly depended on seaweed species, and within the same species it is also influenced by several other factors, such as extraction procedure and solvent, harvest season, and geographic location as well as the maturity of the plant [36,37]. Usov et al. [38] reported that %PS from 25 different brown seaweed species ranged between 0.4% and 20.4%, while in the study of Turan et al. [39] they constituted up to 25–30%. The only commercially available fucoidan is prepared from *Fucus vesiculosus* [7] which is, along with *Fucus evanescens*, especially attractive as the PS source due to the fact of their wide growth area, growth in shallow waters, and large amounts of alginic acid, fucoidan, and mannitol [38]. On the other hand, *F. virsoides* is a species of brown alga endemic to the Adriatic Sea, and it has not been used for PS extraction so far. When comparing the obtained results with the literature [28], PS% of the *Fucus* genus seaweed have been reported to be as high as 21.5% for F. *distichus* [40] and as low as 1.40% for *F. vesiculosus* [41]. The %PS of *C. barbata* obtained in this research was slightly higher than other similar studies performed on different seaweed from *Cystoseira* genus, where it ranged from 2.8% for *C. crinite* [42] to 5.45% for *C. barbata* [43].

A remarkable significant difference ($p \leq 0.05$) in %PS between different solvents was observed. The highest %PS (15.45%) obtained with 0.1 M H_2SO_4 was more than two-fold higher in comparison with %PS obtained with H_2O (5.67%) and 0.1 M HCl (6.80%). PS extraction was probably facilitated due to the cell wall hydrolysis, which is caused by acid application [44]. Similarly, use of H_2O gave more than a two-fold lower %PS than when 1 M HCl was used in research of brown seaweed *Sargassum fusiforme* by Liu et al. [44]. Since %PS obtained by 0.1 M H_2SO_4 with pH 0.7 was much higher than %PS obtained by 0.1 M HCl with pH 1, it appeared that lowering the pH increased the PS% [45]. Similar findings were reported by Ptak et al. [45], who achieved marginally better fucoidan and laminarin yield with 100 mM HCl (pH 2) then with 10 mM H_2SO_4 (pH 4) for *Fucus* seaweed harvested in France. However, they reported opposite observations for the *Fucus* seaweed harvested

in Germany. Considering these results, H_2SO_4 was chosen as optimal solvent and further used for comparison of the extraction techniques.

Table 2. Influence of the algae species, extraction solvent, and extraction treatment on polysaccharide yield (%PS), sulfate group, and fucose content of the extracted polysaccharides.

	N	% PS	Sulfate Group (%)	Fucose (%)
Algae		$p \leq 0.05$ *	$p \leq 0.05$ *	$p \leq 0.05$ *
F. virsoides	30	9.75 ± 0.04^{b}	41.98 ± 0.38^{a}	31.68 ± 0.20^{b}
C. barbata	30	8.86 ± 0.04^{a}	53.60 ± 0.38^{b}	11.19 ± 0.20^{a}
Solvent		$p \leq 0.05$ *	$p \leq 0.05$ *	$p \leq 0.05$ *
H_2O	20	5.67 ± 0.05^{a}	32.50 ± 0.47^{a}	21.33 ± 0.24^{b}
0.1 M HCl	20	6.80 ± 0.05^{b}	38.02 ± 0.47^{b}	25.97 ± 0.24^{c}
0.1 M H_2SO_4	20	15.45 ± 0.05^{c}	72.86 ± 0.47^{c}	17.00 ± 0.24^{a}
Treatment		$p \leq 0.05$*	$p \leq 0.05$ *	$p \leq 0.05$ *
CE	12	11.74 ± 0.06^{e}	42.74 ± 0.61^{a}	32.41 ± 0.31^{e}
UAE 5 min + CE	12	11.41 ± 0.06^{d}	47.92 ± 0.61^{b}	25.73 ± 0.31^{c}
UAE 30 min	12	6.72 ± 0.06^{a}	44.32 ± 0.61^{a}	27.29 ± 0.31^{d}
NTP 5 min + CE	12	9.39 ± 0.06^{c}	42.12 ± 0.61^{a}	11.96 ± 0.31^{b}
NTP 30 min	12	7.29 ± 0.06^{b}	61.87 ± 0.61^{c}	9.76 ± 0.31^{a}
Algae; solvent		$p \leq 0.05$ *	$p = 0.07$	$p \leq 0.05$ *
F. virsoides; H_2O	10	7.18 ± 0.06^{c}	27.44 ± 0.67^{a}	35.41 ± 0.34^{d}
F. virsoides; 0.1 M HCl	10	7.72 ± 0.06^{d}	32.33 ± 0.67^{b}	39.34 ± 0.34^{e}
F. virsoides; 0.1 M H_2SO_4	10	14.35 ± 0.06^{e}	66.19 ± 0.67^{e}	20.29 ± 0.34^{c}
C. barbata; H_2O	10	4.16 ± 0.06^{a}	37.56 ± 0.67^{c}	7.25 ± 0.34^{a}
C. barbata; 0.1 M HCl	10	5.87 ± 0.06^{b}	43.71 ± 0.67^{d}	12.61 ± 0.34^{b}
C. barbata; 0.1 M H_2SO_4	10	16.56 ± 0.06^{f}	79.54 ± 0.67^{f}	13.71 ± 0.34^{b}
Algae; treatment		$p \leq 0.05$ *	$p \leq 0.05$ *	$p \leq 0.05$ *
F. virsoides; CE	6	12.64 ± 0.08^{h}	28.43 ± 0.86^{a}	45.27 ± 0.44^{f}
F. virsoides; UAE 5 min + CE	6	13.27 ± 0.08^{i}	$45.09 \pm 0.86^{c,d}$	40.30 ± 0.44^{e}
F. virsoides; UAE 30 min	6	7.57 ± 0.08^{c}	41.76 ± 0.86^{c}	38.60 ± 0.44^{e}
F. virsoides; NTP 5 min + CE	6	10.17 ± 0.08^{f}	33.66 ± 0.86^{b}	19.22 ± 0.44^{d}
F. virsoides; NTP 30 min	6	5.11 ± 0.08^{a}	$60.98 \pm 0.86^{f,g}$	14.97 ± 0.44^{c}
C. barbata; CE	6	10.85 ± 0.08^{g}	57.04 ± 0.86^{f}	19.54 ± 0.44^{d}
C. barbata; UAE 5 min + CE	6	9.54 ± 0.08^{e}	50.75 ± 0.86^{e}	11.16 ± 0.44^{b}
C. barbata; UAE 30 min	6	5.86 ± 0.08^{b}	$46.88 \pm 0.86^{d,e}$	15.98 ± 0.44^{c}
C. barbata; NTP 5 min + CE	6	8.60 ± 0.08^{d}	50.58 ± 0.86^{e}	4.71 ± 0.44^{a}
C. barbata; NTP 30 min	6	9.47 ± 0.08^{e}	62.76 ± 0.86^{g}	4.54 ± 0.44^{a}

CE = conventional extraction, UAE = ultrasound-assisted extraction, and NTP = non-thermal plasma. Results are expressed as the mean ± SE. * Statistically significant variable at $p \leq 0.05$. Values with different letters within column are statistically different at $p \leq 0.05$.

As it can be seen in Table 2, none of the advanced treatments, alone or in combination with CE, resulted in a yield higher than the one achieved with 3 h long CE (11.74%). Application of UAE and NTP for 30 min, without CE, gave significantly lower ($p \leq 0.05$) %PS, i.e., 6.72% and 7.29%, respectively. UAE and NTP as 5 min pre-treatments in combination with

CE resulted in %PS of 11.41% and 9.39%, respectively. These values were higher than those obtained with treatments alone but still lower than the one obtained when using CE. These results indicate that either too short of an extraction time or the lack of higher temperatures could be the reason for lower efficiency of UAE and NTP in PS extraction when compared to CE. This finding is supported by Okolie et al. [13], who obtained *Ascophyllum nodosum* %PS of 4.56% by UAE (35 min, amplitude 40%) and 11.9% by CE (70 °C for 3 × 3 h). On the contrary, in research by Hanjabam et al. [46], the UAE method (30 min, 50% amplitude) gave higher *Sargassum wightii* %PS (14.61 g 100 g^{-1}) than CE (10.59 g 100 g^{-1}; 85 °C for 2 h). Moreover, in research by Kadam et al. [47], laminarin yield of *L. hyperborea* and *A. nodosum* extracted by 15 min UAE treatment was 15.02–91.76% higher, depending on the solvent.

Table 3. Regression model equations and determination coefficients (R^2) for polysaccharide yield (%PS), sulfate group, and fucose content.

	Regression Model	R^2
%PS	$y = 32.81 - 10.46X_1 - 14.02X_2 + 3.76X_2^2 - 4.86X_3 + 0.27X_3^2 + 2.62X_1X_2 + 1.45X_1X_3 - 0.02X_2X_3$	0.775
Sulfate group (%)	$y = 51.34 + 21.11X_1 - 51.47X_2 + 14.66X_2^2 - 10.55X_3 + 2.18X_3^2 + 1.61X_1X_2 - 4.24X_1X_3 + 3.53X_2X_3$	0.795
Fucose (%)	$y = 103.44 - 55.64X_1 + 7.30X_2 - 6.81X_2^2 - 10.34X_3 - 0.57X_3^2 + 10.79X_1X_2 + 4.52X_1X_3 + 0.53X_2X_3$	0.761

X_1 = algae species (1 = *F. virsoides*; 2 = *C. barbata*), X_2 = solvent (1 = H_2O; 2 = 0.1 M HCl; 3 = 0.1 M H_2SO_4), and X_3 = extraction technique (1 = CE; 2 = UAE 5 min + CE; 3 = UAE; 4 = NTP 5 min + CE; 5 = NTP).

3.2. Influence of Algal Species, Solvent, and Extraction Technique on PS Chemical Composition

As already mentioned, algal PS chemical composition is a key factor in determination of their biological activities; however, it is highly influenced by various parameters. The influence of algal species (*F. virsoides*, *C. barbata*), solvent (H_2O, 0.1 M HCl, 0.1 M H_2SO_4) and extraction method (CE, 30 min UAE, 30 min NTP, 5 min UAE + CE, 5 min NTP + CE) on fucose and sulfate group content is shown in Table 1. All studied parameters had a statistically significant ($p < 0.05$) influence on fucose and sulfate group content.

Sulfate group content is particularly important for biological activities, such as anti-HIV [48], anticoagulant [49], and antioxidant [50], and on levels below 20% it leads to a complete loss of antiproliferative and anticoagulant activity [51]. The average sulfate group content of *F. virsoides* obtained in this research was 41.98%, which is slightly above the range (9–40.3%) reported for the *Fucus* genus [40,41,51–54]. The average *C. barbata* sulfate group content obtained in this research was 53.60%, which is higher than the range (14.65–22.3%) reported for the *Cystoseira* genus in other studies [43,55–57]. The lower amount of sulfate groups found in *F. virsoides* when compared to *C. barbata* indicates the presence of higher amounts of non-sulfated fucose in the linear part of the PS or the lower amount of branching zones that contain sulfated fucose [41]. Acid induces the sulfate ester cleavage so more sulfate groups can be liberated [58,59], which explains the significantly ($p \leq 0.05$) higher sulfate content that was obtained by acid extractions in comparison to water extraction. However, Liu et al. [44] and Saravana et al. [60] reported opposite results for *Sargassum fusiforme* and *Saccharina japonica*, respectively. Average sulfate content obtained by 0.1 M H_2SO_4 was almost two-fold higher than the one obtained by 0.1 M HCl. This is in agreement with previous findings that diluted HCl tends to yield low sulfate fucoidan, while diluted H_2SO_4 tends to yield high sulfate fucoidan as it may interfere with sulfate analysis due to the sulfate group in the sulfuric acid [58,59]. Along with seaweed species and harvesting season, extraction technique is one of the most important factors that influences the sulfate group content [46]. For both examined algae, 30 min NTP treatment resulted in significantly ($p \leq 0.05$) higher sulfate group content. CE gave the lowest sulfate group content in *F. virsoides*, while in *C. barbata* the sulfate group content obtained by CE

was lower than 30 min NPT but higher than any other studied treatment. Okolie et al. [13] reported lower sulfate content in UAE extraction in comparison with CE, while Hanjabam et al. [46] found similar sulfate content obtained with UAE and CE.

Studies have reported that fucose content varies among seaweeds species, extraction techniques [37], extraction conditions [46], harvest time [51,61], plant part [38], and maturity of the seaweed [37], and it was suggested that the higher amount of fucose may contribute to the higher cytotoxic activity of algal PS [62]. The average fucose content obtained in this research was 31.68% and 11.19% for *F. virsoides* and *C. barbata*, respectively. These values are comparable with values reported by other authors [28]. Contrary to the sulfate group content, *C. barbata* fucose content was significantly lower than in *F. virsoides,* which indicates higher degree of sulphation for each fucose in *C. barbata*. The highest fucose content was obtained when using 0.1 M HCl followed by H_2O and 0.1 M H_2SO_4. Higher fucose content, in fucoidan from sporophytes of *Splachnidium rugosum*, was also achieved by 0.2 M HCl than 1% H_2SO_4 [63], and the use of acid (0.03 M HCl) instead of water resulted in a 6% increase in the amount of fucose [64]. However, acid might cause unwanted degradation of functional groups attached to the fucoidan backbone which can result in the failure of fucose detection [58]. In present study, fucose content was significantly ($p \leq 0.05$) higher in extracts obtained by CE than any of the UAE and NTP treatments due to the destructive effect of ultrasound on the structure of fucose [46]. A similar trend of higher fucose content obtained by CE than UAE was previously reported [46], as well as similar fucose content obtained with UAE and conventional hot water extraction [13,65]. Both of the NTP treatments lead to lower fucose content than ones obtained with UAE treatments, suggesting that the effect of OH radicals formed with NTP on glycosidic bonds [66] is inferior in comparison with cavitation effect of ultrasound.

Fucoidan antioxidant and anticoagulant bioactivity [67–69] has been positively correlated with sulfate:fucose (or total sugar) ratio, and it was noted that anticoagulant activity remarkably decreased when the ratio was below 1 [69], while fucoidan with ratio below 0.3 had no anticoagulant activity [68]. The average sulfate:fucose ratio obtained in this research was 1.33 (*F. virsoides*) and 4.79 (*C. barbata*). Previous studies reported that for *F. serratus*, the sulfate:fucose ratio ranged between 0.73 and 1.5 [51,70,71], for *F. vesiculosus* between 1.1 and 2.5 [51,70,72], for *A. nodosum* between 1.1 and 2.7 [51,72,73], for *C. sedoides* between 0.3 and 0.9 [42,58], for *C. compressa* it was 0.27 [42], and for *C. crinite* 0.36 [42]. The highest reported sulfate:fucose ratio was 5.2 for *Ecklonia maxima* [58] fucoidan extracted with 0.15 M HCl. The same *E. maxima* fucoidan but extracted with water had sulfate:fucose ratio of 0.7. Similar to the results of this research, where extracts obtained with 0.1 M H_2SO_4 had a much higher sulfate:fucose ratio than water extracts, *Laminaria pallida* and *Splachnidium rugosum* fucoidan extracted with acid had a significantly higher sulfate:fucose ratio [60]. PS obtained with CE had the lowest sulfate:fucose ratio, followed by UAE treatments, while in NTP treatments this ratio was much higher. As it was previously discussed, this higher ratio was the result of higher sulfate group and lower fucose content obtained by NTP treatments. Hanjabam et al. [46] reported that *Sargassum witghtii* fucoidan had average sulfate:fucose ratio of 0.67 with hot water extraction and 0.72 with UAE.

3.3. Comparison of CE, UAE, and NTP Treatment

As it was mentioned earlier, H_2SO_4 was chosen as the optimal solvent and used for further comparison of 3 h long CE with 30 min UAE and NTP treatments, in terms of yield, chemical composition, molecular properties, and antioxidative capacity. The results are shown in Tables 3 and 4 and Figure 2. CE was considerably longer and performed on higher temperatures than UAE and NTP, which could explain the higher %PS, lower amount of sulfate groups, and higher proportion of uronic acids (Table 4) as described by several studies [74–76]. For *F. virsoides*, %PS obtained by UAE and NTP was respectively two- and three-fold lower than in CE. It seems as if the UAE and NTP treatments were too short or the temperature was too low for higher PS extraction, since PS extraction was generally a lengthy process. *F. virsoides* and *C. barbata* %PS obtained with CE were 18.53% and 16.47%,

while their respective predicted values (from regression model equations) were 18.82% and 17.65%.

Table 4. Polysaccharide yield (%PS) and chemical composition of *Fucus virsoides* and *Cystoseira barbata* polysaccharides obtained with CE, UAE and NTP.

		% PS	Total Sugars (mg g^{-1})	Fucose (%)	Sulfate Group (%)	Uronic Acid (%)
		$p \leq 0.05$ *	$p \leq 0.05$ *	$p \leq 0.05$ *	$p \leq 0.05$ *	$p \leq 0.05$ *
F. virsoides	CE	18.53 ± 0.00 ^{e}	20.17 ± 0.00 ^{e}	41.54 ± 0.01 ^{f}	28.46 ± 0.01 ^{a}	20.06 ± 0.00 ^{e}
	UAE	12.14 ± 0.28 ^{c}	11.58 ± 0.58 ^{d}	14.75 ± 0.12 ^{c}	83.37 ± 0.20 ^{b}	1.77 ± 0.35 ^{b}
	NTP	6.10 ± 0.20 ^{a}	22.95 ± 0.24 ^{f}	9.49 ± 0.16 ^{b}	88.31 ± 5.56 ^{b}	3.68 ± 0.25 ^{c}
C. barbata	CE	16.47 ± 0.18 ^{d}	6.34 ± 0.18 ^{b}	22.53 ± 0.14 ^{d}	35.53 ± 0.80 ^{a}	15.72 ± 0.34 ^{d}
	UAE	11.80 ± 0.07 ^{c}	1.19 ± 0.12 ^{a}	31.70 ± 0.12 ^{e}	90.44 ± 1.40 ^{b}	1.16 ± 0.02 ^{a,b}
	NTP	9.64 ± 0.15 ^{b}	10.120.28 ^{c}	2.83 ± 0.08 ^{a}	88.86 ± 1,73 ^{b}	0.46 ± 0.04 ^{a}

CE = conventional extraction, UAE = ultrasound-assisted extraction, and NTP = non-thermal plasma. Results are expressed as the mean ± SD. * Statistically significant variable at $p \leq 0.05$. Values with different letters within a column are statistically different at $p \leq 0.05$.

Even though longer extraction time and higher temperatures can lead to fucose chain degradation [37] and consequently lower fucose content [75,77], CE which is considerably longer and performed at higher temperature than UAE and NTP resulted with higher fucose content. Possible explanation could be that the bond between fucoses was disrupted due to the intensive treatment of these advanced techniques. On the contrary, sulfate group content was the lowest in extracts obtained by CE indicating that their bonds were disrupted by long extraction at high temperature rather than cavitation or partially ionized gas. Some research showed that lower uronic acids content is more desirable property of algal PSs since higher uronic acids content shows lower anticoagulant [78] and anticomplement activity [79]. Therefore, UAE and NTP showed an advantage in comparison with CE since significant reduction of uronic acids can be observed in those extracts.

The monosaccharide compositions of CE, UAE, and NTP extracted PSs from *F. virsoides* and *C. barbata* are given in Table 4. Generally, in all extracted PSs, L-fucose was the predominant monosaccharide, which was expected and in accordance with most of the previously published studies [65,67,73,80,81]. Other detected monosaccharides were glucose, galacturonic acid (oxidized form of D-galactose), and arabinose, while mannose, rhamnose, and fructose were not detected. Our results showed that the monosaccharides ratio varied according to the algal species and extraction method used, which is in accordance with literature data [81]. For both algae, it can be observed that PS extracted with CE had higher fucose and lower glucose content than PS extracted with UAE and NTP. In both algae, PS extracted with UAE had similar concentration of fucose, glucose and galacturonic acid.

HPSEC was used to analyze molecular properties of the PS extracts, and the results of the weight average molecular weight (M_w), the number average molecular weight (M_n), and the polydispersity index (PDI) are presented in Table 5. M_w is the molecular size of the sample, while M_n represents an average molecular weight of all polymer chains within a sample [82]. M_w is more influenced by high molecular weight chains, whereas M_n is more influenced by the lower molecular weight chains [82]. PDI is the ratio between M_w and M_n, and it measures the heterogeneity of molecular weight distributions of polymers, where larger differences between M_w and M_n (larger PDI) indicate a more heterogeneous molecular weight distribution [82].

Table 5. Monosaccharide composition and molecular properties of *Fucus virsoides* and *Cystoseira barbata* polysaccharides obtained with CE, UAE and NTP.

		Monosaccharide Composition (%)				Molecular Properties		
		Glucose	Fucose	Galacturonic Acid	Arabinose	M_w (kDa)	M_n (kDa)	PDI (M_w/M_n)
		$p \leq 0.05$ *	$p \leq 0.05$ *	$p \leq 0.05$ *	$p \leq 0.05$ *	$p \leq 0.05$ *	$p \leq 0.05$ *	$p \leq 0.05$ *
F. virsoides	CE	18.65 ± 0.24 ^{a}	44.83 ± 0.45 ^{c}	19.48 ± 0.26 ^{b}	17.04 ± 0.25 ^{a}	693.43 ^{b}	264.42 ^{b}	2.62 ^{a,b}
	UAE	31.47 ± 0.32 ^{b}	36.30 ± 0.34 ^{a}	32.23 ± 0.31 ^{d}	n.d.	1133.78 ^{e}	500.16 ^{e}	2.27 ^{a,b}
	NTP	46.20 ± 0.27 ^{c}	39.83 ± 0.41 ^{b}	13.97 ± 0.14 ^{a}	n.d.	16.38 ^{a}	16.38 ^{a}	1.00 ^{a}
C. barbata	CE	n.d.	100 ^{e}	n.d.	n.d.	766.00 ^{c}	322.87 ^{c}	2.37 ^{a,b}
	UAE	31.04 ± 0.29 ^{b}	35.96 ± 0.33 ^{a}	33.00 ± 0.22 ^{d}	n.d.	1651.22 ^{f}	616.65 ^{f}	2.68 ^{b}
	NTP	19.15 ± 0.18 ^{a}	51.08 ± 0.23 ^{d}	29.76 ± 0.25 ^{c}	n.d.	930.83 ^{d}	492.50 ^{d}	1.89 ^{a,b}

M_w = weight average molecular weight, M_n = number average molecular weight, PDI = polydispersity index, CE = conventional extraction, UAE = ultrasound-assisted extraction, and NTP = non-thermal plasma. Results are expressed as the mean ± SD. * Statistically significant variable at $p \leq 0.05$. Values with different letters within column are statistically different at $p \leq 0.05$.

The M_w of CE, UAE, and NTP extracted PSs ranged from 16.38 to 1133.78 kDa for *F.virsoides* and 766 to 1651.22 kDa for *C. barbata.* Obtained values were within the range of reported fucoidan M_w values, 1.4–1323 kDa [41,83]. Algal species, growth conditions, and extraction methodology [82] are some of the factors influencing algal PS molecular weight, therefore explaining the slight difference when comparing measured values with literature ones. According to their molecular weight, fucoidans can be classified as low-molecular-weight fucoidans (<10 kDa), medium-molecular-weight fucoidans (10–10,000 kDa), and high-molecular-weight fucoidans (>10,000 kDa) [84]. For both algae, the highest M_w was achieved in samples obtained by UAE, while the lowest M_w in *C. barbata* was achieved in samples obtained by CE and *F. virsoides* by NTP. Likewise, *N. zanardinii* M_w obtained by UAE was higher (1021 kDa) than one obtained with CE (823 kDa) [81], while M_w of *A. nodosum* fucoidan extracted by CE (40.2 kDa) was significantly lower when compared to samples obtained by UAE (121.1 kDa) [13]. PS was polydispersed with PDIs higher than one unlike natural polymers, such as proteins, which are usually monodispersed with a PDI of approximately one. The PDI of PS extracted in this study ranged from 1 to 2.68, and it was within the 1–6.2 range of reported fucoidan PDI values [56,81]. For *C. barbata*, PDI was higher in the samples obtained by UAE, indicating larger degradation during the extraction process, while for *F. virsoides*, the results were the opposite. Likewise, PDI was lower in *N. zanardinii* fucoidan extracted with UAE (1.27) in comparison with CE (1.56) [81].

3.4. Antioxidant Capacity

The antioxidant capacity of *F. virsoides* and *C. barbata* PS extracts obtained with CE, UAE and NTP, with H_2SO_4 as a solvent was assessed with ORAC and DPPH assays. Obtained results are presented in Figure 3. PS from *F. virsoides* obtained by CE had the highest ORAC value of 37.14 µmol TE g^{-1}, while UAE and NTP extracts had ORAC values of 19.97 and 20.21 µmol TE g^{-1}, respectively. However, the ORAC value was not significantly different among extraction techniques for *C. barbata*. For both algae, the highest DPPH value was determined in CE compared to both of the advanced techniques. Despite the fact that antioxidant capacity of fucoidan has been previously proved, the relationship between antioxidant capacity and chemical structure remained unknown. It is recognized that antioxidant capacity is not influenced only by one factor, but rather a combination of few physicochemical characteristics such as sulfate group content, uronic acid content, protein content, and molecular weight [44]. For this reason, correlation between physicochemical characteristics (i.e., sulfate group, uronic acid, M_w, and PDI) and antioxidant measurements (i.e., ORAC and DPPH) were evaluated. For *F. virsoides* only uronic acid content had strong positive correlation ($r > 0.8$) with ORAC and DPPH values. For *C. barbata*, uronic acid content showed strong positive ($r > 0.8$) and M_w strong negative ($r < -0.8$) correlation with DPPH values. Literature data showed that there was no correlation of ORAC values with the total sulfate content and M_w of brown algae *Lessonia vadosa* and red algae *Gigartina skottsbergii* and *Schizymenia binderi* [85].

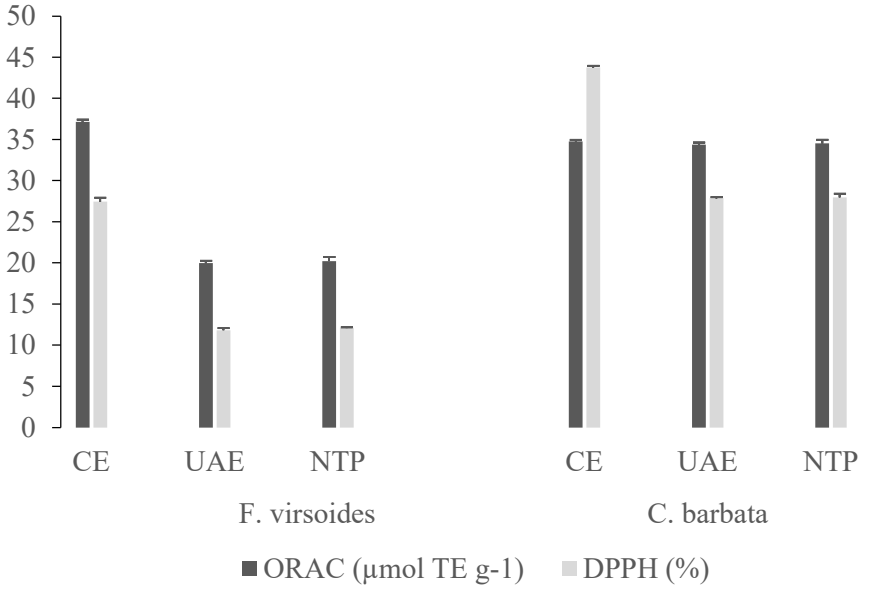

Figure 3. Antioxidant capacity of *F. virsoides* and *C. barbata* polysaccharides obtained by CE, UAE, and NTP as determined by ORAC and DPPH assays.

4. Conclusions

The results of present study showed that the advanced extraction techniques, UAE and NTP, have a great potential for *F. virsoides* and *C. barbata* PS extraction, even though higher %PS was achieved with CE, most likely due to the longer extraction time and higher temperatures. However, a significant time reduction, from 3 h to 30 min, and application of lower temperatures, are beneficial from ecological and economic points of view. Furthermore, both of these advanced techniques resulted in higher amounts of sulfate groups, lower proportions of uronic acids, and higher M_w, which are more desirable properties in terms of PS biological activity. UAE and NTP as 5 min pre-treatments followed by CE did not cause more cell wall breakage, as they gave more or less comparable %PS to that of CE alone.

Diverse biological activities previously attributed to brown algae fucoidan makes them interesting for use in processed and functional food, pharmaceutical, and chemical industries; thus, all these additional improvements in their extraction method could be extremely beneficial from an industrial point of view.

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Chapter 5

General discussion

- *Proximate composition of F. virsoides and C. barbata*
- *Pre-treatment*
- *Influence of algal species, solvent and extraction technique on polysaccharide yield*
- *Optimal parameters for polysaccharide extraction using different extraction techniques*
- *Influence of algal species, extraction technique and solvent on chemical composition and antioxidative capacity*
- *Biological activities of F. virsoides and C. barbata polysaccharides*

1. Proximate composition

Moisture, protein, lipid, carbohydrate, and ash content (Table 3) were analysed in freeze-dried *F. virsoides* and *C. barbata* collected in December 2018 from the southwestern coast of the Novigrad Sea (44°12′02″N; 15°28′51″E) and the coastal region of Zadar (44°12′42″N; 15°09′23″E), respectively.

Table 3. Proximate composition of the *Fucus virsoides* and *Cystoseira barbata*

	Moisture (%)	**Protein (%)**	**Lipids (%)**	**Carbohydrates (%)**	**Ash (%)**
Fucus virsoides	8.47±0.18	8.66±0.31	2.14±0.16	62.30±0.76	18.43±0.14
Cystoseira barbata	5.96±0.07	7.79±0.60	2.82±0.12	59.86±0.81	23.57±0.26

Results are expressed as mean±standard deviation (n=2)

F. virsoides had slightly higher moisture and protein content than *C. barbata*, while the results for lipids, carbohydrate, and ash content were opposite. The ratio of moisture, protein, lipids, carbohydrates, and ash is known to vary among different algal species, geographic areas, seasons, or environmental conditions. Moisture content is reported as a percentage of wet weight in most of the literature and varies from 67% to 88% in the genus Fucus and from 48.99% to 84% in the genus Cystoseira. The moisture content reported as a percentage of dry weight was 11.23% for *F. vesiculosus* (Lorenzo et al., 2017) and 10.14% for *C. trinodis* (Dixit et al., 2018), which is slightly higher than the values obtained in this study. Green and red algae contain higher protein content (10-47%) than brown algae (3-15%), and it is known that protein content varies with geographic location and seasonal conditions and is strongly influenced by seawater temperature, salinity, and available nutrients. In addition, the protein content of algae changes throughout the year, being highest in winter and early spring, and lowest in summer and early fall. Different species of algae from the genus Fucus had between 4.14 and 12.99% protein (Catarino et al., 2018; Heffernan, 2015; Lorenzo et al., 2017; Paiva et al., 2014, 2018; Tibbetts et al., 2016), while the detected protein content in algal species of the genus Cystoseira ranged from 8.91 to 14.14% (Dixit et al., 2018; Oucif et al., 2020; Vizetto-Duarte et al., 2016). Although the lipid content in algae varies greatly throughout the year, it is usually low, ranging from 0.99% to 11.54% in the genus Fucus (Catarino et al., 2018; Heffernan, 2015; Lorenzo et al., 2017; Paiva et al., 2014, 2018; Tibbetts et al., 2016) and from 1.3% to 10.91% for the genus Cystoseira (Dixit et al., 2018; Oucif et al., 2020; Vizetto-Duarte et al., 2016). Carbohydrate synthesis in algae is related to periods of maximum growth, increased photosynthetic activity, and a decrease in protein content, as well as to seawater temperature, salinity, and sunlight

intensity. Algae of the genus Fucus showed carbohydrate contents ranging from 12.77% to 79.11% (Catarino et al., 2018; Heffernan, 2015; Lorenzo et al., 2017; Paiva et al., 2014, 2018; Tibbetts et al., 2016), while it ranged from 33.18% to 73.09% in the genus Cystoseira (Dixit et al., 2018; Oucif et al., 2020; Vizetto-Duarte et al., 2016). Seaweeds generally have higher ash content than terrestrial vegetables, and these high ash levels are associated with high mineral content. The ash content of different seaweeds of the genus Fucus varied from 14% to 29.57% (Catarino et al., 2018; Heffernan, 2015; Lorenzo et al., 2017; Paiva et al., 2014, 2018; Tibbetts et al., 2016) and for the genus Cystoseira it ranged from 7.3% to 35.29% (Dixit et al., 2018; Oucif et al., 2020; Vizetto-Duarte et al., 2016). Among macrominerals, potassium is usually the most abundant element, followed by sodium and calcium, while the content of iron, manganese, magnesium and phosphorus is lower (Lorenzo et al., 2017).

2. Pre-treatment

Pre-treatment is an essential step in the isolation of polysaccharides from brown algae. It is performed before the extraction process to prevent co-extraction of interfering compounds (polyphenols, pigments, lipids, proteins) with similar solubility (Dobrinčić et al., 2020), which are strongly bound to polysaccharides. For this purpose, different solvents and solvent mixtures with different polarity were used, e.g., a mixture of methanol, chloroform, and water in the ratio 4:2:1 (v/v/v), acetone, ethanol, and methanol (Ammar et al., 2016; Guerra Dore et al., 2013; Hentati et al., 2018). The use of a combination of solvents with different polarities instead of a single solvent is necessary because the interfering compounds to be removed include a wide range of compounds, from polar polyphenols to nonpolar lipids.

In *Publication No. 2*, the optimization of the pre-treatment process was carried out with constant stirring of the algal material with solvent in two steps: first at room temperature for 18 h and then at 70°C for 4 h. Five different solvents or solvent mixtures were used: (1) mixture of ethyl acetate and 96% ethanol (1:1) in the first step only; (2) 96% ethanol in both steps; (3) 80% ethanol in both steps; (4) acetone in the first and 96% ethanol in the second step; (5) hexane in the first and 96% ethanol in the second step. The polarity of the solvents used follows this order: 96% ethanol > 80% ethanol > ethyl acetate and 96% ethanol (1:1) > acetone > hexane. The combination of solvents that most effectively removed interfering compounds (polyphenols and pigments) and provided the highest yield of polysaccharides was acetone and 96% ethanol, which was therefore used for all further studies. None of the applied pre-

treatments proved to be significantly more effective than the others with respect to the chemical structure of the polysaccharides (total sugars, sulfate group, fucose and uronic acid content).

3. Influence of algal species, solvent and extraction technique on polysaccharide yield

It is generally believed that the yield of polysaccharides is strongly influenced by the algal species, solvent, extraction technique, and extraction parameters. In this dissertation, several publications optimized different species, solvents and extraction techniques, e.g. CE in *Publication No. 2*, MAE and PLE in *Publication No. 3*, while UAE and NTP were optimized in *Publication No. 4*. To get an overall view of how two different algal species (*F. virsoides* and *C. barbata*), three different solvents (H_2O, 0.1M HCl, and 0.1M H_2SO_4), and five different extraction techniques (CE, MAE, PLE, UAE, and NTP) affect the %PS at different time and temperature settings, all data (384 experiments in total) were combined, statistical analysis was performed, and the results are shown in Figure 3.

The average %PS of *F. virsoides* and *C. barbata*, regardless of the used solvent, extraction technique, and extraction parameters, was 10.23% and 8.01%, respectively. All extracts analysed in this study are crude extracts that could contain other co-extracted compounds such as alginic acid and laminarin (January et al., 2019), thus the term polysaccharide yield is used instead of purified fucoidan yield. Table 5 in *Publication No. 2* shows that algae of the genus Fucus have a polysaccharide content ranging from 1.40% to 21.50% (Fletcher et al., 2017; Imbs et al., 2009; Ptak et al., 2019; Rioux et al., 2007; Rupérez et al., 2002), while the polysaccharide content in algae of the genus Cystoseira ranged from 2.80% to 5.45% (Ammar et al., 2015, 2016; Hentati et al., 2018; Sahera et al., 2015; Sellimi et al., 2014). A higher polysaccharide content in algae of the genus Fucus was somehow expected, since the only commercially available source of fucoidan is the Atlantic Fucus species *F. vesiculosus*. The differences in polysaccharide content and chemical structure could possibly be due to some morphological features of the different species and polysaccharide biosynthesis in the early stage of algal embryo development. *F. virsoides* and *C. barbata* both belong to the same order - Fucales, but to different families - Sargassaceae and Fucaceae, respectively. They differ in some morphological characteristics such as thallus, holdfast, stem, branches, leaf, vesicle, apex, base and margin, length, width and shape. Fucus sp. have a flattened, dichotomous, or subpinnately branched thallus, distinct midrib, and an irregular or disc-shaped holdfast (Linardić, 1949). *Cystoseira* sp. has highly differentiated basal and apical regions, a thallus with many branches that make it look like a tree, and cylindrical, conical, siliceous, or

tuberculate receptors at the ends of the various branches (Nita et al., 2014). Alginic acid, fucoidan, and cellulose are structural components of the cell wall of brown algae in an average weight ratio of 3:1:1 (Mabeau & Kloareg, 1987), formed shortly after fertilization. Alginate and fucoidan are synthesized in the Golgi apparatus, transported to plasma membranes in vesicles, and secreted into the expanding cell wall, while cellulose microfibrils are produced by cellulose synthase complexes and deposited in the plasma membrane (Michel et al., 2010). Alginic acid and cellulose are distributed rapidly, in the first 2 hours, and uniformly over the entire cell wall surface of the embryo, whereas fucoidan is concentrated at the rhizoid end of the cell walls. An alginate epitope rich in guluronic acid is present in stiffer areas, while a sulfated fucan is present in the softer areas (Linardić, 1949). However, a better understanding of the genome sequence and the enzymes that may be involved (glycoside hydrolase and glycosyltransferase) would provide a deeper and more comprehensive insight into polysaccharide biosynthesis in brown algae and the metabolic pathways between the different algal species.

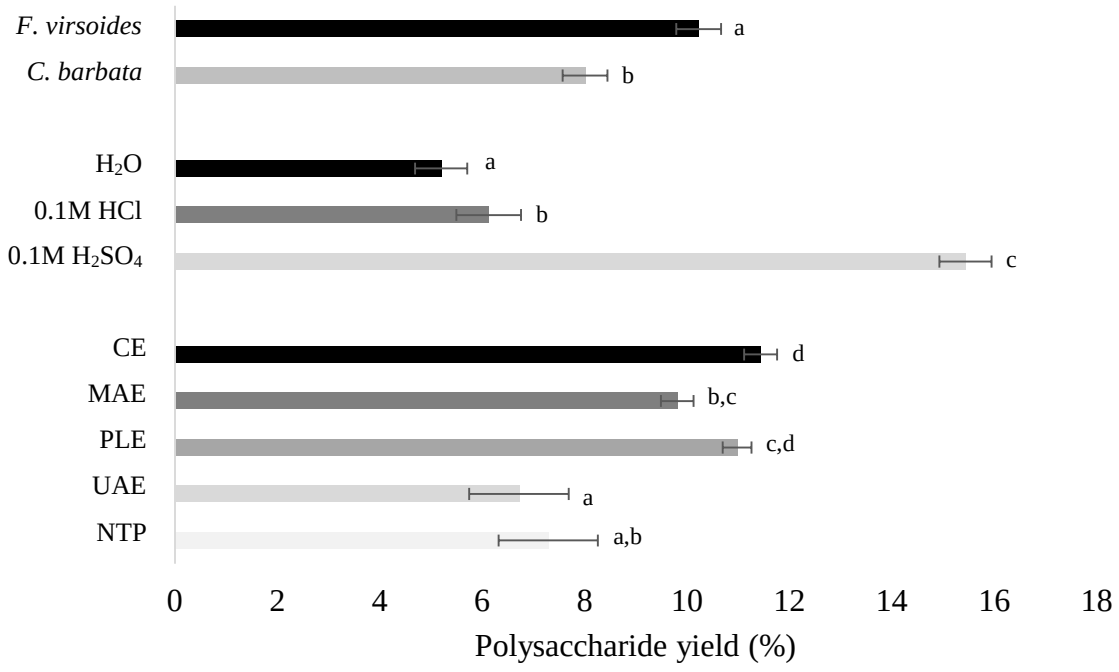

Figure 3. Influence of algae species, solvent and extraction technique on polysaccharide yield from all 384 experiments. Values with different letters within a group are statistically different at $p \leq 0.05$. CE=conventional extraction, MAE=microwave-assisted extraction, PLE=pressurised liquid extraction, UAE=ultrasound-assisted extraction, NTP=non-thermal plasma.

As previously discussed in *Publication No. 2*, *Publication No. 3*, and *Publication No. 4*, the application of 0.1M H_2SO_4 resulted in an almost 3-fold higher %PS than H_2O, regardless of the extraction technique and algal species. Similar results have been reported by other authors (Liu et al., 2019; Ptak et al., 2019; Saleem Ahmad, 2015), as acids facilitated cell wall hydrolysis and thus improved polysaccharide extraction (Liu et al., 2019). However, too high acid concentration can lead to the breaking of glycosidic bonds, causing structural changes, which is not a desirable property in terms of associated biological activities (January et al., 2019). Regardless of which extraction technique and algal species were used, the use of different solvents always resulted in color differences between the extracts, such that the extracts obtained with acid were much lighter in color compared to the water extracts (*Publication No. 2*, Fig. 2.). A darker brown color indicates the presence of algal pigments bound to polysaccharides during the extraction process (Saepudin et al., 2017), as well as a higher total polyphenol content (Bittkau et al., 2020), while a lighter color can be explained by chemical changes that take place in the presence of acids, where pigments are oxidized to colorless compounds (Moss, 1968). All this suggests that darker extracts have more impurities, while lighter polysaccharide extracts have higher purity and thus higher quality (Baba et al., 2018).

Considering the average %PS obtained with the different extraction techniques, regardless of the algal species and solvent, it can be observed that UAE and NTP were the least efficient, while there was no statistically significant difference between PLE and CE and PLE and MAE. Each of these techniques, apart from NTP, has been used before and showed great potential for the extraction of polysaccharides. However, they have different mechanisms of action that can cause cell rapture or change the solvent properties and thus improve the extraction yield. In PLE, high temperature and pressure enhance solvent penetration, capillary action, and cell destruction, resulting in higher extraction yield (Alboofetileh et al., 2019). During MAE, the microwaves are absorbed by the material components and the electromagnetic energy is converted into thermal energy. The temperature rise inside the samples leads to the rupture of the cells in the raw material, which facilitates the diffusion of the intracellular polysaccharides into the solvent (Alboofetileh et al., 2019). In UAE, high cavitation intensity, streaming and microjets lead to faster solvent penetration and solvation of the matrix (Alboofetileh et al., 2019). During NTP discharge, several synergistic effects, including direct chemical interaction of the cell membrane with reactive oxygen (ROS) and nitrogen (RNS) species (O_2, O_3, OH, NO, NO_2) along with charged particles, can damage

cellular components (Leal Vieira Cubas et al., 2020). Different extraction methods have been used for the extraction of *Nizamuddinia zanardinii* fucoidan, including CE, UAE, MAE, and PLE (Alboofetileh et al., 2019). According to their extraction efficiency compared to CE (5.2%), the techniques were classified into two categories: UAE (3.6%) as a low efficiency technique, while MAE (6.17%) and PLE (13.15%) were classified as high efficiency techniques (Alboofetileh et al., 2019). Also, PLE was more efficient than CE in extracting *Saccharina japonica* fucoidan (Saravana et al., 2016, 2018), while MAE was less efficient than CE in extracting *A. nodosum* fucoidan (Okolie et al., 2019; Yuan & Macquarrie, 2015a).

4. Optimal parameters of different extraction techniques

Since different extraction techniques and process parameters have different effects on the algal %PS and chemical composition, one of the objectives of this dissertation, through different publications, was to optimize CE, MAE, PLE, UAE and NTP for the extraction of polysaccharides from the brown algae *F. virsoides* and *C. barbata*. CE was optimized in *Publication No. 2*, MAE and PLE were optimized in *Publication No. 3*, while UAE and PLE were optimized in *Publication No. 4*. and the overview of the optimal parameters for each of the applied techniques is shown in Table 4.

H_2SO_4 was chosen as the optimal solvent because it resulted in an almost 3-fold higher %PS than H_2O, regardless of the extraction technique. The most important difference between CE and all these advanced extraction techniques is the noticeable time saving, as CE is performed for 2 or 3 hours, while MAE takes only 10 minutes. This is extremely important from an economic and environmental point of view, as less energy is consumed with shorter process duration. The optimal extraction temperature, for both algae, of CE and MAE was 80°C, while PLE was most efficient at 140°C. In PLE, the high pressure allows the solvent to remain in a liquid state at high temperature (above the usual boiling point), and under these conditions the solvent has properties that favor the extraction process, such as high diffusion coefficients, low viscosity, and high solvent strength (Mandal et al., 2015). As the name suggests, NTP is a non-thermal technique, while in UAE slight temperature increase (caused by the cavitation effect) is controlled in the cold water bath, and therefore both techniques operate at room temperature. Although this is advantageous from an economic and environmental point of view, as well as for the extraction of some heat-sensitive compounds, it was not useful for the extraction of polysaccharides, since the %PS was lower.

Table 4. Optimal parameters (solvent, time, temperature, and number of cycles) for *F. virsoides* and *C. barbata* polysaccharide extraction using different extraction techniques (CE=conventional extraction, MAE=microwave-assisted extraction, PLE=pressurised liquid extraction, UAE=ultrasound-assisted extraction, NTP=non-thermal plasma).

	ALGAE	SOLVENT	TIME (min)	TEMPERATURE (°C)	NO. CYCLES
CE	*F. virsoides*	0.1M H_2SO_4	180	80	–
	C. barbata		120		
MAE	*F. virsoides*	0.1M H_2SO_4	10	80	–
	C. barbata				
PLE	*F. virsoides*	0.1M H_2SO_4	15	140	2
	C. barbata				
UAE	*F. virsoides*	0.1M H_2SO_4	30	–	–
	C. barbata				
NTP	*F. virsoides*	0.1M H_2SO_4	30	–	–
	C. barbata				

5. Influence of algal species, extraction technique and solvent on chemical composition and antioxidative capacity

After optimizing each of the extraction techniques (CE, MAE, PLE, UAE, NTP) as described in the previous chapter, the extractions were repeated under these optimal conditions (Table 4) for each of the algal species (*F. virsoides*, *C. barbata*). In addition to %PS, chemical composition (sulfate group, fucose and uronic acid content, monosaccharide composition) molecular properties, and antioxidant capacity were analyzed according to the methods described in *Publication No. 2* and *Publication No. 3*. The results of the statistical analysis [multifactor analysis of variance (ANOVA) and Tukey's HSD multiple comparison test] are presented in Tables 5 & 6 and Figure 4.

5.1. Influence of algal species on chemical structure and antioxidative capacity

Since the isolation of "fucoidin" from marine brown algae by Kylin in 1913 and its subsequent renaming as "fucoidan" according to IUPAC rules, numerous studies have been conducted over the years on its chemical composition. The general conclusion is that the chemical composition of most fucoidans is complex and varies considerably from species to species. The structural model of *F. vesiculosus* fucoidan, according to which 1,2-α-fucose is the major component and most of the sulfate groups are located at the C-4 position, was

accepted for forty years. In 1993, Pankter et al. (1993) revised this model and according to their data, the core region of the fucoidan consisted mainly of 1,3-α-fucose units with sulfate groups substituted on some of the fucose residues at the C-4 position and branched points within the chain every 2 to 3 fucose residues (Li et al., 2008).

Fucoidans extracted from different taxonomic orders of brown algae, particularly Fucales, Laminariales, and Chordariales, have different compositions that cannot be categorized or predicted by algal order (Ale & Meyer, 2013). Fucoidans in brown algae of the order Fucales, e.g., *F. evanescens* and *F. serratus*, possess a large proportion of α-(1→3) and α-(1→4) linked L-fucopyranose residues, which may be substituted at C-2 and/or C-4 with sulfate ester groups ($-SO_3^-$) (Bilan et al., 2002, 2006; Cumashi et al., 2007). Fucoidans isolated from the alga *A. nodosum* (Fucales) have a backbone consisting of a repeating structure of α-(1→3) linked L-fucopyranose residues with sulfate at the C-2 position, where the α-(1→3) linked L-fucopyranose residues are linked to α-(1→4) L-fucopyranose residues with disulfate at the C-2 and C-3 positions (Chevolot et al., 1999). Fucoidans isolated from *L. saccharina* (Laminariales) consist mainly of α-(1→3)-linked L-fucopyranose residues with sulfate at both C-2 and C-4 or at C-4 only. Fucoidans from the *Chorda filum* (Laminariales) consist of a core skeleton structure of poly-α-(1→3)-linked L-fucopyranoses sulfated mainly at C-4, sometimes at the C-2 position, with some of the α-(1→3)-linked fucose residues being 2-O-acetylated (Chizhov et al., 1999). Fucoidans from *Cladosiphon okamuranus* (Chordariales) consist essentially of linear backbones of α-(1→3)-linked L-fucopyranose residues, with some of the fucose residues O-acetylated and some sulfates substituted at the C-4 position (Nagaoka et al., 1999).

The polysaccharides of *F. virsoides* extracted under optimal conditions had 34.87% fucose and 57.82% sulfate groups regardless of extraction technique, giving a sulfate to fucose ratio of 1.65, meaning that each fucose unit contained an average of 1.65 sulfate groups. In comparison, *C. barbata* had lower fucose and higher sulfate content, 22.35% and 63.59%, respectively, giving a sulfate-to-fucose ratio of 2.85. According to IUPAC nomenclature and terminology standards, a sulfate polysaccharide containing between 20% and 60% L-fucose can be classified as fucoidan (Ashayerizadeh et al., 2020). While the fucose content of these two algae was within the previously reported range of 17.6 to 61.5% (Ammar et al., 2015; Hadj Ammar et al., 2016), their sulfate group content was higher than the values previously reported in the literature for the genus Fucus (9 - 35.55% (Imbs et al., 2015; Rodriguez-Jasso et al.,

2011)) and the genus Cystoseira (14.65 - 22.51% (Hentati et al., 2018; Sellimi et al., 2014)). This high content of sulfate groups compared to the literature could be due to the use of H_2SO_4 as an extraction solvent, as it has been reported that dilute H_2SO_4 can interfere with sulfate analysis (January et al., 2019) and leads to high sulfate-fucoidan values due to the sulfate group in sulfuric acid (Hamid et al., 2015).

Just like the sulfate group and fucose content, the content of uronic acids in fucoidan is also affected by the algal species and extraction parameters, but unlike these, its lower content is a desirable property due to the lower anticoagulant (Kuznetsova et al., 2018) and anticomplementary (Chaminda Lakmal et al., 2015) activity reported in fucoidan with higher uronic acid content. The uronic acid content in *F. virsoides* was 9.35%, while *C. barbata* had 7.4% uronic acid, which is in a range reported in the literature from 0.59% in *A. nodosum* (Okolie et al., 2019) to 26.12% in *F. evanescens* (Imbs et al., 2015). As mentioned earlier, fucoidans are usually extracted as crude extracts because other components such as alginate, laminaran, mannitol, lipids, and pigments are co-extracted. Despite purification, traces of alginate are often detected in crude fucoidan extracts, and since they consist of β-D-mannuronic and α-L-guluronic acids as building blocks (Catarino et al., 2018), they could interfere with the determination of uronic acids in the chemical characterization of fucoidans. Therefore, a higher uronic acid content could be a consequence of the presence of the co-extracted alginate.

In general, based on the composition of monosaccharides, there are three main types of fucoidan: F-fucoidan or sulfated fucan consists almost entirely of sulfated fucose; G-fucoidan or galactofucan consists of sulfated fucose and sulfated galactose as major components; GA - fucoidan or U-fucoidan consists mainly of fucose, along with other monosaccharides (mainly mannose or galactose, but also glucose, xylose, and rhamnose), with significant amounts of uronic acids and sulfate esters (S. H. Wang et al., 2020). HPLC analysis of the individual monosaccharides (Table 3) revealed that in addition to fucose, which was the predominant monosaccharide in both algal polysaccharides, glucose, galacturonic acid (oxidized form of D-galactose), and arabinose were also detected, whereas fructose, rhamnose, and mannose were not. In most of the previously published studies (Alboofetileh et al., 2019; Alboofetileh, Rezaei, Tabarsa, Rittà, et al., 2018; Foley et al., 2011; J. Wang et al., 2008; Yuan & Macquarrie, 2015b), fucose was the predominant monosaccharide, and different ratios of constituent monosaccharides were reported among different algal species. *F. virsoides* had slightly higher glucose (25.72%) and galacturonic acid (18.44%) content than *C. barbata* (18.87% and

16.50%, respectively). *A. nodosum* fucoidan had 21.3% glucose and 6.1% galactose (Foley et al., 2011), *L. japonica* fucoidan had 1.93% glucose and 24.33% galactose (J. Wang et al., 2008), and *N. zanardinii* fucoidan had 3.19% glucose and 29.95% galactose (Alboofetileh, Rezaei, Tabarsa, & You, 2018).

The molecular properties of the polysaccharide extracts, weight average molecular weight (M_w), the number average molecular weight (M_n), and polydispersity index (PDI), were analysed by HPSEC and the results are shown in Table 6. M_w is the molecular size of the sample and considers the molecular weight of a chain in determining the contributions to the molecular weight average. M_n represents the statistical average molecular weight of all polymer chains within a sample (Fitton et al., 2015). *C. barbata* had a higher M_w (1126.50 kDa) than *F. virsoides* (651.31 kDa) and both values are within the range of 1.4 to 1323 kD reported in the literature (Rioux et al., 2007; Yuan & Macquarrie, 2015a). Based on their M_w, fucoidans can be classified into low molecular weight (< 10 kDa), medium molecular weight (10 - 10 000 kDa), and high molecular weight fucoidans (> 10 000 kDa), so the fucoidans obtained in this study can be considered as medium molecular weight fucoidans. PDI is the ratio between M_w and M_n and measures the heterogeneity of the molecular weight distribution of polymers, with a larger difference between M_w and M_n (larger PDI) indicating a more heterogeneous molecular weight distribution (Fitton et al., 2015). A PDI of about 1 is characteristic of monodisperse natural polymers such as proteins, whereas a PDI greater than 1 is a typical feature of polydisperse polysaccharides. The PDI of fucoidans extracted from *F. virsoides* and *C. barbata* was 2.41 and 2.25, respectively, ranging from 1 to 6.2 of the reported fucoidan PDI values (Alboofetileh, Rezaei, Tabarsa, Rittà, et al., 2018; Hadj Ammar et al., 2016).

The average antioxidant capacity [ORAC (oxygen radical absorbance capacity) and DPPH (2,2-diphenyl-1-picrylhydrazyl) values] was slightly higher in *C. barbata*, regardless of the extraction technique. The reason could be the higher sulfate group content, higher M_w, and lower PDI determined for the polysaccharide of *C. barbata*, although the fucose and uronic acid contents were lower. Although the antioxidant capacity of fucoidan has already been demonstrated, the relationship between the chemical structure and the antioxidant capacity has not yet been clarified. It is known that antioxidant capacity is not influenced by a single factor, but rather by a combination of some physicochemical properties such as sulfate group content, uronic acid content, and molecular weight. Higher sulfate content was shown to positively affect the antioxidant activity of sulfated polysaccharides of *Sargassum fusiforme* (Liu et al.,

2019), *Undaria pinnitafida* (Hu et al., 2010), and *Pleurotus tuberregium* (Tao et al., 2006). Similarly, literature data showed that fucoidan with higher M_w (Falch et al., 2000; Liu et al., 2019; Tao et al., 2006), higher uronic acid content (Hifney et al., 2016), and higher fucose content (Hifney et al., 2016) had a higher antioxidant activity.

5.2. Influence of extraction technique on chemical structure and antioxidative capacity

Fucoidans have been shown to be sensitive to harsh extraction conditions and extraction technique has been shown to affect not only yield but also polysaccharide composition and consequently their biological activities (Ale et al., 2011a). Each of the extraction techniques used has different operating principles that lead to the degradation of brown algal cell walls, where most of the bioactive molecules, including fucoidan, are stored (Okolie et al., 2019). However, these techniques could disrupt the chemical bonds in the fucoidan molecule and thus alter its biological activity. From the results presented in Table 2 it can be seen that the content of sulfate groups was lowest in CE extract while it was significantly higher in the UAE and NTP extracts. NTP also yielded significantly lower fucose content than all other techniques, which means that the ratio of sulfate to fucose was 14.38, which is extremely high compared to CE, MAE and PLE, whose ratio of sulfate to fucose was about 1. It seems that plasma and ultrasound treatment had a deconstructive effect on sulfate ester bonds, so that a higher sulfate group content was observed. *N. zanardinii* fucoidan extracted with PLE (11.57%) had a lower sulfate group content than MAE (24.09%), UAE (22.97%), and CE (18.44%) (Alboofetileh, Rezaei, Tabarsa, Rittà, et al., 2018). The highest uronic acid content was obtained with CE, while the non-thermal extraction techniques UAE and NTP gave very low uronic acid content compared to the other techniques. MAE gave the lowest uronic acid content for *N. zanardinii* fucoidan compared to CE, UAE and PLE, which had the highest uronic acid content (Alboofetileh, Rezaei, Tabarsa, Rittà, et al., 2018).

In all extracts, regardless of algal species, the predominant monosaccharide was fucose, which is consistent with previously published results. In addition to fucose, glucose and galacturonic acid were also detected in all extracts, while arabinose was not detected in the extracts obtained with UAE and NTP. CE yielded extracts with the highest fucose and the lowest glucose content, while the extracts obtained with non-thermal techniques, UAE and NTP, had higher glucose and galacturonic acid content, while their fucose content was lower than the other techniques. Fucoidan of *N. zanardinii* obtained by CE had the lowest fucose content, followed by UAE, MAE, and PLE (Alboofetileh, Rezaei, Tabarsa, Rittà, et al., 2018).

The average M_w of the extracted fucoidans ranged from 473.61 to 1392.65 kDa, while the PDI values ranged from 1.45 to 2.99. The lowest M_w and PDI values were obtained with NTP treatment, while the highest M_w values were obtained with UAE and the highest PDI values were obtained with PLE. Similarly, the average M_w of *N. zanardinii* fucoidan ranged from 444 to1184 kDa and the PDI ranged from 1 to 1.84 (Alboofetileh, Rezaei, Tabarsa, Rittà, et al., 2018). In addition, *N. zanardinii* fucoidan extracted with MAE had higher M_w and PDI than fucoidan extracted with CE and UAE (Alboofetileh, Rezaei, Tabarsa, Rittà, et al., 2018), and although PLE resulted in the lowest M_w, the PDA was almost as high as that of MAE.

The highest ORAC value of polysaccharides of *F. virsoides* was determined in extracts obtained with MAE, while there was no statistically significant ($p \geq 0.05$) difference between PLE, UAE and NTP. The DPPH values of the extracts obtained with MAE, CE and PLE were higher than those obtained with UAE and NTP. The lowest ORAC and DPPH values for *C. barbata* polysaccharides were obtained with PLE, while the other techniques used gave similar antioxidant capacity.

Table 5. Influence of algal species and extraction technique, performed under previously determined optimal parameters, on polysaccharide yield (%PS) and their chemical composition

	N	%PS	Sulfate group (%)	Fucose (%)	Uronic acid (%)
Algae		$p \leq 0.05^{*}$	$p \leq 0.05^{*}$	$p \leq 0.05^{*}$	$p \leq 0.05^{*}$
F. virsoides	10	16.28 ± 0.19^{b}	57.82 ± 0.94^{a}	34.87 ± 0.37^{b}	9.35 ± 0.16^{b}
C. barbata	10	14.35 ± 0.19^{a}	63.59 ± 0.94^{b}	22.35 ± 0.37^{a}	7.40 ± 0.16^{a}
Technique		$p \leq 0.05^{*}$	$p \leq 0.05^{*}$	$p \leq 0.05^{*}$	$p \leq 0.05^{*}$
CE	4	17.41 ± 0.30^{c}	32.00 ± 1.49^{a}	32.04 ± 0.58^{c}	17.89 ± 0.25^{d}
MAE	4	17.85 ± 0.30^{c}	41.35 ± 1.49^{b}	37.55 ± 0.58^{d}	14.23 ± 0.25^{c}
PLE	4	21.50 ± 0.30^{d}	54.70 ± 1.49^{c}	44.07 ± 0.58^{e}	6.24 ± 0.25^{b}
UAE	4	11.97 ± 0.30^{b}	86.91 ± 1.49^{d}	23.23 ± 0.58^{b}	1.47 ± 0.25^{a}
NTP	4	7.87 ± 0.30^{a}	88.59 ± 1.49^{d}	6.16 ± 0.58^{a}	2.07 ± 0.25^{a}
Algae; technique		$p \leq 0.05^{*}$	p=0.43	$p \leq 0.05^{*}$	$p \leq 0.05^{*}$
F. virsoides; CE	2	$18.53\pm0.43^{e,f}$	28.46 ± 2.11^{a}	41.54 ± 0.82^{g}	20.06 ± 0.36^{g}
F. virsoides; MAE	2	20.42 ± 0.43^{f}	$37.13\pm2.11^{a,b}$	48.48 ± 0.82^{h}	15.93 ± 0.36^{f}
F. virsoides; PLE	2	24.22 ± 0.43^{g}	$51.82\pm2.11^{c,d}$	60.08 ± 0.82^{i}	$5.32\pm0.36^{c,d}$
F. virsoides; UAE	2	12.14 ± 0.43^{c}	83.37 ± 2.11^{e}	14.75 ± 0.82^{c}	$1.77\pm0.36^{a,b}$
F. virsoides; NTP	2	6.10 ± 0.43^{a}	88.31 ± 2.11^{e}	9.49 ± 0.82^{b}	$3.68\pm0.36^{b,c}$
C. barbata; CE	2	$16.29\pm0.43^{d,e}$	$35.53\pm2.11^{a,b}$	22.53 ± 0.82^{d}	15.72 ± 0.36^{f}
C. barbata; MAE	2	15.27 ± 0.43^{d}	$45.56\pm2.11^{b,c}$	$26.61\pm0.82^{d,e}$	12.52 ± 0.36^{e}
C. barbata; PLE	2	18.77 ± 0.43^{f}	57.58 ± 2.11^{d}	$28.06\pm0.82^{e,f}$	7.15 ± 0.36^{d}
C. barbata; UAE	2	$11.80\pm0.43^{b,c}$	90.44 ± 2.11^{e}	31.70 ± 0.82^{f}	1.16 ± 0.36^{a}
C. barbata; NTP	2	9.64 ± 0.43^{b}	88.86 ± 2.11^{e}	2.83 ± 0.82^{a}	0.46 ± 0.36^{a}

Results are expressed as mean±standard error. *Statistically significant variable at $p \leq 0.05$. Values with different letters within column are statistically different at $p \leq 0.05$. CE=conventional extraction, MAE=microwave-assisted extraction, PLE=pressurised liquid extraction, UAE=ultrasound-assisted extraction, NTP=non-thermal plasma.

Table 6. Influence of algal species and extraction technique, performed under previously determined optimal parameters, on monosaccharide composition and molecular properties of the extracted polysaccharides

		Monosaccharide composition (%)				Molecular properties		
	N	**glucose**	**fucose**	**galacturonic acid**	**arabinose**	**M_w (kDa)**	**M_n (kDa)**	**PDI**
Algae		$p \leq 0.05^*$	$p \leq 0.05^*$	$p \leq 0.05^*$	$p \leq 0.05^*$	$p \leq 0.05^*$	$p \leq 0.05^*$	$p \leq 0.05^*$
F. virsoides	10	25.72±0.13^b	48.24±0.15^a	18.44±0.10^b	7.60±0.11^b	651.31±11.57^a	252.55±5.26^a	2.41±0.03^b
C. barbata	10	18.87±0.13^a	59.56±0.15^b	16.50±0.10^a	5.06±0.11^a	1126.50±11.57^b	505.82±5.26^b	2.25±0.03^a
Technique		$p \leq 0.05^*$	$p \leq 0.05^*$	$p \leq 0.05^*$	$p \leq 0.05^*$	$p \leq 0.05^*$	$p \leq 0.05^*$	$p \leq 0.05^*$
CE	4	9.33±0.20^a	72.41±0.24^d	9.74±0.16^b	8.52±0.17^b	729.72±18.30^b	293.65±8.32^b	2.49±0.04^c
MAE	4	20.24±0.20^c	69.81±0.24^c	0.00±0.16^a	9.96±0.17^c	1071.72±18.30^c	506.74±8.32^c	2.26±0.04^b
PLE	4	18.00±0.20^b	45.70±0.24^b	23.13±0.16^d	13.17±0.17^d	776.83±18.30^b	282.70±8.32^{a,b}	2.99±0.04^d
UAE	4	31.26±0.20^d	36.13±0.24^a	32.61±0.16^e	0.00±0.17^a	1392.65±18.30^d	558.41±8.32^d	2.47±0.04^c
NTP	4	32.68±0.20^e	45.45±0.24^b	21.87±0.16^c	0.00±0.17^a	473.61±18.30^a	254.44±8.32^a	1.45±0.04^a
Algae; technique		$p \leq 0.05^*$	$p \leq 0.05^*$	$p \leq 0.05^*$	$p \leq 0.05^*$	$p \leq 0.05^*$	$p \leq 0.05^*$	$p \leq 0.05^*$
F. virsoides; CE	2	18.65±0.29^d	44.83±0.34^d	19.48±0.23^c	17.04±0.24^e	693.43±25.88^c	264.42±11.77^c	2.62±0.06^d
F. virsoides; MAE	2	13.26±0.29^b	78.35±0.34^g	0.00±0.23^a	8.39±0.24^b	891.25±25.88^{d,e}	332.14±11.77^d	2.68±0.06^d
F. virsoides; PLE	2	19.04±0.29^d	41.90±0.34^c	26.52±0.23^d	12.55±0.24^{c,d}	521.72±25.88^b	149.64±11.77^b	3.49±0.06^e
F. virsoides; UAE	2	31.47±0.29^f	36.30±0.34^a	32.23±0.23^f	0.00±0.24^a	1133.78±25.88^{f,g}	500.16±11.77^f	2.27±0.06^c
F. virsoides; NTP	2	46.20±0.29^g	39.83±0.34^b	13.97±0.23^b	0.00±0.24^a	16.38±25.88^a	16.38±11.77^a	1.00±0.06^a
C. barbata; CE	2	0.00±0.29^a	100.00±0.34^h	0.00±0.23^a	0.00±0.24^a	766.00±25.88^{c,d}	322.87±11.77^{c,d}	2.37±0.06^{c,d}
C. barbata; MAE	2	27.22±0.29^e	61.27±0.34^f	0.00±0.23^a	11.52±0.24^c	1252.19±25.88^g	681.34±11.77^g	1.84±0.06^b
C. barbata; PLE	2	16.95±0.29^c	49.50±0.34^e	19.75±0.23^c	13.79±0.24^d	1031.94±25.88^{e,f}	415.75±11.77^e	2.48±0.06^{c,d}
C. barbata; UAE	2	31.04±0.29^f	35.96±0.34^a	33.00±0.23^f	0.00±0.24^a	1651.52±25.88^h	616.65±11.77^g	2.67±0.06^d
C. barbata; NTP	2	19.15±0.29^d	51.08±0.34^e	29.76±0.23^e	0.00±0.24^a	930.83±25.88^e	492.50±11.77^f	1.89±0.06^b

Results are expressed as mean±standard error. *Statistically significant variable at $p \leq 0.05$. Values with different letters within column are statistically different at $p \leq 0.05$. CE=conventional extraction, MAE=microwave-assisted extraction, PLE=pressurised liquid extraction, UAE=ultrasound-assisted extraction, NTP=non-thermal plasma, M_w=weight average molecular weight, M_n=number average molecular weight, PDI=polydispersity index.

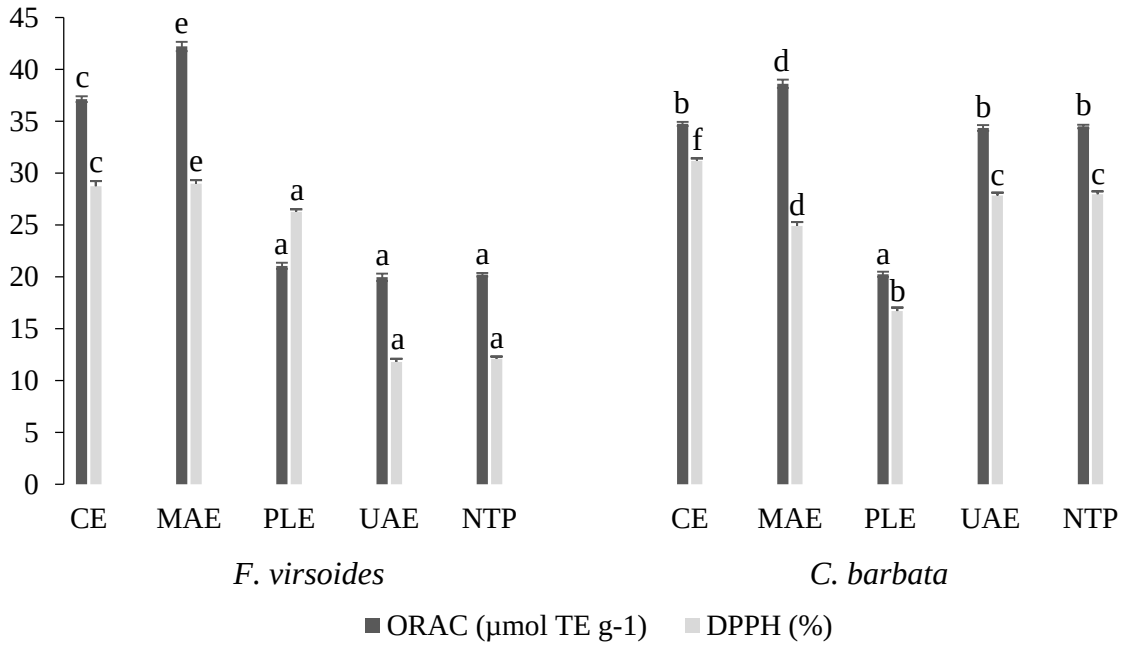

Figure 4. Influence of extraction technique, performed under previously determined optimal parameters, on antioxidative capacity (ORAC and DPPH) of the extracted polysaccharides. Values with different letters within group are statistically different at $p \leq 0.05$. CE=conventional extraction, MAE=microwave-assisted extraction, PLE=pressurised liquid extraction, UAE=ultrasound-assisted extraction, NTP=non-thermal plasma

5.3. Influence of extraction solvent on chemical structure and antioxidative capacity

In *Publication No. 2*, *Publication No. 3* and *Publication No. 4,* different extraction techniques (CE, MAE, PLE, UAE, NTP) were applied for polysaccharide extraction from *F. virsoides* and *C. barbata* using different solvents, H_20, 0.1M HCl and 0.1M H_2SO_4. Along with other parameters (time, temperature, number of cycles), the best solvent was selected mainly based on the best polysaccharide yield and therefore only these extracts were analysed in more detail. The solvent that gave the highest polysaccharide yield regardless of extraction technique and algal species was 0.1M H_2SO_4. Therefore, only extracts obtained with 0.1M H_2SO_4 were used for more detailed analysis of chemical composition, molecular properties, and antioxidant capacity. To compare the influence of other solvents on chemical composition, molecular properties, and antioxidant capacity, additional analyses of extracts obtained by the conventional technique were performed and the results are presented in Tables 7 and 8.

F. virsoides polysaccharides extracted with 0.1M HCl had the highest content of sulfate groups, while in *C. barbata* it was not affected by the solvent. In both algae, the highest fucose content was obtained with 0.1M HCl, while the lowest uronic acid content was obtained in the extracts obtained with H_2O and the highest in those with 0.1M H_2SO_4. Similar results for the content of sulfate groups were also reported previously (January et al., 2019), while the results for the fucose content obtained in this study were opposite to the literature results (Baba et al., 2018; W. W. F. Mak, 2012). The polysaccharides of *Ecklonia maxima*, *Laminaria pallida*, *Splachnidium rugosum*, *U. pinnatifida*, and *Adenocystis utricularis* obtained by hot water extraction also had lower total uronic acid content than the extracts obtained with acid (January et al., 2019; W. W. F. Mak, 2012; Ponce et al., 2003).

M_w was significantly higher in polysaccharides obtained with H_2O compared to the ones obtained with acids. However, this trend was not observed for PDI values. Likewise, *S. fusiforme* polysaccharide extracted with water had M_w 65.34 kDa and 26.63 kDa with acid (Liu et al., 2019). For *A. utricularis* polysaccharide there was no major differences in M_w between acid and water extraction (Ponce et al., 2003). The lowest ORAC value for both algae was achieved in extracts obtained with H_2O while slightly higher value was recorded in extract obtained with 0.1M HCl rather than 0.1M H_2SO_4. Likewise, similar trend can be observed for DPPH values. The lower antioxidant capacity of polysaccharides extracted with water could be explained and connected with their chemical structure, which was also significantly affected by the solvent used. As discussed previously, application of water resulted in polysaccharides with a lower sulfate group, lower uronic acid content, and higher M_w compared to the extracts obtained with acids, which was previously associated with lower antioxidant activity (Hifney et al., 2016; Hu et al., 2010; Liu et al., 2019; Tao et al., 2006). DPPH radical scavenging capacity of *S. fusiforme* polysaccharide extracted with water was much stronger (83.3%) than those extracted with acid (28.1%) (Liu et al., 2019). Apart from the antioxidant activity, the choice of the extraction solvent had an influence on the antiviral activity of the algal polysaccharide as water extracts had lower antiviral activity than extracts obtained with acid (Ponce et al., 2003).

Table 7. Chemical composition of *Fucus virsoides* and *Cystoseira barbata* polysaccharides obtained with conventional extraction using various solvents

		Sulfate group (%)	Fucose (%)	Uronic acid (%)	Monosaccharide composition (%)			
					glucose	fucose	galacturonic acid	arabinose
		$p \leq 0.05^{*}$	$p \leq 0.05^{*}$	$p \leq 0.05^{*}$	$p \leq 0.05^{*}$	$p \leq 0.05^{*}$	$p \leq 0.05^{*}$	$p \leq 0.05^{*}$
F. virsoides	H_20	25.06 ± 2.00^{a}	$28.44\pm0.44^{b,c}$	11.06 ± 0.51^{a}	25.16 ± 0.27^{e}	41.24 ± 0.28^{a}	18.25 ± 0.17^{e}	15.35 ± 0.16^{c}
	0.1M HCl	38.86 ± 2.10^{c}	53.97 ± 0.09^{e}	$18.00\pm1.71^{b,c}$	24.21 ± 0.35^{e}	48.65 ± 0.61^{c}	15.25 ± 0.27^{d}	11.89 ± 0.12^{b}
	0.1M H_2SO_4	$28.46\pm2.11^{a,b}$	41.54 ± 0.82^{d}	20.06 ± 0.36^{c}	18.65 ± 0.29^{d}	44.83 ± 0.34^{b}	19.48 ± 0.23^{f}	17.04 ± 0.24^{d}
C. barbata	H_20	$35.53\pm0.80^{b,c}$	$25.49\pm0.41^{a,b}$	7.43 ± 0.26^{a}	9.23 ± 0.08^{b}	87.56 ± 0.31^{e}	3.21 ± 0.11^{b}	0.00 ± 0.00^{a}
	0.1M HCl	$37.72\pm0.50^{b,c}$	29.94 ± 0.42^{c}	9.25 ± 0.16^{a}	13.02 ± 0.14^{c}	82.42 ± 0.43^{d}	4.56 ± 0.09^{c}	0.00 ± 0.00^{a}
	0.1M H_2SO_4	$35.53\pm2.11^{b,c}$	22.53 ± 0.82^{a}	15.72 ± 0.36^{b}	0.00 ± 0.00^{a}	100.00 ± 0.34^{f}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

Results are expressed as mean±standard error. *Statistically significant variable at $p \leq 0.05$. Values with different letters within column are statistically different at $p \leq 0.05$.

Table 8. Molecular properties and antioxidant capacity of *Fucus virsoides* and *Cystoseira barbata* polysaccharides obtained with conventional extraction using various solvents

		Molecular properties		Antioxidant capacity	
		M_w (kDa)	PDI	DPPH	ORAC
		$p \leq 0.05^{*}$	$p \leq 0.05^{*}$	$p \leq 0.05^{*}$	$p \leq 0.05^{*}$
F. virsoides	H_20	1128 ± 28.07^{d}	$2.21\pm0.17^{a,b}$	27.41 ± 0.41^{a}	33.46 ± 0.26^{b}
	0.1M HCl	646.33 ± 29.91^{a}	1.73 ± 0.13^{a}	$29.47\pm0.45^{a,b}$	39.79 ± 0.34^{e}
	0.1M H_2SO_4	$693.43\pm25.88^{a,b}$	2.62 ± 0.08^{b}	28.74 ± 0.49^{a}	37.14 ± 0.28^{d}
C. barbata	H_20	1621.28 ± 20.78^{d}	2.57 ± 0.20^{b}	$29.64\pm0.37^{a,b}$	30.22 ± 0.19^{a}
	0.1M HCl	916.43 ± 26.33^{c}	2.36 ± 0.16^{b}	32.52 ± 0.42^{c}	$35.91\pm0.22^{c,d}$
	0.1M H_2SO_4	766.00 ± 25.88^{b}	2.37 ± 0.08^{b}	$31.20\pm0.35^{b,c}$	$34.75\pm0.28^{b,c}$

Results are expressed as mean±standard error. *Statistically significant variable at $p \leq 0.05$. Values with different letters within column are statistically different at $p \leq 0.05$. M_w=weight average molecular weight, PDI=polydispersity index.

6. Biological activities – unpublished data

Fucoidan has been reported to possess various biological activities for human health, including antioxidant (Dai et al., 2020; E. A. Kim et al., 2014; Oh et al., 2020; L. Wang et al., 2019), anti-inflammatory (Cumashi et al., 2007; Takahashi et al., 2018), anticoagulant (Athukorala et al., 2006; Cumashi et al., 2007; Jin et al., 2013), antiviral (Krylova et al., 2020; Yim et al., 2021), antitumor (Azuma et al., 2012; E. J. Kim et al., 2010; W. Mak et al., 2014), antimetastatic (Alekseyenko et al., 2007), and antiangiogenic (Koyanagi et al., 2003). In addition, fucoidan has been reported to prevent *Helicobactor pylori* infection (Shibata et al., 2003) and reduce the risk of associated gastric cancer (Maruyama et al., 2006). Various preparations containing fucoidan as a biologically active component are being developed for medical use, for example, in wound dressings (J.-H. Park et al., 2017). The lack of toxicity together with bacteriostatic properties also enabled their application in the food industry, extending the shelf life of the products and not killing the natural human microflora during ingestion (Ayrapetyan et al., 2021).

In collaboration with colleagues from the Ruđer Bošković Institute, the polysaccharides of *F. virsoides* and *C. barbata* obtained by CE at optimal parameters were investigated for various biological activities, e.g., *in vitro* and *in vivo* antioxidant activity, embryotoxicity, cardiotoxicity, behavioural changes, genotoxicity, and antimicrobial activity. The results presented in *Chapter 6* are part of the larger study and will be incorporated into a research article with the working title "Chemical characterization and bioactivity of polysaccharides isolated from 10 marine species of the Adriatic Sea", which is currently in preparation.

6.1. In vitro antioxidant activity

Because of the potential health hazards of consuming synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butylhydroquinone (TBHQ), and propyl gallate (PG), there are strict regulations on their use in the food and pharmaceutical industries (Lourenço et al., 2019). As already mentioned, the antioxidant capacity of fucoidan isolated from various species of algae, which is comparable to synthetic antioxidants, has been widely demonstrated in the literature and the application of fucoidan as a natural and safe antioxidant from renewable and inexpensive sources is considered promising. Since antioxidants are usually involved in multiple mechanisms of action, a single assay cannot accurately reflect the antioxidant potential of complex fractions. Therefore, antioxidant activity should not be concluded based on a single antioxidant assay, but by a combination of at least

two different techniques (Alam et al., 2013). In this study, the *in vitro* antioxidant activity of *F. virsoides* and *C. barbata* polysaccharides obtained by conventional extraction under defined optimal conditions (*Publication No. 2*) was tested by applying four antioxidant assays, ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)), DPPH, FRAP (ferric reducing antioxidant power), and ORAC, according to the procedures described previously (Jerković et al., 2021).

Each of these methods is based on a different mechanism for determining antioxidant capacity, making it difficult to fully compare the results of one method with another (Alam et al., 2013). Therefore, a combination of several methods provides a more comprehensive insight into the antioxidant capacity of the samples under study. ABTS radical cation decolorization assay is based on the reduction of ABTS•+ radicals by antioxidants present in extract. The DPPH method is a rapid, simple (without many steps and reagents), and inexpensive method for free radical scavenging. The FRAP assay was used because it measures antioxidant activity via a single electron transfer (a SET mechanism) but cannot detect compounds that act only by radical quenching (i.e., hydrogen atom transfer (HAT)), so it can be used as a good method to estimate the mechanism of antioxidant activity. Finally, the ORAC assay is based on the scavenging of peroxyl radicals generated by AAPH, which prevent the degradation of the fluorescein probe and, consequently, prevent the loss of fluorescence of the probe (Dudonné et al., 2009).

The results for all tests performed are shown in Figure 5 and expressed as mean±standard deviation (n=3). As it can be observed, the polysaccharides of *C. barbata* had higher antioxidant activity than the polysaccharides of *F. virsoides*, regardless of the assay used. Various antioxidant assays were used to measure the antioxidant capacity of different brown algal polysaccharides, and a wide range of values was reported. The polysaccharides of *C. barbata* previously showed higher antioxidant activity against DPPH, namely 100% inhibition at 1.5 mg mL^{-1} (Sellimi et al., 2014), than some other polysaccharides from brown algae such as *Sargassum vulgare* and *Sargassum pallidum*, which showed DPPH inhibition of 22.5% and 19.1% at concentrations of 2.5 mg mL^{-1} and 3.8 mg mL^{-1}, respectively (Guerra Dore et al., 2013; Ye et al., 2008). *C. compressa* showed a good ability to scavenge DPPH free radical (from 20% to 80% inhibition in the concentration range of 50-500 µg mL^{-1}), while the reported ABTS value was 39 µmol TE g^{-1} and the FRAP value was 77.2 µmol TE g^{-1} (Rashed et al., 2021). In the concentration range from 2.75 to 16.5 mg mL^{-1}, the polysaccharide of *Sargassum*

wightii showed DPPH inhibition ranging from 24.67% to 73.10%, and the FRAP values were in the range of 42.46-139.71 µmol Fe(II) g^{-1} (Linga Prabu et al., 2013). Similarly, *Sargassum hystrix* polsaccharide showed DPPH inhibition from 32.57% to 49.63% at concentrations ranging from 500 to 4000 ppm and the FRAP method gave a value of 89.62 µM g^{-1} Fe(II) (Suhaila et al., 2019). The polysaccharide-rich extracts (1 mg mL^{-1}) of *Laminaria digitata* resulted in DPPH inhibition of 11% and a FRAP value of 8.7 µM TE mg^{-1}, *Laminaria hyperborea* resulted in DPPH inhibition of 44.2% and a FRAP value of 11 µM TE mg^{-1}, while *A. nodosum* resulted in DPPH inhibition of 85.7% and a FRAP value of 104.3 µM TE mg^{-1} (Garcia-Vaquero et al., 2018).

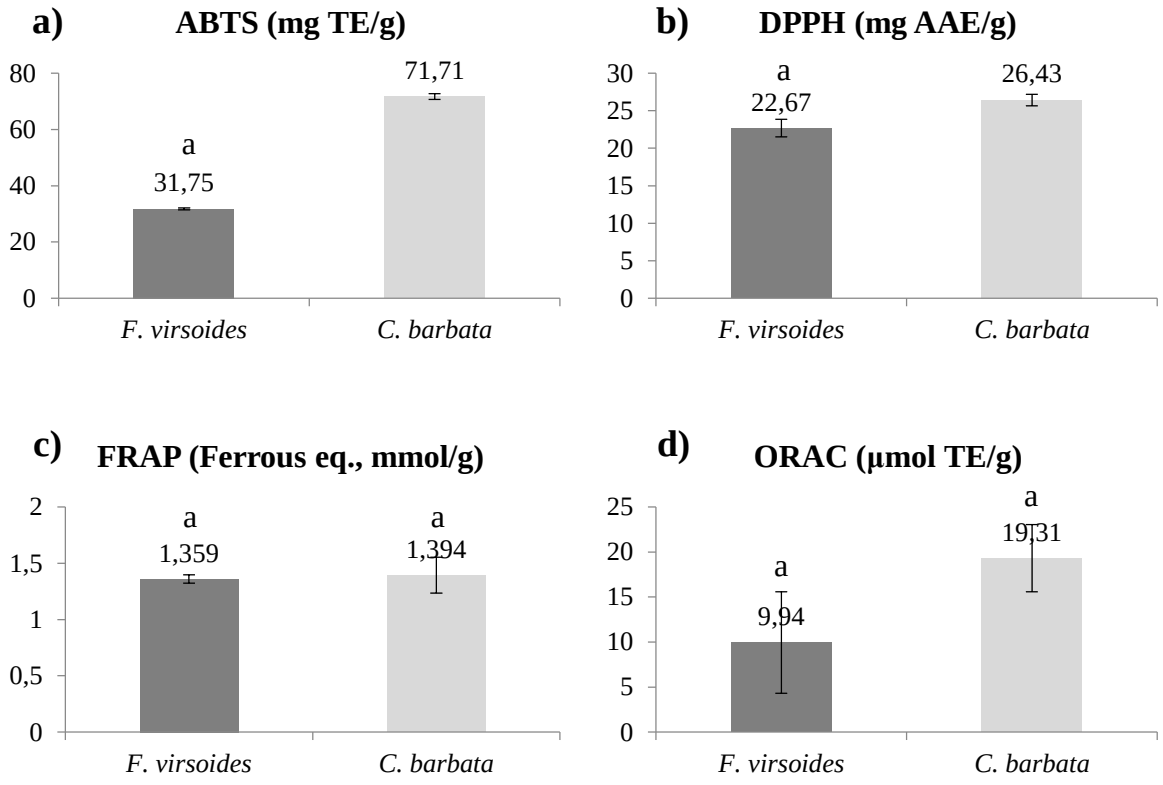

Figure 5. Results of four different antioxidant capacity assays: a) ABTS, b) DPPH, c) FRAP and d) ORAC performed on *Fucus virsoides* and *Cystoseira barbata* polysaccharides. Values with different letters within various assays are statistically different at $p \leq 0.05$.

As mentioned above, the antioxidant capacity of fucoidan is undisputed, but the relationships between its chemical structure and antioxidant capacity have not yet been clarified. This is mainly due to two important factors: the great structural diversity of these polysaccharides and the presence of a number of other molecules (polyphenols and proteins) in the extracts (Ghosh et al., 2015). It has been suggested that the antioxidant properties of

sulfated polysaccharides are related to their molecular weight (Hou et al., 2012), the degree of sulfation and the position of sulfate groups (Cho et al., 2011; J. Wang et al., 2009), and the content of glucuronic acid and fucose (Zhao et al., 2008). The chemical composition (sulfate group content, monosaccharide composition) and molecular properties of the polysaccharides of *F. virsoides* and *C. barbata* obtained by conventional extraction with H_2SO_4 under defined optimal conditions were discussed in a *Chapter 5.3* and presented in Tables 5 and 6. As can be seen, there was no statistically significant ($p \leq 0.05$) difference in sufate group content and M_w between the polysaccharides of *F. virsoides* and *C. barbata*. However, *C. barbata* had significantly ($p \leq 0.05$) lower uronic acid content, which was previously associated with lower anticoagulant (Kuznetsova et al., 2018) and anti-complement activity (Chaminda Lakmal et al., 2015).

Polyphenols and proteins that could interfere with antioxidant assays are usually extracted along with polysaccharides, and it is not possible to completely remove them during purification procedures (Hifney et al., 2016). In a study by Imbs et al. (2015), it was reported that the antioxidant capacity of sulfated polysaccharides from *F. evanescens* was attributed only to the phenolic content and not to the degree of sulfation or the content of uronic acid and fucose (Imbs et al., 2015). However, in most other studies on the antioxidant activity of sulfated polysaccharides extracted from brown algae, the protein and polyphenol content in the crude fucoidans was not determined. Therefore, in this study, we additionally measured the polyphenol content in these polysaccharides from *F. virsoides* and *C. barbata* extracted by conventional technique under optimal conditions. We applied the spectrophotometric method described in *Publication No. 2*. The results showed that the polysaccharide of *F. virsoides* contained 1.52 mg of total polyphenols in one gram of dried polysaccharides, while the polysaccharides of *C. barbata* contained 1.76 mg g^{-1}. Since *C. barbata* also exhibited higher antioxidant capacity, these results are consistent with previous findings in the literature (Imbs et al., 2015).

6.2. Zebrafish embryotoxicity, cardiotoxicity and behavioural changes tests

To test the toxicity potential of the polysaccharide extracts, zebrafish *Danio rerio* embryos (WIK type, obtained from the European Zebrafish Resource Center of the Karlsruhe Institute of Technology) were used. Zebrafish are vertebrates that have special advantages over other experimental animal models, due to their smaller size, high breeding capacity, and short life cycle. They have been traditionally used in the fields of molecular genetics and

development biology, as a model organism for human disease, drug discovery and toxicological studies because of their physiological similarity to mammals (Den Hertog, 2005; Driever et al., 1996; Pichler et al., 2003). Zebrafish maintenance and egg production were performed according to a recent study (Babić et al., 2021). Within zebrafish embryotoxicity test (ZET) zebrafish embryos (4-64 blastomeres) were exposed to a successive dilution of *F. virsoides* and *C. barbata* polysaccharides (1, 0.50, and 0.25 μg L^{-1}) following OECD Guideline 236 (2013). Six embryos were exposed per well containing 1.5 mL of the tested sample in five replicates, amounting to a total of 30 embryos per dilution. During 96 hours of development the embryos were incubated at 26°C (Innova 42 incubator shaker, New Brunswick, Canada) under a regulated light-dark photoperiod (14/10). Mortality and developmental abnormalities were recorded after 96 hours of exposure using an inverted microscope (Leica DMIL LED) equipped with digital camera (Leica EC3). In addition, cardiotoxicity was determined by measuring heartbeats per 15 seconds (N=15 larvae) while behavioral changes were determined by measuring pectoral fin movements per 20 seconds (N=10 larvae). Animal housing and spawning were performed in aquarium facilities approved by the Croatian Ministry of Agriculture (HR-POK -023). All experiments in this study were performed on the non-protected embryonic stages (up to 96 hpf), which do not require approval from the Animal Welfare Commissions (Directive 2010/63/EU, 2010).

The toxic effects of the polysaccharide fractions of *F. virsoides* and *C. barbata* on zebrafish embryonic development are shown in Table 9. The tested polysaccharide fractions had no statistically significant effect on zebrafish survival, developmental abnormalities, and hatching success. The control larvae developed normally (Figure 6) and formed adequate pigmentation of the retina and the entire body. To the best of our knowledge, the embryotoxicity of fucoidan has not been studied. However, several studies have tested the cytotoxicity of fucoidan in various cell lines. Fucoidan from the brown alga *S. cinereum* showed cytotoxic activity against colon cancer cells (Somasundaram et al., 2016), while *F. evanescens* fucoidan had no cytotoxic effects on Jurkat and SC -1 cells in a concentration range of 0.001-100 μg mL^{-1} (Menshova et al., 2016). Purified fucoidan isolated from *S. japonica* had no cytotoxic effects at concentrations up to 25 μg mL^{-1}, as cell proliferation was not significantly affected and the viability of RAW 264.7 macrophage cells was higher than 90% (Ni et al., 2020).

Table 9. Developmental toxicity in zebrafish *Danio rerio* (N=40) after 96 hours of exposure to *Fucus virsoides* and *Cystoseira barbata* polysaccharides

Sample	Concentration (mg mL^{-1})	Mortality (%)	Abnormality (%)	Hatched (%)
	1.00	5.00±10.00	0.00±0.00	100.00±0.00
F. virsoides	0.50	0.00±0.00	0.00±0.00	100.00±0.00
	0.25	0.00±0.00	0.00±0.00	100.00±0.00
	1.00	0.00±0.00	0.00±0.00	90.00±11.55
C. barbata	0.50	0.00±0.00	0.00±0.00	95.00±10.00
	0.25	0.00±0.00	0.00±0.00	95.00±10.00

Results are expressed as mean±standard deviation.

Figure 6. Normally developed larva at 96 hpf

The heart beat rate of zebrafish treated with polysaccharides of *F. virsoides* and *C. barbata* (Figure 7) did not differ from the control values (42.06 beats per 15 sec), from which it can be concluded that these polysaccharides did not show cardiotoxic effects at the concentrations tested. Similarly, the polysaccharides of *F. virsoides* and *C. barbata* showed no behavioral changes, as no statistically significant decrease or increase ($p > 0.01$) in the number of pectoral fin movements was observed at any concentration (Figure 8).

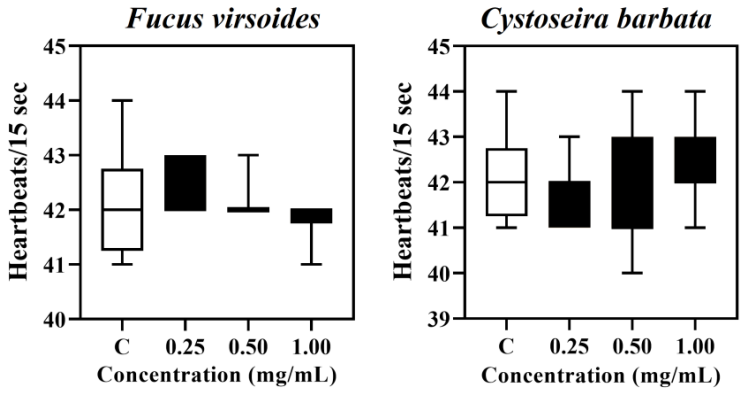

Figure 7. Effect of *Fucus virsoides* and *Cystoseira barbata* polysaccharides on the heartbeat rate of zebrafish model at 96 hours of exposure. The results are presented as box-plots. A line within the box represents the median value, while the boundaries of box-plot indicate 25th and 75th percentiles. Whiskers above and below the box indicate 10th and 90th percentiles.

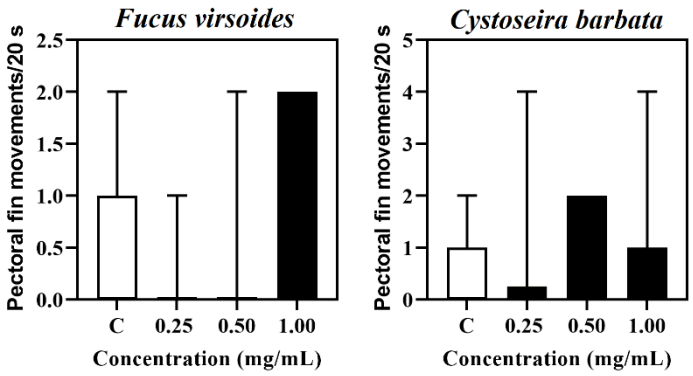

Figure 8. Effect of *Fucus virsoides* and *Cystoseira barbata* polysaccharides on the number of pectoral fin movements of zebrafish model at 96 hours of exposure.

6.3. Microbial mutagenicity assay: Ames test

The bacterial reverse mutation test (Ames test) is a widely used method that utilizes bacteria to test whether a particular chemical can cause mutations in the DNA of the test organism (Hwang et al., 2016). The polysaccharides of *F. virsoides* and *C. barbata* were tested for their mutagenic potential according to ISO 16240 (2005) (Maron & Ames, 1983). Two *Salmonella typhimurium* strains were used: the TA98 strain to test for frameshift mutations and the TA100 strain to detect base pair substitution mutations. The assay was performed on minimal glucose plates containing 100 µL polysaccharide fraction, 2 mL top agar, and 100 µL bacterial culture, both with and without metabolic activation (Aroclor-1254 induced male Sprague Dawley rat liver post-mitochondrial S9 fraction). Fractions at concentrations of 10 and 20 mg/plate were tested in duplicates. Plates were incubated at 37°C for 72 hours, then the number of revertants was counted. Distilled water was used as dilution medium and thus as negative control. The standard mutagen (positive controls) for TA98 and TA100 strains with metabolic activation was 2- AA. The positive control on plates without metabolic activation for the TA98 strain was NOPD and for TA100 MMS.

Treatment with *F. virsoides* and *C. barbata* polysaccharide did not result in a significant dose-dependent increase in revertant colonies compared with spontaneously formed revertant colonies in the control group at any of the doses tested, either for *S. typhimurium* strains TA 98 or TA 100 (Table 10). These data do not indicate a gene mutagenic potential of the polysaccharides of *F. virsoides* and *C. barbata* under the conditions used in this assay. In addition, the polysaccharide treatments showed antimutagenic effects against 2- AA in both TA98 and TA100 with metabolic activation and against NOPD and MMS in TA98 and TA100

without metabolic activation, respectively. Fucoidan extracted from *U. pinnatifida* and *L. japonica* did not cause mutagenicity as there was no dose-dependent increase in the number of reverting colonies in the fucoidan- treated groups (up to 5000 lg/plate) compared to the negative controls (Chung et al., 2010; Hwang et al., 2016; K. J. Kim et al., 2010; Song et al., 2012). Moreover, *U. pinnatifida* fucoidan might even have positive antimutagenic activity, as it showed dose-dependent antimutagenic activity against the mutagen 4-NQO (0.15 µg/plate), up to 71% compared to the control strain TA 98 (Chung et al., 2010).

Table 10. The numbers of revertant colonies induced by polysaccharide fractions in *Salmonella typhimurium* TA98 and TA100 strains with or without metabolic activation (S9)

Sample (concentration)	**Number of His+ revertants**			
	***Salmonella typhimurium* – strain TA 98**		***Salmonella typhimurium* – strain TA 100**	
	(- S9 mix)	**(+ S9 mix)**	**(- S9 mix)**	**(+ S9 mix)**
F. virsoides (20 mg/plate)	13.00±0.00	29.50±3.54	101.00±1.41	88.00±4.24
F. virsoides (10 mg/plate)	12.50±0.07	13.50±2.12	97.00±19.80	89.50±6.77
C. barbata (20 mg/plate)	20.00±1.41	26.50±2.12	97.00±24.04	89.50±10.61
C. barbata (10 mg/plate)	18.00±4.24	19.00±4.24	75.50±4.95	95.50±2.12
Controls of the procedure				
Control without bacteria	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Control-spontaneous revertants	15.50±2.12	19.00±1.00	91.30±6.70	89.30±3.50
Positive Control NOPD (10 µg/ plate)	› 1200			
Positive Control 2-AA (5 µg/plate)		› 1500		› 2000
Positive Control MMS (2µl/ plate)			› 2000	

Results are expressed as means±standard deviation.

6.4. In vivo antioxidant activity using zebrafish model

The antioxidant effect of *F. virsoides* and *C. barbata* polysaccharides in H_2O_2-treated zebrafish was analyzed in agreement with (Jerković et al., 2021). Considering the results obtained in the embryotoxicity test, zebrafish embryos (N = 40) were treated with 1, 0.50, and 0.25 μg L^{-1} of *F. virsoides* and *C. barbata* polysaccharides. After 4 h of pre-treatment, 5 mM H_2O_2 was added to the medium. The negative control groups were exposed to artificial water, while the positive control group was treated with 5 mM H_2O_2. After 96 h of exposure, larvae were observed and mortality and abnormality rates were recorded. To visualize and quantify the extent of oxidative stress, larvae were stained for 1 hour with 10 μM of the fluorogenic dye dichloro-dihydro-fluorescein diacetate (DCFDA), which diffuses into cells. It is then hydrolyzed by intracellular esterases to a non-fluorescent compound, which is then oxidized by ROS to 2′7′ – dichlorofluorescein (DCF) (Eruslanov & Kusmartsev, 2009; W. Wang et al., 2019) and detectable by fluorescence spectroscopy. After rinsing three times with artificial water, DCFDA-stained larvae were visualized and photographed using a fluorescence microscope (Olympus® BX51 binocular light microscope equipped with Microsoft® AnalySIS Soft Imaging System software) with a green fluorescence filter. The fluorescence intensity of the images was quantified using ImageJ software. The antioxidant potential of the fractions should be evident in a decrease in fluorescence intensity in samples exposed to a mixture of the polysaccharides and H_2O_2 compared to samples exposed to H_2O_2.

To confirm the reduction in oxidative stress by polysaccharides, mortality rates (Figure 9) and levels of ROS production (Figure 11) were measured. Although not statistically significant, it can be noted that pre-treatment with 1.00 mg mL^{-1} of *C. barbata* reduced the mortality rate by 17% when compared to the negative control. According to Wang et al. (2020) the cell death of zebrafish treated with H_2O_2 increased to 227.31% compared to control group (100%), but it decreased to 37.58%, 89.46%, and 141.26% in the zebrafish treated with *H. fusiforme* fucoidan at concentrations of 12.5, 25, and 50 μg mL^{-1}, respectively (Wang et al., 2020). Instead of H_2O_2, other free radical-generating compounds such as 2,2`-azobis dihydrochloride (AAPH) could be used. *U. pinnatifida* polysaccharides improved survival rate in AAPH-treated zebrafish to 95% at 250 μg mL^{-1} (Oh et al., 2020) while *Sargassum fulvellum* polysaccharides increased cell viability to 83.62% at 100 μg mL^{-1} (L. Wang et al., 2019) and *Hizikia fusiforme* fucoidan to 80% at 150 μg mL^{-1} (Dai et al., 2020).

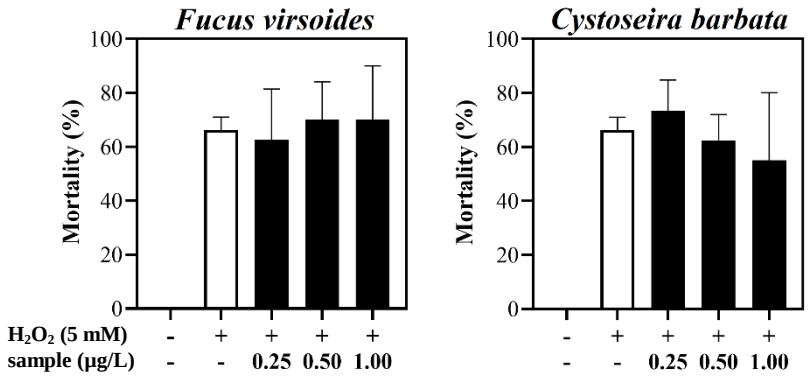

Figure 9. Effects of *Fucus virsoides* and *Cystoseira barbata* polysaccharides on the mortality rate in H_2O_2 - treated zebrafish model

Fluorescent photographs (Figure 10) of zebrafish larvae showed low fluorescence intensity in specimens exposed to the negative control. In contrast, exposure of zebrafish to H_2O_2 resulted in increased fluorescence intensity (296.3%), indicating that ROS was produced in the presence of H_2O_2 (Figure 11). *C. barbata* significantly decreased fluorescence intensity in a concentration-dependent manner, demonstrating its effectiveness in intercepting the production of ROS. Pre-treatment with 1 mg mL^{-1} *C. barbata* reduced the production of ROS in zebrafish by 40.42% ($p < 0.01$) compared to the positive H_2O_2 control group (Figures 7 & 8). The ROS formation in the negative control groups was considered 100%. These results suggest that the polysaccharides of *C. barbata* have stronger antioxidant activity *in vivo* than the polysaccharides of *F. virsoides* and that they could be a potential ingredient for the pharmaceutical and cosmetic industries. Wang et al. (2020) reported that H_2O_2 significantly increased the formation of ROS to 231.33% compared with the control group (100%). However, *H. fusiforme* fucoidan reduced the level of ROS to 187.73%, 154.26%, and 126.89% at concentrations of 12.5, 25, and 50 µg mL^{-1}, respectively (Wang et al., 2020). Similarly, the intracellular ROS level increased by H_2O_2 was effectively down-regulated by *P. boryana* polysaccharides treatment (Jayawardena et al., 2020), and ROS levels increased by AAPH were decreased by *E. cava*, *U. pinnatifida*, *S. fulvellum*, and *H. fusiforme* polysaccharides (Dai et al., 2020; E. A. Kim et al., 2014; Oh et al., 2020; L. Wang et al., 2019).

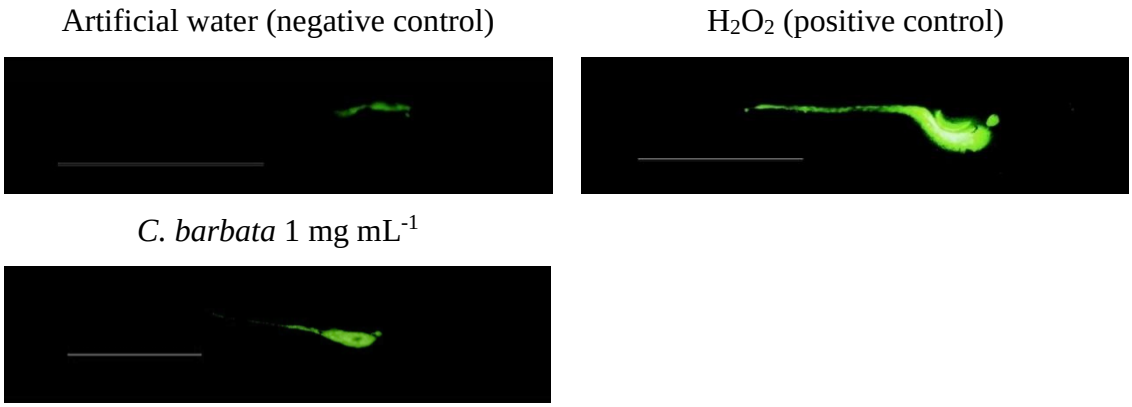

Figure 10. H_2O_2-induced ROS production visualized with DCF-DA staining

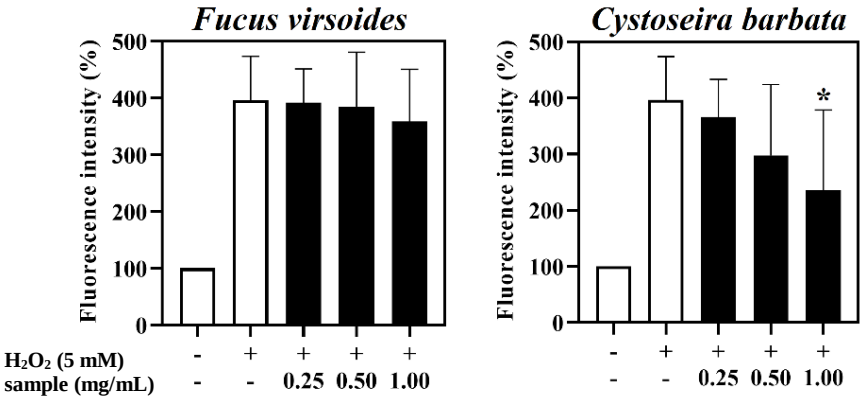

Figure 11. Quantitative results of fluorescence intensities measured using ImageJ software. *Statistically significant difference between tested samples and negative control at $p < 0.01$.

6.5. Anti-microbial activity

For the disk diffusion assays, paper disks 6 mm in diameter (MN 827 ATD, Macherey-Nagel) were preloaded in duplicate with 30 µL (3x10 µL) of sample stock solutions at a concentration of 10 mg mL^{-1} (w/v) and dried in a laminar flow cabinet. Four bacterial species (*Escherichia* coli NCTC 12241, *Bacillus subtilis* subsp. *spizizenii* ATCC 6633, *Pseudomonas aeruginosa* NCTC 12903, and *Staphylococcus aureus* ATCC 6538) and 1 yeast (*Candida albicans* ATCC 90028) were tested. Inocula were prepared from fresh overnight growth on Tryptic Soy Agar (Sigma Aldrich) for bacteria and Yeast Extract-Peptone-Dextrose media (YPD; Sigma-Aldrich) for yeasts in API® 0.85% saline medium (Biomérieux, USA) and diluted to 0.5 McFarland with a nephelometer. Cotton swabs were dipped into the 0.5 McFarland suspensions to completely inoculate the Mueller-Hinton agar (MHA; Merck) plates, and YPD agar for yeast, by sweeping them in three directions at a 60° angle. Six disks were placed per agar plate and then incubated aerobically at 35°C for 18 hours (20 hours for yeast). The distinct halo zones of growth inhibition were measured in mm using a caliper. The

control disks contained the solvents used as well as norfloxacin and nystatin for bacteria and yeast, respectively.

All extracts were also tested using the broth microdilution assay. The minimum inhibitory concentration (MIC) was determined as the highest concentration of extract/drug that completely inhibited visible growth. The highest concentration tested of 1 mg mL^{-1} was diluted 2-fold to the lowest concentration of 1.95 µg mL^{-1}. That is, the wells of the first column of a 96-well plates were prepared by adding 20 µL of the polysaccharide extract (10 mg mL^{-1}) and 80 µL of the cation-adjusted broth MH (CAMHB; Merck) in duplicates. Then, 50 µL of broth was added to all remaining wells (100 µL for the last, 12th column) and 50 µL of the first well was transferred to the second well and mixed by pipetting up and down 6-8 times with a multichannel pipette. This was serially repeated until the 10th column of a microtiter plate when the remaining 50 µL were discarded. Columns 11 and 12 were used as positive controls (growth in wells without extract/drug) and negative controls (wells with media only for validating sterility), respectively. Bacterial inocula were brought to 0.5 McFarland as described above and then diluted with CAMHB to a concentration of $1x10^6$ CFU mL^{-1}. Then, 50 µL of this inoculum was added to all wells except the negative control wells, resulting in a final concentration of $5x10^5$ CFU mL^{-1} in each well. For *C. albicans* testing, YPD medium was used and the inocula were diluted to a final concentration of approximately 0.5 - 2.5 x 10^3 CFU mL^{-1}. For each plate, sterility was checked by spreading 100 µL of the medium with a glass hockey stick, inoculum concentrations of positive controls by CFU counts, and quality controls with norfloxacin and nystatin for bacteria and yeast, respectively. The 96-well plates were incubated aerobically at 35°C for 20 hours (*C. albicans* for 48 hours). Absorbance was measured at 600 nm using an automated plate reader. Before reading, the plate was shaken for 10 seconds in orbital mode with 3 mm amplitude. If MIC was not observed growth at each concentration tested was evaluated relative to positive control wells with no extract present and expressed as relative growth reduction in percentage.

$$\text{Relative growth inhibition } (\%) = \frac{\text{Asample}}{\text{A0}} * 100$$

where A_o is absorbance of the positive growth control (wells with no extracts) for each bacteria or yeast, and A_{sample} is absorbance of the samples treated with polysaccharides, measured after 20 h for bacteria and 48 h for *C. albicans*.

Table 11. Anti-microbial activity of *Fucus virsoides* and *Cystoseira barbata* polysaccharides against *Escherichia* coli, *Bacillus subtilis*, *Staphylococcus aureus* and *Candida albicans* expressed as growth reduction relative to growth controls without extracts using optical absorbance at 600 nm.

	µg mL^{-1}	**Relative growth inhibition (%)**			
		E. coli	***B. subtilis***	***S. aureus***	***C. albicans***
F. virsoides	7.8125	7.0±8.2	3.7±1.1	11.2±3.9	-2.9±0.2
	15.625	6.3±0.3	5.1±3.9	7.7±3.7	-2.8±0.3
	31.25	6.1±4.7	2.4±1.4	4.5±1.3	-2.5±0.4
	62.5	5.2±5.6	4.6±3.6	6.7±2.8	-3.3±1.2
	125	7.3±2.8	2.2±1.7	9.5±7.9	-3.7±0.1
	250	10.0±3.4	10.4±2.9	-28.0±3.7	-5.6±0.7
	500	15.3±0.8	18.2±4.0	-2.6±1.4	-5.1±0.8
	1000	12.6±2.1	37.7±4.7	25.23±7.1	-2.0±1.7
C. barbata	7.8125	12.1±1.2	9.7±2.6	15.5±1.6	-3.1±0.5
	15.625	13.8±3.4	15.8±0.6	13.7±2.0	-0.7±0.4
	31.25	12.4±3.6	17.1±2.2	13.5±1.2	-0.9±0.6
	62.5	6.1±2.5	14.7±2.7	13.6±2.6	0.3±2.2
	125	0.5±8.2	6.8±5.4	11.1±1.4	-1.0±1.2
	250	5.5±4.2	0±1.0	8.9±2.2	-1.9±0.2
	500	7.8±1.3	20.6±2.7	10.4±0.1	-3.7±0.7
	1000	9.2±4.8	45.7±12.4	12.9±0.3	24.0±3.4

Results are expressed as means±standard deviation.

No clear halos were observed in the disk diffusion assay. The MICs of the polysaccharide extracts for *E. coli*, *B. subtilis*, *S. aureus*, and *C. albicans* were not observed at the highest concentration of 1 mg mL^{-1} using broth microdilution assay. However, partial growth inhibition was observed and is summarised in Table 11. Accordingly, *C. barbata* and *F. virsoides* polysaccharides at the highest concentration showed growth reduction of 45.7±12.4% and 37.7±4.7%, respectively, compared to *B. subtilis* positive controls. *C. barbata* also showed partial antifungal activity against *C. albicans* with a growth reduction of 24.0±3.4%. Crude and purified fucoidan from *F. vesiculosus* showed inhibitory activity against the growth of *E. coli*, *S. aureus*, *Bacillus licheniformis*, and *Staphylococcus epidermidis* with MIC of 4 to 6 mg mL^{-1} (Ayrapetyan et al., 2021). *S. polycystum* fucoidan effectively inhibited the growth of *Streptococcus mutans*, *P. aeruginosa*, *S. aureus*, and *E. coli* at concentrations of 100 to 200 µg mL^{-1} (Palanisamy et al., 2019). Similarly, fucoidan isolated from *S. wightii* controls the growth of bacterial pathogens *E. coli*, *Klebsiella pneumoniae*, *P. aeruginosa*, and *Salmonella typhi* (Marudhupandi & Kumar, 2013), while *S. polycystum* crude fucoidan inhibited the growth of *Vibrio harveyi*, *S. aureus*, and *E. coli* at MIC of 12.0, 12.0, and 6.0 mg mL^{-1}, respectively (Chotigeat et al., 2004).

Chapter 6

Conclusions and prospects

- The results of this study show that the brown algae species *F. virsoides*, endemic to the Adriatic Sea, as well as *C. barbata*, can be a good source of sulfated polysaccharides. The data also confirmed that the cell wall polysaccharides of brown algae are complex and that their yield and chemical composition are significantly affected by the algal species and the conditions under which they are extracted.
- *F. virsoides* and *C. barbata* contained 10.23% and 8.01% polysaccharides, respectively. The polysaccharides of *F. virsoides* contained 34.87% fucose and 57.82% sulfate groups, corresponding to a sulfate to fucose ratio of 1.65. In comparison, *C. barbata* had a lower content of fucose and a higher content of sulfate groups, 22.35% and 63.59%, respectively, giving a sulfate-to-fucose ratio of 2.85. The content of uronic acid in the polysaccharides of *F. virsoides* was slightly higher than in the polysaccharides of *C. barbata*. In addition to fucose, which was the predominant monosaccharide in both algal polysaccharides, glucose, galacturonic acid, and arabinose were also detected, whereas fructose, rhamnose, and mannose were not. *C. barbata* had a higher M_w (1126.50 kDa) than *F. virsoides* (651.31 kDa), but both can be considered as medium molecular weight fucoidans.
- The solvent used for pre-treatment affected the %PS, the removal of interfering substances, and the chemical composition of the extracted fucoidan. The combination of acetone and 96% ethanol showed the most promising results in terms of removal of interfering substances and %PS, while none of the applied pre-treatments proved to be significantly more effective than the others in terms of the chemical structure of the polysaccharides (total sugars, sulfate group, fucose and uronic acid content).
- Advanced extraction techniques, MAE, PLE, UAE, and NTP, were successfully applied and optimized to extract sulfated polysaccharides from the brown algae *F. virsoides* and *C. barbata*. PLE under optimal extraction parameters (0.1 M H_2SO_4, for two cycles of 15 min at 140°C) resulted in a significantly higher %PS from *F. virsoides*, while for *C. barbata* a similar yield was obtained with CE and PLE. Likewise, similar yields were obtained with MAE (0.1 M H_2SO_4 for 10 min at 80°C) and CE for both algae, while a slightly higher %PS was obtained with CE than with UAE and NTP (0.1 M H_2SO_4, 30 min). Although the advanced extraction techniques did not excessively improve the polysaccharide yield, the extraction time was reduced from 3 h to 30 min (PLE, UAE, NTP) or 10 min (MAE), which contributed to significant energy savings.

- The chemical structure and antioxidant properties of the extracted polysaccharides were influenced by the extraction technique. The content of sulfate groups was the lowest in the CE extract, while the content of uronic acids was the highest. The non-thermal techniques, UAE and NTP, resulted in significantly higher content of sulfate groups, lower content of fucose, and lower content of uronic acids than any other technique. The polysaccharides of *F. virsoides* obtained by MAE had significantly better antioxidant activity than the extracts obtained by other techniques, while the polysaccharides of *C. barbata* obtained by PLE had significantly lower antioxidant activity compared to the other extracts.
- The use of acid, especially 0.1M H_2SO_4, instead of water significantly improved the %PS and the extracts obtained are much lighter in color compared to the water extract, indicating higher purity. In both algae, the highest fucose content was obtained with 0.1M HCl, while the lowest uronic acid content was obtained in the extracts obtained with H_2O and the highest in those with 0.1M H_2SO_4. The polysaccharides of *F. virsoides* extracted with 0.1M HCl had the highest content of sulfate groups, while it was not affected by the solvent in *C. barbata*. M_w was significantly higher in the polysaccharides obtained with H_2O, while the antioxidant activity (ORAC and DPPH values) of these extracts was lower compared to those obtained with acids.
- Non-thermal techniques, UAE and NTP as 5-min pre-treatments followed by CE, did not result in more cell wall breakage, yielding a comparable %PS as CE alone. However, they did result in polysaccharides with higher sulfate group content and lower fucose content.
- The polysaccharides of *C. barbata* had higher *in vitro* antioxidant activity, as measured by ABTS, DPPH, FRAP, and ORAC assay, then the polysaccharides of *F. vrisoides*. Similarly, the polysaccharides of *C. barbata* have stronger antioxidant activity *in vivo* than the polysaccharides of *F. virsoides*, as they reduced the mortality rate and production of ROS in zebrafish and down-regulated fluorescence intensity.
- The polysaccharides of *F. virsoides* and *C. barbata* did not show embryotoxic and cardiotoxic effects, or behavioural changes, as they did not have a statistically significant effect on zebrafish survival, developmental abnormalities occurrence, hatching success, heart rate, and number of pectoral fin movements at any concentration.

- No gene mutagenic potential was observed for the polysaccharides of *F. virsoides* and *C. barbata*, however they showed positive antimutagenic activity against mutagens 2- AA, NOPD and MMS.
- The polysaccharides of *C. barbata* and *F. virsoides* showed antimicrobial activity against *B. subtilis* at a concentration of 1 mg mL^{-1}. In addition, the polysaccharides of *C. barbata* showed partial antifungal activity against *C. albicans*.
- The results of this dissertation represent a significant contribution to the complex topic of brown algae polysaccharides isolation and provide a fundamental platform that could help scientists further explore this broad field. Moreover, the presented guidelines can be used for industrial production and development of functional foods, nutraceuticals and cosmetic products based on algal polysaccharides.

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Autobiography

Ana Dobrinčić is a research assistant working in the research and higher education since 2018. She holds a Bachelor's degree in Food Technology and a Master's degree in Food Engineering from the Faculty of Food Technology and Biotechnology, University of Zagreb. During her studies, she spent 2 months at Management Center Innsbruck (Austria) as CEEPUS scholar and 5 months at Ghent University (Belgium) as ERASMUS + scholar. In 2017, she won the Rector's Award for her scientific work entitled "Effect of various olive leaf extraction methods on the total phenolic concentration and antioxidant capacity of the extract". She is finishing her Ph.D. study at the Faculty of Food Technology and Biotechnology, University of Zagreb, in the field of Food Technology. She has been employed as an associate at the Scientific Centre of Excellence for Marine Bioprospecting – BioProCro and project Bioprospecting of the Adriatic Sea. Her research is based on the extraction and analysis of the brown algal polysaccharides using conventional and advanced extraction techniques. Her research also involves topics of extraction and isolation of bioactive compounds from various plant materials and their implementation into functional foods using encapsulation methods. Until now, she co-authored 8 scientific papers in journals indexed in Web of Science/Current Contents Connect (134 citations; h-index is 4) and 1 scientific paper indexed in CAB Abstracts. She participated in many international and national congresses where she presented her research results and was awarded two times for the best poster presentation at international conferences. She has participated in the supervision of five final and eight diploma theses. She is a member of the Croatian Society of Food Technologists, Biotechnologists and Nutritionists.

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