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Abstract

Sulfated fucans from brown algae are a heterogeneous group of biologically active molecules. To learn more on their structure and to analyze and exploit their biological activities, there is a growing need to develop reliable and cost effective protocols for their preparation. In the present study, a brown alga *Pelvetia canaliculata* (Linnaeus) was used as a rich source of sulfated fucans. Sulfated fucan preparation methods included neutral and acidic extractions followed by purification with activated charcoal (AC), polyvinylpolypyrrolidone (PVPP), or cetylpyridinium chloride (CPC). Final products were compared in terms of yield, purity, monosaccharide composition and molecular weight. Acidic extractions provided higher yields compared to neutral ones, whereas the AC purification provided sulfated fucan products with the highest purity. Mass spectrometry analyses were done on oligosaccharides produced by the fucanase *Mf*FcnA from the marine bacterium *Mariniflexille fucanivorans.* This has provided unique insight into enzyme specificity and the structural characteristics of sulfated fucans.

Key words: sulfated fucans, brown algae, fucanase

1. Introduction

Brown algae (*Phaeophyceae*) are important primary producers in marine ecosystems, encompassing between 1500 and 2000 species, including kelps and numerous intertidal seaweeds. Besides their great ecological importance, they are also a significant and underexploited renewable resource of different biomolecules with industrial and pharmaceutical potential. A large part of these molecules are present in the brown algal cell wall, a dynamic structure forming a complex three-dimensional matrix which contains different polysaccharides including fucose-containing sulfated polysaccharides (FCSPs), alginate, cellulose, hemicelluloses (e.g. β-(1,3)-glucans, β-(1,3/1,4)-glucans), as well as phenolic compounds and proteins [1, 2]

FCSPs represent the second most abundant cell wall polysaccharide of brown algae after alginate. These complex polymers play important roles in maintaining cell wall structural integrity [3] and preventing algal dehydration [4].

Structurally, brown algal FCSPs are highly heterogeneous, complex, and rarely characterized in-depth [2, 3]. Extensive literature data on FCSPs show considerable variations in monosaccharide composition and structures among different brown algal species [3, 5-7]. FCSPs are divided into two main groups, according to the chemical composition of their polysaccharide backbone. Polysaccharides containing mainly fucopyranosyl residues in their backbones are referred to as sulfated fucans [3]. In *Laminariales*, the fucan backbone is built of (1,3)-linked α-L-fucopyranosyl residues bearing one or two sulfate groups on the fucose residues [8, 9]. In most *Fucales* species, the main fucan chain is made of alternating (1,3)-linked and (1,4)-linked α-L-fucopyranosyl residues bearing one or two sulfate groups on the fucose residues [10, 11]. Sulfated fucans can also be acetylated, and may contain side chains of different length, structure, and monosaccharide composition [3]. The second category of FCSPs are heteropolysaccharides named fucoidans. They have complex polysaccharide backbones, encompassing different monosaccharide residues and heterogeneous structures. Fucoidans can also be acetylated and may contain side chains [3] . Overall, brown algal FCSPs encompass a large spectrum of structurally and compositionally different polysaccharides, ranging from sulfated homofucans to branched and highly heterogeneous fucoidans.

Compared to other brown algae, *Pelvetia canaliculata* (*Linneus*) is shown to be a particularly rich source of FCSPs [6]. This common and edible brown macroalga can be found on many sea coasts of North and Western Europe, including coasts of Norway, Iceland, UK, Ireland, and Atlantic coasts of France, Spain and Portugal [12]. This abundant brown algal species might be considered as a safe and valuable renewable resource of FCSPs. *P. canaliculata*'s FCSPs show relatively high molecular weights (up to $1.7x10^6$ Da), and up to 40% sulfate in the chemical composition [13, 14]. They are mainly composed of L-fucose, but can contain significant amounts of xylose, galactose, mannose and glucose [6, 15]. When compared to other brown algae, *P. canaliculata*'s FCSPs have a relatively high fucose and sulfate content [7].

The marine bacterium *Mariniflexile fucanivorans* SW5 is a member of the *Bacteroidetes* phylum [16], which encompasses numerous polymer degraders in different ecosystems [17]. Marine Bacteroidetes usually produce highly specific polysaccharide-degrading enzymes, notably involved in algal polysaccharide catabolism [18, 19]. Notably, *M. fucanivorans* produces an endo-fucanase (*Mf*FcnA) which is active on *P. canaliculata* FCSPs and hydrolyzes the α-(1,4) backbone linkage [11]. This specific hydrolase is the founding member of the GH107 family and was previously employed to help structurally characterize FCSPs from *P. canaliculata* using ^{1}H-NMR. This demonstrated that the backbone of *P. canaliculata* FCSPs contains alternating (1,3)-linked and (1,4)-linked α-L-fucopyranosyl residues. Sulfate groups were detected at positions O2 and O3 on the fucose moieties [11]. Thus, these FCSPs from *P. canaliculata* are defined as sulfated fucans.

In the last two decades, there has been a growing interest in studying biological activities of brown algal FCSPs for potential pharmaceutical applications [3, 20]. There are numerous studies on their antitumor, antiviral, antioxidant, immunomodulatory and anticoagulant activities [21, 22]. However, the lack of complete information on their structure and the lack of controlled production of fucose-containing sulfated oligosaccharides (FCSOs), are obstacles in studying these biological activities [22, 23]. FCSP structure and chemical compositions vary among different brown algal species [3, 5, 6, 24]. Furthermore, differing degrees of purity of prepared FCSPs may also cause variations in bioactive properties [20, 24]. The absence of a defined universal method for their preparation [25] is therefore another obstacle in FCSP bioactivity research. Obtaining reproducible, highly pure, structurally unmodified polysaccharide is a necessary first step to the fine-structural characterization of FCSPs. This in

turn will lead to a fuller understanding of how these complex structures are related to their biological activities.

Literature reported FCSP extractions are most often performed in water or dilute acid, in the absence or presence of calcium ions, or in buffered solutions in the presence of enzymes [20, 21, 25]. Extractions are usually followed by purification steps due to the inherent presence of alginate, laminarin, and phenolic compounds in raw FCSP extracts, often including anion exchange chromatography or precipitation by cationic detergents [21, 23]. Nevertheless, to the best of our knowledge, none of these studies agree on one universal protocol for preparing FCSPs from brown algae. For quality control purposes, it is important to perform purity tests on final FCSP samples to detect contaminating glycans, phenolic compounds and proteins [7, 13].

The overall aim of this study was to propose a consistent and low cost protocol for obtaining brown algal sulfated fucans in high yield, with an emphasis on preserving fine-structural features for characterization by mass spectrometry. Due to the sulfated fucan variations between species, as well as seasonal variations among single species [5], a single batch of *P. canaliculata* was used to compare different preparation methods. Each of employed methods differed by only one step from at least two other employed methods (Fig. 1), in order to determine how each of the variables affects the quality of the final product. Methods were based on either acidic or neutral extractions and removal of common contaminating brown algal polysaccharides. Additional purifications were done by employing activated charcoal (AC), polyvinylpolypyrrolidone (PVPP), or cationic detergent cetylpyridinium chloride (CPC). The basic extraction method was adapted from the original studies published by Kloareg and coworkers [14, 26]. Purifications using AC or PVPP were introduced in an attempt to achieve higher purity using strategies which are already adopted in products for human consumption [27]. Prepared sulfated fucans were analyzed in terms of yield, purity, molecular weight (Mw), monosaccharide composition, degree of sulfation and digestion profiles introduced by the fucanase *Mf*FcnA from the fucanolytic marine bacterium *Mariniflexile fucanivorans* [11].

2. Material and methods

2.1. Collecting algae and preparing alcohol insoluble residue

Over 30 individuals of *P. canaliculata* were collected during low tide in mid-March 2019 from the rocky shore in Sibiril (48°41'24.7"N 4°04'46.3"W, North Atlantic English Channel coastline of Brittany, France) for use as starting material for testing different FCSP preparation methods. Algae were dried overnight at 50 ˚C in the oven under ventilation, ground to a fine powder using a blender to obtain higher surface to volume ratio and mixed well. Total algal powder was washed twice in 70% ethanol (1:15, w/v), followed by one acetone wash (1:15, w/v) to prepare alcohol insoluble residue (AIR) according to the modified protocol by Salmeán and coworkers [28]. The amount of AIR used per individual extraction corresponded to 10 g of dry and ground algae.

2.2. Sulfated fucan extraction methods

AIR was soaked in extraction liquid (1:15, w/v) during 75 min at RT prior to extraction. Extraction liquid was either water (neutral extraction), or water acidified with H_2SO_4 (pH 2.9), corresponding to a concentration of 0.6 mM (acidic extraction). Extractions were done for 30 min at 98 ˚C, under constant stirring followed by centrifugation (30 min, 14000 g, RT). The supernatant was collected as a raw sulfated fucan extract, whereas the remaining pellet was used for one re-extraction under the same conditions. Following the acidic sulfated fucan extractions, extracts were neutralized. Extractions (E) and re-extractions (R) were processed similarly and done in triplicate for each method (Table 1).

2.3. Removal of alginate from sulfated fucan extracts

The pH of the extractions and re-extractions was adjusted to 6.25. Afterwards, in all samples, 2M $CaCl_2$ was added slowly under constant stirring to reach final concentration of 2% $CaCl_2$. The alginate precipitate was removed by centrifugation (20 min, 14000 g, RT) and the supernatant was kept for further processing.

2.4. Removal of impurities from the extract using activated charcoal or polyvinylpolypyrrolidone

Following the removal of alginate, AC was added to samples of in methods 2 and 5, while PVPP was added to samples in methods 3 and 6. In both cases, AC and PVPP were used at a final concentration of 2% (w/v). Samples were incubated with AC or PVPP overnight at 10 ˚C and 180 rpm shaking. Afterwards, samples were centrifuged (20 min, 14000 g, 4 ˚C) and the pellets

were discarded. The supernatants were filtered using membranes with a 0.45 µm cut-off to remove remaining AC or PVPP particles.

2.5. Ethanol precipitation, removal of low molecular weight molecules and lyophilisation of sulfated fucans

In all samples, sulfated fucans were precipitated from extracts by adding 2 volumes of 96% ethanol and stored overnight at 4 ˚C. Precipitated FCSPs were collected after centrifugation (20 min, 14000 g, 4 ˚C) and dissolved in a minimal volume of water. With the exception of methods 7 and 8, the samples were then dialyzed against water using dialysis tubing with a cut-off of 12000-14000 Da (Sigma Aldrich). All samples were freeze dried. Dialysis in methods 7 and 8 was done after the detergent-based purification, as described in section 2.6.

2.6. Fractionation using cetylpyridinium chloride

Sulfated fucan lyophilized powder for methods 7 and 8 was dissolved in water (1:20, w/v) and an additional 0.5 volumes of 4 M $CaCl_2$ was added. Samples were centrifuged (30 min at 2000 g, RT), the pellets were discarded and an equal volume of 4% CPC was added to reach a final concentration of 2% CPC. Samples were incubated overnight at 30° C then centrifuged (20 min, 1600 g, RT). The supernatants were discarded and the pellets were collected. In order to remove remaining CPC, pellets were dissolved in minimal volume of 3 M $CaCl_2$ and sulfated fucans were precipitated by adding three volumes of absolute ethanol then collected as a pellet after centrifugation (20 min, 1600 g, RT). The removal of CPC by dissolving in minimal volume of 3 M $CaCl_2$ followed by ethanol precipitation of the sulfated fucans was repeated two more times. The FCSP-containing pellet was dissolved in a minimal volume of water and dialyzed against water using dialysis tubing with a cut-off of 12000-14000 Da (Sigma Aldrich), followed by freeze drying.

2.7. Carbohydrate polyacrylamide gel electrophoresis

Sulfated fucans and oligofucans were analyzed by carbohydrate-polyacrylamide gel electrophoresis (C-PAGE), according to the procedure of Zablakis and Perez [29]. Briefly, 0.2-2% (w/v) FCSP/FCSO samples were mixed with the loading buffer (10% sucrose and 0.08% (w/v) phenol red for visualization). Sulfated fucans and oligofucans were resolved on a 0.75 mm thick 6% (w/v) polyacrylamide (PAA) stacking, and 27% (w/v) PAA separating gel in 50 mM Tris-HCl and 2 mM EDTA buffer (pH 8.7) run at 200 V. The glycans were visualized using Alcian blue staining followed by silver nitrate staining [30].

2.8. Monosaccharide compositional analysis

Identification and quantification of neutral monosaccharide units were performed by gas-liquid chromatography (GC) after acidic hydrolysis. To obtain free monosaccharides, 5 mg of FCSPs were dissolved in 0.75 mL of 4 N trifluoroacetic acid (TFA) and incubated for 2 h at 121 °C. Monosaccharides were converted to alditol acetates according to Blakeney and coworkers [31] and chromatographed on a TG-225 GC (Thermo Scientific) column (30 x 0.32 mm ID) using TRACE Ultra Gas Chromatograph (Thermo Scientific) at 205 °C and H_2 as a carrier gas. Standard monosaccharide solutions and inositol were used as internal standards for calibration.

2.9. Determining total sulfate content

Sulfate content in sulfated fucans was determined by turbidimetric method [32]. To determine sulfate content FCSPs were dissolved in 1 M HCl in final concentration 2.5 mg/mL and hydrolyzed at 105 °C for 5 h. Following hydrolysis, 50 µL of each hydrolysate was mixed with 950 µL of 3% (w/v) trichloroacetic acid and 250 µL of barium chloride-gelatin reagent [0.5% (w/v) gelatin and 0.5% (w/v) barium chloride] to precipitate barium sulfate. Samples were incubated at RT for 15 min before measuring A_{360}. Blanks were prepared using 0.5% (w/v) gelatin instead of barium chloride-gelatin reagent. Concentrations of sulfate esters were determined according to a 0-10 mg/mL Na_2SO_4 calibration curve.

2.10. Determining the Mw of sulfated fucans

Molecular weights (Mw) of prepared sulfated fucans from *P. canaliculata* were determined by size-exclusion chromatography (SEC) coupled with multi angle light scattering (MALS). Prior to analysis, sulfated fucan samples were dissolved to 0.2% (w/v) concentration and filtered using

a 0.45 µm membrane. OHpak SB-G guard column (Shodex) was used as a pre-column and SEC was performed using OHpak SB-805 HQ column (Shodex) with the target molecular range 100000 - 1000000 Da followed by OHpak SB-804 HQ column (Shodex) with the target molecular range 5000 - 400000 Da, followed by OHpak SB-803 HQ column (Shodex) with the target molecular range 1000 - 100000 Da. Columns were connected in series to the UltiMate 3000 (Thermo Scientific) chromatography system. SEC was carried out using 0.1 M $LiNO_3$ containing NaN_3 4.6 µM as the mobile phase with a 0.5 mL/min flow rate. Resolved polysaccharides were directed to the MALS detector (Dawn Heleos, Wyatt), followed by a refractive index detector (Optilab Rex, Wyatt). Collected data were analyzed by Astra (Wyatt) software.

2.11. Determination of total phenolic content

Phenolic content was determined based on the reaction of phenolic compounds with the Folin-Ciocalteu's (FC) [33] reagent in the presence of sodium carbonate, resulting in a blue colored complex. In brief, 60 µL of the sulfated fucan sample was dissolved in 900 µL MiliQ H_2O, followed by addition of 60 µL of FC reagent. The reaction mixture was vortexed and left for 5 min at RT. 300 µL of 20% (w/v) Na-carbonate was then added. The reaction mixture was vortexed again and incubated at 30˚ C for 2 h. Phenolic content was determined by measuring the absorbance of the sample solution at 765 nm. Concentrations of phenolic compounds were determined according to 0.04-0.20 mg/mL gallic acid calibration curve.

2.12. Determining protein content

A modified Bradford's method was used [34] to determine protein contamination of prepared sulfated fucans. Bradford's reagent was prepared by dissolving 0.025% (w/v) Coomassie brilliant blue g-250 stain in 5.0% (v/v) ethanol and 8.5% (v/v) orthophosphoric acid. A standard curve for determining protein concentration was determined using bovine serum albumin, in ranges 0.01-0.10 mg/mL. FCSP samples were dissolved and analyzed in concentration of 50 mg/mL and detection was done after mixing 100 µL of each sample with 1 mL of the Bradford's reagent followed by measuring A_{595nm}.

2.13. Cloning of *Mf*FcnA from *M. fucanivorans*

The *fcn*A gene [11] from *M. fucanivorans* SW5 [16] encoding a GH107 endofucanase (locus identifier: Mfuc_340464, GenBankTM accession number AJ877239) was cloned as described

by Groisillier and coworkers [35]. Briefly, primers were designed to amplify the coding region corresponding to the family GH107 catalytic module of *Mf*FcnA with the insertion of a stop codon at the 3' end (forward primer 5'-AAAAAAAGATCTCAAGTACCAGATCCAAACCAAGGA-3', reverse primer 5'-TTTTTTCAATTGTTAATTTCCATCACTAATAATAGTTGCAATT-3'). The gene fragment was amplified by PCR from *M. fucanivorans* SW5 genomic DNA. After digestion with the restriction enzymes BglII and MfeI, the purified PCR product was ligated using the T4 DNA ligase into the pFO4 expression vector [35] linearized with BamHI and EcoRI. This plasmid, after sequencing, was subsequently transformed into *E. coli* DH5α strain for storage and in *E. coli* BL21 (DE3) strain for recombinant protein production with an N-terminal hexa-histidine tag (see below).

2.14. Production of *Mf*FcnA from *M. fucanivorans* and of *Zg*LamA and *Zg*AlyA1 from *Zobellia galactanivorans*

To produce *Mf*FcnA (Uniprot accession number Q08I46_9FLAO), *E. coli* BL21 (DE3) cells harboring the *fcn*A gene in the pFO4 vector were cultivated shaking at 180 rpm and 20°C in 1L auto-induction ZYP 5052 medium supplemented with 100 µg/ml ampicillin to produce His-tagged *Mf*FcnA. All cultures were harvested after 72 hours by centrifugation at 8000 g for 15 minutes. Collected cells were chemically lysed as previously described [36]. Imidazole was added to the clarified lysate at a final concentration of 20 mM. Cell lysate was applied onto an immobilized metal-affinity resin column (IMAC) His Gravitrap (GE Healthcare, V=5 mL) equilibrated in binding buffer (20 mM Tris–HCl, 500 mM NaCl, 20 mM Imidazole, pH 7.5). After washing with binding buffer to remove contaminants, bound proteins were eluted using a linear gradient to 100% of elution buffer (20 mM Tris–HCl, 500 mM NaCl, 500 mM Imidazole, pH 7.5). IMAC purified protein was additionally chromatographically purified with the Superdex 200 (V=125 mL) size exclusion column using an isocratic gradient (20 mM Tris–HCl, 500 mM NaCl, pH 7.5). Purity of the protein was confirmed using 12% SDS PAGE. The production and purifications of the laminarinase A *(Zg*LamA) and the alginate lyase A1 (*Zg*AlyA1) from *Z. galactanivorans* were done as previously described [37, 38].

2.15. Enzymatic digestions

2.15.1. Detection of β-(1,3)- and β-(1,4)-glucans

The dried FCSP samples, prepared by the 8 different methods, were dissolved in 100 mM NaCl and 10 mM Tris buffer (pH 7.5) to a final concentration of 2.5% (w/v). Laminarinase from *Z. galactanivorans* (*Zg*LamA), and commercial cellulase Onozuka R-10 (Yakult, Japan) were used for determining presence of β-(1,3)-glucans and β-(1,4)-glucans, respectively. Each sample was digested with the corresponding enzyme (final concentration 0.1 mg/mL) over 72 hours at RT. Reactions were stopped and visualized by adding equal volume of di-nitrosalycilic acid reagent (DNS) [39], and treated at 95 °C for 5 min. Afterwards, 4 volumes of MilliQ water were added and absorbance at λ=540 nm was measured. The analysis was done in triplicate. A standard curve for maltose was used to determine the concentration of reducing sugars.

2.15.2. Detection of alginate

All sulfated fucans, prepared by the 8 different methods, were dissolved in 100 mM NaCl and 10 mM Tris (pH 7.5) to a final concentration 2% (w/v). *Zg*AlyA1 from *Z. galactanivorans* was added to each sample to a final concentration of 0.1 mg/mL and the samples were incubated overnight at RT. After the digestion was stopped, oligosaccharides released were analyzed by 27% PAA (w/v) C-PAGE. Released reducing sugars were also analyzed using DNS reagent (described in section 2.15.1).

2.15.3. Preparation of oligofucans using *Mf*FcnA

Samples of sulfated fucans, prepared by the 8 different methods, were dissolved in 50 mM ammonium-bicarbonate to a final concentration of 2% (w/v). *Mf*FcnA was added to each sample to a final concentration of 0.005 mg/mL and the samples were incubated overnight at RT. Digestion was stopped by thermal treatment at 95 ˚C over 10 min.

2.16. Characterization of enzymatic products by ultra-high performance liquid chromatography coupled with mass spectrometry

UHLC-MS analysis of each sulfated fucan sample and its enzymatic digest was performed to identify the produced oligosaccharide species. Ion-pair reversed-phase (IP-RP) separation was done on an ultra-high performance liquid chromatography system (UHPLC, Acquity H-Class plus, Waters, Wilmslow, UK), equipped with a BEH C18 column (100 mm x 1 mm) (Waters, Wilmslow, UK) under 0.15 mL/min flow rate at 45 °C. A ternary gradient was used with A, pure water; B, pure acetonitrile; and C, 20 mM Hexylammonium acetate (HxA) in water (pH value adjusted to 6 by addition of acetic acid). The gradient was from 16.6% to 35.0% of solvent B

in first 10 min, then up to 63.4% at 20 min and maintained at 73.4% for 4.5 min. Solvent C was kept constant at 25.0% [40].

Acquisitions were performed through direct coupling of the UHPLC system with a Select Series Cyclic IMS mass spectrometer (Waters, Wilmslow, UK) (Giles et al., 2019). Spectra were acquired in negative Electrospray ionization (ESI) mode on the m/z range 300 – 2000, with the TOF (Time-of-flight) analyzer operating in the V-mode. The source parameters were the following: Capillary voltage 2.5 kV; Cone Voltage: 40 V; Source temperature: 100 °C; Desolvation temperature: 280 °C; Desolvation gas: 500 L/hour; Nebulization gas: 5.5 bar. Data were recorded with the Quartz software (Waters embedded software, release 5). Data were processed using Mass Lynx 4.2 (Waters, Wilmslow, UK).

2.17. Determining antioxidant capacity

To determine the antioxidant capacity of sulfated fucan samples, an assay based on using the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH, Sigma Aldrich) was employed according to the modified protocol by Brand-Williams and coworkers [41]. Samples were dissolved in MilliQ water to concentrations of 0.05 and 0.5 mg/mL, and analyzed in triplicate. The assay was done in a 96-well plate by adding 20 µL of samples to 180 µL of 15 µM DPPH in 90% methanol (v/v). After 30 min incubation in the dark at RT the absorbance at 517 nm was measured. Radical scavenging capacity was calculated according to a 0-500 µM resveratrol calibration curve and water was used as negative control. To evaluate significance of measured radical scavenging capacities, GraphPad t-test calculator was used and samples were compared to a negative control. The difference was considered statistically significant at $p < 0.05$.

2.18. Statistical analysis

All experiments were conducted in triplicate and measurement data are expressed as standard deviation from the mean (SD). The statistical difference among results was analyzed by one-way Analysis of Variance (ANOVA, $p<0.05$ declared significant) and by multiple comparisons using Tukey's test ($p<0.05$ declared significant) for pairwise comparison of means where applicable. Determination of parameters which significantly contributed to the observed differences were done using multi-way ANOVA ($p<0.05$ declared significant). ANOVA and Tukey's test analyses were performed using R software and the ggplot2 package to produce figures [42]. To evaluate significance of measured radical scavenging capacities,

GraphPad Prism 6.0 t-test calculator was used and samples were compared to negative control ($p<0.05$ declared significant). The same software was used to produce figure on determined radical scavenging activity.

3. Results

3.1. Yields of prepared sulfated fucans

For each sulfated fucan preparation, yields were determined in triplicate and expressed as percentages of dry weight. Method 4 (acidic extraction without AC, PVPP or CPC steps, Table 1) provided the highest average yield (14.6% ± 1.1), while method 1 (neutral extraction without AC, PVPP or CPC steps, Table 1) provided an average yield of 12.6% ± 0.3. AC, PVPP and CPC decreased the yields when compared to the corresponding methods without these additional purification steps 3 (Fig. 2 and Table S1).

A single factor analysis of variance (ANOVA) confirmed the statistical significance of differences between the yields obtained by the employed methods at 95% confidence level with ***p=1.3E-7 (*p<0.05, **p<0.033 and ***p<0.01). Relatively higher yields were registered in all acidic extractions (Fig. 2 and Table S1 Method 4, 5, 6 and 8) compared to their corresponding neutral extractions using only water (Fig. 2 and Table S1 Method 1, 2, 3 and 7). Overall, acidic extractions provided significantly higher yields when compared to the neutral extractions (Fig. 2 and Table S1). Multi-way ANOVA confirmed that neutral or acidic extractions significantly contributed to yield differences (***p=3.8E-5). Furthermore, AC, PVPP or CPC also significantly contributed to yield differences (***p=2.8E-8). A pairwise comparison of the means at 95% confidence level is shown in Table S2 and Fig. S1, providing details on the significance between FCSP yields between each method.

3.2. Monosaccharide compositional analysis

Monosaccharide compositional analysis was employed to quantitatively determine presence of different neutral monosaccharide units and express them as percentages of dry weight in dried algal material, AIR and purified sulfated fucans. The most abundant neutral monosaccharide unit in dried algal material, AIR and purified sulfated fucans was fucose (Table 2). Besides fucose, the other monosaccharide units detected were galactose, mannose, xylose,

and glucose (Table 2). The least abundant monosaccharide component was glucose, this was especially pronounced in the sulfated fucan samples prepared by methods 2 and 5 which included an AC purification step. As a general trend, the R fractions contain more fucose (Fig. S2) and less glucose compared to the E fractions (Table 2).

Dried algal material and AIR contained less fucose compared to the purified sulfated fucans, and more glucose. Dried algal material contained a higher percentage of mannose monosaccharide units comparing to AIR and purified sulfated fucans (Table 2). Obtained differences in monosaccharide composition were statistically significant with ***p=4.1E-4 for fucose, ***p=2.3E-4 for xylose, ***p=1.1E-6 for mannose, *p=4.6E-3 for galactose and ***p=5.8E-9 for glucose mean values in sulfated fucans prepared by different methods.

3.3. Sulfate analysis

Sulfate group analyses were done using the turbidimetric method [32] and sulfates were detected in all analyzed samples. The degree of sulfation of each sulfated fucan sample is presented in Fig. 3 and Table S3 as percentages of dry weight. Interestingly, the R fractions contain more sulfate groups than the E fractions, which is in correlation with their fucose content (Fig. S2 and Table 2). Both the AC purification step as well as CPC fractionation lead to enrichment of sulfate group content in purified sulfated fucans. Statistical analysis showed that whether neutral or acidic extractions were used did not significantly contribute to the differences in sulfate content (p=4.9E-1), while the usage of AC, PVPP or CPC did significantly contribute to differences in sulfate content of FCSPs obtained (**p=1.1E-2).

3.4. Mw analysis

MALS-SEC analysis was employed to obtain data on molecular weight and heterogeneity of the prepared sulfated fucans. The MALS output indicates that FCSPs from *P. canaliculata* have high $\overline{Mw}$ values, as well as high heterogeneity which is represented as $\overline{Mw}/\overline{Mn}$ in Table 3.

Regarding the differences between analyzed sulfated fucan samples, R samples obtained by re-extraction show lower $\overline{Mw}$ compared to E samples. R samples also show higher heterogeneity compared to the E samples.

3.5. Purity of prepared sulfated fucans

In order to evaluate the purity of prepared sulfated fucans, the presence of alginate, β-(1,4)-glucans, β-(1,3)-glucans, proteins and phenols were analyzed. Results are summarized in Table 4. Since marine polysaccharide-degrading enzymes are highly specific, they can be employed for the characterization of the structure and/or the purity of complex marine polysaccharides. Besides the *Mariniflexile fucanivorans* fucanase *Mf*FcnA which was used in sulfated fucan structural analyses (below), enzymes from another marine model Bacteroidetes *Zobellia galactanivorans* [18] were used to analyze the purity of obtained FCSPs. The *Z. galactanivorans* alginate lyase *Zg*AlyA1 [36] and laminarinase *Zg*LamA [37] are valuable enzymatic tools for detecting alginate and β-(1,3)-glucan contamination in prepared sulfated fucan samples, respectively.

3.5.1. Presence of β-glucans

Enzymatic digests of the sulfated fucans prepared using the β-(1,4)-glucanase did not provide detectable reducing sugars using the DNS reducing sugar assay. The limit of detection of reducing sugars was determined to be 0.4% (w/w) as determined using a maltose standard curve relative to 5.0% (w/v) of dissolved sulfated fucans. The presence of β-(1,4)-glucans was thus under the limit of detection. After digestion with the *Z. galactanivorans* laminarinase *Zg*LamA β-(1,3)-glucans were detected in E fractions of sulfated fucans prepared by methods 1, 3, 4 and 6 in concentrations below 1% (w/w) of dry weights. No β-(1,3)-glucans were detected in samples prepared by methodologies 2, 5, 7 and 8. The limit of detection of reducing sugars was determined to be 0.4% (w/w) as determined using a maltose standard curve relative to 5.0% (w/v) of dissolved sulfated fucans (Table 4).

3.5.2. Presence of alginate

To determine possible presence of alginate, prepared sulfated fucans were incubated with the alginate lyase *Zg*AlyA1 [36] from *Z. galactanivorans* and analyzed using C-PAGE (Fig. 4) and the DNS reducing sugar assay. Digestion of FCSP samples by *Zg*AlyA1 did not produce detectable oligosaccharides or reducing sugars and therefore the presence of alginate was below the limit of the detection using these methods.

3.5.3. Phenolic content

Phenolic content determination, using gallic acid as a concentration standard, showed that the prepared sulfated fucans contain minor contamination with phenolic compounds (Fig. S3 and Table 4). Data are expressed as percentages of dry weight. A maximum of phenolic compounds was detected in Method 3 E at 1.2% (SD 0.1%). The lowest presence of phenolic compounds was detected in samples treated with AC. Statistical analysis confirmed significant differences between phenolic contents in FCSPs obtained by different methods (***p=3.8E-11). Whether neutral or acidic extractions were used contributed to the differences in phenolic content (*p=1.2E-2), as well as whether AC, PVPP or CPC were used (***p=1.7E-12). A detailed pairwise comparison of the means at 95% confidence level is shown in Table S4.

3.5.4. Protein content

Protein content was analyzed by the Bradford method on 5.0% (w/v) sulfated fucan solutions and expressed as percentages of dry weight. The minor presence of proteins (≤0.3% w/w) was detected in E samples obtained by methods 1 and 4. Proteins were not detected in any of the sulfated fucan fractions prepared by methods which utilized AC, PVPP or CPC purification steps. Protein content was below the limit of detection in all R samples (Table 4). Due to the lower limit of the detection method, it was not possible to measure contamination below 0.2% (w/w) in the samples using the BSA standard curve.

3.6. Enzymatic digestion of sulfated fucans with *Mf*FcnA

Samples digested by *Mf*FcnA were analyzed in order to compare the products of digestion. All prepared sulfated fucan samples gave oligosaccharides when digested with *Mf*FcnA and provided similar C-PAGE profiles but with different intensities of LMW bands (Fig. 5). The C-PAGE results are in agreement with the UHPLC MS analysis results, confirming the highest digestibility of sulfated fucan samples prepared by methodologies 7 and 8 which included a CPC fractionation step. LC-MS indicates the majority of the detected species released by *Mf*FcnA were sulfated oligofucans. The most abundant species detected by LC-MS was a tetrasaccharide containing four fucose units and six sulfate groups [4Fuc+6($-SO_4^-$)], followed by a hexasaccharide containing six fucose units and nine sulfate groups [6Fuc+9($-SO_4^-$)], and next an octasaccharide with eight fucose units and twelve sulfate groups [8Fuc+12($-SO_4^-$)] (Table 5, Fig. 6). The pattern in these most abundant species is n[2Fuc+3($-SO_4^-$)], with n as a

whole number between 1 and 5. Less abundant species which do not follow this pattern were detected, and they have also been identified as sulfated oligofucans (Table 5).

Oligosaccharide species containing monosaccharide units other than fucose were also detected (Table 6 and Table 7). Table 6 shows the main produced oligosaccharide species containing a hexose other than fucose and Table 7 shows the main oligosaccharide species containing a pentose. The observed patterns among the oligofucans shown in Tables 8 and 9 indicate that all major oligofucans which contain a hexose other than fucose have an even number of monosaccharide units, whereas oligofucans which contain a pentose have an odd number of monosaccharide units.

3.7. Antioxidant capacity

Compared to the negative control (H_2O), no significant antioxidant capacity was detected in the first extraction of analyzed FCSP samples using DPPH (E samples, Fig. 7). In contrast, a statistically significant antioxidant activity was detected in some samples obtained by re-extraction (R samples, Fig. 7). Nonetheless, these antioxidant activities are inferior to that of the lowest concentration of Resveratrol (positive control) and thus are relatively weak

4. Discussion

A single lot of the brown alga *P. canaliculata* (Linnaeus) was used for comparison of sulfated fucan extraction methodologies in consideration of expected seasonal and individual variations in sulfated fucan content [5]. Therefore, the differences in quality between the final extracted products depend only on the preparation method. Cell wall phenolic compounds have high affinity for sulfated fucans and alginates and adsorb to them during extractions. Thus, the first preparative steps aimed to remove significant amounts of phenolic compounds prior to sulfated fucan extraction. This pre-treatment was done according to modified protocol of Salmeán and coworkers [28], based on usage of ethanol and acetone washes. Ethanol and acetone are frequently used in removing pigments and lipid compounds from different brown algal material since FCSPs are not soluble in these solvents and remain in the solid algal material due to their high polarity. The resulting washed product is referred to as AIR (alcohol insoluble residue). In terms of monosaccharide composition (Table 2), AIR was enriched in fucose content relative to the dried algal powder. The monosaccharide

compositional analysis also confirmed that the AIR contained significantly lower mannose/mannitol content relative to the dried algal powder. The high abundance of mannose in algal powder might be due to its roles as precursor for extracellular matrix polysaccharide synthesis [43] or because mannitol is a carbon storage compound in brown algae [44]. In any case, the sulfated fucan samples were expected to remain insoluble during the ethanol and acetone wash [28] which is supported by the fucose enrichment after this step (Table 2).

In this study extractions were done in water or in dilute acid, which are the most common conditions used for high yield extraction of sulfated fucans from brown algae [45]. Besides the importance of choosing the suitable extraction solvent, it is important to consider other options such as the use of enzymatic treatments or different physical treatments such as heating with mechanical stirring, microwaves or even ultrasounds [20, 25]. Enzymes increase the cost of the extraction and, for industry, it is important to have cost effective methods with the potential for scaling-up. Sulfated fucans are stable at a wide temperature range in water based extractions and higher temperatures and multiple extractions have been shown to provide higher yields [45]. For these reasons, extractions in the present study were done twice on each sample (E and R), using high temperature and mechanical stirring. Extraction temperature, time and stirring parameters were the same for each method tested to compare the effects of the different purification steps on sulfated fucans preparation.

Acidic extractions provided higher yields compared to the neutral ones for every analyzed combination of methods. One possible explanation, as discussed by Hahn and coworkers [45] is that protons disrupt the hydrogen bonds between the polysaccharides in the cell wall matrix, promoting sulfated fucan release into solution and increasing yield in the acidic extractions. Nevertheless, acidic extraction conditions should be carefully designed not to hydrolyze the target polysaccharides. Glycosidic linkages of desulfated fucose residues are more susceptible to acidic cleavage than sulfated fucose residues and hydrolysis is directly influenced by sulfation structure [46]. Dilute acid has previously been used to obtain HMW sulfated fucans containing 30-40% (w/w) sulfate content from *P. canaliculata* [13]. Sulfate hydrolysis by H_2SO_4 in concentrations under 1 M [13] was only very limited, providing additional support for this approach. Accordingly, the acid extracted FCSPs from our study remained highly sulfated (≥36%, w/w) and the 0.6 mM H_2SO_4 concentration is unlikely to have

led to significant sulfated fucan hydrolysis - which was confirmed by the MALS-SEC Mw analyses (Table 3). Overall, these data support that sulfated fucans are not highly susceptible to acidic hydrolysis in the conditions used in this study.

Alginate is a commonly described contaminant of sulfated fucans [24]. This polysaccharide is highly polar and it is therefore co-extracted with sulfated fucans in water. One option to prevent alginate co-extraction with sulfated fucans, is to extract aqueously in the presence of Ca^{2+} ions; however, these extractions may provide lower sulfated fucan yields compared to simple water and dilute acid extractions [45]. Alginate forms a gel in the presence of Ca^{2+} ions and we suggest this may 'trap' the sulfated fucans in the cell wall matrix during the extraction. In order to avoid a lower sulfated fucan yield, we precipitated the alginate in a separate step after water or dilute acid extraction by the addition of Ca^{2+} ions to the extracts. The volumes of the extractions were significantly higher relative to the volume of original algal material; thus the alginate was also diluted relative to the original algal material. We postulate that the "trapping-effect" on sulfated fucans by the diluted alginate-Ca^{2+} matrix should be decreased by doing the alginate precipitation step after the extraction. There was no alginate detected in any of the final sulfated fucan samples indicating that this approach was successful in removing alginate from the sulfated fucan extracts.

PVPP and AC are common compounds used in purification of different molecules including products for human consumption [27]. Their relative low cost and their re-usability make them useful for both small-scale and large-scale purifications. AC has been previously used successfully in the purification of sulfated fucan fractions from the brown alga *Fucus vesiculosus* [47]. Here, both AC and PVPP were used for adsorbing impurities such as phenolic compounds from our extracts. Aside from being efficient in removal of phenolic compounds, our results show that AC contributed significantly to the removal of β-(1,3)-glucans (Table 4 and Fig. S3). In contrast, the contribution of PVPP in removing β-(1,3)-glucans and phenolic compounds was not significant under the tested conditions (Table 4 and Fig. S3).

Highly sulfated FCSPs can be purified using cationic detergents such as CPC or CTAB, which efficiently precipitate poly-anionic compounds [14, 45]. Methods 7 and 8 use CPC after neutral or dilute acidic extraction, respectively. Following CPC treatment, the detergent was removed from the sulfated fucan precipitate by washing in ethanol with 3 M $CaCl_2$. The removal step had to be repeated to ensure complete removal of the detergent from the sample. This

repetitive detergent removal probably affected the FCSP yield (Table S1). Another factor which could contribute to the lower sulfated fucan yields in Methods 7 and 8 using CPC is the potential loss of less sulfated or LMW sulfated fucans, which would not be precipitated by this polycationic detergent. Statistical analysis has indeed confirmed that CPC treatment significantly contributes to the differences in yields.

The ethanol precipitation of the sulfated fucans was included to reduce the sample volume and to aid in the removal of any remaining pigments or other contaminants. This step also removes Ca^{2+} ions used in alginate precipitation as $CaCl_2$ is soluble in 70% (w/w) ethanol. β-(1,3)-glucans from *P. canaliculata* have significantly lower molecular weights (MW is reported as 2-7*10^3 Da) [48] relative to the sulfated fucans, which are two order of magnitudes larger (Table 3). Thus, a final purification step to remove these smaller polysaccharides was done using dialysis (MWCO 12000-14000 Da) against deionized water. This step also helps remove any remaining LMW contaminants and oligosaccharides. Freeze-drying after the water dialysis provided high-purity, dry and ready-to-use HMW sulfated fucans.

The final sulfated fucan product purity is imperative for in-depth characterization and structural analysis, as well as for potential applications in human and veterinary health care. The laminarinase *Zg*LamA [37] and the endo-guluronate lyase *Zg*AlyA1 [38] from *Z. galactanivorans* were used as valuable enzymatic tools for determining the presence of contaminating β-(1,3)-glucans and alginate, respectively, in the prepared sulfated fucan samples. Alginate and hemicellulose contamination was under the limit of detection in all sulfated fucans prepared; however, sulfated fucans prepared by methods 1, 3, 4, and 6 contained some β-(1,3)-glucans (≤1%, w/w). AC (methods 2 and 5) and CPC (methods 7 and 8) were successful in removal of β-(1,3)-glucans (Table 4). Particularly, method 5 gave high yields and was only moderately time consuming. Since AC is non-toxic and can be easily removed from the solution in a single step by centrifugation or filtration, method 5 is thus a good choice for preparing high purity sulfated fucans for in-depth characterization and structural analysis, and potentially for commercial use.

Due to the tendency of phenolic compounds to associate with FCSPs from brown algae, their concentration in each sample was also examined. Phenolic compounds are present in the prepared sulfated fucan samples, but only in very low concentration (≤1.2% (w/w), Fig. S3 and Table 4). Here, methods using AC were the most successful in removal of phenolic compounds

(Fig. S3 and Table 4) which was confirmed to be statistically significant (Fig. S4 and Table S4). Antioxidant capacity of brown algal FCSP extracts is often stated as one of their bioactive properties [49], but there is conflicting data showing that anti-oxidative capacity correlates with the presence of co-extracted polyphenols [24]. In the present study, none of the sulfated fucan E samples showed significant antioxidant activity (Fig. 7). The higher concentration samples of R samples (0.5 mg/mL) for methods 2 - 7 did demonstrate a statically significant amount of anti-oxidative activity compared to the negative control (H_2O), but these anti-oxidative activities remains weak relatively to the positive control (resveratrol). These results are consistent with the work of Bittkau and coworkers which suggest that sulfated fucans have weak or no anti-oxidative activity and that previous claims of anti-oxidative activity were mainly due to contaminations with polyphenolic compounds [24]. This also highlights the high degree of purity of the sulfated fucans obtained by our extraction methods.

There is minor protein contamination in E samples obtained by methods 1 and 4 (Table 4), in fact protein content was detected only in sulfated fucans obtained by the first extraction, and not purified by AC, PVPP nor CPC. We hypothesize that the high temperature during the extraction (98 ˚C) might thermally denature most proteins resulting in a decreased solubility. These denatured proteins would be removed during the raw extract clarification.

Reported Mw of sulfated fucans from *P. canaliculata* vary widely between 3 *10^4 Da up to 1.7 *10^6 Da [13, 14]. Data obtained in our study show average molecular weight $\overline{Mw}$ values which are comparable with the previously reported values (between 5.18 *10^5 Da and 8.78 *10^5 Da, Table 3). Surprisingly, R samples (second extraction) show lower $\overline{Mw}$ relative to E samples (first extraction). R samples also show higher polydispersity ($\overline{Mw}/\overline{Mn}$) compared to the E samples. We tend to exclude the possibility that the sulfation content contributed to these differences since the sulfate percentages of E and R samples are similar (Table S3). Sulfated fucans are highly heterogeneous biological samples, thus it is not entirely surprising to have high polydispersity indexes. If a less polydisperse sample is required, the lowest $\overline{Mw}/\overline{Mn}$ values were observed among the E samples prepared by methods 7 and 8 (Table 3), which both include a fractionation step with the cationic detergent CPC (Table 1).

Monosaccharide compositional analysis data showed that the extracted sulfated fucans contain fucose, xylose, galactose, mannose, and glucose (Fig. S2 and Table 3) which is in agreement with the available literature [6, 7, 15]. As expected, the most abundant

monosaccharide unit in all extracts was fucose. All R samples showed higher fucose content when compared to their respective E samples. As expected for sulfated fucans [15], the fucose composition also correlated with the sulfation percentages (Fig. 3, Table S3). The hexose moieties of the oligosaccharides released by *Mf*FcnA (Table 6) remain unidentified, though based on the compositional analyses (Table 3) these could potentially be galactose, mannose or glucose (discussed below). The pentose moiety on the oligosaccharides released by MfFcnA is likely to be xylose (discussed below).

The NMR study performed by Colin and coworkers showed that the endofucanase *Mf*FcnA hydrolyzes the α-(1,4) glycosidic linkages within blocks of repeating motifs [→4)-α-L-fucopyranosyl-2,3-disulfate-(1,3)-α-L-fucopyranosyl-2-sulfate-(1→]n in *P. canaliculata* sulfated fucans [11]. However, this previous study did not provide information on how monosaccharide units other than fucose are organized, nor information on the *Mf*FcnA recalcitrant sulfated fucan fraction, nor of any branching. Thus, the mass spectrometry data presented here provides additional insight into the structure of *P. canaliculata* sulfated fucans.

According to our C-PAGE analysis of oligofucans (Fig. 5), a significant part of the *P. canaliculata* sulfated fucans remained undigested after treatment with *Mf*FcnA. This recalcitrance to digestion could be due to branching patterns, acetylation, sulfation patterns and backbone stretches with different repetitive motifs. Among the sulfated fucans prepared in the present study, samples obtained by methods 7 and 8 demonstrated the highest digestibility by fucanase *Mf*FcnA both according to the intensities of oligofucan bands in the C-PAGE analysis (Fig. 5) and according to the intensities of detected oligofucans in UHPLC-MS analysis (Table 5). The UHPLC-MS data provides a full and diverse characterization of a panel of novel released oligofucans for each extraction and re-extraction (E and R) (Table 5-7). *Mf*FcnA mainly releases structures made of n[2Fuc+3(-SO_4^-)] with 1≤n≤5 (Table 5). The highest intensity oligosaccharide species are represented in the Table 5 dataset with the pattern n[2Fuc+3(-SO_4^-)], such as previously described as being released by *Mf*FcnA [11]. These include the oligosaccharides DP4 2[2Fuc+3(-SO_4^-)], DP6 3[2Fuc+3(-SO_4^-)] and DP8 4[2Fuc+3(-SO_4^-)] (Table 5). The smallest oligosaccharide released is 2Fuc+3(-SO_4^-). There are also main oligosaccharide species released by *Mf*FcnA that do not represent the known pattern [11], such as DP4 [4Fuc + 5(-SO_4^-)] and DP6 [6Fuc +7(-SO_4^-)], indicating that all fucose hydroxyl groups do not need to be sulfated for *Mf*FcnA substrate recognition and hydrolysis.

There were also less abundant fucose-containing oligosaccharide species released by *Mf*FcnA that contain either an unidentified hexose or pentose monosaccharide (Table 6 and Table 7, respectively). The oligosaccharide species with hexoses are all even numbered - mainly DP10 with varying degrees of sulfations but also a DP12 (Table 6). As the oligosaccharides containing only hexose monosaccharide units are even numbered, and the sulfated fucans of *P. canaliculata* have repeating disaccharide units of alternating α-(1,3) and α-(1,4) linkages, this might indicate either the presence of hexose units other than fucose in the oligosaccharide main chain of *P. canaliculata* or that these hexose units are part of a specific branching pattern (Table 6). Smaller oligosaccharides containing hexoses were not identified which suggests the hexose units block hydrolysis by *Mf*FcnA. The hexose species released by *Mf*FcnA were most prominent in methods 4, 6, 7 and 8 (Table 6). According to the monosaccharide composition analysis (Table 2), the unidentified hexose is probably galactose, mannose or glucose. This is in good agreement with literature data which show that some brown algal FCSPs named galactofucans contain both galactose and mannose [7, 20].

The pentose-containing oligosaccharide species released by *Mf*FcnA are most prominent in methods 6, 7 and 8 (Table 7). These pentose-containing species are all odd numbered oligosaccharides between DP7 to DP11 with varying degrees of sulfation (Table 7). Since *P. canaliculata* has repeating disaccharide units of alternating α-(1,3) and α-(1,4) linkages, this suggests that there are pentose branches on the oligosaccharides and that *Mf*FcnA is thus likely capable of releasing branched oligosaccharides (Table 7). According to the monosaccharide compositional analysis, the only pentose detected in the samples was xylose (Table 3) making this monosaccharide the likely pentose found in oligofucans. Methods 7 and 8 had a lower xylose content compared to the sulfated fucans obtained by other methods (Table 3); however, unexpectedly methods 7 and 8 also had more oligosaccharide species containing a pentose. This difference might be explained by the structural organization of the pentose groups in the sulfated fucans. For example, the oligosaccharides released by *Mf*FcnA containing pentose (Table 7) all have only one pentose group. These branches are likely to block further degradation by *Mf*FcnA as the smallest DP is DP7 (Table 7) whereas in the main species the smallest oligosaccharide released is a DP2 (Table 5). In turn, the additional xylose residues detected by the compositional analysis in the sulfated fucans in methods 1-6 may be

more concentrated in areas of the polymer, thereby blocking the action of *Mf*FcnA and resulting in less pentose-containing oligosaccharides relative to methods 7 and 8.

Conclusions

These sulfated fucan extraction and purification methods were designed for comparison with one another as each method differed from at least two other methods in not more than one preparation step. The purpose of this approach was to highlight the importance and effect of each preparation step. Besides being successful in obtaining high yields and high purity sulfated fucans, the extraction methods are relatively simple and have the potential to be industrially up-scaled in a cost-effective manner. Sulfated fucan extractions in dilute acid are more efficient compared to the extractions in water (Fig. 2, Fig. S1 and Table S1). The simplest method with the highest yield is the acidic extraction method 4 (Fig. 2, Fig. S1 and Table S1). This method provides sulfated fucans of high diversity (Table 3) with relatively low contamination (Table 4), while requiring less purification steps compared to other methods. For production of oligosaccharides with *Mf*FcnA we highlight method 8 using CPC (Table 5-7), though there seems to be some loss of sulfated fucan diversity (Table 3). Finally, we recommend method 5, which is based on acidic extraction and AC purification, to obtain FCSP samples with high purity and diversity (Table 3 and Table 6). Overall, these approaches are all compatible both with economic production and obtaining high quality sulfated fucan products.

Acknowledgements

This research was supported by Agence Nationale de la Recherche (ANR) for the "Breaking Alg" project (ANR-18-CE43-0003-01). Authors are greatly thankful to Prof. Bernard Kloareg and Dr. Cécile Hervé for their advice on preparation of fucose-containing sulfated polysaccharides. We sincerely thank Dr. Sabine Genicot for helpful discussions and experimental advice. We would also like to acknowledge the Master 2 students from the 'Station Biologique de Roscoff' for producing the recombinant enzymes *Zg*AlyA1 and *Zg*LamA. BIBS Research platform (http://www.bibs.inrae.fr/bibs_eng/, IBISA, Biogenouest) was supported by ANR Phenome-Emphasis-FR (ANR-11-INBS- 0012).

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Table 1. Preparation steps used in the eight different methods to obtain sulfated fucans from the brown alga *P. canaliculata*.

Method	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*
AIR preparation	●	●	●	●	●	●	●	●
Neutral extraction	●	●	●	○	○	○	●	○
Acidic extraction	○	○	○	●	●	●	○	●
Removal of alginate	●	●	●	●	●	●	●	●
Treatment with AC	○	●	○	○	●	○	○	○
Treatment with PVPP	○	○	●	○	○	●	○	○
Treatment with CPC	○	○	○	○	○	○	●	●
Ethanol precipitation	●	●	●	●	●	●	●	●
Dialysis	●	●	●	●	●	●	●	●
Freeze-drying	●	●	●	●	●	●	●	●

● - yes, ○ - no, AIR - alcohol insoluble residue, AC - activated charcoal, PVPP -polyvinylpolypyrrolidone, CPC - cetylpyridinium chloride. Methods 7 and 8 include an additional CPC step for enrichment of highly negatively charged HMW sulfated fucans.

Table 2. Neutral monosaccharide compositional analysis on sulfated fucans from *P. canaliculata* obtained by eight different preparative methods.

Method	E/R	Fuc (%)	SD_{Fuc} (%)	Xyl (%)	SD_{Xyl} (%)	Man (%)	SD_{Man} (%)	Gal (%)	SD_{Gal} (%)	Glc (%)	SD_{Glc} (%)	TNS (%)	SD_{TNS} (%)
1	E	33.7	1.2	3.0	0.1	2.6	0.1	3.2	0.2	1.4	0.2	43.8	1.6
	R	37.0	1.5	2.8	0.2	2.1	0.1	3.0	0.1	0.6	0.1	45.5	1.8
2	E	37.0	1.1	3.2	0.1	2.5	0.1	3.3	0.1	0.3	0.0	46.3	1.2
	R	39.6	1.7	2.6	0.2	1.5	0.2	3.2	0.2	0.3	0.1	47.1	1.9
3	E	35.2	1.5	3.5	0.2	2.6	0.1	3.5	0.2	1.3	0.1	46.1	1.9
	R	36.3	1.9	2.6	0.2	1.9	0.3	2.9	0.2	0.4	0.1	44.2	2.6
4	E	36.5	1.1	3.9	0.3	2.8	0.2	3.5	0.3	1.1	0.4	47.8	0.4
	R	39.6	3.4	3.5	0.5	2.1	0.0	3.4	0.3	0.5	0.0	49.2	4.1
5	E	35.1	0.9	3.0	0.2	1.8	0.1	3.0	0.1	0.2	0.1	43.0	1.0
	R	37.7	1.0	2.6	0.1	1.4	0.2	2.8	0.2	0.0	0.0	44.5	1.1
6	E	32.1	0.5	3.1	0.0	2.2	0.2	2.8	0.1	1.4	0.2	41.6	0.4
	R	36.1	0.1	3.3	0.2	2.0	0.1	3.1	0.2	0.4	0.3	45.0	0.7
7	E	32.7	1.2	2.4	0.2	2.0	0.3	3.1	0.1	0.6	0.1	40.9	1.8
	R	38.6	0.3	2.7	0.2	1.5	0.1	3.5	0.2	0.0	0.0	46.3	0.6
8	E	35.0	1.2	3.0	0.1	1.7	0.1	3.5	0.1	0.0	0.0	43.2	1.2
	R	38.0	0.8	2.9	0.6	1.4	0.2	3.3	0.2	0.0	0.0	45.5	0.9
AIR		14.7	0.9	1.5	0.1	1.5	0.1	1.4	0.1	2.7	0.1	21.8	1.2
P. canaliculata		10.9	0.2	1.2	0.0	4.7	0.1	1.2	0.1	2.2	0.0	20.2	0.4

E - Sulfated fucans *obtained after first extraction, R* - sulfated fucans *obtained after re-extraction, Fuc - fucose, Xyl - xylose, Man - mannose, Gal - galactose, Glc - glucose, TNS – total neutral sugar, SD - standard deviation, AIR - alcohol insoluble residue. *- In case of dry P. canaliculata and AIR detected mannose can as well be mannitol.*

Table 3. MALS-SEC Mw analysis on sulfated fucans from *P. canaliculata* obtained by eight different preparative methods.

Method	E/R	$\overline{\mathrm{Mw}}$ (Da)	SD_{Mw} (%)	$\overline{\mathrm{Mn}}$ (Da)	SD_{Mn}(%)	$\overline{\mathrm{Mw}}/\overline{\mathrm{Mn}}$	$SD_{Mw/Mn}$ (%)
1	E	7.988 E+05	0.3	5.397 E+05	0.4	1.480	0.5
	R	6.682 E+05	0.4	4.028 E+05	0.4	1.659	0.5
2	E	8.722 E+05	0.3	5.604 E+05	0.4	1.556	0.5
	R	7.228 E+05	0.3	4.409 E+05	0.4	1.639	0.5
3	E	8.341 E+05	0.3	5.099 E+05	0.4	1.636	0.5
	R	5.886 E+05	0.3	2.999 E+05	0.7	1.962	0.7
4	E	7.658 E+05	0.4	4.858 E+05	0.5	1.581	0.6
	R	6.273 E+05	0.4	3.578 E+05	0.5	1.753	0.6
5	E	8.634 E+05	0.3	5.901 E+05	0.5	1.463	0.5
	R	6.390 E+05	0.3	3.758 E+05	0.4	1.688	0.5
6	E	8.776 E+05	0.3	5.358 E+05	0.3	1.638	0.4
	R	6.476 E+05	0.6	3.476 E+05	0.6	1.865	0.7
7	E	7.567 E+05	0.4	5.691 E+05	0.4	1.330	0.6
	R	5.640 E+05	0.4	3.759 E+05	0.6	1.500	0.7
8	E	7.219 E+05	0.4	5.130 E+05	0.4	1.407	0.5
	R	5.176 E+05	0.3	3.087 E+05	0.7	1.677	0.8

E - sulfated fucanss obtained after first extraction, R - sulfated fucans obtained after re-extraction, $\overline{Mw}$ - weight-average molar mass, SD - standard deviation, $\overline{Mn}$ - number-average molar mass

$\overline{Mn} = \frac{\sum niMi}{\sum ni} = \frac{\sum ci}{\sum ciMi}$ *(Mi - molar mass (Da) of the i^{th} slice, ni –number of molecules with the molar mass Mi of the i^{th} slice)*

$ci = Mi\ ni$

$\overline{Mw} = \frac{\sum ni\ Mi^2}{ni\ Mi} = \frac{\sum ci\ Mi}{\sum ci}$ *(ci - mass concentration of the i^{th} slice)*

Table 4. Non-FCSP biomolecules (w/w) identified in analyses of *P. canaliculata*'s sulfated fucans prepared by eight different methodologies.

Method	E/R	β-1,4-glucans (%)	β-1,3-glucans (%)	$SD_{\beta\text{-}1,3\text{-}glucans}$ (%)	Alginate (%)	Phenols (%)	$SD_{Phenols}$ (%)	Proteins (%)	$SD_{Proteins}$ (%)
1	E	n. d.	0.57	0.51	n. d.	1.13	0.06	0.28	0.01
	R	n. d.	n. d.	n. d.	n. d.	0.61	0.02	n. d.	n. d.
2	E	n. d.	n. d.	n. d.	n. d.	0.48	0.03	n. d.	n. d.
	R	n. d.	n. d.	n. d.	n. d.	0.24	0.01	n. d.	n. d.
3	E	n. d.	0.96	0.10	n. d.	1.21	0.12	n. d.	n. d.
	R	n. d.	n. d.	n. d.	n. d.	0.59	0.04	n. d.	n. d.
4	E	n. d.	0.55	0.48	n. d.	1.04	0.07	0.30	0.02
	R	n. d.	n. d.	n. d.	n. d.	0.59	0.03	n. d.	n. d.
5	E	n. d.	n. d.	n. d.	n. d.	0.46	0.02	n. d.	n. d.
	R	n. d.	n. d.	n. d.	n. d.	0.26	0.04	n. d.	n. d.
6	E	n. d.	0.99	0.05	n. d.	1.08	0.05	n. d.	n. d.
	R	n. d.	n. d.	n. d.	n. d.	0.51	0.04	n. d.	n. d.
7	E	n. d.	n. d.	n. d.	n. d.	0.90	0.11	n. d.	n. d.
	R	n. d.	n. d.	n. d.	n. d.	0.34	0.01	n. d.	n. d.
8	E	n. d.	n. d.	n. d.	n. d.	0.90	0.09	n. d.	n. d.
	R	n. d.	n. d.	n. d.	n. d.	0.30	0.04	n. d.	n. d.

E – sulfated fucans obtained after first extraction, R - sulfated fucans obtained after re-extraction, SD – standard deviation, n.d. – not detected.

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Table 5. Main oligosaccharide species of *P. canaliculata*'s sulfated fucans after enzymatic digestion with the fucanase *Mf*FcnA from *M. fucanivorans* as detected by UHPLC-MS.

Method	E/R	4 Fuc + 6 ($-SO_4^-$)		6 Fuc + 9 ($-SO_4^-$)	8 Fuc + 12 ($-SO_4^-$)	10 Fuc + 15 ($-SO_4^-$)	2Fuc + 3($-SO_4^-$)		4 Fuc + 5 ($-SO_4^-$)		6 Fuc + 7 ($-SO_4^-$)
		$[M-4.H+2.HxA]^{2-}$	$[M-4.H+2.HxA]^{2-}$	$[M-9.H+7.HxA]^{2-}$	$[M-11.H+8.HxA]^{3-}$	$[M-15.H+12.HxA]^{3-}$	$[M-2.H+1.HxA]^{-}$	$[M-2.H+1.HxA]^{-}$	$[M-3.H+1.HxA]^{2-}$	$[M-3.H+1.HxA]^{2-}$	$[M-7.H+5.HxA]^{2-}$
1	E	5.92E+05	1.88E+05	2.93E+05	1.74E+05	1.08E+04	6.47E+03	1,38E+03	8.53E+03	2,17E+03	4.64E+03
	R	3.96E+05	1.24E+05	2.07E+05	2.05E+05	2.63E+04	4.03E+03	9,71E+02	1.87E+03	5,46E+02	1.45E+03
2	E	3.61E+05	1.09E+05	1.65E+05	1.78E+05	2.77E+04	3.74E+03	9,34E+02	1.73E+03	5,87E+02	1.28E+03
	R	2.21E+05	$7.36*10^4$	1.27E+05	1.75E+05	3.00E+04	1.59E+03	-	-	-	-
3	E	6.46E+05	1.83E+05	4.03E+05	1.96E+05	1.54E+04	-	-	1.22E+04	2,94E+03	8.10E+03
	R	3.10E+05	$8.53*10^4$	1.96E+05	2.24E+05	4.56E+04	2.30E+03	-	1.10E+03	-	-
4	E	5.21E+05	1.32E+05	3.83E+05	2.64E+05	4.59E+04	8.62E+03	1,90E+03	5.26E+03	1,28E+03	4.62E+03
	R	4.08E+05	1.04E+05	3.04E+05	3.04E+05	6.25E+04	2.96E+03	7,03E+02	1.72E+03	5,36E+02	1.44E+03
5	E	3.76E+05	$9.82*10^4$	2.47E+05	2.26E+05	4.25E+04	3.06E+03	6,83E+02	1.84E+03	4,50E+02	1.54E+03
	R	2.46E+05	$6.98*10^4$	1.88E+05	2.45E+05	5.29E+04	1.68E+03	3,92E+02	7.35E+02	-	-
6	E	5.92E+05	1.56E+05	4.40E+05	2.30E+05	2.64E+04	1.81E+04	3,25E+03	9.24E+03	2,16E+03	6.83E+03
	R	3.61E+05	9.96E+04	2.58E+05	2.58E+05	6.38E+04	2.90E+03	6,18E+02	1.58E+03	-	1.45E+03
7	E	6.42E+05	1.66E+05	4.94E+05	2.69E+05	2.38E+04	1.69E+04	3,14E+03	1.03E+04	2,33E+03	8.21E+03
	R	8.09E+05	2.09E+05	6.61E+05	3.69E+05	3.88E+04	2.85E+04	4,91E+03	1.37E+04	3,06E+03	9.56E+03
8	E	7.98E+05	2.04E+05	6.41E+05	3.04E+05	8,66E+03	3.42E+04	5,97E+03	2.02E+04	4,37E+03	1.47E+04
	R	7.06E+05	1.82E+05	5.86E+05	3.88E+05	7.19E+04	1.57E+04	2,93E+03	8.71E+03	2,07E+03	6.84E+03
m/z		641.10	641.11	1159.92	983.96	1296.1212	650.10	650.10	550.57	550.57	978.82
Charge state		2	2	2	3	3	1	1	2	2	2
RT (min)		6.37	6.53	9.66	11.92	13.46	1.55	1.68	4.6	4.73	7.09

E - sulfated fucans obtained after first extraction, R - sulfated fucans obtained after re-extraction, Fuc – fucose, $-SO^{4-}$ - sulfate group, HxA - hexylammonium acetate, m/z – mass-to-charge ratio, RT- retention time.

Table 6. Main oligosaccharide species containing a hexose other than fucose from *P. canaliculata*'s sulfated fucans after enzymatic digestion with the fucanase *Mf*FcnA from *M. fucanivorans* as detected by UHPLC-MS.

Method	E/R	11 Fuc + 1 Hex + 16 (-SO_4^-)		9 Fuc + 1 Hex + 15 (-SO_4^-)		9 Fuc + 1 Hex + 14 (-SO_4^-)	9 Fuc + 1 Hex + 13 (-SO_4^-)		9 Fuc + 1 Hex + 12 (-SO_4^-)
		$[M-16.H+13.HxA]^{3-}$	$[M-15.H+12.HxA]^{3-}$	$[M-9.H+7.HxA]^{2-}$	$[M-11.H+8.HxA]^{3-}$	$[M-12.H+9.HxA]^{3-}$	$[M-12.H+9.HxA]^{3-}$	$[M-12.H+9.HxA]^{3-}$	$[M-12.H+10.HxA]^{2-}$
1	E	-	-	-	-	-	3.13E+03	-	-
	R	-	-	-	-	-	1.63E+03	-	-
2	E	-	-	-	-	-	2.09E+03	-	-
	R	-	-	-	-	-	1.14E+03	-	-
3	E	-	-	8.57E+02	1.05E+03	-	2.69E+03	1.88E+03	2.11E+03
	R	-	-	9.23E+02	7.84E+02	-	1.69E+03	-	-
4	E	-	1.06E+03	1.23E+03	1.23E+03	1.20E+03	2.21E+03	3.19E+03	3.53E+03
	R	-	-	1.14E+03	9.98E+02	1.03E+03	1.87E+03	1.87E+03	1.25E+03
5	E	-	-	7.63E+02	-	-	1.77E+03	1.70E+03	9.74E+02
	R	-	-	8.38E+02	-	6.00E+02	1.11E+03	5.84E+02	-
6	E	-	-	1.25E+03	1.25E+03	1.00E+03	3.40E+03	5.84E+02	4.71E+03
	R	-	-	1.30E+03	1.30E+03	1.08E+03	1.99E+03	1.99E+03	1.22E+03
7	E	-	2.13E+03	1.47E+03	1.82E+03	1.51E+03	3.75E+03	5.53E+03	7.18E+03
	R	-	2.44E+03	3.15E+03	2.73E+03	2.56E+03	6.38E+03	4.31E+03	7.17E+03
8	E	5.67E+02	3.37E+03	2.00E+03	2.22E+03	2.23E+03	4.88E+03	7.56E+03	1.07E+04
	R	6.52E+02	2.37E+03	3.53E+03	-	2.56E+03	6.90E+03	3.73E+03	6.90E+03
m/z		1459.18	1398.82	1267.7423	1267.74	1173.6821	1147.02	1147.02	1086.66
Charge state		3	3	3	3	3	3	3	2
RT (min)		12.97	12.29	13.38	13.78	12.77	11.77	11.90	10.50

E - sulfated fucans obtained after first extraction, R - sulfated fucans obtained after re-extraction, Fuc – fucose, Hex – hexose other than Fuc, -SO^{4-} - sulfate group, HxA - hexylammonium acetate, m/z – mass-to-charge ratio, RT- retention time

Table 7. Main oligosaccharide species containing a pentose from *P. canaliculata*'s sulfated fucans after enzymatic digestion with fucanase *Mf*FcnA from *M. fucanivorans* as detected by UHPLC-MS.

Method	E/R	*10.Fuc + 1.Pent + 13 (-SO_4^-)*	*8.Fuc + 1.Pent + 9(-SO_4^-)*	*8.Fuc + 1.Pent + 8 (-SO_4^-)*	*6.Fuc + 1.Pent + 7 (-SO_4^-)*	*6.Fuc + 1.Pent + 6 (-SO_4^-)*
		$[M-13.H+10.HxA]^{3-}$	*$[M-9.H+7.HxA]^{2-}$*	*$[M-8.H+6.HxA]^{2-}$*	*$[M-6.H+4.HxA]^{2-}$*	*$[M-6.H+4.HxA]^{2-}$*
1	E	-	1.30E+03	-	-	2.13E+03
	R	-	-	-	-	-
2	E	-	-	-	-	-
	R	-	-	-	-	-
3	E	-	-	8.13E+02	1.50E+03	3.10E+03
	R	-	-	-	-	-
4	E	7.13E+02	-	5.97E+02	-	2.18E+03
	R	-	-	-	-	-
5	E	-	-	-	-	-
	R	-	-	-	-	-
6	E	-	1.04E+03	9.30E+02	1.18E+03	3.07E+03
	R	-	-	-	-	-
7	E	1.12E+03	1.30E+03	1.16E+03	1.91E+03	3.66E+03
	R	1.81E+03	1.65E+03	1.68E+03	2.81E+03	4.73E+03
8	E	1.59E+03	1.85E+03	2.02E+03	3.92E+03	5.76E+03
	R	1.80E+03	1.53E+03	1.15E+03	1.58E+03	3.70E+03
m/z		1219.41	1371.97	1281.46	994.28	954.32
Charge state		3	2	2	2	2
RT (min)		12.30	9.65	7.77	7.23	5.93

E - sulfated fucans obtained after first extraction, R - sulfated fucans obtained after re-extraction, Fuc – fucose, Pent – pentose, -SO^{4-} - sulfate group, HxA - hexylammonium acetate, m/z – mass-to-charge ratio, RT- retention time

Captions to illustrations

Figure 1. Step-by-step scheme of the eight methods used to obtain sulfated fucans from the brown alga *P. canaliculata*.

Figure 2. Histogram of sulfated fucan yields for each method compared to initial dry algal weight. E bars represent yields after first extraction, R bars represent yields obtained by re-extraction, and T bars represent total yields (E+R) for each method.

Figure 3. Histogram of sulfate group content in *P. canaliculata* sulfated fucans prepared by eight different methods. E bars represent percentage of sulfate groups (w/w) in sulfated fucans obtained after first extraction, R bars represent percentage of sulfate groups (w/w) in sulfated fucans obtained by re-extraction and T bars represent percentage of sulfate groups (w/w) in total sulfated fucans (E+R) for each method.

Figure 4. C-PAGE profiles of sulfated fucans prepared by 8 different preparation methods, and the enzymatic digests by *Zg*AlyA1 from *Z. galactanivorans*. M1-M8 – methods used for preparing sulfated fucans from *P. canaliculata*. E - sulfated fucans obtained after first extraction, R - sulfated fucans obtained after re-extraction. C1 and C2 are the negative and positive alginate control, respectively. The intensities of the bands of digested alginate in the positive control lane C2 represent 10% alginate contamination as the concentration of the alginate used in lanes C1 and C2 corresponds to 1/10 of the concentration of sulfated fucans applied in the other lanes.

Figure 5. C-PAGE profiles of sulfated fucans prepared by 8 different methods and their enzymatic digests by *Mf*FcnA from *M. fucanivorans*. M1-M8 – methods used for preparing sulfated fucans from *P. canaliculata*. E - sulfated fucans obtained after first extraction, R - sulfated fucans obtained after re-extraction

Figure 6. Main oligosaccharide species released by enzymatic digestion of *P. canaliculata* sulfated fucans with the fucanase *Mf*FcnA and detected by UHPLC-MS. Representation of the crystal structure of *Mf*FcnA (PBD ID 6DLH, [50]) was generated using PYMOL (Schrödinger, version 1.8.2.2).

Figure 7. Histogram of radical scavenging capacities for sulfated fucans prepared by eight different methods. Deionized water was used as a negative control and resveratrol was used

as a positive control. M1-M8 – methods used for preparing sulfated fucans from *P. canaliculata*. E - sulfated fucans obtained after first extraction, R - sulfated fucans obtained after re-extraction. GraphPad Prism t-test calculator was used to evaluate the significance of obtained values compared to the negative control (*$p<0.05$, **$p<0.01$ and ***$p<0.001$).

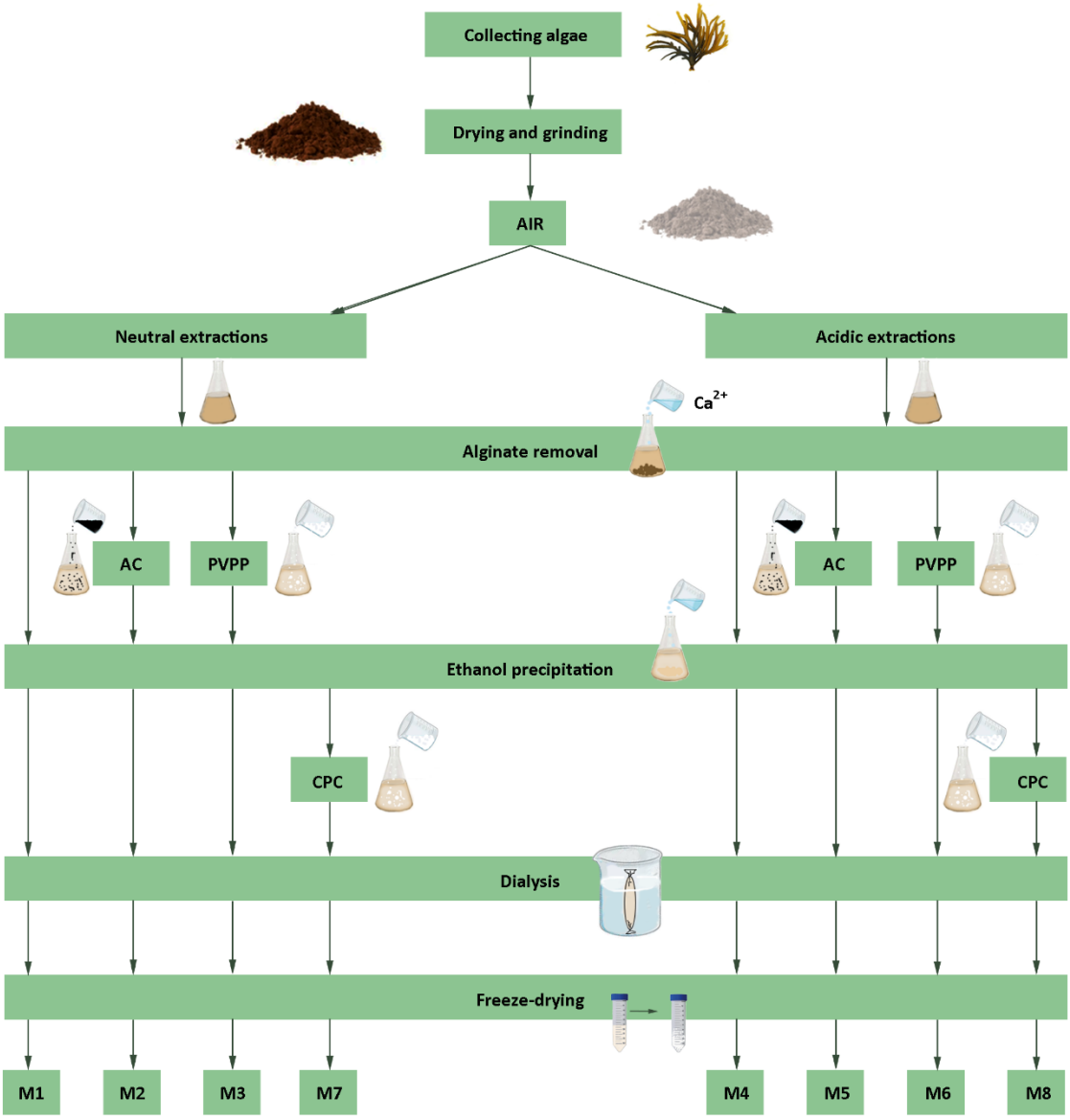
Collecting algae
Drying and grinding
AIR
Neutral extractions
Acidic extractions
Ca2+
Alginate removal
AC
PVPP
AC
PVPP
Ethanol precipitation
CPC
CPC
Dialysis
Freeze-drying
M1
M2
M3
M7
M4
M5
M6
M8

Figure 1

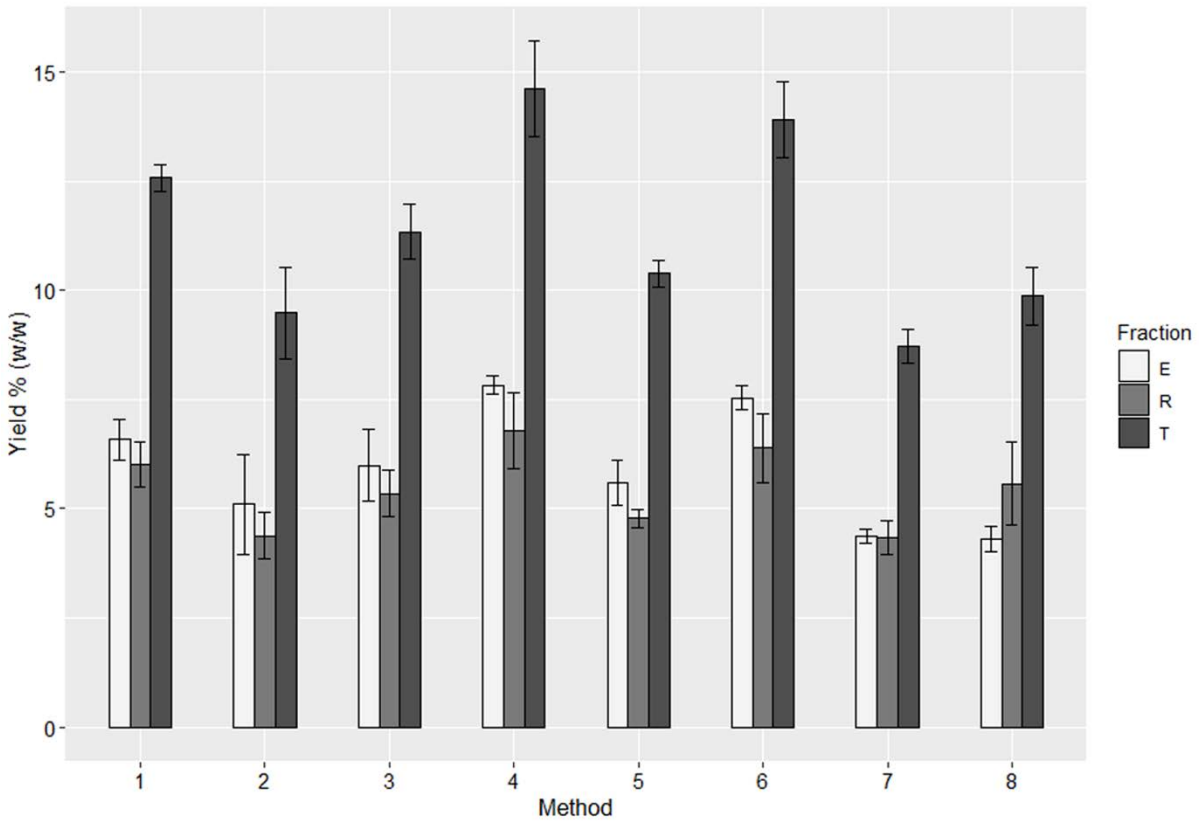

Figure 2

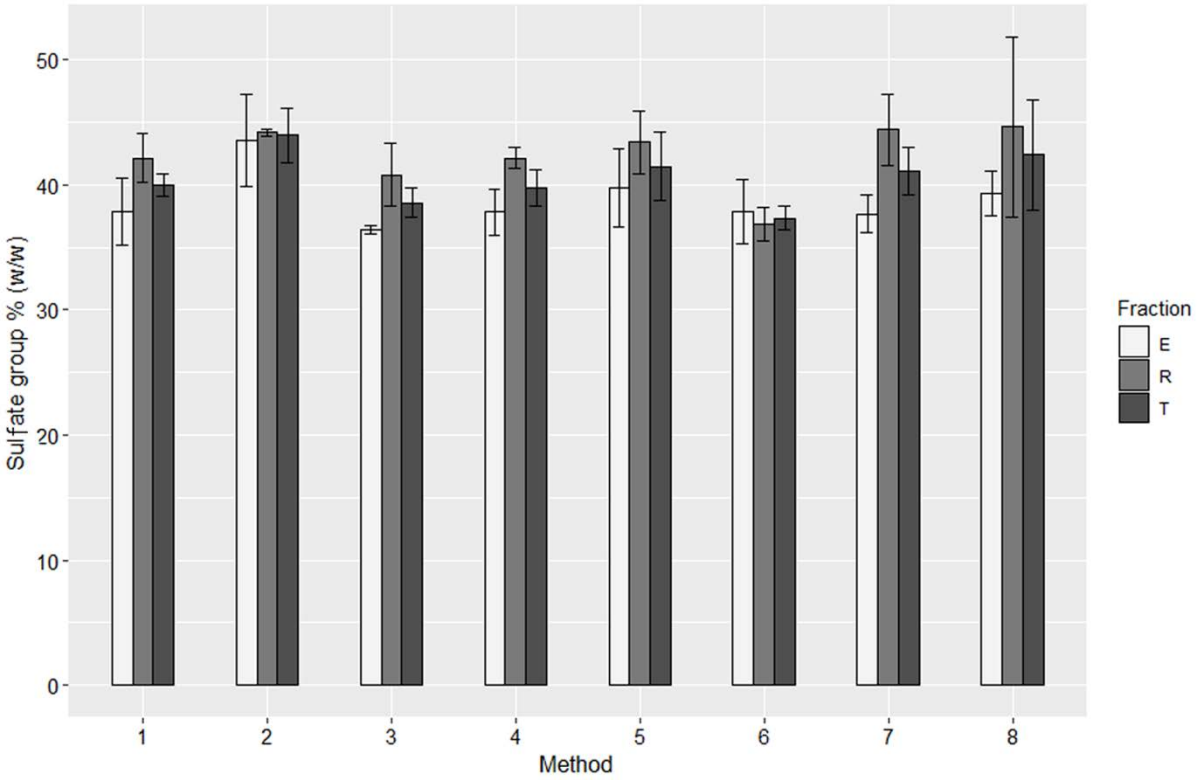
Sulfate group % (w/w)
50
40
30
20
10
0
1
2
3
4
5
6
7
8
Method
Fraction
E
R
T

Figure 3

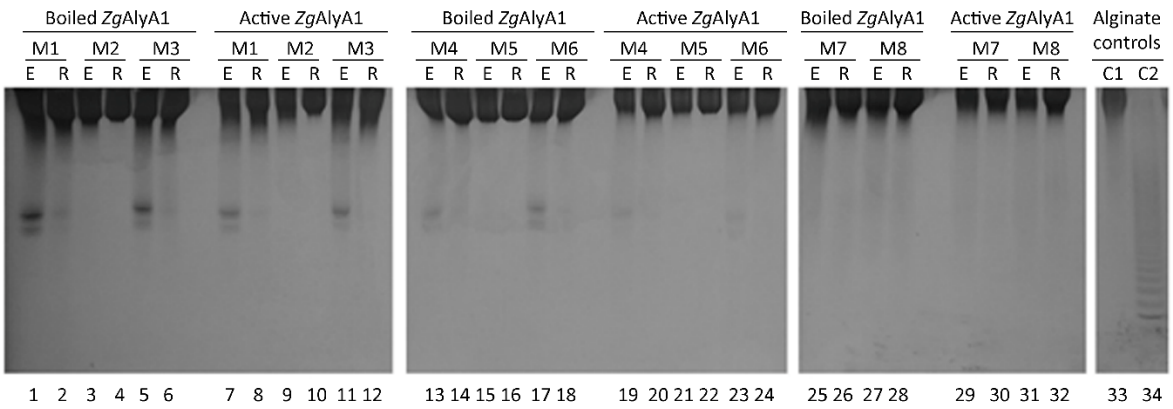
Boiled ZgAlyA1
Active ZgAlyA1
Boiled ZgAlyA1
Active ZgAlyA1
Boiled ZgAlyA1
Active ZgAlyA1
Alginate controls
M1 M2 M3
M1 M2 M3
M4 M5 M6
M4 M5 M6
M7 M8
M7 M8
E R E R E R E R E R E R E R E R E R E R E R E R E R E R E R E R E R E R E R E R E R E R
C1 C2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34

Figure 4

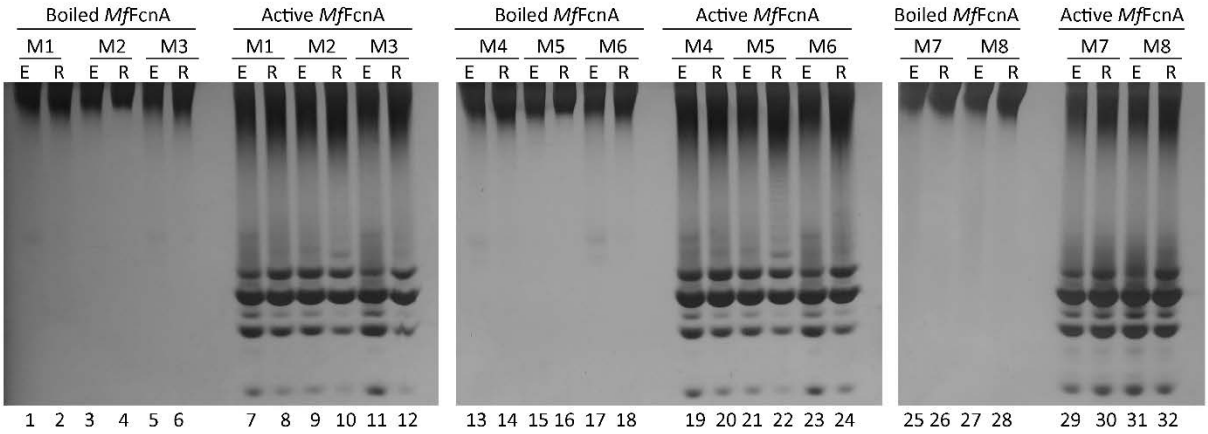
Boiled *Mf*FcnA
Active *Mf*FcnA
Boiled *Mf*FcnA
Active *Mf*FcnA
Boiled *Mf*FcnA
Active *Mf*FcnA
M1 M2 M3
M1 M2 M3
M4 M5 M6
M4 M5 M6
M7 M8
M7 M8
E R E R E R E R E R E R E R E R E R E R E R E R E R E R E R
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32

Figure 5.

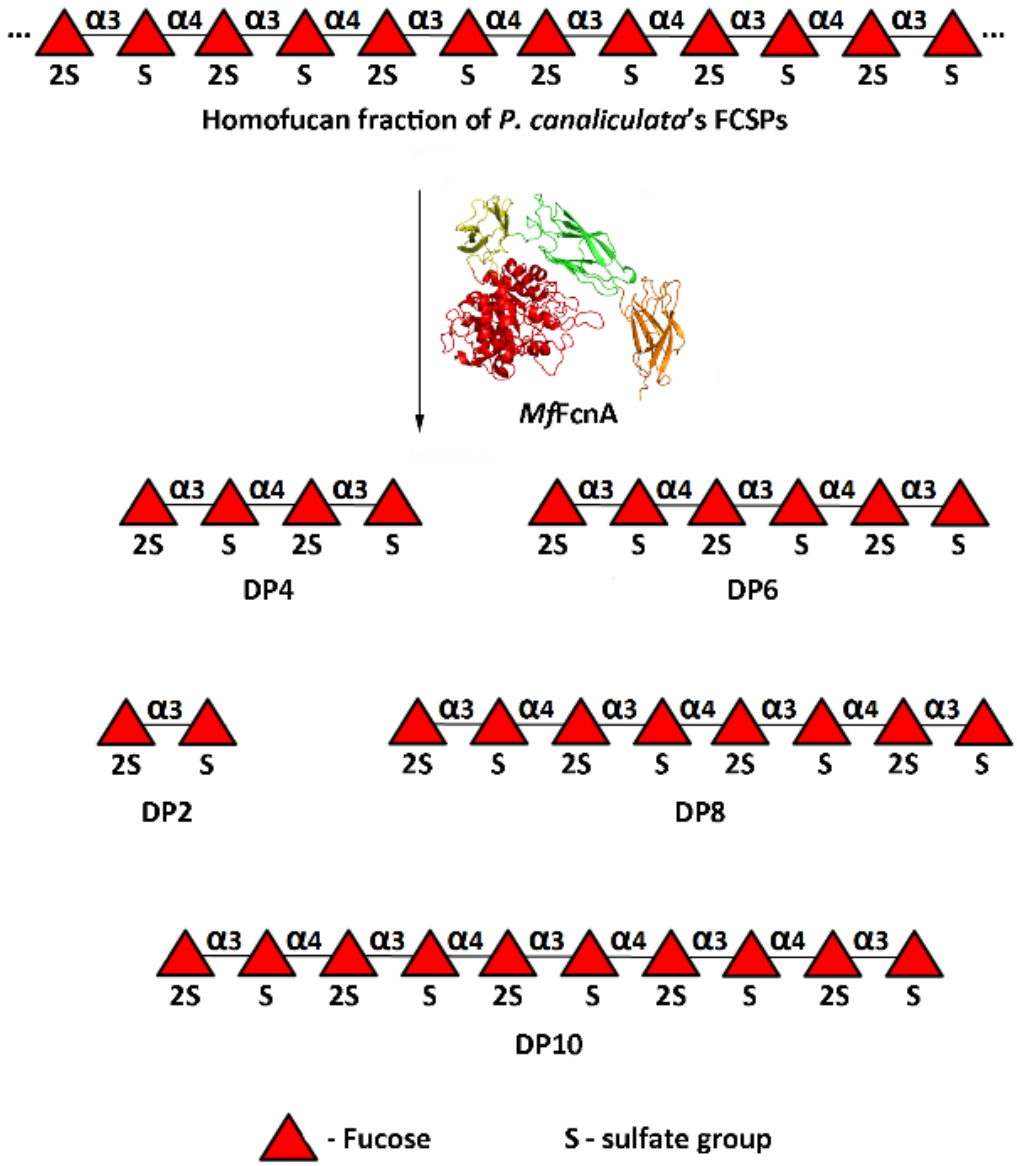
α3 α4 α3 α4 α3 α4 α3 α4 α3 α4 α3
2S S 2S S 2S S 2S S 2S S 2S S
Homofucan fraction of P. canaliculata's FCSPs
MfFcnA
α3 α4 α3
2S S 2S S
DP4
α3 α4 α3 α4 α3
2S S 2S S 2S S
DP6
α3
2S S
DP2
α3 α4 α3 α4 α3 α4 α3
2S S 2S S 2S S 2S S
DP8
α3 α4 α3 α4 α3 α4 α3 α4 α3
2S S 2S S 2S S 2S S 2S S
DP10
- Fucose
S - sulfate group

Figure 6.

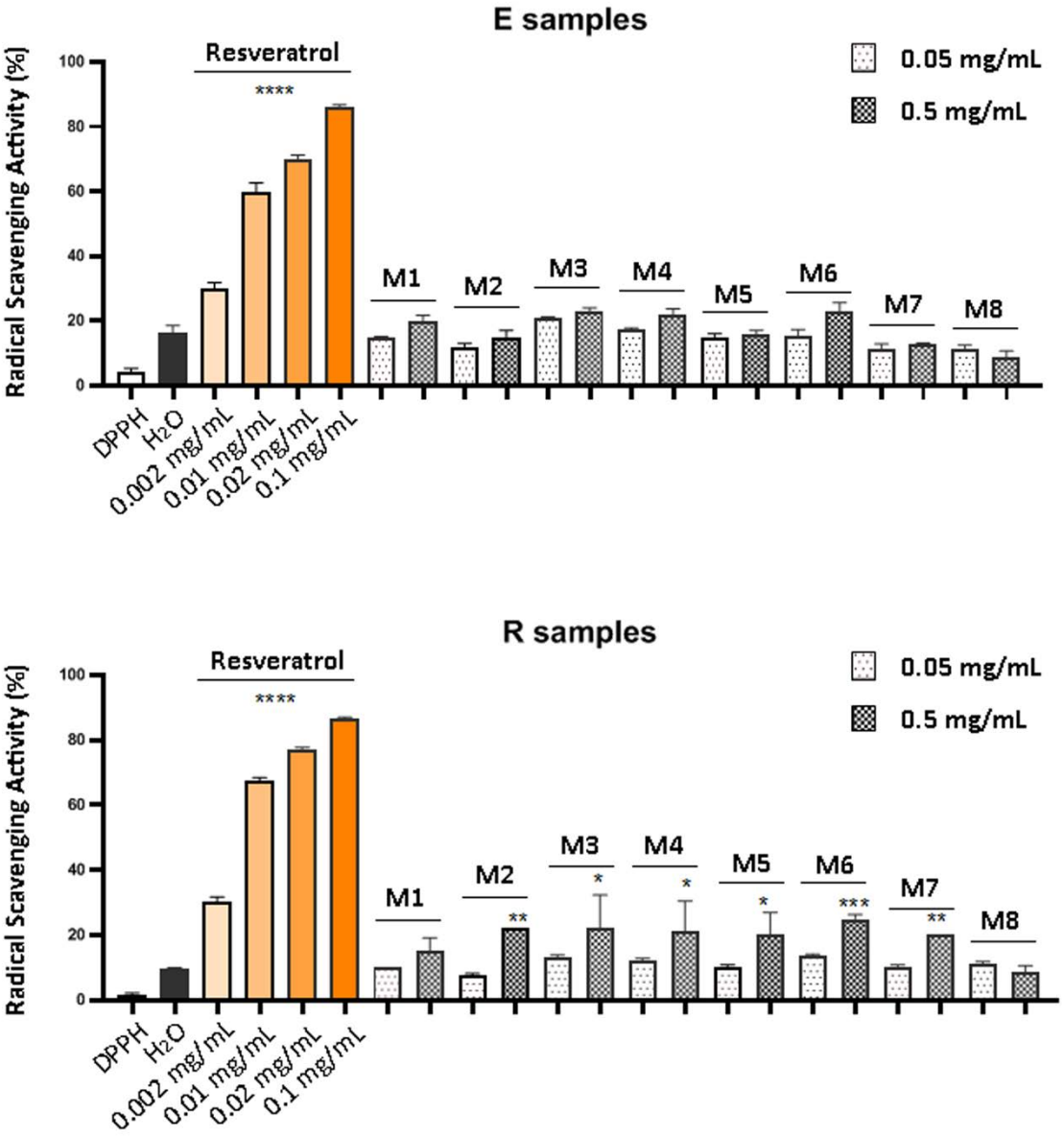
E samples
Resveratrol
****
0.05 mg/mL
0.5 mg/mL
Radical Scavenging Activity (%)
100
80
60
40
20
0
M1
M2
M3
M4
M5
M6
M7
M8
DPPH
H2O
0.002 mg/mL
0.01 mg/mL
0.02 mg/mL
0.1 mg/mL
R samples
Resveratrol
****
0.05 mg/mL
0.5 mg/mL
Radical Scavenging Activity (%)
100
80
60
40
20
0
M1
M2
**
M3
*
M4
*
M5
*
M6
***
M7
**
M8
DPPH
H2O
0.002 mg/mL
0.01 mg/mL
0.02 mg/mL
0.1 mg/mL

Figure 7

Supporting Information

Content:

Table S1. Yield of sulfated fucans obtained by eight methods from initial dry weight of *P. canaliculata.*

Method	E/R	Yield (%)	SD_{yield} (%)	Total yield (%)	$SD_{Total\ yield}$ (%)
1	E	6.6	0.5	12.6	0.3
	R	6.0	0.5		
2	E	5.1	1.1	9.5	1.1
	R	4.4	0.5		
3	E	6.0	0.8	11.3	0.6
	R	5.3	0.5		
4	E	7.8	0.2	14.6	1.1
	R	6.8	0.9		
5	E	5.6	0.5	10.4	0.3
	R	4.8	0.2		
6	E	7.5	0.3	13.9	0.9
	R	6.4	0.8		
7	E	4.4	0.2	8.7	0.4
	R	4.3	0.4		
8	E	4.3	0.3	9.9	0.7
	R	5.6	1.0		

E - sulfated fucans *obtained after first extraction, R - FCSPs obtained after re-extraction, SD – standard deviation.*

Table S2. Pairwise statistical comparison of yield of sulfated fucans obtained by eight methods from initial dry weight of *P. canaliculata*

Pairs			*p value*
M2	-	M1	<0.01 ***
M3	-	M1	0.460
M4	-	M1	0.052
M5	-	M1	<0.033 **
M6	-	M1	0.369
M7	-	M1	<0.01 ***
M8	-	M1	<0.01 ***
M3	-	M2	0.091
M4	-	M2	<0.01 ***
M5	-	M2	0.787
M6	-	M2	<0.01 ***
M7	-	M2	0.898
M8	-	M2	0.997
M4	-	M3	<0.01 ***
M5	-	M3	0.734
M6	-	M3	<0.01 ***
M7	-	M3	<0.01 ***
M8	-	M3	0.275
M5	-	M4	<0.01 ***
M6	-	M4	0.926
M7	-	M4	<0.01 ***
M8	-	M4	<0.01 ***
M6	-	M5	<0.01 ***
M7	-	M5	0.166
M8	-	M5	0.987
M7	-	M6	<0.01 ***
M8	-	M6	<0.01 ***
M8	-	M7	0.548

Table S3. Sulfate group content (w/w) on sulfated fucans from *P. canaliculata* obtained by eight different preparative methods.

Method	E/R	Sulfate groups (%)	$SD_{Sulfate\ groups}$ (%)	Average sulfation (%)	$SD_{Average\ sulfation}$ (%)
1	E	37.9	2.7	40.0	0.9
	R	42.2	2.0		
2	E	43.5	3.7	44.0	2.2
	R	44.2	0.3		
3	E	36.4	0.4	38.6	1.2
	R	40.8	2.5		
4	E	37.8	1.8	39.8	1.5
	R	42.1	0.8		
5	E	39.8	3.1	41.5	2.8
	R	43.4	2.5		
6	E	37.9	2.6	37.3	1.0
	R	36.9	1.4		
7	E	37.7	1.5	41.1	1.9
	R	44.4	2.8		
8	E	39.3	1.8	42.4	4.4
	R	44.6	7.2		

E – sulfated fucans obtained after first extraction, R - sulfated fucans obtained after re-extraction, SD – standard deviation.

Table S4. Pairwise statistical comparison of phenolic content in *P. canaliculata* sulfated fucans prepared by eight different methods.

Pairs			*p value*
M2	-	M1	<0.01 ***
M3	-	M1	0.983
M4	-	M1	0.833
M5	-	M1	<0.01 ***
M6	-	M1	0.703
M7	-	M1	<0.01 ***
M8	-	M1	<0.01 ***
M3	-	M2	<0.01 ***
M4	-	M2	<0.01 ***
M5	-	M2	1.000
M6	-	M2	<0.01 ***
M7	-	M2	<0.01 ***
M8	-	M2	<0.01 ***
M4	-	M3	0.346
M5	-	M3	<0.01 ***
M6	-	M3	0.241
M7	-	M3	<0.01 ***
M8	-	M3	<0.01 ***
M5	-	M4	<0.01 ***
M6	-	M4	1.000
M7	-	M4	<0.01 ***
M8	-	M4	<0.01 ***
M6	-	M5	<0.01 ***
M7	-	M5	<0.01 ***
M8	-	M5	<0.01 ***
M7	-	M6	<0.01 ***
M8	-	M6	<0.01 ***
M8	-	M7	0.766

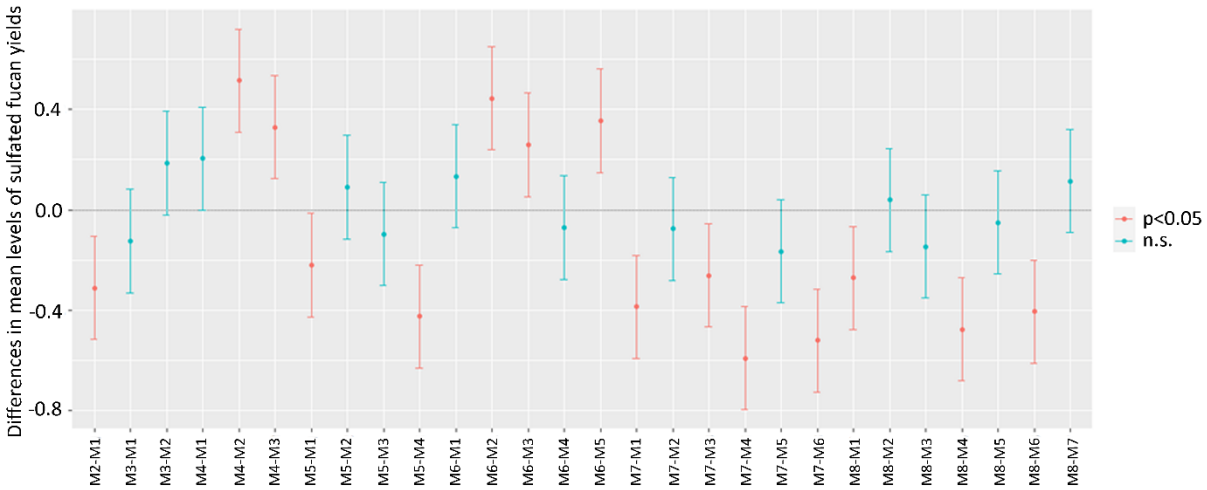

Figure S1. ***Plot representation of pairwise statistical comparison of yield of sulfated fucans obtained by eight methods from initial dry weight of P. canaliculata at 95% confidence level.*** *Analysis was done by Tukey's test at 95% confidence level, with p<0.05 – statistically significant, n.s. –statistically non-significant.*

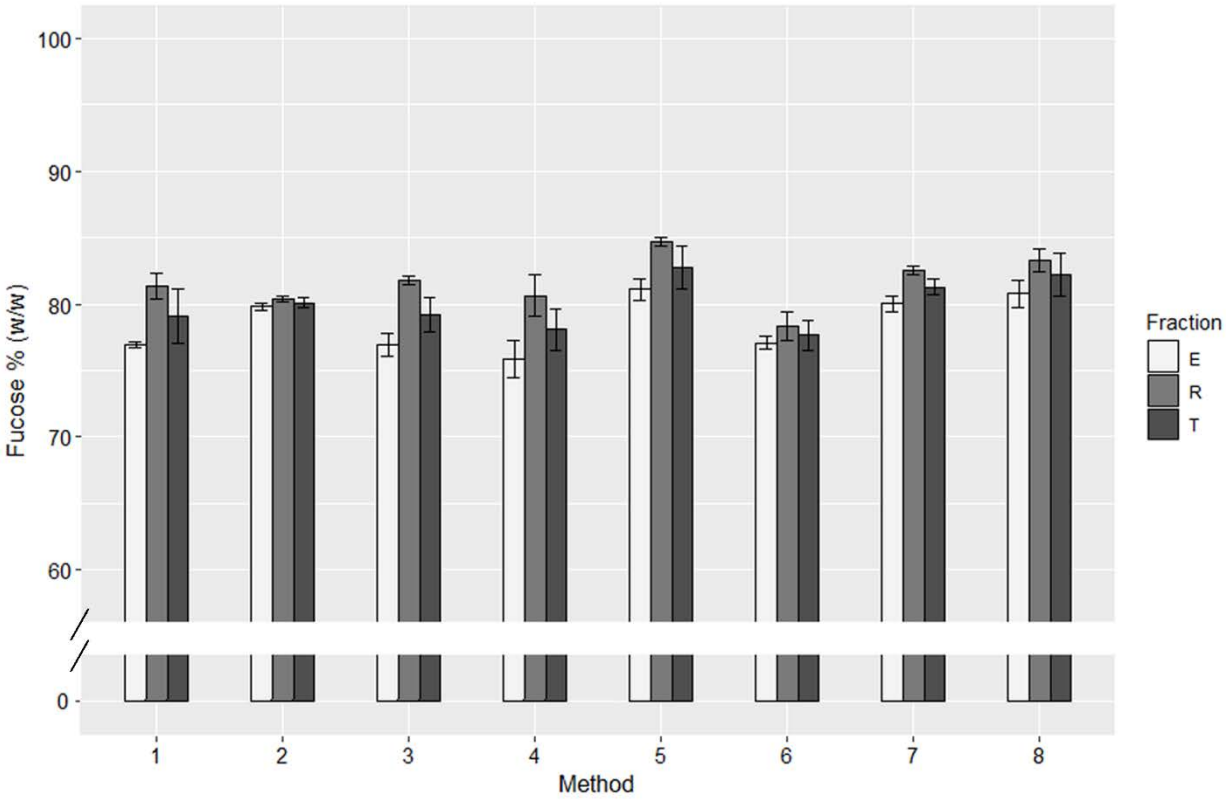

Figure S2. Histogram of fucose content among total neutral sugars detected in *P. canaliculata* sulfated fucans obtained by eight different methods. E bars represent fucose content in total neutral sugar fraction of sulfated fucans obtained by first extraction, R bars represent fucose content in total neutral sugar fraction of sulfated fucans obtained by re-extraction, T bars represent fucose content in total neutral sugar fraction of sulfated fucans obtained by each method (E+R).

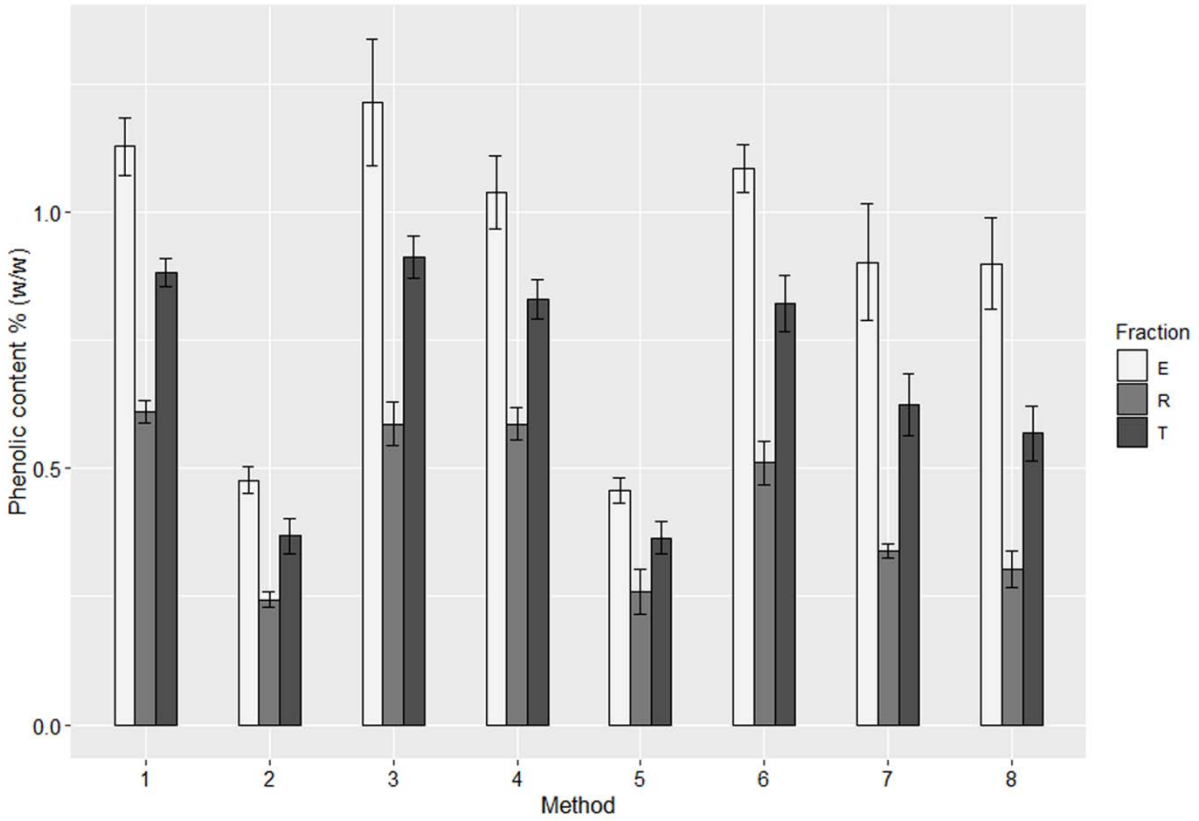

Figure S3. Histogram of phenolic content in *P. canaliculata* sulfated fucans prepared by eight different methods. E bars represent percentage of phenolic content (w/w) in sulfated fucans obtained after first extraction, R bars represent percentage of phenolic content (w/w) in sulfated fucans obtained after re-extraction and T bars represent percentage of phenolic content (w/w) in total sulfated fucans (E + R) obtained by each method.

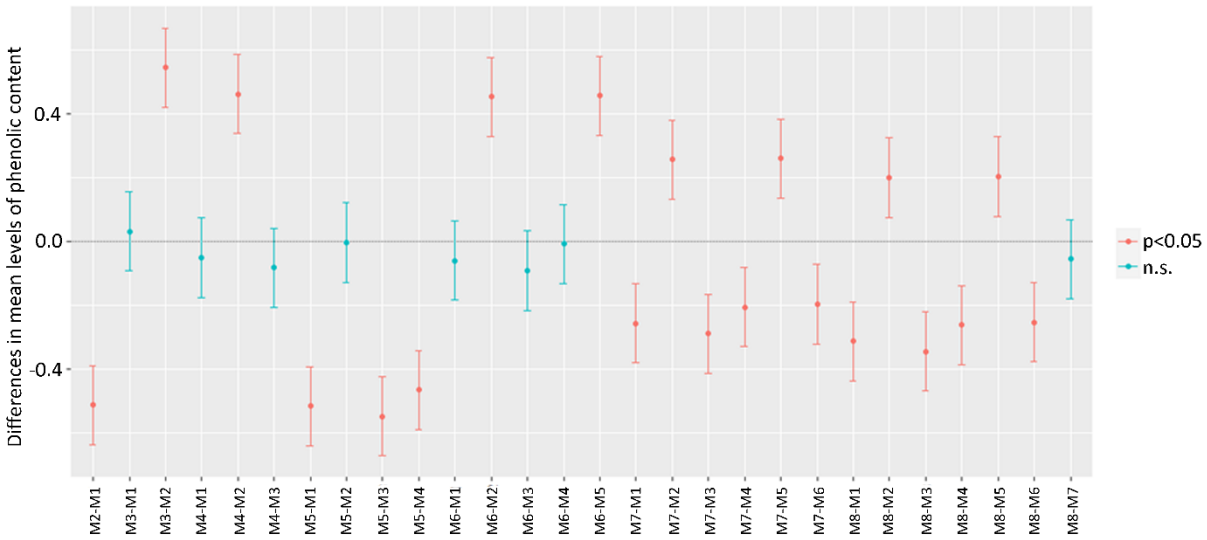

Figure S4. ***Plot representation of pairwise statistical comparison of phenolic content in sulfated fucans obtained by eight methods from initial dry weight of P. canaliculata.*** *Analysis was done by Tukey's test at 95% confidence level, with p<0.05 – statistically significant, n.s. –statistically non-significant.*