Sulfated galactofucan from seaweed *Padina tetrastromatica* attenuates proteolytic enzyme dipeptidyl-peptidase-4: a potential anti-hyperglycemic lead

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**ABSTRACT**

Dipeptidyl-peptidase-4 is a multifunctional ectoenzyme, which is implicated with hyperglycemic pathophysiology. Therefore, dipeptidyl-peptidase-4 inhibitors could be used as an attractive therapeutic strategy in blood-glucose homeostasis to attenuate the pathophysiology of diabetes. A sulfated galactofucan characterized as \([\rightarrow 1)-O-4\text{-sulfonato-}\alpha\text{-fucopyranosyl}(2\rightarrow 1)-O-2\text{-sulfonato-}\alpha\text{-fucopyranose}(3\rightarrow 1)\] along with a branch of \([\rightarrow 1)-6\text{-O-methyl-}\beta\text{-galactopyranosyl}(4\rightarrow 1)\] unit at the C-4 position of \(O-2\text{-sulfonato-}\alpha\text{-fucopyranose},\) isolated from the seaweed *Padina tetrastromatica*, exhibited prospective attenuation property against dipeptidyl-peptidase-4 (IC\textsubscript{50} 0.25 mg mL\textsuperscript{-1}). The studied sulfated galactofucan exhibited potential inhibitory properties against carbolytic enzymes \(\alpha\text{-amylase (IC}\textsubscript{50} 0.98 mg mL\textsuperscript{-1})\) and \(\alpha\text{-glucosidase (IC}\textsubscript{50} 0.87 mg mL\textsuperscript{-1})\) in comparison with the standard antidiabetic agent acarbose, along with radical scavenging activities. The seaweed-originated galactofucan could be developed as a promising natural therapeutic lead against hyperglycemic disorder.
1. Introduction

Dipeptidyl-peptidase-4 (DPP-4) is a homodimeric trans-membrane glycoprotein, which was implicated in the regulation of glucose-dependent incretin hormones, such as insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1). DPP-4 inhibitors were perceived as a key addition to the therapy process in type-2 diabetes (Xie et al. 2016). Bioactive compounds with inhibiting actions on proteolytic DPP-4 and carboxylytic enzymes like α-amylase and α-glucosidase were considered as promising therapeutic approaches to treat hyperglycemia (Makkar and Chakraborty 2017). Attenuation of DPP-4 reduces the degradation of GLP-1 resulting in an increased secretion of insulin in connection with an escalated level of blood glucose (Gallwitz et al. 2013). Diabetes ranks in the top 10 causes of disability, and currently, it was predicted to rise at 25% (578 million) in 2030 and 51% (700 million) during 2045 (Tadesse et al. 2021). Therefore, with several therapeutic treatments, DPP-4 inhibition was found to be an alternative possibility to increase GLP-1, which is involved in regulating glucose homeostasis. Therefore, selective inhibition of DPP-4 could be important for the development new class of antidiabetic agents (Thornberry and Gallwitz 2009). Similarly, compounds with the inhibiting activity of α-glucosidase and pancreatic α-amylase were found to be effective against diabetic pathologies. Marine-derived compounds from brown seaweed *Sargassum fusiforme* (HARV) were characterized as promising anti-diabetic agents to lower blood sugar levels and improve glucose tolerance (Zhang et al. 2008).

The family Dictyotaceae (class Phaeophyceae) covers greater than 300 species in marine habitations. Among numerous Phaeophytan seaweeds, *Padina tetrastromatica* (order Dictyotales) is one of the predominantly abundant species that dispersed all over the temperate and tropical Indo-Pacific region (Guiry and Nic-dhonncha 2003). The pharmacological potential of *Padina tetrastromatica* was previously reported using different in vitro models (Mohsin et al. 2014; Chia et al. 2015; Antony and Chakraborty 2019a). Earlier studies reported the bioactive metabolites belonging to the class of xenicanes, dolabellanes and dolastanes from this seaweed with prospective anti-inflammatory and anti-hyperglycemic properties (Antony and Chakraborty 2019b; Antony et al. 2021). Seaweed-derived polysaccharides were endowed with potential bioactivities to diminish oxidative stress prompted diseases, such as type-2 diabetes (Makkar and Chakraborty 2017). Polysaccharides were demonstrated to possess inhibitory activities against the carboxylytic enzymes (α-glucosidase and α-amylase) and development of glycation end products, which were reported to cause hyperglycemic conditions (Xie et al. 2016). Polysaccharides from marine sources offer diverse therapeutic functions owing to their biocompatibility, biodegradability to harmless products, and non-toxicity (Xie et al. 2016). Polysaccharides isolated from seaweed *Gracilaria opuntia* and *Kappaphycus alvarezii* were found to exhibit significant anti-diabetic activity (Makkar and Chakraborty 2017). Sulfated fucopyranan isolated from the seaweed *Sargassum wightii* was also found to display promising anti-diabetic properties (Anusree and Chakraborty 2018). Polysaccharides from *Inonotus obliquus* were proved to reduce hyperglycemic conditions (Wang et al. 2017). Low molecular weight oligosaccharides (∼3 kDa) isolated from seaweed was reported to stimulate secretion of pancreatic polypeptide insulin from the islets of Langerhans (Zhang et al. 2008).
There were few previous studies that reported the biomedical applications of sulfated polysaccharide isolated from this species (Jose et al. 2015; Lekshmi et al. 2019), whereas limited information was reported about the detailed structural description and effects of polysaccharides on their use to attenuate the hyperglycemic related disorders. Therefore, on account of these facts, our current study envisages the isolation and structural characterization of a sulfated galactofucan, characterized as a repetitive unit of \( \rightarrow 1)-O-4\text{-sulfonato-}\alpha\text{-fucopyranosyl}(2\rightarrow 1)-O-2\text{-sulfonato-}\alpha\text{-fucopyranose}(3\rightarrow \) along with a branch of \( \rightarrow 1)-6\text{-O-methyl-}\beta\text{-galactopyranosyl}(4\rightarrow \) unit at C-4 position of O-2-sulfonato-\alpha\text{-fucopyranose from the brown seaweed Padina tetrastromatica Hauck 1887 (family Dictyotaceae) (Figure 1), and was assessed for its anti-hyperglycemic activities using different in vitro models. The purified polysaccharide was analyzed for its potential to attenuate the proteolytic enzyme DPP-4 and carbolytic enzymes (\( \alpha\text{-amylase and } \alpha\text{-glucosidase). Comprehensive spectroscopic techniques were used for the characterization of the isolated polysaccharide.

2. Results and discussion

2.1. Chemical and monosaccharide compositions of sulfated galactofucan from P. tetrastromatica

The yield of crude sulfated galactofucan (PF) separated from P. tetrastromatica was 5.4% (dry weight basis), and was found to possess 69.01% total sugar and 7.28% of uronic acid, whereas the sulfate and protein contents were 13.01 and 3.83%, respectively (Table S1). The homogenous galactofucan (sub-fraction PF-2) was eluted with 0.2 M NaCl (Figure S1) (through diethylaminoethyl cellulose-52 (DEAE-cellulose-52) anion exchange chromatography), comprising of fucose (61.4%) and galactose (38.6%) as major constituent units (Figure S2A–S2C). Sub-fraction PF-2 recorded a greater yield of carbohydrate (81.32%) and a significant amount of sulfate (12.31%) with lesser protein (0.99%) than other eluted fractions (Table S1). HPLC chromatogram of standard monosaccharides was displayed in Figure S2C. In another study, water extraction of P. tetrastromatica recorded a yield of 8% (Karmakar et al. 2009) with 14% of uronic acid and 54% of fucose content, before precipitation with calcium chloride. Polysaccharide isolated from water extraction of brown seaweed Turbinaria conoides resulted in a yield of 6.2% with 59% of fucose as a major monosaccharide backbone (Chattopadhyay et al. 2010). A total yield of 9% (w/w) was observed in the case of purified sulfated polysaccharide from P. tetrastromatica after acidic extraction and ethanol precipitation, although the amount of carbohydrate content was found to be lesser (Jose et al. 2015) in comparison to that obtained in the present study. Sulfated polygalactopyranosyl fucopyran isolated from a brown seaweed Sargassum wightii was reported 4.5% yield with 56.8% of fucose and 16.4% of galactose as key monosaccharide constituents (Anusree and Chakraborty 2018). A considerable amount of fucose and galactose were found to be present in the polysaccharide isolated from sporophyll of Korean brown seaweed Undaria pinnatifida (Synytsya et al. 2010). Column chromatographic purification with DEAE was used in this study on account of its specificity towards the isolation of negatively charged compounds and devoid of other contaminants (Jose et al. 2015). Based on the percent yield and chemical composition
obtained from column purified fractions (Table S1), PF-2 was subjected to structural characterization (Table S2, Figure S4–S13).

2.2. Spectroscopic description of sulfated galactofucan from P. tetrastromatica

The polysaccharide fraction PF-2 was elucidated as sulfated polygalactofucan (Figure 1) by detailed spectroscopic analyses, such as FTIR, $^1$H-NMR, $^{13}$C-NMR, DEPT-135 coupled with 2D-NMR ($^1$H-$^1$H COSY, HSQC, HMBC, and NOESY), and HR(ESI)MS (Figure S4–S13). Purified polysaccharide fraction (PF-2) was comprised of a repetitive backbone of [→1)-O-4-sulfonato-$\alpha$-fucopyranosyl-(2→1)-O-2-sulfonato-$\alpha$-fucopyranose-(3→] unit, along with a branch of [→1)-6-O-methyl-$\beta$-galactopyranosyl-(4→] entity at the C-4 position of O-2-sulfonato-$\alpha$-fucopyranose isolated from P. tetrastromatica.

![Figure 1](image)

Figure 1. (A) Illustrative photograph of P. tetrastromatica. (B) Polysaccharide characterized as [→1)-O-4-sulfonato-$\alpha$-fucopyranosyl-(2→1)-O-2-sulfonato-$\alpha$-fucopyranose-(3→] along with a branch of [→1)-6-O-methyl-$\beta$-galactopyranosyl-(4→] unit at the C-4 position of O-2-sulfonato-$\alpha$-fucopyranose isolated from P. tetrastromatica.

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$^1$H-NMR spectrum (Figure S5) of hydrolyzed sulfated galactofucan fraction (PF-2) displayed three sets of protons in the region of $\delta_H$ 4.62-5.33, $\delta_H$ 3.10-4.13 and $\delta_H$ 1.11-1.23. The occurrence of these protons in the galactofucan was corroborated by the $^{13}$C-NMR/DEPT/HSQC analyses (Figure S6–S7, and S9). Highly deshielded protons at $\delta_H$ 5.33 ($\delta_C$ 106.7; CH), $\delta_H$ 5.17 ($\delta_C$ 102.5; CH) and $\delta_H$ 4.62 ($\delta_C$ 101.0; CH) were assigned to the anomeric regions (Farias et al. 2000; Ale et al. 2011). The region between $\delta_H$ 4.60-5.70 was associated with C$_1$ anomeric positions of $\alpha$-linked fucopyranose residues (Fucp), and that between $\delta_H$ 4.60-4.80 was attributed to the C$_1$ anomeric position of $\beta$-linked galactopyranose (Galp) units (Anusree and Chakraborty 2018). Therefore, these major signals of sulfated galactofucan ascribed the presence of three glycosidic residues in galactofucan (PF-2). These might be associated with C$_1$ positions of sugar residues of galactofucan. The three sugar residues were labeled as A, B, and C on the origin of their chemical shift values.

On the basis of these data, $\delta_H$ 5.33 ($\delta_C$ 106.7; CH) was assigned to the monosaccharide residue A, which might be associated with the occurrence of Fucp residue. The previous reports have appropriately corroborated these results. For instance, the L-fucopyranosyl residue isolated from echinoderms and polysaccharides from seaweed...
*Turbinaria conoides* exhibited anomeric signals at $\delta_H$ 4.50-5.50 (Mulloy et al. 1994; Chattopadhyay et al. 2010). Similarly, sulfated polysaccharide derived from *Sargassum myriocystum* exhibited signals at $\delta_H$ 5.40 and 4.84 with respect to $\alpha$-anomeric region, and showed the presence of sulfate at position C-4 (Synytsya et al. 2010). The position of these anomeric protons and the various functionalities attached to them were further demonstrated by $^1$H-$^1$H COSY (Figure S8) along with $^1$H-$^1$3C HMBC (Figure S10) experiments. Anomeric H$_1$ proton at $\delta_H$ 5.33 (CH) displayed an intense HMBC correlation with the carbon at $\delta_C$ 84.9 that designated the oxygenated functionality adjacent to H$_1$ anomeric proton. The strong $^1$H-$^1$H COSY correlation was observed between the proton at $\delta_H$ 3.10 (CH; C-2) and $\delta_H$ 3.70 (CH; C-3), which ascertained the attachment of oxygen-bearing adjacent functional group to the CH group at C-2 in unit A. Similarly, the proton at $\delta_H$ 3.75 (CH; C-4) showed a COSY correlation with $\delta_H$ 3.91 (CH; C-5). These correlations together attributed to the presence of a fragment -CH(O)-CH(O)-CH(O)-CH(O)-CH(O)- (from C-1 to C-5) (Figure 1). The deshielded proton at $\delta_H$ 3.75 (CH; C-4) could attribute to the C-4 sulfate in unit A. This data was also supported by FTIR signals at 1254 and 842 cm$^{-1}$ (Figure S4). HMBC correlation from the proton $\delta_H$ 5.33 (H-1) to $\delta_C$ 71.7 (C-5) supported the closure of 6-membered pyranose ring A. HMBC correlation between $\delta_H$ 1.23 (CH$_3$) to $\delta_C$ 71.7 (CH; C-5) attributed to the presence of CH$_3$ group at C-5. This signal at $\delta_H$ 1.23 was characteristic of the methyl group in fucopyranose ring. The normal range of downfield signals of H$_6$ protons in fucopyranose-containing methyl protons was found to reside at $\delta_H$ 1.10-1.25. Thus, the overall COSY correlations along with HMBCs attributed to the presence of sulfate-bearing fucopyranose (-O-4-sulfonato-Fucp) ring A in the sulfated galactofucan, PF-2.

Similarly, the proton at $\delta_H$ 5.17 ($\delta_C$ 102.5; CH) was assigned as an anomeric proton to the monosaccharide residue B. The neighboring functionalities of C-1 in unit B were recognized by COSY and HMBC correlation spectra. The anomeric H$_1$ proton at $\delta_H$ 5.17 (CH) in unit B displayed two HMBC correlations to the carbons at $\delta_C$ 84.9 (C-2 in unit A) and $\delta_C$ 75.5 (CH; C-2 in unit B). HMBC correlation at $\delta_H$ 5.17/$\delta_C$ 84.9 ascribed the connectivity between the C-1 of unit B and C-2 position of unit A. This revealed the (2→1) linkage between the monosaccharide residues A and B. At the same time, HMBC correlation between $\delta_H$ 5.17/$\delta_C$ 75.5 designated the correlation to the adjacent -CH- functionality, which was assigned as C-2 in unit B. $^1$H-$^1$H COSY correlations among the protons $\delta_H$ 4.13/3.80/3.18/3.93 suggested a long continuous fragment in unit B, which was assigned from C$_2$ to C$_5$ \{-CH(O)-CH(O)-CH(O)-CH(O)-CH(O)-\}. Furthermore, unit B exhibited HMBC correlation from $\delta_H$ 3.93 (CH; C-5) to $\delta_C$ 102.5 (CH; C-1), which attributed to the ring closure of pyranose ring B in the galactofucan. Another deshielded proton at $\delta_H$ 4.13 (CH; C-2) was attributable to the sulfate at the position C-2. HMBC correlation from $\delta_H$ 1.11 (CH$_3$) to $\delta_C$ 71.3 (CH; C-5) designated the characteristic methyl at C-5. NMR spectral correlations attributed to the presence of O-2-sulfonato-Fucp residue B in PF-2.

The proton at $\delta_H$ 4.62 ($\delta_C$ 101.0; CH) was assigned as an anomeric proton to the monosaccharide residue C, which might be associated with galactopyranose (Galp) residue (Figure 1). Anomeric signals between $\sim$ $\delta_C$ 101.0 were attributed to the $\beta$-linked galactopyranose residue, whereas other signals at $\delta_C$ 59-84 designated the ring protons of $\beta$-linked galactopyranose units (Rocha et al. 2005). The position of this
anomeric proton and its adjacent functionalities were further corroborated by COSY and HMBC correlations. The C-4 proton at $\delta_H$ 3.18 (CH; C-4) in unit B displayed HMBC correlations to the carbon at $\delta_C$ 101.0 (anomeric C-1) in unit C. This could ascribe the (4→1) linkage between the monosaccharide residues B and C and also recognized that the H$_4$ of -O-2-sulfonato-fucopyranose was connected to C$_1$ of galactopyranose residue. In unison, the COSY correlations between the protons $\delta_H$ 4.62/3.29/3.53; $\delta_H$ 3.39/3.98 recognized a long continuous fragment in unit C, which was assigned as C$_1^{-}$-C$_5$ {−CH(O)-CH(O)-CH(O)-CH(O)-CH(O)-}. Furthermore, unit C exhibited two HMBC correlations from $\delta_H$ 3.98 (CH; C-5) and $\delta_C$ 72.7 (C-6), which indicated the ring closure of galactopyranose C in the galactofucan, and the connection of C-5 proton to neighboring CH$_2$ carbon, respectively. HMBC correlations from the proton at $\delta_H$ 3.32 (CH$_2$; C-6) to carbon at $\delta_C$ 59.6 (-OCH$_3$) could recognize the -OCH$_3$ branching at C$_6$ of unit C. Thus, the COSY correlations along with HMBCs suggested the third monosaccharide galactopyranose residue (Galp) C in PF-2. Therefore, the monosaccharide residues in PF-2 might contain alternate (2→1)/(4→1) linkages in between the sugar residues. NOE correlations (Figure S11) between the protons $\delta_H$ 3.91/5.33/3.10/5.17/3.53/3.93 put forward that these were oriented in the same plane of symmetry, and they were apportioned as $\alpha$-oriented. Similarly, the NOE relationship between the protons $\delta_H$ 3.75/3.18 revealed that those are aligned towards the opposite direction of the $\alpha$-oriented protons, and were assigned as $\beta$-oriented. The linkage pattern of the purified polysaccharide fraction was determined by methylation followed by a series of derivatization reactions. The EI-MS spectrum obtained after partially methylated alditol acetate (PMAA) analysis (Figure S13), recorded three major fragments at $m/z$ 413.07, 385.08 and 350.15, which attributed to the formation of 1,3,4,5-tetra-O-acetyl-fucitol-2-sulfate (unit B), 1,2,5-tri-O-acetyl-3-O-methyl-fucitol-4-sulfate (unit A), 1,4,5-tri-O-acetyl-fucitol-2-sulfate, and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-galactitol (unit C), which corroborated the linkage pattern and structural characterization of the polysaccharide.

UV-vis spectrum of PF-2 showed no characteristic absorption peak between 200-400 nm (Figure S3). FTIR spectrum of galactofucan isolated from P. tetrastromatica exhibited a characteristic broad band at 3407 cm$^{-1}$, which demonstrated the presence of hydroxyl functionalities (Figure S4). Characteristic FTIR absorption band at 2940 cm$^{-1}$ was ascribed to the CH stretching of ring protons of monosaccharide residues (fuco/galacto-pyranose rings). Characteristic FTIR peak at 1254 cm$^{-1}$ was attributed to the sulfate ester functionality in the homogenous galactofucan, whereas two absorption bands at 1200-970 cm$^{-1}$ were caused by C-O-C stretching vibration of the glycosidic bonds along with pyranose C-C and C-O stretching vibrations. Absorption bands at 1037, 819 cm$^{-1}$ could additionally support the structural attribution of presently isolated sulfated galactofucan (Anusree and Chakraborty 2018). Representative absorption bands in PF-2 were in accordance with the polygalactan isolated from Gracilaria caudata (Barros et al. 2013).

ESI-MS analysis of the galactofucan (derived after hydrolysis) was carried out in negative-ion mode owing to the presence of sulfate groups. An intense molecular ion peak occurred at $m/z$ 643.08 could be assigned to the single-charged anion of trisaccharide entities. The spectrum consisted of another two major peaks at $m/z$ 467.02 and $m/z$ 418.08 analogous to the fragments [O-4-SO$_3$-Fucp-O-2-SO$_3$-Fucp]$^{2-}$ and [O-2-
SO3-Fucp-6-OMe-Galp]−, respectively (Figure S12). The spectrum displayed two peaks at m/z 242.01 and m/z 193.07 corresponding to the fragments [O-4-SO3-Fucp]−/[O-2-SO3-Fucp]− and [6-OMe-Galp]−, respectively (Figure S12). The average molecular weight of the sulfated galactofucan (PF-2) was measured from the calibration plot of reference standard dextrans with a range of molecular weight 1-30 KDa, and it was found as 221.0352 kDa (Figure S14). These comprehensive spectroscopic data proposed the structure and the sequence of linkage pattern of glycosidic residues in PF-2 as [→(1)-O-4-sulfonato-α-fucopyranosyl-(2→1)-O-2-sulfonato-α-fucopyranose-(3→] along with a branch of [→(1)-6-O-methyl-β-galactopyranosyl (4→) unit at the C-4 position of O-2-sulfonato-α-fucopyranose (Figure S15).

2.3. Bioactive potential of sulfated galactofucan (PF-2)

The sulfated galactofucan derivative obtained from *P. tetrastromatica* showed considerably greater attenuation potential against DPP-4 (IC50 0.25 mg mL\(^{-1}\)) (Table S3), and was comparable to that exhibited by the positive control (IC50 0.13 mg mL\(^{-1}\)). Likewise, PF-2 exhibited significantly greater inhibition potential against carbohydrate hydrolyzing enzymes α-amylase (IC50 0.98 mg mL\(^{-1}\)) and α-glucosidase (0.87 mg mL\(^{-1}\)) in comparison with positive control acarbose (IC50 1.38 and 1.48 mg mL\(^{-1}\), respectively, p < 0.05). The sulfated galactofucan also exhibited potential quenching properties against the oxidant (DPPH/ABTS) species (IC50 \~0.6 mg mL\(^{-1}\)), which also reinforced its potential capacity to inhibit the oxidant species (Table S3) that could play an essential role in causing hyperglycemia by increasing the advanced glycation end products.

Insulin secretion could be stimulated in a glucose-dependent way by DPP-4 inhibitors, which were also not found to be correlated with hypoglycemia by reason of their glucose-dependent action (Deacon et al. 2012). Also, type-2 diabetes was found to result in excessive glucagon secretion that could be controlled by DPP-4 inhibitors (Alsalim et al. 2018). Therefore, depending upon the stimulatory effect of glucagon and insulin secretion, it might be concluded that DPP-4 inhibitors could effectively attenuate the postprandial hyperglycemia. The correlation between sulfated galactofucan and anti-hyperglycemic activity was found to be related to a complex formation between α-amylase and inhibitor, which might conceivably result in slower diffusion of glucose and its interrupted cellular absorption (Cho et al. 2011). Conspicuously, the electrostatic interface between the enzymes and sulfate group (with a negative charge) of the studied sulfated galactofucan of *P. tetrastromatica* might play a pivotal role to modulate the activities of the carbolytic enzymes. DPP-4 is an oligopeptidase, which was reported to possess a leading role to cause hyperglycemic conditions. The attenuation capacity of seaweed-originated galactofucan against DPP-4 could be significant to ascribe its potential anti-diabetic property.

3. Experimental

3.1. Materials and instrumentation

HPLC and analytical-grade solvents and chemicals were procured from E-Merck (Germany), HiMedia (HiMedia Laboratories, LLC, Kelton, PA), and Sisco Research
Laboratories Pvt. Ltd. (SRL, India). The enzymes α-amylase (from porcine pancreas), α-glucosidase (from yeast), and dipeptidyl-peptidase-4 (from porcine kidney, DPP-4) were obtained from Sigma-Aldrich Chemical Co. Inc. (St. Louis, MO). Fourier transform infrared (FTIR) spectral analysis was carried out in between 4000-500 cm⁻¹ (FTIR Perkin-Elmer 2000; Waltham, MA) with potassium bromide pellets. The UV analysis was performed with a spectrophotometer (UV-VIS Varian Cary 50; Palo Alto, CA). One and two-dimensional nuclear magnetic resonance (NMR) including ¹H-NMR (500 MHz), ¹³C-NMR (125 MHz), distortionless enhancement of polarization transfer (DEPT), homonuclear correlation spectroscopy (¹H-¹H COSY), heteronuclear multiple bond correlation (¹H-¹³C HMBC), heteronuclear single-quantum correlation (HSQC), and nuclear overhauser effect (NOE) were recorded with a Bruker Avance DPX 500 MHz NMR spectrometer (Bruker Co., Billerica, MA) using deuterated water (D₂O) with tetramethylsilane (TMS) as an internal standard (δH 0 ppm). The protons (H) in samples/sulfated galactofucan were substituted with deuterium (D) by sequential freeze-drying in D₂O (>99.9%) (Anusree and Chakraborty 2018). The other acquisition parameters for NMR spectral data were given in Table S4. High-resolution electrospray ionization mass spectral (HRESI-MS) analysis was accomplished in ESI (-) method on the mass spectrometer (Agilent Q/TOF LC-MS/MS, Santa Clara, CA) combined with a high-pressure liquid chromatography (HPLC) fitted out with a RP (reverse-phase) octadecylsilane bonded (C₁₈) column (1.8 μm, 2.1 mm × 5 cm). HPLC analysis (Shimadzu Corp, Nakagyo-Ku, Japan) was accomplished by means of evaporative light scattering detector (ELSD, SoFTA 300S, Teledyne Technologies Inc., Lincoln NE) coupled to an amino column (Luna 250 × 4.6 mm, 5 μm)/C18 reverse phase column (Phenomenex, Torrance, USA) and a binary gradient pump.

3.2. Sample collection and initial processing

The seaweed material, *P. tetrastromatica* (20 kg) (voucher number AB.1.1.2.1) was obtained from the region of south east coastal region of Indian peninsular, specifically, from the Gulf of Mannar region of Mandapam 8°48’ N, 78°9’ E and 9°14’ N, 79°14’ E. The identification of seaweed was confirmed with the sample specimens preserved in the recognized biodiversity repository (Marine Biodiversity Museum, Central Marine Fisheries Research Institute, India). The sample was washed and shade-dried for one week at room temperature before being ground to homogeneity.

3.3. Preparation of crude polysaccharide from brown seaweed *P. tetrastromatica*

The pre-processed and dried seaweed powder of *P. tetrastromatica* was extracted by a previously defined method (Anusree and Chakraborty 2018) with modifications. Briefly, 500 g of powdered seaweed was refluxed in acetone (500 mL) at about 36 °C, for 3-4 h and filtered over Whatman no 1 filter paper, whereas the residual depigmented seaweed material was extracted twice with neutrally reacting water (1:3, w/v) at 80-85 °C for 4-6 h. The extract was filtered over 0.45 μ nylon cloth to obtain the filtrate (2.5 L). The latter was treated with trichloroacetic acid (TCA, 5% w/v) before being kept at 25-
27 °C for 2 h, and clarified by centrifugation (at 8000 × g for 30 min, 4 °C, Sorvall Biofuge Stratos, Thermo Scientific, MA). The crude aqueous extract (2.0 L) was precipitated with calcium chloride (CaCl₂, 1% w/v), and the contents were subjected to centrifugation (10000 g for 15 min, 4 °C) to isolate the calcium salt of residues. The supernatant (∼1200 mL) was concentrated to one-tenth of the initial volume (120 mL) by a rotational vacuum concentrator (RVC-2-33-IR; Martin Christ, Gefriertrocknungsanlagen GmbH) and allowed to cool. Ice-cold ethyl alcohol (∼800 mL) was added to the concentrate and kept at 4-6 °C for about 12 h to precipitate out the crude polysaccharide (PF) (27 g, 5.4% yield).

3.4. Purification and chemical characterization of sulfated galactofucan

Anion exchange chromatography with diethylaminoethyl (DEAE) cellulose was used for the purification of crude polysaccharide on an open glass column (25 cm × 4 cm) (Chakraborty et al. 2021). Slurry was prepared with DEAE-cellulose (4 g) in tris buffer (20 mL, 0.05 M, pH 7.4) before being kept for swelling for at least one hour followed by packing the column with the same using tris-buffer. The aqueous solution (15 mL) of crude polysaccharide (2.5 g) was applied to the pre-packed column, and was washed with several column-volumes of tris buffer followed by initial elution using distilled water and different concentration of NaCl (0-0.5 M) to produce four different fractions (PF-1 through PF-4) (Figure S1), which were subjected to total carbohydrate estimation (Supplementary material S1) for positive chemical reaction of polysaccharide (Miller 1959). Purified fractions were freeze-dried before being subjected to HPLC analysis (with acetonitrile-water, 80:20, v/v as mobile phase) for monosaccharide composition analysis (Figure S2A), and purity by an evaporative light scattering detector (ELSD, SoFA 300S, Teledyne Technologies Inc., Lincoln NE) attached in sequence with an amino column (25 × 0.46 cm, 5 μm) and binary gradient pump (Figure S2B). The polysaccharide fractions (∼20 mg) were hydrolyzed using trifluoroacetic acid (1 M) for monosaccharide compositional analysis (Supplementary material S1). Based on the monosaccharide compositional studies and total carbohydrate content, the fraction PF-2 was further characterized by spectroscopic experiments. Electrospray ionization (negative-ion mode)-coupled mass spectroscopic (ESI-MS) experiment of the hydrolyzed sulfated galactofucan deduced the molecular weight. Molecular weight of the purified fraction was also determined by the gel permeation chromatography (Supplementary material S1). Total carbohydrate content of the purified PF-2 was assessed by the dinitrosalicylic acid method (Miller 1959) and uronic acid by carbazole method (with standard D-glucuronic acid) (Bitter and Muir 1962). Sulfate content was assessed using barium chloride-gelatin turbidimetric experiment (Dodgson and Price 1962) and protein content was analyzed by Lowry’s method with bovine serum albumin as the standard (Lowry et al. 1951) (Supplementary material S1). All analytical parameters were expressed as percent weight of the purified PF-2. Structural elucidation of the sulfated galactofucan was carried out by mass, FT-IR, and NMR spectroscopic analysis. Glycosidic linkage analysis was determined by permethylation followed reduction and acetylation of the purified polysaccharide fraction (Supplementary material S1).
Effect of sulfated galactofucan on attenuating dipeptidyl-peptidase-4 and radical scavenging activities

The sulfated galactofucan (PF-2) was analyzed for its potential to attenuate the proteolytic enzyme dipeptidyl-peptidase-4 (DPP-4) and inhibiting the oxidant species (2, 2-diphenyl-2-picryl-hydrazil, DPPH and 2,2′-azino-bis-3-ethylbenzothiozoline-6-sulfonic acid, ABTS) were evaluated by different in vitro models (Wojdylo et al. 2007; Anusree and Chakraborty 2018). Abilities of the sulfated galactofucan to inhibit the carbolytic enzymes (α-amylase and α-glucosidase) were also assessed (Hamdan and Afifi 2004; Chakraborty and Antony 2019). The activities were expressed as IC50 (mg mL−1), the concentration at which it attenuates 50% of the enzyme (DPP-4 and α-amylase/glucosidase) and quenches 50% of the oxidant species (Supplementary material S2).

Data analysis

The data of bioactive potentials were tabulated as mean of triplicate ± standard deviation. These data were statistically evaluated by using the software SPSS (SPSS Inc., Chicago, USA, version 13.0), by one-way analysis of variance (ANOVA) to obtain the significant differences between the values. The significant differences were considered as p < 0.05.

Conclusions

The phaeophyta seaweed, P. tetrastromatica demonstrated to be a valuable natural resource of bioactive sulfated galactofucan exhibiting potential activities against the proteolytic DPP-4. The present study isolated and assessed anti-hyperglycemic activities of the sulfated galactofucan, which was characterized as [→1)-O-4-sulfonato-α-fucopyranosyl-(2→1)-O-2-sulfonato-α-fucopyranose-(3→] along with a branch of [→1)-6-O-methyl-β-galactopyranosyl-(4→] unit at the C-4 position of O-2-sulfonato-α-fucopyranose as repetitive major structural component, from the aqueous extract of P. tetrastromatica. In vitro studies on DPP-4 and carbolytic enzymes (IC50 lesser than 1 mg mL−1) with the sulfated galactofucan showed its promising attenuation potential against these enzymes with respect to normal control. The isolated sulfated galactofucan could be developed as a novel bioactive agent attenuating the hyperglycemia-related disorders.

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Data availability

The chromatographic and spectroscopic spectral data are included as supplementary item.
Disclosure statement

No potential conflict of interest was reported by the authors.

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