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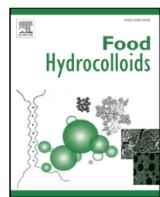


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## Dietary fiber from Indian edible seaweeds and its *in-vitro* prebiotic effect on the gut microbiota

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### ABSTRACT

In the present study, the proximal composition of selected Indian seaweeds such as *S. wightii*, *E. compressa*, *A. spicifera* and their polysaccharide extraction by various enzymes (pancreatin, cellulase and pepsin) was carried out along with its detailed characterisation. In addition, their antioxidant activity and prebiotic score was evaluated with *L. plantarum* and *S. typhimurium*. SCFA composition was analyzed by liquid chromatography. Dietary fiber was found to be maximum in *E. compressa* ( $60.64 \pm 2.2\%$ ). The polysaccharide obtained by combined enzyme assisted extraction exhibited higher antioxidant activity. *E. compressa* (1.44) exhibited the highest prebiotic activity score, followed by *S. wightii* (1.42) and *A. spicifera* (0.84). Plantaricins of *L. plantarum* exhibited equal and enhanced inhibition activity of  $1.3 \pm 0.1$  cm against gut pathogens such as *S. aureus*, *S. flexneri*, *E. coli* and *S. typhimurium*, while compared to MRS broth cultivation. The results indicate that the combined enzyme extract of seaweed polysaccharides could act as a potential prebiotic compound with extraordinary antioxidant activity and prebiotic efficiency.

### 1. Introduction

Decreased and imbalanced level of beneficial gut microbes (probiotics) results in weak immune system, heightened inflammation and other health problems. A high fiber diet can boost beneficial bacterial level in the gut. Marine macroalgal seaweeds are found to be a prime untapped resource of the prebiotic compound called dietary fibers. Polysaccharides constitute about 76% among various bioactive compounds present in seaweeds (Shofia, Jayakumar, Mukherjee, & Chandrasekaran, 2018). Other than polysaccharides, seaweeds are also composed of bioactive compounds like phenolics, flavonoids, proteins, terpenes, phlorotannins, etc. At recent times, investigation on seaweeds has obtained considerable attention for its splendid bioactive components and their properties. Polysaccharides, the biological macromolecule from seaweeds exhibited immune modulating (Okolie et al., 2017), antitumour (Shofia et al., 2018), anti-obese (Sun et al., 2018), anti-inflammatory (Fernando et al., 2018), antimicrobial (Sanjeeva et al., 2018) and antidiabetic effects (Motshakeri, Ebrahimi, Goh, Matanjun, & Mohamed, 2013; Sun et al., 2018). Seaweed polysaccharides, an emerging source of prebiotics, are resistant to digesting enzymes and act as a specific carbon source to enhance gut microbial

activity. Prebiotics boost immune response by modulating gut microbial activity and production of SCFA (short chain fatty acids) (Chen et al., 2018).

The bioactivity of the polysaccharides is correlated to the method of extraction. Enzyme aided extraction (EAE) because of its biocompatibility, high catalytic efficiency, non-toxicity, eco-friendly nature and desired food grade level is mostly preferred in large scale (Okolie, 2018; Charoensiddhi, Conlon, Franco, & Zhang, 2017). Other methods are found to be non-eco-friendly due to their requirement of organic solvents, energy and time consumption. Viscozyme assisted greater yield of polysaccharide from red seaweed *O. pinnatifida* (Rodrigues et al., 2015). Bacteriocins produced by probiotics enhanced the host immunity by providing protection against infective pathogens. Bacteriocins were used in the treatment of head and neck squamous cell carcinoma (HNSCC) because of its increased affinity towards negatively charged cancer cells (Dicks, Dreyer, Smith, & Van Staden, 2018). To the best of our knowledge, there were no recent studies found reporting the characteristics of different seaweed polysaccharides with its antioxidant, prebiotic and plantaricin activity.

The main objective of this study is to estimate the proximal composition of the collected seaweeds such as *S. wightii* (brown), *E.*

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*compressa* (green) and *A. spicifera* (red) to evaluate the extraction efficiency of the seaweed polysaccharides by single and combined enzyme aided extraction (EAE). Characterisation of the extracted polysaccharides is carried out by UV visible spectroscopy, FTIR spectrum, E-SEM, XRD and HPLC. Moreover, we have also evaluated the antioxidant activities and the prebiotic potentiality. Prebiotic score was calculated based on growth stimulating effect towards *L. plantarum* NCIM 2083 and *S. typhimurium* MTCC 3224. Analysis of monosaccharide composition and fermented end products (SCFA) profiling were carried out by HPLC that assist stimulating the host immune response along with the combined action of probiotics. Further, plantaricin activity was studied against gut pathogens like *S. aureus*, *S. flexneri*, *E. coli* and *S. typhimurium* by well diffusion assay.

## 2. Materials and methods

### 2.1. Collection and processing of seaweeds

Seaweeds of different classes such as *S. wightii* (brown), *E. compressa* (green), *A. spicifera* (red) were collected freshly from the southern coastal regions of Tamilnadu (8.7563° N, 78.1791° E) during the month of August 2018 and was authenticated by Central Marine Fisheries Research Institute (CMFRI, Tuticorin, India). They were packed in a fresh sterile polythene bag and brought to lab in sterile condition. The collected seaweeds were shade dried after several washing to remove debris and other salt contents. Then it was fine powdered, sieved to a uniform size of 40 µm and stored. Analytical grade chemicals and reagents were purchased from Sigma Aldrich (USA) and Himedia (India). Enzymes (protease-300 tyrosine Units/ml, α-amylase-3000 Units/ml, amyloglucosidase-3300 Units/ml, pancreatin-8 NF/USP, cellulase- 10 Units/mg, and pepsin-10000NF U/mg) were components of the Megazyme kit (K-TDFR-100A, Ireland).

### 2.2. Proximate composition

The total carbohydrate was measured by the Anthrone method using glucose as standard (Seifter, Dayton, Novic, & Muntwyler, 1950). The protein content was analyzed by Bradford method using bovine serum albumin as standard. The total dietary fibre was estimated by enzyme gravimetric method (AOAC – 991.43) as described by Proskey (1992). Fat content by soxhlet extraction (AOAC – 2003.05), acid and alkaline hydrolysis for crude fibre estimation (AOAC – 962.09), moisture (AOAC – 925.10), ash/mineral (AOAC – 923.03) by hot air furnace combustion was determined as per the standard methods of AOAC (2005).

### 2.3. Extraction with hot water (HWE)

Polysaccharides were extracted by dissolving crude seaweed powder in water (1:10, v/v) with pH adjusted to 2 using 1 N HCl and incubated in boiling water bath for 3 h. The solid biomass was removed after filtration and the filtrate was centrifuged at 7500 rpm at 20 °C for 10 min. Three volume of ethanol was added to the collected supernatant. The polysaccharide pellets were obtained by centrifugation at 7500 rpm at 20 °C for 10 min. The obtained pellets were then dissolved in water and lyophilized. The total polysaccharide content was measured by Anthrone method (Fan et al., 2011).

### 2.4. Enzyme aided extraction (EAE) of seaweed polysaccharides

#### 2.4.1. Pretreatment

The powdered seaweed samples were subjected to cold water extraction for 3 h to remove debris and the collected biomass was air dried in hot air oven. The dried biomass was defatted by treating with petroleum ether and the protein content was removed by treating with sewag reagent (chloroform: t-butanol at ratio 4:1) for 3 h and air dried.

The dried biomass was used for further enzymolysis extraction (Moumita, Das, Hasan, & Jayabal, 2018). The polysaccharide extract of seaweeds (PES) were obtained by enzyme assisted extraction (EAE) using pancreatin as single enzyme (PES-PAE), cellulase along with pepsin (PES-CPAE) and pancreatin-cellulase-pepsin as combined enzymes (PES- PCPAE).

#### 2.4.2. PES-PAE

The pretreated seaweed sample was taken along with buffer solution at the ratio of 1:10. To the mixture, pancreatin (phosphate buffer, pH 7) was added and kept in water bath at 50 °C for 3 h. The enzymes were inactivated by heating at 100 °C for 10 min. The extract was filtered and centrifuged at 7500 rpm at 20 °C for 10 min. Three volume of ethanol was added to the supernatant to precipitate the polysaccharides. The polysaccharide pellets were collected by centrifuging at 7500 rpm for 10 min. The collected polysaccharide pellets were freeze dried and lyophilized (Chiang & Lai, 2018).

#### 2.4.3. PES-CPAE

The pretreated seaweed sample was taken along with buffer solution at the ratio of 1:10. To the mixture, cellulase along with pepsin (acetate buffer, pH 4.5) were added and kept in water bath at 50 °C for 3 h. The enzymes were inactivated by heating at 100 °C for 10 min. The extract was filtered and centrifuged at 7500 rpm at 20 °C for 10 min. Three volume of ethanol was added to the supernatant to precipitate the polysaccharides. The polysaccharide pellets were collected by centrifuging at 7500 rpm for 10 min. The collected polysaccharide pellets were freeze dried and lyophilized (Chiang & Lai, 2018).

#### 2.4.4. PES-PCPAE

The pretreated seaweed sample was taken along with acetate buffer (pH 4.5) at the ratio of 1:10. To the mixture, cellulase along with pepsin (acetate buffer, pH 4.5) was added and kept in water bath at 50 °C for 3 h. Pancreatin was added to the mixture after adjusting the pH to 7 and incubated at 50 °C for 3 h. The enzymes were inactivated by heating at 100 °C for 10 min. The extract was filtered and centrifuged at 7500 rpm at 20 °C for 10 min. Three volume of ethanol was added to the supernatant to precipitate the polysaccharides. The polysaccharide pellets were collected by centrifuging at 7500 rpm for 10 min. The collected polysaccharide pellets were freeze dried and lyophilized (Garcia-Vaquero, Rajauria, O'Doherty, & Sweeney, 2017). The lyophilized samples were dissolved and made into concentration of mg/ml for further analysis and total polysaccharide content was determined by Anthrone method.

## 2.5. Characterisation of seaweed polysaccharides

#### 2.5.1. UV-Vis spectrum scan

The enzymatic seaweed polysaccharide extracts (1 mg/ml) were analyzed using UV-Vis spectrophotometer (UV-Vis; Shimadzu, Japan) for its purity. The polysaccharide extracts were subjected to a spectral scan of 200–400 nm. The graph was plotted from the data obtained to identify other possible absorption.

#### 2.5.2. E-scanning electron microscope (E-SEM)

The morphological characteristics of lyophilized seaweed polysaccharide were examined using Environmental scanning electron microscope (Nova Nanosem 450, USA). The samples were fixed over a black carbon tape and observed under E-SEM to evaluate the morphological characteristics.

#### 2.5.3. X-ray diffraction (XRD)

The X-ray diffraction (XRD) pattern of the lyophilized polysaccharides was measured at room temperature by Ultima IV diffractometer (Japan). The data were collected in the 2-theta range of 5–80° with a step size of 0.05° and a counting time of 20°/min. The

particle size and degree of crystallinity was calculated from the graph obtained by plotting 2-theta value against intensity using Scherrer equation as given in Eq. (1):

$$D_p = \left( \frac{0.94}{\beta} * \frac{\lambda}{\cos\theta} \right) \quad (1)$$

where,  $D_p$  is the average crystallite size in nm,  $\beta$  is the line broadening in radians,  $\theta$  is the Bragg angle in degree,  $\lambda$  is the wavelength of X-ray spectrum in nm.

#### 2.5.4. Fourier transform infrared spectroscopy (FTIR)

FTIR spectroscopy with modern software procedure evidenced to be an esteemed tool for analysis and characterisation of the compounds. The enzymatic seaweed polysaccharide extract were analyzed using Fourier Transform Infrared Spectrophotometer (FTIR; Shimadzu 8201 PC, Japan). The FTIR spectral scan for identification of functional groups was carried out between the frequency  $4000\text{ cm}^{-1}$  to  $400\text{ cm}^{-1}$ . Each functional group produces unique peak at a selective frequency because of its unique conformation. The peaks generated are interpreted with the corresponding functional group associated with the compound.

#### 2.5.5. High performance liquid chromatography (HPLC)

The enzymatic seaweed polysaccharide extract was filtered using a syringe filter of  $0.2\text{ }\mu\text{m}$  and the filtrate volume of  $20\text{ }\mu\text{L}$  was introduced into the Agilent C<sub>18</sub> Eclipse plus column ( $5\text{ }\mu\text{m}^*4.6*250\text{ mm}$ ) of reverse phase liquid chromatography system (Shimadzu, Japan). Sulphuric acid (0.05%) was used as the mobile phase; the flow rate and the temperature were maintained at  $0.7\text{ mL/min}$  and  $35\text{ }^\circ\text{C}$ , respectively. The detection was carried out by refractive index detector over a time period of 60 min. Standards like sucrose, xylose, galactose, arabinose, glucose, maltose, cellobiose, fructose, ribose, butyric acid, ethanol, propionic acid and acetic acid were run and their retention time along with their calibration curve were noted for reference.

#### 2.6. Antioxidant activities

The seaweed polysaccharide extract ( $1\text{ mg/ml}$ ) was prepared to study the antioxidant activities. Total phenolic content (TPC) was determined according to the method described by Waterhouse (2002) using gallic acid as standard and Folin-Ciocalteu reagent. Total flavonoid content (TFC) was estimated as per the method described by Chang, Yang, Wen, and Chern (2002) using quercitin as standard. DPPH and ABTS radical scavenging activity was estimated using the method described by Fan et al. (2011). Ferric reducing power assay (FRAP) was evaluated according to the method of Yildirim, Mavi, and Kara (2001).

#### 2.7. Prebiotic activity assay

The prebiotic activity assay was performed to check the growth promoting effect of the seaweed polysaccharides for the probiotic bacteria *Lactobacillus plantarum NCIM 2083* with respect to enteric pathogen *Salmonella typhimurium MTCC 3224*. The prebiotic study correlates the potentiality of seaweed polysaccharides with glucose to enhance the growth of probiotic strain in relative to the enteric strain. The bacterial sp. *L. plantarum* and *S. typhimurium* was grown in a MRS broth and nutrient broth for 24 h, respectively, and then they were subcultured. The bacterial pellet was collected by centrifuging at 5000 rpm for 20 min and the collected pellet was resuspended in 5 ml of 0.9% saline. The prebiotic assay was carried out by adding 1% of an overnight culture of a probiotic strain to MRS broth containing 1% (wt/vol) glucose and 1% (vol/vol) polysaccharide and kept in shaking incubator at  $37\text{ }^\circ\text{C}$ . Plating was carried out on MRS agar for 0<sup>th</sup> and 24<sup>th</sup> h and the number of colonies was counted. Simultaneously, for enteric strain *S. typhimurium* was added at 1% to a nutrient broth containing 1% (wt/vol) glucose or 1% (vol/vol) prebiotics. They were incubated at  $37\text{ }^\circ\text{C}$  in

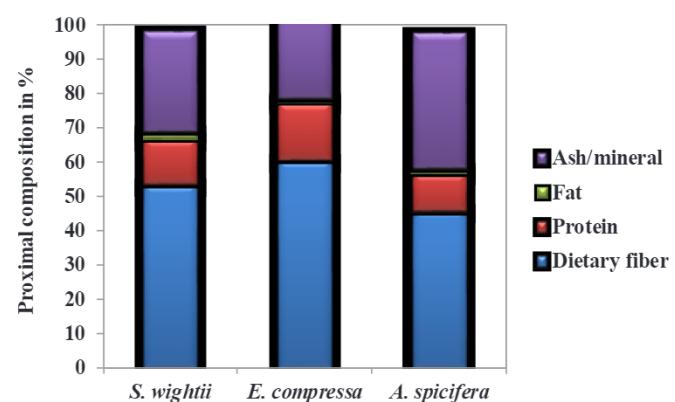


Fig. 1. Proximal composition of the selected Indian seaweed samples.

Table 1

Moisture and crude fiber content of the selected Indian seaweed samples.

Sample	Moisture (%)	Crude fiber (%)
<i>S. wightii</i>	$5.67 \pm 0.55$	$2.49 \pm 0.67$
<i>E. compressa</i>	$4.91 \pm 0.73$	$2.32 \pm 1.22$
<i>A. spicifera</i>	$5.13 \pm 0.49$	$3.19 \pm 0.85$

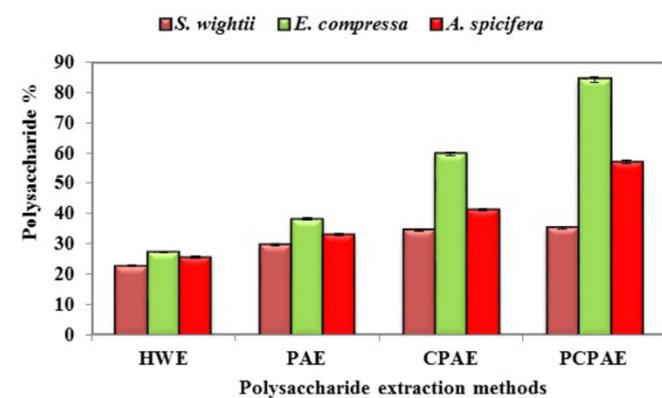


Fig. 2. Yield of polysaccharide from macroalgal seaweeds by various extraction techniques.

an incubator. After 0 and 24 h of incubation, *S. typhimurium* was plated on Hektoen enteric agar and incubated and the colonies were counted. The prebiotic activity score was determined by the following Eq. (2):

$$\text{Prebiotic activity score} = \frac{\log P_X^{24} - \log P_X^0}{\log P_G^{24} - \log P_G^0} - \frac{\log E_X^{24} - \log E_X^0}{\log E_G^{24} - \log E_G^0} \quad (2)$$

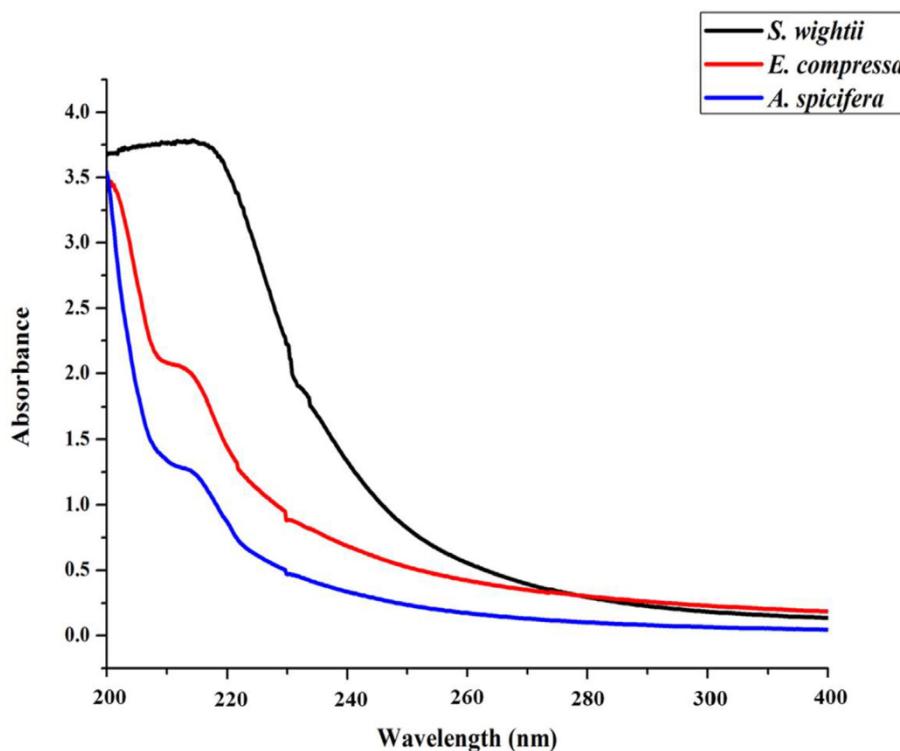
$P_G^0, P_X^0, P_G^{24}, P_X^{24}$  – Probiotic CFU count of glucose (G) and seaweed polysaccharide (X).  $E_G^0, E_X^0, E_G^{24}, E_X^{24}$  – Enteric CFU count of glucose (G) and seaweed polysaccharide (X).

#### 2.7.1. SCFA profiling

The probiotic cultured broth served with seaweed polysaccharides was collected after 24 h incubation time and centrifuged to remove the bacterial pellets. The collected supernatant was syringe filtered and injected into HPLC column of above mentioned conditions (section 2.5.5) to determine the presence of short chain fatty acids.

#### 2.7.2. Plantaricin activity by well diffusion assay

*L. plantarum NCIM 2083* was initially inoculated in MRS broth, where the carbon source was replaced by 1% PES of *S. wightii*, *E. compressa*, and *A. spicifera*, respectively. The 24 h grown culture was used for studying plantaricin activity against various gut pathogens like *S. aureus*, *S. flexneri*, *E. coli* and *S. typhimurium*. The gut pathogens were



**Fig. 3a.** UV-Vis spectra of the polysaccharide extracts.

cultured, maintained and inoculated in their respective agar plates. The zone of inhibition was studied by infusing different concentrations (50, 100, 150 µl) of 24 h grown *L. plantarum* culture. MRS broth without inoculum was used as control.

#### 2.8. Statistical analysis

All experiments were performed in terms of triplicates. Mean and standard deviations were calculated, and the data was reported in standard format with error bar. Statistical calculations were made using the graphpad software, and differences were analyzed.

### 3. Results and discussion

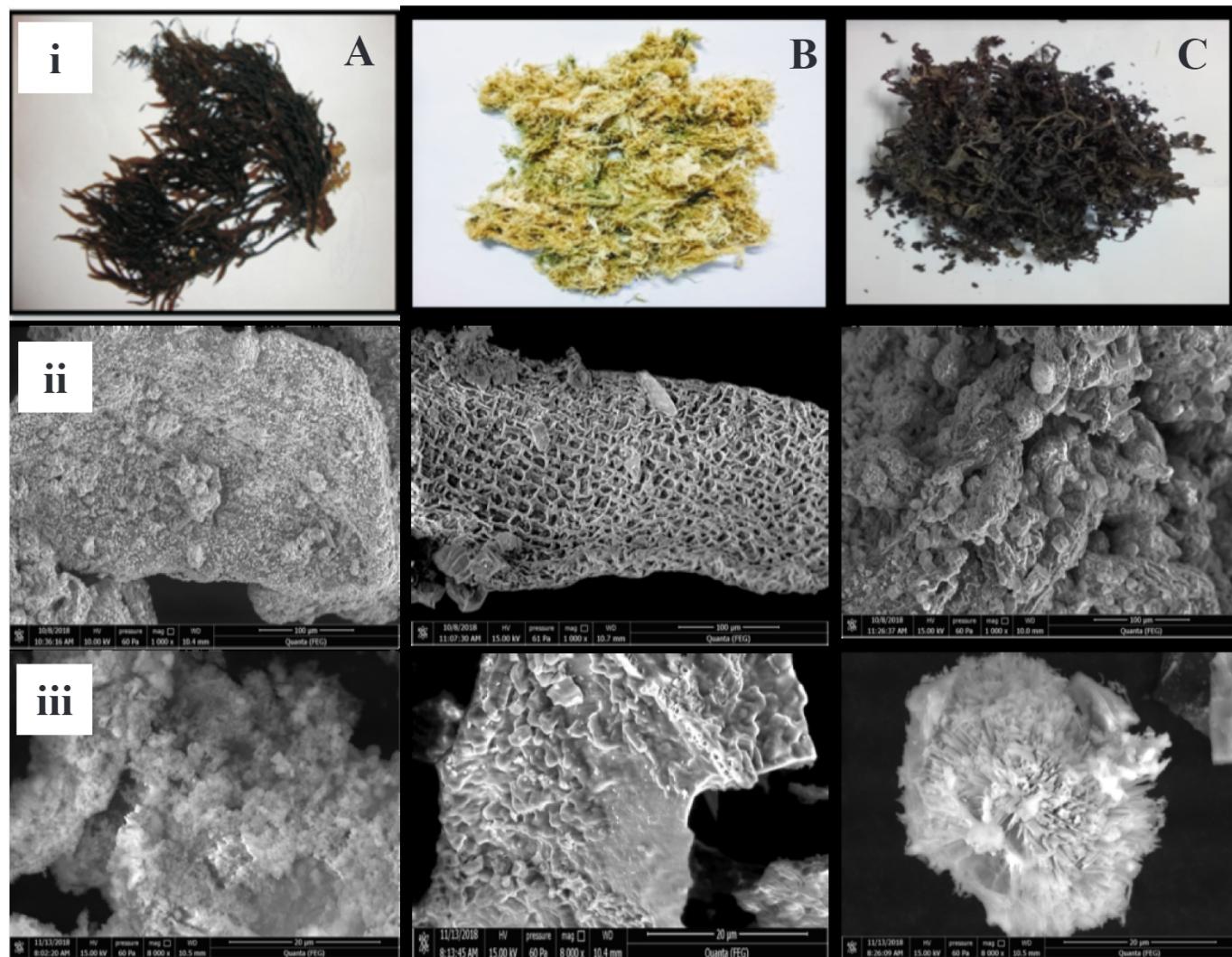
Three species of marine macroalgal seaweeds named *S. wightii*, *E. compressa* and *A. spicifera* belonging to Phaeophyceae (Brown), Chlorophyceae (Green) and Rhodophyceae (Red) family were collected from Tuticorin pearl harbour (southern coastal region of India) for analyzing their proximal composition, as well for determining the extraction efficiency of non-digestible seaweed polysaccharides (dietary fibers) by using digestive enzymes like pancreatin, cellulase and pepsin.

#### 3.1. Proximal composition

Characterisation of the extracted polysaccharides was done by using UV-Vis spectrum for determining their purity, FTIR for analyzing respective functional groups, E-SEM and XRD for structural analysis and HPLC for monosaccharide and SCFA profiling. The antioxidant activity, prebiotic effect and plantaricin activity were also studied. Proximal composition of the selected Indian seaweeds was shown in Fig. 1. Dietary fiber, the non-digestible polysaccharides acts as a potential prebiotic source that boost immune response by enhanced activity of beneficial gut microbes and SCFA production. Dietary fiber present in the collected seaweed samples were evaluated by enzyme gravimetric method. It was noted that *E. compressa*, the green seaweed was exhibiting higher dietary fiber content of about  $60.64 \pm 2.2\%$  followed

by *S. wightii* ( $53.52 \pm 1.5\%$ ) and *A. spicifera* ( $45.79 \pm 3.1\%$ ). Soluble dietary fibers delay the nutrient uptake that results in less production of glucose. Soluble dietary fiber blocks the absorption of bile acids and oestrogen reducing blood cholesterol level and risk of breast cancer (De Jesus Raposo et al., 2016). Insoluble dietary fiber increase stool weight and reduce the waste travelling time eliminating pre-carcinogen at earlier level. Dietary fibers after fermentation increase the biomass of the health beneficial bacteria like *Lactobacillus* sp. and *Bifidobacterium* sp. and also eliminate pathogens. Production of fermented end product like SCFA (propionic acid, butyric acid and acetic acid) reduces the risk of colorectal cancer (Ulmius, 2011). Brown seaweed *Sargassum wightii* contains dietary fiber of range 17–58% (Kumar, Sahoo, & Levine, 2015; Syad, Shunmugiah, & Kasi, 2013) which supports the present study. It was reported that green seaweed *E. compressa* contains total dietary fiber of  $55.4 \pm 2\%$  (Patarra, Paiva, Neto, Lima, & Baptista, 2011) which is quite matching with the dietary fiber of *E. compressa* in this present study. It was reported that red seaweed *Porphyridium* sp. comprised of 45% dietary fiber similar to that of *A. spicifera* ( $45.79 \pm 3.1\%$ ) in the present study (De Jesus Raposo et al., 2016).

The seasonal changes and environmental conditions like tidal variation play an important role in determining the proximal composition of the seaweeds. Protein content was found higher in *E. compressa* ( $16.85 \pm 0.65\%$ ) followed by *S. wightii* ( $13.2 \pm 0.54\%$ ) and *A. spicifera* ( $10.89 \pm 0.78\%$ ). The obtained proximal results are well in accordance with the previously reported studies of Kumar, Bhatla, and Sahoo (2013) and Manivannan, Thirumaran, Devi, Hemalatha, and Anantharaman (2008). The fat or lipid composition of seaweeds is found to be at very low concentration. *S. wightii* was exhibiting the maximum lipid composition of  $2.33 \pm 0.92\%$  followed by *A. spicifera* ( $1.57 \pm 0.77\%$ ) and *E. compressa* ( $1.22 \pm 0.96\%$ ). The ash/mineral content was found maximum in red seaweed *A. spicifera* ( $39.35 \pm 0.73\%$ ) followed by the *S. wightii* ( $28.95 \pm 0.68\%$ ) and *E. compressa* ( $25.42 \pm 0.87\%$ ). Table 1 shows the composition of crude fiber and moisture content among the various seaweed samples.



**Fig. 3b.** Pictorial image of i) A) *S. wightii* B) *E. compressa* C) *A. spicifera* and its E-SEM structural analysis of ii) crude and iii) lyophilized polysaccharides.

### 3.2. Extraction efficiency

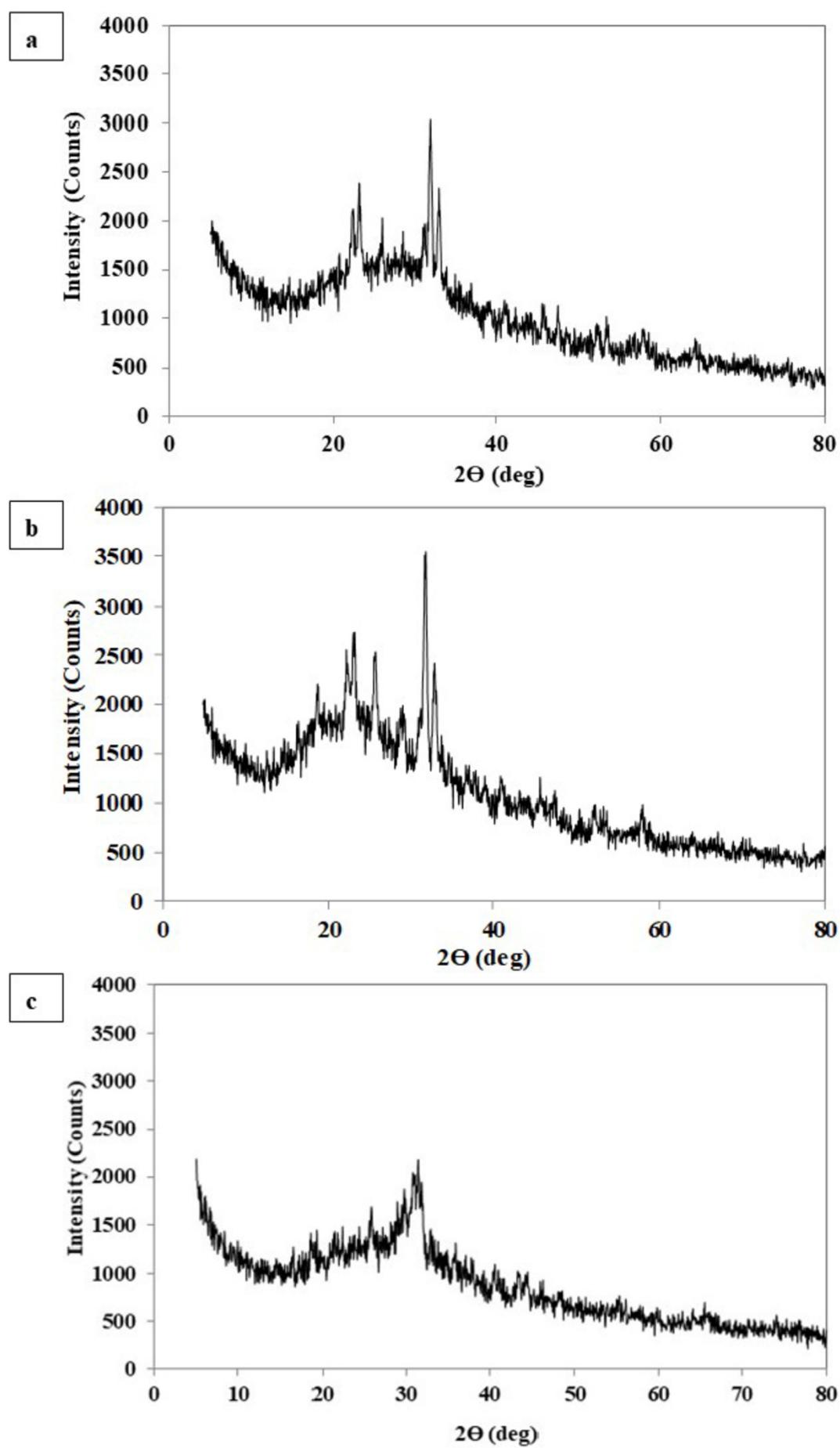
The seaweed polysaccharides were obtained from the three species *S. wightii*, *E. compressa* and *A. spicifera* by hot water extraction and enzyme aided extraction methods. The extraction efficiency was found increasing in an order that follows HWE < PAE < CPAE < PCPAE. The total polysaccharide content was estimated by Anthrone method. Enzymes can degrade the complex cell wall and integral structure that elicits and expands the polysaccharide extraction efficiency. It was found that PCPAE yields higher amount of polysaccharide when compared to the other cases shown in Fig. 2. PCPAE yield polysaccharides of about  $84.31 \pm 0.86\%$  in the green seaweed *E. compressa* which was followed by *A. spicifera* ( $56.95 \pm 0.58\%$ ) and *S. wightii* ( $35.36 \pm 0.36\%$ ). The polysaccharide yield in single enzymolysis by PAE was found to be higher in *E. compressa* ( $38.26 \pm 0.28\%$ ). CPAE yields about  $59.73 \pm 0.63\%$  of polysaccharides from green seaweed *E. compressa* followed by *A. spicifera* ( $41.35 \pm 0.42\%$ ) and *S. wightii* ( $34.43 \pm 0.35\%$ ). In HWE, the polysaccharide content was found higher in *E. compressa* ( $27.32 \pm 0.28\%$ ) followed by *A. spicifera* ( $25.53 \pm 0.26\%$ ) and *S. wightii* ( $22.69 \pm 0.23\%$ ). Pancreatin with protease and lipase degrades protein and lipid content in the seaweeds which in turn improves the extraction efficiency. Rodrigues et al. (2015) in his study reported that cellulase is responsible for the maximum polysaccharide yield of about 60–62% in *C. tomentosum*. Other benefits like enzyme flexibility and hardy tough nature is associated

with the characteristics of cellulase that raise the effectiveness of the extraction. Polysaccharides such as alginates, mannans, carrageenans and agars are non-digestible by human gut enzymes. The proteins are been degraded into polypeptides by the enzymes used which results in high polysaccharide content with less protein (Fan et al., 2011). The selective use of particular digestive enzymes pancreatin, cellulase and pepsin are biocompatible and less toxic that improved the extraction efficiency and at the mean time had little or no impact on extracted polysaccharides. In previous literature, it was reported that the polysaccharide yield from seaweed *A. nodosum* was about 56–90% (Okolie, 2018), *S. longicurvis* 20.0% (Rioix, Turgeon, & Beaulieu, 2007). Okolie (2018) reported that polysaccharide extract of alginate by EAE (90.32%) was expressively higher than the other techniques (56.35%) adopted. Rodrigues et al. (2015) had reported 67, 58 and 55% yield of polysaccharides from *C. tomentosum* (green seaweed), *O. pinnatifida* (red seaweed) and *S. muticum* (brown seaweed) respectively. Charoensiddhi, Franco, Su, and Zhang (2015, 2017) obtained higher yield of polysaccharides of almost 70% by EAE (protease – alkalase and flavourzyme).

### 3.3. Characterisation of EAE polysaccharide extract

#### 3.3.1. UV-Vis spectroscopy

The polysaccharide extract subjected to UV-Vis spectral scan from 200 to 400 nm had shown absorption at 210 and 220 nm which was due



(caption on next page)

**Fig. 3c.** XRD analysis of the polysaccharide extracts of seaweeds from (a) *S. wightii* (b) *E. compressa* and (c) *A. spicifera*.**Table 2**

List of peaks and corresponding functional groups of polysaccharides extracted by EAE method.

List of peaks ( $\text{cm}^{-1}$ )	Bond	Functional group
570–624.04	C-X (Br, Cl, F)	Alkyl halogen group
1072.70	C-S, C=S stretch	Sulfated polysaccharides glycosidic linkages of oligosaccharides
1105.33	C-O stretching	mannuronate and glucuronate carboxyl groups
1301.52	S-O	Sulfated polysaccharides
1529.52	C-O, C=C stretching	Ketone and carbonyl group
1702.87	C=O stretching	aldehyde group of Carbohydrate
1943.51	C-H stretch	Carbohydrate
2184.35	C=C vibrations	Alkynyl group
2351.39	O=C=O stretching	Ketone or carbonyl group
> 3500	O-H stretching	Alcohol, Carboxylic acid

to be presence of phenolic compounds that are responsible for antioxidant activity. No absorption at  $\lambda_{260}$  and  $\lambda_{280}$  were observed further ensure the absence of other contaminants like nucleic acids and proteins (Zhao et al., 2010). Purity of the extracted polysaccharide is confirmed by UV-Vis spectroscopy as represented in Fig. 3a.

### 3.3.2. E-SEM and XRD analysis

The structure of seaweed polysaccharides varies from the structure of polysaccharides from terrestrial plants. Seaweed polysaccharides have strong anionic sulfate groups. Hence seaweed polysaccharides are termed as sulfated polysaccharides. Fucoidan from brown seaweed is a sulfated polysaccharide. The E-SEM images had depicted that lyophilized polysaccharide is with smooth surface and regular in shape shown in Fig. 3b. They could be used as a natural effective bio-carrier without any requirement for nanoparticle coating. The XRD plot was showing peak around the 2-theta value of around  $31^\circ$  for all the PES. The particle sizes of the lyophilized seaweed polysaccharides were calculated from XRD plot as represented in Fig. 3c. The particle size was found to be

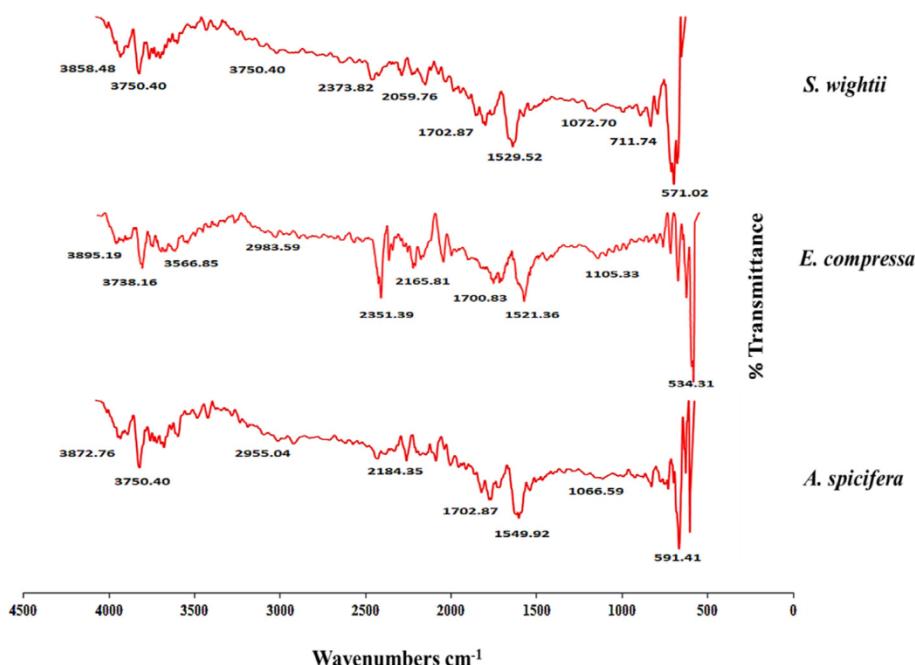
18.51, 19.55 and 19.60 nm for *S. wightii*, *E. compressa* and *A. spicifera* with degree of crystallinity around 35% with reference to Scherrer equation further concluded the semi-crystallinity nature (Raguraman et al., 2018).

### 3.3.3. Fourier transform infrared spectroscopy (FTIR)

The presence of functional groups in the seaweed polysaccharides were identified by subjecting the seaweed polysaccharide extract in FTIR spectral scan. The peaks obtained for the seaweed polysaccharides and its corresponding bond along with functional groups were listed in Table 2. The common peaks were noted around 570–600, 1000–1100, 1300, 1500, 1700, 2100, 2300, after  $3500 \text{ cm}^{-1}$  as depicted in Fig. 3d. The peaks obtained are quite matching with the previously reported literature (Maneesh, Chakraborty, & Makkar, 2017; Mateos-Aparicio, Marterra, Goñi, Villanueva-Suárez, & Redondo-Cuenca, 2018; Shofia et al., 2018). The background peaks noted after  $3500 \text{ cm}^{-1}$  are peaks corresponding to the hydroxyl groups that are present in carbohydrates (Raguraman et al., 2018; Rostami, Tabarsa, You, & Rezaei, 2017).

### 3.3.4. High performance liquid chromatography (HPLC)

The polysaccharide extract of combined EAE and fermented MRS broth extract of *L. plantarum* was subjected to HPLC for monosaccharide composition and SCFA profiling analysis. Identification of compounds was done by comparing the retention time with the standards. The refractive index detector (RID) was used for analysis. In combined EAE, chromatogram peaks of *S. wightii* were noted at a retention time of 4.608, 6.170 and 6.638 min and the corresponding peaks were found to be cellobiose, fructose and glucose. Similarly for *E. compressa*, sugar compounds were found to be cellobiose (4.838), fructose (6.055), glucose (6.502), maltose (9.886) and for *A. spicifera* glucose (6.062) shown in Fig. 3e. In fermented broth, common peaks were noted at retention time of 19.344, 21.978 and 27.544 shown in Fig. 3f. The compounds eluted were found to be butyric acid, propionic acid and acetic acid. Okolie et al. (2019) reported that metabolic activities of gut associated *Lactobacillus* strains (*L. delbrueckii* spp *bulgaricus* and *L. casei*) stimulates higher concentrations of SCFA production such as acetate and propionate.

**Fig. 3d.** FTIR spectra of the combined enzyme aided polysaccharide extract.

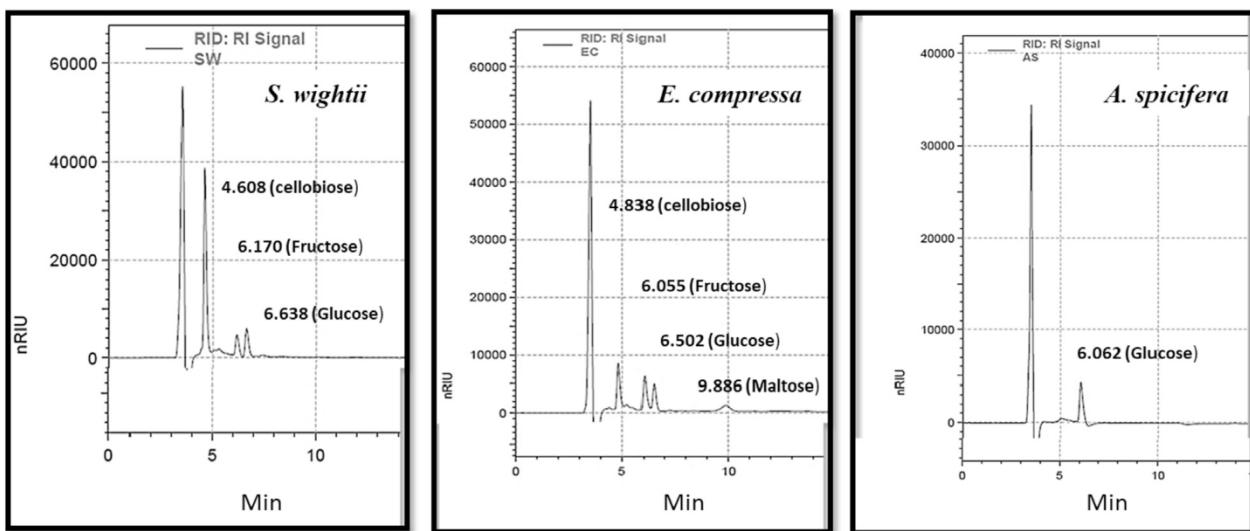


Fig. 3e. HPLC spectra of the polysaccharide extract for monosaccharide composition.

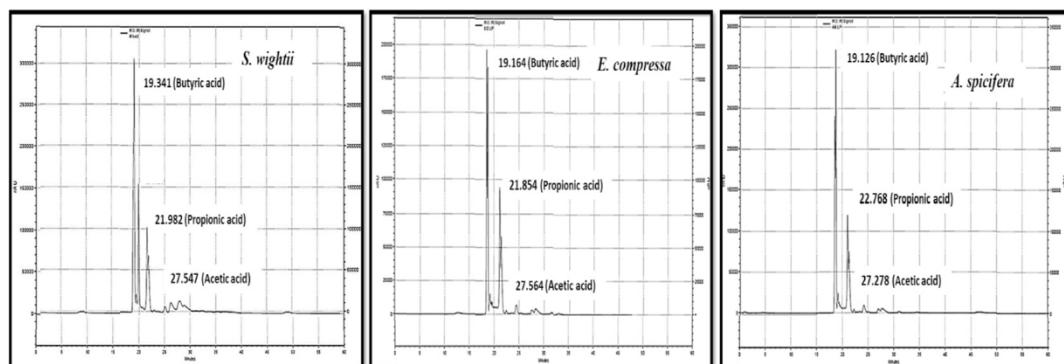


Fig. 3f. HPLC spectra of fermented extract for SCFA detection.

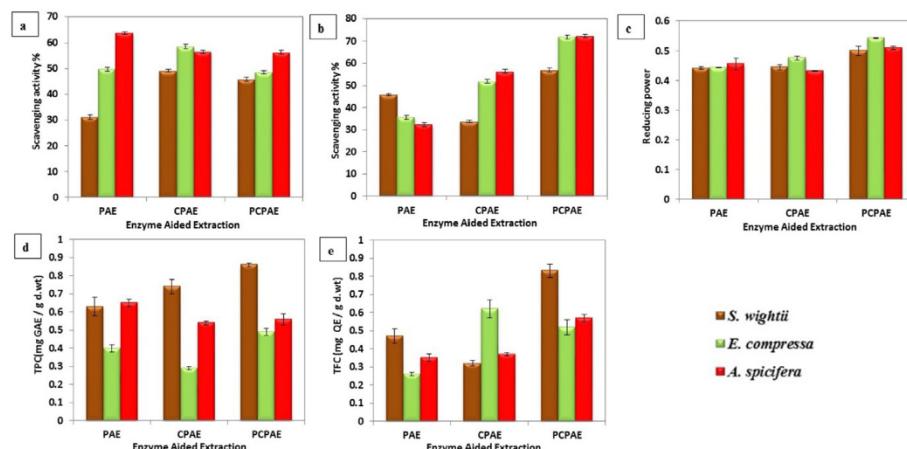


Fig. 4. a) DPPH radical scavenging activity b) ABTS radical scavenging activity c) Ferric reducing power activity d) Total phenolic content and e) Total flavonoid content of the polysaccharide extracts from selected Indian seaweeds.

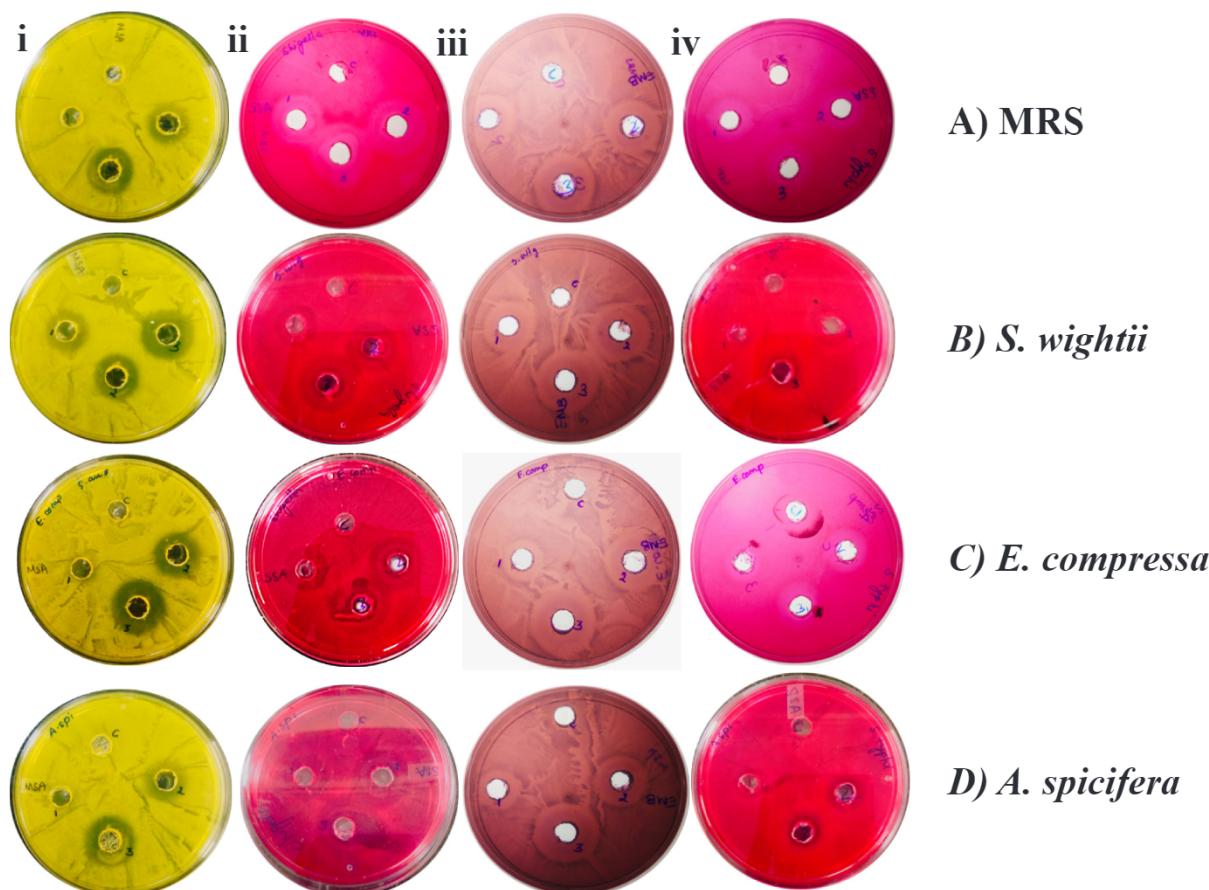
### 3.4. Antioxidant activity

The potential use of antioxidants in therapeutic and functional food industry is because of their crucial role in treating type-2 diabetes, inflammation, atherosclerosis and other diseases. In the present study, the antioxidant activity of the enzymatic extracts of seaweed polysaccharides were analyzed by performing assays such as DPPH, ABTS radical scavenging activity and ferric reducing power assay. *S. wightii*, a

brown seaweed polysaccharide was reported as a potential candidate of anti-oxidative and anti-inflammatory effect (Maneesh et al., 2017).

#### 3.4.1. DPPH radical scavenging activity

DPPH is one of the existing free radical that shows maximum absorption at 517 nm. It is a stable radical and can readily endure scavenging with an antioxidant. Hence DPPH is extensively recognized as a tool for evaluating the radical scavenging activity of the natural



**Fig. 5.** Inhibition of various gut pathogens i) *S. aureus*, ii) *S. flexneri*, iii) *E. coli* iv) *S. typhimurium* by plantaricins.

**Table 3**  
Inhibition of various gut pathogens by plantaricins obtained from several culture.

Gut pathogens	MRS			<i>S. wightii</i>			<i>E. compressa</i>			<i>A. spicifera</i>		
	Zone of inhibition (in cm) with different concentrations ( $\mu$ l)											
	50	100	150	50	100	150	50	100	150	50	100	150
<i>S. aureus</i> MTCC 3160	0.8 ± 0.1	0.9 ± 0.1	1.0 ± 0.2	0.7 ± 0.1	0.9 ± 0.1	1.1 ± 0.2	0.9 ± 0.2	1.0 ± 0.1	1.3 ± 0.1	0.8 ± 0.2	1.0 ± 0.2	1.1 ± 0.1
<i>S. flexneri</i> MTCC 1457	0.9 ± 0.1	1.1 ± 0.2	1.3 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	1.2 ± 0.2	1.4 ± 0.1	1.0 ± 0.2	1.2 ± 0.1	2.0 ± 0.1
<i>E. coli</i> MTCC 1697	0.8 ± 0.1	1 ± 0.1	1.1 ± 0.2	0.9 ± 0.2	1.2 ± 0.1	1.3 ± 0.1	0.9 ± 0.1	1.1 ± 0.2	1.3 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	1.2 ± 0.1
<i>S. typhimurium</i> MTCC 3224	0.5 ± 0.2	0.8 ± 0.1	0.9 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.7 ± 0.2	0.9 ± 0.1	1.0 ± 0.1	0.7 ± 0.1	0.8 ± 0.2	0.9 ± 0.2

Diameter in cm: Z (150  $\mu$ l) > 1 = ++, Z (150  $\mu$ l) < 1 = +.

*S. aureus* (++) , *S. flexneri* (++) , *E. coli* (++) and *S. typhimurium* (+).

Inhibition = (++) strong, (+) Moderate.

potential bioactive compounds. Fig. 4.a indicates that the PES obtained by PCPAE had higher DPPH radical scavenging activity than the extracts prepared through other methods. The DPPH radical scavenging activity of PES *A. spicifera* (63.9 ± 0.51%) obtained by PAE was found to be the highest among all seaweed extracts which was then followed by PES *E. compressa* obtained by CPAE (58.6 ± 0.90%). There was no significant difference in DPPH radical scavenging activity of PES *A. spicifera* by CPAE (56.45 ± 0.61%) and PCPAE (56.29 ± 0.80%).

#### 3.4.2. ABTS radical scavenging activity

ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) is most commonly used to determine the scavenging activity of either a single component or group of compounds exist in plant extract. The PES *A. spicifera* (72.35 ± 0.80%) and *E. compressa* (71.94 ± 0.87%)

obtained by PCPAE exhibited the maximum ABTS free radical scavenging activity. The scavenging activity of PES-PCPAE was about two times higher than the activity of PES-PAE and PES-CPAE represented in Fig. 4b.

#### 3.4.3. Ferric reducing power assay (FRAP)

The ferric reducing power assay indicated that the reducing power of PES *E. compressa* (0.544 ± 0.007) obtained by PCPAE was quite higher than the PES *A. spicifera* (0.51 ± 0.005) and *S. wightii* (0.50 ± 0.016) obtained by PCPAE shown in Fig. 4c. The reducing power of all the polysaccharide extract lies in same range and thereby doesn't show any drastic differences. The reducing power is determined based on the absorbance value. Larger the absorbance value larger the reducing power (Yildirim, Mavi, & Kara, 2001). PES were exhibiting

greater reducing power and have the tendency to reduce free radicals that induce oxidative stress environment which supports the survivalist of cancer cells. Maneesh et al. (2017) had reported that the phenolics such as phlorotannins and flavonoids play a crucial role in promoting  $\text{Fe}^{2+}$  chelating activity. The hydroxyl functional group exists in the polysaccharide helps binding and elimination of Fe, Cu and other ions.

#### 3.4.4. Total phenolic content (TPC)

Phenolics are the bioactive compounds that play a major role for antioxidant activity. The TPC with respect to gallic acid equivalent (GAE) was found maximum in PES *S. wightii* obtained by PCPAE ( $0.86 \pm 0.01$  mg GAE/g d.wt) followed by PES *E. compressa* ( $0.74 \pm 0.04$  mg GAE/g d.wt) obtained by CPAE and *A. spicifera* ( $0.65 \pm 0.02$  mg GAE/g d.wt) obtained by PAE shown in Fig. 4d. Overall, combined PCPAE resulted in maximal extraction of TPC from seaweeds rather than any other extraction procedures adopted in the study. Rodrigues et al. (2015) reported that total phenolic content in respect to standard catechol of three different seaweeds *C. tomentosum* ( $460 \mu\text{g CE/g d.wt}$ ), *S. muticum* ( $250 \mu\text{g CE/g d.wt}$ ) and *O. pinnatifida* ( $169 \mu\text{g CE/g d.wt}$ ) which is quite lesser than the present study. Polyphenol was efficiently extracted using organic solvents like ethanol, methanol, etc. Plaza, Amigo-Benavent, del Castillo, Ibáñez, and Herrero (2010) reported that the maximum phenolic yield was obtained by hot water extraction at 100 and 200 °C due to water being the polar solvent at high temperature enhances the mass transfer, which in turn favors the maximal yield of phenolic content.

#### 3.4.5. Total flavonoid content (TFC)

Flavonoids are the bioactive compounds that also play a major role for antioxidant activity and as a natural colourant. The total flavonoid content with respect to quercetin equivalent (QE) was found maximum in PES *S. wightii* obtained by PCPAE ( $0.83 \pm 0.035$  mg QE/g d.wt) followed by PES *E. compressa* ( $0.62 \pm 0.05$  mg QE/g d.wt) obtained by CPAE and *A. spicifera* ( $0.57 \pm 0.02$  mg QE/g d.wt) obtained by PCPAE shown in Fig. 4e. However it was noticed that PCPAE results in enhancing the TFC comparatively with other adopted methods.

#### 3.5. Prebiotic activity and score

The prebiotic effect of the seaweed polysaccharides was calculated by using them as a substitute of carbon source in replacement of glucose. The key criteria for a potential prebiotics are non-digestible, selective substrate for the probiotic bacteria and should be easily fermentable to produce SCFA. The CFU count of the probiotic *L. plantarum* in 24<sup>th</sup> h of PES culture was slightly higher than that of glucose. Colonies were noted up to  $10^9$  dilution. In case of *S. typhimurium*, colonies were not observed after  $10^5$  dilution. The prebiotic activity score was found positive for all the PES that promotes the growth of *L. plantarum* and suppress the growth of pathogen *S. typhimurium*. Among the extracted polysaccharides, *E. compressa* had exhibited maximum prebiotic activity score of about 1.44 followed by *S. wightii* (1.42) and *A. spicifera* (0.84).

#### 3.5.1. Plantaricin activity by well diffusion assay

A clear zone of strong inhibition was noted against all gut pathogens of order *S. aureus* (+ +), *S. flexneri* (+ +), *E. coli* (+ +) and moderate inhibition over *S. typhimurium* (+) which was shown in Fig. 5. Plantaricin produced by *L. plantarum* sp. can be used as a therapeutic proteins and alternative food preservative. It was noted that PES assist in production of plantaricins with greater zone of inhibition activity (Table 3).

#### 4. Conclusion

The study evaluated the extraction efficiency and characteristics of dietary fiber from Indian edible seaweeds and its *in-vitro* prebiotic effect

on the gut microbiota. *E. compressa* exhibited higher dietary fiber content ( $60.64 \pm 2.2\%$ ) and polysaccharide content up to  $84.31 \pm 0.86\%$  and was extracted by PCPAE method. The structure of polysaccharide was found to be semi-crystalline and the purity was confirmed by UV-Vis spectrum. FTIR and HPLC analysis revealed the presence of polysaccharide functional groups and their monosaccharide and SCFA profile, respectively. Prebiotic score was found to be positive for all the three seaweed samples, which could be the potential candidates for the development of symbiotic functional foods. Plantaricins produced by PES exhibit enhanced inhibition activity against the studied gut pathogens could positively modulate the gut microbiota.

#### Conflicts of interest

The authors declare no conflict of interest.

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