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Brown seaweeds as a source of anti-hyaluronidase compounds

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ABSTRACT

Hyaluronidase enzymes disrupt hyaluronic acid, causing angiogenesis, tumor invasiveness, metastasis, inflammation, and skin aging. Phlorotannin, alginate and fucoidan were extracted successively from the blade and stipe samples of two *Sargassum* seaweeds and *Eisenia arborea* thallus. These were evaluated for their *in vitro* anti-hyaluronidase and antioxidant activities (DPPH and reducing power assay). The crude phlorotannin content was highest in the blade of *S. tenerrimum* (22.405 ± 3.6 μg/mg), followed by *S. vulgare* blade (18.385 ± 3.29 μg/mg) with lower amounts in the stipe portion. The highest yield of alginate and fucoidans was obtained from the blade samples of *S. vulgare* (0.322 ± 0.38 and 0.198 ± 0.016%), followed by *S. tenerrimum* (0.090 ± 0.01 and 0.063 ± 0.005%) and *E. arborea* thallus (0.047 ± 0.008 and 0.032 ± 0.003%). The sulfate content was higher in fucoidan than alginate extracted from the stipe regions of the seaweeds. Phlorotannin, fucoidan and alginate from *S. vulgare*, *S. tenerrimum*, and *E. arborea* possessed anti-hyaluronidase activity as evident by a decrease in the N-acetylglucosamine release. The highest anti-hyaluronidase activity was achieved in the extract of *S. tennerimum* blade (37.67 ± 2.3% inhibition) due to its high phlorotannin content. Alginate and fucoidan extracted from the stipes of *Sargassum* species possess higher bioactivities than the blade samples. The FTIR study ascertained that alginate with a high guluronic acid and high sulfated fucoidan were extracted from the stipe samples compared to the blade samples. This increased viscosity and promoted bioactivity respectively. Further studies to evaluate the emulsifying and viscosity properties of these compounds are required before they can be considered for commercial applications.

1. Introduction

Seaweeds have wide range of constituents that have potential as anti-aging skin care products. Skin composes 15% of the human body and is a primary defense organ against environmental pollution and pathogenic microbes. Skin is susceptible to aging due to environmental (photo-aging, oxidative stress) or age-related issues. Hyaluronic acid is involved in maintaining skin hydration which is largely degraded by the hyaluronidase enzyme or reactive oxygen species (ROS) mediated degradation (Heldin et al., 2019). Ultraviolet radiation regulates the hyaluronidase expression and activity thereby contributing towards photo-aging (Kurdykowski et al., 2011). The hyaluronidase enzyme is involved in migration of cancer, inflammation, allergic effects and also increases permeability of the vascular system (Girish and Kemparaju, 2007). Inhibition of hyaluronidase activity is a promising strategy to alleviate and treat diseases that are associated with this enzyme up-regulation. Inhibition of hyaluronidase enzyme will serve as major determining factor in reducing photo-aging of skin, and is thus a vital cosmeceutical candidate. Natural antioxidants have skin photoprotecting effects and are important components in skin care products.

Marine macroalgae (seaweeds) are a rich source of commercially important compounds including sulfated polysaccharides, polyphenols, bioactive peptides, fatty acids, terpenes, carotenoids (Zhao et al., 2018). These seaweed constituents possess antioxidant activities and act through multiple modes of action including inhibiting ROS generation and anti-hyaluronidase activity (Shibata et al., 2002). Thus, seaweed natural products with beneficial physical and chemical properties could be potentially exploited as cosmeceutics and cosmetics. However, there are only a few studies on the anti-hyaluronidase activity in brown seaweeds (Shibata et al., 2002; Katsube et al., 2003; Ferreres et al., 2012; Li et al., 2017; Sugiura et al., 2018; Catarino et al., 2019). Some flavonoids, metallic salts, polyphenols, metals, clinical drugs and polysaccharides act as inhibitors of hyaluronidase (Kakegawa et al., 1992; Akhtar and Bhakuni, 2003). Seaweeds are a promising resource in the search for nontoxic alternates for anti-hyaluronidase enzymes (Thomas and Kim, 2011; Zhao et al., 2018).

Diverse secondary metabolites like peptides, sugar alcohol, pigments, phenolics, sterols present in seaweeds are usually synthesized

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in response to environmental stress like desiccation, heat, salinity fluctuation, adverse light including UV and heat and grazing (Pereira, 2018). Alginate and fucoidan present in the brown algae cell wall matrix protect against desiccation. They are being exploited as a source of compatible polymers for various pharmaceutical and cosmetical product development due to their gelling and emulsifying properties (Koyanagi et al., 2003; Thomas and Kim, 2011; Pereira, 2018). The monomers composition and sulfation level influences the physio-chemical properties of alginate and fucoidan polymers that are responsible for diverse bioactivity. The monomer composition varies in different parts of the brown algal thallus (Koyanagi et al., 2003; Lee and Mooney, 2012). The phlorotannin of brown algae possess strong antioxidant activity and exhibits inhibitory effects against melanogenesis, protection against photooxidative stress induced by UVB radiation and this activity varies in different parts of the thallus (Koivikko et al., 2005; Morton, 2013). These emulsifying bioactive polysaccharides (alginate and fucoidan) and photoprotective phlorotannins of brown algae thallus are also valued in the food industry (Thomas and Kim, 2011; Pereira, 2018).

The brown algae *Sargassum* naturally accumulates phlorotannins, fucose rich sulfated fucoidan, and equimolar galactose/mannose containing alginate in the cell wall of their large blades, stipe and other parts of the thallus (Sinha et al., 2010). To date, there are no studies on these compounds in *Sargassum tenerrimum* and *S. vulgare* (Haider et al., 2009; Ank et al., 2019). As a food, *Sargassum* has several potential health benefits such as anti-inflammatory, antioxidant, antiviral, antimicrobial, anticoagulant, anti-hyaluronidase, anti-cancer, immunomodulatory and hepatoprotective activity (Yende et al., 2014). The brown alga *Eisenia arborea* is a dominant kelp found in the Northern and Eastern Pacific zones. Phlorotannin is abundantly present in *E. arborea* with significant amounts of fucoidan and alginate (Landa et al., 2017; Sugiura et al., 2018). The aim of this study was to quantify the phlorotannins, alginate and fucoidan extracted successively from the blade and stipe samples of *Sargassum tenerrimum, S. vulgare* and *Eisenia arborea* thallus and to evaluate their *in vivo* anti-hyaluronidase and antioxidant activities to assess their potential applications.

2. Materials and methods

2.1. Source of seaweeds

Fresh and healthy seaweeds *Sargassum tenerrimum* J.Agardh and *Sargassum vulgare* C. Agardh (2 kg each) were collected from the Coasts of Thikkoti (11.4952° N, 75.6239° E), Kerala and Thondi (9.7438° N, 79.0185° E), Tamil Nadu, respectively during January 2018. The *Sargassum* specimens were washed and shade dried for 5 days. The blades and stipes of the two *Sargassum* species were separated. The dried seaweed *Eisenia arborea* Areschoug was purchased from a supermarket at Faro, Portugal during March 2018 and the whole thallus used for further experiments.

2.2. Extraction and estimation of phlorotannins

The powdered samples (100 g DW) of *S. tenerrimum* and *S. vulgare* (blades and stipes) and *E. arborea* thallus were soaked in 300 mL chloroform: methanol (1:1 v/v) in dark for 3 days. The extraction was repeated thrice. The extracts were pooled, filtered through Whatman No 1 filter paper and concentrated by rotary evaporator (Lark, India) at 45 °C. The phlorotannin was extracted from the chloroform: methanol extract (Sugiura et al., 2006). The extract was separated by adding 50 mL water and 25 mL chloroform. The upper layer of the amber colored aqueous solution contained phlorotannin. This was subsequently extracted by 25 mL diethyl ether for 30 mins by vigorous shaking at 10 min intervals. The extraction was repeated thrice and concentrated by rotary evaporator at 45 °C. The crude extract of phlorotannin (0.3 mL) was estimated by Folin-Ciocalteu method where the extract was mixed with 1 mL 10% Folin-Ciocalteu reagent followed by the addition of 2 mL 10% sodium carbonate. The sample was incubated for 1.5 hrs at room temperature in dark and the absorbance was measured at 750 nm using Shimadzu UV-2600 UV-Visible spectrophotometer.

2.3. Extraction of alginate and fucoidan

Alginate and fucoidan were extracted using sodium EDTA (ethylene diamine tetra acetic acid) from the residual sample after extracting phlorotannin by ethanol precipitation (Zhao et al., 2017). The residual sample was suspended in 0.5% EDTA in 20:60 ratios and kept under constant stirring in a water bath for 3 h at 70 °C. The resultant solution was cooled to room temperature and filtered through a Buchner funnel. Alginate was precipitated by adding 20% ethanol to the filtrate so that it became neutral. The precipitated alginate was centrifuged at 6000 rpm for 10 min at 30 °C and collected. The supernatant was taken up for fucoidan extraction. To the supernatant, absolute ethanol was added until a final concentration of 60% to precipitate fucoidan and centrifuged at 6000 rpm for 10 min at 30 °C to obtain fucoidan pellet. The extraction was carried out by using three samples (each 100 g DW) and the average yield of phlorotaninn, alginate and fucoidan was calculated.

2.4. Fractionation of fucoidan

The fucoidan pellets stored at 4 °C were dialyzed by using 3.5 kD membrane cut off against distilled water for 48 hrs at room temperature. The 3.5 kD dialyzed fucoidan fraction was again dialyzed for 48 h in a 14 kD cut off membrane. Fucoidan retained in the dialysis bag was collected as >14 kD HMW (High Molecular Weight) fraction and used for the following investigations.

2.5. FTIR characterization of alginate and fucoidan

The standard and extracted freeze-dried alginate and fucoidan weighing 15 mg DW each were ground uniformly to a fine powder using a glass mortar and pestle. Each powdered sample was divided into three portions. Each portion weighing 5 mg DW was mixed with KBr in 1:20 ratio and used for FTIR analysis in the region between 400 and 4000 cm^{-1} using Perkin Elmer 16 PC spectrometer, Boston, USA. One spectrum among the three consistent spectra of each specimen was taken up for analysis.

2.6. Anti-hyaluronidase assay

Phlorotannin (10 mg/mL 20% DMSO) and polysaccharides (fucoidan and alginate, 10 mg/mL 0.1 M acetate buffer) were used as samples with ascorbic acid (500 μg/mL) as a positive control (Botzki et al., 2004). The hyaluronidase (0.1 mL from 1.88 mg/5 mL 0.1 M acetate buffer (pH 4) was mixed with the extracted compounds (500 μg/mL) and made up to 0.2 mL with 0.1 M acetate buffer. Then the solution was incubated at 37 °C for 20 mins. The NaCl (0.2 mL 0.1 M) was added to the mixture to activate the hyaluronidase enzyme and further incubated at 37 °C for 20 mins. The hyaluronic acid solution (potassium hyaluronate salt 0.4 mg/mL) was added (0.5 mL) to the mixture and incubated for 40 mins. Finally 0.2 mL 0.4 M NaOH was added to terminate the reaction (Fujitani et al., 2001). The percentage of inhibition was calculated using the following formulae:

$$\text{Inhibition}(\%) = [(A - B) - (C - D)]/(A - B) * 100$$

Where A is negative control (inhibitor was replaced by 0.1 M acetate buffer); B is control blank; C is the test sample with inhibitor and enzyme; D is the sample blank with inhibitor but without enzyme.

2.7. Estimation of N-acetylglucosamine

The inhibitory effect was determined by the quantitative analysis of N-acetylglucosamine sugar using a modified Morgan-Elson method (Reissig et al., 1955). N-acetylglucosmine in the sample (0.5 mL each) after treatment with 500 μg/mL of active compounds/ascorbic acid was estimated, where 0.1 mL 0.8 M potassium tetraborate was added. The mixture was heated in boiling water bath for 3 mins and cooled in tap water. The DMAB (p-di-methyl amino benzaldehyde solution, 3 mL) reagent was added and mixed immediately. The reagent was prepared by dissolving 20 g DMAB in an acidic solution (25 mL concentrated hydrochloric acid + 75 mL glacial acetic acid) and then diluted with 400 mL glacial acetic acid immediately before use. The sample mixture was incubated between 36 and 38 °C for 20 min, cooled in tap water and the absorbance was measured at 544 nm in UV-Visible spectrophotometer (Shimadzu UV-2600).

2.8. In vitro *antioxidant activities*

2.8.1. DPPH assay

The antioxidant property of polysaccharide was determined by DPPH assay (Yen and Chen, 1955). The DPPH, 2, 2-diphenyl-1-picrylhydrazyl-hydrate is an electron transfer molecule that produces violet solution in ethanol. It is reduced in the presence of antioxidant and gives rise to colorless ethanol solution. The extracted active compounds (1 mg/mL) were mixed with 0.2 mL 0.16 mM DPPH and vortexed for 1 min followed by incubation at room temperature in the dark for 30 min. Absorbance was measured at 517 nm in UV-Visible spectrophotometer. The scavenging effect of DPPH radical was calculated by the following equation:

$$\text{Scavenging effect}(\%) = [1 - (\text{Ab sample} - \text{Ab sample blank})/\text{Ab control}] \times 100$$

Where Ab sample = Absorbance of test sample (DPPH solution + test sample); Ab sample blank = Absorbance of sample (sample without DPPH solution); Ab control = Absorbance of control (DPPH solution without sample); Ascorbic acid used as positive control.

2.8.2. Reducing power assay

Reducing power property of active compounds was determined as previously described Yen and Chen (1955). Ascorbic acid was used as a positive control. Various concentration of sample extracts (100–1000 μg/mL) was mixed with 2.5 mL 0.2 M phosphate buffer (pH 6.6) and 2.5 mL 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. After immediate cooling in an ice bath, 2.5 mL 10% trichloroacetic acid was added and the extract centrifuged at 3000 rpm for 10 mins. The supernatant (2.5 mL) was mixed with 2.5 mL distilled water + 0.5 mL 0.1% ferric chloride and absorbance was measured at 700 nm.

2.9. Statistical analysis

All experimental analyses were carried out in triplicates and expressed in mean and SD values. Mean values among each variable were grouped by Turkey HSD in one way ANOVA using SPSS 14 and significant differences calculated ($p < 0.05$/ $p < 0.01$).

3. Results

3.1. Yield and proximate composition of phlorotannin, fucoidan and alginate

Significantly higher yields of phlorotannins were obtained from the blade and stipe crude extracts of *Sargassum vulgare*, compared to *S. tenerrimum* and *E. arborea* (Table 1). The same trend occurred in the phlorotannin content extracted from the crude sample (Table 1). Significantly higher polysaccharide (alginate and fucoidan) yields were obtained from the blade of *S. vulgare*, followed by the stipe of *S. vulgare* with significantly lower yields from *S. tenerrimum* and *E. arborea* (Table 1). Since sodium EDTA was used for the extraction, the sulfate content of sodium alginate and fucoidan extracted from the three seaweeds was estimated. The sulfate concentration in the fucoidan polysaccharide was higher than in the alginate. Polysaccharides extracted from the stipe had a significantly higher sulfate content than the polysaccharides extracted from the blade region of the *Sargassum* species, followed by *E. arborea*. In the blade samples, the sulfate content was significantly higher in the polysaccharides of *S. tenerrimum* than *S. vulgare* whereas in stipe samples, *S. vulgare* had the highest sulfate content (Table 1).

3.2. FTIR characterization of alginate and fucoidan

Alginate extracted from the samples of brown seaweeds was confirmed by FTIR spectra (Fig. 1). A broad band centered at 3433 cm^{-1} known for O—H of hydrogen bond and a weak peak at 2929 cm^{-1} for C—H stretching were observed in the standard alginate and alginate extracted from all samples. The bands at 1632 and 1415 cm^{-1} showed the presence of COO^- (carboxyl groups) and a band at 1027 cm^{-1} for O—H bending of guluronic acid were recorded in the spectra of the standard and samples. The C—O stretching vibration of uronic acid appeared at 937 cm^{-1} and C_1—H deformed vibration of β-mannuronic acid at 861 cm^{-1} were found in the extracted alginate with FTIR spectra of the alginate standard. A weak peak at 800 cm^{-1} and strong peak at 1027 cm^{-1} observed in the standard alginate were also noted in the stipe samples of both the *Sargassum* species indicating more guluronic acid (G) in the stipe extracted alginate. Less G and more mannuronic acid (M) were observed in the blade extracted alginate of *S. tenerrimum* and *S. vulgare* and *Eisenia arborea* thallus as the peaks were weak and strong, respectively (Fig. 1).

In the FTIR spectra, O—H stretching was observed in the region of 3260 cm^{-1} and C—H stretching of C6 of fucose and galactose was recorded between 2864 and 2991 cm^{-1}. A peak at 1730 cm^{-1} for CO

Table 1
Yield of phlorotannin and polysaccharides (alginate and fucoidan) and sulfate content of the polysaccharides obtained from the blade and stipe of two brown seaweeds (*S.ten - Sargassum tenerimum; S.vul- Sargassum vulgare*) and thallus of *Eisenia arborea* (*E.arb*).

Seaweed	Compounds (% alga DW)			Estimated phlorotannin (μg/mg crude)	Sulfate content (μg/mg)	
	Crude extract	Alginate	Fucoidan		Alginate	Fucoidan
S.ten (blade)	0.041 ± 0.005^{a}	0.090 ± 0.01^{a}	0.063 ± 0.005^{b}	22.405 ± 3.6^{c}	14.6 ± 2.4^{c}	37.68 ± 3.7^{c}
S.ten (Stipe)	0.035 ± 0.004^{a}	0.081 ± 0.005^{a}	0.052 ± 0.003^{ab}	14.815 ± 2.08^{c}	22.9 ± 1.3^{d}	50.14 ± 2.4^{d}
S.vul (blade)	0.058 ± 0.007^{b}	0.322 ± 0.38^{c}	0.198 ± 0.016^{c}	18.385 ± 3.29^{d}	12.5 ± 0.3^{b}	31.34 ± 3.3^{b}
S.vul (Stipe)	0.057 ± 0.007^{b}	0.250 ± 0.010^{b}	0.158 ± 0.014^{d}	12.51 ± 2.19^{a}	37.61 ± 6.4^{e}	62.28 ± 5.8^{e}
E.arb	0.032 ± 0.003^{a}	0.047 ± 0.008^{a}	0.032 ± 0.003^{a}	13.47 ± 3.01^{b}	8.3 ± 0.6^{a}	27.16 ± 2.1^{a}

Different alphabets superscripted in each column significant as grouped by Tukey HSD ($p < 0.05$).
Among the mean differences in each column significant ($p < 0.05$) by One way ANOVA.

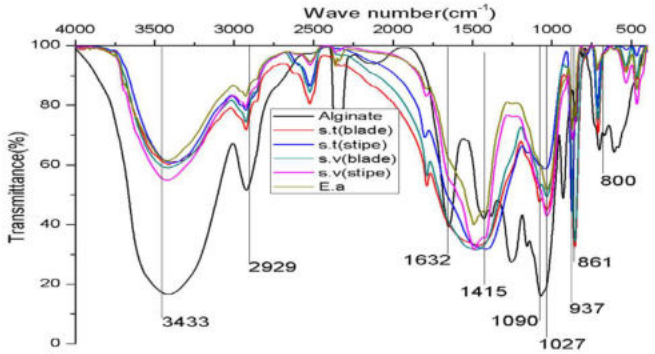

Fig. 1. FTIR spectra of alginate of standard and samples of three brown seaweeds [*S. tenerrimum* blade-S.t (blade); *S. tenerrimum* stipe-S.t (stipe); *S. vulgare* blade-S.v (blade); *S. vulgare* stipe -S.v (stipe); *E. arborea*-E.a].

stretching of O-acetyl groups confirmed the presence of acetyl fucoidans in the two *Sargassum* species and *E. arborea*. The bands around 1462 cm^{-1} with varied positions and intensities indicated the scissoring vibration of CH_2 of galactose and fucose monomers that were recorded in each fucoidan extracted from two *Sargassum* species and *E. arborea*. The stretching vibration around 1140–1050 cm^{-1} showed the presence of RO-SO_3^- bond of the sulfate groups was observed in all the spectra. The peaks at 740 and 716 cm^{-1} showed the presence of C—O—C bending vibrations in glycosidic linkages and were found in all the fucoidans standard and samples. Further, a FTIR spectral peak was observed at 842 cm^{-1} in the stipe samples but not in blade sample and *E. arborea* fucoidans (Fig. 2).

3.3. Anti-hyaluronidase activity

Extracts with increasing anti-hyaluronidase activity decrease the amount of N-acetylglucosamine detected. Among the seaweed extracts tested, phlorotannins exhibited higher anti-hyaluronidase activity followed by fucoidan and alginate extracts. The *S. tenerrimum* blade extract had significantly higher phlorotannin compared to the other extracts (Table 1), and possessed higher anti-hyalurodinase activity of 37.67 ± 2.3% inhibition (Fig. 3). The activity was in the order of *S. tenerrimum* (blade) >*S. vulgare* (blade) >*E. arborea*>*S. tenerrimum* (stipe) >*S. vulgare* (stipe; Fig. 3). The significant increase in the anti-hyaluronidase activity was confirmed by a significant decrease in N-acetylglucosamine production. The polysaccharides isolated from the *S.vulgare* stipe sample possessed high inhibition of hyaluronidase enzyme, followed by *S. tenerrimum* stipe, blade samples of *Sargassum* species and *E. arborea*. In the polysaccharides, fucoidan was more active than the alginate (Fig. 4).

3.4. In vitro *antioxidant assay*

The phlorotannin extracts from the blade samples and sulfated polysaccharides (fucoidan and alginate) extracts of the stipe samples exhibited the highest DPPH activity. The phlorotannin extracted from *S. tenerrimum* blade had the significantly highest DPPH activity followed by the *S. vulgare* blade extraact, *E.arborea* thallus extract and stipe extracts of *S. vulgare* and *S. tenerrimum* (Fig. 5). Fucoidan polysaccharide extracts exhibited higher DPPH activity than the alginate extracts. The stipe extracted polysaccharides possessed high DPPH activity followed by blade extracts of *S. vulgare*, *S. tenerrimum* and *E. arborea* thallus (Fig. 5).

The phlorotannin extracted from *S. tenerrimum* blade showed the highest reducing power ability followed by *S. vulgare* blade extract, *E. arborea* thallus extract and the stipe extracts of *S. vulgare* and *S.*

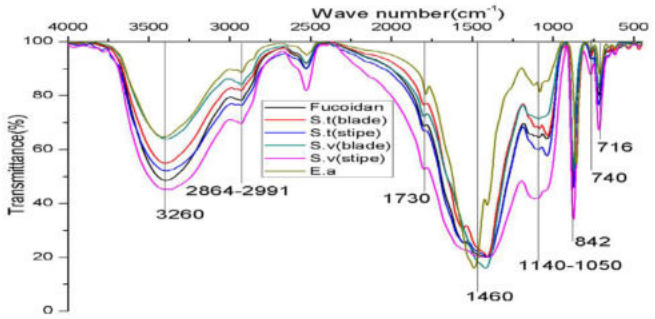

Fig. 2. FTIR spectra of fucoidan of standard and samples of three brown seaweeds [*S. tenerrimum* blade-S.t (blade); *S. tenerrimum* stipe-S.t (stipe); *S. vulgare* blade-S.v (blade); *S. vulgare* stipe -S.v (stipe); *E. arborea*-E.a].

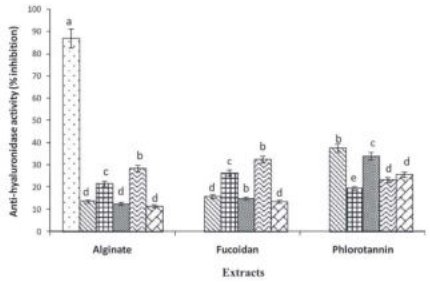

Fig. 3. *In vitro* anti-hyaluronidase activity of phlorotannin, alginate and fucoidan extracted from three brown seaweeds (Ascorbic acid, *S. tenerrimum* (blade), *S. tenerrimum* (stipe), *S. vulgare* (blade), (stipe), *E. arborea*, (Bar values with different alphabet in each group are significantly different with each other and ascorbic acid; $p < 0.01$).

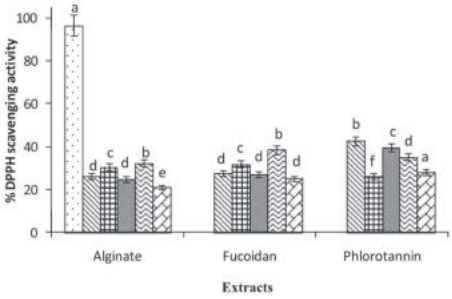

Fig. 5. *In vitro* DPPH assay of phlorotannin, alginate and fucoidan extracted from three brown seaweeds (Ascorbic acid, *S. tenerrimum* (blade), *S. tenerrimum* (stipe), *S. vulgare* (blade), (stipe), *E. arborea*, (Bar values with different alphabet in each groups are significantly different with each other and ascorbic acid; $p < 0.01$).

tenerrimum. The activity was increased with increasing extract concentration. Fucoidan then alginate extracted from the stipe samples possessed higher reducing power ability than the blade extracts of *S. vulgare*, *S. tenerrimum* and *E. arborea* thallus extracts (Fig. 6).

4. Discussion

4.1. Yield and proximate composition of phlorotannin, fucoidan and alginate

In this study, significantly higher amounts of estimated phlorotannins were obtained from the blade samples compared to the stipe samples of *Sargassum vulgare*, followed by *S. tenerrimum* and *E. arborea* thallus. Variations in content will depend on the species and region of the thallus (Samee et al., 2009). A high phlorotannin content in the blade of *Sargassum* species may be due to accumulation in the blade to reduce grazing (Connan et al., 2006). Young blades accumulate more phlorotannin than stipes which are comparatively older parts within the thallus (Ank et al., 2014). Similarly, variations in the polysaccharides (fucoidan and alginate) yields not only depend on the species, extraction methods and age of the thallus (Torres et al., 2007; Rioux et al., 2009; Sinha et al., 2010; Garcia-Rios et al., 2012; Ashayerizadeh et al., 2020) but also on the part of the thallus as observed in the present study where higher amount of fucoidan and alginate were extracted from the blade portion than stipe parts of *Sargassum* species.

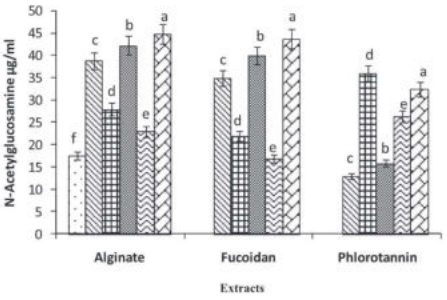

Fig. 4. Amount of N-Acetylglucosamine production (μg/mL) in the assay of phlorotannin, alginate and fucoidan extracted from three brown seaweeds (Ascorbic acid, *S. tenerrimum* (blade), *S. tenerrimum* (stipe), *S. vulgare* (blade), (stipe), *E. arborea*, (Bar values with different alphabet in each groups are significantly different with each other and ascorbic acid; $p < 0.01$).

Sulfate is attached at one or more carbon moieties in the sugar residues, accordingly sulfate content of fucoidan polymers vary depending on the brown algae species (Rioux et al., 2009). Alginate exists as alginic acid in the cell wall of brown algae and is extracted by Na/Ca/K salts of sulfate (Lee and Mooney, 2012). The sulfate content of fucoidan extracted from *Sargassum filipendula* was 5.3and 10.7% in *Padina pavonica* (Garcia-Rios et al., 2012) and in some algae, it may be up to 20% (Rioux et al., 2009). The sulfate content in the fucoidan of Ascophyllum nodosum was increased by decreasing extraction temperature (Yuan and Macquarrie, 2015). Crude galactofucans extracted from brown seaweed Saccharina longicruris contained 19.9–21.5% of sulfate (Rioux et al., 2009). Sodium EDTA used for the extraction in the present study and thus the alginate was extracted as sodium salt. The amount of sulfate recorded was higher in the fucoidan than alginate of the stipe samples of *S. vulgare*, followed by *S. tenerrimum* and the thallus of *E. arborea*. In the blade samples, *S. tenerrimum* contained more sulfate in the polysaccharides than *S. vulgare*. These results suggest that sulfated polysaccharides in the stipe samples are different from that of blade samples and hence may potentially be used for different applications (Koyanagi et al., 2003).

Due to an increase in the sulfate content of seaweed polysaccharides, a higher bioactive activity was observed (Koyanagi et al., 2003; Lee and Mooney, 2012; Ma et al., 2017). The crude yield and estimated phlorotannin concentration revealed that both the blade regions of *S. tenerrimum* and *S. vulgare* had higher activity than the stipe extracts. The food value of brown algae soft blades that contain high amounts of phlorotannin with less sulfated polysaccharides are considered suitable for culinary preparation whereas hard stipe parts containing with high content of sulfated fucoidan and alginate promoting the viscosity of polysaccharides are more suitable for cosmetic products (Afonso et al., 2019).

4.2. FTIR characterization of alginate and fucoidan

In this study, alginate extracted from the blade and stipe samples of the brown seaweeds and an alginate standard were characterized by FTIR spectra and compared to earlier studies (Leal et al., 2008;

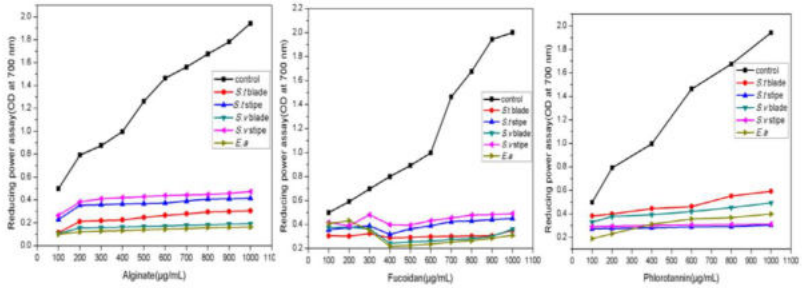

Fig. 6. Reducing power assay of phlorotannin, alginate and fucoidan extracted from three brown seaweeds (Control: Ascorbic acid; *S. t - Sargassum tenerimum; S. v - Sargassum vulgare; E. a - Eisenia arborea*).

Indrani and Budianto, 2013; Rhein-Knudsen et al., 2017; Isnansetyo et al., 2017). Alginate consists of blocks of mannuronic (M) and guluronic acid (G) as well as M alternating with G (Garcia-Rios et al., 2012). Alginate polymers with higher proportion of G than M exhibits strong and rigid gelling properties whereas a higher proportion of M gives soft and elastic gels (Garcia-Rios et al., 2012). The G and M in the alginate polymer in the present study were estimated based on the relative intensity of peaks at the range of 1090 and 790 cm^{-1}, respectively (Sakugawa et al., 2004). A strong peak at 1027 cm^{-1} and weak peak at 800 cm^{-1} observed in the standard alginate were also noted in the stipe samples of both *Sargassum* species indicating more G in the stipe alginate. Less G and more M were observed in the alginate of blade samples of *S. tenerrimum* and *S. vulgare* and *Eisenia arborea* thallus with weak and strong peaks indicating the alginate as soft and elastic gels respectively.

The FTIR spectra of fucoidans extracted in this study were compared with a standard and ascertained by previously described spectral properties (Bilan et al., 2004; Kim et al., 2010; Garcia-Rios et al., 2012; Fernando et al., 2017). Apart from the stretching vibration between 1140 and 1050 cm^{-1} which showed the presence of RO-SO_3^- bond of the sulfate groups in the fucoidan of blade and stipe samples (Fernando et al., 2017), an additional peak at 842 cm^{-1} for sulfate attached at C4 of galactose (Fernando et al., 2017) and fucose residues (Garcio-Rios et al., 2012) was found only in the fucoidan of stipe samples. This suggested that more sulfate groups were found in the fucoidan of stipe samples than the blade samples of *Sargassum* species and *Eisenia arborea*. High sulfate content in the fucoidan of stipe samples would display more biological activity (Patankar et al., 1993; Mandal et al., 2007).

4.3. Anti-hyaluronidase activity

Hyaluronic acid is a polysaccharide with disaccharide units composed of N-acetylglucosamine and glucuronic acid linked by alternating β-1,4 and β-1,3 glycosidic bonds that occurs in the matrix of connective tissues (Pardue et al., 2008). Detection of N-acetylglucosamine residues at the reducing end of hyaluronic acid degradation reaction is one of most commonly used method to assess hyaluronidase activity (Reissig et al., 1955). In this study, phlorotannin extracted from the blade samples exhibited significantly high anti-hyaluronidase activity, followed by fucoidan and alginate extracted from the stipe of *S. tenerrimum, S.vulgare* and *E. arborea*. The release of N-acetylglucosamine upon treatment with these polysaccharides also corroborated with anti-hyaluronidase activity.

Identifying compounds of natural origin having anti- hyaluronidase property active against inflammation and angiogenesis is important for health related products development. Compounds from brown seaweeds have long been used as food and more recently, in pharmaceutical and biomedical applications (Yende et al., 2014). Anti-hyaluronidase property reported from alginate (Asada et al., 1997; Chen et al., 2020) and phlorotannin of brown seaweed species (Shibata et al., 2002; Samee et al., 2009; Ferreres et al., 2012; Fayad et al., 2017) suggest the potential of brown algae for natural anti-allergic medicines or functional foods development. Alginate-hyaluronic acid composite hydrogels are applied as cell delivery vehicles to support cartilage and osteochondral regeneration (Ganesh et al., 2013). Alginate-hyaluronic acid composite powder (Rapid Clot) is used as haemostatic agents with better water absorption capacities and shorter blood clotting time, enabling it be an efficient wound dressing and for topical haemostasis control (Chen et al., 2020). High molecular fucoidan isolated from *Fucus vesiculosus* possessed concentration dependent hyaluronidase inhibition activity with an IC_{50} of 2.9 μg/mL (Pozharitskaya et al., 2020). Due to the limited studies evaluating the anti-hyaluronidase activity of bioactive compounds extracted from different parts of the brown algae (Katsube et al., 2003; Ferreres et al., 2012; Li et al., 2017), this present investigation evaluated the phlorotannin, fucoidan and alginate extracted from the blade and stipe regions of *S. tenerrimum, S.vulgare* and *E. arborea* thallus and indicated that they may have commercial potential.

4.4. In vitro *antioxidant assay*

Oxidation is a chemical reaction that can produce free radicals leading to chain reactions that damage cells. Oxidative damage is a causative agent for chronic diseases such as cancer and atherosclerosis and leads to skin aging. Phlorotannin exhibiting DPPH scavenging activity was extracted in the brown alga *Eisenia bicyclis* (Chowdhury et al., 2014) and *Eisenia arborea* is a source of antioxidant compounds (Raja et al., 2016; Tenorio-Rodriguez et al., 2017; Ito et al., 2018). The polysaccharides of *Sargassum vulgare* displayed good DPPH scavenging activity (Dore et al., 2013) and exhibit enhanced antioxidant activity by limiting lipid peroxidation in obese rats (Kolsi et al., 2017). Fucoidan possessing superoxide radical scavenging and antioxidant properties and capable of reducing the ROS generation and cell death in zebrafish embryos was extracted from the *S. tenerrimum* (Marudhupandi et al., 2014; Raguraman et al., 2019; Ashayerizadeh et al., 2020). There were significant variations in the antioxidant activities like DPPH assay and reducing power ability among the phlorotannin, fucoidan and alginate extracted from the blade and stipe samples of *S. tenerrimum, S. vulgare* and *E. arborea* thallus in the present study. Phlorotannin of blade samples and fucoidan and alginate of stipe samples exhibited good bioactivity, suggesting that the constituents of bioactive compounds found in the blade

are different to those in the stipe regions of the *Sargassum* thallus and thus may have different applications. A detailed study on the emulsifying and viscosity properties of these bioconstituents is required before they can be considered for various commercial applications.

5. Conclusions

In the present investigation, phlorotannin, alginate and fucoidan were extracted successively from the blade and stipe samples of *Sargassum* species (*S. tenerrimum* and *S. vulgare*) and *Eisenia arborea* thallus. It has evaluated for their yield, *in vitro* anti-hyaluronidase and antioxidant activities. High phlorotannin concentration in the blade samples was linked to higher anti-hyaluronidase and anti-oxidant properties. Alginate and fucoidan extracted from the stipe samples of *Sargassum* species possessed higher bioactivity than blade samples. This was linked to more guluronic acid in the alginate and high sulfate in the fucoidan, based on the FTIR spectra characterization. The different compositions of these compounds in the blades and thallus suggest that they may have different commercial applications.

Declaration of Competing Interest

The author does not have any conflict of interest.

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