J Appl Phycol (2017) 29:2521–2537
DOI 10.1007/s10811-017-1086-6

22ND INTERNATIONAL SEAWEED SYMPOSIUM, COPENHAGEN

Total phenolic content and biological activities of enzymatic extracts from *Sargassum muticum* (Yendo) Fensholt

Maya Puspita[1,2] · **Maureen Déniel**[1,3] · **Ita Widowati**[2] · **Ocky Karna Radjasa**[2] · **Philippe Douzenel**[1] · **Christel Marty**[1] · **Laurent Vandanjon**[1,3] · **Gilles Bedoux**[1] · **Nathalie Bourgougnon**[1]

Received: 11 August 2016 / Revised and accepted: 7 February 2017 / Published online: 11 March 2017

Abstract Seaweeds are potentially excellent sources of bioactive metabolites that could represent useful leads in the development of new functional ingredients in pharmaceutical and cosmetic industries. In the last decade, new marine bioprocess technologies have allowed the isolation of substances with biological properties. The brown alga *Sargassum muticum* (Yendo) Fensholt (Ochrophyta) was enzymatically hydrolyzed to prepare water-soluble extracts by using six different commercially available carbohydrate-degrading enzymes and two proteases. Evaluation of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) antioxidant, tyrosinase, elastase, and biofilm inhibition, antibacterial and antiviral activities as well as evaluation of cytotoxicity were realized for each extract. Total phenolic content was measured for extract characterization, and solid-phase extraction was useful to purify the enzymatic extract. Soluble total phenolic content of *S. muticum* Viscozyme extract was highest with 6.4% of dry weight. Enzymatic Celluclast and Viscozyme extracts had the lowest value of DPPH IC_{50} indicating a strong antiradical activity, 0.6 mg mL^{-1}, in comparison with other enzymes. The ferric reducing antioxidant power ranged between 48.7 μM Fe^{2+} Eq, digested with Viscozyme, and 60.8 μM Fe^{2+} Eq, digested with Amyloglucosidase. Tyrosinase inhibition activity of *S. muticum* Neutrase extract was 41.3% higher compared to other enzymes. Elastase inhibition activity of *S. muticum* Shearzyme extract had highest activity (32.8%). All enzymatic extracts showed no cytotoxic effect towards the kidney Vero cells. Meanwhile, only *S. muticum* Neutrase and Alcalase extracts exhibited potential antiviral activity. In addition, *S. muticum* Viscozyme and Shearzyme extracts showed promising activity in suppressing the biofilm formation against *Pseudomonas aeruginosa* and *Escherichia coli*, respectively. Purification of *S. muticum* Viscozyme extracts by solid-phase extraction managed to concentrate the phenolic content and improve the bioactivity. These results indicate the promising potential of enzyme-assisted followed by solid-phase extraction in recovering phenolic content and in improving its bioactivity.

Keywords Bioactivities · Phaeophyceae · Carbohydrase · Enzyme-assisted extraction · Protease · Solid-phase extraction

Introduction

Marine algae have attracted a lot of attention in the last few years. Besides their important ecological role in the ocean, marine algae contain significant quantities of vitamins, minerals, dietary fibers, proteins, polysaccharides, and various functional polyphenols while nutrient contents can vary with species and geographical location, season, and temperature. Algae, rich in nutrition elements of interest, are traditionally consumed as food in East Asian countries, especially in Korea, Japan, China, and Indonesia. Algae contain a large amount of phycocolloids such as agar, alginate, and carrageenan, which have been utilized as gelling agents and emulsifiers in the food and pharmaceutical industries (Liu et al. 2012; Andrade et al. 2013).

✉ Maya Puspita
maya.puspita@hotmail.co.id

[1] Université Bretagne Sud, EA 3884, LBCM, IUEM, 56000, Vannes, France

[2] Faculty of Fisheries and Marine Science, Marine Science Department, Diponegoro University, Semarang 50275, Indonesia

[3] Université de Nantes, GEPEA-UMR CNRS, 6144 Saint-Nazaire, France

Over the last few years, there have been more investigations in the research into new bioactive natural molecules and valuable metabolites from seaweeds having an economic impact. For example, sulfated anionic polysaccharides have been reported to exhibit anti-inflammatory (Mhadhebi et al. 2014), antioxidant (Costa et al. 2011, Chale-Dzul et al. 2015), antiviral (Eom et al. 2015), antimicrobial (Kantachumpoo and Chirapart 2010), and antiproliferative (Lowenthal and Fitton 2015; Ermakova et al. 2016) activities. Proteins also exhibit a potent bioactivity as antioxidant, antityrosinase, antihypertensive, anticoagulant, and antitumor (Suetsuna et al. 2004; Heo et al. 2005; Bondu et al. 2015).

For many years, phenolic compounds from brown algae have been widely studied for their biological activities. Phenolic compounds also called phlorotannins are secondary defense metabolites synthesized during development as components of algal cell walls (Schoenwaelder and Clayton 1999) or as chemical defense in response to abiotic and biotic stress conditions, such as UV radiation, grazing, bacterial infection, and epiphytism, as well as in intra- and interspecific communication (Burtin 2003; Koivikko 2008). Phlorotannins correspond to polymerization of phloroglucinol monomer units containing both phenyl and phenoxy units (Li et al. 2011). Accumulation of these phloroglucinol-based phenolics contributes to the strong antioxidant activity against free radical-mediated oxidation damage (Kang et al. 2012a; Fujii et al. 2013). They also show other potential bioactivity such as a bactericidal, antiviral, and anticancer activity and radio protective and antiallergic effects (Li et al. 2011; Eom et al. 2012; Le Lann et al. 2016).

In the last decades, natural bioactives from plant, fungal, and marine sources are actively investigated as replacements for synthetic antioxidants currently used as food additives. Even if phenolic compounds are sensitive to extraction techniques based on heat or solvent use (Kadam et al. 2013), conventional solid–liquid extraction is frequently used to extract bioactive compounds from algae. There is no standardized protocol for the phenolic compound extraction, as each species produces different chemically structured phenolic compounds. Principal characteristic of solid-liquid extraction method is the utilization of organic toxic solvents and is often time-consuming. As stated by Ibanez et al. (2012), a green chemistry technology concerns and searches for alternatives in order to reduce hazardous chemical exposure to humans and the environment, prevent the waste, and improve the extraction yield. Several methods have appeared, namely supercritical fluid and pressurized liquid extraction and microwave-, ultrasound-, and enzyme-assisted extraction (Michalak and Chojnacka 2014).

Sulfated fucans and alginates encompass the main portion of the wall in brown algae (up to 45% of algal dry weight), while cellulose only accounts for a small fraction (1–8% of algal dry weight). Proteins, phlorotannins, and halide compounds (i.e., iodide, bromide, and chloride) are additional components in brown algal cell walls (Deniaud-Bouet et al. 2014). The chemical and structural complexity of brown algae cell walls and their heterogeneity have become the main drawbacks during the extraction process (Jeon et al. 2012; Wijesinghe and Jeon 2012). These drawbacks decrease the extraction rate and efficiency. As a consequence, the degradation of algal cell wall must be taken into consideration when choosing an extraction method since it is an important step in the release of active compounds (Wijesinghe and Jeon 2012). Application of enzyme in degrading the cell wall of algae has been considered for the past decades as it has been proven to be effective in improving the yield of active compounds and enhancing the bioactivity (Hardouin et al. 2014; Kulshreshtha et al. 2015; Olivares-Molina and Fernández 2016).

As an invasive species that is widely spread along the European Atlantic coasts (Plouguerné et al. 2006; Tanniou et al. 2014), the brown macroalga *Sargassum muticum* is one of the most readily available Sargassaceae species in Europe and has been remarked as a potential sustainable source of bioactive compounds. The genus *Sargassum* is known to produce interesting phenolic compounds of high molecular weight, 5000 and 14,000 Da (Le Lann et al. 2012), which demonstrated biological activities such as antifouling, antiproliferative, antiangiogenesis, and antioxidant.

The aim of this work is to propose the use of nonselective commercial carbohydrase and protease as a tool to improve the extraction efficiency of phenolic compounds from *S. muticum*. Recovery and characterization of total phenolic content and bioactivities of the water-soluble fractions were determined, and results were discussed in relation to the different enzymes used during extraction. Evaluation of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) antioxidant, tyrosinase, elastase, and biofilm inhibition and antibacterial and antiviral activities as well as evaluation of cytotoxicity were performed in vitro. Solid-phase extraction was conducted to obtain a phlorotannin-enriched fraction.

Materials and methods

Algal biomass collection

The brown alga *Sargassum muticum* (Yendo) Fensholt was collected in June 2014 from the rocky shore of St. Gildas de Rhuys peninsula, South Brittany, France (47° 30′ 03″ N, 2° 50′ 12″ W). Apical parts of *S. muticum* thalli were cut and placed in closed boxes to avoid direct contact with sunlight. The collected algal materials were rinsed with tap water to remove the epiphytes and other residual sand. They were ground with a Jupiter's Universal grinder (Wernau, Germany) using a 3-mm perforated grill, lyophilized, and the dry algal materials were stored in the dark for further analysis.

Enzyme-assisted extraction of *S. muticum*

Approximately, 6.5% of dry algal material (19.5 g of dry material) was diluted in 300 mL of distilled water and 2.5% (of dry algal material) commercial enzyme was added. The eight enzymes used were two proteases Neutrase and Alcalase and six carbohydrases Ultraflo, Amyloglucosidase (AMG), Shearzyme, Termamyl, Viscozyme, and Celluclast. Extraction without enzyme or aqueous extract served as negative control. Figure 1 illustrates the enzyme-assisted extraction (EAE) of *S. muticum*.

Sample solutions were incubated for 3 h at 50 °C. Prior to filtration, all samples were denatured for 15 min in 90 °C. Soluble enzymatic extracts were frozen and lyophilized for further analysis. Insoluble enzymatic extracts (residual materials) were stored in −20 °C for total phenolic analysis to determine the insoluble phenolic compounds that may not be digested by the enzymes. All *S. muticum* enzymatic extracts were prepared in triplicates.

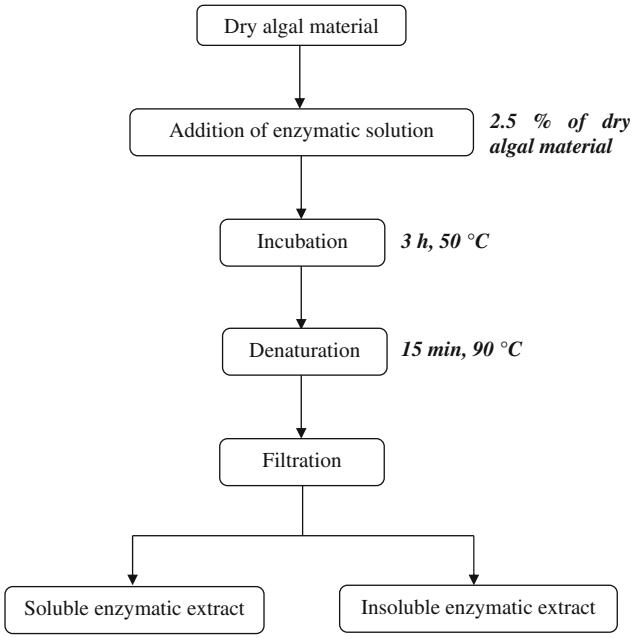

Fig. 1 Enzyme-assisted extraction of *S. muticum*

Total phenolic content analysis

Folin-Ciocalteu method is commonly used for measuring the total phenolic content (TPC). This assay is based on the reduction-oxidation (redox) reactions, which are usually considered to be relatively stoichiometric and on the redox potential of the phenolic hydroxyl group (Hagerman and Butler 1989).

Briefly, 0.5 mL of samples or standard (Phloroglucinol) was added to 0.5 mL 95% ethanol. And 2.5 mL H_2O was then added, followed by 0.25 mL of Folin-Ciocalteu reagent (50%, *v*/*v*). All samples were agitated and left to stand for 5 min. Finally, 0.5 mL of Na_2CO_3 solution (5%) was added. Optical density was read against the blank at 725 nm. Phloroglucinol was used as standard prepared in different concentrations ranging from 0 to 100 μg mL^{-1} (Chandler and Dodds 1983; Heo et al. 2005; Wang et al. 2010).

Free radical *scavenging* activity

DPPH is a stable free radical. DPPH assay is based on the theory that a hydrogen donor is an antioxidant. It measures compounds that are radical scavengers. DPPH shows a strong absorption maximum at 517 nm. This analysis is based on modified method of Yen and Chen (1995) and Chen et al. (2008).

A series of butylated hydroxytoluene (BHA), butylated hydroxyanisole (BHT), and ascorbic acid solutions—serves as standard—in different concentrations was prepared (2–50 μg mL^{-1}). 0.25 mM of DPPH solution was introduced to 100 μL sample solution. Sample solutions of *S. muticum* enzymatic extracts were made in different concentrations by diluting the stock solution in methanol (0–1000 μg mL^{-1}). Before reading the optical density at 517 nm, all samples were incubated at 40 °C for 30 min. Percentage of inhibition was calculated using the following formula:

$$I\,(\%) = \left[{}^{(A_C - A_S)} \Big/ {}_{A_C} \right] \times 100$$

where I (%) = inhibition (expressed in %), A_C = absorbance of control, and A_S = absorbance of samples.

IC_{50}, corresponding to the concentration sufficient to obtain 50% of a maximum scavenging capacity, of samples was determined based on the regression obtained from dose–response curve.

Ferric reducing antioxidant power

This method is based on the reduction of a ferric ion analogue, the complex of tripyridyltriazine $Fe(TPTZ)^{3+}$, to the intensely bluish color complex of $Fe(TPTZ)^{2+}$ by the presence of antioxidants in acidic medium. Then, the results were obtained from the absorbance at 593 nm and expressed as micromolar Fe^{2+} equivalents (Benzie and Strain 1996, 1999). The TPTZ reagent consisted of 300 mM of acetate buffer with pH 3.6, 10 mM 2,4,6-tripyridyl-*s*-triazine (TPTZ), and 20 mM $FeCl_3 \cdot 6H_2O$ solution. Fifty microliter of *S. muticum* enzymatic extract was introduced in a microplate mixed with 150 μL reagent. After 15 min incubation, the absorbance was read at 593 nm. The results were expressed in micromolar equivalent to $FeSO_4 \cdot 7H_2O$ (Shahwar et al. 2012).

Tyrosinase inhibition activity

Inhibition of tyrosinase activity was performed based on Chan et al. (2008) and Lim et al. (2009). Phosphate buffer (80 μL) was introduced to 20 μL of *S. muticum* enzymatic extract (100 μg mL^{-1}) added to 20 μL of 50% (*v*/*v*) dimethyl sulfoxide (DMSO). Later, 40 μL of L-3,4-dihydroxyphenylalanine (L-DOPA) and 40 μL of mushroom tyrosinase enzyme were added to complete the reaction. Before reading the absorbance at 475 nm, all microplates were incubated at 37 °C for 30 min. Quercetin and kojic acid served as standards prepared in different concentrations (0.02–0.4%). All reactions were made in triplicate. Percentage of inhibition was calculated using the following formula:

$$I\ (\%) = \left[{}^{(A_C - A_S)} \Big/ _{A_C} \right] \times 100$$

where $\boldsymbol{I}$ (%) = inhibition (expressed in %), A_C = absorbance of control, and A_S = absorbance of samples.

Elastase inhibition activity

As an inhibitor, trypsin inhibitor type II-O was used, prepared in phosphate buffer in final concentration 1 g L^{-1}. All tested samples were dissolved in 10% (*v*/*v*) DMSO with final concentration same as standard (1 mg mL^{-1}). The activity of porcine pancreatic elastase (type I; Sigma Chem. Co) was examined using substrate (*N*-methoxysuccinyl-ala-ala-proval-*p*-nitroanilide/*N*-Suc-$(Ala)_3$-nitroanilide), and the release of *ρ*-nitroaniline was measured at 405 nm.

The reaction of 20 μL samples (1 μg mL^{-1}) was carried out in 80 μL of 100 mM Tris and 500 mM NaCl pH 8, 50 μL of 0.4 U mL^{-1} elastase solution, and 50 μL of 0.64 mM substrate. Elastase inhibition was assessed every 10 min at 25 °C for 40 min. The same reactions were applied for the standard, but the volume of standard introduced in the well ranged from 0 to 100 μL (C_f = 0–0.5 μg mL^{-1}) in order to obtain the calibration curve. All reactions were made in triplicate (Sallenave et al. 1998).

The percentage of inhibition of elastase was calculated as follows:

$$I\ (\%) = \left[{}^{(A_C - A_S)} \Big/ _{A_C} \right] \times 100$$

where $\boldsymbol{I}$ (%) = inhibition (expressed in %), A_C = absorbance of control, and A_S = absorbance of samples

Biofilm inhibition activity

This test is based on the crystal violet assay (Adetunji and Isola 2011; Welch et al. 2012). *Pseudomonas aeruginosa* and *Escherichia coli* (Gram-negative bacteria) were chosen as biofilm forming bacteria. Prior to analysis, bacterial solution of 1.5×10^8 cells in sterilized 10% (*v*/*v*) saline water was made by incubating the bacteria culture in Lysogeny broth (LB) sterile medium at 37 °C for 24 h. Lysogeny broth medium was composed of Tryptone and yeast extract prepared in sterilized saline water. Bacteria culture was centrifuged for 5 min at 5000 rpm, and the supernatant was discarded. The remaining pellet was dissolved in 10% physiological sterile water.

Bacterial solution (200 μL) was introduced into a sterile 96-well microplate and left for 24 h at 37 °C in order to allow the bacteria to form a film layer in the microplate. After a 24-h incubation, the plate was washed two times with the 10% physiological sterile water. Then, 200 μL of *S. muticum* enzymatic extracts (1 mg mL^{-1}) dissolved in LB was added to the microplate and incubated for 24 h at 37 °C. In the next 24 h, the microplate was rinsed two times with physiological sterile water and 200 μL of 10% crystal violet (*v*/*v*)—prepared in ultrapure water—was added. Fifteen minutes after the crystal violet was added, the microplate was rinsed and the biofilm formed in the microplate was suspended in 200 μL of ethanol. The microplate was read in 600 nm. Negative and positive controls consisted of sterilized saline water and bacteria were applied. Both the control and the samples were made in triplicates.

Antibacterial activity

The agar diffusion method (Bauer et al. 1966) was chosen for evaluating the antibacterial activity of *S. muticum* enzymatic extracts. Two Gram-positive bacteria—*Staphylococcus hominis* and *Bacillus subtilis*—as well as two Gram-negative bacteria—*P. aeruginosa* and *E. coli*—were chosen for this test. These bacteria were prepared in LB medium with bacterial density 1.5×10^8 cfu mL^{-1}. Bacterial suspension was poured on solidified LB agar media and incubated for 1 h at 37 °C.

For screening, *S. muticum* enzymatic extract of 5 mg mL^{-1} was prepared in sterile physiological water. Twenty microliters of sample solution—extract solution or positive control (phosphomycin, ampicillin, and streptomycin at 1 mg mL^{-1})—was added to sterile paper discs. All samples were in triplicate. Petri dishes were then incubated at 37 °C for 48 h. The zone of inhibition was established on the agar media and was then measured and expressed in centimeters.

Antiviral and cytotoxicity activity

Antiviral activity of *S. muticum* enzymatic extracts and their cytotoxicity were analyzed. For cytotoxicity, the kidney cells of African green monkey (Vero cells) were used. Herpes simplex virus type 1 (HSV-1) model was used to determine the antiviral effect.

One hundred microliter of cell suspension (3.5×10^5 cells mL^{-1}) in Eagle's MEM containing 8% FCS was incubated with 50 μL of diluted enzymatic extract (concentration from 10 to 500 μg mL^{-1}) in 96-well plates (72 h, 37 °C, 5% CO_2). Three replicates were infected using 50 μL of medium and a virus suspension at a multiplicity of infection (MOI) of 0.001 ID_{50} $cells^{-1}$. After incubation, antiviral activity was evaluated by the neutral red dye method. Zovirax, a commercial antiherpetic acyclovir (9-2(2-hydroxyethoxymethyl) guanine), was used as reference inhibitor (positive control).

For antiviral, 50% effective antiviral concentration (EC_{50}) was expressed as the concentration that achieved 50% protection of virus-infected cells from virus-induced destruction. The optical density (OD) was related directly to the percentage of viable cells, which was inversely related to the cytopathic effect (CPE). The linear regression was determined for each assay on the basis of cell controls (0% CPE) and virus controls (100% CPE). Data were expressed as a percentage of protection (*%P*):

$$\left[\frac{\left((OD_t)_{virus}-(OD_c)_{virus}\right)}{\left((OD_c)_{MOCK}-(OD_c)_{virus}\right)}\right] \times 100$$

where $(OD_t)_{virus}$ is the OD of the test sample, $(OD_c)_{virus}$ is the OD of the virus control, and $(OD_c)_{MOCK}$ is the OD of the mock-infected control (McLaren et al. 1983; Langlois et al. 1986).

For cytotoxicity, 50% cytotoxic concentration (CC_{50}) was defined as the concentration that reduced the OD of treated cells to 50 % of that of untreated cells. Data were expressed as a percentage of protection (*%D*):

$$\left[\frac{\left((OD_c)_C-(OD_c)_{MOCK}\right)}{(OD_c)_C}\right] \times 100$$

where $(OD_c)_C$ and $(OD_c)_{MOCK}$ are the OD values of untreated cells and treated cells, respectively (Langlois et al. 1986).

Solid-phase extraction of *S. muticum* enzymatic extracts

Solid-phase extraction (SPE) was performed by using C-18 cartridges based on the method of Tanniou et al. (2013), with slight modification. Prior to the fractionation process, the cartridges were conditioned with the following elution: 20 mL methanol and 20 mL ultrapure H_2O successively. Raw enzymatic extract (4 mL) was loaded into the cartridge and left to absorb the column. The phlorotannins were then eluted with 40 mL methanol 50% (*v*/*v*). Prior to elution, the cartridge was washed with 1% acidic water to remove sugars, organic acids, and other water-soluble constituents (Dai and Mumper 2010). All processes were repeated four times in order to accumulate sufficient amount of fractions. The same fractions were pooled and evaporated under reduced pressure at 40 °C on a rotary evaporator. Each fraction was redissolved using acetonitrile-methanol (2:1; *v*/*v*). The phenolic content of methanolic fraction was determined with the Folin-Ciocalteu method, and its antioxidant activity was tested by means of DPPH and FRAP.

Fourier transform infrared spectroscopy (FT-IR)

Spectra of *S. muticum* enzymatic extracts were determined using a Nicolet iS 5 FT-IR spectrometer (Thermo Scientific) with diamond crystal plates. The spectra were collected with subsequent scanning of samples in triplicate ranging from 4000 to 400 cm^{-1}. Dry enzymatic extracts (2 mg) were introduced onto the crystal plate. The spectra were acquired as the percentage of absorbance. The detected peaks were then compared with a standard of phloroglucinol.

High performance liquid chromatography analysis

The chromatographic analysis was performed by UltiMate 3000 Rapid Separation LC (RSLC) System consisting of pump, auto-samplers, and diode array detector (DAD-3000RS) with UV–vis detection (Thermo Scientific). The column was a Nucleodur RP C_{18} ec (250 × 4.6 mm i.d., 5 μm) of Macherey-Nagel. The reversed-phase high performance liquid chromatography (HPLC) analysis was performed based on the modified method of Koivikko et al. (2007) and Sanchez-Camargo et al. (2015). *Sargassum muticum* enzymatic extracts were eluted by a multistep gradient of solvents A (1 % acid formic in water) and B (acetonitrile). The elution was as follows: 0–20 min, 5 % B; 20–35 min, 20 % B; 35–45 min, 80 % B; and 45–55 min, 0 % B.

Phenolic compounds were detected at 280 nm, while the wavelength was set ranging from 240 to 300 nm. Injection volume was 100 μL with 1 mL min^{-1} flow rate. Internal standard range of phloroglucinol was prepared with concentration ranging from 100 to 800 μg mL^{-1}.

Statistical analysis

All results are presented as mean ± standard deviation (SD) with $n = 3$. Analysis of variance (ANOVA) was performed using IBM SPSS Statistics 20. Normality and homogeneity tests were performed on all data. Furthermore, the statistical differences between samples were determined using the Tukey test with significance level at 5 % ($p < 0.05$). Significant differences showed by each sample tested were labeled as different superscript letters. Principal component analysis (PCA) from the FT-IR spectra of *S. muticum* enzymatic extract was performed to transform a number of potentially correlated variables into a number of relatively independent variables that could be ranked based upon their contribution for explaining the variation of the whole data set (Xu and Hagler 2002).

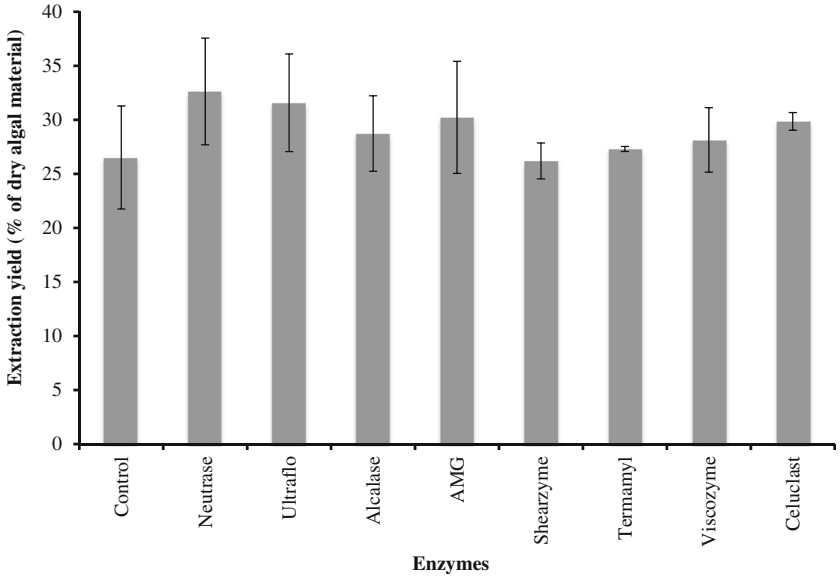

Fig. 2 Yield of enzyme-assisted extraction of *S. muticum* (there is no significant difference between tested samples, $p > 0.05$). Aqueous extraction was used as control

Results

Yield of enzyme-assisted extraction

After the extraction, the insoluble phase was separated from the soluble aqueous phase. Only the soluble aqueous phase was studied. Extraction yields are presented in Fig. 2. There was no significant difference in the yield of enzyme-assisted extraction ($F(8, 18) = 1.127$, $p = 0.392$). In comparison to the aqueous extract (26.5 ± 4.7% of dry algal material), the enzymatic extracts produced higher yield, except for Shearzyme extract (26.2 ± 1.7% dry algal material). Neutrase extract yielded 32.6 ± 4.9% of dry algal material, while Alcalase extract only yielded 28.7 ± 3.5% dry algal material. Second highest yield was obtained from Ultraflo extract, 31.6 ± 4.5% dry algal material. The yield of enzyme-assisted extraction from other enzymes, i.e., AMG, Termamyl, Viscozyme, and Celluclast extracts, ranged between 27.3 ± 0.2 and 30.2 ± 5.2% of dry algal material.

Total phenolic content of *S. muticum* enzymatic extracts

Insoluble and soluble TPC of *S. muticum* enzymatic extracts were determined, and results were expressed as percent of dry algal material and are presented in Table 1. For insoluble TPC, all enzymatic extracts showed the same content of phenolic content, i.e., 0.9 % dry algal material. As for control, there was 1.0 ± 0.0 % dry algal material (data not shown). Despite the

Table 1 Soluble total phenolic content, DPPH IC_{50}, FRAP, tyrosinase, and elastase inhibition activity of *S. muticum* enzymatic extracts

Samples	Soluble TPC (% of dry algal material)	DPPH IC_{50} (mg mL^{-1})		FRAP (μM Fe^{2+} Eq)		Tyrosinase inhibition activity (% of inhibition)		Elastase inhibition activity (% of inhibition)	
		BHA	0.008 ± 0.5	Vitamin C	58.1 ± 0.7	Kojic acid	67.6 ± 6.5	Trypsin inhibitor	50.0 ± 0.0
		BHT	0.012 ± 4.8			Quecertin	42.7 ± 9.4		
Control	2.6 ± 0.0^{a}		0.7 ± 0.2^{a}		65.7 ± 1.0^{a}		14.7 ± 3.1^{a}		23.1 ± 4.8^{ac}
Neutrase	3.8 ± 0.0^{ac}		0.9 ± 0.1^{a}		58.8 ± 0.6^{bd}		6.6 ± 1.5^{b}		23.4 ± 3.3^{ac}
Ultraflo	4.2 ± 0.0^{ac}		2.2 ± 0.9^{c}		58.4 ± 0.8^{b}		24.5 ± 2.6^{c}		24.0 ± 4.2^{ac}
Alcalase	3.8 ± 0.0^{ac}		1.9 ± 0.3^{bc}		56.1 ± 3.0^{c}		10.7 ± 3.5^{a}		30.9 ± 5.9^{ac}
Celluclast	4.5 ± 0.0^{bc}		0.6 ± 0.1^{a}		59.3 ± 1.7^{bd}		19.9 ± 5.1^{a}		29.9 ± 0.5^{ac}
AMG	4.8 ± 0.0^{bc}		0.7 ± 0.3^{a}		60.8 ± 1.8^{ad}		15.4 ± 2.3^{a}		21.6 ± 0.8^{a}
Shearzyme	4.7 ± 0.0^{bc}		1.0 ± 0.3^{ab}		58.4 ± 0.7^{bd}		13.0 ± 0.7^{a}		32.8 ± 0.9^{bc}
Viscozyme	6.4 ± 0.5^{bc}		0.6 ± 0.1^{a}		48.7 ± 0.9^{c}		23.5 ± 2.6^{c}		29.7 ± 1.9^{ac}
Termamyl	5.0 ± 0.1^{bc}		0.8 ± 0.2^{a}		57.4 ± 1.0^{b}		14.2 ± 2.5^{a}		28.7 ± 3.8^{ac}

Each value represented is mean ± standard deviation ($n = 3$), distinguished by different superscript letters in order to indicate a significant difference ($p < 0.05$)

IC_{50} inhibitory concentration 50 %—concentration needed to inhibit 50 % of the reaction, *Eq* equivalent

low value, this result indicated that there were still some traces of phenolic compound in the residues of *S. muticum* enzymatic extracts.

Statistical analysis showed that there was a significant difference in total phenolic content of *S. muticum* enzymatic extract (F (8, 18) = 4.097, p = 0.002). Aqueous extracts of *S. muticum* was significantly different from Celluclast, Shearzyme, AMG, Termamyl, and Viscozyme extracts ($p < 0.05$). Nevertheless, there was no significant difference exhibited in total phenolic content of aqueous, Alcalase, Neutrase, and Ultraflo extracts of *S. muticum* ($p > 0.05$). Neutrase extract (Fig. 2) had the highest yield of dry algal material. However, the highest soluble TPC was shown by Viscozyme extract, 6.4 ± 0.5% of dry algal material, followed by Termamyl extract, 5.0 ± 0.1 % of dry algal material. The proteases, i.e., Neutrase and Alcalase, only extracted 3.8 ± 0.0 % of dry algal material. Nevertheless, in comparison with the aqueous extract (2.6 ± 0.0 % of dry algal material), all eight enzymes enhanced the extraction of phenolic compounds in *S. muticum*.

Bioactivities of *S. muticum* enzymatic extracts

Results for *S. muticum* enzymatic extract bioactivities are presented in Tables 1 and 2. Antioxidant activities of *S. muticum* were determined through the free radical scavenging ability and the reducing power by means of DPPH and FRAP analysis. As summarized in Table 1, certain samples showed a significant difference of DPPH IC_{50} compared to aqueous extract (F (8, 18) = 8.640, p = 0.000). Ultraflo extract of *S. muticum* was significantly different from Shearzyme, Neutrase, Termamyl, AMG, Celluclast, Viscozyme, and aqueous extracts ($p < 0.05$). Alcalase extract also showed a significant difference towards other extracts ($p > 0.05$) except for Shearzyme and Ultraflo extracts ($p > 0.05$).

Having low value of IC_{50} indicates that the sample has a strong antiradical DPPH activity. In this case, Viscozyme and Celluclast extracts (0.6 ± 0.1 mg mL^{-1}) would be mostly effective against the free radical compared to the other. Ultraflo (2.2 ± 0.9 mg mL^{-1}) and Alcalase extracts (1.9 ± 0.3 mg mL^{-1}) were extracts with less effective activity against the free radical as shown by their high value of DPPH IC_{50}. Nevertheless, free radical scavenging activity of *S. muticum* enzymatic extracts was inferior to the commercial antioxidant BHA and BHT (IC_{50} = 0.008 ± 0.5 and 0.012 ± 4.8 mg mL^{-1}).

All samples had significantly different reducing capacity from the aqueous extract (65.7 ± 1.0 μM Fe^{2+} Eq; F (8, 18) = 35.288, p = 0.000). The most effective reducing capacity was shown by AMG extract in 60.8 ± 1.8 μM Fe^{2+} Eq. On the contrary, Viscozyme extract did not show a strong reducing capacity with only 48.7 ± 0.9 μM Fe^{2+} Eq. The reducing power activity of *S. muticum* enzymatic extracts was still inferior to commercial vitamin C, 58.1 ± 0.7 μM Fe^{2+} Eq. Results are presented in Table 1.

For the tyrosinase inhibition activity of *S. muticum* enzymatic extracts, their activity showed significant difference (F (8, 18) = 16.895, p = 0.000). Neutrase, Ultraflo, and Viscozyme extracts were significantly different from all extracts ($p < 0.05$). There was no significant difference between aqueous, Alcalase, Celluclast, AMG, Shearzyme, and Termamyl extracts of *S. muticum* ($p > 0.05$). *S. muticum* enzymatic extract effects on tyrosinase enzyme were still low compared to kojic acid (67.6 ± 6.5 % of inhibition) and

Table 2 Antiviral and biofilm inhibition activity of *S. muticum* enzymatic extract

Samples	Biofilm inhibition activity (% of inhibition)		Antiviral activity		
	P. aeruginosa	*E. coli*		CC_{50} (μg mL^{-1})	EC_{50} (μg mL^{-1})
			Zovirax	>500	0.7 ± 0.3
Control	n.a.	14.7 ± 0.9^{a}		>500	>500
Neutrase	36.1 ± 7.8^{a}	29.1 ± 10.0^{ac}		>500	430.1 ± 16.3
Ultraflo	3.7 ± 2.0^{b}	32.8 ± 3.7^{ac}		>500	>500
Alcalase	n.a.	38.0 ± 7.9^{b}		>500	225.1 ± 23.3
Celluclast	n.a.	33.8 ± 7.7^{a}		>500	>500
AMG	n.a.	43.2 ± 5.9^{be}		>500	>500
Shearzyme	26.9 ± 2.1^{b}	64.3 ± 10.3^{de}		>500	>500
Viscozyme	43.7 ± 4.2^{a}	28.8 ± 9.4^{a}		>500	>500
Termamyl	12.4 ± 3.9^{b}	49.2 ± 13.3^{be}		>500	>500

Each value represented is mean ± standard deviation (n = 3), distinguished by different superscript letters in order to indicate a significant difference ($p < 0.05$)

CC_{50} cytotoxic concentration 50 %—a concentration of product generating 50 % of cell viability compared to control, *EC_{50}* antiviral effective concentration—a concentration of product causing 50 % of inhibition of HSV-induced destruction towards the cells, *n.a.* no activity detected

quercetin (42.7 ± 9.4 % of inhibition)—serving as positive controls. The inhibition activity of *S. muticum* enzymatic extracts ranged from 6.6 ± 1.5 % (Neutrase extract) to 24.5 ± 2.6 % of inhibition (Ultraflo extract).

In elastase inhibition analysis, a significant difference was only showed between the AMG (21.6 ± 0.8 % of inhibition) and Shearzyme extracts (32.8 ± 0.9 % of inhibition) (F (8, 18) = 4.231, p = 0.05). It seems that AMG extract has the lowest activity while Shearzyme extract had the highest. All *S. muticum* enzymatic extracts showed no significant difference with the control (23.1 ± 4.8 % of inhibition) ($p > 0.05$). Furthermore, the elastase inhibition activity of *S. muticum* enzymatic extracts was weaker than that of the trypsin inhibitor (50.0 ± 0.0 % of inhibition).

For the biofilm generated from *P. aeruginosa*, there was no sign of inhibition from the control, *S. muticum* enzymatic extracts of Alcalase, Celluclast, and AMG. Ultraflo and Termamyl extract showed weak inhibition with 3.7 ± 2.0 and 12.4 ± 3.9 % of inhibition, respectively. Highest activity was shown by Viscozyme extract with 43.7 ± 4.2 % of inhibition. Shearzyme and Termamyl extract (64.3 ± 10.3 and 49.2 ± 13.3 % of inhibition, respectively) performed strong biofilm inhibition activity formed by *E. coli*. In addition, their activity was significantly better than the aqueous extract, 14.7 ± 0.9 % inhibition. All *S. muticum* enzymatic extracts were less effective in inhibiting the formation of biofilm formed by *P. aeruginosa* compared to *E. coli*, as can be seen in Table 2. Statistical analysis exhibited a significant difference in biofilm inhibition activity of *S. muticum* against *P. aeruginosa* and *E. coli* (F (8, 18) = 5.870, p = 0.01; F (8, 18) = 6.621, p = 0.000).

Based on the antibacterial activity of *S. muticum* enzymatic extracts against four pathogenic bacteria, i.e., *S. hominis*, *B. subtilis*, *P. aeruginosa*, and *E. coli*, all samples showed no activity (data not shown). It is suggested that the presence of polysaccharides in raw enzymatic extracts provoked the growth of the bacteria instead of suppressing it. On the contrary, phosphomycin, ampicillin, and streptomycin—antibiotics served as positive control—showed substantial antibacterial activity. Inhibition zone formed by phosphomycin in *S. hominis*, *B. subtilis*, *P. aeruginosa*, and *E. coli* was 1.9 ± 0.1, 1.7 ± 0.1, 0.9 ± 0.1, and 2.2 ± 0.1 cm, respectively. Ampicillin showed inhibition zone of 3.6 ± 0.1, 1.6 ± 0.1, 0.5 ± 0.0, and 1.7 ± 0.1 cm in *S. hominis*, *B. subtilis*, *P. aeruginosa*, and *E. coli*. As for streptomycin, the inhibition zone formed was 3.4 ± 0.1, 1.1 ± 0.2, 2.7 ± 0.1, and 1.8 ± 0.2 cm in *S. hominis*, *B. subtilis*, *P. aeruginosa*, and *E. coli*, respectively.

No cytotoxicity effect of the extracts on the Vero cells was observed in the range of the concentrations assayed. After 3 days of treatment, no microscopically visible alteration of normal cell morphology was observed even at 200.0 μg mL^{-1}. As for their potentials as antiviral agent against the HSV-1, only Neutrase and Alcalase extracts showed a potential antiviral activity with EC_{50} at 430.1 ± 16.3 and 225.1 ± 23.3 μg mL^{-1}.

Phlorotannin-rich fraction of *S. muticum* enzymatic extract

Semi-purification of *S. muticum* digested with Viscozyme was performed by means of SPE method. The methanolic fraction of Viscozyme extract reached 51.5 ± 0.6 % of dry weight of phenolic content. It showed that there were tenfold phenolic content compared to the raw extract (6.4 ± 0.5 % dry algal material). Antioxidant activity of this fraction was also improved. The value of DPPH IC_{50} was 0.012 ± 0.6 from 0.6 ± 0.1 mg mL^{-1}. Reducing power capacity of this fraction also improved to 62.4 ± 2.0 from 48.7 ± 0.9 μM Fe^{2+} Eq.

FT-IR and HPLC analysis of *S. muticum* Viscozyme extracts

A preliminary analysis to detect the presence of phenolic compound in *S. muticum* enzymatic extracts was performed via FT-IR analysis. Figure 3 presents the score plot of *S. muticum* enzymatic extract from the FT-IR spectra: (A) the zone at 1800–600 cm^{-1} and (B) wave numbers that are particular to phenolic compounds (3200–3300, 2930–2945, 1605–1620, 1445–1460, 1075–1090, 920–935, and 865–880 cm^{-1}). Figure 2b shows a slight of grouping between Neutrase, Alcalase, Ultraflo, and aqueous extracts while the others were dispersed. It was assumed that they probably had similar characteristic of phenolic compounds that might be related to the chemical structure or numbers of polymers. Samples that are close to each other are similar in terms of what the components represent, in this case, the phenolic compounds based on the selected wave numbers. Unfortunately, as there were no additional measurable variables, it would be delicate to make further perception concerning the components influencing this similarity.

Viscozyme extract was chosen between the eight enzymes as this enzyme had the highest phenolic content based on the Folin-Ciocalteu method. FT-IR managed to detect the presence of phenolic compound based on the identified wavelengths as can be seen in Table 3. The liaison of phenolic compound detected from the raw and purified fraction of Viscozyme extract was at 873.72, 930.47, 1081.72, 1450.96, 2934.75, and 3239.54 cm^{-1}. These wave numbers were in accordance to those detected in phloroglucinol (standard) and from previous studies. Additional bonds which were not present in the purified fraction were detected at 1325 and 899.15 cm^{-1} in raw Viscozyme extract, related to the $C{=}CH_2$ and C–H deformation bonds, respectively.

Chromatogram of HPLC from purified fraction of Viscozyme extract is presented in Fig. 4. Based on the

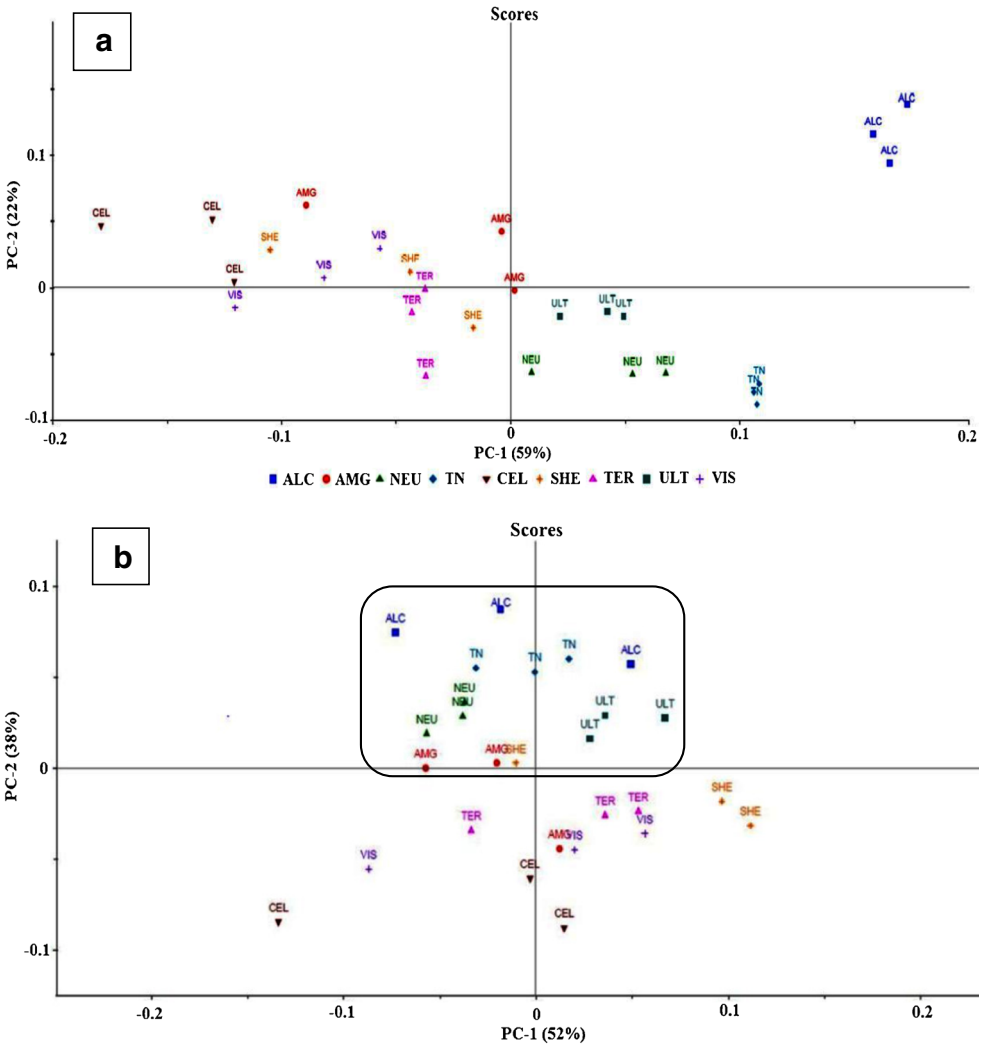

Fig. 3 Principal component analysis of *S. muticum*. **a** From all FT-IR spectra (600–1800 cm^{-1}). **b** Phenolic compound of enzymatic extraction from the selected FT-IR spectra (865–880, 920–935, 1075–1090, 1445–1460, 1605–1620, 2930–2945, 3200–3300 cm^{-1}). *ALC* Alcalase; *AMG* Amyglucosidase, *NEU* Neutrase; *TN* control/aqueous extract, *CEL* Celuclast, *SHE* Shearzyme, *TER* Termamyl, *ULT* Ultraflo, *VIS* Viscozyme

Table 3 Identified wave numbers of Viscozyme extracts (raw and purified sample) correspond to the phenolic compounds

Wave number (cm^{-1})	Bonds	References
873.72[a]	C–H aromatics	Haeuser et al. (1961), standard (phloroglucinol)
887.07[b]		
899.15[a]	$C=CH_2$	Karunakaran et al. (2015), Haeuser et al. (1961)
930.47[a]	Vibration C–O–C	Sekkal et al. (1993)
932.22[b]		
1081.72[a]	C–O stretch	Gómez-Ordóñez and Rupérez (2011)
1075.70[b]		
1325[a]	C–H deformation	Gómez-Ordóñez and Rupérez (2011), Sartori (1997)
1450.96[a]	δ CH_2–CH_3 bend	Sigee et al. (2007)
1493.98[b]		
1618.11[a]	C=C cyclic	Kusumaningsih et al. (2016)
1617.51[b]		
2934.75[a]	C–H stretch	Ahn et al. (2006), Girija et al. (2013)
2935.76[b]		
3239.54[a]	O–H stretch	Standard (phloroglucinol)
3227.27[b]		

[a] Raw hydrolysate

[b] Purified hydrolysate

HPLC analysis, Viscozyme crude extract of *S. muticum* contained a compound with the same characteristic as phloroglucinol. This was due to the peak that was detected at the same retention time as phloroglucinols as shown in Fig. 4a Peak of phloroglucinols was detected at 280 nm. Figure 4b presents the detected peak of phloroglucinol-like compound in purified Viscozyme extract. As can be seen from this figure, the peaks were well separated one to each other, indicating that the purification method applied, i.e., SPE, managed to purify the phenolic compounds. In addition, the chromatogram method successfully elucidated the compound.

Discussion

The focus of this study was to evaluate the bioactivity of *S. muticum* phenolic compound obtained after enzymatic-assisted hydrolysis. This process has been relatively well-known for the past few years as an alternative method to extract the bioactive compounds (Athukorala et al. 2006; Charoensiddhi et al. 2014; Sanchez-Camargo et al. 2015) from algae. EAE is an environmentally friendly extraction method known by its high efficiency and reduced solvent consumption and time. It has been reported to be effective in releasing the bounded compound, increasing the overall yield (Rosenthal et al. 1996), and removing the barriers of water solubility and insolubility for bioactive compounds (Wijesinghe and Jeon 2012; Kadam et al. 2013). Efficiency of EAE particularly depends on the maintenance of its optimal conditions including time and temperature during the process to maximize the extraction yield (Jeon et al. 2012; Wijesinghe and Jeon 2012).

Eight commercial enzymes consisted of two proteases (Neutrase and Alcalase) and six carbohydrases (Ultraflo,

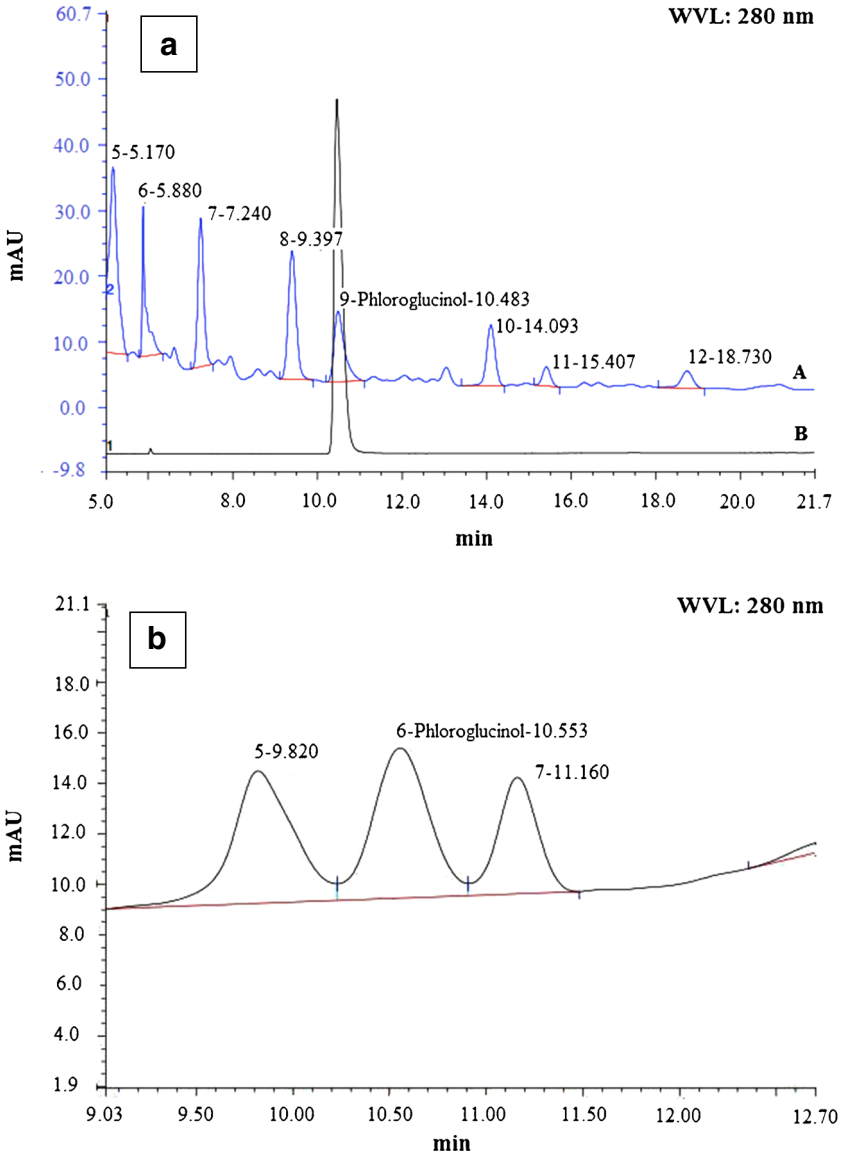

Fig. 4 Chromatogram of *S. muticum* from HPLC analysis. **a** *S. muticum* Viscozyme raw extract overlaid with phloroglucinol detected at 280 nm. **b** Purified fraction of *S. muticum* Viscozyme extract shows a well-separated fraction that is previously assumed to have aromatic rings same as phloroglucinol

Celluclast, AMG, Termamyl, Shearzyme, and Viscozyme) were used to release the phenolic compounds from *S. muticum*. Enzymes used in this study were proven to be effective in increasing the yield of extraction in comparison with water extraction, except for Shearzyme. Furthermore, they have been mostly used in brown algae (Heo et al. 2003; Ahn et al. 2004a; Heo et al. 2005; Athukorala et al. 2006; Kim et al. 2006; Je et al. 2009; Sanchez-Camargo et al. 2015). Highest yield was obtained from *S. muticum* digested with Neutrase, one of proteolytic enzymes, though the value was not significantly different from the other enzymes and water extraction. Enzymes from the protease group have been reported from several studies as an effective enzyme in enhancing the extraction yield in *Ishige okamurae*, *Sargassum coreanum*, *Sargassum fulvellum*, *Sargassum thunbergii*, and *Scytosiphon lomentaria* (Heo et al. 2003; Wang et al. 2010; Hardouin et al. 2014). Nevertheless, the proteolytic enzymes yielding the highest as reported by previous studies were different from the present one; they were Flavourzyme and Alcalase (Heo et al. 2003). Protamex yielded 19% of dry material for *S. fulvellum*, meanwhile for *S. horneri*, *S. coreanum*, and *S. thunbergii* extracted with Alcalase had 30–40% of extraction yield. In addition, *S. coreanum* extracted with Protamex contained 9.9 mg g^{-1} algal material of phenolic content, while Viscozyme extract had 11 mg g^{-1} of algal material. The highest phenolic content in *S. horneri*, *S. fulvellum*, and *S. thunbergii* came from the Alcalase extract with 5.3, 3.6, and 4.16 mg g^{-1} (Heo et al. 2003). In the present study, the highest phenolic content was also shown by Viscozyme extract of *S. muticum* with almost 6% of phenolic (6 mg g^{-1}). Variety in phenolic content obtained from this study and previous one might be due to the different condition of extraction that was applied, i.e., temperature and concentration of enzyme, and also the type of species. It leads to an assumption that the structural heterogeneity and various amounts of algal polysaccharides—alginates, fucoidans, and laminarian in the case of brown algae (Rhein-Knudsen et al. 2015), may influence the efficiency of enzymatic degradation. It is possible that the relative amount and sequence distribution of mannuronic (M) and guluronic (G) residues are two main factors affecting the extraction process since these are the main components in brown algal cell wall constituted as alginates. Mannuronic acids form β-1,4 linkages giving MM blocks a linear and flexible conformation. Meanwhile, guluronic acids generate α-1,4 linkages and introduce a steric hindrance around the carboxyl groups, thereby providing a folded and rigid structure, ensuring the stiffness in the polymer chain (Draget et al. 2005). M/G ratio of *Sargassum* alginates varies from 0.8 to 1.5 (Andriamanantoanina and Rinaudo 2010).

Most of the temperatures suggested for the extraction of marine plant phenolic compounds are below 60 °C (Tanniou et al. 2013; de Andrade et al. 2015; Ismail and Abdullah 2015). This is due to phenolic compounds being easily degraded because of high temperature. Nevertheless, there are several studies that tested the extraction of phenolic compound using temperature above 60 °C depending on the method and type of solvents that were applied for the extraction (Siriwardhana et al. 2008; Park et al. 2012; Shitanda et al. 2014). For marine algae, their maceration into a high level of heat might accelerate the extraction process as the heating softens the tissues and facilitate the depolymerization of polysaccharides (Siriwardhana et al. 2008), phenol-protein, and phenol-polysaccharide linkages (Tan et al. 2013). As shown by Siriwardhana et al. (2008), the brown alga *Hizikia fusiformis* extracted in water at 100 °C for 60 min yielded 4.65 % phenolic content, while at 25, 50, and 75 °C, it was only 1.40, 2.02, and 2.74 % of dry algal material, respectively. An elevated temperature may also help the solvents to reach the sample matrices as the viscosity and the surface tension of the solvents are decreased. In addition, it facilitates the solubility of analytes and promotes higher mass transfer rate (Dai and Mumper 2010). Using high temperature will be mostly compatible with water as solvent, since water becomes a strong source of hydronium (H_3O^+) and hydroxide (OH^-) ions, which can catalyze reactions, including hydrolysis of polysaccharides and proteins into smaller molecules. These molecules are more susceptible to react (Sereewatthanawut et al. 2008). Organic solvents such as methanol, acetone, and many others tend to evaporate at high temperatures (approximately >50 °C); thus, their efficiency decreases due to the shifting of solvent-to-water ratio leading to the change of sample-to-solvent ratio (Tan et al. 2013). As reported by Dai and Mumper (2010), besides temperature, the sample-to-solvent ratio is another one of the crucial factors that influence the yield of chemical extraction.

Antioxidative capacity of phlorotannins, phenolic compounds of brown algae, might also be degraded due to their heat and light sensitivity. The chemistry behind total phenolic assay like Folin-Ciocalteu assay relies on the transfer of electrons from phenolic compounds and other reducing species to molybdenum, forming blue complexes that can be detected spectrophotometrically. It is obvious that other reducing species besides phenolic compounds can also be involved in transferring the required electron (Plaza and Turner 2015). Higher phenolic content from the enzymatic extracts of *H. fusiformis* result from the study by Siriwardhana et al. (2008) is likely due to the reaction of other molecules, such as polysaccharides and proteins. In addition, these molecules are suggested to contribute in the antioxidant activity of *H. fusiformis*. *Hizikia fusiformis* is regarded as a taxonomic synonym of *S. fusiforme* (Harvey) Hetchell (Stiger et al. 2003). Therefore, it implies that the antioxidant activity in *S. muticum* enzymatic extract might also be due to the presence of polysaccharides and proteins.

In relation to algae, the introduction of the enzyme followed by heat treatment can further hydrolyze proteins and laminarin (Siriwardhana et al. 2008). Soluble phlorotannins—phenolics of brown algae—are stored in cell organelles, called physodes observed in the cytoplasm of brown algae (Schoenwaelder 2002). It is suggested that phlorotannins transformed into components of algal cell wall when physodes fuse with the cell membrane and the phlorotannins get secreted into the cell wall, complexing with alginic acid (Arnold and Targett 2003). Phlorotannins are also known to form strong complexes with proteins (Stern et al. 1996). As a consequence, enzyme facilitates the breaking of such complex linkages and this process results in liberating more phenolic compound to the solvents. However, it is important to note that high temperature had an adverse impact on the enzymes but the same case applies for the phenolic extraction (Wijesinghe and Jeon 2012). This is due to the instability of phenolic compounds as the elevated temperature stimulates a degradative reaction which is the oxidation of compound resulting in a decrease of phenolic yields (Dai and Mumper 2010). In summary, the temperature of EAE applied in this study was more favorable and suitable for the extraction of phenolic compound. In addition, this method offers a softer condition compared to other methods that apply higher temperatures as previously described.

This study reveals that the phenolic content of *S. muticum* extracted with carbohydrases was higher compared to the proteases. It leads to an assumption that the carbohydrases disrupt the algal cell wall and release the metabolites linked to the polysaccharides, in this case the phenolic compounds. It is also possible that the phenolic compounds of brown algae are mostly attached to the alginates instead of proteins. As mentioned by previous studies, the phenolic compounds are not only covalently attached to the proteins but also to the alginates (Deniaud-Bouet et al. 2014). The enzymatic degradation of alginate fractions always releases a significant amount of phenolics since phenolic substances form high molecular weight complexes with alginates due to oxidation with haloperoxidases (Bitton et al. 2006). This type of linkage serves as structural component constituting the brown algal cell wall. Phenolic cross-linking of alginate may be an integral part of initial wall formation as shown in the *Fucus* zygote (Arnold and Targett 2003).

From eight commercial enzymes used, Viscozyme—one of the carbohydrases—yielded the highest phenolic content. Viscozyme was also reported effective in extracting the phenolic content of *Ecklonia cava* (Laminariales, Lessoniaceae) and *S. coreanum* (Heo et al. 2005). The result in this study was also in accordance with previous study conducted by Sanchez-Camargo et al. (2015). They reported that *S. muticum* digested with Viscozyme had a higher content of phenolic compounds compared to Alcalase. However, the efficiency of Viscozyme in extracting the phenolic content from the brown algae works differently in every species. Siriwardhana et al. (2004) studied the effect of different commercial enzymes in antioxidant activity of *Hizkia fusiformis*. Based on their result, combination of Alcalase and Ultraflo showed the highest content of phenolic compound. Alcalase also enhanced the phenolic content of *S. thunbergii*, *S. horneri*, *S. fullvelum*, and *I. okamurae* (Heo et al. 2005). The difference in the response obtained in this study compared with others might be due to the optimum conditions that were applied (pH, temperature and time, and/or the carbohydrate compositions in different species leading to different mechanism of breakdown).

From the results of the bioactivities assay presented in Tables 1 and 2, it is apparent that the eight enzymatic extracts of *S. muticum* showed different levels of potential. It indicates that the extracted phenolic compound might function differently in responding to the test applied. In addition, it is also important to consider the possibility of an intervention from other compound as in this study the enzymatic extracts tested were all crude material.

The phenolic compounds are compounds with monomeric, oligomeric, or polymeric rings with an aromatic ring having one or more hydroxyl substituents (Balboa et al. 2013). Phlorotannins belonging to the phenolic compounds are exclusively found in brown algae. They have been known as a promising and strong antioxidant agent since they have up to eight interconnected rings of hydroxyl groups (Wang et al. 2009). The phenolic group is known as an excellent hydrogen donor forming hydrogen bonds with the carboxyl group of protein (Ozdal et al. 2013). Later, the existence of these hydroxyl groups provides the molecule with the ability of donating a proton to radical leading to its role as a possible chain breaking molecule or antioxidant upon secondary oxidation (Franco et al. 2008).

Phenolic compounds are one of the compounds that can upregulate the expression of antioxidant enzymes for examples the superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and reductase (GR). The different types of bioactivity chosen in this study were intended for the possibilities of phenolic compounds to be further applied in the nutraceuticals, cosmeceuticals, and painting industries. Therefore, the antioxidant activity of brown algae is strongly related to their phenolic content. As in this study, *S. muticum* digested with Viscozyme had strongest antiradical activity indicated by the low value of IC_{50}. Reducing the particle size can increase the extraction rate and the yield of phenolic compounds that can be achieved by grinding and enzyme-assisted extraction (Franco et al. 2008).

S. horneri digested with AMG, Celluclast, Termamyl, Ultraflo, and Viscozyme was reported to show DPPH radical scavenging activities at 25 μg mL^{-1} (Park et al. 2004, 2005). Heo et al. (2005) also studied the antioxidant activity via DPPH assay of *S. horneri* enzymatic extracts.

However, their results showed that the Viscozyme, Celluclast, AMG, and Ultraflo extracts with final concentration 2 mg mL^{-1} did not show any inhibition. Instead, the Termamyl extract of *S. horneri* showed 11.2 % inhibition. This activity was poor compared to the commercial standard BHA and BHT with 87 and 56 % inhibition, respectively. The same authors also reported that the antiradical activity of *S. fullvelum*, *S. coreanum*, and *S. thunbergii* extracted with carbohydrate enzymes also showed weak activity ranging from 4 to 38 % compared to the standards. This suggests that there is an interindividual and interspecies variation affecting the performance of antioxidant activity.

The antioxidant effects of *S. muticum* enzymatic extract might also be due to the presence of polysaccharides. The heterofucans of *Sargassum filipendula* have been reported to show antioxidant activity such as hydroxyl and superoxide radical scavenging, chelating effects on ferrous ions, and reducing powers in active concentration at 2 mg mL^{-1} (Costa et al. 2011). Lim et al. (2014) in their study concerning the antioxidant effects of *Sargassum binderi* also reported that the antiradical effect of this sample was at 2 mg mL^{-1}. It indicated that their antioxidant activity was weaker in comparison to the commercial standard, BHA, BHT, and ascorbic acid with 6.0×10^{-4}, 9.6×10^{-4}, and 3.5×10^{-4} mg mL^{-1}. However, it had performed high superoxide (compared to commercial food-grade fucoidan, BHA, and BHT) and hydroxyl radical scavenging activities (compared to BHT) (Lim et al. 2014). The ability of *Sargassum graminifolium* polysaccharides to inhibit 50% of superoxide anions scavenging and antiradical activities was at 1.9 and 0.6 mg mL^{-1}, respectively (Zhang et al. 2012).

Tyrosinase is a metalloenzyme oxidase catalyzing two distinct reactions of melanin synthesis. Melanin helps to protect skin from the damaging ultraviolet radiation of the sun. However, elevated concentrations of melanin in the skin can result in hyperpigmentation such as freckles and moles (Kang et al. 2012b). While tyrosinase is responsible for skin pigmentation, elastase is a proteinase enzyme capable of degrading elastin. Elastin is an extracellular matrix protein providing elasticity to the connective tissues. It forms elastic fiber in skin dermis having an influence on skin elasticity (Kim et al. 2009; Moon et al. 2010). Therefore, inhibition of tyrosinase and elastase activities become a method to protect against skin aging (Kim et al. 2009; Chang 2012). There is interest in the potential of marine algal bioactive compounds in cosmeceutical industries related to their tyrosinase and elastase inhibition activities. In the case of phlorotannins, many studies have reported their potential in inhibiting the tyrosinase activity. Dieckol, isolated phlorotannins from the brown alga *E. cava*, has been examined for its inhibitory effects on mushroom tyrosinase. Dieckol was capable to inhibit the mushroom tyrosinase with IC_{50} 20 μM and it showed no cytotoxicity (Kang et al. 2012b). 7-Phloroekol of *E. cava* has been observed to have an inhibitory effect on mushroom tyrosinase at 50 μg mL^{-1}. This compound was isolated from the ethanol extract of *E. cava* (Yoon et al. 2009). These examples provide knowledge concerning the tyrosinase inhibition activity from marine brown algal phlorotannins. Some results presented in the present study are in accordance with previous studies as previously mentioned. Polyphenol extracts of *S. muticum* extracted in different commercial enzymes showed interesting results regarding the tyrosinase and elastase inhibition activities. Attention was given to the tyrosinase inhibition activity; *S. muticum* Neutrase extract demonstrated the highest activity at 41 % compared to other enzymes. Accordingly, this result indicates another promising potential of enzymatic extracts of *S. muticum* polyphenol in the point of view of cosmeceutical industry.

Enzymatic extracts of *S. muticum* polyphenol did not show expected result against bacteria, as they did not demonstrate any antibacterial activity. It is suspected that the presence of sugar could contribute to the growth of bacteria in the media. However, this assumption will need further verification by evaluating the purified enzymatic extract of *Sargassum* polyphenol. It has been demonstrated that purified phlorotannin showed potent inhibition of cell membrane integrity as well as cell metabolism against *Trichophyton rubrum* (Eom et al. 2012). Phlorotannins from *Ecklonia kurome* were active against food-borne pathogen bacteria. Interaction between protein and brown algal phlorotannin has been considered to play important role in bactericidal action of purified phlorotannins (Ahn et al. 2004b). Even though the enzymatic extract of *Sargassum* polyphenol did not show antibacterial activity, biofilm inhibition activity demonstrated the opposite result. It seems that polyphenol of *Sargassum* enzymatic extracts was more active against biofilm formation. This contradictive result is caused by different mechanism of inhibition. In antibacterial activity, the mechanisms of inhibition are most likely thought to rely on inhibition of extracellular microbial enzymes, deprivation of substrates required for microbial growth, or direct inhibition of oxidative phosphorylation (Scalbert 1991). Regarding biofilm inhibition activity, two main mechanisms have been proposed, i.e., direct inhibition of bacterial settlement or survival of the macrofouler larvae and regulation of macrofouler growth, which in turn affects larval settlement of the macrofoulers (Avelin Mary et al. 1993). Some studies have reported the promising potential of brown algal polyphenol compound as antifouling agent. Water extract of *S. vulgare* polyphenol from its pneumatocysts presented 75 to 99 % inhibition of mussel *Perna perna* attachment. Polyphenol from leaflets of *S. vulgare* had 67 to 80 % inhibition of byssal adhesion (Plouguerné et al. 2012). Phlorotannin extract of *S. tenerrimum* had EC_{50} of larval settlement inhibition of *Hydroides elegans* at 0.526 ppm. It indicated that this

extract was effective in inhibiting the settlement of *H. elegans* larval attachment (Lau and Qian 1997).

Antiviral effects of brown algae are usually shown by their polysaccharides as explained by previous studies (Ahn et al. 2006; Lee et al. 2011; Elizondo-Gonzalez et al. 2012). However, for the past few years, several studies concerning the antiviral effects of brown algae phlorotannins have appeared. Ahn et al. (2004b) reported an inhibitory effect of 8,8′-bieckol and 8-4″-dieckol isolated from *E. cava* on HIV-1 reverse transcriptase and protease in vitro. Phloroglucinol, eckol, 7-phloroeckol, phlorofucoroeckol, and dieckol isolated from *E. cava* were effective against porcine epidemic diarrhea coronavirus at concentration ranging from 10.8 to 14.6 μM (Wang et al. 2008). Ethyl acetate fractions of phlorotannins from brown algae *Eisenia bicyclis* (Lessoniaceae) have been reported to show antiviral effects against genital human papilloma virus (HPV) at 50 μg mL^{-1} (Kim and Kwak 2015). Dichloromethane/methanol extract of *S. cymosum*, *S. polyceratium*, *S. vulgare*, *Padina gymnospora*, *Lobophora variegata*, *Dictyopteris delicatula*, *Sictyota menstrualis*, and *Stypodium zonale* demonstrated herpes simplex virus-1 (HSV-1) inhibition activity with percentage of inhibition ranging from 20 to 96 % at 400 μg mL^{-1}. Spectroscopic analysis of these crude extracts detected the presence of polyphenol compounds along with other active compounds like the halogenated sesquiterpenes obtusol, elatol, meroditerpenoid atomaric acid, triacylglycerols, and fatty acids (Soares et al. 2012). Out of eight, only two enzymatic extracts were found to be effective against the HSV-1; they were *S. muticum* digested with Neutrase and Alcalase. Result in this study regarding the antiviral effect of *S. muticum* enzymatic extract seems to be different from the previous studies. Out of eight, only two enzymatic extracts were found to be effective against the HSV-1; they were *S. muticum* digested with Neutrase and Alcalase. However, the antiviral effects of these two enzymatic extracts might not be provoked by the phlorotannins since the Viscozyme extract of *S. muticum* with high content of phenolic compound did not show any antiviral activity. It is possible that the presence of other compounds such as protein and polysaccharides that are in the enzymatic extracts is involved in disrupting the viral cells. As part of brown algal cell wall, phlorotannins are known to form a complex linkage with alginates and proteins. Early characterization by FT-IR has confirmed the presence of carbohydrates and proteins in *S. muticum* raw extracts. Zone 1800–600 cm^{-1} that was selected for PCA is the type that is usually used for polysaccharides and protein studies in algae (Rodrigues et al. 2015).

In tyrosinase, elastase, and biofilm inhibition activity as well as antiviral effects of *S. muticum* enzymatic extract, other compounds aside from polyphenol might also contribute to these bioactivities such as polysaccharides and proteins. To our best knowledge, there have not been any reported studies concerning the tyrosinase, elastase, biofilm inhibition, and antiviral activity of polyphenol from enzymatic extracts.

The semi-purification fraction of *S. muticum* Viscozyme extract yielded an important percentage of phenolic compounds assumed to be phlorotannins. The antiradical and the reducing capacity of the semi-purified fraction was also improved compared to the raw enzymatic extract. This result suggests that the antioxidant activity shown by *S. muticum* enzymatic extract is generated by the presence of phenolic compounds. This suggestion is supported by FT-IR (Table 3) and HPLC analysis (Fig. 3) that detects the presence of peaks corresponding to phloroglucinol.

FT-IR detects other peaks that appear to be correlated with phenolic bonds characterized by its aromatic and hydroxyl groups with the detection of C–H aromatics, C–O–C vibration, C–O stretch, and C=C. The characteristic of phenolic compounds lies on its aromatic ring (1260, 1620, 2970, and 2850–2970 cm^{-1}) and hydroxyl group (3415 cm^{-1}) (Ganapathi et al. 2013). However, a protein bond is also exposed through this analysis by the presence of amide bond either in the raw or semi-purified enzymatic extracts. On the contrary, the purification method has successfully removed the cellulose and mannuronic acid. Due to the lack of semi-purified extracts, it was impossible to characterize them using mass spectrometry. This will be performed in future work to help to identify the group of phlorotannins that exists in *S. muticum* extracted with enzyme.

This study has demonstrated that the application of enzymes in extracting the phenolic compound in *Sargassum* is quite effective. Based on the results presented in this study, different enzymes used give various results concerning the total phenolic content and their bioactivity. Among the eight enzymes, Viscozyme releases higher content of phenolic compounds and are correlated with strong antiradical activity. This might be supported by the optimum conditions provided, i.e., 50 °C for 3 h, during the extraction process, which is favorable for the Viscozyme, extract activity. Since phenolic compounds are sensitive to heat and easily oxidized by the exposure of light, it is strongly recommended to apply a mild temperature between 40 to 60 °C. Besides the extraction condition, in the case of phenolic compound, it is important to take the period of collection into further consideration. As commonly known, the production of phenolic compound is mostly stimulated by their environmental condition, such as the presence of grazer and direct exposition to sunlight. As a result, the content will vary according to the season.

Another important note to take is that by using enzymes, the active molecules released might not exclusively come from the phenolic groups. It is possible that it also extracts other molecules since the objective of this method is to degrade algae cell walls. Therefore, it will not be surprising that the fucoidan and protein might be responsible for the

bioactivity of *S. muticum*. As a consequence, it will be interesting to further work on the purified samples pretreated by the enzyme-assisted extraction in the future. In addition, the characterization of *S. muticum* phenolic compound might help in understanding more about the mechanisms of their bioactivity.

Acknowledgements This study was a collaboration research between Université Bretagne Sud (UBS), Vannes, France and Diponegoro University (UNDIP), Semarang, Indonesia under Double-Degree programme supported by the Directorate of Higher Education (DIKTI). The authors would like to thank all members of the Laboratoire de Biotechnologie et Chimie Marines (LBCM) who had been very helpful and supportive during the research. Special thanks are due to Dr. Valérie Stiger-Pouvreau from LEMAR, Université de Bretagne Occidentale (UBO), for her help in identifying the sample, Dr. Nolwenn Terme from UBS for her corrections, and Dr. Isuru Wijesekara for his interesting ideas and support during the purification.

References

Adetunji VO, Isola TO (2011) Crystal violet binding assay for assessment of biofilm formation by *Listeria monocytogenes* and *Listeria* spp on wood, steel and glass surfaces. Glob Vet 6:6–10

Ahn CB, Jeon YJ, Kang DS, Shin TS, Jung BM (2004a) Free radical scavenging activity of enzymatic extracts from a brown seaweed *Scytosiphon lomentaria* by electron spin resonance spectrometry. Food Res Int 37:253–258

Ahn MJ, Yoon KD, Min SY, Lee JS, Kim JH, Kim TY, Kim SH, Kim NG, Huh H, Kim J (2004b) Inhibition of HIV-1 reverse transcriptase and protease by phlorotannins from the brown alga *Ecklonia cava*. Biol Pharm Bull 27:544–547

Ahn MJ, Yoon KD, Kim CY, Kim JH, Shin CG, Kim J (2006) Inhibitory activity on HIV-1 reverse transcriptase and integrase of a carmalol derivative from a brown alga, *Ishige okamurae*. Phytother Res 20: 711–713

Andrade PB, Barbosa M, Pedro R, Lopes G, Vinholes J, Mouga T, Valentão P (2013) Valuable compounds in macroalgae extracts. Food Chem 138:1819–1828

Andrade RAMdS, Maciel MIS, Santos AMP, Melo EdA (2015) Optimization of the extraction process of polyphenols from cashew apple agro-industrial residues. Food Sci Tech 35:354–360

Andriamanantoanina H, Rinaudo M (2010) Relationship between the molecular structure of alginates and their gelation in acidic conditions. Poly Int 59:1531–1541

Arnold TM, Targett NM (2003) To grow and defend: lack of tradeoffs for brown algal phlorotannins. Oikos 100:406–408

Athukorala Y, Kim KN, Jeon YJ (2006) Antiproliferative and antioxidant properties of an enzymatic hydrolysate from brown alga, *Ecklonia cava*. Food Chem Toxicol 44:1065–1074

Avelin Mary S, Vitalina Mary S, Rittschof D, Nagabhushanam R (1993) Bacterial-barnacle interaction: potential of using juncellins and antibiotics to alter structure of bacterial communities. J Chem Ecol 19: 2155–2167

Balboa EM, Conde E, Moure A, Falqué E, Domínguez H (2013) In vitro antioxidant properties of crude extracts and compounds from brown algae. Food Chem 138:1764–1785

Bauer AW, Kirby WM, Sherris JC, Turck M (1966) Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol 45:493–496

Benzie IF, Strain JJ (1996) The ferric reducing ability of plasma (FRAP) as a measure of 'antioxidant power': the FRAP assay. Anal Biochem 239:70–76

Benzie IF, Strain JJ (1999) Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Method Enzym 299:15–27

Bitton R, Ben-Yehuda M, Davidovich M, Balazs Y, Potin P, Delage L, Colin C, Bianco-Peled H (2006) Structure of algal-born phenolic polymeric adhesives. Macromol Biosci 6:737–746

Bondu S, Bonnet C, Gaubert J, Deslandes É, Turgeon SL, Beaulieu L (2015) Bioassay-guided fractionation approach for determination of protein precursors of proteolytic bioactive metabolites from macroalgae. J Appl Phycol 27:2059–2074

Burtin P (2003) Nutritional value of seaweeds. Electron J Env Agric Food Chem 2:1579–4377

Chale-Dzul J, Moo-Puc R, Robledo D, Freile-Pelegrín Y (2015) Hepatoprotective effect of the fucoidan from the brown seaweed *Turbinaria tricostata*. J Appl Phycol 27:2123–2135

Chan EWC, Lim YY, Wong LF, Lianto FS, Wong SK, Lim KK, Joe CE, Lim TY (2008) Antioxidant and tyrosinase inhibition properties of leaves and rhizomes of ginger species. Food Chem 109:477–483

Chandler SF, Dodds JH (1983) The effect of phosphate, nitrogen and sucrose on the production of phenolics and solasodine in callus cultures of *Solanum laciniatum*. Plant Cell Rep 2:205–208

Chang T (2012) Biotransformation tyrosinase and tyrosinase inhibitors. J Biocatal Biotransfor 1:1–2

Charoensiddhi S, Franco C, Su P, Zhang W (2014) Improved antioxidant activities of brown seaweed *Ecklonia radiata* extracts prepared by microwave-assisted enzymatic extraction. J Appl Phycol 27:2049–2058

Chen Y-W, Wu S-W, Ho K-K, Lin S-B, Huang C-Y, Chen C-N (2008) Characterisation of Taiwanese propolis collected from different locations and seasons. J Sci Food Agr 88:412–419

Costa LS, Fidelis GP, Telles CBS, Dantas-Santos N, Camara RBG, Cordeiro SL, Costa MSSP, Almeida-Lima J, Melo-Silveira RF, Oliveira RM, Albuquerque IRL, Andrade GPV, Rocha HAO (2011) Antioxidant and antiproliferative activities of heterofucans from the seaweed *Sargassum filipendula*. Mar Drugs 9:952–966

Dai J, Mumper RJ (2010) Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. Molecules 15:7313–7352

Deniaud-Bouet E, Kervarec N, Michel G, Tonon T, Kloareg B, Herve C (2014) Chemical and enzymatic fractionation of cell walls from Fucales: insights into the structure of the extracellular matrix of brown algae. Ann Bot 114:1203–1216

Draget K, Smidsrød O, Skjåk-Bræk G (2005) Alginates from algae. Biopolym Online:1–30

Elizondo-Gonzalez R, Cruz-Suarez LE, Ricque-Marie D, Mendoza-Gamboa E, Rodriguez-Padilla C, Trejo-Avila LM (2012) In vitro characterization of the antiviral activity of fucoidan from *Cladosiphon okamuranus* against Newcastle Disease Virus. J Virol 9:307. doi:10.1186/1743-422X-9-307

Eom SH, Kim YM, Kim SK (2012) Antimicrobial effect of phlorotannins from marine brown algae. Food Chem Toxicol 50:3251–3255

Eom S, Moon S, Lee D, Kim H, Park K, Lee E, Kim TH, Chung Y, Lee M, Kim Y (2015) In vitro antiviral activity of dieckol and phlorofucofuroeckol-A isolated from edible brown alga *Eisenia bicyclis* against murine norovirus. Algae 30:241–246

Ermakova SP, Menshova RV, Anastyuk SD, Vishchuk OSM, Zakharenko AM, Thinh PD, Ly BM, Zvyagintseva TN (2016) Structure, chemical and enzymatic modification, and anticancer activity of polysaccharides from the brown alga *Turbinaria ornata*. J Appl Phycol 28: 2495–2505

Franco D, Sineiro J, Rubilar M, Sánchez M, Jerez M, Pinelo M, Costoya N, Núñez MJ (2008) Polyphenols from plant materials: extraction and antioxidant power. Electron J Env Agric Food Chem 7:3210–3216

Fujii Y, Tanaka R, Miyake H, Tamaru Y, Ueda M, Shibata T (2013) Evaluation for antioxidative properties of phlorotannins isolated from the brown alga *Eisenia bicyclis*, by the H-ORAC method. Food Nutr Sci 4:78–82

Ganapathi K, Subramanian V, Mathan S (2013) Bioactive potentials of brown seaweeds, *Sargassum myriocystum* J. Agardh, *S. plagiaphyllum* C. Agardh and *S. ilicifolium* (Turner) J. Agardh. Int Res J Pharm Appl Sci 3:105–111

Girija K, Hemalatha A, Saranya C, Parthiban C, Anantharaman P (2013) Extraction and isolation of phlorotannins from brown seaweed *Turbinaria Ornata* (Turner) J . Agardh and its antioxidant activity. Int J Bioassays 9:1185–1189

Gómez-Ordóñez E, Rupérez P (2011) FTIR-ATR spectroscopy as a tool for polysaccharide identification in edible brown and red seaweeds. Food Hydrocoll 25(6):1514-1520

Haeuser J, Lombard R, Lederer F, Ourisson G (1961) Isolement et structure d'un nouveau diterpene: L'acide daniellique. Tetrahedron 12(4):205-214

Hagerman AE, Butler LG (1989) Choosing appropriate methods and standards for assaying tannin. J Chem Ecol 15:1795–1810

Hardouin K, Burlot AS, Umami A, Tanniou A, Stiger-Pouvreau V, Widowati I, Bedoux G, Bourgougnon N (2014) Biochemical and antiviral activities of enzymatic hydrolysates from different invasive French seaweeds. J Appl Phycol 26:1029–1042

Heo SJ, Lee KW, Song CB, Jeon YJ (2003) Antioxidant activity of enzymatic extracts from brown seaweeds. Algae 18:71–81

Heo SJ, Park EJ, Lee KW, Jeon YJ (2005) Antioxidant activities of enzymatic extracts from brown seaweeds. Biores Technol 96: 1613–1623

Ibanez E, Herrero M, Mendiola JA, Castro-Puyana M (2012) Extraction and characterization of bioactive compound with health benefits from marine sources: macro and micro algae, cyanobacteria and invertebrates. In: Hayes M (ed) Marine bioactive compounds: sources, characterization and applications. Springer, New York, pp 55–98

Ismail N, Abdullah A (2015) Process optimization for the extraction of polyphenol from Malaysian brown seaweed, *Sargassum* sp. AIP Conference Proceeding 1678(1). doi:10.1063/1.4931322

Je J-Y, Park P-J, Kim E-K, Park J-S, Yoon H-D, Kim K-R, Ahn C-B (2009) Antioxidant activity of enzymatic extracts from the brown seaweed *Undaria pinnatifida* by electron spin resonance spectroscopy. LWT - Food Sci Technol 42:874–878

Jeon YJ, Wijesinghe WAJP, Kim SK (2012) Enzyme-assisted extraction and recovery of bioactive components from seaweeds. In: Kim S-K (ed) Handbook of marine macroalgae: biotechnology and applied phycology. John Wiley & Sons, Ltd, pp 221–226

Kadam SU, Tiwari BK, O'Donnell CP (2013) Application of novel extraction technologies for bioactives from marine algae. J Agric Food Chem 61:4667–4675

Kang M-C, Kim E-A, Kang S-M, Wijesinghe WAJP, Yang X, Kang N-L, Jeon Y-J (2012a) Thermostability of a marine polyphenolic antioxidant dieckol, derived from the brown seaweed *Ecklonia cava*. Algae 27:205–213

Kang SM, Heo SJ, Kim KN, Lee SH, Yang HM, Kim AD, Jeon YJ (2012b) Molecular docking studies of a phlorotannin, dieckol isolated from *Ecklonia cava* with tyrosinase inhibitory activity. Bioorg Med Chem 20:311–316

Kantachumpoo A, Chirapart A (2010) Components and antimicrobial activity of polysaccharides extracted from Thai brown seaweeds. Kasetsart J Nat Sci 44:220–233

Karunakaran C, Christensen CR, Gaillard C, Lahlali R, Blair LM, Perumal V, Miller SS, Hitchcock AP, Dedkov Y, (2015) Introduction of soft X-Ray spectromicroscopy as an advanced technique for plant biopolymers research. PLOS ONE 10(3):e0122959

Kim E, Kwak J (2015) Antiviral phlorotannin from *Eisenia bicyclis* against human papilloma virus in vitro. Planta Med. doi:10.1055/s-0035-1565646

Kim KN, Heo SJ, Song CB, Lee J, Heo MS, Yeo IK, Kang KA, Hyun JW, Jeon YJ (2006) Protective effect of *Ecklonia cava* enzymatic extracts on hydrogen peroxide-induced cell damage. Process Biochem 41:2393–2401

Kim JH, Byun JC, Kumar A, Bandi R, Hyun C, Ho N (2009) Compounds with elastase inhibition and free radical scavenging activities from *Callistemon lanceolatus*. J Med Plants Res 3:914–920

Koivikko R (2008) Brown algal phlorotannins: improving and applying chemical methods. Doctoral Thesis. University of Turku

Koivikko R, Loponen J, Pihlaja K, Jormalainen V (2007) High-performance liquid chromatographic analysis of phlorotannins from the brown alga *Fucus vesiculosus*. Phytochem Anal 18:326–332

Kulshreshtha G, Burlot AS, Marty C, Critchley A, Hafting J, Bedoux G, Bourgougnon N, Prithiviraj B (2015) Enzyme-assisted extraction of bioactive material from *Chondrus crispus* and *Codium fragile* and its effect on Herpes simplex virus (HSV-1). Mar Drugs 13:558–580

Kusumaningsih T, Firdaus M, Wartono MW, Artanti AN, Handayani DS, Putro AE (2016) Ethyl-2-(3,5-Dihidroxyfenol): Phloroglucinol derivatives as potential anticancer material. IOP Conference Series: Mater Sci Eng 107:012059

Langlois M, Allard JP, Nugier F, Aymard M (1986) A rapid and automated colorimetric assay for evaluating the sensitivity of herpes simplex strains to antiviral drugs. J Biol Stand 14:201–211

Lau SCK, Qian PY (1997) Phlorotannins and related compounds as larval settlement inhibitors of the tube-building polychaete *Hydroides elegans*. Mar Ecol Prog Ser 159:219–227

Le Lann K, Connan S, Stiger-Pouvreau V (2012) Phenology, TPC and size-fractioning phenolics variability in temperate Sargassaceae (Phaeophyceae, Fucales) from Western Brittany: native versus introduced species. Mar Env Res 80:1–11

Le Lann K, Surget G, Couteau C, Coiffard L, Cérantola S, Gaillard F, Larnicol M, Zubia M, Guérard F, Poupart N, Stiger-Pouvreau V (2016) Sunscreen, antioxidant, and bactericide capacities of phlorotannins from the brown macroalga *Halidrys siliquosa*. J Appl Phycol 28:3547–3559

Lee JB, Takeshita A, Hayashi K, Hayashi T (2011) Structures and antiviral activities of polysaccharides from *Sargassum trichophyllum*. Carbohydr Polym 86:995–999

Li Y-X, Wijesekara I, Li Y, Kim S-K (2011) Phlorotannins as bioactive agents from brown algae. Process Biochem 46:2219–2224

Lim TY, Lim YY, Yule CM (2009) Evaluation of antioxidant, antibacterial and anti-tyrosinase activities of four *Macaranga* species. Food Chem 114:594–599

Lim SJ, Wan Aida WM, Maskat MY, Mamot S, Ropien J, Mazita Mohd D (2014) Isolation and antioxidant capacity of fucoidan from selected Malaysian seaweeds. Food Hydrocoll 42:280–288

Liu L, Heinrich M, Myers S, Dworjanyn SA (2012) Towards a better understanding of medicinal uses of the brown seaweed *Sargassum* in traditional Chinese medicine: a phytochemical and pharmacological review. J Ethnopharmacol 142:591–619

Lowenthal RM, Fitton JH (2015) Are seaweed-derived fucoidans possible future anti-cancer agents? J Appl Phycol 27:2075–2077

McLaren C, Ellis MN, Hunter GA (1983) A colorimetric assay for the measurement of the sensitivity of herpes simplex viruses to antiviral agents. Antivir Res 3:223–234

Mhadhebi L, Mhadhebi A, Robert J, Bouraoui A (2014) Antioxidant, anti-inflammatory and antiproliferative effects of aqueous extracts of three Mediterranean brown seaweeds of the genus *Cystoseira*. Iran J Pharm Res 13:207–220

Michalak I, Chojnacka K (2014) Algal extracts: technology and advances. Eng Life Sci 14:581–591

Moon J-Y, Yim E-Y, Song G, Lee NH, Hyun C-G (2010) Screening of elastase and tyrosinase inhibitory activity from Jeju Island plants. EurAsian J Biosci 53:41–53

Olivares-Molina A, Fernández K (2016) Comparison of different extraction techniques for obtaining extracts from brown seaweeds and

their potential effects as angiotensin I-converting enzyme (ACE) inhibitors. J Appl Phycol 28:1295–1302
Ozdal T, Capanoglu E, Altay F (2013) A review on protein–phenolic interactions and associated changes. Food Res Int 51:954–970
Park P-J, Fereidoon S, Jeon Y-J (2004) Antioxidant activities of extracts from an edible seaweed *Sargassum horneri* using ESR spectrometry. J Food Lipids 11:15–27
Park P-J, Heo S-J, Park E-J, Kim S-K, Byun H-G, Jeon B-T, Jeon Y-J (2005) Reactive oxygen scavenging effect of enzymatic extracts from *Sargassum thunbergii*. J Agric Food Chem 53:6666–6672
Park JN, Ali-Nehari A, Woo HC, Chun BS (2012) Thermal stabilities of polyphenols and fatty acids in *Laminaria japonica* hydrolysates produced using subcritical water. Korean J Chem Eng 29:1604–1609
Plaza M, Turner C (2015) Pressurized hot water extraction of bioactives. Trends Anal Chem 71:39–54
Plouguerné E, Le Lann K, Connan S, Jechoux G, Deslandes E, Stiger-Pouvreau V (2006) Spatial and seasonal variation in density, reproductive status, length and phenolic content of the invasive brown macroalga *Sargassum muticum* (Yendo) Fensholt along the coast of Western Brittany (France). Aquat Bot 85:337–344
Plouguerné E, Cesconetto C, Cruz CP, Pereira RC, da Gama BAP (2012) Within-thallus variation in polyphenolic content and antifouling activity in *Sargassum vulgare*. J Appl Phycol 24:1629–1635
Rhein-Knudsen N, Ale MT, Meyer AS (2015) Seaweed hydrocolloid production: an update on enzyme assisted extraction and modification technologies. Mar Drugs 13:3340–3359
Rodrigues D, Freitas AC, Pereira L, Rocha-Santos TAP, Vasconcelos MW, Roriz M, Rodríguez-Alcalá LM, Gomes AMP, Duarte AC (2015) Chemical composition of red, brown and green macroalgae from Buarcos bay in central West Coast of Portugal. Food Chem 183:197–207
Rosenthal A, Pyle DL, Niranjan K (1996) Aqueous and enzymatic processes for edible oil extraction. Enzym Microb Technol 19:402–420
Sallenave JM, Xing Z, Simpson AJ, Graham FL, Gauldie J (1998) Adenovirus-mediated expression of an elastase-specific inhibitor (elafin): a comparison of different promoters. Gene Ther 5:352–360
Sanchez-Camargo ADP, Montero L, Stiger-Pouvreau V, Tanniou A, Cifuentes A, Herrero M, Ibanez E (2015) Considerations on the use of enzyme-assisted extraction in combination with pressurized liquids to recover bioactive compounds from algae. Food Chem 192:67–74
Sartori C (1997) The characterisation of alginate systems for biomedical applications. Brunel University
Scalbert A (1991) Antimicrobial properties of tannins. Phytochemistry 30:3875–3883
Schoenwaelder MEA (2002) The occurrence and cellular significance of physodes in brown algae. Phycologia 41:125–139
Schoenwaelder MEA, Clayton MN (1999) The presence of phenolic compounds in isolated cell walls of brown algae. Phycologia 38:161–166
Sekkal M, Huvenne J-P, Legrand P, Sombret B, Mollet J-C, Mouradi-Givernaud A, Verdus M-C (1993) Direct structural identification ofpolysaccharides from red algae by FTIR microspectrometry I: Localization of agar in *Gracilaria verrucosa* sections. Mikrochim Acta 112 (1-4):1-10
Sereewatthanawut I, Prapintip S, Watchiraruji K, Goto M, Sasaki M, Shotipruk A (2008) Extraction of protein and amino acids from deoiled rice bran by subcritical water hydrolysis. Bioresour Technol 99:555–561
Shahwar D, Raza MA, Bukhari S, Bukhari G (2012) Ferric reducing antioxidant power of essential oils extracted from *Eucalyptus* and *Curcuma* species. Asian Pac J Trop Biomed. doi:10.1016/S2221-1691(12)60467-5
Shitanda D, Koide S, Koishi M, Cao W (2014) Enhancing production through optimisation of DPPH and radical scavenging activity of grape seed extracts. Afr Crop Sci J 22:883–896
Sigee DC, Bahrami F, Estrada B, Webster RE, Dean AP (2007) The influence of phosphorus availability on carbon allocation and P quota in *Scenedesmus subspicatus*: A synchrotronbased FTIR analysis. Phycol 46(5):583-592
Siriwardhana N, Jeon YJ, Kim SH, Ha JH, Heo SJ, Lee KW (2004) Enzymatic hydrolysis for effective extraction of antioxidative compounds from *Hizikia fusiformis*. Algae 19:59–68
Siriwardhana N, Kim KN, Lee KW, Kim SH, Ha JH, Song CB, Lee JB, Jeon YJ (2008) Optimisation of hydrophilic antioxidant extraction from *Hizikia fusiformis* by integrating treatments of enzymes, heat and pH control. Int J Food Sci Technol 43:587–596
Soares AR, Robaina MCS, Mendes S, Silva TSL, Gestinari LMS, Pamplona S, Yoneshigue-Valentin Y, Kaiser CR, Romanos MTV (2012) Antiviral activity of extracts from Brazilian seaweeds against herpes simplex virus. Braz J Pharmacog 22:714–723
Stern JL, Hagerman AE, Steinberg PD, Mason PK (1996) Phlorotannin-protein interactions. J Chem Ecol 22:1877–1899
Stiger V, Horiguchi T, Yoshida T, Coleman AW, Masuda M (2003) Phylogenetic relationships within the genus *Sargassum* (Fucales, Phaeophyceae), inferred from it ITS nrDNA, with an emphasis on the taxonomic revision of the genus. Phycol Res 51:1–10
Suetsuna K, Maekawa K, Chen J-R (2004) Antihypertensive effects of *Undaria pinnatifida* (wakame) peptide on blood pressure in spontaneously hypertensive rats. J Nutr Biochem 15:267–272
Tan MC, Tan CP, Ho CW (2013) Effects of extraction solvent system, time and temperature on total phenolic content of henna (*Lawsonia inermis*) stems. Int Food Res J 20:3117–3123
Tanniou A, Leon ES, Vandajon L, Ibanez E, Mendiola JA, Cerantola S, Kervarec N, La Barre S, Marchal L, Stiger-Pouvreau V (2013) Green improved processes to extract bioactive phenolic compounds from brown macroalgae using *Sargassum muticum* as model. Talanta 104:44–52
Tanniou A, Vandanjon L, Incera M, Serrano Leon E, Husa V, Le Grand J, Nicolas J-L, Poupart N, Kervarec N, Engelen A, Walsh R, Guerard F, Bourgougnon N, Stiger-Pouvreau V (2014) Assessment of the spatial variability of phenolic contents and associated bioactivities in the invasive alga *Sargassum muticum* sampled along its European range from Norway to Portugal. J Appl Phycol 26:1215–1230
Wang H, Ooi EV, Ang PO (2008) Antiviral activities of extracts from Hong Kong seaweeds. J Zhejiang Univ Sci B 9:969–976
Wang T, Jónsdóttir R, Ólafsdóttir G (2009) Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic seaweeds. Food Chem 116:240–248
Wang T, Jónsdóttir R, Kristinsson HG, Hreggvidsson GO, Jónsson JÓ, Thorkelsson G, Ólafsdóttir G (2010) Enzyme-enhanced extraction of antioxidant ingredients from red algae *Palmaria palmata*. LWT - Food Sci Technol 43:1387–1393
Welch K, Cai Y, Stromme M (2012) A method for quantitative determination of biofilm viability. J Funct Biomater 3:418–431
Wijesinghe WAJP, Jeon YJ (2012) Enzyme-assistant extraction (EAE) of bioactive components: a useful approach for recovery of industrially important metabolites from seaweeds: a review. Fitoterapia 83:6–12
Xu J, Hagler A (2002) Chemoinformatics and drug discovery. Molecules 7:566–600
Yen G-C, Chen H-Y (1995) Antioxidant activity of various tea extracts in relation to their antimutagenicity. J Agric Food Chem 43:27–32
Yoon NY, Eom TK, Kim MM, Kim SK (2009) Inhibitory effect of phlorotannins isolated from *Ecklonia cava* on mushroom tyrosinase activity and melanin formation in mouse B16F10 melanoma cells. J Agric Food Chem 57:4124–4129
Zhang CY, Wu WH, Wang J, Lan MB (2012) Antioxidant properties of polysaccharide from the brown seaweed *Sargassum graminifolium* (Turn.), and its effects on calcium oxalate crystallization. Mar Drugs 10:119–130