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PRESSURIZED HOT WATER EXTRACTION OF CARRAGEENAN AND PHENOLIC COMPOUNDS FROM *EUCHEUMA COTTONII* AND *GRACILARIA* SP.: EFFECT OF EXTRACTION CONDITIONS

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ABSTRACT

This work was focused on the investigation of the extraction parameters influence on the yield of carrageenan and phenolic components as well as the antioxidant activity of extracts. Pressurized hot water extraction of carrageenan and phenolic components from *Eucheuma cottonii* (*E. cottonii*) and *Gracilaria* sp. was performed in a semi-batch system at various pressures of 1 - 10 MPa and temperatures of 120 - 200 °C. Carrageenan and phenolic compounds were characterized by using FT-IR and UV-vis spectrophotometer. The yields of carrageenan and phenolic components were strongly influenced by the alteration of extraction temperature. The highest extraction yield was resulted at 200 °C for 97% of carrageenan yield and 22 mg gallic acid equivalent (GAE)/g of dried sample for phenolic components extracted from *E. cottonii*. The results confirmed that pressurized hot water extraction is an effective technique to isolate bioactive components from macroalgae and can be an advanced technique for utilization of biomass components.

Keywords: carrageenan; *eucheuma cottonii*; *gracilaria* sp.; phenolic compounds; pressurized hot water extraction.

INTRODUCTION

Generally, algae can be roughly divided into two distinct groups macroalgae and microalgae. Up till now, these were used as sources of bioactive compounds for use as functional food ingredients. Microalgae are already known as attractive raw material for energy production because of their much higher growth rates than agricultural crops. Moreover, due to the high concentration of lipids, microalgae also can improve the production of biodiesel and other biofuels. Similar to microalgae, macroalgae are also generally fast growing and their growth rates exceeded those of terrestrial plants. They had wide products used for food in direct human consumption. Due to its high content in minerals and phytochemicals, macroalgae are utilized as feedstocks for the cosmetics and pharmaceutical industries [1]. Therefore, macroalgae still exhibit prominent and viable sources for functional ingredients. Red macroalgae, including *Eucheuma cottonii* (*E. cottonii*) and *Gracilaria* sp., are rich in dietary fibers, minerals, antioxidants, vitamins, polyphenols, proteins, polyunsaturated fatty acids, and phytochemicals. Moreover, *E. cottonii* and *Gracilaria* sp. have also been used medicinal applications [2]. Namvar *et al.* [3] and Shamsabadi *et al*. [4] reported that the extract of *E. cottonii* may quell tumor and cancer through its antioxidant and antiproliferative properties. *Gracilaria* sp. was also reported as an antiproliferative and anti-inflammatory promoted by its lipid extract [5].

Separation of phytochemical components in macroalgae is necessary to be done in order to upgrade the economic value of macroalgae. One of the methods usually used to separate the valuable compounds from algae is extraction. Here, bioactive compounds which can be found in macroalgae would be extracted by using pressurized hot water. This technique is an effective technique conducted at temperatures in the range of 100 and 374 °C, and pressure high enough to keep the liquid state [6,7]. At these conditions, water is notable as a 'green and environmentally friendly' technique for product extraction and has gained intensely interest as a prominent choice to the conventional separation methods such as hot water extraction, direct solvent, or soxhlet extraction. These conventional methods are using organic solvents and taking longer extraction time [8]. At pressurized hot water, water properties change. Since the network of hydrogen bonding among the molecules is weakened, both hydroxide and hydrogen ions concentrations in the water becomes higher. It results in a lowered surface tension, dielectric constant, and viscosity of solvent. Moreover, at ambient temperature the polarity of water near its critical point is almost equal to that of ethanol, and consequently, the polar compounds were easily extracted from matrices. This technique has been employed to extract amino acids, protein, and phenolic compounds [9]. Several studies reported that pressurized hot water treatment has also been exhibited to successfully change cellulosic [10] and lignocellulosic biomass [11] into valuable compounds.

This research focused on the extract bioactive compounds from *E. cottonii* and *Gracilaria* sp. by using pressurized hot water at subcritical conditions. Extraction was conducted at various temperatures from 120 to 200 °C and pressures from 1 to 10 MPa. Algae contain some bioactive compounds applied in the pharmaceutical, biomedical, and nutraceutical industries [1]. The most of them consisted of carbohydrate, proteins, lipids, minerals and certain vitamins [1,12]. Polysaccharides and polyphenols are the main bioactive compounds which have antifungal, antibacterial, and antiviral properties. They can be extracted from the algae; however, some parts of them can be converted into energy and biological building materials. As the pressurized hot water was utilized in this extraction process, the algae are liquefied,

and thermal cleavage occurs that resulted in numerous radicals. At these situations, the aliphatic and aromatic groups composed of carbon bonds on the algae are easily splatted. Consequently, pressurized hot water is a compatible tool for algae components extraction via the thermal reduction process [6,7,13,14].

EXPERIMENTAL SECTION

Materials

Plant materials (*E. cottonii* and *Gracilaria* sp.) which were obtained from Jepara, located on the Central Java, Indonesia were used as starting materials. The Folin-Ciocalteau's reagent (product no. F9252), 1,1-diphenyl-2-picrylhydrazyl (DPPH, product no. D9132), and gallic acid (product no. G7384) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Sodium carbonate (99.5%), methanol (99.7%), ethanol (99.5%), and kappa carrageenan (product no. 033-09292) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). They were used without further purification.

Methods

In this work, the pressurized hot water extraction was conducted in a semi–batch process. The main apparatus consists of a high–pressure pump (200 LC Pump, Perkin Elmer, Germany), heater (Linn High Therm GmbH, model VMK 1600, Germany), vessel (10 mL; Thar Design Inc., USA) and back–pressure regulators (BPR; AKICO, Japan). Both sides of the vessel were equipped with removable threaded covers included stainless–steel filters (0.1 – 1.0 μm). After the vessel inclusive of 0.5 g of feed was installed to the system, distilled water at room temperature was pumped through the reactor inclusive pre–heater for a few minutes to purge air and completely wet the feed; the system was then pressurized to the set pressure of 1 - 10 MPa through the back-pressure regulator, monitored by a pressure gauge (P, Migishita, Japan). When the system reached the desired pressure and a steady state was achieved, the electric heater was applied to heat the water. The reactor temperature was maintained at 120 - 200 ºC. The temperatures of the pre-heater, reactor and the electric heater were measured using K–type thermocouples and monitored using temperature controller (OMRON E5CJ, Japan). The time required to heat the reactor from room temperature to the desired temperature was 5 - 8 min, after which the reactor temperature equaled the electric heater temperature. After the temperature at the reactor are reached a preset temperature, the pump was used to feed water at 1.0 mL min^{-1}. The time of experiment was 150 min and extracted solution was collected every 30 min.

Next, the total phenolic compounds and the antioxidant activity of the extracts were analyzed by using Genesys 10 UV–Vis scanning spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The Folin-Ciocalteau's and the DPPH reagents were employed to evaluate them [15,16]. The analysis was performed in triplicates. The solid products collected at each operating temperature were analyzed by a Spectrum Two FT-IR spectrophotometer (Perkin-Elmer, Ltd., England) to determine the structure of the solid products after the pressurized hot water treatment. The morphologies of them were also observed by using a scanning electron microscope (SEM; JEOL JSM–6390LV, Japan). The carrageenan in each liquid fraction products was precipitated in 95% ethanol [17]. The yield of carrageenan (%) was determined according to the weight of extracted carrageenan (g) and the weight of dry macroalgae (g) used for extraction.

RESULTS AND DISCUSSIONS

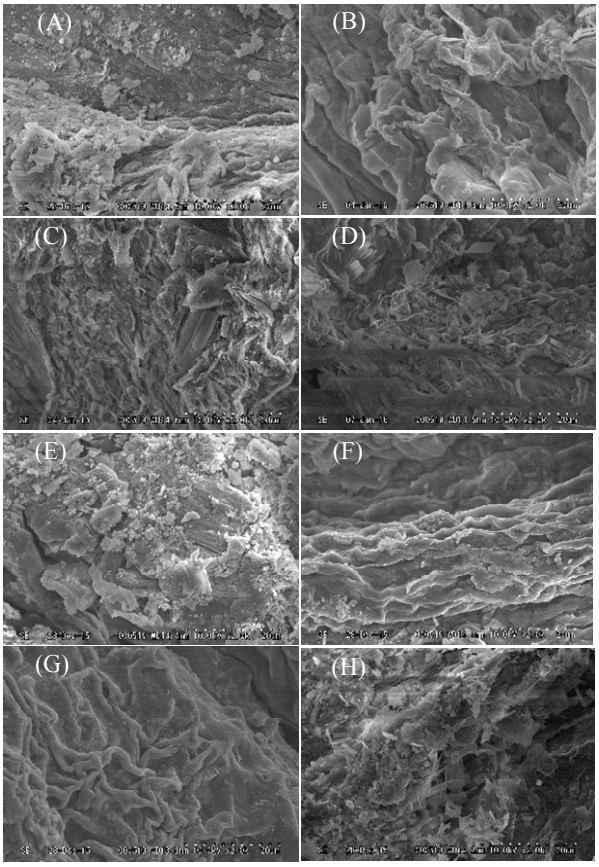


Figure-1. SEM images of macroalgae matrices before and after treatment by pressurized hot water at pressure of 5 MPa (*E. cottonii*: (A) starting material, (B) 120 ºC, (C) 160 ºC, (D) 200 ºC; *Gracilaria* sp.: (E) starting material, (F) 120 ºC, (G) 160 ºC, (H) 200 ºC).

To observe the surface morphology changes of *E. cottonii* and *Gracilaria* sp. as starting materials and the obtained their solid residues, SEM images of them were performed. Figure-1 shows the representative SEM images of *E. cottonii* and *Gracilaria* sp. and their solid residues after treatment by pressurized hot water at 120 - 200 ºC. Before pressurized hot water treatment, the surface morphology of *E. cottonii* and *Gracilaria* sp. were marked by some boundary edges clearly and did not show the

presence of any surface cracks. They showed essentially regular and compact surface structure as an intact morphology. After treatment by pressurized hot water, the physical structures disruption occurred and clearly observed on the surface morphology of *E. cottonii* and *Gracilaria* sp. at each extraction condition. Apparently, this pressurized hot water has disrupted the structure of these macroalgaes that contains carbohydrate, proteins, lipids, minerals and certain vitamins as main components like plants biomass. At 120 ºC, the surface morphology of *E. cottonii* and *Gracilaria* sp. were clearly different from that of the original them due to some substances melted and re–solidified. The surface cracks were clearly found on the morphology of solid residue frameworks and they are very prominent at higher extraction temperatures (160 and 200 ºC). These images exhibited that the cell wall of *E. cottonii* and *Gracilaria* sp. have been destroyed, resulting the removal of their components to dissolve in water at subcritical conditions via autohydrolysis. Based on these SEM images, it could be said that when the pressurized hot water at subcritical conditions were applied on the plants biomass, the components of plant biomass will break down into their backbone units. Toor *et al.* [18] informed that the decomposition products of plants biomass components in subcritical water differ; however, the basic reaction mechanisms can be summarized as follows: first, de–polymerization of the plants biomass following by degradation of plants biomass monomers by cleavage, dehydration, decarboxylation and deamination and finally the recombination of reactive fragments.

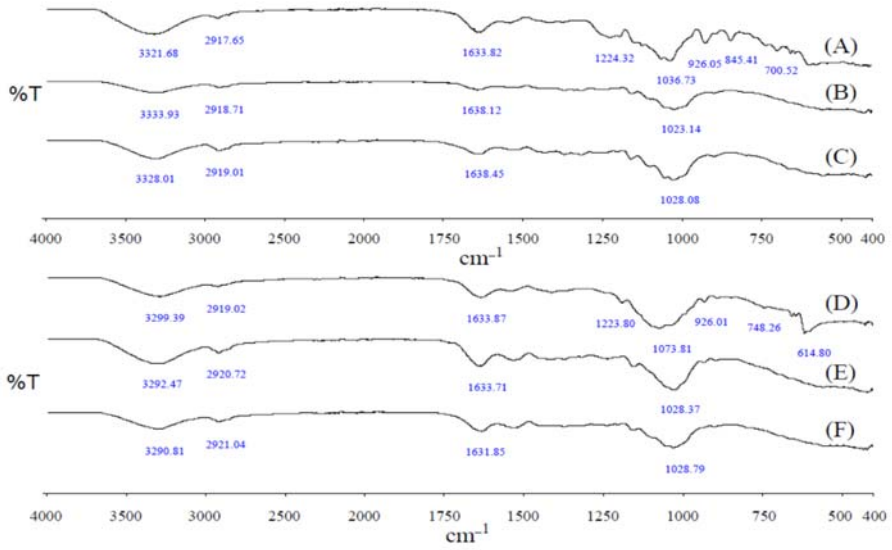


Figure-2. FT-IR spectrum of feed material before and after treatment by pressurized hot water at pressure of 5 MPa (*E. cottonii*: (A) Starting material, (B) 120 ºC, (C) 160 ºC; *Gracilaria* sp.: (D) Starting material, (E) 120 ºC, (F) 160 ºC).

As explained before that during pressurized hot water extraction process, cell wall disruptions of *E. cottonii* and *Gracilaria* sp. occurred, allowing the removal of their constituents via autohydrolysis. Figure-2 shows the characterization of *E. cottonii* and *Gracilaria* sp. and their solid residues after treatment by pressurized hot water at 120 - 160 ºC with pressure of 5 MPa by using FT-IR, respectively. FT-IR is a powerful tool for finding the macroalgae components position in the cell walls of native macroalgae material such as *E. cottonii* and *Gracilaria* sp. [19]. The peak positions of all infrared bands and their functional groups seaweeds used as references were listed in Table-1 [20]. Each molecule is consisted of many different chemical bonds which are slightly elastic: they allow to stretch, bend, or vibrate. Hence, some differences prevail at each FT–IR spectra due to their structural properties.

Table-1. Main functional groups of the seaweed corresponds to infrared absorption frequencies.

Wave number [cm^{-1}]	Functional groups
3500	OH and NH stretching
2900-2960	CHn asymmetric stretching vibration
2845	O-CH_3
1725	COOH
1420, 1640-1695	Amide groups
1605	Carboxylate anion
1210; 1240; 1260	S=O of ester–sulfate
1040-1080	Galactans skeleton
1000-1200	Sulfates and floridean starch
970-975	Galactose skeleton
930-940	Vibrations of the C-O-C of 3,6-anhydrogalactose
805, 905	C-O-SO_4 on 3,6-anhydrogalactose
890-900	Unsulfated β-D-galactose
705, 815-820, 825-867	C-O-SO_4 on galactose
717, 730-750	Bending mode in glycosidic linkages
600-615	C-S stretching

As shown in Figure-2, the main peak of *E. cottonii* and *Gracilaria* sp. constituents seem found in the wavenumbers range from about 1650 to 600 cm^{-1}. The absorbance intensity due to hydrogen bonded OH and NH stretching (3600 - 3000 cm^{-1}) could be recognized in each spectrum. These bands are associated with carbohydrates and proteins of the macroalgae cell wall. The same phenomenon also occurred at 1631.85 - 1638.45 cm^{-1} due to the amide modes of CO–NH stretching from proteins. The intensity of the absorbance in these regions is found in each spectrum and mostly stable, indicating that amide groups in *E. cottonii* and *Gracilaria* sp. have difficulty cleaving under these conditions. The bands in the regions of 2917.65 – 2921.04 cm^{-1} represent the CH stretching in the aliphatic chains present in the carbohydrates. The absorbance intensity corresponds to the existence of carrageenan in the *E. cottonii* and *Gracilaria* sp. could be found clearly in 1036.73 – 1073 cm^{-1} regions [20]. After treatment by pressurized hot water, the intensity of this absorbance disappeared because of the loss of carrageenan groups which were soluble in water. This indicated that pressurized hot water treatment successfully extracted carrageenan groups from *E. cottonii* and *Gracilaria* sp. The solid residues of them only showed absorption peaks at 1028.79 – 1023.14 cm^{-1}, which are the absorption peak of carrageenan groups deformation. The peaks in the 926, 845, 748, 700, and 614 cm^{-1} regions are assigned to the stretching vibrations of the C-O-C of 3,6-anhydrogalactose, C–O–SO_4 on galactose, the bending mode in glycosidic linkages, the stretching of C-O-SO4 on galactose, and the stretching of C-S groups, respectively. These peaks could be obtained obviously in starting materials of *E. cottonii* and *Gracilaria* sp. Next, they disappeared after the pressurized hot water was applied. It showed that these bonds in *E. cottonii* and *Gracilaria* sp. were reacted and consumed in these range temperatures.

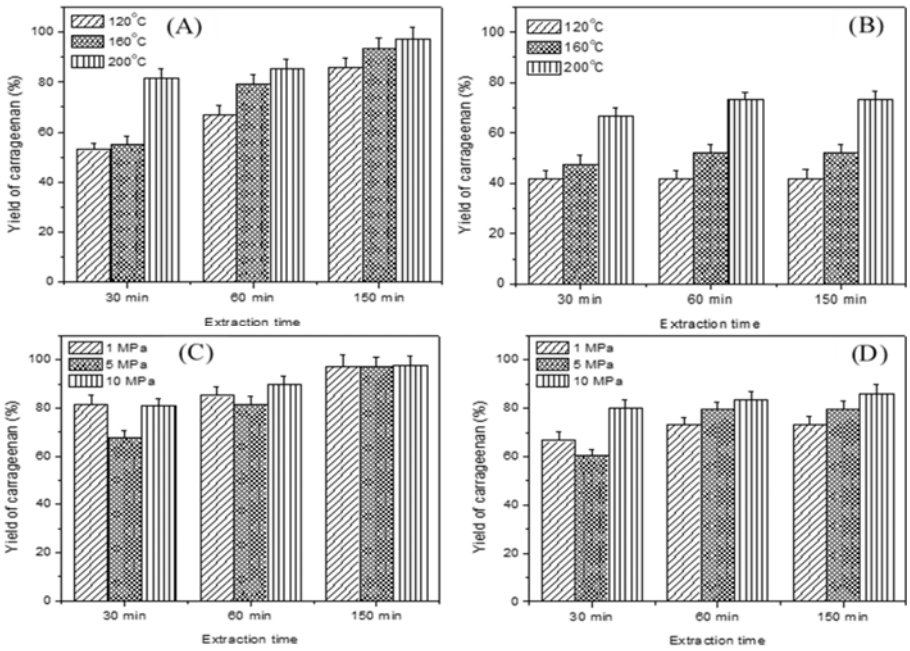


Figure-3. Yield of carrageenan in the extract at various extraction conditions (*E. cottonii*: (A) 1 MPa, (C) 200 ºC; *Gracilaria* sp.: (B) 1 MPa, (D) 200 ºC).

Hot water extraction has been known as the most widely used traditional technology for polysaccharides extraction from plants biomass. Similarly, carrageenan as a one of polysaccharides which is existed in *E. cottonii* and *Gracilaria* sp. also can be extracted by hot water technique. Figure-3 shows the yield of water-soluble carrageenan in the extract obtained from *E. cottonii* and *Gracilaria* sp. at various extraction conditions. The amount of water-soluble carrageenan in the extracts almost increased with increasing extraction time at the same extraction conditions. In Figure-3(A), the yield of water-soluble carrageenan from *E. cottonii* was 53 % at 120 ºC and 1 MPa for 30 min extraction time, then it could approach to 86 % when the extraction time was 150 min at the same extraction temperature. The same results were also found when the extractions were performed at 160 and 200 ºC for the same extraction pressure. As informed before, the substituted time of hot fresh water which was supplied by high pressure pump into the entire volume of the extractor after the feeding sample was around a few seconds. Therefore, the extracted biomass components including carrageenan underwent severe conditions only a short time that can avoid degradation during the semi-batch extraction process [21,22]. On the contrary, the yield of water-soluble carrageenan from *Gracilaria* sp. (see Figure-3(B)) seems hard to increase with expanding of extraction time. When the extraction process was carried out at 120 ºC and 1 MPa extraction pressure, the yield of water–soluble carrageenan from *Gracilaria* sp. looks like stable with expanding extraction time. At this condition, the carrageenan bonds in the *Gracilaria* sp. matrix seemed resistant to hydrolysis. However, the yield of water-soluble carrageenan from *Gracilaria* sp. was 47 % and 66 % at 30 min then increased to 52 % and 73 %, respectively, at 150 min when the extractions were performed at 160 and 200 ºC at the same extraction pressure, respectively. It could be said that extraction time was almost proportional to the yield of water–soluble carrageenan from *E. cottonii* and *Gracilaria* sp. when the extraction processes were conducted at these extraction conditions.

Figure-3 also shows that autohydrolysis could be applied for macroalgae materials lead to the solubilization of their components under subcritical water conditions. In this technique, extraction temperature was known as a key variable of the extraction process. It performed that the extraction temperature had high influence on the yield of extracted carrageenan. The yield of carrageenan from *E. cottonii* and *Gracilaria* sp. increased significantly with the rise of extraction temperature from 120 to 200 ºC. Most of the water-soluble carrageenan released from *E. cottonii* and *Gracilaria* sp. The amount of them could approach to 97 % from *E. cottonii* and 73 % from *Gracilaria* sp. when the extraction conditions were 200 ºC with 1 MPa extraction pressure. Next, the water–soluble carrageenan obtained in the extracts could be assumed to be derived from *E. cottonii* and *Gracilaria* sp. matrix via hydrolysis caused by elevated temperatures [6,21,23]. Figures-3(C) and 3(D) describe the effect of extraction pressures on the yield of water–soluble carrageenan removed from *E. cottonii* and *Gracilaria* sp. at 200 ºC. It was well known that the most important advantages of using pressure in subcritical water extraction are that the water temperature can be maintained above its boiling point, while the water was in the liquid state. The applied of elevated pressures at high temperatures allows the liquid water to pass through the *E. cottonii* and *Gracilaria* sp. matrix and to extract their components. As shown in the Figures-3(C) and 3(D),

apparently, the elevated pressure had effect on the amount of water-soluble carrageenan extracted from *E. cottonii* and *Gracilaria* sp. When the *Gracilaria* sp. was subjected as a feed material to extract carrageenan, the elevated pressure clearly affected the yield of water-soluble carrageenan. At this condition, the solvent power of liquid water provided by the pressure promotes in the disruption of the *Gracilaria* sp. matrix, which enhances the mass transfer of the water-soluble carrageenan from the *Gracilaria* sp. matrix to the liquid water solvent. Nevertheless, the elevated pressure did not give high effect when the *E. cottonii* was fed as a starting material. This result was in agreement with the literatures [6, 21-23] which informed that elevating the pressure in subcritical water extraction has been found to ignore as long as the physical state of the water is not changed.

In general, plant biomass cell walls were mainly constructed by complex biopolymers such as carbohydrates and lignin. Like other plant biomass, most of the *E. cottonii* and *Gracilaria* sp. cell walls were also composed of carbohydrates as major components. Hence, carrageenan as a natural carbohydrate which was located in the cell wall and the intercellular matrix of edible red algae were also found in the *E. cottonii* and *Gracilaria* sp. cell walls. To release the carrageenan from these matrices, the degradation of these cell walls was needed. Consequently, the extraction of carrageenan from the red algae including *E. cottonii* and *Gracilaria* sp. could not produce the pure carrageenan, but more likely composed of carrageenan mix structures. Therefore, the commercial carrageenans were classified into three main types: kappa-, iota-, and lambda–carrageenan [1]. The summary of FT-IR bands assignment for the different types of carrageenan were summarized in Table-2 [24,25]. The structure of them were determined by the number and position of sulphate groups, the presence of 3,6-anhydro-D-galactose, and conformation of the pyranose ring.

Table-2. Assignation of FT-IR absorption bands of carrageenan.

Wavenumber [cm^{-1}]	Absorption bands assignment	Found in carrageenans
1220-1260	Sulphate ester	kappa, iota, lamda
928-933	3,6-anhydro-D-galactose	kappa, iota, lamda
840-850	4-sulphate-D-galactose	kappa, iota
825-830	2-sulphate-D-galactose	lamda
810-820	6-sulphate-D-galactose	lamda
800-805	3,6-anhydro-D-galactose-2-sulphate	kappa, iota

Figure-4 shows FT-IR spectrum of extracted carrageenan from *E. cottonii* and *Gracilaria* sp. at 1 MPa extraction pressure with 30 min extraction time, respectively. In this work, the kappa-carrageenan was used as a standard material and had strong and broad absorption bands in the 845 to 930 cm^{-1} regions [25]. The peaks in the 1215.03 - 1248.13 cm^{-1} regions are assigned to the stretching vibration of sulphate ester (S=O) could be found in spectrum of carrageenan standard and extracted carrageenan from *E. cottonii* and *Gracilaria* sp. at temperatures of 120 and 160 ^{o}C, respectively. The absorbance intensity of extracted carrageenan from *E. cottonii* was found obviously. On the contrary, the weak absorbance intensity occurred on the extracted carrageenan from *Gracilaria* sp. at the same regions. It could be explained that the extraction of carrageenan from *Gracilaria* sp. via autohydrolysis may form reactive carrageenan compounds due to the existence of alkali-labile sulphate groups in the cell walls of *Gracilaria* sp. Next, the elimination of sulphate ester with active compounds which is generated during subcritical water extraction process occurred following with the formation of 3,6-anhydro-D-galactose [26-28]. McLachlan [26] reported that the extracted cell wall components such as agar and carrageenan from *Gracilaria* sp. are generally characterized by forming weak gels, treatment with alkali usually results in strong gels that are commercially valuable. Sekral and Legrand [27] also informed that the spectrum at 1270 - 1200 cm^{-1} region which was assigned to stretching vibration of sulphate ester groups disappear completely after alkali–treatment. This is a reason why the low level absorbance intensity for sulphate ester were found in the extracted carrageenan from *Gracilaria* sp.

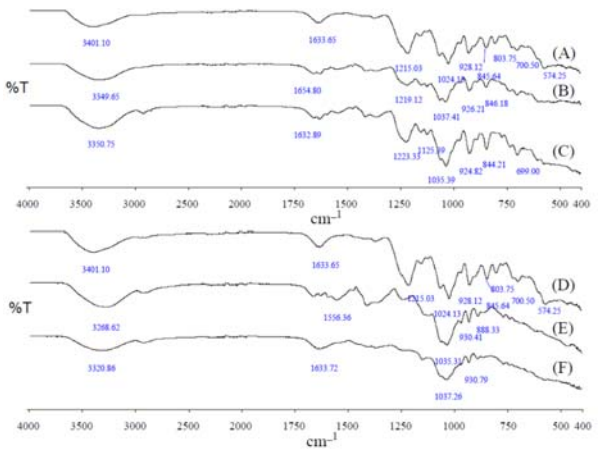


Figure-4. FT-IR spectrum of extracted carrageenan by pressurized hot water at pressure of 1 MPa (*E. cottonii*: (A) carrageenan standard, (B) 120 ºC, (C) 160 ºC; *Gracilaria* sp: (D) carrageenan standard, (E) 120 ºC,(F) 160 ºC).

Similar to the stretching vibration of sulphate ester groups, the peak spectra in all sample extracted carrageenan from *Gracilaria* sp. at the region 840 - 850 cm^{-1} assigned to C-O-C stretching vibration of 4-sulphate-D-galactose groups were also not found. In this case, methyl groups in *Gracilaria* sp. which were located at D-galactose may undergo substitution reaction to form methyl-L-galactose. The absorbance intensities at 1037.41 - 1024.13 and 930.79 - 924.82 cm^{-1}, which were due to stretching mode of O=S=O pseudosymmetric sulphate and 3,6-anhydro-D-galactose groups, respectively, were found in each spectrum. Generally, the strong absorbance intensity at these regions were obtained in kappa-carrageenan type. In the case of iota-carrageenan and lambda-carrageenan, the peak of these bands became broader [25,27]. Based on the FT-IR spectrum of extracted carrageenan from *E. cottonii* and *Gracilaria* sp. with the most probable assignation of the absorption bands given in Table-2, the extracted carrageenans from these macroalgae were almost kappa-carrageenan.

Phenolic compounds are known as a one of the most common classes of secondary metabolites found in marine algae and derived from polymerized phloroglucinol units [1,22]. These compounds possess aromatic structure along with hydroxyl substituents which enable them to protect human tissues from damages caused by oxygen or free radicals, and consequently, reduce the risk of different diseases, and offer beneficial effects against cancers, cardiovascular disease, diabetes, and Alzheimer's disease due to their antioxidant activities [29,30]. Figures-5(A) and 5(B) show the effect of extraction temperature on total phenolic compounds content in the extract obtained from *E. cottonii* and *Gracilaria* sp. when the extraction processes were carried out at pressure of 10 MPa, respectively. The total phenolic compounds content increased obviously with increasing extraction temperature, especially when *E. cottonii* was fed as a starting material. The amount of total phenolic compounds increased with the rise of extraction temperature from 120 to 160 and 200 ºC at the same reaction time. The amount of total extracted phenolic compounds at 200 ºC was almost 2-folds than that obtained at 120 ºC. It was clearly that extraction temperature has a large effect on the solvent properties of water as an extraction media at subcritical conditions. The dielectric constant of water decreased from 50 to 35 when temperature increased from 120 to 200 ºC, which is closed to the dielectric constant of methanol (ε = 33) or ethanol (ε = 25). As a result, the solubility of the phenolic compounds in water increased due to the decrease in water polarity [6,31,32].

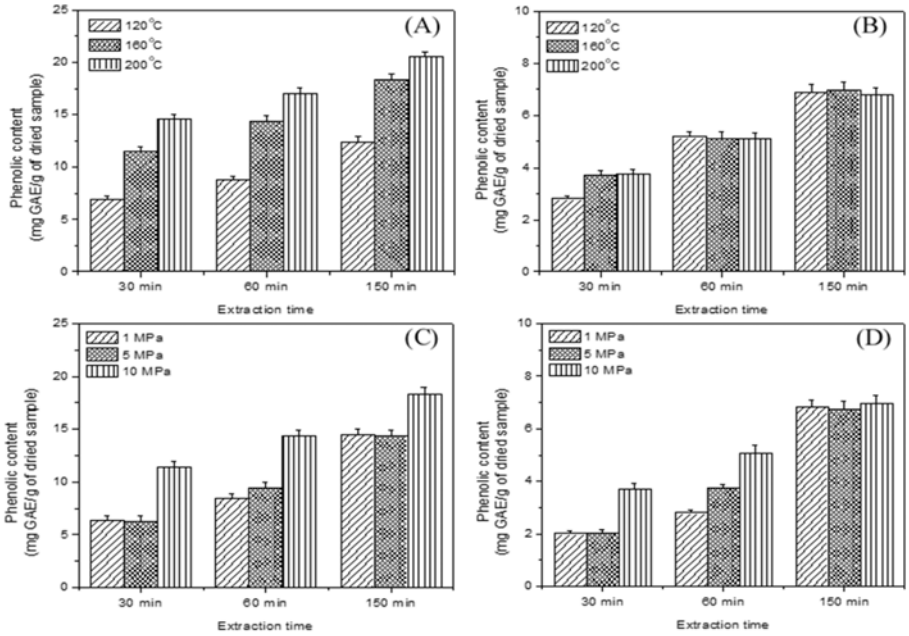


Figure-5. Total phenolic content in the extract at various extraction conditions (*E. cottonii*: (A) 10 MPa, (C) 160 ºC; *Gracilaria* sp.: (B) 10 MPa, (D) 160 ºC).

Apparently, the amount of total phenolic compounds from *Gracilaria* sp. did not linearly increase when the extraction temperatures were increased at the same extraction conditions. However, the information for optimum condition was obtained after which the use of higher temperature and reaction time did not improve the amount of total phenolic compounds in the extract. Similar to the extracted water–soluble carrageenan yield from *E. cottonii* and *Gracilaria* sp. (see Figures-5(C) and 5(D)), the elevated pressures also affected the total phenolic compounds in the extract of them at temperature of 160 ºC. The decrease in water solvent viscosity under subcritical conditions, the elevated pressures may help to disrupt the interaction between water and macroalgae matrices due to the increase of water diffusion coefficients. Furthermore, the water solvent allowed to penetrate easily into macroalgae matrices. This is, of course, a beneficial condition in terms of subcritical water extraction technique where the elevated pressures (~10 MPa) favored the extraction process. This result also explained that the higher pressures may have higher rupture effect on the macroalgae matrices being extracted [22].

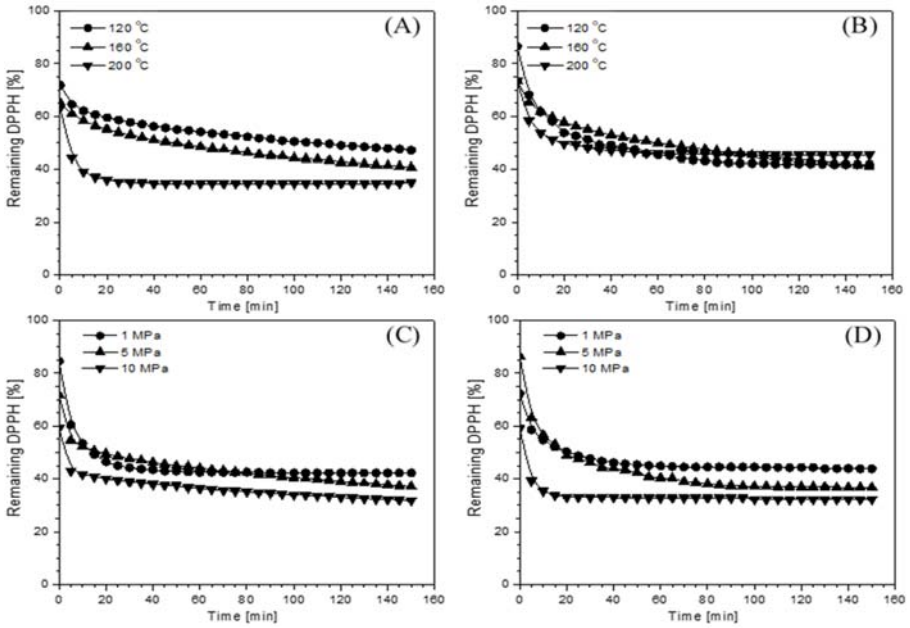
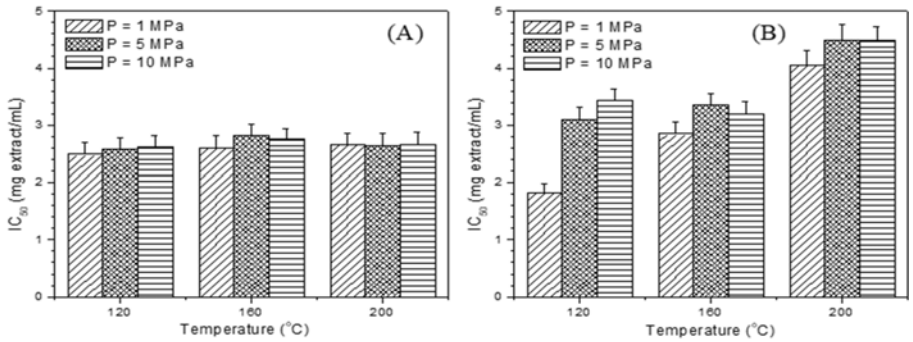


Figure-6. Antioxidant activity in the extract at various extraction conditions (*E. cottonii*: (A) 10 MPa, (C) 160 ºC; *Gracilaria* sp.: (B) 10 MPa, (D) 160 ºC).



Figure–7. IC_{50} of extracted antioxidant activity against DPPH radicals ((A) *E. cottonii*, (B) *Gracilaria* sp.).

DPPH assay is a consistent technique to determine the scavenging activity of biological compounds. This technique is easy and can be applied to measure the overall antioxidant capacity and the free radical scavenging activity of plant biomass including macroalgae. DPPH may react with the whole extracted compounds and sufficient time given in the technique allows DPPH to react slowly even with weak antioxidants. This technique also could be applied in aqueous and nonpolar organic solvents and to examine both hydrophilic and lipophilic antioxidants [33]. When the antioxidant activity of extracted solution was examined at a fixed condition, the kinetic characteristics of the antioxidant became not consider. Hence, the determination of the kinetic behavior may give more information about the antioxidant properties and could be more important than the total antioxidant capacity determined at a fixed condition. Figure-6 illustrated the kinetic behavior of the DPPH-extracted antioxidant activity at various extraction conditions. It should be noted that the compounds in the extract was not observed during the course of this work. Immediately after the loading of the extracted solutions from *E. cottonii* and *Gracilaria* sp. at various extraction conditions to the reaction medium, the absorbance of DPPH at 517 nm decreased, due to the reduce of DPPH concentration in the medium. Apparently, the antioxidant activity of extracted compounds from these macroalgae has the biphasic kinetics. Initially, the fast kinetics on DPPH concentration decompose at the beginning of reaction, followed by a slower kinetics with a gradual reduction of DPPH concentration up to equilibrium state [33,34]. The DPPH concentrations decreased quickly at 20 min reaction time then they seem constant with extending reaction time to 150 min at each extraction condition. Respect to the kinetic behavior of antioxidant activity which had similar stoichiometric trends in fast and slow phases, it could be observed that the hydrogen displacement from extracted solutions occurs by fast or

slow mechanism in equal proportion. The result also suggested the extracted phenolic compounds had responsible for the total antioxidant activity in extracted solutions from *E. cottonii* and *Gracilaria* sp. Figure-7 shows the DPPH radical scavenging activity of extracted compounds from *E. cottonii* and *Gracilaria* sp. at various extraction conditions. The different of scavenging abilities were clearly found at different extraction conditions. A lower IC_{50} value indicates a higher antioxidant activity [33]. Again, this result showed that the extraction temperature as a key variable extraction process had high effect on the contents of extracted solution [23,35].

CONCLUSIONS

Subcritical water extraction of bioactive compounds from *E. cottonii* and *Gracilaria* sp. has been performed at 120 - 200 ºC and 1 - 10 MPa in a semi–batch system. At high temperatures and pressures, thermal softening of these macroalgaes occurred, allowing extraction of bioactive compounds via autohydrolysis. FT–IR spectra showed that the macroalgae components were reacted and consumed in these range temperatures. The extracted carrageenans from *E. cottonii* and *Gracilaria* sp. were kappa-carrageenan. The yields of extracted carrageenan and phenolic compounds from both of them increased with increasing extraction temperature. Based on the results, this method could be an excellent alternative medium for extracting bioactive compounds from other types of biomass due to its temperature–dependent selectivity and environmental acceptability.

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