Extraction of Phytochemical Compounds from *Eucheuma cottonii* and *Gracilaria sp* using Supercritical CO_2 Followed by Subcritical Water

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Abstract. Extraction of phytochemical compounds (such as β-carotene, linoleic acids, carrageenan, and polyphenols) from algae *Eucheuma cottonii* and *Gracilaria sp* with supercritical CO_2 followed by subcritical water has been investigated. Supercritical CO_2 extraction was carried out at pressure of 25 MPa, temperature of 60°C, CO_2 flowrate of 15 ml/min, and ethanol flowrate of 0.25 ml/min. To determine the content of carotenoids and linoleic acids, the extracted compounds were analyzed using a spectrophotometer UV-Vis. The residue of algae starting material was subsequently extracted by subcritical water at pressures of 3, 5, and 7 MPa, and temperatures of 120, 140, 160, and 180 °C. Carrageenan extracted by subcritical water was analyzed using Fourier Transform Infra Red (FTIR), while the total phenolic compound was analyzed with UV-vis spectrophotometer. Moreover, the antioxidant efficiency of extract was also examined by DPPH assay method. Based on the analytical result, β-carotene and linoleic acid content in *Eucheuma cottonii* were 209.91 and 321.025 µg/g sample, respectively. While β-carotene and linoleic acid content in *Gracilaria sp* were 219.99 and 286.52 µg/g sample, respectively. The optimum condition of subcritical water extraction was at 180°C and 7 MPa. At this condition, the highest TPC content in the extract from *Eucheuma cottonii* and *Gracilaria sp* were 18.51 mg GAE/g sample and 22.47 mg GAE/g sample, respectively; while the highest yield of carrageenan extracted from *Eucheuma cottonii* and *Gracilaria sp* were 61.33 and 65.54 g/100 g dried algae, respectively. At the same condition, the antioxidant efficiency was 0.513 min^{-1} for *Eucheuma cottonii* and 0,277 min^{-1} for *Gracilaria sp*. Based on the results the extraction method effectively separated non-polar and polar compounds, then increased the antioxidant efficiency of extract.

1 Introduction

Seaweed is one of the country's sources of foreign exchange and a source of income for coastal communities. In addition, it can be used as food, drinks and medicines. Alginate and carrageenan are important components in the food and pharmaceutical industries. Most of the seaweed in Indonesia is exported in dry form. To increase the added value of seaweed and reduce the imports of its processed products, the processing of seaweed into carrageenan is needed to be developed [1].

One of the most widely grown algae in Indonesian is *Gracilaria sp*, which is a jelly producer. However, the use of this algae was limited for food and medicinal products, and there was no further development effort on other products that have higher economic value [2]. One of the seaweed contents that is useful in the field of industry is phytochemical compounds. The main component of algae is polysaccharides, which can reach 40-70% dry weight depending on the type of algae and its growing environment. In addition to polysaccharides, algae contain some proteins, fats, minerals, and vitamins.

In order to separate the phytochemical compounds of algae, an extraction method was developed by combination of two extraction methods namely supercritical and subcritical fluids extractions.

Supercritical fluid extraction is a process of extraction using supercritical fluids as solvents. This extraction technology utilizes the solvent strength and physical properties of pure components or mixtures at their critical temperatures and pressures in phase equilibrium [3]. The principle of the supercritical fluid extraction method is the process of separating the component above the critical pressure and critical temperature of a solvent fluid. CO_2 is the most solvent used in supercritical fluid extraction method because it is non-toxic, non-flammable, and easy available. Supercritical CO_2 allows non-polar compounds to be extracted from a natural product [4].

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In this work, supercritical CO_2 extraction was carried out at constant temperatures and pressures to extract β-carotene and linoleic acid from *Eucheuma cottonii* and *Gracilaria sp* algae. β-carotene and linoleic acid are valuable compounds with high antioxidant activity.

Subcritical water extraction or hydrothermal extraction or pressurized hot water extraction is one such alternative. Water under subcritical conditions is known as a 'natural and green' method for product extraction and has received increased attention as an important alternative to the conventional separation methods. Water under subcritical conditions can be applied to extract polar organic compounds or to decompose lignocellulosic materials to produce valuable compounds such as saccharides and aromatic organic acids. This method has been applied to recover protein, amino acids, and phenolic compounds [5].

In this work, subcritical water extraction was carried out at various temperatures and pressures to extract carrageenan and total phenolic compounds (TPC) from *Eucheuma cottonii* and *Gracilaria sp* algae residue of supercritical CO_2. The effect of pressure and temperature on the yield of carrageenan and TPC was investigated. The antioxidant efficiency of extract was also investigated.

2 Experimental Section

2.1 Materials

Eucheuma cottonii and *Gracilaria sp* algae used for starting material was purchased from Pamekasan, Madura. Prior to the extraction, *Eucheuma cottonii* and *Gracilaria sp* algae were rinsed by using distilled water to remove the impurities and salts. Then, they were ground into fine using a millser. Next, they were dried at 60^{o}C for one day until no standing moisture was visible. The Folin-Ciocalteau's Reagent, 1,1 diphenyl-2-picrylhidrazil (DPPH) and gallic acid ($C_7H_6O_5$) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Sodium carbonate (Na_2CO_3), methanol (CH_3OH, 99.7%), and ethanol (C_2H_5OH, 99.5%) were supplied by Merck (Germany). Standard carotene (98%) and linoleic acid (98%) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). They were used without further purification.

2.2 Experimental setup

In this work, the supercritical CO_2 and subcritical water extraction were conducted in a semi-batch process. The main apparatus of both processes consist of a high-pressure pump (200 LC Pump, Perkin Elmer, Germany), heater (Linn High Therm GmbH, model VMK 1600, Germany), reactor (10 mL in volume; Thar Design Inc., USA) and back pressure regulators (BPR; AKICO, Japan). Both sides of the reactor were equipped with removable threaded covers included stainless-steel filters (0.1-1.0 μm). The 1/16 in. stainless-steel tube was used to introduce liquid CO_2 or hot water from the pre-heater to the reactor, which was located in the heater.

2.2.1 Supercritical CO_2 extraction

In Supercritical CO_2 extraction process, initially, 12 g of starting material was loaded into the extractor among glass beads put on the both side of extractor inlet and outlet to prevent channeling. Then the extractor was installed in the heater. CO_2 as a solvent was then pumped at a flow rate of 15 mL/min using an HPLC (High Performance Liquid Chromatography) pump to reach an operating pressure. Besides that, ethanol was then pump at a flow rate 0.25 mL/min using an HPLC pump. The mixture of ethanol and CO_2 was flowed into extractor. The pressure was controlled by adjusting back pressure regulator (BPR) that includes a heater. Furthermore, the CO_2 and ethanol was heated using a preheater and heater to achieve an operating temperature. To ensure the temperature in the extractor according to the desired temperature, temperature in the inlet and outlet of extractor were measured by thermocouples (T_1 and T_2). Extraction solution was collected every 30 min in a collection vial. The CO_2 was flowed into environment after passed through gas flow meter. Extract was then stored in a refrigerator. The schematic diagram of supercritical CO_2 extraction apparatus is shown in Figure 1. The extraction was carried out at temperatures of 60^{o}C and pressures of 25 MPa for 3 hours.

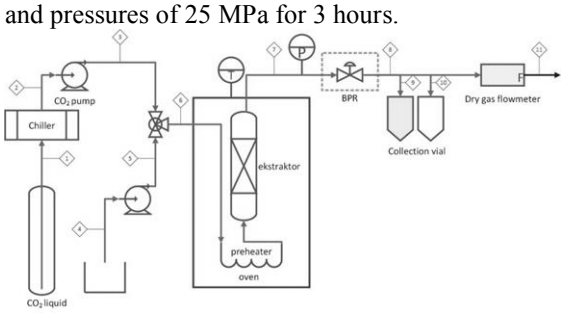

Fig. 1. Apparatus for supercritical CO_2 extraction

2.2.2 Subcritical water extraction

In subcritical water extraction process, initially, 1 g of residue from supercritical CO_2 extraction was loaded into the extractor among glass beads put on the both side of extractor inlet and outlet to prevent channeling. Then the extractor was installed in the heater. Water as a solvent was then pumped at a flow rate of 1 mL/min into the extractor using an HPLC pump to reach an operating pressure. The pressure was controlled by adjusting back pressure regulator (BPR), and was monitored by pressure gauge. Furthermore, the water was heated using a preheater and heater to achieve the operating temperature. To ensure the temperature in the extractor according to the desired temperature, temperature in the inlet and outlet of extractor were measured by thermocouples (T_1 and T_2). The extract solution was cooled by a cooler and then passed the filter and collected in a collection vial every 30 min. Extract solution was then stored in a refrigerator. The schematic diagram of subcritical water extraction apparatus is shown in Figure

2. The extraction was carried out at temperatures of 120 – 180°C and pressures of 1 – 5 MPa for 3 hours.

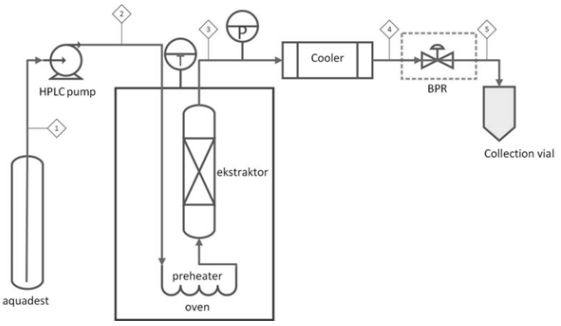

Fig. 2. Apparatus for hydrothermal extraction

2.3 Analytical method

Analysis of phenolic compounds, β-carotene, linoleic acid content, and antioxidant activity in the extracts was conducted using Genesys 10 UV-Vis Scanning Spectrophotometer (Thermo Fisher Scientific, Waltham, MA), allowing spectra between 190 nm to 1,100 nm. Liquid products were analyzed in quartz cuvette with 1 cm path length. The solid products collected at each operating condition were analyzed by a Spectrum One FT-IR Spectrophotometer (Perkin-Elmer, Ltd., England) to determine the structure of the solid products after the subcritical water extraction. The scanning wavenumber ranged was from 4,000 cm^{-1} to 400 cm^{-1}.

2.3.1 β-carotene and linoleic acid Analysis

β-carotene and linoleic acid content in the extract were analyzed using spectrophotometer at a wavelength of 450 nm and 195 nm, respectively. Initially, calibration curve was prepared by measuring a standard solution β-carotene or linoleic acid at concentration of 100 to 500 mg/L. The extract solution was diluted with n-hexane prior to analysis. β-carotene and linoleic acid content in the extract was obtained based on the absorbance of the extract compared with the calibration curve.

2.3.2 Carrageenan analysis

3 ml of the extract was mixed with 90% ethanol in the evaporating disk. The solution then stirred to form a carrageenan fiber (hydrocolloid). And then the mixed solution was leaved for 30 minute at room temperature. After that, it was heated in the heater at 60°C for 24 hours. The results obtained were dry carrageenan sheets. Carrageenan functional group was identified by using FTIR (Fourier Transfer Infra-Red). Equation below is used to calculate the yield of carrageenan:

$$\text{Yield} = \frac{weight\ of\ dried\ carrageenan}{weight\ of\ dried\ seaweed} \text{ x } 100\ \% \quad (1)$$

2.3.3 Total Phenolic Compound (TPC) analysis

TPC content in the extract was determined by Folin Ciocalteu reagent. 1 mL of extract was diluted into 2 mL of aquadest. Then, 1 mL of Folin Ciocalteu reagent was added to the solution followed by the addition of 1 mL Na_2CO_3 solution (7%). And then the mixed solution was leaved for 30 minute at room temperature (in a dark room). Furthermore, the absorbance of the solution was measured with spectrophotometer at the wavelength of 750 nm. TPC content in the extract was determined based on gallic acid standard curve at concentration of 0-200 mg/L. TPC was expressed as mg gallic acid equivalent (GAE)/g sample.

2.4 Antioxidant Efficiency analysis

The efficiency of antioxidant was analyzed using DPPH Assay method. DPPH assay is an easy and accurate method to measure the antioxidant capacity of vegetables, fruits and extracts. DPPH is one of the organic nitrogen (free radical) that is available commercially. The antioxidant efficiency of extract was determined by adding 1000 µL of extract into 2 mL of 25 ppm DPPH in methanol solution. Absorbance of the solution was measured using spectrophotometer at the wavelength of 516 nm every minute until a constant absorbance was obtained. Percentage of the remaining DPPH was calculated with the following equation:

$$\%\text{DPPH}_{rem} = 100\ x\ \frac{[DPPH]_{rem}}{[DPPH]_{t=0}} \quad (2)$$

$[DPPH]_{rem}$ is the absorbance of the extract at a certain time, and $[DPPH]_{t=0}$ is the initial absorbance of DPPH. The efficiency of radical antioxidant was calculated by the following equation:

$$\text{AE} = \frac{1}{EC_{50}\ x\ t_{EC50}} \quad (3)$$

EC_{50} is the concentration of extract that caused 50% decrease in initial DPPH absorbance, and t_{EC50} is time needed to reach steady state at EC_{50} concentration.

3 Result and Discussion

3.1 Extracted compounds from supercritical CO_2 extraction

As the target, the extracted compounds from supercritical CO_2 extraction were β-carotene and linoleic acid. Based on the result of the analysis with UV-VIS spectrophotometer, the content of β-carotene and linoleic acid in the extract from *Eucheuma cottonii* were 209.91 and 321.03 µg/g samples, respectively, while for *Gracilaria sp* the content of β-carotene and linoleic acid in the extract were 219.99 and 286.52 µg/g samples. The extracted carotenoids from *E. cottonii* was higher than the extracted carotenoids reported by Warkoyo and Saati [6]. The reported that the highest carotenoids extracted from 3 types of *E. cottonii* was 1.412 mg/100 g sample

extracted from red *E. cottonii* with ethanol. It indicated that supercritical CO_2 extraction could increase the extracted carotenoids from *E. cottonii* as well as from *Gracilaria sp*.

3.2 Functional group of carrageenan

The FTIR analysis is used to determine the presence of molecular functional groups present in a sample, in which the similarity of functional groups present between the standard and the analyzed sample has a group identical to the carrageenan standard groups.

The spectra of the carrageenan product obtained from the extraction using FTIR shows that the carrageenan product has met the standard carrageenan specification, because the functional groups present in the sample spectrum are identical to the standard spectrum of the carrageenan type as shown in Figure 3. In the FTIR spectra of the carrageenan compounds obtained in Figure 4 shows the presence of sulphate ester in the range 1210-1260 cm^{-1}, glycoside bonds in the range 1010-1080 cm^{-1}, 3,6-anhydro-D-galactose in the range 928-933 cm -1, and D-galactose-4-sulfate in the range 840-850 cm^{-1}. Thus it can be concluded that the extracted carrageenan is a kappa-carrageenan.

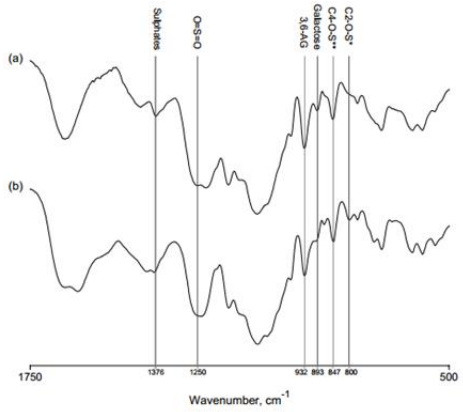

Fig. 3. Standart FTIR spectrum [7]

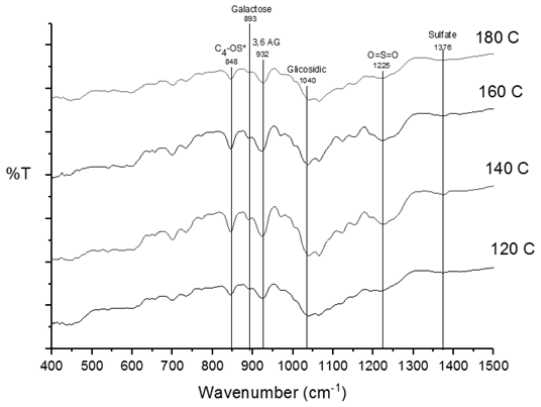

Fig. 4. Spectrum FTIR on *Gracilaria sp* carrageenan at various temperatures and pressure of 3 MPa

3.3 Effect of extraction temperature on the yield of carrageenan

As shown in Figure 5, the carrageenan yields increased as the operating temperature increase. In other words, the solvent can extract optimally when the temperature is increased.

In general, the extracted substance would increase at higher temperatures due to the increasing solubility of the substance at higher temperatures [8]. It can be happened because an increase in operating temperature will result in a decrease in surface tension and the viscosity of the solute, furthermore the extraction efficiency will increase [9]. This is consistent with the experiments which states that the increase of the extraction temperature linear to the yield of carrageenan [10].

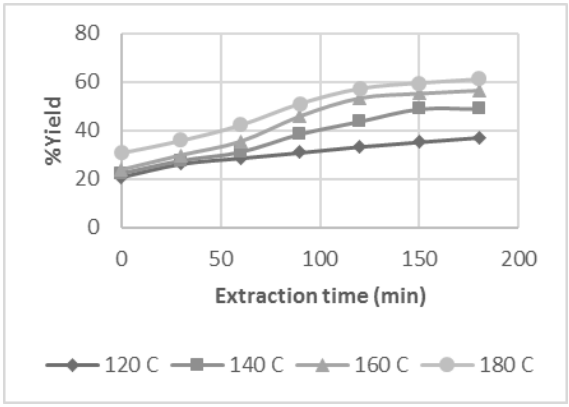

Fig. 5. The Effect of Temperature on *Eucheuma cottonii* Carrageenan at 7 MPa Pressure

3.4 Effect of extraction pressure on the yield of carrageenan

Similar with the temperature, the yield of carrageenan also increased as the increase of the operating pressure. Figure 6 shows the increase of pressure causes the increase of the solubility of the extract in solvent, so the carrageenan will be extracted more. In addition, with the increase in pressure, the number of solvents (subcritical water) per unit volume is greater, and as the result the solubility of the extract in the solvent will increase.

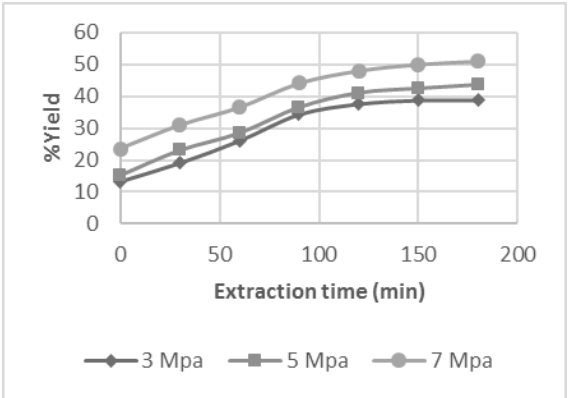

Fig. 6. The Effect of Pressure Against Yield of *Gracilaria sp* Carrageenan at Temperature 140°C

3.5 Effect of extraction temperature on the total phenolic compound (TPC)

Yield of total phenolic compound (TPC) increases as the operating temperature increases as shows in Figure 7.

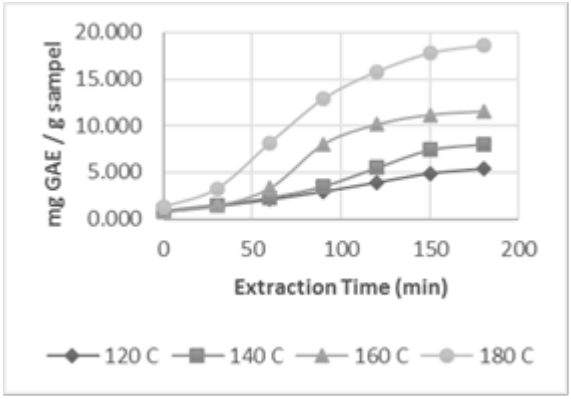

Fig. 7. Effect Analysis of Temperature on Total Phenolic Compound *Eucheuma cottonii* at 5 MPa Pressure

The extracted TPC increased obviously with an increasing extraction temperature. It can be happened because the higher temperature changes affected the solubility of phenolic compounds in water, therefore the solvent (subcritical water) is able to extract seaweed optimally. In addition, As the temperature increases, the permittivity constant of the water falls, so that the phenol becomes more soluble in water.

3.6 Effect of extraction pressure on the Total Phenolic Compound (TPC)

As shown in Figure 8, the effect of operating pressure on the total content of phenol is not very noticeable because the pressure interval taken is too low. In addition, this is also due to the polarity of water that is not influenced by the pressure. For example, at a temperature of 100 ° C, an increase in pressure from 10 MPa to 20 MPa only raises the dielectric constant from 55.9 to 56.2 [11].

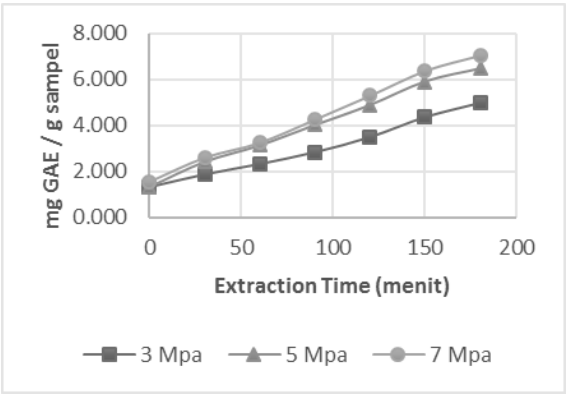

Fig. 8. Effect Analysis of Pressure on Total Phenolic Compound *Gracilaria sp* at Temperature 140°C

3.7 Antioxidant Efficiency

Figure 9 shows %DPPH measurement for antioxidant activity of *E. cottonii*. The decreasing %DPPH in antioxidant activity was caused by the longer incubation time between the extract and DPPH solution as free radical. The seaweed extract acts as an antioxidant that reduce free radical (DPPH) compounds into non-radical compounds. And this can be proved when DPPH concentrations reach 50% of the initial DPPH concentrations.

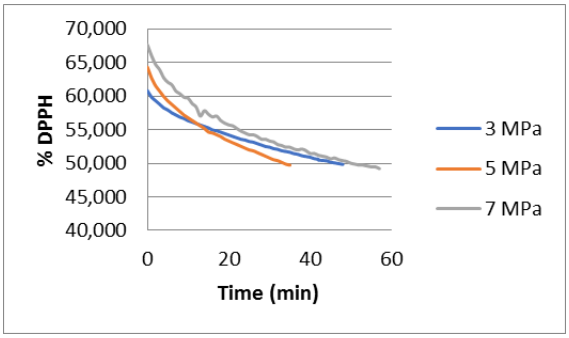

Fig. 9. Decreasing % DPPH Antioxidant Analysis of *Eucheuma cottonii* at 140°C

Eucheuma cottonii contains phytochemicals such as vitamin C, flavonoids, tannins, riboflavin [6]. *Gracilaria sp* are also said to have antioxidant sources such as carotenoids, pigments, flavonoids, terpenes, steroids, tannins, alkaloids, phenols and glycosides in abundant amounts [12].

Table 1 shows effect of subcritical water extraction conditions on the antioxidant efficiency of the extract. The highest antioxidant efficiency (AE) was obtained at the operating temperature of 180 ° C and pressure of 7 MPa. The efficiency of this antioxidant depends on the concentration of antioxidants contained in the extract and the time it takes the extract to reduce the concentration of DPPH per minute. The smaller the concentration of antioxidants needed to react with DPPH, the greater the AE value obtained. The faster the time required by antioxidants to react with DPPH, the greater the AE value obtained. So, the greater the AE value of an extract, the greater the ability of the extract to fight the free radicals.

Tabel 1 Antioxidant Efficiency of Extract at Various Operating Conditions

Seaweed	Temperature (^oC)	Pressure (Mpa)	AE (Min^{-1})
Eucheuma cottoni	120	3	0.0016
	140		0.0181
	160		0.0123
	180		0.0636
Gracilaria sp	120		0.004
	140		0.018
	160		0.08
	180		0.0735
Eucheuma cottonii	120	5	0.0018
	140		0.0186
	160		0.049
	180		0.8266
Gracilaria sp	120		0.0056
	140		0.0193
	160		0.2108
	180		0.2984
Eucheuma cottonii	120	7	0.005
	140		0.026
	160		0.1709
	180		4.9636
Gracilaria sp	120		0.0073
	140		0.023
	160		1.2323
	180		1.404

4. Conclusion

Phytochemical compounds from *E. cottonii* and *Gracilaria sp* have been separated and extracted by supercritical CO_2 and subcritical water. The content of β-carotene and linoleic acid in the extract obtained by supercritical CO_2 were 209.91 and 321.025 μg/g sample, respectively, for *E. cottonii*, and 219.99 and 286.52 μg/g sample, respectively, for *Gracilaria sp*. After supercritical CO_2 extraction, the residue was extracted by subcritical water to obtain high concentration of polar compounds (carrageenan and phenolic compounds) with high antioxidant activity. The maximum yield of carrageenan and the content of TPC extracted by subcritical water were 65.54% and 24.74 mg GAE/g sample, respectively. Based on the results the extraction method effectively separated non-polar and polar compounds, then increased the antioxidant efficiency of extract. Furthermore, the extraction method may be applied in pharmaceutical industry.

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