Metabolite Profiles and Antioxidant Activity of *Caulerpa racemosa* with Different Handlings

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Abstract

Metabolite profiles and antioxidant activity of *Caulerpa racemosa* extract with different handlings were investigated. Three different handlings during transportation were applied, namely samples chilled with ice, stored in liquid nitrogen and soaked in seawater. The different handling significantly affected the yield of ethanolic crude extracts and inorganic fractions but insignificantly to organic fractions. Different handlings resulted in differences of major fractions of *C. racemosa* extracts. Major fractions of the sample that was handled with chilling temperature contained low polar fractions (K_{10}, K_{11}, K_{12}, and K_{13}), while seawater handling extract contained very polar (K_1, K_2 and K_3), polar (K_6, K_7, and K_8) and low polar (K_{13}) fractions. The extract of the sample handled in liquid nitrogen contained balanced fractions. Chilling temperature handling produced highest antioxidant activity (IC_{50} below 2,000 ppm) in ethanolic extract of *C. racemosa.*

Keywords: *antioxidant activity, Caulerpa racemosa, ethanolic extract, handlings, IC_{50}*

1. Introduction

Caulerpa racemosa, also known as sea grape, is a species of *Chlorophyta* that commonly found in lower intertidal and upper subtidal areas which waves and currents action were not strong. The algae lives in habitats with sandy-muddy to rocky-coralline substrates and it is generally distributed in tropical and subtropical marines of Phillipines, Vietnam, Singapore, Malaysia, Thailand, Taiwan, China, Indonesia, and Western Pacific Islands. Sea grape is used for human consumption, eaten fresh or in a salt-preserved form in Japan, Korea, Philippines and other Southeast Asia countries. It is known as a source of active compounds such as caulerpin and caulerpicin. Caulerpin is an active compound with anesthetic activities, while caulerpicin used for medication (antifungal and lowers blood pressure) because of its toxic effect (FAO, 2018).

Secondary metabolites with various categories of natural products such indol derivatives, indan derivatives, sesquiterpenoid derivatives, diphenyl pentadiene derivatives, terpenoids and fatty acids were identified in ethanolic extract of *C. racemosa* (Ornano, Serafini, Sanna, & Bianco, 2014). Various secondary metabolite compounds contained in *C. racemosa* including pseudoephedrine, 5-butyl-2-methyl-δ1-pyrrolidine, 2-myristynoyl pantetheine, tetracontane, deoxyspergualin, hexyl octyl ether (Rahul et al., 2014), phenol, saponin, tannin, flavonoid, reduce sugar, xanthoprotein (Azhagu, Mala, & Prakasam, 2015), sesquiterpenoid and diterpenoid, β-sitosterol, caulerpin, caulerpenin (Gaillande, Payri, Remoissenet,

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& Zubia, 2016), catechin epigallo (Yoshie, Wang, Petillo, & Suzuki, 2000). The secondary metabolite compounds of *C. racemosa* extract have potential usage as antimicrobial compounds against Gram-negative, Gram-positive and pathogenic fungi (Etcherla & Rao, 2014), antimicrobials against *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus* sp, *Salmonella* sp (Perez, Falque, & Domínguez, 2016) *Vibrio harveyi* (Ikbal, 2015), antioxidants (Chew, Lim, Omar, & Khoo, 2008), antiherpes (Ghosh et al., 2004), antitumor (Ayyad & Badria, 1994), anticoagulants and antivirals (Rahul et al., 2014).

Caulerpa racemosa has a high moisture content (88.8-91.5%) so that this commodity is very easily decreased in quality after harvesting process and require immediate processing or preservation (Siah, Aminah, & Ishak, 2013). Bioactive substances in plant materials can be damaged during handling, pre-treatment, processing, drying, extraction, and long-term storage (Mediani, Abas, Tan, & Khatib, 2014). Drying is an eminent method in handling seaweed. Drying will reduce the water activity. Thus inhibits microbial growth, extend shelflife and reduce the volume of seaweeds (Gupta, Cox, & Ghannam, 2011). Unfortunately, the drying method could not be applied to *C. racemosa*, as the quality parameters of *C. racemosa* are the freshness of the thallus, bright green color, and compact texture that indicate the turgor pressure in the cell is still good (IMR, 2012). Drying will change the physical characteristics and active ingredients in seaweeds and resulted in a drastic reduction in the total phenol content and antioxidant activity (Ascorbic acid equivalent antioxidant capacity and ferric reducing power) (Chan et al., 2009). Drying at 25 °C causes a decrease in the total phenol and flavonoid content of 49-51% compared to fresh seaweeds. DPPH scavenging ability of fresh seaweeds is higher than dry seaweeds (Gupta et al., 2011).

The appropriate handling *of C. racemosa* is needed to maintain physical quality and bioactive compounds. The handlings in this study were done by chilling the *C. racemosa* with ice, soaking in seawater and storing in liquid nitrogen. Freezing temperature would preserve the content of several bioactive compounds such as anthocyanin, hydrocinnamic acid, phenol and their antioxidative activity (Oszmian, Wolniak, Wojdyo, & Wawer, 2008; Rapisarda, Bianco, Pannuzzo, &Timpanaro, 2008). Storage at ultra-low temperature in liquid nitrogen (-196 °C) cause process of metabolic, biochemical, cell division, and most physical quality completely preserved, therefore plant material can be stored for long time (Kalaiselvi, Rajasekar, & Gomathi, 2017).

Handling with liquid nitrogen has technical limitations as it could not be applied for large samples. Therefore, it is necessary to find alternative of handling method to preserve the bioactive compounds in *C. racemosa*. Handling by using chilling temperature and storing in seawater is an alternative to overcome the limitation of liquid nitrogen handling. By handling using a chilling temperature of 5 °C, the total phenol content and antioxidant activity can be maintained at 95% (Ali et al., 2018). The storage of seaweeds was more partical when using seawater and cold temperatures (Tiwari & Troy, 2015). Handling by soaking seaweed in seawater aimed to keep it under particularly same condition of its habitat. Hopefully, no changes occur during transportation. The objective of this study was to investigate the effects of different handlings on the metabolic profiles and antioxidant activity of *C.racemosa* ethanolic extracts.

2. Materials and Methods

Technical grade ethanol (96%), 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), methanol (Merck), acetonitrile LC grade (Merck), water was deionized using a Milli-Q water purification system from Millipore (Bedford, MA, USA).

2.1. Sample Collection

The *Caulerpa racemosa* used in this study was collected on 18 January 2018 from the rocky-coralline intertidal area of Binuangeun Coast in Banten Province (6° 50' 40" S, 105° 53' 30" E). The sample was washed with seawater to remove the dirt and impurities. The collected samples were transported to laboratory within 8 hours and was handled in three ways; chilled with ice, stored in seawater, and stored in liquid nitrogen. Chilled with ice handling and seawater handling used coolbox to place the samples, while in liquid nitrogen handling the samples placed in the conical tube and immersed in liquid nitrogen container.

2.2. Preparation of Ethanolic Extracts

Approximatelly 50 g of fresh sample was extracted using 500 mL ethanol (96%) for 24 hours at room temperature then filtered using Whatman no. 42 to obtain crude extracts. The extracts were evaporated under low pressure and then freeze-dried to remove residual solvent. Ethanolic extract was resuspended with methanol and passed through solid phase extraction (SPE) using silica column (C_{18}) to remove inorganic and salt contents. The retained fraction in

SPE was called inorganic fraction that contained salts and an inorganic component. The collected fraction that passes through the SPE was called organic fraction. The organic phase was subsequently evaporated to dry and stored in -20 °C until use. Each method was analyzed in triplicate.

2.3. DPPH Radical-Scavenging Activity of Antioxidant Extract

Antioxidant evaluation of *C. racemosa* crude extract was conducted using 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) method according to Sachindra et al. (2007) with some modifications. DPPH reagent (Merck) was prepared by diluting 3 mg in 5 mL MeOH (p.a). A methanol diluted seaweed dry extract of 160 µL was poured into 96-well microplate then a 40 µL of DPPH reagent was added (A). Extract control (containing 160 µL of seaweed extract and 40 µL of MeOH) (B), negative control (160 µL MeOH and 40 µL of DPPH reagent) (C) and a blank (200 µL MeOH) (D) were also used in this evaluation. The mixture was incubated at 25-28 °C for 30 minutes. Absorbance of each well was measured using microplate reader (Thermo Scientific) at 517 nm. DPPH free radical inhibition percentage was calculated using the following equation: $[(C-D)-(A-B)]/(C-D) \times 100\%$. Inhibition concentration (IC_{50}) value was calculated using probit analysis.

2.4. Preparative HPLC Analysis

The organic fraction of ethanolic extract was resuspended in methanol. The HPLC analysis was performed using Shimadzu LC-20A system model with UV-Vis detector and C_{18} column (20 mm x 250 mm) Phenomenex (particle size 5 µm). The injection volume was 10 µL, and the sample was detected at 254 nm. The mobile phase was composed of 100% water (solvent A) and 100% acetonitrile (solvent B). Linear gradient elution was performed from 20% solvent B and 80% solvent A (t=0 min) to 100% solvent B at t=30 min. Total run time was 30 min. The mobile phase was achieved at 15 mL/ min and the column temperature was 30 °C.

2.5. Statistical Analysis

DPPH measurement and HPLC analysis were carried out in three independent extractions and performed in triplicate for each extraction. Exploratory data analysis was done using box plots to visually summarize and compare groups of data. Computations were conducted by using SPSS Version 16.0 statistical software.

3. Results and Discussion

3.1. Effect of Handling Methods on The Yield of Ethanolic Extracts

The general principle of extraction using solvents is the compounds that extracted have similar polarity with the solvents (Zhang et al., 2007). We used a polar solvent, technical grade ethanol (96%) to extract bioactive compounds in *C. racemosa* since other organic solvents like acetone, methanol, chloroform, and hexane are toxic for human consumption.

Figure 1 shows the effect of handlings on crude extracts, organic and inorganic content in *C. racemosa* extract. Crude extracts were composed of organic and inorganic fractions. The differences in handlings were significantly affected on crude extracts and inorganic fractions, but not significant on the content of organic fractions. Liquid nitrogen handling resulted in yield of extract where the yield of crude extracts and inorganic fractions was higher than the other handlings. According to the results, inorganic fraction content plays a role in determining the amount of the crude extract yield.

The yield of extraction depends on the polarity of solvent, extraction process (pH, temperature, time), and sample compositions. Polarity of solvent and sample constituents is the main condition parameters for extraction process under the same time and temperature (Stalikas, 2007). Organic fractions content in *C. racemosa* extract was 0.8-1.0% (Nagappan & Vairappan, 2013). Ethanol extracts from marine biota have high salt content. The salt content of fresh *C. racemosa* was 4.0 ± 0.2%. That was rapidly decreased when soaked in 50 to 150 mL of aquades at 26 °C for 30 min. Later, it was gradually reduced to 1.2 ± 0.1% when the volume of aquades was rose to 250 mL (Nguyen, 2016).

Liquid nitrogen made the sample freezing quickly at the temperature of -196 °C causing salts and inorganic compounds trapped inside the cell and carried out with crude extracts during extraction process. During chilling temperature handling, some ice melted and carried liquid cells that contained salts and inorganic compounds resulting in the decreased of inorganic fractions of crude extract. Seawater handling aimed to maintain the same condition as the *C. racemosas* habitat, and no changes on compounds during transportation.

3.2. Metabolite Profiles of Ethanolic Extract of *C. racemosa*

The HPLC analysis was performed with a hydrophobic stationary phase (C_{18}) and HPLC grade water and acetonitrile phase with a gradient mode
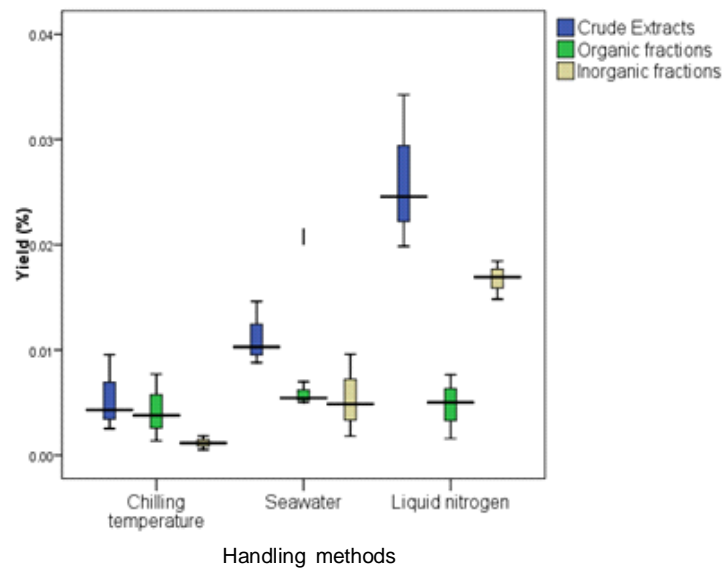
Figure 1. Effect of handling on ethanolic extract of *C. racemosa*

which elutes for 30 minutes. Ethanol extracts from marine biota have high salt content. Therefore, in the early stages of isolation the inorganic contents should be eliminated. The elimination was done by passing the extract on the SPE flash chromatography C_{18} column with the methanol mobile phase. Thereafter, the free extract of inorganic compounds was fractionated and isolated sequentially on preparative, semipreparative and analytical HPLC columns to obtain the target bioactive compound isolates. Reverse Phase HPLC has a broad range of selectivity coupled with a high degree of reproducibility.The retention time of RP-HPLC is longer for less polar molecules; as such, the polar molecules elute more readily (Abubakar, Salleh, Omar, & Wagiran, 2017).

The chromatogram of *C. racemosa* extract with different handlings exhibited similar peak patterns and shapes to each others (Figure 2). The different handlings did not affect the diversity of bioactive compounds in *C. racemosa* extract. The retention time difference was affected by the degree of polarity of the compound, the chemical structure of the compound and the mobile phase of HPLC. The solvent extract used in the preparation of HPLC analysis was a polar pure methanol. At the beginning of the injection, the composition of the water mobile phase was greater than the acetonitrile mobile phase so that the polar compounds were detected earlier, while in the final stages of the injection, the acetonitrile composition was larger than water so that the compounds detected were less polar. The polarity of the compound influenced by the number of oxygen atoms and the length of the chain.

According to the peak and retention time, the quantity of major components in HPLC fractions was measured by dividing HPLC fractions into 13 major fractions namely with fractions of K_1, K_2, K_3, K_4, K_5, K_6, K_7, K_8, K_9, K_{10}, K_{11}, K_{12}, and K_{13}. Each fraction was transferred into a microtube, removed the solvent by freeze-dried and weighted.

As shown in Figure 3, different handlings affected the major compounds in *C. racemosa* extract. Chilling temperature handling contained low polar fractions (K_{10}, K_{11}, K_{12}, and K_{13}), Seawater handling contained very polar (K_1, K_2 and K_3), polar (K_6, K_7 and K_8) and low polar (K_{13}) fractions, while the liquid nitrogen handling contained balanced fractions.

Marine organism contained various polarity metabolite compounds. Polar metabolites represented mainly by alkaloid salts, polyhydroxysteroids, saponins, amino acids and its derivates. The medium polarity metabolites were peptides and depsipeptides, while the low polarity metabolites were hydrocarbons, fatty acids, acetogenins, and terpenes (Riguera, 1997). Polarity of solvent and extraction conditions such as temperature and speed of agitation affect the extraction yield of total phenolic, total flavonoid and antioxidants (Muhamad, Gimbun, & Yusoff, 2014). Polar solvents are frequently used for extracting polyphenols content in plant materials. Polyphenols extraction using ethanol as a solvent gave a high yield of total extract and antioxidant capacity, despite the fact that it was not highly selective for phenols extraction (Dai & Mumper, 2010). Flavonoids and their glycosides, catechols, and tannins contained in plant materials were effectively extracted using ethanol
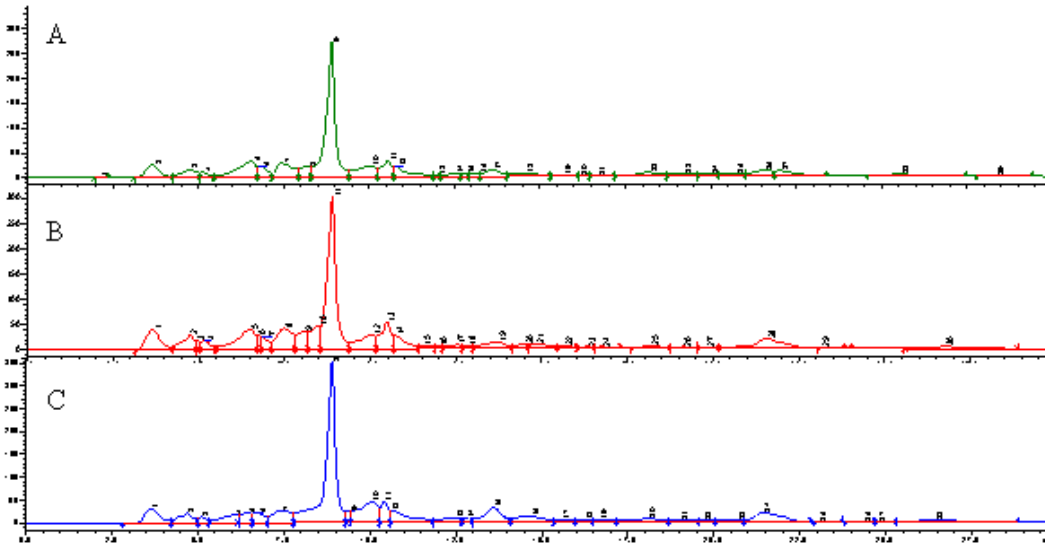
Figure 2. HPLC chromatogram profiles of different handlings of *C. racemosa* ethanolic extracts: (A): Chilling temperature, (B): Seawater, (C): Liquid Nitrogen
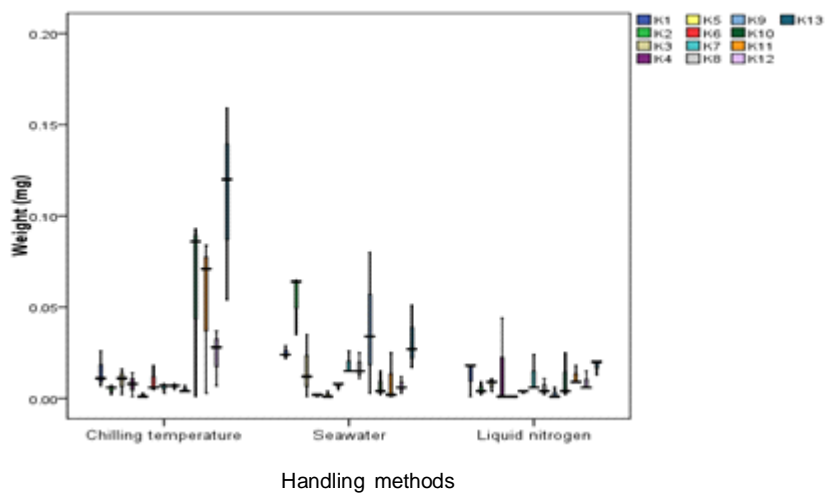

Figure 3. Effect of different handlings to the major compounds of *C. racemosa* ethanolic extract

(Bazykina, Nikolaevskii, Filippenko, & Kaloerova, 2002).

3.3. Effect of Handlings Methods on Antioxidant Activity

The ethanolic extract of *C. racemosa* were evaluated for their antioxidant activity using DPPH radical scavenging activity method. It has been used extensively to evaluate antioxidant activity of substances to neutralize the free radical (Singh & Rajini, 2004). Free-radical scavenger donor an electron or hydrogen to DPPH so it become diamagnetic molecule and paired off, reducing DPPH's ability to absorb colors. The free-radical scavenger capacity determined by the ability of the extract to reduce colors absorption ability of DPPH. The antioxidant activity of extract was measured by IC_{50} value, which is determinated from the linear regression of the % inhibition versus the antioxidant extract concentration. IC_{50} value proportional to the antioxidant activity. (Middha, Usha, & Pande, 2013).

Seaweeds were rich sources of polyphenolic antioxidants such as flavonols, catechins, and phlorotannins (Heo, Park, Lee, & Jeon, 2005). Major compounds of *C. racemosa* ethanolic extract have various biological activities such as lowering the glucose level in blood, reduces inflammation or swelling, and protection against harmfull agents and
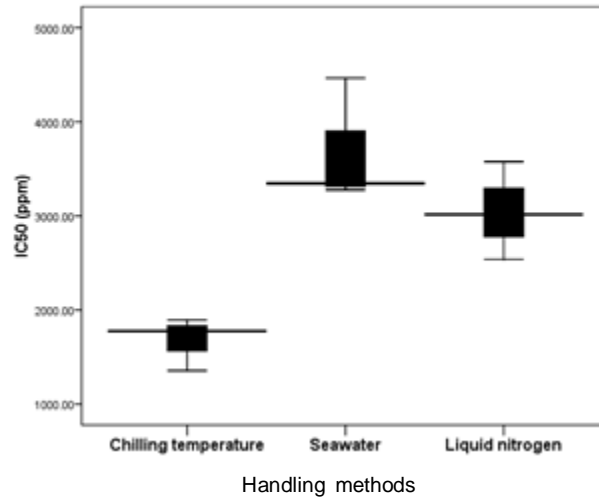
Figure 4. DPPH radical scavenging activity of ethanolic extract of *C. racemosa.*

free radicals. The active compounds were identified as myristynoyl pantetheine, tetracontane, deoxyspergualin, and hexyl octyl ether (Rahul et al., 2014).

Freezing would preserve the content of several bioactive components such as anthocyanin, hydrocinnamic acid, phenol and their antioxidative activity (Oszmian et al., 2008; Rapisarda et al., 2008). Liquid nitrogen protected photosynthetic pigment compounds and tocopherol (Esteban et al., 2009), carbohydrates, proline, phenol components including anthocyanins and flavonoids. Freezing maintained the ability of free radical scavenging capacity of antioxidant compounds. The content of polyphenolic or anthocyanin compounds has a correlation with the antioxidant activity of the material (Magdalena & Macura, 2010).

As shown in Figure 4, different handlings were significantly affected the DPPH radical scavenging activity of ethanolic extract of *C. racemosa.* Chilling temperatures handling resulted in high antioxidant activity compared to the seawater and liquid nitrogen handling. According to the major compounds in the fractions, the compounds of *C. racemosa* extract with the strongest antioxidant activity in the DPPH assay were less polar.

According to the yield of ethanolic extract, extracts with chilling temperature handling had the smallest inorganic fractions content compared to handling with seawater and liquid nitrogen. Inorganic fractions may contain impurities and salts. There was a correlation between the content of inorganic fractions with antioxidant activity. Inorganic fractions in the extract will reduce the proportion of organic fractions that contain bioactive compounds and interfere with an active group in antioxidant structure, so the antioxidant activity of extract will be decreased.

IC_{50} values of extracts with different handling methods showed higher than 1,000 ppm. These values exceeded the IC_{50} maximum limit of a substance that could be called as having antioxidant activity, which is 200 ppm (Blois, 1958). Based on this limit, the antioxidant activity of *C. racemosa* extract tends to be less active. The same results were also shown in a study done by Dwihandita (2009) where the fresh sea grape extract had the best antioxidant activity with the smallest IC_{50} value of 1,115.94 ppm, while the dry seaweed extract have IC_{50} value of 2,716.20 ppm.

4. Conclusion

Chilling temperature handlings resulted in low inorganic fraction of the crude extract, which contain low polar metabolite fractions (K_{10}, K_{11}, K_{12}, and K_{13}) and show the highest antioxidant activity. Therefore, this research suggests that the best way for maintaining the antioxidant activity of *C. racemosa* is by handling with chilling temperature.

5. Acknowledgment

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