

IDENTIFICATION AND TOXICITY TEST OF PHOTOSYNTHETIC PIGMENTS EXTRACTED FROM *CAULERPA RACEMOSA* USING THE ULTRASOUND ASSISTED EXTRACTION (UAE) METHOD

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Abstract: *Caulerpa racemosa* is a green macroalga, also known as sea grapes, which has various bioactive compounds such as pigments. The aim is to isolate pigments of *Caulerpa racemosa* collected at Senapan Island, West Papua Province, Indonesia, and assay their toxicity against *Artemia salina* Leach larvae. Pigments were isolated from *Caulerpa racemosa* using the *Ultrasound Assisted Extraction* (UAE) method with a mixture solvent, namely acetone-methanol (7 : 3; v/v). In addition, the pigment extract was identified using Thin Layer Chromatography (TLC). The pigment spots were also separated using column chromatography and characterized using UV-Vis spectrophotometry at 400 – 800 nm. Furthermore, these pigments were tested for their toxicity using the *Brine Shrimp Lethality Test* (BSLT) method. This result showed six pigment spots in the TLC analysis. Moreover, the major pigments of *Caulerpa racemosa* were identified using column chromatography and UV-Vis spectrophotometry as pheophytin a, chlorophyll a, and β -carotene. Moreover, chlorophyll-a and β -carotene pigment have no toxic towards *Artemia salina* Leach according to BSLT method with LC_{50} is > 1000 ppm. This study provides the pigments composition of *Caulerpa racemosa* and their toxicity where is no toxicity activity given.

Keywords: *chlorophyll a, column chromatography, β -carotene, BSLT, thin layer chromatography*

INTRODUCTION

The oceans are the habitat of a great biodiversity of marine organisms, which have numerous metabolic products [1]. Moreover, most of them have biological activities (like antioxidants, anti-inflammatory, antimicrobial, and so on) that play a positive role in health and have great potential to be exploited for food and nutraceutical applications [2]. Macroalgae, also known as seaweed, are among the highly abundant marine life [3]. They are naturally rich not only in nutrients (i.e. carbohydrates, dietary fiber, minerals, polyunsaturated fatty acids and vitamins) but also in health-beneficial compounds (such as polysaccharides, phenolics, alkaloids, terpenoids, and natural pigments) [3 – 5]. Based on their pigmentation, macroalgae can be classified into three groups, namely brown (*Phaeophyceae*), green (*Chlorophyceae*) and red (*Rhodophyceae*) [6].

Caulerpa sp., also known as sea grapes, is one type of green macroalgae (*Chlorophyceae*) which widely grows in the tropics and subtropics, such as Vietnam, Singapore, Malaysia, Thailand, Philippines, China, and Indonesia. *Caulerpa racemosa* contains protein (3.98 %), fiber (1.36 %), carbohydrate (3.60 %), ash (55.11 %), and secondary metabolites that contain phenolic compounds, triterpenoid, diterpenoid, and glycolipid [7].

In recent years, macroalgae have been identified as important sources of bioactive natural substances, particularly natural pigments, which have been linked to health and food benefits, such as antioxidants [8]. There are three basic classes of natural pigments which found in marine algae such as chlorophylls, carotenoids and phycobiliproteins. In order to increase the yields of bioactive compounds and to improve the efficiency of the extraction procedures, many researches recommend exploring the use of innovative technologies such as ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), enzyme-assisted extraction (EAE) and pressurized liquid extraction (PLE) [6, 9 – 12]. Others benefit of using innovative technologies are reducing the use of chemicals, consumption of energy and generation of waste [9]. Furthermore, ultrasound-assisted extraction (UAE) can improve product quality with the advantage that the processed product can release high amounts of carotenoids and other bioactive compounds due to the rupture of the cell walls by cavitation [13]. Moreover, the ultrasonic waves have a mechanical effect, allowing greater penetration of the solvent into the sample matrix, increasing the contact surface area between the solid and liquid phases, and subsequently, the solute rapidly diffuses from the solid phase to the solvent [14]. In addition, another advantage of the ultrasound-assisted extraction (UAE) method is that it can prevent the chemical degradation of bioactive compounds [15].

Therefore, this study aims to extract photosynthetic pigments from *Caulerpa racemosa*, namely chlorophyll and carotenoid, by using ultrasound-assisted extraction (UAE). After the extraction process, the pigment extracts were identified by thin layer chromatography (TLC) and separated by column chromatography.

MATERIALS AND METHODS

Materials

Caulerpa racemosa collected from Senapan Island, West Papua Province, Indonesia. The used reagents in this study were methanol p.a (Merck, Germany), n-hexane p.a (Merck,

Germany), acetone p.a (Merck, Germany), CaCO_3 (Merck, Germany), TLC plate, silica gel G60-7733 (Merck, Germany).

Sampling of *Caulerpa racemosa*

Fresh seaweed, *Caulerpa racemosa*, was collected by hand from Senapan Island, West Papua Province, Indonesia and was immediately brought to the laboratory in the cooler boxes in order to prevent degradation of chemical contents of seaweed. Moreover, these macroalgae were washed thoroughly with tap water to remove sand, epiphytes, sediment particles and other extraneous materials. Further, the sample was stored in freezer for further step.

Ultrasound Assisted Extraction (UAE) Method

Firstly, the sample was shade dried in the sun for 8 hours until constant weight obtained. Then, dried sample was blended using a mechanical blender to get seaweed powder. Secondly, 200 g of seaweed powder was extracted for each variation of time (5, 10, 15, 20, 25 and 30 minutes) at 35 °C by adding 0.5 g of CaCO_3 . The extraction process was performed based on ultrasound-assisted extraction (UAE) method using an ultrasonic water bath (*Branson, USA*) at a frequency of 42 kHz which has a constant power of 110 W and used mixture organic solvent namely acetone (p.a) and methanol (p.a) in a ratio of 7 : 3 (v/v). All the extraction procedures were performed in triplicate. Furthermore, ultrasound treated samples were concentrated by rotary evaporator to obtain pigment extracts. Then, pigment extracts were stored in dark bottles in the freezer to avoid oxidation.

Thin Layer Chromatography (TLC) Analysis

In order to identify the pigments contained of *Caulerpa racemosa* pigment extracts were analyzed by Thin Layer Chromatography (TLC). TLC analysis on the pigment extract was run with mobile phase, a combination of hexane : acetone (7 : 3, v/v) and stationary phase used silica gel GF 254 plates Merck. The separated pigments were visualized using UV lamp fluorescent at 254 nm. Afterwards, different pigment spots were observed and its corresponding R_f values were calculated.

Pigment Fractionation by Column Chromatography

The extracted pigment which has many spots at TLC analysis was fractionated by column chromatography on silica gel G60-7733 as stationary phase. Combination of hexane and acetone solvent (7 : 3/ v/v) was used as mobile phase. Further, the pigment extracts were evaporated, dissolved in the smallest amount of a given solvent and loaded onto the column. The separation was conducted in dark condition and at room temperature. The fractions were collected and subjected for further analysis [16].

Spectrophotometric Analysis

Identification of the pigment fractions were done by UV-Visible spectrophotometric. The absorbances were read at 400 - 800 nm against the blank containing respective solvents. UV-Vis spectrophotometry measured the interaction between electromagnetic radiation near ultraviolet (190 - 380 nm) and visible light (380 - 780 nm) using a spectrophotometer instrument with a compound. This measurement is based on the absorption of ultraviolet rays or visible light that causes the transition of electrons (electron transfer from a low energy level to the higher energy level) [17].

Brine Shrimp Lethality Test (BSLT) Method

Artemia salina Leach, brine shrimp eggs, were hatched in artificial seawater prepared by dissolving 38 g of sea salt in 1 L of distilled water. The larvae were separated from the eggs after a 24 h incubation period at room temperature (22 - 29 °C) [18].

Toxicities of pigments were tested at 10, 100 and 1000 ppm in 10 mL sea-water solutions with 1 % DMSO (v/v). Ten larvae were added into each bottle and incubated for 24 h. Three replications were used for each concentration. The last bottle was filled with sea salt water and DMSO only, in purpose as a drug-free control or negative control. Afterwards, the bottles were examined, and the number of dead larvae in each bottle was counted after 24 h.

The lethality concentration (LC₅₀) was calculated by using probit analysis. The LC₅₀ (median lethal concentration) values were calculated by using the regression line obtained by plotting the concentration against the death percentage on a probit scale [19].

RESULTS AND DISCUSSION

Caulerpa racemosa Pigment Extracts

Green macroalga, *Caulerpa racemosa*, has branches linked to stolons with length around 27 cm and green colour.



Figure 1. *Caulerpa racemosa*

Crude pigment extracts of *Caulerpa racemosa* was carried out by Ultrasound-Assisted Extraction (UAE) method with six different times of extraction (Table 1) at 42 kHz. The results reported on Table 1 indicate a gradually increasing in extraction yield of *Caulerpa racemosa* pigment extracts. As expected, extraction yields were improved with increased temperatures from 5 to 30 minutes. Higher temperatures obviously enhanced solvation capabilities and higher solubility in the extraction process [20]. The highest yield was achieved at 30 minutes, approximately 55 % (Table 1). The application of ultrasound is

beneficial because the ultrasonic waves break the cells, releasing the cell contents into the extraction medium [21].

Table 1. Extraction yield (%) of *Caulerpa racemosa* pigment extracts

Time Variations [min]	Yield [%]
5	39.5
10	41
15	42
20	51
25	51.5
30	55

Pigment Contents of *Caulerpa racemosa*

In order to identify the pigment contents of *Caulerpa racemosa*, the thin layer chromatography analysis of the pigment extracts (six different times of extraction) was carried out. TLC is used to encourage the identity of a compound in a mixture when the R_f of a compound is compared with the R_f of a known compound [22]. The results showed that there were six pigment spots in 5 and 20 minutes while others variation time (10, 15, 25, 30 minutes) were found five pigment spots.

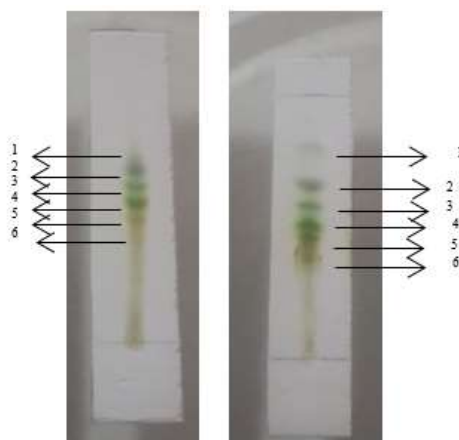


Figure 2. Pigment spots of *Caulerpa racemosa* at 5 minutes (left) and 20 minutes (right)

Chlorophylls and other pigment contents obtained from *Caulerpa racemosa* are presented in Table 2. Comparing R_f values and color for spots of pigments extracted from *Caulerpa racemosa*, these obtained spots were composed of chlorophyll a, chlorophyll c and fucoxanthin.

Table 2. The R_f value of *Caulerpa racemosa* pigment extract

Spot number	R_f Value	Pigment	R_f Value	Pigment	References
	5 minutes		20 minutes		
6	0.14	chlorophyll c	0.11	chlorophyll c	0.01-0.20 [23]
5	0.28	fucoxanthin	0.3	fucoxanthin	0.07-0.33 [23]
4	0.34	xanthophylls	0.44	chlorophyll a	0.28-0.49 [24]
3	0.46	chlorophyll a	0.52	chlorophyll a	0.40-0.65 [25]
2	0.51	chlorophyll a	0.6	chlorophyll a	
1	0.65	chlorophyll a	0.65	chlorophyll a	

Furthermore, pigments were identified based on the combined characteristics of column chromatography and UV-Vis spectrophotometry. The extract pigments of *Caulerpa racemosa* were fractionated by column chromatography using mixture of hexane and acetone (7 : 3/ v/v) as mobile phase. This study aims to isolate photosynthetic pigments namely chlorophyll and carotenoid. They have complementary functions for capturing light [26]. From the fractionation results obtained three main fractions whose colors were vividly different each other (Figure 3) which were expected photosynthetic pigments.

**Figure 3.** Three main unknown pigment fractions

Characterization of Pigment by Spectrophotometry UV-Vis

Pheophytin a

The spectrum pattern of pheophytin-a is shown in Figure 4 (A). Furthermore, the results show that the patterns and maximum wavelengths at 412 nm, 503 nm, 533 nm, 605 nm and 665 nm are the same as in the pheophytin a literature (Figure 4 (B)) [15]. The gray spots found in the sample are thought to be pheophytin-a. According to [3] the color gray is a pheophytin. Pheophytin is a pigment that is formed due to degradation of chlorophyll a. Chlorophyll a is more easily oxidized compared to chlorophyll b and carotenoids. Due to the influence of acid and heat, chlorophyll undergoes a reduction reaction which forms pheophytin through the substitution of Mg^{2+} ions by hydrogen ions [4].

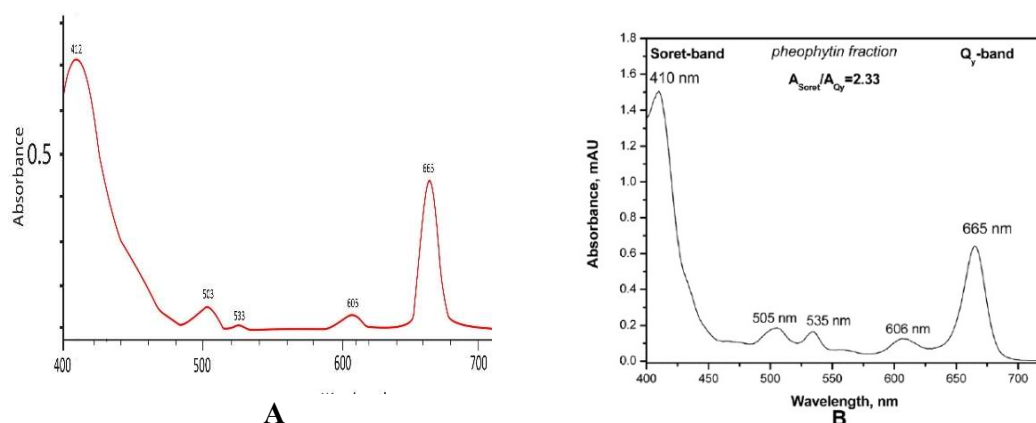


Figure 4. Characteristic UV-Vis spectra of Pheophytin-a from *Caulerpa racemosa* (A); Spectra of standard (B)

Chlorophyll-a

The UV-Vis spectrum Figure 5 (A) showing absorption peaks at 412 nm, 429 nm, 459 nm, 534 nm, 582 nm, 612 nm and 659 nm are similar to standard spectra of pheophytin-a (Figure 5 (B)) [15]. Chlorophyll-a absorbs light in the blue and red parts of the visible electromagnetic spectrum (400 - 700 nm) [27]. It is the most abundant pigment in all photosynthetic organisms [28].

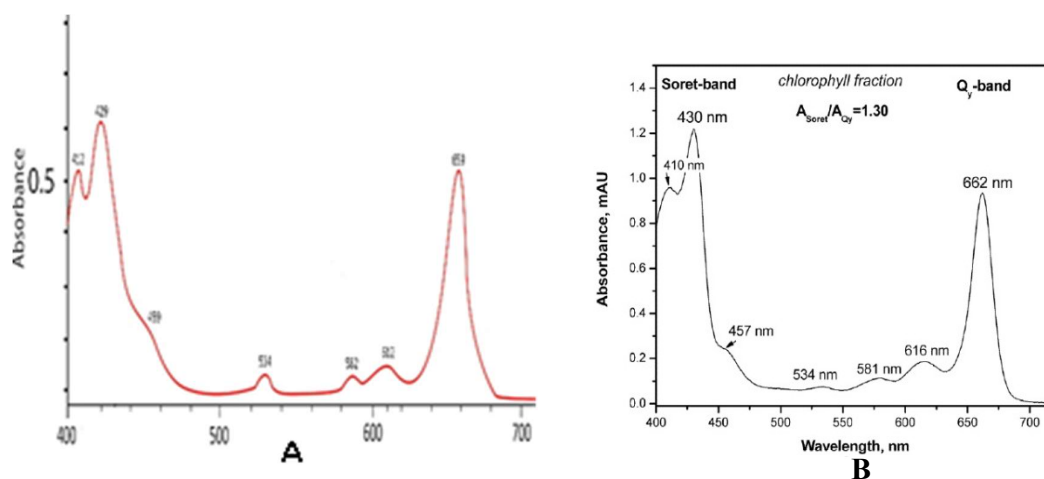


Figure 5. Characteristic UV-Vis Chlorophyll-a from *Caulerpa racemosa* (A); Spectra of standard (B)

β -Carotene

Based on Figure 6, it can be found that the spectral profile of the sample (Figure 6(A)) is similar with that of the standard (Figure 6(B)) [6] and they have the similar absorption signals at that wavelengths. This means that there is β -carotene in the sample. Referring to [2], carotenoid pigments have an absorption peak at a wavelength of 400 - 600 nm (in the visible light region). Carotenoids are a group of lipid-soluble compounds responsible

due to the absence of polar functional groups for yellow and red colors of many plants [29, 30]. Carotenoids were classified into 2 major groups, the carotenes, which are hydrocarbons such as β -carotene and lycopene ($C_{40}H_{56}$), and the xanthophylls, which include oxygen in addition to hydrogen and carbon ($C_{40}H_{56}O_2$).

While, β -carotene is a secondary metabolite synthesized by plants and belongs to an unoxidized compound group of carotenoids [31] with chemical formula $C_{40}H_{56}$ and molecular weight $536.88 \text{ g}\cdot\text{mol}^{-1}$ [32].

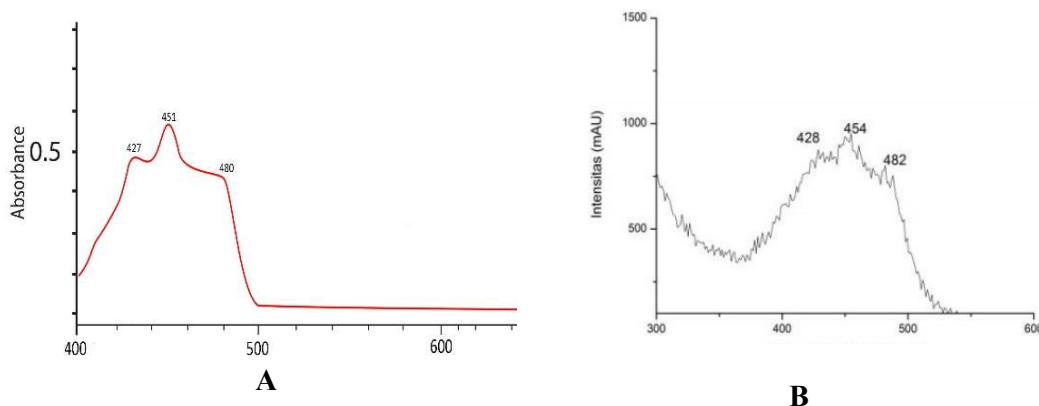


Figure 6. Characteristic UV-Vis β -carotene from *Caulerpa racemosa* (A); Spectra of standard (B)[6]

Toxicity of Pigment

In this study, chlorophyll-a and β -carotene were observed for their toxicity using the brine shrimp lethality test method as they are pigment photosynthetic. This method is conducted to test the toxicity after dosing within 24 hours. Moreover, the toxicity test results of pigment show that pigments extract could kill larvae in series concentrations $10 \mu\text{g}\cdot\text{mL}^{-1}$, $100 \mu\text{g}\cdot\text{mL}^{-1}$ and $1000 \mu\text{g}\cdot\text{mL}^{-1}$. These different variations of concentration were carried out to compare the toxic effects caused by each of these concentrations and to determine which concentration of shrimp larvae underwent LC_{50} [33]. The data for the death rate of brine shrimp larvae at various observation intervals and concentration levels of chlorophyll-a pigment and β -carotene pigment are shown in Tables 3 and 4, respectively.

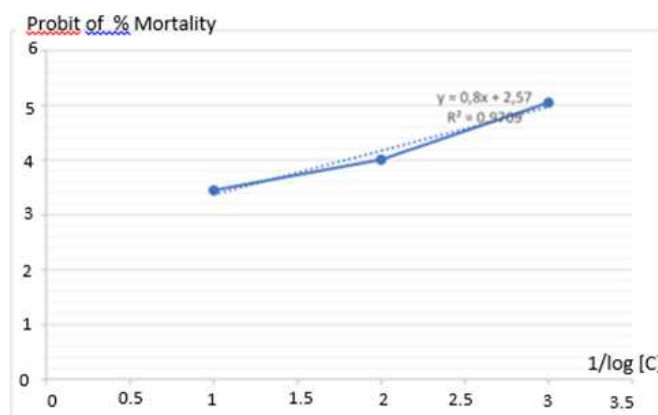
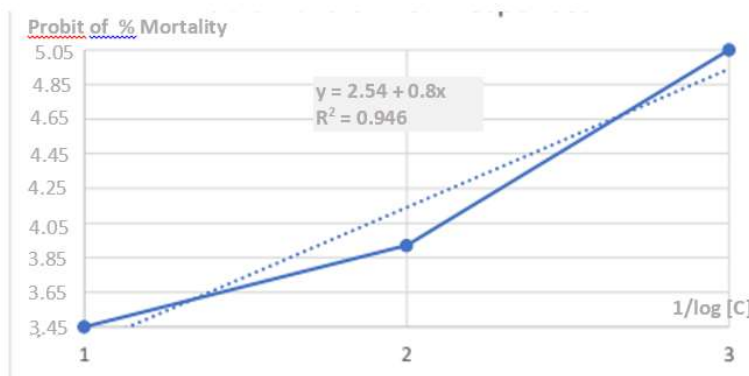
Table 3. Mortality of shrimp larvae after treating with chlorophyll-a

Concentrations [$\mu\text{g}\cdot\text{mL}^{-1}$]	Number of Test Larvae	The number of dead larvae per replication				
		1	2	3	4	5
10	10	1	0	1	0	1
100	10	1	2	1	2	2
1000	10	5	6	5	5	5

Table 4. Mortality of shrimp larvae after treating with β -carotene

Concentrations [$\mu\text{g}\cdot\text{mL}^{-1}$]	Number of Test Larvae	The number of dead larvae per replication				
		1	2	3	4	5
10	10	1	0	0	2	0
100	10	1	1	2	2	1
1000	10	5	6	6	4	5

The total mortality rate was obtained by counting the larvae that died at each concentration. Afterwards, the results of the total deaths after 24 h were analyzed to obtain a LC_{50} value.

**Figure 7.** Linear regression graph of chlorophyll a**Figure 8.** Linear regression graph of β -carotene

Based on Figures 7 and 8, the log concentration of probit values obtained from larvae mortality. From Fig.7, it can be obtained a straight line equation $Y = 0.8x + 2.57$ that means there is a positive correlation because the value of $R^2 = 0.9709$. In addition, Figure 8 described the same results with Figure 7 where is a positive correlation due to $Y = 0.8x + 2.54$ and $R^2 = 0.946$. Furthermore, the results showed that the LC_{50} value of chlorophyll-a and β -carotene pigment was respectively, $1005.629 \text{ mg}\cdot\text{mL}^{-1}$ and $1188.502 \text{ mg}\cdot\text{mL}^{-1}$ (Figures 7 and 8). The criteria for the LC_{50} value are non-toxic if the LC_{50} is >

1000 ppm; the LC₅₀ is low toxic if the LC₅₀ is 500 - 1000 ppm; the LC₅₀ is 100-500 ppm moderate, and the LC₅₀ is 0 - 100 ppm very toxic [34]. Based on these criteria, it can be concluded that both chlorophyll-a and β -carotene pigment have no toxicity since their LC₅₀ values were above 1000 ppm. This result is similar to Hammond's statement that carotenoids are generally nontoxic, even when taken in high doses as purified supplements [30].

CONCLUSIONS

The ultrasound-assisted extraction method yielded the highest yield percentage of *Caulerpa racemosa*, at around 55 % after 30 minutes. Moreover, *Caulerpa racemosa* pigment extract analysis results were separated using column chromatography and identified using UV-Vis Spectrophotometry. Three pigments have been identified, including pheophytin a, chlorophyll a, and β -carotene, each with a different UV-Vis spectrum. According to this study, chlorophyll-a and β -carotene pigment have no toxic towards *Artemia salina* Leach according to BSLT method with LC₅₀ is > 1000 ppm.

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