Correlation between Growth, Phenolic Content and Antioxidant Activity in the Edible Seaweed, *Caulerpa lentillifera* in Open Pond Culture System

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ABSTRACT

In Thailand, *Caulerpa lentillifera* J. Agardh is cultured as a commercial endeavor due to its high rate of growth and its nutrient and antioxidant contents. Of particular interest is the correlation between growth rate in pond culture and the resulting chemical products, such as total phenolic, and antioxidant activity. Samples were randomly collected from ponds once a month from April to June 2017. Morphological features, including dry weight, moisture content and numbers of fronds and branches were measured. Ethanol extraction was used to determine the total phenolic content and antioxidant activity. The results showed that dry weight was highest in April, hence marked the peak of the growth period, and was correlated to the number of fronds and branches. The percentage yield of extracts (16-17%) was not different among months, while phenol content and antioxidant activity showed slight differences among months. The highest phenol content was found in June (73±2.08 mg gallic acid equivalent·g^{-1} DW), while highest antioxidant activity was found in May (29.51±0.78%). The chemical content varied depending on physical environmental factors other than growth. These findings help us to understand the optimal conditions for growth and harvest of seaweed for chemical production and its potential for a variety of applications.

Keywords: Antioxidant, *Caulerpa lentillifera*, Growth, Macroalgae, Phenolic content

INTRODUCTION

Seaweed has been consumed for many decades as a source of food in many Asian countries, such as China, Japan, Korea, Thailand, India, the Philippines and Indonesia (Rabia, 2016). Seaweed is not only a human food, but also used in livestock feed (Garcia-Vaquero and Hayes, 2016). Seaweeds have many other commercial applications, including as bioindicators, in bioremediation of wastewater in agriculture and as soil fertilizers (Hong *et al*., 2007; Sharma *et al*., 2013; Cole *et al*., 2016). Seaweeds are also used in fish aquaria to maintain the nutrient balance and as attractive decoration.

Seaweeds produce unique chemical compounds useful not only as nutrients, but also in specialized industries and especially in medical research and treatments. Seaweed gels are used as emulsifiers in cosmetics and in food to improve taste and texture (Knoshaug *et al*., 2013). Compounds from seaweed have received increased interest

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from scientists in recent years. Both primary bioactive compounds and secondary metabolites are subjects of pharmacological research (Matanjun *et al*., 2008; De Souza *et al*., 2009; Yangthong *et al*., 2009; Lin *et al*., 2012; Pangestuti and Kim, 2015; Santos *et al*., 2015).

The green seaweed *Caulerpa* is widely cultured, especially in ponds, because of its rapid growth rate, high antioxidant activity and nutrition (Kumari *et al*., 2010; Nagappan and Vairappan, 2014). In Thailand, three species have been commercially produced: *C. racemosa* var. *macrophysa* (Sonder ex Kützing) Taylor; *C. corynephora* Montagne; and *C. lentillifera* J. Agardh. *Caulerpa* has an indeterminate prostrate axis, referred to as a stolon, which attaches to a substrate by filiform rhizoids. Upright fronds are the photosynthetic and reproductive units. Fragments of the stolon can regenerate a new thallus. The fronds of the most common species, *C. lentillifera*, have spherical ramuli and have high nutritional value (Nagappan and Vairppan, 2014; Guo *et al*., 2015). As an edible food, *C. lentillifera* is known as Green Caviar or Sea Grape. Its morphology is favorable for marketing and it has a high antioxidant value comparing among species.

Caulerpa lentillifera is widely distributed along the coastal Indo-Pacific region from the subtropical to tropical zones in a wide range of ecological niches. It can be found in mangroves and rocky shores, from the intertidal to subtidal zones (Baleta and Nalleb, 2016; Rabia, 2016). It survives in many locations with fluctuating environmental conditions. *Caulerpa* speies has a highly adaptive morphological plasticity (Collado-Vides 2002; Belton *et al*., 2014), with variation in size, frond morphology, and physiological characters.

The aims of this study were to examine whether there is a correlation between growth, chemical production and antioxidant activity of *C. lentillifera* in pond culture. The results would provide the information on appropriate harvesting time that best serve the application of *C. lentillifera*.

MATERIALS AND METHODS

Sampling and specimen preparation

Caulerpa lentillifera was collected from commercial seawater culture ponds owned by Lamiad Phaochan in Tambon Lampak, Amphur Ban Laem, Phetchaburi Province (13°02'14.4"N 100°05'11.6"E). Collections were made once a month in the same pond from April to June 2017 which is a season of commercial production. The ponds were approximately 50 m × 50 m, and 1 m in depth. At least 30 individual thalli were randomly collected from the same pond during sampling periods. As these were large ponds, biomass could not be measured for the entire pond. Instead, we divided ponds into three zones, and collected samples to represent the population for biomass and moisture content estimates. For each zone, we filled a tray, 30 cm ×50 cm ×10 cm deep, with specimens collected randomly. All specimens were thoroughly cleaned by rinsing with pond seawater before placing them into plastic bags which were kept in the dark and transferred to the laboratory. Salinity, water and air temperatures, and pH were recorded upon collection. Pond seawater measurements of PO_4^{3-}, NH_3 and NO_3^- were carried out at the Faculty of Veterinary Medicine, Kasetsart University Kamphaeng Saen campus. Fresh specimens were separately collected for growth examination, morphological measurements, chemical content and antioxidant activity.

Morphological examination

Approximately 30 cm stolons of *C. lentillifera* were used for morphological examination. The number of fronds and branches was recorded. Some fronds had developed new branches (lateral fronds, Figure 1). The number of fronds was enumerated including those from main branches as well as lateral fronds.

Chemical extraction

Since extraction yield varies with the extraction method, this study used the ethanol-solvent method, which is in wider use (Chakraborty *et al*., 2010; Lin *et al*., 2012; Mohsin *et al*., 2013; Machado *et al*., 2015), and is recommended for high yield in *Caulerpa* species (Yangthoung and Towatana, 2014). Dried algae was ground into a powder. Twenty grams of the powder was placed into a cellulose extraction thimble (30×100 mm). The sample tube was transferred to the extraction chamber in a Soxhlet extractor apparatus with a reflux at 60-70 ºC for 6 h using 170 ml of 95% ethanol as the extraction solvent. The Soxhlet extractions were done in triplicate. After extraction, the samples were left to cool. The solvent was removed using a vacuum rotavapor, followed by freeze-drying the residue and weighing. The dried crude algae was placed in airtight storage containers and stored at -20 ºC. The percentage of yield was calculated by the equation

$$\%\ \text{yield}\left(\frac{w}{w}\right) = \frac{(\text{crude extract (g)})}{(\text{dry weight(g)})}\ \text{X}\ 100$$

Total phenolic content

The total phenolic content was determined following the method of Singleton *et al*. (1999). A 0.5 g sample of dried algae was dissolved in 10 ml 95% ethanol and then centrifuged at 1,300 RCF for 10 min. The crude extract solution (10 µl) was mixed with 0.2 M Folin Ciocalteu reagent (250 µl). The mixture was allowed to settle at room temperature for 8 min, followed by addition of 750 µl 20% Na_2CO_3 and 950 µl distilled water. The solution was mixed and incubated at 30 ºC for 30 min. Absorbance was measured at 765 nm with a spectrophotometer (UV-1800, Shimadzu, Japan). Gallic acid was used as the standard. The absorbance was converted to total phenolic content in terms of mg gallic acid equivalent·g^{-1} DW.

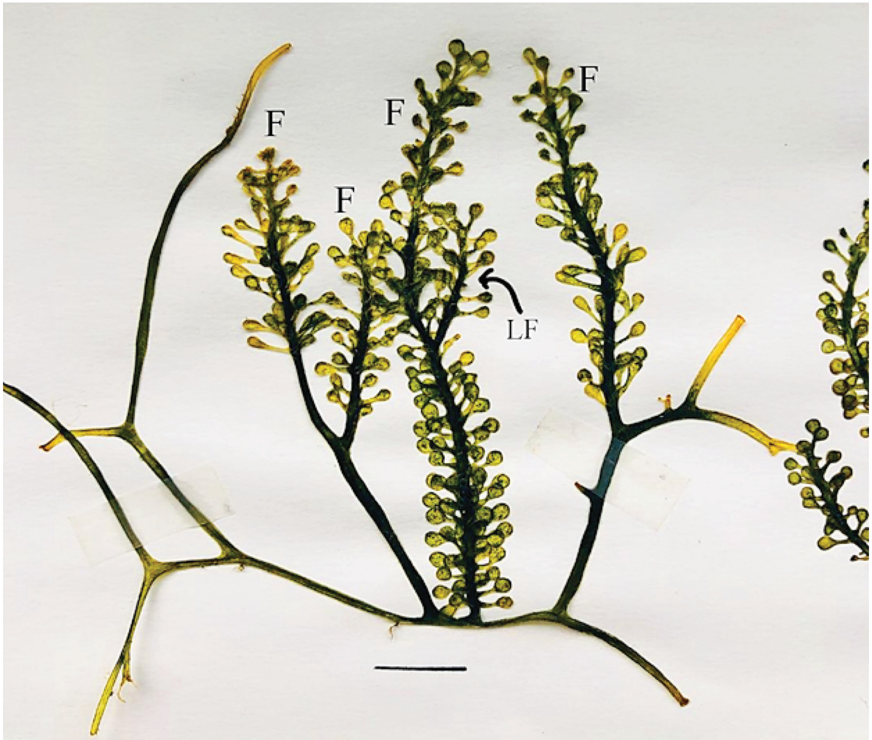

Figure 1. *Caulerpa lentillifera* specimen collected from a culture pond, indicating morphological structures examined (F: frond; LF: lateral frond). Scale bar = 1 cm

DPPH radical scavenging activity

The radical scavenging activity was determined using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method (Brand-Williams *et al*., 1995). Dried crude algae (0.5 g) was extracted with ethanol (10 ml) and the mixture was centrifuged at 5,000 rpm for 10 min. The sample solution (1.5 ml) was mixed with 0.5 ml of 0.5 mM DPPH and stored in the dark at room temperature for 30 min. Spectrophotometric absorbance was then measured at 517 nm. Controls were prepared as for the treatment groups except that the algae solution was replaced with ethanol. The DPPH free radical scavenging activity was calculated by the equation

$$\textit{Radical scavenging}\ (\%) = [(A_0 - A_1)/A_0]\ x\ 100$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

Statistical analysis

Data of phenolic content, extraction yield and DPPH activity were analyzed to evaluate the effect of collection time (month) using one-way analysis of variance (ANOVA). All data were checked for normal distribution before analyses. If not normally distributed, an independent t-test was performed. The correlation between growth-relate traits, phenolic content, %yield, DPPH activity and physical and nutrient parameters of pond water; and between growth-related traits and chemical composition were analyzed using Pearson correlations. All analyses were carried out with SPSS version 16.0 (SPSS Inc., USA).

RESULTS AND DISCUSSION

Correlations between physical parameters and biological measurements

During three months of sampling, the physical environmental parameters fluctuated (Table 1), and some of the physical and nutrient parameters were significantly correlated with growth and chemical activity (Table 2). Growth, as indicated by dry weight and the number of fronds and branches, showed a moderate correlation with pH, NH_3 and NO_3^-. In this study, phenolic content was correlated with salinity, while DPPH activity was correlated with NO_3^-.

Growth study of C. lentillifera

Dry weight varied among the three months of sampling (Figure 2a). The highest dry weight was in April (33.87±0.70 g) whereas the dry weight in May and June (31.40±1.10 g and 29.97±0.80 g, respectively) was not different ($p>0.05$). However, there were no differences of moisture content among months (Figure 2b; April 96.61±0.07%, May 96.86 ±0.11% and June 97.10±0.05%).

Morphological examination of C. lentillifera

Our results showed that the number of fronds was highest in April (19.53±0.97 fronds) and then decreased in May and June (16.80±0.9 and 13.88±0.41, respectively) (Figure 3a). The number of branches showed the same pattern as weight, and was highest in April (14.80±1.30 branches) and decreased in May and June (6.3±

Table 1. Descriptive statistics of physical and nutrient parameters (mean±SD) determined from seawater pond culture of *C. lentillifera.*

Physical and nutrient parameters	Month		
	April	**May**	**June**
salinity (‰)	37.67±0.33	40±0.00	37±0.00
water temperature (°C)	30.33±0.07	28.60±0.00	31.00±0.00
pH	8.5±0.00	8.4±0.00	8.3±0.00
PO_4^{3-} ($mg \cdot l^{-1}$)	0	0.24±0.00	0
NH_3 ($mg \cdot l^{-1}$)	1.41±0.00	0.81±0.00	0.55±0.00
NO_3^- ($mg \cdot l^{-1}$)	1.20±0.00	1.49±0.01	0.80±0.00

0.99 and 5.03±0.57, respectively) (Figure 3b). The correlation analysis showed that the number of fronds was slightly correlated with stolon length (Figure 4a; R^2=0.411, $p<0.01$), but it correlated strongly and positively with number of branches (Figure 4b; R^2=0.824, $p<0.01$).

Caulerpa lentillifera growth was reflected by an increase of both the stolons and branches. In this study, the fast growth pattern of this species showed more branching off the main frond than from the main stolon. This type of short and highly-branched thallus growth with highly-branched ramets is referred to as the compact growth form, and reflects growth under higher light conditions such as in as exposed area (Collado-Vides and Robledo, 1999; Collado-Vides, 2002). Collado-Vides (2002) pointed out that it is also an adaptive feature which may avoid self-shading and allow for the wide distributional range of *C. lentillifera*.

Wide distribution of *C. lentillifera* in different ecosystems with highly fluctuating environmental parameters, such as salinity and temperature, may be reflected in their ability to grow in ponds with variable physical and nutrient parameters. The optimal temperature and light conditions for *C. lentillifera* culture are 27.5 ºC with 40 µmol photons·m^{-2}·s^{-2}, while it is stressed at 30 ºC with 100 µmol photons·m^{-2}·s^{-2} (Guo *et al*. 2014). In this study in open ponds, light was not controlled, and *C. lentillifera* was exposed to the daylight and also high temperature. *C. lentillifera* grew maximally at 30 ºC in the ponds, but growth

Table 2. The correlation between physical and nutrient parameters and growth parameters, %yield, phenolic content and DPPH; ns=non significance, *=p<0.05 and **=p<0.01

Physical and nutrient parameters	Correlation coefficient					
	dry weight	number of fronds	number of branches	%yield	phenolic content	DPPH
salinity (‰)	0.166^{ns}	0.079^{ns}	0.130^{ns}	0.316^{ns}	-1.000**	0.909^{ns}
water temperature (°C)	-0.919^{ns}	-0.105^{ns}	-0.165^{ns}	-0.344^{ns}	0.976^{ns}	-0.921^{ns}
pH	0.553*	0.430**	0.604**	0.994^{ns}	-0.447^{ns}	0.600^{ns}
PO_4^{3-} (mg·l^{-1})	0.070^{ns}	-0.023^{ns}	-0.012^{ns}	0.108^{ns}	-0.895^{ns}	0.800^{ns}
NH_3 (mg·l^{-1})	0.559*	0.452**	0.630**	0.945^{ns}	-0.237^{ns}	0.407^{ns}
NO_3^- (mg·l^{-1})	0.0317^{ns}	0.227*	0.333**	0.655^{ns}	-0.990^{ns}	0.999*

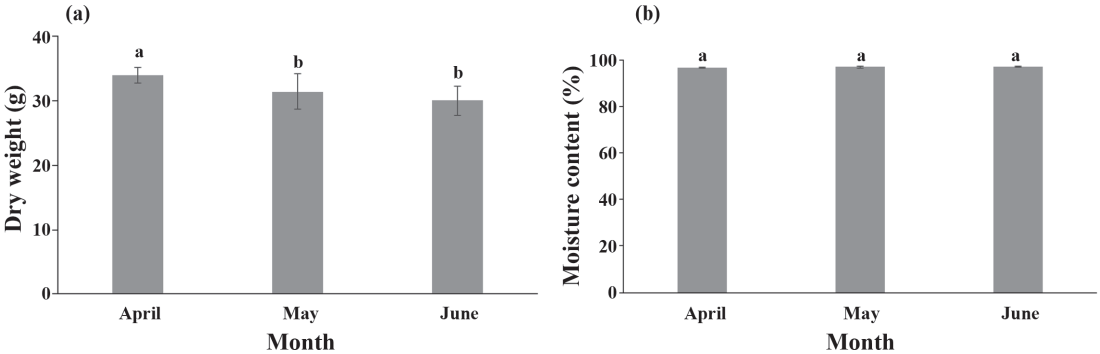

Figure 2. Comparison of *C. lentillifera* (a) dry weight (g), and (b) percentage moisture content (%) during April to June 2017. Data represent mean±SD (n=3 replicates). Different letters above bars indicate significant difference (p<0.05).

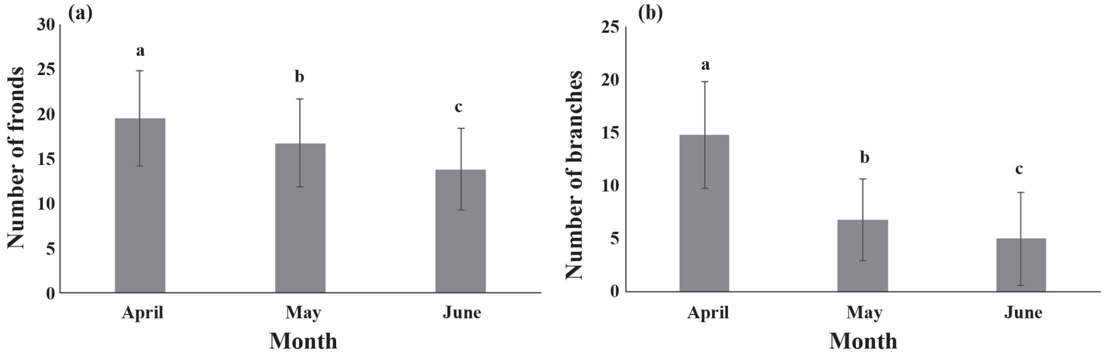

Figure 3. Comparison of (a) the number of fronds and (b) total branches of *Caulerpa lentillifera* from April to June 2017. Data represent mean±SD (n=30 thalli in April and May; 120 thalli in June). Different letters above bars indicate significant differences ($p<0.05$).

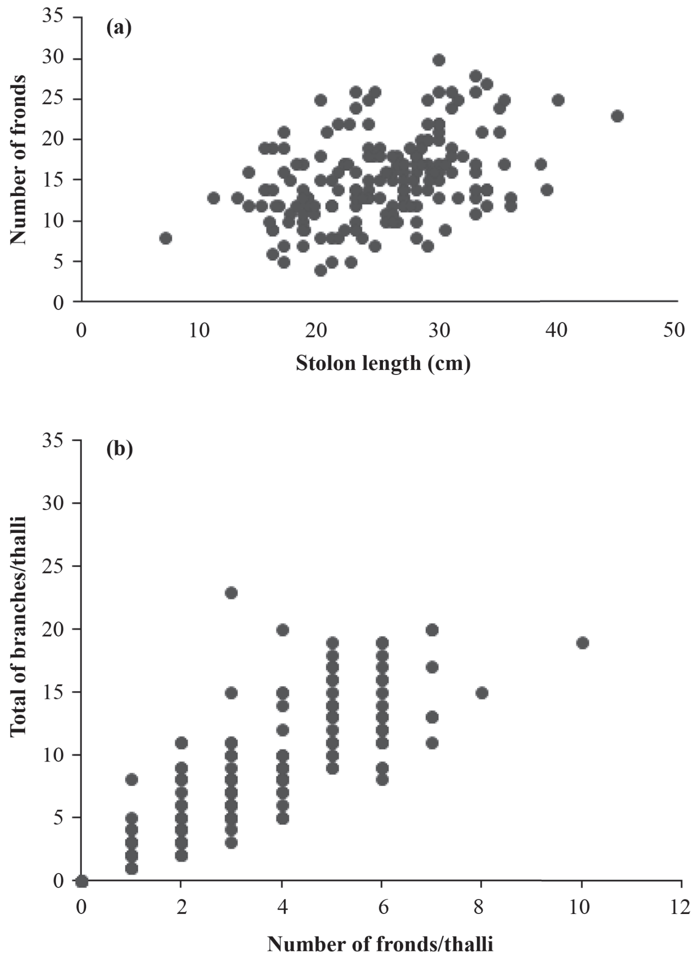

Figure 4. Scatter plot showing the correlation of growth-related parameters of *Caulerpa lentillifera* from April to June: (a) stolon lengths versus number of fronds (n=180; R^2=0.41); (b) number of fronds versus number of total branches (n=180; R^2=0.82).

started to decline at 31 ºC, which might be its point of temperature stress. Therefore, our results show that *C. lentillifera* can grow even in high light and at high temperature. *C. lentillifera* also tolerates salinity between 30-40 ‰ (Guo *et al*., 2015; Pugdeepun, 2001) which agreed with salinity ranged from 35 to 40 ‰ in this study. There was no correlation between temperature or salinity with growth in this study.

The high correlations with dry weight and number of branches and fronds indicated that pH and N (NH_3 and NO_3^-) affected growth. Since pH is a primary factor in cell balance regulation, a pH change would be expected to affect growth. A high pH would indicate the high salt-ion in seawater, which was the case in pond seawater in this study. *C. lentillifera* is a marine seaweed, and pH (in the range of this study) is strongly and positively correlated with growth. However, the upper tolerance to salinity of 40 ‰ affects cell chemical production, as discussed below. NH_3 and NO_3^- are essential nutrients for seaweed (Angell *et al*., 2016). Excess stored N supports the biomass and maximum growth rate of *C. lentillifera* (Liu *et al*., 2016) and *Gracilaria vermiculophylla* (Pedersen and Johnsen, 2017).

Extraction yield, phenolic contents and antioxidant activities

Phenolic content and antioxidant activity varied significantly ($p<0.05$) among the three months studied (Figure 5). The highest phenolic content was in April and June, while the highest DPPH activity was in May. The extraction yield was not different among months. The number of fronds showed high correlation with %yield (R^2= 0.998, $p<0.05$), but not with phenol content or DPPH activity (Table 3).

Caulerpa is one of the most effective bioremediators of chemical elements, and antioxidants are comparable to other edible seaweeds such as *Gracilaria changii*, Sargassum *polycystum* and *Ulva reticulata* (Yangthoung and Towatana, 2014). Total phenolic content and DPPH activity are independent in this study. High phenolic content does not necessarily mean high effective antioxidant activity. This lack of correlation also was reported in brown seaweed (Balboa *et al*., 2013). Yangthogn and Towatana (2014) mentioned that high chemical content does not guarantee high antioxidant activity in *C. microphysa*. N and P are primary nutrients in seaweed (Liu *et al*., 2016; Pedersen and Johnsen, 2017). Phenolic compounds in plants possess antioxidant activity (Sawadogo *et al*., 2006) and also act as a defense mechanism in seaweed correlated with environmental change (de Oliveira *et al*., 2009). High DPPH activity was found in May, and correlation analysis showed a positive relationship between DPPH and NO_3^-, when NO_3^- was high and might have been at toxic levels in the water supply. The amount of NO_3^- detected in the present study ($0.80\ mg{\cdot}l^{-1}$) exceeded the recommended safety level ($0.17\ mg{\cdot}l^{-1}$ at pH 8.4 and 30 ºC). Conversely, salinity showed a negative correlation with phenolic content in May, when the highest salinity was reached (40 ‰). This may have created stress and sub-optimal environmental conditions for *Caulerpa* growth.

Table 3. Pearson correlation coefficient between growth-related parameters and phenolic contents or DPPH determined from cultured *C. lentillifera*.

Growth-related parameters	%yield	phenolic content	DPPH
dry weight	0.966	-0.305	0.471
number of fronds	0.996	-0.464	0.615
number of branches	0.895	-0.11	0.287

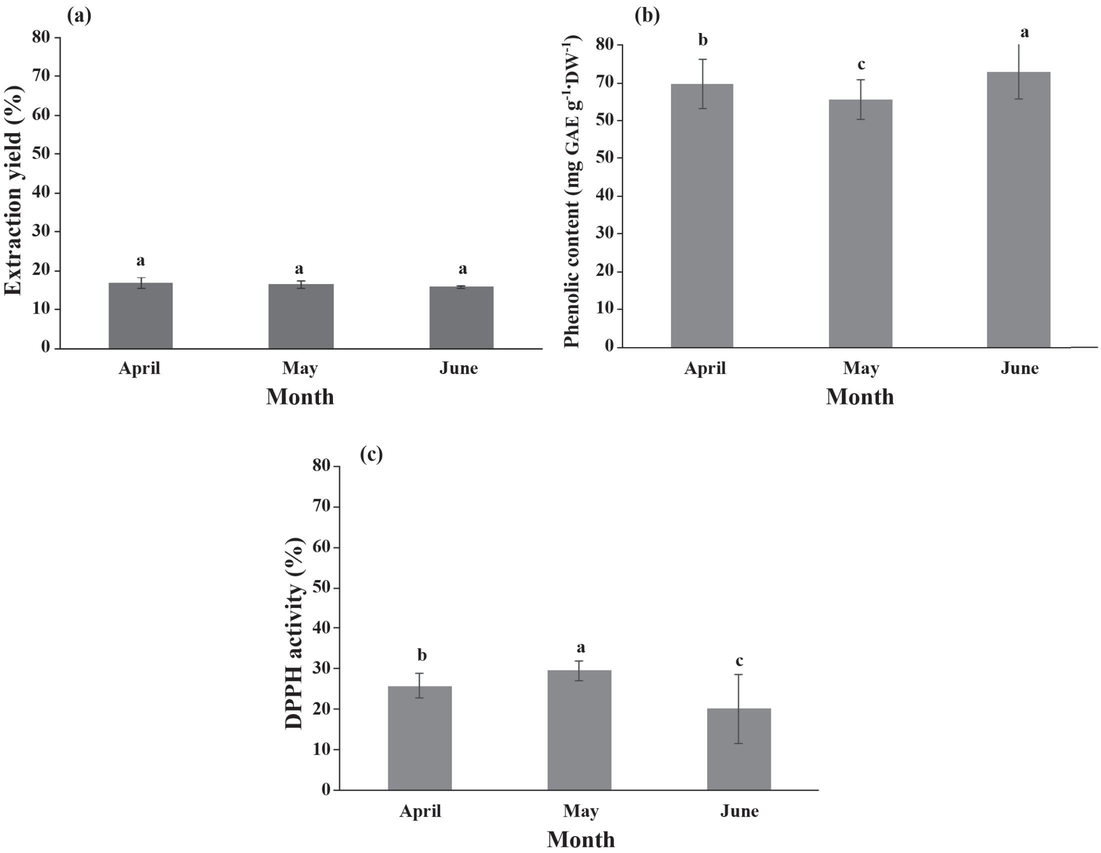

Figure 5. Comparisons of (a) extraction yield, (b) phenolic content, and (c) DPPH activity from *Caulerpa lentillifera* collected in different months. Data represent mean±SD (n=3). Different letters above bars indicate significant difference ($p<0.05$).

CONCLUSION

The biological parameters of growth (by weight), morphological complexity, phenol levels and antioxidant activity were not correlated. This indicated that the fast-growing species *C. lentillifera* did not show increased chemical content nor higher antioxidant activity correlated with growth. Instead, these factors varied according to environmental conditions. Fluctuations of the physical environment can influence the growth of *C. lentillifera*, but affect the levels of phenolic compounds and antioxidant activity differently. When cultured as food, the harvest time should be at the maximum growth of *C. lentillifera*. The concentration of chemical compounds and antioxidant activity may not be at their maximum at the peak of growth, but physical stress is an important factor for culture. All parts of the specimen can be used for chemical extraction, contrary to what is found in most farms. Farmers normally select the thalli showing green pigment and good shape, and discard the unattractive thalli as waste, with this information they can maximize the benefit from their production.

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