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Characteristics of red macroalgae, *Caloglossa beccarii* DeToni from freshwater for food as safe and other applications in Thailand

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Abstract *Caloglossa beccarii* DeToni is a freshwater red algae which contains dark brown filament and lives on surface of rocks at the bottom of shallow stream in different parts of Thailand, especially, in the southern region. The acute toxicity, nutritional value, antioxidant activity and phytochemical properties for high potential edible alga were reported. Acute toxicity was examined with a single oral administration of the extract at a dose of 2 and 5 $g.kg^{-1}$ body weight. Mortality, behavior, body weight, total food intake, and any abnormalities presented in visceral organs, were observed. The extract resulted in no mortality or abnormalities. Among proximate analysis, the algae were rich in mineral (calcium, potassium, manganese, iron and magnesium), carbohydrate (fiber and polysaccharide) and protein (arginine and leucine). It also offered a high level of vitamin C and linolenic acid. The antioxidant activities were shown in all extracts. The highest TPC, DPPH and ABTS activities were in aqueous (20.868 ± 0.68 mgGAE.g $extract^{-1}$), methanolic (IC_{50} = 0.086±0.01 $mg.mL^{-1}$) and ethanolic extract (IC_{50} = 0.178 ± 0.01 $mg.mL^{-1}$). A potential source of phycocyanin and phycoerythrin was shown. These results indicated that *C.beccarii* contains various bioactive compounds which are safe with useful characters.

Keywords: *Caloglossa*, Food, Red algae, Antioxidant

Introduction

Algae have been currently used as food and food additives. It is also useful in other industries such as aquaculture, colorants, cosmetics, pharmaceuticals and nutraceuticals. However, only a minority of all cultivated algal species aims for human use. It is likely that there are more numbers in the thousands of unidentified algal species than ones that have. Hence, algal could be potentially uses in a range of food consumption, health supplements, energy production, and many more. This seems to intensify in the years to come.

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It was found that over 200 species of red algae living in freshwater (Kumano, 2002). All of them were recognized with the naked eyes and noticeable in the nature. The red alga, *Caloglossa* is a most common specie which has been seen either in seawater and freshwater habitats within tropical and temperate zones of both hemispheres (Kamiya and West, 2014). The first record of the *Caloglossa* in Thailand was by Peerapornpisal *et al*. (2006a). *C. beccarii* is freshwater red macroalga belonging to the Rhodophyta, which a locality of this species has been reported as *Caloglossa ogasawaraensis* var. *latifolia* Kumano from Sungai Cherok, Malaysia on the western part of Malay Penninsula (Sato and Akiyama, 2001). This species is naturally grown in stream of watershed area. At the present, in South-East Asia, freshwater red algae study is not efficiently documented for utilization data. The most studies were interested in green and blue green macroalgae. Therefore, utilization of freshwater red macroalgae are still needed to study in order to manage the algae in the future.

Algae have been described as a source of numerous bioactive compounds, and widely used in food and pharmaceutical products. However, some algae contained harmful chemicals and could not be consumed (Quellette and Wilhelm, 2003). Macroalgae possessed various biological activities such as anti-cancer, anti-inflammatory, anti-mutagenic, anti-HIV, and scavenging of free radicals (Shalaby, 2015). The bioactive compounds found in many species of algae, for example, carotenoids, phenolics and sulphated polysaccharides have shown to possess antioxidant activities. In the last decade, the search for antioxidant compounds from the nature has considerably increased commercial potential in many industries, namely, medicine, food production, and cosmetic. However, the utilization of freshwater red algae in Southeast Asia is not properly recorded. The objective of study was to increase the knowledge of red macroalga, *Caloglossa beccarii* as the freshwater edible algae in Thailand through food safety (acute toxicity test), nutritional value, usefulness through phytochemical pigment character and the best antioxidant source.

Materials and methods

Material collection and preparation

Freshwater red alga, *Caloglossa beccarii* (Figure 1) was collected from substrate during the dry season (March to April, 2016) from Nai Tao stream (N 07°57.515; E 099°46.474) Nakhon Si Thammarat province, Thailand. Specimen was maintained at low temperature and preserved in 2 % glutaraldehyde before being classified for species through morphology at a

laboratory. Samples identification was clarified with relevant publications and books (Sato and Akiyama, 2001; Kumano, 2002; Peerapornpisal *et al*., 2006a and Necchi, 2016).

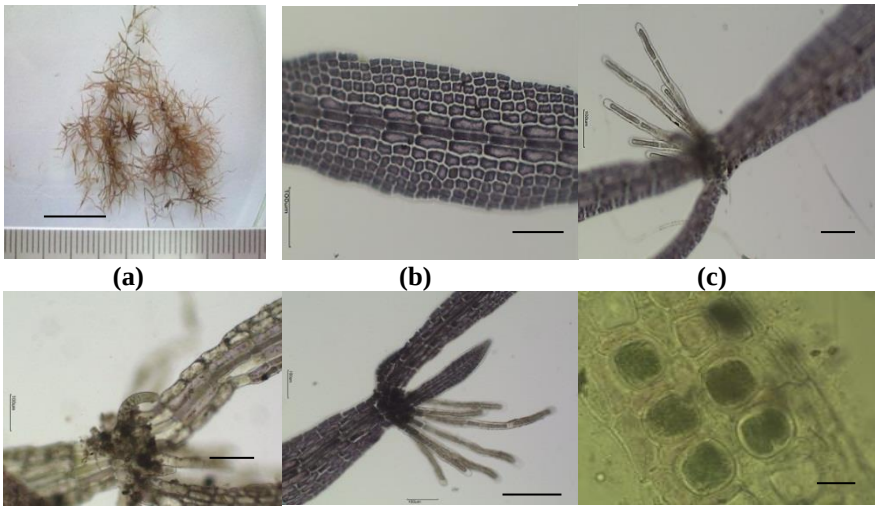

Figure 1. Vegetative morphology of *Caloglossa beccarii* De Toni : plant in natural habit (a), intertidal blade (b) rhizoids at the node (c); adventitious branching (d, e); surface view of plant showing tetrasporangia sorus (f); scale bar = 1.0 cm (fig. a); 100 µm (fig. b,c,d,e); 10 µm (fig. f)

Acute toxicity test

Preparation of algal extract

The dry material (50 g) was extracted in 500 ml of distilled water at 60 ^{o}C for 24 hours in a water bath. The total extract was filtered and the crude extract was concentrated in rotary evaporator at 40 ^{o}C. Subsequently, the extracts were lyophilized for dried powder extract.

Acute toxicity test

The tested animals were healthy females and males, Sprague Dawley (*Rattus norvegicus*) rats with a body weight of 160-200 g. The animals were purchased from the National Laboratory Animal Center, Thailand. They were allowed to acclimatize in the departmental animal facility for one week before the experiment started. Their feed was water and a standard diet. The room temperature was 25±2 ^{o}C approximately with an alternating 12 hours light/dark cycle. All process and protocol involving with the animals were performed following the rules and regulations by the Animal Research Committee of

Faculty of Medicine, Chiang Mai University, Thailand. Initially, the weight of chosen rats was varied ± 20 % of the average weight of the animals in accordance with principles in animals regulated by the OECD (OECD, 2001).

Acute toxicity test, the acute oral toxicity of the crude aqueous extracts of *C. beccarii* was evaluated in rats using the procedures described by Organization for Economic Co-operation and Development. A total of 6 animals were divided into two dosage groups (2 animals per dose). Distilled water was given to the control group while a single dose of 2,000 and 5,000 mg kg^{-1} body weight of dried extract were given to the second and third groups, respectively. Gavage dosing was performed using a curved, ball-tipped intubation needle affixed to a 5 mL syringe. Monitoring on food and water consumption, and body weight was applied daily. Approximately, animals fasted 12 hours before dosing. The animals were observed on the changes in behavior and sings of toxicity in accordance with administration of a single dose of algae preparation. Data were recorded for the first 5, 15, and 30 minutes and at hourly intervals within the next 24 hours and for 14 days afterwards. Body weight was recorded on Day 0 (before dosing), Day 7 and Day 14. At the end of the study, all of the animals were sacrificed for gross necropsy findings.

Chemical and nutritional composition

The nutritional values analysis (carbohydrate, lipid, protein, fiber, vitamin, elements mineral, fatty acid and amino acid) were based on the AOAC International Official Methods of Analysis (AOAC, 2000). The analysis was conducted by the Central Laboratory (Thailand) Co. Ltd., Ministry of Agriculture and Cooperatives, Thailand.

Total phenolic content and antioxidant activity

Preparation of the crude extracts

The sample was dried and powered after washing thoroughly to remove other unwanted materials before being used for extract preparation. In accordance with the method described by Chew *et al.* (2008) with some modification, the milled material was extracted with ethyl acetate, ethanol and methanol by shaking continuously for 24 h. The water extract was extracted at 60 ^{o}C for 24 hours and filtered through Whatman filter paper No. 1. The solvent was evaporated by rotary evaporator. The extract was lyophilized for dried powder extract.

Measurement of total phenolic content

The total phenolic content containing in each extract was measured by using Folin-Ciocalteu method as described by Lopez *et al*. (2011). Shortly, 100 µL of each sample was mixed with 0.5 ml of diluted Folin-Ciocalteu reagent (1:9 v/v; Folin-Ciocalteu reagent: distilled water). The mixture was kept at room temperature for an hour and a microplate reader (EZ Read 2000) was employed to measure absorbance at 720 nm. As standard practice, Gallic acid was used and a gross phenolic content was written in mg Gallic acid equivalents (GAE) per 1 g of extract unit.

Measurement of antioxidant activities

Free radical scavenging activity of the algal extracts was tested with 1, 1-diphenyl-1-picrylhydrazyl (DPPH) through the method proposed by Shimada *et al*. (1992). The principle of this method is the reduction of stable DPPH radical antioxidants in a methalnol solution. In brief, 3.9 ml DPPH solution (0.1 mM) was added to 100 µL of algae extracts at various concentrations. After being incubated at room temperature for 30 min, the absorbance was read at 517 nm with microplate reader (Ez Read, 2000). The measurements were made in triplicates and the percentage scavenging was calculated as shown by the formular : Scavenging (%) = $[(A_{control} - A_{sample}) / A_{control} \times 100]$ where $A_{control}$ is the absorbance of DPPH alone, and A_{sample} is the absorbance of the reaction mixture containing DPPH and sample. The appropriate sample concentration meant to scavenge DPPH radical by 50 % (IC_{50} value) was then calculated from the equation. Ascorbic acid and BHT (butylated hydroxytolueneso) were employed as positive control.

The antioxidant activity of the ABTS (2, 2'-Azino-bis (3-ethylbenothiazoline-6-sulfonic acid) radical cation decolorization assay was measured according to Re *et al*. (1999). ABTS radical cation was firstly prepared by oxidation of 5 mL of 7 mM ABTS with 88 µL of 140 mM potassium persulfate in water and placed in the dark for 12-16 hours at room temperature before use. Absorbance of ABTS radical cation solution at 734 nm was 0.700 ± 0.50 and it was used as absorbance of control. Trolox was used as antioxidant standard. Various concentrations of extracts (10 µL) were added into 1 mL of ABTS radical cation reagent and the mixture underwent incubation period of 6 minutes in the dark. The absorbance of ABTS radical after treatment with algal extracts at 734 nm was measured. In this study, antioxidant activity was expressed as mg of trolox equivalents (TE) per 1 gram of sample. The activity was calculated as follow, Scavenging (%) = $[1-(A_{sample} / A_{control})] \times 100$. IC_{50}, which stands for the concentration required for 50 % scavenging activity, was then calculated from the equation.

Pigment investigation

The sample was dried at 50^{o}C. The examination for the existence of Chlorophyll *a* was processed in 90 % methanol (Bennet and Bogorad, 1973), phycolibiprotein content was extracted in 0.05 M phosphate buffer (pH 6.8) and was calculated using a simultaneous equation with extinction coefficients from Bryant *et al*. (1973). Ethanol was employed to extract carotenoid contents which then was transferred into a solution of diethyl ether: NaCl (1:1) following de Quiros and Costa (2006).

Statistical analysis

Data were tested for normality and expressed statically in terms of means ± deviation. Student's T-Test and One-way analysis of variance (ANOVA) by DMRT test were used for statistical comparison. Differences in mean values were considered significant when $p < 0.05$.

Results

Acute toxicity test

The study on acute toxicity revealed that the animals could tolerate the limit dose of 5,000 mg.kg^{-1} body weight of aqueous extract of *C. beccarii* through oral gavages. No visible signs of acute toxicity were found throughout the 14 days observation period. Number of death at all doses was zero. Compared with baseline or Day 0 values, the rats gained weight significantly after receiving extract treatment at 5,000 mg.kg^{-1} body weight for 14 days. The tested animals did not show any abnormality regarding eating, drinking habit and behavior. The results of average water intake, average food intake, and body weight are shown in Table 1.

Table 1. Feeding pattern of rats in acute toxicity study of *Caloglossa beccarii* De Toni

Parameter	**Control**	**5,000 mg.kg^{-1}**
average water intake (ml.day^{-1})	32.13±6.92^{a}	31.79±11.95^{a}
average food intake (ml.day^{-1})	21.16±1.89^{a}	20.56±5.75^{a}
body weight (g)	212.64±35.34^{a}	220.83±34.82^{a}

Values are expressed as mean ± SD, n=16, different superscript alphabets in each row; mean significantly different ($p< 0.05$)

Determination of nutritional value

Nutritional value of the algae was represented in terms of proximate analysis, amino acids, minerals, vitamins and fatty acids involving energy per serve for 100 g dried weight (dw).The whole results were shown in Table 2. The energy per serve 100 gw was 238.70±0.01 Kcal. From the proximate analysis, the highest to the least number were ash, carbohydrate, protein, moisture, crude fiber and crude fatty. In terms of essential amino acid, 100 g^{-1}dw of *C. beccarii* contained high amount of arginine (2,082±13.48 mg.100g^{-1}dw) and leucine (2,010.18±11.52 mg.100g^{-1}dw). For nonessential amino acids, glutamine (1,301.84±379.59 mg.100g^{-1}dw) was presented. Additionally, the result showed 100 g^{-1}dw of *C. beccarii* offered high level of trace mineral, calcium (24,995.40±331.77 mg); potassium (20,673.65±121.41 mg); iron (7,159.51±86.79 mg) ; magnesium (3,748.73±54.29 mg) and manganese (1,508.06±14.44 mg), while a low level of sodium (193.94±0.58 mg), iron (7,159.51±86.79 mg), and selenium (1.73±0.11 mg) was found.

For vitamins, the highest amount of ascorbic acid (13.1 mg.100g^{-1}dw) was found and vitamin B complex (folic acid, panthothenic, and riboflavin) was ranged between 0.8 and 4.39 mg.100g^{-1}dw. However, the less number was shown for vitamin A and thiamine. Additionally, the algae presented the crude fat for 1.43±0.05 % of 100 g dw and it was represented only fourteen fatty acid. The highest of fatty acid was Palmitic acid (2,634.47±3.66 mg.100 g^{-1} dw).

Total phenolic contents and antioxidant activities

An analysis of phenolic content contains in the extracts, using different solvents showed distinct different chemical profiles of algae. Aqueous extract showed the highest total phenolic content at 20.868±0.68 mg.GAE g^{-1} dry extract while other solvents yielded significant less phenolic content as shown in Table 3.

DPPH radical scavenging activity

DPPH stable free radical method is a rapid and effective assay for measuring antioxidant activity of particular algae extracts (Shon *et al*., 2003). The radical scavenging capability of DPPH radical was examined by the decrease free radical induced by antioxidant compounds, presenting in Table 3. The level of radical scavenger was influenced by extraction method. Among

them, the highest scavenging activity (IC_{50}=0.086±0.01 mg.mL^{-1}) was found in the methanolic extract of *C. beccarii*.

Table 2. Chemical composition and nutritional profile of *Caloglossa beccarii* De Toni (composition by 100 g dw)

Proximate Analysis	
Protein (%)	20.33±0.04
Moisture (%)	8.15±0.01
Crude fat (%)	1.44±0.05
Crude fiber (%)	3.25±0.02
Carbohydrate (%)	19.89±0.98
Ash (%)	49.81±1.52
Energy (Kcal)	238.71±0.01
Amino acid (mg)	
Essential amino acids	
Arginine	2,082.28±13.48
Histidine	220.31±5.06
Isoleucine	953.82±1.69
Leucine	2,010.18±11.52
Lysine	1,904.82±18.36
Methionine	5,395.87±8.43
Phenylalanine	975.24±9.03
Threonine	1,827.22±7.01
Tryptophan	360.43±0.29
Valine	1,441.56±12.60
Nonessential amino acids	
Alanine	1,968.65±3.40
Aspartate	2,017.63±58.60
Cytine	74.78±0.06
Glutamate	1,301.84±379.59
Glycine	1,778.27±9.79
Proline	1,478.18±6.86
Serine	1,160.43±7.38
Tyrosine	201.34±1.56
Other amino acids	
Glutamine	726.13±0.38
Hydroxyproline	47.59±1.39

Minerals (mg)	
Trace minerals	
Calcium	24,995.40±331.77
Sodium	193.94±0.58
Potassium	20,673.65±121.41
Magnesium	3,748.73±54.29
Iron	7,159.51±86.79
Zinc	42.87±0.18
Manganese	1,508.06±14.44
Selenium	1.73±0.11
Chloride	0.19±0.01
Cadmium	0.12±0.01
Cupper	6.98±0.02
Vitamins	
Vitamin A (µg Re)	≤ 5
Ascorbic acid (µg)	13,100±0.08
Folic acid (µg)	4,390±11.85
Panthothenic (µg)	2,370±112.44
Riboflvin (µg)	811±78.25
Thiamine (µg)	≤ 3
Cobalamin (µg)	3.44±0.06
Fatty acids (mg)	
Myristic acid [C14:0]	27.21±0.15
Pentadecannoic acid [C15:0]	17.63±0.30
Palmitic acid [C16:0]	2,634.47±3.66
Palmitoleic acid[C16:1]	32.53±16.97
Stearic acid [C18:0]	91.42±3.51
Oleic acid [C18:1n9c]	176.95±0.45
Linoleic acid [C18:2n6c]	14.98±0.16
ãLinolenic acid [C18:3n6]	1.85±2.62
Linolenic acid (ALA) [C18:3n3]	5.71±8.08
cis-11,14-Eicosadienoic acid [C20:2]	45.86±0.44
cis-8,11,14-Eicosadienoic acid [C20:2]	28.54±0.74
Arachidonic acid [C20:4n6]	184.94±2.63
cis-5,8,11,14,17-Eicosadienoic acid (EPA) [C20:5 n3]	44.48±3.68
cis-13,16-Docosaenoic acid [C22:2]	65.84±8.86

ABTS radical scavenging activity

The algae extract decreased ABTS radical cation by behaving like free radical scavengers or by donating hydrogen to the molecule. This study found that the quantities of antioxidant compounds differed significantly ($p<0.05$) due to different extraction methods. The ethanolic extract obviously showed the highest amount of extract (IC_{50} = 0.178±0.01 $mg.mL^{-1}$) as presented in Table 3.

Table 3. Total phenolic content, DPPH and ABTS radical scavenging activity, expressed in IC_{50} ($mg.mL^{-1}$) for *Caloglossa beccarii* extract

Solvent	Total phenolic content ($mgGAE.g^{-1}$ extract)	DPPH radical scavenging activity	ABTS radical scavenging activity
ethyl acetate	12.734±0.01^{c}	0.286±0.03^{d}	0.532±0.02^{c}
ethanol	7.838±0.11^{b}	0.124±0.01^{b}	0.178±0.01^{b}
methanol	6.735±0.05^{a}	0.086±0.01^{ab}	0.681±0.01^{d}
water	20.868±0.68^{d}	0.183±0.06^{c}	0.709±0.02^{e}
ascorbic acid	-	0.041±0.00^{a}	-
BHT	-	0.038±0.00^{a}	-
Trolox	-	-	0.040±0.00^{a}

Values are expressed as mean ± sd, n=3, different superscript alphabets, mean significantly different; ($p< 0.05$)

Pigment

C. beccarii was rather high pigment content level at 0.497±0.05 and 0.076±0.01 $mg.g^{-1}$cell dw of chlorophyll *a* and total carotenoid. Phycobiliprotein including phycocyanin, phycoerythrin and allophycocyanin were presented at 0.728±0.14, 0.614±0.07 and 0.22±0.03 $mg.g^{-1}$ cell dw, respectively.

Discussion

The animals employed could consume the aqueous extracts of *C. beccarii* up to 5,000 $mg.kg^{-1}$ body weight without any visible signs of abnormality. Based on the chemical labeling and classification of acute systemic toxicity recommended by OECD (1998), the crude extracts of *C. beccarii* were category 5 (LD_{50} > 5,000 $mg.kg^{-1}$) which was the lowest toxicity class. Moreover, it was suggested that the algae could be a potential source of mineral, carbohydrate (fiber and polysaccharide) and protein. It was rich in essential and nonessential amino acids such as arginine (2,082±13.48 $mg.100\ g^{-1}$dw), leucine (2,010.18±11.52 $mg.100\ g^{-1}$ dw) and glutamine (1,301.84±379.59 $mg.g^{-1}$dw). The algae provided the nutrients stated above, it offered adequate daily trace minerals (calcium, potassium, iron, magnesium and manganese) (>100g), which was suggested to adults by Food and Drug Administration of Thailand.

Furthermore, the algae could be shown a rich source of ascorbic acid and believed to be the best antioxidant agent of the algae while the vitamin B complex (folic acid, pantothenic and riboflavin) was varied from 0.8-4.39 mg.100g^{-1}dw. It could indicate that the tested algae was nutritive like macro edible marine algae such as *Porphyra* spp., *Saccharina* sp. (HcHugh, 2003) and microalgae, *Spirulina* sp. (Gabriela *et al*., 2015), thus it could be proposed as a potential candidate for edible algae located in Thailand.

In terms of radical scavenging activity, phenolic compounds are considered as it is one of the most important classes of natural antioxidants. Polyphenols can be divided into several classes including phenolic acids, flavonoids, isoflavonoids, stilbenes, lignans and phenolic polymers (Manach *et al*., 2004). Incomparison with phenolic content on freshwater macroalgae in Thailand, total phenolic content from aqueous extract of *C. beccarii* was higher than aqueous extract of freshwater green algal, *Cladophora* (15.95 mg GAE g^{-1} dry extract) (Amornlerdpison *et al*., 2016) and freshwater blue green alga, *Nostoc commune* was 0.578 mgGAE.g^{-1} dry extract (Yucharoen *et al*., 2015).

Additionally, a variation in radical scavenger level could be influenced by extraction methods and types of red macroalgae. It was found that the antioxidant compounds were significantly differed ($p<0.05$) due to extraction methods. Among the extracts, the methanolic extract of *C. beccarii* showed the highest scavenging activity (IC_{50} = 0.086$\pm$0.01 mg.mL^{-1}). The radical scavenging activity of *C. beccarii* could also be driven by the presence of ascorbic acid, folic acid, and selenium as it was recorded that BHT and ascorbic acid with a little of IC_{50} indicated higher antioxidant activity. The high value of scavenging property of *C. beccarii* possibly dues to hydroxyl groups existing in the phenolic compounds chemical structure that may offer necessary components as radical scavenger. There were low correlation between phenolic contents and DPPH values in algae extract, indicating that antioxidant activity could possibly be influenced by phenolic compounds and other active compounds (Lopez *et al*., 2011). The results of the antioxidant assays indicated that the methanolic extracts of *C. beccarii* were the best source of antioxidant compounds. In addition, solvent selection is a crucial factor for extracting active compounds in algae. Therefore further study can be done to select the best solvent.

Moreover, both aqueous and ethanolic extracts from edible blue green algae, *Nostochopsis lobatus*, showed less ABTS free radical scavenging activities (IC_{50}=25.79 and 5.36 mg.mL^{-1}, respectively) than *C. beccarii*. Also, this aqueous extract had higher antioxidant activity than edible green algae, *Spirogyra neclecta* (IC_{50}=1.584$\pm$0.183 mg.mL^{-1}) (Punyoyai, 2007). However, Trolox, as positive control, recorded lower IC_{50} value indicating that all % ABTS scavenging activities were lower than positive control at the same concentration significantly. Briefly, all extracts from the examined red macroalga, *C. beccarii* contained antioxidant compounds that could effectively

scavenge DPPH and ABTS free radical. Therefore, it is recommended that further studies should focus on the identification and the isolation of the antioxidant components in algae.

Regarding pigment character, the findings indicated that the pigment of this species was lower than amount found in blue green alga, *Nostochopsis lobatus* from Nan river, Thailand (Thiamdao *et al*., 2012). These pigments are valuable as they can be used in proteins labeling as well as biomarker and as food colorant. From this study, *C. beccarii* would be a good source of phycocyanin and phycoerythrin which could be seen as reliable development.

Local people of Thailand have utilized many species of freshwater macroalgae such as *Spirogyra* spp., *Cladophora* spp., *Microspora* spp., *Nostoc* spp. and *Nostochopsis lobatus* (Thiamdao *et al.*, 2009; 2012; Laungsuwan and Chulalaksananukul, 2013; Peerapornpisal *et al.*, 2006b). It is indicated that *C.beccarii* has no visible signs of acute toxicity. This species exhibited high phenolic content and antioxidant activity. It is the first report on the investigation of acute toxicity test, chemical composition and nutritional and antioxidant activity of *C. beccarii*.

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