Natural Carotenoid Pigments of 6 Chlorophyta Freshwater Green Algae Species

Rashidi Othman*, Nur Hidayah Noh, Farah Ayuni Mohd Hatta and Mohd Aizat Jamaludin

International Institute for Halal Research and Training (INHART), Herbarium Unit, Department of Landscape Architecture, Kulliyyah of Architecture and Environmental Design, International Islamic University Malaysia, 53100 Kuala Lumpur, Malaysia

Abstract: Nowadays, halal products are gaining wider recognition as a new benchmark for safety and quality assurance. As a consequence the commercial development of microalgae is established due to their high value chemicals, for examples, β-carotene, astaxanthin, phycobilin pigments and algal extracts for cosmaceutical products. Therefore, many researchers have gained interest to study the potential of microalgae as new valuable chemicals and other product sources. The aim of the research is to explore new sources of pigments to be used as halal food colorants. This quest is not only directed in finding natural alternatives for synthetic dyes, but also to discover new taxons for the carotenoid production. Thus, there is a solid need to investigate the potential of natural pigments, particularly carotenoids in microalgae to be fully utilised and commercialised especially in halal market, health advantages, food products and dye technology. A total of 6 species was evaluated for quantitative and qualitative carotenoid composition, namely, *Chlorella fusca*, *Chlorella vulgaris*, *Selenastrum capricornutum*, *Pandorina morum*, *Botryococcus sudeticus* and *Chlorococcum* sp. The main carotenoids identified in all species through HPLC analysis were lutein, β-cryptoxanthin and β-carotene. The ratio of these carotenoids varies between species. Lutein was detected substantially higher in *Chlorella fusca* (69.54±11.29 µg/g DW); β-cryptoxanthin in *Pandorina morum* species (1.24±0.33 µg/g DW) whereas β-carotene in *Chlorella vulgaris* (18.42±9.2 ug/g DW). The significant outcome of the research will be new findings of new natural carotenoid pigment sources as potential food colorants and bioactive compounds which can be beneficial to halal health promoting products industry.

Keywords: Active pharmaceutical ingredients, natural colorants, carotenoid, freshwater green microalgae, lutein, β-carotene, β-cryptoxanthin.

INTRODUCTION

The market for certified halal food and products is growing robustly, both domestically and internationally. The uses of synthetic food colorant such as Sudan I, II and III as food colorant had raised health issue and in fact the uses of these types of food colorant had been banned in certain country. International Agency for research on cancer had declared that these types of colorant as a category 3 carcinogen which means it can make some alteration in human gene and hence stimulate the growth of cancerous cells [1]. Regardless to that, their consumption is not Shariah compliant for Muslim consumer. Plants produce biochemicals that are of importance in the healthcare, food, flavour and cosmetics industries. Its photosynthetic pigments can be extracted and uses as a natural food colorant, an alternative for the harmful synthetic food colorant. Currently, these and many other natural products are produced solely from massive quantities of whole plant parts. Recent advances in molecular biology, enzymology, physiology and fermentation technology of plant cell cultures suggest that these systems will become a viable source of important natural products [2]. Plant pigments are labile: they can be easily altered, and even destroyed. On the basis of their chemical structures, pigments can be classed into four families which are tetrapyrroles (chlorophyll), carotenoids (β-carotene), polyphenolic compounds (anthocyanins) and alkaloids (betalains) [3]. Colorant from plants commonly is made of carotenoids and anthocyanin. Carotenoids are responsible for the orange and yellow lipid soluble pigments in plastids. Green algae (chlorophyta) is known to benefit in human dietary as they have high nutritional values, related to its composition of biochemical that contain source of proteins, carbohydrates, lipids and vitamins. Recently, studies found that microalgae pigments can be used as an alternative ingredients in food industries [4] such as *Chlorella vulgaris* has been found that having collagen properties [5]. In 1997, 2400 tonnes microalgal biomass were produced and commercialized as “health food” in Japan and indicates that is trending as a fashionable “health food” [6].

Antioxidant that is very crucial in fighting against free radical in our body also can be extracted from algae. That antioxidant has come from the microalgae

*Address correspondence to this author at the International Institute for Halal Research and Training (INHART), Herbarium Unit, Department of Landscape Architecture, Kulliyyah of Architecture and Environmental Design, International Islamic University Malaysia, 53100 Kuala Lumpur, Malaysia; Tel: +6012 6644772; Fax: +603 61964864; E-mail: rashidi@iium.edu.my

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photosynthetic pigments which is named as carotenoid [7-11]. It is one of the bio-constituent that can be extracted from green microalgae due to the resemblance of its characteristic to higher level plants. Membrane bound chloroplast and nuclei presence in this type of green microalgae, and carotenoid can be found in that particular organelle. In Israel and Australia, *Dunaliella salina* was cultured to obtain their β-carotene [12]. Some of the studies reported, used industrial waste water to culture microalgae and extracted their carotenoid [13]. Therefore this study aimed to identify carotenoid profiles of six species of freshwater green microalgae (chlorophyta) as new sources of pigments to be used as halal food colorants.

MATERIALS AND METHODS

Mass Production of Microalgae Cell Culture

Microalgae cell culture was incubated in Bold's Basal Medium in a growth room at 24ºC day and night temperature, with a 16-h photoperiod at 80-85 µmol m-2s-1 under cool white fluorescent light.

Extraction of Carotenoids

The extraction procedure essentially follows the methods described by Othman [14], with some modifications. 0.1 g of each powdered sample was rehydrated with distilled water and extracted with a mixture of acetone and methanol (7:3) at room temperature until colorless. The crude extracted was then centrifuged for 5 min at 10 000 g and stored at 4ºC in the dark prior to analysis. To extract carotenoids an equal volume of hexane and distilled water was added to the combined supernatants. The solution was then allowed to separate and the upper layer containing the carotenoids was collected. The combined upper phase was then dried to completion under a gentle stream of oxygen-free nitrogen.

Saponification

Samples were saponified with a mixture of acetonitrile and water (9:1) and methanolic potassium hydroxide solution (10% w/v). Base carotenoids were then extracted by addition of 2 ml hexane with 0.1% butylated hydroxytoluene (BHT), followed by addition of 10% NaCl until phase separation was achieved. The extracts were washed with distilled water, dried under a gentle stream of oxygen-free nitrogen and re-suspended in ethyl acetate for spectrophotometry and HPLC analysis as described detail in Othman [14].

Determination of Total Carotenoid Content

Total carotenoid concentration was determined by spectrophotometry according to the method described by Othman [14]. The dried carotenoid was re-suspended in 500 µL of ethyl acetate for determination of total carotenoid content. For spectrophotometric analysis, 50 µL of the re-dissolved sample was then diluted with 950 µL chloroform. Three different wavelengths λ; 480 nm, 648 nm, and 666 nm were used in measuring the carotenoid-containing solutions using Varian Cary 50 UV-Vis spectrophotometer. The total carotenoid content in chloroform was obtained by using the Wellburn equation [15] as described below:

$$Ca = 10.91A666 – 1.2A648 \quad (1)$$

$$Cb = 16.36A648 – 4.57A666 \quad (2)$$

$$Cx+c = (1000A480 – 1.42Ca – 46.09Cb) / 202 \ (\mu g / ml) \quad (3)$$

Ca = concentration of carotenoid at 666 nm, Cb = concentration of carotenoid at 648 nm, and Cx+c = total carotenoid concentration at 480 nm.

HPLC Analysis of Carotenoids

The HPLC analysis of carotenoids were performed on an Agilent model 1200 series comprised of a quarternary pump with autosampler injector, micro-degassers, column compartment equipped with thermostat and a diode array detector. The column used was a ZORBAX Eclipse XDB-C_{18} end capped 5 µm, 4.6x150 mm reverse phase column (Agilent Technologies, USA). The eluents used were (A) acetonitrile:water (9:1 v/v) and (B) ethyl acetate. The column separation was allowed via a series of gradient such as follows: 0-40% solvent B (0-20 min), 40-60% solvent B (20-25 min), 60-100% solvent B (25-25.1 min), 100% solvent B (25.1-35 min) and 100-0% solvent B (35-35.1 min) at a flow rate of 1.0 mL min^{-1}. The column would be allowed to re-equilibrate in 100% A for 10 min prior to the next injection. The temperature of the column was maintained at 20°C. The injection volume is 10 µL each. Detection of individual carotenoids was made at the wavelengths of maximum absorption of the carotenoids in the mobile phase: neoxanthin (438 nm), violaxanthin (441 nm), lutein (447 nm), zeaxanthin (452 nm), β -carotene (454 nm), β-cryptoxanthin (450 nm) and α -carotene (456 nm). Compounds were identified by co-chromatography with standards and by elucidation of their spectral characteristics using a photo-diode array detector. Detection for carotenoid peaks was in the range of 350

to 550 nm. Individual carotenoid concentrations were calculated by comparing their relative proportions, as reflected by integrated HPLC peak areas, to total carotenoid content determined by spectrophotometry. The total and individual carotenoid concentration would be expressed in terms of milligram per 1.0 g dry weight of freeze-dried matter (µg/g DW).

RESULTS AND DISCUSSION

Six species of freshwater microalgae which are *Chlorella vulgaris*, *Chlorella fusca*, *Selenestrum capricornutum*, *Botryococcus sudeticus*, *Chlorococcum* sp. and *Pandorina morum* were selected. These 6 species exhibited highly significant differences in total and individual carotenoid content ($p < 0.0001$). *Chlorella vulgaris* was found to have the highest total carotenoid (81.81± 32.60 µg/g DW) substantially higher than all other species tested (Table **1**). In contrast the lowest total carotenoid concentration was found in *Butryococcus sudeticus* (53.96±29.44 µg/g DW). Carotenoid analysis performed by HPLC system detected at least three major carotenoid peaks: lutein, β-cryptoxanthin and β-carotene. As shown in Table **1**, Figure **1** and Figure **2**, lutein and β -carotene were highest in *C. fusca* (63.39 ±5.99 µg/g DW) and *C.*

Table 1: Total and Individual Carotenoid Content (µg/g DW) and Composition of 6 Chlorophyta Freshwater Green Algae Species

Species	Total Carotenoid (µg/g DW)	Lutein (µg/g DW)	β-cryptoxanthin (µg/g DW)	β-Carotene (µg/g DW)
Chlorella vulgaris	81.81± 32.60	63.39 ±5.99	-	18.42 ± 5.31
Chlorella fusca	79.55 ±37.58	69.53 ±9.56	-	10.01 ± 1.27
Selenastrum capricornutum	76.22 ±32.97	62.67 ±18.05	-	13.54 ± 5.36
Chlorococcum sp.	62.66 ±36.18	62.66 ±5.83	-	-
Pandorina morum	56.82 ±28.29	51.61 ±5.68	2.38 ± 0.32	2.82 ±1.43
Botryococcus sudeticus	53.96 ±29.44	51.96 ±3.90	1.99 ±0.21	-

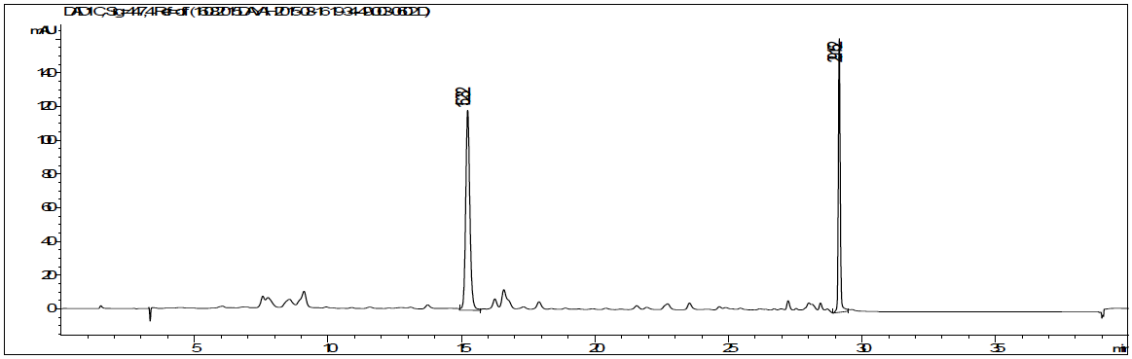

Figure 1: HPLC chromatorgram of lutein and β-carotene in *Chlorella fusca*.

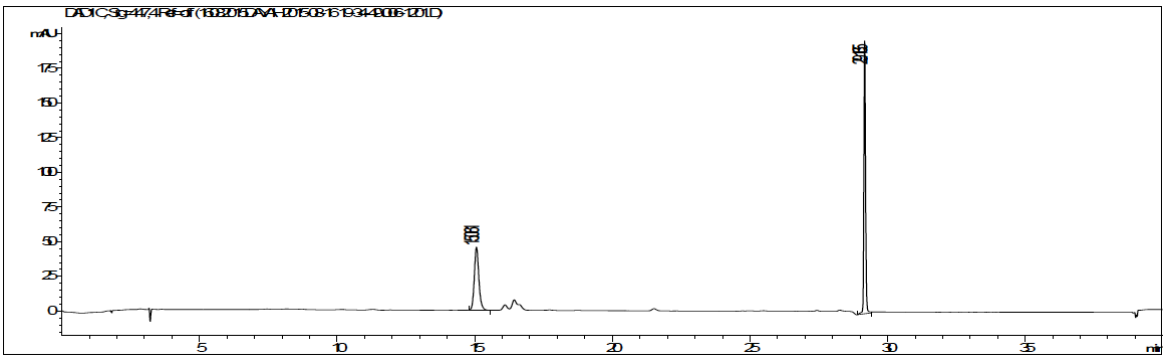

Figure 2: HPLC chromatorgram of lutein and β-carotene in *Chlorella vulgaris*.

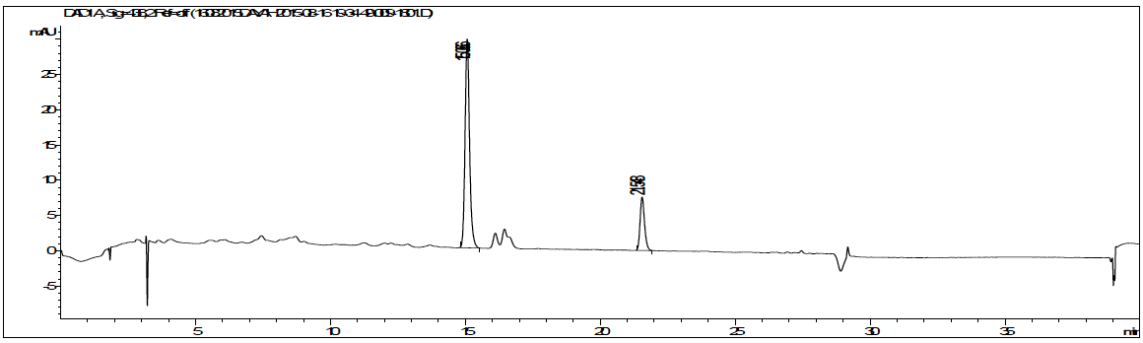

Figure 3: HPLC chromatorgram of lutein and β- cryptoxanthin in *Butryococcus sudeticus*.

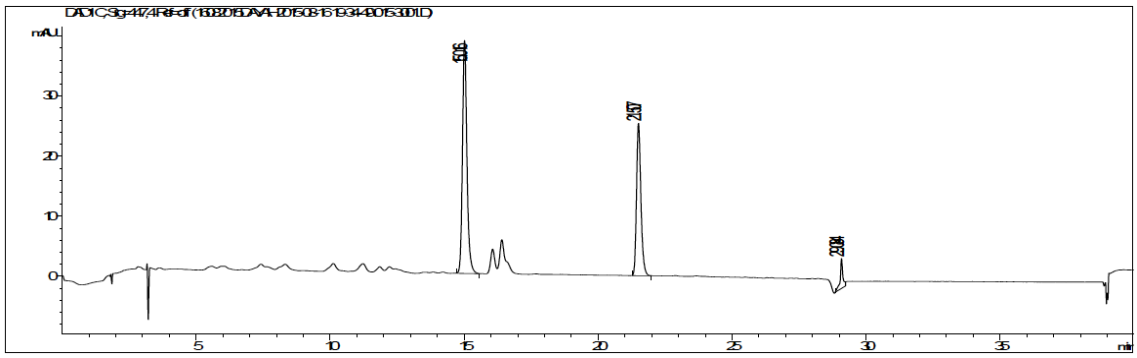

Figure 4: HPLC chromatorgram of lutein, β-cryptoxanthin and β-carotene in *Pandorina morum.*

vulgaris (18.42 ± 5.31 µg/g DW) respectively. Traces of β-cryptoxanthin was found in *B. sudeticus* and *P. morum* ranged from 1.99 ±0.21 to 2.38 ± 0.32 µg/g DW as detailed in Table **1**, Figures **3** and **4**.

All six species could be grouped into 3 classes depending on the accumulation of specific carotenoid pigments (Table **1**). *P. morum* was found to have all three individual carotenoid pigments with a relatively high concentration of lutein. *C. vulgaris*, *C. fusca*, *S. capricornutum* and *B. sudeticus* were detected to have two of the three carotenoid pigments whereas *Chlorococcum* sp.was found with only lutein. This result demonstrates that carotenoid composition and content vary with microalgae species. The identification of such a genetic basis to significant levels of carotenoid levels within this six species or germplasm has provided the impetus to oiptimize carotenoid levels using both breeding and transgenic startegies either as active pharmaceutical ingredients or natural colorants. In terms of health benefits, lutein is beneficial to delay the onset of the cataract hence reducing the risk of cataract occurrence [16]. According to WHO, they estimated that 285 million of people in this world suffered from visually-impaired problems, hence this finding may overcome these problems [16]. Studies reported that the consumption of β -carotene may increase the bone mineral density [17] besides providing cardioprotective effect to reduce the risk of heart disease [18]. In other hands, the anti-proliferative activity of β -cryptoxanthin was discovered via the mitochondrial pathway of apoptosis against the Adenocarcinoma Caco-2 cell [19]. The findings of the natural carotenoids from this study are crucial to Muslim consumer as they seek lawful things to be consumed.

CONCLUSION

Carotenoid can be extracted from the microalgae and from this study *Chlorella vulgaris* recorded the highest carotenoid content. Moreover, lutein can be obtained from all six species of microalgae and furthermore *Pandorina morum* contain all 3 types of carotenoid observed, which are lutein, β-carotene and also β-cryptoxanthin. The carotenoid that extracted from the culture of microalgae not only halal but give diverse health benefits to human. Hence, in a nutshell microalgae have the potential as one of the sources of the halal food colorant.

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