

Chemical Profiles of Methanolic Extracts from Two Species of Microalgae, *Nannochloropsis* sp. and *Spirulina* sp.

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ABSTRACT

Microalgae are potential sources of bioactive compounds that have health promoting effects due to the abundant presence of flavonoids and polyphenols. The goal of this study was to investigate the chemical profiles of microalgae extracts from *Nannochloropsis* sp. and *Spirulina* sp. Total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity (DPPH) were measured. *Nannochloropsis* sp. had a significantly higher TPC value than *Spirulina* sp. (58.43 ± 0.85 mg GAE/g DW vs. 19.64 ± 0.52 mg GAE/g DW, $p < 0.01$) and a significantly higher TFC value than *Spirulina* sp. (79.87 ± 0.12 mg QE/g DW vs. 11.19 ± 0.07 mg QE/g DW, $p < 0.01$). *Nannochloropsis* sp. also exhibited a greater percentage of DPPH inhibition compared to *Spirulina* sp. (EC_{50} 0.195 ± 0.007 mg/mL vs. 0.613 ± 0.003 mg/mL, $p < 0.01$). TPC and TFC were negatively correlated with the EC_{50} of DPPH antioxidant inhibition activity (-0.956 , $p = 0.003$, and -0.899 , $p = 0.015$, respectively). These negative correlations indicate that polyphenol compounds are the major contributors to the antioxidant activity observed in this study. Further analysis is needed to determine how to utilize the health benefits of both microalgae.

Keywords: Antioxidant, microalgae, *Nannochloropsis*, *Spirulina*, total flavonoid content (TFC), total phenolic content (TPC)

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INTRODUCTION

Microalgae are unicellular and multicellular microorganisms that belong to Kingdom Plantae. They are capable of undergoing photosynthesis due to the chlorophyll content in their cells and can be found in both terrestrial and aquatic ecosystems.

Microalgae can be divided into two types, fresh water microalgae and marine microalgae (Chu, Yuen, Wong, Teoh, & Phang, 2002). They were a food source for ancient civilizations in some parts of the world, such as Asia, Africa, and South America (Santoyo et al., 2012). In addition, marine microalgae have been used as medicine to treat illness for centuries (Pooja, 2014).

Marine microalgae contain many bioactive compounds, including lipids that are useful in producing antimicrobial drugs (Lazarus & Bhimba, 2008). Priyadarshani and Rath (2012), and Volk and Furkert (2006) reported that marine microalgae can be a promising source of chemical compounds used in pharmaceutical, aquaculture, agrochemical, and biofuel industries. Microalgae extracts have been developed into cosmetics, anticancer agents, enzymes, pigments, poly-unsaturated fatty acids, and dietary supplements (Demirel, Yilmaz-Koz, Karabay-Yavasoglu, Ozdemir, & Sukatar, 2009; Guedes et al., 2011).

Microalgae are a good source of antioxidant compounds, which have health benefits and are useful in prevention of cardiovascular disorders, age-related diseases, cancer, and Alzheimer's disease (Goiris et al., 2012; Li et al., 2007). Antioxidant compounds, such as carotenoids that are commonly found in microalgae, play a major role in dealing with reactive oxygen species (ROS) and oxidative stress, which can cause multifactorial diseases such as cancer, cardiovascular diseases, and

inflammatory disorders (Laguerre, Lecomte, & Villeneuve, 2007; Sachidanandam, Fagan, & Ergul, 2005).

Some classes of flavonoids, such as isoflavones, flavanones, flavonols, and dihydrochalcones, are also common in microalgae (Klejdus, Lojková, Plaza, Šnóblová, & Štěrbová, 2010). Flavonoid compounds are strong antioxidants that inhibit lipid oxidation by scavenging directly on hydroxyl groups, singlet oxygen, and lipid peroxy radicals. Flavonoids also act as metal chelators and inhibit the activity of lipoxygenase (Pietta, 2000). Inhibition of lipoxygenase activity is important to prevent inflammation-related diseases such as cancer (Wisastra & Dekker, 2014).

Spirulina is a blue green alga that has therapeutic effects such as anticancer properties and lowering blood cholesterol level (Kumar, Bhatnagar, & Srivastava, 2011). It has strong antioxidant activity due to the presence of phenolic compounds such as salicylic acid, chlorogenic acid, and caffeic acid (Pratt, 1992). Salicylic acid found in *Spirulina* was reported to have medicinal benefits that involve disrupting eicosanoic acid metabolism and altering levels of prostaglandins and leukotrienes (Mitchell, Akarasereenont, Thiemermann, Flower, & Vane, 1993). McCarty and Block (2006) showed that salicylic acid regulates molecular signaling through nuclear factor- κ B (NF- κ B), a transcription factor that plays a central role in immunity. Chlorogenic acid is a natural chemical compound formed from esterification of

caffeic acid and quinic acid. Chlorogenic acid has a significant effect on absorption and utilization of glucose from coffee, which may help reduce body mass and body weight in a healthy person (Thom, 2007). Caffeic acid, which is present in many fruits and plants, is well known to have strong antioxidant, anti-inflammatory, and immunomodulatory functions (de Oliveira et al., 2013; Murad, Soares, Brand, Monteiro, & Teodoro, 2015; Wang, Chu, Liang, Lin, & Chiang, 2010). Caffeic acid has been used to treat diseases such as cancers (Lewandowska, Kalinowska, Lewandowski, Stępkowski, & Brzóska, 2016) and upper respiratory tract infections in children (Yuksel & Akyol, 2016).

The green microalga *Nannochloropsis* produces high concentrations of important antioxidants such as zeaxanthin and canthaxanthin. Zeaxanthin and canthaxanthin are valuable nutraceuticals with medicinal applications in food and pharmaceutical industries. Asker, Tarek, Beppu and Ueda (2012) reported that consumption of zeaxanthin helps lower the risk of age-related macular degeneration and prevent glaucoma and cataracts. Carpenter et al. (1997) showed that both zeaxanthin and canthaxanthin are useful in slowing the atherosclerosis process by inhibiting low-density lipoprotein oxidation. Chew, Park, Wong and Wong (1998) showed that canthaxanthin directly inhibits the growth of mammary tumors in mice.

The goal of this study was to determine the chemical properties of methanolic extracts from *Spirulina* sp. and *Nannochloropsis* sp.

The presence of high phenolic and flavonoid contents that contribute to high antioxidant activity may have beneficial health effects that can be further studied.

MATERIALS AND METHODS

Microalgae Samples

Spirulina sp. and *Nannochloropsis* sp. were obtained from the Fisheries Research Institute in Pulau Sayak, Kedah. The microalgae (1000 mL) were cultured in three replicate flasks using sea water (10 ppt) containing Walne medium (Table 1). Media for stock cultures were replaced every 2 weeks, and the cells were maintained at $25 \pm 1^\circ\text{C}$. The cells were cultured under continuous exposure to white fluorescent lamps ($50.05 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) with aeration of normal air. The cells were harvested at the exponential phase of growth (i.e. 1.5×10^6 cells/mL) for the extraction.

Table 1
Ingredients used to prepare 1 L of Walne medium

Chemical	Weight
Potassium nitrate (KNO ₃)	116 mg
Sodium nitrate (NaNO ₃)	100 mg
Disodium salt (EDTA)	45 mg
Boric acid (H ₃ BO ₃)	33.6 mg
Disodium phosphate (NaH ₂ PO ₄)	20 mg
Ferric chloride (FeCl ₃)	1.3 mg
Manganese chloride (MnCl ₂)	0.36 mg
Zinc chloride (ZnCl ₂)	21 mg
Cobalt chloride (COCl ₂)	20 mg
Ammonium molybdate (NH ₄) ₆ Mo ₇ O ₂₄	9 mg
Copper sulphate (CuSO ₄)	20 mg
Thiamine.HCl (B ₁)	10 μg
Cyanocobalamin (B ₁₂)	10 μg
Biotin (H)	0.2 μg

Microalgae Extracts

Harvested *Spirulina* sp. and *Nannochloropsis* sp. cells were extracted following the method of Pereira et al. (2015) with some modifications. Briefly, the cultured cells were freeze dried for 72 h. The dried microalgae were subjected to sonication to disrupt the cell wall and then extracted using 100% methanol at the ratio of one part algae to 40 parts methanol (w/v). The extraction was performed overnight at room temperature (20°C) under continuous stirring. The extracted biomass was centrifuged (10,000 × g, 10 min) and the supernatant removed. The supernatant was filtered and dried using a rotary evaporator at 45°C under vacuum. Dried extracts were resuspended in methanol to a final concentration of 20 mg/mL and stored at -20°C for further use.

Determination of Total Phenolic Content (TPC)

The TPC of extracted microalgae was analyzed according to Chua, Rahaman, Adnan, Tan and Tjih (2013) with some modifications. The extracts were prepared in methanol at three different concentrations (0.1, 0.2, and 0.3 mg/mL). A 10 µL aliquot of each concentration was mixed with 50 µL of Folin-Ciocalteu reagent and incubated for 5 min at room temperature. Next, 40 µL of Na₂CO₃ were added and the solution was incubated for 30 min. After the second incubation, the absorbance of the solution was read at 760 nm wavelength using a spectrophotometer. Gallic acid in a serial dilution from 10 to 0.3125 µg/mL was used as a standard chemical for calibration curve

preparation. The TPC was expressed as gallic acid equivalent (GAE) per gram dry weight (DW) of extract (mg GAE/g DW).

Determination of Total Flavonoid Content (TFC)

The TFC of extracted microalgae was determined based on the method of Isla et al. (2011) with slight modification. Three extracts were prepared in methanol at three different concentrations (0.5, 1.0, and 1.5 mg/mL). A 50 µL aliquot of each concentration was mixed with 50 µL of 2% AlCl₃ and incubated for 10 minutes at room temperature. A flavonoid-aluminium complex was formed and measured using a spectrophotometer at 415 nm. Quercetin was used as the standard compound with a serial dilution ranging from 50 to 1.5625 µg/mL for the calibration curve. The TFC was expressed as quercetin equivalent (QE) per gram dry weight (DW) of extract (mg QE/g DW).

2,2-diphenyl-1-picrylhydrazyl (DPPH) - High performance Liquid Chromatography (HPLC) Antioxidant Assay

DPPH-HPLC analysis was used to measure the free radical scavenging activity of extracted microalgae following Nurdianah, Ahmad Firdaus, Eshaifol Azam and Wan Adnan (2016). Briefly, the radical scavenging activity of DPPH was determined by adding 500 µL of microalgae samples to 500 µL of DPPH (2.5 mM/mL) with the final volume of 1 mL. Next, 10 µL from all extracted samples was injected into the HPLC machine (Varian, Germany). Each extracted sample

was run in triplicate. Trolox was used as the standard, with the standard calibration curve ranging from 0.15625 - 5 µg/mL. Methanol and DPPH were used as a negative control. Analysis of separation was carried out using an Eclipse XDB-C18 4.6 mm × 250 mm, 5µ C18 column (250 mm × 4 mm, 5 µM) (Agilent, Germany). Isocratic elution was carried out with an 80:20 ratio of methanol to water at a flow rate of 1.0 mL/min. The DPPH peaks were measured at 517 nm wavelength. Data analysis was conducted using Galaxie Workstation software. The reduction of DPPH peak area (PA) was used to determine the percentage of radical scavenging activity.

% Radical scavenging activity

$$= (PA_{\text{control}} - PA_{\text{sample}}) / (PA_{\text{control}}) \times 100$$

Statistical Analysis

All results are presented as mean ± standard deviation. Significant difference of the experimental results between the two strains was evaluated using Student's t-test, and $p < 0.05$ was considered to be statistically significant. Spearman correlation analysis was used to evaluate the relationship between TPC or TFC and antioxidant activity. All analyses were conducted using SPSS software version 22.

RESULTS AND DISCUSSION

Microalgae are known to produce bioactive compounds that contain high levels of antioxidants (Pumas & Pumas, 2014).

Examples of these bioactive compounds are β-carotene, xanthophylls, polyphenols, and essential fatty acids (Talero et al., 2015). High levels of antioxidant compounds can prevent or slow down the unfavorable effects of free radicals (Halliwell & Gutteridge, 1999). Antioxidant activity in both microalgae extracts were measured using the DPPH assay. The DPPH radical ions are free radicals, which are reduced in the presence of antioxidants, changing the color of the solution. This change was measured at 517 nm wavelength, and the data is presented as EC₅₀, which is the efficient concentration required to decrease the initial DPPH concentration by 50% (Mishra et al., 2012). In this study, the EC₅₀ value of *Nannochloropsis* sp. was 0.195 ± 0.007 mg/mL, which was significantly lower than that of *Spirulina* sp. (0.613 ± 0.003 mg/mL) ($p < 0.01$) (Table 2).

The antioxidant activity of methanolic extracts of *Nannochloropsis* sp. in our study was much higher than that reported by Safafar, Van Wagenen, Møller and Jacobsen (2015), and Mekdade et al. (2016). This difference was likely due to different methodologies, as we used DPPH- HPLC whereas the other studies used the DPPH assay and a spectrophotometer. Methanolic extracts were better for the DPPH antioxidant assay compared to hexane and water extracts. Kothari and Seshadri (2010) reported that polar extracts were better free radical scavengers than less polar extracts. Custódio et al. (2015) reported higher EC₅₀ values for *Nannochloropsis oculata* hexane (4.93 ± 0.37 mg/mL) and water ($7.31 \pm$

0.71 mg/mL) extracts compared to our methanolic extract. Shalaby and Shanab (2013) analyzed phenolic compounds using HPLC and found that the methanolic extract contained a higher level of phenolic compounds than the water extract, which contributes to its high antioxidant activity.

The antioxidant activity of the methanolic extract of *Spirulina* sp. in our study was slightly lower than that reported by Shalaby and Shanab (2013). They reported that at 0.2 mg/mL, the methanolic extract of *Spirulina platensis* inhibited 89.61% of DPPH, whereas we found that at 0.613 ± 0.003 mg/mL, *Spirulina* sp. showed only 50% inhibition of DPPH. The methanolic extract in our study had lower antioxidant activity than the ethanolic extract of *S. platensis* (EC_{50} 0.1011 mg/mL) studied by Sutanto and Suzery (2016).

Findings from our study and previous studies for *Nannochloropsis* sp. and

Spirulina sp. extracts are summarized in Tables 2 to 4. The use of more polar solvents such as methanol and ethanol are important to extract antioxidant compounds from *Nannochloropsis* sp. and *Spirulina* sp., as they are generally good at extracting phenolic content, which may have a greater effect than other compounds on DPPH activity. Furthermore, Dai and Mumper (2010) reported that methanol was more effective at extracting higher amounts of polyphenols, compared to other solvents.

The TPC data are presented in units of mg GAE/g DW. Gallic acid is a trihydroxybenzoic acid that belongs to the phenolic acid family, and is used as a standard reference in the measurement of phenolic compounds. The TPC of *Nannochloropsis* sp. (58.43 ± 0.85 mg GAE/g DW) was significantly higher than that of *Spirulina* sp. (19.64 ± 0.52 mg GAE/g DW) ($p < 0.01$) (Table 3). Rai and Rajashekar (2015)

Table 2
Antioxidant activity (DPPH inhibition) of *Spirulina* sp. and *Nannochloropsis* sp.

Study	Safafar et al. (2015)	Mekdade et al. (2016)	Shalaby & Shanab (2013)	Sutanto & Suzery (2016)	Custódio et al. (2015)	Custódio et al. (2015)	Our Study
<i>Nannochloropsis</i> sp.	0.25 mg/mL (12.55% inhibition)	3.28 mg/mL (50% inhibition)	–	–	4.93 ± 0.37 mg/mL (50% inhibition)	7.31 ± 0.71 mg/mL (50% inhibition)	0.19475 ± 0.007 mg/mL (50% inhibition)
<i>Spirulina</i> sp.	–	–	0.2 mg/mL (89.61% inhibition)	0.1011 mg/mL (50% inhibition)	–	–	0.6125 ± 0.003 mg/mL (50% inhibition)
Solvent Used	Methanol	Methanol	Methanol	Ethanol	Hexane	Water	Methanol
Cultivation Medium	Industrial waste water	–	–	–	–	–	Walne Medium

Table 3

Comparison of TPC from *Spirulina* sp. and *Nannochloropsis* sp. between our study and others (comparison based on similar standard used in the experiment)

Study	Safafar et al. (2015)	Abd El-Baky et al. (2009)	Agustini et al. (2015)	Custódio et al. (2015)	Rai & Rajashekhar (2015)	This Study
<i>Nannochloropsis</i> sp.	6.45 ± 0.25 mg GAE/g DW	-	-	0.261 ± 0.06 mg GAE/g DW	17.01 ± 0.09 mg GAE/g DW	58.43 ± 0.85 mg GAE/g DW
<i>Spirulina</i> sp.	-	4.51 ± 0.23 mg GAE/g DW	2.117 ± 0.99 mg GAE/g DW	-	7.15 ± 0.07 mg GAE/g DW	19.64 ± 0.52 mg GAE/g DW
Solvent Used	Methanol	Ethanol	Ethanol	Diethyl ether	Methanol	Methanol
Cultivation Medium	Industrial waste water	Zarrouks Medium	-	-	F/2 and Walne Medium	Walne Medium

Table 4

Comparison of TFC from *Spirulina* sp. and *Nannochloropsis* sp. between our study and others (comparison based on similar standard used in the experiment)

Study	Safafar et al. (2015)	Abd El-Baky et al. (2009)	Agustini et al. (2015)	Rai & Rajashekhar (2015)	This Study
<i>Nannochloropsis</i> sp.	3.18 ± 0.59 mg QE/g DW	-	-	9.87 ± 0.25 mg QE/g DW	79.87 ± 0.12 mg QE/g DW
<i>Spirulina</i> sp.	-	1.32 ± 0.03 mg QE/g DW	25.6615 ± 1.62 mg QE/g DW	2.21 ± 0.17 mg QE/g DW	11.19 ± 0.07 mg QE/g DW
Solvent Used	Methanol	Ethanol	Ethanol	Methanol	Methanol
Cultivation Medium	Industrial waste water	Zarrouks Medium	-	F/2 and Walne Medium	Walne Medium

reported a similar pattern for the methanolic extract of *Nannochloropsis* sp. (17.01 ± 0.09 mg GAE/g DW) versus *Spirulina* sp. (7.15 ± 0.07 mg GAE/g DW). However, the TPC value reported in our study was much higher than that found by Rai and Rajashekhar (2015). The differences in TPC values might be due to different media used to culture the microalgae (Walne medium versus F/2 medium, respectively). Safafar et al.

(2015) and El-Baky, El Baz and El-Baroty (2009) also reported that the TPC level of *Nannochloropsis* sp. (6.45 ± 0.25 mg GAE/g DW) was higher than that of *Spirulina* sp. (4.51 ± 0.23 mg GAE/g DW). Natrah, Yusoff, Shariff, Abas and Mariana (2007) reported that higher pigment content in microalgae had been associated with higher production of phenolic compounds. Thus, our results suggest that *Nannochloropsis*

sp. may have higher pigment content than *Spirulina* sp. However, this observation needs to be confirmed by future studies.

However, Agustini, Suzery, Sutrisnanto and Ma'ruf (2015), and Custódio et al. (2015) found that *Nannochloropsis* sp. had a lower TPC value (0.261 ± 0.06 mg GAE/g DW) than *Spirulina* sp. (2.117 ± 0.99 mg GAE/g DW). This difference could be due to the different solvents used to extract the sample, as they used ethanol and diethyl ether, respectively, rather than methanol. Goiris et al. (2012) reported that polarity of the solvent affected the total amount of phenolic content extracted. A polar solvent with high specificity was preferred because it gave a higher yield of phenolic compounds. Thus, in our study, methanol gave a higher yield of TPC because it is a solvent with relatively higher polarity.

Flavonoids are the most abundant polyphenols found in antioxidant compounds (Baxter et al., 1998). Flavonoids found in microalgae are believed to be one of the highest natural phenolics found in natural products (Kumaran & Karunakaran, 2007). The TFC measured in *Nannochloropsis* sp. and *Spirulina* sp. was presented as mg QE/g DW, as quercetin was used as the standard compound in the measurement of TFC. In our study, *Nannochloropsis* sp. contained seven times more TFC than *Spirulina* sp. (79.87 ± 0.12 mg QE/g DW vs. 11.19 ± 0.07 mg QE/g DW, $p < 0.01$) (Table 4). Rai and Rajashekhar (2015) reported a similar trend for methanolic extracts, as the TFC of *Nannochloropsis* sp. was four times greater than that of *Spirulina* sp. (9.87 ± 0.25 mg

QE/g DW vs. 2.21 ± 0.17 mg QE/g DW). The differences between the studies might be due to differences in the culture medium and the drying method used. The TFC values of *Nannochloropsis* sp. and *Spirulina* sp. measured in our study was much higher than those reported by Safafar et al. (2015) (3.18 ± 0.59 mg QE/g DW for *Nannochloropsis* sp.) and El-Baky et al. (2009) (1.32 ± 0.03 mg QE/g DW for *Spirulina maxima*). However, Agustini et al. (2015) reported a TFC value of 25.6615 ± 1.62 mg QE/g DW for the ethanolic extract of *Spirulina* sp. The huge differences in values among studies were probably due to different methods of cultivation and handling of materials. Furthermore, nutrients used in the cultivation of microalgae play an important role in producing flavonoids in both species of microalgae.

The study found a negative correlation between TPC vs. EC_{50} of DPPH antioxidant inhibition activity (-0.956 , $p = 0.003$) and TFC vs. EC_{50} (-0.899 , $p = 0.015$). Similarly, Farasat et al. (2014) reported that TPC and TFC in green seaweeds were negatively correlated with 50% inhibition of DPPH. These significant correlations indicate that polyphenol compounds were the major contributors to antioxidant activity in our methanolic microalgae extracts.

CONCLUSION

Methanolic extracts of *Nannochloropsis* sp. and *Spirulina* sp. possessed different antioxidant activities. The methanol extract from *Nannochloropsis* sp. had higher TPC and TFC compared to *Spirulina* sp. For

antioxidant activity, DPPH inhibition of *Nannochloropsis* sp. was greater than that of *Spirulina* sp. The health promoting effects of both microalgae, especially *Nannochloropsis* sp., should be further studied by evaluating the chemical compositions and biological activities of these species.

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