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Characterization on Phenolic Acids and Antioxidant Activity of *Chlorella* sp. Microalgae using Subcritical Water Extraction

(Pencirian ke atas Asid Fenol dan Aktiviti Antioksidan Mikroalga *Chlorella* sp. menggunakan Pengekstrakan Air Subkritikal)

SITI MAISURAH ZAKARIA, SITI MAZLINA MUSTAPA KAMAL*, RAZIF HARUN, ROZITA OMAR & SHAMSUL IZHAR SIAJAM

ABSTRACT

Chlorella *sp. microalgae contain phenolic compounds with good functional properties. In this study, two extraction methods, soxhlet and subcritical water extraction (SWE) were applied and compared in terms of phenolic compounds recovery from* Chlorella *sp. microalgae and characterization of the phenolic acid components. Phenolic acid analysis demonstrated that the main components of the* Chlorella *sp. extracts were ferulic, caffeic and p-coumaric acids. The comparative study indicated that SWE gave higher extraction yield compared to conventional soxhlet method. High recoveries of phenolic acids were obtained at 175 °C with 3.20, 3.05 and 3.33 mg/100 g of ferulic, p-coumaric and caffeic acid, respectively, compared to soxhlet extraction using methanol with 2.10, 2.29 and 2.37 mg/100 g of ferulic, p-coumaric and caffeic acid, respectively. This proved that subcritical water treatment could effectively be used for the release of phenolic acids from* Chlorella *sp. using safe and green solvent. Analysis by Fourier transform infrared spectroscopy (FTIR) was also performed to observe the effect of subcritical water on the functional groups of the extracts. The current study demonstrated that SWE provided a better way of utilising* Chlorella *sp. as a source of phenolic acids and natural antioxidants.*

Keywords: Extraction; microalgae; phenolic; subcritical; water

ABSTRAK

Mikroalga Chlorella *sp. mengandungi sebatian fenol dengan ciri pelbagai fungsi yang bagus. Dalam kajian ini, dua kaedah pengekstrakan, soxhlet dan pengekstrakan air subkritikal (SWE) telah diaplikasikan dan dibandingkan daripada segi hasil sebatian fenol daripada mikroalga* Chlorella *sp. serta pencirian ke atas komponen asid fenol. Analisis asid fenolik menunjukkan bahawa komponen utama bagi ekstrak* Chlorella *sp. adalah asid ferulik, kafeik dan ρ-kumarik. Kajian perbandingan menunjukkan SWE memberi hasil ekstrak yang lebih tinggi berbanding kaedah konvensional soxhlet. Hasil asid fenolik yang tinggi dicapai pada 175 °C dengan 3.20, 3.05 dan 3.33 mg/100 g masing-masing bagi asid ferulik, ρ-kumarik dan kafeik berbanding pengekstrakan soxhlet menggunakan metanol dengan 2.10, 2.29 dan 2.37 mg/100 g masing-masing bagi asid ferulik, ρ-kumarik dan kafeik. Ini membuktikan bahawa rawatan air subkritikal boleh digunakan secara efektif bagi pengeluaran asid fenol daripada* Chlorella *sp. menggunakan pelarut yang selamat dan hijau. Analisis dengan spektroskopi transformasi Fourier inframerah (FTIR) juga dijalankan untuk melihat kesan air subkritikal ke atas kumpulan berfungsi bagi ekstrak. Kajian menunjukkan bahawa SWE adalah kaedah yang lebih baik bagi penggunaan* Chlorella *sp. sebagai sumber asid fenol dan antioksidan semula jadi. studied.*

Kata kunci: Air; fenol; mikroalga; pengekstrakan; subkritikal

INTRODUCTION

The production of microalgae has been significantly increased due to the demand for its beneficial compounds and nutritive contents. Microalgae have been used in various applications such as food, food additives, aquaculture, colourants, cosmetics, pharmaceuticals and nutraceuticals (Khan et al. 2018; Liang et al. 2004). Many of the unique characteristics that microalgae possess (micronutrient accumulation, carotenoids, phenolic, amino acids) are applicable in a wide range of compounds that are important to human health. Natural antioxidants, originated from microalgae, are significant bioactive compounds that show an important function against several diseases and as defense mechanisms against oxidative injury. It is a valuable source of neuroprotective agents. As microalgae are photosynthetic organisms, they produce free radicals and other oxidative reagents when they are exposed to high oxygen concentrations and light. Due to the absence of structural damage, it is assumed that these organisms are able to produce the essential

compounds to protect themselves from oxidation. Hence, microalgae provide important source for potent antioxidant compounds to protect our bodies from the damaging effect of reactive oxygen species produced as a result of normal metabolism of the body. Microalgae antioxidants consist of many compounds such as carotenoids and vitamin E (α-tocopherol) which are fat-soluble fraction, whereas vitamins, phycobiliproteins and polyphenols are the powerful water-soluble antioxidants (Ina & Kamei 2006).

Chlorella sp. reportedly has the highest total phenolic content among microalgae, which is a major contributor to its antioxidant capacity (Hajimahmoodi et al. 2010). Phenolic compounds have been reported to be responsible for the beneficial health effects of plants and microalgae. These compounds have also been shown to contribute significantly to the antioxidant capacity of the human diet compared with vitamins. Phenolic acids are among the effective antioxidant agents that can be found naturally from plant and microalgae (Liu et al. 2013). A few epidemiological studies associated with free-radical scavenging analysis have shown an inverse relationship between the incidence of certain diseases and malignancies with the intake of phenolic compounds (Rice-Evans et al. 1997). A few studies have reported the effects of phenolic compound intake and their function in preventing or controlling various diseases including cancer, diabetes, heart disease and asthma (Knekt et al. 2002).

Furthermore, there have been several reports on extracting phenolic compounds from *Chlorella* sp. and evaluating their antioxidant activities. Wan Mahmood et al. (2019) recovered polyphenols from *Chlorella* sp. which was subjected to solvent extraction composed of choline chloride and polyols including glycerol, ethylene glycol, 1,3-propanediol and 1,4-butanediol using two conventional solvents, namely ethyl acetate and water. Solvent extracts were analysed for their polyphenolic profiles with major phenolic compounds being gallic acid, caffeic acid, p-coumaric acid and ferulic acid. Hajimahmoodi et al. (2010) evaluated the antioxidant activities and total phenolic contents of 12 soil-isolated strains of microalgae including *Chlorella* sp. Extractions were conducted using several solvents including hexane, ethyl acetate, and water. The results indicated that algal cells contained large amounts of antioxidants. The total phenolic contents varied among different microalgae strains, with *Chlorella* sp. showing values ranging from 0 to 19.15 mg/g. Safafar et al. (2015) investigated the antioxidative properties of some microalgal species from various classes including *Chlorella* sp., grown autotrophically on industrial wastewater. The authors used sonication and four common extraction solvents (methanol, acetone, ethyl acetate, and ethanol) to extract carotenoids and phenolics from *Chlorella* sp., where methanol was shown to produce the highest concentrations of both carotenoids and phenolics. Phenolic acids were also characterised where caffeic, ferulic, p-coumaric and cinnamic acids were found in *Chlorella* sp. extracts.

Various extraction methods have been developed for extracting bioactive compounds such as microwave, ultrasonic and solvent extraction techniques (Dey & Rathod 2013; Hasmida et al. 2014; Kashif et al. 2017). However, these techniques often suffer from low extraction yields, lengthy processing steps, use of toxic organic solvents and may leave traces of toxic solvents in the solute. This is a concern for food and pharmaceutical industries. Since subcritical water extraction (SWE) employs less purity of chemical solvent, and as a promising green process, it is a worthy choice for extracting phenolic compounds from *Chlorella* sp. microalgae. In recent times, SWE has turned out to be increasingly popular for the extraction of bioactive compounds from natural sources (Herrero et al. 2006). SWE is a new technology that uses pressurised hot water to perform a safe, environmentally friendly and rapid extraction (He et al. 2012). A high pressure is used to keep the water liquid, while high temperatures are applied to modify the dielectric constant of the water, which allows polarity tuning and contributes to the selectivity of the extraction process. Therefore, SWE is a good choice for extracting functional compounds from microalgae and is compatible with food and health regulations (Rodríguez-Meizoso et al. 2010; Zakaria & Kamal 2015). This technique has several main advantages compared to other conventional extraction techniques including faster processing, greater yields and reduced solvent usage (Huie 2002).

In the present investigation, phenolic compound extraction from *Chlorella* sp. was performed using subcritical water treatment at temperatures between 100 and 250 °C. HPLC analysis of the extracts was carried out to confirm phenolic compounds release after being subjected to subcritical water. This study detailed the results from the experiments which were carried out to determine phenolic acid composition in subcritical water treated extracts. As a comparison, the analysis on the extracts from conventional soxhlet extraction method has also been carried out. The effects of extraction time and process temperature on extraction of each compound during SWE were analysed and discussed.

Materials and Methods

Chemicals and Materials

Chlorella sp. blue-green microalgae *(Chlorella vulgaris)* was obtained from PureBulk, USA. Acetic acid, caffeic acid, ferulic acid, *p*-coumaric acid and gallic acid were purchased from Sigma Aldrich, Malaysia. Methanol and ethanol (95%) were obtained from R & M Chemicals, Malaysia and acetonitrile (HPLC grade) from BDH Chemicals, Malaysia. Analytical grade of chemicals were received and used without further purification.

Soxhlet Extraction

The extraction was accomplished using Soxhlet apparatus fitted with 500 mL round bottom flask containing 100

mL of extraction solvent. For this process, 5 g of sample were inserted into a filter paper extraction thimble and transfered into 500 mL reflux flask. The extraction was performed for 1 and 4 h and the extraction temperature were kept at the solvent boiling point. The extraction performed by three types of solvent namely distilled water, 95% ethanol and pure methanol. The contents were then centrifuged (Hettich, Germany) at 5000 rpm for 10 min and filtered through Whatman No. 1 filter paper into a conical flask. Samples were then collected for phenolic compound analysis.

SUBCRITICAL WATER EXTRACTION

Extractions were accomplished using a batch fluid bath system (Thomas Kogaku Co Ltd.) at extraction temperatures from 100 to 250 °C, with the pressure inside the reactor estimated from the steam table for subcritical water conditions (0.1014 - 3.9762 MPa). The heating bath was preheated for a few minutes prior to each extraction. The entire extractions were conducted in stainless-steel batch reactor cells (SUS316, 7.5 mm 150.4 mm) (Swagelok, Japan) containing *Chlorella* sp. sample. Figure 1 shows the diagram for (a) reactor and (b) batch fluid bath system used for the experiments.

Extraction was conducted by loading 1.0 g of the sample into the reactor cell, followed by distilled water (5 mL). Air was then removed by purging the reactor cell with argon gas and the reactor was immersed into the preheated bath to start the extraction. After reaching the set extraction time, the reactor cell was removed and rapidly cooled with cold water to stop the reaction. The reactor content was then centrifuged at 5000 rpm for 10 min and filtered into a conical flask using Whatman No.1 filter paper. Both supernatant and residue were collected for the analysis.

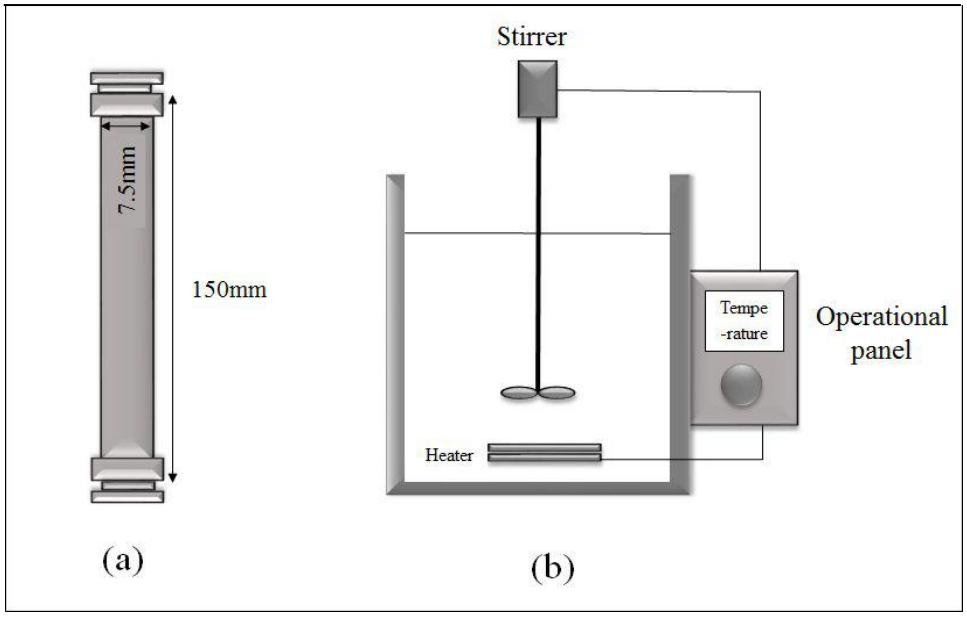

FIGURE 1. (a) Reactor and (b) batch fluid extraction system

ANALYSIS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The HPLC analysis was performed for the identification of phenolic acids from *Chlorella* sp. extracts. The extracts were analysed by Agilent G1310A pumps (Agilent Technologies, USA) that consists of chromatographic separation and diode array detector. LUNA C-18 column (5 µm, 250 mm × 4.6 mm) was used with 0.5 mL/min flow rate to conduct the HPLC analysis. The mixture of solvents which consisted of water/acetic acid (94:6, v/v; pH 2.3) and acetonitrile was used as the mobile phase. The solvent gradient was set at 0-15% B over 40 min, 15-45% B over 40 min, and 45-100% B over 10 min. Prior to HPLC injection, mobile phases and samples were filtered through a Millipore filter (0.22 µm). Each sample was analysed in duplicate. Determination of concentrations for phenolic acid in the samples were done by comparison of their retention times and UV-diode array detection at 280 and 320 nm to known spectral data of standard solutions. The values were means (n=2) given in mg/100 g microalgae (dry weight).

ANTIOXIDANT ANALYSIS

2,2-diphenyl-1-picrylhydrazyl HYDRATE (DPPH) ASSAY

A modified DPPH (2,2-diphenyl-1-picrylhydrazyl hydrate) free radical scavenging assay (Blois 1958) was performed to determine antioxidant activity. One millilitre of 0.1 mM solution of DPPH in methanol was added to 3 mL extracts. The samples were incubated in the dark for 30 min and the color changes in the mixture were observed. The absorbance of each sample was then measured using UV-Vis spectrophotometry at 517 nm. Lower absorbance of the extract mixture showed higher free radical scavenging activity. Percentage of antioxidant activity (%) for DPPH radicals was determined using the following equation:

$$\text{Antioxidant acitivity (\%)} = \frac{A_0 - A_1}{A_0} \times 100\%$$

where A_0 and A_1 are the absorbance of the control sample (mixture which contain all reagents except the extract) and the extracts, respectively. All samples were analysed in duplicate.

FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

FTIR (Perkin Elmer, Spectrum 100) is used for possible chemical functional group detection on *Chlorella* sp. extracts by attenuated total reflectance (ATR) method. The samples were placed onto the ATR sampling accessory crystal and forced applied using the ATR force arm to ensure good contact between the sample and the surface of the crystal. The samples were scanned to acquire data using a minimum of 4 scans with a scan range of 4000-650 cm^{-1}.

RESULTS AND DISCUSSION

PHENOLIC ACIDS FROM *Chlorella* sp. BY CONVENTIONAL SOXHLET EXTRACTION

HPLC analysis of the extracts was performed to identify the major phenolic compounds in the extracts. The most common phenolic acids contained in microalgae are ferulic, *p*-coumaric, protocatechuic, *p*-hydroxybenzoic, vanillic, syringic, caffeic and chlorogenic acid (Safafar et al. 2015). In this study on *Chlorella* sp. extracts, HPLC analysis detected the existence of three free phenolic compounds in the extracts with peaks identified as corresponding to p-coumaric, ferulic and caffeic acid. Other studies have demonstrated the recovery of ferulic acid from microalgae including *C. vulgaris*, *Haematococcus pluvialis*, *Diacronema lutheri*, *Phaeodactylum* sp., *Tetraselmis suecica*, *Porphyridium purpureum* (Goiris et al. 2014) and *Stypocaulon scoparium* (López et al. 2011). *p*-Coumaric acid has been previously extracted from some types of microalgae such as *Stypocaulon scoparium* (López et al. 2011), *Spongiochloris spongiosa* (Onofrejová et al. 2010), *Chlorella vulgaris*, *Haematococcus pluvialis*, *Diacronema lutheri*, *Phaeodactylum* sp., *Tetraselmis suecica* and *Porphyridium purpureum* (Goiris et al. 2014). Detection of caffeic acid has been reported from certain microalgae species including *Spirulina platensis* (Klejdus et al. 2009), *Stypocaulon scoparium* (López et al. 2011), *Spongiochloris spongiosa* and *S. platensis*, *Anabaena doliolum*, *Nostoc* sp. and *Cylindrospermum* sp. (Goiris et al. 2014).

Table 1 shows data on the composition of phenolic acids using soxhlet extraction with methanol, ethanol and water as the solvent. Previous studies found that methanol was the best extraction solvent for obtaining phenolic compounds from most algae and plant matrices. In 1 h of conventional soxhlet extraction, phenolic compounds (caffeic acid: 0.86 mg/100 g, ferulic acid: 0.67 mg/100 g and p-coumaric acid: 0.59 mg/100 g) from *Chlorella* sp. were extracted with pure methanol. The phenolic compounds were also obtained in low quantities by conventional soxhlet extraction with ethanol and water as shown in Table 1. However, ferulic and p-coumaric acids were not detected as part of the extraction with water as a solvent after 1 h of extraction.

TABLE 1. Phenolic acids composition on each extract by soxhlet extraction

Time	Solvent	Phenolic acids (mg/100 g)		
		ferulic	ρ-coumaric	caffeic
1	Methanol	0.67±0.026	0.59±0.026	0.86±0.024
	Ethanol	0.43±0.033	0.34±0.035	0.45±0.037
	Water	n.d.	n.d.	0.24±0.029
4	Methanol	2.10 ±0.031	2.29 ±0.066	2.37 ±0.036
	Ethanol	2.05 ±0.034	1.98 ±0.045	2.26 ±0.034
	Water	0.40 ±0.028	0.31 ±0.046	0.45 ±0.033

All data expressed as mean ± standard deviation n.d – not detected

The amount of all phenolic acids was significantly increased by extending the extraction time to 4 h. Extraction by methanol recovered 2.10 mg/100 g of ferulic acid, 2.29 mg/100 g of *p*-coumaric acid and 2.37 mg/100 g of caffeic acid. Soxhlet extraction using ethanol as the solvent resulted in lower values of extracted compounds with 2.05 mg/100 g of ferulic acid, 1.98 mg/100 g of *p*-coumaric acid and 2.26 mg/100 g of caffeic acid. All of the compounds were detected for water as the solvent and the amounts were higher than with the extraction over 1 h, with 0.40 mg/100 g of ferulic acid, 0.31 mg/100 g of *p*-coumaric acid and 0.45 mg/100 g of caffeic acid. A higher amount of compounds can be extracted if longer time was done for soxhlet extraction. A better and longer exposure of solute to the solvent resulted in a higher yield that also contributed to a higher transfer rate of solutes from *Chlorella* sp. into the solvents used.

The results demonstrated that methanol exhibited a greater ability to extract phenolic compounds over other solvents. The improvement may be attributed to phenolic compounds having a higher level of solubility in methanol than in ethanol and water. The results of this study agreed with those from other studies. Soxhlet extraction from *Xanthium strumarium* L. recovered higher phenolic compounds using methanol (80%) compared to ethanol (80%) for 5 h of extraction (Scherer & Godoy 2014). Extraction of phenolic compounds from *Quercus infectoria* showed that the amount of phenolic compounds obtained from 6 h of conventional solvent extraction using methanol (70%) was better compared to ethanol as an extraction solvent (Hasmida et al. 2014).

This study has shown that in its ambient condition during soxhlet extraction, water has a low capability to extract most of the phenolic acid composition from *Chlorella* sp. However, it still demonstrated that water can be used as the solvent for phenolic compounds extraction. Further study in the next section on the extraction by water in subcritical condition was conducted to explore more about the water properties especially its ability to extract phenolic compounds in its subcritical state. In addition, subcritical water extraction is a convenient and environmentally benign method that could improve the phenolic compounds extracted from this microalgae to be used in food and pharmaceutical industries.

PHENOLIC ACIDS FROM *Chlorella* sp. BY SWE TREATMENT

The phenolic compound composition of the *Chlorella* sp. was tested at several temperatures and extraction times during SWE, and the results are summarised in Figure 2. Experiments were carried out at temperatures of 100 to 250 °C and 5 to 20 min of extraction times.

Figure 3 shows the effect of extraction temperature on the yield of phenolic acids (ferulic, p-coumaric and caffeic) at 10 min of extraction time. According to Figure 3, the amount of phenolic acid (ferulic, p-coumaric and caffeic) recovered varied for each temperature set. Over 10 min of extraction, the amount of ferulic acid at 100 °C was 1.75 mg/100 g and it increased by 50% for a higher temperature of 125 °C to 2.68 mg/100 g. The amount recovered increased further to 3.20 mg/100 g at a 175 °C when the highest amount of ferulic acid was recovered. The amount began to decrease above 175 °C and dropped to 1.50 mg/100 g during the extraction at 250 °C. The effect on p-coumaric acid showed that the amount of the compounds extracted kept increasing from 100 to 175 °C, and dropped by 58% from 3.05 to 1.26 mg/100 g at 250 °C. Caffeic acid was the highest amount of phenolic acid recovered by SWE from *Chlorella* sp. and the highest yield was recovered on extraction at 175 °C (3.33 mg/100 g). The amount extracted then decreased by raising the extraction temperature until it reached 1.84 mg/100 g at 250 °C.

SWE utilises pressurised water of variable polarities under controlled conditions of temperature and pressure. The dielectric constant and polarity of water tend to be close to that of organic solvents such as alcohols (Joana Gil-Chávez et al. 2013). The amount of ferulic acid, p-coumaric acid and caffeic acid were significantly increased by raising the extraction temperature. Research by Safafar et al. (2015) showed that extraction by methanol had the highest concentrations of phenolics with caffeic, ferulic, p-coumaric and cinnamic acids found in *Chlorella* sp. extracts.

A study by Lopez et al. (2011) obtained caffeic acid, ferulic acid, p-coumaric acid, vanillic acid and gallic acid from *Stypocaulon scoparium* algae using ethanol as the extraction solvent. At a very high temperature of 250 °C during SWE, smaller values of ferulic acid, p-coumaric acid and caffeic acid were identified.

The amount of compounds extracted was increased by extending the extraction time from 5 to 20 min for extractions at 100 to 150 °C. At 175 °C, the amount of ferulic, p-coumaric and caffeic acids increased from 5 to 10 min of extraction time. Extending the extraction time to 15 and 20 min slightly decreased the yield of all phenolic acids. The amount of all compounds decreased when the time was extended to 20 min at higher extraction temperatures (200 °C and above). The extended exposure of active compounds to high temperatures led to this decline resulting in decomposition as long as structural damage throughout those longer extraction times. A longer extraction times at a high temperature of extraction raises the risk on reduction of phenolic compounds by way of increasing the loss of phenolics by decarboxylation (Cheng et al. 2014).

ANTIOXIDANT ACTIVITY

After the extracts were collected, the next procedure was determination on its functional characterisation in terms of antioxidant activity. DPPH assay was used to measure the antioxidant activity of the extracts. DPPH is a free radical method of antioxidant assay based on electron-transfer that offers an easy and fast approach to assess

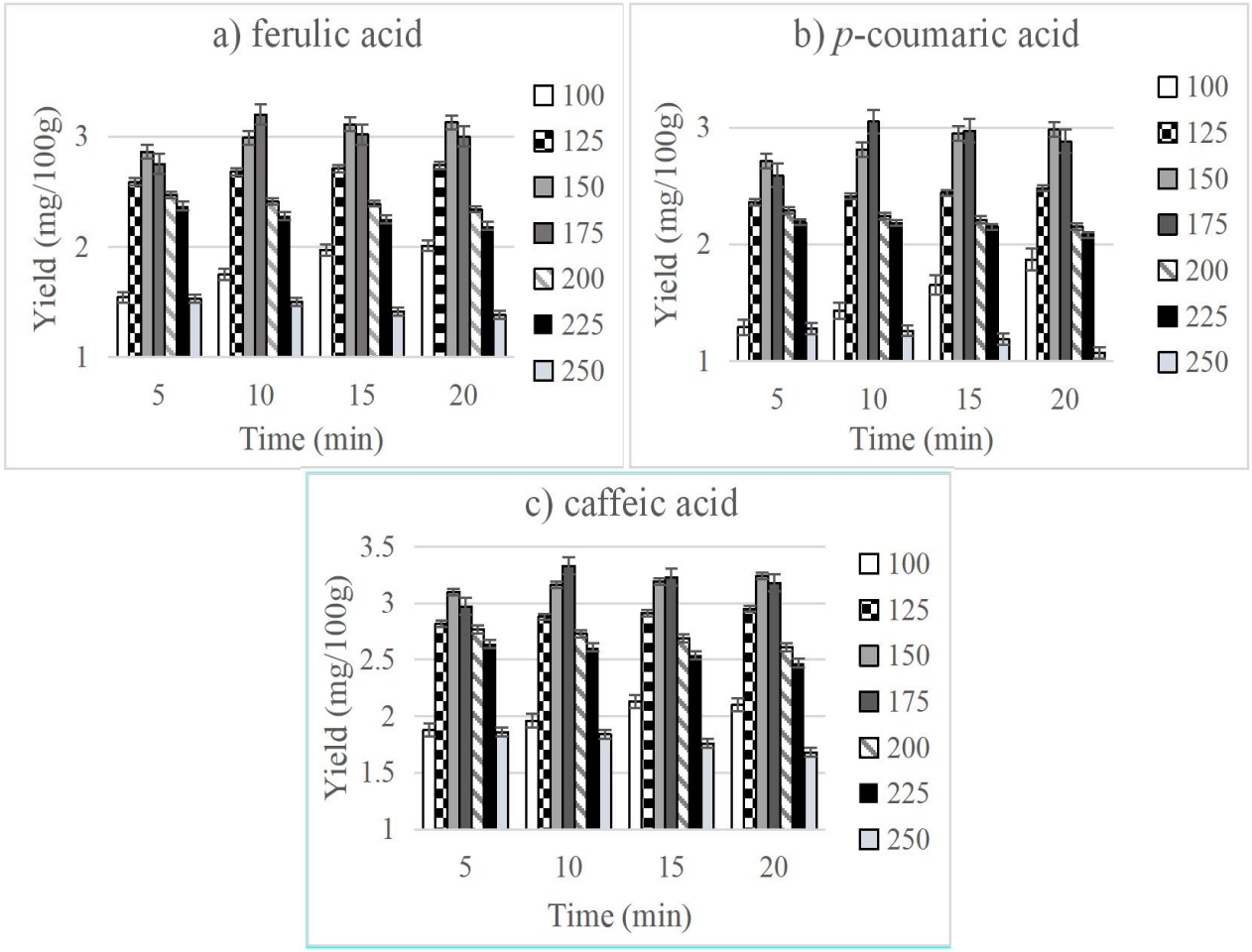

FIGURE 2. Composition of a) ferulic, b) p-coumaric and c) caffeic acids in *Chlorella* sp. extracts at various temperatures and reaction times during SWE system

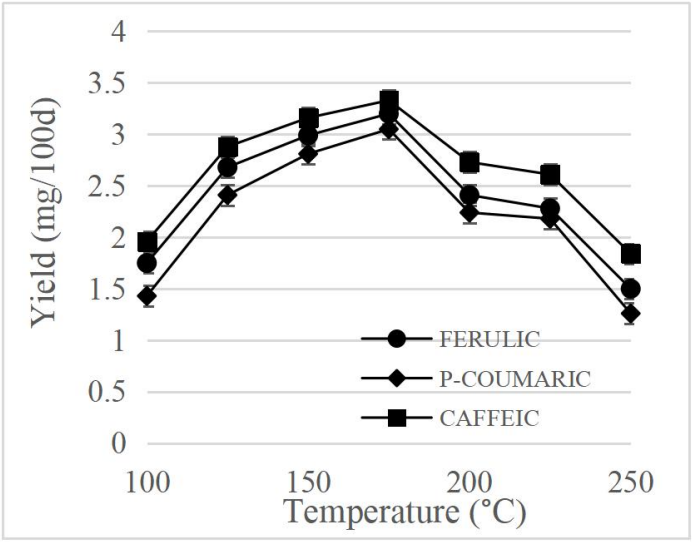

FIGURE 3. The effect of extraction temperature on yield of phenolic acids

antioxidants by spectrophotometry (Shian & Abdullah 2012). Results from this assay are shown in Figure 4. For 4 h of conventional soxhlet extraction method, the extraction with methanol gave the highest antioxidant activity value with 54.83%, compared to ethanol with 48.23% and water with 7.50% of inhibition. From the data, the antioxidant activity in the extract seemed to be correlated with the yield of phenolic compounds recovered. This agreement indicated that the phenolic compounds in *Chlorella* sp. were among the important components in the microalgae that demonstrate the antioxidant activity.

The analysis of extractable phenolic compounds in *Chlorella* sp. extracts and antioxidant activities indicated a large presence of compounds when the extraction were carried out by employing methanol followed by ethanol as solvent. In addition, water as a solvent showed its capability to extract phenolic compounds from *Chlorella* sp. microalgae using conventional soxhlet extraction method.

Figure 4 also shows the effect of SWE temperature on the antioxidant activity of the extracts at 10 min of extraction. The antioxidant activity increased as the temperature increased from 100 to 175 °C. It started to drop when the extraction was set at 200 °C and above. Certain antioxidant compounds might be mobilised at high temperatures, while possibly promoting simultaneous decomposition of antioxidants that were previously mobilised at lower temperatures. Previous study by Wettasinghe and Shahidi (1999) reported that the rate of extraction for thermally stable antioxidants at elevated temperatures is greater than the rate of decomposition of less soluble antioxidants. This finding was suggested by the relatively high percentage of antioxidant activity of the extracts obtained at higher temperatures. Increasing the temperature above 175 °C during SWE treatment reduced the antioxidant activity. These results showed that mobilisation of the antioxidants from the *Chlorella* sp. microalgae might arise up to a certain level of temperature, followed by their potential loss as a result of decomposition at higher temperatures.

The results showed that the yield of phenolic compounds and antioxidant activity increased by increasing temperature from 100 to 175 °C, then decreased by increasing temperature from 200 to 250 °C. It indicated that the phenolic content was significantly correlated with antioxidant capacity, corroborating that this phenolic class is responsible for the beneficial health effects of *Chlorella* sp. These results were in agreement with those published elsewhere (Cao et al. 2007; Hartwig et al. 2012; Zakaria et al. 2017). Furthermore, phenolic compounds act as important antioxidants because of their ability to donate a hydrogen atom or electron to form stable radical intermediates, and they are major contributors to the antioxidant capacities. Therefore, *Chlorella* sp. microalgae may find important and broad applications in the pharmaceutical and food industries because of the high antioxidant activities of their extracellular substances (Hajimahmoodi et al. 2010).

FTIR ANALYSIS

Figure 5 shows the FTIR spectra of the extracts from the SWE method for 10 min of extraction at 100, 150, 200 and 250 °C. For this analysis, the extracts were freeze-dried to remove all the water (solvent) from the samples. FTIR analysis was used to determine the functional groups that existed in *Chlorella* sp. extracts as a result of different conditions. For all spectra, a very strong broad hydroxyl band derived from hydroxyl (-OH) group was identified in the region of 3600-3200 cm^{-1} with 3292.23 cm^{-1}, 3289.77 cm^{-1}, 3288.13 cm^{-1} and 3282.97 cm^{-1} for extraction at 100, 150, 200, and 250 °C, respectively. A broad spectrum can be observed in Figure 5 representing the availability of the O-H bond in the extracts. All extracts have a strong H-bond. H bonds become stronger at lower frequencies. The presence of the O-H bond was important because it indicated the existence of phenolic compounds in the extracts.

A C-H bond of alkanes that had bonded with -COCH3 (3100-2900 cm^{-1}) group frequency was identified in the extracts with frequencies of 2927.29 cm^{-1}, 2927.61 cm^{-1}, 2927.99 cm^{-1} and 2927.31 cm^{-1} for extractions at 100, 150, 200 and 250 °C, respectively. In addition, a carbonyl group (C=O) stretching vibration peak appeared next to two stronger features namely the peak for 100 and 250 °C at 1710-1665 cm^{-1} for the extracts at 150 and 200 °C with frequencies of 1634.85 cm^{-1} and 1638.06 cm^{-1}, respectively. Stronger vibration on C-H bending–OCOCH stretching (1370-1350 cm^{-1}) group frequency was also found in the extracts at 150 and 200 °C with frequencies of 1371.68 cm^{-1} and 1370.72 cm^{-1}, respectively.

As can be seen, the spectra showed an absorption band between 1300-1000 cm and 1 cm^{-1} which corresponded to the characteristic peak of the C-O stretch of the ether functional group in all extracts with frequencies of 1036.31 cm^{-1}, 1034.47 cm^{-1}, 1032.82 cm^{-1} and 1039.07 cm^{-1}, respectively. C-H bonds of aromatic ring (690-900 cm^{-1}) group frequency were also present in the extracts with frequencies of 705.42 cm^{-1}, 706.39 cm^{-1}, 702.92 cm^{-1} and 706.73 cm^{-1} for extraction at 100, 150, 200 and 250 °C, respectively.

From the FTIR spectra, it can be seen that some stronger features occurred for the 150 and 200 °C spectra compared to the 100 and 250 °C spectra and these corresponded to the C=O and CH–OCOCH vibration peaks. The higher efficiency of subcritical water at 150 and 200 °C contributed to better extraction of phenolic compounds from *Chlorella* sp. Thermal degradation of some compounds also occurred when the high temperature of 250 °C was applied during the extraction.

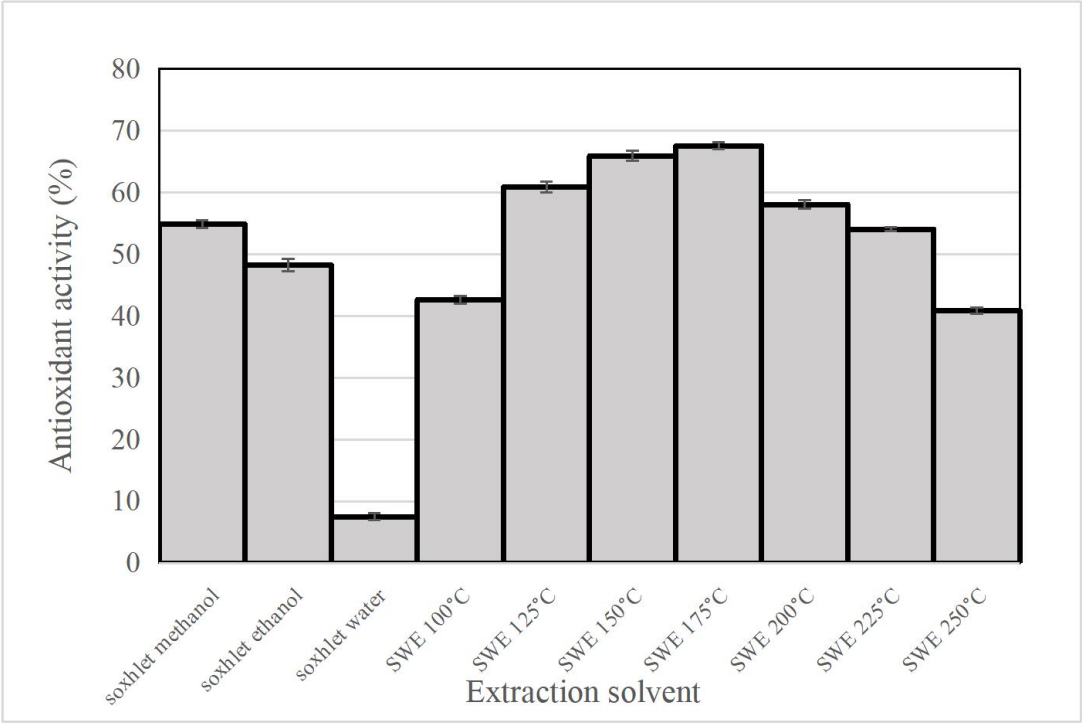

FIGURE 4. Antioxidant activity of the extracts by soxhlet extraction and SWE

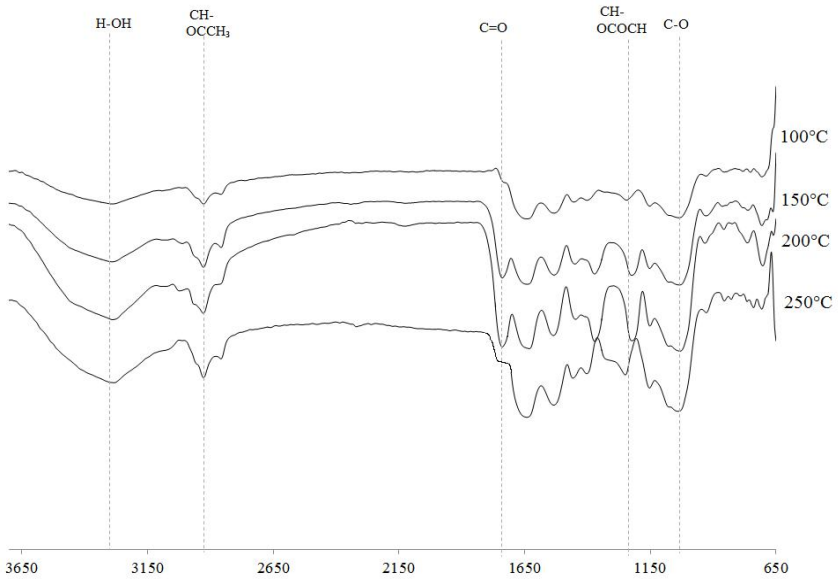

FIGURE 5. FTIR analysis on the 100, 150, 200 and 250 °C SWE extracts

Conclusion

The release of phenolic compounds in the SWE process resulted in a mixture of phenolic acid, which showed significant antioxidant properties. The most concentrated phenolic found in the extracts was caffeic acid followed by ferulic and p-coumaric acids. The maximum concentration of phenolics acids that can be obtained from the extracts was suggested at the temperature of 175 °C, which was based on the release profile of individual phenolics.

Acknowledgements

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