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Antioxidant and Antibacterial Activity of Nutraceutical Compounds from *Chlorella vulgaris* Extracted in Hydrothermal Condition

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Abstract: Water in hydrothermal condition has been used for extraction of nutraceutical compounds from *Chlorella vulgaris*. Hydrothermal extraction was carried out in a semi-batch and a batch extractor at various temperatures (120–200°C), pressures (2–10 MPa), and extraction times (30–300 min) to extract antioxidant and antibacterial compounds. The effect of extraction condition on the yield of extract was investigated. The antioxidant and antibacterial activity of extracts obtained by hydrothermal extraction were examined. The increasing extraction temperature resulted in higher antioxidant activity, but lower antimicrobial activity. As comparison with hot water extraction, the antioxidant activity of extract obtained by hydrothermal extraction was higher than that obtained by hot water extraction, but the antibacterial activity of the extract obtained by hydrothermal extraction was lower.

Keywords: Antimicrobial activity, antioxidant activity, *Chlorella vulgaris*, hydrothermal extraction

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INTRODUCTION

A green microalgae *Chlorella* contains very high concentrations of chlorophyll. It has been reported that *Chlorella* has biological effects in animal and human studies (1–5). A hot water *Chlorella vulgaris* extract that contained glycoprotein-rich has exhibited various immunostimulant activities. The extract exerts an indirect antitumor effect (4,5) and a protective effect on bacterial (6–8). *Chlorella vulgaris* extracted is also known containing large amount of antioxidant compounds, such as carotenoids and phenolic compounds (9–12). Antioxidant properties of blue-green and brown alga have been tested (13–15). The antioxidant properties, including suppression of hemoglobin-induced linoleic acid peroxidation, reducing power, ferrous chelating, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and scavenging of a superoxide anion radical-generated non-enzymatic system were studied. Kuda et al. (14,15) reported that water extract contained phenolic compounds and showed strong antioxidant activity.

Development of an efficient extraction technique would be required for the separation and isolation of nutraceutical and pharmaceutical compounds from natural resources. Water under high-temperature and high-pressure condition called hydrothermal condition, a ''natural and green'' way for product extraction, has received increased attention as an important alternative to conventional separation methods, such as hot water extraction conducted at boiling point temperature and atmospheric pressure. In hot water extraction, water only could extract nutraceutical compounds situated at the outside of plant cells, while the most nutraceutical compounds are located inside of the plant cells. Water in hydrothermal condition can be applied to extract polar organic compounds or to decompose lignocellulosic materials to produce valuable compounds such as saccharides and aromatic organic acids. The method has been applied to recover protein and amino acids (16), and phenolic compounds (17). The hydrothermal treatment has also been demonstrated by several studies to effectively convert cellulosic (18–20) and lignocellulosic biomass (21) into useful products.

As reported by other researchers (9–12), supercritical CO_2 may not be able to extract other antioxidants and antimicrobial present in *C. vulgaris*. To obtain these compounds, the application of high-temperature and high-pressure water (hydrothermal condition) was investigated to extract nutraceutical compounds from *C. vulgaris*. Water is considered generally safe and environmentally benign and can be used in foods and nutraceutical-related extraction process. In this work, extraction in hydrothermal condition was carried out in a semi-batch and a batch type extractor. In addition, physiological

activities of extract, such as antioxidant activity and antibacterial, were also examined.

MATERIALS AND METHODS

Materials and Chemicals

Spray-dried sample of *Chlorella vulgaris* was supplied by Chlorella Industry Co., Ltd., Japan. The sample, which has <5% of moisture content, contains 45% of protein, 20% of total lipid, 20% of carbohydrate, 5% of fiber, and 10% of minerals and vitamins. To prevent degradation, the samples were stored at 0°C in a tightly sealed aluminum bag until use. Chemicals used for analytical of antioxidant activity (1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxyl anisole (BHA) and methanol) and antibacterial of extract (Lactic acid bacteria (LAB)) were also provided by Chlorella Industry Co., Ltd., Japan.

Hydrothermal Extraction

Hydrothermal extraction was carried out in a semi-batch and a batch type extractor. Figure 1 shows a schematic diagram of a continuous-flow pressurized hot water extraction apparatus. About 5 grams of *C. vulgaris* was loaded into a semi-batch type extraction cell (Thar Tech, Inc., USA, 10 ml in volume), and set up in the heater. The experiment was started after the operating conditions were reached. Extraction was conducted at constant water flow rate of 1 ml/min, pressures of 2 to 10 MPa and temperatures (T_2 in Fig. 1) of 120 to 200°C for 120 minutes. After the extraction, the extracted solution was freeze-dried and weighted. Furthermore, the physiological activities of the extract were analyzed.

Batch type hydrothermal extraction was carried out in a batch extractor. About 0.6 grams of *C. vulgaris*, 5.4 ml of water and stainless balls were charged in the extraction cell (AKICO, Japan, 8.8 ml in volume), and air was substituted with argon gas prior to the extraction. Then, the extraction cell was set up in the shaking type furnace after being preheated. The system was held for 15 minutes for heating-up at desired temperature, and then the furnace was shaken. The extraction was conducted at temperatures of 160 and 170°C for 30 to 300 minutes. Extracted sample was filtered and the residue was vacuum-dried and weighed. Furthermore, the physiological activities of the filtrate were evaluated.

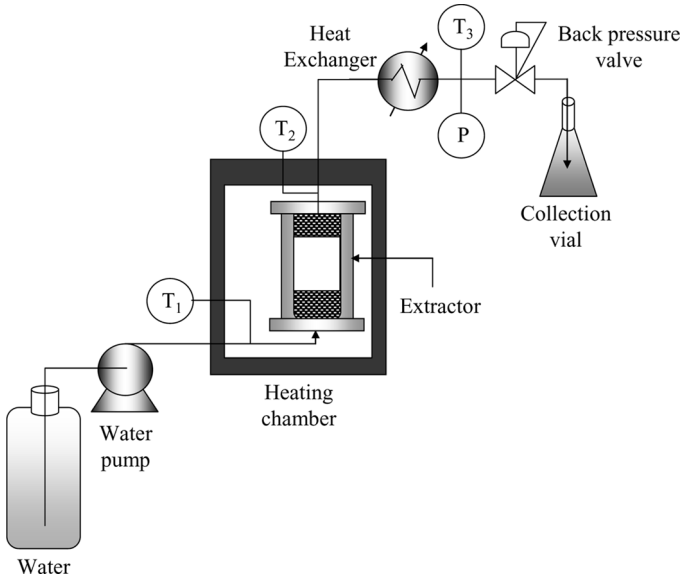

Figure 1. Schematic diagram of semi-batch type hydrothermal extraction apparatus.

In order to compare the hydrothermal extraction with the conventional extraction, hot water extraction was conducted. About 100 grams of *C. vulgaris* and 2.5 L of water were loaded in an autoclave. Extraction was carried out at 95–100°C at ambient pressure for 25 minutes excepting preheating time for 10–15 minutes. Furthermore, chlorella extract obtained by hot water extraction is called as *Chlorella vulgaris* extract (CVE).

Physiological Activity Analysis

Antioxidant Activity

The antioxidant activity is the ability of a compound to act as a donor for hydrogen atoms or electron, with DPPH as a free radical source. Furthermore, the antioxidant activity is described as free radical scavenging efficiency. The free radical scavenging effect of the extracts was assessed by the de-coloration of a methanol solution of DPPH. In this assay, the degree of the de-coloration indicates the free radical scavenging efficiency of the substances. Briefly, aqueous solution (400 μl) of 1 mg/ml test compounds was added to 2 ml methanol solution of 500 μM DPPH. The reaction mixture was shaken vigorously and then kept at room

temperature for 30 min. The absorbance of the remaining DPPH was measured at 517 nm using Shimadzu UV-1200 UV-Vis spectrophotometer. BHA was used as a standard and the samples were prepared using the same dilution procedures (22). The antioxidant activity of samples was described as BHA equivalent in mg/100 ml.

Lactic Acid Bacteria Growth Promotion Examination

The effect of the extract on the promotion of lactic acid bacteria (LAB) growth was investigated. Lactic acid bacteria (LAB) have generally regarded as safe (GRAS) status, due to their ubiquitous appearance in food and their contribution to the healthy micro-flora of human mucosal surfaces. To investigate the effect of chlorella extract on the growth of LAB, 2.5 ml of 4 mg/ml extract was added into the LAB culture medium and incubated at 37°C. And then, the growth was followed by recording absorbance at 660 nm. The amount of LAB growth was measured for 2 to 6 hours incubation.

RESULT AND DISCUSSION

In this work, yield of extract and the antioxidant and antibacterial activity of nutraceutical compounds extracted from *Chlorella vulgaris* will be explained. However, the content of nutraceutical compounds in the extract was not analyzed in detailed.

Yield of total extract was defined as weight of extract after freeze dried (mg) divided by weight of sample (g). Figures 2 and 3 are the yield of total extract obtained by semi-batch and batch extractor, respectively. In the semi-batch extractor, extraction was carried out at various temperatures and pressures, 1 ml/min of water flow rate for 120 min. As shown in Fig. 2, at 2 MPa extraction yield dramatically increased with increasing temperature, while at 10 MPa extraction yield increased up to 170°C and decreased at higher temperature. The increasing temperature causes the increasing ion product of water that result in high penetration of water into *C. vulgaris* to extract the extractible components. On the other hand, the increase in temperature also causes decomposition of extractible components into carbon and as a result the decrease in the yield. As seen in Fig. 2, however, the increasing pressure from 2 to 10 MPa did not affect the extraction yield. It can be explained that the increase in pressure from 2 to 10 MPa almost does not change the properties of water. As a comparison with CVE, the extraction yield of the semi-batch extraction was higher at a temperature of more than 150°C.

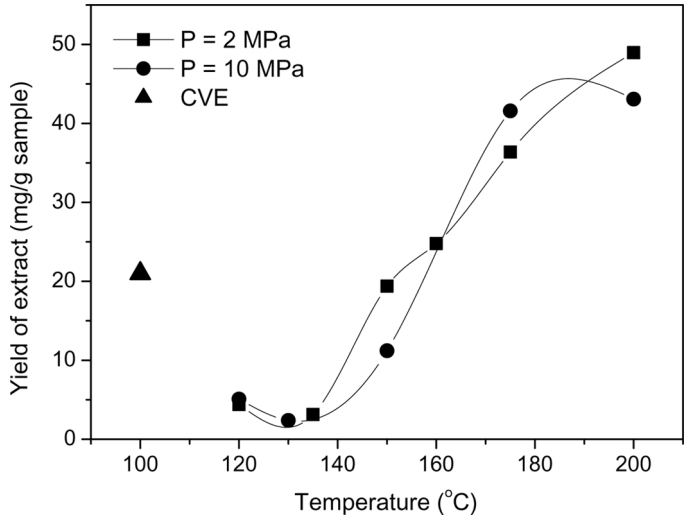

Figure 2. Yield of total extract obtained by semi-batch hydrothermal extraction at various temperatures and pressures, 1 ml/min of water flow rate for 120 min.

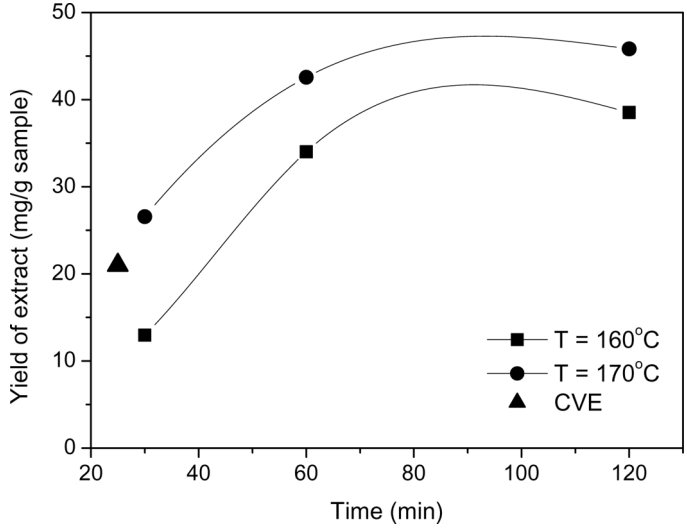

Figure 3. Yield of total extract obtained by batch hydrothermal extraction as function of time at various temperatures for 120 min.

Based on the result, it can be found that the valuable components could be collected from *C. vulgaris* in high yield by this technique.

Figure 3 shows the effect of water on the yield of total extract obtained by hydrothermal batch extractor. Extraction yield significantly increased with increasing temperature and extraction time. As comparison with semi-batch extraction at 160 and 170°C, by using a batch extractor the higher extraction yield was obtained. By using batch extraction, the residence time of water contacting and penetrating with *C. vulgaris* was longer and it resulted in a higher extraction yield. In addition, extraction yield obtained at 170°C and 30 min was higher than CVE.

Antioxidant activities of extracts obtained by semi-batch and batch extraction are listed in Tables 1, 2, respectively. The antioxidant activity of the extract was described as BHA equivalent in mg/100 ml. BHA exhibits antioxidant activity due to its ability to stabilize free radicals, sequestering them. By acting as free radical scavengers, further free radical reactions are prevented. Higher BHA equivalent of extract expresses higher antioxidant activity. The antioxidant activity of *C. vulgaris* extract is especially treated by protein, vitamins, and carotenoids content. Another researcher has reported that microalgae with higher protein content had higher antioxidant activity (2). As shown in Table 1, the antioxidant activity of the extract significantly increased as increasing temperature and pressure. This result can be explained that at higher temperature and pressure extraction yield of nutraceutical components of *C. vulgaris* increased, and as a result the increase in antioxidant content.

Table 1. DPPH radical scavenging activity of extract obtained by semi-batch extraction

	BHA equivalent (mg/100 ml)	
T (°C)	P = 2 MPa	P = 10 MPa
120	0.67	0.94
130	–	0.90
135	0.65	–
150	0.69	1.23
160	1.01	–
170	2.59	–
175	1.53	1.77
200	2.97	2.95
CVE*	1	

*CVE was carried out at T = 100°C; P = 0.1 MPa.

Table 2. DPPH radical scavenging activity of extract obtained by batch extraction

	BHA equivalent (mg/100 ml)		
T (°C)	t = 30 min	t = 60 min	t = 120 min
160 [P = 0.62 MPa]	1.93	0.81	1.79
170 [P = 0.79 MPa]	0.98	0.97	2.16
CVE*		1	

*CVE was carried out at T = 100°C; t = 25 min.

As comparison with CVE, at 2 MPa and 10 MPa, extraction conducted at temperature higher than 160 and 150°C, respectively, were effective to produce higher antioxidant activity components.

In Table 2, at 160°C the increasing extraction time significantly decreased the antioxidant activity. However, for 120 min of extraction time, the antioxidant activity increased. At 170°C the antioxidant activity significantly increased with an increase in extraction time. It can be explained that the increasing extraction time caused the increasing nutraceutical compounds to be extracted due to longer residence time and as a result the increasing antioxidant activity. In addition, the increasing temperature significantly enhanced the antioxidant activity of the extract due to the increasing saturation of vapor pressure of water that caused the increase in extraction yield of nutraceutical components of *C. vulgaris*. As a comparison with semi-batch extraction at 160 and 170°C, extract obtained by batch extractor had higher antioxidant activity. In addition, the antioxidant activity of the extract was also higher than that of CVE for both extraction temperatures. Higher antioxidant activity obtained in batch extraction might be due to the stability of water density and higher residence time to extract nutraceutical components and result in higher yield.

Antibacterial activity of the extract expresses the ability of the extract to promote LAB growth. It was defined as a ratio of LAB growth of extract with the blank (LAB culture medium without extract). Higher ratio of LAB growth indicated higher antibacterial activity. The antibacterial activity of *C. vulgaris* extract is mainly characterized by proteins and polysaccharides content. Table 3 shows the effect of temperature and pressure on the antibacterial activity of extract obtained by semi-batch extraction. At 2 MPa, the increasing temperature tends to decrease in the antibacterial activity of extract. However, at 10 MPa, the antibacterial activity of extract increased with the increase in temperature up to 150°C and then decreased with increasing temperature. The decreasing

Table 3. Antibacterial activity of extract obtained by semi-batch extraction

	Ratio of LAB growth	
T (°C)	P = 2 MPa	P = 10 MPa
120	2.50	1.10
130	–	2.30
135	1.56	–
150	1.56	2.20
160	1.42	–
170	1.23	–
175	1.33	1.60
200	0.89	1.30
CVE*	2.60	

*CVE was carried out at T = 100°C; P = 0.1 MPa.

antibacterial activity with the increase in temperature might be due to decomposition of antibacterial compounds in the extract at higher temperature. Compared with the antibacterial activity of CVE, the antibacterial activity of the extract obtained by semi-batch extraction was lower. It might be due to the fact that higher temperature and longer extraction time was used in the semi-batch extraction.

The antibacterial activity of the extract obtained by batch extraction at various extraction times and temperatures was listed in Table 4. As shown in Table 4, the increasing extraction time did not affect the antibacterial activity of the extract. As same as in semi-batch extraction, the increasing temperature tends to decrease in the antibacterial activity of the extract, and the antibacterial activity of the extract was lower than that of CVE. By comparison with semi-batch extraction, the antibacterial

Table 4. Antibacterial activity of extract obtained by batch extraction

	Ratio of LAB growth		
T (°C)	t = 30 min	t = 60 min	t = 120 min
160 [P = 0.62 MPa]	1.30	1.20	1.40
170 [P = 0.79 MPa]	1.20	0.90	1.10
CVE*	2.60		

*CVE was carried out at T = 100°C; t = 25 min.

activity of the extract obtained by batch extraction was lower. It can be explained that the temperature in the batch extractor was more stable and it caused the decomposition of antibacterial compounds.

CONCLUSION

Nutraceutical compounds from *Chlorella vulgaris* has been extracted using water in hydrothermal condition. Water in hydrothermal condition can be applied to extract polar organic compounds or to decompose lignocellulosic materials. Hydrothermal extraction was carried out in a semi-batch and a batch extractor at various temperatures, pressures, and extraction times to produce antioxidant and antibacterial compounds. The effect of extraction condition on the yield of extract was investigated. The antioxidant and antibacterial activity of extracts obtained by hydrothermal extraction were examined. The increasing extraction temperature resulted in higher antioxidant activity, but lower antibacterial activity. This result indicated that the antioxidant compounds had higher resistance to temperature than the antibacterial compounds. The antibacterial compounds might be decomposed into other compounds, but unfortunately the compounds were not identified in this work. The chemical change in hydrothermal treatment will be studied in the future. As a comparison with hot water extraction, the antioxidant activity of the extract obtained by hydrothermal extraction was higher than that obtained by hot water extraction, but the antimicrobial activity of the extract obtained by hydrothermal extraction was lower.

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