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Optimizing extraction process and characterization of antioxidant ingredients from chlorella sorokiniana

Abstract

This study was to investigate optimized extraction conditions for Chlorella sorokiniana (C. sorokiniana) water extracts with antioxidant functionality and potential key compounds involved. A 2-factor, 5-level response surface methodology was employed using extraction temperature (40-100°C) and time (0.5-6h) as factors. It is indicated that, among the C. sorokiniana extracts examined, the maximal value in yield was 18.0% (w/w) on biomass basis; in 75% ethanol solubility of water extract (WS-E75S%, the potentially major antioxidant fraction), 38.0% (w/w) on extract basis; in total phenolic content (TPC), 3.17 GAEmg/g on extract basis. The highest antioxidant activities were shown by a 50% DPPH□ scavenging concentration (SC50)=7.36mg/mL, 50% Fe2+ chelating concentration (CC50,)=10.4mg/mL, and reducing power increment per unit concentration=0.044mL/mg. By statistical analysis with RSREG program, the obtained polynomials for yield, WS-E75S%, SC50 and CC50 as a function of temperature and time could explain 78.9-82.2% of data variations. An optimal extraction condition was concluded at 100°C for 1h, to give high values in all yield, WS-E75S%, and antioxidant activities (i.e. low SC50 and CC50 values). In WS-E75S, the major compositions were likely nucleic acids and their analogues with ethylene structure, accompanying with detectable amounts of possibly polyunsaturated fatty acids, fatty alcohols, or phytols with acyl dienes, buta-1, 3-diene or ethylene structure. Besides these Phytochemicals, the water extract contained carbohydrates of mainly glucose and ribose (52.4 and 25.9mol%, respectively), followed by galactose and rhamnose, and two molecular fractions. Conclusively, Chlorella water extract at optimally 100 °C for 1h could yield~18% (w/w), contain WS-E75S~37% (w/w) and have statistically predicted SC50 ~3.0mg/mL and CC50 ~11mg/mL.

Keywords: chlorella sorokinian, extraction, antioxidant, DPPH scavenging, response surface design

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Abbreviations: Ara, arabinose; CC_{50}, 50%- Fe^{2+} chelating concentration; CGF, *chlorella* growth factor; ChA, chlorogenic acid; *C. sorokiniana*, *chlorella sorokiniana*; DPPH, 1,1-diphenyl-2-picryl hydrazyl free radical; ECG, (-) epicatechin gallate; EDTA, (-) ethylenediamine tetraacetic acid; EGCG, (-) epigallocatechin gallate; EtOH, ethanol; F1, large molecular weight fraction; F2, small molecular weight fraction; GA, gallic acids; GAE, gallic acid equivalent; Gal, galactose; GCG, (-) gallocatechin gallate; Glc, glucose; HPACE, high performance anion exchange chromatography; HPLC-DAD, high performance liquid chromatography with a photodiarray detector; HPSEC, high performance size exchange chromatography; Man, mannose; M_w, weight-averaged molecular weight; Rha, rhamnose; Rib, ribose; SC_{50}, 50%-scavenging concentration; TPC, total phenolic content; Vit E, α-tocopherol; WS100, water extract at 100°C for 1h; WS-E75S, 75% ethanol soluble of water extract

Introduction

Microalgae *Chlorella* (Chlorophyceae), usually *C. vulgaris*, *C. pyrenoidosa*, and *C. sorokiniana* (previously named as *C. pyrenoidosa*), have been long consumed as nutritional supplements due to containing significant amounts of high-quality proteins with excellent amino acid profiles, polyunsaturated (Ω-3) fatty acids, antioxidant ingredients (e.g. β-carotene, astaxanthin, and chlorophylls), nucleic acids, vitamins, hemagglutinins, starch, and dietary fibers.[1,2] Water extracts from *C. pyrenoidosa* in Japan are regarded as *Chlorella* growth factor (CGF) for human or animals.[3] Oral administrations of *Chlorella* powder, water extracts, proteoglycans or glycoproteins isolates are reported effective for weight management, lipid metabolism control,[3] increasing resistance to *Listeria* infection by preferentially augmenting Th1 responses and antibody levels.[4–7] accelerating dioxin excretion,[8] inhibiting hepatocarcinogenesis,[9] and boosting immunoactivities.[6,7] Accordingly, *C. pyrenoidosa* hot-water extracts (CPE) are patented as Respondin™ as a proprietary immunomodulator.[7,10] Polysaccharides from CPE are reported to activate macrophages via Toll-like receptor 4.[11] Besides, lipid extract from *C. sorokiniana* has been found rich in ω-3 and ω-6 polyunsatured fatty acids and effective to improve short-term memory in rats.[12]

Recently, research focuses have been on new combined processes and condition optimization for producing high value products from both *Chlorella* biomass and residues,[1] e.g. immunomodulator glycoproteins or polysaccharides,[13,14] antioxidant ingredients,[15] glycosidase inhibitors [personnel communications], polyunsaturated (-3) fatty acids,[16] lutein, carotenoids, hydrogen,[1] and bioethanol.[17] Where *Chlorella* water extracts and their immuno stimulatory biopolymer isolates are drawing great attention to enhance their functionalities in anti-ageing (anti-radicals) for skin scare or boosting immunological systems for health purposes. Designing cost-effective processing conditions and looking for chemical indices for quality control are crucial for commercialization of functional resources such as *Chlorella* algae. Accordingly, optimizing extraction conditions for *Chlorella sorokiniana* extract with high antioxidant activities and potential chemical indices for quality control were investigated in this study. Antioxidant activities in scavenging abilities on

DPPH □ radicals and Fe^{2+} ions and reducing power were especially concerned. Antioxidant phytocompounds in *Chlorella* water extracts were identified to a great extent by high performance liquid chromatography-photo di array detector (HPLC-DAD), which were not yet discovered in literature.

Materials and methods

Materials

Chlorella sorokiniana (previously named as *C. pyrenoidosa*) powder was gifted from Taiwan Chlorella Manufacturers, Ltd. (Taipei, Taiwan). It was prepared by high-pressure spray drying and possessed partly broken cell walls for facilitating extraction. Chemical standards used (e.g. chlorogenic acid (ChA), (-) epicatechin gallate (ECG), (-) epigallocatechin gallate (EGCG), (-) gallocatechin gallate (GCG), ethylenediamine tetraacetic acid (EDTA), gallic acids (GA), α-tocopherol (Vit E), and monosaccharides) were purchased from Sigma-Aldrich Co. (USA). Folin & Ciocalteu's phenol reagent, acetonitrile, salts, acids, and ethanol were from Merck Chemical Co. (Germany) or Wako Pure Chemical Industries, Ltd. (Japan).

Extraction of Chlorella ingredients

Chlorella powder was dispersed in 500mL distilled water (*Chlorella*: water: 1:50w/w) in a 1-L screw-capped Erlemeyer flask and then put in a preheated water bath for extraction. The independent factors for extraction, *i.e.* temperature (T) and time (t), were set according to the 2-factor, 5-level central composite experimental design indicated in Table 1 and the extraction conditions in Table 2. The ranges for temperature and time were 40-100°C and 0.5-6h, respectively. The central point was set at 70°C for 3.25h, close to the extraction conditions usually for antioxidant herbal phytocompounds. After extraction, sample was cooled to room temperature and centrifuged (10000rpm, 15min). The supernatant was collected, concentrated *in vacuo*, and freeze-dried. Extract yield (%w/w) was calculated as the percentage of freeze-dried product to *Chlorella* mass on dry basis.

Table 1 Five levels of two independent variables for extraction of antioxidant *C. sorokiniana* ingredients

Independent variable	Coded	Levels				
		-1.414	-1	0	1	1.414
Temperature (°C)	X_1	40	48.8	70	91.2	100
Time (h)	X_2	0.5	1.3	3.25	5.19	6

Solubility in 75% ethanol aqueous solution

The obtained extract was dispersed in 75% ethanol aqueous solution (10-15mg/mL, resembling the solvation conditions in the following antioxidant activity measurements) and stirred gently at ambient temperature for 1h, following by centrifugation (10000rpm, 15min). After removing the supernatant, the insoluble sediment was completely dried in 105°C and weighted. 75%-Ethanol solubility (%w/w) of water extract (termed as WS-E75S%) was calculated as the result of (100%-sediment%) on dry extract basis.

Measurement of total phenolic content (TPC)

Sample was dissolved in 10mL deionized water (5.0mg/mL) by heating to 100°C for 10min and cooled to room temperature for total phenolic content measurement, according to the method of Sato et al.[18] Gallic acid in 50% ethanol aqueous solution (3-100μg/mL) was used as reference. A portion (400μL) of the extract solution or reference was mixed with 400μL of Ciocalteu's phenol reagent for 3min, followed by successively adding with 40μL of 10% Na_2CO_3 aqueous solution and stirring at every 10min. After reaction for one hour, sample mixture was detected for the absorbance at 735nm in a spectrophotometer (Hitachi U-2001, Tokyo, Japan). Data were calculated according to gallic acid standard curve and presented as gallic acid equivalent (GAE) mg/g extract. Three replicated measurements were done.

Measurement of total protein content

Total protein content was measured according to the method of Lowry et al.[19] Sample (60mg) was dissolved in 10mL deionized water by heating, cooled to room temperature, and diluted to 0.6mg/mL before reaction with Lowry reagent and Folin-phenol reagent at room temperature for 45min. The absorbance at 540nm was examined and calibrated with bovine serum albumin (BSA) standard curve. Data were means of three replications.

Measurement of total carbohydrate content

Total carbohydrate content was examined by using the phenol-sulfuric acid method[20] with absorbance at 488nm as an index and glucose as standard. Data were means of three replications.

Characterization of phenolic compounds profile

75%-Ethanol soluble of *Chlorella* water extract (WS-E75S) was examined by high performance liquid chromatography with a photo di array detector (HPLC-DAD) and Polaris C18 column (5μ, 250x46mm, Varian). Sample was filtered through 0.45μm membrane before HPLC analysis. The elution condition was: acetonitrile/0.1% phosphoric acid, flow rate: 1mL/min, at ambient temperature. Phenolic compounds including ChA, ECG, EGCG, GA, and GCG were used as standards.

Analysis of neutral monosaccharide compositions

Sample was subjected to hydrolysis in 2M trifluoroacetic acid (5mg/mL) in a boiling water bath for 6h, followed by vacuum drying in a centrifugal evaporator (Savant Speed-Vac model 100 evaporator, Savant Instruments, Inc. NY). The hydrolysate was re-dissolved in 10mL of de-ionized water (18MΩ) and ion exchanged on Amberlite IRA-400 resins (Cl^- form) to remove acidic residue completely. Sample was then filtered through a 0.45μm membrane for analysis by high performance anion exchange chromatography (HPAEC), using a Dionex DX-500 equipped with an ED40 detector and CarboPac™ PA1 guard (50x4mm ID) and analysis (250x4mm ID) columns (Dionex Co., Sunnyvale, USA). The electric pulses of ED40 detector was set as: output range: 100nA; E_1: +0.05V, t_1: 0.00-0.40s; E_2: +0.75V, t_2: 0.41=0.60s; E_3: -0.15V, t_3: 0.61-1.00s. The elution condition for analysis was 4mM NaOH at 0.75mL/min and at ambient temperature. Sampling size was 100μL. Time for data collection was 30min after injection. Chromatograms were analysed with the Dionex Peak Net System (Dionex Co.). Data were obtained by calibration with monosaccharide standard curves: A: 9.00×10^5□C-8.03×10^4 (R^2: 0.995) for arabinose; A: 5.66×10^5C+2.24×10^6 (R^2: 0.984) for fucose; A: 6.59×10^5C+2.48×10^6 (R^2: 0.995) for glucose; A: 9.42×10^5C-1.48×10^6 (R^2: 0.998) for galactose; A: 9.72×10^5C-4.40×10^6 (R^2:

0.992) for mannose; A: 7.48×10^5□C-2.75×10^6 (R^2:0.995) for rhamnose; A: 9.34×10^5C=4.49×10^6 (R^2: 0.989) for ribose; and A: 1.03×10^6□C-3.81×10^6 (R^2:0.995) for xylose; where A: peak area, C: monosaccharide concentration (μg/mL). All data were measured in three replications and presented as molar percentage (mol%) on total neutral monosaccharide basis.

Molecular distribution analysis

Sample (2mg/mL) was dissolved in deionized water by heating at 95°C for 2h, followed by cooling and pre-filtering through 0.45μm before measurement by high-performance size exclusion chromatography (HPSEC). According to the analysis conditions in our previous report,[21] a high-resolution differential RI detector (Viscotek Co., Texas, USA) and guarded TSK $GMPW_{XL}$ column were employed. Elution was done with 50mM $NaNO_3$ aqueous solution (containing 0.02% (w/w) NaN_3) at a flow rate of 0.5mL/min at 35°C . Chromatograms were collected in triplicate. Data were managed with TriSEC GPC software (Viscotek, Co.) and calibrated with pullulans standards (Shodex Co. Ltd., Kawasaki, Japan).

DPPH Radical scavenging ability assay

The assay was done according to the method of Shimada et al.[22] α, α-Diphenyl--picrylhydrazyl radicals (DPPH□) at 10mM was freshly prepared in methanol in a brown bottle before use. Sample was well dissolved in 75% ethanol solution as mentioned above. One mL of sample (1-20mg/mL) in 75% ethanol solution was mixed with 0.25mL of 10mM DPPH□ solution, followed by stirring for mixing well and staying in the dark at ambient temperature for 30min. The mixture was immediately examined on the absorbance at 517nm (A_{517}) in a spectrophotometer (Hitachi U-2001, Hitachi Instruments, Inc., Japan). DPPH-radical scavenging effect (%) was calculated as Eq. (1). All data were measured in three replications.

$$DPPH-radicalscavengingeffect\,(\%):\left(1-\frac{A_{517,\text{sample}}}{A_{517,\text{control}}}\right)\times100 \quad (1)$$

Chelating ability assay on Fe2+ ions

The method of Dinis et al.[23] was applied. Sample was dissolved in 75% ethanol aqueous solution as mentioned above. A portion (0.2mL) of sample solution (1-30mg/mL) was added with 0.74mL methanol and 0.02mL of 2mM $FeCl_3$ aqueous solution (in deionized water) for 30sec, followed by immediately adding with 0.04mL of 5mM Ferrozine (in deionized water), mixing well, staying for 10min in the dark at ambient temperature, and soon measured on the absorbance at 562nm (A_{562}). Chelating effect on Fe^{2+} was calculated as Eq. (2). All data were measured in three replications.

$$Chelatingeffecton\,Fe^{2+}\,(\%):\left(1-\frac{A_{562,\text{sample}}}{A_{562,\text{control}}}\right)\times100 \quad (2)$$

Reducing power assay

The reducing power assay was done according to the method of Yen et al.[24] Sample was dissolved in 75% ethanol solution as mentioned above. A portion (150μL) of sample (0.039-5.0mg/mL) was mixed well with 150μL of 0.2M sodium phosphate buffer (pH 6.6) and 150μL of 1% (w/w) potassium ferricyanide ($K_3Fe(CN)_6$) (in deionized water), following by reaction at 50°C in a water bath for 20min, cooling in an ice bath for 3 min, and immediately adding with 150μL of 10% (w/w) trichloroacetic acid (in deionized water) to stop reaction. The mixture was then added with 0.6mL deionized water and 120μL of 0.1% (w/w) $FeCl_3$ (in deionized water), mixed well, stayed at room temperature for 14min, and immediately detected on the absorbance at 700nm (A_{700}). All data were measured in three replications. The higher the A_{700}, the greater is the reducing power.

Statistical analysis

A RSREG program and canonical analysis of SAS software 8.12 (SAS Institute, Inc., Cary, USA) was applied to give predicted polynomials. According to the polynomials, counter plots were produced with Sigma Plot 8.0 software (Systat software Inc., CA, USA). Person's correlation analysis was done with SAS software 8.12. General mathematic treatments for data and calibrations were carried out with Excel 2012 (Microsoft Co., USA).

Results and discussion

Yield, 75% ethanol solubility, and total phenolic content

Table 2 illustrates that *C. sorokiniana* water extracts showed a yield in the range of 16.2-18.0% (w/w) on *Chlorella* biomass basis, WS-E75S% (75% ethanol solubility, in the same solvent for antioxidant activities assay) in the range of 30.7-38.0% (w/w) on extract basis, and total phenolic content (TPC) in the range of 2.62-3.17GAEmg/g on extract basis. Generally, high extraction temperatures (91.2 or 100°C) tended to give high values in yield, WS-E75S%, or TPC. The extracts obtained under the repeated central condition (70°C, 3.25h; trials #9-14) exhibited a comparatively low yield (16.6-17.1%w/w), low WS-E75S% (30.7-34.0% (w/w)), and intermediate TPC (2.84-3.08% w/w) among the samples studied. For those extracted at 70°C, prolonging extraction time to 6h (trial #8, 70°C, 6h) resulted in a reduced TPC (2.62GAEmg/mL), as compared with those extracted for 0.5 or 3.25h (trials #7, 9-14). The maximal yields in this study agree closely with that (18%) of 80°C water extract from the same *Chlorella* source.[25]

Antioxidant activities

Figure 1 depicts the concentration dependencies of the antioxidant parameters examined for *Chlorella* water extracts. Generally, the concentration-induced increments in DPPH□ scavenging extent were found the greatest for trials #2 and 7 and least for #8-14 (Figure 1A). Those in Fe^{2+} chelating extent were the greatest for #2 and 6 and least for #1, 3, and 9-14 (Figure 1B). And, those in reducing powers (indicated by A_{700}) were the greatest for #5 and 6 and least for #8-14 (Figure 1C). Table 3 illustrates that the maximal DPPH□ scavenging extent was observed in the range of in average 79.0-95.1% at the extract concentration studied (10-20mg/mL). By interpolating data curves at 50% extent in Figure 1A, 50% DPPH□-scavenging concentration (SC_{50}) was found in the range of 3.85-7.36mg/mL on extract basis (equivalently 1.37-2.40mg/mL on WS-E75S basis). The maximal Fe^{2+} chelating extent was in the range of 55.1-80.9% in average, accompanying with a 50% Fe^{2+} chelating concentration (CC_{50}, obtained from Figure 1B) in the range of 10.4-23.8mg/mL on extract basis (equivalently 3.95-8.95mg/mL on WS-E75S basis). As to reducing power, the concentration (C)-induced increments (A_{700}/C) ranged from 0.028 to 0.044mL/mg. The lowest DPPH□ scavenging SC_{50} values (1.37mg/mL on WS-E75S basis, the same solvent as for

DPPH□ scavenging assay) were about double of that of antioxidative peptide purified from *C. ellipsoidea* protein (Leu-Asn-Gly-Asp-Val-Trp, 50% scavenging concentration:0.92mM, *i.e.* 0.646mg/mL).[15]

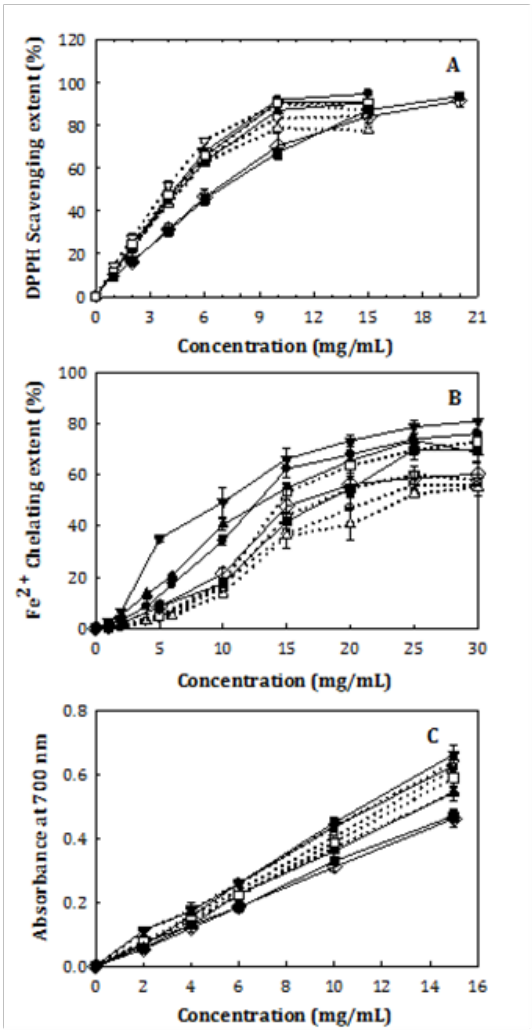

Figure 1 Depicts the concentration dependencies of the antioxidant parameters.

A. Changes in DPPH· scavenging extent. **B.** Fe^{2+} chelating extent. **C.** Reducing power of *C. sorokiniana* water extracts with extract concentration (extraction conditions as indicated in Table 2, where ○ = #1, ●= #2, = #3, ▲= #4, ⍰ = #5, ▼= #6, □= #7, ▓= #8, ⍰= mean of #9-14).

Interactive influences of extraction temperature and time

All data in Table 2 & Table 3 were statistically analyzed by RSREG program of SAS software. The estimated polynomials for yield, WS-E75S%, SC_{50} on DPPH□ radicals and CC_{50} on Fe^{2+} ions, except TPC and reducing power, showed sufficiently high multiple correlation coefficients (R^2:0.789-0.822). From Table 4, it is indicated that yield (% w/w, on dry biomass basis): 16.2+0.525 *T*-0.0323 *t*-0.00479 *T*□*t*-0.0211 T^2+0.000516 t^2*. Prolonging long time (t^2 term) tended to increase yield significantly (P<0.05). WS-E75S% (w/w, on dry extract basis): 55.3-0.695 *T***+0.0953*t*-0.00966 *T*□*t*+0.00525 T^2***+ 0.176 t^2. For scavenging DPPH□, SC_{50} (mg/mL): -9.22+0.387 *T***+1.07 *t*+0.00171 *T*□*t*-0.00278T^2***-0.143 t^2. Both WS-E75S% and SC_{50} values varied with *T* and T^2 significantly (P<0.01 and P<0.001). And, for chelating Fe^{2+} ions, CC_{50}: 22.2+0.00835*T*- 0.,223*t*- 0.00781*T*□-0.00117 T^2*+0.192 t^2, varying significantly and negatively with T^2 (P<0.05).

The counter plots corresponding to the above polynomials were produced and are shown in Figure 2, in order to illustrate the interactive effects of temperature and time concerned. When>70°C, *T* became the dominant variable governing the yield (Figure 2A), accompanying with a saddle point at 58.7°C for 5.75h. The WS-E75S% (Figure 2B) minimized at 67.7°C for 1.59h and increased mainly with increasing or reducing *T* away from 67.7°C or increasing *t*. The SC_{50} (Figure 2C) maximized at 70.8°C for 4.17h and reduced mostly by increasing or decreasing *T* with shortening *t*. And, the CC_{50} reduced mainly with increasing *T* (Figure 2D). Based on the high yield, WS-E75S%, and antioxidant activities (*i.e.* low SC_{50} and CC_{50}), the optimal extraction condition can be concluded at 100°C for 1h.

Characterization of 75% ethanol-soluble phytocompounds

Samples WS-E75S were concerned due to playing a key role for the antioxidant activities. All WS-E75S from 9 extracts with different extraction conditions showed similar HPLC profiles as shown in Figure 3A, exemplified with the WS-E75S from extract at 70 °C for 3.25 h. Typically, there were three major compounds (1-3) and four minor ones (4-7) implied from the chromatographic peaks. The UV spectra (Figures 3B) & (Figure 3C) and maximal wavelengths (λ_{max}) (Table 5) indicate that all compounds showed three absorption peaks in the range of 190-320nm, implying three types of chromophores with λ_{max} at 196-198nm (peak I); 210-213 (for compounds 2-4, 6) or 219-222 nm (compounds 5, 7) (peak II); and 256-259 (compounds 2-4 and 6) or 278-281nm (compounds 5 and 7) (peak III). Compound 1 showed a similar UV spectrum (not shown) to that did compound 2. Generally, compounds 5 and 7 were of different structural features from those of compounds 1-4 and 6.

For structural elucidation, small compounds possibly present in WS-E75S were focused on phenolic compounds, nucleic acids, unsaturated fatty acids such as α-linolenic acid and analogues, based on three considerations. Firstly, the spectra of the compounds in this study (Figure 3B) & (Figure 3C) were identified in reference to several typical phenolic compounds (Figure 3D) and (Table 5). It is indicated that GA displayed a typical UV spectrum of two big absorption peaks with λ_{max}: 225 and 280nm, indicating its phenolic ring ($\pi\rightarrow\pi^*$ transition of C:C bonds in A_1 ring) and free carboxyl group ($n\rightarrow\pi^*$ transition of C:O bonds), respectively. EGCG, GCG, and ECG exhibited similar spectra of a big, broad peak with λ_{max}: 208-210nm and mild broad one with λ_{max}:275-278nm, concerning C: C bonds in 3 phenolic rings (2 A_1 and A_3 rings) and C: O bonds in ester linkage, respectively. And, ChA showed a triplet at 190-278nm and duplet at 278-380nm, contributed by three kinds of C:C bonds in caffeic acid group (A_2) and two types of C: O bonds attached to quinic acid group (B), respectively. Evidently, all sample compounds were different from these phenolic standards on the viewpoints of UV spectra and HPLC retention time. Secondly, WS-E75S samples are reasonably expected to contain small molecules such as nucleotides, monosaccharides, unsaturated (omega-3) fatty acids or derivatives originating from their parent extracts (*C. sorokiniana* and *C. pyrenoidosa*) with known related compositions.[5,13,14,16] Thirdly, the above standard spectra and documented UV spectra.[13, 26–30] conclude that λ_{max} values are generally

185-200 nm for ethylene (RHC:CHR) and C: N groups; 208−225 nm for acylic dienes (phenyl, unsubstituted, conjugated or heteroannular) and buta-1, 3-diene (C:C-C:C); 250-260nm for hexa-1, 3, 5-triene (C:C-C:C-C:C) and nucleic acids; and 270-280nm for carboxyl groups (RRC:O) in aromatic amino acids, proteins, ketones, and fatty acids; and 300-335nm and higher λ_{max} values for conjugated C: O groups in esters, acetals, conjugated or condensed aromatic compounds.

Accordingly, the compounds 1-4 and 6 were likely nucleic acids (λ_{max}:210-220 and 260nm) and analogues with ethylene chromophores (λ_{max}:196198nm).[25] The ethylene group leveled greater in the compounds eluted for a longer retention time. The compounds 5 and 7 were possibly related to Ω-3 fatty acids such as α-linoleic and linolenic acids (λ_{max}:217-219 and 270nm),[29] fatty alcohols, or phytols,[30] those with a significant level of acyl dienes or buta-1, 3-diene (C:C-C:C) (λ_{max}:219-222nm) (compound 5) or ethylene (compound 7) chromophore. Both had carboxyl groups likely conjugated to phenyl rings, ketones, aromatic amino acids, or nucleotides (λ_{max}: 278-281nm) under the bathochromic (λ_{max} red shifting) and hyperchromic (enhanced absorptive) effects by conjugation.[26]

It is interesting to found that in this study the HPLC peak area of only compound 5, rather than the other compounds, correlated significantly and positively with the DPPH□scavenging ability (Person's correlation coefficient: 0.733*, P<0.05). This agrees with the estimation about its Ω-3 fatty acid compositions that are known of high radical scavenging abilities and have been found abundant in *C. sorokiniana* lipid extract.[12] Compound 5 could be a good index responsible for the antioxidant activity in scavenging DPPH□ radicals of *Chlorella* extracts products. As to the compounds 1-4 and 6 (λ_{max}: 260nm), they could be the quality index of functional drinks with CGF, namely *Chlorella* hot water extracts,[3–5] agreeing with the absorbance at 260nm, an old index in *Chlorella* industry.

For WS-E75S compounds, no UV signals about proteins (typically λ_{max}:230 and 280nm),[28] flavonoids (typically λ_{max}~214, 270, and 339nm),[31] β-carotene or chlorophylls (λ_{max} in the range of 400-680nm) that are usually found in *Chlorella* biomass[9] were dateable in this study.

Characterization of biopolymer compositions

According to the optimal extraction condition concluded from Figure 2, the water extract at 100°C for 1h (WS100) was specially prepared for further analysis on carbohydrates, proteins, and molecular property. It is shown that WS100 contained a total protein content: 23.5±0.1% (w/w) (by Lowry assay) and total carbohydrate content: 22±1% (w/w) (by phenol-sulfuric acid method). Figure 4A shows that glucose (Glc) and ribose (Rib) were dominant, followed by galactose (Gal) and rhamnose (Rha) in the neutral monosaccharides of WS100 acid hydrolysate. Mannose (Man), xylose (Xyl), and arabinose (Ara) were almost insignificant. The monosaccharide composition was Glc:Rib:Gal:Rha:Man:Xyl:Ara: 52.4:25.9:8.6:7.3:3.0:2.2:0.7 in mol%, expectedly coming from nucleic acids and biopolymers (polysaccharides and glycoproteins). It possessed two molecular fractions (Figure 4B) as 57.8% F1 fraction (M_w: 25.0±0.6kDa) and 42.2% F2 fraction (M_w:0.95±0.09kDa). From the results of Figure 2, the properties of *Chlorella* water extract could be semi-empirically estimated as yield~18% (w/w), WS-E75S ~37% (w/w), statistically predicted DPPH□ scavenging SC_{50}~3.0mg/mL, and Fe^{2+} chelating CC_{50}~11mg/mL.

Generally, the WS of *C. sorokiniana* (previously classified as *C. pyrenoidosa*) in this study showed a lower content in total carbohydrates (rich in Glc and Rib) or crude proteins than those did *C. vulgaris*.[32] Glc and Rib can be linked to the presence of starches and nucleotides in *Chlorella* biomass.[5,13] Generally, Gal and Rha are the major monosaccharides in cell wall compositions of *C. sorokiniana* and *C. pyrenoidosa*.[33] And, Man and Gal are found dominant in the immunomodulatory polysaccharides or glycoproteins from *C. pyrenoidosa*[7,14,34] and *C. vulgaris*.[4,5] In contrast to the biopolymers in *Chlorella* water extracts at 100°C for 1h in this study, the purified polysaccharide fractions from *C. pyrenoidosa* water extracts at 100°C for 4h show a greater percentage of large molecular weight fraction (76% F1), higher M_w values (82k and 1.7k) and rich in Man.[14] Different results between different studies can be attributed to the differences in *Chlorella* species, cultivation conditions, extraction conditions, and isolation processes applied.

Table 2 Yields, 75% ethanol solubility, and total phenol contents of *C. sorokiniana* water extracts under 14 extraction conditions designed with 2-factor, 5-level central composite experimental design

Trial No	Extraction condition		Yield[1]	WS-E75S[2]	Total phenolic content[3]
	Coded	Practical	(% w/w)	(% w/w)	(GAE mg/g)
1	-1,-1	48.8°C, 1.3h	16.3±1.04	34.3±4.5	2.83±0.02
2	+1, -1	91.2°C, 1.3h	18.0±0.4	34.4±4.3	2.73±0.06
3	-1, +1	48.8°C, 5.19h	16.6±0.2	37.6±2.3	3.04±0.03
4	+1, +1	91.2°C, 5.19h	17.4±0.2	35.9±4.5	3.17±0.07
5	-1.414, 0	40°C, 3.25h	16.6±0.6	35.7±1.7	2.82±0.07
6	+1.414, 0	100°C, 3.25h	17.9±0.9	38.0±3.6	2.87±0.09
7	0, -1.414	70°C, 0.5h	16.2±0.1	32.0±3.4	2.99±0.11
8	0, +1.414	70°C, 6h	17.0±0.2	34.9±4.5	2.62±0.08
9	0, 0	70°C, 3.25h	16.7±0.3	31.9±1.6	3.03±0.09

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Table Continued

Trial No	Extraction condition		Yield[1]	WS-E75S[2]	Total phenolic content[3]
	Coded	Practical	(% w/w)	(% w/w)	(GAE mg/g)
10	0, 0	70°C, 3.25h	16.9±0.6	30.7±1.4	2.84±0.14
11	0, 0	70°C, 3.25h	16.6±0.3	34.0±3.8	2.91±0.32
12	0, 0	70°C, 3.25h	17.1±0.6	33.3±3.1	2.92±0.15
13	0, 0	70°C, 3.25h	16.7±0.7	31.6±1.3	3.08±0.09
14	0, 0	70°C, 3.25h	17.1±0.4	32.6±2.2	2.90±0.06

[1]On dry *Chlorella* biomass basis.
[2]WS-E75S%: 75% ethanol solubility of water extract, on dry extract basis.
[3]GAE, gallic acid equivalent on dry extract basis.
[4]Means±standard deviations (*n*=3).

Table 3 *DPPH Scavenging ability, Fe2+ chelating abilities, and reducing power concentration dependence of C. sorokiniana water extracts*

Trial no	Extraction condition	DPPH ×scavenging ability		Fe^{2+}-chelating ability		Reducing power C-dependence
		Maximum	SC_{50}[1] (mg/mL)	Maximum	CC_{50}[2] (mg/mL)	A_{700}/C[3] (mL/mg)
1	48.8°C, 1.3h	84.5±0.8[4]	4.75	56.1±2.5[4]	22	0.041
2	91.2°C, 1.3h	95.1±1.0	4.35	76.1±0.7	12.8	0.042
3	48.8 °C, 5.19h	79.0±0.5	4.85	55.1±1.7	23.8	0.036
4	91.2°C, 5.19h	90.9±0.1	4.73	73.4±1.4	13.3	0.036
5	40°C, 3.25h	90.6±0.1	3.85	60.0±3.4	18.2	0.043
6	100°C, 3.25h	91.2±0.3	4.55	80.9±0.7	10.4	0.044
7	70°C, 0.5h	90.9±0.4	4.35	72.9±2.3	15	0.039
8	70°C, 6h	93.8±0.1	6.9	69.4±1.3	18.6	0.031
9	70°C, 3.25h	94.8±0.1	5.72	57.3±1.3	15	0.0337
10	70°C, 3.25h	94.9±0.0	6.38	55.8±0.9	15.5	0.034
11	70°C, 3.25h	94.7±0.1	6.4	60.5±1.4	15.8	0.031
12	70°C, 3.25h	87.5±0.5	6.5	58.7±2.1	16	0.03
13	70°C, 3.25h	92.4±0.4	7.15	66.5±1.7	18	0.031
14	70°C, 3.25h	89.4±0.1	7.36	64.4±2.0	19.1	0.028

[1]SC_{50}: 50%-DPPH□ scavenging concentration on the dry basis of extract.
[2]CC_{50}: 50%-Fe^{2+} chelating concentration on the dry basis of extract.
[3]Calculated from the data at C=15mg/mL.
[4]Means±standard deviations (*n*=3).

Table 4 Regression equations for the yield, 75% ethanol solubility (WS-E75S), 50%-DPPH· scavenging concentration (SC50), and 50%-Fe2+ chelating concentration (CC50) of C. sorokiniana water extracts in terms of extraction temperature and time

Variable	Regression Equation[1]	R^{2}[2]
Yield (% w/w)	16.2 + 0.525 T-0.0323 t-0.00479 T×t-0.0211 T^2+0.000516 t^2* (saddle point at 58.7°C, 5.75h)	0.822
WS-E75S (% w/w)	55.3 - 0.695 T**+0.0953 t-0.00966 T×t+0.00525 T^2***+0.176 t^2 (minimal at 67.7°C, 1.59h)	0.831
DPPH× scavenging SC_{50} (mg/mL)	-9.22+0.387 T**+1.07 τt+0.00171 T·t-.00278 T**-0.143 t^2 (minimal at 70.8°C, 4.17h)	0.818
Fe^{2+} chelating CC50 (mg/mL)	22.2+0.00835 T-0.223 t-0.00781 T×t-0.00117 T^2*+0.192 t^2	0.789
Fe^{2+} chelating CC_{50} (mg/mL)	22.2+0.00835 *T*-0.223 *t*-0.00781 *T·t*-0.00117 T^2*+0.192 t^2	0.789

[1]*T*, temperature (°C), *t*, time (h); *,**, and ***, significant levels at *P*<0.05, <0.01, and <0.001, respectively.

[2]R^2, multiple correlation coefficients by least-squared fits.

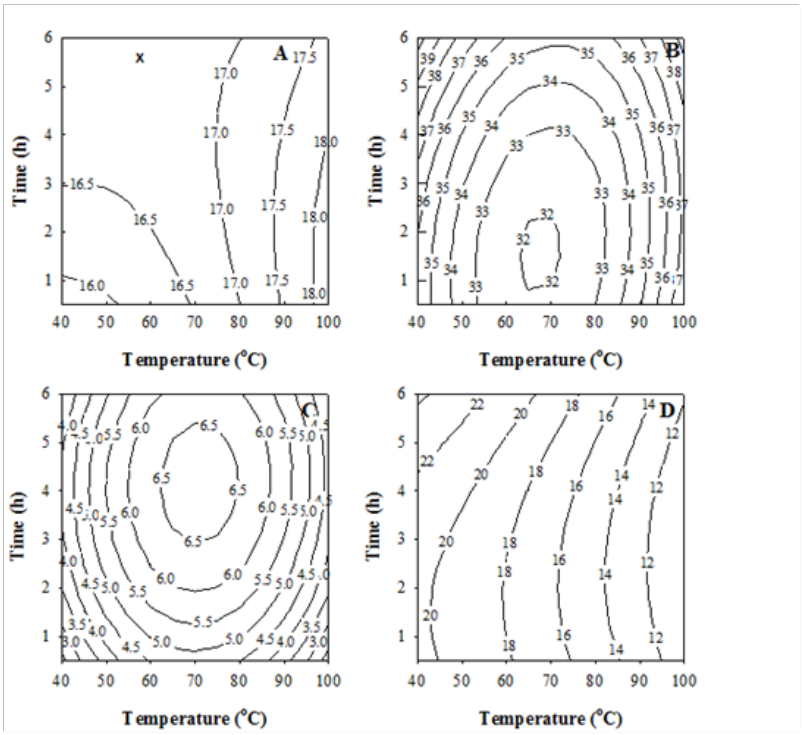

Figure 2 Counter plots for the yield. **(A)** WS-E75S%. **(B)** 50% DPPH□-scavenging concentration (SC_{50}).

(C) 50% Fe^{2+}-chelating concentration (CC_{50}). **(D)** *Chlorella sorokiniana* extracts, with respect to the extraction temperature and time. Numbers indicated on counter lines: response values in the same units as in Table 2 & Table 3.

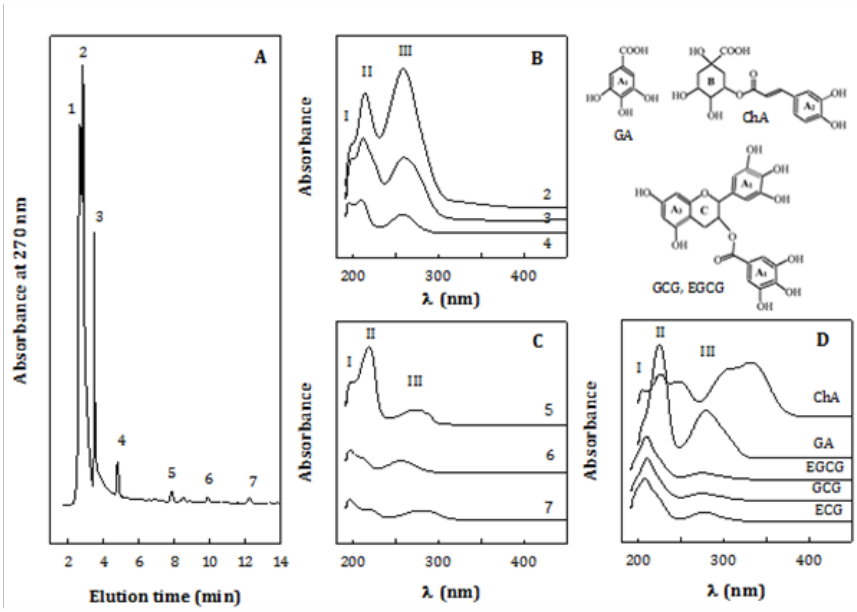

Figure 3 Characterization of 75% ethanol-soluble phytocompounds.

A-HPLC chromatograms. B-C) UV spectra.
D) 75% ethanol soluble (WS-E75S) from Chlorella water extract # 9 (70°C, 3.25h), in reference to UV-spectra of antioxidant phenolic standards.

HPLC-DAD condition: photodiarray detector; Polaris C18 column eluted with acetonitrile/0.1% phosphoric acid; flow rate: 1mL/min. Standards ChA, chlorogenic acid; ECG, (-) epicatechin gallate; EGCG, (-) epigallocatechin gallate; GA, gallic acid; and GCG, (-) gallocatechin gallate.

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Table 5 Retention times and maximal-absorbance wavelengths (□max) of chromatographic fractions from Chlorella WS-E75S and phenolic compound standards eluted byhPLC-DAD1

Peak no.	Retention time (min)	λ_{max} [2] (nm)	Standard[3]	Retention time (min)	λ_{max}(nm)
1, 2	2.62, 2.83	212, 256	GA	4.28	225, 280
3	3.46	198s, 213, 259	ChA	10.07	204, 225, 245, 303, 334
4	4.76	196s, 210, 256	EGCG	10.61	210, 275
5	7.74	198, 219, 278	GCG	12.52	210, 275
6	9.88	197, 210s, 256	ECG	16.06	208, 278
7	12.05	197, 222s, 281			

[1]High performance liquid chromatography with a photodiarray detector and the analysis conditions explained in Figure 3.

[2]The wavelength at which UV absorbance maximized for a peak.

[3]Phenolic standards ChA, chlorogenic acid; ECG, (-) epicatechin gallate; EGCG, (-) epigallocatechin gallate; GA, gallic acid; and GCG, (-) gallocatechin gallate.

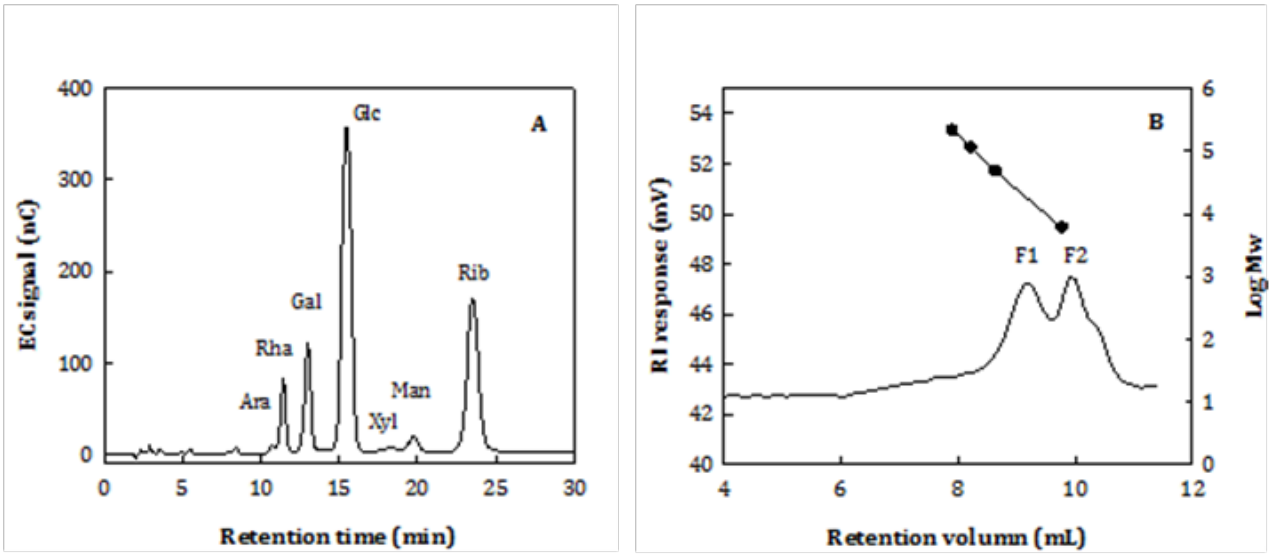

Figure 4 It shows the retention times of glucose and rhibose.

A. Neutral monosaccharide compositions by HPAEC **B.** Molecular distribution by HPSEC. **C.** Hot-water extract from *C. sorokiniana* (extraction condition: 100°C for 1h). Ara, arabinose, Gal, galactose, Glc , glucose, Man, mannose, Rha, rhamnose, and Rib, ribose. Calibration standards: Pullulans (•).

Conclusion

In this study, the estimated polynomials for yield, WS-E75S%, DPPH□ scavenging SC_{50} and Fe^{2+} chelating CC_{50} as a function of temperature and time had been statistically obtained and could explain 78.9-82.2% of data variations. An optimal extraction condition was discovered at 100°C for 1h, associated with the highest yield (~18% (w/w)), WS-E75S% (~38% (w/w)), and antioxidant activities (statistically predicted SC_{50}~3.0mg/mL and CC_{50}~11mg/mL). The antioxidant activities could be mainly related to its phenolic compounds (TPC~3.17GAEmg/g) and WS-E75S, and partly to biopolymers. Where the WS-E75S was mainly composed of compounds like nucleic acids and analogues with ethylene structure and polyunsaturated fatty acids, fatty alcohols, or phytols with acyl dienes, buta-1, 3-diene or ethylene structure. The water extract at 100°C for 1h contained neutral carbohydrates at a ratio of Glc:Rib:Gal:Rha:Man:Xyl:Ara: 52.4:25.9:8.6:7.3:3.0:2.2:0.7 in mol% and two molecular fractions. These results will facilitate developing new combined processes involving counter-current chromatography for continuously collecting functional ingredients from *Chlorella* water extract (CGF), such as antioxidant WS-E75S (ribose, nucleic acids, polyunsaturated fatty acids, and their derivatives) and immunomodulator biopolymers, and from the residues after water extraction. More investigations will be done in the future on examining the functionality *in vitro* and *in vivo* of the isolates and on optimal isolation processes and characterization for more functional ingredients from *Chlorella* residues.

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Conflict of interest

The authors declare that there are no conflicts of interest.

References

1. Skjanes K, Rebours C, Lindblad P. Potential for green microalgae to produce hydrogen, pharmaceuticals and other high value products in a combined process. *Crit Rev Biotech*. 2013;33(2):172–215.
2. Safi C, Zebib B, Merah O, et al. Morphology, composition, production, processing and applications of *Chlorella vulgaris*: a review. *Renew Sustain Energy Rev*. 2014;35:265–278.
3. Hidaka S, Okamoto Y, Arita M. A hot water extract of *Chlorella pyenoidosa* reduces body weight and serum lipids in ovariectomized rats. *Phytother Res*. 2004;18(2):164–168.
4. Hasegawa T, Kimura Y, Hiromatsu K, et al. Effect of hot water extract of *Chlorella vulagris* on cytokine expression patterns in mice with murine acquired immunodeficiency syndrome after infection with *Listeria monocytogenes*. *Immunopharmacology*. 1997;35(3):273–282.
5. Hasegawa T, Ito K, Ueno S, et al. Oral administration of hot water extracts of *Chlorella vulgaris* reduces Ig E production against milk casein in mice. *Int J Immunopharmacol*. 1999;21(5):311–323.
6. Halperin SA, Smith B, Nolan C, et al. Safety and immunoenhancing effect of a *Chlorella*-derived dietary supplement in healthy adults undergoing influenza vaccination: randomized, double-blind, placebo-controlled trial. *CMAJ*. 2003;169(2):111–117.
7. Kralovec JA. AU2001287382B; (2005) US patents 6974576; 6977076; (2003) US patent 6551596; 2006.
8. Morita K, Ogata M, Hasegawa T. Chlorophyll derived from *Chlorella* inhibits dioxin absorption from the gastrointestinal tract and accelerates dioxin excretion in rats. *Environ Health Perspect*. 2001;109(3):289–294.
9. Takekoshi H, Mizoguchi T, Komasa Y, et al. Suppression of glutathione S-transferase placental form-positive foci development in rat hepatocarcinogenesis by *Chlorella pyrenoidosa*. *Oncol Reports*. 2005;14(2):409–414.
10. Kralovec JA, Metera KL, Kumar JR, et al. Immunostimulatory principles from *Chlorella pyrenoidosa*-Part 1: Isolation and biological assessment *in vitro*. *Phytomedicine*. 2007;14(1):57–64.
11. Hsu HY, Jeyashoke N, Yeh CH, et al. Immunostimulatory bioactivity of algal polysaccharides from *Chlorella pyrenoidosa* activates macrophages via Toll-like receptor 4. *J Agric Food Chem*. 2010;58(2):927–936.
12. Morgese MG, Mhillaj E, Francavilla M, et al. *Chlorella sorokiniana* extract improves short-term memory in rats. *Molecules*. 2016;21(10):1311.
13. Lai P, Huang YT, and Jiang KY. Optimization for rapid separation of immunostimulatory *Chlorella* biopolymers. *Gums and stabilizers for the food industry*. Cambridge, UK: Royal Society of Chemistry; 2016;16:123–130.
14. Yang F, Shi Y, Sheng J, Hu Q. *In vivo* immunomodulatory activity of polysaccharides derived from *Chlorella pyrenoidosa*. *Eur Food Res Technol*. 2006;224(2):225–228.
15. Ko SC, Kim D, Jeon YJ. Protective effect of a novel antioxidative peptide purified from a marine *Chlorella ellipsoidea* protein against free radical-induced oxidative stress. *Food Chem Toxicol*. 2012;50(7):2294–2302.
16. Ramanna L, Guldhe A, Rawat I, et al. The optimization of biomass and lipid yields of *Chlorella sorokiniana* when using wastewater supplemented with different nitrogen sources. *Bioresour Technol*. 2014;168:127–135.
17. Lee OK, Oh, YK, Lee EY. Bioethanol production from carbohydrate-enriched residual biomass obtained after lipid extraction of *Chlorella sp*. KR-1. *Bioresour Technol*. 2013;196:22–27.
18. Sato M, Ramarathnam N, Suzuki Y, et al. Varietal differences in the phenolic content and superoxide radical scavenging potential of wines from different sources. *J Agric Food Chem*. 1996;44(1):37–41.
19. Lowery OH, Rosebrough NJ, et al. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951;193(1):265–275.
20. Dubois M, Gilles KA, Revers AP, et al. Calorimetric method for determination of sugars and related substance. *Anal Chem*. 1956;28(3):350–356.
21. Ai L, Chung YC, Jeng KCG, et al. Antioxidant hydrocolloids from herb *Graptopetalum paraguayense* leaves show anti-colon cancer cells and anti-neuroinflammatory potentials. *Food Hydrocoll*. 2017;73:51–59.
22. Shimada K, Fujikawa K, Yahara K, et al. Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *J Agric Food Chem*. 1992;40(6):945–948.
23. Dinis TCP, Madeira VMC, Almeida LM. Action of phenolic derivatives (acetaminophen, salicylate, and 5-amino salicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch Biochem Biophys*. 1994;315(1):161–169.
24. Yen GC, Chen HY. Antoixidant activity of various tea extracts in relation to their antimutagenicity. *J Agric Food Chem*. 1995;43(1):27–32.
25. Suárez ER, Kralovec JA, Noseda MD, et al. Isolation, characterization and structural determination of a unique type of arabinogalactan from an immunostimulatory extract of *Chlorella pyrenoidosa*. *Carbohydr Res*. 2005;340(8):1489–1498.
26. Mistry BD. Ultraviolet spectroscopy. *A Handbook of Spectroscopic Data Chemistry (UV, IR, PMR, ^{13}C NMR and Mass Spectroscopy)*. chapter 1. Jaipur, India: Oxford book company; 2009.
27. Delmonte P, Fardin Kia AR, Aldai N, et al. Analysis of conjugated and other fatty acids. *Conjugated Linoleic Acids and Conjugated Vegetable Oils, C 7*. Cambridge, UK: Royal Society of Chemistry; 2014.
28. Held P. Peptide and amino acid quantification using UV fluorescence in synergy HT multi-mode microplate reader; 2003.
29. Khattab R, Rempel C, Suh M, et al. Quality of canola oil obtained by conventional and supercritical fluid extraction. *Am J Anal Chem*. 2012;3(12A):966–976.
30. Allard B, Templier J. Comparison of neutral lipid profile of various trilaminar outer cell wall (TLS)-containing microalgae with emphasis on algaenan occurrence. *Phytochemistry*. 2000;54(4):369–380.
31. Wang J, Yue YD, Tang F, et al. Screening and analysis of the potential bioactive components in rabbit plasma after oral administration of hot-water extracts from leaves of *Bambusa textilis* mcclure. *Molecules*. 2012;17(8):8872–8885.
32. Hasegawa T, Noda K, Kumamoto S, et al. *Chlorella vulgaris* culture supernatant (CVS) reduces psychological stress-induced apoptosis in thymocytes of mice. *Int J Immunopharmacol*. 2000;22(11):877–885.
33. Burczyk J, Terminska-Pabis K, Smietana B. Cell wall neutral sugar composition of Chlorococcalean algae forming and not forming acetolysis resistant biopolymer. *Phytochemistry*. 1995;38(4):837–841.
34. Sheng J, Yu F, Xin Z, et al. Preparation, identification and their antitumor activities in vitro of polysaccharides from *Chorella pyrenoidosa*. *Food Chem*. 2007;105(2):533–539.

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