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Efficient solvent extraction of antioxidant-rich extract from a tropical diatom, *Chaetoceros calcitrans* (Paulsen) Takano 1968

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ABSTRACT

Objective: To compare the *in vitro* antioxidant capacity of a diatom, *Chaetoceros calcitrans* (*C. calcitrans*) extracted using six types of solvents.
Methods: Each extract was evaluated in terms of extraction yield, total carotenoid, fucoxanthin content, total phenolic and antioxidant capacities (DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging activity and iron chelating activity).
Results: The methanol extract exhibited the highest yield [(22.71 ± 0.96) g/100 g dry weight (DW)], total carotenoid [(4.46 ± 0.36) mg/g DW], total phenolic [(2.49 ± 0.08) mg gallic acid equivalents/g DW] and second highest fucoxanthin content [(2.08 ± 0.03) mg fucoxanthin/g DW] as compared to other solvent extracts. Methanolic extract also exhibited significantly higher ($P < 0.05$) scavenging (DPPH$^{\bullet}$, ABTS$^{\bullet+}$) and iron chelating activities.
Conclusions: Methanol was the recommended solvent for the production of antioxidant rich extract from *C. calcitrans*. Both carotenoids and phenolic acids were found to be positively correlated to the antioxidant capacities of *C. calcitrans*. Lead bioactives confirmed by subsequent high performance liquid chromatography studies were fucoxanthin, gallic acid and protocatechuic acid.

1. Introduction

Lifestyle diseases including atherosclerosis, Alzheimer's disease and cancer are related to chronic oxidative stress [1]. Consequently, the demand for natural antioxidants was reported to exceed supply [2]. In recent years, the concept of antioxidant-rich extract was introduced to the functional food and nutraceuticals sector to fortify and add nutritional value to existing conventional foods (bread, beverages and eggs). It would be a cost and time effective strategy to this industry as it does not require stringent isolation of pure compounds as do drug and pharmaceutical industries. In fact, it is beneficial to extract a group of active compounds rather than single compound because interaction in a combinational group would exhibit synergistic and protagonist effect that contributes to elevated antioxidant capacities [3,4]. In the case of the diatoms, they are not only producers of carotenoids but also phenolic compounds. The preparation of antioxidant rich extract consisting of both carotenoids and phenolic compounds from diatoms would be more time and cost effective, especially if the same amount of biomass used could exert a higher antioxidant activity due to co-extraction of other active compounds.

Diatoms (class Bacillariophyceae) serve as promising sources of sustainable antioxidants because they are effective radical scavengers [5]. In addition, they have the ability to adapt and rapidly grow either in open or closed cultivation facilities [6].

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Controlled culture system enables biomass growth and production of the desired compounds to be closely monitored and manipulated through adjustments of physical (pH, temperature, salinity) and chemical (culture media) parameters [7,8], thus guaranteeing a steady and continuous supply of antioxidants. More importantly, to obtain a substantial amount of antioxidants from diatoms for commercialization purpose does not only depend on the culture conditions but how to effectively yet economically recover the most antioxidant compounds from the biomass. Solvents play an indispensable role in the extraction of bioactive compounds due to their characteristic polarity index *e.g.*, chloroform (4.1) < methanol (MeOH) (5.1) = acetone (ACE) (5.1) < ethanol (EtOH) (5.2) < water (9.0) [9]. Therefore, the selection of solvent closest to polarity of desired compounds is a crucial step to ensure most, if not all of the compounds to be extracted from biomass. This is important for the production of a final extract containing the highest amount of desired bioactives and subsequently higher antioxidant activities. For example, previous scientific reports on carotenoid and phenolic acids extraction from microalgae used a diverse range of solvents with different polarities including chloroform [10], 90% acetone [11], methanol [12] and ethanol [13]. This resulted in different carotenoid yields which could ultimately affect final antioxidant capacities. To date, there are limited standard methods or literature that could recommend the best solvent to recover the highest possible amount of antioxidants and its co-extracts from microalgae.

Therefore, this study aimed to evaluate the effectiveness of six different solvent systems [(MeOH, EtOH, ACE, 9:1 v/v acetone and water (AW), 9:1 v/v acetone and chloroform (AC) and 8:1:1 v/v/v acetone, chloroform and methanol (ACM)] to simultaneously extract major antioxidant compounds (i.e., carotenoids and phenolic compounds) to finally produce an antioxidant rich extract from the tropical marine diatom, *Chaetoceros calcitrans* (*C. calcitrans*). Extracts from each solvent system were compared based on the evaluation of carotenoids and phenolic contents as well as antioxidant activities (radical scavenging and iron chelating ability). Subsequently, major carotenoid and phenolic compounds were profiled to identify lead compounds.

2. Materials and methods

2.1. Chemicals and materials

All chemicals and reagents used were of analytical grade or high performance liquid chromatography (HPLC) grade. Methanol, ethanol, chloroform and acetone were purchased from Merck KGaA (Darmstadt, Germany). Fucoxanthin, 3,4,5-trihydroxybenzoic acid (gallic acid), 3,4-dihydroxybenzoic acid (protocatechuic acid), 3-caffeoylquinic acid (chlorogenic acid), 2,5-dihydroxybenzoic acid (gentisic acid), 4-hydroxybenzoic acid (*p*-hydroxybenzoic acid), 3,4-dihydroxycinnamic acid (caffeic acid), 4-hydroxy-3-methoxybenzoic acid (vanillic acid), O-methylated trihydroxybenzoic acid (syringic acid), 3,5-dimethoxy-4-hydroxycinnamic acid (sinapic acid), 4-hydroxy-3-methoxycinnamic acid (ferulic acid), 3,4-dimethoxybenzoic acid (dimethyl protocatechuic acid), (2″R″)-2-[[(2″E″)-3-(3,4-dihydroxyphenyl)-1-oxo-2-propenyl]]oxy]-3-(3,4-dihydroxyphenyl) propanoic acid (rosmarinic acid), (E)-3-phenylprop-2-enoic acid (cinnamic acid), sodium hydrogen carbonate ($NaHCO_3$), Iron(II) chloride ($FeCl_2$), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), potassium persulfate ($K_2S_2O_8$), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′4″-disulfonic acid monosodium salt (ferrozine), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Folin–Ciocalteu phenol reagent, iron (III) chloride ($FeCl_3 \cdot 6H_2O$), manganese chloride ($MnCl_2 \cdot 4H_2O$), boric acid (H_3BO_3), ethylene diamine tetraacetic acid disodium (Na-EDTA), sodium hydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$), sodium nitrate ($NaNO_3$), zinc chloride ($ZnCl_2$), cobalt chloride ($CoCl_2 \cdot 6H_2O$), ammonium molybdate [$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$], copper sulphate ($CuSO_4 \cdot 5H_2O$), silica ($Na_2SiO_3 \cdot 9H_2O$), cobalamin (vitamin B_{12}) and ammonium formate (NH_4HCO_2) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA).

2.2. Microalgae biomass cultivation and collection

The marine diatom, *C. calcitrans* (UPMC-A0010) was cultured in 120 L capacity annular photobioreactors containing UV-sterilized seawater supplemented with Conway medium [1.3 g/L $FeCl_3 \cdot 6H_2O$, 0.36 g/L $MnCl_2 \cdot 4H_2O$, 33.6 g/L H_3BO_3, 45.0 g/L Na-EDTA, 20 g/L $NaH_2PO_4 \cdot 2H_2O$, 100 g/L $NaNO_3$, 2.1 g/L $ZnCl_2$, 2.0 g/L $CoCl_2 \cdot 6H_2O$, 0.9 g/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 2.0 g/L $CuSO_4 \cdot 5H_2O$, 46.5 g/L $Na_2SiO_3 \cdot 9H_2O$, 1 mL vitamin B_{12}] under constant light (~150 μmol $m^{-2}s^{-1}$, light/dark 12:12 cycle), temperature (23–25 °C) and pH (8–8.5) for 14 days. Microalgae biomass was dewatered with a tubular separator model J-1250 (Hanil Science, Industrial Co. Ltd., Korea) with a final rinse of 1.0 mol/L ammonium formate to remove excess salt crystals. Biomass was collected in clean sample bottles, lyophilized and stored at −80 °C prior to analysis.

2.3. Extraction of antioxidant compounds from *C. calcitrans* biomass

The lyophilized microalgae biomass was sieved through a 250 micron sized sieve. Fifty milliliters of methanol were added to 0.1 g of microalgae biomass and homogenized (Ultra-Turax T25 basic, IKA®-WERKE GmbH & Co. KG, Staufen, Germany) at 9500 r/min for 15 min. This was followed by sonication (Power sonic 505, HwaShin Technology Co., Seoul, Korea) for 30 min at room temperature. Mixtures were filtered through Whatman No. 1 filter paper. Pellet was collected and added with another 50 mL of methanol for the second extraction. This procedure was repeated until the third extraction. Collected filtrates containing solvent and extracts were separated from each other under reduced pressure (Rotavapor R-210, Buchi, Postfach, Flawil, Switzerland) followed by lyophilisation and subsequently stored at −80 °C until further analysis. Extraction procedure was repeated with other solvent systems: 100% EtOH, 100% acetone (ACE), 90% acetone: 10% water (AW), 90% acetone: 10% chloroform (AC), and 80% acetone: 10% chloroform: 10% MeOH (ACM). The experiment was done in triplicates, the yield of the extract determined and expressed as g/100 g dry weight (DW).

2.4. Determination of total carotenoid content

The carotenoid content of *C. calcitrans* extracts was quantified spectrophotometrically at 470 nm, 581 nm, 631 nm and

664 nm. Initially, 1% (w/v) of each extract was diluted with 90% acetone to attain an absorbance of less than one when necessary. Carotenoid content was calculated using equation by Seely *et al.* and results expressed in mg/g DW [14].

2.4.1. Quantification of the major carotenoid fucoxanthin

For accurate quantification of fucoxanthin in the extracts, HPLC was conducted following a modified method by Kim *et al.* [15]. Twenty microliters of standards and samples were injected with an Agilent G1301A auto sampler into an Agilent 1300 series HPLC series (Agilent Technologies Inc., Alpharetta, GA, USA) equipped with a DAD 1400 diode array detector. Carotenoid separations were performed on a Merck chromolith RP-18e (3 mm × 4.6 mm inner diameter 2 μm pore size) at an absorbance of 445 nm. The mobile phase flow rate was 1 mL/min where the gradient was set at 100% water (A) and 100% methanol (B): starting from 0% to 100% A in 2 min, 100% to 50% A in 3 min, 50% to 25% A in 4 min, 25% to 10% A in 6 min, 10% to 5% A in 8 min, and 0% to 100% B in 15 min. All samples were filtered through 0.22 μm polytetrafluorethylene syringe filter before injection. The standard curve and retention times were calibrated using fucoxanthin standard in methanol at six different concentrations (1 000, 500, 250, 125, 62.5, 31.25 μg/mL). All samples were analyzed in triplicates and results were expressed in milligram fucoxanthin per gram biomass dry weight (mg FX/g DW).

2.5. Determination of total phenolic content (TPC)

TPC was determined using Folin–Ciocalteu reagent assay modified from Kabir *et al.* [16]. In brief, 500 μL of 1 mg/mL samples were reacted with 2.5 mL of 10% (v/v) Folin–Ciocalteu reagent and 2.0 mL of 7.5% (w/v) sodium bicarbonate solution in triplicates. After 1 h of incubation at 40 °C, absorbance of each reaction mixture was recorded at 765 nm spectrophotometrically (PharmaSpec UV 1601, Shimadzu, Kyoto, Japan). Gallic acid was used as the standard and TPC values of extracts were expressed in milligram gallic acid equivalent per gram biomass dry weight (mg GAE/g DW).

2.5.1. Quantification of major phenolic compounds

Quantification of major phenolic acids in extracts followed the method by Chan *et al.* [17]. Phenolic acid separations of extracts were performed on an Agilent ZORBAX SB C-18 column (150 × 4.6 mm, 5 μm) at 280 nm and 320 nm. Each sample (n = 3) was filtered through a 0.22 μm polytetrafluorethylene syringe filter prior to HPLC injection at an injection volume of 20 μL into an Agilent 1300 series HPLC (Agilent Technologies Inc., Alpharetta, GA, USA). The flow rate of the mobile phase was set at 1 mL/min with a gradient elution of water–acetic acid (95:5, v/v) (A) and methanol–acetonitrile–acetic acid (95:5:1, v/v/v) (B): starting from 0% to 5% B in 2 min, 5%–24% B in 8 min, 25%–40% B in 10 min, 40%–50% B in 10 min, 50%–100% B in 10 min, 100% B in 5 min, and 100%–5% B in 5 min. Pure reference standards investigated included gallic acid, protocatechuic acid, chlorogenic acid, gentisic acid, *p*-hydroxybenzoic acid, caffeic acid, vanillic acid, syringic acid, sinapic acid, ferulic acid, dimethyl protocatechuic acid, rosmarinic acid, and cinnamic acid. Each standard was fully solubilized in mobile phase B and concentration prepared at 1 000, 500, 250, 125, 62.5, 31.25 μg/mL in order to plot respective standard curves. Results were expressed in μg/g DW.

2.6. Antioxidant activities

2.6.1. DPPH$^{\bullet}$ scavenging activity

DPPH radical scavenging activity of the extracts was conducted in accordance to modified method described by Chan *et al.* [17]. Fifty microliters of sample and trolox standard were reacted with 195 μL of 0.2 mmol/L DPPH methanolic solution in a 96-well micro titre plate. Mixtures were incubated in the dark and at room temperature for an hour and absorbance was read at 540 nm (Multiskan™ GO UV/vis microplate spectrophotometer, Thermo Fisher Scientific, USA). DPPH$^{\bullet}$ scavenging activity of the tested extracts was expressed in milligram trolox equivalent per gram biomass dry weight (mg TE/g DW).

2.6.2. ABTS$^{\bullet+}$ scavenging activity

ABTS$^{\bullet+}$ scavenging activity was determined according to method by Re *et al.* with slight modifications [18]. ABTS$^{\bullet+}$ was generated by reacting 50 mL of 7 mmol/L ABTS stock solution with 50 mL of 2.45 mmol/L potassium persulfate and left for 24 h in the dark. The next day, ABTS$^{\bullet+}$ working solution was obtained by dilution to an absorbance of 0.70 ± 0.05 at 734 nm (Pharmaspec UV-1700, Shimadzu, Kyoto, Japan). Subsequently, 950 μL of working solution was added to 50 μL sample and left for 10 min in the dark. Absorbance of radical-sample mixture was measured at 734 nm. Trolox standard was used and antioxidant activity was expressed in mg TE/g DW.

2.6.3. Iron chelating assay

Ferrous ion-chelating ability followed method by Decker and Welch [19]. One hundred microliters of each extract was added with 135 μL distilled water and 5 μL of 2 mmol/L $FeCl_2$. Reactions were conducted in a 96 well micro titre plate and initiated with the addition of 10 μL of 5 mmol/L ferrozine. The solutions were let to stand for 10 min and read at 562 nm (Multiskan™ GO UV/vis microplate spectrophotometer, Thermo Fisher Scientific, USA). Distilled water (100 μL) was used as negative control. Na-EDTA was used as standard and results were expressed in mg Na-EDTA equivalent/g DW).

2.7. Statistical analyses

Test of normality followed by ANOVA and Duncan's multiple range *post hoc* test was carried out to analyze significant differences ($P < 0.05$) between tested extracts in antioxidant activities using statistical program, SPSS version 21.0 (SPSS Inc., Chicago, USA). Pearson correlation, r was used to test the quantitative correlation between total carotenoid and phenolic content with their antioxidant activities. Values were given as the means ± SD of triplicates.

3. Results

Significant variation in the yield of extraction was observed among different solvent systems (Table 1). The highest extractable yield was obtained by methanolic extraction (22.71 g/100 g DW), significantly different ($P < 0.05$) from

Table 1
The extraction yield, total carotenoid, fucoxanthin and TPC of *C. calcitrans* extract from different solvent systems.

Solvent system	Extract yield (g/100 g DW)	Total carotenoid content (mg/g DW)	Fucoxanthin content (mg FX/g DW)	TPC (mg GAE/g DW)
Acetone	10.66 ± 0.75^{d}	4.43 ± 0.24^{a}	1.73 ± 0.05^{c}	1.56 ± 0.05^{c}
AW	10.41 ± 1.50^{c}	3.07 ± 0.74^{b,c}	0.53 ± 0.09^{e}	1.02 ± 0.16^{d}
MeOH	22.71 ± 0.96^{a}	4.46 ± 0.36^{a}	2.08 ± 0.03^{b}	2.49 ± 0.08^{a}
EtOH	18.06 ± 0.11^{b}	4.11 ± 0.07^{a,b}	2.32 ± 0.03^{a}	1.86 ± 0.09^{b}
AC	3.66 ± 0.76^{e}	2.67 ± 0.52^{c,d}	0.75 ± 0.04^{d}	0.66 ± 0.05^{e}
ACM	10.50 ± 1.08^{d}	1.56 ± 0.04^{d}	0.86 ± 0.02^{d}	0.82 ± 0.07^{d,e}

Results are presented as mean ± SD of three determinations. $^{a, b, c, d, e, f}$Within the same column indicate significant difference ($P < 0.05$).

others. This was followed by ethanol (18.06 g/100 g DW), AW (10.41 g/100 g DW), acetone (10.66 g/100 g DW), ACM (10.50 g/100 g DW) and AC (3.66 g/100 g DW) extracts.

Extracts with higher total carotenoid content were found in methanol (4.46 mg/g DW), acetone (4.43 mg/g DW) and ethanol (4.11 mg/g DW). These values were significantly higher than AW (3.07 mg/g DW), AC (2.67 mg/g DW) and ACM (1.56 mg/g DW). The HPLC representative chromatogram of fucoxanthin standard solution (100 μg/mL) was shown in Figure 1. Results revealed ethanol extract (2.32 mg FX/g DW) and methanol extracts (2.08 mg FX/g DW) to contain more fucoxanthin.

Total extractable phenolic content in *C. calcitrans* was determined via a linear gallic acid standard curve ($y = 0.0082x + 0.0335$; $R^2 = 0.9988$). TPC of all tested extracts ranged from 0.66 mg GAE/g DW to 2.49 mg GAE/g DW (Table 1). The highest was found in the methanol extract (2.49 mg GAE/g DW) which was significantly different ($P < 0.05$) from ethanol extract (1.86 mg GAE/g DW), acetone extract (1.56 mg GAE/g DW), AW extract (1.02 mg GAE/g DW), ACM (0.82 mg GAE/g DW) and AC extract (0.66 mg GAE/g DW). Research reports on brown macroalgae polyphenols revealed types of phenolic compounds found included phlorotannins (eckol, dieckol, phloroeckol, phloroglucinols) [20].

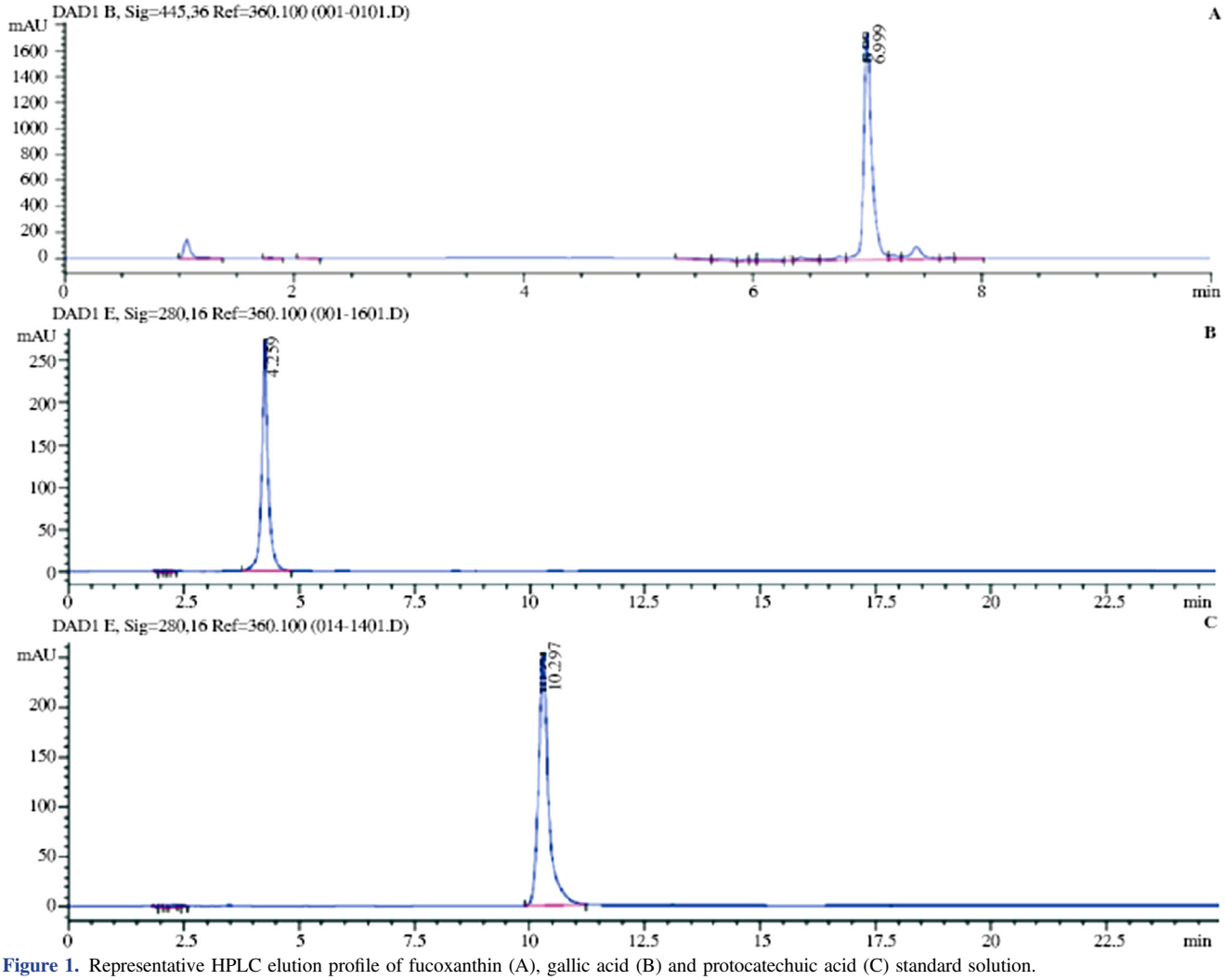

Figure 1. Representative HPLC elution profile of fucoxanthin (A), gallic acid (B) and protocatechuic acid (C) standard solution.

Table 2
Major phenolic composition of different *C. calcitrans* extracts quantified by HPLC-DAD.

Phenolic composition	Concentration of individual phenolic compounds in *C. calcitrans* extracts					
	Acetone (μg/g DW)	AW (μg/g DW)	MeOH (μg/g DW)	EtOH (μg/g DW)	AC (μg/g DW)	ACM (μg/g DW)
Gallic acid	11.05 ± 0.04^{e}	48.51 ± 0.32^{c}	165.77 ± 11.00^{a}	82.28 ± 6.79^{b}	31.62 ± 7.20^{d}	49.13 ± 1.36^{c}
Proto-catechuic acid	79.09 ± 0.11^{c}	73.93 ± 1.36^{c}	326.78 ± 4.45^{a}	192.36 ± 2.25^{b}	48.54 ± 0.03^{d}	32.64 ± 7.23^{e}
Chlorogenic acid	nd	nd	nd	195.16 ± 0.66	nd	nd
Gentisic acid	nd	nd	107.49 ± 8.71	nd	nd	nd
p-Hydroxybenzoic acid	nd	nd	100.30 ± 19.33	nd	nd	nd
Caffeic acid	nd	nd	nd	2.50 ± 0.00	nd	nd
Vanillic acid	nd	nd	348.87 ± 7.70^{a}	nd	1.22 ± 0.01^{c}	12.65 ± 5.00^{b}
Syringic acid	nd	nd	nd	nd	nd	4.19 ± 0.98
Sinapic acid	nd	nd	nd	nd	0.45 ± 0.34	nd
Ferulic acid	nd	nd	nd	nd	nd	nd
Dimethyl protocatechuic acid	nd	nd	nd	63.59 ± 1.27	nd	nd
Rosmaric acid	nd	nd	10.70 ± 2.00^{a}	13.00 ± 6.39^{a}	nd	nd
Cinnamic acid	nd	nd	nd	6.69 ± 0.72	nd	nd
Sum	90.10	122.40	1059.61	555.60	81.83	98.60

nd: Not detected. Results are presented as mean ± SD of three determinations. $^{a, b, c, d, e}$Within the same row indicate significant difference ($P < 0.05$).

However, identification of phenolic compounds especially from the class Bacillariophyceae remained limited. This study presented the phenolic profiles of *C. calcitrans* in Table 2. From the results, hydroxybenzoic acids (protocatechuic and gallic acid) were the major phenolic acids (Figure 1). It was found that in tested samples 30%–80% consisted of proto-catechuic acid while gallic acid accounted 13%–50% of the investigated phenolic compounds. It was also revealed that methanol was the only solvent capable of extracting gentisic acid [(107.5 ± 8.7) μg/g DW], *p*-hydroxybenzoic acid [(100.3 ± 19.3) μg/g DW] and vanilic acid [(348.9 ± 7.7) μg/g DW] in the present study. These compounds were undetected in other solvent systems which might have contributed to the higher antioxidant activity in methanol extracts as shown in Figure 2.

In terms of antioxidant activities, it was observed that methanol extracts exhibited the highest DPPH$^{\bullet}$ scavenging activity (1.435 mg TE/g DW). This was significantly different ($P < 0.05$) from ethanol (0.844 mg TE/g DW), AW (0.665 mg TE/g DW), ACM (0.173 mg TE/g DW), and AC (0.067 mg TE/g DW) extracts (Figure 2). ABTS$^{\bullet+}$ assay demonstrated a similar trend of antioxidant efficacy as DPPH$^{\bullet}$ scavenging assay. It was found that methanol extracts exhibited the highest antioxidant activity (10.593 mg TE/g DW), followed by ethanol (7.210 mg TE/g DW), AW (4.159 mg TE/g DW), acetone (3.046 mg TE/g DW), ACM (2.528 mg TE/g DW) and AC (1.211 mg TE/g DW) extracts. Overall, all extracts exhibited free radical scavenging activity but at different extents.

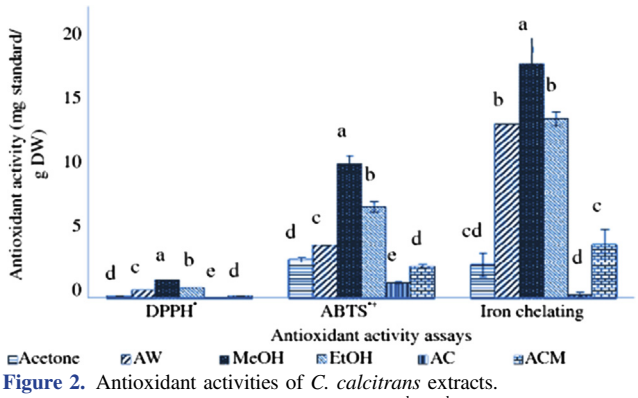

Figure 2. Antioxidant activities of *C. calcitrans* extracts.
Results are presented as mean ± SD ($n = 3$). $^{a, b, c, d, e}$: Indicate significant difference ($P < 0.05$).

Besides demonstrating antioxidant efficacy through primary antioxidant mechanisms, all extracts showed positive effects via secondary antioxidant mechanisms as well. Following the similar trend of radical scavenging activity assays, methanol extracts consistently exhibited the highest iron chelating activity (18.52 mg Na-EDTA/g DW) which was significantly different ($P < 0.05$) from ethanol (14.17 mg Na-EDTA/g DW), AW (13.79 mg Na-EDTA/g DW), ACM (4.25 mg Na-EDTA/g DW), acetone (2.64 mg Na-EDTA/g DW) and AC (0.29 mg Na-EDTA/g DW).

4. Discussion

Methanol extractability of *C. calcitrans* in this study was found to be higher (22.71%) compared to methanolic extracts of commercial microalgae, *Spirulina platensis* (7.3%) [21] or brown macroalgae, *Sargassum polycystum* (4.05%) [22]. Besides that, findings from this study showed that carotenoids in *C. calcitrans* could preferentially dissolve in semi polar solvents (methanol, ethanol) as compared to more polar or less non-polar solvents. Carotenoid contents (4.46 mg/g DW) in this study were found to be two times higher than that from *C. calcitrans* [(2.33 ± 0.14) mg/g DW] methanolic extracts reported by Goiris *et al.* [13]. This may be due to differences in growth phase or culture conditions which directly affect amount of carotenoids produced by the microalgae cells [23]. Additionally, the carotenoid content within methanolic extracts in this study was almost threefold compared to value reported by Xiao *et al.* [24] in brown macroalgae, *Laminaria japonica* extracts (1.5 mg/g DW). It seemed that microscopic algae were better fucoxanthin sources than their larger counterparts, macroalgae with regard to their higher growth rates and fucoxanthin productivity. Furthermore, fucoxanthin content quantified by HPLC was significantly correlated to total carotenoid content ($r = 0.863$, $P < 0.05$). This was supported by Kim *et al.* [15] who evidenced ability of methanol and ethanol to extract a higher amount of fucoxanthin in comparison to hexane, acetone or water from the diatom,

Phaeodactylum tricornutum. Fucoxanthin content from *C. calcitrans* in the methanol and ethanol extracts accounted to 47%–57% with respect to total carotenoid content. This study showed major pigment type in *C. calcitrans* was fucoxanthin whereas remaining pigments were probably diadinoxanthin, diatoxanthin, fucoxanthinol and β-carotene, pigments found exclusively in class Bacillariophyceae [25].

TPC of *C. calcitrans* in this study was on par with report by Goiris *et al.* [13] [(1.8 ± 0.1) mg/g DW]. In addition, Horax *et al.* highlighted the importance of effect of types of solvents that significantly influenced the quantity of phenolic compounds extracted [26]. For example, a higher amount of phenolic acids and flavonoids can be extracted using methanol compared to hexane [27]. Phenolic quantification with HPLC was parallel to TPC assay as both assays concomitantly showed methanol extracts contained the highest amount of phenolic compounds compared to others with gallic and protocatechuic acids as the major phenolic compounds identified. Hydroxybenzoic acids are strong antioxidants in the emulsion and lipid systems frequently used in the food sector to prevent food spoilage [28]. These phenolic compounds demonstrated effective antioxidant activity *in vitro* comparable to Trolox [29], a vitamin E analogue.

Besides that, DPPH scavenging assay follows a free radical scavenging mechanism and is widely accepted as a food, nutraceutical and medicinal antioxidant assay [30]. Lack of activity in scavenging $DPPH^{\bullet}$ in the earlier study by Natrah *et al.* was probably due to low concentration in initial extracts [31]. In this study, the starting concentration of extracts was increased to reflect results in a more accurate manner. Whereas in the correlation studies, compounds responsible for the $DPPH^{\bullet}$ scavenging activity were phenolics ($r = 0.918$, $P < 0.05$) and carotenoids ($r = 0.762$, $P < 0.05$). Phenolic compounds (gallic acid and protocatechuic acid) have hydroxyl groups loosely bonded to their aromatic ring and thus could easily donate a hydrogen atom or electron to reactive radicals making them important radical scavengers.

$ABTS^{\bullet+}$ scavenging activity assay works via single electron transfer mechanism and is proven reliable to test food products containing both hydrophilic and highly pigmented antioxidants [32,33]. The ethanol extracts (7.21 mg TE/g DW) in the current study exhibited higher $ABTS^{\bullet+}$ scavenging activity as compared to findings by Goiris *et al.* who reported a mean value of 6.04 mg TE/g DW in the same species [13]. This discrepancy could be due to differences in culture environment, culture system, duration of culture and even extraction strategies [34]. Parallel to the result from DPPH scavenging activity assay and in agreement to past studies by Goiris *et al.* and Peng *et al.* [13,35], a significantly positive Pearson correlation was obtained through $ABTS^{\bullet+}$ assay between TPC ($r = 0.958$; $P < 0.05$) and carotenoid content ($r = 0.785$; $P < 0.05$) for all tested extracts. The mechanism of action for fucoxanthin as antioxidants in this free radical scavenging assay involves addition of free radicals to its polyene molecule to reduce the pecking order of oxidants as supported by Takashima *et al.* [36]. In terms of metal chelating activity, it was found that all *C. calcitrans* extracts were able to chelate free iron (Fe^{2+}) and consequently preventing further production of reactive oxygen species (peroxyl and alkoxyl radicals).

Overall, a natural and sustainable bioactive extract having both free radical scavenging and iron chelation capability is highly preferable for exploitation as commercial antioxidants. Our findings revealed among the six extracts, methanol extract consistently exhibited the best performance resulting in the highest extractable yield and antioxidant activities. The choice of solvent used during mass extraction of active compounds is a crucial step as different quantities and types of antioxidant compounds can be extracted which will ultimately influence the antioxidant efficacy. This was supported by Airanthi *et al.* who also recommended methanol as the best extract for antioxidant compounds from Japanese brown seaweed [37]. Thus, the findings from this study provide useful information for future extraction processes particularly antioxidant compounds from microalgae, which can be important active ingredients for applications in the functional food, fortified nutraceuticals, cosmeceuticals and supplement sectors.

This study showed methanol was the recommended solvent for the production of an antioxidant-rich extract from *C. calcitrans*. The methanol extract consistently displayed the highest extraction yield [(22.71 ± 0.96) g/100 g DW], total carotenoid [(4.46 ± 0.36) mg/g DW], fucoxanthin content [(2.08 ± 0.03) mg FX/g DW], total phenolic [(2.49 ± 0.08) mg/g DW] with good free radical scavenging and iron chelating activity. Correlation analysis ($P < 0.05$) showed that antioxidant activities were largely influenced by the amount of carotenoids and phenolic acid extracted. In addition, this study identified the major phytochemicals in *C. calcitrans* were the carotenoid (fucoxanthin) and phenolic acids (protocatechuic and gallic acid).

Conflict of interest statement

We declare that we have no conflict of interest.

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