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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