https://doi.org/10.48048/tis.2022.4687

Contribution of the Solvent Extraction Method for Phenolic Compounds, Flavonoids, and Fucoxanthin to the Antioxidative Properties in Extracts of *Chaetoceros tenuissimus* Meunier

Napapat Rattanachitthawat[1,*], Amornrat Kanokrung[2], Sirichet Rattanachitthawat[3], Kanjana Thumanu[4], Waraporn Tanthanuch[4] and Kwanchayanawish Machana[1,5]

[1]Faculty of Pharmaceutical Sciences, Burapha University, ChonBuri 20131, Thailand
[2]The Institute of Marine Sciences, Burapha University, Chonburi 20131, Thailand
[3]Faculty of Agricultural Technology, Burapha University, Sakaeo Campus 27160, Thailand
[4]Synchrotron Light Research Institute (Public Organization); Nakhonratchasima 30000, Thailand
[5]Pharmaceutical Innovations of Natural Products Unit, Faculty of Pharmaceutical Sciences, Burapha University, Chonburi 20131, Thailand

([*]Corresponding author's email: supatta@go.buu.ac.th)

Received: 14 June 2022, Revised: 11 July 2022, Accepted: 21 July 2022, Published: 10 November 2022

Abstract

Chaetoceros sp. has been found to possess significant amounts of valuable fucoxanthin, phenolics, and flavonoids, with enormous nutraceutical and antioxidant potential. The aim of this study was to employ ultrasound-assisted extraction to determine the antioxidant properties of extracts prepared from *Chaetoceros tenuissimus* using different solvents: Propylene glycol (PG), acetone (AC), and methanol (ME), with difference ultrasonication times (15 and 30 min). The total phenolic (TPC), flavonoid (TFC), and fucoxanthin (FUC) contents were evaluated. Antioxidant capacity was evaluated by DPPH and ABTS radical-scavenging assays and lipid peroxidation by TBARS. The FUC content was analyzed by HPLC-DAD. 30-min PG extract (PG-30) exhibited the highest TPC (2.75 ± 0.14 mg GAE.g^{-1} DW), and FUC (8.76 ± 0.06 mg FUC. g^{-1}). Concerning total antioxidant capacity, PG-30 was the most effective against $DPPH^{\bullet}$ (IC_{50} = 970.19 ± 7.70 µg. mL^{-1}) and TBARS (IC_{50} = 717.05 ± 1.45 µg. mL^{-1}), while 15-min PG extract (PG-15) presented the highest inhibitory activity against $ABTS^{+\bullet}$ (IC_{50} = 150.02 ± 6.95 µg. mL^{-1}). The study findings demonstrate that PG extracts of *C. tenuissimus* exhibit high potential antioxidant activity, likely associated with their content of FUC and phenolics, and could be used in cosmetic preparations and as phytonutrients.

Keywords: *Chaetoceros tenuissimus*, Microalgae, Antioxidant, Flavonoid, Fucoxanthin

Introduction

Microalgae biomass and microalgae-derived compounds are promising sources of novel products with extraordinary pharmacological and biological potential, such as animal and fish feed, cosmetics, and natural products with health benefits [1,2]. The naturally derived functional ingredients from microalgae include proteins, lipids, carbohydrates, phenolics, and different pigments, vitamins, and antioxidant compounds used in different sectors, including nutrition, pharmaceuticals, and cosmetics [3,4]. In addition, microalgae are phototropic, and throughout their lifetime, they are exposed to radical stresses and high oxygen tension, and they have a high capacity to produce numerous efficient protective chemicals against oxidative and radical stressors, as well as cytoprotective agents [5]. Microalgae represent an almost natural resource of antioxidants due to their enormous biodiversity. However, not all groups of microalgae can be used as natural sources of antioxidants. Target products, growth rates or yields, ease of cultivation, and/or other microalgae factors vary greatly. Several studies have demonstrated that carotenoids and phenolic substances contribute significantly to the total antioxidant capacity of microalgae [6-9]. Several reports on microalgae evaluated the antioxidant activity belonging to the genera *Thalassiosira, Chlorella, Dunaliella, Nostoc, Phaeodactylum, Spirulina, Haematococcus,* and *Chaetoceros* [10-15]. In the Gulf of Thailand, there is extraordinary biological diversity, including macroalgae, microalgae, and other microorganisms. Of the many types of microalgae, *Chaetoceros* is one of the genera that live and act as primary producers in marine ecosystems. The existence of this microalga as a primary producer is due to the abundance of their living cells in the ocean [16]. *Chaetoceros,* belonging to the class of Bacillariophyceae, is probably

the largest genus of marine plankton, particularly diatoms, with approximately 400 species described. Investigations of the chemical composition and biological activities of *C. tenuissimus* have been limited thus far [10,16]. This species is found in Japanese coastal waters, Narraganset Bay, the Mediterranean Sea, and the San Matías Gulf; has a high growth rate; and often produces blooms during spring and summer [17]. Thus, this marine diatom is considered a key factor in the preservation of coastal primary production. Furthermore, there are limited reports evaluating the antioxidant activity of microalgae, especially concerning the relationship between their phenolic content and antioxidant capacity. Therefore, it was desirable to identify some rich sources of antioxidants from a large group of microalgae and to evaluate the relationship between these 2 parameters [18].

The extraction process is the primary method of obtaining the maximum amount of target compounds and the greatest biological activity from these extracts. Non-conventional extraction methods, such as ultrasound-assisted extraction (UAE), are often applied to recover nutritionally valuable compounds from plant food materials and algae matter [17]. Ultrasounds generate vibrations that mechanically break the cell structure and disrupt the cell wall, as well as enhancing material transfer by stimulating the extraction of lipids and chemical compounds from microalgae [19]. Moreover, these methods have many advantages, including environmental friendliness, with a significant reduction in solvent, temperature, and time consumption [20]. The advantages of ultrasound-assisted lipid extraction from microalgae have been demonstrated in terms of both efficiency and operational costs [17,21]. The chemical compounds and biological activity of the resulting extract are affected not only by the extraction technique but also by the extraction solvent. Many solvents, including methanol, ethanol, acetone, propylene glycol, and water, have been used to extract bioactive compounds from natural products and algae [15]. This is important to produce a final extract containing the highest concentration of the desired bioactive compound and subsequent high antioxidant activities. As previously reported, a range of different solvent extracts prepared from microalgae with different polarities presented different carotenoid and phenolic contents, which could ultimately affect the final antioxidant capacities [22].

Therefore, this study aimed to evaluate the concentrations of chemical substances (i.e., fucoxanthin, flavonoids, and phenolic compounds); antioxidant activities against 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS); and Thiobarbituric acid reactive substances (TBARS) assay in *C. tenuissimus* extracts prepared with 3 different solvents (methanol, acetone, and propylene glycol) by using UAE.

Materials and methods

Materials

Chemical products, analytical grade methanol, acetic acid, and sulfuric acid were purchased from Labscan® (Thailand). 1,1-diphenyl-2-1 picrylhydrazyl (DPPH), fucoxanthin, Trolox, and other compounds were obtained from Sigma-Aldrich® (St. Louis, MO, USA). Gallic acid, quercetin, and Folin-Ciocalteu reagent were purchased from Merck® (Darmstadt, Germany). The Purelink genomic plant DNA purification kit was from QIAGEN GmbH, Germany. Analytical solvents for the HPLC technique were of HPLC grade, including absolute ethanol, acetone, and methanol, which were from Lab Scan. All reagents were of analytical grade.

Microalgae cultivation and harvest

The monoculture of *C. tenuissimus* used in this study was obtained from the culture collection of The Institute of Marine Sciences at Burapha University, Thailand. The sample was identified using light microscopy and an RT-PCR assay. The microalgae were grown in f/2 medium with a salt concentration of 30 g/L. Stock cultures were grown in Erlenmeyer flasks placed on a shaking platform at 25 °C with a 12 h light/dark cycle. The cell abundance was quantified concurrently every day by examining the samples under an optical microscope with a hemocytometer until the death phase. The specific growth rates of microalgae were calculated using Eq. (1), derived from the formula by Andersen [23], below. A microalgae culture was harvested at the stationary phase of growth. One liter of each culture was collected by centrifugation (6,000 g for 10 min) and washed 3 times using normal saline.

$$\mu/\text{ day} = (\ln N_t - \ln N_0)/ (t_2 - t_1) \quad (1)$$

Where N_t is the final algae concentration, N_0 is the initial algae concentration, and Δt is the initial time (t_1) subtracted from the final time (t_2).

DNA extraction and PCR amplification

For molecular identification, DNA was extracted from cultured microalgae cultured strains using the Purelink genomeic plant DNA purification kit (QIAGEN GmbH, Germany). DNA was extracted using a DNA purification kit according to the manufacturer's protocol. The extracted DNA was quantified using gel electrophoresis (0.8 % agarose gel). Five microliters of the extracted DNA were added directly to a mixture of 25 µL ONE PCR PLUS buffer, 18 µL of ultrapure water, and 1 µL of primers for marker regions from the nuclear encoded ribosomal subunit (rDNA). For the partial 28S D1-D3 region, 10 µM of each forward and reverse primer, D1R-F (ACCCGCTGAATTTAAGCATA) and D3B-R (ACGAACGATTTGCACGTCAG), respectively, were used. Amplification was carried out in a thermal cycler (MultiGene Optimax Thermal Cycler, USA) as follows: initially 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1.5 min. After the last cycle, the extension was completed at 72 °C for 5 min. Ten microliters of PCR products were electrophoresed through 1 % agarose at 35 V for 50 min. Ethidium-bromide-stained gels were studied under UV transillumination. Direct sequencing was carried out against DNA electrophoresis results [24]. Sequence alignment of multiple sequences of microalgal isolates with similarities was performed by using the Basic Local Alignment Search Tool (BLASTn tool https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Preparation of *C. tenuissimus* extracts

Freeze-dried microalgal biomass (0.5 g) was extracted with 5 mL of methanol (ME), acetone (AC), and propylene glycol (PG). Each extraction solution was sonicated for 15 or 30 min using an ultrasonic water bath (SK8210HP, Shanghai KUDOS Ultrasonic Instrument Co., Ltd., Shanghai, China) at room temperature. The experiments were performed with the sample at an initial temperature of 18 °C, while the maximum final temperature was lower than 60 °C. Then, the mixture was centrifuged at 5,000 rpm for 5 min as shown in **Table 1**. Then, microalgae extracts were used for the determination of chemical composition and antioxidant activity.

Table 1 Conditions of ultrasound-assisted extraction used to prepare *C. tenuissimus* extracts from different solvents.

Extract	Solvents	Extraction time (min)
AC-15	Acetone	15
ME-15	Methanol	15
PG-15	PG	15
AC-30	Acetone	30
ME-30	Methanol	30
PG-30	PG	30

Determination of total polyphenolic content (TPC)

The total phenolic content of the extracts (AC-15, ME-15, PG-15, AC-30, ME-30, and PG-30) was evaluated by using the Folin-Ciocalteu colorimetric method described by Chandra *et al*. [25], with slight modifications. Twenty microliters of all extracts were mixed with Folin-Ciocalteu reagent and incubated at room temperature. Then, 180 µL of 7.5 % sodium carbonate was added and incubated in the dark at room temperature for 30 min. The absorbance was detected at a wavelength of 765 nm by a microplate reader (M965 Plus, Metertech Inc., Taipei, Taiwan). The concentration of total phenolics was calculated and compared with a gallic acid standard curve. The results were represented as gallic acid equivalents (GAE mg. g^{-1}ext.). All determinations were carried out in triplicate.

Determination of total flavonoid content (TFC)

The aluminum chloride colorimetric method was used to determine the total flavonoid content of the sample [26]. Quercetin was used as the standard for comparison. A 100 µL volume of diluted quercetin standard or sample solution was mixed with 100 µL of 2 % aluminum chloride. The solution mixture was incubated for 30 min at room temperature. The absorbance was measured at a wavelength of 405 nm by a microplate reader. The total flavonoid content was calculated as milligrams quercetin equivalents (QE g^{-1} ext.). All determinations were carried out in triplicate.

Quantification of fucoxanthin by HPLC-DAD

For accurate quantification of fucoxanthin in the extracts, HPLC-DAD was conducted following a modification of the method of Foo *et al.* [27]. Twenty microliters of standards and samples were injected into an Agilent 1,100 series HPLC (Agilent Technologies Inc., Alpharetta, GA, USA) equipped with a photodiode array detector. A C18 column (Hypersil BDS, 4.6×300 mm inner diameter, 5 μm pore size) was used for separations. The absorbance was detected at a wavelength of 450 nm. The flow rate of the mobile phase, 95 % (v/v) methanol: 5 % (v/v) water, was 0.4 mL/min with isocratic elution. The column temperature was set at 28 °C. All samples were filtered through a 0.22 mm syringe microfilter before injection. Fucoxanthin was used as standard. All samples were analyzed in triplicate, and the results were expressed in milligrams of fucoxanthin per gram of biomass dry weight (mg FUC. g^{-1} DW).

DPPH (2,2-Diphenyl-2-picrylhydrazyl) free radical scavenging assay

The DPPH assay measures the capacity of an antioxidant to scavenge the DPPH radical by electron donation. In the presence of radical scavenger, the purple DPPH radical is reduced to a pale-yellow compound, and the discoloration of the radical is measured at 515 nm. The DPPH radical scavenging capacity of microalgae extracts was evaluated by the slightly modified method of Vongsak *et al.* [28]. Trolox was used as a reference compound. Twenty microliters of the extract and a standard Trolox control at varied concentrations (10 - 1,000 μg/mL) were mixed with 180 μL of 1 mM DPPH in ethanol. Then, the solution was incubated at 37 °C for 30 min, and reduced DPPH free radicals were evaluated with a microplate reader at an absorbance of 520 nm. The antioxidant activity of *C. tenuissimus* extracts was set at a threshold of IC_{50}, which is defined as the concentration of extract required to cause a 50 % decrease in the initial absorbance of free radical scavenging activity. The results were expressed as Trolox equivalent antioxidant capacity (TEAC mg. g^{-1} ext.).

ABTS (2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) assay

The ABTS assay followed Gam *et al.* [17], with some modifications. Briefly, 7 mM $ABTS^{\bullet+}$ was produced by the reaction of ABTS solution and 4.9 mM potassium persulfate ($K_2S_2O_8$), following storage in the dark for 16 - 20 h at room temperature. The $ABTS^{\bullet+}$ solution was then diluted with phosphate buffered saline (PBS, pH 7.4) and the absorbance measured (0.70 ± 0.02) before use. The sample solution (20 μL) was pipetted and transferred to 96-well plates. Then, $ABTS^{\bullet+}$ solution was added and incubated for 15 min. After that, the absorbances was recorded at 734 nm by using a microplate reader. Trolox was used as standard for comparison, and the results were reported in terms of Trolox equivalent antioxidant capacity (TEAC mg. g^{-1} ext.).

Thiobarbituric acid reactive substances (TBARS) assay

The TBARS assay is widely used to measure lipid oxidation. The testing procedures followed Uma *et al.* [29]. One hundred microliters of sample or positive control (quercetin) was mixed with 900 μL of egg lecithin (3 mg. mL^{-1}, phosphate buffer, pH 7.4), then 40 μL of 400 mM ferric chloride and 40 μL of ascorbic acid (200 mM) were added to initiate lipid peroxidation. After incubation for 40 min at 37 °C, the reaction was stopped by the addition of 2 mL of 0.25N HCl containing 15 % trichloroacetic acid and 0.375 % thiobarbituric acid. Then, the mixtures were heated at 95 °C for 20 min and cooled to room temperature. Finally, the sample solutions were centrifuged, and the absorbance of the supernatants was measured at 530 nm. Quercetin was used as the reference standard. The results were expressed in terms of IC_{50} and mg QE. g^{-1} ext.

Statistical analysis

The data in the tables are expressed as mean and standard deviation (± SD). Normality and equality of variance were tested, and depending on the results, statistical analyses consisted of analysis of variance (ANOVA) followed by a Tukey test. Differences were considered significant at $p < 0.05$. The correlation between antioxidant assay results and chemical constituents was analyzed using the Pearson correlation test. All tests were performed using SPSS version 16.0.0 software (SPSS Inc., Chicago, IL, USA).

Results and discussion

Morphology, sequence analysis, and microalgae growth rate of *C. tenuissimus*

Microalgae obtained from of The Institute of Marine Sciences, Burapha University, Thailand were cultured and isolated. Sequence analysis was performed with the BLAST algorithm to compare the obtained microalgae sequences with those available in the GenBank database. The complete 28S rDNA repeats of

C. tenuissimus have a total length of 510 bps. The nucleic acid sequences of the 28S rDNA genes obtained in this research were compared with those available in the GenBank database using NCBI/BLAST to search for related sequences. The partial nucleotide sequence of *C. tenuissimus* and that of the database accession no. MK642557.1 are presented in **Figure 1**. Alignment of *Chaetoceros sp.,* which intriguingly has the same base sequence as the *C. tenuissimus* strain, showed 96.27 % sequence identity with the species, reaching as high as 99 % query cover, as shown in **Table 2**.

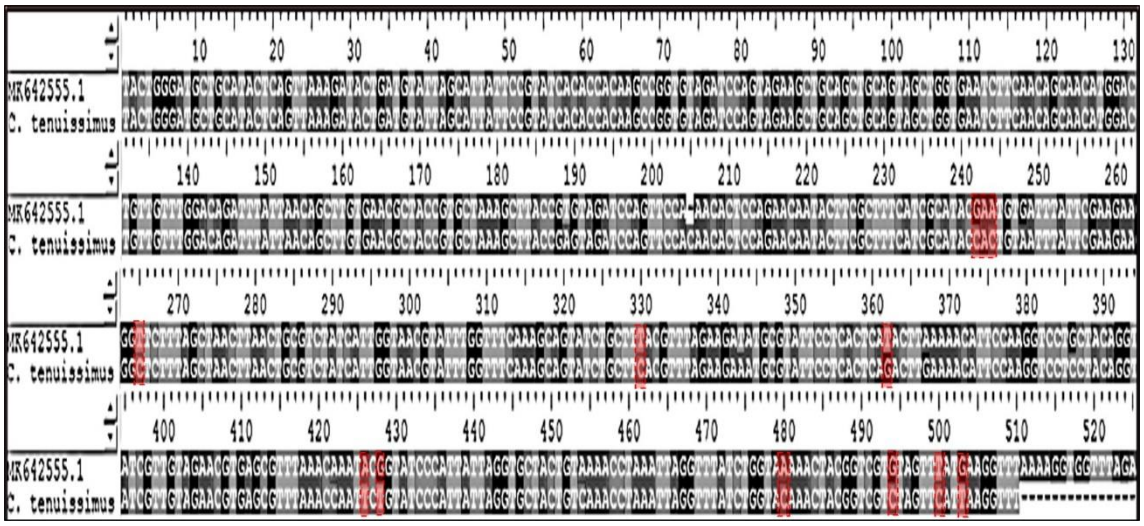
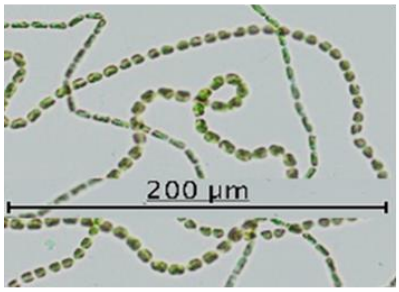


Figure 1 Alignment of the nucleotide sequences of the partial D1F/D3R region of *C. tenuissimus* and database accession No. MK642557.1.

Table 2 Taxonomic identification of screened microalgal strains based on the results of a BLAST search of the NCBI GenBank database.

Name	Length (bp.)	Max score	Total score	Query cover	E value	Ident	Database accession
C. tenuissimus	510	831	831	100 %	0.0	96.27	MK642557.1

The morphology and microalgae growth rate of *C. tenuissimus* were exhibited in both solitary cell and chain forms, as shown in **Figure 2**, with cell dimensions of 5.26 μm wide and 10.18 μm long. The cell had long setae on the edges of all 4 corners. *Chaetoceros tenuissimus* can grow in Guillard f/2 media. A brown color appeared during cultivation, indicated by the increase in optical density (OD).



Class Bacillariophyceae

Subclass Coscinodiscophycidae

Order Chaetocerotanae

Family Chaetocerotaceae

Species *Chaetoceros tenuissimus* Meunier

Figure 2 Morphology of marine *C. tenuissimus* cells under a light microscope at 400×magnification.

Based on **Figure 3**, cell cultures grown in Guillard f/2 media at a salinity of 30 ppt reached a maximum density of 3.4×10^6 cells/mL on the third day.

The marine microalgae *Chaetoceros* sp. is one of the smallest cosmopolitan marine diatoms. It is often confused with morphologically similar species, e.g., *C. tenuissimus, C. neogracilis, C. salsugineus,* and *C. gracilis* [24]. Identification of the cultured Chaetoceros strains was performed according to both microscopic morphological characters and DNA fingerprint analysis. DNA extraction and PCR amplification of the 28S rDNA D1 region of *Chaetoceros* clones was performed using primers D1F and D3R specific to the 28S rDNA D1/D3 region [30] to obtain sequences of microalgae species belonging to *Chaetoceros* sp. The results of our study were consistent with the high homology in 28S rDNA regions (primer D1R/D3F) observed among *Chaetoceros tenuissimus* Meunier (Bacillariophyta, Centrales) strains (96.27 %), with the species reaching as high as 100 % query cover when the BLAST algorithm was used to compare the obtained microalgae sequences with those available in the GenBank database. On the basis of the DNA fingerprint and morphology of *Chaetoceros* sp., it can be concluded that the microalgae used in this study is of the *C. tenuissimus* strain. Then, the growth curve of cultured *C. tenuissimus* was evaluated. The results were similar to those reported by Barros *et al.* and Rahmadi *et al.* [31,32], who showed that optimal growth of *C. tenuissimus* was in culture media with 25 ppt salinity. This is indicated by the increase in optical density (OD) during cultivation. Shirai *et al.* [33] reported that *C. tenuissimus* cell density increased until day 4 in culture. The present study obtained a lower growth rate of *C. tenuissimus* in cell culture than a previous one by Tomaru *et al.* [34].

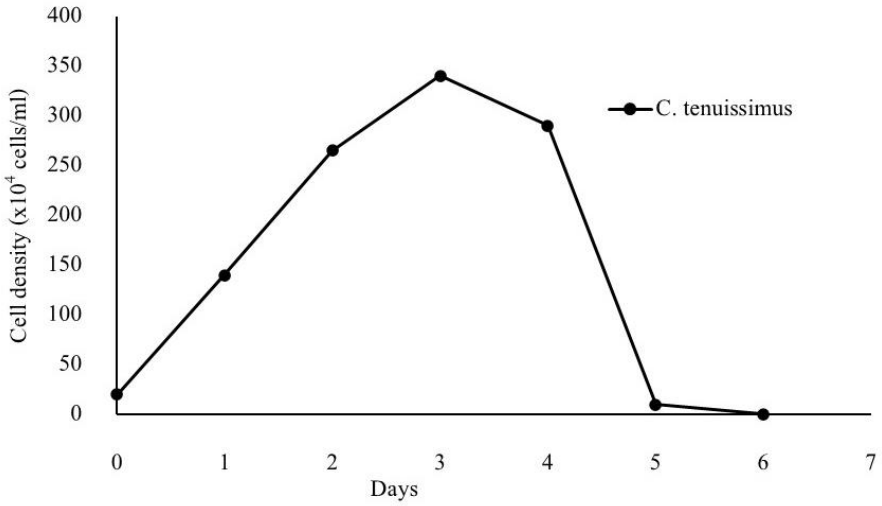


Figure 3 Growth curve of the marine diatom *C. tenuissimus* cultured in Guillard f/2 media.

Physical characteristics of *C. tenuissimus* extracts

The solution extracts of *C. tenuissimus* prepared with different solvents showed a unique dark green color and odor (**Figure 4**), and the pH ranged between 5 and 6, as shown in **Table 3**.

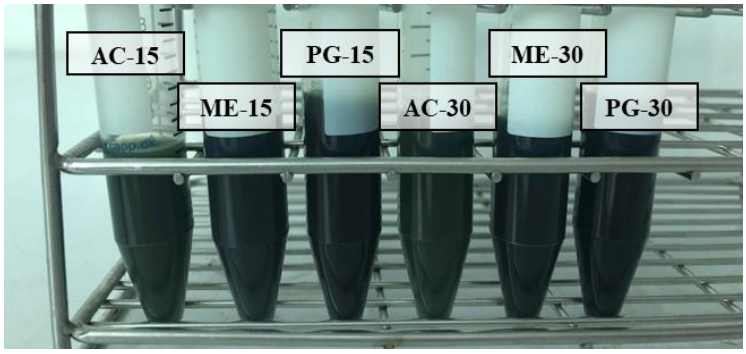


Figure 4 Appearance of *C. tenuissimus* extracts prepared with different solvents by ultrasound-assisted extraction.

The extracts dissolved in PG exhibited the darkest color and a high viscosity, while the supernatant from ME extracts exhibited a dark color and a low viscosity. The supernatant of AC extracts presented the lightest color of all the extracts and low viscosity.

Table 3 Physical properties of *C. tenuissimus* extracts.

Extracts	pH	Viscosity
AC-15	5.0	+
ME-15	5.0	+
PG-15	6.0	++
AC-30	5.0	+
ME-30	5.0	+
PG-30	6.0	++

+ = low, ++ = medium, +++ = high

Total phenolic and flavonoid contents of microalgae extract

The results showed that significant variation in the chemical contents of the extracts occurred among different solvent systems (**Table 4**). The phenolic content of *C. tenuissimus* extracts varied from 1.47 to 2.75 mg GAE. g^{-1}. PG-30 and AC-30 revealed the highest concentrations of phenolic compounds, 2.75 ± 0.14 mg GAE. g^{-1} and 2.73 ± 0.03 mg GAE. g^{-1}, respectively, followed by ME-15 (2.60 ± 0.10 mg GAE. g^{-1}), PG-15 (2.53 ± 0.20 mg GAE. g^{-1}), ME-30 (2.01 ± 0.03 mg GAE. g^{-1}) and AC-15 (1.47 ± 0.04 mg GAE. g^{-1}), respectively. Polyphenols are constituents of plants and present radical scavenging ability owing to their hydroxyl groups [35]. Many studies have reported a highly positive correlation between the total phenolic content and antioxidant activity in various plant species [36,37].

Table 4 Influence of the extraction solvent on TPC, TFC, and fucoxanthin extracted from *C. tenuissimus*.

Sample extracts	Total Phenolic content (mg GAE. g^{-1})	Total Flavonoid content (mg QE. g^{-1})	Fucoxanthin (mg FUC. g^{-1})
AC-15	1.47 ± 0.04^{a}	12.83 ± 0.22^{a}	1.86 ± 0.04^{b}
ME-15	2.60 ± 0.10^{b}	15.39 ± 0.19^{b}	4.65 ± 0.35^{d}
PG-15	2.53 ± 0.20^{c}	13.86 ± 0.21^{c}	1.34 ± 0.00^{b}
AC-30	2.73 ± 0.03^{d}	13.00 ± 0.27^{d}	1.12 ± 0.00^{b}
ME-30	2.01 ± 0.03^{e}	11.40 ± 0.18^{e}	3.31 ± 0.02^{c}
PG-30	2.75 ± 0.14^{d}	5.89 ± 0.08^{f}	8.76 ± 0.06^{e}

Data are presented as mean ± SD, n = 3, $^{a, b, c, d, e, f}$ within the same column indicates a significant difference ($p < 0.05$).

The flavonoid contents of *C. tenuissimus* extracts varied from 5.89 to 15.39 mg QE. g^{-1}. The highest concentration of flavonoids was found in ME-15 (15.39 ± 0.19 mg QE. g^{-1}), which was significantly different ($p < 0.05$) from that in PG-15 (13.86 ± 0.21 mg QE. g^{-1}), AC-30 (13.00 ± 0.27 mg QE. g^{-1}), AC-15 (12.83 ± 0.22 mg QE. g^{-1}), ME-30 (11.40 ± 0.18 mg QE. g^{-1}), and PG-30 (5.89 ± 0.08 mg QE. g^{-1}), respectively (**Table 4**). Klejdus *et al.* (2010) reported the presence of flavonoids capable of inhibiting lipid peroxidation in microalgae [38].

Fucoxanthin content of *C. tenuissimus* extracts

The amount of fucoxanthin was determined by HPLC–DAD using a simple and isocratic elution. The retention time of fucoxanthin was found to be around 14.5 min (**Figure 5**). The fucoxanthin contained in all extracts eluted in the range of 8.76 ± 0.06 and 1.12 ± 0.00 mg FUC. g^{-1} DW, depending on the extraction solvent (**Table 4**). PG-30 showed the highest fucoxanthin concentration (8.76 ± 0.06 mg FUC. g^{-1} DW), which was significantly different ($p < 0.05$) from the others, followed by ME-15 (4.65 ± 0.35 mg FUC. g^{-1} DW) and ME-30 (3.31 ± 0.02 mg FUC. g^{-1} DW), whereas AC and PG extracts showed the lowest fucoxanthin concentration: AC-15 (1.86 ± 0.04 mg FUC. g^{-1} DW), PG-15 (1.34 ± 0.00 mg FUC. g^{-1} DW), and AC-30 (1.12 ± 0.00 mg FUC. g^{-1} DW).

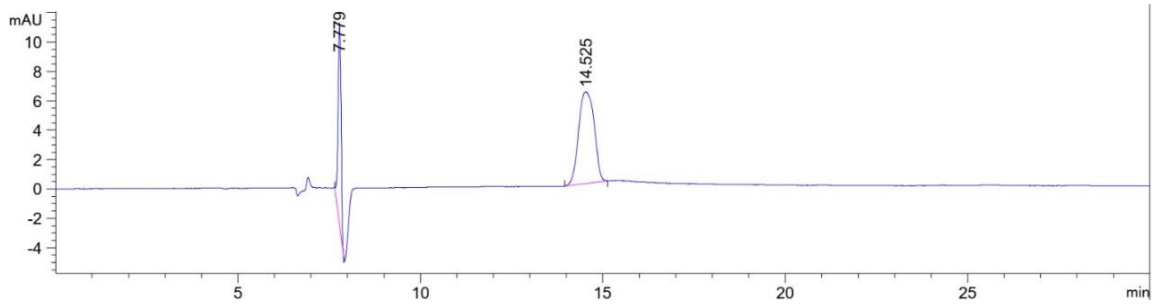


Figure 5 HPLC chromatogram for the fucoxanthin standard (isocratic elution, 95:5-MeOH: Water, column temperature: 28 °C, flow rate: 0.4 mL min^{-1}, absorbance at 450 nm).

It is well known that carotenoids are important contributors to the antioxidant activity in microalgal biomass [10]. Previously, Guo *et al.* [39] and Wang *et al.* [40] reported that the content of fucoxanthin in brown microalgae or diatoms was about 1.0 - 2.5 mg/g (1.00 - 2.50 %) of the dry cell weight. Our study showed a higher value than the abovementioned report and a similar amount of fucoxanthin to that in *C. muelleri* [40]. Furthermore, this study showed that the fucoxanthin and phenolic compounds in *C. tenuissimus* could preferentially dissolve in polar solvents (PG, AC, and ME). The fucoxanthin content in PG-30 (8.76 ± 0.06 mg/g DW) was higher than that in *C. calcitrans* (5.25 ± 0.03 mg FUC. g^{-1} DW) methanolic extracts, as reported by Foo *et al.* (2015). These results supported those of Foo *et al.* (2017), who described microalgae (*Chaetoceros sp.*) as having a higher fucoxanthin content than macroalgae (*Sargassum wightii, Sargassum ilicifolium, Sargassum longifolium, Padina gymnospora,* and *Turbinaria ornate),* in the range of 0.03 - 0.38 mg FUC. g^{-1} alga [41,42] and possibly as high as 18.23 mg FUC. g^{-1} alga in microalgae species *Isochrysis aff. Galbana* [43].

Analysis of the intracellular content of secondary metabolites showed that PG-30 contained the highest phenolic and fucoxanthin concentrations but the lowest concentration of flavonoids (**Table 4**). Furthermore, the results also showed that at 15 min, the efficacy of extraction of phenolics, flavonoids, and fucoxanthin decreased in the order ME > PG > AC. In PG, the extraction efficiency of phenolics and fucoxanthin increased, while the flavonoid content decreased. Previous studies showed that solvent polarity plays a vital role in the extraction process, as it can increase the solubility of antioxidant substances, which is related to this study [13,44]. These results are consistent with those of previous studies [16], which suggested that the choice of solvent significantly affects the extractable substances obtained from polar solvents [44]. The results of this study showed that the longer the ultrasonication extraction time, the higher the levels of phenolics and fucoxanthin obtained. Increasing the temperature naturally improves the solubility of solutes in the solvent and decreases the viscosity and surface tension. This factor boosts the mass transfer to a suitable level and increases the amount of phenolic compounds [44]. PG is non-toxic and widely used in various pharmaceutical formulations as an excipient, serving, for example, as a solvent, diluent, and humectant [45]. Moreover, it should be noted that the chemical composition (fucoxanthin, phenolics, and flavonoids) in some microalgae can be increased by manipulating the environmental conditions [46].

Evaluation of the antioxidant properties of *C. tenuissimus* extracts

The antioxidant potential of all extracts was tested in radical scavenging (DPPH and ABTS) and TBARS assays. The results are shown in **Table 5**. The PG-15 (IC_{50} 998.52 ± 5.23 µg. mL^{-1}/mL) and PG-30 (IC_{50} 970.19 ± 7.70 µg. mL^{-1}) of *C. tenuissimus* presented significantly ($p < 0.005$) higher inhibition than AC and ME in the DPPH assay. Furthermore, ME-15 and ME-30 were found to be inactive against DPPH radicals (IC_{50} > 3 mg. mL^{-1}). For the ABTS assay, the results followed the same trends as those of the DPPH assay, with some exceptions. The IC_{50} value of PG-15 (150.02 ± 6.95 µg. mL^{-1}) showed that it had significantly higher antioxidant ($p < 0.05$) activity than other extracts.

Table 5 Antioxidant capacity values of *C. tenuissimus* from different solvent systems.

Extracts	DPPH· Assay		ABTS$^{\bullet+}$ assay		TBARS	
	TEAC (mg. g^{-1} ext.)	IC_{50} (µg. mL^{-1})	TEAC (mg. g^{-1} ext)	IC_{50} (µg. mL^{-1})	mg QE. g^{-1} ext.	IC_{50} (µg. mL^{-1})
AC-15	2.34 ± 0.02	2560.45 ± 13.72^{a}	1.63 ± 0.01	2221.58 ± 13.57^{a}	37.00 ± 0.42	ND
ME-15	1.64 ± 0.02	ND	2.69 ± 0.03	447.23 ± 7.70^{b}	49.39 ± 0.97	ND
PG-15	3.65 ± 0.02	998.52 ± 5.23^{b}	2.80 ± 0.01	150.02 ± 6.95^{c}	210.61 ± 0.84	865.64 ± 3.19^{a}
AC-30	2.22 ± 0.04	2710.95 ± 49.94^{c}	2.44 ± 0.03	1307.95 ± 8.56^{d}	84.99 ± 0.55	ND
ME-30	2.00 ± 0.04	ND	2.56 ± 0.00	524.36 ± 11.53^{e}	54.25 ± 0.84	ND
PG-30	5.19 ± 0.03	970.19 ± 7.70^{b}	2.76 ± 0.01	312.73 ± 0.76^{f}	249.60 ± 0.48	717.05 ± 1.45^{b}
Quercetin	-	-	-	-	-	139.26 ± 0.53^{c}
Trolox	-	6.17 ± 0.06^{d}	-	3.69 ± 0.03^{g}	-	-

ND: Not detected. Results are presented as mean ± SD of 3 determinations. $^{a, b, c, d, e, f, g}$ within the same column indicates a significant difference ($p < 0.05$).

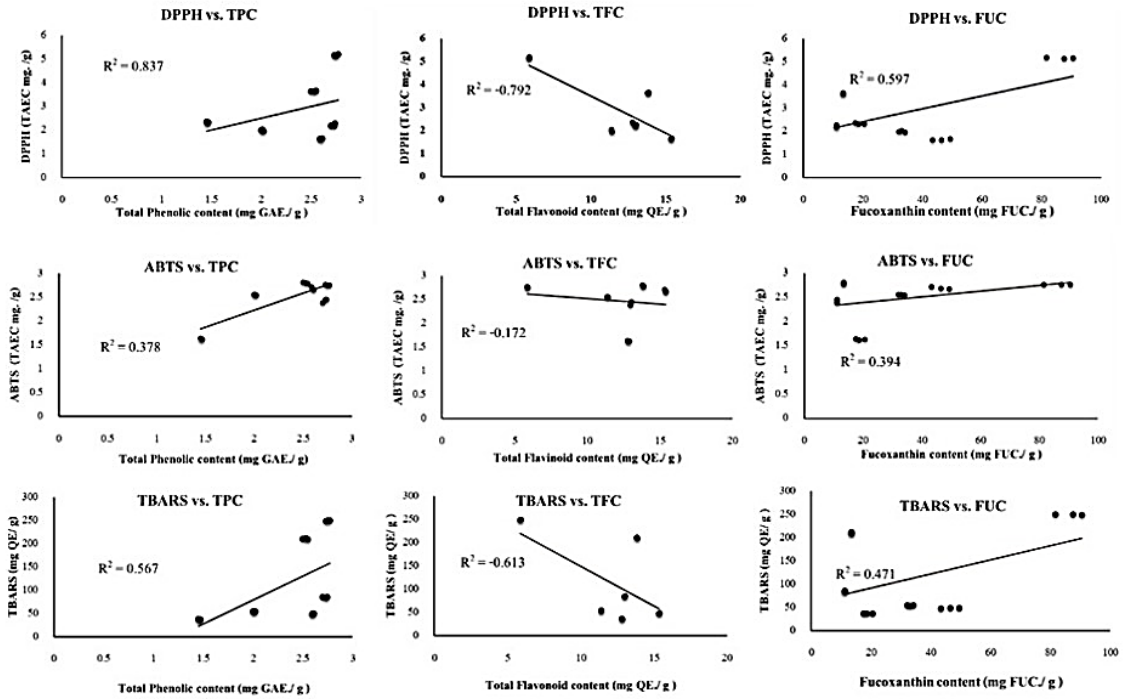
The lipid peroxidation inhibitory effects of different solvent extracts of *C. tenuissimus* are presented in **Table 5**. PG-15 and PG-30 showed that the IC_{50} were 865.64 ± 3.19 µg. mL^{-1} and 717.05 ± 1.45 µg. mL^{-1}. These results indicated that both PG-30 and PG-15 significantly inhibited MDA formation and may have protected cell membranes from lipid oxidation.

The effectiveness of UAE with 3 solvents (ME, AC, and PG) at simultaneously extracting major antioxidant substances (i.e., fucoxanthin, flavonoids, and phenolics) contributes to the antioxidant activity of extracts of the microalgae *C. tenuissimus*. The principles underlying organic solvent extraction of microalgal compounds are based on the basic chemistry concept of "like dissolving like" [47]. Fucoxanthin, one member of the carotenoids, along with phenolics and flavonoids, are considered major contributors to the antioxidant capacity of microalgae. These antioxidant substances also possess diverse biological activities, such as anti-inflammatory, anti-atherosclerotic, and anti-carcinogenic effects [2,5]. This study revealed that increasing the sonication time led to an increased phenolic content in PG and AC extracts. It can be deduced that PG and AC were good solvents for extracting phenolic compounds. This information was concordant with a previous report, which described the effectiveness of polar solvents, such as AC and ME, at extracting phenolics from plants and microalgae [16,18,48]. Moreover, this study also presented a similar phenolic content to that of *C. calcitrans* methanolic extract (2.49 ± 0.08 mg g^{-1} DW) [1]. The polyphenols identified in brown microalgae (*Chaetoceros* sp.) include several different types of phenolic compounds, as well as protocatechuic acid and gallic acid, phlorotannin, and flavonoids such as ferulic acid and apigenin, which are thought to be the foremost contributors of antioxidant capacity [1,18].

Among the microalgae, *Spirulina platensis*, *Isochrysis galbana*, *Chaetoceros calcitrans*, *Amphora montana*, and *Thalassiosira weisflogii* [49] are important sources of antioxidants for human consumption. According to our results, PG-15 (3.65 ± 0.02 TEAC mg. g^{-1} ext.) and PG-30 (5.19 ± 0.03 TEAC mg. g^{-1} ext.) exhibited the highest DPPH-scavenging activity. The UAE technique may help to increase the dissolution of antioxidant substances in solvent extraction. Remarkably, PG is a stable, environmentally friendly solvent that has received growing consideration in the food industry and possesses the advantages of low cost, non-volatility, biodegradation, and non-toxicity. Moreover, PG has the ability to reduce the thickness of many formulations; it has been used as a co-solvent and moisturizer in various pharmaceutical, food, and personal care products [46]. Furthermore, prolonged UAE extraction affected the amount of phenolics and flavonoids, which resulted in antioxidant activity against DPPH and TBARS.

Correlation analysis of total phenolic and flavonoid contents and antioxidant activity

Phenolics, flavonoids, and carotenoids (fucoxanthin) contributed the most to the antioxidant activity of microalgae. The Pearson's correlation coefficient was used to evaluate the correlation between phytochemical contents and antioxidant activities in our research. The results are presented in **Figure 6**. TPC and FUC were significantly related to antioxidant activity. The Pearson correlation analysis approach established a strong positive linear correlation between TPC and FUC, which was correlated with various radical-scavenging assays of *C. tenuissimus* extracts (**Figure 6**). The results were TPC-ABTS: $R^2 = 0.830$ ($p < 0.01$), TPC-TBAR: $R^2 = 0.567$ ($p < 0.05$), FUC-DPPH: $R^2 = 0.597$ ($p < 0.01$), FUC-TBAR: $R^2 = 0.471$ ($p < 0.05$). Only TFC had no correlation with antioxidant activity (DPPH, ABTS, and TBARS) as showed in **Figure 6**.



	TPC	TFC	FUC	DPPH	ABTS	TBAR
TPC	1	−.171	.352	.378	.837**	.567*
TFC		1	−.748**	−.792**	−.172	−.613**
FUC			1	.597**	.394	.471*
DPPH				1	.364	.945**
ABTS					1	.586*

Figure 6 Pearson's correlation coefficients (R^2) between total phenolics, flavonoids and fucoxanthin content and antioxidant activity.

**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

Conclusions

Our results allowed us to conclude that *C. tenuissimus* is a good source of antioxidant bioactive compounds. The UAE technique is a simple method of extracting bioactive substances from *C. tenuissimus.* Using 3 solvents (AC, ME, and PG), we revealed high values of phenolics, flavonoids, and fucoxanthin, which showed antioxidant activity. PG was a good choice of extraction solvent. The long duration of extraction processes by the UAE method allows the recovery of many bioactive compounds. The Pearson's r coefficient between phytochemical contents and antioxidant activities revealed that phenolics and fucoxanthin contributed to the antioxidant activities in this research. Therefore, *C. tenuissimus* is one alternative natural ingredient for application in many products, including foods, cosmetics, and the pharmaceutical industry.

Acknowledgments

This work was financially granted by the Research Grant of Faculty of Pharmaceutical Sciences Burapha University, Thailand (grant No.11/2562) and the research grant of Burapha University, Thailand through National Research Council of Thailand (grant No. 1.1/2562). The authors would like to express their gratitude to the Institute of Marine Sciences, Burapha University for kindly providing the marine microalgae strains in this research. And we thank the Faculty of Pharmaceutical Sciences, Burapha University, Thailand, for their facility and staff support.

References

[1] SC Foo, FM Yusoff, M Ismail, M Basri, NMH Khong, KW Chan and SK Yau. Efficient solvent extraction of antioxidant-rich extract from a tropical diatom, *Chaetoceros calcitrans* (Paulsen) Takano 1968. *Asian Pac. J. Trop. Biomed.* 2015. **5**, 834-40.

[2] A Azizan, M Maulidiani, RRK Shaari, IS Ismail, N Nagao and F Abas. Mass spectrometry-based metabolomics combined with quantitative analysis of the microalgal diatom (*Chaetoceros calcitrans*). *Mar. Drugs* 2020; **18**, 403.

[3] K Skjanes, C Rebours and P Lindblad. Potential for green microalgae to produce hydrogen, pharmaceuticals and other high value products in a combined process. *Crit. Rev. Biotechnol.* 2013; **33**, 172-215.

[4] MB Ariede, TM Candido, ALM Jacome, MVR Velasco, JCMD Carvalho and AR Baby. Cosmetic attributes of algae: A review. *Algal Res*. 2017; **25**, 483-7.

[5] R Tsao and Z Deng. Separation procedures for naturally occurring antioxidant phytochemicals. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci*. 2004; **812**, 85-99.

[6] SH Goh, FM Yusoff and SP Loh. A comparison of the antioxidant properties and total phenolic content in a Diatom, *Chaetoceros* sp. and a green microalga, Nannochloropsis sp. *J. Agric. Sci.* 2010; **2**, 123-30.

[7] K Goiris, K Muylaert, I Fraeye, I Foubert, JD Brabanter and LD Cooman. Antioxidant potential of microalgae in relation to their phenolic and carotenoid content. *J. Appl. Phycol.* 2012; **24**, 1477-86.

[8] T Prartono, K Mujizat and V Katili. Fatty acid composition of three diatom species *Skeletonema costatum*, *Thalassiosira* sp. and *Chaetoceros gracilis. Int. J. Environ. Bioenerg.* 2013; **6**, 28-43.

[9] T Ishika, DW Laird, PA Bahri and NR Moheimani. Co-cultivation and stepwise cultivation of *Chaetoceros muelleri* and Amphora sp. for fucoxanthin production under gradual salinity increase. *J. Appl. Phycol.* 2019; **31**, 1535-44.

[10] CDO Rangel-Yagui, ED Danesi, JCD Carvalho and S Sato. Chlorophyll production from *Spirulina platensis*: Cultivation with urea addition by fed-batch process. *Bioresour. Technol.* 2004; **92**, 133-41.

[11] D Soletto, L Binaghi, A Lodi, JCM Carvalho and A Converti. Batch and fed-batch cultivations of *Spirulina platensis* using ammonium sulphate and urea as nitrogen sources. *Aquaculture* 2005; **243**, 217-24.

[12] SH Lee, KW Lee, JB Lee and YJ Jeon. Antioxidant properties of tidal pool microalgae, *Halochlorococcum porphyrae* and *Oltamannsiellopsis unicellularis* from Jeju Island, Korea. *Algae* 2010; **25**, 45-56.

[13] J Kovacik, B Klejdus and M Backor. Physiological responses of *Scenedesmus quadricauda* (Chlorophyceae) to UV-A and UV-C light. *Photochem. Photobiol.* 2010; **86**, 612-6.

[14] R Sathasivam, R Radhakrishnan, A Hashem and EF Abd Allah. Microalgae metabolites: A rich source for food and medicine. *Saudi J. Biol. Sci*. 2019; **26**, 709-22.

[15] F Chemat, MA Vian, HK Ravi, B Khadhraoui, S Hilali, S Perino and AF Tixier. Review of Alternative solvents for green extraction of food and natural products: Panorama, principles, applications and prospects. *Molecules* 2019; **24**, 3007.

[16] E Roselló-Soto, CM Galanakis, M Brnčić, V Orlien, FJ Trujillo, R Mawson, K Knoerzer, BK Tiwari, and FJ Barba. Clean recovery of antioxidant compounds from plant foods, by-products and algae assisted by ultrasounds processing. Modeling approaches to optimize processing conditions. *Trends Food Sci. Technol.* 2015; **42**, 134-49.

[17] DH Gam, SY Kim and JW Kim. Optimization of ultrasound-assisted extraction condition for phenolic compounds, antioxidant activity, and epigallocatechin gallate in lipid-extracted microalgae. *Molecules* 2020; **25**, 454.

[18] Y Maltsev, K Maltseva, M Kulikovskiy and S Maltseva. Influence of light conditions on microalgae growth and content of lipids, carotenoids, and fatty acid composition. *Biology* 2021; **10**, 1060.

[19] B Singh, N Singh, S Thakur and A Kaur. Ultrasound assisted extraction of polyphenols and their distribution in whole mung bean, hull and cotyledon. *J. Food Sci. Technol.* 2017; **54**, 921-32.

[20] CD Porto, E Porretto and D Decorti. Comparison of ultrasound-assisted extraction with conventional extraction methods of oil and polyphenols from grape (*Vitis vinifera* L.) seeds. *Ultrason. Sonochem*. 2013; **20**, 1076-80.

[21] H Vilakazi, TA Olasehinde and AO Olaniran. Chemical characterization, antiproliferative and antioxidant activities of polyunsaturated fatty acid-rich extracts from chlorella sp. S14. *Molecules* 2021; **26**, 4109.

[22] K Miazek, L Kratky, R Sulc, T Jirout, M Aguedo, A Richel and D Goffin. Effect of organic solvents on microalgae growth, metabolism and industrial bioproduct extraction: A Review. *Int. J. Mol. Sci.* 2017; **18**, 1429.
[23] RA Andersen. Algae culturing technique. Elsevier's Science and Technology, Oxford, U.K., 2005.
[24] SE Hamsher, MM LeGresley, JL Martin and GW Saunders. A comparison of morphological and molecular-based surveys to estimate the species richness of *Chaetoceros* and *Thalassiosira* (bacillariophyta), in the Bay of Fundy. *PLoS One* 2013; **8**, e73521.
[25] S Chandra, S Khan, B Avula, H Lata, MH Yang, MA Elsohly and IA Khan. Assessment of total phenolic and flavonoid content, antioxidant properties, and yield of aeroponically and conventionally grown leafy vegetables and fruit crops: A comparative study. *Evid. Based Complement Alternat. Med.* 2014; **2014**, 253875.
[26] G Miliauskas, PR Venskutonis and TAV Beek. Screening of radical scavenging activity of some medicinal and aromatic plant extracts. *Food Chem.* 2004; **85**, 231-7.
[27] SC Foo, FM Yusoff, M Ismail, M Basri, SK Yau, NM Khong, KW Chan and M Ebrahimi. HPLC fucoxanthin profiles of a microalga, a macroalga and a pure fucoxanthin standard. *Data Brief*. 2017; **10**, 583-6.
[28] B Vongsak, S Kongkiatpaiboon, S Jaisamut, S Machana and C Pattarapanich. In vitro alpha glucosidase inhibition and free-radical scavenging activity of propolis from Thai stingless bees in mangosteen orchard. *Rev. Bras. Farmacogn.* 2015; **25**, 445-50.
[29] R Uma, V Sivasubramanian and SN Devaraj. Evaluation of in vitro antioxidant activities and antiproliferative activity of green microalgae, desmococcus olivaceous and chlorococcum humicola. *J. Algal Biomass* 2011; **2**, 82-93.
[30] K Toyoda, K Nagasaki and Y Tomaru. Application of real-time PCR assay for detection and quantification of bloom-forming diatom *Chaetoceros tenuissimus* Meunier. *Plankton Benthos Res.* 2010; **5**, 56-61.
[31] MUG Barros, AADC Coelho, JWAD Silva, JHC Bezerra, RT Moreira, WRL Farias and RL Moreira. Lipid content of marine microalgae *Chaetoceros muelleri* Lemmermann (Bacillariophyceae) grown at different salinities. *Biotemas* 2014; **27**, 1-8.
[32] A Rahmadi, Y Mulyani and MW Lewaru. Effect of salinity difference on lipid content from *Chaetoceros muelleri* on continuous reactors. *Adv. J. Grad. Res.* 2019; **7**, 3-10.
[33] Y Shirai, Y Tomaru, Y Takao, H Suzuki, T Nagumo and K Nagasaki. Isolation and characterization of a single-stranded RNA virus infecting the marine planktonic diatom *Chaetoceros tenuissimus* Meunier. *Appl. Environ. Microbiol.* 2008; **74**, 4022-7.
[34] Y Tomaru, K Toyoda and K Kimura. Occurrence of the planktonic bloom-forming marine diatom *Chaetoceros tenuissimus* meunier and its infectious viruses in western Japan. *Hydrobiologia* 2018; **805**, 221-30.
[35] T Hatano, L Han, S Taniguchi, T Shingu, T Okuda and T Yoshida. Tannins and related polyphenols of theaceous plants. VIII. camelliatannins C and E, new complex tannins from *Camellia japonica* Leaves. *Cheminform* 2010; **27**.
[36] JA Vinson, Y Hao, X Su and L Zubik. Phenol antioxidant quantity and quality in foods: Vegetables. *J. Agric. Food Chem.* 1998; **46**, 3630-4.
[37] M Oktay, I Gülçin and ÖİKüfrevioğlu. Determination of in vitro antioxidant activity of fennel (*Foeniculum vulgare*) seed extracts. 2003; **36**, 263. *LWT - Food Sci. Technol.* 2003; **43**, 263-71.
[38] B Klejdus, L Lojkova, M Plaza, M Snóblová and DS Štěrbová. Hyphenated technique for the extraction and determination of isoflavones in algae: Ultrasound-assisted supercritical fluid extraction followed by fast chromatography with tandem mass spectrometry. *J. Chromatogr. A* 2010; **1217**, 7956-65.
[39] B Guo, B Liu, B Yang, P Sun, X Lu, J Liu and F Chen. Screening of diatom strains and characterization of *Cyclotella cryptica* as a potential fucoxanthin producer. *Mar. Drugs* 2016; **14**, 125.
[40] LJ Wang, Y Fan, RL Parsons, GR Hu, PY Zhang and FL Li. A rapid method for the determination of fucoxanthin in diatom. *Mar. Drugs* 2018; **16**, 33.
[41] MPSJS Ananthalakshmi and BB Nair. Extraction, purification and study on antioxidant properties of fucoxanthin from brown seaweeds. *J. Chem. Pharm. Res*. 2013; **5**, 169-75.
[42] SC Foo, FM Yusoff, M Ismail, M Basri, SK Yau, NMH Khong, KW Chan and M Ebrahimi. Antioxidant capacities of fucoxanthin-producing algae as influenced by their carotenoid and phenolic contents. *J Biotechnol*. 2017; **241**, 175-83.

[43] S Kim, SW Kang, ON Kwon, D Chung and CH Pan. Fucoxanthin as a major carotenoid in isochrysis aff. galbana: Characterization of extraction for commercial application. *Korean J. Soc. Appl. Biol. Chem.* 2012; **55**.

[44] HB Li, KW Cheng, CC Wong, KW Fan, F Chen and Y Jiang. Evaluation of antioxidant capacity and total phenolic content of different fractions of selected microalgae. *Food Chem*. 2007; **102**, 771-6.

[45] K Ramanauskiene, AM Inkeniene, V Petrikaite and V Briedis. Total phenolic content and antimicrobial activity of different lithuanian propolis solutions. *Evid. Based Complement Alternat. Med*. 2013; **2013**, 842985.

[46] X Mai, Y Liu, X Tang, L Wang, Y Lin, H Zeng, L Luo, H Fan and P Li. Sequential extraction and enrichment of flavonoids from Euonymus alatus by ultrasonic-assisted polyethylene glycol-based extraction coupled to temperature-induced cloud point extraction. *Ultrason Sonochem.* 2020; **66**, 105073.

[47] I Montes, C Lai and D Sanabria. Like dissolves like: A guided inquiry experiment for organic chemistry. *J. Chem. Educ.* 2003; **80**, 447.

[48] HM Amaro, F Fernandes, P Valentao, PB Andrade, I Sousa-Pinto, FX Malcata and AC Guedes. Effect of solvent system on extractability of lipidic components of scenedesmus obliquus (M2-1) and gloeothece sp. on antioxidant scavenging capacity thereof. *Mar. Drugs* 2015; **13**, 6453-71.

[49] NFM Ikhsan, F Yusoff, M Shariff, F Abas and NS Mariana. Screening of malaysian indigenous microalgae for antioxidant properties and nutritional value. *J. Appl. Phycol.* 2007; **19**, 711-18.