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Antioxidant Activities, Total Phenolic Compounds and Fucoxanthin of Marine Benthic Diatoms *Amphora subtropica* BUUC1502 and *Thalassiosira* sp.

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Abstract

This research's aim was to study antioxidant activities, total phenolic compounds and fucoxanthin of crude extract of marine benthic diatoms, *Amphora subtropica* BUUC1502 and *Thalassiosira* sp. These two diatoms were cultured in a Guillard F/2 media, which was prepared from sea water with a salinity at 30 psu for 8 days. It was found that *A. subtropica* BUUC1502 shows better growth than *Thalassiosira* sp. (p<0.05). Whereas the biomass yield of *Thalassiosira* sp. is higher than of *A. subtropica* BUUC1502 (p<0.05). The diatoms were extracted using methanol solvent (99.8%). Crude extracts of *A. subtropica* BUUC1502 and *Thalassiosira* sp. yielded 0.38±0.01 and 0.36±0.00 g/g DW, respectively. The total phenolic compounds and fucoxanthin of them were similar (p>0.05) with 2.92-3.09 mg GAE/g crude extract and 18.85-19.74 mg/g DW, respectively. The IC_{50} of DPPH free radical inhibition of crude extracts from *A. subtropica* BUUC1502 and *Thalassiosira* sp. is 231.75±40.75 and 179.84±27.90 µg/mL, respectively, while the IC_{50} of ABTS free radical inhibition is 68.28±7.31 and 46.90±1.83 µg/mL, respectively. The results of this research show that these two marine benthic diatoms may be an antioxidant source that can be used in various related fields.

Introduction

Diatoms are unicellular algae that live as planktonic or benthic diatoms. They can be found in both freshwater and sea water. Diatoms have many kinds of useful bio-compounds, such as unsaturated fatty acids and pigments. The frustule of diatoms is also used to stimulate blood clotting (Peltomaa et al., 2019; Luo et al., 2021). Diatoms can also be a source of natural antioxidants. During the photosynthesis process in diatom cells, oxygen molecules are released from converting carbon dioxide into starch by using light energy. If diatoms have a high photosynthesis activity, their oxygen production is high, too. Therefore, oxygen may be

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stimulated by ultraviolet radiation or heat from sunlight to form reactive oxygen species, which are toxic to living organisms. Plants including diatoms can create a mechanism to inhibit those free radicals by producing antioxidants (Peltomaa et al., 2019). Rico et al. (2013) reported that the marine diatom *Phaeodactylum tricornutum* contains phenolic compounds such as quercetin, myricetin and rutin. Phenolic compounds and its derivatives contain aromatic rings and hydroxyl groups. Of these, phenolic compounds have a potential to clear the free radicals (Mahfuz et al., 2021). Fucoxanthin is a major type of carotenoids which is an accessory pigment in the chloroplasts and is involved in photosynthesis of diatoms and brown macroalgae. Fucoxanthin has an allenic bond, conjugated carbonyl, epoxide, and acetyl groups in its molecule. Because of this structure, Fucoxanthin is a strong antioxidant (Sies, 1997; Xia et al., 2013). Marine diatoms such as *Cymbella* sp. and *Navicula* sp. produce phenolic compounds that have antioxidant properties and can inhibit oxidation reactions and mutation. Therefore, those compounds from diatoms can be used to prevent various diseases in humans, especially ischemic heart disease and cancer (Natrah et al., 2007). In addition, the diatom *Odontella aurita* contains fucoxanthin, which inhibits cellular oxidation and protects skin cells from UV-B radiation (Mohamed et al., 2012).

Because marine benthic diatoms *Amphora* sp. and *Thalassiosira* sp. have a short production cycle, fast growth rate and high useful biochemical compounds content, they are wildly used in aquaculture hatcheries. Machana et al. (2020) reported that diatom *Amphora* sp. isolated from the East Coast of Thailand comprised ω3-polyunsaturated fatty acids (PUFAs) especially 22: 6n-3 (DHA) and 20:5n-3 (EPA). *Thalassiosira* sp. is rich of PUFAs also (Mai et al., 2021). Khwancharoen et al. (2020) found that Pacific white shrimp postlarvae (*Litopenaeus vannamei*) fed with diatom *A. coffeaeformis* supplemented diet could induce the growth performance of shrimp. Diatom *Thalassiosira* are used as food for shrimp larvae, bivalve larvae, and rotifer (Ortega-Salas & Nava, 2017). *Amphora* sp. and *Thalassiosira* sp. are a great source of natural pigments including chlorophylls and carotenoids (including Fucoxanthin) (Kuczynska et al., 2015). In addition, *Amphora* sp. and *Thalassiosira* sp. are considered for the commercial production (Ortega-Salas & Nava, 2017; Govindan et al., 2021).

In this study, marine centric diatom *Thalassiosira* sp.and pennate diatom *Amphora subtropica* BUUC150 were cultured in the laboratory. Then, diatoms were harvested for crude extracts and determined for total phenolic compounds and fucoxanthin. The diatoms' crude extracts were tested for antioxidant activity, DPPH radical scavenging activity (DPPH) and ABTS radical scavenging activity (ABTS). Therefore, the results of this study can provide information about the potential of diatoms regarding possible uses in medicine and for cosmetic products.

Materials and methods

1. Diatom culture

Diatom *A. subtropica* strain BUUC1502 was isolated from Pacific white shrimp (*Litopenaeus vannamei*) pond, located in Chanthaburi Province (Chenchakhan, 2015) and identified using a partial 18S rRNA sequence analysis. Diatom *Thalassiosira* sp. was obtained from the Center of Excellence for Marine Biotechnology, Department of Marine Science, Faculty of Science, Chulalongkorn University. Diatoms were cultured in axenic Guillard F/2 media (Guillard, 1975), which were prepared from seawater with a salinity at 30 psu. Each of them was cultured at 5 L with 5 replicates. The cultures were placed in a laboratory condition. Temperature was controlled at 26±1˚C. The cultures were exposed to continuous illumination at light intensity of 42.25 µmol/m^2/s using fluorescent lamps and aerated with 0.2 µm filtered air over 8 days. The diatom cells were counted every day using Haemacytometer. Cell density and specific growth rate (µ) were calculated by using the equation (1) of Roleda et al. (2013).

$$\mu\ (day^{-1}) = \ln(N_2\text{-}N_1)/t_2\text{-}t_1 \qquad (1)$$

When N_1 and N_2 equal cell density (cells/mL) at time t_1 and t_2 (day), respectively.

On the final day of the experiment, the diatoms were in stationary phase and then harvested by soaking at 3.4˚C for 16 hours. At this low temperature, the diatom cells take a short time to settle to the bottom. The supernatant was then removed. The concentrated diatoms were centrifuged at 10,000 rpm for 5 minutes and dried by using the lyophilization method.

2. Crude extraction

Diatoms were extracted using absolute methanol with a purity at 99.8% by modifying the method of Sachindra et al. (2007) with a dry diatom and methanol ratio at 1:20 (w/v). Weighed 0.5 g of dry diatoms, then extracted with

10 mL of methanol and soaked overnight for 24 h. The solution was filtered with filter paper and solid residue was extracted with methanol for two times. The methanol solution was dried with a rotary evaporator until the crude extract was formed. The crude extract was weighed and then stored at -20˚C for further study.

3. Total phenolic content determination

The total phenolic content was determined by the Folin-Ciocalteu Colorimetric Method (Sukjamnong & Santiyanont, 2012) by preparing a standard solution of gallic acid (Sigma-Aldrich, United States) at a concentration of 0.625-200 µg/mL and a solution of the sample extract at a concentration of 800 µg/mL. Then 20 µL of the solution was placed in a microtiter plate 96-well and 100 µL of 10% Folin-Ciocalteu solution was added and mixed in. After 6 minutes, 80 µL of 7.5% sodium carbonate were added and left in the dark at room temperature for 30 minutes. Absorbance was determined at 765 nm with a microplate reader (Thermo, Finland). The total phenolic content was calculated as milligrams gallic acid equivalent (mgGAE/g) by using a gallic acid calibration curve.

4. Fucoxanthin content determination

The method of Seely et al. (1972) was improved by using 50 mg dry diatom cells, adding 10 mL of 80% acetone solution, incubating in the dark for 24 hours and centrifuging at 10,000 rpm for 5 minutes. The absorbance of the extracted liquid was determined at 420 nm using the UV–Visible spectrophotometer (Metertech, Taiwan). The fucoxanthin content of the diatom crude extracts was calculated by comparison with the standard fucoxanthin curve (Sigma-Aldrich, United States).

5. Antioxidant activity test of crude extracts from diatoms

5.1 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method

The DPPH radical scavenging method was adapted from Fenglin et al. (2004). Diatom crude extract was diluted to a concentration at 3.125-400 µg/mL with methanol in a 96-well microtiter plate. Then, 0.2 mM of DPPH solution (in methanol) was added at a ratio of 1:1 (v/v) and mixed, incubated in the dark at room temperature for 30 minutes. The absorbance of the extracted liquid was determined at 517 nm by using a microplate reader (Thermo, Finland). The absorbance values were collected and calculated as percentage of DPPH inhibition (% inhibition) at different concentrations as shown in equation (2) with methanol as a blank and DPPH solution as a control unit. The IC_{50} (the concentration of substrate that causes 50% reduction in the DPPH) were calculated to compare with a positive control substance, i.e., vitamin C (Ascorbic acid) and a vitamin E derivative (Trolox).

5.2 2, 2'-azino-bis 3-ethylbenz thiazoline-6-sulfonic acid (ABTS) free radical cation decolorization activity method

The ABTS radical scavenging method was conducted following the method described by Yang et al. (2011), i.e., by mixing 2 mL of 7 mM ABTS with 35.5 µL (in distilled water) of 140 mM potassium per sulfate ($K_2S_2O_8$) and left at room temperature in the dark for 16 hours to get ABTS radical cation ($ABTS^{\bullet+}$) stock. Before testing, $ABTS^{\bullet+}$ stock was diluted to an absorbance in the range of 0.700±0.02 at a wavelength of 734 nm. The diatom crude extract was prepared with a concentration at 3.125-400 µg/mL in a 96-well microtiter plate of 50 µL. Then 100 µL of $ABTS^{\bullet+}$ solution was added and left in the dark at room temperature for 10 minutes. The absorbance was measured at 734 nm with a microplate reader (Thermo, Finland). The absorbance values were collected and calculated as percentage of $ABTS^{\bullet+}$ inhibition with an $ABTS^{+\bullet}$ as control unit. The IC_{50} was calculated to compare with vitamin C and Trolox, which is a standard solution. The percentage of free radical inhibition (% inhibition) was calculated according to the equation (2):

$$\% \text{ inhibition} = [(A_{control}-A_{sample})/A_{control}] \times 100 \quad (2)$$

$A_{control}$ = The absorbance of the control unit (DPPH or ABTS solution)

A_{sample} = The absorbance of crude extract / the standard solution mixed with the DPPH or ABTS solution

6. Data analysis

The mean values of the total phenolic compounds, Fucoxanthin content and antioxidant activity of DPPH• and $ABTS^{+\bullet}$ were calculated. Then, the mean difference was analyzed by T-test at 95% confidence level with SPSS version 15.

Results and discussion

1. Growth and biomass yield of diatoms

From 8 days of diatoms culturing, it was found that A. subtropica BUUC1502's specific growth rate and cell density were higher than *Thalassiosira* sp. ($p<0.05$). However, after harvesting those diatoms on the final day

of this experiment, it was found that *Thalassiosira* sp. had a dry weight at 0.62±0.07 g/L which was significantly higher than *A. subtropica* BUUC1502 (p<0.05) (Table 1). This may be due to *Thalassiosira* sp. having a cell diameter more than 10 µm and a cell volume about 1226-2214 µm^3 (von Dassow et al., 2006), which is greater than *A. subtropica* BUUC1502. de Viçose et al. (2012) reported that *Amphora* have a frustule length and width approximately at 10 and 4 µm, respectively. In addition, during the harvesting process the diatoms were kept at low temperature. This indicates that *Thalassiosira* sp. may precipitate better than *A. subtropica* BUUC1502. Although, some of *A. subtropica* BUUC1502 may have been lost during the harvesting of the cells.

Table 1 Mean±SD (stand deviation) of specific growth rate, maximum cell density and dried weight of marine diatoms, *A. subtropica* BUUC1502 and *Thalassiosira* sp.

Diatom	Specific growth rate (day^{-1})	Maximum cell density (x10^4 cells/mL)	Dried weight (g DW/L)
A. subtropica BUUC1502	0.72±0.07^a (day 0-3)	117.33±11.47^a (day 7)	0.38±0.06^b (day 8)
Thalassiosira sp.	0.45±0.11^b (day 0-3)	45.00±7.05^b (day 8)	0.62±0.07^a (day 8)

Remark: Values in each column followed by different letters denote significant difference at p<0.05

2. Diatom crude extract, total phenolic compounds and fucoxanthin content

When diatoms were extracted with methanol, it was found that *A. subtropica* BUUC1502 had a higher crude extract content than *Thalassiosira* sp. (p<0.05) but total phenolic compounds and fucoxanthin of them were similar (p>0.05) (Table 2). This may be due to the fact that both diatoms belong to the same group of benthic diatoms. Using methanol (absolute methanol, purity 99.8%) to extract diatoms resulted in total phenolic compounds of *A. subtropica* BUUC1502 up to 3.09 mg GAE/g crude extract. When Lee et al. (2008a) extracted *Amphora coffeaeformis* with an 80% methanol solution, a total phenolic compound with 1.05 g GAE/g crude extract was found. This indicates that using methanol with a purity of 99.8% may have caused higher total phenolic compounds than using 80% methanol. The extraction of diatoms by using enzyme is another method that had similar results to this research. According to Lee et al. (2008a), *A. coffeaeformis* has total phenolic compounds at 2.88 mg GAE/g crude extract when extracted with food enzymes in a carbohydrase group with the commercial name Ultraflo, whereas *Thalassiosira* sp. has total phenolic compounds at 2.92 mg GAE/g crude extract. However, Hemalatha et al. (2015), using methanol to extract *Thalassiosira subtilis,* have reported diatoms with a total phenolic compound at 0.48 mg GAE/g DW, which was 6 times less than the results of this study. This indicates that apart from solvents and enzymes, there may be other factors that effect the total phenolic compounds content i.e. certain species or the diatom's growth stage (Rahman et al., 2020).

Fucoxanthin is primary carotenoid pigment in xanthophyll. It is a powerful antioxidant and found in macroalgae such as *Laminaria japonica* and *Undaria pinnatifida* and also in microalgae such as diatom *Phaeodactylum tricornutum,* which has fucoxanthin up to 24.2 mg/g DW (Sies, 1997; Eilers et al., 2016). *A. subtropica* BUUC1502 and *Thalassiosira* sp. have fucoxanthin about 18.85-19.74 mg/g DW (Table 2), which is less than *P. tricornutum.* Hence, there may be other factors that can effect on the Fucoxanthin accumulation as well, i.e., species, nutrients, and culture conditions (Eilers et al., 2016; Rahman et al., 2020). However, *A. subtropica* BUUC1502 and *Thalassiosira* sp. had a similar Fucoxanthin content. This may be because they were cultured under the same conditions and harvested at the same stationary phase as well. Rahman et al. (2020) reported that when microalgae, including diatoms, grow from exponential phase to stationary phase, it has an effect on the antioxidants' accumulation, too (carotenoids, total phenolic compounds and fatty acids).

Table 2 Mean±SD of crude extract, total phenolic compounds and Fucoxanthin contents in marine diatoms *A. subtropica* BUUC1502 and *Thalassiosira* sp.

Diatom	Crude extract (g/g DW)	Total phenolic compounds (mg GAE/g crude extract)	Fucoxanthin (mg/g DW)
A. subtropica BUUC1502	0.38±0.01^a	3.09±1.40^a	19.74±2.46^a
Thalassiosira sp.	0.36±0.00^b	2.92±0.27^a	18.85±2.75^a

Remark: Values in each column followed by different letters denote significant difference at p<0.05

3. DPPH antioxidant activity of diatoms crude extract

DPPH antioxidant activity results of crude extracts from diatoms are shown in Fig. 1. It indicates that DPPH antioxidant activity is increasing when more *A. subtropica* BUUC1502 and *Thalassiosira* sp. crude extracts were added. The concentration of 100 µg/mL crude extract showed the best antioxidant activity, i.e., *A. subtropica* BUUC1502 at 29.32% and *Thalassiosira*

sp. at 53.63%, while 25 µg/mL of Ascorbic and Trolox (vitamin E derivative) inhibited DPPH up to 91.24% and 90.99%, respectively. Although, total phenolic compounds and Fucoxanthin of diatoms' crude extracts from *A. subtropica* BUUC1502 and *Thalassiosira* sp. were similar, but they have different DPPH antioxidant activities. This may be because they contained different compounds such as carotenoids, and the amount and type of fatty acids in the polyunsaturated fatty acid group were different, which affected their antioxidant ability (Rahman et al., 2020). Correlation of crude extract concentrations of *A. subtropica* BUUC1502 and *Thalassiosira* sp. and DPPH radical scavenging values provided linear regression line y = 0.1973x+9.0055, R^2 = 0.9732 and y = 0.3943x+14.124, R^2 = 0.9929, respectively (Fig. 1). Of these results, both linearity models are satisfied regression line. In a previous report, DPPH radical interacts with other radicals, and the time response curve to reach the steady-state is not a good linear with different ratios of antioxidant/DPPH (Brand-Williams et al. 1995; Sanchez-Moreno et al. 1998). In addition, DPPH assay was interfered with other contaminants in the sample such as metal (Lee et al., 2008b). So, in this research, our samples are crude extract form diatoms.

Table 3 shows *A. subtropica* BUUC1502 and *Thalassiosira* sp crude extract which has an IC_{50} value at 231.75±40.71 µg/mL and 179.84±27.90 µg/mL, respectively. Due to complicated composition of crude extract, both crudes show the lower inhibition efficiency of DPPH radicalscavenging activity including pure antioxidant of ascorbic acid and Trolox. The reason why ascorbic acid and Trolox are highly effective in inhibiting DPPH is due to their strong antioxidant properties. Ascorbic acid is a strong natural antioxidant, while vitamin E derivative is a strong synthetic antioxidant with 1,3,4-oxadiazole ring that gives it its antioxidant properties (Rabie et al., 2016). Moreover, ascorbic acid and vitamin E derivative have been purified, while the extract of diatom is only a crude extract. According to Coulombier et al. (2020) microalgae extract has a low DPPH antioxidant capacity. Additionally, if green microalga, *Nephroselmis* sp., was cultured at a low light intensity at 250 µmol/m^2/s, the IC_{50} value (395.93 µg/mL) was better than with a high intensity at 600 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The diatoms *Thalassiosira weissflogi* which were cultured at a low light intensity, the IC_{50} (939.31 µg/mL) was better than others cultured at high light intensity (IC_{50} more than 1,000 µg/mL). In comparison, the results of this research, where *Thalassiosira* was cultured with a light intensity at 42.25 µmol/m^2/s, its extract had a 5.2 times higher DPPH inhibitory ability than the extract prepared during the experiments of Coulombier et al. (2020). This shows that different light intensities in diatom culture conditions and different microalgae species can influence the DPPH antioxidant activity as well.

4. ABTS antioxidant activity of the diatom crude extract

The ABTS antioxidant activity of the diatoms' crude extracts were proportional to the concentration of the extracts. The crude extracts from *A. subtropica* BUUC1502 and *Thalassiosira* sp. at a concentration of 75 µg/mL were able to inhibit ABTS free radicals at 76.30% and 76.25%, respectively (Fig. 2). Linear relationships between ABTS antioxidant activity and crude extract concentrations of *A. subtropica* BUUC1502 and *Thalassiosira* sp. are satisfied. With ABTS assay, good correlation is usually reported with bioactive compounds, and regression factor (R^2) at more than 0.8 (Sadeer et al., 2020). The IC_{50} on ABTS antioxidant of ascorbic acid and Trolox were 5.92 and 11.77 µg/L, respectively. *Thalassiosira* sp. crude extract has an IC_{50} at 46.90±1.83 µg/mL, which was better than *A. subtropica* BUUC1502, which has an IC_{50} at 68.28±7.31 µg/mL (Table 3). However, ascorbic acid and Trolox were able to inhibit free radicals of ABTS better than *A. subtropica* BUUC1502 and *Thalassiosira* sp. extracts. Ascorbic acid is inhibiting free radicals of ABTS better than both diatoms at 12 and 8 times, while Trolox is higher than 6 and 4 times, respectively. Regarding the amount of phenolic compounds and Fucoxanthin, it was found that *Thalassiosira* sp. contains the same amount of these compounds as *A. subtropica* BUUC1502. Therefore, it is possible that the compound may not have a positive effect on the antioxidant activity of ABTS. According to Rahman et al. (2020) the ABTS radical-scavenging activity of diatoms has a positive effect depending on the amount of lutin, beta-carotene and zeaxanthin.

The results of this experiment showed that the extracts from diatom, *A. subtropica* BUUC1502 and *Thalassiosira* sp. had better IC_{50} higher antioxidant effects on ABTS than DPPH. This may be because ABTS is highly soluble in both water and organic solvents, and then quickly reacts and also reacts well at a wide pH range while DPPH free radicals react with antioxidants that are soluble only in solvents such as ethanol. In addition, free radicals ABTS are positively charged free radicals. Thus,

it loses free protons to other molecules with proton receptors, i.e., antioxidants or plant extracts (the extracts from both diatoms). Therefore, it is possible that the extracts from diatoms are highly electronegative which effects their ability to accept protons from free radicals ABTS (Coulombier et al., 2020). Moreover, DPPH is a stable free radical and is not sensitive to reactions like free radicals that occur in the human body. Therefore, the reaction was slow, and the results of the antioxidant activity analysis are less than in reality (Sadeer et al., 2020).

Conclusion

Crude extract of marine benthic diatoms *Thalassiosira* sp. and *A. subtropica* BUUC1502 had similar total phenolic compounds and Fucoxanthin. Additionally, they also showed similar DPPH inhibition. However, crude extraction of centric diatom *Thalassiosira* sp. showed a better ABTS inhibition than pennate diatom *A. subtropica* BUUC1502. This indicates that extracts from marine benthic diatoms, *A. subtropica* BUUC1502 and Thalassiosira sp. produce an antioxidant activity which can be used as a source of natural antioxidants in related fields such as food, cosmetics and medicine.

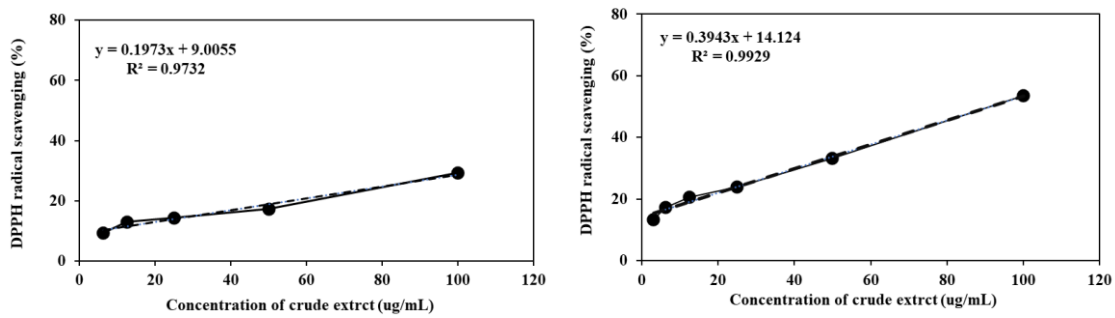

Fig. 1 DPPH radical-scavenging activity of crude extracted from *A. subtropica* BUUC1502 (A) and *Thalassiosira* sp. (B)

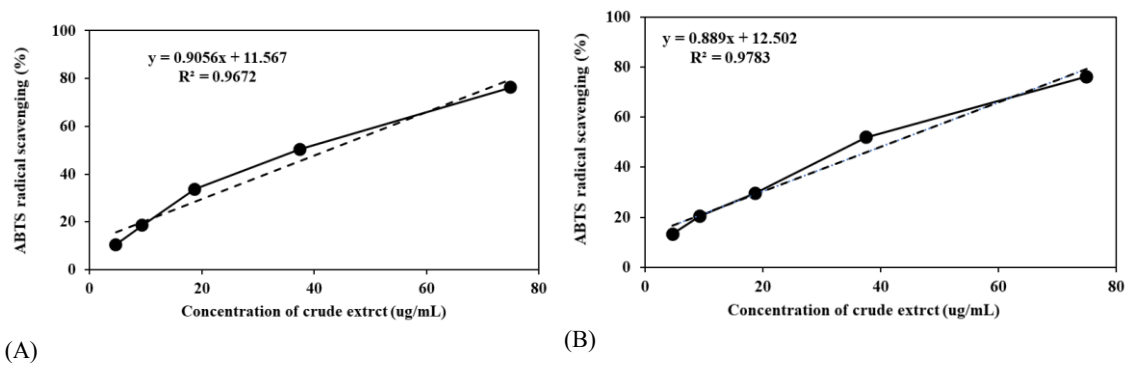

Fig. 2 ABTS radical-scavenging activity of crude extracted from *A. subtropica* BUUC1502 (A) and *Thalassiosira* sp. (B)

Table 3 Mean±SD of the IC_{50} values in the DPPH and ABTS radical-scavenging activity assay of ascorbic acid, Trolox, crude extracted from marine diatoms

Free radical	Ascorbic acid (µg/mL)	Trolox (µg/mL)	Crude extract	
			A. subtropica BUUC1502 (µg/mL)	*Thalassiosira* sp. (µg/mL)
DPPH	5.22±0.42[b]	6.30±0.79[b]	231.75±40.71[a]	179.84±27.90[a]
ABTS	5.92±0.33[d]	11.77±0.41[c]	68.28±7.31[a]	46.90±1.83[b]

Remark: Values in each row followed by different letters denote significant difference at $p<0.05$

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