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Research Article

ANTIOXIDANT POTENTIAL OF BIOPIGMENTS FROM DIATOM MICROALGAE *NAVICULA* SP.

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Abstract

Biopigments known as a colored substance that is commonly generated by the plant also has the primary function of photosynthesis. In addition, the biopigments containing networks of conjugated double bond are potential for antioxidant as they can donate one or more electrons to free radicals. **Aims: T**he antioxidant activities of biopigments isolated from diatom *Navicula* sp. was examined against 2, 2-diphenyl-1-phycrylhydrazil (DPPH) radicals. **Methods:** The diatom *Navicula* sp. was cultured in the seawater medium enriched by urea, phosphate, silicate and iron; the biopigments were isolated from dried algal biomass using acetone as a solvent; biopigments purified by a silica column chromatography with *n*-hexane: acetone (8:2) (v/v) as solvent; the purified biopigments were characterized by Visible spectrophotometer and 2D Thin Layer Chromatography (TLC); and the antioxidant activities of biopigments was assayed against DPPH radicals, with ascorbic acid used as a control. **Results:** The cell density of *Navicula* sp. of 2.56 g/L was observed on the 5th day. There were 6 biopigments showing characteristics of absorption spectrum at 350–800 nm identified as β-carotene, chlorophyll *a,* pheophytin *a*, diatoxanthin, diadinoxanthin and fucoxanthin. The antioxidant activity of ascorbic acid, crude extract, pheophytin *a*, fucoxanthin and β-carotene against DPPH radicals assign an IC_{50} values of 4.9; 107.4; 27.1; 23.3 and 5.32 µg/mL respectively. **Conclusion:** Both crude extract and pure biopigments isolated from *Navicula* sp. exhibited DPPH radical scavenging.

Keywords: Antioxidant, Isolated biopigment, Microalgae, *Navicula* sp.

INTRODUCTION

Free radicals are chemical compounds that have one or more unpaired electrons in the outer shell. This condition causes free radicals to have very high chemical reactivity to other molecules (Valco *et al.,* 2006). The chemical reactivity of free radicals when not inactivated can damage all cell macromolecules including cellular proteins, carbohydrates, lipids and nucleic acids. The effects of protein damage due to free radicals can cause various diseases such as autoimmune, cardiovascular and neurodegenerative diseases as well as cancer (Soeksmanto *et al.,* 2007; Pala and Gurkan, 2008). Antioxidants are compounds that can inhibit or prevent oxidation of easily oxidized substrates by donating one or more electrons to free radicals (Rahman, 2007). In the human body, there are two ways to get antioxidants, namely endogenously (antioxidants are produced from within the body) and exogenous (antioxidants are obtained from outside the body). Examples of endogenous antioxidants are the enzyme superoxide dismutase (SOD), glutathione peroxidase (GSH.Px) and catalase (Amber *et al.,* 2013; Nwosu *et al.,* 2016). However, the human body does not have an excessive amount of antioxidant reserves, so if there is a high enough radical exposure, the body will experience oxidative stress, which can cause damage to genes and cells (Sin *et al.,* 2013). Currently, the intake of antioxidants is exogenously packaged in the form of dietary supplements. Most of the antioxidants contained in this supplement come from synthetic antioxidants, which have carcinogenic effects on the body if consumed at high concentrations and for a long period of time.

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Some natural antioxidant compounds have higher antioxidant activity than synthetic antioxidants, so they can also be used as food additives. Therefore, research for sources of antioxidants that come from nature is widely carried out as an alternative to synthetic antioxidants. Natural antioxidants are mostly obtained from the isolation of bioactive compounds in plants, especially fruits and vegetables. Isolation of antioxidant compounds from fruits and vegetables was focused on the biopigments they contain, one of which is beta-carotene. However, the use of biopigments from these sources will compete with the fulfillment of human food needs. For this reason, it is necessary to find alternative sources of biopigments that do not compete with food, one of which is marine microalgae. Previous research has shown that one of Indonesia's tropical marine microalgae, *Navicula* sp., has good potential as a source of biopigment (Hairunnisa, 2012). This type of diatom microalgae contains a higher diversity of biopigments than other types of microalgae. In addition, this microalgae has advantages over other higher plants, namely that it has a short life span and does not require a large area for cultivation (Barsanti and Gualtieri, 2006). Based on the above background, the exploration of the biopigment content of *Navicula* sp. needs to be done, and to test the antioxidant activity of the biopigments that have been obtained.

METHODOLOGY

Activation and Cultivation of *Navicula* sp.

The culture of *Navicula* sp. was first activated in Walne's medium. The activation conditions were carried out at seawater medium salinity of 25 parts per trillion (ppt), aeration for 24 hours, room temperature, photoperiod of 12:12 (dark:light), and light intensity of 16,000 lux. The activation

process in the Walne's medium was carried out for 2-3 days (Brataningtyas, 2011). It was reactivated in the modified medium for 2 days, then cultivated using the modified medium under the same conditions as the activation in Walne's medium. The composition of the modified medium can be seen in Table 1.

Table 1. Nutrient content in modified medium

Nutrient	Medium Concentration (mg/L culture)
Urea	300
$NaH_2PO_4.2H_2O$	7.5
$FeCl_3$	5
Na_2EDTA	50
$Na_2SiO_3.5H_2O$	450

Before cultivation, the number of microalgae cells was calculated. The cell density value is obtained by entering the calculated number of cells into equation.

$$\text{Cell density (cell/mL)} = \frac{\text{number of cells counted}}{\text{numberof squares counted} \times \text{square volume}}$$

Harvesting of *Navicula* sp.

Harvesting of microalgae *Navicula* sp. performed using a Beckmann J2-HS centrifuge with a large rotor (JA 10) at a speed of 8,670xg (7,000 RPM) for 25 minutes (Brataningtyas, 2011). The supernatant was then separated and the precipitate (wet biomass) was collected and weighed using an analytical balance. The biomass density can then be calculated using the following equation.

$$\text{Biomass density} = \frac{\text{wet biomass mass (gr)}}{\text{Culture volume (L)}}$$

Cell Destruction and Isolation of Biopigment from *Navicula* sp.

Wet biomass *Navicula* sp. freeze-dried using a freeze-dryer under vacuum for approximately 12 hours, until the moisture content in the wet biomass is reduced (Ambati *et al.,* 2010). The dry biomass was then dissolved in cold pure acetone with a ratio of 12:1 (v/v) of acetone to dry biomass. Then, to break the cells, a crusher was used with a cycle of 3 minutes of shaking and 1 minute of rest, in an ice bath. The suspension was filtered using filter paper, then the supernatant was centrifuged using a Boeco U-320R centrifuge at a speed of 3200 rpm for 15 minutes (Gardian *et al.,* 2007). The supernatant in the form of crude extract as a result of centrifugation is separated from the precipitate for further characterization and purification.

Purification and Identification of Biopigment

The purification of biopigments from crude extract was carried out using an chromatographic column with silica gel G_{F60} adsorbent (50-320 mesh). Elution was carried out using acetone : n-hexane (2:8) (v/v) solvent with a flow rate of 0.2 mL/minute. The separated biopigments on column chromatography were characterized by scanning at visible light wavelengths (350–800 nm) using the Biochrom Libra S70 spectrophotometer. Each pigment has a unique absorption pattern so that the type of pigment can be determined.

Qualitative and Quantitative Antioxidant Activity Test

Antioxidant activity was observed by TLC, using silica gel G_{F60} as the stationary phase and acetone: n-hexane (2:8) (v/v) as the solvent. The biopigments in the crude extract were separated using TLC, allowed to dry, then sprayed with a 0.4 mM DPPH solution in methanol. The TLC plate that had been sprayed with DPPH was left for 30 minutes. Furthermore, identification of the antioxidant activity of the biopigment was carried out by the presence of a yellowish glow around the biopigment stain on the TLC plate. The antioxidant activity of each sample of ascorbic acid, crude extract, and pure biopigment is expressed by the percentage of free radical inhibition (percent inhibition), which can be calculated by the following formulation:

$$\% \text{ Inhibition} = \frac{\text{blank absorbance} - \text{sample absorbance}}{\text{blank absorbance}} \times 100\%$$

The DPPH solution was diluted using methanol to show an absorption of 0.70 ± 0.2 at a wavelength of 517 nm on a UV-Vis spectrophotometer. Ascorbic acid was used as a comparison, prepared at a concentration of 2-16 g/mL. A total of 1 mL of ascorbic acid solution was reacted with 1 mL of DPPH solution in a test tube. The mixture was incubated at room temperature for 30 minutes, and then the absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 517 nm. The same procedure was also carried out on other antioxidant compounds in the form of crude extract of *Navicula* sp. and pure biopigments. The absorbance of the blank solution was also measured to calculate the percent inhibition (Williams *et al.,* 1995).

RESULTS AND DISCUSSION

***Navicula* sp. Culture**

Prior to cultivation, microalgae cultures were checked using an optical microscope to ensure that there were no contaminants in the culture. The results obtained (Figure 1) showed that in the culture there was only 1 type of microalgae, namely *Navicula* sp. characterized by oval-shaped cells. This indicates that in the initial culture of *Navicula* sp. in the axenic state, there are no microalgae or other contaminants, such as fungi, bacteria, or protozoa (Barsanti, 2006).

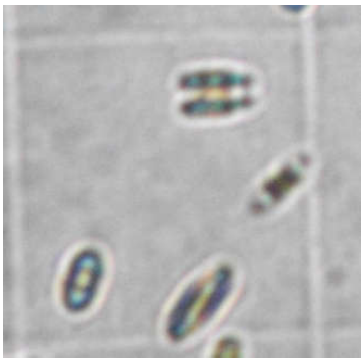

Fig 1. Culture of *Navicula* sp. seen using an optical microscope

Harvesting of microalgae cultures was carried out when the nutrients and physico-chemical conditions of the growing medium were still within normal limits, so that the microalgae condition was still good and no cells had died. Before harvesting, the number of microalgae cells was observed using an optical microscope to determine the density of microalgae cells. In this study, harvesting was carried out on the 5th day of growth (end of the exponential phase to the slowing phase), and the cell density was 42.71 ± 0.3 cells/mL.

Navicula sp. Biomass

Microalgae were harvested after reaching maximum cell density (day-5) using a centrifugation technique. The centrifugation technique is the best harvesting technique because the process is short, the harvesting efficiency is high, and it does not damage the physical and chemical properties of microalgae. Based on previous research, it is known that the wet biomass density of *Navicula* sp. The highest silicate concentration was obtained at 450 mg/L, which is also consistent with this study, which resulted in the highest wet biomass density of 2.56 g/L (Hairunnisa, 2012). This suggests that the increase in silicate content in the growth medium has an effect on biomass production. The obtained microalgae harvesting data is presented in table 2.

Table 2. Microalgae biomass density after cultivation for 5 days

Harvesting	Culture voume (L)	Wet Biomass Weight (g)	Dry Biomass Weight (g)	Wet Biomass Density (g/L)
1	6.78	17.35	3.31	2.56
2	2.46	5.87	1.05	2.39
3	2.65	6.46	1.21	2.44
		Average		2.46 ± 0.08

Wet biomass is obtained from harvesting, then dried first with a freeze-dryer. This drying aims to remove the water content contained in the wet biomass. By comparing the weight of wet biomass to dry biomass, it was found that the moisture content of *Navicula* sp. was 82%.

Biopigments Isolate from *Navicula* sp. Biomass

Extraction of biopigments with acetone solvent was carried out using acetone solvent assisted by a homogenizer in an ice bath. Biopigments in microalgae are located in the thylakoid membrane. For that reason, it is necessary to perform cell lysis first to extract the biopigments contained therein (Henriques, 2000). Extraction was stopped when the resulting supernatant was clear green, which indicated that almost all of the biopigments had been extracted by acetone. The initial characterization of the crude extract was carried out using the TLC technique to determine the best solvent composition for separating the biopigments contained in the crude extract. Figure 2 depicts the best separation pattern.

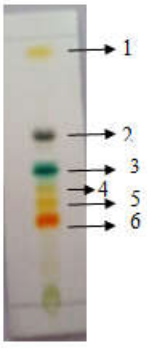

Fig 2. Spot pattern on biopigment separation using n-hexane: acetone (8:2) (v/v) solvent

This characterization indicated the presence of six separate biopigments based on their polarity. Yellow biopigment 1 has the highest Rf, indicating that this biopigment has the lowest polarity because it was first eluted by a nonpolar solvent. Meanwhile, the orange biopigment 6 has the lowest Rf, which indicates that the biopigment has a higher polarity than other biopigments because it is bound for longer by polar silica. To obtain pure biopigment from crude extract, it was separated by column chromatography technique using silica matrix G_{F60} and n-hexane: acetone (8:2) (v/v) solvent, which showed the best separation results on TLC. From the results of column chromatography separation, 44 fractions were obtained, which were then analyzed further to determine the type of biopigment and its level of purity using a spectrophotometer in the visible light region.

Pure Biopigment Absorption Spectrum

The characterization of the obtained biopigment fractions was then carried out by measuring the corresponding spectrophotometrically visible light spectra in the wavelength range of 350-800 nm to be compared with the standard compound spectrum and the pure biopigment spectrum obtained from the literature. From the separation that has been done, obtained fractions identified as ß-carotene, pheophytin-a, chlorophyll-a, diatoxanthin, diadinoxanthin, and fucoxanthin. This is consistent with the results of TLC characterization, which showed the presence of 6 pure biopigments contained in *Navicula* sp. The comparison of the main absorbance peaks in the biopigment spectrum from *Navicula* sp with the main absorbance peaks of biopigment obtained from the literature can be seen in Table 3.

Table 3. Biopigment specific absorption peak

No	Biopigment	Main Absorbance Peak of The Spectrum (nm)	
		Isolate Biopigment	Literature
1	ß- carotene	450.5 ; 475.5	452 ; 478
2	Pheophytin-a	409.6 ; 667	410 ; 666
3	Chlorophyll-a	430 ; 662.5 ; 410	430 ; 662 ; 410
4	Diatoxanthin	456 ; 483	456 ; 483
5	Diadinoxanthin	449.5 ; 478	449 ; 478
6	Fucoxanthin	447 ; 468	446.3 ; 468.3

3.5 Qualitative and Quantitative Antioxidant Activity

From the results of a qualitative test using the free radical DPPH, it was shown that there was antioxidant activity of pure biopigments against free radicals. This is indicated by the presence of luminescence around the biopigment spot on the TLC plate which can be seen in Figure 3.

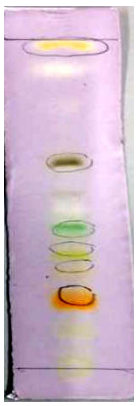

Fig 3. Qualitative TLC chromatogram of biopigment antioxidant activity test

This luminescence is formed due to a shift in the wavelength of DPPH in the form of radicals to a more stable DPPH, which causes a purple color change to yellow luminescence. In the

picture, it can be seen that the ß-carotene pigment has a higher luminescence intensity than other biopigments. This indicates that ß-carotene biopigment has the best antioxidant activity. In addition, the highest abundance of biopigments such as pheophytin-a and fucoxanthin also gave a fairly good luminescence, proving the presence of antioxidant activity against free radicals. In the quantitative antioxidant activity test using the DPPH method, ascorbic acid was used as a control solution. Ascorbic acid, commonly known as vitamin C, has a fairly high antioxidant activity (Weichselbaum and Buttriss, 2011). From the experimental results, the IC_{50} value of ascorbic acid against DPPH was 4.9 g/mL. The biopigments obtained from *Navicula* sp. also have antioxidant activity as well as ascorbic acid, so they can be used as an alternative source of antioxidants other than ascorbic acid. For this reason, antioxidant activity tests were carried out on crude extracts, pheophytin-a, fucoxanthin, and ß-carotene, which gave IC_{50} values of 107.4 ; 27.1 ; 23.3 ; and 5.32 g/mL, respectively (Table 4). IC_{50} value of ß-carotene is classified as a very strong antioxidant (Lung and Destiani, 2017).

Table 4. Antioxidant activity of crude extract and biopigment *Navicula* sp.

Sample	Concentration (mg/L)	% Inhibition	IC_{50} (mg/L)
Ascorbic Acid	2	19.16 ± 3.48	4.9
	4	33.87 ± 2.44	
	6	55.96 ± 1.49	
	8	75.67 ± 1.07	
	10	86.45 ± 1.41	
Crude Extract	91	23.33 ± 2.93	107.4
	121	35.64 ± 3.59	
	136	43.85 ± 4.54	
	152	56.23 ± 5.34	
	228	92.98 ± 0.97	
Pheophytin-a	5	13.20 ± 2.49	27.1
	15	26.87 ± 3.27	
	25	40.19 ± 1.74	
	35	62.69 ± 3.06	
	50	72.85 ± 2.63	
Fucoxanthin	7	11.57 ± 3.67	23.3
	19	33.40 ± 2.58	
	30	57.24 ± 2.88	
	35	76.23 ± 2.28	
	45	90.58 ± 1.16	
ß-Carotene	2	12.59 ± 2.59	5.32
	4	33.74 ± 2.07	
	6	53.43 ± 2.37	
	8	67.43 ± 2.03	
	10	75.69 ± 2.68	

Pure bipigments (pheophytin-a, fucoxanthin, and ß-carotene) showed higher antioxidant activity than crude extract, due to the large amount of pigment still contained in crude extract. It is suspected that there are interactions between the compounds contained in the crude extract, causing the active side of each compound to be less active, so that free radical scavenging activities are more difficult to carry out. ß-carotene biopigment showed better antioxidant activity than other biopigments. It was related to the structure of the ß-carotene compound with a conjugated carbon chain, which had a major role in quenching free radical activity. The conjugated carbon chain in ß-carotene is more stable, so it actively transfers electrons to free radicals and provides the highest antioxidant activity (Berti *et al.,* 2014). When compared with the antioxidant activity provided by ascorbic acid as a standard, ß-carotene, which has similar antioxidant activity, can also be used as a standard in testing antioxidant activity and can be used as an alternative source of antioxidants besides ascorbic acid.

Conclusion

Biopigments from *Navicula* sp. were identified as β-carotene, chlorophyll-a, pheophytin-a, diatoxanthin, diadinoxanthin & fucoxanthin. Both crude extract and purified biopigments (β-carotene, pheophytin-a & fucoxanthin) isolated from *Navicula* sp. exhibited DPPH radical scavenging.

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