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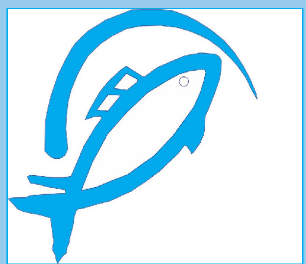

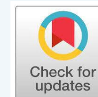

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Short Communication

Phytochemicals and Antioxidant Activity of Microalgae *Dunaliella salina* and *Botryococcus braunii*

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Abstract

Microalgal species such as *Dunaliella salina and Botryococcus braunii* are reportedly rich in natural antioxidants and phytochemicals. This study aimed to determine the phytochemicals and the antioxidant activity of *D. salina and B. braunii*. Microalgal samples were obtained from the Brackish Water Cultivation Fisheries Center (BPBAP), Situbondo, East Java. The extracts were prepared using the multilevel maceration method. The antioxidant activity of the algal species was analyzed using 1,1-diphenyl-2-picrylhydraxyl (DPPH). Quantitative analysis revealed that *D. salina* and *B. braunii* contained antioxidants, indicated by the appearance of yellow spots on the purple background of the TLC plate. The n-hexane extract of *D. salina* exhibited the highest antioxidant activity with a half-maximal inhibitory concentration (IC_{50}) of 443.28 ppm, 61.28 mg GAE/g sample of total phenolics, 0.106 mg/g of chlorophyll a, 0.165 mg/g of chlorophyll b, and 1,697 mol/g of carotenoids. In contrast, the ethyl acetate extract of *B. braunii* exhibited the highest antioxidant activity with an IC_{50} of 634.55 ppm, 46.94 mg GAE/g sample of total phenolics, 18.146 mg/g of chlorophyll a, 12.592 mg/g of chlorophyll b, and 4573 mol/g of carotenoids. The microalgal species used in this study exhibited extremely weak antioxidant activity.

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1. Introduction

Antioxidants can prevent or delay oxidative damage to target molecules such as proteins, fats, and DNA (Singh *et al*., 2016). Presently, the most widely used antioxidants are synthetic in nature, may be carcinogenic, and can cause liver damage if used repeatedly (Ridlo *et al*., 2015). Therefore, further studies are required to identify natural sources of antioxidants, which are safer for health. In this regard, plants and animals can be used as natural sources of antioxidants (Badr *et al*., 2021). Microalgae are thallic organisms that contain several phytopigments, including chlorophylls that absorb sunlight and perform photosynthesis. They are abundant in sea water, where carbon dioxide and sufficient sunlight support their growth (Barkia *et al*., 2019; Stunda-Zujeva *et al*., 2021), making them the primary producers in the aquatic food web. Therefore, most of the photosynthesis occurring in the aquatic ecosystem is attributed to microalgae (Prasad *et al*., 2021). They have been in the fishing industry as food for fish larvae (Vaz *et al*., 2016) and for filtering organisms, along with their applications in the pharmaceutical industry and as food supplements (Saadaoui *et al*., 2021) and anti-malaria in Vitro (Pujiyanto *et al*., 2021). Microalgae are also a storehouse of several active compounds such as proteins, lipids, carotene, provitamins, minerals, phytopigments, and fatty acids (Dineshkumar *et al*., 2017).

Most of the pharmaceutical products, including drugs, are derived from green microalgae (Dragone, 2022) or chlorophytes (Ghasemi *et al*., 2009; Fithriani *et al*., 2015; Koyande *et al*., 2019). Recently, microalgae have been used as a source of vitamins (Vaz *et al*., 2016), suggesting their potential as a promising source of natural antioxidants. Microalgae have also been recognized as a source of valuable pigments such as chlorophyll a, zeaxanthin, and astaxanthin. Furthermore, they can be cultivated on a large scale in bioreactors as a continuous and reliable alternative source of natural products (Elalami *et al*., 2021). In addition, their growth conditions can be regulated using sterile media, which also prevents contamination with herbicides, pesticides, and other toxic substances. Hence, microalgae can be used to extract natural antioxidants.

Green microalgae *Dunaliella salina* and *Botryococcus braunii* contain chlorophyll (Blifernez-Klassen *et al*., 2018) and carotenoids, suggesting the presence of antioxidants in these species (Xu and Harvey, 2019). Zainuddin (2017) reported that *D. salina* cultured on media with a salt concentration of 30 ppt contained 10.96 mg/L of chlorophyll a, 3.636 mg/L of chlorophyll b, and 4.954 mg/L of carotenoids. Singh *et al*., (2016) reported that carotene content enhanced by *D. salina* under stress conditions increased the antioxidant and cytotoxic activity. However, compared to other green microalgal species, there have been fewer studies on the antioxidant activity of *D. salina* and *B. braunii*. In this study, we determine the contents of phytochemicals, including chlorophyll a, chlorophyll b, carotenoids, total phenolics in, and the antioxidant activity of the extracts of *D. salina* and *B. braunii*.

2. Materials and Methods

2.1 Material

The microalgal species of *D. salina* and *B. braunii* were obtained from the Brackish Water Cultivation Fisheries Center (BPBAP), Situbondo, East Java, from July to November 2021. The extraction was performed using the multilevel maceration method based on a study by Setha *et al*. (2013). Briefly, 80 g of dry microalgae was soaked in 400 mL of n-hexane, ethyl acetate, and methanol for 24 hours and then filtered. The use of multilevel extraction was meant to find out detailed information from the results (Prayitno *et al*., 2022). Thereafter, the residue was macerated using 400 mL of the respective solvent and filtered again. The filtrate obtained from the maceration process, which was performed three times, was collected and concentrated using a rotary evaporator at 40°C, to obtain concentrated n-hexane, ethyl acetate, and methanol extracts of the microalgal species. The yields of the concentrated extracts were estimated using the following formula:

Yield (%) = × 100%

2.2 Data Analyses

2.2.1 Phytochemical screening

The extracts for alkaloids, saponins, flavonoids, steroids, and triterpenoids were screened. The presence of alkaloids was determined using Dragendorff and Meyer's reagents, while that of saponins was determined by examining foam formation upon shaking (Faizal and Geelen, 2013). Flavonoids were detected by dissolving the extract in 2 mL of methanol, then adding magnesium powder and five drops of hydrochloric acid. In contrast, steroids and triterpenoids were detected by dissolving the extract in 2 mL of chloroform, followed by the addition of 10 drops of acetic anhydride and three drops of concentrated sulfuric acid (Auwal *et al*., 2014).

2.2.2 Qualitative analysis of antioxidant activity

Thin Layer Chromatography (TLC) was used to analyze the antioxidant present in microalgal extracts.

The extracts of n-hexane, ethyl acetate, and methanol were dissolved in the original solvent, put in a F245 silica-gel plate, and then eluted using n-hexane: n-hexane extracts, ethyl acetate extracts, and methanol extracts with the ratio of 4:6, 6:4, and 3:7, respectively. Subsequently, the plates were sprayed with 0.1 mM 1,1-diphenyl-2-picrylhydraxyl (DPPH) (Munteanu and Apetrei, 2021).

2.2.3 Determination of the absorption maxima of DPPH and the microalgal extracts

A total of 3.94 mg of DPPH was dissolved in 100 mL of 0.1 mM methanol. According to Hidayati *et al*. (2017), 4 mL of 0.1 mM DPPH solution was placed in the cuvette, and the absorption maxima of the solution was recorded at a wavelength of 400–800 nm, which was used as the control. Thereafter, 3 mL of 50 ppm of the extract was incubated for 5–50 minutes, supplemented with 1 mL of 0.1 mM DPPH solution, and placed in the cuvette to measure absorption maxima at 400–800 nm and 515 nm every five minutes.

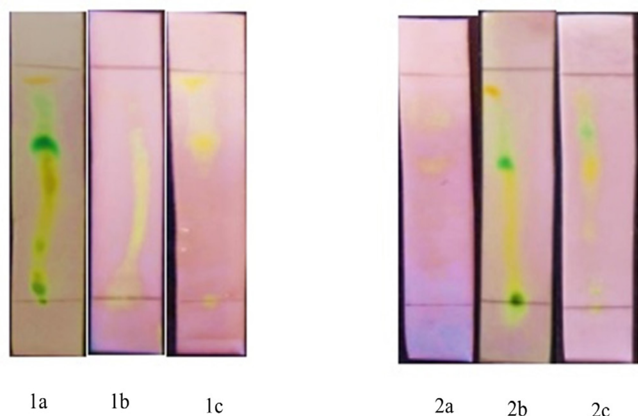

Figure 1. Thin-layer chromatography of *D. salina* (1) and *B. braunii* (2) with solvents (a) n-hexane, (b) ethyl acetate, and (c) methanol

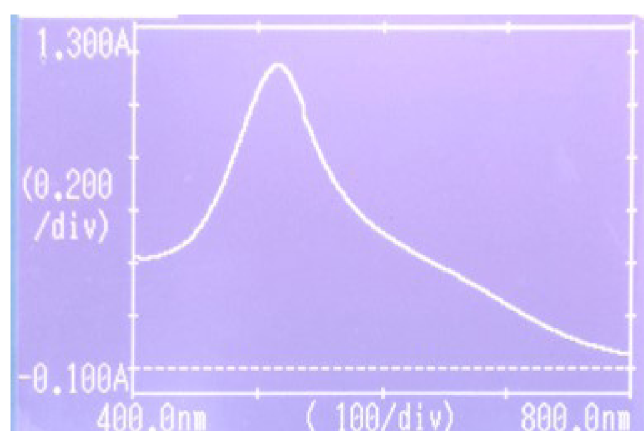

Figure 2. The spectrum of DPPH in methanol

2.2.4 Determination of antioxidant activity

A total of 5 mg microalgal extract was dissolved in 5 mL of methanol to prepare a stock solution with a concentration of 1,000 ppm. The following five concentrations were then prepared for further analysis, namely 50, 100, 200, 400, and 800 ppm. The 3 mL of the diluted solution was added to 1 mL of 0.1 mM DPPH, followed by incubation in a dark room for 30 minutes. The absorption maxima of the solutions were then determined using a UV–Vis spectrophotometer, and the inhibition percentage was calculated using the following formula (Munteanu and Apetrei, 2021):

% inhibition= ×100%

where A = absorbance of DPPH solution; and B = absorbance of DPPH and extract solution

Free radical inhibition (%) was then used to determine the half-maximal inhibitory concentration (IC50). Using y = 50 in the linear regression equation y = bx + a, the value of x was obtained.

2.2.5 Measurement of chlorophyll, carotenoid, and total phenolic content

Approximately 5 mg of the microalgal extract was supplemented with 5 mL of acetone. Chlorophyll and carotenoid contents were measured using a spectrophotometer at wavelengths of 470 nm, 645 nm, and 662 nm, according to Zielewicz *et al*. (2020).

In order to estimate the total phenolic content, 5 mg of the extract was dissolved in 2 mL of ethanol, followed by the addition of 5 mL of distilled water and 0.5 mL of *Folin-Ciocalteau's* reagent, and then incubated for 5 minutes. Then, the mixture was supplemented with 1 mL of 5% Na_2CO_3, incubated for one hour, and its absorbance was measured at 725 nm. Furthermore, serial dilutions of gallic acid (5, 10, 15, 20, and 25 ppm) were prepared to plot a gallic-acid curve for determining the content of phenolic compounds using the regression equation. Total phenolic content was expressed in mg gallic acid equivalence per g extract (mg GAE/g extract) (Trentin *et al*., 2022).

3. Results and Discussion

3.1 Results

Microalgal extracts were successfully prepared using solvents of different polarities (Table 1). It was observed that polar compounds dissolved rapidly in polar solvents, whereas non-polar compounds dissolved rapidly in non-polar solvents. In this study, the highest

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yields of *D. salina* and *B. braunii* were achieved using methanol (5.9% and 7.025% respectively), followed by ethyl acetate (0.375% and 0.761% respectively), and n-hexane (0.053% and 0.203% respectively).

Table 1. Extraction results in Microalgae *D. salina* and *B. braunii*

Sample	Solvents	Rendement (%)	Form	Color
D. salina	n-hexane	0.053	Paste	Yellow
	Ethyl acetate	0.375	Paste	Dark green
	Methanol	5.9	Solid	Light green
B. braunii	n-hexane	0.203	Paste	Tawny
	Ethyl acetate	0.761	Paste	Dark green
	Methanol	7.025	Solid	Dark green

Furthermore, alkaloids, saponins, steroids, and triterpenoids were identified in the extracts of *D. salina* and *B. braunii* (Table 2). Flavonoid compounds were detected in the ethyl acetate and methanol extracts of *D. salina* and *B. braunii*, but not in the n-hexane extracts. However, saponins were detected in the methanolic extracts of *D. salina* and *B. braunii*, but not in their n-hexane and ethyl acetate extracts. Steroids and triterpenoids were also detected in the extracts of *D. salina* and *B. braunii*. As triterpenoids are composed by long chains of C_{30} hydrocarbons that make them non-polar, they are easily extracted using non-polar solvents.

Table 2. Phytochemical test results of the extracts of *D. salina* and *B. braunii*

Phyto-chemical Test		Results		
		n-hexane	Ethyl acetate	Methanol
Alkaloids	Mayer	+	+	+
	Dragen-droff	+	+	+
Flavonoids		-	+	+
Steroids		+	+	+
Triter-penoids		+	+	+
Saponins		-	-	+

The change of color shown by TLC was recorded after sprayed with 0.1 mM DPPH (Figure 1). The presence of antioxidants in the n-hexane, ethyl acetate, and methanol extracts of *D. salina* and *B. braunii* was verified by the appearance of yellow spots on the purple background of the TLC plate. Their appearance can be attributed to the reduction of DPPH to DPPH-H by the hydrogen atoms of the antioxidants present in the extract. The present study also observed the green spots for the ethyl acetate extracts of *D. salina* and *B. braunii* and the methanol extract of *B. braunii*, indicating the presence of chlorophylls.

DPPH was also used to determine the antioxidant activity of the extracts, because it is simple, rapid, and does not require several reagents. The absorption maxima of DPPH was recorded before estimating the antioxidant activity of the extracts in order to determine the absorbance of DPPH with maximum sensitivity (Figure 2). DPPH exhibited absorbance maxima at 515 nm, and also in the range of 510–520 nm.

Antioxidant activity of the extracts of *D. salina* and *B. braunii* were determined using IC_{50} values, which were calculated using linear regression of the extract concentration and the DPPH IC_{50} value (Figure 3). The highest IC_{50} value of *D. salina* was recorded for the n-hexane extract (443.28 ppm), followed by the ethyl acetate (692.51 ppm), and methanol extracts (2175.75 ppm) (Table 3). In contrast, the highest IC_{50} value of *B. braunii* was recorded for the ethyl acetate extract (634.55 ppm), followed by the n-hexane (1300.90 ppm), and methanol extracts (2090.81 ppm).

The total phenolic contents in the *D. salina* and *B. braunii* extracts were 61.28 mg GAE/g extract and 23.75 mg GAE/g extract for n-hexane, 40.17 mg GAE/g extract and 46.94 mg GAE/g extract for ethyl acetate, and 4.08 mg GAE/g extract and 4.38 mg GAE/g extract for methanol, respectively (Table 4). The contents of chlorophyll a, chlorophyll b, and carotenoids in *B. braunii* extracts were 18.146 mg/g, 12.592 mg/g, and 4.573 mol/g, respectively (Table 4). Furthermore, the ethyl acetate extract of *B. braunii* exhibited the maximum content of chlorophyll a, chlorophyll b, and carotenoids, indicating that the antioxidant content of *B. braunii* was influenced by their content. In *D. salina*, the maximum contents of chlorophyll a, chlorophyll b, and carotenoids were observed in the ethyl acetate extract, whereas the maximum antioxidant content was observed in the n-hexane extract. The ethyl acetate extract of *D. salina* contained 3.035 mol/g carotenoids, 11.111 mg/g chlorophyll a, and 7.254 mg/g chlorophyll b. Moreover, the highest content of chlorophyll a and b in the microalgae *D. salina* and *B. braunii* was found in

the ethyl acetate extract. This is because ethyl acetate can extract semi-polar to non-polar compounds; chlorophyll a is non-polar, while chlorophyll b is polar. These results indicate that the antioxidant activity of *D. salina* and *B. braunii* is highly affected by the total phenolic content compared to the content of phytochemicals.

3.2 Discussion

Phytochemicals were more abundant in the methanol extracts than in the n-hexane and ethyl ace

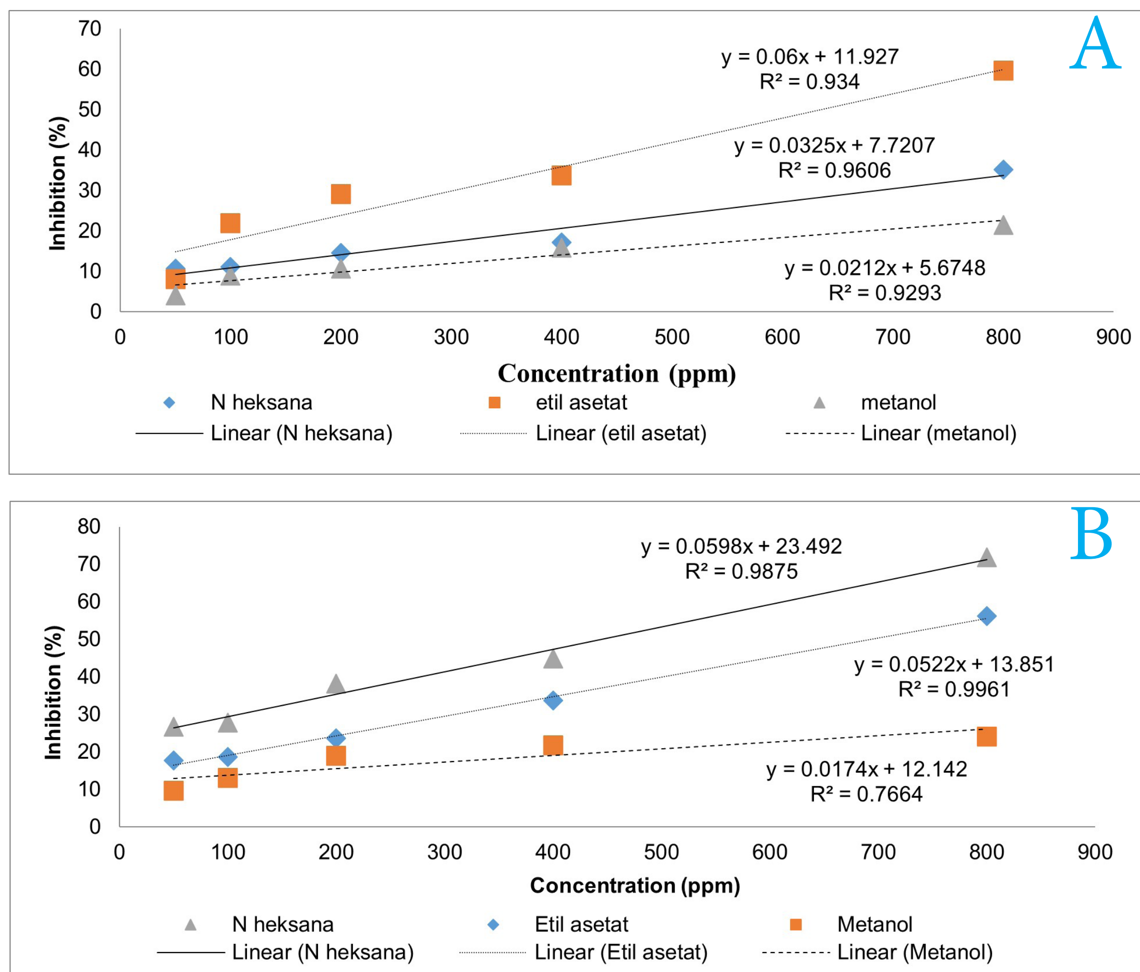

Figure 3. Antioxidant activity graph of *D. salina* (a) and *B. braunii* (b)

Table 4. Content of total phenolates (mg GAE/g sample), chlorophyll a, chlorophyll b, and carotenoids in microalgae *D. salina* and *B. braunii*

Microalgae	Solvent	Total Phenolates (mg GAE/g sample)	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Carotenoid (µmol/g)
	n-hexane	61.28	0.161	0.165	1.697
D. salina	Ethyl acetate	40.17	11.111	7.254	3.035
	Methanol	4.08	2.360	2.736	0.347
	n-hexane	23.75	3.460	4.733	1.091
B. braunii	Ethyl acetate	46.94	18.146	12.592	4.573
	Methanol	4.38	1.480	1.217	0.352

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tate extracts. This is because methanol is a polar solvent and can dissolve compounds with different polarities. Furthermore, the yields of the n-hexane extracts were lowest because n-hexane is a non-polar solvent, which can only dissolve non-polar compounds. However, the ethyl acetate extracts were able to dissolve semi-polar and non-polar compounds. Phytochemical screening and precipitation after the addition of specific reagents (Blainski *et al*., 2013), revealed that various secondary metabolites were present in the extracts.

Table 3. Absorbance value, % inhibition, and IC_{50} extract of *D. Salina* and *B. braunii*

Solvent	Concentration (ppm)	DPPH absorbance	Test Sample Absorbance + DPPH	% Inhibition	IC_{50}
D. Salina					
	50		0.14	26.79	
	100		0.13	27.92	
n-hexane	200	0.19	0.11	38.41	443.28
	400		0.10	45.13	
	800		0.05	71.92	
	50		0.18	17.85	
	100		0.18	18.71	
Ethyl acetate	200	0.22	0.17	23.65	692.51
	400		0.15	33.68	
	800		0.09	56.21	
	50		0.21	9.62	
Methanol	100	0.22	0.19	13.08	2175.75
	200		0.18	18.97	
	400		0.18	21.85	
	800		0.17	24.19	
B. braunii					
	50		0.19	10.66	
	100		0.19	11.15	
n-hexane	200	0.22	0.18	14.66	1300.90
	400		0.18	17.27	
	800		0.14	35.21	
	50		0.20	8.14	
	100		0.17	21.90	
Ethyl acetate	200	0.22	0.15	29.23	634.55
	400		0.14	33.77	
	800		0.09	59.62	
	50		0.21	4.14	
	100		0.20	8.95	
Methanol	200	0.22	0.19	10.66	2090.81
	400		0.18	15.87	
	800		0.17	21.58	

Before estimating the antioxidant activity, appropriate incubation time was determined by the relationship between incubation time and absorbance of the test sample to assess the optimal time as required for the stable reaction between the sample and DPPH. According to Akar *et al.* (2017), samples incubated with DPPH exhibited more stable absorbance values than those without incubation. Although different extracts required different incubation times for stable reaction with DPPH, all extracts were incubated for 30 minutes.

Flavonoids are phenolic compounds that exist freely (aglycone) and are bound as glycosides. Of all flavonoids, polymethoxyaglycones are non-polar and polyhydroxyaglycones are semi-polar, whereas flavonoid glycosides are polar, as they contain multiple hydroxyl groups and sugar (Kumar and Pandey, 2013). Similarly, several triterpenoid compounds, which contain cyclic alcohols, can also be bound to sugar moieties to dissolve in semi-polar and even polar solvents (Kalinowska *et al*., 2005). Thus, triterpenoid and steroid compounds are abundant in the extracts of *D. salina* and *B. braunii*.

Polar saponin compounds were also present in the extracts of *D. salina* and *B. braunii*, as they could dissolve easily in polar solvents. This finding was similar to the study by Podolak *et al.* (2010) who suggested that saponins are polar glycosides derived from sapogenins. The appearance of foam during detection of saponins indicated the presence of glycosides, which have the ability to produce foam in water and are hydrolyzed to glucose and other compounds.

In this study, antioxidant activity test was determined using the electron-transfer method, with DPPH as free radical. Antioxidant activity was estimated based on the ability of microalgal extracts to decrease the absorbance of DPPH free radicals. The principle of this method includes color change from purple to yellow, resulting from the reduction of DPPH to DPPH-H by the donation of hydrogen atoms from the compounds present in the extract and can be measured using a UV-VIS spectrophotometer (Muthia *et al*., 2019). The measured absorbance was the amount of residual DPPH absorbance not dampened by the antioxidant compounds present in the sample. The parameter used to interpret the antioxidant activity using the DPPH method was IC_{50}, which indicated the concentration of the sample solution required to reduce DPPH absorbance by 50%; the smaller the IC_{50} value, the more the antioxidant activity of the sample (Kurniasih *et al*., 2014).

Extremely strong antioxidants exhibit $IC_{50} <$ 50 ppm, strong antioxidants between 50 ppm and 100 ppm, moderate antioxidants between 100 ppm and 150 ppm, weak antioxidants between 150 ppm and 200 ppm, and extremely weak antioxidants > 200 ppm (Kurniasih *et al*., 2014). Based on this classification, the extracts of *D. salina* and *B. braunii* exhibited extremely weak antioxidant activity. The highest IC_{50} value was reported in the n-hexane extract of *D. salina* and the ethyl acetate extract of *B. braunii*. The total phenolic content, which was high in the n-hexane extract of *D. salina* and the ethyl acetate of *B. braunii*, could be the reason for the high antioxidant activity of these extracts. This is in accordance with the findings of Salvador *et al.* (2011), who reported that the antioxidant activity of a sample was closely associated with the content of phenolic compounds.

These results suggest that the phenolic compounds contained in *D. salina* are non-polar, while those contained in *B. braunii* are predominantly semi-polar and non-polar in nature. This was supported by Foo *et al.* (2015) who stated that the phenolic compounds in green microalgae are semi-polar and thus can be easily extracted using ethyl acetate. According to Dai and Mumper (2010), the solubility of phenolic compounds does not always depend on the polarity of the extract but can also depend on the structure of the phenolic compounds. In this study, the maximum total phenolic content was observed in the non-polar n-hexane extract of *D. salina* and the semi-polar ethyl acetate extract of *B. braunii*.

The highest carotenoid content was found in the ethyl acetate extract of *B. braunii*, presumably because the carotenoids present in these microalgae were semi-polar to non-polar in nature. This result was in line with Augustynska *et al.* (2015) who suggested that carotenoids are non-polar. Chlorophyll a, chlorophyll b, and carotenoids have also been reported to exhibit antioxidant activity (Zeb *et al*., 2019). However, in this study, the levels of chlorophyll a, chlorophyll b, and carotenoids in *D. salina* were not associated with antioxidant activity. This can be attributed to antioxidant inhibitors, such as salts, minerals, and other nutrients present in the samples (Akbar *et al*., 2018).

The antioxidant activity of the extracts of *D. salina* and *B. braunii* was relatively weak, presumably because they are crude extracts. This finding was supported by Ridlo *et al.* (2015) who reported that low antioxidant activity could be influenced by the presence of impurities in the extract. Another factor that could have affected the antioxidant activity of the extracts is the dry nature of the samples of *D. salina* and *B. brauni*. As the drying process notably affects and can damage antioxidant compounds, fresh samples will exhibit bet-

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ter antioxidant activity than dry samples (González-Palma *et al*., 2016).

4. Conclusions

The ethyl acetate extracts of *D. salina* and *B. braunii* contained alkaloids, steroids, triterpenoids, and flavonoids, whereas their methanolic extracts contained alkaloids, steroids, triterpenoids, flavonoids, and saponins. The antioxidant activity of all *D. salina* and *B. braunii* extracts was extremely weak. Chlorophyll a, chlorophyll b, and carotenoids were most abundant in the ethyl acetate extracts of *D. salina* and *B. braunii*. In contrast, the maximum total phenolic content was obtained in the n-hexane extract of *D. salina* (61.28 mg GAE/g extract) and the ethyl acetate extract of *B. braunii* (46.94 mg GAE/g extract).

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Authors' Contributions

The contribution of the authors is as follows, AR, DP, RABP, and DA; designed the study, performed the experiments, visualized data, and wrote the manuscript. All authors have discussed, read, approved, and contributed the final manuscript.

Conflict of Interest

The authors declare that there is no competing interests.

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