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Identification of Microalgae Isolates Using 18 S rRNA Markers and Testing Their Antioxidant Capacity

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Abstract. Microalgae are photoautotrophic microorganisms that synthesize bioactive compounds, including antioxidant compounds. Exploratory research on microalgae with strong antioxidant capacity as free radical scavengers is interesting and important to do. This study aims to identify and evaluate the antioxidant capacity of microalgae. Water samples were diluted with multilevel dilution. Microalgae were isolated using the streak plate method, and four microalgae isolates were successfully cultivated in vitro. Identification was carried out by phylogenetic analysis based on 18S rRNA marker gene sequences, namely *Chlorella vulgaris*, *Desmodesmus armatus*, *Dictyosphaerium ehrenbergianum*, and *Vitreochlamys incisa*. Antioxidant capacity was evaluated using three methods, namely DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate)), and FRAP (Ferric Reducing Antioxidant Power). IC50 as the concentration required to inhibit 50% of free radicals showed that methanol extract of *C. vulgaris* had a strong antioxidant capacity in the ABTS test with IC50 of 81.693 ppm and the highest in the DPPH test with IC50 of 297.451 ppm. In the FRAP test, the highest antioxidant capacity was in ethanol extract of *D. ehrenbergianum* isolate of 74.45 mg AAE/g. The *D. armatus* isolate had the lowest antioxidant capacity in the ABTS, DPPH, and FRAP tests.

Keywords: ABTS; Antioxidant capacity; DPPH; FRAP; ITS microalgae isolate

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INTRODUCTION

Microalgae are microscopic photosynthetic organisms in various environments, ranging from freshwater to marine ecosystems. They play a crucial role in ecosystems as primary producers, generating oxygen and biomass that form the foundation of aquatic food chains (Lu et al., 2021). Microalgae are also known for their nutritional content and significant health benefits, including their ability to produce bioactive compounds such as antioxidants. Antioxidants are molecules capable of neutralizing free radicals or reactive oxygen species (ROS), which can potentially damage cells, proteins, and DNA. In this way, antioxidants help protect the body from oxidative stress, which can lead to various diseases, including cancer and heart disease (Sansone & Brunet, 2019)

Exploratory research to obtain microalgae isolates that have strong antioxidant capacity amidst the increasing human health problems caused by free radicals is interesting and important to do. The accurate identification of microalgae is a crucial step in understanding the biodiversity and biological potential of various microalgal species. The 18S rRNA gene and 16S rRNA are often used as a marker for identifying microalgae for several key reasons. The 18S rRNA protein and the 16S rRNA protein are small components of the ribosomal subunit of cells, which are one of the basic components of all eukaryotic cells and prokaryotic cells, respectively. These genes are highly conserved within specific species, almost

reaching 100% conservation, making it a highly reliable tool for distinguishing species (Hajibabaei et al., 2007). Additionally, the universal primers available for the 18S rRNA gene can amplify most of its regions, including the V4 region, which is highly useful for identifying eukaryotic microalgae. The use of these primers enhances identification accuracy by producing amplicons that encompass the most variable regions of the target gene (Kezlya et al., 2023). Extensive 18S rDNA sequence data provide a robust reference for species identification, and metabarcoding methods utilizing this gene are highly valuable for analyzing microbial communities in environmental samples (Fawley & Fawley, 2020)

The antioxidant potential of microalgae has garnered significant attention in biotechnology research due to its ability to produce bioactive compounds capable of combating free radicals and oxidative stress in human cells. Microalgae inhibit free radicals through electron transfer processes and reduction via various mechanisms. Antioxidant enzymes such as superoxide dismutase, catalase, and peroxidase catalyze the reduction of oxygen radicals in water, neutralizing free radicals and preventing oxidative stress (Milrad et al., 2024). Additionally, microalgae produce non enzymatic antioxidants such as carotenoids, flavonoids, and phenolic acids that scavenge free radicals and protect cells from oxidative damage (Vignaud et al., 2023)Electron transfer pathways, such as the Mehler-ascorbate pathway, involve electron transfer from ascorbate to oxygen, reducing it to water (Cardol et al., 2011). During photosynthesis, microalgae generate electrons that are transferred through various electron carriers in the thylakoid membrane, helping to reduce ROS and maintain the balance of electron flow (Milrad et al., 2024). Even in dead microalgae, photoelectrons can be generated from pigments and transferred to bacteria capable of extracellular electron transfer (EET), further reducing ROS in the environment (Chen et al., 2023)

To assess the antioxidant capacity of microalgae, three methods are employed: ABTS, DPPH, and FRAP. The ABTS method measures the ability of extracts to neutralize ABTS•+ radicals, while the DPPH method evaluates their ability to reduce DPPH• radicals. The FRAP method assesses the extract's ability to reduce Fe^{3+} ions to Fe^{2+}. The combined use of these three methods provides a comprehensive overview of the antioxidant capacity of microalgal extracts, enabling accurate comparisons and validation of the results obtained in the context of protection against oxidative stress (Benzie & Strain, 1996; Munteanu & Apetrei, 2021; Xiao et al., 2020). This research aims to identify and evaluate the antioxidant capacity of microalgae isolates from the ITS student dormitory pond using ABTS, DPPH, and FRAP methods to better understand their potential for reducing oxidative stress.

METHODS

Microalgae Sampling and Research Locations

Microalgae sampling was carried out in the waters of the Student Dormitory Pond at the ITS, Sura-baya, East Java, Indonesia. The sampling was conducted at predetermined locations. The isolation of the samples was performed at the Bioscience and Plant Technology Laboratory, Biology Department ITS, while cultivation was conducted at the Aquaculture Laboratory, Biology Department ITS. The identification and antioxidant capacity analysis were carried out at the Biotechnology Laboratory, Biology Department ITS. The freeze drying treatment was conducted at the Life Sciences and Engineering Institute Laboratory, Airlangga University, Surabaya.

Preparation of Agar Medium

The agar medium prepared was Bold's Basal Medium (BBM) for samples collected from the freshwater pond at the ITS Dormitory (Aragaw et al., 2017). The composition of BBM per liter includes 0.25 g of $NaNO_3$, 0.075 g of $MgSO_4 \cdot 7H_2O$, 0.025 g of NaCl, 0.075 g of K_2HPO_4, 0.175 g of KH_2PO_4, 0.025 g of $CaCl_2 \cdot 2H_2O$, 0.0114 g of H_3BO_3, 1 ml of micronutrient solution, 1 ml of EDTA solution, 1 ml of $FeSO_4$ solution, 15 g of agar, and distilled water up to 1 liter. The composition of the micronutrient solution per 100 ml of stock solution includes 0.882 g of $ZnSO_4 \cdot 7H_2O$, 0.144 g of $MnCl_2 \cdot 4H_2O$, 0.071 g of MoO_3, 0.157 g of $CuSO_4 \cdot 5H_2O$, 0.049 g of $Co(NO_3)_2 \cdot 6H_2O$, and distilled water up to 100 ml. The composition of the EDTA solution per 100 ml of stock solution includes 5 g of EDTA, 3.1 g of KOH, and distilled water up to 100 ml. The composition of the $FeSO_4$ solution per 100 ml of stock solution includes 0.498 g of $FeSO_4$, 1 ml of concentrated H_2SO_4, and distilled water up to 100 ml. The final pH of the medium was adjusted to 6.6. The medium was sterilized using an autoclave at 121°C and 1.5 atm pressure for 30 minutes, resulting in a final pH value of 6-8. Trace elements were added as

nutritional supplements. The agar medium was then poured into petri dishes, sealed with plastic wrap, and allowed to solidify before streaking with samples (Andersen, 2005).

Sampling and Isolation of Microalgae

Microalgae samples were collected from the edge of the pond using a plankton net with a 35 µm mesh size. The plankton net was lowered into the water surface to a depth of 20-30 cm and then pulled towards the shore. Samples were transferred into bottles while rinsing the plankton net with water. Each sample bottle contained approximately 50 mL of pond water, which was subsequently used for isolation (Djoru et al., 2020).

The isolation of microalgae samples was conducted through a serial dilution method, where 1 mL of the sample was diluted in 10 mL of medium with serial dilutions ranging from 10^{-1} to 10^{-3}. In each dilution series, the microalgae samples were transferred to a solid agar medium using the streak method with an inoculating loop. The petri dish cultures were incubated at 27°C under a 30-watt fluorescent lamp for 7-14 days. Once the colonies grew, distinct colonies were transferred using the streak method to a new medium. After the growth of single colonies, they were transferred to a liquid medium for further biomass cultivation and microalgae harvesting (Haoujar et al., 2020).

Microalgae Cultivation and Harvesting

Microalgae were cultivated in the laboratory with stepwise volume increases, starting from 20 mL to 100 mL, 500 mL, and finally 2500 mL. The culture consisted of 20% inoculum and 80% fresh liquid media. Cultures were exposed to 30W LED light and aerated during the 100-2500 mL growth stages. Harvesting was conducted at the late log to early stationary phase, using centrifugation at 3500 rpm for 10-15 minutes. Approximately 500 mg of microalgal pellets were freeze-dried, beginning with storage at 60°C. The freeze-drying process involved vacuum drying at <100 mTorr until the samples were fully dried (about 6 hours). Dried samples were sealed under a vacuum and stored at 0°C in darkness (Goss et al., 2020)

Molecular Identification of Microalgae

Genomic DNA was extracted from microalgae cultures harvested during the exponential phase (OD680 ≤ 0.1; ≤5 × 10^5 cells/mL) (Khaw et al., 2020), using the Genomic DNA Mini Kit (Plant) Geneaid™ following the standard protocol. DNA was eluted with 100 µl of preheated elution buffer, and the concentration was measured using a NanoDrop Spectrophotometer. Primer sequences for 18S rRNA and 16S rRNA were retrieved from Chlorella, Navicula, Nitzschia, Picochlorum, Anabaena, and Oscillatoria. Primers were designed using Multalin and OligoAnalyzer, considering Tm, GC content, and homodimer formation. Primers for 18S rRNA were 18SF1 (5'-TKTGAAACTGCGAAYGGCTCAT-3'), 18SF2 (5'-AATTGACGGAAGGGCACCAC-3'), and 18SR (5'-GCGGTGTGTACAAAGGGCAG-3'). PCR was performed with GoTag® Green Master Mix, primers, and DNA templates, using specific thermal profiles for eukaryotic and prokaryotic microalgae. PCR products were visualized on a 1% agarose gel and sequenced by Genetika Science using Sanger sequencing. Sequence homology was assessed via BLAST, and phylogenetic analysis was conducted using MEGA11 with the Kimura 2-parameter model and bootstrap validation.

Microalgae Biomass Extraction

The separation of microalgae cells from the medium begins with centrifugation at 17,000 rpm for 1 minute. The samples are then dried using the freeze-drying method. The freeze-dried microalgae samples are subsequently macerated with acetone, methanol, and ethanol at a ratio of 1:40 w/v. For dissolving 10 mg of microalgae, 400 mL of solvent is required. The samples are stored in the dark at a controlled room temperature (25°C) for 24 hours. The maceration process is repeated three times. The process continues with the removal of the solvent by direct evaporation in the room until the solvent is completely evaporated (Foo et al., 2023).

DPPH (2,2-Diphenyl-1-picrylhydrazyl) Assay

One milligram of microalgae extract from each solvent (methanol, ethanol, and acetone) was dissolved in 1000 µL of methanol, resulting in a 1000 ppm stock solution. Serial dilutions were then prepared to concentrations of 500, 250, 125, 62.5, and 31.25 ppm. The assay was performed in a 96-well plate following the method of Zahratunnisa et al., (2017) with slight modifications. A 20 µL aliquot of the extract at various concentrations and 180 µL of 0.147 mM DPPH solution were added (Sembiring *et al*., 2017). In the 96-well microplate, preparations were made in a dark room, with a correction factor prepared containing 20 µL of sample and 100 µL

of methanol. Additionally, a blank was added to the 96-well microplate, consisting of 20 µL of methanol and 100 µL of DPPH solution. Absorbance was measured at 517 nm using a microplate reader.

The inhibition ratio (%) was calculated using the following equation (Xiao *et al*., 2020):

$$\text{Inhibition ratio (\%)} = \frac{A1-A2}{A1} \times 100$$

A1 is the absorbance of the ethanol substituted for the test sample, and A2 is the absorbance of the sample solution (Xiao *et al*., 2020). The IC_{50} value of DPPH (the concentration of the sample required to inhibit 50% of the DPPH radicals) was obtained through extrapolation from regression analysis. The % inhibition values were plotted on a regression equation with extract concentration (µg/mL) on the x-axis and % antioxidant inhibition on the y-axis. The IC50 value was calculated when the percentage inhibition was 50% using the equation y = ax + b.

ABTS (2,2'-azino-bis(3-etilbenzotiazolin-6-sulfonat)) Assay

One milligram of microalgae extract from each solvent (methanol, ethanol, and acetone) was dissolved in 1000 µL of methanol, resulting in a 1000 ppm stock solution. Serial dilutions were then prepared to concentrations of 500, 250, 125, 62.5, and 31.25 ppm. The assay was performed in a 96-well plate following the method of Zahratunnisa *et al*. (2021) with slight modifications. A 10 µL aliquot of the extract at various concentrations and 190 µL of 0.147 mM ABTS solution were added (Sembiring et al., 2017).

To prepare the ABTS reagent with potassium persulfate, dissolve 0.3841 g of ABTS diammonium salt in 100 mL of phosphate buffer solution at pH 6.8 to create a 7 mM ABTS stock solution. Separately, dissolve 0.0662 g of potassium persulfate (M = 270.322) in 100 mL of the same phosphate buffer to obtain a 2.45 mM potassium persulfate stock solution. Combine equal volumes of these two solutions to achieve final concentrations of 7 mM ABTS and 2.45 mM potassium persulfate. Allow the mixture to react for 12-16 hours at room temperature (25°C) to generate the blue ABTS•+ radical cation. Dilute the activated ABTS solution with deionized water to reach an absorbance of 0.7 ± 0.05 at 734 nm. This method is used in the ABTS assay to assess antioxidant capacity by measuring the scavenging effect of antioxidants on the ABTS•+ radical cation (Tsvetkova et al., 2023). In the 96-well microplate, preparations were made in a dark room, with a correction factor prepared containing 0 µL of sample and 100 µL of methanol. Additionally, a blank was added to the 96-well microplate, consisting of 20 µL of methanol and 100 µL of ABTS solution.

The inhibition ratio (%) was calculated using the following equation (Xiao et al., 2020):

$$\text{Inhibition ratio (\%)} = \frac{A1-A2}{A1} \times 100$$

A1 is the absorbance of the ethanol substituted for the test sample, and A2 is the absorbance of the sample solution (Xiao et al., 2020). The IC_{50} value of ABTS (the concentration of the sample required to inhibit 50% of the ABTS radicals) was obtained through extrapolation from regression analysis. The % inhibition values were plotted on a regression equation with extract concentration (µg/mL) on the x-axis and % antioxidant inhibition on the y-axis. The IC_{50} value was calculated when the percentage inhibition was 50% using the equation y = ax + b.

FRAP (Ferric Reducing Antioxidant Power) Assay

According to Kusumorini et al., (2022) and AquinoBolaos et al., (2016), the FRAP assay protocol for determining antioxidant capacity involves the following steps. The FRAP working solution is prepared by mixing 10 volumes of acetate buffer (300 mM, pH 3.6) with 1 volume of TPTZ (40 mM dissolved in 40 mM HCl) and 1 volume of ferric chloride (20 mM in water). The FRAP working solution is prepared daily and warmed to 37°C for 10 minutes prior to use. Ascorbic acid and AFS standards (1000 µM) are prepared in a 100 mL volumetric flask using double deionized water without any additional precautions. The microplate FRAP assay is performed by adding 20 µL of the sample solution directly to a 96-well microplate, followed by 280 µL of FRAP working solution. The mixture is shaken, incubated at 37°C in the dark for 30 minutes, and then the absorbance at 593 nm is recorded using a microplate reader. Antioxidant capacity is calculated using the results from the linear regression equation of the ascorbic acid standard solution and is computed using the following equations :

$$\text{Ascorbic Acid Equivalent Concentration (AAEC)} = \frac{Corrected\ Absorbance - c}{m}$$

$$\text{Antioxidant capacity (mg AAE/g dry sample)} = AAEC\left(\frac{mg}{mL}\right) \times 1000\frac{mL}{g}$$

(AquinoBolaos et al., 2016)

c represents the y-intercept, indicating the absorbance when the sample concentration is zero, and m represents the slope of the linear regression, indicating the change in absorbance per unit change in ascorbic acid concentration.

Data analysis

Species identification of microalgae was performed using a descriptive qualitative approach. Anti-oxidant capacity was evaluated by determining the IC50 values from the ABTS and DPPH assay. The data distribution was assessed using the Shapiro-Wilk normality test. For non-parametric data, the Kruskal-Wallis test followed by Dunn's post-hoc test was applied. For parametric data, one-way ANOVA followed by Tukey HSD post-hoc test was used to compare antioxidant capacities between microalgal isolates. All analyses were performed using R version 4.0.2 in RStudio version 1.3.1093.

RESULTS AND DISCUSSION

Molecular Identification Result

The DNA concentration of microalgae isolates ranged from 20.22 to 62.52 ng/µl, with an A260/A280 ratio ranging from 1.8 to 1.89, indicating relatively pure DNA with little or no protein contamination. The resulting A260/A230 ratio ranged from 2.01 to 2.17, indicating good purity and indicating little or no organic or salt contamination (Shen, 2023). The results of the gel electrophoresis are shown in **Figure 1**. which shows that the four isolates are at a position of around 1500 bp. This position indicates the size or length of the DNA fragments of the four isolates after the PCR process. The length of the resulting amplicon is determined by the distance or interval between the forward and reverse primers on the target DNA strand. The primer indirectly affects the length of the amplicon, but the primer determines the specificity and initial location of the target DNA amplification which will ultimately affect the length of the resulting amplicon fragment (Debode et al., 2017). The amplification results show that the DNA bands are clearly visible (not faint) so it can be said that the DNA has high purity and low contaminants. This is in accordance with the statement that faint DNA bands in the visualization results indicate an absorption bias at 260 nm due to high contaminants or low purity (Fitriyah et al., 2021).

Molecular identification of the microalgae isolates from the ITS student dormitory pond was performed using 18S rRNA gene sequencing. BLAST analysis confirmed that isolate HDB3a matched 100% with *Dictyosphaerium ehrenbergianum* (KU561148.1), 16HtDB2b with *Vitreochlamys incisa* (99.54%, FR865524.1), 17HtDB2b with *Desmodesmus armatus* (100%, KF673362.1), and KD3a with *Chlorella vulgaris* (100%, LC733217.1). Phylogenetic analysis (Figure 2) further validated these identifications by grouping each isolate into distinct clades that corresponded to their respective genera. Specifically, *D. ehrenbergianum* formed a robust clade with other *Dictyosphaerium* species, supported by a high bootstrap value (96-100%). *V. incisa* clustered closely with other members of the *Vitreochlamys* genus, with a strong bootstrap value of 100%. Similarly, *D. armatus* and *C. vulgaris* were grouped within their respective clades, both of which were supported by high bootstrap values (86%-100%). The phylogenetic tree (Figure 2) was constructed using the maximum-likelihood method with the Kimura 2-parameter model. Bootstrap values above 50% are indicated at the nodes. The identified isolates (*D. ehrenbergianum*, *V. incisa*, *D. armatus*, and *C. vulgaris*) are highlighted in green to distinguish them from other reference sequences. *Bacillus subtilis* was used as an outgroup to root the tree and provide a clear evolutionary context for the microalgae species.

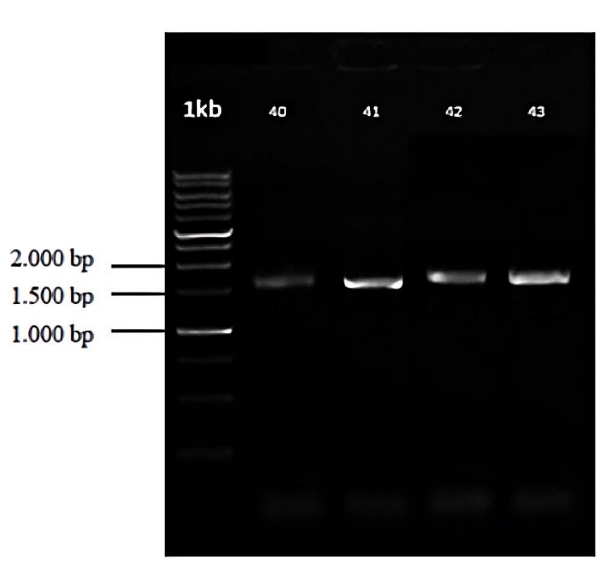

Figure 1. Visualization of amplified DNA on agarose gel. 1kb = DNA ladder, 40 = isolate 16HtDB2b, 41 = isolate 17HtDB2b, 42 = isolate KD3a, and 43 = isolate HDB3a.

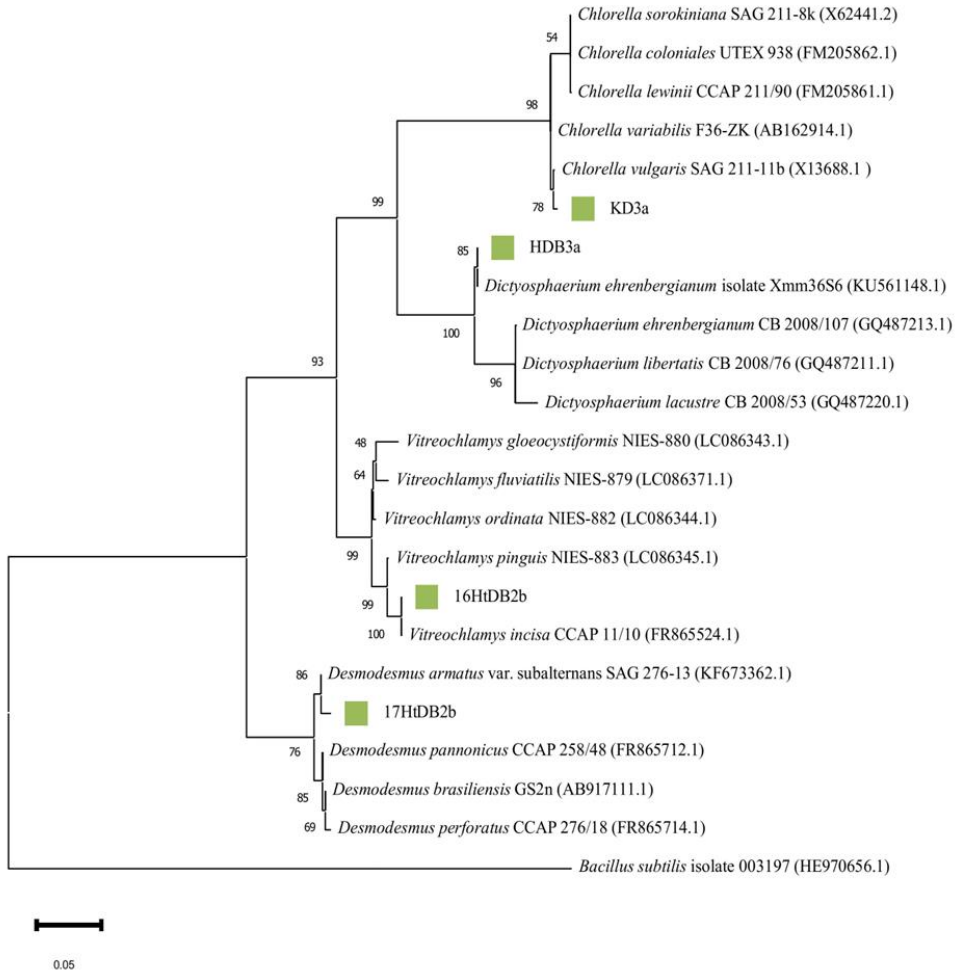

Figure 2. Phylogenetic tree constructed using 18S rRNA sequences. The phylogenetic analysis was performed using the maximum-likelihood method with the Kimura 2-parameter model. Bootstrap values above 50% are indicated at the nodes.

The morphology of the four microalgae isolates that were successfully identified molecularly using the 18S rRNA marker gene is shown in Figure 3 below.

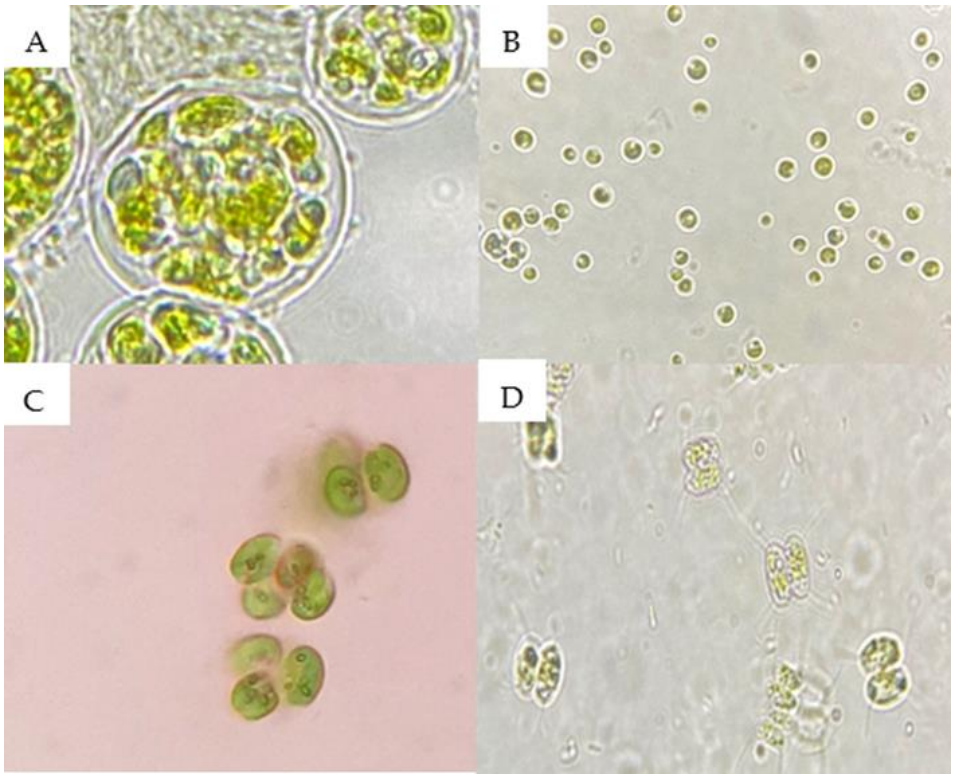

Figure 3. Morphological observation of microalgae isolates under a microscope at 1000× magnification. A. *D. ehrenbergianum*; B. *C. vulgaris*; C. *V. incisa*; D. *D. armatus*.

ABTS, DPPH, and FRAP Assay Results for Antioxidant Capacity of Microalgae Extracts

The antioxidant capacity of four microalgae isolates was assessed using ABTS and DPPH assays, with extracts prepared using acetone, ethanol, and methanol as solvents. IC_{50} values, indicating the concentration required to inhibit 50% of free radicals, were determined through exponential regression of concentration curves (61.25, 125, 250, 500, and 1000 ppm). As shown in Table 1, *C. vulgaris* exhibited the highest antioxidant potential in methanol with an IC_{50} of 81.693 ppm in the ABTS method, categorizing it as a strong antioxidant (50–100 ppm) according to Molyneux (2004). Conversely, *D. armatus* showed the weakest antioxidant capacity with an extremely high IC_{50} in acetone (87,278.031 ppm), indicating low efficacy in all solvents tested. *D. ehrenbergianum* was most effective in methanol (IC_{50} 166.617 ppm), falling within the medium strength antioxidant category (100–150 ppm), while *V. incisa* showed moderate antioxidant capacity in ethanol (IC50 390.167 ppm), classified as weak (150–200 ppm). These findings indicate that methanol consistently outperformed other solvents in extracting antioxidant compounds from microalgae. This result is comparable to findings by Agustini et al. (2022), which demonstrated that ethanol extract of green algae *Cosmarium* sp. exhibited the best antioxidant activity, suggesting that solvent polarity plays a crucial role in extracting bioactive compounds with antioxidant properties.

In the DPPH assay, the IC_{50} values of different solvent extracts further demonstrated significant variation in antioxidant capacity among the microalgae samples. Consistent with the ABTS results, the methanol extract of *C. vulgaris* exhibited the best antioxidant capacity with a relatively low IC_{50} value (297.45 ppm), though this was not as strong as its performance in the ABTS assay. *C. vulgaris* has been shown to enhance superoxide dismutase (SOD) activity, an enzyme that neutralizes superoxide radicals (O_2^-) by converting them to hydrogen peroxide (H_2O_2) and oxygen (O_2) (Hernayanti & Simanjuntak, 2019). In their study, C. vulgaris extract administration to CCl_4-exposed test animals significantly increased SOD activity to levels comparable with healthy controls. This enhancement is attributed to the Cu and Zn content in C. vulgaris, which act as essential cofactors for SOD, demonstrating the specie's role in strengthening enzymatic antioxidant defenses against oxidative stress. Conversely, *D. armatus*

showed a very high IC_{50} value, especially in the methanol extract (78,453.30 ppm), reinforcing its weak antioxidant capacity across both assays. These differences indicate the importance of microalgae species and solvent type in determining antioxidant capacity.

To complement the descriptive findings, statistical analysis was conducted to evaluate the significance of these variations. The normality of the IC_{50} data was assessed using the Shapiro-Wilk test, revealing that the IC_{50} values for acetone ($p = 0.001509$) and ethanol ($p = 0.002574$) extracts did not follow a normal distribution, whereas the methanol extracts exhibited a normal distribution ($p = 0.1301$). Consequently, the Kruskal-Wallis test was applied to acetone and ethanol extracts, while ANOVA was used for methanol extracts. The statistical tests indicated no significant differences in IC_{50} values among microalgae species for acetone ($\chi^2 = 3$, $df = 3$, $p = 0.3916$), ethanol ($\chi^2 = 3$, $df = 3$, $p = 0.3916$), and methanol extracts (ANOVA, $p > 0.05$). Although the statistical analysis showed no significant differences between species for any of the solvents, descriptive analysis highlighted *C. vulgaris* as the most effective in reducing IC_{50} value, particularly with methanol (81.69 ± 4.58 ppm). This observation reinforces the descriptive trends seen across the ABTS and DPPH assays and suggests that methanol is the most effective solvent for extracting antioxidant compounds. Overall, these results imply that while the statistical differences are negligible, methanol consistently provides the best extraction efficiency across microalgae species, aligning with the descriptive analysis findings.

The antioxidant capacity test using the FRAP method is shown in Table 2. The results indicate that *D. ehrenbergianum* extracted with ethanol (74.45 mg AAE/g) and *C. vulgaris* extracted with acetone (53.88 mg AAE/g) exhibited strong antioxidant capacity. In contrast, the *V. incisa* extracts showed moderate antioxidant capacity, with FRAP values ranging from 18.62 mg AAE/g to 20.74 mg AAE/g. *D. armatus* displayed a range of antioxidant activities, from weak to moderate, depending on the solvent used, with FRAP values from 5.58 mg AAE/g to 25.04 mg AAE/g.

The antioxidant capacity of microalgae extracts was evaluated using the ABTS and DPPH assays. Methanol, a highly polar solvent, proved to be the most effective in extracting antioxidant compounds, outperforming ethanol and acetone. Ethanol, though effective, showed lower antioxidant capacity compared to methanol due to its lower polarity, which affects its efficiency in extracting polar and non-polar compounds (Pereira et al., 2024). Methanol, being more polar, can extract a broader range of bioactive compounds, making it superior for obtaining antioxidants from microalgae. In contrast, acetone, with its lower polarity, was more effective at extracting chlorophyll and pigments but less efficient for other bioactive compounds. Additionally, ethanol is considered safer than acetone, which is toxic and poses health risks (Amin et al., 2018).

Table 1. IC_{50} values of ABTS and DPPH antioxidant assays for various solvent extracts of microalgae isolates

Species	*IC_{50} (ppm)*					
	ABTS test			***DPPH test***		
	Aceton	**Ethanol**	**Methanol**	**Aceton**	**Ethanol**	**Methanol**
D. ehrenbergianum	882.54±0.91	389.25±1.99	166.62±1.18	1095.07±0.55	1070.56±0.29	4550.34±1.12
V. incisa	666.79±2.18	390.17±1.05	297.84±1.34	2972.99±0.92	16477.62±0.83	2973.00±1.14
D. armatus	87278.03±0.92	10557.54±0.74	984.94±0.85	1117.84±0.74	285213.01±0.41	78453.30±0.74
C. vulgaris	310.26±3.58	139.63±2.96	81.69±4.58	481.24±0.79	478.30±0.86	297.45±3.03

Table 2. FRAP values for various solvent extracts of microalgae isolates

Species	***FRAP value (mg AAE/g)***		
	Aceton	**Ethanol**	**Methanol**
D. ehrenbergianum	29.90±5.06	74.45±2.11	41.33±7.1
V. incisa	20.57±1.12	18.62±4.23	20.74±2.60
D. armatus	5.58±1.98	7.90±2.12	25.04±0.90
C. vulgaris	53.88±1.51	12.54±1.61	51.78±1.53

The microalgae isolates used in this study, all classified under the Phylum Chlorophyta, displayed notable antioxidant characteristics. For instance, microalgae from the genera *Chlorella* and *Scenedesmus* produce polyphenolic compounds, including flavonoids and phenolic acids, known for their strong antioxidant properties and ability to neutralize free radicals, offering various health benefits (Vignaud et al., 2023). These antioxidants, which include glutathione (GSH) and tripeptides, play a crucial role in maintaining cellular redox balance by scavenging free radicals. The methanol extracts from Chlorophyta microalgae, therefore, contained a diverse range of antioxidants, such as polyphenols, carotenoids, antioxidant enzymes, and glutathione, contributing to their strong antioxidant capacity (Maadane et al., 2015). In Zhao et al. (2022), *Dictyosphaerium* species showed significant antioxidant capacity with high superoxide dismutase (SOD) values. For instance, *Dictyosphaerium* sp. 1A10 exhibited an SOD activity of 940 ± 10 U/g tissue, indicating its potential in mitigating oxidative damage through efficient radical scavenging. Although ABTS, DPPH, and FRAP assays do not directly measure SOD activity, high SOD levels indirectly support the antioxidant efficacy observed in these assays (Kusumorini et al., 2022).

The DPPH assay results indicated that methanol extracts generally showed stronger antioxidant capacity compared to ethanol and acetone. Methanol, being the most polar of the three solvents, was most effective at dissolving polar antioxidant compounds, while acetone, with lower polarity, was less effective. This relationship between solvent polarity and antioxidant capacity reflects the solubility of polar antioxidants like polyphenols, flavonoids, and glutathione, which are soluble in polar solvents and exhibit strong antioxidant properties (Fioroni et al., 2023). On the other hand, nonpolar antioxidants, such as carotenoids and tocopherols, are more soluble in lipid-based solvents and exhibit different extraction efficiencies. *C. vulgaris* extracts showed a direct correlation between solvent polarity and antioxidant capacity, with methanol yielding the highest antioxidant capacity. Despite being categorized as a very weak antioxidant by Molyneux (2004), the methanol extract of *C. vulgaris* demonstrated strong inhibition of DPPH radicals, reflecting its rich content of bioactive compounds. Polar extracts from *C. vulgaris* contained high levels of polyphenolic compounds, flavonoids, and glutathione, contributing to their robust antioxidant properties. Carotenoids in *C. vulgaris*, though generally nonpolar, also include some polar forms, enhancing their solubility and antioxidant effectiveness (Dardavila et al., 2023). Overall, the polar extracts from *C. vulgaris* exhibited substantial antioxidant potential due to their high content of polyphenols, flavonoids, glutathione, and some polar carotenoids.

The inconsistent relationship between solvent polarity and FRAP values across different microalgal isolates can be attributed to several factors. One key factor is the variation in extraction efficiency due to the interaction between the solvent and phytochemicals. Polar solvents like ethanol and methanol are effective at extracting polar compounds, while nonpolar solvents are better suited for extracting nonpolar compounds. This variation can lead to inconsistent FRAP values (Wakeel et al., 2019). Furthermore, the polarity of the phytochemicals themselves plays a crucial role; some are highly polar and thus more efficiently extracted with polar solvents, while others require nonpolar solvents. The conformational structure of phytochemicals also influences their interaction with solvents and their ability to reduce ferric ions in the FRAP assay. The use of solvent combinations with varying polarities can create synergistic or antagonistic effects, further complicating the relationship between solvent polarity and FRAP values (Sulaiman et al., 2011). In the FRAP method, antioxidants reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) through electron transfer, reflecting their antioxidant capacity. Therefore, the variability in FRAP values can be explained by the complex interactions between solvent properties, phytochemical polarity, structural conformation, solvent combinations, and extraction conditions (Yulianti et al., 2023).

This research contributes detailed antioxidant profiles of *Dictyosphaerium ehrenbergianum* and *Vitreochlamys incisa*, which have been understudied compared to more common species. The comparative analysis of three different extraction solvents (acetone, ethanol, and methanol) across multiple antioxidant assessment methods (ABTS, DPPH, and FRAP) provides valuable insights into optimizing extraction procedures for maximum antioxidant yield. The study's findings have significant practical applications, particularly in identifying promising local sources of natural antioxidants, as demonstrated by *C. vulgaris*'s strong antioxidant capacity (IC50 81.69 ppm in methanol). This

research establishes important methodological contributions through its comprehensive protocol for microalgae identification and characterization while also providing baseline data for future comparative studies. The validated molecular identification protocol and detailed biochemical characterization methods presented here can serve as a foundation for future research in microalgae-based biotechnology applications. This work not only advances our understanding of microalgae antioxidant properties but also provides practical insights for developing sustainable sources of natural antioxidants from local water bodies, contributing to both scientific knowledge and potential economic development through microalgae-based products.

CONCLUSION

The conclusion obtained from this study is that four microalgae isolates were successfully identified and characterized, namely, *Dictyosphaerium ehrenbergianum*, *Vitreochlamys incisa*, *Desmodesmus armatus*, and *Chlorella vulgaris*, through 18S gene sequencing. The antioxidant capacity of these isolates was comprehensively assessed, revealing significant variations in IC_{50} values across solvents and methods. Among the isolates, *C. vulgaris* demonstrated the strongest antioxidant potential, particularly with methanol extracts (IC_{50} is 81.69 ppm in ABTS, 297.45 ppm in DPPH, and FRAP value is 51.78 mg AAE/g). In contrast, *D. armatus* consistently exhibited the weakest antioxidant capacity across all solvents and assays, with exceptionally high IC_{50} values. This study underscores the potential of microalgae as sources of natural antioxidants and provides insights into optimizing extraction protocols for enhanced bioactivity.

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