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Determination of Antioxidant Activity, Phenolic Compounds, and Toxicity of Methanolic and Ethanolic Extracts of Pink Pigmented Facultative Methylotrophs (PPFM) Bacteria Pigment

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ABSTRACT

Pink-pigmented facultative methylotrophs bacteria are a plant's surface inhabitant, especially at the leaf. They are known as *Methylobacterium* species. The antioxidant activity, phenolic compounds, and level of toxicity of this bacteria pigment have been studied. Recently, no previous research focused on the same bacterium found in *Melicope lunu-ankenda* (Gaertn.) T. G. Hartley, which is a component of the Malaysian *ulam* leaf. This study employed the 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric ion reducing antioxidant power (FRAP), and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assays, along with total phenolic content determination to assess the antioxidant activities of the methanolic and ethanolic pigment extract. Additionally, the consumption safety level of the pigment extract used brine shrimp lethality assay. From these findings, ethanolic pigment extract has a higher antioxidant capacity than methanolic extract. The DPPH half-maximal inhibitory concentration (IC_{50}) value of methanolic pigment extract is higher than ethanolic extract (0.72 ± 0.04 mg/ml), but the IC_{50} value is *vice versa* for ABTS (4.59 ± 2.17 mg/ml). Furthermore, ethanolic extracts have a high FRAP assay value (1.09 ± 0.19 mg/mg of trolox equivalent at 0.78 mg/ml sample) and phenolic content (1.39 ± 0.07 mg/mg of gallic acid equivalent at 0.78 mg/ml sample) compared to methanolic pigment extracts. Fortunately, the methanolic and ethanolic pigment extract's lethal concentration values

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(4.52 and 9.94 mg/ml) are considered safe for food application since their toxicity level is higher than 1 mg/ml.

Keywords: Antioxidant activity, bacteria pigment, *Methylobacterium* sp., phenolic compound, pink pigmented facultative methylotrophs, toxicity

INTRODUCTION

The bacteria known as pink-pigmented facultative methylotrophs (PPFM) live mostly on the surface of leaves, especially *ulam* leaves, in the phyllosphere. They can solely obtain their energy and carbon from single-carbon molecules or multi-carbon growth substrates (Green, 2014). Their cell body's appearance of reddish pink reflects the presence of carotenoid pigment (Madhaiyan, 2003). However, similar to bacteriochlorophylls, which serve as the light-harvesting pigment, carotenoids in PPFM pigment can also contribute to the process of light absorption (Boronat & Rodrguez-Concepción, 2015). Also, they can withstand more UV radiation than other types of bacteria since too much UV exposure can lead to the formation of oxidants in cells (Dreyer, 2016; Santos et al., 2013). Oxidants, which are unstable and chemically reactive entities, are generally referred to as free radicals, and they need to be managed to prevent unfavourable events in the biological system (Pawar et al., 2015). The greatest significant free radicals in the body are known as reactive oxygen species (ROS). These species interact with nearby macromolecules to stabilise themselves, producing an oxidative stress state that ultimately causes cell death (Cheeseman & Slater, 1993; Dekkers et al., 1996; Halliwell & Gutteridge, 2015).

Bacteria, especially pigmented ones, are constantly exposed to various harmful environmental conditions, including desiccation, freezing, UV radiation, and changes in heavy metal concentrations, which cause ROS to produce and build up in the body gradually (Dring, 2005). They thus turn on their antioxidant defence systems to neutralise ROS since excess ROS damages their cellular structures and function and causes them to die (Fridovich, 1986; Hajam et al., 2022). Hence, the antioxidant compounds found in their cells, including carotenoids, phenolic compounds, and flavonoids, are essential for their survival (Nagy et al., 2018; Photolo et al., 2020; Zeb, 2020). Some examples of antioxidant compounds found in *Methylobactrium* sp. are myxol (carotenoid) and phenol, 2,5-bis(1,1-dimethylethyl) (phenol) (Photolo et al., 2020; Stepnowski et al., 2004). The activity of antioxidant and phenolic compound content of ML8 pigment extract was evaluated through various assays, which are DPPH radical scavenging, ABTS radical scavenging, FRAP, and total phenolic content (TPC) due to the bacteria pigment benefits. Moreover, the toxicity of these pigment extracts was also determined to establish a safe dosage for consumption. In this study, the brine shrimp (*Artemia salina*) lethality bioassay was employed as a quick, easy, and effective method to evaluate the toxicity of a range of compounds such as natural plant extracts, poisonous chemicals, different hazardous

metal substances, and organic compounds, as highlighted in studies conducted by Lu and Yu (2019), Wu (2014), as well as Yu and Lu (2018).

Artemia salina, a class Crustacea and phylum Arthropoda member, can survive in hypersaline surroundings. *Artemia* sp. is a widely used biological model in low-cost toxicity bioassays conducted in laboratory settings, owing to its quick hatching, easy accessibility, and susceptibility to harmful compounds. This organism's small size, well-understood biological and ecological characteristics, and ability to adapt to various testing environments make it easy to operate in labs (Lu & Yu, 2019; Yu & Lu, 2018). Additionally, *Artemia* sp. has a unique ability to reproduce sexually and asexually in water with a 0.4–3.4 M salinity range and varying ionic environments. Its life cycle includes the cyst stage (the most resilient of all animal life history stages) and motile stages (that are among the best osmoregulators in the animal kingdom), making it a popular model for various studies, including toxicity tests (Norouzitallab, 2015; Ríos & Gajardo, 2004). In addition, research has indicated that this species has a similar toxicity mechanism to mammalian creatures. The *A. salina* test and the findings from toxicity studies conducted on a mouse fibroblast cell line, for instance, did not show any appreciable changes, according to Rajabi et al. (2015). However, according to Hamidi et al. (2014), the toxicity test results using mice and *A. salina* have a high correlation in determining the lethal concentration (LC_{50}).

MATERIALS AND METHODS

Materials

The methanol and ethanol pigment extracts of PPFM bacteria were prepared previously in several steps. The PPFM bacteria was initially isolated from *M. lunu-ankenda* leaf, cultured and purified on *Pseudomonas* agar media (BD Difco™, USA). The pure culture of the isolated PPFM bacteria (labelled as ML8) was grown in modified King's B broth media (contained peptone [BD Difco™, USA], magnesium sulphate heptahydrate [Systerms, Malaysia], potassium dihydrogen phosphate and glycerol (anhydrous) [R & M Chemicals, United Kingdom]) for cell enrichment for 5 days to obtain its pigment. Then, the cell was collected through centrifugation, and its pigment was extracted using selected solvents, followed by a drying process with several drying equipment, including a rotary evaporator. Those bacteria pigment extracts were tested on several related antioxidant capacity measurement activities, as well as a toxicity evaluation. They are DPPH, FRAP, ABTS radical scavenging assays, and TPC. Also, the brine shrimp method was used for toxicity level evaluation. In detail, the chemicals used are methanol, 2.0 N Folin-Ciocalteau's phenol reagent, ascorbic acid, potassium dichromate (R & M Chemicals, United Kingdom), sodium carbonate anhydrous (Systerm, Malaysia), trolox, and gallic acid monohydrate (Acros Organics, USA). Then, DPPH (free radical) powder (Alfa Aesar, Thermo Fisher Scientific, USA), ABTS diammonium salt, quercetin dehydrate, sodium acetate

anhydrous, potassium peroxodisulfate, and iron (III) chloride (Sigma-Aldrich, USA), glacial acetic acid, hydrochloric acid (HCl) 37% (EMSURE®, Merck, Germany), 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ) (Merck, Germany), sodium chloride (QRëC, Thailand), and brine shrimp eggs were bought from supplier at Seri Kembangan (Malaysia). A microplate spectrophotometer (BioTek Model EL800, USA), with Gen5™ software (version 1.06.10) was used for absorbance reading in the DPPH assay while microplate spectrophotometer from Benchmark Plus Microplate model (BIO-RAD 170-6930, USA) equipped with Microplate Manager® software (version 5.2.1) was used to read absorbance for ABTS scavenging, FRAP, and TPC assays. All aqueous solutions were prepared using distilled water, and all purchased chemical reagents were of analytical grade. All antioxidant assays were done in minimal light to avoid technical errors during the experiment sessions.

Methods

DPPH Radical Scavenging Assay. In a 96-well microplate, samples were prepared at concentrations of 5–0.04 mg/ml (methanol extract), 2.5–0.02 mg/ml (ethanol extract), and 0.8–0.01 mg/ml for ascorbic acid. Then, 0.2 mM fresh DPPH solution was prepared using DPPH powder (Alfa Aesar, Thermo Fisher Scientific, USA) and methanol (R & M Chemicals, United Kingdom) referring to the modified method of Nor et al. (2023) and Prieto (2012). Next, the volume of the DPPH solution was mixed with the volume of the sample solution with a 1:1 ratio and incubated for 30 min at room temperature. The absorbance was then read at 515 nm. The result was shown by calculating the percentage of scavenging activity according to the equation below:

$$\%\ \text{DPPH scavenging} = \left(\frac{\text{A control} - [\text{A sample} - \text{A colour sample}]}{\text{A control}}\right) \times 100\%$$

where % DPPH scavenging = the percentage of radical scavenging activity, A control = absorbance of solvent with DPPH, A sample = absorbance of the sample with DPPH, and A colour sample = absorbance of the sample.

The results were plotted as the percentage (%) of scavenging activity versus the sample's concentration to obtain the IC_{50} inhibition concentration value. The IC_{50} value represents the sample amount that can reduce 50% of the DPPH radical, as defined by Ismail et al. (2013). The antioxidant capacity was stated in per cent inhibition (%I).

FRAP Assay. By slight modification of Pawar et al. (2015) and Sahib et al. (2012) FRAP method, the preparation of a working FRAP reagent was done by mixing 300 mM acetate buffer (pH 3.6, Sigma-Aldrich, USA), 10 mM TPTZ solution (Merck, Germany, which was diluted in 40 mM HCl [EMSURE®, Merck, Germany]), and 20 mM iron (III) chloride (Sigma-Aldrich, USA) in a ratio of 10:1:1. Then, the working FRAP reagent solution (150

µl) was mixed with a sample (20 µl) in each well of a 96-well microplate. The sample and standard (Trolox, Acros Organics, USA) were prepared as follows: 50–0.4 mg/ml (methanol extract), 5–0.04 mg/ml (ethanol extract), and 0.5–0.004 mg/ml (standard), respectively. After adding the sample to the FRAP reagent, it was placed in the dark at 37°C for incubation at 30 min, and the absorbance was read at 595 nm. The antioxidant activity of the extract was stated as trolox equivalents of 100 mg TE/g of crude sample extract.

ABTS Radical Scavenging Assay. This kind of scavenging assay was conducted based on the protocol described by Hussin et al. (2019), Pawar et al. (2015), and Verma et al. (2009) with slight amendments. The stock solutions of 7 mM ABTS (Sigma-Aldrich, USA) and 2.45 mM potassium peroxodisulfate (Sigma-Aldrich, USA) were prepared freshly before use. Then, those stock solutions were mixed with a 1:1 ratio and placed at room temperature for 16 hr under dark conditions to produce the ABTS radical cation. The working solution was then diluted to an absorbance reading of 0.70 ± 0.2 units at 734 nm, resulting in the $ABTS^{\cdot+}$ solution. A sample (50–0.4 mg/ml) and a standard (quercetin) solution (1.00–0.01 mg/ml) were prepared accordingly. A volume of 195 µl $ABTS^{\cdot+}$ solution was added to react with 5 µl samples for 60 s, and the absorbance was read at 734 nm. The collected data of antioxidant activity for the ABTS scavenging assay was calculated and presented similarly to the DPPH scavenging assay.

TPC Assay. Samples at concentrations of 50–0.4 mg/ml (methanol extract), 5–0.04 mg/ml (ethanol extract), and 0.5–0.004 mg/ml standard (gallic acid) were prepared to conduct the TPC assay adapted from Ismail et al. (2013). The assay was performed by adding a 10 µl sample with 75 µl of 0.2 N Folin-Ciocalteau's phenol reagent (R & M Chemicals, United Kingdom), and the reaction was let to occur at room temperature for 5 min, and subsequently, 75 µl of 6% sodium carbonate (Systerm, Malaysia) was added. The absorbance value at 725 nm was taken after 90 min of incubation at room temperature. A calibration curve was prepared using a series concentration of standard solutions. The TPC result was expressed as 100 mg gallic acid equivalents (GAE)/g of sample weight based on the equation below, as adapted from Singh et al. (2015):

$$C = \frac{V}{m} \times c$$

where C = total phenolic content mg GAE/g dry extract, c = concentration of gallic acid from the calibration curve (mg/ml), V = volume of sample (ml) and reagent mixture, and m = mass of sample (g).

Toxicity Study

The toxicity evaluation in this study was divided into two parts: the hatching of brine shrimp from eggs and the lethally evaluated nauplii. This species can survive on its egg yolk reserves for up to 5 days after hatching (Sanders, 2008), making any lethal effect on them solely due to any foreign compounds' exposure during toxicity studies.

Hatching of Brine Shrimp (*Artemia salina* sp.). The eggs of the species of brine shrimp, namely *A. salina* sp., were obtained from a supplier in Seri Kembangan, Selangor, Malaysia. A method based on Ramli (2018) was used to hatch the eggs with slight modifications. The eggs were hatched using artificial seawater by mixing 10 g of sodium chloride (QRëC, Thailand) with 400 ml of distilled water under good aeration and light conditions for a few days. After incubation for about 24–36 hr, the nauplii were separated from their eggshells by attracting them with light on one side, collected, and transferred into a Petri dish for further action (Figure 1).

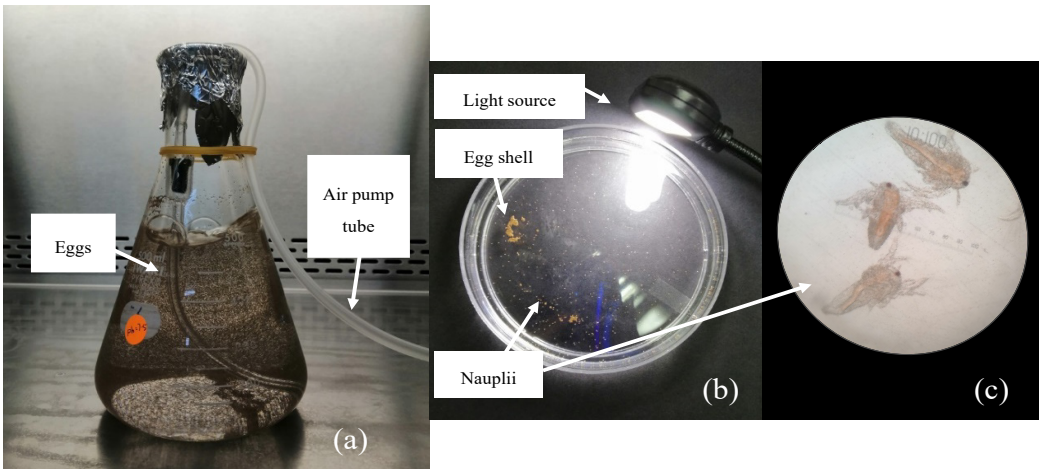

Figure 1. (a) Brine shrimp eggs hatching condition; (b) Nauplii of brine shrimp in a Petri dish; (c) Nauplii at 100× magnification

Brine Shrimp (*Artemia salina* sp.) Lethality Assay. A series of sample concentrations were prepared to range from 0.08–10 mg/ml and 0.01–1.25 mg/ml for potassium dichromate (R & M Chemicals, United Kingdom). Ten nauplii were incubated for 24 hr in each test tube containing 2 ml of solution (Figure 2). The percentage of mortality was calculated using the equation:

$$\% \text{ Mortality} = \left(\frac{\text{Number of dead nauplii}}{\text{Initial number of live nauplii}} \right) \times 100\%$$

The LC_{50} of the sample was calculated based on Probit analysis, and the regression line was achieved by plotting the logarithm of concentration versus the mortality per cent based on the Probit scale (Waghulde et al., 2019). The results were documented by examining the live nauplii for every hour. Potassium dichromate is a suitable reference toxicant for aquatic toxicity testing, as recommended by the U. S. Environmental Protection Agency (2002), with an LC_{50} value of 0.06 to 0.28–0.30 mg/ml (Ramli, 2018; Sahgal et al., 2010; Syahmi et al., 2010).

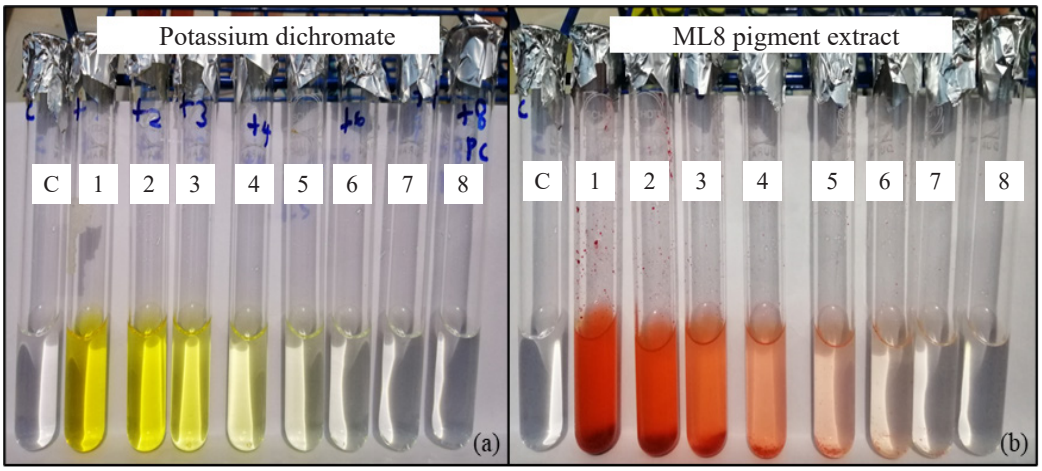

Figure 2. Serial dilution for toxicity analysis. (a) No. 1-8 = control, 1.25, 0.63, 0.31, 0.16, 0.08, 0.04, 0.02, 0.01 mg/ml; (b) No. 1–8 = control, 10.0, 5.00, 2.50, 1.25, 0.63, 0.31, 0.16, 0.08 mg/ml

RESULTS AND DISCUSSION

Using DPPH, FRAP, and ABTS tests, as well as TPC analysis and brine shrimp lethality assay, the antioxidant and toxicity properties of ML8 methanolic and ethanolic pigment extracts were assessed. All the data were examined and documented.

Antioxidant Activities and Phenolic Content of Pigment Extract

The free radical scavenging activities of ML8 pigment extracts were tested against commercial DPPH reagents, demonstrating a linear relationship with concentration. Increased concentration shows the rise of the DPPH scavenging activity. Ethanolic-extract ML8 pigment exhibits 21.34 ± 4.05% of scavenging activity for 0.08 mg/ml of pigment extract and rises to 77.00 ± 3.57% when the concentrations are increased to 0.63 mg/ml with an IC_{50} value of 0.36 ± 0.03 mg/ml. These activities are higher than those of methanol extract, which exhibits 15.50 ± 3.12% and 45.86 ± 1.95%, respectively, with an IC_{50} value of 0.72 ± 0.04 mg/ml (Figure 3). In contrast, methanolic extract ML8 pigment has a good ABTS scavenging activity (13.95 ± 1.57 to 53.81 ± 1.09% with an IC_{50} value of 4.59 ± 2.17 mg/ml) as compared to ethanolic extract (14.96 ± 6.01 to 52.22 ± 4.93 with an IC_{50} value of 6.30 ± 0.31mg/ml, respectively) at 0.78 to 6.25 mg/ml of sample concentration (Figure 4).

However, the analysis of FRAP showed that the ethanolic extract of the ML8 pigment had significant levels of trolox equivalent (TLX Eq.), followed by the methanolic extract of the same pigment. The ethanolic extract showed increasing orders of FRAP values, ranging from 1.09 ± 0.19 for 0.78 mg/ml to 8.77 ± 1.52 mg of TLX Eq./mg for 6.25 mg/ml of sample. In contrast, the methanolic extract exhibited low levels of TLX Eq., despite exhibiting better ABTS scavenging than the ethanolic extract (Figure 5). The TPC of all tested

samples ranged from 0.22 to 11.16 mg/mg of GAE. The ethanol extract of the ML8 pigment had the highest phenolic content (11.16 ± 0.55 mg GAE/mg), whereas the methanol extract had a low value of TPC (Figure 6).

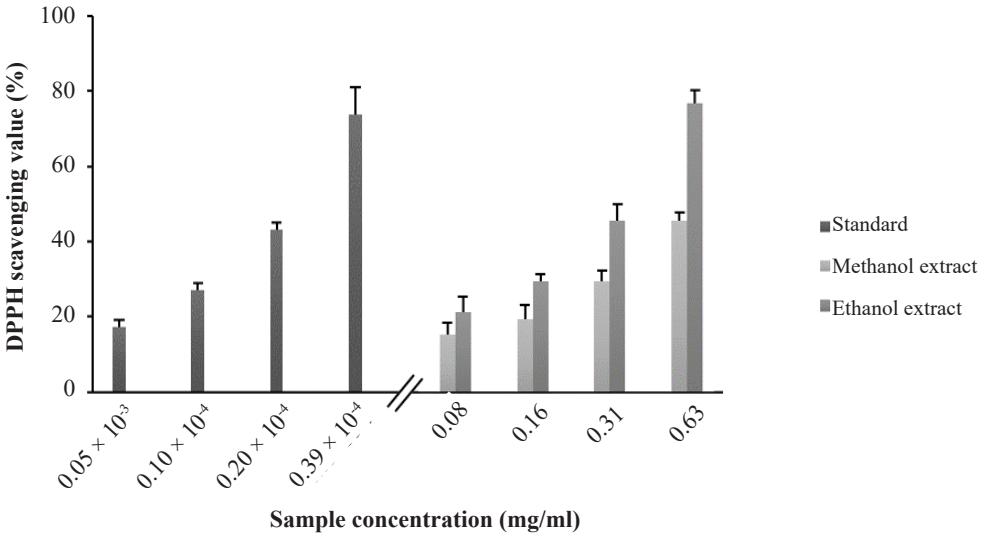

Figure 3. Antioxidant activities of ML8 pigment extract by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

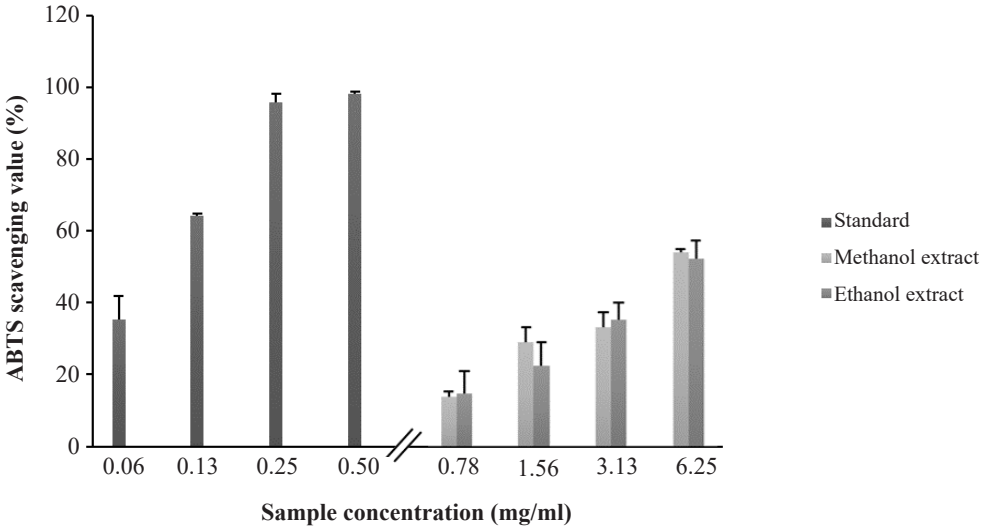

Figure 4. Antioxidant activities of ML8 pigment extract by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay

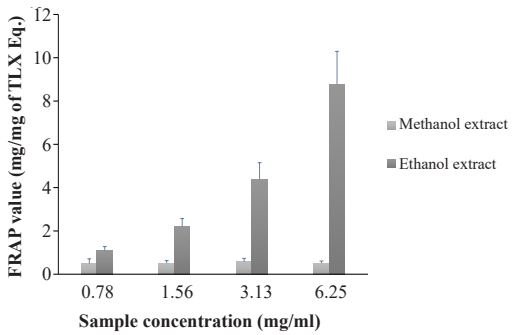

Figure 5. Antioxidant activity of ML8 pigment extract by ferric ion reducing antioxidant power (FRAP) assay

Note. TLX Eq. = Trolox equivalents

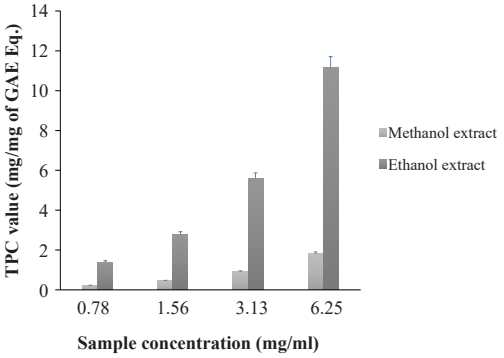

Figure 6. Antioxidant activity of ML8 pigment extract by total phenolic content (TPC) assay

Note. GAE Eq. = Gallic acid equivalents

In contrast, when compared to the IC_{50} values of the pigment extracts of several marine epiphyte species, including *Pseudomonas koreensis* (JX915782), *Serratia rubidaea* (JX915783), and *Pseudomonas argentinensis* (JX915781), high antioxidant activity was observed in both pigment extracts in all DPPH and ABTS scavenging assays (Pawar et al., 2015). Moreover, they displayed a low FRAP value and phenolic content. This finding suggests that various pigmented bacterial species may have various antioxidant properties in response to extract compounds in their pigments.

However, the antioxidant activity levels of the two pigment extracts vary. To be more specific, ethanolic extract ML8 pigment exhibits excellent activity in both DPPH and FRAP assays in addition to having a high phenolic content, whereas methanolic extract ML8 pigment exhibits good antioxidant activity in the ABTS assay but low phenolic content. The somewhat differing polarity index of the solvents (methanol and ethanol) used to extract the ML8 pigment may have an impact on both the type of compounds extracted and the yield value percentage. According to Abarca-Vargas et al. (2016), Torres et al. (2011), and Zullaikah et al. (2019), the polarity indices of methanol and ethanol are 5.1 and 5.2, respectively, making them both polar solvents. Though the polarity index value of ethanol is more polar than methanol, it is more likely to extract phenolic compounds, including polar carotenoids.

The extracted pigment from six microalgae strains from industrial wastewater using methanol and ethanol solvent extraction found that methanol extraction had a slightly lower phenolic content, but a greater carotenoid concentration compared to ethanol extraction (Safafar et al., 2015). Meanwhile, Do et al. (2014) found that the methanol extract of *Limnophilca aromatica* had a lower phenolic concentration than the ethanol extract. *Methylobacterium* sp. has been identified to contain alkaloids, flavonoids, steroids, and various types of

carotenoids, including astaxanthin, lutein, canthaxanthin, -carotene, spheroidene 1,1'- or 2,2'-dihydroxylycopene, and 2'-dehydroxymyxol, which most probably accountable to produce antioxidant activity, according to Photolo et al. (2020) and Stepnowski et al. (2004).

The interaction of chemical reagents with antioxidant molecules in the pigment extract during the antioxidant assay could contribute to the variation in antioxidant activity value. The Folin-Ciocalteu phenol's reagent, used to oxidise phenolic compounds, reacts with a mixture of tungstate and molybdate in a basic media, and any substance or molecule reacting with this solution increases the phenolic concentration. Such substances include organic acids, Fe^{2+}, sodium metabisulphite, sulphite, sugars, proteins, aromatic amines, ascorbic acid, and other enediols and reductones (Cerretani & Bendini, 2010).

The effect of a high phenolics ratio is further supported by Gil et al. (2002) when compared to the carotenoid content of numerous fruit species, including ripe nectarines, peaches, and plums, which generally have high antioxidant activity. Hence, based on the TPC, it is predicted that ethanolic ML8 pigment extracts with higher phenolic content will exhibit a better connection with antioxidant activity than methanolic pigment extracts. Furthermore, the FRAP mechanism that involves a compound's ability to convert Fe^{3+} to Fe^{2+} demonstrates the transfer of an electron rather than a hydrogen atom. In an acidic environment, this test allowed the reaction to decrease the ionisation potential that drives hydrogen atom transfer and increase the redox potential, which is the main reaction mechanism. When TPTZ is present during the reduction of Fe^{3+} to Fe^{2+}, a coloured complex with Fe^{2+} is formed due to the reaction, as described by Cerretani and Bendini (2010).

In phenolic compounds, the extent of conjugation and the degree of hydroxylation are connected to the reducing power. Similar molecules react in the FRAP assay because the reaction picks up substances with redox potentials value (lower than 700 mV) comparable to ABTS. Nevertheless, this assay is not sensitive enough to detect substances that work via hydrogen transfer (radical quenching), such as thiols (as glutathione) and proteins (Cerretani & Bendini, 2010). The lower FRAP score of the methanol extract of ML8 pigment, as compared to the ethanol extract, may be attributed to the possibility of higher concentrations of the mentioned chemicals in the former.

According to Cerretani and Bendini (2010), $ABTS^{\cdot+}$ and $DPPH^{\cdot}$ radicals can be neutralised via direct reduction through electron transfers or radical quenching via hydrogen atom transfers. In the ABTS assay, the decay of the radical cation $ABTS^{\cdot+}$ resulting from the oxidation of ABTS by other substances is spectrophotometrically observed. Although $ABTS^{\cdot}$ is relatively stable, it reacts energetically with molecules and can donate hydrogen atoms or electrons, leading to the disappearance of the blue/ green colour of the radical. A substance

having a lower redox potential than ABTS (680 mV) can reduce the $ABTS^{\cdot+}$. The DPPH radical is one of the few stable organic nitrogen radicals that produces deep purple solutions.

However, the reaction between $ABTS^{\cdot+}$ and $DPPH^{\cdot}$ can proceed slowly in the presence of many individual phenols or phenolic extracts. Additionally, the ABTS radical scavenging assay has some drawbacks, such as poor selectivity of $ABTS^{\cdot+}$ in the interaction with the hydrogen atom donors and ineffective aromatic OH-groups for antioxidation activity, as reported by Cerretani and Bendini (2010). Since these chemicals (aromatic OH-groups) may be abundantly available in methanolic extract based on compound analysis, this circumstance may explain the greater antioxidant activity value in the ABTS experiment compared to ethanol extraction of ML8 pigment.

In contrast, the selectivity of $DPPH^{\cdot}$ in the interaction with hydrogen donors is expected to be higher than $ABTS^{\cdot+}$ as it does not react with only one OH-group in aromatic acids. However, steric accessibility is one of the main limitations since smaller molecules have an easier entry to the radical position, resulting in increased apparent antioxidant action based on Cerretani and Bendini (2010). Due to this, the ethanol extract of the ML8 pigment may show more antioxidant activity compared to methanol in the DPPH experiment.

Nevertheless, research by Photolo et al. (2020) showed substantial DPPH radical scavenging by a pigment extract from a *Methylobacterium* species with an IC_{50} value greater than ascorbic acid. Most often, the EC_{50} (also known as IC_{50}) is the concentration that results in a 50% reduction in the initial DPPH concentration, where it is used to indicate a sample's hydrogen-donating potential measured in the DPPH test (Cerretani & Bendini, 2010). It demonstrates the excellent antioxidant activity of ethanol-extract ML8 pigments.

Toxicity Effect of Extracted Pigment on Brine Shrimp

This study found that the nauplii's mortality rate has a strong positive correlation with the concentration of ML8 pigment extract; the LC_{50} value was obtained through the best-fit line of the percentage of nauplii killed versus the concentration of ML8 pigment extract and potassium dichromate (Supplementary Figures A-C) and is presented in Table 1. Additionally, the brine shrimp lethality assay results revealed that extract ML8 pigments are not toxic to brine shrimp, with LC_{50} values of 4.52 mg/ml (methanol extract) and 9.94 mg/ml (ethanol extract).

Table 1
Toxicity value of ML8 pigment extract using brine shrimp lethality assay

No.	Sample	LC_{50} (mg/ml)
1.	Potassium dichromate	0.05
2.	ML8 pigment methanol extract	4.52
3.	ML8 pigment ethanol extract	9.94

Commonly, potassium dichromate is used as a positive control because of its well-known toxicity and was found to give brine shrimp toxication effect of exposure with an LC_{50} of 0.05 mg/ml. In contrast, brine shrimp in a test tube containing only artificial seawater showed no death and was a negative control. Syahmi et al. (2010) verified that crude extracts with LC_{50} values < 1 mg/ml are considered toxic, while those with values > 1 mg/ml are safe for human consumption. Therefore, the ML8 pigment extract is biologically safe for humans with LC_{50} values of 4.52 mg/ml and 9.94 mg/ml, while potassium dichromate is toxic.

CONCLUSION

The findings of all evaluated antioxidant activities, except for ABTS and phenolic substances, indicated that ethanolic ML8 pigment extract has a greater value than methanolic ML8 pigment extract. Furthermore, neither pigment extract was poisonous and could be used in the food sector for human consumption.

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SUPPLEMENTARY DATA

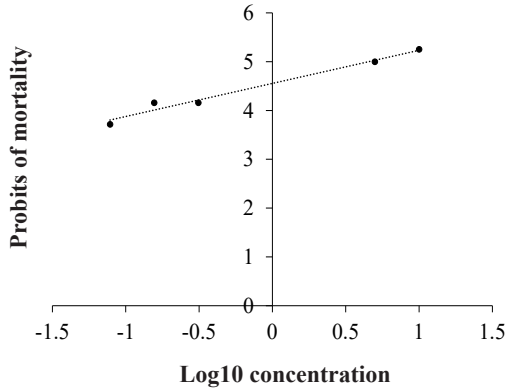

Figure A. Standard curve of brine shrimp lethality assay after being treated with ML8 pigment methanol extract

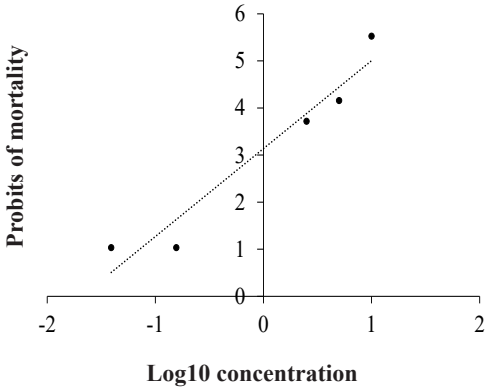

Figure B. Standard curve of brine shrimp lethality assay after being treated with ML8 pigment ethanol extract

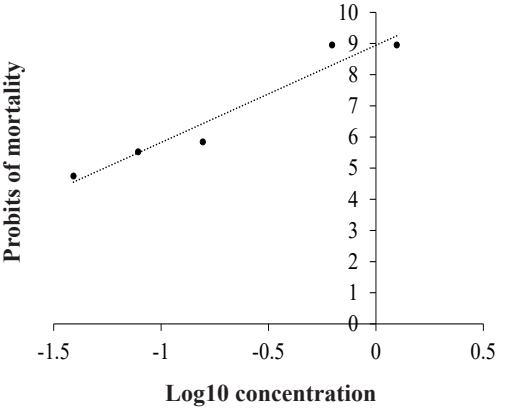

Figure C. Standard curve of brine shrimp lethality assay after being treated with potassium dichromate