Effect of Different Extracting Solvents on Antioxidant Activity and Inhibitory Effect on Diabetic Enzymes of *Chlorella vulgaris* and *Spirulina platensis*

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ABSTRACT

This study aimed to determine the effect of the solvent used in microalgae extraction on the total phenolic content (TPC), total flavonoid content (TFC), antioxidant, and anti-carbohydrate-hydrolyzing enzyme activities of the crude extracts. Algal biomass of *Chlorella vulgaris* and *Spirulina platensis* was extracted by different types of solvent, namely n-hexane, ethanol, methanol, and distilled water. Additionally, the correlations of TPC and TFC with antioxidant activity as well as anti-diabetic activity (anti-α-amylase and anti-α-glucosidase) were investigated. Results demonstrated that *C. vulgaris* and *S. platensis* methanolic extracts had the highest TPC, with values of 40.62±4.13 and 64.38±7.39 mg GAE·g^{-1} (mg of gallic acid equivalent per gram extract), respectively. These extracts also showed the greatest antioxidant activity, with IC_{50} values for DPPH (2,2-diphenyl-1-picrylhydrazyl) of 117.62±4.46 and 97.89±4.64 μg·mL^{-1}, respectively, and ABTS (2,2-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]) of 102.52±2.50 and 46.21±0.88 μg·mL^{-1}, respectively. Furthermore, they strongly inhibited α-amylase activity (IC_{50} of 262.97±9.61 and 275.46±12.44 μg·mL^{-1}) and α-glucosidase activity (IC_{50} of 207.09± 6.97 and 169.01±3.16 μg·mL^{-1}). TPC was positively and significantly correlated with antioxidant and α-glucosidase inhibition activities. Meanwhile, only α-amylase inhibitory activity had a significant positive correlation with TFC. Therefore, crude methanolic extracts of *C. vulgaris* and *S. platensis* might be potential sources of natural antioxidants and anti-diabetic dietary supplements.

Keywords: Anti-α-amylase, Anti-α-glucosidase, Flavonoids, Microalgae, Phenolics

INTRODUCTION

The incidence of type 2 diabetes mellitus (T2DM) is on the rise, and is related to the global childhood obesity crisis and sedentary lifestyles (Abbasi *et al*., 2017; Buttermore *et al*., 2021; Kulshreshta and Shahid, 2021; Sakran *et al*., 2022). According to Saeedi *et al*. (2019), the prevalence of diabetes will continue to increase worldwide, affecting 578 million people in 2030 and 700 million people in 2045. One of the important strategies of T2DM treatment is the inhibition of α-amylase and α-glucosidase, two important digestive enzymes that break down dietary carbohydrates, which results in delaying starch digestion and lowering postprandial blood glucose (Ramos-Romero *et al*., 2021). A prime example of α-glucosidase and α-amylase inhibitors is acarbose, which has been used clinically for the treatment of T2DM (Oboh *et al*., 2016). There are recognized negative effects of anti-diabetic medications, including weight gain, hypoglycemia, and digestive problems (Marín-Peñalver *et al*., 2016). Since dietary treatment and lifestyle changes are safe and effective, they are

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widely applied in the prevention and management of T2DM (Bai *et al*., 2020). A diet rich in certain types of polysaccharides, polyphenols, and flavonoids has been found to be advantageous in the prevention and treatment of T2DM (Sun *et al*., 2020; Zhou *et al*., 2020; Xiao, 2022).

Microalgae are regarded as functional food with significant added value due to their abundance of natural antioxidant molecules and bioactive substances. Currently, *Chlorella* spp. and *Spirulina* (syn. *Arthrospira*) spp. dominate the market of microalgal food supplements (Lucakova *et al*., 2022). According to Yun *et al*. (2011), *Chlorella vulgaris* is rich in antioxidants, chlorophylls, and bioactive compounds like α-carotene, α-tocopherol, β-carotene, ascorbic acid, and lutein. Moreover, some studies reported that treatment with *C. vulgaris* products could significantly reduce DNA damage and blood malondialdehyde level (Aizzat *et al*., 2010) and blood glucose in diabetic rats (Ghwenm *et al*., 2020), and improve some hematological parameters in diabetic rats (Emami and Olfati, 2017). *Spirulina* (*Arthrospira*) spp. contain high protein content and vitamins A, B1, B12, and carotenoids (Spolaore *et al*., 2006). Nasirian *et al*. (2018) found that diabetic rats given *Spirulina platensis* (20-30 $mg \cdot kg^{-1}$ body weight) orally had lowered blood glucose levels and increased activity of antioxidant enzymes. Mallikarjun *et al*. (2015) discovered that the supercritical CO_2 extract of *S. platensis* has antioxidant, anti-diabetic, antihypertensive, and antimicrobial activities. Considering these beneficial effects, both microalgae could be potentially utilized as dietary supplements to manage and prevent a range of disorders, including T2DM.

The bioactive compounds from microalgae biomass can be extracted by organic solvents, e.g., methanol, acetone, ethyl acetate, hexane, and chloroform (Miazek *et al*., 2017). The extracting solvent utilized may impact the quantity and content of bioactive compounds generated by microalgae biomass (Mojzer *et al*., 2016). Secondary metabolites and their antioxidant activities have been studied in relation to the usage of various solvents (Dirar *et al*., 2019; Monteiro *et al*., 2020; Lin *et al*., 2022). However, the effect of solvent type on the antioxidant and anti-diabetic activities of the two microalgae (*C. vulgaris* and *S. platensis*) still needs to be explored. This research aimed to examine the impact of four solvents (n-hexane, ethanol, methanol, and distilled water) on the yield, total phenolic content (TPC), and total flavonoid content (TFC) of the crude extracts of *C. vulgaris* and *S. platensis* along with their antioxidant and anti-diabetic abilities (using carbohydrate-hydrolyzing enzyme inhibition assays). Additionally, the correlations of TPC and TFC with antioxidant as well as anti-diabetic activities were investigated. Selection of the best solvent for extracting the natural products with high antioxidant and anti-diabetic activities would be helpful for the production of functional foods from microalgae for diabetic patients and healthy people as well.

MATERIALS AND METHODS

Materials

The spray-dried microalgae powder of *Chlorella vulgaris* was purchased from Matahari Sdn Bhd, Malaysia, and *Spirulina platensis* was purchased from Polaris Sinar Intan Ltd., Indonesia. The solvent n-hexane (purity≥99.0%), ethanol (purity≥99.9%), and methanol (purity≥99.9%) were purchased from Merck (Darmstadt, Germany). Butylated hydroxytoluene (BHT), Folin-Ciocalteu's phenol reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), α-amylase (EC 3.2.1.1), and α-glucosidase (EC 3.2.1.20) were procured from Sigma-Aldrich (Singapore). *p*-Nitrophenyl-α-D-glucopyranoside (pNPG) was purchased from Santa Cruz Biotechnology (California, USA). Acarbose (Glucobay®, purity 36.15%) was obtained from Bayer Pharmaceuticals (Leverkusen, Germany).

Crude extract preparation of Chlorella vulgaris and Spirulina platensis

The freeze-dried microalgae biomass powders (10 g) of *Chlorella vulgaris* and *Spirulina platensis* were extracted by a reflux apparatus for 3 h with four different solvents (1:10 w/v),

separately: n-hexane (at 69 °C), ethanol (at 78 °C), methanol (at 65 °C), and distilled water (at 100 °C). The boiling point of each solvent was used to establish the extraction temperature. Whatman No. 1 filter paper was used to separate the extracts and concentrate them in a DLAB rotary evaporator RE100-Pro (Beijing, China) at 40 °C (for n-hexane, ethanol, and methanol) or 80 °C (for distilled water). The final crude extracts were blown with nitrogen gas to evaporate the remaining solvent. The dried crude concentrated extracts were kept at -20 °C for further analysis after determining the extraction yield. The yield of the microalgae extraction methods was determined following the formula described by Falleh *et al*. (2008) as % yield = $W_{extr}/W_{samp}\times100$, where W_{extr} is the dry weight of extract after solvent evaporation, and W_{samp} is the dry weight of microalgae.

Determination of total phenolic content (TPC)

The Folin-Ciocalteau method was used to determine TPC according to Ondo *et al*. (2013). Briefly, 0.25 mL of microalgae extract in methanol (1 mg·mL^{-1}) was added to 1.25 mL of 0.2 N Folin-Ciocalteau reagent for 5 min. The mixture was added with 1.0 mL of 7.5% Na_2CO_3 and incubated in darkness at room temperature for 1 h, and the absorbance was analyzed at 765 nm. The TFC was calculated using the linear equation of the standard curve made with gallic acid (GAE) (50 to 500 µg·mL^{-1}) and the result was represented as mg gallic acid equivalent per g extract dry weight cell (mg of GAE·g^{-1} DW cell).

Determination of total flavonoid content (TFC)

The Dowd method was used to analyze total flavonoid content according to Aryal *et al*. (2019). One mL of microalgae extract in methanol (1 mg·mL^{-1}) was mixed with 0.2 mL of 10% $AlCl_3$ solution, 0.2 mL of 1 M potassium acetate, and 5.6 mL distilled water. The mixture was incubated for 30 min at room temperature and the absorbance was determined at 415 nm. The TFC was calculated using the linear equation of the standard curve made with quercetin (QE) (2 to 250 µg·mL^{-1}) and represented as mg quercetin equivalent per g extract dry weight cell (mg of QE·g^{-1} DW cell).

Antioxidant activity

DPPH radical scavenging assay

The DPPH radical scavenging assay was determined as described by Ul-Haq *et al*. (2012). Briefly, 2.8 mL of 0.1 mM methanolic DPPH solution was added to 0.2 mL of microalgae extracts dissolved in methanol (25 to 300 µg·mL^{-1}) at 37 °C for 60 min in the dark. Afterwards, the solution was centrifuged at 5,000×g for 5 min, and the absorbance was measured at 517 nm by UV spectrophotometer. BHT was employed as a positive control. Percentage DPPH radical scavenging activity was determined as: DPPH radical scavenging activity (%) = $[(A_0-A_1)/A_0]\times100$, where the absorbance of the control is represented by A_0 and the absorbance of samples is represented by A_1. The half-maximal inhibitory concentration (IC_{50}) values of the DPPH assay were determined using GraphPad Prism 8 (GraphPad Software, San Diego, California).

ABTS radical scavenging assay

The antioxidant potential of microalgae extracts was also investigated by ABTS assay, as described by Shanab *et al*. (2012). Microalgae extracts with different concentration (10 to 200 µg·mL^{-1}) were prepared in methanol. The solution of radical cation was prepared by mixing a 7 mM ABTS solution and 2.45 mM potassium persulfate. The mixture was then incubated overnight (12-16 h) in the dark at room temperature. Afterwards, the $ABTS^{\cdot+}$ solution was made by adding ethanol until the absorbance at 734 nm reached 0.700. Subsequently, 0.9 mL of $ABTS^{\cdot+}$ solution was mixed with 0.1 mL of the samples for 45 s. After 1 min, the absorbance was determined at 734 nm by UV spectrophotometer; BHT was used as a positive control. Percentage ABTS radical scavenging activity was determined as: ABTS decolorization (%) = $[(A_0-A_1)/A_0]\times100$, where sample absorbance is represented by A_1 and control absorbance is represented by A_0. The IC_{50} values of the ABTS assay were calculated by using GraphPad Prism 8 software.

Carbohydrate-hydrolyzing enzyme inhibition

α-Amylase inhibitory assay

The microalgae extracts were tested for α-amylase inhibitory activity according to Patil and Kaliwal (2019). Briefly, 0.5 mL of 0.02 mM sodium phosphate buffer (pH 6.9 with 6 mM NaCl) and 0.5 mL of α-amylase solution (0.5 $mg \cdot mL^{-1}$) were added to 0.5 mL of microalgae extract. The solution was incubated for 10 min at room temperature. Afterwards, the solution was mixed with the solution of 1% soluble starch of 0.5 mL in 0.02 mM sodium phosphate buffer (pH 6.9 with 6 mM NaCl), and re-incubated for 10 min at room temperature. One mL of 96 mM 3,5-dinitrosalicylic acid (DNS) color reagent was added to stop the reaction and incubated for 5 min at 100 °C, then let to cool until room temperature was attained. The absorbance was then measured at 540 nm after the solution was diluted with 10 mL of deionized water. Acarbose at different concentrations (100 to 500 $\mu g \cdot mL^{-1}$) was used as a positive control. The stock solution of acarbose (1 $mg \cdot mL^{-1}$) was prepared by crushing a tablet of Glucobay® (containing 50 mg acarbose per tablet) and dissolving it in 50 mL phosphate buffer. The inhibition of α-amylase was determined as: Inhibition of α-amylase (%) = [(Abs control-Abs sample)/Abs control]×100, where the absorbance of the phosphate buffer with the α-amylase and without extract is represented by Abs control, and the absorbance of the solution with the extract and α-amylase is represented by Abs sample. The IC_{50} values of the α-amylase inhibitory assay were calculated by using GraphPad Prism 8 software.

α-Glucosidase inhibitory assay

The method of the α-glucosidase inhibitory assay followed Kim *et al*. (2014). Firstly, 5 mL of 67 mM potassium phosphate (pH 6.8 at 37 °C) and 0.2 mL of 3 mM glutathione solution were added into test tubes. Then, α-glucosidase (0.2 mL of 1 $unit \cdot mL^{-1}$) was added into the test tubes. The mixture was mixed with 0.1 mL of microalgae extract and incubated at 37 °C for 20 min. Afterward, 0.5 mL of 10 mM pNPG was added to each tube as a substrate and re-incubated at 37 °C for 20 min. To stop the reaction, 1 mL of the solutions from each tube were combined with 4 mL of 0.1 M Na_2CO_3 in clean test tubes. The absorbance was measured at 400 nm using a UV spectrophotometer. Acarbose was used as a positive control at different concentrations (100 to 500 $\mu g \cdot mL^{-1}$). The inhibition of α-glucosidase was determined as: Inhibition of α-glucosidase (%) = [(Abs control-Abs sample)/ Abs control]×100, where the absorbance of the phosphate buffer with the α-glucosidase and without extract is represented by Abs control, and the absorbance of the solution with the extract and α-glucosidase is represented by Abs sample. The IC_{50} values of the α-glucosidase inhibitory assay were calculated by using GraphPad Prism 8 software.

Statistical analyses

All the experiments were performed in three replicates. One-way analysis of variance (ANOVA) followed by Duncan's new multiple range test were used for the statistical analysis using Prism version 8.0 (GraphPad Software, San Diego, USA). Pearson's correlation was used to perform the correlation analysis in SPSS (version 20, SPSS Inc., Chicago, IL, USA). Statistical significance was considered when a p-value was lower than 0.05.

RESULTS AND DISCUSSION

Effect of solvent type on the extraction yield, TPC, and TFC

The microalgae extractions of *Chlorella vulgaris* and *Spirulina platensis* were done by several types of solvent with different polarities. Based on their polarity indices, the solvents used in this work can be ordered as follows: n-hexane (0.1) < ethanol (4.3) < methanol (5.1) < water (10.2) (Snyder, 1978). The average extraction yields of *C. vulgaris* and *S. platensis* ranged from 2.00 to 5.03 % (Table 1). The *C. vulgaris* methanolic extract produced the highest yield (5.03 %), which was not different ($p>0.05$) from the *C. vulgaris* ethanolic extract (4.73 %), but was significantly ($p<0.05$) higher than the water extract (3.68 %). In general, *S. platensis* produced lower extraction yields than *C. vulgaris*. The yield of the methanolic

extract of *S. platensis* was 3.63 %, slightly higher than the other *S. platensis* extracts. The non-polar solvent (n-hexane) showed a lower yield than the polar solvents for both *C. vulgaris* and *S. platensis*, which were 2.00 and 2.38 %, respectively. Because the content of lipids from microalgae biomass is reportedly lower than hydrophilic substances such as proteins and carbohydrates (Canelli *et al*., 2020; Ramos-Romero *et al*., 2021), it is not surprising that the yields of hexane extracts were the lowest when compared to the polar solvents.

Estimates of TPC and TFC are presented in Table 1. Gallic acid equivalent ($r^2 = 0.9922$) was used to estimate and express the TPC of *C. vulgaris* and *S. platensis* extracts. The TPC of *C. vulgaris* methanolic extract (40.63±4.13 mg of GAE·g^{-1}) was significantly ($p<0.05$) greater than that of other solvents utilized in *C. vulgaris*, but it was significantly ($p<0.05$) lower than that of *S. platensis* methanolic extract (64.38±7.39 mg of GAE·g^{-1}). Similar to the extraction yield, the TPC obtained were also in the order of methanol > ethanol > water > n-hexane for *C. vulgaris* and methanol > water > ethanol > n-hexane for *S. platensis*. The correlation between the yield and TPC for both microalgae species suggests that phenolic compounds might constitute a significant fraction of the extracts. Polar solvents such as methanol, ethanol, and water are commonly used for extracting hydrophilic compounds including phenolic compounds, saponins, and glycosides, while non-polar solvents are frequently utilized for the extraction of lipophilic substances such as steroids and fatty acids (Dirar *et al*., 2019). Our results, showing that TPC in both microalgae was most effectively extracted using methanol (not water), are consistent with previous studies that found polyphenols to be more easily soluble in less polar solvents compared to water (Pinelo *et al*., 2005; Jakopič *et al*., 2009; O'Sullivan *et al*., 2013).

Flavonoids are polyphenolic compounds that are present in a wide variety of natural products, including microalgae (Goiris *et al*., 2014; Mutha *et al*., 2021). The TFC of *C. vulgaris* and *S. platensis* extract were estimated in terms of quercetin equivalent ($r^2 = 0.9970$). In this study, we found dissimilarity between TPC and TFC results in the ranking of microalgae extracts by different solvents. *C. vulgaris* had the highest TFC when extracted with ethanol (48.89±1.25 mg of QE·g^{-1}); however, *S. platensis* had the highest TFC when extracted with hexane (30.80±2.73 mg of QE·g^{-1}) and ethanol (25.28±1.35 mg of QE·g^{-1}). These findings indicate that each solvent has a different solubility in extracting flavonoids and other phenolic compounds. Non-polar solvents such as chloroform or hexane are typically employed for the extraction of flavones, isoflavones, and methylated flavones, whereas polar solvents like methanol and ethanol are used for the extraction of flavonoid glycosides and aglycones (Chávez-González *et al*., 2020).

Table 1. Effect of solvent type on the extraction yield (%), total phenolic content (TPC), and total flavonoid content (TFC) of microalgae extracts.

Microalgae	Solvent	Extraction yield (%)	TPC (mg of GAE·g^{-1} DW cell)	TFC (mg of QE·g^{-1} DW cell)
Chlorella vulgaris	n-Hexane	2.00±0.61^{e}	8.02±1.48^{e}	28.36±2.58^{bc}
	Ethanol	4.73±0.44^{ab}	28.44±2.67^{c}	48.89±1.25^{a}
	Methanol	5.03±0.36^{a}	40.63±4.13^{b}	29.55±2.80^{bc}
	Water	3.68±0.23^{bc}	18.75±1.36^{d}	16.45±1.73^{d}
Spirulina platensis	n-Hexane	2.38±0.52^{de}	11.15±0.95^{de}	30.80±2.73^{b}
	Ethanol	2.99±0.24^{cde}	19.79±2.62^{cd}	25.28±1.35^{c}
	Methanol	3.63±0.31^{bc}	64.38±7.39^{a}	17.64±3.36^{d}
	Water	3.17±0.38^{cd}	47.41±3.78^{b}	8.27±2.36^{e}

Note: Means±SD (n = 3) in the same column superscripted with different lowercase letters are significantly different ($p<0.05$).

Antioxidant activity of Chlorella vulgaris and Spirulina platensis

The investigation of antioxidant activity of *Chlorella vulgaris* and *Spirulina platensis* extract was done by DPPH and ABTS radical scavenging assays (Figure 1). Methanolic extracts of *C. vulgaris* and *S. platensis* exhibited the highest DPPH scavenging activity of 78.37±3.74 % and 74.80±3.65 %, respectively, at concentrations of 300 µg·mL^{-1} in comparison to the other solvent extracts, although still lower than BHT (91.78± 3.14 %) (Figure 1a and b). The percentage of DPPH inhibition at 300 µg·mL^{-1} from the other solvent extractions of *C. vulgaris* was 61.89±2.71 % (water), 58.31±2.90 % (n-hexane), and 58.13± 2.65 % (ethanol), whereas DPPH inhibition by the *S. platensis* extracts was 59.03±3.12 % (water), 53.62±2.92 % (n-hexane), and 51.56±1.21 % (ethanol).

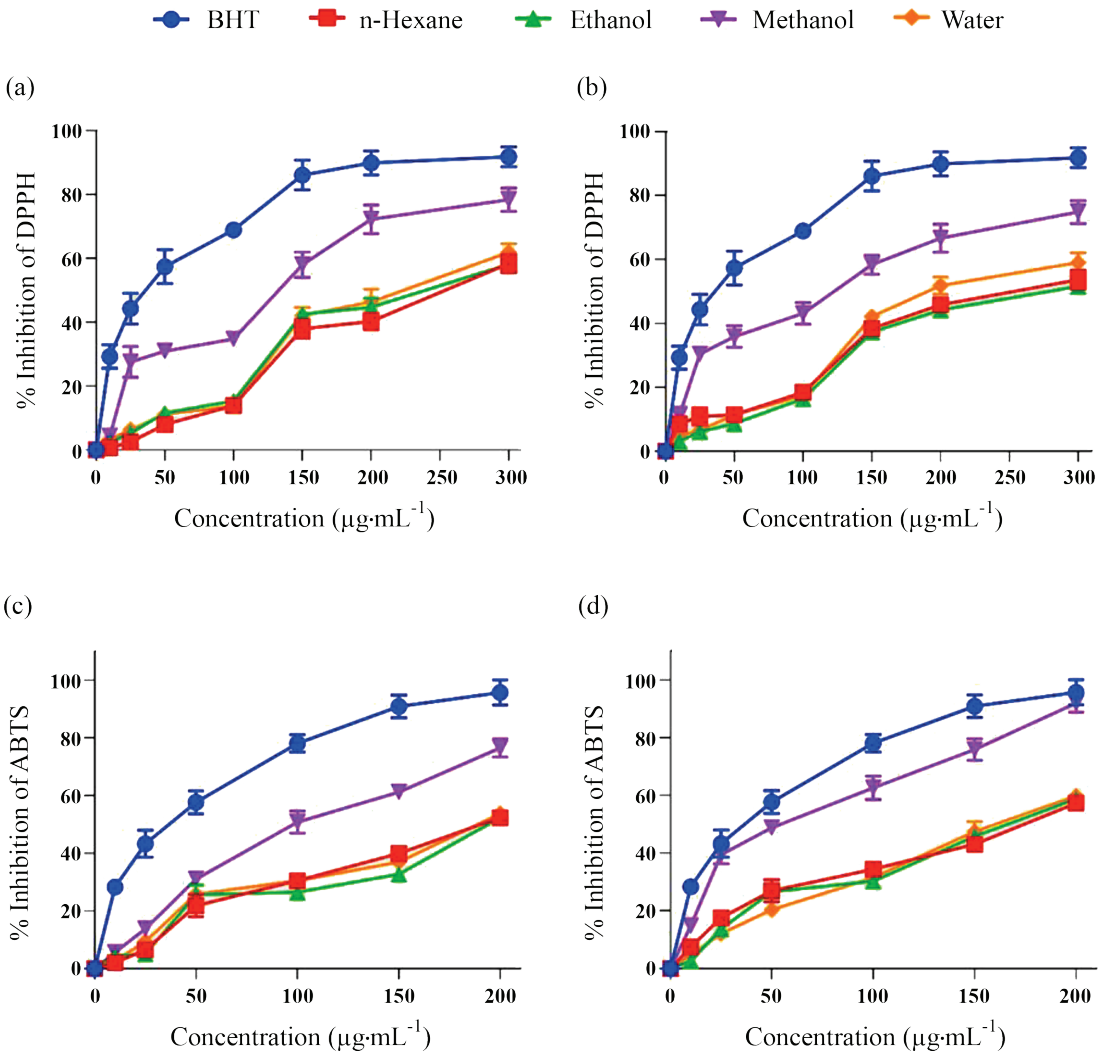

Figure 1. Effect of different extraction solvents on the DPPH radical scavenging activity of *Chlorella vulgaris* (a) and *Spirulina platensis* (b), and ABTS radical scavenging activity of *C. vulgaris* (c) and *S. platensis* (d).

The ABTS free radical scavenging assay recorded higher inhibition activity than the assay of DPPH free radical scavenging. Both *C. vulgaris* and *S. platensis* showed the highest percentage inhibition of ABTS in methanolic extracts, with values of 76.50±3.14 % and 91.96±3.14 %, respectively, at concentrations of 200 $\mu g \cdot mL^{-1}$ (Figure 1c and d), although they were slightly lower than that of the control (BHT). The percentage of ABTS inhibition at concentrations of 200 $\mu g \cdot mL^{-1}$ from the other extractions of *C. vulgaris* were 53.64±1.44 % (water), 52.38±1.48 % (ethanol), and 52.34±1.95 % (n-hexane), whereas those of the *S. platensis* extracts were 59.80±2.66 % (water), 58.74±1.83 % (ethanol), and 57.29±1.94 % (n-hexane).

The IC_{50} values of DPPH and ABTS assays were determined to estimate the amount of extract necessary to inhibit 50 % of free radicals. A lower IC_{50} indicates a higher potency of an extract. The *C. vulgaris* extracts were ranked in decreasing order of DPPH free radical scavenging activity as BHT > methanol > water, ethanol, and hexane, while extracts of *S. platensis* were ranked as BHT > methanol > water > hexane and ethanol (Table 2). Other than the positive control BHT, the methanolic extracts of *S. platensis* and *C. vulgaris* exhibited the most potent antioxidant activity (IC50 = 97.07±10.91 and 118.24± 7.64 $\mu g \cdot mL^{-1}$, respectively), which was significantly higher ($p<0.05$) than the other solvent extractions.

The IC_{50} values of ABTS free radical scavenging activity of *C. vulgaris* extracts decreased in the following order: BHT > methanol > water, hexane, and ethanol. Extracts of *S. platensis* were ranked as BHT and methanol > water, ethanol, and hexane. Other than BHT, the highest potency was observed in the methanolic extract of *S. platensis* (IC_{50} = 45.29±2.30 $\mu g \cdot mL^{-1}$), followed by the methanolic extract of *C. vulgaris* (IC_{50} = 103.15± 9.71 $\mu g \cdot mL^{-1}$). The ABTS scavenging potency of both of these extracts were significantly higher ($p<0.05$) than the other solvent extractions.

The comparatively high antioxidant capacity of the methanolic extracts can be attributed to their rich phenolic contents (Altemimi *et al*., 2017), which naturally possess antioxidant activity by radical scavenging mechanisms through hydrogen atom donation (Kumar and Goel, 2019). Phenolic substances transfer H^+ to free radicals and thus become radicals, but with much greater chemical stability than the initial radicals (Pereira *et al*., 2009; Lin *et al*., 2022). In line with our finding, Abdel-Moneim *et al*. (2022) also demonstrated stronger DPPH and ABTS inhibitory activities of a *S. platensis* methanolic extract than for acetone and hexane extracts. Besides phenolics, alkaloids, glycosides, proteins, terpenoids, tannins, saponin, and coumarin are also found abundantly in *C. vulgaris* methanolic extract (Pradhan *et al*., 2021).

Table 2. IC_{50} values of DPPH and ABTS free radical scavenging activity for *Chlorella vulgaris* and *Spirulina platensis* extracts.

Extract		IC_{50} ($\mu g \cdot mL^{-1}$)	
		DPPH	ABTS
Positive control	BHT	39.73 ± 4.31^{a}	34.45 ± 1.83^{a}
Chlorella vulgaris	n-Hexane	241.38 ± 16.49^{def}	191.81 ± 5.15^{d}
	Ethanol	221.67 ± 9.53^{cde}	196.14 ± 3.02^{d}
	Methanol	118.24 ± 7.64^{b}	103.15 ± 9.71^{b}
	Water	213.56 ± 14.70^{cd}	190.79 ± 4.35^{d}
Spirulina platensis	n-Hexane	248.33 ± 14.57^{ef}	175.41 ± 0.23^{c}
	Ethanol	268.56 ± 15.43^{f}	171.20 ± 0.70^{c}
	Methanol	97.07 ± 10.91^{b}	45.29 ± 2.30^{a}
	Water	197.98 ± 6.40^{c}	164.61 ± 7.88^{c}

Note: Means±SD (n = 3) in the same column superscripted with different lowercase letters are significantly ($p<0.05$) different.

In comparison, the major compounds found in *S. platensis* methanolic extract include phenolics, alkaloids, saponins, steroids, glycosides, and terpenoids (Shalaby and Shanab, 2013; Rahim *et al*., 2021).

Anti-α-amylase and anti-α-glucosidase activities of Chlorella vulgaris and Spirulina platensis

The activities of α-amylase and α-glucosidase inhibition from *Chlorella vulgaris* and *Spirulina platensis* extracted by several solvents is displayed in Figure 2. In this study, acarbose was used as a positive control. Higher concentrations of *C. vulgaris* and *S. platensis* extract showed higher α-amylase inhibitory activity. Methanolic extracts of *C. vulgaris* and *S. platensis* exhibited higher α-amylase inhibitory activity than the other extracts at concentrations of 500 $\mu g \cdot mL^{-1}$, with the values of 93.74±4.18 % and 96.51±3.92 %, respectively, which were nearly as high as the positive control acarbose (97.23±3.03 %). The inhibition of

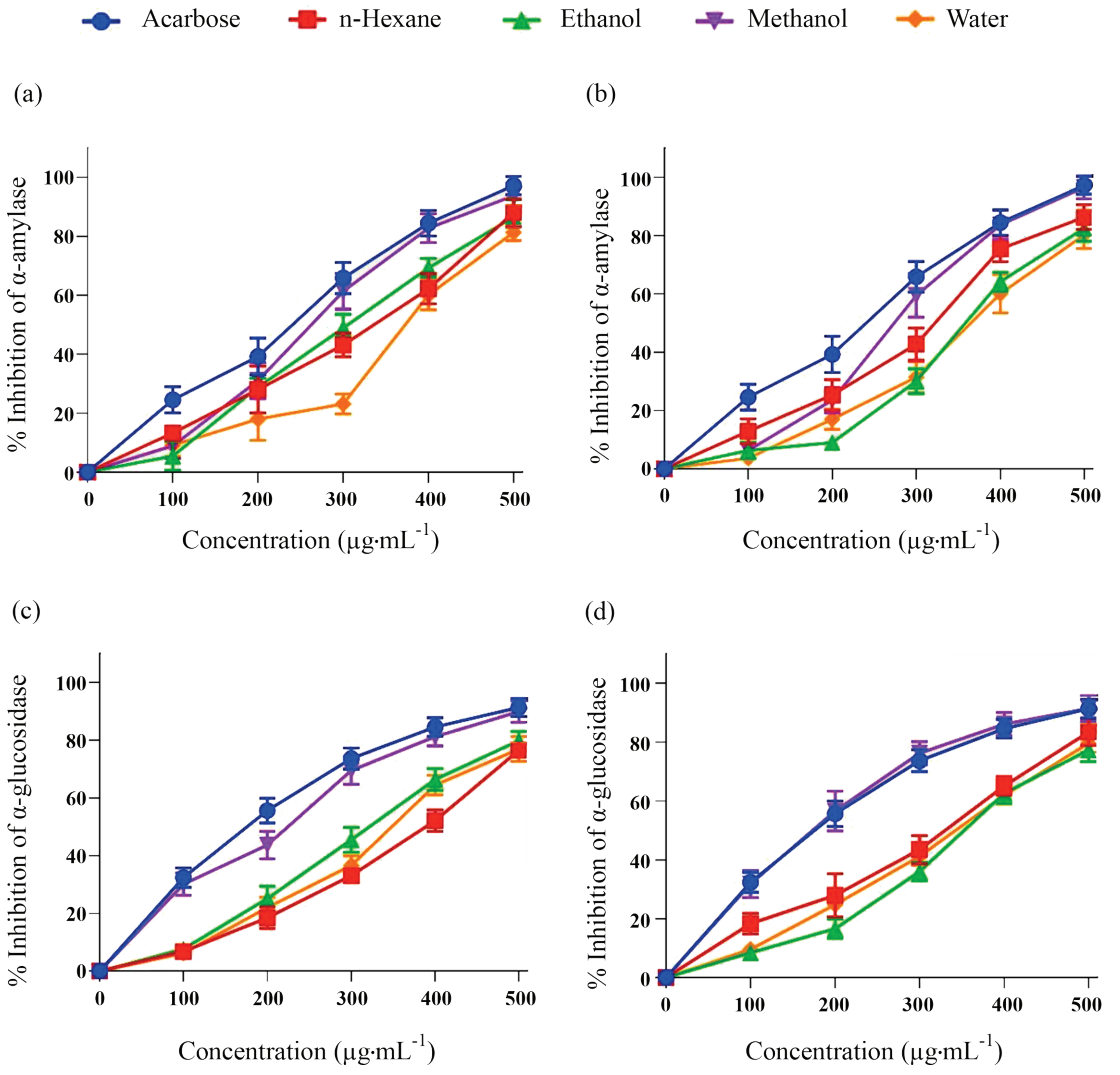

Figure 2. Effect of different extraction solvents on the α-amylase inhibitory activity of *Chlorella vulgaris* (a) and *Spirulina platensis* (b), and α-glucosidase inhibitory activity of *C. vulgaris* (c) and *S. platensis* (d).

α-glucosidase also increased with increasing concentration of *C. vulgaris* and *S. platensis* extracts, and acarbose. The α-glucosidase inhibition of methanolic extracts of both *C. vulgaris* (89.85± 3.77 %) and *S. platensis* (91.40±4.39 %) was highest, and similar to acarbose (91.37±3.17 %) at concentrations of 500 $\mu g \cdot mL^{-1}$.

The IC_{50} values of *C. vulgaris* and *S. platensis* extracts against α-amylase and α-glucosidase were determined and compared with those of acarbose, as shown in Table 3. The methanolic extracts of *C. vulgaris* (IC_{50} = 263.16± 20.98 $\mu g \cdot mL^{-1}$) and *S. platensis* (IC_{50} = 275.53± 9.72 $\mu g \cdot mL^{-1}$) were the most effective in inhibiting α-amylase, and comparable to acarbose (IC_{50} = 230.93±21.19 $\mu g \cdot mL^{-1}$). Likewise, the IC_{50} values for anti-α-glucosidase of *S. platensis* and *C. vulgaris* methanolic extracts (IC_{50} = 170.16±19.3 and 205.88 ±11.63 $\mu g \cdot mL^{-1}$, respectively) were comparable to acarbose (IC_{50} = 170.17±5.05 $\mu g \cdot mL^{-1}$) and significantly lower ($p<0.05$) than the other solvent extractions.

Inhibition of pancreatic α-amylase and intestinal brush border α-glucosidase is a common strategy for the treatment of T2DM to reduce glucose levels in the bloodstream (Krentz and Bailey, 2005). Starch molecules are hydrolyzed by the salivary and pancreatic α-amylases to linear malto-oligosaccharides and α-1,6-branched oligosaccharides (Lee *et al*., 2012). Then, α-glucosidase further hydrolyzes them into monosaccharides in the small intestine before being absorbed (Quan *et al*., 2019). Therefore, suppressing carbohydrate digestion through the inhibition of α-amylase and α-glucosidase enzymes can delay glucose uptake, and consequently reduce blood sugar levels (Alqahtani *et al*., 2020). In fact, this approach is being used in clinical practice (e.g., acarbose) for the treatment of T2DM. Note that acarbose is a competitive inhibitor of pancreatic α-amylase and intestinal brush border α-glucosidase. Due to the presence of glucose residue, which confers the structural similarity to the natural substrates of the enzymes (carbohydrates), acarbose can bind to the enzyme's active sites with high affinity but without being hydrolyzed (Naik and Kokil, 2013; Dirir *et al*., 2022). The use of acarbose as a positive control in the α-amylase and α-glucosidase inhibition assays has been carried out in various studies (Xiong *et al*., 2020; Lankatillake *et al*., 2021; Mechchate *et al*., 2021).

The IC_{50} values for α-amylase inhibition were generally greater than those for α-glucosidase inhibition, suggesting that extracts from *C. vulgaris* and *S. platensis* were more potent against α-glucosidase than α-amylase in the current experimental setting. In both the α-amylase and α-glucosidase assays, the methanolic extracts of *C. vulgaris* and *S. platensis*

Table 3. IC_{50} values of α-amylase and α-glucosidase inhibitory activities of *Chlorella vulgaris* and *Spirulina platensis* extracts.

Extract		IC_{50} ($\mu g \cdot mL^{-1}$)	
		Anti-α-amylase	**Anti-α-glucosidase**
Positive control	BHT	230.93±21.19^{a}	170.17±5.05^{a}
Chlorella vulgaris	n-Hexane	339.83±22.84^{cd}	387.05±16.09^{c}
	Ethanol	304.92±14.45^{bc}	316.78±21.85^{b}
	Methanol	263.16±20.98^{ab}	205.88±11.63^{a}
	Water	389.04±4.62^{d}	343.67±18.81^{b}
Spirulina platensis	n-Hexane	309.03±15.60^{bc}	333.04±14.18^{b}
	Ethanol	364.92±3.39^{d}	353.67±8.77^{bc}
	Methanol	275.53±9.72^{ab}	170.16±19.31^{a}
	Water	366.51±28.17^{d}	340.88±2.11^{b}

Note: Means±SD (n = 3) in the same column superscripted with different lowercase letters are significantly ($p<0.05$) different.

showed the best inhibitory activity. These findings might be attributed to the high amounts of phenolic compounds in the methanolic extracts of *C. vulgaris* and *S. platensis* (Table 1). The phenolic compounds, especially polyphenols, could inhibit the activity of both enzymes through binding interactions between the phenolics with active sites of the enzymes and also the carbohydrates (Sun and Miao, 2020). Similarly, the phenolic compounds that are present in high concentrations in marine algae methanolic extracts are effective inhibitors of α-amylase and α-glucosidase (Nwosu *et al*., 2011). However, this solvent is known to be toxic and has negative impacts on both human health and the environment (Zhou *et al*., 2022). Further research should be done to find safer but effective extraction reagents to replace traditional toxic reagents for microalgae extraction (de Jesus *et al*., 2019).

Correlations between TPC, TFC, and biological activities

The relationship between bioactive compounds (TPC and TFC) and bioactivities (antioxidative and carbohydrate-hydrolyzing enzyme inhibition) of *Chlorella vulgaris* and *Spirulina platensis* extracts was analyzed by Pearson's correlation coefficients (Table 4). A negative correlation coefficient results from an inverse relationship, whereas a positive coefficient indicates a direct relationship. There was a significant positive correlation ($p<0.05$) of TPC with DPPH, ABTS, and α-glucosidase inhibitory activities of *C. vulgaris* and *S. platensis* extracts. The DPPH assay was positively and significantly correlated with ABTS ($r = 0.802$) and α-glucosidase inhibitory assays ($r = 0.794$), and indicated that the antioxidant and α-glucosidase inhibition may be closely related. However, the α-amylase inhibitory assay was not correlated with the α-glucosidase inhibitory assay. The two antioxidant measurements (DPPH and ABTS) are linked because both assays are similarly based on organic radical reactions and colorimetric determination of the final product (Sadeer *et al*., 2020). Specifically, the reaction mechanism of DPPH is hydrogen atom transfer and single electron transfer, while ABTS involves only hydrogen atom transfer (Liang and Kitts, 2014). In contrast, anti-α-amylase and anti-α-glucosidase activities in this study were not significantly correlated, as the two enzymes hydrolyze carbohydrate molecules at different steps: α-amylase breaks down long starch chains, whereas α-glucosidase breaks down oligosaccharides and disaccharides (Nair *et al*., 2013).

The fact that TPC, but not TFC, correlated significantly with the antioxidant activity suggests that the major antioxidant compounds in the *C. vulgaris* and *S. platensis* extracts are phenolic compounds other than flavonoids. Furthermore, TPC was significantly correlated with α-glucosidase inhibitory activity ($r = 0.734$, $p<0.01$), although not correlated with α-amylase inhibitory activity ($r = 0.027$, $p>0.05$). This result implies that the phenolic compounds found in *C. vulgaris* and *S. platensis* exerted their anti-diabetic activity through α-glucosidase inhibition even if the identities of the phenolic compounds responsible for the α-glucosidase inhibitory action are yet to be elucidated.

Table 4. Pearson's correlation coefficients between TPC, TFC, and biological activities.

	TPC	**TFC**	**DPPH**	**ABTS**	**AA**
TFC	-0.344	-	-	-	-
DPPH	0.694**	-0.107	-	-	-
ABTS	0.816**	-0.260	0.802**	-	-
AA	0.027	0.450*	0.387	0.139	-
AG	0.734**	-0.132	0.794**	0.894**	0.348

Note: * significant ($p<0.05$; $n = 3$); ** highly significant ($p<0.01$); TPC = total phenolic content; TFC = total flavonoid content; DPPH = 2,2-diphenyl-1-picrylhydrazyl assay; ABTS = 2,20-azino-bis assay; AA = anti-α-amylase assay; AG = anti-α-glucosidase assay

Several studies of plant extracts have previously indicated that phenolic compounds have anti-diabetic action, particularly inhibiting α-glucosidase activity (Shobana *et al*., 2009; Hemalatha *et al*., 2016; Pradeep and Sreerama, 2018). According to a molecular docking study, phenolic compounds from different plants show strong binding affinity to the α-glucosidase active sites, causing structural modifications and inhibiting action (Swargiary *et al*., 2022). Natural α-glucosidase inhibitors derived from plants have gained importance in the study of T2DM treatment due to their relatively low toxicity and promising efficacy. It is worth mentioning that in another study, the phenolic contents of plant extracts did not show a significant correlation with either α-amylase or α-glucosidase (Quan *et al*., 2019). Such dissimilarity might be related to the differences in the amounts and compositions of the extracted phenolic compounds.

In addition to the α-glucosidase inhibitory activity of TPC, a significant correlation ($r = 0.450$, $p<0.05$) between TFC and anti-α-amylase was observed, suggesting that flavonoids might be responsible for the α-amylase inhibition of the *C. vulgaris* and *S. platensis* extracts. Some studies similarly reported the anti-diabetic action of plant flavonoids, particularly α-amylase inhibition (Hargrove *et al*., 2011; Ibrahim *et al*., 2017; Hua *et al*., 2018; Proença *et al*., 2019). The mechanism of α-amylase inhibition by flavonoid compounds is related to their ability to bind to the enzyme's active site and/or substrate-binding sites through hydrogen and hydrophobic bindings (Takahama and Hirota, 2018; Martinez-Gonzalez *et al*., 2019). Hydroxylation at hydrogen atom sites of flavonoids was found to enhance the inhibitory effect against α-amylase since the hydroxyl group could interact with the amino acid residues in the enzyme's active sites (Sun and Miao, 2020). Surprisingly, no relationship between TFC and antioxidant was detected, despite the fact that the antioxidant property of flavonoids is well-recognized (Panche *et al*., 2016). Finally, the lack of correlation between TPC and TFC suggests that flavonoids do not dominate the phenolic group present in *C. vulgaris* and *S. platensis* methanolic extracts in our study.

CONCLUSION

Different types of solvent affected the bioactive compound composition, antioxidant, and carbohydrate-hydrolyzing enzyme inhibition activities of the microalgae extracts differently. Our findings demonstrated that methanol was the most effective extraction solvent for producing the highest TPC, antioxidant, anti-α-amylase, and anti-α-glucosidase activities from *Chlorella vulgaris* and *Spirulina platensis*. TPC was found to have a significant positive correlation with antioxidant and α-glucosidase inhibition activities. Meanwhile, only α-amylase inhibitory activity had a significant positive correlation with TFC. We concluded that methanolic extracts of *C. vulgaris* and *S. platensis* show promise as health-promoting dietary supplements. However, further research is needed to elucidate the secondary metabolites that are responsible for the antioxidant and anti-diabetic effects. The development of dietary supplements with pharmacological benefits against oxidative stress-related disorders may be facilitated by a more comprehensive characterization of the extract and its biological effects in cells and in vivo animal experiments.

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