Original Article

Phytochemical Profiling and *in vitro* Screening for Neuritogenic and Antioxidant Activities of *Spirulina platensis*

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ABSTRACT

Background: In neurological diseases, neuronal loss is frequently associated with overproduction of free radicals and reduced level of endogenous neurotrophic factors. The blue-green microalga, *Spirulina platensis* is a well-known superfood with a high content of diverse nutrients and possesses several therapeutic properties. Here, we aimed to study the neuritogenic and antioxidant activities of *Spirulina platensis* UMACC 159. **Materials and Methods:** PC-12Adh (rat pheochromocytoma) cell was used to investigate the cytotoxicity effect of *S. platensis* UMACC 159 extracts (water, methanol, and ethanol) via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Neuritogenic activity of the extracts towards PC-12Adh cell line was studied using neurite outgrowth assay and immunofluorescence imaging of neurofilaments. The extracts were screened for the phytochemical contents, and antioxidant activities using 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-Diphenyl-1-pircrylhydrazyl (DPPH) and reducing power. **Results:** Ethanol extract was found to exhibit the highest neuritogenic effect and enhanced the cytoskeleton formation in PC-12Adh cells at 6.25 µg/mL. Ethanol extract also showed the highest total phenolic content (49.09 ± 1.35 mg GAE/g), ABTS (EC_{50} of 1.34 ± 0.01 mg/mL) and DPPH (EC_{50} of 0.45 ± 0.04 mg/mL) scavenging activities ($P \leq 0.05$), suggesting that the neuritogenic effect of ethanol extract was attributed to the phenolic compound(s) via antioxidant activity. **Conclusion:** Ethanol extract contains bioactive compound(s) with similar neuritogenic activity as nerve growth factor for neuronal survival, growth, and axonal regeneration. *S. platensis* has been proposed as a promising cognitive supplement.

Key words: *Spirulina platensis,* Antioxidants, Cytotoxicity, Neuronal outgrowth, Phytochemicals.

INTRODUCTION

Deaths caused by dementia increased more than doubled within 16 years, making dementias the 5th leading cause of global deaths in 2016.[1] Currently, drugs approved for Parkinson's Disease (PD) by the Food and Drug Administration (FDA), such as levodopa and rivastigmine are symptomatic therapies. These drugs come with adverse effects, for example motor fluctuations and dyskinesias for levodopa; nausea, dyspepsia, and asthenia for rivastigmine.[2,3] Therefore, there is a need to discover novel compounds to counter against neurodegenerative diseases.

In various neurodegenerative diseases, the loss of neuron is a common hallmark accompanied by excess free radicals and insufficient endogenous neurotrophic factors. Reactive oxygen species (ROS) are byproduct of aerobic metabolism, and are highly reactive whereby overproduction can induce oxidation of biomolecules including

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proteins and nucleic acid, eventually result in neuronal death.[4] Nerve growth factor (NGF) is a neurotrophic factor crucial for neuronal growth, survival and regeneration.[5,6] NGF has potent neuroprotective property via promoting neuronal survival and stimulating neurite outgrowth.[6] Neurite outgrowth or neuritogenesis is the process where neurons produce and elongate the neurites to construct neuronal communications network.[5] Diminutions in the level of NGF cause major declines in brain cell performance resulting in neurodegenerative diseases.[6] Although direct administration into AD patient's brain showed encouraging results,[7] NGF failed to travel across the blood-brain barrier due to high molecular weight polypeptides.[6] Hence, the unearthing of NGF-potentiating substances with low molecular weight from natural sources are believed to be the new alternative therapeutic drugs.

Different species of microalgae, namely *Cylindrotheca closterium*, *Odontella mobiliensis*, *Pseudo-nitzschia pseudo delicatissima*, *Skeletonema marinoi*, *Leptocylindrus danicus*, and *Leptocylindrus aporus* have exhibited therapeutic activities such as anti-inflammatory, anticancer, antibacterial, and anti-biofilm activities.[8] *Nannochloropsis* sp. also had neuroprotective activity against neuroinflammation and oxidative DNA damage.[9,10] In fact, eicosapentaenoic acid in *Nannochloropsis oceanica* defends against oxidative stress by upregulating the levels of antioxidant enzymes in Neuro-2A cells.[11] The blue-green microalga, *Spirulina platensis* is a prokaryotic filamentous cyanobacterium that belongs to the genus of *Arthrospira*. *Spirulina* is commonly known as a superfood due to its high content of diverse nutrients. In 2012, *Spirulina* has been nominated as a safe nutritional supplement by both the World Health Organization (WHO) and FDA.[12,13] *S. platensis* is known to possess several therapeutic properties, including immunomodulation,[14] wound healing enhancement,[15] antiallergic,[16] anticancer,[17] antimicrobial,[18,19] antioxidative, antidiabetic, antihypertensive,[20] and anti-inflammation.[21,22] Antioxidative property of *S. platensis* gives rise to various type of protective effects, such as UV-protection,[21] renal protection,[23] hepatoprotection[22] and neuroprotection.[24,25] *S. platensis* protects dopaminergic neurons via anti-oxidation and anti-inflammatory mechanisms[24,25] and also promotes recovery of spinal cord injury in animal model.[26]

Numerous studies have reported the neuroprotective effect of Spirulina, but neuritogenic effect of *S. platensis* remains unknown. Hence, we aimed to examine antioxidant and neuritogenic activities of *S. platensis* extracts in rat pheochromocytoma cells (PC-12Adh). PC-12Adh cell line is commonly used to study the neuronal differentiation in both neurobiological and neurotoxicological studies. PC-12Adh cells have the morphology of noradrenergic adrenal chromaffin cells, but undergo neurite outgrowth like sympathetic neurons upon NGF stimulation.[27]

MATERIALS AND METHODS

Preparation of solvent extracts

Fresh *S. platensis* UMACC 159 was procured from the University of Malaya Algae Culture Collection (UMACC), Algae Research Laboratory, University Malaya. It was cultivated in Kosaric medium for approximately 7-10 days prior harvesting. The biomass was freeze-dried using vacuum concentrator (LaboGene, Brigachtal, Germany). The dried biomass was soaked in water, methanol, and ethanol separately (1:50 w/v) at 37°C, agitated for 48 h, and the extracts were then centrifuged (2,688 x g, 4°C, 20 min). By using a rotary evaporator (Fisher Scientific EYELA N-1200A, Tokyo), the supernatant was dried and further concentrated under vacuum (SpeedSan 40, Korea) and then kept at -20°C for future use. The extraction yield (%) was determined using the equation (1):

$$\text{Extraction yield (\%)} = \frac{\text{Weight of concentrated extract (g)}}{\text{Dry weight of powder (g)}} \times 100\% \quad (1)$$

Cell culture

PC-12Adh cell line (ATCC® CRL-1721.1TM) was cultured in Ham's F-12 (F-12 K) medium (Kaighn's modification) supplemented with horse serum (15%), fetal bovine serum (2.5%), and penicillin-streptomycin (1%) (Sigma-Aldrich, MO, US) and incubated at 37 ± 2°C under 5% CO_2.

In vitro cell viability measurement

PC-12Adh cells were inoculated in 96-well plate with 5 x 10^3 cells/well. The cells were treated with 0-8 mg/mL of extracts and incubated for 24 h. Ten microliter of 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Merck and Co, NJ, US) was added and incubated for 4 h. To solubilize formazan formed in viable cells, dimethyl sulfoxide (DMSO) was used. The absorbance at 570 nm (measuring) and 630 nm (reference) wavelengths was measured with the UV-Vis spectrophotometer microplate reader (Infinite 200 Pro, Tecan, Mannedorf, Switzerland). The cell viability (%) was calculated using the equation (2):

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of sample-blank}}{\text{Absorbance of negative control}} \times 100\% \quad (2)$$

Half-maximal inhibition concentration (IC_{50}) was determined from the graph of cell viability (%) against treatment concentration.

Neuritogenic effect of *Spirulina platensis* extracts

Neurite outgrowth activity

Neuritogenic activity was determined according to Pang *et al.*[28] with minor alterations. PC-12Adh cells were inoculated in 24-well plate with 5 x 10^3 cells/well and treated with NGF (70 ng/mL, positive control), culture medium (negative control) or extracts (6.25-37.5 µg/mL). The cells were incubated at 37 ± 2°C under 5% CO_2 for 48 h. Five random fields (300-600 cells/well) from each well were examined using Nikon Eclipse Ti-S (10 × 10.5 objective) and photographed with Digital Sight DS-Fi2 camera using NIS-Elements imaging software (Nikon, Tokyo, Japan). Number of neurite-bearing cells (cells with axon-like extension that is at least equal to diameter of the cell body) was counted and the percentage was calculated using the equation (3):

$$\text{Neurite–bearing cells (\%)} = \frac{\text{Neurite–bearing cell number per well}}{\text{Total cell number per well}} \times 100\% \quad (3)$$

Neuronal cytoskeleton analysis by immunofluorescence imaging

PC-12Adh cells were inoculated in 12-well cell culture chamber with 5 x 10^3 cells/well. The cells were treated with the NGF (70 ng/mL, positive control), culture medium (negative control) or optimal concentration of extracts (6.25 µg/mL) and incubated at 37 ± 2°C under 5% CO_2 for 48 h. Paraformaldehyde (4%) was used to fix the cells for 20 min. Cells were incubated with rabbit anti-neurofilament 200 polyclonal antibody (1:80, Sigma-Aldrich, cat. no. N4142) for 1 h, followed by anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) antibody (1:80, Sigma-Aldrich, cat. no. F7512) in the dark for 2 h. The slide was mounted with Prolong® gold antifade mounting reagent with 4'-6-diamidino-2-phenylindole (DAPI) (Life Technologies Corporation, California, US) to counterstain the nuclei. The stained cells were observed with DAPI and FITC filters under fluorescence illumination using inverted microscope. The neuronal cytoskeleton was photographed using the NIS-elements Imaging Software.

Phytochemicals screening

Quantification of total phenolic content (TPC)

TPC of extracts was measured according to Singleton *et al.*[29] with minor alterations. Gallic acid (0-1000 µg/mL) (Sigma-Aldrich, Shanghai, China) served as the standard. The extracts or gallic acid (5 µL) was mixed with Folin-Ciocalteu reagent (25 µL) (EMD Millipore Corporation, Darmstadt, Germany) and double-distilled water (ddH_2O) (350 µL), and incubated in dark for 4 min. Sodium carbonate (75 µL, 20%) and ddH_2O (45 µL) were added with the mixture and incubated in dark for 60 min. The absorbance at 750 nm was measured using the microplate reader. TPC was expressed as mg of gallic acid equivalent (GAE) per g of extract (mg GAE/g).

Quantification of total flavonoid content (TFC)

TFC of extracts was quantified according to Pekal and Pyrzynska[30] with minor alterations. Quercetin (0-1000 µg/mL) (Sigma-Aldrich, Bangalore, India) served as the standard. The quercetin or extract (10 µL) was mixed with methanolic aluminum chloride (250 µL, 2%) (Sigma-Aldrich, Munich, Germany), sodium acetic acid (250 µL, 1 M) and ddH_2O (490 µL). The mixture was incubated in dark for 15 min. The absorbance at 425 nm was measured using the microplate reader. TFC was expressed as mg of quercetin equivalent (QE) per g of extract (mg QE/g).

Qualitative determination of carotenoid and terpenoid

Presence of carotenoid was identified according to Sharma *et al.*[31] with minor alterations. Extract (0.01 g) was mixed vigorously with 1 mL of chloroform and filtered prior added with 85% sulphuric acid. Blue colour formation at the interfere indicates the presence of carotenoid. Meanwhile, the presence of terpenoid was identified according to Batista-Gonzalez *et al.*[32] Extract (0.01 g) was mixed with acetic anhydride and sulphuric acid. Greenish blue colour formation indicates the presence of terpenoid.

Antioxidant assays

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) scavenging activity

ABTS scavenging activity acts on the electron transfer (ET) reaction,[33] was measured according to Miller *et al.*[34] and Re *et al.*[35] with minor alterations. Trolox (0-1 mg/mL) (Sigma-Aldrich, St. Gallen, Switzerland) served as the positive control. Seven-millimolar ABTS (Roche, Baden-Wurttemberg, Germany) was activated through incubation with equal volume of potassium persulfate (2.45 mM) in dark for 16 h. Ethanol (1:10 v/v) was used to dilute the activated ABTS to the absorbance of 0.7 ± 0.02 at 734 nm. The trolox or extract (0.1 mL) was incubated with diluted ABTS (1 mL) in dark for 6 min. The absorbance at 734 nm was measured using the

microplate reader. ABTS scavenging activity (%) was calculated using the equation (4):

$$\text{ABTS scavenging activity (\%)} = 1 - \frac{\text{Absorbance of samples at 734 nm}}{\text{Initial absorbance of ABTS at 734 nm}} \times 100\% \quad (4)$$

Half-maximum effective concentration (EC_{50}) was determined from the graph of ABTS scavenging activity (%) against the treatment concentration.

2,2-Diphenyl-1-pircrylhydrazyl (DPPH) scavenging activity

DPPH scavenging activity is based on ET reaction, with hydrogen transfer as an alternate pathway.[33] The activity was determined according to Brand-Williams *et al*.[36] with minor alterations. Ascorbic acid (0-25 µg/mL) (Sigma-Aldrich, Tokyo, Japan) served as the positive control. The ascorbic acid or extract (50 µL) was incubated with DPPH (1 mL, 0.1 mM, Alfa Aesar) in dark for 30 min. The absorbance at 518 nm was measured using the microplate reader. DPPH scavenging activity (%) was calculated using the equation (5):

$$\text{DPPH scavenging activity (\%)} = (1 - \frac{\text{Absorbance of samples at 518nm}}{\text{Absorbance of samples at 0 µg/mL at 518nm}} \times 100\% \quad (4)$$

The EC_{50} was determined from the graph of DPPH scavenging activity (%) against treatment concentration.

Reducing power

Reducing power of antioxidant that acts on the ET reaction[37] was quantified according to Oyaizu[38] with minor alterations. Ascorbic acid (0-300 µg/mL) served as the positive control. The ascorbic acid or extract (100 µL) was mixed with phosphate buffer (250 µL, 0.2 M) and potassium ferricyanide (250 µL, 1 %) and incubated at 50°C for 20 min. Trichloroacetic acid (250 µL, 10%) was added and centrifuged at 250 x g for 10 min. Supernatant (250 µL) was mixed with iron (III) chloride (250 µL, 0.1%) and ddH_2O (250 µL). The absorbance at 700 nm was measured using the microplate reader. The reducing power was expressed in EC_{50}, calculated by adopting from the graph of absorbance against treatment concentration.

Statistical analysis

All experimental data ($n \geq 3$) were presented as mean ± standard error (SE). One-way analysis of variance (ANOVA) with Post Hoc test was performed using GraphPad Prism ver. 5.02 with a significant value of $P \leq 0.05$.

RESULTS

Cytotoxicity effect of *S. platensis* UMACC 159 extracts on PC-12Adh cells

Cytotoxicity effect on PC-12Adh cells was determined using the reduction of MTT to formazan in viable cells. A natural product is considered as non-toxic when the LD_{50} or IC_{50} is more than 5 mg/mL.[39] Our findings on cell viability showed that all extracts are non-toxic and having >50% cell viability for cell cultures dosed with more than 5 mg/mL (Table 1).

The neuritogenic effect of *S. platensis* UMACC 159 extracts on PC-12Adh cells

All solvent extracts demonstrated neuritogenic activity, with ethanol extract having the highest percentage of neurite-bearing cells (Figure 1). Methanol (12.11 ± 1.31%) and ethanol (17.13 ± 2.31%) extracts showed maximum percentage at 6.25 µg/mL, which are significantly ($P \leq 0.001$) higher than the negative control by 2.24- and

Table 1: Effect of *S. platensis* UMACC 159 extracts on the viability of PC-12Adh cells.

Extract	IC_{50} (mg/mL)
Water	7.18 ± 0.58 [a]
Methanol	9.63 ± 0.71 [ab]
Ethanol	10.37 ± 0.78 [b]

Data are expressed as mean ± SE (n = 3). Mean with different alphabet indicate significant difference ($P \leq 0.05$) between each group by Tukey's test. A lower IC_{50} indicates higher cell viability inhibition.

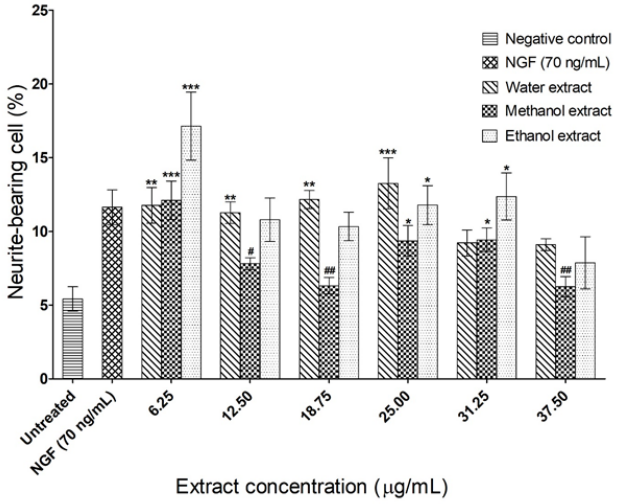

Figure 1: Percentage of neurite-bearing cells treated with different treatments. Data are expressed as mean ± SE (n = 3). * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ when compared to the negative control; # $P \leq 0.05$; ## $P \leq 0.01$ when compared to the nerve growth factor (NGF) by Dunnett's Multiple Comparison Test.

3.16-fold respectively. As the concentration increased to 25 μg/mL, water extract showed a maximum percentage of 13.26 ± 1.73%, which is significantly ($P \leq 0.001$) higher than negative control by 2.45-fold.

NGF (70 ng/mL, positive control) significantly ($P \leq 0.05$) increased the percentage of neurite-bearing cells by 11.63 ± 1.18%, which is 2.15-fold higher than the negative control (5.42 ± 0.83%). All extracts at their respective optimal concentration had higher percentage than NGF, specifically 1.14- (water extract), 1.04- (methanol extract), and 1.47-fold (ethanol extract) higher. At other concentrations, water (6.25 and 18.75 μg/mL) and ethanol extract (25 and 31.25 μg/mL) also had slightly higher percentage (1.01- to 1.06-fold) than NGF. Notably, only neurite-bearing cells in methanol extract showed significant ($P \leq 0.05$) decreased (0.54- to 0.67-fold) at 12.5, 18.755, and 37.5 μg/mL.

Neuronal cytoskeleton analysis by immunofluorescence imaging

Immunofluorescence imaging of neurofilament showed that neurite outgrowth was stimulated by the positive control (NGF) and all extracts of *S. platensis*. Enhanced neurite elongation of PC-12Adh cells was observed in the NGF and extracts treatments (Figure 2).

Phytochemical contents of *S. platensis* UMACC 159 extracts

Water, methanol, and ethanol with different solvent polarities were selected for extracting a wide range of components from the samples. The extraction yield decreased with polarity of a solvent in the following order: water (71.94 ± 10.38 %) > methanol (17.14 ± 2.20 %) > ethanol (13.78 ± 0.70 %) (Table 2). Water extract has the highest yield, with approximately 4- and 5-fold higher than methanol and ethanol extracts, respectively.

The amount of phenolic and flavonoid contents extracted in the organic solvents were significantly ($P \leq 0.05$) higher than the water extract (Table 2). TPC ranged from 32.69 ± 1.73 (water extract) to 49.09 ± 1.35 mg GAE/g (ethanol extract), while TFC ranged from 7.65 ± 0.12 (water extract) to 151.84 ± 3.95 mg QE/g (methanol extract). All the extracts contain carotenoids and terpenoids (Table 2).

Antioxidant activities of *S. platensis* UMACC 159 extracts

The lower EC_{50} value, the higher antioxidant activity of an extract. Overall, antioxidant activities of the organic solvent extracts were significantly ($P \leq 0.05$) higher than the water extract (Table 3). Ethanol extract showed significant ($P \leq 0.05$) higher ABTS (1.34 ± 0.01 mg/mL) and DPPH (0.45 ± 0.04 mg/mL) scavenging activities. However, methanol extract (3.49 ± 0.05 mg/mL) had significant ($P \leq 0.05$) higher reducing power than ethanol extract (4.31 ± 0.09 mg/mL). Water extract gave the lowest antioxidant activities in scavenging ABTS (2.26 ± 0.04 mg/mL) as well as DPPH (1.91 ± 0.01 mg/mL), and the reducing power (15.28 ± 0.10 mg/mL).

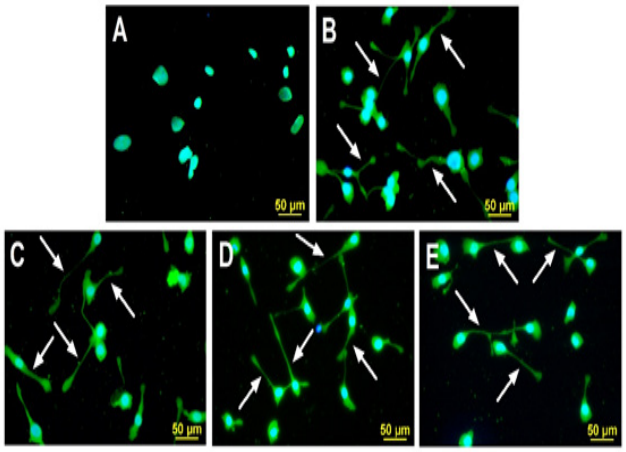

Figure 2: Morphology of PC-12Adh cells stained with anti-NF-200 antibody. Cells were incubated with (A) negative control: F-12K culture medium, (B) positive control: 70 ng/mL nerve growth factor (NGF), or 6.25 μg/mL of extract ((C) water, (D) methanol and (E) ethanol extracts) for 48 h. Nuclei stained blue by DAPI; neurofilament stained green by anti-neurofilament-200 labelled with FITC. Arrows indicate neurite outgrowth. Photomicrographs were taken with 20x magnification; Scale bar: 50 μm.

DISCUSSION

Natural products hold paramount potential since they were traditionally consumed as dietary supplements. Recently, marine algae and their constituents have been gaining more interest as nutraceuticals with neuroprotective activity.[40] Antioxidative and anti-inflammatory activities of *S. platensis* protect dopaminergic neurons in the 6-OHDA-lesioned striatum of the PD rat model,[25] possibly contributed by the polysaccharide.[24] *S. platensis* also alleviates morphological impairment after spinal cord injury and promotes recovery.[26] However, there is no report regarding the neuritogenic effect of *S. platensis*. This study is the first to report the neuritogenic effect of *S. platensis*. All *S. platensis* UMACC 159 extracts promoted neuritogenesis in PC-12Adh cells, and the effect is comparable with the positive control (NGF). In addition, *S. platensis* UMACC 159 extracts possess phytochemicals such as phenolic, flavonoid, carotenoid, and terpenoid that exhibited promising antioxidant activity.

Table 2: Extraction yield and the phytochemical contents of *S. platensis* UMACC 159 extracts.

Solvent extract	Yield (%)	TPC (mg GAE/g)	TFC (mg QE/g)	Carotenoids	Terpenoids
Water	71.94 ± 10.38	32.69 ± 1.73[a]	7.65 ± 0.12[a]	++	++
Methanol	17.14 ± 2.20	40.48 ± 2.16[b]	151.84 ± 3.95[b]	++	++
Ethanol	13.78 ± 0.70	49.09 ± 1.35[c]	85.96 ± 1.80[c]	++	++

Data are expressed as mean ± SE (n = 3). Mean with different alphabet indicate significant difference ($P \leq 0.05$) between each group by Tukey's test. ++ indicates present. *TPC - total phenolic content; TFC - total flavonoid content; mg GAE/g - mg of gallic acid equivalent (GAE) per g of extract; mg QE/g - mg of quercetin equivalent (QE) per g of extract.*

Table 3: *In vitro* antioxidant activities of *S. platensis* UMACC 159 extracts.

Extract/control	EC_{50} (mg/mL)		
	ABTS	DPPH	Reducing power
Extract			
Water	2.26 ± 0.04[a]	1.91 ± 0.01[a]	15.28 ± 0.10[a]
Methanol	1.65 ± 0.01[b]	0.86 ± 0.06[b]	3.49 ± 0.05[b]
Ethanol	1.34 ± 0.01[c]	0.45 ± 0.04[c]	4.31 ± 0.09[c]
Control			
Trolox	0.008 ± 0.0001[d]	-	-
Ascorbic acid	-	0.001 ± 0.0000[d]	0.009 ± 0.0003[d]

Data are expressed as mean ± SE (n = 3). Mean with different alphabet indicate significant difference ($P \leq 0.05$) between each group by Tukey's test. Trolox serves as the positive control for ABTS; ascorbic acid serves as the positive control for DPPH and reducing power. A lower EC_{50} indicates a higher antioxidant activity. *ABTS - 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); DPPH - 2,2-Diphenyl-1-pircrylhydrazyl.*

Exogenous neurotrophic factors help in the development of neuronal networks via neurite outgrowth.[41] Our findings showed that the percentage of neurite-bearing cells of ethanol extract is 1.47- and 3.16-fold higher than the NGF and the negative control respectively, indicating its enhancing effect on the formation of complex neuronal network. *S. platensis* UMACC 159 ethanol and methanol extracts exhibit neuritogenic activity in PC-12Adh cells in a hormetic dose-response relationship, with the optimal concentration identified at 6.25 µg/mL. C-phycocyanin, the bioactive compound in *S. platensis* water extract, has several therapeutic activities including antioxidative, anti-inflammatory, antibacterial and anticancer.[42-44] However, our results suggest that the neuritogenic activity of *S. platensis* were attributed by unknown bioactive compound(s). To our best knowledge, very few studies have been conducted on the neuritogenic activity of microalgae. Besides our study, only the antillatoxin extracted from the cyanobacterium *Lyngbya majuscula* was reported to enhance neurite outgrowth by activating voltage-gated sodium channels.[45] In accordance with our results, antillatoxin also promoted neurite outgrowth in a hormetic dose-response. Jabba's group proposed that this relationship was primarily attributed to the regulation of intracellular calcium level,[45] of which the optimal calcium level is essential for the neurite outgrowth.[46,47] Antillatoxin significantly enhanced the neurite outgrowth by more than 2-fold at 30 nM, while our ethanol extract required a higher concentration of 6.25 µg/mL. This is possibly due to the crude extract was used in comparison with the pure compound antillatoxin.

Bioactive compound(s) in *S. platensis* UMACC 159 ethanol extract remained unknown, but the neuritogenic effect may be attributed to the phenolic compound(s). Currently, two phenolic compounds, sargachromenol and sargaquinoic acid isolated from methanol extract of *Sargassum macrocarpum* have been reported to enhance NGF-dependent neuritogenesis in PC12 cells.[48,49] Besides, pheophytin a, a chlorophyll-related compound isolated from methanol extract of *Sargassum fulvellum* had the same neuritogenic activity.[50] It is noteworthy that the neuritogenic activity of these isolated bioactive compounds are NGF-dependent. In contrast, our ethanol extract is independent of NGF, suggesting a more prominent effect. Other than PC-12 cells, hippocampal neuron was also used to study the neuritogenic activity of Phaeophyta. Ethanol extract of *S. fulvellum* and *Undaria pinnatifida* were reported to enhance neuronal maturation and neuronal survival of hippocampal

neurons.[51,52] Neuritogenic activity of ethanol extract of Rhodophyta, such as *Gracilariopsis chorda*, *Gelidium amansii, Kappaphycus alvarezii* and *Porphyra yezoensis,* had also been reported in hippocampal neurons.[53-56] In comparison, our ethanol extract has higher neuritogenic activity than all the Rhodophyta (15 μg/mL), except for *K. alvarezii* which had optimal activity at 1 μg/mL.

Most microalgae are well-adapted to survive under a wide spectrum of environmental stresses. As a unicellular or simple multicellular microorganism, they can divide rapidly and react to the changes in an environment. Metabolic processes in algal cells allow the production of numerous metabolites, for example phenolic compounds, carotenoids, phycobiliproteins and alkaloids.[57] Phenolic compounds are able to combat free radicals and suppress AD pathogenic cascade.[58,59] For example, ethanol extracts of *Chlorella sorokiniana* and *Chlorella minutissima* with TPC of 14.21 and 13.35 mg GAE/g respectively, exhibited radical scavenging activities and neuroprotective activities which include inhibition on cholinesterase activity and $A\beta_{1-42}$ aggregation.[60] The amount of phenolic and flavonoid produced by *S. platensis* can be easily affected by environment factors, including the light irradiance intensity and culture medium.[61,62] Kepekci's group[62] reported that increase in light irradiance intensity significantly increase the amount of phenolic compound from 6.32 to 49.83 mg GAE/g. In comparison, all our extracts had considerably high phenolic content (32.69 ± 1.73 to 49.09 ± 1.35 mg GAE/g), indicating the potential of *S. platensis* UMACC 159 in the production of phenolic compounds.

Methanol and ethanol have been known as ideal solvents for polyphenol extraction, as these solvent systems aid in the denaturation of cell membranes, simultaneously dissolve and stabilize the polyphenols.[63] Based on our results, the best TPC extracting solvent was ethanol as TPC decreased with increasing solvent polarity index. This could be caused by the presence of phenolic compounds that are more soluble in methanol and ethanol compared with water.[63] In agreement with our findings, Shalaby and Shanab also reported that methanol extract of *S. platensis* has higher TPC than water extract.[64] We also investigated the TFC as flavonoids are the most abundant polyphenols found in human diets. Our findings showed that the best TFC extracting solvent was methanol. In addition, in the present study, carotenoids and terpenoids are present in all the extracts. Carotenoids are a group of terpenoids with antioxidative properties that have displayed a diverse scope of therapeutic activities, including anti-inflammatory, immunomodulatory, neuroprotective, and anticarcinogen properties.[65] Fucoxanthinol, a carotenoid isolated from *Nitzschia laevis* protects against neuroinflammatory response in BV-2 microglia.[66] Terpenoids extracted from other *S. platensis* strains have also been reported for its neuroprotective activities against oxidative stress.[67,68]

According to our results, organic solvent extracts showed stronger antioxidant activities than water extract. In regard to ABTS and DPPH scavenging activities, the potency decreased as solvent polarity index increased, with ethanol extract having the highest activities. Shalaby and Shanab also reported that methanol extract of *S. platensis* had higher ABTS and DPPH scavenging activities than water extract.[64] In fact, studies on red seaweed also reported a similar trend,[69,70] but comparatively our ethanol extract (EC_{50} = 0.45 ± 0.04 mg/mL) had higher DPPH scavenging activity than the red seaweed *Gracilaria changii* (EC_{50} = 2.36 ± 0.13 mg/mL).[69] Overall, our results suggest that the phenolic compounds in ethanol extract may be responsible for the DPPH and ABTS scavenging activities, while the flavonoid compounds in methanol extract may contribute to the reducing power. Phenolic compounds are one of the key metabolites that contribute to the DPPH scavenging activity in *S. platensis* ethanol extracts.[71]

CONCLUSION

In conclusion, we found that all *S. platensis* UMACC 159 extracts promote neuritogenesis in PC-12Adh cells, with ethanol extract possessing the highest neuritogenic activity. Our findings suggest the role of *S. platensis* UMACC 159 polyphenol as free radical scavengers, particularly flavonoid as ferric ions reducing agent. Bioactive compound(s) that regulates the neuritogenic activity remained unknown. However, our results demonstrated that the neuritogenic activity may be contributed by phenolic compound(s) present in *S. platensis* UMACC 159. These findings not only suggest the importance of the *S. platensis* in a healthy diet, but also trigger further research to provide insight on the molecular mechanisms involved the neuritogenic activity, which are beneficial in the production of functional food and drug for neurodegenerative disease.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

ABBREVIATIONS

%: percentage; **°C:** Degree Celsius; **µg/mL:** micrograms per millilitre; **µL:** microliter; **6-OHDA:** 6-hydroxydopamine; **ABTS:** 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); **AD:** Alzheimer's disease; **ANOVA:** analysis of variance; **Aβ:** amyloid beta; **CO_2;** carbon dioxide; **DAPI:** 4'-6-diamidino-2-phenylindole; **ddH_2O:** double-distilled water; **DMSO:** dimethyl sulfoxide; **DNA:** deoxyribonucleic acid; **DPPH:** 2,2-Diphenyl-1-pircrylhydrazyl; **EC_{50}:** Half-maximum effective concentration; **ET:** electron transfer; **FDA:** Food and Drug Administration; **FITC:** Fluorescein isothiocyanate; **g:** gram; **GAE:** gallic acid equivalent; **h;** hour; **IC_{50}:** Half-maximal inhibition concentration; **LD_{50}:** median lethal dose; **M:** moles per liter; **mg GAE/g:** mg of GAE per g of extract; **mg QE/g:** mg of QE per g of extract; **mg/mL:** milligrams per millilitre; **min:** minute; **mL:** millilitre; **mM:** millimolar; **MO;** Missouri; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **ng/mL:** nanograms per millilitre; **NGF:** nerve growth factor; **NJ:** New Jersey; **nm:** nanometer; **nM:** nanomolar; **PD:** Parkinson's disease; **QE:** quercetin equivalent; **ROS:** reactive oxygen species; **SE:** standard error; **sp.:** species; **TFC:** total flavonoid content; **TPC:** total phenolic content; **UMACC:** University of Malaya Algae Culture Collection; **US:** United States; **UV:** ultraviolet; **v/v:** volume by volume; **ver.:** version; **w/v:** weight by volume; **WHO:** World Health Organization; **xg:** times gravity.

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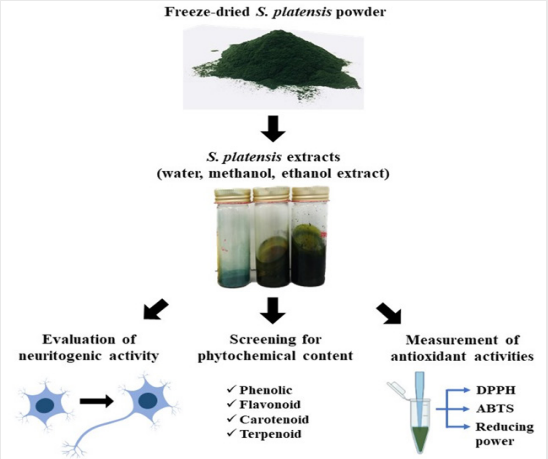
PICTORIAL ABSTRACT



SUMMARY

The blue-green microalga, *Spirulina platensis* is well-known for the high content of diverse nutrients and has been reported with several therapeutic properties. Current study demonstrated that all *S. platensis* UMACC 159 extracts exhibit neuroprotective activity by promoting neuritogenesis in PC-12Adh cells, with ethanol extract possessing the highest neuritogenic activity. In addition, our findings suggest the role of phenolic compound(s) in the antioxidant and neuritogenic activities of *S. platensis* UMACC 159 ethanol extract. These findings further emphasise the importance of *S. platensis* as a food supplement. Nevertheless, further research on the molecular mechanisms involved in neuritogenic activity would be beneficial in the production of functional food and drug for neurodegenerative disease.

About Authors

Ms. Ee-Ling Ngu is a postgraduate student at the Department of Biological Sciences, School of Medical and Life Sciences, Sunway University. Her research interest is to explore the potential of natural product, particularly marine algae in the development of functional food or treatment for neurological disease. Her research has been focussing on the neuroprotective effect of blue-green microalga, *Spirulina platensis* using cellular models.

Ms. Chen-Lin Ko is a student graduated from Sunway University in 15 September 2017. She is graduated with Bachelor of Science in Medical Biotechnology. Her Final Year Project on Preliminary study of phytochemical and *in vitro* antioxidant activities of superfood *Spirulina platensis*. She is currently a senior product specialist at local pharmaceutical company to promote and share company's products to the Healthcare Professional.

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Dr. Yoon-Yen Yow is a Senior Lecturer at the School of Medical and Life Sciences, Sunway University. She is an algae biotechnologist and her research focuses on developing of high-value products from algae. She is exploring the pharmacological effects of algae in areas including neuroprotection, cosmeceuticals and their anti-microbial effects against infectious diseases. She is also interested in the study of molecular phylogeny and genetic diversity of algae, and invasive Pomacea apple snails in Malaysia. In line with Sunway University's commitment to the United Nations Sustainable Development Goals (UNSDGs), she looks forward to discovering how algae play a role in the SDGs with the aim to promote well-being for all.

Emeritus Professor Dr. Siew-Moi Phang is the pioneered Applied Phycological Research in Malaysia. Her research group focuses on the areas including Taxonomy, Biotechnology, Bioremediation and Algal Renewable Energy. She has produced 210 ISI and 40 Scopus journal publications, 17 books and 57 book chapters; with 6 patents granted, 11 filed; 4 copyright filed; produced 32 PhDs and 68 Masters graduates. She is presently working as an Emeritus Professor, University of Malaya and was recently appointed the Deputy Vice Chancellor (Research & Postgraduate), UCSI University, Malaysia.

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