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Antioxidative, Anticholinesterase and Anti-Neuroinflammatory Properties of Malaysian Brown and Green Seaweeds

Siti Aisya Gany, Swee Ching Tan, Sook Yee Gan

***Abstract*—**Diminished antioxidant defense or increased production of reactive oxygen species in the biological system can result in oxidative stress which may lead to various neurodegenerative diseases including Alzheimer's disease (AD). Microglial activation also contributes to the progression of AD by producing several proinflammatory cytokines, nitric oxide (NO) and prostaglandin E2 (PGE2). Oxidative stress and inflammation have been reported to be possible pathophysiological mechanisms underlying AD. In addition, the cholinergic hypothesis postulates that memory impairment in patient with AD is also associated with the deficit of cholinergic function in the brain. Although a number of drugs have been approved for the treatment of AD, most of these synthetic drugs have diverse side effects and yield relatively modest benefits. Marine algae have great potential in pharmaceutical and biomedical applications as they are valuable sources of bioactive properties such as anticoagulation, antimicrobial, antioxidative, anticancer and anti-inflammatory. Hence, this study aimed to provide an overview of the properties of Malaysian seaweeds (*Padina australis, Sargassum polycystum* and *Caulerpa racemosa*) in inhibiting oxidative stress, neuroinflammation and cholinesterase enzymes. These seaweeds significantly exhibited potent DPPH and moderate superoxide anion radical scavenging ability ($P<0.05$). Hexane and methanol extracts of *S. polycystum* exhibited the most potent radical scavenging ability with IC_{50} values of 0.157±0.004mg/ml and 0.849±0.02mg/ml for DPPH and ABTS assays, respectively. Hexane extract of *C. racemosa* gave the strongest superoxide radical inhibitory effect (IC_{50} of 0.386±0.01mg/ml). Most seaweed extracts significantly inhibited the production of cytokine (IL-6, IL-1 β, TNFα) and NO in a concentration-dependent manner without causing significant cytotoxicity to the lipopolysaccharide (LPS)-stimulated microglia cells ($P<0.05$). All extracts suppressed cytokine and NO level by more than 50% at the concentration of 0.4mg/ml. In addition, *C. racemosa* and *S. polycystum* also showed anti-acetylcholinesterase activities with the IC_{50} values ranging from 0.086-0.115 mg/ml. Moreover, *C. racemosa* and *P. australis* were also found to be active against butyrylcholinesterase with IC_{50} values ranging from 0.118-0.287 mg/ml.

***Keywords*—**Anticholinesterase, antioxidative, neuroinflammation, seaweeds.

Siti Aisya Gany and Swee Ching Tan are with the School of Postgraduate Studies, International Medical University, Jalan Jalil Perkasa 19, 57000 Kuala Lumpur, Malaysia (e-mail: aisyagany@gmail.com, sweeching_tan89@yahoo.com).

Sook Yee Gan is with School of Pharmacy, International Medical University, Jalan Jalil Perkasa 19, 57000 Kuala Lumpur, Malaysia (corresponding author: phone: 603-27317518; fax: 603-86567228; e-mail: sookyee_gan@imu.edu.my).

I. Introduction

ALZHEIMER disease (AD) is the most common type of dementia that effects the brain of elderlies at the age of 65 years and above. This disease has become a serious public health issue and it affects the quality of life of patients and their family members. Oxidative stress and reduced acetylcholine (ACh) levels have been implicated in AD pathology [1]. Enhancement of acetylcholine levels by acetylcholinesterase (AChE) inhibitor drugs to address the cholinergic deficit in AD patient's brain has proven to be the most viable therapeutic target for symptomatic improvement in AD. To date, donepezil and galanthamine are approved AChE inhibitors. However, these drugs can cause undesirable side effects and they are largely ineffective for treating severe AD cases [2].

The free radical and oxidative stress theories of aging suggest that oxidative damage is a major player in the degeneration of cells [3]. It is one of the earliest events in the pathogenesis of AD and oxidative markers were found to appear prior to β-amyloid deposition [4]. Hence, the anti-oxidative properties of natural products could be beneficial for the prevention and treatment of AD.

Microglial cells represent the first line of defense against invading pathogens or other types of brain tissue injury [5]. They are the immune effector cells in the central nervous system (CNS) and their activation by lipopolysaccharide (LPS) and β-amyloid could lead to detrimental effects such as release of neurotoxins, proinflammatory factors and reactive oxygen species (ROS) [6],[7]. Thus, regulating microglial activation may have therapeutic potential to reduce neuronal injury or death in AD.

Seaweeds are divided into three different classes, namely Rhodophyta (red), Phaeophyta (brown) or Chlorophyta (green). They are potential resources of bioactive compounds as they produce a great variety of secondary metabolites with a broad spectrum of biological activities [8]. The present study aimed to review the inhibitory activities of three Malaysian seaweeds (*Padina australis, Sargassum polycystum and Caulerpa racemosa*) against cholinesterase, oxidation and neuroinflammation.

II. Procedure

A. Preparation of Seaweed Extracts

Fresh green seaweed *C. racemosa* was collected from Cape Rachado, Port Dickson in May 2013 and fresh brown

seaweeds (*P. australis* and *S. polycystum*) were collected from Semporna, Sabah in June 2013. The seaweed samples were identified, authenticated and voucher specimens of each species were deposited as herbarium. The fresh samples were cleaned and dried. The dried samples were ground to fine powder and subjected to solvent extraction using *n*-hexane, followed by dichloromethane (DCM) and methanol at ambient temperature.

B. DPPH (1, 1-Diphenyl-2-Picrylhydrazyl) Free Radical Scavenging Assay

The antioxidant activity of seaweed extracts was measured on the basis of the scavenging activity of the stable 1, 1-diphenyl 2-picrylhyorazyl (DPPH) free radical according to the method described by Molyneux [9]. A total of 100µL of extract at various concentrations were added to 100µL of 0.2mM DPPH (Sigma-Aldrich, USA) in methanol. After 30 min of incubation at ambient temperature, the reduction of the DPPH free radical was measured by reading the absorbance at 520 nm using a microplate reader (Infinite F200, Tecan). Ascorbic acid (Sigma-Aldrich, USA) was used as positive control. The inhibitory activity was calculated in percentage as: % inhibition= [(absorbance of control-absorbance of sample)/absorbance of control] ×100.

C. ABTS (2, 2'-Azino-Bis (3-Ethylbenzthiazoline-6-Sulfonic Acid) Radical Scavenging Assay

The determination of antioxidant activity of seaweed extracts based on the ABTS radical scavenging activity was conducted according to Re *et al.* [10]. ABTS (Sigma-Aldrich, USA) at 14mM was mixed with an equal volume of 4.95mM potassium persulphate (Sigma-Aldrich, USA). The mixture was incubated at ambient temperature in the dark for 17 hrs. The ABTS radical solution was then adjusted with phosphate buffered saline, PBS (MP Biomedicals, France) to an absorbance of 0.7 ± 0.02 at 734nm. Various concentrations of extracts in PBS (20µl) were mixed with 180µL of ABTS radical solution. The mixture was incubated for 10 min at ambient temperature and measured at 734nm. Ascorbic acid was used as positive control. The inhibitory activity was calculated as described for DPPH assay (B).

D. Superoxide Anion Radical Scavenging Activity

The determination of superoxide scavenging activity of the seaweed samples was conducted as described by Hsia-Yin *et al.* [11]. The reaction mixture consisted of 50µl extract at various concentrations in PBS, 50µL of 300 µM nitrobluetetrazolium (NBT, Sigma-Aldrich, USA), 50µL of 936µM NADH and 50µL of 120µM phenazine methosulphate (PMS, Sigma-Aldrich, USA). The mixture was incubated at ambient temperature for 5 min. The activity was measured at 560nm. Ascorbic acid was used as positive control. The inhibitory activity was calculated as previously described.

E. Cholinesterase Inhibition Assays

Cholinesterase inhibitory activities of seaweed extracts at various concentrations (0.0125, 0.025, 0.05, 0.1 and 0.2 mg/ml) were determined using the Ellman's colorimetric method [12]. The reaction mixture contained: 140µl of 0.1M PBS (pH8.0), 20µl of algal extracts and 20µl of either acetylcholinesterase (AChE) or butyrylcholinesterase (BChE) solution (Sigma-Aldrich, USA). The mixture was incubated for 15 min at room temperature. Thereafter, 10µl of 0.5 mM DTNB was added and the reaction was initiated with the addition of 10µl of 0.5 mM acetylthiocholine iodide (ATCI) or butyrylthiocholine iodide (BTCI). The hydrolysis of ATCI or BTCI was monitored by following the formation of yellow 5-thio-2-nitrobenzoate anion at 405 nm using microplate reader (Infinite F200, Tecan). Galanthamine served as positive control. The percentage of AChE/BChE inhibition was calculated using the formula (E-S)/E x 100, where E is the absorbance of blank (without test sample) while S is the absorbance of test wells with sample extracts.

F. Cytotoxicity Assay

The C8-B4 microglia cells (ATCC® CRL-2540) were cultured in Dulbecco's modified Eagle medium(DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100µg/ml streptomycin. The culture was maintained in a humidified incubator with 5% CO_2. Approximately 2 × 10^4/ 200 µL/ well of microglia cells were treated with various concentrations of seaweed extracts (0.05, 0.1, 0.2 or 0.4 mg/ml) or lipopolysaccharide [(LPS 1µg/ml) *Escherichia coli* serotype 055:B5, Sigma-Aldrich, USA] in serum-free DMEM. The culture was incubated for 24 hrs prior to the addition of 20µl 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (0.5 mg/ml MTT, Merck, USA). After incubation for 3 hours at 37 °C and 5% CO_2, the supernatant was removed and DMSO (100µl) was added to each well to dissolve the purple crystal (formazan). Absorbance was determined at a test wavelength of 570 nm and a reference wavelength of 630 nm using a microplate reader.

G. Measurement of Cytokines, PGE_2 and NO Level

Microglial cells (2 × 10^4/200 μL/well in a 96-well plate) were pre-treated with seaweed extracts for 30 min and stimulated with LPS (1 µg/mL). The supernatants of the cultured microglia were collected 24 hrs after LPS stimulation, and the concentrations of TNF-α, IL-1β, IL-6 and PGE_2 were measured by enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies as described by the manufacturer's instruction (R&D systems, MN, USA). Accumulated nitrite was measured in the cell supernatant using the Griess reagent assay (R&D systems, MN, USA). The conditions of cell culture and treatment were similar to those reported for ELISA.

H. Statistical Analysis

Values of all experiments were represented as mean ± SEM of more than one experiment. Values were compared using one way ANOVA with Tukey's test (multiple comparisons). The level of significance was set at $P < 0.05$.

III. Results

A. Antioxidative Activity of Seaweed Extracts

Three commonly used antioxidant evaluation methods, namely DPPH radical scavenging activity, ABTS radical scavenging activity and superoxide anion radical scavenging activity were used to determine the antioxidant capacity of *P. australis*, *S. polycystum* and *C. racemosa*. The IC_{50} value obtained for each extract in respective assay is shown in Table I.

All of the seaweed extracts reduced DPPH radicals significantly ($P<0.05$). The antioxidant activity recorded for each extract was dose-dependent; with the highest concentration showing the highest activity. Based on the DPPH assay, SH has the lowest IC_{50} (0.157±0.004mg/ml) followed by PD, SD and CH (Table I). CM exhibited the least potent antioxidant activity by only scavenging 50% of DPPH radical at 0.7087 ± 0.02 mg/ml. The seaweed extracts showed lower inhibition activities against ABTS radicals compared to DPPH radicals. All extracts inhibited ABTS radicals in a dose-dependent manner ($P<0.05$). SM showed the strongest ABTS radical scavenging activity with an IC_{50} value of 0.849 ±0.02 mg/ml, followed by CM and PM. Presence of the extracts also caused significant decrease of the superoxide anion radical activity in a dose dependent manner ($P<0.05$). As shown in Table I, the highest activity was recorded for CH with an IC_{50} value of 0.386 ± 0.01mg/ml followed by CD and SH.

TABLE I
Antioxidative Activities of Seaweed Extracts

Samples	IC_{50} (mg/ml)		
	DPPH	$ABTS^+$	O_2^-
Standard			
Ascorbic acid	0.0031±0.00	0.0365±0.0	0.0091±0.0
P. australis			
Dichloromethane (PD)	0.276±0.01	>1.4	0.458±0.01
Methanol (PM)	0.649±0.03	1.392±0.01	0.873±0.03
S. polycystum			
Hexane (SH)	0.157±0.004	>1.4	0.426±0.01
Dichloromethane (SD)	0.337±0.01	>1.4	0.57±0.03
Methanol (SM)	0.611±0.02	0.849±0.02	0.498±0.01
C. racemosa			
Hexane (CH)	0.476±0.01	>1.4	0.386±0.01
Dichloromethane (CD)	0.541±0.03	> 1.4	0.408±0.01
Methanol (CM)	0.709±0.02	1.31±0.04	>1

Data are expressed as the mean ± standard deviation (SD) of 2 independent studies of three replicates.

B. Acetyl- and Butyrylcholinesterase Inhibitory Activities of Seaweed Extracts

All the seaweed extracts showed moderate to good inhibitory effects on AChE and BChE in a dose-dependent manner. At the highest tested concentration (0.2 mg/ml), most of the extracts showed inhibition greater than 50% for AChE while for BChE only two extracts, namely CM and CH recorded inhibition over 50% with values of 80.41±1.09 and 57.89±0.26%, respectively. The highest AChE inhibition activity at 0.2mg/ml of extracts was recorded for CM (87.98±0.75%), followed by SH and CH. Among all the extracts tested, PM (0.2mg/ml) had the lowest inhibition activity for both AChE and BChE with values of 49.7±0.98% and 11.03±1.43% respectively. The IC_{50} values of the extracts for each respective enzyme inhibition assay are recorded in Table II. For AChE, CH extract had the lowest IC_{50} value of 0.086±0.012 mg/ml, whereas for BChE, CM extract recorded the lowest IC_{50} value of 0.118±0.041 mg/ml.

C. Effects of Seaweed Extracts on Cell Viability

The effect of seaweed extracts and LPS on the cell viability of microglial cell is shown in Fig. 1. These tested samples did not demonstrate any toxicity at the concentrations tested (0.05, 0.1, 0.2, 0.4 mg/ml).

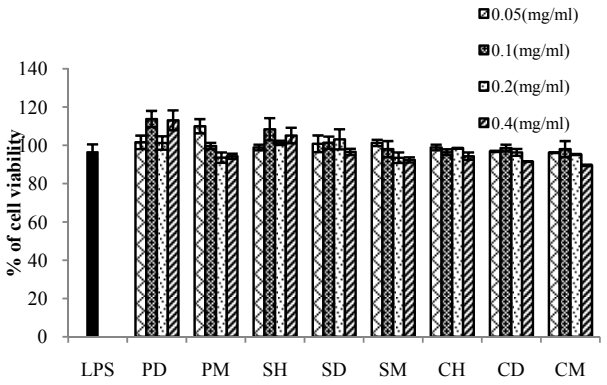

Fig. 1 Effect of seaweed extracts on cell viability of microglial cell. Cells were treated with extracts at indicated concentrations or 1 μg/ml LPS for 24 hrs. Cell viability was examined with MTT assay. Results were expressed as the percentage of surviving cells relative to control cells and presented as mean ± SD of three independent experiments; each was performed in triplicate. Cell viability of treated cells was compared to the respective control sample (cell treated with same amount of vehicle). '∗' indicates $P < 0.05$

D. Effects of Seaweed Extracts on NO Production in LPS-Stimulated Microglial Cells

The LPS-induced production of nitrite decreased with increased concentrations of seaweed extracts (Fig. 2). At 0.4mg/ml, PD and PM caused the most significant reduction of NO level ($P<0.05$) by 73.63±3.81% and 75.67 ±0.21%, respectively. However, at 0.4mg/ml, CH and CD only reduced the NO level by less than 50%. Pretreatment of cells with the highest concentration (Control: 0.4mg/ml) of seaweed extracts for 24 hrs without any exposure to LPS did not induce NO production.

TABLE II
ACETYLCHOLINESTERASE AND BUTYRYLCHOLINESTERASE INHIBITORY ACTIVITIES OF SEAWEED EXTRACTS

Samples	IC_{50} (mg/ml)	
	AChE	BChE
Standard		
Galanthamine	$1.128 \times 10^{-4} \pm 0.01$	$1.248 \times 10^{-3} \pm 0.02$
P. australis		
Dichloromethane (PD)	0.149±0.046	>0.2
Methanol (PM)	>0.2	>0.2
S. polycystum		
Hexane (SH)	0.115±0.025	>0.2
Dichloromethane (SD)	0.180±0.036	>0.2
Methanol (SM)	0.162±0.017	>0.2
C. racemosa		
Hexane (CH)	0.086±0.012	0.156±0.037
Dichloromethane (CD)	0.089±0.036	>0.2
Methanol (CM)	0.095±0.028	0.118±0.041

Data are expressed as the mean ± standard deviation (SD) 3 independent studies in three replicates

E. Effects of Seaweed Extracts on PGE_2 Production in LPS-Stimulated Microglia Cells

As shown in Fig. 3, pretreatment of cells with the highest concentration (Control: 0.4mg/ml) of seaweed extract for 24 hrs without any exposure to LPS did not induce PGE_2 production. At the lowest concentration (0.05mg/ml), all extracts did not suppress the LPS-induced PGE_2 production significantly except for PM (13.07±0.1%) ($P<0.05$). Presence of seaweed extracts resulted in moderate inhibition of secreted PGE_2. The inhibition recorded for the highest extract concentration (0.4mg/ml) ranged from 20-30%.

F. Effects of Seaweed Extracts on TNF-α, IL-1β and IL-6 Production in LPS-Induced Microglial Cells

The levels of all cytokines were increased in the culture media of LPS-induced microglia. The production of these cytokines was inhibited significantly in a concentration-dependent manner when pretreated with seaweed extracts (Fig. 4). As shown in Figs. 4 (a)-(c) *P. australis* methanol extracts displayed the most potent inhibitory activities by inhibiting 94.45±1.92%, 89.82±1.3% and 92.07±1.99% of TNF-α, IL-1β and IL-6 production, respectively. Pretreatment of cells with the highest concentration (Control: 0.4mg/ml) of seaweed extracts for 24 hrs without any exposure to LPS did not induce cytokine production.

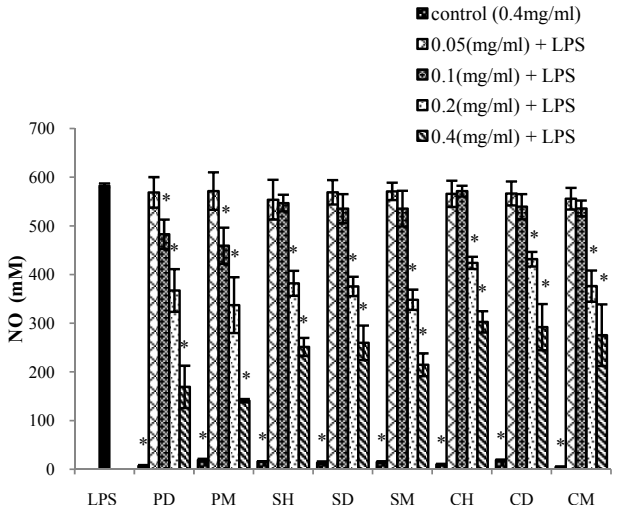

Fig. 2 Effect of seaweed extracts on NO production in LPS-induced microglial cells. The Griess reagent assay was carried out to measure the production of nitrite. Data are presented as mean ± SD of two independent experiments performed in triplicate. The NO level of each treatment was compared with the LPS treated control group. '∗' indicates $P < 0.05$

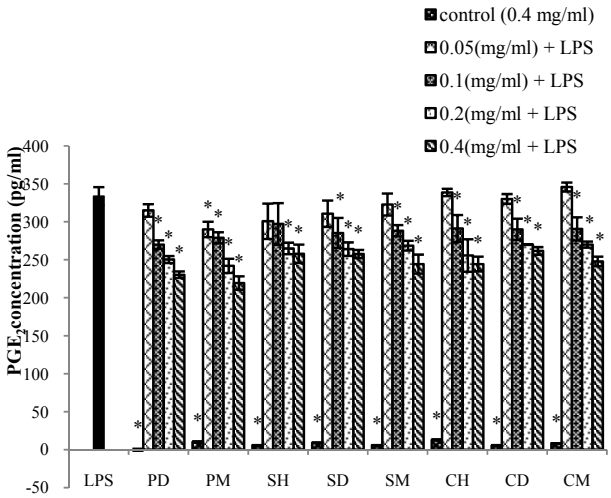

Fig. 3 Effects of seaweed extracts on LPS-induced PGE_2 production in microglial cells. The amount of secreted PGE_2 was measured after simultaneous 24 hours treatment with 1μg/ml LPS and seaweed extracts. Data are represented as mean ± SD of two independent experiments performed in triplicate. The PGE_2 level of each treatment was compared with the LPS treated control group. '∗' indicates $P < 0.05$

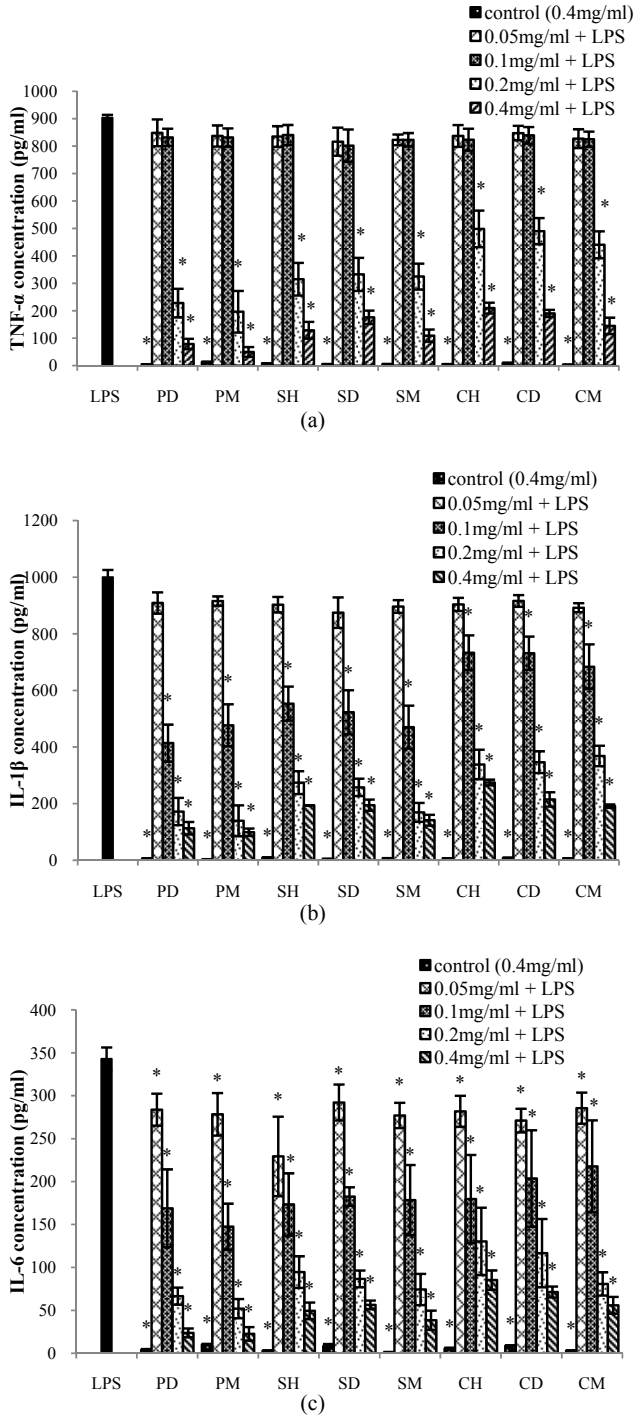

Fig. 4 Effects of seaweed extracts on the production of TNF-α (a), IL-1β (b) and IL-6(c) in LPS-induced microglial cells. The amount of secreted cytokines was measured after simultaneous 24 hours treatment with 1μg/ml LPS and seaweed extracts. Cytokines concentration was measured in culture media using a commercial ELISA kit. Date is represented as mean ± SD of two independent experiments performed in triplicate. The cytokine levels of each treatment were compared with the LPS treated control group. '∗' indicates $P < 0.05$

IV. Discussion

The central nervous system is vulnerable to free radical and oxidative damage due to the high oxygen consumption [13] that leads to high generation of ROS such as superoxide anion radical, hydrogen peroxide, hydroxyl radical and peroxyl radicals subsequently results in oxidative stress [14],[15]. Active antioxidants are used to scavenge this harmful radical. In the present study, the brown seaweeds, *S. polycystum* and *P. australis* exhibited a stronger DPPH radical scavenging activity compared to the green seaweed, *C. racemosa*. It was suggested that the high DPPH radical scavenging activity of brown seaweeds was due to high amounts of polyphenols [16], [17]. In addition, radical scavenging activities were reported to be associated with the total phenolic content in *Sargassum* sp. [18]-[20], *Padina* sp. [21], [22] and *Caulerpa* sp. [23], [24]. The non-polar extracts of each seaweed showed better DPPH scavenging activity and the activity got weaker as the polarity of the solvent extracts increased. Altogether, hexane extract of *S. polycystum* gave the most potent DPPH radical scavenging activity compared to other seaweed extracts. According to a study done by Sanaa, the high antioxidant activity demonstrated by *Sargassum dentifolium* and *Laurencia papillosa* of non-polar extracts might be attributed to the high content of the lipid soluble total chlorophylls and high content of total carotenoids of the seaweeds [25]. On the contrary, methanol extract of the seaweeds, particularly *S. polycystum*, was the most potent ABTS radical scavenger compared to the other solvent extracts. However, the scavenging activities of ABTS radical by the seaweed extracts were weaker compared to their DPPH scavenging activities. Even though both assays used the same mechanisms in the radical-antioxidant reactions, it is still difficult to compare antioxidant activity based on antioxidant assays because of the different test systems used [26]. Movahedinia and Heydari also reported that *S. tenerrimum* and *Gracilaria corticata* exhibited strong DPPH radical scavenging activity, but did not inhibit ABTS radicals [27]. It was also observed in the present study that *C. racemosa* has stronger superoxide anion radical scavenging ability compared to both *S. polycystum* and *P. australis*. Similar activity displayed in a study by Yangthong *et al*. that showed *Caulerpa* species exhibited stronger superoxide anion radical scavenging activity compared to *Sargassum* species [28]. These observations may be due to the presence of high level of superoxide dismutase (SOD) in *C. racemosa* [29]. In a biological system, toxicity of superoxide radicals can be eliminated by this enzyme which degrades superoxide.

Acetylcholine is the most important neurotransmitter involved in the regulation of cognitive functions [30]. AChE inhibitors boost the endogenous levels of acetylcholine in the brain and thus increasing cholinergic transmission, whereas, BChE inhibitors believed to decrease neuritic plaques in the brain [31]. Cholinesterase inhibitors are the standard therapeutic approach in treating AD [32]. Dual anti-cholinergic activity (AChE and BChE) was observed for hexane and methanol extract of *C. racemosa*, which is an important finding in this study. Plant extracts which have dual anticholinergic activity, is deemed appropriate to treat patients

in moderate stage of AD [33]. The IC_{50} values recorded for AChE inhibition activity ranged from 0.086±0.012 to 0.210±0.019 mg/ml. As for BChE inhibition activity, the IC_{50} values ranged from 0.118±0.041 to 0.629±0.020 mg/ml (Table II). Although IC_{50} values of BChE inhibition is generally higher than that observed for AChE, BChE inhibition is still an advantage as it is regarded as a valid approach to restore cholinergic functions in AD [34]. Extracts of *C. racemosa* had the lowest IC_{50} values against both AChE (0.086 mg/ml) and BChE (0.118 mg/ml). In another report, AChE inhibition activities were also recorded for other species of *Sargassum* (*S. boveanum* and *S. oligocystum)* and *P. australis* [35].

Excessive production of inflammatory mediators and cytokines from activated microglia contributes to uncontrolled inflammation in neurodegenerative diseases [36]. In this study, all extracts of *P. australis, S. polycystum* and *C. racemosa* inhibited the production of NO, TNF-α, IL-β and IL-6. At 0.4mg/ml, the extracts inhibited more than 70% of TNF-α, IL-1β and IL-6. Although the reduction of PGE_2 level was not as dramatic as what was observed for cytokines and NO level, the seaweed extracts reduced the level of PGE_2 dose-dependently. However, at the highest tested concentration, these extracts could only reduce approximately 20-30% of PGE_2 level. High levels of NO are produced from L-arginine by inducible nitric oxide synthase (iNOS) enzyme whereas; PGE_2 are generated from arachidonic acid *via* the action of cyclooxygenase (COXs) in prolonged activation of microglial cells [37]-[39]. Activation of the transcription factor, nuclear factor-kappaB (NF-κB) also plays a critical role in the induction of iNOS and COX-2 by LPS stimulation as well as coordinates the expression of cytokines, including TNF-α, IL-1β, and IL-6 [40], [41]. Previous studies have proven that seaweed extracts had reduced cytokine level *via* the suppression of NF-κB pathway and reduction of NO and PGE_2 level *via* suppression of iNOS and COX-2 expression, respectively [42]-[45].

Based on the observations of this study, the brown seaweeds (*Sargassum* and *Padina*) exhibited a stronger activity compared to the green seaweed, *Caulerpa*. It was reported that fucoidan, a sulfated polysaccharide which could be isolated from *Padina* and *Sargassum* [46], [47], was able to reduce the production of NO and PGE_2 in LPS-induced BV2 microglia. It also attenuated expression of iNOS, COX-2, and proinflammatory cytokines, IL-1β and TNF-α [48], [49]. Furthermore, fucoxanthin (carotenoids), pheophytin A and pheophorbide A (chlorophyll derivatives) were identified as the key compounds responsible for the seaweed's anti-inflammatory activity in many studies [50]-[52]. Cytokines and proinflammatory mediator generation have become therapeutic targets in neurodegenerative diseases, and thus their down-regulation might assist in preventing or delaying the onset of AD.

V. Conclusion

The three seaweeds, namely *P. australis, S. polycystum* and *C. racemosa* exhibited antioxidative, anticholinesterase as well as anti-neuroinflammatory properties. These seaweeds showed potential applications for the prevention of neurodegenerative diseases.

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