

Cholinesterase inhibitory activities of neuroprotective fraction derived from red alga *Gracilaria manilaensis*

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Abstract

Anti-cholinesterase (ChE)s are commonly prescribed as the symptomatic treatment of Alzheimer's disease. They are applied to prevent the breakdown of neurotransmitter acetylcholine (ACh) that bind to muscarinic and nicotinic receptors in the synaptic cleft. Seaweeds are one of the richest sources of bioactive compounds for both nutraceuticals and pharmacognosy applications. This study aimed to determine the anti-ChEs activity of *Gracilaria manilaensis*, one of the red seaweeds notables for its economic importance as food and raw materials for agar production. Methanol extracts (GMM) of *G. manilaensis* were prepared through maceration, and further purified with column chromatography into a semi-pure fraction. Ellman assay was carried out to determine the anti-acetylcholinesterase (AChE) and anti-butyrylcholinesterase (BuChE) activities of extracts and fractions. Lineweaver-Burk plot analysis was carried out to determine the inhibition kinetic of potent extract and fraction. Major compound(s) from the most potent fraction was determined by liquid chromatography-mass spectrometry (LCMS). GMM and fraction G (GMMG) showed significant inhibitory activity AChE with EC_{50} of 2.6 mg/mL and 2.3 mg/mL respectively. GMM and GMMG exhibit mixed-inhibition and uncompetitive inhibition respectively against AChE. GMMG possesses neuroprotective compounds such as cynerine A, graveoline, militarinone A, eplerenone and curumenol. These findings showed a promising insight of *G. manilaensis* to be served as a nutraceutical for neuronal health care in the future.

Keywords: *Gracilaria manilaensis*, Alzheimer's disease, Graveoline, Mixed-inhibition, Uncompetitive inhibition, Cholinesterases

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Introduction

Acetylcholine (ACh) is the major neurotransmitter synthesized from acetyl CoA and choline, by choline acetyltransferase (ChAT) in the cytoplasm (Ferreira-Vieira et al., 2016). It is uptaken by the vesicular acetylcholine transporter (VACHT) into the synaptic vesicles to convey a synaptic signal to the postsynaptic neurons through its binding to nicotinic ACh receptors (nAChR) or muscarinic ACh receptors (mAChR) (Silverthorn et al., 2016). In the synaptic cleft, ACh is promptly hydrolyzed to acetate and choline through the catalysis of acetylcholinesterase (AChE) enzyme and recycled into the presynaptic neuron (Colovic et al., 2013). Alzheimer's disease damages the brain cells that produce or use ACh, thereby reducing the amount of neurotransmitters available to carry neurological signals; whereas the amount of ChAT, nAChR, and mAChR are also abnormally downregulated in Alzheimer's patients (Kihara & Shimohama, 2004; Lombardo & Maskos, 2015). Furthermore, the natural process of ACh metabolism facilitated by AChE further reduces the amount of ACh and weakens the transportation of electrical impulses in the cholinergic system (Hung & Fu, 2017). This results in impairments in motor function, memory and attention in patients.

While there is currently no cure for Alzheimer's, clinicians have applied cholinesterases inhibitors (ChEIs) to prevent the hydrolysis of ACh by cholinesterases (ChEs) (Hung & Fu, 2017). The drugs indirectly aid in maintaining the ACh level that is important for learning and memory, and compensating the loss of functioning brain cells; thus, they enhance the symptoms related to memory thinking, language, judgment and other cognitive processes (Alzheimer's Association Report, 2021). To date, three out of five U.S. Food and Drug Administration (FDA) approved symptomatic medications for Alzheimer's disease are ChEIs, namely Aricept (donepezil), Exelon (rivastigmine), and Razdyne (galantamine) (Alzheimer's Association Report, 2021). Certain ChEIs seems to offer other benefits, for example, galantamine appears to stimulate the release of ACh and improve the way certain message-receiving neurons respond to it (Alzheimer's Association Report, 2021); while rivastigmine is able to intensify the cholinergic activity by providing dual inhibition of AChE and butyrylcholinesterase (BuChE), which further extend its usage in subcortical vascular and Parkinson's disease dementia (Kandiah et al., 2017). Besides, Donepezil was reported with several beneficial impacts on behavioral and neuropsychiatric symptoms, such as hallucinations, distractibility, aberrant motor behavior and apathy (Tsuno, 2009). Despite a small degree of improvement, clinical trials of all ChEIs

are overall showing enhanced memory and thinking compared to placebo, as well as delay or slow worsening of Alzheimer's disease symptoms in up to 40% of individuals who taken the medication (Mielke et al., 2012). Despite all the benefits above, ChEIs do not reverse Alzheimer's nor stop the underlying degeneration of nerve cells. In fact, there is still risk of side effects that are unavoidable such as nausea, vomiting, gastrointestinal bleeding, bronchospasm and skin patches (Ruangritchankul et al., 2021). Knowing that nearly 10 million dementia patients are increasing every year (World Health Organisation, 2021) and the effect of current drugs are still in a large space to be improved, growing demand of getting better ChEIs with greater effect and lower side effects are gradually catching up, which has led us to explore any possibilities from the novel resources such as marine algae.

Consumption of algae has been revealed to prevent neurodegeneration. Studies showed that a higher seaweed intake in the Japanese diet and other East Asia countries compared to the European countries poised a lower neurodegenerative disorder risk (Okubo et al., 2017; Pangestuti & Kim, 2011). In Malaysia, red seaweed *Gracilaria* is commonly used in traditional dishes for the Malay natives along the east coast of Peninsular Malaysia as Kelantanese seaweed salad named 'Kerabu Sare'. *Gracilaria* species including *Gracilaria changii*, *Gracilaria firma*, and *G. manilaensis* Yamamoto & Trono are potential agarophytic species that provide raw materials for agar production in the food industry and as a food source for shellfish and shrimp (Ng et al., 2017; Phang et al., 2019; Yow et al., 2013). Current findings on *G. manilaensis* showed that it possesses various bioactive compounds with potential therapeutic effects. This includes hexadecanoic acid and 2-hydroxymyristic acid that portray anticancer property, 1-(4'-methoxyphenyl)-3-(2,4,6-trihydroxyphenyl)-3-hydroxypropanone that portrays antioxidant property, as well as cholesteryl myristate that portrays antibacterial property (Andriani et al., 2016). *G. manilaensis* from Malaysia also possesses the neurotogenic effect and *in vitro* antioxidant activities (Pang et al., 2018). The proven neurological benefits have driven our interest to further investigate the anti-AChE activity of *G. manilaensis* and to determine the bioactive substance(s) that is responsible for this activity.

Materials and Methods

Specimen collection and processing

Fresh specimens of *G. manilaensis* were collected from Kuala Muda, Kedah. The specimens were washed with salt water to re-

move sands, mud and impurities, followed by the final rinse in distilled water and later froze. The frozen specimens were then freeze dried (Labogene, Germany) and pulverised. *G. manilaensis* powder was kept at -20°C prior to extracts preparation.

G. manilaensis extracts preparation

G. manilaensis extracts were prepared from the powder by maceration as described in Pang et al. (2018). Briefly, 1 g of seaweed powder was dissolved into hot boiling water, methanol and ethanol at the ratio of 1:50 (w/v) respectively. For hot aqueous extract (GMHW), the mixture was incubated in roller mixer for 30 min at room temperature. The mixture was centrifuged at $2,688\times g$ for 20 min at 4°C . After that, the supernatant was frozen and concentrated with a vacuum concentrator (Labogene, Germany). The dried product, GMHW was stored at -20°C until used. Meanwhile, for methanol and ethanol extracts (GMM and GME), the solvent-powder mixtures were incubated in an incubator shaker at $9\times g$ for 36–48 h at room temperature. The mixture was centrifuged at $2,688\times g$ for 20 min at 4°C . Subsequently, the supernatants were further concentrated using a rotary evaporator and vacuum concentrator. Both extracts were stored at -20°C until used.

In vitro anti-cholinesterase assay

Ellman assay was used to determine the AChE and BuChE activities using the method described by Yoon et al. (2009) with slight modification. Each reaction was scaled to 200 μL reaction in a 96-well microplate. To prepare GMHW, distilled water was used as the vehicle. For GMM and the positive controls (eserine and isoxazole), 10% aqueous methanol was used as the vehicle. Meanwhile, 10% aqueous ethanol was used to dissolve GME. Briefly, 20 μL of the sample was added into 140 μL of sodium phosphate buffer (pH 8.0) and 20 μL of AChE (0.036 U/mL, C2888, Sigma-Aldrich, St. Louis, MO, USA) or BuChE (0.036 U/mL, C1057, Sigma-Aldrich). After 15 min of incubation, 20 μL of substrate ACh or BuCh (3 mM, Sigma-Aldrich) and 20 μL of 5-5'-thiobis-2-nitrobenzoic acid (DTNB, 5 mM, Sigma-Aldrich) were added into the reaction mixture. The absorbance of each reaction mixture was read at 412 nm at 37°C by the kinetic loop of 3 min intervals for 15 min. The rate of reaction was obtained by the slope of absorbance against the time in minutes. The unit was expressed in absorbance/min. The AChE or BuChE inhibitory activity (%) was obtained by the formula:

$$\text{AChE or BuChE inhibitory activity \%}$$

$$= \left(1 - \frac{\text{Rate of reaction of samples}}{\text{Rate of reaction of the negative control}} \right) \times 100\%$$

The half-maximum effective concentration (EC_{50}) values of the positive controls and the extracts were measured by adapting from the dose-depending relationship curve of AChE or BuChE activity % against the concentration of respective treatment. Three independent experiments were carried out to obtain the data in mean \pm SD.

Enzyme kinetic study

Enzyme kinetic study was also carried out using the method described by Yoon et al. (2009) with slight modification. The concentrations of GMM and GMMG were fixed at their respective EC_{25} or EC_{50} . Substrates of ascending concentration from 3, 6, 9, 12, and 15 mM were added after 15 min of incubation. The reciprocal value of the rate of reaction ($1/V$) and the reciprocal value of the substrate concentration ($1/[S]$ mM) was used to plot the line-weaver Burk plot. The values of enzyme activity (K_m , mM), the maximum rate of reaction (V_{max} , Absorbance/min) and inhibition constant (K_i , mg/mM) were obtained by three independent experiments. These experiments were repeated for its quarter-maximum EC_{25} .

Isolation of active fractions

GMM was selected as the most potent extract to be further investigated in this study. To produce fractions, 200 g powder was extracted with 2 L of absolute methanol for five times. After the removal of powder by filtration, approximate 10 g of GMM was concentrated with a rotary evaporator, and then dissolved in absolute chloroform. The GMM (10 g) was subjected to column chromatography over silica gel 60, elution successively with hexane-chloroform (4:6–1:9), chloroform, chloroform-methanol (99:1–90:1) and ethanol to produce 10 main fractions based on thin-layer chromatography (TLC) profile: GMMA (75.1 mg), GMMB (46.6 mg), GMMC (41.9 mg), GMMD (129.6 mg), GMME (97.6 mg), GMMF (283.4 mg), GMMG (272.6 mg), GMMH (85.6 mg), GMMI (339.2 mg), and GMMJ (1,227.3 mg).

Liquid chromatography-mass spectrometry (LCMS) analysis

GMMG was subjected to LCMS analysis with Agilent 1290 Infinity LC system coupled to Agilent 6520 Accurate-Mass Q-TOF mass spectrometer with dual ESI source (Agilent Technologies, Santa Clara, CA, USA). The column used was Agilent

Zorbax Eclipse XDB-C18, Narrow-Bore 2.1 × 150 mm, 3.5 micron (P/N: 930990-902) with mobile phase 0.1% formic acid in water and 0.1% formic acid in acetonitrile. 1 µL of sample (1 mg/mL) was injected at the flow rate of 0.5 mL/min. The result of chromatogram data was analysed with Agilent MassHunter Qualitative Analysis B.07.00 (Method: Metabolomic-2019.m) matched with Metiln database based on mass over charge (m/z). Compounds were shortlisted by elimination of blank, include all the compounds with Score Db or Score MFG ≥ 90, and with Difference (Db.ppm) or Difference (MFG.ppm) that is ± 5. Activities of the known compound were matched with publication and database on Web of Science and PubChem.

Statistical analysis

The means of data were subjected to one-way analysis of variance (ANOVA). Dunnett and Tukey Post Hoc were implemented to test for the significant difference between extracts. All the analysis was done by Graph Pad Prism ver. 7.

Results

ChEs inhibitory activities of *G. manilaensis*

All *G. manilaensis* extracts possessed anti-AChE activity in majority of the tested concentrations (Fig. 1). Meanwhile, none of the extracts but isoxazole showed significant anti-BuChE activ-

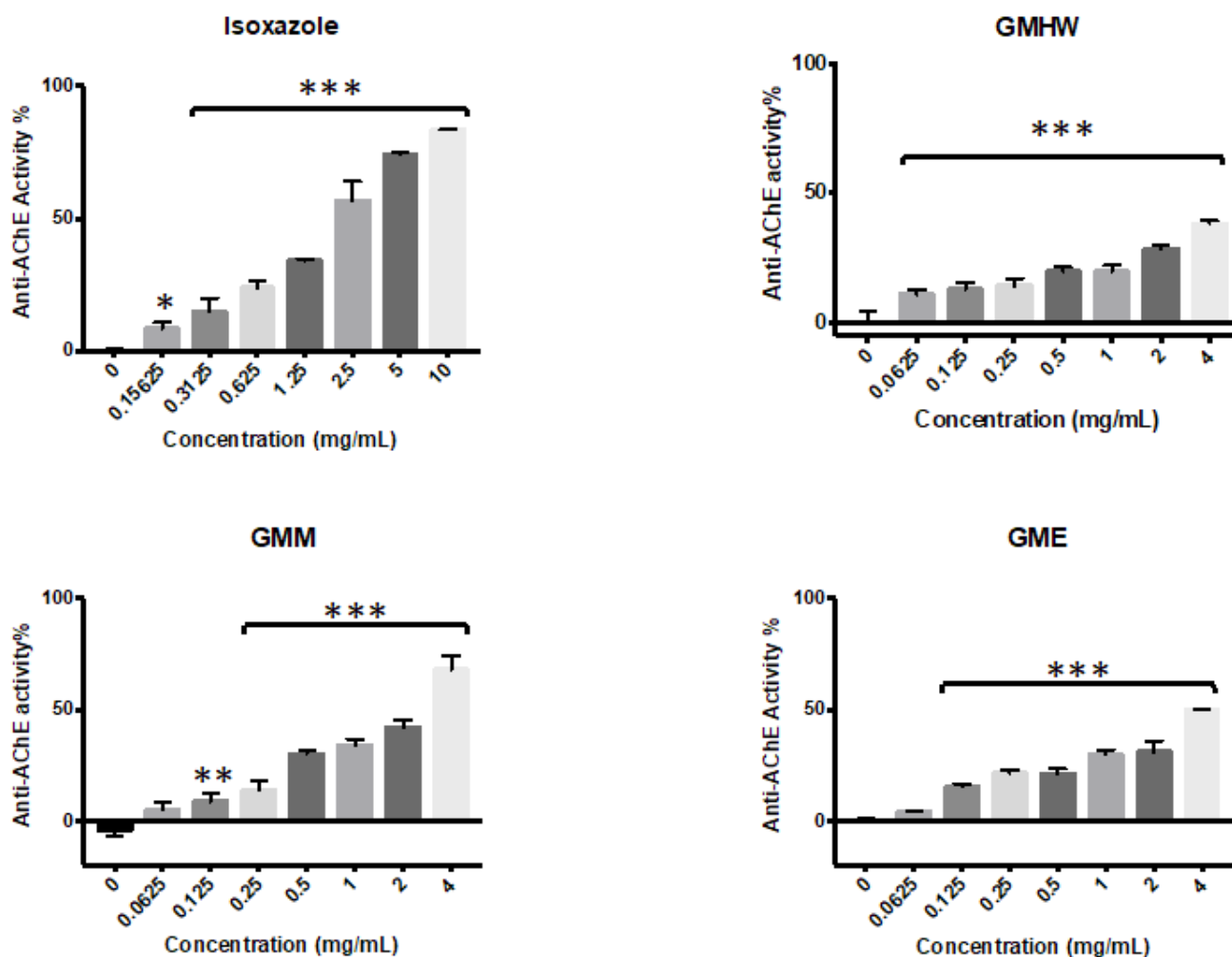


Fig. 1. AChE inhibition activity of *Gracilaria manilaensis* extracts and the positive controls. Data are expressed in mean ± SD (n = 3). Arterisk symbol indicates significant differences (0 mg/mL) (* p < 0.05, ** p < 0.01, *** p < 0.005; Dunnett) against the negative control. Isoxazole was used as the positive control. AChE, acetylcholinesterase; GMHW, hot water extract; GMM, methanol extract; GME, ethanol extract.

ity ($p < 0.05$) (Fig. 2). GMHW produced significant anti-AChE activity of $10.98 \pm 1.67\%$ at concentration as low as 0.0625 mg/mL ($p < 0.005$). The highest activity of GMHW observed was $38.23 \pm 1.05\%$ at 4 mg/mL . On the other hand, GMM and GME only showed significant anti-AChE activity at 0.125 mg/mL , with $8.62 \pm 3.73\%$ and $15.21 \pm 1.28\%$ respectively ($p < 0.01$ and $p < 0.005$). However, anti-AChE activity of GMM and GME observed a rapid increment from 2 mg/mL to 4 mg/mL , which resulted in a higher anti-AChE activity of $69.3 \pm 7.16\%$ and $49.94 \pm 0.23\%$ at 4 mg/mL respectively. Since GMM was the only extract that showed anti-AChE activity higher than 50% at 4 mg/mL , it was selected for the downstream fractionation process.

GMM was fractionated into 10 fractions according to the TLC profile (Fig. 3). All fractions except GMMD possessed significant anti-AChE activity ($p < 0.05$) against the negative control (Fig. 4). GMMA and GMME showed the lowest anti-AChE activity at $33.8 \pm 4.6\%$ and $37.1 \pm 3.8\%$ respectively. Meanwhile, GMMF, GMMH, and GMMJ showed moderate activity at $43.4 \pm 7.7\%$, $45.3 \pm 1.5\%$ and $41.7 \pm 1.9\%$ respectively. Furthermore, GMMB, GMMC, and GMMI showed moderate-high anti-AChE activity at $54.4 \pm 4.8\%$, 62.0 ± 1.1 and $53.6 \pm 1.5\%$ respectively. GMMG showed the highest anti-AChE activity at $69.2 \pm 5.1\%$; thus, GMMG was selected for subsequent assay of cholinesterase inhibitory activities.

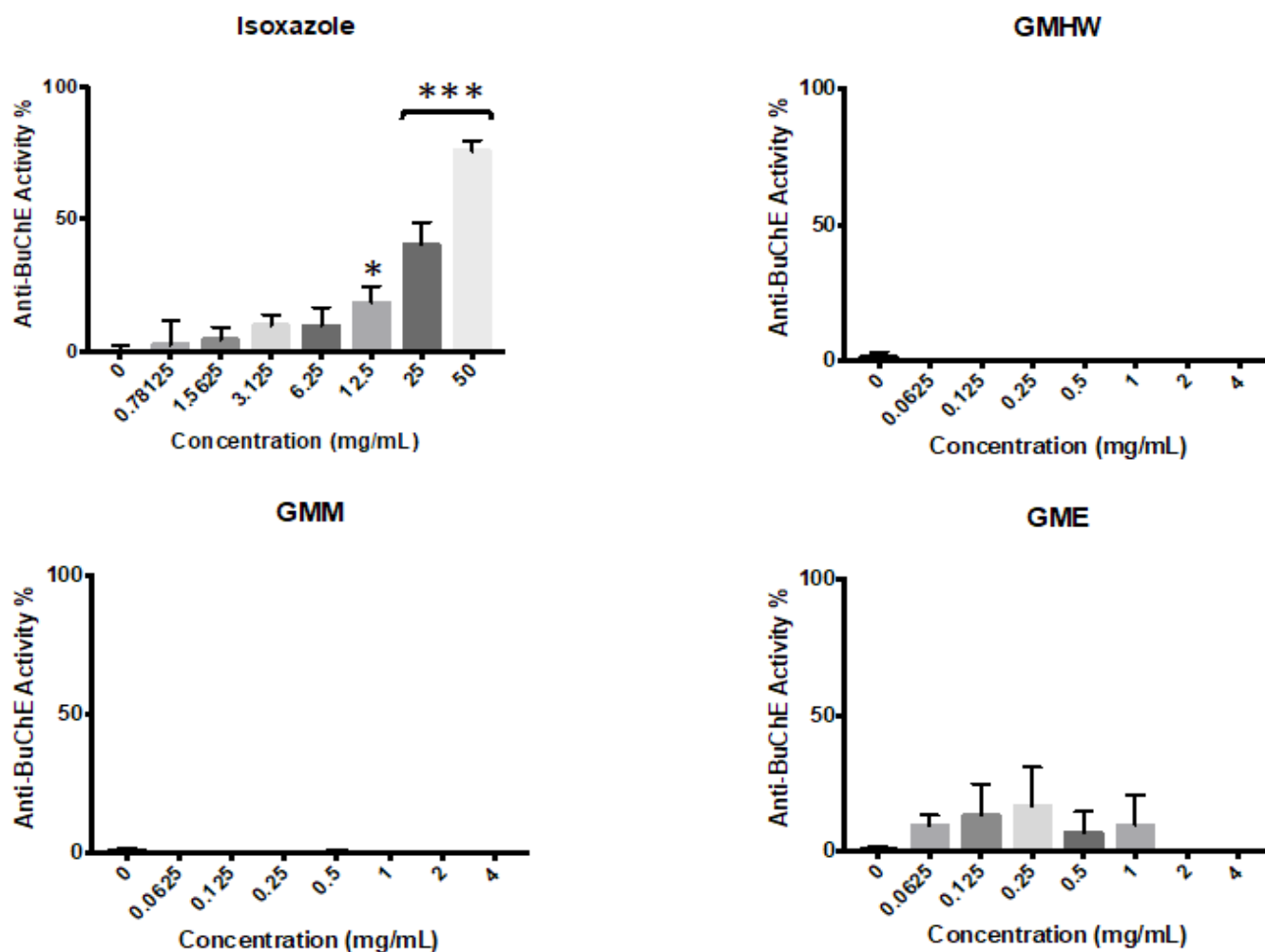


Fig. 2. BuChE inhibition activity of *Gracilaria manilaensis* extracts and the positive controls. Data are expressed in mean \pm SD ($n = 3$). * Arterisk symbol indicates significant difference (0 mg/mL) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$; Dunnett) against the negative control. Isoxazole was used as the positive control. BuChE, butyrylcholinesterase; GMHW, hot water extract; GMM, methanol extract; GME, ethanol extract.

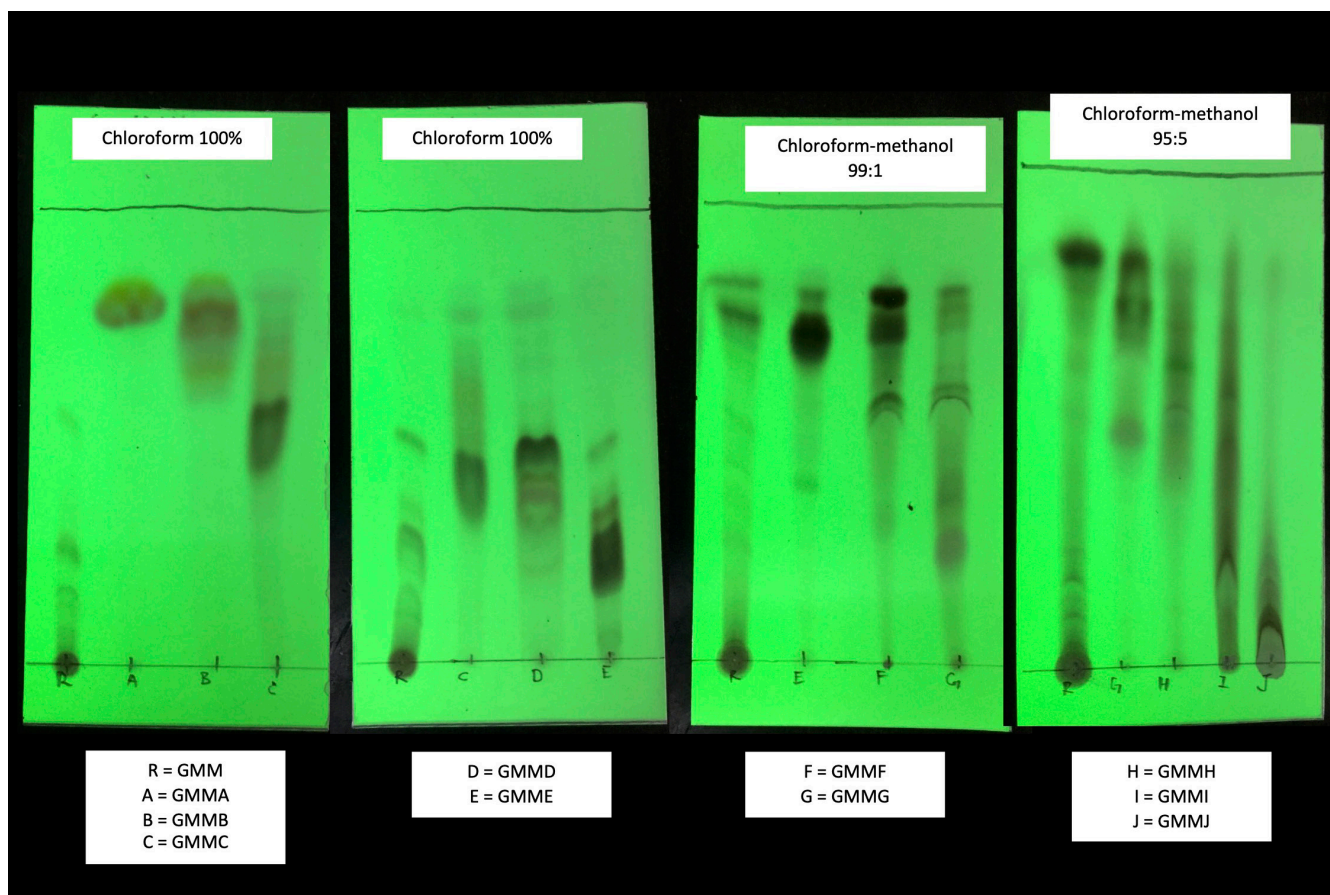


Fig. 3. Thin layer chromatography (TLC) profile of *Gracilaria manilaensis* methanol fraction GMMA to GMMJ. Each TLC plate from left to right was separated with different ratio of chloroform-methanol solvent system as labeled. GMM was used as the reference, marked as R in every TLC plate. GMM, methanol extract; GMMA-J, fraction A to J.

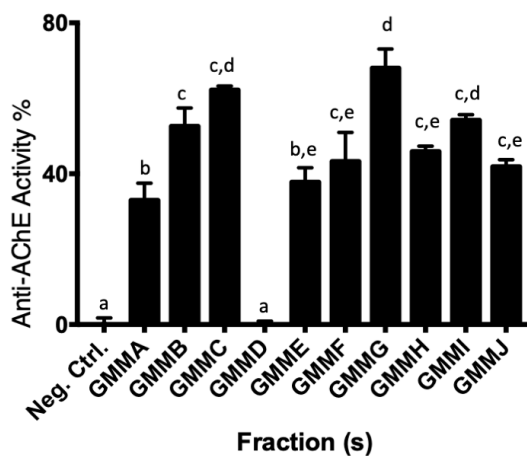


Fig. 4. AChE inhibition activity of 10 methanol fractions of *Gracilaria manilaensis* (GMMA to GMMJ) at 30 mg/mL with 10% aqueous methanol as the negative controls. Data are expressed as the mean \pm SD (n = 3). Different alphabets indicated significant different to other data ($p < 0.05$; Tukey). AChE, acetylcholinesterase; GMMA-J, fraction A to J.

GMMG showed positive AChE activity but negative BuChE activity (Fig. 5), similar to its predecessor, GMM. At the concentration of 0.375 mg/mL, GMMG started to show significant anti-AChE activity with $20.89 \pm 0.27\%$. The anti-AChE activity of GMMG increased with the concentration, where it reached the highest at 3 mg/mL with $61.09 \pm 6.15\%$.

Anti-AChE and BuChE activities of *G. manilaensis* and the positive controls (eserine and isoxazole) expressed in EC_{50} (Table 1). In this study, eserine and isoxazole were used as the positive control. Eserine has been optimised *in vitro* in the Ellman assay as described in Yoon et al. (2008, 2009), whereby isoxazole is a novel AChE inhibitor described in Gutiérrez et al. (2013) which is effective against AChE from *Electroporus electricus*, the similar AChE used in our study model (C2888, Sigma-Aldrich). Notable, eserine showed the lowest EC_{50} at 10.6 ± 2.0 ng/mL, whereas GMM and GMMG showed anti-AChE activity with

EC_{50} at 2.60 ± 0.16 mg/mL and 2.22 ± 0.27 mg/mL respectively, significantly lower than isoxazole and GME ($p < 0.05$). For BuChE, all samples were negative within the tested range, hence no EC_{50} value was shown. Based on Andriani et al. (2016), methanol was the best solvent to extract the anti-AChE compound(s) from *G. manilaensis*, which was similar to our findings. GMM and GMMG warrant a high potential for further testing.

AChE inhibitory kinetic of GMM and GMMG

GMM and GMMG were tested for their enzyme inhibition kinetic using the Lineweaver-Burk plot (Figs. 6 and 7). The presence of GMM increased the value of K_m from 1.48 mM to 1.68 mM at EC_{25} (1.3 mg/mL) and 2.03 mM at EC_{50} (2.6 mg/mL). However, the value of V_{max} was decreased from 0.0281 mM to 0.0231 mM at EC_{25} (1.3 mg/mL) and 0.0213 mM at EC_{50} (2.6 mg/mL) by GMM (Table 2). This matched the mixed-inhibition

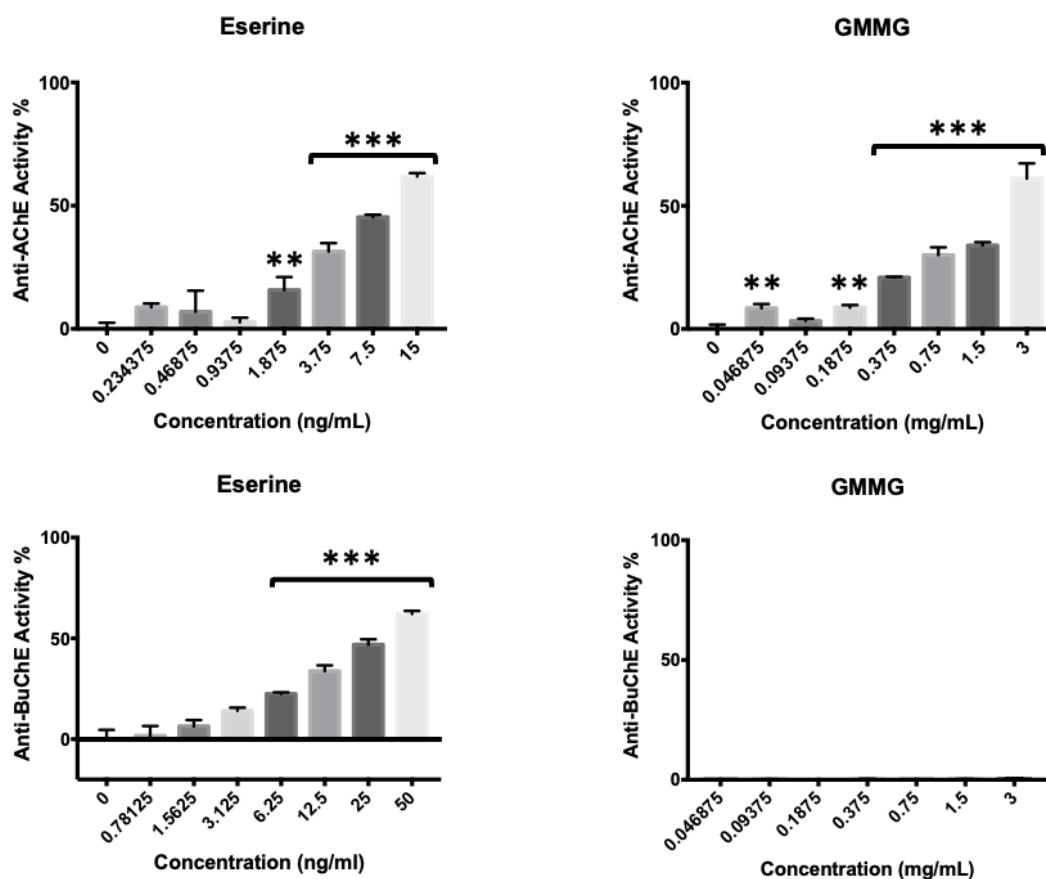


Fig. 5. AChE and BuChE inhibition activities of GMMG and the positive controls. Data are expressed in mean \pm SD ($n = 3$). *Arterisk indicates significant (0 mg/mL) ($p < 0.05$, ** $p < 0.01$, *** $p < 0.005$; Dunnett) differences against the negative control. Eserine was used as the positive control. AChE, acetylcholinesterase; GMMG, fraction G; BuChE, butyrylcholinesterase.

Table 1. Anti-AChE and BuChE activities of GMHW, GMM, GME, GMMG and the positive controls in EC₅₀ (mg/mL)

Samples	EC ₅₀ (mg/mL)	
	Anti-AChE activity	Anti-BuChE activity
Isoxazole*	4.10 ± 0.23 ^a	32.47 ± 3.35 ^a
Eserine*	1.06 × 10 ⁻⁵ ± 2.00 × 10 ^{-7b}	3.41 × 10 ⁻⁵ ± 1.20 × 10 ^{-6b}
GMHW	Negative	Negative
GMM	2.60 ± 0.16 ^d	Negative
GME	3.77 ± 0.20 ^a	Negative
GMMG	2.30 ± 0.18 ^d	Negative

Data are expressed as mean ± SD (n = 3).

^{a-d} Mean with different alphabet within the same column indicate significant differences (p < 0.05; Tukey).

*Asterisk symbol indicates the positive controls.

AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; GMHW, hot water extract; GMM, methanol extract; GME, ethanol extract; GMMG, fraction G.

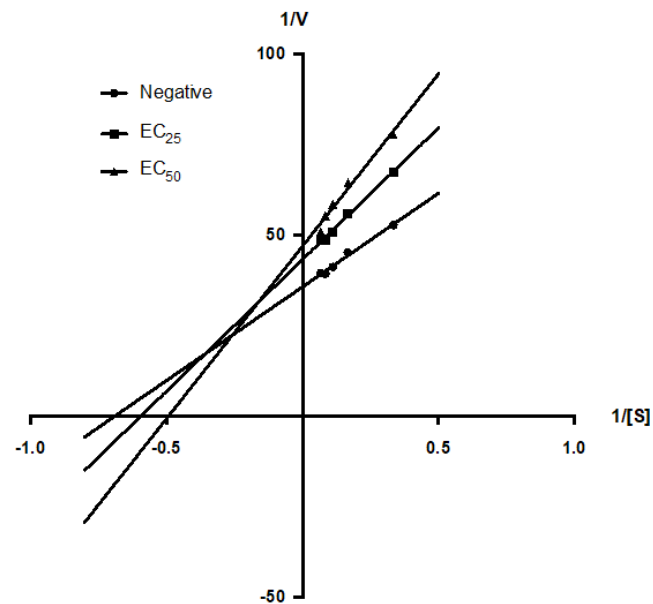


Fig. 6. Line-weaver Burk plot of AChE activity in the presence of GMM. AChE, acetylcholinesterase; GMM, methanol extract.

pattern of the Lineweaver-Burk plot, suggested that the GMM might possess bioactive compound(s) which can bind with both the enzyme and enzyme-substrate complex, with a higher affinity to the enzyme. GMM could be a mixed inhibitor to the anti-AChE activity.

On the other hand, GMMG decreased the value of K_m from 1.78 mM to 1.55 mM at EC₂₅ (1.15 mg/mL) and 1.33 mM at EC₅₀ (2.30 mg/mL). The value of V_{max} was decreased from 0.026 mM to 0.02 mM at EC₂₅ (1.15 mg/mL) and 0.016 mM at EC₅₀

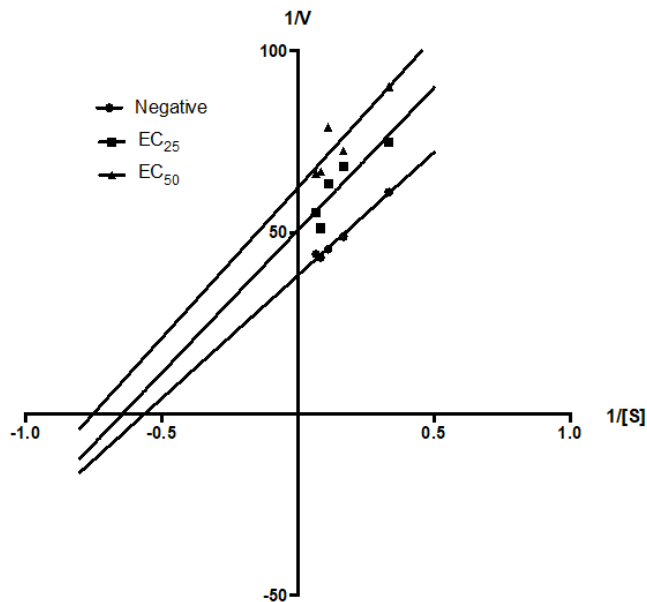


Fig. 7. Line-weaver Burk plot of AChE activity in the presence of GMMG isolated from methanol extract. AChE, acetylcholinesterase; GMMG, fraction G.

Table 2. The kinetic parameter of AChE activity in the presence of GMM

Inhibitors	K_m (mM)	V_{max} (mM)	K_i
Without inhibitor	1.48	0.028	
EC ₂₅ (1.3 mg/mL)	1.68	0.023	3.36
EC ₅₀ (2.6 mg/mL)	2.03	0.021	3.18

AChE, acetylcholinesterase; GMM, methanol extract.

(2.30 mg/mL) by GMMG (Table 3). This matched the uncompetitive inhibition pattern of the Lineweaver-Burk plot, suggested that the GMMG possess bioactive compound(s) which can bind with the enzyme-substrate complex specifically. GMMG could be an uncompetitive inhibitor to the anti-AChE activity.

Identification of compound(s) in GMMG

LCMS analysis was carried out and the results detected 136 peaks in the positive ion mass spectra. Of these, 28 peaks were matched with the identity of known molecules on the Metlin database, with Molecular Formula Generator (MFG) scores above 90% and ± 5 differences in MFG scores. The proposed compounds were searched and presented in Table 4. Interestingly, cynerine A, graveolinine, militarinone A, eplerenone and curumenol are the neuroprotective-related compounds

Table 3. The kinetic parameter of AChE activity in the presence of GMMG

Inhibitor	K_m (mM)	V_{max} (mM)	K_i
Without inhibitor	1.78	0.026	
EC ₂₅ (1.15 mg/mL)	1.55	0.020	7.21
EC ₅₀ (2.30 mg/mL)	1.33	0.016	10.32

AChE, acetylcholinesterase; GMMG, fraction G.

detected among the 28 known compounds. In contrast, 10 other compounds that had MFG scores above the cut-off did not match with any molecules in the library, indicating a large family of new unknown compounds that are yet to be explored in GMMG (Table 5). The chromatograms and MS spectra of compounds were shown in Figs. 3 and 8.

Table 4. Identified compounds in GMMG fraction through LCMS analysis

No	Name	Formula	m/z	Mass	Group	Activity	Related compound(s)	Activity
1	4-(2-hydroxypropoxy)-3,5-dimethyl-phenol	C ₁₁ H ₁₆ O ₃	197.12	196.11	Alcohol	• No record		
2	Dioscoretine	C ₁₃ H ₂₃ NO ₃	242.17	241.17	Fatty acid	• No record		
3	2-Phenylethyl 3-methylbutanoate	C ₁₃ H ₁₈ O ₂	207.14	206.13	Carboxylic acid	• No record		
4	ML-236C	C ₁₈ H ₂₆ O ₃	291.20	290.19	Carbocyclic compound	• Anticholesteremic (Endo et al., 1976)		
5	C16 Sphinganine	C ₁₆ H ₃₅ NO ₂	274.27	273.27	Lipid: Phospholipid	• Antibacteria (Reid et al., 2019) • Anticancer (Bagheri et al., 2018)		
6	2,6-Nonadien-1-ol	C ₉ H ₁₆ O	158.15	140.12	Fatty alcohol	• Flavour compound (Kemp et al., 1974; Flath et al., 1983; McGinty et al., 2010; Api et al., 2015, 2019)		
7	(-)-trans-C75	C ₁₄ H ₂₂ O ₄	272.19	254.15	Carboxylic acid	• No record		
8	Cyrneine A [†]	C ₂₀ H ₂₈ O ₃	317.21	316.20	Terpene: Diterpene	• Neurite outgrowth (Marcotullio et al., 2006; Obara et al., 2007)	Cyathane diterpene group	• Anti-oxidant (Marcotullio et al., 2008) • Anti-nitric oxide (Wang et al., 2014) • Neurite outgrowth (Cao et al., 2018) • Anti-bacterial (Nitthithanasilp et al., 2018)
9	7beta-Hydroxy-lathyrol	C ₂₀ H ₃₀ O ₄	335.22	334.21	Lipid: Diterpene	• No record		
10	Graveolinine [†]	C ₁₇ H ₁₃ NO ₃	297.12	279.09	Quinoline	Anti-angiogenesis (An et al., 2010) Anti-tumor and anti-platelet aggregation (Wu et al., 2003) Anti-AChE and anti-β amyloid (Luo et al., 2020)		
11	3α-Hydroxy-4,4-bis-nor-8,11,13-podocarpatriene	C ₁₅ H ₂₀ O	217.16	216.15	Phenol	• No record		
12	Militarinone A [†]	C ₂₆ H ₃₇ NO ₆	460.27	459.26	Pyridine alkaloid	• Neurite outgrowth (Schmidt et al., 2002; Riese et al., 2004; Küenzi et al., 2008)	4-hydroxy-2-pyridone (+)-N-Deoxymilitarinone A	• Neurite outgrowth (Schroder et al., 2015) • Neurite outgrowth (Cheng et al., 2006)

Table 4. Continued

No	Name	Formula	m/z	Mass	Group	Activity	Related compound(s)	Activity
13	Eplerenone*	C ₂₄ H ₃₀ O ₆	432.24	414.21	Lactone	<ul style="list-style-type: none"> • Anti-inflammation (Suzuki et al., 2006; Xiao et al., 2009; Bayorh et al., 2012; Chen et al., 2013, 2018; Łabuzek et al., 2014; Wada et al., 2017; Vecchiola et al., 2020) • Neuroprotective (Dong et al., 2012; Wang et al., 2020) 		
14	Curcumenol*	C ₁₅ H ₂₂ O ₂	235.17	234.16	Curcumin	<ul style="list-style-type: none"> • Anti-neuroinflammation (Tanaka et al., 2008; Lo et al., 2015; Hsiao et al., 2020; Pintatum et al., 2020) • Antioxidant • Neuroprotective (Hamdi et al., 2015) 	Oxycurcuminol	• Anti-stroke (Chen et al., 2018)
15	(+)-Trihydroxy-decidiene	C ₂₀ H ₃₂ O ₃	321.24	320.23	Lipid: Isoprenoid	<ul style="list-style-type: none"> • No record 		
16	alpha-Tocopheronolactone	C ₁₆ H ₂₂ O ₄	301.14	278.15	Lipid: Isoprenoid	<ul style="list-style-type: none"> • No record 		
17	N-cis-Tetradec-9Z-enoyl-L-Homoserine lactone	C ₁₈ H ₃₁ NO ₃	310.24	309.23	Lactone	<ul style="list-style-type: none"> • No record 		
18	Stigmatellin Y	C ₂₉ H ₄₀ O ₆	502.32	484.28	Phenol	<ul style="list-style-type: none"> • No record 		
19	Anandamide (20:l, n-9)	C ₂₂ H ₄₃ NO ₂	376.32	353.33	Fatty amide	<ul style="list-style-type: none"> • No record 		
20	2-Amino-14,16-dimethyloctadecan-3-ol	C ₂₀ H ₄₃ NO	336.32	313.33	Amino alcohol	<ul style="list-style-type: none"> • No record 		
21	2-Ethyl-dodecanoic acid	C ₁₄ H ₂₈ O ₂	229.22	228.21	Fatty acid	<ul style="list-style-type: none"> • No record 		
22	(-)-Isoamijiol	C ₂₀ H ₃₂ O ₂	322.27	304.24	Lipid: Isoprenoid	<ul style="list-style-type: none"> • No record 		
23	Palmitic amide	C ₁₆ H ₃₃ NO	256.26	255.26	Fatty amide	<ul style="list-style-type: none"> • No record 		
24	Coproporphyrin IV	C ₃₆ H ₃₈ N ₄ O ₈	655.28	654.27	Porphyrin	<ul style="list-style-type: none"> • No record 		
25	Cassaidine	C ₂₄ H ₄₁ NO ₄	408.31	407.30	Tricyclic diterpenoids	<ul style="list-style-type: none"> • No record 		
26	Haplophytine	C ₃₇ H ₄₀ N ₄ O ₇	653.30	652.29	Indole alkaloid	<ul style="list-style-type: none"> • No record 		
27	24,25-Epoxy-cholesterol	C ₂₇ H ₄₄ O ₂	401.34	400.33	Desmosterol	<ul style="list-style-type: none"> • No record 		
28	MG-DG(18:1(9Z)/18:1(9Z))	C ₄₅ H ₈₂ O ₁₀	805.58	782.59	Glycosyl-diradylglycerols	<ul style="list-style-type: none"> • No record 		

*Compound marked with asterisk symbol indicates compound with previously identified neuroprotective properties. GMMG, fraction G; LCMS, liquid chromatography-mass spectrometry; AChE, acetylcholinesterase.

Discussion

To date, there are many red algae with anti-AChE activity have been reported, including *Hypnea valentiae* (Suganthi et al., 2010), *Gracilaria edulis* (Suganthi et al., 2010), *Gelidiella acerosa* (Syad et al., 2012, 2016), *Hypnea musciformis* (Machado et al., 2015), *Ochtodes secundiramea* (Machado et al., 2015), *Pterocladia capillacea* (Machado et al., 2015), *Gelidium foliaceum* (Kannan et al., 2013), *Amphiroa beauvoisii* (Kannan et al.,

2013), *Laurencia complanata* (Kannan et al., 2013), *Rhodomeleopsis africana* (Kannan et al., 2013), *Porphyra tenera* (Senvirathne et al., 2010), *G. changii* (Andriani et al., 2016) and *G. manilaensis* (Andriani et al., 2016). In contrast, this is the first comprehensive study of Malaysian *G. manilaensis* whereby the anti-AChE and BuChE activities and their inhibition kinetic were reported. In the present study, GMM and GMMG possess anti-AChE activity closely compared to the ethanol extracts of *H. valentiae* and *G. edulis*, with IC₅₀ of 2.6 mg/mL and 3.5 mg/mL

Table 5. Unidentified compounds in GMMG

Formula	m/z	mass
C ₁₅ H ₂₉ N ₅ O ₄	344.2285	343.2213
C ₂₀ H ₃₇ N ₁₅ O ₂	520.3326	519.3255
C ₂₇ H ₅₃ N ₅ O ₁₀	608.3857	607.379
C ₂₂ H ₃₂ N ₆ O ₄	435.1767	434.1696
C ₂₁ H ₂₉ N ₃ O ₈	452.2022	451.1949
C ₂₈ H ₃₂ N ₂ O ₃ S	477.2227	476.2139
C ₁₁ H ₁₈ O ₃	199.1329	198.1255
C ₂₂ H ₄₁ NO ₂	352.3236	351.3139
C ₃₁ H ₃₆ N ₁₄ S	654.3324	636.2978
C ₃₇ H ₃₄ N ₈ O ₃	639.2822	638.2753

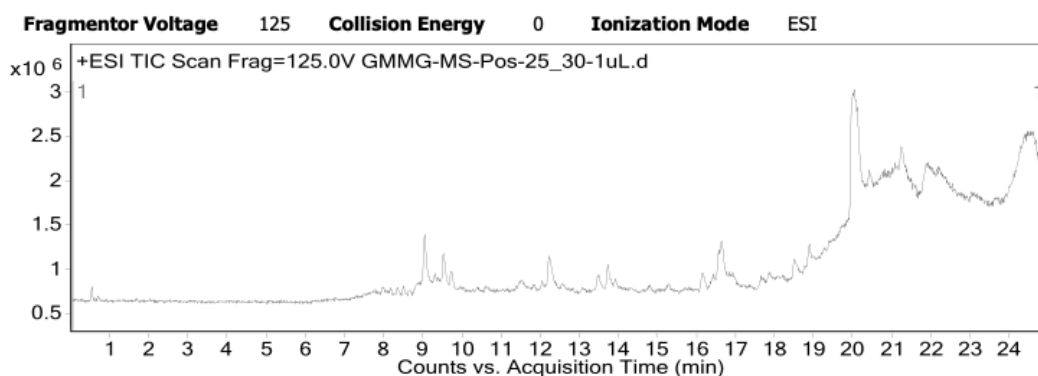
GMMG, fraction G.

may have a lower tendency to cause toxicity compared to other red algae according to Jońca et al. (2015). BuChE is also known as plasma cholinesterase, which is most abundant in plasma, pancreas and liver (Jońca et al., 2015). Jońca et al. (2015) stated that “BuChE serves as the first line of defence to the toxic compounds that reach the bloodstream that might inhibit AChE, the sister enzyme that located in the nervous system”. Hence, *G. manilaensis* could be a weaker but safer anti-AChE agent compared to other algae.

Andriani et al. (2016) has reported hexanoic acid as the main contributor to the positive AChE inhibitory activity in their preliminary screening. In contrast, we have only discovered two fatty acids, 2-ethyl-dodecanoic acid and dioscoretine

Data File	GMMG-MS-Pos-25_30-1uL.d	Sample Name	GMMG
Sample Type	Sample	Position	P1-A4
Instrument Name	Instrument 1	User Name	
Acq Method	U-ESI-E-XDB-C18-NB-MS-20(30)-Pos-July2015.m	Acquired Time	4/21/2021 9:19:43 PM
IRM Calibration Status	Success	DA Method	Metabolomics-2019.m
Comment			

Sample Group		Info.	
Stream Name	LC 1	Acquisition SW Version	6200 series TOF/6500 series Q-TOF B.06.01 (B6172 SP1)

**Fig. 8. LCMS chromatogram of GMMG.** LCMS, liquid chromatography-mass spectrometry; GMMG, fraction G.

respectively (Suganthi et al., 2010). However, *G. manilaensis* did not show anti-BuChE activity compared to other red algae, postulating that the bioactive compounds that are responsible for anti-AChE activity in *G. manilaensis* are selective towards AChE but not BuChE. This finding revealed that *G. manilaensis*

which do not have relevant activities to AChE and BuChE, besides dioscoretine for its hypoglycaemic effect (Loizzo et al., 2017). On the other hand, ML-236C, C16 Sphingosine and 2,6-Nonadien-1-ol in GMMG also do not contain neuroprotective and ChEs inhibitory activity. ML-236C, also known

as (4R,6R)-6-[2-[(1S,2S,8aR)-2-methyl-1,2,6,7,8,8a-hexahydronaphthalen-1-yl]ethyl]-4-hydroxyoxan-2-one, is a carbobicyclic compound with anti-cholesteremic activity that was isolated from fungus *Pythium ultimum* (Endo et al., 1976). C16 Sphingosine is a phospholipid that possesses anti-bacteria (Reid et al., 2019) and anti-cancer activities (Bagheri et al., 2018). 2,6-Nonadien-1-ol is a volatile fatty alcohol that function as a flavour compound described in many papers (Api et al., 2015, 2019; Flath et al., 1983; Kemp et al., 1974; McGinty et al., 2010).

The five neuroprotective compounds discovered in GMMG were drawn to our attention. Graveolinine is the only compound in GMMG that has been reported to positively inhibit AChE (Luo et al., 2020). Besides, graveolinine and its derivative also demonstrated neuroprotective activity against the neurotoxicity of β -amyloid and scopolamine (Luo et al., 2020). Meanwhile, cyneirne A (Marcotullio et al., 2006; Obara et al., 2007) and militarinone A (Küenzi et al., 2008; Riese et al., 2004; Schmidt et al., 2002) possess neurite outgrowth activity against PC12 cells. Interestingly, their respective derivatives, cyathane diterpene (Cao et al., 2018), as well as 4-hydroxy-2-pyridone (Schrüder et al., 2015) and (+)-N-deoxymilitarinone A (Cheng et al., 2006) also displayed neurite outgrowth activity as their precursors. Curcumenol (Hamdi et al., 2015) and Eplerenone (Dong et al., 2012; Wang et al., 2020) are also neuroprotective towards neuronal cell lines and mouse brain. Curcumenol also shown anti-neuroinflammatory activity against LPS-stimulated murine BV2 microglia (Hsiao et al., 2020; Lo et al., 2015; Pintatum et al., 2020; Tanaka et al., 2008), whereby eplerenone shown anti-inflammatory activity to a variety of illnesses and conditions such as viral myocarditis (Xiao et al., 2009), heart-related inflammations (Chen et al., 2018; Łabuzek et al., 2014; Suzuki et al., 2006), renal inflammation (Bayorh et al., 2012; Chen et al., 2013) and obesity-associated inflammasome (Vecchiola et al., 2020; Wada et al., 2017). These compounds made GMMG a very promising neuroprotective fraction to be used as nutraceutical agents for neuronal health.

Our findings disclosed that GMM and GMMG of *G. manilaensis* possessed inhibitory activity to AChE but not BuChE. GMM and GMMG showed mixed inhibition and uncompetitive inhibition to the AChE activity respectively. The anti-AChE activity of GMMG is likely to be contributed by graveolinine. Taking together, graveolinine from *G. manilaensis* can be a promising lead compound for the development of anti-Alzheimer medicine in future. However, it is crucial to further validate the cholinesterase inhibitory activities of *G. manilaensis* in ani-

mal cell culture *in vitro* and *in vivo* assays.

Competing interests

No potential conflict of interest relevant to this article was reported.

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Availability of data and materials

Upon reasonable request, the datasets of this study can be available from the corresponding author.

Ethics approval and consent to participate

This research has been approved by Sunway University Research Ethics Committee (Ethical Application Ref: PGSU-REC2017/030).

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