Discovery of potential neuroprotective compounds from marine sponges and algae

by
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SUMMARY

Alzheimer's disease (AD) is the major neurodegenerative disease responsible for more than 60% of dementia cases globally. One of the main hallmarks of AD is the presence of amyloid beta (Aβ) protein that forms plaques in the brain. $A\beta_{1-40}$ and $A\beta_{1-42}$ are two major forms generated from the cleavage of amyloid precursor protein (APP) by β-secretase and γ-secretase. Current treatment strategies for AD mostly target acetylcholinesterase and the N-methyl-D-aspartate (NMDA) receptor. However, these treatments can only mitigate some of the cognitive and memory loss symptoms and are not considered disease-modifying. Hence, the development of new treatments for AD is required. This study aimed to find new neuroprotective compounds from marine sponges and algae with bioactivity against the neurotoxicity and aggregation of $A\beta_{1-42}$.

Ninety-two extracts from marine sponges and algae (43, 13, 16, and 20 extracts from sponge, green algae, brown algae, and red algae, respectively) from South Australia have been screened initially for neurotoxicity. Only extracts that did not show cytotoxicity (45) were used for further study to characterise their bioactivity against the cytotoxicity induced by $A\beta_{1-42}$. This study showed that one-third of 92 extracts screened (29) were found to reduce neurotoxicity induced by $A\beta_{1-42}$ in PC-12 cells. *In silico* modelling was also used to screen the potential of compounds isolated from marine sponges and algae that showed activities in reducing the aggregation propensity of $A\beta_{1-42}$. Astaxanthin and fucoxanthin were selected as promising compounds due to their high docking scores to $A\beta_{1-42}$ and their commercial availability. It was subsequently demonstrated that both compounds inhibited the cytotoxicity induced by both $A\beta_{1-42}$ and hydrogen peroxide. In addition, these compounds demonstrated anti-aggregation effects in the Thioflavin T (ThT) assay for inhibition of $A\beta_{1-42}$ fibrillisation kinetics and via transmission electron microscopy (TEM) for morphological alteration of fibrils and aggregates. Molecular docking studies of the binding of these compounds were also performed in order to find the optimal binding regions of these compounds with $A\beta_{1-42}$. Furthermore, these compounds were shown to inhibit the apoptosis induced by $A\beta_{1-42}$, and also to enhance neurite outgrowth activity.

To explore the potential of South Australia-algae-derived functional food and nutrient manipulation for neuroprotection, six different fractions of the brown macroalgae *Ecklonia radiata* and five fucoidan samples have been studied for their neuroprotective potential. These samples were screened for their neuroprotective activities against the cytotoxicity and aggregation of $A\beta_{1-42}$; antioxidant activity in reducing the cytotoxicity induced by hydrogen peroxide and apoptosis induced by $A\beta_{1-42;}$ and neurite outgrowth activity. The results showed that these samples have different neuroprotective activities and impacts on neurite outgrowth. The fractionation process has significant impact on

neuroprotective activities of the fraction samples produced, highlighting the significance of process development on bioactivities. In general, astaxanthin and fucoxanthin demonstrated the highest neuroprotective activities among other compounds/extracts used in this study with significant results (one way ANOVA) in all assays.

Overall, this project has demonstrated great potential of marine sponges, especially algae as sources of neuroprotective compounds in both functional food and pharmaceutical applications toward modifying and alleviating neurodegenerative diseases.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Mousa Alghazwi
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LIST OF PUBLICATIONS

- Published

1- Alghazwi, M, Kan, Y, Zhang, W, Gai, W & Yan, X 2016, 'Neuroprotective activities of marine natural products from marine sponges', *Current Medicinal Chemistry*, vol. 23, no. 4, pp. 360-82.

2- Alghazwi, M, Kan, YQ, Zhang, W, Gai, WP, Garson, MJ & Smid, S 2016, 'Neuroprotective activities of natural products from marine macroalgae during 1999–2015', *Journal of applied phycology*, vol. 28, no. 6, pp. 3599–616.

- Publications under revision

1- Alghazwi, M, Smid, S & Zhang, W 2017, '*In vitro* protective activity of South Australian marine sponge and macroalgae extracts against amyloid beta (Aβ1-42) induced neurotoxicity in PC-12 cells'.

- Publications under preparation

1- Alghazwi, M, Charoensiddhi, S, Smid, S & Zhang, W 2017, 'Impact of extraction processes on the neuroprotective activities of *Ecklonia radiata* against amyloid beta (Aβ1-42) toxicity and aggregation'.

2- Alghazwi, M, Smid, S, Musgrave, I, Karpiniec, S, & Zhang, W 2017, 'Comparative study on neuroprotective activities of fucoidans from *Fucus vesiculosus* and *Undaria pinnatifida*', *Carbohydrate Polymers*.

3- Alghazwi, M, Smid, S, Musgrave, I, & Zhang, W 2017, '*In silico* and *in vitro* studies of the neuroprotective activities of astaxanthin and fucoxanthin against amyloid beta (Aβ1-42) toxicity and aggregation'.

- Conference poster

1- Mousa Alghazwi, Wei Zhang, Scott Smid "Neuroprotective activities of fucoidan-enriched seaweed extracts from *Fucus vesiculosus* and *Undaria pinnatifida* against Aβ1–42-induced cytotoxicity in PC12 cells" presenting a poster display in Bioprocessing Network conference 23rd-25th October, 2017 Adelaide, Australia.

2- Mousa Alghazwi, Wei Zhang, Scott Smid "*In silico* and *in vitro* studies of the neuroprotective effects of astaxanthin and fucoxanthin against amyloid beta ($A\beta_{1-42}$) toxicity" presenting a poster display in The Australasian Neuroscience Society (ANS) 36th Annual Scientific Meeting 4th -7th of December, 2016 in Hobart, Australia.

3- Mousa Alghazwi, Wei Zhang, Scott Smid "Neuroprotective effect of *Undaria pinnatifida* fucoidan extracts against $A\beta_{1-42}$-induced cytotoxicity in PC12 cells" presenting a poster display in:

•The Centre for Neuroscience (CNS) Collaborators Day 2016, 22nd September 2016 at Flinders Medical Center, Adelaide.

• 1st Australia New Zealand Marine Biotechnology Society symposium 14th -15th of April, 2016 at Flinders University, Adelaide.

4- Qi Yang, Suvimol Charoensiddhi, Mousa Alghazwi, Wei Zhang "Sponges and seaweeds South Australia's untapped economic resource" presenting a poster display in AMSA-SA 9th Annual Symposium 16th October, 2014 at SARDI Aquatic Sciences, Adelaide.

1. CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Neurodegenerative diseases

Neurodegenerative diseases pose one of society's most significant health challenges in the 20th century (Selkoe, 2008). These diseases are characterized by cognitive dysfunction due to neuronal loss (Liu et al., 2017), and have at least one abnormally misfolded and aggregated protein associated with disease progression. These include beta amyloid (Aβ) and tau proteins in Alzheimer's disease (AD), α-synuclein protein in Parkinson's disease and huntingtin protein in Huntingtin's disease (Soto, 2003), amongst others.

One of the most challenging issues is to understand the mechanisms underlying the disease occurrence. During the 1970s, some clues appeared using biochemical and molecular genetic techniques. However, one of the most challenging issues has been identifying disease causation (Selkoe, 2008), and this has arguably led to limited clinical management, particularly with pharmacotherapeutic treatment options. Until now, there are no approved drugs that are disease modifying, rather acting to reduce some symptomatic effects and with limited effectiveness (Cummings et al., 2014).

1.1.1 Introduction to Alzheimer's disease (AD)

Rapidly progressing memory loss was one of the characteristics of Alzheimer's diseases (AD) in Alois Alzheimer's diagnostic report of the patient that he was taking care of in 1907. In addition, the patient was disoriented regarding the place and time. The patient was forgetting everything immediately and was not able to write or read correctly. In addition, Alois Alzheimer reported the two main hallmarks of the disease which were beta amyloid plaques and neurofibrillary tangles (Stelzmann et al., 1995, Serrano-Pozo et al., 2011).

Alzheimer's disease is the most common causes of dementia in the elderly, and the fourth leading cause of death in developing countries (Galimberti and Scarpini, 2012, Ray and Lahiri, 2009). This disease contributes to about 60-70% of all dementia cases. About 5% of people who are older than 65 years are diagnosed with AD (Florent-Béchard et al., 2007). The risk of getting AD doubles every 5 years after the age of 65, and researchers suggested that half of the people who are older than 85 years are AD patients (Florent-Béchard et al., 2007). Even though AD has been reported for more than a century, investigations on the risk factors, causes and possible treatments has only commenced in the last 30 years (Thies and Bleiler, 2013).

The incidence of AD has increased gradually with a prevalence of 27.7 million people globally in 2003 (Wimo et al., 2006). The number of AD patients in the world increased in 2010 to reach 35.56 million cases, and is predicted to reach 115 million cases in 2050 (Prince et al., 2013). This is of great concern to society as the population is aging, thus increasing the number of AD cases. In Australia there were about 321,000 reported cases in 2012, and this number is predicted to increase to 900,000 by 2050. It is the second leading cause of death in Australia (AustralianBureauofStatistics, 2017). A world Alzheimer's report in 2010 stated that there were about 35 million people with dementia costing US$600 billion in health care and related costs (Weiner et al., 2010).

Usually the diagnosis of AD occurs at more than 65 years of age. This type is called sporadic or late-onset AD (LOAD) which contributes more than 99% of all AD cases. The other type occurs in a very small percentage (less than 1%) between the age of 30

and 60 and is due to family inheritance associated with a number of gene mutations in APP such as PSEN1, PSEN2, and MMP3, and for that reason it's known as familial AD (FAD) (Goate et al., 1991, Holtzman et al., 2011, Wallon et al., 2012, Han et al., 2017).

There are three stages of AD which include preclinical AD, mild cognitive impairment (MCI) and dementia. Diagnosing AD at an early stage can favour the patient before his/her situation worsens (Thies and Bleiler, 2013). Mild AD dementia usually last between 2 and 5 years, whereas the moderate stage lasts between 2 and 4 years (Holtzman et al., 2011). There is another rating stage which is called the Clinical Dementia Rating (CDR), which was used to rate the severity of dementia. The rating according to cognitive function is as follows: normal cognitive function is rated CDR=0, very mild loss of cognitive CDR=0.5, mild cognitive impairment CDR=1, moderate cognitive impairment CDR =2, severe cognitive impairment CDR=3 (Holtzman et al., 2011).

Other researchers divided the AD into seven stages (Takashima, 2009). The first stage is when there is no cognitive dysfunction. In stage 2 the person will have a very mild cognitive function, such as forgetting names and places. The person in stage 3 will have mild cognitive decline with difficulties in in solving issues. Moderate cognitive decline is a characteristic of stage 4. Stages 5, 6, and 7 are characterized by moderately severe, severe and very severe cognitive decline, respectively.

1.1.2 Diagnosis of AD

Alzheimer's disease can be diagnosed by a physician who can check the history of the patient to see whether the patient has AD in a family member. In addition, the

physician can look to the history of the patient as to any cognitive loss. They can conduct a neurological examination and use diagnostic imaging magnetic resonance and PET scanning to investigate any brain alterations and pathological markers, such as beta amyloid deposition (Thies and Bleiler, 2013).

Biomarkers are a critical element in diagnosis and clinical management of Alzheimer's disease. Biomarkers should be reliable with high sensitivity and specificity. These biomarkers could be compounds derived from tissue or fluid, or it could be pathological marker identifiable from neuroimaging (Hampel et al., 2010).

Currently, there are many methods of diagnosing AD, which include cerebrospinal fluid (CSF) analysis for both beta amyloid and tau (phosphorylated and total). The patient should show low CSF level of $A\beta_{42}$ and high CSF levels of both total and phosphorylated tau (Strozyk et al., 2003, Buerger et al., 2006). Beta amyloid plaques can be measured using positron emission tomography (PET) as this method uses radiolabelled compound that can bind directly to beta amyloid plaques such as fluorodeoxyglucose (FDG), ^{18}F-florbetaben, and florbetapir (^{18}F) (Richards and Sabbagh, 2014, Nakamura et al., 2016, Payoux and Salabert, 2017). It seems that both Aβ tests from CSF level and PET scan can give an early diagnosis of the disease as the deposition of these plaques can occur as early as 15 years before the onset of the disease. Tau levels and FDG are the second step after the deposition of beta amyloid. On the other hand, MRI is the best tool for diagnosing the severity of the disease when the patient develops symptoms (Jack Jr et al., 2010). Recently, a new method called protein misfolding cyclic amplification assay (Aβ-PMCA) has been suggested to be used to diagnose AD, which can detect a small quantity of misfolded Aβ

oligomer in CSF with 90% sensitivity and 93% specificity (Salvadores et al., 2014). There are also some genetic tests which can be used to detect Familial Alzheimer's disease (FAD) such as detecting the mutation in presenilin-1 (PSEN1), presenilin-2(PSEN2), APP, and APOE (Hampel et al., 2010).

1.1.3 Risk Factors for AD

There are many factors which can be linked to AD. These factors can include family history as the most important factor for many people who diagnosed with the familial form of AD. Genetic factors include the APOε4 gene as a factor that can contribute to increasing AD rate. Traumatic brain injury (TBI) is another factor which can be described as a disruption in brain function. Education can play an important role in slowing the development of AD; it is believed that education can build cognitive function and increases the connection between neurons (Thies and Bleiler, 2013). Women have a higher risk of developing AD after the age of 80, while men having higher risk before the age of 80 (Letenneur et al., 1999).

Cardiovascular disease can increase the possibility of developing AD. Smoking, diabetes, hypertension, and obesity can enhance cardiovascular risk factors and tend to increase AD risk (Luchsinger and Mayeux, 2004, Thies and Bleiler, 2013).

Physical exercise can delay the weakening of cognitive function and provide protective effects to the elderly brain (Rolland et al., 2008). Even though life style factors such as physical exercise and a healthy diet can reduce the incidence of AD, but will not prevent people from developing AD (Knopman, 2009).

1.1.4 Current issue of treating AD

Finding new therapeutic compounds that can be used as candidate drugs to treat AD is a hot area of research. However, most compounds to date have failed in clinical trials

or are still under trial (Cummings et al., 2014, Sperling et al., 2011a). This raises questions as to whether the selected targets for these compounds are matched to the pathophysiological mechanisms of the disease, whether these drugs are not effectively targeted, or are right targets selected, but at a wrong stage of the disease (Sperling et al., 2011b).

Multiple targets still provide researchers and clinicians with therapeutic opportunities even in the absence of a clearly defined disease causation (Bateman et al., 2011). The intensive collective research effort may still provide for an optimistic vision in preventing AD by 2025 (Gandy and DeKosky, 2013).

1.1.5 Beta Amyloid: features, folding and neurotoxicity

Beta amyloid (Aβ) is a 40-42 amino acid peptide (which referred to it as $A\beta_{1-40}$ or $A\beta_{1-42}$) that forms senile plaques as one of Alzheimer's disease hallmarks (Thal et al., 2006). It is believed that Aβ aggregation occurs upstream of the formation of tau abnormalities causing neurofibrillary tangles (Hardy and Selkoe, 2002). The production of Aβ is generated from the amyloid precursor protein (APP) by β and γ secretase cleavage, respectively. Many studies suggest that Aβ aggregation is the first sign of AD. In addition, all the genetic studies, and CSF or PET studies on Aβ from patients with mild cognitive impairment (MCI) showed that accumulation of Aβ is the main pathogenic feature of AD (Forsberg et al., 2008, Hansson et al., 2006). There are over 100 missense mutations found in presenilins which are linked to the increased production of $A\beta_{42}$ (Selkoe, 2001, Martinez, 2010).

The accumulation of Aβ due to the failure in regulating the production and removing the excess amount can lead to toxicity and neuronal damage (Bharadwaj et al., 2009).

The amyloidosis is formed by accumulation of the toxic form of the protein. This toxic protein results from a misfolding and changing the conformation from an α-helical coil to β-sheet enabling aggregation to the stable β-sheet fibrillar protein. The formation of Aβ oligomer leads to the formation of Aβ fibrils which eventually form plaques in the parenchyma (Selkoe, 2008). These aggregations lead to memory loss and cognitive impairment in AD patients as a result of neuron death that may also occur due to inflammation, formation of ROS, or microglial activation (Lesné et al., 2006, Shankar et al., 2008, Butterfield et al., 2001, Lull and Block, 2010, Rubio-Perez and Morillas-Ruiz, 2012).

Biomarker studies on pathological and biochemical changes in AD demonstrate that pathology may start years before the symptomatic development of the disease. For that reason these studies are important to provide therapeutic tools at early stage of the disease which will be more effective in delaying or preventing further development. Therapeutic strategies for Aβ have been hypothesized such as inhibiting the production or aggregation of Aβ (Sisodia and George-Hyslop, 2002). Even though many drugs in clinical trials have targeted the cycle of amyloid beta, most of these drugs have failed (Selkoe, 2011). Compounds that can inhibit the production of Aβ by inhibiting β and/or γ secretases or by enhancing α secretase rather than targeting the accumulation of Aβ were also considered as therapeutic targets (Sperling et al., 2011b), but have had some noticeable failures in clinical trials (Doody et al., 2013, Vandenberghe et al., 2016).

1.1.5.1 β-site APP cleaving enzyme 1 (BACE-1)

Compounds inhibitng BACE-1 will result in decreasing Aβ production (Citron, 2010), but it would be a challenge to develop a compound that targets BACE-1 as it has

many substrates, and therefore some considrations shoould be taken as blocking BACE-1 might result in some side effects (De Strooper et al., 2010). The other challenges are that the compound should pass through the blood-brain barrier, and have drug likeness properites which include favourable absorption, distribution, metabolism and excretion (ADME) (Mangialasche et al., 2010, Ghosh et al., 2012).

1.2 Current treatment for AD

One of the most difficult issues about AD is finding a treatment method. Even though, five drugs (Figure 1-1) have been developed to target cholinesterase and NMDA have been approved to be used to treat AD's patents, these patients will only show marginal improvements that are usually only temporary (Cummings et al., 2014). There are four cholinesterase inhibitors drugs available for treating AD namely tacrine, donepezil, rivastigmine, and galanthamine. These drugs are used for mild cognitive impairment (MCI) patients (Doody et al., 2001). All drugs inhibit acetylcholinesterase. However, rivastigmine can inhibit butyrylcholinesterase. Each of these drugs have different pharmacokinetic and pharmacodynamics. For instance, donepezil is a non-competitive inhibitor for acetylcholinesterase with high selectivity. Rivastigmine is a reversible inhibitor of acetylcholinesterase and butyrylcholinesterase. Galanthamine is a reversible inhibitor to acetylcholinesterase (Scarpini et al., 2003). Tacrine is a reversible inhibitor to acetylcholinesterase and was the first approved drug for AD treatment (Jann et al., 2002). Recently, another compound (huperzine-A) targeting acetylcholinesterase was approved in China (Xing et al., 2014).

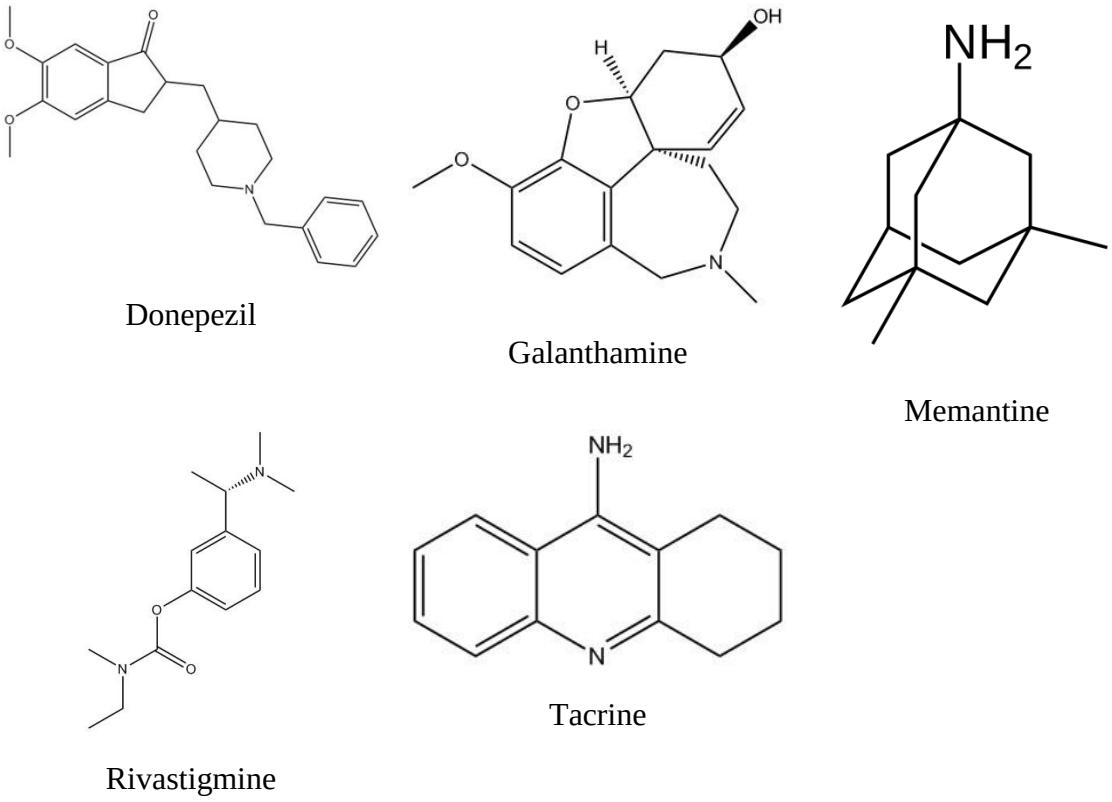


Figure 1-1 Structure of five approved drugs for AD treatment: acetylcholinesterase inhibitors of donepezil, galanthamine, rivastigmine and tacrine, and NMDA inhibitor memantine.

On the other hand memantine is the approved drug targeting the NMDA type of glutamate receptor. It is a non-competitive inhibitor that is used to treat moderate to severe cognitive impairment (Scarpini et al., 2003).

1.3 Natural products: experimental treatments with clinical potential

Curcumin is one among many natural derived compounds that demonstrated potential effects to be used as therapeutic agent for AD (Ray and Lahiri, 2009). It showed anti-inflammatory effects by inhibiting lipoxygenase, cyclooxygenase 2 and inducible nitric oxide synthase (iNOS) (Ammon et al., 1993, Pan et al., 2000). Interestingly, curcumin provides an anti-amyloidogenic effect by inhibiting the formation and

aggregation of Aβ (Ono et al., 2004). Animal studies using a low dosage of curcumin (160 ppm) have demonstrated that it can reduce soluble and insoluble amyloid beta and plaques by 43-50% (Lim et al., 2001). One of the reasons curcumin has failed in clinical trials is due to the poor bioavailability (Mancuso et al., 2011, Baum et al., 2008).

Many compounds have been synthesized and used to treat major diseases based on a natural product chemical entity. Examples such as galanthamine, which inhibits cholinesterase activity, was derived from a natural source (*Caucasian snowdrop*) and is now synthesized as a treatment for AD (Heinrich and Teoh, 2004, Loy and Schneider, 2006). Secondary metabolites are those compounds not involved in growth or survival of an organism, but those compounds that increase the life of the organism and that have roles in defence against pathogens or predation. Most of the active compounds from phytochemicals in plant come from secondary metabolites which include alkaloids, terpenoids, and phenolics (Harbome, 1993). Many natural products derived from plants have demonstrated anti-ageing activities such as curcumin, quercetin, tamarixetin, and kaempferol (Argyropoulou et al., 2013).

1.3.1 (−)-epigallocatechin-3-gallate (EGCG)

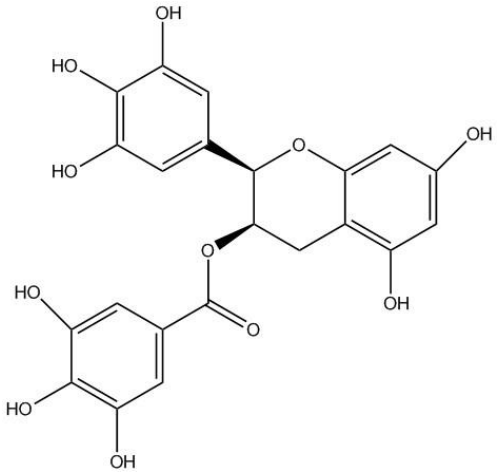
Flavonoids are a group of polyphenolic compounds that can be found in fruits, vegetables, tea, and wine (Beecher, 2003). Many researchers have shown the relationship between flavonoid compounds and prevention of dementia (Orhan et al., 2015, Baptista et al., 2014, Williams and Spencer, 2012). Several flavonoids compounds have demonstrated to protect PC-12 cells from the toxicity induced by $A\beta_{1-42}$ in PC-12 cells (Sasaki et al., 2015, Marsh et al., 2017).

Among these flavonoid compound is EGCG (Figure 1-2) which was found to prevent the toxicity induced by Aβ and decrease the level of malondialdehyde (MDA) in

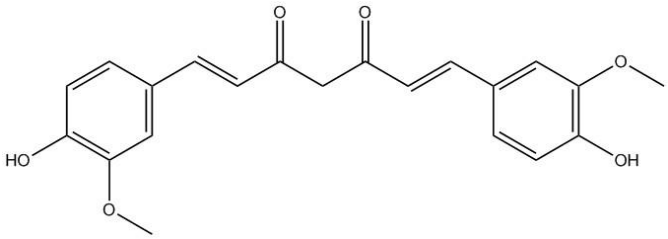
hippocampal neuronal cells (Choi et al., 2001). EGCG was able to bind to the native unfolded polypeptide and prevent it from converting to the aggregated form of Aβ (Ehrnhoefer et al., 2008). EGCG was also shown to convert pre-formed Aβ fibrils into smaller proteins with no toxic effect (Bieschke et al., 2010).

1.3.2 Curcumin

Curcumin (Figure 1-2) is polyphenolic compound derived from *Curcuma Long Lin*. It shows anti-oxidant activity by reducing lipid peroxidation in animal model (Reddy and Lokesh, 1992). Curcumin was able to inhibit toxicity of Aβ in SH-SY5Y cell and to inhibit the aggregation of Aβ animal model using Tg2576 mice brain (Yang et al., 2005). In addition, curcumin was found to protect PC-12 cells from $A\beta_{25-35}$ induced cytotoxicity, and also decrease oxidative stress and DNA damage (Park et al., 2008). Recent study showed that curcumin did not inhibit the aggregation of Aβ but rather increase the non-toxic oligomer form, which suggests curcumin is working through modifying the pathway of Aβ aggregation to form non-toxic aggregates (Thapa et al., 2015). However, curcumin has a low bioavailability as it showed very poor solubility and degraded quickly (Wang et al., 1997). Recently, curcumin analogues have been synthesised and demonstrated to be more soluble and more active in reducing the aggregation of $A\beta_{1-42}$ than curcumin (Endo et al., 2014).



(−)-epigallocatechin-3-gallate



Curcumin

Figure 1-2 Structure of some of natural products that showed neuroprotective activities

1.4 Marine sources of natural compounds for AD treatment and prevention

Natural products account for about 70% of all drugs in the market compared to only 30% of synthetic drugs (Newman, 2008). Plant-based natural products have been used since antiquity as traditional medicines. Natural products history was reported for the first time from 2900 BCE from Egyptian medicine with the best known medicine *Ebers Papyrus* dated from 1500 BCE (Butler and Newman, 2008). Marine natural products have gained the attention of researchers, as the oceans cover more than 70% of the earth's surface and marine organisms have a huge diversity exceeding 300,000 species. Natural products from marine organisms have not be explored as intensively as plant-based products, as there are limitations on access and collection of the samples, as well as a lack of information of the chemical diversity (Bowling et al., 2007). Marine organisms offer the potential to treat a range of human and animal diseases (Aneiros and Garateix, 2004).

1.4.1 Marine macroalgae

A comprehensive review on neuroprotective compounds discovered from marine macroalgae has recently been published (Alghazwi et al., 2016b). It will form part of this Chapter 1 Literature review but is presented in full as the Appendix 1.

Marine macroalgae (seaweed) compose 90% of marine plant species and are responsible for providing 50% of photosynthesis in the ocean (Dhargalkar and Neelam, 2005). Usually macroalgae occur in seawater rich in nutrients, enabling nutrient absorption (Dhargalkar and Neelam, 2005). They usually occur either in shallow water or up to a maximum depth of 200 meters (Carté, 1996). In addition, macroalgae can adapt for survival in high salinity, temperature shifting and low nutrient environments (Plaza et al., 2008). There are more than 15,000 species of macroalgae around the world (Waters et al., 2012). Marine algae are a primary source of food, with many marine organisms depending on its availability (Samarakoon and Jeon, 2012) including as a source of vitamins, minerals, and folic acid (Darcy-Vrillon, 1993). Macroalgae has been used as a human food source as fresh or dried especially in Asian countries. They have been used as animal feed and fertilizer as well. Seaweed can be classified according to the colour: brown, green, and red, which are known as Ochrophyta, Chlorophyta, and Rhodophyta, respectively (Carté, 1996). Compared to flowering plants, macroalgal chemical composition is relatively understudied (Ratana-arporn and Chirapart, 2006). Macroalgae-derived chemicals are varied according to the classes of macroalgae, the species type and environmental conditions (Ito and Hori, 1989). These chemicals have been recorded for their biological activities, including anti-viral, antioxidant, anti-allergic, anti-cancer, anti-obesity, and many other activities, including neuroprotection as potential drug

candidates (Mayer and Hamann, 2002, Mayer and Gustafson, 2004, Mayer and Hamann, 2005, Mayer et al., 2007).

1.4.2 Marine sponges

A comprehensive review on neuroprotective compounds discovered from marine sponges has been recently published (Alghazwi et al., 2016a). It will form part of this Chapter 1 Literature review but is also presented in the Appendix 2.

Sponges (phylum *Porifera*) have evolved for more than 700 million years with about 15,000 species (Belarbi et al., 2003). Sponges are an organism that can feed on organic particles (Osinga et al., 1999). Sponges produce toxins as a defence mechanism against predators and infection, as well as for communication (Uriz et al., 1996, Belarbi et al., 2003). Sponges grow slowly but can reach a large size depending on the species and environment, where the time for doubling biomass can be varied from a month to a year (Belarbi et al., 2003). Porifera are divided in three classes, 25 orders, 127 families, and 682 genera (Hooper and Van Soest, 2002)

Sponges provide pharmacologically active compounds with different applications in oncology, diabetes, antibiotic and antiviral treatments (Mayer et al., 2013). Moreover, marine sponges were found to contribute to about 30% of marine natural products discovered, making them inarguably the single best source of marine natural products for drug discovery (Mehbub et al., 2014).

1.5 Prospective Therapy

Only a small number of compounds can make it through clinical trials due to many reasons, including specificity, selectivity, efficacy, pharmacokinetics and off target toxicity. In neurodegenerative diseases, using one drug may be insufficient to treat a patient; however multiple-medication therapy (MMT) may also cause drug interactions. Another approach is that of multiple-compound medication (MCM). This approach is based on combining different compounds in one drug, or using one compound that can bind to different targets. This approach seems to be the most effective among these three approaches (Cavalli et al., 2008). Many compounds have been screened for their multifunctional activities as potential drugs for treating AD. As a good example, pyrimidinylthiourea derivatives have demonstrated acetylcholinesterase inhibition, antioxidant, anti-aggregatory effects against $A\beta_{1-42}$ and ROS inhibition (Li et al., 2016). In addition, some donepezil derivatives have demonstrated multifunctional activities such as acetylcholinesterase inhibition , anti-aggregatory activity against $A\beta_{1-42}$, neuroprotection against $A\beta_{1-42}$ in PC-12 cells and antioxidant activity (Wang et al., 2016b).

Marine derived compounds have demonstrated clinical efficacy in treating some diseases (Haefner, 2003). Over 1200 compounds derived from marine organisms that have demonstrated different biological activities in preclinical phase during 1998-2006 (Mayer et al., 2010). Currently, there are seven approved drugs by FDA from marine sources, one over the counter drug, and many other compounds at different clinical phases (Meyer, 2017, Martins et al., 2014). Interestingly, three of these approved drugs are derived from sponges (Meyer, 2017). An example of that is

cytarabine, Ara-C which is approved for cancer therapy (Mayer et al., 2010). For these reason, marine compounds is an important sources of future drugs.

1.6 Hypotheses

The hypotheses of the project are:

1- Marine sponges and algae collected in South Australia contain neuroprotective compounds especially against the neurotoxicity of $A\beta_{1-42}$.

2- Different extracts/compounds from algae will demonstrate different neuroprotective activities in multiple neuroprotective assays.

The rationales of these hypotheses are based on our reviews of marine sponge and algae as potential sources of neuroprotective compounds. However, there are only few studies about the neuroprotective activities of marine sponge and algae collected in Australia.

1.7 Aims and scope of this thesis

Marine sponges and algae provide a promising source of neuroprotective compounds (Alghazwi et al., 2016a, Alghazwi et al., 2016b). However, more research is needed in order to find their potential to be used for neuroprotective activity. The schematic overview of the project plan can be found in Figure 1-3. One of the main objectives of this study is to evaluate the potential of marine sponges and algae collected in South Australia as source of compounds to reduce the cytotoxicity induced by $A\beta_{1-42}$ (Chapter 2). Then, an *in silico* model has been used to select potential compounds from these two sources (Chapter 3). After that, six fractions of *Ecklonia radiata* and five fucoidan samples were investigated for their inhibition of the cytotoxicity and aggregation of $A\beta_{1-42}$. Additionally, these samples were investigated for their antioxidant and neurite outgrowth activities (Chapter 4 and 5). Astaxanthin and

fucoxanthin were investigated for their binding with $A\beta_{1-42}$, and then for their potential in reducing the cytotoxicity induced by $A\beta_{1-42}$ and hydrogen peroxide (H_2O_2). These compounds were also investigated for their anti-aggregatory effects against $A\beta_{1-42}$. In addition, their neurite outgrowth activity and inhibition of apoptosis induced by $A\beta_{1-42}$ were investigated (Chapter 6).

The aims of this project are outlines below:

1- To understand the potential of sponge and algae collected in South Australia as sources of neuroprotective compounds especially against neurotoxicity and aggregation induced by $A\beta_{1-42}$.

2- To apply an *in silico* model to identify potential compounds that are likely to inhibit the aggregation of $A\beta_{1-42}$.

3- To investigate the impact of extraction processes on the neuroprotective activities of the alga *Ecklonia radiata*, the most abundant brown algae species in South Australia.

4- To do a comparative study on neuroprotective activities of fucoidans extracts from the algae *Fucus vesiculosus* and *Undaria pinnatifida* against amyloid beta ($A\beta_{1-42}$) toxicity and aggregation.

5- To do *in silico* and *in vitro* studies of the neuroprotective activities of astaxanthin and fucoxanthin.

There was no report about using an *in silico* model for selecting neuroprotective compounds from these two organisms especially against the aggregation of Aβ. In addition, there is no comparable study about neuroprotective activities of fucoidans from different sources and with different composition.

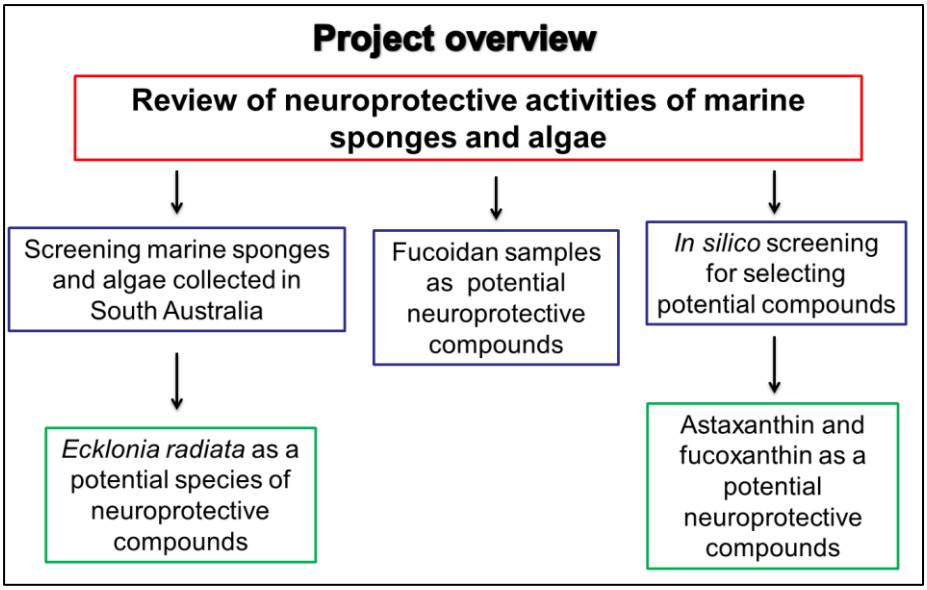


Figure 1-3 Schematic overview of the project

1.8 Significance

This is the first study conducted on both marine sponges and algae collected on South Australia for their neuroprotective activities against the neurotoxicity of $A\beta_{1-42}$. The results of this study will highlight the potential of these organisms as sources of neuroprotective compounds. The study provides knowledge on the multiple neuroprotective activities of compounds and extracts based on different extraction methods, different chemical compositions as indicated from *E. radiata* fractions and from fucoidans that can contribute to developing new marine-derived neuroprotective compounds in preventing and treatment of Alzheimer's disease.

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2. CHAPTER 2: *IN VITRO* PROTECTIVE ACTIVITY OF SOUTH AUSTRALIAN MARINE SPONGE AND MACROALGAE EXTRACTS AGAINST AMYLOID BETA (AB_{1-42}) INDUCED NEUROTOXICITY IN PC-12 CELLS

Contribution: Mousa Alghazwi has done all the experiments, analysed all the data, and wrote the manuscript. Wei Zhang and Scott Smid provide feedback to improve the work and helped in editing the manuscript. The Australian Institute of Marine Science provided all the extract samples.

2.1 Introduction

Alzheimer's disease (AD) is a neurodegenerative disease responsible for 60-80% of dementia cases (Alzheimer'sAssociation, 2014). Current treatment strategies for AD mostly target acetylcholinesterase and the N-methyl-D-aspartate (NMDA) receptor. However, these treatments can only mitigate some of the cognitive and memory loss symptoms and are not considered disease-modifying. Hence, the development of new treatments for AD are required (Scarpini et al., 2003).

One of the main hallmarks of AD is the presence of amyloid beta (Aβ) protein that forms plaques in the brain. $A\beta_{1-40}$ and $A\beta_{1-42}$ are major forms generated from the cleavage of amyloid precursor protein (APP) by β-secretase and γ-secretase (Hussain et al., 1999). It is suggested that the aggregation and diminished clearance are pathogenic factors of AD (Hardy and Selkoe, 2002). Animal studies demonstrate that amyloid plaques are correlated with memory defects (Hsiao et al., 1996). For that reason, targeting Aβ may be considered an effective approach in the treatment of AD (Hardy and Selkoe, 2002).

Marine sponges, one of the oldest multicellular animals on the planet (Hentschel et al., 2002), are a rich source of natural compounds contributing more than 30% of all compounds discovered from marine organisms (Mehbub et al., 2014). These compounds possess a spectra of biological activities including anti-viral, anti-bacterial, and anti-inflammatory properties (Mayer et al., 2013). A recent review of neuroprotective compounds from marine sponges ascribed a variety of mechanisms to their neuroprotection, including glutamate and serotoninergic receptor activity, kinase inhibition, neuritogenic and anti-oxidant activity (Alghazwi et al., 2016a).

Interestingly, seven out of 90 neuroprotective compounds were reported as sourced from Australian species.

Macroalgae (or seaweeds) have been known for their uses in food and as potential drug sources. Macroalgae can be classified based on the pigment colours into different phyla such as Chlorophyta, Ochrophyta (class Phaeophyceae), and Rhodophyta which are commonly named as the green, brown and red algae, respectively (Lobban and Harrison, 1994, Guiry, 2012). Macroalgae present a range of biological activities such as anti-viral, anti-bacterial, antioxidant, anti-cancer and neuroprotective activity (Wang et al., 2008, Lima-Filho et al., 2002, Kang et al., 2003, Kang et al., 2004, Aisa et al., 2005, Pangestuti and Kim, 2011). A recent review reported a total of 99 compounds isolated from macroalgae demonstrating neuroprotective activities (Alghazwi et al., 2016b). The mechanisms ascribed to these effects included inhibiting Aβ aggregation and acetylcholinesterase inhibition, decreasing oxidative stress and kinase activity, enhancing neurite outgrowth, anti-inflammatory activity and protecting dopaminergic neurons.

South Australian waters have more than 1000 different species of sponges that belong to 200 genera (Bergquist and Skinner, 1982). South Australia hosts one of the highest diversity of macroalgae, as it is home to over 1200 species with 62% of them as endemic (Phillips, 2001, Womersley, 1996). Few studies have reported neuroprotective activities of sponges and macroalgae collected in Australian waters, with only seven neuroprotective compounds from sponges. Esmodil was shown to inhibit acetylcholinesterase (Capon et al., 2004), while debromohymenialdisine inhibited CDK5/p25, CK1, and GSK-3β (Zhang et al., 2012c). Four compounds

(Lamellarins O1, Ianthellidone F, lamellarins O2 and O) were shown to inhibit β-site amyloid precursor protein cleaving enzyme (BACE) (Zhang et al., 2012a), in addition to Dictyodendrin J (Zhang et al., 2012b). Moreover, only 3 compounds isolated from macroalgae collected in Australia were shown to have demonstrated neuroprotective activity. Spiralisone A, spiralisone B, and chromone 6 showed inhibition of CDK5/p25, CK1δ and GSK3β kinases (Zhang et al., 2012d). Therefore the present study was conducted to evaluate the potential of South Australia marine sponge and macroalgae extracts as a source of neuroprotective compounds, with a focus on reducing the cytotoxicity of Aβ in neuronal PC-12 cells.

2.2 Materials and methods

2.2.1 Samples collection

The Australian Institute of Marine Science (AIMS) provided all the samples used in this study. These samples were collected by hand whilst scuba diving or from shallows at low tide. They were frozen after a representative taxonomy sample was taken. All samples were collected in South Australia. The details of collections sites can be found in the Table 2-1.

The taxonomy information was provided by AIMS. Phylogenetic trees of these samples were conducted according to their class, order, family, genus, and species with a guide from http://www.algaebase.org/ (for algae samples) (Guiry and Guiry, 2014) and http://www.marinespecies.org/porifera/ (for sponge samples) (Van Soest et al., 2017).

The marine samples were divided in four different categories based on their class for sponges (Demospongiae) or phyla for macroalgae (Chlorophyta, Ochrophyta, and Rhodophyceae). For each category a separate phylogenetic distribution was constructed to distribute the class into order, family, genus, and species, respectively.

All the sponge samples were from Demospongiae class with a broad distribution of 8 orders and 17 families (Figure 2-1). In Chlorophyta, there were 3 orders and 4 families (Figure 2-2). In Phaeophyceae, there were 6 orders and 8 families (Figure 2-3). In Rhodophyceae, there were 5 orders and 10 families (Figure 2-4).

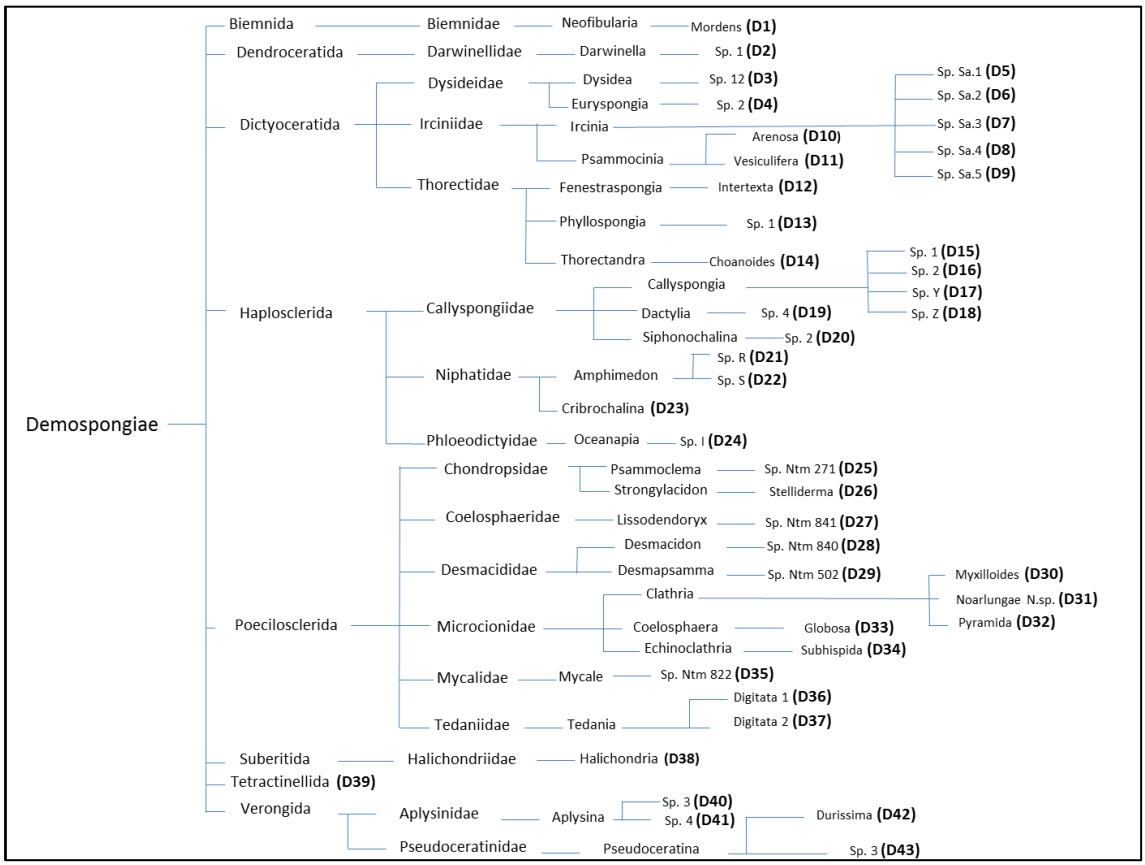

Figure 2-1 Phylogenetic distribution of sponge species among order, family, and genus from South Australia used in this study

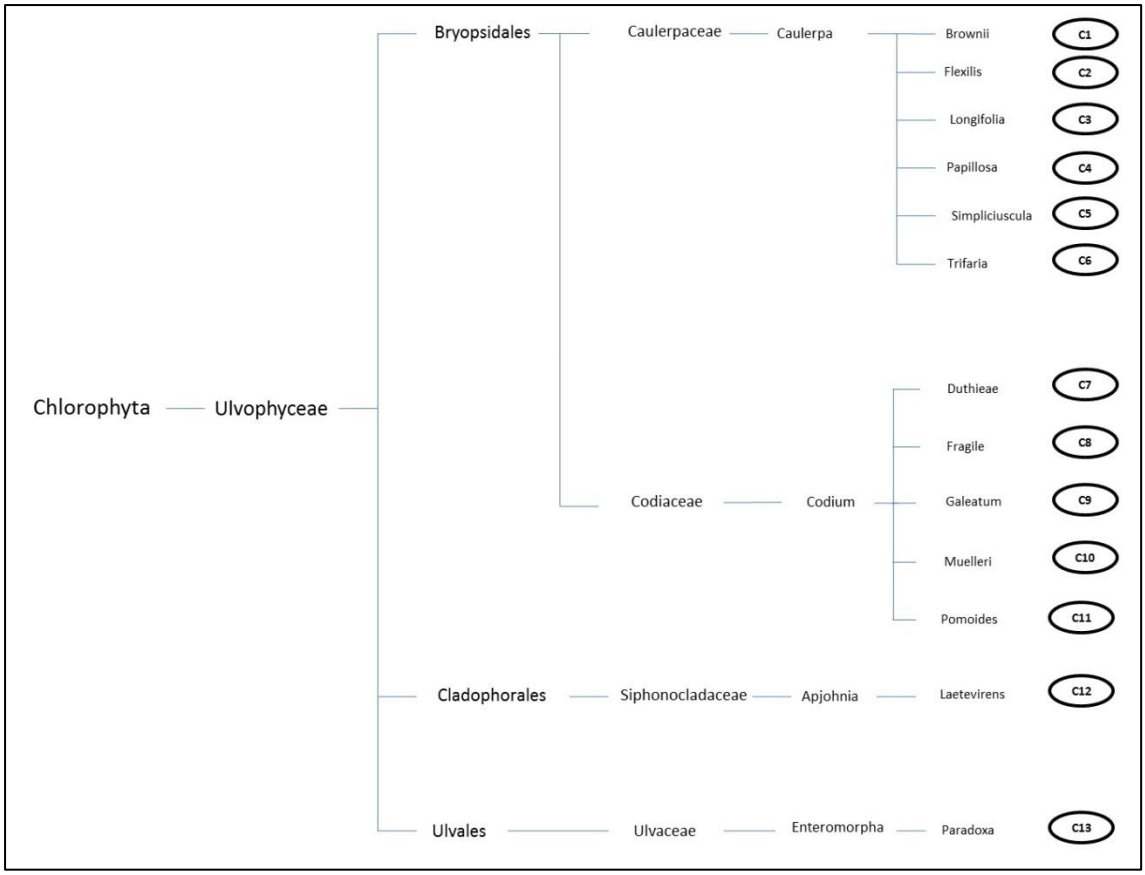

Figure 2-2 Phylogenetic distribution of green algae species among order, family, and genus from South Australia used in this study

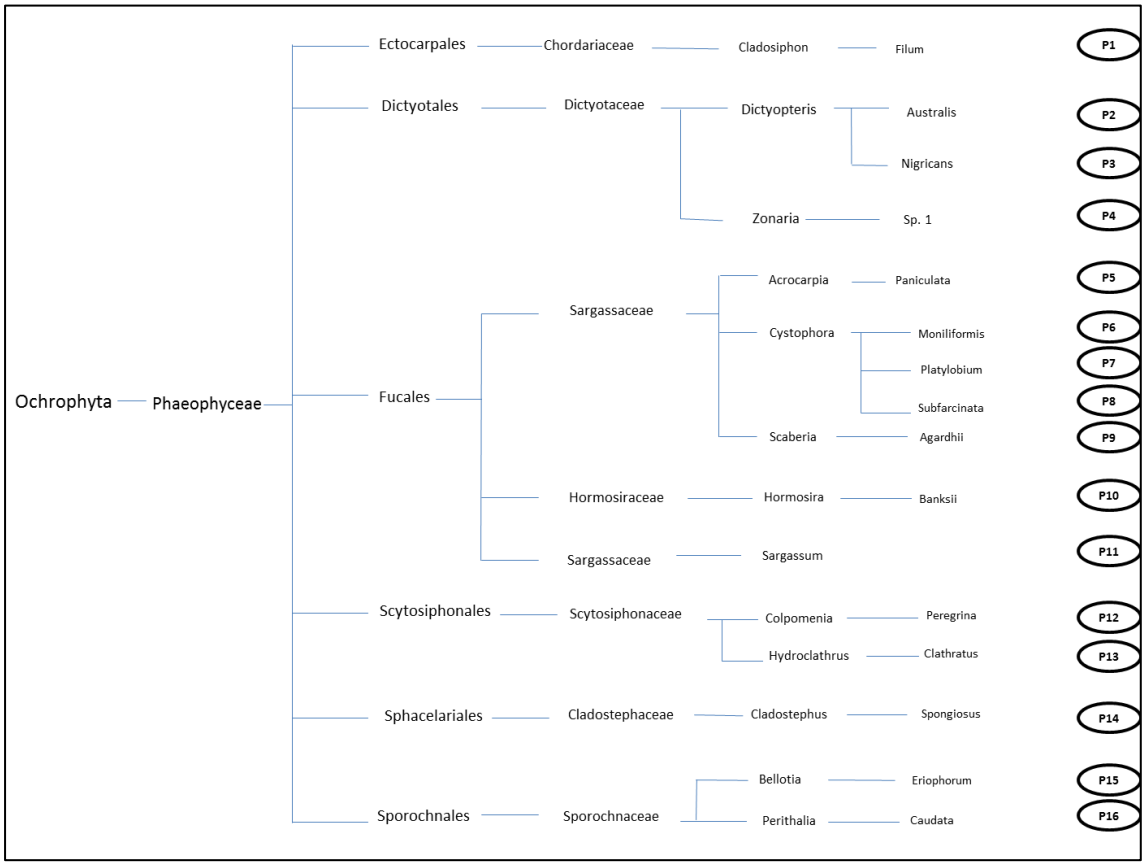

Figure 2-3 Phylogenetic distribution of brown algae species among order, family, and genus from South Australia used in this study

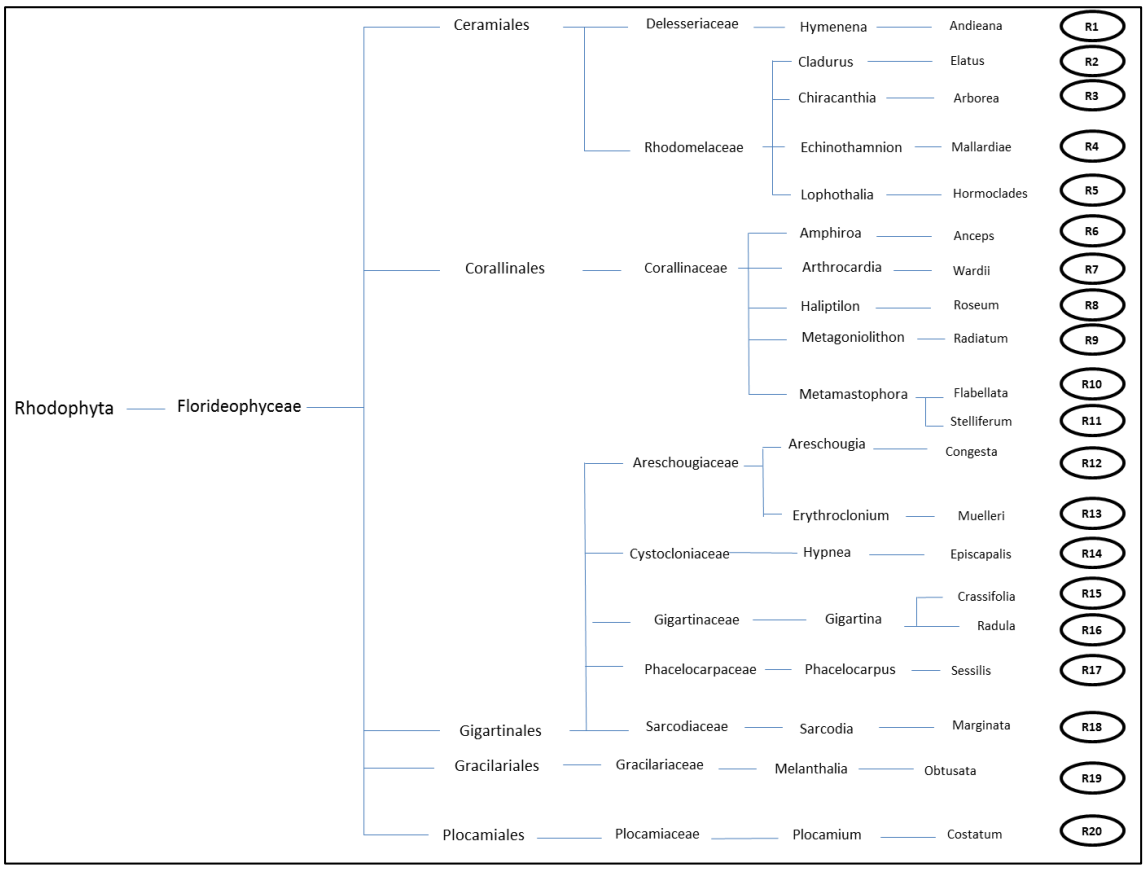

Figure 2-4 Phylogenetic distribution of red algae species among order, family, and genus from South Australia used in this study

Table 2-1 The location sites of the different algal and sponges species collected in South Australia

Sample code	Location	Sample code	Location
C9, C12 R17	Third way from Cape Jaffa to Margaret Brock Reef; near Kingston; S.A.	P1 R8, R11	Nore Creina Beach near Robe; South-East S.A.
D15, D19, D26 P2 R4, R5	Marion Reef off Edithburgh; Southern. Yorke Peninsula; S.A.	D27, D41	Kingston Jetty, far end; Kingston; SE S.A.
C2, C3	Jetty Piles; Cape Jaffa; near Kingston; S.A.	D5 P9, P11 R18, R19	D'estree's Bay; Kangaroo Island; South Australia
C11 D24 R1	Horseshoe Reef-0.65 miles; 355 degrees to Margaret Brock Light; near Kingston; S.A.	C6 D2, D6, D11, D32, D35, D36	American River; Green side of channel west of Strawbridge Point; Kangaroo Island; S.A
C5, C13	Coobowie Bay; Southern. Yorke Peninsula; S.A.	D1, D17, D29, D33, D42	Smith Bay; West of Cape D'estaing; Kangaroo Island; S.A.
C7, C10 D16, D31 P12	Margaret Brock Reef just near lighthouse (ne); near Kingston; S.A.	D7, D12, D13, D23 R6	Between Knob Point and Cape Cassini; Kangaroo Island; S.A.
C8	Cape Thomas; half way between Kingston and Robe; S.A.	D10, D21, D37 P7 R16	Point Ellen, Vivonne Bay; Kangaroo Island; S.A.
R15, R16	Port Mcdonnell Breakwater; Southern; S.A.	D3, D25	Cape D'estaing; North of Reef; Emu Bay; Kangaroo Island; S.A.
P13 R2, R3	Old Jetty Piles; Kingston Jetty; Kingston; SE; S.A.	D18, D20, D22	Smith Point; West of D'estaing; Kangaroo Island; S.A.
D40 P5, P15, P16 R10, R19, R15, R20	Godfrey Island; between Kingston and Robe; STHN. S.A.	D30, D34, D43	West of Cape D'estaing; Emu Bay; Kangaroo Island; S.A.
P3	Beachport Jetty; Beachport; S.E; S.A.	C4 P6, P8 R7, R9	Pandalowie Bay; South of lookouts; STHN Yorke Peninsula; S.A.
D14, D28, D39	1KM off Margaret Brock Lighthouse; Cape Jaffa; near Kingston; S.A.	P4, P10	Edithburgh Jetty, North of Jetty; Yorke Peninsula, S.A.
D9	Horseshoe Reef 3km wnw of Margaret Brock Lighthouse; Cape Jaffa; Kingston; S.A.	D8	Edge of Marine Reserve - Pelican Lagoon; American River; Kangaroo Island; S.A.
D38	Outside Port Mcdonnell - Deep Creek (old dairy factory creek); under bridge; S.A.	C1 P14	Point Turton Jetty; STHN. Yorke Peninsula; S.A.
D4	PORT GILES JETTY; Southern, YORKE PENIN.;S.A.		

2.2.2 Extract preparation

A small subsample was removed and placed in a glass vial. The sub-samples were freeze dried. After the samples were dried they were steeped in either ethanol or methanol then stored at -20°C. The extracts were frequently refreshed with either ethanol or methanol.

2.2.3 Sample Preparation for the Queensland Compound Library (QCL) /Compounds Australia

The extract collection was removed from the freezer one day prior to use and refreshed with methanol to the top of the vial and well mixed. A 975 µL aliquot was removed from the extract vial and pipetted into a 96 deep well Macrotitre plate. The solvent was removed under vacuum in a Savant Drier. An adhesive foil lid was placed on the samples and they were stored at -20°C until delivered to QCL/Compounds Australia on dry ice. QCL/Compounds Australia reconstituted the samples in DMSO to a final concentration of 5 mg/mL.

2.2.4 Preparation of sponge and macroalgae extracts samples for biological assays

Samples received from QCL were prepared in three different concentrations (0.05, 0.5 and 5 mg/ml) by dissolving the AIMS sample in DMSO. All samples were kept cold in the freezer at -20 °C.

2.2.5 Reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 97.5%) was purchased from Sigma-Aldrich (USA). The MTT was dissolved in 1×phosphate buffered saline (PBS) at 5 mg/ml, then filtered through 0.22 µm sterile filter (Sartorius Stedim Biotech, France) prior to use and then was stored in the freezer at -

20°C. RPMI-1640 medium was purchased from Sigma-Aldrich (USA). Foetal bovine serum (FBS) was purchased from Bovogen Biologicals (East Keilor, VIC, Australia). Penicillin/streptomycin and 10× trypsin EDTA were obtained from Thermo Fisher Scientific (Scoresby, VIC, Australia). Human amyloid-β_{1-42} protein ($A\beta_{1-42}$) was obtained from Mimotopes (Melbourne, VIC, Australia).

2.2.6 Cell lines and cell culture

PC-12 cells were grown in RPMI-1640 media with 10% Foetal Bovine Serum and 50 µg/ml penicillin and streptomycin. These cells were maintained in an incubator at 37 °C temperature and 5% CO_2.

PC-12 cells were treated with different concentrations of marine extract samples (0.25, 2.5, and 25 µg/mL) for 48 hours. The control group was treated with 1×PBS for the same amount of time. In addition, a solvent control contains 0.5% of DMSO in media was used to make sure that DMSO was not causing cell death. All marine extract samples were dissolved in DMSO with the final concentration less than 0.5% (v/v). All experiments were carried out in triplicate.

2.2.7 Cell viability assessment using MTT assay

The MTT assay is widely used to measure mitochondrial activity as an indicator of cell viability. PC-12 cells were seeded at 2×10^4 cells per well in 100 µl media in 96-well plates. The plates were kept in the incubator to attach the cells overnight. On the second day, the marine extracts were applied to the cells at final concentrations of 0.25, 2.5, and 25 µg/mL. After the treatment, the plates were incubated for 48 hours.

On the fourth day, the plate was taken out and the media removed before adding 100 µl of MTT solution at a concentration of 0.5 mg/ml in 1×PBS. The plate was kept in aluminium foil and incubated for further 2 hours at 37 °C. Then, the MTT mixture was removed from the wells and 100µl of DMSO (Sigma Aldrich) was added to each

well to dissolve formazan into a coloured product that can be measured by a plate reader. The plate was read in a microplate reader (μQuant) at 570 nm. The cytotoxicity of marine extracts calculated by comparing the absorbance to cells which were treated with PBS (these cells were considered as 100% cell viability).

2.2.8 Preparing Aβ for treating cells

DMSO at 1% was used to dissolve non-fibrillar $A\beta_{1-42}$ to yield a protein concentration of 3.8 mM. After that, sterile PBS was added to prepare a final concentration of 100 μM. Amyloid was then dispensed into aliquots and immediately frozen at −70 °C until required.

2.2.9 Neuroprotection study

To assess the neuroprotective activity, only extracts that showed more than 90% cell viability at three different concentration tested earlier were selected for this assay, as treating cells with 1 μM $A\beta_{1-42}$ causing 20-30% of cell death.

PC-12 cells were used to evaluate the potential neuroprotective effects of the extracts using the MTT assay. Cells were plated at 2×10^4 cells per well in RPMI-1640 and 10% fetal bovine serum. Cells were then treated with the extracts at two different concentrations (0.25 and 25 μg/ml), either alone or 15 minutes prior to the addition of 1 μM $A\beta_{1-42}$. All cells were incubated for 48 hours. MTT was then added (0.5 mg/ml for 2 hours) and cells were lysed with DMSO. Spectrophotometric absorbance at 570 nm was then measured to assess cell viability.

2.2.10 Statistical analysis

All results were based on at least three independent experiments (n = 3 different samples). The effects of marine extracts on the viability of PC12 cells were analysed using one way ANOVA, followed by Tukey's honestly significant difference (HSD)

post-hoc test for equal and unequal variances as appropriate. All data were analysed using SPSS software (Version 22). Differences were considered statistically significant when *p*-values were less than 0.05.

2.3 Results

2.3.1 Cytotoxicity of extracts of marine sponges and macroalgae from South Australia

The cytotoxicity of 93 marine extract samples were screened against PC-12 cells using the MTT assay at three different extract concentrations (0.25, 2.5, and 25 µg/ml). The results were analysed to determine which extracts did not kill more than 10% of cells in any concentration. Tables 2-2 – 2-5 shows the cytotoxicity screening results after treating with marine sponge extracts, green algae, brown algae and red algae, respectively. Twenty six out of 43 sponge extracts, six out of 13 green algae extracts, five out of 16 brown algae extracts, and ten out of 20 red algae extracts showed cytotoxicity, with cell viability being less than 90% at one of the three concentrations tested (Tables 2-2 – 2-5). Overall, there was nearly 50% of all extracts exhibited cytotoxicity at one of the three concentrations tested, with marine sponges the most toxic (60.5% of extracts) and brown algae the least toxic extracts (only 31.25% of extracts) (Table 2-2 – 2-4). Based on the taxonomic evaluation of sponges' species (Figure 2-1), all the species of Ircinia genus were toxic with the exception of D8. Two third of the species from Dictyoceratida and Haplosclerida orders were toxic. Six extracts out of 13 did not show cytotoxicity in Poecilosclerida order, with all of the extracts being cytotoxic in Chondropsidae, Coelosphaeridae, and Desmacididae families. In green algae (Figure 2-2), all the extracts from Codiaceae family were safe to PC-12 cells. On the other hand, Caulerpaceae family was the most

toxic family, as all of the species showed cell viability below 90% with the exception of C3. The taxonomic distribution of brown algae (Figure 2-3) showed that all the species of Sporochnales and Sphacelariales were not toxic to the cells, while almost one third of Fucales order species showing cytotoxic to PC12 cells. The taxonomic evaluation of red algae (Figure 2-4) showed that both Gracilariales and Plocamiales orders were cytotoxic, while all the species of Ceramiales order were not cytotoxic to PC-12 cells with the exception of R5.

Table 2-2 Summary of sponge extract toxicity against neuronal PC-12 cells at three different extract concentrations (n=3) (* p <0.05, ** p <0.01, and ***p <0.005 versus control PBS)

Sample No	Cell viability at different extract concentrations (%)			Sample No	Cell viability at different extract concentrations (%)		
	0.25 µg/mL	2.5 µg/mL	25 µg/mL		0.25 µg/mL	2.5 µg/mL	25 µg/mL
D1	78.7 ± 1.2 ***	78.7 ± 3.5 ***	75.2 ± 2.6 ***	D23	89.2 ± 1.7 *	96.5 ± 2.4	87.8 ± 2.1 *
D2	99.2 ± 2.1	96.6 ± 1.4	90.5 ± 5.1	D24	93.1 ± 3.5	92 ± 2.1	86.2 ± 2.6 *
D3	90.9 ± 3.6	90.7 ± 2.5	94.6 ± 1.1	D25	95.6 ± 1.3	93.3 ± 0.5	84.7 ± 2.9***
D4	92 ± 3.6	76.8 ± 9 *	84 ± 1.2	D26	81.1 ± 6.3 *	87.3 ± 2.2	80.9 ± 1.7 *
D5	99.8 ± 3.6	92.4 ± 1.6	88.1 ± 3.5	D27	79.5 ± 5.2 **	79.9 ± 1.8 **	80.2 ± 0.3 **
D6	81.9 ± 2.3 **	76 ± 3.3 ***	76.8 ± 2.6 ***	D28	88.5 ± 2.3 *	87.4 ± 2.6 *	89.9 ± 0.5 *
D7	93.3 ± 6.9	95.1 ± 1.5	77.3 ± 3.6 *	D29	80.7 ± 1.1 **	78.9 ± 4.9***	82.8 ± 1.2 *
D8	90.3 ± 2.6	90.2 ± 3.3	93 ± 4.4	D30	90.4 ± 0.4	90.9 ± 3.5	90.5 ± 2.3
D9	75.1 ± 1.7 ***	74.6 ± 1.1 ***	68.8 ± 2 ***	D31	97.3 ± 1.6	99..2 ± 0.2	96.1 ± 1.6
D10	95.7 ± 0.9	100.9 ± 3.1	94.4 ± 2.6	D32	96 ± 5.3	93.3 ± 1.4	82.4 ± 6.6

D11	96.4 ± 1.7	96.1 ± 0.5	94.2 ± 0.8	D33	95.7 ± 2.6	92.9 ± 2.5	91.3 ± 0.9
D12	84.6 ± 3.7 *	84.6 ± 2.2 *	78.5 ± 1.6 ***	D34	89.9 ± 2.4	89.7 ± 1.2	92.2 ± 3.2
D13	92.5 ± 3.6	85.8 ± 2.4	87.4 ± 1.9	D35	98.5 ± 1.8	100.6 ± 2.9	91.7 ± 0.8 *
D14	71.9± 8 **	74 ± 1.3 *	70.7 ± 1 **	D36	93.2 ± 3.9	90.3 ± 1.9	93.2 ± 0.3
D15	83.5 ± 1.9 ***	82.9 ± 1.4 ***	86.7 ± 1.3 **	D37	88.1 ± 0.9 *	89.1 ± 1.4 *	87.9 ± 2.8 *
D16	95.4 ± 2.1	92.5 ± 4.8	94.8 ± 3.6	D38	75.9 ± 1.5 *	71.6 ± 5.4 **	74.6 ± 0.9 **
D17	92.9 ± 1.3	95.7 ± 2.6	29.9 ± 1.3 ***	D39	101.7 ± 2.2	100.6 ± 2.3	96.3 ± 0.7
D18	99.4 ± 3.8	99.6 ± 2.8	39.4 ± 2.5	D40	105.8 ± 0.8	95.2 ± 2.8	95.4 ± 3.2
D19	96.4 ± 2	90.8 ± 2.1	96.3 ± 2.7	D41	91.5 ± 1.8	87.4 ± 2.9 *	87.6 ± 1.1 *
D20	80.2 ± 2.9 ***	80.7 ± 2.6 ***	80.3 ± 1.5 ***	D42	96.9 ± 3.5	94.3 ± 3.2	102.2 ± 2.5
D21	95.8 ± 4.8	96.5 ± 2.3	94.2 ± 2.5	D43	95.6 ± 1	100.1 ± 2.3	97.6 ± 0.2
D22	84 ± 0.8 ***	72.1 ± 3.3 ***	7.7 ± 0.5 ***				

Table 2-3 Summary of green algae extract toxicity against neuronal PC-12 cells at three different extract concentrations (n=3) (* p <0.05, ** p <0.01, and ***p <0.005 versus control PBS)

Sample No	**Cell viability at different extract concentrations (%)**			**Sample No**	**Cell viability at different extract concentrations (%)**		
	0.25 µg/mL	**2.5 µg/mL**	**25 µg/mL**		**0.25 µg/mL**	**2.5 µg/mL**	**25 µg/mL**
C1	83.9 ± 1.2 **	87.5 ± 2.1 *	80.4 ± 2.8 ***	C8	94.9 ± 1.3	92.4 ± 2.9	92.2 ± 2.3
C2	86.6 ± 4.8	82.5 ± 8.9	80.3 ± 5.5	C9	90 ± 2.7	91.9 ± 2.7	91.2 ± 2.2
C3	101.2 ± 0.2	98.9 ± 2.1	100.6 ± 0.9	C10	90.3 ± 1.4	91.3 ± 2.1	90.4 ± 2.9

C4	87.2 ± 1.3 ***	87.4 ± 1.1 ***	85.9 ± 1 ***	C11	96.9 ± 3.2	92.9 ± 1.4	94.7 ± 2.3
C5	80.9 ± 2.2 ***	81.3 ± 2 ***	78.3 ± 1.4 ***	C12	99.9 ± 2.3	98.7 ± 0.6	91.1 ± 3.2
C6	82.8 ± 2.9 ***	79.6 ± 0.2 ***	72.9 ± 1 ***	C13	91.3 ± 3.1	88.5 ± 2.3	85.9 ± 2.2 *
C7	101.9 ± 3.9	98.6 ± 4.6	98.7 ± 0.4				

Table 2-4 Summary of brown algae extract toxicity against neuronal PC-12 cells at three different extract concentrations (n=3) (* p <0.05, ** p <0.01, and ***p <0.005 versus control PBS)

Sample No	Cell viability at different extract concentrations (%)			Sample No	Cell viability at different extract concentrations (%)		
	0.25 µg/mL	2.5 µg/mL	25 µg/mL		0.25 µg/mL	2.5 µg/mL	25 µg/mL
P1	88.9 ± 0.7	86.5 ± 4.8	84.2 ± 3.9 *	P9	95.7 ± 3.6	92.5 ± 2.7	90.4 ± 1.7
P2	102.1 ± 0.6	104.2 ± 2	101.2 ± 1.8	P10	96.2 ± 0.8	91.9 ± 2.2	98.9 ± 2.1
P3	92 ± 2.7	92.4 ± 2.3	92.1 ± 2.1	P11	88 ± 2.8 *	83.6 ± 0.5***	81.7 ± 2.1***
P4	85.3 ± 2.9 **	89.4 ± 1.3 **	84.4 ± 1.6 **	P12	88.9 ± 3.2	91.8 ± 1.7	87.9 ± 2.1 *
P5	97.3 ± 2	93.5 ± 3	92.1 ± 1.9	P13	97.7 ± 2.7	92.5 ± 2.6	98.1 ± 2.4
P6	91.3 ± 1.9	94.2 ± 2.7	92.8 ± 3.7	P14	91.8 ± 0.9 *	90.4 ± 1.3 *	91.9 ± 1.3 *
P7	97.4 ± 1.5	96.9 ± 1.1	108.1 ± 2.5	P15	98.9 ± 1.7	95.4 ± 2	92.8 ± 1.7
P8	87.3 ± 1.5 *	87.9 ± 1.6 *	82.9 ± 2.1 ***	P16	90.9 ± 0.8 *	96.3 ± 2.2	92.7 ± 1.5

Table 2-5 Summary of red algae extract toxicity against neuronal PC-12 cells at three different extract concentrations (n=3) (* p <0.05, ** p <0.01, and ***p <0.005 versus control PBS)

Sample No	Cell viability at different extract concentrations (%)			Sample No	Cell viability at different extract concentrations (%)		
	0.25 µg/mL	2.5 µg/mL	25 µg/mL		0.25 µg/mL	2.5 µg/mL	25 µg/mL
R1	94.4 ± 3.3	95.2 ± 2.7	97.9 ± 1.3	R11	93.6 ± 1.5	94.3 ± 2.7	90 ± 1.3 *
R2	90.7 ± 0.3 *	90.3 ± 0.5 *	90.3± 1.3 *	R12	94.5 ± 0.5	92.6 ± 2.2	92.3 ± 0.7
R3	94.9 ± 0.9	94.3 ± 1.6	97.5 ± 2.4	R13	87.4 ± 0.8*	84.9 ± 1.4 **	82.9 ± 2.9***
R4	90.3 ± 5.9	100.6 ± 2.3	90.4 ± 1.3	R14	98.2 ± 1.4	98.7 ± 4.6	85.9 ± 2.8*
R5	86.2 ± 0.6 ***	87.7 ± 0.6 ***	86.4 ± 2 ***	R15	77.9 ± 0.7***	82.5 ± 1.6 **	83.5 ± 4.2 **
R6	77.4 ± 0.5 ***	78.9 ± 1.1 ***	76.2 ± 0.9 ***	R16	86.2 ± 0.6 *	87.8 ± 3.7	88.6 ± 3.2
R7	91.7 ± 3.2	91.8 ± 1.3	96 ± 1.4	R17	89.6 ± 3.3	88.5 ± 1.5 *	88.9 ± 2.1
R8	93.9 ± 1.4	96.5 ± 2.8	92.6 ± 3.3	R18	75.4 ± 0.5***	74.1 ± 0.4***	81 ± 1***
R9	89.6 ± 1.1 *	85 ± 1.9 ***	86.6 ± 1.4 **	R19	85.1 ± 2.2 *	85.9 ± 0.3 *	83.6 ± 3.3 **
R10	94.6 ± 2	93 ± 1.7	95.1 ± 2.7	R20	99 ± 2.9	97.6 ± 3.6	99.9 ± 5.6

2.3.2 Neuroprotective activity of extracts of marine sponges and macroalgae from South Australia

Only these extracts at any of these three concentrations showing cell viability of more than 90% were used in this assay. The sponge and macroalgae extracts were applied to PC-12 cells 15 minutes before adding $A\beta_{1-42}$ (1 µM) and the cells were incubated for 48 hours before assessing cell viability. Treating the cells with $A\beta_{1-42}$ alone resulted in 25-35% reduction in cell viability (Figure 2-5 – 2-8; $P<0.05$). Sponge extracts demonstrated a varied degree of cell protection from the toxicity induced by

$A\beta_{1-42}$ (Figure 2-5). 76% of the sponge extracts (13) showed neuroprotection at the highest concentration (25 µg/ml). More than half of the green algal and brown algal extracts significantly reduced the toxicity of $A\beta_{1-42}$ (Figure 2-6 and Figure 2-7), while only half of the red algae extracts showed neuroprotective activity (Figure 2-8). Interestingly, five extracts (D8, D16, D19, D21, and D40) from marine sponges showed neuroprotective activities in both tested concentrations, while ten extracts from algae [2 from green algae (C9 and C10), 4 from brown algae (P2, P3, P9, and P10), and 4 from red algae (R1, R3, R4, and R11)] were active in protecting the cells from the toxicity of Aβ in both tested concentrations. Based on the taxonomic distribution of sponges' species, extracts from all nontoxic species of Dendroceratida, Dictyoceratida, and Haplosclerida orders were neuroprotective to cells against the toxicity induced by of $A\beta_{1-42}$. Three species of the Haplosclerida order were active in both concentrations, which highlighted the potential of these orders for providing neuroprotective compounds. In green algae, extracts from half of nontoxic species of Codiaceae family showed significant neuroprotective activity. In brown algae, both extracts from Dictyopteris genus showed significant neuroprotective activities in both tested concentrations. 80% of nontoxic extracts from Fucales order were shown to protect PC-12 cells against $A\beta_{1-42}$, while all extracts from Sporochnales order failed to provide any protection. In red algae, 75% of nontoxic extracts from Ceramiales order showed neuroprotective activities, while extracts from half of species from Corallinales order were neuroprotective. Gigartinales order did not provide any neuroprotective extracts.

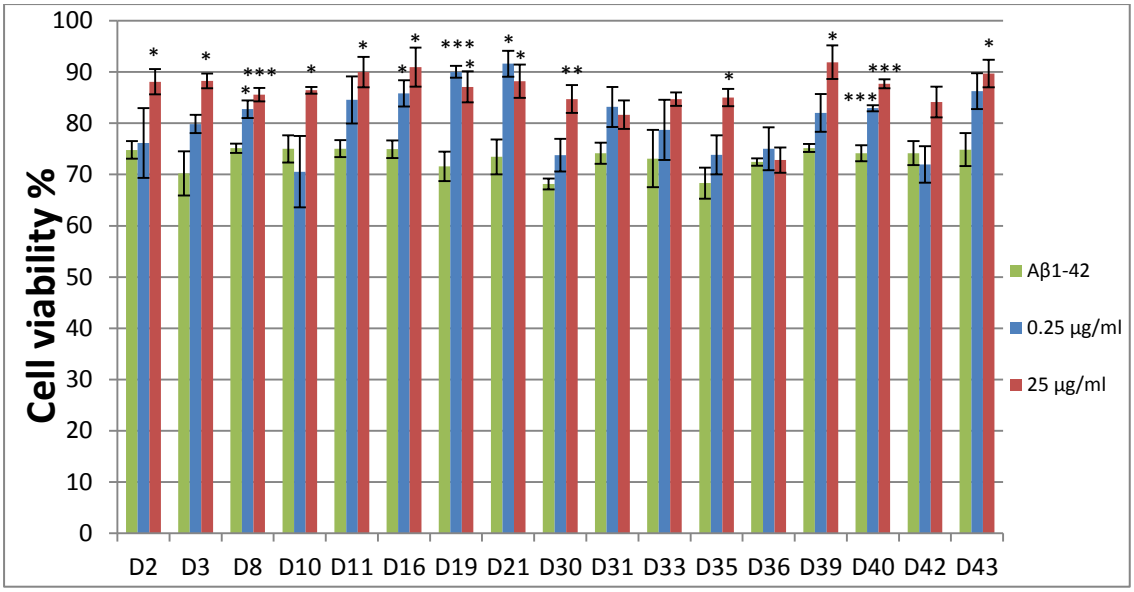

Figure 2-5 Neuroprotection activity of sponge extracts against $A\beta_{1-42}$ induced cytotoxicity in PC-12 cells using MTT assay showing relative cell viability (%). Each value is the mean ± SEM of three independent experiments (* p <0.05, ** p <0.01, and ***p <0.005 versus control Aβ)

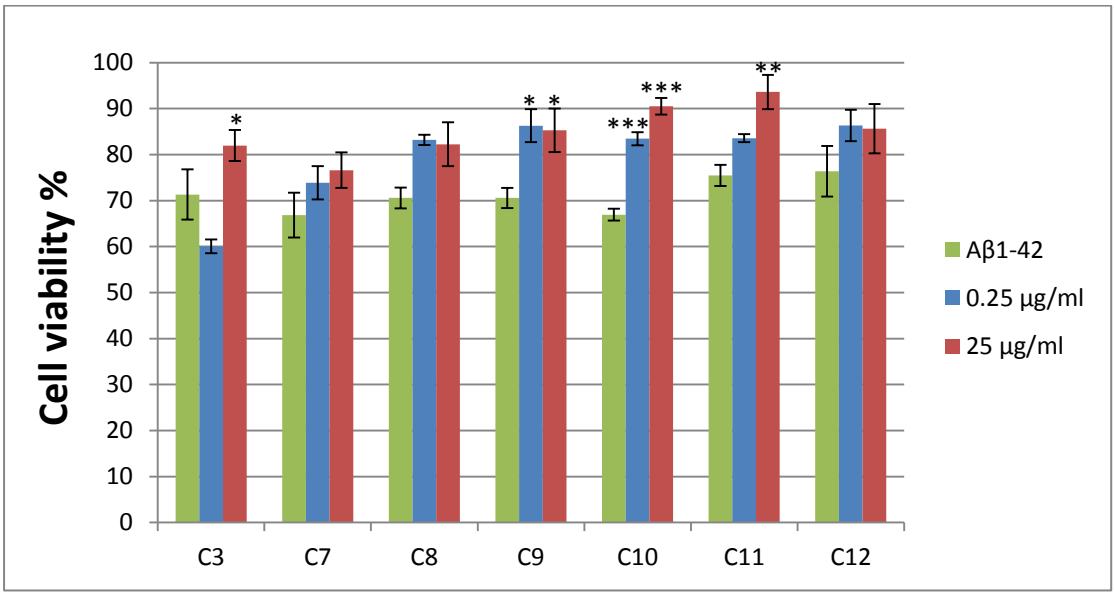

Figure 2-6 Neuroprotection activity of green algae extracts against $A\beta_{1-42}$ induced cytotoxicity in PC-12 cells using MTT assay showing relative cell viability (%). Each value is the mean ± SEM of three independent experiments (* p <0.05, ** p <0.01, and ***p <0.005 versus control Aβ)

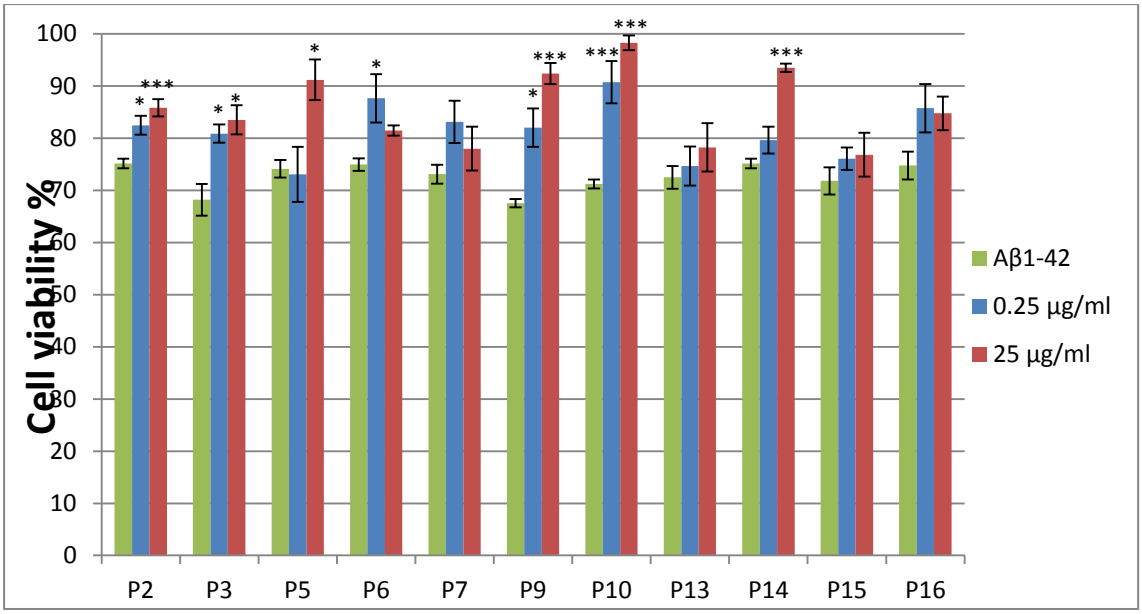

Figure 2-7 Neuroprotection activity of brown algae extracts against $A\beta_{1-42}$ induced cytotoxicity in PC-12 cells using MTT assay showing relative cell viability (%). Each value is the mean ± SEM of three independent experiments (* *p* <0.05, ** *p* <0.01, and ****p* <0.005 versus control Aβ)

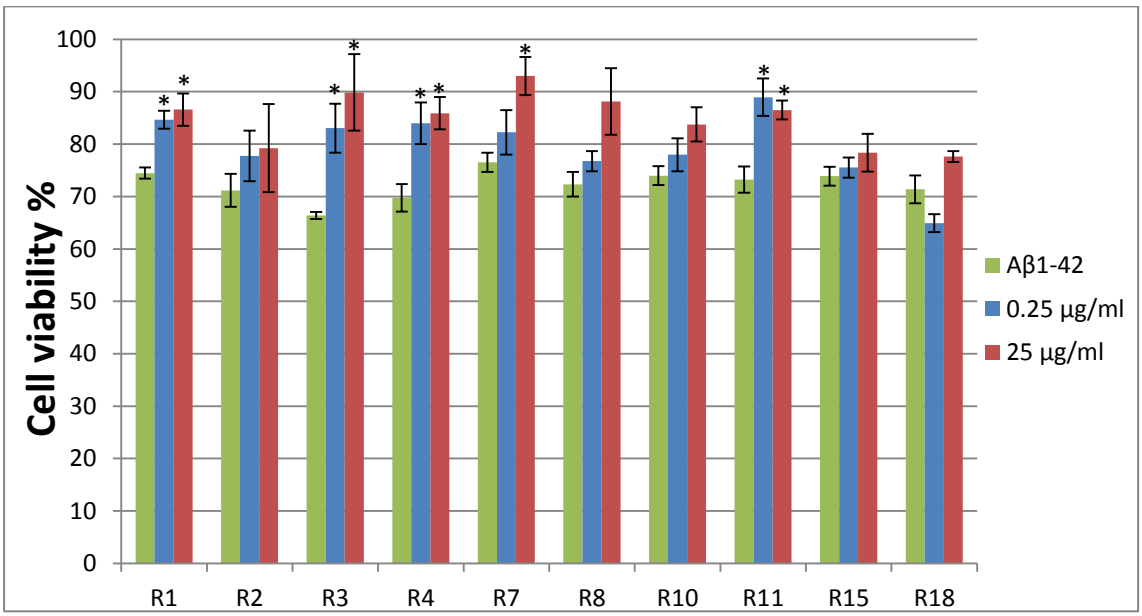

Figure 2-8 Neuroprotection activity of red algae extracts against $A\beta_{1-42}$ induced cytotoxicity in PC-12 cells using MTT assay showing relative cell viability (%). Each value is the mean ± SEM of three independent experiments (* *p* <0.05, ** *p* <0.01, and ****p* <0.005 versus control Aβ)

2.4 Discussion

60% of the marine sponge extracts tested (26 out of 43 extracts) at a concentration range of 0.25-25µg/ml were cytotoxic to PC-12 cells. Cytotoxic compounds in sponges are common given that half of the anticancer compounds discovered during 2001 to 2010 were isolated from sponges (Mehbub et al., 2014). In addition, a study showed that four dictyodendrins isolated from *Ianthella* sp. were shown to be active in reducing the activity of beta-site amyloid precursor protein cleaving enzyme (BACE); however three of them were cytotoxic to SW620 cells (Zhang et al., 2012b). Three extracts (D17, D18, and D22) belonging to the Haplosclerida order showed the highest cytotoxicity, with cell viability less than 40%. The lethal concentration 50 (LC50) of samples D17, D18, and D22 are 17.7, 20.9, and 10.8 µg/mL, respectively.

It is significant though that 76% (13 out of 17) of those non-toxic sponge extracts demonstrated promising neuroprotective activity, by reducing the cytotoxicity induced by Aβ.

For all the three algae classes combined, 43% of the algae extracts (21 out of 49 algae extracts) were shown to reduce cell viability to less than 90%. The algae extracts are less toxic when compared with the sponge extracts. Among these three algae classes, brown algae extracts showed the least toxic, with only 31%, when compared with 46% for green algae and 50% for red algae.

In contrast to the cytotoxicity trend, the highest percentage (44%) of brown algae extracts demonstrated neuroprotective activity against Aβ-induced cytotoxicity to PC-12 cells, followed by 31% green algae and 25% red algae.

Based on the location sites, extracts with demonstrated neuroprotective activities were collected from 19 location sites. Two location sites were shown to have three

neuroprotective extracts, which was American River with three sponge extracts (D2, D11, and D35) and Marion Reef with 1 sponge and 2 macroalgae extracts (D19, P2, R4). In addition, six locations which include Point Ellen (D10 and D20), Margrete Brock (D16, C10), West of Cape (D30 and D43), Godfrey Island (D40 and P5), Horseshoe Reef-0.65 miles (C11 and R1), and Pandalowie Bay (P6 and R7) showed to have 2 extracts with neuroprotective activities. This highlights the diverse activities among different location sites.

Based on sponge taxonomy, all of the orders in this study with the exception Biemnida order have been reported before to have compounds with neuroprotective activities. In addition, 7 families (Dysideidae, Ircinidae, Thorectidae, Niphatidae, Desmacididae, Halichondriidae, and Aplysinidae) have been reported before to have neuroprotective compounds (Alghazwi et al., 2016a).

Table 2-6 Summary of sponge, green algae, brown algae, and red algae showing cytotoxicity and neuroprotective activity against PC-12 cells

Marine organism	Total screened extracts	Total toxic extracts	Total neuroprotective extracts
Sponge	**43**	**26 (60%)**	**13 (30%)**
Green algae	**13**	**6 (46%)**	**4 (30.7%)**
Brown algae	**16**	**5 (31.25%)**	**7 (43.75%)**
Red algae	**20**	**10 (50%)**	**5 (25%)**
Total	**92**	**47 (51.1%)**	**29 (31.5%)**

Even though many sponges and macroalgae extracts were reported to show neuroprotective activity previously from similar families to the one used in our studies, many unreported families in this study were found to possess neuroprotective activity for the first time. From sponge samples, only four of these extracts were

reported previously from a similar family, and nine of these extracts were reported for the first time. Interestingly, all of the green algae extracts were reported before by other researchers from a similar family to show neuroprotective activity. In red algae, 2 families were reported previously, and 3 families were yet to reported (Table 2-7). All brown algal extracts are reported here to show neuroprotective activity for the first time. This highlights the diverse species in South Australia, and also their great potential for neuroprotective drug discovery, as all the species are reported for the first time.

In contrast, lack of neuroprotective activity or cytotoxicity was presented in some extracts from similar family or genus to the reported neuroprotective compounds in the literature. This include *Cribrochalina* genus, as extract D23 was toxic and excluded from further studies. Two compounds, namely cribronic acid (2S,4R,5R)-5-hydroxy-4- sulfooxypiperidine-2-carboxylic acid and (2S,4S)-4-sulfooxypiperidine-2-carboxylic acid, isolated from *Cribrochalina olemda* have reported to have glutamate receptor inhibition activity (Sakai et al., 2003). Hymenialdisine isolated from *Halichondriidae* demonstrated kinase inhibition by reducing the activity of GSK- 3β and CK1 (Meijer et al., 2000). However, in our screening, extract D14 isolated from *Halichondriidae* was toxic, with observed cell viability of less than 80%. Three compounds; spiralisone A, spiralisone B and chromone 6 were isolated from *Zonaria spiralis* and found to inhibit kinase activity (Zhang et al., 2012d). Extract P3 is from the same genus *Zonaria* and was found to be toxic to PC-12 cells. Many compounds isolated from *Sargassum* genus demonstrated neuroprotective activities. Compounds such as (5E,10Z)-6,10,14-Trimethylpentadeca-5,10-dien-2,12-dione and (5E,9E,13E)-6,10,4-trimethylpentadeca-5,9,13-trien-2,12-dione were previously shown to reduce cholinesterase activity (Ryu et al., 2003). Fucoxanthin demonstrated DPPH radical-

scavenging activity (Yan et al., 1999) and inhibited cell toxicity induced by hydrogen peroxide (Heo et al., 2008). Three plastoquinone and nineteen meroditerpenoid compounds demonstrated antioxidant activity by reducing lipid peroxidation and radical scavenging against DPPH (Mori et al., 2005), (Jung et al., 2008). Alginic acid has shown anti-inflammatory activity (Sarithakumari and Kurup, 2013) and four compounds; MC14 (Tsang et al., 2001), sargaquinoic acid (Kamei and Tsang, 2003), sargachromenol (Tsang et al., 2005) and pheophytin A (Ina et al., 2007) have shown enhanced neuronal growth. Only one extract, P11 of *Sargassum* genus tested in our study reduced cell viability to less than 90%.

The most promising orders from sponge taxonomy are the Haplosclerida order, as both provided three neuroprotective extracts at both tested concentrations. Moreover, the Dictyoceratida order provided four neuroprotective extracts. From green algae, *Codium* genus seems to be promising as it provides three neuroprotective extracts. From brown algae, two neuroprotective extracts were isolated from *Dictyopteris* genus and three others from the Sargassaceae family. The Ceramiales order provided three neuroprotective extracts, while Corallinaceae family provided two in the red algae. For that reason, more research should be conducted in these families and orders to derive neuroprotective compounds.

Table 2-7 Summary of reported compounds/extracts from sponge, green algae, brown algae, and red algae from similar family or genus in this study with their reported activities

Genus/ Family	Extract number	Reported compound/ extract	Reported activity	Reference
Dysidea genus	D3	Dysiherbaine	kainic acid (KA) receptor and [3H]α-amino-3-hydroxy-5-methyl-4- isoxazolepropionic acid (AMPA) receptor inhibitors	(Sakai et al., 2001b)
		Neodysiherbaine A	Inhibiting KA and AMPA receptors	(Sakai et al., 2001a)
		Dysibetaine CPa and dysibetaine CPb	Inhibiting N-methyl-D-aspartic acid-type (NMDA) and KA receptors	(Sakai et al., 2004)
		Dysideamine	Reduce reactive oxygen species (ROS) formation	(Suna et al., 2009)
Ircinia genus	D8	Methanol extracts from *Ircinia spinulosa* and *Ircinia fasciculate*	Acetylcholinesterase inhibitor activity	(Orhan et al., 2012)
		Isopropanolic extract from *Ircinia dendroides*	GSK-3β	(Bidon-Chanal et al., 2013)
Amphimedon genus	D21	Ethyl acetate extract from Amphimedon navalis	Acetylcholinesterase inhibition activity	(Beedessee et al., 2013)
Aplysina genus	D40	NP04634 from Aplysina cavernicola	Reduce to toxicity in bovine adrenal medullary chromaffin induced by calcium	(Valero et al., 2009)
Caulerpa genus	C3	Racemosin A and racemosin B	BACE inhibitors	(Liu et al., 2013)

		α-tocospirone, (23E)-3β-hydroxystigmasta-5,23-dien-28-one, and (22E)-3β-hydroxycholesta-5,22-dien-24-one	BACE inhibitors	(Yang et al., 2015)
		Caulerpin	Anti-inflammatory effects	(de Souza et al., 2009)
Codium genus	C9, C10, and C11	Clerosterol isolated from *Codium fragile*	Anti-inflammatory activity	(Lee et al., 2013)
Rhodomelaceae family	R3 and R4	3-(2,3-Dibromo-4,5-dihydroxybenzyl)pyrrolidine-2,5-dione, methyl 4-(2,3-Dibromo-4,5-dihydroxybenzylamino)-4-oxobutanoate, 4-(2,3-Dibromo-4,5-dihydroxybenzylamino)-4-oxobutanoic acid, 3-Bromo-5-hydroxy-4-methoxybenzamide, and 2-(3-Bromo-5-hydroxy-4-methoxyphenyl) acetamide	Reduce oxidative stress	(Li et al., 2012)

The extracts that have shown positive results by inhibiting Aβ toxicity in PC-12 cells are likely to work via different and possibly multiple mechanisms. These mechanisms include anti-aggregation effects against Aβ, anti-oxidant activity, or kinase inhibitory activity. Another possible mechanism is that the extracts are able to reduce reactive oxygen species (ROS) which occurs as a consequence of Aβ exposure (Butterfield

and Boyd-Kimball, 2004). For example, pinostrobin was found to inhibit the toxicity of Aβ via inhibiting intracellular ROS (Xian et al., 2012). Aβ can induce apoptosis as well, so these extracts might inhibit caspase 3 and 9, or inhibit Bcl and Bax. Previously, astaxanthin has been found to inhibit Aβ toxicity via the apoptosis pathway, inhibiting Bax and upregulating Bcl-2, while also upregulating HO-1 expression (Wang et al., 2010).

One limitation in this study is the use of MTT assay as earlier research revealed that Aβ can effect cell cultures whereas more complex tissue such as hippocampal slices are more resistant to it (Rönicke et al., 2008). As this study was an initial study to evaluate the potential of South Australia marine sponges and algae as sources of neuroprotective compounds, future work will validate these results using *in vivo* model.

Further studies are required to isolate and purify the active compounds from these active extracts identified. Novel compounds are expected to be discovered by further mechanistic insight into cellular pathways for neuroprotection.

2.5 Conclusion

This study has demonstrated that marine sponges and macroalgae collected in South Australia are a potential source of neuroprotective compounds, with one-third of 92 extracts screened (29 extracts) found to reduce neurotoxicity induced by Aβ in PC-12 cells. More than half of these extracts (15 extracts out of 29 extracts) were active in both tested concentrations. 65% of these sponge and macroalgae extracts from genus that have not been reported before, to demonstrate protection against Aβ-neurotoxicity. These results are of significance for further studies isolating and characterizing the active compounds from these extracts and understand their mechanisms of actions for potential neuroprotective drug discovery and development.

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3. CHAPTER 3: *IN SILICO* SCREENING OF NEUROPROTECTIVE COMPOUNDS REPORTED FROM MARINE SPONGE AND ALGAE AGAINST AMYLOID BETA (AB) AGGREGATION

Contribution: Mousa Alghazwi has done all the computational experiments, analysed all the data, and wrote the manuscript. Wei Zhang and Scott Smid provide feedback to improve the work and helped in editing the manuscript.

3.1 Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease responsible for 70% of dementia cases (Florent-Béchard et al., 2007). AD can be characterized by the presence of senile plaques and neurofibrillary tangles (NFTs) (Selkoe, 1991). One of the main hallmark of AD is the presence of amyloid beta (Aβ) protein that can induce neurotoxicity in neuronal cells (Tang et al., 2006). The second hallmark of AD is the presence of neurofibrillary tangles, which are produced by the accumulation of hyperphosphorylated tau protein (Avila et al., 2006).

Several strategies have been suggested to enable disease-modifying AD treatments, which include modulation of Aβ production either by inhibiting γ-secretase and β-secretase, or by enhancing α-secretase. The other strategies include inhibiting Aβ aggregation and promoting Aβ clearance. For tau protein-targeting strategies, this includes inhibiting tau hyperphosphorylation and targeting kinases such as glycogen synthase kinase 3 (GSK3), MAP/ microtubule affinity-regulating kinase 1 (MARK1), and cyclin-dependent kinase 5 (CDK5) (Citron, 2010).

Typically, drug discovery starts with screening different compounds for specific bioactivities. The bioactive compounds identified are further studied for their toxicity, pharmacokinetic properties, and metabolism. Most drug candidate failures at this stage come from poor pharmacokinetics (39%) followed by lack of efficacy, animal toxicity, adverse effects in human and other commercial reasons (Van De Waterbeemd and Gifford, 2003). Many compounds have neuroprotective activity in reducing the toxicity of Aβ and/or inhibiting the fibril formation, however these compounds have generally failed in further development for a variety of reasons

related to lack of efficacy and poor pharmacokinetics or toxicity. A study examining candidate drugs for AD in clinical trials between the period 2002 and 2012 found that there were 413 trials (30% in phase 1, 50% in phase 2, and 20% in phase 3). The amount of candidates proceeding to the next phase was found to be low (0.4%) (Cummings et al., 2014).

In addition, drug discovery can be expensive and time consuming. Therefore, *in silico* modelling using computational methods can reduce these disadvantages of traditional drug discovery, as these methods can find promising candidate compounds in a more timely and cost-effective manner (Jorgensen, 2004).

The aim of this study was therefore to identify promising candidate compounds in inhibiting Aβ and tau proteins by evaluating *in silico* the drug-likeness, the potential to pass the blood-brain barrier, and the docking scores of neuroprotective compounds reported from marine sponges and algae in other neuroprotective assays. These promising candidate compounds can be tested in future *in vitro* and *in vivo* assays to accelerate the discovery and development of preventive and therapeutic treatments for AD.

3.2 Materials and methods

3.2.1 Compounds selected from marine sponges and macroalgae

Five natural products were used as positive reference compounds, while 57 compounds from marine sponges and 26 compounds from marine algae based on their reported neuroprotective activities and their 3D structure availability.

3.2.2 Ligand preparation

Chemical 3D structures of the selected neuroprotective compounds reported for marine sponges and macroalgae were taken from (https://pubchem.ncbi.nlm.nih.gov/) or (http://www.chemspider.com/). Then, the SMILE files of ligand were translated using the cactus website (https://cactus.nci.nih.gov/translate/). These compounds were docked with 3 forms of Aβ which are Aβ monomer (PDB: 1IYT), Aβ pentamer (PDB: 2BEG), and Aβ fibrils (PDB: 2LMQ).

3.2.3 Molecular docking

The binding interactions of these ligands with their target compounds were studied using CLC Drug Discovery Workbench, version 1.5.1 (http://www.clcbio.com/products/clc-drugdiscovery-workbench). All compounds structures were taken from either (https://pubchem.ncbi.nlm.nih.gov/) or (http://www.chemspider.com/) and then the file was translated to PDB format using (https://cactus.nci.nih.gov/translate/). Then, the ligands were extracted from these compounds by using the docking function on each form of Aβ (monomer, pentamer, and polymer) with 300 iterations. The binding site of each protein was set as maximum where pharmacophores were not known. The results then were summarized in the Table 3-1 - 3-3.

3.2.4 Screening for Drug-likeness

Screening the compounds for drug-likeness was performed using DruLiTo software (http://www.niper.gov.in/pi_dev_tools/DruLiToWeb/DruLiTo_index.html), which was based on Lipinski's rule of five, which are molecular weight ≤ 500, LogP ≤5, H-bond donor ≤5, and H-bond acceptor ≤10 (Lipinski, 2004). In addition, blood-brain barrier (BBB) likeness were analysed, which is based on molecular weight ≤ 400, H-

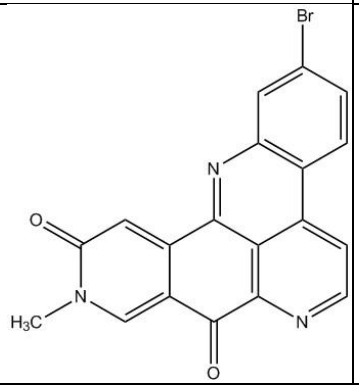
bond total ≤8, and absence of acidic groups. The results of the drug-likeness were summarized in Table 3-1. The detailed results can be found in appendix 3.

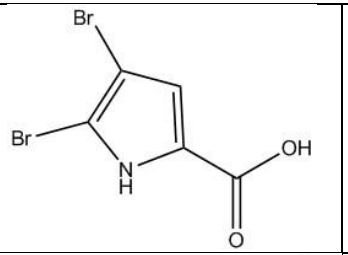
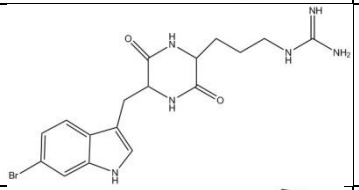
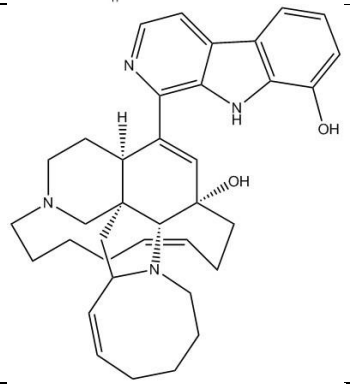
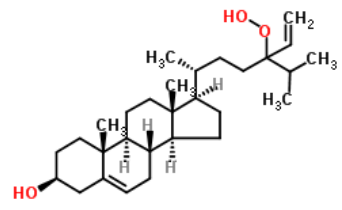
3.3 Results and discussion

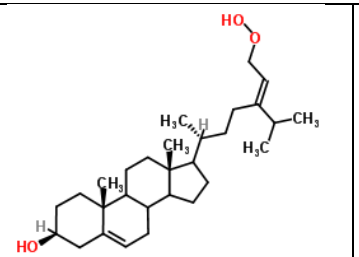
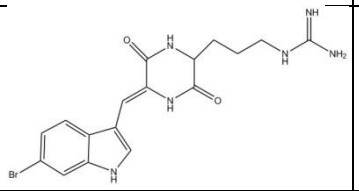
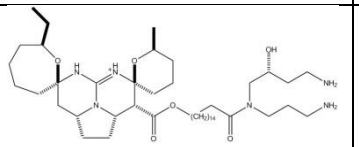
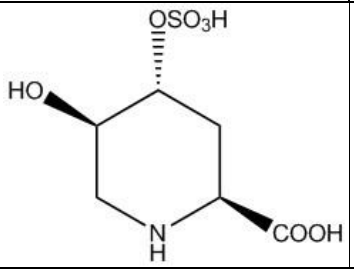
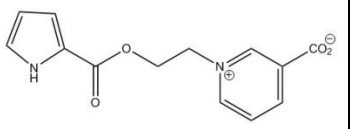
3.3.1 Molecular docking results

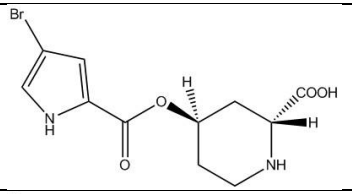
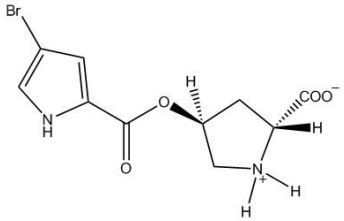
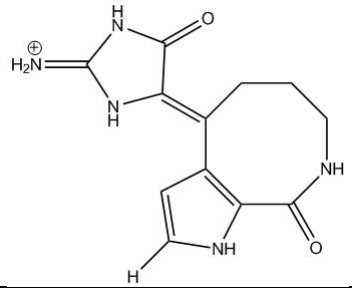
Molecular docking results are presented below for sponges (Table 3-1), algae (Table 3-2) and natural product references (Table 3-3).

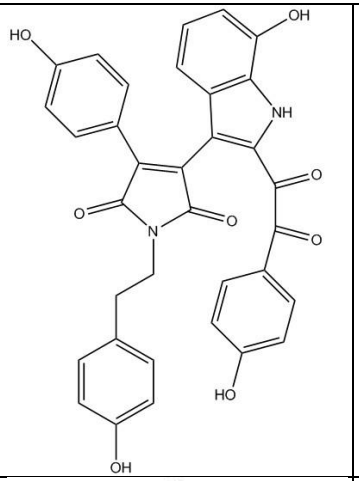
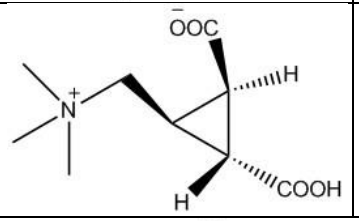
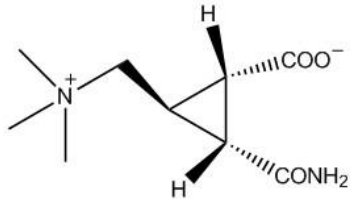
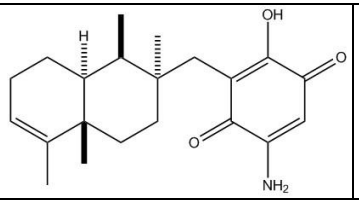
Table 3-1 Summary of the docking scores results of 57 marine sponge derived compounds. The information includes, the compound name, source sponge species, structure, PubChem CID number, molecular weight, neuroprotective activity, docking scores against three forms of Aβ, Lipinski rules and BBB likeness.

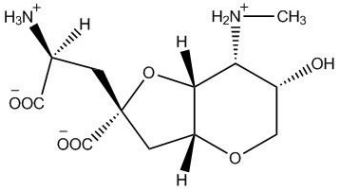
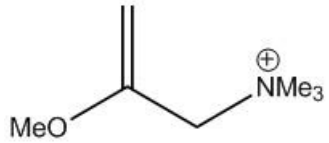
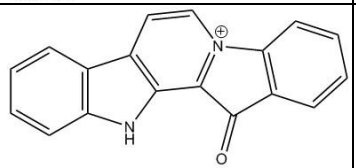
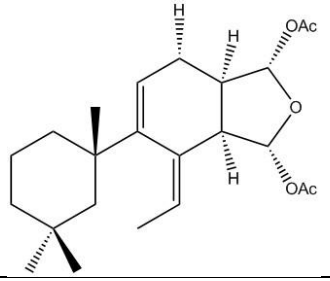
Compound	Source	Structure	ID number	Molecular weight	Biological activity	Docking score	Lipinski rule/ BBB likeness	References
2-bromoamphimedine (1)	*Petrosia n. sp.*		PubChem CID: 44561204	393.22	Inhibition of acetylcholinesterase (>300μM)	With Aβ monomer: -41.62 With Aβ pentamer: -50.48 With Aβ polymer: -157.23	Yes / Yes	(Nukoolkarn et al., 2008)

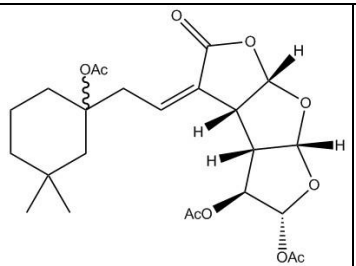
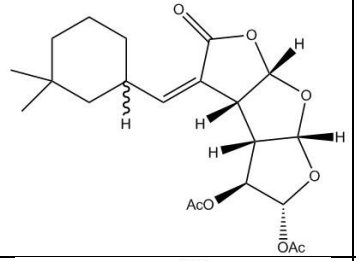
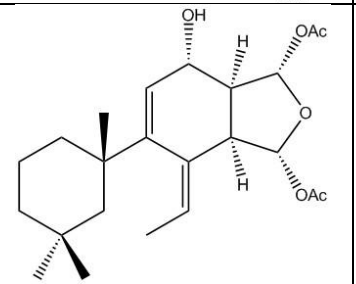
4,5-dibromo-2-pyrrolecarboxylic acid (2)	*Agela swieden mayeri*		ChemSpider ID142131	268.89	Inhibition of oxidative stress (>40mg/mL)	With Aβ monomer: -28.17 With Aβ pentamer: -36.34 With Aβ polymer: -79.55	Yes / No	(Lysek et al., 2003)
8,9-dihydrobarettin (3)	*Geodia barretti*		ChemSpider ID10479977	422.31	Enhancement of serotonin receptor (4.63 µM for HT2c)	With Aβ monomer: -37.08 With Aβ pentamer: -46.52 With Aβ polymer: -133.79	Yes / No	(Hedner et al., 2006)
8-Hydroxymanzamine (4)	*Acanthostrongylophora aff. ingens*		PubChem CID: 101838106	564.77	Enhancement of serotonin receptor (IC50 of 3.2 µM for Neuro 2a, and IC50of 3.0 µM for HL-60)	With Aβ monomer: -50.86 With Aβ pentamer: -56.83 With Aβ polymer: -192.74	No / No	(Zhang et al., 2008)
24-hydroperoxy-24-vinylcholesterol (5)	*Xestospongi testudinaria*		ChemSpider ID20169511	444.70	Inhibition of acetylcholinesterase (11.45 µM)	With Aβ monomer: -42.77 With Aβ pentamer: -61.83 With Aβ polymer: -218.26	No / No	(Zhou et al., 2011)

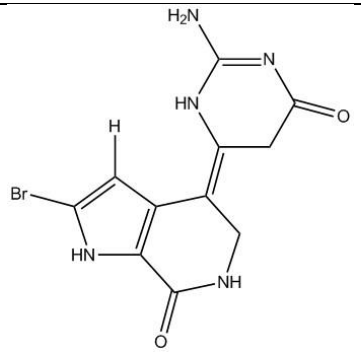
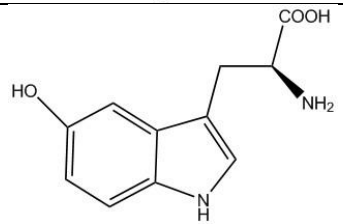
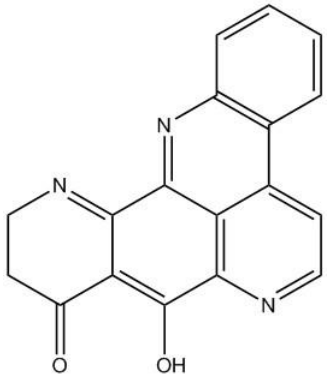
29-hydroperoxystigmasta-5,24(28)-dien-3 (6)	*Xestospongi testudinaria*		ChemSpider ID8609035	444.70	Inhibition of acetylcholinesterase (14.5 μM)	With Aβ monomer: -41.85 With Aβ pentamer: -52.15 With Aβ polymer: -194.11	No / No	(Zhou et al., 2011)
Barettin (7)	*Geodia barretti*		PubChem CID: 11177588	420.29	Enhancement of serotonin receptor (1.93, 0.34, and 1.91 μM for 5-HT_{2A}, 5- HT_{2c}, and 5- HT_4)	With Aβ monomer: -35.25 With Aβ pentamer: -38.49 With Aβ polymer: -219.11	Yes / No	(Hedner et al., 2006)
Crambescidin 800 (8)	*Monanchoraungiculata*		ChemSpider ID2280672	803.19	Inhibition of oxidative stress (Protect HT22 cells against glutamate induced toxicity at 0.06μM)	With Aβ monomer: -52.72 With Aβ pentamer: -75.71 With Aβ polymer: -240.08	No / No	(Suna et al., 2007)
Cribronic acid (9)	*Cribrochalinaolemda*		PubChem CID: 10444353	241.21	Inhibition of glutamate receptors (83 nM *in vivo*)	With Aβ monomer: -20.72 With Aβ pentamer: -24.21 With Aβ polymer: -113.88	Yes / No	(Sakai et al., 2003)
Daminin (10)	*Axinella damicornis*		ChemSpider ID9371310	260.25	Inhibition of oxidative stress (Reduced Ca2+ level via NMDA receptor at >40 μg/mL)	With Aβ monomer: -43.13 With Aβ pentamer: -65.61 With Aβ polymer: -144.03	Yes / Yes	(Aiello et al., 2005)

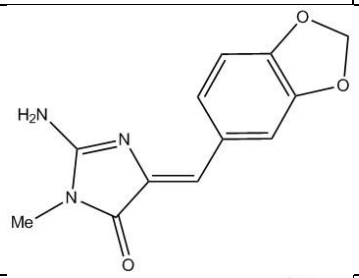
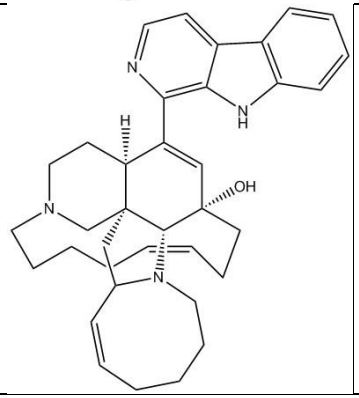
Damipipecoline (11)	*Axinella damicornis*		PubChem CID: 16742594	317.14	Enhancement of serotonin receptor (Ca2+ influx inhibition at 10μg/ml)	With Aβ monomer: -35.03 With Aβ pentamer: -41.18 With Aβ polymer: -121.28	Yes / No	(Aiello et al., 2007)
Damituricin (12)	*Axinella damicornis*		PubChem CID: 23653240	331.17	Enhancement of serotonin receptor (Ca2+ influx inhibition at 10μg/ml)	With Aβ monomer: -38.48 With Aβ pentamer: -42.95 With Aβ polymer: -127.15	Yes / Yes	(Aiello et al., 2007)
Debromohymenialdisine (13)	*Axinella sp.*		PubChem CID: 3037580	249.27	Inhibition of kinase (inhibits CDK5/p25, CK1, and GSK-3β with 0.4, 0.1, and 0.2 μM, respectively)	With Aβ monomer: -32.86 With Aβ pentamer: -35.44 With Aβ polymer: -155.61	Yes / No	(Zhang et al., 2012c)

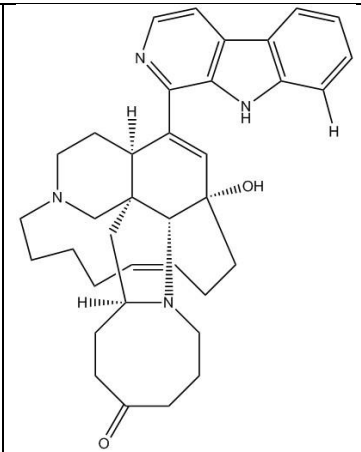
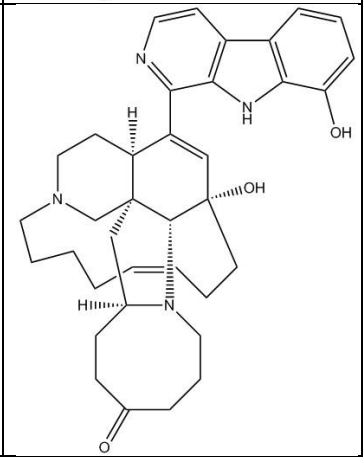
Dictyodendrin J (14)	*Ianthella sp.*		ChemSpider ID29214618	589.58	Inhibition of BACE at 2 μM	With Aβ monomer: -54.45 With Aβ pentamer: -57.61 With Aβ polymer: -223.79	No / No	(Zhang et al., 2012a)
Dysibetaine CPa (15)	*Dysidea herbacea*		PubChem CID: 72969641	202.23	Inhibition of glutamate receptors (CPa inhibits both kainic acid receptor and CGP396 53 with13 and 10 μM)	With Aβ monomer: -26.08 With Aβ pentamer: -28.77 With Aβ polymer: -97.12	Yes / No	(Sakai et al., 2004)
Dysibetaine CPb (16)	*Dysidea herbacea*		PubChem CID: 72770440	201.25	Inhibition of glutamate receptors (CPb inhibits Kainic acid with 4.9 μM)	With Aβ monomer: -26.17 With Aβ pentamer: -29.82 With Aβ polymer: -94.69	Yes / No	(Sakai et al., 2004)
Dysideamine (17)	*Dysidea sp.*		PubChem CID: 44188455	345.48	Inhibition of oxidative stress (Inhibited production of reactive oxygen species (ROS) that	With Aβ monomer: -32.49 With Aβ pentamer: -37.86 With Aβ polymer: -140.11	No / Yes	(Suna et al., 2009)

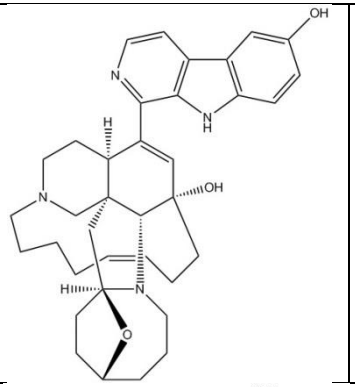
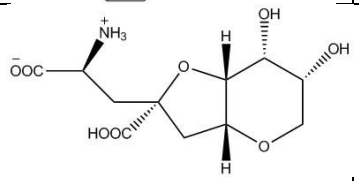
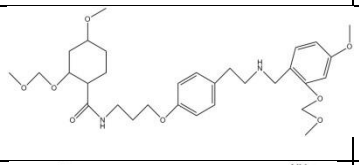
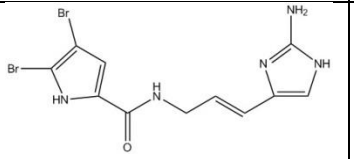
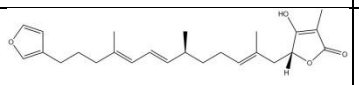
					trigger MAPK pathway, and protect HT22 cells against iodoacetic acid at 0.06μM)			
Dysiherbaine (18)	*Dysidea herbacea*		ChemSpider ID8015154	305.31	Inhibition of glutamate receptors (66 nM *in vivo*)	With Aβ monomer: -29.21 With Aβ pentamer: -35.77 With Aβ polymer: -99.11	Yes / No	(Sakai et al., 2001b)
Esmodil (19)	*Raspailia*		PubChem CID: 20055494	130.21	Inhibition of acetylcholinesterase	With Aβ monomer: -17.48 With Aβ pentamer: -20.34 With Aβ polymer: -83.04	Yes / Yes	(Capon et al., 2004)
Fascaplysin (20)	*Fascaplysinopsis Bergquist sp.*		PubChem CID: 73293	272.31	Inhibition of acetylcholinesterase (1.49 μM)	With Aβ monomer: -42.49 With Aβ pentamer: -53.74 With Aβ polymer: -255.67	Yes / Yes	(Bharate et al., 2012)
Gracilin A (21)	*Spongionella sp.*		ChemSpider ID10233474	392.54	Inhibition of oxidative stress (protect mitochondria against oxidative stress through Nrf2/ARE pathway at 0.1 μM)	With Aβ monomer: -37.47 With Aβ pentamer: -42.48 With Aβ polymer: -172.48	No / Yes	(Leirós et al., 2014)

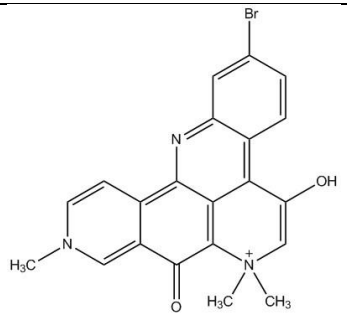
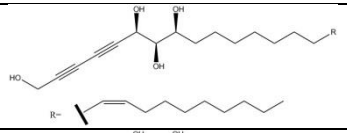
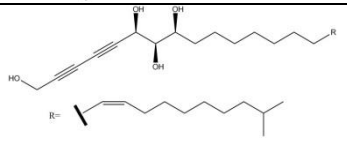
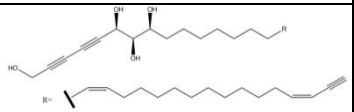
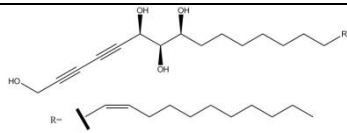
Gracilin j (22)	*Spongionella sp.*		ChemSpider ID24675190	482.53	Inhibition of oxidative stress (protect mitochondria against oxidative stress through Nrf2/ARE pathway at 0.1 μM)	With Aβ monomer: -34.95 With Aβ pentamer: -52.61 With Aβ polymer: -182.36	Yes / No	(Leirós et al., 2014)
Gracilin K (23)	*Spongionella sp.*		PubChem CID: 44254082	408.45	Inhibition of oxidative stress (protect mitochondria against oxidative stress through Nrf2/ARE pathway at 0.1 μM)	With Aβ monomer: -29.10 With Aβ pentamer: -42.10 With Aβ polymer: -170.9	Yes / No	(Leirós et al., 2014)
Gracilin L (24)	*Spongionella sp.*		ChemSpider ID24654576	410.55	Inhibition of oxidative stress (protect mitochondria against oxidative stress through Nrf2/ARE pathway at 0.1 μM)	With Aβ monomer: -43.88 With Aβ pentamer: -51.04 With Aβ polymer: -198.75	No / No	(Leirós et al., 2014)

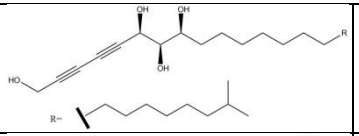
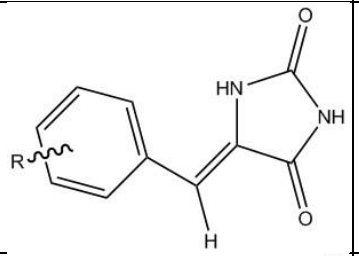
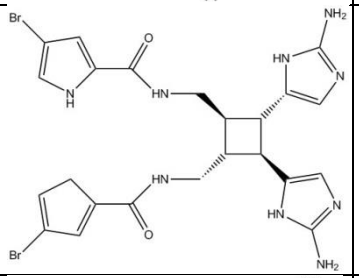
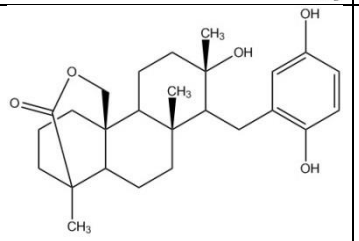
Hymenialdisine (25)	*Halichondriidae*		ChemSpider ID2299698	326.15	Inhibit GSK-3β and CK1with 10 and 35nM, respectively	With Aβ monomer: -31.58 With Aβ pentamer: -43.42 With Aβ polymer: -107.16	Yes / No	(Meijer et al., 2000)
L-5-hydroxytryptophan (26)	*Hymeniacidonhelioophila*		PubChem CID: 439280	221.24	Inhibition of oxidative stress	With Aβ monomer: -30.77 With Aβ pentamer: -50.39 With Aβ polymer: -108.57	Yes / No	(Lysek et al., 2003)
Labuanine A (27)	*Biemna fortis*		ChemSpider ID10188942	302.31	Enhancement of neuronal growth (at 1 μM)	With Aβ monomer: -40.38 With Aβ pentamer: -50.85 With Aβ polymer: -152.96	Yes / Yes	(Aoki et al., 2003)
Lembehyne A (28)	*Haliclona sp.*		ChemSpider ID8250618	510.89	Enhancement of neuronal growth (at 2 μg/mg in PC12 cell line, and 0.1	With Aβ monomer: -60.16 With Aβ pentamer: -80.07 With Aβ polymer: -271.17	No / No	(Aoki et al., 2000)

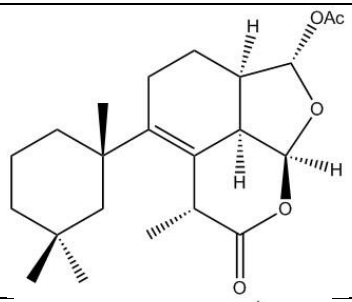
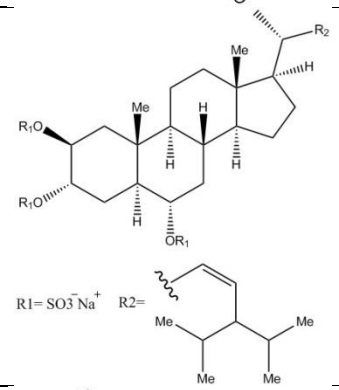
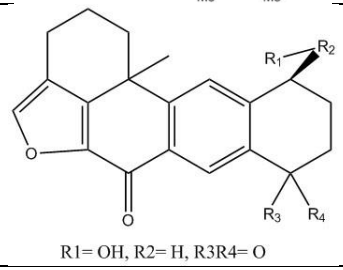
					μg/mg in Neuro 2A cell)			
Leucettamine B (29)	*Leucetta microraphis*		PubChem CID: 10037501	246.25	Inhibit different Kinases such as CDK1, CDK2, CDK5, CDK7, CDK9, and GSK-3β with IC50 of >10 μM	With Aβ monomer: -34.54 With Aβ pentamer: -57.36 With Aβ polymer: -126.14	Yes / Yes	(Tahtouh et al., 2012)
Manzamine A (30)	*Acanthostrongylophora*		ChemSpider ID5006904	548.76	Noncompetitive inhibitor of ATP binding against GSK-3β, and CDK5 with 10.2 and 1.5μM,respectively	With Aβ monomer: -46.80 With Aβ pentamer: -58.56 With Aβ polymer: -230.97	No / No	(Hamann et al., 2007)

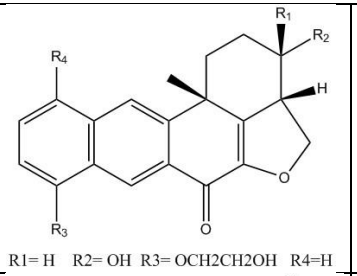
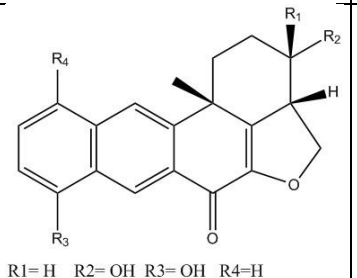
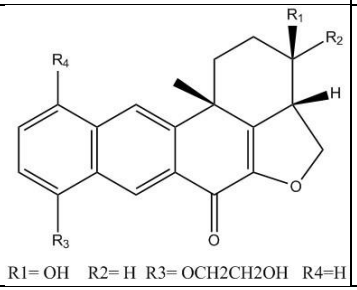
Manzamine E (31)	*Acanthostrongylophora aff. ingens*		PubChem CID: 44445401	564.76	Enhancement of neuronal growth (IC50 for Neuro 2a at 5μM, IC50 for HL-60 at 8.9 μM)	With Aβ monomer: -47.85 With Aβ pentamer: -49.55 With Aβ polymer: -194.42	No / No	(Zhang et al., 2008)
Manzamine F (32)	*Acanthostrongylophora aff. ingens*		PubChem CID: 44445402	580.76	Enhancement of neuronal growth (IC50 for Neuro 2a at >15 μM, IC50 for HL-60 at >10 μM)	With Aβ monomer: -45.69 With Aβ pentamer: -48.52 With Aβ polymer: -192.01	No / No	(Zhang et al., 2008)

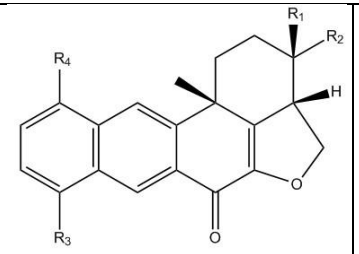
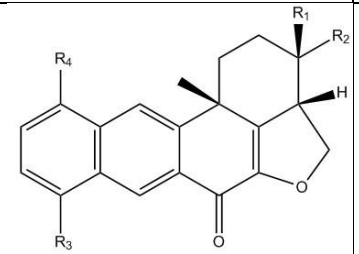
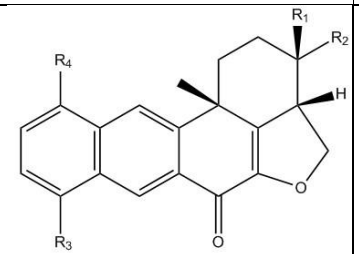
Manzamine X (33)	*Acanthostrongylophora aff. ingens*		PubChem CID: 101855965	580.76	Enhancement of neuronal growth (IC50 for Neuro 2a at >15 μM, IC50 for HL-60 at >10 μM)	With Aβ monomer: -49.78 With Aβ pentamer: -60.63 With Aβ polymer: -199.79	No / No	(Zhang et al., 2008)
Neodysiherbaine A (34)	*Dysidea herbacea*		PubChem CID: 11460505	292.26	Inhibition of glutamate receptors (at 227 nM inhibition of Kainic acid glutamate receptors and AMPA)	With Aβ monomer: -24.07 With Aβ pentamer: -33.02 With Aβ polymer: -98.73	Yes / No	(Sakai et al., 2001a)
NP04634 (35)	*Aplysina cavernicola*		PubChem CID: 17747558	582.65	Inhibition of oxidative stress (inhibit the toxic Ca2+ via VDCC pathway)	With Aβ monomer: -50.13 With Aβ pentamer: -50.66 With Aβ polymer: -217.59	No / No	(Valero et al., 2009)
Oroidin (36)	*Agelas oroides*		PubChem CID: 6312649	389.05	Inhibition of oxidative stress (reduced Ca2+ level by binding with NMDA recepto at >40 mg/mL)	With Aβ monomer: -38.57 With Aβ pentamer: -50.28 With Aβ polymer: -204.33	Yes / No	NA
Palinurin (37)	*Ircinia dendroides*		PubChem CID: 54686465	398.54	GSK-3β inhibitor by binding to an allosteric site located at the N-terminal lobe	With Aβ monomer: -45.23 With Aβ pentamer: -55.78 With Aβ polymer: -200.62	No / Yes	(Bidon-Chanal et al., 2013)

					with 1.9 and 1.6 μM for GSK-3β and GSK-3α			
Petrosamine (38)	*Petrosia n. sp.*		PubChem CID: 360199	424.30	Inhibition of acetylcholinesterase at 0.091 μM	With Aβ monomer: -46.8 With Aβ pentamer: -55.49 With Aβ polymer:-179.96	Yes / No	(Nukoolkarn et al., 2008)
Petrosiol A (39)	*Petrosia strongylata*		ChemSpider ID28536122	406.61	Enhancement of neuronal growth (2 μM)	With Aβ monomer: -61.1 With Aβ pentamer: -64.41 With Aβ polymer: -187.03	No / No	(Horikawa et al., 2013)
Petrosiol B (40)	*Petrosia strongylata*		ChemSpider ID29216354	420.63	Enhancement of neuronal growth (2 μM)	With Aβ monomer: -50.86 With Aβ pentamer: -68.99 With Aβ polymer: -220.32	No / No	(Horikawa et al., 2013)
Petrosiol C (41)	*Petrosia strongylata*		ChemSpider ID29216355	484.72	Enhancement of neuronal growth (2 μM)	With Aβ monomer: -52.69 With Aβ pentamer: -82.28 With Aβ polymer: -255.89	No / No	(Horikawa et al., 2013)
Petrosiol D (42)	*Petrosia strongylata*		ChemSpider ID29216356	420.63	Enhancement of neuronal growth (2 μM)	With Aβ monomer: -53.88 With Aβ pentamer: -59.63 With Aβ polymer: -234.05	No / No	(Horikawa et al., 2013)

Petrosiol E (43)	*Petrosia strongylata*		ChemSpider ID29216357	394.60	Enhancement of neuronal growth (2 μM)	With Aβ monomer: -45.94 With Aβ pentamer: -61.28 With Aβ polymer: -184.78	No / Yes	(Horikawa et al., 2013)
Phenylmethylene (44)	*Hemimycalearabica*		PubChem CID: 687302	206.26	Inhibition of kinase (GSK-3β inhibitor at 4.2 μM)	With Aβ monomer: -33.12 With Aβ pentamer: -55.49 With Aβ polymer: -115.85	Yes / Yes	(Khanfar et al., 2009)
Sceptrin (45)	*Agela swiedenmayeri*		PubChem CID: 157394	620.31	Inhibition of oxidative stress (>40 mg/mL)	With Aβ monomer: -46.23 With Aβ pentamer: -44.20 With Aβ polymer: -166.46	No / No	(Bickmeyer et al., 2004)
Strongylophorine-8 (46)	*Petrosia Strongylophoracorticata*		ChemSpider ID23283193	428.57	Inhibition of oxidative stress (increase glutathione And activate Nrf2/ARE pathway to prevent oxidative stress at 4.1 μM)	With Aβ monomer: -36.55 With Aβ pentamer: -50.82 With Aβ polymer: -177.50	Yes / No	(Sasaki et al., 2011)

Tetrahydroaplysulphurin-1 (47)	*Spongionella sp.*		PubChem CID: 23247763	376.49	Inhibition of oxidative stress (protect mitochondria against oxidative stress through Nrf2/ARE pathway at 0.1 μM)	With Aβ monomer: -37.34 With Aβ pentamer: -45.69 With Aβ polymer: -168.8	No / Yes	(Leirós et al., 2014)
Topsentinol K trisulfate (48)	*Topsentia*		PubChem CID: 46938754	700.92	1.2 μM for BACE	With Aβ monomer: -43.64 With Aβ pentamer: -66.13 With Aβ polymer: -197.32	No / No	(Dai et al., 2010a)
Xestosaprol D (49)	*Xestospongia sp.*		PubChem CID: 45140079	322.35	30 μg/ml for BACE	With Aβ monomer: -38.28 With Aβ pentamer: -47.43 With Aβ polymer: -152.96	Yes / Yes	(Millán-Aguiñaga et al., 2010)

Xestosaprol F (50)	*Xestospongia* sp		PubChem CID: 46848865	366.41	135 μM for BACE	With Aβ monomer: -43.27 With Aβ pentamer: -54.19 With Aβ polymer: -250.28	Yes / Yes	(Dai et al., 2010b)
Xestosaprol G (51)	*Xestospongia* sp		PubChem CID: 46849021	322.35	155 μM for BACE	With Aβ monomer: -43.61 With Aβ pentamer: -57.13 With Aβ polymer: -154.16	Yes / Yes	(Dai et al., 2010b)
Xestosaprol H (52)	*Xestospongia* sp		PubChem CID: 46849022	366.41	82 μM for BACE	With Aβ monomer: -43.66 With Aβ pentamer: -56.56 With Aβ polymer: -174.83	Yes / Yes	(Dai et al., 2010b)

Xestosaprol I (53)	*Xestospongia* sp		PubChem CID: 46849023	306.36	163 μM for BACE	With Aβ monomer: -41.44 With Aβ pentamer: -63.78 With Aβ polymer: -155.20	Yes / Yes	(Dai et al., 2010b)
Xestosaprol J (54)	*Xestospongia* sp		PubChem CID: 46849024	336.38	90 μM for BACE	With Aβ monomer: -42.24 With Aβ pentamer: -57.32 With Aβ polymer: -163.32	Yes / Yes	(Dai et al., 2010b)
Xestosaprol K (55)	*Xestospongia* sp		PubChem CID: 46849025	322.36	93 μM for BACE	With Aβ monomer: -43.73 With Aβ pentamer: -57.70 With Aβ polymer: -153.45	Yes / Yes	(Dai et al., 2010b)

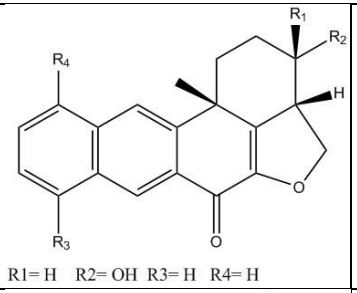
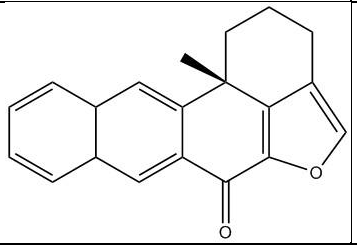
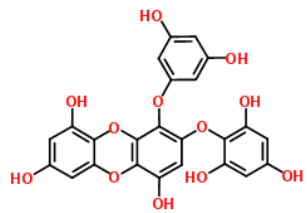
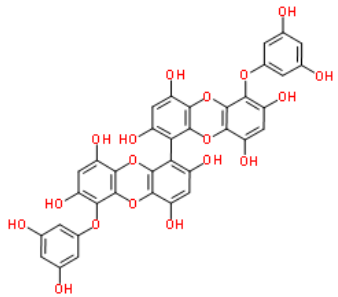
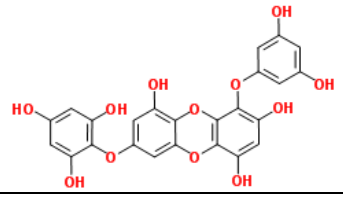
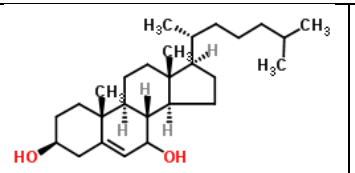
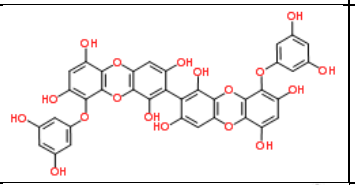
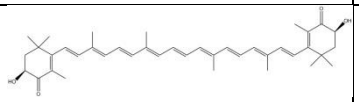
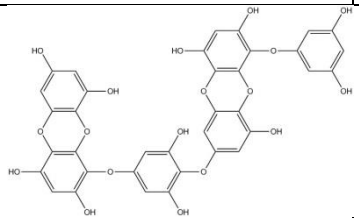
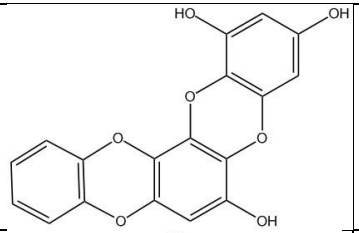
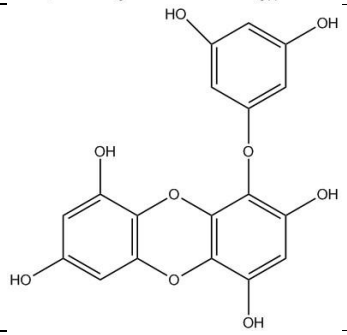
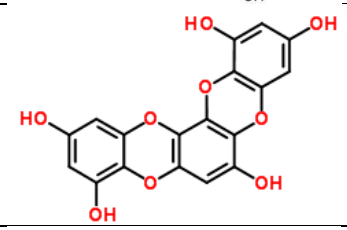
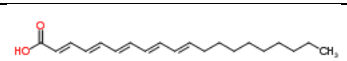
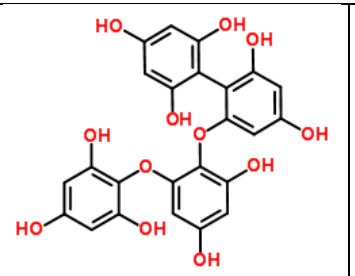
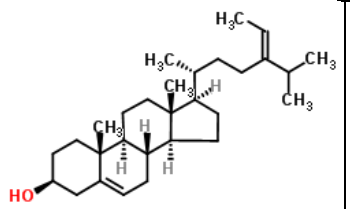
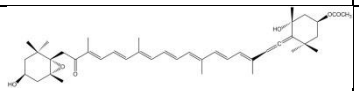
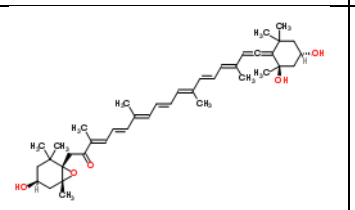
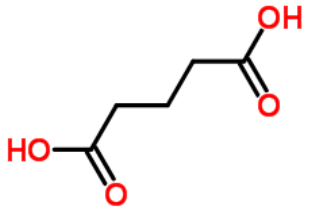
Xestosaprol L (56)	*Xestospongia* sp		PubChem CID: 46849026	306.36	98 μM for BACE	With Aβ monomer: -42.11 With Aβ pentamer: -57.56 With Aβ polymer: -153.3	Yes / Yes	(Dai et al., 2010b)
Xestosaprol M (57)	*Xestospongia* sp		PubChem CID: 46849027	288.34	104 μM for BACE	With Aβ monomer: -43.34 With Aβ pentamer: -61.49 With Aβ polymer: -161.13	Yes / Yes	(Dai et al., 2010b)

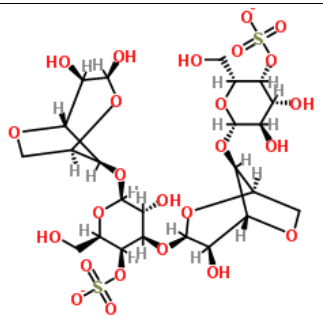
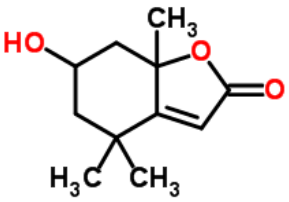
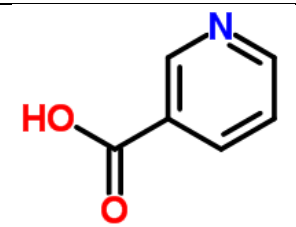
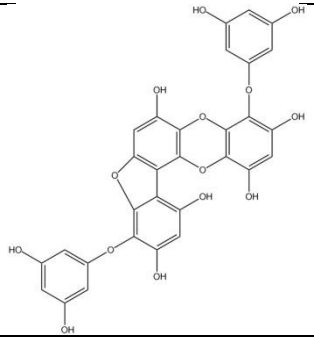
Table 3-2 Summary of the Docking scores results of 26 macroalgae derived compounds. The information includes, the compound name, source sponge species, structure, PubChem CID number, molecular weight, neuroprotective activity, docking scores against three forms of Aβ, Lipinski rules and BBB likeness

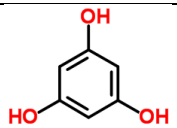
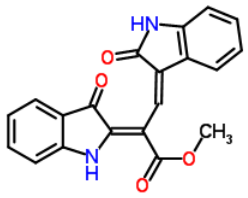
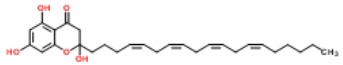
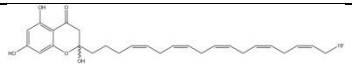
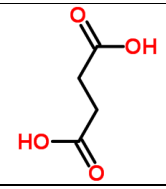
Compound	Source	Structure	ID number	Molecular weight	Biological activity	Docking score	Lipinski rule/ BBB likeness	References
2-phloroeckol (1)	Brown algae *Ecklonia stolonifera*		ChemSpider ID4478591	498.39	ROS inhibiting activity by inhibiting nitric oxide with IC50 of 85.3 μM/L	With Aβ monomer: -47.27 With Aβ pentamer: -58.13 With Aβ polymer: -259.71	No / No	(Wei et al., 2016)
6,6'-Bieckol (2)	Brown algae: *Ecklonia cava*		ChemSpider ID121062	742.55	BACE-1 inhibitor activity of 18.6% with 1 μM	With Aβ monomer -58.04 With Aβ pentamer: -54.98 With Aβ polymer: -301.90	No / No	(Choi et al., 2015)
7-Phloroeckol (3)	Brown algae: *Eisenia bicyclis*		ChemSpider ID8656347	499.40	Suppression of BACE-1 enzyme activity with 8.59 μM	With Aβ monomer: -47.39 With Aβ pentamer: -63.50 With Aβ polymer: -239.59	No / No	(Jung et al., 2010)

7-Hydroxycholesterol (4)	Red algae: *Gloiopeltis furcata*		ChemSpider ID: 20021193	402.66	Inhibit AChE and BuChE with IC50 of 2.35 and 5.57 μg/mL	With Aβ monomer -48.26 With Aβ pentamer: -63.65 With Aβ polymer: -201.86	No / No	(Fang et al., 2010)
8,8'-Bieckol (5)	Brown algae: *Ecklonia cava*		ChemSpider ID2278310	743.56	BACE-1 inhibitor activity of 35% with 1 μM	With Aβ monomer -54.76 With Aβ pentamer: -66.93 With Aβ polymer: -309.81	No / No	(Choi et al., 2015)
Astaxanthin (6)	Microalgae		PubChem CID: 5281224	596.84	Increase the cell viability when it was treated at 0.1 μM by partially down-regulating the activation of caspase 3 and by reducing the expression of Bax	With Aβ monomer -46.02 With Aβ pentamer: -88.83 With Aβ polymer: -387.44	No / No	(Chang et al., 2010)
Dieckol (7)	Brown algae: *Eisenia bicyclis*		ChemSpider ID2278311	744.56	GSK-3β inhibitory of 15.1% with 50 μM, Suppression of BACE-1enzyme activity with 2.21 μM, Increase cell viability from 50% to 70% with 20 μM	With Aβ monomer: -55.99 With Aβ pentamer: -67.51 With Aβ polymer: -335.39	No / No	(Choi et al., 2015) (Jung et al., 2010) (Ahn et al., 2012)

Dioxinodehydroeckol (8)	Brown algae: *Eisenia bicyclis*		PubChem CID: 10429214	370.27	5.35 µM for BACE	With Aβ monomer: -42.68 With Aβ pentamer: -59.57 With Aβ polymer: -127.62	Yes / No	(Jung et al., 2010)
Eckol (9)	Brown algae: *Eisenia bicyclis*		PubChem CID: 145937	372.28	12.20 µM for BACE and 50 µM for GSK-3β	With Aβ monomer: -39.33 With Aβ pentamer: -43.93 With Aβ polymer: -215.93	No / No	(Jung et al., 2010)
Eckstolonol (10)	Brown algae: *Ecklonia stolonifera*		PubChem CID: 10429214	370.27	Inhibit AChE and BuChE with IC50 of 42.66 and 230.27 µM	With Aβ monomer -57.21 With Aβ pentamer: -59.66 With Aβ polymer: -127.73	Yes / No	(Yoon et al., 2008)
Eicosapentaenoic acid (11)	Red algae: *Gloiopeltis furcata*		PubChem CID: 446284	302.45	Inhibit AChE and BuChE with IC50 of 11.53 and 6.56 µg/mL Stimulate IL-4 which decrease the effect of Aβ in male Wistar rats	With Aβ monomer -55.02 With Aβ pentamer: -84.88 With Aβ polymer: -293.42	No / No	(Fang et al., 2010) (Lynch et al., 2007)

Fucodiphloroethol G (12)	Brown algae: *Ecklonia cava*		PubChem CID: 44590821	498.40	Scavenging activity against DPPH with IC50 of 14.72 µM	With Aβ monomer -34.78 With Aβ pentamer: -43.46 With Aβ polymer: -147.72	No / No	(Li et al., 2009)
Fucosterol (13)	Brown algae: *Ecklonia stolonifera*		ChemSpider ID: 4444705	412.7	Inhibit BuChE with IC50 of 421.72 µM	With Aβ monomer -50.3 With Aβ pentamer: -59.71 With Aβ polymer: -220.06	No / No	(Yoon et al., 2008)
Fucoxanthin (14)	Brown algae		PubChem CID: 5281239	662.94	Inhibit $A\beta_{25-35}$ cytotoxicity in cerebral cortex neurons completely at 6.25 µg/mL	With Aβ monomer: -46.86 With Aβ pentamer: -72.12 With Aβ polymer: -414.03	No / No	(Zhao et al., 2015)
Fucoxanthinol (15)	Brown algae: *Undaria pinnatifida*		ChemSpider ID9448555	622.93	DPPH inhibition activity with IC50 of 153.78 µM	With Aβ monomer -54.56 With Aβ pentamer: -73.48 With Aβ polymer: -250.13	No / No	(Sachindra et al., 2007)
Glutaric acid (16)	Red algae: *Gloiopeltis furcata*		PubChem CID: 743	132.1	Inhibit AChE and BuChE with IC50 of 5.65 and 41.52 µg/mL	With Aβ monomer -27.2 With Aβ pentamer: -34.8 With Aβ polymer: -72.69	Yes / No	(Fang et al., 2010)

Kappa-carrageenan (17)	Red algae: *Kappaphycus alvarezii*		ChemSpider ID: 10140242	788.65	Inhibit superoxide radicals with IC50 of 0.112 mg/mL	With Aβ monomer -42.46 With Aβ pentamer: -34.1 With Aβ polymer: -151.91	No / No	(de Souza et al., 2007a)
Loliolide (18)	Red algae: *Gloiopeltis furcata*		PubChem CID: 100332	196.25	Inhibit AChE with IC50 of 7.57 µg/mL	With Aβ monomer -32.56 With Aβ pentamer: -32.75 With Aβ polymer: -105.62	Yes / Yes	(Fang et al., 2010)
Nicotinic acid (19)	Red algae: *Gloiopeltis furcata*		PubChem CID: 938	123.11	Inhibit AChE and BuChE with IC50 of 1.14 and 20.86 µg/mL	With Aβ monomer -24.12 With Aβ pentamer: -34.37 With Aβ polymer: -75.95	Yes / No	(Fang et al., 2010)
Phlorofucofuroeckol A (20)	Brown algae: *Eisenia bicyclis*		PubChem CID: 130976	602.46	2.13 µM for BACE and 10 µM for Increasing cell viability from 50% to 75%	With Aβ monomer: -48.31 With Aβ pentamer: -58.23 With Aβ polymer: -271.50	No / No	(Jung et al., 2010, Ahn et al., 2012)

Phloroglucinol (21)	Brown algae: *Eisenia bicyclis*		PubChem CID: 359	126.11	36.47 μM for BACE	With Aβ monomer: -26.01 With Aβ pentamer: -36.35 With Aβ polymer: -76.67	Yes / Yes	(Jung et al., 2010)
Racemosin A (22)	Green algae: *Caulerpa racemosa*		ChemSpider ID30900727	350.37	10 μM for BACE	With Aβ monomer -46.06 With Aβ pentamer: -58.8 With Aβ polymer: -184.84	Yes / Yes	(Liu et al., 2013)
Spiralisone A (23)	Brown algae: *Zonaria spiralis*		ChemSpider ID29215449	454.61	Kinases inhibitory to CDK5/p25, CK1δ, and GSK3β with 10.0, <10, and <10 μM, respectively	With Aβ monomer: -61.1 With Aβ pentamer: -72.73 With Aβ polymer: -227.48	No / No	(Zhang et al., 2012d)
Spiralisone B (24)	Brown algae: *Zonaria spiralis*		ChemSpider ID29215450	452.58	Kinases inhibitory to CDK5/p25, CK1δ, and GSK3β with 3, 5, and 5.4 μM, respectively	With Aβ monomer: -63.5 With Aβ pentamer: -77.7 With Aβ polymer: -235.26	No / No	(Zhang et al., 2012d)
Succinic acid (25)	Red algae: *Gloiopeltis furcata*		PubChem CID: 1110	118.09	Inhibit AChE with IC50 of 5.74 μg/mL	With Aβ monomer -21.15 With Aβ pentamer: -28.58 With Aβ polymer: -68.16	Yes / No	(Fang et al., 2010)

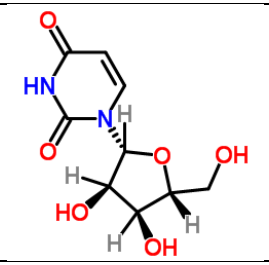
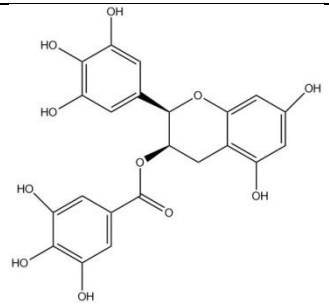
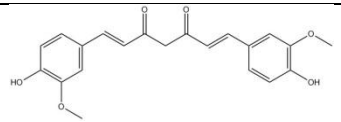
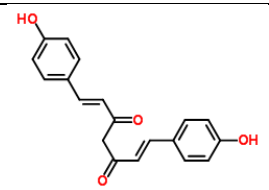
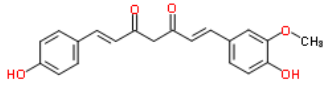
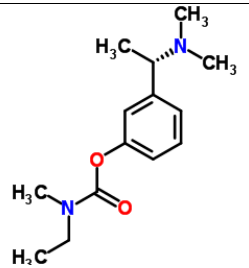
Uridine (26)	Red algae: *Gloiopeltis furcata*		PubChem CID: 6029	245.21	Inhibit AChE and BuChE with IC50 of 1.63 and 35.83 μg/mL	With Aβ monomer -32.41 With Aβ pentamer: -35.56 With Aβ polymer: -98.23	Yes / No	(Fang et al., 2010)

Table 3-3 Summary of the docking scores results of five natural products used as positive references. The information includes, the compound name, source sponge species, structure, PubChem CID number, molecular weight, neuroprotective activity, docking scores against three forms of Aβ, Lipinski rules and BBB likeness. Four compounds meet all the rules

Compound	Source	Structure	ID number	Molecular weight	Biological activity	Docking score	Lipinski rule/ BBB likeness	References
(-)-Epigallocatechin gallate	Green tea polyphenol		PubChem CID: 65064	458.37	Treating cells with 100 µM can reduce toxicity induced by Aβ from 50% to 85%. EGCG pre-treatment completely prevented the formation of fibrils and aggregates at 100 µM	With Aβ monomer: -47.32 With Aβ pentamer: -55.54 With Aβ polymer: -239.59	No / No	(Harvey et al., 2011)
Curcumin	Turmeric: *Curcuma longa*		PubChem CID: 969516	368.38	Cell protective effect against $A\beta_{1-42}$ insult with IC_{50} of 7.1 µg/ml	With Aβ monomer: -47.44 With Aβ pentamer: -68.86 With Aβ polymer: -262.83	Yes / Yes	(Kim et al., 2001)
Bisdemethoxycurcumin (Curcumin III)	Turmeric: *Curcuma longa*		PubChem CID: 5315472	308.33	Cell protective effect against $A\beta_{1-42}$ insult with IC_{50} of 3.5 µg/ml	With Aβ monomer: -47.74 With Aβ pentamer: -79.66 With Aβ polymer: -268.06	Yes / Yes	(Kim et al., 2001)
Demethoxycurcumin	Turmeric: *Curcuma longa*		PubChem CID: 5469424	338.35	Cell protective effect against $A\beta_{1-42}$ insult with IC_{50} of 4.7 µg/ml	With Aβ monomer: -48.46 With Aβ pentamer: -78.04 With Aβ polymer: -266.55	Yes / Yes	(Kim et al., 2001)

(Curcumin II)								
Rivastigmine	Semi-synthetic of a parasympathomimetic alkaloid (Calabar bean)		PubChem CID: 77991	250.34	Acetylcholinesterase Inhibitor for AD	With Aβ monomer: -34.26 With Aβ pentamer: -56.40 With Aβ polymer: -221.37	Yes / Yes	(Birks, 2006)

3.3.1.1 Compounds targeting cholinesterase

In this study, 6 compounds from sponges and 9 from macroalgae with cholinesterase inhibiting activity were screened by *in silico* modelling for their potential as anti-aggregation against Aβ fibrillization. From the results shown in Tables 3-1 – 3-3, cholinesterase inhibitors did not have high scores in binding with Aβ in all three forms. For sponge-derived compounds, there was a correlation between IC50 and docking scores in four compounds. For instance, 2-bromoamphimedine, 29-hydroperoxystigmasta-5,24(28)-dien-3, 24-hydroperoxy-24-vinylcholesterol, and fascaplysin that have an IC50 of 300, 14.5, 11.45, and 1.49 μM, respectively demonstrated correlated docking scores of -157, -194, -218, and -255 with Aβ polymer. However, petrosamine did not follow the relationship as it has IC50 of 0.091 μM but gave a docking score of -179 against the same protein form. Five compounds (succinic acid, glutaric acid, nicotinic acid, uridine, and 7-hydroxycholesterol) from macroalgae show a correlation between IC50 and docking score. Succinic acid, glutaric acid, nicotinic acid, uridine, and 7-hydroxycholesterol that have IC50 of 48.6, 42.77, 9.26, 6.6, and 5.8 μM possess docking scores of -68, -72.69, -75.95, -98.23, and -201.86 against Aβ polymer. Rivastigmine is an approved drug targeting acetylcholinesterase with a high docking score of -221.4 against Aβ polymer. However, eicosapentaenoic acid demonstrated a higher docking score of -293, which highlights the importance of this compound for use as Aβ aggregation inhibitor. Many tacrine hybrid compounds have demonstrated IC50 against cholinesterase activity at low concentration (ranging from 2.6-113 nM) and IC50 against $A\beta_{1-42}$ aggregation at higher concentration (at 20 μM) as the differences between these two concentrations is more than 176 times (Luo et al., 2011). Similarly, isaindigotone derivatives demonstrated cholinesterase activity in 23-360 nM concentration and $A\beta_{1-}$

$_{40}$ aggregation inhibition at 10 μM (Yan et al., 2012). This indicates cholinesterase inhibitor compounds may demonstrate an anti-aggregation effect but at very high concentration. For that reason, they might provide low binding scores in the *in silico* results.

3.3.1.2 Compounds targeting BACE

There are 11 compounds targeting BACE activity from sponges, but there was no correlation found between their docking scores and IC50. These compounds showed moderate binding scores in a range of -153.3 to -250 for binding with Aβ polymer. On the other hand, 8 compounds from algae targeting BACE activity, and 6 of them (eckol, phlorofucofuroeckol A, phloroglucinol, 7-phloroeckol, 6,6'-bieckol, and 8,8'-bieckol) showed a correlation between their docking scores with Aβ polymer and BACE activity. The lowest active compound was phloroglucinol with an active concentration at 36.47 μM, which showed the lowest docking score of -76.6, while both 6,6'-bieckol, and 8,8'-bieckol reported to be highly active against BACE at 1 μM concentration showing the highest docking score of -301 and -309, respectively. These high docking scores of phlorotannin compounds are not surprising as the *Ecklonia radiata* rich fraction of phlorotannin demonstrated the highest anti-aggregation effect (Chapter 4). It is likely that compounds targeting BACE-1 are also associated with anti-aggregation activity. For example, a butanol extract of *Ecklonia cava* and a compound named AP2469 were shown to have BACE-1 activity and anti-aggregation against $A\beta_{1-42}$ (Kang et al., 2011, Tarozzi et al., 2014).

3.3.1.3 Compounds targeting serotoninergic activity

Five compounds from sponges (8,9-dihydrobarettin, 8-hydroxymanzamine, barettin, damipipecoline, and damituricin) showed serotoninergic activity. The relationship

between active concentration and docking scores was comparable, as lower active concentration showed a higher binding score (Table 3-1). For instance barettin with the lowest active concentration at 1.9 μM showed the highest binding score with Aβ polymer at -219.1, while damipipecoline with the highest active concertation of 31.5 μM showed the lowest binding score at -121 with Aβ polymer. It has been suggested that a serotonin receptor might play a role in Aβ aggregation (Madsen et al., 2011). Also, another study found that administrating serotonin inhibitors to mice and human can result in decreasing the accumulation of Aβ (Cirrito et al., 2011). This can indicate that compounds demonstrating serotoninergic activity might also associate with anti-aggregation against Aβ activity. An example of that is amoxapine, which was demonstrated to reduce the production of Aβ through different serotonin targets such as serotonin receptor 6 (HTR6) (Li et al., 2017).

3.3.1.4 Compounds targeting GSK-3β activity

Six sponge compounds targeting GSK-3β have no apparent relationship between the docking score and its biological activity. Among four algal compounds targeting GSK-3β, three compounds (eckol, spiralisone A, and spiralisone B) demonstrated a correlation between biological activity and docking score. Spiralisone B with the lowest active concentration at 5.4 μM showed docking score of -235, while eckol with the highest active concentration at 50 μM demonstrated the lowest binding score of -215.9. Overall, compounds that are targeting GSK-3β might also have Aβ anti-aggregatory properties. An earlier animal study demonstrated that GSK-3 has a role in developing Aβ pathology, and inhibiting GSK-3 can result in decreasing Aβ aggregation (Avrahami et al., 2013). Another study also demonstrated that inhibiting GSK-3β can decrease the expression of BACE-1, which will result in decreasing the production of Aβ (Ly et al., 2012). Exposure to the oligomeric form of Aβ was found

to increase the activity of GSK-3β, and inhibiting GSK-3β can decrease the amount of oligomeric Aβ (DaRocha-Souto et al., 2012). These findings suggest that inhibiting GSK-3β can result in decreasing Aβ aggregation, which supports our assertion that compounds inhibiting GSK-3β might also have Aβ aggregation inhibition activity. An example of that is honokiol, a polyphenolic compound (lignin) that was found to decrease the neurotoxicity and aggregation of $A\beta_{1-42}$ and also to inhibit the activation of GSK-3β (Xian et al., 2016, Das et al., 2016).

3.3.1.5 Compounds targeting glutamate activity

Six sponge-derived compounds (crambescidin 800, cribronic acid, dysibetaine CPa, dysibetaine CPb, dysiherbaine, and neodysiherbaineA) that inhibit the glutamate receptor demonstrated low binding with Aβ polymer, with a binding score of less than -115, except crambescidin 800 with a high docking score of -240. There is a correlation between active concentration and docking scores in three compounds. For instance, cribronic acid has the highest docking score (-113.88) with active concentration at 83 nM, while dysibetaine CPb has docking score of 94.69 and active concentration at 4.9 µM. Aβ has shown to disrupt two glutamate receptors 2-amino-3-(3-hydoxy-5-methylisoxazol-4-yl) propionic acid (AMPA) and N-methyl-D-aspartate receptors (NMDARs), which results in synaptic dysfunction (Parameshwaran et al., 2008). A good correlation between these two activities could not be predicted, as there is a lack of papers reporting compounds to have both activities. A report demonstrated that an ethanolic extract from *Rhinacanthus nasutus* reduced the cytotoxicity of $A\beta_{1-42}$ and glutamate in hippocampal HT-22 cells (Brimson et al., 2012). For that reason, more studies on compounds that have dual activities against inhibiting glutamate and Aβ aggregation are needed.

3.3.1.6 Compounds enhancing neurite outgrowth activity

There were 10 sponge-derived compounds that demonstrated an enhancement of neurite outgrowth activity. Most of these compounds showed moderate to high binding scores ranging from -152.9 to -255. There was no correlation observed in their docking scores with their active concentration. Other compounds have been shown to have both activities. For example, senegenin is a saponin compound that reduced the neurotoxicity of $A\beta_{25-35}$ and also enhanced neurite outgrowth in PC-12 cells (Jesky and Chen, 2015). However, α-mangostin a polyphenolic compound was found to block the fibril at 10 μM and also to enhance neurite outgrowth that was reduced by $A\beta_{1-40}$ and $A\beta_{1-42}$ at 5 nM (Wang et al., 2012b). A molecule named J2326 was found to decrease the fibril formation at 10 mM, while it was able to enhance neurite outgrowth at 10 μM (Chang et al., 2015). This means that some compounds that enhance neurite outgrowth activity could also inhibit the aggregation of Aβ but at higher concentrations.

3.3.1.7 Compounds reducing oxidative stress activity

Thirteen sponge-derived compounds were demonstrated to have oxidative stress inhibition activity. These compounds showed low docking scores with Aβ polymer ranging from -79 to -217. Four algae-derived compounds (2-phloroeckol, fucoxanthinol, fucodiphloroethol G, and kappa-carrageenan) showed oxidative stress inhibition activity. Fucodiphloroethol G and kappa-carrageenan demonstrated weak binding with Aβ polymer, with binding scores of -147.7 and -151.9 respectively, while the other two compounds showed moderate binding scores. No correlation was observed between their docking scores and their active concentration. Aβ was found to induces oxidative stress, which can contribute to neuronal death (Butterfield et al., 2002, Butterfield et al., 2001). A flavonoid compound named rutin was found to

inhibit the production of ROS and malondialdehyde (MDA) at a concentration of 0.8 μM, while it only inhibits 25% of $A\beta_{1-42}$ aggregation at 50 μM (Wang et al., 2012a). However, some xanthone compounds from *Garcinia mangostana* (mangosteen) demonstrated to have ROS inhibiting activity and inhibit the aggregation of $A\beta_{1-42}$ at comparable concentrations (Wang et al., 2016a). On the other hand, EGCG was not found to have antioxidant activity against hydrogen peroxide and *tert* butyl hydroperoxide, while it was able to reduce the aggregation of $A\beta_{1-42}$ (Harvey et al., 2011). Also, several benzimidazole derivatives were demonstrated to have an effective activity against hydrogen peroxide; however they demonstrated only weak anti-aggregation against Aβ (Ozadali-Sari et al., 2017). This is likely due to the different binding regions of these compounds with Aβ polymer form, which also might be indicated by their docking scores.

3.3.1.8 Compounds reducing amyloid beta neurotoxicity

Both astaxanthin and fucoxanthin reduced the neurotoxicity of Aβ, and here they showed very high binding scores with Aβ polymer (>-387). In comparison, the positive compounds used in this study such as EGCG, showed a docking score of -239.6 with Aβ polymer, while curcumin, curcumin II, and curcumin III showed docking scores of -262.8, -266.5, and -268, respectively. Astaxanthin and fucoxanthin showed much higher binding scores compared to these positive reference compounds. Recently, fucoxanthin was found to inhibit the aggregation induced by $A\beta_{1-42}$ (Xiang et al., 2017). Promising drug candidates are those that have dual activities i.e. that can inhibit both neurotoxicity and aggregation of Aβ. Not all compounds that inhibit the neurotoxicity of Aβ can inhibit the aggregation of Aβ. For instance, fucoidan from *Fucus vesiculosus* was found to reduce the neurotoxicity of Aβ, however it was not found to have anti-aggregation effect (Jhamandas et al., 2005). Another study showed

that grape skin extract and EGCG can reduce the neurotoxicity of Aβ, but only EGCG was able to disrupt the fibril formation of Aβ (Harvey et al., 2011).

3.3.2 Drug likeness and likelihood of BBB penetration

Thirty-four compounds from sponges and 10 compounds from macroalgae follow the Lipinski rules. This is mainly due to the high molecular weight of many macroalgae-derived compounds where 9 compounds have molecular weight of more than 500 kDa, and 8 compounds have more than 10 H-bond acceptors or having more than 5 H-bond donors. Additionally, nine macroalgae-derived compounds had log p values of more than 5.

A total of 21 and 4 compounds from sponges and algae respectively are likely to pass through the blood brain barrier (BBB). The low number of algal compounds is mainly due to their high molecular weight, where 16 compounds have a molecular weight of more than 400 kDa. Moreover, high H-bond values may also attribute to a lower BBB permeability, where 12 compounds have more than 8 H-bonds, such as phlorotannins.

Drug and BBB likeness has been analysed in this study to understand the different "druggable" properties of compounds derived from marine sponges and algae. A low likeness score however does not predict CNS access with 100% accuracy. For instance, astaxanthin is predicted not to pass through the BBB, however a previous study found that astaxanthin is CNS accessible (Tso and Lam, 1996). Additionally, conforming to Lipinski's rule of five does not guarantee the success of a drug (Lipinski, 2004). Only 51% of FDA-approved drugs complied with these rules, and this does not cover natural product-based drugs (Zhang and Wilkinson, 2007). For

that reason, more studies on the drug and BBB likeness of such compounds are required.

Many compounds have been reported to exhibit certain neuroprotective bioactivity. However, these activities are not necessary associated with reducing the aggregation of Aβ. Compounds that inhibited acetylcholinesterase did not show promising results in the docking software for binding with Aβ. All these compounds showed less than 43, 62, and 218 docking scores with Aβ monomer, pentamer, and polymer, respectively. One of the acetylcholinesterase inhibitors criteria is to have a polar surface area (Gupta and Mohan, 2014) which means that compounds targeting acetylcholinesterase need to ne polar to bind to the active site, whereas Aβ has hydrophobic groove that is likely to be responsible for the aggregation (Wang et al., 2013) which will decrease the likeliness of these compounds to bind to the beta sheet in the hydrophobic groove. Also, compounds targeting serotonin and glutamate receptors did not show high docking scores. Compounds targeting oxidative stress and neurite outgrowth showed moderate scores with the Aβ polymer. On the other hand, compounds targeting BACE activity showed some promising results as anti-aggregation inhibitors, especially compounds derived from algae. It is interesting that compounds targeting BACE could inhibit aggregation of Aβ, as BACE is the enzyme that cleaves APP to produce Aβ. Furthermore compounds targeting GSK-3β especially from algae showed very high docking scores (>-215.9) with Aβ polymer. It may be difficult to find compounds that have multiple sites of neuroprotective action with anti-aggregation activity against Aβ, especially in relation to concomitantly targeting oxidative stress, neurotransmitter dynamics (acetylcholine, glutamate, and serotonin), and neurite outgrowth activity. It may be easier to find compounds that

can target BACE or GSK-3β and have anti-aggregation activity against Aβ. In addition, many researchers have been suggesting different methods for screening compounds that have dual targets against BACE-1 and GSK-3β for treating AD (Kumar et al., 2017). Curcumin was found to inhibit both BACE-1 and GSK-3β and for that reason many curcumin analogues have been synthesised in order to find the best dual inhibitor (Di Martino et al., 2016). In this study, compounds that showed docking scores of more than -250 with Aβ polymer include fascaplysin, lembehyne A, petrosiol C, and xestosaprol F from sponges, and 2-phloroeckol, 6,6'-bieckol, 8,8'-bieckol, astaxanthin, dieckol, eicosapentaenoic acid, fucoxanthin, fucoxanthinol, and phlorofucofuroeckol A from algae. However, only one sponge-derived compound from (lembehyne A) and six compounds from algae (6,6'-bieckol, 8,8'-bieckol, astaxanthin, dieckol, fucoxanthin, and phlorofucofuroeckol A) demonstrated higher binding scores than curcumin III (the highest binding score) against Aβ polymer. This highlights the potential of carotenoid and phlorotannin compounds as possible Aβ aggregation inhibitors.

3.4 Conclusion

In this study, an *in silico* modelling approach has been effective in identifying three sponge-derived, and seven algae-derived promising candidate compounds against the aggregation of Aβ from many neuroprotective compounds reported. With respect to compounds with six different neuroprotective activities, good correlation was observed between compounds targeting cholinesterase, serotonin receptors (for sponges), BACE-1 (for algae), GSK-3β (for algae), and glutamate receptors (for sponges). Of note, lembehyne A from sponges and 6,6'-bieckol, 8,8'-bieckol, astaxanthin, dieckol, fucoxanthin and phlorofucofuroeckol A from algae showed better or equivalent activities than the highest docking reference control curcumin III,

and therefore warrant future tests in various *in vitro* and in vivo models to validate efficacy and selectivity against Aβ.

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4. CHAPTER4: IMPACT OF EXTRACTION PROCESSES ON THE NEUROPROTECTIVE ACTIVITIES OF *ECKLONIA RADIATA* AGAINST AMYLOID BETA (AB_{1-42}) TOXICITY AND AGGREGATION

Contribution: Mousa Alghazwi has done most of the experiments, analysed all the data, and wrote the manuscript. Suvimol Charoensiddhi has done all extraction process. Wei Zhang, Scott Smid, and Ian Musgrave provide feedback to improve the work and helped in editing the manuscript.

4.1 Introduction

Alzheimer's disease (AD) is the main neurodegenerative disease responsible for dementia that is predicted to affect more than 130 million people worldwide by 2050 (Pratchett, 2015). AD can be characterized by memory loss and cognitive decline (Glass et al., 2010). Amyloid plaques, a hall mark of AD, contain the extracellular amyloid beta (Aβ) peptide (Selkoe, 2001), the deposition of which results from an imbalance between Aβ production and clearance leading to aggregate formation (Querfurth and LaFerla, 2010). Aggregated Aβ is neurotoxic and is thought to play a key role in the pathogenesis and neurological damage in AD. To date, there is no effective drug to treat or prevent AD (Yiannopoulou and Papageorgiou, 2013). One approach for new pharmacotherapies is to inhibit the aggregation and/or toxicity of Aβ (Citron, 2010).

Macroalgae-derived compounds have been shown to possess antibacterial (Lima-Filho et al., 2002), anticancer (Lowenthal and Fitton, 2015), antioxidant (Castro et al., 2014) and neuroprotective activities (Alghazwi et al., 2016b). In particular, neuroprotective activities include inhibition of amyloid beta neurotoxicity (Jhamandas et al., 2005), inhibition of oxidative stress (Heo et al., 2008) and enhancement of neurite outgrowth (Kamei and Tsang, 2003). There are more than 1200 different species of macroalgae reported in Southern Australia. Among these species, there are 231 species of brown algae of which 57% are endemic (Womersley, 1990). *Ecklonia radiata* (C. Agardh) J. Agardh is one of the most abundant brown seaweed species in South Australia (Wiltshire et al., 2015). A recent review summarized the different biological activities reported from *Ecklonia* species, which include antioxidant, anti-inflammatory, anti-bacterial, and neuroprotective properties (Koirala et al., 2017).

However, little is known regarding the impact of extraction methods of macroalgae on neuroprotective activities, and indeed there is no report on neuroprotective activities of *Ecklonia radiata*.

In this study, we investigated six different fractions of *Ecklonia radiata*, prepared by different extraction methods, for their multiple neuroprotective activities against $A\beta_{1-42}$ toxicity in PC-12 cells, anti-aggregation effect against $A\beta_{1-42}$ using thioflavin T (ThT) assay, the antioxidant activity against reactive oxygen species (ROS) generated by H_2O_2, and their ability to enhance neurite outgrowth in PC-12 cells.

4.2 Materials and Methods

4.2.1 Reagents and materials

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 97.5%), hydrogen peroxide, and Roswell Park Memorial Institute 1640 (RPMI) were purchased from Sigma-Aldrich (USA). Penicillin/streptomycin and 10 × trypsin EDTA were obtained from Thermo Fisher Scientific (Scoresby, VIC, Australia). Foetal bovine serum (FBS) was purchased from Bovogen Biologicals (East Keilor, VIC, Australia). Human amyloid-β_{1-42} protein ($A\beta_{1-42}$) was obtained from rpeptide (Bogart, Georgia, USA).

4.2.2 Seaweed extraction and fractionation process

All six fractions were prepared by the extraction processes described in previous papers with some modifications (Charoensiddhi et al., 2017a, Charoensiddhi et al., 2017b). A summary of the extraction process for the six fractions is shown in Figure 4-1.

4.2.2.1 Crude extract (CE)

The dried and ground seaweed was dispersed in pH-adjusted water in the ratio 1:10 (w/v). The pH was adjusted using 1 M HCl to achieve the optimum pH of Viscozyme at 4.5. The enzyme solution was added at 10% (v/w), and the enzymatic hydrolysis was performed under optimal conditions at 50°C for 3 h under continuous shaking. The enzyme was inactivated by boiling the sample at 100°C for 10 min and cooling immediately in an ice bath. The extract was centrifuged, and the supernatant was collected, adjusted to pH 7.0, freeze dried, and stored at −20°C until analysis and use.

4.2.2.2 Phlorotannin (PT), polysaccharide (PS), and free sugar fractions (FS)

The dried seaweed powder was firstly extracted with 90% (v/v) ethanol at a seaweed solid to solvent ratio of 1:10 (w/v) to obtain PT from the seaweed biomass. The residue was further processed by enzymatic hydrolysis (the same as CE). Ethanol was then added to the supernatant of CE to a concentration of 67% (v/v) to precipitate PS. PS was precipitated at 4°C overnight, and then collected FS (supernatant) by centrifugation. The ethanol in PT and FS was evaporated in a rotary evaporator. PT, PS, and FS were freeze dried and stored at −20°C until analysis and use.

4.2.2.3 Low molecular weight (LM) and high molecular weight fractions (HM)

These fractions were prepared using the same process as for CE, but the supernatant of CE without phlorotannin removal was subsequently separated into two fractions based on their MWs. Ethanol was added to CE at the concentration of 67% (v/v) to precipitate HM. HM was precipitated at 4°C overnight before centrifugation. LM was collected as the supernatant.. The ethanol in the supernatant fraction (LM) was

evaporated in a rotary evaporator. LM and HM were freeze dried and stored at −20°C until analysis.

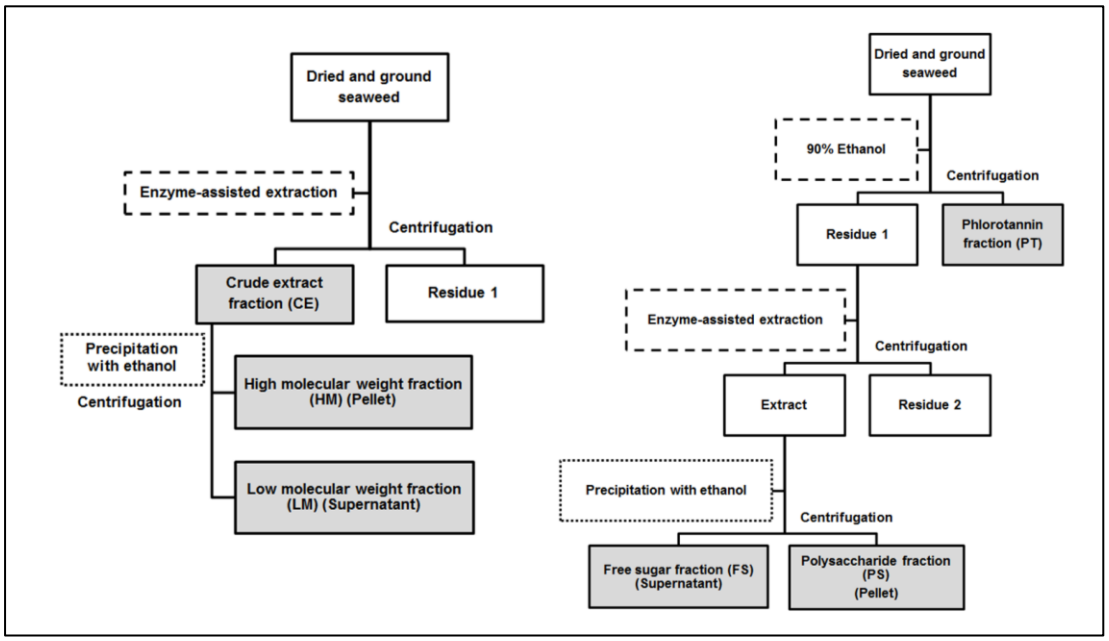


Figure 4-1 The extraction processes for the preparation of the six fractions used in this study.

4.2.3 PC12 cell culture

Rat pheochromocytoma PC-12 cells displaying a semi-differentiated neuronal phenotype with neuronal projections were used and maintained in RPMI-1640 media with 10% (v/v) foetal bovine serum (FBS), and 1% (w/v) penicillin/ streptomycin.

4.2.4 Aβ preparation and treatment in PC12 cells

Native, non-fibrillar $A\beta_{1–42}$ was prepared by dissolving dry $A\beta_{1–42}$ in 1% DMSO to yield a protein concentration of 3.8 mM. Sterile PBS was added to prepare a final concentration of 100 μM. Amyloid was then dispensed into aliquots and immediately frozen at −70 °C until required.

4.2.5 MTT assay for neuronal cell viability

The MTT assay was used to evaluate the cytotoxicity of the seaweed fractions, by measuring mitochondrial activity as an index of cell viability. Cells were seeded at 2 ×

10^4 cells per well in 100 µl media into 96 well tissue culture plates. *E. radiata* fractions were diluted in PBS to their final stock concentrations prior to addition to cells. PC12 cells were treated with each of these fractions at six different concentrations (3.125, 6.25, 12.5, 25, 50, and 100 µg/ml) and then incubated for 48 hours at 37 °C. After incubation, the media was removed and replaced with 0.5 mg/ml of MTT (diluted in 1 × PBS). The plate was then incubated for 2 hours at 37 °C. After that, the MTT solution was removed from the cells and cells lysed with 100 µl of DMSO. The absorbance of the plate was measured at 570 nm with a reference wavelength of 630 nm using a microplate reader (Bio-Tek Instruments Inc, USA). The same procedure for cytotoxicity was followed as before. After treating the cells with different concentrations of *E. radiata* fractions, cells were incubated for 15 minutes prior to the addition of $A\beta_{1-42}$ (1 µM). Cells were then incubated for 48 hours at 37°C prior to measurement of cell viability.

4.2.6 Thioflavin T assay of Aβ fibril formation

Thioflavin T (ThT; 10 µM in PBS) was added with $A\beta_{1-42}$ (10 µM), alone or in combination with the highest non-toxic concentration of each *E. radiata* fraction. The plate was incubated at 37^oC in a fluorescence microplate reader (Bio-Tek, Bedfordshire, UK) with excitation at 446 nm and emission at 490 nm every 10 minutes for 48 hours to assess inhibiting effects on $A\beta_{1-42}$ fibril kinetics.

4.2.7 MTT assay for protecting PC-12 cells against H_2O_2-induced cytotoxicity

PC-12 cells were incubated for 15 mins after the *E. radiata* fractions treatment, then the cells were treated with H_2O_2 (100 µM). Cells were then incubated for 24 hours at 37°C prior to the measurement of cell viability by MTT assay.

4.2.8 Nuclear staining for assessment of apoptosis

PC-12 cells were seeded at a density of 2×10^4 overnight. Then, the cells were treated at with different *E. radiata* fractions at different concentrations (ranging from 3.125 - 50 µg/mL) for 15 minutes before adding $A\beta_{1-42}$ (1 µM). The cells were incubated for 48 hours and then 5 µg/mL of Hoechst 33258 stain was added and incubated for 10 minutes in the dark to stain the cell nuclei. The cells were then washed with 1×PBS and the plates were examined/imaged using EVOS FL Cell Imaging System (Thermo Fisher) fluorescence microscope. The percentage of apoptotic cells (n=400 cells per well) was calculated as followed:

Apoptotic rate % = apoptotic cells ÷ total cells (viable cells + apoptotic cells) ×100

Three independent experiments were performed for every treatment.

4.2.9 Neurite outgrowth assay

PC-12 cells were seeded at a density of 2×10^3 /well in 6-well plates and incubated overnight at 37°C. After that, the media was replaced with serum-free media and the cells treated with different *E. radiata* fractions at different concentrations ranging from 3.125 to 100 µg/mL for 24 hours. Then, cells were visualized using an inverted microscope (Olympus CK2) at × 400 magnification and images were taken from at least six random fields and then analysed using ImageJ software. Neurite outgrowth was considered positive when the length of projections on cells were equal to or longer than the size of cells, and the amount of neurite outgrowth was calculated from at least 300 cells per treatment. Neuronal outgrowth was expressed as:

Neuronal outgrowth% = (Number of cells with neurites / total number of cells) x 100.

4.2.10 Statistical analysis

All results were based on at least three independent experiments (n = 3). The effects of *E. radiata* fractions on the cell viability of PC12 cells were analysed using one-way ANOVA, followed by Tukey's honestly significant difference (HSD) post hoc test using SPSS software (Version 22). Area under the curve (AUC) analysis was measured by comparing the different treatment against $A\beta_{1-42}$ using one-way ANOVA with post hoc test. Differences were considered statistically significant at $p < 0.05$.

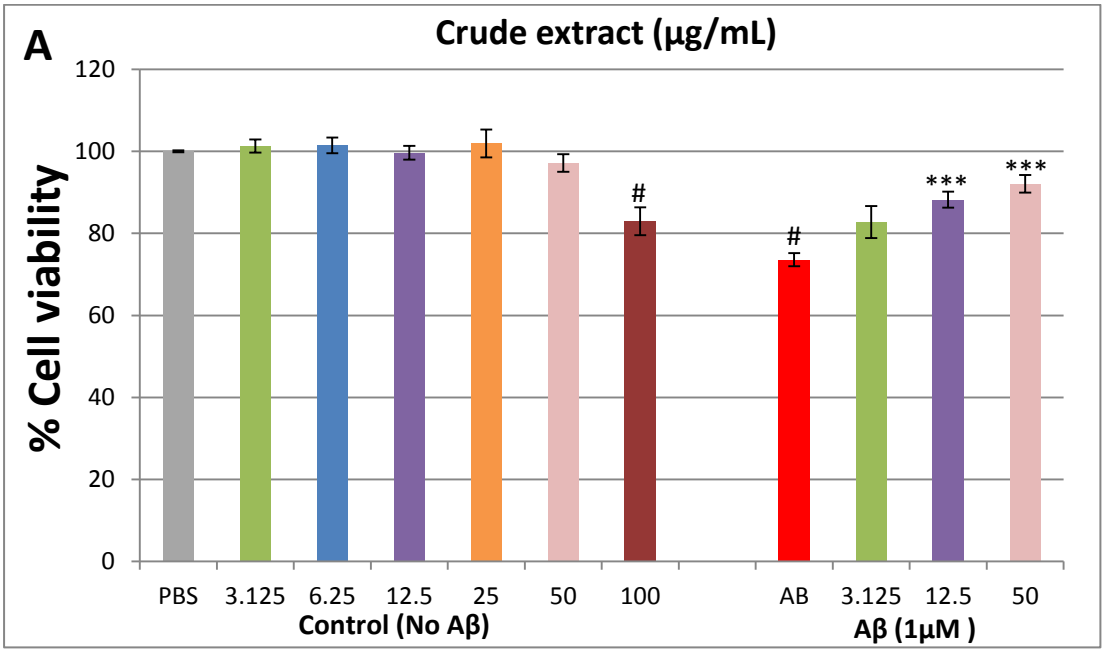
4.3 Results and discussion

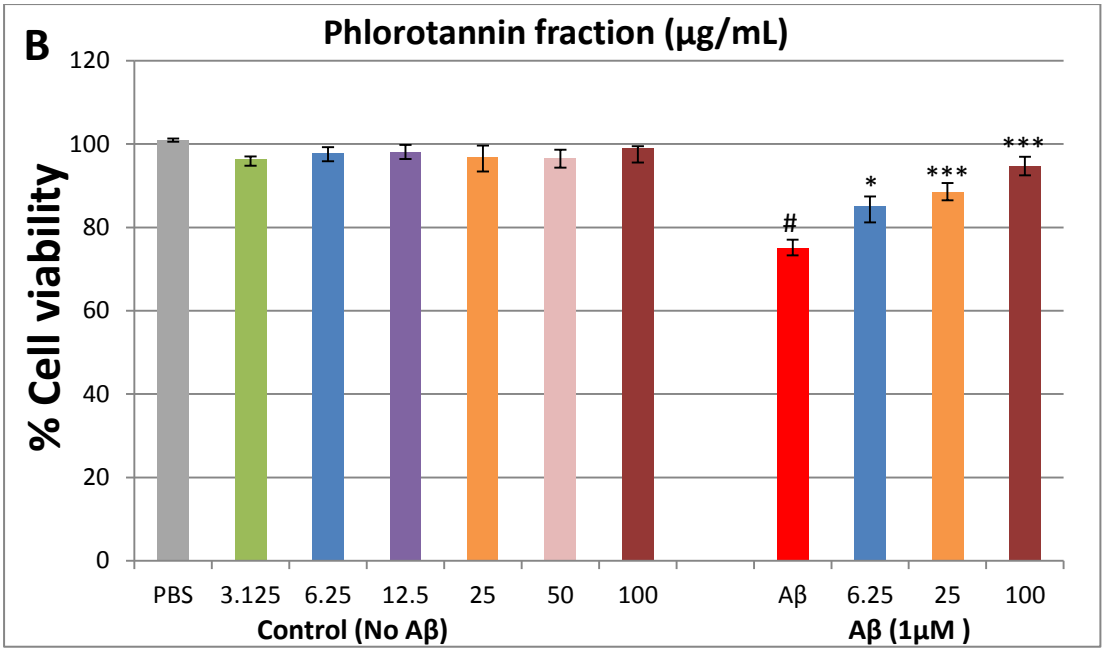
4.3.1 Neurotoxicity and neuroprotective activity of *E. radiata* fractions against $A\beta_{1-42}$

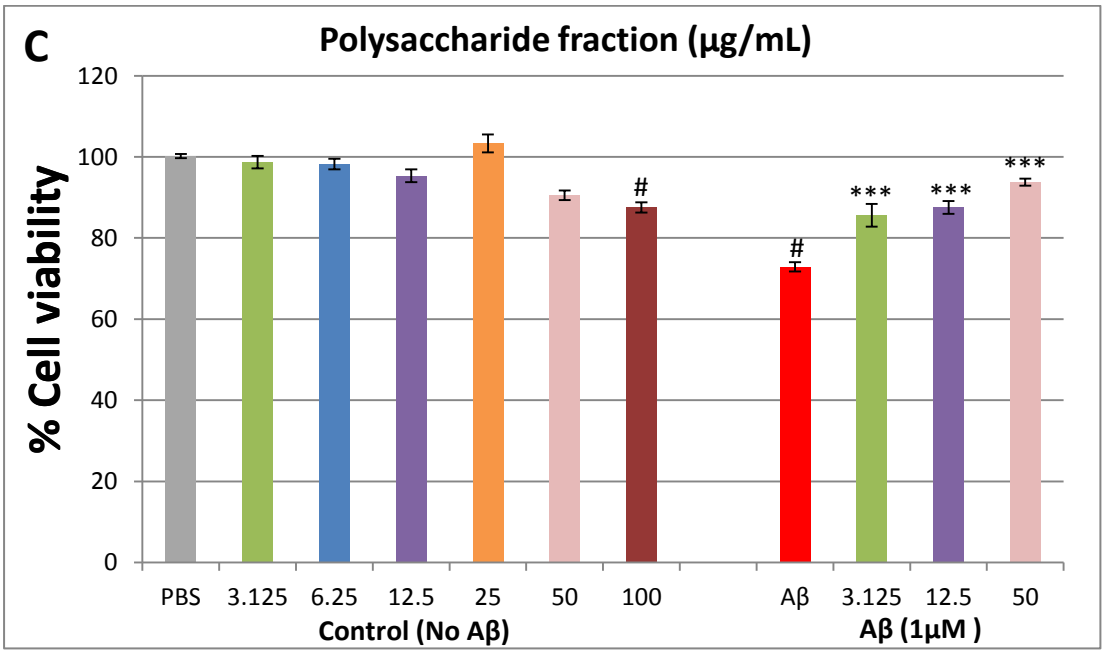
In order to evaluate the neurotoxicity of these fractions, the MTT assay was used. Three fractions (PT, FS, and LM) of *E. radiata* did not show significant cytotoxicity to PC-12 cells even at the highest concentration of 100 μg/mL (Figure 4-2B, 4-2D, and 4-2F). On the other hand, the HM fraction showed the highest cytotoxicity at 25 μg/mL and above, with cell viability below 68% (Figure 4-2E). CE and PF fractions showed cytotoxic effects to cells only at the highest concentration of 100ug/mL, with cell viability less than 88% (Figure 4-2A and 4-2C). The cytotoxicity of some of these fractions might be attributed to the presence of some toxic components in these fractions in addition to neuroprotective components.

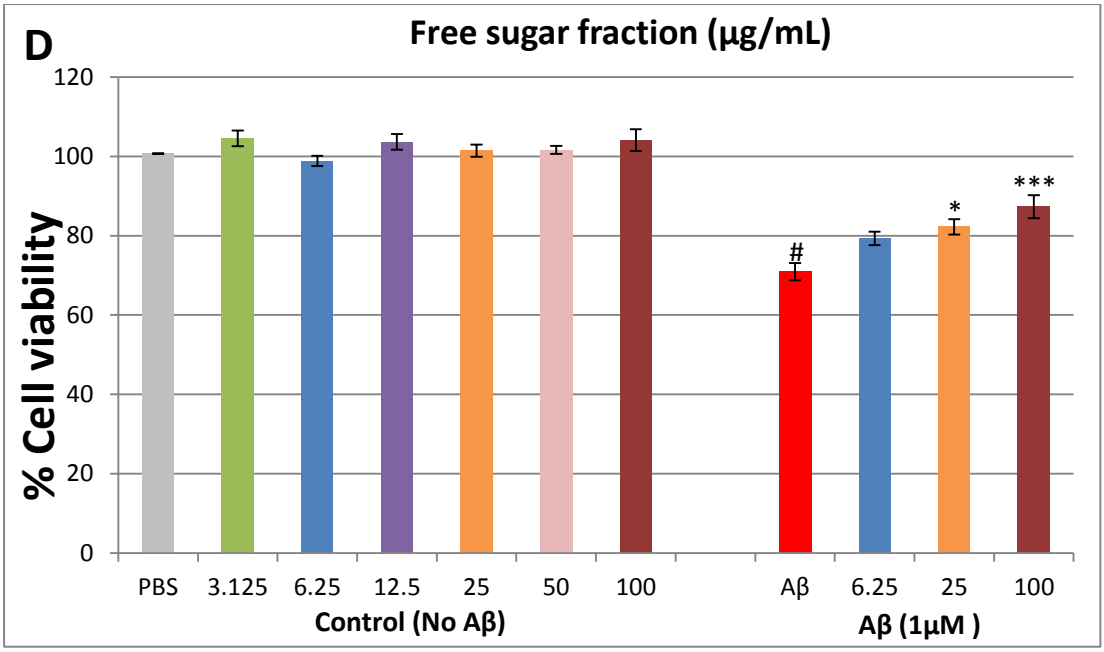
Interestingly, all fractions showed a concentration-dependent neuroprotective activity against the cytotoxicity induced by $A\beta_{1-42}$. All the fractions demonstrated neuroprotective activities by increasing the cell viability to more than 78% in the lowest tested concentrations. Interestingly, the CE, PT, and PS fractions demonstrated the highest neuroprotective activities, with cell viability exceeding 92% in the highest

tested concentrations. On the other hand, the HM fraction did not demonstrate significant neuroprotective activities even at the highest tested concentration (12.5 µg/mL). The results of *E. radiata* fractions are promising, as other studies have reported similar results. For example, 50 µg/mL of butanol extract of *Ecklonia cava* resulted in increasing cell viability from 75% in the $A\beta_{1-42}$ oligomer treatment to about 90% in primary rat cortical neurons (Kang et al., 2011). This highlights the potential of macroalgae as sources of neuroprotective compounds, as several derived compounds reduce the cytotoxicity induced by Aβ, such as fucoidan (Jhamandas et al., 2005) and an acidic oligosaccharide sugar chain (Hu et al., 2004).




B
Phlorotannin fraction (µg/mL)
% Cell viability
120
100
80
60
40
20
0
PBS
3.125
6.25
12.5
25
50
100
Aβ
6.25
25
100
Control (No Aβ)
Aβ (1µM)
#
*
***
***


C
Polysaccharide fraction (µg/mL)
% Cell viability
120
100
80
60
40
20
0
PBS
3.125
6.25
12.5
25
50
100
Aβ
3.125
12.5
50
Control (No Aβ)
Aβ (1µM)
#
#
***
***
***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Free sugar fraction (µg/mL)
% Cell viability
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100
80
60
40
20
0
#
*
***
PBS
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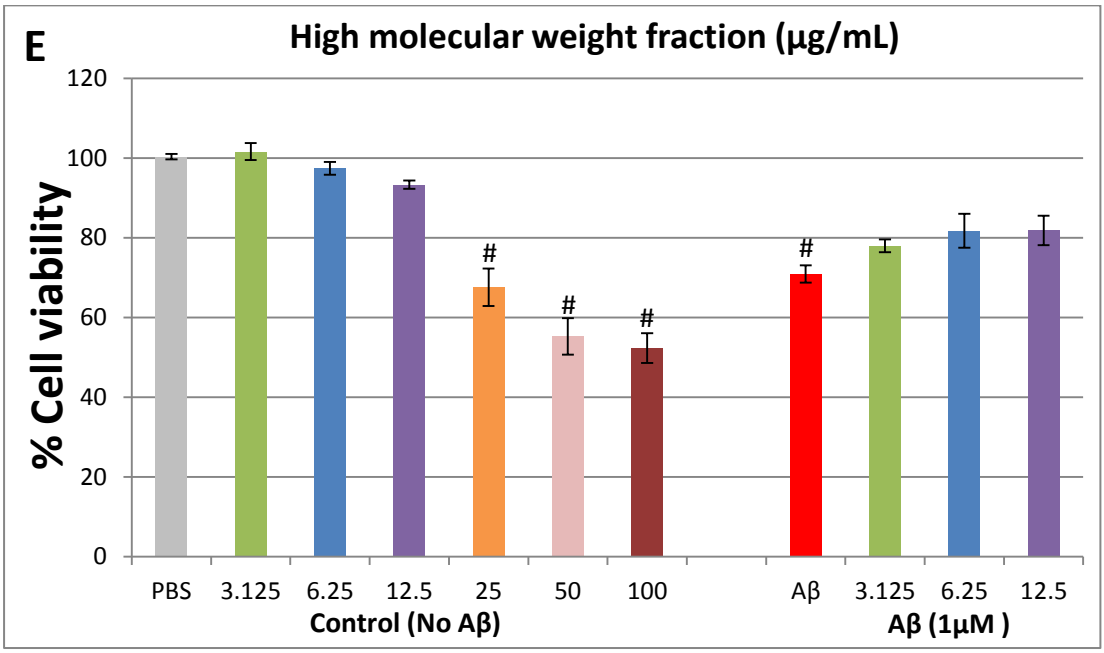
100
Aβ
6.25
25
100
Control (No Aβ)
Aβ (1µM)


E
High molecular weight fraction (µg/mL)
% Cell viability
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100
80
60
40
20
0
#
#
#
#
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3.125
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50
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Aβ
3.125
6.25
12.5
Control (No Aβ)
Aβ (1µM)

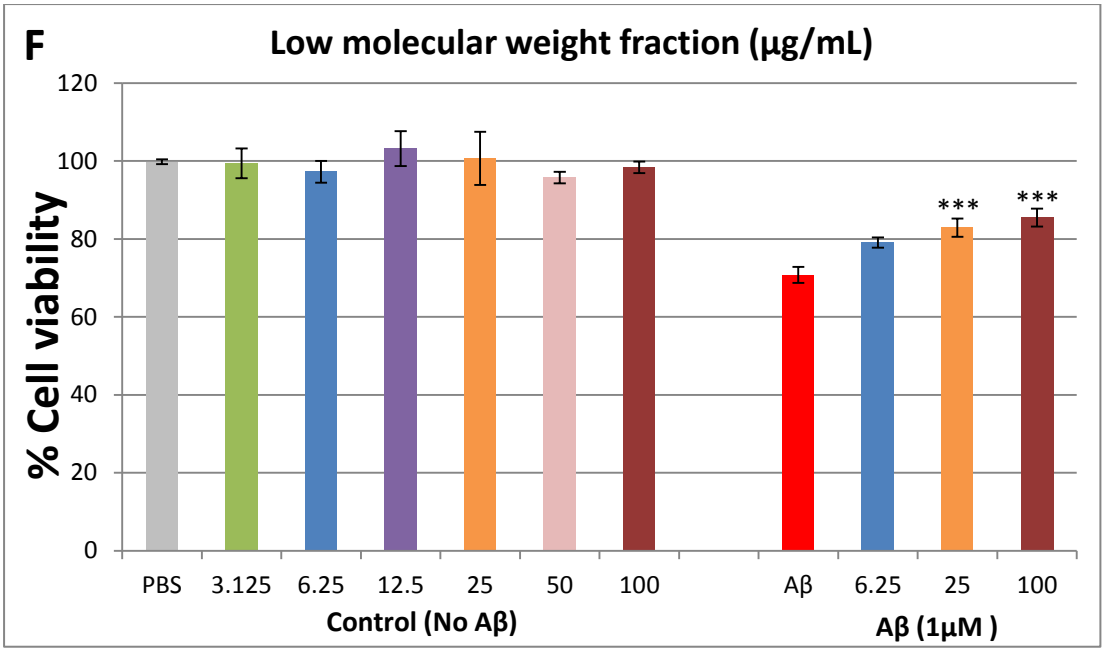
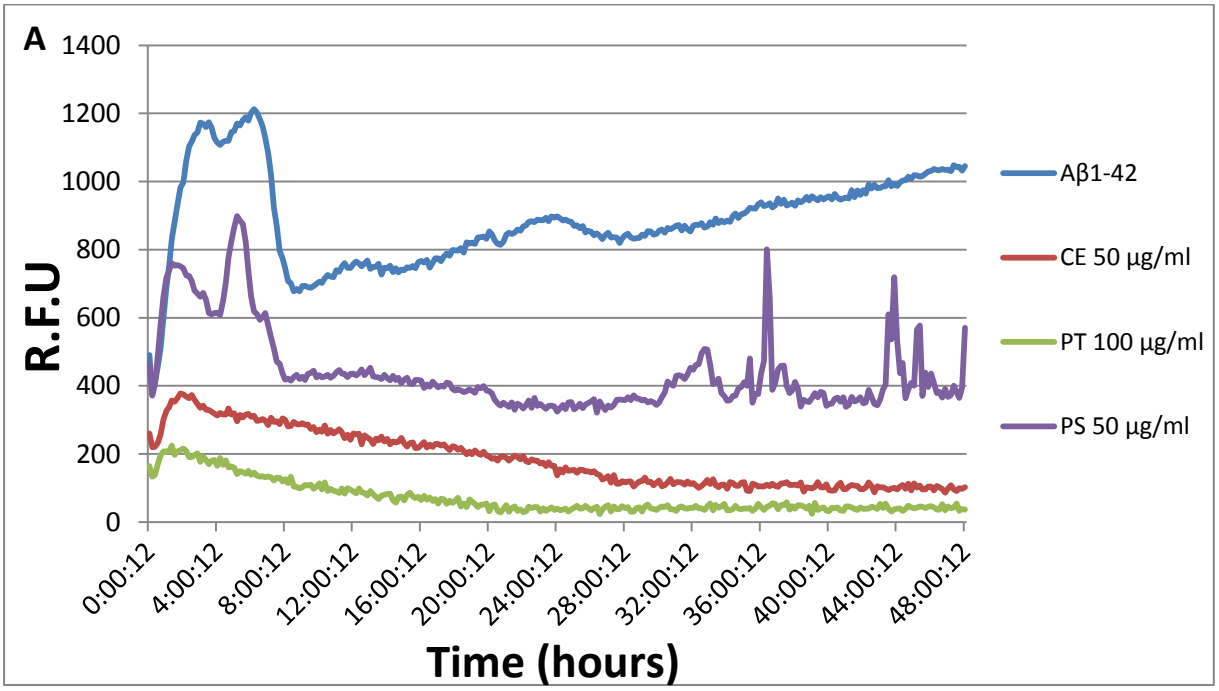


Figure 4-2 Relative cell viability (%) of PC12 cells estimated by MTT assay representing different concentrations of *E. radiata* fractions treatment alone, $A\beta_{1-42}$ alone, or $A\beta_{1-42}$ (1 µM) with *E. radiata* fractions [crude extract CE (A), phlorotannin fraction PT (B), polysaccharide fraction PS (C), free sugar fraction FS (D), high molecular weight fraction HM (E), and low molecular weight fraction LM (F)] treatment after 48 hours of incubation (n=4) (# p <0.05 vs PBS, * p <0.05, ** p <0.01, and ***p <0.005 vs control 1 µM $A\beta_{1-42}$)

4.3.2 Effects of *E. radiata* fractions against $A\beta_{1-42}$ fibril and aggregate formation

Anti-aggregation effects of six *E. radiata* fractions against $A\beta_{1-42}$ were investigated using the thioflavin T (ThT) fluorescent assay of fibril kinetics. All the fractions inhibited the aggregation of $A\beta_{1-42}$ significantly ($p<0.05$), with the exception of the HM fraction (Figure 4-3A - 4-3C). Four fractions: CE, PT, FS, and LM fractions showed the highest anti-aggregation effects. Among these four fractions, the PT fraction showed the highest anti-aggregation effect, inhibiting more than 90% of $A\beta_{1-42}$ formation ($p<0.005$). The PS fraction (the lowest phlorotannin content) demonstrated moderate inhibition of 50% of $A\beta_{1-42}$ formation ($p<0.05$), while the HM fraction demonstrated the lowest inhibition, which might be due to the low concentration used in this study due to cytotoxicity ($p>0.05$) (Figure 4-3C). These

results suggest phlorotannin compounds play a role in inhibiting $A\beta_{1-42}$ fibril formation. We have also seen that fucoidan rich polyphenolic samples derived from *Fucus vesiculosus* show higher anti-aggregation effects than fucoidans from the same species (Alghazwi et al., 2018b). Phlorotannins are the most common polyphenolic compounds in brown seaweed (Ragan and Glombitza, 1986). While no one has reported that seaweed-derived phlorotannins could inhibit $A\beta_{1-42}$ aggregation, some plant-based natural polyphenolic compounds were reported to possess anti-aggregatory activity against $A\beta_{1-42,}$ such as (−)-epigallocatechin-3-gallate (EGCG) and curcumin (Hudson et al., 2009, Yang et al., 2005). In comparison, acidic oligosaccharide sugar chain (AOSC), a compound derived from *Ecklonia kurome*, was demonstrated to inhibit around 50% of the $A\beta_{1-40}$ formation at 100 µg/mL (Hu et al., 2004). In addition, 250 µg/mL of acetone extract of *Padina gymnospora* inhibited 80% of $A\beta_{25-35}$ aggregation over 48 hours of treatment (Shanmuganathan et al., 2015). These results highlight the potential of phlorotannins as promising neuroprotective compounds that have anti-aggregation effects against $A\beta_{1-42}$ formation.



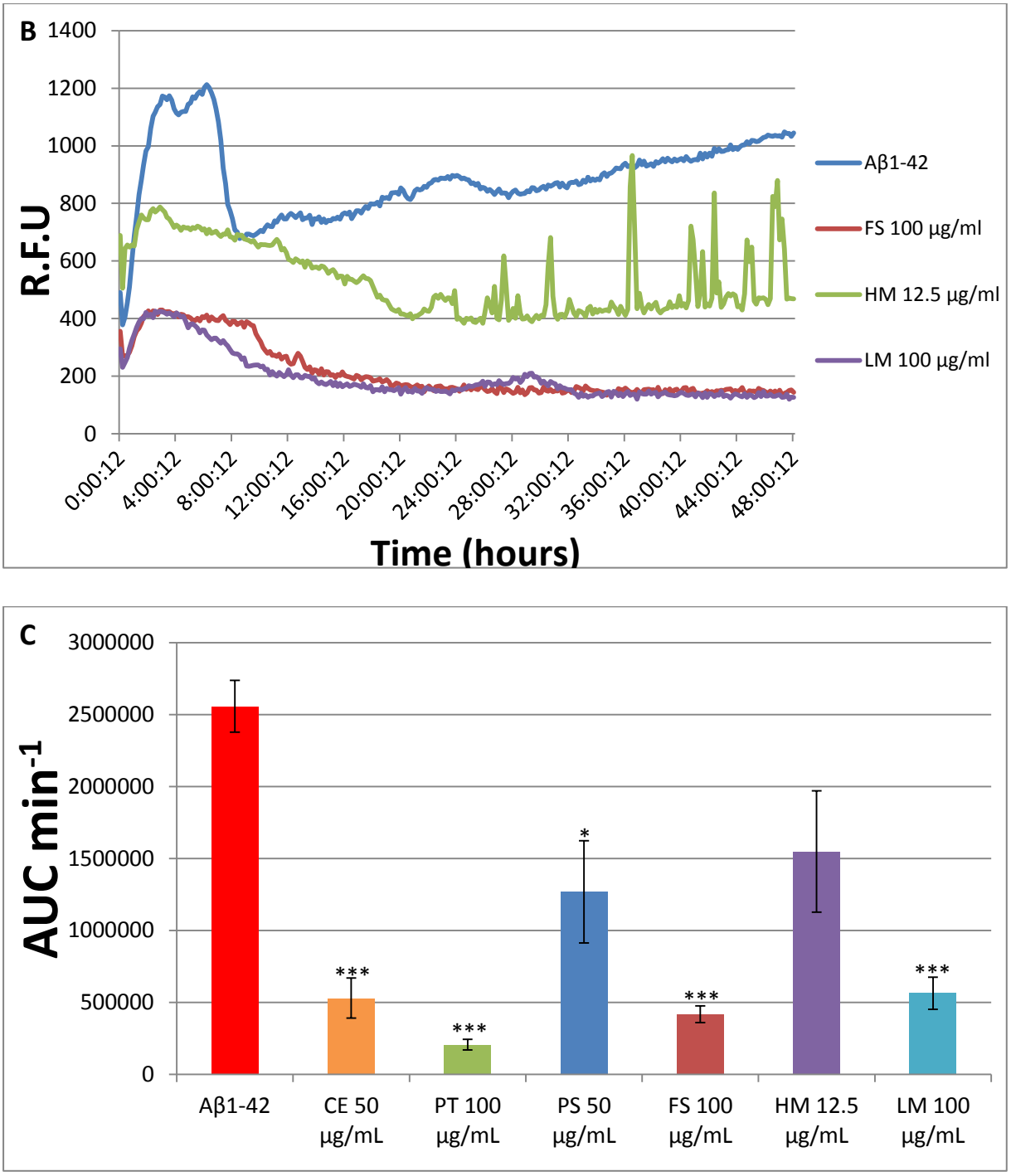
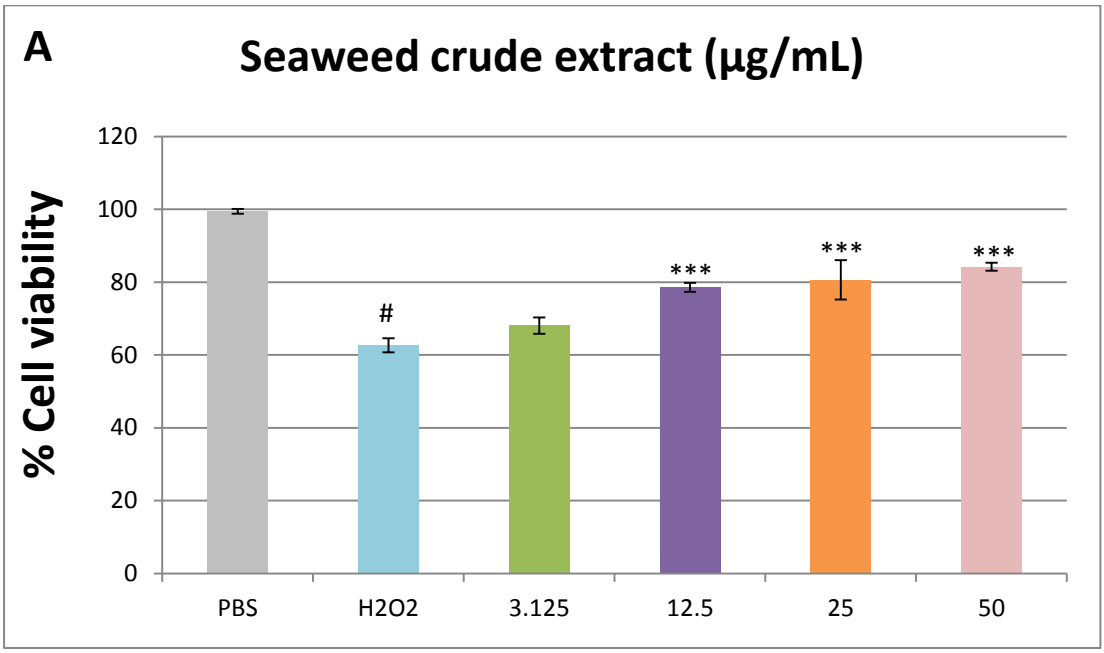


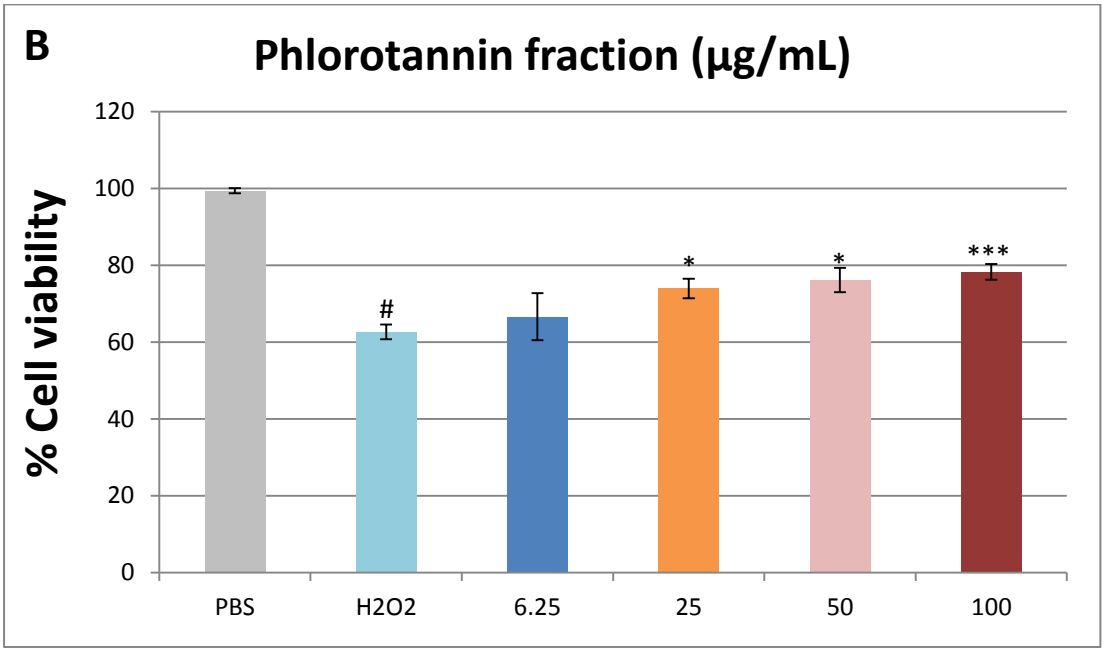
Figure 4-3 Thioflavin T (ThT) fluorescence assay demonstrating amyloid $A\beta_{1-42}$ fibrillization kinetics over 24 h in PBS, alone or in the presence of the highest non-toxic concentration of crude extract (CE), phlorotannin (PT), and polysaccharide (PS) fractions (A), free sugar (FS), high molecular weight (HM), and low molecular weight (LM) fractions (B). Area under the curve (AUC) was measured to quantitate the effects of these fractions against overall kinetics of $A\beta_{1-42}$ fibrillization (C) (n=3) (* p <0.05, ** p <0.01, and ***p <0.005 vs control 10 µM $A\beta_{1-42}$)

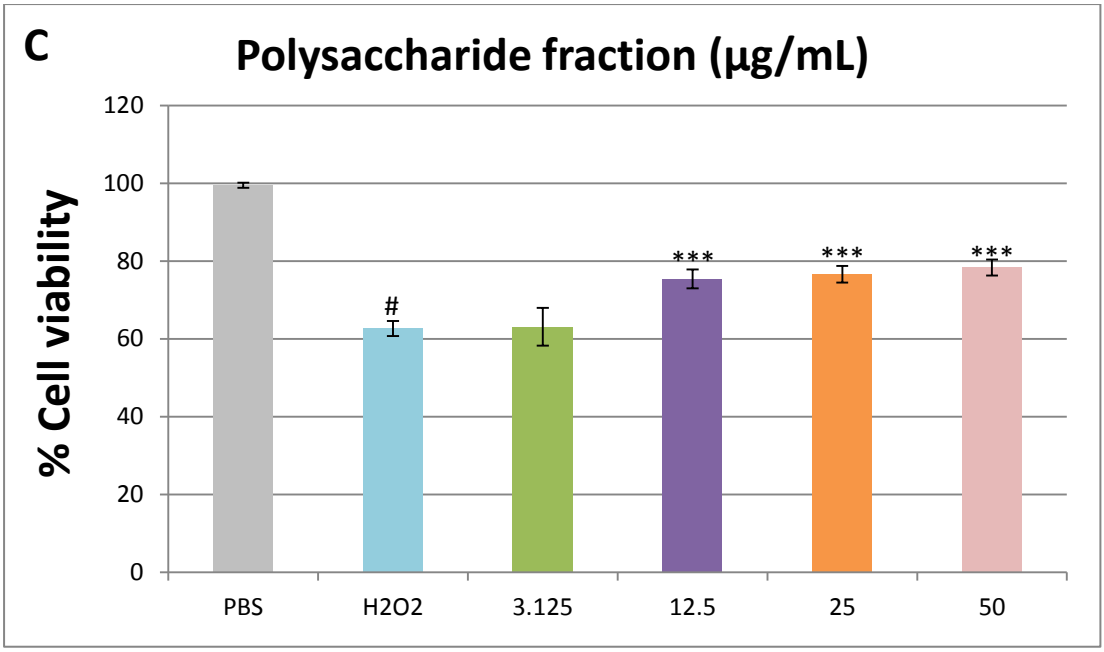
4.3.3 Bioactivity of *E. radiata* fractions against H_2O_2

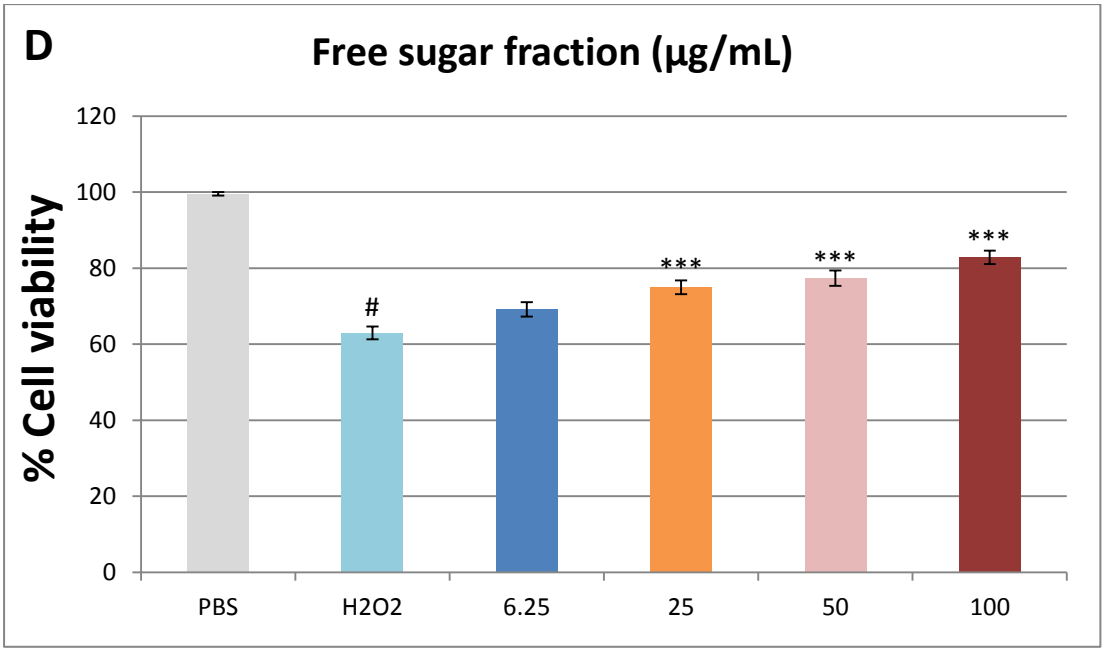
Reactive oxygen species (ROS) induced by Aβ has previously been demonstrated in *in vitro* and *in vivo* models (Li et al., 2008b, Peng et al., 2009). Therefore, the neuroprotective activity of these fractions was evaluated against hydrogen peroxide exposure (100 μM) for 24 hours in PC-12 cells. Hydrogen peroxide at 100 μM caused about 38% reduction in cell viability (Figure 3). Overall, all the fractions demonstrated neuroprotective activities by reducing the neurotoxicity of hydrogen peroxide, with the exception of the HM fraction. Two fractions (PT and FS) showed significant antioxidant activity at 25 μg/mL ($p<0.05$), CE and PS showed significant antioxidant activity at 12.5 μg/mL ($p<0.05$), and LM fraction showed significant antioxidant activity at 50 μg/mL ($p<0.05$). The CE fraction showed the highest antioxidant activity, as the cell viability of PC-12 cells could be recovered from 62% in the presence of 100 μM H2O2 to about 84% when treated at 50 μg/mL CE (Figure 4-4A), while the LM fraction showed the lowest protection, with 100 μg/mL LM resulting in a recovery from 62% to 75% cell viability (Figure 4-4F). The other three fractions (PS, PT, and FS) showed varying protection, with a recovery of 63-82% viability in the range of tested concentrations (3.125 – 100 μg/mL) (Figure 4-4B, 4-4C, and 4-4D). It is not surprising that these fractions demonstrated antioxidant activity, as algae are known to have different antioxidant compounds (Munir et al., 2013), while many extracts from different *Ecklonia* species demonstrate antioxidant activity (Koirala et al., 2017). Furthermore, an enzymatic extract of *E. radiata* demonstrated antioxidant activity in ORAC and FRAP assays (Charoensiddhi et al., 2015). Many algae derived compounds demonstrate antioxidant effects, such as fucoidan (de Souza et al., 2007b) and five phlorotannin compounds (dieckol, eckstolonol, eckol, triphloroethol A and phloroglucinol) (Kang et al., 2013), but we

are yet to analyse the constituent phlorotannins in our samples to confirm their presence.






C
Polysaccharide fraction (µg/mL)
% Cell viability
120
100
80
60
40
20
0
#
***
***
***
PBS
H2O2
3.125
12.5
25
50


D
Free sugar fraction (µg/mL)
% Cell viability
120
100
80
60
40
20
0
#
***
***
***
PBS
H2O2
6.25
25
50
100

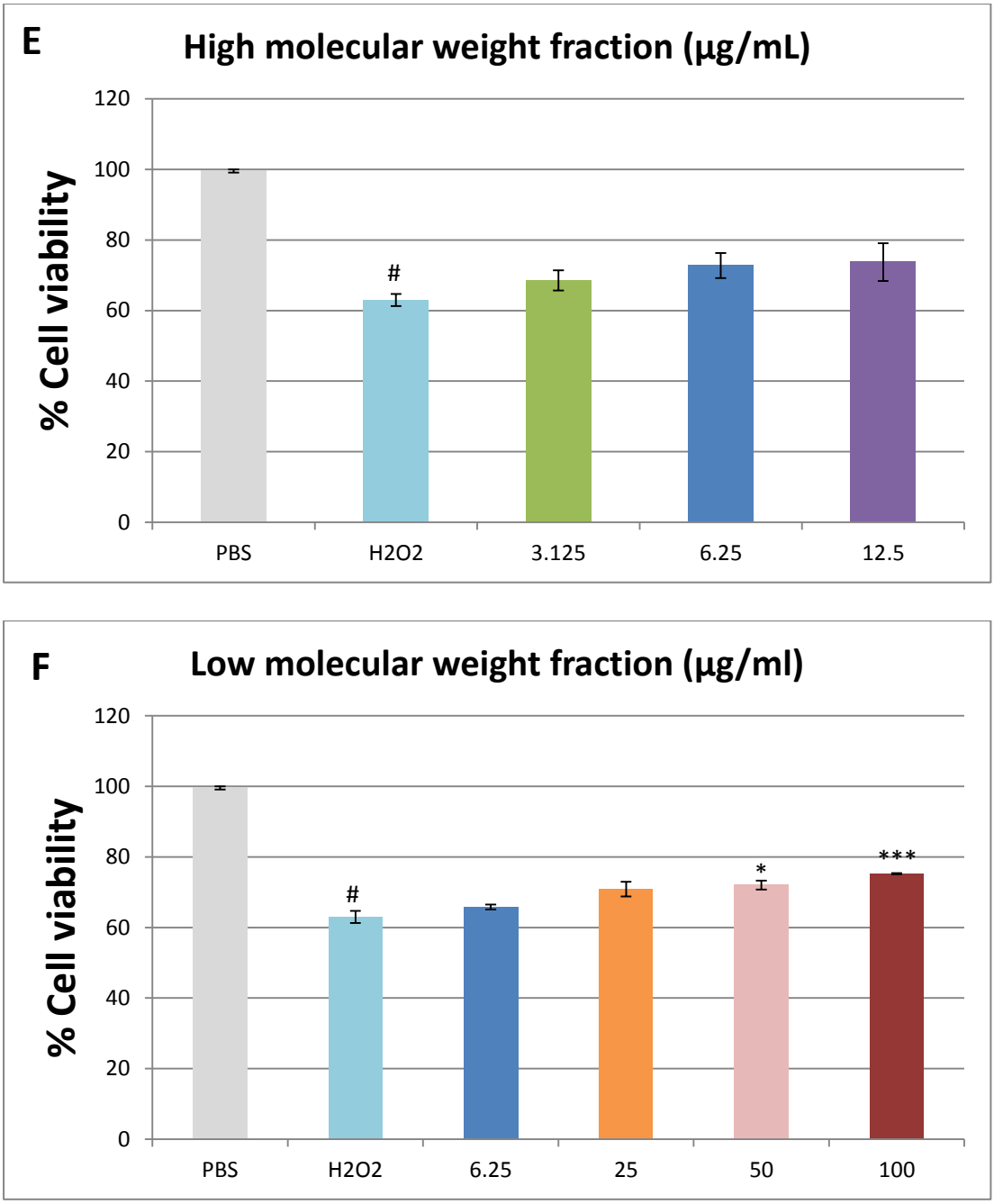
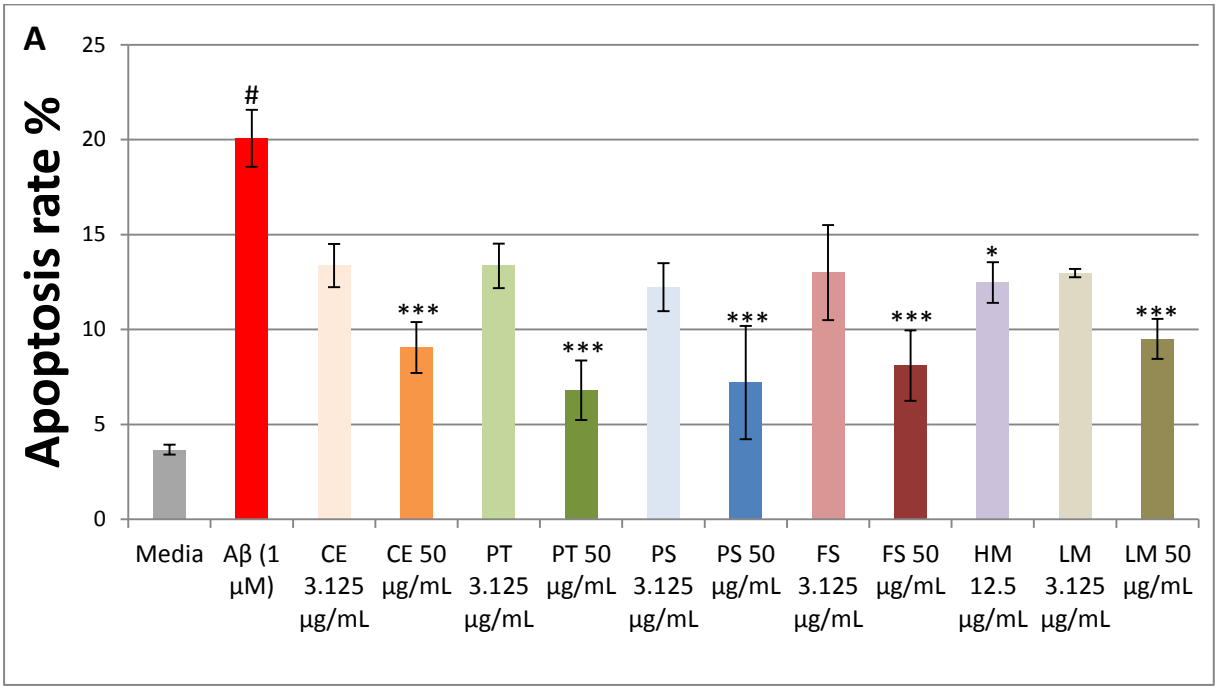


Figure 4-4 Relative cell viability (%) of PC12 cells estimated by MTT assay representing different concentrations of *E. radiata* fractions [crude extract (A), phlorotannin (B), polysaccharide (C), free sugar (D), high molecular weight (E), and low molecular weight fraction (F)] treatment with H_2O_2 (100 µM) after 24 hours of incubation (n=4). (# p <0.05 vs PBS, * p <0.05, ** p <0.01, and ***p <0.005 vs control 100 µM H_2O_2)

4.3.4 Neuroprotective activities of *E. radiata* fractions in reducing the PC12 cell apoptosis induced by $A\beta_{1-42}$

Hoechst 33258 stain is a dye commonly used to stain double-stranded DNA and thus can be used to stain apoptotic cells. Six fractions were investigated to determine

whether they can prevent apoptosis induced by $A\beta_{1-42}$. Treating PC-12 cells with 1 μM of $A\beta_{1-42}$ resulted in approximately 20% apoptosis. However, treating the cells with these different fractions resulted in anti-apoptotic effects. All the fractions showed singificant neuroprotective activity ($p<0.05$) at the highest concentration used in this study, with apoptosis rates less than 12.5% (Figure 4-5A). The phlorotanin fraction demonstrated the highest anti-apoptosis activity with an apoptosis rate of less than 7%, while HM demonstrated the lowest activity with an apoptosis rate of 12.5%. This might be due to the high content of the hydroxyl group in phlorotanin compounds as an earlier study demonstrated neuroprotective activity of 6,6′-bieckol against high glucose-induced cytotoxicty in INS-1 cells (Park et al., 2015). The PS fraction demonstrated high activity, which might be attributed to the presence of high fucoidan content, as fucoidan from *F. vesiculosus* has been shown to inhibit apoptosis through inhibiting caspase 3 and 9 (Jhamandas et al., 2005). These results indicate that both the phlorotannin and fucoidan content of these fractions can influence the anti-apoptotic effects.



B

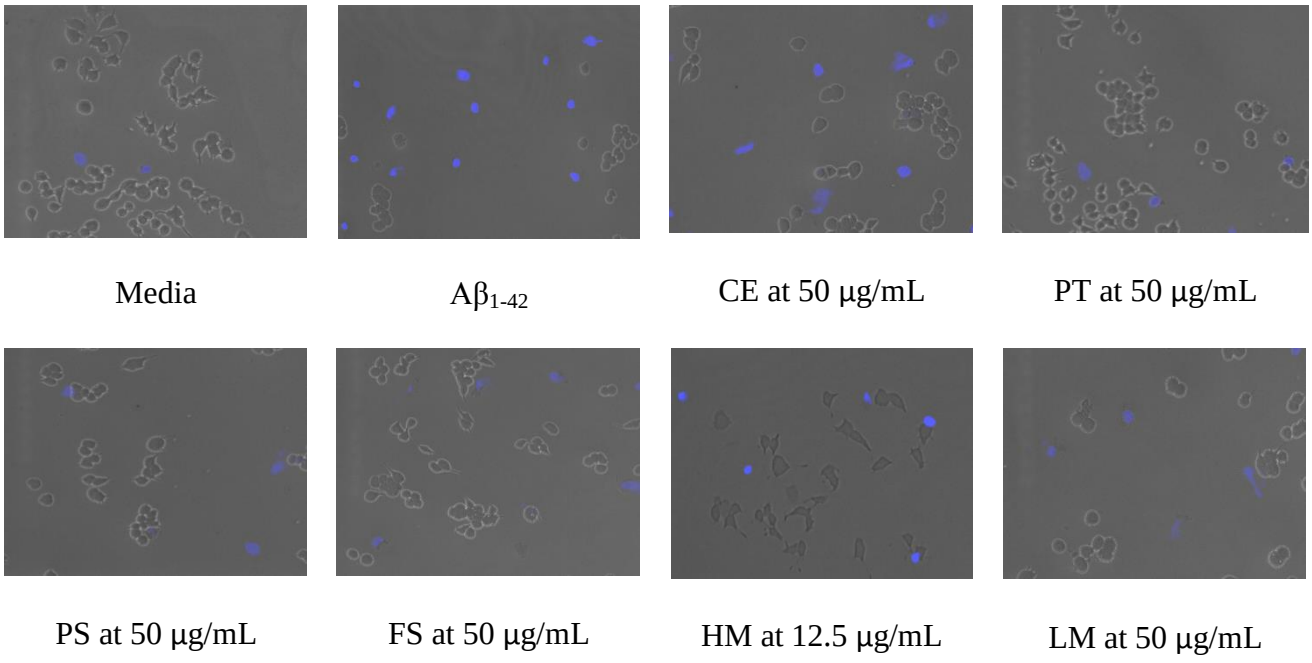


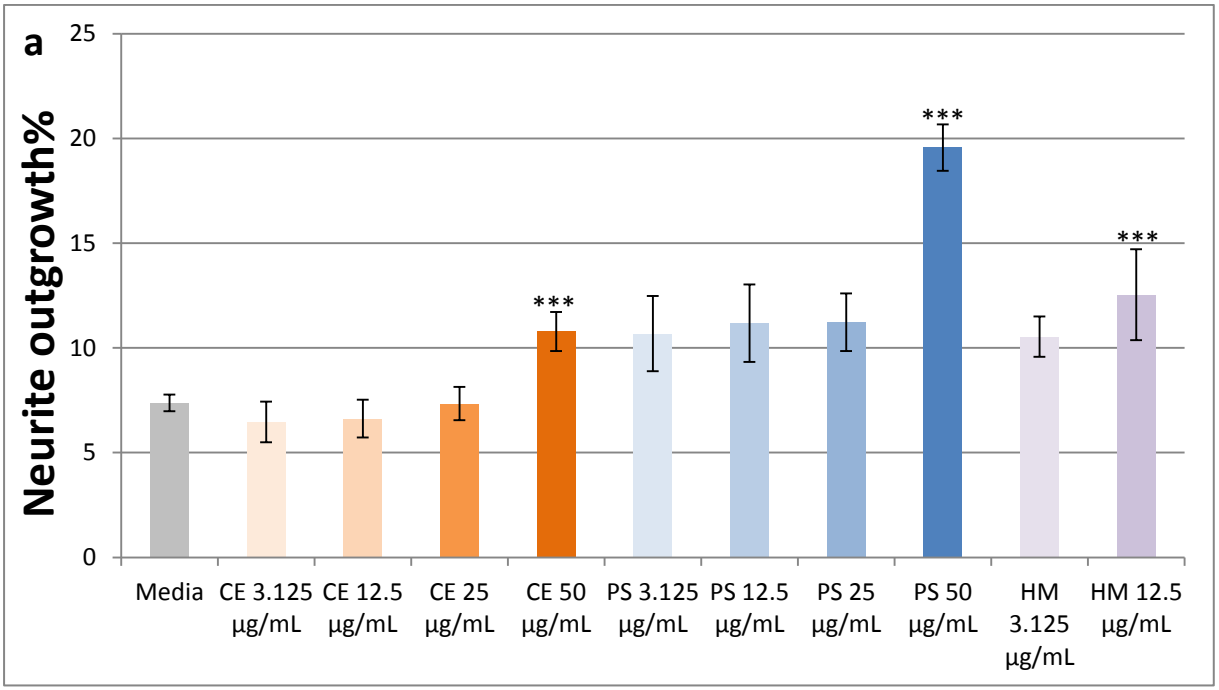
Figure 4-5 (A**) Effect of different concentrations of crude extract (CE), phlorotannin (PT), and polysaccharide (PS), free sugar (FS), high molecular weight (HM), and low molecular weight (LM) fractions on apoptosis induced by $A\beta_{1-42}$ (1 µM) in PC12 cells for 48 hours. (**B**) Cells were stained using Hoechst 33258 stain and then visualized using fluorescent microscopy. At least 400 cells were counted for every treatment. Each value is the mean ± SEM of four independent experiments (# *p* <0.005 versus PBS, * *p* <0.05, ** *p* <0.01, and ***p* <0.005 versus control $A\beta_{1-42}$)**

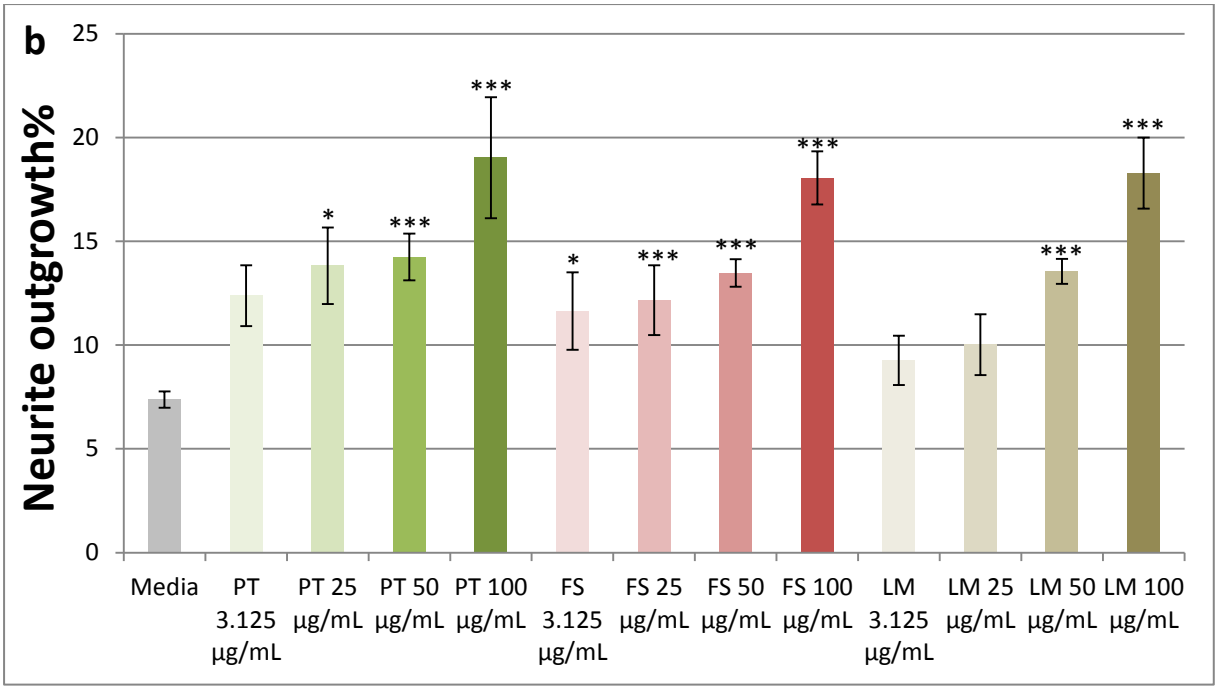
4.3.5 Neuroprotective activities of *E. radiata* fractions in enhancing neurite outgrowth

All the six fractions of *E. radiata* demonstrated to enhance neurite outgrowth significantly at least at the highest tested concentration ({3.125 µg/mL for FS}, {12.5 µg/mL for HM}, {25 µg/mL for PT}, and {50 µg/mL for CE, PS, and LM}) (Figure 4-6A and 4-6B). Four fractions (PS, PT, FS, and LM) enhanced neurite outgrowth by more than 18% in the highest concentration used. Among these four fractions, the PS fraction was the most potent as it enhanced the neurite outgrowth in PC-12 cells to more than 19% at 50 µg/mL, compared to the other 3 fractions which showed similar activity but at higher concentration (100 µg/mL). This might be due to the high

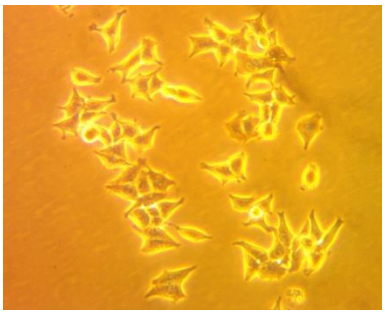
content of fucose in the PS fraction compared to others, as high fucose content was responsible for enhancing neurite outgrowth activity (Shida et al., 2017). In addition, phlorotannin compounds might play a role in this activity, where fractions rich in phlorotannin content demonstrated high activity. On the other hand, CE and HM fractions showed the lowest activity among all the fractions.

It was reported that some extracts isolated from algae have been reported to enhance neurite outgrowth, such as an ethanol extract from the red algal species *Kappaphycus alvarezii* (Tirtawijaya et al., 2016) and *Porphyra yezoensiswhich* (Mohibbullah et al., 2016), which showed enhanced neurite outgrowth in hippocampal neurons which highlight the potential of algae derived compounds in this activity.

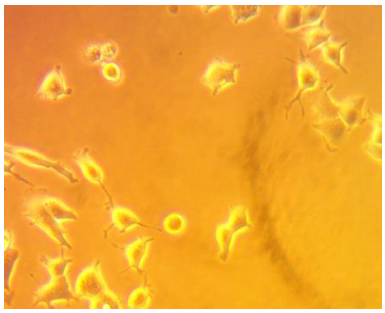




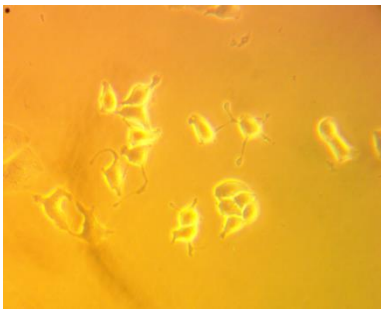
C



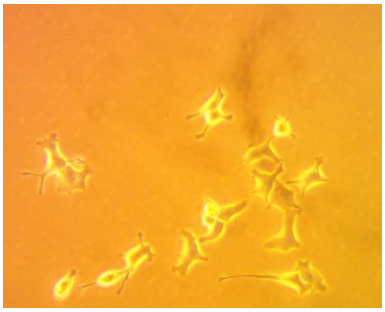
Media



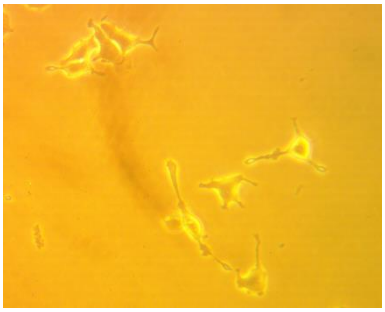
CE at 50 µg/mL



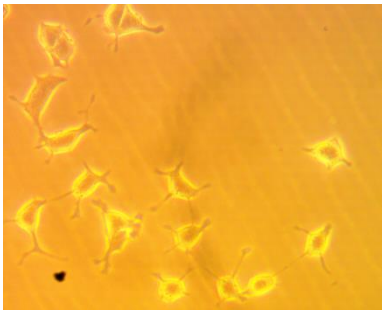
PT at 100 µg/mL



PS at 50 µg/mL



FS at 100 µg/mL



LM at 100 µg/mL

Cell without neurite Cell with neurie outgrowth

Figure 4-6 Effect of different concentrations of crude extract (CE), phlorotannin (PT), and polysaccharide (PS) fractions (A**), free sugar (FS), high molecular weight (HM), and low molecular weight (LM) fractions (**B**) on enhancing neurite outgrowth in PC12 cells. (**C**) Cells were visualized using light microscopy. At least 300 cells were counted for every treatment. Each value is the mean ± SEM of four independent experiments (* *p* <0.05 and ****p* <0.005 versus control media)**

4.4 Conclusion

In this study, six different *Ecklonia radiata* extract fractions prepared by different extraction processes were tested for their multiple neuroprotective activity. Neurotoxicity in PC-12 cells is associated with exposure to the crude extract, the high molecular weight and polysaccharide fractions at the highest tested concentration (100 μg/mL) at least. All extracts were shown to inhibit neurotoxicity and apoptosis induced by $A\beta_{1-42}$ in PC12 cells. The phlorotannin content is likely to play a role in $A\beta_{1-42}$ anti-aggregation activity, as the phlorotannin fraction demonstrated the highest activity, while the polysaccharide fraction was most effective in enhancing neurite outgrowth, which suggests fucose content plays a role in this activity. In addition, all fractions inhibited cytotoxicity induced by H_2O_2 in PC-12 cells, with the exception of the high molecular weight fraction. These findings support further investigation into neuroprotective constituents derived from *Ecklonia radiata* as potential leads in the development of neurodegenerative disorders such as Alzheimer's disease.

4.5 References

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5. CHAPTER 5: COMPARATIVE STUDY ON NEUROPROTECTIVE ACTIVITIES OF FUCOIDANS FROM *FUCUS VESICULOSUS AND UNDARIA PINNATIFIDA*

Contribution: Mousa Alghazwi has done most of the experiments, analysed all the data, and wrote the manuscript. Samuel Karpiniec has done all fucoidan analysis. Wei Zhang, Scott Smid, and Ian Musgrave provide feedback to improve the work and helped in editing the manuscript.

5.1 Introduction

Alzheimer's disease (AD) accounts for more than 80% of dementia cases (Anand et al., 2014). Histopathological studies of the AD-affected brains demonstrate the presence of amyloid beta (Aβ) plaques and neurofibrillary tangles (NFT) (Querfurth and LaFerla, 2010). Aβ accumulation is thought to play a role in the neuronal damage found in AD. Currently there is no available disease-modifying drug for AD, the five approved AD therapy drugs only alleviate some symptoms of the disease (Cummings et al., 2014). Thus drugs which act on the mechanism(s) of AD are needed.

Fucoidan is a group of sulphated polysaccharides derived mainly from brown algae. Fucoidan can also be found in other marine organisms such as sea urchin and sea cucumber (Mulloy et al., 1994). The structures of fucoidan polymers vary from one algae species to another (Li et al., 2008a). In addition, the molecular weight of fucoidan varies among different species and is usually very high (>20 kDa). For example, fucoidan extracted from *Fucus vesiculosus* was reported to have a molecular weight of 82.5 kDa, while fucoidan from *Undaria pinnatifida* was reported to be 51.7 kDa (Fitton et al., 2015). Moreover, the molecular weight of fucoidan from the same species may vary, depending on the preparation methods, where the molecular weight of fucoidan extracted from *Undaria pinnatifida* and its commercially sourced fucoidan was found to be 171 kDa and 54 kDa, respectively (Mak et al., 2013). Another group reported that fucoidan from *U. pinnatifida* has a molecular weight of 2100 kDa. The difference in molecular weights was suggested to be attributable to different extraction methods (Kim et al., 2007).

Fucoidan has been shown to exhibit anticoagulant, anti-inflammatory, antiangiogenic, gastric protection, antibacterial, antiviral and anti-tumour activities (Cumashi et al., 2007, Li et al., 2008a, Morya et al., 2012). In addition, fucoidan has been shown to exhibit different neuroprotective activities that include inhibiting reactive oxygen species (ROS) (de Souza et al., 2007b), anti-inflammatory activity (Cui et al., 2010, Cui et al., 2012), promoting brain-derived neurotrophic factor release (BDNF) (Lee et al., 2012) and inhibiting both 1-methyl-4- phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Luo et al., 2009) and 6-hydroxydopamine (6-OHDA)-mediated toxicity (Zhang et al., 2014). For that reason, fucoidan was identified as one of the most promising neuroprotective compounds isolated from macroalgae (Alghazwi et al., 2016b), and may have utility as a mechanism based treatment for AD.

Fucoidan isolated from *Fucus vesiculosus* demonstrated neuroprotective activity against Aβ, but did not inhibit its aggregation (Jhamandas et al., 2005). Fucoidan isolated from *Laminaria japonica* can improve learning and memory in Aβ-treated rats by inhibiting the activity of acetylcholine esterase (AChE), reducing oxidative stress and promoting anti-apoptotic activity at a dosage of 200 mg/kg (Gao et al., 2012). Recently, fucoidan isolated from *Undaria pinnatifida* was investigated in PC-12 cells for neuroprotective activity against toxicity induced by D-galactose and $A\beta_{25-35}$ (Wei et al., 2017). Fucoidan from *U. pinnatifida* can increase cell viability by inhibiting the apoptosis induced by D-galactose and $A\beta_{25-35}$, while also improving learning and memory in impaired mice (Wei et al., 2017).

The aim of this current study was to directly compare the neuroprotective activities of five fucoidan samples with different chemical compositions prepared from two

commonly studied brown seaweed species, *Fucus vesiculosus* and *Undaria pinnatifida,* in multiple assays protecting against the neurotoxicity and aggregation of $A\beta_{1\text{-}42}$, including inhibition of apoptosis induced by $A\beta_{1\text{-}42}$ and mitigating hydrogen peroxide toxicity and enhancing neurite outgrowth.

5.2 Materials and Methods

5.2.1 Reagents and materials

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 97.5%), hydrogen peroxide, and Roswell Park Memorial Institute 1640 (RPMI) were purchased from Sigma-Aldrich (USA). Foetal bovine serum (FBS) was purchased from Bovogen Biologicals (East Keilor, VIC, Australia). Penicillin/streptomycin and 10 × trypsin EDTA were obtained from Thermo Fisher Scientific (Scoresby, VIC, Australia). Human amyloid-$\beta_{1\text{–}42}$ protein ($A\beta_{1\text{–}42}$) was obtained from Mimotopes (Melbourne, VIC, Australia).

5.2.2 Fucoidan extraction and analysis

Three fucoidan samples (FE, FF, and S) from *F. vesiculosus* and two fucoidan samples (UE and UF) from *U. pinnatifida* were provided kindly by Marinova (Tasmania, Australia). The extraction and isolation was done according to previous study (Fitton et al., 2015). The fucoidan component, molecular weight distribution and carbohydrate profile can be found in Table 5-1, Table 5-2, and Table 5-3, respectively. Fucoidan FF has a lower content of uronic acid than fucoidan FE. Fucoidan S contains the highest phlorotannins content (28.2%) compared to the other four samples (< 2.5%). It also has the lowest total carbohydrates of 50.21% when compared with other four samples ranging from 57.3-67.5%. Fucoidan UE has less total carbohydrate content and less sulfate content than fucoidan UF, but it has higher

uronic acid content than UF. The molecular weights of the samples varied between 32 to 203 kDa. Most of the molecular weight distribution of fucoidan FE and FF is in the range (5-200 kDa) and (20-1100 kDa), accounting for 61% and 75% of total molecular weight, respectively. In addition, fucoidan S has highest molecular weight peak, as most of the molecular weight distribution is in the range (60-1100 kDa) that accounts for 41% of the total molecular weight, while most of polyphenol content is in the range (<5 kDa). Fucoidan UE has lower molecular weight than fucoidan UF, due to the fact that most of molecular weight distribution in the range (>5, and 20-200 k) versus (20-200 k) accounting for 57.5% and 68.4% of total molecular weight. Fucoidan S has the lowest fucose content and highest glucose content, while fucoidan FE highest xylose content in *F. vesiculosus*. On the other hand fucoidan UF has the higher galactose content than fucoidan UE, while UE has higher glucose content than UF.

Table 5-1 Mass percentage of fucoidan samples components determined by Marinova Pty. Ltd.

Analyses	**Total Carbs (%)**	**Uronic Acid (%)**	**Sulfate (%)**	**Cations (%)**	**Polyphenol (%)**
FE	65.6	4.4	28.6	5.1	2.5
FF	67.5	1.4	26.6	11.9	<2
S	50.21	1.74	12.1	4.05	28.2
UE	57.3	5.0	23.2	7.05	<2
UF	64	0.9	31	6.8	<2

Table 5-2 Molecular weight distribution profile of fucoidan samples determined by Marinova Pty. Ltd.

Molecular weight distribution (%)								
Sample code	**Max peak MW**	**%>1600 k**	**%1100-1600**	**%200-1100**	**%60-200**	**%20-60**	**%5-20**	**%<5 k**
FE	**32948**	**7.4**	**0.8**	**10.0**	**19.8**	**24.6**	**16.7**	**20.7**
FF	**56901**	**5.8**	**1.3**	**17.4**	**30.9**	**27**	**6.6**	**11**
S	**203583**	**9.4**	**4**	**25.1**	**16.3**	**8**	**5.9**	**31.2**
UE	**48172**	**17.6**	**0**	**17.7**	**21.4**	**20.5**	**7.3**	**15.6**
UF	**84317**	**17.8**	**0**	**25.4**	**26.7**	**16.3**	**3.4**	**10.4**

Table 5-3 Carbohydrate profile (Weight %) of fucoidan samples using standardised methods by Marinova Pty. Ltd.

Sample code	Fucose	Xylose	Galactose	Glucose	Arabinose	Rhamnose
FE	45.7	6.8	2.6	0.0	0.0	0.0
FF	50.7	2.2	2.1	0.2	0.4	0.3
S	30.6	3.7	2.6	4.4	0.0	0.0
UE	21.7	1.4	16.1	1.3	0.8	0.4
UF	23.7	1.9	21.4	0.0	0.0	0.0

5.2.3 PC-12 cell culture

Rat pheochromocytoma PC12 cells displaying a semi-differentiated phenotype with neuronal projections were used as model neuronal cells and maintained in RPMI-1640 media with 10% (v/v) foetal bovine serum (FBS), and 1% (w/v) penicillin/ streptomycin.

5.2.4 Preparation and treatment of $A\beta_{1\text{-}42}$ in PC-12 cells

1% (v/v) DMSO was added to non-fibrillar $A\beta_{1–42}$ to dissolve it at a stock concentration of 3.8 mM. To prepare a final concentration of the protein at 100 μM, sterile PBS was added. Then, the protein was aliquoted, frozen immediately and stored at −70 °C until required.

5.2.5 MTT assay for cytotoxicity

Cell viability was determined using the MTT assay to evaluate the cytotoxicity of fucoidan samples. Initially, cells were seeded at 2×10^4 cells per well. PBS was used to dilute fucoidan samples to their final stock concentrations prior to addition to the cells. PC12 cells were treated with each of these fucoidan samples at six different concentrations (ranging from 3.125 to100 µg/ml) and then incubated for 48 hours at 37 °C. Then, the media was removed and replaced with 0.5 mg/ml of MTT. The plate was then incubated for 2 hours at 37 °C. After that, the cells were lysed with DMSO. The absorbance of the plate was measured at 570 nm with a reference wavelength of 630 nm using a Microplate Reader (Bio-Tek Instruments Inc, USA).

5.2.6 MTT assay for determining protection from $A\beta_{1-42}$-induced loss of P-C12 cell viability

The same procedure as above was used with minor modification. PC-12 cells were incubated for15 minutes with different concentrations of fucoidan, prior to the addition of $A\beta_{1–42}$ (1 µM). Cells were then incubated for 48 hours at 37°C prior to measurement of cell viability.

5.2.7 Thioflavin T assay of Aβ fibril

ThT (10 µM in PBS) was added with $A\beta_{1–42}$ (10 µM), alone or in combination with 12.5 or 100 µg/mL of each of the fucoidan samples. The plate was incubated at 37 °C in a fluorescence microplate reader (Bio-Tek, Bedfordshire, UK) with excitation at 446 nm and emission at 490 nm every 10 minutes for 24 hours to assess effects on $A\beta_{1–42}$ fibril kinetics.

5.2.8 Transmission Electron Microscopy (TEM) assay for $A\beta_{1-42}$ aggregate formation morphology

Transmission electron microscopy (TEM) was used to visualize the aggregation of $A\beta_{1-42}$ and to investigate the effects of the fucoidan samples on $A\beta_{1-42}$ aggregate morphology. Native $A\beta_{1-42}$ (10 μM) in PBS was prepared by incubating either alone or with the fucoidan samples (100 μg/mL) for the period of 24 hours at 37 °C. Then, 5 μl of the solution was loaded onto a 400 mesh formvar carbon-coated nickel electron microscopy grid (Proscitech, Kirwan, QLD, Australia) for 2 minutes and then filter paper was used to blot off the sample. Subsequently, 5 μl of contrast dye containing 2% uranyl acetate was added for two minutes to the grid and then blotted off. Finally, the grids were loaded and viewed using FEI Tecnai G2 Spirit Transmission Electron Microscope (FEI, Milton, QLD, Australia) and representative images were taken.

5.2.9 MTT assay for determining protection of P-C12 cells from H_2O_2 cytotoxicity

PC-12 cells were incubated with fucoidan samples for 15 mins prior to the addition of H_2O_2 (100 μM and 200 μM), then incubated for 24 hours at 37°C prior to measurement of cell viability as per the MTT assay (*2.6*).

5.2.10 Nuclear staining for assessment of apoptosis

PC-12 cells were seeded at a density of 2×10^4 per well overnight. Then, the cells were treated with different fucoidan samples at different concentrations for 15 minutes before adding $A\beta_{1-42}$. The cells were incubated for 48 hours and then 5 μg/mL of Hoechst 33258 stain was added and incubated for 10 minutes in the dark to stain the cell nuclei. The cells were then washed with 1×PBS and the plates were monitored using EVOS FL Cell Imaging System (Thermo Fisher) fluorescence microscope. The

percentage of apoptotic cells (n=400 cells at least per well) were calculated as followed:

Apoptotic rate % = apoptotic cells ÷ total cells (viable cells + apoptotic cells) ×100

Three independent experiments were performed for every treatment.

5.2.11 Neurite outgrowth assay

PC-12 cells were seeded at a density of 2×10^3 /well and were incubated overnight at 37°C. Media was then replaced with serum-free media and the cells were treated with different fucoidan samples at different concentrations ranging from 3.125 to 100 µg/mL for 24 hours. Then, the cells were visualized using an inverted microscope (Olympus CK2) at × 400 magnification and images were taken from at least six random fields and then analysed using ImageJ software. Neurite outgrowth was considered positive when the measured length of neurites was equal or longer than the size of cells, and the amount of neurite outgrowth was calculated from at least 200 cells per treatment. Neurite outgrowth% was calculated as number of cells positive for neurites (regardless of number of neurites per cell) / total number of cells.

5.2.12 Statistical analysis

All results were based on at least three independent experiments (n = 3). The effects of fucoidan samples on the cell viability of PC12 cells were analysed using one way ANOVA, followed by Tukey's honestly significant difference (HSD) post hoc test using SPSS software (Version 22). Area under the curve (AUC) analysis was measured by comparing the different treatment against $A\beta_{1-42}$ using one-way ANOVA with post-hoc test. Differences were considered statistically significant at $p < 0.05$.

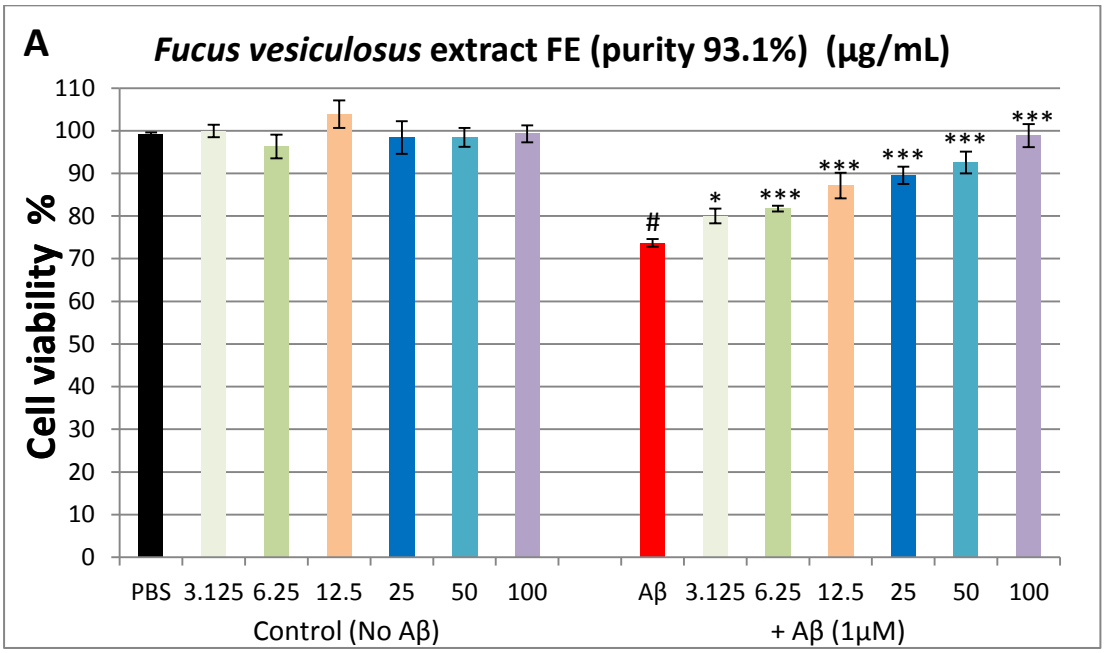
5.3 Results and Discussion

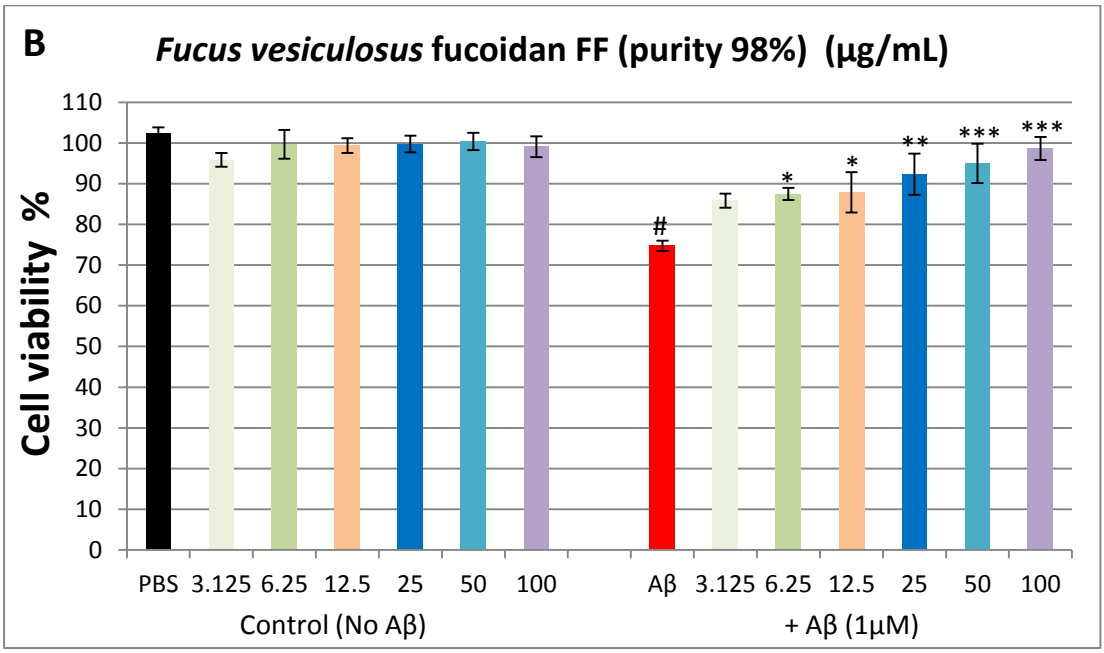
5.3.1 Cytotoxicity and neuroprotection of fucoidan samples against $A\beta_{1-42}$-induced cytotoxicity

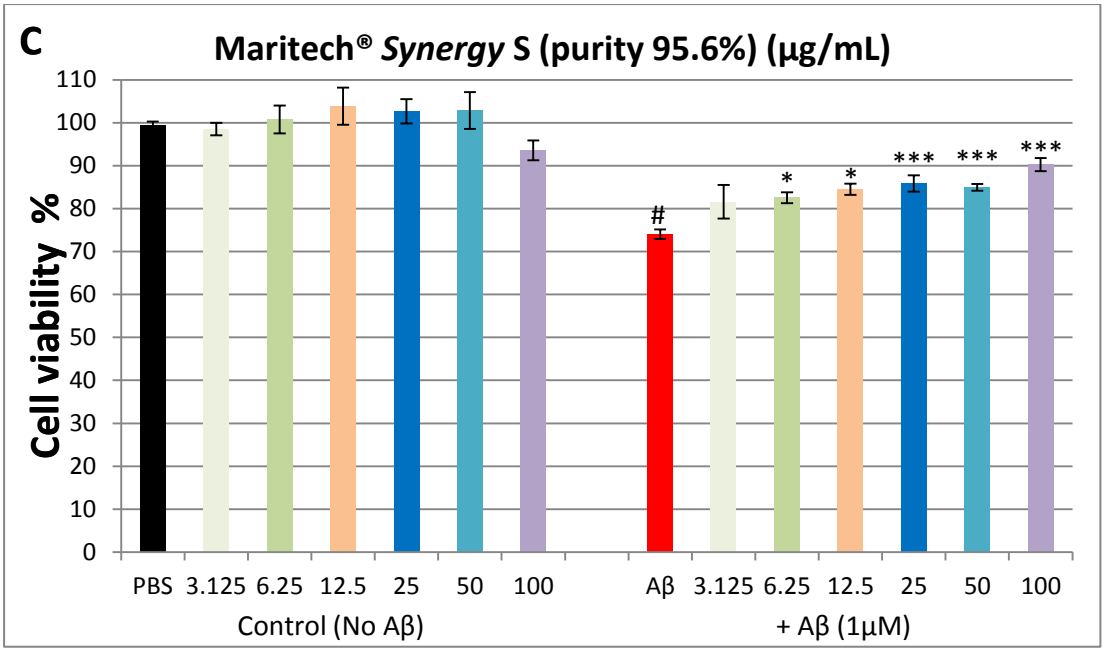
The cytotoxicity of fucoidan samples was measured using the MTT assay in PC12 cells. Five types of fucoidan samples (FE, FF, and S from *F. vesiculosus*, UE, and UF from *U. pinnatifida*) were used in this study. Generally, all five fucoidan samples at the range of concentrations up to 100 µg/mL did not show any statistically significant cytotoxicity to PC-12 cells over 48 hours, with cell viability typically more than 90% (Figure 5-1A – 5-1E).

The results are not surprising, as fucoidan at such concentrations was also reported to have no cytotoxic effects in other *in vitro* and *in vivo* models (Chung et al., 2010, Fitton, 2011). $A\beta_{1-42}$, incubation (1µM) reduced cell viability to 75% (Fig. 1A- 1E). All five fucoidan samples provided protection to PC-12 cells against $A\beta_{1-42}$-induced cytotoxicity to varying extents, while both fucoidan samples (FE and FF) from *F. vesiculosus* inhibited cytotoxicity induced by $A\beta_{1-42}$, with cell viability exceeding 80% at the lowest concentration (3.125 µg/mL), while at the highest concentration (100 µg/mL) it can reach more than 98% (Fig. 1A and 1B). In contrast, fucoidan S provided the lowest neuroprotective activity, where cell viability did not exceed 90% even at the highest concentration (Fig. 1C). Fucoidan (UE*)* showed higher activity than fucoidan from the same species (UF), which might be attributable to the loss of acetylation when fucoidan was further purified. These results were supported by an earlier study which demonstrated the protective effects of fucoidan from *F. vesiculosus* against the neurotoxicity of $A\beta_{1-42}$ (Jhamandas et al., 2005) and fucoidan from *U. pinnatifida* against $A\beta_{1-42}$ and D-Galactose (Wei et al., 2017). The higher

neuroprotective activity of fucoidan FE, FF, and UE may be due to the low molecular weight of these samples.



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Cell viability %
110
100
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PBS 3.125 6.25 12.5 25 50 100
Aβ 3.125 6.25 12.5 25 50 100
Control (No Aβ)
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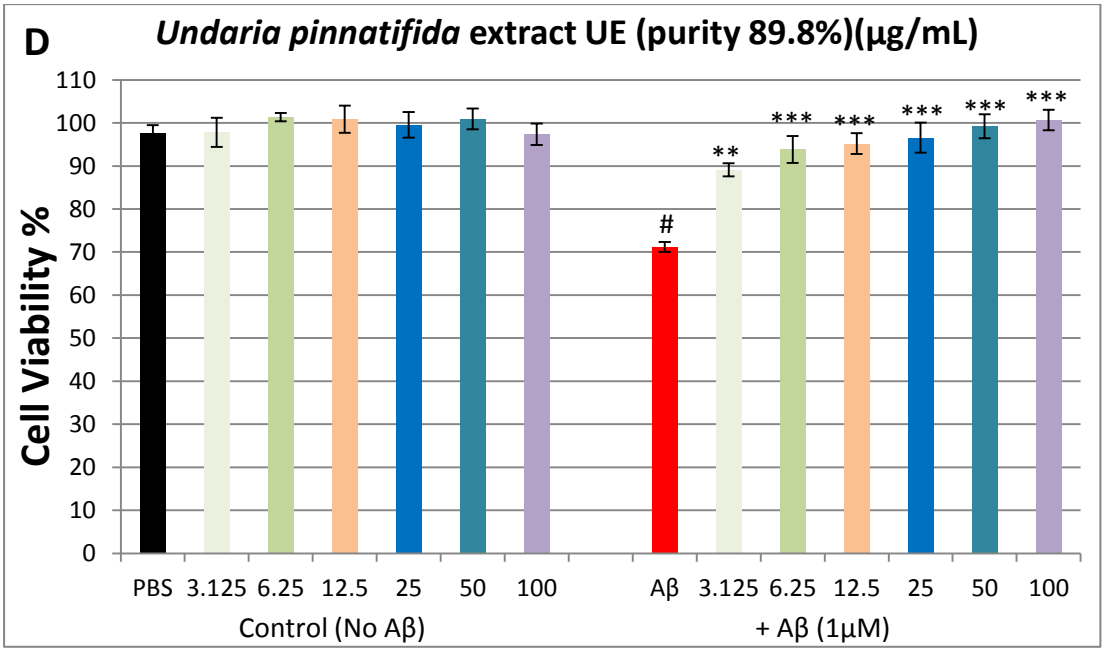
D
Undaria pinnatifida extract UE (purity 89.8%)(µg/mL)
Cell Viability %
110
100
90
80
70
60
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PBS 3.125 6.25 12.5 25 50 100
Aβ 3.125 6.25 12.5 25 50 100
Control (No Aβ)
+ Aβ (1µM)
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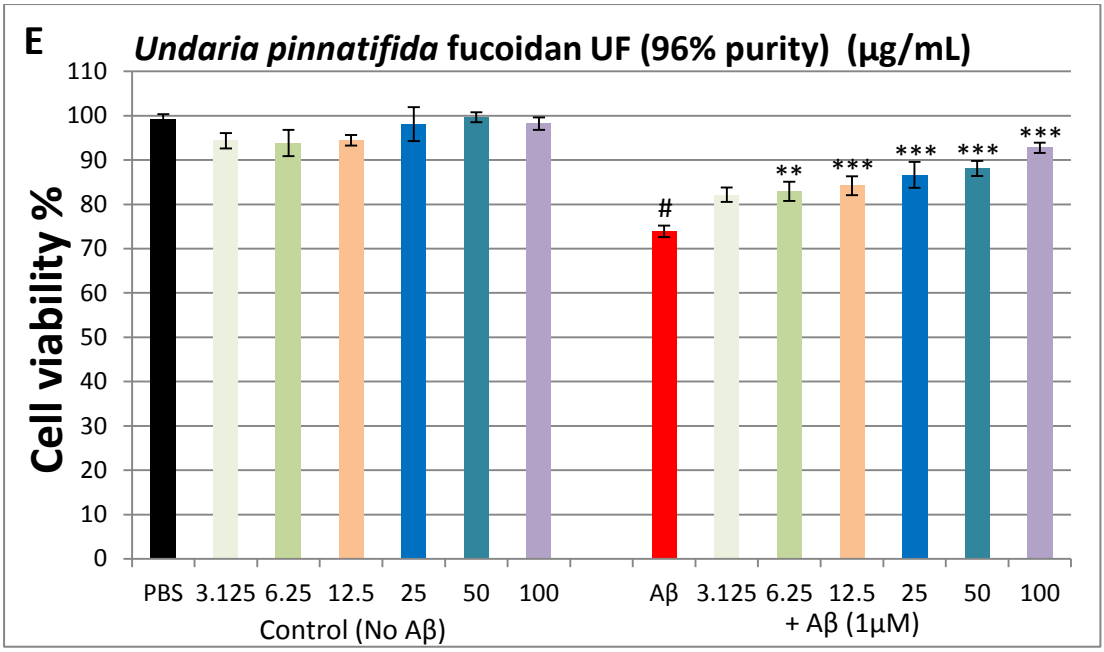
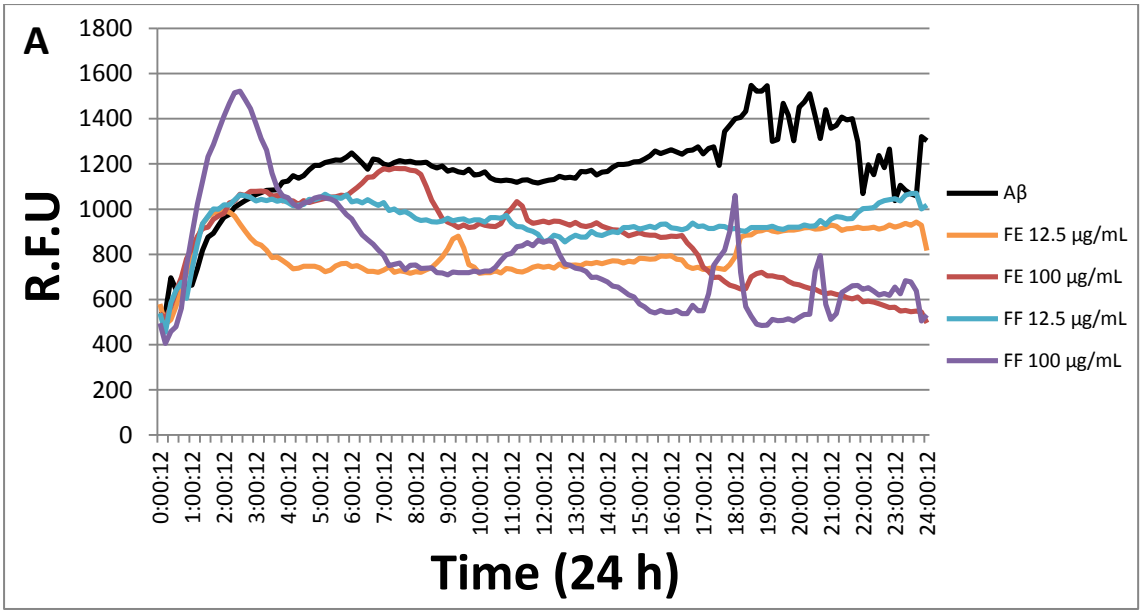


Figure 5-1 Relative cell viability (%) of PC12 cells estimated by MTT assay following 48 h treatment with different concentrations of five fucoidan samples, and neuroprotection activity of fucoidan samples [*F. vesiculosus* extract FE (purity 93.1%) (A), *F. vesiculosus* fucoidan FF (purity 98%) (B), Maritech® Synergy S (purity 95.6%) (C), *U. pinnatifida* extract UE (purity 89.8%) (D), *U. pinnatifida* fucoidan UF (purity 96%) (E)] against $A\beta_{1-42}$ (1 µM) induced cytotoxicity. Each value is the mean ± SEM of four independent experiments (# p <0.005 vs PBS, * p <0.05, ** p <0.01, and ***p <0.005 vs control 1 µM $A\beta_{1-42}$)

5.3.2 Thioflavin T assay for measuring $A\beta_{1-42}$ fibril and aggregate formation

The anti-aggregation effect of five fucoidan samples was investigated using the thioflavin T (ThT) assay of amyloid fibrillisation kinetics. The FF sample demonstrated higher inhibition than the FE sample (Figure 5-2A and 5-2B) as the ThT fluorescence, indicative of $A\beta_{1-42}$ fibril formation, was clearly impeded. Area under the curve analysis demonstrated that there was a modest anti-aggregatory effect of the fucoidan samples, with the highest concentration reducing aggregation-based fluorescence by 25% (Fig. 2B).


A
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1800
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0
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3:00:12
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22:00:12
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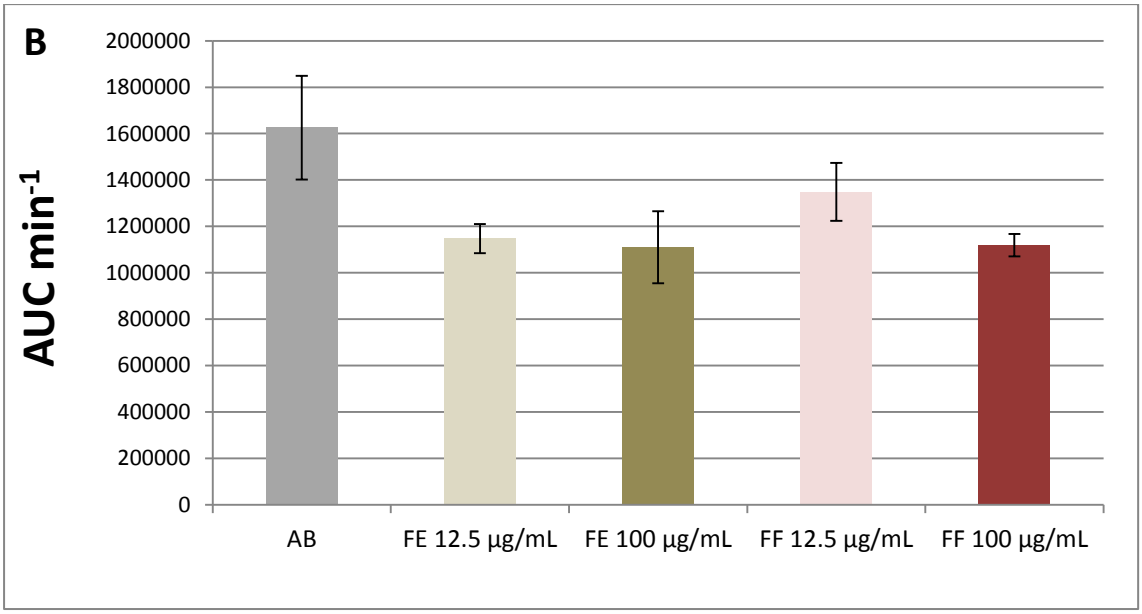
Time (24 h)
Aβ
FE 12.5 µg/mL
FE 100 µg/mL
FF 12.5 µg/mL
FF 100 µg/mL


B
AUC min-1
2000000
1800000
1600000
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1200000
1000000
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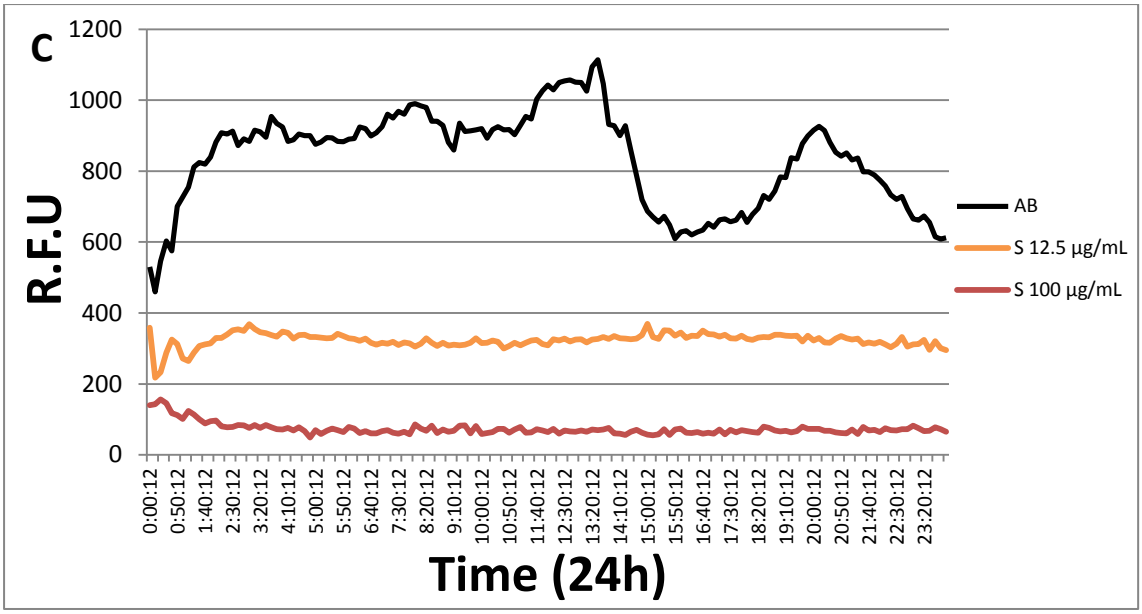
AB
FE 12.5 µg/mL
FE 100 µg/mL
FF 12.5 µg/mL
FF 100 µg/mL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4:10:12
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17:30:12
18:20:12
19:10:12
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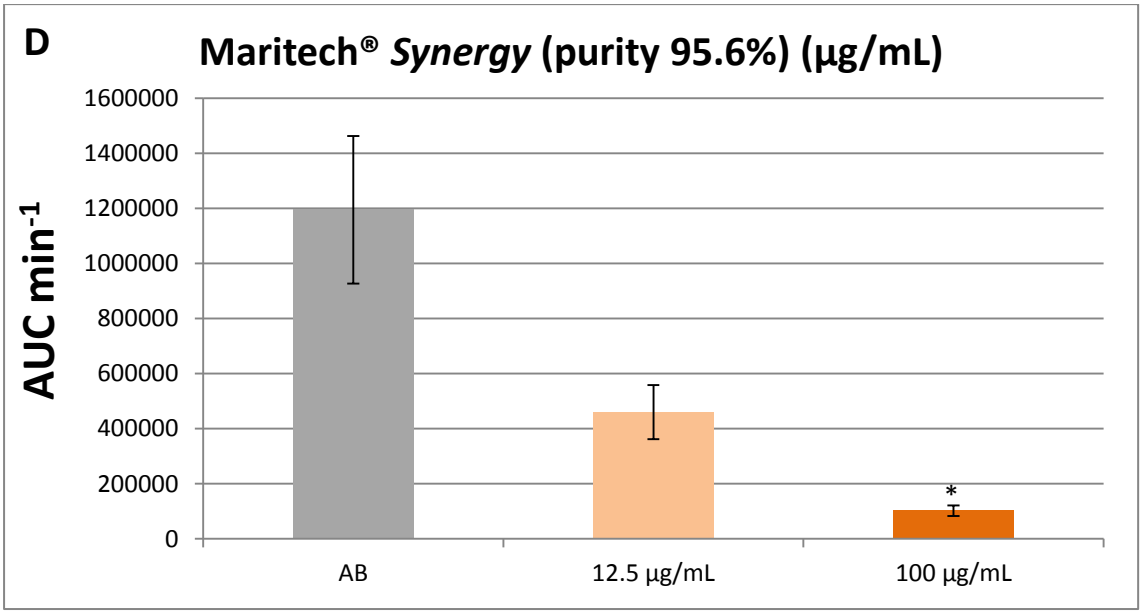
Time (24h)
AB
S 12.5 µg/mL
S 100 µg/mL


D
Maritech® Synergy (purity 95.6%) (µg/mL)
AUC min-1
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
*
AB
12.5 µg/mL
100 µg/mL

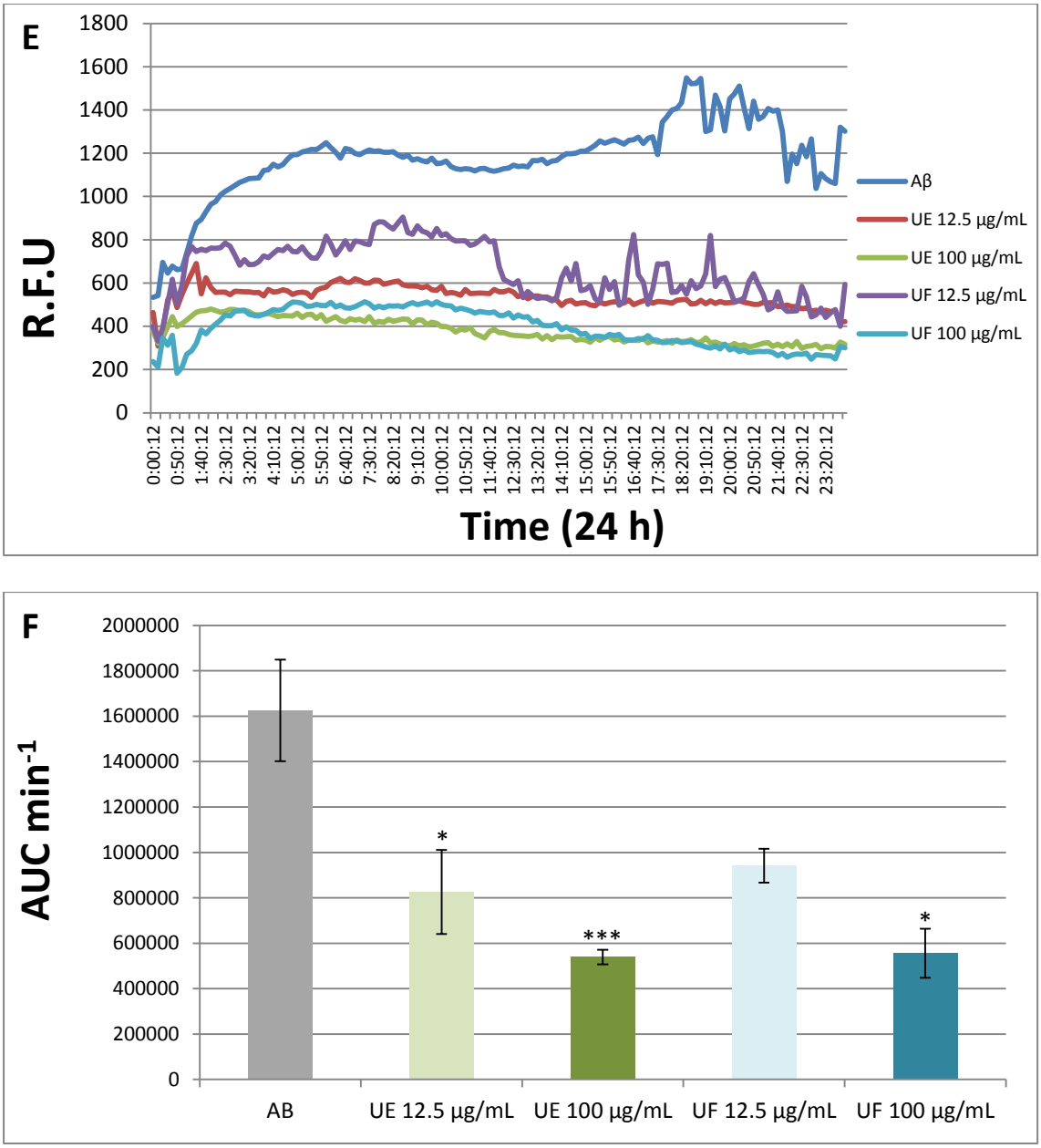


Figure 5-2 Thioflavin T (ThT) fluorescence assay demonstrating amyloid $A\beta_{1-42}$ fibrillization kinetics over 24 h in PBS, alone or in the presence of different concentrations of five fucoidan samples *F. vesiculosus* extract (purity 93.1%) FE and *F. vesiculosus* fucoidan (purity 98%) FF (A), Maritech® Synergy (purity 95.6%) S (C), and *U. pinnatifida* extract (purity 89.8%) UE and *U. pinnatifida* fucoidan (purity 96%) UF (E) at two concentrations 12.5 and 100 µg/mL. Area under the curve (AUC) was measured to quantitate the effect of fucoidan samples FE and FF (B), S (D), and UE and UF (F) (n=3) on amyloid aggregation. The values are average of three independent replicates and the error bars represents the SEM.

The phlorotannin- rich sample of fucoidan (S) from *F. Vesiculosus* demonstrated the highest activity in reducing the aggregation of $A\beta_{1-42}$ (Figure 5-2C). Area under the curve analysis demonstrated that this sample was able to reduce the aggregation to almost 20% (Figure 5-2D). On the other hand, *Undaria pinnatifida* extract and fucoidan showed a greater anti-aggregation effect against $A\beta_{1-42}$ (Fig. 2E). Area under the curve analysis demonstrated that both of these samples were able to decrease the aggregation $A\beta_{1-42}$ (Fig. 2F).

5.3.3 TEM assay for confirming $A\beta_{1-42}$ fibril and aggregate formation

The morphology of $A\beta_{1-42}$ aggregates was examined using transmission electron microscopy (TEM). $A\beta_{1-42}$ formed fibrils and aggregates after 48 hours (Figure 5-3A). The presence of FE and FF, each at 100 µg/mL, did not affect the amount of fibril formation (Figure 5-3A and 5-3C). Fucoidan S was shown to reduce $A\beta_{1-42}$ fibrillization, as the filamentous fibrils appeared less dense (Figure 5-3D). Both UE and UF at 100 µg/mL) were able to reduce the aggregation of $A\beta_{1-42}$ (Figure 5-3C and 5-3F).

Different fucoidan samples have different anti-aggregation activities and they can be varied in their activity depending on the species and chemical composition. For example, fucoidan isolated from *F.s Vesiculosus* showed only a weak anti-aggregation effect, while fucoidan from *Undaria pinnatifida* to have anti-aggregatory effects against $A\beta_{1-42}$. A previous study showed that fucoidan at 1 µM was not able to decrease the aggregation of $A\beta_{1-42}$ (Jhamandas et al., 2005). This might due to the fact that they used a lower concentration (30-50 µg/mL if MW is 30000-50000) compared to our results at 100µg/mL. However, we found that a polyphenol-rich sample (S) from *F. Vesiculosus* showed the greatest anti-$A\beta_{1-42}$ aggregation effects, so we cannot

exclude the possibility that the polyphenols in the sample may account for this. Many polyphenolic compounds have been reported for their anti-aggregation effects against Aβ, such as curcumin and (−)-epigallocatechin-3-gallate (EGCG) (Yang et al., 2005, Hudson et al., 2009). In addition, other extracts rich in polyphenolic compounds were shown to inhibit this aggregation (Wong et al., 2013, Kang et al., 2011). Phlorotannins are the most common polyphenolic compounds in brown algae (Ragan and Glombitza, 1986). In our earlier study of different fractions from *Ecklonia radiata*, phlorotannin showed the highest anti-aggregation effects (Alghazwi et al., 2018a).

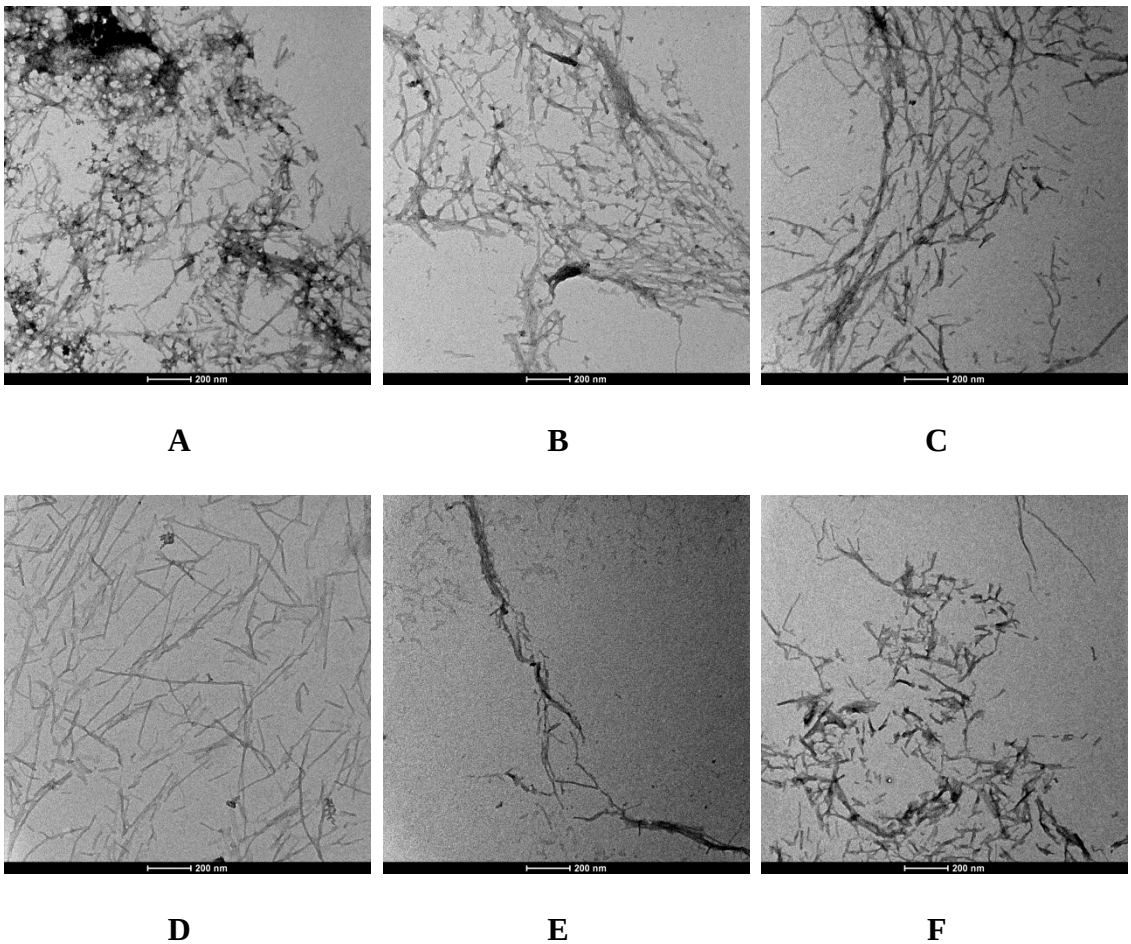


Figure 5-3 Representative images of TEM of $A\beta_{1-42}$ fibril formation, alone and following 48 h incubation with five fucoidan samples: (A) **$A\beta_{1-42}$ (**B) **$A\beta_{1-42}$ and *F. vesiculosus* extract (purity 93.1%) FE (100 µg/mL) (**C) **$A\beta_{1-42}$ and *F. vesiculosus* fucoidan (purity 98%) FF (100 µg/mL) (**D) **$A\beta_{1-42}$ and Maritech® Synergy (purity 95.6%) S (100 µg/mL) (**E) **$A\beta_{1-42}$ and *U. pinnatifida* extract (purity 89.8%) UE (100 µg/mL) (**F) **$A\beta_{1-42}$ and *U. pinnatifida* fucoidan (96% purity) UF (100 µg/mL). Scale bar: 200 nm.**

5.3.4 Neuroprotection of PC-12 cells against H_2O_2-induced cytotoxicity

Aβ has been shown to induce ROS production previously in PC-12 cells and in animal models (Li et al., 2008b, Peng et al., 2009). For that reason, the antioxidant activity of fucoidan samples against hydrogen peroxide was evaluated using PC-12 cells. Hydrogen peroxide (at 100 µM) induced cytotoxicity to about 34 % when cells were treated for 24 hours (Figure 5-4A). Treating the cells with fucoidan samples reduced the cytotoxicity in a concentration-dependent manner. Fucoidan samples did not show neuroprotective activities in the lowest concentrations used (6.25 µg/mL), while the highest concentration (100 µg/mL) showed significant neuroprotective activities, with cell viability exceeding 75%. On the other hand, hydrogen peroxide (at 200 µM) induced cytotoxicity to about 74% of cells (Figure 5-4B). Only two fucoidan samples from *F. vesiculosus* (FE and S) showed neuroprotective activities at the highest tested concentration (100 µg/mL). An earlier study showed that fucoidan can inhibit ROS generation using 2',7'-dichlorofluorescein (DCF) fluorescence (Jhamandas et al., 2005). Also, fucoidan from *Sargassum crassifolium* was shown to possess radical scavenging activity against DPPH and antioxidant activity against hydrogen peroxide (Yang et al., 2017). As antioxidant activity can provide protection against oxidative stress generated in AD (Zhang et al., 2013a), fucoidan samples may scavenge free radicals to provide an antioxidant defence.

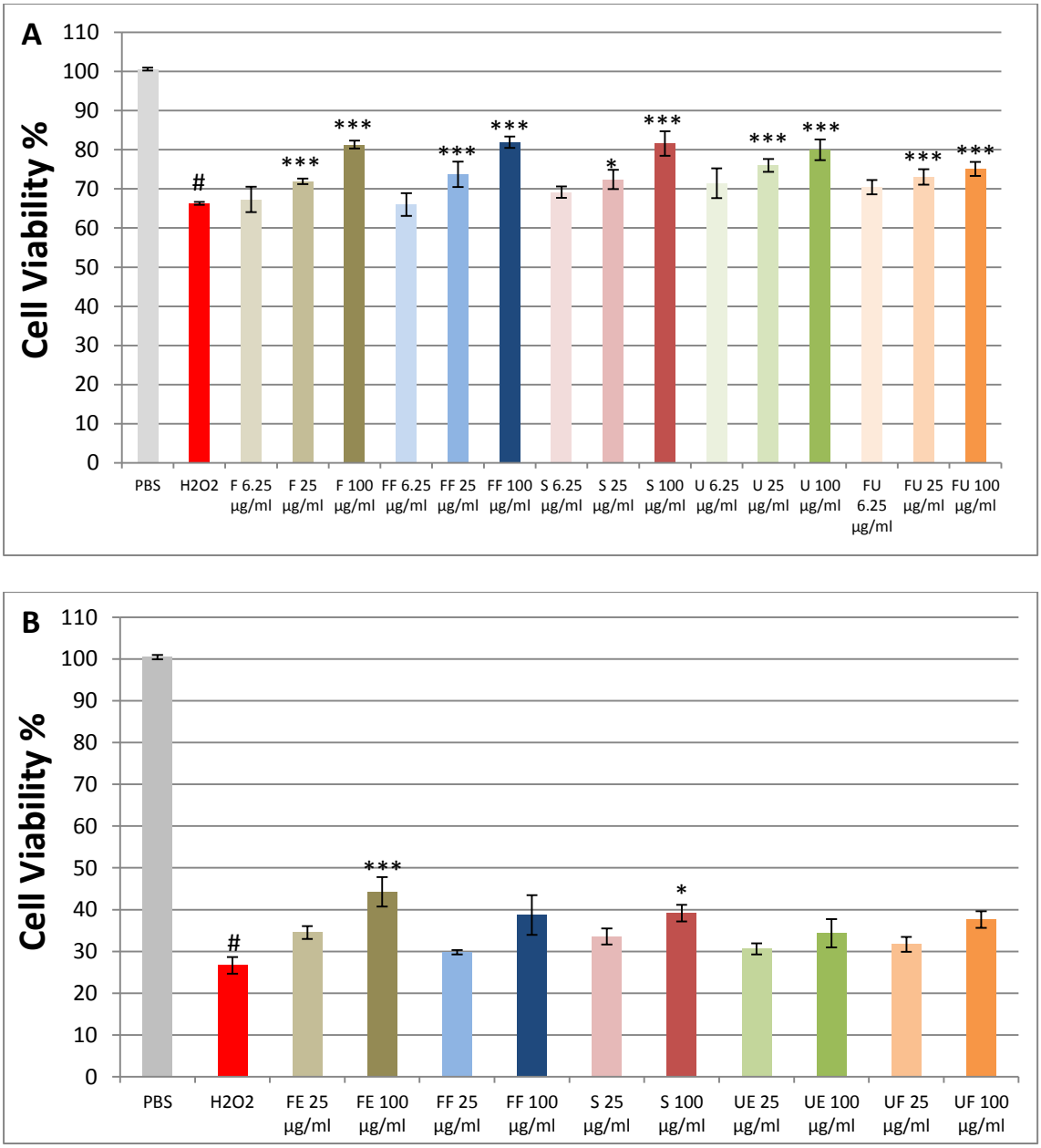


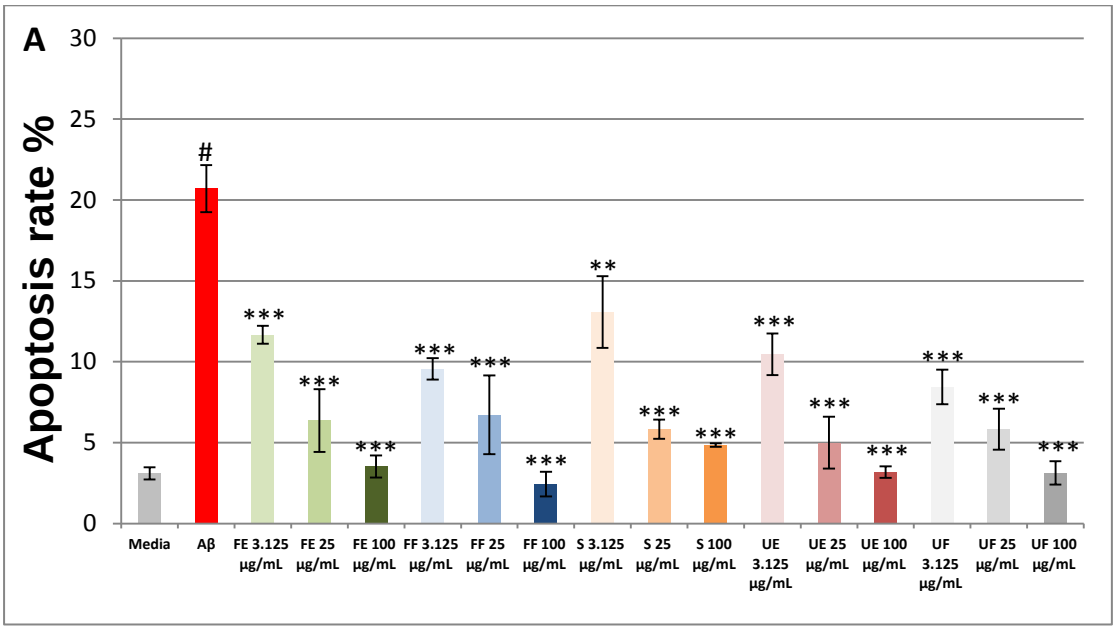
Figure 5-4 Effect of different concentrations of fucoidan samples on viability of PC12 cells. (A) **Fucoidan samples [*F. vesiculosus* extract (purity 93.1%) FE, *F. vesiculosus* fucoidan (purity 98%) FF, Maritech® Synergy (purity 95.6%) S, *U. pinnatifida* extract (purity 89.8%) UE, *U. pinnatifida* fucoidan (purity 96%) UF] exposed to H_2O_2 at 100 µM** (B) **and fucoidan samples exposed to H_2O_2 at 200 for 24 h. Each value is the mean ± SEM of four independent experiments (# *p* <0.005 versus PBS, * *p* <0.05 and ***p* <0.005 versus control H_2O_2)**

5.3.5 Fucoidan inhibited Aβ$_{1-42}$ –induced apoptosis

The percentage of apoptosis was investigated using Hoechst 33258 stain after 48 hours incubation with Aβ$_{1-42,}$ alone or in the presence of different fucoidan samples at different concentrations (ranging from 3.125 to 100 µg/mL). 1 µM Aβ$_{1-42}$ was shown

to induce apoptosis in about 20% of cells (Figure 5-5A and 5-5B). However, treating PC-12 cells with different concentrations of fucoidan samples decreased the percentage of apoptosis significantly in a concentration-dependent manner.

Fucoidan may inhibit apoptosis induced by $A\beta_{1\text{-}42}$ through inhibition of caspase 3 and 9, as was confirmed in a previous study of fucoidan from *F. vesiculosus* (Jhamandas et al., 2005, Wei et al., 2017). Fucoidan from *Laminaria japonica* Aresch inhibited the apoptosis pathway by increasing Bcl-2/Bax ratio and reducing caspase-3 activity (Gao et al., 2012).



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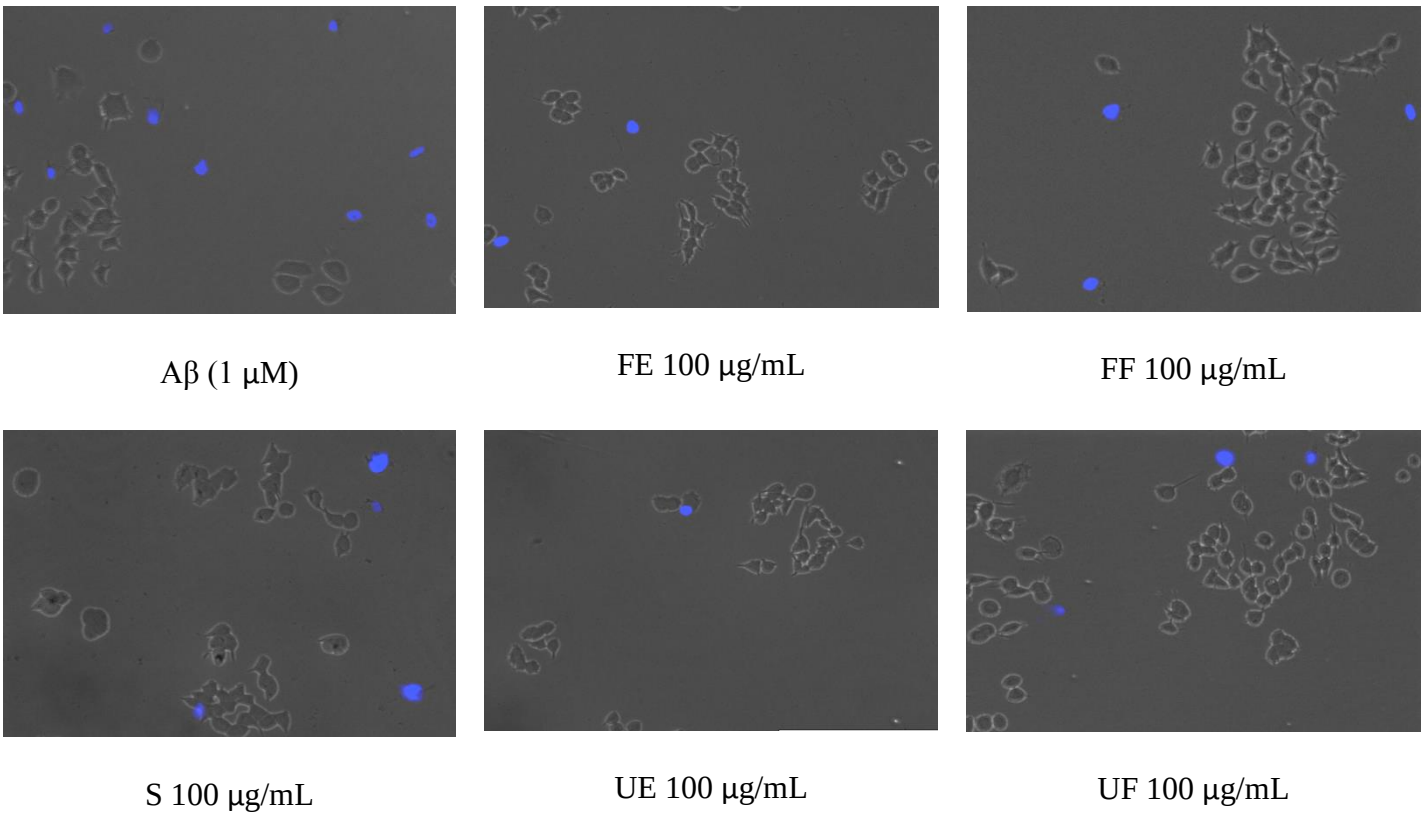


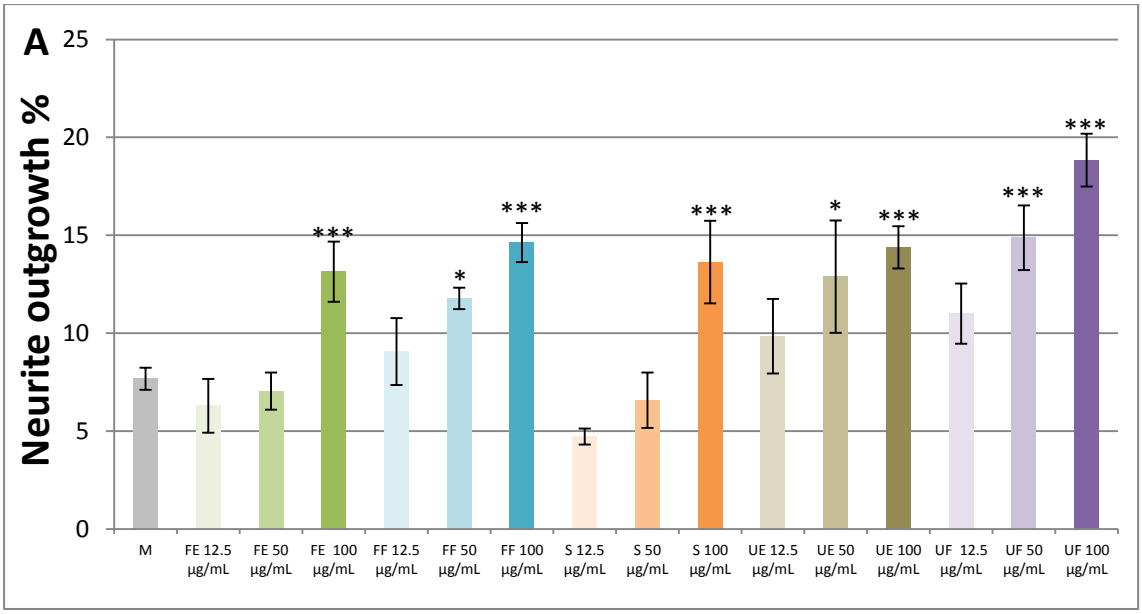
Figure 5-5 (A**) Effect of different concentrations of fucoidan samples [*F. vesiculosus* extract (purity 93.1%) FE, *F. vesiculosus* fucoidan (purity 98%) FF, Maritech® Synergy (purity 95.6%) S, *U. pinnatifida* extract (purity 89.8%) UE, *U. pinnatifida* fucoidan (purity 96%) UF] on apoptosis induced by $A\beta_{1-42}$ (1 μM) in PC12 cells for 48 hours. (**B**) Cells were stained using Hoechst 33258 stain and then visualized using fluorescent microscopy. At least 300 cells were counted for every treatment. Each value is the mean ± SEM of four independent experiments (# *p* <0.005 versus PBS, * *p* <0.05 and ***p* <0.005 versus control $A\beta_{1-42}$)**

5.3.6 Fucoidan enhanced neurite outgrowth

One of the strategies to treat neurodegenerative diseases is to enhance neurite outgrowth, as it plays an important role in maintaining neuronal communication (Koliatsos et al., 1991). Nerve growth factor can enhance neuronal survival (Kromer, 1987) as decreasing its level in the brain can lead to neuronal dysfunction (Hefti and Weiner, 1986). This study investigated whether fucoidan samples can enhance the neurite outgrowth in PC-12 cells. All fucoidan samples showed significant activity in

enhancing neurite outgrowth to more than 13% at the highest concentration (100 µg/mL) (Figure 5-6A and 5-6B). Fucoidan samples (FF and UF) were shown to enhance neurite outgrowth even at 50 µg/mL concentrations. The enhanced neurite outgrowth could be due to the highest purity of fucoidan in these samples. Also, FF and UF have less uronic acid content and higher fucose content than FE and UE, respectively. Polysaccharides from *Lignosus rhinocerotis* were reported to enhance neurite outgrowth through the MEK/ERK1/2 pathway (Seow et al., 2015), and administrating fucoidan from *F. vesiculosus* orally to Sprague-Dawley rats was shown to regulate brain-derived neurotrophic factor (BDNF) (Lee et al., 2012). To our knowledge this is the first report of an enhancement in neurite outgrowth activity from fucoidan samples.

One of the major concerns about fucoidan samples is their bioavailability and their ability to cross blood-brain barrier (BBB). One technical challenge in evaluating the bioavailability of fucoidan is the lack of analytical method that can be used to quantify fucoidan in blood circulation (Zhao et al., 2016). Even with limited analytical techniques available to measure fucoidan, the bioavailability was found to be low (Fitton et al., 2015). A recent study demonstrated that the bioavailability of fucoidan can be improved by decreasing the fucoidan molecular weight as low molecular weight fucoidan showed higher bioavailability than medium molecular weight fucoidan (Zhao et al., 2016). Another study also suggested that the bioavailability of fucoidan depended on the molecular mass, and fucoidan with lower molecular weight has higher absorption and bioavailability than fucoidan with high molecular weight (Zuo et al., 2015).



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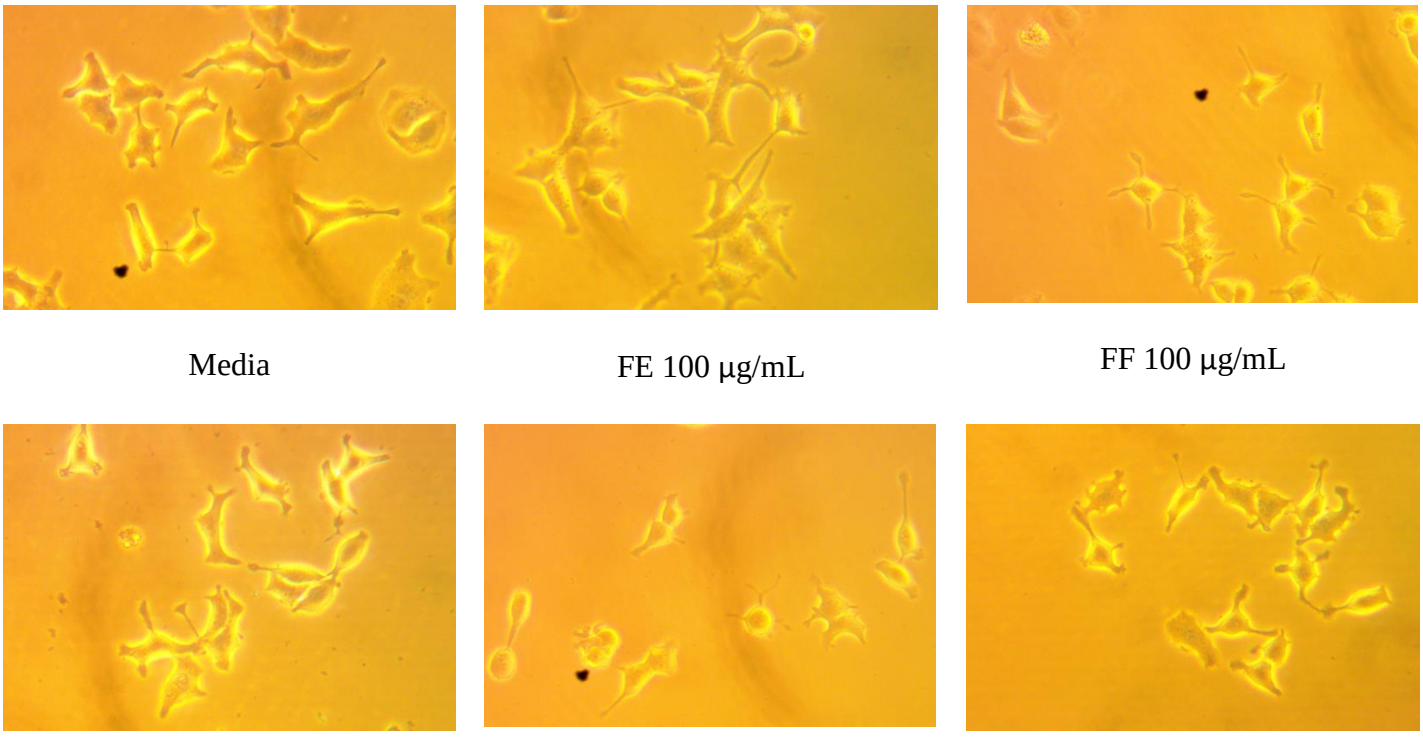

Figure 5-6 (A) **Effect of different concentrations of fucoidan samples [*F. vesiculosus* extract (purity 93.1%) FE, *F. vesiculosus* fucoidan (purity 98%) FF, Maritech® Synergy (purity 95.6%) S, *U. pinnatifida* extract (purity 89.8%) UE, *U. pinnatifida* fucoidan (purity 96%) UF] on enhancing neurite outgrowth in PC12 cells.** (B) **Cells were visualized using light microscopy after treatment with different fucoidans. At least 300 cells were counted for every treatment. Each value is the mean ± SEM of four independent experiments (* *p* <0.05 and ****p* <0.005 versus control media)**

5.4 Conclusion

Distinctive neuroprotective activities of five commercially available, well-characterized and standardized fucoidan samples with different biochemical profiles were found between two brown seaweed species, *Fucus Vesiculosus* and *Undaria pinnatifida*. While all fucoidan samples were able to reduce the cytotoxicity induced by $A\beta_{1-42}$ and H_2O_2 in PC-12 cells, a phlorotannin-rich fucoidan sample from *F. Vesiculosus* and fucoidans from *U. pinnatifida* showed higher anti-aggregation effects against $A\beta_{1-42}$ than *Fucus Vesiculosus* in the ThT assay and TEM as compounds targeting Aβ aggregation may provide a treatment option for AD patients. All fucoidan samples reduced apoptosis levels induced by $A\beta_{1-42}$ and enhanced neurite outgrowth activity at higher concentrations. The results highlight that the neuroprotective activities of fucoidan samples are highly dependent on the purity, biochemical composition, other co-existing compounds, sources of seaweed species and the preparation methods.

5.5 References

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6. CHAPTER 6: *IN SILICO* AND *IN VITRO* STUDIES OF THE NEUROPROTECTIVE ACTIVITIES OF ASTAXANTHIN AND FUCOXANTHIN AGAINST AMYLOID BETA (AB_{1-42}) TOXICITY AND AGGREGATION

Contribution: Mousa Alghazwi has done all of the experiments, analysed all the data, and wrote the manuscript. Wei Zhang, Scott Smid, and Ian Musgrave provide feedback to improve the work and helped in editing the manuscript.

6.1 Introduction

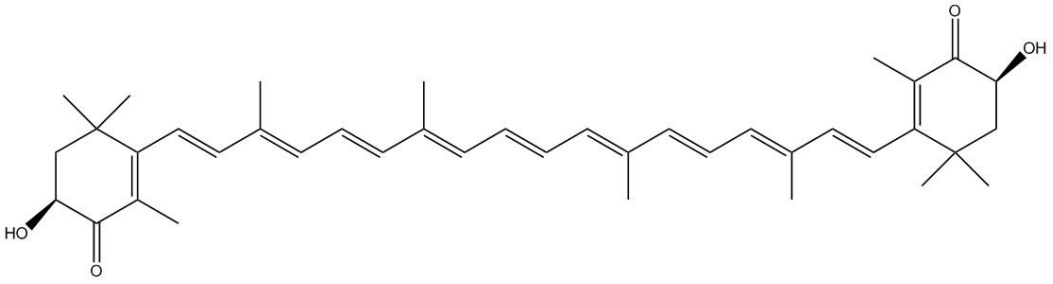
Alzheimer's disease (AD) is the major neurodegenerative disorder responsible for more than 60% of dementia cases globally (Alzheimer'sAssociation, 2014). Aggregates of the protein Amyloid beta (Aβ) form the amyloid plaques that are one of the main hallmarks of AD pathology (Hussain et al., 1999). The amyloid hypothesis suggests Aβ as a pathognomonic feature of AD (Hardy and Selkoe, 2002). Currently, only four compounds targeting acetylcholinesterase (donepezil, tacrine, rivastigmine and galantamine) and one drug targeting the N-methyl-D-aspartate (NMDA) receptor (memantine) have been approved to reduce some symptoms of AD patients (Cummings et al., 2014). An effective disease-modifying treatment is urgently needed for AD and drugs which target Aβ aggregation may be a pathway to such treatments.

Astaxanthin (Figure 6-1a) is a red carotenoid pigment found in different marine organisms such as microalgae, crustaceans, and krill (Miki, 1991, Higuera-Ciapara et al., 2006). It can also be found in yeasts, plants, and feathers of some birds (Hussein et al., 2006). Astaxanthin belongs to the xanthophyll family and is available commercially from microalgae *Haematococcus pluvialis* and yeast *Phaffia rhodozyma* (Ambati et al., 2014, Wu et al., 2015). However, it can vary in composition depending on the natural source and extraction method. Synthetic astaxanthin is a mixture of three isomers (3-R,3′-R), (3-R,3′-S) and (3-S,3′-S), whereas naturally-sourced astaxanthin from *Haematococcus pluvialis* contains only the isomer (3-S,3′-S) (Fassett and Coombes, 2011).

On the other hand, fucoxanthin (Figure 6-1b) is a carotenoid (also known as tetraterpenoid) compound derived from brown algae and microalgae (Peng et al.,

2011). It is an orange coloured pigment found in high content in classes such as Phaeophyceae, Haptophyta, Bacillariophyceae and Chrysophyceae. It is also found in minor levels in these classes: Rhodophyta, Raphidophyceae, and Dinophyta (Takaichi, 2011). Fucoxanthin belongs to the xanthophyll class, as it contains an oxygen in the functional group (Mikami and Hosokawa, 2013). The structure of fucoxanthin was illustrated for the first time in 1990 (Englert et al., 1990) and contains a unique allenic bond in its structure. In addition, the polyene chain contains an epoxide and conjugated carbonyl group (Mikami and Hosokawa, 2013).

a



b

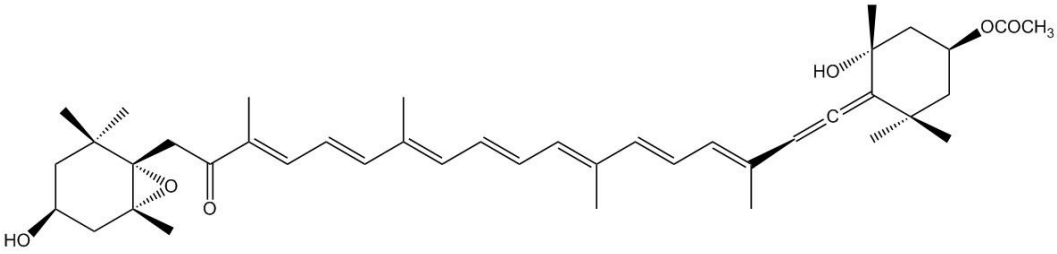


Figure 6-1 The chemical structure of astaxanthin (a**) and fucoxanthin (**b**)**

Astaxanthin has been shown to possess different biological activities, especially as an antioxidant. Earlier research on the antioxidant activity of astaxanthin against single oxygen species has revealed that it has over ten times the activity than other carotenoids such as lutein and β-carotene, and 100 more times activity than α-tocopherol (Miki, 1991). As for the interactions with the amyloid beta protein, astaxanthin has been shown to protect neuronal PC-12 cells from the toxicity induced by $A\beta_{25\text{-}35}$ (Chang et al., 2010). Another study demonstrated that astaxanthin can

attenuate the toxicity of $A\beta_{25-35}$ in SH-SY5Y cells through decreasing the Bcl-2/Bax ratio (Wang et al., 2010). Astaxanthin exhibited stronger activity than β-carotene and canthaxanthin in reducing the cytotoxicity induced by $A\beta_{25-35}$ when treated at 10 μM (Chang et al., 2013).

In all the previous studies, there was no direct comparison of these two promising neuroprotective carotenoids. The aim of this study was to undertake a systematic comparative study of the neuroprotective activities of both fucoxanthin and astaxanthin in a number of bioassays to understand their relative neuroprotective capacity. Using *in silico* approaches to search for promising marine-derived compounds that may reduce the aggregation and toxicity of amyloid beta (data are not included), astaxanthin and fucoxanthin demonstrated promising docking scores using these modelling approaches. Subsequently, a range of *in vitro* assays were carried out to test these two compounds in a cell-based neuroprotective assay in reducing the toxicity of amyloid beta and their anti-aggregation effects using the Thioflavin T (ThT) assay of fibril kinetics and transmission electron microscopy (TEM) of direct fibril morphology. The antioxidant and protective effects against reactive oxygen species, inhibition of apoptosis induced by $A\beta_{1-42}$ and enhancing neurite outgrowth activity were also assessed.

6.2 Materials and Methods

6.2.1 Reagents and materials

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 97.5%), Roswell Park Memorial Institute 1640 (RPMI), astaxanthin, and fucoxanthin were purchased from Sigma-Aldrich (USA). Foetal bovine serum (FBS) was purchased

from Bovogen Biologicals (East Keilor, VIC, Australia). Penicillin/streptomycin and 10 × trypsin EDTA were obtained from Thermo Fisher Scientific (Scoresby, VIC, Australia). Human amyloid-β 1–42 protein ($A\beta_{1–42}$) was obtained from rPeptide (Bogart, Georgia, USA).

6.2.2 In silico study

The 3D structures of astaxanthin (PubChem CID: 5281224) and fucoxanthin (PubChem CID: 5281239) were obtained. By using ligand structures of these compounds, the interaction between each compound and either $A\beta_{1–42}$ monomer (PDB ID: 1IYT), oligomeric pre-fibrillar pentamer β-sheet structure (PDB ID: 2BEG), or fibril form (PDB ID: 2NAO) were studies using CLC Drug Discovery Workbench, version 1.5.1. Interaction was set to 100 interactions for all docking simulations. The software calculated all docking scores and these data were summarized in Table 6-1.

6.2.3 PC-12 cell culture

Rat pheochromocytoma cells PC-12 displaying a semi-differentiated phenotype with neuronal projections were used and maintained in RPMI-1640 media with 10% foetal bovine serum (FBS), and 1% (w/v) penicillin/ streptomycin.

6.2.4 $A\beta_{1-42}$ preparation and treatment in PC-12 cells

Native, non-fibrillar $A\beta_{1–42}$ was prepared by dissolving in 1% (v/v) DMSO to yield a protein concentration of 3.8 mM. A final concentration of 100 μM was made up by adding sterile PBS, and was aliquoted immediately and stored at −70 °C until required.

6.2.5 MTT assay for cytotoxicity study

The cytotoxicity of these compounds was measured using the MTT assay. Initially, cells were plated at 2×10^4 cells per well in 100 μl media and incubated at 37°C with

5% CO_2 for 24 hours. Both astaxanthin and fucoxanthin were diluted in DMSO to their final stock concentrations. PC-12 cells were treated with each of these carotenoid compounds at different concentrations (astaxanthin 0.1-50 µM, and fucoxanthin 0.1-20 µM), and then incubated for 48 hours at 37°C. The media was subsequently removed and replaced with 0.5 mg/ml of MTT (diluted in 1× PBS). The plate was then incubated for 2 hours at 37°C. Then, the MTT solution was removed and the cells lysed with 100 µl of DMSO. The absorbance of the plate was measured at 570 nm with a reference wavelength of 630 nm using a microplate reader (Bio-Tek Instruments Inc, USA).

6.2.6 MTT assay for protecting cells against $A\beta_{1-42}$ -induced cytotoxicity

The procedure for cytotoxicity testing was followed as previously (section 4.5) with some modifications. After treating the cells with different concentrations of carotenoid compounds (astaxanthin 0.1-50 µM, or fucoxanthin 0.01-2 µM), cells were incubated for 15 minutes prior to the addition of $A\beta_{1–42}$ (1 µM). Cells were then incubated for 48 hours at 37°C prior to measurement of cell viability.

6.2.7 Thioflavin T assay of $A\beta_{1-42}$ fibril and aggregate formation

ThT (10 µM in PBS) was added with $A\beta_{1–42}$ (10 µM), alone or in combination with three different concentrations of each of the carotenoid compounds (astaxanthin 0.1-50 µM, or fucoxanthin 0.1-10 µM). The plate was incubated at 37 ^{o}C in a fluorescence microplate reader (Bio-Tek, Bedfordshire, UK) with excitation at 446 nm and emission at 490 nm every 10 minutes for 48 hours to assess effects on $A\beta_{1–42}$ fibril kinetics.

6.2.8 Transmission electron microscopy (TEM) of $A\beta_{1-42}$ fibril formation

In order to visualize the aggregation of $A\beta_{1-42}$, transmission electron microscopy (TEM) was used to investigate the effects of the carotenoid compounds on $A\beta_{1-42}$ morphology. The samples were prepared by incubating native $A\beta_{1-42}$ (10 μM) in PBS, either alone or with astaxanthin (50 μM) or with fucoxanthin (2 μM) for 48 hours at 37 °C. Subsequently, 5 μl of each sample was placed onto a 400 mesh formvar carbon-coated nickel electron microscopy grid (Proscitech, Kirwan, QLD, Australia) for 2 minutes before the sample was blotted off using filter paper. After that, 10 μl of contrast dye containing 2% uranyl acetate was placed onto the grid, left for two minutes and blotted off with filter paper. Finally, the grids were loaded onto a specimen holder and then into a FEI Tecnai G2 Spirit Transmission Electron Microscope (FEI, Milton, QLD, Australia). The sample grids were then viewed using a magnification of 34,000–92,000× and representative images were taken.

6.2.9 MTT assay for protecting cell against H_2O_2 cytotoxicity

The same procedure was undertaken according to cytotoxicity assay procedure (section 4.6). Cells were incubated for 15 minutes after carotenoid treatment, then treated with H_2O_2 (100 μM and 200 μM) (Sigma). Cells were then incubated for 24 hours at 37°C prior to measurement of cell viability.

6.2.10 Nuclear staining for assessment of apoptosis

PC-12 cells were seeded at a density of 2×10^4 for overnight. Then, the cells were treated with different astaxanthin and fucoxanthin samples at different concentrations for 15 minutes before adding $A\beta_{1-42}$. The cells were incubated for 48 hours and then 5 μg/mL of Hoechst 33258 stain was added and incubated for 10 minutes in the dark to

stain the cell nuclei. The cells were then washed with 1×PBS and the plates were monitored using EVOS FL Cell Imaging System (Thermo Fisher) fluorescence microscope. The percentage of apoptotic cells (n=350 cells at least per well) were calculated as followed:

Apoptotic cells % = apoptotic cells ÷ total cells (viable cells + apoptotic cells) × 100

Three independent experiments were performed for every treatment.

6.2.11 Neurite outgrowth assay

PC-12 cells were seeded at a density of 2×10^3 and were incubated overnight at 37°C. Media was then replaced with serum-free media and the cells were treated with different astaxanthin and fucoxanthin samples at different concentrations for 24 hours. Then, cells were visualized using an inverted microscope (Olympus CK2) at × 400 magnifications and images were taken from at least six random fields and then analysed using ImageJ software. Neurite outgrowth was considered positive when the cell projections were measured to be equal or longer than the size of cells, and the amount of neurite outgrowth was calculated from at least 200 cells per treatment. Results were expressed as percentage of cells expressing neurites.

6.2.12 Statistical analysis

All results were based on at least three independent experiments (n = 3). The effects of carotenoid compounds on PC-12 cell viability were analysed using one way ANOVA followed by Tukey's honestly significant difference (HSD) post-hoc test using SPSS software (Version 22). Area under the curve (AUC) analysis was measured by comparing the different treatments against $A\beta_{1\text{-}42}$ using one-way ANOVA with post-hoc test. Differences were considered statistically significant at $p < 0.05$.

6.3 Results and Discussion

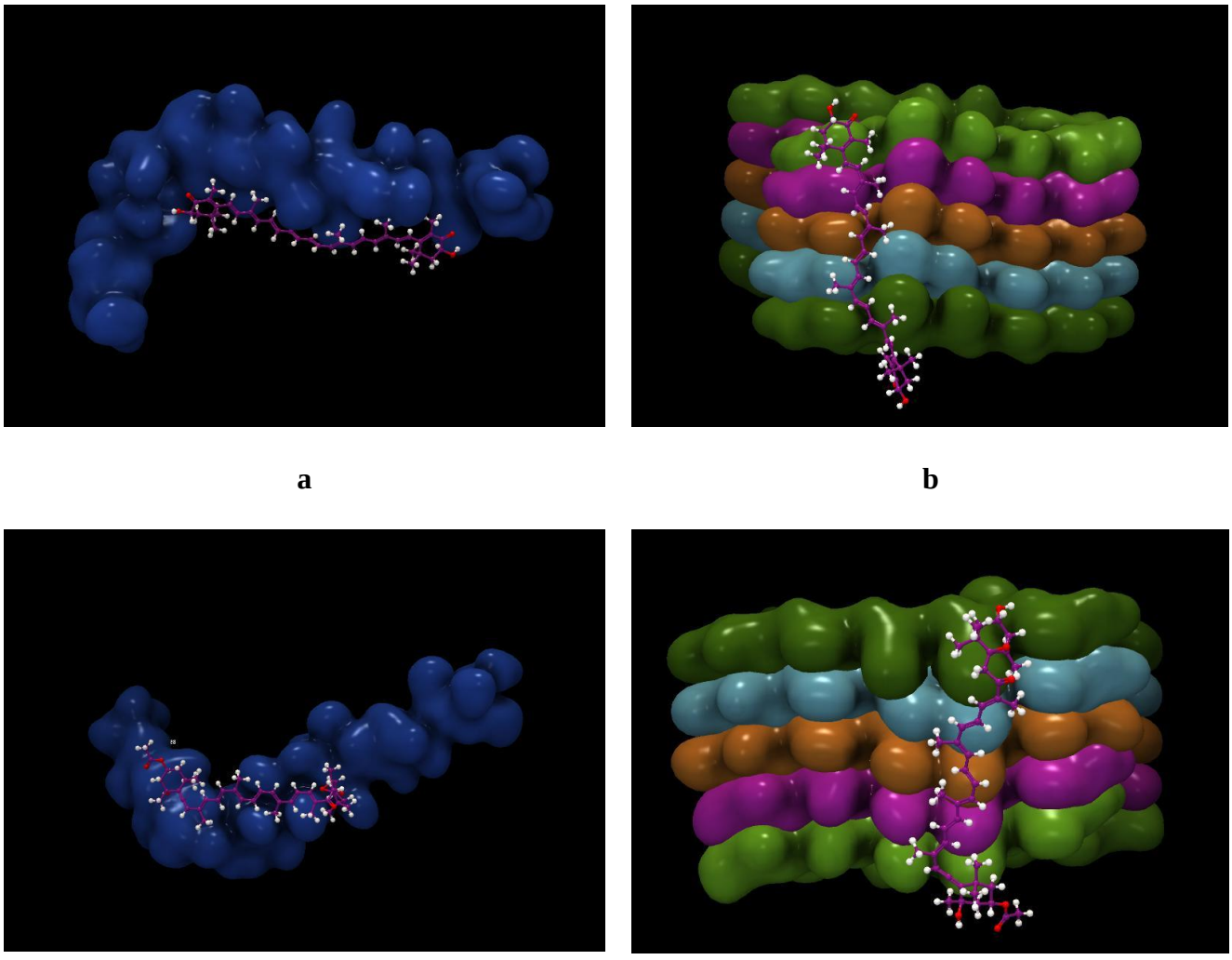
6.3.1 *In silico* studies of astaxanthin and fucoxanthin

Both astaxanthin and fucoxanthin were docked to $A\beta_{1-42}$ monomer, pentamer, and fibril forms. The docking scores of both compounds with these $A\beta_{1-42}$ conformations are shown in Table 6-1. The interaction of astaxanthin with $A\beta_{1-42}$ monomer and pentamer are shown in Figure 6-2a and 6-2b, while the interactions of fucoxanthin with $A\beta_{1-42}$ are shown in Figure 6-2c and 6-2d. *In silico* studies of both astaxanthin and fucoxanthin showed that they have strong binding affinity with the target $A\beta_{1-42}$ monomer, pentamer or fibril. In fact, they are among the best binding affinity tested in *in silico* studies from many marine-derived neuroprotective compounds discovered from marine algae in a recent review (data not shown) (Alghazwi et al., 2016b).

The high docking scores of both astaxanthin and fucoxanthin with $A\beta_{1-42}$ monomer might be justified as they bind to the middle of the protein with the binding area alongside the protein surface (Figure 6-2a and 6-2c). Even though both astaxanthin and fucoxanthin have a relatively high molecular weight, their aliphatic chains have an advantage of binding to more sites of $A\beta_{1-42}$. Most of the docking score arises from steric interactions. Astaxanthin binds to certain amino acids which include Ala21, Gly9, Gly25, His6, His13, His14, Leu17, Tyr10, and Val24. On the other hand, fucoxanthin binds to amino acids Ala30, Asp23, Gly33, Gly37, Leu34, Lys16, Phe19, Phe20, and Val24. Some amino residues in $A\beta_{1-42}$ have been found previously to contribute to hydrophobic interactions; these include Lys16, Val24, Phe20, and Val24 residues with fucoxanthin and Ala21, Gly25, Leu34 residues with astaxanthin (Shuaib and Goyal, 2017). Fucoxanthin binds to Phe19, an important amino acid in forming fibrils; previous research found replacing it can result in decreasing fibril formation by 15% (Wood et al., 1995). Fucoxanthin also binds to Lys16, which has previously

resulted in reducing the toxicity and aggregation of Aβ (Usui et al., 2009). Similarly, Asp23 is an important amino acid responsible for initiating neurotoxicity and forming fibrils (Kocis et al., 2017). The higher score of fucoxanthin supports the neuroprotective activity of it in reducing the cytotoxicity and aggregation of $A\beta_{1-42,}$ and potentially fucoxanthin may be more potent than astaxanthin. On the other hand, astaxanthin binds to Val24, an important amino acid which was suggested to be targeted as an approach to treat Alzheimer's disease (Lazo et al., 2005). His13 and Tyr10 are known for their contribution in $A\beta_{1-42}$ aggregation and toxicity (Zhang et al., 2013b).

The $A\beta_{1-42}$ pentamer contains 5 monomers, and as shown in Figure 6-2b and 6-2d, both astaxanthin and fucoxanthin bind to all five regions, contributing to higher docking scores compared to reported compounds such as epigallocatechin gallate (EGCG), rivastigmine, and curcumin (data are not included). Both astaxanthin and fucoxanthin bind to amino acids Val18 and Phe20. Phe20 is also one amino acid known for its roles in cytotoxicity and aggregation of $A\beta_{1-42}$ (Zhang et al., 2013b).



a **b**

c **d**

Figure 6-2 The interaction between carotenoid compounds with their target protein $A\beta_{1-42}$, astaxanthin with $A\beta_{1-42}$ monomer (PDB ID: 1IYT) (a) and pentamer (PDB ID: 2BEG) (b), fucoxanthin with $A\beta_{1-42}$ monomer (c) and pentamer (d)

Table 6-1 Molecular docking results of astaxanthin and fucoxanthin with Aβ monomer, pentamer, and fibril

	Docking score	Steric interaction score	RMSD (Å)
	$A\beta_{1-42}$ monomer		
Astaxanthin	-46.02	-58.6	9.45
Fucoxanthin	-46.86	-54.74	18.32
	$A\beta_{1-42}$ pentamer		
Astaxanthin	-88.83	-95.65	21.29
Fucoxanthin	-72.12	-93.59	22.96
	$A\beta_{1-42}$ fibril 2NAO		
Astaxanthin	-274.97	-295.83	36.67
Fucoxanthin	-376.69	-400.01	38.12

6.3.2 Cytotoxicity of astaxanthin and fucoxanthin and protection against $A\beta_{1-42}$ -induced cytotoxicity

Astaxanthin did not show any sign of cytotoxicity when it was incubated with PC-12 cells even at the highest concentration (50 μM) tested (Figure 6-3). Astaxanthin (up to 50 μM) did not show any significant cytotoxicity to PC-12 cells for 48 hours. It was reported that astaxanthin is very safe for human consumption (Guerin et al., 2003) and was approved to be used as a food supplement by United States Food and Drug Administration (Stewart et al., 2008, Guerin et al., 2003).

Fucoxanthin showed a significant reduction in PC-12 cell viability at ≥5 μM, and has negligible toxicity below 2 μM. In our study, fucoxanthin was shown to be cytotoxic to PC-12 cells at ≥ 5 μM. This is similar to a previous study in which fucoxanthin was

toxic at 10 µM in human lymphocytes for 24 hours, reducing cell viability to 40% (Molina et al., 2014). Fucoxanthin was also toxic to HaCaT cells (keratinocytes) at 40 µM or more when treated for 16 hours (Zheng et al., 2013). As both the incubation times and cell line are different, this may explain the variation in higher toxic concentrations. In contrast, fucoxanthin did not show any sign of toxicity on ICR mice administrated at 1000 mg/kg and 2000 mg/kg doses (Beppu et al., 2009). Fucoxanthin did not show any toxicity to HUVEC cells or zebrafish, but it was protective against high glucose-induced intracellular reactive oxygen species (ROS) even at 50 µM (Kang et al., 2014). Using weanling female Wistar rats, fucoxanthin did not show any toxicity up to 100 mg/kg body weight (Ravi et al., 2015). It is likely that some cell lines are more sensitive than others, such as the PC-12 cells in the current study, and also exposure to this compound is varied between animals and cell lines.

Treating PC-12 cells with $A\beta_{1-42}$ (1 µM) resulted in cell viability decreasing to 71%, with astaxanthin pre-treatment provided significant neuroprotection against $A\beta_{1-42}$ at all tested concentrations ($p<0.05$, Figure 6-3). This protective activity was even significant at the lowest concentration used (0.1 µM; $p<0.05$). This result is consistent with earlier studies reporting that astaxanthin at 0.1 µM can reduce the toxicity of $A\beta_{25-35}$ in PC-12 cells when treated for 48 hours (Chang et al., 2010), and that treating SH-SY5Y cells with astaxanthin at 10 µM for 24 hours can rescue cell viability lost to $A\beta_{25-35}$ from 75% to 87% (Wang et al., 2010).

Treating PC-12 cells with fucoxanthin < 2µM significantly reduced the cytotoxicity of $A\beta_{1-42}$ in a concentration-dependent manner (Figure 6-4). A recent study on the neuroprotective activity of fucoxanthin showed that it was able to protect cerebral

cortical neurons against the toxicity induced by $A\beta_{25-35}$ (Zhao et al., 2015). In addition, fucoxanthin inhibited BACE1 with an IC_{50} of 5.31 µM (Jung et al., 2016). Fucoxanthin showed significant neuroprotection to PC-12 cells against $A\beta_{1-42}$ at all tested concentrations (0.01–2 µM) in this study ($p<0.05$).

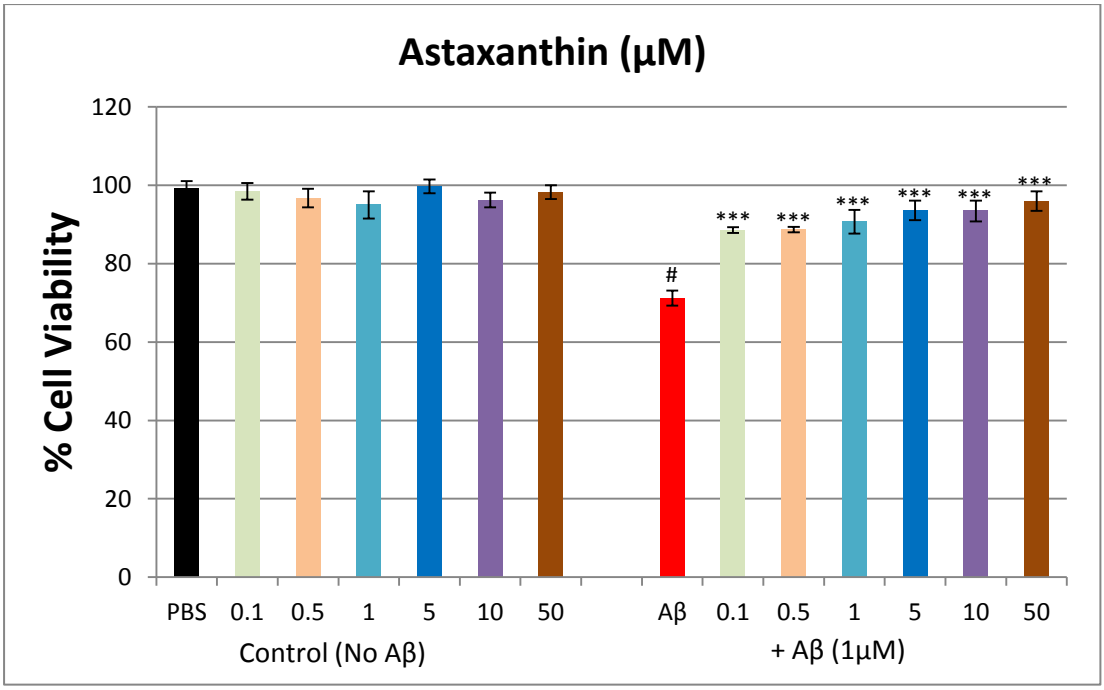


Figure 6-3 Cell viability (%) of PC12 cells measured by MTT assay following 48 h treatment with astaxanthin, and neuroprotection activity of astaxanthin against $A\beta_{1-42}$ (1 µM) induced cytotoxicity. Each value is the mean ± SEM of four independent experiments (# *p* <0.005 vs PBS, * *p* <0.05, ** *p* <0.01, and ***p* <0.005 vs control 1 µM $A\beta_{1-42}$)

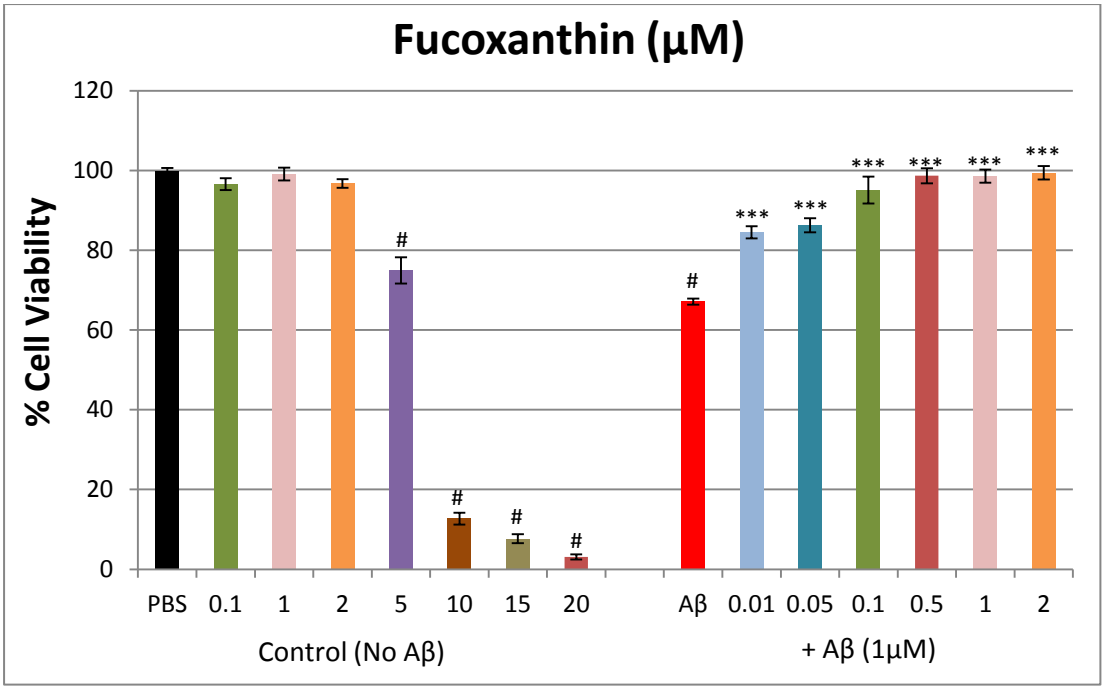


Figure 6-4 Cell viability of PC12 cells measured by MTT assay following 48 h treatment with fucoxanthin, and neuroprotection activity of fucoxanthin against $A\beta_{1-42}$ (1 µM) induced cytotoxicity. Each value is the mean ± SEM of four independent experiments (# *p* <0.005 vs PBS, * *p* <0.05, ** *p* <0.01, and ***p <0.005 vs control 1 µM $A\beta_{1-42}$)

6.3.3 ThT assay for measuring $A\beta_{1-42}$ fibril and aggregate formation

The ThT assay was used to study the kinetics of any anti-aggregative effect of astaxanthin and fucoxanthin against Aβ. The ThT assay demonstrated that $A\beta_{1-42}$ formed fibrils, as the fluorescence increased substantially over 48 hours, while astaxanthin inhibited $A\beta_{1-42}$ fibril formation in a concentration-dependent manner over this period (Figure 6-5a). Area under the curve analysis showed that 1 µM astaxanthin was able to reduce $A\beta_{1-42}$ aggregation by 50% (Figure 6-5b). Astaxanthin at 50 µM showed the highest anti-aggregation activity, with almost 80% reduction in the intensity of ThT fluorescence.

On the other hand, fucoxanthin inhibited $A\beta_{1-42}$ fibril formation even at a low concentration (0.1 µM). Moreover, 1-10 µM fucoxanthin inhibited fibril formation dramatically (Figure 6-6a). Fucoxanthin at 2 µM was able to reduce the aggregation

of $A\beta_{1-42}$ by 75% (Figure 6-6b). The strongest activity was at 10 µM, but this concentration was toxic to PC-12 cells.

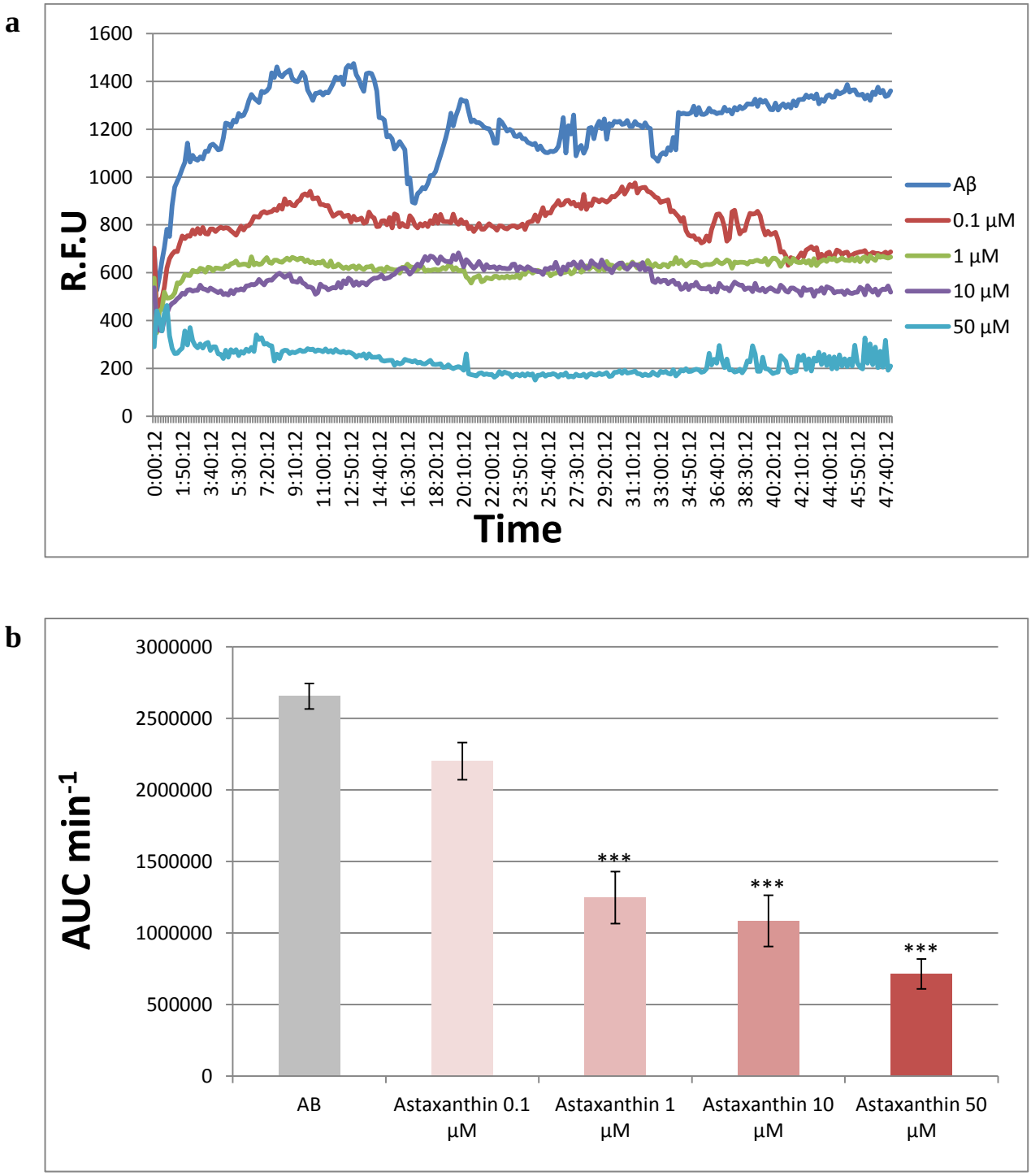


Figure 6-5 (a) **Thioflavin T (ThT) fluorescence assay demonstrating amyloid $A\beta_{1-42}$ fibrillization kinetics over 48 h in PBS, alone or in the presence of different concentrations of astaxanthin (0.1–50 µM).** (b) **Area under the curve (AUC) was measured to quantitate the effect of astaxanthin on $A\beta_{1-42}$ (* p <0.05, ** p <0.01, and ***p <0.005 vs control $A\beta_{1-42}$) of astaxanthin at different concentrations (n=4)**

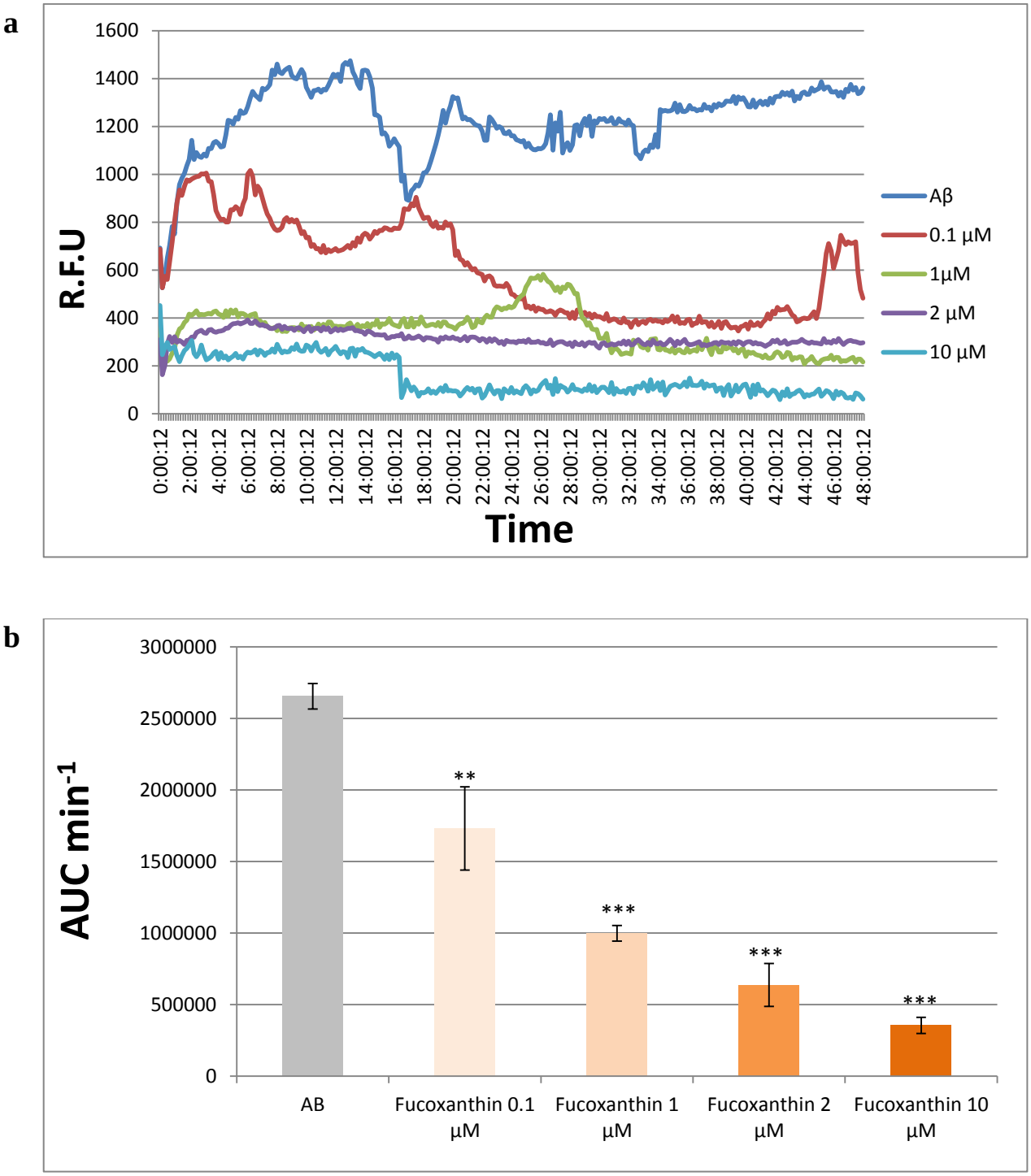
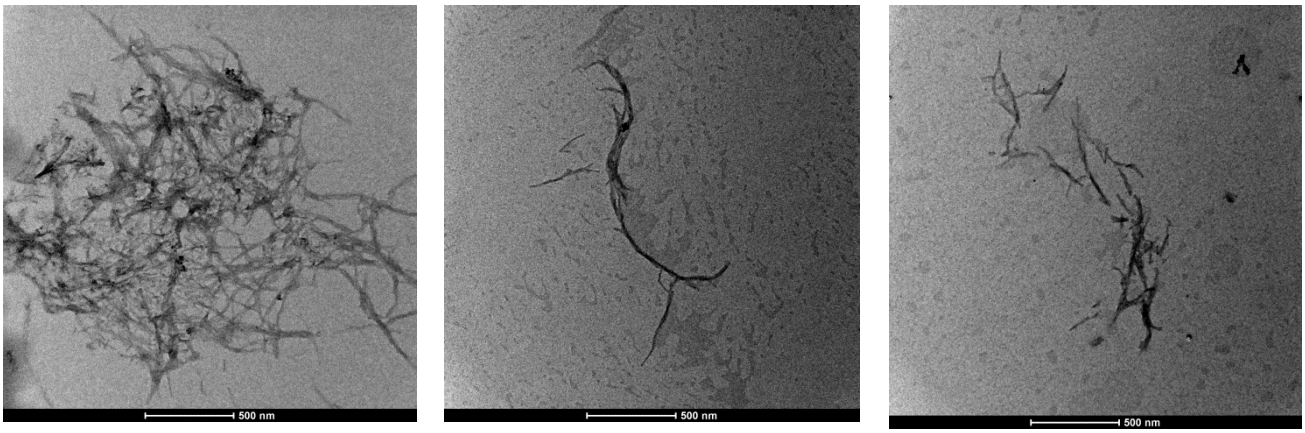


Figure 6-6 (a) **Thioflavin T (ThT) fluorescence assay demonstrating amyloid $A\beta_{1–42}$ fibrillization kinetics over 48 h in PBS, alone or in the presence of different concentrations of fucoxanthin (0.1–10 µM).** (b) **Area under the curve (AUC) was measured to quantitate the effect of fucoxanthin on $A\beta_{1-42}$ (* p <0.05, ** p <0.01, and ***p <0.005 vs control $A\beta_{1-42}$) of fucoxanthin at different concentrations (n=4)**

6.3.4 Electron microscopy for $A\beta_{1-42}$ fibril and aggregate formation

Examining the morphology of $A\beta_{1-42}$ aggregates using transmission electron microscopy (TEM) showed that it formed fibrils and aggregates after 48 hours (Figure 6-7a). In the presence of astaxanthin (50 µM), fibril formation was observed to be less dense than the control $A\beta_{1-42}$ (Figure 6-7b).

TEM analysis showed that fucoxanthin can inhibit the formation of fibrils and also truncates fibril length (Figure 6-7c). The ThT assay results were confirmed by TEM for fucoxanthin, where it inhibited aggregation of $A\beta_{1-42}$.This finding supports a previous study which showed that fucoxanthin was able to inhibit the aggregation of $A\beta_{1-42}$ and can also alleviate cognitive impariment in an animal model (Xiang et al., 2017).

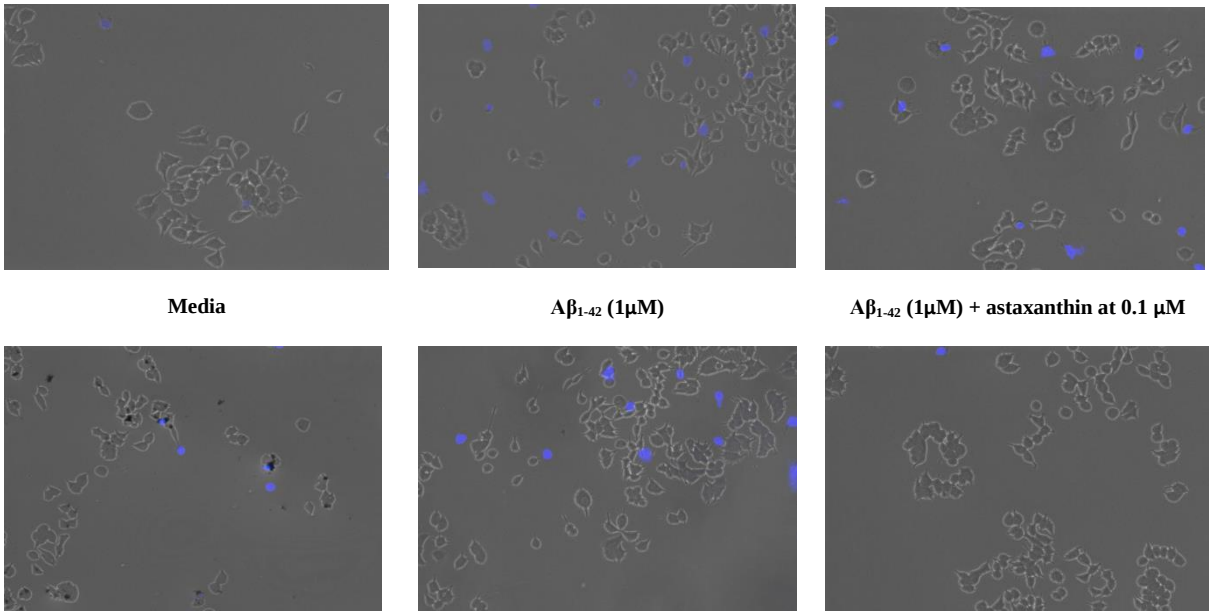


a **b** **c**

Figure 6-7 Representative images of transmission electron microscope (TEM) of $A\beta_{1-42}$ fibril formation, alone and following 48 h incubation with astaxanthin and fucoxanthin: (a) **$A\beta_{1-42}$ (**b) **$A\beta_{1-42}$ and astaxanthin (50 µM) (**c) **$A\beta_{1-42}$ and fucoxanthin (2 µM). Scale bar: 500 nm.**

6.3.5 Astaxanthin and fucoxanthin inhibited $A\beta_{1-42}$ –induced apoptosis

Treating PC-12 cells with $A\beta_{1-42}$ (1μM) for 48 hours and measuring apoptosis using the Hoechst 33258 nuclear stain resulted in about 20% of apoptotic cells (Figure 6-8). However, cells treated with astaxanthin or fucoxanthin at different concentrations resulted in a reduction in the number of apoptotic cells in a concentration-dependent manner. This result is consistent with a report that astaxanthin inhibited apoptosis induced by H_2O_2 in Mouse Neural Progenitor Cells (Kim et al., 2009), 6-OHDA in SH-SY5Y cells (Ikeda et al., 2008), $A\beta_{25-35}$ in SH-SY5Y cells (Wang et al., 2010). Similarly, fucoxanthin has been previously shown to reduce apoptosis induced by both H_2O_2 (Yu et al., 2017) and Aβ oligomer in SH-SY5Y cells (Lin et al., 2017).



Media | **$A\beta_{1-42}$ (1μM)** | **$A\beta_{1-42}$ (1μM) + astaxanthin at 0.1 μM**

$A\beta_{1-42}$ (1μM) + astaxanthin at 50 μM | **$A\beta_{1-42}$ (1μM) + fucoxanthin at 0.01 μM** | **$A\beta_{1-42}$ (1μM) + fucoxanthin at 2 μM**

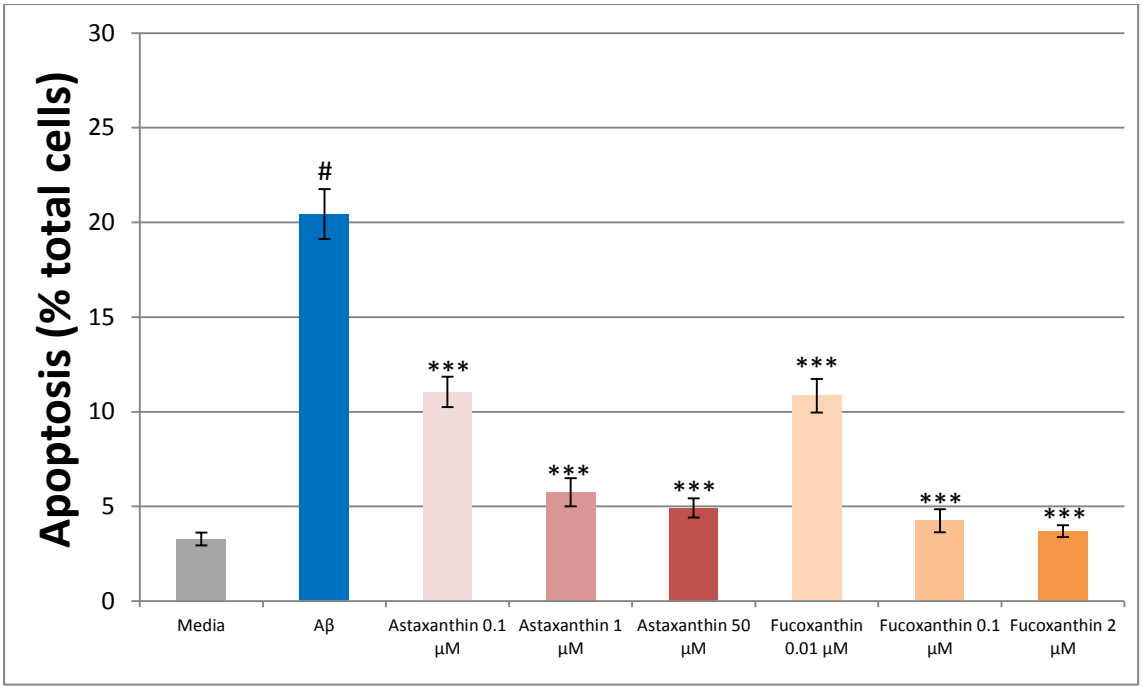


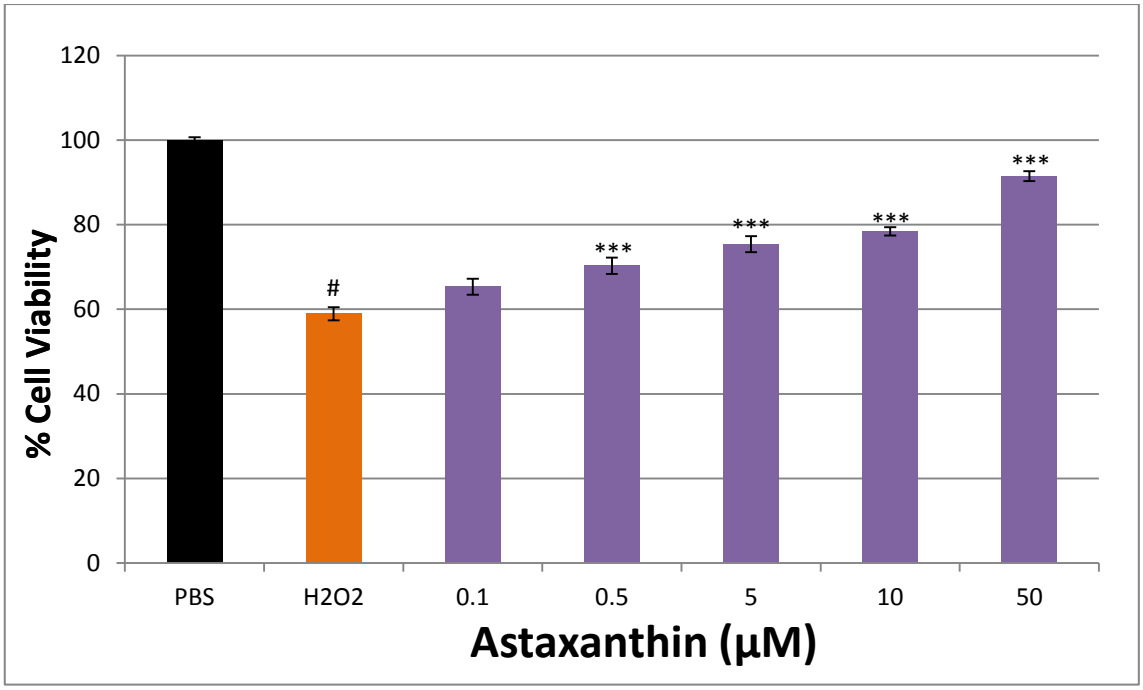
Figure 6-8 Effect of different concentrations of astaxanthin and fucoxanthin on apoptosis induced by $A\beta_{1-42}$ (1 µM) in PC12 cells for 48 hours. Cells were stained using Hoechst 33258 stain and then visualized using fluorescent microscopy. At least 350 cells were counted for every treatment. Each value is the mean ± SEM of four independent experiments (# p <0.005 versus PBS, * p <0.05 and ***p <0.005 versus control $A\beta_{1-42}$).

6.3.6 Protection of PC-12 cells against H_2O_2-induced cytotoxicity

Cell viability was significantly decreased after treating PC-12 cells with 100-200 µM hydrogen peroxide (H_2O_2) (Figures 6-9 and 6-10). At 100 µM H_2O_2, cell viability was reduced to 60%, while at 200 µM H_2O_2 the cell viability was only 30% (Figure 6-9 and 6-10). Astaxanthin provided significant neuroprotection to PC-12 cells against H_2O_2-induced cytotoxicity, with cell viability exceeding 65% ($p<0.05$, Figure 6-9a), and 36% ($p<0.05$, Figure 6-9b) against 100 and 200 µM H_2O_2, respectively. Astaxanthin is a known antioxidant against ROS, reportedly 10 times stronger than other carotenoids and 100 times stronger than α-tocopherol (Miki, 1991). In addition, astaxanthin has been shown previously to protect differentiated PC-12 cells from H_2O_2-induced cytotoxicity (Chan et al., 2009). Our results demonstrated that astaxanthin was a strong antioxidant, reducing the cytotoxicity induced by H_2O_2 in PC-12 cells significantly and at low concentrations.

While fucoxanthin did not show a significant protection against H_2O_2 to PC-12 cells at the lowest concentration tested (0.01–0.1 μM), it showed a significant protection at higher concentrations (0.5 - 2 μM) as shown in Figure 6-10a ($p<0.05$). Fucoxanthin at the highest concentrations of 2 μM demonstrated a significant increase in cell viability against H_2O_2 at 200 μM (Figure 6-10b). Fucoxanthin was reported to have strong antioxidant activity by inhibiting DPPH (2,2-diphenyl-1-picrylhydrazyl) formation with an IC_{50} of 164.60 μM, and showed 13.5 times higher activity than previously reported for α-tocopherol in hydroxyl radical-scavenging assay (Sachindra et al., 2007). Additionally, a previous study found that fucoxanthin could reduce the toxicity induced by H_2O_2 in Vero cells in a concentration-dependent manner (5-200 μM) (Heo et al., 2008). Recently, fucoxanthin was found to inhibit the cytotoxicity induced by H_2O_2 in SH-SY5Y cells and to activate the PI3-K/Akt cascade and inhibit the ERK pathway (Yu et al., 2017). Our study found that fucoxanthin at 0.1 to 4 μM is able to increase PC-12 cell viability against H_2O_2 at 100 μM. However, fucoxanthin at 1 and 2 μM was only able to significantly reduce the cytotoxicity induced by 200 μM H_2O_2.

a



b

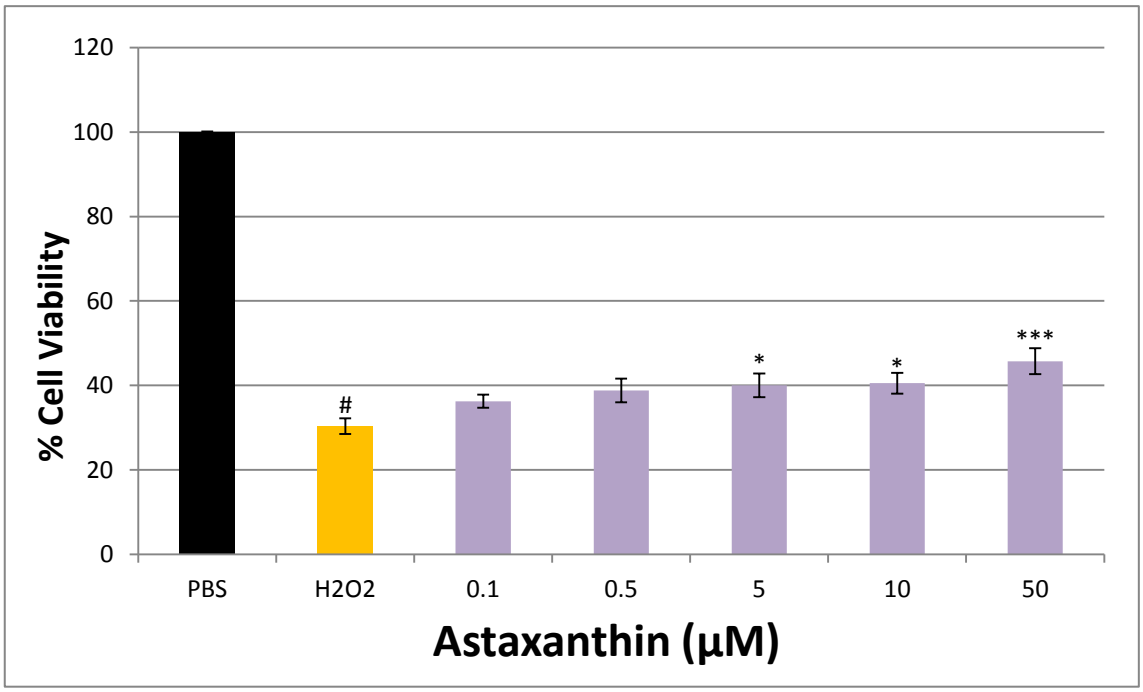
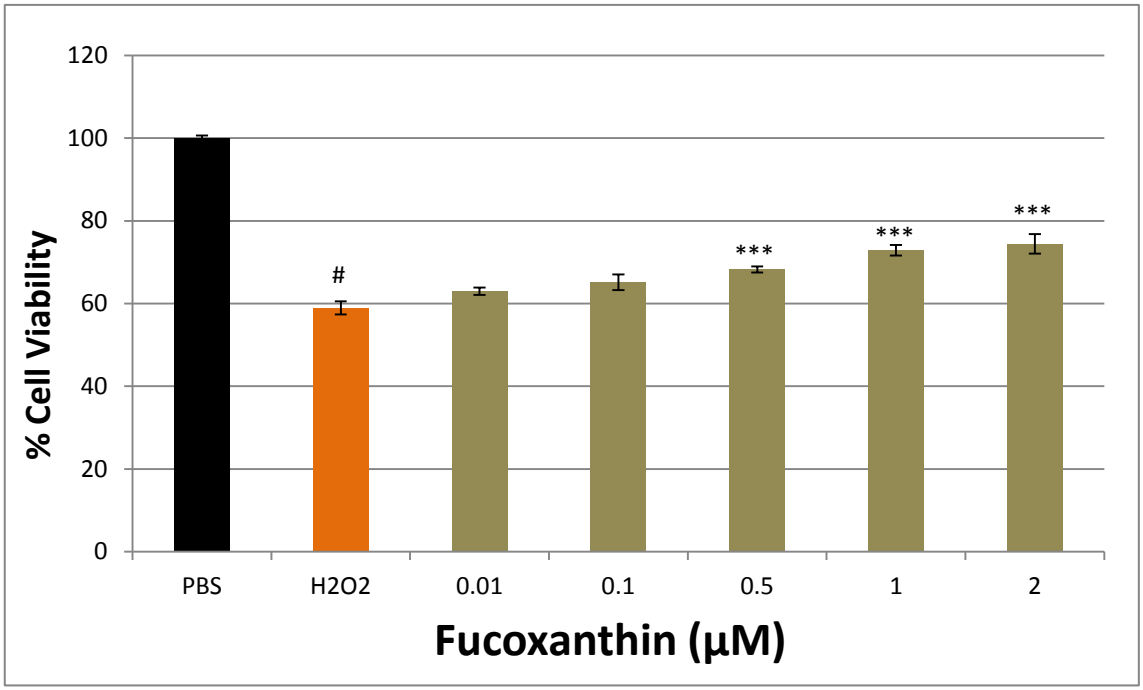


Figure 6-9 Effect of different concentrations of astaxanthin on viability of PC12 cells exposed to H_2O_2 at 100 µM (a) **and 200 µM** (b) **for 24 h. Each value is the mean ± SEM of four independent experiments (# *p* <0.005 versus PBS, * *p* <0.05 and ***p* <0.005 versus control H_2O_2)**

a



b

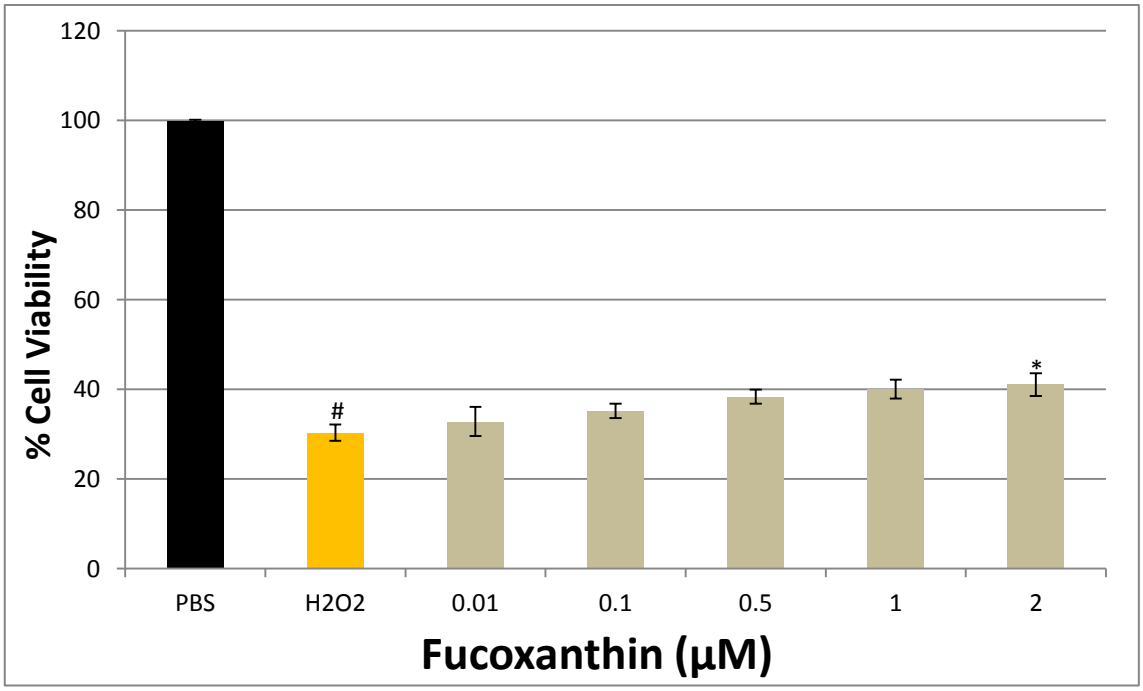
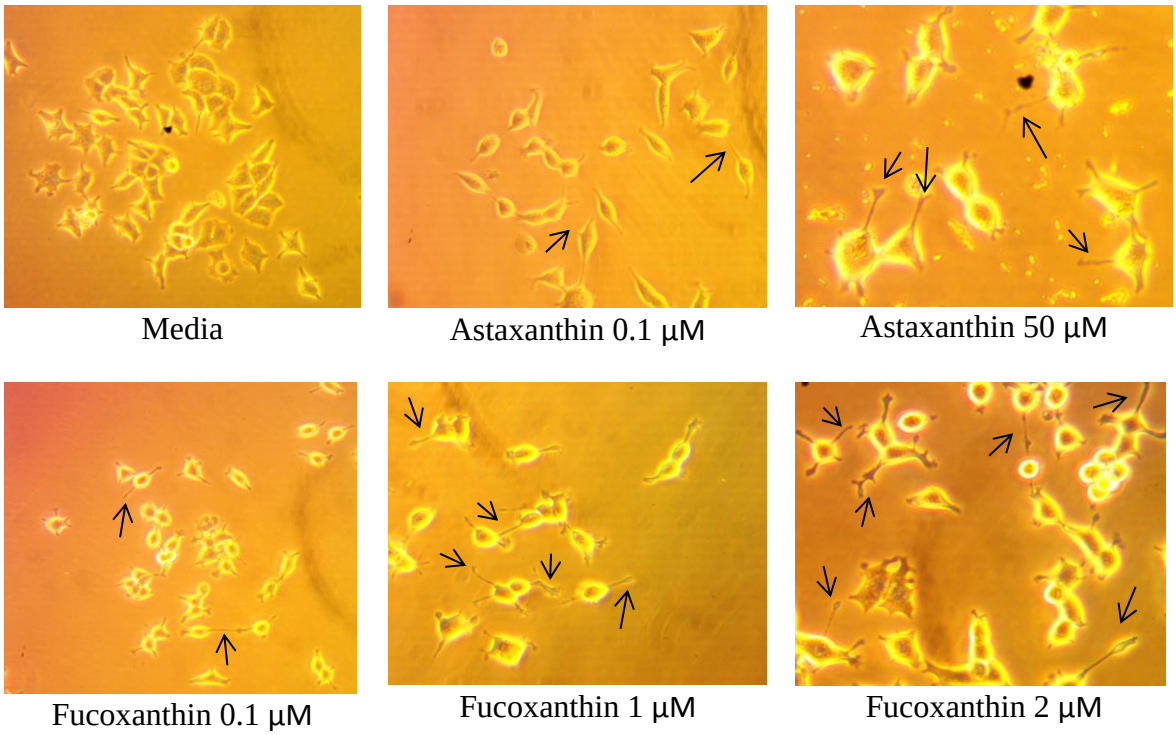


Figure 6-10 Effect of different concentrations of fucoxanthin on viability of PC12 cells exposed to H_2O_2 100 µM (a) and 200 µM (b) for 24 h. Each value is the mean ± SEM of four independent experiments (# *p* <0.005 versus PBS, * *p* <0.05 and ***p* <0.005 versus control H_2O_2)

6.3.7 Astaxanthin and fucoxanthin can enhance neurite outgrowth activity

Enhancing neurite outgrowth is considered as one strategy to treat neurodegenerative diseases, as it can help maintain neuronal plasticity and communication (Koliatsos et al., 1991). It was found that decreasing the level of nerve growth factor (NGF) can result in neuronal dysfunction (Hefti and Weiner, 1986), while NGF can increase neuronal survival (Kromer, 1987). Also, NGF was found to enhance neurite outgrowth (Huang and Reichardt, 2001). In this study, astaxanthin and fucoxanthin enhanced neurite outgrowth activity in PC-12 cells (Figure 6-11). There was around 8% of PC-12 cells developed neurites in the control group over 24 hours, while astaxanthin and fucoxanthin increased the number of neurites in a concentration-dependent manner. Astaxanthin was able to promote 13.6 - 23.7% of neurite outgrowth in a concentration ranging from 0.1 to 50 μM. On the other hand, fucoxanthin showed more potent neurite outgrowth activity, as it was able to promote 15.7 - 31% of neurite outgrowth in a concentration ranging from 0.1 to 2 μM. This is the first report of these compounds possessing such activity. Previously, a methanolic extract *Sargassum macrocarpum* (brown algae) and a PBS extract from *Jania adharens* (red algae) elicited neurite outgrowth activity in PC-12 cells (Kamei and Sagara, 2002). A recent review summarizes 5 compounds from algae that were also able to enhance neurite outgrowth activity in literature (Alghazwi et al., 2016b).



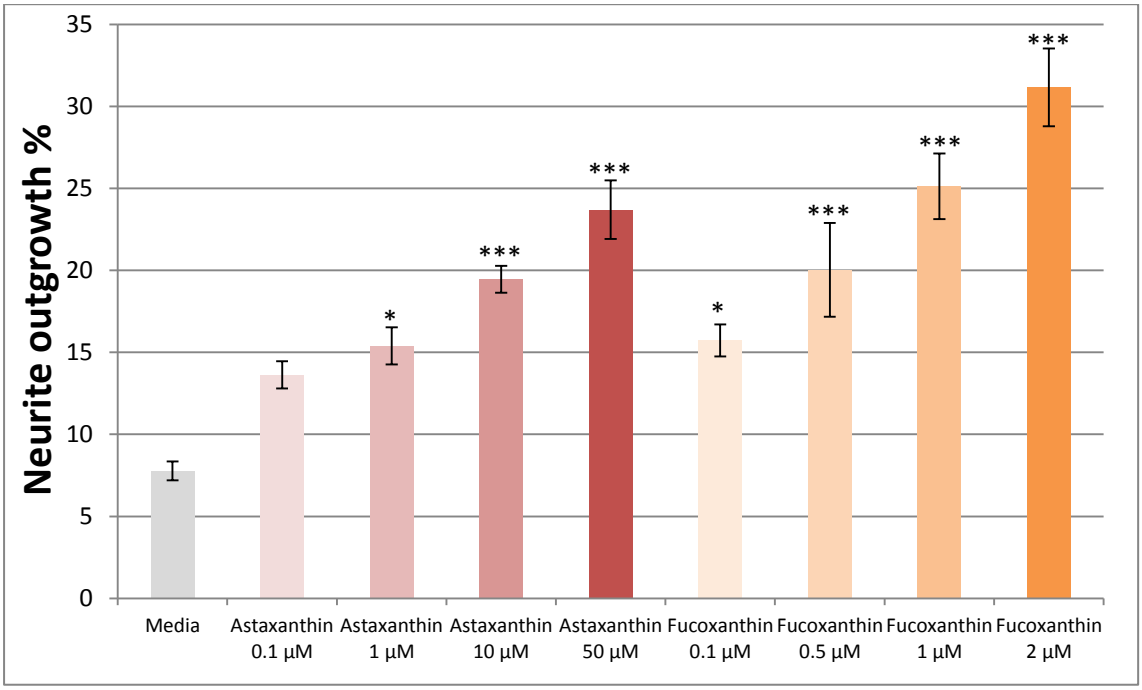


Figure 6-11 Effect of different concentrations of astaxanthin and fucoxanthin samples on enhancing neurite outgrowth in PC12 cells. Cells were visualized using light microscopy. At least 200 cells were counted for every treatment. Each value is the mean ± SEM of six independent experiments (* *p* <0.05 and ****p* <0.005 versus control media)

Astaxanthin may work through inhibiting apoptosis via caspase 3, the expression of Bax and ROS (Chang et al., 2010, Zhang et al., 2015). Alternatively, fucoxanthin could possess neuroprotective capacity through antioxidant activity (Zhao et al., 2015). Fucoxanthin was previously investigated for its activity against beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) (Jung et al., 2016). Molecular docking studies revealed that two hydroxyl groups of fucoxanthin interact with two residues of the BACE1 enzyme (Gly11 and Ala127) (Jung et al., 2016), thereby raising the possibility that it may also inhibit Aβ production. Our study suggests that fucoxanthin is able to decrease the fibrillization and cytotoxicity of $A\beta_{1\text{-}42,}$ inferring that fucoxanthin could be a promising therapeutic agent for Alzheimer's disease.

In a review on neuroprotective compounds derived from macroalgae, fucoxanthin was considered to be a promising compound due to its multiple neuroprotective pathways (Alghazwi et al., 2016b). Astaxanthin has also been considered to be a promising compound for the treatment of neurological diseases (Wu et al., 2015). Our work supports their neuroprotective activities, as both compounds reduced the toxicity of $A\beta_{1\text{-}42}$ and H_2O_2. Moreover, this study showed the anti-aggregation effect against fibrils formed by $A\beta_{1\text{-}42}$. These data are consistent with molecular docking results and show a favourable affinity for binding of both compounds to amyloid beta. In addition, both astaxanthin and fucoxanthin were shown to inhibit apoptosis induced by $A\beta_{1\text{-}42}$ and promote neurite outgrowth activity. As fucoxanthin demonstrated higher neuroprotective activity than astaxanthin, more focus on fucoxanthin in the prevention and treatment of neurodegenerative diseases is recommended, but with consideration of its potential neurotoxicity. Astaxanthin may be considered as a complementary treatment, possibly within nutraceutical applications.

6.4 Conclusion

The current study demonstrated that both astaxanthin and fucoxanthin possess multiple neuroprotective activities but with different potency in a range of bioassays, consistent with molecular docking results of high binding affinity to $A\beta_{1-42}$.. Both compounds exhibited neuroprotective activities by reducing $A\beta_{1-42}$ and ROS toxicity to PC-12 cells, reducing apoptosis and inhibiting the aggregation of $A\beta_{1-42}$. At 10-fold less concentration, fucoxanthin demonstrated both higher neuroprotective and neurite-promoting activity against $A\beta_{1-42}$ than astaxanthin. In conclusion, astaxanthin and fucoxanthin are promising neuroprotective marine-derived compounds that warrant further development as food supplements or drugs for treating neurodegenerative diseases such as Alzheimer's disease.

6.5 References

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7. CHAPTER 7: CONCLUSION AND FUTURE DIRECTIONS

7.1 Major findings

Marine sponge and algae are rich sources of bioactive compounds with a diverse range of biological activities such as anti-viral, anti-bacteria, anti-inflammatory, and anti-cancer actions. Our comprehensive reviews of the literature from 1999-2014 identified 92 and 99 pure compounds with neuroprotective activities from marine sponge and algae, respectively (Alghazwi et al., 2016a, Alghazwi et al., 2016b). The reviews revealed that marine sponges and algae may be promising sources of neuroprotective compounds, however the researches are very scattered and limited in scope and depth, especially targeting multiple neuroprotective pathways. Therefore, this study was conducted to further understand and discover the potential of marine sponge and algae-derived compounds with multiple neuroprotective activities.

There are only few reported compounds that demonstrated neuroprotective activities from either sponges or algae collected in South Australia. Our study evaluated the potential neuroprotective activities of 92 extracts from marine sponges (43) and macroalgae (49) (13 green algae, 16 brown algae, and 20 red algae) collected in South Australia. It was found that 51.1% were neurotoxic to PC-12 cells for 48 hours exposure at the concentrations up to 25 μg/mL by MTT assay. Over 64% of the non-toxic extracts (45) up to 25 μg/mL showed neuroprotective activities by reducing the neurotoxicity induced by $A\beta_{1-42}$. Overall, over 30% of all the sponge and algal extracts were identified to reduce the neurotoxicity induced by $A\beta_{1-42}$, which highlight the important of South Australia marine organisms especially sponges and algae as potential sources of neuroprotective compounds.

In silico modelling of 57 and 26 compounds from sponges and algae with reported neuroprotective activities and available 3D structures in one or more assays predicted the binding between these compounds and therapeutic target protein Aβ in three forms (monomer, pentamer, and polymer). Only three and seven compounds from sponge and algae, respectively showed high docking scores with all forms of Aβ when compared with reference drugs. Further consideration of drug likeness by following Lipinski rule of five, and likelihood to pass through BBB should be applied for these compounds. Majority of the algae-derived candidate compounds are phlorotannins, with astaxanthin and fucoxanthin being commercially available and showing the highest binding scores, worthy of further experimental investigation for the activity in anti-aggregation and protecting neurotoxicity against $A\beta_{1-42}$.

The study on the effect of different extraction processes and therefore six different fractions of the most abundant brown algae in South Australia, *Ecklonia radiata* on neuroprotective activities showed varied bioactivity profiles. Three fractions (crude extract, polysaccharide, and high molecular weight) showed low to high cytotoxicity while the other three fractions did not show any cytotoxicity up to 100 μg/mL when treating PC-12 cells for 48 hours. At the safe doses, crude extract, phlorotannin, and polysaccharide fractions showed the best neuroprotective activity against the toxicity and apoptosis induced by $A\beta_{1-42}$, followed by free sugar and low molecular weight fractions, while high molecular weight fraction showed the lowest activity. Phlorotannin fraction demonstrated the highest anti-aggregation effects against $A\beta_{1-42}$ aggregates formation followed by free sugar, crude extract and low molecular weight fraction with various

amount of phlorotannins. Polysaccharide fractions showed the highest neurite outgrowth activity followed by phlorotannin fraction, free sugar, and low molecular weight fraction. These results highlight impact of different extraction process and different fraction compositions on different neuroprotective activities. Multiple neuroprotective assays are critical to identify neuroprotective mechanisms, and differentiate specific compounds responsible for these activities.

Fucoidans are sulphated polysaccharide compounds that possess many biological activities including neuroprotective activities. A comparative study reported different neuroprotective activities of fucoidan extracts prepared from two different macroalgae sources of *Fucus vesiculosus* and *Undaria pinnatifida* and with different chemical compositions from the same species. Fucoidan UE showed higher neuroprotective activities against $A\beta_{1\text{-}42}$ than fucoidan UF with acetylation lost when further purified. Fucoidans from *U. pinnatifida* showed higher anti-aggregation activity against $A\beta_{1\text{-}42}$ than *F. vesiculosus*. Phlorotannins content in these fucoidan extracts showed good correlation with the anti-aggregation activity, and the fucoidan S with the highest phlorotannin content from *F. vesiculosus* showed the highest anti-aggregation activity. This conclusion was supported by our early study as phlorotannin-rich extract from *E. radiata* showed the highest anti-aggregation activity. Fucoidans from *U. pinnatifida* have higher neurite outgrowth enhancement activity than *F. vesiculosus*.

The two most promising neuroprotective compounds astaxanthin and fucoxanthin selected from our *in silico* study bound to very important regions in Aβ monomer form. Fucoxanthin bounds to Asp23, Lys16, and Phe19; while astaxanthin bounds

to His14 and Tyr10, which are important in aggregation formation and toxicity initiation. Astaxanthin did not show any cytotoxicity up to 50 μM, while fucoxanthin demonstrated cytotoxicity at 5 μM and above. Fucoxanthin demonstrated higher neuroprotective activity against $A\beta_{1-42}$ induced cytotoxicity and apoptosis. Fucoxanthin demonstrated higher anti-aggregation activity against $A\beta_{1-42}$ and higher enhancement of neurite outgrowth than astaxanthin. In contrast, astaxanthin showed higher antioxidant activity than fucoxanthin. This comparative study clearly differentiates multiple neuroprotective mechanisms of these two compounds.

The key results from chapter 4 to chapter 6 are summarized in Table 7-1.

In this study, different marine sponges and algae extracts/compounds have demonstrated their ability to inhibit the neurotoxicity and aggregation of $A\beta_{1-42}$. One possible mechanism is their ability to reduce apoptosis induced by $A\beta_{1-42}$ via different pathways such as inhibiting Bcl-2, Bax, caspase 3 and 9, or cytochrome C expression while further studies will be required. The other possible mechanism is by inhibiting ROS, as $A\beta_{1-42}$ was demonstrated to increase the production of ROS. Hydrogen peroxide is not toxic by itself as it is produced normally in the body, but it can produce toxicity due to the reactive species generated via Fenton reaction (Halliwell et al., 2000). The hydrogen peroxide was released in early stage of Aβ aggregation, while mature Aβ fibril did not form any hydrogen peroxide (Tabner et al., 2005). Future study should investigate the bioavailability of these extracts/compounds. Some compounds have already been investigated such as fucoidan, astaxanthin, and fucoxanthin. Fucoidan was found to have poor

bioavailability, but reducing the molecular weight can increase its bioavailability (Fitton et al., 2015, Zhao et al., 2016). Similarly, astaxanthin has low bioavailability, but the presence of fat can increase the bioavailability of astaxanthin. An example is that polysorbate 80 can enhance the bioavailability almost 4-fold (Odeberg et al., 2003). Astaxanthin showed the highest bioavailability than other carotenoids compounds (β-carotene and lutein) (Rao et al., 2013). The bioavailability of fucoxanthin can be increased using different agents such as carrier oils and skimmed milk (Salvia-Trujillo et al., 2015, Mok et al., 2018).

Table 7-1 Summary of the multiple neuroprotective activities of extracts tested in Chapter 4-6

Sample/Activity	Not-toxic to PC-12	Neuroprotective against Aβ1-42 induced neurotoxicity	Anti-aggregation against Aβ1-42	Anti-apoptotic against Aβ1-42	Antioxidant against hydrogen peroxide	Enhance neurite outgrowth
CE fraction	×	✓	✓	✓	✓	✓
PT fraction	✓	✓	✓	✓	✓	✓
PS fraction	×	✓	✓	✓	✓	✓
FS fraction	✓	✓	✓	✓	✓	✓
LM fraction	✓	✓	✓	✓	✓	✓
HM fraction	×	×	×	✓	×	✓
FE fucoidan	✓	✓	×	✓	✓	✓
FF fucoidan	✓	✓	×	✓	✓	✓
S fucoidan	✓	✓	✓	✓	✓	✓
UE fucoidan	✓	✓	✓	✓	✓	✓
UF fucoidan	✓	✓	✓	✓	✓	✓
Astaxanthin	✓	✓	✓	✓	✓	✓
Fucoxanthin	×	✓	✓	✓	✓	✓

Overall, this thesis has clearly demonstrated high potential of macroalgae as great sources of neuroprotective activities acting by multiple mechanisms.

7.2 Current challenges for treating AD

Many compounds targeting Aβ have failed to show significant beneficial outcomes to AD patients. An example is that tramiprosate with promising pre-clinical results showed no clinical efficacy in clinical trial III (Gauthier et al., 2009). Similarly, some compounds targeting tau have failed in clinical trials such as valproate that failed in

clinical trial III due to lack of efficacy (Fleisher et al., 2011). Targeting Aβ and tau may not be sufficient to develop effective drugs and AD could be more complex than the early understanding. Therefore, multiple drugs or drug targeting multiple pathways are needed in the new approach to treat AD (Piau et al., 2011). Another possible reason of the clinical trials failure is the use of symptomatic AD patients in the clinical trials. In order to prevent false negative results, asymptomatic patients are needed in clinical trials as amyloid hypothesis is based on accumulation of Aβ years before the appearing of the symptoms (Sperling et al., 2011a, Hampel et al., 2015). Moreover, the mechanism of AD development and the causing agents of Aβ and tau are still required to be identified, in order to find the efficient therapeutic targets (Winblad et al., 2016).

7.3 Future directions

There were some limitations in this study that include time limitations which prevent us from identifying the active compounds presented in the extracts and fractions (Chapter 2 and Chapter 4), and commercial unavailability of the potential compounds from sponges (Chapter 3). This gap should be filled in the future by conducting more research to explore the different compounds presented in these two groups of marine organisms. For that reason, the future direction of this work will be identifying and characterizing the compounds that are responsible for the neuroprotective activities from the extracts (Chapter 2) and the fractions (Chapter 4). Different sources of fucoidans can be used such as from different species or even from different marine organisms such as sea cucumber (Yu et al., 2014).

This study focused on discovering neuroprotective compounds and extracts with multiple neuroprotective activities. As a multi-target ligand compound is favourable to treat AD, many researchers are looking for compounds that can show different neuroprotective activities. As an example of that is (-)-epigallocatechin gallate (EGCG) which demonstrated recently to inhibit the formation and cytotoxicity of tau (Wobst et al., 2015). The second hallmark of AD is the presence of neurofibrillary tangles (aggregation of hyperphosphorylated tau), further investigation should target this protein. It can start with *in silico* modelling and then different screening strategies for tau. One of the strategies is by stabilizing microtubule (MT) as phosphorylated tau can lead to MT disruption (Yoshiyama et al., 2012). Administrating BMS-241027 to rTg4510 mice showed beneficial effects as it stabilized MT which suggest it might be a useful treatment for AD (Barten et al., 2012). Other strategies include targeting kinases such as glycogen synthase kinase 3β (GSK-3β) and cyclin dependent kinase 5 (CDK-5), and inhibiting tau fibrillization (Yoshiyama et al., 2012). Based on our reviews, only 9 compounds from sponges (Alghazwi et al., 2016a) and only 3 compounds from algae have demonstrated to inhibit kinases (Alghazwi et al., 2016b).

Another neurodegenerative disease model can be used such as Parkinson's disease (PD) by targeting for instance α-synuclein (Dehay et al., 2015). PD can be characterized by 1-methyl-4-phenyl- 1,2,3,6-tetrahydropyridine (MPTP) (Langston et al., 1999), In our review of neuroprotective compounds derived from macroalgae, only 1 compounds which is fucoidan found to inhibit MPTP (Luo et al., 2009).

The overview of the future direction of this work can be found in Figure 7-1.

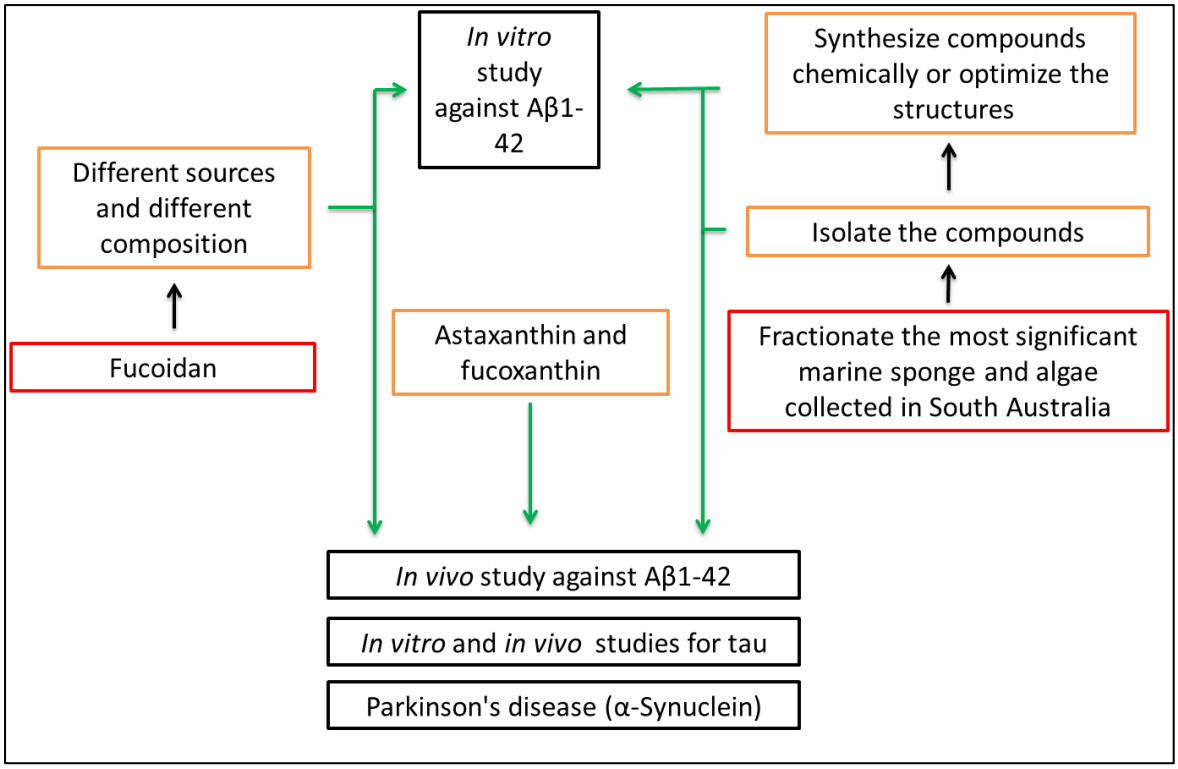

Figure 7-1 Schematic overview of the future direction of this work.

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Appendices

Appendix 1: Published review 1

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Candidate's Contribution to the publication:

Research Design 75%

Data Collection 80%

Writing and editing 85%

Outline your (the candidate's) contribution to the publication:

My contribution was collecting most of the data and wrote most part of this paper. Also, make all the table and figures and draw all chemical structures.

✕ I confirm that the details above are an accurate record of the candidate's contribution to the work.

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Neuroprotective Activities of Natural Products from Marine Macroalgae during 1999-2015

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Abstract

This review focuses on macroalgae-derived compounds with neuroprotective activity that may provide lead compounds for the prevention and treatment of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Huntington's disease. Coverage is provided from 1999-2015. A total of 99 pure compounds have been reported. The main *in vitro* bioactivities of these compounds include inhibition of beta amyloid protein (Aβ), inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), reduction in oxidative stress, anti-inflammatory activity, inhibition of kinases, enhancement of neurite outgrowth, and the reduction in dopaminergic neurotoxicity. The majority of these bioactive compounds are derived from Phaeophyceae (57.6%), followed by Rhodophyta (28.3%) and Chlorophyta (14.1%), respectively. This review presents valuable knowledge on macroalgae-derived compounds and their known pathways of neuroprotection to further explore the potential of these compounds in preventing and treating neurodegenerative diseases.

Keywords: Macroalgae, seaweed, neuroprotective activity, natural products, neurodegenerative diseases

Introduction

Macroalgae, also known as seaweeds have been widely recognized as food, functional food and potential drug sources since the beginning of civilization. Based on the colour of pigments, macroalgae can be classified in many different phyla which include Ochrophyta (class Phaeophyceae), Chlorophyta, and Rhodophyta which are brown, green, and red algae, respectively (Guiry, 2012). Macroalgae can be found in diverse habitats from arctic shores to tropical coral reefs, which have evolved by developing a variety of protective enzymes and metabolites under diverse environments.

Over several decades scientists have recognized the medicinal potential of macroalgae-derived bioactive metabolites and mineral elements, including but not limited to antibacterial (Lima-Filho et al. 2002), antiviral (Wang et al. 2008), antioxidant (Kang et al. 2003; Kang et al. 2004), anticancer (Aisa et al. 2005) and neuroprotective activities (Pangestuti and Kim 2011b).

The world population increasingly suffers from aging-related neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD). The search for novel compounds for effective prevention and/or treatment of neurodegenerative diseases is intensely pursued worldwide, much of it utilising natural products as templates for neuroprotective drug discovery and development. The term neuroprotection can be defined as any activity that is related to the protection of neurons in the central nervous system (CNS) or peripheral nervous system (PNS) from dysfunction, degeneration, injury and apoptosis (Zarros 2009).

Understanding the potential of using macroalgae-derived compounds for the prevention and treatment of aging-related neurodegenerative diseases is therefore very important. Systematic scan of the literature has revealed scattered information in several reviews of marine natural products and their bioactivities. These include reports of six neuroprotective pigments (Pangestuti and Kim 2011a), twelve neuroprotective compounds from algae that inhibit AChE, BuChE, GSK-3β, and Aβ (Choi and Choi 2015), one compound inhibiting acetylcholinesterase activity (Pinho et al. 2013), and four phlorotannins (eckol, phlorofucofuroeckol A, dieckol, and 8,8'-

bieckol) exhibiting antioxidant activity (Wijesekara et al. 2010). Only two reviews focused on neuroprotective compounds isolated from macroalgae (Pangestuti and Kim 2011b; Barbosa et al. 2014), Pangestuti and Kim (2011b) reviewed a total of 21 extracts and 21 compounds demonstrating antioxidant, anti-inflammatory, acetylcholinesterase inhibition, inhibition of neuronal death and other neuroprotective actions. Barbosa et al. (2014) recently presented 36 compounds under three different bioactive categories: preventing neuroinflammation, oxidative damage, and synaptic loss. These compounds were classified in eight different chemical classes, namely phlorotannins, alkaloids, terpenes, pigments, sterols, oligo- and polysaccharides, fatty acids, and other compounds. However, these two reviews have provided a very limited rather than comprehensive summary of macroalgae-derived neuroprotective compounds reported in the literature.

Here we present a comprehensive review of potential neuroprotective compounds from macroalgae during the last 15 years, with a total of 99 pure unique compounds reported during the period 1999-2015 (Figure 1). These compounds were searched using key words of "algae" or "seaweed" combined with "neuroprotection", "neuroprotective", or "Alzheimer" in three major databases (ScienceDirect, PubMed, and Scopus). Some compounds have been reported to have multiple activities and are assigned according to the activity that was first reported. In such instances, the compound is assigned to the activity with the highest target IC_{50}. Fucoidan compounds are the only exception, as they have been counted as different compounds for the same activity because of their structural variants from different algal species (Li et al. 2008). All other compounds were checked to make sure that they have a unique structure, so compounds that exhibit more than one activity but have the same structure are counted as one single compound.

There are only 6 compounds reported during 1999-2004. The number of active compounds discovered increased from 2005 onwards with the exception of 2006, 2011, and 2014, with a peak of 27 compounds reported in 2008.

In terms of the macroalgae phyla from which these compounds are isolated (Figure 2), 57 compounds were found from Ochrophyta (class Phaeophyceae) (57.6% of the

total), followed by 28 and 14 compounds from Rhodophyta and Chlorophyta, respectively.

For this review, these macroalgae-derived compounds are classified into seven categories based on their neuroprotective mechanisms of action (Figure 3). These activities are: inhibition of amyloid beta (8 compounds), inhibition of cholinesterases (27 compounds), enhancement of neurite outgrowth (5 compounds), inhibition of oxidative stress (49 compounds), anti-inflammation (6 compounds), inhibition of kinase activity (3 compounds) and protection of dopaminergic neurons (1 compound).

Neuroprotective Activities of Macroalgae–derived Compounds

A comprehensive summary of macroalgae-derived pure compounds with different neuroprotection activities is presented in Tables 1-7.

Inhibition of aggregation of amyloid-beta (Aβ) protein

The presence of amyloid-beta (Aβ) protein is one of the main hallmarks of AD. The aggregation of Aβ peptides comprising 39-43 amino acids, and derived from amyloid precursor protein (APP) can induce neurotoxicity and increase protein oxidation in neuronal cells (Tang et al. 2006). Aβ is formed via an amyloidogenic pathway in which APP is cleaved by beta-site APP cleaving enzyme 1 (BACE1) to form a 99-amino acid C-terminal stub, which is then cleaved by γ-secretase to form Aβ (Hussain et al. 1999; LaFerla et al. 2007). Amyloid plaques have been found to be correlated with memory defects in transgenic mice (Hsiao et al. 1996). BACE inhibitors are an excellent target for AD, as BACE knockout mice do not show any side effects (Roberds et al. 2001). This evidence supports inhibiting or slowing down the oligomerization and accumulation of Aβ protein as one of the possible approaches to treat or prevent AD (John 2006).

Eight compounds (1-8) have been found from macroalgae with neuroprotective activity against Aβ (Table 1). Five compounds (4-8) are reported from the Chlorophyta *Caulerpa racemosa*, and three compounds (1-3) isolated from Phaeophyceae. Two compounds (4-5) are alkaloids and two compounds (7-8) are sterols based on the chemical classes. Other chemical classes include oligosaccharides, polysaccharides, fatty acids, terpenoids, and phlorotannins.

Fucoidan is a class of sulfated polysaccharides rich in fucose, and fucoidan from different species have different structures (Li et al. 2008). Fucoidan (2) derived from Phaeophyceae has shown neuroprotective activity by blocking Aβ-mediated toxicity in primary cell culture at a dosage of 1 μM. However, fucoidan did not inhibit the aggregation of Aβ as shown by electron microscopic images. Signalling pathway studies showed that fucoidan can block caspase 3 and 9 associated in initiating apoptosis (Jhamandas et al. 2005). A further study on the effect of fucoidan was performed using Sprague–Dawley rats, where it was found that fucoidan could improve Aβ-mediated impairments of learning and memory in rats via an increase of the Bcl-2/Bax ratio, reducing caspase-3 activity, regulating cholinergic nerve activity and decreasing oxidative stress (Gao et al. 2012b). Dioxinodehydroeckol (3) (Figure 4) is a phlorotannin compound isolated from Phaeophyceae *Eisenia bicyclis* in Korea, which was found to suppress BACE-1 enzyme activity, with an IC_{50} of 5.35 μM (Jung et al. 2010).

Inhibition of acetylcholinesterase (AChE) and butylcholineterase (BuChE)

Acetylcholine (ACh) is a neurotransmitter hydrolysed by the enzyme acetylcholinesterase (AChE) in order to regulate the synaptic concentration of acetylcholine (Soreq and Seidman 2001). Another type of cholinesterase (ChE) in the nervous system is butyrylcholinesterase (BuChE), which can be found within glial cells and specific cholinergic nerve tracts (Darvesh and Hopkins 2003). BuChE was recently found to play an important role in the regulation of ACh levels in brain. However, the level of BuChE did not decrease in AD (Greig et al. 2002). Defects in cholinergic transmission have been found to correlate with memory loss (Bartus et al. 1982). In addition, the level of ACh was reduced in AD patients (Tohgi et al. 1994). Moreover, AChE, but not BuChE, was shown to accelerate the formation of Aβ protein aggregation (Inestrosa et al. 1996). This means that AChE inhibitors could have another role in reducing the aggregation of Aβ (Rees and Brimijoin 2003). Further studies have shown that BuChE inhibitors can reduce Aβ levels in overexpressed human mutant APP transgenic mice and improve cognitive function (Greig et al. 2005). The cholinergic strategy to treat AD is based on symptomatic treatment by improved memory and associated behaviours (Grutzendler and Morris 2001). For that reason both AChE and BuChE have been utilised as therapeutic targets for AD to improve functioning of the cholinergic system (Greig et al. 2002).

Twenty seven compounds (9-35) have been reported to have cholinesterase inhibition activity (Table 2). Fifteen and twelve compounds were isolated from Rhodophyta and Phaeophyceae, respectively. *Gloiopeltis furcata* is the only species of Rhodophyta that provided all 15 compounds, which highlights the potential of discovering neuroprotective compounds from other Rhodophyta species. In Phaeophyceae, several compounds were isolated from *Ecklonia stolonifera*, *Sargassum sagamianum*, *Ecklonia maxima*, and *Ishige okamurae*. The most common chemical class of these cholinesterase inhibitors is phlorotannins, with additional sterol, terpene, and fatty acid classes. More than 96% of the compounds were isolated from macroalgae collected from Korean waters, highlighting great opportunity in discovering new compounds with cholinesterase inhibition activity from macroalgae collected from other regions.

Of interest from all these compounds studied by far, 2-(3-hydroxy-5-oxotetrahydrofuran-3-yl)acetic acid (20) (Figure 5) isolated from the Rhodophyta *Gloiopeltis furcata* has multiple inhibition activities, with IC_{50} values of 1.4 µg mL^{-1} and 12.6 µg mL^{-1} for AChE and BuChE respectively (Fang et al. 2010). For comparison, the known cholinesterase inhibitor nicotinic acid has an IC_{50} value of 1.14 µg mL^{-1} against AChE and 20.9 µg mL^{-1} against BuChE (Fang et al. 2010).

Phlorofucofuroeckol A (14) (Figure 5) isolated from Phaeophyceae was found to possess the strongest BuChE inhibition activity, with an IC_{50} activity of 0.95 µM. This compound also showed a low IC_{50} of 96.3 µM against AChE (Choi et al. 2015a). In contrast, another group of researchers reported that phlorofucofuroeckol A has an IC_{50} of 136.7 and 4.89 µM against BuChE and AChE, respectively (Yoon et al. 2008). The different methods of measuring the activity of cholinesterase might account for this difference.

The commonly used positive control compounds include eserine (IC_{50}: 0.004 and 0.06 µM for AChE and BuChE, respectively), tacrine (IC_{50}: 0.006 and 0.001 µM for AChE and BuChE, respectively), berberine (IC_{50}: 0.006 – 0.22 and 1.29 – 11.74 µM for AChE and BuChE, respectively), and galanthamine (IC_{50}: 1.8 µM for AChE) (Yoon et al. 2008). Donepezil, galanthamine, and rivastigmine are used clinically as AChE inhibitors for patients with mild to moderate Alzheimer's disease to reduce some

symptoms (Blennow et al. 2006). In comparison with these positive controls, algae-derived compounds showed promising results, as they demonstrated lower or comparative IC_{50} values *in vitro* which are worthy of further development as AChE inhibitors clinically.

Decreasing oxidative stress

Oxidative stress is defined as the damage that occurs to cells and later to affects microglia due to the presence of reactive oxygen species (ROS). One mechanism involved is lipid peroxidation, when reactive oxygen removes hydrogen from lipid, and the resulting products can lead to damage of cell macromolecules and ensuing neuronal death. Another possible mechanism is the presence of reactive oxygen can break DNA strand, which can cause genotoxicity (Cai and Yan 2007). Therefore, the use of antioxidants may be beneficial in the treatment of neurodegenerative diseases (Bonda et al. 2010).

Calcium plays a crucial role in cellular homeostasis (Pascale and Etcheberrigaray 1999). Aβ may influence calcium levels by increasing it above the normal range (Pascale and Etcheberrigaray 1999). Aβ containing senile plaques can deposit calcium in layers in neurons and lead to an increase in the levels of calcium, resulting in neuronal death (Fukuyama et al. 1994). Mitochondrial dysfunction in neurons that results from oxidative stress could be another cause of increased calcium levels (Bezprozvanny 2009). Many potential compounds targeting Aβ have failed in clinical trials; therefore finding new targets such as reducing calcium level will increase the chance of finding new treatment tools, even if such treatments only delay the development of the disease (Bezprozvanny 2009).

Forty nine compounds (36-84) are active in reducing ROS activity (Table 3), accounting for nearly half of all the neuroprotective compounds reported. Thirty of these compounds have been found in Phaeophyceae, while twelve and seven compounds are from Rhodophyta and Chlorophyta, respectively. The majority of these compounds can be classified as terpenoids, polysaccharides and phlorotannins with 19, 10, and 6 compounds, respectively. Thirty compounds have been isolated from macroalgae collected from Korea. Other countries include China with 11 compounds, and Japan with 4 compounds.

Of significant interest for further investigation, eight meroditerpenoids (62-69) isolated from the Phaeophyceae *Sargassum thunbergii* showed 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) inhibition activity, with IC_{50} values less than 0.35µg mL^{-1} (Jung et al. 2008). Fucoxanthin (36) (Figure 6) showed scavenging activity of 18.4, 34.7, and 57.7% in a dose dependent manner with concentrations of 5, 50, 100 µM, respectively on scavenging intracellular ROS. In addition, fucoxanthin showed protection of Vero cells from H_2O_2 induced damage (reducing cell viability to 43.7%) with cell survival increased to 63.6, 69.4, 78.5, and 89.2% when the cells were treated with 5, 50, 100, and 200 µM of fucoxanthin, respectively. Moreover, fucoxanthin showed DNA protective activity and anti-apoptotic effect induced by H_2O_2 (Heo et al. 2008). Further studies on fucoxanthin showed promising results *in vivo* against oxidative stress by inhibiting lipid peroxidation, with a target site concentration of 0.83 µM (Sangeetha et al. 2009).

Anti-inflammatory activity

Glial cells consisting of microglial cells and astrocytes are involved in brain inflammation (Kreutzberg 1996). Activating microglial cells can produce neurotoxic factors such as cytokines, proteinases, and free radicals (Banati et al. 1993). It has been found that glial cells are activated in many diseases such as Alzheimer's disease and Parkinson's disease (McGeer et al. 1988). It has been suggested that aggregation of Aβ activates microglial cells via receptor for advanced glycation end-product (RAGE) receptors (Meraz-Ríos et al. 2013). For that reason, microglial activation was believed to be involved in neurodegenerative diseases (Lull and Block 2010).

Six compounds (85-90) isolated from macroalgae have been found to have anti-inflammatory activity (Table 4). Three of these compounds (two of which are polysaccharides) have been isolated from Phaeophyceae. The other chemical classes include phlorotannins, alkaloids, sterols, and glycosides. Three compounds have been isolated from Korean algae, and only one compound each from China, Brazil, and India.

The Rhodophyta *Laurencia undulata* contains one compound floridoside (87) (Figure 7) having anti-inflammatory activity. This compound works by inhibiting the

production of nitric oxide (NO) and ROS and blocks the phosphorylation of p38 and ERK in BV-2 cells, with IC_{50} of 10 μM (Kim et al. 2013). Clerosterol (88) isolated from green algae *Caulerpa racemosa* has shown to reduce the activity of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and Tumor necrosis factor alpha (TNF- α) with an IC_{50} of 3μg mL^{-1} (Lee et al. 2013). These compounds (87 and 88) are worthy of further investigation.

Inhibition of kinases

Tau is a microtubule associated protein (MAP) found in axons (Goedert et al. 1988; Hirokawa et al. 1996). Tau is responsible for regulating the stability of microtubules (Drechsel et al. 1992). Hyperphosphorylation of tau results in its dissociation from microtubules and aggregation in the form of neurofibrillary tangles (Avila et al. 2006). Hyperphosphorylated tau protein is a major component in neurofibrillary tangles which is a hallmark of AD (Selkoe 1997). In addition, accumulation of these hyperphosphorylated tau proteins can trigger synaptic dysfunction (Hoover et al. 2010). Dysregulation of kinases and phosphatases has been found to increase tau hyperphosphorylation levels (Hanger et al. 2009). Some examples of kinases found to play an important role in phosphorylating tau include MAP/ microtubule affinity-regulating kinase 1 (MARK1), cyclin-dependent kinase 5 (CDK5), and GSK3 (Dolan and Johnson 2010). For that reason an approach for treating AD is to target tau phosphorylation (Götz et al. 2012). This approach can be achieved through the targeted discovery and development of tau kinase inhibitors (Noble et al. 2011).

Only three compounds (91-93) from macroalgae have kinase inhibitory activity (Table 5). All of these compounds have been isolated from Phaeophyceae. Moreover, these compounds were isolated from a single species *Zonaria spiralis* collected in Australia, and all of them are phloroglucinols. The most active compound is spiralisone B (92) (Figure 8), inhibiting the kinases cyclin-dependent kinase 5 (CDK5/p25), casein kinase 1 (CK1δ) and glycogen synthase kinase 3 beta (GSK3β), with IC_{50} values of 3, 5, and 5.4 μM, respectively (Zhang et al. 2012).

Enhancement of neurite outgrowth

Neurotrophic factors are polypeptides, which have an essential role in the nervous system as regulators of neuronal differentiation (Hefti 1997). There are many types of

neurotrophic factors including nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), neurotrophin 4/5 (NT4/5) and many others (Hefti 1997). They work by sending signals to neurons to change their morphology and function through binding to a specific region of a gene, such as TrkA and TrkB for NGF (Yuen et al. 1996). Supplying endogenous NGF can enhance the survival of neurons and thereby be used as a treatment strategy for treating neurodegenerative diseases (Kromer 1986). In order to provide a suitable treatment for diseases like AD, small molecules with neurotrophic activity are desirable to maximize penetration across the blood-brain barrier (Murakami et al. 2001).

Five compounds (94-98) from macroalgae can enhance neurite outgrowth (Table 6). All of these compounds (94-98) have been isolated from Phaeophyceae. Interestingly, three compounds (94-96) (Figure 9) were isolated from one species *Sargassum macrocarpum*. These compounds represent different chemical classes including carotenoids, terpenoids, and polysaccharides. One of the interesting compounds is sargaquinoic acid (95), *Sargassum macrocarpum* collected in Japan. It was found to enhance neurite outgrowth at a concentration of 3 μg mL^{-1} via a TrkA-dependent MAP kinases-mediated signalling pathway (Kamei and Tsang 2003).

Protection of dopaminergic neurons

Parkinson's disease is usually characterized by the loss of dopaminergic neurons, and the presence of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) can induce parkinsonism (Langston et al. 1999). Administration of MPTP can result in motor dysfunction similar to that which occurs in Parkinson's disease, which makes it a favourable experimental model for this disease (Gerlach et al. 1991).

One compound (99) has been found to attenuate the neurotoxicity of MPTP activity (Table 7). Fucoidan (99) is derived from the Phaeophyceae *Saccharina japonica*. Fucoidan (99) has been demonstrated in mice models to be effective at a dosage of 25 mg kg^{-1} in protecting the cells from MPTP-induced neurotoxicity by reduceing the behavioural deficits and cell death, and increasing the level of dopamine (Luo et al. 2009).

Macroalgae-derived compounds exhibiting multiple activities

A new strategy called multitarget-directed ligands (MTDL's), involving the design of a chemical compound that can modulate more than one target, has been proposed in the search for compounds to treat AD (Cavalli et al. 2008; Fang et al. 2015). An example of this strategy is memoquin. It has been shown *in vitro* that memoquin is capable of targeting Aβ, AChE, ROS, and BACE1, and is effective in reducing Aβ aggregation, inhibiting tau hyperphosphorylation and improving behavioural deficits and cholinergic activity *in vivo* (Bolognesi et al. 2009). From this perspective, it is interesting to note that 23 macroalgae-derived compounds exhibited multiple neuroprotective activities (Table 8). For instance, phlorofucofuroeckol-A suppressed BACE-1 enzyme activity, inhibited AChE and BuChE, scavenged ROS (which include DPPH, hydroxyl, superoxide, and peroxyl radicals), and inhibited GSK-3β kinase. Dieckol (Figure 5) similarly reduced BACE-1 activity, inhibited AChE and BuChE, reduced NO and inducible nitric oxide synthase (iNOS) expression, scavenged ROS, and decreased GSK-3β levels. These compounds are thus promising multitarget-directed drug candidates. Given that most of these compounds have only been tested in a single assay, it will be important in the future to examine these compounds in multiple assays, including other new targets such as γ or α secretases, and targeting tau via the prevention of tau phosphorylation or misfolding.

In vivo studies of macroalgae-derived neuroprotective compounds

Five macroalgae-derived compounds have been tested in animal models (Table 9). This section will discuss these compounds and their activity in more detail, as these compounds warrant further investigation in assessing their clinical potential.

Fucoidan has been tested for three activities: anti-Aβ, anti-inflammatory effect and inhibiting MPTP-evoked toxicity. Fucoidan (2) was shown to improve memory and learning impeded by the administration of Aβ in rats, and this was associated with inhibiting apoptosis, regulating cholinergic activity and decreasing ROS (Gao et al. 2012b). Sprague-Dawley rats were injected with lipopolysaccharide to activate microglia and cause dopaminergic neuronal injury, and subsequently behaviourally examined after treating the animals with fucoidan (86). The results showed that fucoidan (86) can promote the survival of injured neurons by a reduction in ROS and the pro-inflammatory cytokine TNF-α expression (Cui et al. 2012). Treating C57BL/6

mice with MPTP resulted in behavioural defects; however treating these animals with fucoidan (99) resulted in behavioural improvement. The mechanism of fucoidan (99) reducing MPTP-derived neurotoxicity is believed to occur via antioxidative activity (Luo et al. 2009). As fucoidan is of significant structural diversity, it would be very interesting to investigate the structure-activity relationship, furthering rational design and modification of fucoidan toward efficient clinical development.(Fang et al. 2015)

Fucoxanthin (36) showed antioxidant activity by reducing lipid peroxidation in albino rats (Sangeetha et al. 2009). Caulerpin (85) (Figure 7) was also examined for anti-inflammatory activity in the capsaicin-induced ear oedema model following administration at 100μmol kg^{-1} to mice. A notable increase in plasma extravasation usually seen in this model was decreased by 55.8% following caulerpin treatment (de Souza et al. 2009).

Overall, the *in vivo* studies of these macroalgae-derived neuroprotective compounds are very limited, and it is difficult to draw a useful conclusion. The preliminary promising results however warrant more investigation.

Conclusion & future perspectives

In the last 15 years from 1999-2015, at least 99 compounds have been discovered from macroalgae with a diverse range of neuroprotective activities. These findings demonstrate that macroalgae, are rich sources of novel compounds which could become potential agents for the prevention and treatment of neurodegenerative diseases.

Even though these compounds have been tested for various targets related to neurodegenerative diseases, some targets may afford a greater utility than others as potentially disease-modifying treatments. Alzheimer's disease is the major neurodegenerative disease which has been characterized by the presence of senile plaques and neurofibrillary tangles (Blennow et al. 2006). A recent review emphasised that decreasing Aβ and preventing the formation of tangles may be the most effective strategy for approaching AD treatment (Sperling et al. 2011). A therapeutic strategy for treating AD by targeting β and γ secretases, inhibiting Aβ aggregation, or using immunological approaches may therefore offer more potential

than a symptomatic strategy targeting cholinesterase activity as one example (Thorsett and Latimer 2000). For that reason, compounds targeting either BACE or kinase activities may be preferred. Compounds targeting BACE include fucoidan (2) and some phlorotannins compounds such as 6,6'-Bieckol, 8,8′-bieckol, dieckol, phlorofucofuroeckol A, and dioxinodehydroeckol (3) with IC_{50} values of 1, 1, 2.21, 2.13 and 5.35 μM, respectively should be further investigated in more detail to clinically translate their neuroprotective potential. On the other hand, only a few compounds have been screened for kinase activities, with the highest activity being noted for spiralisone B (92) with IC_{50} values of 5.4, 3, and 5 μM for inhibiting GSK3β, CDK5/p25, and CK1δ, respectively. Kinases such as GSK3β and CDK5 have been suggested to be used to target the production of neurofibrillary tangles. So, more compounds derived from macroalgae should be screened for activity against these two kinases.

Another review has highlighted the neuroprotective compounds isolated from marine sponges with a total of 90 compounds (Alghazwi et al. 2016). Among these compounds, 16 were targeting BACE and 9 were targeting kinases activities. The highest activity of BACE comes from prenylated hydroxybenzoic acid 1 and topsentinol K trisulfate, with IC_{50} values of 1 and 1.2 μM, respectively. The highest kinase activity comes from a compound named as hymenialdisine, with an IC_{50} of 10 and 35nM for GSK-3β and CK1, respectively. Algae-derived compounds showed comparative results for BACE activity, but not for kinases. This might due to the fact that all the algal compounds which have been tested for kinase activities come from only two studies, compared to seven studies on sponge compounds. For that reason, more studies on algal compounds targeting tau protein through kinase or phosphatase activities, or inhibiting tau hyperphosphorylation should be undertaken. This becomes even more apparent when considering that more macroalgae-derived compounds have been shown to exhibit multiple activities when compared to sponge-derived compounds. As the strategy of one compound targeting one target may be limited, compounds directed at multiple targets could be the solution for these complex diseases (Leon, 2013).

The world's oceans have a great diversity of macroalgae with a total of 1792, 7000 and 8000 known species for brown, red, and green macroalgae, respectively (Guiry

2012). The number of macroalgae which have been studied for neuroprotective activities is still very limited. Therefore the discovery of new macroalgae-derived neuroprotective compounds with highly potent activities could be a prolific field of research towards developing therapeutic strategies for neurodegenerative diseases in an aging society. Currently, there is only one compound (acidic oligosaccharide sugar chain) which has passed Phase I clinical trials in China as an anti- Alzheimer's therapy (Liu et al. 2008). This review calls for more studies to progress from *in vitro* studies into *in vivo* animal studies, and then into clinical trials.

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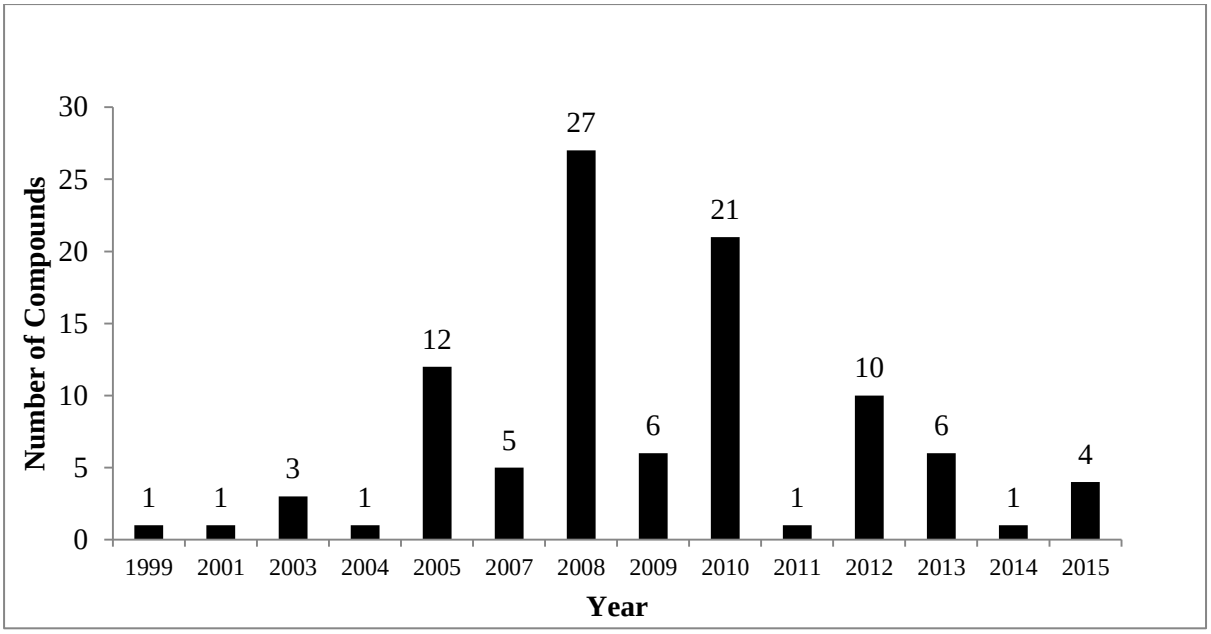


Figure 1: Yearly discovery of neuroprotective compounds from macroalgae during the years 1999-2015. The review was based on three major databases of ScienceDirect, PubMed, and Scopus

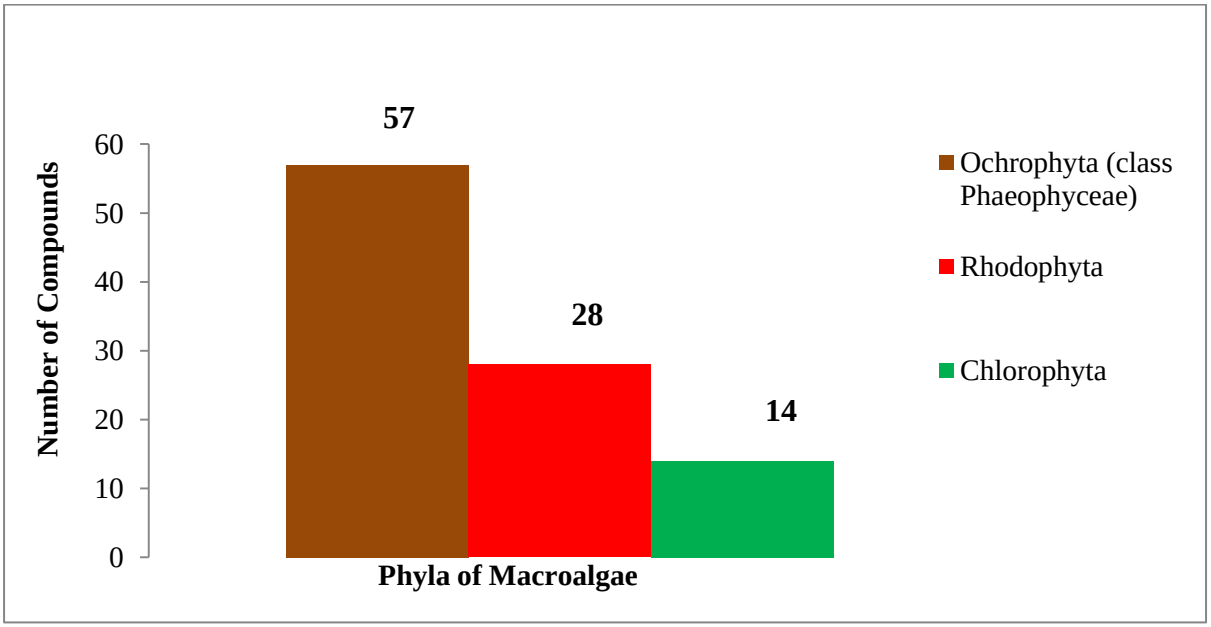


Figure 2: Distribution of neuroprotective compounds identified by macroalgae phyla from year 1999-2015. The review was based on three major databases of ScienceDirect, PubMed, and Scopus

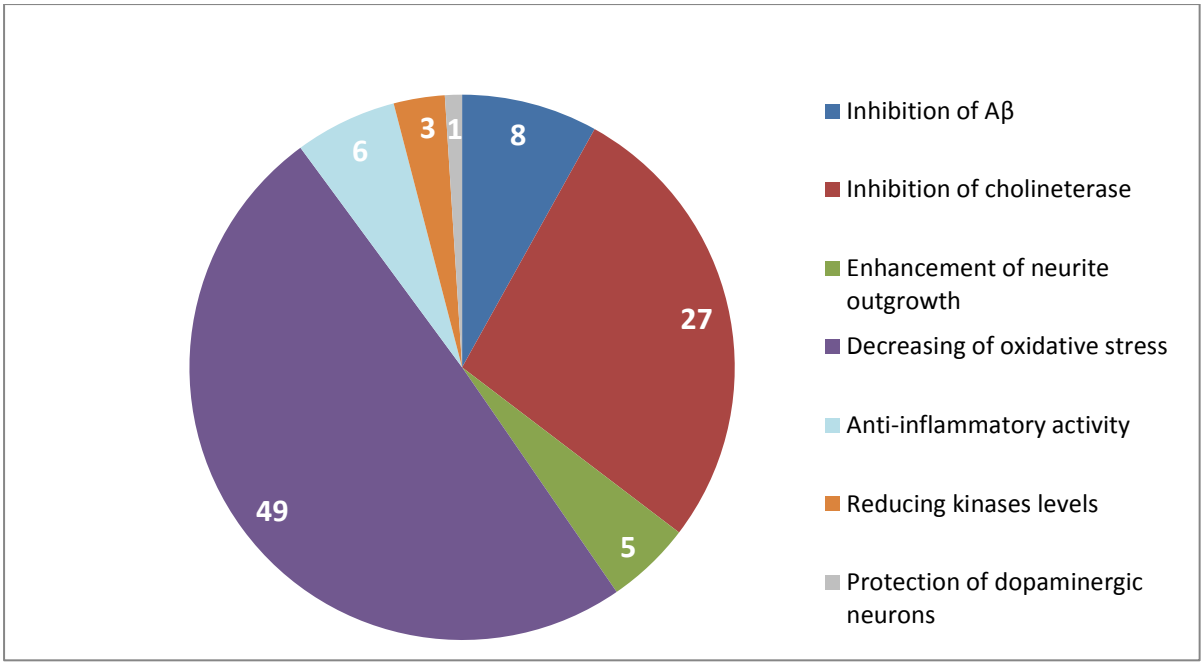


Figure 3: Distribution of macroalgae-derived neuroprotective compounds according to their neuroprotection activity types from year 1999-2015. The total number of the compounds is 99, and the labelled number showed the number of compounds in each activity. The review was based on three major databases of ScienceDirect, PubMed, and Scopus

Table 1: Summary of macroalgae-derived compounds targeting BACE activity

Compound/ number	Macroalgae phyla/class and species	Class of chemistry	Mode of action	IC_{50}	References
Acidic oligosaccharide sugar chain (AOSC) (1)	Phaeophyceae: *Echlonia kurome*	Oligosaccharide	Blocking the fibril formation of Aβ and reducing the toxicity of by increasing cell viability from 55% to 85% and from 65% to 80% in primary cortical neurons and SH-SY5Y cells, respectively	100 µg mL^{-1}	Hu et al. 2004
Fucoidan (2)	Phaeophyceae	Sulfated polysaccharides	Blocking Aβ neurotoxicity in primary cell culture	1 µM	Jhamandas et al. 2005
			Increasing of Bcl-2/Bax ratio, reducing caspase-3 activity	200 mg kg^{-} in Sprague–Dawley rats	Gao et al. 2012b
Dioxinodehydroeckol (3)	Phaeophyceae: *Ecklonia bicyclis*	Phlorotannins	Suppression of BACE-1 enzyme activity	5.35 µM	Jung et al. 2010
Racemosin A (4)	Chlorophyta: *Caulerpa racemosa*	Bisindole alkaloid	Increase 14.6% of cell viability in SH-SY5Y cells	10 µM	Liu et al. 2013
Racemosin B (5)	Chlorophyta: *Caulerpa racemosa*	Bisindole alkaloid	Increase 5.5% of cell viability in SH-SY5Y cells	10 µM	Liu et al. 2013
α-tocospirone (6)	Chlorophyta: *Caulerpa racemosa*	Terpenoid	13.85% increases in cell viability in SH-SY5Y cells	10 µM	Yang et al. 2015
(23E)-3b-hydroxy-stigmasta-5,23-dien-28- one (7)	Chlorophyta: *Caulerpa racemosa*	Sterol	11.31% increases in cell viability in SH-SY5Y cells	10 µM	Yang et al. 2015
(22E)- 3b-hydroxy-cholesta-5,22-dien-24-one (8)	Chlorophyta: *Caulerpa racemosa*	Sterol	15.98% increases in cell viability in SH-SY5Y cells	10 µM	Yang et al. 2015

Table 2: Summary of macroalgae-derived compounds targeting cholinesterase activity

Compound/ number	Macroalgae phyla/class and species	Class of chemistry	Mode of action	IC_{50}	References
(5E, 10Z)-6,10,14-Trimethylpentadeca-5,10-dien-2,12-dione (9)	Phaeophyceae: *Sargassum sagamianum*	Sesquiterpenes	Inhibiting AChE	65 µM	Ryu et al. 2003
(5E,9E, 13E)-6,10,4-trimethyl-pentadeca-5,9,13-trien-2,12-dione (10)	Phaeophyceae: *Sargassum sagamianum*	Sesquiterpenes	Inhibiting AChE	48 µM	Ryu et al. 2003
Eckstolonol (11)	Phaeophyceae: *Ecklonia stolonifera*	Phlorotannin	Inhibiting AChE and BuChE	42.66 and 230.27 µM	Yoon et al. 2008
2-Phloroeckol (12)	Phaeophyceae: *Ecklonia stolonifera*	Phlorotannin	Inhibiting AChE	38.13 µM	Yoon et al. 2008
Eckol (13)	Phaeophyceae: *Ecklonia stolonifera*	Phlorotannin	Inhibiting AChE	20.56 µM	Yoon et al. 2008
	Phaeophyceae: *Ecklonia cava*		Inhibiting BuChE	29 µM	Choi et al. 2015a
Phlorofucofuroeckol A (14)	Phaeophyceae: *Ecklonia stolonifera*	Phlorotannin	Inhibiting AChE and BuChE	4.89 and 136.71 µM	Yoon et al. 2008
	Phaeophyceae: *Ecklonia cava*		Inhibiting BuChE	0.95 µM	Choi et al., 2015
Dieckol (15)	Phaeophyceae: *Ecklonia stolonifera*	Phlorotannin	Inhibiting AChE	17.11 µM	Yoon et al. 2008
	Phaeophyceae: *Ecklonia cava*		Inhibiting BuChE	2.7 µM	Choi et al. 2015a
7-Phloroeckol (16)	Phaeophyceae: *Ecklonia stolonifera*	Phlorotannin	Inhibiting AChE	21.11 µM	Yoon et al. 2008
Fucosterol (17)	Phaeophyceae: *Ecklonia stolonifera*	Sterol	Inhibiting BuChE	421.72 µM	Yoon et al. 2008
24-Hydroperoxy 24-vinylcholesterol (18)	Phaeophyceae: *Ecklonia stolonifera*	Sterol	Inhibiting BuChE	176.46 µM	Yoon et al. 2008
6,6'-Bieckol (19)	Phaeophyceae: *Ishige okamurae*	Phlorotannin	Inhibiting AChE	46.42 µM	Yoon et al. 2009
	Phaeophyceae: *Ecklonia cava*		Inhibiting BuChE	27.4 µM	Choi et al. 2015a
2-(3-Hydroxy-5-oxotetrahydrofuran- 3-yl)acetic acid (20)	Rhodophyta: *Gloiopeltis furcata*	NA	Inhibiting AChE and BuChE	1.4 and 12.61 µg mL^{-1}	Fang et al. 2010
Glutaric acid (21)	Rhodophyta: *Gloiopeltis furcata*	NA	Inhibiting AChE and BuChE	5.65 and 41.52 µg mL^{-1}	Fang et al. 2010
Succinic acid (22)	Rhodophyta: *Gloiopeltis furcata*	NA	Inhibiting AChE	5.74 µg mL^{-1}	Fang et al. 2010
Nicotinic acid (23)	Rhodophyta: *Gloiopeltis furcata*	Alkaloid	Inhibiting AChE and BuChE	1.14 and 20.86 µg mL^{-1}	Fang et al. 2010

(E)-4-Hydroxyhex-2-enoic acid (24)	Rhodophyta: *Gloiopeltis furcata*	NA	Inhibiting AChE and BuChE	12.29 and 31.49 µg mL^{-1}	Fang et al. 2010
Cholesterol (25)	Rhodophyta: *Gloiopeltis furcata*	Sterol	Inhibiting AChE	1.15 µg mL^{-1}	Fang et al. 2010
7-Hydroxycholesterol (26)	Rhodophyta: *Gloiopeltis furcata*	Sterol	Inhibiting AChE and BuChE	2.35 and 5.57 µg mL^{-1}	Fang et al. 2010
Uridine (27)	Rhodophyta: *Gloiopeltis furcata*	Nucleoside	Inhibiting AChE and BuChE	1.63 and 35.83 µg mL^{-1}	Fang et al. 2010
Glycerol (28)	Rhodophyta: *Gloiopeltis furcata*	NA	Inhibiting AChE and BuChE	1.61 and 8 µg mL^{-1}	Fang et al. 2010
5-(Hydroxymethyl)-2-methoxybenzene- 1,3-diol (29)	Rhodophyta: *Gloiopeltis furcata*	Phenolic	Inhibiting AChE and BuChE	7.40 and 32.66 µg mL^{-1}	Fang et al. 2010
(Z)-3-Ethylidene-4-methylpyrrolidine-2,5-dione (30)	Rhodophyta: *Gloiopeltis furcata*	Pyrrole/alkaloid	Inhibiting AChE and BuChE	4.17 and 75.25 µg mL^{-1}	Fang et al. 2010
Loliolide (31)	Rhodophyta: *Gloiopeltis furcata*	Degradation product of carotenoids	Inhibiting AChE	7.57 µg mL^{-1}	Fang et al. 2010
Cholesteryl stearate (32)	Rhodophyta: *Gloiopeltis furcata*	Sterol	Inhibiting AChE	6.34 µg mL^{-1}	Fang et al. 2010
Cis-5,8,11,14,17-Eicosapentaenoic acid (33)	Rhodophyta: *Gloiopeltis furcata*	Fatty acid	Inhibiting AChE and BuChE	11.53 and 6.56 µg mL^{-1}	Fang et al. 2010
α-Linolenic acid (34)	Rhodophyta: *Gloiopeltis furcata*	Fatty acid	Inhibiting AChE and BuChE	12.50 and 15.89 µg mL^{-1}	Fang et al. 2010
Dibenzo [1,4] dioxine- 2,4,7,9-tetraol (35)	Phaeophyceae: *Ecklonia maxima*	Phlorotannin	Inhibiting AChE	84.48 µM	Kannan et al. 2013

Table 3: Summary of macroalgae-derived compounds targeting oxidative stress activity

Compound/ number	Macroalgae phyla/class and species	Class of chemistry	Mode of action	IC_{50}	References
Fucoxanthin (36)	Phaeophyceae: *Hizikia fusiformis*	Carotenoids	DPPH radical scavenging activity	NA	Yan et al. 1999
	Phaeophyceae: *Sargassum siliquastrum*		Inhibit hydrogen peroxide in Vero cells	100 µM	Heo et al. 2008
	Phaeophyceae: *Padina tetrastromatica*		Reduce lipid peroxidation in rats	0.83 µM	Sangeetha et al. 2009
Triphlorethol-A (37)	Phaeophyceae: *Ecklonia cava*	Phlorotannin	Scavenging activity against ROS and DPPH via activation of ERK protein	NA	Kang et al. 2005
Three compounds (38-40)	Phaeophyceae: *Sargassum micracanthum*	Plastoquinones	Lipid peroxidation and DPPH assays	0.95, 44.3, and 1.15 µg mL^{-1} for lipid peroxidation and 3, 52.6, and 32.3 µg mL^{-1} for DPPH assay	Mori et al. 2005
HU (41)	Chlorophyta: *Ulva australis*	Sulfated heteropolysaccharide	Scavenging activity for superoxide radicals	20.0 µg mL^{-1}	Qi et al. 2005
HU1 (42)	Chlorophyta: *Ulva australis*	Sulfated heteropolysaccharide	Scavenging activity for superoxide radicals and hydroxyl radicals	5.8 µg mL^{-1} and 1.43 mg mL^{-1}	Qi et al. 2005
HU2 (43)	Chlorophyta: *Ulva australis*	Sulfated heteropolysaccharide	Scavenging activity for superoxide radicals	6.1 µg mL^{-1}	Qi et al. 2005
HU3 (44)	Chlorophyta: *Ulva australis*	Sulfated heteropolysaccharide	Scavenging activity for superoxide radicals and hydroxyl radicals	11.4 µg mL^{-1} and 0.82 mg mL^{-1}	Qi et al. 2005
HU4 (45)	Chlorophyta: *Ulva australis*	Sulfated heteropolysaccharide	Scavenging activity for superoxide radicals and hydroxyl radicals	11.5 µg mL^{-1} and 0.53 mg mL^{-1}	Qi et al. 2005
HU5 (46)	Chlorophyta: *Ulva australis*	Sulfated heteropolysaccharide	Scavenging activity for superoxide radicals and hydroxyl radicals	10.9 µg mL^{-1} and 0.46 mg mL^{-1}	Qi et al. 2005
Fucoidan (47)	Phaeophyceae: *Fucus vesiculosus*	Polysaccharides	Inhibit superoxide radicals, hydroxyl radicals, and lipid peroxidation.	0.058, 0.157, and 1.250 mg mL^{-1}, respectively	de Souza et al. 2007
	Saccharina japonica		Reduce the toxicity of H_2O_2 in PC12 cells via activation of PI3K/Akt pathway	60 µg mL^{-1}	Gao et al. 2012a
Kappa-carrageenan (48)	Rhodophyta: *Eucheuma alvarezii*	Polysaccharides	Inhibit superoxide radicals, hydroxyl radicals, and lipid peroxidation	0.112, 0.335, and 0.323 mg mL^{-1}	de Souza et al. 2007
Iota-carrageenan (49)	Rhodophyta: *Eucheuma denticulatum*	Polysaccharides	Inhibit superoxide radicals, hydroxyl radicals, and lipid peroxidation	0.332, 0.281, and 0.830 mg mL^{-1}, respectively	de Souza et al. 2007
Lambda carrageenans (50)	Rhodophyta: *Chondracanthus acicularis*	Polysaccharides	Inhibit superoxide radicals, hydroxyl radicals, and lipid	0.046, 0.357, and 2.2697 mg mL^{-1},	de Souza et al. 2007

			peroxidation	respectively	
Nineteen meroditerpenoids (51-69)	Phaeophyceae: *Sargassum thunbergii*	Meroditerpenoids	Scavenging activity in DPPH assay	0.10 - 23.23 µg mL^{-1}	Jung et al. 2008
Phloroglucinol (70)	Phaeophyceae: *Ecklonia cava*	Phlorotannins	Scavenging activity against hydroxyl, superoxide, and peroxyl radicals	392.5, 115.2, and 128.9 µM, respectively	Li et al. 2009
	Phaeophyceae: *Eisenia bicyclis*		Decrease ROS from 140% to 100%	800 µM	Ahn et al. 2012
	Phaeophyceae: *Ecklonia cava*		Suppressing the overproduction of intracellular ROS induced by hydrogen peroxide	50 µM	Kang et al. 2012
	Phaeophyceae: *Fucus vesiculosus*		Reduce advanced glycation end products level	0.068 mg mL^{-1}	Liu and Gu 2012
	Phaeophyceae: *Ecklonia cava*		Reduce the toxicity ROS induced by hydrogen peroxide	10 µg mL^{-1}	Kim et al. 2012
Fucodiphloroethol G (71)	Phaeophyceae: *Ecklonia cava*	Phlorotannins	Scavenging activity against DPPH, hydroxyl, superoxide, and peroxyl radicals	14.72, 33.5, 18.6, 18.1 µM, respectively	Li et al. 2009
7- Phloroglucinol eckol (72)	Phaeophyceae: *Ecklonia cava*	Phlorotannins	Scavenging activity against DPPH, hydroxyl, superoxide, and peroxyl radicals	18.64, 39.6, 21.9, 22.7 µM, respectively	Li et al. 2009
Phycoerythrobilin (73)	Rhodophyta: *Porphyra* sp.	Tetrapyrrole	An antioxidant's reducing capacity by in Folin-Ciocalteu reagent (FCR) and peroxy radical scavenging capacity by crocin bleaching assay (CBA)	0.048 mmol g^{-}	Yabuta et al. 2010
(5E,7E)-9-Oxodeca-5,7-dienoic acid (74)	Rhodophyta: *Gloiopeltis furcata*	Fatty acid	Scavenging against peroxynitrite	39.80 µg mL^{-1}	Fang et al. 2010
Dehydrovomifoliol (75)	Rhodophyta: *Gloiopeltis furcata*	Terpene	Scavenging against peroxynitrite	46.75 µg mL^{-1}	Fang et al. 2010
Palmitic acid (76)	Rhodophyta: *Gloiopeltis furcata*	Fatty acid	Scavenging against peroxynitrite	7.30 µg mL^{-1}	Fang et al. 2010
Pheophorbide A (77)	Chlorophyta: *Ulva prolifera*	Pigment	DPPH scavenging activity	71.9 µM	Cho et al. 2011
3-(2,3-Dibromo-4,5-dihydroxybenzyl) pyrrolidine-2,5-dione (78)	Rhodophyta: *Rhodomela confervoides*	Bromophenols	DPPH scavenging activity	5.22 µM	Li et al. 2012
Methyl 4-(2,3-Dibromo-4,5-dihydroxybenzylamino)-4-oxobutanoate (79)	Rhodophyta: *Rhodomela confervoides*	Bromophenols	DPPH scavenging activity	5.70 µM	Li et al. 2012
4-(2,3-Dibromo-4,5-dihydroxybenzylamino)-4-oxobutanoic acid	Rhodophyta: *Rhodomela confervoides*	Bromophenols	DPPH scavenging activity	5.43 µM	Li et al. 2012

(80)					
3-Bromo-5-hydroxy-4-methoxybenzamide (81)	Rhodophyta: *Rhodomela confervoides*	Bromophenols	DPPH scavenging activity	23.60 μM	Li et al. 2012
2-(3-Bromo-5-hydroxy-4-methoxyphenyl)acetamide (82)	Rhodophyta: *Rhodomela confervoides*	Bromophenols	DPPH scavenging activity	20.81 μM	Li et al. 2012
Diphlorethohydroxycarmalol (DPHC) (83)	Phaeophyceae: *Ishige okamurae*	Phlorotannin	Upregulation of cleaved Bcl-xL and caspase-9 and -3-mediated PARP cleavage in HT22 cells	50 μM	Heo et al. 2012
974-B (84)	Phaeophyceae: *Eisenia bicyclis*	Phlorotannin	Scavenging activity of DPPH, peroxynitrite, and total ROS	0.86, 1.80, and 6.45 μM, respectively	Choi et al. 2015b

Table 4: Summary of macroalgae-derived compounds targeting anti-inflammatory activity

Compound/ number	Macroalgae phyla/class and species	Class of chemistry	Mode of action	IC_{50}	References
Caulerpin (85)	Chlorophyta: *Caulerpa racemosa*	Alkaloid	Inhibition of nociception	100 µM kg^{-1} in Swiss albino mice	de Souza et al. 2009
Fucoidan (86)	Phaeophyceae: *Saccharina japonica*	Polysaccharides	Inhibiting microglia which inhibits LPS-induced NO production via suppression of p38 and ERK phosphorylation	125 µg mL^{-1}	Cui et al. 2010
	Phaeophyceae: *Saccharina japonica*		Reducing oxidative stress and tumor necrosis factor-alpha (TNF-a) in animal model	125 µg mL^{-1}	Cui et al. 2012
Floridoside (87)	Rhodophyta: *Chondrophycus undulatus*	Glycerol glycosides	Inhibiting the production of NO and ROS, and blocking the phosphorylation of p38 and ERK in BV-2 cells	10 µM	Kim et al. 2013
Clerosterol (88)	Chlorophyta: *Codium fragile*	Sterol	Exhibit reducing activity to COX-2, iNOS, and TNF- α	3µg mL^{-1}	Lee et al. 2013
Alginic acid (89)	Phaeophyceae: *Sargassum swartzii*	Polysaccharide	Inhibition activities to COX-2, lipoxygenase (5-LOX), xanthine oxidase (XO) and myeloperoxidase (MPO) in type II collagen induced arthritic rats	100 mg kg^{-}	Sarithakumari and Kurup 2013
8,8′-Bieckol (90)	Phaeophyceae: *Ecklonia cava*	Phlorotannin	Reduce COX-2, NO and prostaglandin E2 (PGE2)	100 µM	Yang et al. 2014

Table 5: Summary of macroalgae-derived compounds targeting kinase inhibition

Compound/ number	Macroalgae phyla/class and species	Class of chemistry	Mode of action	IC_{50}	References
Spiralisone A (91)	Phaeophyceae: *Zonaria spiralis*	Phloroglucinol	Kinases inhibitory to CDK5/p25, CK1δ, and GSK3β	10.0, <10, and <10 µM, respectively	Zhang et al. 2012
Spiralisone B (92)	Phaeophyceae: *Zonaria spiralis*	Phloroglucinol	Kinases inhibitory to CDK5/p25, CK1δ, and GSK3β	3, 5, and 5.4 µM, respectively	Zhang et al. 2012
Chromone 6 (93)	Phaeophyceae: *Zonaria spiralis*	Phloroglucinol	Kinases inhibitory to CDK5/p25, CK1δ, and GSK3β	10.0, <10, and <10 µM, respectively	Zhang et al. 2012

Table 6: Summary of macroalgae-derived compounds targeting enhancing neuronal growth activity

Compound/ number	Macroalgae phyla/class and species	Class of chemistry	Mode of action	IC_{50}	References
MC14 (94)	Phaeophyceae: *Sargassum macrocarpum*	Carotenoids	Promote neurite outgrowth activity to 0.4 in PC12 cells	6.25 µg mL^{-1}	Tsang et al. 2001
Sargaquinoic acid (95)	Phaeophyceae: *Sargassum macrocarpum*	Meroterpenoid	Signalling pathway of TrkA-MAP kinase pathway	3 µg mL^{-1}	Kamei and Tsang 2003
Sargachromenol (96)	Phaeophyceae: *Sargassum macrocarpum*	Meroterpene	Promote survival of PC-12 cells and neurite outgrowth through activation of cAMP and MAP kinase pathways	9 µM	Tsang et al. 2005
Pheophytin A (97)	Phaeophyceae: *Sargassum fulvellum*	Pigments	Produce neurite outgrowth (from 20% to 100% in the present of 10 ng mL^{-1} of NGF) and activate MAPK pathway	3.9 µg mL^{-1} in PC12 cells	Ina et al. 2007
Fucoidan (98)	Phaeophyceae: *Fucus vesiculosus*	Polysaccharide	Stimulate cAMP-response elementbinding protein (CREB) and BDNF expressions in brain	50 mg kg^{-1} in male Sprague-Dawley rats	Lee et al. 2012

Table 7: Summary of macroalgae-derived compounds targeting inhibiting MPTP activity

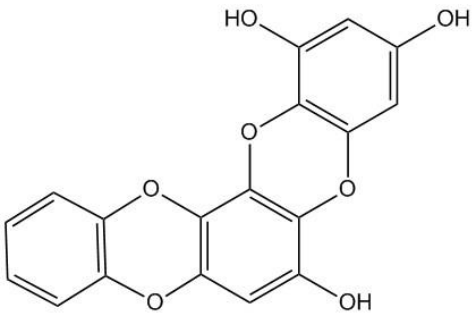
Compound/ number	Macroalgae phyla/class and species	Class of chemistry	Mode of action	IC_{50}	References
Fucoidan (99)	Phaeophyceae: *Saccharina japonica*	Polysaccharide	Protective effect in MPTP-induced neurotoxicity. In addition, reduce behavioural deficits and cell death, and increase dopamine	25 mg kg^{-1}, once per day in mice	Luo et al. 2009
			Reduced 6-hydroxydopamine (6-OHDA) and reduced the loss of dopaminergic in neurons	20 mg kg^{-1} in rats	Zhang et al. 2014

Table 8: Summary of macroalgae-derived compounds that show multiple neuroprotective activities

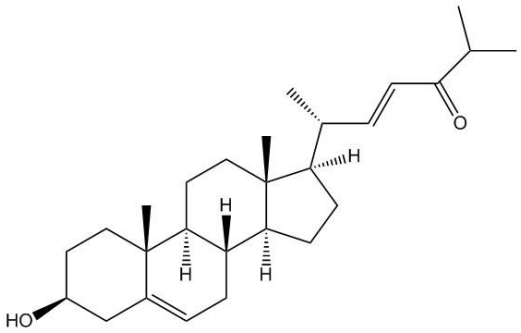
Compound	Activity
Acidic oligosaccharide sugar chain (1)	Inhibiting Aβ, ROS and neuronal growth enhancement
Eckstolonol (11)	Inhibiting ChE and ROS
Eckol (13)	Inhibiting Aβ, ChE, ROS, and GSK-3β
Phlorofucofuroeckol A (14)	Inhibiting Aβ, ChE, ROS, and GSK-3β
Dieckol (15)	Inhibiting Aβ, ChE, ROS, GSK-3β and anti-inflammatory
7-Phloroeckol (16)	Inhibiting Aβ, ROS, and AChE
6,6'-Bieckol (19)	Inhibiting Aβ, ChE, ROS, and GSK-3β
(E)-4-Hydroxyhex-2-enoic acid (24)	Inhibiting ChE and ROS
5-(Hydroxymethyl)-2-methoxybenzene- 1,3-diol (29)	Inhibiting ChE and ROS
Cis-5,8,11,14,17-Eicosapentaenoic acid (33)	Inhibiting ChE and ROS
α-Linolenic acid (34)	Inhibiting ChE and ROS
Fucoxanthin (36)	Inhibiting ROS and anti-inflammatory
Triphlorethol-A (37)	Inhibiting Aβ and ROS
Phloroglucinol (70)	Inhibiting Aβ, AChE, and ROS
(5E,7E)-9-Oxodeca-5,7-dienoic acid (74)	Inhibiting ChE and ROS
Dehydrovomifoliol (75)	Inhibiting ChE and ROS
Palmitic acid (76)	Inhibiting AChE and ROS
Diphlorethohydroxycarmalol (DPHC) (83)	Inhibiting BuChE and ROS
974-B (84)	Inhibiting ChE and ROS
Caulerpin (85)	Inhibiting Aβ and anti-inflammatory
8,8′-Bieckol (90)	Inhibiting Aβ, BuChE, GSK-3β and anti-inflammatory
Sargaquinoic acid (95)	Inhibiting ChE, ROS and neuronal growth enhancement
Sargachromenol (96)	Inhibiting AChE and neuronal growth enhancement

Table 9: Summary of macroalgae-derived compounds that show *in vivo* neuroprotective activity

Compound	Target activity
Fucoidan (2)	Aβ
Fucoxanthin (36)	ROS
Caulerpin (85)	Anti-inflammatory
Fucoidan (86)	Anti-inflammatory
Fucoidan (99)	Inhibiting MPTP activity

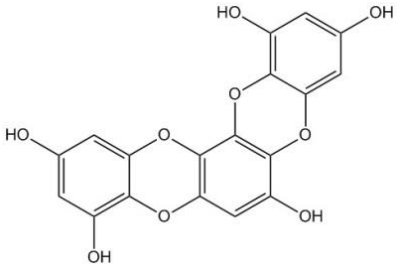


Dioxinodehydroeckol (3)

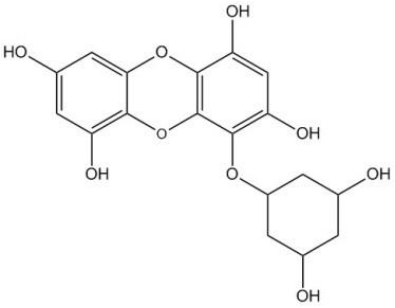


(22E)-3β-hydroxy-cholesta-5,22-dien-24-one (8)

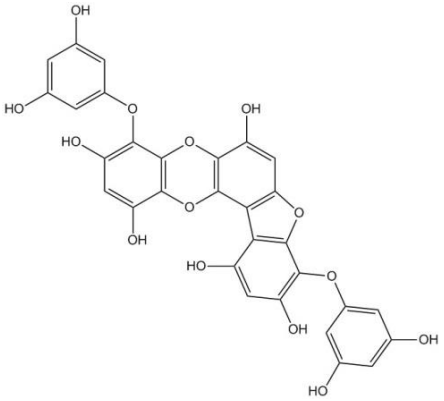
Figure 4: Chemical structures of selected macroalgae-derived compounds with BACE activity



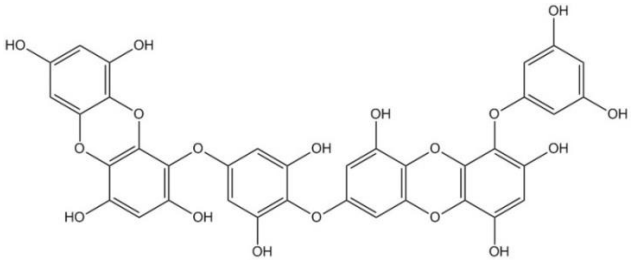
Eckstolonol (11)



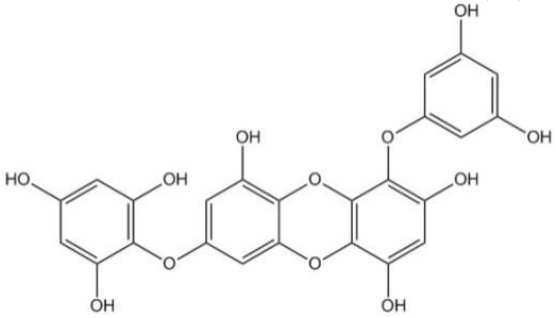
Eckol (13)



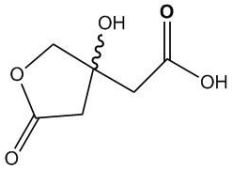
Phlorofucofuroeckol A (14)



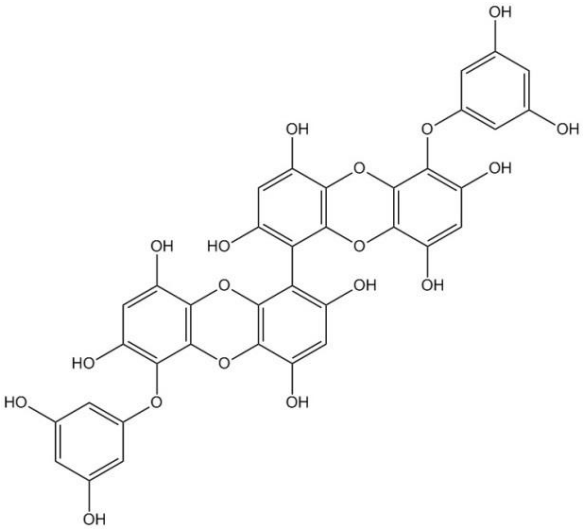
Dieckol (15)



7-phloroeckol (16)

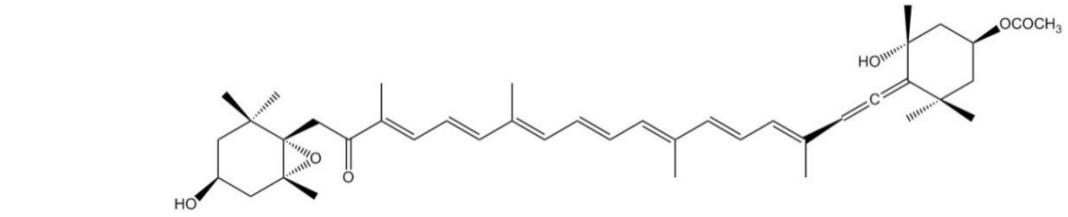


2-(3-Hydroxy-5-oxotetrahydrofuran- 3-yl)acetic acid (20)

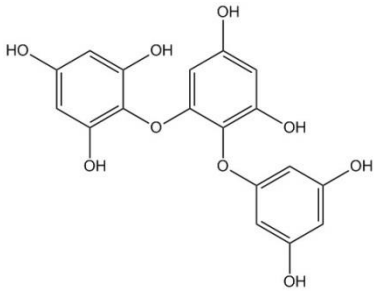


6,6'-Bieckol (19)

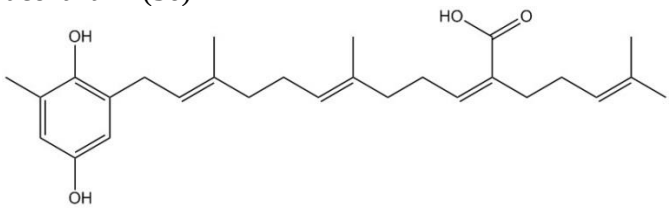
Figure 5: Chemical structures of selected macroalgae-derived compounds with cholinesterase activity



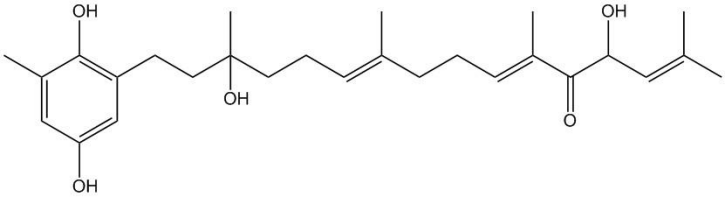
Fucoxanthin (36)



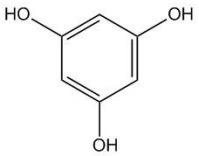
Triphlorethol-A (37)



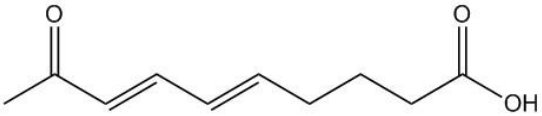
Compound (68)



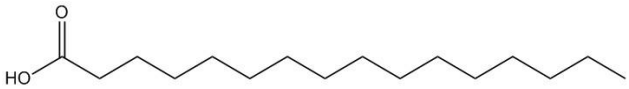
Compound (69)



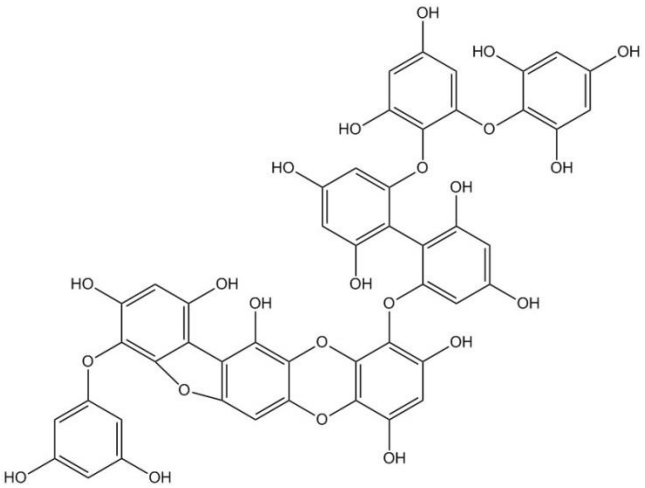
Phloroglucinol (70)



(5E,7E)-9-oxodeca-5,7-dienoic acid (74)

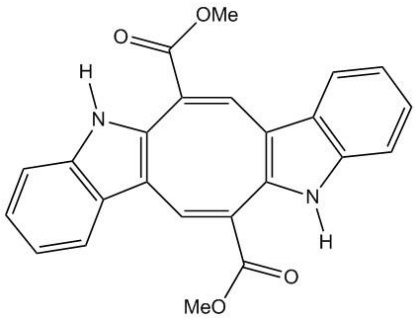


Palmitic acid (76)

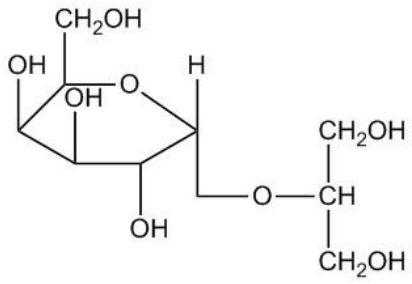


974-B (84)

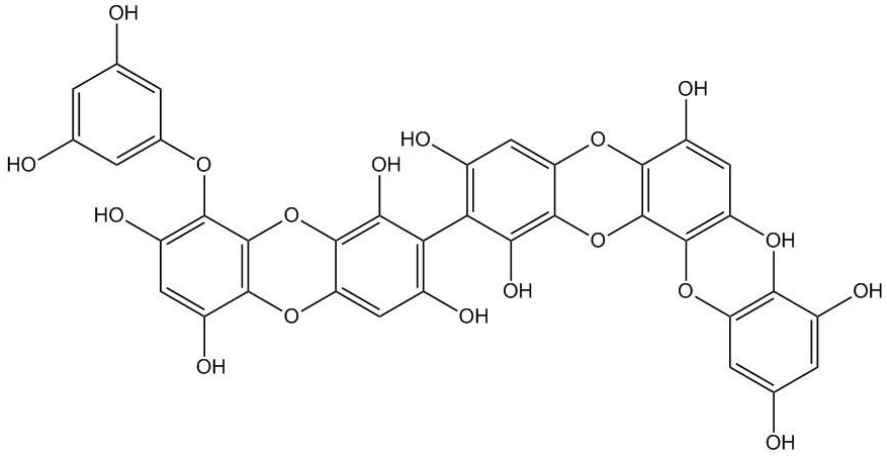
Figure 6: Chemical structures of selected macroalgae-derived compounds with oxidative stress activity



Caulerpin (85)

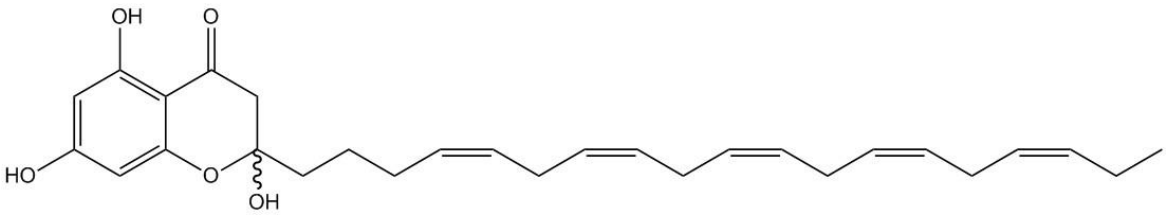


Floridoside (87)



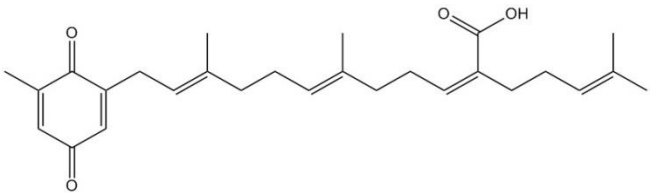
8,8′-Bieckol (90)

Figure 7: Chemical structures of selected macroalgae-derived compounds with anti-inflammatory activity

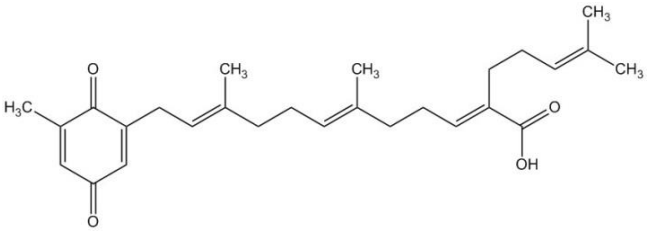


Spiralisone B (92)

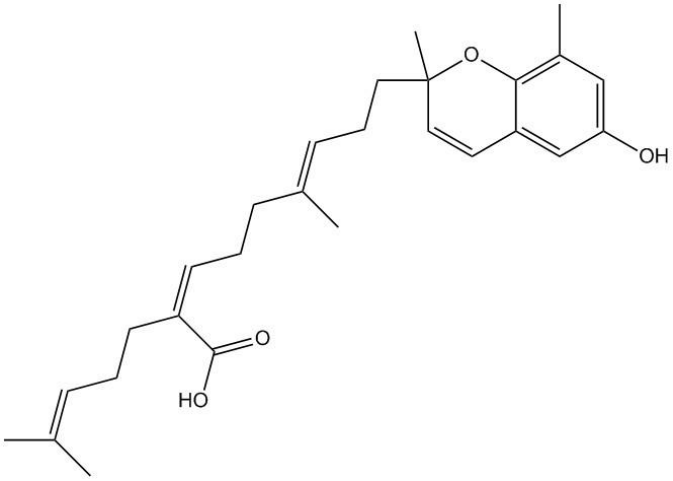
Figure 8: Chemical structure of a selected macroalgae-derived compound with kinases activity



MC14 (94)



Sargaquinoic acid (95)



Sargachromenol (96)

Figure 9: Chemical structures of selected macroalgae-derived compounds with enhancing neuronal growth activity

Appendix 2: Published review 2

Publication Details: Alghazwi, M., Kan, Y.Q., Zhang, W., Gai, W.P.,Yan, X.X. (2016). Neuroprotective Activities of Marine Natural Products from Marine Sponges, Current Medicinal Chemistry, 23, 360-382.

Section of the thesis where the publication is referred to chapter 1

Candidate's Contribution to the publication:

Research Design 75%

Data Collection and analysis 70%

Writing and editing 80%

Outline your (the candidate's) contribution to the publication:

My contribution was collecting most of the data and wrote most part of this paper. Also, make all the table and figures and draw all chemical structures.

× I confirm that the details above are an accurate record of the candidate's contribution to the work.

Name of Co-Author 1: Yen Qi Kan Signed:  Date: 20/01/2018

× I confirm that the details above are an accurate record of the candidate's contribution to the work

Name of Co-Author 2: Wei Zhang Signed:  Date: 18/01/2018

❑ I confirm that the details above are an accurate record of the candidate's contribution to the work

Name of Co-Author 3: Wei Ping Gai Signed: Date:____/____/___

× I confirm that the details above are an accurate record of the candidate's contribution to the work

Name of Co-Author 4: Xiao-Xin Yan Signed:  Date: 19/01/2018

Neuroprotective Activities of Marine Natural Products from Marine Sponges

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Abstract: This review covers the compounds isolated from marine sponges with neuroprotective activities during the period between 1999 and 2014 based on their chemical structures, collections sites, sponge taxonomy and neuroprotective effects. These compounds were isolated from marine sponges collected from 18 countries, most of them in Indonesia, followed by Japan. A total of 90 compounds were reported to exhibit a range of neuroprotective efficacy. These compounds were shown to modulate the synthesis or activity of some neurotransmitters such as acetylcholinesterase and glutamate, enhancement of serotonin, reducing oxidative stress, inhibition of kinases and proteases, and enhancement of neurite growth. None of them have progressed into any marine pharmaceutical development pipeline, sustained researches will be required to enhance the potential of utilizing these compounds in the future prevention and therapeutic treatment of neurodegenerative diseases.

Keywords: Acetylcholinesterase, Alzheimer's disease, amyloid beta, marine sponge, natural products, nerve growth factor, neurodegenerative diseases, neuroprotection, oxidative stress, tau protein

1. Introduction

Neurodegenerative diseases have become increasingly common in the last decades with the total number of patients expected to increase to more than 115 million by 2050 [1]. Neurodegenerative diseases affect cells of the central and peripheral nervous systems resulting in neuronal death [2]. Among the neurodegenerative diseases, Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) are most common. These diseases share similar features including abnormal protein aggregation [3], however their origin and mechanism are far from well-understood. With the aging society coming in the developed countries and very soon the developing countries, the health care costs for the prevention and treatment of neurodegenerative diseases will be skyrocketing.

Marine sponges (Porifera) are evolutionarily oldest multicellular sessile animals with a high biodiversity across vastly different marine environments [4-6]. In addition, marine sponges have been demonstrated to contribute over 30% of all marine natural products discovered by far, being the single richest source of potential marine drugs [7]. Many compounds with vastly different biological activities have been isolated from sponges. These activities include antibacterial, antifungal, antimalarial, antiprotozoal, antituberculosis, antiviral, anti-inflammatory, targeting immune system, and targeting nervous system [8].Over the past decades, the neuroprotective activity of natural compounds from marine sponges have been shown to protect the neuron cells from neuronal dysfunction, degeneration, injury and apoptosis [9], with the activities manifested at neurophysical, neurochemical, pharmacological and molecular levels.

In this review, the marine sponge-derived compounds during 1999-2014 with preventive and therapeutic potentials for neurodegenerative diseases have been comprehensively reviewed. A total of 90 compounds are discussed with regard to their origins of collection, taxonomic distribution, chemical features, neuroprotection activities, structure-activity relationship (SAR), and their potential on prevention and treatment of neurodegenerative diseases.

2. Trend of neuroprotective compounds discovery and overview from sponges

There have been several excellent reviews on bioactive compounds isolated from marine organisms. Most of these reviews focus on different bioactivities for different diseases such as anticancer, antibacterial, antifungal, anti-inflammatory, antiviral activities [8, 10-15].

There have been very few previous reviews covered the neuropharmacological application of marine natural products in neurodegenerative diseases. Bourguet-Kondracki and Kornprobst (2005) reviewed marine compounds for the treatment of infectious diseases, Osteoporosis and Alzheimer's disease, with only one compound reported from marine worm *Amphiporus lactifloreus*, and five synthesized derivatives having potentials in treating Alzheimer's disease [16].

Another review in 2005 focused on patented marine compounds for the treatment of neurological diseases from the period of 1998 to 2004, which reported 31 compounds with only two compounds from sponges [17]. Three recent reviews in 2014 on neuroactive compounds from marine sources [18-20] reported 247 compounds with about 48 compounds from sponges [18];184 compounds with 35 compounds from sponges [19]; and 104 compounds with 42 compounds from sponges [20]. Despite sponges are the richest sources of marine natural products, there is no comprehensive review on marine sponge-derived compounds targeting neurodegenerative disease.

With a total number of 90 compounds covered in this review, Figure 1 illustrates the yearly distribution of marine sponge-derived compounds with neuroprotective activities reported from 1999 to 2014. Only five compounds are reported between 1999 and 2001, and a significant increase from 2003 with a peak of 11 compounds reported in 2006.

Figure 2 shows the distribution of these compounds according to the collection sites. These compounds have been isolated from sponges collected in 18 sites, with the highest number of 18 compounds discovered from sponges of Indonesia, followed by Japan, Corsica and Australia with 15, 7 and 7 compounds, respectively. There are 9 compounds of which publications did not give any information on the collection sites. This trend of discovery across collection sites is similar to the overall trend of any bioactive compounds from marine sponges [7], reflecting the R&D activities across these different countries.

These marine sponge-derived compounds can be classified into seven categories according to their reported biological activities (Figure 3). These activities include: enhancement of neurotrophic activities (19 compounds), inhibition of oxidative stress (17 compounds), inhibition of the beta-site APP cleaving enzyme (BACE) (16 compounds), inhibition of acetylcholinesterase (15 compounds), enhancement of serotonin neurotransmission (4 compounds), inhibition of glutamate receptor (10 compounds), and inhibition of kinases (9 compounds). These sponge-derived compounds did show a high diversity of neuroprotective activities, demonstrating high potential for further development. However the reports on multiple neuroprotective activities of one compound are rare, calling for more future studies in this area.

It is more important to understand the taxonomic contribution of these neuroprotective compounds from sponges as this knowledge will guide further search of valuable compounds. Over the 90 compounds reported, 85 compounds (94.4%) have been isolated from the Demospongiae Class, two compounds from the Calcarea Class and one compound from the Homoscleromorpha Class.

At sponge order level, these compounds have been isolated from eleven different orders, with 35, 15 and 11

compounds from the Orders of Haplosclerida Dictyoceratida and Halichondrida, respectively. The Order Haplosclerida has produced active compounds in six categories of activities except enhancement of serotonin receptor. Compounds attenuating oxidative stress are from highest diversity of sponges of seven orders, while compounds presenting enhancement of serotonin activity are from sponges of only two orders (Figure 4).

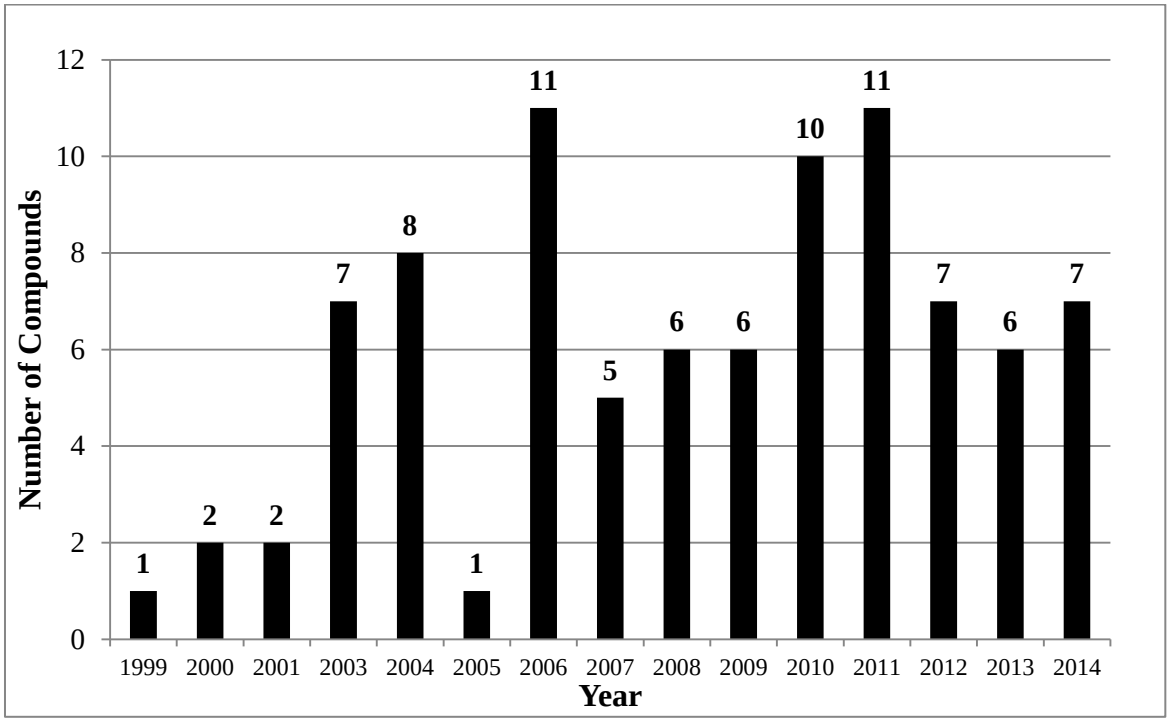

Figure 1: The yearly distribution of marine sponge-derived-compounds reported with neuroprotective activities from 1999-2014. The data were collected by searching ScienceDirect, PubMed, and Scopus

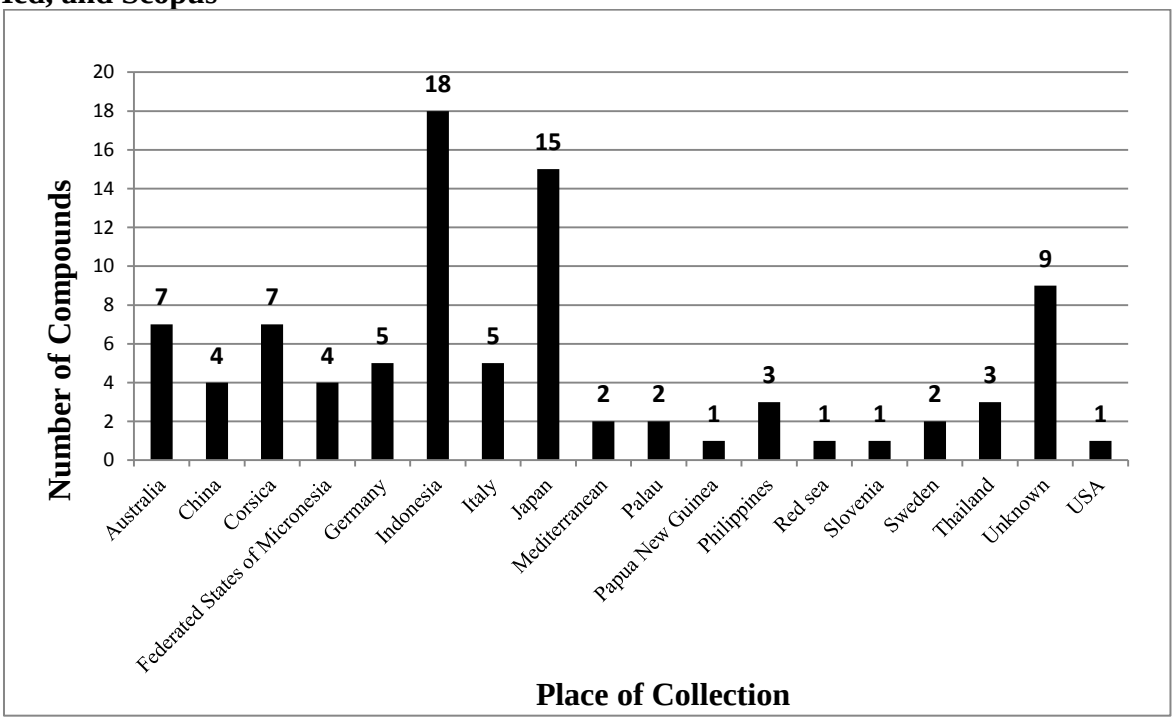

Figure 2: Distribution of the neuroprotective compounds based on collection sites of marine sponges from 1999-2014. The data were collected by searching ScienceDirect, PubMedicine, and Scopus

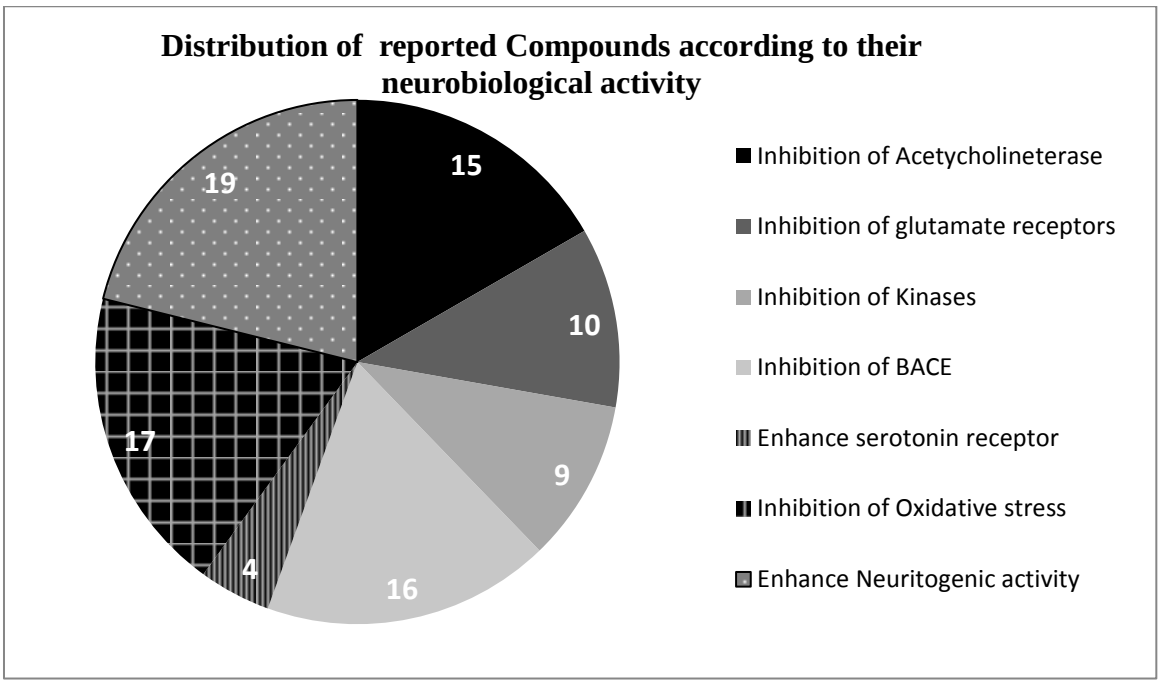

Figure 3: Distribution of marine sponge-derived compounds with seven different neuroprotective activities from 1999-2014. The data were collected by searching ScienceDirect, PubMedicine, and Scopus

At the sponge family level, Figure 5 shows that all these 90 compounds are isolated from 20 families of sponges. The highest number of compounds (26) is derived from sponges of Petrosiidae family, followed by 8 compounds from the family of Axinellidae. The rest of the sponge family produced from 1 to 7 compounds.

3. Neuroprotective Activities of Marine Sponge -derived Compounds

A summary of marine sponge-derived neuroprotective compounds with seven different activities is presented in Table 1.

3.1. Compounds targeting neurotransmitters

3.1.1. Inhibition of Acetylcholinesterase (AChE)

Acetylcholinesterase (AChE) is an enzyme that hydrolyses the neurotransmitter acetylcholine. AChE is distributed widely in conductive tissues especially in the nervous system. Its function in neurons is to terminate the signal transmission between nerve cells by degrading acetylcholine to prevent it from interacting with their receptors on post-synaptic membranes. The concentration of acetylcholine in synaptic cleft is highly regulated. When there is excess acetylcholine, it will be hydrolyzed by AChE. On the other hand, acetylcholine concentration can be increased by inhibiting the AChE in synaptic cleft [21]. However, if irreversible inhibition of AChE occurs, the excess acetylcholine may cause muscular paralysis and convulsion. Deficiency of acetylcholine in basal forebrain is one of the major characteristics in Alzheimer's disease [22].

Sixteen compounds are isolated from five sponge Orders (Agelasida, Haplosclerida, Homosclerophorida, Poecilosclerida, and Thorectidae) and six sponge families with AChE inhibition activities with different IC_{50} (Table 1). The majority (12 compounds) are isolated from Haplosclerida order. The chemical classes of these compounds include 10 alkaloids, 2 brominated and 2 sterols, 1 pyridines and 1 quaternary amine.

One of the first compounds with AChE inhibition activity, 3-alkylpyridium polymers (poly-APS) (1) was isolated in 1998 from the water soluble extract of marine sponge *Reniera sarai*, collected from North Adriatic Sea of Slovenia [23]. It has been shown to inhibit AChE activity with the half maximal inhibitory concentration (IC_{50}) of 0.57 μM, and led to irreversible inhibition of AChE [24].

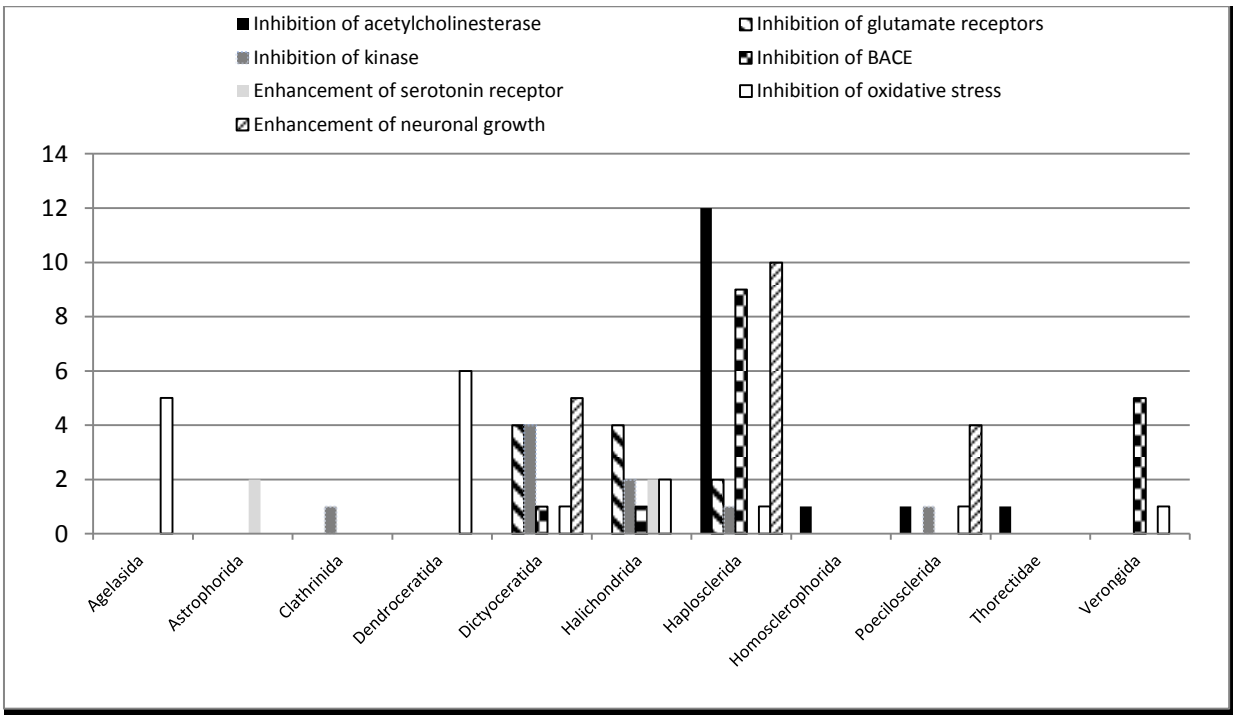

Figure 4: Distribution of compounds with neuroprotective activities isolated from marine sponge from 1999-2014 within different sponges' orders

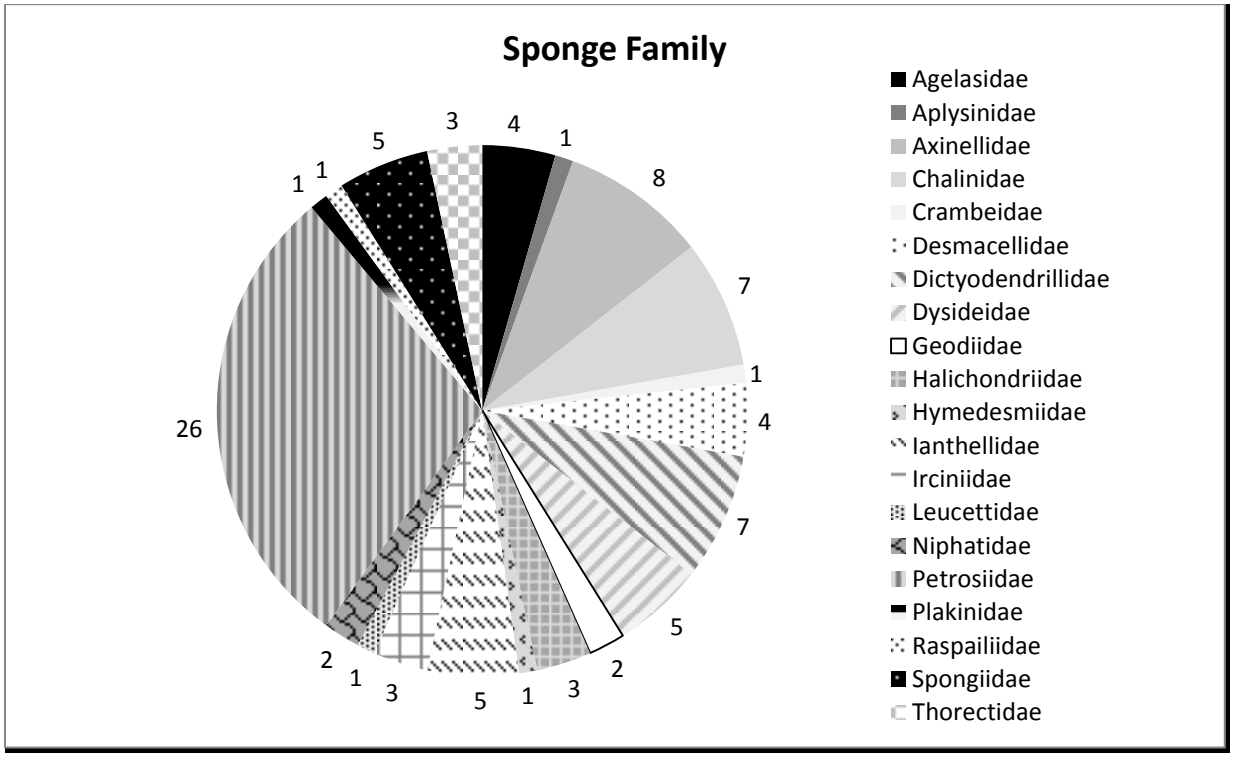

Figure 5: Distribution of compounds with neuroprotective activities isolated from marine sponge from 1999-2014 within different sponges' families

Petrosamine (4) isolated from a Thai sponge *Petrosia* n. sp. demonstrated the highest AChE inhibition activity with IC_{50} of 0.091 µM, more potent than the positive control galanthamine (IC_{50} of 0.590 µM) [25].

With regard to the AChE inhibition assay, several different assays have been used in all these tests. Most of these compounds are tested using the AChE inhibition assay called Ellman's colorimetric method which is

based on hydrolyzing acetylcholine to form thiocholine which in turn react with the Ellman reagent (5,5′-dithio-bis(2-nitrobenzoic acid or DTNB) to form a yellow product [26].

Thin-layer chromatography (TLC) assay in the combination of microplate assay is also used [25, 27], in addition to kinetic studies of various inhibitors by using molecular docking method [25]. The commonly used positive control compounds include galanthamine and tacrine. The IC_{50} of galanthamine and tacrine is 0.59 µM [25] and 0.41 µM [28]. Several AChE inhibitors including donepezil, galanthamine, and rivastigmine have been used clinically in patients with mild to moderate Alzheimer's disease and showed some benefit in symptomatic relief [29]. These drugs have high IC_{50} in AChE inhibition assay of 4.3 and 6.7 nM for rivastigmine and donepezil, respectively [30]. In comparison with these clinically used drugs, sponge-derived compounds with low IC_{50} *in vitro* could be promising candidates for further development as AChE inhibitors for clinical use.

3.1.2. Inhibition of glutamate receptors

Glutamate receptors are the synaptic receptors for the excitatory amino acid neurotransmitter glutamate in mammalian central nervous system (CNS) to mediate the glutamate neurotransmission between neurons [31]. Glutamate receptors play important roles in the formation of memory, learning and other neuronal functions. Glutamate receptors are categorized in two major types, metabotropic and ionotropic receptors. Metabotropic receptors are activated by the calcium ion (Ca^{2+}) through GTP-binding protein-dependent mechanisms. Ionotropic receptors are activated and this leads to the opening of sodium, potassium or calcium ion channels [32]. There are three types of ionotropic receptors commonly recognized as NMDA (N-methyl-D-aspartate) receptor, and non-NMDA classes, AMPAR (2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) proprionate receptor and kainite receptor subtypes [33]. Treatment therapy for AD targeting glutamate receptor is aimed to prevent memory loss by blocking glutamate receptor [34].

Ten compounds isolated from three sponge orders (Dictyoceratida, Haplosclerida, and Halichondrida) and three families (Axinellidae, Dysideidae, and Niphatidae) exhibit inhibitory activity of glutamate receptors (Table 1). There are four, four, and two compounds isolated from the families of Axinellidae, Dysideidae, and Niphatidae, respectively. The chemical classes of these compounds include 4 amino acids, 4 bromopyrrole alkaloids, and 2 betaines.

One of the first compounds with kainic acid and mGluR5 glutamate receptor inhibition activity (IC_{50} at 66 nM), dysiherbaine (DH) (16) was isolated from *Dysidea herbacea* [35]. Another compound, cribronic acid (2S,4R,5R)-5-hydroxy-4-sulfooxypiperidine-2-carboxylic acid) (18) was isolated from the water-soluble extract of *Cribrochalina olemda*, which was found to bind NMDA receptors with IC_{50} of 83 nM [36].

These compounds have been primarily screened by radioligand binding assay in electrophysiology study in order to study the specific receptors of these compounds such as AMPA, NDMA, or kainic acid. The animal behavior responses of these compounds are also investigated [35], with several studies using MTT assay to calculate IC_{50} of the compounds tested [37].
Memantine is an inhibitor targeting NMDA and has been approved to use for moderate to severe cases of Alzheimer's disease [29]. It protects cortical neurons and cerebellar granule cells from apoptosis induced by S-Nitrosoglutathione, and from oxygen-glucose deprivation with an effective dose dependent range of 1, 10, and 50 µM [38].

The compounds from sponges are more potent in glutamate receptor inhibition than memantine in *in vitro* assay, therefore warrant further investigation.

Table 1: Summary of compounds with neuroprotective activities isolated from marine sponges from year 1999-2014

Neuroprotective activity	Compound/ Extracts Identified	Sponge Species	Collection Site	Chemical Class	Mode of Action (MOA)	IC_{50}	References
Inhibition of acetylcholinesterase	3-Alkyl-pyridinium (1)	*Reniera Sarai*	Slovenia	Alkaloid	Irreversible inhibition of the enzyme	0.57 µM	[24]
	Esmodil (2)	*Raspailia*	Australia	Quaternary amine	NA	NA	[39]
	4-Acetoxy-plakinamine B (3)	*Corticium*	Thailand	Stigmastane-type steroidal alkaloid	Mixed-competitive inhibition	3.75 µM	[21]
	Two compounds: Petrosamine and 2-bromoamphimedine (4-5)	*Petrosia* n. sp.	Thailand	Alkaloid	NA	0.091 and >300 µM	[25]
	Four compounds: Mutafuran H, xestospongic acid, 24-hydroperoxy-24-vinylcholesterol and 29-hydroperoxystigmasta-5,24(28)-dien-3 (6-9)	*Xestospongia testudinaria*	China	Brominated and sterols	NA	0.64, 12.65, 11.45, and 14.51µM, respectively	[28]

Five compounds: saraines A–C, protonated saraines A–C, saraine 1, saraine 3, and saraine C (10-14)	*Reniera* (*Haliclona*) *sarai*	Italy	Saraines - diamine alkaloids	Competitive inhibition	5.7, 7.7, 6.4, 6.3, and 8.4 µM, respectively	[40]
Fascaplysin (15)	*Fascaplysinopsis Bergquist* sp.	NA	Alkaloid	Non-competitive	1.49 µM	[41]
Two methanol extracts and one compound oroidin	*Ircinia spinulosa*, *Ircinia fasciculate*, and *Agelas oroides*	Mediterranean Sea- Turkey	Two extracts, and one alkaloid	NA	50 µg/ml for both extracts, and 200 µg/ml for oroidin	[42]
Two ethyl acetate extracts	*Pericharax heteroraphis* and *Amphimedon navalis*	Mauritius	Extract	Competitive/non-competitive inhibition	0.018 and 0.016 mg/ml	[27]
Two methanol extracts	*Latrunculia cf. lendenfeldi and Latrunculia cf. bocagei*	Antarctic	Extract	NA	1.3 and 9 ng/ml	[43]

Neuroprotective activity	Compound Identified	Sponge Species	Collection Site	Chemical Class	Mode of Action (MOA)	IC_{50}	References
Inhibition of glutamate receptors	Dysiherbaine (DH) (16)	*Dysidea herbacea*	Federated States of Micronesia	Amino acid	Non-NMDA agonist	66 nM *in vivo*	[35]
	Neodysiherbaine A (17)	*Dysidea herbacea*	Federated States of Micronesia	Amino acid	Inhibition of Kainic acid glutamate receptors and AMPA	227 nM	[44]
	Two compounds: Cribronic acid (2S,4R,5R)-5-hydroxy- 4-sulfooxypiperidine-2-carboxylic acid and (2S,4S)-4-sulfooxypiperidine-2-carboxylic acid (18-19)	*Cribrochalina olemda*	Palau	Amino acid	Binding to NMDA-type glutamate receptor	83and 214 nM *in vivo*, respectively	[36]
	Two compounds: Dysibetaine CPa and dysibetaine CPb (20-21)	*Dysidea herbacea*	Federated States of Micronesia	Betaine	Inhibition of kainic acid receptor and CGP39653 receptor which is a type of NDMA	CPa inhibits both kainic acid receptor and CGP39653 with13 and 10 µM, CPb inhibits Kainic acid with 4.9 µM	[45]
	Four Bromopyrrole alkaloids (22-25)	*Axinella verrucosa*	Bay of Calvi (Corsica)	Bromopyrrole alkaloid	NA	NA	[37]

Neuroprotective activity	Compound Identified	Sponge Species	Collection Site	Chemical Class	Mode of Action (MOA)	IC_{50}	References
Enhancement of serotonin receptor	Two compounds: Barettin and 8,9-dihydrobarettin (26-27)	*Geodia barretti*	Sweden	Brominated cyclodipeptides	Binding to serotonin 5-HT_{2C}	Barettin: 1.93, 0.34, and 1.91 µM for 5-HT_{2A}, 5-HT_2c, and 5-HT_4, 8,9-dihydrobarettin: 4.63 µM for HT_2c	[46]
	Four Bromopyrrole alkaloids	*Axinella verrucosa*	Bay of Calvi (Corsica)	Bromopyrrole alkaloid	NA	NA	[37]
	Two compounds: Damipipecolin and damituricin (28-29)	*Axinella damicornis*	Bay of Calvi (Corsica)	Alkaloid	Ca^{2+} influx inhibition	10 µg/ml	[47]

Neuroprotective activity	Compound Identified	Sponge Species	Collection Site	Chemical Class	Mode of Action (MOA)	IC_{50}	References
Inhibition of oxidative stress	l-5-hydroxytryptophan (l-5-HTP) (30)	*Hymeniacidon heliophila*	USA	Amino acid	Suppresses UV induced apoptosis in human monocytes at the same concentrations as it occurs in the sponge tissue	NA	[48]
	Five compounds: Dibromosceptrin, sceptrin, oroidin, hymenidin and 4,5-dibromopyrrole-2-carboxylic acid (31-35)	*Agela swiedenmayeri, A. conifer* and *A. sventres*	Germany	Pyrrole alkaloids	Reduced Ca2+ level by binding with NMDA receptor	More than 40 mg/mL	[49]
	Daminin (36)	*Axinella damicornis*	Corsica	Pyrrole alkaloids	Reduced Ca2+ level via NMDA receptor	More than 40 µg/mL	[50]
	Crambescidin 800 (37)	*Monanchora ungiculata*	Indonesia	Pentacyclic guanidine alkaloid	Protect HT22 cells against glutamate induced toxicity	0.06 µM	[51]
	Dysideamine (38)	*Dysidea* sp.	Indonesia	Aesquiterpene aminoquinone	Inhibited production of reactive oxygen species (ROS) that trigger MAPK pathway, and protect HT22 cells against iodoacetic acid	0.06 µM	[52]

NP04634 (39)	*Aplysina cavernicola*	Mediterranean	Amides	Inhibit the toxic Ca^{2+} via VDCC pathway	NA	[53]
Strongylophorine-8 (40)	*Petrosia Strongylophoracorticata*	Papua New Guinea	Para-hydroquinone-type pro-electrophilic compound (terpenes)	Increase glutathione And activate Nrf2/ARE pathway to prevent oxidative stress	4.1 µM	[54]
Six compounds: Gracilin A, gracilin H, gracilin J, gracilin K, gracilin L, and tetrahydroaplysulphurin-1 (41-46)	*Spongionella* sp.	NA	Diterpenes	Protect mitochondria against oxidative stress through Nrf2/ARE pathway	0.1 µM	[55]

Neuroprotective activity	Compound Identified	Sponge Species	Collection Site	Chemical Class	Mode of Action (MOA)	IC_{50}	References
Inhibition of kinase	Hymenialdisine (47)	*Halichondriidae*	NA	alkaloid	Inhibition of cyclin-dependent kinase, GSK-3β and CK1 by competing with ATP for binding, and blocking the *in vivo* phosphorylation of tau	10 and 35nM, respectively (vary on different kinases tested)	[56]
	Manzamine A (48)	*Acanthostrongylophora*	Indonesia	Alkaloid	Non-competitive inhibitor of ATP binding against GSK-3β, and CDK5	10.2 and 1.5μM, respectively	[57]
	Phenylmethylene hydantoin (49)	*Hemimycale arabica*	Red sea	Imidazolidine	GSK-3β inhibitor	4.2 μM	[58]
	Three compounds: Carteriosulfonic acids A, B, and C (50-52)	*Carteriospongia* sp.	Philippines	Acids	GSK-3β inhibitor	2.5, 6.8, and 6.8 μM, respectively	[59]
	Leucettamine B (53)	*Leucetta microraphis*	Japan	Alkaloid	Potent inhibitor of CLKs and DYRKs, two families of kinases involved in alternative pre-mRNA splicing and Alzheimer's disease	0.4 and 2.8μM	[60]
	Leucettamine B	*Leucetta microraphis*	NA	Alkaloid	Inhibit different kinases such as CDK1, CDK2, CDK5, CDK7, CDK9, and GSK-3β	>10 μM	[61]

	Debromohymenialdisine (54)	*Axinella* sp.	Australia	Alkaloid	Inhibits CDK5/p25, CK1, and GSK-3β	0.4, 0.1, and 0.2 µM, respectively	[62]
	Isopropanolic extracts --> Palinurin (55)	*Ircinia dendroides*	Mediterranean	Terpene	GSK-3β inhibitor by binding to an allosteric site located at the N-terminal lobe	50 mg/ml. 1.9 and 1.6 µM for GSK-3β and GSK-3α	[63]

Neuroprotective activity	Compound Identified	Sponge Species	Collection Site	Chemical Class	Mode of Action (MOA)	IC_{50}	References
Inhibition of BACE	Xestosaprol D (56)	*Xestospongia* sp.	Indonesia	Pentacyclic quinone	NA	30 µg/ml	[64]
	Eight compounds: Xestosaprols F, G, H, I, J, K, L, and M (57-64)	*Xestospongia* sp.	Indonesia	Pentacyclic quinone	NA	135, 155, 82, 163, 90, 93, 98, and 104 µM, respectively	[65]
	Topsentinol K trisulfate (65)	*Topsentia*	Indonesia	Steroid	Nonspecific inhibitor	1.2 µM	[66]
	Four compounds: Ianthellidones O1, Ianthellidones F, O2, and O (66-69)	*Ianthella* sp.	Australia	Alkaloid	NA	< 10 µM for Ianthellidones O1, and >10 µM for the rest	[67]
	Dictyodendrin J (70)	*Ianthella* sp.	Australia	Alkaloid	NA	2 µM	[68]
	Prenylated hydroxybenzoic acid 1 (71)	*Sarcotragus* sp.	NA	Phenyl derivatives	Decrease Aβ production	1 µM	[69]

Neuroprotective activity	Compound Identified	Sponge Species	Collection Site	Chemical Class	Mode of Action (MOA)	Effective Concentration	References
Enhancement of neuronal growth	Lembehyne A (72)	*Haliclona* sp.	Indonesia	Polyacetylene	NA	2 µg/mg in PC12 cell line, and 0.1 µg/mg in Neuro 2A cell	[70]
	Labuanine A and three pyridoacridine alkaloids (73-76)	*Biemna fortis*	Indonesia	Pyridoacridine alkaloids	Induce more than 70% of neuritogenesis	1, 3, 0.03, and 1µM, respectively	[71]
	Five sestertepenes (77-81)	*Spongia* sp.	Japan	Sesterterpene	Induce more than 50% of neuritogenesis	50 µg/ml	[72]
	Four compounds: Manzamine A, 8-hydroxymanzamine (82), manzamine E (83), manzamine F (84) and manzamine X (85)	*Acanthostrongylophora aff. ingens*	Japan	Alkaloid (Manzamines)	Treating Neuro 2A with these compounds can enhance the growth rate after 48 hours and 72 hours at 1 µM concentration	IC_{50} for Neuro 2a: 3.3, 3.2, 5.7, >15, and >15 µM IC_{50} for HL-60: 4.2, 3.0, 8.9, >10, and >10 µM	[73]
	Five compounds: Petrosiols A-E (86-90)	*Petrosia strongylata*	Japan	Acetylenic alcohol	Induce more than 30% of neuritogenesis	2 µM	[74]

3.1.3. Up-regulation of serotonin

Serotonin (5-hydroxytryptamine; 5-HT) is an important neuromodulator in neurotransmission, which has multiple functions in controlling mood, sleeping, memory and learning [75-77]. Dysfunction or depletion of serotonin can occur in different neurological conditions such as Alzheimer's disease (AD, Parkinson's disease (PD), sleep disorders and depression [78]. Targeting serotonin system has been considered as one of the treatment strategies in neurodegeneration diseases [75, 76].

Eight compounds have been isolated from Demospongiae Class of two sponge orders (Halichondrida and Astrophorida) and two families (Axinellidae and Geodiidae) with enhancing activity of serotonin receptor (Table 1). Six compounds are of alkaloids isolated from Axinellidae, and two compounds are of brominated cyclodipeptides isolated from Geodiidae. Two brominated cyclodipeptides called barettin (cyclo[(6-bromo-8-entryptophan)arginine]) (26) and 8,9-dihydrobarettin (cyclo[(6- bromotryptophan)arginine]) (27), are invistgated for their ineractions with serotonin recepors on HEK-293 cells after transfecting the cells with human serotonin recptors. Barettin has been found to interact with serotonin receptors 5-HT_{2A}, 5-$HT_{2c,}$ and 5-HT_4 with IC_{50} of 1.93, 0.34, and 1.91 µM, respectivley. Wheras 8,9-dihydrobarettin only interact with serotonin recport 5-HT_{2c} with IC_{50} of 4.63 µM [46]. Two other interestingly compounds are damipipecolin (28) and damituricin (29) isolated from *Axinella damicornis*, which act on serotonin receptors and maybe useful candidate for developing drugs for the treatment of depression [47].

For screening compounds acting on serotonin system, human serotonin receptors are transfected in HEK-293 cell line, followed by radioligand binding assay [46]. Some studies measure the calcium levels in these transfected cells after treating these cells with the compounds and serotonin [37, 47]. MTT assay is used in some studies to detect the IC_{50} of these compounds using primary neuron cells and PC-12 cells or PC-12, L5178y and HeLa cells [37, 47].

3.2. Inhibition of Oxidative stress

Oxidative stress results in excess reactive oxygen species (ROS) and causes damage to cells and especially neurons. Oxidative stress increases in ageing and is involved in many ageing related diseases [79].

Increased oxidative stress in AD may potentiate protein oxidation, lipid peroxidation, DNA oxidation and free radical formation [79, 80]. An example of lipid peroxidation is the lipoprotein in which free radicals attack and remove hydrogen atom from lipid [79, 81]. Oxidative stress has been shown to increase the secretion of amyloid-β peptides from primary chick brain neurons [82]. Oxidative stress can also increase the formation of amyloid beta via activation of β and γ secretases, while reducing α secretase activity [83, 84]. Protein oxidation occurs when a large numbers of oxidized proteins such as low density lipoprotein (LDL) accumulate in the brain, and contributes to neurodegeneration including AD [79, 85]. DNA oxidation is one of the major contributing factors in AD as reactive oxygen species attack DNA and resulted in DNA damage [86].

Calcium is an important element for regulation in the brain and plays a role in gene expression [87, 88]. Calcium can be found at a higher level in AD, while the level of calcium binding protein is found to be lower [88]. Increased level of calcium can result in neurons dysfunction, and this has been recognized as one of the earliest cellular changes in AD [87]. Oxidative stress plays an important role in disease progression in AD and many other neurodegenerative diseases; and targeting oxidative stress should be integral part of treatment for these diseases [79]. As all treatments available for Alzheimer's disease are to reduce the symptoms, the needs to find a cure for this disease become important. Oxidative stress is an early sign of this disease which means that it will provide a good target therapy [89].

Seventeen compounds with anti-oxidative stress activities have been isolated from Demospongiae Class of seven sponge orders (Agelasida, Dendroceratida, Dictyoceratida, Halichondrida, Haplosclerida, Poecilosclerida, and Verongida) and eight families (Agelasidae, Aplysinidae, Axinellidae, Crambeidae, Dictyodendrillidae, Dysideidae, Halichondriidae, and Petrosiidae) (Table 1). These two orders of Dendroceratida and Agelasida have produced 11 compounds. These compounds belong to a wide range of chemical classes, including alkaloids, amino acids, aminoquinoline, and Terpenes.

There are several highly active compounds reported to be protective of neurons against various oxidative stresses at concentration below 0.1 µM [51, 52, 55]. Crambescidin 800 (37), isolated from *Monanchora ungiculata* in Indonesia, can protect the neuron cells by reducing glutamate-induced and NO-induced oxidative stress toxicities at 0.1 µM and 0.01 – 0.1 µM, respectively [51]. Six compounds (Gracilin A, Gracilin H, Gracilin J, Gracilin K, Gracilin L, and Tetrahydroaplysulphurin-1) (41-46) isolated from *Spongionella* sp. have been tested with cultured cortical neurons and evaluated for their neuroprotective activity against H_2O_2 using LDH assay. Two compounds (Gracilin H and Tetrahydroaplysulphurin-1) have showed neuroprotective activity at a concentration of 0.1 µM. Moreover, all compounds have showed to inhibit ROS production when tested at 0.1 µM concentration in the non-enzymatic antioxidant glutathione (GSH) [55]. These active compounds warrant further investigation for their *in vivo* neuroprotective activities, and may represent a rich source of marine compounds with anti-oxidative stress property useful for neurodegenerative conditions.

3.3. Inhibition of Kinases

Tau is a microtubule-associated protein highly expressed in axons and important in regulation of microtubules by stabilizing it. Hyperphosphorylation of tau decreases the affinity of tau binding to microtubule and leads to microtubule destabilization in AD [90]. Hyperphosphorylated tau has been shown to be a major element in paired helical filamentous (PHF) which form neurofibrillary tangles in AD brains [91, 92]. It has been showed that neurofibrillary tangles correlate to the severity of dementia in AD [93]. Studies suggested that increasing kinase activity or decreasing phosphatase activity can lead to tau hyperphosphorylated. Most common kinase include glycogen synthase kinase-3 (GSK-3), cyclin-dependent kinase-5 (CDK5), mitogen-activated protein kinases (MAPK), microtubule affinity-regulated kinases (MARK), casein kinase 1 (CK1), cyclic AMP-dependent protein kinase (c-AMP), and tyrosine kinases [94]. For that reason finding kinase inhibitors can be a potential prevention and treatment strategy for AD [95].

Ten compounds with inhibiting kinase activity have been isolated from two sponge Classes (Demospongiae and Calcarea) of five sponge orders (Clathrinida, Dictyoceratida, Halichondrida, Haplosclerida, and Poecilosclerida) and seven families (Axinellidae, Halichondriidae, Hymedesmiidae, Irciniidae, Leucettidae, Petrosiidae, and Thorectidae) (Table 1). Eight compounds are isolated from Demospogiae, with four from the Dictyoceratida order. Most of these compounds are of alkaloids. Several highly active kinase inhibitors have been reported at concentrations below 2 µM [56, 57, 60, 62, 63].

Hymenialdisine (47) found in *Axinelliadae, Agelasidae and Halichondriidae sp.* has an inhibition activity to GSK-3β and CK1 *in vivo* study with IC_{50} of 10 and 35 nM, respectively [56]. This compound has been isolated again from *Callyspongia* sp. and has been tested for different kinases which include CK1, CDK5/p25, and GSK-3β with IC_{50} of 0.03, 0.16 and 0.07 µM [96].

Leucettamine B (53) is another alkaloid compound isolated from a Calcarea sponge *Leucetta microraphis* with a significant inhibition activity of cdc-2 like kinase (CLK) and tyrosine phosphorylation regulated kinase (DYRKs) that is involved in alternative pre-mRNA processing and AD with

IC_{50} of 0.4 and 2.8 µM, respectively [60]. Isopropanolic, extract of a Mediterranean sponge *Ircinia dendroides*, is found to inhibit 90% of GSK-3β activity at 50mg/ml. Bioassay guided fractionation led to the isolation of palinurin (55), which inhibits GSK-3β and GSK-3α with IC_{50} of 1.9 and 1.6 µM, respectively [63].

3.4. Inhibition of BACE1 (Beta-site APP Cleaving Enzyme1)

BACE1 is an enzyme that cleaves the amyloid precursor protein (APP) to form a soluble N-terminal fragment of about 100 kD, named as APPsβ, and a C-terminal fragment of about 12 kD at carboxyl-terminus, named as APPβ-CTF or C99. The latter is further proteolyzed by γ-secretase, releasing amyloid beta (Aβ) that may accumulate and deposit in the brain as amyloid plaques. Overexpression of BACE1 is found to increase the level of amyloid beta [97]. Initial studies suggest that BACE knockout does not cause severe neurological phenotype, while late studies show morphological and behavioral deficits in BACE1 null mice [98]. BACE mRNA is mostly expressed in the brain [99]. BACE gene is located in chromosome 11, while its homology BACE2 gene is located in chromosome 21 [100]. BACE2 shared about 62% of amino acid with BACE1. In addition, BACE2 does not have the specificity to cleave BACE1. For that reason BACE1 inhibitors are considered as promising Aβ-lowering drugs for potential use in Alzheimer's disease therapy [101].

Sixteen compounds with BACE1 inhibitory activity have been isolated from Demospongiae Class of four sponge orders (Dictyoceratida, Halichondrida, Haplosclerida, and Verongida.) and four families (Halichondriidae, Ianthellidae, Irciniidae, and Petrosiidae) (Table 1). Among them, eleven compounds are isolated from the Haplosclerida order, and five compounds from the Verongida order. Nine of these compounds are of pentacyclic, with other compounds as alkaloids, phenyl derivative, and steroid.

Several compounds with IC_{50} in BACE1 inhibitoion equal or below 2 µM were reported [66, 67, 69]. Topsentinol K trisulfate (65) isolated from *Topsentia* has been shown to inhibit BACE1 with IC_{50} of 1.2 µM with the activity being attributed to the presence of sulphate esters [66]. Dictyodendrin J (70) compound isolated from *Ianthella sp.* shows BACE1 inhibition activity with IC_{50} of 2 µM [68]. Prenylated hydroxybenzoic acid 1 (71) was isolated from *Sarcotragus* sp. with BACE-1 inhibition of 45±21% at 1 µM [69].

Most of these studies have used BACE1 assay kit [62, 65, 66, 68], and FRET (fluorescence resonance energy transfer) assay kit, cell-based assay for inhibition of Aβ (1–40) peptide (APP) production, and TRF (continuous time-resolved fluorescence assay for identification of BACE1) assay have also been applied for the screening studies [69, 102].

3.5. Neurotrophic activity

There are four types of neurotrophins that include nerve growth factor (NGF) , neurotrophin 3 (NT-3) , neurotrophin 4 (NT-4) and brain derived neurotrophic factor (BDNF) which are responsible for the regulation and proliferation of neurons [103]. Depletion of these neurotrophins factors were found in patients with neurodegenerative diseases such as Alzheimer disease [103, 104]. NGF has two receptors which are TrkA and P75 of high and low affinity, respectively [105, 106]. NGF is an important factor in cholinergic neuron which is an important element in transmitting the neuron signals and messages, critical in diseases such as AD [107]. The level of NGF in AD patients increases when compared to the control except one region of nucleus basalis with NGF level decreased [108]. Therefore, neurotrophins has been considered as a treatment target [103, 104].

Nineteen compounds isolated from Demospongiae Class of three sponge orders (Dictyoceratida, Haplosclerida, and Poecilosclerida) and four families (Chalinidae, Desmacellidae, Spongiidae, and Petrosiidae), have been shown to have neurotrophic effect (Table 1). Ten compounds

are isolated from the Haplosclerida order, and five compounds from the Dictyoceratida, order. Eight of these compounds are of alkaloids, with five being both acetylenic alcohol and sesterterpene [70-74].

Some examples of such compounds with good activities are: lembehyne A (72) isolated from *Haliclona sp* being highly active at 2 μg/ml in PC-12 cells and 0.1μg/ml in Neuro 2A cell line [70]; labuanine A and three different compounds (73-76) isolated from *Biemna fortis* showed over 50% of differentiation of Neuro 2A cell line treated at 1, 3, 0.03 and 1 μM, respectively [71]; five petrosiols A-E compounds (86-90) isolated from *Petrosia strongylata* induced neuronal differentiation in PC12 cell line at 2 μM [74].

4. Structure-Activity Relationship and *In Vivo* Test

Among 90 neuroprotective compounds identified from marine sponges, 50% are of alkaloids (45 compounds), and nearly 15% of terpenes (13 compounds), with the rest spreading over quinolone, acid, peptides, sterols etc (Table 1). In terms of neuroprotective activity, alkaloids should be the future focus of discovery and development. This is also reflected in the proportion of alkaloid compounds being subjected to structure-activity relationship (SAR) studies for structure optimization toward lead compound development. Out of 23 compounds being studied for SAR, 13 are alkaloids accounting for over 56% of the total. The 13 alkaloids showed four different neuroprotective activities.

The structures of 13 alkaloids exhibit a high diversity. For the acetylcholinesterase inhibitory activity, three alkaloids from different sponges have unique structures. A new stigmastane-type steroidal alkaloid, 4-acetoxy-plakinamine B (3) isolated from sponge *Corticium* sp is the first steroidal alkaloid bearing a stigmastane skeleton, showing significant acetylcholinesterase inhibitory activity [21]. The 3-D QSAR studies showed that the inhibitory activity was primarily favored by the negative density surrounding C-3 and C-4, where the stigmastane skeleton distances the nitrogen atom on the side chain with a 4-carbon bridge farther than those in the pregnane skeleton.Petrosamine (4) is the second potent anti-cholinesterase pyridoacridine alkaloid from sponge *Petrosia* n. sp. Using a computational docking study of petrosamine with the enzyme from the electric eel Torpedo californica (TcAChE), the quaternaryammonium group of petrosamine was identified as the major contribution to the petrosamine-TcAChE interaction [25].

The third potent cholinesterase inhibitor, a bis-indole alkaloid fascaplysin (15) has been chemically synthesized. Molecular docking studies revealed that fascaplysin accommodates within a peripheral anionic site and inner linings of the AChE active site gorge [41].

Five brominated pyrrole alkaloids (31-35) from *Agelas* sponges reduced the depolarization-induced cellular calcium elevation [49]. The structure–activity relationship of bromopyrrole alkaloids has been studied by comparing with six other derivatives to test whether these alkaloids generally act as feeding deterrents by its taste and smell. The aminoimidazole group and the degree of bromination of the pyrrole moiety appeared to have a significant effect on voltage dependent calcium elevation [49]. Similarly, two other bromopyrrole alkaloids (28-29) isolated from the Mediterranean sponge *Axinella damicornis* have unique structures lacking the commonly found short linear aliphatic segment linking the bromopyrrole 2-carboxylic acid moiety to the common imidazole nucleus [47]. This study supports the important roles of the aminoimidazole group and the degree of bromination of the pyrrole moiety underlining the serotonin receptor activity *in vitro* [47].

Hymenialdisine (47), a sponge alkaloid is a potent inhibitor of CDK2 and GSK-3β. Using a CDK2–hymenialdisine complex crystal structure, the key interacting residues

underlying the activity and selectivity have been identified to be three hydrogen bonds link hymenialdisine to the Glu81 and Leu83 residues of CDK2. *In vivo* inhibition studies of CDK5/p35 and GSK-3 demonstrated the lack of phosphorylation/down-regulation of Pak1 kinase in E18 rat cortical neurons, and the inhibition of MAP-1B phosphorylation, respectively. Hymenialdisine also blocks the *in vivo* phosphorylation of the microtubule-binding protein tau at sites that are hyperphosphorylated by GSK-3 and CDK5/p35 in Alzheimer's disease [56].

Manzamine A (48) is a promising new alkaloid class of GSK-3β inhibitors from which more potent and selective GSK-3 inhibitors could be designed as potential therapeutic agents for Alzheimer's disease [57]. A comprehensive SAR study by Hammann *et al.* demonstrated that manzamine A constitutes a promising scaffold and specifically inhibits on GSK-3_ and CDK-5, the two kinases involved in tau pathological hyperphosphorylation [57].

Debdab *et al.* [60] has conducted a detailed SAR study of sponge-derived alkaloids leucettines with the identification of the first potent inhibitors of two families of serine/threonine kinases, dual-specificity, tyrosine phosphorylation regulated kinases (DYRKs), and cdc2-like kinases (CLKs). This study has demonstrated that leucettine B (53) modulates alternative pre-mRNA splicing, most probably through phosphorylation of serine/arginine-rich proteins (SRp) [60]. A further study from the same group by Tahtouh *et al.* [61] have reported the selectivity, cocrystal structures, and neuroprotective properties of leucettines B (53) as inhibitors of protein kinases implicated in the onset and development of Alzheimer's disease and Down syndrome. Their study clearly demonstrated this group of alkaloids and other similar inhibitors shared structure-activity relationship, with possible further improved inhibitors synthesized from the leucettamine B scaffold [61].

Two brominated cyclodipeptides barettin (cyclo[(6-bromo-8-entryptophan)arginine]) and 8,9-dihydrobarettin (cyclo[(6-bromotryptophan)arginine]) (26-27) isolated from the marine sponge *Geodia barretti* have been studied to establish the molecular target and mode of action in terms of their affinity to human serotonin receptors. The study has identified that the tryptophan residue in the barettins resembles that of endogenous serotonin [5-hydroxytryptamine]. Barettin selectively interacted with the serotonin receptors 5-HT2A, 5-HT2C, and 5-HT4, while 8,9-dihydrobarettin interacted exclusively with the 5-HT2C receptor. The results suggest that the double bond between the tryptophan and arginine residue plays an important role in the interaction with the receptor proteins [46].

Khanfar *et al.* (2009) used molecular docking modelling study and identified the molecular target and binding pocket of marine sponge-derived inhibitors phenylmethylene hydantoin (49) of glycogen synthase kinase-3β (GSK-3β). This has led to the development improved leads for potential drug development [58]. Bidon-Chanal *et al.* (2013) have used molecular dynamics simulations to identify a novel allosteric mechanism by which binding of a sponge-derived furanoterpenoid compound, palinurin (55) leads to GSK-3β inhibition. This is the first compound described to target this allosteric site [63].

Lopez-Ogalla *et al.* (2014) reported that the prenylated phenyl rings of Prenylated hydroxybenzoic acid 1 (71) are important scaffolds for the potent and selective inhibiting activity of β-secretase-1 (BACE-1) by a preliminary SAR analysis of a group of bioactive prenylated phenyl derivatives [69]. Horikawa *et al.* (2013) has reported five novel diyne polyols (petrosiols A-E) (86-90), and suggested that the rare 2,4-diyne-1,6,7,8-tetraol fragment plays an important role for the neurotrophic activity of the petrosiols [74].

While there are so many SAR studies to optimize the structure-activity relationship toward lead development, however only four compounds have been tested *in vivo* using animals. Hymenialdisine (47) is an example of that as it has been tested in an *in vivo* model for inhibitory activity of GSK-3β and CK1 with IC50 of 10 and 35 nM, respectively [56]. Dysiherbaine (DH) was non-NMDA agonist *in vivo* by inhibiting glutamate receptor with IC50 of 66 nM [35[. Cribronic acid (2S,4R,5R)-5-hydroxy- 4-sulfooxypiperidine-2-carboxylic acid and (2S,4S)-4-sulfooxypiperidine-2-carboxylic acid (18-19) inhibit glutamate receptor *in vivo* with IC50 of 83 and 214 nM, respectively [36].

One of the reasons for lack of *in vivo* studies at present may reflect that all these studies are still in the early stage of development given this is still an emerging area of research and development. However the supply problem of these sponge-derived compounds for further tests is the main issue as for all the other sponge-derived drug development. It is commonly reported that majority of these sponge-derived compounds are in very low content from limited supply of sponge biomass from the sea [7]. Further progress into *in vivo* animal test and clinical trials requires sustainable supply of these bioactive compounds in quantity, and solutions including chemical synthesis, sponge cell culture and transgenic production has been attempted [7, 109, 110].

5. Conclusion

In the search for neuroprotective marine sponge-derived compounds since 1999, Class of Demospongiae contributes to 85 bioactive compounds out of 90 compounds identified by far. These compounds were isolated from eleven orders which include Agelasida, Astrophorida, Clathrinida, Dendroceratida, Dictyoceratida, Halichondrida, Haplosclerida, Homosclerophorida, Poecilosclerida, Thorectidae, and Verongida. Nearly 68% of these compounds (61 compounds) were found from three Orders of Haplosclerida (35 compounds), Dictyoceratida (15 compounds) and Halichondrida 11 compounds). Twenty families contribute to these compounds, with 26 compounds from the Petrosiidae family. Six compounds (oroidin, four bromopyrrole alkaloids, and manzamine A) have showed two target activities potentially acting as multitarget-directed ligands (MTDL's) for treatment of AD [111]. Oroidin (33) showed to inhibit AChE and calcium level. Four bromopyrrole alkaloids (22-25) exhibited reducing glutamate receptor and enhancing serotonin receptor, while manzamine A (48) showed to inhibit kinase activity and enhance neuronal growth. Out of these limited studies, a number of novel highly active compounds in a range of neuroprotective assays have been identified targeting diverse mechanisms of actions with potential for the prevention and treatment of neurodegenerative diseases. While these sponge-derived compounds are yet to progress into clinical trial, it is encouraging to see that 26% of these 90 compounds have been undergone active SAR studies. These efforts will generate improved lead compounds that are expected to enter *in vivo* animal studies and clinical trial pipelines in the near future. The limited *in vivo* studies of only four bioactive compounds may be hindered by the supply problem, however provide great opportunities for synthetic chemistry and subsequently *in vivo* and clinical trials when this issue is solved. For that purpose, their efficacy and safety will be extensively assessed in the course of drug development. This review highlights some of the bioactive compounds discovered by far warrant further investigation for potential new drug development. The huge number of sponges (over 15,000) and their richest chemical diversity available for neuroprotective screening could become a fruitful field of research into the discovery and development of new generation of marine drugs effective for the prevention and treatment of neurodegenerative diseases.

CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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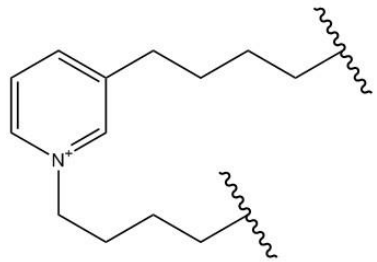
Inhibition of acetylcholinesterase:



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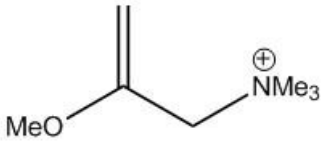

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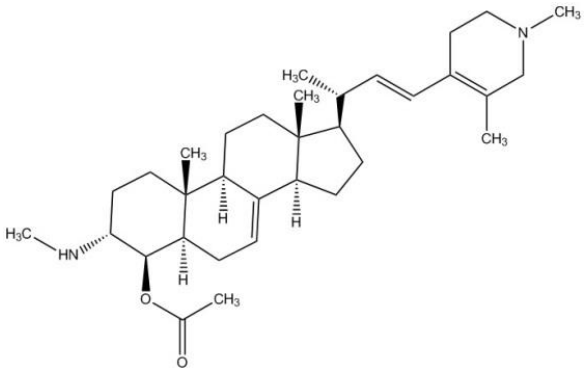

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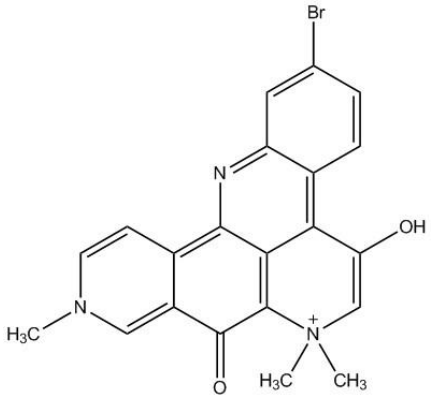

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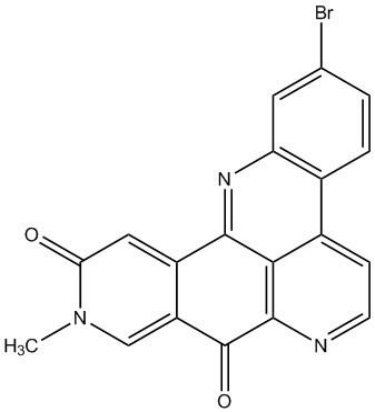

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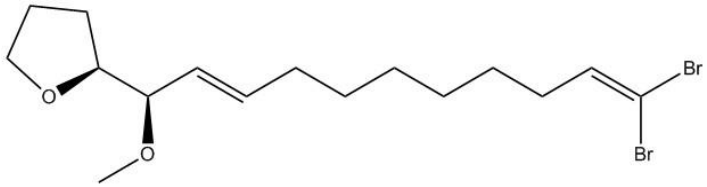

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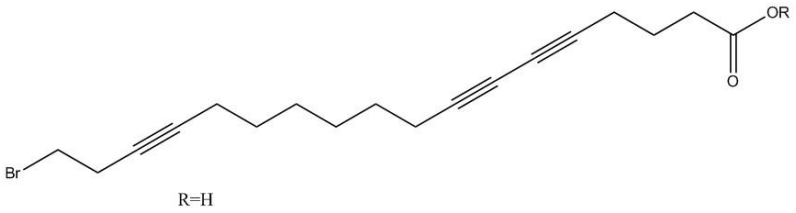

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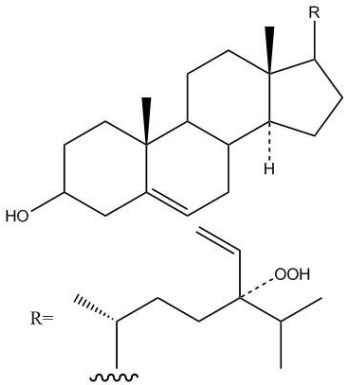

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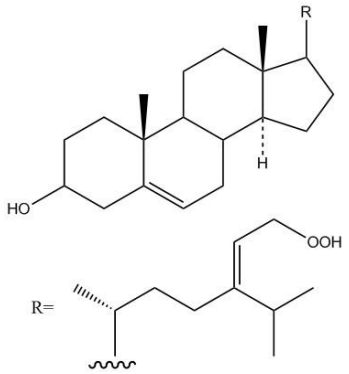

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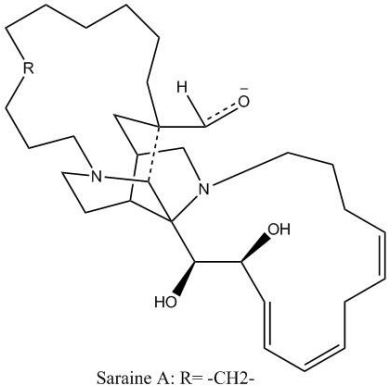
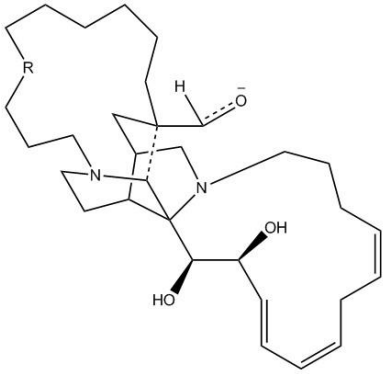


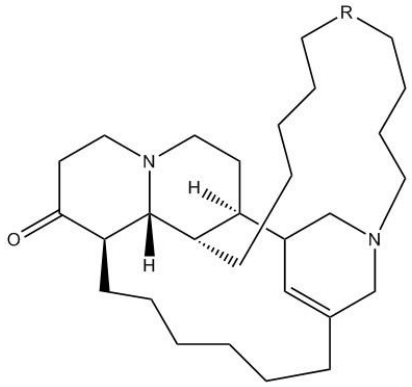
Saraine A: R= -CH2-
Saraine B: R= -CH=CH-
Saraine C: R= -CH=CHCH2-

(10)



Protonated saraine A: R= -CH- +H
Protonated saraine B: R= -CH=CH- +H
Protonated saraine C: R= -CH=CHCH2- +H

(11)



R= -CH=CH-

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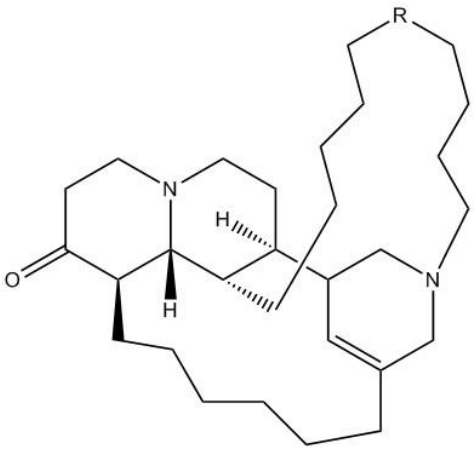

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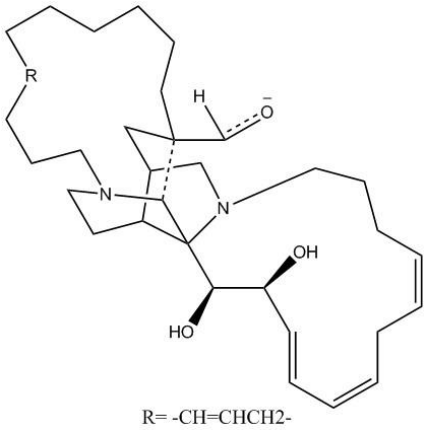

R= -CH=CHCH2-

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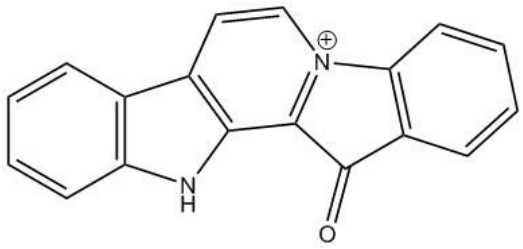
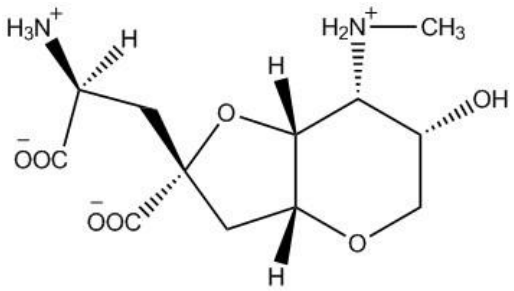


(15)

Inhibition of glutamate receptors:



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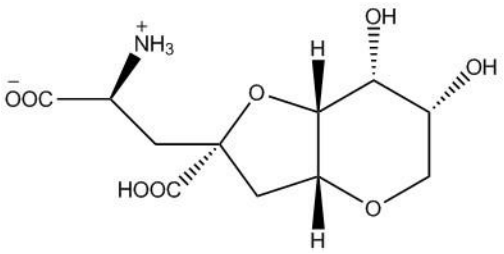

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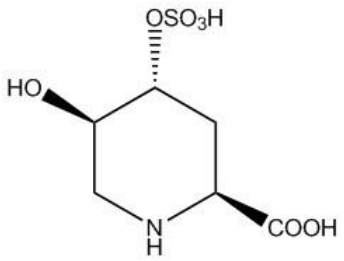

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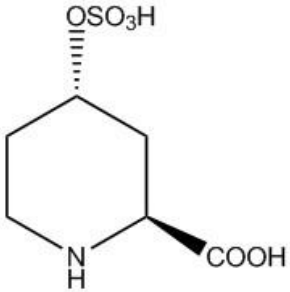

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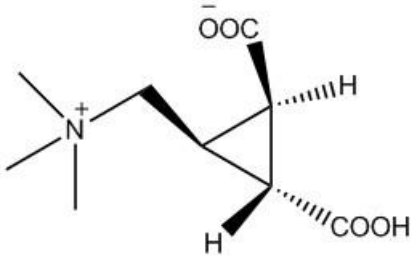

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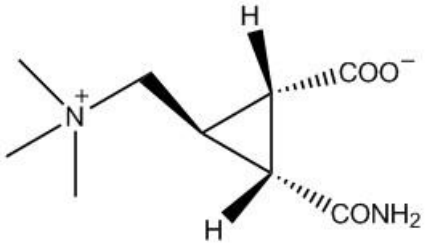

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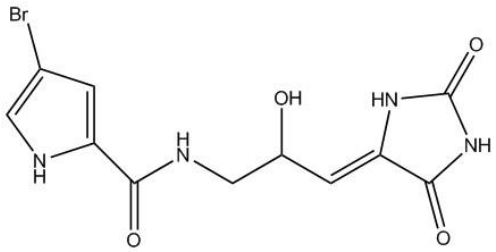

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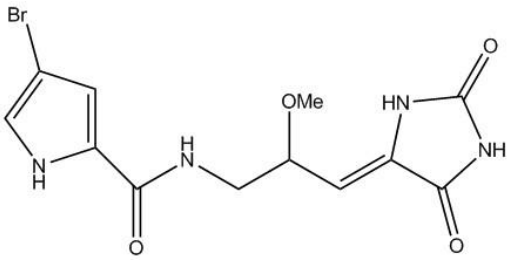

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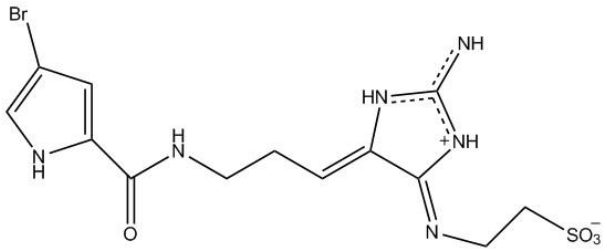

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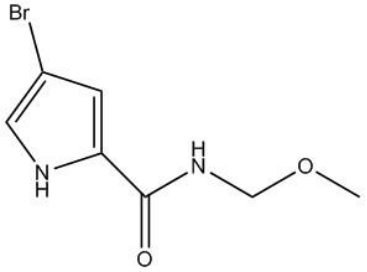

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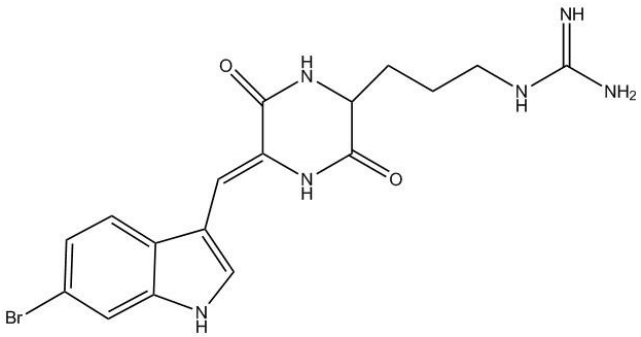
Enhancement of serotonin receptor:



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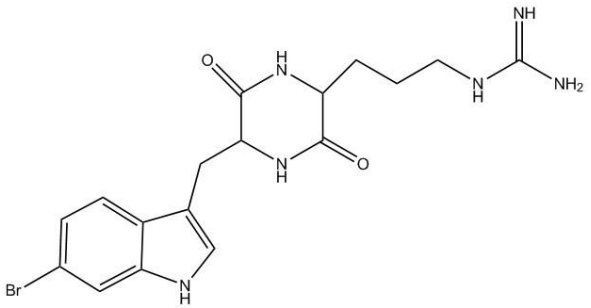

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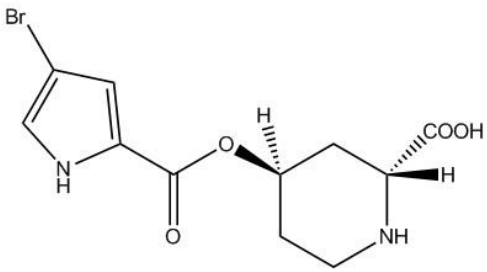

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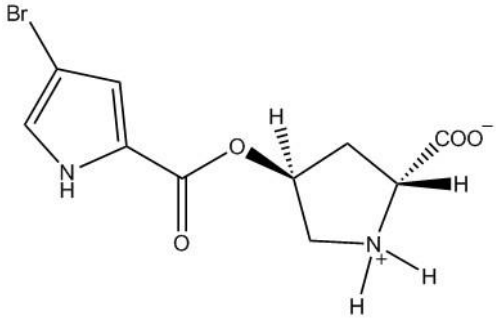

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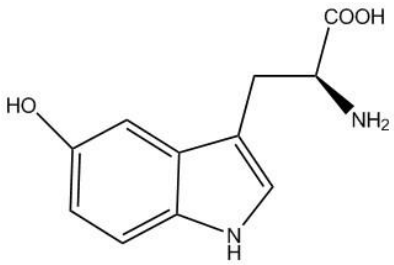
-Inhibition of oxidative stress:



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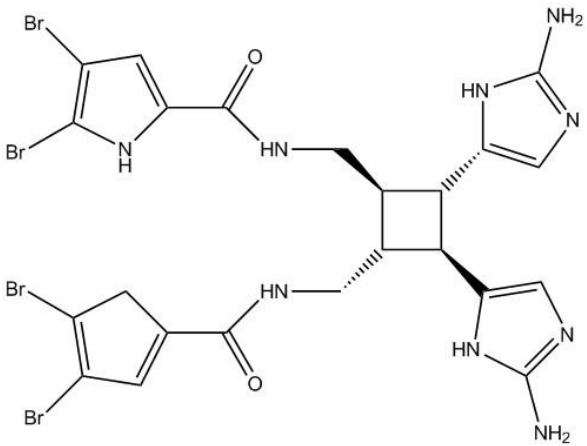

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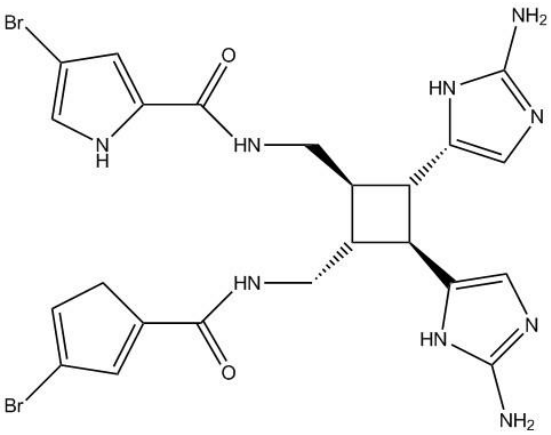

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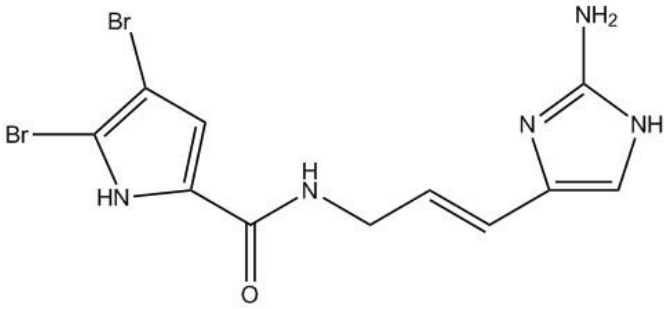

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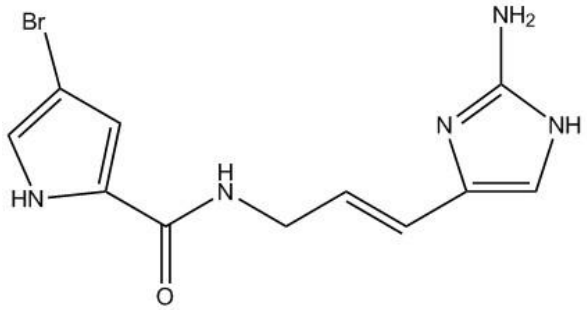

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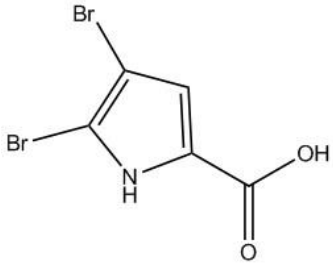

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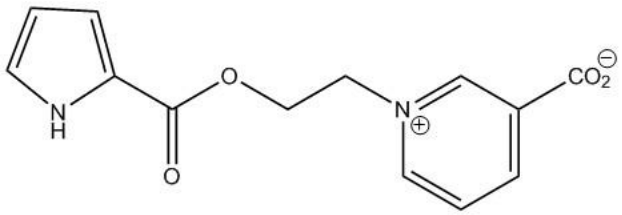

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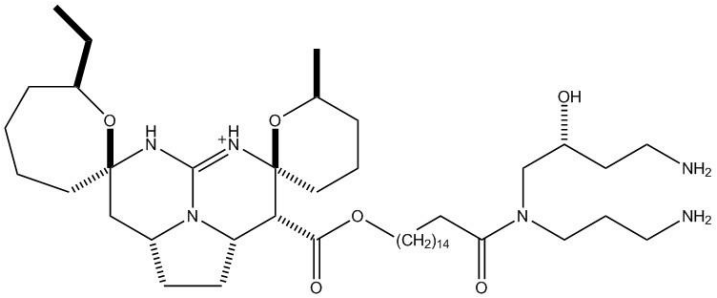

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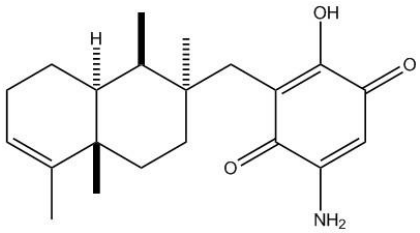

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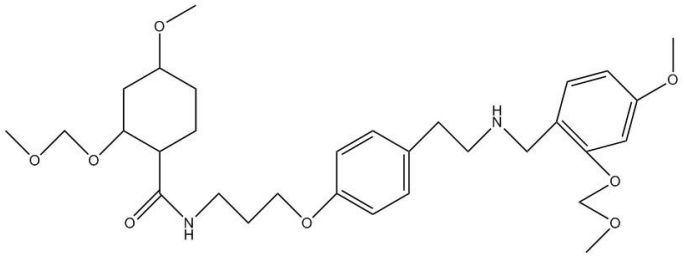

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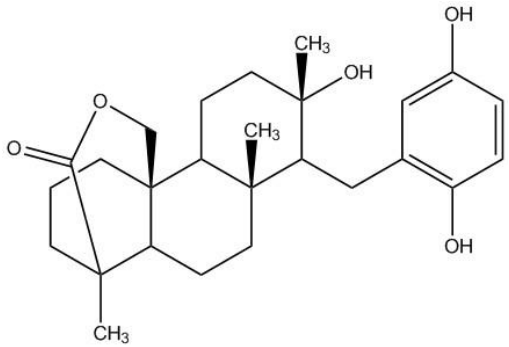

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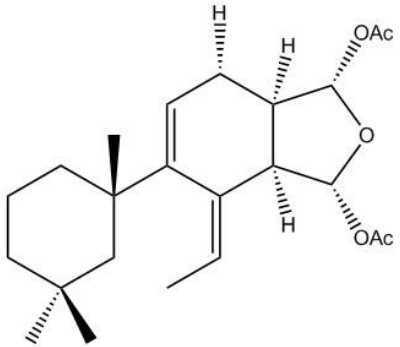

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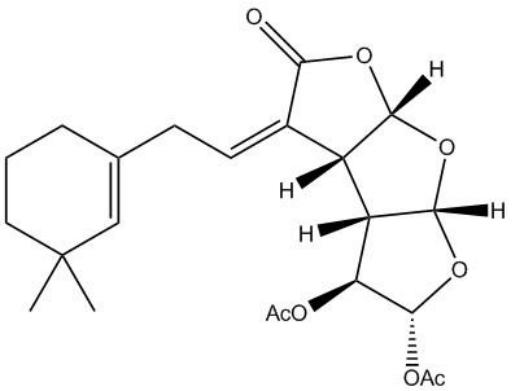
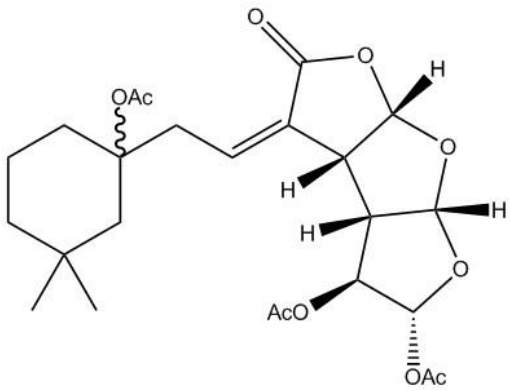
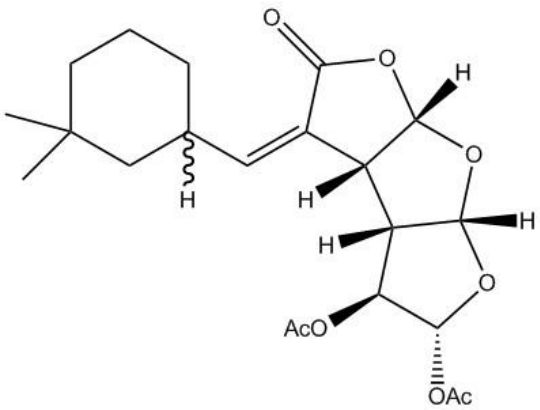

O
O
H
O
H
H
O
AcO
OAc

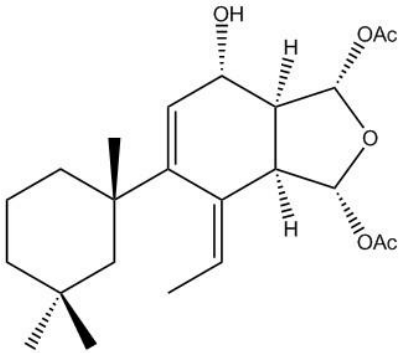
(42)


OAc
O
O
H
O
H
H
O
AcO
OAc

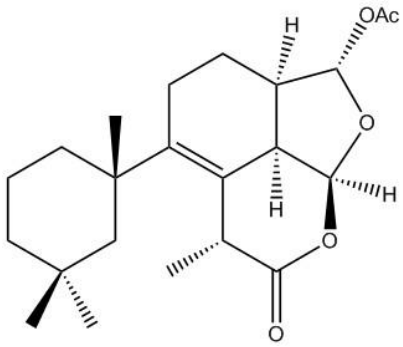
(43)


O
O
H
O
H
H
H
O
AcO
OAc

(44)

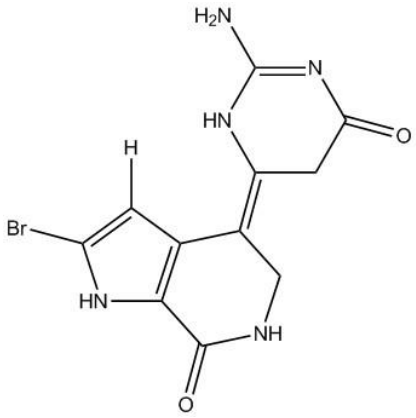

OH
H
OAc
O
H
OAc

(45)


H
OAc
O
H
H
O
O

(46)

-Inhibition of kinase:



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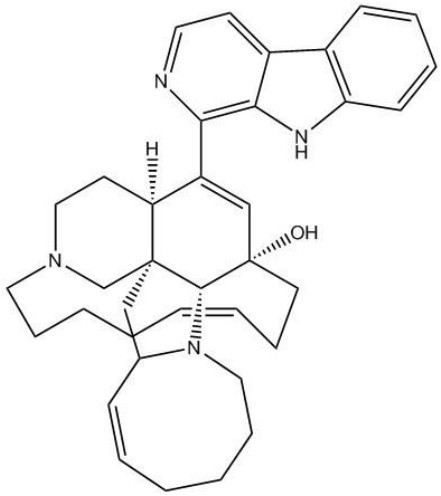

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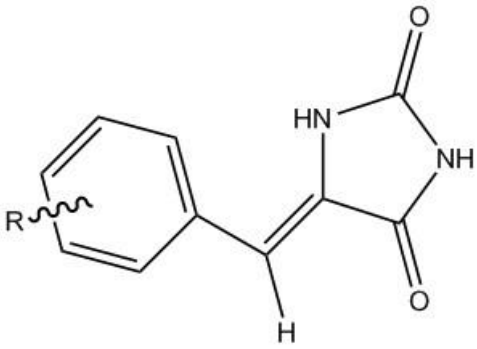

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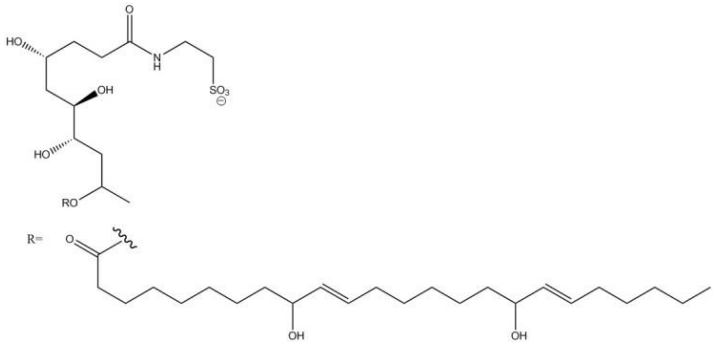

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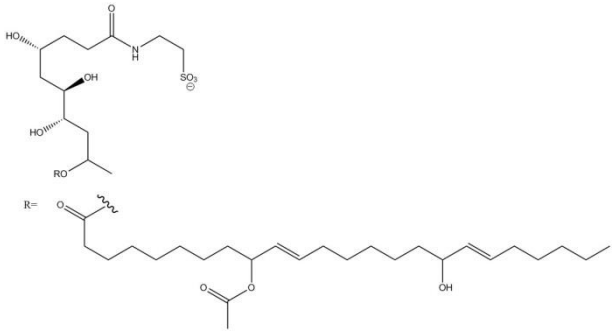

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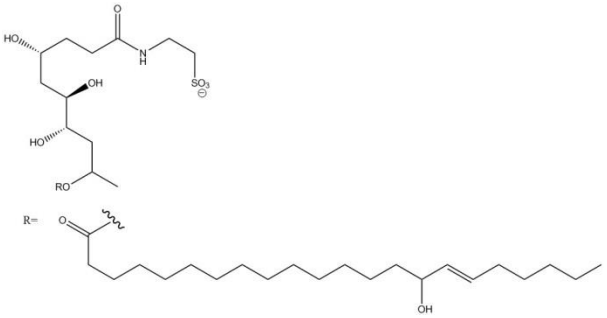

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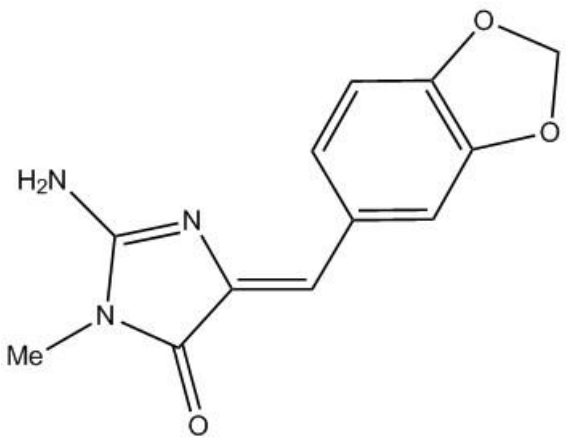

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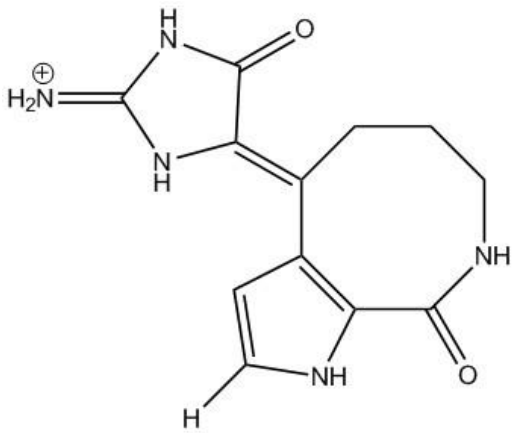

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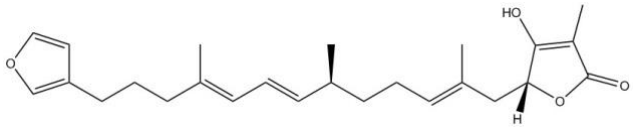

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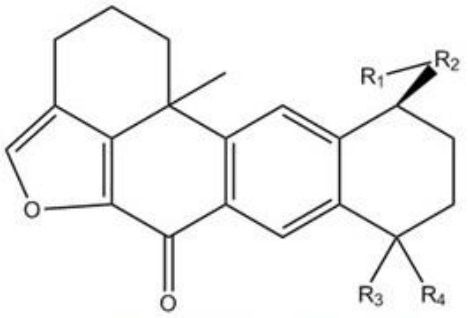
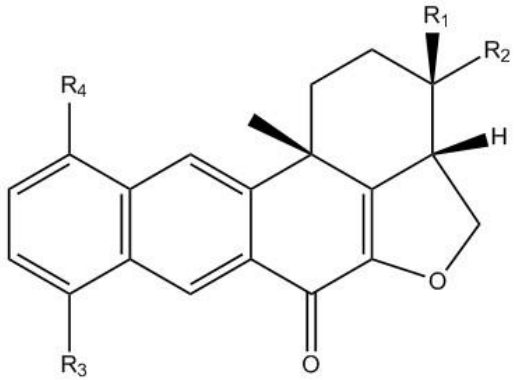
Inhibition of BACE:



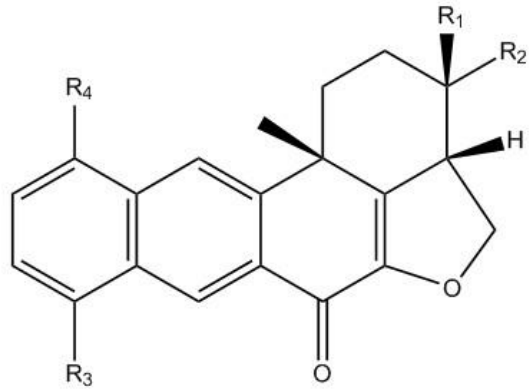
R1 = OH, R2 = H, R3R4= O

(56)



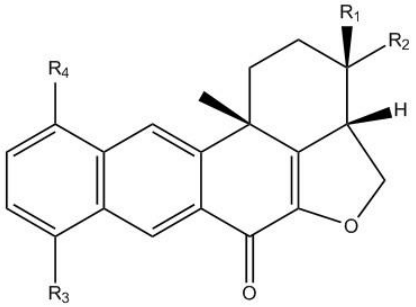
R1= H R2= OH R3= OCH2CH2OH R4=H

(57)



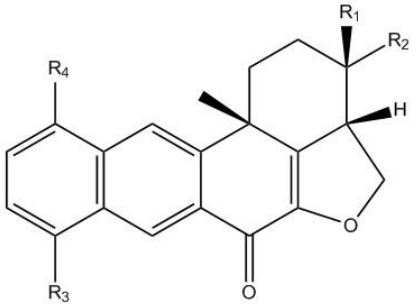
R1= H R2= OH R3= OH R4=H

(58)



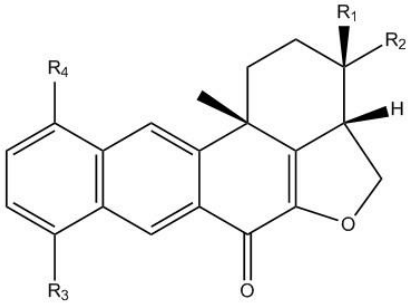
R1= OH R2= H R3= OCH2CH2OH R4=H

(59)



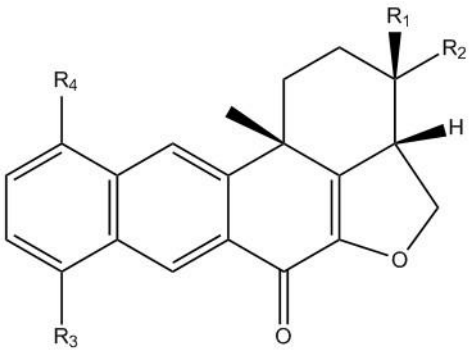
R1= OH R2= H R3= H R4=H

(60)



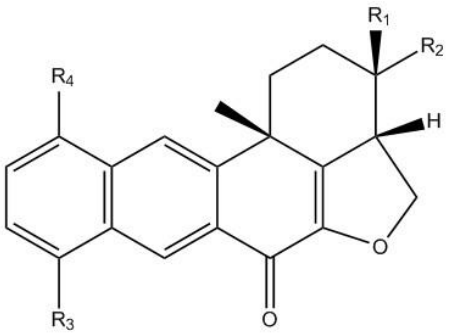
R1= H R2= OH R3= H R4= OCH3

(61)



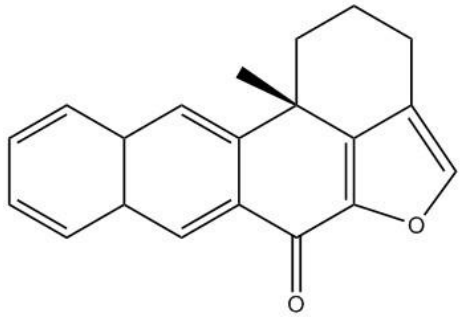
R1= H R2= OH R3= H R4= OH

(62)



R1= H R2= OH R3= H R4= H

(63)



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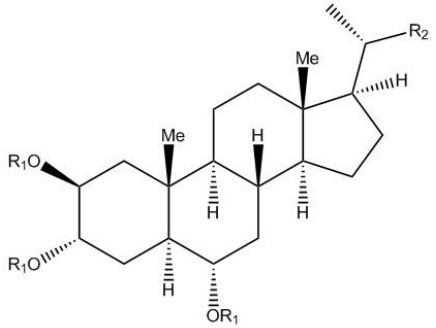
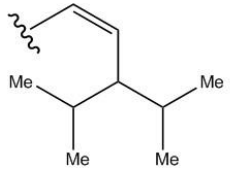


R1= $SO_3^-Na^+$ R2=



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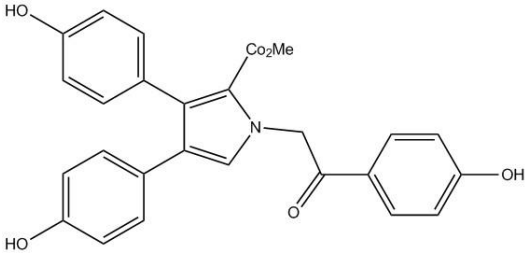

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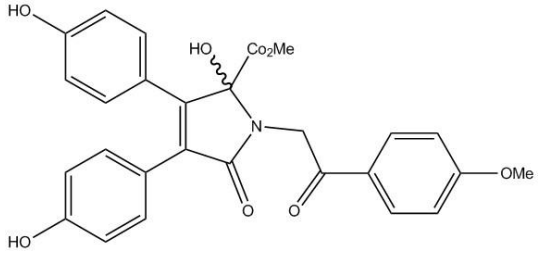

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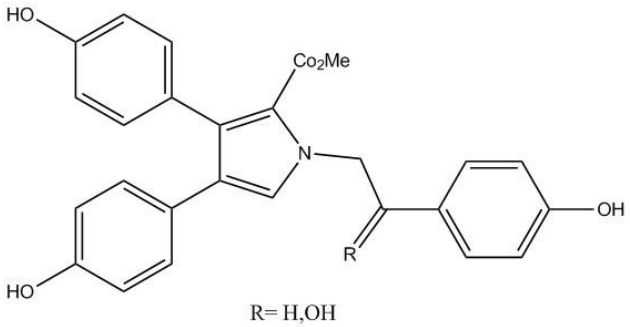
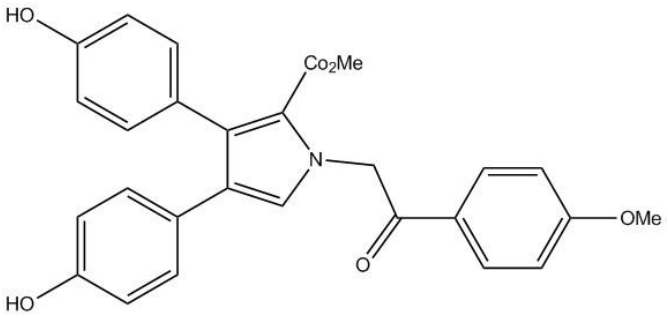
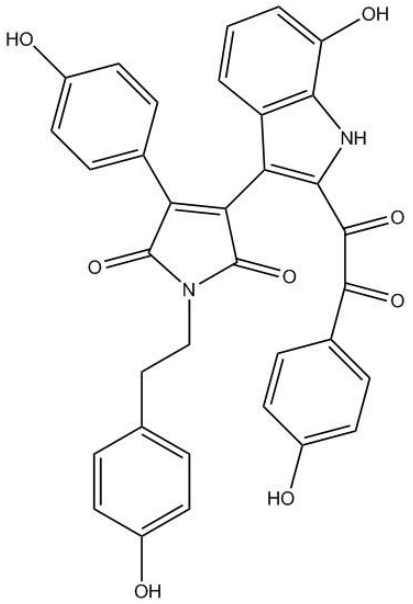

HO
Co2Me
N
OH
R
HO
R= H,OH

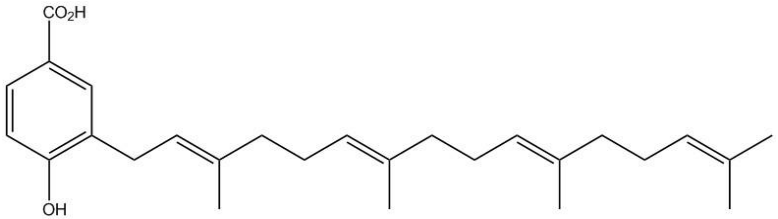
(68)


HO
Co2Me
N
OMe
O
HO

(69)

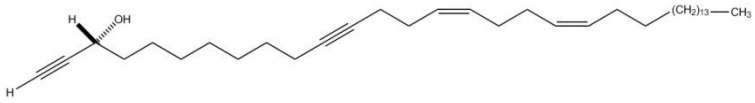

HO
OH
NH
O
O
N
O
O
HO
OH

(70)


CO2H
OH

(71)

Enhancement of neuronal growth:



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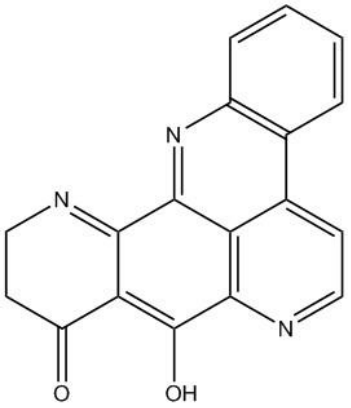

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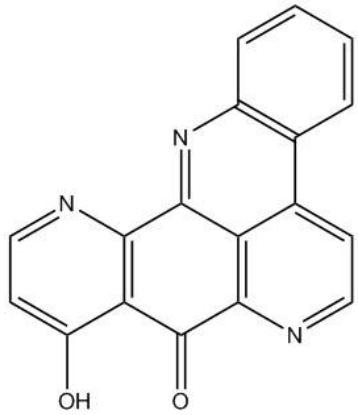

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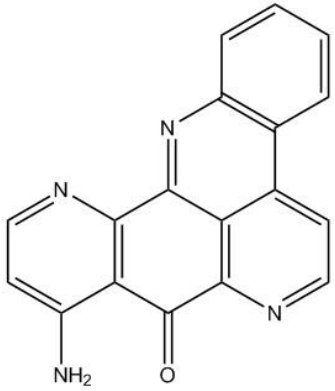

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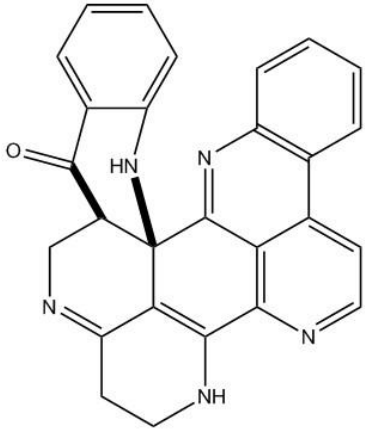

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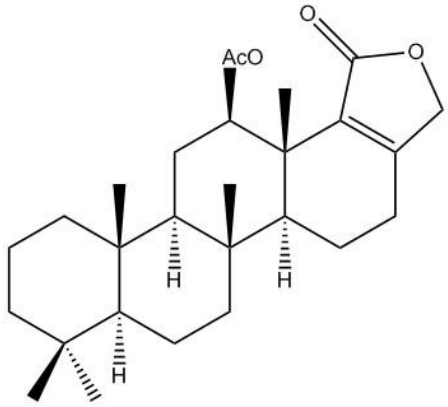

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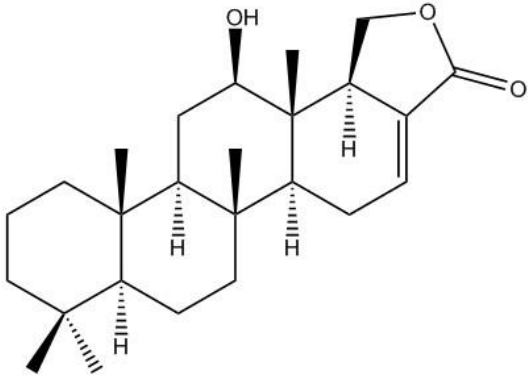

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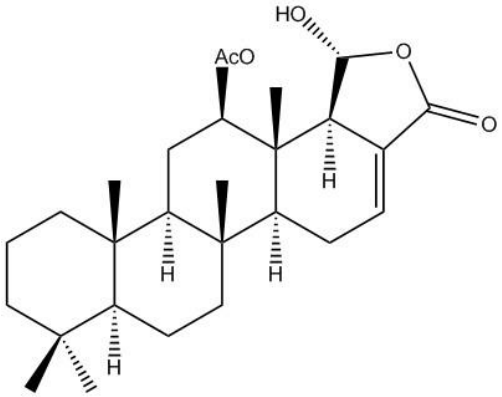

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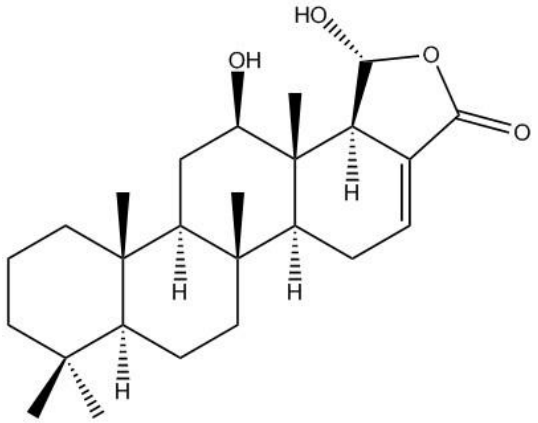

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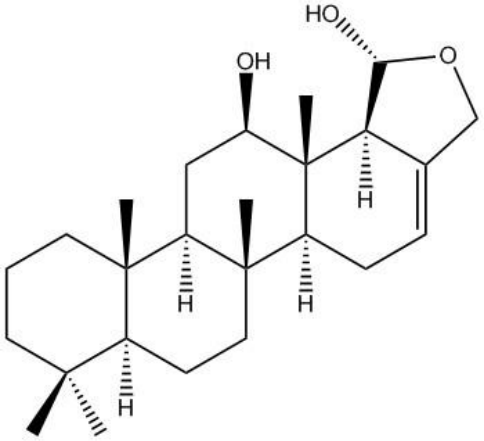

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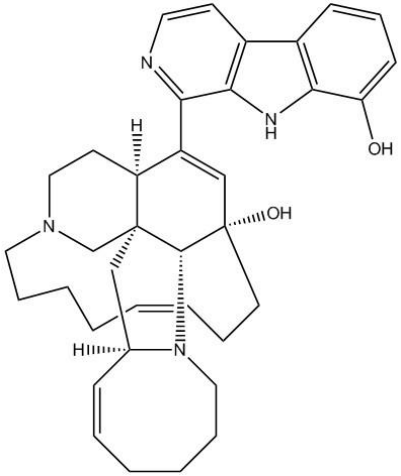

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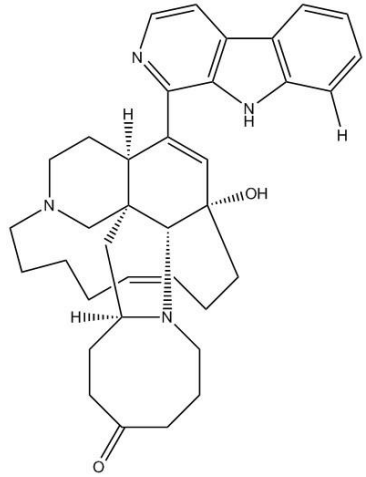

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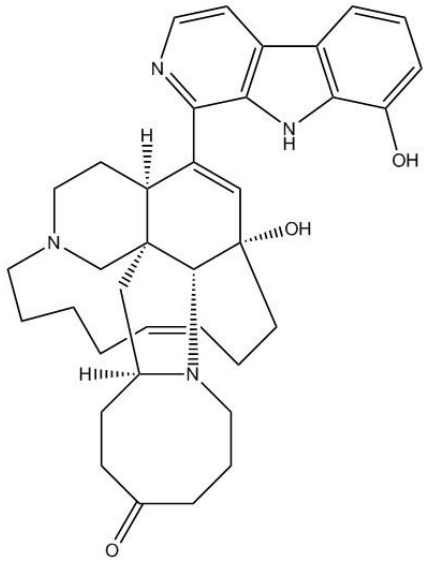

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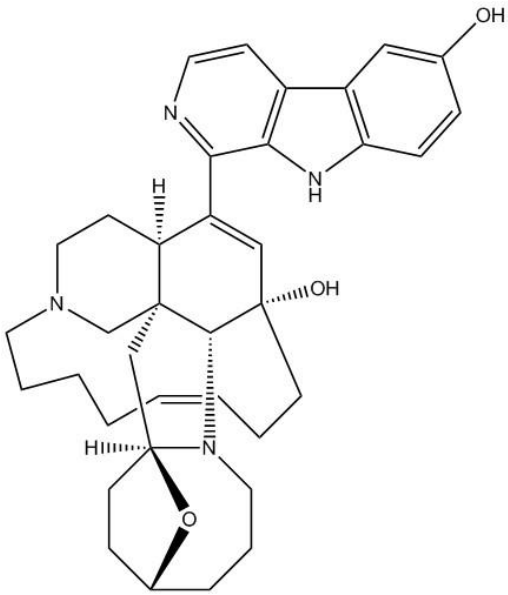

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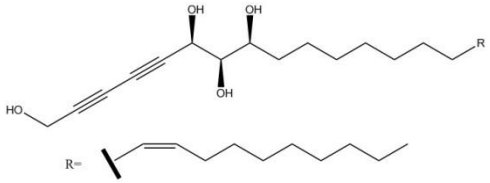

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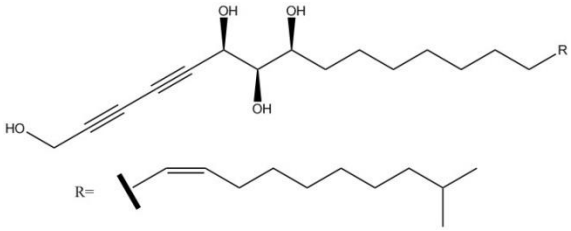

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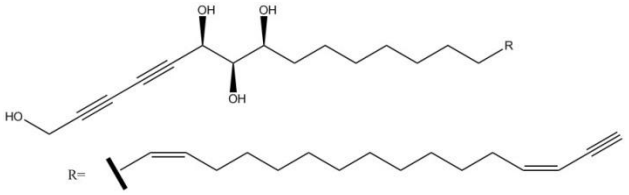

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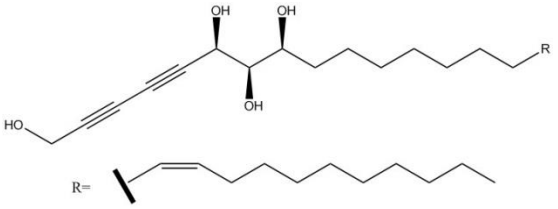

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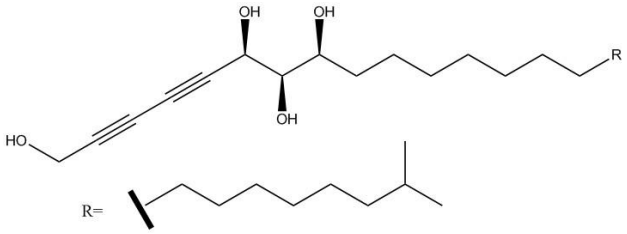

(90)

Appendix 3: Drug-likeness results:

The Table below summarizes the results of the drug-likeness of the compounds.

Appendix 3 Table 1: The drug-likeness test results of sponge-derived compounds by Lipinski's rule and BBB likelihood analyses.

	Lipinski's rule					BBB likeness			
Compound	**Molecular weight (=<500)**	**LogP (=<5)**	**H-bond donor (=<5)**	**H-bond acceptor (=<10)**	**Follow this rule?**	**Molecular weight (=<400)**	**H-bond (total) (=<8)**	**No acids (0)**	**Follow this rule?**
2-bromoamphimedine	391	0.008	0	5	Yes	391	5	0	Yes
4,5-dibromo-2-pyrrolecarboxylic acid	266.85	2.008	2	3	Yes	266.85	5	1	No
8-Hydroxymanzamine	564.35	4.5	3	6	No	564.35	9	0	No
8,9-dihydrobarettin	420.09	0.836	5	8	Yes	420.09	13	0	No
24-hydroperoxy-24-vinylcholesterol	400.02	8.64	0	3	No	400.02	3	0	No

29-hydroperoxystigmasta-5,24(28)-dien-3	444.36	9.009	2	3	No	444.36	5	0	No
Barettin	418.08	1.224	5	8	Yes	418.08	13	0	No
Crambescidin 800	800.61	8.571	4	12	No	800.61	16	0	No
Cribronic acid	241.03	-2.326	4	8	Yes	241.03	12	2	No
Daminin	260.08	0.527	1	5	Yes	260.08	6	0	Yes
Damipipecoline	316.01	1.163	3	6	Yes	316.01	9	1	No
Damituricin	330.02	0.372	1	5	Yes	330.02	6	0	Yes
Debromohymenialdisine	245.09	0.755	4	7	Yes	245.09	11	0	No

Dictyodendrin J	588.15	2.046	5	10	No	588.15	15	0	No
Dysibetaine CPa	202.11	-1.286	2	4	Yes	202.11	6	2	No
Dysibetaine CPb	201.12	-2.014	2	4	Yes	201.12	6	1	No
Dysideamine	343.21	5.599	2	4	No	343.21	6	0	Yes
Dysiherbaine	304.296	-4.677	5	9	Yes	304.296	14	2	No
Esmodil	130.12	0.394	0	1	Yes	130.12	1	0	Yes
Fascaplysin	271.09	2.347	1	2	Yes	271.09	3	0	Yes
Gracilin A	359	6.578	0	5	No	359	5	0	Yes
Gracilin j	480.2	0.406	0	10	Yes	480.2	10	0	No

Gracilin K	408.18	4.23	0	8	Yes	408.18	8	0	No
Gracilin L	406.24	5.392	1	6	No	406.24	7	0	No
Hymenialdisine	323	1.563	4	7	Yes	323	11	0	No
L-5-hydroxytryptophan	220.08	-2.315	4	5	Yes	220.08	9	1	No
Labuanine A	290	-0.097	0	5	Yes	290	5	0	Yes
Lembehyne A	510.48	16.05	1	1	No	510.48	2	0	No
Leucettamine B	245.08	0.23	1	6	Yes	245.08	7	0	Yes
Manzamine A	548.35	4.363	2	5	No	548.35	7	0	No
Manzamine E	564.35	2.963	2	6	No	564.35	8	0	No

Manzamine F	580.34	3.1	3	7	No	580.34	10	0	No
Manzamine X	580.34	3.52	3	7	No	580.34	10	0	No
Neodysiherbaine A	291.1	-5.0	5	9	Yes	291.1	14	2	No
NP04634	582.26	2.1	2	11	No	582.26	13	0	No
Oroidin	386.93	1.738	4	6	Yes	386.93	10	0	No
Palinurin	398.25	6.246	1	4	No	398.25	5	0	Yes
Petrosamine	422.05	0.931	0	4	Yes	422.05	4	0	No
Petrosiol A	406.31	7.339	4	4	No	406.31	8	0	No
Petrosiol B	420.35	7.847	4	4	No	420.35	8	0	No

Petrosiol C	484.36	9.504	4	4	No	484.36	8	0	No
Petrosiol D	420.32	7.908	4	4	No	420.32	8	0	No
Petrosiol E	394.31	7.225	4	4	No	394.31	8	0	Yes
Phenylmethylene	204.4	2.166	2	3	Yes	204.4	5	0	Yes
Sceptrin	618.05	0.75	8	12	No	618.05	20	0	No
Strongylophorine-8	428.26	4.379	3	5	Yes	428.26	8	0	No
Tetrahydroaplysulphurin-1	376.22	5.077	0	5	No	376.22	5	0	Yes
Topsentinol K trisulfate	700.26	6.049	3	12	No	700.26	15	3	No
Xestosaprol D	322.12	1.302	1	4	Yes	322.12	5	0	Yes

Xestosaprol F	366.15	1.016	2	5	Yes	366.15	7	0	Yes
Xestosaprol G	322.12	1.404	2	4	Yes	322.12	6	0	Yes
Xestosaprol H	366.15	1.016	2	5	Yes	366.15	7	0	Yes
Xestosaprol I	306.13	2.54	1	3	Yes	306.13	4	0	Yes
Xestosaprol J	336.14	1.514	1	4	Yes	336.14	5	0	Yes
Xestosaprol K	322.12	1.193	2	4	Yes	322.12	6	0	Yes
Xestosaprol L	306.13	2.54	1	3	Yes	306.13	4	0	Yes
Xestosaprol M	288.12	4.057	0	2	Yes	288.12	2	0	Yes
Xestospongic acid	348.07	6.309	1	2	No	348.07	3	1	No

Appendix 3 Table 2: The drug-likeness test results of algae-derived compounds by Lipinski's rule and BBB likelihood analyses

	Lipinski's rule					BBB likeness			
Compound	**Molecular weight (=<500)**	**LogP (=<5)**	**H-bond donor (=<5)**	**H-bond acceptor (=<10)**	**Follow this rule?**	**Molecular weight (=<400)**	**H-bond (total) (=<8)**	**No acids (0)**	**Follow this rule?**
2-phloroeckol	496.06	3.052	8	12	No	496.06	20	0	No
6,6'-Bieckol	742.08	4.33	12	18	No	742.08	30	0	No
7-Phloroeckol	496.37	3.05	8	12	No	496.37	20	2	No
7-Hydroxycholesterol	402.65	8.699	0	2	No	402.65	0	2	No
8,8'-Bieckol	742.08	4.33	12	18	No	742.08	30	0	No
Amarouciaxanthin A	614.4	8.445	3	5	No	614.4	8	0	No
Astaxanthin	596.39	9.69	2	4	No	596.39	6	0	No

Dieckol	742.08	4.768	11	18	No	742.08	29	0	No
Dimethylpropiothetin	134.04	0.578	0	2	Yes	134.04	2	0	Yes
Dioxinodehydroeckol	370.03	2.479	5	9	Yes	370.03	14	0	No
Eckol	372.05	2.289	6	9	No	372.05	15	0	No
Eckstolonol	370.269	2.479	5	9	Yes	370.269	14	0	No
Eicosapentaenoic acid	302.22	8.022	1	2	No	302.22	3	1	No
Fucodiphloroethol G	498.396	2.426	10	12	No	498.396	22	0	No
Fucosterol	412.691	10.629	0	1	No	412.691	1	0	No
Fucoxanthin	658.42	9.874	2	6	No	658.42	8	0	No

Fucoxanthinol	616..41	9.134	3	5	No	616..41	8	0	No
Glutaric acid	132.04	-0.352	2	4	Yes	132.04	6	2	No
Kappa-carrageenan	788.65	-9.011	0	25	No	788.65	25	0	No
Loliolide	196.246	0.952	1	3	Yes	196.246	4	0	Yes
Phlorofucofuroeckol A	602.07	3.65	9	14	No	602.07	23	0	No
Phloroglucinol	126.03	0.573	3	3	Yes	126.03	6	0	Yes
Racemosin A	346.1	2.089	2	6	Yes	346.1	8	0	Yes
Spiralisone A	454.27	8.725	3	5	No	454.27	8	0	No
Spiralisone B	452.26	8.398	3	5	No	452.26	8	0	No

Succinic acid	118.088	-0.71	2	4	Yes	118.088	6	2	No
Nicotinic acid	123.111	-0.508	1	3	Yes	123.111	4	1	No
Uridine	244.203	-2.117	4	8	Yes	244.203	12	0	No

Appendix 3 Table 3: The drug-likeness test results of the natural product references compounds for Lipinski's rule and BBB likelihood analyses

	Lipinski's rule					BBB likeness			
Compound	**Molecular weight (=<500)**	**LogP (=<5)**	**H-bond donor (=<5)**	**H-bond acceptor (=<10)**	**Follow this rule?**	**Molecular weight (=<400)**	**H-bond (total) (=<8)**	**No acids (0)**	**Follow this rule?**
(-)-Epigallocatechin gallate	458.08	2.984	8	11	No	458.08	19	0	No
Curcumin	368.13	1.945	2	6	Yes	368.13	8	0	Yes
Bisdemethoxycurcumin (Curcumin III)	308.1	1.887	2	4	Yes	308.1	6	0	Yes
Demethoxycurcumin (Curcumin II)	338.12	1.916	2	5	Yes	338.12	7	0	Yes
Rivastigmine	250.17	1.363	0	4	Yes	250.17	4	0	Yes