Neuroprotective activities of industry-grade fucoxanthin and fucoidan alone and in combination against beta-amyloid and hydrogen peroxide-induced neurotoxicity in PC12 cell line

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List of Abbreviations

Abbreviation	Explanation
AD	Alzheimer's Disease
Aβ	Amyloid-beta
Aβ1-42	Amyloid-beta peptide 1-42
APP	Amyloid Precursor Protein
BDNF	Brain-Derived Neurotrophic Factor
CI	Combination Index
CNS	Combination Index
DMSO	Dimethyl Sulfoxide
FD	Fucoidan
FX1	Fucoxanthin (95.6% purity)
FX4	Fucoxanthin (5.6% purity)
H2O2	Hydrogen Peroxide
LDH	Lactate Dehydrogenase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenenyltetrazolium Bromide
NDDs	Neurodegenerative Diseases
PBS	Phosphate Buffered Saline
PI3K	Phosphoinositide 3-Kinase
ROS	Reactive Oxygen Species
SEM	Standard Error of the Mean
SD	Standard deviation
WHO	World Health Organization

Abstract

Introduction: Alzheimer's disease (AD) is a chronic neurodegenerative disorder marked by gradual loss of memory and cognitive decline. It is featured pathologically by beta-amyloid ($A\beta_{1-42}$) plaques accumulation, oxidative stress and mitochondrial dysfunction in the brain cells. The treatment options available only relieve the symptoms but do not prevent the disease progression. Thus, marine-derived natural products like fucoxanthin and fucoidan are gaining popularity due to their antioxidant, anti-inflammatory and neuroprotective effects. But most of the previous research are done with highly pure analytical grade compounds, which are not only expensive but also impractical for large scale use. We hypothesized that these compounds would show neuroprotection against beta-amyloid and hydrogen peroxide induced stresses in PC12 cells and their combination would show synergistic effects. Methods: PC12 cells were treated with different concentrations of industrial-grade of FX1, FX4 and FD. The cells were then exposed to amyloid-beta (1 μM and 2 μM) and hydrogen peroxide (100 μM and 150 μM) induced cytotoxicity. Cell viability was measured by the MTT assay. The Chou-Talalay method was used to determine the combined neuroprotective potential of the compounds. The CI value obtained helped to determine the effects are synergistic (CI <1), additive (CI=1) or antagonistic (CI >1). Results: FX1, FX4 and FD significantly provided dose-dependent neuroprotective effects when tested individually against both Aβ and H_2O_2 induced cytotoxicity. FX4 (20 μg/mL), being lower in purity (5.6%), showed protection like FX1(95.6%) at 10 μg/mL. FD in all concentrations showed protection against both the stresses. Combination treatments, restored of up to 93% viability for FX1+FD at 5+25 μg/mL, 10+25 μg/mL and FX4+FD at 20+25 μg/mL, respectively, with CI < 1 showing a synergistic effect. The combinations restored cell viability at higher concentrations of the stress (2 μM of Aβ and 150 μM of H_2O_2). Though under the high stress, fewer concentrations were found synergistic, which might be due to any previous cellular damage. Conclusion: This study showed that industry-grade fucoxanthin and fucoidan was able to show neuroprotection against beta-amyloid and hydrogen peroxide-induced cytotoxicity in PC12 cells. They have shown neuroprotection when used individually against the induced stress. However, in combination, the protection was better than the sum of their individual effects. This result supported our hypothesis that, in combination, they will show synergistic effect. Moreover, FX4, being of low purity, was found to be as potent as higher purity FX1. This might be due to the presence of other crude extracts that are contributing to offer neuroprotection. These findings align with the earlier research on pure compounds. Therefore, these results demonstrate the potential of industrial-grade purity of

fucoxanthin and fucoidan as an affordable neuroprotective agent for early-stage prevention of Alzheimer's disease.

Declaration

I confirm that this thesis is my own work and has not been submitted for any other academic qualification at this or any other institution. To the best of my knowledge, all sources of information, ideas, or content taken from other authors have been clearly referenced throughout the document.

Raisa Mahboob

09/06/2025

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1 Introduction

1.1 Increasing challenge of Alzheimer's disease

Alzheimer's disease (AD) is a progressive, neurodegenerative condition known to be one of the most significant public health challenges of the 21st century worldwide. It is responsible for 60-80% of the total cases of the primary cause of dementia (Abeysinghe et al., 2020; Sonkusare et al., 2005; Vogt et al., 2023). AD is marked by a gradual and permanent loss of memory and cognitive functions, leading to significant behavioral changes and increasing difficulties in everyday activities. This condition leads to a gradual loss of independence as patients experience increasing challenges in processing information and managing personal activities (Mobaderi et al., 2024; van der Flier et al., 2023).

The World Health Organization reports that more than 55 million individuals suffer from symptoms of dementia (Korczyn & Grinberg, 2024), which is projected to rise to 152.8 million by 2050 (Nichols & Vos, 2021). Dementia was ranked as the 7th leading cause of death in 2019 globally, contributing to about 1.6 million (17.3%) of neurological deaths (Avan & Hachinski, 2021; Collaborators et al., 2021). For people over 70 years old, AD is the 7th cause of death worldwide (Wang et al., 2024). This growth highly affects not only patients and families but also the healthcare system. Because the need for long-term care and additional treatment resources is crucial (Matthews et al., 2019).

1.2 Pathophysiology of Alzheimer's disease

Alzheimer's disease is a complex neurodegenerative disease. It is characterized by the buildup of beta amyloid plaques, tau proteins phosphorylation, and impaired synaptic function. Other problems that might be associated with the progression of AD are chronic inflammation in the brain, oxidative damage, impaired mitochondrial function and disrupted cholinergic signalling (Goedert, 2009; Jain et al., 2024). Though the exact cause of AD is still uncertain, beta amyloid accumulation and Tau protein phosphorylation are the most widely studied Alzheimer's model for AD pathogenesis (Goedert, 2009). Some new insights into detecting and managing AD include blood-based biomarkers, advanced brain imaging and personalized treatment approaches, which are gaining popularity. Moreover, a healthy lifestyle like physical activity and proper nutrition plays a key role in slowing memory loss to improve the quality of life (Mishra et al., 2025).

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Figure 1. 1 Pathophysiology of the Alzheimer's disease (Lau et al., 2023).

1.3 The role of beta-amyloid in AD

Beta-amyloid accumulation in the brain is one of the most important precursors for the development of Alzheimer's disease. Two enzymes, β-secretase and γ-secretase break down the amyloid precursor protein (APP) to generate the beta-amyloid peptides (Blennow et al., 2006; Neuner et al., 2020). Aβ1-42 is the most prominent among all the peptides generated. It normally clumps outside the neuron and forms plaques. Thus, it disrupts the normal signalling and synaptic functions of the neurons (Abeysinghe et al., 2020; Alberdi et al., 2013; Barret, 2010; Sehar et al., 2022). This clump is further responsible for any damage to the brain, for example oxidative stress, inflammation and cell death as well (Abeysinghe et al., 2020; Kumar et al., 2016; Mattson, 2004). Recent studies have found that Aβ oligomers (small soluble forms of beta-amyloid) are more toxic and harmful for the brain than large plaques. These oligomers are mainly responsible for disrupting synaptic functions, initiating phosphorylation of tau proteins and triggering neuroinflammation. Altogether, they further lead towards memory loss and cognitive decline (Benilova et al., 2012; Cleary et al., 2005; Haass & Selkoe, 2007; Sehar et al., 2022). Along with this, they also boost up oxidative stress by producing reactive oxygen species (Asai et al.) in brain cells leading the brain cells towards apoptosis (Mattson, 2004; Pereira et al., 2005; Xu et al., 2017). These large amounts of amyloid-beta plaques, along with tau-protein tangles in the brain, persist for a long period of time, serving as a precursor to aging brain and AD (DeFina et al., 2013; Wang et al., 2012).

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Figure 1. 2 Comparison between a healthy brain and an Alzheimer's disease brain highlighting Aβ plaques and tau protein tangles (Breijyeh & Karaman, 2020).

1.4 Current treatment options for AD

Alzheimer's disease is a slow and progressive brain disorder. It causes memory loss, cognitive decline, confusion, and changes in behavior. There are no definite treatment options available to slow down the progression of AD. Thus, multiple treatments have been developed and are emerging to slow down the progression of AD.

Cholinesterase inhibitors are one of the most prominent treatments available for AD. They prevent the breakdown of acetylcholine (a brain chemical responsible for learning and memory) (Francis et al., 2010). Another drug, Donepezil, is effective for all stages of AD. It works by improving cholinergic signalling and thus elevates cognitive functions. In earlier stages of AD, rivastigmine and galantamine have been found to offer moderate improvements. However, the main purpose of these medications is to delay the symptoms of the disease, rather than to halt its progression. Also, they come with a lot of side effects, including nausea, vomiting and decreased heart rate, which may limit their use (Baakman et al., 2022; Singh et al., 2024). Memantine is another approved drug for the treatment of moderate to severe AD. It targets NMDA (N-methyl-D-aspartate) receptors and thus lowers the damage induced by

excessive glutamate release in brain cells (Keiski, 2017; Liu et al., 2019; Melnikova, 2007). It also helps to improve memory and thinking when used in conjunction with cholinesterase inhibitors (DeFina et al., 2013; Pardo-Moreno et al., 2022; Santos et al., 2016; Singh et al., 2024). As discussed previously, treatment options are now shifting towards targeting the root cause of AD, especially beta-amyloid and tau protein buildup in brain cells. Monoclonal antibody therapy, Aducanumab was first approved by the FDA in 2021 (Sevigny et al., 2016; Singh et al., 2024). It works by clearing amyloid plaques and slowing down cognitive decline. Though its approval was a bit controversial because of inconsistency in trial results and potential side effects including observation of swelling and bleeding in brain cells during imaging studies (Guardado Yordi et al., 2024; Leisher et al., 2023; Reish et al., 2023).

Despite this, newer drugs like Lecanemab and Donanemab, which target earlier forms of amyloid-beta, have shown more promising results in clinical trials, demonstrating reductions in plaque load and modest improvements in cognitive performance (Shi et al., 2022; Söllvander et al., 2015). In addition to these therapies, anti-tau therapies are also under development to block tau aggregation and reduce cognitive deterioration (Godyń et al., 2016; Panza et al., 2016).

Though pharmacological treatments play a key role in Alzheimer's disease management, non-drug interventions like cognitive exercises, physical activities, natural compound-based nutritional supplements, and dietary approaches (Aschettino et al., 2021), are gaining recognition for their positive impact on brain health due to their anti-inflammatory and neuroprotective properties (Gardener et al., 2012; McGrattan et al., 2019; Scarmeas et al., 2009). It is of special interest that natural compounds such as astaxanthin, fucoxanthin, fucoidan, and polyphenols have demonstrated significant neuroprotective activities in multiple mechanisms (Alghazwi, Smid, Musgrave, et al., 2019), hence holding great potential in developing preventive and management strategies for alleviating and slowing Alzheimer's disease such as functional food and nutritional supplements (Abraham et al., 2021).

1.5 Marine natural compounds used in neuroprotection and managing AD

Oceans cover over 70% of the Earth's surface and host a diverse range of marine species comprising nearly half of global biodiversity (Boeuf, 2011; Kim & Wijesekara, 2010). Terrestrial organisms have traditionally been the main source of natural products, but there has been a notable shift in recent years towards exploring bioactive compounds from marine environments (Bălașa et al., 2020). Till now, more than 440,000 natural products have been

identified, yet only about 10% of these have been commercially utilized (Pereira, 2019). These environments are abundant with various health-promoting natural compounds. Many of the organisms from the ocean produce polysaccharides, bioactive peptides, PUFAs (polyunsaturated fatty acids), minerals pigments, polyphenols, vitamins and enzymes (Lonikar et al., 2021; Shahidi, 2008). These compounds can be incorporated into the development of functional foods. Moreover, these compounds have gained attention for providing new insights into preventive strategies for curing the progression of diseases. They have already shown their potential to manage cancer, wound healing, high blood pressure, diabetes, inflammation, and very recently, neurodegenerative diseases (Abachi et al., 2019; Lonikar et al., 2021; Ozogul et al., 2021; Phadke et al., 2021; Unnikrishnan & Jayasri, 2018).

A growing amount of research is now focusing on marine compounds particularly on neuroprotective effects (Agrawal, 2020). Among all marine organisms, marine algae-derived bioactive compounds are extensively used for food, functional food, and nutritional supplements globally (Ghaliaoui et al., 2024; Ghosh et al., 2022; Tiwari & Troy, 2015). During the last 20 years, there have been increasing scientific studies from in vitro, in vivo, and clinical studies of marine algae-derived compounds for their activities in managing neurodegenerative conditions through various mechanisms, mostly their neuroprotective properties (Šimat et al., 2024). More than 100 compounds have been identified from marine algae for their neuroprotective activities. Among these compounds studied, the top five compounds showing the most promising activities, with potential to be further developed into preventive and treatment products, include fucoxanthin, astaxanthin, polyphenols, fucoidans, and Eckols (Alghazwi et al., 2016). Among these highly potent neuroprotective compounds, fucoxanthin and fucoidan have been chosen for this project as they represent two different types of compounds: carotenoids (Anjana & Arunkumar, 2024) and polysaccharides (Karim et al., 2017). By the 2040s, neurodegenerative diseases are expected to overtake cancer as the second leading cause of death among the elderly, following cardiovascular diseases (Gammon, 2014). Consequently, there is a rapidly increasing demand to discover and develop novel, effective prevention and treatment of AD from marine natural products of hugely unexplored resources (Deepika et al., 2023; Rahman et al., 2021).

Fucoxanthin, a prominent carotenoid found in brown seaweeds, accounts for more than 10% of the total carotenoid production in nature (Kim & Wijesekara, 2017). Fucoxanthin, isolated from *Undaria pinnatifida* and *Ecklonia bicyckis*, has demonstrated β-secretase inhibitory activity, suggesting its potential against Alzheimer's disease (Jung et al., 2016). It was found

in previous research that it effectively reduces Aβ toxicity in PC12 cells (Alghazwi, Smid, Musgrave, et al., 2019).

Fucoxanthin is a marine carotenoid mainly sourced from *Sargassum horneri*. Research has shown that fucoxanthin can help to reduce amyloid-beta accumulation and improve cognitive function (Xiang et al., 2017). Animal studies have also reported that fucoxanthin has the potential to reverse the memory loss caused by scopolamine (Lin et al., 2016).

Fucoxanthins unique structural and chemical features including an allenoic bond, epoxide ring and hydroxyl group help to exert its antioxidant properties (Bae et al., 2020). These properties are mitigating free radicals. Moreover, fucoxanthin activates the PI3K/Akt pathways, which then stimulate the Nrf2 pathway. Moreover, this helps to protect nerve cells from oxidative damage (Kim et al., 2022).

Another marine-derived compound, fucoidan is a sulphated polysaccharide, which has been used in health supplements due to its wide range of biological effects (Šimat et al., 2020). Studies have shown that fucoidan exhibits neuroprotective activities, along with other seaweed-derived polysaccharides, including carrageenan and ulvan(Xiang et al., 2017). It helped to lower oxidative stress and apoptosis in the HT-22 hippocampal cell line (Olasehinde et al., 2020). Fucoidan extracted from *Fucus vesiculosus* and *Undaria pinnatifida* was also found to reduce nerve cell damage caused by beta-amyloid and hydrogen peroxide in PC12 cell lines Alzheimer's disease (Alghazwi, Smid, Karpiniec, et al., 2019).

As both fucoxanthin and fucoidan have been shown to be promising individually in providing neuroprotection, there is still very limited research available on how they might work together. Thus, a clear gap remains in exploring their combined or synergistic effects, which serves a key role for future studies.

1.6 Cell lines tested for neuroprotective assays

Different cell lines have been widely used to study neurodegeneration in Alzheimer's disease. These cell lines differ from one another in terms of species of origin, ability to differentiate, and, most importantly, their relevance to brain biology. Commonly used models include PC12, SH-SY5Y and HT-22 cells, which are summarized below in Table 1.1

Table 1. 1 Common cell lines and their properties used in AD-related research

Cell line	Origin	Species	Key features	Common applications	Limitations	References
PC12	Pheochromocytoma (adrenal)	Rat	Differentiates from NGF, dopaminergic traits	Oxidative stress, Aβ toxicity, and drug screening	Lacks BBB and human-specific context	(Alghazwi, Smid, Musgrave, et al., 2019; Wiatrak et al., 2020)
SH-SY5Y	Neuroblastoma	Human	Differentiates from RA/BDNF, human origin	Tauopathy, mitochondrial dysfunction	Variable differential protocol	(Encinas et al., 2000; Pandey et al., 2024; Xicoy et al., 2017)
HT-22	Hippocampal neurons	Mouse	High sensitivity to oxidative stress	Hydrogen peroxide-induced toxicity models	Poor differential capacity	(Liu et al., 2025; Olasehinde et al., 2019; Sillapachaiyaporn et al., 2022)
SK-N-SH	Neuroblastoma	Human	Parent of SH-SY5Y, moderate differentiation ability	Limited neurodegeneration studies	Limited use due to immature phenotype	(Chen et al., 2022; Djiokeng Paka et al., 2016)

The two most common cell types for in vitro studies for Alzheimer's disease research are PC12 and SH-SY5Y cell lines. They are popular due to their ability to mimic neuronal damage under experimental settings (Encinas et al., 2000; Xicoy et al., 2017). Though PC12 cells are generated from rat adrenal tumors, they are highly studied due to their ability to differentiate into neuron-like features. Neuroprotective studies have shown that they have predictable growth and stable responses against beta-amyloid and hydrogen peroxide induced toxicity (Alghazwi, Smid, Musgrave, et al., 2019). Moreover, as they originated from rats, they are also easy to grow and handle. Their consistent behavior under lab conditions and observing cellular mechanisms like oxidative stress, apoptosis or mitochondrial dysfunction help the researchers to rely on this cell line.

In contrast, SH-SY5Y cells originated from human neuroblastoma. Though they offer better relevance to human neuron characteristics, still they are tough to maintain. Specific differentiation protocols and growth conditions are critical to maintain that can further affect the reproducibility among experimental batches (Xicoy et al., 2017).

Considering these factors, PC12 cells were chosen for this research project. They have been already well-established in previous research involving fucoxanthin and fucoidan against beta-amyloid and hydrogen peroxide induced toxicity (Alghazwi, Smid, Karpiniec, et al., 2019; Alghazwi, Smid, Musgrave, et al., 2019). Thus, their applicability for assessing neuroprotective potential of these two compounds is justified for the current project.

1.7 Neuroprotective potential of Fucoxanthin and fucoidan

Marine derived compounds, especially fucoxanthin and fucoidan, have been studied extensively for their ability to protect neuronal cells from different types of stress models including amyloid-beta and hydrogen peroxide. Table 1.2 shows a structured summary of current literature, outlining key experimental models and findings to detail the rationale behind this project.

Table 1. 2 A summary of previous neuroprotective research on fucoxanthin and fucoidan at different purities

Compound	Study model	Cell line/model used	Key findings	Purity of the compounds	References
Fucoxanthin	Aβ-induced toxicity	PC12	Reduced ROS, activated Nrf2, improved viability	≥95% (Analytical grade)	(Kim & Wijesekara, 2017)
	Oxidative stress	SH-SY5Y	Activated Nrf2/ARE pathway, reduced apoptosis	≥95% (Analytical grade)	(Liu et al., 2019)
	APP/PS1 Alzheimer's model	APP/PSI mice	Improved cognition, reduced plaque load	≥95% (Analytical grade)	(Jiang et al., 2025)
	Mitochondrial dysfunction	Primary hippocampal neurons	Stabilized mitochondria, downregulated apoptosis	≥90% (Analytical grade)	(Wu et al., 2021)
	Aβ-induced apoptosis	PC12	Dose-dependent increase in cell viability (0.01-2mM), reduced Aβ aggregation	Crude extract	(Alghazwi, Smid, Karpiniec, et al., 2019)
Fucoidan	Parkinson's model	MPTP & Rotenone PD models	Enhanced dopaminergic survival, increased antioxidants	≥90% (Standardized extract)	(Nagata et al., 2021)

	Amyloid toxicity	SH-SY5Y	Reduced ROS, protected against Aβ induced death	≥90% (Analytical grade)	(Kang et al., 2024)
	$A\beta_{42}$ induced apoptosis	SH-SY5Y	Activated PI3K/Akt signaling pathway and MAPK cascades, ameliorated neuronal apoptosis	≥90% (Analytical grade)	(Thulin et al., 2016)
	Aβ and H2O2 induced toxicity	PC12	Reduced cytotoxicity, preserved neuronal structure	≥85% (Standardized extract)	(Alghazwi et al., 2020)
	Aβ25-35 and D-Gal induced toxicity	PC12 and AD mice model	Protected against apoptosis, improved learning and memory impairments	≥85% (Standardized extract)	(Park et al., 2019)
	Neuroinflammation in vivo (AD)	Mice	Lowered cytotoxicity, preserved neuronal structure	Crude extract	(Wang et al., 2022)
	Zinc-induced neurotoxicity	HT-22	Reduced oxidative damage, enhanced cholinergic markers	Crude extract	(Olasehinde et al., 2020)
	6-OHDA induced neurotoxicity	Rat model	Inhibited nigral microglial activation, suppressed Nox1-triggered oxidative stress	Crude extract	(Luo et al., 2009)
	LPS-induced neuronal damage	Mice	Reduced expression of IL-8, TNF-α, iNOS, and MPO, decreased infarct size in ischemic brain injury	Crude extract	(Jiang et al., 2012)

	Monocrotophos-induced AD model	*Drosophila melanogaster*	Dose and time dependent inhibition of cholinergic and monoamine metabolized enzymes, improved cognitive function	Crude extract	(Thakare et al., 2018)
	Combined with Cerebro lysin	Rat model	Enhanced suppression of proinflammatory markers and oxidative stress protein compared to individual treatments	Crude extract	(Ardah et al., 2019)

Fucoxanthin and fucoidan have shown individual neuroprotective effects, but their combined potential is still underexplored. This research addresses the gap by studying their combined effects in PC12 cell models under beta amyloid and hydrogen peroxide induced stress.

1.8 Purity of fucoxanthin and fucoidan in neuroprotective studies

Most of the previous studies have focused on high-purity compounds. Purity of compounds vary from any one of the three from either analytical grade (>95%), or reagent grade (>90%) or crude extracts. It is very important to evaluate the purity of the compounds before going for real world applications into various formulations. It is also necessary to assess whether the purity of the compounds is related to the neuroprotective potential of the compounds. Thus, in this review, we will be looking into the types and purities of fucoxanthin and fucoidan, which were used in previous studies to understand any underlying knowledge gaps.

1.8.1 Analytical-grade compounds

Many studies use analytical grade of fucoxanthin and fucoidan with purity over 95% (Nagata et al., 2021),. These were sourced from reputed suppliers like Sigma-Aldrich (Alghazwi, Smid, Musgrave, et al., 2019; Liu et al., 2022). This grade of compounds come with a lot of limitations. These purified forms do not contain the natural components present in crude extracts, which could act together to enhance biological activity (Lourenço-Lopes et al., 2022). Moreover, these compounds cannot replicate the natural complexity and interactions that occurs in food matrices. Thus, it makes them less suitable for real-world use, especially in nutraceutical development (Din et al., 2022). In addition to these, the higher price of the compounds makes it impractical for commercial product development (Menaa et al., 2021). That is why they are mostly suitable for lab-based studies rather than any food or therapeutic products.

1.8.2 Highly Pure Compounds

Some studies used highly pure compounds of around 90% purity than the analytical grade. Fucoxanthin of 90% pure grade has been studied for its ability to support mitochondrial function in neuroblastoma cells (Zhang et al., 2017). In another research, Alghazwi et al. (2019) assessed 90% pure fucoidan in Alzheimer's disease model and found promising neuroprotective effects. Studying highly pure compounds help the researchers to understand the specific actions and safety in controlled lab settings (Alghazwi, Smid, Karpiniec, et al., 2019). Though, it has some limitations over the advantages, like this compound are costly, has lower bioavailability

and stability during storage or formulation. These are the reasons that makes them less practical for developing any nutraceutical or functional foods (Doolaanea et al., 2023).

1.8.3 Crude extracts

Crude extracts are rich in bioactive compounds of fucoxanthin and fucoidan. But they also come with a lot of limitations. And that is why they are not suitable for neuroprotective applications. Their inconsistent composition, variable active compounds in between batches make it very tough to maintain precise dosing (Mohibbullah et al., 2022). These inconsistencies can further affect the reproducibility of the experiments (Li et al., 2022). Again, this grade of compounds come with a lot of impurities that might have affected the cellular functions. Moreover, the purification of the compounds are challenging, time consuming and not a scalable solution for any nutraceutical or functional food development (Li et al., 2022; Zayed et al., 2020).

1.9 Research gaps, challenges, and future directions

This review extensively highlights on the neuroprotective potential of two marine derived compounds from brown seaweed, fucoxanthin and fucoidan. Several in vitro studies have confirmed that they were promising against beta-amyloid and hydrogen peroxide induced damage in neuronal cells. Though, more research is needed to confirm their lab-based results in real world settings. More in vivo studies and clinical trials can bridge this gap and help to develop preventive measures from fucoxanthin and fucoidan for early-stage prevention of Alzheimer's. These treatment options include cholinesterase inhibitors that help to alleviate memory and thinking (Francis et al., 2010). Researchers are aiming to treatments that are focused on the root causes of AD. And this work is focused mostly on two major causes of AD, amyloid-beta plaques and tau protein tangles (Abeysinghe et al., 2020; Golde, 2006).

1.9.1 The synergistic effects of fucoxanthin and fucoidan have not been studied

Although fucoxanthin and fucoidan have been individually studied for their neuroprotective properties, no research has explored their potential synergistic effects. Moreover, there is variability in the purity of commercially available compounds, which affects their bioavailability and efficacy. Standardizing these compounds is crucial for consistent outcomes in nutraceutical development. Therefore, this project hypothesizes that the combination of fucoxanthin and fucoidan can lead to synergistically improved neuroprotection activities, specifically against Aβ and H_2O_2-induced neurotoxicity, when compared to their individual use.

1.9.2 Limited understanding of the effect of the compound's purity

While there are different purity grades of fucoxanthin and fucoidan used in previous studies, however, there is no study to compare directly on the effect of the purity of these two compounds, hence leaving a question on how the previous studies primarily using high-purity or analytical-grade compounds like those from Sigma-Aldrich or crude extracts can be translated into commercial product development and production. The project hypothesizes that the purity of fucoxanthin and fucoidan (or the impurities) have a significant impact on the neuroprotective activities, recognizing that these products may differ in bioavailability, bioactivity, and safety compared to highly purified forms. As the commercial-grade products, more likely used in functional food and nutraceuticals, will use commercially available industrial-grade fucoxanthin and fucoidan, therefore this project will use these industrial-grade compounds with different purities to test this hypothesis.

1.9.3 Limited studies using commercially available industrial-grade compounds

To ensure the neuroprotective results can be applied to commercial product development, the project proposes to assess the neuroprotective activities of commercially available industrial grade fucoxanthin and fucoidan. Unlike analytical-grade compounds that are highly refined, industrial-grade products contain certain and often unknown impurities that could influence their efficacy and bioavailability. It is therefore valuable to study the industrial-grade compounds that are more likely to be used in formulating nutraceuticals and functional foods. This research project will bridge the gap between lab-based findings and practical, market-ready applications for neuroprotection in conditions like AD.

1.9.4 Need for developing functional foods and nutraceuticals for AD prevention and management

The growing prevalence of Alzheimer's disease and other neurodegenerative conditions emphasizes the urgent need for preventive and management measures before their onset, and during their progression. Functional food and nutraceuticals formulated from marine compounds like fucoxanthin and fucoidan offer promising benefits such as accessibility, and safety compared to synthetic drugs for long-term use. Therefore, this project will provide the scientific understanding of how to translate into practical commercial functional foods and nutraceutical products for prevention and management of neurodegenerative risks such as AD.

1.9.5 Lack of clinical trial

While many in vitro and some in vivo animal studies demonstrate the neuroprotective potentials of fucoxanthin and fucoidan, there is a significant gap in human clinical trials, large-scale, randomized studies are necessary to confirm their safety, efficacy, and appropriate dosage guidelines in human populations.

1.9.6 Future directions

To effectively translate lab-based research into practical applications, future studies on fucoxanthin and fucoidan should focus on several critical areas. Firstly, further *in vivo* and clinical trials are crucial to confirm the neuroprotective effects observed in *in vitro* studies, such as with PC12 cells. This step is vital for determining the compound's safety, efficacy, and appropriate dosage for real-world use in neurodegenerative diseases like Alzheimer's. Moreover, we are studying industrial grade fucoxanthin and fucoidan for research aiming to support their use in any functional food or supplement development in future. Also studying their cellular mechanisms like PI3K/Akt and Nrf2 will help us to explain how they are providing the protection. Thus, this could provide further guidance in developing more targeted therapies for Alzheimer's prevention. Lastly, using industrial grade of fucoxanthin and fucoidan in drug formulation could be tested more to improve their stability, absorption and considering them more suitable for everyday prevention of Alzheimer's disease.

1.10 Aim, hypothesis, and novelty of this research project

1.10.1 Project aim

The aim of this project was to explore how two natural compounds from marine sources, fucoxanthin and fucoidan can help protect PC12 nerve cells from damage caused by beta-amyloid and hydrogen peroxide. The effects of different purity levels of fucoxanthin and their combinational effects fucoidan were also studied.

1.10.2 Project objectives

The research objectives for this study included

i. To find safe, non-toxic doses of fucoxanthin (95.6% and 5.6%) and fucoidan (20%), which were tested on PC12 cells for their cytotoxic effects
ii. To evaluate if fucoxanthin and fucoidan can protect neurons from beta-amyloid and hydrogen peroxide-induced damage
iii. To assess whether their combination results in synergistic, additive, or antagonistic effects using the Chou-Talalay method

iv. To compare the effects of different purity levels of the same compounds to determine which of the purities can retain a broader therapeutic potential

1.10.3 Hypothesis

Fucoxanthin and fucoidan, when combined, exhibit a synergistic neuroprotective effect against beta-amyloid and hydrogen peroxide-induced damage in PC12 cells. The different purities of these two compounds will exhibit varying neuroprotective activities, not in proportion to their purity, due to the effects of different excipients.

1.10.4 Rationale behind the hypothesis

The neuroprotective potential of two marine-derived compounds, fucoxanthin and fucoidan, has already been established in recent studies. Fucoxanthin protects the neuronal cells from beta-amyloid induced toxicity by activating the antioxidant pathway and reducing oxidative damage. Thus, fucoxanthin was found to be protecting the PC12 cells from amyloid-beta-induced toxicity (Alghazwi, Smid, Musgrave, et al., 2019). In contrast, fucoidan was found to be promising to protect the neuronal cells by limiting the oxidative stress and inhibiting apoptosis caused by hydrogen peroxide (Gao et al., 2012).

While both compounds were found to be promising individually in protecting neuronal cells, their combined effects in protecting brain cells have not been studied extensively. Given that they work through different mechanisms, like fucoxanthin primarily through antioxidant activity and fucoidan through anti-inflammatory pathways. It was predicted that their combination could potentially offer enhanced neuroprotection. Moreover, the purity of these compounds may influence their efficacy. Lower-purity extracts might retain additional bioactive components that contribute to their overall effect.

This study aims to explore the synergistic effects of fucoxanthin and fucoidan at varying purity levels on neuronal cells subjected to oxidative stress and amyloid-beta toxicity. By doing so, it aims to provide insights into the potential of these compounds as cost-effective, natural therapeutic agents for neurodegenerative diseases, such as Alzheimer's.

1.10.5 Research novelty

Almost all the studies conducted before used mostly highly pure or analytical-grade extracts to study neurotoxicity and neuroprotection models. However, we were studying industry-grade of different purities of fucoxanthin and fucoidan. This makes the research more practical, as it

is not only cheaper but also provides real-world insight into the applicability of the purity grades studied in commercial products, yielding tangible benefits. Again, we were studying the combined effect of the compound using the Chou-Talalay method to compare the individual and combined effects. We were also studying two different types of cell damage methods: one generated by beta-amyloid and another by hydrogen peroxide, to better reflect the level of protection that can be gained from the purities of the compounds studied. Thus, the study was novel and provided useful information that could be applied in the future to incorporate these compounds into health supplements.

1.10.6 Significance of the project to human health and biotechnology

This project aims to offer a new and more natural approach to prevent neurodegenerative diseases like Alzheimer's. Alzheimer's is becoming very common nowadays. The most common treatments available only manage the symptoms and come with side effects. In contrast, marine-derived compounds such as fucoxanthin and fucoidan are known for being safe and capable of protecting various forms of cell damage, including inflammation and oxidative stress. If these were proven effective, they could help improve brain health and maintain quality of life for everyone, especially older individuals. Moreover, the research investigates how varying levels of purity of these compounds affect their activity. This is important because we were studying affordable, commercial-grade compounds to evaluate whether they could be used in future functional food development, making them easily accessible to everyone. This approach would also be a sustainable and innovative, ensuring health and nutrition in adults, particularly the elderly. In the future, this study could also focus on how these compounds work at the molecular level, their potential for drug synergy, and how they can be proven more efficacious in combination, inspiring researchers to work on this area. Thus, this kind of research could open the arena of natural neuroprotective products to offer a more scalable, reproducible, and sustainable solution in biotechnology.

2 Materials and Methods

2.1 Chemicals and materials

Unless otherwise stated, analytical or cell-culture grade chemicals and consumables were used in this experiment. The chemicals were selected based on the previous literature review and past studies in our laboratory (Alghazwi et al., 2016). The materials used, including culture media, supplements, cell culture assay reagents, and chemicals, sourced from manufacturers are summarized and listed in **Table 2.1.**

Table 2. 1 Summary of chemicals, reagents, and consumables used in the project experiments

Materials / Grade	**Supplier**	**Catalogue No.**	**Purpose and use**
Dulbecco's Modified Eagle Medium (DMEM)	Merck Life Science Pty Ltd	SLM-020-A	Nutrient-rich basal medium for cell growth
Fetal Bovine Serum (FBS)	Merck Life Science Pty Ltd	TMS-016-B	Provides growth factors and nutrients
Penicillin (10,000 U/ml)-Streptomycin (10,000 µg/ml) (100X)	Merck Life Science Pty Ltd	516106	Prevents bacterial contamination during cultivation
Non-essential amino acids (NEAA, 100X)	Merck Life Science Pty Ltd	TMS-001-C	Supports metabolic activity and protein synthesis in cultured cells
Trypsin-EDTA (10X)	Merck Life Science Pty Ltd	T4174-20ML	Used for cell detachment and passaging of adherent cells
MTT reagent	Merck Life Science Pty Ltd	CT01-5	Viability assay for quantifying cell metabolic activity
Dimethyl Sulfoxide (DMSO)	Merck Life Science Pty Ltd	20-139	Solubilizing MTT formazan crystals and dissolving hydrophobic compounds

Phosphate Buffer Saline (PBS)	Thermo-Fisher Scientific	10010023	Isotonic buffer used for washing and preparing dilutions
Hydrogen Peroxide (30%)	Merck Life Science Pty Ltd	Not specified	Oxidative induced cytotoxic, inducer for neurotoxicity in cultured cells
Amyloid beta peptide ($A\beta_{1-42}$)	Thermo-Fisher Scientific	Not specified	Used for inducing amyloid-beta-related neurotoxicity in vitro
Culture flasks, pipettes, tips, etc.	Adelab Scientific	Not specified	General cell culture handling and liquid transfer
Fucoxanthin (≥95%)	Shandong Jiejing Group	Not specified	Sourced from brown seaweed (*Laminaria japonica*), it is a marine carotenoid used to assess antioxidant and neuroprotective activity in vitro
Fucoxanthin (5.6%)	Shandong Jiejing Group	Not specified	Sourced from brown seaweed (*Laminaria japonica*), it is a marine carotenoid used to assess antioxidant and neuroprotective activity in vitro
Fucoidan (20%)	(Shandong Jiejing Group)	Not specified	Sourced from brown seaweed (*Laminaria japonica*), a sulphated polysaccharide used to assess neuroprotection and anti-inflammatory properties

2.2 Cell line

PC12 cell lines is widely used for neurobiological studies (Westerink & Ewing, 2008). It was sourced from rat adrenal medulla pheochromocytoma cells (Xie et al., 2023). Table 2.2 below outlines the main characteristics and basic requirements for culturing PC12 cells in the laboratory. When stimulated, these cells can differentiate and develop neuron-like features. This makes them ideal for neurodegenerative assays and oxidative induced cytotoxicity studies (Chua & Lim, 2021; Wiatrak et al., 2020).

Table 2. 2 Biological features and standardized laboratory culturing requirements for PC12 cells in neuroprotection assays (Matsuzaki et al., 2019; Xie et al., 2023).

Characteristics	Description
Origin	Rate adrenal medulla pheochromocytoma
Morphology	Small, rounded, and semi-adherent with neuron-like projections when stimulated
Medium	DMEM, supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% non-essential amino acid (NEAA)
Culture conditions	At 37°C incubator with 5% CO_2
Sub-culture method	1x Trypsin-EDTA treatment when 70-80% confluency was reached
Passage range for use	5-7 passages
Observation protocol	Daily at almost a fixed time for morphology and contamination
Contamination control	Following aseptic technique, sterile handling using a class II biosafety cabinet, and media replacement every 2-3 days

2.3 Cell culturing protocol

2.3.1 Stock solution preparation

A Class II biosafety cabinet was used to maintain aseptic conditions throughout the experimental procedure. Moreover, we followed manufacturer guidelines and standardized protocols from previous research to ensure consistency across all the replicates (Alghazwi et

al., 2016). The preparation of different stock solutions to be used during the experiment is described below

2.3.1.1 Preparation of Phosphate Buffer Saline (PBS)

PBS was used in this experiment as a buffer for key cell culturing and handling procedures. It is widely chosen due to its isotonicity and pH stability. During the experiments, it was used to wash the PC12 cells during the sub-culturing process. It also helped to remove any residual serum and media component present in the flask that can interfere with the sub-culturing procedure. Moreover, it was used as a vehicle control in the treatment group to dissolve fucoidan (20%). Additionally, it was used to dissolve reagents, such as MTT. PBS was prepared by dissolving one commercially available PBS tablet in 200 mL of sterile Milli-Q water. It was done by following the manufacturer's guidelines to achieve a 1x working solution (Sigma-Aldrich, 2022). The solution was then sterilized by autoclaving it. It was stored at 4°C in properly labelled aliquots following the standard protocols of maintenance for in vitro applications (Alghazwi, 2018; Scientific, n.d.-a).

2.3.1.2 MTT reagent preparation

MTT assay is one of the simplest and widely used methods to observe cell viability. Living cells convert the MTT reagent into purple crystals and thus help to determine alive and healthy cells in the culture plate (Li et al., 2023; Van Meerloo et al., 2011). The MTT reagent (5 mg/mL) was prepared by dissolving Thiazolyl Blue Tetrazolium Bromide in sterile 1x PBS. The solution was vortexed and filter-sterilized through a 0.2 µM syringe. It was then aliquoted into light-protected tubes. Later, it was stored at -20°C and thawed before use (Alghazwi, 2018).

2.3.1.3 Complete culture media preparation

Complete culture media was prepared by supplementing 10% (v/v) fetal bovine serum (FBS), 1% (w/v) penicillin-streptomycin, and 1% (w/v) non-essential amino acids to Dulbecco's Modified Eagle Medium (DMEM). Then the complete media was aliquoted in small sterile tubes and stored at 4°C. Before each use, the media were pre-warmed in a 37°C water bath to avoid thermal shock (Shrestha et al., 2022; Wang et al., 2025).

2.3.1.4 Trypsin-EDTA preparation for adherent cell detachment

Trypsin-EDTA was used to detach the cells from the bottom of the flasks. EDTA does this by breaking down the calcium and magnesium needed for cell attachment (Freshney, 2015). Trypsin-EDTA (1x) was prepared by diluting the 10x stock solution. It was diluted in sterile

PBS at a 1:10 (v/v) ratio under aseptic conditions. It was then stored at -20°C and thawed before use (Solutions, 2017).

2.4 Sub-culturing PC12 cells

2.4.1 Cell harvesting and routine passaging protocol

The protocol for cell sub-culturing was adopted from Alghazwi et al. (2018) and Shrestha et al. (2022). PC12 cells were grown in complete culture media. When the confluence reached approximately 70-80%, they were sub-cultured into a new flask for regular passaging. Cells were kept at 37°C in a 5% CO_2 incubator for growth. The cell monolayer was gently washed twice with sterile10mL 1x PBS to remove excess traces of the fetal bovine serum. Then, 1x Trypsin-EDTA (1:1) was used to detach the cells after incubation at 37°C with 5% CO_2. The cell pellet was obtained by centrifugation. The pellet was resuspended in 5 mL of culture media, which was used for cell counting. Then, 1.5-2 mL of the cell suspension was transferred to a new flask for experimental use (Alghazwi et al., 2018; Shrestha et al., 2022).

2.4.2 Trypan Blue assay for cell counting

PC12 cells were harvested and resuspended in culture medium according to the protocol outlined in Section 2.4.1. Cell viability was evaluated by preparing a 1:4 dilution in which 10 µL of the cell suspension was mixed with 40 µL of 0.4% Trypan Blue solution. From this mixture, 10 µL of solution was transferred to a sterilized hemocytometer. The number of viable and non-viable cells, as well as viability (%), was calculated in all four squares of a hemocytometer (Kamiloglu et al., 2020; Scientific, n.d.-b) using the equation below

$$Viable\ or\ non-viable\ cells\ per\ mL = Average\ no.of\ viable\ or\ non-viable\ cells\ per\ square\ \times Dilution\ factor\ \times 10^4$$

Cell viability (%) was calculated using the formula below:

$$Viability\ (\%) = (No.of\ Viable\ cells \div total\ no.of\ cells) \times 100$$

2.4.3 Freezing and cryopreservation protocol for PC12 cells

Following the protocol described by Yamatoya et al. (2022), PC12 cells (ATCC: CRL-1721.1) (Collection, n.d.) were cryopreserved in a -80°C freezer. It was done for long-term maintenance of cell integrity and to continue the experiments with almost similar passage numbers. Once the cultures reached 80-90% confluency, the cells were harvested and centrifuged according to the protocols outlined in Section 2.3.1. Then the cells were resuspended in freezing medium

containing 90% FBS and 10% DMSO at a density of 1-2 x 10^6 cells/mL. One mL of the resuspended solution was aliquoted and transferred into labelled cryovials which were then stored in a -80°C freezer until further use (Yamatoya et al., 2022).

2.4.4 Thawing PC12 cells from frozen stocks

Frozen vials of PC12 cells were gently agitated and thawed rapidly for 1 minutes in a warm water bath at 37°C. At room temperature, the cell suspension was transferred into a sterile tube containing 10 ml of complete DMEM medium. Viable cells were obtained as a pellet, followed by centrifugation. The supernatant was carefully removed, and the pellet was then gently resuspended in fresh complete culture medium. Then, the suspension was transferred to a new culture flask and kept at 37°C under a humidified 5% CO_2 incubator (Yamatoya et al., 2022).

2.5 MTT assay

It is the most common method for determining cell viability in viable cells. PC12 cells were seeded into a 96-well plate in 100 µL of culture medium at a density of 1×10^4 cells/well. The plates were incubated at 37°C and 5% CO2 for 24 hours for cell attachment. After incubation, the plate was aspirated to remove excess media and treated with different concentrations of the compounds. Again, the plate was incubated for 48 hours. After the incubation was over, the cells were treated with 100 µL of MTT solution (5 mg/mL in PBS) prepared and thawed before (mentioned in the protocol 2.3.1.2) and incubated for 4 hours at 37°C, 5% CO_2 incubator. This time was allowed for the viable cells to convert MTT into purple formazan crystal. The MTT was aspirated after incubation and 100 µL of DMSO was added to dissolve the formazan. The absorbance was read at 570 nm in a microplate reader (Bahuguna et al., 2017; Kumar et al., 2018; Riss et al., 2016).

2.6 Investigating the cytotoxicity of industry-grade fucoxanthin and fucoidan of different purities and concentrations against PC12 cells

2.6.1 Determining the safe concentration of DMSO to be used as a solvent control

The objective of this experiment was to assess the maximum non-toxic concentration of DMSO that can be used as a solvent vehicle control in treatment preparation. This was essential to ensure that results were observed only from the compounds tested, not from the solvent (Galvao et al., 2014; Santos et al., 2003). The cytotoxicity of DMSO on PC12 cells was evaluated by seeding at 1×10^4 cells/well (3 biological replicates, 4 technical replicates) in a 96-well plate. The plate was incubated overnight at 37°C under a humidified 5% (v/v) CO_2 incubator. After

24 hours of incubation, the cells were treated with predetermined concentrations of DMSO, ranging from 0.01% to 10% (v/v) for 24 hours. The plate was further incubated overnight. After 24 hours of incubation, the MTT assay was performed according to the protocol outlined in section 2.5, and the absorbance was read at 570 nm using a microplate reader. The safe DMSO concentration will be determined for use in the treatments with fucoxanthin dissolved in DMSO (Galvao et al., 2014).

2.6.2 Preparation of fucoxanthin and fucoidan stock solutions

Stock solutions of fucoxanthin (95.6% and 5.6%, labelled FX1 and FX4, respectively) and Fucoidan (20%, labelled FD) were prepared to assess the cytotoxicity of these compounds on PC12 cells to determine safe doses for use in the neuroprotection experiments. 200 mg of each of these compounds was measured and taken into sterile microfuge tubes. FX1 and FX4 were dissolved in 1 mL of 0.01% DMSO, and FD was dissolved in 1 mL of sterile PBS solution. All stock solutions were vortexed and sonicated to dissolve the compounds and a homogeneous mixture was prepared. The mixture was then stored at -20°C until use.

2.6.3 Prepare the standard curve

This experiment was conducted to create a standard curve correlating PC12 cell densities, thereby validating the accuracy of cell seeding in subsequent assays. Following the protocol in 2.4.1 and 2.4.2 cells were grown and counted. Cells were seeded at a density of 1×10^4 cells/well into a sterile flat-bottom 96-well plate. A cell suspension of PC12 cells with media was prepared and adjusted to a density of 400,000 cells/mL in a sterile tube. Serial two-fold dilutions were prepared to achieve final densities ranging from 0 (media-only blank control), 62.5, 1250, 2500, 5000, 10000, 20000 and 40000 cells/well. The final well volume was 100 µL and all the dilutions were added in 4 replicates. The plate was incubated overnight at 37°C under a humidified 5% (v/v) CO_2 incubator. After the 24-hour incubation period, the MTT assay was performed as described in Section 2.5. A standard curve was generated to correlate the cell number and OD readings at 570 nm during the experiments. A linear regression analysis was performed to derive an equation for predicting cell viability in future experiments.

2.6.4 Cell viability assessment of PC12 cells treated with industry-grade FX1, FX4 and FD to establish safe doses

This experiment aimed to determine the safe doses of fucoxanthin (FX1 and FX4) and fucoidan (FD) to be used in the neuroprotection assays. As shown in Figure 2.1, PC12 cells were seeded into a 96-well plate in 100 µL of culture media at a density of 1×10^4 cells/well. The plates

were incubated at 37°C and 5% (v/v) CO2 for 24 hours to promote cell attachment. The cells were treated with different purities of fucoxanthin (95.6% denoted as FX1, and 5.6% denoted as FX4) and fucoidan (20% denoted as FD) on the next day. To evaluate the safe doses, the concentrations tested for FX1 and FX4 ranged from 2.5-40 µg/mL and 6.25-100 µg/mL for FD. Later, the plates were incubated again for an additional 48 hours. The MTT assay was performed according to the protocol outlined in Section 2.5. Microsoft Excel was used for preliminary data analysis and viability calculations. Later, we used GraphPad Prism 9 to do statistical analysis, graph generation and visualize the viability trends.

The following equation was used to calculate the cell viability (%)

$$Viability\ (\%) = (No.of\ Viable\ cells \div total\ no.of\ cells) \times 100$$

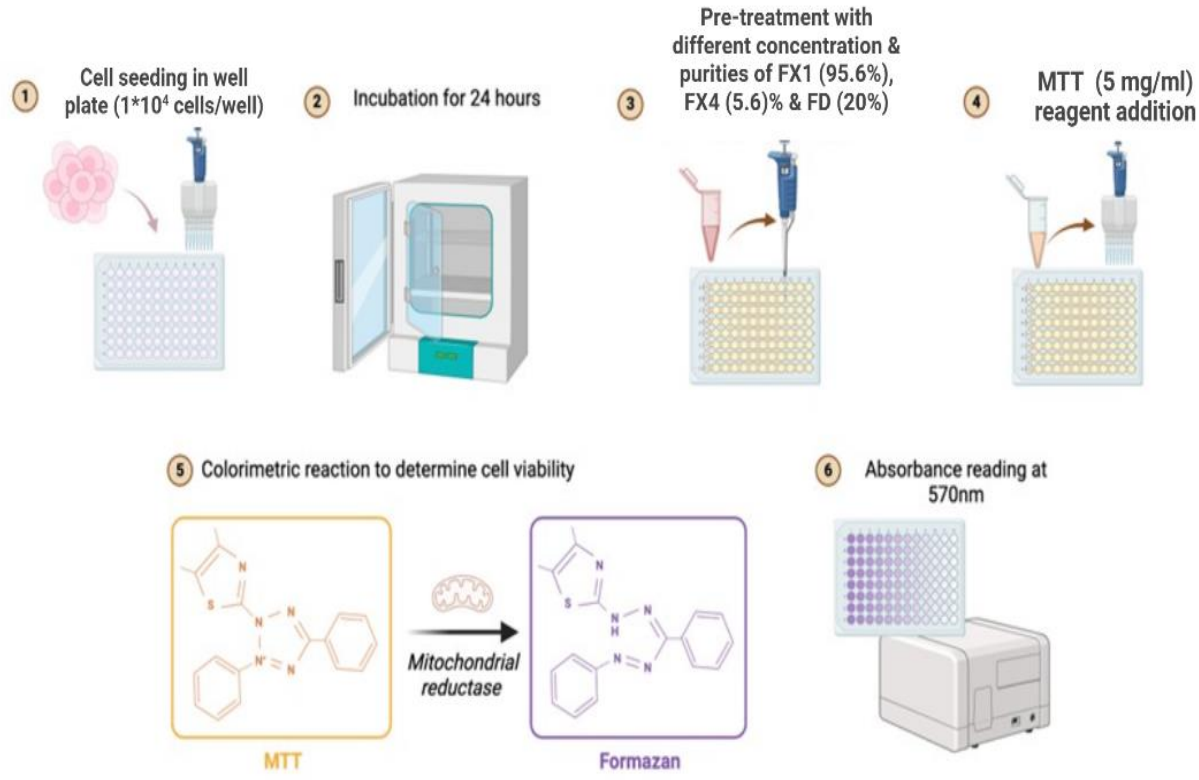


Figure 2. 1 Experimental process flow for cytotoxicity testing of fucoxanthin and fucoidan to determine the safe doses for neuroprotection experiments (Image credit: generated by Biorender).

2.7 Testing the neuroprotective activities of industry-grade FX1, FX4 and FD (alone) against amyloid-beta induced toxicity

2.7.1 Preparation of Amyloid beta ($A\beta_{1-42}$) stock solution

Lyophilized $A\beta_{1-42}$ peptide was dissolved in 0.01% (v/v) DMSO to achieve a concentrated stock solution of 1-2 mM. 1x PBS was then used for diluting the stock solutions to achieve a 100 µM working solution. The solution was filter-sterilised using a 0.2 µM syringe and aliquoted into sterile microfuge tubes. The tubes were labelled properly and stored at -70°C (Alghazwi, 2018).

2.7.2 Determining the neuroprotective activities of industry-grade fucoxanthin and fucoidan (alone) against amyloid-beta induced toxicity

As shown in **Figure 2.2,** PC12 under regular subculture maintenance were harvested and grown according to the protocol outlined in Section 2.3.1. In each experiment, the cells were seeded into the treatment plate and treated with predetermined concentrations of fucoxanthin and fucoidan. The concentrations tested for the compounds were as follows: FX1 at 0.25, 0.5, 2.5, 5, and 10 µg/mL; FX4 at 0.25, 0.5, 5, 10, and 20 µg/mL; and FD at 0.5, 1, 6.25, 25, and 100 µg/mL. The plate was then incubated for 15 minutes at 37°C under a humidified 5% (v/v) CO_2 incubator. 100 µL of 1µM or 2µM of pre-aggregated $A\beta_{1-42}$ solution was added to the cells. The plate was further incubated for 48 hours. The MTT assay was performed after the incubation period of 4 hours by following the methods described in Section 2.5. The results collected were processed through Microsoft Excel. Then GraphPad Prism 9 for statistical analysis, preparing dose-dependent graphs and visualization of the viability trends (Alghazwi et al., 2018; Shrestha et al., 2022).

2.7.3 Determining the neuroprotective activities of industry-grade fucoxanthin and fucoidan in different combinations against amyloid-beta induced cytotoxicity

The cell harvesting, passaging, and cell seeding protocols for treatment plates, as well as incubation and data analysis, were followed according to the protocols outlined in Section 2.3.1. Two forms of Fucoxanthin with different purities, FX1 (95.6%) and FX4 (5.6%), were tested in combination with Fucoidan (20%). These combinations were tested to evaluate the neuroprotective effects against amyloid-beta-induced toxicity in PC12 cells. The treatments were prepared by mixing appropriate volumes of FX1 and FX4 with FD from a 50 mg/mL stock solution in sterile tubes. Sterile fresh media were used to adjust the final well volume to 100 µL. The combinations tested for FX1 with FD included 0.25+0.5 µg/mL, 0.25+1 µg/mL, 0.5+0.5 µg/mL, 0.5+1 µg/mL, 5+25 µg/mL, 10+25 µg/mL, 5+50 µg/mL, and 10+50 µg/mL.

The concentrations texted for FX4, and FD were 0.25+0.5 µg/mL, 0.25+1 µg/mL, 0.5+0.5 µg/mL, 0.5+1 µg/mL, 5+25 µg/mL, 20+525 µg/mL, 5+50 µg/mL, and 20+50 µg/mL. All treatments were done in 4 replicates on the plate. After adding the treatment combination, the plates were incubated for 15 minutes at 37°C under a humidified 5% CO_2 incubator. Then the amyloid-beta induced cytotoxicity was introduced to cells following the protocol mentioned in section 2.7.2 followed by MTT assay in section 2.5. The experiments were conducted in three independent replicates. The results collected were processed through Microsoft Excel. Then GraphPad Prism 9 for statistical analysis, preparing dose-dependent graphs and visualization of the viability trends.

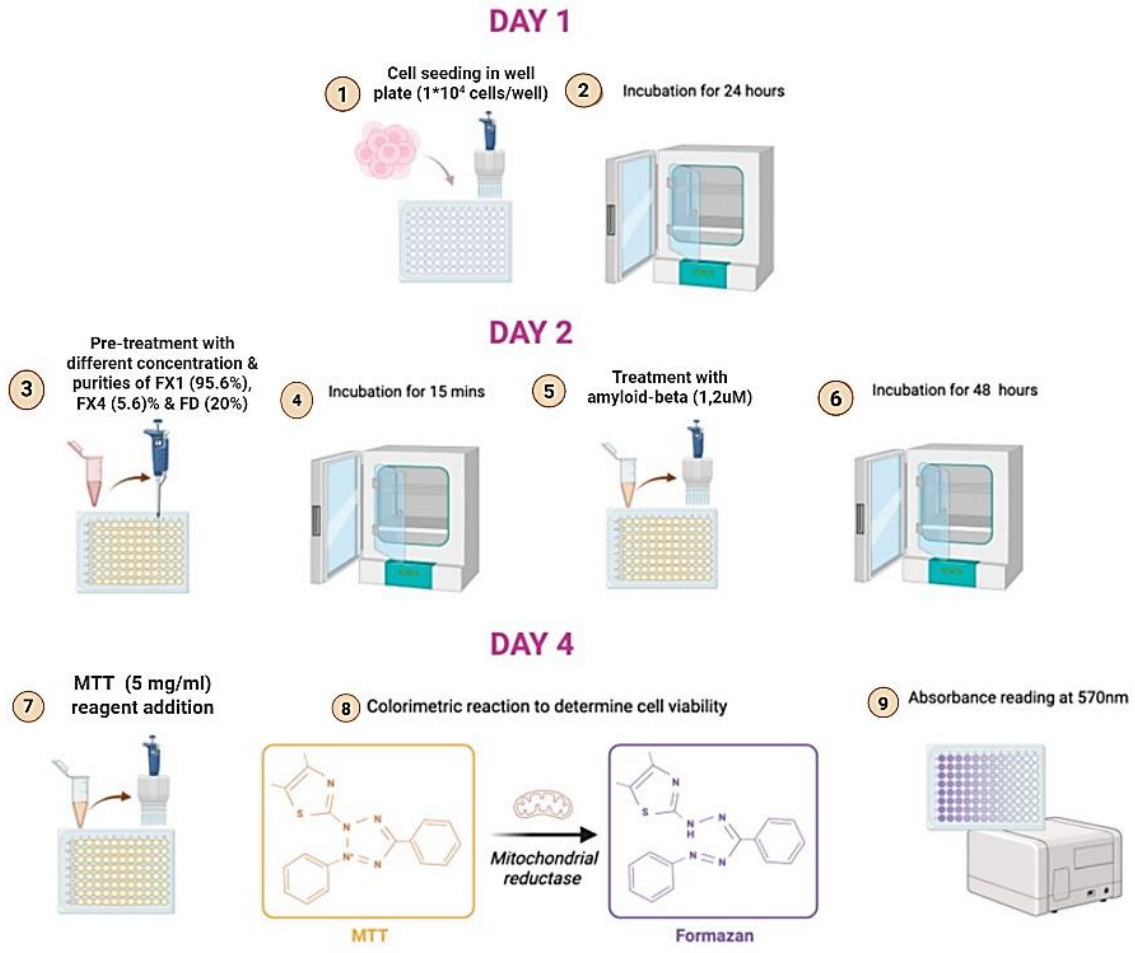


Figure 2. 2 Pictorial experimental process flow for testing the neuroprotective activities of industry-grade fucoxanthin and fucoidan alone against amyloid-beta induced toxicity (image credit: generated by Biorender).

2.8 Determining the neuroprotective activities of industry-grade fucoxanthin and fucoidan against hydrogen-peroxide induced toxicity

2.8.1 Preparation of H_2O_2 stock solution

Hydrogen peroxide stock solutions were prepared fresh on the day of the experiment to maintain their stability. A 30% (w/v) H_2O_2 solution (approximately 9.8 M) was diluted in complete media to achieve a working concentration of 100 mM. Specifically, 102 µL of 30% H_2O_2 was mixed with 9.898 mL of complete media to make a stock solution of 10 mL in total. The prepared solution was kept in the dark to avoid degradation. Complete culture media was used to prepare serial dilutions from the stock solutions to obtain a final concentration of 200 µM and 100 µM. This was expected to cause a moderate level of toxicity in PC12 cells as reported in the literature (Liu et al., 2007).

2.8.2 Determine the batch and concentration of hydrogen peroxide to be used for the neuroprotection experiments

Initially, the cytotoxicity of hydrogen peroxide was assessed using a range of concentrations (50-300 µM) against PC12 cell lines, following the protocols described in Section 2.3.1. Two batches of hydrogen peroxide were assessed in four replicates to determine their stability and the concentration that causes approximately 50% cytotoxicity. From the results, fresh batch of hydrogen peroxide with two hydrogen peroxide concentrations of 100 µM and 150 µM was selected for further experiments.

2.8.3 Determining the neuroprotective effects of fucoxanthin and fucoidan alone against hydrogen peroxide-induced toxicity

As shown in **Figure 2.3**, PC12 cells after regular subculture, were harvested, seeded, and pre-treated with different concentrations and purities of fucoxanthin and fucoidan, following the protocol outlined in Section 2.3.1. In each experiment, the cells were seeded into the treatment plate and treated with predetermined concentrations of fucoxanthin and fucoidan. The concentrations tested for the compounds were as follows: FX1 at 0.25, 0.5, 2.5, 5, and 10 µg/mL; FX4 at 0.25, 0.5, 5, 10, and 20 µg/mL; and FD at 0.5, 1, 6.25, 25, and 100 µg/mL. The plate was then incubated for 15 minutes at 37°C under a humidified 5% CO_2 incubator. 100 µL of 100 µM or 150 µM of hydrogen peroxide solution was added to the cells. The plate was further incubated for 24 hours. The MTT assay was performed after a 4-hour incubation period, following the methods described in Section 2.5.

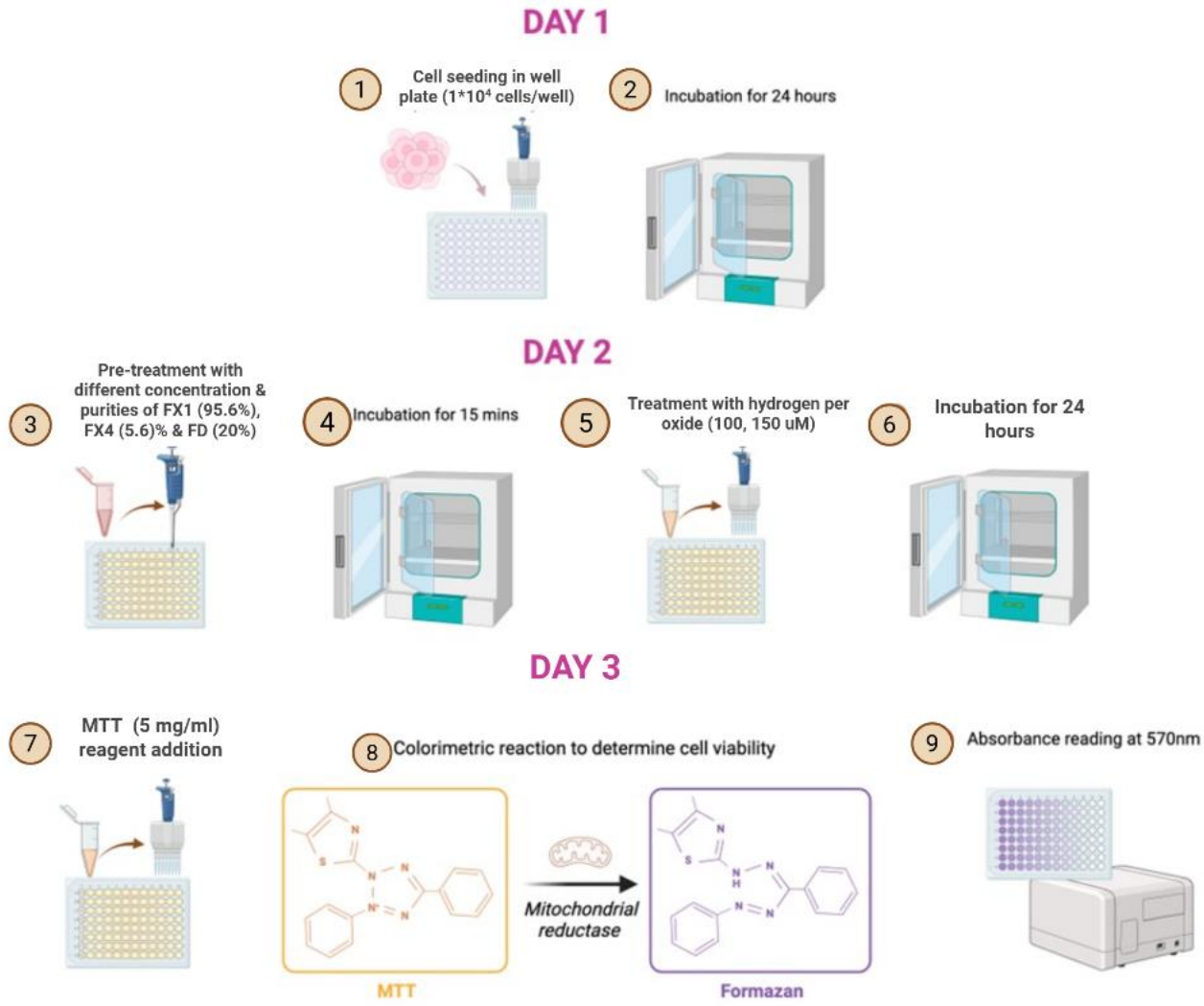


Figure 2. 3 Experimental process flow for testing the neuroprotective activities of industry-grade fucoxanthin and fucoidan alone against hydrogen peroxide-induced toxicity (Image credit: generated by Biorender).

2.8.4 Determining the neuroprotective activities of industry-grade fucoxanthin and fucoidan in combination against hydrogen-peroxide induced toxicity

PC12 cells were harvested, seeded, and treated with various combinations of industry-grade fucoxanthin and fucoidan, as described in Section 2.3.1. The combination of fucoxanthin and fucoidan was prepared fresh on the day of treatment following the protocol outlined in Section 2.8.1. The treatments were prepared by mixing appropriate volumes of FX1 and FX4 with FD from a 50 mg/mL stock solution in sterile tubes. Sterile complete media were used to adjust the final well volume to 100 µL. The combinations tested for FX1 with FD included 0.25+0.5 µg/mL, 0.25+1 µg/mL, 0.5+0.5 µg/mL, 0.5+1 µg/mL, 5+25 µg/mL, 10+25 µg/mL, 5+50 µg/mL, and 10+50 µg/mL. The combinations tested for FX4 and FD were 0.25+0.5 µg/mL,

0.25+1 µg/mL, 0.5+0.5 µg/mL, 0.5+1 µg/mL, 5+25 µg/mL, 20+525 µg/mL, 5+50 µg/mL, and 20+50 µg/mL. All treatments were done in 4 replicates on the plate. After adding treatment, the plates were incubated for 15 minutes at 37°C under a humidified 5% CO_2 incubator. Then the hydrogen peroxide induced cytotoxicity was introduced to cells following the protocol mentioned in section 2.8.3 followed by MTT assay at section 2.5. The experiments were conducted in three independent replicates.

2.9 Determination of Total Polyphenol Content (TPC) in fucoxanthin of different purities

Total Polyphenol Content (TPC) of two fucoxanthin samples, FX1 (95.6%), and FX4 (5.6%), was tested following the Folin-Ciocalteu method (Wan-Ibrahim et al., 2010). At first, each sample of fucoxanthin (1 mg) was dissolved in 1 mL of methanol. This was added to three individual wells (counted as 3 replicates) in a 96-well plate. Later, 160 µL of distilled water was added to the wells, followed by 10 µL of Folin- Ciocalteu reagent and then 20 µL of 10% sodium carbonate solution. After gently mixing of contents into the well, it was allowed to incubate for 1 hour in the dark at room temperature. After incubation was over, the absorbance was recorded using a UV-Vis spectrophotometer at 765 nm.

To generate a standard curve, gallic acid standards with concentrations ranging from 0 to 200 µg/mL were used to plot a standard curve. The polyphenol contents were then calculated using this curve. Each sample was tested in three replicates and the results were calculated as mean ± standard deviation (Noviendri et al., 2023).

2.10 Data Analysis

Statistical comparisons among multiple experimental groups of three individual replicates were performed using one-way ANOVA, followed by Tukey's multiple comparison post hoc test. A p-value less than 0.05 was considered statistically significant in all the experiments. GraphPad Prism (version 9) and Microsoft Excel were used for data processing and graph generation (Alghazwi et al., 2018). For the assessment of the combined effect of fucoxanthin with fucoidan, the Chou-Talalay method was used to evaluate the drug interaction dynamics. According to the author, the combination index (CI) value less than 1 denoted a synergistic effect (CI = <1), a value equal to 1 indicated an additive effect (CI = 1), and a value greater than 1 showed antagonism (CI = >1) (Chou, 2010).

The interaction between fucoxanthin (FX) and fucoidan (FD) under different concentration of beta-amyloid and H_2O_2 induced cytotoxicity were evaluated and the combination index (CI)

was manually calculated using Chau-Talalay method. Firstly, the average cell viability (%) for FX (1 and 4) alone, FD alone and in combination at their corresponding doses was measured form triplicates. The viability values were converted to fraction affected (Fa) using the following formula:

$$\boldsymbol{Fraction\ Affected\ (Fa) = 1 - (average\ viability\ (\%)/100)}$$

The combination index (CI) was calculated using the following equation:

$$\boldsymbol{Combination\ Index\ (CI) = \left(\frac{Fa\ combo}{Fa\ FX1}\right) - \left(\frac{Fa\ combo}{Fa\ FD}\right)}$$

Where,

Fa combo is the fraction affected by FX (1 and 4) and FD respectively

The final CI values calculated were used to interpret the interaction type – CI < 1 indicating synergism, CI = 1 indicating an additive effect and CI > 1 indicating antagonistic effect. This method allowed us to perform a precise assessment of the potential synergistic neuroprotective effect of FX (1 and 4) and FD.

3 Results

3.1 Identifying the safe concentration for DMSO to be used as a solvent control

The primary objective of this experiment was to determine the non-toxic concentration of Dimethyl Sulfoxide (DMSO) to be used in the experiment for dissolving fucoxanthin, specifically FX1 (95.6%) and FX4 (5.6%). Figure 3.1 shows the effect of different concentrations of DMSO ranging from 0.01% – 10% (v/v) on PC12 cells. 0% DMSO was considered the control for the experiment. At the lowest concentration (0.01%), the relative growth was found to be close to the control (~99%). With increasing concentration, the relative growth decreased gradually, indicating toxicity. This result indicates that 0.01% (v/v) DMSO is suitable as a solvent control for dissolving FX1 and FX4.

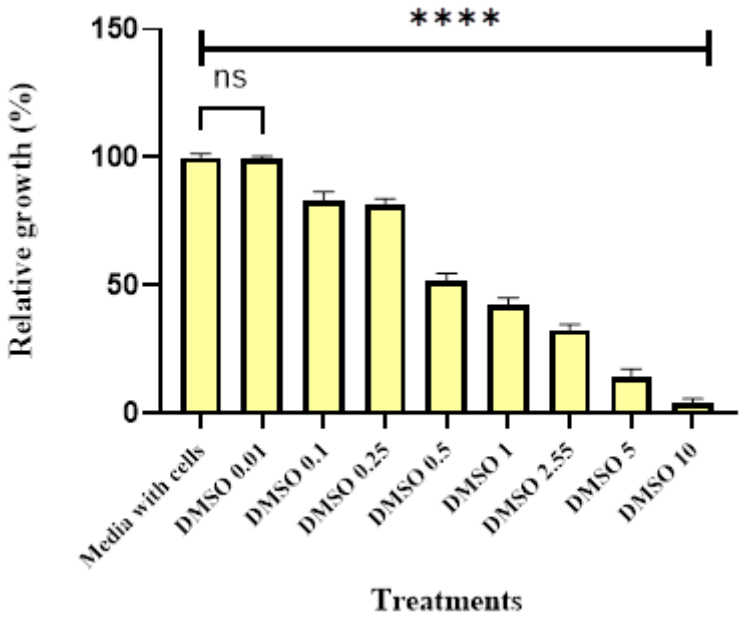


Figure 3. 1 The effect of different concentrations of Dimethyl Sulfoxide (DMSO) on relative growth (%) of PC12 cells to determine the safe concentration used in the experiment to dissolve fucoxanthin, FX1 (95.6%) and FX4 (5.6%) as a solvent control. PC12 cells were seeded at 1×10^4 cells/well. The plate was incubated for 24 hours at 37˚C and 5% CO_2 incubator. The cells were then treated with different concentrations of DMSO (0.01-10% v/v) in a final well volume of 100 µL with media and incubated for an additional 24 hours. After the incubation, MTT assay was used to determine the relative growth (%). Media with cells and 0% DMSO were used as a control. Data was represented by the mean ± SD from three independent replicates, each of which was carried out in quadruplicate wells. The error bars represented the variability among the three independent experiments. Statistical significance was analysed using one-way Anova with Tukey's post-hoc test (**** $p < 0.0001$, ns = non-significant).

3.2 Determination of safe doses of industry-grade fucoxanthin and fucoidan by MTT assay

The objective of this experiment was to determine the safe doses of two purities of fucoxanthin, FX1 (95.6%) and FX4 (5.6%), and Fucoidan, FD (20%) for use in neuroprotective assays. The PC12 cells were treated with varying concentrations of FX1, FX4, and FD for 48 hours, and cell viability was assessed using the MTT assay.

3.2.1 Determination of safe doses of industry-grade fucoxanthin, FX1 (95.6%)

Figure 3.2 shows the effect of different concentrations of FX1 (2.5, 5, 10, 20 and 40 µg/mL) on the relative growth (%) of PC12 cells, with media as a control and DMSO (0.01%) as a solvent control. Concentrations up to 10 µg/mL were found to be safe, with a statistically non-significant difference to the media control, along with the solvent control. FX1 20 µg/mL and 40 µg/mL were found statistically significant with * $p < 0.05$, and *** $p < 0.001$, respectively. The results indicated that the safe (non-toxic) doses of FX1 were below 10 µg/mL (2.5, 5 and 10 µg/mL), which will be used for further neuroprotective assays.

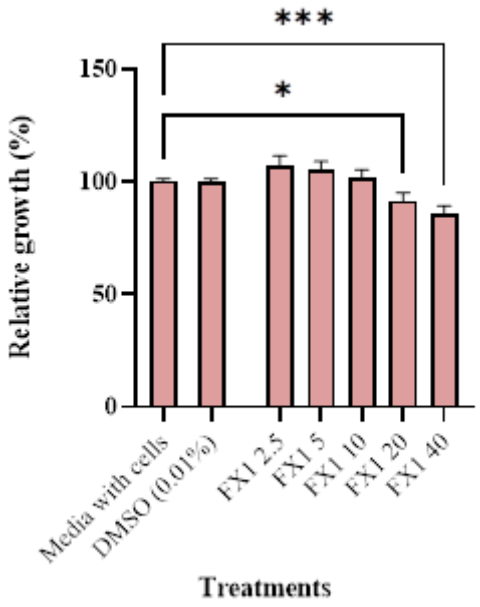


Figure 3. 2 Determining the safe doses of FX1 (95.6% Fucoxanthin) to be used in neuroprotective assays based on the cytotoxicity in PC12 cells. PC12 cells after 72 hours of regular subculture was harvested, resuspended and seeded at 1×10^4 cells/well. The plate was incubated for 24 hours at 37˚C and 5% CO_2 incubator. The cells were then treated with different concentrations of FX1, specifically at 2.5, 5, 10, 20, and 40 µg/mL for 48 hours. After the incubation, the MTT assay was used to determine the relative growth (%). The culture conditions maintained constant throughout the experiment, and each well contained 100 µL of

media. Media and the solvent DMSO (0.01%) used to dissolve the FX1 were used as the control. Data were represented by the mean ± SD from three independent replicates. Each treatment was carried out in quadruplicate wells. Statistical significance was analysed using one-way Anova with Tukey's post-hoc test (* $p < 0.05$, *** $p < 0.001$, ns = non-significant).

3.2.2 Determination of safe doses of industry-grade fucoxanthin, FX4 (5.6%)

Figure 3.3 shows the effect of fucoxanthin, FX4 (5.6%), at different concentrations ranging from 2.5, 5, 10, 20, and 40 µg/mL on the relative growth (viability) of PC12 cells, with media control and DMSO (0.01%) as the solvent control. No statistically significant cytotoxicity was observed for any of the tested doses of FX4 on PC12 cells, even at the highest dose of 40 µg/mL. Therefore, safe doses below 20 µg/mL (2.5, 5. 10 and 20 µg/mL) of FX4 were selected for the following neuroprotective studies. Concentrations higher than 10 µg/mL, a decreasing trend is observed, hence, the safe doses were selected below 20 µg/mL.

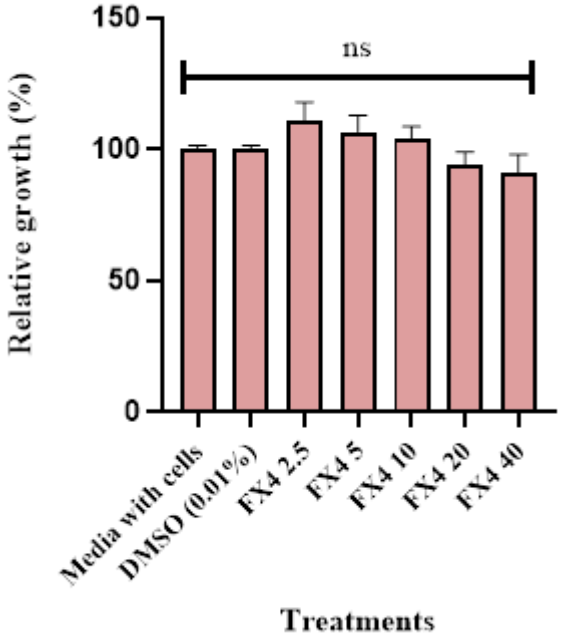


Figure 3. 3 Determining the safe doses of FX4 (5.6% Fucoxanthin) to be used in neuroprotective assays based on the cytotoxicity in PC12 cells. PC12 cells after 72 hours of regular sub-culture were harvested, resuspended and seeded at 1×10^4 cells/well. The plate was incubated for 24 hours at 37˚C and 5% CO_2 incubator. The cells were then treated with different concentrations of FX4, specifically at 2.5, 5, 10, 20, and 40 µg/mL for 48 hours. After the incubation, the MTT assay was used to determine the relative growth (%). The culture conditions maintained constant throughout the experiment, and each well contained 100 µL of media. Media and the solvent DMSO (0.01%) used to dissolve the FX1 were used as the

control. Data were represented by the mean ± SD from three independent replicates. Each treatment was carried out in quadruplicate wells. Statistical significance was analysed using one-way Anova with Tukey's post-hoc test (ns = non-significant).

3.2.3 Determination of safe doses of industry-grade fucoidan, FD (20%)

Figure 3.4 shows the effect of fucoidan (FD, 20%) at different concentrations on the cell viability of PC12 cells. PC12 cells were treated with 6.25, 12.5, 25, 50, and 100 µg/mL of FD. Media with cells and PBS were used as a control and solvent control, respectively for the experiment. A significant increase in relative growth was observed at 12.5 µg/mL with *$p < 0.05$, and a more pronounced effect at 6.25 µg/mL, with ***$p < 0.001$. Relative growth remained unchanged across the higher doses. Thus, all doses up to 100 µg/mL were safe for the neuroprotective assays, as they showed similar relative growth compared to the control. But for experimental purposes, three concentrations were selected for further experiments ranging from low to high concentrations, including 6.25, 25 and 100 µg/mL.

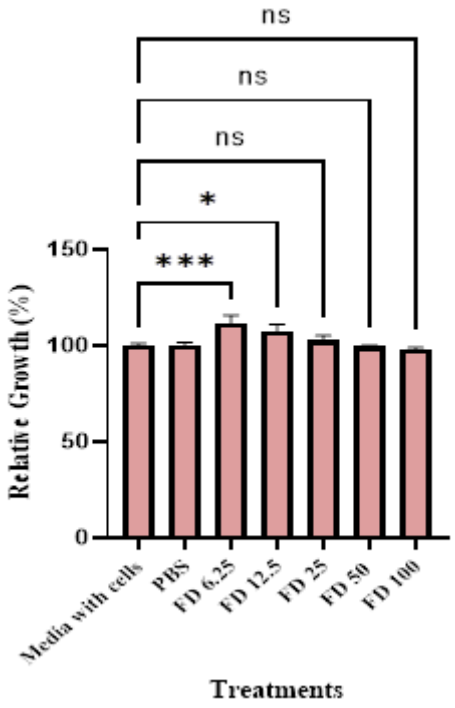


Figure 3. 4 Determining the safe doses of FD (20% Fucoidan) to be used in neuroprotective assays based on the cytotoxicity in PC12 cells. PC12 cells after 72 hours of regular subculture were harvested, resuspended and seeded at 1×104 cells/well. The plate was incubated for 24 hours at 37˚C and 5% CO2 incubator. The cells were then treated with different concentrations of FD, specifically at 6.25, 12.5, 25, 50, and 100 µg/mL for 48 hours. After the incubation, the MTT assay was used to determine the relative growth (%). The culture conditions maintained

constant throughout the experiment, and each well contained 100 µL of media. Media and the solvent DMSO (0.01%) used to dissolve the FX1 were used as the control. Data were represented by the mean ± SD from three independent replicates. Each treatment was carried out in quadruplicate wells. Statistical significance was analysed using one-way Anova with Tukey's post-hoc test (* $p < 0.05$, *** $p < 0.001$, ns = non-significant).

3.3 Neuroprotective effect of industry-grade fucoxanthin and fucoidan alone against beta-amyloid induced cytotoxicity in PC12 cells

This experiment aimed to investigate the neuroprotective potential of industry-grade fucoxanthin, with two different purities, FX1 (95.6%), and FX4 (5.6%), and Fucoidan (20%), alone against beta-amyloid ($A\beta_{1-42}$)-induced cytotoxicity in PC12 cells. Two concentrations of amyloid-beta (1 µM and 2 µM) were used to cause neurotoxicity in PC12 cells. FX1, FX4, and FD were tested alone in concentrations decided at section 3.2.1 for FX1 (2.5, 5, and 10 µg/mL), 3.2.2 for FX4 (5, 10 and 20 µg/mL) and 3.2.3 for FD (6.25, 25, and 100 µg/mL) to determine the level of neuroprotection provided against the beta-amyloid induced cytotoxicity. Relative growth was measured using the MTT assay after a 48-hour incubation period.

3.3.1 Neuroprotection of industry-grade fucoxanthin, FX1 (95.6%) alone against 1 µM amyloid-beta ($A\beta_{1-42}$) induced cytotoxicity of PC12 cells

Figure 3.5 shows the neuroprotection effect of FX1 (95.6%) at concentrations of 2.5, 5, and 10 µg/mL on PC12 cell viability followed by exposure to 1 µM amyloid-beta ($A\beta_{1-42}$). Media with cells and DMSO (0.01%) were used as a blank and solvent controls, respectively. Amyloid-beta ($A\beta_{1-42}$) has shown a significant reduction in cell viability to approximately 74.44%, when compared to the media control. Pre-treatment with FX1 has shown a significant dose-dependent increase in cell viability to ~84.3% for 2.5 µg/mL (*** $p < 0.001$), to ~84.3% for 5 µg/mL (**** $p < 0.0001$) and the highest to ~91% 10 µg/mL (**** $p < 0.0001$).

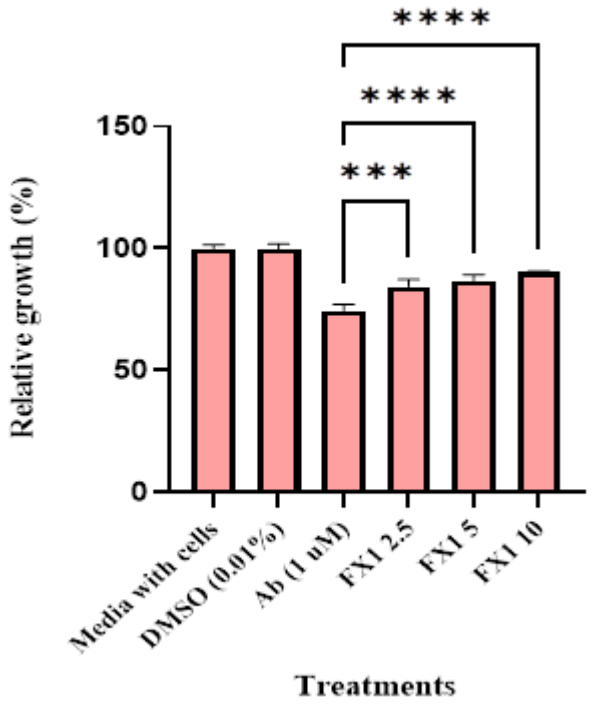


Figure 3. 5 Determining the neuroprotective effect of FX1 (95.6%) at different concentrations against beta-amyloid (1 µM)-induced cytotoxicity in PC12 cells. PC12 cells after 72 hours regular subculture was harvested, resuspended and seeded at 1×10^4 cells/well. The plate was incubated for 24 hours at 37˚C and 5% CO_2 incubator. The cells were then pre-treated with FX1 at concentrations of 2.5, 5, and 10 µg/mL for 15 minutes, followed by exposure to 1 µM of amyloid-beta to induce the induced cytotoxicity. After 48 hours of exposure, cell viability was assessed using the MTT assay. The total well volume was 100 µL with media. Media with cells and the solvent DMSO (0.01%) used to dissolve the FX1 were used as the control. Data was represented by the mean ± SD from three independent replicates, each of which was carried out in quadruplicate wells. Statistical significance was analysed using one-way Anova with Tukey's post-hoc test (*** $p < 0.001$ and **** $p < 0.0001$).

3.3.2 Neuroprotection of industry-grade fucoxanthin, FX4 (5.6%) alone against 1 µM amyloid-beta ($A\beta_{1-42}$) induced cytotoxicity of PC12 cells

Figure 3.6 shows the effect of FX4 (5.6%) at different concentrations on the neuroprotection of PC12 cells when exposed to 1 µM amyloid-beta ($A\beta_{1-42}$). Media and 0.01% of DMSO were used as the negative and solvent control. Treatment with $A\beta_{1-42}$ showed a significant level of reduction in cell viability compared to control to an average of ~74% (**** $p < 0.0001$). However, when the cells were pre-treated with FX4 at 5, 10, and 20 µg/mL, cell viability

significantly improved in a dose-dependent manner, reaching approximately 90%, 92% and 93%, respectively, with statistical significance (**** $p < 0.0001$).

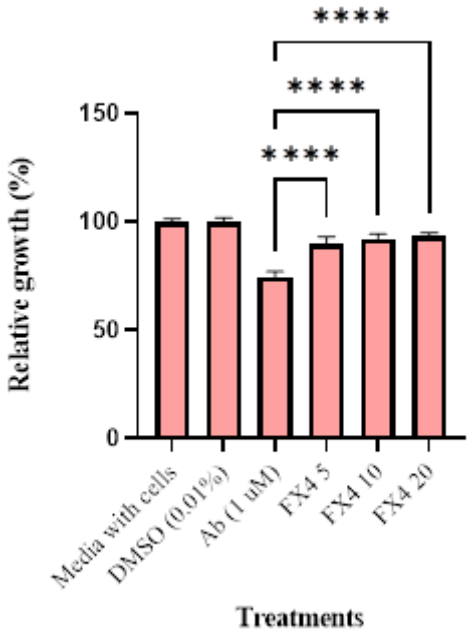


Figure 3. 6 Determining the neuroprotective effect of FX4 (5.6%) against beta-amyloid (1 µM)-induced cytotoxicity in PC12 cells. PC12 cells after 72 hours of regular sub-culture were harvested, resuspended and seeded at 1×10^4 cells/well. The plate was incubated for 24 hours at 37˚C and 5% CO_2 incubator. The cells were then pre-treated with FX4 at concentrations of 5, 10, and 20 µg/mL for 15 minutes, followed by exposure to 1 µM of amyloid-beta to induce the induced cytotoxicity. After 48 hours of exposure, relative growth was assessed using the MTT assay. The total well volume was 100 µL with media. Media with cells and the solvent DMSO (0.01%) used to dissolve the FX4 were used as the control. Data was represented by the mean ± SD from three independent replicates, each of which was carried out into quadruplicates wells. Statistical significance was analysed using one-way Anova with Tukey's post-hoc test (**** $p < 0.0001$).

3.3.3 Neuroprotection of industry-grade fucoidan, FD (20%) alone against 1 µM amyloid-beta ($A\beta_{1-42}$) induced cytotoxicity of PC12 cells

Figure 3.7 shows the neuroprotective effect of fucoidan (20%) at concentrations of 6.25, 25, and 100 µg/mL on PC12 cell viability when exposed to 1 µM amyloid-beta ($A\beta_{1-42}$). Media and sterile PBS were used as the blank and solvent controls. Treatment with $A\beta_{1-42}$ resulted in a significant reduction (**** $p < 0.0001$) in relative growth, averaging ~74% compared to the control. However, when the cells were pre-treated with FD at 6.25, 25, and 100 µg/mL, the cell

viability significantly improved to ~ 91%, 93% and 92% respectively with statistical significance of **** $p < 0.0001$. The optimal results were mainly observed between 25-100 µg/mL.

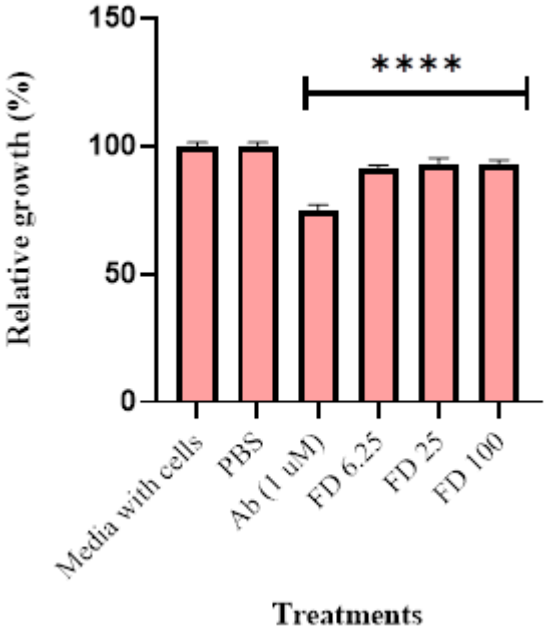


Figure 3. 7 Determining the neuroprotective effect of FD (20%) against beta-amyloid (1 µM)-induced cytotoxicity in PC12 cells. PC12 cells after 72 hours of regular sub-culture were harvested, resuspended and seeded at 1×10^4 cells/well. The plate was incubated for 24 hours at 37˚C and 5% CO_2 incubator. The cells were then pre-treated with FD at concentrations of 6.25, 25, and 100 µg/mL for 15 minutes, followed by exposure to 1 µM of amyloid-beta to induce the induced cytotoxicity. After 48 hours of exposure, relative growth was assessed using the MTT assay. The total well volume was 100 µL with media. Media with cells and the solvent PBS used to dissolve the FD were used as the control. Data was represented by the mean ± SD from three independent replicates, each were carried out into quadruplicates wells. Statistical significance was analysed using one-way Anova with Tukey's post-hoc test (**** $p < 0.0001$).

3.3.4 Neuroprotection of industry-grade fucoxanthin, FX1 (95.6%) alone against 2 µM amyloid-beta ($A\beta_{1-42}$) induced cytotoxicity

As indicated in sections 3.3.1-3.3.3 of the results, the doses selected for neuroprotection effects have shown very similar effects without an obvious dose-dependent manner. Additionally, the 1uM amyloid-beta has shown only 26% cytotoxicity. To further understand the neuroprotective effects, the experiments have been extended to test lower doses and the higher cytotoxicity of approximately 50% induced by 2uM amyloid-beta.

Figure 3.8 shows the effect of FX1 (95.6%) at 0.25, 0.5, 2.5, 5, and 10 µg/mL on PC12 cell viability followed by exposure to 2 µM amyloid-beta ($A\beta_{1-42}$), which induced cytotoxicity. Media with cells and 0.01% DMSO were served as blank and solvent controls respectively. Amyloid-beta alone resulted in a strong reduction in relative growth (%) compared to the control group, to around 51%. With increasing concentration of FX1, the level of neuroprotection against $A\beta_{1-42}$ increased. A slight increase to ~56% was observed for 0.25 µg/mL which increased further to ~73% for 5 µg/mL. The highest neuroprotection was found to be ~81% for 10 10 µg/mL, highlighting a dose-dependent neuroprotection for FX1 with statistical significance of ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$, respectively, for 2.5, 5 and 10 10 µg/mL. The rest of the concentrations were found to be non-significant different statistically.

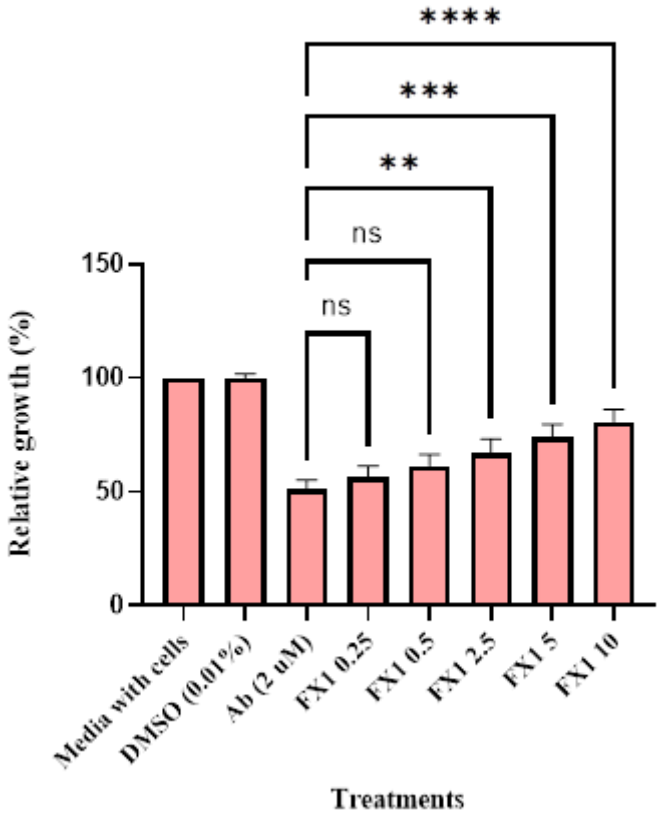


Figure 3. 8 Determining the neuroprotective effect of FX1 (95.6%) against beta-amyloid (2 µM)-induced cytotoxicity in PC12 cells. PC12 cells after 72 hours of regular sub-culture were harvested, resuspended and seeded at 1×10^4 cells/well. The plate was incubated for 24 hours at 37˚C and 5% CO_2 incubator. The cells were then pre-treated with FX1 at concentrations of 0.25, 0.5, 2.5, 5, and 10 µg/mL for 15 minutes, followed by exposure to 2 µM of amyloid-beta to induce the induced cytotoxicity. After 48 hours of exposure, relative growth was assessed

using the MTT assay. The total well volume was 100 µL with media. Media with cells and the solvent DMSO (0.01%) used to dissolve the FX1 were used as the control. Data was represented by the mean ± SD from three independent replicates, each of which was carried out in quadruplicate wells. Statistical significance was analysed using one-way Anova with Tukey's post-hoc test (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and ns = non-significant).

3.3.5 Neuroprotection of industry-grade fucoxanthin, FX4 (5.6%) alone against 2 µM amyloid-beta ($A\beta_{1-42}$) induced cytotoxicity of PC12 cells

Figure 3.9 shows the effect of FX4 (5.6%) at 0.25, 0.5, 5, 10 and 20 µg/mL on PC12 cell viability followed by exposure to 2 µM amyloid-beta ($A\beta_{1-42}$), which induced cytotoxicity. Media with cells and 0.01% DMSO were served as blank and solvent controls respectively. 2 µM Amyloid-beta alone resulted in a strong reduction in cell viability compared to the control group to around 51%. With increasing concentration of FX4, the level of neuroprotection against 2 µM $A\beta_{1-42}$ increased. FX4 at 0.25, 0.5 and 5 µg/mL showed moderate protection of ~55% and ~62%, while 10 and 20 µg/mL showed stronger and significant neuroprotection of approximately 71% and 82% (** $p < 0.01$ and *** $p < 0.001$, respectively). This result indicated that, FX4 can show neuroprotection against severe amyloid-beta (2 µM)-induced neurotoxicity in a dose-dependent manner in PC12 cells.

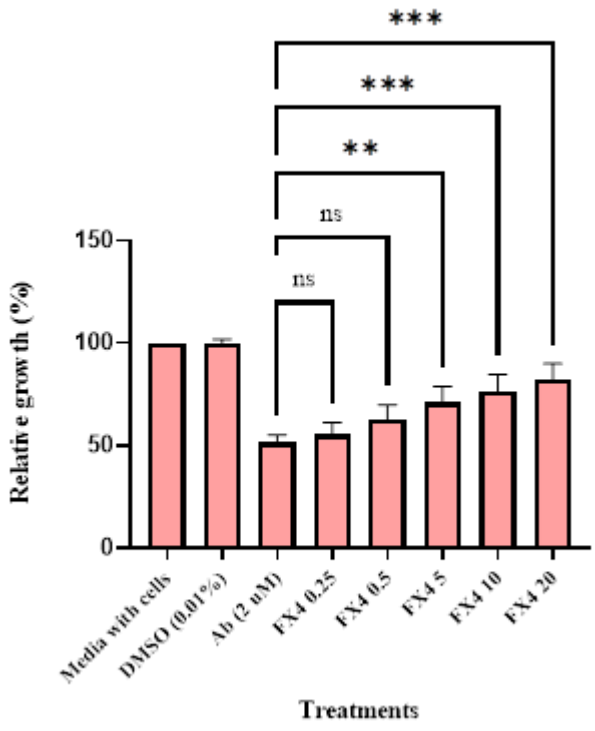


Figure 3. 9 Determining the neuroprotective effect of FX4 (5.6%) against beta-amyloid (2 µM)-induced cytotoxicity in PC12 cells. PC12 cells after 72 hours of regular sub-culture were

harvested, resuspended and seeded at 1×10^4 cells/well. The plate was incubated for 24 hours at 37˚C and 5% CO_2 incubator. The cells were then pre-treated with FX4 at 0.25, 0.5, 5, 10 and 20 µg/mL for 15 minutes, followed by exposure to 2 µM of amyloid-beta to induce the induced cytotoxicity. After 48 hours of exposure, relative growth was assessed using the MTT assay. The total well volume was 100 µL with media. Media with cells and the solvent DMSO (0.01%) used to dissolve the FX4 were used as the control. Data was represented by the mean ± SD from three independent replicates, each were carried out into quadruplicates wells. Statistical significance was analysed using one-way Anova with Tukey's post-hoc test (** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$).

3.3.6 Neuroprotection of industry-grade fucoxanthin, FD (20%) alone against 2 µM amyloid-beta ($A\beta_{1-42}$) induced cytotoxicity of PC12 cells

Figure 3.10 shows the effect of fucoidan (FD, 20%) at 0.5, 1, 6.25, 25, and 100 µg/mL on PC12 cell viability, followed by exposure to 2 µM amyloid-beta ($A\beta_{1-42}$), which induced cytotoxicity. Media with cells and PBS were served as blank and solvent controls respectively. Amyloid-beta alone resulted in a significant reduction in relative growth compared to the control group, approximately 51%. Pre-treating the cells with lower concentrations of FD (0.5 and 1 µg/mL) did not show a significant improvement in viability (%) on PC12 cells (~59%), though an upward trend was observed. Though, higher concentrations (6.25, 25, and 100 µg/mL) showed significant protection with ~64%, 77% and 86% respectively (*** $p < 0.001$ and **** $p < 0.0001$). Thus, a dose-dependent trend of neuroprotection against 2 µM amyloid-beta ($A\beta_{1-42}$) induced cytotoxicity of PC12 cells was observed for fucoidan (20%).

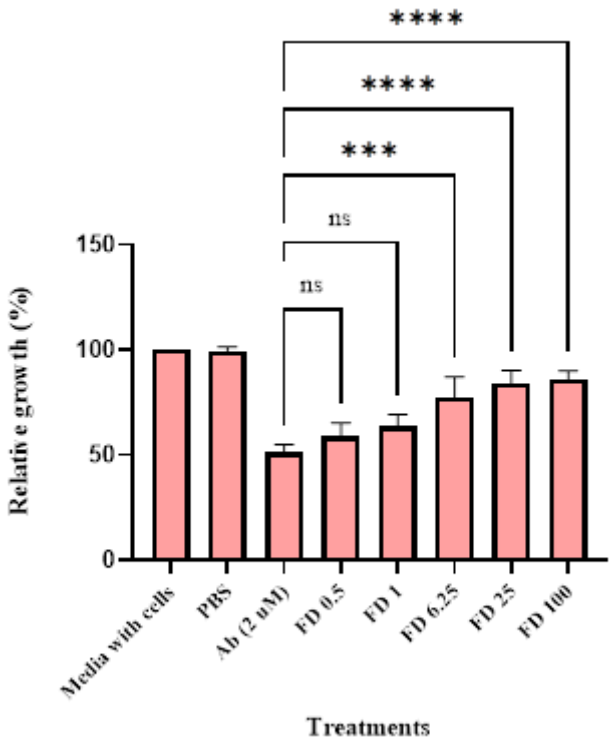


Figure 3. 10 Determining the neuroprotective effect of FD (20%) against beta-amyloid (2 µM)-induced cytotoxicity in PC12 cells. PC12 cells after 72 hours of regular subculture were harvested, resuspended and seeded at 1×104 cells/well. The plate was incubated for 24 hours at 37˚C and 5% CO2 incubator. The cells were then pre-treated with FD at 0.5, 1, 6.25, 25 and 100 µg/mL for 15 minutes, followed by exposure to 2 µM of amyloid-beta to induce the cytotoxicity. After 48 hours of exposure, cell viability was assessed using the MTT assay. The total well volume was 100 µL with media. Media with cells and the solvent DMSO (0.01%) used to dissolve the FX4 were used as the control. Data was represented by the mean ± SD from three independent replicates, each of which was carried out in quadruplicate wells. Statistical significance was analysed using one-way Anova with Tukey's post-hoc test (*** $p < 0.001$, **** $p < 0.0001$ and ns = non-significant).

3.4 Neuroprotective effect of industry-grade fucoxanthin and fucoidan in combination against beta-amyloid induced cytotoxicity of PC12 cells

The experiment aimed to test the neuroprotective effects of different combinations of industry-grade fucoxanthin of two different purities, FX1 (95.6%) and FX4 (5.6%) with fucoidan, FD (20%), against beta-amyloid ($A\beta_{1-42}$)-induced cytotoxicity in PC12 cells. Two concentrations of amyloid-beta (1 µM and 2 µM) were used to cause neurotoxicity in PC12 cells. FX1, FX4 and FD were combined at different concentrations to observe the level of neuroprotection

provided against the induced cytotoxicity. Cell viability was measured using the MTT assay after a 48-hour incubation period.

3.4.1 Neuroprotection of industry-grade fucoxanthin, FX1 (95.6%) in combination with fucoidan, FD (20%) against 1 µM amyloid-beta ($A\beta_{1-42}$) induced cytotoxicity of PC12 cells

Figure 3.11 shows the effects of combined treatments of industry-grade FX1 (95.6%) with fucoidan (20%) at various combinations of concentrations (FX1+FD) 5+25, 5+50, 10+25, and 10+50 µg/mL, on PC12 cell viability followed by exposure to 1 µM amyloid-beta ($A\beta_{1-42}$) induced cytotoxicity. Media with cells and DMSO+PBS were served as blank and solvent controls, respectively. Amyloid-beta alone resulted in a reduction in relative growth compared to the control group of around 74%. The combinations tested with FX1+FD showed a significant level of neuroprotection of to reach approximately 95% viability for the combination of 5+25 µg/mL, with the highest protection observed achieving around 99.5% viability for 10+25 µg/mL. Combinations of 10+50 µg/mL of FD did not show better protection compared to 10+25 µg/mL. All the combinations were found statistically significant for neuroprotection with **** $p < 0.0001$.

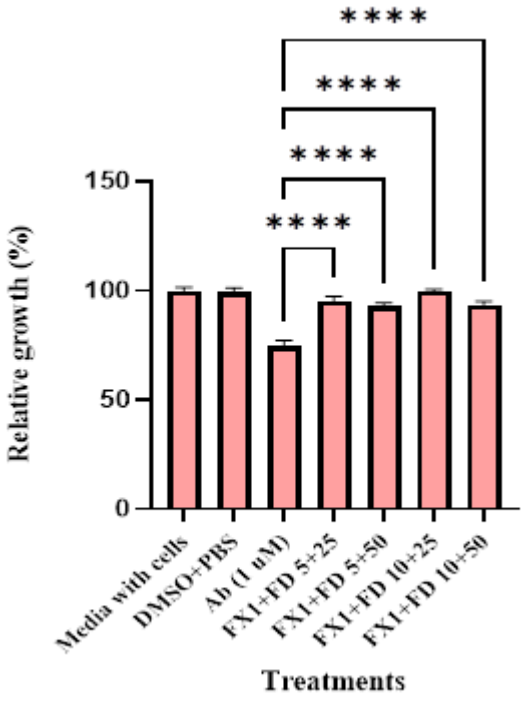


Figure 3. 11 Determining the neuroprotective effect of the combinations of FX1 (95.6%) with FD (20%) at different concentrations against beta-amyloid (1 µM)-induced cytotoxicity in

PC12 cells. PC12 cells after 72 hours regular sub-culture was harvested, resuspended and seeded at 1×104 cells/well. The plate was incubated for 24 hours at 37˚C and 5% CO2 incubator. The cells were then pre-treated with FX1+FD at concentrations of 5+25, 5+50, 10+25, and 10+50 µg/mL for 15 minutes, followed by exposure to 2 µM of amyloid-beta to induce the induced cytotoxicity. After 48 hours of exposure, cell viability was assessed using the MTT assay. The total well volume was 100 µL with media. Media with cells and the solvent DMSO (0.01%) + PBS used to dissolve the FX1+FD were used as the controls. Data was represented by the mean ± SD from three independent replicates, each of which was carried out into quadruplicates wells. Statistical significance was analysed using one-way ANOVA with Tukey's post-hoc test (**** $p < 0.0001$).

3.4.2 Neuroprotection of industry-grade fucoxanthin, FX4 (5.6%) in combination with fucoidan, FD (20%) against 1 µM amyloid-beta ($A\beta_{1-42}$) induced cytotoxicity of PC12 cells

Figure 3.12 shows the effects of combined treatments of industry-grade FX4 (5.6%) with fucoidan (20%) at various combinations of FX4+FD concentrations 5+25, 5+50, 20+25, and 20+50 µg/mL on PC12 cell viability, followed by exposure to 1 µM amyloid-beta ($A\beta_{1-42}$), which induced cytotoxicity. Media with cells and DMSO+PBS were served as blank and solvent controls, respectively. Amyloid-beta alone resulted in a reduction in relative growth compared to the control group of around 74%. The combinations tested with FX4+FD showed a significant level of additional neuroprotection. Combinations with 5+25 µg/mL showed around 96% of relative growth, with better protection achieved with 20+25 µg/mL at approximately 99.9%. Although combinations with 50 µg/mL FD also showed a level of increased neuroprotection of around 93%, this was lower than that of 25 µg/mL FD. All the concentrations tested were found statistically significant (**** $p < 0.0001$) against the 1 µM amyloid-beta (Aβ1-42) -induced cytotoxicity, indicating a likely strong synergistic or additive effect.

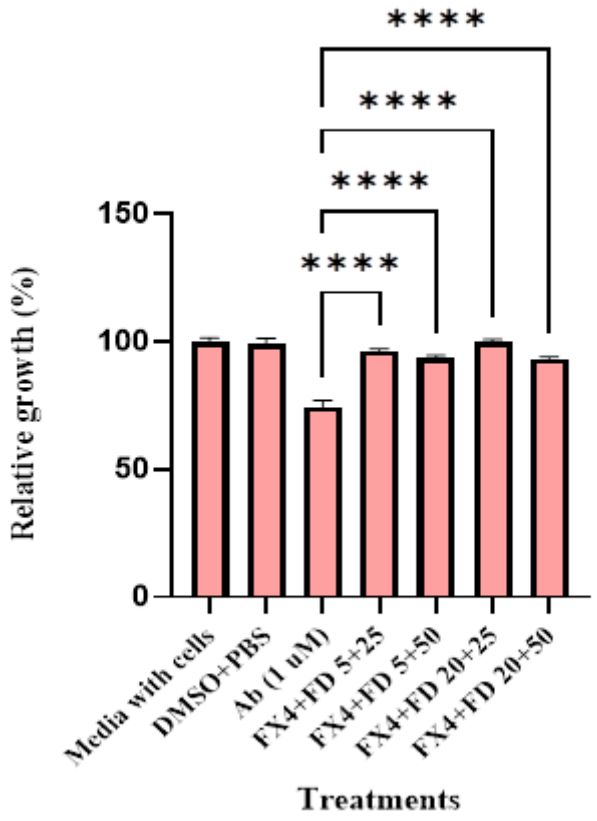


Figure 3. 12 Determining the neuroprotective effect of the combined effect of FX4 (5.6%) with FD (20%) at different concentrations against beta-amyloid (1 μM)-induced cytotoxicity in PC12 cells. PC12 cells after 72 hours of regular sub-culture were harvested, resuspended and seeded at 1×10^4 cells/well. The plate was incubated for 24 hours at 37˚C and 5% CO_2 incubator. The cells were then pre-treated with FX4+FD at concentrations of 5+25, 5+50, 20+25, and 20+50 μg/mL for 15 minutes, followed by exposure to 1 μM of amyloid-beta to induce the induced cytotoxicity. After 48 hours of exposure, relative growth was assessed using the MTT assay. The total well volume was 100 μL with media. Media with cells and the solvent DMSO (0.01%) + PBS used to dissolve the FX4+FD, were used as the control. Data was represented by the mean ± SD from three independent replicates, each of which was carried out into quadruplicate wells. Statistical significance was analysed using one-way ANOVA with Tukey's post-hoc test (**** $p < 0.0001$).

3.4.3 Neuroprotection of industry-grade fucoxanthin, FX1 (95.6%) in combination with fucoidan, FD (20%) against 2 μM amyloid-beta ($A\beta_{1-42}$) induced cytotoxicity of PC12 cells

Figure 3.13 represents the effect of combined effect of treatments of industry-grade FX1 (95.6%) with fucoidan (20%) at concentrations of 0.25+0.5, 0.25+1, 0.5+0.5, 0.5+1, 5+25,

5+50, 10+25, and 10+50 µg/mL on PC12 cell viability followed by exposure to 2 µM amyloid-beta ($A\beta_{1-42}$) induced cytotoxicity. Media with cells and DMSO+PBS were served as negative and solvent control, respectively. Amyloid-beta alone resulted in a substantial reduction in cell viability compared to the control group to about 51%. At the lowest combination dose (FX1+FD 0.25+0.5 µg/mL), a slight increase in viability was observed (~63%), although it was not statistically significant (Lin et al.). Other combinations of FX1+FD tested also showed significant protection ranging from ~68-93% against the beta-amyloid induced cytotoxicity. The highest protection was observed for FX1+FD at 10+25 µg/mL, with approximately 93%, protection, while a drop in growth was observed at 86% for the combination of 10+50 µg/mL. Statistical significance was observed for ** $p < 0.01$ for 0.25+1 µg/mL, *** $p < 0.001$ for 0.5+0.5 µg/mL and **** $p < 0.0001$ for the rest of the combinations tested.

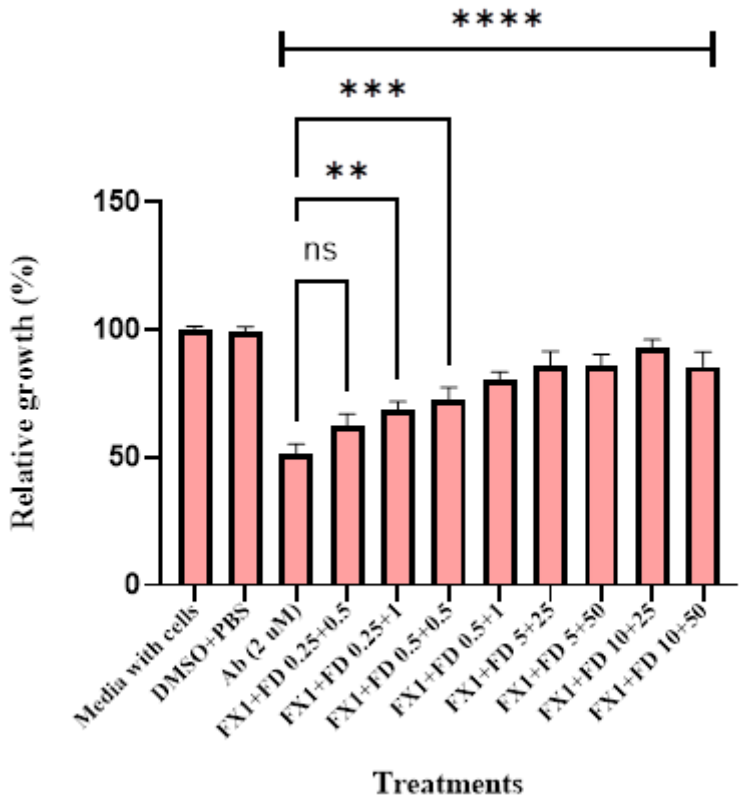


Figure 3. 13 Determining the neuroprotective effect of the combined treatment of FX1 (95.6%) with FD (20%) at different concentrations against beta-amyloid (2 µM)-induced cytotoxicity in PC12 cells. PC12 cells after 72 hours of regular sub-culture were harvested, resuspended and seeded at 1×10^4 cells/well. The plate was incubated for 24 hours at 37˚C and 5% CO_2 incubator. The cells were then pre-treated with FX1+FD at concentrations of 0.25+0.5, 0.25+1, 0.5+0.5, 0.5+1, 5+25, 5+50, 10+25, and 10+50 µg/mL for 15 minutes, followed by exposure to 2 µM of amyloid-beta to induce cytotoxicity. After 48 hours of exposure, relative growth

was assessed using the MTT assay. The total well volume was 100 µL with media. Media with cells and the solvent DMSO (0.01%) + PBS used to dissolve the FX1+FD were used as the control. Data was represented by the mean ± SD from three independent replicates, each of which was carried out into quadruplicates wells. Statistical significance was analysed using one-way Anova with Tukey's post-hoc test (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ and ns = non-significant).

3.4.4 Determining neuroprotection of industry-grade fucoxanthin, FX4 (5.6%) in combination with fucoidan, FD (20%) against 2 µM amyloid-beta ($A\beta_{1-42}$) induced cytotoxicity

Figure 3.14 shows how different combinations of FX4 (5.6%) and fucoidan (20%) at concentrations of 0.25+0.5, 0.25+1, 0.5+0.5, 0.5+1, 5+25, 5+50, 10+25, and 10+50 µg/mL affected the viability (%) of PC12 cells treated with 2 µM amyloid-beta ($A\beta_{1-42}$) induced cytotoxicity. Media with cells and DMSO+PBS were served as negative and solvent control, respectively. Amyloid-beta alone resulted in a substantial reduction in cell viability compared to the control group to around 51%. Though, the combination of FX4+FD showed a significantly improved cell viability (%) in a dose-dependent manner. At lower doses (0.25+0.5 µg/mL, 025+1 µg/mL, 0.5+0.5 µg/mL and 0.5+1 µg/mL), the relative growth (%) was low to moderate, ranging from 61-81%. But it started to significantly improve from 5+25 µg/mL and 20+25 µg/mL, reaching towards ~93% and ~97% with statistical significance of **** $p < 0.0001$. At higher concentrations of FD, the combinations 5+50 µg/mL and 20+50 µg/mL resulted in a drop in relative growth to ~90% and ~89%, respectively, which is may be due to the saturation of the compound. The statistical significance was found to be *$p< 0.05$, ** $p < 0.01$, *** $p < 0.001$ for 0.25+0.5 µg/mL, 0.25+1 µg/mL, 0.5+0.5 µg/mL respectively and for rest of the compounds **** $p < 0.0001$.

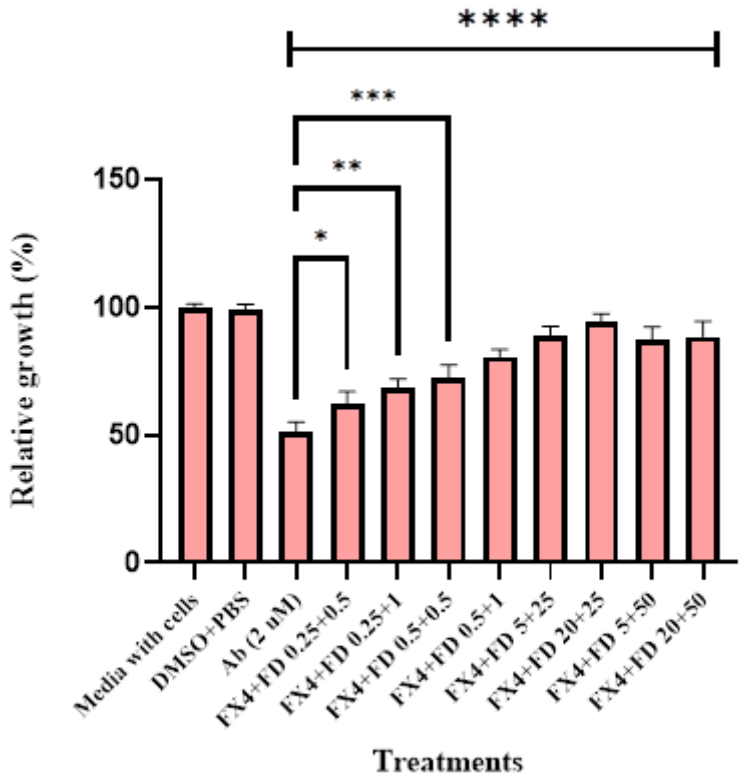


Figure 3. 14 Determining the neuroprotective effect of combined effect of FX4 (5.6%) with FD (20%) at different concentrations against beta-amyloid (2 µM)-induced cytotoxicity in PC12 cells. PC12 cells after 72 hours regular sub-culture was harvested, resuspended and were seeded at 1×10^4 cells/well. The plate was incubated for 24 hours at 37˚C and 5% CO_2 incubator. The cells were then pre-treated with FX1+FD at concentrations of 0.25+0.5, 0.25+1, 0.5+0.5, 0.5+1, 5+25, 5+50, 20+25, and 20+50 µg/mL for 15 minutes, followed by exposure to 2 µM of amyloid-beta to induce the induced cytotoxicity. After 48 hours of exposure, relative growth was assessed using the MTT assay. The total well volume was 100 µL with media. Media with cells and the solvent DMSO (0.01%) +PBS used to dissolve the FX4+FD were used as the control. Data was represented by the mean ± SD from three independent replicates, each were carried out into quadruplicates wells. Statistical significance was analysed using one-way Anova with Tukey's post-hoc test (*$p< 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$).

3.5 Neuroprotective effect of industry-grade fucoxanthin and fucoidan alone against hydrogen peroxide-induced cytotoxicity of PC12 cells

3.5.1 H_2O_2 sample screening for use in neuroprotective studies

Figure 3.15 shows the effect of two different H_2O_2 samples (A and B) at concentrations ranging from 50-300 µM on the viability of PC12 cells. PC12 cells grown in media only were used as a control. With an increase in concentration, both samples showed a gradual reduction in cell viability in a dose-dependent manner. Sample B showed better cytotoxicity compared to sample A and was thus chosen to be used for this experiment (100 and 150 µM).

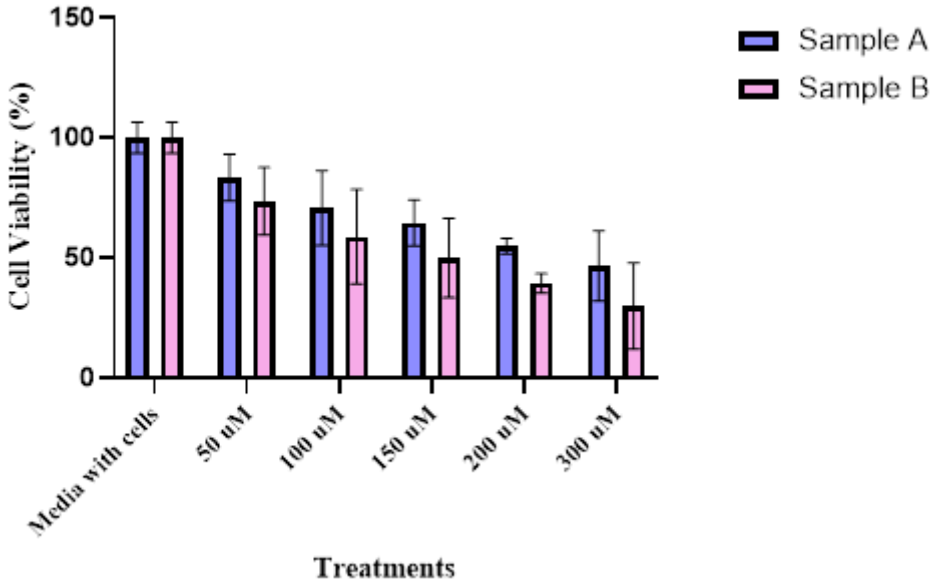


Figure 3. 15 Determining the cytotoxicity shown by two different samples of hydrogen peroxide at concentrations ranging from 50-300 µM on the viability of PC12 cells. PC12 cells after 72 hours of regular subculture were harvested, resuspended and seeded at 1×10^4 cells/well. The plate was incubated for 24 hours at 37˚C and 5% CO_2 incubator. The cells were then treated with predetermined concentrations of 50-300 µM H2O2, with two samples of H_2O_2 to induce cytotoxicity. After 24 hours of exposure, cell viability was assessed using the MTT assay. The total well volume was 100 µL with media. Media with cells was considered as the control. Data was represented by the mean ± SD (representing the variability among three replicates) from three independent replicates, each of which was carried out in quadruplicate wells.

3.5.2 Determining the level of neuroprotection of industry-grade fucoxanthin, FX1 (95.6%) alone against hydrogen peroxide-induced (100 µM) induced cytotoxicity of PC12 cells

Figure 3.16 shows the effect of industry-grade FX1 (95.6%) at concentrations of 2.5, 5, and 10 µg/mL on PC12 cell viability, followed by exposure to 100 µM hydrogen peroxide (H2O2), which induced cytotoxicity. Media with cells and 0.01% DMSO were used as the negative and solvent control, respectively. H_2O_2 alone resulted in a stronger reduction in cell viability compared to the control group to around~ 63%. However, with increasing concentration of FX1, the level of protection against H_2O_2 increased in a dose-specific manner, showing very significant neuroprotection of ~75%, ~84% and ~90%, with statistical significance (**** $p < 0.0001$).

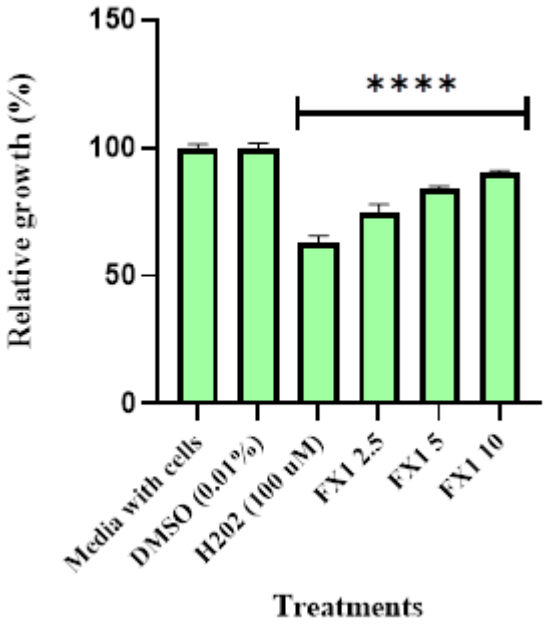


Figure 3. 16 Determining the neuroprotective effect of FX1 (95.6%) against hydrogen peroxide (100 µM)-induced cytotoxicity in PC12 cells. PC12 cells after 72 hours regular sub-culture was harvested, resuspended and were seeded at 1×10^4 cells/well. The plate was incubated for 24 hours at 37˚ C and 5% CO_2 incubator. The cells were then pre-treated with FX1 at concentrations of 2.5, 5, and 10 µg/mL for 15 minutes, followed by exposure to 100 µM of amyloid-beta to induce the induced cytotoxicity. After 24 hours of exposure, relative growth was assessed using the MTT assay. The total well volume was 100 µL with media. Media with cells and the solvent DMSO (0.01%) used to dissolve the FX1 were used as the control. Data was represented by the mean ± SD from three independent replicates, each of which was carried

out in quadruplicate wells. Statistical significance was analysed using one-way ANOVA with Tukey's post-hoc test (***** $p < 0.0001$).

3.5.3 Determining neuroprotection of industry-grade fucoxanthin, FX4 (95.6%) alone against hydrogen peroxide (100 µM) induced cytotoxicity of PC12 cells

Figure 3.17 shows the effect of industry-grade FX4 (5.6%) at concentrations of 5, 10 and 20 µg/mL on PC12 cell viability, followed by exposure to 100 µM hydrogen peroxide (H_2O_2), which induced cytotoxicity. Media with cells and 0.01% DMSO were served as negative and solvent control respectively. H_2O_2 alone resulted in a strong reduction in relative growth (%) compared to the control group to around 63%. However, with increasing concentration of FX4, the level of protection against H_2O_2 increased. FX4 at low doses (5 µg/mL) showed ~88% relative growth. Whereas at higher concentrations (20 µg/mL),it was tested and showed highly significant neuroprotection reaching up to ~93% (**** $p < 0.0001$). This result indicated that, FX4 can show neuroprotection against hydrogen peroxide (100 µM)-induced neurotoxicity in a dose-dependent manner in PC12 cells.

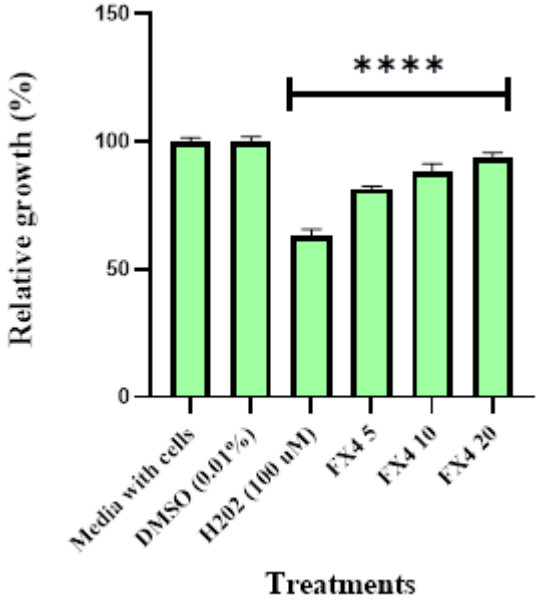


Figure 3. 17 Determining the neuroprotective effect of FX4 (5.6%) against hydrogen peroxide (100 µM)-induced cytotoxicity in PC12 cells. PC12 cells after 72 hours regular sub-culture was harvested, resuspended and were seeded at 1×10^4 cells/well. The plate was incubated for 24 hours at 37° C and 5% CO_2 incubator. The cells were then pre-treated with FX4 at concentrations of 5, 10 and 20 µg/mL for 15 minutes, followed by exposure to 100 µM of amyloid-beta to induce the induced cytotoxicity. After 24 hours of exposure, relative growth was assessed using the MTT assay. The total well volume was 100 µL with media. Media with

cells and the solvent DMSO (0.01%) used to dissolve the FX4 were used as the control. Data was represented by the mean ± SD from three independent replicates, each were carried out into quadruplicates wells. Statistical significance was analysed using one-way Anova with Tukey's post-hoc test (***p< 0.001 **** p < 0.0001 and ns= non-significant).

3.5.4 Determining protection provided by industry-grade fucoidan, FD (20%) alone against hydrogen peroxide (100 µM) induced cytotoxicity of PC12 cells

Figure 3.18 represents the effect of industry-grade FD (20%) at concentrations of 6.25, 25 and 100 µg/mL on PC12 cell viability followed by exposure to 100 µM hydrogen peroxide (H_2O_2), which induced cytotoxicity. Media with cells and PBS were served as blanks and solvent controls, respectively. H_2O_2 alone resulted in a stronger reduction in relative growth compared to the control group to approximately 63%. However, with increasing concentration of FD, the level of protection against H_2O_2 increased. All tested concentrations of FD against oxidative-induced cytotoxicity induced by H_2O_2 showed significant neuroprotection of ~80%, ~85% and ~92%, respectively (**** p < 0.0001). This result indicated that, FD can show neuroprotection against hydrogen peroxide (100 µM)-induced neurotoxicity in a dose-dependent manner in PC12 cells.

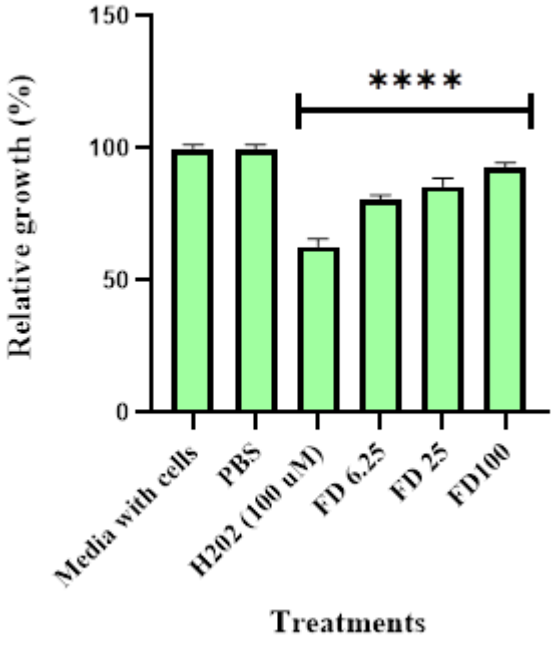


Figure 3. 18 Determining the neuroprotective effect of FD (20%) against hydrogen peroxide (150 µM)-induced cytotoxicity in PC12 cells. PC12 cells after of 72 hours of regular sub-culture was harvested, resuspended and were seeded at 1×10^4 cells/well. The plate was incubated for 24 hours at 37˚C and 5% CO_2 incubator. The cells were then pre-treated with FD

at concentrations of 0.5, 1, 6.25, 25 and 100 µg/mL for 15 minutes, followed by exposure to 150 µM of amyloid-beta to induce the induced cytotoxicity. After 24 hours of exposure, relative growth was assessed using the MTT assay. The total well volume was 100 µL with media. Media with cells and the solvent PBS used to dissolve the FD were used as the control. Data was represented by the mean ± SD from three independent replicates, each were carried out into quadruplicates wells. Statistical significance was analysed using one-way Anova with Tukey's post-hoc test (**** $p < 0.0001$).

3.5.5 Determining neuroprotection of industry-grade fucoxanthin, FX1 (95.6%) alone against hydrogen peroxide (150 µM) induced cytotoxicity of PC12 cells

Figure 3.19 shows the effect of industry-grade FX1 (95.6%) at concentrations of 0.25, 0.5, 2.5, 5, and 10 µg/mL on PC12 cell viability followed by exposure to 150 µM hydrogen peroxide (H_2O_2) induced cytotoxicity. Media with cells and 0.01% DMSO were served as controls respectively. H_2O_2 alone resulted in a substantial reduction in relative growth compared to the control group of ~49%. However, with increasing concentration of FX1, the level of protection against H_2O_2 increased. FX1 at 0.25 µg/mL showed mild protection to around 56% (** $p < 0.01$), while 0.5 - 10 µg/mL showed more significant neuroprotection from ~68% to ~86% respectively (** $p < 0.01$ and **** $p < 0.0001$, respectively). This result indicated that, FX1 can show neuroprotection against hydrogen peroxide (150 µM)-induced neurotoxicity in a dose-dependent manner in PC12 cells.

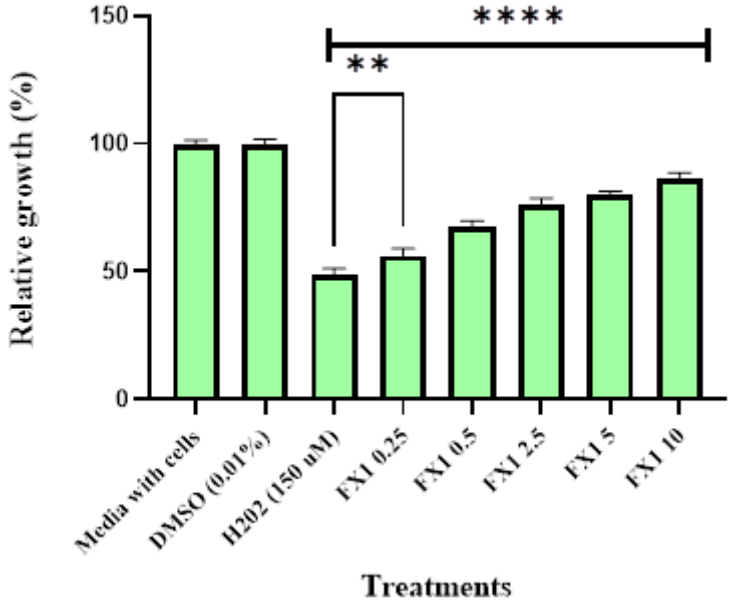


Figure 3. 19 Determining the neuroprotective effect of FX1 (95.6%) against hydrogen peroxide (150 µM)-induced cytotoxicity in PC12 cells. PC12 cells after 72 hours regular sub-culture was harvested, resuspended and were seeded at 1×10^4 cells/well. The plate was incubated for 24 hours at 37˚ C and 5% CO_2 incubator. The cells were then pre-treated with FX1 at concentrations of 0.25, 0.5, 2.5, 5, and 10 µg/mL for 15 minutes, followed by exposure to 150 µM of amyloid-beta to induce cytotoxicity. After 24 hours of exposure, cell viability was assessed using the MTT assay. The total well volume was 100 µL with media. Media with cells and the solvent DMSO (0.01%) used to dissolve the FX1 were used as the control. Data was represented by the mean ± SD from three independent replicates, each of which was carried out into quadruplicate wells. Statistical significance was analysed using one-way Anova with Tukey's post-hoc test (** $p < 0.01$ and **** $p < 0.0001$).

3.5.6 Determining neuroprotection of industry-grade fucoxanthin, FX4 (95.6%) alone against hydrogen peroxide (150 µM) induced cytotoxicity of PC12 cells

Figure 3.20 shows the effect of industry-grade FX4 (5.6%) at concentrations of 0.5, 1, 5, 10 and 20 µg/mL on PC12 cell viability followed by exposure to 150 µM hydrogen peroxide (H_2O_2), which induced cytotoxicity. Media with cells and 0.01% DMSO were served as blank and solvent control respectively. H_2O_2 alone resulted in a substantial reduction in relative growth compared to the control group to around ~49%. However, with increasing concentration of FX4, the level of protection against H_2O_2 increased from ~60% to till the highest at ~92%. FX4 at all the concentrations tested showed highly significant neuroprotection (**** p <

0.0001). This result indicated that, FX4 can show neuroprotection against hydrogen peroxide (150 µM)-induced neurotoxicity in a dose-dependent manner in PC12 cells.

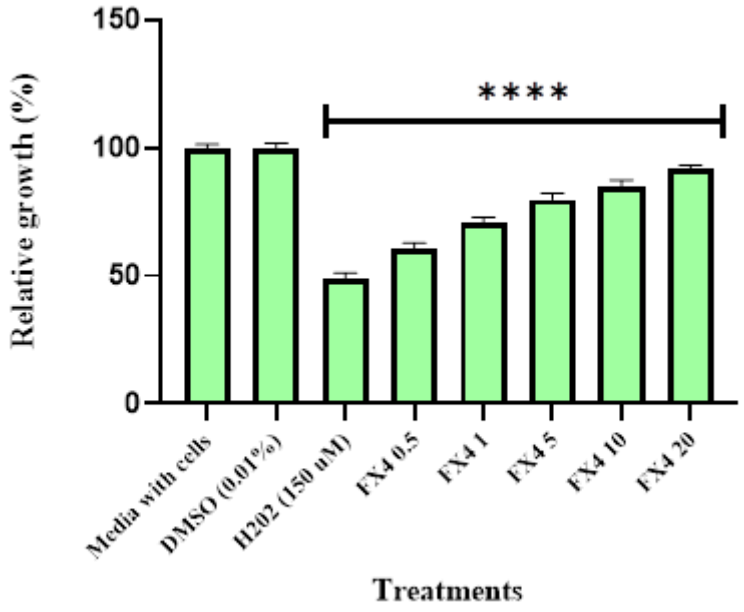


Figure 3. 20 Determining the neuroprotective effect of FX4 (5.6%) against hydrogen peroxide (150 µM)-induced cytotoxicity in PC12 cells. PC12 cells after 72 hours of regular sub-culture were harvested, resuspended and were seeded at 1×10^4 cells/well. The plate was incubated for 24 hours at 37˚ C and 5% CO_2 incubator. The cells were then pre-treated with FX4 at concentrations of 0.5, 1, 5, 10 and 20 µg/mL for 15 minutes, followed by exposure to 150 µM of H_2O_2 to induce the induced cytotoxicity. After 24 hours of exposure, cell viability was assessed using the MTT assay. Media with cells and the solvent DMSO (0.01%) were used as the control. Data was represented by the mean ± SD from three independent replicates, each were carried out into quadruplicates wells. Statistical significance was analysed using one-way Anova with Tukey's post-hoc test (**** $p < 0.0001$).

3.5.7 Determining neuroprotection of industry-grade fucoidan, FD (20%) alone against hydrogen peroxide (150 µM) induced cytotoxicity on PC12 cells

Figure 3.21 represents the effect of industry-grade FD (20%) at concentrations of 0.5, 1, 6.25, 25 and 100 µg/mL on PC12 cell viability followed by exposure to 150 µM hydrogen peroxide (H_2O_2), which induced cytotoxicity. Media with cells and PBS served as controls, respectively. H_2O_2 alone resulted in a substantial reduction in relative growth compared to the control group at ~49%. However, with increasing concentration of FD, the level of protection against H_2O_2 increased. FD at the concentration 0.5 µg/mL showed mild neuroprotection to around ~62%

(**p < 0.01), and concentrations from 1 – 100 µg/mL showed significant neuroprotection at around ~72% - ~89% respectively (**** p < 0.0001). This result indicated that, FD can show neuroprotection against hydrogen peroxide (150 µM)-induced neurotoxicity in a dose-dependent manner in PC12 cells.

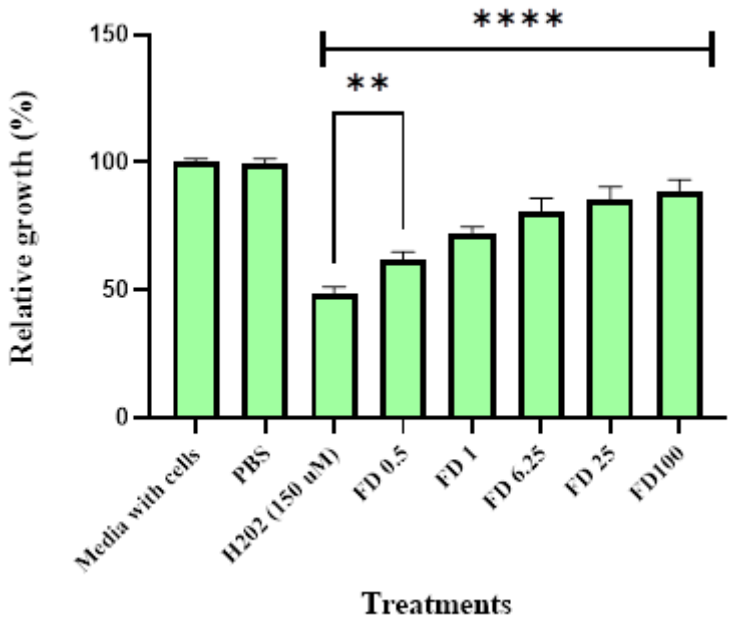


Figure 3. 21 Determining the neuroprotective effect of FD (20%) against hydrogen peroxide (150 µM)-induced cytotoxicity in PC12 cells. PC12 cells after 72 hours of regular sub-culture were harvested, resuspended and were seeded at 1×10^4 cells/well. The plate was incubated for 24 hours at 37˚ C and 5% CO_2 incubator. The cells were then pre-treated with FX4 at concentrations of 0.5, 1, 6.25, 25 and 100 µg/mL for 15 minutes, followed by exposure to 150 µM of amyloid-beta to induce the induced cytotoxicity. After 24 hours of exposure, relative growth was assessed using the MTT assay. Media with cells and the solvent PBS used to dissolve the FD were used as the control. Data was represented by the mean ± SD from three independent replicates, each were carried out into quadruplicates wells. Statistical significance was analysed using one-way ANOVA with Tukey's post-hoc test (**p < 0.01 and **** p < 0.0001).

3.6 Determining neuroprotection of industry-grade fucoxanthin and fucoidan in combination against hydrogen peroxide (100 µM) induced cytotoxicity in PC12 cells

The objective of this experiment was to observe the combined neuroprotective effects of industry-grade fucoxanthin of two different purities, FX1 (95.6%) and FX4 (5.6%) with fucoidan, FD (20%), against hydrogen peroxide-induced cytotoxicity in PC12 cells. Two

concentrations of hydrogen peroxide (100 µM and 150 µM) were used to induce neurotoxicity in PC12 cells. FX1, FX4 and FD were combined at different concentrations with to observe the level of neuroprotection provided against the induced cytotoxicity. Relative growth was measured using the MTT assay after a 48-hours of the incubation period.

3.6.1 Determining neuroprotection of industry-grade fucoxanthin, FX1 (95.6%) in combination with fucoidan, FD (20%) against 100 µM hydrogen peroxide (H_2O_2) induced cytotoxicity of PC12 cells

Figure 3.22 represents the effects of combined treatments of industry-grade FX1 (95.6%) with fucoidan (20%) at concentrations 5+25, 5+50, 10+25, and 10+50 µg/mL on PC12 relative growth followed by exposure to 100µM hydrogen peroxide (H_2O_2), which induced cytotoxicity. Media with cells and DMSO+PBS were served as blank and solvent controls, respectively. H_2O_2 alone resulted in a substantial reduction in cell viability compared to the control group at around ~62%. The combinations tested with FX1+FD showed significant level of additional neuroprotection against the 100 µM hydrogen peroxide (H_2O_2) induced cytotoxicity, indicating a strong synergistic or additive effect. The highest protection was observed by FX1 10 µg/mL, with FD 25 µg/mL showing the highest protection of around ~95% with significance **** $p < 0.0001$, reaching viability nearly the negative control (media with cells). FX1 10 µg/mL when combined with 50 µg/mL FD, showed a drop in protection (~90%), which may be due to saturation.

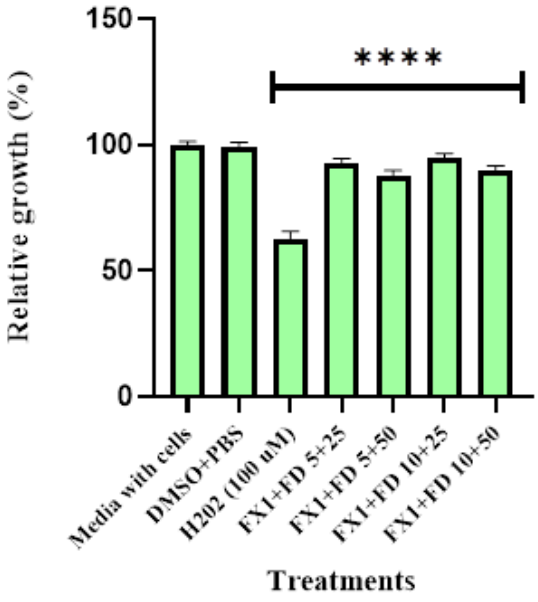


Figure 3. 22 Determining the neuroprotective effect of combined effect of FX1 (95.6%) with FD (20%) at different concentrations against hydrogen peroxide (150 µM)-induced

cytotoxicity in PC12 cells. PC12 cells after 72 hours of regular sub-culture was harvested, resuspended and were seeded at 1×10^4 cells/well. The plate was incubated for 24 hours at 37˚ C and 5% CO_2 incubator. The cells were then pre-treated with FX1+FD at concentrations of 5+25, 5+50, 10+25, and 10+50 µg/mL for 15 minutes, followed by exposure to 100 µM of hydrogen peroxide to induce the induced cytotoxicity. After 24 hours of exposure, cell viability was assessed using the MTT assay. Media with cells and the solvent DMSO (0.01%) +PBS used to dissolve the FX1+FD were used as the blank and solvent control respectively. Data was represented by the mean ± SD from three independent replicates, each were carried out into quadruplicate wells. Statistical significance was analysed using one-way ANOVA with Tukey's post-hoc test (**** $p < 0.0001$).

3.6.2 Determining neuroprotection of industry-grade fucoxanthin, FX4 (5.6%) in combination with fucoidan, FD (20%) against 100 µM hydrogen peroxide (H_2O_2) induced cytotoxicity

Figure 3.23 represents the effects of combined treatments of industry-grade FX1 (95.6%) with fucoidan (20%) at concentrations 5+25, 5+50, 10+25, and 10+50 µg/mL on PC12 cell viability followed by exposure to 100µM hydrogen peroxide (H_2O_2) induced cytotoxicity. Media with cells and DMSO+PBS were served as blank and solvent control, respectively. H_2O_2 alone resulted in a strong reduction in relative growth (%) compared to the control group, at around ~63%. The combinations tested with FX4+FD showed significant level of additional neuroprotection at about ~96.5% (5+25 µg/mL) and ~87% (5+50 µg/mL) against the 100 µM hydrogen peroxide (H_2O_2) induced cytotoxicity, indicating a strong synergistic or additive effect. The highest protection was observed by FX4 20 µg/mL, with FD 25 µg/mL (~96%) showing the highest protection with significance **** $p < 0.0001$, reaching viability nearly the negative control (media with cells).

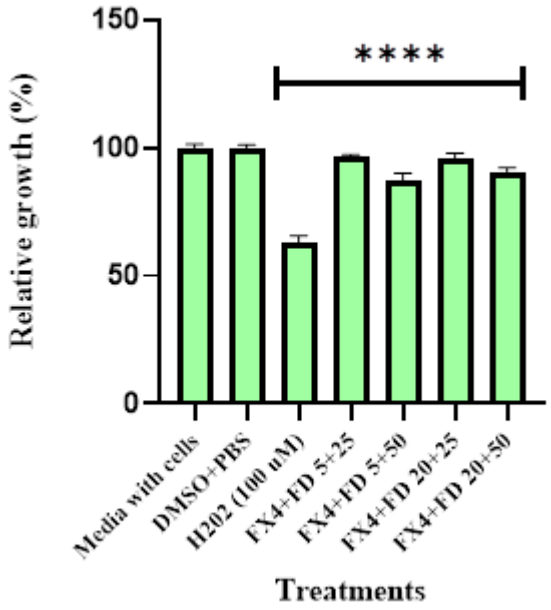


Figure 3. 23 Observing the neuroprotective effect of FX4+FD against hydrogen peroxide (100 µM)-induced cytotoxicity in PC12 cells. PC12 cells after 72 hours regular sub-culture was harvested, resuspended and were seeded at 1×10^4 cells/well. The plate was incubated for 24 hours at 37˚ C and 5% CO_2 incubator. The cells were then pre-treated with FX4+FD at concentrations of 5+25, 5+50, 20+25 and 20+50 µg/mL for 15 minutes, followed by exposure to 100 µM of hydrogen peroxide to induce the induced cytotoxicity. After 24 hours of exposure, relative growth was assessed using the MTT assay. Media with cells and the solvent DMSO+PBS used to dissolve the FX4+FD were used as the blank and solvent control, respectively. Data was represented by the mean ± SD from three independent replicates, each were carried out into quadruplicate wells. Statistical significance was analysed using one-way Anova with Tukey's post-hoc test (**** $p < 0.0001$).

3.6.3 Determining the level of protection provided by industry-grade fucoxanthin, FX1 (95.6%) in combination with fucoidan, FD (20%) against 150 µM hydrogen peroxide (H_2O_2) induced cytotoxicity

Figure 3.24 represents the effects of combined treatments of industry-grade FX1 (95.6%) with fucoidan (20%) at concentrations 5+25, 5+50, 10+25, and 10+50 µg/mL on PC12 relative growth followed by exposure to 150µM hydrogen peroxide (H_2O_2) induced cytotoxicity. Media with cells and DMSO+PBS were served as negative and solvent control, respectively. H_2O_2 alone resulted in a significant reduction in relative growth compared to the control group, at ~49%. The combinations tested with FX1+FD showed a significant level of additional

neuroprotection of approximately ~91% for 5+25 µg/mL against the 150 µM hydrogen peroxide (H_2O_2) stress, indicating a strong synergistic or additive effect. The highest protection was observed by FX1 10 µg/mL, with FD 25 µg/mL showing the highest protection (~94%) with significance **** $p < 0.0001$, reaching viability nearly the negative control (media with cells).

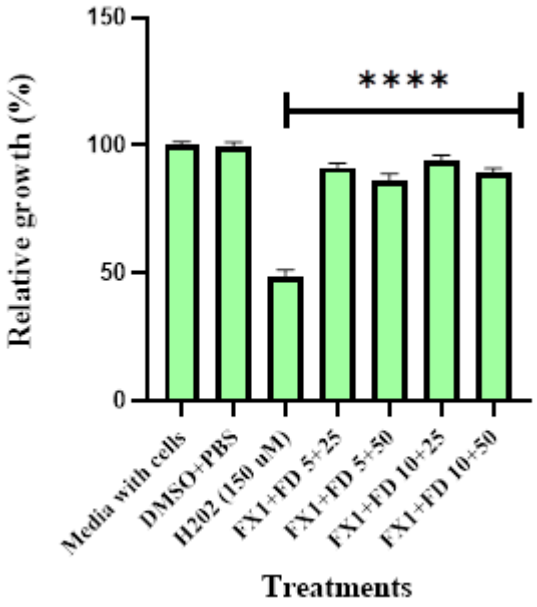


Figure 3. 24 Determining the neuroprotective effect of combined effect of FX1 (95.6%) with FD (20%) at different concentrations against hydrogen peroxide (150 µM)-induced cytotoxicity in PC12 cells. PC12 cells after 72 hours of regular sub-culture was harvested, resuspended and were seeded at 1×104 cells/well. The plate was incubated for 24 hours at 37˚ C and 5% CO2 incubator. The cells were then pre-treated with FX1+FD at concentrations of 5+25, 5+50, 10+25, and 10+50 µg/mL for 15 minutes, followed by exposure to 150 µM of hydrogen peroxide to induce the induced cytotoxicity. After 24 hours of exposure, cell viability was assessed using the MTT assay. Media with cells and the solvent DMSO (0.01%) +PBS used to dissolve the FX1+FD were used as the control. Data was represented by the mean ± SD from three independent replicates, each were carried out into quadruplicates wells. Statistical significance was analysed using one-way Anova with Tukey's post-hoc test (**** $p < 0.0001$).

3.6.4 Determining the level of protection provided by industry-grade fucoxanthin, FX4 (5.6%) in combination with fucoidan, FD (20%) against 150 µM hydrogen peroxide (H_2O_2) induced cytotoxicity

Figure 3.25 represents the effects of combined treatments of industry-grade FX1 (95.6%) with fucoidan (20%) at concentrations 5+25, 5+50, 10+25, and 10+50 µg/mL on PC12 cell viability followed by exposure to 150µM hydrogen peroxide (H_2O_2) induced cytotoxicity. Media with cells and DMSO+PBS were served as blank and solvent control, respectively. H_2O_2 alone resulted in a significant reduction in cell viability compared to the control group, at around ~49%. The combinations tested with FX4+FD showed significant level of additional neuroprotection of around ~91% (5+25 µg/mL) against the 150 µM hydrogen peroxide (H_2O_2) induced cytotoxicity, indicating a strong synergistic or additive effect. The highest protection was observed by FX4 20 µg/mL with FD 25 µg/mL, which showed the highest protection (~96%) with significance **** $p < 0.0001$, reaching relative growth nearly the negative control (media with cells).

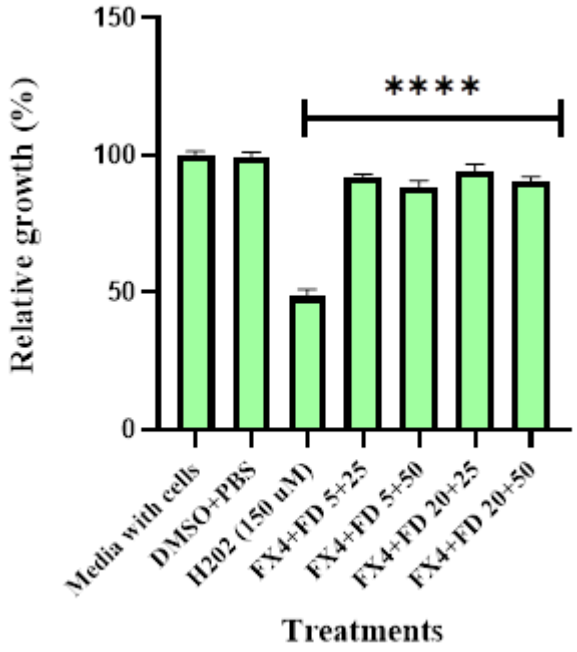


Figure 3. 25 Determining the neuroprotective effect of FX4+FD against hydrogen peroxide (150 µM)-induced cytotoxicity in PC12 cells. PC12 cells after 72 hours of regular sub-culture was harvested, resuspended and were seeded at 1×10^4 cells/well. The plate was incubated for 24 hours at 37˚C and 5% CO_2 incubator. The cells were then pre-treated with FX4+FD at concentrations of 5+25, 5+50, 20+25 and 20+50 µg/mL for 15 minutes, followed by exposure to 150 µM of hydrogen peroxide to induce the induced cytotoxicity. After 24 hours of exposure,

cell viability was assessed using the MTT assay. Media with cells and the solvent DMSO+PBS used to dissolve the FX4+FD were used as the blank and solvent control, respectively. Data was represented by the mean ± SD from three independent replicates, each of which was carried out int quadruplicate wells. Statistical significance was analysed using one-way ANOVA with Tukey's post-hoc test (**** $p < 0.0001$).

4 Discussion

This research project aimed to understand the neuroprotective effects of two marine-derived compounds, of industry-grade, rather than analytical grade, which have been using in the majority of the past studies. Fucoxanthin (FX1 95.6% and FX4 5.6%) and fucoidan (20%) alone and in combinations in PC12 cells. These two compounds were chosen because they have been demonstrated to have significant and the highest neuroprotective activities among many other natural compounds in past studies with potential uses in, with potential uses in the prevention and management of the progression of neurodegenerative diseases, such as Alzheimer's disease (AD). Fucoxanthin, being a carotenoid, was reported by Alghazwi et al. (2019) to improve the survival of PC12 cells exposed to cytotoxic amyloid-beta (Aβ1-42). The study found that fucoxanthin reduced oxidative stress by decreasing in the generation of reactive oxygen species (Asai et al.). It also activated the Nrf2 antioxidant pathway. Thus, by activating both the pathways it helped to protect the brain cells from damage in Alzheimer's disease (Alghazwi, Smid, Karpiniec, et al., 2019). Similarly, fucoidan which is a sulphated polysaccharide, has shown similar protective effects in brain cells. Liu et al. (2022) demonstrated that fucoidan reduced apoptosis and inflammation while enhancing mitochondrial function in both cell culture and animal models of Alzheimer's disease. As both of the compounds targeted several key mechanisms involved in Alzheimer's progression, like oxidative stress, mitochondrial dysfunction and inflammation. That is why they were considered as strong candidates for our research to study further neuroprotection.

However, there are two significant gaps in the previous studies: (1) there is little scientific studies on how the purity and purity of industry-grade fucoxanthin and fucoidan will impact on their neuroprotective activities to facilitate their commercial applications; and (2) there is little studies if any to understand the neuroprotection activities of the combination treatments of fucoxanthin and fucoidan of specifically at industry-grade, whether additive or synergistic effects can be achieved. Hence, our study was designed to address these two critical research gaps, althrough it is preliminary, given that this project is only a 9-month master's research project.

4.1 Determination of safe doses of industry-grade fucoxanthin and fucoidan by MTT assay

One of the primary objectives of this project was to determine the safe and non-toxic concentrations of industry-grade fucoxanthin and fucoidan of different purities, FX1 (95.6%), FX4 (5.6%) and FD (20%) in PC12 cell line. This was crucial for assessing the neuroprotective

potential of these compounds against the amyloid-beta and hydrogen peroxide-induced cytotoxicity in PC12 cells. PC12 cells were extensively used as an in vitro model for studying neuronal differentiation and neuroprotective mechanisms. These cells undergo differentiation into neuron-like projections, marked by neurite outgrowth and neuronal marker expression. PC12 cells were already established in previous literature as a reliable in vivo model for neuroprotective assays (Tominami et al., 2024).

Fucoxanthin is lipophilic and DMSO was selected as a solvent control for this experiment based on previous research done by the laboratory and literature review (Alghazwi et al., 2018). DMSO is known for inducing cytotoxicity at higher concentrations in neuronal cells, including PC12 cells. Recent studies have highlighted the cytotoxic effects of DMSO, even at very low concentrations (Alanazi et al., 2025; Dessì et al., 2025). Galvao et al. (2014) showed that even 0.05% DMSO can induce apoptosis and reduce cell viability (%) in neuronal and retinal cell model (Galvao et al., 2014). Another study by Dessi et al. (2025) and Alanazi et al. (2025) validated that the PC12 cell viability was not affected by DMSO concentrations at below 0.05% (Alanazi et al., 2025; Dessì et al., 2025). These findings align with our results, which show that DMSO concentrations above 0.1% cause a decrease in cell viability (%), whereas 0.01% DMSO was found to be non-toxic to the cells (**Figure 3.1**). Based on that, we selected 0.01% DMSO as a solvent control and the final concentration for all the treatments prepared.

Determining the safe, non-toxic concentrations of industrial grade fucoxanthin (FX1, 95.6% and FX4 5.6%) and fucoidan (FD 20%) was an important aspect of this project. As we were studying industrial-grade compounds, there were no previous data available on the safety of the compounds. This step was crucial so that, in the neuroprotective studies, the safe doses could be assessed without any doubt that the effect was due to the biological actions of the compounds, rather than from any toxicity.

. Doses above 20 µM were found to be linked with stress responses in brain cells (Liu et al., 2022; Pruccoli et al., 2024). According to purity, FX4 is less pure than FX1, but it offers a wider safety margin than FX1. Figure 3.3 showed that it was safe up to 40 µg/mL. It gave a good direction for food supplement development in terms of safety and cost priorities. These findings are linked to previous research conducted by Zhang et al. (2017). They found that though lower purity of fucoxanthin extracts showed weak effects but were less likely to be toxic (Zhang et al., 2017). The broader safety margin of the FX4 was also beneficial for product development perspectives, particularly for chronic or preventive strategies (nutraceuticals/functional foods), where long-term safety margins are crucial.

For fucoidan (20%), all doses tested, ranging from 6.25 to 100 µg/mL, were found to be safe and non-toxic to the cells, showing relative growth of between 90% and 100% compared to the control (**Figure 3.2**). At low doses, 6.25 µg/mL and 12.5 µg/mL were found to show an increase in viability (110% and 106.6%, respectively). Statistical significance was found with ***$p < 0.001$ and *$p < 0.05$, respectively, suggesting a possibility of mild proliferative or neuroprotective effects (Figure 3.4). This observation aligns with the findings of the previous literature review. Fucoidan showed protection against β-amyloid-induced neurotoxicity in neuronal cells through an antioxidant defence mechanism. (Nagata et al., 2021). Again, another study showed that fucoidan has shown promising results in decreasing oxidative stress through the apoptosis regulation pathway. This finding suggests that at low doses, fucoidan can provide protection against mild oxidative stress, albeit with some signs of damage. (Wang et al., 2021). Batista et al. (2023) also found some similar findings in their research, reporting that fucoidan has the potential to increase viability against oxidative stress without inducing any cytotoxicity, showing a correlation with our findings. (Batista et al., 2023). Although the purity of our fucoidan sample was lower than the analytical grade, the result was consistent with the previous studies, supporting its potential in further neuroprotective assays.

These findings confirmed that industry-grade fucoxanthin and fucoidan of different purities could be used safely in PC12 cells to study the Alzheimer's model. However, FX1 was purer but offered a narrower margin of safety. Although less pure, FX4 was found to be non-toxic at higher doses. This contrast offered us an insight that FX1 could be used for pharmaceutical purposes while FX4 could be used for dietary approaches. Fucoidans' broad safety range also supports their use in both pharmaceutical and food applications.

4.2 Neuroprotective effect of industry-grade fucoxanthin and fucoidan alone in PC12 cells

4.2.1 Neuroprotective effect of industry-grade fucoxanthin and fucoidan alone against beta-amyloid induced cytotoxicity in PC12 cells

Amyloid-beta ($A\beta_{1-42}$) is a toxic protein known to be responsible for Alzheimer's disease progression. It is known to form a plaque that causes oxidative stress, inflammation and mitochondrial damage, contributing to nerve cell damage (Ashford et al., 2021; Brand et al., 2022). To mimic that model, Amyloid-beta ($A\beta_{1-42}$) is widely used in the laboratory to study early neurodegeneration.

In this study, we selected two different concentrations of amyloid-beta (1 µM and 2 µM) to study the level of stress induced on the PC12 cell line and then the level of protection provided

by FX1, FX4, and FD alone. These concentrations cause a significant reduction in cell viability (%) and we aimed to observe whether these compounds can reverse or reduce the level of damage. Based on the previous section, we have already identified three safe doses for each of FX1, FX4 and FD. So, we will test those concentrations against amyloid-beta (1 μM and 2 μM) stress.

All concentrations tested for FX1, FX4, and FD were able to demonstrate neuroprotection against beta-amyloid cytotoxicity. At 10 μg/mL, FX1 exhibited relative growth of approximately 90% (Figure 3.5). This showed similarity aligning with previous findings that pure fucoxanthin was able to reduce amyloid-beta induced toxicity. This aligned with prior research that pure fucoxanthin could mitigate amyloid-beta induced cytotoxicity by activating the Nrf2/HO-1 antioxidant pathway and reduce the chances for apoptosis (Zhao et al., 2022).

FX4 had shown better relative growth at its highest concentrations tested. A relative growth of up to ~93% was observed for the highest concentration tested (20 μg/mL) (Figure 3.6). This result was surprising for such a low purity of fucoxanthin content. One reason could be the presence of other critical marine compounds, like polyphenols or oxidized carotenoids. These compounds may have contributed to providing better neuroprotection than the higher-purity one, FX1. Previous studies also observed that the whole extracts mostly work better in comparison to purified compounds because of the presence of other beneficial compounds (Lomartire & Gonçalves, 2023).

FD restored relative growth to its highest for the concentrations tested, around 93% at 25 μg/mL (**Figure 3.7**). Previous research had found that fucoidan was able to protect the nerve cells by activating survival pathways like PI3K/Akt and ERK1/2 through inhibiting the inflammatory signals (Nagata et al., 2021; Wang et al., 2021).

Once these results were achieved, we increased the concentration of amyloid-beta to 2 μM to mimic advanced-level neurodegeneration. This was featured with more severe oxidative stress, inflammation and apoptosis (Kumar & Singh, 2015). A sharp reduction in relative growth was observed for 2 μM beta-amyloid, reaching ~51%. To assess neuroprotection under such high stress, a few lower concentrations were added to determine the extent of their protection and whether they could go provide dose-dependent protection. FX1 showed improvement in relative growth at 2.5 μg/mL and peaked at 10 μg/mL (**Figure 3.8**). A similar type of neuroprotection was also observed for FX4. FX4 also restored relative growth at its highest concentration tested (20 μg/mL) to around 82% (**Figure 3.9**). FD was as effective as the previous study, which found it to be the most effective, achieving approximately 86% for 100 μg/mL (**Figure 3.10**).

These findings were supported by the previous research conducted by Liu et al. (2022) and D'Orazio et al. (2023). They reported that pure fucoxanthin was shown to lower the amyloid-beta toxicity and oxidative damage in nerve cells (Kumar & Singh, 2015; Liu et al., 2022). Analytical grade or pure fucoidan had been found to protect both the brain cells and animal models designed to mimic AD (Alghazwi, Smid, Karpiniec, et al., 2019; Li et al., 2023).

4.2.2 Neuroprotective effect of industry-grade fucoxanthin and fucoidan alone against hydrogen peroxide induced cytotoxicity in PC12 cells

This study aimed to observe the neuroprotective effects of industry-grade fucoxanthin (FX1, 95.6% and FX4, 5.6%) and fucoidan (FD, 20%) against hydrogen peroxide-induced stress in PC12 cells. Two different concentrations of H_2O_2 were chosen, 100 and 150 μM to mimic moderate to severe oxidative stress respectively and determine the level of protection exerted by FX1, FX4 and FD.

Initially, we compared two batches of H_2O_2 that we had purchased previously. Therefore, two different samples (A & B) from different batches were tested across a range of concentrations (50-300 μM) to address the potential inconsistencies that might arise from different degradation rates. Figure 3.15 shows that, despite both samples exhibiting dose-dependent cytotoxicity to PC12 cells, sample B was found to have significantly better damage potential. Thus, sample B was chosen to generate oxidative stress in PC12 cells and ensure consistency and reproducibility of the experiments.

At 100 μM H_2O_2 induced oxidative stress, relative growth was dropped to 63%. This was restored to ~90% at 10 μg/mL for FX1. FX4, being lower in purity, was found to be similarly effective at a higher dose of 20 μg/mL, achieving a relative growth of around 90%. FD concentrations had showed strong protection against the oxidative stress with relative growth of ~92% at 100 μg/mL. These results were supported by previous research showing that both fucoxanthin and fucoidan were effective in lowering ROS and protecting against mitochondrial dysfunction (Alghazwi et al., 2020; Chen et al., 2021; Heo et al., 2008).

In contrast, oxidative stress induced by 150 μM H_2O_2 has lowered the relative growth to approximately 48.5%, though all three compounds have shown a significant level of protection. FX1 was found to offer neuroprotection of ~86% at 10 μg/mL, and ~90% at 20 μg/mL for FX4 (**Figure 3.20-3.21**), and ~93% at 100 μg/mL for FD (Figure 3.22). Previous studies using the purified compounds have shown similar levels of protection against oxidative stress. Fucoxanthin was found to activate the PI3K/Akt signalling pathway (Yu et al., 2017). Fucoidan

was found to be defending against oxidative stress by preventing apoptosis and promoting antioxidant defence (Gao et al., 2012).

Thus, our research with industry-grade compounds was able to support the data found for previous research with pure compounds. This leads us to explore cost-effective and less pure compounds for reproducible neuroprotective interventions. Comparing the results between FX1 and FX4 revealed a similar trend, as mentioned in Section 2.4.1. It was evident from previous literature that whole seaweed extract was found to show better protection because of the presence of other bioactive compounds (Wang et al., 2021) This provided us with a firm ground to use cost-effective and lower-purity compounds with neuroprotective potential, which could be used in any nutraceutical or functional food development.

4.3 Neuroprotective effect of industry-grade fucoxanthin and fucoidan in combination against hydrogen peroxide-induced cytotoxicity in PC12 cell

After observing the individual neuroprotective potentials of industry-grade fucoxanthin and fucoidan of different purities and concentrations, we aimed to assess the combined neuroprotective potential of these compounds. The rationale behind this study was to determine whether the combination could provide better protection than when used alone. This was important because there were more than one damaging factor that acted through different mechanisms. Thus, to offer better protection by combining FX1 and FX4 with varying concentrations of FD to observe the level of neuroprotection offered. This rationale was supported by a few of the previous studies stating that combining the natural antioxidants could show better protection than they were used individually (Daverey & Agrawal, 2018).

We used the Chou-Talalay method to determine how much protection each concentration of FX1 or FX4 with FD provides against amyloid-beta and hydrogen peroxide-induced cytotoxicity in PC12 cells **(Appendix A-H)**. The most promising synergistic result (CI<1) was observed for FX1+FD combinations at 5+25 µg/mL, 10+25 µg/mL and FX4+FD at 20+25 µg/mL. These combinations showed increased relative growth in comparison to when used alone. A similar trend of results was observed for 100 µM of hydrogen peroxide stress for the similar combinations.

However, a higher level of stress induced by 2 µM amyloid-beta in PC12 cells resulted in less synergistic effects when the combinations were tested. Only FX1+FD (10+25 µg/mL) and FX4+FD (20+25 µg/mL) showed synergistic effects, though the rest of the combinations mainly were additive or antagonistic. The rest of the combinations were found either additive or antagonistic. This could be due to the excessive protective capacity of the compounds.

Another possible reason might be the fully activated cellular defence pathway, which left a limited room for improvement.

4.4 Total Polyphenol Content and activity correlation

FX4, being less pure, was showing better protection than FX1 in all the experiments conducted. To get to know more about this, we did one biological replicate with two technical replicates of total polyphenol content analysis of the compounds **(Appendix I - J)**. From the results we found that FX4 contained three times more phenolic compounds (~33%) than the phenolic compounds for FX1 (~11%). During the steps of purification, a spectrum of bioactive compounds, like polyphenols might get lost. These compounds were known to enhance antioxidant activity by scavenging reactive oxygen species (Asai et al.) and thus reduces oxidative damages (Chandini et al., 2008; Zorov et al., 2014). Previous studies had shown that crude or semi-purified seaweed extracts demonstrate stronger antioxidant and anti-inflammatory effects compared to isolated compounds (Wang et al., 2009). Thus, our results showed its significance and supported the results for FX4 observed.

4.5 Physiological relevance and translational potential

Though all the tested concentrations for fucoxanthin (FX1, FX4), and fucoidan (FD) were effective in vitro, their in vivo applicability might be limited due to several pharmacokinetic factors. Fucoidan has been known for its low oral bioavailability. Though modifications such as its transformation into fucoidan-derived carbon dots have been found promising to be improving blood brain barrier permeability (Han et al., 2025). But fucoxanthin undergoes metabolic conversion into bioactive derivatives like fucoxanthinol and amarouciaxanthin A, which were detectable in the blood plasma after the oral intake (Asai et al., 2004; Hashimoto et al., 2009). However, it was still unclear to what extent these compounds could cross the blood-brain. Thus, further investigations are needed for in vivo pharmacokinetic studies using advanced in vitro BBB models.

4.6 Limitations of this study

Although the project yielded some interesting and encouraging findings, it still has some limitations. PC12 cells lines were used for in vitro analysis of neuroprotection of marine-derived compounds, fucoxanthin and fucoidan of different purities and concentrations. This is a widely used cell line in neurobiology. However, this study does not accurately mimic how a real brain cell functions in the body. Thus, there is a need for in vivo or animal model testing to observe the actual effects of these compounds.

Moreover, we only assessed relative growth in this experiment. However, we could not assess the underlying cellular mechanisms by which these compounds provide protection. Flow cytometry, ROS analysis, HPLC profiling of the compounds due to time constraints. These could have provided a better understanding of how these compounds work at the molecular level. Another limitation was the lack of chemical profiling of fucoxanthin and fucoidan. These compounds were tested without confirming their structural composition. As our experiments were carried out using the industrial grade of compounds which might have impurities within it. This could have affected the unidentified results.

The Chou-Talalay method was used to understand whether the combined effects were synergistic, additive or antagonistic. However, this analysis was conducted solely through viability analysis. The interaction remains unclear without additional molecular-level studies underlying this synergy. Investigating the pathways involved like Nrf2/ARE, PI3K/Akt, or MAPK signalling by western blotting or gene expression studies could provide stronger mechanistic support. Lastly, the concentrations used in this study might not include any direct evidence related to achieving bioavailability issues. Factors such as solubility, GI absorption, systemic metabolism, and BBB permeability were not evaluated. Thus, pharmacokinetic profiling in animal model validation, which could lead us towards further clinical applications, was critical.

4.7 Future directions

Future studies should aim to understand the molecular pathways (oxidative stress, apoptosis or inflammation) by which fucoxanthin and fucoidan provide neuroprotection. Exploring these mechanisms will help us to understand how these compounds can show better protection beyond simply increasing relative growth. Detailed advanced chemical profiling of the different purity grades of fucoxanthin and fucoidan by high performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR) or liquid chromatography-mass spectrometry (LC-MS) will help to determine the effect of impurities on their action. This will help to identify or quantify the presence of polyphenols or co-active compounds, especially for FX4. Further flow cytometry analysis will facilitate determining how these compounds exert protection in cellular level. For translational relevance of this study, in vivo animal models of AD can be a promising area of research in future to assess the bioavailability, metabolism and BBB permeability. Moreover, mechanistic studies can be conducted to clarify how these compounds provide neuroprotection by using different assays to determine whether pathways like Nrf2/ARE, PI3K/Akt, or MAPK are involved or not. If these results are promising, then

the compounds may be used in developing affordable functional foods or supplements for preventing early signs of neurodegenerative diseases.

4.8 Conclusion

This study was designed to investigate the neuroprotective effects of industry-grade fucoxanthin of two different purities (FX1, 95.6%), (FX4, 5.6%) and fucoidan (FD, 20%) in against the cytotoxicity of P12 cells induced by two different stresses, amyloid beta (1 μM and 2 μM) and hydrogen peroxide (100 μM and 150 μM). The findings support the hypothesis as all three compounds showed neuroprotection activity against these stresses in a dose-dependent manner. FX4, with only 5.6% fucoxanthin, showed better neuroprotection than the higher-purity 95.6% fucoxanthin (FX1). This may be due to the presence of other neuroprotective compounds that contribute to the neuroprotection of FX4. For the combination of the compounds, a synergistic effect was observed for only two sets of combinations, indicating their potential for practical applications. The novelty of this study lies in comparing the industrial-grade purity of fucoxanthin and fucoidan with the costly analytical-grade purity to develop scalable alternatives for early-stage Alzheimer's prevention, offering affordable options. This research also serves as a baseline for studying the synergism these compounds in combination against oxidative and amyloid-beta-induced stresses. Despite the limitations, this research has shed a light into the differential and surprisingly better neuroprotective responses of industry-grade fucoxanthin and fucoidan as a neuroprotective agent for Alzheimer's prevention by further pharmacokinetic profiling, exploring molecular mechanisms and in vivo studies.

Appendices

Appendix A: Manual CI calculation for FX1+FD combination under 1 µM beta-amyloid induced cytotoxicity

Replicates	Treatment	Relative Growth (%)	Fa (Combo)	Fa (FX1)	Fa (FD)	CI (Manual)	Interaction type
1	FX1+FD 5+25	94.96	0.0504	0.1484	0.0653	1.111	Antagonistic
2	FX1+FD 5+50	92.94	0.0706	0.1484	0.0653	1.558	Antagonistic
3	FX1+FD 10+25	99.64	0.0036	0.0942	0.0653	0.093	Synergistic
4	FX1+FD 10+50	93.19	0.0681	0.0942	0.0653	1.765	Antagonistic

Appendix B: Manual CI calculation for FX4+FD combination under 1 µM beta-amyloid induced cytotoxicity

Replicates	Treatment	Relative Growth (%)	Fa (Combo)	Fa (FX4)	Fa (FD)	CI (Manual)	Interaction type
1	FX4+FD 5+25	96.47	0.0353	0.0712	0.0653	1.035	Antagonistic
2	FX4+FD 5+50	93.77	0.0623	0.0712	0.0653	1.828	Antagonistic
3	FX4+FD 20+25	99.96	0.0004	0.0468	0.0653	0.015	Synergistic
4	FX4+FD 20+50	93.42	0.0658	0.0468	0.0653	2.414	Antagonistic

Appendix C: Manual CI calculation for FX1+FD combination under 2 µM beta-amyloid induced cytotoxicity

Replicates	Treatment	Average Viability (%)	Viability FX1 (%)	Viability FD (%)	Fa (Combo)	Fa (FX1)	Fa (FD)	CI (Manual)	Interaction type
1	FX1+FD 0.25+0.5	70.85	56.273	58.923	0.2915	0.4373	0.4108	1.376	Antagonistic
2	FX1+FD 0.25+1	82.88	56.273	63.853	0.1712	0.4373	0.3615	0.865	Synergistic
3	FX1+FD 0.5+0.5	74.46	61.483	58.923	0.2554	0.3852	0.4108	1.285	Antagonistic
4	FX1+FD 0.5+1	82.01	61.483	63.853	0.1799	0.3852	0.3615	0.965	Synergistic
5	FX1+FD 5+25	85.81	74.177	84.033	0.1419	0.2582	0.1597	1.438	Antagonistic
6	FX1+FD 5+50	86.04	74.177	85.867	0.1396	0.2582	0.1413	1.528	Antagonistic
7	FX1+FD 10+25	93.02	80.783	84.033	0.0698	0.1922	0.1597	0.801	Synergistic
8	FX1+FD 10+50	85.62	80.783	85.867	0.1438	0.1922	0.1413	1.766	Antagonistic

Appendix D: Manual CI calculation for FX4+FD combination under 2 µM beta-amyloid induced cytotoxicity

Replicates	Treatment	Average Viability (%)	Viability FX4 (%)	Viability FD (%)	Fa (Combo)	Fa (FX4)	Fa (FD)	CI (Manual)	Interaction type
1	FX4+FD 0.25+0.5	55.41	55.41	58.92	0.4459	0.4459	0.4108	2.085	Antagonistic
2	FX4+FD 0.25+1	62.67	55.41	63.85	03733	0.4459	0.3615	1.87	Antagonistic
3	FX4+FD 0.5+0.5	71.04	62.67	58.92	0.2896	0.3733	0.4108	1.481	Antagonistic
4	FX4+FD 0.5+1	76.07	62.67	63.85	0.2393	0.3733	0.3615	1.303	Antagonistic
5	FX4+FD 5+25	82.11	71.04	84.03	0.1789	0.2896	0.1597	1.738	Antagonistic

Appendix E: Manual CI calculation for FX1+FD combination under 100 µM H_2O_2 induced cytotoxicity

Replicates	Treatment	Average Viability (%)	Fa (Combo)	Fa (FX1)	Fa (FD)	CI (Manual)	Interaction type
1	FX1+FD 5+25	93.07	0.0693	0.1613	0.1442	0.91	Synergistic
2	FX+FD 5+50	87.72	0.1228	0.1613	0.0756	2.385	Antagonistic
3	FX1+FD 10+25	95.08	0.0492	0.0975	0.1442	0.845	Synergistic
4	FX1+FD 10+50	89.7	0.103	0.0975	0.0756	2.419	Antagonistic

Appendix F: Manual CI calculation for FX4+FD combination under 100 µM H_2O_2 induced cytotoxicity

Replicates	Treatment	Average Viability (%)	Fa (Combo)	Fa (FX4)	Fa (FD)	CI (Manual)	Interaction type
1	FX4+FD 5+25	96.54	0.0346	0.1839	0.1442	0.428	Synergistic
2	FX4+FD 5+50	87.14	0.1286	0.1839	0.0756	2.4	Antagonistic
3	FX4+FD 20+25	96.12	0.0388	0.0655	0.1442	0.861	Synergistic
4	FX4+FD 20+50	90.65	0.0935	0.0655	0.0756	2.663	Antagonistic

Appendix G: Manual CI calculation for FX1+FD combination under 150 µM H_2O_2 induced cytotoxicity

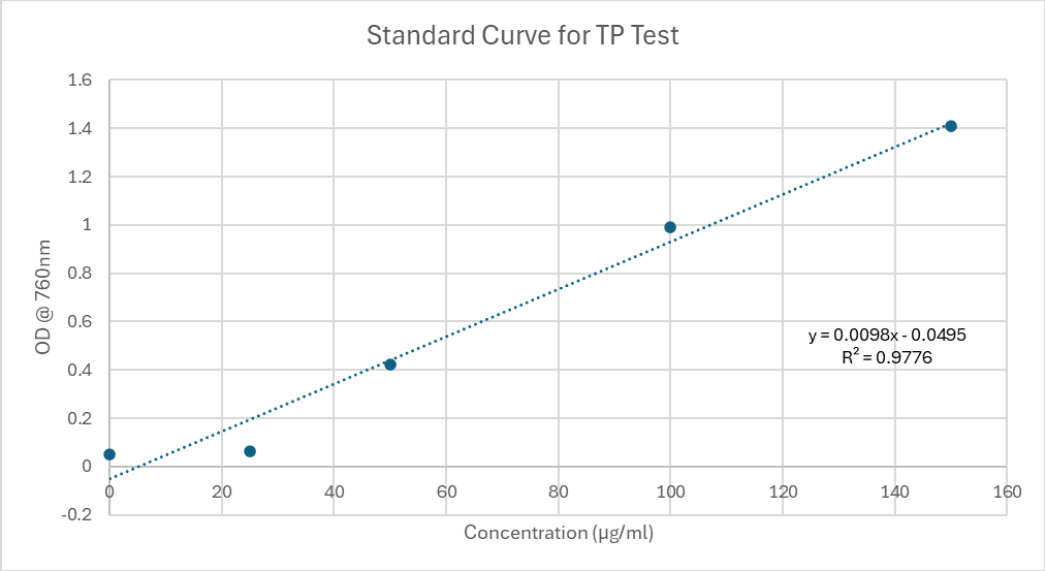
Replicates	Treatment	Average Viability (%)	Fa (Combo)	Fa (FX1)	Fa (FD)	CI (Manual)	Interaction type
1	FX1+FD 5+25	91.21	0.0879	0.1981	0.145	1.05	Antagonistic
2	FX+FD 5+50	86.32	0.1368	0.1981	0.1115	1.918	Antagonistic
3	FX1+FD 10+25	93.9	0.061	0.1356	0.145	0.87	Synergistic
4	FX1+FD 10+50	88.88	0.1112	0.1356	0.1115	1.818	Antagonistic

Appendix H: Manual CI calculation for FX4+FD combination under 150 µM H_2O_2 induced cytotoxicity

Replicates	Treatment	Average Viability (%)	Fa (Combo)	Fa (FX4)	Fa (FD)	CI (Manual)	Interaction type
1	FX4+FD 5+25	92.05	0.0795	0.202	0.145	0.941	Synergistic
2	FX4+FD 5+50	88.16	0.1184	0.202	0.1115	1.648	Antagonistic
3	FX4+FD 20+25	94.33	0.0567	0.0806	0.145	1.094	Antagonistic
4	FX4+FD 20+50	90.53	0.0947	0.0806	0.1115	2.024	Antagonistic

Appendix I: Total Polyphenol (TP) content for FX1 and FX4

Concentration	FX1 (0.5 mg/ml)	FX1 (1 mg/ml)	FX4 (0.5 mg/ml)	FX4 (1 mg/ml)
Absorbance at 760 nm	0.070	0.085	0.253	0.349
	0.062	0.062	0.283	0.325
Average Absorbance	0.066	0.074	0.268	0.337
TP content (1 µg/ml)	12.19	13.72	30.87	40.66
TP content (2 µg/ml)	11.38	11.38	33.93	38.21
Average TP content	11.79	12.55	32.4	39.44



Appendix J: Standard Curve for Total Polyphenol (TP) Test

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