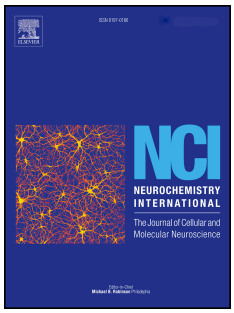
Accepted Manuscript



In vitro studies of the neuroprotective activities of astaxanthin and fucoxanthin against amyloid beta ($A\beta_{1-42}$) toxicity and aggregation

Mousa Alghazwi, Scott Smid, Ian Musgrave, Wei Zhang

PII: S0197-0186(18)30249-3

DOI: https://doi.org/10.1016/j.neuint.2019.01.010

Reference: NCI 4378

To appear in: *Neurochemistry International*

Received Date: 6 June 2018

Revised Date: 31 December 2018

Accepted Date: 8 January 2019

Please cite this article as: Alghazwi, M., Smid, S., Musgrave, I., Zhang, W., *In vitro* studies of the neuroprotective activities of astaxanthin and fucoxanthin against amyloid beta ($A\beta_{1-42}$) toxicity and aggregation, *Neurochemistry International* (2019), doi: https://doi.org/10.1016/j.neuint.2019.01.010.

In vitro studies of the neuroprotective activities of astaxanthin and fucoxanthin against amyloid beta ($A\beta_{1-42}$) toxicity and aggregation

Mousa Alghazwi[1, 2, 3*], Scott Smid[4], Ian Musgrave[4], Wei Zhang[1, 2*]

[1] Centre for Marine Bioproducts Development (CMBD), and [2] Medical Biotechnology, College of Medicine and Public Health, Flinders University, GPO Box 2100, Adelaide 5001, South Australia, Australia;

*Corresponding authors, Emails: algh0068@flinders.edu.au , mossa309@hotmail.com (Mousa Alghazwi); Wei.zhang@flinders.edu.au (Wei Zhang)

[3] Ministry of Higher education in Saudi Arabia, King Faisal Hospital Street, Riyadh 11153

[4] Discipline of Pharmacology, School of Medicine, Faculty of Health Sciences, The University of Adelaide, Adelaide, South Australia, Australia;

E-mails: scott.smid@adelaide.edu.au (Scott Smid), ian.musgrave@adelaide.edu.au (Ian Musgrave)

Abstract

Amyloid beta (Aβ) can aggregate and form plaques, which are considered as one of the major hallmarks of Alzheimer's disease. This study aims to directly compare the neuroprotective activities *in vitro* of two marine-derived carotenoids astaxanthin and fucoxanthin that have shown a spectrum of biological activities, including neuroprotection. The *in vitro* neuroprotective activities were investigated against $A\beta_{1-42}$-mediated toxicity in pheochromocytoma (PC-12) neuronal cells using the MTT cell viability assay, anti-apoptotic, antioxidant and neurite outgrowth activities; as well as inhibition against $A\beta_{1-42}$ fibrillization in the Thioflavin T (ThT) assay of fibril kinetics and via transmission electron microscopic (TEM) evaluation of fibril morphology. The results demonstrated that both astaxanthin and fucoxanthin exhibited multi-neuroprotective effects favouring fucoxanthin over astaxanthin supporting neuroprotective roles of marine-derived carotenoids as potential novel dementia prevention or therapeutic strategies.

Keywords: Astaxanthin; fucoxanthin; neuroprotection; amyloid beta; Alzheimer's disease

1. Introduction

Alzheimer's disease (AD) is a major neurodegenerative disorder responsible for more than 60-80% of dementia cases globally (Alzheimer'sAssociation, 2018). Aggregates of the protein amyloid beta (Aβ) form the amyloid plaques that are one of the main hallmarks of AD pathology (Hussain et al., 1999). The amyloid hypothesis suggests Aβ as a pathognomonic feature of AD (Hardy and Selkoe, 2002). Currently, only four compounds targeting acetylcholinesterase (donepezil, tacrine, rivastigmine and galantamine) and one drug targeting the N-methyl-D-aspartate (NMDA) receptor (memantine) have been approved to reduce some symptoms experienced by AD sufferers (Cummings et al., 2014). It was suggested that small molecules are favorable to target Aβ (Re et al., 2010) and could be the strategy to treat AD (Nie et al., 2011). Many of such molecules have demonstrated neuroprotective activities against Aβ cytotoxicity and aggregation such as Alzhemed, EGCG, and SEN304 (Doig and Derreumaux, 2015). An effective disease-modifying treatment is urgently needed, and drugs that target Aβ aggregation were proposed as a pathway to such treatments. Even though, no drug that is targeting Aβ aggregation and toxicity has been approved yet. The possible reason includes targeting the right target but at the wrong stage as the Aβ is accumulated in the body for more than 10 years before the symptoms. It will be difficult to treat these patients as they are in late stage (Hampel et al., 2015; Sperling et al., 2011). In addition, it might be insufficient to target Aβ alone to find effective therapeutic drug as AD is a complex disease. For that reason, drug targeting multiple pathways are needed in the new approach to treat AD (Piau et al., 2011).

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

On the other hand, fucoxanthin (Figure 1b) is a carotenoid (also known as tetraterpenoid) derived from brown algae and microalgae (Peng et al., 2011). It is an orange coloured pigment found in high content in classes such as Phaeophyceae, Haptophyta, Bacillariophyceae and Chrysophyceae. It is also found in minor levels in Rhodophyta, Raphidophyceae, and Dinophyta (Takaichi, 2011). Fucoxanthin belongs to the xanthophyll class, as it contains an oxygen in the functional group which differentiate it from carotenes (Mikami and Hosokawa, 2013). The structure of fucoxanthin was illustrated for the first time in 1990 (Englert et al., 1990) and contains a unique allenic bond in its structure. In addition, the polyene chain contains an epoxide and conjugated carbonyl group (Mikami and Hosokawa, 2013).

Astaxanthin has been shown to possess different biological activities, especially as an antioxidant. Earlier research on the antioxidant activity of astaxanthin against singlet oxygen species has revealed that it has over ten times the activity of other carotenoids

such as lutein and β-carotene, and 100 more times activity than α- tocopherol (Miki, 1991). As for the interactions with the amyloid β protein, astaxanthin has been shown to protect neuronal PC-12 cells from the toxicity induced by $A\beta_{25-35}$ (Chang et al., 2010). Another study demonstrated that astaxanthin can attenuate the toxicity of $A\beta_{25-35}$ in SH-SY5Y cells through decreasing the Bcl-2/Bax ratio (Wang et al., 2010). Astaxanthin (at 10 μM) exhibited stronger activity than β-carotene and canthaxanthin in reducing the cytotoxicity induced by $A\beta_{25-35}$ (Chang et al., 2013).

Fucoxanthin has shown to exhibit several bioactivities which include anti-cancer (Kumar et al., 2013), cardioprotective activity (Matsumoto et al., 2010), anti-obesity (Okada et al., 2008), anti-diabetic activity (Nishikawa et al., 2012), and antioxidant (Sachindra et al., 2007). Fucoxanthin was found to inhibit the neurotoxicity induced by $A\beta_{1-42}$ in cerebral cortex neurons (Zhao et al., 2015). A recent study showed that fucoxanthin can inhibit the aggregation of $A\beta_{1-42}$ and also was found to protect the SH-SY5Y cells from the neurotoxicity induced by $A\beta_{1-42}$ (Xiang et al., 2017). Fucoxanthin was found to protect SH-SY5Y cells against Aβ oligomer via inhibiting ERK pathway and activating PI3K/Akt pathway (Lin et al., 2017).

In all the previous studies, there was no direct comparison of these two promising neuroprotective carotenoids. Our aim was to undertake a systematic comparative study of the neuroprotective activities of both fucoxanthin and astaxanthin in a number of bioassays to understand their relative neuroprotective capacity. A range of *in vitro* assays were carried out to test these two compounds in a cell-based neuroprotective assay in reducing the toxicity of amyloid β and their anti-aggregation effects using the Thioflavin T (ThT) assay of fibril kinetics and transmission electron

microscopy (TEM) of direct fibril morphology. The antioxidant and protective effects against reactive oxygen species, inhibition of apoptosis induced by $A\beta_{1-42}$ and enhancing neurite outgrowth activity were also assessed.

2. Material and methods

2.1 Reagents and materials

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 97.5%), Roswell Park Memorial Institute 1640 (RPMI), astaxanthin (SML0982) with 97% purity, and fucoxanthin (F6932) with 95% purity were purchased from Sigma-Aldrich (USA). Foetal bovine serum (FBS) was purchased from Bovogen Biologicals (East Keilor, VIC, Australia). Penicillin/streptomycin and 10×trypsin EDTA were obtained from Thermo Fisher Scientific (Scoresby, VIC, Australia). Human amyloid-β 1–42 protein ($A\beta_{1-42}$) was obtained from rPeptide (Bogart, Georgia, USA).

2.2 PC-12 cell culture

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

2.3 $A\beta_{1-42}$ preparation and treatment in PC-12 cells

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

2.4 MTT assay for astaxanthin and fucoxanthin cytotoxicity, induced-protection of cells against Aβ1-42 -induced cytotoxicity, and induced-protection of cells against H_2O_2 cytotoxicity

The cytotoxicity of these compounds was measured using the MTT assay. Initially, cells were plated at 2×10^4 cells per well in 100 µl media and incubated at 37°C with 5% CO_2 for 24 hours. Both astaxanthin and fucoxanthin were diluted in DMSO to their final stock concentrations. PC-12 cells were treated with each of these carotenoid compounds at different concentrations (astaxanthin 0.1-50 µM; fucoxanthin 0.1-20 µM) and then incubated for 48 hours at 37°C. The media was subsequently removed and replaced with 0.5 mg/mL of MTT (diluted in PBS). The plate was then incubated for 2 hours at 37°C. The MTT solution was then removed and the cells lysed with 100 µl of DMSO. The absorbance of the plate was measured at 570 nm with a reference wavelength of 630 nm using a microplate reader (Bio-Tek Instruments Inc, USA).

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

For induced-protection of cells against H_2O_2 cytotoxicity procedure, cells were incubated for 15 minutes after carotenoid treatment, then treated with H_2O_2 (100 µM

and 200 µM). Cells were then incubated for 24 hours at 37°C prior to measurement of cell viability.

2.5 Thioflavin T (ThT) assay of $A\beta_{1-42}$ fibril and aggregate formation

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

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

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

2.7 Nuclear staining for assessment of apoptosis

PC-12 cells were seeded at a density of 2×10^4 overnight. Cells were then treated with astaxanthin and fucoxanthin at different concentrations for 15 minutes before

adding $A\beta_{1-42}$ (1 µM). The cells were incubated for 48 hours and then 5 µg/mL of Hoechst 33258 stain was added and incubated for 10 minutes in the dark to stain the cell nuclei. The cells were then washed with PBS and the plates monitored using an EVOS FL Cell Imaging System (Thermo Fisher) fluorescence microscope. The percentage of apoptotic cells (n=350 cells at least per well) were calculated as followed: $Apoptotic\ cells\ \% = apoptotic\ cells\ \div total\ cells\ (viable\ cells + apoptotic\ cells) \times 100$

Three independent experiments were performed for every treatment.

2.8 Neurite outgrowth assay

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

2.9 Statistical analysis

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

ANOVA with Tukeys HSD post-hoc test. Differences were considered statistically significant at $p < 0.05$.

3. Results and Discussion

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

Astaxanthin did not show any cytotoxicity when it was incubated with PC-12 cells, even at the highest concentration (50 µM) tested (Figure 2a). It was reported that astaxanthin is very safe for human consumption (Guerin et al., 2003) and was approved to be used as a food supplement by United States Food and Drug Administration (Guerin et al., 2003; Stewart et al., 2008).

Fucoxanthin showed a significant reduction in PC-12 cell viability at ≥5 µM and has negligible toxicity below 2 µM (Figure 2b). This is similar to a previous study in which fucoxanthin was toxic at 10 µM in human lymphocytes for 24 hours, reducing cell viability to 40% (Molina et al., 2014). Fucoxanthin was also toxic to HaCaT cells (keratinocytes) at 40 µM or more when treated for 16 hours (Zheng et al., 2013). As both the incubation times and cell lines are different, this may explain the variation in toxic concentrations. By contrast, fucoxanthin did not show any sign of toxicity in ICR mice administrated 1000 mg/kg and 2000 mg/kg doses (Beppu et al., 2009). Fucoxanthin did not show any toxicity to HUVEC cells or zebrafish and was protective against high glucose-induced intracellular reactive oxygen species (ROS) at 50 µM (Kang et al., 2014). Using weanling female Wistar rats, fucoxanthin did not show any toxicity up to 100 mg/kg dosage(Ravi et al., 2015). It is likely that some cell lines are more sensitive than others, such as the PC-12 cells in the current study.

Treating PC-12 cells with $A\beta_{1-42}$ (1 µM) resulted in cell viability decreasing to 71%, with astaxanthin pre-treatment providing significant neuroprotection against $A\beta_{1-42}$ at all tested concentrations (p<0.05, Figure 2a). This protective activity was even significant at the lowest concentration used (0.1 µM; p<0.05). This result is consistent with earlier studies reporting that astaxanthin at 0.1 µM can reduce the toxicity of $A\beta_{25-35}$ in PC-12 cells when treated for 48 hours (Chang et al., 2010), and that treating SH-SY5Y cells with astaxanthin at 10 µM for 24 hours can rescue cell viability lost to $A\beta_{25-35}$ (Wang et al., 2010).

Treating PC-12 cells with fucoxanthin < 2µM significantly reduced the cytotoxicity of $A\beta_{1-42}$ in a concentration-dependent manner (Figure 2b). In fact, fucoxanthin showed significant neuroprotection to PC-12 cells against $A\beta_{1-42}$ at all tested concentrations (0.01–2 µM) in this study (p<0.05). These results are comparable to recent studies as 1 µM of fucoxanthin demonstrated the ability to increase cell viability compromised by $A\beta_{1-42}$ in SH-SY5Y cells from 50% to almost 80% (Xiang et al., 2017), and increase cell viability from 55% to 87% comparing to control Aβ oligomer-induced toxicity in SH-SY5Y cells (Lin et al., 2017). In our study 1 µM of fucoxanthin was able to increase cell viability compromised by $A\beta_{1-42}$ from around 67% to 98.5% in PC12 cells. A recent study on the neuroprotective activity of fucoxanthin showed that it was able to protect cerebral cortical neurons against the toxicity induced by $A\beta_{25-35}$ (Zhao et al., 2015). In addition, fucoxanthin inhibited BACE1 with an IC_{50} of 5.31 µM (Jung et al., 2016). Earlier studies and the current results together suggest that both carotenoid compounds are neuroprotective to cell lines against the cytotoxicity induced by Aβ (all forms).

3.2 Effects of astaxanthin and fucoxanthin on $A\beta_{1-42}$ fibril and aggregate formation

The ThT assay was used to study the kinetics of any anti-aggregative effect of astaxanthin and fucoxanthin against Aβ. The ThT assay demonstrated that $A\beta_{1-42}$ formed fibrils, as the fluorescence increased substantially over 48 hours, while astaxanthin inhibited $A\beta_{1-42}$ fibril formation in a concentration-dependent manner over this period (Figure 3a). Area under the curve analysis showed that 1 µM astaxanthin was able to reduce $A\beta_{1-42}$ aggregation by 50% (Figure 3b). Astaxanthin at 50 µM showed the highest anti-aggregation activity, with almost 80% reduction in the intensity of ThT fluorescence.

Similarly, fucoxanthin inhibits $A\beta_{1-42}$ fibril formation in a concentration-dependent manner. However, 1-10 µM fucoxanthin inhibited fibril formation dramatically (Figure 3c). Fucoxanthin at 2 µM was able to reduce the aggregation of $A\beta_{1-42}$ by 75% (Figure 3d). The strongest activity was at 10 µM, but this concentration was toxic to PC-12 cells. Earlier research demonstrated that fucoxanthin at more than 1 µM was able to reduce 50% of $A\beta_{1-42}$ aggregation (Xiang et al., 2017), while in this study 1 µM of fucoxanthin was able to reduce more than 50% of $A\beta_{1-42}$ aggregation.

To our knowledge, this evidence collectively is the first report of anti-aggregation effects of astaxanthin against $A\beta_{1-42}$.

3.3 Effects of astaxanthin and fucoxanthin on $A\beta_{1-42}$ fibril and aggregate morphology

Examining the morphology of $A\beta_{1-42}$ aggregates using transmission electron microscopy (TEM) showed that $A\beta_{1-42}$ formed fibrils and aggregates after 48 hours incubation (Figure 4a). In the presence of astaxanthin (50 µM), fibrils were observed to be similar in morphology but less prevalent than control $A\beta_{1-42}$ sample (Figure 4b).

TEM analysis showed that fucoxanthin inhibited the formation of fibrils and truncated fibril length (Figure 4c). The ThT assay results were confirmed by the electron micrographs for fucoxanthin, where it inhibited aggregation of $A\beta_{1-42}$. This finding supports a previous study showing that fucoxanthin was able to inhibit the aggregation of $A\beta_{1-42}$ while concomitantly alleviating cognitive impariment in an animal model (Xiang et al., 2017).

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

Treating PC-12 cells with $A\beta_{1-42}$ (1 µM) for 48 hours and measuring apoptosis using the Hoechst 33258 nuclear stain resulted in approximately 20% of apoptotic cells (Figure 5). However, cells treated with astaxanthin or fucoxanthin at different concentrations resulted in a reduction in the number of apoptotic cells in a concentration-dependent manner. Lin et al. demonstrated that Aβ oligomer induced about 60% of apoptosis cells in SH-SY5Y cells, and treating the cells with 3 µM fucoxanthin reduced apoptotic cells to 20% (Lin et al., 2017). This result is consistent with a report that astaxanthin inhibited apoptosis induced by H_2O_2 in mouse neural progenitor cells (Kim et al., 2009), 6-OHDA in SH-SY5Y cells (Ikeda et al., 2008) and $A\beta_{25-35}$ in SH-SY5Y cells (Wang et al., 2010). Similarly, fucoxanthin has been previously shown to reduce apoptosis induced by both H_2O_2 (Yu et al., 2017) and Aβ oligomer in SH-SY5Y cells (Lin et al., 2017). These results suggested that both compounds demonstrate a pleitropic neuroprotective effect *in vitro,* by inhibiting overt toxicity, apoptosis and aggregation of $A\beta_{1-42}$.

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

Cell viability was significantly decreased after treating PC-12 cells with 100-200 µM hydrogen peroxide (H_2O_2) (Figures 6a- 6d). At 100 µM H_2O_2, cell viability was reduced to 60%, while at 200 µM H_2O_2 cell viability was only 30% (Figure 6a - 6b).

Astaxanthin provided significant neuroprotection to PC-12 cells against H_2O_2-induced cytotoxicity, with cell viability exceeding 65% (p<0.05, Figure 6a), and 36% (p<0.05, Figure 6b) at both H_2O_2 concentrations, respectively. Astaxanthin is a known antioxidant, reportedly 10 times stronger than other carotenoids and 100 times stronger than α-tocopherol (Miki, 1991). In addition, astaxanthin has been shown previously to protect differentiated PC-12 cells from H_2O_2-induced cytotoxicity (Chan et al., 2009). Our results also demonstrated that astaxanthin was an effective antioxidant, reducing the cytotoxicity induced by H_2O_2 in PC-12 cells significantly and at low concentrations.

While fucoxanthin did not show significant protection against H_2O_2 to PC-12 cells at the lowest concentrations tested (0.01–0.1 µM), it demonstrated significant protection at higher concentrations (0.5–2 µM; Figure 6c (p<0.05)). Fucoxanthin at the highest concentration (2 µM) demonstrated a significant increase in cell viability against H_2O_2 at 200 µM (Figure 6d). These results are supported by an earlier study when fucoxanthin was found to inhibit the cytotoxicity of H_2O_2 in SH-Sy5Y cells (Yu et al., 2017). However, this study demonstrated lower protection as the other study showed increase in cell viability from 50% to 79% and 98% when treated at 1 and 3 µM fucoxanthin, respectively. These differences might account for different cell lines used. Fucoxanthin was reported to have strong antioxidant activity by inhibiting DPPH (2,2-diphenyl-1-picrylhydrazyl) formation with an IC_{50} of 164.60 µM and showed 13.5 times higher activity than previously reported for α-tocopherol in hydroxyl radical-scavenging assay (Sachindra et al., 2007). Additionally, a previous study found that fucoxanthin could reduce the toxicity induced by H_2O_2 in Vero cells in a concentration-dependent manner (5-200 µM) (Heo et al., 2008). Recently,

fucoxanthin was found to inhibit cytotoxicity induced by H_2O_2 in SH-SY5Y cells and to activate the PI3K/Akt cascade and inhibit the ERK pathway (Yu et al., 2017). Our study found that fucoxanthin at 0.1 - 4 µM was able to increase PC-12 cell viability against H_2O_2 at 100 µM. However, fucoxanthin at 2 µM was only able to significantly reduce cytotoxicity induced by 200 µM H_2O_2.

It is clear from our results that astaxanthin was more effective than fucoxanthin in reducing the cytotoxicity induced by hydrogen peroxide.

3.6 Astaxanthin and fucoxanthin can enhance neurite outgrowth activity

Enhancing neurite outgrowth is considered as one strategy to treat neurodegenerative diseases, as it can help maintain neuronal plasticity and communication (Koliatsos et al., 1991). It was found that decreasing the level of nerve growth factor (NGF) can result in neuronal dysfunction (Hefti and Weiner, 1986), while NGF can increase neuronal survival (Kromer, 1987). Also, NGF was found to enhance neurite outgrowth (Huang and Reichardt, 2001).

In this study, astaxanthin and fucoxanthin enhanced neurite outgrowth activity in PC-12 cells (Figure 7). Around 8% of cells developed neurites in the control group over 24 hours, while treatment with astaxanthin and fucoxanthin increased the number of neurites in a concentration-dependent manner. Astaxanthin was able to promote 13.6 - 23.7% of cells to develop neurite outgrowth at concentrations ranging from 0.1 to 50 µM.

On the other hand, fucoxanthin demonstrated more potent neurite outgrowth activity, as it was able to promote 15.7 - 31% of cells to develop neurite outgrowth in a concentration dependent manner at much lower concentrations (0.1 to 2 µM). This is

the first report of these compounds possessing such activity, which further highlights the potential neuroprotective activities of these compounds. Previously, a methanolic extract of *Sargassum macrocarpum* (brown algae) and a PBS extract from *Jania adharens* (red algae) elicited neurite outgrowth activity in PC-12 cells (Kamei and Sagara, 2002). Five compounds (MC14, sargaquinoic acid, sargachromenol, pheophytin A, and fucoidan) from algae were also shown to be able to enhance neurite outgrowth activity from previous studies (Alghazwi et al., 2016).

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

In a review on neuroprotective compounds derived from macroalgae, fucoxanthin was considered to be a promising compound due to its multiple neuroprotective pathways (Alghazwi et al., 2016), while astaxanthin has also been considered to be a promising compound for the treatment of neurological diseases (Wu et al., 2015). Our work supports their neuroprotective activities, as both compounds reduced the toxicity of

$A\beta_{1-42}$ and H_2O_2. Moreover, this study showed an anti-aggregation effect against fibrils formed by $A\beta_{1-42}$.. In addition, both astaxanthin and fucoxanthin were shown to inhibit apoptosis induced by $A\beta_{1-42}$ and promote neurite outgrowth. As fucoxanthin demonstrated higher neuroprotective activity than astaxanthin at much lower concentration, more focus on fucoxanthin in the prevention and treatment of neurodegenerative diseases is recommended, but with consideration of its potential neurotoxicity at higher concentrations. Astaxanthin may be considered as a complementary treatment, possibly within nutraceutical applications.

Astaxanthin has low bioavailability, but the presence of fat can increase the bioavailability of astaxanthin. An example is that polysorbate 80 can enhance the bioavailability of astaxanthin by almost 4-fold (Odeberg et al., 2003). Astaxanthin showed the highest bioavailability than other carotenoids (β-carotene and lutein) (Rao et al., 2013). Similarly, the bioavailability of fucoxanthin can be increased using different agents such as carrier oils and skimmed milk (Mok et al., 2018; Salvia-Trujillo et al., 2015). Further studies on the bioavailability and pharmacokinetics will be required for practical applications of these compounds.

Even though some of the neuroprotective activities have been reported before in various biological models for astaxanthin and fucoxanthin, this is the first study that used a range of neuroprotective assays to compare different neuroprotective activities in one model. The current study unambiguously demonstrated that both astaxanthin and fucoxanthin possess multiple neuroprotective activities but with different potency in a range of bioassays testing different protection mechanisms. At 10-fold less

concentration, fucoxanthin demonstrated both higher neuroprotective and neurite-promoting activity against $A\beta_{1-42}$ than astaxanthin.

4. Conclusion

This study contributes to advance our knowledge in systematic understanding of neuroprotective activities and relative potencies of astaxanthin and fucoxanthin for its future development. Both astaxanthin and fucoxanthin exhibited neuroprotective activities by reducing $A\beta_{1-42}$ toxicity and hydrogen peroxide in PC-12 cells. In addition, these two compounds inhibited the aggregation of $A\beta_{1-42}$. Fucoxanthin showed higher neuroprotective activity against $A\beta_{1-42}$ than astaxanthin. Astaxanthin and fucoxanthin are promising neuroprotective marine-derived compounds, and further research on pharmacokinetics and clinical trials would be required for the development as food supplements or drugs for treating neurodegenerative diseases such as Alzheimer's disease.

Conflict of interest:

The authors confirm that this article content has no conflict of interest.

Acknowledgements

The authors acknowledge the Ministry of Higher Education in Saudi Arabia for their scholarship support and Flinders University for research support to Mousa Alghazwi. The authors would like to thanks Lyn Waterhouse from Adelaide Microscopy for her assistance with TEM and Sukanya Das for assistance in the ThT assay.

References

Alghazwi, M., Kan, Y.Q., Zhang, W., Gai, W.P., Garson, M.J., Smid, S., 2016. Neuroprotective activities of natural products from marine macroalgae during 1999–2015. J Appl Phycol 28, 3599-3616.

Alzheimer'sAssociation, 2018. 2018 Alzheimer's disease facts and figures. Alzheimer's & Dementia 14, 367-429.

Ambati, R.R., Phang, S.-M., Ravi, S., Aswathanarayana, R.G., 2014. Astaxanthin: Sources, extraction, stability, biological activities and its commercial applications—A review. Mar. Drugs 12, 128-152.

Beppu, F., Niwano, Y., Tsukui, T., Hosokawa, M., Miyashita, K., 2009. Single and repeated oral dose toxicity study of fucoxanthin (FX), a marine carotenoid, in mice. The Journal of Toxicological Sciences 34, 501-510.

Chan, K.c., Mong, M.c., Yin, M.c., 2009. Antioxidative and anti‐inflammatory neuroprotective effects of astaxanthin and canthaxanthin in nerve growth factor differentiated PC12 cells. J. Food Sci. 74, H225-H231.

Chang, C.-H., Chen, C.-Y., Chiou, J.-Y., Peng, R.Y., Peng, C.-H., 2010. Astaxanthine secured apoptotic death of PC12 cells induced by β-amyloid peptide 25–35: Its molecular action targets. J. Med. Food 13, 548-556.

Chang, C.-S., Chang, C.-L., Lai, G.-H., 2013. Reactive oxygen species scavenging activities in a chemiluminescence model and neuroprotection in rat pheochromocytoma cells by astaxanthin, beta-carotene, and canthaxanthin. The Kaohsiung Journal of Medical Sciences 29, 412-421.

Cummings, J.L., Morstorf, T., Zhong, K., 2014. Alzheimer’s disease drug-development pipeline: few candidates, frequent failures. Alzheimers Res. Ther. 6, 37.

Doig, A.J., Derreumaux, P., 2015. Inhibition of protein aggregation and amyloid formation by small molecules. Curr. Opin. Struct. Biol. 30, 50-56.

Englert, G., Bjørnland, T., Liaaen‐Jensen, S., 1990. 1D and 2D NMR study of some allenic carotenoids of the fucoxanthin series. Magn. Reson. Chem. 28, 519-528.

Fassett, R.G., Coombes, J.S., 2011. Astaxanthin: a potential therapeutic agent in cardiovascular disease. Mar. Drugs 9, 447-465.

Guerin, M., Huntley, M.E., Olaizola, M., 2003. Haematococcus astaxanthin: applications for human health and nutrition. Trends Biotechnol. 21, 210-216.

Hampel, H., Schneider, L.S., Giacobini, E., Kivipelto, M., Sindi, S., Dubois, B., Broich, K., Nistico, R., Aisen, P.S., Lista, S., 2015. Advances in the therapy of Alzheimer's disease: targeting amyloid beta and tau and perspectives for the future. Expert Rev. Neurother. 15, 83-105.

Hardy, J., Selkoe, D.J., 2002. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297, 353-356.

Hefti, F., Weiner, W.J., 1986. Nerve growth factor and Alzheimer's disease. Ann. Neurol. 20, 275-281.

Heo, S.-J., Ko, S.-C., Kang, S.-M., Kang, H.-S., Kim, J.-P., Kim, S.-H., Lee, K.-W., Cho, M.-G., Jeon, Y.-J., 2008. Cytoprotective effect of fucoxanthin isolated from brown algae Sargassum siliquastrum against H2O2-induced cell damage. European Food Research and Technology 228, 145-151.

Higuera-Ciapara, I., Felix-Valenzuela, L., Goycoolea, F., 2006. Astaxanthin: a review of its chemistry and applications. Crit. Rev. Food Sci. Nutr. 46, 185-196.

Huang, E.J., Reichardt, L.F., 2001. Neurotrophins: roles in neuronal development and function. Annu. Rev. Neurosci. 24, 677-736.

Hussain, I., Powell, D., Howlett, D.R., Tew, D.G., Meek, T.D., Chapman, C., Gloger, I.S., Murphy, K.E., Southan, C.D., Ryan, D.M., Smith, T.S., Simmons, D.L., Walsh, F.S., Dingwall, C., Christie, G., 1999. Identification of a novel aspartic protease (Asp 2) as β-secretase. Molecular and Cellular Neuroscience 14, 419-427.

Hussein, G., Sankawa, U., Goto, H., Matsumoto, K., Watanabe, H., 2006. Astaxanthin, a carotenoid with potential in human health and nutrition. J. Nat. Prod. 69, 443-449.

Ikeda, Y., Tsuji, S., Satoh, A., Ishikura, M., Shirasawa, T., Shimizu, T., 2008. Protective effects of astaxanthin on 6‐hydroxydopamine‐induced apoptosis in human neuroblastoma SH‐SY5Y cells. J. Neurochem. 107, 1730-1740.

Jung, H.A., Ali, M.Y., Choi, R.J., Jeong, H.O., Chung, H.Y., Choi, J.S., 2016. Kinetics and molecular docking studies of fucosterol and fucoxanthin, BACE1 inhibitors from brown algae *Undaria pinnatifida* and *Ecklonia stolonifera*. Food Chem. Toxicol. 89, 104-111.

Kamei, Y., Sagara, A., 2002. Neurite outgrowth promoting activity of marine algae from Japan against rat adrenal medulla pheochromocytoma cell line, PC12D. Cytotechnology 40, 99-106.

Kang, M.-C., Lee, S.-H., Lee, W.-W., Kang, N., Kim, E.-A., Kim, S.Y., Lee, D.H., Kim, D., Jeon, Y.-J., 2014. Protective effect of fucoxanthin isolated from Ishige okamurae against high-glucose induced oxidative stress in human umbilical vein endothelial cells and zebrafish model. Journal of Functional Foods 11, 304-312.

Kim, J.-H., Choi, W., Lee, J.-H., Jeon, S.-J., Choi, Y.-H., Kim, B.-W., Chang, H.-I., Nam, S.-W., 2009. Astaxanthin Inhibits H2O2-Mediated Apoptotic Cell Death in Mouse Neural Progenitor Cells via Modulation of P38 and MEK Signaling Pathways. Journal of Microbiology and Biotechnology 19, 1355–1363.

Koliatsos, V.E., Clatterbuck, R.E., Nauta, H.J., Knüsel, B., Burton, L.E., Hefti, F.F., Mobley, W.C., Price, D.L., 1991. Human nerve growth factor prevents degeneration of basal forebrain cholinergic neurons in primates. Ann. Neurol. 30, 831-840.

Kromer, L.F., 1987. Nerve growth factor treatment after brain injury prevents neuronal death. Science 235, 214-217.

Kumar, S.R., Hosokawa, M., Miyashita, K., 2013. Fucoxanthin: A marine carotenoid exerting anti-cancer effects by affecting multiple mechanisms. Mar. Drugs 11, 5130-5147.

Lin, J., Yu, J., Zhao, J., Zhang, K., Zheng, J., Wang, J., Huang, C., Zhang, J., Yan, X., Gerwick, W.H., 2017. Fucoxanthin, a marine carotenoid, attenuates β-amyloid oligomer-induced neurotoxicity possibly via regulating the PI3K/Akt and the ERK pathways in SH-SY5Y cells. Oxid. Med. Cell. Longev. 2017, 1-10.

Matsumoto, M., Hosokawa, M., Matsukawa, N., Hagio, M., Shinoki, A., Nishimukai, M., Miyashita, K., Yajima, T., Hara, H., 2010. Suppressive effects of the marine carotenoids, fucoxanthin and fucoxanthinol on triglyceride absorption in lymph duct-cannulated rats. Eur. J. Nutr. 49, 243-249.

Mikami, K., Hosokawa, M., 2013. Biosynthetic pathway and health benefits of fucoxanthin, an algae-specific xanthophyll in brown seaweeds. International Journal of Molecular Sciences 14, 13763-13781.

Miki, W., 1991. Biological functions and activities of animal carotenoids. Pure Appl. Chem. 63, 141-146.

Mok, I.-K., Lee, J.K., Kim, J.H., Pan, C.-H., Kim, S.M., 2018. Fucoxanthin bioavailability from fucoxanthin-fortified milk: *In vivo* and *in vitro* study. Food Chem. 258, 79-86.

Molina, N., Morandi, A.C., Bolin, A.P., Otton, R., 2014. Comparative effect of fucoxanthin and vitamin C on oxidative and functional parameters of human lymphocytes. Int. Immunopharmacol. 22, 41-50.

Nie, Q., Du, X.-g., Geng, M.-y., 2011. Small molecule inhibitors of amyloid β peptide aggregation as a potential therapeutic strategy for Alzheimer's disease. Acta Pharmacol. Sin. 32, 545.

Nishikawa, S., Hosokawa, M., Miyashita, K., 2012. Fucoxanthin promotes translocation and induction of glucose transporter 4 in skeletal muscles of diabetic/obese KK-Ay mice. Phytomedicine 19, 389-394.

Odeberg, J.M., Lignell, Å., Pettersson, A., Höglund, P., 2003. Oral bioavailability of the antioxidant astaxanthin in humans is enhanced by incorporation of lipid based formulations. Eur. J. Pharm. Sci. 19, 299-304.

Okada, T., Nakai, M., Maeda, H., Hosokawa, M., Sashima, T., Miyashita, K., 2008. Suppressive effect of neoxanthin on the differentiation of 3T3-L1 adipose cells. Journal of Oleo Science 57, 345-351.

Peng, J., Yuan, J.-P., Wu, C.-F., Wang, J.-H., 2011. Fucoxanthin, a marine carotenoid present in brown seaweeds and diatoms: metabolism and bioactivities relevant to human health. Mar. Drugs 9, 1806-1828.

Piau, A., Nourhashémi, F., Hein, C., Caillaud, C., Vellas, B., 2011. Progress in the development of new drugs in Alzheimer’s disease. J. Nutr. Health Aging 15, 45-57.

Rao, A.R., Baskaran, V., Sarada, R., Ravishankar, G., 2013. *In vivo* bioavailability and antioxidant activity of carotenoids from microalgal biomass—A repeated dose study. Food research international 54, 711-717.

Ravi, H., Arunkumar, R., Baskaran, V., 2015. Chitosan-glycolipid nanogels loaded with anti-obese marine carotenoid fucoxanthin: Acute and sub-acute toxicity evaluation in rodent model. J. Biomater. Appl. 30, 420-434.

Re, F., Airoldi, C., Zona, C., Masserini, M., Ferla, B.L., Quattrocchi, N., Nicotra, F., 2010. Beta amyloid aggregation inhibitors: small molecules as candidate drugs for therapy of Alzheimer's disease. Curr. Med. Chem. 17, 2990-3006.

Sachindra, N.M., Sato, E., Maeda, H., Hosokawa, M., Niwano, Y., Kohno, M., Miyashita, K., 2007. Radical scavenging and singlet oxygen quenching activity of marine carotenoid fucoxanthin and its metabolites. J. Agric. Food Chem. 55, 8516-8522.

Salvia-Trujillo, L., Sun, Q., Um, B., Park, Y., McClements, D., 2015. *In vitro* and *in vivo* study of fucoxanthin bioavailability from nanoemulsion-based delivery systems: Impact of lipid carrier type. Journal of Functional Foods 17, 293-304.

Sperling, R.A., Jack, C.R., Aisen, P.S., 2011. Testing the right target and right drug at the right stage. Sci. Transl. Med. 3, 111cm133-111cm133.

Stewart, J.S., Lignell, Å., Pettersson, A., Elfving, E., Soni, M., 2008. Safety assessment of astaxanthin-rich microalgae biomass: Acute and subchronic toxicity studies in rats. Food Chem. Toxicol. 46, 3030-3036.

Takaichi, S., 2011. Carotenoids in algae: distributions, biosyntheses and functions. Mar. Drugs 9, 1101-1118.

Wang, H.-Q., Sun, X.-B., Xu, Y.-X., Zhao, H., Zhu, Q.-Y., Zhu, C.-Q., 2010. Astaxanthin upregulates heme oxygenase-1 expression through ERK1/2 pathway and its protective effect against beta-amyloid-induced cytotoxicity in SH-SY5Y cells. Brain Res. 1360, 159-167.

Wu, H., Niu, H., Shao, A., Wu, C., Dixon, B.J., Zhang, J., Yang, S., Wang, Y., 2015. Astaxanthin as a Potential Neuroprotective Agent for Neurological Diseases. Mar. Drugs 13, 5750-5766.

Xiang, S., Liu, F., Lin, J., Chen, H., Huang, C., Chen, L., Zhou, Y., Ye, L., Zhang, K., Jin, J., 2017. Fucoxanthin inhibits β-amyloid assembly and attenuates β-amyloid oligomer-induced cognitive impairments. J. Agric. Food Chem. 65, 4092-4102.

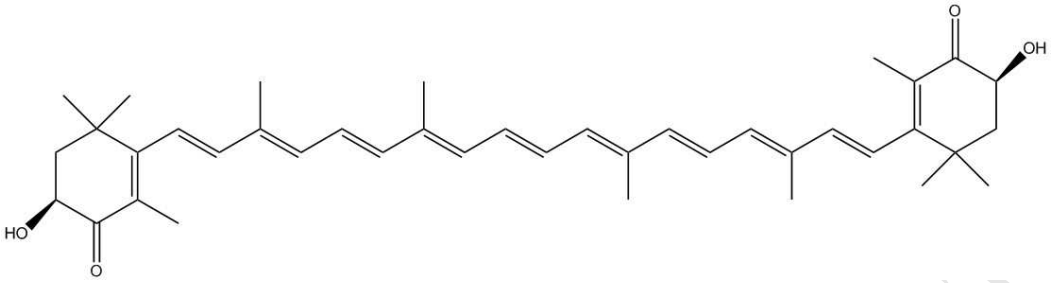
Yu, J., Lin, J.-J., Yu, R., He, S., Wang, Q.-W., Cui, W., Zhang, J.-R., 2017. Fucoxanthin prevents H2O2-induced neuronal apoptosis via concurrently activating the PI3-K/Akt cascade and inhibiting the ERK pathway. Food & Nutrition Research 61, 1304678.

Zhang, Y., Wang, W., Hao, C., Mao, X., Zhang, L., 2015. Astaxanthin protects PC12 cells from glutamate-induced neurotoxicity through multiple signaling pathways. Journal of Functional Foods 16, 137-151.

Zhao, X., Zhang, S., An, C., Zhang, H., Sun, Y., Li, Y., Pu, X., 2015. Neuroprotective effect of fucoxanthin on β-amyloid-induced cell death. Journal of Chinese Pharmaceutical Sciences 24, 467–474.

Zheng, J., Piao, M.J., Keum, Y.S., Kim, H.S., Hyun, J.W., 2013. Fucoxanthin protects cultured human keratinocytes against oxidative stress by blocking free radicals and inhibiting apoptosis. Biomol. Ther. (Seoul) 21, 270-276.

a



b

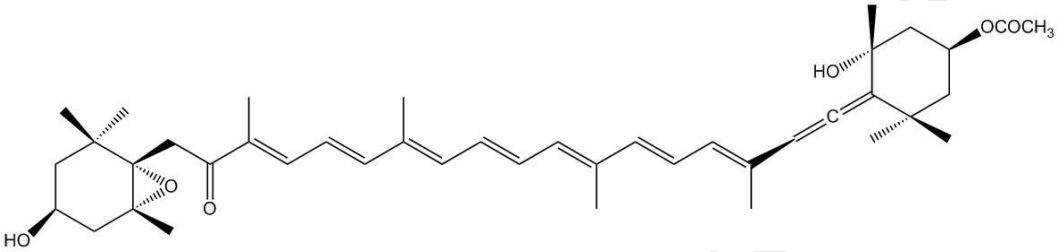


Figure 1: The chemical structure of astaxanthin (**a**) and fucoxanthin (**b**)

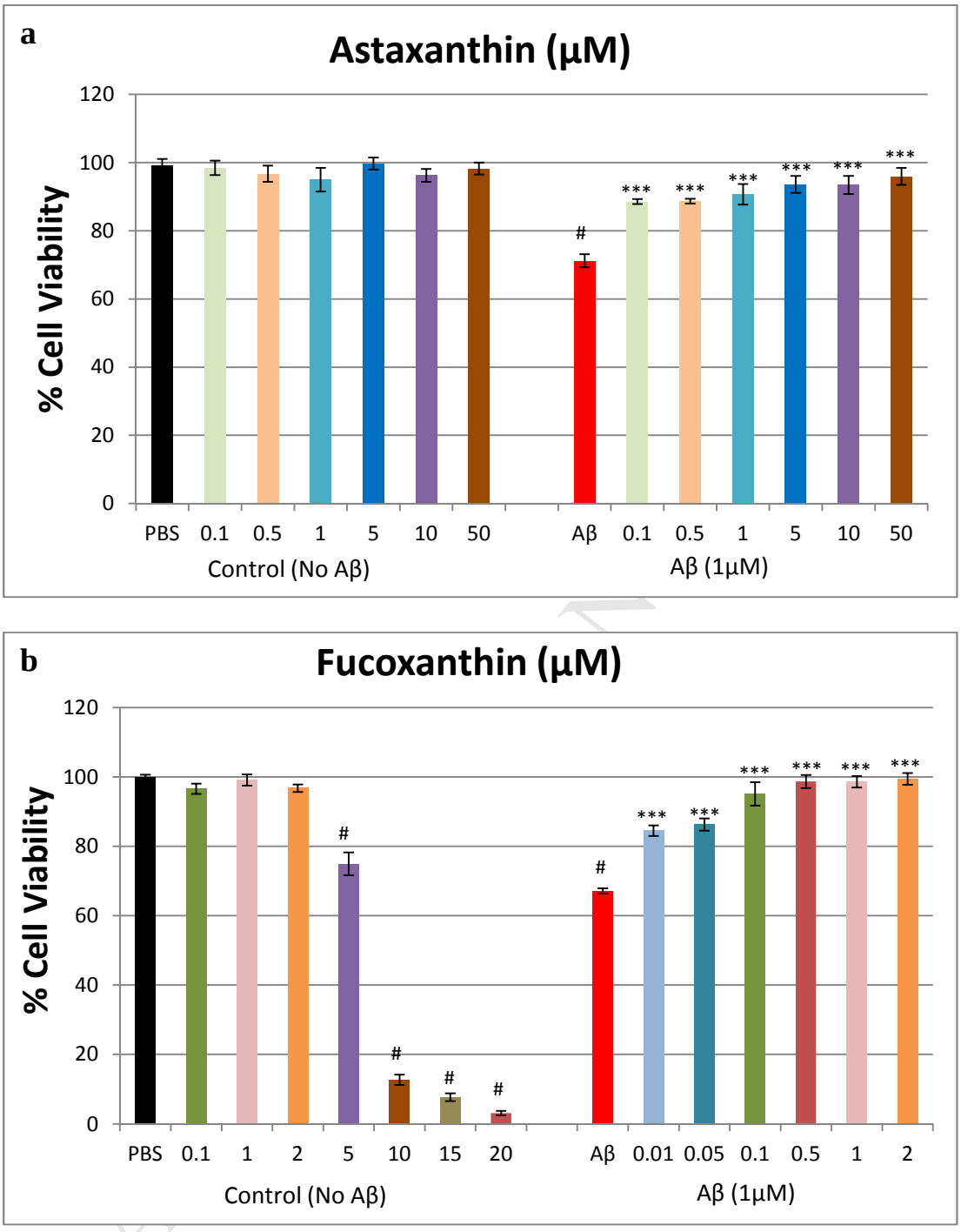
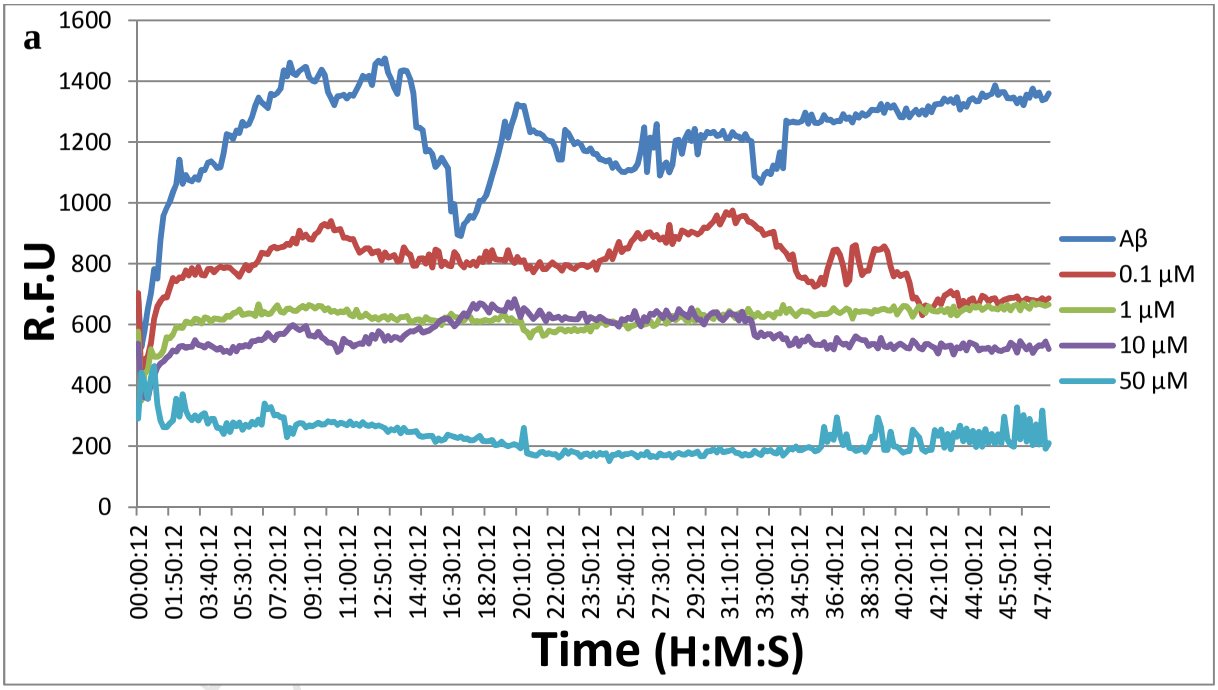
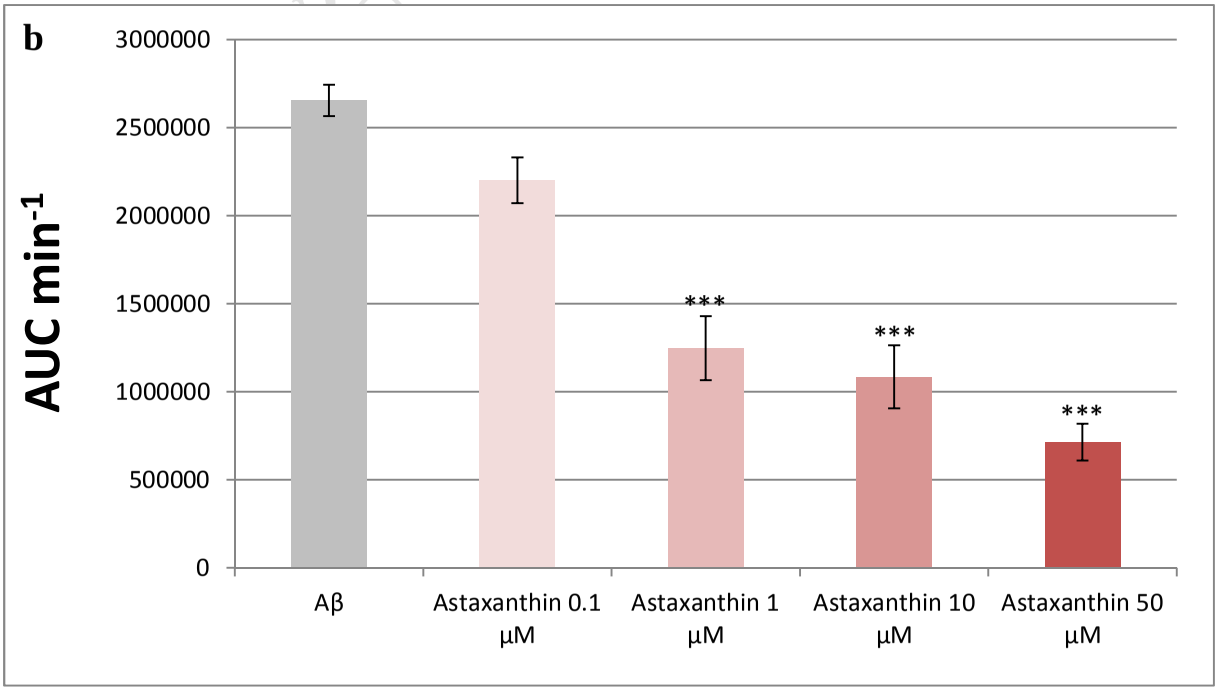


Figure 2: Cell viability (%) of PC12 cells measured by MTT assay following 48 h treatment with astaxanthin (**a**) or fucoxanthin (**b**), and neuroprotection activity of astaxanthin and fucoxanthin against $A\beta_{1\text{-}42}$ (1 µM) induced cytotoxicity. Each value is the mean ± SEM of four independent experiments (#p <0.005 vs PBS, *p <0.05, **p <0.01, and ***p <0.005 vs control 1 µM $A\beta_{1\text{-}42}$)





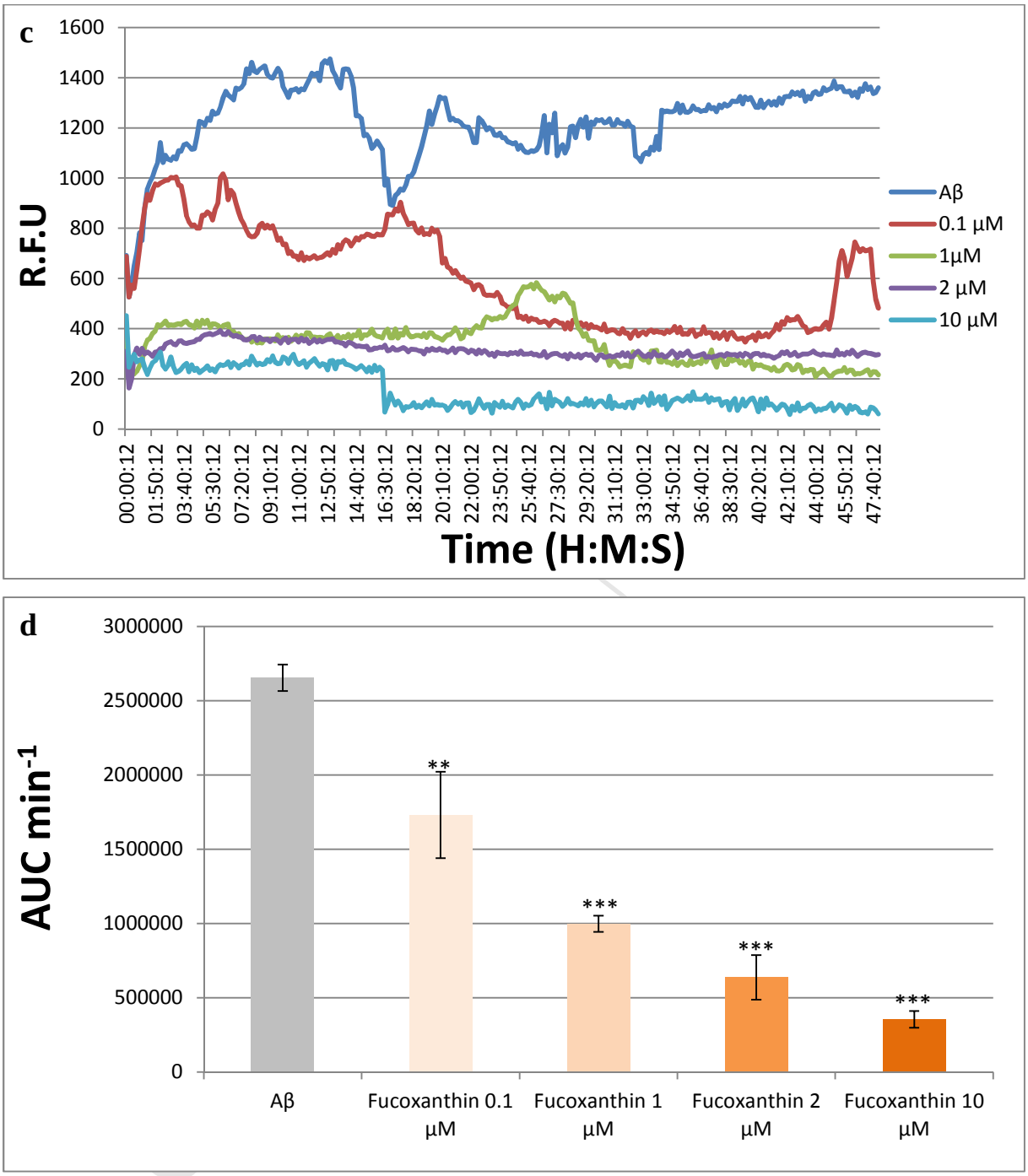
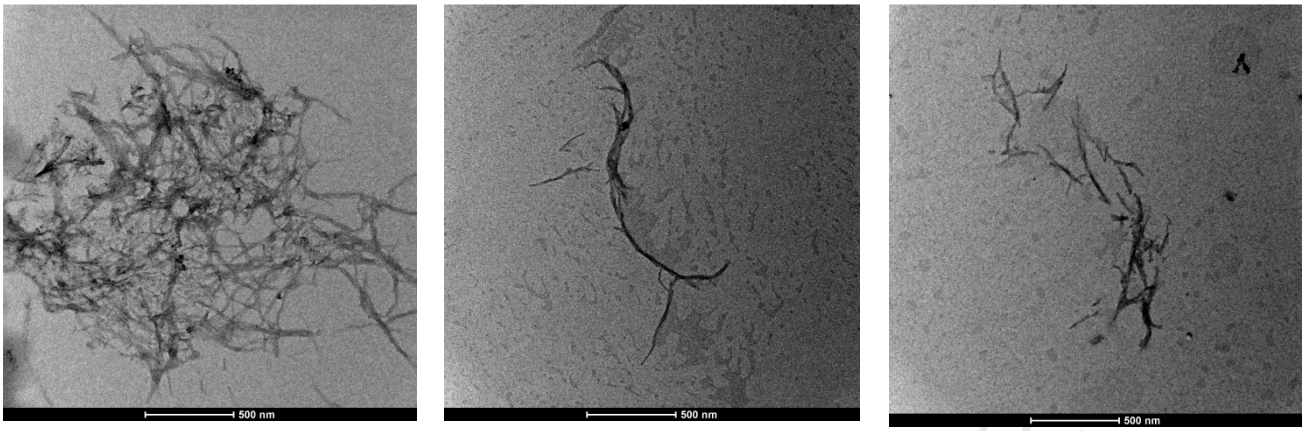
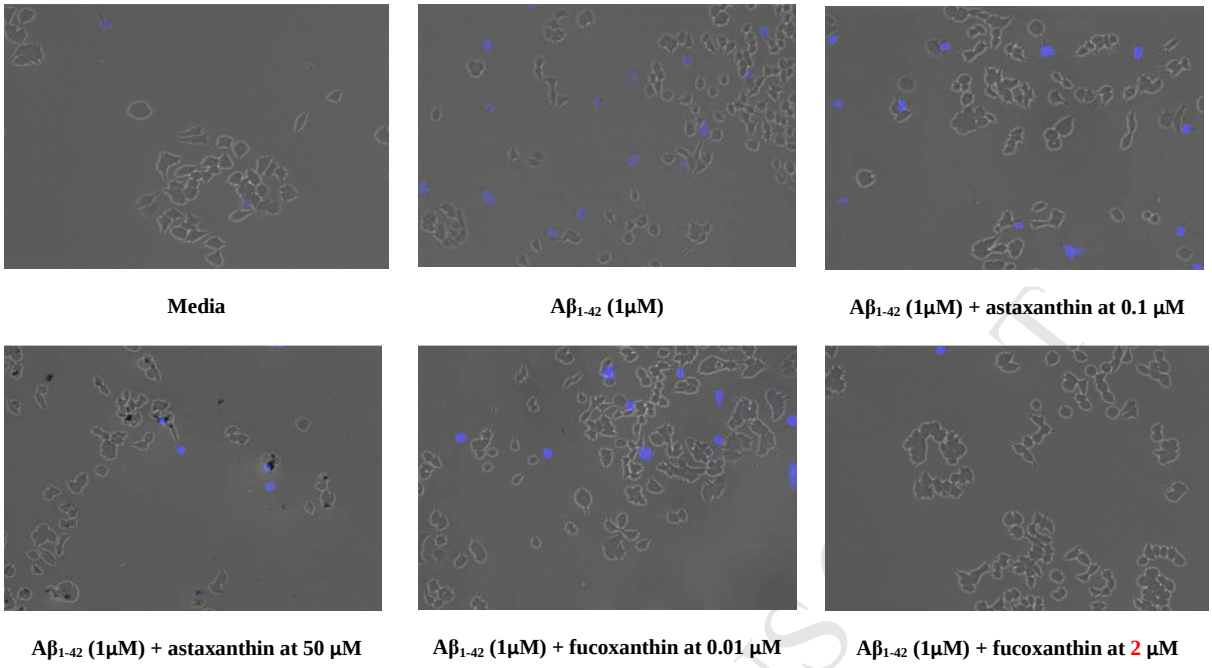


Figure 3: Thioflavin T (ThT) fluorescence assay demonstrating amyloid $A\beta_{1-42}$ fibrillization kinetics over 48 h in PBS, alone or in the presence of different concentrations of astaxanthin (0.1–50 µM) (**a**) or fucoxanthin (0.1–10 µM) (**c**). Area under the curve (AUC) was measured to quantitate the summative effect of astaxanthin (**b**) or fucoxanthin (**d**) on $A\beta_{1-42}$ kinetics (*p <0.05, **p <0.01, and ***p <0.005 vs control $A\beta_{1-42}$) of astaxanthin or fucoxanthin at different concentrations (n=4)



a **b** **c**

Figure 4: Representative TEM micrographs of $A\beta_{1-42}$ fibrils, alone and following 48 h incubation with astaxanthin or fucoxanthin: (**a**) $A\beta_{1-42}$; (**b**) $A\beta_{1-42}$ and astaxanthin (50 μM) and (**c**) $A\beta_{1-42}$ and fucoxanthin (2 μM). Scale bar: 500 nm.



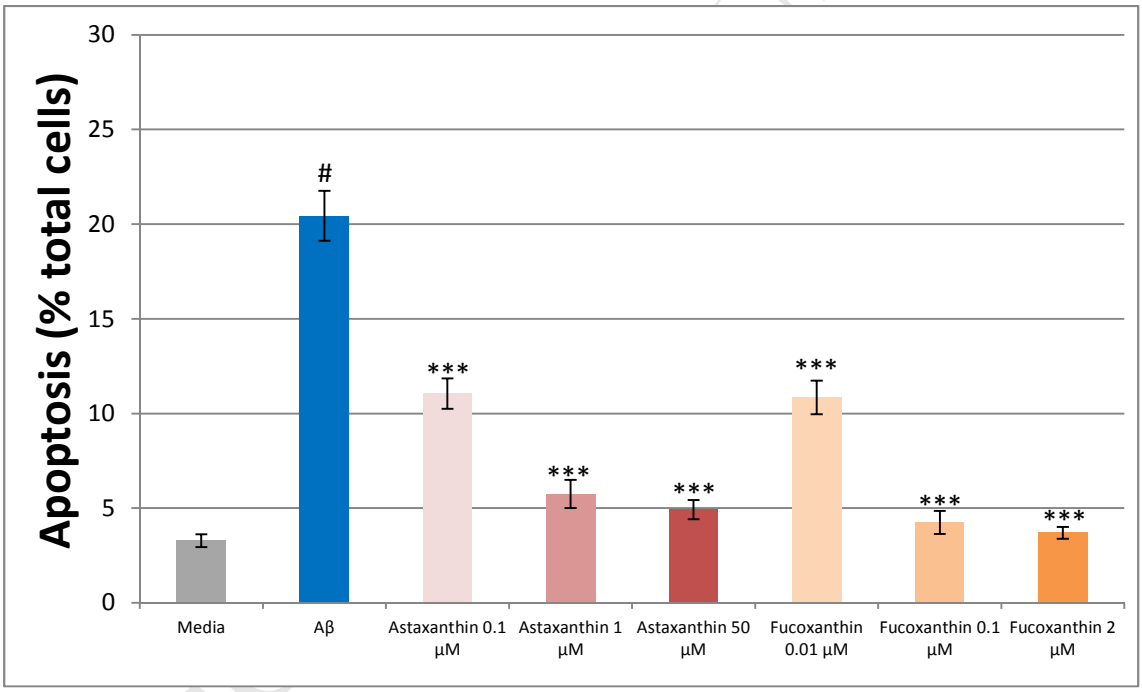
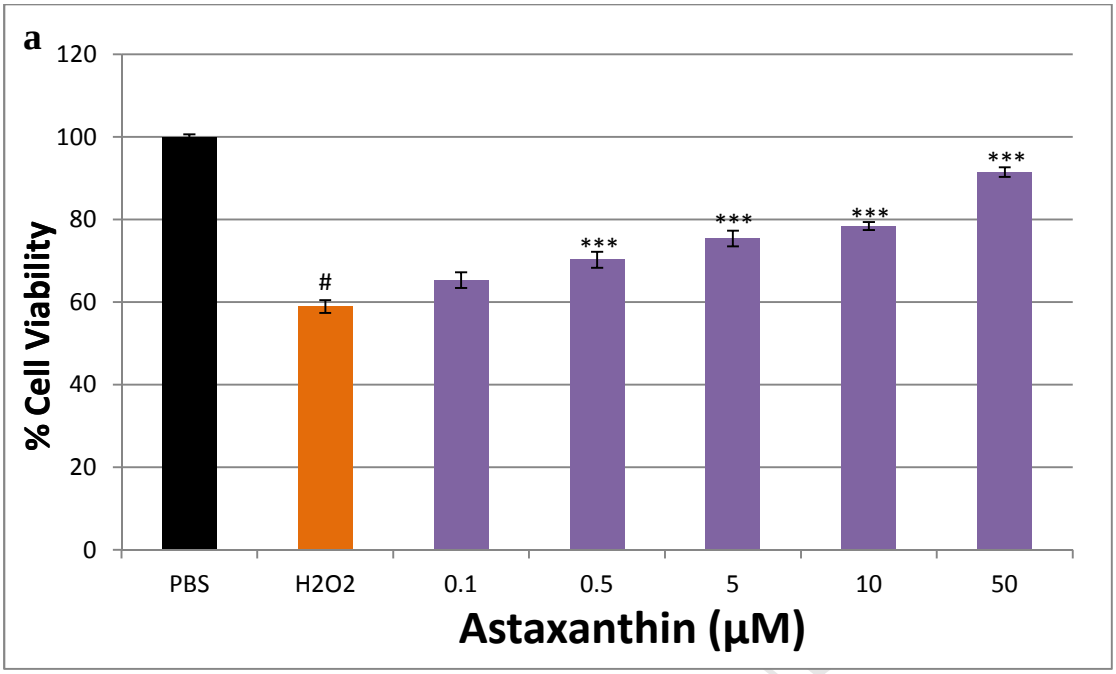
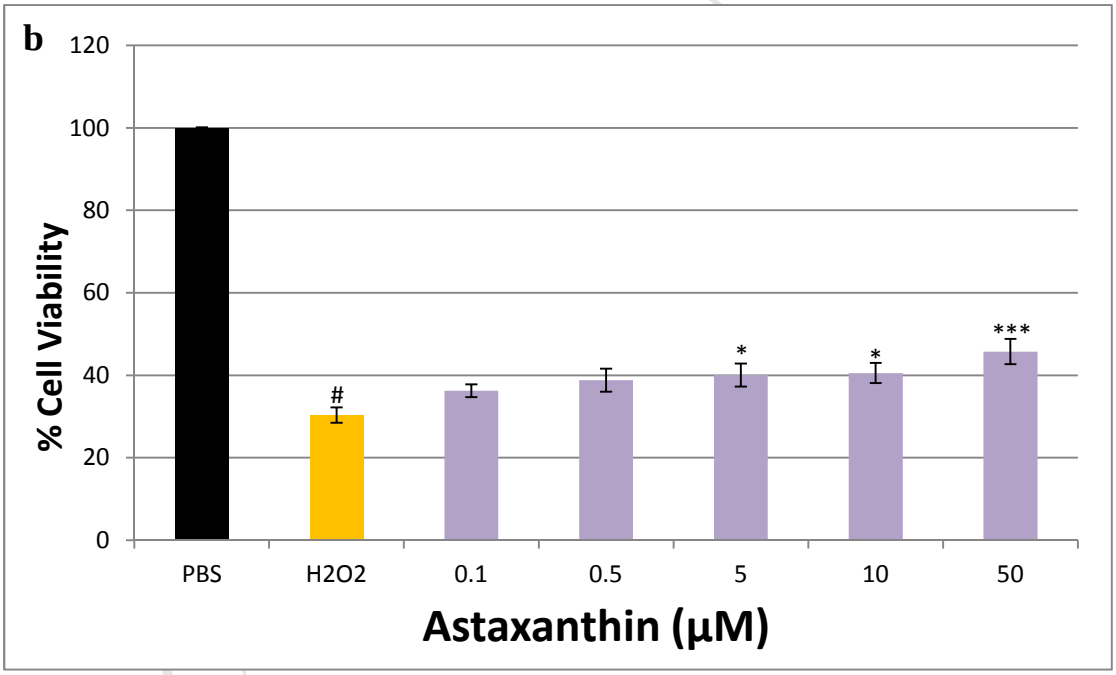


Figure 5: Effect of different concentrations of astaxanthin and fucoxanthin on apoptosis induced by $A\beta_{1-42}$ in PC12 cells over 48 hours. Cells were stained using Hoechst 33258 and then visualized using fluorescence microscopy. At least 350 cells were counted for every treatment. Each value is the mean ± SEM of four independent experiments (#p <0.005 versus PBS, *p <0.05 and ***p <0.005 versus control $A\beta_{1-42}$).


a
120
100
80
60
40
20
0
% Cell Viability
#
***
***
***
***
PBS
H2O2
0.1
0.5
5
10
50
Astaxanthin (µM)


b
120
100
80
60
40
20
0
% Cell Viability
#
*
*
***
PBS
H2O2
0.1
0.5
5
10
50
Astaxanthin (µM)

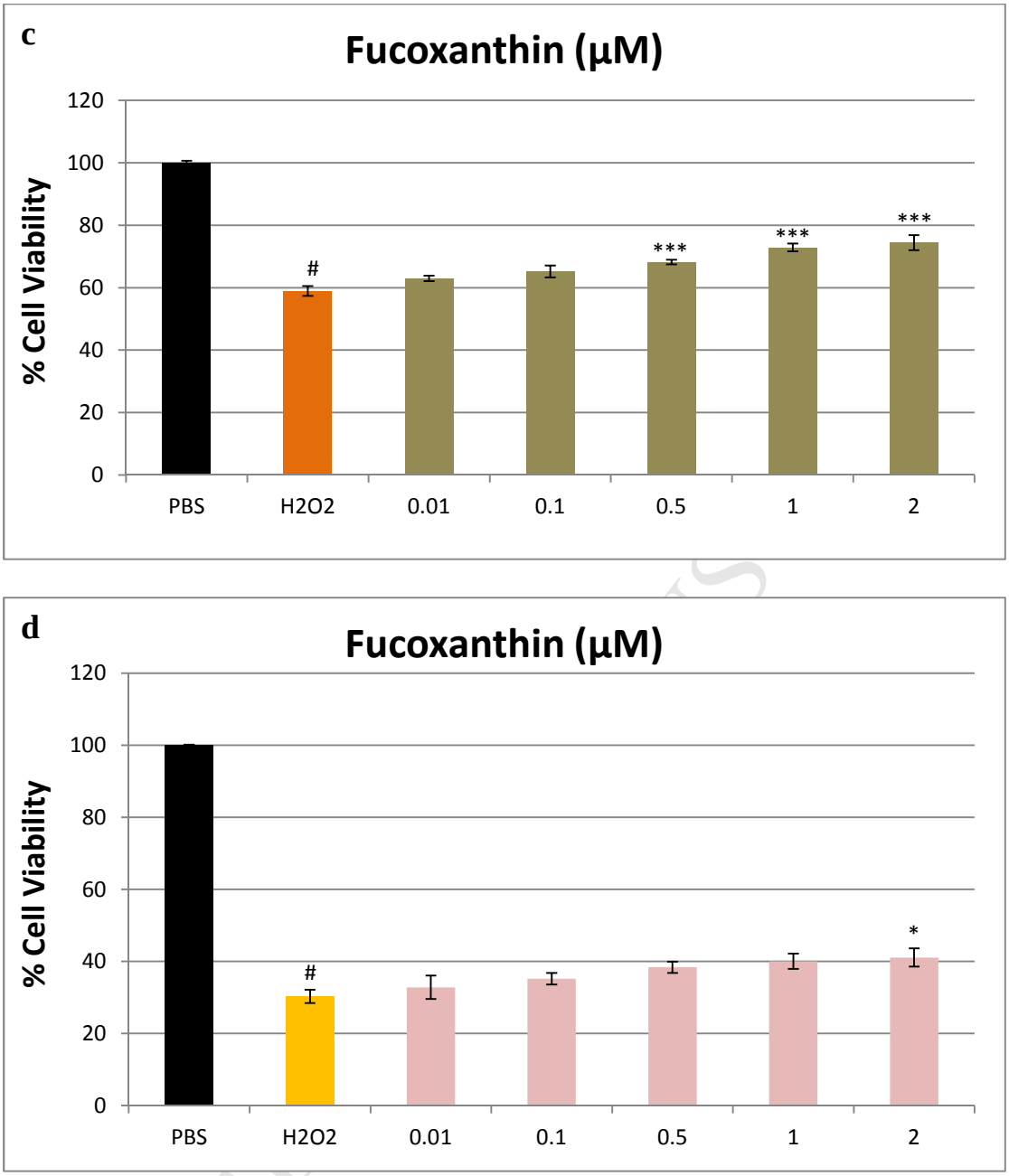
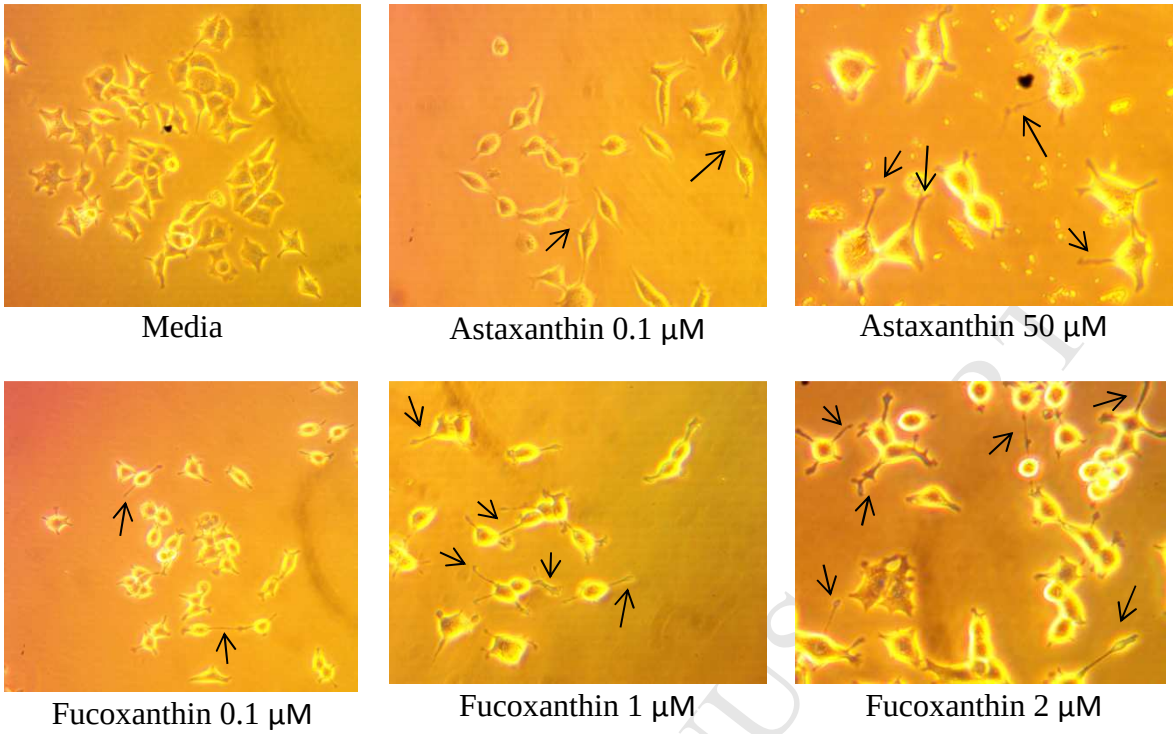


Figure 6: Effect of different concentrations of astaxanthin (**a**, **b**) and fucoxanthin (**c, d**) on viability of PC12 cells exposed to H_2O_2 at 100 µM (**a**, **c**) and 200 µM (**b**, **d**) for 24 h. Each value is the mean ± SEM of four independent experiments (#p <0.005 versus PBS, *p <0.05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



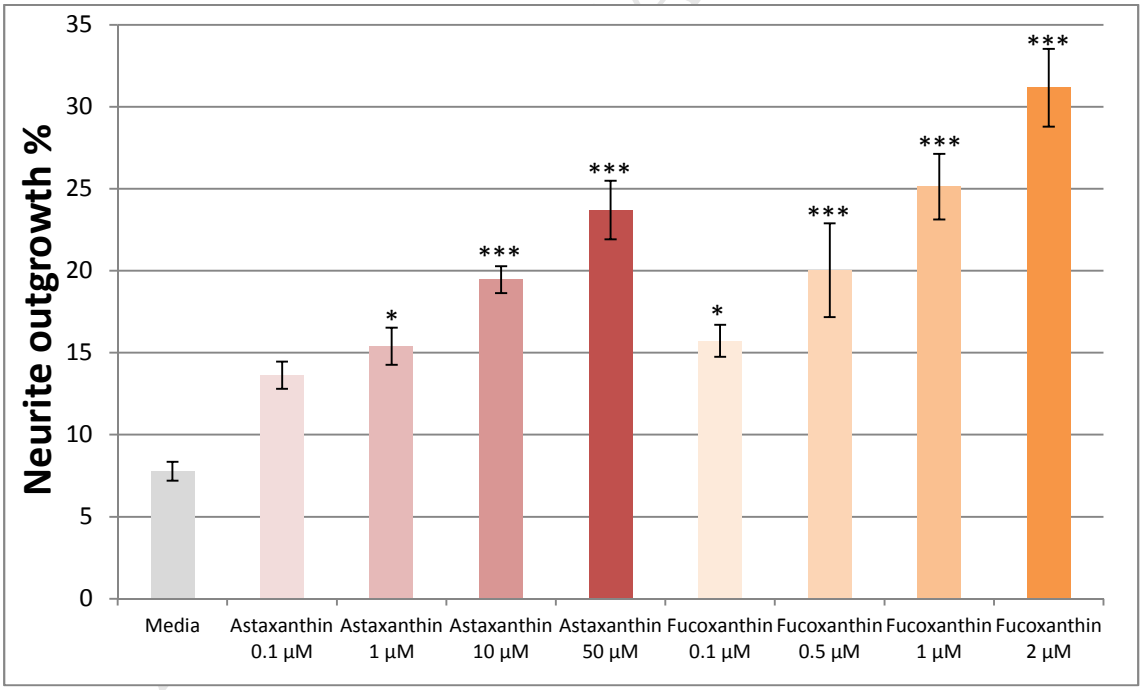


Figure 7: Effect of different concentrations of astaxanthin and fucoxanthin on enhancing neurite outgrowth in PC12 cells. Cells were visualized using light microscopy. At least 200 cells were counted for every treatment. Each value is the mean ± SEM of six independent experiments (*p <0.05 and ***p <0.005 versus control media)

- Astaxanthin and fucoxanthin provided neuroprotection against $A\beta_{1-42}$
- Both compounds showed anti-aggregation effects against $A\beta_{1-42}$
- Both compounds demonstrated multiple neuroprotective activities
- Fucoxanthin showed higher anti-aggregation and neurite outgrowth activity