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ORIGINAL ARTICLE

Reactive oxygen species scavenging activities in a chemiluminescence model and neuroprotection in rat pheochromocytoma cells by astaxanthin, beta-carotene, and canthaxanthin

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Abstract The objective of this study was to determine chemiluminescence (CL) antioxidant activities and neuroprotective effects of astaxanthin, beta-carotene (β-carotene), and canthaxanthin on undifferentiated rat pheochromocytoma (PC12) cells. We performed three CL antioxidant assays, and the three carotenoids showed varying degrees of antioxidant activity, with astaxanthin exhibiting the highest antioxidant activity than the other two samples. Results of a pyrogallol–luminol assay revealed β-carotene to have higher antioxidant activity than canthaxanthin, whereas cupric sulfate–Phen–Vc–hydrogen peroxide (H_2O_2) assay showed canthaxanthin to have higher antioxidant activity than β-carotene. Luminol–H_2O_2 assay showed the antioxidant activity series as canthaxanthin > β-carotene at 62.5–1000 μg/mL and β-carotene > canthaxanthin at 1000–4000 μg/mL. Astaxanthin exhibited partial neuroprotective activity against H_2O_2 and the strongest neuroprotective activity against amyloid beta-peptide$_{(25-35)}$ [(Aβ)$_{(25-35)}$]-induced undifferentiated PC12 cell deaths at 0.5–5.0 μM. Canthaxanthin showed partial neuroprotective activity in Aβ$_{(25-35)}$-induced undifferentiated PC12 cell deaths at 1.0–5.0 μM. Astaxanthin protected undifferentiated PC12 cells from the damaging effects of H_2O_2 and Aβ$_{(25-35)}$ by the following ways: (1) scavenging superoxide anion radicals, hydroxyl radicals, and H_2O_2; (2) securing cell viability; (3) suppressing the production of reactive oxygen species; and (4) eliminating calcium ion influx. Our results conclusively show that astaxanthin has the merit as a potential neuron protectant.
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Introduction

Chemiluminescence (CL) is a method that offers high sensitivity and selectivity, has a wide linear range, and is simple to use for studying the reactive oxygen species (ROS) scavenging activities [1]. In an earlier study, astaxanthin was evaluated using lucigenin and luminol as chemiluminogenic probes for the superoxide anion radicals ($^{\bullet}O_2^-$) and hydrogen peroxide (H_2O_2) species, respectively; the study results showed that astaxanthin had scavenging activities for both $^{\bullet}O_2^-$ and H_2O_2 [2]. In another study, methoxylated Cypridina luciferin analog-dependent CL, when treated with $^{\bullet}O_2^-$, was effectively quenched by vitamin C, beta-carotene (β-carotene), and astaxanthin. In addition, β-carotene and alpha-tocopherol (vitamin E) effectively suppressed the CL activity when the ROS were hydroxyl radicals ($^{\bullet}OH$) [3].

The rat pheochromocytoma cell (PC12 cell, CRL-1721) a useful model for studying specific neurodegenerative diseases caused by free radical damage and the neuroprotective effects of puerarin (isoflavone) in PC12 cells were evaluated by Jiang and co-workers [4]. The 11-amino-acid fragment of the amyloid beta-peptide (Aβ), $A\beta_{(25-35)}$, which is located in the hydrophobic domain at the C-terminal end of $A\beta_{(1-42)}$, was shown to mimic certain pathological processes in brains affected by Alzheimer's disease (AD) [5]. Neuroprotective studies on the activities of astaxanthin and canthaxanthin present the following information: pretreatment with 0.5 μM astaxanthin significantly inhibited 50 μM H_2O_2-induced apoptosis when the primary culture of cortical neurons was measured by Hoechst 33342 staining [6]. The antioxidative and anti-inflammatory neuroprotective effects of astaxanthin and canthaxanthin were evaluated by pretreating nerve growth factor (NGF)-differentiated PC12 cells with astaxanthin or canthaxanthin at 10 or 20 μM, followed by exposing the cells to 100 μM H_2O_2 or 1-methyl-4-phenylpyridinium ions in order to induce cell injury. The results showed that the cellular activities of glutathione peroxidase and catalase in the pretreated PC12 cells were significantly retained, while the production of malonyldialdehyde and ROS decreased [7]. A study by Chang et al. showed that 0.1-μM dose of astaxanthin protected undifferentiated PC12 cells from the damaging effects of $A\beta_{(25-35)}$ present at a concentration of 30 μM [8].

Although various antioxidant methods have been developed and used in different systems, the results of these methods presented were inconsistent. A simple universal method by which antioxidant capacity can be assessed accurately and quantitatively does not exist. Because only a few reports are currently available on the assessment of the CL antioxidant assay and the neuroprotection activities of astaxanthin, β-carotene, and canthaxanthin, it is difficult to compare the correlations between the CL antioxidant assays and neuroprotection. Therefore, in this study, the correlations between the CL antioxidant assays and neuroprotection are examined and presented. In a previous study, we examined a series of CL systems to evaluate the ROS scavenging abilities of *Terminalia chebula* Retzius [9,10]. In this study, we evaluated the quenching effects of astaxanthin, β-carotene, and canthaxanthin against ROS, such as $^{\bullet}O_2^-$, $^{\bullet}OH$, and H_2O_2 using modified CL methods. The three CL systems used are not exclusively selective to individual ROS. We also examined the effects of astaxanthin, β-carotene, and canthaxanthin on H_2O_2 and $A\beta_{(25-35)}$-induced neurotoxicity. We primarily focused on the neurotoxic effects of H_2O_2 and $A\beta_{(25-35)}$ and analyzed the neuroprotection provided by astaxanthin, β-carotene, and canthaxanthin. Our results show that these carotenoids induce neuroprotection by increasing cell viability, decreasing ROS production, and reducing calcium influx. To our knowledge, this is the first study to evaluate the antioxidant activities of astaxanthin, β-carotene, and canthaxanthin analyzed in an antioxidant assay in a modified CL model.

Materials and methods

Materials

Vitamin C, astaxanthin, β-carotene, canthaxanthin, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), Pluronic F-127, *N*-acetyl—Asp—Glu—Val—Asp—CHO (Ac-DEVD-CHO), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), and poly-L-lysine hydrobromide were obtained from Sigma-Aldrich (Shanghai, China). Vitamin C and trolox were used as positive control samples over an optimized concentration range (62.5, 125, 250, 500, 1000, 2000, and 4000 μg/mL). Vitamin C is a water-soluble antioxidant, trolox is a hydrophilic analog of vitamin E, and vitamin E is a fat-soluble antioxidant. Thirty-five percent of H_2O_2 was purchased from Riedel-de Haën (Seelze, Germany), dimethyl sulfoxide (DMSO) was purchased from Merck (Darmstadt, Germany), 2′,7′-dichlorofluorescein 3′,6′-diacetate (2′,7′-DCFH-DA) was purchased from ECHO Chemical Corporation Ltd. (Taichung, Taiwan, R.O.C.), $A\beta_{(25-35)}$ was purchased from Kelowna International Scientific Inc. (Taipei, Taiwan, R.O.C.), and fluo-3-AM was purchased from Quantum Biotechnology Inc. (Taichung, Taiwan, R.O.C.). Y.C. Shen of the National Research Institute of Chinese Medicine (Taipei, Taiwan, R.O.C.) provided the PC12 cells used in this study. Dulbecco's modified Eagle's medium (DMEM) was purchased from Tseng Hsiang Life Science Ltd. (Taipei, Taiwan, R.O.C.).

Pyrogallol—luminol, $CuSO_4$—Phen—Vc—H_2O_2, and luminol—H_2O_2 systems

The assay methods used in this study for the pyrogallol—luminol, $CuSO_4$—Phen—Vc—H_2O_2, and luminal—H_2O_2 systems were the same as those in our previous studies [9,10]. Samples of vitamin C, trolox, astaxanthin, β-carotene, canthaxanthin, and DMSO as control were prepared with three replicates, with concentrations in the range of 62.5—4000 μg/mL.

Preparation of the $A\beta_{(25-35)}$ stock solution

The Aβ oligomers are more toxic to neurons than monomers (soluble form) or fibrils [11]. $A\beta_{(25-35)}$ exhibited neurotrophic and neurotoxic activities similar to those

exhibited by $A\beta_{(1-40)}$ [12]. Therefore, the $A\beta_{(25-35)}$ oligomers used in this study were preaggregated before use. Synthetic $A\beta_{(25-35)}$ was prepared at a concentration of 1 mM in water, aggregated at 4 °C for 60 hours and then at 37 °C for 8 hours with regular mixing every 2 hours before use [13–15].

Protective effects

Undifferentiated PC12 cell cultures were identical to those in our previous study [10]. The cells were seeded in poly-L-lysine hydrobromide-coated 24-well cell-culture plates (1.25×10^5 cells/well) with complete DMEM for 24 hours. H_2O_2 or $A\beta_{(25-35)}$ was then added to induce undifferentiated PC12 cell death. To study the protective effects of the test samples on the undifferentiated PC12 cells, we renewed the media and preincubated the cells for 12 hours, both with and without 500 μL test samples, in order to obtain sample concentrations of 0, 0.5, 1, 5, and 10 μM. The media were replaced, and 500 μL of H_2O_2 was added to a concentration of 40 μM H_2O_2, and the mixture was incubated for an additional 12 hours. The undifferentiated PC12 cells were exposed to 0.01 μM $A\beta_{(25-35)}$ with various concentrations of test samples (0, 0.5, 1, 5, and 10 μM) for 24 hours. The control was incubated without the addition of other samples, H_2O_2, or $A\beta_{(25-35)}$ solution. All samples were dissolved in a final DMSO concentration of 0.5% in the culture medium, which had no observable effect on cell viability, as determined by the MTT reduction assay. Cell viability was measured in the same culture by the MTT reduction assay [10].

Assay for intracellular ROS

The intracellular ROS assay of undifferentiated PC12 cells was performed as follows: (1) To study the effects of ROS on undifferentiated PC12 cells in the test samples and H_2O_2, the media were renewed, and the cells were preincubated for 12 hours, with and without the presence of 500 μL test samples, in order to obtain sample concentrations of 0, 0.5, 1, 5, and 10 μM. The media were removed, and 500 μL of 2′,7′-DCFH-DA was added to a concentration of 50 μM. The culture solution was then incubated in the dark at 37 °C for 1 hour. The 2′,7′-DCFH-DA fluorescent probe was removed, 500 μL of H_2O_2 was added to a concentration of 40 μM, and the mixture was incubated for an additional 2 hours. (2) To study the effects of ROS on undifferentiated PC12 cells in the presence of test samples and $A\beta_{(25-35)}$, the media were removed, and 500 μL of 2′,7′-DCFH-DA was added to a concentration of 50 μM. The culture solution was then incubated in the dark at 37 °C for 1 hour. The 2′,7′-DCFH-DA fluorescent probe was removed, and the undifferentiated PC12 cells were exposed to 0.01 μM $A\beta_{(25-35)}$ with various concentrations of test samples (0, 0.5, 1, 5 and 10 μM) for an additional 2 hours. (3) The control was incubated without the samples, H_2O_2, or $A\beta_{(25-35)}$ solution. After completing Steps 1 and 2, the undifferentiated PC12 cells were washed two times with phosphate-buffered saline (PBS) and resuspended in 450 μL PBS. The suspension was seeded onto a 96-well plate (250 μL/well). Fluorescence intensity was measured using a fluorescence spectrophotometer at an excitation wavelength of 485 nm and emission wavelength of 520 nm. The acquired data were expressed in relative fluorescence units.

Assay for intracellular calcium

The calcium-sensitive fluo-3-AM dye was dissolved in DMSO solution (supplied as 1 mM DMSO solution) and mixed with an equal volume of Pluronic F-127, which was also prepared with DMSO (25% w/v). The intracellular calcium assay of the undifferentiated PC12 cells was completed using the following procedures: (1) To study the effects of H_2O_2 on undifferentiated PC12 cell calcium expression in the presence of the test samples, the media were renewed, and the cells were preincubated for 12 hours, with and without the presence of 500 μL test samples, in order to obtain sample concentrations of 0, 0.5, 1, 5, and 10 μM. The media were removed, 500 μL of fluo-3-AM was then added to a concentration of 1 μM fluo-3-AM, and the culture solution was incubated in the dark at 37 °C for 1 hour. After removing the fluo-3-AM fluorescent probe, 500 μL of H_2O_2 was added to a concentration of 40 μM H_2O_2, and the mixture was incubated for an additional 4 hours. (2) To study the effects of $A\beta_{(25-35)}$ on undifferentiated PC12 cell calcium expression in the presence of test samples, the media were removed, 500 μL fluo-3-AM fluorescent probe was added to a concentration of 1 μM fluo-3-AM, and the culture solution was incubated in the dark at 37 °C for 1 hour. The fluorescent probe was then removed, and the undifferentiated PC12 cells were exposed to 0.01 μM $A\beta_{(25-35)}$ with various concentrations of test samples (0, 0.5, 1, 5, and 10 μM) for an additional 2 hours. (3) The control was incubated without the samples, H_2O_2, or $A\beta_{(25-35)}$ solution. After completing Steps 1 and 2, fluorescence intensity was determined as described for intracellular ROS.

Statistical analysis

Each CL system was tested three times using the same sample to determine reproducibility and provide a mean ± standard deviation (SD) using Microsoft Excel 2003. To evaluate the protective effect of each sample, suppression of the ROS production, and inhibition of the calcium ion influx against the effects of H_2O_2 or $A\beta_{(25-35)}$ on undifferentiated PC12 cells, only data on the exposure of undifferentiated PC12 cell group to H_2O_2 and $A\beta_{(25-35)}$ solution were considered. All data are represented as means ± SD based on triplicate determinations. Data were analyzed for statistical significance using one-way analysis of variance, followed by a *post hoc* Tukey test using SPSS software (version 10; SPSS for Windows, SPSS Inc., Chicago, IL, USA).

Results

Antioxidant activity of the pyrogallol–luminol system

We evaluated the antioxidant activity of five samples, including trolox and vitamin C for comparison, using the

pyrogallol–luminol assay. The scavenging effect of $^{\bullet}O_2^-$ was observed in the concentration range of 62.5–4000.0 μg/mL when the five samples were added (Fig. 1). The antioxidant properties, ranked alongside vitamin C and trolox, were in the order of trolox > astaxanthin > β-carotene > vitamin C > canthaxanthin, demonstrating various antioxidant properties at concentrations of 500–4000 μg/mL.

Antioxidant activity of the $CuSO_4$–Phen–Vc–H_2O_2 system

The antioxidant activity of five samples was evaluated by the $CuSO_4$–Phen–Vc–H_2O_2 assay. The effects of five samples were tested between concentrations of 62.5 and 4000 μg/mL, and marked inhibitions of $^{\bullet}OH$ were obtained (Fig. 2). The antioxidant properties of the five samples, including vitamin C and trolox, were as follows: trolox > astaxanthin > canthaxanthin > β-carotene > vitamin C, indicating varying $^{\bullet}OH$ scavenging activities for the five samples at concentrations of 125–1500 μg/mL.

Antioxidant activity of the luminol–H_2O_2 system

The antioxidant activities of the five samples were evaluated using the luminol–H_2O_2 assay (Fig. 3). The antioxidant activities of the five samples, including vitamin C and trolox, were observed in the decreasing order of trolox > vitamin C > astaxanthin > canthaxanthin > β-carotene at concentrations of 62.5–1000 μg/mL, and trolox > vitamin C > astaxanthin > β-carotene > canthaxanthin at concentrations of 1000–4000 μg/mL, demonstrating that the five samples had varied antioxidant properties against H_2O_2.

In vitro protection against oxidative damage

Fig. 4 shows the $A\beta_{(25-35)}$-induced cytotoxicity in undifferentiated PC12 cells. Cell viability decreased to 28% when exposed to 0.01 μM $A\beta_{(25-35)}$ for 48 hours. To obtain a detectable effect, the cells were treated with 0.01 μM $A\beta_{(25-35)}$ for 24 hours, causing cell viability to decrease to 48.4% (Fig. 4). Table 1 shows the results of our protective study. Cell viability decreases to 40.7 ± 7.4% when exposed to H_2O_2 (40 μM) for 12 hours, and decreases to 39.4 ± 8.5% when exposed to $A\beta_{(25-35)}$ (0.01 μM) for 24 hours. The undifferentiated PC12 cell protection of the three samples and Ac-DEVD-CHO against H_2O_2 decreases in the following order: astaxanthin > β-carotene > Ac-DEVD-CHO > canthaxanthin. The undifferentiated PC12 cell protection of the three samples and Ac-DEVD-CHO against $A\beta_{(25-35)}$ decreases in the following order: astaxanthin > Ac-DEVD-CHO > canthaxanthin > β-carotene.

Assay for intracellular ROS

PC12 cells were incubated with 40 μM H_2O_2 or 0.01 μM $A\beta_{(25-35)}$ with and without samples for 12 and 2 hours, respectively. Table 2 shows that the intensity of 2′,7′-dichlorofluorescein (DCF) fluorescence increased significantly after undifferentiated PC12 cells were treated with 40 μM H_2O_2 or 0.01 μM $A\beta_{(25-35)}$ for 2 hours. However, pre-incubation or co-incubation of the cells with sample concentrations less than 40 μM H_2O_2 and 0.01 μM $A\beta_{(25-35)}$ significantly reduced the DCF intensity. The various decreases in the production of H_2O_2-induced ROS in undifferentiated PC12 cell for the three samples and Ac-DEVD-CHO were in the following order: canthaxanthin > β-carotene > astaxanthin > Ac-DEVD-CHO (in the decreasing order of activity). The various reductions in the production

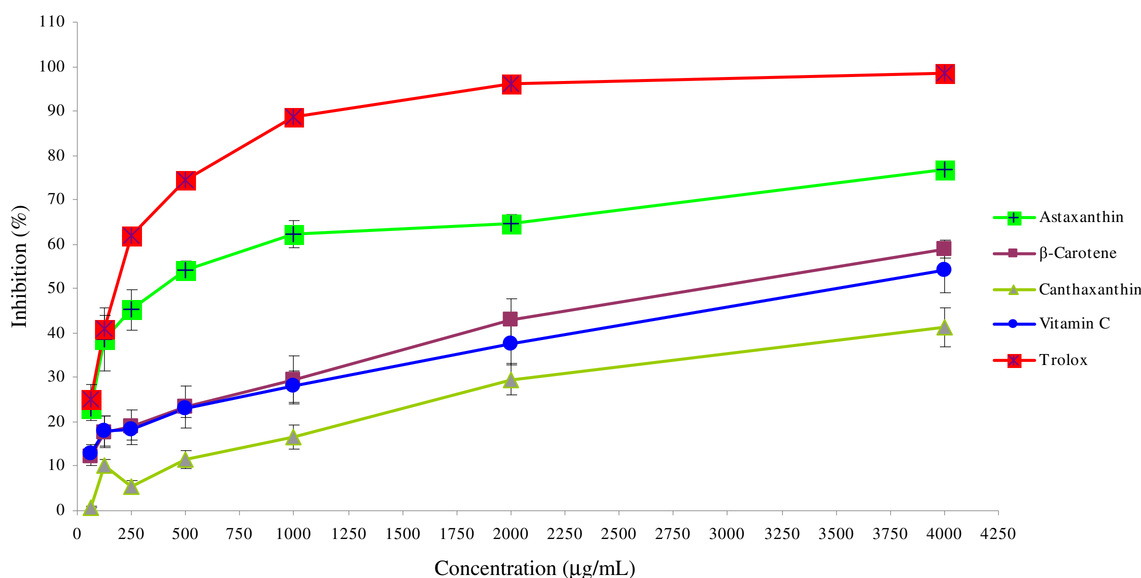

Figure 1. Superoxide anion radical scavenging activity of five samples at various concentrations using the pyrogallol–luminol system.

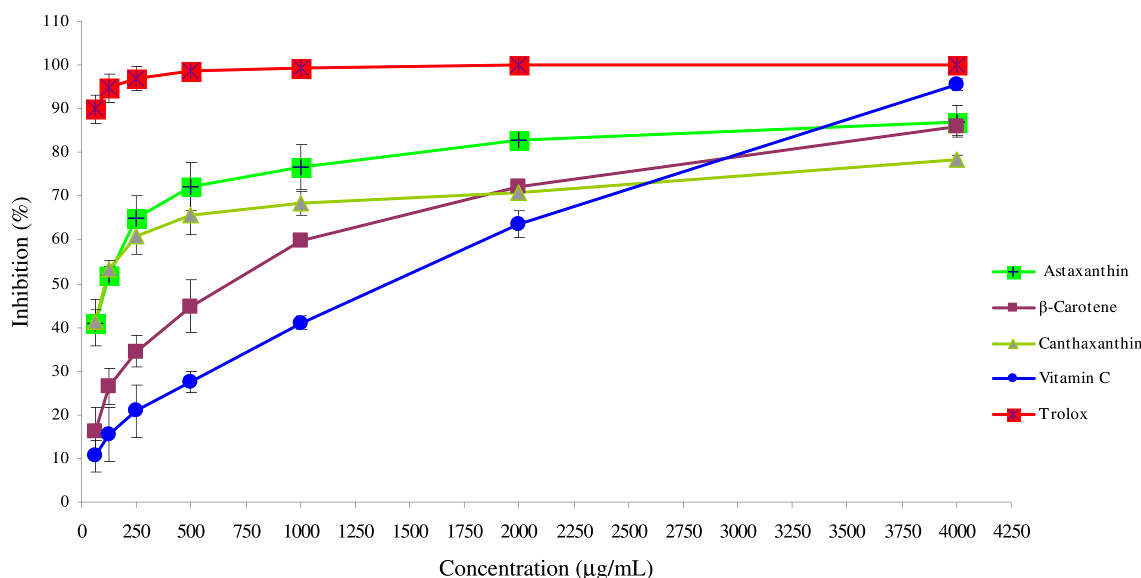

Figure 2. Hydroxyl radical scavenging activity of five samples at various concentrations using the $CuSO_4$—Phen—Vc—H_2O_2 system.

activities of ROS induced by $A\beta_{(25-35)}$ in undifferentiated PC12 cells for the three samples and Ac-DEVD-CHO were in the following decreasing order: Ac-DEVD-CHO > canthaxanthin > β-carotene > astaxanthin.

Assay for intracellular calcium

Table 3 shows the effects of preincubation or co-incubation of the cells with 40 μM H_2O_2 or 0.01 μM $A\beta_{(25-35)}$ on intracellular calcium. The various decreases in the calcium-production activities in undifferentiated PC12 cells in response to H_2O_2 for the three samples and Ac-DEVD-CHO in the decreasing order of activity were astaxanthin > Ac-DEVD-CHO > β-carotene > canthaxanthin. The different decreases in calcium-production activities in undifferentiated PC12 cell activities, induced by $A\beta_{(25-35)}$, for the three samples and Ac-DEVD-CHO in the decreasing order of activity were astaxanthin > canthaxanthin > Ac-DEVD-CHO > β-carotene.

Discussion

Pyrogallol, autoxidized under alkaline conditions, generated $^{\bullet}O_2^-$ radical ions, and $^{\bullet}O_2^-$ scavenging occurred with the addition of each of the five samples. In this reaction, luminol was excited by $^{\bullet}O_2^-$, and the decay from the excited state back to the ground state is accompanied by an emission of light (luminescence). Among the samples tested, astaxanthin led to an inhibition (76.7%) at the 4000 μg/mL concentration and exhibited more effective radical scavenging activity, which was also stronger than that of vitamin C in the pyrogallol—luminol assay. The

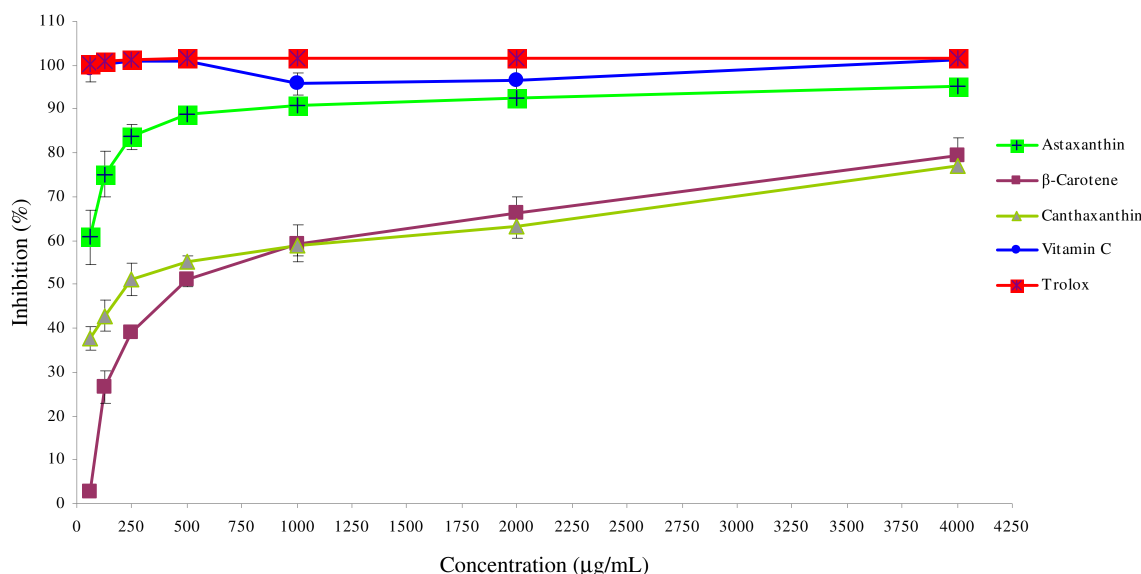

Figure 3. Hydrogen peroxide scavenging activity of five samples at different concentrations using the luminol—H_2O_2 system.

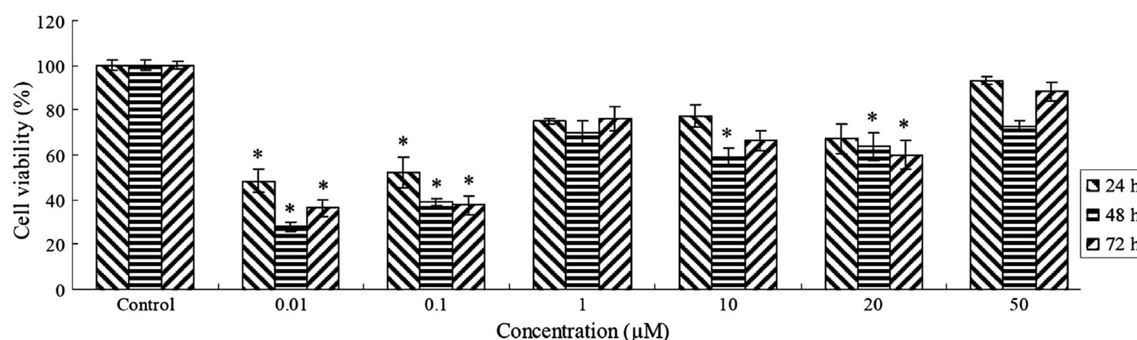

Figure 4. $A\beta_{(25-35)}$-induced cytotoxicity in undifferentiated PC12 cells. Cell viability was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay. $*p < 0.001$ versus control (0 μM).

greater $^{\bullet}O_2^-$ scavenging effects exhibited by astaxanthin were related to the presence of hydroxyl and ketone groups in the compound structure, and the relatively low $^{\bullet}O_2^-$ scavenging effects exhibited by canthaxanthin were because of its ketone group. Our results show astaxanthin as a powerful $^{\bullet}O_2^-$ scavenger.

The $^{\bullet}OH$ scavenging effect was observed with the addition of each of the five samples. In the $CuSO_4$—Phen—Vc—H_2O_2 system, Cu(II) strongly mediated the oxidation of the L-ascorbate solution, producing Cu(I) through a Cu(II)/Cu(I) redox mechanism. The generation of $^{\bullet}OH$ required the presence of Cu(I) and H_2O_2 in the Fenton reaction. 1,10-Phenanthroline was excited when oxidized by $^{\bullet}OH$ and its decay from the excited state back to the ground state is accompanied by CL. Among the studied samples, astaxanthin led to an inhibition (87.0%) at the 4000 μg/mL concentration and showed higher scavenging activity. Experimental evidence, numerous chemical-level details, and mechanisms are assigned to different ROS uptakes based on functional groups. Both astaxanthin and canthaxanthin, in which the 4- and 4′-positions are occupied by oxo groups, react more slowly than β-carotene, in which this position is free. The initial rates of autocatalytic oxidation were in the order of β-carotene > astaxanthin/canthaxanthin [16]. These results suggest that both astaxanthin and canthaxanthin are more effective antioxidants than β-carotene in stabilizing the trapped radicals [17]. Therefore, we attributed the greater $^{\bullet}OH$ scavenging activity of astaxanthin to its hydroxyl and ketone groups. The lower scavenging activity shown by β-carotene can be attributed to the absence of any hydroxyl and ketone groups in its molecular structure.

Table 1 Protective effects of the three samples and positive control on H_2O_2 and $A\beta_{(25-35)}$-induced PC12 cell death.

Sample	Concentration (μM)	Cell viability (%) by H_2O_2	Cell viability (%) by $A\beta_{(25-35)}$
Control	0	100.0 ± 4.7	100.0 ± 6.1
H_2O_2	40	40.7 ± 7.4*	—
$A\beta_{(25-35)}$	0.01	—	39.4 ± 8.5*
Astaxanthin	0.5	61.8 ± 8.9	87.2 ± 8.6**
	1.0	66.8 ± 7.0	85.1 ± 3.9**
	5.0	54.2 ± 3.7	124.8 ± 10.6***
	10.0	39.9 ± 6.4	146.3 ± 12.9****
β-Carotene	0.5	58.4 ± 8.4	43.7 ± 8.8
	1.0	44.0 ± 6.2	41.2 ± 5.5
	5.0	38.5 ± 2.9	43.8 ± 5.7
	10.0	49.5 ± 4.5	54.2 ± 7.8
Canthaxanthin	0.5	40.8 ± 5.4	71.2 ± 8.5
	1.0	35.6 ± 2.8	59.5 ± 10.0
	5.0	27.3 ± 5.1	71.3 ± 7.7
	10.0	32.7 ± 2.6	61.7 ± 9.0
Ac-DEVD-CHO	0.5	55.3 ± 8.5	103.1 ± 10.9***
	1.0	51.4 ± 5.5	81.1 ± 6.8**
	5.0	41.3 ± 3.8	82.2 ± 9.9**
	10.0	29.7 ± 5.5	92.7 ± 8.8**

These data are presented as mean ± SD for three replicates.
$*p < 0.01$ versus the control group without the addition of the sample and H_2O_2 or $A\beta_{(25-35)}$ solutions.
$**p < 0.05$.
$***p < 0.01$.
$****p < 0.001$ versus the 0.01 μM $A\beta_{(25-35)}$-treated group without the addition of the sample solution. Ac-DEVD-CHO = *N*-acetyl—Asp—Glu—Val—Asp—CHO.

Table 2 Protective effects of the three samples and positive control on the accumulation of intracellular ROS induced by H_2O_2 or $A\beta_{(25-35)}$ in undifferentiated PC12 cells.

Sample	Concentration (μM)	DCF fluorescence intensity (% of control) by H_2O_2	DCF fluorescence intensity (% of control) by $A\beta_{(25-35)}$
Control	0	100.0 ± 7.2	100.0 ± 6.2
H_2O_2	40	183.7 ± 7.2*	—
$A\beta_{(25-35)}$	0.01	—	205.3 ± 8.3*
Astaxanthin	0.5	140.2 ± 6.3	140.9 ± 9.1**
	1.0	147.8 ± 8.0	172.6 ± 10.8
	5.0	122.9 ± 1.5**	140.4 ± 11.4**
	10.0	120.9 ± 10.4**	159.7 ± 12.3
β-Carotene	0.5	90.0 ± 5.7**	149.4 ± 8.8**
	1.0	138.0 ± 9.4	157.2 ± 11.5
	5.0	117.1 ± 8.0**	145.3 ± 5.3**
	10.0	112.5 ± 10.7**	138.2 ± 7.3**
Canthaxanthin	0.5	92.7 ± 7.5**	137.8 ± 10.7**
	1.0	108.0 ± 8.8**	130.6 ± 2.3**
	5.0	113.9 ± 10.9**	113.5 ± 8.6**
	10.0	112.9 ± 7.0**	98.2 ± 3.8**
Ac-DEVD-CHO	0.5	170.3 ± 9.0	98.7 ± 7.8**
	1.0	175.9 ± 5.7	103.6 ± 10.1**
	5.0	134.5 ± 10.1	119.8 ± 7.8**
	10.0	124.2 ± 8.2**	105.7 ± 2.6**

These data are presented as mean ± SD for three replicates.
*$p < 0.001$ versus the control group without the addition of the sample and H_2O_2 or $A\beta_{(25-35)}$ solutions.
**$p < 0.001$ versus the 40-μM H_2O_2 or 0.01-μM $A\beta_{(25-35)}$-treated groups without the addition of the sample solution. Ac-DEVD-CHO = *N*-acetyl—Asp—Glu—Val—Asp—CHO; DCF = 2′,7′-dichlorofluorescein; ROS = reactive oxygen species.

Table 3 Preventive effects of the three samples and positive control on the elevation of intracellular calcium as induced by H_2O_2 or $A\beta_{(25-35)}$ in undifferentiated PC12 cells.

Sample	Concentration (μM)	Intracellular calcium (% of control) by H_2O_2	Intracellular calcium (% of control) by $A\beta_{(25-35)}$
Control	0	100.0 ± 8.1	100.0 ± 9.1
H_2O_2	40	143.3 ± 9.2*	—
$A\beta_{(25-35)}$	0.01	—	148.7 ± 8.0*
Astaxanthin	0.5	115.3 ± 1.9	109.6 ± 8.1
	1.0	123.8 ± 4.1	71.2 ± 10.0**
	5.0	110.2 ± 2.8	73.2 ± 3.4**
	10.0	101.4 ± 8.0 **	144.9 ± 8.8
β-Carotene	0.5	110.5 ± 7.4	117.3 ± 8.8
	1.0	128.2 ± 8.6	121.0 ± 3.7
	5.0	137.4 ± 8.0	126.5 ± 9.9
	10.0	127.7 ± 10.4	197.6 ± 10.2**
Canthaxanthin	0.5	122.7 ± 6.1	101.1 ± 9.8**
	1.0	115.7 ± 9.0	99.7 ± 0.3**
	5.0	140.2 ± 3.4	92.7 ± 5.2**
	10.0	130.4 ± 2.8	104.6 ± 5.5**
Ac-DEVD-CHO	0.5	130.5 ± 9.6	112.4 ± 10.6
	1.0	114.9 ± 8.4	132.5 ± 6.7
	5.0	112.8 ± 3.0	129.5 ± 9.5
	10.0	125.7 ± 6.1	136.7 ± 11.2

These data are presented as mean ± SD for three replicates.
*$p < 0.001$ versus the control group without the addition of the sample and H_2O_2 or $A\beta_{(25-35)}$ solutions.
**$p < 0.001$ versus the 40-μM H_2O_2 or 0.01-μM $A\beta_{(25-35)}$-treated groups without the addition of the sample solution.
Ac-DEVD-CHO = *N*-acetyl—Asp—Glu—Val—Asp—CHO.

In the oxygen and alkaline conditions, H_2O_2 oxidizes luminol to induce luminescence. The reaction with antioxidant species can quench this luminescence in the luminol–H_2O_2 assay. The highest H_2O_2 scavenging activity, exhibited by astaxanthin at concentrations of 62.5–4000 μg/mL, is a consequence of its hydroxyl and ketone groups. Astaxanthin led to a nearly complete inhibition (95.2%) at the 4000 μg/mL concentration and exhibited a more powerful effect than vitamin C.

Vitamin C can abolish $A\beta_{(25-35)}$-induced calcium increase and cell death in undifferentiated PC12 cells [18]. Vitamin E can prevent H_2O_2-induced cell apoptosis in undifferentiated PC12 cells [19]. However, vitamin C and vitamin E are antioxidants. Oxidative stress-induced cell death was significantly attenuated in amyloid precursor protein PC12 cells by pretreatment with caspase-3 inhibitors [20]. Cortical neuronal cultures treated with $A\beta_{(25-35)}$ demonstrate increased caspase-3 activity but not increased caspase-1 activity. Furthermore, significant neuroprotection is elicited by selective inhibition of caspase-3 in cortical neurons administered with $A\beta_{(25-35)}$, whereas selective caspase-1 inhibition has no effect [21]. These findings indicate that Ac-DEVD-CHO, a caspase-3 inhibitor, can be used as a positive control in protective/preventive intracellular assays. To ascertain the neuroprotective effects of astaxanthin, β-carotene, canthaxanthin, and Ac-DEVD-CHO, we applied an *in vitro* inhibition of H_2O_2 or $A\beta_{(25-35)}$-induced PC12 cell death model. However, none of the four compounds showed signs of cytotoxicity up to 48 hours at 0.5–10 μM (data not shown).

Application of neither H_2O_2 nor $A\beta_{(25-35)}$ induced undifferentiated PC12 cell death. Cell viability decreased to 12.2% when exposed to 80 μM H_2O_2 for 12 hours. To obtain a detectable effect, cells were treated with 40 μM H_2O_2 for 12 hours, causing cell viability to decrease to 47.3% [10]. Many laboratories have invested significant resources in establishing and validating cell-culture models of Aβ toxicity. In a separate report, 20 μM $A\beta_{(1-40)}$, $A\beta_{(1-38)}$, and $A\beta_{(1-28)}$ were shown to have short-term neurotrophic effects on immature rat hippocampal neurons cultured at low density in serum-containing media. Evaluation of a series of overlapping peptides spanning the Aβ sequence revealed that the fragment $A\beta_{(25-35)}$ exhibited neurotrophic and neurotoxic activities similar to those of $A\beta_{(1-40)}$ [12]. Other workers also reported that the aggregated $A\beta_{(25-35)}$ is cytotoxic to a clone of PC12 cells at concentrations above 0.001 μM and to several other cell lines at higher concentrations [22]. The aggregated $A\beta_{(25-35)}$ is cytotoxic to undifferentiated PC12 cells at 0.1–0.001 μM, and reduces cell viability to 40–50% [22,23]. Fig. 4 shows that low doses of $A\beta_{(25-35)}$ (0.01 and 0.1 μM) for 24, 48, and 72 hours significantly reduced undifferentiated PC12 cell viability (cell viability $< 60\%$). However, the graph shows that significantly higher doses (1, 10, 20, and 50 μM) of $A\beta_{(25-35)}$ for 24, 48, and 72 hours lead to reduced cell toxicity (cell viability $> 60\%$). The result of this experiment was contradictory to the dose–response observation. Perhaps the most important factor, which was found to influence toxicity, was the aggregation state of Aβ [23]. The 40-μM H_2O_2 for 12 hours and 0.01-μM $A\beta_{(25-35)}$ for 24 hours were thus selected for use in the experiments.

Astaxanthin partially inhibited H_2O_2-induced cytotoxicity at 0.5–5.0 μM (cell viability = 61.8–54.2%). Both 0.5 μM β-carotene (cell viability = 58.4%) and 0.5–1.0 μM Ac-DEVD-CHO (cell viability = 55.3–51.4%) provided low-cell protection against H_2O_2, but canthaxanthin did not provide any observable protection for the undifferentiated PC12 cells against H_2O_2. Moreover, increased dosages of the three samples and Ac-DEVD-CHO failed to show better effects (Table 1). Astaxanthin clearly inhibited $A\beta_{(25-35)}$-induced cytotoxicity at 0.5–10.0 μM (cell viability = 87.2–146.3%), and canthaxanthin partially inhibited $A\beta_{(25-35)}$-induced cytotoxicity at 1.0–5.0 μM (cell viability = 59.5–71.3%). Ac-DEVD-CHO inhibited $A\beta_{(25-35)}$-induced cytotoxicity at 0.5–1.0 μM (cell viability = 103.1–81.1%). The 10.0 μM β-carotene (cell viability = 54.2%) provided low protection of undifferentiated PC12 cells against $A\beta_{(25-35)}$. However, the information presented in Table 1 shows that between the three samples that are compared with the control cells, only astaxanthin significantly attenuated $A\beta_{(25-35)}$ toxicity, and none of the samples were able to modulate H_2O_2 toxicity significantly. Astaxanthin at 5.0 and 10.0 μM induces 124.8% and 146.3% cell viability, which is higher than in the control cells because it alone did not show any cytotoxicity at 0.5–10.0 μM. The total time of this protective experiment was 24 hours, and astaxanthin could induce cell proliferation with cell viability above 100.0% at 0.5–10.0 μM for 48 hours (data not shown). We propose that the neuroprotective activity exhibited by astaxanthin is due to its $^{\bullet}O_2^-$, $^{\bullet}OH$, and H_2O_2 scavenging activities, and the presence of hydroxyl and ketone groups in its structure. The neuroprotective activity exhibited by canthaxanthin against $A\beta_{(25-35)}$ toxicity is owing to its $^{\bullet}OH$ and H_2O_2 scavenging activities and the presence of ketone group in its structure. However, β-carotene exhibited low neuroprotective activities in the undifferentiated PC12 cells against H_2O_2 and $A\beta_{(25-35)}$ because no hydroxyl and ketone groups are present in its structure. Our studies clearly demonstrate that the neuroprotection of undifferentiated PC12 cells against H_2O_2 and $A\beta_{(25-35)}$ is related to the presence of hydroxyl and ketone groups in the three samples.

The degree of ROS generation in cells was measured by the fluorescence assay using a 2′,7′-DCFH-DA probe. The 2′,7′-DCFH-DA can be deacetylated in cells, where it can quantitatively react with intracellular radicals, such as H_2O_2, and convert them into DCF-fluorescent products, which are retained within the cells. This assay provides an index of cell cytosolic oxidation. DCF fluorescence results evaluated by fluorescent spectrometer demonstrated that H_2O_2 and $A\beta_{(25-35)}$ induced the production of ROS. A tremendous amount of ROS was evoked by 40 μM H_2O_2 and 0.01 μM $A\beta_{(25-35)}$, reaching net elevations of 83.7% and 105.3%, respectively. After treatment with four samples, approximately 7.8–93.7% and 32.7–107.1% of H_2O_2 and $A\beta_{(25-35)}$-induced ROS were reduced, respectively. However, these results were not dose-dependent responses except for canthaxanthin under the 0.01 μM $A\beta_{(25-35)}$ condition (Table 2). The high activity of canthaxanthin in reducing the production of ROS has a low dependence on its antioxidant activity and medium dependence on its neuroprotection against $A\beta_{(25-35)}$ toxicity to undifferentiated PC12 cells, and is independent of its neuroprotection against H_2O_2. Astaxanthin exhibits the highest antioxidant activity, good

neuroprotection against undifferentiated PC12 cell A$\beta_{(25-35)}$-induced toxicity, and medium neuroprotection against H_2O_2 toxicity in undifferentiated PC12 cells, but it shows the lowest decrease in ROS production. Compared with other published results, the protection of both astaxanthin and canthaxanthin against 100 μM H_2O_2 toxicity was 10 and 20 μM in NGF-differentiated PC12 cells ($p < 0.05$). The differentiated PC12 cell protection of astaxanthin and canthaxanthin against H_2O_2 decreased in the order of astaxanthin > canthaxanthin. However, the various decreases in differentiated PC12 cell H_2O_2-induced ROS production for astaxanthin and canthaxanthin in the decreasing order of activity were canthaxanthin > astaxanthin [7]. Thus, neuroprotection data (Table 1) are not related to the capacity of astaxanthin and canthaxanthin in reducing ROS in undifferentiated PC12 cells (Table 2). Table 2 shows that β-carotene and canthaxanthin are able to modulate ROS production, but neither of the compounds is fully able to mitigate H_2O_2 or A$\beta_{(25-35)}$ toxicity.

Undifferentiated PC12 cells exposed to 40 μM H_2O_2 and 0.01 μM A$\beta_{(25-35)}$ for 4 hours and 2 hours, respectively, exhibited increased fluorescence intensity because of enhanced intracellular calcium levels. The control cells showed weak cytoplasmic labeling. The influx of calcium into undifferentiated PC12 cells was significantly enhanced by 43.3% and 48.7%, respectively. Approximately 3.1—41.9% and 3.8—77.5% of H_2O_2 and A$\beta_{(25-35)}$-induced cytosolic calcium influx were reduced by the addition of the four samples to the culture medium, respectively. However, A$\beta_{(25-35)}$-induced cytosolic calcium influx was enhanced by 97.6% after the addition of 10.0 μM β-carotene to the culture medium. The high activity of astaxanthin in decreasing calcium production is highly dependent on its antioxidant activity and on its neuroprotection activity against A$\beta_{(25-35)}$ toxicity to undifferentiated PC12 cells and has medium dependence on its neuroprotection activity against H_2O_2 toxicity to undifferentiated PC12 cells. The scavenging abilities of both β-carotene and canthaxanthin against free radicals were weaker than astaxanthin. Table 3 shows that β-carotene and canthaxanthin are able to modulate calcium production, but neither compound is fully able to mitigate H_2O_2 or A$\beta_{(25-35)}$ toxicity. Moreover, these findings show that reduction of undifferentiated PC12 cell calcium-production activities in response to H_2O_2 and A$\beta_{(25-35)}$ exposure by the three samples is related.

For the first time, we demonstrated and compared *in vitro* CL antioxidant activities and the neuroprotective effects of astaxanthin, β-carotene, and canthaxanthin. Recent evidence suggests a lack of correlation between activities determined on the same material by different antioxidant assays and between activities determined by the same antioxidant assay in different laboratories [24]. This study intends to demonstrate similar antioxidant activities by using three different CL antioxidant methods. However, the study shows varying degrees of antioxidant activity for the three carotenoids in the three CL antioxidant assays. The three carotenoids present various ROS scavenging activities because of differences between the three ROS CL mechanisms, including the scavenging effects of $^\bullet O_2^-$, $^\bullet OH$, and H_2O_2. Astaxanthin had a powerful free radical scavenging activity in the three different CL antioxidant assays. The three carotenoids are antioxidants obtained from natural sources and have potential in food applications and nutraceutical products. For a homogeneous solution, the effects of the structure of the astaxanthin, β-carotene, and canthaxanthin were also investigated, and the stability of the three carotenoids in the oxidizing system was different, with an order of decreasing stability being astaxanthin > canthaxanthin > β-carotene [25]. Carotenoid aggregation may reduce the potential for radical—pigment interaction and may also favor carotenoid—carotenoid auto-oxidation in the aggregation [26]. Therefore, the protective effects of astaxanthin, β-carotene, and canthaxanthin at different concentrations were not dose dependent in undifferentiated PC12 cells. In addition, astaxanthin shows partial neuroprotective activity against H_2O_2-induced toxicity toward undifferentiated PC12 cells and exhibits the highest neuroprotective activity against A$\beta_{(25-35)}$-induced toxicity toward undifferentiated PC12 cells. Canthaxanthin inhibits only A$\beta_{(25-35)}$-induced cytotoxicity and exhibits partial neuroprotective activity. Our findings suggest that both astaxanthin and canthaxanthin reverse H_2O_2 and A$\beta_{(25-35)}$-induced toxicity by scavenging free radicals, and decreasing intracellular ROS production and calcium levels. Thus, astaxanthin may have the potential to ameliorate the effects of AD, which is in part caused by ROS and Aβ. Further study of astaxanthin is required to elucidate the precise *in vivo* mechanisms of its antioxidant and neuroprotective effects.

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