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Enhanced Antioxidant and Protective Activities on Retinal Ganglion Cells of Carotenoids-Overexpressing Transgenic Carrot

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Abstract: Carotenoids are considered to act as antioxidants and protect humans from serious disorders such as skin degeneration and ageing, cardiovascular disease, certain types of cancer, and age-related diseases of the eye. In this study, these chemopreventive activities of a carotenoids-overexpressing transgenic carrot were evaluated. The results of DPPH, hydroxyl, and superoxide radical scavenging tests demonstrate that the acetone extract obtained from the taproots of the carrot plants exhibits significant antioxidant activity. A higher activity was detected in the transgenic carrot extract compared with the wild-type extract. A chemopreventive activity test for degenerative diseases of the eye revealed that pretreatment with the carrot extract reduced cell death in a retinal ganglion cell line, RGC-5 cells exposed to 1-buthionine-(*R*,*S*)-sulfoximine and L-glutamic acid.

Keywords: Carotenoids, transgenic carrot, antioxidant activity, RGC-5 cells.

INTRODUCTION

Carotenoids are a class of natural pigments, which range in color from yellow to red that are found ubiquitously in plants and photosynthetic microorganisms. Carotenoids are considered to act as antioxidants and protect humans from serious disorders such as skin degeneration and ageing, cardiovascular disease, certain types of cancer, and age-related diseases of the eye [1, 2]. Because carotenoids generally cannot be biosynthesized in animals, animals must ingest these compounds in their regular diet. Carrots (*Daucus carota* L.) are consumed as an important dietary source of carotenoids, particularly *β*-carotene and *α*-carotene. Carotenoids can be categorized into two classes: xanthophylls and carotenes. As a result, *β*-carotene belongs to the class of carotenes, which are purely hydrocarbons and contain no oxygen atoms [3]. It is known that *β*-carotene has a higher retinol activity equivalent than all of the other provitamin A carotenoids, and this carotenoid is associated with a lower risk for various cancers and the scavenging of certain reactive oxygen species [4, 5]. In contrast, ketocarotenoids constitute a group of xanthophylls that contain at least one keto group, which is found either in the linear chain or on the *β*-ionone ring(s). Astaxanthin (3,3′-dihydroxy-*β*,*β*-carotene-4,4′-dione), which is a ketocarotenoid, is found in the natural diet of many aquatic animals and is widely used as a food supplement, natural colorant, and feed additive. It has been reported that astaxanthin possesses various biological activities including strong antioxidant, immune-potentiating, hypotensive, neuroprotective, and hepatoprotective activities [6-10]. In a previous study, we reported that HpBkt-transgenic carrot plants had been generated to overproduce carotenoids including astaxanthin, which is a non-natural ketocarotenoid in this plant, using a storage root (*ibAGP1*) promoter, an amyloplast targeting sequence (TP1) and a single *β*-carotene ketolase (*HpBkt*) gene [11].

The reactive oxygen species (ROS) that induce autooxidation and the thermal oxidation of lipids are associated with ageing and membrane damage in living organisms. The production of ROS includes the superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$) and singlet oxygen ($^1O_2$) which causes degenerative human diseases such as cancer, heart disease, and cerebrovascular disease through multiple mechanisms [12, 13].

Primary open angle glaucoma (POAG) is a chronic disease characterized by a progressive reduction in the numbers of retinal ganglion cells (RGCs) and a loss of vision. Moreover, RGC death is a common feature of many ophthalmic disorders, such as optic neuropathies and various retinovascular diseases including diabetic retinopathy and retinal vein occlusions [14]. It is known that oxidative stress plays a ma-

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jor role in RGC death, which results in the pathogenesis of POAG [15, 16].

Therefore, in this study, we evaluated the antioxidant and protective activities on retinal ganglion cells (RGCs) of the acetone extracts obtained from the taproots of wild-type carrot plant and a transgenic carrot plant, HpBkt-8, which exhibits high contents of *β*-carotene, astaxanthin and lutein.

MATERIALS AND METHODS

Preparation of Carotenoid Extracts from Carrot

Lyophilized roots (1 g) obtained from a wild type and a transgenic plant, HpBkt-8 of *Daucus carota* were homogenized in a pre-chilled mortar and a pestle with 5 ml of acetone, sea sand, Na_2SO_4, and $NaHCO_3$. The mixture was transferred to a 50-ml conical tube with additional acetone and extracted twice with ultrasonication for 30 min. The extract (80 ml) was then centrifuged at 5,000 rpm for 10 min and the supernatant was filtered through a 0.45-μm membrane filter (Whatman, New York, NY, USA; PTFE, 13 mm). The filtrate was concentrated *in vacuo* and dried with N_2 gas prior to its analysis and various bioassays. All of the extraction procedures were performed under subdued light to avoid any degradation in the stability of the pigments. The carotenoid analysis was performed using an HPLC-DAD system according to the external calibration method described in our previous report [11]. The methanol, water and *tert*-butyl methyl ether used in the HPLC system were all of HPLC grade, and the other chemicals used in the extraction and analysis of carotenoids were of extra-pure grade.

Assessment of Antioxidant Activities

The scavenging activities of the samples on the DPPH (1,1-diphenyl-2-picrylhydrazyl), hydroxyl and superoxide radicals were measured according to our previously reported method with a slight modification [17]. Briefly, the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of the samples was measured using the method developed by Brand-Williams with slight modifications [18].The scavenging activity of the samples toward the hydroxyl radical was evaluated using the deoxyribose method [19]. Superoxide radicals were generated using the method developed by Chun *et al.* to determine the superoxide anion radical scavenging activity with slight modifications [20]. Each sample was dissolved in acetone and then diluted in ethanol to obtain various sample concentrations (0, 5, 10, 20, 50 and 100 μg/ml). L-Ascorbic acid, butylated hydroxyanisole (BHA) and quercetin were purchased from Sigma Co. (St. Louis, Mo, USA) and used as positive controls for these radical scavenging activity tests.

Measurement of Total Phenolic and Total Flavonoid Contents

The total phenolic contents of the extracts were determined spectrophotometrically according to our previous method, which is based on the Folin-Ciocalteu colorimetric method with slight modifications [17]. Using catechin standard for the calibration (Sigma, USA), the results were expressed as μg catechin equivalents/gram extract (μg CE/g). The total flavonoid content was determined through the aluminum chloride colorimetric method using quercetin as the standard, and the results are expressed as μg quercetin equivalents/gram extract (μg QE/g) [17].

Culture of RGC-5 Retinal Ganglion Cells

RGC-5 cells were cultured and maintained in DMEM (Sigma-Aldrich, MO, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Paisley, UK), 25 mM glucose, 100 U/ml penicillin (Invitrogen, Paisley, UK) and 100 μg/ml streptomycin (Invitrogen, Paisley, UK) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Confluent cultures of RGC-5 cells from 75-cm^2 filter-capped cell culture flasks were generally passaged using a trypsin-EDTA solution at a ratio of approximately 1:8 to obtain a cell density of approximately 4 to 5×10^4 cells/ml. One hundred microliters of these cells were placed in individual 96-well plates. After the cells were allowed to settle for approximately 24 h, the medium was replaced with DMEM supplemented with 1% FBS to avoid the possibility of antioxidant activity arising from serum protein. H_2O_2 or 1-buthionine-(*R*,*S*)-sulfoximine (BSO) and L-glutamic acid (Sigma-Aldrich, MO, USA) were added to the samples in the 96-well plates to obtain final concentrations of 300 μM or 0.5 mM and 10 mM, respectively. The samples (final concentrations of 1 and 5 μg/ml in 0.5% DMSO) were added 60 min before the addition of these toxicants. The control samples received equivalent amounts of vehicle (0.5% DMSO). An antagonist, *N*-acetyl cysteine (NAC), was used as a positive reference compound. The cell viability of the cells in the 96-well plates was analyzed after 24 h. The cell viability was assessed using the modified MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma) reduction assay [21].

Statistical Analysis

All of the measurements used for the assessment of the antioxidant activities were repeated six times, and all of the cell-based assays were repeated three times. The results are shown as the mean values with the standard deviation. The statistical comparisons of the results were made using analysis of variance (ANOVA), and differences with a P value less than 0.05 were considered significant.

RESULTS

Carotenoid Contents of Carrot Extracts

The carotenoid contents of the acetone extracts obtained from the wild-type (WT) and a transgenic carrot plant (HpBkt-8) are listed in Table **1**. On average the total carotenoid content of the transgenic carrot was 1.6-fold higher than that of the wild-type. Astaxanthin, which is not usually found in carrot plants, was detected in the transgenic carrot, at a level of 0.54± 0.15 mg/g extract, but was not detected in the wild-type carrot plants (Table **1** and Fig. **1**). The major carotenoid of carrot, *β*-carotene and the third major carotenoid component, lutein, were detected in the transgenic carrot extract at levels that were 1.7- and 2.9-fold higher, respectively, than those in the wild-type. There was a slight difference in the *α*-carotene content between the two extracts. HpBkt exhibited a distinctively higher violaxanthin content (0.67 ± 0.14 mg/g extract) than the wild-type

Table 1. Carotenoid Contents in the Acetone Extracts Obtained from Taproots of Wild-Type and a Transgenic (HpBkt-8) Carrot Plants (mg/g extract)[a].

Samples	Violaxanthin	Astaxanthin	Lutein	Zeaxanthin	*β*-Cryptoxanthin	*α*-Carotene	*β*-Carotene	Total
WT	0.21 ± 0.11	ND[b]	0.60 ± 0.10	ND	0.16 ± 0.08	7.75 ± 0.84	13.02 ± 1.23	21.73 ± 2.18
HpBkt-8	0.67 ± 0.14	0.54 ± 0.15	1.74 ± 0.24	ND	0.10 ± 0.02	9.35 ± 0.62	22.71 ± 1.26	35.11 ± 2.42

[a]The Data are expressed as the mean (average value) and SD (the standard deviation value) of two independent experiments (each with three replicate samples).
[b]Not detected.

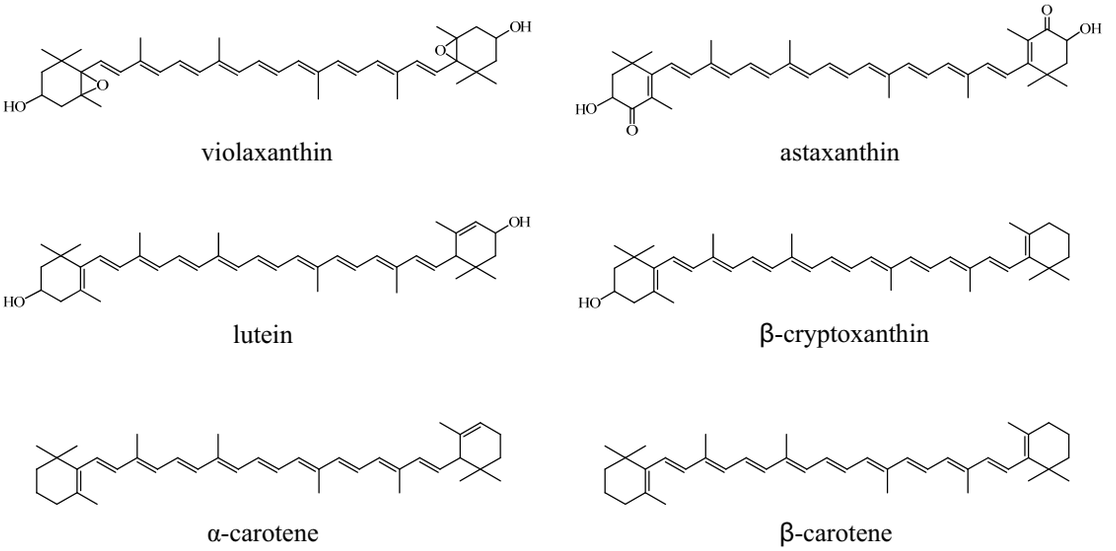

Fig. (1). The chemical structures of the carotenoids analyzed in this study.

(0.21 ± 0.11 mg/g extract). Zeaxanthin was not detected in the two samples tested.

Anti-oxidant Activity of Carrot Extracts

Whereas the WT extract failed to exhibit any DPPH radical scavenging activity even at the concentration of 100 μg/ml, HpBkt-8 exhibited significant antioxidant activity at concentrations of 50 and 100 μg/ml (Fig. **2A**). The analysis of the hydroxyl radical scavenging activity revealed that both extracts at the concentration of 5 μg/ml scavenged the radical more efficiently than the other three positive controls (L-ascorbic acid, BHA and quercetin). The extracts showed antioxidant activity in a dose-dependent manner, and the activities of the extracts were higher than that of quercetin at all of the tested concentrations. The lipophilic acetone extract of the transgenic carrot (HpBkt-8) exhibited higher radical scavenging activity than that of the wild-type carrot. The activity of HpBkt-8 at the concentrations of 50 and 100 μg/ml was comparable to that of the potent antioxidant BHA (Fig. **2B**). In fact, the two samples at concentrations of 50 and 100 μg/ml exhibited more than twofold higher superoxide anion radical scavenging activity than the three positive controls. At a concentration of 20 μg/ml, the extracts presented superoxide anion radical scavenging activity that was similar to that exhibited by the potent antioxidants, L-ascorbic acid and BHA. This observed scavenging effect was also higher in HpBkt-8 compared with the WT (Fig. **2C**).

Total Phenolic and Flavonoid Contents

To investigate the effect of phenolic and flavonoid compounds on the antioxidant activities of carrot extract, the total phenolic and flavonoid contents of the lipophilic extracts from wild-type and a transgenic carrot were evaluated. The results showed that there was no significant difference in the total phenol and flavonoid contents between the two extracts (Table **2**). The phenolic contents were 22.70 ± 1.05 and 23.68 ± 1.02 μg catechin/g extract in the WT and theHpBkt-8 extracts, respectively. The flavonoid contents were 25.18 ± 0.03 and 25.30 ± 0.08 μg quercetin/g extract in the WT and the HpBkt-8 extracts, respectively. The total phenolic content was expressed as microgram catechin equivalents/gram extract (μg CE/g extract) because catechin is a common polyphenol compound. The results for the total flavonoid content were described as microgram quercetin equivalents/gram extract (μg QE/g extract) because quercetin is a ubiquitous flavonoid in plant tissue.

Protective Activity of Carrot Extracts against Oxidative Stress Induced Retinal Ganglion Cell Injury

These two lipophilic extracts from carrot plants were tested for their capacity to counteract the oxidative stress induced by H_2O_2 or BSO plus glutamate on RGC-5 cells in culture. The poor solubility of the extracts resulted in a maximum final concentration of 5 μg/ml in 0.5% DMSO.

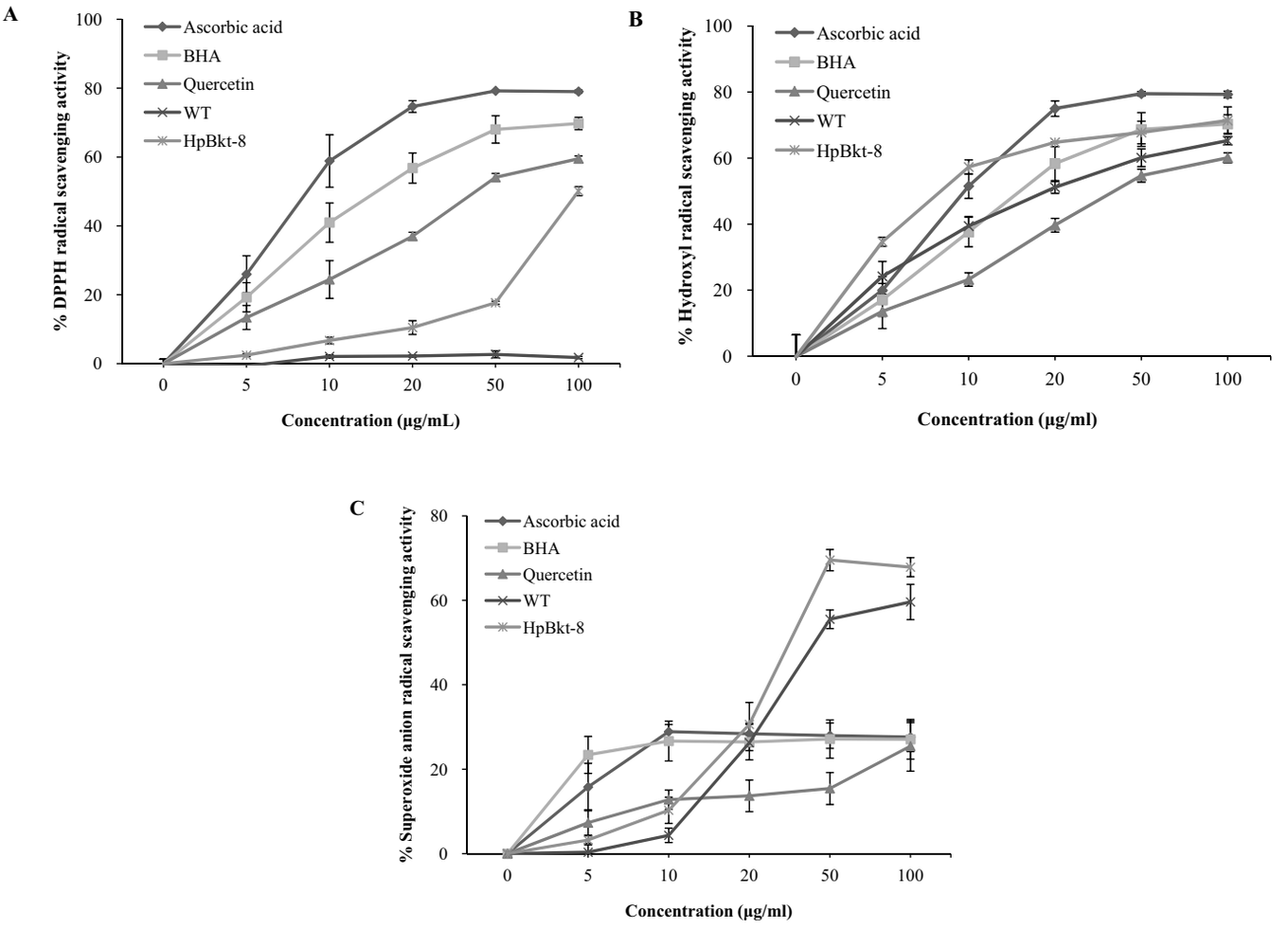

Fig. (2). Antioxidant activities of carrot extracts at different concentrations (0, 5, 10, 20, 50, and 100 μg/mL). The radical scavenging effects against the DPPH radical (A), hydroxyl radical (B) and superoxide anion radical (C) were evaluated. The values represent the mean ± standard deviation of triplicate determinations (n = 6). *p < 0.05, **p < 0.01: the values obtained with the same concentration are significantly different.

Table 2. Total Phenolic and Flavonoid Contents of the Carrot Extracts.

Samples	Total phenolic content (μg catechin/g extract)	Total flavonoid content (μg quercetin/g extract)
WT	22.70 ± 1.05	25.18 ± 0.03
HpBkt-8	23.68 ± 1.02	25.30 ± 0.08

[a]The Data are expressed as the mean (average value) and SD (the standard deviation value) of two independent experiments (each with three replicate samples).

The viability of the RGC cells decreased to approximately 69% and 54%, 24 h after oxidative stress induced by H_2O_2 or BSO plus glutamate, respectively. Although HpBkt-8 and WT failed to show any significant protective effect against H_2O_2- induced cell damage (Fig. **3A**), both two types of carrot extracts at the concentrations of 1 and 5 μg/ml significantly inhibited the negative effect of BSO and glutamate on RGC-5 cells (Fig. **3B**). The viability of the cells increased from approximately 54% to 64% and 63% after the addition of 1 and 5 μg/ml of the WT extract, respectively. In contrast, the viability of the cells exposed to HpBkt-8 at concentrations of 1 and 5 μg/ml increased to 66% and 72%, respectively.

DISCUSSION

The aim of this study was to evaluate the antioxidant and protective activities on retinal ganglion cells (RGCs) of the acetone extract obtained from the taproot of a transgenic carrot plant (HpBkt-8), which has higher contents of β-carotene, astaxanthin and lutein than the wild-type carrot extract.

The DPPH radical scavenging test is a non-enzymatic method that is currently used to provide basic information on the ability of extracts to scavenge free radicals. The reduction of DPPH radicals by an antioxidant results in a loss of absorbance at approximately 515 nm [22]. Our results from the DPPH assay indicate that the lipophilic extract of HpBkt-8 has significant antioxidant potential, whereas the WT extract did not exhibit any potential antioxidant activity. The hydroxyl radical is known to be the most reactive radical because it can attack and damage almost every biomolecule

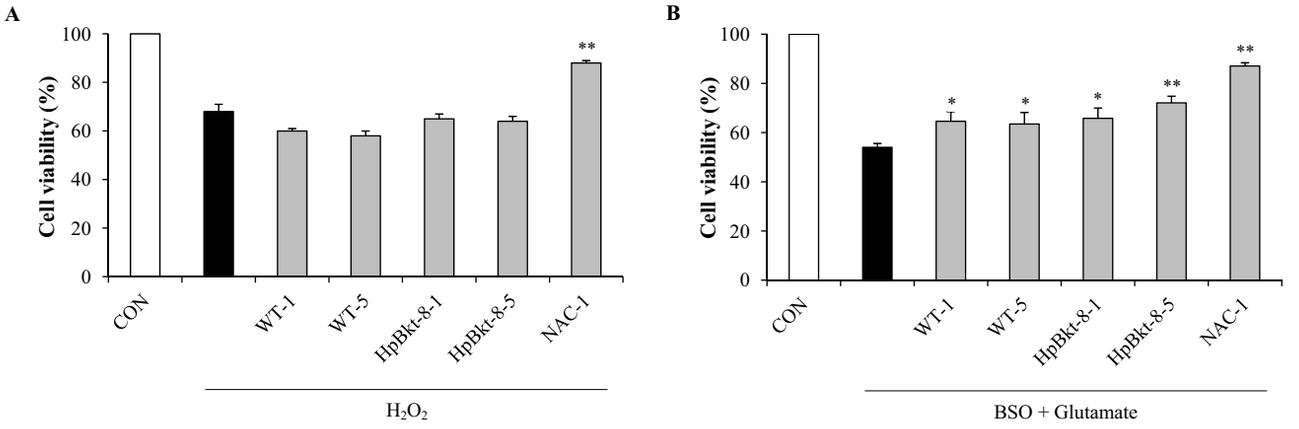

Fig. (3). Effect of carrot extracts on the cell viability of RGC-5 cells exposed to H_2O_2 (A) or BSO and glutamate (B). The results represent mean values, and the error bars indicate the S.E.M., where $n = 3$ (*$p < 0.05$, **$p < 0.01$). Twenty-four hours after the RGC-5 cells were plated, the cells were preincubated with NAC (1 μM) or the carrot extracts (1 and 5 μg/ml). One hour later, H_2O_2 (300 μM) or BSO (0.5 mM) and glutamate (5 mM) were added. The cell viability was determined 24 h after exposure and is expressed as the percentage of cells surviving compared with the control.

found in living cells. The reactivity of $^{\bullet}OH$ include its ability to interact with the purine and pyrimidine bases of DNA and its ability to abstract hydrogen atoms from biomolecules, which leads to the formation of sulfur radicals and oxysulfur radicals that damage biomolecules [23]. The best-characterised biological damage caused by the hydroxyl radical is its capacity to stimulate lipid peroxidation, which occurs when $^{\bullet}OH$ is generated close to membranes and attacks the fatty acid side chains of the membrane phospholipids [23]. In the work herein, the two carrot extracts exhibited scavenging activity for the hydroxyl radical in a dose-dependent manner. The superoxide radical is produced *in vivo* by activated phagocytes, by electron leakage from the mitochondrial electron transport chain [23] and in the conversion of xanthine to uric acid [24]. Although the reactivity of this radical is limited, the superoxide radical is considered to be toxic. In fact, much of the molecular damage that can be induced by the superoxide radical is due to its conversion into much more reactive species, namely, the hydroxyl radical and peroxynitrite [25]. In the present study, the two carrot extracts exhibited comparable or even superior superoxide anion radical scavenging activity compared with the three positive control compounds. According to the results obtained in the three antioxidant assays, the transgenic carrot extract proved to have higher antioxidant potential than the wild-type carrot extract. In addition, the effects observed were concentration-dependent. These findings are not surprising because HpBkt-8 extract exhibited a higher content of carotenoids, including *β*-carotene, astaxanthin, and lutein, which have been reported to have high radical scavenging ability [5]. Moreover, the total content of phenolic and flavonoid compounds, which are generally known to have antioxidant activity [26], was too low compared with the carotenoid content to affect the antioxidant activity of the extracts. In addition, there was no significant difference in the total phenolic and flavonoid contents between the two samples. Therefore, the observed scavenging activities of the extracts and the different activities between two samples might be due to the presence of carotenoids and the difference in the carotenoid content, respectively.

The antioxidant activity of the hydrophilic ethanol extract obtained from the taproots of the carrot plant has been reported without any compositional data [27]. The water extract of carrot has been found to exhibit a protective effect against lindane-induced hepatotoxicity in rats [28]. However, the acetone extract of carrot, which is mostly composed of more lipophilic compounds, such as carotenoids (different from flavonoids, phenols, and other more hydrophilic natural antioxidants), has not been widely studied [26]. Moreover, the radical scavenging activity of the lipophilic fraction in some orange-fleshed sweet potato cultivars has been reported, and the results indicated that the most predominant contributor to the activity is be *β*-carotene [29, 30].

Based on the role of reactive oxygen and the degeneration of RGCs in the pathogenesis of glaucoma [15-16, 31], the protective activity of the carrot extract with radical scavenging activity *in vitro* was evaluated using two models of RGCs exposed to oxidative stress. Direct oxidative stress was induced by excessive H_2O_2, which can enter the cells and induce cytotoxicity due to its high membrane permeability and trigger the apoptosis of RGC cells through a caspase-independent pathway [32, 33]. Another oxidative stress to RGC-5 cells in a culture was delivered indirectly by exposure to a combination of BSO and glutamate. It is known that BSO causes a reduction in glutathione (GSH) biosynthesis and that extracellular glutamate can enhance this action by reducing the availability of cysteine, which is a precursor for GSH synthesis [34].

A recent study showed that astaxanthin, which is a powerful biological antioxidant [35], protected cultured retinal ganglion cells from the neurotoxicity induced by H_2O_2 or serum deprivation and reduced the intracellular oxidation induced by various reactive oxygen species [36]. This keto-carotenoid cannot be biosynthesized in wild-type carrot plants, and its presence has been detected in the transgenic line, HpBkt-8, in our previous study. Lutein, which is a xanthophyll, is predominately present in the macular region and acts as an efficient pigment for the absorption of high energy blue light and a direct free radical scavenger to prevent

macular damage. Lutein has been reported to protect RGC-5 cells against both $CoCl_2$-induced chemical hypoxia and H_2O_2-induced oxidative stress [37]. However, the neuroprotective activities of astaxanthin and lutein were observed at concentrations higher than 10 μM, and their activity against the oxidative stress induced by BSO plus glutamate has not been reported. In this study, whereas 5 μM astaxanthin and 5 μM lutein both failed to exhibit activity in both models (data not shown), the lipophilic carrot extract protected RGCs from BSO plus glutamate toxicity at this concentration. This result suggests that other compounds in addition to carotenoids might contribute to the antioxidant activity and protective activities of the extracts on retinal ganglion cells. For example, lutein has been reported to exhibit increased bioavailability and retinal accumulation in the presence of lipid substances or fetal calf serum [38, 39]. In fact, each pure carotenoid compound has poor aqueous solubility, but the solubility is increased to some extent in the mixture state of the acetone extract. A higher activity was also detected in the transgenic carrot extract compared with the wild-type extract. This result indicates that the carotenoid content and the composition are correlated with the protective capacity of the lipophilic extract of carrot.

To the best of our knowledge, this is the first study that demonstrates the antioxidant and protective activities of the lipophilic extract of carrot. The results of this study suggests that the taproot extract can be used as a good sources of antioxidants and a protective agent for eye diseases in which free radicals are involved, such as POAG. Further investigations using an *in vivo* model and for the identification of its action mechanism should be performed.

CONCLUSIONS

The significant antioxidant activity of the lipophilic extracts obtained from the taproots of the carrot plant was demonstrated in DPPH, hydroxyl and superoxide radical scavenging tests. As determined through a chemopreventive activity test for degenerative diseases of the eye, pretreatment with the carrot extract attenuated the death of a retinal ganglion cell line (RGC-5 cells) by exposed to 1-buthionine-(*R*,*S*)-sulfoximine and L-glutamic acid. Higher activities were detected in the transgenic carrot extract compared with the wild-type extract. Because the acetone extract of the carrot plant includes several carotenoids with antioxidant activity, these biological activities are most likely due to the presence of these compounds, which contribute to the protective effect that was observed against oxidative stress on retinal cells.

CONFLICT OF INTEREST

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

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