RESEARCH ARTICLE

Annatto prevents retinal degeneration induced by endoplasmic reticulum stress in vitro and in vivo

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Scope: Annatto (*Bixa orellana*) seeds have been used as a colorant in butter and in a variety of other foods. In this study, we investigated the amelioration of retinal damage by an acetone extract of annatto (A-ext.), bixin (a main component of annatto), and four bixin derivatives (Bx-1, Bx-2, Bx-3, and Bx-4) that we have synthesized.

Methods and results: We used cultured retinal ganglion cells (RGC-5) to examine in vitro effects of A-ext. on stress pathways, focusing on intracellular oxidation induced by reactive oxygen species, expression of endoplasmic reticulum (ER) stress-related proteins, caspase-3 activation, and cell membrane damage. In vivo retinal damage in mice following intravitreous injection of tunicamycin was evaluated by counting the cell numbers in the ganglion cell layer (GCL) and measuring the thickness of outer nuclear layer (ONL). A-ext., bixin, and Bx-1 treatment inhibited both tunicamycin- and H_2O_2-induced cell death. Bixin derivatives also inhibited tunicamycin-induced cell death. Treatment with A-ext., bixin, and Bx-1 reduced tunicamycin-induced caspase-3 activity and inhibited the inversion of phosphatidylserine, an early apoptotic event without antioxidant effect or reduction of ER stress itself. A-ext., bixin, and Bx-1 significantly inhibited the tunicamycin-induced loss of cells from the GCL, and these materials also suppressed the tunicamycin-induced thinning of ONL.

Conclusion: A-ext., its main component bixin, and bixin derivatives may therefore be useful for preventive and therapeutic treatment of retinal-related diseases.

Keywords:
Annatto / Apoptosis / ER stress / Neuroprotection / Retina

1 Introduction

Retinal ganglion cell (RGC) death is a common feature of many ophthalmic disorders such as glaucoma. Its underlying mechanisms may involve oxidative stress [1], excitatory amino acid (glutamate) [2], or endoplasmic reticulum (ER) stress [3], which is caused by ocular hypertension, low blood flow or genetic factors. For example, recent reports have shown that ER stress is involved in a variety of experimental retinal neurodegenerative models, such as diabetic retinopathy [4] and retinitis pigmentosa [5], which is mainly damaged in photoreceptors.

The ER is a cellular organelle in which proteins (destined for secretion or for diverse subcellular localization) are not only synthesized, but where they acquire their correct folded conformation. When the ER environment is perturbed by several biochemical and physiological stimuli, the accumulation of unfolded proteins in the ER lumen is occurred, known as ER stress [6]. As a response to ER stress, quality control mechanisms such as induction of chaperones (immunoglobulin heavy-chain protein [BiP]), translational attenuation, and ER-associated degradation are mobilized, which is called unfolded protein response [7]. However, if excessive stress was caused in ER, the unfolded protein response ultimately

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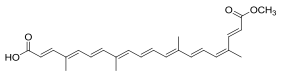
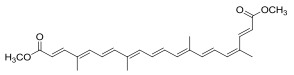
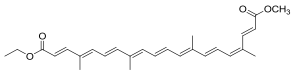
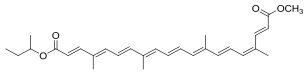
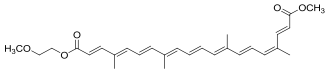
Abbreviations: A-ext, acetone extract of annatto; **BiP**, immunoglobulin heavy-chain binding protein; **CHOP**, C/EBP homologous protein; **GCL**, ganglion cell layer; **ONL**, outer nuclear layer; **PS**, phosphatidylserine; **RGC**, retinal ganglion cell; **ROS**, reactive oxygen species

*These authors contributed equally to this work.

Table 1. Chemical structures and formulas of bixin and its derivatives

Compounds	Chemical structures	Chemical formulas
Bixin		$C_{25}H_{30}O_4$
Bx-1		$C_{26}H_{32}O_4$
Bx-2		$C_{27}H_{34}O_4$
Bx-3		$C_{29}H_{38}O_4$
Bx-4		$C_{28}H_{36}O_5$

activates an apoptotic pathway [8] that involves a C/EBP homologous protein (CHOP) [9].

Carotenoids including apocarotenoids have many physiological functions in vertebrates, such as free radical scavenging and enhancement of the immune system. For this reason, many carotenoids such as annatto (*Bixa orellana*) constituents, bixin and norbixin, are widely used as food additives, drugs, and cosmetics. Annatto has various stress suppressing effects, such as serving as an effective singlet molecular-oxygen quencher and as a protective agent against radiation- and cisplatin-induced clastogenicity in rodents [10]. However, the effects of annatto against stress-induced retinal degeneration have not yet been reported. Saffron, which also contains an apocarotenoid of crocetin, has been extensively used in traditional medicine [11], and an antiapoptotic characterization of crocetin in the retina has been reported [12]. We have previously showed the protective effect of crocetin against light-induced retinal damage [13].

In the present study, we conducted in vitro and in vivo investigations of the protective effects of an acetone extract of annatto (A-ext.), bixin, and four bixin derivatives (Table 1) on retinal damage. Our retinal damage models included in vitro tunicamycin- and H_2O_2-induced neurotoxicity in RGC cultures and in vivo tunicamycin-induced retinal damage in mice.

2 Materials and methods

2.1 Materials

The RGC line [14] was a kind gift from Dr. Neeraj Agarwal (UNT Health Science Center, Fort Worth, TX). Drugs and sources were as follows: H_2O_2 (Wako, Osaka, Japan), tunicamycin (Wako), Hoechst 33342 (Invitrogen, Carlsbad, CA), propidium iodide (PI; Invitrogen), and Annexin-V-FLUOS Staining Kit (Roche Applied Science, Penzberg, Germany). Seeds of *Bixa orellana* were purchased from Jairamdass Khushiram (Navi Mumbai, Maharashtra, India). A voucher specimen was deposited in the Gifu Pharmaceutical University.

2.2 Extraction and isolation of annatto

The dried and ground *Bixa orellana* seeds (1 kg) were extracted with acetone (2 L) three times at room temperature. The acetone solution was evaporated in vacuo to give a dark red extract (102 g). The crude extract (60 g) was dissolved in acetone, and left to stand overnight at room temperature, which generated red precipitation of bixin (21 g). The chemical structures and formulas of bixin and its derivatives are shown in Table 1.

2.3 Synthesis of bixin derivatives

A solution of bixin (300 mg) in methanol (150 mL) containing concentrated sulfuric acid (2 mL) was refluxed at 80°C for 9 h. The reaction mixture was added to H_2O, and extracted with ethyl acetate. The ethyl acetate layer was purified by column chromatography on silica gel 60 F_{254} (Merck, Whitehouse Station, NJ) to give Bx-1 (35.2 mg). A solution of bixin (300 mg) in ethanol (30 mL) containing concentrated sulfuric acid was refluxed at 90°C for 6 h. The reaction mixture was purified in a similar manner as described for Bx-1 to give Bx-2 (27.5 mg). A similar protocol using 2-butanol or ethylene glycol monomethyl ether in place of ethanol generated Bx-3 and Bx-4, respectively. The yields were 4.3 mg (Bx-3) and 17.6 mg (Bx-4) from bixin (300 mg).

2.4 Retinal ganglion cell culture

RGC cultures were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin (Meiji Seika Kaisha Ltd., Tokyo, Japan), and 100 μg/mL streptomycin (Meiji Seika) under a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The cells were passaged by trypsinization every 3 to 4 days, as described in our previous report [15].

2.5 Tunicamycin or H_2O_2-induced cell death assay

RGC cultures were seeded at 1×10^3 cells per well in 96-well plates and then incubated for 24 h. The entire medium was then replaced with fresh medium containing 1% FBS, and a carotenoid [A-ext. (0.1–3 μg/mL), bixin (1–10 μM) or its derivatives (0.1–10 μM)]. After a 1 h pretreatment, tunicamycin (at a final concentration of 2 μg/mL) or H_2O_2 (0.3 mM) was added. A-ext., bixin, and its derivatives were dissolved in dimethyl sulfoxide (DMSO) and diluted with

phosphate buffered saline (PBS) containing 1% DMSO (final concentration 0.1%).

Nuclear staining assays were carried out after an additional 24 h of incubation. At the end of the culture period, Hoechst 33342 ($\lambda_{ex} = 360$ nm, $\lambda_{em} > 490$ nm) and PI ($\lambda_{ex} = 535$ nm, $\lambda_{em} > 617$ nm) were added to the culture medium for 15 min at final concentrations of 8.1 μM and 1.5 μM, respectively. Hoechst 33342 freely enters living cells and then stains the nuclei of viable cells, as well as those that have suffered apoptosis or necrosis. PI is a membrane-impermeable dye that is generally excluded from viable cells. Images were collected using an Olympus IX70 inverted epifluorescence microscope (Olympus, Tokyo, Japan). The total number of cells was counted in a blind manner (by M.Y.) and calculated the percent of PI positive cells.

2.6 Radical scavenging-capacity assay

RGC cultures were seeded at 2×10^3 cells per well in 96-well plates, and then incubated. After incubation for 1 day, cells were washed with 1% FBS DMEM. Radical species (H_2O_2 and $O_2\cdot^-$) oxidize nonfluorescent dichlorofluorescein (DCFH) to fluorescent dichlorofluorescein (DCF). The cells were loaded with radical probe 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) (10 μM) by incubation for 20 min at 37°C. The cell-culture medium was then replaced to remove excess probe. To generate radical species, we added H_2O_2 at 1 mM (H_2O_2 radical) or KO_2 at 1 mM ($O_2\cdot^-$) to the radical probe loading-medium. Following exposure of the cells to reactive oxygen species (ROS)-generating compounds for various time intervals, fluorescence was measured using excitation/emission wavelength of 485/535 nm (SkanIt RE for Varioskan Flash 2.4; Thermo Fisher Scientific, Waltham, MA), as previously reported [16].

2.7 Western blot analysis

RGC cultures were washed with PBS, harvested, and lysed in RIPA buffer (Sigma-Aldrich) supplemented with protease inhibitor cocktail (Sigma-Aldrich) and phosphate inhibitor cocktails 1 and 2 (Sigma-Aldrich). Lysates were centrifuged at 12 000×g for 15 min at 4°C. Supernatants were collected and boiled for 5 min in SDS sample buffer (Wako). Equal amounts of protein were subjected to 10% SDS-PAGE gradient gels and then transferred to poly vinylidene difluoride membranes. After being blocked with Block Ace (Snow Brand Milk Products Co. Ltd., Tokyo, Japan) for 30 min, the membranes were incubated with the primary antibody (mouse anti-BiP/GRP78 antibody (BD Transduction Laboratories, Lexington, KY), mouse anti-GADD 153 (B-3) (Santa Cruz Biotechnology, CA), and mouse anti-β-actin antibody (Sigma-Aldrich)). Subsequently, the membrane was incubated with the secondary antibody (HRP-conjugated goat anti-mouse IgG or rabbit anti-goat IgG, (H+L), peroxidase conjugated (Thermo Fisher Scientific). The immunoreactive bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and the LAS-4000 luminescent image analyzer (Fuji Film Co., Ltd., Tokyo, Japan).

2.8 Caspase-3 assay

Caspase-3 activity was measured using the CaspACE™ Assay System, Fluorometric (Promega, Madison, WI). RGC cultures were seeded at 2×10^6 cells per 10 cm dishes and then incubated for 24 h at 37°C. Cells were treated with tunicamycin in the presence or absence of 3 μg/mL of A-ext. or 10 μ M of bixin or 10 μM of Bx-1 for 24 h. The cells were then collected by trypsinization and suspended in cell lysis buffer. Cell lysates were mixed in CaspACE™ Assay substrates and incubated for 1 h at 37°C. The fluorescence was measured using SkanIt RE for Varioskan Flash 2.4 with excitation/emission wavelengths of 360/460 nm.

2.9 Annexin V staining

RGC cultures were seeded at 1×10^3 cells per well in 96-well plates and incubated for 24 h. The entire medium was then replaced with fresh medium containing 1% FBS, and cells were pretreated with A-ext. (3 μg/mL), bixin (10 μM) and Bx-1 (10 μM) for 1 h. Tunicamycin was then added (at a final concentration of 2 μg/mL). After 24 h incubation, Hoechst 33342, PI and Annexin V ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 518$ nm) were added to the culture medium for 15 min. The number of cells undergoing early stages of apoptosis (Annexin V positive and PI negative) was counted in a blinded manner and expressed as a percentage of the total cell count.

2.10 Animals

Male adult ddY mice (Japan SLC, Hamamatsu, Japan) were kept under 12 h light/12 h dark conditions and had ad libitum access to food and water. All experimental procedures were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University (permission number: 2010-108, -176 and -252).

2.11 Tunicamycin-induced retinal damage

Tunicamycin induced retinal damage was produced as previously reported [17, 18]. Briefly, mice were anesthetized with 3.0% isoflurane and maintained with 1.5% isoflurane in 70% N_2O and 30% O_2 via an animal general anesthesia machine (Soft Lander; Sin-ei Industry Co. Ltd., Saitama, Japan). The body temperature was maintained between 37.0 and 37.5°C with the aid of a heating pad. Retinal damage was induced by the injection (2 μl/eye) of at tunicamycin at 1 μg/mL

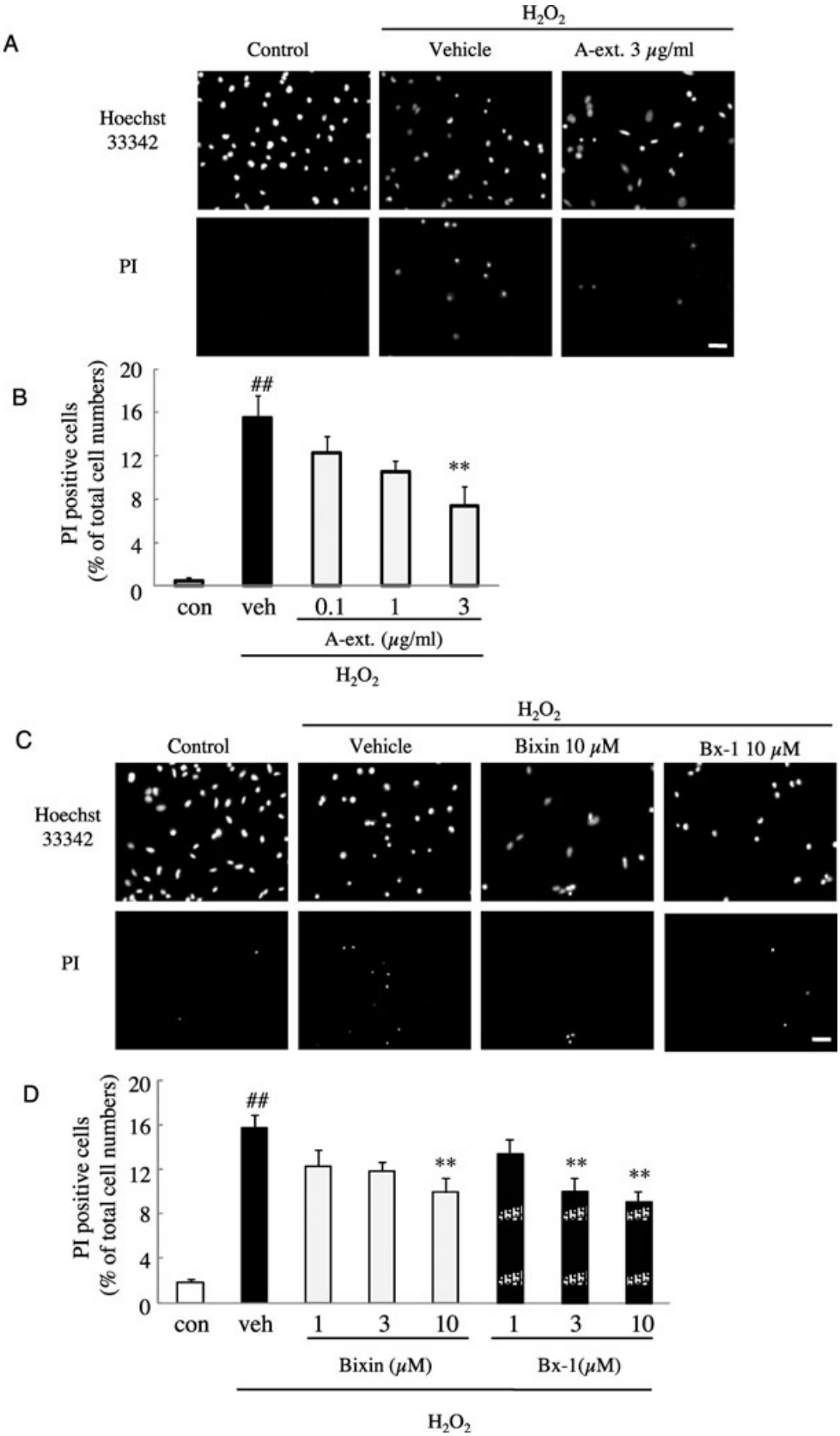


Figure 1. Effects of A-ext., bixin, and Bx-1 on cell damage induced by H_2O_2 in RGC culture. (A, C) Representative fluorescence microscopy of Hoechst 33342 and PI staining (added at 27 h after H_2O_2). (B, D) Apoptotic cell death was quantitatively evaluated by the number of PI positive cells at 27 h after H_2O_2 treatment. Data are shown as mean ± SEM (n = 6 or 8). $^{*}p < 0.05$, $^{**}p < 0.01$ versus H_2O_2 plus the vehicle-treated group (vehicle), $^{\#\#}p < 0.01$ versus Control. The scale bar represents 50 μM.

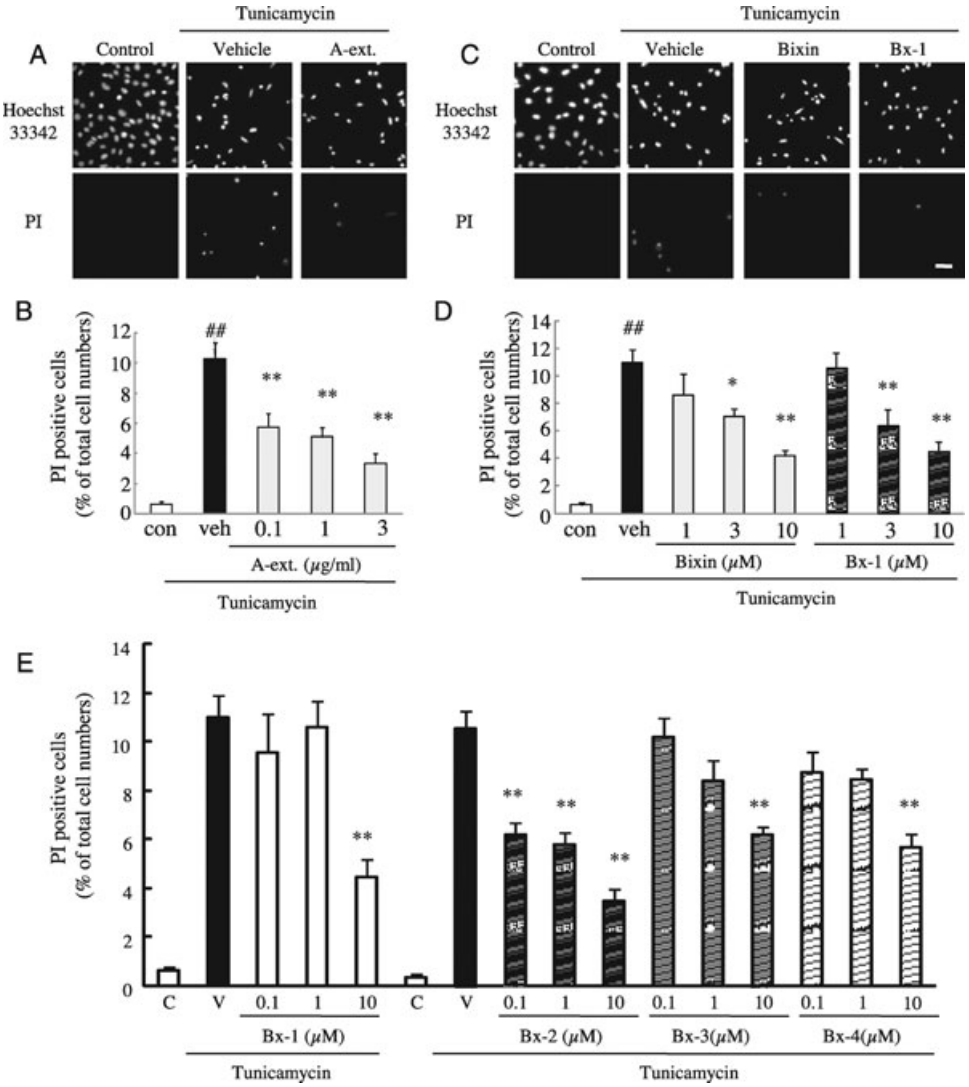


Figure 2. Effects of A-ext., bixin, and Bx-1 on cell damage induced by tunicamycin in RGC culture. (A, C) Representative fluorescence microscopy of Hoechst 33342 and PI staining (added at 24 h after tunicamycin). (B, D, E) Apoptotic cell death quantitatively evaluated by the number of PI positive cells at 24 h after tunicamycin treatment. Data are shown as mean ± SEM ($n = 6$ or 8). $^{*}p < 0.05$, $^{**}p < 0.01$ versus tunicamycin plus the vehicle-treated group (vehicle), $^{\#\#}p < 0.01$ versus Control. The scale bar represents 50 μM. con: control, veh: vehicle.

dissolved in 0.01 M PBS with 5% DMSO. Solutions were injected into the vitreous body of the left eye. A-ext. (1.0 μg) or bixin (1.0 nmol) or Bx-1 (1.0 nmol) or vehicle (5% DMSO in PBS) was coadministered with the tunicamycin. One drop of 0.01% levofloxacin ophthalmic solution (Santen Pharmaceuticals Co. Ltd., Osaka, Japan) was applied topically to the treated eye immediately after the intravitreal injection. Seven days after the injection, eyeballs were enucleated for histological analysis. For comparative purposes, nontreated retinas from each mouse strain were also investigated.

2.12 Histological analysis

Mice under anesthesia produced by an intraperitoneal injection of sodium pentobarbital (80 mg/kg, Nembutal®, Dainihon Sumitomo, Co., Ltd., Tokyo, Japan), each eye was enucleated and kept immersed for at least 24 h at 4°C in a fixative solution containing 4% paraformaldehyde. Six paraffin-embedded sections (thickness, 5 μM), cut through the optic disc of each eye, were prepared in the standard manner, and stained with hematoxylin and eosin. The damage induced by tunicamycin was then evaluated, with three sections from each eye used for the morphometric analysis, as described below. Light-microscope images were photographed, and the cells in the ganglion cell layer (GCL), at a distance between 375 and 625 μM from the optic disc, were counted on the photographs in a masked fashion by a single observer (H.S.). The thickness of the outer nuclear layer (ONL) was also measured. Data from three sections (selected randomly from the six sections) were averaged for each eye and used to evaluate the cell count in the GCL and the thickness of ONL.

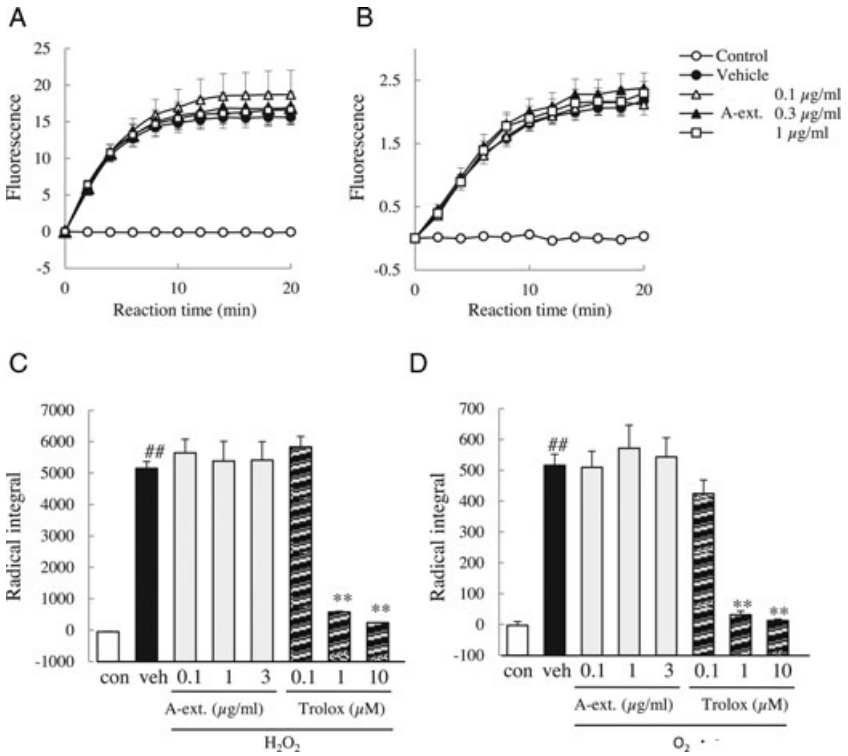


Figure 3. Kinetic and concentration response data for antioxidant activities of A-ext. against various ROS (H_2O_2, $O_2{\cdot}^-$) measured by fluorescence intensity. (A) Kinetics of DCFH oxidation by H_2O_2 in RGC cultures. (B) Kinetics of DCFH oxidation by $O_2{\cdot}^-$ in RGC cultures. (C, D) Integral of ROS-mediated oxidation from kinetics curves. The radical integral was calculated from A and B. Radical species were H_2O_2 (C), $O_2{\cdot}^-$ (D). Data are shown as mean ± SEM ($n = 8$). $^{\#\#}p < 0.01$ versus Control. con: control, veh: vehicle.

2.13 Statistical analysis

Data are presented as the means ± SEM Statistical comparisons were made using Student's *t*-test and one-way ANOVA followed by Dunnett's test [using STAT VIEW version 5.0 (SAS Institute, Cary, NC)]. $p < 0.05$ was considered to indicate statistical significance.

3 Results

3.1 A-ext., bixin, and Bx-1 suppressed H_2O_2-induced cell death in RGC cultures

Representative photographs of Hoechst 33342 and PI staining are shown in Fig. 1A and C. Hoechst 33342 stains all cells (live and dead cells), whereas PI stains only dead cells. Pretreatment with A-ext. at a concentration of 3 μg/mL protected against H_2O_2-induced cell death (Fig. 1B). Bixin at a concentration of 10 μM protected against H_2O_2-induced cell death, and Bx-1 at 3 and 10 μM protected against H_2O_2-induced cell death (Fig. 1D).

3.2 A-ext., bixin, and bixin derivatives suppressed tunicamycin-induced cell death in RGC cultures

Representative photographs of Hoechst 33342 and PI staining are shown in Fig. 2A and C. A-ext. at concentrations of 0.1–3 μg/mL protected against tunicamycin-induced cell death in a concentration-dependent manner (Fig. 2B). Bixin at concentrations of 3 and 10 μM and Bx-1 at 3 and 10 μM protected against tunicamycin-induced cell death in a concentration-dependent manner (Fig. 2D). Bx-2 at concentrations of 0.1–10 μM and Bx-3 and Bx-4 at a concentration of 10 μM also protected against tunicamycin-induced cell death (Fig. 2E).

3.3 Effects of A-ext. on the intracellular oxidation of DCFH induced by H_2O_2 or $O_2{\cdot}^-$

To investigate the effect of A-ext. on H_2O_2 or $O_2{\cdot}^-$ production, we employed a radical scavenging assays using reactive oxygen species (ROS)-sensitive probes, CM-H_2DCFDA. The time-kinetics of ROS reactivity (monitored as fluorescence generation) are shown in figure 3A (H_2O_2) and B ($O_2{\cdot}^-$). H_2O_2 was added at a concentration of 1 mM. Trolox addition at concentrations of 1–100 μM significantly scavenged H_2O_2 radicals, but A-ext. at 3 μg/mL did not show any scavenging effect (Fig. 3C). When $O_2{\cdot}^-$ radicals were generated by treatment with KO_2 at 1 mM, trolox at concentrations of 1 to 10 μM again significantly scavenged the $O_2{\cdot}^-$ radicals, but A-ext. did not show any scavenging effect (Fig. 3D).

3.4 Effects of A-ext., bixin, and Bx-1 on the expression of ER stress-related proteins

The ER stress pathway was investigated by examining expression of the ER-stress associated proteins, BiP and CHOP, by

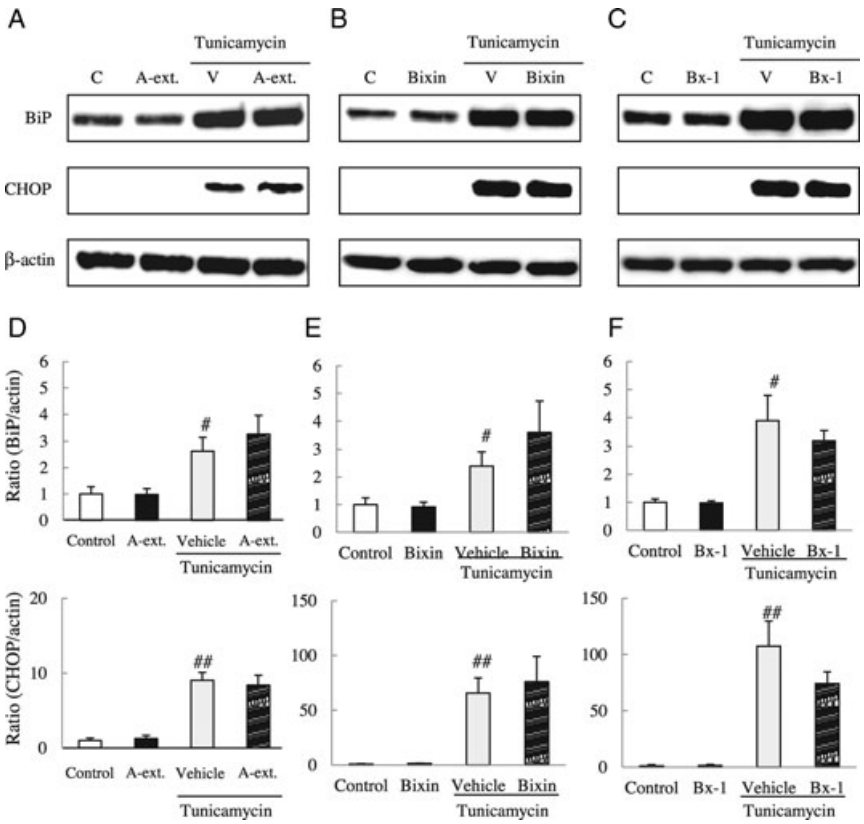


Figure 4. Western blots showing effects of A-ext., bixin, and Bx-1 on expressions of BiP and CHOP in RGC cultures. (A, B, C) Representative band images show immunoreactivities against BiP and CHOP. (D, E, F) Quantitative analysis of band densities. Data are shown as mean ± SEM ($n = 5$ or 6). $^{\#}p <$ 0.05 versus Control. C: control, V: vehicle.

Western blot analysis (Fig. 4). Tunicamycin treatment significantly up-regulated BiP and CHOP expressions, but A-ext. or bixin treatment had no effect on this up-regulation.

3.5 A-ext., bixin, and Bx-1 suppressed tunicamycin-induced caspase-3 activation

Tunicamycin significantly increased caspase-3 activation. A-ext. at 3 μg/mL, bixin at 10 μM, and Bx-1 at 10 μM significantly attenuated the activity of caspase-3 (Fig. 5).

3.6 A-ext., bixin, and Bx-1 reduced the tunicamycin-induced inversion of phosphatidylserine

Cell membrane alteration was evaluated with the Annexin-V-FLUOS Staining Kit according to the manufacturer's instructions. In the early stages of apoptosis, changes occur on the cell surface [19]. Necrotic cells were distinguished from early apoptotic cells using PI. Tunicamycin significantly increased annexin V positive and PI negative cells, and A-ext. at 3 μg/mL, bixin at 10 μM, and Bx-1 at 10 μM significantly inhibited the annexin V positive and PI negative cells (Fig. 6).

3.7 A-ext., bixin, and Bx-1 protected against tunicamycin-induced retinal damage

Representative photographs of retinal images were taken at 7 days after the intravitreal injection of tunicamycin (Fig. 7A). The cell number in the GCL was markedly decreased in the vehicle-treated group (Fig. 7Ab) versus the normal group (Fig. 7Aa). The cell number per 1 mm was counted; the data were averaged and are reported in Fig. 7B. A-ext. at 1.0 μg/eye suppressed the cell number reduction in the GCL (Fig. 7A-c and 7B). Bixin at 1.0 nmol and Bx-1 at 1.0 nmol treated group also suppressed the cell number reduction seen in the GCL of the vehicle treated group (Fig. 7B). The thickness of ONL was reduced by tunicamycin treating, and A-ext. and bixin significantly suppressed the thinning of ONL (Fig. 7C). The tendency of protective effect was also observed in Bx-1 treated group.

4 Discussion

In the present study, we evaluated the effects of A-ext. and its constituents, bixin and bixin derivatives (Bx-1, Bx-2, Bx-3, and Bx-4), against tunicamycin- and H_2O_2-induced retinal

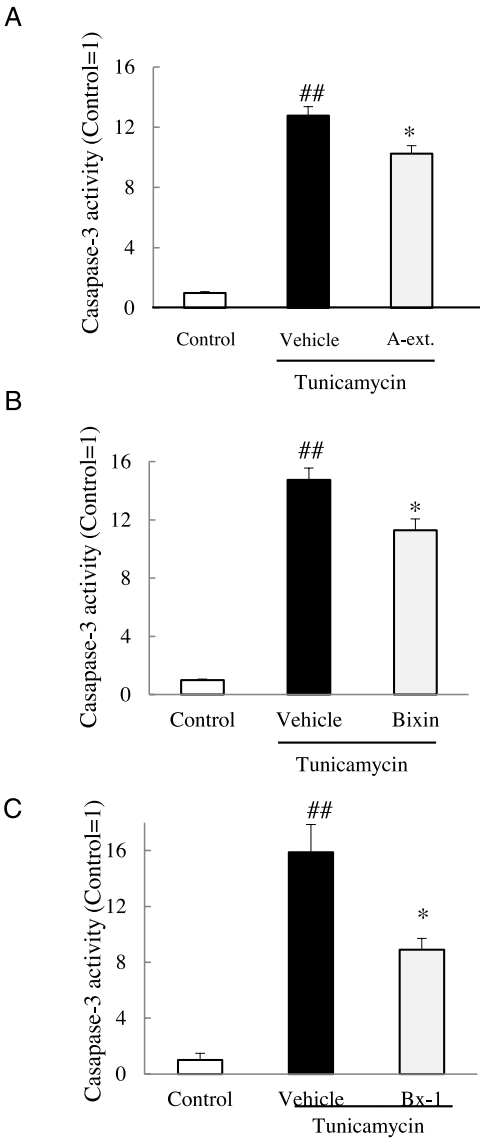


Figure 5. Effects of A-ext., bixin, and Bx-1 on tunicamycin-induced caspase-3 activity in RGC cultures. Caspase-3 activity was evaluated by the CaspACE™ Assay System Fluorometric after a 24 h pretreatment with tunicamycin. Cells were treated with 3 μg/mL A-ext. (A), 10 μM of bixin (B), or 10 μM Bx-1 (C). Data are shown as mean ± SEM ($n = 3$–6). $^{*}p < 0.05$, versus tunicamycin plus the vehicle-treated group (vehicle), $^{\#\#}p < 0.01$ versus Control.

degeneration, and clarified their protective mechanism against tunicamycin-induced cell death.

In vitro, A-ext., bixin, and Bx-1 inhibited retinal cell death induced by tunicamycin and H_2O_2 in a concentration-dependent manner (Figs. 1 and 2). A protective effect was observed for bixin derivatives (Bx-1, Bx-2, Bx-3, and Bx-4) (Fig. 2E), with Bx-2 showing the most effect against tunicamycin-induced cell death. Jianfei et al., who examined 6-OHDA induced SH-SY5Y neurotoxicity, reported that a methylated resveratrol derivative exerted a stronger protective effect at ten-fold lower concentration than the effective concentration for resveratrol, whereas trimethylated resveratrol did not show a protective effect [20]. These data imply that while methylation is desirable to improve cellular uptake of the purported neuroprotective agents, it is important that a balance be maintained between hydrophobicity and neuroprotective effects for good neuroprotective activity in multicellular systems. Bixin has been reported as a ligand for peroxisome proliferator-activated receptor γ (PPARγ) [21]. We investigated the involvement with PPARγ on protective effects of bixin using a PPARγ antagonist, T0070907. Although T0070907 did not attenuate the protective activity of bixin or derivatives against the tunicamycin-induced cell death (data not shown), the strong cell protective effect of Bx-2 may be not only due to improving the cellular uptake but also changing affinities to such receptors. Bixin has been detected in human plasma, and has also been demonstrated to be absorbed into the blood stream after ingestion of a single dose of annatto food color [22]. Our present in vitro study confirmed that methylation of bixin to Bx-1 results in a maximal protective effect at 10 μM. Although further study is necessary, these data indicated that methylation may accelerate absorption and tissue uptake of bixin, thereby providing a maximal effect. In the present study, A-ext. inhibited ER stress-induced cell death more effectively than it inhibited oxidative stress-induced cell death (Figs. 1 and 2). These results may relate to differences in the death signal pathways in RGC cultures [17] .

Annatto has been reported to have antioxidant activity [23]; however, A-ext. had no effect on ROS production under our experimental conditions (Fig. 3). This result suggests that the protective effects of A-ext. against tunicamycin- and H_2O_2-induced cell death were independent of its antioxidant activity in our experiments.

Since A-ext., bixin, and bixin derivatives protected against an ER stressor, tunicamycin, we thought that they might alter the expressions of ER stress-related proteins (BiP and CHOP). However, no alterations in tunicamycin-induced expression of either BiP or CHOP were observed following A-ext., bixin, or Bx-1 treatment (Fig. 4). These data suggest that the defensive effects of A-ext., bixin, and Bx-1 against ER stress may be independent of alterations in the levels of protein expression induced by ER stress; i.e. these effects may not involve the unfolded proteins.

Next, we focused on caspase-3, which is activated following ER stress or oxidative stress [17, 24, 25]. A-ext. at 10 μg/mL, bixin at 3 μM, and Bx-1 at 3 μM inhibited caspase-3 activation after tunicamycin treatment (Fig. 5). Previously, we have reported that crocetin, a carotenoid, has an inhibitory effect on caspases [13]. Further studies will be needed to determine how A-ext. inhibits caspase-3 activity.

Cell membrane alteration occurs during early apoptosis. A-ext., bixin, and Bx-1 significantly reduced the number of apoptotic cells induced by tunicamycin (Fig. 6). Suzuki et al. have reported that the inversion of phosphatidylserine is

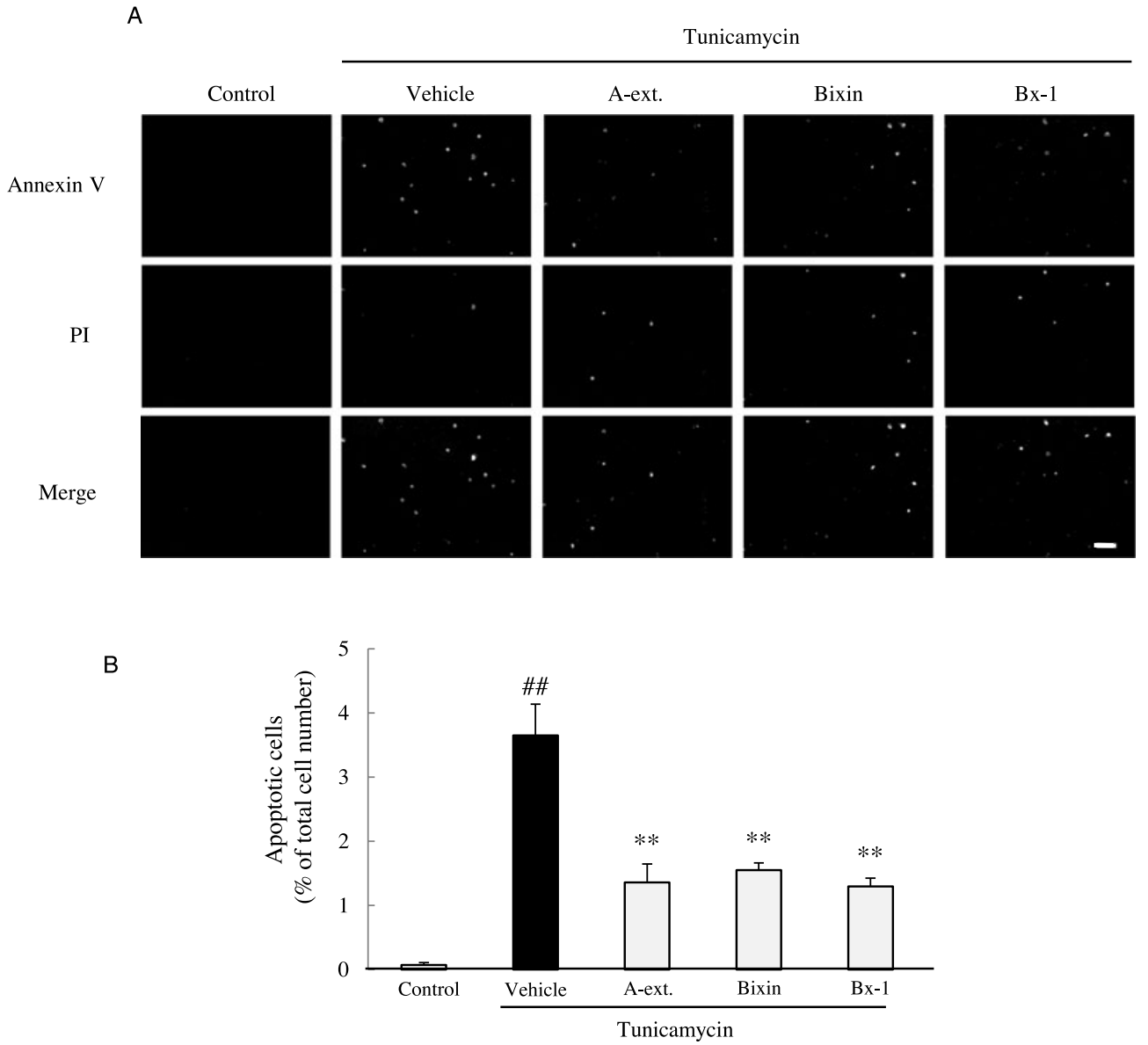
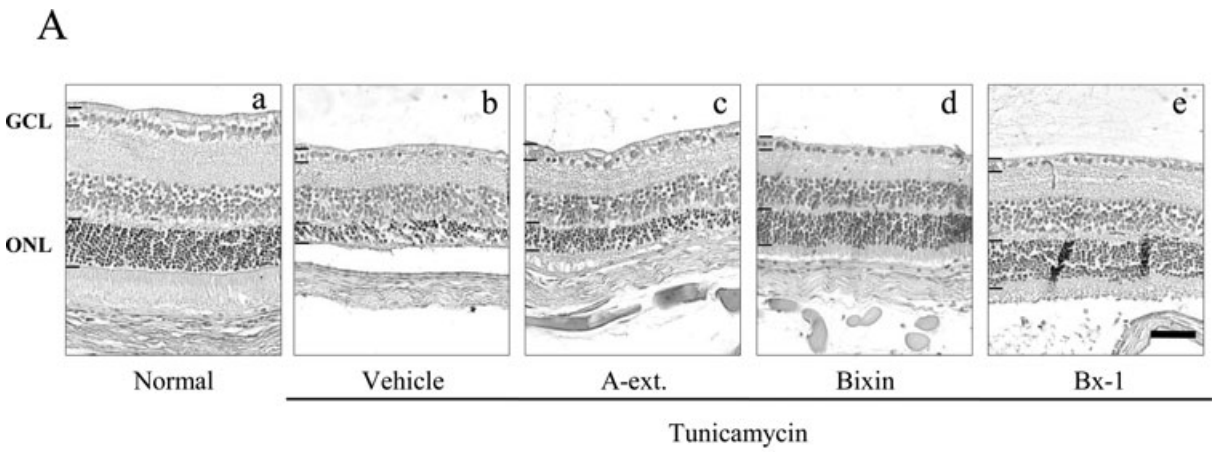
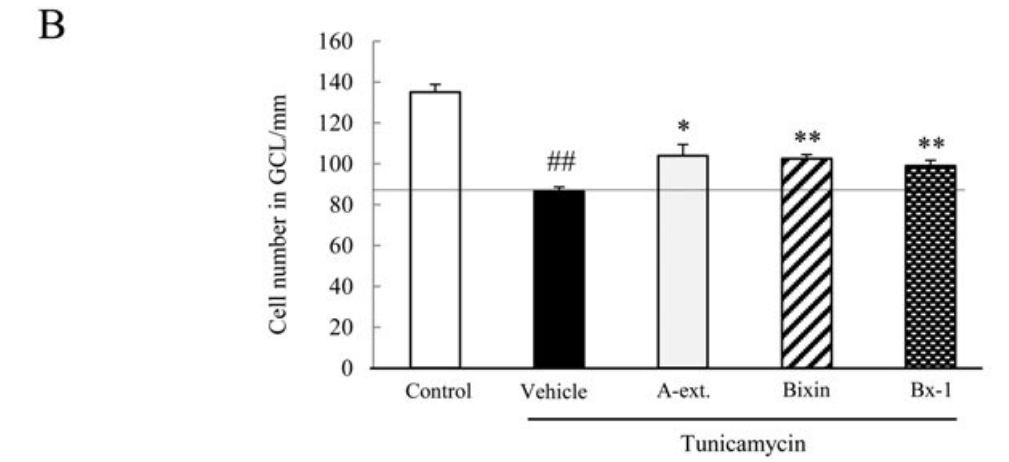


Figure 6. Effects of A-ext., bixin, and Bx-1 against tunicamycin-induced cell membrane alteration in RGC cultures. (A) Representative fluorescence microscopy images of Annexin V (green) and PI (red) staining (added at 24 h after tunicamycin). (B) Early stage of apoptotic cell death quantitatively evaluated by the number of Annexin V positive and PI negative cells at 24 h after tunicamycin treatment. Data are shown as mean ± SEM ($n = 6$). $^{**}p < 0.01$ versus tunicamycin plus the vehicle-treated group (vehicle), $^{\#\#}p < 0.01$ versus Control. The scale bar represents 50 μM.

dependent on intracellular Ca^{2+} [26]. A-ext. may therefore be involved in calcium homeostasis and may reduce apoptosis at an early stage. On the other hand, Ochiai et al. have reported that crocin, another carotenoid, inhibited cell membrane damage, and crocin showed protective effects against peroxidation of cell membrane lipids and inhibited caspase-8 activation during serum/glucose deprivation [27]. Therefore, annatto may also inhibit cell membrane alteration by a similar mechanism.

We have previously reported that increased expressions of XBP-1 splicing, BiP, and CHOP could be detected in mouse retinas after the induction of retinal damage by tunicamycin. Moreover, ER stress was detected in mouse *N*-methyl-D-aspartate injection model and high intraocular pressure model [18]. Therefore, ER stress could quite conceivably have caused the retinal damage observed following tunicamycin intravitreal injection. Our histological analysis indicated that A-ext., bixin, and Bx-1 reduced tunicamycin





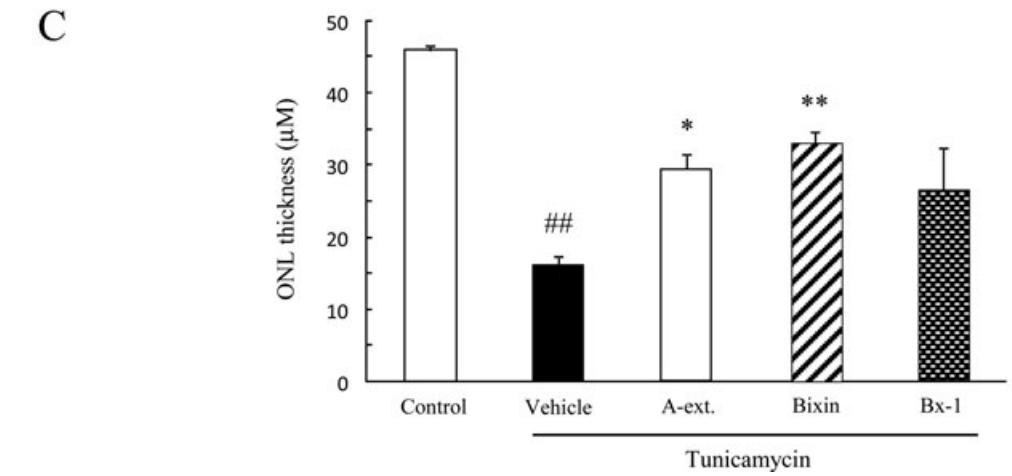


Figure 7. Effects of A-ext., bixin, and Bx-1 on *in vivo* tunicamycin-induced retinal damage in mice. (A) Representative photographs (hematoxylin and eosin staining) showing retinal sections from eyes (a) non-treated, (b) intravitreally injected with tunicamycin (1.0 μg/eye) plus vehicle, (c) intravitreally injected with tunicamycin plus A-ext. at 1.0 μg/eye, (d) intravitreally injected with tunicamycin plus bixin at 1.0 μM, and (e) intravitreally injected with tunicamycin plus Bx-1 at 1.0 μM. Retinal damage was evaluated by counting cell numbers in the GCL (B) and by measuring the thickness of ONL (C) at 7 days after intravitreous injection. The cell numbers in the GCL at a width of 1 mm or the thickness of ONL was measured at a distance between 375 and 625 μM from the optic disc. Data are shown as mean ± SEM (B, $n = 8$–18; C, $n = 5$). $^{*}p < 0.05$, $^{**}p < 0.01$ versus tunicamycin plus the vehicle-treated group (vehicle), $^{\#\#}p < 0.01$ versus Normal. The scale bar represents 50 μM.

induced retinal damage in the GCL and ONL (Fig. 7). Light irradiation induces ER stress in photoreceptor cells in vitro and in ONL, which may be associated with photoreceptor cell death (data not shown). Thus, A-ext. and bixin may reduce the photoreceptor degeneration, which is related to retinitis pigmentosa and age-related macular degeneration. Our in vitro studies showed that A-ext. at 3 μg/mL, bixin at 10 μM, and Bx-1 at 10 μM had strong protective effects against tunicamycin-induced damage in RGC cultures (Fig. 2). If a typical vitreous volume for mice is considered to be approximately 10 μL, then the effective A-ext. concentration was approximately 100 μg/mL, and bixin and Bx-1 concentrations were approximately 100 μM in the vitreous body after injection. These concentrations might be not far from our in vitro experimental concentrations.

Takanishi et al. have reported that bixin induces mRNA expression of PPAR target genes such as adipocyte-specific fatty acid-binding protein (aP2) and lipoprotein lipase (LPL) [28]. On the other hand, Zhang et al. have reported that carotenoids increase in the expression of connexin43 mRNA and protein, a gene that encodes a major gap junction protein [29]. Up-regulation of Connexin43 shows chemopreventive action. These reports indicate that A-ext. induces expression of various factors that can exert protective effects against cell damage.

In conclusion, our findings indicate that A-ext., bixin, and bixin derivatives exert protective effects against tunicamycin-induced retinal damage by inhibiting an early apoptotic event and following caspase-3 activation. Annatto may be a good candidate as a possible prophylactic agent for degenerative diseases of the retina.

The authors have declared no conflict of interest.

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