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ORIGINAL ARTICLE

# Edible Seaweed, *Eisenia bicyclis*, Protects Retinal Ganglion Cells Death Caused by Oxidative Stress

**Kyung-A Kim · Sang Min Kim · Suk Woo Kang · Sang Il Jeon · Byung Hun Um · Sang Hoon Jung**

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**Abstract** The purpose of the present study was to determine whether edible seaweed, *Eisenia bicyclis*, is effective in blunting the negative influence of *N*-methyl-D-aspartate (NMDA) on rat retinas and of oxidative stress-induced transformed retinal ganglion cell (RGC-5 cell line) death. The ethanol extract of *E. bicyclis* (EEEB) significantly attenuated the negative insult of L-buthionine-(S,R)-sulfoximine plus glutamate on RGC-5 cells. Treatment of the RGC-5 cells with EEEB reduced the reactive oxygen species and recovered the reduced glutathione level caused by various radical species such as $H_2O_2$, OH·, or $O_2^{\cdot -}$. Moreover, EEEB inhibited lipid peroxidation on rat brain homogenates caused by sodium nitroprusside. Applying NMDA to the retina affected the thickness of the inner plexiform layer (IPL) and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) produced a positive effect on ganglion cells. Importantly, EEEB protected the thinning of IPL and increased TUNEL positive cells in the ganglion cell layer (GCL). Five phlorotannin derivatives were isolated using chromatographic methods and liquid chromatography-mass spectroscopy analysis which has been known as an antioxidant. In conclusion, EEEB has a neuroprotective effect in vitro and in vivo. Furthermore, the major constituents of this extract, phlorotannins, could possibly be active compounds due to their antioxidative potency.

**Keywords** *Eisenia bicyclis* · Neuroprotection · NMDA · RGC-5 · ROS

**Abbreviations**

| | |
|---|---|
| BSO | L-buthionine-(S,R)-sulfoximine |
| DCFH-DA | 2′,7′-dichlorodihydrofluorescein diacetate |
| EEEB | ethanol extract of *Eisenia bicyclis* |
| GCL | ganglion cell layer |
| GSH | glutathione |
| $H_2O_2$ | hydrogen peroxide |
| IPL | inner plexiform layer |
| MDA | malonedialdehyde |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NMDA | *N*-methyl-D-aspartate |
| PI | propidium iodide |
| RGCs | retinal ganglion cells |
| ROS | reactive oxygen species |
| SNP | sodium nitroprusside |
| TUNEL | terminal deoxynucleotidyl transferase dUTP nick-end labeling |

## Introduction

Glaucoma is a chronic neurodegenerative disease characterized by the progressive degeneration of retinal ganglion cells (RGCs) and visual field damage. There are several hypotheses regarding the mechanisms of glaucoma, such as mechanical compression and vascular ischemia; however,

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pathophysiological mechanisms remain unclear (Fechtner and Weinreb 1994).

Elevated intraocular pressure, caused by impaired outflow of aqueous humor, is an important risk factor for glaucoma, which can damage RGCs axons at the optic nerve head and alter the appropriate nutritional requirements for ganglion cell survival (Varma et al. 2008).

However, in cases of normal tension, glaucoma in a significant number of patients has shown optic nerve damage and vision loss, and all ocular hypertensive patients do not develop glaucoma. Therefore, it would seem desirable that neuroprotection, in this case preventing the death of neurons (RGCs and their axon), would be an adjunctive therapeutic paradigm for use with intraocular pressure-lowering agents (Weinreb and Levin 1999).

Neuroprotection refers to the use of any therapeutic modality that prevents, retards, or reverses neuronal cell death resulting from primary neuronal lesions (Barkana and Belkin 2004). Multiple targets for neuroprotection are suggested, including: preventing apoptosis, preventing tumor necrosis factor activation, stabilizing $Ca^{2+}$ homeostasis, blocking glutamate excitotoxicity, inhibiting nitric oxide production, modulating expression of heat shock proteins, supplying neurotrophins, and improving blood flow to the optic nerve, etc. (Marcic et al. 2003).

In particular, various hypotheses have been proposed with regard to reasons for RGC death in glaucoma. These hypotheses include ischemic insults to the ganglion cell axons (Osborne et al. 2001), neutrophin deprivation (Meyer-Franke et al. 1995), malfunction in non-neuronal components in the optic nerve head (Quigley 1999), mechanical damage to ganglion cell axons (Morgan 2000), and retinal glial cell dysfunctions (Zhong et al. 2007).

Substantial evidence shows that oxidative stress plays a major role in the pathogenesis of glaucoma or glaucomatous optic neuropathy (Tezel 2006). For example, an increase in the reactive oxygen species (ROS) level in the aqueous humor alters trabecular cell integrity, and a fairly significant correlation between oxidative DNA damage in the trabecular meshwork and intraocular pressure increase and visual field defects in glaucomatous patients has been demonstrated (Izzotti et al. 2006). In the epidemiological study, patients with glaucoma exhibited lower levels of circulating glutathione and a more elevated plasma malondialdehyde level than the controls (Gherghel et al. 2005; Yildirim et al. 2005). For these reasons, the ROS inhibitory materials, antioxidants, could be beneficial for therapeutic prevention of glaucoma.

*Eisenia bicyclis* (Kjellman) Setchell, which is a common perennial brown alga of the family of Laminariaceae, inhabits the middle Pacific coast around Korea, Japan, and Russia. It has been consumed as the raw material for sodium alginate. Since *E. bicyclis* has been taken by oral route as food, it might be a useful source for the treatment of diseases, without side effects, when taken regularly.

A variety of bioactive compounds such as phlorotannins (Jung et al. 2010) and phloroglucinol derivatives (Okada et al. 2004) have been reported from this algae, and the experimental studies suggest that the extract or its isolated compounds have several useful pharmacological properties such as anti-inflammatory (Whitaker and Carlson 1975) and anti-diabetic complications (Okada et al. 2004). Previous studies showed that phlorotannins isolated from *E. bicyclis* have an inhibitory effect on β-site amyloid precursor protein cleaving enzyme 1 and would, thus, have a beneficial use in Alzheimer's disease (Jung et al. 2010). However, to our knowledge, there have been no reports focusing *E. bicyclis* and its potential affect on eye-related disease such as glaucoma.

This study screened the protective effect of the ethanol extract of *E. bicyclis* (EEEB) collected from the eastern coast of South Korea, on the degeneration of oxidative stress-induced transformed RGCs (RGC-5), as well as the scavenging effect on ROS production. Moreover, the effects of EEEB on *N*-methyl-D-aspartate (NMDA)-induced retinal damage were evaluated.

## Materials and Methods

### Animals

All animal studies were carried out in a pathogen-free barrier zone at KIST Gangneung Institute in accordance with the procedure outlined in the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Procedures used in this study were approved by the Animal Care and Use Committee of KIST.

Male adult Sprague–Dawley rat weighing 250–300 g (8 weeks old) was used in the present study and were acclimated at least for 1 week, caged in group of five or less, and fed with a diet of animal chow and water ad lib. They were housed at 23±0.5 °C and 10 % humidity in a 12 h light–dark cycle.

### Chemicals

First-grade solvents were used for extraction, fractionation, and column chromatography. Kiesel gel 60 (70–230 mesh, Art, 7734 Merck) was used for the column packing material. 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) was from Molecular Probes (Eugene, OR, USA), and Zoletil and Rompun were used as anesthesia purchased from Virbac Laboratories and Bayer Korea, respectively. All other chemicals and reagents were from Sigma-Aldrich (St. Louis, MO, USA).

### Seaweed Samples

Seaweed samples were harvested from the eastern coast of South Korea, washed with fresh water, and stored at −20 °C until use (Table 1). The frozen samples were thawed at room temperature and extracted three times with 6 l of 95 % ethanol. The extracted solution was then concentrated in a rotary vacuum evaporator to obtain the crude extract.

### Liquid Chromatography-Mass Spectroscopy

EEEB (1 mg/ml in methanol) was injected into a Varian 1,200 L liquid chromatography-mass spectroscopy (LC-MS) system (Walnut Creek, CA, USA). Separation of each compound with a reversed-phase hydrosphere-$C_{18}$ [150×4.6 mm i.d., 5 μm particle size (YMC Co., Kyoto, Japan)] was performed under ambient conditions using a Prostar 230 ternary gradient pump, a Prostar 430 autosampler, and a Prostar 335 photodiode array detector. The mobile phase consisted of acetonitrile and water was used with 0.4 ml/min flow rate. Acetonitrile was gradually increased from 0 to 50 % for 50 min. The chromatographic profile was recorded at 280 nm. Mass data was collected using a triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source. The positive ion ESI mass spectra were acquired from *m/z* 50–1,000. The mass spectrometer conditions were as follows: drying gas of 12 psi at 150 °C, nebulizing gas ($N_2$) of 60 psi, auxiliary gas of 17 psi, corona current of 5 μA, and housing temperature of 50 °C.

### Isolation of Phlorotannins Compounds from EEEB

The EEEB (60 g) was suspended in distilled water (1 l) and partitioned with ethyl acetate (1 l×3). Then, the ethyl acetate was collected and evaporated in a rotary vacuum evaporator to obtain the ethyl acetate portion (24 g). The ethyl acetate portion (20 g) was fractionated by normal phase silica gel chromatography (400 g, chloroform/methanol/water=80:18:2, *v*/*v*→chloroform/methanol/water=50:49:1, *v*/*v*) to yield 13 fractions. Fraction 8 (2.73 g) was further fractionated by sephadex LH-20 chromatography with methanol to obtain 54 fractions. Each fraction was analyzed by Agilent high-performance liquid chromatography (HPLC) system equipped with a reversed-phase Pack Pro-$C_{18}$ [150×4.6 mm i.d., 3 μm particle size (YMC Co., Kyoto, Japan)]. The mobile phase consisted of acetonitrile (A) and 0.1 % TFA in water (B) with 1 ml/min flow rate. The gradient program was 0–10 min, 10 % A; 10–50 min, 10–50 % A. The chromatogram was detected at 280 nm. The fractions showing similar HPLC chromatogram were collected together (G1–G5) and subjected to reverse-phase preparative HPLC on a 250×20 mm i.d. YMC Pack Ph $C_{18}$ column to obtain pure compounds. The mobile phase was same with analytical HPLC with 10 ml/min flow rate. Eckol (35 mg) was purified from G1 (fraction 26–30, 83 mg) with 20 % A and 6,6′-bieckol (63 mg) was purified from G2 (fraction 38–44, 133 mg) with 15 % A. 8,8′-bieckol (28 mg) and dieckol (49 mg) were separated from G4 (fraction 53, 103 mg) in the condition of 20 % A for 40 min, then 20–40 % A for the next 40 min. Phlorofucofuroeckol A (12 mg) was purified from G5 in the condition of 20 % A for 3 min, then 20–45 % A to 80 min. All isolated compounds were analyzed by $^1$H–NMR (500-MHz Varian NMR system) and compared with the literature data for identification (Jung et al. 2010).

### Culture of RGC-5 Cells

These cells have been previously shown to express ganglion cell markers and exhibit ganglion cell-like behavior in culture (Krishnamoorthy et al. 2001). RGC-5 cells were kindly gifted by Alcon Research, Ltd (Fort Worth, TX, USA).

Cells were cultured in Dulbecco's modified eagle medium (DMEM) containing 5 mM glucose, 100 U/ml penicillin/streptomycin, 2 mM glutamine, 5 mg/ml active geneticin, and 10 % (*v*/*v*) heat-inactivated fetal bovine serum (FBS) in a humidified incubator with 5 % $CO_2$ at 37 °C. To examine the effects of the samples, RGC-5 cells were seeded at a density of $5\times10^3$ cells per well into 96-well plates. After incubating for 24 h, cells were washed in DMEM containing 1 % FBS. Cells were then exposed to DMEM containing 1 % FBS plus samples or plus vehicle. After 1 h pretreatment with samples, L-buthionine-(S,R)-sulfoximine (BSO) plus glutamate was added to cultures, which were then maintained for 24 h.

**Table 1** The marine algae in this study

| Samples | Family | Species | Harvest place (East Sea) | Harvest date |
|---|---|---|---|---|
| 1 | Laminariaceae | *Laminaria japonica* | Aninjin, Gangneung | 03/07/2006 |
| 2 | Ceramiaceae | *Delesseria serrulata* | Sacheon, Gangneung | 01/03/2006 |
| 3 | Desmarestiales | *Desmarestia viridis* | Aninjin, Gangneung | 03/25/2006 |
| 4 | Desmarestiales | *Desmarestia ligulata* | Aninjin, Gangneung | 03/25/2006 |
| 5 | Laminariaceae | *Eisenia bicyclis* | Hyunpo, Ulleung | 06/20/2006 |
| 6 | Rhodomelaceae | *Laurencia nipponica* | Saguenjun, Gangneung | 05/15/2006 |

### Cell Viability

After incubation for 24 h after treatment with or without BSO plus glutamate, cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay with slight modification of Mosmann (1983). Briefly, MTT was added at a final concentration of 0.5 mg/ml for 1 h at 37 °C and reduced MTT was solubilised by adding 100 μl of DMSO to each well. Solubilised formazan product in each well was measured using a spectrophotometer (BioTek Instruments, VT, USA) with a 570 nm test wavelength and a 690 nm reference wavelength.

### Microscopic Analysis for Cell Viability by PI and Hoechst 33342 Double Staining

Apoptotic or necrotic cell death caused by BSO plus glutamate was characterized by double staining the cells with Hoechst 33342 and PI (Hong et al. 2009). Cells were stained with 8 μM Hoechst 33342 and 1.5 μM PI for 30 min at 37 °C. After being washed twice with serum-free media, cells were imaged using a fluorescence microscope.

### Assessment of ROS

In order to quantify intracellular ROS, DCFH-DA was used as a radical probe (Shimazawa et al. 2009). RGC-5 cells were seeded at a density of $5.0\times10^3$ cells per well into 96-well plates, and then incubated in a humidified atmosphere of 95 % air and 5 % $CO_2$ at 37 °C. Twenty-four hours later, the cell culture medium was replaced with DMEM containing 1 % FBS. Radical species ($H_2O_2$, OH·, and $O_2{\cdot}^-$) oxidize nonfluorescent dichlorofluorescein to fluorescent dichlorofluorescein. The cells were loaded with radical probe, DCFH-DA (10 μM) by incubation for 20 min at 37 °C. Then, the cell culture medium was replaced to remove the extra probe. To generate radical species, we added $H_2O_2$ at 1 mM ($H_2O_2$ radical), $H_2O_2$ at 1 mM plus 100 μM iron (II) perchlorate hexahydrate (OH radical), or $KO_2$ at 1 mM ($O_2{\cdot}^-$) to the radical probe-loading medium. Fluorescence was measured after ROS-generating compounds had been present for various time periods, using excitation/emission wavelengths of 485/535 nm (Luminescence Spectrometer LS50B, Perkin-Elmer Ltd. England).

### Assessment of Total Intracellular GSH

Total glutathione concentration in the cell extract was determined using a kinetic assay in which amounts of glutathione reductase (GR) brought about the continuous reduction 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) to 5-thio-2-nitrobenzoic acid (TNB) formation by nicotinamide adenine dinucleotide phosphate (NADPH; Rahman et al. 2006).

Briefly, RGC-5 cells were lysed with cold lysis buffer containing 143 mM sodium phosphate, 0.1 % triton-X and 8 mM EDTA. 5 % sulfosalicylic acid was then added to the cell extract followed by shaking for 5 min. A 120-μl DTNB solution containing 1.68 mM DTNB and 3.33 U/ml GR was subsequently added to 25 μl of the cell extract. Generation of TNB was initiated by adding 50 μl of 895 μM NADPH and followed spectrophotometrically at 412 nm using a Synergy HT Multi-microplate reader (Bio-Tek Instruments, VT, USA)

### Lipid Peroxidation Assay

The amount of formation for thiobarbituric acid reactive species (TBARS) was used as the rate of membrane lipid peroxidation and the previously described method was used with slight modification (Zhang and Osborne 2006). Sprague–Dawley rat brain was homogenized in 10 volumes of ice-cold 0.9 % saline (pH 7.0) using a motor-driven polytetrafluoroethylene-glass homogeniser. The homogenate was centrifuged at 1,000×*g* for 10 min at 4 °C and the resulting low-speed supernatant was used for the lipid peroxidation assays. The homogenate from a single animal was used for each set of lipid peroxidation experiments. Aliquots of supernatant (0.5 ml) were preincubated at 37 °C for 5 min with 0.3 ml of 0.9 % saline (pH 7.0) containing various concentrations of EEEB or vehicle. Lipid peroxidation in rat homogenate was evaluated by the TBARS method as described by previously report (Jung et al. 2008). Absorbance of the samples was measured at a 532 nm and the amount of TBARS was determined using a standard curve of the malondialdehyde derivative 1,1,3,3-tetraethoxypropane (0.63–100 μM). Protein concentration in whole-brain homogenate supernatant was determined with Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard.

### NMDA-Induced Retinal Damage

After anesthesis with a Zoletil (1.6 μg/g, Verbac Laboratories 06515, France) and Rompun (0.05 μl/g, Bayer) mixture, retinal damage was induced by an injection (2 μl/eye) of NMDA (2.5 mM dissolved in 0.01 M PBS) to the left eye using 30 gauge of Hamilton syringe (Inokuchi et al. 2006). One drop of 0.5 % moxifloxacin ophthalmic solution (Alcon Laboratories Inc., Fort Worth, USA) was applied topically to the treated eye immediately after the intravitreous injection. Seven days after the injection, eyeballs were enucleated for histological analysis. EEEB (10 mg/kg) or vehicle (0.5 % CMC in water) was injected intraperitoneally 1 h before NMDA injection and immediately after NMDA

injection. Animals were killed 1 day after NMDA injection and fixed for TUNEL assay, and 7 days after NMDA injection fixed for histological analysis.

### Immunostaining

In order to observe the retina layer thickness caused by NMDA, we used hematoxylin and eosin staining. Animals were killed on the 7 days of the experiment, and each eye was enucleated. Eyes were fixed in 10 % formalin for 24 h, embedded in paraffin, and sectioned. The sections were stained with hematoxylin and eosin stain according to standard procedures and were evaluated under a light microscope (Olympus, Tokyo, Japan).

### TUNEL Staining

To analyze NMDA-induced apoptosis, we used a TUNEL assay using an ApopTag Red in situ Apoptosis Detection kit (Chemicon/Millipore, USA) according to the manufacturer's protocol. Briefly, sections were submitted to enzymatic digestion with 20 μg/ml of proteinase K for 15 min, then washed with PBS and incubated with 3 % hydrogen peroxide in PBS for 5 min at room temperature and twice rinsed in PBS. They were then immersed, incubated with a stock solution of terminal deoxynucleotidyl transferase reaction enzyme in a humidified chamber at 37 °C for 1 h, then washed three times in PBS for 1 min. Sections were subsequently incubated with antidigoxygenin peroxidase conjugate and peroxidase substrate to detect signs of apoptosis, staining brown. The histological aspect of apoptosis was observed using a light microscope (Olympus, Tokyo, Japan).

### Statistical Analysis

Data are expressed as a mean percentage of the control value plus SEM. $p<0.05$ was considered significant. A Student's $t$ test was used to compare the data obtained.

## Results

### Effect of Ethanol Extracts of Six Marine Algae on the Viability of RGC-5 Cells Caused by an Insult of BSO plus Glutamate

The ethanol extracts of six marine algae were tested for their protective effect on BSO plus glutamate-induced retinal degeneration in RGC-5 cells. The results are shown in Fig. 1. From these six marine algae, only EEEB was significantly attenuated the negative effect of BSO plus glutamate (Fig. 1a) at 10 μg/ml concentration, and this was in a concentration-dependent manner (Fig. 1b).

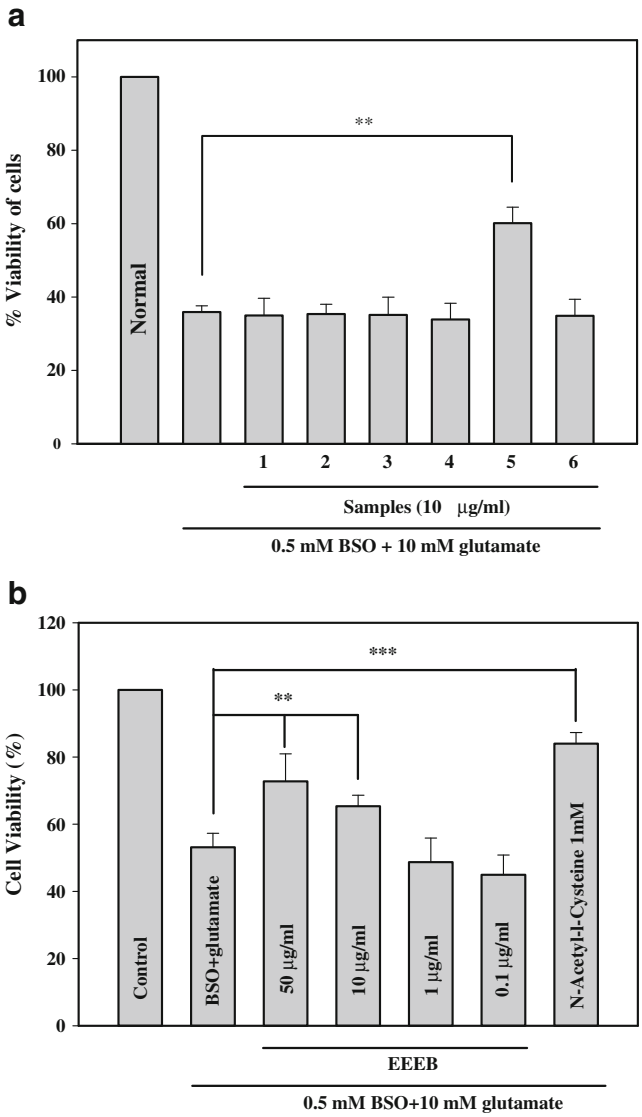


**Fig. 1** The effect of the six marine algae (**a**) and the sample *5* (EEEB) (**b**) in a concentration dependent on BSO plus glutamate for 24 h on the viability of RGC-5 cells in the culture as measured by the MTT assay. Results are expressed as percentage of viable cells with *error bars* indicating ±SEM where $n=4$ (**$p<0.01$, ***$p<0.001$). *1 Laminaria japonica*, *2 Delesseria serrulata*, *3 Desmarestia viridis*, *4 Desmarestia ligulata*, *5 Eisenia bicyclis*, *6 Laurencia nipponica*

These results were reconfirmed by microscopic analysis, using a Hoechst 33342/PI double staining method, as shown in Fig. 2. Hoechst 33342 is a cell permeable blue fluorescence dye that stains live cells, whereas PI, a red fluorescence dye, is only permeable in cells with damaged membranes. Cells under the control condition displayed normal nuclear morphology with few red-stained nuclei. It can be seen that BSO plus glutamate showed many red-stained nuclei, which were thought to be apoptotic or necrotic cells. Importantly, the numbers of dead red cells are clearly reduced in exposed cultures where EEEB is present (Fig. 2).

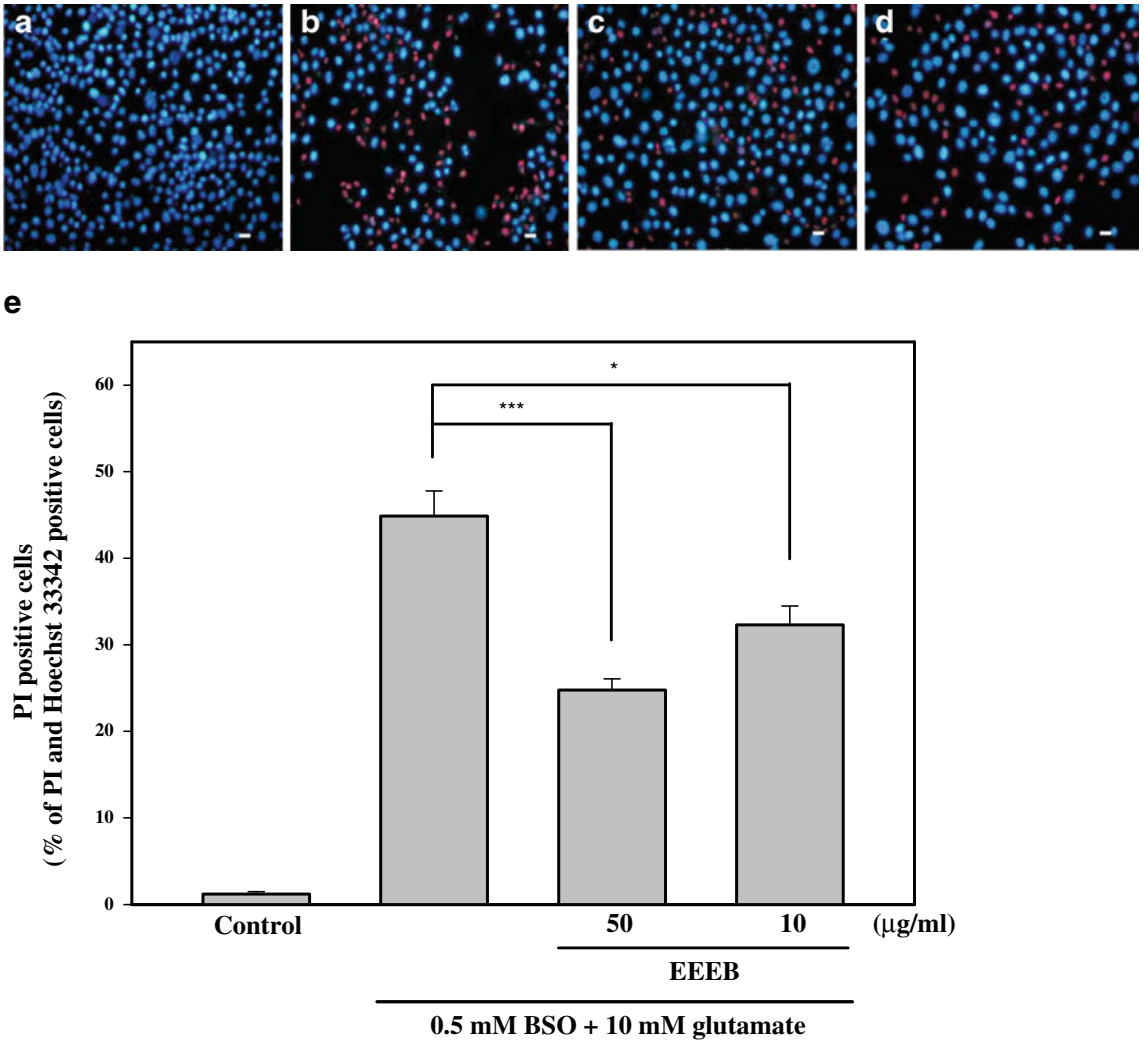


**Fig. 2** Representative fluorescence microscopy of propidium iodide (*red*) and Hoechst 33342 (*blue*) staining. Control RGC-5 cells in culture (**a**). BSO plus glutamate causes many of the cells to stain by early apoptosis and necrosis indicated by red staining (**b**); however, EEEB clearly blunts the red-stained cells as shown in **c**. PI-positive cells were counted using a cell counter under a fluorescence microscope at 100 times magnification and four representative images were use to estimate the percent of PI-positive cells of total cell numbers (minimum 200 cells/well were counted) as shown in **e**. Results are mean values with *error bars* indicating ±SEM where $n=4$ (*$p<0.05$, ***$p<0.001$). *Scale bar* 50 μm

From quantification, it can be seen that BSO plus glutamate to RGC-5 cells elevated the percentage of PI positive cells (44.9±5.8; according to the percent of the total number of cells) relative to cultures in which serum was present. This increase was reduced by the inclusion of 50 μg/ml of EEEB to the culture (24.8±2.5) as shown in Fig. 2e.

## Effect of EEEB on Oxidative Stress-Induced ROS Production in RGC-5 Cells

ROS quantifications were tested using DCFH-DA as a fluorescence probe caused by various oxidative insults $H_2O_2$, $O_2\cdot^-$, or OH·. Exposure of cultures to $H_2O_2$ at 1 mM, and ROS activity (relative DCF fluorescence compared to the control) was increased up to 939.7±10.5 from the basal level of 100. This increase in ROS was reduced by EEEB in a concentration-dependent manner (Fig. 3a). Similar results were obtained caused by $O_2\cdot^-$ or OH· radicals, respectively (Fig. 3b and c).

## Effect of EEEB on Oxidative Stress-Induced Total GSH Depletion in RGC-5 Cells

Similar to the result obtained in oxidative-stress induced ROS production in RGC-5 cells, EEEB reduced GSH depletion caused by three different ROS, $H_2O_2$ (Fig. 4a), $O_2\cdot^-$ (Fig. 4b), or OH· (Fig. 4c) in a concentration-dependent manner.

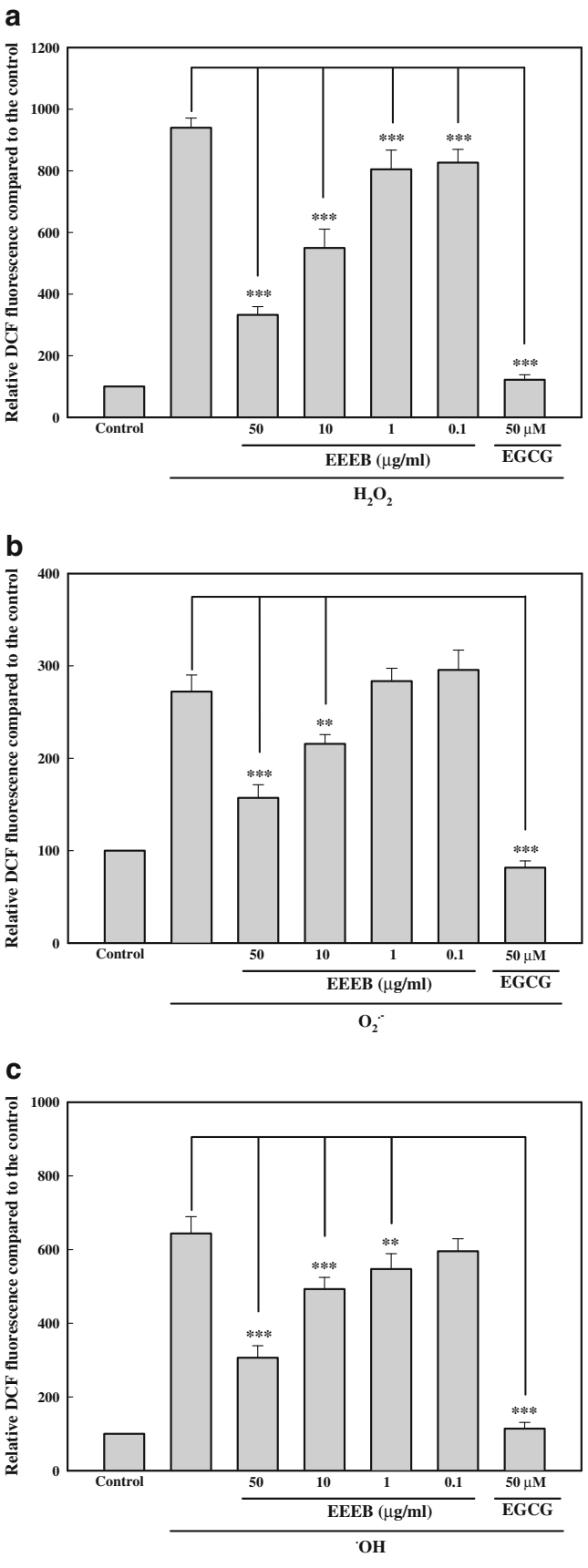


**Fig. 3** Antioxidative activities of EEEB against the production of various radical species ($H_2O_2$, $O_2^{\cdot -}$, OH·) in RGC-5 cells (**a**–**c**). ROS production was stimulated with $H_2O_2$ at 1 mM (**a**), or with $KO_2$ at 1 mM (**b**), or with $H_2O_2$ at 1 mM plus ferrous perchlorate (II) at 100 μM (**c**) in RGC-5 cells. Results are mean values with *error bars* indicating ±SEM. where $n=6$ (**$p<0.01$, ***$p<0.001$)

## Effect of EEEB on SNP-Induced Lipid Peroxidation in Rat Brain Homogenate

Lipid peroxidation was assessed by determining the production of MDA in rat brains. In the previous study, we examined the dose–response effect of sodium sodium nitroprusside (SNP) on TBARS formation in rat brain homogenates in order to find a relevant concentration (Jung et al. 2008). We found out that SNP, at a concentration of 20 μM, caused a submaximum stimulation of TBARS; therefore, this concentration of SNP was used to determine the inhibitory effect. TBARS in homogenates of the rat brains was stimulated by 20 μM; however, EEEB inhibited the production of TBARS in a concentration-dependent manner with the $IC_{50}$ value of 5.07 μg/ml (Table 2).

## Effect of EEEB on NMDA-Induced Retinal Damage

Retinal tissues were tested for ganglion cell numbers and for inner plexiform layer (IPL) thickness. Seven days after intravitreal injection of NMDA, the retinal tissues showed clear changes when compared with the controls (Fig. 5a). A clear thinning of IPL can be observed after NMDA injection (365.0±35.2 μm as shown in Fig. 5b); however, EEEB-treated rats significantly inhibited the thinning of the IPL thickness (410.5±43.4 μm as shown in Fig. 5c). The IPL thickness in the nontreated group was 422.0±42.4 μm, as shown in Fig. 5b.

The effect of EEEB on NMDA-induced apoptosis was tested using TUNEL staining (Fig. 6). Twenty-four hours after the intravitreal injection of NMDA, increased TUNEL-positive cells could be seen in the ganglion cell layer (GCL), as shown in Fig. 6b; however, the change seen in these GCL caused by NMDA was significantly attenuated by EEEB (Fig. 6c).

## Identification of the Compounds from EEEB Using LC-MS

EEEB was assessed by LC-MS in order to estimate the possible constituents. Within 50 min, a distinct separation of each constituent was obtained by a linear gradient system of 0–50 % acetonitrile in $H_2O$. The chromatogram, recorded at 280 nm, is shown in Fig. 7a. The mass spectra from several peaks (peaks 1–6) were obtained in the positive mode with an ESI source. As shown in Fig. 7b, the molecular ions were identical with the phlorotannins, tannin

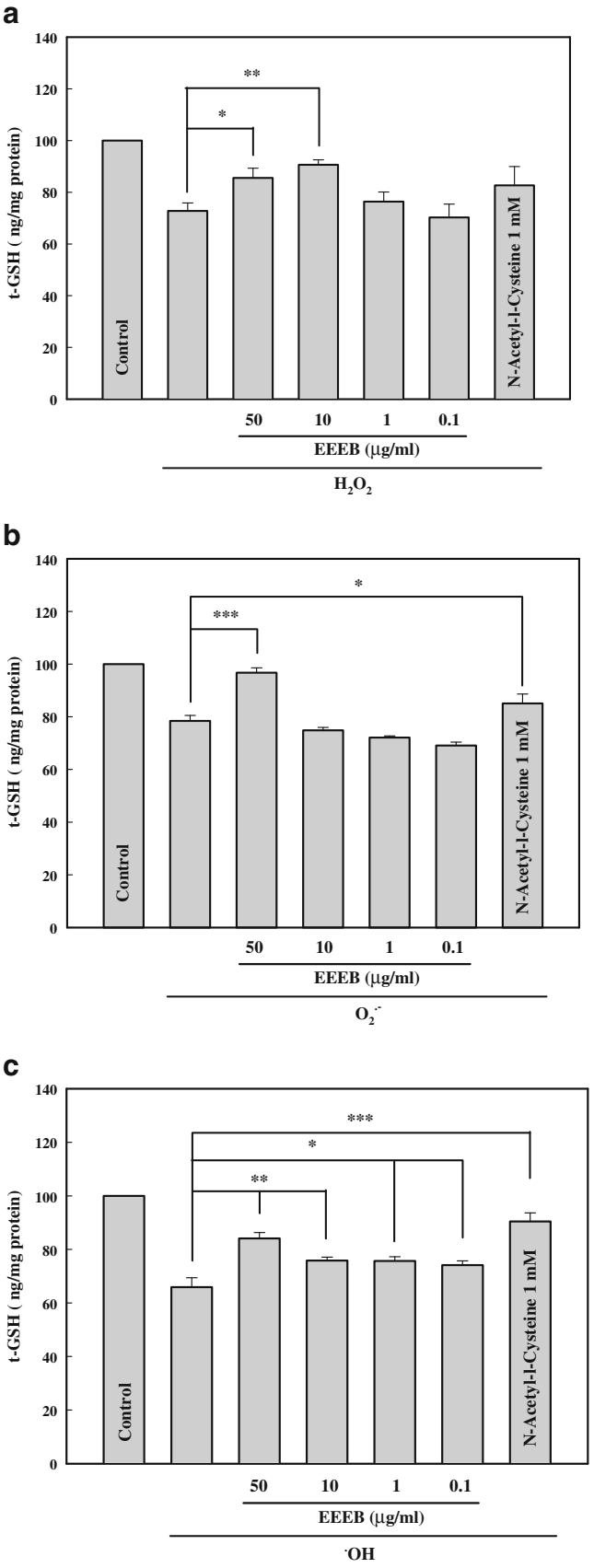


**Fig. 4** Inhibitory effect of GSH depletion of EEEB against the production of various radical species ($H_2O_2$, $O_2\cdot^-$, OH·) in RGC-5 cells. ROS production was stimulated with $H_2O_2$ at 1 mM (**a**) or with $KO_2$ at 1 mM (**b**), or with $H_2O_2$ at 1 mM plus ferrous perchlorate (II) at 100 μM (**c**) in RGC-5. Results are mean values with *error bars* indicating ±SEM where $n=3$ (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

derivatives, composed of several phloroglucinol units linked to each other in different ways, which were previously reported from this algae (Shibata et al. 2004).

## Discussion

In the present study, we examined the effects of EEEB against BSO plus glutamate as an oxidative stress model with the RGC-5 cell line, and its inhibitory effect on lipid peroxidation in rat brain homogenates. Moreover, the present study clearly shows that EEEB protects retinal damage in the in vivo model.

Glutamate is the most abundant excitatory neurotransmitter in the central nervous system and retina. Glutamate plays an important role in neuronal damage, which is associated with the excessive release of glutamate and subsequent influx of $Ca^{2+}$ via NMDA receptor of glutamate and has been implicated as a mechanism of RGCs death in glaucoma (Levin 2003; Osborne et al. 1999).

BSO is an inhibitor of γ-glutamylcysteine synthetase, which plays an important role in the proposed γ-glutamylcycle synthesizing glutathione from amino acid (Drew and Miners 1984). Glutamate plus BSO is an induction of oxidative stress by GSH depletion that inhibits cystein uptake and relies on ROS, produced endogenously by mitochondria (Nakajima et al. 2007; Maher and Hanneken 2005b).

We used a negative insult, BSO plus glutamate, in this study for the protective effect of EEEB caused by oxidative stress. From the activity screening of marine algae, EEEB was able to attenuate BSO plus glutamate-induced RGC-5 cells death at a 10 μg/ml concentration, and its inhibition was concentration dependent with a concentration range of 1–50 μg/ml, in which 50 μg/ml was the most effective concentration (Fig. 1a and b). The neuroprotective potency of EEEB was supported by the Hoechst 33342/PI double-staining method (Fig. 2).

ROS are reactive molecules which include superoxide anion, $H_2O_2$, hydroxyl radical, nitric oxide, peroxyl radical, and singlet oxygen (Galaris and Pantopoulos 2008). They are highly reactive due to their reduced metabolites of oxygen atom and have important role in various signal transduction pathways.

It has been suggested that ganglion cell death caused by overproduction of ROS plays a key role in the pathogenesis leading to neurodegenerative disease like glaucoma (Aslan

**Table 2** Inhibitory effect of EEEB in SNP induced lipid peroxidation

| Samples | Lipid peroxidation | | |
|---|---|---|---|
| | Concentration (μg/ml) | Inhibition (%) | $IC_{50}$ (μg/ml) |
| EEEB | 20.0 | 63.2±1.7 | 5.07 |
| | 10.0 | 55.7±0.64 | |
| | 5.00 | 49.6±0.66 | |
| | 2.50 | 43.4±3.4 | |
| | 1.25 | 37.4±0.47 | |

SNP at a concentration of 20 μM caused a submaximum stimulation of TBARS in the previous study; this concentration of SNP was therefore used to determine the inhibitory effects of samples.

et al. 2008; Tezel 2006). A number of experimental data support the hypothesis that oxidative damage is an important step in the pathogenesis of primary open angle glaucoma, and thus might be a relevant target for both prevention and therapy (Izzotti et al. 2006). Natural products have been shown to combat oxidative stress in several models using RGC-5 cells (Jung et al. 2008; Maher and Hanneken 2005a; Matsunaga et al. 2009; Nakajima et al. 2009; Lee et al. 2010). In our previous study, we suggested that luteolin 6-*C*-(6″-O-*trans*-caffeoylglucoside), isolated from *Phyllostachys nigra*, significantly attenuated the negative effects of BSO plus glutamate, or hydrogen peroxide, to RGC-5 cells and this was due to its antioxidative properties (Lee et al. 2010). In the present study, the EEEB scavenged intracellular radical production in RGC-5 cells caused by extracellular $H_2O_2$, $O_2^{\cdot-}$, or ·OH in a concentration dependent manner between a concentration range of 0.1–50 μg/ml (Fig. 3).

GSH is a tripeptide with L-cysteine, L-glutamic acid, and glycine. GSH contains a sulfhydroxyl (−SH) functional group, which can function as a nucleophile to donate electrons and act as an antioxidant (Meyer and Hell 2005). GSH has been detected as aqueous humor (Richer and Rose 1998)

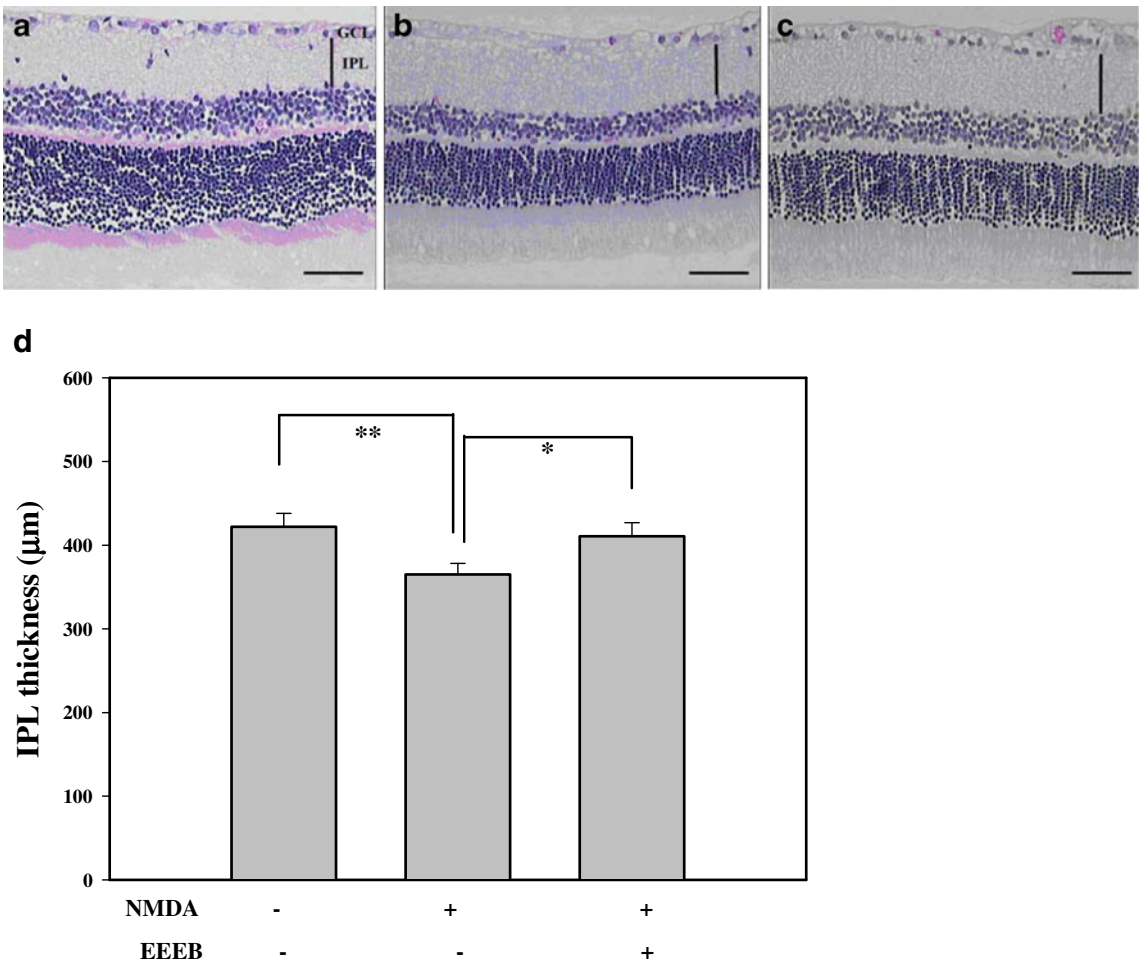


**Fig. 5** Effect of EEEB on retinal damage induced by intravitreal injection of *N*-methyl-D-aspartate in rat. **a** Non-treated, **b** NMDA-treated, **c** NMDA (5 nmol) plus EEEB (10 mg/kg)-treated retinal cross-sections at 7 days after with or without NMDA. *Scale bar* 50 μm. **d** The thickness of IPL. Results are mean values with *error bars* indicating ±SEM where $n=7$ (*$p<0.05$, **$p<0.01$)

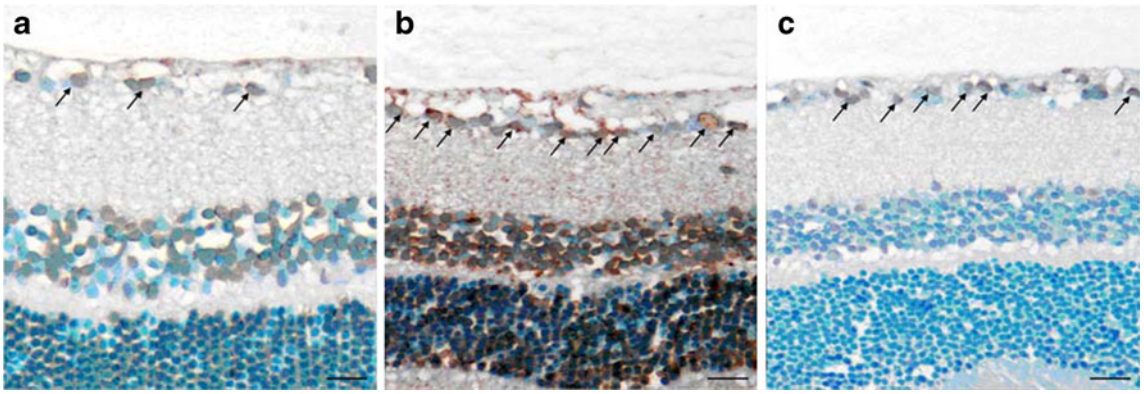


**Fig. 6** Figure shows the effect of EEEB in NMDA induced expression of TUNEL-positive cells. **a** Non-treated, **b** NMDA-treated, **c** NMDA (5 nmol) plus EEEB (10 mg/kg)-treated retinal cross-sections at 1 day after with or without NMDA. *Arrows* indicate TUNEL-positive cells. *Scale bar* 50 μm

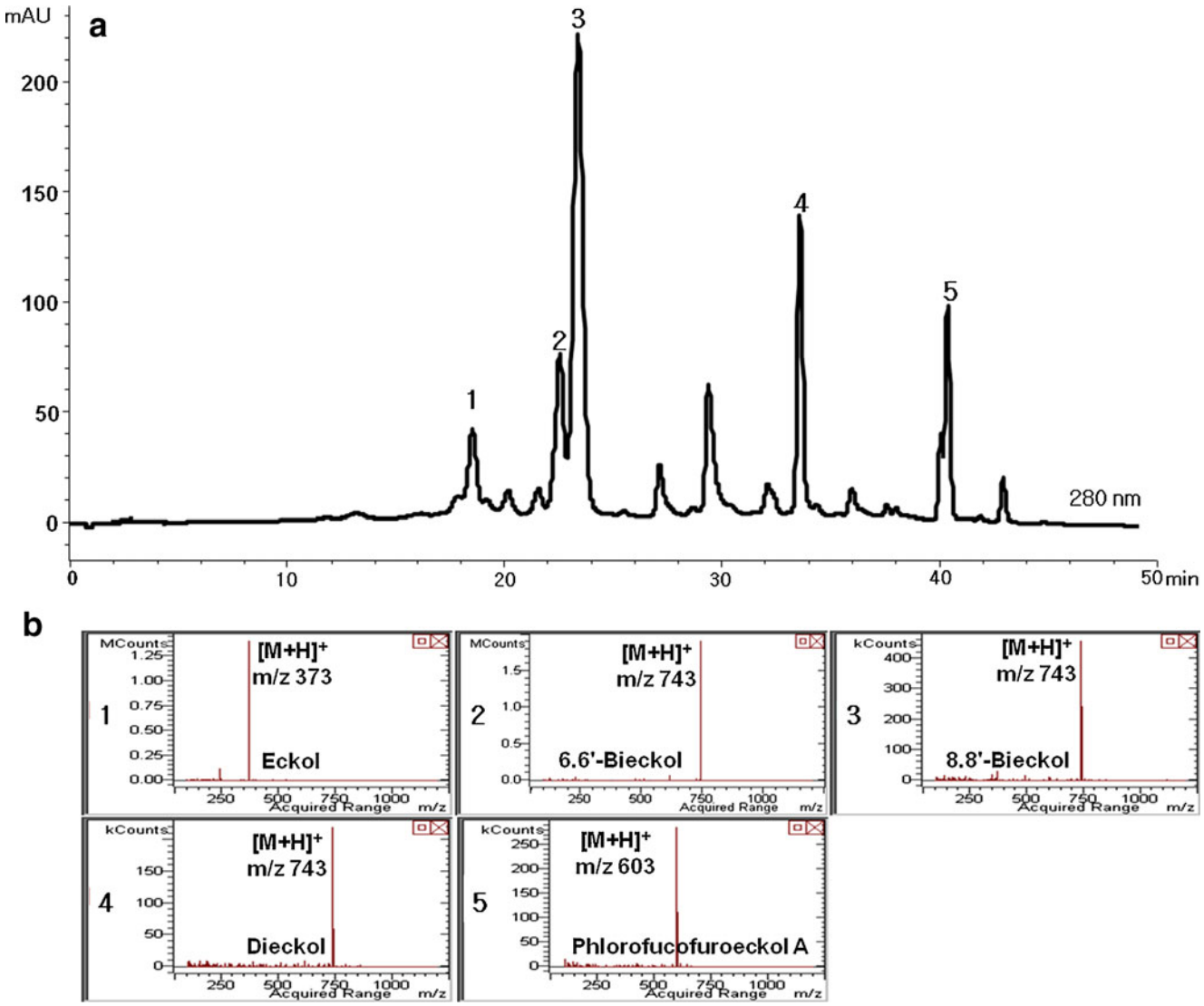


**Fig. 7** The HPLC chromatogram of EEEB (**a**) and MS spectra of each peak (**b**). The EEEB was analyzed by LC-MS with mobile phase of acetonitrile and water. The chromatogram was detected at 280 nm, and the MS spectra of the separated peaks (*1–5*) were archived in positive mode with the ESI ion source. The collected MS data from those peaks were identified as phlorotannin derivatives

and was found to be depleted, even at the earliest stages of the glaucoma disease (Bunin et al. 1992). In clinical study, patients with primary open angle glaucoma exhibited low levels of circulating glutathione, suggesting a general compromise of the antioxidative defense (Gherghel et al. 2005). In our study, EEEB prevented the decrease of cellular GSH levels caused by various ROS, such as extracellular $H_2O_2$, $O_2^{\cdot -}$, or ·OH (Fig. 4).

In this study, we tested whether or not EEEB inhibited lipid peroxidation. Lipid peroxidation was assessed by determining the production of MDA in rat brains. In biological systems, MDA is a very reactive species and plays a role in cross-linking DNA with proteins and damaging the liver cells and measurement of TBARS is generally considered to provide an index for lipid peroxidation (Carbonneau et al. 1990). SNP, which we used in this study, is a potent stimulator of lipid peroxidation (Kishnani and Fung 1996), and EEEB was shown to exhibit significant inhibition of MDA production in a concentration-dependent manner (Table 2). From these results, we concluded that EEEB, when used to blunt the negative effect of BSO plus glutamate on RGC-5 cells in a culture, seems to be related to its antioxidative properties.

Glutamate receptors comprise four different receptor families, including NMDA, kainate, α-amino-3-hydroxy-5-methylisoxazole-4-propionate, and metabotropic receptors (Trist 2000). NMDA is a synthetic substance and mimics the action of the neurotransmitter glutamate receptor. NMDA is thought to activate intracellular $Ca^{2+}$ influx, which increase ROS production and causes neurotoxicity

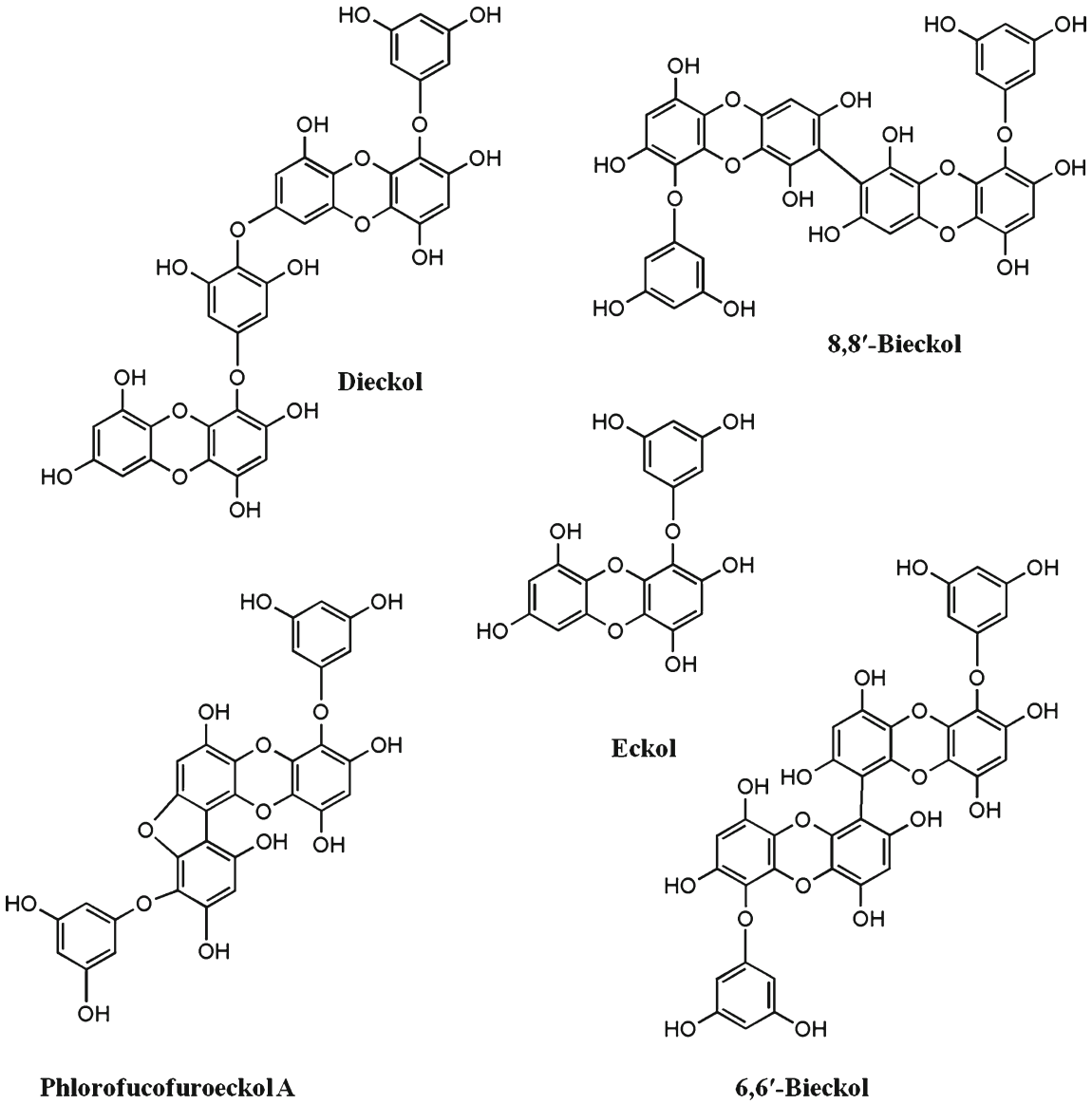


**Fig. 8** Chemical structures of the compounds isolated from EEEB

(Choi 1992; Inokuchi et al. 2009). It has also been shown that increased efflux of glutathione could be a factor in initiating nerve cell death via a change in intracellular redox potential and/or a decrease in the intracellular capacity for inactivation of reactive oxygen species (Wallin et al. 1999).

The present studies show that EEEB, when administered via intraperitoneal injection into rats, provides protection from retinal neuronal death caused by NMDA insult to the retina. It is known that the inner retina is particularly affected by NMDA with ganglion cell death and thickness of IPL thinning occurring (Inokuchi et al. 2009), and EEEB attenuated the thinning of the IPL thickness (Fig. 5). Moreover, a reduction in TUNEL-positive cells (apoptotic marker) in GCL, caused by NMDA injection in rats, provides its protective effects on RGC death (Fig. 6).

In order to identify constituents of EEEB, LC-MS analysis was performed, and five compounds were isolated from EEEB using chromatographic methods (Shibata et al. 2004). Their chemical structures were elucidated by spectral analysis, and their structures were identified as eckol (peak 1), 6,6′-bieckol (peak 2), 8,8′-bieckol (peak 3), dieckol (peak 4), phlorofucofuroeckol A (peak 5), respectively (Figs. 7 and 8). These phlorotanins are common polyphenol compounds that have been found to exist only within brown algae (Shibata et al. 2008). It is well-known that phlorotannins contain distinctive antioxidant and free radical-scavenging activities (Li et al. 2009; Kang et al. 2003). Phlorotannins such as phloroglucinol, eckstolonol, eckol, phlorofucofuroeckol A, and dieckol isolated from *Ecklonia stolonifera* inhibited total ROS generation (Kang et al. 2004), and eckol from *Ecklonia cava* protected oxidative stress-induced lung fibroblast cells death by enhancing cellular antioxidant activity and modulating cellular signal pathways (Kang et al. 2005).

Moreover, components of phlorotannin can defend intestinal stem cells against the oxidative damage caused by gamma irradiation, and this might be due to their antioxidative effects (Moon et al. 2008).

Overall, the findings suggest that the EEEB had a protective effect on BSO plus glutamate-induced retinal ganglion cell death in vitro and EEEB attenuated retinal degeneration induced by NMDA in vivo. In particular, major constituents of this extract, phlorotannins, could possibly be the acting compounds due to their antioxidative potency. The protective effects of five isolated phlorotannins, isolated from the EEEB, on oxidative stress-induced RGC-5 cell death and their mechanisms of the action will be investigated.

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