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A standardized extract of *Rhynchosia volubilis* Lour. exerts a protective effect on
benzalkonium chloride-induced mouse dry eye model

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ABSTRACT

Ethnopharmacological relevance: In contrast to other leguminous plants generally used as food, *Rhynchosia volubilis* Loureiro, a small soybean with a black seed coat, has been used as a traditional oriental remedy for various human diseases in Eastern Asia. In this study, we demonstrated the protective effect of *R. volubilis* against dry eye disease.

Aim of the study: We aimed to investigate whether a standardized ethanol extract of *R. volubilis* (EERV) can protect the cornea in a benzalkonium chloride (BAC)-induced mouse dry eye model.

Materials and Methods: Experimental dry eye was induced by the instillation of 0.2% BAC on mouse cornea. A standardized ethanol extract of *R. volubilis* (EERV) was orally administered following BAC treatment. The positive control group was treated with commercial eye drops. Fluorescein staining, tear break-up time (BUT), and hematoxylin and eosin staining were evaluated on the ocular surface. Squamous metaplasia and apoptosis in the corneal epithelial layer were detected by immunostaining. Furthermore, the protein expression of cytochrome *c*, Bcl-2, and Bax was determined.

Results: EERV treatment significantly improved fluorescein scoring, BUT, and smoothness in the cornea compared to the vehicle group. In addition, EERV inhibited squamous metaplasia and apoptosis in the cornea. The expression of cytochrome *c* and Bax was upregulated, while that of Bcl-2 was downregulated in the vehicle group compared with that in the control group. However, EERV treatment inhibited the expression of cytochrome *c* and Bax, while that of Bcl-2 was improved.

Conclusion: Standardized EERV could be a beneficial candidate for the treatment of dry eye disease.

Abbreviations:

BAC, Benzalkonium chloride; BSA, bovine serum albumin; BUT, tear break-up time; CMC, carboxymethyl cellulose; DE, dry eye; DEWS, dry eye workshop; ECL, enhanced chemiluminescent; EERV; ethanol extract of *R. volubilis*; H&E, hematoxylin and eosin; HLA, human leukocyte antigen; IL, interleukin; LOD, limits of detection; LOQ, limits of quantification; MCP-1, monocyte chemoattractant protein-1; PBST, phosphate-buffered saline with Triton-X100; PFA, paraformaldehyde; ROS, reactive oxygen species; RSDs, relative standard deviations; TFOS, tear film and Ocular surface society; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling; UV, ultraviolet;

CLASSIFICATIONS: 1.09: Eye

KEYWORDS: Small black bean, dry eye disease, cornea, benzalkonium chloride, natural products 2.444: ophthalmology; 2.642: Traditional medicine Asia & Oceania; 2.348: inflammation

Chemical compounds (PubChem CID) studied in this article

Ethanol (702); Acetonitrile (6342); Cyanidin 3-*O*-glucoside (44256715); (-)-Epicatechin (72276); Daidzin (107971); Genistin (5281377); 6''-*O*-malonyldaidzin (9913968); 6''-*O*-malonylgenistin (15934091); Formic acid (284); Benzalkonium chloride (24854040); Carboxymethylcellulose (24748); Fluorescein (16850); Zolazepam hydrochloride (71416); Tiletamine hydrochloride (26534); Xylazine hydrochloride (68554); Paraformaldehyde (712); Hematoxylin (442514); Eosin Y (11048); Triton-X100 (5590); DAPI (2954).

1. Introduction

Dry eye (DE) is a disorder of the tear film and ocular surface, which results in increased osmolality and inflammation as updated by the 2017 report of Tear Film & Ocular Surface Society (TFOS) and the Dry Eye Workshop II (DEWS II) (Craig et al., 2017). DE can cause discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface owing to inadequate tear secretion and excessive evaporation of the tear film (Lemp et al., 2007). The specific mechanism of DE is unclear, but inflammation may have a major role in the pathogenesis of DE. It can lead to DE-related ocular complications, such as structural damage of the corneal epithelial layer, squamous metaplasia, and neovascularization (Bhavsar et al., 2011; Chotikavanich et al., 2009; Lam et al., 2009; Pflugfelder et al., 2000; Solomon et al., 2001; Tishler et al., 1998; Xu et al., 1996; Yoon et al., 2007).

Oxidative stress is commonly known to be a risk factor associated with anterior eye disorders, such as DE, benzalkonium chloride (BAC)-induced as well as tobacco smoke-induced ocular surface damage (Kruk et al., 2015; Ogawa, 2013; Rosin and Bell, 2013). Commercial ophthalmic solutions contain BAC, which is the most commonly used preservative for preventing microbial contamination (Yu et al., 2008). However, chronic exposure to BAC leads to the induction of excessive generation of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{\bullet-}$) (Guenoun et al., 2005). High levels of ROS accelerate the generation of pro-inflammatory cytokines, chemokines, and metalloproteinases that appear to initiate an inflammatory cascade and apoptotic cell death in the cornea (Luo et al., 2004; Nita and Grzybowski, 2016; Padgett et al., 2013). These processes were observed in DE owing to a change in the quality and quantity of tear fluid. (Sonawane et al., 2012; Stevenson et al., 2012).

Several studies have reported that antioxidants may prevent ocular surface damage

induced by oxidative stress, thereby reducing the symptoms of DE (Andrade et al., 2014; Gatell-Tortajada, 2016; McCusker et al., 2016).

Rhynchosia volubilis Loureiro, a small black bean, is a perennial climbing herb growing in mountains and fields in Korea, China, Japan, and Vietnam. While other members of the leguminous family are generally utilized as food, *R. volubilis* is used as a traditional oriental remedy for neuralgia, kidney disease, postmenopausal osteoporosis, and senile dementia (Kim, S.J. et al., 2007; Kinjo et al., 2001; Park et al., 2016; Yim et al., 2009). *R. volubilis* was previously reported to exhibit strong anti-oxidative activities, anti-obesity effect, proliferative activities on human osteoblastic cells, and anti-proliferative activities against gastric adenocarcinoma (Jeon et al., 2014; Kim, H.J. et al., 2007; Kim et al., 2005; Kim, S.J. et al., 2007; Kinjo et al., 2001; Shin et al., 2008). Moreover, it has been considered a major medication in contraceptive prescriptions owing to its spermicidal activity (Guan et al., 2014).

In Eastern Asia, *R. volubilis* was eaten for obtaining a clearer and better vision (JXUTCM, 1970). The activity of *R. volubilis* against eye diseases such as eye pain and blurred vision has been reported in the Herbal Handbook (Quattrocchi, 2012). *R. volubilis* contains an abundance of bioactive constituents including anthocyanins (cyanidin-3-glucoside and delphinidin), isoflavonoids (daidzein, calycosin, biochanin A, and genistin), flavonoids (quercetin, epicatechin, tricetin, and apigenin), polysaccharides, and peptides (Guo Yanyan, 2011; Hong et al., 2016; Jeon et al., 2014; Kim, H.J. et al., 2007; Yoshida et al., 1996).

Anthocyanins from the seed coat of black soybean has been reported to be effective in reducing retinal degeneration and cyanidin-3-*O*-glucoside, the dominant anthocyanin component in *R. volubilis*, has been reported to increase regeneration of rhodopsin in rod photoreceptors (Matsumoto et al., 2003; Paik et al., 2012; Tirupula et al., 2009; Yanamala et al., 2009). In addition, isoflavonoids and flavonoids have been previously shown to exhibit

protective effects against DE and corneal neovascularization (Joussen et al., 2000; Kim, K.A. et al., 2016; Kruse et al., 1997; Yang et al., 2016). From these results, black soybean could be a beneficial agent for various ocular diseases. Despite being a potential candidate, the protective effect of *R. volubilis* against DE has not yet been reported.

Therefore, we investigated whether a standardized ethanol extract of *R. volubilis* (EERV) was able to protect the cornea in a BAC-induced mouse DE model.

2. Materials and methods

2.1 Reagents

All solvents were purchased from Fisher Scientific (Pittsburgh, PA). The standard, cyanidin 3-*O*-glucoside (1), was obtained from J & H chemical (Hangzhou, China). (-)-Epicatechin (2), daidzin (3), genistin (4), and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO). The mounting medium was purchased from Vector Laboratories (Burlingame, CA). Bcl-2, β -actin, and HRP-conjugated IgG antibodies were purchased from Santa Cruz (Santa Cruz, CA). Alexa 488-conjugated and Alexa 594-conjugated antibodies were purchased from Invitrogen (Carlsbad, CA). Cytochrome *c* and Bax antibodies were purchased from Cell Signaling Technology (Beverly, MA). Cytokeratin-10 was purchased from Abcam (Cambridge, MA). HLA-DR was purchased from Biorbyt (San Francisco, CA). Zoletil and Rumpun were purchased from Virbac Laboratories (Fort Worth, TX) and Bayer (Newbury, UK), respectively.

2.2 Plant material

The seeds of *R. volubilis* were provided from Highland Agriculture Research Center, National Institute of Crop Science, Pyeongchang, South Korea. *R. volubilis* has been checked with <http://www.theplantlist.org> (Record 38957). A voucher specimen (KISTGN-RN-2016-

003) has been deposited at the KIST Gangneung Institute. Dried *R. volubilis* seeds were ground into powder (1 kg) and extracted twice with 5 L of 70% ethanol at 25°C for 3 h in an ultrasonic cleaning bath (model RK 158s, Bandelin, Berlin, Germany). The extracts were filtered through Whatman no. 1 filter paper, and the combined filtrate was concentrated to dryness by rotary evaporation at 40°C to obtain 50.4 g of EERV. Then, EERV was stored at -20°C.

2.3 Malonate constituents

Separation of EERV was performed by reverse-phase HPLC chromatography on a Gilson semi-preparative HPLC system (Middleton, WI) equipped with a 321 pump and UV/VIS-155 HPLC detector. EERV (1 g) was dissolved in 5 mL MeOH and separated on a YMC packpro C₁₈ column (5 µm particle size, 250 × 20 mm, YMC Co., Japan) with an acetonitrile (A)-water (B) gradient (25–56% A for 0–20 min, 56–95% A for 20–25 min, 10 mL/min, 260 nm) to yield 4.2 mg of 6''-*O*-malonyldaidzin (5) (t_R = 13.9 min) and 7.8 mg of 6''-*O*-malonylgenistin (6) (t_R = 17.2 min). These compounds were identified by the Varian 500 MHz NMR system (Palo Alto, CA) and Thermo Fisher Scientific LCQ FLEET ion trap mass spectrometer (San Jose, CA).

2.4 HPLC- diode array detector (DAD)/MS analysis

HPLC-DAD/MS was used for the identification of the major constituents of EERV as well as the quantitative analysis of compounds 1-6. Chromatographic separation was performed with a YMC pack pro C₁₈ column (3 µm particle size, 150 × 4.6 mm I.D., YMC Co., Japan) using the Agilent Series 1200 liquid chromatography system (Agilent, CA). The HPLC solvent consisted of 0.1% formic acid in acetonitrile (solvent A) and 0.1% formic acid in water (solvent B). The separation was performed with a linear gradient (5% A for 0–1 min, 5–40% A for 1–26 min, 40–95% A for 26–30 min, 95% A for 30–33 min, 95–5% A for 33–35

min) at a flow rate of 1 mL/min. The absorbance was monitored at 260, 280, and 520 nm. The mass spectra were obtained using LCQ FLEET ion trap mass spectrometer with an electrospray ionization source (Thermo Fisher Scientific Inc., CA). The mass spectrometer conditions were as follows: positive ion mode; mass range, m/z 200–800; capillary voltage, 10.0 V; tube lens, 45 V; sheath gas flow rate (N₂), 50 arbitrary units; auxiliary gas flow rate (N₂), 12 arbitrary units; capillary temperature, 350°C.

2.5 Experimental DE model

This study was approved by the Animal Care and Use Committee of KIST (No. 2016-063). All the procedures were performed at the Korea Institute of Science and Technology (KIST) Gangneung Institute in agreement with the Association for Research and Vision in Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research guidelines. We used male BALB/c mice (6-week-old, 20–25 g) purchased from Central Lab (Gyeonggi-do, Korea). The animals were housed in cages with aspen bedding at $23 \pm 0.5^\circ\text{C}$ and 10% humidity in a 12:12 h light-dark cycle. Pelleted food and tap water were provided *ad libitum*.

An experimental DE model was induced by the topical treatment of BAC (Kim, K.A. et al., 2016; Lin et al., 2011; Xiao et al., 2012). The left eyes of the mice were instilled with 5 μL of 0.2% BAC, twice daily (10 AM and 10 PM) for 14 days. Thirty mice were randomly divided into control, DE (0.2% BAC-treated, alone), 10 mg/kg EERV (with 0.2% BAC), 50 mg/kg EERV (with 0.2% BAC), and positive control (commercial eye drops with 0.2% BAC) groups, comprising six mice each. The control group was instilled with an equal volume (5 μL) of physiological saline.

DE was pre-induced by BAC for 3 days and administrative processing was conducted. EERV, physiological saline, and commercial eye drops were administered three days later for

11 days. EERV (10 or 50 mg/kg body weight) was dissolved in distilled water with 0.5% carboxymethyl cellulose (CMC) and administered daily via oral gavage. The positive control group was instilled with commercial eye drops (Refresh Plus[®] preservative-free, Allergan, CA) four times daily (11 AM, 3 PM, 7 PM, and 11 PM). The control, DE, and positive control groups orally received 0.5% CMC solution alone *via* gavage throughout the experimental period.

After evaluation of BUT and fluorescein staining, the animals were sacrificed by cervical dislocation at 14 days. The left eyes were enucleated and immediately frozen in liquid nitrogen (Western blot analysis; n= 3 eyes per group) or fixed with 4% paraformaldehyde solution (Histological analysis; n= 3 eyes per group) at room temperature.

2.6 Tear break-up time (BUT)

After anesthesia of the mice with an intraperitoneal injection of a mixture of Zoletil and Rompun, the tear break up time (BUT) was measured on the ocular surface.

To examine BUT, 2% fluorescein (Alcon Research Ltd, PA) was instilled in the conjunctival sac, and the eyelids were closed manually 3–4 times. Then, the ocular surface was evaluated under a slit lamp microscope (Haag–Streit, Germany). At the first appearance of a dry sign, BUT was recorded (in seconds).

2.7 Fluorescein staining

Fluorescein staining was performed by applying fluorescein solution in the conjunctival sac after recording the BUT score. The corneal surface was divided into four areas that were scored separately from 0 to 4 in a blind fashion using the 2017 TFOS DEWS II-recommended grading system (Wolffsohn et al., 2017; Zhang et al., 2014).

2.8 Histological examination

Whole eye tissue was fixed in 4% paraformaldehyde solution for 24 h. The tissues were embedded with paraffin and cut into 4 μm sections. The section tissues were deparaffinized in xylene, rehydrated in 95, 70, 50, and 30% ethanol, and dipped in distilled water for 10 min. To stain the nuclei, the sections were dipped in 0.1% hematoxylin buffer for 8 min at room temperature, and then washed 3 times with PBS. 1% Eosin Y buffer was used for staining the cytoplasm. The sections were dipped in 1% Eosin Y buffer for 1 min at room temperature. After washing with tap water, the section slides were dehydrated through 30, 50, 70, and 95% ethanol, followed by xylene. The section slides were subsequently mounted in mounting medium and sealed. Hematoxylin and eosin (H&E) staining was performed under a light microscope (Olympus, Japan) to evaluate the morphological changes.

2.9 Immunostaining

Paraffin was removed from the corneal sections with xylene. Then, the sections were rehydrated in 95, 70, 50, and 30% ethanol, and dipped in distilled water for 10 min. The sections were incubated in 10 mM citrate buffer (pH 6.0) for 3 min and heated in a microwave oven for three exposure cycles of 5 min each. Next, the sections were cooled in an ice bath and incubated with 5% hydrogen peroxide in PBS for 10 min at room temperature. After washing 3 times with PBS, the sections were blocked in blocking solution for 1 h and incubated with cytokeratin-10 (diluted 1:200) and human leukocyte antigen DR (HLA-DR; diluted 1:200) primary antibodies at 4°C overnight. Subsequently, the sections were washed with PBS containing 0.05% Triton-X100 and incubated with anti-rabbit Alexa 488 (diluted 1:500) and anti-mouse Alexa 594 (diluted 1:500) secondary antibodies. The sections were washed 3 times, and mounted in Vector mounting medium with DAPI. Immunostaining was examined using confocal microscopy (Leica, Wetzlar, Germany).

2.10 TUNEL staining

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) assay was performed using an *in situ* TUNEL detection kit (Roche, UK), according to the manufacturer's instructions. The apoptotic cells were detected by their dark nuclear staining (TUNEL-positive; Green) using confocal microscopy (Leica, Wetzlar, Germany).

2.11 Western blot analysis

Protein extraction from the corneal and conjunctival tissues was performed according to the reagent instruction (RIPA buffer, Sigma, MO). Equal amounts of total proteins were separated on SDS-PAGE gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, CA). Nonspecific binding sites were blocked with 5% skim milk, and then incubated overnight with cytochrome *c* (diluted 1:2000), Bcl-2 (diluted 1:2000), Bax (diluted 1:2000), and β -actin (diluted 1:3000) primary antibodies. The membranes were washed and developed with HRP-conjugated secondary antibodies (diluted 1:5000) at room temperature. For chemiluminescence studies, immunoreactivity was detected using an enhanced chemiluminescent (ECL) solution (Thermo Scientific, IL). Band densities were examined using the Multi Gauge 3.1 software (Fuji Photo Film, Tokyo, Japan).

2.12 Statistical analysis

All data were assessed with a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. The obtained data are presented as the mean \pm standard error of mean (SEM). A statistical probability of $p < 0.05$ was considered significant using GraphPad Prism version 7.00.

3. Results

3.1 Identification and quantification of the major constituents of *R. volubilis*

EERN was injected into the HPLC system and six compounds were detected in the HPLC profile with a DAD chromatogram (Figure 1A). The compounds were identified according to the mass spectra and retention times relative to the standards [tR 9.0 (1), 13.1 (2), 14.4 (3), 17.5 (4), 18.2 (5), and 21.0 min (6)]. The mass spectra of the six compounds showed a molecular ion $[M]^+$ and protonated molecular ions $[M+H]^+$ of the standards (Figure 1B). The retention time of compounds 1-6 was identical to that of the corresponding standard. The six compounds were identified as cyanidin 3-*O*-glucoside (1), (-)-epicatechin (2), daidzin (3), genistin (4), 6''-*O*-malonyldaidzin (5), and 6''-*O*-malonylgenistin (6) (Figure 1C). The concentration of each constituent was evaluated from the peak area based on the calibration curves derived for each compound. The method validation parameters are presented in Table 1. Five levels of each standard solution were injected in triplicate to establish the calibration curves. The value of correlation coefficients (r^2) was in the range of 0.9998–1.0000, indicating good linearity between the peak areas and concentrations. The limits of detection (LOD) and quantification (LOQ) were expressed as $LOD = 3.3\sigma/S$ and $LOQ = 10\sigma/S$, where σ is the standard deviation of the response and S is the slope of the calibration curve of the analyte. Intra- and inter-day variability was determined three times a day and on three separate days. Variations are indicated by relative standard deviations (RSDs). The accuracy of the method was measured by the recovery of a spiked sample. The recovery value was between 96.76 and 100.42%, which is completely acceptable for analysis of plant extracts. The amount of each compound was expressed as mg/g of EERV in Table 1. The extract of *R. volubilis* has been standardized to a minimum of 80% to a maximum of 120% of the amount of each compound (1–6).

3.2 Tear film stability and corneal surface damage

To observe corneal damage caused by BAC treatment, we investigated fluorescein

staining score and BUT of the ocular surface (Figure 2A–C). The fluorescein staining scores increased significantly in the mouse group treated with BAC alone (DE group). However, the groups administered EERV (10 and 50 mg/kg) showed reduction in BAC-induced corneal damage. Similarly, BUT decreased in the DE group compared to that in the control group (Figure 2C). BUT results showed statistical significance, and the 50 mg/kg EERV-treated group showed effects similar to the positive control. Histological analysis of the corneal tissue was conducted after H&E staining (Figure 2D). Compared to the control, the DE group presented corneal epithelial cells with irregular shapes (red circle and arrow). The positive control and 50 mg/kg EERV-treated groups ameliorated the irregularity of ocular surface and changes in the epithelial cells. According to these results, the oral administration of EERV and positive control showed a protective effect in an experimental mouse DE model.

3.3 Inflammation and squamous metaplasia

To detect inflammation and squamous metaplasia of the corneal tissue, we performed immunofluorescence staining (Figure 3). The inflammatory cells in the cornea were stained for human leukocyte antigen-DR (HLA-DR). The DE group exhibited increased expression of HLA-DR (HLA-DR-stained; red) compared to the control group. The protein level of HLA-DR in EERV-treated groups increased dose dependently. We next investigated squamous metaplasia induced by inflammatory mediators. To detect squamous metaplasia, immunostaining with cytokeratin-10 was performed. The distribution of cytokeratin-10 expression in the corneal tissue of DE group increased significantly when compared to that of the control. Similar to the positive control group, the 50 mg/kg EERV-treated group showed protective effects against BAC-induced squamous metaplasia. These findings suggest that EERV treatment could inhibit inflammation and squamous metaplasia in an experimental mouse DE model.

3.4 Apoptosis

We performed TUNEL assay, which is a useful method for detecting cell death via apoptosis (Figure 4A and 4B). The apoptotic cells were detected by their dark nuclear staining (TUNEL-positive cells; Green). The DE group showed significant number of TUNEL-positive cells (34.25 ± 5.0 cells) compared to the control group. Statistically, few TUNEL-positive cells (15.00 ± 4.2 cells) were observed in the 50 mg/kg EERV-treated group, and they showed effects similar to the positive control (10.00 ± 2.2 cells). These data demonstrated that EERV treatment was effective for cell survival in an experimental mouse DE model.

3.5 Expression of apoptosis-associated proteins

We investigated whether the expression of apoptosis-associated proteins (cytochrome *c*, Bcl-2, and Bax) is regulated by EERV treatment (Figure 5). Western blot analysis was conducted in protein lysates from the corneal and conjunctival tissues. The expression of cytochrome *c* and Bax appeared to be higher in the DE group than that in the control group. However, these protein levels were significantly downregulated in the EERV-treated group. The expression of Bcl-2 in protein lysates was downregulated in the DE group. EERV treatment upregulated the expression of Bcl-2 compared to the DE group. These results suggested that EERV treatment was effective in protecting against BAC-induced cell death.

4. Discussion

DE is a multifactorial ocular surface disease related to oxidative stress (Nakamura et al., 2007; Wakamatsu et al., 2008). Redox systems are maintained in the corneal epithelial cells and tear film (Higuchi et al., 2012). To protect the ocular surface from oxidative stress, anti-

oxidative enzymes are upregulated in the corneal epithelial cells (Offord et al., 1999). However, an imbalance of pro-oxidants/antioxidants leads to the overproduction of ROS on the ocular surface (Kruk et al., 2015; Ogawa, 2013; Ray et al., 2012). Several researchers suggested that natural products used as dietary supplements can minimize the development of DE (Andrade et al., 2014; Gatell-Tortajada, 2016; McCusker et al., 2016). Many studies have shown that the active constituents of natural products strongly inhibit ROS, which could provide beneficial effects against DE. Our previous study has shown that the leaves of persimmon (*Diospyros kaki*) protect the cornea in a BAC-induced DE model (Kim, K.A. et al., 2016). Since *R. volubilis* contains plentiful active constituents including anthocyanins, isoflavones, and polyphenols, we expected that it would be beneficial for the treatment or prevention of DE. In this study, we showed that EERV protected the cornea in a murine DE model.

The chromatogram of EERV revealed six major peaks (Figure 1A), and the major components of EERV were identified as cyanidin 3-*O*-glucoside, (-)-Epicatechin, daidzin, genistin, 6''-*O*-malonyldaidzin, and 6''-*O*-malonylgenistin. The chemical composition of a plant extract may be affected by various factors, such as seasonal changes, cultivation location, harvesting time, post-harvesting process, and extracting procedures. Therefore, extract standardization is an important process, where the amount of one or more selected compounds can be guaranteed. We carried out the standardization of EERV to compounds 1-6 as marker compounds. One gram of EERV contains 1.14, 3.11, 1.99, 3.69, 4.84, and 8.95 mg of compounds 1-6, respectively.

BAC is the most widely used preservative to prevent the microbial contamination of eye drops (Yu et al., 2008). However, long-term exposure to BAC can lead to severe ocular surface changes and has been shown to cause DE in humans (Pauly et al., 2011).

We used a BAC-induced DE model that showed signs similar to human DE, including

corneal epithelial apoptosis, instability of tear film, and inflammation (Baudouin et al., 2010; Lin et al., 2011). Our results have shown that EERV treatment significantly decreased the fluorescein staining score in an experimental mouse DE model (Figure 2A and 2B).

Instability of the tear film can trigger the overexpression of inflammatory cytokines, such as interleukin-6 (IL-6), interleukin- α (IL- α), and HLA-DR in DE (Calonge et al., 2010; Epstein et al., 2009; Rolando et al., 2005). HLA-DR is a biomarker of inflammation, and upregulated expression of HLA-DR was observed on the ocular surface of DE (Baudouin et al., 2000; Lopin et al., 2009; Pisella et al., 2000). We showed that EERV treatment dose-dependently reduced the expression of HLA-DR compared with the DE group (Figure 3, red).

Inflammatory cytokines are risk factors associated with the formation of squamous metaplasia, which is usually observed in patients with chronic DE (Chen et al., 2009; Murube and Rivas, 2003). Increased expression of cytokeratin-10 indicates the presence of squamous metaplasia, a condition in which the epithelial cells are converted into a type of keratinizing cells in the cornea (Kim, K.A. et al., 2016). The DE group showed increased expression of cytokeratin-10 compared to the control group (Figure 3, green). However, EERV treatment inhibited the increased expression of cytokeratin-10 in the corneal epithelial layer. These results suggested that EERV would reduce inflammation and squamous metaplasia in DE.

Inflammatory signaling promotes the apoptosis of corneal cells (Li et al., 2012; Strong et al., 2005). Inflammation and squamous metaplasia lead to apoptosis signaling cascades (Li et al., 2008). Apoptotic cell death is indicated by TUNEL-positive cells (green). In the present study, higher number of apoptotic cells was observed in the DE group than in the control group (Figure 4A and B). In the positive-control and EERV (50 mg/kg)-treated groups, apoptotic cells in the corneal and stromal layers were less than that in the DE group. This phenomenon was observed in a previous study, indicating that BAC is a small molecule that easily penetrated into the stromal layer from the corneal layer causing apoptotic cell death

(Kim, K.A. et al., 2016; Kim, Y.H. et al., 2016; Tseng et al., 2016). This study showed that EERV treatment at 50 mg/kg was effective in inhibiting apoptosis of the corneal epithelial layer, and it would be able to prevent damage to the cornea caused by DE (Figure 4A and B).

The main apoptotic signals associated with programmed cell death are release of cytochrome, upregulation of Bax, and downregulation of Bcl-2 (Katiyar et al., 2005; Sarkar et al., 2003). These signals lead to the induction of cell death (Delbridge and Strasser, 2015; Kim et al., 2013; Naseri et al., 2015). In a previous study, the expression of the pro-apoptotic proteins Bax and cytochrome *c* increased, while that of Bcl-2, an anti-apoptotic protein, decreased in DE (Luo et al., 2007; Sun and Wang, 2006). From the results of this study (Figure 5), we can suggest that EERV treatment at 50 mg/kg decreased cytochrome *c* and Bax expression compared with the DE group. Furthermore, Bcl-2 expression was increased by EERV treatment (Figure 5). It is suggested that EERV could improve DE because EERV treatment regulated the expression of apoptosis-associated proteins.

R. volubilis is reported to have strong antioxidant activity compared to other soybean cultivars (Nam et al., 2014). Previous studies revealed that epicatechin was a major antioxidant present in *R. volubilis* extracts (Jeon et al., 2014; Nam et al., 2014). Epicatechin showed more than 100 times stronger hydroxyl radical ($\cdot\text{OH}$) scavenging effect than mannitol, a typical $\cdot\text{OH}$ scavenger, and it exerted inhibitory effect on superoxide anion (O_2^-) generation in hypoxanthin-xanthin oxidase system (Hanasaki et al., 1994).

R. volubilis has an abundance of anthocyanins, which are water-soluble natural pigments with strong antioxidant activity (Malencic et al., 2012; Song et al., 2013; Xu and Chang, 2008a, b). The common anthocyanins are cyanidin, delphinidin, petunidin, peonidin, malvidin, and pelargonidin. The characteristics of anthocyanins are dependent on the position of hydroxylation, methoxylation, and bonded sugar residues. Previous studies showed that the hydroxylation position of B (benzene) and C (pyran) rings in anthocyanins play a role in

increasing antioxidant activity (Lim et al., 2013). Cyanidin and its glycoside derivatives inhibited lipid peroxidation, and their antioxidant activities were superior to those of vitamin E (Wang et al., 1999).

Therefore, epicatechin and cyanidin-3-glucoside, being the predominant components present in *R. volubilis*, can either donate hydrogen atoms or transfer a single electron to free radicals, which results in ROS scavenging in DE model. However, it is difficult to identify the active compounds responsible for the beneficial effects of EERV in DE. Further studies are necessary to determine the active compounds of EERV that are effective in DE treatment.

5. Conclusions

In conclusion, the clinical signs of DE were effectively ameliorated by EERV in an experimental mouse DE model. EERV stabilized the ocular surface and maintained the integrity of the epithelium. It also provided a protective effect against squamous metaplasia and apoptosis in the cornea. Therefore, EERV could be a beneficial agent for the treatment of DE diseases.

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Author contributions

Suk Woo Kang, Kyung-A Kim, Tae Kyeom Kang, and Je Hyeong Jung conducted the research; Chung Hyun Lee, Sung Jae Yang, and Tae-Jin Kim analyzed the data; Suk Woo Kang, Kyung-A Kim, and Sang Hoon Jung compiled the data and wrote the manuscript; Sang-Rok Oh provided supervised the project and material collection. Sang Hoon Jung supervised the project and provided financial support. All authors helped design the study, provided editorial comments and assistance, and have read and approved the submitted manuscript.

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Conflict of interest statement

The authors have declared no competing financial or commercial interests

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FIGURE LEGENDS

Figure 1. HPLC-UV chromatogram of EERV (A); MS spectra of major peaks (1–6) acquired by positive LC-ESI/MS (B); Identified compounds (C)

Figure 2. Protective effect of EERV on BAC-induced corneal damage. (A) Corneal fluorescein staining, (B) fluorescein staining score (C) tear break-up time (BUT), and (D) H&E staining were evaluated in all groups. Original magnification: 400 X, scale bars: 50 μm . Experimental values are expressed as the mean \pm SEM from three independent experiments (** $p < 0.01$, *** $p < 0.001$).

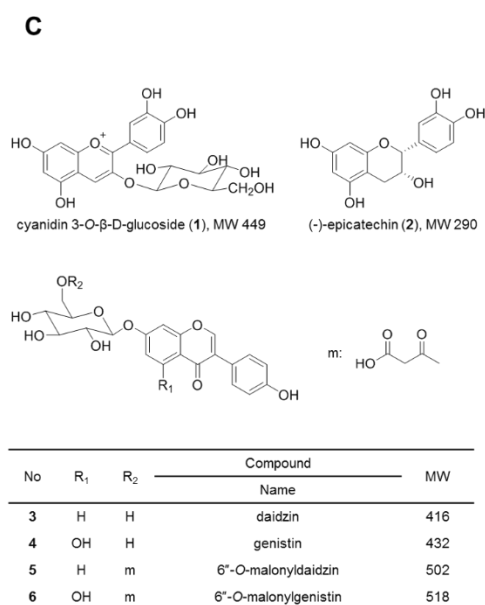
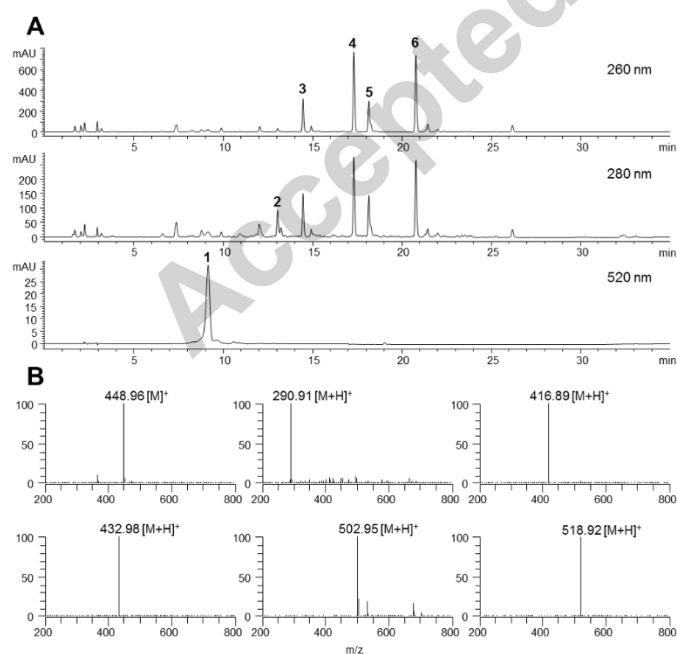
Figure 3. Expression of cytokeratin-10 and human leukocyte antigen DR (HLA-DR) in an experimental mouse model of dry eye (DE). The corneal sections were stained with anti-cytokeratin-10 (green) and anti-HLA-DR (red). The nuclei were stained with DAPI, and the expression of proteins was examined under a confocal microscope (Original magnification: 630 X). The results shown are representative of three independent experiments.

Figure 4. Apoptotic cell death in an experimental mouse model of dry eye (DE). (A) The corneal sections were stained with a TUNEL kit (Nuclei were stained with DAPI) and examined under a confocal microscope (Original magnification, 630 X). TUNEL-positive cells (green) indicate apoptotic cell death of the section slides (B) Quantification of TUNEL-positive cells. Scale bar = 50 μm . Experimental values are expressed as the mean \pm SEM from three independent experiments (*** $p < 0.001$).

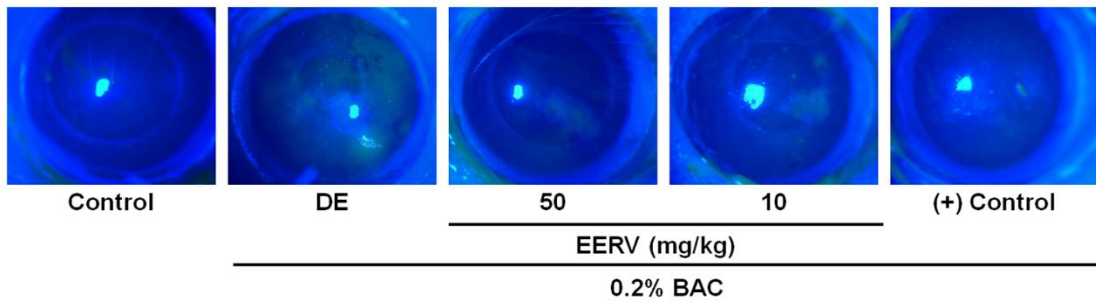
Figure 5. Expression of apoptosis-associated proteins. (A) Total protein and immunoreactivities of cytochrome *c*, Bcl-2, Bax, and β -actin (loading control) were examined by western blot analysis. (B) Quantification of relative protein levels compared to β -actin (loading control). Experimental values are expressed as the mean \pm SEM from three independent experiments (* p <0.05, ** p <0.01).

Table 1. Calibration curve, precision, LOD, LOQ, and contents of Compounds 1-6 in EERV

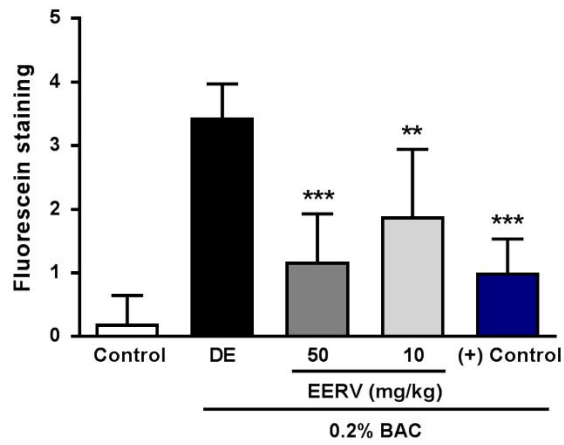
Compound	Linear range ($\mu\text{g/mL}$)	Calibration curve		Precision, RSD (%)		LOD ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)	Content (mg/g EERV)
		Regression equation	r^2	Intraday (n = 3)	Interday (n = 3)			
1	5–160	$y = 19.076x - 8.469$	1.0000	1.68	1.90	0.58	1.93	1.14
2	5–160	$y = 7.0251x + 6.772$	0.9999	0.34	1.35	0.30	1.00	3.11
3	20–160	$y = 44.375x + 78.322$	0.9999	0.26	1.21	0.31	1.04	1.99
4	20–160	$y = 60.109x + 136.530$	0.9998	0.13	1.17	0.20	0.66	3.69
5	20–320	$y = 25.180x + 80.011$	0.9998	3.29	8.05	3.13	10.43	4.84
6	20–320	$y = 32.745x + 113.100$	0.9998	2.50	10.61	0.29	0.95	8.95



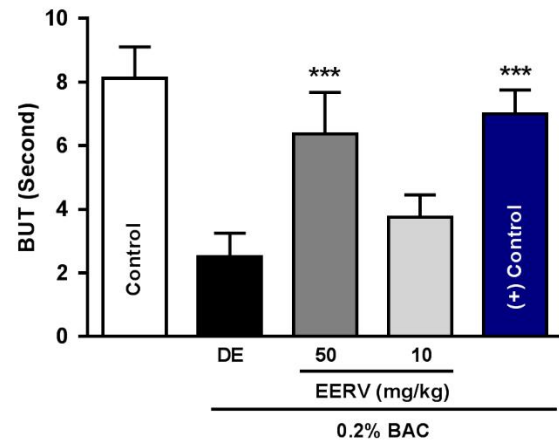
A



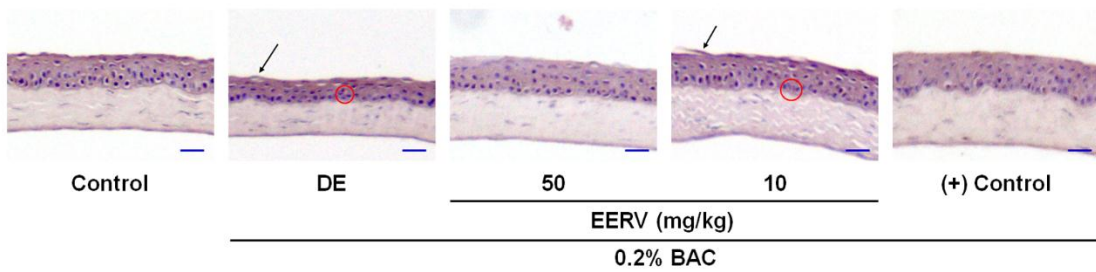
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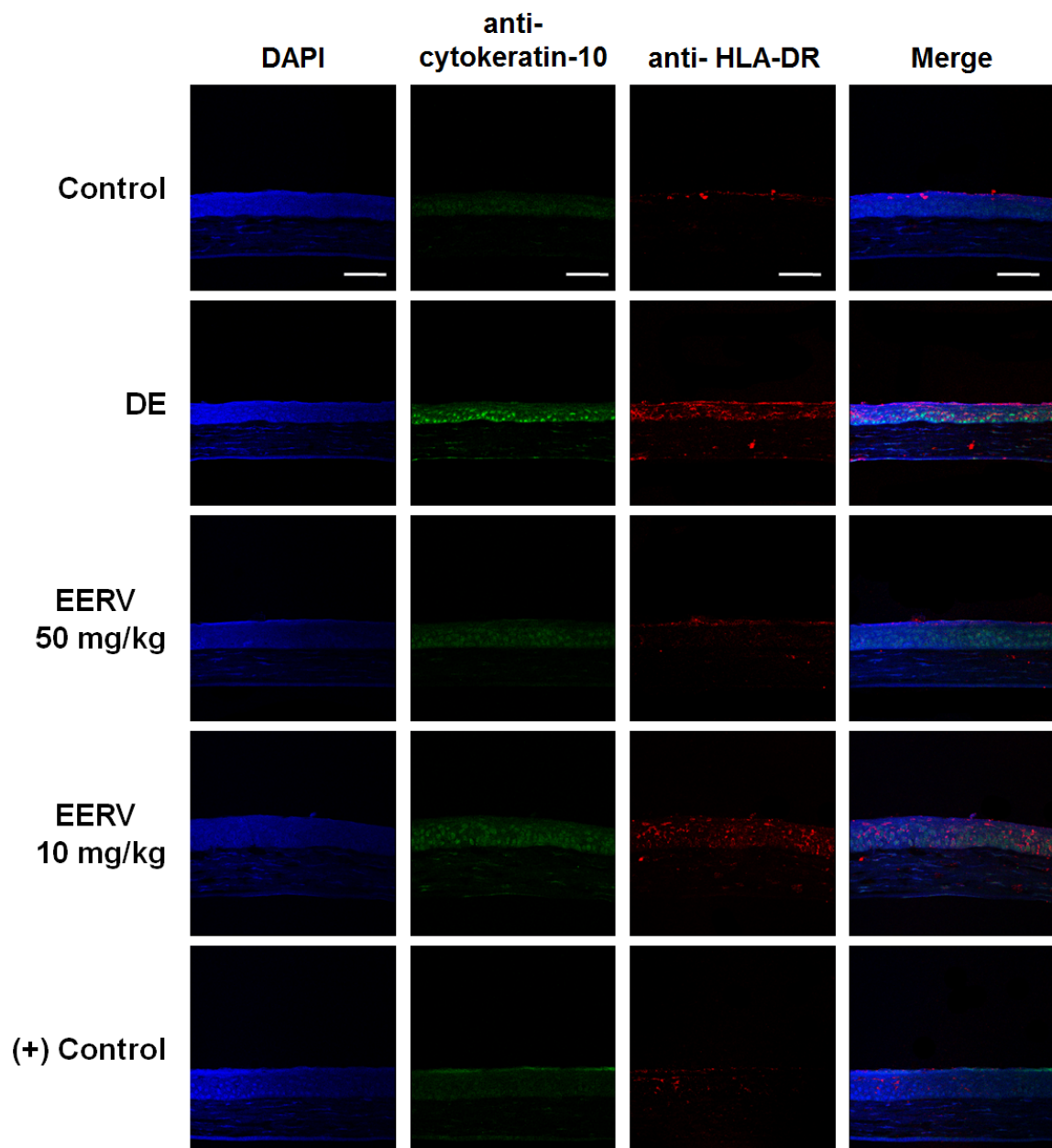
C



D

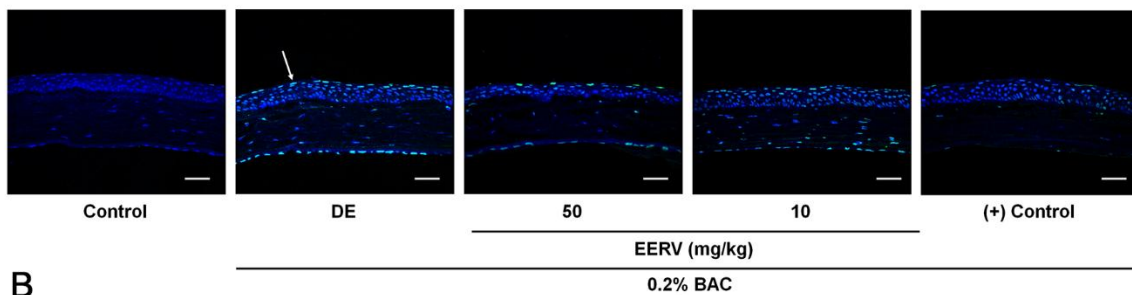


ACC

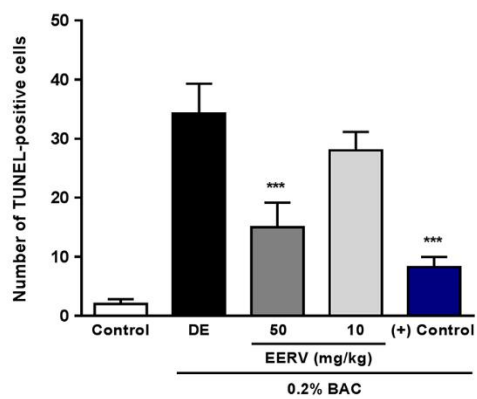


A

A

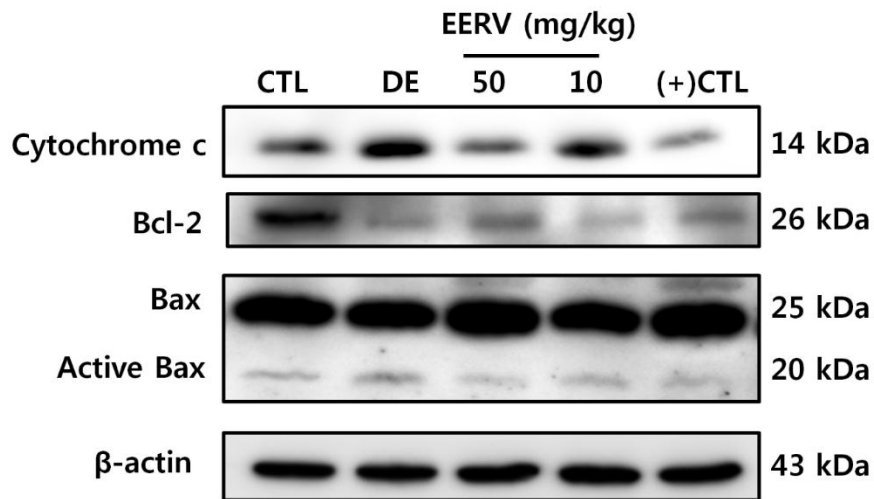


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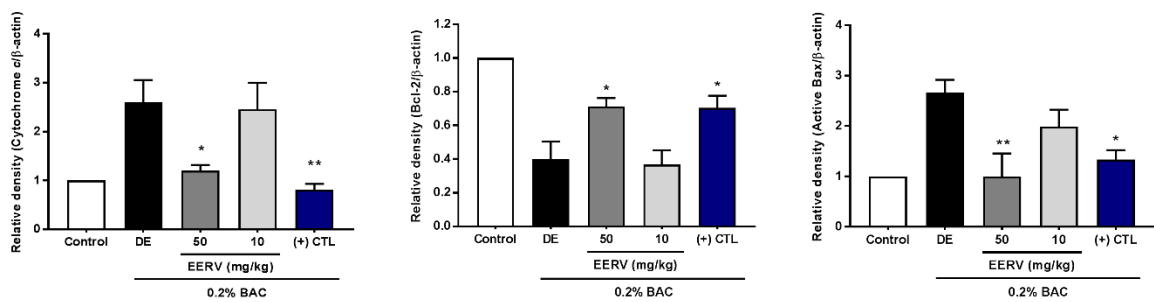


Accepted

(A)



(B)



Graphical abstract:

