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Original Article

Homonoia riparia and its major component, myricitrin, inhibit high glucose-induced apoptosis of human retinal pericytes

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ABSTRACT

Background: The loss of retinal pericytes is one of the earliest changes associated with diabetic retinopathy (DR). Chronic hyperglycemia induces apoptosis of these cells, leading to the onset and progression of DR. In this study, we investigated the effects of *Homonoia riparia* (*H. riparia*) and its major component, myricitrin, on high glucose (HG)-induced apoptosis of primary human retinal pericytes (HRPs).
Methods: The effects of an ethanol extract of *H. riparia* leaves and of myricitrin on HRP viability and apoptosis were investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry. Reactive oxygen species (ROS) levels were measured using 2′,7′-dichlorofluorescein diacetate. The activity of specificity protein 1 (Sp1), a transcription factor, was measured using a luciferase reporter assay and western blot analyses were performed to measure the expression of proteins involved in signaling and apoptosis.
Results: HG produced cytotoxic effects on HRPs, which showed increased Sp1 expression and ROS levels. *H. riparia* extract and myricitrin significantly inhibited HG-induced apoptosis and ROS generation, and also inhibited Sp1 activity. This was evidenced by an attenuation of the HG-mediated increase in extracellular signal-regulated kinase phosphorylation.
Conclusion: These data indicate that HG-mediated induction of Sp1 is one of a number of key signaling pathways involved in HRP apoptosis, and that *H. riparia* extracts or myricitrin may provide useful approaches to preventing and treating DR.

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1. Introduction

Diabetic retinopathy (DR) is a retinal microvascular disease that is characterized by diabetic macular edema and retinal neovascularization. It is a frequent complication of diabetes and a leading cause of vision loss in adults.[1,2] Effective regulation of blood glucose, as well as early detection and treatment of diabetes, are effective means of preventing this cause of

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severe visual impairment and blindness.[3] In addition, early DR is characterized by increased endothelial cell degeneration, microvascular destabilization, vascular permeability, perfusion alterations, and loss of retinal pericytes.[4,5] One of the earliest changes observed in retinal capillaries affected by DR is the loss of pericytes; this may make the capillaries vulnerable.[2] According to several reports, exposure of pericytes to high glucose (HG) levels reduced their proliferation and induced apoptosis.[2,6–9] Hyperglycemia is known to increase the generation of reactive oxygen species (ROS), especially in retinal vessels.[10–13] Accordingly, diabetes-related dysfunction of the retinal vasculature may be ameliorated by blocking the generation of ROS.

Homonoia riparia (*H. riparia*) is a mangrove species that belongs to the small genus of shrubs or small trees within the *Euphorbiaceae* family.[14–16] This plant usually grows in wet soil close to riverbanks and is widespread from India to China, Laos, Philippines, Taiwan, and throughout Malaysia to Papua New Guinea.[14–16] Different parts of *H. riparia* have been used for efficient and effective treatment of a number of diseases. For example, the roots produce laxative, diuretic, refrigerant, depurative, and emetic effects, whereas the leaves and fruits have been used to treat wounds, ulcers, and skin diseases, as well as showing depurative and antiseptic properties.[14,15] As we reported previously, cycloartane-type triterpenes from the leaves of *H. riparia* inhibited vascular endothelial growth factor-induced angiogenesis in human umbilical vein endothelial cells. Furthermore, we found that myricitrin (3-O-rhamnoside-myricitrin) was one of the major components of *H. riparia* using high-performance liquid chromatography. Several plants containing myricitrin have shown anxiolytic, anti-nociceptive, anti-inflammatory, and antioxidant activities.[17–19] Previous studies have suggested that myricitrin affords protection from oxidative stress-induced apoptotic cell death *in vitro* and *in vivo*.[20] Furthermore, the specificity protein 1 (Sp1) transcription factor has been shown to regulate the expression of genes implicated in cell growth, proliferation, and angiogenesis via physiological and pathological stress pathways regulated by extracellular signal-regulated kinase (ERK), Akt, cyclin-dependent kinase, and other protein kinases.[21]

In the present study, we investigated the roles of ROS generation, ERK/Sp1 pathway activation, and apoptotic mechanisms in the protective effects of *H. riparia* or myricitrin against HG-induced apoptosis in human retinal pericytes (HRPs).

2. Methods

2.1. *H. riparia* extraction and analysis

H. riparia leaves were collected in Hanoi (Vietnam) in June 2009, and identified by Professor J.-H. Kim, Gachon University, Republic of Korea. A voucher specimen (No. TBRC-VN-118) was deposited in the Herbarium of the Diabetic Complications Research Team, Korea Institute of Oriental Medicine (KIOM), Republic of Korea.[14] The leaves (10 g) were air-dried, extracted with 80% ethanol at room temperature for 24 hours, filtered, and concentrated to yield 1.3 g. This 80% ethanol extract (20 mg) was dissolved in methanol (10 mL) and the solution was filtered through a 0.2-μm syringe filter (Millipore, Bedford, MA, USA) prior to analysis by high-performance liquid chromatography (HPLC). A myricitrin standard stock solution of 1 mg/mL was prepared in MeOH. Calibration standard solutions at five levels were prepared by serially diluting the stock solution to concentrations of 6.25 μg/mL, 12.5 μg/mL, 25 μg/mL, 50 μg/mL, and 100 μg/mL. The solutions were filtered through a 0.2-μm syringe filter (Millipore) prior to injection. Each analysis was repeated three times, and the calibration curves were fitted by linear regression. HPLC-grade acetonitrile and water were obtained from J. T. Baker (Phillipsburg, NJ, USA). Analytical-grade formic acid was obtained from Wako (Tokyo, Japan). HPLC analysis was performed using an Agilent 1200 HPLC instrument (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump, vacuum degasser, autosampler, column compartment, and diode array detector. The column used was a Luna C_{18} (250 × 4.6 mm, 5.0 μm; Phenomenex, Torrance, CA, USA). The mobile phase was a mixture of Solvent A (water with 0.1% formic acid) and Solvent B (acetonitrile). A linear gradient elution was performed using 90–73% A over 30 minutes, 73–45% A over 15 minutes, and 45% B for 15 minutes, followed by column washing and reconditioning. The column temperature was maintained at 30 °C. The injection volume of sample was 5 μL and analysis was performed at a flow rate of 1.0 mL/min, with signal monitoring at 330 nm.

2.2. Cell culture and reagents

The primary HRPs (ACBRI 183) used in this study were purchased from Cell Systems Corporation (Kirkland, WA, USA). HRPs were maintained at 37 °C in a humidified incubator with a 5% CO_2 atmosphere and cultured in complete medium with 10% serum and Attachment Factor (CS-4Z0-500; Cell Systems, Kirkland, WA, USA), supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). Antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). Most other biochemical reagents, including myricitrin, were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified.

2.3. Determination of cell viability

Cells were seeded into 96-well plates at a density of 1×10^3 cells/well and incubated overnight prior to exposure to HG, *H. riparia* extract, or myricitrin. Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, MTT solution (0.5 mg/mL) was added to each well following the indicated treatment, and incubated for 4 hours at 37 °C. The supernatant was then removed and the formazan product was dissolved by adding 100 μL dimethylsulfoxide and placing the plate on a shaker for 15 minutes; the absorbance at 570 nm was then measured using a microtiter plate reader (BIO-TEK Synergy HT; Winooski, VT, USA). Cell viability was calculated using the optical density of each treatment group, expressed as a percentage of the control cell optical density.

2.4. Determination of apoptosis

The ratio of apoptotic cells was analyzed by flow cytometry using an annexin V-fluorescein isothiocyanate (FITC) and propidium iodide apoptosis detection kit (BD Bioscience, San Jose, CA, USA) following the manufacturer's protocol. HRPs were plated into 6-well plates at a density of 2.5×10^5 cells/well and exposed to HG (30 mM) in the presence or absence of *H. riparia* extract or myricitrin for 24 hours. Following digestion with trypsin and washing twice with phosphate-buffered saline, the cells were incubated with 5 μL annexin V-FITC and 5 μL propidium iodide for 5 minutes at room temperature in the dark. Binding buffer (400 mL) was then added and the percentage of apoptotic cells was analyzed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

2.5. Measurement of ROS

The cellular ROS level was measured using 2',7'-dichlorofluorescein diacetate (DCF-DA) as a substrate (Invitrogen). Briefly, HRPs were seeded into 6-well plates at a density of 2.5×10^5 cells/well and incubated for 24 hours. HRPs were then exposed to HG (30 mM) in the presence or absence of *H. riparia* extract and/or myricitrin for 24 hours. The cells were then incubated with 10-μM DCF-DA at 37 °C for 30 minutes before washing with phosphate-buffered saline and analyzing the intensity of green fluorescence by flow cytometry using 10,000 cells (FACSCalibur). The DCF-DA fluorescence in the HRPs was also visualized using an Olympus fluorescence microscope (BX81, Olympus, Tokyo, Japan).

2.6. Determination of HG-mediated changes in Sp1-driven gene expression

The effects of HG on gene expression in HRPs were investigated using the Cignal Finder 45-Pathway Reporter Array kit (SA Biosciences, Fredrick, MD, USA), according to the manufacturer's instructions. HRPs were seeded into 96-well cell culture plates containing luciferase reporters and lipofectamine transfection reagent, and incubated in the presence or absence of HG for 24 hours. Each experiment was performed in duplicate. Luciferase activity was then measured using the Dual-Glo luciferase reporter assay system (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. The signal was measured using a Centro XS3 LB960 microplate luminometer (Berthold Technology, Bad Wildbad, Germany). Data were normalized using the ratio of firefly to renilla luciferase activity. The fold change was calculated by dividing the normalized luciferase activities of each pathway-focused reporter in cells treated with HG by the normalized luciferase activity of the respective pathway-focused reporter in untreated cells.

2.7. Determination of protein levels

HRPs were plated in 60-mm culture dishes at 2×10^5 cells/dish 16–24 hours before exposure to HG (30 mM) in the presence or absence of *H. riparia* extract or myricitrin for 24 hours. Cells were then lysed with Laemmli sample buffer (Bio-Rad, Hercules, CA, USA), incubated at 100 °C for 5 minutes, and electrophoresed at 25 μg protein/lane on denaturing sodium dodecyl sulfate-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (GE Healthcare UK Ltd., Buckinghamshire, England) using a tank blotting apparatus (Bio-Rad). The membranes were then probed with the indicated specific primary antibodies, washed, and incubated with horseradish peroxidase-linked secondary antibodies. The membranes were washed three times prior to signal detection using the EzWestLumiOne enhanced chemiluminescence solution (Atto Corporation, Tokyo, Japan) and Fujifilm LAS-3000 (Fuji Photo, Tokyo, Japan).

2.8. Statistical analysis

All experiments were repeated at least three times and all values are represented as the mean ± the standard deviation (SD). The significance of group differences was determined using an analysis of variance and Tukey test (PRISM5 software, Graph Pad, La Jolla, CA, USA). A p value <0.05 was considered statistically significant.

3. Results

3.1. Myricitrin is a major constituent of the H. riparia extract

HPLC was applied to the quantitative analysis of myricitrin in the 80% ethanol extract of *H. riparia* leaves. The identification and quantitative determination of myricitrin in the extract were accomplished by comparing the peak retention times and areas with those of a myricitrin standard (Fig. 1). The myricitrin peak appeared at a retention time of 21.7 minutes, and this was identified as a major component within the *H. riparia* extract. The linearity of the HPLC method was checked by injecting five concentrations of myricitrin standard solutions. The calibration curve showed a good linearity ($R^2 = 0.9999$) in the range 6.25–100 μg/mL of a myricitrin standard. The content of myricitrin in the *H. riparia* extract was 12.8 mg/g.

3.2. H. riparia and myricitrin inhibit HG-induced cytotoxicity in HRPs

As shown in Fig. 2A, HRPs were exposed to a range of HG conditions (10–30 mM) for 24 hours and cell viability was decreased in the presence of 30-mM glucose, as compared with the control cells. Cell viability was also investigated in the presence of *H. riparia* extract or myricitrin in order to identify the non-toxic concentrations of these treatments. As shown in Fig. 2B and C, the average viabilities of HRPs exposed to *H. riparia* extract or myricitrin were >90% at the tested concentrations. Furthermore, pretreatment with *H. riparia* extract or myricitrin significant decreased the HG-induced cytotoxicity in HRPs, as shown in Fig. 2D and E. These results demonstrated that *H. riparia* extract or myricitrin could protect HRPs from HG-induced cytotoxicity.

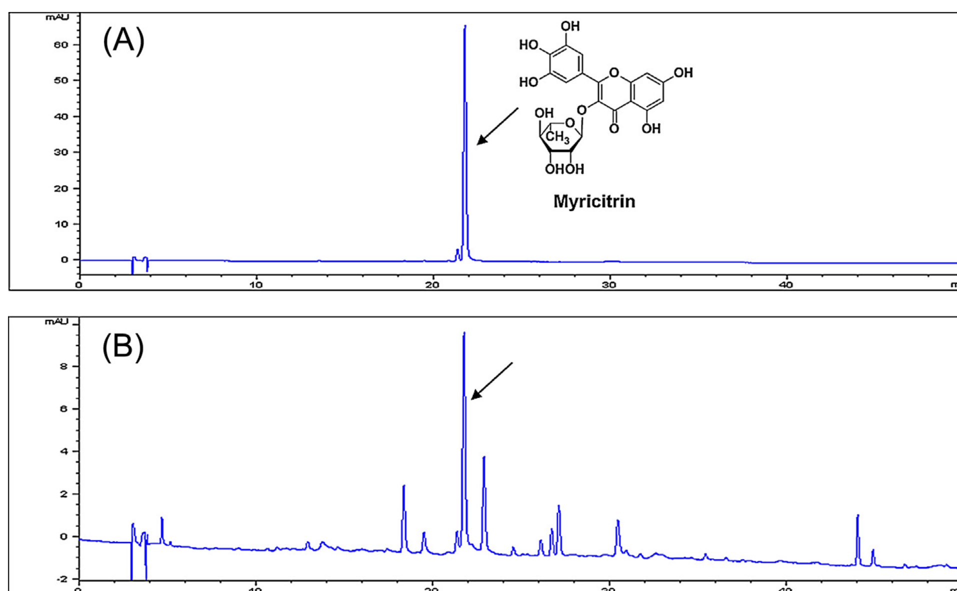

Fig. 1 – HPLC chromatographs. (A) A myricitrin standard. (B) The 80% ethanol extract of *Homonoia riparia* leaves with detection at 330 nm.

3.3. *H. riparia and myricitrin inhibit HG-induced apoptosis in HRPs*

Fig. 3 presents the results of flow cytometric analysis of apoptosis in HRPs exposed to HG in the presence or absence of *H. riparia* extract or myricitrin for 24 hours. Early and late apoptotic cells are shown in the lower and upper-right quadrant of the scatter plot, respectively, and live cells are in the lower-left quadrant. As shown in Fig. 3A and B, the proapoptotic effect of HG in HRPs was reversed by *H. riparia* extract or myricitrin. In addition, analysis of the effects of *H. riparia* extract or myricitrin on the expression of HG-induced apoptotic proteins [cleaved caspase 3 and poly (ADP-ribose) polymerase (PARP)] in HRPs by western blot indicated that these treatments decreased the levels of these proteins (Fig. 3C and D). These results indicated that *H. riparia* extract and myricitrin attenuated HG-induced apoptosis in HRPs.

3.4. *H. riparia and myricitrin inhibit HG-induced ROS generation in HRPs*

ROS levels were measured in HRPs that were pretreated with *H. riparia* extract, myricitrin, or a ROS scavenger [N-acetyl-l-cysteine, (NAC)] for 30 minutes prior to exposure to HG for 24 hours. These results showed that pretreatment with *H. riparia* extract, myricitrin, or NAC significantly attenuated the HG-induced increase in ROS levels in HRPs (Fig. 4A and B). These data suggest that ROS generation was involved in the HG-mediated changes in cell viability and apoptosis in HRPs.

3.5. *H. riparia- and myricitrin-mediated inhibition of HG-induced apoptosis of HRPs involves ERK-Sp1 signaling*

As shown in Fig. 5A, HRPs exposed to HG showed elevated levels of Sp1 activity. The phosphorylation of ERK was also examined as a measure of ERK signaling pathway activation. In HRPs that were pretreated with *H. riparia* extract or myricitrin, slight attenuations of HG-induced ERK phosphorylation and Sp1 upregulation were observed (Fig. 5B and C). Taken together, these results show that HG-induced apoptosis in HRPs might be partially mediated by the upregulation of Sp1 and activation of ERK signaling.

4. Discussion

The results of the present study showed that HG-induced apoptosis in HRPs involved ERK-Sp1 signaling and ROS. The loss of retinal cells, including retinal pericytes, in individuals with hyperglycemia is important for the pathogenesis of visual system changes in DR.[9,22] According to previous studies, HG increases the intracellular nitric oxide and superoxide levels, resulting in endothelial cell and mitochondrial dysfunction, which eventually leads to apoptosis.[22–24] Some mechanistic studies have shown that antiapoptotic effect of retinal pericytes is mediated via the inhibition of nicotinamide adenine dinucleotide phosphate oxidase activation, glyceraldehyde 3-phosphate dehydrogenase activity, and ROS generation.[2,10]

Our data showed decreased cell viability in the presence of a high concentration of glucose (30 mM) and also indicated that *H. riparia* extract or myricitrin significantly inhibited this HG-induced effect on HRP viability (Fig. 2). The balance between cell survival and death in retinal pericytes is closely associated with DR. These data show that the loss of HRPs due to hyperglycemia might underlie the progression of DR. Our previous study found that cycloartane-type triterpenes extracted from *H. riparia* leaves protected against vascular endothelial growth factor-induced angiogenesis in human umbilical vein endothelial cells. Similarly, the present data indicated that an *H. riparia* extract and one of its major components may afford protection against DR-related pathology.

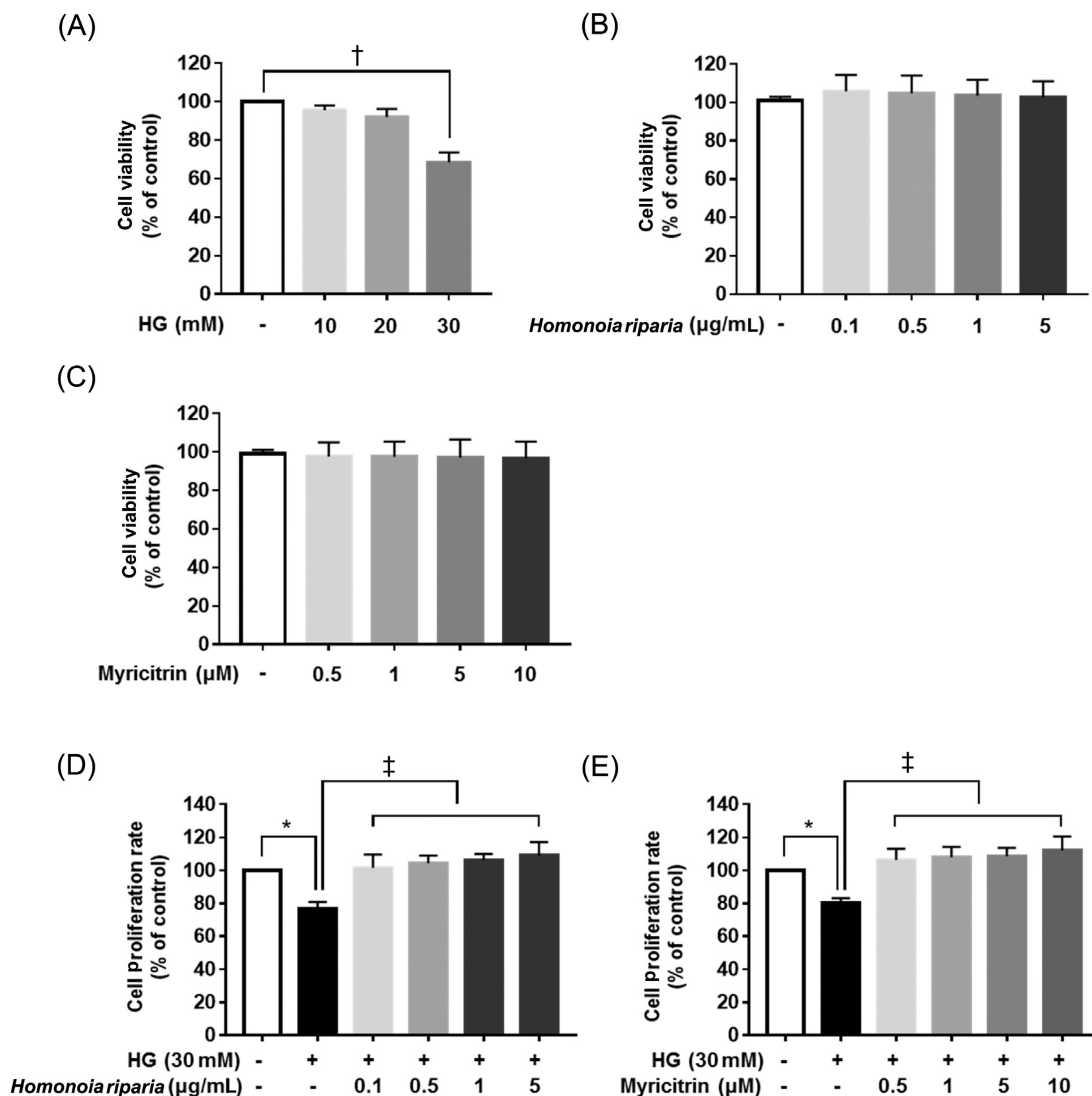

Fig. 2 – Effects of HG and/or *Homonoia riparia* extract/myricitrin on HRP viability. (A, B, and C). HRPs were incubated in the presence of the indicated concentrations of glucose (HG), *H. riparia* extract, or myricitrin for 24 hours. (D and E) HRPs were pretreated for 30 minutes with *H. riparia* extract or myricitrin prior to exposure to 30-mM glucose for 24 hours.
Cell viability was determined by MTT assay and measurement of absorbance at 560 nm. Data were representative of three independent experiments, and expressed as the mean ± SD.
****p* < 0.001.**
†*p* < 0.01 versus control.
‡*p* < 0.001 versus HG.
(-), untreated cells; (+), HG-treated cells.
HG, high glucose; HRP, human retinal pericytes; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SD, standard deviation.

The present study found that HG-induced apoptosis was associated with ROS accumulation, and that both apoptosis and ROS levels were significantly reduced by *H. riparia* extract or myricitrin. We also demonstrated that HG-induced apoptosis involved a caspase-3- and PARP-dependent pathway, raising the possibility that *H. riparia* and myricitrin may act on this pathway in HRPs (Figs. 3 and 4).

Mitogen-activated protein kinase signaling is involved in the upregulation and activation of Sp1, a critical transcriptional regulator of gene expression.[21,25] Furthermore, Sp1 activation dependent on ERK1/2 phosphorylation exerts reg-

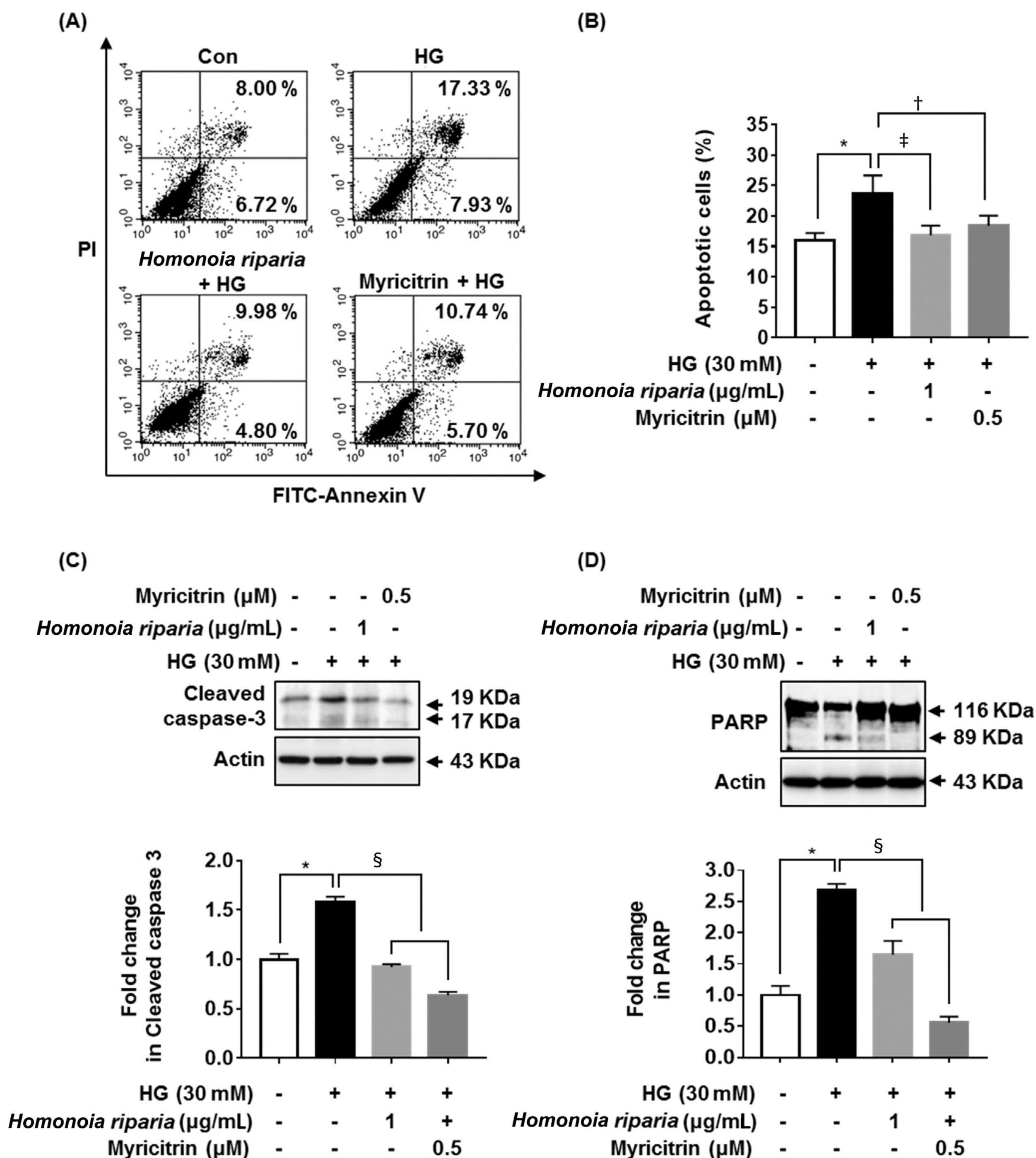

Fig. 3 – Effects of HG and/or *Homonoia riparia* extract/myricitrin on HRP apoptosis. HRPs were pretreated with *H. riparia* extract (5 μg/mL) or myricitrin (0.5 μM) for 30 minutes prior to exposure to 30-mM glucose (HG) for 24 hours. (A and B) Apoptotic cells were detected using FITC-labeled annexin V and flow cytometry. (C and D) The expression levels of caspase-3 and PARP were investigated by western blotting. Data were representative of three independent experiments, and expressed as the mean ± SD.

***$p < 0.001$ versus Control.**

†$p < 0.05$.

‡$p < 0.01$.

§$p < 0.001$ versus HG.

(-), untreated cells; (+), HG-treated cells.

Con, control;

FITC, V-fluorescein isothiocyanate; HG, high glucose; HRP, human retinal pericytes; PARP, poly (ADP-ribose) polymerase; SD, standard deviation.

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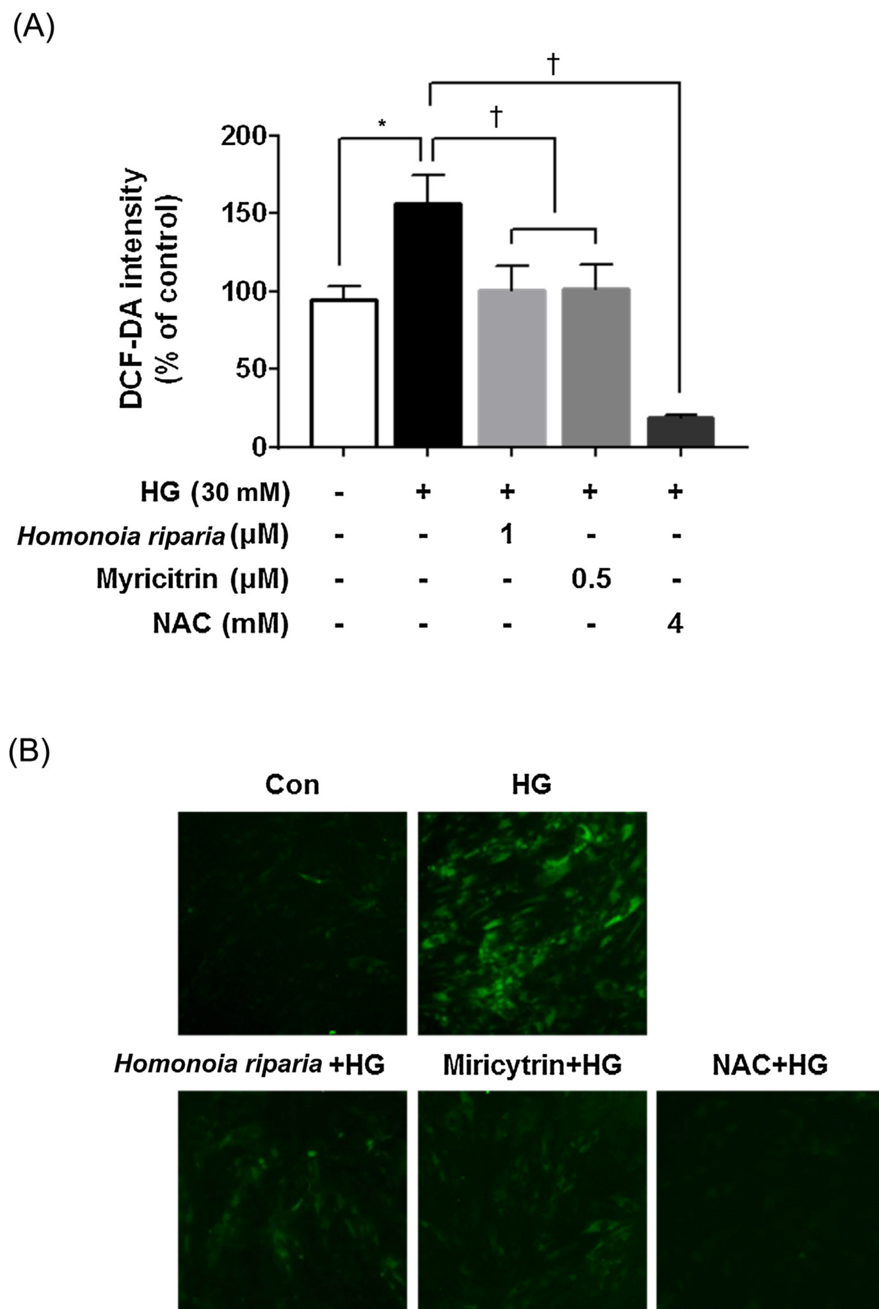

Fig. 4 – Involvement of ROS generation in HG-induced cytotoxicity and apoptosis in HRPs. HRPs were pretreated with *Homonoia riparia* extract, myricitrin, or N-acetyl-l-cysteine (NAC) for 30 minutes prior to exposure to 30-mM glucose (HG) for 24 hours. Intracellular ROS was detected as DCF-DA fluorescence using (A) flow cytometry and (B) fluorescence microscopy. Data were representative of three independent experiments, and expressed as the mean ± SD.
****p* < 0.001 versus Control.**
†*p* < 0.001 versus HG.
(-), untreated cells; (+), HG-treated cells.
Con, control;
DCF-DA, 2′,7′-dichlorofluorescein diacetate; HG, high glucose; HRP, human retinal pericytes; NAC, N-acetyl-l-cysteine; PI, propidium iodide; ROS, reactive oxygen species; SD, standard deviation.

ulating vascular endothelial growth factor expression.[21] The present study found that HG-induced apoptosis in HRPs was associated with ERK phosphorylation and increased Sp1 levels. These changes were attenuated by *H. riparia* extract or myricitrin (Fig. 5). However, further research will be required to study the specific mechanisms underlying the cell death observed, and to determine whether the mitochondrial pathway is involved.

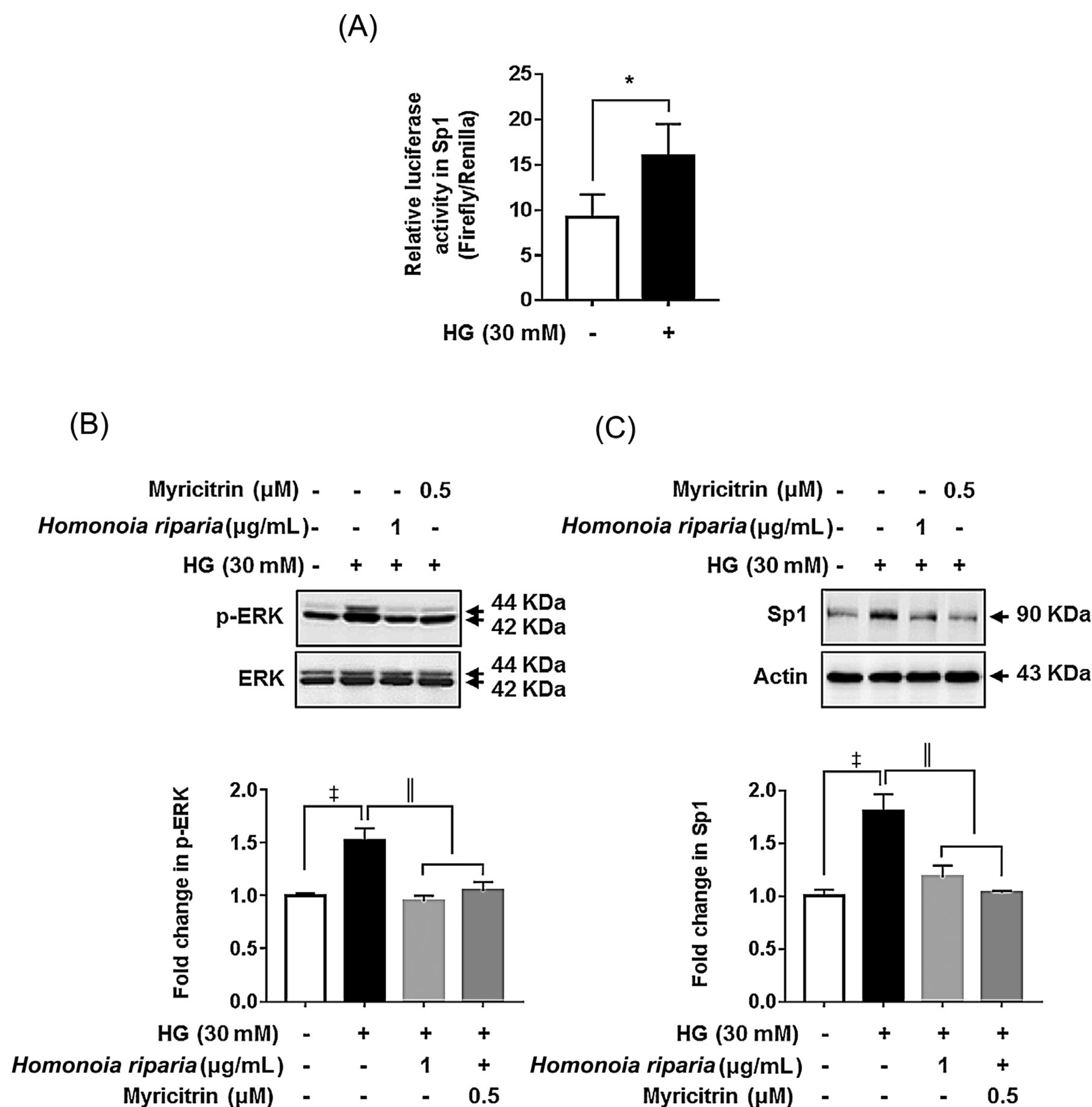

Fig. 5 – Effects of HG and *Homonoia riparia* extract/myricitrin on Sp1 and ERK in HRPs. (A) HG-driven gene expression in HRPs, treated as indicated. Firefly and Renilla luciferase activity was measured using a dual luciferase reporter assay system. (B and C) HRPs were pretreated with *H. riparia* extract (1 μg/mL) or myricitrin (0.5 μM) for 30 minutes prior to exposure to 30-mM glucose (HG) for 24 hours.
Total cell lysates were analyzed by western bloting using specific antibodies for phosphorylated ERK1/2 and Sp1. Total ERK and actin served as loading controls. Data were representative of three independent experiments, and expressed as the mean ± SD.
$^*p < 0.05$.
†$p < 0.01$.
‡$p < 0.001$ versus control.
§$p < 0.01$.
‖$p < 0.001$ versus HG.
(-), untreated cells; (+), HG-treated cells.
ERK, extracellular signal-regulated kinase; HG, high glucose; HRP, human retinal pericytes; SD, standard deviation; Sp1, specificity protein 1.

In conclusion, the present study demonstrated for the first time that HG-induced apoptosis of HRPs was independently activated by the ROS-mediated ERK-Sp1 signaling pathway and the caspase-3-PARP pathway, and that an *H. riparia* extract or myricitrin inhibited this cytotoxicity by reducing the activation of this pathway (Fig. 6). Therefore, these results suggested

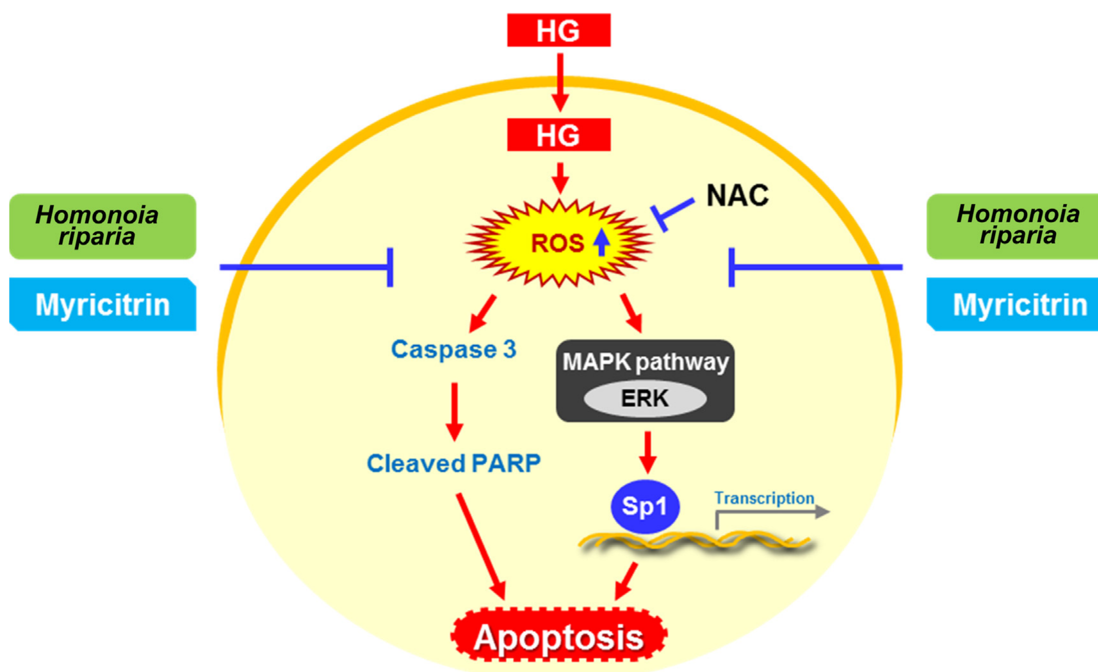

Fig. 6 – Proposed model of the ROS-ERK-Sp1 and ROS-caspase 3-PARP apoptotic pathway in HG-induced apoptosis of HRPs, indicating the protective effects of *Homonoia riparia* extract or myricitrin.
The HRPs stress induced by HG induces an increased generation of ROS. This increase in ROS production triggers activation of ERK and caspase 3- PARP, resulting in apoptosis.
ERK, extracellular signal-regulated kinase; HG, high glucose; HRP, human retinal pericytes; NAC, N-acetyl-l-cysteine; PARP, PARP, poly (ADP-ribose) polymerase; ROS, reactive oxygen species; Sp1, specificity protein 1.

that *H. riparia* extracts or myricitrin could be developed as phytochemicals and used to prevent the development of DR.

Conflicts of interest

The authors have no conflicts of interest to declare.

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