Effect of Baechu Kimchi Added Ecklonia cava Extracts on High Glucose-induced Oxidative Stress in Human Umbilical Vein Endothelial Cells

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ABSTRACT: Endothelial cell dysfunction is considered to be a major cause of vascular complications in diabetes. In the present study, we investigated the protective effect of a baechu kimchi added Ecklonia cava extract (BKE) against high glucose induced oxidative damage in human umbilical vein endothelial cells (HUVECs). Treatment with a high concentration of glucose (30 mM) induced cytotoxicity, whereas treatment with BKE protected HUVECs from high glucose induced damage; by restoring cell viability. In addition, BKE reduced lipid peroxidation, intracellular reactive oxygen species and nitric oxide levels in a dose dependent manner. Treatment with high glucose concentrations also induced the overexpression of inducible nitric oxide synthase, cyclooxygenase-2 and NF-κB proteins in HUVECs, but BKE treatment significantly reduced the overexpression of these proteins. These findings indicate that BKE may be a valuable treatment against high glucose-induced oxidative stress HUVECs.

Keywords: baechu kimchi, Ecklonia cava, high glucose, oxidative stress, human umbilical vein endothelial cells (HUVECs)

INTRODUCTION

Hyperglycemia induces oxidative stress via several mechanisms, including autoxidation of glucose formation of advanced glycation end-products (AGEs), and activation of the polyol pathway. Factors such as free fatty acids and leptin, also contribute to increased reactive oxygen species (ROS) generation in diabetics (1). Increased endothelial cell ROS concentrations are responsible for pathophysiological changes, such as vascular inflammation, and leukocyte adhesion, that lead to vascular complications in diabetes mellitus (2,3). Diabetic vascular complications such as atherosclerosis, retinopathy and neuropathy contribute to the increased morbidity and mortality of this disease (4). Cellular antioxidant activity is related to a reduction of diabetic vascular complications (3,5). Therefore, antioxidants can protect against cell damage caused by the hyperglycemia-induced oxidative stress associated with diabetes (6).

Sea algae contain an abundance of bioactive compounds and are potential sources of functional food sources (7). Brown algae, in particular, contain a variety of polyphenolic compounds, such as eckol, dieckol, and phloretin, which have influential biological activities (8,9). Ecklonia cava Kjellman (class, Phaeophyceae; family, Lessoniaceae; order, Laminariales), a brown algae, is popular in Korea and Japan as a food ingredients and as a folk medicine. Bioactive polyphenolic compounds isolated from Ecklonia cava have anti-inflammatory, antioxidative, anti-diabetic, and immunomodulatory activities (10-14).

Baechu kimchi, the most well-known Korean traditional food, is made by fermenting vegetables such as Chinese cabbage mixed with garlic, salt, chili peppers, and other spices (15). Recently, global interest in the use of kimchi as a side dish has increased. This increase in consumption is believed to be due to kimchi’s taste and its health-promoting characteristics [i.e., antioxidant activity (16), immunomodulatory effect (17), anti-cancer effect (18) and anti-diabetic effects (19)].

Our previous work indicates that baechu kimchi added Ecklonia cava extract (BKE) is a natural food that may alleviate postprandial hyperglycemia by inhibiting α-glucosidase activity (20). However, the effect of BKE on the function of endothelial cells experiencing hyperglycemia-related oxidative stress has not been examined. In the present study, we investigate the protective effect of BKE...
against high glucose-induced oxidative stress in human umbilical vein endothelial cells (HUVECs).

**MATERIALS AND METHODS**

**Materials**

The *Ecklonia cava* was collected along the coast of Jeju Island, Korea. The samples were washed three times with tap water to remove the salt, epiphytes, and sand attached to the surface, and then carefully rinsed with fresh water. The rinsed *Ecklonia cava* was then soften by boiling with 0.3% citric acid for 10 min. The baechu (i.e., Chinese cabbage) was divided into four pieces, and 3% (w/w) salt was evenly sprinkled on it. The four pieces of baechu were pickled in 10% salt water. The salted cabbages were washed twice under running water, and then were naturally dehydrated for 2 h.

The baechu kimchi was prepared by mixing the dehydrated, salted cabbage with spices, and softened *Ecklonia cava* [15% w/w, (Table 1)] and then fermenting the mixture at 5°C for 20 days.

Our previous study exploring the effect of fermentation time on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of baechu kimchi with *Ecklonia cava* revealed that DPPH radical scavenging activity was strongest in baechu kimchi with *Ecklonia cava* (pH 4.28, acidity 0.71) that had been fermented for 20 days at 5°C. Thus, optimally ripened baechu kimchi with *Ecklonia cava* was used in this study. The fermented baechu kimchi with *Ecklonia cava* was extracted with 80% methanol for 12 h three times. The resulting extracts were filtered through Whatman No. 1 filter paper and evaporated under a vacuum at 40°C. The resulting evaporated baechu kimchi with *Ecklonia cava* extract filtrates (i.e., BKE) were stored in a deep freezer (Nihon freezer Co., Tokyo, Japan).

**Cell culture**

HUVECs and endothelial cell basal medium-2 (EBM-2) bullet kits were purchased from Clonetics Corp. (San Diego, CA, USA). According to the supplier’s recommendation, cells were cultured in EBM-2 containing 2% fetal bovine serum (FBS; GIBCO Inc., Rockville, MD, USA) at 37°C in a humidified atmosphere containing 5% CO2. HUVEC passages 3 through 6 were used for this study.

**Assay of cell viability**

Cell viability was assessed by measuring HUVEC uptake of the neutral red, a supravital dye. Cells (4×10⁴ cells/well) were cultured in 24-well plates in a humidified atmosphere containing 5% CO₂ at 37°C. Cells, were pre-incubated with glucose (5.5 mM or 30 mM) for 48 h, and then co-treated with various concentrations (0.05 mg/mL, 0.1 mg/mL, 0.25 mg/mL, or 0.5 mg/mL) of BKE for 20 h. Thereafter, the medium was carefully removed from each well, and replaced with 0.5 mL of fresh medium containing neutral red (1.14 mmol/L). After 3 h of incubation, the neutral red medium was removed and the cells were washed twice with phosphate buffered saline (PBS, pH 7.4). The incorporated neutral red was released from the HUVEC cells by incubation in 1 mL of cell lysis buffer [50 mM/L Tris-HCl (pH 7.4), 150 mM/L NaCl, 5 mM/L dithiothreitol (DTT), and Triton X-100 (1%, v/v)], acetic acid (1%, v/v), ethanol (50%, v/v) at room temperature for 15 min. The cell lysates were then centrifuged and dye uptake was determined by spectrophotometrically measuring the absorbance of the resulting supernatant at 540 nm.

**Assay of lipid peroxidation**

In this study, lipid peroxidation, was caused by the ROS generated from high glucose-induced oxidative damage of HUVECs. Lipid peroxidation was measured by thiobarbituric acid reactive substances (TBARS) production. Cells (4×10⁴ cells/well) were seeded in a 24-well plate and pre-incubated with glucose (5.5 mM or 30 mM) in a humidified atmosphere containing 5% CO₂ at 37°C. After 48 h of incubation, the cells were treated with various concentrations (0.05 mg/mL, 0.1 mg/mL, 0.25 mg/mL, or 0.5 mg/mL) of BKE and incubated for an additional 20 h. Then a 200 μL sample of each medium supernatant was mixed with 400 μL of TBARS solution and boiled at 95°C for 20 min. The absorbance of the resulting solution was measured at 532 nm. TBARS concentrations were extrapolated from a 1,1,3,3-tetraethoxy-propane serial dilution standard curve and expressed as equivalent nmoles of malondialdehyde (MDA).

**Assay of intracellular ROS levels**

Intracellular ROS levels were measured by the 2′,7′-dichlorofluorescin diacetate (DCF-DA) assay. DCF-DA can be deacetylated in cells, where it reacts quantitatively with intracellular radicals to produce a fluorescent

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**Table 1. Recipes of baechu kimchi added Ecklonia cava and baechu kimchi used in this study (%)**

| Ingredients                        | Baechu kimchi added Ecklonia cava |
|------------------------------------|-----------------------------------|
| Korean cabbage                     | 85.00                             |
| *Ecklonia cava*                    | 12.75                             |
| Green onion                        | 2.30                              |
| Garlic                             | 2.50                              |
| Ginger                             | 0.50                              |
| Red pepper powder                  | 2.60                              |
| Fermented shrimp juice             | 1.70                              |
| Sand eel fermented juice           | 1.30                              |
| Glutinous rice paste               | 3.60                              |
| Sugar                              | 0.50                              |
| Total                              | 100.00                            |

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USA) at 37°C in a humidified atmosphere containing 5% CO₂ at 37°C.
product, DCF, that is retained within the cells. Therefore, DCF-DA was used to evaluate the generation of ROS in HUVECs with high glucose-induced oxidative damage. Cells \((2 \times 10^4 \text{ cells/well})\) were seeded in a 96-well plate and pre-incubated with glucose \((5.5 \text{ mM or 30 mM})\) in a humidified atmosphere containing \(5\% \text{ CO}_2\) at 37°C. After 48 h of incubation, the cells were treated with various concentrations \((0.05 \text{ mg/mL}, 0.1 \text{ mg/mL}, 0.25 \text{ mg/mL}, \text{ or } 0.5 \text{ mg/mL})\) of BKE and incubated for an additional 20 h. Thereafter, the medium was removed and the cells were washed twice with PBS. HUVECs were then incubated with 100 μM DCF-DA for 30 min at room temperature. Fluorescence was measured with a fluorescence plate reader.

**Assay of nitric oxide (NO) levels**

High glucose-induced (i.e., the end product of NO generation), accumulation in HUVECs was assessed by the Griess reaction. Cells \((2 \times 10^4 \text{ cells/well})\) were seeded in a 96-well plate and pre-incubated with glucose \((5.5 \text{ mM or 30 mM})\) in a humidified atmosphere containing \(5\% \text{ CO}_2\) at 37°C. After 48 h of incubation, the cells were treated with various concentrations \((0.05 \text{ mg/mL}, 0.1 \text{ mg/mL}, 0.25 \text{ mg/mL}, \text{ or } 0.5 \text{ mg/mL})\) of BKE and incubated for an additional 20 h. Thereafter, each 50 μL of each culture supernatant was mixed with an equal volume of Griess reagent \([0.1\% \text{ N}-\text{(1-naphthyl)-ethylenediamine, } 1\% \text{ sulfanilamide in 5% phosphoric acid}]\) and incubated at room temperature for 10 min. The absorbance of the resulting solution was measured at 550 nm with a microplate reader. A series of known concentrations of sodium nitrite was used as a standard.

**Total and nuclear protein extracts**

To obtain total protein extracts, cells were homogenized in an ice-cold lysis buffer containing 250 mM NaCl, 25 mM Tris-HCl (pH 7.5), 1% v/v NP-40, 1 mM DTT, 1 mM PMSF, and a protease inhibitor cocktail \((10 \mu\text{g/mL aprotinin, } 1 \mu\text{g/mL leupeptin})\). The homogenates were then centrifuged at 20,000 g for 15 min at 4°C, and the resulting supernatant was collected for further use.

To obtain nuclear protein extracts, cells were homogenized in an ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 15 mM CaCl₂, 1.5 mM sucrose, 1 mM DTT, and a protease inhibitor cocktail \((10 \mu\text{g/mL aprotinin, } 1 \mu\text{g/mL leupeptin})\). Then, the homogenates were centrifuged at 11,000 g for 20 min at 4°C. The resulting supernatants were mixed with an extraction buffer containing 20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 0.42 M NaCl, 0.2 mM EDTA, 25% (v/v) glycerol, 10 mM DTT, and a protease inhibitor cocktail \((10 \mu\text{g/mL aprotinin, } 1 \mu\text{g/mL leupeptin})\). The samples were shaken gently for 30 min and centrifuged at 21,000 g for 5 min at 4°C. The resulting pellets were used as nuclear protein extracts. A Bio-Rad (Hercules, CA, USA) protein kit was used to determine total and nuclear protein content. Bovine serum albumin was used as the standard.

**Immunoblotting**

iNOS and COX-2 expression and NF-κB p65 DNA-binding activity were determined by western blot analysis. Briefly, 20 μg of total protein \((\text{iNOS, COX-2, or NF-κB})\) were electrophoresed through a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Separated proteins were electrophoretically transferred to a pure nitrocellulose membrane, blocked with a 5% skim milk solution for 1 h, and then incubated with primary antibodies \((1:1000; \text{Abcam, Cambridge, UK})\) overnight at 4°C. After washing, the blots were incubated with a goat anti-rabbit or a goat anti-mouse IgG HRP-conjugated secondary antibody for 1 h at room temperature. Each antigen-antibody complex was visualized using ECL Western Blotting Detection Reagents and detected by chemiluminescence with an LAS-1000 plus (FUJIFILM, Tokyo, Japan). Band densities were determined by an image analyzer (Multi Gauge V3.1) and normalized to β-actin for total protein and nuclear protein.

**Statistical analysis**

The data are represented as the mean±standard error (SE) of triplicate experiments. SAS 9.0 software (SAS Institute, Cary, NC, USA) was used for statistical analysis. The values were evaluated by one-way analysis of variance (ANOVA), followed by post-hoc Duncan’s multiple range tests. P values less than 0.05 were considered statistically significant.

**RESULTS AND DISCUSSION**

**Cell viability**

Fig. 1 shows the effect of BKE on viability of HUVECs treated with high levels of glucose (i.e., 30 mM). Cells were treated with BKE for 20 h under oxidative stress-inducing conditions and then cell viability was examined with a neutral red assay. When HUVECs were treated with high glucose levels without BKE for 48 h, cell viability was significantly decreased to 26.34%. BKE treatment protected HUVECs from high glucose-induced damage. Treatment with 0.05 mg/mL, 0.1 mg/mL, 0.25 mg/mL, or 0.5 mg/mL dose of BKE significantly increased cell viability to 26.34%, 44.82%, 58.91%, 67.90%, and 74.52%, respectively.

These results suggest that BKE protects endothelial cells from high glucose-induced oxidative damage. Previous studies have reported that high concentrations of glucose can cause endothelial cell damage, leading to in-
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Fig. 1. Effect of BKE on cell viability in high glucose-treated HUVECs. Cells in 24-well plates (4x10^4 cells/well) were preincubated in medium containing 5.5 mM glucose (i.e., low glucose concentration) or medium containing 30 mM glucose (i.e., high glucose concentration) for 48 h and then incubated with 0 mg/mL, 0.05 mg/mL, 0.1 mg/mL, 0.25 mg/mL, and 0.5 mg/mL doses of BKE for 20 h. Each value is expressed as mean±SD (n=3). Values with different letters (a-f) are significantly different at P<0.05 as analyzed by a Duncan’s multiple range test. BKE, baechu kimchi added Ecklonia cava extract.

Fig. 2. Effect of BKE on TBARS generation in high glucose-treated HUVECs. Cells in 24-well plates (4x10^4 cells/well) were preincubated with glucose in the absence or presence of BKE, as described in the legend to Fig. 1. Each value is expressed as mean±SD (n=3). Values with different letters (a-e) are significantly different at P<0.05 as analyzed by a Duncan’s multiple range test. BKE, baechu kimchi added Ecklonia cava extract.

creased cell apoptosis in diabetes (21). The extracts from natural products such as vegetables and seaweeds significantly increase cell viability under conditions of oxidative damage (22,23). In this study, BKE treatment significantly increased endothelial cell viability.

Lipid peroxidation
The effect of BKE on high glucose-induced lipid peroxidation in HUVECs was determined by measuring TBARS as a lipid peroxidation product. When HUVECs were treated with 30 mM glucose for 48 h, the TBARS level was significantly greater than the TBARS level of cells treated with 5.5 mM glucose. The TBARS level of 5.5 mM glucose-treated cells was 0.13 nmol MDA, whereas the TBARS level of 30 mM glucose treated cells was 0.27 nmol MDA (Fig. 2). However, the TBARS levels of cells treated with BKE were significantly decreased. The TBARS levels of HUVECs that were treated with 0.05 mg/mL, 0.1 mg/mL, 0.25 mg/mL, or 0.5 mg/mL dose of BKE were 0.23 nmol MDA, 0.19 nmol MDA, 0.15 nmol MDA, and 0.13 nmol MDA, respectively.

Lipid peroxidation is associated with oxidative damage, increased cell injury, and increased cell death (24). Lipid peroxidation is begins when free radicals attack membrane lipids. This attack generates, large amounts of reactive products, that have been implicated the initiation and promotion of cell damage (25). MDA is a thiobarbituric acid reactive substance that is the end-product of lipid peroxidation and is widely used as a biomarker of oxidative stress (8). Increased production of MDA has been reported in the plasma membranes of diabetic patients (26), and high-lipid peroxide levels have been observed in diabetic rats (27). Previous work suggests that increased MDA levels lead to cell membrane damage, which is associated with the progression of diabetes and the development of diabetic complications (28). Vitamin E and α-lipoic acid from natural oils and vegetables have been used as antioxidants and to prevent lipid peroxidation (29,30). In our study, BKE treatment had an inhibitory effect against lipid peroxidation. This implies that the antioxidant phytochemical compounds in BKE contribute to inhibition of lipid peroxide production. The results of the present study also confirmed that baechu kimchi and Ecklonia cava contain various antioxidant compounds such as phloroglucinol, eckol and 3-(4'-hydroxyl-3',5'-dimethoxyphenyl) propionic acid (31-33) which may have the an inhibitory effect on lipid peroxidation.

Intracellular ROS generation
The exposure of endothelial cells to high glucose concentrations increases ROS production at the mitochondrial level, leading to increased cellular apoptosis (34). As shown in Fig. 3, the intracellular ROS level in HUVECs that had been treated with 30 mM glucose was significantly greater than that of HUVECs that had been treated with 5.5 mM (230.71% vs. 100%). However, BKE significantly inhibited the production of ROS in HUVECs that had been treated with high glucose concentrations. The ROS levels of HUVECs that had been treated with 0.05 mg/mL, 0.1 mg/mL, 0.25 mg/mL, or 0.5 mg/mL dose of BKE were significantly decreased in a dose dependent manner to 174.19%, 152.15%, 124.23% and 112.46%, respectively.

The major sources of ROS in hyperglycemia are the mitochondria and increased protein glycation, PKC activation, NAD(P)H oxidase activation and nitric oxide synthase (NOS) uncoupling (35). Increased ROS accumulation causes damage to DNA, proteins, and cell membranes, which might be responsible for the endothe-
Fig. 3. Effect of BKE on intracellular ROS generation in high glucose-treated HUVECs. Cells in 24-well plates (4×10^4 cells/well) were preincubated with glucose in the absence or presence of BKE, as described in the legend to Fig. 1. Each value is expressed as mean±SD (n=3). Values with different letters (a-f) are significantly different at P<0.05 as analyzed by a Duncan’s multiple range test. BKE, baechu kimchi added Ecklonia cava extract.

Fig. 4. Effect of BKE on NO level in high glucose-treated HUVECs. Cells in 24-well plates (4×10^4 cells/well) were preincubated with glucose in the absence or presence of BKE, as described in the legend to Fig. 1. Each value is expressed as mean±SD (n=3). Values with different letters (a-f) are significantly different at P<0.05 as analyzed by a Duncan’s multiple range test. BKE, baechu kimchi added Ecklonia cava extract.

Gliarial dysfunction observed in diabetes (36,37). Furthermore, the increased ROS generation associated with high concentrations of glucose stimulates death pathways leading to endothelial cell apoptosis, which subsequently results in microvascular barrier dysfunction (38). As a result, ROS are a major trigger in the development of diabetic complications (39). Therefore, inhibition of ROS generation is necessary to protect endothelial cells from oxidative damage and prevent diabetic complications. Natural antioxidants such as phenolic compounds from vegetables may be useful for the prevention of ROS generation (40,41). In the present study, BKE significantly reduced the production of ROS in HUVECs treated with high glucose concentration. This reduction in ROS production may due to the presence of antioxidant compounds such as result polyphenols [e.g., eckol (32) and dieckol (42)] and flavonoids in BKE.

NO generation
Hyperglycemia has been reported to accelerate atherosclerosis by inducing endothelial dysfunction (43). It is well known that high glucose concentrations stimulate the overproduction of superoxide anions (O_2^-) and NO (44). NO interacts with O_2^- to form peroxynitrite (ONOO^-) that leads to highly reactive oxidative damage associated with diabetes (45,46). As a result, NO is a mediator of endothelial dysfunction in diabetes (47). As shown in Fig. 4, NO production was significantly higher in HUVECs treated with 30 mM glucose than in HUVECs treated with 5.5 mM glucose. In contrast, NO production in HUVECs treated with BKE decreased in a dose dependent manner. The level of NO in high glucose-treated HUVECs was 315.91%, but treatment with 0.05 mg/mL, 0.1 mg/mL, 0.25 mg/mL, or 0.5 mg/mL of BKE resulted in a significant decrease in NO levels to 302.27%, 242.41%, 208.36%, and 163.26%, respectively.

Excess NO causes oxidative stress, DNA damage, and mitochondrial dysfunction, which can lead to cell death by apoptosis or necrosis. Inhibition of NO production may help prevent cell death and help cell recover from damage (48). Previous reports indicate that flavonoids and polyphenols from herbal tea (49), bamboo (50) and red grapes (51) inhibit NO production and iNOS expression. Our study demonstrated that BKE significantly reduced NO production in HUVECs that had been exposed to high glucose concentration. These results suggest that BKE may be an effective inhibitor of NO production and may protect against the cellular injury caused by NO generation.

Immunoblotting
iNOS and COX-2 expression: Inflammatory genes, such as iNOS and COX-2, appear to be upregulated in high glucose-stimulated HUVECs (52). To determine whether BKE inhibits high glucose-induced overexpression of the iNOS and COX-2 proteins, HUVECs were treated with 0.10 mg/mL or 0.50 mg/mL doses of BKE. As shown in Fig. 5, the protein levels of iNOS and COX-2 were greater in the 30 mM glucose-treated HUVECs than in the 5.5 mM glucose-treated HUVECs. However, treatment with BKE significantly reduced the expression of iNOS and COX-2.

In endothelial cells, high levels of NO are released from iNOS under conditions of oxidative stress. Precious work indicates that excess NO is responsible for diabetic-vascular complications (53). COX-2 is not expressed under normal physiological conditions but it can be rapidly expressed under certain circumstances, including hyperglycemia (54). A recent study revealed that COX-2 is highly expressed in a type 2 diabetic mouse model.
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In our study, high glucose concentrations induced the overexpression of iNOS and COX-2 proteins. However, treatment with BKE reduced the expression of iNOS and COX-2 proteins. These results suggest that BKE alleviates oxidative stress by inhibiting the expression of the iNOS and COX-2 enzymes.

**NF-κB activation:** To determine whether BKE inhibits high glucose-induced overexpression of NF-κB, we used nuclear extracts of HUVECs that had been treated with a high glucose concentration in the absence or presence of 0.10 mg/mL or 0.50 mg/mL doses of BKE. NF-κB activity was significantly higher in 30 mM glucose-treated HUVECs compared to 5.5 mM glucose-treated HUVECs (Fig. 6). However, the expression of NF-κB was significantly decreased by BKE treatment.

Transcription factor NF-κB is present in the cytoplasm in a latent inactive state due to its binding to an inhibitory protein, I-κB. Under high glucose conditions, I-κB phosphorylation is increased, allowing NF-κB to translocate into the nucleus, and activate, the expression of immune, inflammatory, growth, and adhesion genes (57,58). NF-κB has been shown to regulate a variety of genes that are induced in atherosclerotic lesions, including iNOS and COX-2 (59,60). Many studies have supported the notion that NF-κB activation is a key mechanism for the acceleration of vascular complications (61-63). In the present study, BKE reduced NF-κB protein expression in HUVECs that had been treated with a high concentration of glucose. This suggests that BKE may protect HUVECs against the oxidative stress induced by high levels of glucose and might also be useful for inhibiting NF-κB activation.

In summary, we demonstrated that BKE alleviates high glucose-induced oxidative stress by reducing lipid peroxidation, ROS production and NO production. In addition, BKE inhibited iNOS, COX-2 and NF-κB proteins expression under oxidative stress-inducing conditions. These findings reveal the potential benefits of BKE as a valuable treatment for the reduction of oxidative stress induced by high glucose concentration.

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**AUTHOR DISCLOSURE STATEMENT**

The authors declare no conflict of interest.
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