



## ANTIOXIDANT AND MODULATION OF APOPTOSIS-RELATED PATHWAYS OF MAKIANG FRUIT EXTRACT IN ARPE-19 CELLS EXPOSED TO GLUCOSE-INDUCED OXIDATIVE STRESS

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### ABSTRACT

Diabetic retinopathy (DR) is a leading cause of visual loss resulting from microvascular complications of diabetes mellitus. In DR, retinal pigment epithelial (RPE) cells are exposed to elevated blood sugar levels for prolonged periods, leading to oxidative stress and apoptosis. Makiang fruit (*Cleistocalyx nervosum* var. *paniala*) is rich in anthocyanins (ACN), which possess antioxidant and anti-apoptotic properties. However, its protective effects on RPE cells against glucose-induced oxidative stress have not been studied. This study aimed to examine the effects of Makiang fruit extract on human retinal pigment epithelial (ARPE-19) cells exposed to glucose-induced oxidative stress. Makiang fruits were extracted with 95% ethanol (MKE95). The results showed that MKE95 exhibited antioxidant capacity as measured by the DPPH, ORAC, and FRAP assays. For the cell-based experiments, ARPE-19 cells were co-treated with MKE95 (50–200 µg/mL) and 0.5% DMSO in DMEM/F-12 (5.5 mM glucose), with D-glucose adjusted to 30 mM. At the highest concentration, MKE95 significantly ( $p < 0.05$ ) improved cell viability by 15.4%, reduced intracellular ROS by 37.8%, and decreased caspase-9 and -3 activities (1.66- and 1.64-fold, respectively) compared to the glucose-treated group. It also enhanced antioxidant defenses, increasing SOD, CAT, GPx activities, and GSH levels by 22.4%, 26.3%, 24.7%, and 24.4%, respectively, in a dose-dependent manner. However, these findings are derived from an *in vitro* model. Although Makiang extract demonstrates protective effects against glucose-induced oxidative stress in RPE cells, further *in vivo* and clinical studies are necessary to confirm its potential role in preventing diabetic retinopathy.

**Keywords:** Makiang fruit, diabetic retinopathy, human retinal pigment epithelial ARPE-19 cells, oxidative stress, anthocyanin, antioxidant defense

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## Introduction

Diabetic retinopathy (DR) is one of the most common complications of long-duration diabetes mellitus (DM), affecting approximately 22% of individuals with diabetes worldwide, with about 10% progressing to vision-threatening stages such as proliferative DR.<sup>1-4</sup> The increasing global burden of DR presents significant clinical and economic challenges.<sup>2,5,6</sup> The retinal pigment epithelium (RPE) is essential for maintaining retinal homeostasis, facilitating nutrient transport, and supporting photoreceptor function.<sup>7,8</sup> In DR, chronic hyperglycemia disrupts the blood-retinal barrier, heightens RPE permeability, and leads to retinal damage.<sup>9</sup>

Oxidative stress is primarily driven by the overproduction of reactive oxygen species (ROS) that overwhelm endogenous antioxidant defenses. The intracellular antioxidant defense system plays a crucial role in maintaining redox homeostasis and protecting cells from oxidative stress, which induces cellular damage. Key enzymatic antioxidants, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), function cooperatively to detoxify ROS. SOD catalyzes the dismutation of superoxide radicals to form hydrogen peroxide, which is subsequently converted to water by CAT and GPx. In parallel, glutathione (GSH) serves as a major non-enzymatic antioxidant, maintaining cellular redox balance and supporting GPx activity. Therefore, the inhibition of these intracellular antioxidant defenses results in excessive oxidative stress within cells, compromising cellular function and leading to both structural and functional changes.<sup>10-12</sup> This accumulation of oxidative stress affects essential cellular processes, promotes mitochondrial dysfunction, activates key regulatory proteins in the intrinsic apoptotic pathway, particularly via caspase-9 and caspase-3, which promotes RPE cell death and progression of DR.<sup>13,14</sup> Given this pathological mechanism, restoring redox

balance between oxidants and antioxidants through modulated apoptosis-related pathways may be proposed as a potential approach for DR prevention or therapy.<sup>15-17</sup>

Makiang fruit (*Cleistocalyx nervosum* var. *paniala*), a member of the Myrtaceae family, is widely consumed as a local fruit in Southeast Asia, particularly in northern Thailand, where it is valued for its distinctive taste and natural pigmentation. Its ripe fruit contains low energy and a low glycemic index. Traditionally, fruits rich in anthocyanins (ACN) from the Myrtaceae family have been associated with various health-promoting properties, largely attributed to their high levels of phenolic compounds and antioxidants.<sup>18-19</sup> Previous studies using both *in vitro* and *in vivo* models have reported that Makiang extracts exhibit neuroprotective effects in PC12 cells<sup>20</sup>, protect *Caenorhabditis elegans* from oxidative stress via the Nrf-2 pathway, and prevent hydrogen peroxide-induced DNA damage in human monocyte U937 cells.<sup>21</sup> Notably, previous studies by Panritdum P. et al (2024) have identified that one of the major bioactive ACN constituents in *C. Nervosum* was cyanidin-3-glucoside (C3G).<sup>22</sup> The ACN has represented diverse biological and pharmacological effects, with remarkably strong antioxidant properties.<sup>23</sup> Research indicates that ACN can significantly reduce the risk of diabetes by lowering oxidative stress levels and protecting adult retinal pigment epithelial ARPE-19 cells from oxidative damage.<sup>24</sup> Moreover, recent articles have revealed that ACN possesses therapeutic potential for improving ocular health in clinical studies.<sup>25</sup> Despite growing interest in natural antioxidants for retinal protection<sup>26,27</sup>, the potential of Makiang fruit extract to protect human RPE cells from oxidative stress associated with DR remains unexplored. Therefore, this study aimed to address this gap by investigating the protective effects of Makiang fruit extract against glucose-induced oxidative stress in human retinal pigment epithelial (ARPE-19) cells.

## Materials and Methods

### 1. Chemicals and reagents

All supplements for ARPE-19 cells were obtained from Gibco (Grand Island, NY 14072, USA). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) (CAT. # T0793) was received from Bio Basic (NY, USA). 2',7'-dichlorofluorescein diacetate (DCFH-DA) assay (CAT. #D6883), dimethyl sulfoxide (DMSO), (CAT. #D1435), folin & ciocalteu's phenol reagent (CAT. # F9252), 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) (CAT. # T1253), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (CAT. #D9132) and (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) (CAT. #648471) were obtained from Sigma-Aldrich (Dorset, UK). The SOD (CAT. #706002), CAT (CAT. #707002), GPx (CAT. #703102), and GSH (CAT. #703002) assay kits were from Cayman Chemical (Ann Arbor, MI, USA). The caspase-9 (CAT. #MBS9718959) and caspase-3 (CAT. #MBS9718955) assay kits were obtained from MyBioSource (San Diego, USA).

### 2. Sample preparation

Makiang fruits were obtained from the Plant Genetic Conservation Project initiated by Her Royal Highness Princess Maha Chakri Sirindhorn (RSPG) and harvested in Lampang province, Thailand, during July–August. Botanical identification and authentication of the plant material were performed by Assistant Professor Dr. Thaya Jenjittikul, Department of Plant Science, Faculty of Science, Mahidol University, and a voucher specimen (No. 9428) was deposited at Suan Luang Rama IX Herbarium, Bangkok, Thailand. Only ripe and undamaged fruits were selected, washed with DI water, and their pulp was separated and blended. The homogenized sample was lyophilized until dry. The freeze-dried sample was ground, vacuum-sealed in aluminum foil, and stored at -20 °C.

### 3. Sample extraction

The freeze-dried sample (1g) was extracted with 15 mL of 95% ethanol (1:15, w/v). The 95% ethanol was chosen for its effectiveness in extracting phenolic compounds and anthocyanins, major bioactive constituents of Makiang fruit. Moreover,

ethanol is also widely used as a food-grade solvent for the extraction of phytochemicals. The extraction was conducted at room temperature overnight with agitation to ensure sufficient extraction efficiency and recovery of bioactive compounds. The mixture was centrifuged (4600 rpm, 10 min), and the supernatant was collected. The extraction was repeated twice, and the pooled extracts were evaporated using a vacuum evaporator (Buchi Rotavapor-Re, U.S.A.) at 40 °C, then dissolved in 95% ethanol and dried under nitrogen. The final extract (MKE95) was stored at -20 °C until use. The extraction yield was approximately 30%.

$$\text{Extraction Yield (\%)} = \left( \frac{\text{Weight of the dried extract}}{\text{Initial weight of dried sample}} \right) \times 100$$

### 4. Determination of antioxidant properties in MKE95

#### • The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The DPPH assay assesses the antioxidant capacity of a substance by evaluating its ability to neutralize free radicals, which can be activated by a hydrogen atom from antioxidant molecules.<sup>28</sup> The experimental procedure followed the protocols outlined in previous studies.<sup>22,29</sup> Briefly, MKE95 at 5000, 10000, and 20000 µg were added to a 96-well plate, followed by DPPH solution in 90% ethanol. After incubation in the dark at room temperature for 30 min, the absorbance was measured at 520 nm using a microplate reader (BioTek®, Vermont, USA). To correct for potential interference from the DPPH reagent's inherent color, wells containing DPPH solution without sample (Abs<sub>Control</sub>) were included in each assay plate, and their absorbance values were subtracted from those of the sample wells. Trolox was used as a positive control and served as the reference standard.

$$\% \text{ Inhibition} = \left( \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Extract}}}{\text{Abs}_{\text{Control}}} \right) \times 100$$

The % inhibition was reported as IC<sub>50</sub> values in milligrams per milliliter (mg/mL), calculated from the standard curve.

- Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was performed to evaluate antioxidant capacity by measuring the inhibition of peroxy radical-induced oxidation, generated by 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH).<sup>30</sup> Trolox standards (7.8-500  $\mu$ M) and MKE95 at 10, 50, and 100  $\mu$ g were added to a 96-well plate, followed by fluorescein solution, and the plate was incubated at 37°C for 30 min. Subsequently, AAPH (153 mM) was added to initiate the reaction, and fluorescence was measured for 3 h at 485 nm excitation and 520 nm emission using a microplate reader. To account for potential background fluorescence, blank wells containing all reagents except the sample were included in each plate. Antioxidant activity was calculated from the area under the curve (AUC) and represented as  $\mu$ mol Trolox equivalents per gram of dry weight ( $\mu$ mol TE/g).

- Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was performed to evaluate antioxidant capacity based on the reduction of  $\text{Fe}^{3+}$ -TPTZ to  $\text{Fe}^{2+}$ -TPTZ.<sup>31,32</sup> Briefly, 20  $\mu$ L of MKE95 at 100, 500, and 1000  $\mu$ g was added to 150  $\mu$ L of FRAP reagent (TPTZ and ferric chloride in acetate buffer) in a 96-well plate. After incubation in the dark at room temperature for 8 min, the absorbance was measured at 600 nm using a microplate reader. Blank wells containing only FRAP reagent, without a sample, were included on each plate. The absorbance values from the blank wells were subtracted from the readings of all sample wells. Antioxidant capacity was calculated from the Trolox standard curve and expressed as  $\mu$ mol Trolox equivalents per gram of dry weight ( $\mu$ mol TE/g).

## 5. Cell culture

Human retinal pigment epithelial (ARPE-19) cells (CAT. #CRL-2302) were acquired from the American Type Culture Collection (ATCC) (Manassas, VA 20110-2209, USA). Cells were cultured in a complete medium composed of DMEM/F-12 (CAT. #12500062) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (CAT.

#16000044) along with 100 units/mL penicillin and 100  $\mu$ g/mL streptomycin (CAT. #15140122). The basal glucose concentration of the DMEM/F-12 medium used in this study was 5.5 mM. All cultures were maintained under standard sterile conditions in a 5%  $\text{CO}_2$  atmosphere at 37°C. The cells reached more than 90% in passages 11–15, which were included in all experiments. Cell morphology and growth characteristics were routinely monitored to confirm the typical cobblestone appearance of epithelial cells. No signs of contamination, such as abnormal morphology or altered growth patterns, were observed during routine culture. Mycoplasma testing was not performed in the present study, which represents a limitation.

## 6. Cytotoxicity of MKE95 and glucose on the cell viability of ARPE-19 cells

ARPE-19 cells were cultured at  $1.5 \times 10^5$  cells/well in a 48-well plate for 24 h. After incubation, cells were washed once with DMEM/F-12 medium. For the extract's cytotoxicity testing, cells were treated with MKE95 (10, 50, 100, 200, and 500  $\mu$ g/mL) containing 0.5% DMSO (v/v) in DMEM/F-12 for 24 h. The final concentration of DMSO was maintained at 0.5% in all treatment groups, which also served as the vehicle control, and was used to dissolve poorly soluble compounds from the extract without causing significant cytotoxicity, as confirmed by a cell viability assay. For glucose cytotoxicity testing, cells were treated with a final concentration of glucose (10-50 mM). After treatment with MKE95 or glucose, cells were washed with PBS, incubated with MTT solution (0.5 mg/ml in PBS) for 3 h, and then dissolved in DMSO. The absorbance was measured at 540 nm using a microplate reader. Cell viability was expressed as a percentage relative to the control group. To account for potential extract interference, blanks containing culture medium with the extract (without cells) were included and processed according to the MTT protocol. Their absorbance values were subtracted from all samples to eliminate non-cellular background interference. Experiments were

performed in three independent experiments. The highest non-cytotoxic concentration of MKE95 was selected for subsequent studies, whereas the glucose concentration that yielded approximately 50% cell viability relative to the control was selected to induce oxidative stress.

### 7. Effects and mechanisms of MKE95 on glucose-induced oxidative stress in ARPE-19 cells

ARPE-19 cells were seeded at a density of  $1.5 \times 10^5$  cells/well in 48-well plates for determination of cell viability, seeded at a density of  $5 \times 10^4$  cells/well in black 96 well plates for measurement of ROS production, and seeded at a density of  $1.5 \times 10^6$  cells/well in 6-well plates for measurement of intracellular antioxidant defenses (SOD, CAT, GPx activity, and amount of GSH) and related-apoptosis markers (caspase-3 and caspase-9 activity). After incubation for 24 h, cells were co-treated with MKE95 at 50, 100, and 200  $\mu\text{g/mL}$  (without toxicity, as in section 6), which contained 5.5 mM glucose, and D-glucose was consistently added to achieve a final concentration of 30 mM (resulting in osmolarity equivalent to that of the glucose condition without affecting glucose metabolism from section 6). After co-incubation for 24 h, cells were collected for the determination of cell viability using the MTT assay, ROS production using the DCFH-DA assay, and intracellular antioxidant defenses and related apoptosis markers using the kit assay.

### 8. ROS production

ARPE-19 cells in a black 96-well plate after co-treatment with MKE95 and glucose for 24 h. The cells were washed with DMEM/F-12 and incubated with 10  $\mu\text{M}$  DCFH-DA in DMEM/F-12 at  $37^\circ\text{C}$  for 20 minutes. ROS production was determined using a fluorescence plate reader for 3 h with excitation at 485 nm and emission at 530 nm.

$$\% \text{ ROS production} = \left( \frac{\text{Mean Fluorescence of Treated group}}{\text{Mean Fluorescence of Control group}} \right) \times 100$$

### 9. Intracellular antioxidant and apoptotic markers assay

ARPE-19 cells in a 6-well plate after co-treatment with MKE95 and glucose for 24 h. Cells were soaked and scraped with PBS containing 1% (v/v) Triton X-100. The cell suspension was then sonicated at  $4^\circ\text{C}$  for 10 minutes using an ultrasonic sonicator. The cell lysates were centrifuged at  $12,500 \times g$ ,  $4^\circ\text{C}$  for 10 minutes. Supernatants were collected, and total protein concentration in each sample was determined using the bicinchoninic acid (BCA) assay. The activities of SOD, CAT, and GPx, as well as GSH levels and caspase-9 and -3 activities, were measured from the supernatants using commercial assay kits, with all enzymatic assay results normalized to total protein content to ensure accurate comparison among samples.

### 10. Statistical analysis

SPSS Statistics Version 30 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. All data were collected from three independent biological replicates, each conducted on separate occasions. Within each experiment, samples were analyzed in triplicate (technical replicates). Data are presented as mean  $\pm$  standard deviation (SD) of the three independent experiments. Statistical differences among groups were analyzed using one-way analysis of variance (ANOVA) followed by Scheffé's post hoc test. A  $p$ -value less than 0.05 was considered statistically significant.

## Results

### 1. The antioxidant properties in MKE95

As shown in Table 1, the antioxidant capacity of MKE95 was evaluated using DPPH, ORAC, and FRAP assays. In the DPPH assay, MKE95 exhibited an  $\text{IC}_{50}$  of  $8.02 \pm 0.50$  mg/mL, corresponding to approximately 80% radical scavenging activity. The ORAC and FRAP values were  $672.20 \pm 54.51$  and  $186.57 \pm 9.50$   $\mu\text{mol TE/g DW}$ , respectively. These results indicate that MKE95 exhibits antioxidant

activity through multiple mechanisms, including radical scavenging and reducing capacity.

## 2. Effects of MKE95 and glucose on cell viability of ARPE-19 cells

ARPE-19 cells were incubated with MKE95 (10 – 500 µg/mL) for 24 h to evaluate cytotoxicity, and the final concentration of glucose (10 – 50 mM) to establish optimal conditions for inducing oxidative stress. MKE95 showed no cytotoxicity at 10–200 µg/mL, with cell viability not different from the control group, whereas 500 µg/mL significantly reduced viability ( $p < 0.05$ ) (Figure 1A). Incubation with glucose 10–50 mM for 24 h resulted in a concentration-dependent reduction in cell viability in ARPE-19 cells, with 30 mM glucose decreasing cell viability to approximately 50% compared to the control (Figure 1B). Based on these results, the incubation with MKE95 at 200 µg/mL and final

glucose concentration at 30 mM for 24 h was selected for subsequent experiments.

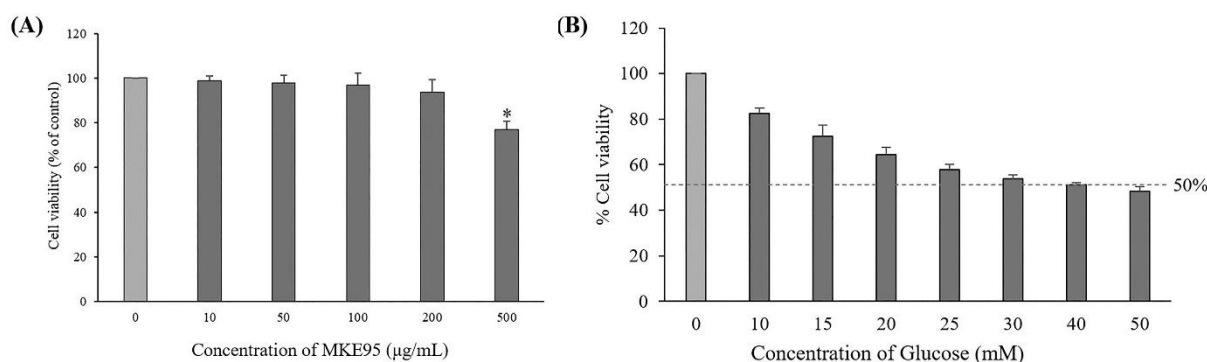
## 3. Effects of MKE95 on cell viability in glucose-induced ARPE-19 cells

As shown in Figure 2, ARPE-19 cells were co-incubated with MKE95 at 50, 100, or 200 µg/mL and 30 mM glucose for 24 h to determine its protective effect. The results showed that the induced-glucose group reduced approximately 50% of cell viability compared to the control group ( $p < 0.05$ ) (Figure 2A). Viability was recovered to 57.4%, 60.7%, and 69.2% after MKE95 co-treatment, respectively (Figure 2A). Notably, MKE95 alone showed no cytotoxicity. Morphology observation in Figure 2B supported these results. These results indicate that MKE95 potentially protects ARPE-19 cells against glucose-induced oxidative stress.

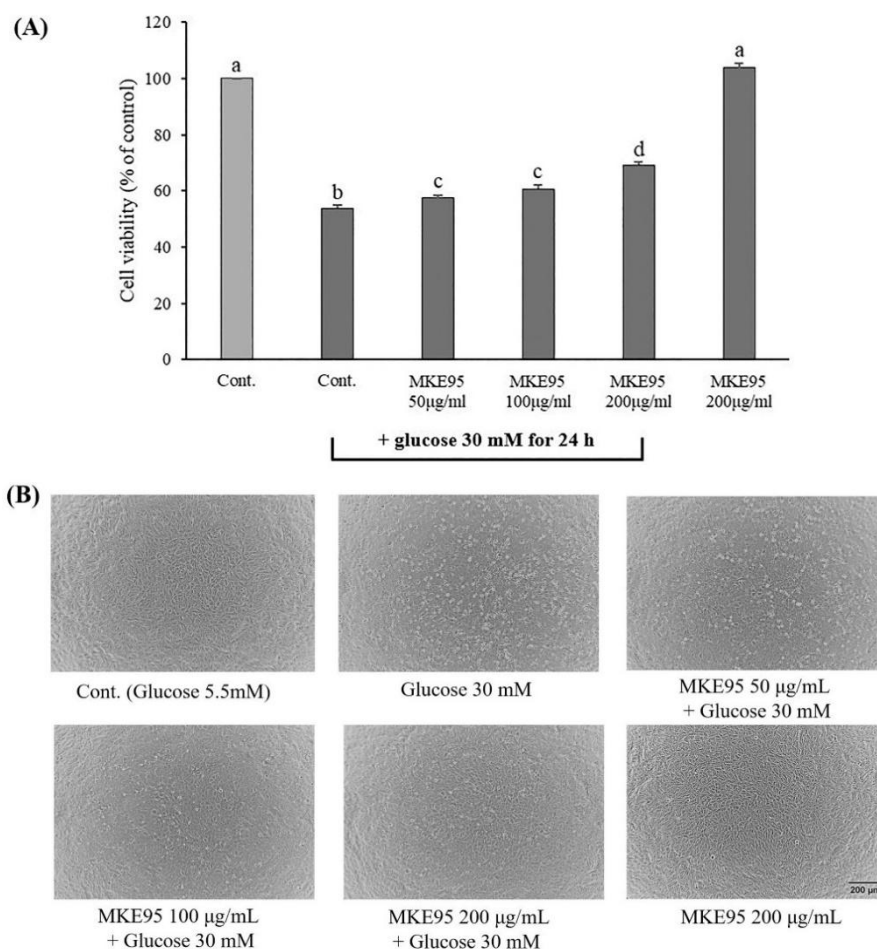
**Table 1** The antioxidant activities in MKE95

| Antioxidant Activity          |                     |                     |
|-------------------------------|---------------------|---------------------|
| DPPH IC <sub>50</sub> (mg/mL) | ORAC (µmol TE/g DW) | FRAP (µmol TE/g DW) |
| 8.02 ± 0.50                   | 672.20 ± 54.51      | 186.57 ± 9.50       |

Note: Data are represented as mean ± SD (n = 3).



**Figure 1** Effects of MKE95 and glucose on cell viability of ARPE-19 cells. (A) ARPE-19 cells were treated with different concentrations of MKE95 (10 – 500 µg/ml) in DMEM/F-12 for 24 h. A 0.5% DMSO in DMEM/F-12 was used as a control. (B) ARPE-19 cells were incubated with different concentrations of glucose (10–50 mM) in DMEM/F-12 medium for 24 h. Cell viability was measured by the MTT assay. Graphs represent average cell viability (mean ± SD; n = 3). The symbol \* indicates significant differences compared to the control group ( $p < 0.05$ ).



**Figure 2** Effects of MKE95 on cell viability in glucose-induced ARPE-19 cells. (A) ARPE-19 cells were co-treated with MKE95 (50, 100, or 200 µg/mL) and a final glucose concentration of 30 mM for 24 h. Cell viability was measured by the MTT assay. Values are expressed as mean  $\pm$  SD ( $n = 3$ ). Different superscript letters (a-d) above the bars indicate significant differences between groups at  $p < 0.05$ . (B) Morphology of ARPE-19 cells under all experimental conditions, detected by phase-contrast microscopy at 10x magnification. Scale bar represents 200 µm.

#### 4. Effects of MKE95 on ROS production in glucose-induced ARPE-19 cells

As shown in Figure 3, incubation with 30 mM glucose for 24 h increased ROS production by 1.75-fold compared to the control group ( $p < 0.05$ ). The ROS production fold decreased after co-treatment with MKE95 at 100 and 200 µg/mL (1.54-fold and 1.33-fold, respectively), whereas no significant effect was observed at 50 µg/mL. Notably, treatment with MKE95 alone did not differ from the control group. These results show that MKE95 effectively attenuates glucose-induced oxidative stress in ARPE-19 cells.

#### 5. Effects of MKE95 on caspase-9 and caspase-3 activity in glucose-induced ARPE-19 cells

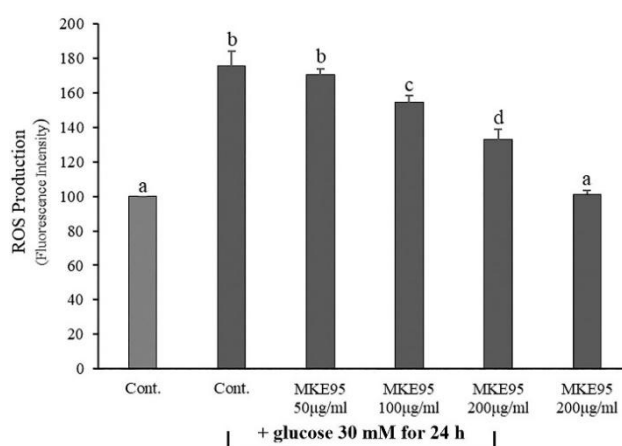
As shown in Figure 4, the incubation of ARPE-19 cells with 30 mM glucose for 24 h resulted in a 4.44-fold increase in caspase-9 and a 5.70-fold increase in caspase-3 activity compared to the control group ( $p < 0.05$ ). While co-incubation with MKE95 at 50, 100, and 200 µg/mL, changing the fold of both caspase activity in a dose-dependent manner (Figure 4A-B). The caspase-9 activity was 4.08, 3.50, and 2.67-fold, and the caspase-3 activity was 5.20, 4.26, and 3.47-fold, respectively. Notably, treatment with MKE95 alone did not significantly differ from the

control group. These results indicate that MKE95 attenuates caspase-9 and caspase-3 activities, suggesting a reduction of apoptosis-associated signaling in ARPE-19 cells exposed to glucose-induced oxidative stress.

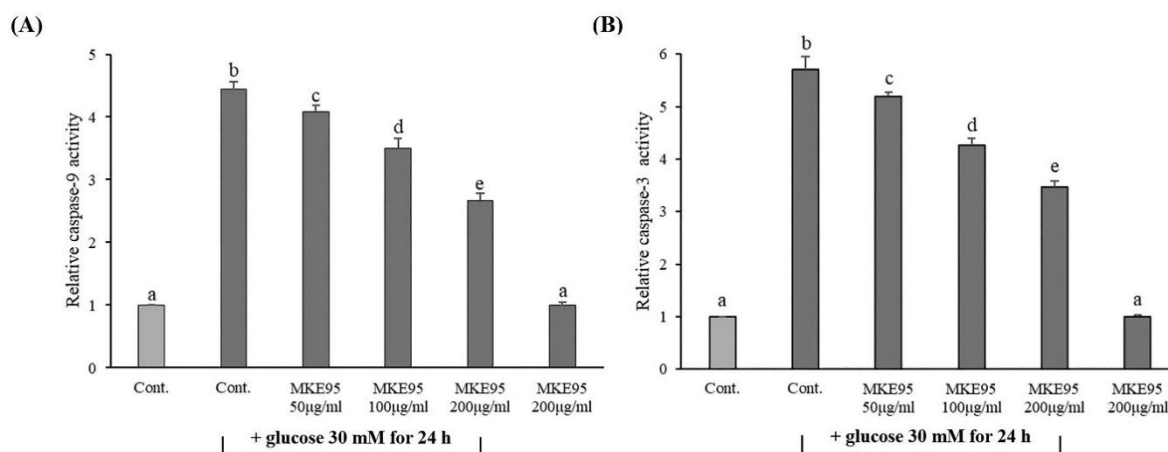
#### 6. Effects of MKE95 on intracellular antioxidant defenses in glucose-induced ARPE-19 cells

As shown in Figure 5, incubating ARPE-19 cells with 30 mM glucose for 24 h impairs intracellular

antioxidant defenses, as indicated by marked reductions in SOD (57.40%), CAT (49.95%), GPx (50.85%), and GSH (51.91%). These findings suggest that glucose might disrupt redox homeostasis and promote oxidative imbalance. Co-treatment with MKE95 at 100 and 200  $\mu\text{g}/\text{mL}$  tended to restore SOD, CAT, GPx activity, and GSH levels, whereas no significant effect was observed at 50  $\mu\text{g}/\text{mL}$  (Figure 5A-D). Importantly, treatment with MKE95 alone did



**Figure 3** Effects of MKE95 on ROS production in glucose-induced ARPE-19 cells. ARPE-19 cells were co-treated with MKE95 (50, 100, or 200  $\mu\text{g}/\text{mL}$ ) and a final glucose concentration of 30 mM for 24 h. ROS production was then measured using the DCFH-DA assay. Data are expressed as mean  $\pm$  SD ( $n = 3$ ). Different lowercase letters (a–d) above the bars indicate significant differences among groups ( $p < 0.05$ ).



**Figure 4** Effects of MKE95 on caspase-9 and caspase-3 activity in glucose-induced ARPE-19 cells. ARPE-19 cells were co-treated with MKE95 (50, 100, or 200  $\mu\text{g}/\text{mL}$ ) and a final glucose concentration of 30 mM for 24 h. (A) Caspase-9 activity and (B) caspase-3 activity are expressed as mean  $\pm$  SD ( $n = 3$ ). Different superscript letters above (a–e) indicate significant differences between groups at  $p < 0.05$ .

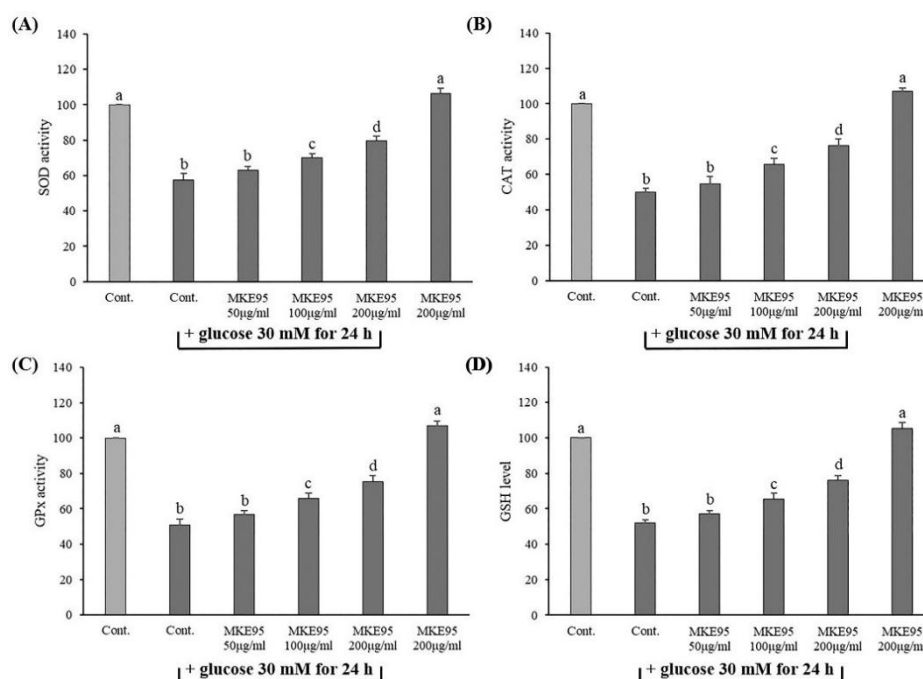
not significantly alter these parameters compared to the control, indicating no adverse effect on normal antioxidant status. These results demonstrate that MKE95 partially restored intracellular antioxidant defense parameters, including SOD, CAT, GPx, and GSH, in glucose-induced oxidative stress in ARPE-19 cells.

## Discussion

Diabetic retinopathy, the most common microvascular complication of diabetes, results from chronic hyperglycemia and can lead to retinal damage, visual impairment, and blindness, respectively.<sup>33</sup> Increasing evidence indicates that glucose-induced oxidative stress plays a role in DR pathogenesis.<sup>8,9</sup> Extensive clinical investigations have established that persistent hyperglycemia induces structural and functional deterioration within the retinal microvasculature.<sup>34</sup> These elevates the accumulation of ROS and oxidative stress to retinal cells which caused the breakdown of the blood-

retinal barrier (BRB), primary contributing to the decreasing the tightness of the RPE cell layer in immortalized ARPE-19 cell cultures, maintenance of visual cycle, synthesis and secretion numerous growth factors, cytokines and cellular structure related proteins that play key roles in the regulation of the structures and functions of the retina. These situations highlight oxidative stress as a fundamental mechanism.<sup>35-38</sup> Our present findings are consistent with the notion that oxidative stress associated with glucose may be an early event in the development of DR. Further investigation is needed to clarify the specific mechanism.

Dietary fruits and vegetables are abundant sources of phytochemicals, renowned for their antioxidant capacities, which have been demonstrated to counteract oxidative damage in *in vitro* and *in vivo* models.<sup>39,40</sup> Given that RPE cells orchestrate homeostasis vital to photoreceptor function and structural maintenance, mitigating oxidative stress emerges as a critical therapeutic target



**Figure 5** Effects of MKE95 on intracellular antioxidant defenses in glucose-induced ARPE-19 cells. ARPE-19 cells were co-treated with MKE95 (50, 100, or 200 µg/mL) and final glucose concentration at 30 mM for 24 h. (A) SOD and (B) CAT (C) GPx and (D) GSH content are expressed as mean  $\pm$  SD ( $n = 3$ ). Different superscript letters above (a-d) the bars indicate significant differences between groups at  $p < 0.05$ .

to prevent retinal degeneration.<sup>12,41</sup> To our knowledge, this study is the first to report the protective effects of Makiang extract against glucose-induced oxidative stress in ARPE-19 cells. The results indicate that MKE95 confers cryoprotection, likely by directly neutralizing reactive oxygen species, as evidenced by DPPH, ORAC, and FRAP antioxidant assays. Besides the multifaceted benefits of ACN to retinal cells, the C3G, a predominant ACN found in the edible portions of purple-hued fruits and vegetables, is particularly noted for its ability to cross the BRB and enhance its function by protecting RPE cells from stress-induced damage.<sup>42-44</sup> Previous phytochemical studies have reported that *Cleistocalyx nervosum* var. *paniala* contains several bioactive phenolic compounds and ACN.<sup>22</sup> These compounds are known to possess antioxidant and cytoprotective properties and may contribute to the biological effects observed in the present study. However, these constituent results may not correspond to variations in cultivation areas, geographic locations, possible breeding developments over time, or across research methodologies.

In this study, a co-treatment model was used to mimic hyperglycemia associated with DR progression. Our findings demonstrate that MKE95 exerts significant cytoprotective effects by attenuating glucose-induced cytotoxicity (Figure 2A) and reducing ROS production (Figure 3), thereby mitigating oxidative stress and activating caspase-dependent apoptotic pathways, ultimately increasing initiator caspase-9 and executioner caspase-3 activity.<sup>10,17</sup> Consequently, several *in vitro* studies have identified caspase-9 and caspase-3 as key mediators of mitochondrial-dependent apoptosis under oxidative stress via the intrinsic pathway.<sup>17</sup> This process leads to DNA damage, nuclear fragmentation, and cytoskeletal disruption, thereby compromising cellular function and structural integrity in ARPE-19 cells.<sup>45</sup> Previous studies have also reported increased immunoreactivity of active

caspase-3 in the photoreceptor inner segments of rodent RPE tissue.<sup>46</sup> Consistent with these findings, our results demonstrated that glucose treatment significantly increased caspase-9 and caspase-3 activities, whereas MKE95 dose-dependently attenuated their activation (Figure 4A–B) and improved ARPE-19 cell morphology (Figure 2B).

SOD, CAT, GPx, and GSH are key components of the cellular antioxidant defense system, functioning as first-line mechanisms to neutralize ROS and maintain redox homeostasis.<sup>46</sup> In RPE cells, intrinsic antioxidant capacity is largely attributed to enzymes such as SOD, CAT, and GSH, which protect retinal integrity.<sup>45</sup> Supporting this, *in vivo* studies have shown that anthocyanidin extracts from *Aronia melanocarpa* enhance antioxidant capacity by upregulating SOD, CAT, and GPx activities in rat retinas.<sup>48</sup> Therefore, strengthening endogenous antioxidant defenses is essential for protection against oxidative stress. Our MKE95, particularly at concentrations exceeding 100 µg/mL, restored both enzymatic (SOD, CAT, and GPx) and non-enzymatic (GSH) antioxidant levels in ARPE-19 cells under glucose-induced oxidative stress (Figure 5A–D). MKE95 treatment alone did not significantly alter SOD, CAT, GPx activities, or GSH levels compared to the control group, suggesting that the extract does not directly stimulate antioxidant enzyme systems under basal conditions. Instead, the observed restoration of antioxidant enzyme activities under oxidative stress conditions may be attributed to MKE95's ability to reduce intracellular ROS levels and attenuate oxidative damage. Therefore, the effects of MKE95 are more likely associated with the preservation of endogenous antioxidant defenses rather than direct activation of antioxidant pathways. Nevertheless, further studies investigating upstream regulatory mechanisms, such as Nrf2 signaling, are warranted.

Previous studies have demonstrated that Makiang fruit (*C. nervosum*) extract reduces intracellular ROS in various models, including

trophoblast cells, human monocyte U937 cells, and *C. elegans* under oxidative stress.<sup>20-21,49</sup> In addition to its direct radical-scavenging activity, the extract enhances the expression and activity of antioxidant enzymes, including SOD, CAT, and GPx, as observed in mouse hippocampal neurons.<sup>50,51</sup> Furthermore, *in vivo* studies in rats have shown that ethanolic extracts of *C. nervosum* decrease lipid peroxidation and increase glutathione peroxidase activity, likely due to their anthocyanin-rich composition.<sup>52,53</sup> These effects are associated with activation of the Nrf2 signaling pathway, leading to enhanced antioxidant responses and cellular defense mechanisms. Overall, the protective effects of Makiang extract against oxidative stress and apoptosis may be attributed to its phytochemical constituents. Although the present study did not directly characterize the extract, previous studies have reported that *Cleistocalyx nervosum* var. *paniala* is rich in anthocyanins, particularly cyanidin-3-glucoside (C3G), which was identified by HPLC analysis.<sup>22</sup> C3G is widely recognized for its antioxidant and cytoprotective effects through ROS scavenging and modulation of apoptosis-related pathways. Thus, the observed biological activities may be partly attributed to these compounds. Notably, anthocyanins such as C3G regulate mitochondrial-mediated apoptosis by influencing the Bax/Bcl-2 balance and caspase activation, consistent with our findings. However, further chemical characterization and standardization of the extract are warranted to strengthen these conclusions in future studies.

A limitation of this study is the absence of an osmotic control group (e.g., mannitol or D-glucose), which is important for distinguishing the effects of hyperglycemia from increased osmolarity.<sup>54,55</sup> Although not included, previous studies in ARPE-19 cells under similar conditions have shown that 30 mM mannitol, an osmotic equivalent to 30 mM D-glucose, does not induce oxidative stress markers such as ROS or MDA.<sup>56</sup> These observations suggest that the cellular damage we report is attributable to

glucose-induced metabolic stress rather than to nonspecific effects of increased osmolarity. While referencing such literature supports our interpretation, future studies including a parallel osmotic control group in our specific experimental setting would further strengthen the validity of our conclusions regarding the protective effects of MKE95.

Although this study demonstrates the protective effects of MKE95 against oxidative stress and apoptosis in ARPE-19 cells, it does not evaluate RPE-specific functional outcomes. Given that RPE cells are essential for retinal homeostasis, including barrier integrity, phagocytosis, and regulation of angiogenic and inflammatory responses, future studies should assess whether MKE95 preserves these functions under hyperglycemic conditions, further supporting its potential role in diabetic retinopathy.

## Conclusion

Our study reveals that MKE95 protects against glucose-induced oxidative stress in ARPE-19 cells. The results showed that MKE95 has strong antioxidant capabilities. It is forceful and able to scavenge radicals induced by glucose in our cell model. These effects are associated with reduced oxidative cytotoxicity, reduced ROS production, modulation of apoptotic-related pathways, as evidenced by decreased caspase-9 and caspase-3 activities, and enhanced SOD, CAT, GPx activity, and GSH levels. Our findings indicate that Makiang fruit has antidiabetic properties, highlighting its potential for further *in vivo* and well-designed clinical studies to confirm its efficacy, safety, and potential to prevent or delay the progression of DR.

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