Research article

Protective Effects of Extraction and Bioaccessible Fraction of Mulberry on Hydrogen Peroxide-Induced Oxidative Damage in Human Retinal Pigment Epithelial (ARPE-19) Cells

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ABSTRACT

Age-related macular degeneration (AMD) is a condition affecting the macula in the retina that can lead to vision impairment and blindness. Oxidative stress is an important factor in AMD development. Mulberry (*Morus alba* L.) is a rich source of anthocyanins, a class of naturally occurring biologically active compounds containing a powerful radical-scavenging antioxidant that can neutralize free radicals. This study investigated the effects of mulberry extract (ME) and bioaccessible fraction of mulberry (BFM) on H_2O_2-induced oxidative damage in human retinal pigment epithelial (ARPE-19) cells. Cells were pre-treated with ME or BFM for 24 h and then exposed to H_2O_2 to induce oxidative damage. Results indicated that both ME and BFM significantly (p<0.05) protected ARPE-19 cells from induced oxidative damage by decreasing the level of intracellular reactive oxygen species (ROS). Furthermore, after simulated digestion, mulberry demonstrated a statistically significant capability to mitigate oxidative damage in retinal cells. These findings suggest that both ME and BFM can protect human retina cells from oxidative damage induction and AMD development. Since this study was limited to *in vitro* research, further investigations into the impact of mulberry on AMD are warranted encompassing both experimental animal models and human subjects.

Key words: *Morus alba* L.; ARPE-19 cells; Age-related macular degeneration; Oxidative stress; Simulated digestion

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บทความวิจัย

ผลการปกป้องของสารสกัดและส่วนที่ผ่านกระบวนการย่อยภายในร่างกายของลูกหม่อนต่อเซลล์จอประสาทตามนุษย์ที่ถูกเหนี่ยวนำให้เกิดความเสียหายจากภาวะเครียดออกซิเดชันด้วยไฮโดรเจนเปอร์ออกไซด์

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บทคัดย่อ

ภาวะจอประสาทตาเสื่อมตามอายุ เป็นโรคที่เกี่ยวข้องกับจุดรับภาพในชั้นเรตินาของดวงตา ซึ่งเป็นสาเหตุความบกพร่องทางการมองเห็น ภาวะเครียดออกซิเดชันเป็นสาเหตุสำคัญของการเกิดภาวะจอประสาทตาเสื่อมนี้ ลูกหม่อนเป็นผลไม้ที่เป็นแหล่งของสารแอนโทไซยานินซึ่งเป็นสารออกฤทธิ์ทางชีวภาพที่มีฤทธิ์ต้านอนุมูลอิสระ การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาประสิทธิผลของสารสกัดและส่วนที่ผ่านกระบวนการย่อยของลูกหม่อนต่อเซลล์จอประสาทตาที่ถูกเหนี่ยวนำให้เกิดความเสียหายจากภาวะออกซิเดชันด้วย H_2O_2 เซลล์ถูกเลี้ยงด้วยสารสกัดจากลูกหม่อน (ME) หรือลูกหม่อนที่ผ่านกระบวนการย่อยภายในร่างกาย (BFM) เป็นเวลา 24 ชั่วโมงก่อนเหนี่ยวนำด้วย H_2O_2 การศึกษาพบว่า ME และ BFM สามารถป้องกันเซลล์จอประสาทตาที่ถูกเหนี่ยวนำให้เกิดความเสียหายได้ โดยการลดระดับของอนุมูลอิสระภายในเซลล์ อย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) ผลการศึกษานี้แสดงให้เห็นว่า ทั้งสารสกัดจากลูกหม่อนและลูกหม่อนที่ผ่านกระบวนการย่อยของร่างกายแล้ว สามารถปกป้องเซลล์จอประสาทตาจากการเหนี่ยวนำให้เกิดความเสียหายจากภาวะเครียดออกซิเดชันซึ่งเป็นสาเหตุของการเกิดภาวะจอประสาทตาเสื่อมได้ อย่างไรก็ตาม การศึกษานี้เป็นเพียงการศึกษาในหลอดทดลองเท่านั้น ผลของลูกหม่อนต่อภาวะจอประสาทตาเสื่อมควรศึกษาเพิ่มเติมในสัตว์ทดลองและมนุษย์ต่อไป

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INTRODUCTION

Age-related macular degeneration (AMD) is an ocular disease that causes damage to the retinal macula, the part of the eye that controls sharp, straight-ahead (central) vision. Associated with aging, AMD usually occurs in elderly persons and, in severe cases, can lead to blindness thus impacting daily life routines. According to reports, the prevalence and incidence of AMD worldwide, including in Thailand[1], have increased rapidly with the estimated number of affected individuals reaching 196 million in 2020 and a projected increase to 288 million by 2040[2]. Furthermore, considering that Asia accounts for over half of the world's population, it is anticipated that the number of AMD cases in this region alone will rise to approximately 113 million by 2040[3]. AMD can be classified into two types. Dry AMD is a slowly developing nonexudative form that may develop over a long period and proceed slowly but can also cause severe central vision loss over time. Wet AMD is a severely developing exudative form that can cause permanent and rapid central vision loss. Furthermore, dry AMD can progress to wet AMD and blindness. Although AMD is a major health issue, the therapeutics that exist for AMD are restricted and limited largely to its wet form with no current suitable treatment for its dry form. Consequently, the identification and development of effective treatments are critically needed to delay or prevent AMD development[4]. While the pathogenesis and development of AMD are not fully understood, the dysfunction of retinal pigment epithelium (RPE) plays a significant role in the progression of dry AMD and represents a key feature in its development and pathogenesis[5-7]. Oxidative stress is a leading cause of dysfunction and death in RPE cells, contributing to the progression and development of AMD[8]. Oxidative stress is defined by the heightened presence of reactive oxygen species (ROS), resulting from an imbalance between ROS production and accumulation within cells, and the efficiency of biological systems to neutralize ROS. These ROS cause detrimental effects to lipids, proteins, and nucleic acids, leading to subsequent cellular dysfunction and death. RPE cells, characterized by a high metabolic rate, reside in an environment abundant in endogenous ROS, including H_2O_2 and OH^{-4}. The accumulation of long-term oxidative damage results in the dysfunction and eventual death of RPE cells, contributing to the progression of AMD. Consequently, the protection of RPE cells from oxidative damage may be a protective strategy against AMD development[9-11]. Dietary bioactive compounds, particularly those belonging to the polyphenol group, have emerged as promising molecules capable of exerting a beneficial effect against AMD[12-14]. Mulberry (*Morus alba* L.) is a fruit that is commonly found in Thailand and is high in anthocyanins (ACNs), a class of water-soluble flavonoids. Many studies have reported the free radical scavenging activities of anthocyanins and their benefits in protecting visual health through antioxidant, anti-inflammatory, and anti-apoptotic properties[14-17] in cell models[1,18,19] and animal models[20-23]. Consequently, this study evaluated the

antioxidant activity of ethanol extraction and bioaccessible fraction of mulberry on hydrogen peroxide-induced oxidative damage in human retinal pigment epithelial (ARPE-19) cells.

MATERIALS AND METHODS

Chemicals and Reagents

DMEM: F12 medium containing DMEM/F-12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) powder was obtained from Life Technologies Limited, Paisley, United Kingdom. Fetal bovine serum was obtained from Merck (Darmstadt, Germany). Penicillin-streptomycin solution was obtained from Caisson Laboratories (USA). Sodium bicarbonate ($NaHCO_3$) powder, dimethyl sulfoxide (DMSO), and measurement chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Sample preparation

Mulberry (*Morus alba* L.) samples were obtained from two representative farms located in Nakhon Pathom and Rayong provinces, Thailand. The samples from the two farms were thoroughly washed with tap water, dried at room temperature, freeze-dried, packed in aluminum foil under a vacuum, and then stored at -20 °C.

Sample extraction

Sample extraction protocols were followed according to Panritdum P., et al[38] and Wang Y., et al[39]. Samples from the two farms were mixed equally and extracted with 70% ethanol (the dried 1 g/15 ml) and soaked overnight. The solution was mixed for 1 min, sonicated for 15 mins, and centrifuged at 4600 revolutions per minute (rpm) for 10 mins. The supernatant was transferred to an amber flat-bottom flask for evaporation until dry. The extraction procedure was repeated 3 times and the upper layer was combined. The clear supernatant was dried using a vacuum evaporator at a temperature of 35-40˚C and dissolved with absolute ethanol. The dissolved sample was applied to the amber vial and blown with nitrogen gas until dry. The mulberry extract (ME) was stored at -20˚C. The 70% ethanolic extract of ME was measured by the yield percentage to determine the efficiency of extraction (% extraction yield = 81.74 ± 0.21).

Stimulated digestion

Simulated gastrointestinal digestion was carried out according to a prior protocol [24,25]. Briefly, in the gastric phase, 0.6-0.8 g of freeze-dried mulberry was homogenized with 20 ml of 120 mM NaCl in a 50 ml polypropylene tube using a homogenizer (model T25D, Germany), then adjusted for pH to 2.0 ± 0.1 with 1 M HCl. Pepsin (40 mg/ml in 100 mM HCl) 2 ml was then added. Thereafter, the volume was adjusted to 40 ml with 120 mM NaCl, blanked the remaining tube with nitrogen gas, tightly capped and sealed with parafilm, and incubated in a shaking water bath for 1 h at 37ºC, 95 rpm. After incubation for 1 h, the small intestinal phase was followed by raising the pH to 6.0 ± 0.1 with 1 M Na_2HCO_3. Crude bile extract (40 mg/ml in 100 mM Na_2HCO_3) 3 ml, pancreatin (10 mg/ml in 100 mM Na_2HCO_3) 2 ml, and lipase (5 mg/ml in 100 mM Na_2HCO_3) 2 ml were added to the reaction tube. The pH of the sample was increased to 6.5 ± 0.1 using 1 M Na_2HCO_3. Thereafter, the tube was filled with nitrogen gas, tightly capped, and sealed with parafilm before placing it in a shaking water bath for 2 h at 37ºC, 95 rpm. After completion of the simulated small intestinal phase of digestion, the

sample solution was centrifuged (Becton Dickinson Dynac Centrifuge, Sparks, MD, U.S.A.) at 4,000g for 1 h at room temperature to isolate the aqueous fraction (supernatant). Control digestion without mulberry was also conducted to assess the possible cytotoxic effects of digested compounds in the aqueous fraction. The supernatant was filtered (0.22 µm pores; polytetrafluoroethylene [PTFE] membrane; Millipore Corp., Cork, Ireland) to obtain the fraction with mixed micelles or bioaccessible fraction of mulberry (BFM). The BFM was used to assess anti-apoptosis in ARPE-19 cells.

ARPE-19 cell culture

Human retinal pigment epithelial (ARPE-19) cells were obtained from the American Type Culture Collection (ATCC) (CRL-2302 TM, Manassas, VA 20110-2209, USA). Cells were cultured in complete media consisting of DMEM: F-12 medium containing 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were grown in 5% CO_2 at 37ºC until more than 90% confluence.

Effect of ME on cell viability of ARPE-19 cells

ARPE-19 cells were seeded into a 48-well plate at a density of 1.0 x 10^5 cells/well in media. Thereafter, the media was removed and washed with serum-free media. Cells were then treated with various concentrations of ME (10, 100, 200, 500, 1000 µg/ml) in 0.5% DMSO in serum-free media for 24 h. After incubation, the media were removed and washed with phosphate-buffered saline (PBS), and a 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) solution (0.5 mg/ml in PBS) was added to each well and incubated for 3 h. Next, the MTT solution was removed before adding DMSO and then shaken to dissolve formazan crystals. Cell viability directly depends on the density absorption of the formazan crystal product. Experiments were performed in three independent experiments. The absorbance of formazan was measured at 540 nm by a microplate reader. Results were presented as % cell viability relative to the control group, and 0.5% DMSO in serum-free media was used as a control. The highest concentration of ME, in which cell viability did not have statistically significant differences relative to the control, was selected for further study.

Effect of ME and BFM on cell viability of ARPE-19 cells induced oxidative damage with H_2O_2

ARPE-19 cells were seeded into a 48-well plate at a density of 1.0 x 10^5 cells/well in media. Thereafter, the media was removed and washed with serum-free media, and the cells were treated with ME at 50, 100, and 200 µg/ml in 0.5% DMSO in serum-free media or BFM diluted 1:3 (v/v) with serum-free media. After incubation for 24 h, cells were washed 1 time with serum-free media and then treated with H_2O_2 in serum-free media at 800 µM for 1 h. Cell viability was determined by MTT assay.

Effect of ME and BFM on intracellular ROS production of ARPE-19 cells induced oxidative damage with H_2O_2

ARPE-19 cells were seeded at a density of 3.0 x 10^4 cells/well in a 96-black well plate to determine intracellular ROS production using 2',7-dichlorofluorescein diacetate (DCFH-DA) assay. Experiments were performed in three independent stages. The media was removed and washed with serum-free media. Cells were treated with ME at 50, 100, and 200 µg/ml in

0.5% DMSO in serum-free media or BFM diluted 1:3 (v/v) with serum-free media. After incubation for 24 h, the media was removed and washed 1 time with serum-free media. Cells were treated with 10 µM of DCFH-DA for 30 mins. After treatment, cells were washed 2-3 times with serum-free media. Cells were treated with H_2O_2 at 800 µM in serum-free media for 1 h. Thereafter, washing was conducted before the determination of fluorescent intensity using a fluorescence microplate reader at excitation and emission wavelengths of 485 nm and 530 nm, respectively.

Antioxidant capacity

ORAC assay

ORAC was measured according to the method described by Carvalho J., et al[26]. For testing, the final volume of the reaction mixture was at 200 µl. Briefly, serial dilution of ME or BFM diluted 1:3 (v/v) with serum-free media was placed into a 96-well black plate solid bottom with fluorescein working solution per each sample. This was followed by the addition of 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) substrate (freshly prepared). Trolox was used as the antioxidant standard. Absorbance was measured after incubating for 30 min at dark room temperature at 485 nm for λ excitation and 528 nm for λ emission measured by fluorescence microplate reader Gen5 (Bio-Tek Instruments) software.

FRAP assay

FRAP was measured according to the method described by Xiao F., et al[27]. For testing, the final volume of the reaction mixture at 170 µl. Briefly, serial dilution of ME or BFM diluted 1:3 (v/v) with serum-free media was added into the 96-well plate. Thereafter, 150 µl of FRAP reagent was added which was freshly prepared by mixing a volume ratio of 10:1:1 of the following stock solutions: 300 mM acetate buffer, 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ solution. Trolox was used as the antioxidant standard. Absorbance was measured after incubation for 8 min at room temperature at 595 nm measured by a microplate reader.

DPPH assay

DPPH assay was measured according to the method described by Xiao F., et al[27]. For testing, the final volume of the reaction mixture at 220 µl was used. Briefly, serial dilution of ME or BFM diluted 1:3 (v/v) with serum-free media was added into the 96-well plate. Thereafter, 200 µl of DPPH reagent was added which was freshly prepared. Trolox was used as the antioxidant standard. Absorbance was measured after incubation for 30 min at a dark room temperature of 520 nm measured by a microplate reader.

Statistical analysis

All data were presented as the mean ± SD (n=3) for a minimum of three independent experiments and shown by bar graph and histogram using One-way ANOVA and post hoc multiple comparison tests (Bar graph). The unpaired t-test was employed for the statistical analysis of antioxidant capacity. Data were analyzed using the publicly accessible software SPSS (Version 16.0). P-value < 0.05 is considered statistically significant.

RESULTS

Effect of ME on cell viability of ARPE-19 cells

The cytotoxic concentration of ME was determined for cell viability using MTT assay. ARPE-19 cells were treated with ME at 10, 100, 200, 500, and 1000 µg/ml for 24 h. The results showed that the viability of cells treated with ME at more than 200 µg/ml for 24 h was significantly lower than that of the control group ($p < 0.05$) (**Figure 1**). These results indicated that a concentration of ME more than 200 µg/ml was toxic to ARPE-19 cells. Consequently, the highest concentration of ME at 200 µg/ml was used for subsequent experiments to evaluate their protective effects on ARPE-19 cells-induced oxidative damage.

Effect of ME and BFM on cell viability of ARPE-19 cells with induced oxidative damage with H_2O_2

The protective effect of ME (three serial dilution concentrations) and BFM on ARPE-19 cells with induced oxidative damage by H_2O_2 was determined by cell viability using MTT assay. The cells were treated with ME at 50, 100, and 200 µg/ml or BFM diluted 1:3 (v/v) with serum-free media for 24 h before receiving induced oxidative damage with H_2O_2. The results indicated that ARPE-19 cells receiving induced oxidative damage with H_2O_2 at 800 µM for 1 h significantly decreased the viability of ARPE-19 cells (about 50%) compared to the control group ($p < 0.05$) (**Figure 2**). Pre-treatment with ME at 50, 100, and 200 µg/ml could significantly protect the viability of ARPE-19 cells compared to ARPE-19 cells treated with H_2O_2 alone ($p < 0.05$) in a dose-dependent manner without toxicity (**Figure 2A**). In addition, the pre-treatment of BFM (diluted 1:3 [v/v] with serum-free media) could significantly protect the viability of ARPE-19 cells compared to ARPE-19 cells treated with H_2O_2 alone without toxicity (**Figure 2B**). These results demonstrate that ME can protect ARPE-19 cells from oxidative damage induction in a dose-dependent manner without toxicity. Moreover, the mulberry constituents that had gone through simulated digestion could still protect ARPE-19 cells from oxidative damage induction.

Effect of ME and BFM on intracellular ROS production of ARPE-19 cells with induced oxidative damage with H_2O_2

Intracellular ROS production was determined using DCFH-DA assay. The cells were treated with ME at 50, 100, and 200 µg/ml or BFM diluted 1:3 (v/v) with serum-free media for 24 h before incubation with DCFH-DA for 30 min. After incubation, cells were exposed to induced oxidative damage with H_2O_2 at 800 µM for 1 h. The results showed that ARPE-19 cells receiving induced oxidative damage with H_2O_2 at 800 µM for 1 h significantly increased the intracellular ROS production of ARPE-19 cells compared to the control group ($p < 0.05$) (**Figure 3**). Pre-treatment with ME at 50, 100, and 200 µg/ml could significantly reduce the intracellular ROS production of ARPE-19 cells compared to ARPE-19 cells treated with H_2O_2 alone ($p < 0.05$) in a dose-dependent manner (**Figure 3A**). In addition, the pre-treatment of BFM (diluted 1:3 [v/v] with serum-free media) could significantly decrease the intracellular ROS production of ARPE-19 cells compared to ARPE-19 cells treated with H_2O_2 alone (**Figure 3B**). These findings indicate that ME could protect ARPE-19 cells from oxidative damage induction by suppressing the production

of intracellular ROS in a dose-dependent manner. Moreover, the mulberry constituents that went through simulated digestion could still protect ARPE-19 cells from oxidative damage induction by suppressing the production of intracellular ROS.

The antioxidant capacity of ME and BFM

ME and BFM solutions were evaluated for antioxidant capacities using chemical assays, namely ORAC, FRAP, and DPPH. All results are shown in **Table 1**. Both ME and BFM showed antioxidant capacity using ORAC, FRAP, and DPPH assays. ME had a significantly higher antioxidant capacity than BFM.

DISCUSSION

Several studies have demonstrated that oxidative stress is a significant contributor to cellular dysfunction and death in RPE cells, playing a pivotal role in the progression and development of AMD[6,28]. Consequently, safeguarding RPE cells from oxidative damage may serve as an effective protective strategy against AMD development. Consistent consumption of dietary anthocyanins may decrease the risk of AMD development due to their antioxidant properties[17,29]. On its part,

Table 1. The antioxidant capacity of ME and BFM

Compounds	ORAC assay	FRAP assay	DPPH assay
	μmoles TE/g extract	μmoles TE/g extract	μmoles TE/g extract
The ethanolic mulberry extraction (ME)	85.48 ± 3.34 [a]	23.59 ± 0.88 [a]	23.45 ± 1.22 [a]
The bioaccessible fraction mulberry extraction (BFM)	41.69 ± 1.80 [b]	12.65 ± 0.69 [b]	13.03 ± 0.62 [b]

Different superscript letters (a, b) indicate significant differences between values in the column ($p < 0.05$).

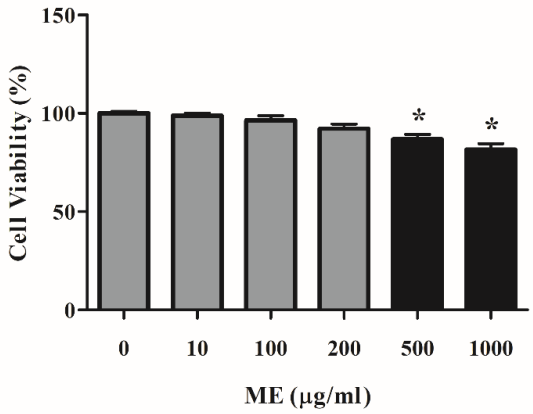

Figure 1. Effect of ME on cell viability of ARPE-19 cells. ARPE-19 cells were treated with ME in serum-free media containing 0.5% DMSO at various concentrations as 10, 100, 200, 500, and 1000 μg/ml for 24 h. Cell viability was determined using MTT assay. Graphs show the mean of cell viability (mean ± SD; n = 3). The symbol "*" indicates significant differences between values in the column ($p < 0.05$).

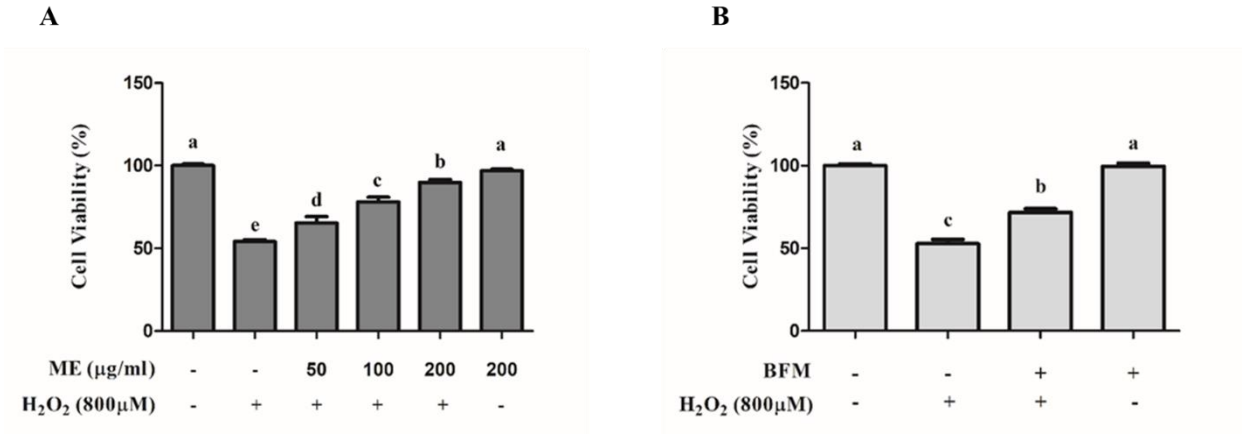

Figure 2. Effect of ME and BFM on cell viability of ARPE-19 cells induced oxidative damage with H_2O_2. ARPE-19 cells were treated with **(A)** ME at 50, 100, and 200 µg/ml or **(B)** BFM diluted 1:3 (v/v) with serum-free media for 24 h and induced oxidative damage with H_2O_2 at 800 µM for 1 h. Cell viability was determined using MTT assay. Graphs show the mean of cell viability (mean ± SD; n = 3). Different superscript letters (a-e) indicate significant differences between values in the column ($p < 0.05$).

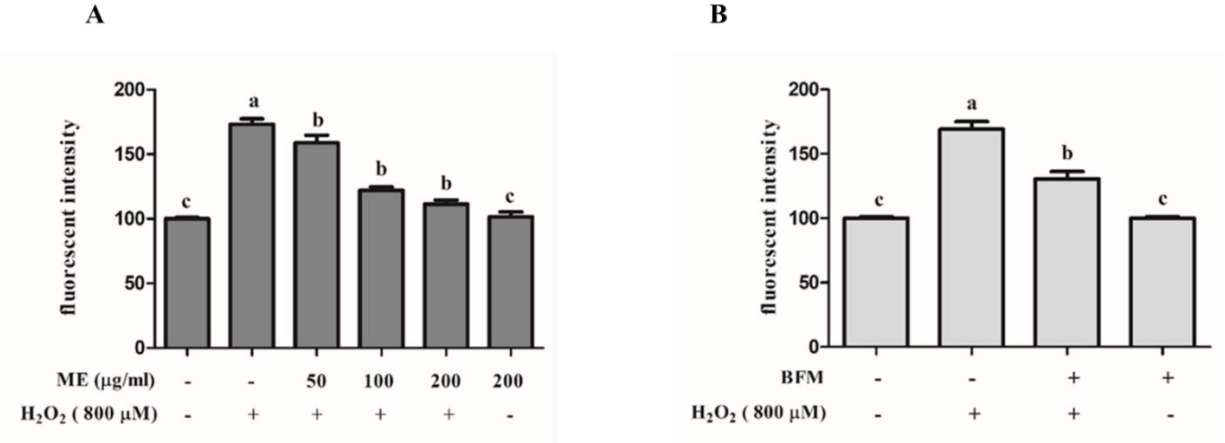

Figure 3. Effect of ME and BFM on ROS production of ARPE-19 cells induced oxidative damage with H_2O_2. ARPE-19 cells were treated with **(A)** ME at 50, 100, and 200 µg/ml or **(B)** BFM diluted 1:3 (v/v) with serum-free media for 24 h and induced oxidative damage with H_2O_2 at 800 µM for 1 h. Intracellular ROS was determined by DCFH-DA assay. Graphs show the mean of fluorescent intensity (mean ± SD; n = 3). Different superscript letters (a-e) indicate significant differences between values in the column ($p < 0.05$).

mulberry is a rich source of natural bioactive compounds, particularly anthocyanins, which exhibit potent antioxidant activity[30,31]. The mulberry anthocyanins content was 3.2 mg/g fresh weight and the main anthocyanins in mulberry are cyanidin-3-O-glucoside (C3G) and cyanidin-3-O-rutinoside (C3R)[40,41]. To our knowledge, the present study is the first to show the protective effect of ethanol extract and bioaccessible fraction following simulated digestion from mulberry on hydrogen peroxide-induced oxidative damage in ARPE-19 cells. We found that ME demonstrated the ability to shield ARPE-19 cells from oxidative damage induction by inhibiting the generation of intracellular ROS in a dose-dependent manner without toxicity.

Additionally, even after undergoing simulated digestion (BFM), the constituents of mulberry retained their capacity to safeguard ARPE-19 cells from oxidative damage induction by suppressing intracellular ROS production without toxicity. The efficacy of ME and BFM in safeguarding retinal cells from induced damage likely stems from their ability to directly neutralize free radicals, attributed to their antioxidant properties (Table 1). The simulated digestion process decreased the antioxidant capacities of mulberry due to the high temperature, pH levels, and enzymes involved, which impacted the stability and efficacy of anthocyanins, consequently resulting in the loss of their biological activities[32]. Nevertheless, the pretreatment of BFM was still able to reduce the production of intracellular ROS in ARPE-19 cells-induced oxidative damage. In addition, ME and BFM may increase the antioxidant system in ARPE-19 cells to eradicate intracellular ROS, which will be further studied. Several studies have shown that anthocyanins from mulberry could activate the Nrf2 transcription factor which leads to the activation of gene expression for several antioxidant enzymes and phase I and II detoxification enzymes which could eradicate ROS from oxidative stress[33-37] Consequently, mulberry is a potentially valuable fruit for use in food and supplement development for AMD prevention.

CONCLUSION

The present study indicated that both the ethanol extract of mulberry and the bioaccessible fraction following simulated digestion of mulberry could protect human retina cells from oxidative damage induction which causes AMD development by suppressing the production of intracellular ROS without toxicity. However, this study is limited to *in vitro* research. Further investigation into the effects of mulberry on AMD is warranted, including studies involving experimental animal models and human subjects.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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