



Water extract of mangosteen suppresses H₂O₂-induced endothelial apoptosis by inhibiting oxidative stress

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ABSTRACT

Excessive production of reactive oxygen species (ROS) is a major cause of endothelial apoptosis. Mangosteen extract has been shown to possess antioxidant properties. Mangosteen is commonly extracted either with semi-polar solvent, yielding virtually pure α -mangostin, or with water, yielding a low α -mangostin concentration but including a wide variety of other polyphenols present in the fruit. However, the effect of a water extract of mangosteen (ME) on ROS induced cell death is not yet known. This study evaluated whether ME suppresses H₂O₂-induced endothelial cell death and ROS production in human endothelial cell lines. The concentrations of ME and H₂O₂ were determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. Intracellular ROS levels were determined by 2', 7' dichlorodihydrofluorescein diacetate assay, and cell death rates by MTT and Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling assays. Mitogen-activated protein kinase (MAPK) and apoptotic proteins were analyzed by western blot. Results showed that ME concentrations of 1, 5, and 10 μ g/ml were non-toxic. ME significantly attenuated ROS formation and cell death, both in a dose-dependent manner. ME also reduced phosphorylation of p38 MAPK as well as cleavage of caspase 3 and poly(ADP-ribose) polymerase-1. In summary, ME demonstrates anti-apoptotic effects against H₂O₂-induced endothelial cell death by inhibiting ROS formation and suppressing p38 MAPK.

INTRODUCTION

Excessive production of vascular reactive oxygen species (ROS) plays a central role in the development and progression of cardiovascular diseases (CVD), including hypertension, atherosclerosis, and myocardial infarction (Kockx and Knaepen, 2000). In normal arteries, there is a low turnover of endothelial cells because endothelial cells produce nitric oxide (NO) to protect against endothelial apoptosis (Kang-Decker *et al.*, 2007; Nematbakhsh *et al.*, 2008). ROS act as a cellular mediator of endothelial dysfunction and apoptosis (Hsu *et al.*, 2010; Niwa *et al.*, 2003). Previous research has shown that excessive production

of H₂O₂ impairs cell proliferation, reduces cell viability, and decreases the bioavailability of nitric oxide (Antunes and Cadenas, 2001; Price *et al.*, 2000; Stone and Collins, 2002).

There are three mitogen-activated protein kinases (MAPKs): c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38-MAPK. All three are serine/threonine kinases. MAPK signaling is a response to stress. ERK is usually associated with cell survival, whereas JNK and p38 are usually associated with cell apoptosis (Carvalho *et al.*, 2004; Colavitti *et al.*, 2002; Peshavariya *et al.*, 2009). It has been shown that H₂O₂ induces apoptosis through MAPK (Ramachandran *et al.*, 2002).

Mangosteen (*Garcinia mangostana* L.) is a tropical fruit found extensively in Southeast Asia, including in Thailand. Many parts of the mangosteen tree have been used in traditional medicine, for example, the leaves and bark are used to treat eczema, and the pericarp is used to treat dysentery. The peel of the mangosteen fruit has been found to contain more than 50 xanthenes (such as

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α -mangostin, γ -mangostin, β -mangostin, and garcinone), and it also contains flavonoids and proanthocyanidins (Fu *et al.*, 2007). The xanthenes are nonpolar, while the proanthocyanidins are polar. Xanthenes have been shown to possess anti-inflammatory (Gutierrez-Orozco *et al.*, 2013), anti-tumor (Hung *et al.*, 2009; Kaomongkolgit *et al.*, 2011; Krajang *et al.*, 2011; Kosem *et al.*, 2013; Kritsanawong *et al.*, 2016; Kwak *et al.*, 2016; Lee *et al.*, 2016; Li *et al.*, 2014), and antioxidant properties (Jung *et al.*, 2006; Kosem *et al.*, 2007; Kondo *et al.*, 2009). The polar fraction of mangosteen extract (ME) is also antioxidant. Moongkarndi *et al.* (2014) showed specifically that polar compounds extracted from mangosteen peel are more antioxidant than the nonpolar compounds. Previous studies have also shown that ME has neuroprotective effects during oxidative stress in SK-N-SH neuronal cells (Moongkarndi *et al.*, 2010; Sattayasai *et al.*, 2013). In addition, ME suppresses amyloid peptide-induced neural injury and prevents memory impairment in mice. However, very little research exists on the effects of ME on H₂O₂-induced endothelial cell injury. The current study examines the antioxidant effects of ME on H₂O₂-induced endothelial cell death.

MATERIALS AND METHODS

Reagents

Dulbecco's Modified Eagle's Medium (DMEM), trypsin-EDTA, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), penicillin-streptomycin, and fetal bovine serum (FBS) were purchased from Gibco-Invitrogen (Grand Island, NY, USA). 2', 7' dichlorodihydrofluorescein diacetate (DCF-DA) was obtained from Sigma-Aldrich (St. Louis, MO) and the apoptosis assay kit was purchased from Merck (Mendota Heights, MN).

Plant material and extraction

Mangosteen extraction followed the procedure previously described by Moongkarndi *et al.* (Moongkarndi *et al.*, 2010; 2014). Briefly, the peel of the mangosteens was chopped, dried, crushed, and then macerated with ethanol. This crude ethanolic extract was then partitioned with ethyl acetate and water to fractionate the low polar and high polar fractions, respectively. That step separated xanthone and α -mangostin away from the water-soluble constituents. The water-soluble fraction was then spray dried to yield a powder extract. This powder extract is the mangosteen extract referred to as ME in this study. A preliminary analysis of the chemical constituents and biological activities of the ME were performed by thin-layer chromatography and high-performance liquid chromatography.

Cell culture

Human endothelial cell lines (EA.hy926; ATCC number CRL-2922) were cultured in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, and incubated with 5% CO₂ at 37°C. This same incubation atmosphere and temperature were used throughout the study. This medium is hereafter referred to as "complete medium" (CM). At 70% confluence, the cells were prepared for subsequent experiments.

Cell cytotoxicity assay

Cytotoxicity of the ME and H₂O₂ were separately determined by MTT assay. 1×10^3 cells were incubated with CM in each well of a 96-well plate for 24 hours. After that, the original CM medium was removed and replaced with a low serum medium (LSM) consisting of DMEM supplemented with 1% FBS and 0.5% penicillin-streptomycin for 8 hours. The cells were then treated by adding various concentrations of ME (0, 1, 5, 10, 20, or 40 μ g/ml) or H₂O₂ (0, 50, 100, 200, or 300 μ M) to the existing medium and incubating them for another 24 hours. After that incubation, the medium was suctioned off and into each well was added fresh LSM and 5 mg/ml of MTT. The cells were then incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 4 hours. The cells were then again drained of their medium and DMSO was added to dissolve the formazan crystals. Finally, the optical density of each well's contents was measured at 540 nm using a microplate reader.

Intracellular ROS assay

In order to first find the most effective concentration of H₂O₂ to produce ROS, cells were incubated with CM in 24 well plates. At 70% confluence, the CM was replaced with LSM and incubated for another 8 hours. Then to each well was added 20 μ M DCF-DA. The cells were incubated for another 30 minutes and then washed with PBS and drained. Fresh LSM was added, along with H₂O₂ at concentrations of 0, 50, 100, 200, or 300 μ M. The fluorescence intensity was immediately determined by a microplate reader with excitation 504 nm and emission 529 nm. Next, in order to determine the effect of ME on H₂O₂-induced ROS production, cells were prepared as per the above procedure, except just before adding DCF-DA, three different concentrations (1, 5, and 10 μ g/ml) of ME were added as a pretreatment, followed by an additional 30 minutes of incubation and another PBS wash and replenishment of fresh LSM. Then DCF-DA was added just as before and the original procedure continued as above, except that only the optimal concentration of H₂O₂ was used (200 μ M). Finally, fluorescence intensity was determined as before.

Cell viability assay

In order to determine the effect of ME on H₂O₂-induced cell death, cells were cultured and then pretreated with three non-toxic concentrations of ME (1, 5, and 10 μ g/ml) for 30 minutes. After that, the cells were treated with 200 μ M H₂O₂ and then incubated for 24 hours. MTT was added, followed by 4 hours of additional incubation. Finally, DMSO was added. The optical density was measured using a microplate reader at 540 nm.

Apoptosis assay

In brief, cells were seeded onto an 18-mm coverslip in a 12-well plate and incubated until 70% confluence. The cells were then pretreated with ME for 30 minutes and then treated with H₂O₂ for 24 hours. Apoptotic cells were detected using the Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling (TUNEL) assay according to the manufacturer's protocol.

Western blot analysis

Cells were pretreated with ME for 30 minutes and then treated with H₂O₂ for 15 minutes. Cells were washed with PBS and then extracted with RIPA buffer. 20 µg of protein was separated using 8%–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene difluoride membrane. After washing and blocking, the membrane was incubated with the primary antibodies (p-p38, t-p38, p-JNK, t-JNK, p-ERK, t-ERK, cleaved caspase3, cleaved poly(ADP-ribose) polymerase-1 (PARP-1), and β-actin) overnight at 4°C. The membranes were then incubated again with horseradish peroxidase-conjugated secondary antibodies against the primary antibodies (1:2,000) for 1 hour more at room temperature. Blots were visualized by enhanced chemiluminescence and detected using the Gel Doc XR+ system. Band densities were analyzed with Image J software.

Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). Statistical comparisons were analyzed using one-way ANOVA with the Tukey Post hoc test. A *p*-value of less than 0.05 was considered as statistically significant.

RESULTS

Cell cytotoxicity and intracellular ROS production

Results of the cell cytotoxicity assay on ME (Fig. 1) showed that ME concentrations of 20 and 40 µg/ml significantly reduced the percentage of viable cells, by 10% and 60%, respectively, whereas ME concentrations of 1, 5, and 10 µg/ml did not significantly reduce cell viability. These three concentrations are, therefore, considered non-toxic and were used in the subsequent experiments. Similarly, results of H₂O₂ cytotoxicity are shown in Figure 2a. Only H₂O₂ concentrations of 200 and 300 µM significantly increased cell death. The results of the intracellular ROS assay for selecting the optimal H₂O₂ concentrations are seen in Figure 2b. H₂O₂ enhanced ROS production in a concentration-dependent manner. Specifically, H₂O₂ concentrations of 200 and 300 µM significantly increased ROS production and cell death.

200 µM H₂O₂ was used in the subsequent experiments. Since 200 µM was the minimum concentration that caused both maximum ROS production and significant cell death.

Effect of ME on H₂O₂-induced intracellular ROS production

Next, the results of the intracellular ROS assay for determining the effect of ME on H₂O₂-induced ROS production are displayed in Figure 3. ME at 5 and 10 µg/ml significantly reduced H₂O₂-induced ROS. Both of these ME concentrations yielded a 30% reduction.

Effect of ME on H₂O₂ induced cell death

Figure 4 shows that ME at 5 and 10 µg/ml significantly inhibited H₂O₂-induced cell death. Both of these ME concentrations enhanced cell viability by 10%.

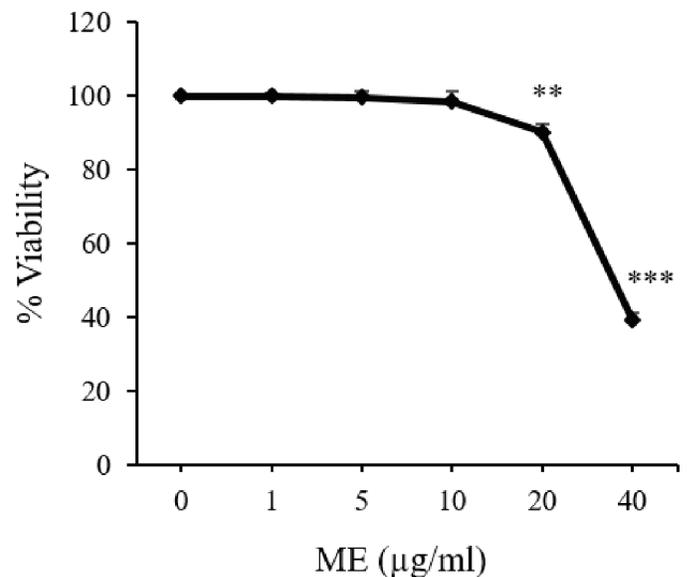


Figure 1. Effect of ME on cell viability. Cells were treated with various concentrations of ME. Cell viability was assessed by MTT assay (*n* = 3, ** *p* < 0.01, *** *p* < 0.001 vs. control).

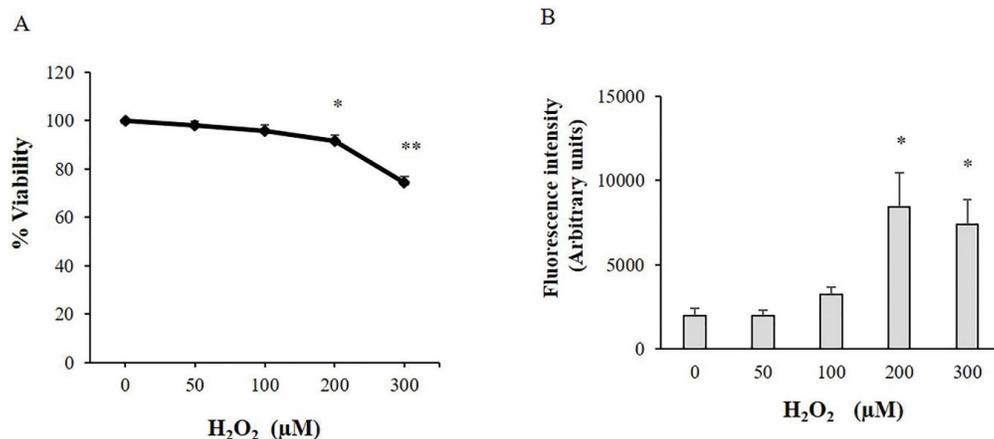


Figure 2. Effects of H₂O₂ on cell death and ROS formation. Cells were treated with different concentrations of H₂O₂ for 24 hours. (A) Cell viability was determined by MTT assay and (B) ROS production was detected by DCF-DA (*n* = 3, * *p* < 0.05, ** *p* < 0.01 vs. control).

Effect of ME on H₂O₂-induced apoptosis

Mangosteen extract's inhibition of H₂O₂-induced apoptosis can be seen in Figure 5. ME concentrations of 5 and 10 µg/ml significantly inhibited cell apoptosis. The inhibition by either concentration was 33%.

ME suppresses H₂O₂-induced apoptosis through p38 MAPK

The western blot results are displayed in Figure 6. H₂O₂ significantly activated p-p38 MAPK, cleaved caspase 3, and cleaved PARP-1. ME significantly inhibited these three processes.

DISCUSSION

Endothelial cells are the innermost layer of blood vessels and they regulate vascular tone. Endothelial cells produce and release not only vasorelaxant substances such as NO and endothelium-derived hyperpolarizing factors but also vasoconstrictors, including endothelin, thromboxane A₂, and prostaglandin H₂. In normal physiology, there is a low turnover of endothelial cells in arteries because of the action of NO against apoptosis. However, excessive vascular ROS can induce endothelial dysfunction and apoptosis, leading to the development and progression of CVD. Therefore, ROS can greatly affect the molecular signaling pathway of endothelial apoptosis. Previous studies have established that H₂O₂ induces apoptosis in endothelial cells (Niwa *et al.*, 2003; Hsu *et al.*, 2010), epithelial cells (Carvalho *et al.*, 2004), and neuronal cells (Sattayasai *et al.*, 2013).

Polyphenols from mangosteen have been shown to possess antioxidant properties. For example, α -mangostin prevented cardiac reperfusion injury by reducing oxidative stress in the Lagendorff-reperfused heart model (Buelna-Chontal *et al.*, 2011). In other studies, α -mangostin also attenuated ROS production induced by hypoxia in retinal endothelial cells (Jittiporn *et al.*, 2014) by H₂O₂ in human retinal pigment epithelial cells (Fang *et al.*, 2016) and by high glucose in endothelial cells (Jittiporn *et al.*, 2017). Also, α -mangostin reduced total ROS and mitochondrial ROS in high fat-induced hepatic steatosis (Tsai *et al.*, 2016). The mangosteen extracts used in the above-mentioned studies were all semi-polar solvent extracts with purified α -mangostin (>98%), while the studies mentioned below all used a powdered ME. The finished ME powder contained less than 2% α -mangostin but included a broad spectrum of other polyphenols from the fruit. A previous study by Moongkarndi *et al.* (2010) showed that ME suppresses beta-amyloid-induced oxidative stress in SK-N-SH neuroblastoma cells (Moongkarndi *et al.*, 2010). In addition, Sattayasai *et al.* (2013) found that ME demonstrates antioxidant action in H₂O₂-induced or polychlorinated-biphenyl induced oxidative stress in human neuroblastoma cells. In the same study, ME also protected against memory deficit in mice. Suthammarak *et al.* (2016) studied the antioxidant action of ME in healthy volunteers aged 20–60. That study showed that ME enhances the antioxidant ability of red blood cells (RBC) and decreases oxidative damage of proteins within RBCs. Jaisupa *et al.* (2018) showed that ME attenuated H₂O₂-induced intracellular ROS production through enhancement of catalase and heme oxygenase-1 in human neuroblastoma cells and human embryonic kidney cells. Dovetailing with the findings of the previous ME studies mentioned here, the results of the

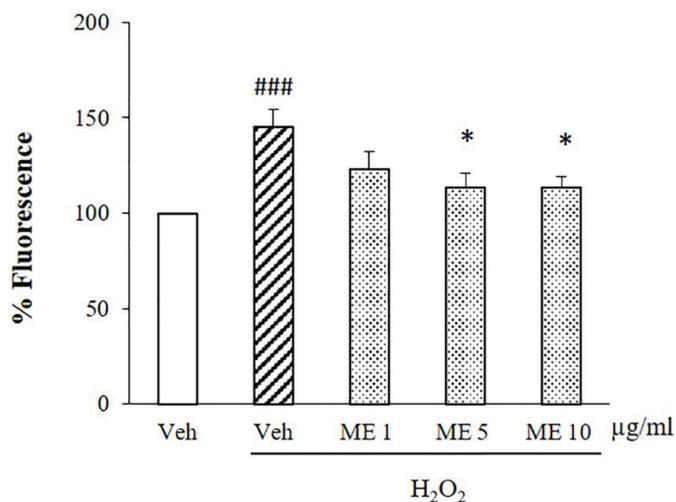


Figure 3. Impact of ME on H₂O₂-induced ROS formation. Human endothelial cells were treated with or without H₂O₂ in the presence or absence of ME. ROS production was detected by DCF-DA (n = 3, ###p < 0.001 vs. vehicle, * p < 0.05 vs. vehicle + H₂O₂).

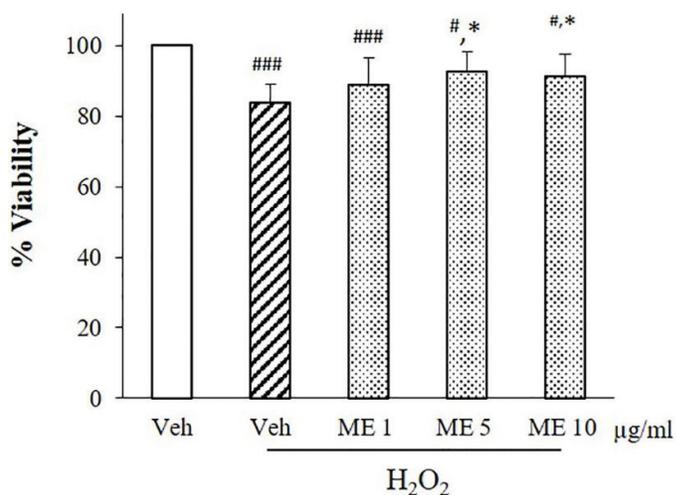


Figure 4. Impact of ME on H₂O₂-induced cell damage. Cells were treated with or without H₂O₂ in the presence or absence of ME. Cell viability was determined by MTT assay (n = 3, #p < 0.05, ###p < 0.001 vs. vehicle, *p < 0.05 vs. vehicle + H₂O₂).

present study demonstrated that ME attenuated intracellular ROS in endothelial cells.

Specifically, this study showed that ME significantly suppressed H₂O₂-induced endothelial apoptosis in a dose-dependent manner by inhibiting ROS production. The results indicate that ME significantly suppressed TUNEL positive cell death and MTT cell death induced by H₂O₂. The signaling pathway of ROS induced apoptosis relies on MAPKs (Carvalho *et al.*, 2004; Matsuzawa and Ichijo, 2008; Son *et al.*, 2011). This study showed that ME inhibits H₂O₂-induced phosphorylation of p38 but does not inhibit ERK, JNK, or Akt (data not shown). ME also significantly suppressed apoptotic downstream signaling including cysteine-

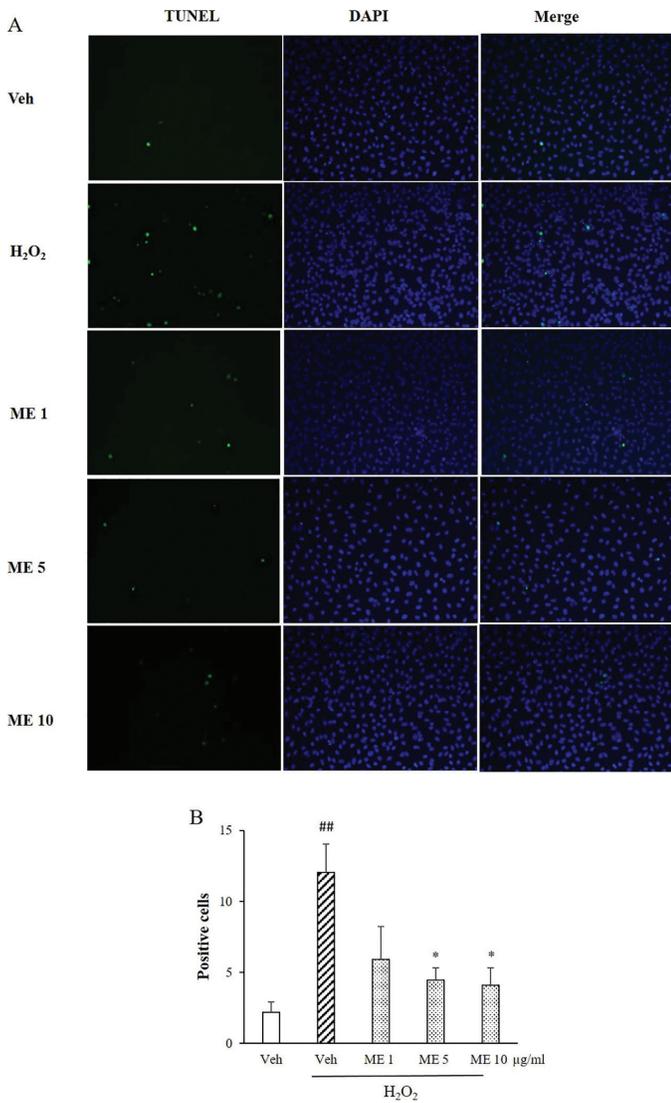


Figure 5. Impact of ME on H₂O₂-induced cell apoptosis. (A) Cells were treated with or without H₂O₂ in the presence or absence of ME. Apoptotic cells detected by TUNEL assay. (B) Quantification of apoptotic cells was determined manually (n = 3, ##p < 0.01 vs. vehicle, *p < 0.05 vs. vehicle + H₂O₂).

dependent aspartate-specific proteases 3 (caspase 3) and PARP-1. Caspase 3, which belongs to the cysteine proteases group, is considered the main terminal cleavage enzyme in the apoptotic cascade. Activation of caspase 3 results in the cleaving of PARP-1. Cleavage of PARP-1 is another hallmark of apoptosis (Mallat and Tedgui, 2000; Nicholson *et al.*, 1995). Previous studies have shown that α -mangostin attenuated hepatic cell apoptosis in high fat-induced hepatic steatosis in rats (Tsai *et al.*, 2016) as well as high glucose-induced apoptosis in endothelial cells through inhibition of phosphorylation of JNK and p38 MAPK (Jittiporn *et al.*, 2017) and inhibition of acid sphingomyelinase (Luo and Lei, 2017). ME also suppressed amyloid and H₂O₂-induced neuronal cell apoptosis (Moongkarndi *et al.*, 2010; Sattayasai *et al.*, 2013) and decreased scopolamine-induced memory impairment in mice (Sattayasai *et al.*, 2013). The current study indicates that by downregulating the p38 MAPK, caspase3, and PARP-1 signaling pathway, ME is able to protect against H₂O₂-induced apoptosis in endothelial cells.

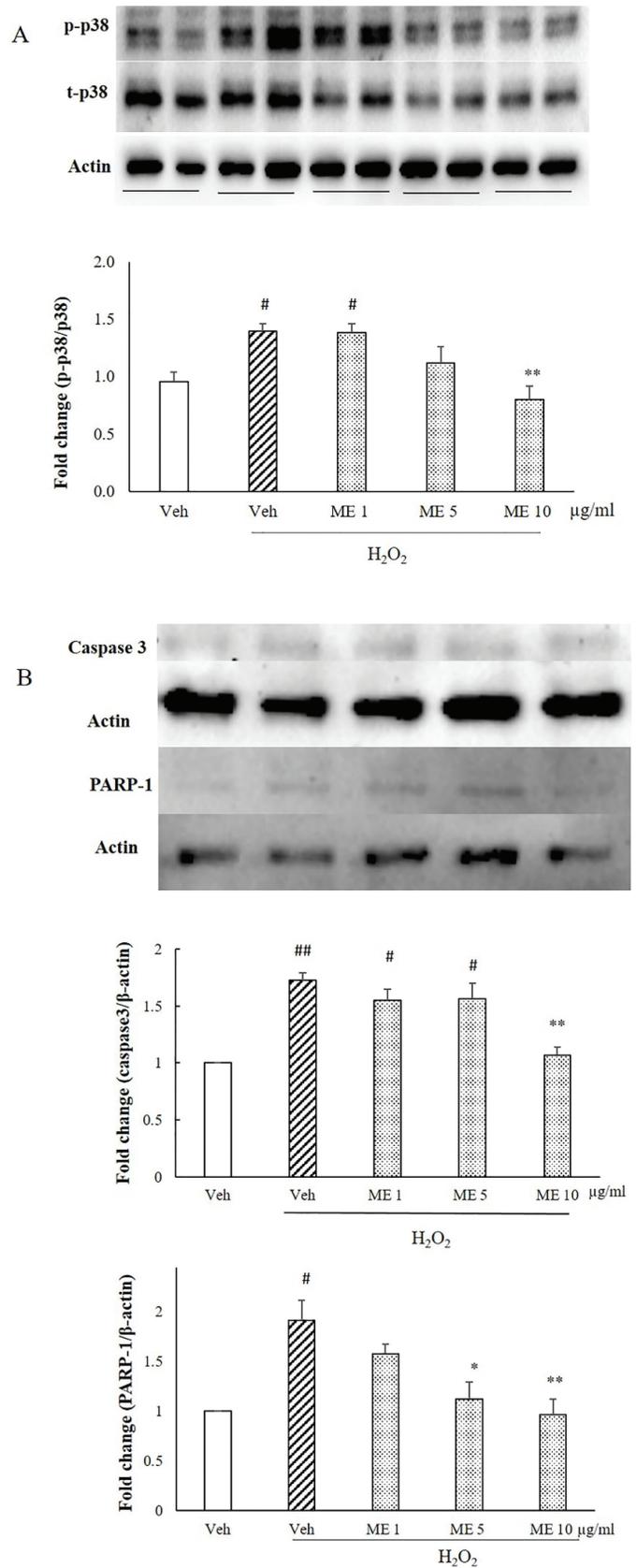


Figure 6. Determination of the apoptosis signaling in response to treatment of ME. Cells were treated with or without H₂O₂ in the presence or absence of ME for 15 minutes. (A) p38, (B) caspase3 and PARP-1 were detected by Western blot (n = 3, # p < 0.05, ## p < 0.01 vs. vehicle, *p < 0.05, **p < 0.01 vs. vehicle + H₂O₂).

CONCLUSION

This study has demonstrated that water extracts of mangosteen exert antioxidant and anti-apoptotic actions on endothelial cells through p38 MAPK, and these abilities make ME a good candidate for use as a natural substance in the prevention of CVD.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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