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# Dihydromyricetin protects endothelial cells from hydrogen peroxide-induced oxidative stress damage by regulating mitochondrial pathways

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ABSTRACT

*Heading aims:* Dihydromyricetin (DMY) is the most abundant ingredient in vine tea. Here, we investigated the cytoprotective effects and possible mechanisms of DMY on hydrogen peroxide ($H_2O_2$)-induced oxidative stress damage in human umbilical vein endothelial cells (HUVECs).
*Materials and methods:* The percentage of cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. We determined the antioxidant properties of DMY by measuring the activity of superoxide dismutase (SOD) and malondialdehyde (MDA). Flow cytometry was used to measure apoptosis in HUVECs that were double stained with Hoechst 33342 and propidium iodide (PI). The generation of intracellular reactive oxygen species (ROS) was measured in 2′,7′-dichlorofluorescin diacetate (DCFH-DA)-loaded HUVECs using a fluorescent microscope. Moreover, the expression of apoptosis-related proteins was determined by Western blotting. In addition, the release of nitric oxide (NO) was analyzed using a commercial kit.
*Key findings:* HUVECs treated with $H_2O_2$ had a notable decrease in cell viability that was attenuated when cells were pretreated with DMY (37.5–300 μM). DMY pretreatment significantly attenuated $H_2O_2$-induced apoptosis in HUVECs and inhibited intracellular ROS overproduction. Finally, pretreatment of cells with DMY prior to $H_2O_2$ exposure resulted in the inhibition of p53 activation, followed by the regulation of the expression of Bcl-2 and Bax, the release of cytochrome c, the cleavage (activation) of caspase-9 and caspase-3, and then the suppression of PARP cleavage in $H_2O_2$-induced HUVECs.
*Significance:* Our study is the first to report that DMY can protect HUVECs from oxidative stress damage, an effect that is mediated by the mitochondrial apoptotic pathways.

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## 1. Introduction

The vascular endothelium is thought to be the biggest endocrine organ in the human body and is known to play a critical role in the entire cardiovascular system [35,45]. The loss of endothelial cell function is a critical event in the initiation and progression of vascular diseases such as arteriosclerosis, hypertension and diabetes—ultimately leading to complications including stroke or myocardial infarction [38,40]. Several lines of evidence demonstrate that oxidative stress is a major initiator of endothelial cell injury in the pathogenesis of cardiovascular diseases and disorders [8,15]. For example, endothelial cell function is impaired by oxidative stress [15], decreased nitric oxide (NO) production, increased production of reactive oxygen species (ROS), and depletion of the antioxidant systems [7,11]. Previous studies have also indicated that the continuous release of NO inhibits apoptosis in vascular endothelial cells, while high levels of ROS induce cellular apoptosis and thus contribute to the progression of vascular diseases such as atherosclerosis [9,19]. In addition, oxidative stress can mediate cellular apoptosis through regulation of both mitochondria-dependent and mitochondria-independent pathways [34].

*Ampelopsis grossedentata* (Hand.-Mazz.) W. T. Wang, an indigenous plant in South China, is widely distributed in several provinces in the southern region of the Yangtze River, such as Hubei, Fujian, Guangdong, Guangxi, Guizhou, Hunan, Jiangxi and Yunnan. The tender stems and leaves of *A. grossedentata*, commonly known as vine tea, are used as a medicinal herb in traditional Chinese medicine [10]. As recorded in the *Chinese Materia Medica*, vine tea can clear away heat, promotes diuresis and blood circulation, and removes channel obstructions (Editorial Committee of *Chinese Materia Medica*, 1999). Indigenous people including the Tujia, Lahu, Yao, and Dong in China have used vine tea to treat hypertension and other diseases such as the common cold, fever, chronic pharyngitis, scabies, and constipation for hundreds of years [26].

As the most abundant ingredient in vine tea, dihydromyricetin (DMY) can reach levels of more than 30% in the tender stems and leaves [43]. A growing body of research suggests that the bioactivity of vine tea is due to the various beneficial effects of DMY; also considered to be the most important bioactive ingredient. Although the practices of

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traditional Chinese medicine as well as evidence from modern experimental studies suggest that vine tea has cardiovascular benefits, the direct protective effects of DMY on endothelial cells remain unknown. Therefore, this paper explores the protective effects of DMY on $H_2O_2$-induced oxidative stress damage and cell apoptosis of human umbilical vein endothelial cells (HUVECs). Furthermore, we investigated the underlying molecular mechanisms of action involved in this process.

## 2. Materials and methods

### 2.1. Materials

DMY (purity > 99%) was isolated and provided by the Department of Pharmacy, Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology. The identity and purity of this compound were determined by high-performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) as previously described [2]. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, USA). The Hydrogen peroxide ($H_2O_2$), RPMI 1640 medium, fetal bovine serum (FBS) and trypsin were purchased from GIBCO-BRL (Grand Island, NY); NO, SOD and MDA assay kits were procured from Nanjing Jiangcheng Bioengineering Institute (Nanjing, China); and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) was purchased from Acros Organics (New Jersey, USA). The anti-GAPDH, anti-β-actin, anti-Bax, anti-Bcl-2, anti-PARP, anti-cleaved-PARP, anti-cleaved caspase-3, anti-p53, anti-p-p53, anti-cleaved-caspase-9 and anti-cytochrome c antibodies were obtained from Cell Signaling Technology (1:1000 dilution; Boston, MA). All other chemicals and reagents in this work were commercially available and of analytical grade.

### 2.2. Cell culture and treatments

HUVECs were purchased from American Type Culture Collection (ATCC) (Washington, DC, USA). Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and maintained at 37 °C in a humidified 5% $CO_2$ incubator with media replenished every 2 days. HUVECs between passage numbers 4 and 12 were used in this study. The DMY stock solution was prepared in DMSO and diluted with PBS prior to the experiment. The cells were then incubated in a serum-free medium for 24 h and treated with $H_2O_2$ (400 μM) in the absence or presence of DMY at different intervals, after which they were harvested for further analysis [23].

### 2.3. Cell viability assay

HUVECs were seeded at a density of $5 \times 10^4$ cells/mL in 96-well plates. DMY was dissolved in DMSO. Once cells were 80% confluent, they were washed with RPMI 1640 medium then incubated with or without DMY of varying concentrations for 2 h, then exposed to $H_2O_2$ for an additional 24 h. An MTT assay was used to measure cell viability as previously described [5]. In brief, MTT was dissolved in RPMI 1640 medium, added to each well, and incubated at 37 °C for another 4 h at a final concentration of 5 mg/mL. The medium was subsequently replaced with 150 μL of DMSO at the indicated time points. The absorption values were measured on a universal microplate reader (Elx 800, Bio-TEK Instruments Inc., USA) at 490 nm (630 nm as a reference). Cell viability is expressed as a percentage of the control value. All data are expressed as the mean ± SD of three experiments with six wells per treatment group.

### 2.4. SOD and MDA

Cells were plated in 6-well plates ($1 \times 10^6$ cells/well). After incubation for 24 h, the cells were treated with various concentrations of DMY (ranging from 37.5–300 μM) for 2 h, followed by exposure to $H_2O_2$ (400 μM) for 24 h. Then the cells were washed three times with ice cold PBS and centrifuged at 1000 rpm for 5 min. The concentrations of SOD and MDA were measured by using the xanthine oxidase and thiobarbituric acid methods on a microplate reader (Salzburg, Austria) at 532 nm and 450 nm, respectively [22]. All of the procedures were performed according to the manufacturer's instructions (Jiancheng, Nanjing, China). Values for different groups are expressed as a percentage of the control.

### 2.5. Hoechst 33342/PI double staining

The nuclear chromatin morphological changes indicative of apoptosis were detected by fluorescent microscopy using Hoechst 33342/PI double fluorescent staining as previously described [32]. Cells were seeded in 24-well plates at a density of $2 \times 10^5$ per well. After treatment, cells were stained with PI (10 μg/mL) and Hoechst 33342 (10 μg/mL) for 10 min, and then fixed with 4% paraformaldehyde for 10 min. Morphological changes of the nuclear chromatin were observed under a fluorescent microscope. The PI and Hoechst 33342 were excited at 340 and 620 nm (Li et al., 2013).

### 2.6. Apoptosis detection by flow cytometry

To determine whether or not DMY has a protective effect on $H_2O_2$-exposed HUVECs over the range of concentrations studied, we used an Annexin V-FITC apoptosis detection kit (Bestbio, China) to measure apoptosis. Briefly, HUVECs were harvested (floating and attached cells) following treatment with increasing concentrations of DMY, washed twice with PBS, double-stained using the Annexin V-FITC apoptosis detection kit according to the manufacturer's protocol [24], then analyzed by flow cytometry (Beckman-Counter, USA).

### 2.7. Measurement of ROS production

The effect of DMY on $H_2O_2$-induced ROS production in HUVECs was determined by performing a fluorometric assay, using 2′,7′-dichlorofluorescin diacetate (DCFH-DA) as a probe for the presence of hydroxyl radicals. Confluent HUVECs ($2 \times 10^5$ cells/well) in 6-well plates were preincubated with or without DMY at increasing concentrations for 2 h, followed by exposure to $H_2O_2$ (400 μM) for 2 h. The cells were then washed with PBS and subsequently incubated with 20 μM DCFH-DA in PBS at 37 °C for 1 h. Free radicals generated within the cells cleave the diacetate from DCFH-DA to form DCFH, which can then be detected by fluorescent microscopy (ix71, Olympus, USA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm and subsequently photographed [28,44]. The results are expressed as a percentage of the control fluorescence intensity.

### 2.8. The effect of DMY on NO release

NO is a signaling molecule that plays a key role in the regulation of endothelial functions. The production of NO was determined by using a total NO detection assay kit in cultured HUVECs. Cells were treated with various concentrations of DMY (ranging from 37.5–300 μM) for 2 h, followed by exposure to $H_2O_2$ (400 μM) for 24 h. Then the supernatant was collected from plates and the NO released into the media was assessed according to the supplied manual. The concentration of nitrites was determined by measuring the absorbance with a microplate reader at 540 nm [5].

### 2.9. Western blot analysis

HUVECs were grown to confluence, pretreated with DMY (37.5–300 μM) for 2 h, and then exposed to $H_2O_2$ (400 μM)) for an additional 1 h or 24 h. After treatment, cells were washed, scraped from culture dishes, and lysed in ice-cold RIPA buffer. The debris was

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