

Protective Effect of Dealcoholized Persimmonwine on H₂O₂ - Induced Oxidative Injury in H9c2 Cardiomyocytes

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Abstract

In this study, we investigated the antioxidant capacity of persimmon wine (PW) and dealcoholized persimmon wine (DPW). Both PW and DPW showed radical scavenging activity in the DPPH (1-diphenyl-2-picrylhydrazyl) assay. We next analyzed the phenolic content and major compounds present in PW using high-performance liquid chromatography (HPLC). Phenolic compounds, including gallic acid, catechin, and epicatechin, were found in PW. Gallic acid was the most abundant phenolic compound (157.5 µg/ml) in PW. In addition, the protective effects of DPW and gallic acid against H₂O₂-induced cell injury in H9c2 cardiomyocytes were investigated. Pretreatment with DPW or gallic acid strongly inhibited H₂O₂-induced cell death in a dose-dependent manner. These results suggested that PW and its major phenolic component, gallic acid, were effective inhibitors of oxidative stress and oxidative stress-induced cardiomyocyte injury.

Keywords: dealcoholize dpersimmon wine, oxidative stress, H9c2 cardiomyocytes, phenolic compounds, H₂O₂

1. Introduction

Persimmon is widely grown in oriental countries, such as China, Japan, and Korea. Persimmon fruit showed some lipid lowering effects in animal models (Matsumoto, Watanabe, Ohya, & Yokoyama, 2006). Furthermore, it is abundant in nutrients, including vitamins A, B, and C; carotenoids; glucose, and fructose. Persimmon also contains other active compounds, such as polyphenols, which have been reported to have protective effects against oxidative stress, and exerted benefits on diabetes, obesity, cardiovascular disease, and even cancer (Matsumoto, Watanabe, Ohya, & Yokoyama, 2006; Wojcik, Burzynska-Pedziwiatr, & Wozniak, 2010). Phenolic composition is an important factor affecting the functionality of natural ingredients or products (Cabrera, Artacho, & Giménez, 2006). A number of studies have identified a correlation between the phenolic components and physiological activities of natural ingredients or products (Kılıçgün & Altınar, 2010).

Reactive oxygen species (ROS) have been reported to be associated with the development of various diseases, including cardiovascular disease. In particular, heart ischemia and reperfusion lead to the generation of ROS, thereby resulting in cellular injury (Varela, Rolo, & Palmeira, 2011). A number of studies have suggested that antioxidants have protective effects on ischemia-reperfusion-induced cell death. For example, administration of antioxidant protected against ischemia-reperfusion injury in cardiomyocytes (Braunersreuther & Jaquet, 2012). Several antioxidants found in food sources also exerted protective effects against ROS-induced cellular injury in various cell types (Rodrigo, Prieto, & Castillo, 2013; López-Miranda et al., 2012).

Persimmon wine (PW) contains various chemical compounds, including polyphenols. Although PW has bioactive compounds and is believed to influence various physiological functions, its ability to protect against oxidative stress has not yet been investigated. To determine the antioxidant activity of PW in vitro, either PW or dealcoholized persimmon wine (DPW) were used. Dealcoholized persimmon wine (DPW) was used to investigate cardioprotection in H9c2 cells because the alcohol in PW can affect the cell system and was therefore not added directly to the cells.

2. Material and Methods

2.1 Reagents and Cell Culture

1,1-Diphenyl-2-picrylhydrazyl (DPPH), (+)-catechin, (-)-epicatechin, gallic acid monohydrate, hydrogen peroxide (H₂O₂), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and

2,7-dichlorofluorescein-diacetate were purchased from Sigma (Sigma-Aldrich, St. Louis, Mo., USA.). H9c2 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from WelGene (Daegu, South Korea). Cells were grown in DMEM containing 10% fetal bovine serum, 100 U/ml of penicillin, 100 µg/ml of streptomycin and maintained in a 5% CO₂ incubator at 37 °C (Hwang, Kwon, Park, & Kim, 2008).

2.2 Wine Making and Sample Preparation

PW was produced by Yangchon Persimmon Wine Farm Corporation (Choosi wine, 2010). Briefly, harvested persimmons were randomly crushed and incubated with 1.5% citric acid, 2.5% tartaric acid, and 250 ppm sulfite in sterile conditions. After incubation, 0.2% *Monascus* (KCCM 60170, Korean Culture Center of Microorganisms, Seoul, South Korea) was added and the temperature was maintained at approximately 26 °C for 10 days. Thereafter, the temperature was increased to 50 °C for an additional 3 days to induce alcoholic fermentation. Once fermentation was complete, the fermented liquid was transferred to a new tank, inoculated with red wine yeast and pectin lyase (Pascal Biotech, Paris, France), and maintained at 26 °C for 14 days. Cellulase (Lot No. CTD1150106, Amano Enzyme Inc., Nagoya, Japan) was added for 30 days, and thereafter, batonnage was performed for 50 days to remove the yeast. Finally, PW was filtered (Papeleradel Besós Placas Filtrantes SI, Barcelona, Spain) and stored at 18 °C for 1 year. DPW was prepared by evaporation in a rotary evaporator (Laborota 4000, Heidolph Instruments Inc., Schwabach, Germany), and freeze-drying to remove the alcoholic and aqueous phases. The resulting wine powder was re-suspended in distilled water prior to the cell culture experiments.

2.3 DPPH (1-diphenyl-2-picrylhydrazyl) Radical-Scavenging Assay

DPPH radical-scavenging activity was investigated according to the method of Hou et al (Hou et al., 2001). DPPH was dissolved in methanol (spectrophotometric grade) at various concentrations. PW and DPW samples (0.1 ml) were mixed with 0.1 ml of 300 µM DPPH solution for 30 min in the dark. The absorbance at 517 nm (A₅₁₇) was determined, using methanol as the blank. DPPH radical-scavenging activity was calculated according to the following equation: scavenging activity (%) = 100 × (A₅₁₇blank - A₅₁₇sample)/A₅₁₇blank. IC₅₀ values denote the concentration of sample required to scavenge 50% of DPPH free radicals.

2.4 Quantitative Analysis of Phenolic Compounds

Analysis was conducted using a high-performance liquid chromatography (HPLC) system (Jasco, Japan) with a Bondapak C18 (10 µm, 3.9 × 300 mm) column, and a mobile phase of distilled water containing 2% acetic acid (solvent A) and 50% acetonitrile containing 0.5% acetic acid (solvent B). Gradient elution of 10-80% solvent B over 70 min was used at a flow rate of 0.8 mL/min. The column temperature was maintained at 40 °C, and the signal was detected at 280 nm. Gallic acid, catechin, epicatechin, gallic acid, gallic acid, gallic acid, gallic acid, and gallic acid were used as standards for quantification and were purchased from Sigma (Sigma-Aldrich, St. Louis, Mo., U.S.A.).

2.5 MTT Assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed as described previously (Hwang et al., 2006). Briefly, H9c2 cells (3 × 10⁵) were seeded in 24-well culture plates and incubated for 48 h. Cells were pre-treated with DPW (10, 25, 50, 100, 200, 400, or 800 µg/ml) or gallic acid (10, 25, 50, 100, or 200 µg/ml) for 1 h before exposure to 500 µM H₂O₂ for 4 h. Two hours prior to the end of the H₂O₂ incubation, 30 µl MTT solution (5 mg/ml in PBS) was added to the cells. Thereafter, the solution was removed, the cells were dissolved in 150 µl DMSO and the absorbance at 570 nm read using a plate reader (Molecular Devices Corp., Sunnyvale, CA, USA).

2.6 ROS Measurement

Cellular ROS were measured using a fluorescence microscope as described previously (Hwang et al., 2006). Briefly, cells were pre-treated with DPW for 1 h, and then exposed to 500 µM H₂O₂ for 4 h. After an additional 30-min treatment with 10 µM 2',7'-dichlorofluorescein diacetate (DCFH-DA), cells were washed with PBS and the fluorescence was analyzed by fluorescence microscopy.

2.7 Statistical Analysis

All data are presented as means ± SD. Statistical analysis by one-way ANOVA was carried out using SPSS 9.0 (SPSS Inc., Chicago, IL). All experiments were performed in triplicate and repeated at least 3 times.

3. Results and Discussion

We analyzed the phenolic contents of PW (Figure 1A) using HPLC. As shown in Table 1, we analyzed several

phenolic compounds, including gallic acid, catechin, epicatechin, gallic acid, gallo catechin gallate, and catechingallate. Gallic acid was the most abundant phenolic compound in PW (157.5 µg/ml), whilst gallo catechin, gallo catechin gallate, and catechingallate were not detected.

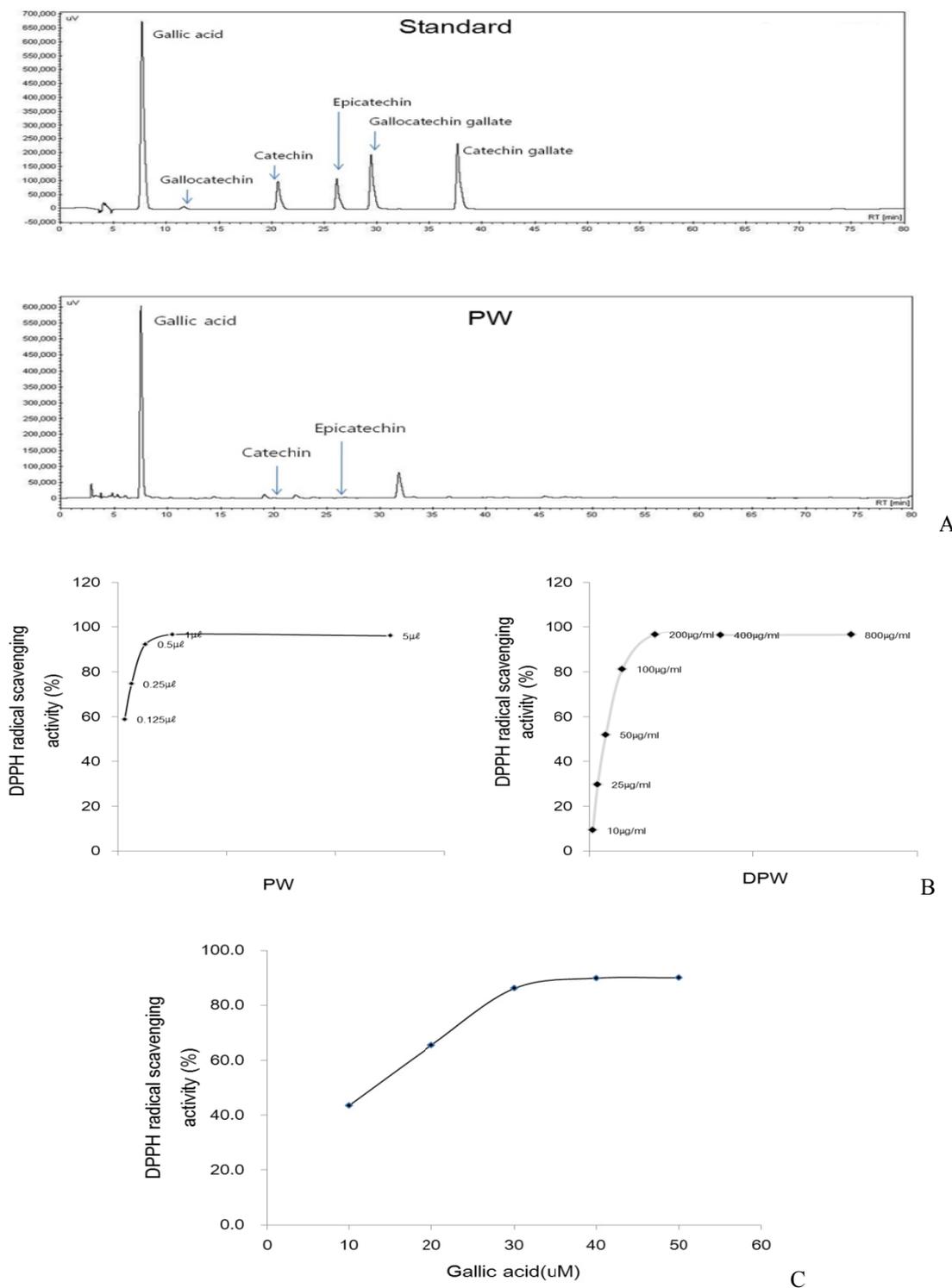


Figure 1. Key components of persimmon wine and their antioxidant activities

High-performance liquid chromatography (HPLC) analysis of phenolic compounds in persimmon wine (PW) (A). Radical-scavenging activities of PW and dealcoholized PW (DPW) measured using the DPPH method (B, C)

Table 1. Polyphenolic components of persimmon wine

Components	Contents ($\mu\text{g/mL}$)
Gallic acid	157.5 \pm 3.5
Catechin	1.6 \pm 0.1
Epicatechin	1.4 \pm 0.0

All measurements were repeated at least 3 times and the compounds expressed as $\mu\text{g/ml}$ of persimmon wine. We next examined whether PW or gallic acid had free radical scavenging capacity by using the DPPH assay. As shown in Fig. 1B, PW and DPW showed free radical-scavenging capacity in a dose-dependent manner. It was previously reported that persimmon fruit contained bioactive compounds such as gallic acid and tannin, and had the potential to prevent diseases involving oxidative stress (Chen, Fan, Yue, Wu, & Li, 2008; Loizzo et al., 2009). The present study confirmed that PW has antioxidant properties. Furthermore, we showed that gallic acid, a major constituent of PW, possessed radical-scavenging activity under these experimental conditions (Figure 1C). These results demonstrated that PW had antioxidant effects, and suggested that gallic acid may play an important role in these effects. ROS have been reported to be associated with the development of diseases, including cardiac injury (Varela, Rolo, & Palmeira, 2011).

Natural compounds have been proposed to reduce ROS levels in cell cultures (Quiñones, Miguel, & Aleixandre, 2013). DCFH-DA (2',7'-dichlorofluorescein diacetate) was used to examine the effect of DPW on ROS production in H9c2 cardiac muscle cells. As shown in Figure 2, H_2O_2 significantly increased the production of ROS (green color) as compared to control. Under the same conditions, treatment with DPW (100-800 $\mu\text{g/ml}$) decreased ROS production, similar to DPPH results. These results showed that DPW had a scavenging activity against intracellular ROS.

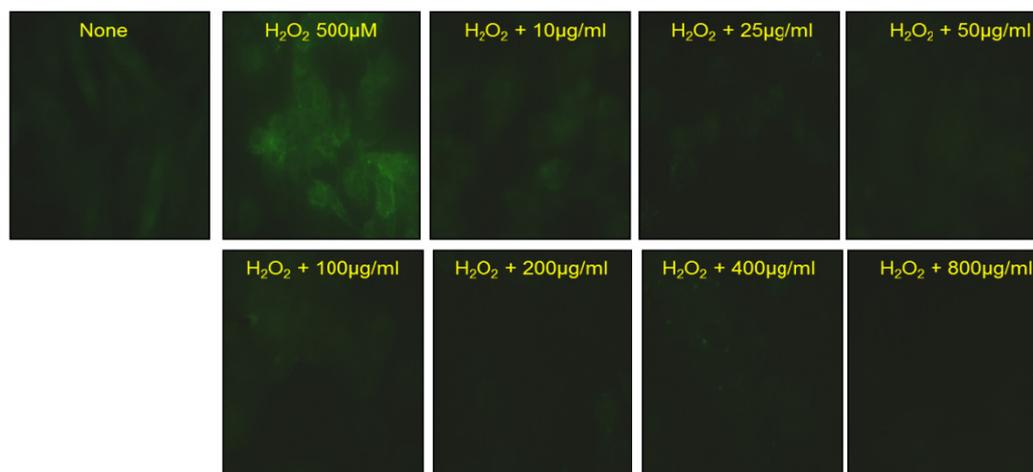


Figure 2. Effects of dealcoholized persimmon wine on reactive oxygen species generated by H_2O_2

Reactive oxygen species (ROS) generation was determined by DCFH-DA as described in Materials and Methods. Cells were pretreated with DPW for 1 h at the concentrations indicated, and then exposed to 500 μM H_2O_2 for 4 h. After an additional 30-min treatment with 10 μM 2', 7'- dichlorofluorescein diacetate (DCFH-DA), the fluorescence-activated cells were analyzed by fluorescence microscopy.

We next examined whether DPW or gallic acid protected against oxidative injury in cardiac muscle cells. H9c2 cells were pretreated with a range of DPW concentrations for 1 h, and then exposed to 500 μM H_2O_2 for 4 h. As shown in Figure 3A and B, exposure to H_2O_2 induced cell death, as compared to control. DPW protected the cells against H_2O_2 induced-cellular injury in a dose-dependent manner. In addition, we also investigated the effects of gallic acid on H_2O_2 induced-cellular injury. We found that gallic acid significantly decreased cell death under these conditions (Figure 3C). Our previous study supported the effect of resveratrol, a naturally occurring antioxidant, on cardiac cell injury in cell culture (Hwang, Kwon, Park, & Kim, 2008). The present study showed that DPW had a protective effect against the ROS stimulated by H_2O_2 in H9c2 cells. These results showed that

DPW and gallic acid had the ability to protect cardiac muscle cells from ROS. It is well known that red wine and its polyphenolic compounds prevent cardiovascular diseases. In particular, gallic acid is abundant in red wine and plays a central preventive role in cardiovascular disease by modulating ROS scavenging mechanisms such as catalase, superoxide dismutase (SOD), and glutathione (GSH) (Karthikeyan, SaralaBai, Gauthaman, & NiranjaliDevaraj, 2005; Seifried, Anderson, Fisher, & Milner, 2007). This finding is in agreement with our present study, where gallic acid was also the most abundant polyphenolic compound in PW and exerted a protective effect on ROS-induced cardiac injury.

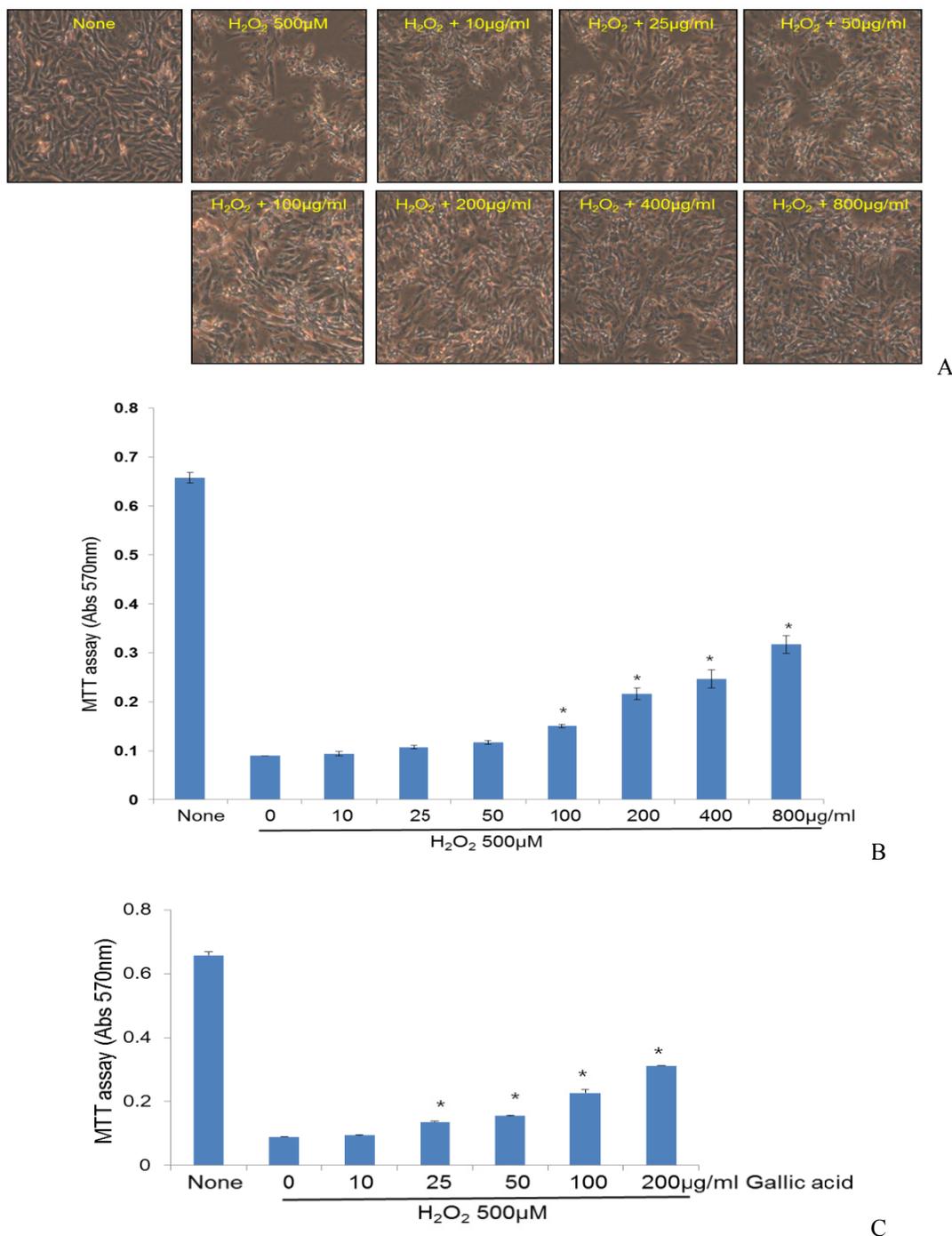


Figure 3. Effects of dealcoholized persimmon wine and gallic acid on H₂O₂-induced cardiac cell injury

Cells were pretreated with dealcoholized persimmon wine (DPW) or gallic acid for 1 h at the concentrations indicated, and then exposed to 500 μM H₂O₂ for 4 h. After finishing treatments, cell morphology and cell viability were studied by microscopy (A) or MTT assay (B,C).

4. Conclusion

The present study demonstrated that DPW exhibited antioxidant effects and protected against H₂O₂-stimulated cell death in H9c2 cells. Moreover, our findings implicated gallic acid as a key antioxidant constituent in DPW. Further studies should investigate the precise mechanism by which DPW and gallic acid exert protective effects against oxidative stress and cardiac injury.

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