Original Article

Chlorogenic acid analogues from *Gynura nepalensis* protect H9c2 cardiomyoblasts against H₂O₂-induced apoptosis

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Aim: Chlorogenic acid has shown protective effect on cardiomyocytes against oxidative stress-induced damage. Herein, we evaluated nine caffeoylquinic acid analogues (**1–9**) isolated from the leaves of *Gynura nepalensis* for their protective effect against H₂O₂-induced H9c2 cardiomyoblast damage and explored the underlying mechanisms.

Methods: H9c2 cardiomyoblasts were exposed to H₂O₂ (0.3 mmol/L) for 3 h, and cell viability was detected with MTT assay. Hoechst 33342 staining was performed to evaluate cell apoptosis. MMPs (mitochondrial membrane potentials) were measured using a JC-1 assay kit, and ROS (reactive oxygen species) generation was measured using CM-H₂DCFDA. The expression levels of relevant proteins were detected using Western blot analysis.

Results: Exposure to H₂O₂ markedly decreased the viability of H9c2 cells and catalase activity, and increased LDH release and intracellular ROS production; accompanied by a loss of MMP and increased apoptotic rate. Among the 9 chlorogenic acid analogues as well as the positive control drug epigallocatechin gallate (EGCG) tested, compound **6** (3,5-dicaffeoylquinic acid ethyl ester) was the most effective in protecting H9c2 cells from H₂O₂-induced cell death. Pretreatment with compound **6** (1.56–100 μmol/L) dose-dependently alleviated all the H₂O₂-induced detrimental effects. Moreover, exposure to H₂O₂ significantly increased the levels of Bax, p53, cleaved caspase-8, and cleaved caspase-9, and decreased the level of Bcl-2, resulting in cell apoptosis. Exposure to H₂O₂ also significantly increased the phosphorylation of p38, JNK and ERK in the H9c2 cells. Pretreatment with compound **6** (12.5 and 25 μmol/L) dose-dependently inhibited the H₂O₂-induced increase in the level of cleaved caspase-9 but not of cleaved caspase-8. It also dose-dependently suppressed the H₂O₂-induced phosphorylation of JNK and ERK but not that of p38.

Conclusion: Compound **6** isolated from the leaves of *Gynura nepalensis* potently protects H9c2 cardiomyoblasts against H₂O₂-induced apoptosis, possibly by inhibiting intrinsic apoptosis and the ERK/JNK pathway.

Keywords: chlorogenic acid analogues; *Gynura nepalensis*; cardiomyocytes; oxidative stress; ROS; apoptosis; epigallocatechin gallate; ERK/JNK pathway

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Introduction

Oxidative stress-induced cardiomyocyte apoptosis plays a critical role in the pathological progress of multiple types of heart conditions, including coronary artery disease, cardiomyopathy, myocarditis, and hypertension^[1, 2]. Apoptosis of cardiomyocytes generally begins with the generation of ROS in the ischemic myocardium. Excessive ROS accumulation in

a cell can lead to DNA damage, mitochondrial dysfunction, further ROS generation and cellular injury, contributing to the development and progression of myocardial remodeling^[2]. The mechanisms underlying apoptosis are highly complex and sophisticated, involving an energy-dependent cascade of molecular events^[3]. Over the past few decades, it has become evident that apoptosis is mediated by two central pathways, an extrinsic pathway involving cell surface receptors and an intrinsic pathway involving mitochondria and the endoplasmic reticulum^[4]. In contrast to the extrinsic pathway that transduces a specialized set of death stimuli, the intrinsic pathway integrates stimuli from a broad spectrum of extracellular and intracellular stresses^[5]. Changes to the inner

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mitochondrial membrane caused by ROS stimulation result in an opening of the mitochondrial permeability transition pore and the release of multiple death-promoting factors (including apoptosomes, which are assembled from recombinant Apaf-1, procaspase-9, cytochrome *c*, and dATP) residing in the mitochondrial intermembrane space into the cytosol^[6]. Pro- and anti-apoptotic Bcl-2 family proteins, which are regulated by the tumor suppressor protein p53, can control the release of these mitochondrial proteins by inducing or preventing changes to the permeability of the outer mitochondrial membrane^[7].

Members of the MAPK family regulate multiple cell functions, including proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis^[8]. Three MAPK subfamilies, p42/p44 extracellular signal-regulated kinase (ERK1/2), p38 MAPK, and *c*-Jun-NH₂ terminal protein kinase (JNK), are activated when oxidative stress occurs^[9, 10].

Gynura nepalensis DC, belonging to the family Compositae, is widely distributed in China, and its leaves have been used as a folk medicine for the treatment of hyperglycemia and hypertension. Phytochemical studies have revealed chlorogenic acid and its derivatives to be the major secondary metabolites of this plant species^[11]. Basic and clinical investigations indicate that chlorogenic acid may alleviate type 2 diabetes^[12], obesity^[13], and cardiovascular disease^[14, 15], as well as other diseases. *In vitro* studies have also demonstrated a protective effect of chlorogenic acid on cardiomyocytes against damage induced by oxidative stress^[16]. However, the molecular mechanisms involved in protecting cardiomyocytes, such as H9c2 cells, from ROS-induced apoptosis are still not clear. In this study, chlorogenic acid and some of its derivatives isolated from *G. nepalensis* were selected to evaluate their protective effect against H₂O₂-induced damage in H9c2 cardiomyoblasts, and the possible molecular mechanisms of anti-apoptosis were also investigated.

Materials and methods

Materials and reagents

All general reagents for cell culture were purchased from Gibco Life Technology, Shanghai, China. Hydrogen peroxide was from Sinopharm Chemical Reagent Co, Ltd. Hoechst 33342, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H₂DCFDA) were obtained from Sigma-Aldrich, Shanghai, China. The mitochondrial membrane potential was tested using a JC-1 assay kit (Beyotime, Haimen, China). Lactate dehydrogenase release and catalase activity were measured using kits from Beyotime, Haimen, China.

Plant material

G. nepalensis leaves were collected in November 2014 from a suburb of Shanghai, China, and identified by Prof Zhen-yu LI, Beijing Institute of Botany, Chinese Academy of Sciences. A voucher specimen (No WMZ-20140920-MYC) was deposited at the Shanghai Institute of Materia Medica.

Extraction and Isolation

Fresh *G. nepalensis* leaves (10 kg) were percolated with 95% ethanol. The extract was further separated via chromatography through a silica gel, followed by preparative HPLC to give nine pure chlorogenic acid derivatives: chlorogenic acid (**1**, 1.4 g), isochlorogenic acid B (**2**, 0.1 g), 3,4-dicaffeoylquinic acid methyl ester (**3**, 0.42 g), isochlorogenic acid A (**4**, 0.83 g), 3,5-dicaffeoylquinic acid methyl ester (**5**, 0.23 g), 3,5-dicaffeoylquinic acid ethyl ester (**6**, 0.05 g), isochlorogenic acid C (**7**, 0.06 g), 4,5-dicaffeoylquinic acid methyl ester (**8**, 0.048 g), and 3-*O*-*cis*-*p*-coumaroylquinic acid (**9**, 0.016 g) (Figure 1). The purity of these compounds was determined to be greater than 95% based on HPLC analysis [HPLC column, Athena C18-WP, 21 mm×50 mm; solvent system, acetonitrile-0.2% HCOOH with gradient elution; flow rate, 0.3 mL/min; UV detection, 327 nm; Shimadzu LC-MS-2020, Shimadzu Corporation, Kyoto, Japan].

Figure 1. Structures of chlorogenic acid derivatives (**1–9**) isolated from the leaves of *Gynura nepalensis*.

Cell culture

H9c2 cardiomyoblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 1.5 g/L glucose and 10% (*v/v*) fetal bovine serum (FBS). All cells at passages 10–15 were kept at 37°C with 5% CO₂ in humidified air.

Cell viability assay

Cell viability was determined using an MTT assay as previously described^[17]. In brief, H9c2 cardiomyoblasts were first pretreated with 0.1% dimethyl sulfoxide (DMSO) or the test compounds at different concentrations (0, 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 μmol/L) for 1 h. After pre-incubation, an H₂O₂ solution at a final concentration of 0.3 mmol/L was added, and cells were incubated for 3 h. The cells were then

supplemented with 0.5 mg/mL MTT at 37°C for another 4 h. The medium was removed, and the resulting formazan was dissolved in 100 μ L DMSO. The absorbance of the samples at a wavelength of 492 nm was measured with a FlexStation 3 system (Molecular Devices, Sunnyvale, CA, USA).

Hoechst 33342 staining

Apoptosis in H9c2 cardiomyoblasts was evaluated via Hoechst 33342 staining. After pre-incubation with different concentrations of compound **6** for 1 h and then exposure to 0.3 mmol/L H₂O₂, the cells were fixed in 4% paraformaldehyde and stained with 10 μ g/mL Hoechst 33342. Photos were obtained via fluorescence microscopy (DP70, Olympus, Japan), and the apoptotic ratio in each group was assessed.

LDH release and CAT activity assays

LDH release and CAT activity were both measured using a colorimetric assay kit according to the manufacturers' instructions.

Measurement of intracellular ROS production

After reaching 70%–80% confluence in 96-well plates, the cells were washed twice with PBS buffer after being loaded with 10 μ mol/L CM-H₂DCFDA for 30 min at 37°C. The cells were treated with different concentrations of compound **6** for 1 h and then co-incubated with H₂O₂ (0.3 mmol/L) for 3 h. After washing with PBS, the ROS levels were assessed by determining their 2',7'-dichlorofluorescein (DCF) content, which was produced from CM-H₂DCFDA through oxidation. The formation of fluorescent DCF in each well was quantified at an excitation wavelength of 488 nm and emission wavelength of 525 nm using a FlexStation 3 system. Fluorescence photomicrographs were taken using fluorescence microscopy (DP70, Olympus, Japan), with the cells prepared as described above in 24-well plates.

Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

The mitochondrial membrane potential was tested using a JC-1 assay kit according to the manufacturer's instructions. In brief, after treatment as mentioned above, H9c2 cells were washed twice with a Krebs-Ringers phosphate HEPES buffer (20 mmol/L HEPES, 5 mmol/L NaH₂PO₄, 1.25 mmol/L MgSO₄, 1.25 mmol/L CaCl₂, 128 mmol/L NaCl, 4.7 mmol/L KCl, pH=7.4). Then, the cells were incubated with JC-1 for 30 min at 37°C. The fluorescent values were obtained using a FlexStation 3 system, with 485 nm and 530 nm as the green excitation and emission wavelengths, respectively, and 530 nm and 580 nm as red excitation and emission wavelengths, respectively. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was used as a positive control. The cells were treated as mentioned above in 6-well plates while the fluorescence photos were taken.

Western blot analysis

After cells were pretreated with compound **6** for 1 h and co-incubated with 0.3 mmol/L H₂O₂ for the indicated time,

proteins from each sample were collected. The cell lysates were prepared in a RIPA buffer containing 1 mmol/L PMSE, proteinase, and a phosphatase inhibitor cocktail. The protein samples were separated via 10% or 12% SDS-PAGE and electrotransferred to PVDF membranes. After blocking with 1% BSA or 5% defatted milk in 0.01 mol/L Tris-buffer saline (TBS) with 0.1% tween 20 for 1 h at room temperature, the membranes were incubated with primary antibodies overnight at 4°C and then incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1.5 h. The primary antibodies used included rabbit anti-cleaved caspase-3 (Asp175, 1:1000), rabbit anti-cleaved caspase-8 (Asp387, 1:1000), rabbit anti-cleaved caspase-9 (Asp353, 1:1000), rabbit anti-Bcl-2 (1:1000), rabbit anti-Bax (1:1000), rabbit anti-phospho-c-Jun N-terminal kinase (JNK, Thr183/Tyr185, 1:1000), rabbit anti-JNK (1:1000), rabbit anti-phospho-p38 (Tyr180/Tyr182, 1:1000), rabbit anti-p38 (1:1000), rabbit anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204, 1:3000), rabbit anti-p44/42 MAPK (Erk1/2, 1:3000), rabbit anti-phospho-Akt (Ser473, 1:1000), rabbit anti-phospho-nuclear factor Kappa B (NFkB, Ser536, 1:1000), rabbit anti-phospho-p53 (Ser15, 1:1000, all from Cell Signaling Technology, Danvers, MA, USA), and mouse anti- β -actin (Sigma-Aldrich, St Louis, MO, USA, 1:10000). The secondary antibodies used were anti-mouse IgG and anti-rabbit IgG (Jackson Laboratory, USA, 1:10000). Protein expression levels were determined based on optical density using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

All data are expressed as the mean $\pm$ SEM. A one-way analysis of variance (ANOVA) followed by Dunnett's analysis was performed, and values of $P<0.05$ were considered significant.

Results

Protective effect of chlorogenic acid derivatives (1–9) on cell viability

All of the chlorogenic acid derivatives (**1–9**) were evaluated for their capacity to protect against H₂O₂-induced H9c2 cardiomyoblast injury via MTT assay. The preliminary tests of these pure isolates at 100 μ mol/L revealed that compounds **3**, **5**, **6**, and **8** significantly increased the viability of H9c2 cells. Among them, compound **6** exhibited a more potent cytoprotective effect than EGCG (epigallocatechin gallate) (Figure 2A), which suggests that compound **6** deserves further evaluation as a potential therapeutic agent for protection against H₂O₂-induced H9c2 cell injury. No cytotoxic effect of compound **6** on H9c2 cells was found at up to 200 μ mol/L (Supplementary Figure S1). Pretreatment of H9c2 cells with compound **6** at concentrations ranging from 3.12 to 100 μ mol/L for 1 h prior to a treatment with 0.3 mmol/L H₂O₂ dose-dependently increased cell viability (cell viability rates were 100.13% $\pm$ 1.88% at 100 μ mol/L, 98.77% $\pm$ 4.33% at 50 μ mol/L, 93.01% $\pm$ 2.93% at 25 μ mol/L, 68.97% $\pm$ 3.52% at 12.5 μ mol/L, 59.27% $\pm$ 5.20% at 6.25 μ mol/L, and 58.19% $\pm$ 3.36% at 3.12 μ mol/L. Viability was 43.89% $\pm$ 4.16% for the H₂O₂-

Figure 2. Protective effects of compounds 1–9 and EGCG against H₂O₂-induced decrease in the viability of H9c2 cells. (A) H9c2 cells were pretreated with 100 μmol/L 1–9 or EGCG for 1 h prior to treatment with the vehicle or H₂O₂ (0.3 mmol/L) for 3 h, the cell viability was detected by MTT assay. The cell viability was shown as inhibitory ratios (% of control). (B) H9c2 cells were treated with the vehicle or 0.3 mmol/L H₂O₂ for 3 h and compound 6 (0, 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 μmol/L for 1 h before exposure to H₂O₂). Cell viability was detected by MTT assay. All data are expressed as mean±SEM (*n*=6). **P*<0.05, ****P*<0.001 versus the H₂O₂-treated alone group.

induced untreated group, Figure 2B).

Compound 6 attenuates H₂O₂-induced oxidative stress in H9c2 cells

LDH is an enzyme found extensively in tissues throughout the body and can be measured as a surrogate for cell injury. The protective effect of compound 6 was also demonstrated by detecting the LDH leakage caused by H₂O₂, which was significantly increased in untreated cells compared with that observed in the control group. By contrast, when cells were pretreated with compound 6 at concentrations of 3, 12.5, 25.0, and 50.0 μmol/L and then co-incubated with H₂O₂ (0.3 mmol/L) for 3 h, LDH leakage decreased by 21%, 57%, 72%, and 100%, respectively (Figure 3A). CAT is a cellular antioxidant enzyme that protects cell against oxidative damage caused by ROS^[18]. As shown in Figure 3B, compound 6 significantly prevented the H₂O₂-induced decrease in CAT activity at doses of 25.0 μmol/L and higher. These results indicate that compound 6 has a protective effect against H₂O₂-

Figure 3. Effect of compound 6 on LDH release and catalase activity in H₂O₂-treated H9c2 cells. After H9c2 cells were pretreated with 6 for 1 h and co-incubated in presence/absence of 0.3 mmol/L H₂O₂ for 3 h, the LDH and CAT assay were performed. (A) The production of LDH release was measured (*n*=5). (B) The catalase activity was evaluated (*n*=4). All data were shown as mean±SEM. **P*<0.05, ****P*<0.001 versus the H₂O₂-treated alone group.

induced damage in H9c2 cardiomyoblasts by reducing LDH release and increasing the activity of CAT.

Compound 6 protects H9c2 cells from H₂O₂-induced apoptosis

To further investigate the protective effect of compound 6, the apoptotic H9c2 cells were examined via Hoechst 33342 staining. The fluorescence images of the normal cells revealed oval-shaped nuclei with homogeneous fluorescence, whereas heterogeneous intensities and chromatin condensation were observed in the nuclei of the H₂O₂-induced H9c2 cells. The nuclei of apoptotic cells were counted for statistical analysis. The results revealed that, after being exposed to 0.3 mmol/L H₂O₂ for 3 h, the percentage of H9c2 cardiomyoblasts showing an apoptotic nuclear morphology was 45.03%±12.05%; meanwhile, the percentages of apoptotic cells in the samples pretreated with different concentrations of compound 6 (12.5, 25.0, and 50.0 μmol/L) for 1 h and then co-incubated with 0.3 mmol/L H₂O₂ for 3 h, were 22.17%±9.37%, 15.15%±9.79%, and 14.29%±8.02%, respectively (Figures 4A and 4B).

Cleaved caspase-3 is an important executioner caspase in the process of apoptosis. To further evaluate the anti-apoptotic effect of compound 6, the protein level of cleaved caspase-3 was determined via Western blot analysis. As shown in Figures 4C and 4D, compound 6 significantly decreased the

Figure 4. Compound 6 protected H9c2 cells from H₂O₂-induced apoptosis. (A) Representative fluorescent photomicrographs of Hoechst 33342 staining, the brighter nuclei was indicated as Hoechst 33342-positive apoptotic cells. Scar bar=50 μm and refers to all panels; (B) The statistical data of apoptotic rate ($n=5$). (C) The Western blot analysis of cleaved caspase-3 and β -actin. (D) The density of each blot band of cleaved caspase-3 was detected and adjusted by the density of internal β -actin; the results were expressed at least three independent experiments. All data were shown as mean $\pm$ SEM. * $P<0.05$, ** $P<0.01$ versus the H₂O₂-treated alone group.

expression of cleaved caspase-3 at concentrations of 12.5 and 25.0 $\mu\text{mol/L}$ in H₂O₂-injured H9c2 cardiomyoblasts. These results suggest that compound 6 dose-dependently suppresses H₂O₂-triggered apoptosis in H9c2 cells.

Compound 6 attenuates H₂O₂-induced ROS generation and mitochondria membrane potential ($\Delta\Psi\text{m}$) disruption in H9c2 cells

Apoptosis in cardiomyocytes generally begins with the generation of ROS in the ischemic myocardium. Whether compound 6 shows a potent ROS-scavenging effect was determined by using a fluorescence assay. As shown in Figure 5A, when the cells were exposed to H₂O₂, the generation of ROS increased significantly, whereas treatment with compound 6 alone did not induce any ROS generation compared with the levels in the control condition. A statistical analysis of fluorescence intensity revealed that compound 6 significantly decreased ROS production in H₂O₂-treated cells, even at a concentration of 0.78 $\mu\text{mol/L}$ (Figure 5B). Cardiac mitochondria are the major site of ROS generation, and ROS overload leads to leakage through the mitochondrial outer membrane and mitochondrial dysfunction. A reduction in the mitochondrial

membrane potential ($\Delta\Psi\text{m}$) is regarded as a hallmark of the early apoptotic period. The effect of compound 6 on $\Delta\Psi\text{m}$ damage induced by H₂O₂ was assayed via JC-1 staining. The results show that the exposure of H9c2 cells to H₂O₂ caused a significant increase in the green/red fluorescence ratio relative to that of the control group. By contrast, the green/red fluorescence ratios of cells cultured with compound 6 (12.5, 25.0, and 50.0 $\mu\text{mol/L}$) were markedly reduced (Figure 5C). A statistical analysis of the fluorescence intensity revealed that the $\Delta\Psi\text{m}$ was decreased to 63% when cells were exposed to H₂O₂ alone relative to that of the vehicle control, while the $\Delta\Psi\text{m}$ increased by 27%, 31%, and 32% in the presence of different concentrations of compound 6 (12.5, 25.0, and 50.0 $\mu\text{mol/L}$, respectively) (Figure 5D). These results suggest that the anti-apoptotic effect of compound 6 was most likely due to the elimination of ROS and the maintenance of mitochondrial function.

Compound 6 inhibits H₂O₂-triggered H9c2 apoptosis through intrinsic apoptotic pathways

As mentioned above, apoptosis pathways commonly consist of extrinsic and intrinsic pathways. To understand the

Figure 5. Compound 6 inhibited H₂O₂-induced intracellular ROS generation and attenuated MMP decrease. (A) The cells were treated with compound 6 for 1 h before H₂O₂ exposure for 3 h, ROS fluorescence was taken photos. Scar bar=50 μm and refers to all panels. (B) Quantified ROS generation in the different treated cells was detected with CM-H₂DCFDA (*n*=6), all data are expressed as mean±SEM. Asterisks denote significant difference versus the H₂O₂-treated alone group (****P*<0.001). (C) Representative fluorescent images of JC-1 with compound 6 in the presence/absence H₂O₂ were shown in the change of mitochondrial membrane high potential (red) and low potential (green). (D) The mitochondrial membrane potential was quantitatively determined with JC-1, CCCP as positive control. All data are expressed as mean±SEM (*n*=5). ****P*<0.001 versus the control group. ###*P*<0.001 versus the H₂O₂-treated group.

mechanism by which compound 6 inhibits H₂O₂-induced H9c2 apoptosis, several key apoptosis-associated proteins

were evaluated via Western bolt analysis. Cleaved caspase-8 is an inducer of the extrinsic apoptotic pathway, while cleaved

Figure 6. Compound 6 protected H9c2 cell from H₂O₂-induced apoptosis via intrinsic pathway. After H9c2 cells were treated with compound 6 at 12.5 μmol/L or 25 μmol/L for 1 h and co-incubated with 0.3 mmol/L H₂O₂ for 3 h, the cell lysates were collected and the expressive levels of followed proteins were detected by Western blot analysis. (A) The Western blot of p53, Bcl-2, Bax, cleaved caspase-8 and cleaved caspase-9, β-actin was used as internal control. (B) The relative density of the cleaved caspase-3 protein band was calculated. (C) The relative density of the cleaved caspase-9 protein band was calculated. (D) The relative density of the phosphorylation of p53 was calculated. (E) The relative density of Bcl-2 was calculated. (F) The relative density of Bax was calculated. **P*<0.05 versus the control group. #*P*<0.05, ##*P*<0.01 versus the H₂O₂-treated alone group. *n*=3. Mean±SEM

caspase-9 is related to the intrinsic pathway. As shown in Figures 6A, 6B, and 6C, cleaved caspase-8 and cleaved caspase-9 expression levels were significantly increased in H9c2 cells after treatment with H₂O₂, and compound 6 (at the concentrations of 12.5 and 25.0 μmol/L) only suppressed the activation of caspase-9 in H₂O₂-treated cells. Next, we investigated the expression levels of Bax and Bcl-2. As expected, the expression level of the pro-apoptotic protein Bax was increased and that of the anti-apoptotic protein Bcl-2 was decreased in H₂O₂-induced H9c2 cells, and compound 6 significantly attenuated these effects (Figures 6D and 6E). In addition, we evaluated the phosphorylation of p53, which regulates Bcl-2 and Bax. We found that compound 6 significantly inhibited the phosphorylation of p53 at a concentration of 25.0 μmol/L (Figure 6F). These results indicate that compound 6 inhibits H₂O₂-triggered H9c2 apoptosis through intrinsic apoptotic pathways.

Compound 6 attenuates the H₂O₂-induced phosphorylation of ERK and JNK in H9c2 cells

Studies have revealed that ROS activate three MAPK subfamilies when apoptosis occurs in cardiomyocytes or heart fibroblasts^[19]. To understand which subfamily was activated by compound 6, Western blot assays were performed. As shown in Figures 7, the phosphorylation of ERK, JNK, and p38 were increased in the H₂O₂-stimulated cells. Pretreatment with compound 6 (at concentrations of 12.5 and 25.0 μmol/L)

significantly attenuated the phosphorylation of JNK and ERK in H₂O₂-treated H9c2 cells.

Discussion

In our continuing effort to find natural beneficial bioactive compounds, a phytochemical investigation of *G nepalensis* leaves was undertaken. As a result, nine chlorogenic acid derivatives (1–9) were identified. H9c2 is a permanent rat-derived cardiomyoblasts cell line that has been applied in the study of heart function. H9c2 cells show similar morphological characteristics to those of undifferentiated embryonic cardiomyocytes. In addition, studies have revealed that H9c2 cells maintain some elements of the electrical and hormonal signal pathway which is also found in adult cardiomyocytes^[20, 21]. Winstead demonstrated that H₂O₂ induced the same oxidative stress in H9c2 cells as in primary cultured rat cardiomyocytes^[22]. Moreover, the cell-based antioxidant activity assay by using H9c2 cells is common in many studies^[23–25]. Therefore, H9c2 cardiomyoblasts may be feasible as a model for oxidative stress-induced cardiomyocyte damage considering the signal transduction and applicable to antioxidant investigate. Through a series of experiments, we found that H9c2 cell viability decreased in a dose-dependent manner with increasing H₂O₂ concentration. H9c2 cell viability decreased by nearly 50% at 0.3 mmol/L H₂O₂ vs the control treatment. Thus, 0.3 mmol/L H₂O₂ might be an appropriate concentration to use for the study of oxidative

Figure 7. Effect of compound **6** on H₂O₂-induced MAPKs activation in H9c2 cells. After the cells were pretreated with/without different concentrations of compound **6** and co-incubated with 0.3 mmol/L H₂O₂, the cell lysates were collected. (A–C) The phosphorylation level of ERK (p-ERK), JNK (p-JNK) and p38 (p-p38), the expression level of total ERK, total JNK (JNK) and total p38 (p38) were detected by Western blot analysis, β-actin was used as internal control. (D–F) The phosphorylation ratio of ERK, JNK, p38 were measured by the expression of phosphorylated protein divided by total protein expression. All experiments were repeated at least three times. The statistical results are shown as the mean±SEM. **P*<0.05 versus the control group. ##*P*<0.01 versus the H₂O₂-treated alone group.

stress-induced apoptosis in H9c2 cells (data not shown). We then examined the protective effects of these isolates on the cytotoxicity induced by H₂O₂ in H9c2 cardiomyoblasts. Among the isolates, compound **6** exhibited a more potent protective effect than EGCG. Further evaluation of the effects of compound **6** on LDH release and CAT activity revealed that compound **6** dose-dependently protected H9c2 cells against H₂O₂-triggered damage. Compound **6** exerted its protective effects partially through the scavenging of ROS, inhibiting the intrinsic apoptotic pathway, and regulating MAPK signaling pathways in H9c2 cells. The cellular and molecular mechanisms underlying the actions of compound **6** have not been fully elucidated to date. Our present results suggest that several mechanisms play roles in the protective effects of compound **6** in against H₂O₂-induced H9c2 apoptosis.

The precise cellular mechanisms underlying H₂O₂-induced cardiac apoptosis have not been fully described. However, it is widely accepted that the generation of excessive ROS is the key factor^[26]. ROS are by-products of cellular oxygen metabolism and are used in cellular signaling at low concentrations. However, uncontrolled increases in ROS often lead to free radical-mediated chain reactions, which indiscriminately target proteins, lipids, polysaccharides, and DNA, and trigger the intrinsic apoptotic pathway^[27]. In the assays performed here, compound **6** decreased ROS production more than chlorogenic acid and EGCG at the same concentrations (Supplementary Figure S2).

Studies have revealed that the activation of intrinsic apoptosis represents a major response of p53 signaling^[28]. This is one of the pathways of programmed cell death, and the key step in this pathway is the permeabilization of the outer mitochondrial membrane, which is regulated by members of

the Bcl-2 family^[29]. This family contains both pro-apoptotic proteins, including the Bax-like and BH3-only subfamilies, and anti-apoptotic proteins, including Bcl-2, Bcl-XL, and Mcl-1. p53 directly activates the protein Bax in the absence of other proteins to permeabilize the mitochondria and initiate the apoptotic program. It also releases both pro-apoptotic multidomain proteins and BH3-only proteins^[30]. When apoptosis is activated, the BH3-only proteins inhibit Bcl-2 proteins and induce Bax oligomerization, the permeabilization of the mitochondria, and the release of cytochrome *c*. Subsequently, cytochrome *c* forms a complex with caspase-9 and Apaf-1, leading to the activation of caspase-9. Caspase-9 activates the executioner caspases (caspase-3, -6, and -7), inducing cell death^[31]. In terms of the direct effects of ROS on the Bcl-2 family members, H₂O₂ reportedly induces Bax dimerization, which promotes its translocation from the cytoplasm to the outer mitochondrial membrane^[32]. According to our experimental data, compound **6** inhibits the H₂O₂-induced phosphorylation of p53 (Figure 6D) and increases the mitochondrial membrane potential (ΔΨ_m) (Figure 5D). In addition, after incubation with compound **6**, the expression of Bcl-2 was increased significantly, whereas that of Bax was decreased (Figures 6E and 6F). Western blotting also showed that compound **6** inhibits the H₂O₂-induced increase in the expression of cleaved caspase-9 (Figure 6C) and caspase-3 (Figures 4C and 4D), protecting the H9c2 cells from H₂O₂-induced apoptosis.

Previous studies have stated that three major MAPK pathways (ERK1/2, p38, and JNK) are pivotal in the regulation of cell survival and apoptosis. The activation of ERK is believed to regulate a cellular protective response to oxidative stress, but the mechanism is complex and diverse^[33]. Some

natural products inhibit ERK activation in response to H₂O₂-elicited apoptosis in H9c2 cells^[34, 35]. The role of JNK and p38 in apoptosis remains controversial because in different cell types, these proteins can have the opposite effects in response to apoptotic stimuli. Herein, our results show that pretreatment with compound 6 inhibits the activation of ERK and JNK rather than of p38 (Figure 7C), which suggests that compound 6 regulates the phosphorylation of ERK and JNK in protecting against ROS-induced apoptosis in H9c2 cardiomyoblast. Meldrum *et al* confirmed that H₂O₂ alone induces TNF- α -mediated myocardial injury via a p38 MAPK-dependent mechanism^[36]. We found that compound 6 did not inhibit the H₂O₂-induced activation of caspase-8 (Figure 6B), which is in accordance with the findings that compound 6 protects H9c2 cells against ROS-induced apoptosis via intrinsic pathways. In addition, the activation of JNK via oxidative stress is linked with DNA fragmentation and caspase activation^[37]. JNK is commonly believed to be upstream of NF κ B and to regulate its transcription level. Therefore, the expression of some downstream pro-inflammatory genes was measured in this study. We found that compound 6 significantly reduced the H₂O₂-induced phosphorylation of NF κ B (Supplementary Figure S3), which may relate to its strong ROS scavenging ability. Based on our findings, further investigations into the mechanisms of the protective effects of compound 6 should be performed.

In summary, our study supports a mechanism for the protective effect of compound 6 against H₂O₂-induced apoptosis in H9c2 cells. Further studies with compound 6 are warranted and may lead to the development of novel agents for the treatment of some heart diseases.

Supplementary information

Supplementary Figures are available on the website of Acta Pharmacologica Sinica.

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Author contribution

He-yao WANG, Wei-min ZHAO and Hui-min FAN designed the research and revised the manuscript; Bang-wei YU and Jin-long LI performed the research; Jin-long LI isolated the compounds; Bang-wei YU analyzed the data and wrote the manuscript; and Bin-bin GUO performed part of the experiments.

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