Difference in protective effects of three structurally similar flavonoid glycosides from *Hypericum ascyron* against H_2O_2-induced injury in H9c2 cardiomyoblasts

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Abstract. According to previous studies, hyperoside possesses myocardial protective effects. To investigate whether isoquercitrin and isohyperoside have similar functions, the protective effects of isoquercitrin and isohyperoside against H_2O_2-induced injury in H9c2 rat cardiomyoblasts were evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The mechanism of action was investigated by assessing the leakage of lactate dehydrogenase (LDH) of hyperoside and isoquercitrin-pretreated H9c2 cardiomyocytes following H_2O_2-induced injury, and examining their effects on the levels of malondialdehyde (MDA) and superoxide dismutase (SOD) activity. The isolation of two flavonoid glycosides from *H. ascyron* was performed, following extraction, using semi-preparative high performance liquid chromatography. Using the spectral characteristics, the structures of these compounds were identified as isoquercitrin and isohyperoside. This was the first time, to the best of our knowledge, that isohyperoside has been identified from *H. ascyron*. The results revealed that isoquercitrin and isohyperoside possessed similar protective effects to hyperoside against H_2O_2-induced injury in H9c2 cells. The half maximal inhibitory concentration values of hyperoside, isoquercitrin and isohyperoside were 0.0008, 0.0017 and 0.0002 μM, respectively. Based on these results, isohyperoside possessed more marked protective effects against H_2O_2-induced injury in the H9c2 cardiomyoblasts. The significant reduction in LDH leakage, decrease in MDA level and increase in SOD activity also provided evidence of the cardioprotective effects of isoquercitrin and isohyperoside. The present study reported for the first time, to the best of our knowledge, the myocardial protective effects of isoquercitrin and isohyperoside. The mechanism of action may involve protection of the cell membrane from oxidative damage.

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Abbreviations: H_2O_2, hydrogen peroxide; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; NMR, nuclear magnetic resonance; DMSO, dimethyl sulfoxide; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase; MDA, malondialdehyde; SOD, superoxide dismutase

Key words: Hypericum ascyron, cardioprotective effect, isolation, structure identification, structurally similar flavonoid glycosides

Introduction

Hypericum ascyron is recorded as Hong-Han-Lian in the Compendium of Materia Medica and Dictionary of Medicinal Plants (1). *H. ascyron* has been used in Chinese medicine for the treatment of wounds, swelling, headaches, nausea and abscesses (2). The aerial sections of *H. ascyron* have also been used in Chinese medicines for the treatment of abnormal menstruation and to promote lactation (2). Previous studies have suggested that *H. ascyron* has several pharmacological activities, including inhibition of histamine-release (3), anti-inflammatory and analgesic effects (4), antioxidant activity (5), glucosidase inhibitory and antioxidant properties, and antidiabetic activity (6). Various constituents of *H. ascyron* have been identified, including flavonoids and xanthones.

Flavonoids are secondary metabolites, which are found ubiquitously in plants and are the most common group of polyphenolic compounds consumed by humans as dietary constituents (7,8). They are common dietary components of

fruits, vegetables, beverages, tea and herbs, and are usually present in plant tissues in the form of glycosides (9-11). Flavonoids exhibit a multitude of biological activities, including antibacterial, anti-inflammatory, anti-allergy, anti-oxidative and cardioprotective effects, as well as anti-tumor activity (12-15). Epidemiological investigations have demonstrated that the dietary intake of flavonoids is inversely associated with the incidence of coronary heart disease and cancer (16,17). Due to their abundance in Chinese herbal medicine and their potential beneficial pharmacological and nutritional effects, flavonoids have received considerable interest as drug treatments and health food supplements (18).

According to the literature, hyperoside possesses myocardial protective effects, however, the cardioprotective effects of isoquercitrin and isohyperoside on the oxidative damage of cardiomyocytes have not been reported. Therefore, the purpose of the present study was to evaluate whether two structural analogues, isoquercitrin and isohyperoside from *H. ascyron* provide similar cardioprotective effects as hyperoside on oxidative damage of cardiomyocytes. The structural form of the compounds responsible for the observed effects were also investigated. The underlying mechanism of action was also examined to provide evidence and a guide for the utilization of *H. ascyron* as a potential cardioprotective drug and health food supplement.

Materials and methods

Reagents. Analytical grade ethanol (Tianjin, China) was used for sample preparation. Acetonitrile, of high performance liquid chromatography (HPLC) grade, was purchased from Concord Technology (Tianjin, China). High purity water was obtained using a Millipore Milli-Q water purification system (EMD Millipore, Billerica, MA, USA). Hyperoside was purchased from the National Institutes for Food and Drug Control (Beijing, China).

Plant material. *H. ascyron* was collected from Hailaer (Mongolia, China) in July 2012. All specimens, which were authenticated by Professor Ya-hong Sun (Genhe City of Inner Mongolia Agriculture and Animal Husbandry Bureau, Genhe, China), were dried in the shade until weight remained constant.

Extraction. The aerial parts of the dried *H. ascyron* were crushed and passed through a 60-mesh sieve (Guangzhou Jiajun Experimental Apparatus Co., Ltd., Guangzhou, China). Each powder sample (1.00 g) was accurately weighed (LE323S; Sartorius AG, Göttingen, Germany) and extracted using 30 ml 60% ethanol solution by ultrasonic extraction using the HF-10B Ultrasonic Circulation Extractor (Beijing Hongxianglong Biotechnology Co. Ltd., Beijing, China) for 30 min at 30˚C. Following centrifugation at 5,000 x g for 10 min, the eluate was evaporated by rotary evaporation at 40±2˚C. Prior to use, 1 g extract was dissolved in 10 ml 60% ethanol solution and filtered through a 0.22 μm nylon acrodisc (Agela Technologies, Inc, Tianjin, China).

Isolation. The crude extract of *H. ascyron* (2 ml, 100 mg/ml in 60% ethanol), was loaded onto an Agilent semi-preparative C18 250x9.4 mm column. The semi-preparative HPLC system used was an Agilent 1200 series (Agilent Technologies, Inc., Santa Clara, CA, USA). The solvents used were: Solvent A, ultrapure H_2O containing 0.1% HAC, and solvent B, CH_3CN. The conditions for gradient elution were as follows: 0-15 min, 0-8% B; 15-20 min, 8-15% B; 20-40 min, 15-18% B; 40-60 min, 18-25% B; 60-90 min, 25-30% B. The flow rate was 2.5 ml/min and peaks were detected at 275 nm. The extract was subjected to semi-preparative HPLC, and compounds 1 and 2 were obtained, as shown in Fig. 1. The compounds were concentrated under reduced pressure, lyophilized with the LGJ-10 Freeze Dryer (pressure, 1.0 Pa; Beijing Yuechengjiaye Technology Co., Ltd., Beijing, China) and stored at -20˚C prior to use.

Structural identification. HPLC-Diode Array Detector (HPLC-DAD) analysis was performed on an Agilent 1200 Series liquid-chromatograph system, equipped with a binary pump, auto sampler, photo-diode array detector and column temperature controller. The analytical column used was a Kromasil C18 column (250x4.6 mm inner diameter; 5 μm; Akzo Nobel, Alby, Sweden) with the oven temperature maintained at 30˚C. Acetic acid aqueous solution (0.1%; V/V, solvent A) and acetonitrile (solvent B) were used in the mobile phase for the liquid chromatogram analysis. The conditions for linear gradient elution were as follows: 0-10 min, 20-23% B; 10-30 min, 23-25% B. The flow rate was 1.0 ml/min and peaks were detected at 200-400 nm. Mass spectrometry (MS) analysis was performed on an Agilent Technologies Ion Trap 6120, coupled to a UV absorption detector, LC-electrospray ionization (ESI)-MS-UV. The ^{1}H-(400 MHz) and ^{13}C-nuclear magnetic resonance (NMR) spectra (100 MHz) were recorded on a Bruker Avance 400 spectrometer (Bruker Daltonik GmbH, Leipzig, Germany).

Cell culture. H9c2 rat cardiomyoblasts (Cell Bank of the Molecular Pharmacology Laboratory, College of Biotechnology, Tianjin University of Science and Technology, Tianjin, China) were grown in high-glucose Dulbecco's modified Eagle's medium (Gibco Life technologies, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (Tianjin Kang Yuan Biological Technology Co., Ltd., Tianjin, China), penicillin G (100 U/ml) and streptomycin (100 mg/ml) in a humid 5% CO_2 atmosphere at 37˚C.

Cytotoxicity assay. The cytotoxicity was evaluated by measuring the cell viability following treatment with the extract of *H. ascyron*, hyperoside, isoquercitrin, isohyperoside and vehicle (untreated cells), respectively, using an MTT assay. H9c2 cells in the exponential growth phase were seeded at a seeding density of $4x10^5$/well into a 96-well plate and incubated for 12 h to allow attachment. The H9c2 cells were then pretreated with various concentrations of the extract of *H. ascyron* (12.5, 25, 50, 100 or 200 μg/ml), hyperoside (0.0054, 0.054, 0.54, 5.40 or 54.00 μM), isoquercitrin (0.0054, 0.054, 0.54 μM, 5.40 or 54.00 μM) and isohyperoside (0.0054, 0.054, 0.54, 5.40 or 54.00 μM), in the medium containing 0.1% dimethyl sulfoxide (DMSO) for 24 h. Following treatment, MTT (5 mg/ml) was added to each well to produce a final concentration of 0.25 mg/ml, and the plates were incubated for 4 h at 37˚C. The supernatant was then removed and the MTT

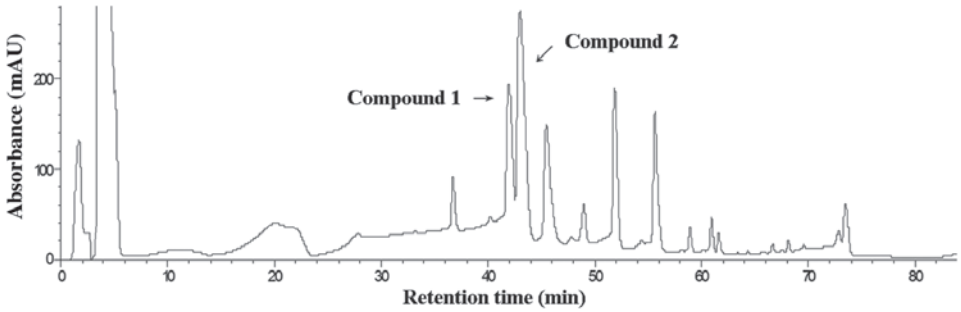

Figure 1. Semi-preparative high-performance liquid chromatography chromatogram of *Hypericum ascyron*.

metabolic product, formazan, was resuspended in DMSO and detected at 490 nm on a plate reader (Synergy 4; BioTek Instruments, Inc., Winooski, VT, USA). Cell viability (%) was calculated as follows: (Absorbance of the H_2O_2 treatment group / absorbance of the normoxic control) x 100.

Cell viability assay. Cells in the exponential growth phase were dissociated with 0.25% trypsin (Sigma-Aldrich, St. Louis, MO, USA) and were seeded into a 96-well plate at a seeding density of $4x10^4$/well prior to incubation for 24 h at 37˚C. Initially, the dose-dependent effects of H_2O_2-induced injury in H9c2 cardiomyoblasts were examined, and then the protective effects of hyperoside, isoquercitrin and isohyperoside were investigated. Briefly, the H9c2 cells seeded in the 96-well plate at a density of $4x10^5$/well were pre-incubated with various concentrations (0.0054, 0.054, 0.54, 5.40 or 54.00 μM) of hyperoside, isoquercitrin, isohyperoside or vehicle at 37˚C for 24 h. Following incubation, the supernatant was removed, the cells were washed with phosphate-buffered saline (Solarbio, Beijing, China) three times and were exposed to 200 μM H_2O_2, which was established as the optimal dose from dose-dependent effect analysis, in the culture medium for 24 h at 37˚C to induce cell injury. The surviving cells in the 96-well plate were then detected using an MTT assay. Cell viability (%) was calculated as follows: (Absorbance of injured group / absorbance of normoxic control) x 100.

Determination of the level of malondialdehyde (MDA) and activities of superoxide dismutase (SOD) and lactate dehydrogenase (LDH). The level of MDA, the activity of SOD in the cell lysate and the activity of LDH in the medium were measured using MDA, SOD and LDH kits (Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's instructions. The level of MDA was assayed using a thiobarbituric acid (Jiancheng Bioengineering Institute) method and determined at 532 nm. The absorbance of each well was measured using Synergy 4 Microplate Reader (BioTek Instruments, Inc.). The activity of SOD was determined by measuring the optical density at 550 nm, as the degree of inhibition on nitro blue tetrazolium produced by superoxide radicals from the xanthine/xanthine oxidase system. The level of LDH leakage was measured as the LDH activity in the culture medium, and detected at the wavelength of 450 nm.

Statistical analysis. The results are expressed as the mean ± standard deviation Statistical analyses were performed using one-way analysis of variance followed by Dunnett's test to determine significant differences among groups. Statistical analysis was performed using SPSS software, version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results and Discussion

Structural identification. A full scan of compound 1 and compound 2 between 200 and 400 nm was performed, and the wavelengths and retention time of hyperoside, compound 1 and compound 2 were recorded. MS analysis was performed on an Agilent Technologies Ion Trap 6120, coupled to a UV absorption detector LC-ESI-MS-UV, and the ^{1}H-(400 MHz) and ^{13}C-NMR spectra (100 MHz) were recorded on a Bruker Avance 400 spectrometer. Chemical shifts are expressed in ppm referenced to the residual solvent signals. Coupling constants (J) are reported in Hz.

The data for compound 1, an amorphous yellow powder, were as follows: Structure, $C_{21}H_{20}O_{12}$; UV λmax (MeOH), 254 and 346 nm; retention time, 8.683 min; ESI-MS m/z, 463[M-H]$^-$; ^{1}H NMR (DMSO-*d*6, 400 MHz) δ12.64 (1 H, s), 7.68 (2 H, d, J=2 Hz, H-2', 6'), 6.82 (1 H, d, J=8. 4 Hz, H-5'), 6.41 (1 H, s, H-8), 6.20 (1 H, s, H-6), and 5. 38 (1 H, d, J=7.6 Hz, H-1"). The ^{13}C NMR data are presented in Table I. Compound 1 was identified as isoquercitrin (Fig. 2) by comparing the physical characteristics and data of the ESI-MS and NMR spectra. The results are consistent with that of previous studies (19-22).

The data for compound 2, an amorphous yellow powder were as follows: Structure, $C_{21}H_{20}O_{12}$; UV λmax (MeOH), 255 and 357 nm; retention time, 8.927 min. However, based on the same HPLC/DAD conditions of hyperoside, the UV λmax (MeOH) was 258 and 360 nm and the retention time was 8.747 min. The results provided evidence for elucidating the structure of isohyperoside. ESI-MS m/z: 463[M-H]-; ^{1}H NMR (DMSO-*d*6, 400 MHz) δ12.64 (1 H, s), 7.59 (1 H, d, J=3.2 Hz, H-2'), 7.58 (1 H, dd, J=2 Hz, H-6'), 6.85 (1 H, d, J=8.8 Hz, H-5'), 6.41 (1 H, d, J=2 Hz, H-8), 6.20 (1 H, d, J=2 Hz, H-6), 5.47 (1 H, d, J=6.0 Hz, H-1"), 3.23-4.25 (6 H, H-2", H-6"). The ^{13}C NMR data are presented in Table I. Compound 2 was identified as isohyperoside (Fig. 2) by comparing the physical characteristics and data of the ESI-MS and NMR spectra. The results are consistent with those of a previous study (23).

Cytotoxicity. Cytotoxicity evaluation revealed that the extract of *H. ascyron* was not toxic. Hyperoside, isoquercitrin and

Table I. ^{13}C nuclear magnetic resonance data of compound 1 and compound 2 in dimethyl sulfoxide-d6.

	δC (chemical shift)/ppm	
Position	Compound 1	Compound 2
2	156.71	156.62
3	133.94	133.78
4	177.93	177.90
5	161.66	161.70
6	99.16	99.14
7	164.65	164.67
8	94.01	93.97
9	156.78	156.79
10	104.35	104.41
1'	122.39	122.06
2'	115.66	115.67
3'	145.27	145.28
4'	148.94	148.94
5'	116.43	116.67
6'	121.54	121.62
1"	102.30	101.34
2"	71.67	74.65
3"	73.65	76.97
4"	68.38	70.41
5"	76.27	78.03
6"	60.59	61.44

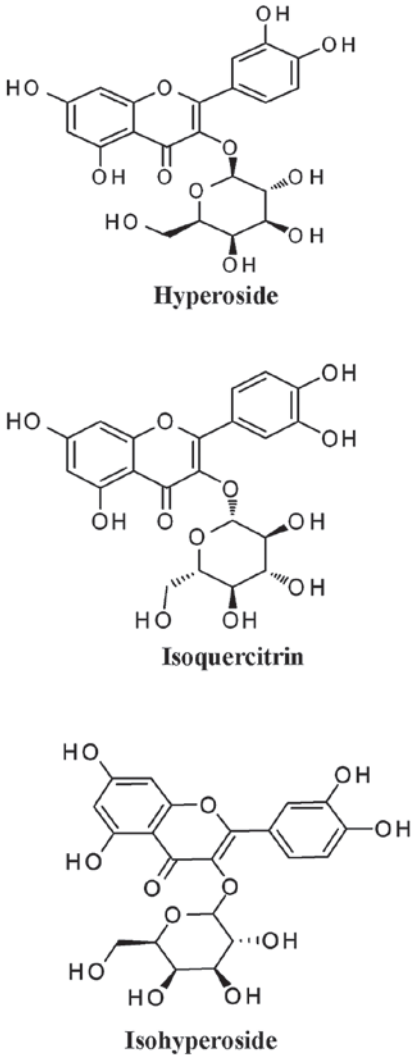

Figure 2. Chemical structures of hyperoside, isoquercitrin and isohyperoside.

isohyperoside were not cytotoxic <54 μM. All subsequent experiments were performed below these concentrations for the evaluation of their biological effects.

Protective effects of three structurally similar flavonoid glycosides on H_2O_2-induced injury of H9c2 cells

Effects on cardiomyocyte viability. As shown in Fig. 3A, compared with the normal control, H_2O_2 significantly reduced the viability of the cardiomyocytes in a dose-dependent manner. The survival rate of the H9c2 cells decreased to 93.32, 80.40, 56.44 and 53.75% when they were treated with H_2O_2 at 50, 100, 150 or 200 μM, respectively. To analyze the protective effects of the flavonoid glycosides against this oxidative damage, 200 μM was selected as the optimal dose for the establishment of the injury model. As shown in Fig. 3B, hyperoside restored cell survival rate to 60.43, 63.67, 71.23, 78.01 and 79.03%, respectively, at 0.0054, 0.054, 0.54, 5.40 or 54.00 μM, indicating that its protective effect on H_2O_2-induced damage was dose-dependent. Similar effects were also observed for isoquercitrin and isohyperoside (Fig. 3C and D), where 0.0054, 0.054, 0.54, 5.40 or 54.00 μM of isoquercitrin restored cell survival rate to 60.41, 63.26, 70.35, 77.92 and 78.04%, respectively. At the same concentrations, isohyperoside restored cell survival rate to 60.45, 64.32, 72.28, 79.84 and 80.74%, respectively. These observations suggested that hyperoside, isoquercitrin and isohyperoside attenuated H_2O_2-induced injury. These results were consistent with the hypothesis that flavonoids interact with cell death/survival signaling pathways, which, depending on the dose, may promote or inhibit apoptosis. The IC_{50} values of hyperoside, isoquercitrin and isohyperoside were 0.0008, 0.0017 and 0.0002 μM, respectively. Based on these results, it was suggested that the three structurally similar flavonoid glycosides possessed protective effects against H_2O_2-induced injury in the H9c2 cells. Isohyperoside had the most marked protective effects of the three structurally similar flavonoid glycosides.

Effects on LDH leakage. The protective effects of the three structurally similar flavonoid glycosides against H_2O_2-induced damage were further investigated through examination of their effects on LDH leakage. The leakage of LDH was measured as LDH activity in the culture medium. As shown in Fig. 4A, LDH leakage significantly increased following exposure to H_2O_2 for 24 h. However, marked reductions in LDH leakage were observed in the cardiomyocytes pretreated with 0.54-54 μM hyperoside, isoquercitrin and isohyperoside in a dose-dependent manner. The LDH leakage reduced to almost the same level as that of the normoxic control group when the cardiomyocytes were pretreated with 54 μM isohyperoside ($P<0.001$, vs. H_2O_2 group).

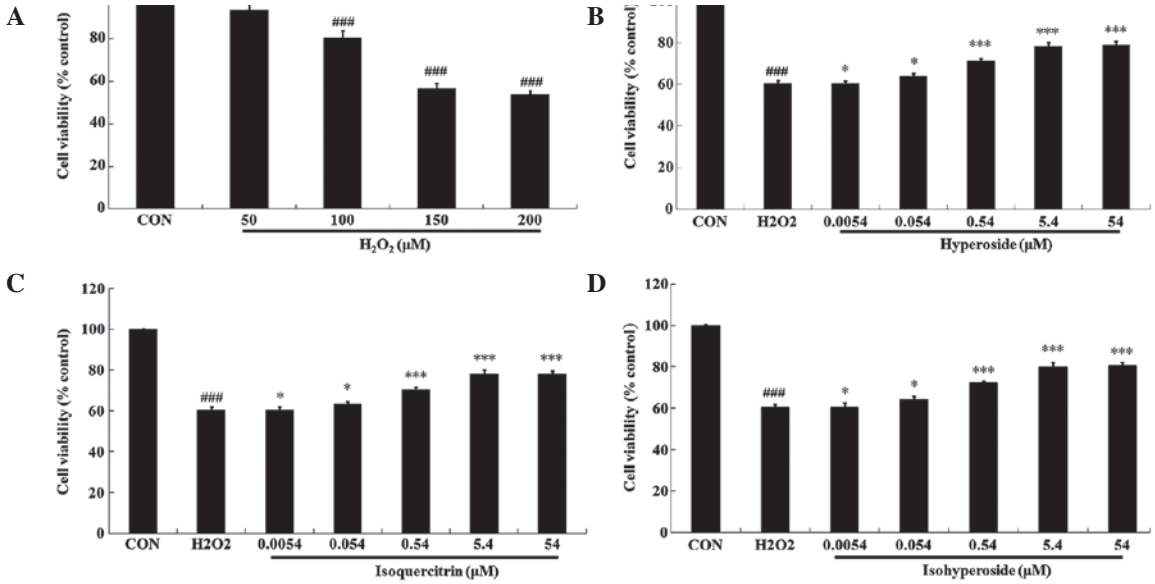

Figure 3. Effects of (B) hyperoside, (C) isoquercitrin and (D) isohyperoside on the viability of H9c2 cells following (A) H_2O_2-induced injury. Data are expressed as the mean ± standard deviation (n=3). $^{###}$P<0.001 vs. control group; $^{##}$P<0.001, vs. control group; $^{#}$P<0.001, vs. control group, ***P<0.001, vs. H_2O_2 group; **P<0.01, vs. H_2O_2 group; *P<0.05, vs. H_2O_2 group.

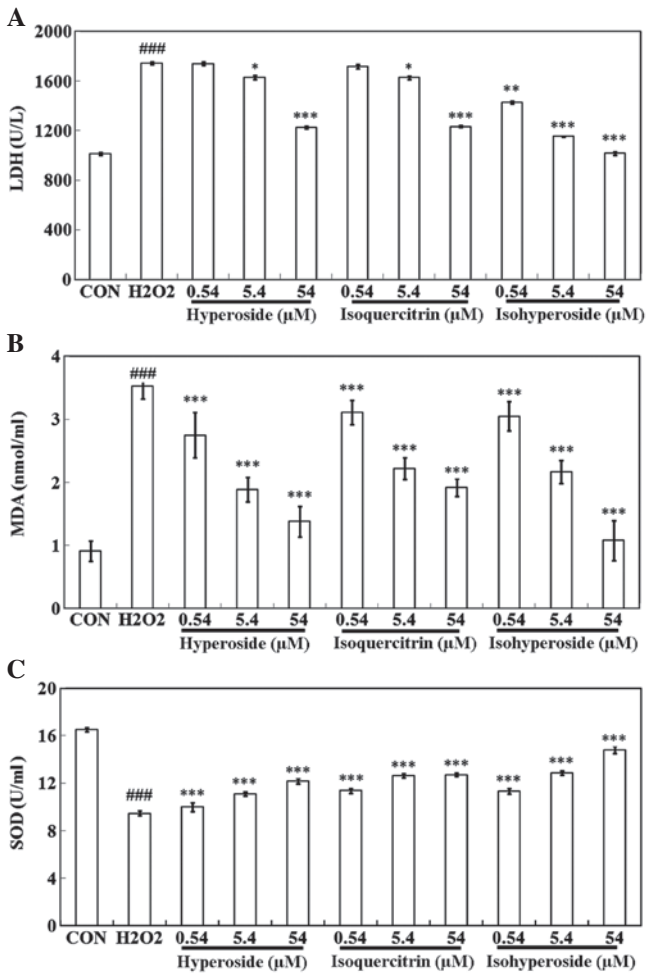

Figure 4. Effects of hyperoside, isoquercitrin and isohyperoside on (A) LDH leakage, (B) levels of MDA and (C) SOD activity ofthe H9c2 cells following H_2O_2-induced injury. Data are expressed as the mean ± standard deviation (n=3). $^{###}$P<0.001, vs. control group; $^{##}$P<0.001, vs. control group; $^{#}$P<0.001, vs. control group; ***P<0.001, vs. H_2O_2 group; **P<0.01, vs. H_2O_2 group; *P<0.05, vs. H_2O_2 group. SOD, superoxide dismutase; LDH, lactate dehydrogenase; MDA, malondialdehyde; CON, control.

Effects on the levels of MDA. To examine how hyperoside, isoquercitrin and isohyperoside triggered the defense mechanisms of the H9c2 cardiomyocytes against the oxidative damage generated following H_2O_2-induced injury, the levels of MDA in the cell lysate of the H9c2 cells were determined. As is shown in Fig. 4B, treatment with isohyperoside significantly (P<0.001, vs normoxic control) increased the level of MDA. The results also indicated that, although hyperoside, isoquercitrin and isohyperoside exhibited pronounced protective effects against H_2O_2-induced cell damage at non-cytotoxic concentrations, isohyperoside appeared favorable, as it elicited its protective effects at lower concentrations.

Effects on the activity of SOD. To examine how hyperoside, isoquercitrin and isohyperoside triggers the defense mechanisms of the H9c2 cardiomyocytes against oxidative damage following the H_2O_2-induced injury, the SOD activity of the cell lysate of H9c2 cells was determined. As shown in Fig. 4C, treatment of isohyperoside significantly (P<0.001, vs. normoxic control) reduced the activity of SOD in the cell lysate of the H9c2 cells.

In conclusion, the present study isolated and identified isoquercitrin and isohyperoside from *H. ascyron*, and isohyperoside was identified. This was the first report, to the best of our knowledge, to identify isohyperoside in *H. ascyron* and to demonstrate the myocardial protective effects of isoquercitrin and isohyperoside. It was demonstrated that the protective efficacy of isohyperoside against H_2O_2-induced injury was more marked, compared with that of hyperoside and isoquercitrin. The significant reductions in LDH leakage, decrease in the level of MDA and increase in SOD activity also provided evidence of the cardioprotective effects of isoquercitrin and isohyperoside.

The results of the present study demonstrated that *H. ascyron* contains cardioprotective agents against chemically-induced damage, and isoquercitrin and isohyperoside were identified as two active compounds. Continued investigations to clarify

the detailed mechanism of action may improve further understanding of the cardioprotective effects of *H. ascyron*.

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