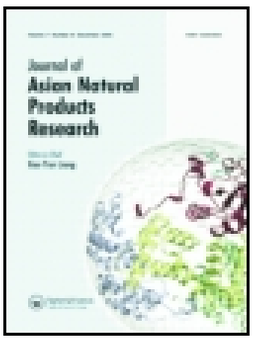

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# Oleanane triterpene saponins with cardioprotective activity from *Clinopodium polycephalum*

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**ABSTRACT**

Two new triterpene saponins, clinopodiside VI (**1**) and saikosaponin c (**2**), along with six known saikosaponins (**3**–**8**), were isolated from the plant of *Clinopodium polycephalum*. Compounds **1**–**3** showed moderate inhibition against H9c2 cell damage induced by $H_2O_2$.

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**KEYWORDS**
*Clinopodium polycephalum*; triterpene saponins; cardioprotective activity

## 1. Introduction

The crude drug "Clinopodii" is the dried aerial part of *Clinopodium polycephalum* (Van.) C. Y. Wu et Hsuan ex Hsuan, and is used as a folk remedy for treatment of various uterine bleeding, hematuria, epistaxis, gingival bleeding and bleeding wounds [1]. Many phytochemical researches on *C. polycephalum* have been reported that triterpene saponins and flavonoids are the main active ingredients, and they exhibited different pharmacological activities including anti-inflammatory and immunity [2], anti-hyperglycemic [3] and anti-tumor [4]. Our previous chemical studies led to the isolation of triterpene saponins. Further investigations of the extracts from the aerial parts of *C. polycephalum* resulted in the isolation of three new triterpene saponins. This paper deals with the isolation and identification of three triterpene saponins with their moderate inhibition against H9c2 cell damage induced by $H_2O_2$, and *five known triterpene saponins*.

## 2. Results and discussion

The alcohol extracts of *C. polycephalum* were subjected to silica gel column chromatography, ODS and Sephadex LH-20 gel chromatography, then semi-preparative HPLC, and yielded three new triterpene saponins clinopodiside VI (**1**), saikosaponin c (**2**), along with six known

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[1] Yu-xia Hu and Wei Zhang contributed equally to this work.
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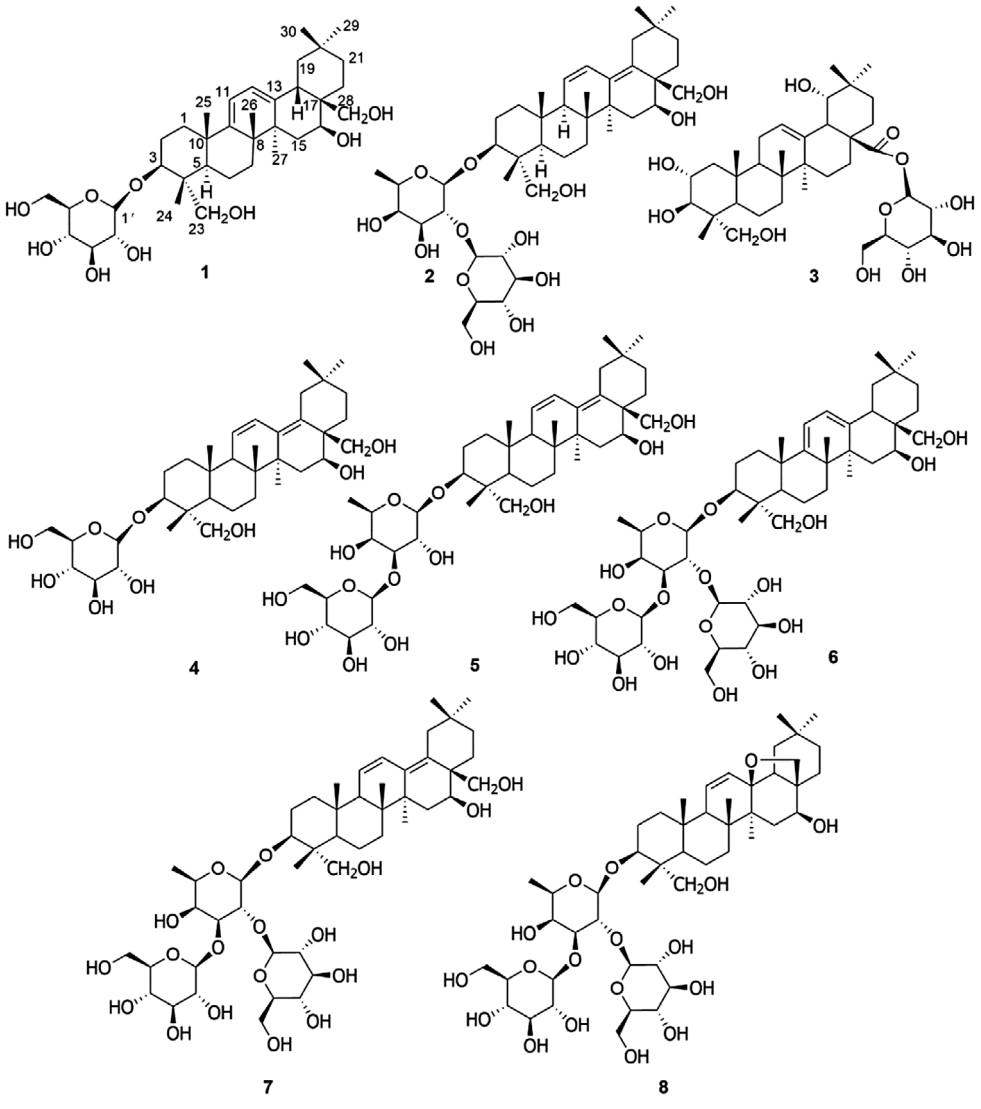


**Figure 1.** Structures of compounds **1–8**.

triterpene saponins (**3**–**8**) (Figure 1). Their structures were elucidated by 1D and 2D NMR and MS analysis. Compounds **3**–**8** were identified by comparing their spectroscopic data with published data for arjunglucoside I (**3**) [5], clinopodiside I (**4**) [6], saikosaponin b1 (**5**) [7], clinopodiside II (**6**) [8], buddlejasaponin IVb (**7**) [9] and buddlejasaponin IV (**8**) [10].

Compound **1** was assigned the molecular formula $C_{36}H_{58}O_9$, determined on the basis of its positive HR-ESI-MS at *m/z* 657.3981 $[M + Na]^+$. The type and absolute configuration of the sugar was identified as D-glucose on the basis of the TLC method comparison with authentic monosaccharides ($CHCl_3$: MeOH: $H_2O$ = 3: 2: 0.2), followed by gas chromatography. In the $^1H$ NMR spectrum, the coupling constants between the anomeric proton ($\delta_H$ 6.37, d, $J$ = 7.8 Hz, H-1′) and the adjacent proton suggested that the glucose residue was $\beta$-orientation. In the $^1H$ NMR spectrum, signals were observed for six methyl singlets

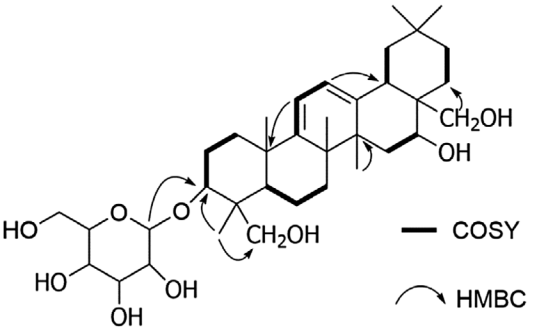


**Figure 2.** Key COSY and HMBC correlations of **1**.

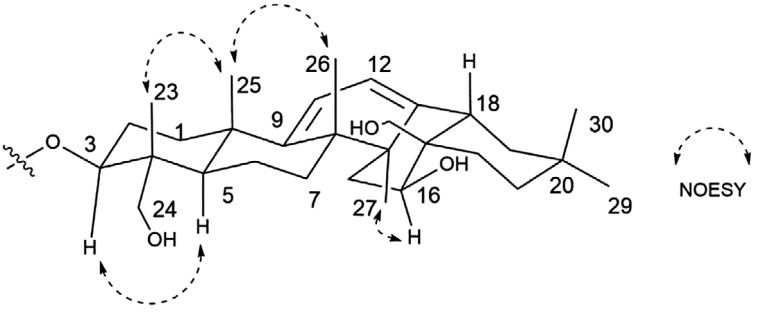


**Figure 3.** Key NOESY correlations of **1**.

($\delta_H$ 0.86, 0.97, 1.04, 1.24, 1.25, and 1.28), one anomeric proton signal at $\delta_H$ 5.10 (d, $J$ = 7.8 Hz, H-1′), two olefinic protons at $\delta_H$ 5.62 (d, $J$ = 6.0 Hz, H-12) and 5.70 (d, $J$ = 6.0 Hz, H-11). The $^{13}$C-ATP spectrum showed 36 carbons including six methyl carbons at $\delta_C$ 14.0, 21.5, 21.7, 24.4, 26.5, and 33.5, four olefinic carbons at $\delta_C$ 116.4, 123.5, 145.7, and 155.8. The data above indicated that **1** was an oxygenated oleanolic-type triterpenoid saponin derivative with one sugar moiety. Comparison of the $^1$H NMR and $^{13}$C-APT data between **1** [$\delta_H$ 5.62 (H-12), 5.70 (H-11), $\delta_C$ 116.4 (C-11), 123.5 (C-12), 145.7 (C-13), 155.8 (C-9)] and clinopodiside I [$\delta_H$ 5.67 (H-12), 6.50 (H-11), $\delta_C$ 127.0 (C-11), 125.6 (C-12), 136.4 (C-13), 133.2 (C-18)] (Compound **4**) [6] indicated that the conjugated double bonds at C-11/13 in **4** were changed to C-9/12. The HMBC (Figure 2) correlations of H-3/C-4, C-23, and C-24, of H-23/C-3 and C-4 suggested the locations of the hydroxyl groups at C-3 and C-23. The HMBC correlations from H-18 to C-16, C-17, and C-28 confirmed the locations of oxygenated groups at C-16. The NOESY (Figure 3) correlations of $CH_3$-24/$CH_3$-25, H-9/$CH_3$-27, and H-16/$CH_3$-27 indicated that the hydroxymethylene group of C-23 was $\alpha$-oriented and the hydroxyl of C-16 was $\beta$-oriented. The cross-peaks of $CH_3$-24/$CH_3$-25, $CH_3$-25/$CH_3$-26, and H-18/$CH_3$-30 indicated that 24-$CH_3$, 25-$CH_3$, 26-$CH_3$, and 30-$CH_3$ methyl groups were $\beta$ orientation. The arrangement of sugar unit was determined by HMBC experiment. Based on the above spectral evidence, the structure of **1**, clinopodiside VI, was defined as 3$\beta$, 16$\beta$, 23, 28-tetrahydroxyoleana-9(11), 12-dien-3-O-$\beta$-D-glucopyranoside.

Compound **2** possessed a molecular formula of $C_{42}H_{68}O_{13}Na$ based on HR-ESI-MS (*m/z* 803.4167 $[M + Na]^+$, calcd for 803.4558). The $^1H$ NMR spectrum displayed seven methyl signals at $\delta_H$ 0.85, 0.86, 0.96, 0.97, 1.07, 1.07, 1.52, two coupled olefinic signals at $\delta_H$ 5.70 (1H, d, $J$ = 10.8 Hz), 6.52 (1H, dd, $J$ = 10.8, 3.0 Hz), and sugar anomeric proton signals at $\delta_H$ 4.95 (d, $J$ = 7.8 Hz, H-1′) and 5.22 (d, $J$ = 7.8 Hz, H-1′). The positions of two double bonds at $\Delta^{11,12}$ and $\Delta^{11,38}$ were also secured by HMBC correlations of H-11 ($\delta_H$ 6.52, d, $J$ = 10.8, 3.0 Hz) with C-10 ($\delta_C$ 36.7), C-8 ($\delta_C$ 40.7) and C-13 ($\delta_C$ 136.6), H-12 ($\delta_H$ 5.70, d, $J$ = 10.8 Hz) with C-9 ($\delta_C$ 54.7), C-14 ($\delta_C$ 44.5), and C-18 ($\delta_C$ 133.5). On hydrolysis, the sugar units of **2** were identified as D-glucose and D-fucose by TLC and GC analyses. The linkage positions of sugars were determined by comparing its NMR spectral data with that of saikosaponin b1 (**5**) [10], clinoposaponin XV [11] and were further established by HMBC cross-peaks between H-1′ and C-3, H-1″ and C-2′, respectively. Based on the above results, it was concluded that **2** has a similar structure to that of saikosaponin b1, except for the different linkage positions of sugars. Thus, the structure of **2** was assigned as 3*β*, 16*β*, 23, 28-tetrahydroxyoleana-11,13(18)-dien-3-*O*-[*β*-D-glucopyranosyl(1→2)]-*β*-D-fucopyranoside and named saikosaponin c.

In the *in vitro* cardioprotective assays, **1**–**3** exhibited moderate inhibition against H9c2 cell damage induced by $H_2O_2$. Compared with the cell viability of 63.3 ± 2.4% in the model, **1**–**3** showed increased viability of 77.8 ± 2.6%, 80.9 ± 4.4% and 79.8 ± 2.7% at 100.0 μg/ml, respectively, using quercetin as a positive control (cell viability of 84.1 ± 2.7%).

## 3. Experimental

### 3.1. General experimental procedures

Optical rotations were obtained on a Perkin-Elmer 341 digital polarimeter (Perkin Elmer, Norwalk, USA) in MeOH at 20 °C. IR data were measured on FTIR-8400S spectrometer (Shimadzu, Kyoto, Japan). UV data were run on a Shimadzu UV2550 spectrometer (Shimadzu, Kyoto, Japan). HR-ESI-MS were performed on a LTQ-Obitrap XL spectrometer. NMR spectra were recorded on a Bruker AVIII 600 NMR spectrometer (Bruker Spectrospin, Fällanden, Switzerland, chemical shift values are presented as $\delta$ values with TMS as the internal standard). The GC was run on an Agilent 6890 N Gas Chromatograph (Agilent Technology, Santa Clara, USA) using a HP-5 capillary column (30 m × 0.25 mm, i.d.) (Agilent, Santa Clara, USA), with detection by FID. The initial temperature was set to 150 °C for 2 min and then increased to 210 °C at a speed of 5 °C/min and the carrier gas was $N_2$. ODS reversed phase silica gel (40–63 μm, Merk, Darmstadt, Germany), Sephadex LH-20 (Pharmacia, Uppsala, Sweden) and silica gel (100–200 mesh, Qingdao Haiyang Chemical Co., Ltd, Qingdao, China) were used for column chromatography. HPLC separation was performed on a CXTH LC-3000 HPLC system with a CXTH LC-3000 UV spectrophotometric detector (Beijing Chuangxintongheng Science and Technology Co., Ltd., Beijing, China) and a YMC (250 × 10 mm) semi-preparative column packed with C18 (5 μm, YMC CO., Ltd., Kyoto, Japan). TLC was carried out on silica gel precoated plates (Yantai Chemical Co. Ltd, Yantai, China), and spots were detected by spraying with 10% $H_2SO_4$ in EtOH followed by heating. All solvents used were of analytical grade (Beijing Chemical Works, Beijing, China).

### 3.2. Plant material

The aerial part of *Clinopodium polycephalum* was provided from Anhui Institute for Sciinno Drug Research, Anhui Province, China, in October 2013. The specimen was identified by Jianli Zhou (Anhui University of Chinese Medicine, Anhui, China). A voucher specimen (No. 20131028) has been deposited in the Anhui Science Institute for Drug Research Co. Ltd.

### 3.3. Extraction and isolation

The air-dried and aerial parts of *C. polycephalum* (11.6 kg) were cut into small pieces and then extracted three times with 70% aqueous EtOH (870 g). The solution was evaporated under reduced pressure to obtain an extract without alcohol. The crude extract was suspended in $H_2O$ and respectively partitioned with petroleum ether, ethyl acetate, and n-butanol. The n-BuOH extract (204 g) was subjected to column chromatography over silica gel, and eluted with a gradient of $CHCl_3$-MeOH (1:0 to 0:1) to give three fractions (A–C). Fraction B was subjected to ODS column, which was eluted with MeOH–$H_2O$ (30:70, 50:50, 75:25, 90:10, 100:0) to give twelve fractions (B1–B12). B3 (1.2 g) was repeatedly separated by Sephadex LH-20 (MeOH) and further purified by semi-preparative HPLC (flow rate 2.0 ml/min; UV detection at 210 nm; eluent MeOH–$H_2O$ 60:40; $t_R$ = 24.6 min) to offer compound **3** (39.3 mg). B8 (0.8 g) was separated by Sephadex LH-20 (MeOH) and then isolated by semi-preparative HPLC (flow rate 2.0 ml/min; UV detection at 210 nm; eluent MeOH–$H_2O$ 80:20) to yield compounds **1** (9.2 mg; $t_R$ = 27.5 min), and **4** (35.2 mg; $t_R$ = 25.4 min). B9 (3.2 g) was separated by Sephadex LH-20 (MeOH) and further purified by semi-preparative HPLC (flow rate 2.0 ml/min; UV detection at 210 nm; eluent MeOH–$H_2O$ 80:20) to get compounds **2** (14.5 mg; $t_R$ = 21.7 min), and **5** (60.6 mg; $t_R$ = 23.1 min). Fraction C (15.6 g) was subjected to ODS column, which was eluted with MeOH–$H_2O$ (30:70, 50:50, 75:25, 90:10, 100:0) to give fifteen fractions (C1–C15). Fractions C8 (131.3 mg), C10 (178.8 mg), and C12 (21.5 mg) precipitated solids after one week. Removing the supernatant and repeatedly recrystallizing solids with acetone and MeOH provided white solids and yielded compounds **6** (40.6 mg), **7** (90.4 mg), and **8** (7.5 mg), respectively.

#### 3.3.1. Clinopodiside VI

White amorphous powder; $[\alpha]_D^{20}$ + 78.5 (c 0.05, MeOH); UV (MeOH) $\lambda$max (log$\varepsilon$) 256 (3.71), 262 (3.70), 281 (3.72) nm; IR (film) $\nu_{max}$ 3367, 2942, 2875, 1457, 1067 $cm^{-1}$. $^1H$ and $^{13}C$-APT spectral data see Table 1; HR-ESI-MS: *m/z* 657.3981 $[M + Na]^+$ (calcd for $C_{36}H_{58}O_9Na$, 657.3979).

#### 3.3.2. Saikosaponin c

White amorphous powder; $[\alpha]_D^{20}$ + 1.67 (c 0.05, MeOH); UV (MeOH) $\lambda$max (log$\varepsilon$) 251 (3.48), 256 (3.53) nm; IR (film) $\nu_{max}$ 3391, 2934, 2880, 1730, 1070 $cm^{-1}$. $^1H$ and $^{13}C$-APT spectral data see Table 1; HR-ESI-MS: *m/z* 803.4167 $[M + Na]^+$ (calcd for $C_{42}H_{68}O_{13}Na$, 803.4558).

### 3.4. Acid hydrolysis

A solution of each saponin (3 mg) was heated in 3 M TFA (4 ml) on a water bath for 3 h, and then extracted with EtOAc. The aqueous layer was evaporated to dryness with

**Table 1.** $^{1}H$ and $^{13}C$ NMR spectral data for **1** and **2** (600 MHz, Pyridine-$d_5$).

| | **1** | | **2** | |
|---|---|---|---|---|
| No. | $\delta_C$ | $\delta_H$ (*J* in Hz) | $\delta_C$ | $\delta_H$ (*J* in Hz) |
| 1 | 38.0 | 1.93–1.95, m | 38.6 | 1.81–1.83, m |
| | | 1.47–1.48, m | | 1.09–1.10, m |
| 2 | 26.6 | 2.38–2.42, m | 26.2 | 2.30–2.32, m |
| | | 2.02–2.03, m | | 2.08–2.09, m |
| 3 | 82.4 | 4.30, dd, (10.8, 3.6) | 82.4 | 4.13–4.15, m |
| 4 | 41.0 | | 44.5 | |
| 5 | 44.1 | 1.81, d, (11.4) | 48.2 | 1.54–1.56, m |
| 6 | 18.5 | 1.80–1.82, m | 18.5 | 1.80–1.82, m |
| | | 1.78–1.79, m | | 1.40–1.41, m |
| 7 | 31.4 | 1.95–1.97, m | 32.9 | 1.40–1.42, m |
| | | 1.37–1.38, m | | 1.32, m |
| 8 | 43.7 | | 40.7 | |
| 9 | 155.8 | | 54.7 | 2.07, s |
| 10 | 39.2 | | 36.7 | |
| 11 | 116.4 | 5.70, d, (6.0) | 127.3 | 6.52, dd, (10.8, 3.0) |
| 12 | 123.5 | 5.62, d, (6.0) | 125.9 | 5.70, d, (10.8) |
| 13 | 145.7 | | 136.6 | |
| 14 | 44.0 | | 44.5 | |
| 15 | 36.6 | 2.34, br t, (12.6) | 35.1 | 2.19, br t, (12.6) |
| | | 1.35–1.36, m | | 1.54–1.55, m |
| 16 | 67.2 | 4.57–4.59, m | 75.7 | 4.06–4.08, m |
| 17 | 43.6 | | 44.6 | |
| 18 | 43.1 | 2.53, dd, (13.8, 4.8) | 133.5 | |
| 19 | 47.3 | 1.78–1.81, m | 38.5 | 2.49, d, (13.2) |
| | | 1.17–1.18, m | | 1.81, d, (13.2) |
| 20 | 27.0 | | 32.9 | |
| 21 | 32.5 | 1.92–1.94. m | 35.3 | 1.71–1.73, m |
| | | 1.34–1.35, m | | 1.41–1.43, m |
| 22 | 34.5 | 2.83, br d, (12.0) | 30.2 | 2.93, br d, (13.2) |
| | | 1.59–1.60, m | | 1.54–1.55, m |
| 23 | 65.3 | 4.38, d, (10.2) | 65.0 | 4.40, br s |
| | | 3.70, d, (10.2) | | 3.73–3.75, m |
| 24 | 14.0 | 1.04, s | 13.1 | 1.07, s |
| 25 | 26.5 | 1.25, s | 19.0 | 0.86 s |
| 26 | 21.5 | 1.24, s | 17.6 | 0.97, s |
| 27 | 21.7 | 1.28, s | 22.2 | 1.07, s |
| 28 | 69.8 | 3.74, d, (10.2) | 64.3 | 4.37–4.39, m |
| | | 4.51, d, (10.2) | | 4.17–4.19, m |
| 29 | 33.5 | 0.86, s | 32.5 | 0.85, s |
| 30 | 24.4 | 0.97, s | 25.0 | 0.96, s |
| 1′ | 106.3 | 5.10, d, (7.8) | 104.3 | 4.95, d, (7.8) |
| 2′ | 76.3 | 4.04, br t, (7.8) | 82.6 | 4.49, br t, (8.4) |
| 3′ | 79.1 | 4.14, br t, (9.6) | 77.8 | 4.16–4.19, m |
| 4′ | 72.1 | 4.24, br t, (9.6) | 72.6 | 3.98, br s |
| 5′ | 78.6 | 3.98, m | 71.2 | 3.69, q, (6.0) |
| 6′ | 63.3 | 4.58, dd, (11.4, 5.4) | 17.3 | 1.52, d, (6.0) |
| | | 4.39, dd, (11.4, 3.6) | | |
| 1″ | | | 106.3 | 5.22, d, (7.8) |
| 2″ | | | 76.9 | 4.06–4.08, m |
| 3″ | | | 78.4 | 4.17–4.19, m |
| 4″ | | | 71.5 | 4.26, br t, (9.0) |
| 5″ | | | 78.3 | 3.73–3.76, m |
| 6″ | | | 62.7 | 4.40, br s |
| | | | | 4.40, br s |

ethanol in vacuo at 50 °C until neutral. The residues were determined in comparison with authentic monosaccharides using TLC ($CHCl_3$:MeOH:$H_2O$ = 3:2:0.2). For the sugars of **1**–**3**, the Rf values of glucose and fucose by TLC were 0.25 and 0.34, respectively. Also, the

absolute configurations of the sugars were determined by gas chromatography [12]. By this method, L-cysteine methyl ester hydrochloride (0.06 mol/L) and hexamethyldisilazane–trimethylchlorosilane (HMDS-TMCS, 3:1) were added to the aqueous residue for derivatization. The solution was then centrifuged and the precipitate was removed. After these processes, n-hexane was used to extract derivate and analyzed by GC. D-Glucose ($t_R$ = 12.163, 13.800 min) and D-fucose ($t_R$ = 8.250, 8.930 min) were detected by comparing with authentic monosaccharides.

### 3.5. H9c2 Cell protection assay

H9c2 cells at a density of $5 \times 10^4$ cells per well in 96-well plates were cultured in dulbecco modified eagle medium media (Hyclone, Los Angeles, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Los Angeles, USA), and L-glutamine (2 mM). Cultures were maintained at 37 °C in 5% $CO_2$ in a humidified incubator for 24 h. After 12 h of treatment with different concentrations of drugs, followed by incubation with 150 mM $H_2O_2$ for 2 h, 20 µl of 5 mg/ml MTT solution was added to each well (0.1 mg/well), and incubated for 4 h. The supernates were aspirated, and the formazan crystals in each well were dissolved in 150 ml of DMSO. The absorbance was measured at 570 nm on a microplate reader (BioTek, Vermont). The survival rate of H9c2 cells was evaluated and the inhibition (%) was obtained by the following formula: Inhibition (%) = [(OD(sample) – OD(control))/(OD(normal) – OD(control))] × 100.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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