Phytochemistry Letters 14 (2015) 178–184

Contents lists available at ScienceDirect

Phytochemistry Letters

journal homepage: www.elsevier.com/locate/phytol

Seven new triterpenoids from the aerial parts of *Ilex cornuta* and protective effects against H_2O_2-induced myocardial cell injury

Shanshan Li[a,b], Jianping Zhao[c], Wenlian Wang[b], Yuchen Lu[b], Qiongming Xu[b,*], Yanli Liu[b,*], Xiaoran Li[b], Ikhlas A. Khan[b,c], Shilin Yang[b]

[a] *Children's Hospital of Soochow University, Suzhou 215003, China*
[b] *College of Pharmaceutical Science, Soochow University, Suzhou 215123, China*
[c] *National Center for Natural Products Research, and Department of Pharmacognosy, Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi, Mississippi 38677, United States*

ARTICLE INFO

Article history:
Received 29 June 2015
Received in revised form 3 October 2015
Accepted 10 October 2015
Available online 10 November 2015

Keywords:
Ilex cornuta
Aquifoliaceae
Triterpenoids
Structure identification
Cardiomyocytes oxidative injury
DPPH radical scavenging activity

ABSTRACT

Seven new triterpenoids (**1**–**7**), together with two known ones (**8**–**9**), were isolated from the aerial parts of*Ilex cornuta*. The leaves of *I. cornuta* are the major source of "Kudingcha", a popular herbal tea consumed in China and other countries. The structures of compounds **1**–**7** were determined as 20-*epi*-urs-12,18-dien-28-oic acid 3β-*O*-α-L-arabinopyranoside (**1**), 20-*epi*-urs-12,18-dien-28-oic acid 2′-*O*-acetyl-3β-*O*-α-L-arabinopyranoside (**2**), 20-*epi*-urs-12,18-dien-28-oic acid 3β-*O*-β-D-glucuronopyranoside-6-*O*-methyl ester (**3**), 3β,23-dihydroxy-20-*epi*-urs-12,18-dien-28-oic acid (**4**), 23-hydroxy-20-*epi*-urs-12,18-dien-28-oic acid 3β-*O*-α-L-arabinopyranoside (**5**), 23-hydroxy-20-*epi*-urs-12,18-dien-28-oic acid 3β-*O*-β-D-glucuronic acid (**6**), 23-hydroxy-20-*epi*-urs-12,18-dien-28-oic acid 3β-*O*-β-D-glucuronopyranoside-6-*O*-methyl ester (**7**), on the basis of spectroscopic analyses (IR, ESI–MS, HR-ESI–MS, 1D and 2D NMR) and chemical reactions. Protective effects against H_2O_2-induced H9c2 cardiomyocyte injury were tested in vitro for compounds **1**–**9**, and the data showed that compound **4** had significant cell-protective effect. Compounds **1**-**9** did not show significant DPPH radical scavenging activity.

1. Introduction

Ilex cornuta Lindl. et Paxt. (Aquifoliaceae family), a slow-growing evergreen shrub or small tree, is cultivated widely in South China. The leaves of *I. cornuta* are used to make the popular herbal tea, called as "Kudingcha" or bitter tea (Chau and Wu, 2006) in China. Commercially, Kudingcha is sold in the form of tea-bags packed with ground leaves (1 to 2 g). The leaf extracts are also used as ingredients in food or dietary supplement industries. Traditionally, Kudingcha has been used for treatment of sore throat, as an agent for weight loss (Ahai, 1985), and for the relief of hypertension (Jiangsu Medical College, 1975). It is believed that Kudingcha has potential benefits for reducing cardiovascular and oxidative stress-related diseases (Thuong et al., 2009). Recently, *I. cornuta* has been gaining research attention because of its potential health benefits. Studies showed that Kudingcha possesses antioxidant, antidiabetic, hepatoprotective, neuroprotective, anti-inflammatory, and diuretic effects (Jiangsu New Medical College, 1986; Qin et al., 1988; Puangpraphant and De Mejia, 2009). Previous phytochemical investigations showed that the whole plant of *I. cornuta* is a rich source of triterpenoids and flavonoids as well as their corresponding glycosides (Wang et al., 2014; Liao et al., 2013; Li et al., 2006). Previous studies on the aerial parts of *I. cornuta* have resulted in the isolation of a series of new triterpenoidal saponins, and some triterpenoidal saponins showed potential benefit for inhibition of peroxidative damage associated with ischemia and reperfusion (Li et al., 2014). As part of a continuous search for potentially active substances for the prevention of coronary artery disease, seven new triterpenoids (**1**-**7**) (Fig. 1), along with two known ones were obtained from the aerial parts of *I. Cornuta*. The isolation and structural elucidation of the new compounds, as well as the protective effects of the isolates against cardiomyocytes injury induced by H_2O_2 are reported herein. In addition, their radical scavenging activity was evaluated by DPPH assay.

* Corresponding authors. Fax: +86 512 65882089.
E-mail addresses: xuqiongming@suda.edu.cn (Q. Xu), liuyanli@suda.edu.cn (Y. Liu).

http://dx.doi.org/10.1016/j.phytol.2015.10.010
1874-3900/

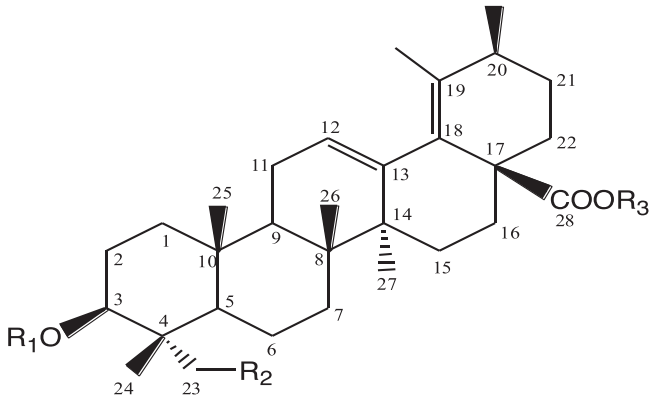

Fig. 1. Structures of the compounds **1**–**9**.

2. Results and discussion

2.1. Structure Identification of the isolates

A 50% EtOH extract of the aerial parts of *I. cornuta* was subjected to column chromatographic separations on macroporous resin D101, silica gel and octadecylsilane (ODS) silica gel, yielding seven new triterpenoids (**1**–**7**) and two known ones which were identified as 3β-hydroxy-20α(H)-urs-12,18-dien-28-oic acid (**8**) (Ali and Srivastava, 1990), 3β-*O*-α-L-arabinopyranosyl-20α(H)-urs-12,18-dien-28-oic acid 28-*O*-β-D-glucopyranoside (**9**) (Che et al., 2012) (Fig. 1).

Compound **1** was isolated as white powder, and its negative-ion HR-ESI–MS showed a quasimolecular $[M+Na]^+$ ion peak at *m*/*z* 609.3796, attributed to the molecular formula of $C_{35}H_{54}O_7Na$ (calc'd. 609.3767). The ^{1}H NMR spectrum showed the singlet resonances of six tertiary methyl groups at δ 0.97 (3H, s, Me-25), 1.06 (3H, s, Me-24), 1.12 (3H, s, Me-26), 1.23 (3H, s, Me-27), 1.38 (3H, s, Me-23), 1.97 (3H, s, Me-29), one methyl doublet at 1.21 (3H, d, *J* = 6.5 Hz Me-30), an olefinic proton signal at δ 5.73 (1H, m, H-12), and the signal of a oxygen-bearing methine at δ3.49 (1H, dd, *J* = 4.0, 12.0 Hz, H-3). The NMR spectra indicated that the aglycone of **1** was a 3β-hydroxy-20-*epi*-urs-12,18-dien-28-oic acid by comparing its spectroscopic data with those of compound **8** (Ali and Srivastava, 1990). In the ^{1}H NMR spectrum, an anomeric proton was observed at δ_H 4.90 (1H, d, *J* = 7.0 Hz), which showed HSQC correlation with the anomeric carbon at δ_C 107.7 (C-1 of arabinose (Ara)), indicating the presence of one sugar unit in the structure of compound **1**. The sugar residue yielded from acid hydrolysis of **1** was identified as L-arabinose by GC analysis. The location of arabinopyranosyl group was assigned at C-3 of the aglycone on the basis of the observations of the down field shift of H-3 at δ3.49, the correlation between δ_H 3.49 (H-3) and δ_C 107.7 (C-1 of Ara), as well as the correlation between δ_H 4.90 (H-1 of Ara) and δ_C 89.0 (C-3 of the aglycone) in the HMBC spectrum (Fig. 2). The α-configuration of arabinopyranosyl unit was inferred from the NOESY correlations between δ 4.90 (H-1 of Ara) and δ 4.26 (H-3 of Ara), 4.42 (H-4 of Ara) (Fig. 2). Thus, compound **1** was established as 20-*epi*-urs-12,18-dien-28-oic acid 3β-*O*-α-L-arabinopyranoside.

Compound **2** was obtained as white powder. Its molecular formula was determined to be $C_{37}H_{56}O_8$ according to the $[M-H]^-$ ion peak at *m*/*z*627.3891(calc'd. 627.3897). An anomeric proton observed at δ_H 4.83 (1H, d, *J* = 7.5 Hz) showed HSQC correlation with the anomeric carbon at δ_C 104.8 (C-1 of Ara), Which indicated the presence of one sugar unit in the structure of compound **2**. The structure of the sugar residue yielded from acid hydrolysis of **2** was identified as L-arabinose by GC analysis. Comparing the NMR spectrum of compound **2** with that of compound **1**, it was suggested that **2** was derived from the acetylation of **1**, The downfield shift of C-2 (+1.4 ppm) of Ara at δ_C 74.5 suggested that the acetyl (Ac) group was located at C-2 of arabinose, and the assignment was supported by the HMBC correlation between δ_H 5.98 (H-2 of Ara) and δ_C 170.1 (C-1 of Ac) (Fig. 2). Thus, compound **2** was elucidated as 20-*epi*-urs-12,18-dien-28-oic acid 3β-*O*-(2′-*O*-acetyl)-α-L-arabinopyranoside.

Compound **3** was obtained as white powder. Its molecular formula was determined to be $C_{37}H_{56}O_9$ based on the positive HR-ESI–MS. An anomeric proton observed at δ_H 5.13 (1H, d, *J* = 14.0 Hz, H-1 of GlcA) showed HSQC correlation to the anomeric carbon at δ_C 107.5 (C-1 of GlcA), indicating the presence of a sugar unit in the structure of compound **3**. The sugar residue obtained from the acid hydrolysis of **3** was identified as D-glucuronic acid by the GC analysis. The ^{13}C NMR spectrum of **3** was very similar to that of urs-12,18-dien-28-oic acid 3β-*O*-β-D-glucuronopyranoside-6-*O*-methyl ester, and the main differences arising from the significant downfield shifts of C-18 (+11.4) at δ 134.8 and C-22 (+1.1) at δ 35.9 due to the γ-effect of the 30-β (axial) methyl group that were observed when compared with urs-12,18-dien-28-oic acid 3β-*O*-β-D-glucuronopyranoside-6-*O*-methyl ester which possesses a 30-*α* (equatorial) methyl group instead (Hidaka et al., 1987). Moreover, the β orientation assignment of Me-30 was also confirmed by comparing its spectroscopic data with those of compound **1**. Therefore, the structure of **3** was elucidated as 20-*epi*-urs-12,18-dien-28-oic acid 3β-*O*-β-D-glucuronopyranoside-6-*O*-methyl ester.

Compound **4** was obtained as white powder. Its molecular formula was assigned as $C_{30}H_{46}O_4$ on the basis of its positive HR-ESI–MS $[M+Na]^+$ ion peak at *m*/*z*493.3318 (calc'd. 493.3294). The ^{1}H NMR spectrum showed the singlet resonances of five tertiary methyl groups at δ 1.08(3H, s, Me-27), 1.89 (3H, s, Me-29), 1.09 (3H, s, Me-26), 1.07 (3H, s, Me-24), 1.01 (3H, s, Me-25), one methyl doublet at 1.12 (3H, d, *J* = 10.0 Me-30), an olefinic proton signal at δ 5.69 (1H, m, H-12), and the signals of a hydroxymethylene group at δ 4.23 (1H, m, H-23), 3.76 (1H, d, *J* = 15.0 Hz, H-23), as well as the signal of a oxygen-bearing methine at δ4.28 (1H, dd, *J* = 5.0, 10.0 Hz, H-3). The ^{13}C NMR showed resonances for 30 carbon atoms, whose multiplicity patterns were revealed from the DEPT and HSQC experiments as six methyls, ten methylenes, five methines, and nine quaternary carbons. It showed four olefinic C-atoms at δ_C 126.4, 134.5, 135.6, 139.6, one oxymethylene group at δ_C 67.6, corresponding to C-23, and one oxymethine carbon at δ 73.1, corresponding to C-3, as well as one carboxyl group at δ_C 178.8, corresponding to C-28. The ^{13}C NMR spectrum of compound **4** was similar to that of compound **8** except for the significant chemical shift change of C-23 (+38.5 ppm) at δ_C 67.6, suggesting the methyl group in compound **8** was replaced by the hydroxymethylene group in compound **4.** The assignment was confirmed by the observation of HMBC correlations between C-23 at δ_C 67.6 and Me-24 at δ_H 1.07, and between C-24 at δ_C 13.2 and Me-23 at δ_H 3.76. Thus, compound **4** was identified as 3β,23-dihydroxy-20-*epi*-urs-12,18-dien-28-oic acid.

Compound **5** was isolated as white powder. Its HR-ESI–MS $[M+HCOOH-H]^-$ ion peak at *m*/*z* 647.3801 (calc'd. 647.3795)

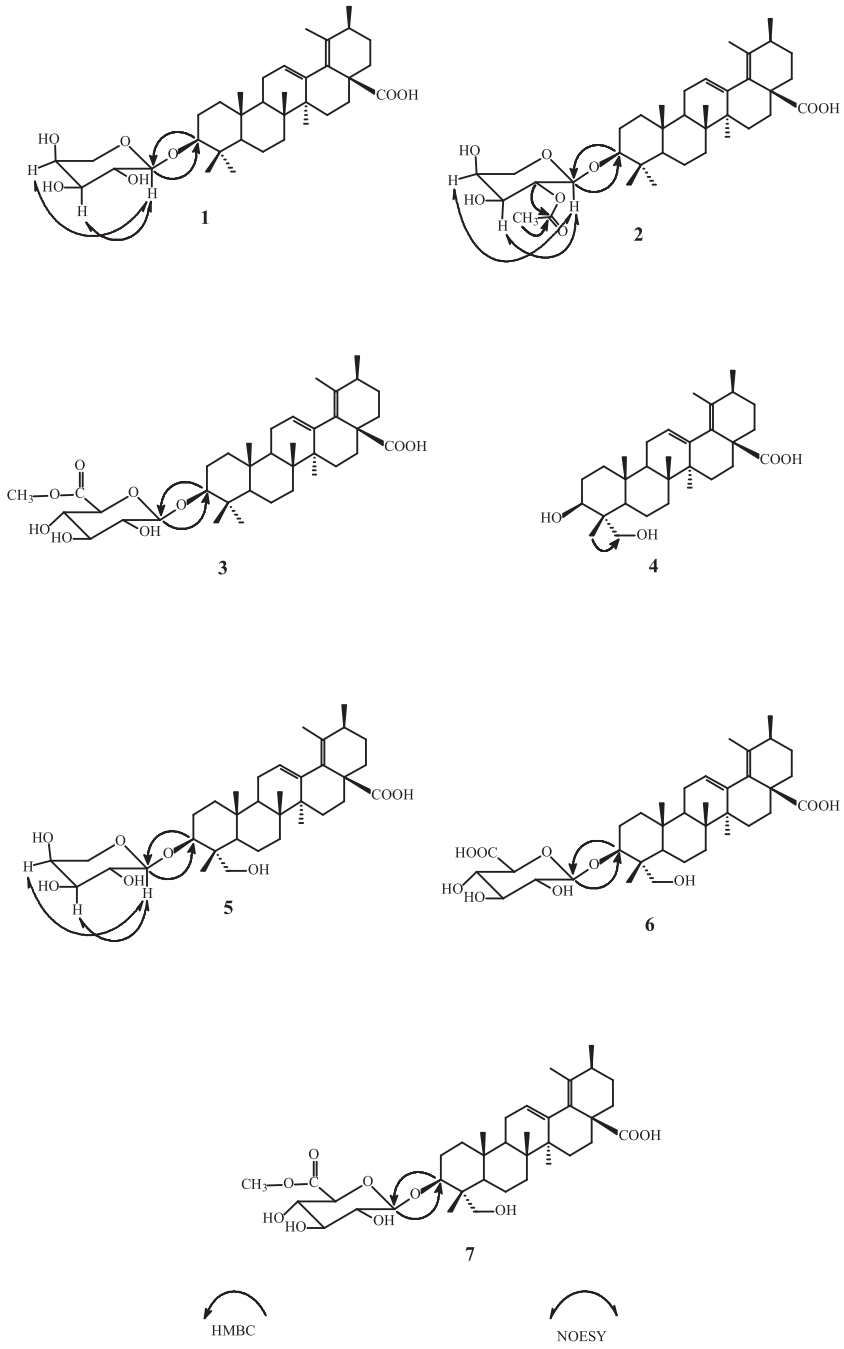

Fig. 2. Key HMBC and NOESY correlations for **1**–**7**.

suggested the molecular formula $C_{35}H_{54}O_8$. An anomeric proton observed at δ_H 5.09 (1H, d, J = 10.0 Hz) showed the HSQC correlation with the anomeric carbon at δ_C 106.8 (C-1 of Ara), indicating the presence of one sugar unit in the structure of compound **5**. The sugar residue yielded from acid hydrolysis of **5** was identified as L-arabinose by GC analysis. Comparing the NMR spectral data between compound **5** and compound **4**, it was suggested that **5** was derived from the glycosylation of compound **4**. The downfield shift of C-3 (+8.8 ppm) of aglycone at δ_C 81.9 suggested that the arabinopyranosyl group was located at C-3 of aglycone, and the assignment was confirmed by the observation of the HMBC correlations between δ_H 5.09 (H-1 of Ara) and δ_C 81.9 (C-3 of aglycone), and between δ_H 4.38 (H-3 of aglycone) and δ_C 106.8 (C-1 of arabinose) (Fig. 2). The α-configuration of arabinopyranosyl unit was assigned according to the NOESY correlations between δ 5.09 (H-1 of arabinose) and δ 4.15 (H-3 of arabinose), 4.33 (H-4 of arabinose) (Fig. 2). Thus, compound **5** was established as 23-hydroxy-20-*epi*-urs-12,18-dien-28-oic acid 3β-*O*-α-L-arabinopyranoside.

Compound **6** was obtained as white powder. The positive HR-ESI–MS showed a quasi-molecular ion peak at m/z 645.3694, indicating the molecular formula of $C_{36}H_{53}O_{10}$ ($[M\text{-}H]^-$, calc'd. 645.3639). The presence of a sugar unit in the structure of compound **6** was confirmed by the observation of an anomeric proton at δ_H 5.31 (1H, d, J = 10.0 Hz, H-1 of GlcA), which showed HSQC correlation to the anomeric carbon at δ_C 106.4 (C-1 of GlcA). The sugar residue obtained from the acid hydrolysis of **6** was identified as D-glucuronic acid by the GC analysis. Compound **6** had a similar structure as compound **5** with comparison of the NMR spectral data between them, and the only difference was that

compound **5** had an arabinopyranosyl group while compound **6** had a GlcA group. The β configuration of GlcA group was confirmed on the basis of the large $3J_{H\text{-}1,H\text{-}2}$ coupling constant. Therefore, the structure of compound **6** was elucidated as 23-hydroxy-20-*epi*-urs-12,18-dien-28-oic acid 3β-*O*-β-D-glucuronic acid.

Compound **7** was obtained as white powder. The positive HR-ESI–MS showed a quasi-molecular ion peak at *m*/*z* 683.3771, indicating the molecular formula of $C_{37}H_{56}O_{10}Na$ ($[M+Na]^+$, calcd. 683.3771). In the ^1H NMR spectrum, an anomeric proton was observed at δ_H 5.28 (1H, d, J = 10.0 Hz, H-1 of GlcA), which showed the correlation to the anomeric carbon at δ_C 106.9 (C-1 of GlcA) in the HSQC spectrum, indicating the presence of a sugar unit in the structure of compound **7**. The sugar residue obtained from the acid hydrolysis of **7** was identified as D-glucuronic acid by the GC analysis. The ^{13}C NMR data of **7** were similar to those of **6** except for the additional signal at δ_C 52.5 corresponding to one OCH_3 group. The OCH_3 group was determined to attach at C-6 of GlcA group according to the HMBC correlation between δ_C 171.3 (C-6 of GlcA) and δ_H 3.74 (OCH_3). Therefore, the structure of **7** was elucidated as 23-hydroxy-20-*epi*-urs-12,18-dien-28-oic acid 3β-*O*-β-D-glucuronopyranoside-6-*O*-methyl ester.

2.2. Protective effects of the isolates against H_2O_2-induced myocardial cell injury

Many chronic diseases such as cardiovascular diseases, may be caused by intracellular oxidative damage of biomolecules via ROS (Fearon and Faux, 2009). Oxidative stress plays an important role in the pathogenesis of cardiomyocytes ischemic injury. A number of studies showed that antioxidants reduced oxidative stress-mediated cellular injury (Yousuf et al., 2009). Among ROS, H_2O_2 readily crosses cellular membranes and gives rise to the highly reactive hydroxyl radical, which is the most reactive and induces severe damage to adjacent biomolecules(Valko et al., 2007). H_2O_2, superoxide and hydroxyl radicals, have been used frequently in models of oxidative stress in H9c2 cardiomyocytes to investigate heart dysfunction(Hescheler et al., 1991; Yasuoka et al., 2004). The previous investigation suggested that *I. cornuta* extract might have a protective effect on myocardial ischemia (Liu et al., 2009; Li et al., 2014). For the investigation of the protective effects against H_2O_2-induced myocardial cell injury, compounds **1**–**9** were tested. Among them compound **4** (12.5–200 μM) significantly increased the viability of H9c2 induced by H_2O_2 in a dose-dependent manner with concentrations ranging from 12.5 to 200 μM (Table 3). According to the results, Compound **4** exhibited significant protective effects on H_2O_2 induced myocardial cell injury. Vitamin E, which was reported to have effects on inhibiting peroxidative damage associated with ischemia and reperfusion, was used as a positive control (Yamamoto et al., 1983; Fujimoto et al., 1984). Vitamin E can incorporate into the membrane lipids of endothelial cells to protect against membrane lipid peroxidation by quenching cytotoxic ROS (Martin et al., 1996). We speculated that the active compounds isolated from *I. cornuta* might play a similar antioxidant role in the prevention of peroxidative damage. Therefore, the radical scavenging capacities of the isolates were evaluated in the present study.

2.3. DPPH radical scavenging capacity assay

Compounds **1**–**9** were evaluated for antioxidant activity by DPPH radical scavenging capacity assay. As shown in Table 4, the maximal scavenging rates of compounds **1**–**9** were less than 20% at the concentration of 200 μM. The test results showed that compounds **1**–**9** were found to have no significant DPPH radical scavenging capacity. Combined with H_2O_2-induced myocardial cell injury experiment, the DPPH radical scavenging capacity assay suggested that protective effects on H_2O_2 induced myocardial cell injury were not directly associated with their radical scavenging capacity.

Previous studies on the cardioprotective potential of natural products or herbal medicines have revealed that except for radical scavenging, other cellular and molecular anti-oxidation mechanisms may be involved in their protective effects against myocardial ischemia/reperfusion (I/R) injury (Liu et al., 2013). For instance, it was found that senticoside B, a triterpenoidal saponin exhibiting antioxidant action and cardiac protection, could attenuate oxidative stress and protect cardiomyocytes from I/R injury by enhancing the actions of catalase (CAT), glutathione peroxidase (GSHPx), and superoxide dismutase (SOD) (Shi et al., 2014; Liang et al., 2010; Luo et al., 2011). Since the compounds isolated in this study belong to triterpenoidal saponins, we speculated that these active compounds might exert their protection on myocardial cells through enhancing the level of antioxidant enzymes (CAT, GSHPx, and SOD) in cardiomyocyte, thereby increasing the contents of endogenous antioxidants. Nevertheless, further investigations are needed to explore the mechanisms of protective effects of the saponins from *I. cornuta* on myocardial cell injury.

Our previous investigation of the structure-activity relationship of these natural triterpenoidal saponins revealed that the ursane-

Table 1
^{13}C NMR data (δ) of compounds **1**–**7** (125 MHz in pyridine-*d*5).

No.	1	2	3	4	5	6	7
1	39.5	39.2	39.4	39.2	39.4	39.7	39.6
2	27.0	26.7	26.9	27.7	26.4	26.6	26.6
3	89.0	89.0	89.3	73.1	81.9	82.1	82.5
4	39.8	39.4	39.7	42.9	43.7	44.0	44.0
5	56.3	56.1	56.2	48.2	47.8	48.1	48.1
6	18.7	18.6	18.6	18.4	18.3	18.7	18.6
7	35.1	35.0	35.1	34.6	34.8	35.2	35.1
8	39.5	39.4	39.4	39.2	39.5	39.9	39.8
9	48.5	48.4	48.4	48.5	48.5	48.8	48.8
10	37.1	36.9	36.9	37.0	36.9	37.2	37.2
11	23.8	23.7	23.7	23.5	23.7	24.1	24.0
12	126.7	126.6	126.6	126.4	126.7	127.1	127.0
13	139.8	139.7	139.8	139.6	139.8	140.1	140.1
14	45.1	45.0	45.0	44.8	45.0	45.4	45.3
15	29.5	29.1	29.5	29.2	29.4	29.8	29.8
16	35.1	35.0	35.1	34.8	35.1	35.5	35.4
17	50.5	50.5	50.5	50.2	50.5	50.8	50.8
18	134.9	134.6	134.8	134.5	134.7	135.6	135.0
19	136.1	135.7	135.7	135.6	135.8	135.7	136.2
20	37.7	37.7	37.7	37.4	37.6	38.0	38.0
21	29.1	29.4	29.1	28.9	29.1	29.5	29.5
22	35.9	35.8	35.9	35.6	35.9	36.2	36.2
23	28.5	28.2	28.4	67.6	64.6	64.9	64.7
24	17.3	17.0	17.2	13.2	13.9	14.2	14.2
25	16.5	16.2	16.3	16.6	17.0	17.4	17.3
26	18.3	18.2	18.3	18.2	18.4	18.7	18.7
27	22.5	22.4	22.5	22.2	22.5	22.9	22.8
28	179.1	179.2	179.0	178.8	179.0	179.3	179.3
29	20.5	20.4	20.5	20.2	20.4	20.8	20.7
30	20.8	20.7	20.8	20.5	20.7	21.1	21.1
C_3-O-GlcA-1			107.5			106.4	106.9
2			75.6			75.7	75.9
3			78.1			78.7	78.3
4			73.3			74.0	73.7
5			77.4			78.0	77.7
6			171			174.0	171.3
Methyl-1			52.2				52.5
Ara-1	107.7	104.8			106.8		
2	73.1	74.5			73.3		
3	74.8	72.6			74.9		
4	69.7	69.9			69.8		
5	66.9	67.3			67.1		
$COOCH_3$		21.4					
$COOCH_3$		170.1					

type triterpenoidal saponins with one double bond located at C-12 is essential for the protective activity, and the glucuronic acid methyl ester at C-3 of aglycone were active in protecting myocardial cell injury (Li et al., 2014). In this paper, we found that the ursane type triterpenoidal saponins with two conjugated double bonds located at C-12 and C-18 also showed protective activity, suggesting that the two conjugated double bonds did not significantly alter the activity. Compound **4** with significant activity was just a triterpene with no glycolic chain, suggesting the glucosylation might diminish the activity.

3. Experimental

3.1. General experimental procedures

^{1}H, ^{13}C NMR and 2D NMR spectra were measured in C_5D_5N with a Varian Inova 500 spectrometer (Varian Inc., Palo Alto, CA, USA) using tetramethylsilane (TMS) as the internal standard. HR-ESI–MS spectra were recorded on a Micromass Q-TOF2 spectrometer (Micromass Corp., London, UK).

HPLC analysis and purification were performed on a Shimadzu HPLC system equipped with a LC-20AT pump, a SPD-20A detector (Shimadzu Corp., Kyoto, Japan), and the wavelength for detection was 203 nm. The HPLC column (250 × 9.4 mm i.d., 5 μm, Agilent Zorbax SB-C18 semipreparative) was purchased from Agilent Corp. (Palo Alto, CA, USA). MPLC purification was taken on a Büchi Flash Chromatography system composed of a C-650 pump and a flash column (460 mm × 26 mm i.d., Büchi Corp., Flawil, Switzerland). The ODS column for MPLC was purchased from Merck KGaA (Darmstadt, Germany). Silica gel (60–100 mesh) used for open column chromatography and precoated silica gel TLC plates were purchased from Qingdao Marine Chemical Factory (Qingdao, China). Macroporous resin (D101, Xi'an Sunresin New Materials Co., Ltd., Xi'an, China) was used for column chromatography. TLC was colored on 10% sulfuric acid alcohol solution. GC was carried out on a GC-14C (Shimadzu Corp., Kyoto, Japan) with a flame ionization detector (FID).

3.2. Plant material

The aerial parts of *I. cornuta*were collected from Liuan City, Anhui province of China in April 2011, and authenticated by Professor Xiao-Ran Li at Soochow University. A voucher sample (No.11-11-06-01) was kept in the herbarium of the College of Pharmaceutical Science, Soochow University.

3.3. Extraction and isolation

Dried aerial parts (10 kg) of *I. cornuta* were extracted with 50% EtOH. Evaporation of the solvent under reduced pressure yielded a residue (325 g), which was dissolved in distilled water and subjected to a D101 macroporous resin column (200 × 30 cm i.d.), then eluted with 0%, 30%, 60% and 90% EtOH. The 90% EtOH eluate was dried in vacuo to yield a fraction (30 g), which was separated by a silica gel column (60-100 mesh) using $CHCl_3$-MeOH (90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 0:100, each 6.0 L) for elution. The $CHCl_3$-MeOH (80:20) eluate (4.9 g) was further separated by MPLC over an ODS column to yield 5 fractions. Fraction 1 (225 mg) was separated by semi-preparative HPLC, eluted with MeOH-H_2O (75:25) to yield compounds **1** (31 mg, t_R 37.24 min), **7** (7 mg, t_R 39.10 min). In the same way, compound **8** (25 mg, t_R 49.21 min) was obtained from fraction 2 (170 mg). The

Table 2
^{1}H NMR data of compounds **1**–**7** (500 MHz in pyridine-*d*5).

No.	1	2	3	4	5	6	7
1	1.03m, 1.70m	0.98m, 1.65m	0.90m, 1.56m	1.12m, 1.69m	1.13m, 1.75m	0.99m	0.94m, 1.57m
2	1.98m, 2.28m	1.91m, 2.17m	1.95m, 2.24m	1.97m, 2.02m	2.11m, 2.36m	2.41m	2.02m, 2.26m
3	3.49dd (4.0,12.0)	3.35dd (4.5,11.8)	3.51dd (4.0,12.0)	4.28dd (5.0,10.0)	4.38m	4.44m	4.35m
5	0..94m	0.90m	1.10m	1.65m	1.79m	1.75m	1.70m
6	1.35m, 1.57m	1.36m, 1.58m	1.35m, 1.56m	1.43m, 1.71m	1.39m, 1.78m	1.39m	1.28m, 1.67m
7	1.55m, 1.72m	1.55m, 1.70m	1.56m, 1.74m	1.50m, 1.75m	1.51m, 1.81m	1.52m	1.45m, 1.74m
9	1.60m	1.59m	1.56m	1.58m	1.71m	1.66m	1.58m
11	2.04m	1.96m	2.00m	2.03m	2.03m	2.01m	1.95m
12	5.73m	5.73m	5.73m	5.69m	5.74m	5.72m	5.66m
15	1.70m, 1.79m	1.35m, 2.31m	1.73m, 1.81m	1.63m, 1.72m	1.70m, 1.79m	1.73m	1.65m, 1.72m
16	1.65m, 2.56m	1.61m, 2.58m	1.67m, 2.59m	1.62m, 2.51m	1.67m, 2.53m	1.68m	1.62m, 2.47m
20	2.27m	2.26m	2.27m	2.18m	2.24m	2.26m	2.20m
21	1.35m, 2.31m	1.73m, 1.80m	1.35m, 2.30m	1.27m, 2.26m	1.29m, 2.29m	1.31m	1.24m, 2.22m
22	1.68m, 2.45m	1.67m, 2.46m	1.71m, 2.45m	1.57m, 2.38m	1.60m, 2.44m	1.67m	1.60m, 2.37m
23	1.38s	1.21s	1.41s	3.76d(15.0), 4.23m	3.79m, 4.38m	3.79m	3.71m, 4.37m
24	1.06s	0.98s	1.06s	1.07s	1.01s	1.03s	0.95s
25	0.97s	0.92s	0.92s	1.01s	1.05s	1.02s	0.93s
26	1.12s	1.11s	1.10s	1.09s	1.13s	1.12s	1.05s
27	1.23s	1.22s	1.23s	1.08s	1.15s	1.18s	1.09s
29	1.97s	1.95s	1.98s	1.89s	1.96s	1.96s	1.89s
30	1.21d(6.5)	1.19d(7.0)	1.21d(7.0)	1.12d(10.0)	1.18d(10.0)	1.20d(10.0)	1.12d(8.5)
C_3-O-GlcA-1			5.13d(14.0)			5.31d(10.0)	5.28d(10.0)
2			4.18t(8.0)			4.21m	4.13t(10.0)
3			4.36t(9.0)			4.29 m	4.20t(10.0)
4			4.57t(9.5)			4.60 m	4.48m
5			4.69d(10.0)			4.62 m	4.51m
Methyl-1			3.85s				3.74s
Ara-1	4.90d(7.0)	4.83d(7.5)			5.09d(10.0)		
2	4.52m	5.98t(8.8)			4.52t(10.0)		
3	4.26m	4.26dd (3.0.9.5)			4.15m		
4	4.42m	4.37m			4.33m		
5	3.92m, 4.41m	4.39mchar_dot 3.88d(11.0)			3.81m, 4.37m		
$COOCH_3$		2.20s					

*Data in parentheses are *J* values (in Hz). Ara referred to arabinose, GlcA referred to gluconic acid.

$CHCl_3$-MeOH (50:50) eluate (3.7 g) was separated into 4 fractions by MPLC over the ODS column in a similar way described above, and offered compounds **2** (12 mg), **3** (10 mg) and **6** (10 mg) after semi-preparative HPLC purification. Moreover, compounds **4** (18 mg), **5**(11 mg) and **9** (15 mg) were obtained from the $CHCl_3$-MeOH (40:60) eluate (2.9 g) after undertaking the similar steps of MPLC and semi-preparative HPLC separations as described above.

3.4. Acid hydrolysis and sugar analysis of compounds *1-3* and *5-7*

The acid hydrolysis and sugar analysis were carried out according to the method described previously (Gao et al., 2011). Using the method, the following sugar units in compounds **1**–**3** and **5**–**7** were identified by comparison with authentic samples: D-glucose (t_R 10.49 min), L-glucose (t_R 11.10 min), D-glucuronic acid (t_R 9.21 min), L-glucuronic acid (t_R 9.66 min), D-arabinose (t_R 8.52 min), L-arabinose (t_R 8.33 min).

3.5. Assay for testing the protective effects against myocardial cell injury induced by H_2O_2

H9c2 cells (the Cell Bank of the Chinese Academy of Sciences Shanghai, China) were seeded into 96-well flat microtiter plates at a density of 1×10^5 per well and allowed 24 h to adhere before drugs were introduced. After incubation with different concentrations of compounds **1**–**9** (12.5–200 μM) for 12 h, the cells were treated with 200 μM H_2O_2 for 24 h. (Konorev et al., 1999; Xu et al., 2005). The cells in the control groups were treated with the same volume of phosphate-buffered saline (PBS). Cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. Absorbance was read on an ELISA plate reader at 540 nm as a measure of quantity of formazan. The percentage cell viability was calculated as a ratio of optical density (OD) value of sample to the OD value of control (Shi et al., 2013). All experiments were done for three times.

3.6. DPPH radical scavenging capacity assay

1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity of compounds **1**–**9** were measured in triplicate according to a described method (Hatano et al., 1988). Briefly, a 0.5 mM of methylated DPPH solution was prepared. An aliquot (100 μL) of tested compound at final concentrations (5–200 μM) in methanol was mixed with 100 μL of methylated DPPH radical solution and incubated for 30 min in the dark. Vitamin E served as a positive control. A freshly prepared DPPH solution exhibits a deep purple color with an absorption maximum at 517 nm. The DPPH free radical scavenging rate in percent (*I*%) was calculated in the following way:

$$I\% = 100 \times \left[1 - \left(\frac{A_{sample} - A_{blank}}{A_{Control}}\right)\right]$$

$A_{Control}$ is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound, A_{blank} is the absorbance of a blank sample.

Table 4
Maximal scavenging rate of *1*–*9* against DPPH in vitro.

Compound[a]	Scavenging rate (200 μM)
1	7.06 ± 5.08
2	5.48 ± 1.26
3	9.35 ± 1.21
4	3.85 ± 2.03
5	11.03 ± 6.69
6	6.78 ± 3.45
7	4.73 ± 2.63
8	8.28 ± 2.11
9	3.52 ± 1.90
V_E	92.74 ± 1.09

[a] For **1–9**, the test concentration range from 5 to 200 μM; for V_E, the test concentration was 200 μM.

3.7. Statistical analysis

The results were expressed as the mean ± SD. Differences were evaluated with one way ANOVA. The *post hoc* test was done with student's Dunnett test. *P* Values less than 0.05 were considered statistically significant. Statistical calculations were performed using the SPSS 16.0 for Windows software package.

3.7.1. Compound *1*

white amorphous powder; $[\alpha]_D^{25}$ +17.3 (c 0.11, MeOH); UV (MeOH)λ_{max} (logε) 223 (2.76) nm; IR ν_{max} (KBr) cm^{-1} 3429, 2934, 2787, 1756, 1458, 1348, 1071; ^{1}H NMR and ^{13}C NMR, see Tables 1 and 2; HR-ESI–MS *m/z* 609.3796 ([M + Na]$^+$, calc'd. for $C_{35}H_{54}O_7Na$, 609.3767).

3.7.2. Compound *2*

white amorphous powder; $[\alpha]_D^{25}$ +14.2 (c 0.12, MeOH); UV (MeOH)λ_{max} (logε) 226 (2.78); IR ν_{max} (KBr) cm^{-1} 3425, 2961, 2707, 1742, 1435, 1325, 1060; ^{1}H NMR and ^{13}C NMR, see Tables 1 and 2; HR-ESI–MS *m/z* 627.3891 ([M-H]$^-$, calc'd. for $C_{37}H_{55}O_8$, 627.3897).

3.7.3. Compound *3*

white amorphous powder; $[\alpha]_D^{25}$ +15.0 (c 0.10, MeOH); UV (MeOH)λ_{max} (logε) 229 (2.87); IR ν_{max} (KBr) cm^{-1} 3345, 2972, 2734, 1797, 1472, 1363, 1083; ^{1}H NMR and ^{13}C NMR, see Tables 1 and

Table 3
Protective Effects of Triterpenoidal Saponins from *Ilex cornuta* on H_2O_2-induced H9c2Cell Injury.

Compound	Concentration(μM)					
	0	12.5	25	50	100	200
1	53.52 ± 7.22	41.04 ± 1.21	50.61 ± 4.31	53.58 ± 9.86	15.35 ± 0.66	16.05 ± 0.60
2	55.37 ± 3.26	58.25 ± 6.54	59.38 ± 4.20	59.46 ± 2.12	42.77 ± 4.27	12.10 ± 0.69
3	61.81 ± 1.18	53.62 ± 1.42	53.51 ± 0.14	43.60 ± 1.34	25.27 ± 0.14	26.22 ± 0.55
4	61.81 ± 1.18	61.94 ± 1.24	65.37 ± 1.24	71.75 ± 2.22*	76.76 ± 2.79**	82.00 ± 2.10**
5	63.86 ± 1.67	59.02 ± 1.69	61.73 ± 0.70	64.09 ± 0.43	66.11 ± 1.26	72.06 ± 0.81
6	55.37 ± 3.26	49.25 ± 3.45	53.08 ± 0.77	52.62 ± 0.77	11.06 ± 0.54	11.28 ± 0.11
7	55.37 ± 3.26	37.10 ± 2.73	36.06 ± 2.73	38.83 ± 5.40	37.36 ± 5.64	41.08 ± 4.65
8	53.52 ± 7.22	44.00 ± 0.88	47.23 ± 2.99	50.44 ± 2.99	57.30 ± 6.17	49.42 ± 7.34
9	56.68 ± 0.77	49.20 ± 2.71	51.74 ± 2.71	51.21 ± 4.52	51.43 ± 4.12	48.12 ± 2.63
Vitamin E[a]	62.56 ± 1.14	64.99 ± 2.50	68.28 ± 3.24	70.27 ± 1.86	75.27 ± 1.09*	82.49 ± 0.96**

2; HR-ESI–MS *m/z* 679.3643 ([M + Cl]$^-$, calc'd. for $C_{37}H_{56}O_9Cl$, 679.3612).

3.7.4. Compound **4**

white amorphous powder; $[\alpha]_D^{25}$ +23.4 (c 0.12, MeOH); UV (MeOH)λ_{max} (logε) 225 (2.78); IR ν_{max} (KBr) cm^{-1} 3398, 2892, 2714, 1674, 1463, 1327, 1052; ^{1}H NMR and ^{13}C NMR, see Tables 1 and 2; HR-ESI–MS *m/z* 493.3318 ([M + Na]$^+$, calc'd. for $C_{30}H_{46}O_4Na$, 493.3294).

3.7.5. Compound **5**

white amorphous powder; $[\alpha]_D^{25}$ +16.7 (c 0.15, MeOH); UV (MeOH)λ_{max} (logε) 226 (2.79); IR ν_{max} (KBr) cm^{-1} 3413, 2969, 2760, 1772, 1446, 1369, 1049; ^{1}H NMR and ^{13}C NMR, see Tables 1 and 2; HR-ESI–MS *m/z* 647.3801 ([M + HCOOH-H]$^-$, calc'd. for 647.3795).

3.7.6. Compound **6**

white amorphous powder; $[\alpha]_D^{25}$ +15.2 (c 0.12, MeOH); UV (MeOH)λ_{max} (logε) 224 (2.77); IR ν_{max} (KBr) cm^{-1} 3312, 2852, 2709, 1789, 1415, 1374, 1100; ^{1}H NMR and ^{13}C NMR, see Tables 1 and 2; HR-ESI–MS *m/z* 645.3694 ([M-H]$^-$, calc'd. for $C_{37}H_{55}O_{10}$, 645.3639).

3.7.7. Compound **7**

white amorphous powder; $[\alpha]_D^{25}$ +11.8 (c 0.13, MeOH); UV (MeOH)λ_{max} (logε) 223 (2.77); IR ν_{max} (KBr) cm^{-1} 3421, 2961, 2767, 1790, 1402, 1358, 1070; ^{1}H NMR and ^{13}C NMR, see Tables 1 and 2; HR-ESI–MS *m/z* 683.3771 ([M + Na]$^+$, calc'd. for $C_{37}H_{56}O_{10}Na$, 683.3771).

Acknowledgement

We sincerely thank Dr. Jon Parcher at the University of Mississippi for helpful discussions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2015.10.010.

References

Ahai, R.K., 1985. Iconographia Cormophytorum Sinicoru. Beijing Sci. 641.
Chau, C.F., Wu, S.H., 2006. The development of regulations of Chinese herbal medicines for both medicinal and food uses. Trends. Food. Sci. Tech. 17, 313–323.
Jiangsu Medical College, 1975. Dictionary of Chinese Medicine. Shanghai People's Press, Shanghai, pp. 441–443.
Jiangsu New Medical College, 1986. Dictionary of Chinese Herbal medicines. Shanghai Science and Technology Press, Shanghai, pp. 441–443.
Thuong, P.T., Su, N.D., Ngoc, T.M., Hung, T.M., Dang, N.H., Thuan, N.D., Oh, W.K., 2009. Antioxidant activity and principle constituents of Vietnam bitter tea *Ilex kudingcha*. Food. Chem. 113, 139–145.
Qin, W.J., Zhao, J.J., Fukuyama, Y., 1988. Chemical constituent of the leaves of *Ilex corunta*. Chin. Tradit. Herbal. Drugs 19, 434–439.
Puangpraphant, S., De Mejia, E.G., 2009. Saponins in yerba mate tea (*Ilex paraguariensis* A. St. -Hil) and quercetin synergistically inhibit iNOS and COX-2 in lipopolysaccharide-induced macrophages through NFκB pathways. J. Agric. Food Chem. 57, 8873–8883.
Wang, W.L., Zhou, X., Liu, Y.L., Xu, Q.M., Li, X.R., Yang, S.L., 2014. Two new 20α (H)-ursane-type triterpenoids from *Ilex cornuta* and their cytotoxic activities. J. Asian. Nat. Prod. Res. 16, 175–180.
Liao, L., Zhou, X., Liu, Y.L., Xu, Q.M., Li, X.R., Yang, S.L., 2013. Four new triterpenoidal saponins from *Ilex cornuta* and their cytotoxic activities. Phytochem. Lett. 6, 429–434.
Li, Y., Wu, T., Chen, Z.H., Wang, Z.T., 2006. New triterpene saponins and flavonol glycosides from the leaves of *Ilex cornuta*. Chin. J. Chem. 24, 577–579.
Li, S.S., Zhao, J.P., Liu, Y.L., Chen, Z., Xu, Q.M., Khan, I., Yang, S.L., 2014. New Triterpenoid saponins from *Ilex cornuta* and their protective effects against H_2O_2-induced myocardial cell injury. J. Agric. Food Chem. 62, 488–496.
Ali, M., Srivastava, T.N., 1990. Triterpenoids from *Symplocos racemosa* bark. Phytochemistry 29, 3601–3604.
Che, Y.Y., Zhang, L., Li, N., Zeng, K.W., Tu, P.F., 2012. Triterpenoid saponins from *Ilex mamillata*. Nat. Prod. Res. 26, 1991–1995.
Hidaka, K., Ito, M., Matsuda, Y., Kohda, H., Yamasaki, K., Yamahara, J., Chisaka, T., Kawakami, Y., Sato, T., Kagei, K., 1987. New triterpene saponins from *Ilex pubescens*. Chem. Pharm. Bull. 35, 524–529.
Fearon, I.M., Faux, S.P., 2009. Oxidative stress and cardiovascular disease: novel tools give (free) radical insight. J. Mol. Cell. Cardiol. 47, 372–381.
Yousuf, S., Atif, F., Ahmad, M., Hoda, N., Ishrat, T., Khan, B., Islam, F., 2009. Resveratrol exerts its neuroprotective effect by modulating mitochondrial dysfunctions and associated cell death during cerebral ischemia. Brain. Res. 1250, 242–253.
Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M., Telser, J., 2007. Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. Cell. B. 39, 44–84.
Hescheler, J., Meyer, R., Plant, S., Krautwurst, D., Rosenthal, W., Schultz, G., 1991. Morphological, biochemical, and electrophysiological characterization of a clonal cell (H9c2) line from rat heart. Circ. Res. 69, 1476–1486.
Yasuoka, C., Ihara, Y., Ikeda, S., Miyahara, Y., Kondo, T., Kohno, S., 2004. Antiapoptotic activity of Akt is downregulated by Ca^{2+} in myocardiac H9c2 cells: Evidence of Ca^{2+}-dependent regulation of protein phosphatase 2Ac. J. Biol. Chem. 279, 51182–51192.
Liu, L.X., Sun, Y., Laura, T., Liang, X.F., Ye, H., Zeng, X.X., 2009. Determination of polyphenolic content and antioxidant activity of kudingcha made from *Ilex kudingcha*. Food Chem. 112, 35–41.
Yamamoto, M., Shima, T., Uozumi, T., Sogabe, T., Yamada, K., Kawasaki, T., 1983. A possible role of lipid peroxidation in cellular damages caused by cerebral ischemia and the protective effect of alpha tocopherol administration. Stroke 14, 977–982.
Fujimoto, S., Mizoi, K., Yoshimoto, T., Suzuki, I., 1984. The protective effect of vitamin E on cerebral ischemia. Surg. Neurol. 22, 449–454.
Martin, A., Zulueta, J., Hassoun, P., Blumberg, J.B., Meydani, M., 1996. Effect of vitamin E on hydrogen peroxide production by human vascular endothelial cells after hypoxia/reoxygenation. Free. Radical. Bio. Med. 20, 99–105.
Liu, Q., Li, J., Wang, J., Li, J., Janicki, J.S., Fan, D., 2013. Effects and mechanisms of chinese herbal medicine in ameliorating myocardial ischemia-reperfusion injury. Evid-Based Compl. Alt. 2013, 925625–925639.
Shi, Y.N., Jennifer, K.N., Jiang, B., Rong, T., Yoshinori, M., 2014. Antioxidant activity of enzymatic hydrolysates from eggshell membrane proteins and its protective capacity in human intestinal epithelial Caco-2 cells. J. Funct. Foods 10, 35–45.
Liang, Q.M., Yu, X.F., Qu, S.C., Xu, H.L., Sui, D.Y., 2010. Acanthopanax senticosides B ameliorates oxidative damage induced by hydrogen peroxide in cultured neonatal rat cardiomyocytes. Eur. J. Pharmacol. 627, 209–215.
Luo, H., Huang, J., Liao, W.G., Huang, Q.Y., Gao, Y.Q., 2011. The antioxidant effects of garlic saponins protect PC12 cells from hypoxia-induced damage. Brit. J. Nutr. 105, 1164–1172.
Gao, L., Zhang, L., Liu, J.Y., Cai, P.L., Yang, S.L., 2011. New triterpenoid saponins from Patrinia scabiosaefolia. Carbohydr. Res. 346, 2881–2885.
Konorev, E.A., Kennedy, M.C., Kalyanaraman, B., 1999. Cell-permeable superoxide dismutase and glutathione peroxidase mimetics afford superior protection against doxorubicin-induced cardiotoxicity: the role of reactive oxygen and nitrogen intermediates. Biochem. Biophys. 368, 421–428.
Xu, S.M., Wang, P.Y., Ma, H.Y., 2005. Preparation of hypoxic surroundings with sodium dihionite for cell culture. Acta. Acad. Med. Mil. Tert. 27, 359–360.
Shi, M.F., He, W.J., Liu, Y.L., Li, X.R., Yang, S.L., Xu, Q.M., 2013. Protective effect of total phenylethanoid glycosides from *Monochasma savatieri* Franch on myocardial ischemia injury. Phytomedicine 20, 1251–1255.
Hatano, T., Kagawa, H., Yasuhara, T., Okuda, T., 1988. Two new flavonoids and other constituents in licorice root: their relative astringency and radical scavenging effects. Chem. Pharm. Bull 36, 2090–2097.