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Hepatoprotective glycosides from *Leonurus japonicus* Houtt.

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ABSTRACT

Two new phenylethanoid glycosides **1** and **2** named leonoside E and leonoside F, and one new sesquiterpene glycoside (**3**) identified as 7α (*H*)-eudesmane-4,11 (12)-diene-3-one-2β-hydroxy-13-β-D-glucopyranoside, together with seven known glycosides (**4**–**10**), were isolated from the aerial part of *Leonurus japonicus* Houtt. Their structures were elucidated on the basis of spectroscopic data and chemical evidence. When tested in in vitro assays, compounds **1**, **2**, **4**, and **6** exhibited potent hepatoprotective activity against D-galactosamine-induced toxicity in HL-7702 cells at concentration of 1×10^{-5} M.

1. Introduction

The genus *Leonurus* (labiatae) comprises more than 20 species widespread in Eurasia, from Western Europe to China, and is used as a traditional Chinese Medicine for regulating menstrual disturbance, invigorating blood circulation, diuretics, and dispel edema.[1] Previous investigations of this genus revealed the presence of labdane-type diterpenoids,[2,3] phenylethanoid glycosides,[4,5] iridoids,[5] flavonoids,[6] and cyclic peptides,[7] some of them have shown to exhibit anti-inflammatory,[5] anti-platelet aggregation,[8] and cytotoxic activities.[9] To systematically search the phytochemistry and bioactivity of the high-polarity fraction of the plants of the genus *Leonurus*, we investigated *Leonurus japonicus* Houtt., a species growing Hebei Province of the People's Republic of China.[10] Two new phenylethanoid glycosides, namely leonoside E and leonoside F, and one new sesquiterpene glycoside identified as 7α (*H*)-eudesmane-4,11 (12)-diene-3-one-2β-hydroxy-13-β-D-glucopyranoside, were isolated, along with seven known compounds. The hepatoprotective activities evaluation of the isolated compounds has been investigated.

2. Results and discussion

The plant extract was subjected to HP-20 macroporous resin using a H_2O–EtOH step gradient to give six fractions. The 15% and 30% fractions were further subjected to column chromatography and preparative HPLC to yield ten compounds (Fig. 1). The new compounds mentioned above included two new phenylethanoid glycosides, and one new sesquiterpene glycoside. The known compounds were identified as verbascoside (**4**),[11] 2-(3,4-dihydroxyphenethy)-*O*-α-arabinopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→3)-6-*O*-β-D-glucopyranoside (**5**),[12] cistanoside E (**6**),[13] lavandulifolioside (**7**),[4] staphylionoside E (**8**),[14] citroside A (**9**),[15] 9-hydroxy-megastigma-4,7-dien-3-one-9-*O*-glucopyranoside (**10**),[16] respectively.

Compound **1** was obtained as a white powder, its molecular formula, $C_{26}H_{40}O_{16}$, was established on the basis of HRESIMS at *m*/*z* 631.2209 $[M+Na]^+$ (calcd for 631.2214). Its IR spectra showed absorption bands for hydroxyl groups (3372 cm^{-1}), $-CH_2-$ (2932 cm^{-1}), aromatic rings (1592, 1513, and 1440 cm^{-1}). The ^1H NMR spectrum (Table 1) of **1** exhibited characteristic signals belonging to 3-methoxyl-4-hydroxyphenylethanol moieties: one set of ABX system [δ 6.68 (1H, d, *J* = 2.0 Hz, H-2), 6.81 (1H, d, *J* = 8.0 Hz, H-5), and 6.63 (1H, dd, *J* = 8.0, 2.0 Hz, H-6)], two diastereotopic protons at δ 3.56 (1H, dd, *J* = 11.5, 3.5 Hz) and δ 3.89 (1H, dd, *J* = 11.5, 3.0 Hz) of the side-chain of the aglycon moiety, and one methoxy group at δ 3.72 (3H, s). In addition, three signals of anomeric protons appearing as *d* at δ 4.21 (1H, *J* = 8.0 Hz), 5.25 (1H, *J* = 1.0 Hz), 4.26 (1H, *J* = 7.0 Hz), which were attributed to Glu-1′, Rha-1″ and Ara-1‴, respectively. This monosaccharide analysis was confirmed by acid hydrolysis, which afforded glucose, rhamnose, and arabinose, identified by TLC. The ^{13}C NMR spectrum (Table 1) of **1** showed twenty-six carbon signals including nine carbons that could be assigned to the aglycon moiety, and seventeen for three monosaccharide groups. An unambiguous determination of the sequence and linkage sites was obtained from the HMBC correlations (Fig. 2), in which the correlations of H-7 (δ 2.70) with C-2 (δ 116.2), C-6 (δ 119.3), of H-1′ (δ 4.21) with C-8 (δ 69.8), of H-1″ (δ 5.25) with C-3′ (δ 81.6), and of H-1‴ (δ 4.26) with C-2″ (δ 80.8)

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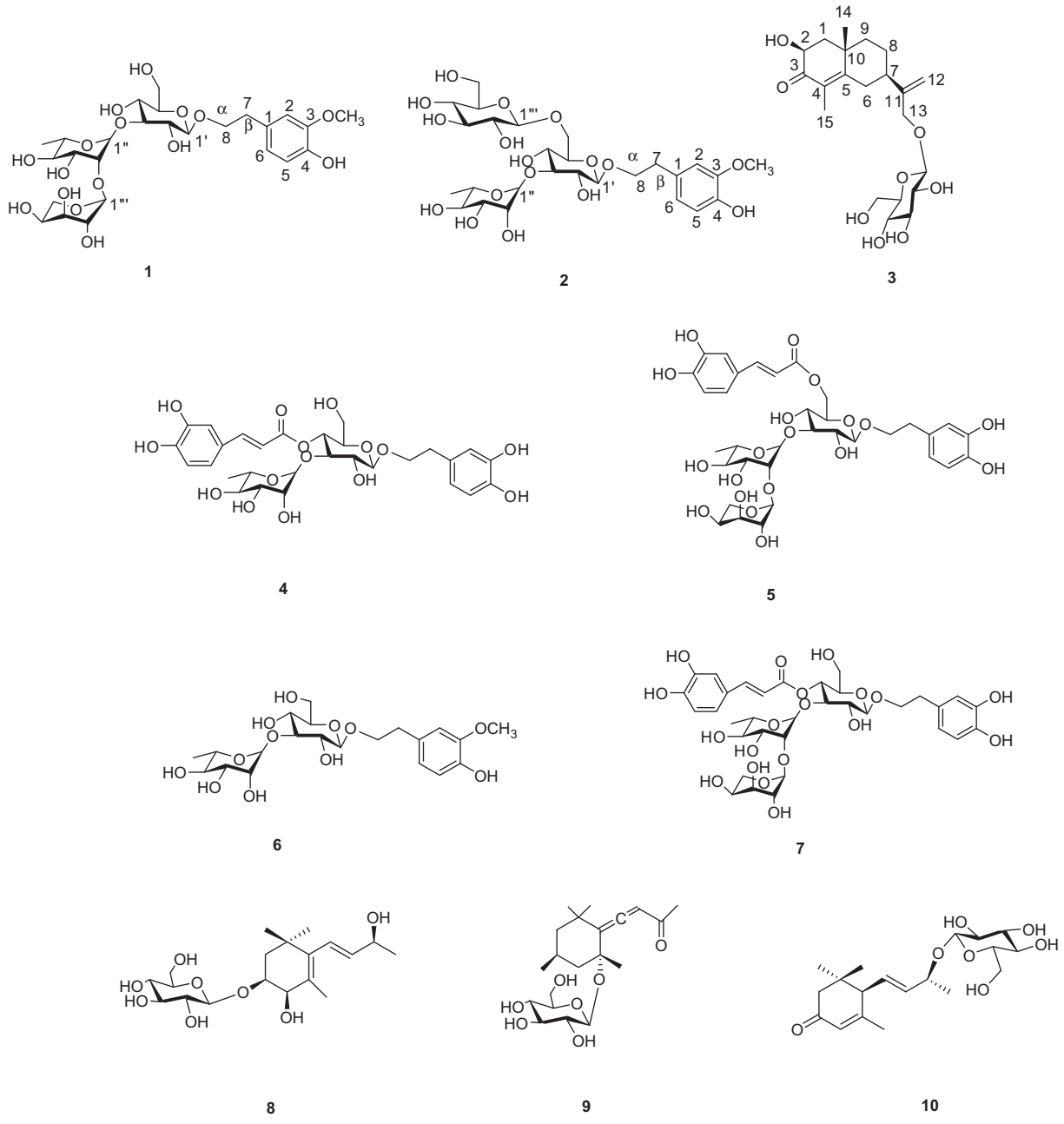

Figure 1. The structures of **1–10**.

were observed. On the basis of above evidence and related literature,[17] the structure of **1** was deduced to 2-(3-methoxyl-4-hydroxyphenyl)-ethanol-*O*-α-L-arabinopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→3)-β-D-glucopyranoside (Fig. 1), and named leonoside E.

Compound **2** was obtained as a white powder. The molecular formula was $C_{27}H_{42}O_{17}$, as indicated by HRESIMS. The IR spectra similarly indicated hydroxyl, -CH_2-, and aromatic groups. The ^{13}C NMR and ^{1}H NMR data (Table 1) were closely similar to those of **1**, except for a α-L-arabinopyranosyl unit in **1** was replaced by a β-D-glucopyranoside group, which was further confirmed based on the observation that acid hydrolysis of **2** gave only glucose and rhamnose, identified by TLC. Furthermore, the location of latter one was confirmed at C-6′ of inner glycosyl unit by +7.6 ppm shift of C-6′ observed from ^{13}C NMR spectrum and the HMBC correlation from H-1‴ (δ 4.23) to C-6′ (δ 68.5). In addition, in an HMBC experiment, a correlation between H-1″ (δ 5.02) and C-3′ (δ 81.7) was observed (Fig. 2). On the basis of above evidence and related literature,[18] the structure of compound **2** was determined as 2-(3-methoxyl-4-hydroxyphenyl) ethanol-*O*-α-L-rhamnopyranosyl-(1→3) [β-D-glucopyranosyl-(1→6)]-β-D-glucopyranoside, and named leonoside F (Fig. 1).

Compound **3** was isolated as colorless oil, the molecular formula $C_{21}H_{32}O_8$ of **3** was determined on the basis of the HRESIMS at *m*/*z* 435.2002 $[M+Na]^+$ (calcd for 435.1989). The IR showed signals for hydroxyl groups (3380 cm^{-1}) and a terminal double bond (1665 cm^{-1}). In the ^{1}H NMR spectrum of **3** (Table 1), two methyl groups including a vinyl methyl signal at δ 1.26, five methylene signals including a O-bearing methylene group at δ 4.29/4.08, two methine signals, one pair of olefinic proton signals at δ 5.13/5.02, and an anomeric proton at δ 4.15 (1H, d, J = 7.5) indicating a β-linkage, were observed. The ^{13}C NMR spectrum of **3** (Table 1)

Table 1
^{1}H NMR (500 Hz) and ^{13}C NMR (125 Hz) data of compounds **1–3** (in DMSO-d_6)[a]

No.	Compound **1**		Compound **2**		No.	Compound **3**	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C		δ_H (J in Hz)	δ_C
1		131.1		131.6	1	1.94dd (13.0, 5.5)	46.5
2	6.68d (2.0)	116.2	6.68d (2.0)	116.7		1.59t (13.0)	
3		146.2		146.6	2	4.19dt (13.0, 5.0)	68.5
4		146.2		146.4	3		200.4
5	6.81d (8.0)	112.2	6.81d (8.0)	112.8	4		125.8
6	6.63dd (8.0, 2.0)	119.3	6.64dd (8.0, 2.0)	119.8	5		162.1
7	2.70 br s	35.4	2.71td (6.5, 3.0)	35.4	6	2.68d (11.5)	33.1
8	3.56m	69.8	3.61m	71.0		2.12overlap	
	3.89m		3.87m		7	2.14 br s	41.7
–OMe	3.72s	55.8	3.73s	56.1	8	1.66m	27.3
	Glc		Glc (inner)		9	1.66m	42.7
1′	4.21d (8.0)	103.3	4.21d (7.0)	102.9		1.38m	
2′	3.09dd (8.0, 2.5)	73.9	3.09m	74.5	10		37.4
3′	3.36t (5.0)	81.6	3.38m	81.7	11		149.7
4′	3.87 br s	68.4	3.19 br s	68.8	12	5.13d (1.5)	111.5
5′	3.15	76.7	3.19 br s	76.0		5.02d (1.5)	
6′	3.46dd (11.5, 5.0)	60.9	3.60m	68.5	13	4.29d (13.0)	70.4
	3.65m		3.97d (11.0)			4.08d (13.0)	
	Rha		Rha		14	1.71s	23.0
1″	5.25d (1.0)	99.2	5.02d (1.0)	101.1	15	1.26s	11.3
2″	3.77dd (2.0, 3.0)	80.8	3.50m	71.0		Glc	
3″	3.57m	70.5	3.17m	70.5	1′	4.15d (7.5)	102.6
4″	3.16m	72.5	3.16m	72.5	2′	2.99m	74.2
5″	3.60m	67.8	3.91m	68.8	3′	3.13m	77.3
6″	1.09d (6.0)	17.7	1.10d (6.0)	18.3	4′	3.06m	70.7
	Ara		Glc (terminal)		5′	3.06m	77.6
1‴	4.26d (7.0)	105.7	4.23d (8.0)	103.8	6′	3.44dd (12.0, 5.0)	61.8
2‴	3.17m	71.1	2.97dd (6.5, 5.5)	74.0		3.68m	
3‴	3.18m	72.6	3.15m	77.1			
4‴	3.66m	67.6	3.09m	70.3			
5‴	3.60m	65.5	3.10m	77.3			
	3.39m						
6‴			3.44dd (11.5, 5.0)	61.5			
			3.66m				

[a] NMR data (δ) were measured in DMSO-d_6 at 500 MHz for ^{1}H and 125 MHz for ^{13}C. Coupling constants (J) in Hz are given in parentheses. The assignments are based on HMBC experiments.

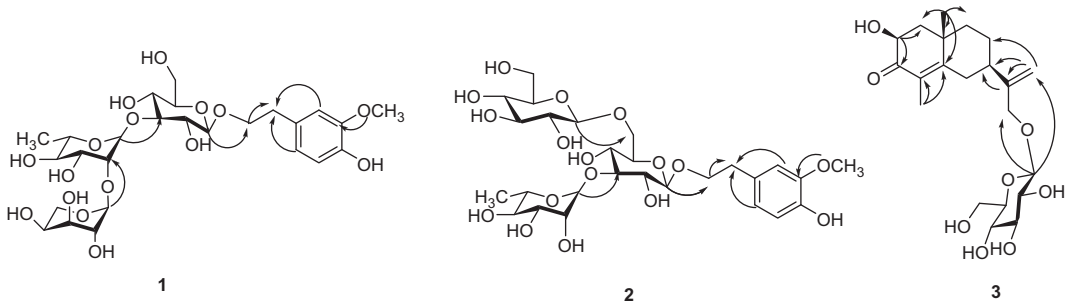

Figure 2. Key HMBC correlations in compounds **1–3**.

exhibited twenty-one carbon resonances, six of which were assigned to a glucopyranosyl moiety, fifteen for the sesquiterpene aglycon moiety. A ketone carbonyl (δ 200.4), two pairs of olefinic signals with one cyclic and one exo-cyclic double bond at δ 125.8 (162.1) and 149.7 (111.5), which were assigned C-4 (5), and C-11 (12) respectively, and a quaternary carbon signal at δ 37.4, were observed in the ^{13}C NMR spectrum of **3**. Thus, these data above allowed the skeleton of **3** to be deduced as an eudesmanolide-type sesquiterpene[19] derivative. Furthermore, an HMBC experiment showed ^{1}H/^{13}C correlations between C-3 and H-2, H-1, and between Glc-1′ and H-13. The relative stereochemistry of **3** was determined by ROESY experiment, in which the correlation of H_α-C (9) with H_α-C (1), and H-C (7), of 14-Me with H_β-C (9) and H_β-C (6), were observed. Because the 14-Me of eudesmanolide-type sesquiterpene is defined in the β-configuration, H-C (7) was deduced to be α-oriented, which was further supported by the fact that correlation between H-C (7) and H_α-C (6), the latter has no correlation with 14-Me. Additionally, a strong correlation between H-C (2) and H_α-C (1) suggested the H-C (2) has the α-configuration. As a result, the structure of compound **3** can be concluded to be 7α (*H*)-eudesmane-4,11 (12)-diene-3-one-2β-hydroxy-13-β-D-glucopyranoside (Fig. 2).

The hepatoprotective activities for compounds **1–10** were evaluated against D-galactosamine-induced toxicity in HL-7702 cells. Compounds **1**, **2**, **4**, and **6** displayed a promising hepatoprotective activity at 1×10^{-5} M in vitro by comparison of the cell survival rate and inhibitory rate with those of positive control substance bicyclol as shown in Table 2.

Table 2
Hepatoprotective effects of compounds **1**, **2**, **4** and **6** against D-galactosamine-induced toxicity in HL-7702 cells[a]

Compounds	Cell survival (%)	Inhibition (%)
Normal	100	
Control	55***	
Bicyclol[b]	66**	25.3
Compound **1***	68**	30.0
Compound **2***	69**	30.9
Compound **4**	66*	24.4
Compound **6**	69**	31.7

[a] Results are expressed as mean ± SD (n = 3; for normal and control, n = 6).
[b] Positive control substance.
* p <0.05.
** p <0.01.
*** p <0.001.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a Jasco P-2000 polarimeter. The UV spectra were scanned by a Jasco V650 spectrophotometer. IR spectra were recorded on an IMPACT 400 (KBr) spectrometer. ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), and 2D NMR spectra were run on an INOVA-500 spectrometer and values were given in ppm. HRESIMS spectra were performed on a Finnigan LTQ FT mass spectrometer. The ESI mass spectra were recorded by an Agilent 1100 series LC/MSD TOF from Agilent Technologies. Column chromatography was performed with Macroporous resin (Diaion HP-20, Mitsubishi Chemical Corp. Tokyo, Japan), Rp-18 (50 μm; YMC, Kyoto, Japan), and Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden). Preparative HPLC was carried out on a Shimadzu LC-6AD instrument with a SPD-20A detector, using a YMC-Pack ODS-A column (250 × 20 mm, 5 μm). TLC was carried out with glass precoated silica gel GF254 plates. Spots were visualized under UV light or by spraying with 10% sulfuric acid in EtOH followed by heating.

3.2. Plant material

The dried aerial part of *Leonurus japonicus* Houtt. was purchased from Tongrentong Pharmacy, and identified by Professor Lin Ma of the Chinese Academy of Medical Sciences. A voucher specimen (NO. ID-S-2387) was deposited at the Institute of Materia Medica, Chinese Academy of Medical Sciences.

3.3. Extraction and isolation

The dried aerial parts of *Leonurus japonicus* Houtt. (23 kg) were extracted with 30% EtOH under reflux for 2 × 2 h. After evaporation of EtOH in vacuo, the concentrated extract (3020 g) was suspended in H_2O (1000 mL), then 20 L of 95% EtOH were added. The resulting precipitate was removed and the supernatant solution was concentrated to give residue (2000 g). The residue was subjected to column chromatography over macroporous resin two times, eluting successively with H_2O, 15% EtOH, 30% EtOH, 50% EtOH, 70% EtOH, and 95% EtOH (20 L each). After removing the solvents, 15% EtOH fraction (62 g) was subjected to chromatography over ODS eluting with H_2O–MeOH mixtures of increasing ratio of MeOH gave five fractions ($B_{1\text{-}5}$) on the basis of HPLC-DAD analysis. Fraction B_2 (28 g) was further separated by Sephadex LH-20 column chromatography with 15%MeOH/H_2O as the mobile phase to yield four fractions ($B_{2\text{-}1}$–$B_{2\text{-}4}$). Subfractions $B_{2\text{-}3}$ (10 g) were further purified by reversed-phase preparative HPLC, using different MeOH–H_2O (32:68, 32:68, 34:66, and 33:67) as the mobile phase, respectively, to yield compound **1** (10 mg), **6** (15 mg), **8** (15 mg), **9** (12 mg). The 30% EtOH fraction (99 g) was subjected to chromatography over Sephadex LH-20 column using a H_2O–MeOH step gradient to give 10 fractions (C_1–C_{10}), fraction C_2 (40 g) was repeatedly applied to chromatography over Sephadex LH-20 column and further preparative HPLC using MeOH–H_2O (40:60) to afford compound **3** (10 mg) and **10** (30 mg). Fraction C_4 (0.8 g) was further purified by reversed-phase preparative HPLC with MeOH–H_2O (30:70) to give compound **2** (12 mg). Fraction C_7 was further subjected to chromatography over Sephadex LH-20 column to give 20 subfractions ($C_{7\text{-}1}$–$C_{7\text{-}20}$). Subfraction $C_{7\text{-}7}$ (10 g) was purified by reversed-phase preparative HPLC with MeOH–H_2O (30:70, 40:60, 40:60) to give compound **4** (25 mg), **5** (15 mg), and **7** (30 mg).

3.4. Characterization data

3.4.1. Leonoside E (1)

White powder; $[\alpha]_D^{20}$ −32.67 (c 0.056, MeOH); UV (MeOH) λ_{max} 218, 330 nm; IR ν_{max} 3372, 2932, 1647, 1592, 1513, 1440, 1129, 1074, 1039 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz), and ^{13}C NMR (DMSO-d_6, 125 MHz) data, see Table 1; ESIMS: m/z 631[M+Na]$^+$, 607[M−H]; (+)-HRESIMS: calcd for $C_{26}H_{40}O_{16}Na$ [M+Na]$^+$, m/z 631.2214; found m/z 631.2209.

3.4.2. Leonoside F (2)

White powder; $[\alpha]_D^{20}$ −73.2 (c 0.05, MeOH); UV (MeOH) λ_{max} 220, 330 nm; IR ν_{max} 3367, 2929, 1624, 1512, 1440, 1273, 1073, 1041 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz), and ^{13}C NMR (DMSO-d_6, 125 MHz) data, see Table 1; (+)-HRESIMS: calcd for $C_{27}H_{42}O_{17}Na$ [M+Na]$^+$, m/z 661.2314; found m/z 661.2322.

3.4.3. 7α (*H*)-Eudesmane-4,11 (12)-diene-3-one-2β-hydroxy-13-β-D-glucopyranoside (3)

Colorless oil; $[\alpha]_D^{20}$ −6.65 (c 0.124, MeOH); UV (MeOH) λ_{max} 253 nm; IR ν_{max} 3380, 2928, 2877, 1665, 1611, 1411, 1294, 1075, 1043 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz), and ^{13}C NMR (DMSO-d_6, 125 MHz) data, see Table 1; (+)-HRESIMS: calcd for $C_{21}H_{32}O_8Na$ [M+Na]$^+$, m/z 435.1989; found m/z 435.2002.

3.5. Acid hydrolysis of leonurus *D–E* (1–2)

Based on the reported procedure,[20] each (5 mg) of the compounds **1** and **2** were dissolved 0.2 N H_2SO_4 (5 mL) and heated at 100 °C for 2 h. After cooling, the reaction mixture was extracted with EtOAc. The aqueous layer was concentrated to dryness, then analyzed on silica gel TLC (EtOAc–MeOH–H_2O–AcOH 13:3:3:4) by comparison with authentic samples.

3.6. Bioassays for hepatoprotective activity

The hepatoprotective activities for compounds **1–10** were determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay in HL-7702 cells.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2011.10.034.

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