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Iridoid glycosides from *Harpagophytum procumbens* D.C. (devil's claw)

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Abstract

Iridoid glycosides, harprocumbide A (6″-*O*-α-D-galactopyranosyl harpagoside, **1**) and harprocumbide B (6″-*O*-(*cis*-*p*-coumaroyl)-procumbide, **2**) were isolated from the tubers of *Harpagophytum prucumbens* D.C., along with nine known iridoid glycosides 6-*O*-α-D-galactopyranosylharpagoside (**3**), and harpagoside (**4**), harpagide (**5**), 8-cinnamoylmyoporoside (**6**), 8-*O*-feruloylhapagide (**7**), procumbide (**8**), 6″-*O*-(*p*-coumaroyl)-procumbide (**9**), 8-*O*-(*p*-coumaroyl)-harpagide (**10**) and 8-*O*-(*cis*-*p*-coumaroyl)-harpagide (**11**). Compound **10** showed marginal inhibition activity against macrophages respiratory burst.

Keywords: *Harpagophytum procumbens* D.C.; Pedaliaceae; Iridoid glycosides; Harprocumbide A; Harprocumbide B

1. Introduction

Hapagophytum procumbens D.C. is a native plant distributed in Africa, especially in Namibia Steppes of Southern Africa and the Kalahari Desert. Its dried tubers are traditionally used as a folk medicine by natives for treatment of fever, indigestion, malaria, allergies, skin cancer, rheumatism and arthritis for centuries (Watt and BreYer-Brandwijk, 1962; Stewart and Cole, 2005). Since its introduction to Europe from Africa in the early 1900s, dried tubers of the plant have been used to restore appetite, relieve heartburn, and to reduce pain and inflammation (Costa De Pasquale et al., 1985; Grahame and Robinson, 1981; Occhiuto et al., 1985; Soulimani et al., 1994). In fact, mounting evidence has suggested that devil's claw tubers may help relieve pain and inflammation in people with arthritis and other painful disorders, although the mechanism of action (Whitehouse et al., 1983; Baghdikian et al., 1997; Lanhers et al., 1992) is not yet well understood. Several phytochemical investigations had led to isolation of some iridoid glycosides from the plant (Kikuchi et al., 1983; Boje et al., 2003; El-Naggar and Beal, 1980). A thorough investigation of the tubers of this title plant led us to the isolation of 11 iridoid glycosides of which two are new. Except for compound **6**, due to a paucity of material, all of the compounds were tested for their inhibitory activity against macrophages respiratory burst (raw 264.7 macrophages) following chemiluminescence detection (Pasmans et al., 2001). Only compound **10** showed significant inhibition activity against macrophages respiratory burst with an IC_{50} value of approximately 32 μM.

2. Results and discussion

The water suspension of the hot EtOH extract of the powdered tubers of the plant was partitioned successively against EtOAc and *n*-BuOH. The *n*-BuOH-soluble fraction

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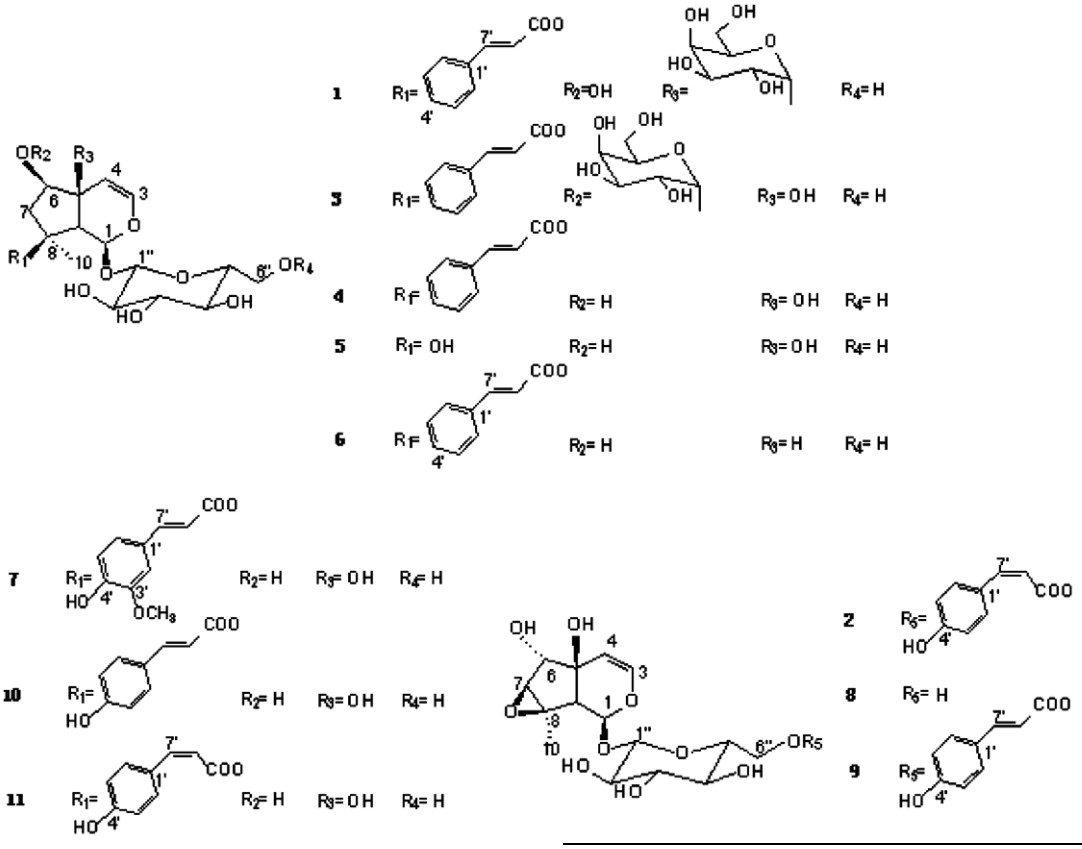
was subjected to silica gel, Sephadex LH-20, and ODS column chromatography, and yielded two new iridoid glycosides (**1** and **2**), named harprocumbide A (**1**) and B (**2**), as well as nine known iridoid glycosides (**3**–**11**). The NMR data of compounds **3**–**11** were in excellent agreement with those reported for 6-*O*-α-D-galactopyranosyl harpagoside (**3**), which is reported presently in the title plant for the first time (Li et al., 1999), harpagoside (**4**), harpagide (**5**), 8-cinnamoyl-myoporoside (**6**), 8-*O*-feruloylhapagide (**7**), procumbide (**8**), 6″-*O*-(*p*-coumaroyl)-procumbide (**9**), 8-*O*-(*p*-coumaroyl)-harpagide (**10**) and 8-*O*-(*cis*-*p*-coumaroyl)-harpagide (**11**) (El-Naggar and Beal, 1980; Kikuchi et al., 1983; Boje et al., 2003; Seger et al., 2005).



Harprocumbide A (**1**) was isolated as white amorphous powder with the molecular formula $C_{30}H_{40}O_{16}$, as established by HR-ESI-FTMS. Preliminary inspection of the ^{1}H NMR spectra of harprocumbide A (**1**) led to the identification of the following representative signals: δ 1.70 (3H, *s*), 2.10 (1H, *dd*, $J = 14.8$, 4.2 Hz), 2.60 (1H, *d*, $J = 14.8$ Hz), 3.60 (1H, *s*), 4.66 (1H, *d*, $J = 4.2$ Hz) as well as three anomeric protons δ 5.15 (1H, *d*, $J = 6.4$ Hz), 6.47 (1H, *d*, $J = 6.4$ Hz) and 6.86 (1H, *s*). In addition, seven proton signals due to a cinnamoyl group were also observed. The ^{13}C NMR spectra displayed 28 signals, of which 13 resonated in the region 60–90 ppm, seven carbon resonances for a cinnamoyl group, and two methine signals at δ 99.2 and 101.6 suggested the presence of two anomeric carbons of two hexose residues. All the information mentioned above supports harprocumbide A (**1**) be an iridoid diglycoside.

Acid hydrolysis of **1** with 1 M HCl produced two monosaccharides. The monosaccharides were determined to be D-glucose and D-galactose by optical rotation and high performance TLC analysis comparison with authentic samples. Complete ^{1}H and ^{13}C NMR spectroscopic assignments of (**1**) (Table 1) were made by HSQC combined with HMBC spectra. The NMR data of the aglycone of **1** were found to be similar to those of the aglycone of a known iridoid derivative, harpagoside (**4**), which was also isolated from the plant, including the same glycosidic position at C-1. That is to say two monosaccharides were attached to C-1 to form a monodesmosidic glycoside. The only diagnostic difference between **1** and **4** is the carbon signal at C-6 of the glucose, which is about 6 ppm downfield from those of **4** due to the known effects of *O*-glycosylation, indicating that **1** contained a terminal α-D-galactopyranosyl and a C-6 substituted β-D-glucopyranosyl group. The anomeric proton δ 5.54 (1H, *d*, $J = 2.4$ Hz, H-1‴) of the galactose giving a three-bond correlation with the C-6″ (δ 69.1) in the HMBC spectra confirmed the position of the glycosidic linkage (see Fig. 1) Finally, the structure of harprocumbide A (**1**) was elucidated as 6″-*O*-α-D-galactopyranosylharpagoside.

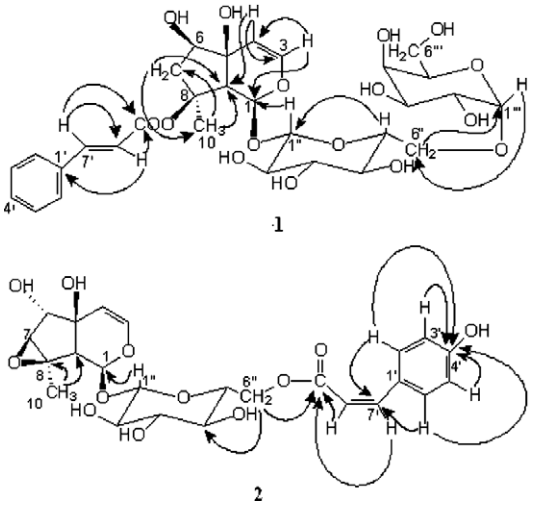


Fig. 1. Selected HMBC correlation (H → C) for harprocubide (A) and (B).

Harprocumbide B (**2**) and compound **9** (6″-*O*-(*p*-coumaroyl)-pro-cumbide) were obtained as an inseparable mixture, which could hardly be separated by various solvent systems and reverse phased materials. However, the ESI-MS spectrometry showed two near peaks had the same $[M-H]^-$ ion, thus indicating a pair of isomers. This was clarified by the proton and carbon signals in pairs observed in the ^1H and ^{13}C NMR spectra of the mixture (the ratio of **2** and **9** was estimated as *ca*. 3: one based on the intensity of ^{13}C NMR signals). Acid hydrolysis of the mixture afforded D-glucose as the sole sugar. The ^{13}C NMR data for the sugar moieties of the mixture of **2** and **9** were superimposed and most of the signals for the aglycone were coincidental except for the observed difference in the double bond (C-7′ and C-8′) due to the *cis*- or *trans*-isomers. Although the previous study did not address the geometry of the double bond, the proton signals of the *p*-coumaroyl moiety [δ 6.57 and 7.89 (1H each, *d*, $J = 16.0$ Hz)] of **9** reported by Kikuchi et al. (1983), indicated the *trans* geometry. So, except for the similar signals observed in **9** (Kikuchi et al., 1983), two additional signals obviously arising from a *cis*-*p*-coumaroyl moiety [δ 5.92 and 6.79 (1H each, *d*, $J = 12.8$ Hz)] (for olefinic bond), and δ 7.12 and 8.02 (2H each, *d*, $J = 8.4$ Hz) (for 1,4-disubstituted benzene ring) (Takeda et al., 1987), were observed in **2**. Correlation signals based on the combined detailed analysis of the HSQC and HMBC spectra provided good support for the structures of **2** and **9**. The cross peak between C-9′ (δ 166.8) and H-6″ δ 4.83 (1H, *dd*, $J = 12.0$, 5.2 Hz) in the HMBC spectra showed the *cis*-*p*-coumaroyl group attached at C-6″ just like in **9** (see Fig. 1). Thus, based on the above evidence, the structure of harprocumbide B (**2**) was elucidated as 6″-*O*-(*cis*-*p*-coumaroyl)-procumbide, which is the *cis*-isomer of compound **9** (Zhang and Cheng, 2000).

Compound **11** was decided by LC-DAD-MS/SPE-NMR technique by Seger et al. (2005), which is the *cis*-isomer of compound **10** (Kikuchi et al., 1983). By preparative HPLC, we obtained the compound and measured its physicochemical data.

Table 1
NMR spectroscopic data for harprocumbide A (**1**) and **4** (C_5D_5N, 400 MHz) (δ in ppm, *J* in Hz)

Atom	Harprocumbide A (**1**)			**4**
	HSQC (δ_H)	δ_C	HMBC correlations	δ_C
Aglycone				
1	6.86 (*s*)	95.0	3	94.8
3	6.47 (*d*, 6.4)	143.0	1,4	142.5
4	5.15 (*d*, 6.4)	109.1	3,9	108.1
5	–	74.4		73.4
6	4.66 (*d*, 4.2)	77.6		77.0
7	2.10 (*dd*, 14.8, 4.2)	47.0	10	46.1
	2.60 (*d*, 14.8)		9	
8	–	88.3		87.6
9	3.60 (*s*)	55.9	1,5,8,1″	55.3
10	1.70 (3H, *s*)	23.7	7,9	22.8
Cinnamoyl				
1′	–	136.0		135.0
2′,6′	7.50 (2H, *m*)	130.0	3′,4′,5′,7′	129.3
3′,5′	7.25 (2H, *m*)	129.5	2′,6′	128.6
4′	7.25 (*m*)	131.2	2′,6′	130.5
7′	7.82 (*d*, 16.0)	145.3	2′,4′,6′,8′,9′	144.5
8′	6.53 (*d*, 16.0)	121.1	1′,9′	120.3
9′	–	167.6		166.9
Glucose				
1″	5.26 (*d*, 8.0)	99.2	1	99.3
2″	3.99 (*t*, 8.4)	75.6		74.9
3″	4.17 (*t*, 8.4)	77.8		78.8
4″	4.08 (*m*)	72.1		71.7
5″	4.05 (*d*, 6.4)	79.5	1″,6″	78.5
6″	4.52 (*dd*, 10.0, 4.8)	69.1		62.9
	4.32 (*d*, 10.0)		1‴	
Galactose				
1‴	5.54 (*d*, 2.4)	101.6	6″	
2‴	4.57 (*m*)	71.7		
3‴	4.65 (*d*, 10.0)	73.1		
4‴	4.67 (*m*)	72.6		
5‴	4.75 (*t*, 6.0)	73.6	1‴	
6‴	4.47 (2H, *d*, 5.8)	63.8		

Signals were assigned using ^1H–^1H COSY and HSQC.

The iridoid glycosides have been considered as the active constituents in devil's claw for long time, but their anti-inflammatory mechanism remains unclear. Macrophages respiratory burst plays an important role in specific and nonspecific immune-inflammatory processes, which links to many inflammatory mediators such as cytokines, chemokines, nitric oxide synthase, phospholipases and free radical generation. And inhibition of macrophages respiratory burst has been one of the well-documented methods for the evaluation of anti-inflammatory activity for various synthetic compounds and natural products (Cho et al., 2000; Pasmans et al., 2001). In the present study, we chose macrophages respiratory burst assay as a model to evaluate the compounds isolated from *Harpagophytum procumbens* D.C. for their activity. All isolated compounds except compound **6** were tested for their inhibitory activity against macrophages respiratory burst (raw 264.7 macrophage cell line) with chemiluminescence detection. As shown in Table 4, only a known compound **10** showed considerable activ-

ity with IC_{50} value of 32.4 μM. And the others compounds showed inhibitory effect with higher IC_{50}. The bioassay data were showed in Table 4.

3. Experimental

3.1. General

Optical rotations were measured on a JASCO P-1020 polarimeter. ^1H and ^{13}C NMR spectra were recorded at 300 MHz and 400 MHz on Brucker AV-300, 400 spectrometer. Standard pulse sequences and parameters were used for the experiments. Chemical shift references were obtained from the solvent resonances of C_5D_5N at ^1H 8.60 and ^{13}C 149.8 and CD_3OD at ^1H 3.30 and ^{13}C 50.4, relative to TMS. The ESIMS data were obtained from an Agilent 1100 LC API MSD instrument. HR-ESI-FTMS was measured on Bruker Daltonics, APEX III Instrument. Column chromatography was carried out with silica gel (200–300 mesh, Qingdao Marine Chemistry Company, People's Republic of China). LiChroprep Rp-18 (60–80 mesh, BÜCHI) and Sephadex LH-20 (Pharma-cia, Sweden). Preparative HPLC was performed using a Zorbax ODS column (200 × 21.2 mm, i.d.; 7 μm) coupled to an Agilent Prep-1100 pump with flow rate of the mobile phase [MeOH–H_2O (4.5:5.5)] set at 20 mL/min; the column temperature set at 30 °C; and the Agilent 1100 multiple wavelength detector set at 310 nm. The chemiluminescence value was recorded by BPCL-1-G-C Ultra-weak Luminescence Analyzer (Beijing Institutes for Biophysics, Chinese Academy of Science). The murine macrophage-like cell line RAW 264.7 was obtained from the Institute of Biochemistry and cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Science. RPMI-1640, Phorbol 12-myristate 13-acetate (PMA) and fetal calf serum (FCS) were obtained from GIBCO (USA), Promega (USA) and Hyclone (USA), respectively.

3.2. Plant material

Tubers of *Harpagophytum procumbens* (Burch.) D.C. ex Meissner. Subsp-procumbens were collected from Namibia Steppes of Southern Africa in April 2002 and authenticated by Dr. Samuel X. Qiu (Chemistry Department, Washington University). A voucher specimen (Q2002-D-3209) was deposited in Department of Complex Prescription of TCM, China Pharmaceutical University.

3.3. Extraction and isolation

Air dried powdered tubers of the plant (4 kg) were extracted with hot EtOH–H_2O (4:1) for 3 h each time under refluxing (×5). The combined EtOH extracts were evaporated to dryness in vacuum and the residue was suspended in water and extracted successively with EtOAc and *n*-BuOH. The *n*-BuOH layer residue (283 g) was subjected to silica gel CC eluted successively with $CHCl_3$–CH_3OH–H_2O (8.5:1.5:0.15), $CHCl_3$–CH_3OH–H_2O (8:2:0.2), and $CHCl_3$–CH_3OH –H_2O (7:3:0.3) to afford three fractions (I–III).

Fraction I (12 g) was subjected to silica gel CC (350 g) eluted with $CHCl_3$–CH_3OH–H_2O (9:1:0.1) to give a residue (4 g), which was then applied to a silica gel column twice that was eluted with $CHCl_3$–CH_3OH–H_2O (9:1:0.1) to give **4** (2 g) and **6** (1.3 mg).

Fraction II (7.4 g) was subjected to silica gel CC eluted with $CHCl_3$–CH_3OH–H_2O (8.5:1.5:0.15) to give three portions. The first position (2.3 g) was applied to a silica gel column eluted with EtOAc–CH_3OH–H_2O (9:1:0.1) to give **5** (20 mg) and **8** (345 mg). The second portion was purified on RP-18 CC eluted with CH_3OH–H_2O (6:4) to give **7** (8 mg), then the remainder was finally purified by preparative HPLC to give **10** (20 mg) and **11** (7 mg). The last portion was subjected to silica gel CC eluted with EtOAc–CH_3OH–H_2O (9:1:0.1), then passed through a RP-18 col-

Table 2
NMR spectroscopic data for harprocumbide B (**2**) and **9** (C_5D_5N, 400 MHz) (δ in ppm, J in Hz)

Atom	Harprocumbide B (**2**)			**9**	
	HSQC (δ_H)	δ_C	HMBC correlations	HSQC (δ_H)	δ_C
Aglycone					
1	6.05 (*d*, 9.2)	96.0	3,1′	6.07 (*d*, 9.2)	96.1
3	6.67 (*d*, 6.2)	142.7		6.67 (*d*, 6.2)	142.7
4	5.23 (*d*, 6.2)	105.1		5.21 (*d*, 6.2)	105.1
5	–	79.6		–	79.6
6	4.35 (*s*)	77.4		4.35 (*s*)	77.4
7	3.66 (*s*)	65.4	6	3.69 (*s*)	65.4
8	–	66.2		–	66.1
9	2.62 (*d*, 9.2)	52.6	5	2.63 (*d*, 9.2)	52.6
10	1.81 (3H, *s*)	17.6	8, 9	1.84 (3H, *s*)	17.6
Coumaroyl					
1′	–	126.3		–	125.9
2′,6′	8.02 (2H, *d*, 8.4)	133.6	4′, 7′	7.52 (2H, *d*, 8.4)	130.5
3′,5′	7.12 (2H, *d*, 8.4)	115.7	4′	7.06 (2H, *d*, 8.4)	116.5
4′	–	160.4		–	161.2
7′	6.79 (*d*, 12.8)	144.5	9′	7.89 (*d*, 16.0)	145.0
8′	5.92 (*d*, 12.8)	115.8	9′	6.57 (*d*, 16.0)	114.9
9′	–	166.8		–	167.2
Glucose					
1″	5.37 (*d*, 8.0)	100.1	1	5.40 (*d*, 8.0)	100.2
2″	4.07 (*t*, 8.4)	74.7	1″	4.10 (*t*, 8.4)	74.6
3″	4.22 (*t*, 8.8)	78.0		4.26 (*t*, 8.8)	78.0
4″	4.14 (*m*)	71.0		4.19 (*t*, 9.2)	71.0
5″	3.97 (*dd*, 5.2, 2.4)	75.2		4.00 (*dd*, 5.2, 2.4)	75.4
6″	4.89 (*dd*, 12.0, 2.3)	63.7	4″,9′	4.97 (*dd*, 12.0, 2.3)	63.8
	4.83 (*dd*, 12.0, 5.2)			4.91 (*dd*, 12.0, 5.2)	

Signals were assigned using ^1H–^1H COSY and HSQC.

umn eluted with CH_3OH–H_2O (5:5) to give the mixture of **2** and **9** (7 mg).

Fraction III (5.3 g) was applied to a silica gel (200 g) column eluted with $CHCl_3$–CH_3OH–H_2O (9:1:0.1) to give a residue (2 g), which was further successively chromatographed on Sephadex LH-20 eluted with $CHCl_3$–CH_3OH (1:1) and RP-18 eluted with CH_3OH–H_2O (5:5) to give **1** (9 mg) and **3** (7 mg).

3.3.1. 6″-O-α-galactopyranosylharpagoside (*1*)

White amorphous powder; m.p. 246–247 °C, $[\alpha]_D^{22}$ + 16.31 (MeOH; c 0.10); for NMR spectroscopic data, see Table 1; ESI-MS *m*/*z*: 655 $[M-H]^-$; HR-ESI-FTMS *m*/*z*: 679.2202 $[M + Na]^+$ (calcd. for $C_{30}H_{40}O_{16}Na$: 679.2209).

3.3.2. 6″-O-(cis-p-coumaroyl)-procumbide (*2*)

White amorphous powder; for NMR spectroscopic data, see Table 2; ESI-MS *m*/*z*: 507 $[M-H]^-$.

3.3.3. 8-O-(cis-p-coumaroyl)- harpagide (*11*)

White amorphous powder; m.p. 230–231 °C, $[\alpha]_D^{22}$ − 43.60 (MeOH; c 0.10), NMR data in Table 3.

3.4. Acid hydrolysis of (*1*) and (*2*) + (*9*)

A solution of (**1**) (7 mg) or (**2**) + (**9**) (5 mg) in MeOH (2 ml) with 1 M HCl was refluxed for 4 h. Each reaction

Table 3
NMR spectroscopic data for **11** and **10** (CD_3OD, 300 MHz) (δ in ppm, *J* in Hz)

Atom	**11**		**10**	
	δ_H	δ_C	δ_H	δ_C
Aglycone				
1	6.09 (*s*)	95.0	6.18 (*s*)	95.1
3	6.36 (*d*, 6.4)	144.2	6.41 (*d*, 6.4)	144.4
4	4.90 (*dd*, 6.3, 1.2)	107.7	4.94 (*dd*, 6.4, 1.5)	107.3
5	–	73.6	–	73.9
6	3.66 (*d*, 3.9)	78.1	3.74 (*d*, 3.9)	78.1
7	2.17 (*d*, 15.1)	46.6	2.26 (*d*, 15.2)	46.7
	1.97 (*dd*, 14.9, 4.5)		1.98 (*dd*, 15.1, 4.3)	
8	–	88.8	–	88.9
9	2.89 (*s*)	56.3	2.93 (*s*)	56.0
10	1.94 (3H, *s*)	23.1	1.51 (3H,*s*)	23.2
Coumaroyl				
1′	–	128.2	–	133.8
2′,6′	7.54 (2H, *d*, 8.6)	133.9	7.46 (2H, *d*, 8.7)	131.6
3′,5′	6.74 (2H, *d*, 8.8)	116.4	6.81 (2H, *d*, 8.7)	117.3
4′	–	160.4	–	161.6
7′	6.74 (*d*, 12.7)	144.4	7.58 (*d*, 15.9)	146.8
8′	5.74 (*d*, 12.7)	118.9	6.33 (*d*, 15.9)	116.9
9′	–	169.0	–	169.8
Glucose				
1″	4.58 (*d*, 7.8)	100.2	4.63 (*d*, 7.8)	100.4
2″	3.23 (*t*, 8.3)	75.1	3.23 (*t*, 8.0)	75.0
3″	3.27–3.86 (5H, m)	78.7	3.30–3.95 (5H, m)	78.6
4″		72.2		72.2
5″		78.2		78.2
6″		63.3		63.4

Table 4
Inhibition of MP-CL by iridoid glycosides from *Hapagophytum procumbens* D.C.

	Samples									Control test reagents	
	1	2 + 9	3	4	5	7	8	10	11	Dexamethasone	Rutin
IC_{50} (μmol/L) mean ± SD	164.23 ± 36.01	n.d.	n.d.	310.39 ± 24.49	433.79 ± 37.s56	293.83 ± 36.59	n.d.	32.41 ± 3.26	114.58 ± 7.78	354.57 ± 46.55	14.53 ± 2.47

Note. PMNs were stimulated by PMA. Chemiluminescence was measured with luminol.

mixture was evaporated in vacuo, and partitioned with Et_2O. Each H_2O layer was neutralized with 2% KOH and concentrated in vacuo. Each residue was washed with $CHCl_3$ (3× 4 mL). The monosaccharide fractions were then concentrated in vacuo. Each syrup was subjected to silica gel preparative TLC using *iso*-propanol–MeOH–H_2O (25:1:2) as eluting solvent to yield D-glucose (0.5 mg) and D-galactose (1.4 mg) from **1** or D-glucose (1.2 mg) from **2** + **9**. D-glucose and D-galactose were identified by optical rotation and TLC compared with authentie samples. Compound **1**: D-glucose: $[\alpha]_D^{20} + 54.12$ (H_2O; c 0.05), TLC $R_f = 0.61$, solvent systems: *iso*-propanol–MeOH–H_2O (25:1:2). D-galactose: $[\alpha]_D^{20} + 86.53$, (H_2O; c 0.14), TLC ($R_f = 0.46$, solvent systems: *iso*-propanol–MeOH–H_2O (25:1:2)). Compound **2** + **9**: D-glucose: $[\alpha]_D^{20} + 55.44$ (H_2O; c 0.12), TLC ($R_f = 0.60$, solvent systems: *iso*-propanol–MeOH–H_2O (25:1:2)).

3.5. Macrophages respiratory burst inhibitory activity

The murine macrophage-like cell line RAW 264.7 was routinely cultivated at 37 °C, 5% CO_2 in RPMI-1640 supplemented with 10% FCS (Hyclone, America), 100 μg/mL streptomycin, 118 μg/mL ampicillin, and 2 mg/mL sodium bicarbonate. After 72 h, the macrophage cells formed a confluent monolayer. The monolayer cells were digested with trypsin. After washed with PBS without Ca^{2+} and Mg^{2+}, the deposited cells were suspended with RPMI-1640 without FCS (approximately 2×10^6 cell/mL) in the vitreous culture flask (Cho et al., 2000).

The details of chemiluminescence assay procedure were according to the method described in the literature (Pasmans et al., 2001). Tested compounds were prepared as 10 mM top stocks, dissolved in DMSO, and stored at 4 °C. Phorbol 12-myristate 13-acetate (PMA) was applied as triggering agent. Data were collected at a frequency of 6 s/min and chemiluminescence was recorded for up to 30 min. The IC_{50} values were obtained by linear regression analysis of the dose response curves, which were plots of % inhibition versus concentration (Chadfield and Olsen, 2001).

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