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Hepatoprotective triterpenes from traditional Tibetan medicine *Potentilla anserina*

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ABSTRACT

A methanol extract from the tuberous roots of *Potentilla anserina* (Rosaceae) exhibited hepatoprotective effects against D-galactosamine (D-GalN)/lipopolysaccharide-induced liver injuries in mice. Six triterpene 28-*O*-monoglucopyranosyl esters, potentillanosides A–F, were isolated from the extract along with 32 known compounds, including 15 triterpenes. The structures of potentillanosides A–F were determined on the basis of spectroscopic properties and chemical evidence. Four ursane-type triterpene 28-*O*-monoglycosyl esters, potentillanoside A (IC_{50} = 46.7 μM), 28-*O*-β-D-glucopyranosyl pomolic acid (IC_{50} = 9.5 - μM), rosamutin (IC_{50} = 35.5 μM), and kaji-ichigoside F1 (IC_{50} = 14.1 μM), inhibited D-GalN-induced cytotoxicity in primary cultured mouse hepatocytes. Among these four triterpenes, potentillanoside A, rosamutin, and kaji-ichigoside F1 exhibited *in vivo* hepatoprotective effects at doses of 50–100 mg/kg, p.o. The mode of action was ascribable to the reduction in cytotoxicity caused by D-GalN.

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Introduction

The plant *Potentilla anserina* L. (Rosaceae) is widely distributed in the western areas of China, particularly in the Qinghai-Tibetan Plateau (Wang et al., 2010; Xia and You, 2011). In traditional Tibetan medicine, roots of this plant have been used to treat malnutrition, anemia, diarrhea, and haemorrhage (Chen et al., 2010). Several chemical constituents of this plant, such as tannins (Schimmer and Lindenbaum, 1995), flavan-3-ols and flavonoids (Kombal and Glasl, 1995), triterpenes (Chu et al., 2008; Li et al., 2003), triterpene glycosides (Zhao et al., 2008), polysaccharides (Chen et al., 2010; Wang et al., 2010), and amino acids (Xia and You, 2011) have been reported. In addition, biological activities such as antimutagenic (Schimmer and Lindenbaum, 1995), anti-hepatitis B virus (Zhao et al., 2008), and immunomodulatory activities (Chen et al., 2010) of the extracts and/or constituents have been reported. During our studies on medicinal herbs in Tibet and Xinjiang autonomous regions in China, such as *Cistanche tubulosa* (Morikawa et al., 2010a,b; Pan et al., 2010; Xie et al., 2006; Yoshikawa et al., 2006), *Punica granatum* (Xie et al., 2008), and *Poacynum hendersonii* (Morikawa et al., 2012), a methanol extract of the tuberous roots of *P. anserina* was found to have a protective effect against liver injuries induced by D-galactosamine (D-GalN)/lipopolysaccharide (LPS) in mice. From this methanol extract, we have isolated six new triterpene 28-*O*-monoglucopyranosyl esters named potentillanosides A–F (**1**–**6**) along with 32 compounds, including 15 triterpenes (**7**–**21**). This study deals with the isolation and structural elucidation of these new triterpenenes (**1**–**6**) and their hepatoprotective effects and their possible mode of action.

Results and discussion

Effects of P. anserina methanol extract and its fractions on D-GalN/LPS-induced liver injuries in mice

Dried tuberous roots of *P. anserina* were extracted with methanol under conditions of reflux to yield a methanol extract (23.0% from dried material). The methanol extract at a dose of 500 mg/kg, p.o. in mice showed inhibitory effects against an increase in serum levels of aspartate aminotransaminase (sAST) and alanine transaminase (sALT), which are the markers of liver injuries induced by D-GalN/LPS (Table 1). Following this, the methanol extract was partitioned into ethyl acetate (EtOAc)–H_2O mixture (1:1, v/v) to furnish an EtOAc-soluble fraction (0.58%) and an aqueous phase. The latter was subjected to Diaion HP-20 column chromatography (H_2O → MeOH) to yield H_2O- and MeOH-eluted fractions (21.5% and 0.73%, respectively). A bioassay-guided fractionation established that the EtOAc-soluble and MeOH-eluted fractions were active (percentage inhibition at 250 mg/kg, p.o., 95.8% and 85.1%, respectively, for sAST and 97.0% and 99.1%,

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Table 1
Inhibitory effects of the methanol extract from the tuberous roots of *P. anserina* and its fractions on D-GalN/LPS-induced liver injuries in mice.

Treatment	Dose (mg/kg, p.o.)	*n*	sAST		sALT	
			(Karmen unit)	Inhibition (%)	(Karmen unit)	Inhibition (%)
Normal (vehicle)	–	8	107 ± 9[b]	–	20 ± 2[b]	–
Control (D-GalN/LPS)	–	12	5572 ± 768	–	3703 ± 515	–
MeOH extract	250	6	4231 ± 994	24.5	2649 ± 765	28.6
	500	8	2029 ± 673[a]	64.8	1158 ± 336[b]	69.1
Control (D-GalN/LPS)	–	12	5344 ± 1100	–	3600 ± 881	–
EtOAc-soluble fraction	250	7	328 ± 71[b]	95.8	129 ± 47[b]	97.0
MeOH-eluted fraction	250	6	886 ± 426[b]	85.1	394 ± 225[b]	89.6
H_2O-eluted fraction	500	6	2906 ± 1396	46.6	2445 ± 1428	32.3
Normal (vehicle)	–	5	95 ± 5[b]	–	19 ± 1[b]	–
Control (D-GalN/LPS)	–	8	9126 ± 1477	–	9830 ± 1650	–
Hydrocortisone[c]	10	7	627 ± 262[b]	94.2	247 ± 123[b]	97.7

Each value represents the mean ± S.E.M.
Significantly different from the control.
[a] $p < 0.05$.
[b] $p < 0.01$.
[c] Commercial hydrocortisone was purchased from Sigma–Aldrich Chemical (St. Louis, MO, USA) (Morikawa et al., 2010a).

respectively, for sALT), whereas the H_2O-eluted fraction showed no notable activity (Table 1).

Isolation

The active fractions, the EtOAc-soluble and MeOH-eluted fractions, were subjected to normal-phase silica gel and reversed-phase ODS column chromatographic purification steps, and finally by HPLC to furnish potentillanosides A (**1**, 0.013%), B (**2**, 0.00067%), C (**3**, 0.00005%), D (**4**, 0.00046%), E (**5**, 0.0015%), and F (**6**, 0.00047%), respectively. Additionally, 15 triterpenes, i.e., pomolic acid (**7**, 0.0027%) (Amimoto et al., 1992; Kuang et al., 1989), 28-*O*-*β*-D-glucopyranosyl pomolic acid (**8**, 0.00033%) (Amimoto et al., 1992), tormentic acid (**9**, 0.0074%) (Kuang et al., 1989; Taniguchi et al., 2002), rosamutin (**10**, 0.063%) (Jia et al., 1993), euscaphic acid (**11**, 0.00037%) (Guang-Yi et al., 1989; Kuang et al., 1989), kaji-ichigoside F1 (**12**, 0.0085%) (Guang-Yi et al., 1989; Seto et al., 1984), 2*α*,19*α*-dihydroxy-3-oxours-12-en-28-oic acid (**13**, 0.00088%) (Taniguchi et al., 2002), 2-oxopomolic acid (**14**, 0.00006%) (D'Abrosca et al., 2005), 2-oxopomolic acid 28-*O*-*β*-D-glucopyranosyl ester (**15**, 0.00049%) (Jia et al., 1993), 2*α*-hydroxyursolic acid (**16**, 0.00020%) (Kuang et al., 1989; Taniguchi et al., 2002), cecropiacic acid (**17**, 0.00047%) (Lontsi et al., 1987), maslinic acid (**18**, 0.00029%) (Kuang et al., 1989; Taniguchi et al., 2002), 24-deoxy-sericoside (**19**, 0.011%) (Zhou et al., 1992), arjunglucoside I (**20**, 0.00010%) (Abe and Yamauchi, 1987), and alphitolic acid (**21**, 0.00004%) (Kuang et al., 1989; Yagi et al., 1978), were isolated (Fig. 1) together with gallic acid (0.00032%) (Nawwar et al., 1982), gallic acid methyl ester (0.00017%) (Khalid et al., 1989), ellagic acid (0.00081%) (Khac et al., 1990; Nawwar et al., 1994), ellagic acid 4-*O*-*α*-L-arabinofuranoside (0.0011%) (Zafrilla et al., 2001), ducheside B (0.0010%) (Ye and Yang, 1996), (+)-catechin (0.00058%) (Davis et al., 1996; Jia et al., 1993; Khalid et al., 1989), (+)-gallocatechin (0.0029%) (Davis et al., 1996), (+)-catechin 7-*O*-*β*-D-glucopyranoside (0.0015%) (Kashiwada et al., 1986), quercetin 3-*O*-*β*-D-glucuropyranosiduronic acid (0.00018%) (Möhle et al., 1985), quercetin 3-*O*-sambubioside (0.00015%) (Webby, 1991), quercetin 3-*O*-*β*-D-xylopyranosyl-(1 → 2)-*β*-D-glucopyranoside-3′-*O*-*β*-D-glucopyranoside (0.00014%) (Hübner et al., 1999), 6-*O*-*p*-coumaroylsucrose (0.00067%) (Gouda et al., 2006), 6-*O*-feruloylsucrose (0.00011%) (Bokern et al., 1991), and L-tryptophan (0.016%) (Fig. S1).

*Structures of potentillanosides A–F (**1**–**6**)*

Potentillanoside A (**1**) was obtained as an amorphous powder and had a positive optical rotation ($[\alpha]_D^{26}$ + 20.4 in MeOH). Its IR

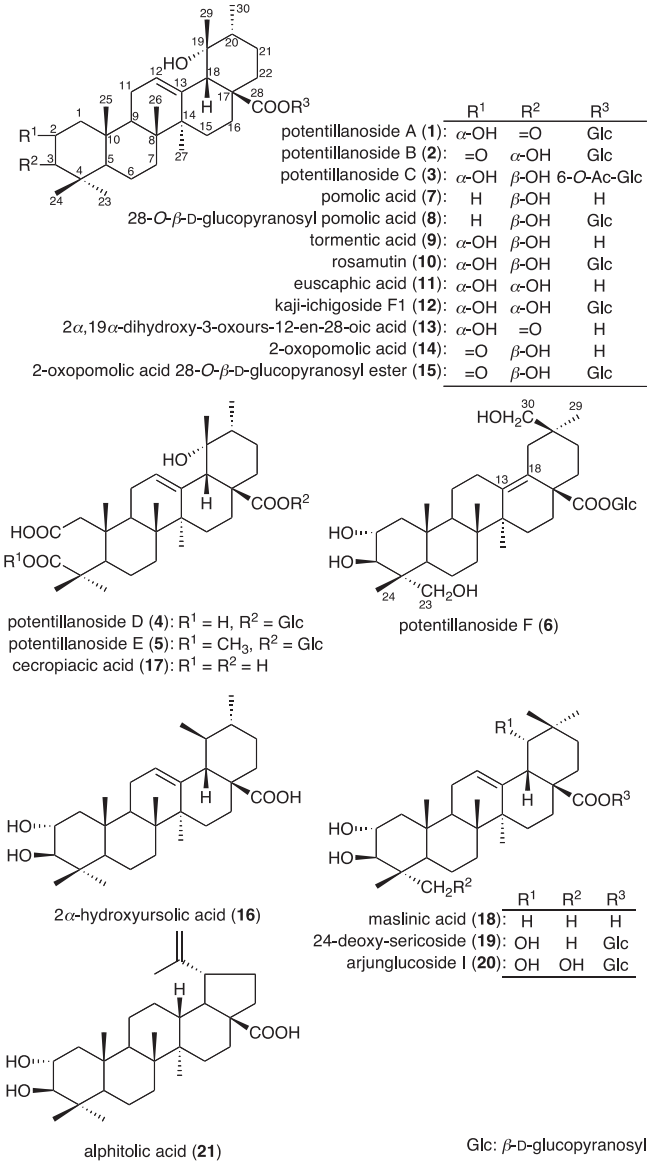

Fig. 1. Triterpene constituents (**1**–**21**) from the tuberous roots of *P. anserina*.

spectrum showed absorption bands at 1725, 1686, and 1655 cm^{-1} ascribable to carbonyl, ester carbonyl, and olefin functionalities, and broad bands at 3470 and 1073 cm^{-1} suggestive of a glycoside structure. In the positive-ion FABMS, a quasimolecular ion peak was observed at *m/z* 671 $[M+Na]^+$, and HRFABMS analysis indicated the molecular formula to be $C_{36}H_{56}O_{10}$. The ^1H and ^{13}C NMR spectra (pyridine-d_5, Table 2), which were assigned with the aid of DEPT, ^1H–^1H COSY, HMQC, and HMBC experiments (Fig. S2), showed signals assignable to seven methyls [δ 1.03, 1.16, 1.20, 1.20, 1.37, 1.57 (3H each, all s, H_3-24, 26, 25, 23, 29, 27), 1.06 (3H, d, J = 6.7 Hz, H_3-30)], a methine bearing an oxygen function [δ 4.79 (1H, dd, J = 6.5, 12.4 Hz, H-2)], an olefin [δ 5.51 (1H, dd, J = 3.5, 3.5 Hz, H-12)], and a ester carbonyl group [δ_C 176.9 (C-28)] together with a β-glucopyranosyl part [δ 6.21 (1H, d, J = 8.1 Hz, H-1′)]. The ^1H and ^{13}C NMR spectroscopic properties of the aglycone part were quite similar to those of **13**. Only a group of signals due to the 28-*O*-β-D-glucopyranosyl ester moiety of **13** was lacking in the spectrum of **1**. In the HMBC experiment, a long-range correlation was observed between the anomeric proton of the glucopyranosyl part and the ester carbonyl carbon. The stereostructure was characterized by a nuclear Overhauser enhancement spectroscopy (NOESY) experiment (Fig. S3). The absolute stereostructure of **1** was determined by derivatizing **1** to **10** and **12** using sodium borohydride ($NaBH_4$) as shown in Fig. 2. Acid hydrolysis of **1** with 5% aqueous H_2SO_4–1,4-dioxane (1:1, v/v) liberated D-glucose, which was identified by HPLC analysis (Morikawa et al., 2010a,b). On the basis of this evidence, the absolute stereostructure of potentilllanoside A was determined as 2α,19α-dihydroxy-3-oxours-12-en-28-oic acid 28-*O*-β-D-glucopyranosyl ester (**1**).

Potentillanoside B (**2**) was obtained as an amorphous powder. Its molecular formula, $C_{36}H_{56}O_{10}$, was found to be the same as that of **1** by HRFABMS measurement. The ^1H and ^{13}C NMR (Table 2) spectroscopic properties were also quite similar to those of 2-oxopomolic aicd 28-*O*-β-D-glucopyranosyl ester (**15**) (Table S1), except for signals due to the A ring part of the aglycone [δ 2.31, 2.97 (1H each, both d, J = 12.4 Hz, H_2-1), 3.92 (1H, s, H-3); δ_C 51.6 (C-1), 213.3 (C-2), 83.1 (C-3)]. The connectivities of the hydroxy and carbonyl moieties in the A ring of the aglycone were characterized on the basis of the HMBC spectrum, in which long-range correlations were observed between: H_2-1 and C-2/C-3/C-10 (δ_C 42.8); H-3 and C-1/C-2/C-4 (δ_C 42.3); and H_3-23 [δ 1.22 (3H, s)]/H_3-24 [δ 0.96 (3H, s)] and C-3/C-4/C-5 (δ_C 50.3). Reduction of **2** with $NaBH_4$ yielded **12**

Table 2
^1H and ^{13}C NMR spectroscopic data (pyridine-d_5) of **1** and **2**.

	1		**2**	
Position	δ_H	δ_C	δ_H	δ_C
1	1.35 (m)	50.2	2.31 (d, 12.4)	51.6
	2.48 (dd, 6.5, 12.5)		2.97 (d, 12.4)	
2	4.79 (dd, 6.5, 12.4)	69.5		213.3
3		216.4	3.92 (s)	83.1
4		48.0		42.3
5	1.23 (br d, *ca.* 12)	69.5	1.99 (dd, 1.6, 12.2)	50.3
6	1.38 (m)	19.6	1.41 (m)	19.5
	1.44 (m)		1.50 (m)	
7	1.43 (m)	33.1	1.48 (m)	33.2
	1.54 (m)		1.72 (m)	
8		40.6		41.1
9	1.83 (dd, 6.9, 10.7)	47.3	2.20 (dd, 6.9, 10.9)	47.8
10		37.9		42.8
11	2.06 (m)	24.1	2.00 (m)	24.0
	2.09 (m)		2.06 (m)	
12	5.51 (dd, 3.5, 3.5)	127.6	5.53 (dd, 3.8, 3.8)	128.0
13		139.3		139.4
14		42.1		42.1
15	1.20 (m)	29.1	1.25 (m)	29.2
	2.40 (ddd, 3.9, 13.1, 13.6)		2.45 (m)	
16	1.98 (m)	26.0	2.03 (m)	26.1
	3.04 (ddd, 3.9, 13.1, 13.3)		3.07 (ddd, 4.3, 13.1, 13.2)	
17		48.5		48.7
18	2.89 (br s)	54.3	2.93 (br s)	54.5
19		72.6		72.7
20	1.35 (m)	42.0	1.39 (m)	42.1
21	1.23 (m)	26.6	1.23 (m)	26.7
	1.96 (m)		1.99 (m)	
22	1.85 (m)	37.6	1.88 (m)	37.6
	2.02 (m)		2.05 (m)	
23	1.20 (3H, s)	25.2	1.22 (3H, s)	27.5
24	1.03 (3H, s)	21.7	0.96 (3H, s)	21.8
25	1.20 (3H, s)	16.0	1.06 (3H, s)	16.9
26	1.16 (3H, s)	17.5	1.16 (3H, s)	17.5
27	1.57 (3H, s)	24.5	1.64 (3H, s)	24.4
28		176.9		176.9
29	1.37 (3H, s)	27.0	1.38 (3H, s)	27.0
30	1.06 (3H, d, 6.7)	16.6	1.06 (3H, d, 7.2)	16.6
28-*O*-Glc				
1′	6.21 (d, 8.1)	95.7	6.27 (d, 8.0)	95.7
2′	4.16 (dd, 7.1, 8.1)	73.9	4.21 (dd, 8.0, 8.6)	74.1
3′	4.24 (dd, 7.1, 8.5)	78.8	4.28 (dd, 8.6, 8.8)	79.0
4′	4.27 (dd, 7.9, 8.5)	71.3	4.32 (dd, 8.8, 9.1)	71.4
5′	4.00 (m)	79.0	4.04 (m)	79.2
6′	4.34 (dd, 4.8, 11.6)	62.4	4.38 (dd, 4.7, 11.9)	62.4
	4.43 (dd, 2.3, 11.6)		4.46 (dd, 2.4, 11.9)	

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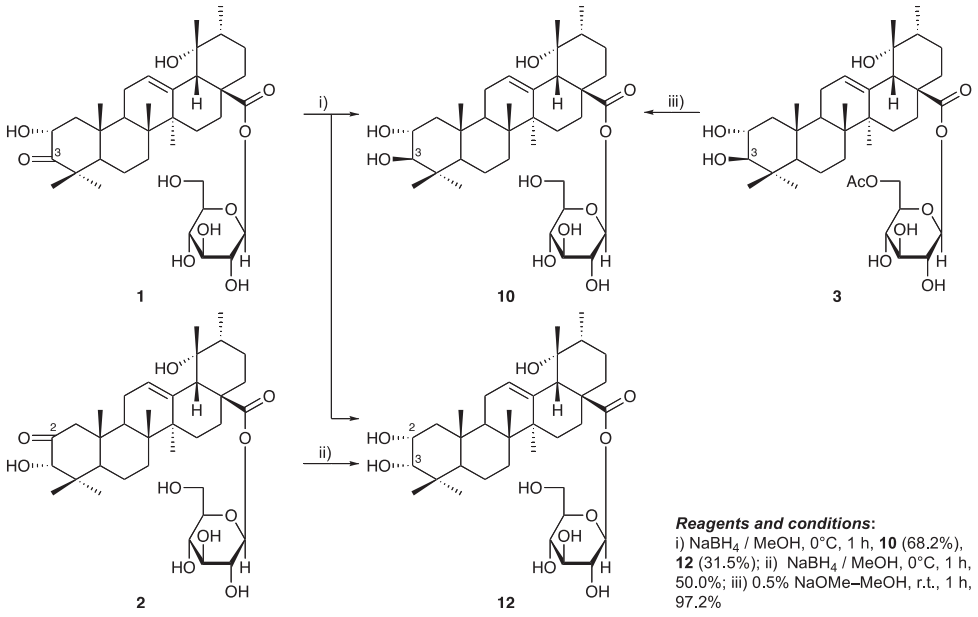

Fig. 2. Absolute stereostructures of **1**–**3**.

(Fig. 2), so that the absolute stereostructure of **2** was determined to be 3α,19α-dihydroxy-2-oxours-12-en-28-oic acid 28-*O*-β-D-glucopyranosyl ester.

Potentillanoside C (**3**) was also obtained as an amorphous powder. Its molecular formula, $C_{38}H_{60}O_{11}$, was determined by a HRFABMS measurement. Its ^{1}H and ^{13}C NMR (Table 3) spectroscopic properties were quite similar to those of rosamutin (**10**) (Table S2), except for signals due to an acetyl group [δ 1.95 (3H, s); δ_C 170.8 and 20.7]. The connectivity of the acetyl group was clarified by HMBC experiments, which showed a long-range correlation between the 6′-position of the glucopyranosyl part [δ 4.76 (1H, m), 4.86 (1H, dd, J = 1.7, 12.2 Hz)] and the acetyl carbonyl carbon. As shown in Fig. 2, treatment of **3** with 0.5% sodium methoxide (NaOMe)–MeOH provided **10**. Consequently, the stereostructure of **3** was elucidated to be 6′-*O*-acetylrosamutin.

Potentillanoside D (**4**) was obtained as an amorphous powder with a positive optical rotation ($[\alpha]_D^{27}$ + 22.6 in MeOH). Its positive-ion FABMS showed a quasimolecular ion peak at *m*/*z* 703 $[M+H]^+$, and its molecular formula was determined as $C_{36}H_{56}O_{16}$ by HRFABMS measurement. The ^{1}H and ^{13}C NMR (Table 4) spectra showed signals assignable to seven methyls [δ 1.02 (3H, d, J = 6.6 Hz, H_3-30), 1.26, 1.27, 1.30, 1.55, 1.59, 1.80 (3H each, all s, H_3-25, 26, 29, 23, 24, 27)], an olefin [δ 5.55 (1H, dd, J = 3.5, 3.5 Hz, H-12)], two carboxy and a ester carbonyl groups [δ_C 174.3, 177.0, 182.3 (C-2, 28, 3)] together with a glucopyranosyl part [δ 6.24 (1H, d, J = 8.3 Hz, H-1′)]. As shown in Fig. 3, alkaline hydrolysis of **4** with 5% aqueous potassium hydroxide (KOH)–1,4-dioxane (1:1, v/v) furnished cecropiacic acid (**17**). Finally, the position of the β-D-glucopyranosyl ester was determined on the basis of a HMBC experiment, in which a long-range correlation was observed between H-1′ and C-28. Thus the connected position was unambiguously clarified, and structure **4** was elucidated to be cecropiacic acid 28-*O*-β-D-glucopyranosyl ester. The ^{1}H and ^{13}C NMR (Table 4) spectroscopic properties of potentillanoside E (**5**) were quite similar to those of **4**, except for signals due to the carbomethoxy group [δ 3.68 (3H, s)]. In the HMBC experiment, a long-range correlation was observed between the carbomethoxy proton and C-3 (δ_C 179.8). As shown in Fig. 3, methylation of **4** and **5** with trimethylsilyldiazomethane ($TMSCHN_2$) gave the common dimethyl ester (**4a**). Thus, the stereostructure of **5** was clarified to be as shown.

Potentillanoside F (**6**) was obtained as an amorphous powder with a positive optical rotation ($[\alpha]_D^{26}$ + 46.9 in MeOH). Its molecular formula, $C_{36}H_{58}O_{11}$, was determined by HRFABMS measurement {*m*/*z* 689.3877 $[M+Na]^+$ (calcd for $C_{36}H_{58}O_{11}Na$, 689.3883)}. The acid hydrolysis of **6** liberated D-glucose, which was identified by HPLC analysis. The ^{1}H and ^{13}C NMR (Table 5) spectra of **6** showed signals assignable to five methyls [δ 0.98, 1.00, 1.04, 1.12, 1.14 (3H each, all s, H_3-25, 29, 24, 27, 26)], two hydroxymethyls {δ 3.60 (2H, s, H_2-30), [3.70, 4.17 (1H each, both d, J = 10.4 Hz), H_2-23]}, and two methines bearing oxygen functions [δ 4.18 (1H, d, J = 9.4 Hz, H-3), 4.24 (1H, m, H-2)] together with a glucopyranosyl part [δ 6.32 (1H, d, J = 8.1 Hz, H-1′)]. As shown in Fig. 4, a ^{1}H–^{1}H COSY experiment indicated the presence of partial structures written in bold lines. In the HMBC experiment, long-range correlations were observed between the following protons and carbons; (H-3 and C-4/C-23/C-24; H-5 and C-4/C-10/C-23/C-24; H_2-7 and C-8; H-9 and C-10; H_2-12 and C-13; H_2-15 and C-14; H_2-16 and C-17; H_2-19 and C-13/C-18/C-20; H_2-21 and C-20; H_2-22 and C-17/C-28; H_2-23 and C-3/C-4/C-5/C-24; H_3-24 and C-3/C-4/C-5/C-23; H_3-25 and C-1/C-5/C-9/C-10; H_3-26 and C-7/C-8/C-9/C-14; H_3-27 and C-8/C-13/C-14; H_3-29 and C-19/C-20/C-21/C-30; H_2-30 and C-19/C-20/C-21/C-29; H-1′ and C-28), respectively. Next, the stereostructure was characterized by a NOESY experiment, which showed NOE correlations between the following proton pairs; {H-1α [δ 1.36 (m)] and H-3; H-2 and H_3-24/H_3-25; H-3 and H-5/H_2-23; H-5 and H-7α [δ 1.54 (m)]/H-9/H_2-23; H-6β [δ 1.37 (m)] and H_3-26; H-9 and H-12α [δ 1.88 (m)]/H_3-27; H-11β [δ 1.28 (m)] and H_3-25; H-12α and H_3-27; H-15β [δ 2.10 (m)] and H_3-26; H-16α [δ 1.61 (m)] and H-22α [δ 1.50 (br dd, J = *ca.* 13, 14 Hz)]/H_3-27/H_3-29; H-22α and H_3-29; H_3-24 and H_3-25; H_3-25 and H_3-26}. Consequently, the structure of **6** was elucidated as 2α,3β,30-trihydroxy-olean-13(18)-en-28-oic acid 28-*O*-β-D-glucopyranosyl ester.

Effects on D-GalN-induced cytotoxicity in primary cultured mouse hepatocytes

D-GalN/LPS-induced liver injuries are known to develop through immunological responses (Freudenberg and Galanos, 1991) that progress *via* two steps. First, expression of inhibitors against apoptosis (IAPs) is inhibited by administration of D-GalN through

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Table 3
^{1}H and ^{13}C NMR spectroscopic data (pyridine-d_5) of **3**.

	3	
Position	δ_H	δ_C
1	1.27 (br d, *ca.* 13)	48.1
	2.26 (dd, 4.5, 12.5)	
2	4.09 (dd, 4.5, 9.4)	68.7
3	3.37 (d, 9.4)	84.0
4		39.9
5	1.07 (m)	56.1
6	1.45 (m)	19.1
	1.56 (m)	
7	1.47 (m)	33.6
	1.66 (m)	
8		40.7
9	1.94 (m)	47.9
10		38.6
11	2.13 (2H, m)	24.2
12	5.55 (dd, 3.5, 3.5)	128.5
13		139.3
14		42.2
15	1.25 (m)	29.2
	2.43 (ddd, 4.7, 13.0, 14.1)	
16	2.00 (m)	26.2
	3.06 (ddd, 4.4, 13.0, 13.2)	
17		48.8
18	2.92 (br s)	54.4
19		72.7
20	1.33 (m)	42.1
21	1.22 (m)	26.7
	1.96 (m)	
22	1.96 (m)	37.8
	2.07 (m)	
23	1.25 (3H, s)	29.4
24	1.09 (3H, s)	17.6
25	1.10 (3H, s)	17.1
26	1.19 (3H, s)	17.6
27	1.66 (3H, s)	24.6
28		176.9
29	1.37 (3H, s)	27.0
30	1.04 (3H, d, 6.5)	16.6
28-*O*-Glc		
1′	6.22 (d, 7.9)	95.7
2′	4.21 (dd, 7.9, 8.9)	74.0
3′	4.25 (dd, 8.0, 8.9)	78.7
4′	4.11 (m)	71.2
5′	4.11 (m)	76.1
6′	4.76 (m)	64.5
	4.86 (dd, 1.7, 12.2)	
6′-*O*-Ac		170.8
	1.95 (3H, s)	20.7

depletion of uridine triphosphate in hepatocytes. Second, pro-inflammatory mediators, such as nitric oxide (NO) and tumor necrosis factor-α (TNF-α), are released from LPS-activated macrophages (Kupffer's cells). Apoptosis of hepatocytes by TNF-α is reported to play an important role in this D-GalN/LPS-induced liver injury (Josephs et al., 2000). In a previous investigation of compounds from natural medicines possessing hepatoprotective activity, it was reported that sesquiterpenes and diarylheptanoids from *Curcuma zedoaria* (Morikawa et al., 2002), acid amides from *Piper chaba* (Matsuda et al., 2009) and acylated phenylethanoids from *C. tubulosa* (Morikawa et al., 2010a) exhibited significant protective effects against liver injuries induced by D-GalN/LPS in mice. In addition, these constituents were found to reduce D-GalN-induced cytotoxicity in primary cultured hepatocytes. Because a methanol extract of the tuberous roots of *P. anserina* also exhibited hepatoprotective effects in this study, the inhibitory effects of its constituents on D-GalN/LPS-induced cytotoxicity were examined in primary cultured mouse hepatocytes by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Thus, we found that the following four triterpenes: potentillanoside A (**1**, IC_{50} = 46.7 μM), tormentic acid (**9**, 9.5 μM), rosamutin (**10**, 35.5 μM), and kaji-ichigoside F1 (**12**, 14.1 μM) and gallic acid methylester (53.7 μM), (+)-catechin (17.7 μM), (+)-gallocatechin (18.4 μM), (+)-catechin 7-*O*-β-D-glucopyranoside (55.4 μM), and quercetin 3-*O*-β-D-glucopyranoside (34.2 μM) exhibited significant activity. Among the triterpene constituents, the potency of **9**, **10**, and **12** was higher than that of silybin (38.8 μM), a commercially available positive control (Fehér et al., 1989; Skottová and Krecman, 1998) (Table 6).

Effects on LPS-activated NO production in mouse peritoneal macrophages

Effects of the isolates on NO production were examined to provide an index for estimation of macrophage activating levels in LPS-treated mouse peritoneal macrophages. Thus, it was found that pomolic acid (**7**, IC_{50} = 33.1 μM), 28-*O*-β-D-glucopyranosyl pomolic acid (**8**, 91.9 μM), **9** (68.4 μM), 2α-hydroxyursolic acid (**16**, 21.1 μM), and maslinic acid (**18**, 30.1 μM) significantly inhibited NO production (Table 7). The potencies of **7**, **16**, and **18** were equivalent to that of N^G-monomethyl-L-arginine (L-NMMA, 36.0 μM), a NO synthase inhibitor, but were lower than that of caffeic acid phenethyl ester (CAPE, 11.0 μM), an inhibitor of nuclear factor-κB activation (Morikawa et al., 2011). Thus, these compounds were found to prevent overproductions of NO from LPS-activated macrophages.

Effects on TNF-α-induced cytotoxicity in L929 cells

To examine the effects of the constituents on TNF-α-induced cytotoxicity, the viability of L929 cells, a TNF-α-sensitive cell line (Kouroku et al., 2000), under the presence of the constituents was examined. Thus, it was found that only **8** (IC_{50} = 25.5 μM) significantly improved cell viability (Table 8).

*Effects of principal triterpenes (**1**, **7**, **9**, **10**, and **12**) on D-GalN/LPS-induced liver injuries in mice*

Finally, the effects of the principal triterpenes: potentillanoside A (**1**), pomolic acid (**7**), tormentic acid (**9**), rosamutin (**10**), and kaji-ichigoside F1 (**12**) on D-GalN/LPS-induced liver injuries in mice were examined. Three ursane-type triterpene 28-*O*-monoglycosyl esters (**1**, **10**, and **12**) were found to significantly inhibit the increase in both sAST and sALT levels induced by D-GalN/LPS in mice at doses of 50–100 mg/kg, p.o. In particular, the inhibitory activity of **1** was significant and was as potent as curcumin, a positive control (Morikawa et al., 2002) (Table 9).

Concluding remarks

In conclusion, a methanol extracts from the tuberous roots of *P. anserina* exhibited protective effects against liver injuries induced by D-GalN/LPS in mice. From the extract, six new triterpene 28-*O*-monoglucopyranosyl esters, potentillanosides A–F (**1**–**6**) were isolated along with 32 known compounds, including 15 triterpenes (**7**–**21**). Among the isolated constituents, three ursane-type triterpene 28-*O*-monoglycosyl esters (**1**, **10**, and **12**) showed *in vivo* hepatoprotective effects at doses of 50–100 mg/kg, p.o. On the basis of *in vitro* studies, the following plausible mechanisms of action for the hepatoprotective effect can be proposed: (i) reduction of both D-GalN and TNF-α-induced cytotoxicity (**8**), (ii) reduction of D-GalN induced cytotoxicity (**1**, **10**, and **12**), and (iii) inhibition of LPS-activated macrophage activation (**7**, **9**, **16**, and **18**). However, the detailed mechanisms of action for the hepatoprotective effect need to be studied further.

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Table 4
^{1}H and ^{13}C NMR spectroscopic data (pyridine-d_5) of **4**, **5**, and **4a**.

	4		**5**		**4a**	
Position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	2.74 (d, 17.8)	43.0	2.65 (d, 18.2)	42.5	2.45 (d, 17.9)	42.1
	3.07 (d, 17.8)		2.69 (d, 18.2)		2.69 (d, 17.9)	
2		174.3		174.3		171.9
3		182.3		179.8		179.6
4		47.0		46.8		46.7
5	3.14 (dd, 3.2, 11.5)	49.0	2.96 (dd, 2.5, 12.3)	49.2	2.69 (m)	49.3
6	1.73 (2H, m)	22.2	1.54 (m)	21.9	1.48 (m)	21.7
			1.62 (m)		1.55 (m)	
7	1.52 (m)	33.0	1.49 (ddd, 2.8, 12.9, 13.0)	32.9	1.45 (m)	32.8
	1.91 (m)		1.82 (m)		1.74 (m)	
8		40.7		40.6		40.5
9	3.42 (dd, 6.4, 11.4)	39.6	3.34 (dd, 6.4, 11.4)	39.5	2.98 (m)	39.8
10		42.4		42.1		42.2
11	2.19 (ddd, 3.5, 11.4, 18.1)	24.6	2.15 (ddd, 3.6, 11.4, 18.1)	24.5	2.08 (m)	24.4
	2.38 (ddd, 3.5, 6.4, 18.1)		2.32 (ddd, 3.6, 6.4, 18.1)		2.12 (m)	
12	5.55 (dd, 3.5, 3.5)	128.0	5.60 (dd, 3.6, 3.6)	128.9	5.57 (dd, 3.9, 3.9)	128.6
13		139.0		139.0		139.1
14		43.0		43.0		42.9
15	1.27 (m)	29.4	1.27 (m)	29.4	1.26 (m)	29.3
	2.43 (ddd, 4.4, 13.2, 14.0)		2.43 (ddd, 4.4, 13.2, 14.0)		2.40 (m)	
16	1.99 (m)	26.2	2.00 (m)	26.3	2.00 (m)	26.3
	3.01 (ddd, 4.4, 13.2, 13.2)		3.01 (ddd, 4.4, 13.2, 13.2)		3.02 (m)	
17		48.7		48.7		48.8
18	2.94 (br s)	54.5	2.94 (br s)	54.5	2.94 (br s)	54.5
19		72.7		72.8		72.7
20	1.34 (m)	42.1	1.35 (m)	42.1	1.36 (m)	42.1
21	1.22 (m)	26.7	1.28 (m)	26.7	1.23 (m)	26.7
	1.95 (m)		1.95 (m)		1.96 (m)	
22	1.84 (m)	37.6	1.85 (m)	37.6	1.85 (m)	37.6
	2.04 (m)		2.04 (m)		2.05 (m)	
23	1.55 (3H, s)	27.5	1.39 (3H, s)	27.6	1.33 (3H, s)	27.5
24	1.59 (3H, s)	25.1	1.42 (3H, s)	24.7	1.34 (3H, s)	24.4
25	1.26 (3H, s)	19.6	1.13 (3H, s)	19.4	1.06 (3H, s)	19.2
26	1.27 (3H, s)	17.5	1.24 (3H, s)	17.4	1.20 (3H, s)	17.4
27	1.80 (3H, s)	24.2	1.78 (3H, s)	24.2	1.72 (3H, s)	24.1
28		177.0		177.0		177.0
29	1.30 (3H, s)	27.1	1.30 (3H, s)	27.1	1.34 (3H, s)	27.1
30	1.02 (3H, d, 6.6)	16.6	1.04 (3H, d, 6.6)	16.6	1.05 (3H, d, 6.6)	16.6
28-*O*-Glc						
1′	6.24 (d, 8.3)	95.9	6.26 (d, 8.2)	95.9	6.26 (d, 8.2)	95.9
2′	4.18 (dd, 8.3, 8.8)	74.1	4.19 (dd, 8.2, 8.6)	74.1	4.19 (dd, 8.2, 8.4)	74.1
3′	4.27 (dd, 8.8, 9.0)	79.0	4.27 (dd, 8.6, 8.8)	79.0	4.27 (dd, 8.4, 8.6)	79.0
4′	4.30 (dd, 9.0, 9.2)	71.4	4.31 (dd, 8.8, 9.2)	71.4	4.31 (dd, 8.6, 9.4)	71.4
5′	4.03 (ddd, 2.6, 4.6, 9.2)	79.1	4.03 (ddd, 2.6, 4.6, 9.2)	79.2	4.03 (m)	79.2
6′	4.36 (dd, 4.6, 12.0)	62.5	4.37 (dd, 4.6, 12.0)	62.5	4.36 (m)	62.5
	4.45 (dd, 2.6, 12.0)		4.45 (dd, 2.6, 12.0)		4.45 (m)	
2-$COOCH_3$					3.54 (3H, s)	50.6
3-$COOCH_3$			3.68 (3H, s)	51.8	3.62 (3H, s)	51.7

Experimental

General experimental procedures

The following instruments were used to obtain physical data: specific rotations, Horiba SEPA-300 digital polarimeter (l = 5 cm); UV spectra, Shimadzu UV-1600 spectrometer; IR spectra, Shimadzu FTIR-8100 spectrometer; ^{1}H NMR spectra, JNM-ECA600 (600 MHz) and JNM-ECS400 (400 MHz) spectrometers; ^{13}C NMR spectra, JNM-ECA600 (150 MHz) and JNM-ECS400 (100 MHz) spectrometers with tetramethylsilane as an internal standard; FABMS and HRFABMS, JEOL JMS-SX 102A mass spectrometer; EIMS and HREIMS, JEOL JMS-GCMATE mass spectrometer; HPLC detector, Shimadzu RID-6A refractive index and SPD-10A UV–VIS detectors and Shodex OR-2 optical rotation detector; HPLC column, Cosmosil $5C_{18}$-MS-II (Nacalai Tesque, Inc., Kyoto, Japan, 4.6 mm i.d. × 250 mm and 20 mm i.d. × 250 mm) for analytical and preparative purposes, respectively, and Kaseisorb LC NH_2-60-5 (Tokyo Kasei Co., Ltd., Tokyo, Japan, 4.6 mm i.d. × 250 mm) for identification of sugar part.

The following experimental conditions were used for column chromatography (CC): highly porous synthetic resin, Diaion HP-20 (Mitsubishi Chemical Co., Tokyo, Japan); normal-phase silica gel CC, silica gel 60N (Kanto Chemical Co., Ltd., Tokyo, Japan; 63–210 mesh, spherical, neutral); reversed-phase ODS CC, Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., Aichi, Japan; 100–200 mesh); TLC, pre-coated TLC plates with silica gel $60F_{254}$ (Merck, Darmstadt, Germany, 0.25 mm) (normal-phase) and silica gel RP-18 WF_{254S} (Merck, Darmstadt, Germany, 0.25 mm) (reversed-phase); reversed-phase HPTLC, pre-coated TLC plates with silica gel RP-18 WF_{254S} (Merck, Darmstadt, Germany, 0.25 mm); detection was carried out by spraying 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4, followed by heating.

Plant material

Tuberous roots of *P. anserina* collected at Tibet Autonomous Region, China. The plant material was identified by one of the authors (M. Y.). A voucher specimen (20080109 Tibet-01) of this plant is on file in our laboratory.

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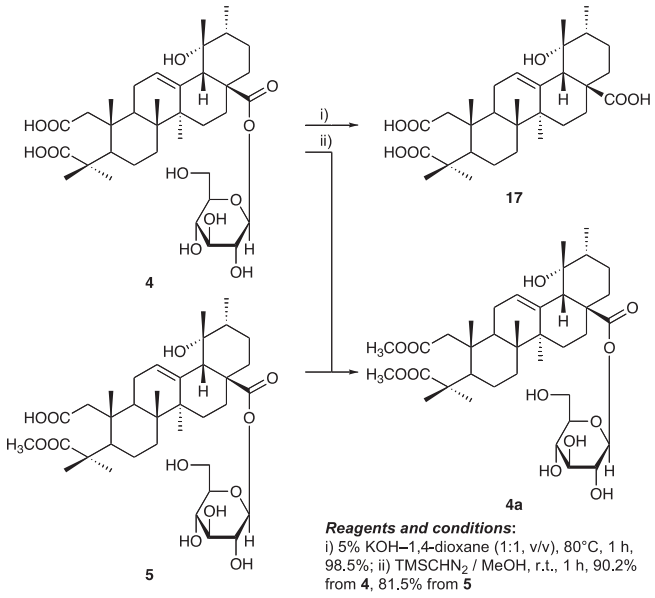

Fig. 3. Absolute stereostructures of **4** and **5**.

Extraction and isolation

Dried tuberous roots of *P. anserina* (9.7 kg) were finely cut and extracted 3 times with MeOH (20 L) under conditions of reflux for 3 h. Evaporation of the solvent under reduced pressure provided a MeOH extract (2.23 kg, 23.0%). An aliquot (2.13 kg) was partitioned with EtOAc–H_2O (1:1, v/v) to furnish an EtOAc-soluble fraction (53.3 g, 0.58%) and an aqueous phase. The latter was subjected to Diaion HP-20 CC (3.0 kg, H_2O → MeOH, twice) to give H_2O-eluted (1990.0 g, 21.50%) and MeOH-eluted (67.3 g, 0.73%) fractions. An aliquot (43.3 g) of the EtOAc-soluble fraction was subjected to normal-phase silica gel CC [1.3 kg, hexane–acetone (10:1 → 2:1 → 2:5, v/v) → MeOH] to give 11 fractions [Fr. 1 (1.11 g), Fr. 2 (5.08 g), Fr. 3 (7.12 g), Fr. 4 (871.1 mg), Fr. 5 (702.2 mg), Fr. 6 (943.2 mg), Fr. 7 (465.5 mg), Fr. 8 (2.74 g), Fr. 9 (3.58 g), Fr. 10 (8.00 g), and Fr. 11 (8.79 g)]. Fraction 4 (871.1 mg) was subjected to reversed-phase ODS CC [26 g, MeOH–H_2O (70:30 → 80:20 → 90:10, v/v) → MeOH → acetone] to give five fractions [Fr. 4-1 (68.7 mg), Fr. 4-2 (113.1 mg), Fr. 4-3 (345.2 mg), Fr. 4-4 (142.6 mg), and Fr. 4–5 (142.9 mg)]. Fraction 4-2 (113.1 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (80:20, v/v)] to give pomolic acid (**7**, 9.5 mg, 0.00013%), tormentic acid (**9**, 5.1 mg, 0.00007%), and 2-oxopomolic acid (**14**, 4.6 mg, 0.00006%). Fraction 5 (702.2 mg) was subjected to reversed-phase ODS CC [23 g, MeOH–H_2O (60:40 → 70:30 → 80:20 → 90:10, v/v) → MeOH → acetone] to give five fractions [Fr. 5-1 (101.5 mg), Fr. 5-2 (104.5 mg), Fr. 5-3 (217.8 mg), Fr. 5-4 (167.5 mg), and Fr. 5-5 (110.0 mg)]. Fraction 5-2 (104.5 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (70:30, v/v)] to give cacropiacic acid (**17**, 7.2 mg, 0.00010%). Fraction 5–3 (217.8 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (70:30, v/v)] to give euscaphic acid (**11**, 17.8 mg, 0.00024%), 2α,19α-dihydroxy-3-oxours-12-en-28-oic acid (**13**, 57.2 mg, 0.00077%), and **17** (5.3 mg, 0.00007%). Fraction 5-4 (167.5 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (80:20, v/v)] to give **7** (50.8 mg, 0.00068%), **11** (10.0 mg, 0.00013%), **13** (8.3 mg, 0.00011%), 2α-hydroxyursolic acid (**16**, 15.0 mg, 0.00020%), maslinic acid (**18**, 21.6 mg, 0.00029%), and alphitolic acid (**21**, 3.2 mg, 0.00004%). Fraction 8 (2.74 g) was subjected to reversed-phase ODS CC [240 g, MeOH–H_2O (10:90 → 30:70 → 70:30, v/v) → MeOH → acetone] to give eight fractions [Fr. 8-1 (368.0 mg), Fr. 8-2 (331.2 mg), Fr. 8-3 (240.0 mg), Fr. 8-4 (111.9 mg), Fr. 8-5 (550.1 mg), Fr. 8-6 (636.0 mg), Fr. 8-7 (297.2 mg), and Fr. 8-8 (168.7 mg)]. Fraction 8-1 (368.0 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (10:90, v/v)] to give gallic acid methyl ester (7.8 mg, 0.00010%) and (+)-gallocatehin (86.9 mg, 0.00116%). Fraction 8-3 (240.0 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (10:90, v/v)] to give (+)-catechin (37.2 mg, 0.00050%) and (+)-gallocatehin (20.0 mg, 0.00027%). Fraction 8-5 (550.1 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (50:50, v/v)] to give rosamutin (**10**, 112.3 mg, 0.00150%) and 24-deoxy-sericoside (**19**, 37.1 mg, 0.00050%). Fraction 8-6 (636.0 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (70:30, v/v)] to give **10** (203.3 mg, 0.00272%), kaji-ichigoside F1 (**12**, 143.9 mg, 0.00193%), and **19** (11.6 mg, 0.00016%). Fraction 9 (3.58 g) was subjected to reversed-phase ODS CC [130 g, MeOH–H_2O (50:50 → 70:30 → 80:20, v/v) → MeOH] to give six fractions [Fr. 9-1 (344.4 mg), Fr. 9-2 (111.1 mg), Fr. 9-3 (1.85 g), Fr. 9-4 (246.3 mg), Fr. 9-5 (61.6 mg), and Fr. 9-6 (360.2 mg)]. Fraction 9-1 (344.4 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (15:85, v/v)] to give

Table 5
^1H and ^{13}C NMR spectroscopic data (pyridine-d_5) of **6**.

	6	
Position	δ_H	δ_C
1	1.36 (m)	48.1
	2.39 (dd, 4.4, 12.4)	
2	4.24 (m)	69.2
3	4.18 (d, 9.4)	78.4
4		43.6
5	1.75 (br d, *ca.*11)	48.2
6	1.37 (m)	18.6
	1.67 (m)	
7	1.32 (br d, *ca.* 13)	34.9
	1.54 (m)	
8		41.9
9	1.71 (dd, 3.1, 12.7)	51.1
10		38.7
11	1.28 (m)	22.2
	1.54 (m)	
12	1.88 (m)	25.6
	2.81 (m)	
13		139.0
14		44.9
15	1.04 (m)	27.7
	2.10 (m)	
16	1.61 (m)	33.2
	2.25 (ddd, 3.2, 3.3, 13.4)	
17		49.4
18		127.9
19	2.48 (br d, *ca.* 14)	36.4
	2.80 (br d, *ca.* 14)	
20		38.4
21	1.41 (m)	32.0
	1.97 (br dd, *ca.* 13, 14)	
22	1.50 (br dd, *ca.* 13, 14)	35.7
	2.56 (br d, *ca.* 14)	
23	3.70 (d, 10.4)	66.7
	4.17 (d, 10.4)	
24	1.04 (3H, s)	14.2
25	0.98 (3H, s)	18.4
26	1.14 (3H, s)	18.1
27	1.12 (3H, s)	21.3
28		175.9
29	1.00 (3H, s)	20.3
30	3.60 (2H, s)	73.4
28-*O*-Glc		
1′	6.32 (d, 8.1)	96.2
2′	4.15 (dd, 8.1, 8.5)	74.3
3′	4.23 (m)	78.9
4′	4.24 (dd, 8.8, 9.2)	71.5
5′	4.00 (m)	79.2
6′	4.31 (dd, 4.8, 11.8)	62.5
	4.40 (dd, 2.4, 11.8)	

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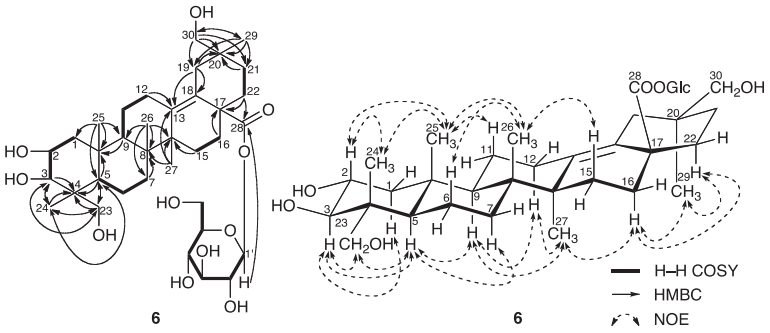

Fig. 4. Selected ^{1}H–^{1}H COSY, HMBC, and NOESY correlations of **6**.

Table 6
Inhibitory effects of the methanol extract, its fractions, and constituents on D-GalN-induced cytotoxicity in primary cultured mouse hepatocytes.

	Inhibition (%)					
	0 μg/mL	3 μg/mL	10 μg/mL	30 μg/mL	100 μg/mL	IC_{50} (μg/mL)
MeOH extract	0.0 ± 2.4	4.5 ± 1.1	6.2 ± 2.1	5.3 ± 3.1	16.2 ± 2.4[b]	
EtOAc-soluble fraction	0.0 ± 6.6	36.8 ± 6.4[b]	69.7 ± 6.0[b]	93.3 ± 4.2[b]	91.9 ± 5.6[b]	4.9 ± 1.1
MeOH-eluted fraction	0.0 ± 2.0	9.3 ± 1.5[a]	19.4 ± 1.9[b]	25.6 ± 1.7[b]	51.6 ± 2.6[b]	95.1 ± 7.7
H_2O-eluted fraction	0.0 ± 1.3	2.2 ± 2.1	−10.8 ± 9.6	−9.2 ± 7.0	−3.3 ± 10.4	

	Inhibition (%)					
	0 μM	3 μM	10 μM	30 μM	100 μM	IC_{50} (μM)
Potentillanoside A (**1**)	0.0 ± 3.7	18.5 ± 2.0[a]	25.0 ± 1.9[b]	38.5 ± 3.3[b]	65.6 ± 8.6[b]	46.7 ± 9.0
Potentillanoside D (**4**)	0.0 ± 0.6	0.1 ± 1.2	−0.2 ± 0.7	−0.9 ± 0.6	−0.2 ± 0.5	
Potentillanoside E (**5**)	0.0 ± 1.2	3.3 ± 0.5	5.5 ± 1.0	6.9 ± 0.6	14.6 ± 1.5[b]	
Pomolic acid (**7**)	0.0 ± 2.4	2.2 ± 4.1	−43.4 ± 1.2[c]	–[c]	–[c]	
28-*O*-Glc pomolic acid (**8**)	0.0 ± 2.5	32.3 ± 2.1[b]	52.3 ± 2.3[b]	66.2 ± 3.7[b]	–[c]	9.5 ± 1.3
Tormentic acid (**9**)	0.0 ± 0.7	9.2 ± 3.5	2.6 ± 1.6	2.7 ± 0.7	−5.8 ± 1.3	
Rosamutin (**10**)	0.0 ± 1.5	15.8 ± 0.6[b]	21.9 ± 1.7[b]	42.4 ± 4.7[b]	95.3 ± 3.3[b]	35.5 ± 9.8
Euscaphic acid (**11**)	0.0 ± 3.3	15.4 ± 3.5	11.0 ± 2.8	5.9 ± 2.1	3.8 ± 11.0	
Kaji-ichigoside F1 (**12**)	0.0 ± 3.3	39.1 ± 2.4[b]	45.3 ± 1.0[b]	56.9 ± 3.8[b]	51.1 ± 10.0[b]	14.1 ± 2.6
13	0.0 ± 1.7	−4.3 ± 5.1	−12.0 ± 3.4	−11.3 ± 0.5	−7.2 ± 4.4	
2-Oxopomolic acid 28-*O*-Glc (**15**)	0.0 ± 0.9	1.6 ± 0.7	4.0 ± 0.8	7.1 ± 1.0	19.1 ± 2.5[b]	
2α-Hydroxyursolic acid (**16**)	0.0 ± 2.0	14.3 ± 2.9	11.7 ± 4.0	–[c]	–[c]	
Cecropiacic acid (**17**)	0.0 ± 1.1	−0.5 ± 1.6	0.9 ± 1.9	5.5 ± 1.2	19.3 ± 2.6[b]	
Maslinic acid (**18**)	0.0 ± 0.6	−9.2 ± 3.2	−13.7 ± 3.0	−9.3 ± 2.0	–[c]	
24-Deoxy-sericoside (**19**)	0.0 ± 3.0	11.1 ± 1.1[a]	24.5 ± 0.7[b]	25.2 ± 4.2[b]	15.6 ± 1.5[b]	
Gallic acid	0.0 ± 1.2	10.8 ± 1.8	14.2 ± 2.8[a]	26.0 ± 5.6[b]	35.9 ± 7.5[b]	
Gallic acid methyl ester	0.0 ± 0.5	5.8 ± 0.5	14.2 ± 1.3[b]	33.1 ± 1.7[b]	65.8 ± 4.1[b]	53.7 ± 7.1
Ellagic acid	0.0 ± 0.5	6.0 ± 0.7	7.8 ± 0.4	6.0 ± 1.1	14.7 ± 1.4[b]	
Ellagic acid 4-*O*-Ara(f)	0.0 ± 1.1	5.2 ± 1.4	6.9 ± 0.2	8.4 ± 1.2	16.2 ± 3.3[b]	
(+)-Catechin	0.0 ± 2.6	14.1 ± 2.8[a]	34.9 ± 1.3[b]	66.6 ± 6.2[b]	104.8 ± 1.2[b]	17.7 ± 1.1
(+)-Gallocatechin	0.0 ± 3.2	18.3 ± 4.3[a]	26.7 ± 4.3[b]	76.2 ± 2.9[b]	99.0 ± 4.0[b]	18.4 ± 0.4
(+)-Catechin 7-*O*-Glc	0.0 ± 4.6	13.0 ± 2.8	19.1 ± 2.5[b]	34.2 ± 4.0[b]	64.5 ± 4.3[b]	55.4 ± 5.6
Quercetin 3-*O*-GlcA	0.0 ± 5.6	17.2 ± 5.8	34.8 ± 5.6[b]	46.7 ± 2.0[b]	63.1 ± 1.4[b]	34.2 ± 8.8
Quercatin 3-*O*-Xyl(1 → 2)Glc	0.0 ± 1.8	0.4 ± 3.8	7.6 ± 2.0	13.9 ± 4.3[a]	32.2 ± 2.1[b]	
6-*O*-*p*-Coumaroylsucrose	0.0 ± 10.6	1.1 ± 6.5	6.3 ± 6.4	12.1 ± 7.6	17.3 ± 4.9	
6-*O*-Feruloylsucrose	0.0 ± 0.4	2.8 ± 0.9	4.5 ± 1.1	10.9 ± 0.5[b]	26.1 ± 2.4[b]	
Silybin[d]	0.0 ± 0.3	4.8 ± 1.1	7.7 ± 0.7	45.2 ± 8.8[b]	77.0 ± 5.5[b]	38.8 ± 4.6

Each value represents the mean ± S.E.M. ($N = 4$).
Significantly different from the control.
[a] $p < 0.05$.
[b] $p < 0.01$.
[c] Cytotoxic effects were observed.
[d] Commercial silybin was purchased from Funakoshi Co., Ltd. (Tokyo, Japan) (Morikawa et al., 2010a).

gallic acid (23.9 mg, 0.00032%), gallic acid methyl ester (5.5 mg, 0.00007%), (+)-catechin (6.3 mg, 0.00008%), and (+)-gallocatechin (81.7 mg, 0.00110%). Fraction 9-3 (530.0 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (55:45, v/v)] to give potentillanosides A (**1**, 273.9 mg, 0.01282%) and B (**2**, 14.3 mg, 0.00067%), **10** (46.1 mg, 0.00216%) and **12** (57.0 mg, 0.00267%). Fraction 9-4 (246.3 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (60:40, v/v)] to give potentillanoside C (**3**, 3.8 mg, 0.00005%), 28-*O*-β-D-glucopyranosyl pomolic acid (**8**, 24.6 mg, 0.00033%), **10** (115.8 mg, 0.00155%), and **19** (14.6 mg, 0.00020%). Fraction 9-5 (61.6 mg) was subjected to HPLC [Cosmosil $5C_{18}$-MS-II, MeOH–1% aqueous AcOH (70:30, v/v)] to give **10** (9.2 mg, 0.00012%) and **12** (10.8 mg, 0.00014%). Fraction 10 (8.00 g) was subjected to reversed-phase ODS CC [240 g, MeOH-H_2O (50:50 → 60:40 → 80:20, v/v) → MeOH] to give seven fractions [Fr. 10-1 (967.4 mg), Fr. 10-2 (233.0 mg), Fr. 10-3 (195.8 mg), Fr. 10-4 (22.7 mg), Fr. 10-5 (5.97 g), Fr. 10-6 (308.1 mg), and Fr. 10-7 (191.1 mg)]. Fraction 10-3 (195.8 mg) was subjected to HPLC

Table 7
Inhibitory effects of the methanol extract, its fractions, and constituents on LPS-activated NO production in mouse peritoneal macrophages.

	Inhibition (%)					
	0 μg/mL	3 μg/mL	10 μg/mL	30 μg/mL	100 μg/mL	IC_{50} (μg/mL)
MeOH extract	0.0 ± 2.3	9.7 ± 2.5	7.5 ± 2.9	11.2 ± 3.5	14.9 ± 2.8[b]	
EtOAc-soluble fraction	0.0 ± 3.3	4.0 ± 1.1	11.4 ± 5.7	11.2 ± 5.5	58.2 ± 4.3[b]	70.1 ± 3.8
MeOH-eluted fraction	0.0 ± 6.4	10.3 ± 7.3	32.6 ± 4.7[b]	32.5 ± 3.2[b]	41.4 ± 4.4[b]	
H_2O-eluted fraction	0.0 ± 4.2	9.7 ± 5.3	−12.0 ± 13.0	−25.6 ± 19.3	−0.1 ± 4.3	
	Inhibition (%)					
	0 μM	3 μM	10 μM	30 μM	100 μM	IC_{50} (μM)
Potentillanoside A (**1**)	0.0 ± 3.1	2.5 ± 3.2	0.9 ± 2.8	5.1 ± 2.6	6.2 ± 1.1	
Potentillanoside D (**4**)	0.0 ± 3.0	−1.5 ± 1.6	1.2 ± 1.4	1.4 ± 2.7	12.2 ± 2.1	
Potentillanoside E (**5**)	0.0 ± 4.1	3.6 ± 1.6	4.7 ± 3.8	8.2 ± 0.6	17.6 ± 2.0[b]	
Pomolic acid (**7**)	0.0 ± 3.0	2.9 ± 0.6	13.0 ± 2.0[b]	45.2 ± 2.2[b]	83.9 ± 1.6[b,c]	33.1 ± 2.0
28-*O*-Glc pomolic acid (**8**)	0.0 ± 2.5	0.5 ± 6.6	-2.2 ± 4.9	13.1 ± 0.9	55.3 ± 2.4[b]	91.9 ± 2.8
Tormentic acid (**9**)	0.0 ± 2.9	1.0 ± 1.6	1.9 ± 5.3	17.1 ± 1.6[b]	82.7 ± 0.3[b,c]	68.4 ± 2.2
Rosamutin (**10**)	0.0 ± 3.2	−1.3 ± 2.0	−3.1 ± 3.3	−7.7 ± 3.0	−0.1 ± 0.9	
Euscaphic acid (**11**)	0.0 ± 4.0	−3.2 ± 3.5	−6.4 ± 2.2	−4.0 ± 3.4	17.9 ± 0.8[b]	
Kaji-ichigoside F1 (**12**)	0.0 ± 4.7	−0.5 ± 0.8	2.1 ± 2.1	0.8 ± 1.5	8.7 ± 2.6	
13	0.0 ± 2.2	3.6 ± 1.0	4.4 ± 1.3	9.6 ± 1.1	26.1 ± 0.5[b]	
2-Oxopomolic acid 28-*O*-Glc (**15**)	0.0 ± 2.5	0.5 ± 2.2	4.5 ± 1.9	3.4 ± 2.8	10.9 ± 0.7[b]	
2α-Hydroxyursolic acid (**16**)	0.0 ± 0.6	7.5 ± 0.4	−3.8 ± 1.8	51.8 ± 0.9[b]	91.3 ± 1.1[b,c]	21.1 ± 3.0
Cecropiacic acid (**17**)	0.0 ± 3.1	−0.5 ± 2.9	−2.6 ± 3.2	4.5 ± 3.1	21.0 ± 2.4[b]	
Maslinic acid (**18**)	0.0 ± 6.2	−6.4 ± 3.0	2.5 ± 7.9	51.6 ± 2.5[b]	100.0 ± 1.1[b,c]	30.1 ± 2.7
24-Deoxy-sericoside (**19**)	0.0 ± 5.0	10.0 ± 10.4	8.9 ± 1.9	7.9 ± 3.4	15.3 ± 0.9[b]	
Gallic acid	0.0 ± 2.5	−1.4 ± 1.2	−8.0 ± 2.0	−4.2 ± 2.5	17.1 ± 2.4[b]	
Gallic acid methyl ester	0.0 ± 2.7	−1.5 ± 3.9	3.2 ± 2.2	12.8 ± 2.7[a]	31.0 ± 1.6[b]	
Ellagic acid	0.0 ± 2.7	6.5 ± 2.0	1.5 ± 2.3	−6.7 ± 1.7	8.5 ± 2.5	
Ellagic acid 4-*O*-Ara(f)	0.0 ± 2.9	3.6 ± 2.8	3.6 ± 2.2	1.9 ± 0.8	11.0 ± 1.4[a]	
Ducheside B	0.0 ± 2.7	1.7 ± 1.8	−3.0 ± 1.8	0.8 ± 3.0	0.3 ± 1.6	
(+)-Catechin	0.0 ± 3.0	−5.9 ± 3.2	-−3.6 ± 1.4	−0.3 ± 0.4	6.7 ± 1.6	
(+)-Gallocatechin	0.0 ± 2.9	0.4 ± 3.3	0.4 ± 1.9	0.4 ± 2.2	13.3 ± 0.9[b]	
(+)-Catechin 7-*O*-Glc	0.0 ± 2.9	5.8 ± 1.3	4.6 ± 1.9	1.8 ± 0.7	4.5 ± 1.3	
Quercetin 3-*O*-GlcA	0.0 ± 0.4	3.5 ± 2.4	3.3 ± 2.1	0.4 ± 2.0	7.3 ± 2.9	
Quercatin 3-*O*-Xyl(1 → 2)Glc	0.0 ± 2.5	2.0 ± 1.0	0.3 ± 2.4	2.4 ± 2.8	7.4 ± 1.5	
6-*O*-*p*-Coumaroylsucrose	0.0 ± 2.0	4.6 ± 2.2	6.1 ± 2.3	7.9 ± 1.7	7.3 ± 2.3	
6-*O*-Feruloylsucrose	0.0 ± 3.1	−0.9 ± 4.0	4.0 ± 1.9	4.7 ± 2.2	9.0 ± 0.6	
L-NMMA[d]	0.0 ± 3.1	1.4 ± 2.8	19.9 ± 2.8[b]	43.0 ± 2.1[b]	70.9 ± 1.6[b]	36.0 ± 2.9
CAPE[d]	0.0 ± 2.1	5.9 ± 5.2	44.4 ± 3.2[b]	86.2 ± 1.1[b]	99.6 ± 0.1[b,c]	11.0 ± 2.1

Each value represents the mean ± S.E.M. ($N = 4$).
Significantly different from the control.
[a] $p < 0.05$.
[b] $p < 0.01$.
[c] Cytotoxic effects were observed.
[d] Commercial L-NMMA and CAPE were purchased from Sigma–Aldrich Chemical (St. Louis, MO, USA) (Morikawa et al., 2011).

[MeOH–1% aqueous AcOH (35:65, v/v)] to give ellagic acid (21.3 mg, 0.00029%). Fraction 10-5 (125.0 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (55:45, v/v)] to give **10** (79.8 mg, 0.05114%), **12** (5.9 mg, 0.00378%), and **19** (15.4 mg, 0.00987%).

The MeOH-eluted fraction (55.0 g) was subjected to normal-phase silica gel CC [2.0 kg, $CHCl_3$–MeOH–H_2O (15:3:0.3 → 10:3:0.4 → 6:4:1 → 5:5:1, v/v/v) → MeOH] to give eight fractions [Fr. 1 (20.44 g), Fr. 2 (8.47 g), Fr. 3 (2.21 g), Fr. 4 (4.05 g), Fr. 5 (3.49 g), Fr. 6 (5.49 g), Fr. 7 (3.11 g), and Fr. 8 (2.53 g)]. Fraction 1 (20.44 g) was subjected to reversed-phase ODS CC [620 g, MeOH–H_2O (60:40 → 70:30 → 75:25 → 90:10, v/v) → MeOH → acetone] to give 10 fractions [Fr. 1-1 (300.2 mg), Fr. 1-2 (104.9 mg), Fr. 1-3 (552.2 mg), Fr. 1-4 (118.8 mg), Fr. 1-5 (737.4 mg), Fr. 1-6 (492.1 mg), Fr. 1-7 (3.03 g), Fr. 1-8 (6.31 g), Fr. 1-9 (3.84 g), and Fr. 1-10 (2.14 g)]. Fraction 1-2 (104.9 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (55:45, v/v)] to give potentillanoside D (**4**, 28.8 mg, 0.00038%). Fraction 1-3 (552.2 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (60:40, v/v)] to give potentillanoside E (**5**, 44.6 mg, 0.00059%), **10** (216.5 mg, 0.00287%), 2-oxopomolic acid 28-*O*-β-D-glucopyranosyl ester (**15**, 36.8 mg, 0.00049%), and **17** (22.3 mg, 0.00030%). Fraction 1-4 (118.8 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (60:40, v/v)] to give **4** (5.9 mg, 0.00008%). Fraction 1-5 (737.4 mg) was recrystalized with MeOH to give **9** (553.2 mg, 0.00734%). Fraction 1-6 (492.1 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (80:20, v/v)] to give **7** (141.3 mg, 0.00188%). Fraction 2 (8.47 g) was separated by reversed-phase ODS CC [255 g, MeOH-H_2O (10:90 → 30:70 → 40:60 → 50:50 → 60:40, v/v) → MeOH → acetone] to give eight fractions [Fr. 2-1 (379.5 mg), Fr. 2-2 (581.0 mg), Fr. 2-3 (376.5 mg), Fr. 2-4 (123.6 mg), Fr. 2-5 (331.3 mg), Fr. 2-6 (204.5 mg), Fr. 2-7 (3.37 g), and Fr. 2-8 (2.21 g)]. Fraction 2–3 (376.5 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (20:80, v/v)] to give ducheside B (75.0 mg, 0.00100%). Fraction 2–5 (331.3 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (50:50, v/v)] to give **5** (58.4 mg, 0.00077%) and arjunglucoside I (**20**, 7.6 mg, 0.00010%). Fraction 2–6 (204.5 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (55:45, v/v)] to give **5** (9.4 mg, 0.00012%), **10** (57.0 mg, 0.00076%), and **19** (11.4 mg, 0.00015%). Fraction 3 (2.21 g) was separated by reversed-phase ODS CC [70 g, MeOH–H_2O (10:90 → 30:70 → 40:60 → 60:40 → 90:10, v/v) → MeOH → acetone] to give eight fractions [Fr. 3-1 (230.4 mg), Fr. 3-2 (252.7 mg), Fr. 3-3 (42.4 mg), Fr. 3–4 (82.1 mg), Fr. 3–5 (114.0 mg), Fr. 3–6 (159.3 mg), Fr. 3–7 (381.1 mg), and Fr. 3–8 (268.7 mg)]. Fraction 3–1 (230.4 mg) was subjected to HPLC

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Table 8
Inhibitory effects of the methanol extract, its fractions, and constituents on TNF-α-induced cytotoxicity in L929 cells.

	Inhibition (%)					
	0 μg/mL	3 μg/mL	10 μg/mL	30 μg/mL	100 μg/mL	IC_{50} (μg/mL)
MeOH extract	0.0 ± 1.4	9.7 ± 1.3	5.4 ± 2.1	3.6 ± 1.0	2.7 ± 2.0	
EtOAc-soluble fraction	0.0 ± 0.9	4.1 ± 0.6	6.7 ± 2.8	58.3 ± 1.8[b]	59.0 ± 2.6[b]	30.8 ± 3.3
MeOH-eluted fraction	0.0 ± 1.8	9.4 ± 1.8	9.3 ± 2.0	16.5 ± 1.3[b]	33.8 ± 3.3[b]	
H_2O-eluted fraction	0.0 ± 1.4	2.9 ± 1.0	2.9 ± 1.3	2.3 ± 1.1	0.2 ± 0.7	
	Inhibition (%)					
	0 μM	3 μM	10 μM	30 μμM	100 μM	IC_{50} (μM)
Potentillanoside A (**1**)	0.0 ± 0.2	2.9 ± 1.6	3.0 ± 0.4	5.9 ± 2.0	12.9 ± 1.4[b]	
Potentillanoside D (**4**)	0.0 ± 1.0	1.0 ± 0.4	1.3 ± 0.2	2.0 ± 0.3	3.0 ± 0.7	
Potentillanoside E (**5**)	0.0 ± 0.7	2.0 ± 0.9	0.3 ± 0.3	1.8 ± 0.3	3.4 ± 0.4	
Pomolic acid (**7**)	0.0 ± 0.6	0.5 ± 1.7	−9.9 ± 0.4	−11.3 ± 0.4	−10.7 ± 0.5	
28-*O*-Glc pomolic acid (**8**)	0.0 ± 1.1	4.8 ± 2.0	12.8 ± 0.4[b]	61.9 ± 2.3[b]	13.1 ± 2.7[b]	25.5 ± 1.6
Tormentic acid (**9**)	0.0 ± 0.4	1.1 ± 0.2	2.2 ± 0.4	4.8 ± 0.7	−3.8 ± 0.6	
Rosamutin (**10**)	0.0 ± 0.9	0.8 ± 1.2	1.8 ± 1.3	2.0 ± 1.0	7.2 ± 1.3	
Euscaphic acid (**11**)	0.0 ± 0.5	0.6 ± 0.7	6.5 ± 1.3	8.2 ± 1.7	12.9 ± 1.2[b]	
Kaji-ichigoside F1 (**12**)	0.0 ± 0.3	1.3 ± 0.5	1.7 ± 0.4	2.3 ± 1.6	7.1 ± 1.1	
13	0.0 ± 1.1	2.1 ± 0.7	3.8 ± 1.2	5.6 ± 1.0	14.3 ± 1.8[b]	
2-Oxopomolic acid 28-*O*-Glc (**15**)	0.0 ± 0.5	2.4 ± 0.5	1.9 ± 0.6	2.7 ± 0.6	4.1 ± 0.5	
2α-Hydroxyursolic acid (**16**)	0.0 ± 0.3	0.5 ± 0.8	3.9 ± 1.3	−7.6 ± 0.4	−8.0 ± 0.5	
Cecropiacic acid (**17**)	0.0 ± 0.7	1.1 ± 0.6	0.7 ± 0.4	2.2 ± 0.9	5.3 ± 0.4	
Maslinic acid (**18**)	0.0 ± 0.5	1.9 ± 1.0	3.1 ± 0.6	10.7 ± 2.5	−8.6 ± 0.1	
24-Deoxy-sericoside (**19**)	0.0 ± 1.3	−1.0 ± 0.9	3.7 ± 0.7	3.1 ± 0.9	5.9 ± 1.4	
Gallic acid	0.0 ± 0.9	2.1 ± 1.4	0.2 ± 1.6	2.8 ± 0.5	−8.5 ± 0.1	
Gallic acid methyl ester	0.0 ± 1.4	−0.2 ± 0.9	3.1 ± 1.4	14.8 ± 2.0[b]	17.1 ± 1.1[b]	
Ellagic acid	0.0 ± 0.3	1.1 ± 0.2	13.7 ± 1.5[b]	21.8 ± 2.7[b]	6.7 ± 0.3	
Ellagic acid 4-*O*-Ara(f)	0.0 ± 0.2	2.1 ± 0.5	2.0 ± 0.4	2.0 ± 0.4	0.1 ± 0.8	
Ducheside B	0.0 ± 0.2	1.8 ± 0.5	0.9 ± 0.6	2.5 ± 0.9	2.1 ± 0.8	
(+)-Catechin	0.0 ± 1.1	2.1 ± 0.5	3.4 ± 0.4	4.6 ± 0.9	13.4 ± 1.9[b]	
(+)-Gallocatechin	0.0 ± 1.1	1.7 ± 0.9	2.6 ± 1.1	11.3 ± 2.4	−6.9 ± 0.1	
(+)-Catechin 7-*O*-Glc	0.0 ± 0.5	3.6 ± 0.9	4.4 ± 0.6	6.9 ± 0.8	10.2 ± 0.7[a]	
Quercetin 3-*O*-GlcA	0.0 ± 1.3	0.8 ± 0.6	−0.2 ± 0.9	0.6 ± 1.2	28.5 ± 3.5[b]	
Quercatin 3-*O*-Xyl(1 → 2)Glc	0.0 ± 0.6	0.0 ± 1.2	3.7 ± 0.5	2.9 ± 0.7	4.0 ± 0.6	
6-*O*-*p*-Coumaroylsucrose	0.0 ± 0.4	2.5 ± 0.5	2.6 ± 1.6	3.5 ± 0.5	1.8 ± 0.5	
6-*O*-Feruloylsucrose	0.0 ± 0.6	2.6 ± 1.0	2.2 ± 0.4	2.6 ± 0.5	2.6 ± 0.3	
Silybin[c]	0.0 ± 2.6	5.3 ± 2.8	22.0 ± 3.8[b]	48.0 ± 4.1[b]	50.8 ± 3.9[b]	60.4 ± 3.8

Each value represents the mean ± S.E.M. ($N = 4$).
Significantly different from the control.
[a] $p < 0.05$.
[b] $p < 0.01$.
[c] Commercial silybin was purchased from Funakoshi Co., Ltd. (Tokyo, Japan) (Morikawa et al., 2010a).

[MeOH–1% aqueous AcOH (5:95, v/v)] to give (+)-gallocatechin (24.0 mg, 0.00032%). Fraction 4 (4.05 g) was subjected to reversed-phase ODS CC [125 g, MeOH–H_2O (20:80 → 30:70 → 40:60 → 60:40 → 95:5, v/v) → MeOH → acetone] to give seven fractions [Fr. 4-1 (238.5 mg), Fr. 4-2 (334.3 mg), Fr. 4-3 [= L-tryptophan (1.17 g, 0.0155%)], Fr. 4-4 (295.6 mg), Fr. 4–5 (334.9 mg), Fr. 4–6 (128.6 mg), and Fr. 4–7 (1.30 g)]. Fraction 4-4 (295.6 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (20:80, v/v)] to give 6-*O*-*p*-coumaroylsucrose (50.6 mg, 0.00067%) and 6-*O*-feruloylsucrose (8.2 mg, 0.00011%). Fraction 4–5 (334.9 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (30:70, v/v)] to give quercetin 3-*O*-sambubioside (11.0 mg, 0.00015%). Fraction 4–6 (128.6 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (30:70, v/v)] to give potentillanoside F (**6**, 18.3 mg, 0.00024%) and ellagic acid 4-*O*-α-L-arabinofuranoside (22.6 mg, 0.00030%). Fraction 5 (3.49 g) was separated by reversed-phase ODS CC [110 g, MeOH–H_2O (5:95 → 20:80 → 50:50 → 80:20, v/v) → MeOH → acetone] to give eight fractions [Fr. 5-1 (210.8 mg), Fr. 5-2 (223.7 mg), Fr. 5-3 (523.8 mg), Fr. 5-4 (358.5 mg), Fr. 5-5 (275.2 mg), Fr. 5–6 (345.4 mg), Fr. 5–7 (402.2 mg), and Fr. 5–8 (448.5 mg)]. Fraction 5–3 (523.8 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (5:95, v/v)] to give (+)-catechin 7-*O*-β-D-glucopyranoside (114.3 mg, 0.00152%). Fraction 5-4 (358.5 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (30:70, v/v)] to give ellagic acid 4-*O*-α-L-arabinofuranoside (10.4 mg, 0.00014%) and quercetin 3-*O*-β-D-glucuronopyranosiduronic acid (13.7 mg, 0.00018%). Fraction 5-5 (275.2 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (30:70, v/v)] to give **6** (10.4 mg, 0.00014%), ellagic acid (24.3 mg, 0.00032%), ellagic acid 4-*O*-α-L-arabinofuranoside (14.5 mg, 0.00019%), and quercetin 3-*O*-β-D-xylopyranosyl-(1 → 2)-β-D-glucopyranoside-3′-*O*-β-D-glucopyranoside (10.5 mg, 0.00014%). Fraction 6 (5.49 g) was separated by reversed-phase ODS CC [180 g, MeOH–H_2O (10:90 → 30:70 → 50:50, v/v) → MeOH → acetone] to give seven fractions [Fr. 6-1 (2.36 g), Fr. 6-2 (490.0 mg), Fr. 6-3 (757.1 mg), Fr. 6-4 (409.5 mg), Fr. 6-5 (155.0 mg), Fr. 6-6 (1.03 g), and Fr. 6-7 (423.0 mg)]. Fraction 6-3 (500.0 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (35:65, v/v)] to give ellagic acid 4-*O*-α-L-arabinofuranoside (9.4 mg, 0.00019%). Fraction 6-4 (409.5 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (30:70, v/v)] to give ellagic acid (10.0 mg, 0.00013%), and ellagic acid 4-*O*-α-L-arabinofuranoside (14.0 mg, 0.00019%). Fraction 6-5 (155.0 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (35:65, v/v)] to give **6** (6.8 mg, 0.00009%), ellagic acid (5.0 mg, 0.00007%), and ellagic acid 4-*O*-α-L-arabinofuranoside (4.7 mg, 0.00006%).

*Potentillanoside A (**1** = 2α,19α-dihydroxy-3-oxours-12-en-28-oic acid 28-O-β-D-glucopyranosyl ester)*

Amorphous powder, $[\alpha]_D^{26}$ + 20.4 (*c* 1.14, MeOH); IR (KBr) υ_{max} - cm^{-1}: 3470, 1725, 1686, 1655, 1073; For ^1H and ^{13}C NMR spectro-

Table 9
Inhibitory effects of potentillanoside A (**1**), pomolic acid (**7**), tormentic acid (**9**), rosamutin (**10**), and kaji-ichigoside F1 (**12**) on D-GalN/LPS-induced liver injuries in mice.

Treatment	Dose (mg/kg, p.o.)	*n*	sAST		sALT	
			(Karmen Unit)	Inhibition (%)	(Karmen Unit)	Inhibition (%)
Normal (vehicle)	–	8	107 ± 9[b]	–	20 ± 2[b]	–
Control (D-GalN/LPS)	–	14	12579 ± 1795	–	7118 ± 1077	–
Potentillanoside A (**1**)	50	7	5670 ± 1817[a]	55.0	3137 ± 1106[b]	56.8
	100	8	2931 ± 959[b]	76.8	1618 ± 568[b]	78.4
Rosamutin (**10**)	50	6	9821 ± 2097	22.0	5434 ± 1163	24.0
	100	8	4386 ± 1339[a]	65.2	2134 ± 684[b]	71.1
Control (D-GalN/LPS)	–	12	10236 ± 1360	–	5595 ± 760	–
Pomolic acid (**7**)	50	7	6593 ± 1470	36.0	3998 ± 750	28.6
	100	8	6443 ± 1976	37.4	4338 ± 1477	22.5
Tormentic acid (**9**)	50	6	8505 ± 3212	17.1	5869 ± 2348	−4.9
	100	6	9733 ± 1710	5.0	5702 ± 1043	−1.9
Kaji-ichigoside F1 (**12**)	50	7	7365 ± 2434	28.3	4412 ± 1399	21.2
	100	8	4191 ± 681[a]	59.7	2408 ± 449[a]	57.2
Control (D-GalN/LPS)	–	10	6605 ± 1985	–	6033 ± 1647	–
Curcumin[c]	12.5	10	5024 ± 1189	24.0	4770 ± 1218	21.1
	25	10	3253 ± 981	50.9	3177 ± 979	47.8
	50	9	1916 ± 483[a]	71.2	2220 ± 563[a]	63.8

Each value represents the mean ± S.E.M.
Significantly different from the control.
[a] $p < 0.05$.
[b] $p < 0.01$.
[c] Morikawa et al. (2002).

scopic data, see Tables 2 and 3; Positive-ion FABMS *m/z*: 671 $[M+Na]^+$; HRFABMS *m/z*: 671.3767 $[M+Na]^+$ (calcd for $C_{36}H_{56}O_{10}Na$, 671.3771).

*Potentillanoside B (**2** = 3α,19α-dihydroxy-2-oxours-12-en-28-oic acid 28-O-β-D-glucopyranosyl ester)*

Amorphous powder, $[\alpha]_D^{21}$ + 29.8 (*c* 0.62, MeOH); IR (KBr) υ_{max} cm^{-1}: 3470, 1719, 1686, 1655, 1075; For ^1H and ^{13}C NMR spectroscopic data, see Tables 2 and 3; Positive-ion FABMS *m/z*: 671 $[M+Na]^+$; HRFABMS *m/z*: 671.3764 $[M+Na]^+$ (calcd for $C_{36}H_{56}O_{10}Na$, 671.3771).

*Potentillanoside C (**3** = 2α,3β,19α-trihydroxyurs-12-en-28-oic acid 28-O-6-O-acetyl-β-D-glucopyranosyl ester)*

Amorphous powder, $[\alpha]_D^{21}$ + 12.8 (*c* 0.13, MeOH); IR (KBr) υ_{max} cm^{-1}: 3470, 1721, 1686, 1655, 1076; For ^1H and ^{13}C NMR spectroscopic data, see Tables 2 and 3; Positive-ion FABMS *m/z*: 715 $[M+Na]^+$; HRFABMS *m/z*: 715.4042 $[M+Na]^+$ (calcd for $C_{38}H_{60}O_{11}Na$, 715.4033).

*Potentillanoside D (**4** = 19α-hydroxy-2,3-secours-12-en-2,3,28-trioic acid 28-O-β-D-glucopyranosyl ester)*

Amorphous powder, $[\alpha]_D^{28}$ + 22.6 (*c* 0.15, MeOH); IR (KBr) υ_{max} cm^{-1}: 3470, 1725, 1655, 1075; ^1H and ^{13}C NMR spectroscopic data, see Tables 2 and 3; Positive-ion FABMS *m/z*: 703 $[M+Na]^+$; HRFABMS *m/z*: 703.3676 $[M+Na]^+$ (calcd for $C_{36}H_{56}O_{12}Na$, 703.3669).

*Potentillanoside E (**5** = 19α-hydroxy-2,3-secours-12-en-2,3,28-trioic acid 3-methyl-28-O-β-D-glucopyranosyl ester)*

Amorphous powder, $[\alpha]_D^{29}$ + 22.2 (*c* 0.13, MeOH); IR (KBr) υ_{max} cm^{-1}: 3470, 1720, 1655, 1075; For ^1H and ^{13}C NMR spectroscopic data, see Tables 2 and 3; Positive-ion FABMS *m/z*: 717 $[M+Na]^+$; HRFABMS *m/z*: 717.3822 $[M+Na]^+$ (calcd for $C_{37}H_{58}O_{12}Na$, 717.3826).

*Potentillanoside F (**6** = 2α,3β,30-trihydroxy-olean-13-en-28-oic acid 28-O-β-D-glucopyranosyl ester)*

Amorphous powder, $[\alpha]_D^{24}$ + 46.9 (*c* 0.10, MeOH); IR (KBr) υ_{max} cm^{-1}: 3400, 1725, 1655, 1073; ^1H and ^{13}C NMR spectroscopic data, see Tables 2 and 3; Positive-ion FABMS *m/z*: 689 $[M+Na]^+$; HRFABMS *m/z*: 689.3877 $[M+Na]^+$ (calcd for $C_{36}H_{58}O_{11}Na$, 689.3883).

*$NaBH_4$ Reduction of potentillanosides A (**1**) and B (**2**)*

To a solution of **1** (9.5 mg) in MeOH (1.0 mL) was added $NaBH_4$ (0.6 mg) and the mixture was stirred at 0 °C for 1 h. The reaction was quenched with acetone, and following removal of the solvent under reduced pressure gave a residue, which was purified by HPLC [Cosmosil $5C_{18}$-MS-II, MeOH–1% aqueous AcOH (60:40, v/v)] to give rosamutin (**10**, 6.5 mg, 68.2%) and kaji-ichigoside F1 (**12**, 3.0 mg, 31.5%). In a similar manner, **12** (2.0 mg, 50.0%) and its 2-epimer (0.8 mg, 19.9%) were obtained from **2** (4.0 mg) using $NaBH_4$ (0.3 mg).

*Acid hydrolysis of potentillanosides A (**1**), B (**2**), D (**4**), and F (**6**)*

A solution of either **1**, **2**, **4** or **6** (each 2.0 mg) in 5% aqueous H_2SO_4–1,4-dioxane (1:1, v/v, 1.0 mL) was heated at 80 °C for 3 h. After being cooled, the reaction mixture was neutralized with Amberlite IRA-400 (OH^- form) and the resins were filtered. In each case removal of the solvent under reduced pressure gave a residue, which was partitioned in an EtOAc–H_2O (1:1, v/v) mixture to give an EtOAc-soluble fraction and an aqueous phase. The aqueous layer was subjected to HPLC analysis under following conditions: column, Kaseisorb LC NH_2-60-5, 4.6 mm i.d. × 250 mm (Tokyo Kasei Co., Ltd., Tokyo, Japan); detection, optical rotation [Shodex OR-2 (Showa Denko Co., Ltd., Tokyo, Japan); mobile phase, CH_3CN-H_2O (80:20, v/v); flow rate 1.0 mL/min]. Identification of D-glucose present in the aqueous layer was carried out by comparing the retention time and the optical rotation with the standard [t_R: 18.7 min (positive optical rotation)].

*Deacetylation of potentillanoside C (**3**)*

Solution of **3** (2.3 mg) in 0.5% NaOMe–MeOH (1.5 mL) was stirred at room temperature for 1 h. The reaction mixture was neutralized with Dowex HCR-W2 (H^+ form) and the resins were removed

by filtration. Evaporation of the solvent from the filtrate under reduced pressure gave a residue, which was purified by HPLC [Cosmosil $5C_{18}$-MS-II, MeOH–1% aqueous AcOH (60:40, v/v)] to give **10** (2.1 mg, 97.2%).

*Alkaline hydrolysis of potentillanoside D (**4**)*

A mixture of **4** (6.8 mg), 1,4-dioxane (1.0 mL) and 5% aqueous KOH (1.0 mL) was stirred at 80 °C for 1 h. After being cooled, the reaction mixture was neutralized with Dowex HCR W2 (H^+ form), and the resins were removed by filtration. Removal of the solvent under reduced pressure gave a residue, which was partitioned in an EtOAc–H_2O (1:1, v/v) mixture to give an EtOAc-soluble fraction and an aqueous phase. The EtOAc-soluble fraction was subjected to HPLC [Cosmosil $5C_{18}$-MS-II, MeOH–1% aqueous AcOH (70:30, v/v)] to give cecropiacic acid (**17**, 5.1 mg, 98.5%).

*Methylation of potentillanosides D (**4**) and E (**5**) with $TMSCHN_2$*

A solution of **4** (3.3 mg) and trimethylsilyldiazomethane ($TMSCHN_2$, 10% in hexane, *ca.* 0.2 mL) in MeOH (0.5 mL) was stirred at room temperature for 1 h. Removal of the solvent under reduced pressure gave **4a** (3.1 mg, 90.2%). In a similar manner, **5** (3.3 mg) wad derived to **4a** (2.8 mg, 81.5%).

*Compound **4a***

An amorphous powder, $[\alpha]_D^{21}$ + 7.2 (*c* 0.32, MeOH); for ^1H and ^{13}C NMR spectroscopic data, see Tables 2 and 3; Positive-ion FABMS *m/z*: 731 $[M+Na]^+$; HRFABMS *m/z*: 731.3979 $[M+Na]^+$ (calcd for $C_{38}H_{60}O_{12}Na$, 731.3982).

Bioassay

Reagents

LPS (from *Salmonella enteritidis*), minimum essential medium (MEM), and William's E medium were purchased from Sigma–Aldrich Chemical (St. Louis, MO, USA); fetal bovine serum (FBS) was from Life Technologies (Rockville, MD, USA); and other chemicals were from Wako Pure Chemical Industries, Co., Ltd. (Osaka, Japan). 96-Well microplates were purchased from Sumitomo Bakelite Co., Ltd. (Tokyo, Japan).

Animals

Male ddY mice were purchased from Kiwa Laboratory Animal Co., Ltd., (Wakayama, Japan). The animals were housed at a constant temperature of 23 ± 2 °C and were fed a standard laboratory chow (MF, Oriental Yeast Co., Ltd., Tokyo, Japan). All the experiments were performed with conscious mice unless otherwise noted. The experimental protocol was approved by the Experimental Animal Research Committee of Kinki University.

Effects on D-GalN/LPS-induced liver injuries in mice

The method described by Tiegs et al. was modified and used for this study (Tiegs et al., 1989). Briefly, male ddY mice weighing about 25–30 g were fasted for 20 h before the experiment. D-GalN (350 mg/kg) and LPS (10 μg/kg) dissolved in saline were injected intraperitoneally to produce liver injuries. Each test sample was given orally 1 h before the D-GalN/LPS injection. Blood samples were collected from the infraorbital venous plexus 10 h after D-GalN/LPS injection. sAST and sALT levels were determined using Transaminase CII Test Wako (Wako Pure Chemical Industries, Co., Ltd.). Hydrocortisone was used as a reference compound. Test samples were suspended with 5% arabic gum solution, and the suspension was administered orally at 10 mL/kg in each experiment, while the vehicle was given orally at 10 mL/kg in the corresponding control group.

Effects on cytotoxicity induced by D-GalN in primary cultured mouse hepatocytes

The hepatoprotective effects of the constituents were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay using primary cultured mouse hepatocytes (Matsuda et al., 2009; Morikawa et al., 2010a). Hepatocytes were isolated from male ddY mice (30–35 g) by a collagenase perfusion method. A cell suspension at 4×10^4 cells in 100 μL William's E medium containing FBS (10%), penicillin G (100 units/mL), and streptomycin (100 μg/mL) was inoculated in a 96-well microplate and pre-incubated for 4 h at 37 °C under 5% CO_2 atmosphere. After pre-incubation, 100 μL of fresh medium containing D-GalN (2 mM) with or without the test sample was added to the medium. After 44 h incubation, the medium was exchanged with 100 μL of fresh medium, and 10 μL of MTT [5 mg/mL in phosphate buffered saline (PBS(−))] solution was added to the medium. After 4 h incubation, the medium was removed, and 100 μL of isopropanol containing 0.04 M HCl was added to dissolve formazan produced in the cells. The optical density (O.D.) of the formazan solution was measured by microplate reader at 570 nm (reference: 655 nm). Inhibition (%) was obtained by following formula.

$$\text{Inhibition}\,(\%) = [(\text{O.D.(sample)} - \text{O.D.(control)})/(\text{O.D.(normal)} - \text{O.D.(control)})] \times 100$$

Effects on production of NO in LPS-activated mouse peritoneal macrophages

Screening tests for NO production using TGC-induced mouse peritoneal macrophages were performed as described previously (Matsuda et al., 2009). Briefly, peritoneal exudate cells were collected from the peritoneal cavities (100 μL) of male ddY mice and were suspended in RPMI 1640 supplemented with 10% FBS, penicillin G (100 units/mL) and streptomycin (100 μg/mL), and pre-cultured in 96-well microplates (5×10^5 cells/well) at 37 °C in 5% CO_2 in air for 1 h. Nonadherent cells were removed by washing with PBS(−), and the adherent cells (10 μg/mL) were cultured in 200 μL of a fresh medium containing LPS and various concentrations of test compounds for 20 h. NO productions in each well were assessed by measuring the accumulation of nitrite (NO_2^-) in the culture medium using Griess reagent. Cytotoxicity was determined by the MTT colorimetric assay, after 20 h incubation with test compounds. Each test compound was dissolved in DMSO, and the solution was added to the medium (final DMSO concentration was 0.5%). N^G-Monomethyl-L-arginine (L-NMMA) and caffeic acid phenetyl ester (CAPE) were used as reference compounds. Inhibition (%) was calculated using the following formula ($N = 4$).

$$\text{Inhibition}\,(\%) = [(A - B)/(A - C)] \times 100$$

A–C: NO_2^- concentration (μM) [A: LPS (+), sample (−); B: LPS (+), sample (+); C: LPS (−), sample (−)].

Inhibitory effects against TNF-α-induced cell death in L929 cells

L929 cells (RIKEN) were maintained in a Minimum Essential Medium Eagle (MEM, Sigma–Aldrich) containing 10% FBS, 1% MEM Non-Essential Amino acids (Invitrogen), penicillin G (100 units/mL), and streptomycin (100 μg/mL) at 37 °C under 5% CO_2 atmosphere. Cells were inoculated in a 96-well tissue culture plate [5×10^3 cells/well in 100 μL/well in MEM]. After 20 h incubation, 100 mL/well of medium containing TNF-α (2 ng/mL) and a test sample. After 44 h incubation, viability of the cells was assessed by the MTT colorimetric assay (*vide ante*) (Matsuda et al., 2009; Morikawa et al., 2010a). Each test compound was dissolved in DMSO, and the solution was added to the medium (final concentration in DMSO 0.5%).

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Statistics

Values are expressed as means ± S.E.M. One-way analysis of variance (ANOVA) followed by Dunnett's test was used for statistical analysis. Probability (p) values less than 0.05 were considered significant.

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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.phytochem. 2014.03.002.

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