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NOTE

Ellagic acid glycosides with hepatoprotective activity from traditional Tibetan medicine *Potentilla anserina*

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Abstract Two new gallic acid glycosides, potentillanosides G (**1**) and H (**2**), were newly isolated from the methanol extract of the tuberous roots of *Potentilla anserina* (Rosaceae), together with a known compound, ellagic acid 3-*O*-α-L-rhamnopyranoside (**3**). Their structures were elucidated on the basis of chemical and physicochemical evidence. Among the constituents, potentillanoside H (**2**, IC_{50} = 99.5 μM) was found to show hepatoprotective activity.

Keywords Potentillanoside · Ellagic acid glycoside · *Potentilla anserina* · Hepatoprotective activity · Rosaceae

Introduction

Potentilla anserina L. is a Rosaceae plant which is widely distributed in the western areas of China, particularly in the Qinghai–Tibetan Plateau. The roots of *P. anserina* have been used to treat malnutrition, anemia, diarrhea, hemorrhage, cough, and sputum in traditional Tibetan medicine [1–5]. For thousands of years, the roots have also been used in food, such as congee with rice, which is a principal food of the local people [4, 5]. Previous chemical studies on this plant material revealed the presence of several tannins [6], flavonoids [7], triterpenes [8–10], polysaccharides [2, 3], and amino acids [4]. In addition, pharmacological activities of the extracts and/or constituents, such as anti-mutagenic [6], anti-hepatitis B virus [10], immunomodulatory [3], antitussive [5], and expectorant activities [5], have been reported. In the course of our studies on bioactive constituents from the tuberous roots of *P. anserina*, we have reported the isolation and structure determination of 21 triterpenes as well as the protective effects of the methanol extract and several triterpenes against liver injuries induced by D-galactosamine (D-GalN)/lipopolysaccharide (LPS) in mice [1]. Further separation of the constituents in the extract allowed us to isolate two new ellagic acid glycosides, potentillanosides G (**1**) and H (**2**), and a known compound, ellagic acid 3-*O*-α-L-rhamnopyranoside (**3**). Here, we describe the isolation and structure elucidation of **1** and **2** as well as the protective effects of the isolates on D-GalN-induced cytotoxicity in primary cultured mouse hepatocytes.

Results and discussion

Isolation

In the present study, we isolated three ellagic acid glycosides, potentillaosides G (**1**, 0.00098%) and H (**2**, 0.00033%) and ellagic acid 3-*O*-α-L-rhamnopyranoside (**3**, 0.00048%) [11], from the ethyl acetate (EtOAc)-soluble and methanol (MeOH)-eluted fractions using normal-phase silica gel and reversed-phase ODS CC, and finally preparative HPLC (Fig. 1).

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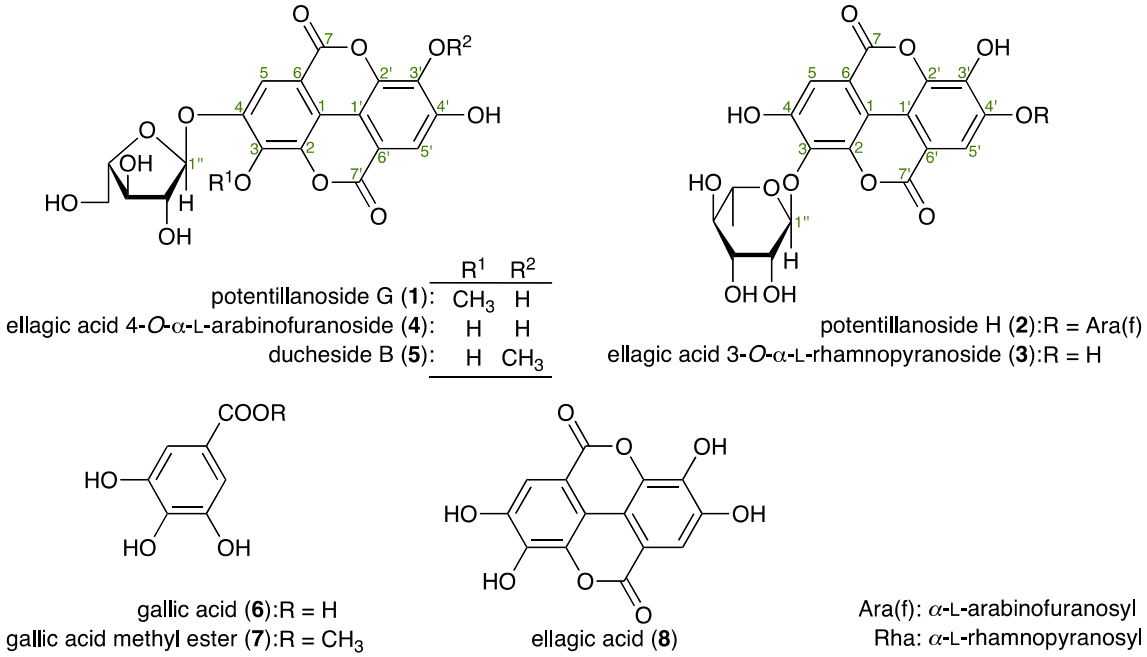


Fig. 1 Structures of the isolates (**1**–**8**) from the tuberous roots of *P. anserina*

Structure determination of potentillanosides G (1) and H (2)

Potentillanoside G (**1**) was obtained as an amorphous powder with negative optical rotation ($[\alpha]_D^{27}$—21.0 in MeOH). The UV spectrum of **1** exhibited absorption maxima at 254 and 354 nm, while the IR spectrum showed absorption bands at 3385, 1710, 1611, and 1078 cm^{-1} ascribable to hydroxy, carbonyl, and aromatic functionalities. In the positive-ion FABMS profile, a quasimolecular ion peak was observed at *m/z* 471 $[M+Na]^+$, and HRFABMS analysis revealed the molecular formula to be $C_{20}H_{16}O_{12}$. The acid hydrolysis of **1** using 1 M hydrochloric acid (HCl)-1,4-dioxane (1:1, v/v) produced 3-*O*-methylellagic acid (**1a**) [12] together with L-arabinose, which was identified by HPLC analysis [1, 13–21]. The ^1H (Table 1) and ^{13}C NMR (Table 2) spectra (DMSO-d_6) of **1**, which were assigned using DEPT, ^1H–^1H COSY, HSQC, and HMBC experiments, showed signals assignable to a methoxy methyl [δ 4.00 (3H, s, 3-OCH_3)] and two aromatic protons [δ 7.42, 7.50 (1H each, both s, H-5, 5′)] together with an arabinofuranosyl moiety [δ 5.53 (1H, br s, Ara(f)-H-1)]. The ^1H and ^{13}C NMR spectroscopic properties of **1** were quite similar to those of ducheside B (**5**) [22]. As shown in Fig. 2, the connectivities of the methoxy and arabinofuranosyl moieties in **1** were characterized on the basis of the HMBC experiment. Long-range correlations were observed between H-5 (δ 7.42) and C-1 (δ_C 115.4), C-3 (δ_C 137.5), and C-7 (δ_C 159.6); H-5′ (δ 7.50) and C-1′ (δ_C 114.0), C-3′ (δ_C 139.9), and C-7′ (δ_C 160.4); H-1″ and C-4 (δ_C 149.2), and the methoxy methyl proton (δ 4.00) and C-3 (δ_C 137.5). In the nuclear Overhauser enhancement spectroscopy (NOESY) experiment, the nuclear Overhauser effect (NOE) correlation was observed between H-1″ and H-5, as shown in Fig. 2. In addition, methylation of **1** with iodomethane (CH_3I) in the presence of potassium carbonate (K_2CO_3) gave **1b**, which was also obtained by the same methylation of **5** (Scheme 1). Therefore, the configuration of the L-arabinofuranosyl linkage in **1** was the same as that of **5**, i.e., α-form. Acid hydrolysis of **1b** produced 3,3′,4′-tri-*O*-methylellagic acid (**1c**) [12, 23, 24] as the aglycone, thus the structure of **1b** was elucidated to be 3,3′,4′-tri-*O*-methylellagic acid 4-*O*-α-L-arabinofuranoside. On the basis of the above-mentioned evidence, the structure of potentillanoside G was determined to be 3-*O*-methylellagic acid 4-*O*-α-L-arabinofuranoside (**1**).

Potentillanoside H (**2**), $C_{25}H_{24}O_{16}$, was obtained as an amorphous powder with negative optical rotation ($[\alpha]_D^{28}$—52.9 in MeOH). Acid hydrolysis of **2** released ellagic acid (**8**) [12, 23, 25, 26] together with L-rhamnose and L-arabinose, which were identified by HPLC analysis. The ^1H and ^{13}C NMR (DMSO-d_6, Tables 1, 2) spectra of **2** showed signals ascribable to an ellagic acid moiety [δ 7.60, 7.73 (both 1H, each s, H-5, 5′)] along with a rhamnopyranosyl [δ 5.56 (1H, br s, Rha-H-1), 1.09 (3H, d, $J = 6.2$ Hz, Rha-H_3-6)] and an arabinofuranosyl moiety [δ 5.65 (1H, br s, Ara(f)-H-1)]. Methyl ether derivatives **2a** and **3a** were obtained by the methylation of **2** and **3**, respectively, using the above-mentioned procedure. The NOE correlations of **2a** and **3a** in the NOESY experiment were observed between the following proton pairs: 4-OCH_3 [**2a**: δ 4.02 (3H, s); **3a** δ 4.02 (3H, s)]

Table 1 ^{1}H NMR spectroscopic data (600 MHz, DMSO-d_6) for potentillanosides G (**1**) and H (**2**), **1b**, **1c**, **2a**, and **3a**

Position	**1**	**1b**	**1c**	**2**	**2a**	**3a**
5	7.42 (s)	7.79 (s)	7.60 (s)	7.60 (s)	7.70 (s)	7.68 (s)
5′	7.50 (s)	7.66 (s)	7.51 (s)	7.73 (s)	7.80 (s)	7.67 (s)
3-OCH_3	4.00 (3H, s)	4.11 (3H, s)	4.04 (3H, s)			
4-OCH_3					4.02 (3H, s)	4.02 (3H, s)
3′-OCH_3		4.06 (3H, s)	4.02 (3H, s)		4.11 (3H, s)	4.07 (3H, s)
4′-OCH_3		4.02 (3H, s)	3.98 (3H, s)			4.02 (3H, s)
3-*O*-Rha						
1				5.56 (br s)	5.48 (d, 1.7)	5.47 (br s)
2				4.08 (br s)	4.06 (dd, 1.7, 3.5)	4.08 (m)
3				3.81 (dd, 3.1, 9.3)	3.78 (dd, 3.5, 9.5)	3.78 (m)
4				3.34 (dd, 9.3, 9.5)	3.33 (dd, 9.4, 9.5)	3.33 (m)
5				4.17 (dq, 9.5, 6.2)	4.10 (m)	4.11 (m)
6				1.09 (3H, d, 6.2)	1.09 (3H, d, 6.2)	1.09 (3H, 6.2)
4 or 4′-*O*-Ara(f)						
1	5.53 (br s)	5.67 (d, 1.4)		5.65 (br s)	5.68 (d, 1.6)	
2	4.13 (dd, 1.4, 3.8)	4.25 (dd, 1.4, 3.9)		4.34 (br d, ca. 4)	4.25 (dd, 1.6, 4.0)	
3	3.78 (dd, 3.8, 6.1)	3.88 (dd, 3.9, 6.4)		3.88 (dd, 3.8, 5.9)	3.88 (dd, 4.0, 6.4)	
4	4.00 (ddd, 3.5, 5.7, 6.1)	3.98 (ddd, 3.4, 5.4, 6.4)		3.99 (ddd, 3.7, 5.5, 5.9)	3.99 (ddd, 3.4, 5.5, 6.4)	
5	3.46 (dd, 5.7, 11.7) 3.52 (dd, 3.5, 11.7)	3.52 (dd, 5.4, 12.0) 3.63 (dd, 3.4, 12.0)		3.51 (dd, 5.5, 11.9) 3.61 (dd, 3.7, 11.9)	3.52 (dd, 5.5, 12.0) 3.62 (dd, 3.4, 12.0)	

and H-5 [**2a**: δ 7.70 (1H, s); **3a**: δ 7.68 (1H, s)]; and 4′-OCH_3 [**3a**: δ 4.02 (3H, s)] and H-5′ [**3a**: δ 7.67 (1H, s)]. In addition, acid hydrolysis of **2a** and **3a** gave the corresponding aglycones 4,3′-di-*O*-methylellagic acid (**2b**) [26] and 4,3′,4′-tri-*O*-methylellagic acid (**3b**) [23, 27], respectively. The proton and carbon signals due to the 3-*O*-α-L-rhamnopyranosyl part in **2a** were superimposable on those of **3a**, while due to the 4′-*O*-α-L-arabinofuranosyl part in **2a** also resembled those of **1b**. Based on these findings and comparison of the NMR data with those of the corresponding derivatives, the structure of potentillanoside H was determined to be ellagic acid 3-*O*-α-L-rhamnopyranosyl-4′-*O*-α-L-arabinofuranoside (**2**).

Inhibitory effects on D-GalN-induced cytotoxicity in primary cultured mouse hepatocytes

As a part of our efforts to characterize hepatoprotective compounds from natural medicines, we have investigated several constituents that showed inhibitory effect on D-GalN-induced cytotoxicity in primary cultured hepatocytes [1, 14, 28–49]. As a continuation of the above study, hepatoprotective compounds from *P. anserina* were explored. The results revealed that potentillanoside H (**2**, IC_{50} = 99.5 μM) shows hepatoprotective activity (Table 3).

In conclusion, two new ellagic acid glycosides, potentillanosides G (**1**) and H (**2**), were newly isolated from the methanol extract of the tuberous roots of *P. anserina* together with the known compound, ellagic acid 3-*O*-α-L-rhamnopyranoside (**3**). Among them, **2** was investigated as a hepatoprotective constituent. The detailed mechanisms of action as well as the structural requirements of these ellagic acid derivatives for hepatoprotective activity should be further examined.

Materials and methods

General

The following instruments were used to obtain spectroscopic data: specific rotation, 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; HPLC detectors, Shimadzu RID-6A refractive index and SPD-10A UV–VIS detectors and Shodex OR-2 optical rotation detector; HPLC columns, 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 the sugar part.

Table 2 ^{13}C NMR spectroscopic data (150 MHz, DMSO-d_6) for potentillanosides G (**1**) and H (**2**), **1b**, **1c**, **2a**, and **3a**

Position	**1**	**1b**	**1c**[a]	**2**	**2a**	**3a**
1	115.4	113.5	111.0	111.3	112.8	113.1
2	142.6	142.0	141.0	142.7	142.1	141.2
3	137.5	141.2	139.9	136.8	137.7	137.7
4	149.2	151.0	152.5	153.1	154.8	154.6
5	114.4	112.0	111.6	111.5	107.8	107.9
6	112.9	112.2	112.3	113.9	112.2	112.8
7	159.6	158.2	157.5	159.1	158.2	158.3
1′	114.0	112.9	112.3	114.6	113.7	113.4
2′	142.6	141.1	141.5	136.3	141.4	142.0
3′	139.9	141.0	140.5	142.8	142.2	141.2
4′	152.1	154.3	153.7	146.8	151.2	154.3
5′	110.6	107.7	107.5	112.4	112.1	107.9
6′	112.9	112.5	114.5	106.5	113.6	112.3
7′	160.4	158.3	157.8	158.7	158.2	158.2
3-OCH_3	60.9	61.4	60.9			
4-OCH_3					57.1	56.9
3′-OCH_3		61.3	61.2		61.6	61.4
4′-OCH_3		56.8	56.8			57.1
3-*O*-Rha						
1				102.6	103.3	103.3
2				70.2	70.2	70.2
3				70.5	70.5	70.5
4				71.6	71.5	71.5
5				70.7	70.9	70.9
6				17.7	17.7	17.7
4 or 4′-*O*-Ara(f)						
1	107.5	107.6		107.9	107.7	
2	80.7	82.1		81.5	82.2	
3	77.1	76.6		76.8	76.7	
4	87.1	86.2		86.3	86.3	
5	62.0	61.0		61.2	61.2	

[a]Measured in DMSO-d_6 [12]

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, 0.25 mm) (reversed-phase); reversed-phase HPTLC, pre-coated TLC plates with silica gel RP-18 WF_{254S} (Merck, 0.25 mm). Detection was carried out by spraying 1% $Ce(SO_4)_2$—10% aqueous H_2SO_4, followed by heating.

Plant material

This item has been described in a previous report [1].

Extraction and isolation

Potentillanosides G (**1**) and H (**2**) and ellagic acid 3-*O*-α-L-rhamnopyranoside (**3**) were isolated from previously reported fractions: fraction 10–3 (195.8 mg), originally obtained from the EtOAc-soluble fraction (0.58%) of the methanol extract (23.0%) from the dried tuberous roots of *P. anserina* and fractions 4–5 (334.9 mg) and 4–6 (128.6 mg) obtained from the MeOH-eluted fraction (0.73%) [1]. Fraction 10–3 (195.8 mg), obtained from the EtOAc-soluble

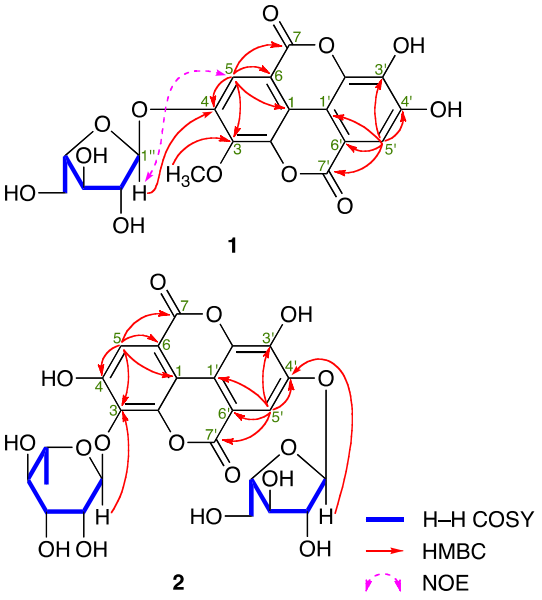
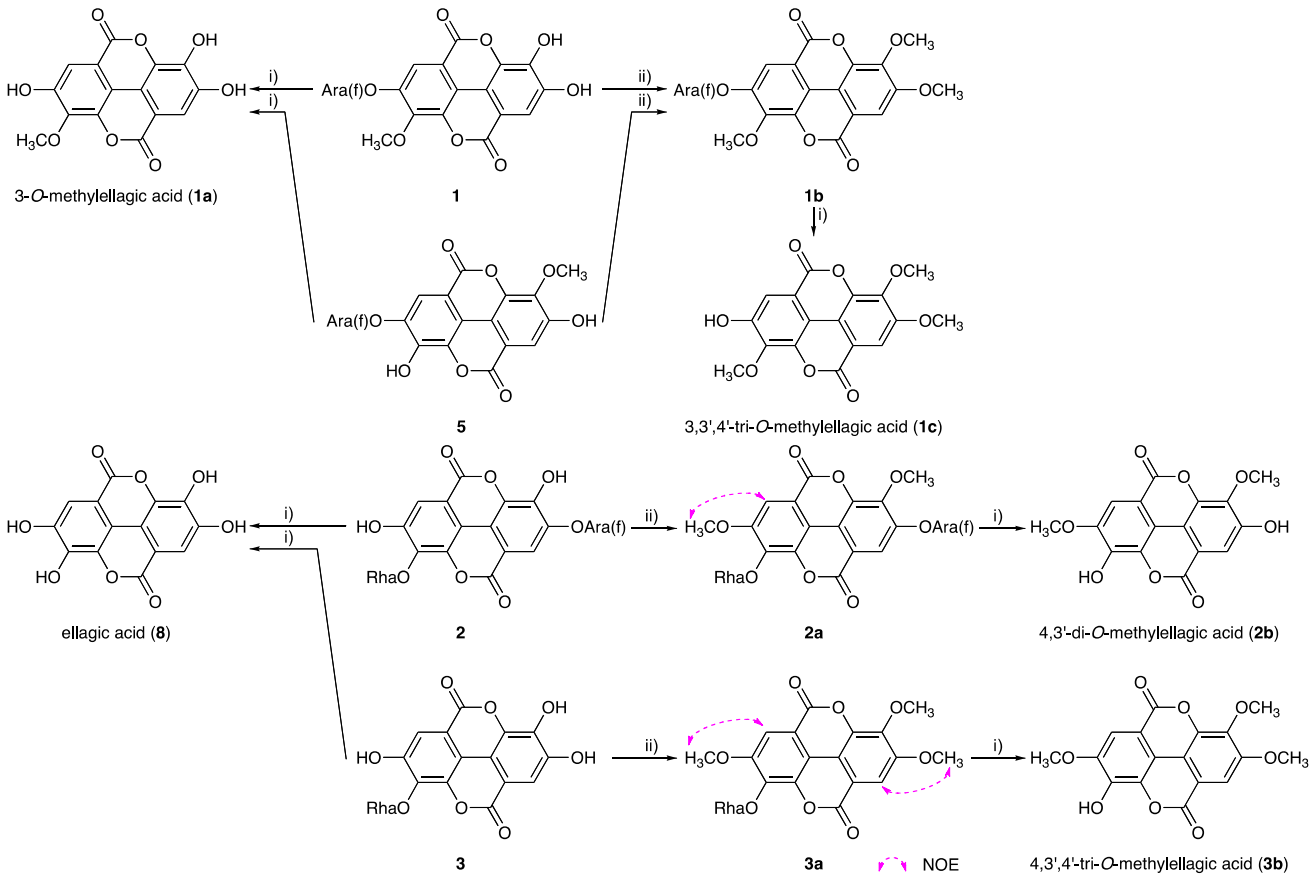


Fig. 2 ^{1}H–^{1}H COSY, HMBC, and NOE correlations of **1** and **2**

fraction, was subjected to HPLC [MeOH—1% aqueous acetic acid (AcOH) (35:65, v/v)] to give potentillanoside G (**1**, 73.4 mg, 0.00098%) together with ellagic acid (**8**, 21.3 mg). Fraction 4 (4.05 g), obtained from the MeOH-eluted fraction, 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)], as described previously [1]. Fraction 4–5 (334.9 mg) was subjected to HPLC [MeOH–1% aqueous AcOH (30:70, v/v)] to give potentillanoside H (**2**, 24.7 mg, 0.00033%) together with quercetin 3-*O*-sambubioside (11.0 mg). Fraction 4–6 (128.6 mg) was subjected to HPLC [MeOH—1% aqueous AcOH (30:70, v/v)] to give ellagic acid 3-*O*-α-L-rhamnopyranoside (**3**, 35.5 mg, 0.00048%) [11] along with potentillanoside F (18.3 mg) and ellagic acid 4-*O*-α-L-arabinofuranoside (**4**, 22.6 mg).



Scheme 1 Conversion of ellagic acid derivatives. Reagents and conditions: i) 1 M HCl-1,4-dioxane (1:1, v/v), 80 °C, 1 h, **1a** (78% from **1**, 85% from **5**), **1c** (88% from **1b**), **8** (77% from **2**, 86% form **3**), **2b** (88% from **2a**), **3b** (73% from **3a**); ii) CH_3I, K_2CO_3/DMSO, r.t., 45 min, **1b** (94% from **1**, 94% from **5**), **2a** (99% from **2**), **3a** (91% from **3**)

Table 3 Inhibitory effect of the constituents of ellagic acid derivatives (**1**–**8**) on D-GalN-induced cytotoxicity in primary cultured mouse hepatocytes

Treatment	Inhibition (%)[a]					IC_{50} (μM)
	0 μM	3 μM	10 μM	30 μM	100 μM	
Potentillanoside G (**1**)	0.0 ± 2.2	− 1.4 ± 1.2	− 1.6 ± 1.1	− 0.7 ± 0.6	2.4 ± 0.5	
Potentillanoside H (**2**)	0.0 ± 0.6	3.1 ± 0.5	6.6 ± 2.0	11.2 ± 2.8[c]	50.7 ± 3.6[c]	99.5
Ellagic acid 3-*O*-α-L-rhamnopyranoside (**3**)	0.0 ± 0.5	2.4 ± 0.9	4.9 ± 0.5	11.1 ± 1.0[c]	26.3 ± 2.1[c]	
Ellagic acid 4-*O*-α-L-arabinofuranoside (**4**) [1]	0.0 ± 1.1	5.2 ± 1.4	6.9 ± 0.2	8.4 ± 1.2	16.2 ± 3.3[c]	
Ducheside B (**5**)	0.0 ± 0.7	3.7 ± 0.6	0.0 ± 2.2	2.3 ± 1.4	3.7 ± 0.9	
Gallic acid (**6**) [1]	0.0 ± 1.2	10.8 ± 1.8	14.2 ± 2.8[b]	26.0 ± 5.6[c]	35.9 ± 7.5[c]	
Gallic acid methyl ester (**7**) [1]	0.0 ± 0.5	5.8 ± 0.5	14.2 ± 1.3[c]	33.1 ± 1.7[c]	65.8 ± 4.1[c]	53.7
Ellagic acid (**8**) [1]	0.0 ± 0.5	6.0 ± 0.7	7.8 ± 0.4	6.0 ± 1.1	14.7 ± 1.4[c]	
Silybin[d] [1]	0.0 ± 0.3	4.8 ± 1.1	7.7 ± 0.7	45.2 ± 8.8[c]	77.0 ± 5.5[c]	38.8

[a]Each value represent the mean ± SEM, ($n = 4$)

Significantly different from the control group, [b]$p < 0.05$, [c]$p < 0.01$

[d]Commercial silybin was purchased from Funakoshi Co., Ltd. (Tokyo, Japan)

*Potentillanoside G (**1**)*

An amorphous powder, $[\alpha]_D^{27}$—21.0 (*c* 0.10, MeOH); UV [MeOH, nm (log ε)]: 254 (4.53), 354 (4.03); IR (KBr) ν_{max}: 3385, 1710, 1611, 1078 cm^{-1}; ^1H (600 MHz, DMSO-d_6) and ^{13}C NMR (150 MHz, DMSO-d_6): shown in Tables 1 and 2; Positive-ion FABMS *m/z*: 471 $[M+Na]^+$; HRFABMS *m/z*: 471.0547 $[M+Na]^+$ (calcd for $C_{20}H_{16}O_{12}Na$, 471.0539).

*Potentillanoside H (**2**)*

An amorphous powder, $[\alpha]_D^{28}$—52.9 (*c* 0.10, MeOH); UV [MeOH, nm (log ε)]: 247 (4.98), 352 (4.17); IR (KBr) ν_{max}: 3385, 1744, 1613, 1078 cm^{-1}; ^1H (600 MHz, DMSO-d_6) and ^{13}C NMR (150 MHz, DMSO-d_6): shown in Tables 1 and 2; positive-ion FABMS *m/z*: 603 $[M+Na]^+$; HRFABMS *m/z*: 603.0967 $[M+Na]^+$ (calcd for $C_{25}H_{24}O_{16}Na$, 603.0962).

Acid hydrolysis of 1–3, 5, 1b, 2a, and 3a

A solution of **1** or **2** (each 5.0 mg) in 1 M HCl–1,4-dioxiane (1:1, v/v, 1.0 mL) was stirred at 80 °C for 1 h. After cooling, the reaction mixture was neutralized with Amberlite IRA-400 (OH^- form) and the resin was removed by filtration. After removal of the solvent from the filtrate under reduced pressure, the residue was partitioned in EtOAc–H_2O (1:1, v/v), and the solvents removed in vacuo from the EtOAc-soluble fraction and an aqueous phase, respectively. The EtOAc-soluble fraction was purified by normal-phase silica gel CC [500 mg, hexane–EtOAc (3:1, v/v)] to furnish 3-*O*-methylellagic acid (**1a**, 2.8 mg, 78%, from **1**) [12] or ellagic acid (**8**, 2.0 mg, 77% from **2**) [12, 23, 25, 26]. In turn, the aqueous layer was subjected to HPLC analysis under the following conditions: HPLC column, Kaseisorb LC NH_2-60-5, 4.6 mm i.d. × 250 mm (Tokyo Kasei Co., Ltd.); detection, optical rotation [Shodex OR-2 (Showa Denko Co., Ltd., Tokyo, Japan); mobile phase, CH_3CN–H_2O (85:15, v/v); flow rate 0.5 mL/min]. Identification of L-rhamnose [t_R: 12.0 min (negative optical rotation)] from **2** and L-arabinose [t_R: 16.2 min (positive optical rotation)] from **1** and **2** in the H_2O-eluted fraction was carried out by comparing the retention time and optical rotation with those of authentic samples [1, 13–21]. Through a similar procedure, **1a** [3.0 mg, 85% from **5** (5.0 mg)], 3,3′-di-*O*-methylellagic acid [**1c**, 1.9 mg, 88% from **1b** (3.0 mg)] [12, 23], 4,3′-di-*O*-methylellagic acid [**2b**, 1.0 mg, 88% from **2a** (2.0 mg)] [26], 4,3′,4′-tri-*O*-methylellagic acid [**3b**, 1.0 mg, 73% from **3a** (2.0 mg)] [23, 27], and **8** [2.9 mg, 86% from **3** (5.0 mg)] were purified from each EtOAc-soluble fraction by normal-phase silica gel CC.

Methylation of 1–3 and 5

A solution of **1** (5.0 mg) in DMSO (1.0 mL) was treated with iodomethane (CH_3I, 0.1 mL) in the presence of K_2CO_3 (5.0 mg), and the mixture was stirred at room temperature for 45 min. The reaction mixture was poured into ice water and was extracted with EtOAc. The EtOAc layer was successively washed with 5% aqueous HCl, saturated aqueous $NaHCO_3$, and brine, then dried over $MgSO_4$ powder, and filtrated under reduced pressure. The residue was purified by HPLC [MeOH–1% aqueous AcOH (60:40, v/v)] to give **1b** (5.0 mg, 94%). Through a similar procedure, **1b** [5.0 mg, 94% from **5** (5.0 mg)], **2a** [3.1 mg, 99% from **2** (3.0 mg)] and **3a** [3.0 mg, 91% from **3** (3.0 mg)] were obtained.

*Compound **1b***

An amorphous powder, $[\alpha]_D^{27}$—9.2 (*c* 0.12, MeOH); UV [MeOH, nm (log ε)]: 248 (4.70), 365 (4.11); IR (KBr) ν_{max}: 3380, 1744, 1609, 1098 cm^{-1}; ^1H (600 MHz, DMSO-d_6) and ^{13}C NMR (150 MHz, DMSO-d_6): shown in Tables 1 and 2; Positive-ion FABMS *m/z*: 499 $[M+Na]^+$; HRFABMS *m/z*: 499.0886 $[M+Na]^+$ (calcd for $C_{22}H_{20}O_{12}Na$, 499.0852).

*Compound **2a***

An amorphous powder, $[\alpha]_D^{28}$—49.9 (*c* 0.11, MeOH); UV [MeOH, nm (log ε)]: 251 (4.69), 363 (4.05); IR (KBr) ν_{max}: 3385, 1734, 1618, 1072 cm^{-1}; ^1H (600 MHz, DMSO-d_6) and ^{13}C NMR (150 MHz, DMSO-d_6): shown in Tables 1 and 2; positive-ion FABMS *m/z*: 631 $[M+Na]^+$; HRFABMS *m/z*: 631.1267 $[M+Na]^+$ (calcd for $C_{27}H_{28}O_{16}Na$, 631.1275).

*Compound **3a***

An amorphous powder, $[\alpha]_D^{27}$—18.0 (*c* 0.11, MeOH); UV [MeOH, nm (log ε)]: 247 (4.74), 370 (4.17); IR (KBr) ν_{max}: 3380, 1739, 1609, 1063 cm^{-1}; ^1H (600 MHz, DMSO-d_6) and ^{13}C NMR (150 MHz, DMSO-d_6): shown in Tables 1 and 2; positive-ion FABMS *m/z*: 513 $[M+Na]^+$; HRFABMS *m/z*: 513.1003 $[M+Na]^+$ (calcd for $C_{23}H_{22}O_{12}Na$, 513.1009).

Bioassay

Reagents

LPS (from *Salmonella enteritidis*), minimum essential medium, and Williams' E medium were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Fetal calf serum (FCS) was obtained from Life Technologies (Rockville, MD, USA). Other chemicals were procured from Wako Pure Chemical Industries, Co., Ltd. (Osaka, Japan), and 96-well microplates were purchased from Sumitomo Bakelite Co., Ltd. (Tokyo, Japan).

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

The hepatoprotective effects of the isolated constituents were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay using primary cultured mouse hepatocytes [1, 48, 49]. Hepatocytes were isolated from male ddY mice (30–35 g) by the collagenase perfusion method. A cell suspension at 4×10^4 cells in 100 µL Williams' E medium containing FCS (10%), penicillin G (100 units/mL), and streptomycin (100 µg/mL) was inoculated in a 96-well microplate and precultured for 4 h at 37 °C under a 5% CO_2 atmosphere. The medium was added to 100 µL of the fresh medium containing D-GalN (2 mM), with or without the test sample, and the hepatocytes were cultured for 44 h. The medium was exchanged with 100 µL of the fresh medium, and 10 µL of MTT (5 mg/mL in phosphate-buffered saline) solution was added to the medium. After 4 h of cultivation, the medium was removed, and 100 µL of isopropanol containing 0.04 M HCl was then added to dissolve the formazan produced in the cells. The optical density (O.D.) of the formazan solution was measured by a microplate reader at 570 nm (reference: 655 nm). Inhibition (%) was obtained using the following formula:

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

Statistical analysis

Values are expressed as mean ± SEM. One-way analysis of variance (ANOVA), followed by Dunnett's test, was used for statistical analysis. Probability (*p*) values <0.05 were considered significant.

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