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NOTE

Flavonoids isolated from the flowers of *Pulsatilla flavescens* and their anti-piroplasm activity

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Abstract

Pulsatilla species are known as "Yargui", and their flowers are traditionally used in Mongolia as a tonic and for the treatment of inflammatory diseases. By chemical investigation of *P. flavescens* flowers, 21 flavonoids, including a new chalcone *C*-glucoside, chalconaringenin 2′-*O*-β-D-glucopyranosyl-5′-β-D-glucopyranoside, and two new flavanone *C*-glucosides, (2*R*)- and (2*S*)-naringenin 8-β-D-glucopyranosyl-4′-*O*-β-D-glucopyranoside, were isolated. The absolute configurations of the seven flavanone glucosides were elucidated by ECD spectra. For the isolated compounds, inhibitory activity against *Babesia caballi* and *Theileria equi*, which cause fatal diseases in horses, was estimated. Although most of the isolated chalcone and flavanone derivatives did not show any anti-piroplasm activity, all the isolated flavone and flavonol derivatives showed moderate effects against *B. caballi* and/or *T. equi*.

Keywords *Pulsatilla flavescens* · Yargui · Flavonoids · Anti-piroplasma activity

Introduction

Pulsatilla genus plants are called "Yargui" in Mongolia, and their flowers bloom in early spring on the East Asian plateau. The flowers are traditionally welcomed by nomads because are thought of as tonic resources for livestock that have survived the harsh winter. Goats like the flowers, and the meat and blood of goats that have eaten the flowers are thought to produce a healthy life for these nomadic people. *Pulsatilla flavescens* (Zucc.) Juz. (synonym: *Anemone flavescens* Zucc.) is a Ranunculaceae perennial herb commonly called yellow Yargui by the nomadic people. Flowers of *P. flavescens* are used not only as a grass providing a tonic effect for animals but also as a folk medicine for the treatment of infectious diseases, inflammation, and swelling [1]. Ranunculin and its derivative protoanemonin are known to be characteristic contact toxic constituents of *Ranunculus* and *Pulsatilla* plants. It was thought that *P. flavescens* also includes these compounds because of the presence of anemonin, which is a degradation product of the toxic components [2]. Our previous studies focused on the traditional benefits of feeding *P. flavescens* to animals and their toxicity against infectious diseases, including evaluation of the antiprotozoal and antibacterial in vitro activities of the plant

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extracts and constituents. *Babesia caballi* and *Theileria equi* infect horses via tick bites and cause equine piroplasmosis, leading to an increased risk of morbidity and mortality. In Mongolia, an epidemiological survey showed a high rate of *B. caballi* and *T. equi* infections in horse populations [3]. For the Mongolian economy, the livestock sector is very important, with horses, cattle, sheep, goats, and camels being the five main livestock species, all of whihch can be hosts of piroplasm parasites, with apparent or unapparent infections [4, 5]. In this study, three new flavonoids glucosides and 18 known compounds were isolated from the flowers of *P. flavescens*. The structures of the new compounds were established based on their spectroscopic data, while the absolute configurations of seven flavanone glucosides were elucidated by ECD spectra. To identify anti-piroplasm compounds from this native Mongolian plant, the isolated flavonoids were tested for their anti-piroplasm activities against *B. caballi* and *T. equi*.

Results and discussion

An acetone–water (3:2) extract of the flowers of *P. flavescens* was dissolved in H_2O and fractionated with diethylether. The aqueous fraction was applied to an open column using ODS and four fractions were obtained. Further fractionation and purification using preparative HPLC gave 21 flavonoids. The known flavonoids, kaempferol (**1**) [6], apigenin 7-*O*-(6″-*O*-*p*-coumaroyl)-β-D-glucopyranoside (**2**) [7], kaempferol 3-*O*-(6″-*O*-*p*-coumaroyl)-β-D-glucopyranoside (**3**) [8], kaempferol 7-*O*-(6″-*O*-*p*-coumaroyl)-β-D-glucopyranoside (**4**) [9], apigenin 7-*O*-(6″-*O*-*p*-coumaroyl)-β-D-glucopyranoside (**5**) [10], apigenin 7-*O*-[6″-*O*-(3‴-hydroxy-3″-methylglutarate)]-β-D-glucopyranoside (**6**) [11], quercetin 3-*O*-β-D-glucopyranoside (**7**) [12], kaempferol 3-*O*-β-D-glucopyranoside (**8**) [12], quercetin 3-*O*-rutinoside (**9**) [12], kaempferol 3-*O*-rutinoside (**10**) [12], kaempferol 3-*O*-[6″-*O*-(*Z*)-*p*-coumaroyl]-β-D-glucopyranoside (**11**) [13], quercetin 3-*O*-β-D-glucuronopyranoside (**12**) [14], kaempferol 3-*O*-β-D-glucuronopyranosyl methyl ester (**13**) [15], (2*R*)- and (2*S*)-naringenin 6,8-di-β-D-glucopyranosides (**17**, **18**) [16], (2*R*)- and (2*S*)-isohemipholins (**19**, **20**) [17, 18], and naringenin 7-*O*-[6″-*O*-(*p*-coumaroyl)-β-D-glucopyranoside (**21**) [19], were identified based on their spectroscopic values compared with previously published data.

The molecular formula of **14** was determined as $C_{27}H_{32}O_{15}$ based on HR-FAB-MS *m/z* 597.1818 $[M+H]^+$ (calcd for $C_{27}H_{33}O_{15}$, 597.1819). In the aromatic region of the ^{1}H NMR spectrum of **14** (Table 1), a singlet (δ 6.28, 1H, s, H-3′) and two sets of coupling proton resonances [(δ 7.69, 1H, d, J = 16.0 Hz, H-7 and 8.02, 1H, d, J = 16.0, H-8) and (δ 6.83, 2H, d, J = 9.0 Hz, H-3 and 5 and 7.61, 2H, d, J = 9.0 Hz, H-2 and 6)] were observed. Their corresponding carbons were assigned as δ 95.7 (C-3′), 144.5 (C-7), 125.9 (C-8), 116.9 (C-3 and 5), and 131.9 (C-2 and 6) using the HMQC spectrum. In the HMBC spectrum (Fig. 2), the H-3′ resonance was long-range coupled with C-2′ (δ 161.2), C-4′ (δ 164.8), C-5′ (δ 107.0), and C-1′ (δ 107.3), which showed the ring A moiety of a chalcone. Similarly, the HMBC correlations from olefinic proton (H-2) to C-1 (δ 128.5), C-2, and C-6; from H-2 and H-6 to C-1 and C-4 (δ 161.4); from H-7 and H-8 to the carbonyl carbon at δ 194.7 (C-9) were observed, which showed the ring B moiety of the chalcone. Collectively, the above-assigned protons and carbons suggested the presence of a 4,2′,4′,6′-tetraoxygenated chalcone moiety. The coupling constant between H-7 and H-8 (J = 16.0 Hz) showed that the C-7 and C-8 double bond is *trans* [20]. Twelve oxygenated sp^3 carbon resonances were observed in the ^{13}C NMR spectrum, which suggested the presence of two hexose moieties. The anomeric proton at δ 5.12 (1H, d, J = 7.0 Hz) and six carbons (δ 101.9, C-1‴; 75.0, C-2‴; 78.5, C-3‴; 71.8, C-4‴; 78.6, C-5‴; 62.5, C-6‴) were assigned as an *O*-glucosyl moiety [12]. Sugar identification using HPLC indicated that the glucosyl moiety was D-glucose, and the coupling constant J = 7.0 showed its β-orientation. The anomeric proton (H-1‴) was HMBC long-range coupled with C-2′, showing a 2′-*O*-β-D-glucopyranosyl moiety similar to isosalipurposide [20]. For another glycosyl moiety, the anomeric proton at δ 4.87 (H-1″) and six oxygenated sp^3 carbons (δ 75.7, C-1″; 72.8, C-2″; 80.2, C-3″; 71.2, C-4″; 82.6, C-5″; 62.8, C-6″) were assigned as a *C*-glucosyl moiety [17]. The anomeric proton (H-1″) had HMBC long-range correlations with C-4′, C-5′, and C-6′, showing the 5′-*C*-glucopyranosyl partial structure. The coupling constant between H-1″ and H-2″ was J = 9.5 Hz and between H-2″ and H-3″ was J = 9.0 Hz. Although the configuration was not confirmed by sugar analysis, the NMR data compared with the synthesized flavonoid-*C*-β-D-glucoside and the coupling constant suggested that **14** has an 5′-*C*-β-D-glucoside moiety [17]. From these data, **14** was determined as chalconaringenin 2′-*O*-β-D-glucopyranosyl-5′-β-D-glucopyranoside, as shown in Fig. 1.

The molecular formulae of **15** and **16** were determined as $C_{27}H_{32}O_{15}$ based on HR-FAB-MS *m/z* 597.1810 $[M+H]^+$ (calcd for $C_{27}H_{33}O_{15}$, 597.1819) and 619.1631 $[M+Na]^+$ (calcd for $C_{27}H_{32}O_{15}Na$, 619.1638), respectively. The ^{1}H and ^{13}C NMR spectroscopic data of **15** and **16** were very close to those of 8-(β-D-glucopyranosyl) naringenin (**19** and **20**) except for the presence of additional hexose resonances [**15**: δ 4.93 (1H, d, J = 7.5 Hz, H-1‴); δ 102.2 (C-1‴), 74.9 (C-2‴), 78.0 (C-3‴), 71.8 (C-4‴), 78.2 (C-5‴), 62.5 (C-6‴); **16**: δ 4.93 (1H, d, J = 7.5 Hz, H-1‴); δ 102.3 (C-1‴), 74.9 (C-2‴), 78.0 (C-3‴), 71.9 (C-4‴), 78.2 (C-5‴), 62.5 (C-6‴)]. In the HMBC spectra of **15** and **16**, the anomeric proton

Table 1 NMR spectroscopic data of compounds **14–16**

Position	**14**		**15**		**16**	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1		128.5				
2	7.61, d (9.0)	131.9	5.42, dd (12.5, 3.0)	80.2	5.49, dd (13.0, 3.0)	80.1
3	6.83, d (9.0)	116.9	2.79, dd (17.5, 3.0)	43.9	2.78, dd (17.0, 3.0)	44.3
			3.11, dd (17.5, 12.5)		3.02, dd (17.0, 13.0)	
4		161.4		197.8		198.0
5	6.83, d (9.0)	116.9		164.0		163.3
6	7.61, d (9.0)	131.9	5.98, s	96.4	5.96, s	95.8
7	7.69, d (16.0)	144.5		167.3		167.2
8	8.02, d (16.0)	125.9		106.1		105.7
9		194.7		164.2		164.9
10				103.3		103.6
1′		107.3		134.0		134.4
2′		161.2	7.42, d (9.0)	128.8	7.50, d (9.0)	128.5
3′	6.28, s	95.7	7.13, d (9.0)	117.8	7.14, d (9.0)	117.8
4′		164.8		159.3		159.1
5′		107.0	7.13, d (9.0)	117.8	7.14, d (9.0)	117.8
6′		166.8	7.42, d (9.0)	128.8	7.50, d (9.0)	128.5
1″	4.87[a]	75.7	4.78, d (9.5)	75.2	4.75, d (10.0)	75.4
2″	4.10, dd (9.5, 9.0)	72.8	4.11, dd (9.5, 9.0)	72.6	4.05, dd (10.0, 8.5)	72.5
3″	3.51, dd (9.0, 8.5)	80.2	3.35–3.48[a]	80.0	3.35–3.48[a]	80.0
4″	3.45–3.53[a]	71.2	3.35–3.48[a]	71.4	3.35–3.48[a]	71.4
5″	3.41, m	82.6	3.32[a]	82.5	3.30[a]	82.4
6″	3.73, dd (12.0, 5.0)	62.8	3.70[a]	62.9	3.69[a]	63.1
	3.85, dd (12.0, 2.0)		3.89, dd (12.0, 2.0)		3.87[a]	
1‴	5.12, d (7.0)	101.9	4.93, d (7.5)	102.2	4.93, d (7.5)	102.3
2‴	3.56, dd (9.0, 7.0)	75.0	3.35–3.48[a]	74.9	3.35–3.48[a]	74.9
3‴	3.45–3.53[a]	78.6	3.35–3.48[a]	78.0	3.35–3.48[a]	78.0
4‴	3.45–3.53[a]	71.8	3.35–3.48[a]	71.8	3.35–3.48[a]	71.9
5‴	3.49[a]	78.6	3.35[a]	78.2	3.36[a]	78.2
6‴	3.74, dd (12.0, 5.0)	62.5	3.70[a]	62.5	3.69[a]	62.5
	3.92, dd (12.0, 2.0)		3.85, dd (12.0, 2.0)		3.87[a]	

[a]Unclear signal pattern due to overlapping signals

(H-1‴) was long-range coupled with C-4′ (**15**: δ 159.3; **16**: δ 159.1); in their differential NOE spectra, the H-1‴ resonance also correlated with H-3′ and H-5′ (**15**: δ 7.13, 2H, d, *J* = 9.0 Hz; **16**: δ 7.14, 2H, d, *J* = 9.0 Hz) (Fig. 2). These correlations showed the presence of a 4′-*O*-β-D-glucopyranosyl moiety of **15** and **16**, and their molecular structure was determined as naringenin 8-β-D-glucopyranosyl-4′-*O*-β-D-glucopyranoside.

In the ECD spectrum of **15** (Fig. 3), the positive Cotton effect at 288 nm showed the (2*R*)-absolute configuration of **15** [21]. On the other hand, the negative Cotton effect at 288 nm of **16** showed the (2*S*)-absolute configuration of **16**. Therefore, **15** and **16** were determined as (2*R*)-naringenin 8-β-D-glucopyranosyl-4′-*O*-β-D-glucopyranoside and (2*S*)-naringenin 8-β-D-glucopyranosyl-4′-*O*-β-D-glucopyranoside, respectively.

Similarly, the 2*R*-absolute configurations of **17** and **19** were determined using the positive Cotton effect at 290 nm, and the 2*S*-absolute configurations of **18**, **20** and **21** were determined using the negative Cotton effect at 290 nm.

Four different concentrations (1, 10, 50, and 100 μg/mL) of all isolated flavonoids were evaluated against in vitro growth of *B. caballi* and *T. equi*, and the half maximal growth inhibitory concentration (IC_{50}) values are shown in Table 2. The isolated chalcone and flavanone derivatives including new compounds **14–16** did not show an anti-piroplasm activity, except for **21**. In addition, all flavone and flavonol derivatives (**1–13**) showed moderate effects against *B. caballi* and/or *T. equi*. Compound **11** had the highest growth inhibitory effect (IC_{50} 17.7 μM) against *B. caballi*, and the relatively high-activity compounds among those isolated here had a *p*-coumaroyl moiety in their structures.

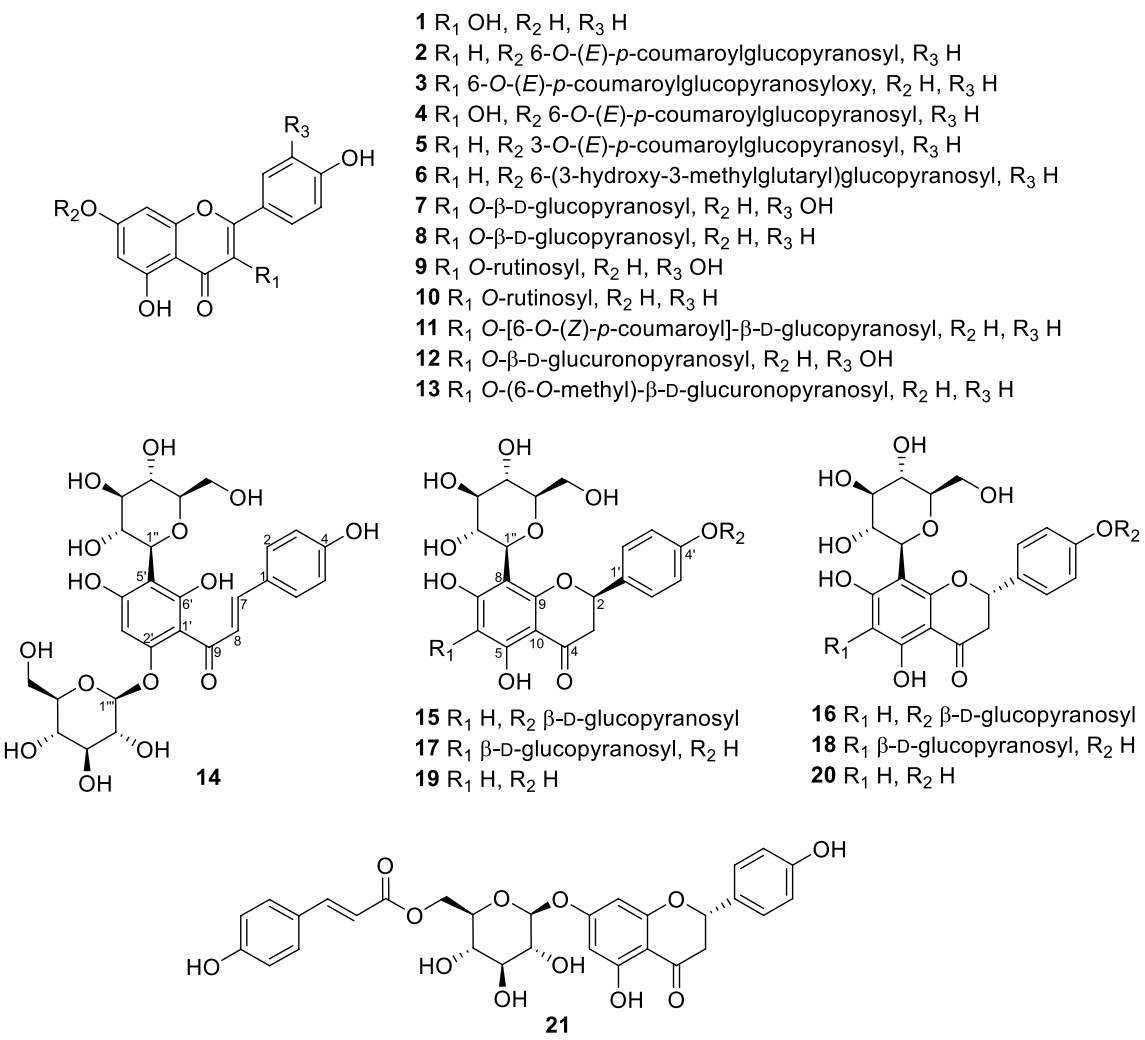

Fig. 1 Structures of compounds **1**–**21**

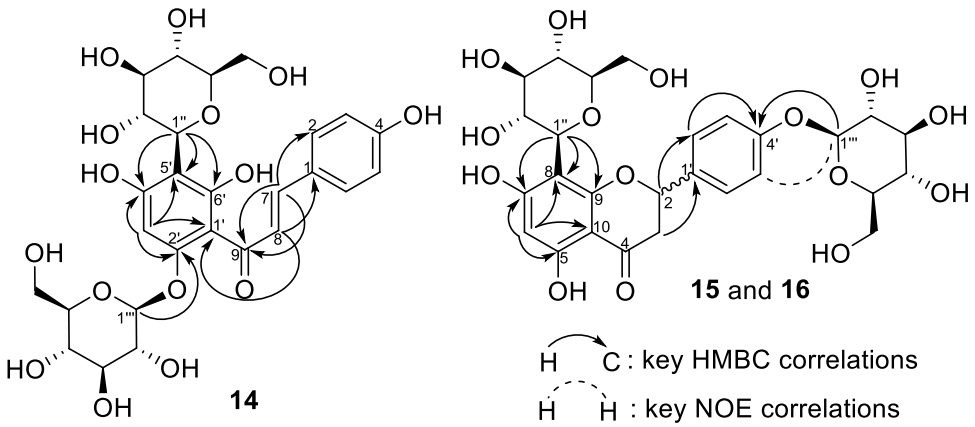

Fig. 2 Key HMBC and NOE correlations of compounds **14–16**

Previous studies [21] also showed that the galloyl moiety on glycosides was a key structure for anti-piroplasma active compounds. The findings of the previous and present studies suggested that further investigations of the effect by acyl groups against piroplasm parasites are needed. Each active compound was weak, but Yargui includes over 20 of these

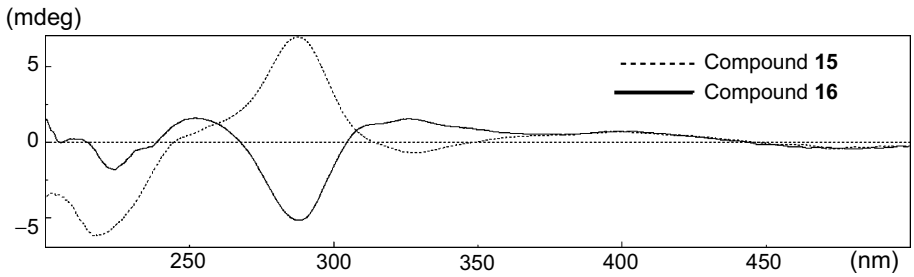

Fig. 3 ECD spectra of compounds **15** and **16**

Table 2 Anti-piroplasma activity of compounds **1–21**

Compound	*Babesia caballi* IC_{50} (μM)[a]	*Theileria equi* IC_{50} (μM)[a]
1	220 ± 0.2	201 ± 0.5
2	18.8 ± 0.3	55.6 ± 0.1
3	91.7 ± 0.2	99.9 ± 0.03
4	26.2 ± 1.0	76.0 ± 0.8
5	57.0 ± 0.4	45.2 ± 0.2
6	165 ± 0.3	130 ± 0.1
7	52.9 ± 0.1	113 ± 0.1
8	135 ± 0.1	222 ± 0.6
9	94.3 ± 0.5	–[b]
10	134 ± 0.03	164 ± 0.5
11	17.7 ± 0.3	139 ± 0.6
12	147 ± 0.3	–[b]
13	144 ± 0.1	–[b]
14	–[b]	–[b]
15	–[b]	–[b]
16	–[b]	–[b]
17	–[b]	–[b]
18	–[b]	–[b]
19	–[b]	–[b]
20	–[b]	–[b]
21	118 ± 0.7	52.7 ± 0.1
Diminazene aceturate	0.03	0.67

10 mg/ml of each compound were diluted with culture medium to give concentrations of 1, 10, 50, and 100 μg/mL

[a]Mean ± SD; the treatment was replicated two times for each concentration

[b]$IC_{50} > 100$ μg/mL

flavonoids as typical constituents. These flower flavonoids could be contributing to prevent piroplasmosis in herbivores.

Experimental

General experimental procedures

Optical rotations, UV, ECD, and IR spectra were measured using a JASCO P-2300 polarimeter (Tokyo, Japan), a Shimadzu MPS-2450 (Kyoto, Japan), a JASCO J-700 spectropolarimeter, and a PerkinElmer Spectrum One FT-IR spectrometer (Waltham, MA, USA), respectively. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded using a JEOL JNM-AL400 FT-NMR spectrometer (Tokyo, Japan), and chemical shifts are given as δ values with TMS as an internal standard (measured in methanol-d_4, chloroform-d, and pyridine-d_5). HMQC (optimized for $^1J_{C-H} = 145$ Hz) and HMBC (optimized for $^nJ_{C-H} = 8$ Hz) pulse sequences with a pulsed field gradient. HR-FAB-MS data were obtained using a JEOL JMS700 mass spectrometer, with a glycerol matrix. Preparative and analytical HPLC was performed using a JASCO 2089 with UV detection at 210 nm, using the following columns: Ultra Pack ODS-SM-50C-M (37 × 100 mm; Yamazen, Osaka, Japan), TSKgel ODS-120T (21.5 × 300 mm; Tosoh, Tokyo, Japan), Mightysil RP-18 GP (10 × 250 mm; Kanto Chemical, Tokyo, Japan), Cosmosil 5C_{18} AR-II (20 × 250 mm; Nacalai Tesque, Kyoto, Japan) and Develosil C_{30}-UG-5 (20 × 250 mm; Nomura Chemical, Aichi, Japan).

Plant material

Flowers of *P. flavescens* were collected in May 2011 at Selkhiin am, Ullanbaatar, Mongolia and identified by Prof. Ch. Sanchir, Institute of Botany, Mongolian Academy of Sciences. A voucher specimen (No. 20110523) was deposited at the Laboratory of Bioorganic Chemistry and Pharmacognosy, National University of Mongolia.

Extraction and isolation

Dried flowers of *P. flavescens* (480 g) were extracted with acetone–water (3:2) (5 × 2 L). The dried extract (130 g) was suspended in H_2O (1.5 L) and fractionated separately with diethylether. The aqueous fraction was concentrated to a blown solid, and subjected to column chromatography (Mitsubishi Diaion HP-20, 500 mL) to yield fraction 1A [1 L MeOH:H_2O (1:4, v/v), 5.87 g], fraction 1B [1 L MeOH:H_2O (1:1, v/v), 9.66 g], fraction 1C [1 L MeOH:H_2O (4:1, v/v), 7.80 g], and fraction 1D (MeOH, 1.22 g). Fraction 1B was loaded onto a reverse-phase ODS-SM-50C-M column, and eluted with MeOH–H_2O (gradient system from 1:4 to 1:1,

v/v) as the mobile phase to yield fractions 2A–2I. Fraction 2F (317 mg) was subjected to preparative HPLC to isolate compounds **14** (13.8 mg), **19** (4.4 mg), **20** (24.2 mg) [TSK-gel ODS-120T, CH_3CN–H_2O (3:17, v/v) containing 0.2% TFA; Develosil C_{30}-UG-5, CH_3CN–H_2O (3:17, v/v) containing 0.2% TFA] and fraction 2C (317 mg) was subjected to preparative HPLC to obtain compounds **15** (16.9 mg), **16** (4.2 mg), **17** (3.0 mg), and **18** (3.6 mg) [TSKgel ODS-120T, CH_3CN–H_2O (3:17, v/v) containing 0.2% TFA; Develosil C_{30}-UG-5, CH_3CN–H_2O (3:17, v/v) containing 0.2% TFA]. Fraction 1C (7.8 g) was loaded onto a reverse-phase ODS-SM-50C-M column, and eluted with MeOH–H_2O (gradient system from 3:2 to 4:1, v/v) as the mobile phase to yield fractions 3A–3Q. Fractions 3H–J (291 mg) was subjected to preparative HPLC to isolate compound **13** (10.6 mg) [TSKgel ODS-120T, CH_3CN–H_2O (1:3, v/v) containing 0.2% TFA; Develosil C_{30}-UG-5, CH_3CN–H_2O (1:3, v/v) containing 0.2% TFA] and fraction 3L (281 mg) was subjected to preparative HPLC to isolate compound **11** (1.7 mg) [TSKgel ODS-120T, CH_3CN–H_2O (3:7, v/v) containing 0.2% TFA; Develosil C_{30}-UG-5, CH_3CN–H_2O (3:7, v/v); containing 0.2% TFA]. Fraction 1D (1.2 g) was loaded onto a reverse-phase ODS-SM-50C-M column, and eluted with MeOH–H_2O (gradient system from 3:2 to 4:1, v/v) as the mobile phase to yield fractions 4A–4J. Fraction 4D (91.3 mg) was subjected to preparative HPLC to isolate compounds **2** (7.4 mg), **3** (5.9 mg), **5** (14.8 mg), and **21** (10.8 mg) [Cosmosil 5C_{18} AR-II, CH_3CN–H_2O (3:7, v/v)], fraction 4C (167 mg) was subjected to preparative HPLC to obtain compounds **4** (81.5 mg), **7** (9.5 mg), **8** (24.7 mg), **9** (6.2 mg), **10** (9.7 mg), and **12** (10.7 mg) [Cosmosil 5C_{18} AR-II, CH_3CN–H_2O (3:7, v/v); Develosil C_{30}-UG-5, CH_3CN–H_2O (3:17, v/v) containing 0.2% TFA], fraction 4B (322 mg) was subjected to preparative HPLC to obtain compounds **1** (3.3 mg) and **6** (13.3 mg) [Mightysil RP-18 GP, CH_3CN–H_2O (3:7, v/v)].

Chalconaringenin 2′-*O*-β-D-glucopyranosyl-5′-β-D-glucopyranoside (14) Yellowish, amorphous solid; $[\alpha]_D^{28}$ +6.2° (c 1.4, MeOH); UV (MeOH) λ_{max} (log ε) 373 (3.64); ECD (c 0.20, MeOH) nm ([θ]) 215 (−8800); IR ν_{max} (KBr) cm^{-1}: 3380, 1607, 1076; ^1H NMR: (CD_3OD, 400 MHz) Table 1; ^{13}C NMR: (CD_3OD, 100 MHz) Table 1; HRFABMS (positive) *m/z* 597.1818 $[M+H]^+$ (Calcd for $C_{27}H_{33}O_{15}$: 597.1819).

(2*R*)-Naringenin 8-β-D-glucopyranosyl-4′-*O*-β-D-glucopyranoside (15) Colorless, amorphous solid; $[\alpha]_D^{22}$ −81° (c 0.97, MeOH); UV (MeOH) λ_{max} (log ε) 292 (4.13), 329 (4.06); ECD (c 0.20, MeOH) nm ([θ]) 217 (−22,900), 288 (25,900), 329 (−2600), 397 (2700); IR ν_{max} (KBr) cm^{-1}: 3380, 1648, 1615, 1077; ^1H NMR: (CD_3OD, 400 MHz) Table 1; ^{13}C NMR: (CD_3OD, 100 MHz) Table 1; HRFABMS (positive) *m/z* 597.1810 $[M+H]^+$ (Calcd for $C_{27}H_{33}O_{15}$: 597.1819).

(2*S*)-Naringenin 8-β-D-glucopyranosyl-4′-*O*-β-D-glucopyranoside (16) Colorless, amorphous solid; $[\alpha]_D^{22}$ −150° (c 0.38, MeOH); UV (MeOH) λ_{max} (log ε) 292 (4.18), 326 (4.02); ECD (c 0.20, MeOH) nm ([θ]) 224 (−5600), 252 (5000), 288 (−16,000), 326 (4900), 380 (1600); IR ν_{max} (KBr) cm^{-1}: 3394, 1645, 1615, 1078; ^1H NMR: (CD_3OD, 400 MHz) Table 1; ^{13}C NMR: (CD_3OD, 100 MHz) Table 1; HRFABMS (positive) *m/z* 619.1631 $[M+Na]^+$ (Calcd for $C_{27}H_{32}O_{15}Na$: 619.1638).

Compound 17 Colorless, amorphous solid; $[\alpha]_D^{22}$ −101° (c 0.33, MeOH); UV (MeOH) λ_{max} (log ε) 292 (3.92), 334 (4.05); ECD (c 0.17, MeOH) nm ([θ]) 218 (−15,300), 290 (23,000), 331 (−400), 403 (3500).

Compound 18 Colorless, amorphous solid; $[\alpha]_D^{22}$ −84° (c 0.40, MeOH); UV (MeOH) λ_{max} (log ε) 292 (3.91), 334 (4.04); ECD (c 0.20, MeOH) nm ([θ]) 219 (14,000), 240 (−700), 257 (4700), 290 (−8900), 330 (5400).

Compound 19 Colorless, amorphous solid; $[\alpha]_D^{22}$ +27° (c 0.22, MeOH); UV (MeOH) λ_{max} (log ε) 226 (3.69), 291 (3.41), 329 (3.29); ECD (c 0.20, MeOH) nm ([θ]) 217 (−24,400), 290 (26,900), 330 (−3900), 398 (2100).

Compound 20 Colorless, amorphous solid; $[\alpha]_D^{22}$ +14° (c 1.67, MeOH); UV (MeOH) λ_{max} (log ε) 226 (3.68), 291 (3.43), 329 (3.21); ECD (c 0.20, MeOH) nm ([θ]) 218 (20,000), 238 (3400), 251 (5000), 290 (−16,000), 330 (5000).

Compound 21 Colorless, amorphous solid; UV (MeOH) λ_{max} (log ε) 211 (4.53), 286 (4.39), 314 (4.29); ECD (c 0.20, MeOH) nm ([θ]) 216 (26,000), 236 (4800), 249 (9400), 290 (−26,000), 337 (6500).

Sugar identification

Glucosidic moiety identification was carried out in accordance with a previously described method [22]. Compounds **14** (1.0 mg), **15** (2.0 mg), **16** (4.6 mg) were separately hydrolyzed with 7% HCl (1 mL) at 60 °C for 2 h, then the mixtures were subjected to the aqueous sugar fractionation. The concentrated sugar fraction was stirred with L-cysteine methyl ester (3 mg) in pyridine (0.5 mL) at 60 °C, 1 h. Then, *o*-tolyl isothiocyanate (3 μL) was added to each solution (60 °C, 1 h). The reaction mixtures were analyzed by high-performance liquid chromatography (HPLC) (Siseido, Capcel Pak C_{18}, 4.6 × 250 mm; CH_3CN–H_2O (1:3, v/v) containing 0.2% TFA, 1.0 mL/min; detection at 250 nm). The peaks of authentic L-glucose (*t*R = 15.8 min) and D-glucose (*t*R = 17.0 min) derivatives were used to identify the

O-glucosidic moieties in **14**–**16** as D-glucose based on the corresponding retention times of 17.0 min.

Growth inhibitory effects of compounds against *Babesia* and *Theileria* parasites

The USDA strains of *B. caballi* and *T. equi* were maintained in culture media with horse erythrocytes in an atmosphere of 5% O_2 and 5% CO_2 at 37 °C, as described previously [23]. Culture media (medium 199 used for *T. equi* and RPMI-1640 used for *B. caballi*) were purchased from Sigma-Aldrich (Tokyo, Japan) and prepared with 40% horse serum and 1× antibiotic antimycotic solution containing 100 U/mL penicillin G, 100 µg/mL streptomycin and 0.25 µg/L amphotericin B, as described previously [18]. Horse erythrocytes and serum were obtained from an adult horse at the horse field center of Obihiro University of Agriculture and Veterinary Medicine (Hokkaido, Japan). These parasite cultures were used in further experiments to evaluate the growth inhibitory effects of the isolated compounds. Tests with parasites were performed using a previously described method [23, 24]. Briefly, percent parasitized erythrocytes (PE) were diluted with fresh erythrocytes to afford 1% PE. From this stock, 5 (equine piroplasms) µL of 1% PE were dispensed into a 96-well culture plate (Nunc, Roskilde, Denmark) with 95 µL of culture medium containing four different concentrations of the compounds (1, 10, 50 and 100 µg/mL). Cultures were incubated as described above without changing the medium. On day 4 of culture, 100 µL of lysis buffer with SYBR Green I nucleic acid stain (2×; Lonza, Walkersville, MD, USA: 10,000×) was added and incubated for 8 h in the dark at room temperature. Subsequently, relative fluorescence values were determined using a fluorescence plate reader (Fluoroskan Ascent; Thermo Labsystems, Philadelphia, PA, USA), and the IC_{50} values of the compounds against the growths of the parasites were calculated using a curve-fitting method.

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Compliance with ethical standards

Conflicts of interest The authors indicate that there is no conflict of interest.

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