A NEW HYDROLYZABLE TANNIN FROM *GEUM JAPONICUM* AND ITS ANTIVIRAL ACTIVITY

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Abstract — A new hydrolyzable tannin, geponin, in addition to six known compounds were isolated from the aqueous extract of whole plant of *Geum japonicum* and their structures were determined by the use of 2D nmr. Geponin and gallic aldehyde showed potent antiviral activity against HSV-1 among the isolated seven compounds. These may contribute antiviral activity of the aqueous extract of *G. japonicum.*

In searching for natural products as antiviral agents, we have screened more than 140 different crude drugs for their antiviral activity against herpes simplex virus type 1 (HSV-1), poliovirus type 1, and meales virus by the plaque reduction assay.[1] By the result of screening, *Geum japonicum* Thunb., a perennial herb and the flowering plant of the Rosaceae family, was selected for preliminary study because the aqueous extract from it showed a strong antiviral activity against HSV-1 *in vitro* and *in vivo*.[1] This plant has been used in Japan and China as diuretics and astringents. Several chemical constituents, including triterpenoids, glycosides[2] and tannins[3] have been isolated from this plant, but there have been very few pharmacological and biological studies on this subject, especially antiviral activity.

In this paper, we describe the isolation and the structure elucidation of a new hydrolyzable tannin along with

six known compounds from the EtOAc-soluble fraction of the aqueous extract of *G. japonicum*. We also report antiviral activity of these isolated compounds against HSV-1 *in vitro*.

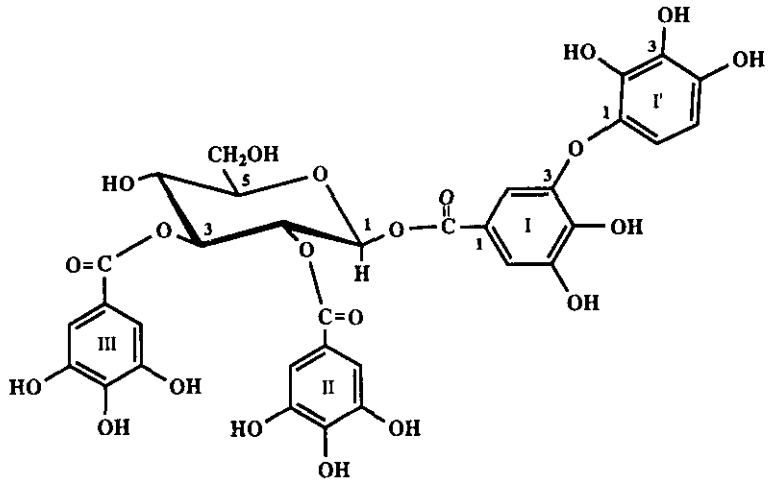
Results and Discussion

The dried whole plant of *G. japonicum* was extracted by refluxing with water to get aqueous extract which was fractionated to give EtOAc-, *n*-BuOH-, and water-soluble fractions, and these fractions were assayed for their antiviral activity against HSV-1 *in vitro*. The EtOAc-soluble fraction showed a strong inhibitory activity at a concentration of 200 μg/ml and no cytotoxity was noted, while the *n*-BuOH- and water-soluble fractions were found to be inactive.

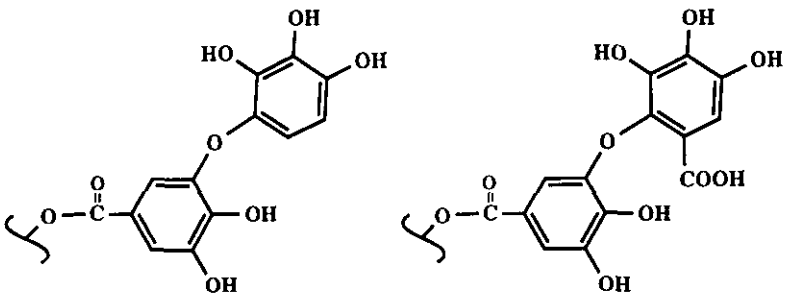
The active EtOAc-soluble fraction was then separated by silica gel column chromatography followed by Sephadex LH-20 chromatography to yield a new hydrolyzable tannin, named geponin (**1**), together with six known compounds, 1,2,3-tri-*O*-galloyl-β-D-glucopyranside (**2**),[4] blumenol A (**3**),[5] gallic aldehyde (**4**), 3,4-dihydroxybenzoic acid (**5**),[6] caffeic acid (**6**), and kaempferol-3-*O*-glucopyranside (**7**).[7] These known compounds were identified by comparing the ms, ^{1}H- and ^{13}C-nmr data of the previous literature.

Geponin (**1**) was a white amorphous powder, found to be optically active $[\alpha]_D$ +84.75° (*c* 0.4, MeOH), and showed a positive $FeCl_3$ reaction. The positive ion FAB-ms spectrum showed the quasi-molecular ion peak at *m/z* 783 $(M+Na)^+$ corresponding the molecular formula to be $C_{33}H_{28}O_{21}$. A number of hydrolyzable tannins have been reported from this plant,[3] and the uv and ir absorptions spectra of **1** also supported that the compound (**1**) to be a tannin. In the ^{1}H-nmr spectrum, two sets of equivalent galloyl protons at δ 6.96 (2H, s) and 6.85 (2H, s) and a set of two non-equivalent galloyl protons at δ 7.10 (1H, d, *J*=2.5 Hz) and 6.75 (1H, d, *J* =2.5 Hz) were observed. In addition, a set of two *ortho*-coupling protons in the aromatic region at δ 6.34 (1H, d, *J* =8.5 Hz) and 6.28 (1H, d, *J*=8.5 Hz) were also observed. Only three sugar protons were observed at low field [δ 6.03 (d, *J*=8.5 Hz), 5.47 (t, *J*=9.5 Hz), and 5.24 (dd, *J*=9.5, 8.5 Hz)], suggesting three galloyl groups were bonding with the sugar. One of the galloyl groups with two *meta*-coupling protons was found to be non-equivalent due to the phenyl group bonding at C-3 of ring-I. ^{13}C-Nmr and DEPT spectra showed a six membered sugar unit and by analyzing the coupling constant value with the aid of ^{1}H-^{1}H COSY spectrum the sugar is found to be the β-glucopyranoside.

Next we measured the HMBC spectrum for **1** to confirm the position of the substituents in the glucose ring. The anomeric proton at δ 6.03 (1H, d, *J*=8.5 Hz) and both the *meta*-coupling protons gave correlation with the carbonyl carbon at δ 164.06 (s), suggesting that C-1 is linked with galloyl group containing phenyl group. A



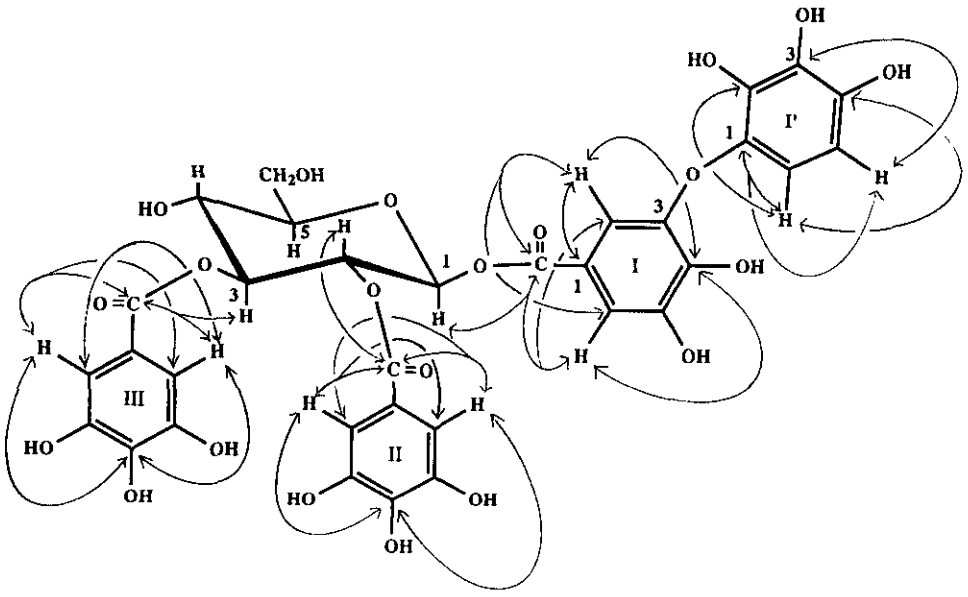
1



a b

Scheme 1

diphenyl ether substituent (**a**) containing a set of two *ortho*-coupling protons in one phenyl ring [δ 6.28, 1H, d, J=8.5 Hz and 6.34, 1H, d, J=8.5 Hz] were confirmed by HMBC spectrum. A double doublet signal at δ 5.24 (1H, dd, J=9.5, 8.5 Hz) assigned for 2-H showed the correlation with the carbonyl carbon at δ 164.70, similarly the triplet signal at δ 5.47 (1H, t, J=9.5 Hz) showed correlation with the carbonyl carbon at δ 165.27. In addition, the two singlet peaks of each for two protons at δ 6.85 and 6.96 showed correlation with the carbonyl carbon at δ 164.70 and 165.27, respectively. Considering all these facts two galloyl groups were found to be substituted at C-2 and C-3 position of the glucose. The other significant long-range correlations observed in



Scheme 2

the HMBC spectrum was also shown by the arrow in Scheme 2 . On the basis of above findings, the structure of **1** was fully established and it was named geponin.

All these compounds isolated from the EtOAc-soluble fraction were tested for their *in vitro* antiviral activity against HSV-1 by plaque reduction assay in Vero cell culture. As shown in Table I, **1** and **4** showed good antiviral activity and was not cytotoxic at the tested concentrations, while other compounds were found to be little or inactive.

Tannins are widely distributed in the plant kingdom and are believed to have many biological and pharmacological activities, such as antiviral activity,[8] antibacterial activity,[9] and anti-allergic activity.[10] Several plant extracts and commercial tannins were investigated for their antiviral activity against HSV-1, using a plaque reduction assay. Among them, the monomeric hydrolyzable tannins, oligomeric ellagitannins and condensed tannins, having galloyl groups, have been reported to have an anti-HSV activity.[8]

Geponin (**1**) showed an anti-HSV-1 activity, but 1,2,3-tri-*O*-galloyl-β-D-glucopyranside (**2**) had weak activity at the tested concentrations, although it has three galloyl groups. This suggests that the activities of tannins are different from each other depending on their chemical structures. It should be mentioned that some of known

Table I. Antiviral activity of compounds (**1-7**) isolated from ethyl acetate soluble fraction of *Geum japonicum* against HSV-1

	Plaque formation (%)		
	Concentration (μg/ml)		
Compound	12.5	25.0	50.0
1	95.5	50.8	40.5
2	91.7	91.8	62.2
3	88.0	102.6	104.1
4	42.6	0.5	0.0
5	98.9	101.1	104.9
6	94.7	93.2	98.9
7	108.2	105.6	107.9

hydrolyzable tannins contain dehydrodigalloyl (DHDG) substituent as **b**. In contrast, the carboxylic group of DHDG (**b**) substituent is displaced by hydrogen in **a** (Scheme 1). So that **1** seems to be a biogenetically interesting compound.

We have reported that the aqueous extract of *G. japonicum* has antiviral activity against HSV-1 both *in vitro* and *in vivo*.[1] Our studies indicate that geponin (**1**) and gallic aldehyde (**4**) may contribute antiviral activity of the aqueous extract of *G. japonicum*. Further isolation and purification of other fractions from aqueous extract of *G. japonicum* are currently in progress, in order to find other possible antiviral compounds and elucidate the relationship between structure and activity against HSV-1 and other viruses.

EXPERIMENTAL

All melting points were determined with a Kofler type apparatus and were uncorrected. Infrared (Ir) spectra were recorded on a Hitachi 260-10 infrared spectrophotometer in KBr disc and absorbance frequency is expressed in cm^{-1}. Uv spectra were taken on a Shimadzu Uv 2200 ultraviolet-visible spectrophotometer in MeOH and the λ_{max} is expressed in nanometer (nm). Optical rotation were measured on a JASCO DIP-4 automatic polarimeter at 26 °C. ^{1}H- and ^{13}C-nmr spectra were taken on a JEOL GX-400 Nmr spectrometer taking TMS as an internal standard for ^{1}H-nmr and chemical shifts were expressed in δ-value. ^{1}H-^{1}H COSY, and ^{1}H-detected heteronuclear multiple-bond multiple-quantum coherence (HMBC) spectra were obtained with

the usual pulse sequence and data processing was performed with the standard JEOL software. Mass and high resolution mass spectra were taken on JEOL JMX DX-300L mass spectrometer using a direct inlet system and glycerol was used as matrix in fast atomic bombardment Ms measurements. Column chromatography was done with Wako gel C-200 (Wako Pure Chemical Co., Osaka, Japan) and tlc and preparative tlc were carried out on precoated Merck Kieselgel F_{254} plates (0.25 or 0.5 mm). All the chemicals were used as analytical grade.

Extraction and isolation: Dried whole plant of *G. japonicum* was purchased from Tochimoto Tenkaido Co.(Osaka, Japan). The reference sample was deposited in the Herbarium of Materia and Medica of Toyama Medical and Pharmaceutical University, Toyama, Japan. The drugs (5 kg) were chopped into small pieces and refluxed with water (20 l x 2) for 2 h. The extract was filtered, and the filtrate was evaporated under reduced pressure and lyophilized to give a aqueous extract (1027 g), which was suspended in H_2O and extracted with EtOAc (1 l x 6) and *n*-BuOH (1 l x 6) successively to obtain EtOAc (30.6 g), BuOH(182 g), and water soluble fractions (800 g). The EtOAc-soluble fraction (30 g) was then fractionated by column chromatography on silica gel (400 mesh, Merck), eluting first with $CHCl_3$ and then with an increasing amount of MeOH to give seven fractions [fr. 1 ($CHCl_3$) 7 mg, fr. 2 (5% MeOH~$CHCl_3$) 5.15 g, fr. 3 (10% MeOH~$CHCl_3$) 14.40 g, fr. 4 (15% MeOH~$CHCl_3$) 850 mg, fr. 5 (20% MeOH~$CHCl_3$) 2.20 g, fr. 6 (30% MeOH~$CHCl_3$) 3.07 g, fr. 7 (MeOH) 1.10 g].

Fraction 6 was further purified by Sephadex LH-20 column chromatography, eluting with H_2O:MeOH (1:1) followed by reversed-phase preparative tlc (RP-18) running three times with the solvent system MeOH:H_2O (3:7) to give two tannins, **1** and **2**. **3**, **4~6**, and **7** were isolated from frs 2, 3, and 4, respectively, after rechromatography or/and preparative TLC.

Geponin (1): Pale brown amorphous powder, positive ion FAB-ms *m/z* 783 $(M+Na)^+$. $[\alpha]_D$ +84.8° (*c* 0.40, MeOH). Uv (MeOH) 276 nm (ε 45600). Ir (KBr) 3300, 1720, 1600, 1520, 1440, 1430, 1200, 1025 cm^{-1}. ^{1}H-Nmr (DMSO-d_6) δ 3.60~3.77 (4H, 4-, 5- and 6-H), 5.24 (1H, dd, *J*=9.5, 8.5 Hz, 2-H), 5.47 (1H, t, *J*=9.5 Hz, 3-H), 6.03 (1H, d, *J*=8.5 Hz, 1-H), 6.28 (1H, d, *J*=8.5 Hz, I'-5-H), 6.34 (1H, d, *J*=8.5 Hz, I'-6-H), 6.75 (1H, d, *J* =2.5 Hz, I-2-H), 6.85 (2H, s, II-2, 6-H), 6.96 (2H, s, III-2, 6-H), 7.10 (1H, d, *J*=2.5 Hz, I-6-H). ^{13}C-Nmr (DMSO-d_6) δ 60.11 (t, C-6), 67.61 (d, C-5), 70.74 (d, C-2), 74.90 (d, C-3), 77.90 (d, C-4), 92.14 (d, C-1); ring I: 109.02 (s, C-2), 111.24 (d, C-6), 118.41 (s, C-1), 141.29 (s, C-4), 146.18 (d, C-5), 147.50 (s, C-3), 164.06 (s, C=O); ring I': 105.95 (d, C-6), 110.93 (d, C-5), 134.76 (s, C-4), 136.43 (s, C-2), 138.53 (s, C-3), 143.26 (s, C-1); ring II: 109.02 (d, C-2 and -6), 117.61 (s, C-1), 139.11 (s, C-4), 145.57 (s, C-3 and -5), 164.70 (s, C=O); ring III: 109.02 (d, C-2 and -6), 119.28 (s, C-1), 138.71 (s, C-4), 145.57 (s, C-3 and -5), 165.27 (s, C=O).

1,2,3-Tri-*o*-galloyl-β-D-glucopyranside (2): Pale brown amorphous powder, FAB-ms m/z 659 $(M+Na)^+$. $[\alpha]_D$ +41° (*c* 0.40, MeOH). Uv (MeOH) 278 nm (ε 34852). Ir (KBr) 3400, 1700, 1600, 1530, 1440, 1200, 1080, 1025 cm^{-1}. ^{1}H-Nmr (DMSO-d_6) δ 5.24 (1H, dd, *J*=9.5, 8.5 Hz, Glc-2), 5.44 (1H, t, *J*=9.5 Hz, Glc-3), 6.04 (1H, d, *J*=8.5 Hz, Glc-1), 6.81, 6.91, 6.92 (each 2H, s, galloyl-H). ^{13}C-Nmr (DMSO-d_6) δ 60.05 (t, C-6), 67.58 (d, C-5), 70.77 (d, C-2), 74.90 (d, C-3), 77.78 (d, C-4), 91.98 (d, C-1), 164.21, 164.70, 165.18 (each s, ester C=O).

Blumenol A (3): Colorless crystals, mp 108-109°C. $[\alpha]_D$ +192.9° (*c* 0.4, $CHCl_3$). Ir (KBr) 3360, 2960 and 1660 cm^{-1}. Ms *m/z* 224 $(M)^+$, 206, 168, 150, 135, 124. ^{1}H-Nmr (pyridine-d_5) δ 1.13 (3H, s, 11-H_3), 1.28 (3H, s, 12-H_3), 1.45 (d, *J*=6.5 Hz, 10-H_3), 2.03 (3H, d, *J*=1.5 Hz, 13-H_3), 2.41, 2.69 (each 1H, ABq, *J*=16.5 Hz, 2-H), 4.70 (1H, m, 9-H), 6.11 (1H, t, *J*=1.5 Hz, 4-H), 6.24 (1H, dd, *J*=16.0, 1.5 Hz, 7-H), 6.33 (1H, dd, *J* =16.0, 5.0 Hz, 8-H). ^{13}C-Nmr (pyridine-d_5) δ 19.41(q, C-13), 23.51 (q, C-12), 24.45 (q, C-11), 24.51 (q, C-10), 41.60 (s, C-1), 50.26 (t, C-2), 67.41 (d, C-9), 78.94 (s, C-6), 126.61 (d, C-4), 129.09 (d, C-7), 137.17 (d, C-8), 164.61 (s, C-5), 197.86 (s, C-3).

Gallic aldehyde (4): Colorless crystals, mp 218° (decomp.). Ms *m/z* 154 $(M)^+$. ^{1}H-Nmr (DMSO-d_6) δ 6.85 (2H, s, 2- and 6-H), 9.60 (-CHO). ^{13}C-Nmr (DMSO-d_6) δ 108.84 (d, C-2 and -6), 127.43 (s, C-1), 140.37 (s, C-4), 146.17 (s, C-3 and -5), 191.24 (-CHO). The ms, ^{1}H- and ^{13}C-nmr spectra were identified with those of an authentic sample.[11]

3,4-Dihydroxybenzoic acid (5): ^{1}H-Nmr (CD_3OD) δ 6.77 (1H, d, *J*=8.0 Hz, 5-H), 7.28 (1H, dd, *J*=8.0, 2.0 Hz, 6-H), 7.33 (1H, d, *J*=2.0 Hz, 2-H), 9.27 (1H, s, OH), 9.65 (1H, s, OH). ^{13}C-Nmr (CD_3OD) δ 115.12 (d, C-5), 116.52 (d, C-2), 121.66 (d, C-6), 121.86 (s, C-1), 144.87 (s, C-3), 144.97 (s, C-4), 167.28 (C=O).

Caffeic acid (6): ^{1}H-Nmr (CD_3OD) δ 6.18 (1H, d, *J*=16.5 Hz, 2-H), 6.77 (1H, d, *J*=8.0 Hz, 5'-H), 6.96 (1H, dd, *J*=8.0, 2.0 Hz, 6'-H), 7.05 (1H, d, *J*=2.0 Hz, 2'-H), 7.42 (1H, d, *J*=16.5 Hz, 3-H). ^{13}C-Nmr (CD_3OD) δ 114.64 (d, C-5'), 115.24 (d, C-6'), 115.83 (d, C-2'), 121.20 (d, C-2), 125.80 (s, C-1'), 144.60 (d, C-3), 145.63 (s, C-3'), 148.17 (s, C-4'), 168.00 (COOH).

Kaempferol-3-*o*-glucopyranside (7): ^{1}H-Nmr (DMSO-d_6) δ 5.45 (1H, d, *J*=7.5 Hz 1''-H), 6.22 (1H, d, *J*=2.0 Hz, 6-H), 6.45 (1H, d, *J*=2.0 Hz, 8-H), 6.90 (2H, d, *J*=8.5 Hz, 3', 5'-H), 8.04 (2H, d, *J*=8.5 Hz, 2', 6'-H). ^{13}C-Nmr (DMSO-d_6) δ 60.84 (t, C-6''), 69.89 (d, C-4''), 74.23 (d, C-2''), 76.45 (d, C-5''), 77.48 (d, C-3''), 93.69 (d, C-8), 98.76 (d, C-6), 100.95 (d, C-1''), 104.01 (s, C-10), 115.15 (d, C-3', 5'), 120.92 (s, C-1'), 130.88 (d, C-2', 6'), 133.25 (s, C-3), 156.40 (s, C-2, 9), 160.02 (s, C-4'), 161.23 (s, C-5), 164.30 (s, C-7), 177.61 (s, C-4).

Cells and viruses: Vero (E6 strain) cells were grown and maintained at 37 °C in Eagle's minimal essential medium supplemented with 5% and 2% calf-serum, respectively. HSV-1 (Seibert strain[12]) was propagated in Vero cells. The infected cultures were frozen and thawed three times, and centrifuged at 3,000 rpm for 15 min. Their supernatants were stored at -80 °C until use.[13]

Antiviral assay: Antiviral activity of compounds was examined by plaque reduction assay. Duplicate cultures of Vero cells in 60 mm plastic dishes were infected with 100 plaque forming units of HSV-1 in 0.2 ml of medium for 1 h. Then the cells were overlaid with 5 ml of nutrient methylcellulose (0.8%) medium containing samples, and incubated for 2 days. The infected cells were fixed and stained, and the number of plaques were counted.[12]

The cytotoxicity of the samples was evaluated by the extent of omission of uninfected cells from the surface of stained dishes in the plaque reduction assay.

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