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## A NEW GALLOYLBERGENIN FROM *BERGENIA CRASSIFOLIA* WITH ANTI-LIPID DROPLET ACCUMULATION ACTIVITY

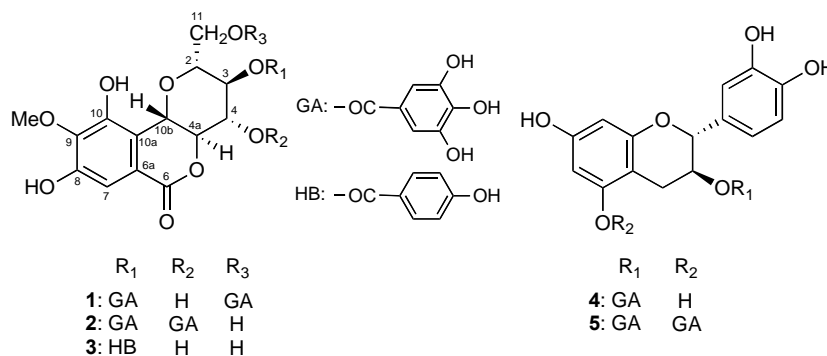
Jenis Janar,<sup>a,b</sup> Liang Fang,<sup>a</sup> Chin Piow Wong,<sup>a</sup> Toshio Kaneda,<sup>a</sup> Yusuke Hirasawa,<sup>a</sup> Burasheva Gauhar Shahmanovna,<sup>b</sup> Abilov Zharylkasyn Abduahitovich,<sup>b</sup> and Hiroshi Morita<sup>a,\*</sup>

<sup>a</sup>Faculty of Pharmaceutical Sciences, Hoshi University, Ebara 2-4-41 Shinagawa-ku, Tokyo 142-8501, Japan, <sup>b</sup>Department of Chemistry and Technology of Organic Matters, Natural Compounds and Polymers, Al-Farabi Kazakh National University, al-Farabi ave. 71, Almaty 050038, Kazakhstan

**Abstract** – A new bergenin derivative, 3,11-*di-O*-galloylbergenin (**1**) was isolated from the roots of *Bergenia crassifolia* together with four known related compounds. Their structures were elucidated by spectroscopic and chemical analysis. The new galloylbergenin showed a moderate anti-lipid droplet accumulation activity.

*Bergenia crassifolia* (Saxifragaceae) has been used for treatments of bronchitis, gastroenteritis, diarrhea, hemostasia, and metrorrhagia in Kazakh traditional medicine.<sup>1</sup> The phytochemical constituents including bergenin, tannins, flavonoids, phenols, polysaccharide, and coumarins with some pharmacological actions such as antioxidant, antimicrobial, antiviral, anti-inflammatory, diuretic, immunostimulating, and lipase inhibiting activities have also been reported.<sup>2-6</sup>

Adipogenesis is a process from fibroblast-like preadipocytes to mature adipocytes. Increases in the mass and accumulation of lipid droplet of adipocytes are observed in cases of severe human obesity which

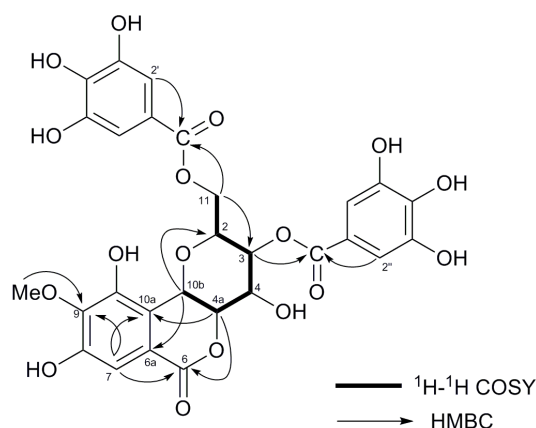


<sup>†</sup>Dedicated to Professor Ei-ichi Negishi, Purdue University, on the occasion of his 77<sup>th</sup> birthday.

presents a risk to health and may lead to the development of hypertension, hyperlipidemia, diabetes, and cardiovascular disease under the number of pathological disorders.<sup>7,8</sup>

Our research for novel lead natural products from Kazakh medicinal plants led to isolation of a new galloylbergenin, 3,11-*di-O*-galloylbergenin (**1**) to show anti-lipid accumulation active along with 4,11-*di-O*-galloylbergenin (**2**),<sup>9</sup> 11-*O*-(*p*-hydroxybenzoyl)bergenin (**3**),<sup>10</sup> (+)-catechin 3-*O*-gallate (**4**),<sup>6</sup> and (+)-catechin 3,5-*di-O*-gallate (**5**)<sup>6</sup> from the roots of *B. crassifolia*. In this paper, we describe the isolation and structure elucidation of **1**, and anti-lipid accumulation activities of isolated compounds.

3,11-*Di-O*-galloylbergenin (**1**), amorphous powder,  $[\alpha]_D^{32} -49.6$  (*c* 0.53, MeOH), showed a molecular formula,  $C_{28}H_{24}O_{17}$ , which was determined by HRESIMS [ $m/z$  655.0921 ( $M+Na$ )<sup>+</sup>]. The presence of a carbonyl and a hydroxy group were inferred by the absorptions observed in the IR spectrum of **1** appearing at 1740 and 3380  $cm^{-1}$ . The <sup>1</sup>H NMR spectrum of **1** (Table 1) suggested the presence of a methoxy group at  $\delta_H$  3.91 (s), five oxymethine groups at  $\delta_H$  4.24 (m), 5.26 (t), 4.22 (m), 4.26 (m), and 5.14 (d), one aromatic proton at  $\delta_H$  7.12 (1H, s), and two 3,4,5-trisubstituted benzoyl groups characteristic signals at 7.11 (2H, s) and 7.15 (2H, s). Furthermore, <sup>13</sup>C NMR spectrum showed the signals at  $\delta_C$  165.5, 168.0, and 167.4 which could be attributed to ester carbonyls. The gross structure of **1** was deduced from extensive analyses of two-dimensional NMR data, including <sup>1</sup>H–<sup>1</sup>H COSY, HSQC, and HMBC spectra in CD<sub>3</sub>OD (Figure 1). The <sup>1</sup>H–<sup>1</sup>H COSY and HSQC spectra revealed the presence of a partial structure (C-11, C-2 – C-4a, C-10b) as shown in Figure 1. The connectivity between aromatic rings and this partial structure was revealed by the HMBC correlations of H-10b to C-2 and C-6a, and H-4a to C-6 and C-10a. The locations of methoxy group and aromatic proton at  $\delta_H$  7.12 (1H, s) proved to be C-9 and C-7, respectively, by HMBC correlations of 9-OMe to C-9 and H-7 to C-6 ( $\delta_C$  165.5), C-9, and C-10a. Further analysis of the <sup>1</sup>H, <sup>13</sup>C and 2D-NMR data indicated the structure of **1** was virtually identical to those of a bergenin derivative, 4,11-*di-O*-galloylbergenin (**2**) except for difference in downfield chemical shift of H-3 at  $\delta_H$  5.26 (t) and up field shift of H-4 at  $\delta_H$  4.22 (m) in **1**.



**Figure 1.** Selected 2D NMR Correlations for 3,11-*Di-O*-galloylbergenin (**1**).

The location of two galloyl groups at C-3 and C-11 of bergenin were confirmed by  $^1\text{H}$  NMR and HMBC spectra as shown in Figure 1. The typical downfield shift of H-3 ( $\delta_{\text{H}}$  5.26) and H<sub>2</sub>-11 ( $\delta_{\text{H}}$  4.25 and 4.67) due to esterification indicated the position of C-3 and C-11 linked with the galloyl groups (Table 1).<sup>11</sup> Moreover, the HMBC correlations of H-11 and H-2' to 1'-CO ( $\delta_{\text{C}}$  168.0), and H-3 and H-2'' to 1''-CO ( $\delta_{\text{C}}$  167.4) also served to conclude positions of the galloyl groups to be at C-3 and C-11. The absolute configuration of **1** was determined by the hydrolysis of gallate group. On enzymatic hydrolysis with tannase, **1** gave bergenin to confirm the deduced structure. UV and CD spectral data of a hydrolysate of **1** was in good agreement with the authentic sample.

**Table 1.**  $^1\text{H}$  &  $^{13}\text{C}$  NMR Data of 3,11-*Di-O*-galloylbergenin (**1**) in CD<sub>3</sub>OD at 300K<sup>a</sup>

| Position | $\delta_{\text{H}}$ (J,Hz) | $\delta_{\text{C}}$ | HMBC      |
|----------|----------------------------|---------------------|-----------|
| 2        | 4.24, m                    | 81.2                |           |
| 3        | 5.26, t, $J=8.8$           | 72.0                | 1'-CO     |
| 4        | 4.22, m                    | 73.4                |           |
| 4a       | 4.26, m                    | 78.7                | 6, 10a    |
| 6        |                            | 165.5               |           |
| 6a       |                            | 119.4               |           |
| 7        | 7.12, s                    | 111.4               | 6, 9, 10a |
| 8        |                            | 152.4               |           |
| 9        |                            | 142.4               |           |
| 10       |                            | 149.3               |           |
| 10a      |                            | 116.8               |           |
| 10b      | 5.14, d, $J=10$            | 74.4                | 2, 6a     |
| 11       | 4.25, m                    | 63.7                | 3, 1'-CO  |
|          | 4.67, dd, $J=10, 6$        |                     |           |
| 9-OMe    | 3.91, s                    | 61.0                | 9         |
| 1'       |                            | 120.6               |           |
| 2', 6'   | 7.11, s                    | 110.4               | 1'-CO     |
| 3', 5'   |                            | 146.5               |           |
| 4'       |                            | 140.1               |           |
| 1'-CO    |                            | 168.0               |           |
| 1''      |                            | 120.8               |           |
| 2'', 6'' | 7.15, s                    | 110.5               | 1''-CO    |
| 3'', 5'' |                            | 146.6               |           |
| 4''      |                            | 140.3               |           |
| 1''-CO   |                            | 167.4               |           |

<sup>a</sup>  $\delta$  in ppm

Anti-lipid droplet accumulation activity<sup>12</sup> for **1** - **5** was evaluated. 3,11-*Di-O*-galloylbergenin (**1**) and 4,11-*di-O*-galloylbergenin (**2**) exhibited moderate anti-lipid accumulation activities with IC<sub>50</sub> values of 38.4  $\mu\text{M}$  and 60.5  $\mu\text{M}$ , respectively. As a comparison, berberine used as a positive control showed anti-lipid accumulation activity with IC<sub>50</sub> values of 14.2  $\mu\text{M}$ . On the other hand, (+)-catechin 3-*O*-gallate

(4) and (+)-catechin 3,5-di-*O*-gallate (5), which also possessed one or two galloyl groups, showed practically no anti-lipid accumulation activity. It is interesting to note that the presence of galloyl group in bergenin may play an important role to show anti-lipid accumulation activity.

## EXPERIMENTAL

**General Experimental Procedures.** CD spectra were measured on a JASCO J-820 spectropolarimeter, and IR spectra were recorded on a JASCO FT/IR-4100 spectrophotometer. <sup>1</sup>H and 2D NMR spectra were recorded on a JEOL ECA600 and Bruker AV 600 spectrometers, and chemical shifts were referenced to the residual solvent peaks ( $\delta_{\text{H}}$  3.31 and  $\delta_{\text{C}}$  49.0 for MeOD). Standard pulse sequences were employed for the 2D NMR experiments. High-resolution ESI MS were obtained on a LTQ Orbitrap XL (Thermo Scientific). HPLC was performed on a CAPCELL PAK C<sub>18</sub> MG-II, 5  $\mu\text{m}$  ( $\phi$ 10 x 250 mm).

**Plant Material.** The roots of *Bergenia crassifolia* was collected in Katon-karagai (Oskemen region, Kazakhstan) in 2009. The botanical identification was made by pharmacist Bahargul Konirhan, Institute of Medicine Inspection Department of Altay City, Xinjiang, China.

**Extraction and Isolation.** The roots of *B. crassifolia* (900 g) were extracted with 70% EtOH, and a part (35 g) of the extract (750 g) was partitioned with hexane, CHCl<sub>3</sub>, *n*-BuOH, and H<sub>2</sub>O. The *n*-BuOH fraction was subjected to an HP-20 column (H<sub>2</sub>O/MeOH, 0:1 to 1:0), and the 80% MeOH fraction (3.2 g) was further separated by using an ODS column (H<sub>2</sub>O/MeOH, 8:2 to 0:1) to give 5 fractions. Fractions 3-5 were separated by an ODS HPLC (25% MeCN/0.1% formic acid) and a Sephadex LH-20 column (CHCl<sub>3</sub>/MeOH, 1:1) to afford 3,11-*di-O*-galloylbergenin (1, 7.2 mg), 4, 11-*di-O*-galloylbergenin (2, 2.1 mg), 11-*O*-(*p*-hydroxybenzoyl)bergenin (3, 1.7 mg), (+)-catechin 3-*O*-gallate (4, 2.0 mg) and (+)-catechin 3,5-*di-O*-gallate (5, 9.5 mg).

**3,11-*Di-O*-galloylbergenin 1.** Colorless amorphous powder;  $[\alpha]_{\text{D}}^{32}$  -49.6 (*c* 0.53, MeOH), IR  $\nu_{\text{max}}$  (KBr) 3380 and 1740  $\text{cm}^{-1}$ ; UV (MeOH)  $\lambda_{\text{max}}$  219 ( $\epsilon$  69000), and 278 (25400) nm; CD (MeOH)  $\lambda_{\text{max}}$  213 ( $\Delta\epsilon$  -10.6), 243 ( $\Delta$  0), 264 ( $\Delta$  -2.25), and 288 ( $\Delta$  4.96) nm; <sup>1</sup>H and <sup>13</sup>C NMR data, see Table 1; ESIMS *m/z* 655 M+Na<sup>+</sup>; HRESIMS [*m/z* 655.0921 M+Na; calcd for C<sub>28</sub>H<sub>24</sub>O<sub>17</sub>Na, 655.0911].

**Enzymatic hydrolysis of 1:** A solution of 3,11-*di-O*-galloylbergenin (1, 2.0 mg) in H<sub>2</sub>O (3 mL) was incubated with tannase (0.5 mg) at 40 °C for 8 h. After evaporation, the residue was suspended in MeOH and was subjected to an ODS HPLC (25% MeCN/0.1% formic acid) to give bergenin, whose spectral data were identical with those of authentic sample.

**Anti-Lipid Droplet Accumulation Activity.** Cell culture and adipocyte differentiation. MC3T3-G2/PA6 murine preadipocytes (Riken Cell Bank) were maintained in basal medium (alpha minimum essential medium ( $\alpha$ -MEM) supplemented with 10% FBS). For the induction of adipocyte differentiation, cells

were pre-cultured for two days, and followed by addition of inducers [0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 0.5  $\mu$ M dexamethasone (DEX) and 5  $\mu$ g/mL insulin (INS)] and samples. Cells were maintained with inducer and samples for 6-days. Basal medium containing inducers and sample were changed every three days. The degree of samples anti-lipid accumulation was measured based on the amount of lipid droplet after 6-days cultivation with inducers. Berberine (Sigma) was used as a positive control.

Nile red staining and quantitation. To quantify lipid accumulation, Nile red fluorescent staining method with slight modification was employed. Cells were washed twice with PBS, and stained with Nile red (1 mg/mL) for 1 h at room temperature. Fluorescence was measured with Promega GloMax<sup>®</sup>-Multi Detection System ( $\lambda$ excitation 525 nm;  $\lambda$ emission 580-640 nm).

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