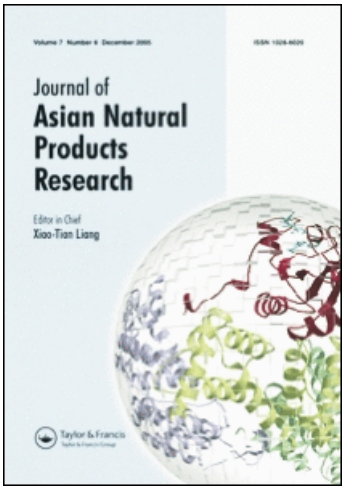


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# Inhibitory activity of isoflavones of *Pueraria* flowers on nitric oxide production from lipopolysaccharide-activated primary rat microglia

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Microglial activation plays an important role in alcohol-induced neuroinflammation. In search for natural medicines that may be of therapeutic potential for alcoholism, two new natural isoflavone glycosides, 6-hydroxybiochanin A-6,7-di-*O*-β-D-glucopyranoside (**1**) and 6-hydroxygenistein-7-*O*-β-D-glucopyranoside (**2**), were isolated from the ethanolic extract of the flowers of *Pueraria thomsonii* Benth., together with the seven known isoflavones, genistein (**3**), tectorigenin (**4**), irisolidone (**5**), genistin (**7**), tectoridin (**8**), tectorigenin-7-*O*-β-D-xylosyl-(1 → 6)-β-D-glucopyranoside (**9**), and 6-hydroxygenistein-6,7-di-*O*-β-D-glucopyranoside (**11**). Moreover, gehuain (**6**) and kakkalide (**10**) were obtained from the flowers of *Pueraria lobata* (Willd.) Ohwi. The structures of the new compounds were elucidated by UV, IR, HR-MS, and 1D and 2D NMR spectroscopic methods. Compounds **3**–**5** substantially inhibited the lipopolysaccharide-induced nitric oxide release from primary cultured rat cortical microglia ($IC_{50}$: 1.3–9.3 μM). The inhibitory effects of compounds **6**, **8**, **9**, and **11** ($IC_{50}$: 38–62 μM) were significant but weaker than the above aglycones. However, compounds **1**, **2**, **7**, and **10** showed little inhibitory activity. With regard to the structure–activity relationships of the isoflavonoids for the inhibition of microglial activation, the glycosylation at the C-7 hydroxyl group reduces the inhibitory activity. The methoxylation of 4′-hydroxyl group of 7-glycosylated isoflavonoids reduces the inhibitory activity, while the methoxyl group at the 6-position enhances the activity. The results suggest that isoflavonoids of *Pueraria* flowers may be of therapeutic potential in alcoholism related to microglial activation.

**Keywords:** isoflavonoids; flowers of *Pueraria thomsonii* and *Pueraria lobata*; NO production; microglia



## 1. Introduction

Microglia are the primary immune cells in the central nervous system. They play a key role in inflammatory processes related to the brain injury. Increasing evidence suggests that microglia activation is implicated in neurodegeneration and the regulation of alcoholic toxication [1], which causes the release of nitric oxide (NO) and several proinflammatory cytokine [2]. *Pueraria* flowers that are botanically from the flowers of *Pueraria thomsonii* or *Pueraria lobata* have been traditionally used to relieve toxic symptoms caused by excess drinking of alcohol, such as hangover, nausea, headache, and red face, in China and Japan [3]. Many phytoestrogenic isoflavones, saponins as well as a tryptophan glucoside were isolated from the flower herb [4–7]. They were reported to possess the

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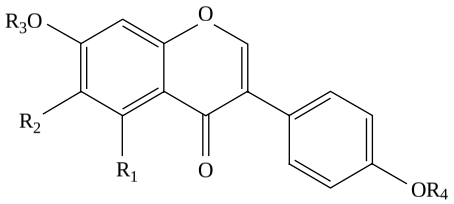
protective effect of hepatic injury and ethanol-induced toxication, inhibitory effect of aldose reductase and prostaglandin $E_2$ production, hypoglycemia, and hypolipidemic and anti-allergic effects [8–13]. Recently, the inhibitory activity of the irisolidone, tectorigenin, and glycitein on lipopolysaccharide (LPS)-activated microglial cells have been reported [14]. They were the human intestinal microflora-induced metabolites of kakkalide, tectoridin, and glycitin that are the active constituents of this herbal medicine. Genistein is neuroprotective in murine models of familial amyotrophic lateral sclerosis and stroke, and is protective for dopaminergic neurons by inhibiting microglial activation [15,16].

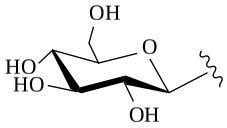
For the purpose of searching for natural medicines that may be applied to the development of pharmaceutical treatments for alcohol-induced neurodegenerative diseases, polyhydroxylated isoflavonoids were isolated, including 6-hydroxybiochanin A-6,7-di-*O*-β-D-glucopyranoside (**1**), 6-hydroxygenistein-7-*O*-β-D-glucopyranoside (**2**), genistein (**3**), tectorigenin (**4**), irisolidone (**5**), genistin (**7**), tectoridin (**8**), tectorigenin-7-*O*-β-D-xylosyl-(1 → 6)-β-D-glucopyranoside (**9**), and 6-hydroxygenistein-6,7-di-*O*-β-D-glucopyranoside (**11**) from the flowers of *P. thomsonii*, and gehuain (**6**) and kakkalide (**10**) from those of *P. lobata* (Figure 1). Among them, compounds **1** and **2** are two new compounds, whose structures were elucidated using UV, IR, MS, and 1D and 2D NMR techniques. The known compounds were identified by comparing their NMR and MS data with the reported values [5,17–21]. Their inhibitory activity of the isolates on NO release in LPS-activated microglial cells was assayed.

## 2. Results and discussion

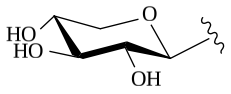
The *n*-hexane-, EtOAc-, *n*-BuOH-soluble fractions and water fraction of the EtOH extracts were obtained by partitioning the crude EtOH extracts of the flowers of *P. thomsonii* and *P. lobata* separately. Compounds **3**, **4**, **5**, **7**, and **8** were isolated from the EtOAc-soluble fraction of the extract of *P. thomsonii*, and compounds **1**, **2**, **9**, and **11** from the water fraction. On the other hand, compounds **6** and **10** were isolated from EtOAc-soluble fraction of that of *P. lobata*.

Compound **1** was isolated as colorless needle crystals. The molecular formula was determined to be $C_{28}H_{33}O_{16}$ from the molecular ion peak at *m/z* 625.1802 $[M + H]^+$ in HR-FAB-MS. The UV absorption maxima at 211.3, 264.5, and 330.8 nm revealed an isoflavonoid skeleton. A positive reaction in $AlCl_3$ reagent suggests that it is a hydroxyl-substituted isoflavonoid. The IR spectra showed absorption bands due to hydroxyl group ($3420\,cm^{-1}$), chelated carbonyl ($1654\,cm^{-1}$), and aromatic ring (1580 and $1514\,cm^{-1}$). PC analysis of the acid hydrolysis of compound **1** exhibited only the presence of glucose. The $^1H$ NMR spectrum of **1** (Table 1) indicated six aromatic proton signals due to aglycone moiety, a singlet at $\delta$ 8.50 due to H-2, a singlet at $\delta$ 6.95 (H-8) indicating A-ring with three substituents, a pair of doublet at $\delta$ 7.52 (d, $J = 8.4$ Hz, H-2′, 6′) and 7.02 (d, $J = 8.4$ Hz, H-3′, 5′) indicating a C-4′ substitute at B-ring, and a singlet at $\delta$ 3.80 due to methoxyl protons. The location of the methoxyl at C-4′ was deduced from the HMBC correlation (Figure 2) between its protons and C-4′. The resonance for the carbons due to aglycone moiety (Table 1) had a close resemblance to the reported values of those of the known kakkalide [17] except for the signal of C-6 at $\delta_C$ 129.2 showing a upfield shift, and they were assigned according to the literature values of the $^1H$ and $^{13}C$ data for kakkalide and its own HMQC spectrum. The proton signals of sugar moiety appeared as a pair of doublet at $\delta$ 5.02 (d, $J = 7.2$ Hz) and 4.88 (d, $J = 7.2$ Hz) due to two anomeric




β-D-glucopyranosyl




β-D-xylpyranosyl

| Compounds | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| 6-hydroxybiochanin A-6,7-di-*O*-β-D-glucopyranoside (**1**) | OH | O-glc | -glc | Me |
| 6-hydroxygenistein-7-*O*-β-D-glucopyranoside (**2**) | OH | OH | -glc | H |
| genistein (**3**) | OH | H | H | H |
| tectorigenin (**4**) | OH | OMe | H | H |
| irisolidone (**5**) | OH | OMe | H | Me |
| gehuain (**6**) | H | OMe | -glc-xyl | Me |
| genistin (**7**) | OH | H | -glc | H |
| tectoridin (**8**) | OH | OMe | -glc | H |
| tectorigenin-7-*O*-β-D-xylosyl-(1→6)-β-D-glucopyranoside (**9**) | OH | OMe | -glc-xyl | H |
| kakkalide (**10**) | OH | OMe | -glc-xyl | Me |
| 6-hydroxygenistein-6,7-di-*O*-β-D-glucopyranoside (**11**) | OH | O-glc | -glc | H |

Figure 1. Structures of compounds **1**–**11**.

Table 1. $^{1}H$ and $^{13}C$ NMR spectroscopic data for compounds **1**, **11**, and **2**.

| | **1** | | | **11** | | **2** | |
|---|---|---|---|---|---|---|---|
| Position | $\delta_C$[a], mult. | $\delta_H$[a] (*J* in Hz) | HMBC[c] | $\delta_C$[b], mult. | $\delta_H$[b] (*J* in Hz) | $\delta_C$[b], mult. | $\delta_H$[b] (*J* in Hz) |
| 2 | 155.1, CH | 8.50 s | 3 | 154.8, CH | 8.46 s | 154.6, CH | 8.42 s |
| 3 | 122.8, C | | | 122.3, C | | 121.4, C | |
| 4 | 180.8, C | | | 180.9, C | | 180.9, C | |
| 5 | 152.9, C | | | 152.8, C | | 149.5, C | |
| 6 | 129.2, C | | | 129.2, C | | 130.4, C | |
| 7 | 156.4, C | | | 156.4, C | | 157.5, C | |
| 8 | 94.5, CH | 6.95 s | 7, 9 | 94.5, CH | 6.94 s | 93.8, CH | 6.89 s |
| 9 | 153.2, C | | | 152.9, C | | 151.6, C | |
| 10 | 106.7, C | | | 106.7, C | | 106.6, C | |
| 1′ | 122.8, C | | | 121.1, C | | 121.8, C | |
| 2′, 6′ | 130.3, CH | 7.52 d (8.4) | 1′ | 130.3, CH | 7.40 d (8.4) | 130.2, CH | 7.36 d (7.8) |
| 3′, 5′ | 113.8, CH | 7.02 d (8.4) | 4′ | 115.2, CH | 6.83 d (8.4) | 115.2, CH | 6.81 d (7.8) |
| 4′ | 159.3, C | | | 157.6, C | | 157.6, C | |
| 4′-$CH_3$ | 55.3, $CH_3$ | 3.80 d | | | | | |
| 5-OH | | 12.96 s | | | 13.00 s | | 12.69 s |
| 1″ | 101.1, CH | 5.02 d (7.2) | 7 | 101.1, CH | 5.00 d (7.5) | 101.2, CH | 4.98 d (6.0) |
| 2″ | 73.4, CH | 3.37 m | 1″ | 73.4, CH | 3.35 m | 73.2, CH | 3.35 m |
| 3″ | 76.0, CH | 3.30 m | 2″, 4″ | 75.9, CH | 3.31 m | 75.9, CH | 3.35 m |
| 4″ | 69.8, CH | 3.15 m | 3″, 5″ | 69.8, CH | 3.16 m | 69.9, CH | 3.18 m |
| 5″ | 76.4, CH | 3.22 m | 4″, 6″ | 76.4, CH | 3.22 m | 75.9, CH | 3.18 m |
| 6″ | 60.8, $CH_2$ | 3.73 m, 3.46 m | 5″ | 60.8, $CH_2$ | 3.72 m, 3.48 m | 60.8, $CH_2$ | 3.74 m<br>3.45 m |
| 1‴ | 103.4, CH | 4.88 d (7.2) | 6 | 103.4, CH | 4.88 d (7.2) | | |
| 2‴ | 74.2, CH | 3.29 m | 1‴ | 74.2, CH | 3.28 m | | |
| 3‴ | 77.3, CH | 3.06 m | 2‴, 4‴ | 77.3, CH | 3.08 m | | |
| 4‴ | 69.8, CH | 3.15 m | 3‴, 5‴ | 69.8, CH | 3.14 m | | |
| 5‴ | 77.5, CH | 3.43 m | 4‴, 6‴ | 77.5, CH | 3.42 m | | |
| 6‴ | 60.9, $CH_2$ | 3.42 m, 3.59 m | 5‴ | 60.8, $CH_2$ | 3.61 m 3.45 m | | |

[a] Measured separately at 600 MHz.
[b] Measured separately at 300 MHz.
[c] HMBC correlations, optimized for 8 Hz, are from proton(s) stated to the indicated carbon. Some chemical shift assignments were done on the basis of HMQC and HMBC techniques.

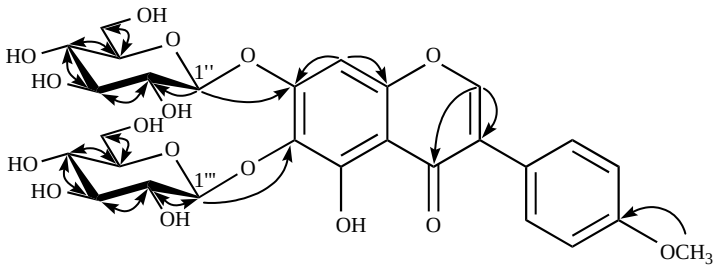



Figure 2. Key HMBC correlations of compound **1**.



protons as well as 12 protons at $\delta$ 3.73–3.06, which suggested the presence of two glucosidic moieties. The $^{13}$C NMR chemical shifts of the sugar moiety were consistent with two glucose units. The coupling constants of both anomeric protons indicated that the sugar moieties were both β-oriented configuration. Non-existence of interglucosidic linkage was confirmed by the signals of C-6″ and C-6‴ at $\delta_C$ 60.8 and 60.9. The locations of two sugar moieties separately at C-7 and C-6 were deduced from HMBC spectra (Figure 2). The HMBC correlations were observed between H-1″ and C-7 and between H-1‴ and C-6. Hence, compound **1** was assigned as 6-hydroxybiochanin A-6,7-di-*O*-β-D-glucopyranoside, a new isoflavonoid glycoside. The possibility of **1** as an artifact was ruled out by the fact that HPLC analysis confirmed its presence in the extract from the outset.

Compound **2** was obtained as a yellowish amorphous powder. The molecular formula was determined to be $C_{27}H_{31}O_{16}$ from the molecular ion peak at *m/z* 449.1037 $[M + H]^+$ in HR-FAB-MS, suggesting the addition of a hexose unit (162 mass units) to 4′,5,6,7-tetrahydroxyisoflavone. The resonances for carbons and protons of the aglycone had a close resemblance to those of 4′,5,6,7-tetrahydroxyisoflavone [22] except for C-7 as well as the proton of H-8 (Table 1). The downfield shift of C-7 ($\delta_C$ 157.5, + 3.9 ppm) and the upfield shift of H-8 ($\delta_H$ 6.89, + 0.42 ppm) relative to the corresponding signals of 4′,5,6,7-tetrahydroxyisoflavone revealed the glycosidation of C-7. The signals of an anomeric proton ($\delta_H$ 4.98, d, $J = 6.0$ Hz) and five protons ($\delta_H$ 3.18–3.74) together with the $^{13}$C NMR spectral data indicated the presence of a β-D-glucosyl moiety [23]. Hence, compound **2** was assigned as 6-hydroxygenistein-7-*O*-β-D-glucoside, which was reported as a natural component for the first time.

Compound **11** was isolated as yellowish needle crystals. The molecular ion peak at *m/z* 611.1615 $[M+H]^+$ in HR-FAB-MS data imparted its molecular formula of $C_{27}H_{31}O_{16}$. EI mass spectrum (MS) displayed a molecular ion at *m/z* 611, indicating the loss of a methyl group (14 mass units) from compound **1** (*m/z* 625). Its NMR spectroscopic data (Table 1) indicated that only the signals of the protons and carbons of B-ring were different from those of compound **1**. The downfield shifts of the H-2′, 6′ and H-3′, 5′ in $^1$H NMR spectrum suggested the hydroxylation of C-4′, which was further confirmed by its $^{13}$C NMR spectral data and the reported values of tectoridin [21]. Hence, compound **11** was confirmed as 6-hydroxygenistein-6,7-di-*O*-β-D-glucoside. Although it was reported to be isolated from *P. thomsonii* [24], the spectral values of the compound has not been reported as yet.

It is generally regarded that the inhibition of microglia activation would be an effectively therapeutic approach to alleviate the progress of neurodegeneration and

alcoholism [1,2]. Thus, the inhibitory activity of the 11 constituents on microglial activation was assayed. The cytotoxic activity of the tested compounds on primary cultured rat cortical microglial cells in the presence of LPS was tested with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium promide (MTT) assay. Co-treatment of stimulated microglial cells with all samples did not affect the cell viability at the tested concentrations, except for irisolidione at a concentration more than 10 μM and genistein or tectorigenin at a concentration of 100 μM (data not shown). As shown in Table 2, compounds **3**, **4,** and **5** showed potency of inhibiting NO release from LPS-activated microglia with $IC_{50}$ values of 1.3, 9.3, and 2.3 μM, respectively. The inhibitory activity of glycosidic compounds **6**, **8**, **9,** and **11** also was significant, with their $IC_{50}$ values being 43, 62, 45, and 38 μM, respectively. The other glycosidic compounds **1**, **2**, **7,** and **10** showed little inhibitory activity. In the parallel experiment, resveratrol, a naturally occurring polyphenol present in red wine, significantly suppressed NO production induced by LPS, which was consistent with our previous report [25]. The inhibitory effect of irisolidone, tectorigenin, and their glycoside on NO production in LPS-stimulated microglial cells in our experiment was similar to Park's report [14]. Our results suggest that irisolidone, tectorigenin, genistein and some isoflavone glycosides of *Pueraria* flowers may be of therapeutic potentials for the treatment of neurodegeneration and alcoholism related to microglial activation.

With regard to the structure–activity relationships, the glycosylation of 7-hydroxy group reduces the inhibitory activity due to such a result that the $IC_{50}$ values of compounds **3**–**5** are less than those of their related glycosides, compounds **7**, **8,** and **10**. For the C-7 glycosylated isoflavonoids, the methoxylation of 4′-hydroxyl group reduces the inhibitory activity, according to such a result, the inhibitory potency of compounds **9** and **11** are more significant than those of **10** and **1**, respectively. On the other hand, the methoxylation of 6-hydroxyl group enhances the inhibitory activity according to such a result that the inhibitory potency of compound **2** is less than those of compound **8** or **9**.

## 3. Experimental

### *3.1 General experimental procedures*

Melting points were measured using a micro-melting point apparatus and are uncorrected (Yanamoto Manufactory Co., Kyoto, Japan). Optical rotations were recorded on Perkin-Elmer 241MC automatic polarimeter. The UV spectra were obtained in Shimadzu UV-2201 spectrophotometer and IR spectra were obtained with a Bruker IFS-55 infrared spectrometer. 1D and 2D NMR spectra were recorded separately on a Bruker ARX-600 or ARX-300 spectrometer. HR-FAB-MS were acquired on a JE JMS-AX505W mass spectrometer with a resolution of 25,000 (10% valley). HPLC was carried out on a Shimadzu's LC-2010AHT. Column chromatography was carried out on silica gel (200–300 mesh, Qingdao Haiyang Chemical Group Co., Qingdao, China), polyamide gel (Taizhou Luqiao Sijia Biochemistry Plastic Co., Taizhou, China), DIAION HP-20 (Mitsubishi Chemical Co., Tokyo, Japan), Sephadex LH-20 column (GE Healthcare, Upassala, Sweden), ODS (YMC Co., Ltd, Kyoto, Japan), and MDS-5 reverse phase packings (200–300 mesh, Beijing Medicine Technology Center, Beijing, China).

### *3.2 Plant material*

Flowers of *P. thomsonii* Benth. and *P. lobata* (Willd.) Ohwi. were collected in Hangzhou/Zhejiang Province in October 2005 and Dandong/Liaoning Province in August 2006 (China), respectively. They

Table 2. Inhibitory effects of compounds **1**–**11** on NO release from LPS-activated microglia.

| | Inhibition (%) | | | | | |
|---|---|---|---|---|---|---|
| Compounds | Conc. 0[a] | 1 | 3 | 10 | 100 (μM) | $IC_{50}$ (μM) |
| **1** | 0.0 ± 7.0 | 10.2 ± 4.5 | 9.1 ± 2.4 | 41.0 ± 4.2** | 39.8 ± 3.8* | >100 |
| **2** | 0.0 ± 1.4 | −1.3 ± 3.7 | 0.1 ± 1.6 | 10.7 ± 1.0 | 42.6 ± 2.1** | >100 |
| **3** | 0.0 ± 6.6 | 43.5 ± 2.9** | 74.8 ± 2.4*** | 95.4 ± 1.0*** | –[b] | 1.3 |
| **4** | 0.0 ± 4.4 | 4.6 ± 2.2 | 19.7 ± 5.3 | 61.1 ± 4.2*** | –[b] | 9.3 |
| **5** | 0.0 ± 4.5 | 27.9 ± 1.0 | 56.4 ± 6.4*** | –[b] | –[b] | 2.3 |
| **6** | 0.0 ± 1.8 | −3.2 ± 1.0 | 13.3 ± 1.1 | 48.3 ± 1.4** | 56.7 ± 10.0*** | 43 |
| **7** | 0.0 ± 2.8 | −18.9 ± 0.3 | −25.7 ± 2.4* | −13.5 ± 2.1 | 2.7 ± 0.6 | >100 |
| **8** | 0.0 ± 3.6 | −8.4 ± 3.4 | 6.9 ± 3.1 | 16.8 ± 1.6 | 56.3 ± 3.2*** | 62 |
| **9** | 0.0 ± 3.8 | 2.2 ± 5.6 | 21.6 ± 3.3* | 28.9 ± 4.7* | 64.5 ± 4.9*** | 45 |
| **10** | 0.0 ± 5.6 | −11.9 ± 2.9 | 11.3 ± 2.7 | 19.0 ± 6.9 | 21.4 ± 0.1* | >100 |
| **11** | 0.0 ± 2.3 | 15.8 ± 10.5 | 25.1 ± 4.7* | 27.6 ± 3.9* | 67.1 ± 6.6*** | 38 |
| | 0 | 3 | 10 | 30 (μM) | | |
| Resveratrol | 0.0 ± 1.3 | 11.9 ± 1.4* | 41.1 ± 2.6** | 84.2 ± 0.7*** | | 12 |

Primary microglial cells were treated with serial dilutions of the compounds in the presence of LPS (1 μg/ml) and then incubated for 48 h. Absorbance of 540 nm was determined after mixing the culture supernatants with Griess reagent as described in the Experimental Section. Data were represented as mean ± SEM of triplicate cultures. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the groups treated with LPS alone. Resveratrol was used as positive control.

[a] Stimulation of microglial cells with the LPS resulted in a marked increase in $NO_2^-$ production (18.1 ± 0.4 μM).

[b] Significant toxicity for primary cells were showed at the concentrations.

were authenticated by Prof. Qishi Sun, Shenyang Pharmaceutical University. Two voucher specimens (Nos A0270606B and A0270609C) are kept in herbarium of the Department of Shenyang Pharmaceutical University.

### 3.3 Biological materials

Fetal bovine serum (FBS) and Iscove's modified Dulbecco's medium (IMDM) were purchased from Gibco BRL (Grand Island, NE, USA). LPS (E5:055) was purchased from Sigma (St Louis, MO, USA). Resveratrol was purchased from Xi'an Guanyu Biotech Co., Ltd (Xi'an, China), and its purity is 98% according to HPLC area purity method. Compounds **1**–**11** with their purities being more than 97% by HPLC method and resveratrol were dissolved initially in DMSO and then were diluted with PBS for experiments. DMSO at the highest concentration possibly present under experimental conditions used (0.1%) was not toxic to cells.

### 3.4 Microglial cell cultures

Primary rat microglia were prepared from the cortex of newborn Wistar rats (1 day) [26]. Briefly, meninges and blood vessels were removed from the rat cortex. Tissues were dissociated with 0.25% trypsin at 37°C for 15 min, and then the cell suspension was filtered through 50-μm diameter nylon mesh. The cells were collected by centrifugation at 1200 rpm for 10 min, resuspended in IMDM supplemented with 5% FBS, and then plated in culture flasks. After 11–14 days, the flasks were placed in a rotary shaker at 250 rpm for 1 h. The resulting cell suspension rich in microglia was placed on culture dishes to which the cells adhered after 30 min at 37°C. The purity of cells obtained was more than 95%. The cells were plated at density of $5 \times 10^5$-cells/cm$^2$ onto 96-well microtiter plates for MTT and nitrite assay.

### 3.5 Cell viability

Cell viability was determined in microglial cells by the MTT reduction assay [27]. In brief, cells were seeded in 96-well microtiter plates and treated with various test sample solutions with LPS (1 μg/ml) for 48 h. The treated cells were incubated with MTT (0.25 mg/ml) at 37°C for 3 h. The formazan crystals in the cells were solubilized with a solution containing 50% dimethylformamide and 20% sodium dodecyl sulfate (pH 4.7). The level of MTT formazan was assayed by measuring the absorbance at the wavelength of 490 nm with Spectra (shell) Reader (Tecan, Austria).

### 3.6 Nitrite assays

Accumulated nitrite in the culture supernatants, an indicator of NO synthase activity, was measured by Griess reaction [26]. Briefly, cells were seeded in 96-well microtiter plates and treated with the test sample solutions with LPS (1 μg/ml) for 48 h. Then, 50 μl culture supernatants were mixed with 50 μl Griess reagent (part I: 1% sulfanilamide; part II: 0.1% naphthylethylene diamide dihydrochloride and 2% phosphoric acid) at room temperature. Fifteen minutes later, the absorbance was determined at 540 nm using the SPECTRA (shell) Reader. Nitrite concentration was calculated with reference to a standard curve of sodium nitrite using known concentrations. The inhibition (%) of the releases of NO by the test sample was calculated by the following equation, and $IC_{50}$ values were determined on the basis of linear or nonlinear regression analysis of the concentration response curves data ($n = 3$). Dunnet's $t$-test was used for statistic analysis:

$$\text{Inhibition}\,(\%) = [1 - (T - N)/(C - N)] \times 100,$$

where control ($C$): LPS (+), test sample (−); test ($T$): LPS (+), test sample (+); and normal ($N$): LPS (−), test sample (−).

### 3.7 Extraction and isolation

The air-dried flowers of *P. thomsonii* (850 g) were extracted with EtOH (5, 3.5, 3.5 l) using reflux method. The condensed extract (160 g) was dissolved in water (0.5 l), and successively partitioned with *n*-hexane (3 × 0.5 l), EtOAc (3 × 0.5 l), and *n*-BuOH (3 × 0.5 l) to give *n*-hexane fraction (13.3 g), EtOAc fraction (23.3 g), *n*-BuOH fraction (46 g), and water fraction (32 g), respectively. A half of water fraction was subjected to DIAION HP-20 column chromatography eluting with a MeOH–$H_2O$ gradient. The fraction (880 mg) eluted with MeOH–$H_2O$ (50:50) was passed through silica gel column chromatography eluting with $CHCl_3$–MeOH (9:2) to yield four major fractions (5–8). Fraction 8 (540 mg) was repeatedly recrystallized with MeOH to give compound **11** (50 mg). The fraction (450 mg) eluted with MeOH–$H_2O$ (60:40) was subjected to a polyamide gel column chromatography (3.0 × 60 cm) eluting with a $CHCl_3$–MeOH gradient to yield four major fractions (1–4). Fraction 1 (280 mg) eluted with $CHCl_3$–MeOH (11:1) was further purified with Sephadex LH-20 column eluting with $CHCl_3$–MeOH (50:50), and then further subjected to an ODS open column chromatography eluting with MeOH–$H_2O$ (30:70) to yield compound **2** (8 mg). Fraction 3 (50 mg) eluted with $CHCl_3$–MeOH (8:1) was repeatedly recrystallized with MeOH to give compound **9** (14 mg). The crude crystals from the fraction (240 mg) eluted with MeOH–$H_2O$ (70:30) were repeatedly recrystallized with MeOH to give compound **1** (17 mg). The EtOAc-soluble fraction (14 g) was subjected to silica gel column chromatography eluting with $CHCl_3$–MeOH gradient. The fraction (228 mg) eluted with $CHCl_3$–MeOH (70:1) was further separated with an open column chromatography packed with MDS-5 reverse phase packing particles eluting with MeOH–$H_2O$ gradient to yield seven major fractions (9–15). Fraction 13 (27 mg) eluted with MeOH–$H_2O$ (80:20) was purified by an ODS open column chromatography eluting with MeOH–$H_2O$ (80:20) to give compound **5** (20 mg). The fraction (2.3 g) eluted with $CHCl_3$–MeOH (30:1) was recrystallized with $CHCl_3$ to give compound **4** (1.77 g). The fraction (3 g) eluted with $CHCl_3$–MeOH (25:1) was passed through a polyamide gel column chromatography eluting with $CHCl_3$–MeOH (6:1) to give nine major fractions (16–24). Fraction 20 (279 mg) was further separated on silica gel column chromatography eluting with $CHCl_3$–MeOH (10:1) to yield compound **3** (40 mg). The fraction (2 g) eluted with $CHCl_3$–MeOH (5:1) was repeatedly recrystallized with MeOH–$H_2O$ (20:80) to give compound **8** (650 mg). The dried mother liquor (1.2 g) was subjected to a silica gel column eluting with $CHCl_3$–MeOH–$H_2O$ (30:10:1) to give fractions 25–28. Fraction 26 (160 mg) was further purified by an ODS open column chromatography eluting with MeOH–$H_2O$ (50:50) to give compound **7** (16 mg).

The flowers of *P. lobata* (0.5 kg) were percolated with EtOH. The precipitates filtered from the condensed extract were recrystallized with MeOH–$H_2O$ (20:80) to give crude crystals (1.5 g). They were further subjected to a DIAION HP-20 column eluting with MeOH–$H_2O$ gradient. The fraction (100 mg) eluted with MeOH–$H_2O$ (50:50) was subjected to an ODS open column to yield compound **6** (16 mg). The fraction (1 g) eluted with MeOH–$H_2O$ (60:40) gave compound **10** (375 mg).

#### 3.7.1 *6-Hydroxybiochanin A-6,7-di-*O-*β-D-glucopyranoside (**1**)*

Colorless needle crystals (MeOH); mp >300°C; $[\alpha]_D^{24}$ −45 ($c = 0.1$, MeOH). UV (MeOH) $\lambda_{max}$ (nm): 213, 264, 330. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3420, 1654, 1580, 1456, 1076. $^1H$ and $^{13}C$ NMR spectral data, given in Table 1. EI-MS *m/z* (%): 625

(8) $[M+H]^+$, 460 (10), 307 (74), 300 (14), 289 (33), 259 (10), 176 (12), 154 (100), 136 (100), 107 (40), 89 (34), 77 (30). HR-FAB-MS *m/z*: 625.1802 $[M+H]^+$ (calcd for $C_{28}H_{33}O_{16}$, 625.1768).

### *3.7.2 6-Hydroxygenistein-6,7-di-*O-*β-D-glucopyranoside (**11**)*

Yellowish needle crystals; $[\alpha]_D^{24}$ −78 ($c = 0.1$, MeOH). UV (MeOH) $\lambda_{max}$ ($\log \varepsilon$; nm): 211 (3.64), 263 (4.21), 332 (4.32). $^1$H and $^{13}$C NMR data, given in Table 1. EI-MS *m/z* (%): 611 (8) $[M+H]^+$, 449 (16), 356 (4), 318 (12), 302 (20), 274 (100), 246 (25), 218 (3). HR-FAB-MS (positive) *m/z*: 611.1615 $[M + H]^+$ (calcd for $C_{27}H_{31}O_{16}$, 611.1612).

### *3.7.3 6-Hydroxygenistein-7-*O-*β-D-glucopyranoside (**2**)*

Yellowish amorphous powder; mp >300°C; $[\alpha]_D^{24}$ −28 ($c = 0.2$, MeOH). UV (MeOH) $\lambda_{max}$ ($\log \varepsilon$; nm): 212 (3.89), 269 (4.64), 345 (4.21). $^1$H and $^{13}$C NMR (DMSO-$d_6$) spectral data, given in Table 1. EIMS *m/z* (%): 471 (100) $[M+Na]^+$, 441 (19), 413 (39), 381 (10), 346 (11), 318 (37), 274 (68), 246 (6), 154 (100), 136 (100), 107 (40), 89 (34), 77 (30). HR-FAB-MS (positive) *m/z*: 449.1037 $[M+H]^+$ (calcd for $C_{21}H_{21}O_{11}$, 449.1084).

### *3.8 Acid hydroxylysis of 1*

Compound **1** (2 mg) was refluxed at 100°C for 1 h with 2 N HCl in MeOH (10 ml). The acid hydrolysate was extracted with EtOAc, the sugar in the aqueous layer was identified as glucose by co-paper chromatography (*n*-BuOH–AcOH–$H_2O$, 4:1:5, $R_f$ 0.19, aniline phthalate spray).

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