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# Steroidal constituents from the leaves of *Hosta longipes* and their inhibitory effects on nitric oxide production

Chung Sub Kim [a], Sun Yeou Kim [b], Eunjung Moon [c], Mi Kyeong Lee [d], Kang Ro Lee [a,*]

[a] Natural Products Laboratory, School of Pharmacy, Sungkyunkwan University, 300 Chonchon-dong, Jangan-ku, Suwon, Gyeonggi-do 440-746, Republic of Korea
[b] College of Pharmacy, Gachon University, #191 Hambakmoe-ro, Yeonsu-gu, Incheon 406-799, Republic of Korea
[c] Graduate School of East-West Medical Science, Kyung Hee University Global Campus, #1732 Deogyeong-daero, Giheung-gu, Yongin, Gyeonggi-do 446-701, Republic of Korea
[d] College of Pharmacy, Chungbuk National University, #52 Naesudong-ro, Heungdeok-gu, Cheongju 361-763, Republic of Korea

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ABSTRACT

*Hosta longipes* (FR. et SAV.) MATSUMURA (Liliaceae) is an edible vegetable in Korea. This study was conducted with the aim of evaluating the potential of *H. longipes* as a functional food for the treatment of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. In this respect, the study resulted in the identification of three new steroidal compounds, longipenane (**1**), longipenane 26-*O*-β-D-glucopyranoside (**2**) and neogitogenin 3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→4)]-β-D-galactopyranoside (**3**), along with two known steroidal saponins (**4** and **5**). The identification and structural elucidation of these compounds were based on 1D and 2D NMR measurements, high-resolution FAB mass spectroscopy (HR-FAB-MS), and chemical methods. A proinflammatory mediator, nitric oxide (NO), in murine microglial BV-2 cells was used to assess the anti-neuroinflammatory effect of the isolated compounds from *H. longipes*. Among them, compounds **4** and **5** showed strong inhibitory effects on NO production without high cell toxicity in lipopolysaccharide-activated BV-2 cells ($IC_{50}$ = 17.66 and 13.16 μM, respectively).



Neuroinflammation is a defense mechanism in the central nervous system against various harmful infections and injuries. However, substantial evidence supports the idea that neuroinflammation by activated microglia is strongly involved in the pathogenesis and progression of neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease, and Parkinson's disease.[1] Microglia cells are the resident immune cells of the central nervous system. Under normal conditions, these cells play a role in homeostasis regulation and defense against injury.[2] However, in response to abnormal stimuli including environment toxins and neurotoxins, microglia are activated and release various proinflammatory factors including nitric oxide (NO),[3] leading to possible exacerbation of neuronal cell death resulting in neurodegenerative diseases.[4]

*Hosta longipes* (FR. et SAV.) MATSUMURA (Liliaceae) is an edible vegetable in Korea and widely distributed throughout Korea, China, and Japan.[5] It is called "Bi-Bi-Chu" in Korea, and its young leaves are consumed raw as a salad. This plant has been used as a Korean traditional medicine for the treatment of swelling, inflammation and snake bites.[5,6] In previous phytochemical and biological activity studies, its EtOH extract showed antioxidant activity[7] and steroidal saponins isolated from this plant showed cytotoxic activity towards HeLa cells.[8] However, little phytochemical and biological investigations on *H. longipes* have been conducted. In our screening procedures, the MeOH extract of leaves of *H. longipes* showed an inhibitory effect on NO production in lipopolysaccharide (LPS)-activated BV-2 cells, a murine microglial cell line. Therefore, as part of a continuing search for bioactive constituents from Korean medicinal plant sources, we attempted to investigate the active constituents of this herb.

The MeOH extract from the leaves of *H. longipes* was suspended in distilled water and then partitioned with *n*-hexane, $CHCl_3$, EtOAc and BuOH. The $CHCl_3$ layer showed a considerable inhibitory effect on nitric oxide (NO) production in LPS-activated BV-2 cells, and EtOAc and BuOH layers displayed moderate inhibitory effects in our screening procedures. Chemical investigation of these layers using successive column chromatography over silica gel, Sephadex LH-20, and preparative HPLC resulted in the isolation and identification of three new steroidal compounds **1**-**3** and two known steroidal saponins **4** and **5** (Fig. 1).

Compound **1** was obtained as a colorless gum. The molecular formula was determined to be $C_{27}H_{44}O_5$ from the molecular ion peak $[M+H]^+$ at *m*/*z* 449.3266 (calcd for 449.3267) in the positive-ion HR-FAB-MS. The IR spectrum displayed strong OH vibrations at 3382 $cm^{-1}$ and a carbonyl band at 1660 $cm^{-1}$. The $^1H$ NMR spectrum showed the presence of four methyl groups [$\delta_H$ 1.08, 0.93 (each 3H, d, *J* = 7.0 Hz), 1.24 and 1.09 (each 3H, s)], three

* Corresponding author. Tel.: +82 31 290 7710; fax: +82 31 290 7730.
*E-mail address:* krlee@skku.ac.kr (K.R. Lee).

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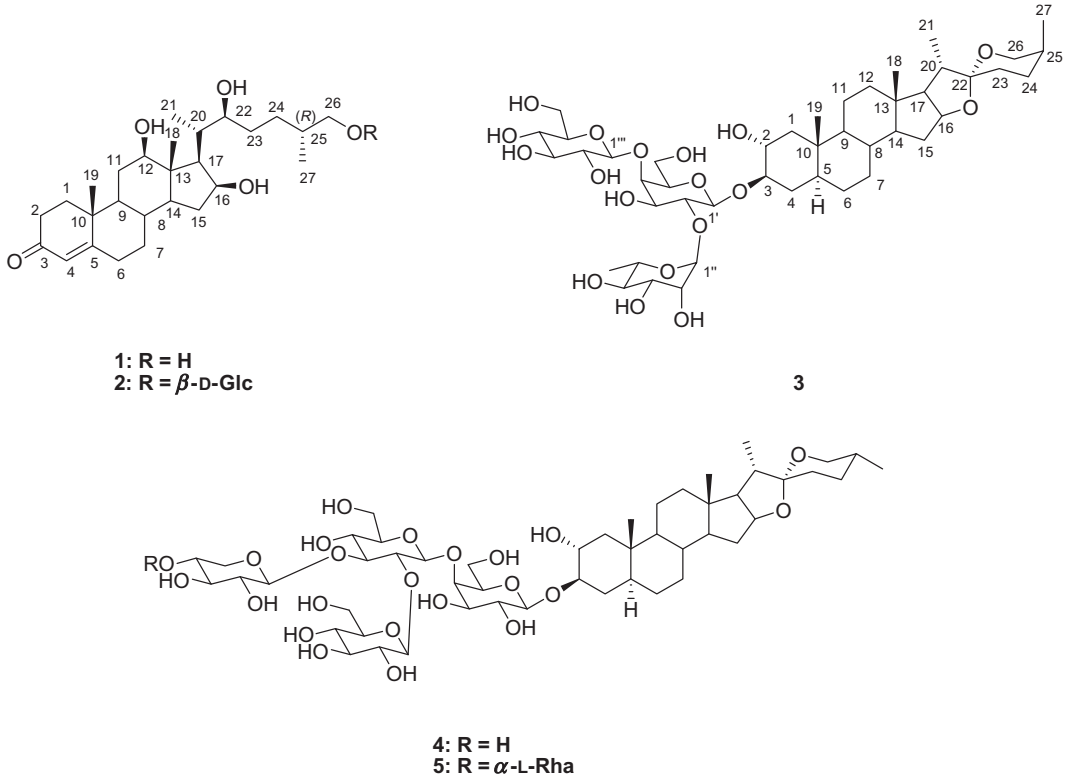


**Figure 1.** Structures of **1**–**5** from the leaves of *H. longipes*.

oxygenated methine [$\delta_H$ 4.34 (1H, dt, $J$ = 7.0, 4.0 Hz), 4.04 (1H, t, $J$ = 6.0 Hz) and 3.35 (1H, m)], one oxygenated methylene [$\delta_H$ 3.44 (1H, dd, $J$ = 5.5, 10.5 Hz), and 3.34 (1H, m)], and one olefinic proton [$\delta_H$ 5.71 (1H, s)] signals (Table 1). The $^{13}$C NMR spectrum indicated 27 carbon resonances, which were classified by DEPT and HMQC experiments as one carbonyl carbon ($\delta_C$ 201.0), one trisubstituted double bond ($\delta_C$ 173.4 and 123.1), three oxygenated methines ($\delta_C$ 78.1, 72.9 and 70.9), one oxygenated methylene ($\delta_C$ 67.2), four methyls, eight methylenes, six methines and two quaternary carbons. These NMR data were very similar to those of (25*S*)-16(*S*), 22(*S*), 26-trihydroxycholest-4-en-3-one,[9] except for the absence of one methylene proton and carbon resonances and the presence of one oxygenated methine [$\delta_H$ 3.35 (1H, m); $\delta_C$ 78.1]. The HMBC correlation of H-18/C-12 and NOE correlations of H-12/H-9 and H-14 (Fig. 2) confirmed the presence of an OH group at C-12. The relative stereochemistry was assumed to be the same as that of (25*S*)-16(*S*),22(*S*),26-trihydroxycholest-4-en-3-one[9] based on the $J$ values and confirmed by the NOE correlations of H-8/H-18 and H-19, H-9/H-12 and H-14, H-16/H-14, H-17 and H-21, and H-18/H-20 (Fig. 2). The β-orientiation of the OH group at C-12 was confirmed by the NOE correlations of H-12/H-9 and H-14. The relative configuration of the OH group at C-22 was anti to the Me group at C-20 by comparing the $J$ value of H-22 (t, $J$ = 6.5 Hz) with that of (22*S*)-bethosides B and C [t, $J$ = 6.9 Hz for H-22 (22$\beta$); ddd, $J$ = 11.1, 3.3, 2.1 Hz for H-22 (22$\alpha$)].[10] The absolute configuration at C-25 was confirmed as *R* by examination of the Mosher esters of **1**; *R* ester **1a** showed a larger difference ($\Delta\delta$ 0.211) between two H-26 signals than that of *S* ester **1b** ($\Delta\delta$ 0.081 ppm).[11] Thus, compound **1** was characterized as (25*R*)-*12β,16β,22β,26*-tetrahydroxycholest-4-en-3-one and named longipenane.

Compound **2** was obtained as a colorless gum. The molecular formula was determined to be $C_{33}H_{54}O_{10}$ from the molecular ion peak $[M+H]^+$ at $m/z$ 611.3795 (calcd for 611.3795) in the positive-ion HR-FAB-MS. The IR spectrum displayed strong OH vibrations at 3382 cm$^{-1}$ and a carbonyl band at 1656 cm$^{-1}$. The $^1$H NMR spectrum of **2** showed an anomeric proton at $\delta_H$ 4.23 (1H, d, $J$ = 8.0 Hz, H-1′), which is a characteristic signal for a β-glucopyranosyl unit, and six oxygenated protons attributed to a sugar at $\delta_H$ 3.87 (1H, dd, $J$ = 12.0, 2.0 Hz, H-6′a), 3.66 (1H, dd, $J$ = 12.0, 10.5 Hz, H-6′b), 3.34 (1H, m, H-3′), 3.29 (1H, m, H-4′), 3.27 (1H, m, H-5′) and 3.18 (1H, dd, $J$ = 9.0, 8.0 Hz, H-2′). The $^{13}$C NMR spectrum of **2** showed signals for a β-glucopyranosyl unit ($\delta_C$ 103.4, 76.8, 76.6, 73.9, 70.5 and 61.5) and the other signals in the $^1$H and $^{13}$C NMR spectra were fairly similar to those of **1**. The chemical shifts of C-26 in **2** [$\delta_H$ 3.74, 3.38 (each 1H, dd $J$ = 10.5, 6.5 Hz); $\delta_C$ 74.9] were downfield shifted compared with those of C-26 in **1**, indicating the location of the β-glucopyranosyl unit at C-26. This was confirmed by the HMBC correlation of H-1′/C-26 (Fig. 3). The enzymatic hydrolysis of **2** with β-glucosidase (Almonds) yielded the aglycone **2a**, whose $^1$H NMR spectrum was in good agreement with that of **1**, and D-glucose {$[\alpha]_D^{25}$ +62.2 ($c$ = 0.05, $H_2O$)}. Thus, compound **2** was characterized as (25*R*)-12$\beta$,16$\beta$,22$\beta$,26-(β-D-glucopyranosyl)-tetrahydroxycholest-4-en-3-one and named longipenane 26-*O*-β-D-glucopyranoside.

Compound **3** was obtained as a white powder. The molecular formula was determined to be $C_{45}H_{74}O_{18}$ from the molecular ion peak $[M+H]^+$ at $m/z$ 903.4954 (calcd for 903.4953) in the positive-ion HR-FAB-MS. The $^1$H NMR spectrum indicated the presence of two tertiary methyl groups [$\delta_H$ 0.93 and 0.81 (each 3H, s)], two secondary methyl groups [$\delta_H$ 1.14 and 1.08 (each 3H, d, $J$ = 7.0 Hz)], typical steroid methyls, and three anomeric proton [$\delta_H$ 6.27 (1H, br s), 5.20 and 4.97 (each 1H, d, $J$ = 7.5 Hz)] signals showing HSQC correlations with three anomeric carbon signals [$\delta_C$ 102.8, 107.6 and 101.4, respectively] (Table 2). Comparison of the signals from the aglycone part of **3** in the $^1$H and $^{13}$C NMR spectra with those from the aglycone part of cistocardin[12] showed that the aglycone part of **3** was the same as that of cistocardin, and this was confirmed

**Table 1**
$^1$H (500 MHz) and $^{13}$C NMR (125 MHz) data of compounds **1** and **2** in $CD_3OD$ ($\delta$ in ppm, $J$ values in parentheses)[a]

| Position | 1 | | 2 | |
|---|---|---|---|---|
| | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
| 1 | 2.07 m, 1.71 m | 35.5 t | 2.08 m, 1.71 m | 35.5 t |
| 2 | 2.50 m, 2.30 m | 33.4 t | 2.50 m, 2.30 m | 33.5 t |
| 3 | | 201.0 s | | 201.1 s |
| 4 | 5.71 s | 123.1 d | 5.72 s | 123.1 d |
| 5 | | 173.4 s | | 172.8 s |
| 6 | 2.51 m, 2.33 m | 32.7 t | 2.51 m, 2.33 m | 32.7 t |
| 7 | 1.89 m, 1.01 m | 31.4 t | 1.89 m, 1.01 m | 30.7 t |
| 8 | 1.68 m | 33.9 d | 1.68 m | 33.8 d |
| 9 | 1.07 m | 53.0 d | 1.05 m | 53.0 d |
| 10 | | 38.6 s | | 38.6 s |
| 11 | 1.66 m, 1.46 m | 30.1 t | 1.66 m, 1.46 m | 30.1 t |
| 12 | 3.35 m | 78.1 d | 3.36 m | 78.1 d |
| 13 | | 47.3 s | | 47.5 s |
| 14 | 0.84 m | 52.5 d | 0.82 m | 52.5 d |
| 15 | 2.26 m, 1.35 m | 35.5 t | 2.27 m, 1.36 m | 35.8 t |
| 16 | 4.32 dt, (7.0, 4.0) | 70.9 d | 4.33 dt, (7.5, 4.0) | 70.8 d |
| 17 | 1.44 m | 62.9 d | 1.44 m | 63.0 d |
| 18 | 1.09 s | 9.5 q | 1.10 s | 9.5 q |
| 19 | 1.24 s | 16.3 q | 1.25 s | 16.3 q |
| 20 | 2.44 m | 37.0 d | 2.45 m | 36.9 d |
| 21 | 1.08 d (7.0) | 12.1 q | 1.07 d (7.0) | 12.0 q |
| 22 | 4.04 t (6.0) | 72.9 d | 4.05 t (6.0) | 72.6 d |
| 23 | 1.52 m | 32.9 t | 1.52 m | 32.7 t |
| 24 | 1.12 m | 30.0 t | 1.66 m, 1.15 m | 30.5 t |
| 25 | 1.57 m | 35.9 d | 1.77 m | 33.5 d |
| 26a | 3.44 dd (10.5, 5.5) | 67.2 t | 3.74 dd (10.0, 6.5) | 74.9 t |
| 26b | 3.34 m | | 3.38 dd (10.0, 6.5) | |
| 27 | 0.93 d (6.5) | 15.9 q | 0.95 d (6.5) | 16.1 q |
| 1′ | | | 4.23 d (8.0) | 103.4 d |
| 2′ | | | 3.18 dd (9.0, 8.0) | 73.9 d |
| 3′ | | | 3.34 m | 76.8 d |
| 4′ | | | 3.29 m | 70.5 d |
| 5′ | | | 3.27 m | 76.6 d |
| 6′a | | | 3.87 dd (12.0, 2.0) | 61.5 t |
| 6′b | | | 3.66 dd (12.0, 10.5) | |

[a] The assignments were based on DEPT, $^1$H–$^1$H COSY, HMQC and HMBC experiments.

through 1D and 2D NMR spectra ($^1$H and $^{13}$C NMR, DEPT, $^1$H–$^1$H COSY, HSQC, HMBC and NOESY). The sugar parts were assumed to be *O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→4)]-β-D-galactopyranosyl unit by comparing the $^1$H and $^{13}$C NMR spectra with those of gitogenin 3-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→4)]-β-D-galactopyranoside}[8] and confirmed through HMBC correlation of H-1′/C-3, H-1″/C-2′ and H-1‴/C-4′ (Fig. 4) and TOCSY (Supplementary data). Acid hydrolysis of **3** gave neogitogenin (**3a**), which was identified through $^1$H NMR and MS spectra with previously reported values,[12,13] and three monosaccharides identified by GC/MS[14] analysis were L-rhamnose, D-glucose and D-galactose. The stereochemistries of the aglycone part were determined by NOE correlations (Fig. 4). The 25*S* configuration was confirmed through the comparison characteristic chemical shifts for C-23, C-24, C-25, C-26 and C-27 with those for C-23, C-24, C-26 and C-27 (27.3 ± 0.3, 26.1 ± 0.3, 65.1 ± 0.1, and 16.2 ± 0.2 ppm, respectively, 25*S*) and for C-23, C-24, C-25 and C-26 (31.3 ± 0.3, 28.8 ± 0.3, 30.3 ± 0.3 and 66.9 ± 0.2, respectively, 25*R*) in previously reported data.[15–17] Thus, the structure of **3** was established as neogitogenin 3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→4)]-β-D-galactopyranoside.

Compounds **4** and **5** were identified by comparing the $^1$H and $^{13}$C NMR, and MS spectra with the literature. They were determined to be gitogenin 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside} (**4**) and gitogenin 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[*O*-α-L-rhamnopyranosyl-(1→4)-β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside} (**5**),[18] which were suggested as the main constituents of *H. longipes*.

Neuroinflammation by overactivated microglia has been known to contribute to progressive neuronal damage in many neurodegenerative diseases.[1] In this study, we tested the anti-neuroinflammatory effects of compounds (**1**–**5**) isolated from *H. longipes* via measurement of NO levels using a bacterial endotoxin, LPS, in murine microglia BV-2 cells.[19,20] The inhibitory activities of the isolated compounds on NO production were expressed as 50% inhibition concentration ($IC_{50}$). As shown in Table 3, compounds **2**, **3**, **4**, and **5** decreased NO production. Among them, the $IC_{50}$ values of compounds **3**, **4**, and **5** were determined to be below than 20 μM. They exhibited inhibitory activity with an $IC_{50}$ of 2.70, 17.66, and 13.16 μM, respectively. It was shown that compound **3** strongly reduced the cell viability of BV-2 cells [38.9 ± 2.2% at a concentration of 20 μM compared with LPS only treatment group, median lethal dose ($LD_{50}$) of 3.12 μM]. We suggest that the inhibitory effect of compound **3** on NO production may be influenced by its high cytotoxic activities, although the percentage of inhibition of NO production was more than that of cell cytotoxicity. Compounds **4** and **5** had no influence on cell viability at concentrations up to 20 μM. These compounds had shown the cell viability of BV-2 cells with 89.6 ± 5.5% and 86.4 ± 5.8% at a concentration of 20 μM, respectively. The results indicate that statistically significant differences were not shown between LPS-only treatment group and each compound at 20 μM. Therefore, compounds **4** and **5** inhibited LPS-induced NO production in BV-2 cells without the influence of cytotoxicity. And these results indicate that steroidal saponins (**4** and **5**) may be the main active compounds of *H. longipes*, exhibiting strong anti-neuroinflammatory properties. Moreover, these compounds isolated from *H. longipes* can be candidates for treatment of various neurodegenerative diseases associated with neuroinflammation via regulation of activated microglia.

In conclusion, this study indicates that steroidal constituents are the main components of the leaves of *H. Longipes*. Additionally,

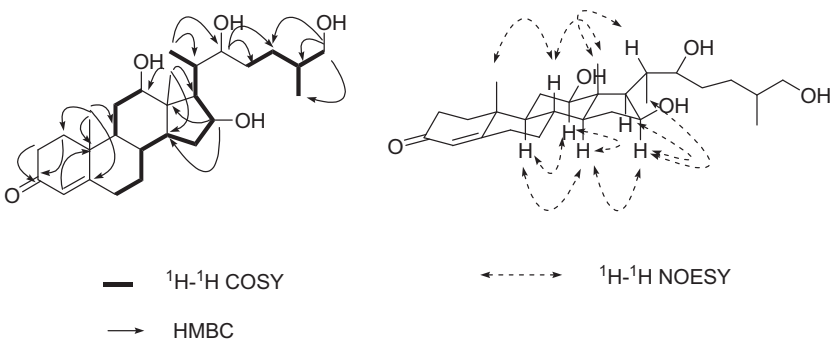


**Figure 2.** HMBC, $^1$H–$^1$H COSY and NOESY correlations of **1**.

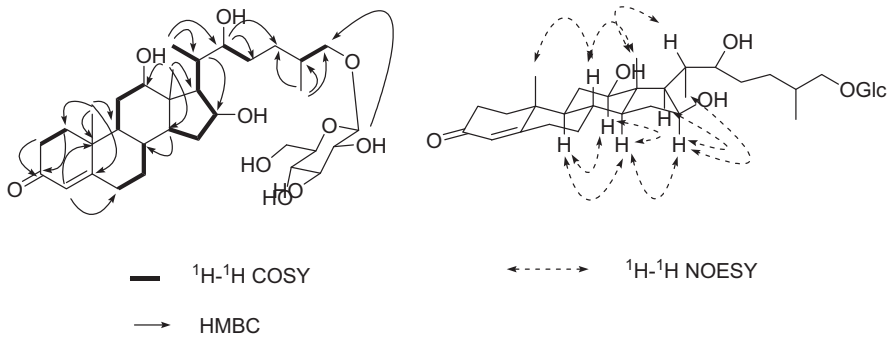


**Figure 3.** HMBC, $^1$H–$^1$H COSY and NOESY correlations of **2**.

**Table 2**
$^1$H (500 MHz) and $^{13}$C NMR (125 MHz) data of compounds **3** in pyridine-$d_5$ ($\delta$ in ppm, $J$ values in parentheses)[a]

| Position | Aglycone | | Position | Sugar | |
|---|---|---|---|---|---|
| | $\delta_H$ | $\delta_C$ | | $\delta_H$ | $\delta_C$ |
| 1 | 2.27 m, 1.19 m | 46.2 t | 1′(Gal) | 4.97 d (7.5) | 101.4 d |
| 2 | 4.13 m | 71.0 d | 2′ | 4.58 m | 77.2 d |
| 3 | 3.89 m | 85.4 d | 3′ | 4.37 m | 76.9 d |
| 4 | 2.02 m, 1.83 m | 33.9 t | 4′ | 4.59 m | 81.0 d |
| 5 | 1.03 m | 45.1 d | 5′ | 4.14 m | 76.1 d |
| 6 | 1.24 m, 1.13 m | 28.6 t | 6′ | 4.60 m, 4.32 m | 61.5 t |
| 7 | 1.53 m, 0.82 m | 32.6 t | 1″(Rha) | 6.27 br s | 102.8 d |
| 8 | 1.42 m | 35.1 d | 2″ | 4.81 m | 72.8 d |
| 9 | 0.62 br t (11.0) | 54.8 d | 3″ | 4.60 m | 73.2 d |
| 10 | | 37.3 s | 4″ | 4.33 m | 74.5 d |
| 11 | 1.50 m, 1.25 m | 21.9 t | 5″ | 4.87 m | 69.9 d |
| 12 | 1.63 m, 1.03 m | 40.5 t | 6″ | 1.63 d (6.5) | 19.0 q |
| 13 | | 41.2 s | 1‴(Glc) | 5.20 d (7.5) | 107.6 d |
| 14 | 1.02 m | 56.8 d | 2‴ | 4.15 m | 76.0 d |
| 15 | 2.03 m, 1.41 m | 32.7 t | 3‴ | 4.23 m | 79.4 d |
| 16 | 4.57 m | 81.8 d | 4‴ | 4.11 m | 72.6 d |
| 17 | 1.79 m | 63.3 d | 5‴ | 4.00 m | 79.1 d |
| 18 | 0.81 s | 17.0 q | 6‴ | 4.59 m, 4.22 m | 63.5 t |
| 19 | 0.93 s | 14.0 q | | | |
| 20 | 1.91 m | 42.9 d | | | |
| 21 | 1.14 d (6.5) | 15.3 q | | | |
| 22 | | 110.2 s | | | |
| 23 | 1.24 m, 1.12 m | 28.6 t | | | |
| 24 | 1.90 m, 1.45 m | 26.8 t | | | |
| 25 | 1.36 m | 26.7 d | | | |
| 26 | 4.07 m, 3.38 m | 65.5 t | | | |
| 27 | 1.08 d (7.0) | 16.8 q | | | |

[a] The assignments were based on DEPT, $^1$H–$^1$H COSY, HSQC and HMBC

**Table 3**
Effects of compounds **1**–**5** on NO production and cell viability in LPS-activated BV-2 cells

| Compound | $IC_{50}$[a] (μM) | Cell viability[b] (%) |
|---|---|---|
| 1 | >200 | 93.4 ± 2.0 |
| 2 | 46.30 | 94.8 ± 1.5 |
| 3 | 2.70 | 38.9 ± 2.2* |
| 4 | 17.66 | 89.6 ± 5.5 |
| 5 | 13.16 | 86.4 ± 5.8 |
| L-NMMA[c] | 14.29 | 102.3 ± 3.7 |

[a] $IC_{50}$ value of each compound was defined as the concentration (μM) causing 50% inhibition of NO production in LPS-activated BV-2 cells.
[b] Cell viability after treatment with 20 μM of each comound was expressed as a percentage (%) of the LPS only treatment group. The results are averages of three independent experiments, and the data are expressed as mean ± SD. (Student's $t$-test, *$p$-value <0.05)
[c] L-NMMA as a positive control.

three new steroidal compounds, longipenane (**1**), longipenane 26-*O*-β-D-glucopyranoside (**2**), and neogitogenin 3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→4)]-β-D-galactopyranoside (**3**) were isolated from this plant source. Two main steroidal saponins (**4** and **5**) of this plant, which significantly inhibited nitric oxide (NO) production in LPS-activated BV-2 cells, were also investigated. The present study thus indicates that these compounds would be good candidates for further research as anti-neuroinflammatory agents and show the potential of *H. longipes* as a functional food for the treatment of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease.

## Acknowledgements

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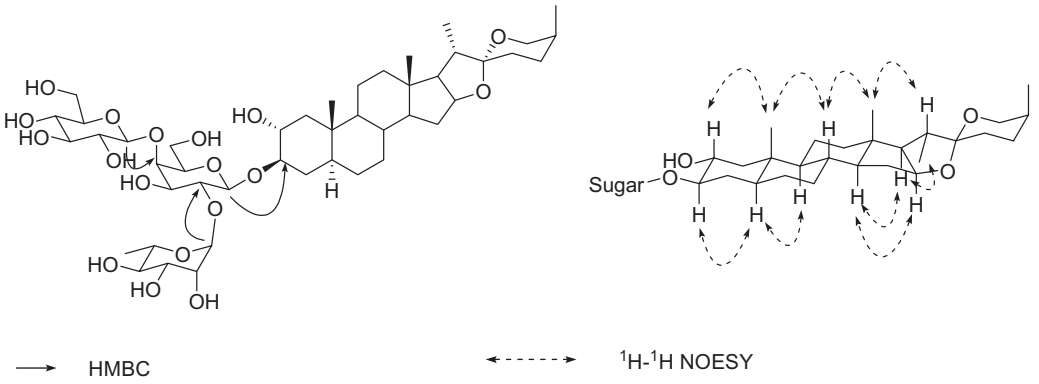


**Figure 4.** Key HMBC and NOESY correlations and of **3**.

## Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.01.050.

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