SHORT REPORT

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Stemona alkaloids isolated from *Stemona tuberosa* roots and their inhibitory activity on lipopolysaccharide-induced nitric oxide production

Ki Yong Lee[1,2], Eun Ju Jeong[2,3], Sang Hyun Sung[2] and Young Choong Kim[*2]

[1]*College of Pharmacy, Korea University, Sejong 339-700, South Korea*
[2]*College of Pharmacy and Research Institute of Pharmaceutical Science, Seoul National University, Seoul 151-742, South Korea*
[3]*Department of Agronomy & Medicinal Plant Resources, College of Life Sciences and Natural Resources, Gyeongnam National University of Science and Technology, Jinju, South Korea*

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Abstract: A methanol extract of the roots of *Stemona tuberosa* (Stemonaceae) significantly inhibited lipopolysaccharide-induced nitric oxide production in murine BV2 microglial cells. Eight alkaloids tuberostemonine K (**1**), neotuberostemonine (**2**), oxotuberostemonine (**3**), bisdehydroneotuberostemonine (**4**), epibisdehydrotuberostemonine J (**5**), bisdehydrotuberostemonine (**6**), tuberostemoninol (**7**), and neostenine (**8**) were isolated from the methanolic extract using bioactivity-guided fractionation. Among them, compounds **4**, **5** and **6** showed significant inhibitory effect on lipopolysaccharide-induced nitric oxide production in BV2 microglia at 100 μM.

Keywords: *Stemona tuberose*; alkaloids; lipopolysaccharide; BV2 microglia; nitric oxide.

1. Plant Source

The roots of *Stemona tuberosa* ("Bai-Bu" in Chinese) has been applied in traditional Chinese medicine for centuries to manage respiratory diseases, e.g., bronchitis, pertussis, and tuberculosis, and to prevent human and cattle parasites, agricultural pests, and domestic insects. The extract of the roots was found to have antitussive, antibacterial, antifungal, antiviral, insecticidal and neuroprotective activities [1-3].

The roots of *S. tuberosa* (15kg) were purchased from Kyungdong Oriental Herbal Market, Seoul, Korea and identified by Dr. Jong Hee Park, a professor of the College of Pharmacy, Pusan National University (Busan, Korea). A voucher specimen (SNUPH-0822) has been deposited in the Herbarium of the Medicinal Herb Garden, College of Pharmacy, Seoul National University.

2. Previous Studies

Main active constituents in these roots are *Stemona* alkaloids which have been shown to constitute a unique chemical feature of the small monocotyledonous family Stemonaceae, not detected so far in any other plant family. The chemical structure of this alkaloidal class is characterized by a pyrrolo[1,2-a]azepine core usually linked with two carbon chains forming mostly terminal lactone

[*] Corresponding author: E- Mail:youngkim@snu.ac.kr; Phone +82-1-880-7842.

rings [1, 2, 4]. Recent studies have demonstrated that *Stemona* alkaloids are the antitussive activity [1, 5]. To date, however, there have been no reports on the anti-inflammatory constituents of the plant. Therefore, we attempted to investigate the active constituents for anti-inflammation. In the present study, we report the isolation and structural elucidation of eight alkaloids from the roots of *S. tuberosa* (Fig. 1). Their effects on NO production were evaluated using LPS-stimulated murine microglial BV2 cells as an assay system.

3. Present Study

LPS-stimulated BV2 microglia offers an excellent model for the screening and subsequent evaluation of the effects of candidate drugs on the inflammatory pathway. Thus we tried to find compounds modulating NO production from natural products using LPS-stimulated BV2 microglia. In this screening system, the methanol extract of *S. tuberosa* roots significantly inhibited LPS-induced NO production in murine BV2 microglial cells at the concentration of 100μg/mL.

The dried roots (15 kg) of *S. tuberosa* were extracted with 80% MeOH in an ultrasonic apparatus. Upon removal of solvent *in vacuo*, the MeOH extract yielded 8 kg. After evaporation of most of the solvent, the residue was acidified with dilute HCl (4%) and centrifuged at 5°C, 3000 rpm for 40 min. The supernatant was adjusted to pH 9 with 25% NH_4OH and extracted with CH_2Cl_2. The CH_2Cl_2 (150g) fraction was subjected to column chromatography (CC) over silica gel (600g, 12 × 100cm) eluted with *n*-Hexane-EtOAc-MeOH mixtures (50:1:0, 20:1:0, 10:1:0, 5:1:0, 3:1:0, 2:1:0, 1:1:0, 0:1:0, 0:50:1, 0:20:1, 0:10:1, 0:5:1, 0:3:1, 0:2:1, 0:1:1, 0:0:1, 2L of each solvent) to afford 15 fractions (F1 – F15). F9 was subjected to CC over silica gel (200g, 5 × 60cm) eluted with $CHCl_3$/MeOH/H_2O mixtures (50:1:0, 20:1:0, 10:1:0, 5:1:0, 3:1:0, 2:1:0, 1:1:0, 0:1:0, 0:50:1, 0:20:1, 0:10:1, 0:5:1, 0:3:1, 0:2:1, 0:1:1, 0:0:1, 500mL of each solvent) to afford 19 fractions (F9_1 – F9_19). F9_2 was recrystallized from MeOH to give white needles (**4**, 110mg). F9_3 was recrystallized from MeOH to give colorless crystals (**2**, 3.4g). F9_8 was recrystallized from MeOH to give white amorphous powders (**7**, 10mg). F9_9 was subjected to reversed-phase HPLC chromatographic separation (acetonitrile:H_2O = 60:40, flow rate 2.0ml/min) to give **3** (Rt = 13.6 min, 18 mg). HPLC was performed with an L-6200 pump (Hitachi, Japan), an L-4000 UV detector (Hitachi, Japan), and an YMC-Pack Pro C18 column (YMC Co., Ltd., Japen). F13 was subjected to CC over silica gel (200g, 5 × 60cm) eluted with $CHCl_3$/MeOH mixtures (100:0, 50:1, 25:1, 10:1, 5:1, 2:1, 1:1, 0:1, 1L of each solvent) to afford 15 fractions (F13_1 – F13_15). F13_2 was subjected to reversed-phase HPLC chromatographic separation (acetonitrile:H_2O = 53:47, flow rate 2.0ml/min) to give **5** (Rt = 27.8 min, 25 mg) and **6** (Rt = 30.6 min, 18 mg), respectively. F14 was subjected to CC over silica gel (200g, 5 × 60cm) eluted with $CHCl_3$/MeOH mixtures (100:0, 50:1, 25:1, 10:1, 5:1, 2:1, 1:1, 0:1, 1L of each solvent) to afford 12 fractions (F14_1 – F14_12). F14_5 was recrystallized from MeOH to give white amorphous powders (**1**, 610 mg). F14_8 was subjected to silica gel CC (100g, 3 × 60cm) and eluted with $CHCl_3$/MeOH/H_2O mixture (25:4:1) to give **8** (588 mg).

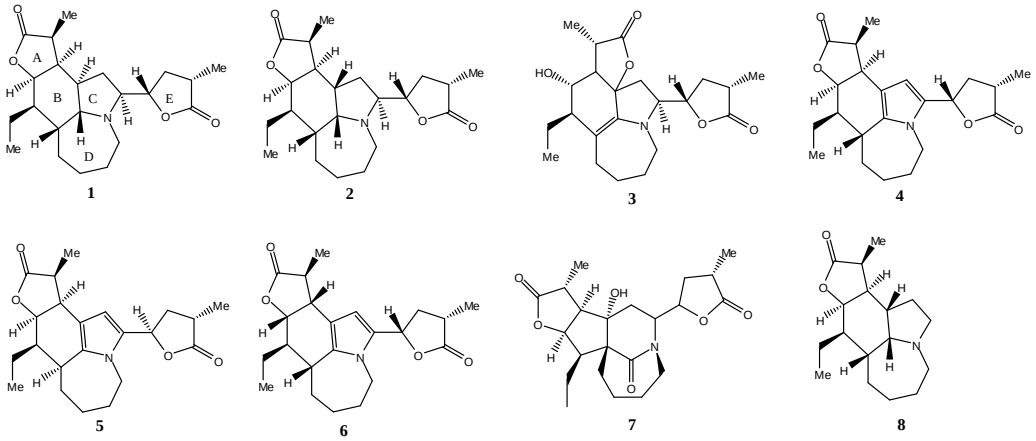

Figure 1. Chemical structures of **1 - 8** isolated from *S. tuberosa*.

Table 1. Inhibitory activities of the methanol extract and the fractions of *S. tuberosa* roots against LPS-induced NO production in BV2 microglial cells.

Fractions	Relative NO (%)[a]	Viability (%)
Control	0.0 ± 3.8	100.0 ± 4.5
LPS	100.0 ± 9.5	94.5 ± 4.3
Methanol extract	50.0 ± 5.1***	99.1 ± 5.5
CH_2Cl_2 fraction	61.0 ± 4.2***	97.0 ± 2.2
H_2O fraction	92.7 ± 9.7	101.1 ± 6.6

Control is the value of BV2 cells which were not treated with LPS. LPS is the value of BV2 cells which were treated with 100 ng/mL LPS for 24 hr. Nitrite concentrations of control and LPS-treated cultures were 2.89 ± 0.20 μM and 8.03 ± 0.49 μM, respectively. Test fractions (100μg/ml) were added 1 hr before the LPS treatment. [a] Relative NO production (%) was calculated as 100 x (nitrite concentration of LPS + sample-treated – nitrite concentration of control)/(nitrite concentration of LPS-treated – nitrite concentration of control). The values are expressed as the means ± S.D. (n=3). Mean value is significantly different (* $p< 0.05$, ** $p<0.01$, *** $p<0.001$) from the value of the LPS-treated.

As shown in Table 1, among the partitioned fractions, the CH_2Cl_2 fraction showed the most potent NO production inhibitory activity. This fraction rich in alkaloids as determined by TLC (Dragendorff test) were used for the isolation of active compounds. Eight *Stemona* alkaloids were identified as tuberostemonine K (**1**), neotuberostemonine (**2**), oxotuberostemonie (**3**), bisdehydroneotuberostemonine (**4**), epibisdehydrotuberostemonine J (**5**), bisdehydrotuberostemonine (**6**), tuberostemoninol (**7**), and neostenine (**8**), respectively by comparison of physicochemical and spectroscopic data with previously reported data [5-10]. Compounds **1**-**8** were evaluated for their inhibitory activity against LPS-induced NO production in BV2 microglial cells using Griess assay. The detailed of assay is described in the supporting information. Among them, compounds **4**, **5** and **6** showed significant inhibitory effect on LPS-induced NO production in BV2 microglia at 100 μM without cytotoxicity. More derivatives should be assessed to clarify the relationship between the structure and activity, nevertheless our study suggests the importance of double bonds in C ring and its orientation for the inhibitory activity of *Stemona* alkaloids against LPS-induced NO production.

Table 2. Inhibitory activities of compounds **1**-**8** isolated from *S. tuberosa* against LPS-induced NO production in BV2 microglial cells.

Compounds	1 μM		10 μM		100 μM	
	Relative NO (%)[a]	Viability (%)	Relative NO (%)[a]	Viability (%)	Relative NO (%)[a]	Viability (%)
1	94.9 ± 1.5	94.7 ± 6.1	116.8 ± 6.8	92.2 ± 5.1	114.8 ± 13.1	92.5 ± 3.5
2	79.7 ± 7.4**	94.8 ± 4.7	78.3 ±12.7*	94.9 ± 1.2	89.9 ± 10.3	90.8 ± 1.9
3	76.3 ± 13.3*	100.2 ± 4.9	73.2 ± 8.3**	96.9 ± 8.9	71.2 ± 4.9***	99.0 ± 5.5
4	108.0 ± 15.8	100.5 ± 4.0	92.0 ± 14.1	105.9 ± 5.3	51.2 ± 4.3***	105.3 ± 2.6
5	85.8 ± 5.1	101.4 ± 2.9	78.2 ± 4.2**	105.9 ± 3.1	61.7 ± 1.1***	99.2 ± 3.7
6	80.8 ± 16.7	101.8 ± 3.7	78.1 ± 14.5*	106.6 ± 4.0	58.5 ± 4.9***	103.0 ± 8.4
7	104.6 ± 5.3	104.3 ± 6.5	80.1 ± 10.3*	98.5 ± 5.9	81.4 ± 5.5**	102.4 ± 2.9
8	92.6 ± 9.4	97.7 ± 3.5	79.1 ± 2.5*	101.4 ± 3.3	87.8 ± 12.0	105.0 ± 1.7
NAME[b]	58.5 ±3.9*	99.1 ± 3.2	29.5 ±1.4***	89.6 ± 4.9	11.2 ± 3.4***	98.3 ± 3.9

Control is the value of BV2 cells which were not treated with LPS. LPS is the value of BV2 cells which were treated with 100 ng/mL LPS for 24 hr. Nitrite concentrations of control and LPS-treated cultures were 2.89 ± 0.20 μM and 8.03 ± 0.49 μM, respectively. Compounds were added 1 hr before the LPS treatment. [a] Relative NO production (%) was calculated as 100 x (nitrite concentration of LPS + sample-treated – nitrite concentration of control)/(nitrite concentration of LPS-treated – nitrite concentration of control). The values are expressed as the means ± S.D. (n=3). Mean value is significantly different (* $p< 0.05$, ** $p<0.01$, *** $p<0.001$) from the value of the LPS-treated. [b] NAME (ω-nitro-L-arginine methyl ester) was used as a positive control.

In conclusion, our present study suggests that CH_2Cl_2 fraction of the roots of *S. tuberosa* and compounds **4**, **5**, and **6** significantly inhibited LPS-induced NO production in murine BV2 microglial cells. Precisely excessive NO has been reported to play a causative role in the development of inflammatory neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and other disorders of the CNS [11]. Therefore, this will provide further insight into the design of new approaches for prevention and/or treatment of neurodegenerative diseases.

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Supporting Information

Supporting information accompanies with this paper on http://www.acgpubs.org/RNP

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