Lipopolysaccharide-Induced Nitric Oxide and Prolyl Oligopeptidase – Inhibitory Activities of Triterpenoids from *Sanguisorba officinalis*

Hongyu Ma, Xizhe Li, Yuchen Zhang, Guangsong Chu, Zhong Yuan

School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, Shenyang, People's Republic of China

Abstract
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Lipopolysaccharide-induced nitric oxide and the protease prolyl oligopeptidase (EC 3.4.21.26) have been proposed as targets for the treatment of cognitive disturbances such as dementia. In this study, nine triterpenoids isolated from the roots of *Sanguisorba officinalis*, including arjunic acid (**1**), rosamultic acid (**2**), haptadienic acid (**3**), 1β-hydroxyeuscaphic acid (**4**), euscaphic acid (**5**), tormentic acid (**6**), pomolic acid (**7**), ursolic acid (**8**), and oleanolic acid (**9**), were tested *in vitro* for their inhibitory activities on lipopolysaccharide-induced nitric oxide in N9 microglia cells and on prolyl oligopeptidase. Among them, **7** exhibited the strongest inhibitory activity on lipopolysaccharide-induced nitric oxide (IC$_{50}$, 21.9 μM), while **8** and **9** exhibited a significant prolyl oligopeptidase inhibitory activity (IC$_{50}$ 18.1 and 24.0 μM, respectively). The results could provide a clue that these triterpenoids are responsible for the reported neuro-protective effect of *S. officinalis*.

Key words

Sanguisorba officinalis · Rosaceae · triterpenoids · lipopolysaccharide-induced nitric oxide · prolyl oligopeptidase

The dried roots of *Sanguisorba officinalis* L. (Rosaceae) are used in traditional Chinese medicine for the treatment of disorders such as bleeding, burns, ulcerative colitis, and chronic intestinal infections, due to their hemostatic, anti-infective, antibacterial, and anti-inflammatory activities [1–8]. Previous chemical investigations of *S. officinalis* revealed the existence of triterpenes, triterpenoids, saponins and polyphenols [3–6]. Moreover, *S. officinalis* and its active component (catechin) have been reported to possess potential neuroprotective effects and antioxidant activities [3–8]. Our interest in the constituents possibly responsible for these beneficial properties inspired us to undertake a further biological investigation of *S. officinalis*.

Nitric oxide (NO) released by N9 microglia (stimulated by lipopolysaccharide; LPS) is known as a neurotoxic factor and has a great influence on the cell viability of microglia. Microglia, the resident macrophage-like cells in the brain, have been proposed to play a pivotal role in the immune surveillance of the central nervous system [9]. Furthermore, lines of evidence showed that microglia-mediated neuroinflammation contributes to the pathology of both acute pathologies such as stroke or traumatic brain injury and chronic neurodegenerative diseases [10–12].

The protease prolyl oligopeptidase (POP; EC 3.4.21.26) has been proposed to play an important role in the neuropeptide metabolism and to be a target for the treatment of cognitive disturbances such as dementia [13–15]. It has been postulated that specific POP inhibitors could protect nerve cells, prevent memory loss, and enhance cognition [16–18]. Therefore, POP inhibitors have been developed as candidate drugs for the treatment of various clinical conditions of the brain [19]. Some natural POP inhibitors, especially triterpenoids from medical plants, have also been reported [1, 20–22].

Identifying LPS-induced NO and POP inhibitors might lead to a better understanding of the potential neuroprotective benefits of *S. officinalis*. In our present work, dried roots of *S. officinalis* were extracted with 70% ethanol and the concentrated extract was partitioned with ethyl acetate (EtOAc) and water. From the EtOAc-soluble fraction, nine triterpenoids (**1**–**9**) were prepared and then tested *in vitro* for LPS-induced NO in N9 microglia cell and POP inhibitory activities.

The dried roots (8 kg) of *S. officinalis* were extracted with 70% ethanol and the concentrated extract was partitioned with EtOAc and water. In an investigation of the LPS-induced NO and POP inhibitory activities, the EtOAc-soluble fraction showed noticeable activities (NO inhibition rate of 100 μg/mL, 68.3 ± 0.63%; POP inhibition rate of 100 μg/mL, 93.40 ± 0.52%). Compounds **1**–**9** were obtained from the EtOAc-soluble fraction after purification by polyamide, ODS C-18, Sephadex LH 20, and silica gel column chromatography. By comparison with published spectral data, the isolated triterpenoids were determined as arjunic acid (**1**), rosamultic acid (**2**), haptadienic acid (**3**), 1β-hydroxyeuscaphic acid (**4**), euscaphic acid (**5**), tormentic acid (**6**), pomolic acid (**7**), ursolic acid (**8**), and oleanolic acid (**9**), respectively (○ **Fig. 1**) [23–24]. HPLC analysis also demonstrated that the EtOAc-soluble fraction was triterpenoid-rich (○ **Fig. 2**).

Compounds **1**–**9** (purity > 95% as indicated by HPLC analysis) and resveratrol (a natural polyphenol that potently inhibits the production of NO by LPS-activated microglia; purity ≥ 98.0%) were evaluated for their inhibitory activities against LPS-induced NO [25]. Among them, compound **7** exhibited a moderate activity (IC$_{50}$, 21.9 μM) comparable to the positive control resveratrol (IC$_{50}$, 15.1 μM), while the activities of the other compounds were negligible. Meanwhile, compounds **1**–**9** and bacitracin [a bacteria peptide and natural POP inhibitor; potency (as bacitracin A): min. 40 units/mg] were measured for their POP inhibitory activities. Compared with the positive control bacitracin (IC$_{50}$, 14.9 μM), **8** and **9** exhibited a moderate activity (IC$_{50}$ 18.1 and 24.0 μM, respectively) and showed no significant difference to the reported data [21]. Ursolic acid (**8**) and oleanolic acid (**9**) are isomers, only different at the 29-CH$_3$ linkage and their POP inhibitory activities were also similar. However, the hydroxyl substituted derivatives of ursolic acid, such as 1β-hydroxyeuscaphic acid (**4**), euscaphic acid (**5**), and pomolic acid (**7**) had lost these activities (○ **Table 1**), which suggests that the hydroxyl groups substituted on ursolic acid or oleanolic acid are the obstacles of their POP inhibitory activities. It also suggests that the triterpenoid-rich fraction (the EtOAc-soluble fraction) is responsible for the reported neuroprotective effect of *S. officinalis*, and deserves to be further investigated.

Materials and Methods
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General

The NMR spectra were recorded on a Bruker ARX-600 (600 MHz) instrument with TMS as internal standard. The absorbance was obtained on microplate reader (Synergy HT, BioTek). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and LPS from *Escherichia coli* 026:B6 were purchased from Sigma
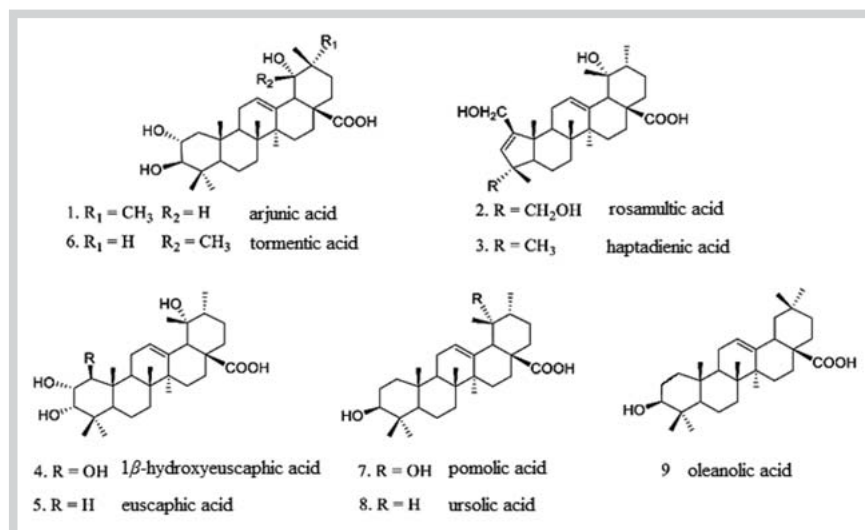
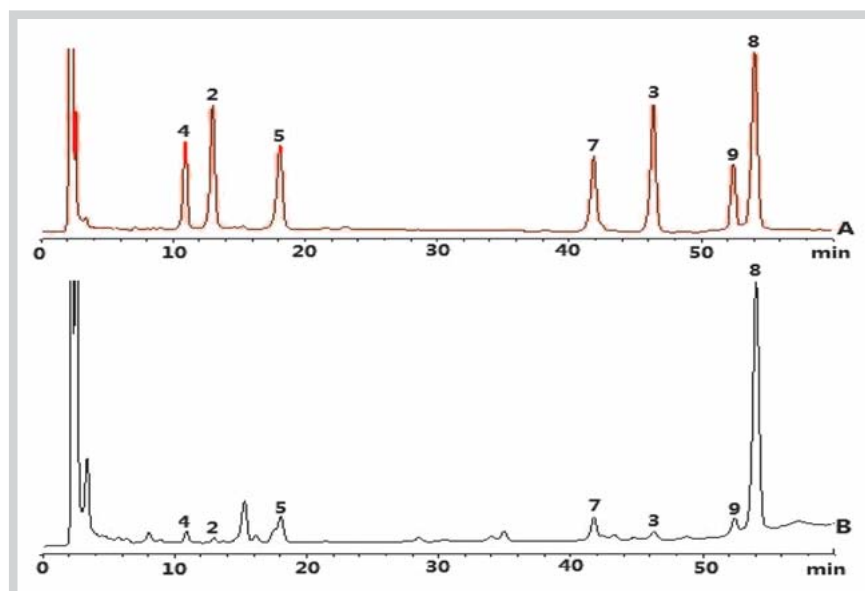**Fig. 1** Structures of compounds **1**–**9**.

Fig. 2 HPLC analysis of EtOAc-soluble extract. **A** standards; **B** EtOAc-soluble extract.

Chemical Co. Z-Gly-Pro-AMC was purchased from Bachem AG Chemical Co. HPLC analysis was performed using a Shimadzu HPLC system.

Plant material

S. officinalis roots were collected in November 2011 in Benxi, Liaoning Province, China and identified by Prof. Weichun Wu of the Shenyang Phamaceutical University, Shenyang, China. A voucher specimen (NO. 20111015) was deposited in the School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University.

Extraction and isolation

Dried and powdered plant material (8 kg) was refluxed with 70% ethanol for two hours (10 L × 3 times) and the ethanol extract was concentrated under reduced pressure to give a residue (2130 g). The residue was suspended in water and partitioned with EtOAc and then exhaustively evaporated to yield an EtOAc extract (425 g) and water extract (542 g). The EtOAc-soluble extract was subjected to polyamide column chromatography and

Table 1 Anti-inflammatory activities of compounds **1**–**9** and controls.

Compound	POP inhibition IC$_{50}$ (µM)	NO inhibition IC$_{50}$ (µM)
Arjunic acid (**1**)	> 100	> 100
Rosamultic acid (**2**)	> 100	> 100
Haptadienic acid (**3**)	93.2 ± 1.3	> 100
1β-hydroxyeuscaphic acid (**4**)	> 100	> 100
Euscaphic acid (**5**)	> 100	> 100
Tormentic acid (**6**)	> 100	> 100
Pomolic acid (**7**)	> 100	21.9 ± 0.3
Ursolic acid (**8**)	18.1 ± 1.3	> 100
Oleanolic acid (**9**)	24.0 ± 1.4	> 100
bacitracin	14.9 ± 1.2	–
resveratrol	–	15.1 ± 0.5

eluted with an EtOH-H$_2$O gradient system. The 25% ethanol eluted fraction (50.5 g) was then subjected to column chromatography over silica gel combined with Sephadex LH-20 and ODS

column chromatography and recrystallization to give compounds **1** (5 mg), **2** (9 mg), **3** (10 mg), **4** (15 mg), **5** (20 mg), **6** (5 mg), **7** (20 mg), **8** (30 mg), **9** (19 mg).

HPLC analysis

HPLC was carried out on a Kromasil C_{18} column (200 mm × 4.6 mm i.d., 5 µm) under the following chromatographic conditions: sample injection volume, 20 µL; column temperature, 30 °C; flow rate, 0.8 mL/min; mobile phase, acetonitrile (A) and 1.25% aqueous phosphoric acid (v/v, B). A gradient program was used according to the following profile: 0–16 min, 47–48% A; 16–63 min, 48–74% A. The wavelength of UV detection was set at 206 nm. Sample was dissolved in acetonitrile and filtered through an ultramembrane filter (pore size 0.45 µm) before being used. Compounds **2**–**5**, **7**–**9** were dissolved in acetonitrile as mixture standards.

Microglial cell culture

The murine microglia cell line N9 was a kind gift from Dr. P. Ricciardi-Castagnoli (Universita Degli Studi di Milano-Bicocca, Milan, Italy). The cells were similar to primary microglia in producing NO and various cytokines after stimulation. N9 cells were cultured in IMDM supplemented with 5% FBS, 2 mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37 °C in humidified 5% CO_2 [26].

Measurement of cell viability

Cell viability was evaluated by MTT reduction assay [27]. In brief, N9 cells at 1×10^5 cells/well were seeded into 96-well plates. After various treatments for 24 h, the medium was removed and the cells were incubated with MTT (0.25 mg/mL) for 3 h at 37 °C. The formazan crystals in the cells were solubilized with DMSO. The level of MTT formazan was determined by measuring its absorbance at 490 nm using a microplate reader.

Nitric oxide assay

Accumulation of nitrite (NO_2^-), an indicator of NO synthase activity, in cell culture supernatant was measured by the Griess reaction [28]. N9 cells (1×10^5 cells/well) were seeded into 96-well plates and treated with each sample (dissolved in DMSO) for 2 h and then stimulated with 1 mg/mL LPS. After incubation for 24 h, 50 µL of culture supernatant was mixed with equal volume of Griess reagent (part I: 1% sulfanilamide; part II: 0.1% naphthyl-ethylene diamide dihydrochloride and 2% phosphoric acid) at room temperature. Fifteen minutes later, the absorbance was determined at 540 nm using a microplate reader.

Prolyl oligopeptidase assay

The inhibitory activity against POP from porcine brain was measured by fluorescence spectrophotometer as described by Wallén et al. [29–30]. Z-Gly-Pro-AMC was used as the substrate. Formation of 7-amino-4-methylcoumarin (AMC) was determined fluorometrically and the excitation and emission wavelengths were 380 and 460 nm, respectively.

Data analysis

Results were expressed as mean ± S.E.M. of three independent experiments performed in triplicates. One-way ANOVA followed by Dunnett's t-test was used for statistical analysis (SPSS 20.0 software). The IC_{50} value was defined as the concentration of compound required to inhibit 50% of NO and POP activity, and determined by non-linear regression using GraphPad Prism 5.0 software.

Conflict of Interest
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The authors declare no conflict of interest.

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Correspondence
Zhong Yuan
Department of Analysis of Traditional Chinese Medicine
School of Traditional Chinese Materia Medica
Shenyang Pharmaceutical University
103 Wenhua Road
Shenyang 110016
People's Republic of China
Phone: + 86 0 24 23 98 64 69
Fax: + 86 24 24 82 43 52
yuanzhong@syphu.edu.cn