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Nitric Oxide Inhibitory Constituents from Fruits of *Opuntia humifusa*

You-Jeng Kang[1], Hae-Young Kim[1], Chul Lee[2], and So-Young Park[1,3,*]

[1]*World Class University, Department of Nanobiomedicine, Dankook University, Cheonan 330-714, Korea*
[2]*Eco-friendly Material Research Center, Korea Research Institute of Bioscience and Biotechnology, Jeonbuk 580-185, Korea*
[3]*Laboratory of Pharmacognosy, College of Pharmacy, Dankook University, Cheonan 330-714, Korea*

Abstract – *Opuntia humifusa*, also called as Cheonnyuncho, is a cactus widely cultivated in southern regions of Korea. It has been known to have diverse biological activities, but most of the studies were performed with the MeOH extracts or solvent-partitioned fractions. Furthermore, the efforts to identify the responsible compounds for the biological activities are very limited. In this study, we tested the inhibitory effect of extracts and solvent-partitioned fractions of *O. humifusa* against LPS-induced nitric oxide (NO) production in Raw264.7 cells. The butanol fractions of *O. humifusa* efficiently inhibited the production of NO in Raw264.7 cells, but it was not due to the reduction of cell viability. Bioassay-guided isolation of butanol fractions of *O. humifusa* allowed the isolation of three flavonoids isorhamnetin 3-*O*-β-D-galactosyl-4'-*O*-β-D-glucoside (**1**), isorhamnetin 3,4'-di-*O*-β-D-glucoside (**2**) and isorhamnetin 3-*O*-β-D-(6-*O*-α-L-rhamnosyl)glucoside (**3**), and one lignan syringaresinol *O*-β-D-glucopyranoside (**4**). Among them, isorhamnetin 3-*O*-β-D-galactosyl-4'-*O*-β-D-glucoside (**1**) and isorhamnetin 3,4'-di-*O*-β-D-glucoside (**2**) exhibited the moderate inhibitory effects against LPS-induced NO production. This is the first time to report anti-inflammatory effects of these compounds.
Keywords – *Opuntia humifusa*, Nitric oxide, Isorhamnetin derivatives, Raw264.7 cells

Introduction

Cacti in the genus *Opuntia* are members of the Cactaceae family, and these species are widely distributed in countries with semiarid climate, especially in the Mediterranean and Central America.[1,2] Among cacti in the genus *Opuntia*, *Opuntia ficus-indica* (L.) Mill., Indian-fig prickly pear, is one of the species which are intensively studied for its chemical constituents and biological activities. It has been reported that *Opuntia ficus-indica* has anti-inflammatory, anti-ulcer, anti-diabetic and neuroprotective effects.[3] In Korea, *O. ficus-indica* is widely cultivated in the Jeju Island, and fruits and stems of *O. ficus-indica* are used for the production of functional foods. Another cactus, *Opuntia humifusa* Raf., eastern prickly pear cactus, which is locally called as Cheonnyuncho, is also cultivated in Korea, particularly southern coastal area. *O. humifusa* is different from *O. ficus-indica* with respect of plant shapes. *O. humifusa* is a prostrate succulent and stems are covered with tiny hair-like bristles and spines. The stems become shrunk in winter, but new stems grow in spring because *O. humifusa* is a perennial plant, which is resistant against frost and the cold under −20 °C.[4] Compared to *O. ficus-indica*, the studies on chemical and biological activities of *O. humifusa* are limited. Anti-oxidant, anti-bacterial, anti-cancer and hepatoprotective[5-8] effects are reported, but these effects are mainly by the total extracts or solvent-partitioned fractions of *O. humifusa*. Particularly, the major parts used from *O. humifusa* are stems, and studies on the fruits of *O. humifusa* are very limited. Therefore, in this study, chemical constituents of fruits of *O. humifusa* having anti-inflammatory effects were investigated.

*Author for correspondence
So-Young Park, Laboratory of Pharmacognosy, College of Pharmacy, Dankook University, San29, Anseo-dong, Dongnam-gu, Cheonan-si, Chungnam 330-714, Korea
Tel: +82-41-550-1434; E-mail: soypark23@dankook.ac.kr

Experimental

Plant materials and chemicals – Fruits of *O. humifusa* were harvested from Cheonan, Chungchungnam-Do in May 2012 and supplied by NaSaRang Eco&Bio. A voucher specimen has been deposited in Pharmacognosy lab of Dankook University. MTT, LPS (lipopolysaccharide), and rosmarinic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell cultures – Raw264.7 mouse macrophage cells were purchased from Korean Cell Line Bank. The cells

were routinely cultured in RPMI medium supplemented with 10% fetal bovine serum (Equitech-Bio, TX, USA) at 37 °C under 5% CO_2. These cells were utilized for experiments during the exponential growth phase.

Cytotoxicity test – The cytoxic effects of extracts, fractions or isolated pure compounds were tested by MTT assay. Briefly, cells were plated on a 96 well plate. Following morning, cells pre-treated with samples for 1 h were further incubated with LPS for 24 h. Then, cells were treated with MTT (5 mg/mL) for 3 h and followed by lysis buffer for overnight. Absorbance was measured at 590 nm using Emax Precision microplate reader (Molecular Devices, CA, USA). All determinations were carried out in triplicates and repeated at least three times.

Determination of NO production – The NO production was determined using Griess reagents. The cells pre-treated with samples for 1 h were incubated with LPS. After 24 h treatment, the supernatants were transferred to EP tubes and centrifuged at 12,000 rpm for 10 min in order to remove the cell debris. Then, same amount of Griess reagent and supernatant was mixed and incubated at 37 °C for 30 min in the dark. The absorbance was determined at 510 nm using a microplate reader.

Extraction and isolation – The fruits of *O. humifusa* (4.0 kg) were extracted with the MeOH (18 L × 3) to give the MeOH extract (196.5 g). The MeOH extract was suspended in distilled water and partitioned with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH, progressively. The *n*-BuOH soluble fraction (9.6 g) was subjected to silica gel column chromatography, eluted with $CHCl_3$-MeOH (30 : 1 to 1 : 1), to give 13 subfractions (OFB1 - OFB13). The subfraction OFB4 (0.3 g) was purified by semi-preparative HPLC eluting with ACN-H_2O (25 to 70% ACN, gradient) to yield compounds **1** (3.2 mg), **2** (2.0 mg), **3** (2.5 mg), and **4** (2.0 mg).

Structure analysis – NMR spectra were recorded on a Bruker Advanced III 700 MHz NMR spectrometer using CD_3OD and DMSO-d_6 as solvents with the tetramethyl-silane as an internal standard. Chemical shift are presented in ppm. Electrospray ionization (ESI) mass spectra were measured on a Thermo Scientific LCQ Fleet spectrometer. Semipreparative HPLC was performed using a Shimadzu Prominence UFLC with UV detector. Open column chromatography was performed using silica gel (70 - 230 mesh, Merck) and Lichroprep RP-18 (40 - 63 μm, Merck). Thin-layer chromatography (TLC) was performed using precoated silica gel 60 F254 (0.24 mm, Merck) plates.

Isorhamnetin 3-*O*-*β*-D-galactosyl-4'-*O*-*β*-D-glucoside (1) – Yellow amorphous powder; ESI-MS *m/z* 639 $[M-H]^-$; ^{1}H-NMR (700 MHz, CD_3OD) δ_H 8.08 (1H, d, *J* = 2.1 Hz), 7.63 (1H, dd, *J* = 2.1, 9.1 Hz), 7.26 (1H, d, *J* = 9.1 Hz), 6.40 (1H, br s), 6.21 (1H, d, *J* = 2.1 Hz), 5.39 (1H, d, *J* = 7.7 Hz), 5.03 (1H, d, *J* = 7.7 Hz), 3.96 (3H, s); ^{13}C-NMR (177 MHz, CD_3OD) δ_C 178.2, 164.7, 161.7, 157.1, 156.5, 148.7, 148.6, 134.4, 124.6, 121.7, 115.2, 113.7, 104.5, 102.8, 100.6, 98.5, 93.4, 76.9, 76.4, 75.9, 73.5, 73.4, 71.7, 69.8, 68.7, 61.0, 60.8, 55.8.[9]

Isorhamnetin 3,4'-di-*O*-*β*-D-glucoside (2) – Yellow amorphous powder; ESI-MS *m/z* 639 $[M-H]^-$; ^{1}H-NMR (700 MHz, DMSO-d_6) δ_H 7.98 (1H, d, *J* = 2.1 Hz), 7.55 (1H, dd, *J* = 2.1, 9.1 Hz), 7.24 (1H, d, *J* = 9.1 Hz), 6.45 (1H, br s), 6.21 (1H, d, *J* = 1.4 Hz), 5.59 (1H, d, *J* = 7.0 Hz), 5.05 (1H, d, *J* = 7.7 Hz), 3.85 (3H, s); ^{13}C-NMR (177 MHz, DMSO-d_6) δ_C 177.9, 161.7, 156.9, 156.1, 148.9, 148.5, 133.8, 124.1, 121.8, 114.9, 113.9, 104.4, 101.8, 99.9, 94.3, 78.0, 77.6, 77.3, 76.8, 74.8, 73.6, 70.2, 70.0, 61.1, 61.0, 56.1.[9]

Isorhamnetin 3-*O*-*β*-D-(6-*O*-*α*-L-rhamnosyl)glucoside (3) – Yellow amorphous powder; ESI-MS *m/z* 623 $[M-H]^-$; ^{1}H-NMR (700 MHz, CD_3OD) δ_H 7.95 (1H, d, *J* = 2.1 Hz), 7.63 (1H, dd, *J* = 2.1, 9.1 Hz), 6.92 (1H, d, *J* = 9.1 Hz), 6.42 (1H, d, *J* = 2.1 Hz), 6.21 (1H, d, *J* = 2.1 Hz), 5.24 (1H, d, *J* = 7.7 Hz), 4.52 (1H, d, *J* = 1.4 Hz), 3.90 (3H, s), 1.09 (3H, d, *J* = 6.3 Hz); ^{13}C-NMR (177 MHz, CD_3OD) δ_C 177.9, 164.7, 157.5, 157.1, 149.5, 146.9, 134.3, 122.6, 121.6, 114.7, 113.1,104.3, 102.9, 101.1, 98.5, 93.5, 76.8, 75.9, 74.5, 72.4, 70.8, 70.6, 70.2, 68.4, 67.1, 62.9, 55.3, 16.5.[9]

(+)-Syringaresinol *O*-*β*-D-glucopyranoside (4) – ESI-MS *m/z* 580 $[M-H]^-$; ^{1}H-NMR (700 MHz, CD_3OD) δ_H 6.72 (2H, s), 6.65 (2H, s), 4.77 (1H, d, *J* = 4.2 Hz), 4.71 (1H, d, *J* = 4.2 Hz), 4.27 (2H, m), 3.92 (1H, m), 3.91 (1H, m), 3.86 (6H, s), 3.84 (6H, s), 3.77 (1H, m), 3.75 (1H, m); ^{13}C-NMR (177 MHz, CD_3OD) δ_C 153.2, 147.9, 138.1, 134.8, 134.1, 131.6, 103.9, 103.4, 103.1, 86.2, 85.8, 76.9, 76.4, 74.3, 71.6, 71.5, 69.9, 62.9, 55.7, 55.4, 54.3, 54.2.[10]

Data analysis – All experiments were performed in triplicate. The results are presented as mean ± SD. Statistical analysis was carried out using SPSS 18.0 software. The probability was considered significant at $p < 0.05$.

Result and Discussion

As part of a research program searching for anti-inflammatory agents from Korean medicinal plants, we discovered that the MeOH extract of the fruits of *O. humifusa* showed the inhibitory effect on NO production. In order to determine the responsible fractions and compounds for the inhibition of NO production, the

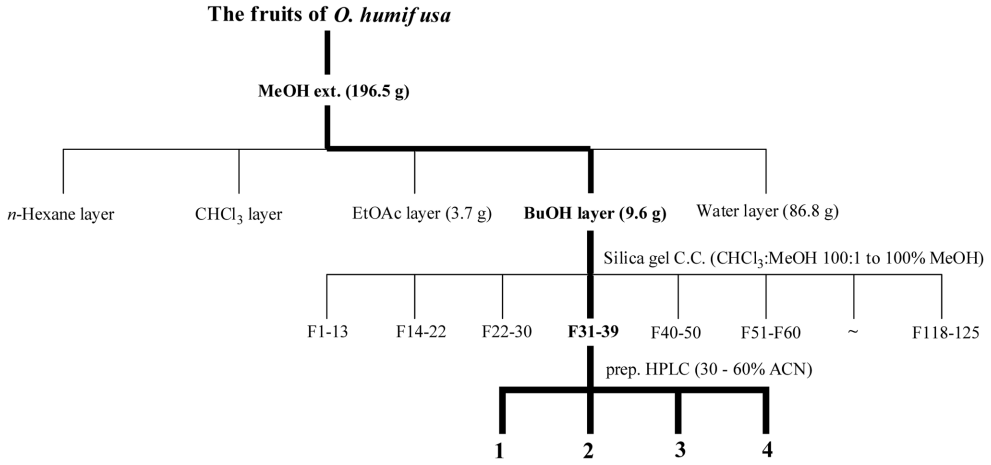

Fig. 1. Solvent-partitioned fractions and compound isolation scheme of *O. humifusa*.

Table 1. The effects of *O. humifusa* fractions on the cell viability and NO production in LPS-stimulated Raw264.7 cells

Samples	Concentration (mg/mL)	Cell viability (% of control)	NO production (% of LPS)
Control		100.0 ± 0.0	11.4 ± 5.4
LPS treated		100.6 ± 1.1	100.0 ± 0.0
MeOH ext + LPS	2.0	105.5 ± 14.8	78.6 ± 6.6
	1.0	113.0 ± 18.4	84.3 ± 6.4
	0.5	108.5 ± 2.1	93.6 ± 4.6
BuOH fraction + LPS	2.0	107.4 ± 4.5	64.3 ± 8.1
	1.0	97.7 ± 1.9	123.0 ± 17.9
	0.5	95.4 ± 1.5	113.0 ± 14.1
Water fraction + LPS	2.0	99.3 ± 6.7	120.4 ± 23.5
	1.0	103.4 ± 5.9	102.2 ± 19.8
	0.5	103.1 ± 2.0	94.7 ± 8.5

MeOH extract of the fruits of *O. humifusa* were solvent-partitioned. Each fraction (hexane, chloroform, ethylacetate, butanol and water fractions) was then first tested for its cytoxicity using MTT. As the results, the viability of cells treated with solvent-partitioned fractions was not significantly different from the DMSO-treated control cells, indicating that the fractions were not cytotoxic to the cells. Then, the cells treated with LPS in the presence or absence of 5 fractions were tested for NO production (Table 1). Among 5 fractions, 2 mg/ml of butanol fraction reduced the production of NO to 50% compared to the controls, but other fractions didn't. Then, in order to isolate the constituents responsible for the inhibition of NO production, butanol fraction was further investigated.

Bioassay-guided fractionation of butanol fraction of *O. humifusa* by sequential column chromatography using silica gel, MPLC and finally HPLC (Fig. 1) led to the isolation of three known flavonoid glycosides (**1 - 3**) and a known lignan glycoside (**4**). These isolated compounds were identified using spectroscopic analysis including ^{1}H-, ^{13}C-NMR and ESI-MS. The structures were identified as isorhamnetin 3-*O*-*β*-D-galactosyl-4'-*O*-*β*-D-glucoside (**1**), isorhamnetin 3,4'-di-*O*-*β*-D-glucoside (**2**), isorhamnetin 3-*O*-*β*-D-(6-*O*-*α*-L-rhamnosyl)glucoside (**3**), and syringaresinol O-*β*-D-glucopyranoside (**4**) (Fig. 2) by comparison of their ^{1}H-, ^{13}C-NMR and ESI-MS data with those of the literature.

Table 2. The inhibitory effects of compounds isolated from *O. humifusa* on LPS-induced NO production

Compounds	IC50 (μg/mL)
Isorhamnetin 3-*O*-*β*-D-galactosyl-4'-*O*-*β*-D-glucoside (**1**)	239
isorhamnetin 3,4'-di-*O*-*β*-D-glucoside (**2**),	114
isorhamnetin 3-*O*-*β*-D-(6-*O*-*α*-L-rhamnosyl)glucoside (**3**),	> 500
syringaresinol *O*-*β*-D-glucopyranoside (**4**)	> 500

The information about the chemical constituents of *O. humifusa* is limited. Furthermore, compounds responsible for the biological activities of *O. humifusa* are not fully elucidated because the studies for biological activities of *O. humifusa* are mainly performed with the MeOH extracts or solvent-partitioned fractions. Reported compounds from *O. humifusa* are polyphenols such as ferulic acid, protocatechuic acid, taxifolin, myricetin, isorhamnetin 3-*O*-*β*-D-galactosyl-4'-*O*-*β*-D-glucoside, isorhamnetin 3,4'-di-*O*-*β*-D-glucoside, isorhamnetin 3-*O*-*β*-D-(6-*O*-*α*-L-rhamnosyl)glucosyl-4'-*O*-*β*-D-glucoside, isorhamnetin-3-*O*-*β*-D-(6-*O*-*α*-L-rhamnosyl)glucoside, and isorhamnetin 3-*O*-*β*-D-(6-*O*-*α*-L-rhamnosyl)galactoside.[9,10] These compounds are all isolated from stems of *O. humifusa*, but none of the

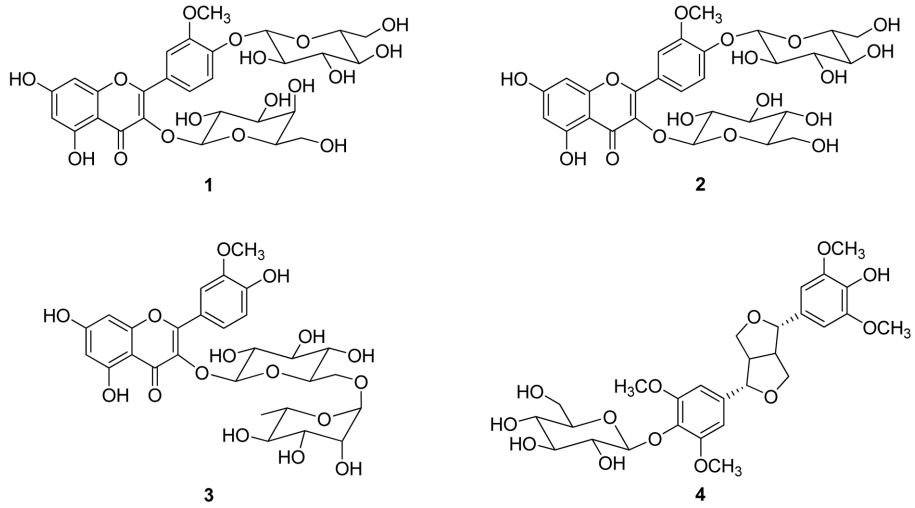

Fig. 2. The structures of compounds isolated from *O. humifusa*. Isorhamnetin 3-*O*-*β*-D-galactosyl-4'-*O*-*β*-D-glucoside (**1**), isorhamnetin 3,4'-di-*O*-*β*-D-glucoside (**2**), isorhamnetin 3-*O*-*β*-D-(6-*O*-*α*-L-rhamnosyl)glucoside (**3**), and syringaresinol *O*-*β*-D-glucopyranoside (**4**).

report is available for the isolation of compounds from the fruits of *O. humifusa*. Therefore, we first report the isolation of isorhamnetin 3-*O*-*β*-D-galctosyl-4'-*O*-*β*-D-glucoside (**1**), and isorhamnetin 3,4'-di-*O*-*β*-D-glucoside (**2**), and isorhamnetin 3-*O*-*β*-D-(6-*O*-*α*-L-rhamnosyl) glucoside (**3**) from the fruits of *O. humifusa*. Furthermore, the isolation of syringaresinol *O*-*β*-D-glucopyranoside (**4**) from *O. humifusa* is also first reported here.

The effect of isolated compounds on the cell viability was tested with MTT assay. The viability of cells treated with up to 200 μg/ml of isolated compounds was not significantly altered compared to the DMSO-treated controls. Then, in order to determine the effect on NO production, cells were pretreated with different concentrations of isolated compounds. Among four isolated compounds, compound **1** and **2** significantly reduced the production of NO induced by LPS in Raw264.7 cells. The 200 μg/mL of compound **1** and 100 μg/mL of compound **2** reduced the production of NO down to 60% of control levels. Their IC50 values for the inhibition of LPS-induced NO production were 239 and 114 μg/mL, respectively. However, this inhibitory effect is not due to the decrease of cell viability.

O. humifusa locally called as Cheonnyuncho is a cactus, which is widely cultivated in southern regions of Korean. The most well-known activity of *O. humifusa* is the antioxidant effect. The stems of *O. humifusa* efficiently scavenged the free radicals.[5,11,12] In addition, stems of *O. humifusa* also exhibited the anti-inflammatory effect in LPS-activated macrophage cells.[5] Furthermore, *O. humifusa* stems decreased the levels of blood glucose and cholesterol in diabetic rats.[13] Stems of *O. humifusa* also have anti-cancer and anti-bacterial activities.[11,12] Most of the studies about biological activities are done with the extracts of *O. humifusa* stems. However, the biological activities of *O. humifusa* fruits are not reported.

Isorhamnetin is known to have anti-inflammatory effects. Isorhamnetin reduced the acute inflammation in the paw carrageenan-induced edema.[14] Isorhamnetin-3-*O*-galactoside reduced HMB1-induced inflammatory responses in HUVECs and CLP-induced septic mice.[15] However, isorhamnetin 3-*O*-*β*-D-galactosyl-4'-*O*-*β*-D-glucoside (**1**) and isorhamnetin 3,4'-di-*O*-*β*-D-glucoside (**2**) which are isolated from fruits of *O. humifusa* in this study are not reported to have anti-inflammatory effects. However, these compounds are known to have antioxidant effects.

In this study, we isolated 3 flavonoids and one lignan from the fruits of *O. humifusa.* Even though the flavonoids are previously isolated from stems of *O. humifusa,* this is the first report regarding the isolation of three flavonoids from the fruits of *O. humifusa* having the inhibitory effect on the production of NO induced by LPS. In addition, this is the first time to report the isolation of lignan syringaresinol-4'-*O*-*β*-D-glucoside from *O. humifusa*.

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