Short Communication

Harpagophytum procumbens Suppresses Lipopolysaccharide-Stimulated Expressions of Cyclooxygenase-2 and Inducible Nitric Oxide Synthase in Fibroblast Cell Line L929

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Abstract. *Harpagophytum procumbens* (*Pedaliaceae*) has been used for the treatment of pain and arthritis. The effect of *Harpagophytum procumbens* against lipopolysaccharide-induced inflammation was investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, reverse transcription-polymerase chain reaction, prostaglandin E_2 (PGE_2) immunoassay, and nitric oxide detection on mouse fibroblast cell line L929. The aqueous extract of *Harpagophytum procumbens* was shown to suppress PGE_2 synthesis and nitric oxide production by inhibiting lipopolysaccharide-stimulated enhancement of the cyclooxygenase-2 and inducible nitric oxide synthase (iNOS) mRNAs expressions in L929 cells. These results suggest that *Harpagophytum procumbens* exerts anti-inflammatory and analgesic effects probably by suppressing cyclooxygenase-2 and iNOS expressions.

Keywords: *Harpagophytum procumbens*, lipopolysaccharide, cyclooxygenase-2

The medicinal plant *Harpagophytum procumbens* (*Pedaliaceae*), commonly known as devil's claw, has been used for the control of pain and treatment of arthritis, and it has been reported to possess anti-inflammatory and analgesic effects (1, 2).

Lipopolysaccharide (LPS) initiates a number of major cellular responses that play a vital role in the pathogenesis of inflammatory responses including activation of inflammatory cells and production of cytokines and other mediators. Prostaglandin E_2 (PGE_2) is a key inflammatory mediator, converted from arachidonic acid by cyclooxygenase. There are two isoforms of cyclooxygenase: cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). While COX-1 is a constitutively expressed form in normal physiologic functions, COX-2 is expressed only in response to inflammatory signals such as cytokines and bacterial endotoxin LPS. COX-2 produces a large amount of PGE_2 that induces inflam-

mation (3, 4).

Nitric oxide (NO), endogenously generated from L-arginine by NO synthase (NOS), plays an important role in the regulation of many physiological processes (5). Several isoforms of NOS exist and fall into three major classes: inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS). Of these, iNOS is an important enzyme involved in regulation of inflammation (6).

In the present study, the effect of *Harpagophytum procumbens* against LPS-stimulated expressions of COX-2 and iNOS in cells of the mouse fibroblast cell line L929 was investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, reverse transcription-polymerase chain reaction (RT-PCR), PGE_2 immunoassay, and NO detection.

Cells of the mouse fibroblast cell line L929 were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). Cells were cultured in RPMI-1640 (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated FBS (Gibco BRL) at 37°C

in $5\% CO_2 - 95\% O_2$ in a humidified cell incubator. *Harpagophytum procumbens* used in this study is the same herb that was collected and identified by G.J.R. Betti in Namibia, and its voucher specimen number is *Harpagophytum procumbens* 4-93 (7 – 9). This herb was deposited in the Department of Herbal Pharmacology, College of Oriental Medicine, Kyung Hee University. To obtain the water extract of *Harpagophytum procumbens*, 200 g of *Harpagophytum procumbens* was added to distilled water, and extraction was performed by heating at 80°C for 12 h, concentrating with a rotary evaporator, and lyophilizing (10). The resulting powder, weighing 35 g (a yield of 20.59%), was diluted to the concentrations needed with normal saline solution and filtered through a 0.45-μm syringe filter before use.

Cell viability was determined using the MTT assay kit (Boehringer Mannheim GmbH, Mannheim, Germany) as per the manufacturer's protocols. In order to determine the cytotoxicity of *Harpagophytum procumbens*, cells were treated with *Harpagophytum procumbens* at concentrations of 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, and 1 mg/ml for 24 h. Cultures of the control group were left untreated. Ten microliters of the MTT labeling reagent was added to each well, and the plates were incubated for 4 h. A 100-μl aliquot of solubilization solution was then added to each well, and the cells were incubated for another 12 h. The absorbance was then measured with a microtiter plate reader (Bio-Tek, Winooski, VT, USA) at a test wavelength of 595 nm and a reference wavelength of 690 nm. Optical density (O.D.) was calculated as the difference between the absorbance at the reference wavelength and that at the test wavelength. Percent viability was calculated as (O.D. of drug-treated sample/control O.D.) $\times$ 100.

To identify expressions of COX-2 and iNOS mRNAs, RT-PCR was performed. Total RNA was isolated from L929 cells using RNAzol™B (TEL-TEST, Friendswood, TX, USA). Two micrograms of RNA and 2 μl of random hexamers (Promega, Madison, WI, USA) were added together, and the mixture was heated at 65°C for 10 min. One microliter of AMV reverse transcriptase (Promega), 5 μl of 10 mM dNTP (Promega), 1 μl of RNasin (Promega), and 5 μl of 10 $\times$ AMV RT buffer (Promega) were then added to the mixture, and the final volume was brought up to 50 μl with diethyl pyrocarbonate (DEPC)-treated water. The reaction mixture was then incubated at 42°C for 1 h.

PCR amplification was performed in a reaction volume of 40 μl containing 1 μl of the appropriate cDNA, 1 μl of each set of primers at a concentration of 10 pM, 4 μl of 10 $\times$ RT buffer, 1 μl of 2.5 mM dNTP, and 2 units of *Taq* DNA polymerase (Takara, Ohtsu). For mouse *COX-1*, the primer sequences were 5'-AGTGCGGTCCAACCTTATCC-3' (a 20-mer sense oligonucleotide) and 5'-CCGCAGGTGATACTGTC GTT-3' (a 20-mer anti-sense oligonucleotide). For mouse *COX-2*, the primer sequences were 5'-TGCAT GTGGCTGTGGATGTCATCAA-3' (a 25-mer sense oligonucleotide) and 5'-CACTAAGACAGACCCGTC ATCTCCA-3' (a 25-mer anti-sense oligonucleotide). For mouse *iNOS*, the primer sequences were 5'-GTGT TCCACCAGGAGATGTTG-3' (a 21-mer sense oligonucleotide) and 5'-CTCCTGCCCACTGAGTTCGTC-3' (a 21-mer anti-sense oligonucleotide). For *cyclophilin*, the internal control used in the study, the primer sequences were 5'-ACCCCACCGTGTTCTTCGAC-3' (a 20-mer sense oligonucleotide starting at position 52) and 5'-CATTTGCCATGGACAAGATG-3' (a 20-mer anti-sense oligonucleotide starting at position 332). The expected sizes of the PCR products were 382 bp (for *COX-1*), 583 bp (for *COX-2*), 500 bp (for *iNOS*), and 299 bp (for *cyclophilin*).

For *COX-1*, *COX-2*, and *iNOS*, the PCR procedure was carried out using a GeneAmp 9600 PCR system (Perkin Elmer, Norwalk, CT, USA) under the following conditions: initial denaturation at 94°C for 5 min, followed by 40 amplification cycles, each consisting of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, with an additional extension step at the end of the procedure at 72°C for 5 min. For *cyclophilin*, the PCR procedure was carried out under identical conditions except that 25 amplification cycles were executed. The final amount of RT-PCR product for each of the mRNA species was calculated densitometrically using Molecular Analyst™ version 1.4.1 (Bio-Rad, Hercules, CA, USA).

Assessment of PGE$_2$ synthesis was performed using a commercially available PGE$_2$ competitive enzyme immunoassay kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). Cells were lysed and cell lysates and standards were put into different wells on the goat anti-mouse IgG-coated microtiter plate provided in the kit. Mouse anti-PGE$_2$ antibody and peroxidase-conjugated PGE$_2$ were added to each well, and the plate was incubated at room temperature and shook for 1 h. The wells were drained and washed, and 3,3',5,5'-tetramethylbenzidine/hydrogen peroxide solution was added. The plate was incubated at room temperature with shaking, and the reaction was stopped after 30 min through the addition of H_2SO_4. The absorbance of the content of each well was then measured at 450 nm.

In order to determine the effect of *Harpagophytum procumbens* on NO synthesis, the amount of nitrite in the cell-free culture supernatant was measured using a commercially available NO detection kit (iNtRon, Inc., Seoul, Korea). After collection of 100 μl of

supernatant, 50 μl of N1 buffer was added to each well, and the plate was incubated at room temperature for 10 min. N2 buffer was then added, and the plate was incubated at room temperature for 10 min. The absorbance of the content of each well was measured at 450 nm. The nitrite concentration was calculated from a nitrite standard curve.

Results are expressed as the mean ± S.E.M. Data were analyzed by one-way ANOVA followed by Duncan's post-hoc test using SPSS. Differences were considered statistically significant at *P*<0.05.

The viabilities of cells incubated with *Harpagophytum procumbens* at 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, and 1 mg/ml for 24 h were 91.06 ± 2.86%, 91.66 ± 2.33%, 89.06 ± 1.53%, 94.25 ± 3.41%, 91.18 ± 4.50%, 91.98 ± 2.61%, and 105.06 ± 3.74% of the control value, respectively. The MTT assay revealed that *Harpagophytum procumbens* exerted no significant cytotoxicity in the fibroblast cell line L929 (Fig. 1).

RT-PCR analysis of the mRNA levels of COX-1, COX-2, and iNOS was performed in order to provide an estimate of the relative levels of expressions of these genes. In the present study, the mRNA levels of COX-1, COX-2, and iNOS in the control cells were used as the control value (1.00). The level of COX-1 mRNA following treatment with 5 μg/ml LPS for 24 h was 1.06 ± 0.08, while this number was 1.01 ± 0.01 and 0.67 ± 0.05 in cells treated with *Harpagophytum*

procumbens at 0.1 and 1 mg/ml, respectively. LPS treatment did not enhance COX-1 expression. Treatment with 0.1 mg/ml of *Harpagophytum procumbens* exerted no significant effect on the COX-1 expression, while 1 mg/ml of *Harpagophytum procumbens* suppressed COX-1 expression. The level of COX-2 mRNA following treatment with 5 μg/ml LPS for 24 h was significantly increased to 11.96 ± 0.96, while it decreased to 4.59 ± 0.56 and 0.95 ± 0.68 in cells treated with *Harpagophytum procumbens* at 0.1 and 1 mg/ml, respectively. The level of iNOS mRNA was markedly increased to 9.47 ± 0.58 following treatment with 5 μg /ml LPS for 24 h, while it decreased to 8.01 ± 0.67 and 6.39 ± 0.28 in cells treated with *Harpagophytum procumbens* at 0.1 and 1 mg/ml, respectively (Fig. 2).

From the PGE$_2$ immunoassay, after 24 h of exposure to LPS, the amount of PGE$_2$ was increased from 5.00 ± 1.24 to 71.00 ± 3.27 pg/well, while it decreased to 38.00 ± 2.94 and 22.00 ± 3.51 pg/well by the treatment with *Harpagophytum procumbens* at 0.1 and 1 mg/ml, respectively. From the NO detection assay, after 24 h of exposure to LPS, the amount of nitrite was increased from 0.11 ± 0.01 to 16.02 ± 0.59 μM, while it decreased to 10.54 ± 0.62 and 7.41 ± 0.49 μM by treatment with *Harpagophytum procumbens* at 0.1 and 1 mg/ml, respectively (Fig. 3).

Elevation of COX-2 activity is closely associated with the occurrence of cancer, arthritis, and several types of neurodegenerative disorders. Specific COX-2 inhibitors attenuate the symptoms of inflammation (4, 11). NO exerts diverse and multifunctional effects in the host cells. After exposure to endogenous and exogenous stimulators such as LPS and viral infections, iNOS is induced quantitatively in the various cells, and triggers several deleterious cellular responses inducing inflammation, sepsis, and stroke (6, 12). COX activity and subsequent production of PGE$_2$ are closely related to the generation of NO radicals. Salvemini et al. (13) reported that NO modulates the activity of COX-2 in a cGMP-independent manner and plays a critical role in the release of PGE$_2$ by direct activation of COX-2. Inhibition of the iNOS expression in murine macrophages was suggested as another possible mechanism of non-steroidal anti-inflammatory drugs (14).

Harpagophytum procumbens comprises iridoid glycosides, mainly harpagide, harpagoside, procumbide, and procumboside. Of these, harpagide and harpagoside were suggested as active components of the *Harpagophytum procumbens* purified from the root of the *Harpagophytum procumbens* by preparative liquid chromatography (7 – 9). *Harpagophytum procumbens* has been used for the suppression of inflammatory and degenerative disorders in the skeletal muscle system.
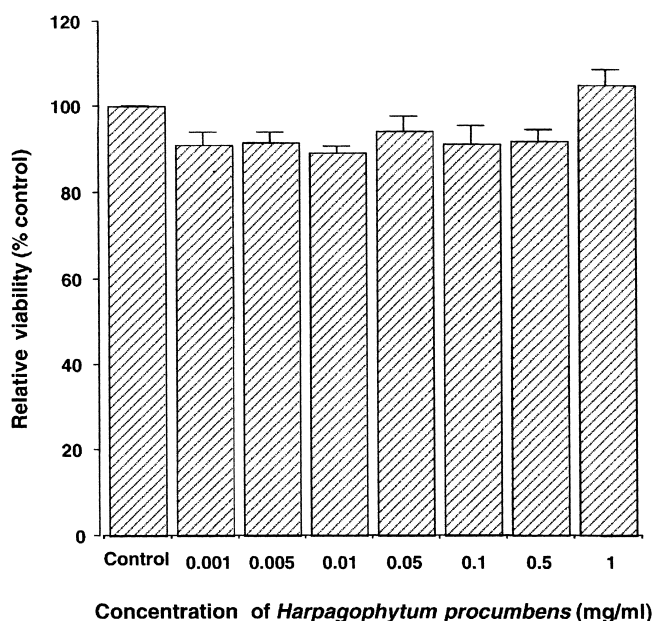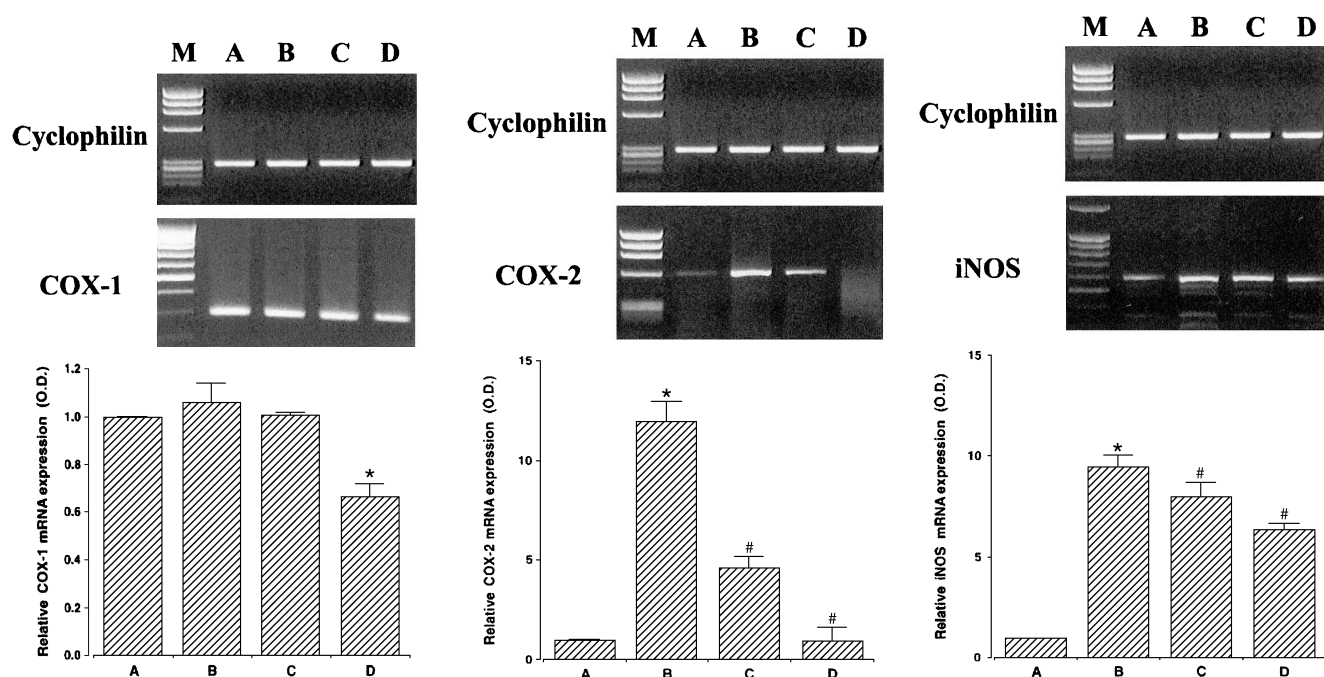

Fig. 1. Effect of *Harpagophytum procumbens* on L929 cell viability. Viability was determined via the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results are presented as the mean ± S.E.M.

Fig. 2. Results of reverse transcription-polymerase chain reaction (RT-PCR) analysis of the mRNA levels of cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS). As the internal control, cyclophilin mRNA was also reverse-transcribed and amplified. * represents $P<0.05$ compared to the control. # represents $P<0.05$ compared to the lipopolysaccharide (LPS)-treated group. M, Marker; A, control; B, LPS-treated group; C, LPS- and 0.1 mg/ml *Harpagophytum procumbens*-treated group; D, LPS- and 1 mg/ml *Harpagophytum procumbens*-treated group.
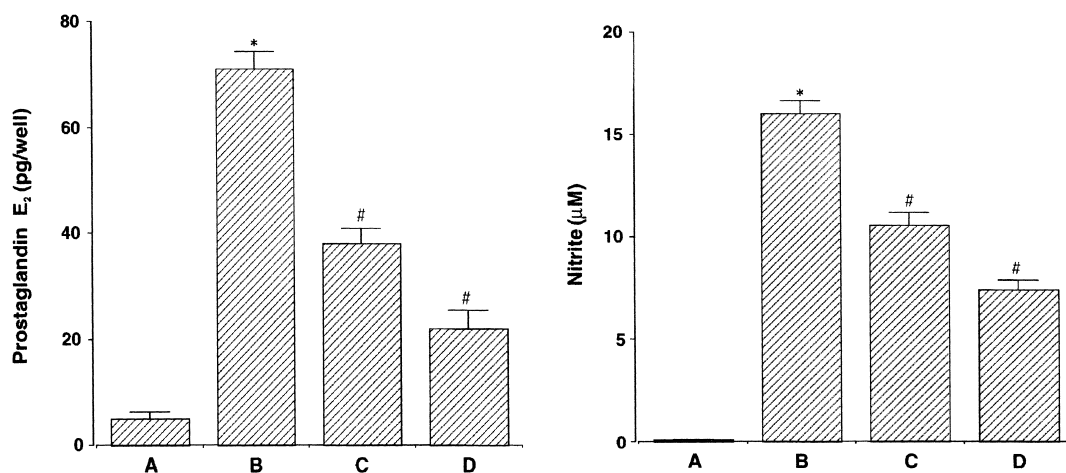
Fig. 3. Measurement of prostaglandin E_2 and nitric oxide (NO) production in L929 cells. * represents $P<0.05$ compared to the control. # represents $P<0.05$ compared to the lipopolysaccharide (LPS)-treated group. A, control; B, LPS-treated group; C, LPS- and 0.1 mg/ml *Harpagophytum procumbens*-treated group; D, LPS- and 1 mg/ml *Harpagophytum procumbens*-treated group.

Harpagophytum procumbens is known to be effective in the treatment of osteoarthritis of the knee and hip (15) and to show an anti-inflammatory effect by inhibiting the tumor necrosis factor (TNF)-alpha synthesis in LPS-stimulated primary monocytes (2). In the present study, the aqueous extract of *Harpagophytum pro-*

cumbens was shown to suppress PGE_2 production by inhibition of the LPS-stimulated enhancement of COX-2 enzyme activity and iNOS expression in the L929 cell lines. These results suggest that *Harpagophytum procumbens* exerts anti-inflammatory and analgesic effects probably by suppressing of COX-2 and iNOS

expressions, resulting in inhibition of PGE$_2$ synthesis.

Acknowledgments

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