

Original Article

Harpagophytum Procumbens Suppresses Lipopolysaccharide Induced Expressions of Cyclooxygenase-2 and Inducible Nitric Oxide Synthase in Mouse BV2 Microglial Cells

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The excessive release of proinflammatory products by activated microglia causes neurotoxicity, and this has been implicated in the pathogenesis of neurodegenerative diseases. *Harpagophytum procumbens* (Pedaliaceae) has been widely used for the treatment of pain and arthritis in the clinical field. In this study, we investigated the effect of *Harpagophytum procumbens* against lipopolysaccharide-induced inflammation. From the present results, the aqueous extract of *Harpagophytum procumbens* was shown to suppress prostaglandin-E₂ synthesis and nitric oxide production by inhibiting the lipopolysaccharide-stimulated enhancement of cyclooxygenase-2 and inducible nitric oxide synthase expressions in mouse BV2 microglial cells. These results suggest that *Harpagophytum procumbens* may offer a valuable means of therapy for the treatment of brain inflammatory diseases by attenuating lipopolysaccharide-induced prostaglandin-E₂ synthesis and nitric oxide production.

Key Words: *Harpagophytum procumbens*, Lipopolysaccharide, Prostaglandin-E₂, Nitric oxide

Introduction

Neuroinflammation is known to be a key event in neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease, and amyotrophic lateral sclerosis (ALS)¹⁻³⁾.

Microglia is currently accepted as being the sensor cells in the central nervous system that responds to injury and brain disease. The main function of microglia is for

defense of the brain, because these cells scavenge invading microorganisms and dead cells, and they also act as immune or immuno-effector cells. Microglia are also thought to contribute to the onset of or the exacerbation of neuronal degeneration and/or inflammation in many brain diseases by their production of deleterious factors including superoxide anions, nitric oxide and inflammatory cytokines.

During neuroinflammation, the microglia are activated and the activated microglial cells secrete numerous products including prostaglandins (PGs) and nitric oxide (NO) that are involved in the neuroinflammatory responses⁴⁾.

PGs are derived from arachidonic acid by the cyclooxygenase (COX) pathway, and they act as a key

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inflammatory mediator⁴). There are two isoforms of cyclooxygenase: cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). While COX-1 is a constitutively expressed form involved in normal physiologic functions, COX-2 is expressed only in response to such inflammatory signals as cytokines and bacterial endotoxin lipopolysaccharide (LPS). COX-2 produces a large amount of PG-E₂ and this induces the process of inflammation.

Nitric oxide (NO) is endogenously generated from L-arginine by nitric oxide synthase (NOS), and NO plays an important role in the regulation of many pathophysiological processes. The NO that is secreted by activated microglia can rapidly react with O₂- to produce the peroxynitrite anion⁵. The production of excessive NO and reactive oxygen species (ROS) in the brain is known to contribute to the neurodegenerative processes⁶. Several isoforms of NOS exist and these fall into three major classes: inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS). Both nNOS and eNOS are constitutively expressed, whereas iNOS is inducible in response to immunologic activation and their subsequent transcription⁷. iNOS is responsible for the overproduction of NO in inflammation⁸).

Harpagophytum procumbens (Pedaliaceae) is popularly known as “devil’s claws”, and it is a medicinal plant that originates from Southern Africa. The plant has been traditionally used as a digestive tonic and to relieve headaches, fevers, and allergies; it is also used as an ointment to alleviate pain during childbirth.⁹

Harpagophytum procumbens be known to an anti-inflammatory effect by the inhibition of the synthesis of inflammatory cytokines in LPS-stimulated primary monocytes and in LPS-stimulated fibroblast cell line 929, in IL-1 β -stimulated human chondrocytes¹⁰⁻¹²). Although many effects of the *Harpagophytum procumbens* have been studied on biological system, the

approach that reveals the relationship between *Harpagophytum procumbens* and the effect on brain inflammatory, immune and degenerative processes associated with PGs and NO has not yet to be elucidated.

Here in this study, we examine effect of *Harpagophytum procumbens* on the LPS-stimulated expressions of COX-1, COX-2, and iNOS, and on production of PG-E₂ and NO in the mouse BV2 microglial cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, reverse transcription-polymerase chain reaction (RT-PCR), PG-E₂ immunoassay and NO detection assay.

Materials and Methods

1. Cell culture

The mouse BV2 microglial cells used in this experiment were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in 5% CO₂, 95% O₂ in a humidified cell incubator.

2. Drugs

To obtain the water extract of *Harpagophytum procumbens*, 200g of *Harpagophytum procumbens* was added to distilled water, and extraction was performed by heating the mixture at 80°C for 24 h, and then it was concentrated with a rotary evaporator and lyophilized. The resulting powder weighed 35g (a yield of 20.59%), and this was diluted to the required concentrations with normal saline solution and filtered through a 0.45 μ m syringe filter before use.

3. MTT assay for cell viability

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*, the cells were treated with *Harpagophytum procumbens* at concentrations of 0.001 mg/ml, 0.005 mg/ml, 0.01 mg/ml, 0.05 mg/ml, 0.1 mg/ml, 0.5 mg/ml and 1 mg/ml for 24 h. Cultures of the control group were left untreated. 10 μ l of the MTT labeling reagent was added to each well, and the plates were incubated for 4 h. Solubilization solution 100 μ l 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. The optical density (O.D.) was calculated as the difference between the absorbance at the reference wavelength and the absorbance at the test wavelength. The percent of cell viability was calculated as (O.D. of drug-treated sample/O.D. of control) \times 100.

4. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

To identify expressions of COX-1, COX-2, and iNOS mRNA, RT-PCR was performed. The total RNA was isolated from the BV2 microglial cells using RNAzolTMB (TEL-TEST, Friendswood, TX, USA). 2 μ g of RNA and 2 μ l of random hexamers (Promega, Madison, WI, USA) were added together, and the mixture was heated at 65 $^{\circ}$ C for 10 min. 1 μ l of AMV reverse transcriptase (Promega), 5 μ l of 10 mM dNTP (Promega), 1 μ l of RNasin (Promega), and 5 μ l of 10 x AMV RT buffer (Promega) were then added to the mixture, and the final volume was brought up to 50 μ l with diethyl pyrocarbonate (DEPC)-treated water. The reaction mixture was then incubated at 42 $^{\circ}$ C for 1 h.

PCR amplification was performed in a reaction volume of 40 μ l containing 1 μ l of the appropriate cDNA, 1 μ l of each set of primers at a concentration of 10 pM, 4 μ l of 10 x RT buffer, 1 μ l of 2.5 mM dNTP,

and 2 units of Taq DNA polymerase (TaKaRa, Shiga, Japan). For mouse COX-1, the primer sequences were 5'-AGTGCGGTCCAACCTTATCC-3' (a 20-mer sense oligonucleotide) and 5'-CCGCAGGTG-ATACTGTCGTT-3' (a 20-mer anti-sense oligonucleotide). For mouse COX-2, the primer sequences were 5'-TGCATGTGGCTGTGGATGT-CATCAA-3' (a 25-mer sense oligonucleotide) and 5'-CACTAAGACAGACCCGTCATCTCCA-3' (a 25-mer anti-sense oligonucleotide). For mouse iNOS, the primer sequences were 5'-GTGTTCCACCAGG-AGATGTTG-3' (a 21-mer sense oligonucleotide) and 5'-CTCCTGCCACTGAGTTTCGTC-3' (a 21-mer anti-sense oligonucleotide). For cyclophilin, the internal control used in the study, the primer sequences were 5'-ACCCCACCGTGTCTTCGAC-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 size of the PCR product was 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: an initial denaturation step at 94 $^{\circ}$ C for 5 min, and this was followed by 35 amplification cycles, each consisting of denaturation at 94 $^{\circ}$ C for 30 sec, annealing at 58 $^{\circ}$ C for 30 sec, and extension at 72 $^{\circ}$ C for 30 sec, with an additional extension step at the end of the procedure at 72 $^{\circ}$ 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 AnalystTM version 1.4.1 (Bio-Rad, Hercules, CA, USA).

5. PG-E₂ assay

The assessment of PG-E₂ synthesis was performed using a commercially available PG-E₂ competitive enzyme immunoassay kit (Amersham Pharmacia Biotech. Inc., Piscataway, NJ, USA). The cells were lysed, and then the cell lysates and standards were put into different wells on the goat anti-mouse IgG-coated microtiter plate that was provided in the kit. Mouse anti PG-E₂ antibody and peroxidase-conjugated PG-E₂ were added to each well and the plate was then shaken and incubated at room temperature and for 1 h. The wells were drained, washed, and 3,3',5,5'-tetramethylbenzidine/hydrogen peroxide solution was next added. The plate was incubated at room temperature with shaking, and the reaction was stopped after 30min through the addition of H₂SO₄. The absorbance of the

content of each well was then measured at 450nm.

6. Measurement of NO generation

The amount of nitrite (NO₂-) in cell-free culture supernatant was measured by using a commercially available NO detection kit (Intron, Inc., Seoul, Korea). After collection of 100 μ l of supernatant, 50 μ l 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 another 10min. The absorbance of the content of each well was measured at 540nm. The nitrite concentration was calculated from a nitrite standard curve.

7. Statistical analysis

The results are expressed as the mean \pm standard

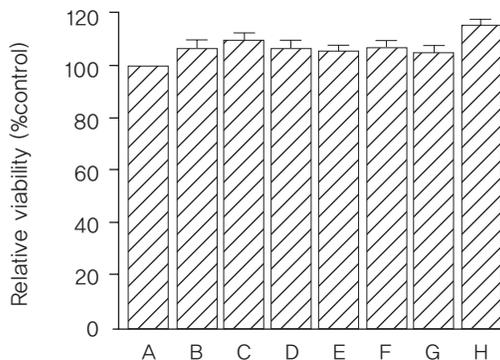


Fig. 1. Effect of *Harpagophytum procumbens* on BV2 cell viability. Cell viability was determined via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The results are presented as the mean \pm standard error mean (S.E.M).

A : Control

B : 0.001 mg /ml *Harpagophytum procumbens*-treated group

C : 0.005 mg /ml *Harpagophytum procumbens*-treated group

D : 0.01 mg /ml *Harpagophytum procumbens*-treated group

E : 0.05 mg /ml *Harpagophytum procumbens*-treated group

F : 0.1 mg /ml *Harpagophytum procumbens*-treated group

G : 0.5 mg /ml *Harpagophytum procumbens*-treated group

H : 1 mg /ml *Harpagophytum procumbens*-treated group.

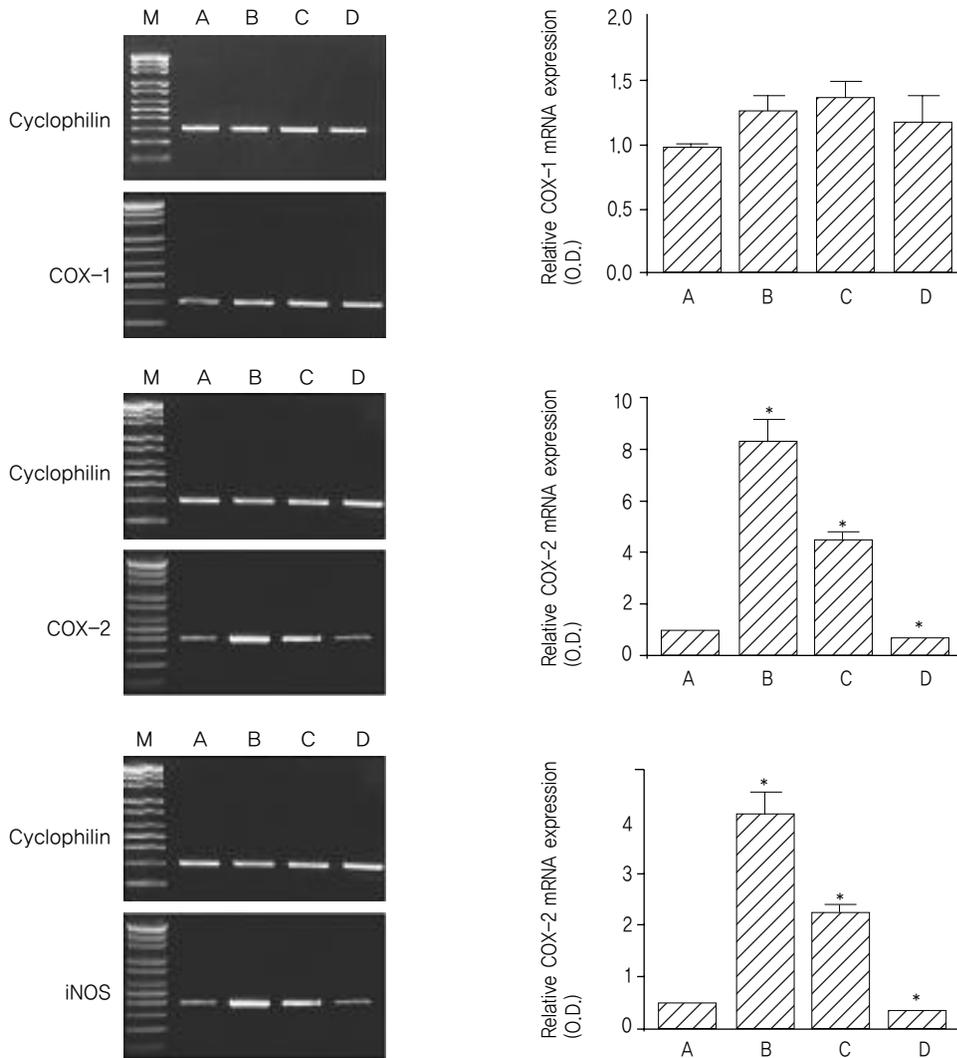


Fig. 2. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of the mRNA levels of cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS). Cyclophilin mRNA was used as the internal control.

* represents $p < 0.05$ compared to the normal group.

represents $p < 0.05$ compared to the control group ; lipopolysaccharide (LPS)-treated group).

M : Marker

A : Normal group

B : Control group ; LPS-treated group

C : LPS- and 0.1 mg/ml *Harpagophytum procumbens*-treated group

D : LPS- and 1 mg/ml *Harpagophytum procumbens*-treated group

error mean (S.E.M.). The data were analyzed by one-way ANOVA followed by Duncan's post-hoc test. Differences were considered significant at $p < 0.05$.

Results

1. Effect of *Harpagophytum procumbens* on cell viability

The viabilities of cells incubated with *Harpagophytum procumbens* at 0.001 mg/ml, 0.005 mg/ml, 0.01 mg/ml, 0.05 mg/ml, 0.1 mg/ml, 0.5 mg/ml, and 1 mg/ml for 24h were $106.73 \pm 2.64\%$, $110.10 \pm 3.16\%$, $107.21 \pm 3.02\%$, $106.44 \pm 2.42\%$, $107.62 \pm 2.35\%$, $106.35 \pm 2.11\%$ and $116.46 \pm 2.14\%$ of the control value, respectively. The MTT assay revealed that *Harpagophytum procumbens* exerted no significant cytotoxicity on the mouse BV2 microglial cells (Fig. 1).

2. Effect of *Harpagophytum procumbens* on the expressions of COX-1, COX-2, and iNOS mRNA

RT-PCR analysis of the mRNA levels of COX-1, COX-2 and iNOS was performed. In the present study, the mRNA level of COX-1, COX-2, and iNOS in the control cells was set as 1.00.

The level of COX-1 mRNA following treatment with 1 $\mu\text{g/ml}$ LPS for 24 h was 1.27 ± 0.12 , while it was 1.38 ± 0.14 and 1.19 ± 0.28 for the cells treated with 0.1 mg/ml *Harpagophytum procumbens* and 1 mg/ml *Harpagophytum procumbens* 1 h prior to the 1 $\mu\text{g/ml}$ LPS exposure, respectively. LPS treatment exerted no significant effect on the COX-1 mRNA expression in the mouse BV2 microglial cells. Pretreatment with 0.1 mg/ml *Harpagophytum procumbens* and 1 mg/ml *Harpagophytum procumbens* also exerted no significant effect on the COX-1 mRNA expression.

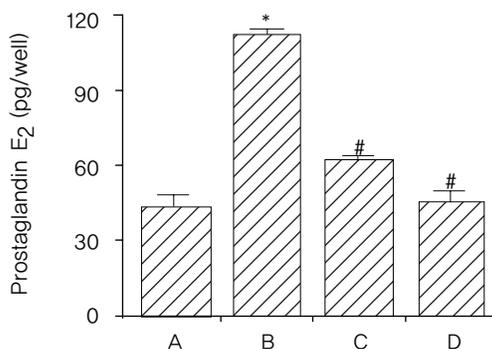


Fig. 3. Measurement of prostaglandin-E2 synthesis in BV2 cells.

* represents $p < 0.05$ compared to the normal group .

represents $p < 0.05$ compared to the control group ; lipopolysaccharide (LPS)-treated group

A : Normal group

B : Control group ; LPS-treated group

C : LPS- and 0.1 mg/ml *Harpagophytum procumbens*-treated group

D : LPS- and 1 mg/ml *Harpagophytum procumbens*-treated group

The level of COX-2 mRNA following $1\mu\text{g/ml}$ LPS treatment for 24 h was significantly increased to 8.30 ± 0.98 , while it was decreased to 4.50 ± 0.38 and 0.75 ± 0.03 for the cells treated with 0.1mg/ml *Harpagophytum procumbens* and 1mg/ml *Harpagophytum procumbens* 1 h prior to the $1\mu\text{g/ml}$ LPS exposure, respectively. LPS treatment significantly enhanced COX-2 mRNA expression in the mouse BV2 microglial cells. Pretreatment with 0.1mg/ml *Harpagophytum procumbens* and 1mg/ml *Harpagophytum procumbens* suppressed the LPS-induced COX-2 mRNA expression.

The level of iNOS mRNA was significantly increased to 3.34 ± 0.36 following $1\mu\text{g/ml}$ LPS treatment for 24 h, while it was decreased to 3.16 ± 0.41 and 0.49 ± 0.16 for cells treated with 0.1mg/ml *Harpagophytum procumbens* and 1mg/ml *Harpagophytum procumbens* 1 h prior to the $1\mu\text{g/ml}$ LPS exposure, respectively. LPS treatment significantly

enhanced iNOS mRNA expression in the mouse BV2 microglial cells. Pretreatment with 0.1mg/ml *Harpagophytum procumbens* and 1mg/ml *Harpagophytum procumbens* suppressed the LPS-induced iNOS mRNA expression (Fig. 2).

3. Effect of *Harpagophytum procumbens* on PG-E₂ synthesis

From the results of the PG-E₂ immunoassay, after 24 h of exposure to $1\mu\text{g/ml}$ LPS, the amount of PG-E₂ from the culture medium was increased from $43.33 \pm 4.80\text{ pg/ml}$ to $112.75 \pm 1.84\text{ pg/ml}$. It was decreased to $63.00 \pm 0.00\text{ pg/ml}$ and $46.25 \pm 3.60\text{ pg/ml}$ by treatment with 0.1 mg/ml *Harpagophytum procumbens* and 1 mg/ml *Harpagophytum procumbens*, respectively. LPS treatment increased PG-E₂ synthesis in the mouse BV2 microglial cells. Pretreatment with 0.1mg/ml *Harpagophytum procumbens* and 1mg/ml *Harpagophytum procumbens* significantly suppressed

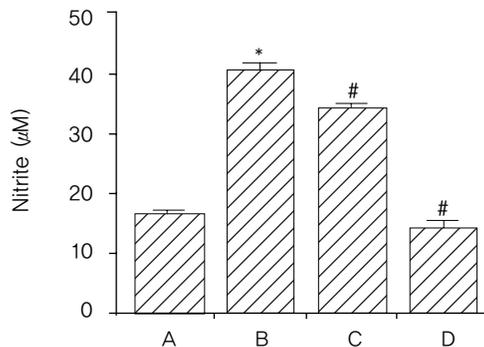


Fig. 4. Measurement of nitric oxide (NO) production in BV2 cells.

* represents $p < 0.05$ compared to the normal group.

represents $p < 0.05$ compared to the control group ; lipopolysaccharide (LPS)-treated group.

A : Normal group

B : Control group ; LPS-treated group

C : LPS- and 0.1 mg/ml *Harpagophytum procumbens*-treated group

D : LPS- and 1 mg/ml *Harpagophytum procumbens*-treated group.

the LPS-induced PG-E₂ synthesis (Fig. 3).

4. Effect of *Harpagophytum procumbens* on NO production

From the results of the NO detection assay, after 24 h of exposure to 1 $\mu\text{g/ml}$ LPS, the amount of nitrite was increased from $16.77 \pm 0.15 \mu\text{M}$ to $40.72 \pm 1.04 \mu\text{M}$. The amount of nitrite was decreased to $34.60 \pm 0.57 \mu\text{M}$, and $14.64 \pm 1.14 \mu\text{M}$ by treatment with 0.1mg/ml *Harpagophytum procumbens*, and 1mg /ml *Harpagophytum procumbens*, respectively. LPS treatment increased NO production in the mouse BV2 microglial cells. Pretreatment with 0.1mg /ml *Harpagophytum procumbens* and 1mg /ml *Harpagophytum procumbens* significantly suppressed the LPS-induced NO production(Fig. 4).

Discussion

Harpagophytum procumbens has been used as a supportive treatment of inflammatory and degenerative diseases of the skeletal system^{9,13-15}.

Jang *et al*⁽¹⁰⁾ examines the anti-inflammatory and analgesic effects of *Harpagophytum procumbens* by the inhibition of PG-E₂ synthesis and NO production in fibroblast cell Line L929 model.

Here in this study, the anti-inflammatory property of the *Harpagophytum procumbens* was tested on the LPS-induced inflammatory responses in mouse BV2 microglial cells, which is a useful model of neuroinflammation.

The present results show that *Harpagophytum procumbens* suppressed PG-E₂ synthesis and NO production by inhibiting the LPS-stimulated expressions of the COX-2 and iNOS genes in the mouse BV2 microglial cells.

LPS is derived from the cell walls of gram-negative bacteria, and it mediates inflammatory sequences

induced by infection¹⁶. LPS initiates major cellular responses that play a vital role in the pathogenesis of inflammation including the activation of inflammatory cells and the production of cytokines and other mediators.

In the present study, the microglial cells activated by LPS produced a large amounts of PGs and NO, and both of which are known be critical factors in determining the final outcome of a microglial reaction to pathological stimuli. PGs synthesis and NO production are markedly increased when the inducible isoforms of COX and NOS are expressed.

It has been firmly established that the COX-1 isozyme is a housekeeping protein in most tissues, and it catalyzes the synthesis of PGs used by the body for normal physiological functions. Thus, COX-1 is expressed at essentially a constant level, and this level does not fluctuate in response to various stimuli. As a constant inducible isoform, COX-2 expression is rapidly stimulated by tumor promoters, growth factors, cytokines, and pro-inflammatory molecules in various cell types⁹. COX-2 is known to be responsible for the production of high levels of PGs in several pathological conditions such as inflammation, and it is a major isoform expressed by inflammatory cells, including the microglia¹⁷.

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, inhibit both isoforms of COX, and these drugs exert both their beneficial effects (inhibition of COX-²) and their potentially deleterious effects (inhibition of COX-1). The recent identification and our better understanding of COX-1 and COX-2 may lead to new approaches for the development of anti-inflammatory therapies. When we consider that NSAIDs constitute one of the largest groups of prescribed drugs worldwide, the search for selective inhibitors of COX-2 may well provide extensive clinical benefits in the future¹⁸.

The present study shows that the expression of COX-2 mRNA in the mouse BV2 microglial cells was significantly increased by LPS and that *Harpagophytum procumbens* inhibited LPS-induced COX-2 mRNA expression in the microglial cells. *Harpagophytum procumbens* did not inhibit COX-1 mRNA. The present results show that *Harpagophytum procumbens* selectively inhibits COX-2 mRNA expression, and doses used in this study did not incur side effects such as peptic ulcer formation and renal dysfunction are sometimes associated with COX-1 inhibition.

NO production through the iNOS pathway is also increased in inflammatory diseases, and excessive NO production induces cellular injury. Expression of the iNOS gene is increased in several pathophysiological conditions, and this produces large amounts of NO in response to inflammatory signals including cytokines and LPS^{4,18}). The present study shows that iNOS mRNA expression in microglial cells was increased by LPS, and that treatment with *Harpagophytum procumbens* inhibited the LPS-induced iNOS mRNA expression in microglial cells.

Here in our study, we have shown that *Harpagophytum procumbens* probably exerts its anti-inflammatory and analgesic effects by the suppression of the COX-2 and iNOS mRNA expressions, and the final result is the inhibition of PGE₂ synthesis and NO production in mouse BV2 microglial cells. microglial cells are believed to play an active role in brain inflammatory, immune and degenerative processes with secrete numerous products including PGs and NO⁹).

Based on our present results, it is possible that *Harpagophytum procumbens* can offer a valuable means of therapy for the treatment of brain inflammatory diseases by attenuating LPS-induced PGE₂ and NO synthesis.

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