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Antiinflammatory Activity of Hyperin from *Acanthopanax chiisanensis* Roots

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The chloroform and the ethyl acetate fractions from the roots of *Acanthopanax chiisanensis* exhibited a significant inhibition of prostaglandin E_2 (PGE_2) production in rat peritoneal macrophages stimulated by the protein kinase C activator, 12-*O*-tetradecanoylphorbol 13-acetate (TPA). Hyperin was isolated as an active principle from the ethyl acetate fraction. It suppressed not only PGE_2 production but also nitric oxide (NO) production *in vitro* in a concentration dependent manner, their IC_{50}, being 24.3 and 32.9 μM, respectively. Hyperin also caused a significant inhibition of increase in acetic acid-induced vascular permeability in mice *in vivo*.

Key words: *Acanthopanax chiisanensis*, Araliaceae, Hyperin, Anti-inflammatory activity

INTRODUCTION

Acanthopanax chiisanensis is an indigenous plant belonging to the family Araliaceae self-grown wild in its vertical distribution ranges from 200 to 1400 m altitude stretching from Mt. Jiri across the Korean peninsula. In Chinese medicine, it has been used for the alleviation and treatment of various diseases such as rheumatism, palsy, hypertension and diabetes (Perry, 1980). Approximately fifteen species of the genus *Acanthopanax* are known to be self-grown in the Korean peninsula. Of them, *A. chiisanensis* is one of the most abundant species.

In the course of our continued attempts to evaluate anti-inflammatory principles from the roots of this plant, hyperin was isolated as an active principle. In present study, anti-inflammatory activities of this compound were evaluated by determining PGE_2 production and NO production in rat peritoneal macrophages *in vitro* and vascular permeability in mice *in vivo*.

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MATERIALS AND METHODS

Plant materials

The roots of *Acanthopanax chiisanensis* Nakai were collected at Gongju, Korea, and authenticated by Prof. S. H. Cho, Gongju National University of Education, Korea. The voucher specimen (Shin2000-03) was deposited at the Herbarium of Natural Products Research Institute (NPRI), Seoul National University, Korea.

Extraction and isolation

The air-dried powdered roots (1.5 kg) were extracted with methanol (3×3000 mL). The extract thus obtained (159 g) was partitioned between equal volumes of *n*-hexane (4 g) and water, and the aqueous layer was again partitioned successively with chloroform (26 g), ethyl acetate (15 g) and *n*-butanol (24 g). Repeated silica gel column chromatography (7×60 cm) of the ethyl acetate fraction with a gradient of ethyl acetate/methanol (90:10) gave hyperin (1.13 g, 0.08% from the dry roots). The compound isolated was identified by co-TLC and comparison of the spectral data (NMR, IR and MS) of an authentic sample (Lee *et al.*, 2003).

Hyperin (quercetin-3-*O*-β-D-galactopyranoside): IR ν_{max}

(KBr): 3316 (OH), 2900, 1655, 1607, 1060 cm^{-1}; FABMS: m/z 465 $[M + H]^+$; ^{1}H-NMR (400 MHz, DMSO-d_6): δ 12.64 (1H, s, 5-OH), 7.67 (2H, dd, *J* = 2.0, 8.5 Hz, H-6'), 7.53 (1H, d, *J* = 2.0 Hz, H-2'), 6.82 (1H, d, *J* = 8.5 Hz, H-5'), 6.41 (1H, d, *J* = 1.9 Hz, H-8), 6.21 (1H, d, *J* = 1.9 Hz, H-6), 5.38 (1H, d, *J* = 7.8 Hz, galactosyl H-1"); ^{13}C-NMR (100 MHz, DMSO-d_6): δ 177.9 (C-4), 164.5 (C-7), 161.6 (C-5), 156.6 (C-2, C-9), 148.9 (C-4'), 145.2 (C-3'), 133.9 (C-3), 122.4 (C-6'), 121.5 (C-1'), 116.3 (C-5'), 115.6 (C-2'), 104.3 (C-10), 102.2 (C-1"), 99.1 (C-6), 93.9 (C-8), 76.2 (C-5"), 73.6 (C-3"), 71.6 (C-2"), 68.3 (C-4"), 60.5 (C-6").

Measurement of TPA-induced PGE_2 production *in vitro*

Preparation of rat peritoneal macrophages

A solution of soluble starch (Wako Pure Chemicals, Osaka, Japan) and Bactopeptone (Difco, Detroit, MI, USA), 5% each, was injected intraperitoneally into male Sprague-Dawley rats (300-350 g, specific pathogen-free, Charles River Japan, Kanagawa, Japan) at a dose of 5 mL per 100 g body weight. Four days later, the rats were killed by cutting the carotid artery under diethylether anesthesia and peritoneal cells were harvested (Ohuchi *et al.*, 1985). The animal experiments were done in accordance with the procedure approved by the Animal Ethics Committee of the Graduate School of Pharmaceutical Sciences (Tohoku University, Japan).

Macrophage culture

The peritoneal cells were suspended in Eagles minimal essential medium (EMEM, Nissui, Tokyo, Japan) containing 10% calf serum (Flow Laboratories, North Rydge, Australia), penicillin G potassium (18 g/mL) and streptomycin sulphate (50 g/mL) (Meiji Seika, Tokyo, Japan), then seeded at a density of 7.5×10^5 cells per well in 24-well plastic tissue culture dishes (Corning Glass Works, Corning, NY, USA) in 0.5 mL of medium, and incubated at 37°C for 2 h. The wells were then washed three times with medium to remove non-adherent cells, and the adherent cells were further incubated at 37°C for 20 h. After three washes, the adherent cells were used for the subsequent experiments.

Incubation of macrophages with hyperin and determination of PGE_2

The adherent cells were incubated at 37°C for 8 h in 0.5 ml of medium containing 10% calf serum and various concentrations of hyperin or indomethacin (Sigma) in the presence or absence of TPA (Sigma). Each reagent was dissolved in ethanol and added to the medium. The final concentration of ethanol was adjusted to 0.1% (v/v). Eight hours later after incubation, the conditioned medium was obtained and PGE_2 concentrations in the supernatant fraction were radio-immunoassayed (Nishizuka, 1992).

Measurement of LPS-induced NO production *in vitro*

Cell culture

RAW 264.7 cells obtained from RIKEN Gene Bank (Tsukuba, Japan) were seeded in 24-well plates at a density of 0.75×10^6 cells per well, and incubated for 20 h at 37°C in 0.5 mL of Eagle's minimal essential medium (MEM), phenol red-free (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 1% (v/v) MEM non-essential amino acid solution (Sigma, St. Louis, MO, USA), and 10% (v/v) heat-inactivated fetal bovine serum (Flow Laboratories, North Rydge, N.S.W., Australia). After three washes with medium, the cells were incubated for the indicated periods at 37°C in 0.5 mL of medium containing drugs.

Drugs

Drugs used were lipopolysaccharide (from *Escherichia coli* 0111:B4, Difco Laboratories, Detroit, MI, USA). Lipopolysaccharide was directly dissolved in medium. Other drugs were dissolved in ethanol and an aliquot of each ethanol solution was added to the medium. The final concentration of ethanol was adjusted to 0.1% (v/v). Control medium contained the same amount of the vehicle. After treatment with drugs, the viability of the cells was examined by the Trypan blue exclusion test; no cytotoxic effects were observed.

Determination of nitrite

Nitrite concentrations in the conditioned medium were determined by using Griess reagent according to the method described by Green *et al.* (1982). Briefly, 100 μL aliquots of the conditioned medium were mixed with equal volume of Griess reagent [1 part 0.1% (w/v) *N*-1-naphthylethylenediamine dihydrochloride to 1 part 1% (w/v) sulphanilamide in 2.5% (v/v) phosphoric acid]. The absorbance at 540 nm was measured with a microplate reader (Model 450, Bio-Rad Laboratories, Hercules, CA, USA) and the nitrite concentration was determined by interpolation of a calibration curve of standard sodium nitrite concentrations against absorbance. All assays were performed on three to four replicates and repeated at least three independent experiments.

Western blot analysis for NO synthase

Protein concentrations in the supernatant fractions were determined (Wang and Smith, 1975). The fractionated proteins resolved by SDS-PAGE were transferred to nitrocellulose membranes and blotted in blocking solution (Block Ace, Dainippon Pharmaceutical, Osaka, Japan) for 1 h. The membranes were then incubated at 4 overnight

with the primary antibody, a rabbit immunoglobulin G to inducible NO synthase purified from RAW 264.7 cells (Upstate Biotechnology, NY, USA), at a dilution of 1:2000. Thereafter the membranes were washed and incubated for 3 h at 4°C with rabbit anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA, USA) at a dilution of 1:2000. The reaction products were incubated for 30 min at room temperature with Vectastatin ABC reagent (Vector Laboratories) and were visualized by using a chemiluminescence detection system (ECL system, Amersham, Arlington Heights, IL, USA). The membranes were exposed to Kodak X-Omat AR film (Eastman Kodak, Rochester, NY, USA) at room temperature for 30 s and photographed. The levels of inducible NO synthase protein were quantified by scanning densitometry and expressed as relative densities signals.

Measurement of acetic acid-induced vascular permeability in mice *in vivo*

Fifty minutes later after the administration of hyperin (100 mg/kg, i.p. and 200, 500 mg/kg, p.o.) or aspirin (200 mg/kg, p.o.), 0.15 mL of an aqueous solution of 4% Brilliant blue was administered intravenously. Ten minutes later after treatment, the mice were injected with 0.25 mL of an aqueous solution of 0.6% acetic acid intraperitoneally. Twenty minutes later, the mice were killed and leakage of dye into peritoneal cavity was measured at 560 nm.

Cell viability assay

MTT was used as an indicator of cell viability as determined by the mitochondrial-dependent reduction to formazone. RAW 264.7 cells were plated at a density of 1×10^5 cells/well into 96-well plates for 12 h, followed by treatment with different concentrations of each compound for a further 16 h. Cells were washed with PBS three times and MTT (50 mg/mL) was added to the medium for 4 h. Furthermore, the supernatant was removed, and the formazone crystals were dissolved using 0.04 N HCl in isopropanol. The absorbance was read at 600 nm with an ELISA analyzer.

Statistical analysis

The statistical significance of the results was analyzed by Student's *t*-test for unpaired observation.

RESULTS AND DISCUSSION

The activity-guided systematic fractionation of the root extracts were carried out and various fractions obtained were subjected to test for their inhibitory effects on TPA (16.2 nM)-induced PGE_2 production in rat peritoneal macrophages. The chloroform and the ethyl acetate fractions were found to cause almost complete inhibition of PGE_2 production at a concentration of 100 μg/mL (Table I). Further fractionation of ethyl acetate fraction eluting with ethyl acetate/methanol on silica gel column led to the isolation of hyperin (Fig. 1), which caused a significant inhibition of TPA-induced PGE_2 production (Fig. 2). Under the same condition, indomethacin, a non-specific inhibitor of COX-1

Table I. Effects of the various fractions from the roots on the PGE_2 production in rat peritoneal macrophages stimulated by TPA

Treatments	PGE_2 (ng/mL)
None	2.10 ± 0.04***
TPA	8.48 ± 0.57
TPA + *n*-Hexane fraction	8.28 ± 0.59
TPA + Chloroform fraction	2.11 ± 0.12***
TPA + Ethyl acetate fraction	3.58 ± 0.33***
TPA + *n*-Butanol fraction	8.63 ± 0.75

Rat peritoneal macrophages (7.5×10^5 cells) were incubated at 37°C for 8 h in 0.5 μL of medium with or without TPA (16.2 nM) and each fraction (100 mg/mL), and PGE2 concentrations in the conditioned medium were determined. Values are the means ± S.E.M. from four separate determinations. Statistical significance; ***p<0.001 vs. TPA control.

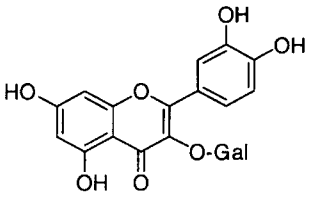

Fig. 1. Chemical structure of hyperin

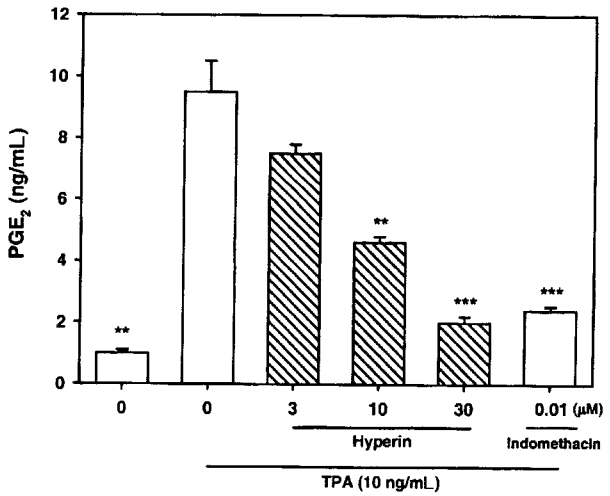

Fig. 2. Effect of the various concentration of hyperin and the indicated concentration of indomethacin on the TPA-induced PGE2 production in rat peritoneal macrophages. Values are the means from four separate determinations with ± S.E.M. shown by vertical bars. Statistical significance: ***p<0.001, **p<0.01 vs. TPA alone.

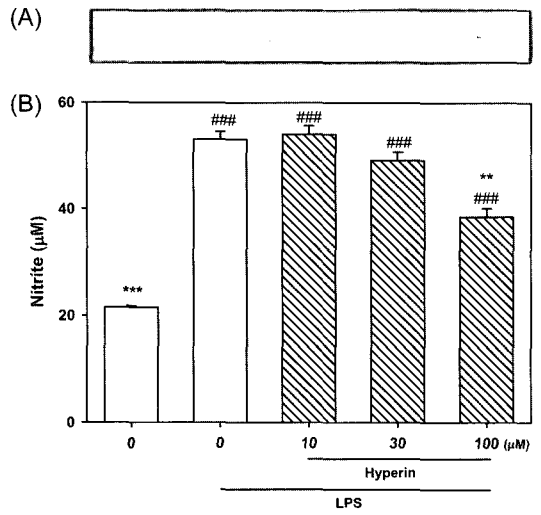

Fig. 3. Effects of hyperin on the iNOS protein expression (A) and the LPS-induced NO production (B) in rat peritoneal macrophages. Rat peritoneal macrophages (7.5×10^5 cells) were incubated at 37°C for 4 h in 0.5 or 2 mL of medium in the presence of the indicated concentration of hyperin, and then stimulated with LPS (1 μg/mL) at 37°C for 24 h. iNOS protein was detected by Western-blot analysis. Nitrite in the conditioned medium was determined using Griess reagent. Values are the means from four separate determinations with ± S.E.M. shown by vertical bars. Statistical significance: $^{**}p<0.01$, $^{***}p<0.001$ vs. LPS control; $^{\#\#\#}p<0.001$ vs. unstimulated control.

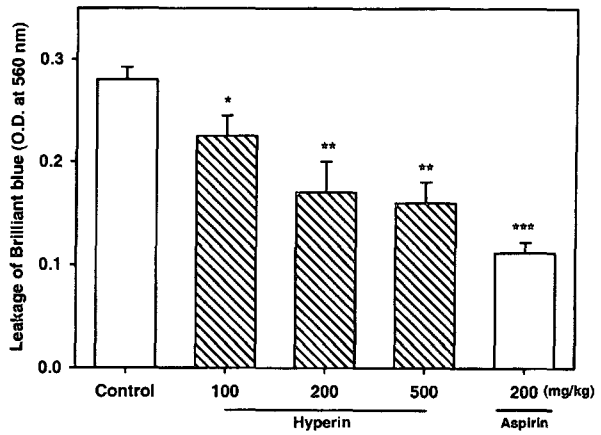

Fig. 4. Effect of administration of hyperin (100 mg/kg, i.p. and 200, 500 mg/kg, p.o.) on the acetic acid-induced vascular permeability in mice. Values are the means from four separate determinations with ± S.E.M. shown by vertical bars. Statistical significance: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ vs. control.

and -2, showed 72.3% inhibition of PGE_2 production at 0.01 μM. In compliance with the inhibitory effect on PGE_2 production, hyperin was also demonstrated to exhibit a inhibition of NO production as well as iNOS protein expression in murine macrophages activated by LPS. As shown in Fig. 3, treatment of hyperin at 10-100 μM in LPS-induced rat peritoneal macrophages for 4 h caused not only inhibition of NO production but also decrease in inducible NO synthase protein expression in a concentration dependent manner. The IC_{50} values of hyperin on PGE_2 production and NO production were calculated to be 24.3 and 32.9 μM, respectively. In agreement with *in vitro* effects, hyperin when administered orally at 200 and 500 mg/kg and i.p. at 100 mg/kg caused a significant inhibition of the increase in acetic acid-induced vascular permeability in mice (Fig. 4). The cytotoxic effect of hyperin was measured using the MTT assay. Hyperin did not demonstrate any significant cytotoxic effects on RAW 264.7 cells up to 700 μM (data not shown).

The results indicated that hyperin possessed anti-inflammatory properties and suggested that its mode of action might be partly due to the inhibition of PGE_2 production as well as NO production immune cells such as macrophages.

There are several reports describing the effects of various flavonoids on purified COX-1 (Bucar *et al.*, 1998; Kalkbrenner *et al.*, 1992). We reported previously that tectorigenin, an isoflavone isolated from the rhizomes of *Belamcanda chinensis*, inhibited TPA-induced PGE_2 production in rat peritoneal macrophages by inhibiting the induction of COX-2 protein but not the direct inhibition of COX-1 and -2 (Kim *et al.*, 1999).

Therefore, the anti-inflammatory potencies of flavonoids might be different with the structural moieties such as the differences in the sugar moieties and in the position of the sugars in flavonoids, and the possibility remains that the inhibition by hyperin of PGE_2 production might be due to the direct inhibition of COX-2 enzyme or to the inhibition of induction of COX-2 protein. We have also found that hyperin was one of major constituents from the roots of *A. chiisanensis*. These findings provide support for the use of hyperin as a potential prototype that will lead to the discovery of very effective anti-inflammatory agents.

In conclusion, hyperin was considered to contribute as one of the main anti-inflammatory principles of this plant part. Further studies on the mechanism of action of hyperin are in progress.

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REFERENCES

Bucar, F., Jachak, S. M., Noreen, Y., Nartnig, T., Perera, P., Bohlin, L., and Schubert-Zsilavecz, M., Amentoflavone from *Biophytum sensitivum* and its effect on COX-1/COX-2 catalysed prostaglandin biosynthesis. *Planta Med.*, 64, 373-

374 (1998).

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. R., Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. *Anal. Biochem.*, 126, 131-138 (1982).

Kalkbrenner, F., Wurm, G., and Bruchhausen, F., *In vitro* inhibition and stimulation of purified prostaglandin endoperoxide synthase by flavonoids: structure-activity relationship. *Pharmacol.*, 44, 1-12 (1992).

Kim, Y. P., Yamada, M., Lim, S. S., Lee, S. H., Ryu, N., Shin, K. H., and Ohuchi, K., Inhibition by tectorigenin and tectoridin of prostaglandin E_2 production and cyclooxygenase-2 induction in rat peritoneal macrophages. *Biochim. Biophys. Acta*, 1438, 399-407 (1999).

Lee, S., Jung, S. H., Lee, Y. S., and Shin, K. H., Hyperin, an aldose reductase inhibitor from *Acanthopanax senticosus. Nat. Prod. Sci.,* 9, 4-6 (2003).

Nishizuka, Y., Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science*, 258, 607-614 (1992).

Ohuchi, K., Watanabe, M., Yoshizawa, K., Tsurufuji, S., Fujiki, H., Suganuma, M., Sugimura, T., and Levine, L., Stimulation of prostaglandin E_2 production by 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-type and non-TPA-type tumor promoters in macrophages and its inhibition by cycloheximide. *Biochim. Biophys. Acta*, 834, 42-47 (1985).

Perry, L. M., "Medical Plants of East and Southeast Asia," MIT Press, Cambridge, Massachusetts and London, 1980, p. 41.

Wang, C. and Smith, R. L., Lowry determination of protein in the presence of Triton X-100. *Anal. Biochem.*, 63, 414-417 (1975).