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Anti-Inflammation Activity of *Actinidia polygama*

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The fruit of *Actinidia polygama* (AP) has long been used as a folk medicine in Korea for treating pain, rheumatic arthritis and inflammation. The present investigation was carried out to determine the *in vivo* and *in vitro* anti-inflammatory activity of AP using several animal models of inflammation. The 70% ethanol extract of the fruit of AP significantly inhibited acetic acid-induced, vascular permeability in a dose dependent manner (23%, 38%, and 41% inhibition at doses of 200 mg/kg, 500 mg/kg and 1000 mg/kg, respectively). This effect was maintained in AP water-soluble fraction (APW). The APW fraction also showed significant inhibitory activity against the rat paw edema induced by a single treatment of carrageenan. *In vitro* experiments were performed to demonstrate the inhibitory activities of APW (100 μg/ml) on lipopolysaccharide (LPS)-induced nitric oxide (NO) and prostaglandin E_2 (PGE_2) production. The results showed that APW dose-dependently suppressed LPS-induced NO production in RAW 264.7 macrophages without a notable cytotoxic effect and also decreased inducible NO synthase (*i*NOS) protein expression. APW also showed a significant inhibitory effect in LPS-induced PGE_2 production and cyclooxygenase-2 (COX-2) expression.

Key words: *Actinidia polygama*, Anti-inflammation, Vascular permeability, Carrageenan, LPS, iNOS, COX-2, PGE_2

INTRODUCTION

Inflammatory diseases, including different types of rheumatic disease, are very common throughout the world. Although rheumatism is one of the oldest known diseases of mankind affecting the majority of the population, no substantial progress has been made in achieving a permanent cure. The greatest disadvantage in presently available potent synthetic drugs lies in their toxicity and reappearance of symptoms after discontinuation. Therefore, the screening and development of drugs for their anti-inflammatory activity continues and there is much hope for finding anti-inflammatory drugs from indigenous medicinal plants (Srinivasan *et al*, 2001).

During the inflammatory process, large amounts of the pro-inflammatory mediators nitric oxide (NO) and prostaglandin E_2 (PGE_2) are generated by the inducible isoforms of NO synthase (*i*NOS) and cyclooxygenase-2 (COX-2), respectively (Lee *et al*, 1992). NO is produced in large amounts from the amino acid, L-arginine. In mammalian cells, three isoforms of NOS, types I, II and III, have been identified on the basis of the physical and biochemical characteristics of the purified enzymes. Types I (neuronal NOS, *n*NOS) and III (endothelial NOS, *e*NOS) have been classified as constitutive NOS (cNOS) because they are continuously present in the cells, whereas type II, an iNOS, is expressed only after exposure to specific stimulants such as cytokines, bacterial endotoxic lipopolysaccharide (LPS), and calcium ionophore in some cells. COX is the enzyme which converts arachidonic acid (AA) to prostaglandins (PGs). Nonsteroidal anti-inflammatory drugs (NSAIDs) produce their therapeutic activities through inhibition of COX. They share, to a greater or lesser degree, the same side effects, including gastric and renal toxicity. Recent research has shown that there are at least two COX isoenzymes. COX-1 is constitutive and makes PGs that protect the stomach and kidney from damage. COX-2 is induced by inflammatory stimuli, such as cytokines, and produces PGs that contribute to the pain and swelling of inflammation (Vane and Botting, 1998). COX-2 is mainly an inducible enzyme and is involved primarily in the regulation of inflammation. PGs, and in particular PGE_2,

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are regarded as a potent pro-inflammatory molecule; however, increasing evidence has indicated that they can also exert anti-inflammatory functions which are important for the resolution of the inflammatory response (Herschman, 1996). Activation of macrophages by LPS enhances the production and release of inflammatory mediators, including cytokines, AA metabolites and NO (Wink and Mitchell, 1998).

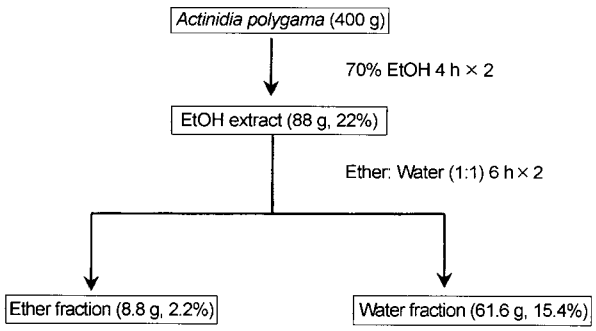
Actinidia polygama (Actinidiaceae; AP) is a deciduous tuberous plant, which is distributed in oriental countries. The fruit of AP has been called Ge-darae in Korea and used in traditional medicine to treat abdominal pain, rheumatic arthritis, and stroke. A number of chemical constituents like actinidine and matatabilactone have been identified from the species.

In this study, we evaluated the anti-inflammatory effect of the 70% ethanol extract of AP using an acetic acid-induced, vascular permeability test. After solvent fractionation, we compared the anti-inflammatory effects of AP ether and water-soluble fractions (APE and APW). The APW fraction was found to have significant anti-inflammatory effect in both vascular permeability and the carrageenan-induced edema test. This finding prompted us to conduct *in vitro* experiments into the anti-inflammatory action of this plant material to determine its anti-inflammatory mechanism. We evaluated the effects of APW on LPS-induced NO, PGE_2 release by the macrophage cell line RAW 264.7 and examined the protein expression of iNOS and COX-2.

MATERIALS AND METHODS

Plant materials

A 400 g powder sample of AP, obtained from the Korea Herbal Science Institute, was mixed with 3 L of 70% EtOH and extracted for 4 h twice at 90°C. The whole mass was filtered twice and concentrated to one third in volume by evaporation. As shown in Scheme 1, the same volume of ether and water was added and the mixture was fractionized by using a separating funnel. The resulting water and ether fractions were freeze-dried. The ethanol extract (88 g) was partitioned with ether to give a dried ether soluble fraction (8.8 g) and a water-soluble fraction (61.6 g). The resulting powder was stored in a deep freezer until use.



Scheme 1. Fractionation of Actinidia polygama.

In vivo study

Animals and treatment

Male Sprague-Dawley rats (160~200 g, Han-lim Lab. animal, Hwasung, Korea) and male ICR mice (25~30 g, Han-lim Lab. animal, Hwasung, Korea), aged 6 weeks, were used. All animals were acclimatized to the laboratory environment for one week before the experiment. They were maintained on a 12h/12h light-dark cycle with controlled temperature (25 ± 1°C) and humidity (50 ± 5%) and standard laboratory food (Samyangsa, Kangwon, Korea) and tap water available *ad libitum*. AP extracts were given orally to animals after being suspending in a mixture of distilled water and 0.5% sodium carboxymethyl cellulose (CMC). The control animals received the same experimental handling as those of the test groups except that the drug treatment was replaced with appropriate volumes of the dosing vehicle. Naproxen (200 mg/kg) dissolved in 0.5% CMC was used as a reference drug.

Acetic acid-induced vascular permeability in mice

In order to evaluate the inhibitory activity of AP against animal models of acute inflammation, an acetic acid-induced permeability test was employed. through a modification to the method of Lee (2001). ICR mice weighing 23-28 g were fasted for 10 h prior to experiment and the test samples were given orally. Thirty minutes later, each animal was given an intravenous injection of a 4% solution of methylene blue (Shinyo pure chemical Co., Osaka, Japan) as 0.1 mL/10 g (b.w.). Sixty minutes after administration of the sample, 0.1 mL/10g of 0.7% acetic acid in physiological saline was injected intraperitoneally. Twenty minutes later, the mice were killed by dislocating the neck and 10 mL of normal saline was injected intraperitoneally, after which the washing solution was collected in test tubes. To clear turbidity due to protein, 0.1 mL of 1 N NaOH solution was added to each tube and the absorbance was read at 595 nm in a spectrophotometer (U3210, Hitachi, Japan). The absorbance was entered as the Standard Curve and the vascular permeability was expressed in terms of the amount of total dye (ng/mL) which was leaked into the intraperitoneal cavity.

Carrageenan-induced hind paw edema in rats

To measure the anti-inflammatory activity in an animal model of subchronic inflammation, carrageenan-induced paw edema was carried out using a slight modification to the procedure of Lee (2001). The edema was induced on the right hind foot of the rat by subplantar injection of 0.1

mL/rat of 1.0% carrageenan (type IV, Sigma) in saline. The test samples were given orally 1 h before carrageenan injection. Swelling of the paw was measured before (time 0) and at 1-7 h after injection of carrageenan. The degree of paw edema was determined by measuring the hind paw volume by plethysmometer (Ugo Basile, Varese, Italy).

In vitro study

Reagents

DMEM medium, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies Inc. (Grand Island, NY, USA). Sulfanilamide, 3-(4,5-dimethlythiazol-2-yl)-2,5-diphenyltertazolium bromide (MTT), aprotinin, leupeptin, phenylmethylsulfonyl fluoride, dithiothreitol, *N*-mono-methylarginine (NMA) and *E. Coli* lipopolysaccharide (LPS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). COX-2 and *i*NOS monoclonal antibodies and the peroxidase conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). ELISA kit for PGE_2 was obtained from R&D systems (Minneapolis, MN, USA).

RAW 264.7 cell culture and treatment

RAW 264.7 murine macrophage cell line was obtained from the Korea Cell Line Bank (Seoul, Korea). These cells were grown at 37°C in DMEM medium supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin sulfate (100 μg/mL) in a humidified atmosphere of 5% CO_2. Cells were incubated with APW at increasing concentrations (25-100 μg/mL) and stimulated with 1 μg/mL of LPS for 24 h.

Nitrite assay

Nitrite accumulation, an indicator of NO synthesis, was measured in the culture medium by Griess reaction. Briefly, 100 μL of cell culture medium was mixed with 100 μl of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylethylenediamine-HCl] and incubated at room temperature for 10 min, after which the absorbance at 550 nm was measured in a microplate reader. Fresh culture medium was used as the blank in all experiments. The amount of nitrite was calculated from a sodium nitrite standard curve freshly prepared in culture medium.

PGE_2 assay

The PGE_2 level in macrophage cultured medium was quantified by ELISA kit according to the manufacturer's instruction.

Western blot analysis

Cellular proteins were extracted from control and APW treated RAW 264.7 cells. The washed cell pellets were resuspended in extraction lysis buffer (50 mM HEPES pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 mM NaF, 0.5 mM Na-orthovanadate) containing 5 μg/mL each of leupeptin and aprotinin and incubated for 15 min at 4°C. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. Protein concentration was determined by Bio-Rad protein assay reagent according to the manufacturer's instruction. Cellular proteins, 40-50 μg, from treated and untreated cell extracts were electroblotted onto nitrocellulose membrane following separation by 10% SDS-polyacrylamide gel electrophoresis. The immunoblot was incubated overnight with 5% skim milk at 4°C, followed by incubation for 4 h with a 1:500 dilution of monoclonal anti-*i*NOS and COX-2 antibody. Blots were washed twice with Tween 20/Tris-buffered saline (TTBS), incubated with a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody for 1 h at room temperature, washed again three times in TTBS and then developed by enhanced chemiluminescence.

Statistical analysis

All data are expressed as mean± SEM. The data were analyzed using Students *t*-test. Mean values were considered significantly different at $P<0.05$.

RESULTS

Effect of AP on acetic acid-induced vascular permeability in mice

The effect of 70% ethanol extract of AP on acetic acid-induced vascular permeability in mice is shown in Fig. 1.

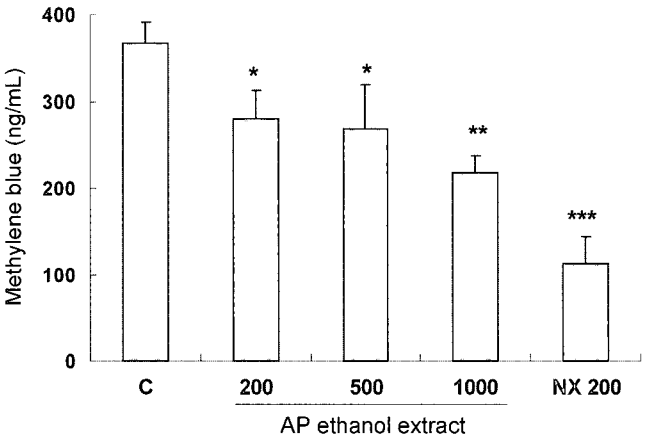


Fig. 1. Effect of AP on acetic acid-induced vascular permeability in mice. Control (C), vehicle treated control; 200, 500 and 1000 mg/kg of *Actinidia polygama* (AP) ethanol extract; NX200, 200 mg/kg of naproxen. Values are means ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001, significantly different from the control.

The oral administration of AP at a dose of 200 mg/kg significantly inhibited the increase of dye leakage into the peritoneal cavity in mice ($P<0.05$, 23% inhibition) and at doses of 500 and 1000 mg/kg showed inhibition of 38 and 41%, respectively, while naproxen (200 mg/kg) demonstrated 70% inhibition.

Comparison of APE and APW on acetic acid-induced vascular permeability in mice

Fig. 2 shows a comparison of APE and APW) obtained from the ethanol extract on acetic acid-induced vascular permeability in mice. APE (100 mg/kg) did not exert any inhibition of vascular permeability, whereas APW (700 mg/kg) showed a significant inhibition of vascular permeability ($P<0.05$, 27% inhibition). The doses administered were proportional to the yield of the fractions obtained from the extracts. This result suggests that active principle(s) of the 70% ethanol extract might be transferred to the water-soluble fraction, so we continued our experiment with APW to examine the anti-inflammatory activity.

Effect of APW on carrageenan-induced hind paw edema in rats

The effect of APW on carrageenan-induced paw edema is shown in Fig. 3. The rats' footpad became swollen soon after the injection of carrageenan. The edema rate of the right footpad reached its peak at 5 h. Administration of APW at a dose of 350 mg/kg significantly inhibited the development of pad swelling from 5 to 7 h after carrageenan injection (-$P<0.01$) and APW at 700 mg/kg showed further inhibition of paw swelling.

Inhibition by APW of LPS-induced NO_2^- production

The inhibitory effect of APW on NO synthesis was not due to the cytotoxicity of APW at concentrations up to 100 mg/mL. As an indicator of cell viability, the mitochondrial respiratory activity of the cells was assessed (data not shown). NO_2^- production was increased by LPS (1 μg/mL) from 2.6 ± 0.1 μM to 31.0 ± 0.7 μM. However, NO production was significantly suppressed by APW. NO production at 25 μg/mL of APW was 26.8 ± 3.8 μM ($P<0.05$), and that at 50 and 100 μg/ml of APW was 24.9 ± 2.1 μM ($P<0.01$), and 16.5 ± 1.2 μM ($P<0.001$), respectively. Therefore, APW showed an inhibitory effect on LPS-induced NO production in a concentration-dependent manner (Fig. 4).

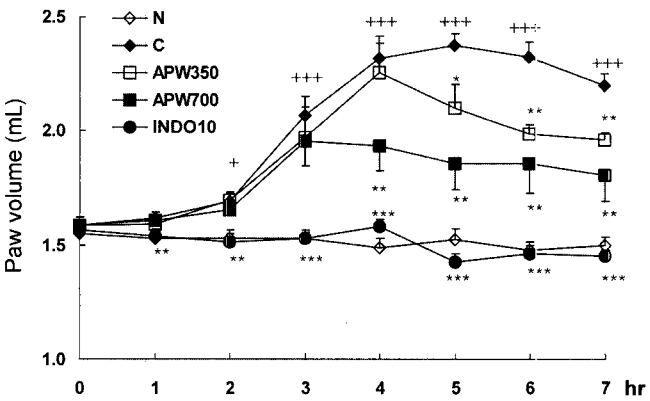


Fig. 3. Effect of APW on carrageenan-induced hind paw edema in rats. APW350 and APW700, 350 and 700 mg/kg of APW, respectively; INDO10, 10 mg/kg of indomethacin. Values are means ± SEM. $^{+}P<0.05$, $^{+++}P<0.001$, significantly different from the normal; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, significantly different from the control.

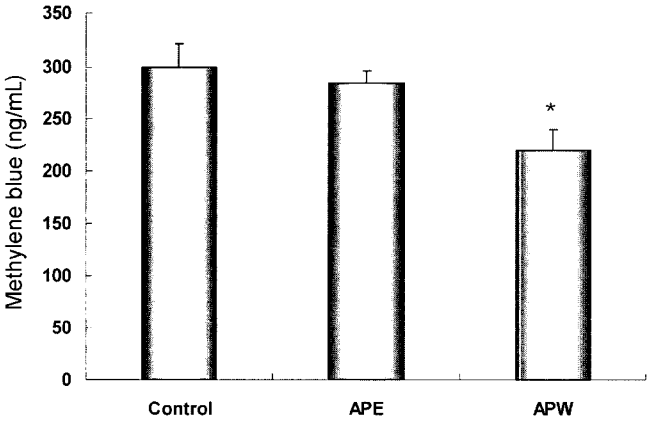


Fig. 2. Comparison of APE and APW on acetic acid-induced vascular permeability in mice. APE, ether fraction of AP; APW, water fraction of AP. Values are means ± SEM. $^{*}P<0.05$, significantly different from the control.

Effect of APW on LPS-induced iNOS expression

To determine whether the inhibitory effect of APW on NO release was related to a modulation of *i*NOS induc-

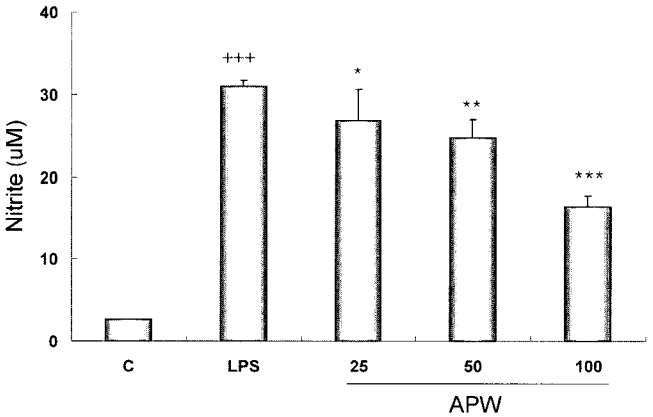


Fig. 4. Evaluation of nitrite production by RAW 264.7 cells stimulated for 24 h with LPS alone or in combination with increasing concentrations (25-100 μM) of APW. APW 25, 50, and 100 μg/mL represent treatment with APW 25, 50, and 100 μg/mL, respectively. Values are means ± SEM. $^{+++}P<0.001$, significantly different from the normal; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, significantly different from the control.

tion, we examined the protein expression level of *i*NOS by western blot analysis. In response to LPS, the expression of *i*NOS was markedly induced (Fig. 5), and APW significantly inhibited the iNOS induction in a concentration-dependent manner (77% inhibition at a dose of 100 μg/mL).

Effect of APW on PGE_2 production

PGE_2 production was increased by LPS (1 μg/mL) from

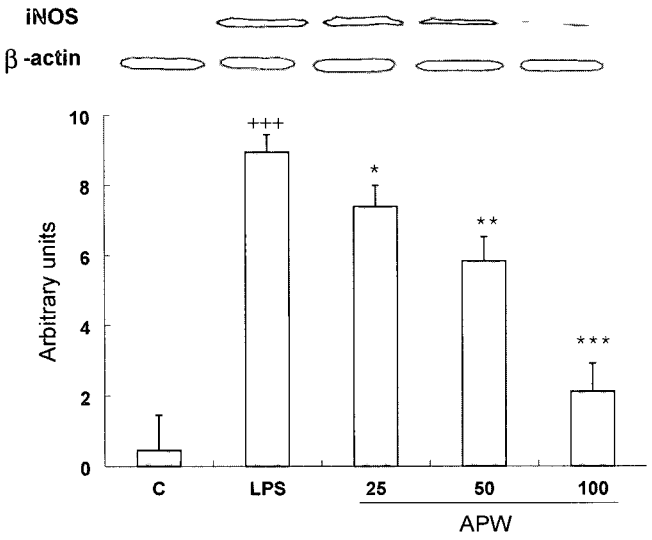


Fig. 5. Effect of APW on LPS-induced iNOS expression in RAW 264.7 cells. Lysates were prepared from control or 24 h-LPS (1 μg/mL) stimulated cells alone or in combination with increasing concentrations (25-100 μg/mL) of APW. APW 25, 50, and 100 represent treatment with APW 25, 50, and 100 mg/kg, respectively. Values are means ± SEM. $^{+++}P<0.001$, significantly different from the normal; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, significantly different from the control.

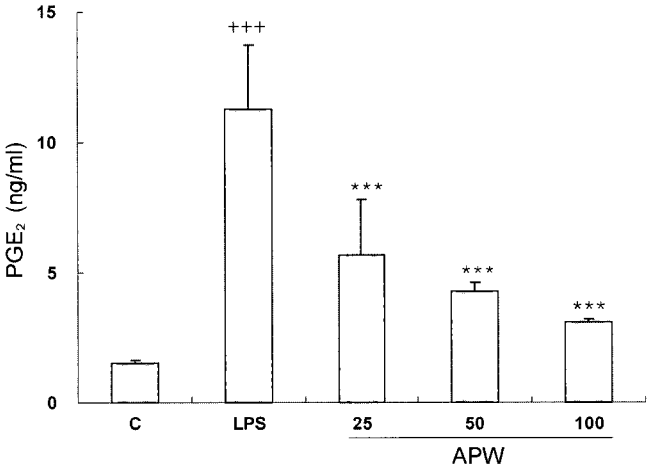


Fig. 6. Effect of APW on PGE_2 production by LPS-induced RAW 264.7 cells for 24 h. Values are means ± SEM. $^{+++}P<0.001$, significantly different from the normal, $^{***}P<0.001$, significantly different from the control.

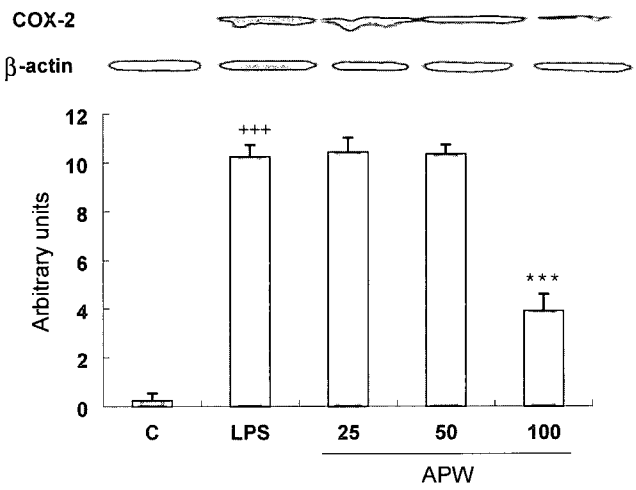


Fig. 7. Effect of APW on LPS-induced COX-2 expression in RAW 264.7 cells. Lysates were prepared from control or 24 h-LPS (1 μg/mL) stimulated cells alone or in combination with increasing concentrations (25-100 μg/mL) of APW. Values are means ± SEM. $^{+++}P<0.001$, significantly different from the normal; $^{***}P<0.001$, significantly different from the control.

1.0 ± 0.1 μM to 11.0 ± 2.5 μM, but was significantly suppressed by all concentrations of APW ($P<0.001$). PGE_2 production at 25 μg/mL of APW was 6.0 ± 2.1 μM ($P<0.05$), and that at 50 and 100 μg/mL of APW was 5.0 ± 0.4 μM and 3.0 ± 0.1 μM, respectively. Therefore, APW showed an inhibitory effect on LPS-induced PGE_2 production and this effect was concentration-dependent (Fig. 6.).

Effect of APW on LPS-induced COX-2 expression

To determine whether the inhibitory effect of APW on PGE_2 level was related to a modulation of COX-2 induction, we examined the expression level by western blot analysis. LPS-induced COX-2 expression was not markedly inhibited by APW at doses of 25 and 50 μg/mL. However, APW at a dose of 100 μg/mL significantly inhibited the COX-2 induc-tion (Fig. 7).

DISCUSSION

In accordance with the ethnomedical use of AP, the present study was carried out to evaluate the anti-inflammatory activity of the ethanol extract of this plant and to identify the active fraction from the extract for further isolation of the active principle(s).

In our experiments, the ethanol extract of AP showed significant inhibition of vascular permeability in mice. Moreover, it showed significant inhibition of carrageenan edema in rat paws. A carrageenan test has significant predictive value for anti-inflammatory agents that act by inhibiting the mediators of acute inflammation. Carrageenan-induced inflammation is useful in detecting orally active

anti-inflammatory agents. Edema formation due to carrageenan in the rat paw is a biphasic event. The initial phase is attributed to the release of histamine and serotonin; the second phase to the release of PGs, protease and lysosome. The second phase is sensitive to most clinically effective anti-inflammatory drugs (Olajide *et al.*, 2000). These results indicate that the mechanism of the anti-inflammatory activity of AP extract is similar to that of the known anti-inflammatory drugs like ibuprofen and aspirin, which exert a potent anti-edematous activity.

Macrophages play a crucial role in both non-specific and acquired immune responses and macrophage activation by LPS leads to a functionally diverse series of responses, including the production of pro-inflammatory cytokines (IL-2, TNF-α and IL-6), the activation of phospholipase A_2 which produces lipid metabolites of AA such as PGs, and NO production. In *in vitro* experiments, we demonstrated that exposure of RAW 264.7 macrophage to LPS for several hours was associated with an accumulation of nitrite in the medium, implying an enhanced NO production. This LPS-induced NO production was significantly inhibited by APW in a concentration-dependent manner without notable cytotoxicity. Results from western blotting analysis further indicated that LPS-induced *i*NOS expression in RAW 264.7 macrophages was significantly blocked by APW. As demonstrated in this study, *i*NOS, which is responsible for long-lasting NO production, is strikingly induced by LPS. Therefore, these results suggest that inhibition of LPS-induced NO production by APW occurs mainly through the regulation of iNOS gene expression.

The inhibition of PG synthesis, which is mediated by COX, is at least shared by the mechanisms of various anti-inflammatory drug actions. COX exists in two isoforms, COX-1 and COX-2, each with a distinct expression pattern in various cell types. COX-1 has been suggested to provide a physiologic level of prostaglandins for normal platelets, stomach and kidney function. In contrast, COX-2 has been found to be highly induced at inflammatory sites in animals as well as patients with inflammatory diseases, and is thus considered to be responsible for pro-inflammatory PG formation (Kim *et al.*, 2002). In addition to inhibition of NO release and *i*NOS induction, APW also significantly inhibited PGE_2 production and COX-2 gene expression in LPS-treated RAW 264.7 macrophages.

In conclusion, AP ethanol extract elicited anti-inflammatory activity in vascular permeability and carrageenan tests. Solvent fractionation results showed that the activity of the 70% ethanol extract was shifted to the water-soluble fraction, which is therefore suspected to contain the anti-inflammatory principles. It is noted that the active principles in the water fraction remain to be isolated.

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