Biomolecules & Therapeutics, 16, 9-13(2008)

Antioxidant and Anti-Nociceptive Activities of *Ulmus davidiana* var. *japonica*

Hyun-Joo Jung[1,¶], Hyun-Jung Kang[2,¶], Yun Seon Song[1], Chang-Jin Lim[2], and Eun-Hee Park[1,*]

[1]*College of Pharmacy, Sookmyung, Women's University, Seoul 140-742, Korea*
[2]*Division of Life Sciences and Research Institute of Life Sciences, Kangwon National University, Chuncheon 200-701, Korea*

(Received December 13, 2007; Accepted March 5, 2008)

Abstract – Some pharmacological activities of *Ulmus davidiana* var. *japonica* were evaluated using its methanol extract (UDE). An acute anti-inflammatory activity of UDE was assessed using carrageenan-induced hind paw edema in rats. UDE exhibited an antioxidant activity when assayed by a stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). Dose-dependent anti-nociceptive activity of UDE was assessed using the acetic acid-induced writhing test in mice. UDE was able to diminish the reactive oxygen species (ROS) level in the lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage cells. UDE also suppressed production of nitric oxide and induction of inducible nitric oxide synthase and cyclooxygenase-2 in the stimulated macrophages cells. Collectively, the results imply that *U. davidiana* var. *japonica* has antioxidant and anti-nociceptive activities in addition to anti-inflammatory activity.

Keywords □ *Ulmus davidiana* var. *japonica*, anti-nociceptive, anti-inflammatory, antioxidant, nitric oxide, reactive oxygen species

INTRODUCTION

Ulmus davidiana Planchon var. *japonica* Nakai (Ulmaceae) is a deciduous broad-leaved tree widely distributed in Oriental countries, and its stem and root barks have been used as a traditional medicine for the treatment of edema, mastitis, cancer, inflammation, and rheumatoid arthritis for a long time (Lee, 1966). Until recently, several glycoproteins and terpenoids have been identified in the stem and root barks of *U. davidiana* var. *japonica*, and their pharmacological actions were documented. G-120, a 120 kDa glycoprotein isolated from the ethanol extract of *U. davidiana* var. *japonica*, exerted an important role in the induction of apoptosis, suppression of NF-κB activation, and induction of c-Jun/Fra-1 or c-Jun/Fra-2 dimerization in MCF-7 human breast caner cells, leading to the inhibited proliferation of the cancer cells (Lee *et al.*, 2005). It suppressed hydroxyl radical-mediated activation of NF-κB and AP-1 in mouse thymocytes (Lim *et al.*, 2002), and also inhibited the activation of matrix metalloproteinases, indicating that it would prevent excessive breakdown of extracellular matrix occurring in many pathological conditions (Son *et al.*, 2004). A glycoprotein of *U. davidiana* Nakai (UDN glycoprotein), as a potential modulator of apoptotic signal pathways, had inhibitory effects on protein kinase Cα translocation, NF-κB DNA binding activity, and apoptosis in 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-stimulated NIH3T3 cells (Lee *et al.*, 2004). UDN glycoprotein also inhibited glucose/glucose oxidase-induced apoptosis in BNL CL2, an embryonic murine hepatocyte cell line, by inhibiting activation of caspase-3 and poly(ADP-ribose) polymerase cleavage (Ko *et al.*, 2005). In this article, we demonstrate that *U. davidiana* var. *japonica* possesses antioxidant and anti-nociceptive activities in addition to its anti-inflammatory activity.

¢"These authors equally contributed to this work.
*Corresponding author
Tel: +82-2-710-9565, Fax: +82-2-3273-5847
E-mail: ehpark@sookmyung.ac.kr

MATERIALS AND METHODS

Chemicals

Carrageenan, indomethacin, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), L-ascorbic acid, *E. coli* lipopolysaccharide (LPS) and Griess reagent were purchased from Sigma Chemical Co. (St. Louis, MI, USA). 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) was obtained from

Invitrogen (Carlsbad, CA, USA). All other chemicals used were of reagent grade or better.

Experimental animals

The experiments were performed using male ICR mice (20-25 g) or male Sprague-Dawley rats (130-150 g), which were obtained from Samtaco Animal Farm, Osan, Korea. The animal room was maintained at 23±2°C with a 12-h light/dark cycle. Food and tap water were supplied *ad libitum*. The ethical guidelines described in the NIH Guide for Care and Use of Laboratory Animals were followed throughout the experiments.

Plant material

The stem and root barks of *U. davidiana* var. *japonica* were purchased at Kyungdong Folk Medicine Market, Seoul, Korea in March, 2005, and authenticated by Prof. Ki-Oug Yoo, Division of Life Sciences, Kangwon National University, Chuncheon, Korea. The voucher specimen of the plant material was deposited in the herbarium of the Division of Life Sciences, College of Natural Sciences, Kangwon National University under the acquisition number KWNU56515.

Preparation of methanol extract (UDE)

The barks were ground under liquid nitrogen and extracted for one month with 80% methanol at room temperature. The methanol extract (UDE) was evaporated *in vacuo* as described in the previous study (Park *et al.*, 2003). The yield was measured to be 11.6%.

Cell culture

The RAW264.7 cells, a murine macrophage cell line, were obtained from American Type Culture Collection (Manassas, VA, USA). The mammalian cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum (FBS), 25 mM HEPES (pH 7.5), 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were plated at a density of 1×10^6 and preincubated for 24 h at 37°C, and maintained in a humidified atmosphere containing 5% CO_2. For all experiments, the cells were grown to 80-90% confluence, and subjected to no more than 20 cell passages.

Assay for DPPH radical scavenging activity

DPPH scavenging activity of UDE was examined according to the method previously described (Song *et al.*, 2004). In brief, the reaction mixtures containing various concentrations of UDE and 100 μM DPPH solution in a 96-well microtiter plate were incubated at 37°C for 30 min, and absorbance was measured at 490 nm.

Carrageenan-induced rat paw edema

According to a modification of the method of Winter *et al.* (1962), acute inflammation was induced in the right hind paw of rats by subcutaneous injection of 0.1 ml/rat of 1% freshly prepared suspension of carrageenan in saline. UDE (100 or 200 mg/kg) or a positive control (indomethacin, 10 mg/kg) was administered orally 1 h before the carrageenan injection. The control group received only saline. The percent increase of paw volume in swelling % was calculated based on the volume of the pre-injection paw.

Acetic acid-induced writhing response

According to the procedure described by Koster *et al.* (1959), the response to an intraperitoneal injection of acetic acid solution, manifesting as a contraction of the abdominal muscles and stretching of hind limbs, was measured. Nociception was induced by intraperitoneal injection of 0.7% acetic acid solution at the dose of 0.1 ml/10g body weight. Each experimental group of mice was treated orally with vehicle (saline), UDE 100, 200 or 400 mg/kg) or phenylbutazone (PB, 100 mg/kg) as a positive control. From 10 min later, the number of writhes during the following 10 min period was counted.

Nitrite analysis

Accumulated nitrite (NO_2^-) in the exudates prepared from the carrageenan-induced air pouches and the culture media was determined using a colorimetric assay based on the Griess reaction (Sherman *et al.*, 1993).

Immunoblot analysis

The RAW264.7 cells were incubated with LPS (1 μg/ml) in the presence or absence of UDE for 24 h and then washed twice with ice-cold phosphate-buffered saline (PBS). The cells were lysed in a buffer containing 20 mM HEPES (pH 7.9), 0.1 M KCl, 0.3 M NaCl, 10 mM EDTA, 1% SDS, 1 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml pepstatin. For immunoblotting, anti-inducible nitric oxide synthase (anti-iNOS; Transduction Laboratories, Lexington, KY, USA) and anti-cyclooxygenase-2 (anti-COX-2; Transduction Laboratories, Lexington, KY, USA) and anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA) antibodies were used.

Determination of intracellular reactive oxygen species (ROS)

For analysis of intracellular ROS, the redox-sensitive fluorescent probe DCFH-DA was used, as previously described (Royall and Ischiropoulos, 1993). The RAW264.7 macrophage cells were pretreated with 0, 0.1, 0.5 or 1.0 mg/ml UDE for 24 h. Then, the cells were incubated with 5 μM DCFH-DA for 30 min at 37°C. The harvested cells were immediately analyzed by a flow cytometry.

Statistical analysis

The results were expressed as mean±S.E. Comparison between experimental groups was performed by ANOVA followed by the Tukey's multiple range tests. *P* values less than 0.05 were considered to be significant.

RESULTS AND DISCUSSION

Acute anti-inflammatory activity

In order to assess an anti-inflammatory activity of *U. davidiana*, carrageenan-induced acute paw edema and carrageenan-induced air pouch models have been used. UDE at the doses of 100 and 200 mg/kg, p.o., gave rise to an inhibition of 22.7% and 35.5% against acute paw edema induced by carrageenan at 3 h after its injection, respectively (Fig. 1). The treatment of indomethacin (10 mg/kg, p.o.) showed an inhibition on the edema formation by 53.6% at 3 h after the injection of carrageenan (Fig. 1). In the carrageenan-induced air pouch model, dexamethasone (0.01 mg/pouch) reduced the volumes of the exudates (data not shown). Treatment with UDE (0.5 or 1.0 mg/pouch) diminished the carrageenan-induced increase in the exudate volumes (data not shown). In brief, UDE is confirmed to possess an acute anti-inflammatory activity. Recently, it has been reported that the water extract of *U. davidiana* Planchon diminishes the progression of collagen-induced arthritis and inhibits the production of pro-inflammatory cytokines, such as TNF-α and IL-1β, in the paws of mice with collagen-induced arthritis (Kim *et al.*, 2005). On the contrary, the water extract was able to increase the concentrations of anti-inflammatory cytokines, such as IL-4 and IL-10, in the serum of mice with collagen-induced arthritis (Kim *et al.*, 2005).

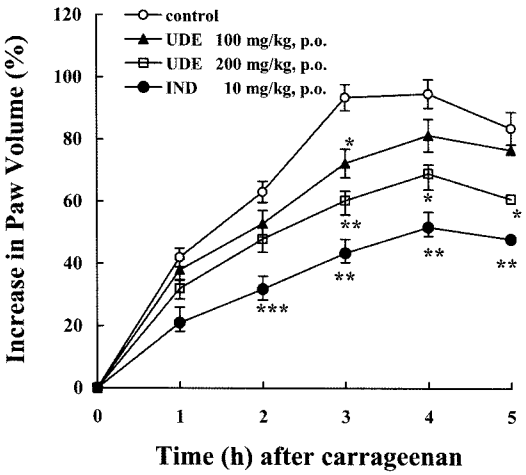

Fig. 1. Inhibitory effect of orally administered UDE on carrageenan-induced acute paw edema. Each point represents the mean±S.E. (n=7). UDE at the oral doses of 100 and 200 mg/kg body weight was given to rats 1 hour prior to carrageenan administration. Indomethacin (IND, 10 mg/kg, p.o.), as a positive control, was used, whereas the control rats received the appropriate amounts of saline. *, $P< 0.05$; **, $P< 0.01$; ***, $P<0.001$.

Antioxidant activity

Antioxidant activities are assumed to be useful for the prevention of oxidative damage in aging and age-related disorders. Since ROS play an important role in the pathogenesis of inflammatory diseases, antioxidants may also be efficient therapeutic tool used as anti-inflammatory agents. The free radical scavenging activity of UDE was examined to bleach a stable radical DPPH, which provided information on the reactivity of UDE with free radicals. UDE was able to directly scavenge the stable DPPH radical up to a concentration of 1000 μg/ml (85.2 ±0.2%

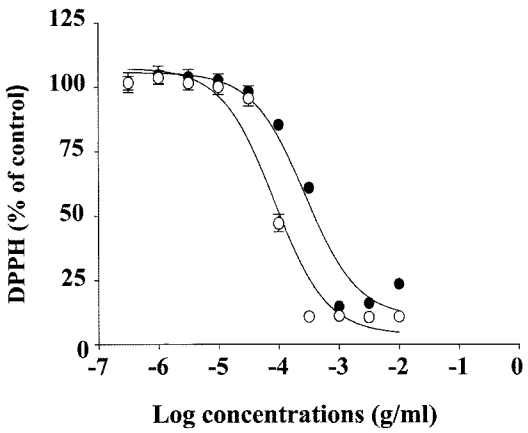

Fig. 2. The DPPH scavenging activity of the methanol extract (UDE, -●-) prepared from the stem and root barks of *U. davidiana* var. *japonica*. Vitamin C (-○-) was used as a positive control. DPPH concentration used was 100 μM. A representative of three independent experiments is shown. Each point represents the mean±S.E.

inhibition) in a concentration-dependent manner (Fig. 2). As shown in Fig. 2, the free radical scavenging activity of UDE is comparable to that of vitamin C. Pure active component(s) contained in UDE would exert stronger free radical scavenging activity than vitamin C. In addition to UDE's ability to scavenge the stable radical DPPH *in vitro* (Fig. 2), UDE was also able to decrease the ROS level in RAW264.7 macrophage cells (Fig. 3). This *in vivo* antioxidant activity could be related with the DPPH-scavenging activity of UDE. The potent antioxidant activity of UDE possibly supports the therapeutic effects of *U. davidiana* var. *japonica* against various disorders.

Anti-nociceptive activity

Since UDE was identified to have anti-inflammatory activity in carrageenan-induced rat paw edema (Fig. 1), its anti-nociceptive activity was examined using acetic acid-induced writhing response. In the acetic acid -induced writhing response which is the visceral pain model, the anti-nociceptive mechanism of abdominal writhing induced by acetic acid involves the process or release of arachidonic acid metabolites via cyclooxygenase, and prostaglandin biosynthesis (Franzotti *et al.*, 2000). As shown in Fig. 4, UDE at 100, 200 and 400 mg/kg, p.o., caused an inhibition by 69.4%, 78.8% and 90.7%, respectively, on the writhing response induced by acetic acid. This finding proposes that UDE also contains strong anti-nociceptive activity, subsequently suggesting that prostaglandin biosynthesis might be involved in the activities of UDE.

Inhibitory activity on NO production

Since iNOS-derived NO is involved in various pathological conditions such as inflammation and autoimmune diseases and leads to cellular injury (Singh *et al.*, 2000), suppression of iNOS is closely linked with anti-inflammatory action. Inhibitory effect of UDE was convinced on LPS-induced NO production in the RAW264.7 macrophages. When the macrophage cells were treated with 0.1, 0.5 and 1.0 mg/ml UDE, NO production induced by LPS was suppressed in a dose-dependent manner (Fig. 5A). UDE dose-dependently suppressed iNOS induction without changes in the levels of β-actin, an internal control, indicating the specific inhibition of iNOS expression by UDE (Fig. 5B). No significant cytotoxic effects on the macrophages were observed at the used concentrations of UDE, which was determined by MTT assay (data not shown). Suppressive effect of UDE on the production of NO was confirmed in the *in vivo* experiment. UDE also gave rise to a marked decrease on the content of nitrite in the exudates obtained from the carrageenan-induced air-pouch model (data not shown), which corresponded with *in vitro* results obtained with using the macrophages. Various natural products of plant and marine origin contain their anti-inflammatory activities through suppression of inducible cyclooxygenase-2 (COX-2) (Jachak, 2006). In a dose-dependent manner, UDE was also able to suppress COX-2 expression in LPS-stimulated RAW264.7 macrophage cells (Fig. 5B).

In conclusion, the methanol extract (UDE) of *Ulmus davidiana* var. *japonica* contains acute anti-inflammatory and anti-nociceptive activities. UDE also possesses potent antioxidant activity and inhibitory activity on *in vitro* and *in vivo* NO production. It is capable of diminishing expression

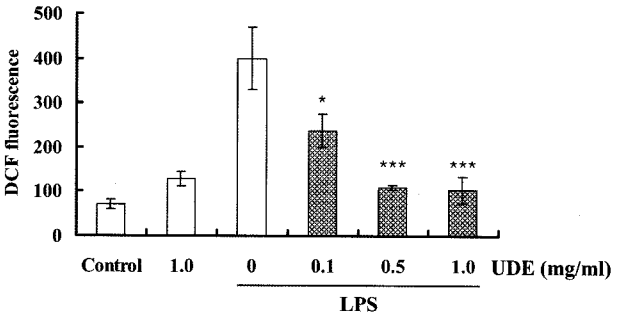

Fig. 3. Effect of UDE on the ROS level of RAW264.7 macrophage cells. The mammalian cells were treated with indicated concentrations of UDE for 24 h. ROS levels were determined by FACS analysis of the cells loaded with DCFH-DA and given as fluorescence units. **, $P<0.01$: ***, $P<0.001$

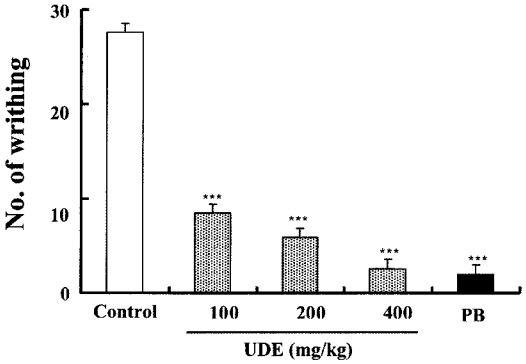

Fig. 4. Effect of UDE on the acetic acid-induced writhing response in mice. UDE (100, 200 or 400 mg/kg) was orally administered. Phenylbutazone (PB, 100 mg/kg body weight) was used as a positive control. Experiments were performed in triplicate. Each column represents mean±S.E. (n=8). ***, $P< 0.001$.

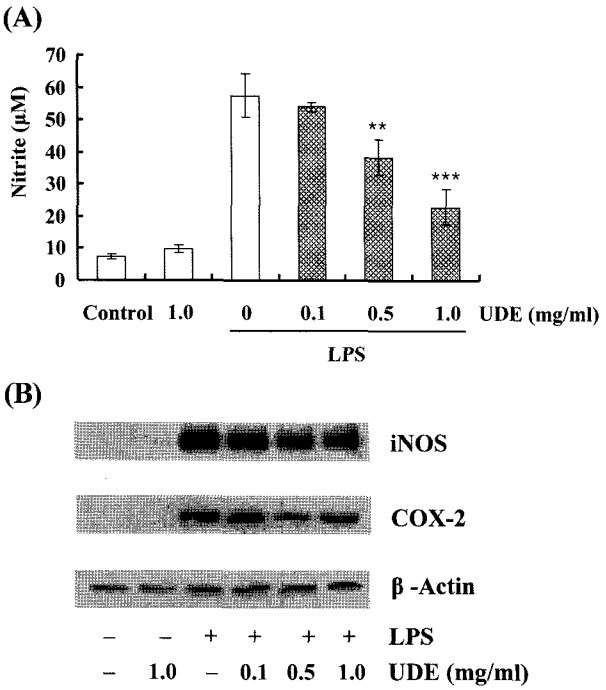

Fig. 5. Inhibitory effect of UDE on LPS-induced NO production (A) and expression (B) of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in RAW 264.7 macrophage cells. The values are mean ± S.E. of the three independent experiments. **, $P<0.01$; ***, $P<0.001$. In western blotting, the cell lysates (30 μg protein) were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane and blotted with anti-iNOS antibody or anti-COX-2 antibody. β-Actin was used an internal control. This blot is a representative of the three independent experiments.

of iNOS and COX-2 in the LPS-stimulated RAW264.7 cells. It decreased the ROS level in the same macrophage cells. These findings provide additional pharmacological information on the therapeutic efficacy of *Ulmus davidiana* var. *japonica*.

ACKNOWLEDGMENTS

This work was supported by the SRC program of MOST/KOSEF (R11-2005-017) to EHP.

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