BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 19(4), 425-430 (2011)

Forsythiae Fructus and Its Active Component, Arctigenin, Provide Neuroprotection by Inhibiting Neuroinflammation

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Abstract

In this study, we found that Forsythiae fructus (FF) and one of its main compounds, arctigenin, significantly inhibited nitric oxide production in lipopolysaccharide (LPS)-stimulated BV-2 microglial cells. Arctigenin also suppressed the expression of inducible nitric oxide synthase and cyclooxygenase-2, and inhibited the activation of extracellular signal-regulated kinase, c-Jun N-terminal kinase and p38. Moreover, it also reduced levels of proinflammatory cytokines, interleukin 1β, tumor necrosis factor α and prostaglandin E2, and inhibited neuronal death in LPS-treated organotypic hippocampal cultures. Therefore, we suggest that arctigenin may confer a neuroprotective effect *via* the inhibition of neuroinflammation.

Key Words: Forsythiae fructus, Arctigenin, Microglia, Neuroprotection, Neuroinflammation

INTRODUCTION

Microglia are the resident immune cells of the brain. Under normal conditions, they play important roles in the regulation of homeostasis and defense against injury (Perry and Gordon, 1988). However, in pathological conditions, microglia are over-activated and produce reactive proinflammatory mediators, including nitric oxide (NO), interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α) and prostaglandin E_2 (PGE_2) (Minghetti and Levi, 1998; Hanisch, 2002). These also play a deleterious role in neuronal death and neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease (Boje and Arora, 1992; Banati *et al.*, 1993; Gehrmann *et al.*, 1995). Therefore, much attention has been focused on materials that could effectively regulate the activation of microglia. Previously, we demonstrated that apigenin (Ha *et al.*, 2008), falcarindiol (Kim *et al.*, 2003a) and 15,16-dihydrotanshinone (Lee *et al.*, 2006) can act as inhibitors of LPS-induced overstimulation in microglia.

In Korea, Forsythiae fructus (FF; *Forsythia koreana* Nakai, Oleaceae) is a well-known traditional herbal medicine that has been used for the treatment of inflammation, as well as a diuretic and an antibacterial. Some phytochemical investigations of FF have previously been conducted. Several constituent substances of FF were reported, including lignans, phenylethanoid glycosides and flavonols (Guo *et al.*, 2007; Piao *et al.*, 2008).

Arctigenin is a phenylpropanoid dibenzylbutyrolactone lignan found in FF with anti-virus and anti-tumor pharmacological activities (Yang *et al.*, 2005; Awale *et al.*, 2006). Anti-inflammatory activities of FF and arctigenin have previously been reported, and FF inhibits allergic inflammatory reactions through the downregulation of mast cell activation (Kim *et al.*, 2003b; Choi *et al.*, 2007). Moreover, it inhibits NO production and inducible nitric oxide synthase (iNOS) expression in Raw264.7 cells (Kim *et al.*, 2000). Arctigenin also has significant anti-inflammatory and analgesic activities in animal models of acute inflammation (Kang *et al.*, 2008). It also inhibits B- and T-cell mediated allergic inflammation, as well as pro-inflammatory enzymes, such as cyclooxygenase-1 (COX-1) and COX-2, 5-lipoxygenase, phospholipase A2 and phosphodiesterase (Lee and Kim, 2010).

The purpose of this study was to investigate the inhibitory activities of FF and arctigenin on neuroinflammation and to determine the neuroprotective effects of arctigenin.

MATERIALS AND METHODS

Materials

FF was obtained from Omniherb (Daegu, Korea). Dulbecco's modified Eagle's medium (DMEM), minimum essential medium (MEM), fetal bovine serum (FBS), horse serum (HS), penicillin-streptomycin (PS), Hank's buffered salt solution

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Open Access **http://dx.doi.org/10.4062/biomolther.2011.19.4.425**

pISSN: 1976-9148 eISSN: 2005-4483

Received Jun 3, 2011 **Revised** Aug 9, 2011 **Accepted** Aug 9, 2011

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(HBSS) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Invitrogen (Carlsbad, CA, USA). Lipopolysaccharide (LPS) and arctigenin were obtained from Sigma Chemical Company (St. Louis, MO, USA).

Sample preparation and high performance liquid chromatographic analysis

FF (1 kg) were extracted with 85% ethanol in water using an ultrasonic apparatus and then concentrated under vacuum. In total, 110 g of ethanol extracts (FF-T) were isolated. FF-T was suspended in water and partitioned with ethylacetate. The aqueous layer was partitioned with *n*-butanol. The total yields of ethylacetate-soluble (FF-E) and *n*-butanol-soluble fraction (FF-B) from FF were 6.7% (67.3g) and 1.1% (11.0 g), respectively. The water-soluble fraction (FF-W) was 18.7 g. Arctigenin (Sigma-Aldrich Co. Ltd., St. Louis MO, USA) in FF-E was quantified by high performance liquid chromatographic analysis (HPLC; Agilent Technologies Inc., Santa Clara, CA, USA) under the following conditions: column, J'sphere ODS-H80 column (250×4.6 mm, 4 μm, YMC Co. Ltd., Ishikawa, Japan); detector wavelength, 280 nm; flow rate, 1.0 ml/min; and retention time, 17.94 minutes. The mobile phase consisted of (A) acetonitrile and (B) water, and each contained 0.02% of trifluoroacetic acid. The following gradient elution was used: 0 to 10 minutes, 90% B; 20 minutes, 70% B; and 30 minutes, 0% B. The arctigenin content in FF-E was determined using the linear regression equation from the calibration graph, and the arctigenin content in FF-E was 36.80 (± 0.07) mg/g (Fig. 1C).

Nitric oxide (NO) and cell viability assay

The BV-2 mouse microglial cell line was maintained in DMEM supplemented with 5% FBS and 1% PS. To measure NO production, cells were plated in a 96-well plate (3×10^4 cells/well). After 24 h, cells were pretreated with samples for 30 minutes and then stimulated with 100 ng/ml of LPS for another 24 h. LPS triggers an innate immune responses in microglia through Toll-like receptor 4 (TLR4), which participates in pathogen recognition (Miyake, 2004). Therefore, it has been widely used as an activator of microglia.

Nitrite, a soluble oxidation product of NO, was measured in the culture media using Griess reagents (1% sulfanilamide, 0.1% N-1-napthylethylenediamine dihydrochloride in 5% phosphoric acid). Cell viability was assessed by a 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT) assay.

Western blot analysis

We performed Western blot analysis to assess the effects of arctigenin on the protein expression of iNOS and COX-2 and the phosphorylation of ERK, JNK and p38. BV-2 cells were seeded in a six-well plate (2×10^6 cells/well). After 24 h, cells were pretreated with arctigenin for 30 minutes and then exposed to LPS (100 ng/ml) for 6 h (iNOS and COX-2) and 30 minutes (ERK, JNK and p38). Protein samples from cell extracts were separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk and then incubated with primary antibodies (Cell Signaling, Danver, MA, USA) and horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blots were developed using ECL Western Blotting Detection Reagents (Amersham Bioscience, Piscataway, NJ, USA).

Enzyme-linked immunosorbent assay

For determining the effects of arctigenin on proinflammatory cytokines, BV-2 cells were pretreated with arctigenin for 30 minutes, and then inflammation of cells was induced with LPS (100 ng/ml). After 24 h, the supernatant from the culture medium was used to determine the levels of IL-1β, TNF-α and PGE_2 as measured by enzyme immunoassay kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

Organotypic hippocampal culture

The experimental procedure was approved by the Animal Research Committee of the Kyung Hee University (2008-006). All efforts were made to minimize the number of animals used in this study and their suffering. We isolated the hippocampus of the brains of one-day-old Sprague-Dawley rats, after which the dorsal halves were sectioned into 350 μm-thick slices using a tissue chopper (Mickle Laboratory Engineering Co., Surrey, UK). The slices were placed on a membrane insert (0.4 μm, Millicell-CM, Milliopore, Bedford, MA, U.S.A) that was set into six-well plates filled with MEM medium containing 1% PS, 25% HS, 25% HBSS, 6 g/L D-glucose, 1 mM L-glutamine and 20 mM HEPES. After two weeks, the tissues were pretreated with arctigenin for 30 minutes and then exposed to 10 μg/ml of LPS for 72 h. We stained the tissues with propidium iodide (PI; Piao *et al.*, 2008) in order to assess neuronal cell death. PI fluorescent imaging analysis was performed using a fluorescence microscope (Carl Zeiss, Goettingen, Germany) at 514 nm.

Statistical analysis

All data are expressed as the mean (± SE) of at least three independent experiments. Statistical comparisons between the different treatments were performed using one-way ANOVA with Student's *t*-test. Results with *p*-values <0.05 were considered statistically significant.

RESULTS

FF and arctigenin inhibited NO production in BV-2 cells

We measured NO production after treatment with FF extract (FF-T), its subfractions (FF-E, FF-B and FF-W) and arctigenin in LPS-activated BV-2 cells. NO production was inhibited in cells treated with FF-T compared to cells treated with LPS alone without cell toxicity. FF-B had no effect on NO production, and FF-W did not influence NO levels in the culture medium. However, FF-W at 20 μg/ml was cytotoxic in BV-2 cells. Among the subfractions of FF extract, only FF-E significantly reduced the levels of secreted NO without cell toxicity (Fig. 1A). Moreover, concentrations of arctigenin ranging from 1 to 5 μM also inhibited NO production in a dose-dependent manner (Fig. 1B). To determine whether a large quantity of arctigenin may be contained in FF-E, the most active subfraction of FF-T, we measured the arctigenin content in FF-E by HPLC analysis. Arctigenin was detected as one of the most abundant compounds in FF-E (Fig. 1C).

Arctigenin suppressed the expression of iNOS and COX-2, the secretion of IL-1β, TNF-α and PGE_2, and the activation of MAPKs in BV-2 cells

Two important inflammatory mediators, NO and PGE_2, are

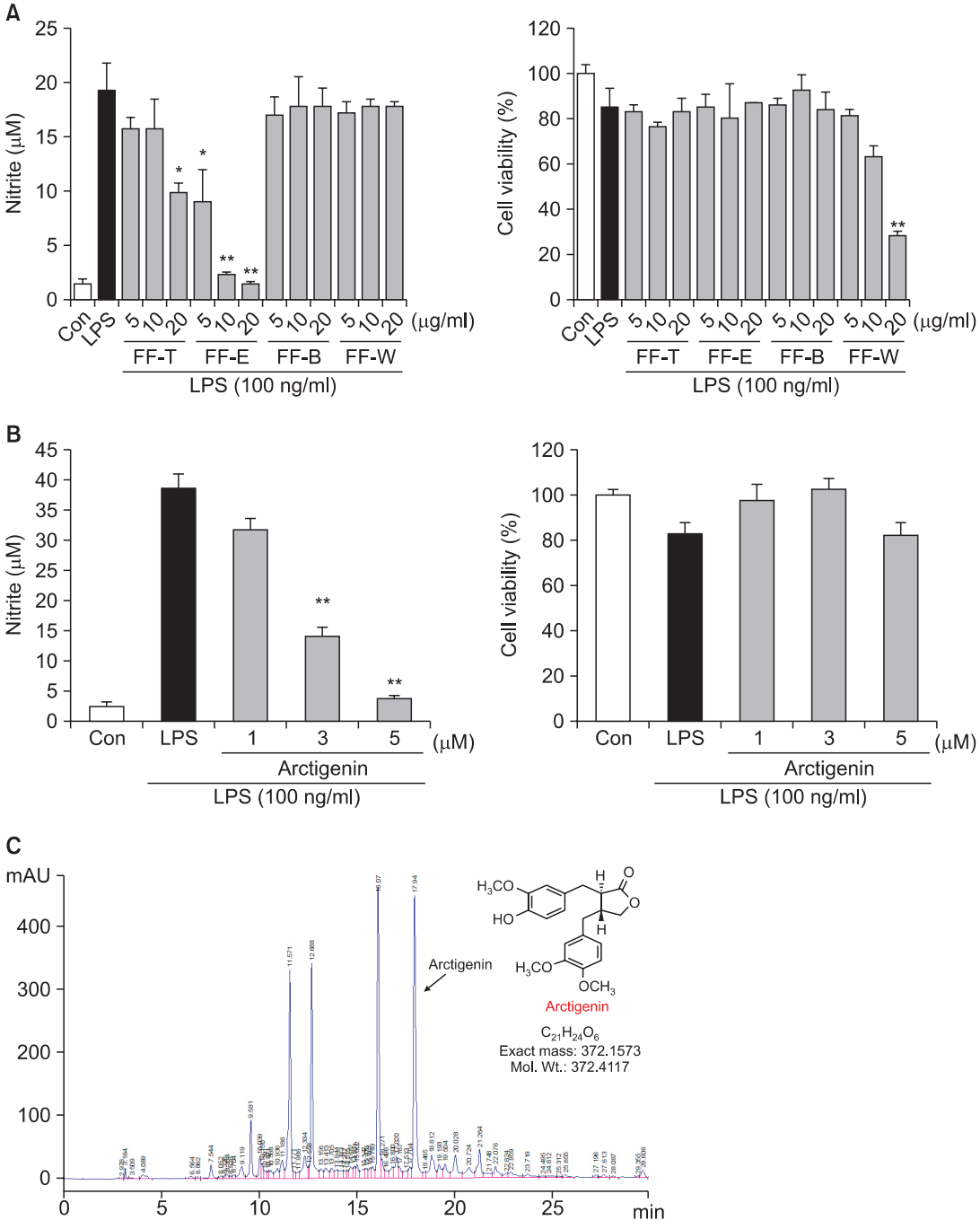

Fig. 1. The effects of FF and arctigenin on NO production in BV-2 cells. The effects of FF (A) and arctigenin (B) on NO production and cell viability. BV-2 cells were pretreated with FF extracts for 30 minutes and then stimulated with LPS (100 ng/ml) for 24 h. Nitrite was measured using a Griess reaction in culture medium. Cell viability was evaluated using an MTT assay. FF-T, total extract of FF; FF-E, ethylacetate-soluble fraction of FF-T: FF-B, *n*-butanol-soluble fraction of FF-T: and FF-W, water-soluble fraction of FF-T. All data are presented as the mean (± SE) of three independent experiments. **p*<0.05 and ***p*<0.001 indicate significant differences when compared to treatment with LPS alone. (C) The HPLC spectrum of arctigenin in FF-E.

produced by iNOS and COX-2, primarily in response to proinflammatory stimulation. We performed Western blot analysis to determine whether arctigenin had an effect on the expression levels of iNOS and COX-2. The pretreatment of cells with arctigenin led to a significant decrease in iNOS protein levels at 3 μM in LPS-activated BV-2 cells. Similarly, pretreatment with arctigenin inhibited the expression of COX-2 protein at 5 μM (Fig. 2A).

Mitogen-activated protein kinases (MAPKs) signaling regulates the expression of many genes encoding inflammatory mediators (Kaminska, 2005). Thus, we intended to determine the effect of arctigenin on the phosphorylation of three

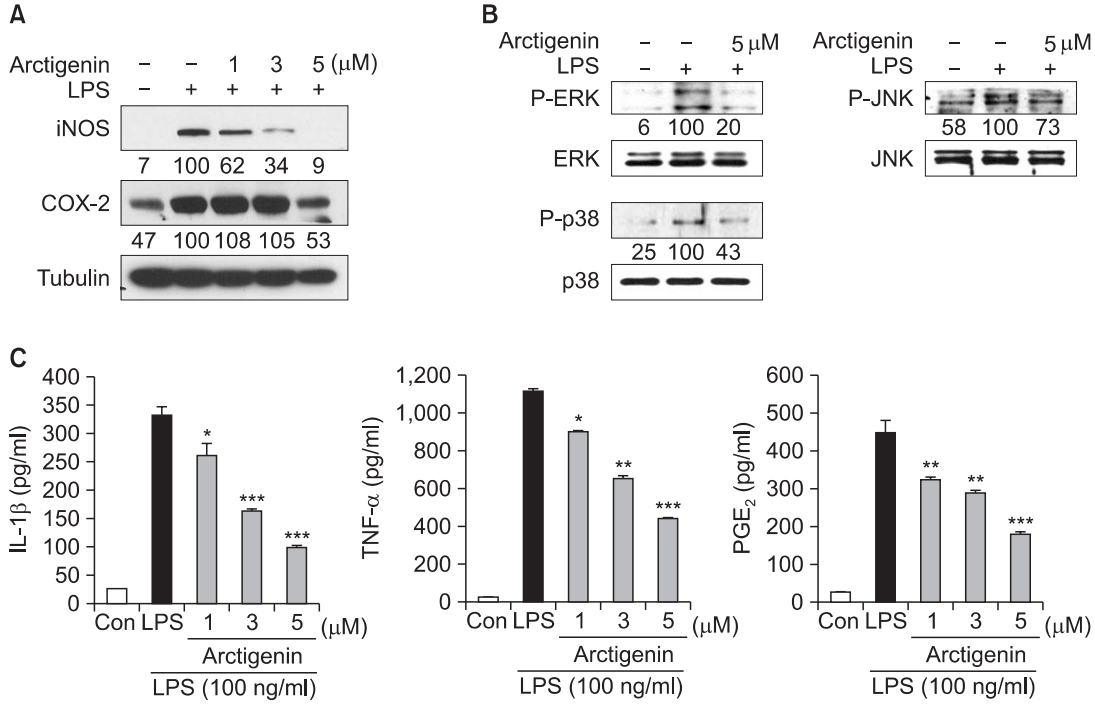

Fig. 2. The effects of arctigenin on the expression of iNOS and COX-2, the activation of MAPKs, and secretion levels of IL-1β, TNF-α and PGE_2 in BV2 cells. BV-2 cells were pretreated with samples for 30 minutes and then stimulated with LPS (100 ng/ml) for 6 h (iNOS and COX-2) and 30 minutes (MAPKs). The effects of arctigenin on LPS-induced iNOS and COX-2 expression (A) and MAPKs activation (B) were determined using Western blot analysis. Densitometry analysis of bands was performed as described in the methods section. The levels of secreted IL-1β, TNF-α and PGE_2 (C) were assessed using an enzyme immunoassay kit after treatment of BV-2 cells with LPS (100 ng/ml) for 24 h (IL-1β and TNF-α) and 6 h (PGE_2) in the presence or absence of arctigenin. All data are presented as the mean (±SE) of three independent experiments. **p*<0.05, ***p*<0.01 and ****p*<0.001 indicate significant differences as compared to treatment with LPS alone.

MAPKs: ERK, JNK and p38. Treatment with LPS stimulated the phosphorylation of p38, ERK and JNK. However, LPS-induced MAPKs activation was inhibited by 5 μM of arctigenin in BV-2 cells (Fig. 2B).

We also measured the levels of proinflammatory cytokines, such as IL-1β, TNF-α and PGE_2, after treatment with LPS alone and LPS plus arctigenin. The stimulation of microglia by LPS led to an increase in IL-1β, TNF-α and PGE_2 production. The levels of these cytokines were significantly decreased in a dose-dependent manner when pretreated with arctigenin (Fig. 2C).

Arctigenin reduced neuronal cell death in organotypic hippocampal cultures

We prepared rat hippocampal slice cultures, treated them with arctigenin after induction of neuronal damage by LPS, and measured the levels of cell death by detecting the level of PI uptake. Excessive PI uptake was only evident in the LPS-treated cultures. In contrast, arctigenin inhibited uptake at 0.1 and 0.5 μM in a dose-dependent manner (Fig. 3).

DISCUSSION

Neuroinflammation is associated with neuronal cell death and various neurodegenerative diseases. Our study showed that arctigenin inhibited neuroinflammation induced by over-activated microglia by suppressing NO production, expression of iNOS and COX-2, activation of MAPKs and secretion of pro-inflammatory cytokines. Moreover, arctigenin also significantly reduced neuronal cell death. Therefore, arctigenin may act as a neuroprotector by inhibiting neuroinflammation, and could be a candidate for treating neurodegenerative diseases.

In this study, we investigated the inhibitory activities of FF and arctigenin on neuroinflammation using the murine microglial cell line BV-2. A previous study showed that arctigenin reduced neuronal death and secondary inflammation and oxidative stress resulting from microglial activation in mice with Japanese encephalitis (Swarup *et al.*, 2008). These results cannot be directly compared to those of our study; however, the effects of arctigenin on inflammation in LPS-induced neuroinflammation animal models are analogous.

In order to determine the effect of FF and arctigenin on neuronal cell death, we used LPS in organotypic hippocampal culture. LPS is a potent activator of microglia and an inducer of neuroinflammation in many *in vitro* and *in vivo* experimental models. The organotypic hippocampal slice culture preserves the neuronal environment with the interaction between glial and neuronal cells. Thus, it can be used as a suitable experimental model that reflects *in vivo* conditions (Frotscher *et al.*, 1990; Frotscher *et al.*, 1995). Therefore, we think the organotypic hippocampal culture treated with LPS is adequate for estimating the anti-neuroinflammatory activity of arctigenin *in vivo*. In our study, lower concentrations of arctigenin reduced neuronal cell death in primary organotypic hippocampal cultures than were necessary in BV-2 cells. This could be be-

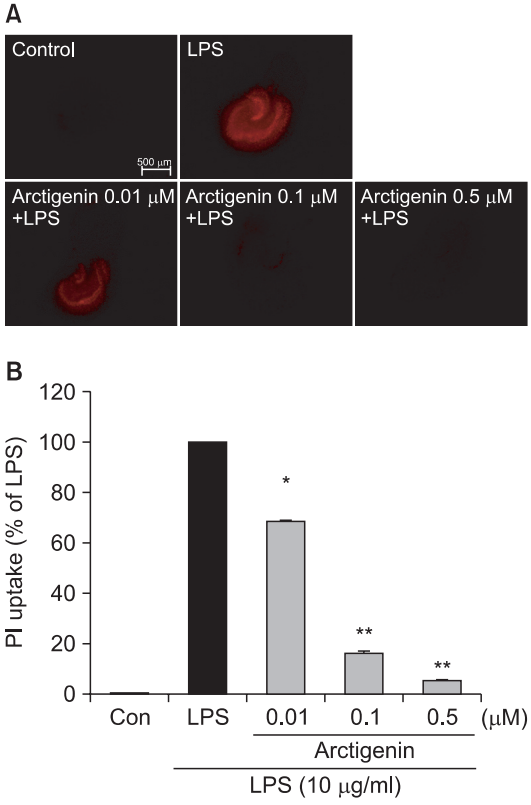

Fig. 3. The effect of arctigenin on neuronal cell death in organotypic hippocampal cultures. Hippocampal slices were pretreated with arctigenin for 30 minutes and stimulated with LPS (10 μg/ml). To investigate the effect of arctigenin on neuronal cell death, we performed PI staining. (A) Neuronal cell death was photographed by PI fluorescence imaging under a confocal microscope at 514 nm. A representative picture from one of the three different experiments is shown. Bar=500 μm. (B) Neuronal cell death was assessed by the PI uptake values. All data are presented as the mean (± SE) of three independent experiments. *p<0.01 and **p<0.001 indicate statistically significant differences as compared to treatment with LPS alone.

cause of the systematic difference between a primary cultured tissue and an immortalized cell line. Nevertheless, we suspect that arctigenin, as a neuroprotective agent, may be more potent in an *ex vivo* system than in an *in vitro* system. However, investigation in an *in vivo* system is necessary to confirm these results.

In this study, FF-T also inhibited NO production. Among the FF-T subfractions, FF-E was the only effective fraction. We deduced that a large amount of arctigenin may be contained in FF-E, and, as expected, arctigenin was found to be one of the most abundant compounds in FF-E on HPLC analysis. Therefore, arctigenin may play an important role in the suppression of NO production by FF treatment in over-activated microglia. However, the anti-inflammatory effects of other compounds isolated from FF have also been reported. Phylligenin and rengyolone had an anti-inflammatory effect in LPS-stimulated Raw264.7 cells (Kim *et al.*, 2006; Lim *et al.*, 2008). Pinoresinol suppresses inflammatory responses in LPS-activated microglia of rat cerebral cortices (Jung *et al.*, 2010). Therefore, it is possible that the inhibitory activities of FF on NO production may be a result of the synergy between many compounds in FF.

Because FF inhibits the binding of platelet activating factor to platelets (Iwakami *et al.*, 1992) and can cause extra bleeding, treatment with FF may need to be avoided during and after surgery. Moreover, arctigenin suppresses heat shock response in mammalian cells (Ishihara *et al.*, 2006). Therefore, we suggest that FF and arctigenin should be used carefully.

In conclusion, arctigenin, the active compound in FF, may reduce neuronal cell death by suppressing neuroinflammation in over-activated microglia. Ultimately, arctigenin could be used as a candidate treatment for neuronal damage and various neurodegenerative diseases.

ACKNOWLEDGMENTS

This research was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (iPET) (No.810006-03-1-SB120). We thank May Kim at Korea International School and Jinhwa Hong at Kyung Hee University for technical assistance.

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