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In Vitro Antioxidant and Anti-inflammatory Activities of Jaceosidin from *Artemisia princeps* Pampanini *cv.* Sajabal

Min-Jung Kim[1,2], Jong-Min Han[1], Yue-Yan Jin[1], Nam-In Baek[3], Myun-Ho Bang[3], Hae-Gon Chung[4], Myung-Sook Choi[5], Kyung-Tae Lee[6], Dai-Eun Sok[2], and Tae-Sook Jeong[1]

[1]National Research Laboratory of Lipid Metabolism & Atherosclerosis, KRIBB, Daejeon 305-806, Korea [2]College of Pharmacy, Chungnam National University, Daejeon 305-764, Korea, [3]Graduate School of Biotechnology & Plant Metabolism Research Center, Kyung Hee University, Suwon 449-701, Korea, [4]Gangwha Agricultural R&D Center, Incheon 417-830, Korea, [5]Department of Food Science & Nutrition, Kyungpook National University, Daegu 702-701, Korea, and [6]College of Pharmacy, Kyung Hee University, Seoul 130-701, Korea

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Oxidized low-density lipoprotein (oxLDL) plays a key role in the inflammatory processes of atherosclerosis. Jaceosidin isolated from the methanolic extracts of the aerial parts of *Artemisia princeps* Pampanini *cv.* Sajabal was tested for antioxidant and anti-inflammatory activities. Jaceosidin inhibited the Cu^{2+}-mediated LDL oxidation with IC_{50} values of 10.2 μM in the thiobarbituric acid-reactive substances (TBARS) assay as well as the macrophage-mediated LDL oxidation. The antioxidant activities of jaceosidin were exhibited in the conjugated diene production, relative electrophoretic mobility, and apoB-100 fragmentation on copper-mediated LDL oxidation. Jaceosidin also inhibited the generation of reactive oxygen species (ROS) concerning in regulation of NF-κB signaling. And jaceosidin inhibited nuclear factor-kappa B (NF-κB) activity, nitric oxide (NO) production, and suppressed expression of inducible nitric oxide synthase (iNOS) in lipopolysaccharide (LPS)-induced RAW264.7 macrophages.

Key words: *Artemisia princeps* Pampanini *cv.* Sajabal, Jaceosidin, Antioxidant, Anti-inflammation, Macrophages, Atherosclerosis

INTRODUCTION

Artemisia princeps Pampanini (Family Asteraceae) has been widely cultivated in Korea, Japan, and China, and the leaves of *A. princeps* Pamp. have long been used as tea, food, and traditional medicine in Korea. The recent studies have reported that extracts of *A. princeps* Pamp. possess anti-diabetic and antiallergic activities (Jung *et al*., 2007; Lee *et al*., 2006). Various flavonoids, sterols, and a lot of volatile chemicals were isolated from the aerial parts of *A*. *princeps* Pamp. (Bang *et al*., 2005, 2006; Umano *et al*., 2000). Sajabalssuk, a variant of *A. princeps* Pamp., is particularly cultivated in Ganghwa County, a place located in the west coast of Korea. It contains a high-content of flavonoids such as eupatilin and jaceosidin compared to the *Artemisia* herbs from other regions in east and south coast of Korea (Ryu *et al*., 2005).

Bioflavonoids from citrus fruit peel, naringin and hesperidin, are known to have antioxidant and anti-inflammatory activities and their anti-atherogenic effects in hypercholesterolemic rats and rabbits also are well known (Jeong *et al*., 2001; Lee *et al*., 2002; Jeon *et al*., 2007; Wilmsen *et al*., 2005). Four flavonoids, jaceosidin, eupatilin, eupafolin, and apigenin, were isolated from the aerial parts of *A. princeps* Pamp. *cv.* Sajabal (Bang *et al*., 2005). We investigated the inhibitory activities of four flavonoids on atherogenic targets, low-density lipoprotein (LDL)-oxidation, acyl-CoA: cholesterol acyltransferase (ACAT), lipoprotein-associated phospholipase A_2 (Lp-PLA_2), and cholesteryl ester transfer protein (CETP). Among them, jaceosidin exhibited significant LDL-antioxidant activity. LDL is susceptible to oxidative damage, and oxidized LDL (oxLDL) plays a key role in the development of atherosclerotic lesions (Steinberg, 1997). OxLDL in vessel wall is subjected to uptake rapidly by scavenger receptors on monocyte-derived macrophages, leading to the formation of foam

Correspondence to: Tae-Sook Jeong, National Research Laboratory of Lipid Metabolism & Atherosclerosis, Korea Research Institute of Bioscience and Biotechnology (KRIBB), 52 Eoeun, Yuseong, Daejeon 305-806, Korea
Tel: 82-42-860-4558, Fax: 82-42-861-2675
E-mail: tsjeong@kribb.re.kr

cells that accumulate cholesterol (Glass *et al*., 2001). LDL oxidation is associated with the formation of a number of highly reactive molecules which cause vascular inflammation (Steinberg, 2002). Experimental studies in animal models and humans have indicated that reactive oxygen species (ROS) mediate or enhance virtually every aspect of atherosclerotic lesion formation (Griendling and FitzGerald, 2003). Atherogenesis is accompanied by increasing lipid peroxidation in vivo. ROS promotes lipid peroxidation and oxidative damage to LDL. Several lines of investigation suggest that suppression of LPS-induced ROS results in diminution of NF-κB activity and subsequent inhibition of NF-κB responded gene expression (Park *et al*., 2005). NO derived from iNOS protein plays a significant role in regulating macrophage activation and proliferation *in vitro* through regulation of NF-κB activity (Zingarelli *et al*., 2002).

In this study, the antioxidant activities of jaceosidin were evaluated using several methods: Cu^{2+}-mediated and macrophage-mediated LDL oxidation in the thiobarbituric acid-reactive substances (TBARS) assay; measurement of the formation of conjugated diene; relative electrophoretic mobility (REM); fragmentation of ApoB-100 on LDL oxidation; and 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity. And we examined the effects of jaceosidin on LPS-mediated ROS, NO, iNOS, and NF-κB activation in murine RAW264.7 macrophages.

MATERIALS AND METHODS

Plant materials

The aerial parts of *A. princeps* Pamp. *cv.* Sajabal were provided from Ganghwa County Agricultural Technology Center, Incheon, Korea, which were harvested at Gangwha County in 2003 and stored for 2 years in the air. The *A. princeps* Pamp. *cv.* Sajabal was identified by Prof. Ryu S. N. (Department of Agricultural Science, Korea National Open University, Seoul 110-791, Korea).

Chemicals and reagents

Silica gel (63-200 mesh) for column chromatography was purchased from Merck Korea. Human monocyte THP-1 cells and murine RAW264.7 cells obtained from American Type Culture Collection (ATCC, Manassas, VA). The RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM), and antibiotics used in this study were purchased from Gibco Laboratories (Grand Island, NY). The fetal bovine serum (FBS) was obtained from Hyclone Laboratories (Logan, UT). All of the reagent grade chemicals were purchased from the Sigma-Aldrich Korea.

Extraction and isolation of flavonoids

The aerial parts of *A. princeps* Pamp. *cv.* Sajabal (4 kg) were ground and extracted two times with 80% methanol (15 L) for 24 h at room temperature. The combined methanolic extracts were concentrated *in vacuo*, and the resulting aqueous suspension was successively partitioned with EtOAc and n-BuOH. The EtOAc-extracted residue (47 g) was chromatographed on silica gel (ϕ4×20 cm) with a step gradient of *n*-hexane-EtOAc (7:1, 5:1, 3:1, 1:1, v/v) to give 20 fractions (SSE-1~SSE-20) at the first column. The SSE-16 fraction (1.53 g) was rechromatographed on silica gel (ϕ4×20 cm) with $CHCl_3$-MeOH solvent pairs (30:1, v/v) to obtain compound **1** (SSE-16-4, 127 mg) and compound **3** (SSE-16-8, 13 mg). Among the second column, SSE-16-6 (558 mg) was applied to ODS column (ϕ3×20 cm) eluting with MeOH-H_2O (2:1, v/v) to obtain compound **2** (57 mg). The other fraction, SSE-17 (409 mg) was purified by silica gel column (ϕ5×12 cm) eluting with $CHCl_3$-MeOH (3:1, v/v) to obtain compound **4** (42 mg). The compounds **1**-**4** were identified as eupatilin (**1**), jaceosidin (**2**), apigenin (**3**), and eupafolin (**4**) on the basis of the physicochemical properties and spectroscopic analysis (Bang *et al*., 2005) and by comparison with values reported in the literatures (Ryu *et al*., 1997; Aguinaldo *et al*., 2003; Miyazawa *et al*., 2003; Jin *et al*., 2005).

In vitro assay of ACAT, Lp-PLA_2, and CETP

ACAT and Lp-PLA_2 inhibitory activities of four flavonoids were examined as described (Kim *et al*., 2005). CETP inhibitory activity of four flavonoids was investigated as described (Kim *et al*., 1996).

In vitro antioxidant assay

Blood was collected from normalipidaemic volunteers with permission according to the 'Guidelines of Blood Donation Program for a Research' of the Korea Red Cross Blood Center and LDL was isolated from the plasma by preparative ultracentrifugation as described (Lee *et al*., 2005).

The LDL oxidation was determined spectrophotometrically by measuring the amount of TBARS (Yagi, 1982). A LDL solution (120 μg/mL) in phosphate buffered saline (PBS) was supplemented with 5 μM $CuSO_4$ as an oxidation initiator. The oxidation was performed in a screw-capped 5 mL glass vial at 37°C in the presence or absence of test compounds. After 4 h incubation, the reaction was terminated by addition of 1 mL of 20% trichloroacetic acid. Following precipitation, 1 mL of 0.67% TBA in 0.05 N NaOH was added and vortexed, and the final mixture was heated for 5 min at 95°C, cooled on ice, and centrifuged for 2 min at 1,000 × *g*. The optical density of the produced malondialdehyde was measured at 532 nm. Calibration was done with a standard curve of malonaldehyde bis (dimethyl acetal) (MDA) equivalents generated by acid-catalyzed hydrolysis of 1,1,3,3-tetramethoxypropane.

The formation of conjugated dienes was measured by

monitoring of the absorbance at 234 nm (Esterbauer *et al*., 1989) using an UV-visible spectrophotometer (Hewlett Packard model 8453, Agilent Technologies, Germany). Three mL of an LDL solution (120 μg/mL) in PBS was incubated with 5 μM $CuSO_4$ at 37°C in the presence or absence of jaceosidin, thereafter the absorbance at 234 nm was measured every 10 min. The plot of absorbance against time produces three phases: (a) a lag phage, (b) a propagation phase, and (C) a decomposition phase. The lag time (the extent to which the compounds protected LDL from oxidation was reflected by prolongation of the lag phase compared to that of control) was measured as the intercept between the baseline and the tangent of the absorbance curve during the propagation phase.

The REM of native or oxidized LDL was detected by agarose gel electrophoresis (Reid and Mitchinson, 1993). After LDL oxidation, samples were loaded onto 0.7% agarose gel and electrophoresed in TAE buffer [40 mM Tris, 40 mM acetic acid, and 1 mM ethylenediaminetetraacetic acid (EDTA)]. After electrophoresis, lipoprotein bands were stained with Coomassie Brilliant Blue R250, and REM was defined as ratio of the distances migrated from the origin by ox-LDL *vs* native LDL.

After the oxidation with or without jaceosidin, samples were denatured with 3% SDS, 10% glycerol, and 2-mercaptoethanol at 95°C for 10 min. SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 4%) was performed to detect the apoB-100 fragmentation (Miura *et al*., 1994). The electrophoresis was processed at 100 V for 80 min. After the electrophoresis, the gel was dried and stained with Coomassie Brilliant Blue R250. Density of each spot was measured at 550 nm by a Shimadzu Dual-wavelength flying-spot scanner CS-9000.

Radical DPPH scavenging activity

The radical DPPH scavenging activity was measured according to the procedure described (Bursa and Oleszek, 2001). In brief, freshly made radical DPPH solution (2 mL, final conc. 100 μM) was added to 1 mL of jaceosidin (final conc. 100 μM) in methanol. The absorbance of DPPH radical remaining was measured at 517 nm against a blank of pure methanol including only DPPH radical for 35 min using the UV-visible spectrophotometer at room temperature. The radical DPPH scavenging capacity was calculated from the difference in the absorbance with tested compounds and expressed as percent DPPH radical remaining.

Human macrophage-mediated LDL Oxidation

Human monocyte THP-1 cells were cultured in RPMI 1640 medium with phenol red containing 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% FBS at 37°C under 5% CO_2 in air. THP-1 monocytes were incubated at 1×10^6 cells/well in PMA (150 ng/mL) for 4 day to induce differentiation into macrophages. THP-1 macrophages were replaced with phenol red-free and serum-free RPMI 1640 media. To examine the effect of jaceosidin on macrophage-mediated LDL oxidation, cells were incubated with 100 μg/mL of LDL in the serum-free culture medium with or without jaceosidin, supplemented with 2 μM $CuSO_4$ for 24 h at 37°C. The extent of LDL oxidation was determined directly in the harvested medium using the TBARS assay (Lesnik *et al*., 1997).

Culture of RAW264.7 cells

RAW264.7 cells were cultured in DMEM containing 2 mM L-glutamine, 100 U/mL of penicillin, 100 μg/mL of streptomiycin, and 10% FBS at 37°C under 5% CO_2 in air.

Measurement of intracellular ROS generation

To investigate the effect of jaceosidin on LPS-induced ROS generation in RAW264.7 cells, a fluorometric assay using 2',7'-dichlorofluorescein diacetate ($DCFH_2$-DA, Molecular Probes) as a probe was used for the presence of hydroxyl radical. The intracellular production of ROS was measured as described (Lesnik *et al*., 1997) with modification. ROS have been implicated in the pathophysiology of many vascular disorders. Confluent RAW264.7 cells (1×10^4 cells/well) in 96-well plates were treated or not with various concentrations of jaceosidin for 2 h, followed by incubation with 1 μg/mL of LPS for 16 h. After the removal of media from wells, the cells were incubated with 10 μM $DCFH_2$-DA for 1 h. The fluorescence was measured on a spectrofluorometer (Wallac 1420, Perkin-Elmer, Turku, Finland) at 485 nm excitation and 530 nm emission wavelength.

Measurement of NO production and iNOS expression

Nitrite accumulation, as an indicator of NO production, was measured in culture medium (Eigler *et al*., 1995). RAW264.7 cells were plated at 1×10^6 cells/mL and treated or not various concentrations of jaceosidin for 2 h, followed by incubation with 1 μg/mL of LPS for 16 h. The isolated supernatants were mixed with an equal volume of Griess reagent [1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine in 2% phosphoric acid] and incubated for 10 min at room temperature. Sodium nitrite was used to generate a standard curve, and the nitrite concentration was determined by measuring the optical density at 540 nm with a Microplate reader (Bio-Rad, Hercules, CA).

The expression of iNOS in the cell lysates was measured as described (Yadav *et al*., 2003) by Western blot analysis. RAW264.7 cells were treated or not with jaceosidin for 2 h, followed by incubation with 1 μg/mL of LPS for 16 h. The cells were washed twice in ice-cold PBS. The cell lysates were prepared by suspending 1×10^6 cells in 100

μL of lysis buffer [140 mM NaCl, 15 mM ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 0.1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 100 mM PMSF, and 20 mM leupeptin, pH 7.5], disrupted by sonication, and extracted for 30 min at 4°C. The cytoplasmic extracts were centrifuged at 15,000 g for 10 min. Samples of equal amounts of proteins were separated through 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred to an Immobilon-P membrane (Millipore Co., Bedford, MA). The membrane was incubated for 2 h at room temperature with iNOS antibody (BD Transduction Laboratories, Lexington, KY). Immunoreactive proteins were detected with the ECL Western blotting kit (Amersham).

NF-κB activity assay

NF-κB activity was determined by using RAW264.7 cells stably transfected with a plasmid containing 8 copies of κB elements linked to secreted alkaline phosphatase (SEAP) gene (Lee *et al.*, 2002). The cells were kindly provided by Dr. Jung Joon Lee (KRIBB, Daejeon, Korea). NF-κB activity was performed as described (Xu *et al.*, 2006).

Data analysis

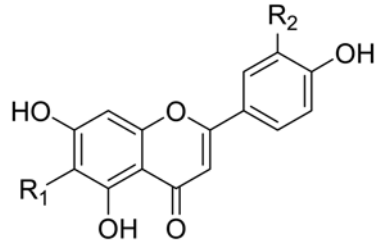
All values are expressed as mean ± standard deviations (S.D.) of two independent experiments performed in duplicate. Statistical analysis was done using Student's *t*-test. A value of $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

As described in the extraction and isolation procedures, phytochemical investigation on the aerial parts of *A. princeps* Pamp. *cv.* Sajabal led to the isolation of four flavonoids, jaceosidin, eupatilin, eupafolin, and apigenin (Fig. 1) (Bang *et al.*, 2005). Four flavonoids were investigated the inhibitory activities on atherogenic targets, LDL-oxidation, ACAT, Lp-PLA_2, and CETP. From the screening results, jaceosidin exhibited significant LDL-antioxidant activity (64.6 ± 0.4% inhibition at 5 μg/mL) and eupatilin has mild ACAT1 inhibitory activity (56.9 ± 7.6% inhibition at 100 μg/mL (Table I). And so, jaceosidin was first evaluated for its potential to inhibit LDL-oxidation using several methods. At first, jaceosidin showed potent Cu^{2+}-induced LDL-antioxidant activities with IC_{50} value of 10.2 μM in TBARS assay (Fig. 2).

Oxidation of LDL was assessed by the formation of conjugated dienes at 234 nm. The lag time of conjugated diene production, indicating of the resistance of LDL to oxidation, was prolonged when LDL was incubated with jaceosidin. As shown in Fig. 3, the LDL (120 μg/mL) was incubated with 5 μM $CuSO_4$ alone to have a lag time of 67 min. In the presence of 1, 3, and 5 μM jaceosidin, the lag time was extended to 75, 80, and 89 min, respectively. Thus, jaceosidin delayed LDL oxidation much greater than that of control.

To evaluate another parameter that is affected by LDL oxidation, jaceosidin was applied to REM assay. As shown in Fig. 4, The LDL was incubated with 5 μM $CuSO_4$ for 12 h



jaceosidin : $R_1 = OCH_3$, $R_2 = OCH_3$
eupatilin : $R_1 = OCH_3$, $R_2 = CH_3$
eupafolin : $R_1 = OCH_3$, $R_2 = OH$
apigenin : $R_1 = H$, $R_2 = H$

Fig. 1. Chemical structures of jaceosidin, eupatilin, eupafolin, and apigenin isolated from the aerial parts of *A. princeps* Pamp. *cv.* Sajabal

Table I. Inhibitory activities of jaceosidin, eupatilin, eupafolin, and apigenin on LDL- oxidation, ACAT, Lp-PLA_2, and CETP

Flavonoids	Inhibition (%)				
	LDL-oxidation[a] (5 μg/mL)	hACAT1[b] (100 μg/mL)	hACAT2[b] (100 μg/mL)	Lp-PLA_2[c] (100 μg/mL)	CETP[d] (50 μg/mL)
Jaceosidin	64.6 ± 0.4	20.1 ± 1.0	22.9 ± 8.6	10.3 ± 5.1	0
Eupatilin	25.8 ± 1.6	56.9 ± 7.6	34.8 ± 7.4	5.3 ± 1.5	0
Eupafolin	30.8 ± 4.2	0	0.8 ± 0.1	4.4 ± 0.3	9.2 ± 0.5
Apigenin	5.5 ± 1.2	11.6 ± 1.0	0	11.5 ± 1.1	20.5 ± 2.0

[a]The inhibitory activity of low-density lipoprotein (LDL) oxidation was measured in thiobarbituric acid-reactive substances (TBARS) assay. [b]In vitro acyl-CoA: cholesterol acyltransferase (ACAT) inhibitory activity was measured using expressed hACAT-1 and -2. [c]Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) inhibitory activity was measured using isolated human LDL (15 μg protein). [d]Cholesteryl ester transfer protein (CETP) inhibitory activity was measured using partially purified protein sources isolated from human plasma. Data are shown as mean ± S.D. from two independent experiments performed in duplicate.

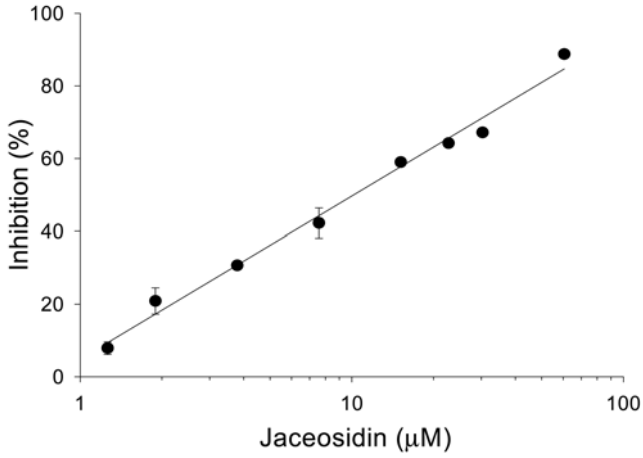


Fig. 2. Inhibitory activity of jaceosidin on Cu^{2+}-induced LDL-oxidation in TBARS assay. LDL (120 μg/mL) was incubated for 4 h at 37°C in PBS buffer (pH 7.4, 10 mM) with 5 μM Cu^{2+} in various concentrations of jaceosidin.

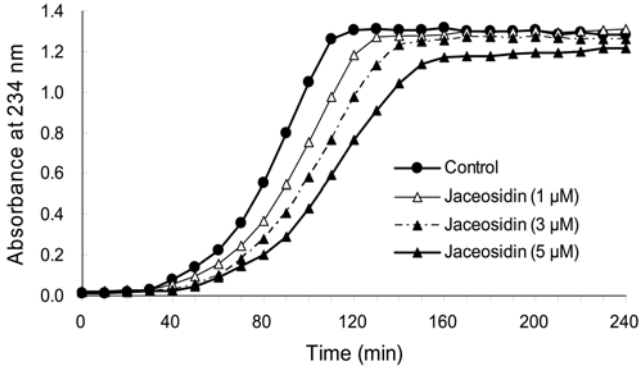


Fig. 3. Effect of jaceosidin on the generation of conjugated diene during the copper-mediated oxidation of LDL. Conjugated diene formation was measured by determining the absorbance at 234 nm every 10 min for 4 h. One of three representative experiments is shown.

to induce the oxLDL (lane 2). The REM of LDL in jaceosidin was reduced dose dependently. In the presence of each 40, 20, 10, and 5 μM jaceosidin, the LDL oxidation was protected in 81.0 ± 0.5, 31.0 ± 0.8, 7.1 ± 1.0, and 4.8 ± 1.6%, respectively, as compared to that of oxLDL.

It has been reported that products of lipid peroxidation such as malondialdehyde (MDA) could cause fragmentation of apoB-100, a major component of LDL (Tanaka *et al.*, 1999). The inhibition of the oxidative process by jaceosidin was evaluated by the densitometric area of band of apoB-100 through SDS-PAGE (Fig. 5). The band of apoB-100 was observed on native LDL (lane 2), which had been incubated without 5 μM $CuSO_4$ for 12 h at 37°C, but the band completely disappeared when the LDL was incubated with 5 μM $CuSO_4$ (lane 3). Jaceosidin at 10, 20, and 40 μM protected the fragmentation of apoB-100 in 11.5 ± 0.5, 58.3 ± 0.3, and 86.3 ± 0.7%, respectively.

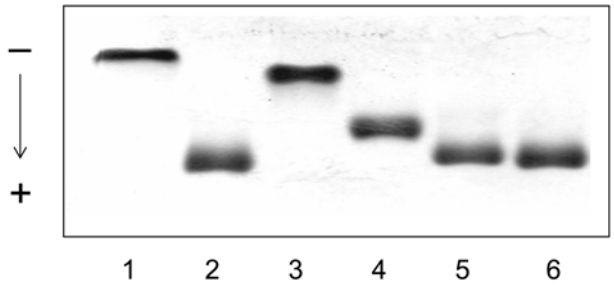


Fig. 4. Effect of jaceosidin on changes in REM of LDL. Lane 1, native LDL (absence of $CuSO_4$); lane 2, ox-LDL; lane 3, jaceosidin (40 μM); lane 4, jaceosidin (20 μM); lane 5, jaceosidin (10 μM); lane 6, jaceosidin (5 μM).

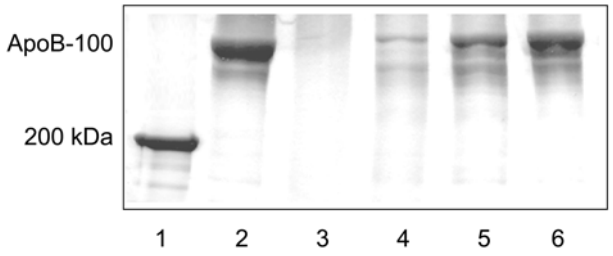


Fig. 5. Effect of jaceosidin on apoB-100 fragmentation in Cu^{2+}-mediated LDL oxidation. Lane 1, marker; Lane 2, native LDL (absence of $CuSO_4$); lane 3, ox-LDL; lane 4, jaceosidin (10 μM); lane 5, jaceosidin (20 μM); lane 6, jaceosidin (40 μM).

The antioxidant activity of jaceosidin in macrophage-mediated oxidation of LDL was examined (Table II). The cellular oxidative modification of LDL to a form recognized by the scavenger receptor requires the presence of transition metal ions in the medium. In the case of the LDL incubated in the presence of THP-1 macrophages without $CuSO_4$, the MDA-like product formation (32.3 ± 1.5 MDA nmol/mg LDL protein) was too low. The copper-induced LDL oxidation in the absence of the cells slightly increased

Table II. Effect of jaceosidin on macrophage-mediated LDL oxidation

Incubation conditions[a)]	MDA nmol/mg LDL protein[b)]
LDL + Cu^{2+}	56.4 ± 4.1
LDL + cell + Cu^{2+} (control)	277.1 ± 6.5
LDL + cell + Cu^{2+} + 20 μM jaceosidin	107.3 ± 0.1
LDL + cell + Cu^{2+} + 10 μM jaceosidin	204.0 ± 17.6*
LDL + cell + Cu^{2+} + 5 μM jaceosidin	270.0 ± 6.1*

[a)]LDL (100 μg/mL) was incubated for 24 h at 37°C in serum-free RPMI 1640 medium with 2 μM Cu^{2+} in the absence (control) or presence of each 5-20 μM jaceosidin.

[b)]The extent of LDL oxidation was directly determined in the harvested medium using the TBARS assay. Data are shown as mean ± S.D. from two independent experiments performed in duplicate. *$p<0.05$ *vs.* control.

the MDA-like product formation (56.4 ± 4.1 MDA nmol/mg LDL protein). Surprisingly, the copper-induced plus macrophage-mediated LDL oxidation (277.1 ± 6.5 MDA nmol/mg LDL protein) was 5-fold higher as compared with only the copper-induced LDL oxidation. Therefore, the antioxidant activity of jaceosidin was tested by macrophage-mediated LDL oxidation with 2 μM $CuSO_4$. The content of the MDA-like product in the presence of 20, 10, and 5 μM jaceosidin was reduced 69.3%, 29.9%, and 2.9%, respectively. These results indicated that jaceosidin has the significant antioxidant activities on macrophage-mediated LDL oxidation. Macrophages have essential functions in all phases of atherosclerosis, from development of the fatty streak to processes that ultimately contribute to plaque rupture and myocardial infarction. Although macrophages may not be required to initiate LDL oxidation, they are likely to amplify oxidative reactions in macrophage-rich areas of atherosclerotic lesions (Li and Glass, 2002). The antioxidant activity of jaceosidin on macrophage-mediated LDL oxidation provides a basis for *in vivo* anti-atherogenic efficacy test of jaceosidin.

The radical DPPH scavenging activity is an important parameter to determine antioxidant activity. The activity of jaceosidin was measured as decolorizing activity following the trapping of the unpaired electron of DPPH (Fig. 6). After 35 min, 82.0 ± 4.9% DPPH radicals remained at the presence of 100 μM jaceosidin. Therefore, jaceosidin showed mild radical scavenging capacity.

Since ROS plays an important role in atherosclerosis and other cardiovascular diseases, we determined whether jaceosidin inhibited ROS generation in LPS-stimulated RAW264.7 macrophages using $DCFH_2$-DA. Prior to our study on the anti-inflammatory effect of jaceosidin, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide bromide (MTT) assay was performed to determine the potential toxicity of jaceosidin to RAW264.7 macrophages. The jaceosidin at a concentration up to 40 μM had no effect on cell viability (data not shown). RAW264.7 cells were incubated with LPS for 16 h to increase ROS generation about 5.6-fold as compared to control (LPS absence). Pretreatment with various concentrations of jaceosidin for 2 h was significantly reduced LPS-induced ROS generation in a dose-dependent manner (Fig. 7).

We next examined the effect of jaceosidin on NO production and iNOS expression in activated macrophages. NO production was monitored in RAW264.7 cells stimulated by LPS in the presence or absence of jaceosidin for 16 h. LPS (1 μg/mL) increased the level of nitrite in culture medium by 6-fold as compared to control (LPS absence), and 40 μM jaceosidin inhibited 90.7% NO production (Fig. 8A). Western blot analysis confirmed that 40 μM jaceosidin markedly suppressed the expression of iNOS protein stimulated by LPS (Fig. 8B).

Transcription factors of the NF-κB proteins play an important role in the regulation of genes involved in pro-inflammatory and prothrombotic responses in human atherosclerosis (Monaco *et al.*, 2004a). Therefore, many authors addressed NF-κB as an important therapeutic target in atherosclerosis and thrombosis (Monaco *et al.*, 2004b). To investigate a molecular mechanism in the anti-inflammatory effect of jaceosidin, NF-κB transcriptional activity was monitored using RAW264.7 cells stably transfected with a plasmid containing 8 copies of κB elements linked to SEAP gene. The LPS-induced cells increased

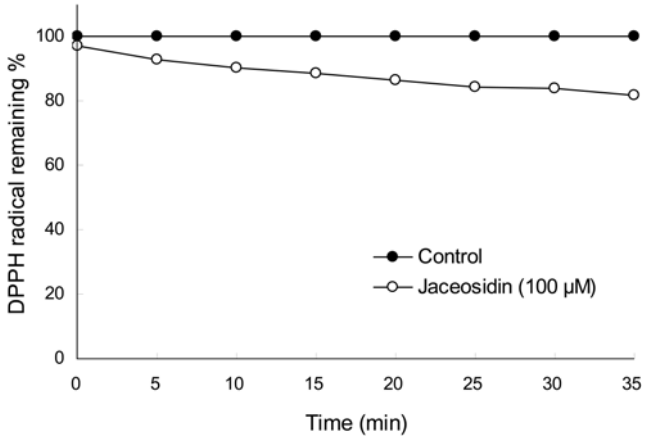


Fig. 6. Effect of jaceosidin on radical DPPH scavenging. Jaceosidin (100 μM) was incubated with 100 μM DPPH in methanol for 35 min at room temperature. The absorbance of jacoesidin solution was measured at 517 nm. The antiradical activity was expressed by the percentage of remaining DPPH.

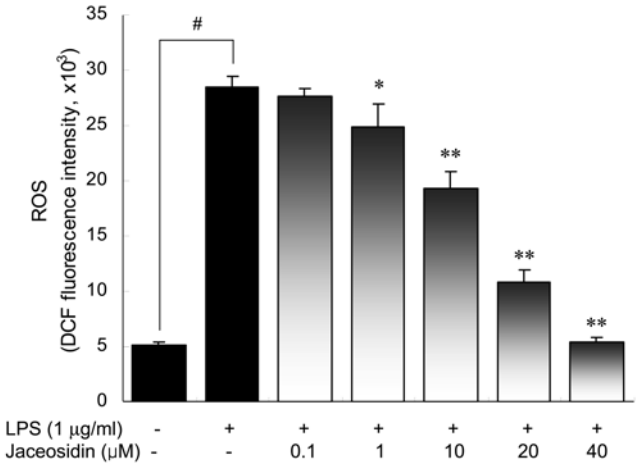


Fig. 7. Effect of jaceosidin on the intracellular ROS level in LPS-induced RAW264.7 macrophages. RAW264.7 cells were pretreated with 5-40 μM jaceosidin for 2 h, followed by incubation 1 μg/mL of LPS for 16 h. Cells were washed twice with PBS, and the intracellular levels of ROS were analyzed by VICTOR3 (485/538 nm). The data represent mean ± S.D. (n = 4). $^{\#}p<0.01$ *vs.* media-treated group. * $p<0.05$, $^{**}p<0.01$ *vs.* LPS-treated group.

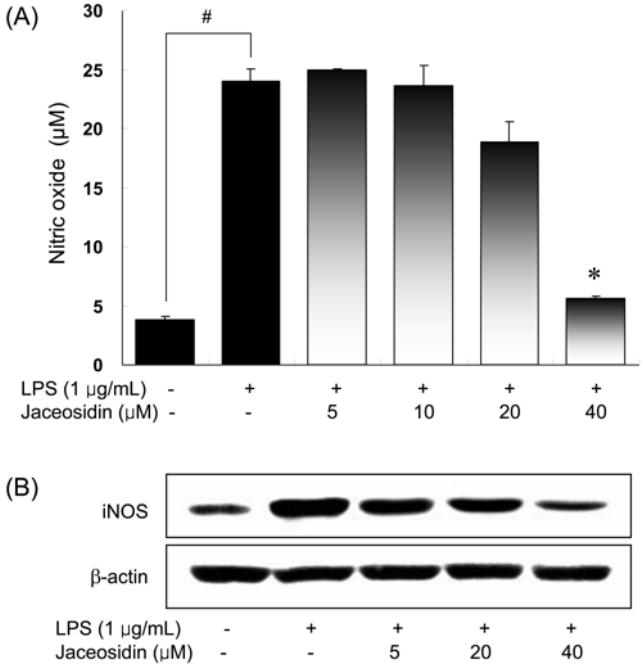


Fig. 8. Effect of jaceosidin on NO production and iNOS expression in LPS-induced RAW264.7 macrophages. RAW264.7 cells were pretreated with jaceosidin for 2 h, followed by incubation 1 μg/mL of LPS for 16 h. (**A**) Inhibitory effect of jaceosidin on LPS-inducible NO production. Nitrite accumulation, as an indicator of NO production, was measured in culture medium. (**B**) Inhibitory effect of jacoesidin on LPS-inducible iNOS protein expression. The data represent mean ± S.D. (n = 4). $^{\#}p$<0.01 *vs.* media-treated group. * p<0.05 *vs.* LPS-treated group.

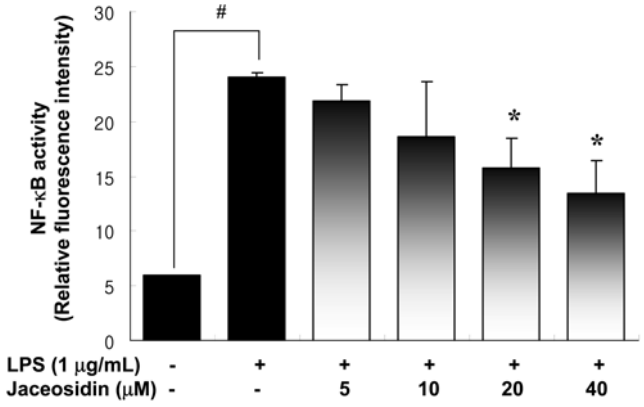


Fig. 9. Inhibition of NF-κB-mediated transcription of the reporter gene by jaceosidin. RAW264.7 cells transfected with a NF-κB reporter plasmid were pretreated with jaceosidin for 2 h, and then stimulated with LPS (1 μg/mL) for 24 h. NF-κB activity in the culture medium was measured using SEAP assay. $^{\#}p$<0.01 *vs.* media-treated group. *p<0.05 *vs.* LPS-treated group.

SEAP expression to about three-fold over the basal levels. As shown in Fig. 9, jaceosidin inhibited the SEAP expression in a dose-dependent manner. This is the first report demonstrating NF-κB inhibitory activity of jaceosidin in LPS-induced RAW264.7 macrophages.

In summary, jaceosidin, isolated from the methanolic extracts of the aerial parts of *A. princeps* Pamp. *cv.* Sajabal, showed potent antioxidant activities in the Cu^{2+}-mediated LDL oxidation in TBARS assay as well as the macrophage-mediated LDL oxidation. In addition, jaceosidin inhibited the conjugated diene formation, REM of oxLDL, and fragmentation of apoB-100 on Cu^{2+}-induced LDL. There has been known that NO and ROS are responsible for regulation on the transcriptional pathways of NF-κB. Jaceosidin inhibited ROS and NO production and iNOS expression in LPS-stimulated RAW264.7 cells. In addition, jaceosidin inhibited the LPS-induced NF-κB activation. These results are suggested that jaceosidin may constitute an additional antioxidant and anti-inflammatory agent for anti-atherosclerosis. Further studies remain to be elucidated how jaceosidin regulates the NF-κB activation. And the anti-atherogenic efficacy test of jaceosidin in high cholesterol diet-fed LDL receptor-deficient mice is underway.

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