Inhibitory Effects of *Curcuma xanthorrhiza* Supercritical Extract and Xanthorrhizol on LPS-Induced Inflammation in HGF-1 Cells and RANKL-Induced Osteoclastogenesis in RAW264.7 Cells

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Received: March 29, 2018

Revised: May 24, 2018

Accepted: May 25, 2018

First published online
June 6, 2018

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pISSN 1017-7825, eISSN 1738-8872

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Periodontal disease is triggered by the host immune response to pathogens in the microbial biofilm. Worsening of periodontal disease destroys the tooth-supporting tissues and alveolar bone. As oral inflammation can induce systemic diseases in humans, it is important to prevent periodontal disease. In this study, we demonstrated that *Curcuma xanthorrhiza* supercritical extract (CXS) and its active compound, xanthorrhizol (XAN), exhibit anti-inflammatory effects on lipopolysaccharide (LPS)-treated human gingival fibroblast-1 cells and anti-osteoclastic effects on receptor activator of nuclear factor kappa B ligand (RANKL)-treated RAW264.7 cells. LPS-upregulated inflammatory factors, such as nuclear factor kappa B p65 and interleukin-1 β , were prominently reduced by CXS and XAN. In addition, RANKL-induced osteoclastic factors, such as nuclear factor of activated T-cells c1, tartrate-resistant acid phosphatase, and cathepsin K, were decreased in the presence of CXS and XAN. CXS and XAN inhibited the mitogen-activated protein kinase (MAPK)/activator protein-1 (AP-1) signaling pathway. Collectively, these results provide evidence that CXS and XAN suppress LPS-induced inflammation and RANKL-induced osteoclastogenesis by suppressing the MAPK/AP-1 pathway.

Keywords: *Curcuma xanthorrhiza*, xanthorrhizol, gingival inflammation, osteoclastogenesis, periodontitis

Introduction

Periodontal disease is an inflammatory disease characterized by alveolar bone destruction and loss of tooth-supporting tissue and is due to plaque accumulation. Periodontopathogens, such as *Streptococcus mutans* and *Porphyromonas gingivalis*, are the major etiologic pathogens of periodontitis [1]. *S. mutans* forms a plaque biofilm that creates anaerobic conditions for microbes, such as *P. gingivalis*, to grow rapidly [2]. In particular, continuous secretion of lipopolysaccharide (LPS) by *P. gingivalis* initiates host immune responses in the periodontal pocket by binding to the Toll-like receptor (TLR) [3]. The interaction between LPS and TLR activates the nuclear factor kappa B (NF- κ B) or mitogen-activated protein kinase (MAPK)/activator protein-1 (AP-1) signaling pathway [4]. The NF- κ B and MAPK/AP-1 pathways

subsequently upregulate interleukin (IL)-1 β , a major cytokine, and matrix metalloproteinases (MMPs), which are protein degradation enzymes, consequently destroying the periodontal ligament [4, 5].

Additionally, the receptor activator of nuclear factor kappa-B (RANK) ligand (RANKL) is highly secreted from osteoblasts or gingival fibroblasts by inflammatory responses [6]. The major role of RANKL is to stimulate osteoclastogenesis or the process to differentiate pre-osteoclasts into mature osteoclasts or multinucleated cells to resorb alveolar bone. At the molecular level, the binding of RANK with RANKL stimulates osteoclastogenesis by activating MAPK [7]. MAPK increases the major osteoclast-specific factors, such as cathepsin K, nuclear factor of activated T-cell c1 (NFATc1), and tartrate-resistant acid phosphatase (TRAP), by stimulating the AP-1 complex [8, 9].

Fig. 1. Chemical structure of xanthorrhizol.

Curcuma xanthorrhiza Roxb., known as Java turmeric, is indigenous to Indonesia and possesses several biological properties, such as antibacterial, anticancer, and anti-diabetes activities [10]. *C. xanthorrhiza* and its active compound, xanthorrhizol (XAN, Fig. 1), have been presented to have anti-inflammatory activities in hydrogen peroxide-treated HT22 hippocampal cells [11] and LPS-treated RAW264.7 monocytes [12]. In addition, many previous studies have shown that XAN reduced the phosphorylation of MAPK in CT26-bearing mice [13], 7,12-dimethylbenz[a]anthracene-treated mouse skin [14], and the liver of cisplatin-treated mice [15]. Based on previous studies, we hypothesized that *C. xanthorrhiza* and XAN have anti-periodontitis effects. To confirm our hypothesis, we evaluated whether *C. xanthorrhiza* extract and XAN inhibited LPS-induced inflammatory responses in human gingival fibroblast-1 (HGF-1) and RANKL-induced osteoclastogenesis in RAW264.7 monocytes.

Materials and Methods

Preparation of Standardized *C. xanthorrhiza* Supercritical Extract

The rhizomes of *C. xanthorrhiza* were collected from Jakarta, Indonesia and a voucher specimen was deposited at Yonsei University (Korea). The dried rhizomes were extracted using a supercritical CO₂ fluid extraction system (SCFE-P400; Ilshin Autoclave Co., Ltd., Korea). The ground rhizomes were loaded into a high-pressure vessel, the oven was allowed to reach a temperature of 50°C, and then CO₂ was pressurized using a high-pressure pump and charged into the vessel at a rate of 3.2 l/min to maintain the pressure at 40 MPa. The *C. xanthorrhiza* supercritical extracts (CXS) was obtained at a yield of 8.0% (w/w). The standardized CXS contained approximately 30.0% (w/w) XAN as the bioactive compound [16].

Cell Culture

Both HGF-1 cells and RAW264.7 cells (American Type Culture Collection, USA) were cultured in Dulbecco's modified Eagle's medium (HyClone, USA) containing 10% fetal bovine serum (FBS;

HyClone, USA) and antibiotics (120 U/ml penicillin and 75 µg/ml streptomycin; Invitrogen, USA) in a 5% CO₂ atmosphere at 37°C. For differentiation into osteoclasts, RAW264.7 cells were maintained in alpha minimum essential Eagle's medium (Gibco, USA) containing 10% FBS and antibiotics (120 U/ml penicillin and 75 µg/ml streptomycin). The HGF-1 cells and RAW264.7 cells were stimulated in 1 µg/ml LPS (Sigma-Aldrich, USA) and 25 ng/ml RANKL (Peprotech Inc., USA), respectively. The HGF-1 cells were incubated with LPS in the presence or absence of CXS and XAN for 24 h. The RAW264.7 cells were incubated with the medium containing RANKL with or without CXS and XAN for 4 days to induce osteoclastogenesis.

Cell Viability

To evaluate the cell viability of CXS and XAN, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Sigma-Aldrich, USA) was used. HGF-1 cells and RAW264.7 cells were cultured in 24-well plates (2 × 10⁵ cells/well) with various concentrations of CXS and XAN for 24 h. Then, the medium was changed to serum-free medium containing MTT, followed by incubation for 4 h. After removing the medium, the formazan granules were dissolved in dimethyl sulfoxide. The absorbance of the solution was detected at 540 nm using a VERSAmax tunable microplate reader (Molecular Devices Inc., USA).

TRAP Staining and Activity

TRAP staining was performed using the Leukocyte Acid Phosphatase Kit (Sigma-Aldrich, USA) according to the manufacturer's protocol. The cells were stained with a TRAP solution consisting of tartrate, naphthol AS-Bi phosphate, sodium nitrite, acetate, and Fast Garnet GBC base solutions. Stained cells were analyzed using an Eclipse TE2000U inverted microscope with twin CCD cameras (magnification, ×100; Nikon, Japan). To quantify the activity of TRAP, the lysates were extracted from the cells using 0.1% Triton X-100 (Oriental Chemical Industries, Japan) and centrifuged at 18,000 ×g for 10 min at 4°C. The supernatant and TRAP solution were mixed at 1:1 ratio at 37°C for 1 h. The absorbance of the solution was determined at 540 nm using a VERSAmax tunable microplate reader.

Western Blotting

Lysate was extracted from the cells using NP-40 lysis buffer (Elpis Biotech, Korea) containing a protease inhibitor cocktail (Sigma-Aldrich, USA). The concentration of protein was quantified using a Bradford protein assay kit (Bio-Rad Laboratories Inc., USA), and the lysate was separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins transferred onto a nitrocellulose membrane (Whatman GmbH, Germany) were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at 25°C. After washing with TBST, the membranes were exposed to primary antibodies against MMP-2, MMP-8, NF-κB p65, IL-1β, TRAP, NFATc1,

cathepsin K, c-Fos, phospho (p)-c-Jun N-terminal kinase (p-JNK), p-extracellular signal-regulated kinase (p-ERK), p-p38 (1:1,000; Santa Cruz Biotechnology Inc., USA), ERK, JNK, p-c-Jun, c-Jun, p38, and α -tubulin (Cell Signaling, USA) overnight at 4°C. The membranes bound with primary antibodies were exposed to horseradish peroxidase-conjugated immunoglobulin G antibodies (Bethyl Laboratories, Inc., USA) at 4°C for 2 h. Proteins on the membrane were visualized using the enhanced chemiluminescence detection system (Amersham Biosciences, UK) and then detected using the G:BOX EF imaging system (Syngene, UK) and the GeneSys program.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated using Trizol reagent (Takara, Japan) following the manufacturer's instruction. The concentration of total RNA was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., USA). Equal amounts of RNA were mixed with reverse transcriptase premix (Elpis Biotech, USA) to synthesize the cDNA. Reverse transcription was performed as follows: initiation for 55 min at 42°C and termination for 15 min at 70°C. The cDNA was amplified with specific primers (Bioneer, Korea) (Table 1) using the GeneAmp PCR system 2700 (Applied Biosystems, USA) with the following procedure: denaturation for 30 sec at 94°C, annealing for 1 min, extension for 1 min at 72°C, and final extension for 5 min at 72°C. The PCR products were stained with Loading STAR (DynaBio, Korea) and electrophoresed

on a 1.5% agarose gel. The PCR product band was detected with the G:BOX EF imaging system and the GeneSys program.

Statistical Analysis

All assays were performed in triplicate and the mean $\pm$ standard deviation (SD) are presented in the graphs. The statistical analysis was carried out with the Statistical Package for the Social Sciences ver. 24.0 (SPSS Inc., USA). Differences between groups were assessed using a one-way analysis of variance, followed by the Scheffe's test. [#] $p < 0.05$, ^{##} $p < 0.01$, ^{*} $p < 0.05$, and ^{**} $p < 0.01$ were defined as a statistical significance.

Results

Effects of CXS and XAN on Cell Viability

The effects of CXS and XAN on cell viability were evaluated by MTT assay. HGF-1 cells were co-treated with LPS and CXS (1, 5, 10, and 20 μ g/ml) or LPS and XAN (1, 5, 10, and 20 μ M). Additionally, RAW264.7 cells were co-treated with LPS and CXS (1, 5, 10, and 20 μ g/ml) or LPS and XAN (1, 5, 10, and 20 μ M). No significant cytotoxicity at concentrations of up to 10 μ g/ml CXS and 10 μ M XAN was shown in HGF-1 cells (Figs. 2A and 2B). Similarly, there was no cytotoxicity at concentrations of up to 10 μ g/ml CXS and 10 μ M XAN (Figs. 2C and 2D). Therefore, CXS and

Table 1. Primer sequences used in the reverse transcription-polymerase chain reaction analysis.

Origin	Gene	Direction	Sequence (5'-3')
Mouse	TRAP	Forward	AAA TCA CTC TTT AAG ACC AG
		Reverse	TTA TTG AAT AGC AGT GAC AG
	Cathepsin K	Forward	ATC TCT CTG TAC CCT CTG CA
		Reverse	CCT CTC TTG GTG TCC ATA CA
	NFATc1	Forward	CCT GGA GAT CCC GTT GCT TC
		Reverse	TCC CGG TCA GTC TTT GCT TC
	c-Fos	Forward	CAG CTC AGT AAC AGT CCG CC
		Reverse	TCA CTA TTG GCA ACG AGC GG
	β -Actin	Forward	CAG CTC AGT AAC AGT CCG CC
		Reverse	TCA CTA TTG GCA ACG AGC GG
Human	IL-1 β	Forward	CCT GAG CTC GCC AGT GAA AT
		Reverse	GCA TCG TGC ACA TAA GCC TC
	NF- κ B p65	Forward	CAT CAA GAT CAA TGG CTA CAC AGG A
		Reverse	CTC TTC TTG GAA GGG GTT GTT GT
	MMP-2	Forward	CAT ACA AAG GGA TTC CCA GG
		Reverse	ATC GCT CCA GAC TTG GAA GG
	MMP-8	Forward	AGA AAG CCA GGA GGG GTA GA
		Reverse	TCC ACA GCG AGG CTT TTT CA
	GAPDH	Forward	CTC CTG TTC GAC AGT CAG CC
		Reverse	TCG CCC CAC TTG ATT TTG GA

Fig. 2. Effects of *Curcuma xanthorrhiza* supercritical extract (CXS) and xanthorrhizol (XAN) on cell viability.

Cell viability was measured by MTT assay. HGF-1 cells were co-treated with 1 µg/ml LPS and (A) CXS (1–20 µg/ml) or (B) XAN (1–20 µM) for 24 h. RAW264.7 cells were co-treated with 25 ng/ml RANKL and (A) CXS (1–20 µg/ml) or (B) XAN (1–20 µM) for 4 days. Data are expressed as the mean ± SD (% control) of three individual experiments. ** $p < 0.01$ vs. untreated control.

XAN were investigated in this study at concentrations less than 10 µg/ml and 10 µM, respectively, using HGF-1 cells and RAW264.7 cells.

CXS and XAN Inhibit Inflammation in LPS-Treated HGF-1 Cells

We treated HGF-1 cells with LPS for inducing inflammation in order to evaluate the inhibitory effects of CXS and XAN on gingival inflammation. LPS treatment increased the mRNA levels of gingival inflammatory factors, such as NF-κB p65, IL-1β, MMP-2, and MMP-8, whereas co-treatment with LPS and CXS (1 and 10 µg/ml) or LPS and XAN (1 and 10 µM) decreased the mRNA expression (Figs. 3A and 3B). In particular, the mRNA expression of IL-1β and MMP-2 was decreased by 84.58% and 69.92%, respectively, in the co-treatment with LPS and 10 µg/ml CXS group. The protein expression of NF-κB p65, IL-1β, MMP-2, and MMP-8 was reduced by co-treatment with LPS and CXS or LPS and XAN, compared with that of the

LPS-treated group (Figs. 3C and 3D).

CXS and XAN Inactivate the MAPK/AP-1 Signaling Pathway in LPS-Treated HGF-1 Cells

AP-1, which is activated by MAPK, is a key regulator for the activation of inflammatory gene expression [17]. Compared with the LPS-treated group, the 10 µg/ml CXS- and 10 µM XAN-treated groups showed decreased phosphorylation of ERK, JNK, and p38 (Figs. 4A and 4B). In addition, CXS and XAN treatments dose-dependently downregulated the protein expression of c-Fos and p-c-Jun (Figs. 4C and 4D).

CXS and XAN Inhibit Osteoclastogenesis in RANKL-Treated RAW264.7 Cells

To assess the anti-osteoclastic activities of CXS and XAN, we used RAW264.7 cells with RANKL as an osteoclastogenesis inducer [5]. RANKL treatment elevated both the protein and mRNA levels of the osteoclastic transcription factors

Fig. 3. Effects of *C. xanthorrhiza* supercritical extract (CXs) and xanthorrhizol (XAN) on inflammatory factors in LPS-treated HGF-1 cells.

(A) The mRNA levels of IL-1β, NF-κB p65, MMP-2, and MMP-8 following the CXS treatment were measured using RT-PCR, with GAPDH as the housekeeping gene. (B) The mRNA levels of IL-1β, NF-κB p65, MMP-2, and MMP-8 with the XAN treatment were measured using RT-PCR, with GAPDH as the housekeeping gene. The protein levels of IL-1β, NF-κB p65, MMP-2, and MMP-8 with (C) CXS and (D) XAN treatment were measured using western blotting, with α-tubulin as the housekeeping gene. The relative expression levels are expressed as the mean ± standard deviation (% control) of three independent experiments. #*p* < 0.05, ##*p* < 0.01 vs. untreated control; and **p* < 0.05, ***p* < 0.01 vs. LPS control.

Fig. 4. Effects of *C. xanthorrhiza* supercritical extract (CXS) and xanthorrhizol (XAN) on the MAPK signaling and AP-1 complex formation pathways in LPS-treated HGF-1 cells.

Protein activation of p38, JNK, and ERK following (A) CXS and (B) XAN treatment was measured using western blotting with specific antibodies. Effects of (C) CXS and (D) XAN treatment on protein expression of the AP-1 complex components (p-c-Jun, c-Jun, and c-Fos) were evaluated using western blotting, with α -tubulin as the housekeeping gene. The relative expression levels are expressed as the mean $\pm$ standard deviation (% control) of the three independent experiments. ^{##} $p < 0.01$ vs. untreated control; and ^{*} $p < 0.05$, ^{**} $p < 0.01$ vs. LPS control.

Fig. 5. Effects of *C. xanthorrhiza* supercritical extract (CXS) and xanthorrhizol (XAN) on osteoclastic expression in RANKL-treated RAW264.7 cells.

The mRNA levels of NFATc1, c-Fos, TRAP, and cathepsin K following (A) CXS and (B) XAN treatment were measured using RT-PCR, with β -actin as the housekeeping gene. The protein levels of NFATc1, c-Fos, TRAP, and cathepsin K following (C) CXS and (D) XAN treatment were measured using western blotting, with α -tubulin as the housekeeping gene. The relative expression levels are expressed as the mean $\pm$ standard deviation (% control) of three independent experiments. [#] $p < 0.05$, ^{##} $p < 0.01$ vs. untreated control; and ^{*} $p < 0.05$, ^{**} $p < 0.01$ vs. RANKL control.

and bone degradation enzymes, NFATc1, c-Fos, cathepsin K, and TRAP, whereas CXS and XAN treatments decreased the expression levels (Figs. 5A and 5B). Specifically, the transcripts of TRAP and c-Fos were decreased by 73.68% and 82.98%, respectively, in the 10 $\mu\text{g/ml}$ CXS-treated group compared with that in the RANKL-treated group (Fig. 5A).

CXS and XAN Inactivate the MAPK Signaling Pathway in RANKL-Treated RAW264.7 Cells

The MAPK signaling pathway, which is stimulated by RANKL, is involved in osteoclastogenesis or the process to

develop the maturation of osteoclasts [7]. Activated MAPKs bind to NFATc1 and upregulate the expression of osteoclastic factors. The protein levels of p-ERK, p-JNK, and p-p38 were highly expressed in the RANKL-stimulated RAW264.7 cells (Figs. 6A and 6B). However, CXS and XAN treatments reduced the phosphorylation of ERK, JNK, and p38 (Figs. 6A and 6B).

CXS and XAN Prevent the Osteoclastic Maturation of RANKL-Treated RAW264.7 Cells

To evaluate whether CXS and XAN inhibited osteoclast

Fig. 6. Effects of *C. xanthorrhiza* supercritical extract (CXS) and xanthorrhizol (XAN) on the MAPK signaling pathway in RANKL-treated RAW264.7 cells.

Protein activation of p38, JNK, and ERK following (A) CXS and (B) XAN treatment was measured using western blotting with specific antibodies. α -Tubulin was used as the housekeeping gene. The relative expression levels are expressed as the mean $\pm$ standard deviation (% control) of three independent experiments. [#] $p < 0.05$, ^{##} $p < 0.01$ vs. untreated control; and ^{*} $p < 0.05$, ^{**} $p < 0.01$ vs. RANKL control.

formation, we stained RANKL-stimulated RAW264.7 cells with TRAP solution and measured the activity of TRAP. The RANKL-treated group showed the formation of large and multinucleated osteoclasts, whereas the 10 µg/ml CXS- and 10 µM XAN-treated groups showed morphology similar to that of the normal group (Figs. 7A and 7B). Compared with the RANKL-treated group, 10 µg/ml CXS and 10 µM XAN treatments reduced the TRAP activity by 27.0% and 54.3%, respectively (Figs. 7C and 7D).

Discussion

Periodontal disease can be divided into two phases, gingivitis and periodontitis. Gingivitis is an acute inflammation induced by oral pathogens, and is classified as the initial stage of gingival inflammation [18]. Chronic inflammation causes periodontitis, which results in teeth loss via destruction of the gingival tissue and alveolar bone [19]. Since periodontal disease is correlated with systemic

Fig. 7. Effects of *C. xanthorrhiza* supercritical extract (CXS) and xanthorrhizol (XAN) on TRAP staining and TRAP activity in RANKL-stimulated RAW264.7 cells.

Morphological changes following (A) CXS and (B) XAN treatment were observed microscopically (magnification, $\times 100$). Blue arrow, multinucleated cells. Effects of (C) CXS or (D) XAN on TRAP activity were determined by measuring the absorbance at 540 nm wavelength. The relative TRAP activity is expressed as the mean $\pm$ standard deviation (% control) of three independent experiments. $^{##}p < 0.01$ vs. untreated control; and $^{**}p < 0.01$ vs. RANKL control.

diseases such as diabetes, cardiovascular diseases, and Alzheimer's disease [20–22], it is necessary to prevent periodontal disease in its initial stage. Therefore, the present study investigated a natural ingredient to prevent periodontal disease. Specifically, we provide evidence of the anti-periodontitis effects of CXS and XAN using LPS-stimulated HGF-1 cells and RANKL-stimulated RAW264.7 cells.

C. xanthorrhiza contains several phytochemicals, such as α -pinene, camphor, and curcumin [23]. The anti-inflammatory effect of α -pinene was revealed by demonstrating that α -pinene downregulated inducible nitric oxide synthase, which is the enzyme that generates a major inflammatory mediator in IL-1 β -treated primary chondrocytes [24]. Camphor reduces the phorbol-12-myristate-13-acetate/ionomycin-triggered secretion of the pro-inflammatory cytokines IL-6 and IL-8 in HGF-1, suggesting its anti-inflammatory property [25]. In addition, the anti-inflammatory effect of curcumin on LPS-treated HGF-1 cells was revealed by its inhibition of NF- κ B expression [26]. NF- κ B acts as a major transcriptional factor to upregulate pro-inflammatory cytokines once it translocates to the nucleus from the cytosol in gingival fibroblasts [27, 28]. XAN inhibited 12-O-tetradecanoylphorbol-13-acetate-triggered acute inflammation by blocking NF- κ B activity [14]. In this study, both CXS and XAN reduced the mRNA and protein expression of NF- κ B p65, consequently downregulating IL-1 β in LPS-treated HGF-1 cells (Fig. 3). XAN is the most abundant compound in *C. xanthorrhiza* [23], and the CXS used in this study contained 30% (w/w) of XAN. Thus, the anti-inflammatory effect of CXS in LPS-treated HGF-1 cells is attributable to XAN as well as its other compounds.

In periodontitis, MAPKs are the major proteins responsible for gingival inflammation and alveolar bone loss. MAPKs increase MMP-2, MMP-8, and IL-1 β in gingival tissue by activating the heterodimerization of c-Jun and c-Fos [4]. CXS and XAN reduced gingival inflammation by downregulating the MAPK pathway. (Figs. 3 and 4). Additionally, MAPKs also affect osteoclastogenesis, a process that stimulates bone resorption in alveolar bone. Activated MAPKs increase the expression of NFATc1, which stimulates TRAP and cathepsin K [8]. Since CXS and XAN downregulated LPS-induced IL-1 β and NF- κ B in HGF-1 by inactivating the MAPK signaling pathway, the current study evaluated whether CXS and XAN prevented RANKL from stimulating osteoclastogenesis. CXS and XAN inactivated MAPKs and downregulated TRAP and NFATc1 in RANKL-stimulated RAW264.7 cells (Figs. 5 and 6). In addition, the RANKL-

induced formation of multinucleated cells was suppressed by CXS and XAN (Fig. 7). Thus, the inactivation of MAPKs by CXS and XAN prevented osteoclast formation by downregulating TRAP and NFATc1.

Previously, it was reported that antioxidant and anti-inflammatory properties of XAN were exerted in vitro at concentrations of 1, 5, and 10 μ M [11]. Significant cytotoxicity was observed at concentrations of up to 10 μ M XAN in this study (Fig. 2). Therefore, in this research, XAN was used at concentrations of 1 and 10 μ M (low and high, respectively). Since the CXS contained approximately 30% XAN, which has a molecular mass of 218.34 g/mol, 1 and 10 μ M XAN correspond to 0.73 and 7.28 μ g/ml CXS, respectively. Thus, we used CXS at 1 and 10 μ g/ml (low and high, respectively) in this study. Inflammation-related factors such as NF- κ B and IL-1 β in LPS-treated HGF-1 cells, and osteoclastic factors such as cathepsin K and TRAP in RANKL-stimulated RAW264.7 cells were remarkably attenuated by CXS in a concentration-dependent manner (Figs. 3C and 5C). Similarly, XAN significantly and dose-dependently reduced NF- κ B p65 or a pivotal mediator of inflammatory cytokines in LPS-treated HGF-1 cells and osteoclastic factors in RANKL-stimulated RAW264.7 cells (Figs. 2B and 6D). In particular, 10 μ g/ml CXS and 10 μ M XAN reduced gingival inflammation and multinucleated cell formation to normal levels. Thus, other concentration between 1 and 10 μ g/ml CXS or 1 and 10 μ M XAN would have anti-inflammatory and anti-osteoclastogenic effects.

Homeostasis of alveolar bone is determined by a balance between osteoclasts and osteoblasts, the cells related to bone formation [29]. The inhibitory effects of CXS and XAN on osteoclastogenesis were demonstrated, and they prevented and delayed bone loss in periodontitis. However, the stimulatory effects of CXS and XAN on osteoblastogenesis or the process of bone formation have not been demonstrated yet. Thus, the osteoblastogenic effects and underlying molecular mechanisms of these compounds in biological systems, such as animal models, require further elucidation. It is anticipated that the demonstration of the anti-periodontitis effects of CXS and XAN in an animal model and clinical trials would support their industrial and practical application to human oral health as natural anti-periodontitis agents.

Acknowledgments

This research was supported by the Yonsei University Future-leading Research Initiative of 2016 (RMS2 2016-11-

1540) and partially by the World Class 300 Project R&D Program (S2435140) funded by the Small and Medium Business Administration (SMBA), Republic of Korea.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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