Inhibitory effects of Cynaropicrin from *Cynara scolymus* L. on *Porphyromonas gingivalis* LPS-induced production of inflammatory cytokines and RANKL-induced osteoclast differentiation

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Contents

This doctoral thesis was prepared using the original article "Cynaropicrin from *Cynara scolymus* L. suppresses *Porphyromonas gingivalis* LPS-induced production of inflammatory cytokines in human gingival fibroblasts and RANKL-induced osteoclast differentiation in RAW264.7 cells" (Journal of Natural Medicines, *in press*. https://doi.org/10.1007/s11418-018-1250-6.) with new unpublished data (Fig. 5).

Abstract

The negative impact of tooth loss from periodontal disease on quality of life is a growing global issue as the percentage of elderly individuals continues to increase. Lipopolysaccharide (LPS) produced by the periodontopathic bacterium *Porphyromonas gingivalis*, a gram-negative black-pigmented anaerobe induces the expression of inflammatory cytokines that promote inflammatory bone destruction. Mounting evidence supports that periodontal diseases are involved in the onset and progression of several systemic diseases, such as aspiration pneumonia and diabetes. Although treatment of periodontal diseases by removing the periodontopathic bacteria by brushing is a standard practice, it has limitations and is not effective in all cases. Therefore, a new method to replace or complement brushing is needed for the treatment of periodontal diseases. *Cynara scolymus* L., also known as artichoke, belongs to the family *Compositae* and grows to a height of approximately 2 m and blooms violet flowers, which are used as traditional medicine in Europe. This study investigated the anti-inflammatory effects of artichoke extract and its pharmacologically effective compound cynaropicrin, a sesquiterpene lactone, on human gingival fibroblasts (HGFs) stimulated by LPS and the potential anti-osteoclastogenic effects on RAW264.7 cells induced by receptor activator of NF-κB ligand (RANKL). Overall, the results suggest that cynaropicrin inhibited IL-8 and IL-6 mRNA and protein synthesis induced by LPS in HGFs without

affecting cell viability at pharmacologically effective doses. *P. gingivalis* LPS-induced degradation of IκBα and phosphorylation of NF-κB p65 were also suppressed by cynaropicrin, as was LPS-stimulated NF-κB transactivation. Thus, cynaropicrin's inhibition of *P. gingivalis* LPS-induced IL-8 and IL-6 expression may be due to the inhibition of the NF-κB pathway. Furthermore, cynaropicrin and artichoke extract behaved similarly with respect to the osteoclast differentiation induced by RANKL, although cynaropicrin appeared to be more effective than artichoke extract. These results suggest that cynaropicrin may be useful for preventing periodontal diseases and could prove valuable in the development of more effective preventative approaches for periodontal diseases.

Keywords: Cynaropicrin; Periodontal diseases; Inflammatory cytokine; Human gingival fibroblasts; Osteoclast differentiation

Introduction

Human periodontal diseases are inflammatory conditions that cause the destruction of the supporting tissues of teeth and are a major public health problem affecting more than half of the adult population worldwide [1]. Dental biofilms are considered to be the principal etiological factor in the onset and progression of periodontal diseases [1, 2]. A specific periodontopathic bacterium in dental biofilms is *Porphyromonas gingivalis*, a gram-negative black-pigmented anaerobe that colonizes periodontal pockets and spreads deeper into tissues such as connective and alveolar bone tissues [2]. It is well understood that lipopolysaccharide (LPS), a key component of the outer membrane of *P. gingivalis*, is a potent stimulator of inflammatory cytokines that eventually induce connective tissue breakdown, including alveolar bone resorption [3]. Both LPS and inflammatory cytokines promote osteoclastogenesis and enhance osteoclast-mediated bone resorption by promoting the expression of the receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) [3, 4].

Evidence so far supports that periodontal diseases are involved in the onset and progression of several systemic diseases such as aspiration pneumonia, heart disease, diabetes, and pre-term birth [1, 2]. Thus, prevention of periodontal diseases is important for maintaining not only oral cavity health but also that of the whole body. In fact, numerous studies have demonstrated that oral care is

effective against pneumonia and diabetes [2, 5-7]. Conventional activities, such as brushing and flossing, are used to remove dental plaque and prevent periodontal disease progression. Although these procedures are effective in managing the majority of periodontitis patients, there is a limit to the removal of dental plaque by mechanical cleaning alone and this does not always achieve the desired clinical outcome. In addition, use of adjunct antibiotics, including tetracycline and amoxicillin, is associated with a rise in resident bacterial strains. Since therapeutic options for periodontal diseases are limited, a new method to replace or complement brushing is needed for the treatment of periodontal diseases.

Recently, interest in using natural products from plants and vegetables has increased, as has the need to discover safe and efficient new drugs to treat inflammatory conditions. The anti-inflammatory effects of natural products such as curcumin, catechin, and resveratrol on inflammatory diseases, including periodontal diseases, have been reported in animal studies [8-10]. *Cynara scolymus* L., also known as artichoke, belongs to the family *Compositae* and grows to a height of about 2 m and blooms violet flowers, which are used as a traditional medicine in Europe. Its extract has inhibitory effects on liver and acute gastric mucosal injuries [11, 12]. In addition, artichoke contains bioactive compound cynaropicrin, sesquiterpene lactone, which has anti-inflammatory activity [13-15]. Thus, cynaropicrin was isolated from the leaves of artichoke using

preparative column chromatography as a potent candidate of preventive agent for periodontal diseases.

The present study investigated the anti-inflammatory effects of the artichoke extract and cynaropicrin on *P. gingivalis* LPS-induced expression of inflammatory cytokines in human gingival fibroblasts (HGFs) that are the major cells in periodontal connective tissues, provide a tissue framework for tooth anchorage, and function as inflammation regulators. In addition, the potential of artichoke extract and cynaropicrin to inhibit differentiation of RAW264.7 into osteoclast-like cells was determined.

Materials and methods

Cell culture

HGFs were maintained at 37 °C in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific, Rockford, IL, USA), penicillin (100 U/ml), and streptomycin (100 μg/ml) as described previously [16]. The Ethics Committee of Nihon University School of Dentistry at Matsudo approved the study (EC03-041, EC10-040, EC14-023), and a consent form was obtained from each patient after the entire the protocol had been explained. The mouse macrophage-like cell line RAW264.7 was purchased from Dainippon pharmaceutical (Osaka, Japan) and maintained in α-minimal essential medium (Gibco BRL, Rockville, MD, USA) supplemented with 10% FBS, and penicillin/streptomycin.

Reagents and Plasmid

An artichoke extract was prepared from the leaves of artichoke, *Cynara scolymus* L. The leaves were soaked in hot water (15 times the leaf weight) for 45 min at 95 °C and then filtered (pore size 0.45μm) and concentrated using an evaporator. The concentrate was then freeze-dried (FD) and adjusted with dextrin to have 1.5% (w/w) cynaropicrin content. The content of the final

artichoke extract is 60%, and dextrin is 40%. Cynaropicrin was purified from the leaves of artichoke. The leaves were soaked in 50% ethanol (10 times the leaf weight) for 7 days and subsequently filtered. The crude extract was purified using polyamide C-200 (Wako, Osaka, Japan) with methanol. Subsequently, it was purified it using preparative HPLC with Xbridge prep C18 with an acetonitrile gradient of 17%–100%. Finally, purified cynaropicrin (98.9% of purity) was obtained after washing with polyamide C-200 with hexane/ethyl acetate. HPLC analysis for cynaropicrin was carried out under the following conditions: column, Mightysil RP-18GP (5 μm, 250 mm× 4.6 mm, Kanto chemical Co., Inc., Tokyo, Japan); column temperature, 40 °C; flow rate, 1 ml/min; solvent, CH_3CN: 0.1% H_3PO_4 = 18:72; and detection, UV absorption at 210 nm. *P. gingivalis* LPS was obtained from InvivoGen (San Diego, CA, USA). Antibodies against IκBα and phospho-p65 (Ser536) (Cell Signaling Technology, Danvers, MA, USA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used. Horseradish peroxidase-conjugated secondary antibodies were obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK). BAY11-7082, an inhibitor of IκBα phosphorylation, was purchased from Sigma. A reporter plasmid expressing firefly luciferase under the control of NF-κB (pGL3-5xκB-luc) was used as described previously [17].

mRNA preparation and real-time polymerase chain reactions

To measure mRNA expression of various genes, HGFs (4×10^5 cells/ml) were cultured, washed once with phosphate-buffered saline, and homogenized with QIAshredder (QIAGEN, Hilden, Germany). Total RNA was purified using RNeasy (QIAGEN) according to the manufacturer's protocol. For cDNA synthesis, total RNA was reverse-transcribed using an RNA PCR kit (PrimeScript; Takara Bio, Shiga, Japan). The resulting cDNA mixture was subjected to real-time PCR analysis using SYBR Premix Ex *Taq* solution (Takara Bio) containing sense and antisense primers. The following primer sequences were used: IL-8 forward (5′-CTTGTCATTGCCAGCTGTGT-3′) and reverse (5′-TGACTGTGGAGTTTT GGCTG-3′); IL-6 forward (5′-TTCGGTCCAGTTGCCTTCTC-3′) and reverse (5′- GAGGTGAGTGGCTGTCTGTG-3′); and GAPDH forward (5′-TGCACCACCAACTGCTTAGC-3′) and reverse (5′-GGCATGGACTGTGGTCATGAG-3′). PCR assays were performed using a TP-800 Thermal Cycler Dice Real-Time System (Takara Bio) and analyzed using the software provided by the device manufacturer. Thermal cycling conditions were 40 cycles at 95 °C for 5 s and 60 °C for 30 s. All real-time PCR experiments were performed in triplicates; the specificity of each product was verified by melting curve analysis. The level of gene expression was normalized to that of GAPDH mRNA.

Measurement of cell viability

To assess the cytotoxicity of cynaropicrin and artichoke extracts, a WST-1 assay (Roche Diagnostics, Basel, Switzerland) was performed as described previously [18]. Briefly, HGFs were incubated with cynaropicrin or artichoke extract in a 96-well plate (1×10^5 cells/ml) for 24 h, and then incubated for a further 4 h in the presence of WST-1. The absorbance of the soluble formazan was measured at 450 nm.

Cytokine measurements

Cytokine concentrations were determined in HGF culture supernatants using cytokine-specific enzyme-linked immunosorbent assay (ELISA) kits for IL-8 and IL-6 (Quantikine ELISA kits; R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Three independent experiments with triplicates were performed for this measurement.

Western blotting

Experimental procedures for western blotting were performed as described previously [19, 20]. Briefly, equal amounts of protein (20 μg) were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane (EMD Millipore Corporation, Billerica, MA, USA). Proteins on the membrane were probed with specific antibodies

and visualized using a SuperSignal West Pico enhanced chemiluminescence kit (Thermo Fisher Scientific).

Transient luciferase assay

HGFs (4×10^5 cells/ml) were transfected with reporter plasmids using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Two hundred ng of 5xκB-luc, a plasmid in which luciferase gene expression is under the control of NF-κB, and 10 ng of an internal control plasmid pRL-TK, which expresses *Renilla reniformis* luciferase under the control of TK promoter, were used for each transfection. Twenty-four hours after transfection, the cells were treated with cynaropicrin for 1 h and then stimulated with 0.5 μg/ml LPS for 24 h. Cells were harvested using Passive Lysis Buffer (Promega, Madison, WI, USA) and the extracts were assessed for luciferase activity using the Dual-Luciferase Assay System (Promega) as described previously [17]. Luciferase activity was normalized to *R. reniformis* luciferase activity, which acted as an internal control for transfection efficiency.

Osteoclast formation and tartrate-resistant acid phosphatase (TRAP) staining

RAW264.7 cells were seeded in 24-well culture plates (1.2×10^4 cells/well). After culturing overnight, medium was replaced with 500 μl of α-minimal essential medium (Wako) containing

10% fetal bovine serum and 100 ng/ml soluble RANKL (R&D Systems) in the absence or presence of the indicated concentrations of cynaropicrin or artichoke extract; the cultures were then incubated at 37 °C for 4 days. Cells were fixed with 4% paraformaldehyde and stained for TRAP activity as described previously [21]; the plates were then scanned using an EPSON GT-X800 scanner (EPSON, Suwa, Japan). TRAP-positive giant cells with three or more nuclei were counted. These cells were also examined at higher magnification using a Digital Sight Ds-Fi1 (Nikon, Tokyo, Japan).

Statistical analysis

Mean values ± standard deviation (SD) were calculated. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test. $P < 0.05$ was considered to be statistically significant.

Results

Effect of artichoke extract on the expression of IL-8 and IL-6 mRNA by LPS-stimulated HGFs

To examine the effect of artichoke extract on the expression of inflammatory cytokines by HGFs stimulated by LPS, real-time PCR analysis was employed. LPS induced the expression of IL-8 and IL-6 by 236.5- and 16.7-fold, respectively (Fig. 1A). When HGFs were pretreated with artichoke extract for 1 h before LPS treatment, the extent of IL-8 and IL-6 mRNA expression was reduced in a concentration-dependent manner. No cytotoxicity was observed at the artichoke extract concentrations used (Fig. 1B).

Cynaropicrin inhibits LPS-induced production of inflammatory cytokines by HGFs

Cynaropicrin is one of the sesquiterpene lactones found in artichoke extract (Fig. 2A). To determine its effects on the expression of inflammatory cytokines, cynaropicrin was purified from artichoke extract (See Materials and methods for details). Cynaropicrin was calculated to represent 1.5% of the artichoke extract by weight. Real-time PCR analysis was employed to test whether cynaropicrin purified from artichoke extract have similar effects on inflammatory cytokine expression as the artichoke extract. This showed that cynaropicrin also dramatically suppressed LPS-induced expression of IL-8 and IL-6 mRNAs in HGFs (Fig. 2B). Cynaropicrin and artichoke

extract behaved similarly with respect to the expression of both inflammatory cytokines induced by LPS, although cynaropicrin appeared to be more effective than artichoke extract (Fig. 2C). It was confirmed this effect by measuring the secretion of both cytokines by specific ELISA (Fig. 2D). Cynaropicrin inhibited LPS-induced production of IL-8 and IL-6 in HGFs, and at 5 and 10 μM of cynaropicrin, the LPS-induced production of IL-8 was 60.2 and 98.2%, respectively. These concentrations were not cytotoxic (Fig. 2E). These results indicate that cynaropicrin suppresses the inflammatory cytokine levels induced by LPS without cytotoxicity.

Cynaropicrin inhibits LPS-induced NF-κB activity in HGFs

A number of highly inducible genes encoding chemokines and cytokines contain NF-κB-binding sites in their proximal promoters [22], and activation of NF-κB plays a central role in LPS-dependent cytokine expression and pathology. NF-κB is an inducible cellular transcription factor that regulates a wide variety of cellular genes involved in controlling inflammatory and immune responses, and is usually sequestered in the cytoplasm as an inactive complex with inhibitor κB (IκBα). Treatment with LPS induces IκBα phosphorylation, leading to its ubiquitination and subsequent degradation of IκBα by the 26S proteasome; this results in transport of NF-κB p65/p50 into the nucleus. In addition to nuclear translocation, phosphorylation of the NF-κB p65 subunit at Ser536 is also essential for its maximal transcriptional activity [22]. Because previous studies have

reported that NF-κB is important in mediating IL-8 and IL-6 expression and the NF-κB inhibitor, BAY11-7082, inhibited LPS-induced production of IL-8 and IL-6 in HGFs (Fig. 3A), whether cynaropicrin can inhibit NF-κB in HGFs was then examined.

LPS stimulation caused IκBα degradation and phosphorylation of the NF-κB p65 subunit at Ser536 five minutes after stimulation of HGFs (Fig. 3B). However, treatment with cynaropicrin blocked IκBα degradation and NF-κB p65 phosphorylation in a concentration-dependent manner (Fig. 3B). GAPDH levels were unchanged, indicating that these effects were treatment-specific. To determine whether LPS-induced inhibition of NF-κB in HGFs occurs at the transcriptional level, luciferase assay using a reporter plasmid whose expression is controlled by NF-κB was employed. LPS increased transcription by NF-κB by 3.9 ± 0.7-fold (Fig. 3C). However, when cells were pretreated with cynaropicrin, this increase in transcription was inhibited in a concentration-dependent manner and abolished almost completely at 10 μM cynaropicrin (Fig. 3C). These data suggest that cynaropicrin can inhibit *P. gingivalis* LPS-induced cytokine expression, and that this could be due to inhibition of the NF-κB signaling pathway.

Cynaropicrin inhibit RANKL-induced osteoclast differentiation

Osteoclasts originated from monocyte/macrophage lineage demineralized and degrade the bone matrix [4, 23]. To examine the effect of cynaropicrin on RANKL-induced osteoclastogenesis,

macrophage-like RAW264.7 cells were treated with different concentrations of cynaropicrin in the presence of recombinant RANKL. Although RANKL stimulation induced the formation of osteoclast-like TRAP-positive multinuclear giant cells, treatment with cynaropicrin dramatically reduced their number (Fig. 4A and 4B). Higher magnification images also revealed that cynaropicrin decreased both the number and size of osteoclast-like cells in a dose-dependent manner (Fig. 4C). RANKL-induced osteoclastogenesis, was inhibited, on average, by 79%, by 5 μM cynaropicrin. In addition, artichoke extract inhibited osteoclast differentiation induced by RANKL (Fig. 5), although cynaropicrin appeared to be more effective than artichoke extract.

Discussion

The negative impact of tooth loss from periodontal disease on quality of life is a growing global issue as the percentage of elderly individuals continues to increase. Since it is well known that *P. gingivalis* LPS is involved in destruction of periodontal tissues by promoting production of proinflammatory cytokines by HGFs and monocytes [2, 3], *P. gingivalis* is one of the clinical parameters of periodontal diseases, such as probing pocket depth, bleeding on probing, and alveolar bone loss. In addition, several clinical studies have demonstrated that improved oral hygiene and receiving periodontal intervention reduces the number of oral bacteria, thereby reducing the risk of aspiration pneumonia, diabetes, and chronic obstructive pulmonary disease [5-7, 24]. Thus, it is important to understand that the suppression and prevention of periodontal diseases also leads to the prevention of systemic diseases. Since mechanical treatment of periodontal disease is not always sufficient to terminate disease progression, other adjunct treatments are sometimes needed. These can include use of antibiotics, but they risk the development of resistant strains of bacteria. Therefore, logically, various investigators have focused on inhibiting inflammatory responses, as it could lead to even more improvement in patients with periodontal diseases. As a result, the study sought to identify a novel and safe alternative agent present in natural plant extracts that can target the inflammation caused by LPS of periodontopathic bacteria. If this agent also target the

subsequent osteoclastogenesis, it would be more preferable. The present study showed for the first time that cynaropicrin inhibit *P. gingivalis* LPS-induced production of inflammatory cytokines in HGFs as well as inhibit RANKL-induced osteoclast differentiation, indicating an important aspect of the anti-inflammatory activities of this compound. Artichoke extract also inhibited osteoclast differentiation induced by RANKL in RAW264.7 cells (Fig. 5).

Cynaropicrin and artichoke extract inhibited the expression of IL-8 and IL-6 induced by LPS without affecting cell viability at pharmacologically effective doses (Figs. 1 and 2). The expression of other inflammatory cytokines, namely IL-1β and tumor necrosis factor alpha (TNF-α), was also inhibited by cynaropicrin (data not shown). The inhibitory effects of cynaropicrin were more potent than those of artichoke extract (Fig. 2C). Artichoke extract contains many other ingredients, as plants in general contain many compounds that exhibit various physiological activities. Thus, the presence of some other components in the extract may have attenuated the effects investigated.

IL-8 is a potent neutrophil chemoattractant and activator and has been associated with the pathogenesis of periodontal diseases. It brings about accumulation and degranulation of neutrophils with subsequent destruction of normal tissue [25, 26]. IL-6 is involved in stimulation of acute phase protein synthesis, leukocyte recruitment, B-cell differentiation, and T-cell activation in many chronic inflammatory diseases [27]. In addition, these cytokines can also stimulate bone resorption by promoting the formation of osteoclasts [4, 28]. It has been demonstrated that IL-8 and IL-6 are

excessively and/or continuously produced in response to the amount of accumulated periodontopathic bacteria and their products, such as LPS in periodontal pockets, and their etiological correlation with periodontal diseases has been clearly demonstrated [29-31]. Brandolini *et al.* reported that after priming by IL-1β, IL-8 strikingly upregulates elastase release by neutrophils, giving rise to a significant increase in periodontal inflammation [32]. In addition, it has been reported that since IL-6 levels in periodontal pockets of patients with periodontitis are higher than IL-1 levels, IL-6 plays an important role in the progression of periodontal disease [33]. Since these inflammatory cytokines are multifunctional and exert their effects in a paracrine and autocrine fashion to modulate inflammatory and immune responses of HGFs, these findings suggest that cynaropicrin may be effective as a therapeutic agent for periodontal disease.

A number of highly inducible genes encoding cytokines and chemokines contain NF-κB-binding sites in their proximal promoters [22], and activation of NF-κB plays a central role in LPS-dependent cytokine expression and pathology. Fig. 3 shows that LPS could stimulate the degradation of IκBα, phosphorylation of NF-κB p65, and NF-κB transactivation. In addition, an inhibitor of NF-κB reduced LPS-induced production of IL-6 and IL-8 in HGFs. Thus, it is possible that cynaropicrin inhibits LPS-induced IL-8 and IL-6 production in HGFs via regulation of the NF-κB signaling pathway. Previous studies have demonstrated that sesquiterpene lactones suppress NF-κB activation [13, 15]. However, since sesquiterpene lactones affect other signaling pathways, such

as that of mitogen-activated protein kinase (MAPK), which is known to be activated by LPS [14], further studies are needed, including an evaluation of the effect of cynaropicrin on the activation of MAPK and other transcriptional factors.

A major clinical symptom of periodontal diseases is alveolar bone loss due to excessive resorption by osteoclasts. Differentiation of osteoclasts is regulated by binding of RANK on the surface of pre-osteoclasts to its ligand RANKL [23], which leads to the activation of NF-κB. Deletion of both NF-κB subunits p50 and p52 results in defective osteoclast differentiation and osteopetrosis in mice [34], indicating that the NF-κB pathway controls osteoclastogenesis. *P. gingivalis* affects osteoclast formation in diverse ways, such as by interacting directly with osteoclast precursors, such as bone marrow macrophages, or indirectly by activation of gingival fibroblasts and osteoblasts that can support osteoclast formation. LPS is also involved in osteoclast differentiation via inducing RANKL expression [4, 35]. Since cynaropicrin inhibits NF-κB activity, its inhibitory effect on osteoclast differentiation may be because of its suppression of the activation of NF-κB by RANKL. Further studies are needed into how cynaropicrin inhibits osteoclast differentiation.

Although there are no reports evaluating the effect of cynaropicrin and artichoke extract on periodontal diseases *in vivo*, a previous study investigated the potential benefits of cynaropicrin *in vivo* [36]. Preliminary studies have shown that cynaropicrin inhibits the expression of IL-8 in

gingival tissues in a mouse experimental model of periodontal disease (data not shown). Although there are concerns that sesquiterpene lactones are cytotoxic, significant cytotoxicity of cynaropicrin using two kinds of cells was not observed. In addition, no unusual weight changes or morphological abnormality were found during autopsy in previously preclinical safety trials using mice that orally ingested cynaropicrin [36]. Moreover, no increase in the number of colonies was observed in a reverse-mutation test using two *Salmonella typhimurium* strains TA98 and TA100. More comprehensive safety evaluations need to be conducted in the future, but these initial results indicate that cynaropicrin is safe and highly tolerable.

Although additional basic, clinical, and animal studies are required, this study suggests that cynaropicrin represents a promising tool for controlling periodontal diseases as a complementary approach to conventional periodontal therapy. Further development of feasible and affordable drug therapies or combined mechanical treatment/drug therapies against periodontal diseases should be pursued.

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References

1. Pihlstrom BL, Michalowicz BS, Johnson NW (2005) Periodontal diseases. The Lancet 366:1809–1820
2. Hajishengallis G (2015) Periodontitis: from microbial immune subversion to systemic inflammation. Nat Rev Immunol 15:30–44
3. Mysak J, Podzimek S, Sommerova P, Lyuya-Mi Y, Bartova J, Janatova T, Prochazkova J, Duskova J (2014) *Porphyromonas gingivalis*: major periodontopathic pathogen overview. J Immunol Res 2014:476068
4. Amarasekara DS, Yun H, Kim S, Lee N, Kim H, Rho J (2018) Regulation of osteoclast differentiation by cytokine networks. Immune Netw 18:e8
5. Darré L, Vergnes JN, Gourdy P, Sixou M (2008) Efficacy of periodontal treatment on glycaemic control in diabetic patients: A meta-analysis of interventional studies. Diabetes Metab 34:497–506
6. Sjögren P, Nilsson E, Forsell M, Johansson O, Hoogstraate J (2008) A systematic review of the preventive effect of oral hygiene on pneumonia and respiratory tract infection in elderly people in hospitals and nursing homes: effect estimates and methodological quality of randomized controlled trials. J Am Geriatr Soc 56:2124–2130
7. Munenaga Y, Yamashina T, Tanaka J, Nishimura F (2013) Improvement of glycated hemoglobin in Japanese subjects with type 2 diabetes by resolution of periodontal inflammation using adjunct topical antibiotics: results from the Hiroshima Study. Diabetes Res Clin Practice 100:53–60
8. Corrêa MG, Pires PR, Ribeiro FV, Pimentel SZ, Casarin RC, Cirano FR, Tenenbaum HT, Casati MZ (2017) Systemic treatment with resveratrol and/or curcumin reduces the progression of experimental periodontitis in rats. J Periodont Res 52:201–209
9. Noorafshan A, Ashkani-Esfahani S (2013) A review of therapeutic effects of curcumin. Curr Pharm Des 19:2032–2046
10. Yun HJ, Yoo WH, Han MK, Lee YR, Kim JS, Lee SI (2008) Epigallocatechin-3-gallate suppresses TNF-α-induced production of MMP-1 and -3 in rheumatoid arthritis synovial fibroblasts. Rheumatol Int 29:23–29
11. Mehmetçik G, Ozdemirler G, Koçak-Toker N, Cevikbaş U, Uysal M (2008) Effect of pretreatment with artichoke extract on carbon tetrachloride-induced liver injury and oxidative stress. Exp Toxicol Pathol 60:475–480

12. Ishida K, Kojima R, Tsuboi M, Tsuda Y, Ito M (2010) Effects of artichoke leaf extract on acute gastric mucosal injury in rats. Biol Pharm Bull 33:223–229
13. Siedle B, García-Piñeres AJ, Murillo R, Schulte-Mönting J, Castro V, Rüngeler P, Klaas CA, Da Costa FB, Kisiel W, Merfort I (2004) Quantitative structure-activity relationship of sesquiterpene lactones as inhibitors of the transcription factor NF-κB. J Med Chem 47:6042–6054
14. Sun X, Jiang C, Ma L, Zhao X, Chang J, Zheng B, Li L, Xie W, Li X (2015) 3β-Angeloyloxy-8β,10β-dihydroxyeremophila-7(11)-en-12,8α-lactone inhibits lipopolysaccharide-induced nitric oxide production in RAW264.7 cells. Biol Pharm Bull 38:836–843
15. Tamura R, Chen Y, Shinozaki M, Arao K, Wang L, Tang W, Hirano S, Ogura H, Mitsui T, Taketani S, Ando M, Kataoka T (2012) Eudesmane-type sesquiterpene lactones inhibit multiple steps in the NF-κB signaling pathway induced by inflammatory cytokines. Bioorg Med Chem Lett 22:207–211
16. Ogata Y, Niisato N, Sakurai T, Furuyama S, Sugiya H (1995) Comparison of the characteristics of human gingival fibroblasts and periodontal ligament cells. J Periodontol 66:1025-1031
17. Imai K, Okamoto T, Ochiai K (2015) Involvement of Sp1 in butyric acid-induced HIV-1 gene expression. Cell Physiol Biochem 37:853–865
18. Victoriano AF, Imai K, Togami H, Ueno T, Asamitsu K, Suzuki T, Miyata N, Ochiai K, Okamoto T (2011) Novel histone deacetylase inhibitor NCH-51 activates latent HIV-1 gene expression. FEBS Lett 585:1103–1111
19. Imai K, Kamio N, Cueno ME, Saito Y, Inoue H, Saito I, Ochiai K (2014) Role of the histone H3 lysine 9 methyltransferase Suv39 h1 in maintaining Epsteinn-Barr virus latency in B95-8 cells. FEBS J 281:2148–2158
20. Kamio N, Imai K, Shimizu K, Cueno ME, Tamura M, Saito Y, Ochiai K (2015) Neuraminidase-producing oral mitis group streptococci potentially contribute to influenza viral infection and reduction in antiviral efficacy of zanamivir. Cell Mol Life Sci 72:357–366
21. Zhao N, Tsuda H, Murofushi T, Imai K, Ochiai K, Yang P, Suzuki N (2015) 1-7 Chaetocin inhibits RANKL-induced osteoclast differentiation through reduction of Blimp1 in Raw264.7 cells. Life Sci 143:1–7
22. Hayden MS, Ghosh S (2004) Signaling to NF-κB. Genes Dev 18:2195–2224
23. Mundy GR (2007) Osteoporosis and inflammation. Nutr Rev 65:S147–S151

24. Zhou X, Han J, Liu Z, Song Y, Wang Z, Sun Z (2014) Effects of periodontal treatment on lung function and exacerbation frequency in patients with chronic obstructive pulmonary disease and chronic periodontitis: a 2-year pilot randomized controlled trial. J Clin Periodontol 41:564–572
25. Takashiba S, Takigawa M, Takahashi K, Myokai F, Nishimura F, Chihara T, Kurihara H, Nomura Y, Murayama Y (1992) Interleukin-8 is a major neutrophil chemotactic factor derived from cultured human gingival fibroblasts stimulated with interleukin-1β or tumor necrosis factor α. Infect Immun 60:5253–5258
26. Bickel M (1993) The role of interleukin-8 in inflammation and mechanisms of regulation. J Periodontol 64:456–460
27. Hunter CA, Jones SA (2015) IL-6 as a keystone cytokine in health and disease. Nat Immunol 16:448–457
28. Hwang YS, Lee SK, Park KK, Chung WY (2012) Secretion of IL-6 and IL-8 from lysophosphatidic acid-stimulated oral squamous cell carcinoma promotes osteoclastogenesis and bone resorption. Oral Oncol 48:40–48
29. Tsai CC, Ho YP, Chen CC (1995) Levels of interleukin-1β and interleukin-8 in gingival crevicular fluids in adult periodontitis. J Periodontol 66:852–859
30. Jin L, Söder B, Corbet EF (2000) Interleukin-8 and granulocyte elastase in gingival crevicular fluid in relation to periodontopathogens in untreated adult periodontitis. J Periodontol 71:929–939
31. Bozkurt FY, Berker E, Akkuş S, Bulut S (2000) Relationship between interleukin-6 levels in gingival crevicular fluid and periodontal status in patients with rheumatoid arthritis and adult periodontitis. J Periodontol 71:1756–1760
32. Brandolini L, Sergi R, Caselli G, Boraschi D, Locati M, Sozzani S, Bertini R (1997) Interleukin-1β primes interleukin-8-stimulated chemotaxis and elastase release in human neutrophils via its type I receptor. Eur Cytokine Netw 8:173–178
33. Reinhardt RA, Masada MP, Kaldahl WB, DuBois LM, Kornman KS, Choi JI, Kalkwarf KL, Allison AC (1993) Gingival fluid IL-1 and IL-6 levels in refractory periodontitis. J Clin Periodontol 20:225–231
34. Iotsova V, Caamaño J, Loy J, Yang Y, Lewin A, Bravo R (1997) Osteopetrosis in mice lacking NF-κB1 and NF-κB2. Nat Med 3:1285–1289
35. Kikuchi T, Matsuguchi T, Tsuboi N, Mitani A, Tanaka S, Matsuoka M, Yamamoto G, Hishikawa T, Noguchi T, Yoshikai Y (2001) Gene expression of osteoclast differentiation factor is

induced by lipopolysaccharide in mouse osteoblasts via Toll-like receptors. J Immunol 166:3574–3579

36. Tanaka YT, Tanaka K, Kojima H, Hamada T, Masutani T, Tsuboi M, Akao Y (2013) Cynaropicrin from *Cynara scolymus* L. suppresses photoaging of skin by inhibiting the transcription activity of nuclear factor-κB. Bioorg Med Chem Lett 23:518–523

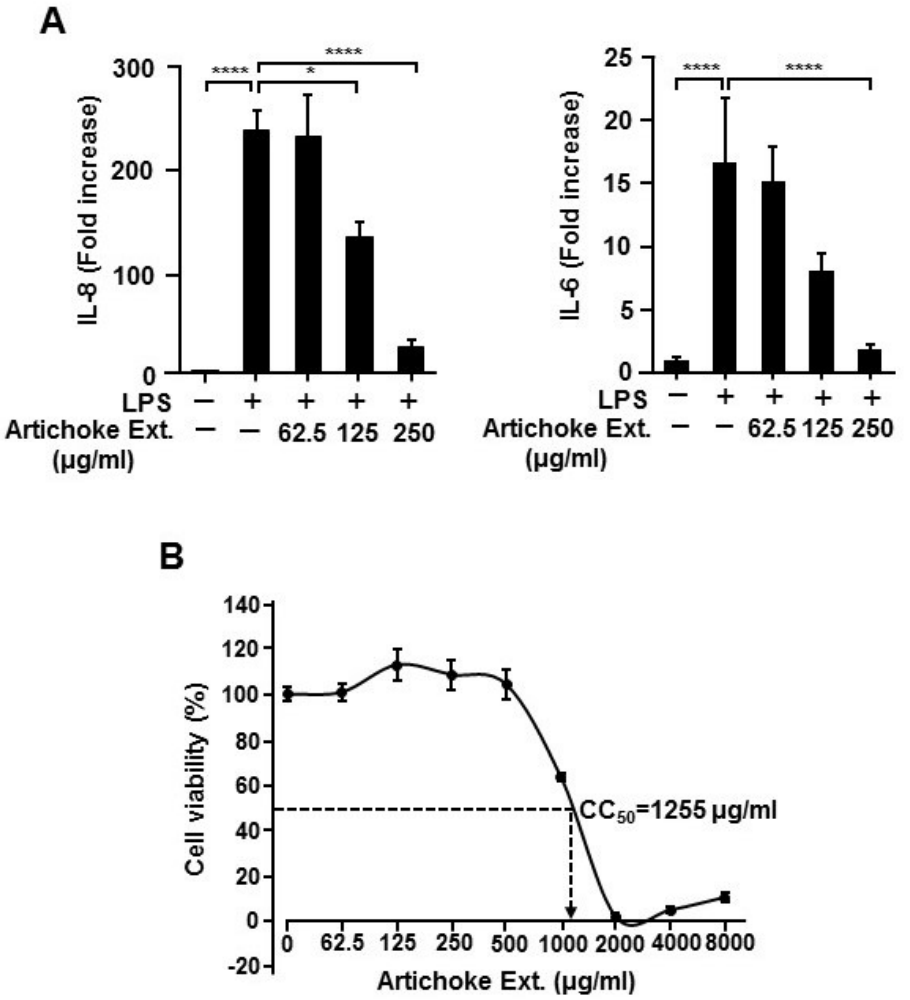

Fig. 1. Inhibitory effects of artichoke extract on LPS-induced IL-8 and IL-6 mRNA expression

A. HGFs were stimulated by *P. gingivalis* LPS (0.5 μg/ml) for 3 h with or without pretreatment of the indicated concentrations of artichoke extract (Artichoke Ext.) that had been added 1 h prior to LPS. Total RNA was extracted, and real-time PCR analysis was performed to detect IL-8 and IL-6 mRNA expression using specific primers. IL-8 and IL-6 mRNA levels were normalized to GAPDH mRNA levels and expressed as fold-increase. **B.** Cell viability in the presence of various concentrations of artichoke extract was determined by WST-1 assay. The mean 50% cytotoxic concentration (CC_{50} value) of 1255.2 μg/ml was extrapolated from this measurement. The values are presented as mean ± SD; $n = 3$ (*, $p < 0.05$; ****, $p < 0.0001$).

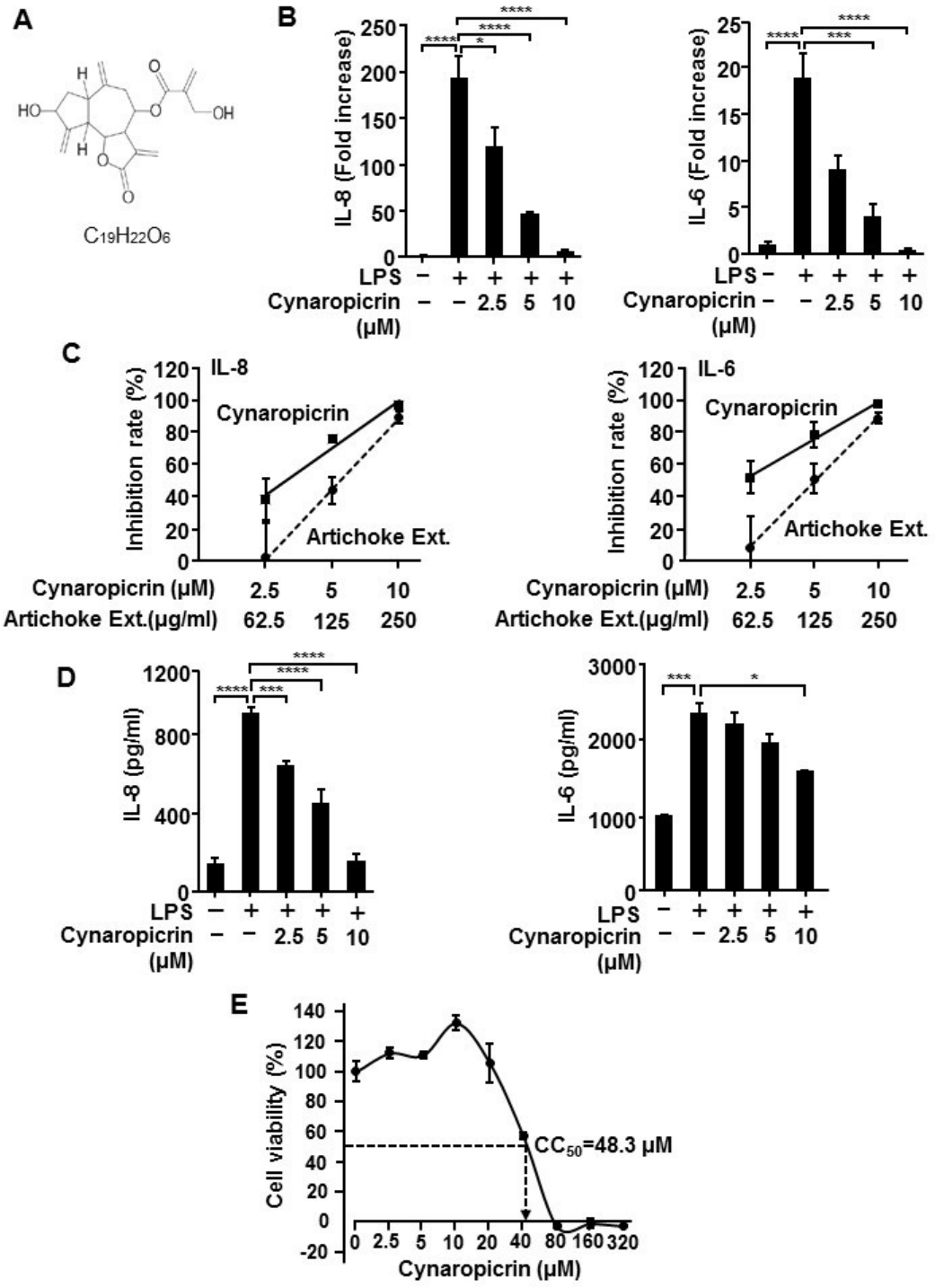

Fig. 2. Inhibitory effects of cynaropicrin on LPS-induced production of IL-8 and IL-6

A. Chemical structure of cynaropicrin. **B.** HGFs were stimulated by *P. gingivalis* LPS (0.5 μg/ml) for 3 h with or without pretreatment with the indicated concentrations of cynaropicrin 1

h prior to LPS. Real-time PCR analysis was then performed as described in Fig. 1A. **C.** Comparison with the anti-inflammatory activity of artichoke extract. **D.** HGFs were pre-treated with the indicated concentrations of cynaropicrin for 1 h and were then stimulated with or without LPS (0.5 μg/ml). After a further 24 h incubation, IL-8 and IL-6 concentrations in culture supernatants were determined using ELISA. **E.** Cytotoxic effects of cynaropicrin. HGFs' viability was determined by WST-1 assay. CC_{50} value of 48.3 μM was extrapolated from this measurement. The values are presented as mean ± SD; $n = 3$ (*, $p < 0.05$; *** $p < 0.005$; ****, $p < 0.0001$).

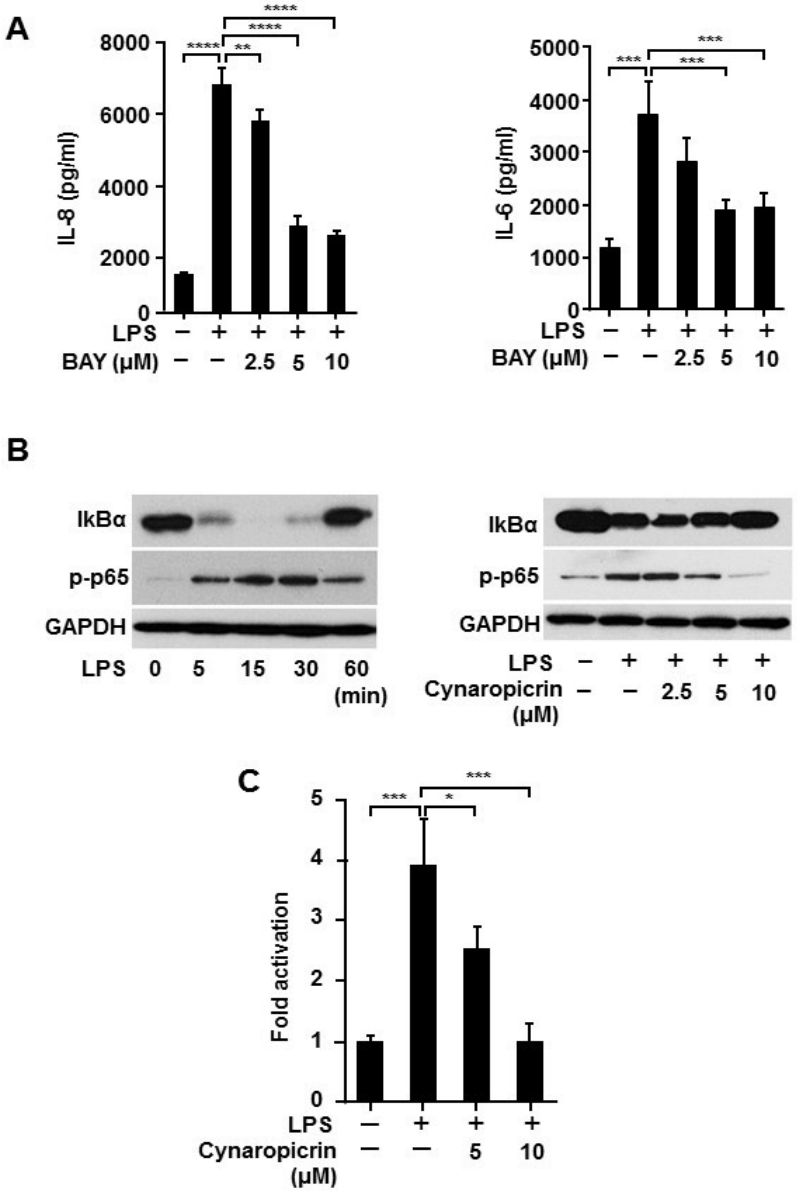

Fig. 3. Inhibition of NF-κB by cynaropicrin

A. HGFs were pre-treated with the indicated concentrations of BAY11-7082 for 1 h and then stimulated with or without LPS (0.5 μg/ml). After a further 24 h incubation, IL-8 and IL-6 production was determined using ELISA. **B.** HGFs were stimulated by LPS (0.5 μg/ml) for the indicated time (min) (left panel). Cells were pre-treated with the indicated concentrations of cynaropicrin for 1 h and subsequently stimulated with LPS (0.5 μg/ml) for 5 min (right panel).

Whole cell extracts were prepared and subjected to immunoblotting with anti-IκBα and anti-phospho-p65 (Ser536) antibodies. **C.** HGFs were transfected with a 5xκB-luc reporter plasmid together with an internal pRL-TK control plasmid. Twenty-four hours after transfection, cells were pre-treated with the indicated concentrations of cynaropicrin for 1 h and stimulated with 0.5 μg/ml of LPS for 24 h. Luciferase activity in the whole cell lysate was then determined. The data are presented as the fold-increase in luciferase activity relative to control transfected cells (no stimulation). Values are the means ± SD of three independent transfections. BAY, treated with BAY11-7082. The values are presented as mean ± SD; $n = 3$ (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.0001$).

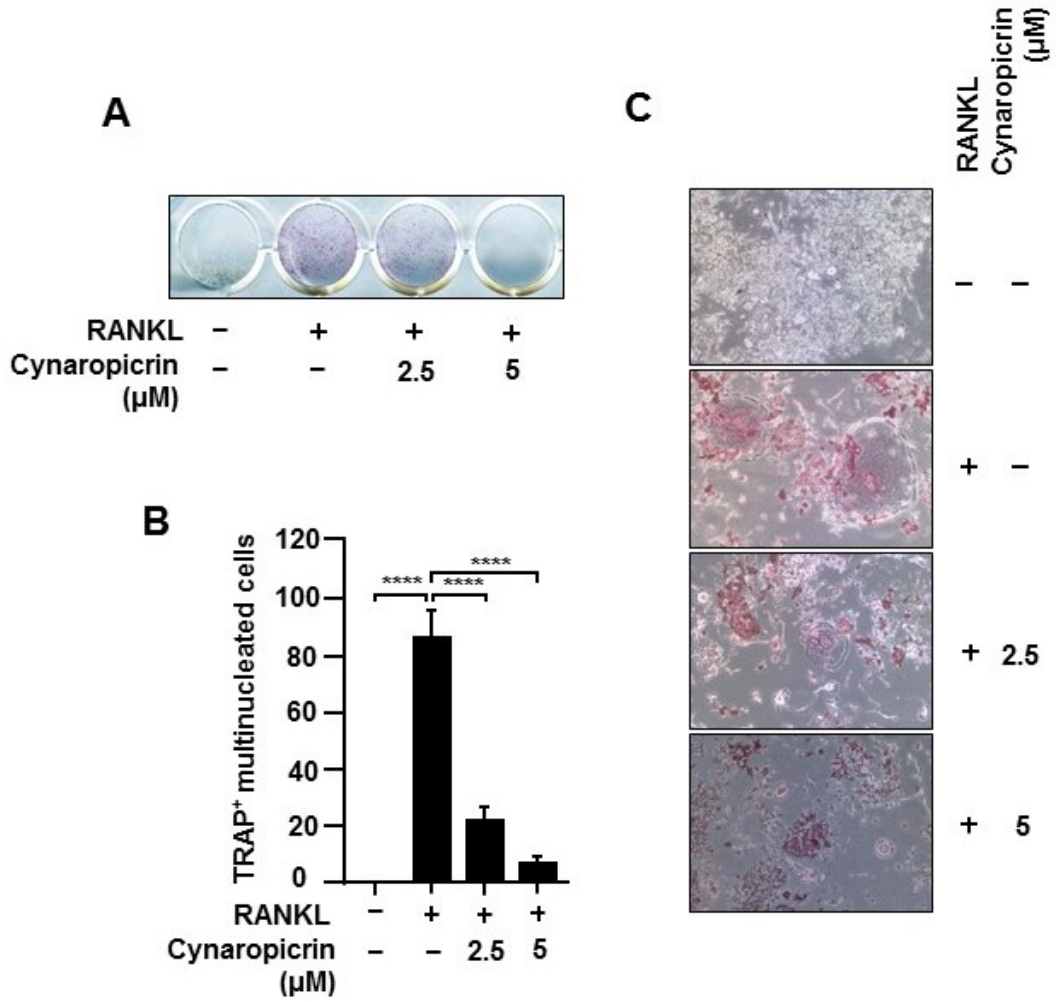

Fig. 4. Inhibition of osteoclast differentiation by cynaropicrin

A. RAW264.7 cells were seeded in a 24-well plate and treated with recombinant RANKL (100 ng/ml) in the absence or presence of the indicated concentrations of cynaropicrin for 4 days, followed by TRAP staining. **B.** The number of TRAP-positive multinucleated cells present in each well was counted. **C.** The cells were observed under higher magnification. The values are presented as mean ± SD; $n = 4$ (****, $p < 0.0001$).

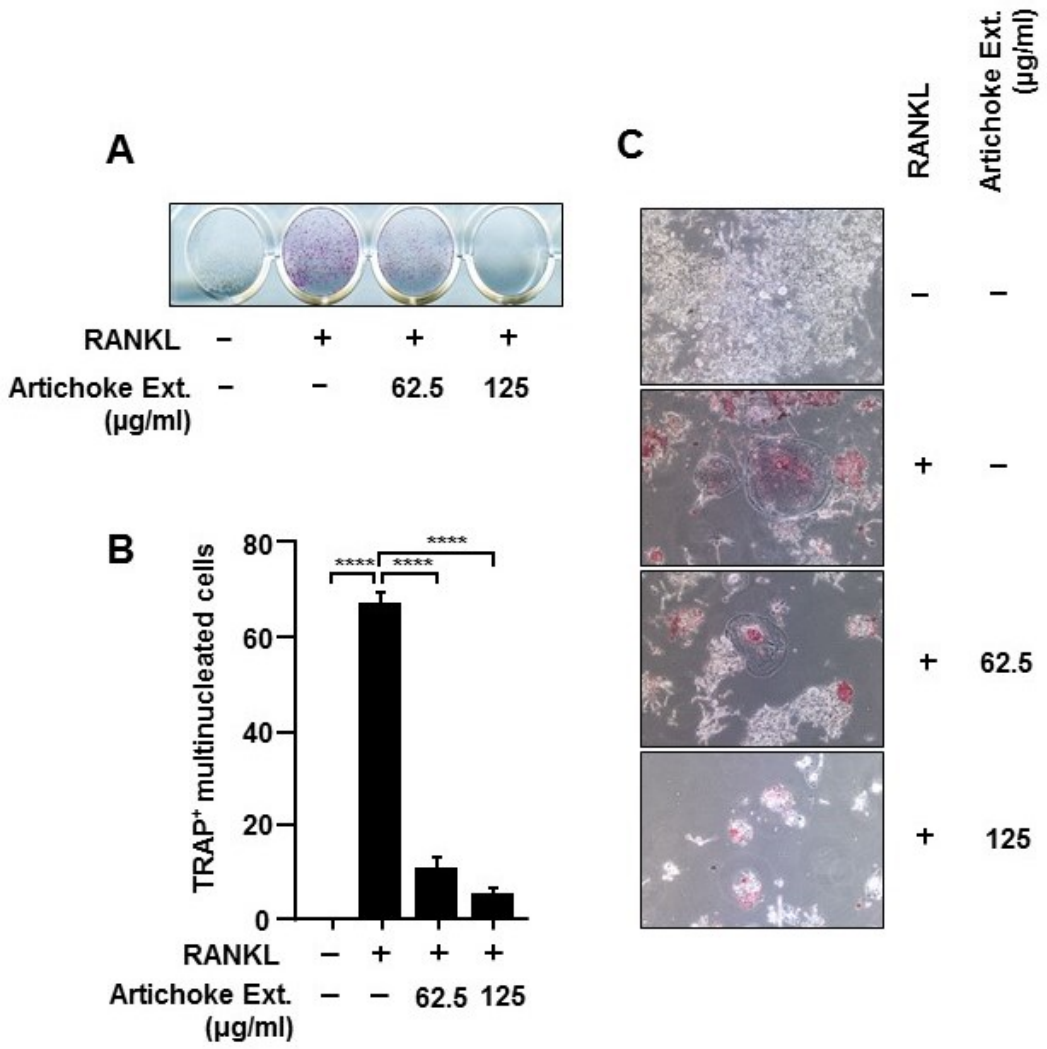

Fig. 5. Inhibitory effects of artichoke extract on osteoclast differentiation

A. RAW264.7 cells were treated with recombinant RANKL (100 ng/ml) in the absence or presence of the indicated concentrations of artichoke extract for 4 days, followed by TRAP staining. **B.** The number of TRAP-positive multinucleated cells was counted. **C.** The cells were observed under higher magnification. The values are presented as mean ± SD; $n = 4$ (****, $p < 0.0001$).