

Suppressive effect of ethanolic *Kaempferia pandurata* Roxb. extract on matrix metalloproteinase-2 expression in *Porphyromonas gingivalis*-treated human gingival fibroblasts *in vitro*

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Abstract: In periodontal disease, gingival fibroblasts activated by the Gram-negative anaerobic bacterium *Porphyromonas gingivalis* induce overexpression of matrix metalloproteinase-2 (MMP-2), which is involved in inflammatory progression. This process is followed by tissue destruction and bone loss. In the present study, we investigated the *in vitro* effect of the ethanolic *Kaempferia pandurata* Roxb. extract on expression of MMP-2 in *P. gingivalis*-treated human gingival fibroblast-1 (HGF-1) cells. In addition, we utilized gelatin zymography, Western blotting, and reverse transcription-PCR analysis to elucidate the molecular mechanisms underlying MMP-2 inhibition via the mitogen-activated protein kinase (MAPK) and cyclic AMP response element-binding protein (CREB) signaling pathways. Treatment with *K. pandurata* extract (1-10 µg/ml) dose-dependently suppressed the activity, secretion, and protein expression of MMP-2 in HGF-1 cells exposed to *P. gingivalis*. At the transcriptional level, inhibition of MMP-2 gene expression by *K. pandurata* was mediated by phosphorylation of c-Jun N-terminal kinase (JNK) and CREB signaling pathways in *P. gingivalis*-treated HGF-1 cells. These results suggest that *K. pandurata* extract suppresses MMP-2 expression at the protein and

gene levels via downregulation of the principal JNK and CREB signaling pathways. Due to its efficacy in inhibiting MMP-mediated periodontal destruction, *K. pandurata* might represent a new, potent periodontal therapy. (J Oral Sci 52, 583-591, 2010)

Keywords: *Kaempferia pandurata* Roxb.; matrix metalloproteinase-2; *Porphyromonas gingivalis*; human gingival fibroblasts.

Introduction

The matrix metalloproteinases (MMPs), a family of zinc- and calcium-dependent endopeptidases, are host-derived proteolytic enzymes involved in remodeling of the extracellular matrix and basement membrane during the progression of periodontal disease. In chronic periodontitis, levels of active MMP-2 (72/67-kDa; gelatinase A) and MMP-9 (92/84-kDa; gelatinase B) are elevated in gingival tissue, gingival crevicular fluid, and saliva (1-2), and thus serve as biomarkers of the clinical severity of periodontal disease. These enzymes are mainly produced by gingival epithelial cells, fibroblasts, polymorphonuclear leukocytes, monocytes/macrophages, and endothelial cells (3). They act principally by degrading basement membrane type IV collagen and gelatin (4).

Gingival fibroblasts are the predominant cell type in periodontal connective tissue. They produce components of the extracellular matrix and enzymes for matrix degradation that lead to inflammatory periodontal disease. Considerable evidence from *in vitro* studies has demon-

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strated that fibroblasts obtained from healthy and inflamed periodontal tissues differ in cell size and shape, proliferative rate, collagen production, and expression of growth factor receptors (5-6). It has been reported that Gram-negative periodontopathogens colonize gingival pockets including fibroblasts and form gingival plaque during periodontal disease progression. *Porphyromonas gingivalis*, a major etiologic agent of chronic periodontitis, possesses several virulence factors (e.g., gingipain, lipopolysaccharide, and fimbriae) and causes periodontal tissue destruction by triggering secretion and activation of MMPs, including MMP-2 and MMP-9 (7-11).

MMP activity is tightly regulated by gene expression, proenzyme activation, and inhibition of active enzymes by specific tissue inhibitors of metalloproteinase (TIMPs). An imbalance between activated MMPs and their respective endogenous TIMPs promotes extracellular matrix degradation during periodontitis (4). Thus, inhibition of MMP expression is an attractive strategy in chemical periodontal therapy. A synthetic MMP inhibitor, doxycycline, has entered development, but its clinical efficacy remains unclear (12). Therefore, the evaluation of natural therapeutic candidates offers an opportunity to identify MMP inhibitors. *Kaempferia pandurata* Roxb. is an edible medicinal plant known as temu kunci in Indonesia and krachai in Thailand. It has been traditionally used in culinary and folk medicine for treatment of dental caries, colic disorder, fungal infection, dry cough, rheumatism, and muscle pain (13). However, its effect on controlling periodontal inflammation via inhibition of MMP expression has not been explored. We investigated the inhibitory effect of *K. pandurata* extract on MMP-2 expression in human gingival fibroblasts exposed to *P. gingivalis* *in vitro*. Then, we examined if *K. pandurata* extract interfered with signaling pathways involved in MMP-2 expression in this system.

Materials and Methods

Plant materials and reagents

Dried rhizomes of *K. pandurata* Roxb. were collected in Jakarta, Indonesia, and identified by Dr. Nam-In Baek, Department of Oriental Medicinal Materials and Processing, Kyunghee University (Yongin, Korea). A voucher specimen (H082) was deposited in the Department of Biotechnology, Yonsei University (Seoul, Korea). The ground *K. pandurata* Roxb. (100 g) was extracted twice with 95% ethanol (400 ml), and the extract (11.95 g) was used for the experiments. *P. gingivalis* ATCC 33277 was purchased from the American Type Culture Collection (Manassas, VA, USA). The protease inhibitors 4-(2-aminoethyl) benzenesulfonyl fluoride, N-ethylmaleimide, o-phenanthroline, and ethylenediaminetetraacetic acid

were purchased from Sigma-Aldrich (St. Louis, MO, USA). Inhibitors of MMP (GM6001), p38 (SB203580), and JNK (SP600125) were obtained from Calbiochem (San Diego, CA, USA). Inhibitors of MEK1/2 (U0126) and PI3K (LY295002) were purchased from Cell Signaling Technology (Beverly, MA, USA). All inhibitors were first dissolved in dimethyl sulfoxide (DMSO) and then diluted with culture medium with a total concentration of less than 0.1% (v/v). The primary anti-rabbit polyclonal antibodies against MMP-2, ERK1/2, p38, and JNK, and the anti-mouse monoclonal antibodies to p-ERK1/2, p-p38, and p-JNK were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-rabbit polyclonal antibodies to p-CREB and CREB, and the secondary antibody peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG were obtained from Cell Signaling Technology (Beverly, MA, USA).

Cell culture and cell viability

Human gingival fibroblasts (HGF-1, ATCC CRL-2014) were obtained from the American Type Culture Collection (Manassas, VA, USA) and were cultured in a 5% CO₂ atmosphere at 37°C in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 100 U/ml of penicillin A, 100 U/ml of streptomycin, and 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). The cells were seeded at a concentration of 2×10^5 cells/ml per 75-cm² flask (SPL, Seoul, Korea) and cultured for 24 h. Cell cultures from passages 4-10 were used. Cell age did not change cell morphology or structure and had no effects on response to stimuli in *P. gingivalis* supernatant or protein expression in MMP-2.

The effects of *P. gingivalis* supernatant and *K. pandurata* extract on cell viability were evaluated with the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay (Sigma-Aldrich, St. Louis, MO, USA). *K. pandurata* extract was dissolved in 100% DMSO, and the stock solution of the extract at a concentration of 1,000 µg/ml was prepared in 10% DMSO. The final concentrations of the extract ranged from 1 to 50 µg/ml in the culture media, and all cells were treated with DMSO at a final concentration of 0.1%. This DMSO concentration had no effect on cell viability.

Preparation of bacterial supernatant and sample treatment

P. gingivalis supernatant was prepared using a slight modification of the technique described by Chang et al. (11). *P. gingivalis* strain was maintained in brain-heart infusion (BHI; Difco, Detroit, MI, USA) broth. The

inoculum density was prepared in BHI broth and adjusted to a turbidity equivalent to 2 McFarland standard (6×10^8 CFU/ml), in order to concentrate the bacterial products in *P. gingivalis* supernatant. After centrifugation at 13,000 rpm for 20 min at 4°C, supernatants were filter-sterilized using a 0.2- μ m filter and stored at -80°C until used. *P. gingivalis* supernatants were directly diluted with culture medium to a final concentration of 10%.

Cells were seeded at a concentration of 2×10^5 cells/ml in 6-well plates and cultured for 24 h in DMEM-FBS. After washing with Dulbecco's phosphate-buffered saline (DPBS), the cells were incubated in serum free-DMEM without *P. gingivalis* supernatant (negative control group), with 10% *P. gingivalis* supernatant (positive control group), or with 10% *P. gingivalis* supernatant plus treatment for 48 h. The treatment groups in this study included *K. pandurata* extract (1-10 μ g/ml), MAPK inhibitors (U0126, SB203580, and SP600125 at a total concentration of 10 μ M), PI3K inhibitor (LY295002 at 10 μ M), and standard MMP inhibitor (GM6001 at 10 μ M). Cell lysates and conditioned media were collected for these experiments.

Gelatin zymography

MMP-2 secretion and activity in the conditioned media were evaluated by gelatin zymography. To determine MMP-2 secretion, cells were treated with *P. gingivalis* supernatant plus treatment (*K. pandurata* extract or MAPKs/PI3K/MMP inhibitors) for 48 h. Conditioned media were collected and subjected to electrophoresis with 10% SDS polyacrylamide gels containing 0.2% gelatin. Electrophoresis was run at 90 V for 1.5 h in an electrophoretic apparatus (Bio-Rad Mini Protean 3 Cell, Bio-Rad Laboratories, Hercules, CA, USA). After electrophoresis, gels were washed twice with 25 ml of 2.5% Triton X-100 on a gyratory shaker for 1 h at room temperature to remove SDS. The gel was then incubated in 25 ml of reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM CaCl₂, 0.15 M NaCl) at 37°C for 24 h, stained with Coomassie Brilliant Blue R-250, and destained with methanol-acetic acid in water.

To detect MMP-2 activity, the conditioned media from cells treated or not treated with *P. gingivalis* supernatant were directly subjected to gelatin zymography. After electrophoresis, gelatin-containing gels were further treated with *K. pandurata* extract or protease inhibitors in 25 ml of reaction buffer and incubated at 37°C for 24 h, followed by staining and destaining treatment. The MMP-2 gelatinolytic band was detected at 67 kDa as a clear zone against the dark background. Relative band densities were analyzed by using Gel-Doc Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Western blotting

To determine MMP-2 protein expression, the conditioned media from cells treated with *P. gingivalis* supernatant plus *K. pandurata* extract were concentrated with the Fast-Con Protein Concentration kit (Corebio, Belmont, CA, USA) and subjected to Western blotting. To determine the expression of MAPK and CREB phosphorylation, cellular lysates from the negative control, positive control, and treatment group (*K. pandurata* extract) were prepared and assayed by Western blot analysis. Proteins (50 μ g) were resolved by 10% SDS-PAGE and transferred to nitrocellulose transfer membranes. The membranes were blocked with 5% skim milk for 1 h at room temperature and then probed with the primary anti-rabbit polyclonal antibodies to MMP-2 and p-CREB and anti-mouse monoclonal antibodies against p-ERK1/2, p-p38, and p-JNK at a 1:1000 dilution overnight at 4°C. After 3 washes, the blots were subsequently incubated with the secondary antibody peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG at a 1:4000 dilution for 2 h at room temperature. The blots were stained with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL, USA) and visualized using an LAS 3000 Bio Imaging Analysis System (Lab Science, Fuji Film, Tokyo, Japan). Equal loading of blots was demonstrated by stripping blots and reprobing with anti-rabbit polyclonal antibodies for ERK1/2, p38, JNK, and CREB.

Reverse transcription-PCR

Total RNA from *P. gingivalis* supernatant-treated HGF-1 cells was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and quantified spectrophotometrically at 260/280 nm. The cDNA was synthesized by using 5 μ g total RNA, oligo(dT) primers, and SuperScript III First-Strand (Invitrogen, Carlsbad, CA, USA) in a 10- μ l reaction. Reverse transcription was started at 50°C for 50 min and terminated at 85°C for 5 min. The cDNA products were diluted with sterile water up to 100 μ l, and PCR was conducted using 5 μ l cDNA in a 25- μ l reaction system with 0.5 U Takara LA Tag (Takara Bio, Shiga, Japan). The human oligonucleotide primers for MMP-2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed according to a PCR primer selection program at the website of the Virtual Genomic Center from the GenBank database. MMP-2 primers were designed as 5'GACAGTGGATGATGCCTTTGCTCGTGCCCTCC3' for forward and 5'ATCGGCGTTCCCATACTTCA CACGGACC3' for reverse. GAPDH primers were 5'ATTGTTGCCATCAATGACCC3' for forward and 5'AGTAGAGGCAGGGATGAT3' for reverse. PCR

consisted of 35 amplification cycles, and each cycle was carried out for 30 s at 94°C, 30 s at annealing temperature (69°C for MMP-2 and 48°C for GAPDH), and 1 min at 72°C in a thermal cycler (Gene Amp PCR System 2700, Applied Biosystems, CA, USA). The human GAPDH housekeeping gene was used as an internal control to standardize the relative expression levels of MMP-2. PCR products were separated electrophoretically on a 2% agarose DNA gel and stained with ethidium bromide. The stained gel was visualized by using Gel-Doc Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Results

Stimulating effect of *P. gingivalis* supernatant on MMP-2 activity and secretion in HGF-1 cells

Gelatin zymography revealed a main gelatinolytic band corresponding to an approximate molecular mass of 67 kDa. To identify whether the gelatinolytic enzyme belonged to the MMP class, enzyme activity was tested against several protease inhibitors, including 4-(2-aminoethyl) benzenesulfonyl fluoride, *N*-ethylmaleimide, *o*-phenanthroline, and ethylenediaminetetraacetic acid (Fig. 1A). The results demonstrated that, as compared with untreated cells, the enzyme was totally abolished by specific metalloproteinase

inhibitors (*o*-phenanthroline and ethylenediaminetetraacetic acid). In contrast, the serine protease inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride and thiol protease inhibitor *N*-ethylmaleimide did not inhibit gelatinolytic activity. These results confirm that the gelatinolytic enzyme was MMP-2. HGF-1 cells primarily produced MMP-2, and the MMP-2 secretion was upregulated by *P. gingivalis* supernatant in the cells during a 48-h culture period (Fig. 1B). At 10%, *P. gingivalis* supernatant optimally increased the level of MMP-2 expression, as compared with unstimulated cells.

Inhibitory effect of *K. pandurata* extract on MMP-2 expression in HGF-1 cells treated with *P. gingivalis* supernatant

The use of 10% *P. gingivalis* supernatant to induce MMP-2 secretion in HGF-1 cells did not affect cell viability. Also, *K. pandurata* at concentrations up to 10 µg/ml was not cytotoxic to the viability of HGF-1 cells, or to unstimulated or *P. gingivalis* supernatant-stimulated cells, which suggests that the inhibitory effect of *K. pandurata* extract on MMP-2 expression was not attributable to its cytotoxicity (data not shown). In addition, *K. pandurata* extract modulated the *in vitro* activity and secretion of

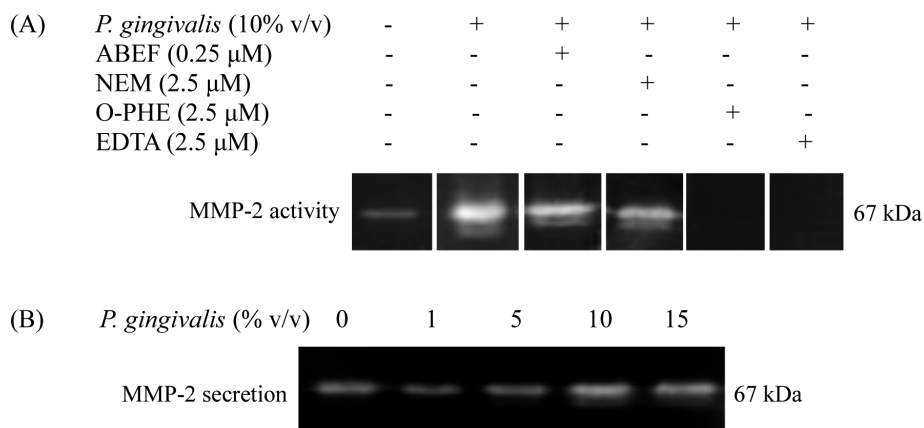


Fig. 1 Effect of protease inhibitors on the expression of MMP-2 activity in *P. gingivalis* supernatant-treated HGF-1 cells (A). Cells treated with *P. gingivalis* supernatant (10%) were cultured for 48 h. Cells not treated with *P. gingivalis* supernatant were used as controls. Culture media (with or without *P. gingivalis* supernatant) were directly assayed by gelatin zymography. After electrophoresis, gelatin-containing gels were further treated with various protease inhibitors – 4-(2-aminoethyl) benzenesulfonyl fluoride (ABEF), *N*-ethylmaleimide (NEM), *o*-phenanthroline (O-PHE), and ethylenediaminetetraacetic acid (EDTA) – and incubated for 24 h at 37°C. Dose effect of *P. gingivalis* supernatant on upregulation of MMP-2 secretion in HGF-1 cells (B). Cells treated with *P. gingivalis* supernatant (10%) were cultured for 48 h. Cells not treated with *P. gingivalis* supernatant were used as controls. MMP-2 secretion in the culture media was assayed by gelatin zymography.

MMP-2 from the conditioned media of *P. gingivalis* supernatant-treated HGF-1 cells. As shown in Fig. 2A, *K. pandurata* dose-dependently inhibited MMP-2 activity in HGF-1 cells exposed to *P. gingivalis* supernatant. Incubation with the extract for 18 h effectively reduced the activity of MMP-2. *K. pandurata* significantly suppressed MMP-2 secretion and protein expression in *P. gingivalis* supernatant-treated HGF-1 cells (Figs. 2B and C). Furthermore, *K. pandurata* had an inhibitory effect on transcriptional level by decreasing MMP-2 mRNA expression in HGF-1 cells in response to *P. gingivalis* supernatant (Fig. 2D).

Inhibitory effect of *K. pandurata* extract on *P. gingivalis* supernatant-induced activation of MAPK and CREB phosphorylation in HGF-1 cells

The decrease in MMP-2 mRNA caused by *K. pandurata* extract suggests that the inhibitory effect of the extract is tightly regulated at the transcriptional level. Inhibition of MMP-2 expression via signaling pathways in *P. gingivalis* supernatant-treated HGF-1 cells was preliminarily examined with gelatin zymography by using the specific mitogen-activated protein kinase (MAPK) inhibitors U0126, SB203580, and SP600125 and the phosphatidylinositol-3 kinase (PI3K) inhibitor LY294002. The results showed that all inhibitors significantly reduced MMP-2

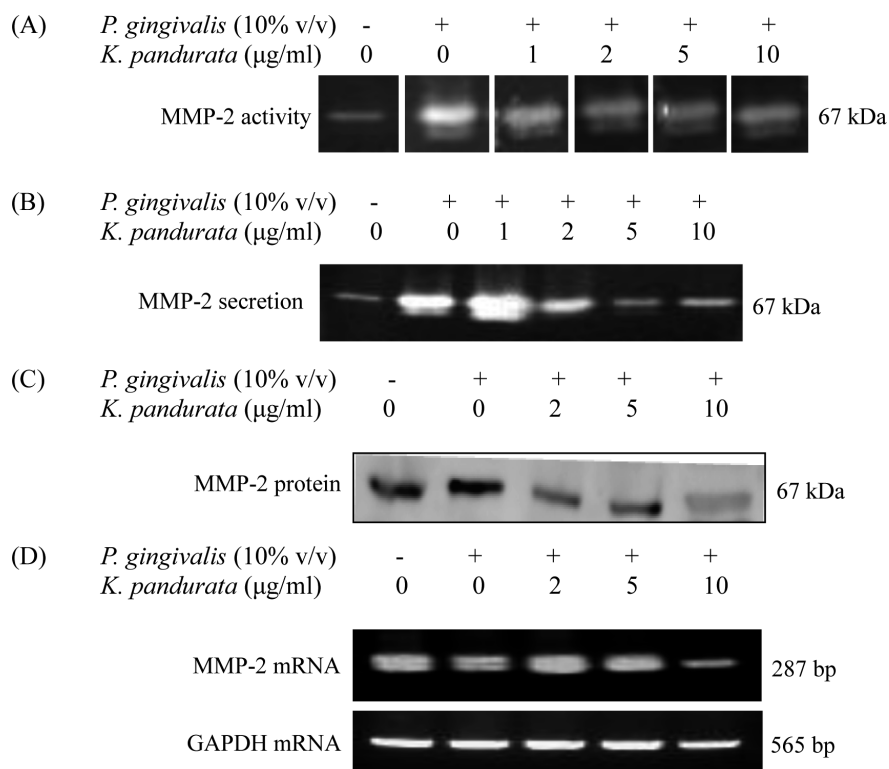


Fig. 2 Effects of *K. pandurata* extract on MMP-2 activity (A), secretion (B), and protein (C), and mRNA (D) in *P. gingivalis* supernatant-treated HGF-1 cells. Cells treated with *P. gingivalis* supernatant (10%) and *K. pandurata* extract (1, 2, 5, and 10 $\mu\text{g/ml}$) were cultured for 48 h. Cells not treated with *P. gingivalis* supernatant were used as controls. (A) To measure MMP-2 activity, culture media from cells treated or not treated with *P. gingivalis* supernatant were directly assayed by gelatin zymography. After electrophoresis, gelatin-containing gels were further treated with *K. pandurata* extract (1, 2, 5, and 10 $\mu\text{g/ml}$) and incubated for 24 h at 37°C. (B) To determine MMP-2 secretion, culture media were assayed by gelatin zymography. (C) To measure MMP-2 protein, culture media were concentrated and analyzed by Western blotting, using MMP-2 antibody. (D) To determine MMP-2 mRNA, total RNA was extracted from cell lysates, and MMP-2 mRNA was measured by RT-PCR. GAPDH mRNA was used as an internal control.

secretion in HGF-1 cells exposed to *P. gingivalis* supernatant (Fig. 3A). The reference MMP inhibitor, GM6001, also specifically attenuated MMP-2 expression in *P. gingivalis* supernatant-treated HGF-1 cells.

Next, we used a Western blot assay to elucidate the molecular mechanisms underlying *K. pandurata* inhibition of MAPK signaling pathways involved in MMP-2 gene expression in *P. gingivalis* supernatant-treated HGF-1

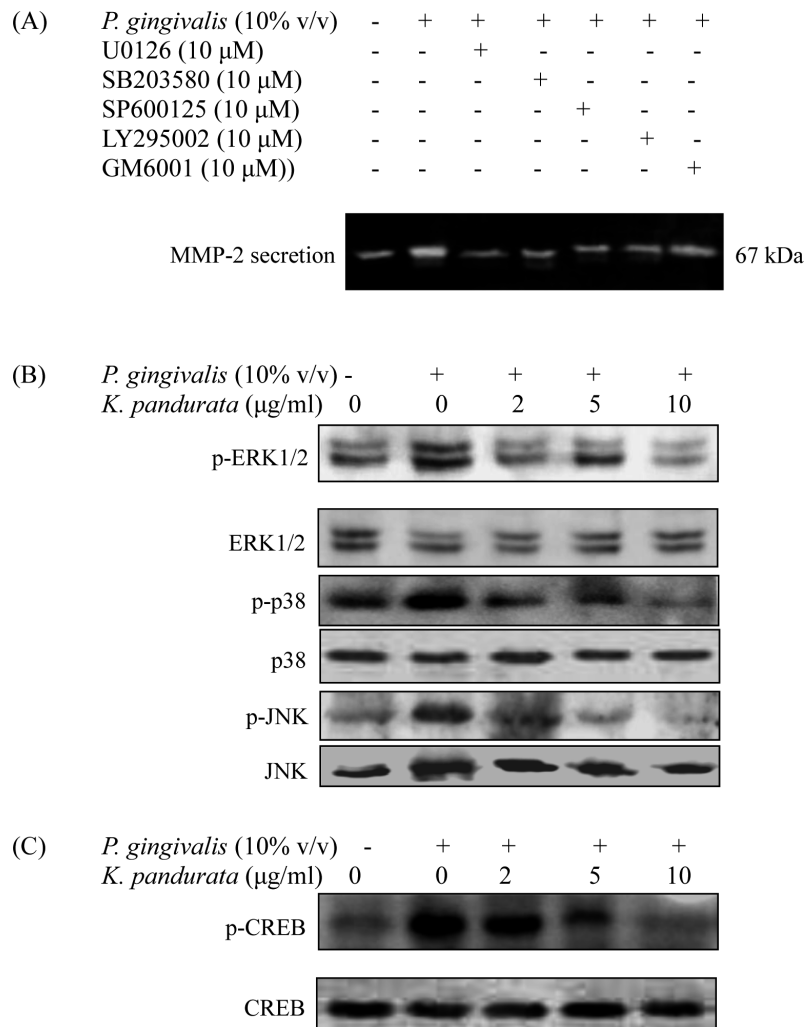


Fig. 3 Effects of signaling inhibitors on MMP-2 expression in *P. gingivalis* supernatant-treated HGF-1 cells (A). Cells treated with *P. gingivalis* supernatant (10%) and a MAPK inhibitor (U0126, SB203580, and SP600125), PI3K inhibitor (LY600125), or standard MMP inhibitor (GM600125) at a total concentration of 10 μ M were cultured for 48 h. Cells not treated with inhibitor or *P. gingivalis* supernatant were used as controls. The level of MMP-2 secreted from culture media was assayed by gelatin zymography. Effect of *K. pandurata* extract on *P. gingivalis* supernatant-induced activation of MAPK and CREB phosphorylation in HGF-1 cells (B-C). Cells treated with *K. pandurata* extract (2, 5, and 10 μ g/ml) and *P. gingivalis* supernatant (10%) were cultured for 48 h. Cells not treated with *K. pandurata* extract or *P. gingivalis* supernatant were used as controls. To investigate MAPK and CREB phosphorylation, cell lysates were analyzed by Western blotting using (B) p-ERK1/2, p-p38, p-JNK, and (C) p-CREB antibodies. Membranes were stripped and reprobed using an antibody against each protein as a control for equal loading.

cells. Exposure of HGF-1 cells to *P. gingivalis* supernatant (10%) enhanced ERK1/2, p38, and JNK phosphorylation, as compared with the untreated controls. Treatment with *K. pandurata* extract dose-dependently suppressed expression of all MAPK phosphorylation in *P. gingivalis* supernatant-treated HGF-1 cells, as compared with untreated control cells (Fig. 3B). It has been reported that *K. pandurata* extract has a stronger inhibitory effect on JNK phosphorylation than on ERK1/2 and p38 phosphorylation. CREB is a regulatory element in the promoter regions of human MMP genes, including the MMP-2 gene (14). A Western blot assay was used to test the effect of *K. pandurata* extract on *P. gingivalis* supernatant-stimulated activation of CREB phosphorylation in HGF-1 cells. *K. pandurata* extract significantly decreased phosphorylated CREB in HGF-1 cells treated with *P. gingivalis* supernatant (Fig. 3C).

Discussion

Considerable research has been devoted to the use of therapeutic agents in the management of periodontitis. Chronic periodontitis caused by the interaction between host responses and periodontopathogens is associated with excess production of inflammatory mediators, including MMPs (15). Only a few synthetic MMP inhibitors, such as the tetracycline groups, have been reported as possible treatments for periodontitis (12,16). However, their clinical efficacy has not been established. Some natural compounds, including *Lupinus albus* extract, cranberry constituents, and hop polyphenols from *Humulus lupulus* L. (Cannabaceae), have been effective in the *in vitro* treatment of periodontal inflammation by attenuating expression of MMPs, prostaglandin, and cytokines (17-20).

In this study, we observed an inhibitory effect of ethanolic *K. pandurata* extract on signaling pathways mediating MMP-2 expression in *P. gingivalis* supernatant-treated human gingival fibroblasts. Gingival fibroblasts naturally produce MMPs, the synthesis of which is stimulated by periodontopathogens and their products, including *P. gingivalis* lipopolysaccharide and supernatant, *Aggregatibacter actinomycetemcomitans* lipopolysaccharide, and *Treponema denticola* lipopolysaccharide (21-24). *P. gingivalis* is a Gram-negative anaerobic bacterium found in periodontal pockets and has been identified as an important pathogen in periodontal disease. Secreted virulence factors, including lipopolysaccharide and supernatant from *P. gingivalis*, alter MMP secretion and activate host cells, resulting in tissue degradation. HGF-1 cells, i.e., normal human gingival fibroblasts, also produce a small amount of MMP-2; thus, in this study, *P. gingivalis* supernatant was added to culture cells to increase the level

of MMP-2. Gelatin zymography revealed that *P. gingivalis* supernatant treatment of HGF-1 cells enhanced major MMP-2 expression (Fig. 1), suggesting that *P. gingivalis* supernatant is involved in periodontitis progression via the production of MMP-2. Our results were similar to those of a previous study, which demonstrated that MMP-2 expression was elevated after treatment with *P. gingivalis* supernatant in human periodontal ligament, oral epithelial, and osteosarcoma cells (9,11,25). However, the specific mechanisms by which these factors mediate cellular MMP-2 secretion are unclear. A previous report described a mechanism by which periodontal pathogens might mediate connective tissue degradation in periodontal disease. The researchers suggested that cell wall antigens stimulate circulating mononuclear phagocytes to produce cytokines, which in turn induce MMP synthesis, thereby initiating extracellular matrix degradation (26).

The ethanolic *K. pandurata* extract reduced MMP-2 activity and secretion in a dose-dependent manner (Figs. 2A and B). Increased MMP activity is associated with various pathological conditions during periodontitis progression. *K. pandurata* extract has the potential to directly inhibit enzyme activity (Fig. 2A). Because of its potent inhibitory effects against both MMP-2 activity and secretion, *K. pandurata* extract is a potential agent for treating and preventing periodontal inflammation. Furthermore, *K. pandurata* extract decreased MMP-2 protein and gene expression in *P. gingivalis*-treated HGF-1 cells (Figs. 2C and D). It is thought that *K. pandurata* extract may modulate the regulation of MMP-2 expression at the transcriptional level. However, MMP regulation is complex, due to the involvement of multiple pathways, including cell responses to regulatory signals, signal transduction, transcription induction, post-transcriptional processing, MMP activation, transport, and secretion (14).

The MAPK signaling pathways play a key role in regulating expression and activating major transcription factors in several MMP genes. To date, only a few studies have observed involvement of MAPK and PI3K signaling pathways in the regulation of MMP-2 expression in human periodontal cell lines (11,27). However, the mechanisms and signaling pathways involved in MMP-2 expression in HGF-1 cells exposed to *P. gingivalis* supernatant remain unclear. In the present study, gelatin zymography revealed that the specific kinase inhibitors MAPK and PI3K attenuated MMP-2 secretion, indicating that these signaling pathways partially mediate MMP-2 expression in *P. gingivalis*-treated HGF-1 cells (Fig. 3A). *P. gingivalis* also activated phosphorylation of MAPK members, including ERK1/2, p38, and JNK, in HGF-1 cells (Fig. 3B). We noted that *K. pandurata* treatment more strongly

inhibited phosphorylated JNK than ERK1/2 and p38 phosphorylation, in HGF-1 cells exposed to *P. gingivalis* supernatant (Fig. 3B). These results suggest that *K. pandurata* extract suppresses MMP-2 gene expression by downregulating major JNK signaling. This resembles the mechanisms of action of other naturally occurring anti-inflammatory flavonoid compounds, such as luteolin, quercetin, genistein, and quercetagenin, which inhibit *E. coli* lipopolysaccharide-stimulated MAPK activation in human gingival fibroblasts (28-29).

At the transcriptional level, *P. gingivalis* supernatant effectively enhanced expression of CREB phosphorylation, one of the regulatory elements in the promoter regions of the MMP-2 gene. It has been noted that activation of p38 kinase results in phosphorylation of CREB (30), suggesting that *P. gingivalis* supernatant induces expression of phosphorylated CREB via p38 kinase signaling in HGF-1 cells. Activation of PI3K also leads to phosphorylation of Akt, which plays a critical role in the activation of transcription factor CREB. In the present study, the PI3K inhibitor LY295002 partially inhibited MMP-2 secretion in HGF-1 cells treated with *P. gingivalis* supernatant (Fig. 3A). Chan et al. (31) reported that CREB signaling was stimulated by protease-activated receptors during wound healing and inflammatory responses in human gingival tissues. In the present study, treatment with *K. pandurata* extract dose-dependently decreased phosphorylated CREB (Fig. 3C), suggesting that *K. pandurata* extract reduced MMP-2 gene expression via inhibition of the CREB and JNK signaling pathways. The importance of the CREB element in the promoter of the MMP-2 gene in *P. gingivalis* supernatant-treated HGF-1 cells highlights the need to elucidate the mechanisms underlying CREB/MAPKs and PI3K/Akt signaling and identify the transcription factors affected by *P. gingivalis* and *K. pandurata* treatment.

In sum, our findings provide insight into the molecular mechanisms by which ethanolic *K. pandurata* extract blocks *P. gingivalis* supernatant-stimulated MMP-2 expression in human gingival fibroblasts. *K. pandurata* extract inhibited *P. gingivalis* supernatant-stimulated MMP-2 protein and gene expression via modulation of CREB signaling, which may be facilitated by attenuation of JNK phosphorylation. Thus, *K. pandurata* Roxb. appears to be a potent MMP inhibitor that may prove useful in controlling the destruction of periodontal tissue.

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